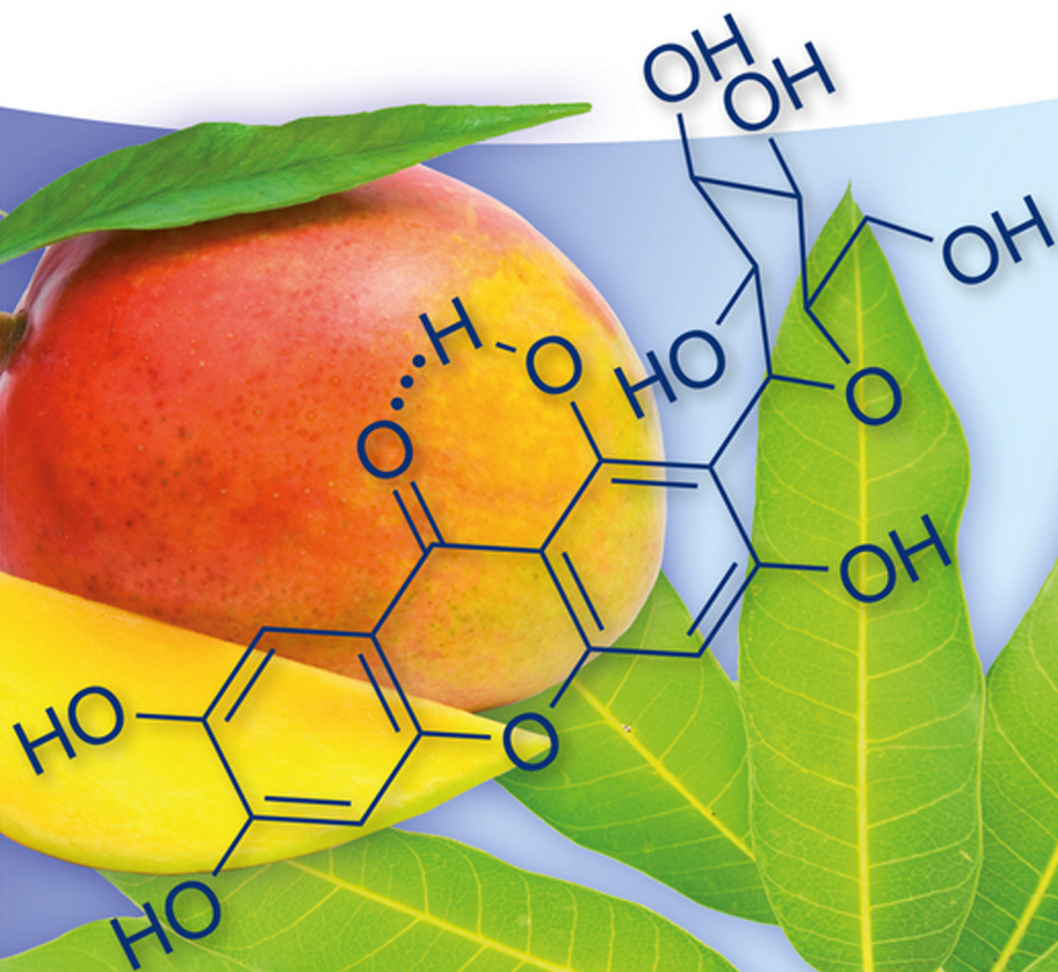


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*All those who are working globally with bioactive natural products for the cause of human welfare*





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## Foreword

*Bioactive Natural Products: Chemistry and Biology* edited by Professor (Dr.) Goutam Brahmachari and published by Wiley-VCH is a timely, highly significant, and useful book for readers engaged in chemical, biological, pharmacological, and medicinal study as well as research in these emerging areas.

Natural product science was one among the very few key areas of research in ancient times. Despite substantial progress in many other areas, research on natural product chemistry remains in the fuzzy region between chemical and biological research with undefined boundaries. It is argued that natural product chemistry is not a separate subject any longer: it is a hybrid discipline. In reality, the success in natural product research and its applications has opened up many other subdomains in science that are widely accepted in the academic world as well as in the modern chemical and pharmaceutical industries. For example, owing to the availability of many bioactive natural products, synthetic organic chemistry, computational chemistry, medicinal chemistry, biochemistry, and analytical chemistry as well as molecular biology, pharmacognosy, biotechnology, and clinical science have all become major areas of scientific research in recent decades. It has been extensively demonstrated that the search for natural products, or products obtained from natural sources through synthesis, is directed toward identifying new molecules for diseases and investigating their mechanisms of action and their specific targets of interaction (for example, DNA, RNA, proteins, and enzymes).

Natural products are classified on the basis of their origins, biological functions, and structures. Plants are a vast source of many natural drugs. A number of drugs are also synthesized from natural products through different chemical reactions. The most important of these natural products are terpenoids, alkaloids, steroids, phenolic compounds, vitamins, carbocyclics and hetrocyclic aromatic compounds, proteins, and carbohydrates. Bacteria and fungi are microorganisms that are also extremely useful in the search for and identification of organic molecules that have the potential to serve as drugs and highly active compounds. Many different drugs other than antibiotics have been isolated for medicinal

use from microorganisms. Marine sources are relatively unexplored for the identification of bioactive compounds. However, there is considerable interest now in the isolation of novel molecules from various marine sources and the possibilities here are endless. The ocean is a huge source for natural biological and chemical products. In their search for natural products in the ocean's fish, snails, algae, sponges, reefs, bacteria, and microorganisms, it would appear that the journey of chemists and biologists to explore unknown, diverse, structurally unique, and potentially useful amazing naturally occurring molecules has only just begun. In addition to these sources, animals, venoms, and toxins are also screened for bioactive organic natural compounds.

Most of the natural products are found in combination with many other active or nonactive components. The first objective of research in natural product chemistry is to isolate, purify, determine the structure and structural alteration, and test these molecules as entities that can be used in medicine to enhance the quality of animal and human life. This is achieved through total or partial synthesis of bioactive natural products. In fact, the combined and tireless efforts of chemists and other scientists including clinicians have solved numerous complex medical problems through research on natural products. The second objective of research in this area is to study the mechanisms of action of natural products that may find use as drugs or lead molecules by identification of biochemical pathways using modern methods including genomic and proteomic analyses. The biodiversity of nature is available to us. Clearly, nature remains the most valuable source of chemical and biological investigation of molecules with novel stereochemistry. Therefore, new natural products and established bioactive natural products will remain a key for our well-being.

This book, edited by Professor Brahmachari, has a depth of knowledge and information. I believe expert scientists, researchers, and students will use this book for a tremendous learning and research experience. The quality and timeliness of this book in a market of competitive research will stimulate the present and future generations of scientists who are interested in improving the quality of human life through working on natural products and derivatives stemming from them. The book contains a wealth of features that include information on a diverse number of bioactive natural products presented by prominent authors with many pertinent references. This will help even persons without sufficient knowledge in natural products to undertake research in the area in the future.

The editor has a long history of publishing many books that have received tremendous scientific attention. The dedication of the editor in the selection of the authors, subject matter, and organization of the chapters proves his experience and scholarly activity in this particular area. He has chosen numerous contributors who are active in this specific field. Each chapter is presented in a highly elegant and precise manner. Scientists working on natural product chemistry will find this a handy resource book that will help them with their work. The enduring work of many scientists on biologically active natural products in

the isolation, detection, and structure elucidation along with biological study as described in the content of every chapter will prove to be very useful for the current and future generations of scientists.

I strongly recommend this state-of-the-art book on the chemistry and biology of bioactive natural products to students and researchers who are engaged in natural product research as well as synthesis and biological evaluation of novel molecules for different types of medical disorders and for those who are pursuing their activities to improve the quality of our lives.

*Bimal K. Banik*  
*The University of Texas-Pan American*  
*Edinburg, USA*



## Preface

This single volume entitled *Bioactive Natural Products: Chemistry and Biology* is an endeavor to focus the recent cutting-edge research advances in the field of bioactive natural products, very particularly operating at the interface of chemistry and biology, and also to underline how natural product research continues to make significant contributions in the domain of discovery and development of new medicinal entities. This book consists of a total of 17 chapters contributed by eminent researchers from several countries in response to my personal invitation. I am most grateful to the contributors for their generous and timely response in spite of their busy and tight schedules with academics, research, and other responsibilities.

Natural products usually refer to chemical substances produced by a living organism or found in nature that have distinctive biological and pharmacological effects; they encompass a wide variety of chemical compound classes, including alkaloids, antibiotics, terpenoids, flavonoids, xanthonoids, phenolics, carbohydrates, lipids, proteins and amino acids, and nucleic acids. The huge diversity in chemical structures of natural products is an outcome of biosynthetic processes that have been modulated over the millennia through genetic effects. Such chemical entities have played a crucial role in modern drug development and still constitute a prolific source of novel lead compounds, or pharmacophores, for ongoing drug discovery programs. Most significantly, natural products operate at the interface of chemistry and biology. Hence, search for bioactive molecules from nature (plants, animals, microflora) continues to play an important role in fashioning new medicinal agents. With the advent of modern techniques, very particularly the rapid improvements in spectroscopic as well as accompanying advances in high-throughput screening techniques, it has become possible to have an enormous repository of bioactive natural compounds, thus opening up exciting new opportunities in the field of new drug development to the pharmaceutical industry.

Medicinal chemistry of such bioactive compounds encompasses a vast area that includes their isolation and characterization from natural sources; structure modification for optimization of their activity and other physical properties;

and also total and semisynthesis for a thorough scrutiny of structure–activity relationships. It has been well documented that natural products played a significant role in modern drug development, especially for antibacterial and antitumor agents; however, their uses in the treatment of other epidemics such as AIDS and cardiovascular, cancerous, neurodegradative, infective, and metabolic diseases have also been extensively explored. The need for leads to solve such health problems threatening the world population makes all natural sources important for the search of novel molecules, diversified and unique structural architectures of which inspired scientists to pursue new chemical entities with completely different structures from known drugs. Researchers around the globe are deeply engaged in exploring the detailed chemistry, pharmacology, and biology of such potent and naturally occurring efficacious bioactive compounds. Current research trends in the field suggest an optimistic future for natural products in drug discovery; however, novel strategies and innovative approaches in addition to the introduction of more sophisticated technical requirements are still needed today for the development of natural products into new drugs.

This book, which comprises a variety of 17 chapters written by active researchers and leading experts working in the field of chemistry of biologically active natural products, brings together an overview of current discoveries and trends in this remarkable field. Chapter 1 presents an overview of the book and summarizes the contents of the other chapters so as to offer glimpses of the subject matter covered to the readers before they go in for a detailed study. Chapters 2 through 17 are devoted to exploring the ongoing chemical, biological, and pharmacological advances in naturally occurring organic compounds and describe their biosynthesis, semisynthesis, total synthesis, chemical transformations, structure–activity relationships, nucleic acid and protein-binding aspects, biodiversity, and bioproduction, including mechanism of action, high-throughput drug screening, and drug design.

This timely volume encourages interdisciplinary work among chemists, biologists, pharmacologists, botanists, and agronomists with an interest in bioactive natural products. It is also an outstanding source of information with regard to the industrial application of natural products for medicinal purposes. The broad interdisciplinary approach dealt with in this book would surely make the work much more interesting for scientists deeply engaged in the research and/or use of bioactive natural products. It will serve as a valuable resource for researchers in their own fields not only to predict promising leads for developing pharmaceuticals to treat various ailments and disease manifestations but also to motivate young scientists to the dynamic field of bioactive natural products research.

Representation of facts and their discussions in each chapter are exhaustive, authoritative, and deeply informative; hence, the book would serve as a key reference for recent developments in the frontier research on bioactive natural products at the interface of chemistry and biology, and would also be of much utility to scientists working in this area. I would like to express my sincere thanks once



again to all the contributors for the excellent reviews on the chemistry, biology, and pharmacology of bioactive natural products. It is their participation that makes my effort to organize such a book possible. Their masterly accounts will surely provide the readers with a strong awareness of current cutting-edge research approaches being followed in some of the promising fields of biologically active natural products.

I would like to express my sincere thanks and deep sense of gratitude to Professor Bimal K. Banik, Department of Chemistry, The University of Texas-Pan American, United States, for his keen interest in the manuscript and for writing foreword to the book.

Finally, I would like to express my deep sense of appreciation to all of the editorial and publishing staff members associated with Wiley-VCH, Weinheim, Germany, for their keen interest in publishing the work and also for their all-round help so as to ensure that the highest standards of publication are maintained in bringing out the book.

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## About the Editor



**Professor (Dr.) Goutam Brahmachari** was born at Barala in the district of Murshidabad (West Bengal), India, on April 14, 1969. He received his high school degree in scientific studies in 1986 at Barala R. D. Sen High School under the West Bengal Council of Higher secondary Education (WBCHSE). Then he moved to Visva-Bharati (a central University founded by Rabindranath Tagore at Santiniketan, West Bengal, India) to study chemistry at the undergraduate level. After graduating from this University in 1990, Prof. Brahmachari completed his masters in 1992 with specialization in organic chemistry and thereafter received his Ph.D. degree in 1997 in chemistry from the same University. He was appointed as assistant professor of organic chemistry at Visva-Bharati University, Department of Chemistry, in 1998, then became associate professor in 2008. In 2011, he became full professor of organic chemistry in the same faculty. At present, he is responsible for teaching courses in organic chemistry, natural products chemistry, and physical methods in organic chemistry. Several students have received their Ph.D. degree under the supervision of Prof. Brahmachari during this period, and a couple of research fellows are presently working with him both in the fields of natural products and synthetic organic chemistry. He serves as a member of the Indian Association for the Cultivation of Science (IACS) and Indian Science Congress Association (ISCA), Kolkata. He also serves as an Editor-in-Chief, *Signpost Open Access Journal of Organic and Biomolecular Chemistry*, and as editorial board member of several journals.

Prof. Brahmachari's research interests include (i) isolation, structural determination, and/or detailed NMR study of new natural products from medicinal plants; (ii) synthetic organic chemistry with special emphasis on green chemistry; (iii) semisynthetic studies with natural products; and (iv) evaluation of biological activities and pharmacological potential of natural and synthetic compounds. With more than 16 years of teaching experience, he has produced so far nearly 120 publications including original research papers, review articles, and invited book

chapters in edited books in the field of natural products and organic synthesis from internationally reputed presses. Prof. Brahmachari has authored/edited a number of text and reference books that include *Organic Name Reactions: A Unified Approach* (Narosa Publishing House, New Delhi; Co-published by Alpha Science International, Oxford, 2006), *Chemistry of Natural Products: Recent Trends & Developments* (Research Signpost, 2006), *Organic Chemistry Through Solved Problems* (Narosa Publishing House, New Delhi; Co-published by Alpha Science International, Oxford, 2007), *Natural Products: Chemistry, Biochemistry and Pharmacology* (Narosa Publishing House, New Delhi; Co-published by Alpha Science International, Oxford, 2009), *Handbook of Pharmaceutical Natural Products – 2 volume-set* (Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim, Germany, 2010), *Bioactive Natural Products: Opportunities & Challenges in Medicinal Chemistry* (World Scientific Publishing Co. Pte. Ltd., Singapore, 2011), *Chemistry and Pharmacology of Naturally Occurring Bioactive Compounds* (CRC Press, Taylor & Francis group, USA, 2013), and *Natural Bioactive Molecules: Impacts & Prospects* (Narosa Publishing House, New Delhi; Co-published by Alpha Science International, Oxford, 2014), *Green Synthetic Approaches for Biologically Relevant Heterocycles* (Elsevier Inc., USA, 2014).

He is regularly consulted as a referee by leading international journals including Elsevier, Royal Society of Chemistry, American Chemical Society, Wiley, Taylor & Francis, Springer, Bentham Science, Indian Chemical Society, Korean Chemical Society, Brazilian Chemical Society, Bulgarian Academy of Sciences and so on, and also various financial commissions.

Goutam Brahmachari enjoys songs of Rabindranath Tagore, and finds interests in Literature as well!

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# 1

## An Overview

Goutam Brahmachari

### 1.1

#### Introduction

The endeavor of this book entitled *Bioactive Natural Products: Chemistry and Biology* is to present details of cutting-edge research in the chemistry and biology of bioactive natural products and it helps the reader understand how natural product research continues to make significant contributions in the discovery and development of new medicinal entities. This is a reference book meant for phytochemists, synthetic chemists, combinatorial chemists, as well as other practitioners and advanced students in related fields. The book, comprising of 16 technical chapters, highlights the chemical and biological aspects of potential natural products with an intention to unravel their pharmaceutical applicability in modern drug discovery processes. The book covers the synthesis, semisynthesis as well as biosynthesis of potentially bioactive natural products. It also features chemical and biological advances in naturally occurring organic compounds, describing their chemical transformations, modes of action, and structure–activity relationships.

This introductory chapter (Chapter 1) presents an overview of the book, and summarizes the contents and subject matter of each chapter so as to offer certain glimpses of the coverage of discussion to the readers before they go for detailed study.

### 1.2

#### An Overview of the Book

The present book contains a total of 16 technical chapters – Chapters 2–17; this section summarizes the contents and subject matter of each of these chapters.

## 1.2.1

**Chapter 2**

In Chapter 2, Golshani and his group have discussed the use of chemical genomics to investigate the mechanism of action (MOA) for inhibitory bioactive natural compounds. Understanding the specific MOA of small molecules is considered one of the most significant hurdles in developing new drugs. Traditional pathway-specific mechanistic approaches are time consuming and expensive. Global genome-wide single-deletion array (GDA) technology nowadays provides a more feasible alternative to laborious metabolic pathway-specific assays and has the added advantage of working on a global scale. The use of GDA technology to screen natural substances for intriguing inhibitory compounds can help probe the biological complexity of intracellular networks or identify leads for promising novel antimicrobials. GDA technology can both identify direct target and off-target effects of a novel compound or expand our understanding of previously studied compounds. This illuminating and useful chapter offers an extensive overview on the use of GDAs in *Saccharomyces cerevisiae* and *Escherichia coli* as well as combinatorial haploinsufficiency mutant profiling/homozygous mutant profiling (HIP/HOP) as genomic tools to investigate MOA in naturally derived inhibitory compounds. The present chapter offers an impetus to the practitioners deeply engaged in this remarkable field.

## 1.2.2

**Chapter 3**

Yang and his group have discussed on the application of high-throughput screening (HTS) of potential natural products based on cancer-signaling strategies including EGFR, P13K, Wnt, and STATs in Chapter 3. With the advances in molecular biology, human genetics, and functional genomics, HTS involves continuous invention and improvement in methods. With the assay of HTS, targeting the cancer-signaling pathways has experienced a more significant impact on drug discovery and development in recent times. More attentions is being focused on the selection of cancer molecular targets between generality and specificity, that is, cell proliferation and survival peculiar to a tumor and anticancer drug research with advanced HTS assays is expected to be a revolutionary technological advance in coming years. The present chapter would surely motivate researchers working in this area of interest.

## 1.2.3

**Chapter 4**

Chapter 4, by Demain and his group, is dedicated to potential microbial immunosuppressants including antifungal peptides and antibiotics cyclosporin,

tacrolimus, sirolimus, mycophenolic acid, and ascomycin. Discovery, fermentation, strain improvement, mode of action, and biosynthesis of the representative immunosuppressants are discussed in detail. The biosynthetic pathway of such microbial products involves a series of complex reactions carried out by multi-enzyme polypeptides that catalyze reactions in a belt-like manner, first forming a chain, then undergoing elongation and cyclization. Genes encoding these enzymes have been cloned and studied as well. The present chapter highlights the applications of immunosuppressants not only in organ transplantation, but also in the treatment of many other life-threatening diseases such as autoimmune disorders, cancer, AIDS, asthma, skin diseases, respiratory ailments, and malaria. Continuing research offering more insights into the genetics, biosynthesis, and molecular mode of action of these drugs would open new windows for their further applications as effective therapeutics. This illuminating review on immunosuppressants would obviously enrich the readers and would motivate them in undertaking in-depth research in immunosuppressants coupled with medicinal chemistry.

#### 1.2.4

##### **Chapter 5**

In Chapter 5, Mallik and his group have presented a comprehensive discussion on the activators and inhibitors of ADAM-10 for management of cancer and Alzheimer's disease. A disintegrin and metalloproteinase (ADAM) family of proteolytic enzymes is known for "shedding" of membrane-bound proteins and are unique among cell surface proteins as they possess an adhesion domain and a protease domain. The deviation from normal levels of ADAMs is also observed in various pathological conditions such as cancer and Alzheimer's disease. The enzyme is downregulated in Alzheimer's disease while over-expressed in various cancers. Involvement of ADAM-10 in progression of cancer and Alzheimer's disease is now well established. Compounds from natural and synthetic origins involved in selective activation or inhibition of ADAM-10 possess tremendous potential as therapeutics for treating cancer and Alzheimer's disease. The present chapter offers an up-to-date development in this field.

#### 1.2.5

##### **Chapter 6**

Chapter 6, by Huczyński and his group, deals with the structure and biological activity of polyether ionophores and their semisynthetic derivatives. Polyether ionophores, which belong to a large group of antibiotics, are unique natural compounds because they exhibit a broad spectrum of biological activities including antibacterial, antiviral, and anticancer activity. Natural polyether ionophores have been found to exhibit potent activity against those cancer cells that display

multidrug resistance (MDR) and also against cancer stem cells (CSCs). It has been demonstrated that biological potency of such polyether ionophores is related to their unique chemical structure, as well as their ability to form complexes with mono- and divalent metal cations facilitating their transport across lipid membranes. This phenomenon results in a disturbance of the natural cation concentration gradient and intracellular pH change, leading to mitochondrial injury, cell swelling, and vacuolization and, as a consequence, programmed cell death (apoptosis). The authors have discussed all these issues in detail in the present chapter highlighting their structural and chemical properties, semisynthetic derivatives, and the mechanisms of cation transport.

#### 1.2.6

##### Chapter 7

Désaubry and coauthors have reviewed the synthesis and pharmacology of bioactive flavaglines in Chapter 7. Flavaglines represent a family of cyclopenta[*b*]benzofurans found in medicinal plants of the genus *Aglaiia*, and have been reported to display potent anti-inflammatory, neuroprotective, cardioprotective, and anti-cancer activities. It has been revealed that flavaglines have the ability to kill cancer cells without affecting normal cells. Such a selective cytotoxicity to cancer cells and cytoprotection to normal cells, both of which occur at nanomolar concentrations, is unprecedented. In the present chapter, the authors have offered an excellent overview on the synthetic routes to flavaglines, MOA, and evaluation of biological potency of the target compounds with the objective of discovering certain novel therapeutic agents from this class of bioactive natural products.

#### 1.2.7

##### Chapter 8

In Chapter 8, Sil and his group have described the beneficial effect of naturally occurring antioxidants against oxidative stress-mediated organ dysfunctions. The metabolism of oxygen by cells generates potentially harmful reactive oxygen species (ROS). In recent times, oxidative stress or imbalance between pro-oxidants and antioxidants is a comparatively new issue that has extensively troubled research in biomedical sciences. It has now been established that it significantly contributes to the pathophysiology of various prevalent diseases such as hypertension, diabetes, asthma, allergies, autism, lupus, acute renal failure, atherosclerosis, rheumatoid, Alzheimer's, Parkinson's, and cardiovascular diseases. Oxidative stresses in the cells have a considerable impact leading to defective cellular function, aging, or disease. Consequently, a better thoughtful role of ROS-mediated signaling in normal cellular function as well as in disease is necessary for developing therapeutic tools for oxidative stress-related pathogenesis. The present chapter has a detailed discussion on the



multifunctional therapeutic applications and signaling properties of naturally occurring antioxidants, which obviously play a number of beneficial roles in oxidative stress-induced organ dysfunctions. The authors have been successful in unraveling the potential use of naturally occurring antioxidants as novel promising therapeutic strategies.

#### 1.2.8

#### **Chapter 9**

Chapter 9, by Gopinatha Suresh Kumar, deals with the nucleic acid and protein-binding aspects of isoquinoline alkaloids and their analogs, and their therapeutic potential for drug design. Isoquinoline alkaloids and analogs represent an important class of molecules that have attracted attention for their various potential pharmacological activities. Specific binding to cellular biomacromolecules such as DNA and RNA has been thought to be one of the most important routes for their therapeutic action. In this chapter, an up-to-date knowledge on the binding aspects of some of the most important isoquinoline alkaloids and their analogs are presented. Elucidation of the recognition mechanism and accumulation of a large volume of recent research outcomes have been covered in the present chapter, which serves as a useful guide to researchers working in the development of potential therapeutic agents.

#### 1.2.9

#### **Chapter 10**

Jean Fotie has presented an exhaustive discussion on the potential of peptides and depsiptides from terrestrial and marine organisms in the fight against human protozoan diseases such as malaria, trypanosomiasis, leishmaniasis, amebiasis, toxoplasmosis, cryptosporidiosis, sarcocystis, coccidiosis, babesiosis, and giardiasis in Chapter 10. Peptides and depsiptides are a widely distributed family of naturally occurring molecules, usually found in fungi, actinomycetes, cyanobacteria, higher plants, and marine organisms, with a broad window of biological and pharmacological activities ranging from antibacterial to anticancer, some of which are currently in clinical use or have entered human clinical trials as antibiotic or anticancer agents. This family of compounds should be given a serious and careful consideration for their antiprotozoan activity. The present chapter would act as a stimulus in this direction.

#### 1.2.10

#### **Chapter 11**

Chapter 11, by Chaturvedi and his group, is devoted to naturally occurring sesquiterpene lactones and their semisynthetic analogs as potential anticancer agents. Wide structural diversity coupled with potential biological

activities of sesquiterpene lactones has attracted a great deal of attention from medicinal chemists around the world. Although, the exact MOA of SLs is not well known, it has been documented through various published reports that the biological activity displayed by majority of sesquiterpene lactones is due to the presence of  $\alpha$ -methylene- $\gamma$ -lactones and the  $\alpha,\beta$ -unsaturated cyclopentenone ring. In the present chapter, the authors have focused on an up-to-date and comprehensive account on the sesquiterpenes lactones as anticancer agents.

1.2.11

#### **Chapter 12**

Brahmachari has focused on the chemistry and biology of naturally occurring calanolides in Chapter 12. Natural calanolides occupy a significant position in the pyranocoumarin class of compounds, and are well known for their anti-HIV potential. In addition, these pyranocoumarins have also been found to exhibit anti-tuberculosis activity as well. Such promising pharmaceutical activity coupled with low availability of natural calanolides has evoked tremendous interest among the organic chemists to undertake systematic chemical studies toward the total synthesis of this class of compounds. Preclinical and clinical results of both natural and synthetic calanolides have been found to be quite encouraging, and consequently they are being regarded as potential “leads” in the development of future anti-HIV and antituberculosis drugs. The present chapter covers up-to-date literature of naturally occurring calanolides in view of their anti-HIV and antituberculosis potential, their chemical analogs, and total syntheses.

1.2.12

#### **Chapter 13**

Chapter 13, by Lakshmanan and Sadasivan, deals with certain plant-derived selective estrogen receptor modulators (SERMs). Phytoestrogens are markedly similar in chemical structure to the mammalian estrogen and estradiol; they and bind to estrogen receptors, with a preference for ER $\beta$ . Different physiological functions of the body such as reproduction, behavior, and neuroendocrine function are regulated by estrogen through estrogen receptor subtypes. These receptors have tissue-specific functions with respect to each other. For example, ER $\alpha$  induces cell proliferation, whereas ER $\beta$  antagonizes this action. Thus, their differential expression and activation in a balanced manner is necessary for normal functioning of the body and any imbalance in this expression leads to oncogenesis and several autoimmune diseases. Hence, phytoestrogens which mimic the function of endogenous estrogen can be judiciously exploited for regulating this imbalance and reverting back the normal functions of the body. SERMs may, thus be considered as potential lead compounds for the development of drugs in the treatment of estrogen-mediated cancers and autoimmune diseases.

The present chapter would be very useful to readers whose interests lie in the study of potential phytoestrogens.

1.2.13

#### **Chapter 14**

Modolo and her group have discussed biosynthesis and biological activities of phenylpropanoids in Chapter 13. Phenylpropanoids compounds, which bear a  $C_6-C_3$  phenolic scaffold, have received particular attention not only because of their function in plants but also because of their wide spectrum of biological activities. Flavonoids, coumarins, and stilbenes have been considered as the main subclass of phenylpropanoids in the present discussion. This illuminating overview provides valuable information about the biosynthesis and pharmacological potential of such medicinally important phenylpropanoid compounds.

1.2.14

#### **Chapter 15**

Chapter 15 by Serafini and his group is devoted to neuropeptides which are the active neuromodulators involved in the pathophysiology of suicidal behavior and major affective disorders. Neuropeptidergic circuits seem to act as fundamental mediators of human behavior. These molecules may represent interesting mediators of stress-related disorders, major affective conditions, and suicidal behavior. From their detailed literature survey, it has been demonstrated that there remains an association between suicidality and corticotrophin-releasing factor (CRF), nerve growth factor (VGF), cholecystokinin (CCK), orexin, substance P, and Neuropeptide Y (NPY); these molecules play a key role in many biological functions and act as important neuromodulators of emotional processing. Although many studies identified a positive association between neuropeptide alterations and major depressive disorders/suicidal behavior, it is, however, unlikely that neuropeptides may currently represent definitive biomarkers of suicidality/depression. Further studies are needed in order to elucidate the complex nature of neuropeptidergic abnormalities underlying suicidal behavior and major affective conditions. The authors of this present chapter have performed the job in this direction to offer an insight into the cause and the search for possible remedies.

1.2.15

#### **Chapter 16**

Chapter 16 by Miller and his group is devoted to the discussion on some innovative techniques to identify and characterize novel compounds from marine organisms

as potential drug molecules. Marine organisms are a rich source of natural products, having potential as lead compounds in drug discovery. The path from discovery of a novel compound to clinical use, however, is a long and complex one in which many lead structures drop out along the way. In the present chapter, the authors have focused on the approach used in their chemistry and biology laboratories to find new biologically active compounds (NMR-based screening) along with providing clues on their mode of action (chemical genetics and proteomics), and validation of their effects in mammalian cells (biochemical analysis of target responses). This chapter would provide useful information to researchers deeply involved in the drug discovery process.

1.2.16

### Chapter 17

Chapter 17, by Leal and Calado, deals with the biodiscovery, biodiversity, and bioproduction of marine natural products (MNPs). MNPs are acknowledged as the “blue gold,” as they hold a vast reservoir of promising leads for drug development. Critical survey of literature on the biodiscovery, biodiversity, and bioproduction of MNPs reveals that although new technologies have promoted significant advances in the collection, screening, and identification of a whole new range of molecules, marine chemical ecology is still several decades behind its terrestrial counterpart; there is still a vast fraction of marine biodiversity yet to be screened, as well as regions in the world’s oceans that remain poorly explored. In this chapter, the authors have overviewed past and current trends of MNP biodiscovery, both taxonomically and geographically, and discuss them in view of marine biodiversity and biogeography. In addition, they have also discussed the bioproduction of secondary metabolites of marine organisms, particularly through *in toto* aquaculture.

1.3

### Concluding Remarks

This introductory chapter summarizes the technical chapters of this book, each of which is exhaustive in its representation of facts and with discussions that are authoritative and deeply informative. The readers will find discussions that provoke interest in each of the chapters, which practically cover wide area of bioactive natural product research, particularly on their chemical and biological aspects. The references encourage interdisciplinary work among chemists, pharmacologists, biologists, botanists, and agronomists with an interest in bioactive natural products. Hence, the present book should definitely serve as a key reference for recent developments in frontier research on bioactive natural products, and also would find much utility for the scientists working in this area.

## 2

# Use of Chemical Genomics to Investigate the Mechanism of Action for Inhibitory Bioactive Natural Compounds<sup>1</sup>

*Daniel Burnside, Houman Moteshareie, Imelda G. Marquez, Mohsen Hooshyar, Bahram Samanfar, Kristina Shostak, Katayoun Omid, Harry E. Peery, Myron L. Smith, and Ashkan Golshani*

### 2.1

#### Introduction: Antibiotic Resistance and the Use of Natural Products as a Source for Novel Antimicrobials

A significant fraction of current therapeutic agents for all diseases were originally derived from natural sources. Between 1981 and 2006, 45% of newly approved small molecule drugs were either natural products or a derivative/mimic of a natural product [1]. Naturally derived compounds are a promising source of new antibiotic scaffolds which have not yet seen induced resistance develop [2]. Given the immense number of bioactive natural compounds found in nature, cost- and time-permissible assays which help to characterize strong leads are crucial when sifting through expanding chemical libraries. The biodiversity on Earth has the potential to introduce a nearly endless library of novel naturally derived therapeutic molecules, indicating that many promising findings have not been thoroughly examined [3].

A major obstacle in the development of biologically derived molecules to treat disease is the lack of understanding of the specific mechanism(s) by which pathway inhibition produces effects that ultimately cumulate in cell death/stasis [4]. Understanding how an inhibitory natural compound perturbs microbes helps determine if the molecule works in a manner similar to antimicrobials already in use or if a novel mechanism is at work. Possessing a thorough understanding of the mechanism of action (MOA) can predict safety issues and expedite the development phase [5]. Regulatory approval is more rapidly acquired if a thorough mechanistic understanding of the molecule is available.

Traditionally, after isolating a compound showing inhibitory activity from a crude extract, its MOA was investigated using a series of target-specific

<sup>1</sup> This work is dedicated to the loving memory of Minoos Rajabian.

independent assays artificially reconstituted *in vitro*. The goal of these assays was to test the potential effects of a compound on specific essential pathways and deductively infer its MOA. Examples include specific enzyme inhibition assays [6], DNA and RNA polymerization assays [7, 8], protein synthesis assays [9], and cell wall/membrane stability assays [10], among others. An example of target-specific inhibition is whole cell labeling. This method quantifies a compound's inhibition of core anabolic pathways by introducing modified precursors. Inhibition of phospholipids, DNA, RNA, proteins, and cell wall synthesis can be probed by incubation with 2-[ $^3H$ ]glycerol, 6-[ $^3H$ ]thymidine, 5,6-[ $^3H$ ]uracil, 4,5-[ $^3H$ ]leucine, and 2,3-[ $^3H$ ]alanine (or 2-[ $^3H$ ]glycine) respectively. The general impact of the compound on these processes can be quantified through scintillation counting but must be adjusted for variations in penetration and efflux and should be verified by a demonstration of specific enzyme inhibition [11].

The downfall of these assays is that they are limited in scope, time, and resource intensive, and often *in vitro*, meaning vital factors such as intracellular localization/concentration and drug metabolism are not considered [12]. A more effective approach is the concurrent *in vivo* analysis of the response of all genes/proteins in a cell to a compound of interest. Gene deletion arrays (GDAs), global transposon mutagenesis, proteome-wide protein–protein interaction analysis through yeast two-hybrid or affinity purification tagging, microarrays, RNA sequencing, and bioinformatic approaches, among others, are capable of examining the molecular biology of a cell as a system and can study chemical perturbation from a variety of angles. In the following sections, we primarily discuss chemical genetic analysis using variations of the GDAs first developed in the budding yeast *Saccharomyces cerevisiae* as functional genomic tools for inferring the MOA of inhibitory natural compounds and how to augment results with data from these other techniques to infer meaningful relationships.

## 2.2

### Chemical Genetics and Genomics

Chemical genetics often refers to the study of the alterations in the genetic network of a biological system in the presence of cell-permeable bioactive small molecules [13]. There are two branches of this field known as *forward* and *reverse chemical genetics*. In forward chemical genetics, phenotypic screening of chemical libraries is performed in living organisms to identify compounds or drug targets that cause a desirable effect (i.e., screening for antimicrobial activity). This forward approach is followed by employing various biochemical and molecular biological tools to identify protein targets.

In reverse chemical genetics, proteins of interest are used as targets against chemical libraries to validate drug targets, provide experimental models of disease, and to observe the effects of the chemical compounds *in vivo* for interesting

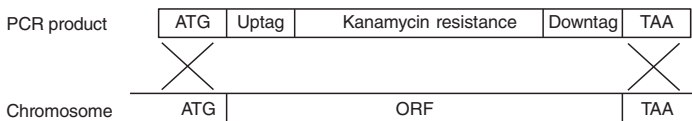
biological responses [14]. In other words, in the “forward” approach, the pathway goes from phenotype to protein, whereas in the “reverse” approach, the pathway is from protein to phenotype. New technologies have expanded reverse chemical genetics into *chemical genomics*; the systematic assaying of the relationships between a bioactive compound and individual gene products in the genome [13]. By isolating systematic gene mutants previously, one can bypass the linking of the phenotype to the corresponding gene. Such systematic gene deletion libraries are referred to as *gene deletion arrays (GDAs)* [15] or *gene knockout collections* [16].

### 2.3

#### Development of GDA Technology

In 1996, the *Saccharomyces* Genome Project identified ~6000 open reading frames (ORFs) [17] but more than 10 years later, 1000 of these remained uncharacterized [18]. The *Saccharomyces* Genome Deletion Project hoped to cross this knowledge gap through the systematic production of deletion mutants for every gene in the genome. The endeavor successfully disrupted >96% of these ORFs using a PCR-based strategy to replace each gene with a Kanamycin resistance cassette (KanMX) flanked by two unique barcode sequences causing a complete loss of function [19]. The method relied on homologous recombination to replace the endogenous genetic sequence with a marker, effectively deleting the gene. A PCR product containing a KanMX resistance marker flanked by a region homologous to the upstream and downstream sequence of the target gene is transformed into the yeast cell. These homologous regions bind the complementary sequence in the yeast genome. The yeast machinery then recognizes this homology and induces a crossover event resulting in gene conversion (Figure 2.1). The ultimate result was the production ~6000 heterozygous diploid and ~5000 viable haploid mutants [20].

Use of yeast as a model is beneficial because it can exist in either haploid or diploid form, allowing for one or two copies of an allele to be present. In its haploid



**Figure 2.1** Schematic of a homologous recombination event which replaces an endogenous ORF on the yeast chromosome with a PCR product containing a kanamycin resistance cassette (*kan<sup>r</sup>*) flanked by two barcode sequences (Uptag and Downtag)

inside two regions with sequence homology to the regions directly upstream and downstream of the target ORF. The PCR product will replace the target ORF, deleting the gene.

form, the disruption of one gene can abolish function entirely but in a diploid background, the gene dosage can be lowered from two copies to one. *S. cerevisiae* GDAs are available in four backgrounds, haploid (*S. cerevisiae* mating type a (MATa) or *S. cerevisiae* mating type  $\alpha$  (MAT $\alpha$ )), heterozygous diploid, or homozygous diploid. You can also use a collection containing only essential genes (heterozygous diploid background).

### 2.3.1

#### The Use of Gene Deletion Arrays (GDAs) to Investigate MOA

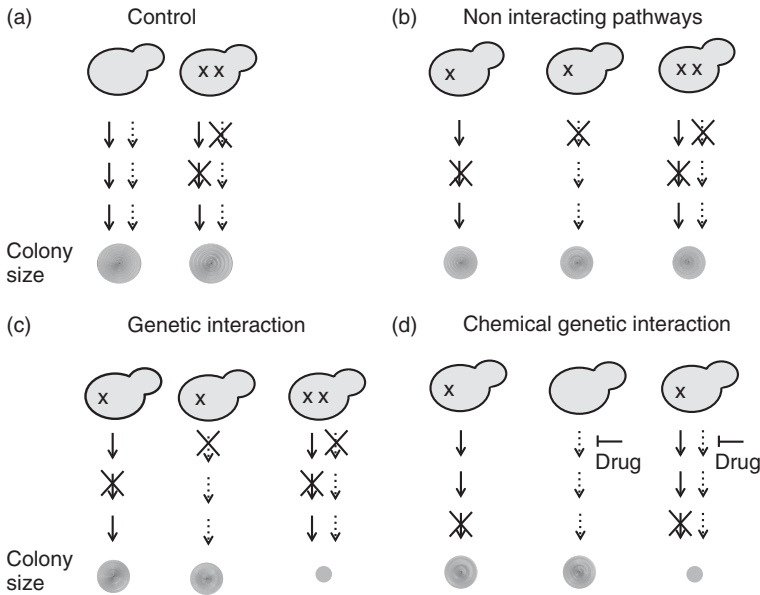
Deleting a gene can provide insights into the functional activity of the gene product. In this way, the sensitivity/resistance to biologically active compounds can be assessed by comparing the phenotype of a wild-type strain to a strain lacking a single gene (gene deletion) to determine whether the activity of the compound is influenced by the presence of the target gene. The theory behind GDA-based chemical genomic technology is that in the presence of inhibitory bioactive compounds, certain nonessential genes may become conditionally essential in the way that their deletion causes a new phenotype. The profiles obtained from such conditional sensitivities/essentialities phenotypes can help to elucidate an MOA activity for the target bioactive compound. GDA technology was pioneered in *S. cerevisiae* [16] and later expanded to *Escherichia coli* [21], *Bacillus subtilis* [22], *Acinetobacter baylyi* ADP1 [23], and *Candida albicans* [24] among others. GDAs constructed in well-characterized common laboratory strains can be utilized to readily screen the phenotypic response of different gene deletion mutants to a query compound. The potential of chemical genomics has only been recently recognized by pharmaceutical companies as a valuable tool for drug target identification/validation process [25]. They are an effective preliminary tool in the drug development process [26] because of their ability to examine drug sensitivity/resistance in a global (genome-wide) manner and identify functionally related pathways which help buffer the inhibitory effects of a compound on a target.

### 2.3.2

#### Chemical Genetic Interactions

When the products of two independent genes or pathways function in a similar manner or produce the same outcome, the absence of one gene may be compensated by the presence of the other in a bidirectional manner. In this context, the masking of the phenotype of a gene deletion by the phenotypic effects of a mutation at another locus is termed *epistasis* [27]. Epistasis is not limited to an interaction between two genes but can also include multiple loci interacting to create unexpected phenotypes [28]. A genetic interaction is defined as an unexpected phenotype resulting from a combination of mutations in relation to a single mutant's individual effects [29]. If the disruption of two genes results in





**Figure 2.2** Representation of genetic and chemical genetic interactions of in *S. cerevisiae* demonstrated using colony size as a phenotype on solid media. (a) Colony size in the wild-type strain and a noninteracting double-deletion strain are identical. (b) Single mutants of two genes in independent unrelated pathways have the same colony size as the double mutant. (c) A double mutant of genes in functionally related (parallel) pathways shows unexpected growth defects

(synthetic sick) or cell death via a negative genetic interaction. (d) Through a mechanism analogous to the genetic interaction in (c), the concurrent independent inhibition of functionally related (parallel) pathways by a single-gene deletion and the presence of an inhibitory chemical. An unexpected growth defect occurs when the single mutant is grown in the presence of an inhibitory compound via a negative chemical genetic interaction.

a double mutant, whose phenotype is significantly more profound/unexpected compared to the phenotype of either single mutant, a genetic interaction has occurred (Figure 2.2). In this way, genetic interactions can report functional relationships between genes and often demonstrate a higher degree of complex interactions between different pathways [30]. Genetic interactions can be broadly characterized as either positive (alleviating) or negative (aggravating). A negative genetic interaction results from two independent deletions each of which decreases fitness beyond the predicted multiplicative fitness of individual mutants through a reinforcing/synergistic/aggravating overlapping process [31].

Assaying for functional relationships across the entire complement of genes within a cell is a powerful tool for understanding the interconnectedness of a genome and can provide important clues as to how a cell functions as a system. A recent project examining global genetic interactions in *S. cerevisiae* identified 170 000 functional relationships involving 75% of the genome (~40 interaction

partners/gene) [32]. This not only validates the vast connectivity, plasticity, and redundancy apparent in numerous organisms [33, 34], it also demonstrates how compensatory genes act as buffers to perturbation and accentuates biological complexity. Only 307 (7%) of *E. coli* genes are essential for cell viability under normal laboratory growth conditions [35]. This functional buffering extends from single-cell *E. coli* to multicellular organisms including worms, mouse, and humans [36, 37]. Functional genomic redundancy may help to explain why affective monotherapeutic antibiotics almost never target the product of a single gene [38]. Such a buffering system can further complicate the study of the direct effects of bioactive compounds on specific gene pathways within a cell.

Chemical genetic relationships are a variation of genetic interactions where the inhibitory effects of a molecule on a specific physical target is considered theoretically analogous to a gene disruption. Instead of studying a combination of gene deletions, one can study the phenotype resulting from a single-deletion mutation and the chemical inhibition of a second physical target(s). The phenotype observed in these chemical genetic interactions is a synergistic combination of a gene disruption and an inhibitory compound acting on targets in parallel pathways (Figure 2.2). Assaying a single or double deletion(s) for sensitivity to a natural compound provides information on the pathways affected by the presence of a compound. Such profiles offer insights into inferring the potential MOA of the target compound. This approach can identify the direct targets of, resistance mechanism against, or mechanisms which compensate for the inhibition of an essential pathway by a target compound. It also provides valuable insight into the molecular complexity of microbes, knowledge which is essential when attempting to intelligently facilitate cell death [32].

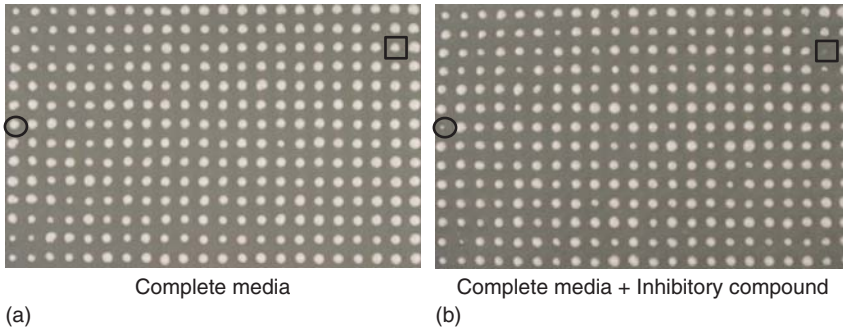
### 2.3.3

#### Quantifying Genetic and Chemical Genetic Interactions

Quantifying the phenotype produced by a known combination of two alleles or an allele and an inhibitory compound is crucial in understanding genetic and chemical interactions. GDAs depend on the ability to study fitness as a phenotype by scoring allele frequency in batch cultures or quantitatively examining growth rate in isogenic microbial cultures. Defining quantitative interactions requires two components: (i) a quantitative measure of a phenotype and (ii) a normalization function predicting the phenotype of strains possessing two noninteracting mutations [29]. If no genetic interaction exists between the two genes, the fitness of the double mutant does not vary from the fitness of either single-deletion mutant [31].

The various *S. cerevisiae* GDAs can use three primary high-throughput methods for performing chemical sensitivity profiling using fitness as a proxy [39]:

- 1) Individual strains can be grown in liquid media on microplates. Spectrophotometric analysis of the microcultured mutants compares growth curves with and without a query compound being present.



**Figure 2.3** Example of chemical genetic screening using a *S. cerevisiae* haploid GDA. (a) A subsection of haploid deletion strains growing on complete medium (yeast extract + peptone + dextrose; YPD). (b) The same deletions grown on complete

medium + a subinhibitory concentration of hydroxyurea. The square represents a chemical genetic interaction resulting in cell death while the circle represents an interaction resulting in a synthetic sick phenotype.

- 2) Individual strains can be grown on solid media. Fitness with and without a compound present are compared by analyzing relative colony size (Figure 2.3) [10].
- 3) Coculturing a pool of single-mutant strains in the presence of a compound and subsequent quantification of the relative abundance of each unique deletion as a proxy for fitness in this competitive environment. Microarray hybridization or direct sequencing of the unique barcodes quantifies the fitness of each mutant based on abundance [40].

#### 2.3.4

##### Data Analysis

Analyzing the size of colonies by visual inspection is a challenging task. Not only will small differences be missed but the approach is also not quantitative. Therefore, various software have been developed to analyze the colony size differences. These programs include Growth Detector [15], ScreenMill [41], and Plate Analyzer [42] all of which offer significant improvements over manual inspection in terms of both sensitivity and specificity. Growth Detector (GD) automatically acquires quantitative and comparative information for yeast colony growth. Through code written in MATLAB, GD identifies true yeast colonies from a digital image and provides an accurate coordinate-oriented map of the colony areas. This program compares the size and shape of colonies treated with various compounds to the untreated ones which are used as control. Since an inhibitory compound often causes a general reduction in average growth of all yeast colonies, the GD method also takes into account the average size of the colonies grown on each individual plate for each experimental condition. In this

way, the rate of false positives are significantly reduced. More recent methods are written in languages that are freely available for downloading, making them more accessible to most researchers [42]. SGAtools is the most recent algorithm developed for colony-size measurement [43]. It is a versatile and user-friendly web-based tool which simplifies colony size analysis.

Once a high-confidence chemical genetic profile of a compound is generated, the results can be mined for functional relationships within the affected genes [44]. Hits must be categorized to identify pathways/processes that exhibit enrichment in the data. The most enriched category often refers to the main (dominant) mechanism of activity of the inhibitory compound [39]. This can be accomplished using a variety of clustering techniques employing any number of parameters associated with the function of the affected genes and the cellular processes in which they participate. Genes can be clustered in a hierarchical manner by expression profile, essentiality, colocalization, gene function, and cellular process, just to name a few. Genetic interaction and protein–protein interaction data can provide valuable information when integrated with clustering tools. For example, it is expected that the deletion of the different members of a known protein complex would result in similar sensitivity/resistance to a target compound. For example, given a known protein complex, A–B–C–D, it is generally accepted that individual deletions of genes A, B, C, and D will result in a similar degree of sensitivity. If all members of the same protein complex are identified as hits in the chemical genomics analysis, a higher degree of confidence can be assigned. Similarly, the deletion of genes in the same pathway is expected to produce similar sensitivity profiles. Since genes in a given pathway are expected to form a genetic interaction with members of a compensating parallel pathway, combining genetic interaction data with chemical genomics data can be used as a powerful tool to predict the MOA of a bioactive compound.

Many compounds have multiple or controversial MOA and any number of potential targets. The clustering of chemical genomic data can identify additional functional target groups beyond the primary target which may or may not contribute to the observed phenotype. Identifying likely target pathways by clustering data from chemical genomics can help predict whether the side effects are due to effects on the primary target or other “off-target” effects [45]. Cross talk between unrelated processes may also be identified through clustering [46]. Gene ontology (GO) terms were developed so that precise terminology is available to annotate a gene on the basis of biological process, cell component, or molecular function across different species [47]. A number of software tools have been developed to perform GO analysis on gene lists but the information is more powerful if integrated with additional data from other sources. Accessible web-based tools can perform functional profiling and network integration to make sense of gene lists produced in large-scale experiments such as chemogenomic GDA screens. Notable examples include GeneMANIA (<http://www.genemania.org>), g:Profiler (<http://biit.cs.ut.ee/gprofiler/>), and Cytoscape (<http://www.cytoscape.org>).

GeneMANIA performs biological network integration between a gene list and networks generated from available genomic and proteomic data. It can be used to suggest gene function, illustrate relationships between genes and the data sets, identify other genes related to the list, or prioritize genes for secondary assays and spur hypothesis generation [48]. The chemogenetic profile generated by the target compound can be inputted into GeneMANIA as a gene list. Parameters can then be selected to adjust how data from public databases including co-expression data, physical and genetic interaction data, colocalization data, predicted protein–protein interaction data, and pathway and molecular interaction data will be integrated. The program can also expand the list with additional genes which share specified properties or functions with the inputted genes. For long lists, GeneMANIA “learns” from the data set and creates a network where genes on the list interact with each other as much as possible. For shorter lists, the program tries to reproduce GO process co-annotation patterns [48].

An alternative tool is g:Profiler, another publicly available web server, which characterizes gene lists and produces informative, visually appealing results. Benefits of g:Profiler include the ability to support ranked gene lists, recognition of multiple gene identifiers, and the ability to find orthologs and search for co-expressed genes from microarray databases [49]. Cytoscape offers a similar platform but is largely driven by a community of users and developers who help expand functionality through plug-ins, core improvements, and parallel versions [50]. The extensive information on yeast and *E. coli* contained in countless databases prove extremely beneficial when using analytical tools to infer meaningful relationships, decipher networks, and cluster measures of similarity to decipher likely MOA from the GDA profile.

Chemical genomic GDA analysis is prone to yield a false positive rate owing to both technical errors and the propensity to select for genes involved in general stress response or multidrug-resistant genes such as efflux pumps. Data from GDAs will not determine if a drug will be digested, degraded, or delivered as desired. They also will not account for potential metabolic degradation of the compound only possible in higher organisms. The concept of “drug likeness” refers to the potential of the compound to be orally administered [51] which cannot be predicted using GDA technology. For this reason, chemical genomic GDAs are good screening tools capable of providing information on likely MOA and potential side effects but do not adequately predict medical effectiveness or bioavailability.

### 2.3.5

#### Platforms for Chemical Genomic GDA Studies

The two most commonly used species in chemical genomics are the eukaryotic budding yeast *S. cerevisiae* and the prokaryotic bacterium *E. coli* whose single-mutant collections can be purchased (GE Dharmacon, Life Technologies) and maintained in standard level 1 laboratories with ease. Both *S. cerevisiae* and *E. coli*

strains grow quickly on solid or liquid culture, can be stored for long periods of time and transformed easily, and have multiple known selection markers which can be used in genetic manipulations. They also both possess relatively small genomes, among the first to be sequenced. Importantly, they are among the most extensively studied species making them ideal for use as model organisms.

There are inherent advantages and disadvantages of using *E. coli* over *S. cerevisiae* GDA and vice versa. The primary difference between the two species is that *E. coli* is prokaryotic while yeast is a eukaryote, albeit a relatively simple one. Yeast is also a member of the fungal kingdom and can act as a proxy for antifungal drug targets [52]. An ideal antimicrobial agent targets only the intended species and avoids unintended targets such as host tissue or agricultural crops. Yeast aids in helping to understand this specificity. Despite having a genome which is relatively smaller, yeast shares significant functional conservation with higher eukaryotes including humans [53]. This conservation can help identify potential off-target interactions (side effects) the compound may have on eukaryotic cells. *E. coli* on the other hand is useful as a representative of the prokaryotic domain and can offer information on antibacterial MOA. The *E. coli* GDA (Keio collection) has been useful in screening for both tolerance and susceptibility to common antibiotics [54].

Many MOA studies have been performed successfully using the yeast haploid GDA [44, 55] and there are variations of this platform which can prove useful when attempting to truly examine the genome globally. Homozygous mutant profiling (HOP) can be performed in yeast diploid strains to achieve a similar endpoint. However, both methods are limited to genes whose presence is not required for vitality on complete media (nonessential genes). Essential genes comprise 17% of the roughly 6000 ORFs in yeast [16] and represent the most basal of metabolic functions. An alternative to complete gene abolition would be the lowering of the gene dosage from two copies to one. In this way, essential genes can also be investigated. Sensitivity to a compound in diploid yeast heterozygous strain is known as a *haploinsufficiency*. In this approach, the product of a deleted gene is identified as a likely target [56]. Identification and quantification of chemically induced growth defects in a heterozygous deletion strain is known as chemogenomic *haploinsufficiency mutant profiling* (HIP). The combinatorial use of HIP/HOP screening can identify both essential gene products that are probable targets using HIP, and compensatory pathways which act to buffer the perturbation caused by the compound of interest using HOP. This information can comprehensively cover the potentially large number of pathways affected by the compound [57].

Following the success of the Genome Deletion Project, Datsenko and Wanner [58] developed a new and highly efficient method for directed inactivation of chromosomal genes in *E. coli* using a PCR-based transformation. This approach represented a simple and efficient method for gene deletion very similar to that in yeast [59]. It utilized the  $\lambda$ -Red recombinase system which enabled *E. coli* transformation with linear PCR products. Using this approach, the first set of *E. coli*

K-12 gene deletion mutants was generated containing precisely defined single-gene deletion strains for all 3985 nonessential genes in *E. coli* [60].

Working with *S. cerevisiae* or *E. coli* can provide beneficial and extensive information on gene/protein function, regulation, and interaction. Arguably, *S. cerevisiae* possesses the most characterized genome of any species [61]. This information has helped to make *S. cerevisiae* a proven model for understanding the effects of compounds on eukaryotic and fungal cells [52]. It was the first species to have a complete loss-of-function gene deletion set made and is the most widely utilized organism for chemical genomic studies. More is known about the *E. coli* K12 genome than any other prokaryote [62]. Genome-scale investigations of bacteria remain in their infancy [32] and need to be expanded to help better understanding of the unique interactions of other microbes. An underutilized approach is the systematic high-throughput production of mutants which can be screened for alterations to chemical sensitivity as a chemical genetic tool and should be expanded to other species of interest [20].

Knockdown is used in the transparent roundworm *C. elegans* to mediate gene silencing using RNA interference (RNAi). This reverse genetic approach is accomplished by delivery of dsRNA coding for the sense and antisense RNA corresponding to the target gene. Of interest is that *C. elegans* RNAi interference collection is maintained in an *E. coli* host/vector. *E. coli* housing a plasmid expressing RNAi for a target gene is introduced to laboratory strains of *C. elegans* through feeding, soaking, or microinjection [63]. The key advantage in using *C. elegans* is the ability to examine development, behavior, and tissue-specific effects, including the presence of inhibitory compounds, in this primitive multicellular organism grown under highly controlled conditions [64].

### 2.3.6

#### Why Screen Natural Products in GDAs?

In view of the rapid emergence of antibiotic-resistant microbial strains, there is an immediate – and in some cases desperate – need for new and efficacious antibiotics [65]. The potential epidemic spread of antibiotic-resistant bacterial infectious diseases poses a severe risk to global public health and has become a major area of focus for medical research [66]. New antibiotics which act on previously unexploited targets are vital in advancing the fight against the potentially pandemic spread of antibiotic-resistant bacteria [67] and can be successfully identified using chemical GDA technology. Identifying these new compounds with a desired MOA and entering them into an antibiotic development pipeline to become available when other options prove ineffective is necessary to outpace increased resistance. Novel compounds optimized for safe therapeutic use through medicinal chemistry and progressed through an efficient research program may establish unprecedented new classes of antibiotics in roughly 20 years [68].

The United States Food and Drug Administration approved 16 new antibiotics between 1983 and 1987 but 25 years later, only one new antibiotic gained approval between 2008 and 2012 [69]. Perhaps even more startling is the fact that of the 167 antibiotics in development in 2008, only 15 appear to act through a novel MOA [70]. More than 80 years after Alexander Fleming first reported on the effect of *Penicillium notatum* on bacterial colonies, a greater number of Americans die each year from hospital-acquired methicillin-resistant *Staphylococcus aureus* (MRSA) than from reported AIDS and tuberculosis cases combined [71]. Acquired and induced drug-resistant infectious disease causes the death of nearly 100 000 people per year in the United States [72].

Global GDA techniques are an optimal platform for identifying promising new inhibitory compounds isolated from natural sources. The ability of GDA-based chemical genomics to yield new targets has been doubted [65], but these technologies have yet to be exploited to their full potential and the data they have produced and will produce can be subject to any number of new methods for interpretation. The recent lack of promising leads in the antibiotic pipeline is due in large part to poor quality/quantity of isolates and lack of appropriate high-quality screens capable of predicting successful clinical outcomes in the early stages of study [72]. It is misleading to assume that chemical genomic tools have failed at the identification of novel antibacterial targets because we have yet to fully exploit both the potential of these technologies and the biodiversity of our planet.

Plants are a great source of unique natural inhibitory compounds because they must protect themselves from predators (including microbes) while being fundamentally immobile. Many plant metabolites produced by normal metabolic processes persist in plant cells acting as a chemical barrier to parasitic or microbial infectious disease attack. Of more than 350 000 identified plant species, each possessing ~500–800 metabolites, only between 5% and 10% have been investigated for bioactivity [73]. The constitutive presence of secondary metabolites (phytoanticipins) and the inducible presence of by-products from pathways under transcriptional or translational regulation (phytoalexins) function together to ward off threats. Both phytoanticipins and phytoalexins are widely studied as potential disease-controlling agents for plants and humans [74] but the bioactivity of phytoalexins may not appear in screens if not induced before extraction. There is still a lack of understanding about the MOA of the majority of plant-based antimicrobials [74, 75].

Many plant extracts are generally regarded as safe (GRAS) [12] and hence are already widely utilized in food production as additives for livestock feed [76], seed disinfectants [77], and preservatives [75]. Using compounds which are GRAS allows food producers to advertise their products as natural or antibiotic free, fueling the development of antimicrobials that are effective on a large scale but are also safe and natural [78]. There are many cases of known antimicrobial activity from natural products with no understanding of the underlying mechanisms. For example, the methanol extracts of 12 traditional Iranian medicinal plants used in



the treatment of gastrointestinal illness were tested against *Helicobacter pylori*, the primary etiologic organism in gastric peptic ulcer disease and a risk factor in gastric cancer [79]. Metronidazole-resistant *H. pylori* showed considerable antibiotic sensitivity with *Salvia metronidazole* extract, a very common folk medicine, exhibiting the strongest activity against this microbe [80]. The active components have not yet been identified and the MOA is not understood. Extracts from *Satureja hortensis* applied to lettuce and tomato seeds reduce the disease severity of common plant pathogens and increase seed germination, plant height, and root/shoot weights but again, through an ambiguous MOA [77]. Grupo Omega of Spain has commercialized a product composed of various citrus fruit extracts rich in citric acid and flavonoids known as BIOLL<sup>+</sup>® which can be used to supplement poultry and swine feed/drinking water to improve digestion and prevent pathogenic gastrointestinal bacterial infection. Studies on BIOLL<sup>+</sup>'s MOA using metabolic assays have been unable to definitively deduce a MOA [81].

The salicylates which include aspirin and the common topical antibiotic, salicylic acid, have been used in the treatment of disease for over 4000 years. First extracted from plant leaves, they continue to be invaluable topical treatments for many dermatological and gastrological diseases without a thorough understanding of the underlying MOA. Their demonstrations of antibiofilm and antibiotic metabolizing effects are not well understood [82]. The aforementioned are limited examples of the promising potential of naturally derived plant metabolites which are best studied using chemogenomic technologies in the early stages to suggest likely MOAs.

Global microbiota are also a plentiful source of understudied but naturally produced inhibitory compounds, which include peptides, polyketides, carbohydrates, sterols, terpenoids, and alkaloids [83]. A large number of secondary metabolites synthesized by microbes appear to enhance survival in niches where competition is present by eliminating other species [52]. A remarkable number of bioactive compounds are produced by bacteria and fungi, many of which possess medicinal properties useful in infection control, cancer treatment, and autoimmune disease therapy [83]. There has also been a growing emphasis on investigating the synthesis of microbial secondary metabolites for use as potential antibiotics [84].

### 2.3.7

#### Successful Applications of GDA Technology

A very recent effort utilized an optimized HIP/HOP platform in yeast to profile nearly 1800 bioactive compounds. This successful endeavor provided unique insights into pathways that are sensitive and resistant to both known and novel compounds [53]. Hypotheses generated by global two-way hierarchical clustering of profiles of this chemical library identified novel compounds with a similar MOA acting upon microtubules or vacuolar ATPases. The success of

this endeavor demonstrates how the unique chemical profile of a compound can drive hypothesis-driven experimentation and lead to MOA determination.

Chitosan has been acknowledged as an antifungal for over 30 years without its MOA being fully understood [85–87]. Screening ~4600 yeast haploid deletion mutants on a subinhibitory concentration of chitosan identified 107 sensitive mutants, 31% of which were involved in protein synthesis. Enrichment-guided secondary assays confirmed that both the newly identified inhibition of protein synthesis and previously identified minor membrane disruption contributed to chitosan antimicrobial activity [88].

The antifungal activity of eugenol has also been extensively studied. Like chitosan, disruption of the structural and functional integrity of the plasma membrane originally appeared to be eugenol's MOA [89–91]. Chemical genetic profiling using the *S. cerevisiae* haploid GDA identified 21 sensitive deletion mutants. The profile was enriched (13/21) for genes participating in the synthesis and cross-membrane transport of aromatic and branch-chained amino acids [10]. 10/13 sensitive mutants involved in aromatic amino acid biosynthesis showed poor growth in an auxotrophic supplementation assay. Secondary genetic and biochemical assays showed that eugenol interfered with dual-function amino acid permeases and expanded the understanding of the MOA of this compound, demonstrating the power of GDA analysis to identify multiple target pathways of an inhibitory compound.

Chemogenomic profiles expand beyond the primary target. Paromomycin is a known inhibitor of protein biosynthesis through its inhibitory binding to the ribosome. As expected, the majority of the 352 yeast mutants sensitive to paromomycin (134) are known protein synthesis genes but other sensitive genes are involved in metabolism (45), compartmentalization (28), DNA maintenance (21), transport (20), unknown (39), or other functions (38) [39]. These may represent minor (side effects) of paromomycin or novel links to protein synthesis such as the previously unannotated *ORF YBR261C* which was termed *translated associated element 1* (*Tae1*) following an investigation of this drug.

The bacterial metabolite 2,4-diacetylphloroglucinol (DAPG) exhibits broad-spectrum activity against bacteria and fungi [92, 93]. One hundred and eighty-one tolerant mutants were identified when screened on a subinhibitory concentration of DAPG using *S. cerevisiae* GDA. One mutant *ycd1Δ* showed increased tolerance through an apparently unique mechanism. This helped identify the disruption of the transmembrane protein gradient and subsequent loss of mitochondrial function as the principal MOAs of DAPG [52].

## 2.4

### Concluding Remarks

Global GDA technology provides a more feasible alternative to laborious metabolic pathway-specific assays and has the added advantage of working

on a global scale. The use of GDA technology to screen natural substances for intriguing inhibitory compounds can help probe the biological complexity of intracellular networks or identify leads for promising novel antimicrobials. GDA technology can identify direct target as well as off-target effects of a novel compound or expand our understanding of previously studied compounds. The use of well-characterized *S. cerevisiae* and *E. coli* GDA variations to screen for sensitivity/resistance directly correlated to gene function is a robust and proven global approach in recognizing the potentially vast interactions caused by compounds of interest. The power of this approach is increased through the integration of other biological network data using *in silico* tools.

Chemical genomic GDA technology should be expanded into additional platforms spanning a spectrum from multiple representatives in the prokaryotic domain all the way to higher eukaryotes including humans. Greater coverage will develop our knowledge of genetic interconnectedness and should be furthered. The mouse conditional knockout collection is progressing toward completion [94] as global chemical genetic analysis makes its way to mammalian cell lines. There is currently no heterozygous deletion collection in human cells but genome-wide RNAi techniques are being performed to screen for therapeutic targets in disease models [95–97]. It is important to perform cross-referencing between these various platforms to identify inhibitory compounds with strong antimicrobial activity and minimal off-target interactions with host tissues. A popular approach for characterizing compounds in the near future will be to identify targeted pathways in yeast and then model inferred pathways using RNAi in mammalian tissue with a subset of orthologous genes [98].

We anticipate increased large-scale RNAi (knockdown) libraries in mice and human tissue cultures to gain prevalence in the coming years [99] which when combined with data from simpler organisms may provide novel insights. Human disease phenotypes often do not map to molecular defects which may be detected in yeast models. For example, mouse angiogenesis genes map to yeast stress response and cell wall biogenesis genes [100]. The highly conserved Schwann-Bodia-Diamond syndrome protein (linked to bone marrow failure/leukemia predisposition in humans) was identified in yeast as a key activator of translation at ribosomes [101].

Crossing the threshold into genome-wide studies in human cells will be difficult because of sheer technical complexity but is further burgeoned by the ~4 million genetic variants and polymorphisms unique in each individual [102]. This makes the mapping of genetic interactions in simple model systems such as yeast a vital reference when attempting to enable experimental and comparative analysis of genetic interactions in higher organisms. Specifically, network hubs and genes involved in multiple genetic interactions appear to be highly conserved and capable of predicting hubs in more technically challenging environments [103]. The described methods all come with limitations which mean the integration of all multiple approaches will lead to a more thorough understanding of the

biological response to a compound and ultimately help us design more effective drugs by incorporating a broad understanding of MOA.

### Abbreviations

MOA	mechanism of action
GDA	gene deletion array
HIP	haploinsufficiency mutant profiling
HOP	homozygous mutant profiling
KanMX	kanamycin resistance cassette
MATa	<i>S. cerevisiae</i> mating type a
MAT $\alpha$	<i>S. cerevisiae</i> mating type $\alpha$
ORF	open reading frame
GRAS	generally regarded as safe
YPD	yeast extract + peptone + dextrose; yeast complete media
GD	growth detector software
GO	gene ontology
RNAi	RNA interference
MRSA	methicillin resistant <i>Staphylococcus aureus</i>
DAPG	2,4-diacetylphloroglucinol

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## 3 High-Throughput Drug Screening Based on Cancer Signaling in Natural Product Screening

*Xinxin Zhang, Yuping Du, and Jinbo Yang*

### 3.1

#### Introduction

Nature is an attractive source of new therapeutic candidates with a tremendous chemical diversity found in plants, animals, marine organisms, and microorganisms. For many living organisms, this chemical diversity reflects the impact of evolution in the selection and conservation of self-defense mechanisms that represent the strategies employed to repel or destroy predators. More than half of currently available drugs [1] are natural compounds or are related to them. This situation is accompanied by increasing interest in the search for new drugs [2, 3]. Despite changing strategies in research on natural products, encompassing concerned sample selection and collection, isolation techniques, structure elucidation, biological evaluation, semisynthesis, de-replication, biosynthesis, as well as optimization of downstream processing [4, 5], the rate of discovery of truly novel natural products has, however, actually decreased. The reasons for this fact are related to high costs and the time consuming nature of conventional programs in natural products research. This has led to the exploitation of modern high-throughput screening (HTS) to generate new drugs [6].

HTS typically refers to a process in which large numbers of chemicals are tested (i.e., screened) with high efficiency to identify biologically active small molecules as candidates for further validation in additional biological or pharmacological experiments. Following the advances in molecular biology, human genetics and functional genomics continue to produce increasing numbers of molecular targets available for therapeutic intervention. As a result, the pharmaceutical industry has made considerable capital investment in fueling rapid advances in HTS technologies in terms of automation, miniaturization, and assay methodology. Assay methodology can be divided broadly into two categories: biochemical assays and cell-based assays [7, 8]. Biochemical assays are target based and have been the mainstay of HTS campaigns in the pharmaceutical industry. Such *in vitro* assays include assessment of enzymatic activity (e.g., for kinases, proteases,

and transferases), receptor–ligand binding (e.g., for G protein-coupled receptors (GPCRs), ion channels, and nuclear receptors) or protein–protein interactions [9–15]. In contrast to biochemical target-based assays, many cell-based assays aim to identify modulators of a pathway of interest in the more physiological environment of a cell, complete with intact regulatory networks and feedback control mechanisms. Examples of cell-based assays include functional assays (e.g., second messenger mobilization after GPCR activation), reporter gene assays, and phenotypic assays for cellular processes (e.g., cell migration and cytokinesis) [16–21]. The steps of HTS commonly followed are choosing the target, then choosing the library, followed by assay methods and detection [22]. Continuous invention and improvement of methods, more potential targets, and larger chemical libraries have greatly stimulated adoption of HTS as the primary tool for the early stages of drug discovery.

On the basis of the aforementioned concept, we introduce an effective technology that allows HTS to search for new drugs from nature, which has rich resources. Once the goal is set, finding the targets and determining the significance of the drugs that are discovered will follow. In considering a target for drug discovery, one of the most important issues to be addressed is whether a target hypothetically associated with a disease necessarily represents an appropriate point for new drug intervention. Cancer represents one of the most severe health problems worldwide and the development of new anticancer drugs and more effective treatment strategies are areas of utmost importance in drug discovery and clinical therapy. During the course of tumor progression, cancer cells acquire a number of characteristic alterations. These include the capacities to proliferate independently of exogenous growth-promoting or growth-inhibitory signals, to invade surrounding tissues and metastasize to distant sites, to elicit an angiogenic response, and to evade mechanisms that limit cell proliferation, such as apoptosis and replicative senescence. These properties reflect alterations in the cellular signaling pathways that in normal cells control cell proliferation, motility, and survival [23]. On the basis of significant advances in cancer biology, much of the research in these areas is currently focused on cancer-specific mechanisms and the corresponding molecular targets [24]. Traditional cytotoxic drugs often have low selectivity and high toxicity because they play a major role in interfering DNA, RNA, and protein microtubules that are the shared components for a cell's survival. In contrast, in many signal transduction pathways there are such huge differences between normal cells and tumor cells or different types of tumor cells that the existence of these differences gives us a historic opportunity to find antitumor drugs with high selectivity, high efficiency, and low toxicity [25]. Signaling pathways such as EGFR (growth-factor-induced epidermal growth factor receptor), P13Ks (phosphatidylinositol 3-kinases), Wnt, and STAT (signal transducers and activators of transcription) signaling have always been associated with loss of control of cell cycle, apoptosis, growth, and survival [26], and at the end of the signal pathways are the transcription factors and DNA binding that activate the downstream gene

expression. Applications of signaling pathway approaches have had a significant impact on drug discovery and development, especially in the areas of tumorigenesis and metastasis [27]. This chapter aims to introduce some common cancer signals with their own drug-screening assays in HTS, especially in the screening and finding of natural products.

### 3.2

#### Cancer Signaling Pathways with Their Own Drug Screening Assays in HTS

##### 3.2.1

##### $\beta$ -Galactosidase Enzyme Complementation Assays for EGFR Signaling Drug Screening

EGFR signaling is essential for many normal morphogenic processes and involved in numerous additional cellular responses. Its aberrant activity plays a key role in the development and growth of tumor cells and have become a crucial class of targets for the development of small-molecule anticancer agents [28–30].

The phenomenon of enzyme complementation and its use in monitoring protein–protein interactions in bacterial and mammalian cells has been widely described. Alpha complementation is a naturally occurring process in bacteria and in engineered cells, and can also occur in eukaryotic cells. Two forms of alpha complementation have been used in HTS, in which interacting fragments complement each other with either low or high affinity. Low-affinity complementation, used to monitor protein–protein interactions, provides a robust screen for detection of EGFR inhibitors. High-affinity complementation provides the basis for several HTS assays, in which analyses, compounds such as cAMP (cyclic adenosine monophosphate) or IP3 (inositol trisphosphate), are detected in crude cell lysates [31]. As already reported, a lot of work has been done on EGFR inhibitors with the GlaxoSmithKline corporate compound in these assays [32, 33], a few screening processes with the natural products have been carried out, but natural product-based libraries have been used. So far, several mechanisms contributing improved local tumor control after radiation combined with EGFR inhibitors have been identified in preclinical studies. These include direct killing of cancer stem cells by EGFR inhibitors, cellular radiosensitization through modified signal transduction, inhibition of repair of DNA damage, reduced repopulation, and improved reoxygenation during fractionated radiotherapy [34, 35].

##### 3.2.2

##### Fluorescence Superquenching Assays for PI3Ks Signaling Drug Screening

PI3Ks are lipid kinases responsible for the phosphorylation of phosphatidylinositol on the D3 position of their inositol ring [36]. The family is made up of 14 enzymes that can be separated into four classes, of which classes I, II, and

III are lipid kinases and class IV are related protein kinases. The most studied are the class I PI3Ks, which are subdivided into class IA and class IB [37–41]. Over recent years, studies have established the central role of PI3K signaling in several cellular processes critical for cancer progression, including metabolism, growth, survival, and motility. Consequently, significant efforts have been made to generate inhibitors of the PI3K pathway to treat cancers.

Fluorescence superquenching of a conjugated polymer upon metal ion-mediated association of phosphorylated and dye-labeled substrates can be used to screen inhibitors for PI3K activities. Because of phosphorylation, the quencher, and polymer are brought into proximity, and fluorescent energy transfer occurs. The PI3K inhibitors can be divided into isoform-specific inhibitors or pan-PI3K inhibitors. Pan-PI3K inhibitors target all class I PI3K in cancers. Most of the small molecule PI3K inhibitors developed to date are ATP competitive inhibitors. Inhibitors and enhancers of PI3K activities were screened in these assays with an 84-kinase/phosphatase inhibitor library of Biomol [42]. The first-described PI3K inhibitors, which were considered as relatively specific, were the Wm (Wortmannin) series of products of the fungus *Talaromyces wortmannin*, that inhibit signal transduction pathways by forming a covalent complex with an active-site residue of PI3K, inhibiting PI3K activity [43, 44].

### 3.2.3

#### TOP Flash Reporter Gene Assays for Wnt Signaling Drug Screening

Cell signaling cascades activated by Wnt proteins (collectively, the Wnt signaling pathways) have been well conserved throughout evolution. In addition to regulating cellular processes including proliferation, differentiation, motility, and survival and/or apoptosis, the Wnt signaling pathways play key roles in embryonic development and maintenance of homeostasis in mature tissues. Among the described Wnt signaling pathways, the canonical pathway (Wnt/ $\beta$ -catenin pathway) and the noncanonical pathways (the planar cell polarity pathway, the Wnt/ $\text{Ca}^{2+}$  pathway, and the protein kinase A pathway), the Wnt/ $\beta$ -catenin signaling pathway is by far the best characterized [45–48].

In HTS, many of the cell-based assays rely on reporter gene technology that measure cell proliferation, toxicity, production of markers, motility, activation of specific signaling pathways, and changes in morphology [49]. They are based on the splicing of transcriptional control elements to a diversity of reporter genes (e.g., green fluorescent protein,  $\beta$ -galactosidase, and luciferases) and have been fruitfully used to monitor the cellular events associated with signal transduction and gene expression [50, 51]. The most-applied assay technology for HTS on Wnt/ $\beta$ -catenin signaling is the TOP flash reporter gene assay. The TOP flash reporter consists of a luciferase gene preceded by a minimal promoter and several TCF (T-cell factor)-binding sites [52]. A number of natural compounds have been identified as inhibitors and/or modulators of



Wnt/ $\beta$ -catenin signaling pathway such as nonsteroidal anti-inflammatory drugs, vitamins, and polyphenols [53]. Most of these are generally protein–protein interactions for which no or insufficient structural information is available and the structure–activity-relationship (SAR) studies are consequently problematic [54]. Inhibitors of the Wnt/ $\beta$ -catenin signaling pathway can be grouped into two classes, that is, small-molecule inhibitors and biologic inhibitors. Small-molecule inhibitors include existing drugs such as nonsteroidal anti-inflammatory drugs (NSAIDs). Biologic inhibitors include antibodies, RNA interference (RNAi), and recombinant proteins [55].

#### 3.2.4

#### Luciferase Reporter Gene Assays for STATs Signaling Drug Screening

STATs are a family of cytoplasmic proteins acting as signal messengers and transcription factors that participate in normal cellular responses to cytokines and growth factors. Frequently, however, abnormal activity of certain STAT family members, particularly STAT3 and STAT5, is associated with a wide variety of human malignancies, including hematologic, breast, head and neck, and prostate cancers. Application of molecular biology and pharmacology tools in disease-relevant models has confirmed STAT3 as having a causal role in oncogenesis and provided validation of STAT3 as a target for cancer drug discovery and therapeutic intervention [56–60].

Most of these cell-based assays based on reporter gene technology rely on the use luciferase from *Photinus pyralis*, which is by far the best-characterized bioluminescent protein. The most-applied assay technology for STAT signaling has been the luciferase reporter gene assay. There is a report of a novel natural product, brevilin A, which inhibits Janus Kinase activity, which is also among the achievements of our lab research [61]. With these methods, drugs may play roles in tyrosine or serine kinase as inhibitors, physiological protein modulators of STAT activation, modulation of phosphatases, disrupters of STAT dimerization, inhibitors of STAT translocation, direct blocking of STAT DNA-binding and transcriptional activity [62, 63].

### 3.3

#### Concluding Remarks

Over the past years, natural product-derived compounds have led to the discovery of many drugs to treat human diseases; these compounds are also an attractive source as well as foundation of synthetic drugs which can be considered as a big treasure in improving product libraries. With HTS assays, targeting cancer signaling pathways has a more significant impact on drug discovery and development., for example, the  $\beta$ -Galactosidase enzyme complementation assays

for EGFR signaling drug screening, fluorescence superquenching assays for PI3Ks signaling drug screening, TOP flash reporter gene assays for Wnt signaling drug screening and luciferase reporter gene assays for STATs signaling drug screening.

In the future, more attention should be focused on the selection of cancer molecular targets between generality and specificity, namely, on cell proliferation and survival that are peculiar to a specific type of tumor. Anticancer drug research with advanced HTS assays is expected to become a revolutionary technological advance. We should also pay more attention to the formation of a natural-based product compounds library.

### Abbreviations

HTS	high-throughput screening
GPCRs	G protein-coupled receptors
EGFR	growth-factor-induced epidermal growth factor receptor
PI3Ks	Phosphatidylinositol 3-kinases
STATs	Signal transducers and activators of transcription
cAMP	camp cyclic adenosine monophosphate
IP3	inositol trisphosphate
Wm	wortmannin
TCF	T-cell factor
SAR	structure–activity-relationship
NSAID	non-steroidal anti-inflammatory drugs
RNAi	RNA interference

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## 4

### Immunosuppressants: Remarkable Microbial Products

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#### 4.1

##### Introduction

Immunosuppressants are valuable drugs that greatly decrease the risks of rejection of a transplanted organ, protecting the new organ, and preserving its function [1]. These drugs act by blocking the recipient's immune system so that it is less likely to react against the transplanted organ. They are also employed to slow down the immune response in patients suffering from certain immune disorders. In addition to being used to prevent organ rejection, immunosuppressant drugs are also used to treat severe skin disorders such as psoriasis and other diseases such as rheumatoid arthritis, Crohn's disease (chronic inflammation of the digestive tract), and patchy hair loss (alopecia areata). Some of these conditions are termed *autoimmune* diseases, indicating that the immune system is reacting against the body itself. Many immunosuppressive drugs are produced by microorganisms; they were originally isolated as antifungal antibiotics [2]. A wide variety of immunosuppressants is available and each of them works in a different way. Immunosuppressants can be classified broadly according to their specific molecular mode of action. The main immunosuppressant drugs currently used include ascomycin (FR900520, immunomycin, FK520), sirolimus (rapamycin, Rapamune<sup>®</sup>), tacrolimus (FK506, Fujimycin, Prograf), cyclosporins (Neoral, Sandimmune, SangCya), mycophenolate (CellCept), azathioprine (Imuran), monoclonal antibodies, including basiliximab (Simulect), daclizumab (Zenpax), and muromonab (Orthoclone OKT3), and corticosteroids such as prednisolone (Deltasone, Orasone).

Cyclosporins act by binding to immunophilins, thereby inhibiting T-cell activation and preventing T-cells from attacking the transplanted organ, while azathioprine disrupts the synthesis of DNA and RNA and, therein, the process of cell division. Cyclosporin A is used in heart, liver, kidney, pancreas, bone marrow, and heart/lung transplantations. The neoral form of cyclosporin has been used to treat psoriasis, rheumatoid arthritis, multiple sclerosis, diabetes, and myasthenia

gravis. It has antiparasitic and antiviral activities and is active as an anti-HIV drug. However, it is cytotoxic, causing damage to the kidney, liver, and nerves, as well as causing hypertension, diabetes, and abnormal hair growth. Thus, drugs with fewer side effects, such as sirolimus, are used in combination with corticosteroids in kidney transplants. Corticosteroids suppress the inflammation associated with transplant rejection. Sirolimus is an immensely valuable drug, and is ever finding new uses in almost every sphere of medicine as research into its activity continues. Sirolimus and its derivatives (“rapamycins”) have been shown to have anticancer activities including prevention of angiogenesis. It is also used to treat patients with psoriasis and to coat cardiac stents used in angioplasty. Tacrolimus is used in liver and kidney transplants. However, it has some of the same aforementioned toxicities that cyclosporin A has.

Azathioprine (Imuran) is used not only to prevent organ rejection in kidney transplants but also in the treatment of rheumatoid arthritis. It has been used to treat chronic ulcerative colitis, although for this use it has proved to be of limited value.

Most patients are prescribed a combination of drugs after their transplant, one from each of the aforementioned main groups; for example, they may be given a combination of cyclosporin, azathioprine, and prednisolone. Over a period of time, the dose of each drug and the number of drugs taken may be reduced as the risk of rejection decreases. Most transplant patients, however, will need to take at least one immunosuppressive medication for the rest of their lives.

Monoclonal antibodies act by inhibiting the binding of interleukin-2 (IL-2), which in turn slows down the production of T-cells in the patient’s immune system. Some, such as basiliximab (Simulect) and daclizumab (Zenapax), are also used in combination with cyclosporin and corticosteroids in kidney transplants. Muromonab CD3 (Orthoclone OKT3) is used along with cyclosporin in kidney, liver, and heart transplants.

The focus of this article shall be on the immunosuppressants that act on immunophilins, such as cyclosporin, tacrolimus, sirolimus, and ascomycin.

## 4.2

### Discovery

A variety of immunosuppressants are produced by microorganisms and were first isolated as antibiotics. For example, sirolimus, tacrolimus, ascomycin, and cyclosporin A were discovered as antifungal antibiotics. They found their medical niche, however, as immunosuppressants and are responsible for the success of the organ transplantation field. The antifungal and immunosuppressive activities may be unrelated as non-immunosuppressive derivatives have been obtained with good antifungal activity [3].

For years, the only natural product used as an immunosuppressant was the peptide antibiotic cyclosporin A. It was originally developed as a narrow-spectrum



antifungal agent in 1970 by Sandoz Pharmaceutical Co. It is produced by the fungus *Beauveria nivea* (also known as *Tolypocladium nivenum* and previously as *Tolypocladium inflatum*) [4] and *Trichoderma polysporum* [5]. Discovery of the immunosuppressive activity of cyclosporin A led to its use in heart, liver, and kidney transplants. Cyclosporin A suffers from various side effects such as kidney, nerve and liver damage, hypertension, diabetes, and abnormal hair growth. Although cyclosporin A had been the only product on the market for many years, the more potent compounds, sirolimus, tacrolimus, and ascomycin, produced by actinomycetes, provided new opportunities [6–8]. Sirolimus [9] and the independently discovered tacrolimus [10] are both narrow-spectrum polyketide antifungal agents, which are 100-fold more potent than cyclosporin A as immunosuppressants and less toxic [11]. The annual market for cyclosporin A has reached \$1.5 billion.

The discovery of sirolimus has an interesting history. A name that needs to be in the forefront when discussing the discovery of sirolimus is that of Surendra Nath Sehgal [12], who started working in Claude Vézina's microbiology department at the Ayerst Canada laboratory in Montreal, in 1959. In 1964, a Canadian scientific expedition traveled to Easter Island (Rapa Nui) to gather plant and soil samples. The expedition shared the soil samples with the Ayerst microbiology team. In 1972, they isolated a new compound produced by the actinomycete *Streptomyces hygroscopicus* that exhibited potent activity against the pathogenic yeast, *Candida albicans*, and other yeasts. In 1975, the discovery of sirolimus as an antifungal antibiotic was published [9]. Also published were data regarding its fermentation, isolation, and characterization [13] and the evaluation of its activity [14]. The compound was found to be active *in vivo* in mice and rats but was toxic in dogs. Ayerst workers also discovered that the compound suppressed the immune system, thus explaining dog toxicity as dogs are supersensitive to immunosuppressive drugs. In addition to his research on the compound, Sehgal was in charge of the pilot manufacture of sirolimus and sent a sample of the drug to the National Cancer Institute for testing where it was discovered that the drug had activity against solid tumors. The US patent for sirolimus was awarded in 1975 [15–17]. Owing to a different mode of action, sirolimus has advantages over cyclosporin A and tacrolimus [18, 19]. Sirolimus is 100-fold more potent than cyclosporin as an immunosuppressant and is less toxic. It does not exhibit the nephrotoxicity of cyclosporin A and is synergistic with cyclosporin or tacrolimus in immunosuppressive action. By combining sirolimus with either, kidney toxicity is markedly reduced. Sirolimus was approved by FDA in 1999 as an immunosuppressant.

Ascomycins (immunomycins) were discovered earlier than the structurally related sirolimus and tacrolimus. They act as immunosuppressants, antitumor agents, and antifungals. Ascomycin was first isolated in 1962, 25 years before the isolation of tacrolimus, as an antifungal antibiotic of unknown structure. It was produced by and isolated from *S. hygroscopicus* subsp. *ascomyceticus* (ATCC

14891, MA6475) [20, 21]. Later, screening programs for novel immunosuppressants resulted in the isolation of the ascomycin derivatives immunomycin and FK520 from *S. hygroscopicus* subsp. *ascomyceticus* and subsp. *yakushiaensis* (FERM BP-928, MA6531), respectively [22, 23]. It has since been confirmed that ascomycin, FK520, and immunomycin are structurally identical [24–26]. Although ascomycins never were commercialized as immunosuppressants, they are used for inflammatory skin diseases (see Section 4.7).

Tacrolimus was first discovered in 1984 by scientists at the Fujisawa Pharmaceutical Co. while screening a multitude of compounds for antibacterial activity [10, 27]. Tacrolimus is a macrolide produced by *Streptomyces tsukubaensis* 9993, which also produces ascomycin as a coproduct [8]. This bacterium is found in the soil near Tsukuba, Japan, and its taxonomy was described [20, 26]. Tacrolimus was almost abandoned when initial animal studies showed dose-related toxicity. However, Thomas Starzyl of the University of Pittsburgh, realizing that the immunosuppressant was 30–100 times more active than cyclosporin, tried lower doses, which proved to be very effective and nontoxic. This discovery saved the drug as well as many patients, especially those that were not responding to cyclosporin [19]. It was approved in Japan in 1993 and in the United States by the FDA in 1994 to prevent graft rejection in patients undergoing liver transplantation [28, 29]. It was later extended to other organ transplants (kidney, heart, pancreas, lung, and intestine) and for the prevention of graft-versus-host disease. Tacrolimus had a market of \$2 billion in 2007.

A very old broad-spectrum antibiotic, actually the first antibiotic ever discovered, is mycophenolic acid, which has an amazing history. The unsung hero of the story is Bartolomeo Gosio (1863–1944), an Italian physician who discovered the compound in 1893 [30]. Gosio isolated a fungus from spoiled corn which he named *Penicillium glaucum* and was later reclassified as *Penicillium brevicompactum*. He isolated crystals of the compound from culture filtrates in 1896 and found it to inhibit growth of *Bacillus anthracis*. This was the first time an antibiotic had been crystallized and the first time that a pure compound had ever been shown to have antibiotic activity. The work was forgotten, but the compound was fortunately rediscovered by Alsberg and Black [31] and given the name mycophenolic acid. They used a strain originally isolated from spoiled corn in Italy called *Penicillium stoloniferum*, a synonym of *P. brevicompactum*. The chemical structure was elucidated many years later by Raistrick and coworkers in England [32]. Mycophenolic acid has antibacterial, antifungal, antiviral, antitumor, antipsoriasis, and immunosuppressive activities. It was never commercialized as an antibiotic because of its toxicity, but its 2-morpholinoethylester was approved as a new immunosuppressant for kidney transplantation in 1995 and for heart transplants in 1998 [33]. The ester is called *mycophenolate mofetil* (CellCept) and is a prodrug which is hydrolyzed to mycophenolic acid in the body. It is sometimes used along with cyclosporin in kidney, liver, and heart transplants. Mycophenolic acid also appears to have antiangiogenic activity [34].

In addition to the aforementioned compounds, red pigments called *prodigiosins*, produced by *Serratia marcescens*, have immunosuppressive and anticancer activities [35]. Pigments produced by fungi of the genus *Monascus* also have immunosuppressive activity in addition to their antibiotic and hypotensive properties [36].

### 4.3

#### Mode of Action

Studies on the mode of action of the immunosuppressive agents have markedly enhanced our knowledge of T-cell activation and proliferation [37]. Immunosuppressants sirolimus, tacrolimus, ascomycin, and cyclosporin A act intracellularly by binding to intracellular enzymatic proteins known as *immunophilins*, which are nonessential for life. These enzymes are rotamases (*cis-trans* peptidylprolyl isomerases) allowing them to act as protein foldases. As one would expect from the structural similarity of tacrolimus and sirolimus, they bind to the same enzyme, that is, FK506-binding protein 12 (FKBP12) [38]. So does ascomycin, whereas cyclosporin A binds to a different enzyme, cyclophilin [39]. Once the immunosuppressant binds to its immunophilin, the enzyme becomes inactive, but the novel immunosuppressant-immunophilin complex inhibits the action of other enzymes involved in signal transduction pathways of lymphocyte activation which normally lead to activation of T-cells [40–42].

The targets of immunosuppressants are highly conserved from microbial eukaryotes to humans [43]. In humans, the signal transduction pathway is required for the activation of T-cells. When these immunosuppressants enter cells, they form complexes with their immunophilins and can inhibit the protein-folding ability of the latter's prolyl isomerase. More importantly, tacrolimus binds to a receptor known as *FKBP12*. Both the tacrolimus-FKBP12 complex and the cyclosporin A-cyclophilin complex inhibit calcineurin, a serine-threonine-specific protein phosphatase which is normally activated by calmodulin in response to increases in intracellular  $\text{Ca}^{2+}$ . Inhibition of calcineurin results in T-cells which cannot respond to antigen presentation, cannot transcribe the IL-2 gene, and cannot undergo IL-2-dependent proliferation [10].

Sirolimus combined with its immunophilin (FKBP12) acts at a later step in T-cell activation and inhibits mTOR (mammalian target of rapamycin) which is a member of a family of lipid/protein kinases and part of the sirolimus-sensitive signal transduction pathway. The mTOR kinase normally transduces growth-promoting signals that are sent in response to nutrients, for example, amino acids and growth factors to regulate translation, transcription, and cell cycle progression [44]. mTOR has phosphatidylinositol kinase activity which is involved in cell cycle regulation. Thus, sirolimus interferes with growth factor-mediated signaling in the G1 to S phases of the eukaryotic cell cycle, a step later than that inhibited by cyclosporin or tacrolimus [45]. TOR (target of rapamycin) proteins of yeasts and

mammals share sequence similarity to protein and lipid kinases, although their predominant activity is thought to be that of phosphatidylinositol kinase, and TORs might be unusual protein kinases [46]. TORs respond to nitrogen sources and other growth factors to regulate translation, transcription, and cell cycle progression [44]. The sirolimus-FKBP12 complex prevents the IL-2 from stimulating T-cell proliferation. It inhibits FRAP (FK506-binding protein 12-*rapamycin*-associated protein, also known as *FK506-binding protein* sirolimus-associated protein), which is a human phosphatidylinositol kinase. FRAP is also known as RAFT (*rapamycin* and FKBP12 target), mTOR, SEP, RAPT1, and P210 [47, 48]. These homologs of FRAP exist in yeast and mammalian cells. FRAP is inhibited by sequestration via formation of a ternary complex with FKBP12-sirolimus. The FKBP12-sirolimus complex also inhibits other enzymes of signal transduction pathways which are normally involved in T-cell activation. The complex inhibits a unique regulation path utilized by lymphocytes in responding to several cytokines.

The sensitivity of yeasts to cyclosporin A and tacrolimus is due to the need for calcineurin to promote yeast survival during cation stress. Thus, the inhibition of calcineurin by the cyclosporin A-cyclophilin complex or by the tacrolimus-FKBP12 complex is responsible for antifungal activity [49].

Having a different mode of action, sirolimus has advantages over cyclosporin A and tacrolimus [15]. Sirolimus and its less immunosuppressive analogs are effective against *C. albicans* and *Candida neoformans* via FKBP12-dependent inhibition of TOR kinases [50]. These enzymes are necessary for stationary phase entry, expression of ribosomal protein genes, nitrogen catabolite repression, and translation.

Since TOR proteins are involved negatively in nutrient repression, addition of sirolimus induces certain yeast genes whose transcription is repressed by nitrogen abundance, glucose abundance, and also genes involved in the diauxic shift [51–53].

In *Cryptococcus neoformans*, calcineurin is needed for virulence. Other filamentous fungi inhibitable by cyclosporin and tacrolimus are *Coccidioides immitis*, *Aspergillus niger*, *Aspergillus fumigatus*, and *Neurospora crassa*, suggesting that calcineurin may be necessary for viability in these species. Two non-immunosuppressive analogs of cyclosporin are active against *C. neoformans* [3]. Similar to the immunosuppressive cyclosporin, they act by binding to cyclophilin A and inhibiting the action of the fungal calcineurin. A non-immunosuppressive tacrolimus derivative, a C18 hydroxy C21 ethyl analog called *L-685,818*, inhibits *C. neoformans* by inhibition of calcineurin. It is non-immunosuppressive because its combination with human immunophilin (human FKBP12) does not inhibit vertebrate calcineurin, but when used in combination with fungal FKBP12, it does [54, 55]. Thus, it is possible to exploit subtle differences in the structures of human and fungal FKBP12 [56]. Cyclosporin A and tacrolimus inhibit virulence of *C. neoformans* by inhibiting growth at 37 °C but not at 24 °C [57–59]. Calcineurin action is required for growth at 37 °C and is inhibited by the drugs complexed to their binding proteins.

*Saccharomyces cerevisiae* produces at least eight cyclophilins and four FKBP s which are the target of cyclosporin A, tacrolimus, and sirolimus, but none are essential for life [60]. However, the absence of some is associated with slow growth. The main site for inhibition of yeast growth by cyclosporin A and tacrolimus is the inhibition of calcineurin which is essential for stress responses or viability in yeast, whereas growth inhibition by sirolimus is due to the inhibition of TOR2, an essential phosphatidyl 3-kinase required for progression from G1 to S phase in the yeast cell cycle [61].

Sirolimus has been the basis for chemical modification to yield clinically important products such as everolimus, temsirolimus (CCI-779), and deforolimus (A23573). A review on the use of sirolimus analogs and other small molecules to control mammalian gene expression is that of Clackson [61]. The sirolimus-producing *S. hygroscopicus* ATCC 29253 culture also produces nigericin and another polyketide [62, 63] which is evidently elaiophylin. This second type-I PKS (polyketide synthase) gene cluster was isolated and found not to be involved in production of sirolimus or nigericin [63, 64].

## 4.4

### Biosynthesis

#### 4.4.1

#### Acetate, Propionate, Butyrate, Methionine, and Valine as Precursors of the Macrolide Rings of Sirolimus, Ascomycin, and Tacrolimus

The structure of sirolimus was confirmed by X-ray crystallography [64], chemical degradation, and high-field nuclear magnetic resonance spectroscopy studies [65]. The major structural feature is the very large 31-membered macrolide ring containing three conjugated double bonds (Figure 4.1). Ascomycin is a novel 23-membered macrolide lactone with many structural similarities to sirolimus. In fact, with the exception of the differing oxidation state at C27–C28 and a methoxy substituent at C13, the ascomycin structure extending from the substituted furan ring through to the  $\alpha$ -ketoamide functionality and heterocyclic system to the substituted cyclohexane ring is identical to sirolimus. Both *S. hygroscopicus* subsp. *yakushiaensis* and subsp. *ascomyceticus* produce a minor ascomycin analog (FK523, L-683795) that has a methyl, rather than ethyl, side chain at C21 (Figure 4.2) [22, 23, 40–42].

The large 23-membered ring of the ascomycin family and the 31-membered ring of sirolimus are similar to many macrolide antibiotics [66]. The biosynthetic precursors of the carbon backbone of macrolide antibiotics, which include compounds such as erythromycin and tylosin, have been shown to be acetate, propionate, and occasionally butyrate [67]. Considerable biochemical and genetic evidence has been obtained to show that such processes occur with the

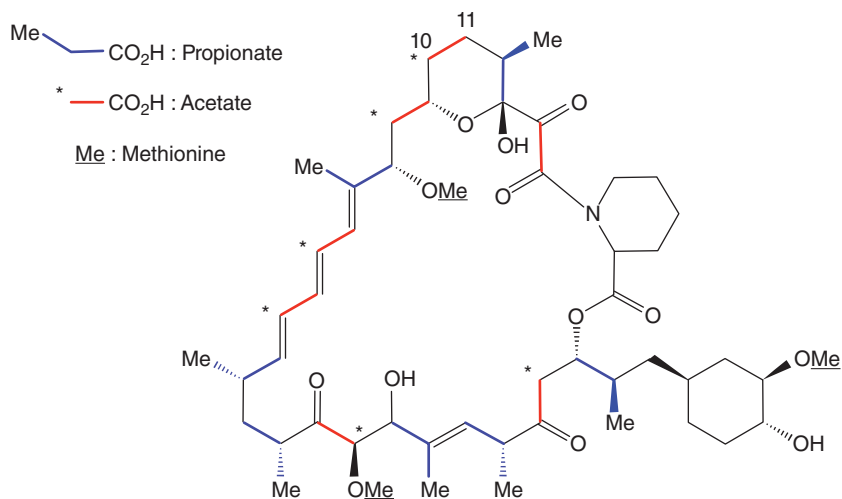


Figure 4.1 Origin of the carbon backbone of sirolimus.

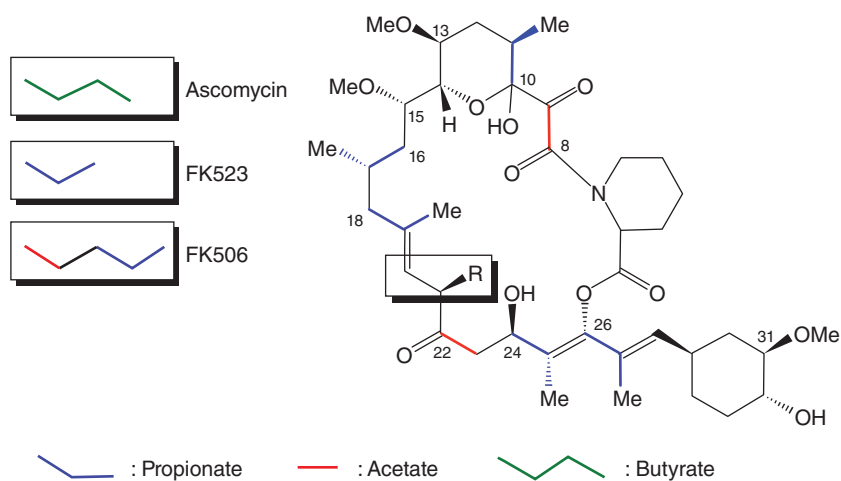


Figure 4.2 Origin of the carbon backbone of ascomycin, tacrolimus (FK506), and FK523.

condensation of these precursors, activated as malonyl-CoA, methylmalonyl-CoA, and ethylmalonyl-CoA, in a sequential manner with a specific primer unit in a manner analogous in some ways to fatty acid biosynthesis. Tacrolimus differs in structure from sirolimus and ascomycin by the presence of an allyl group derived from allylmalonyl-CoA [68]. Biosynthesis of this group involves a discrete PKS acting in coordination with the fatty acid synthase (FAS) of the tacrolimus-producing strain.

The origin of the macrolide ring of sirolimus has been investigated using  $^{13}\text{C}$ -labeled acetate and propionate [69–71]. Monitoring the isotopic enrichment in the  $^{13}\text{C}$  NMR of sirolimus following incorporation of  $[1-^{13}\text{C}_1]$ -,  $[2-^{13}\text{C}_1]$ -, and  $[1,2-^{13}\text{C}_2]$  acetate into producing cultures of *S. hygroscopicus* revealed both the location and orientation of six acetate-derived positions (Figure 4.1). Similar incorporation experiments with  $[1-^{13}\text{C}]$ propionate and  $[2-^{13}\text{C}]$ propionate revealed the location and orientation of the seven propionate-derived positions. The origin of the C10 and C11 in sirolimus remains a mystery, although the domain organization of its PKS clearly suggests they are derived from an acetate unit. Efficient incorporation of  $^{13}\text{CH}_3$  from L- $[^{13}\text{C}$ -methyl]methionine into all three methoxy groups of sirolimus was also observed.

Tacrolimus biosynthesis involves the PKS-catalyzed condensation of 4,5-dihydroxycyclohex-1-enecarboxylic acid (a shikimic acid-derived starter unit) with 10 extender units. The extender units are two malonyl-CoAs, five methylmalonyl-CoAs, two methoxymalonyl-ACPs, and one allylmalonyl-CoA. The condensation forms a nascent polyketide chain. Then, a lysine-derived pipercolic acid unit is incorporated, followed by a cyclization catalyzed by FkbP peptide synthetase, forming the earliest macrolactone intermediate. Finally, a specific methyltransferase and oxidoreductase act to form tacrolimus [72].

The five carbons (C20, C21, C35, C36, and C37) of tacrolimus are labeled by an acetate and a propionate [24–26]. Presumably, these two building blocks are condensed as CoA thioesters to form a pentanoate/pentenoate starter unit in a process analogous to butyryl-CoA formation. The resulting pentanoyl-CoA could be incorporated into tacrolimus either after initial oxidation to 4-pentenoyl-CoA or directly with the formation of the double bond occurring at a later step in the biosynthetic process.

Incorporation studies were conducted to examine the carbon backbone of the ascomycin family of immunosuppressants [24–26]. For all three of these compounds (ascomycin, FK523, and tacrolimus) the three methoxy carbons at C13, C15, and C31 were shown to be derived from methionine. All three immunosuppressants were enriched at C10, C16, C18, C24, and C26 by  $[1-^{13}\text{C}]$  propionate and at C8 and C22 by  $[1-^{13}\text{C}]$  acetate (Figure 4.2). Interestingly, in the acetate incorporation study, no  $^{13}\text{C}$  enrichment was observed at either the C12 of the furan ring (analogous to C11 in sirolimus) or at C14. The C12–C15 portion of the ascomycin family is derived from the unusual PKS extender unit methoxymalonyl acyl carrier protein (ACP) [72]. In FK523, carbons C20, C21, and C35 were shown to be derived from propionate. The four carbons of ascomycin at C20, C21, C35, and C36 were labeled by  $[1-^{13}\text{C}]$  acetate, although the enrichment at these positions was lower than at other acetate-derived positions, indicating a more direct butyrate precursor. This hypothesis was confirmed by incorporation experiments with  $[1-^{13}\text{C}]$  butyrate and  $[4-^{13}\text{C}]$  butyrate which led to efficient  $^{13}\text{C}$  enrichment at C20 and C36, respectively.

C21 was enriched by an incorporation experiment with [2-<sup>13</sup>C] D-L valine. These results are consistent with (i) the role of butyrate, presumably activated as ethylmalonyl-CoA, as the precursor to C20, C21, C35, and C36 of ascomycin and (ii) two alternate pathways for butyrate formation functioning in *Streptomyces*. The role of valine as a precursor to butyrate units in streptomycete metabolism has been seen in the biosynthesis of many secondary metabolites, including tylosin and monensin [73, 74]. In such a pathway, the valine is thought to be degraded to isobutyryl-CoA which is first isomerized to *n*-butyryl-CoA before being converted to ethylmalonyl-CoA by propionyl-CoA carboxylase or butyryl-CoA carboxylase [72]. The formation of butyrate from two acetyl-CoA molecules has not been fully addressed, although it seems to be an operational pathway in many streptomycetes. It is possible that two acetyl-CoA molecules are condensed together to form an acetoacetyl-CoA molecule which is converted to hydroxybutyryl-CoA and subsequently to crotonyl-CoA by three enzymes usually associated with the  $\beta$ -oxidation of fatty acids [75, 76]. An NAD(P)H-dependent reductase could then catalyze the conversion of crotonyl-CoA to butyryl-CoA. It is reasonable to consider that *S. hygroscopicus* subsp. *ascomyceticus* may also have a crotonyl-CoA reductase responsible for formation of the butyrylate-derived unit of ascomycin from two acetyl-CoA molecules. Cell-free extracts of *S. hygroscopicus* subsp. *ascomyceticus* have been shown to exhibit crotonyl-CoA reductase activity [77]. Furthermore, a 348 base-pair fragment of *S. hygroscopicus* subsp. *ascomyceticus* DNA was amplified using oligonucleotide primers based on the *Streptomyces collinus* crotonyl-CoA reductase sequence. The PCR product was cloned and sequenced in *Escherichia coli* and the predicted amino acid sequence was shown to be 88% identical to a predicted 116 amino acid residue fragment of the *S. collinus* crotonyl-CoA reductase [78]. Recent results by Alber and colleagues [79] suggest a subtle adjustment to this model. It has been shown that crotonyl-CoA reductase can catalyze the conversion of crotonyl-CoA to ethylmalonyl-CoA more efficiently than the conversion of crotonyl-CoA to butyryl-CoA. Therefore, a crotonyl-CoA reductase is actually a crotonyl-CoA carboxylase/reductase. It is difficult to predict which of the butyrate pathways plays a more significant role in ascomycin biosynthesis. The labeling of the butyrate unit (~2.5-fold enrichment) relative to the labeling of the positions directly obtained from acetate (~5-fold) in the acetate incorporation study, however, suggest that both pathways play a significant role in butyrate production.

#### 4.4.2

##### Pipecolate Moiety of the Macrolide Ring of Sirolimus, Ascomycin, and Tacrolimus

The macrolide structures also contains a heterocyclic moiety that can be isolated as L-pipecolate by either acid- or base-catalyzed hydrolysis. Early findings that pipecolic acid is a precursor of sirolimus and that its source is lysine [80] were followed up by a study on lysine cyclodeaminase and its purification. The enzyme



catalyzes the reaction converting lysine to pipercolic acid [81]. The ability of likely inhibitors of the enzyme to yield analogs of sirolimus was shown with ( $\pm$ ) thiozolidine-2-carboxylic acid (T2CA) [82]. Gatto *et al.* [81] showed that T2CA was more active than two compounds used by others, that is, (*S*)-(+)-nipecotic acid and (*R*)-(-)-nipecotic acid.

Pipercolic acid has been shown to be the direct precursor of the heterocyclic ring of both sirolimus [80] and the ascomycin family. Radioactive sirolimus was produced by incorporation of both L-[U- $^{14}$ C]lysine and DL-[U- $^3$ H] pipercolic acid in producing a culture of *S. hygroscopicus* (strain AY-B-1206) in a low-lysine fermentation medium [80]. Hydrolysis of the isolated sirolimus afforded radioactive pipercolic acid, clearly demonstrating that radioactivity had been incorporated into this portion of the compound. Unlabeled pipercolic acid reduced the incorporation of radioactive lysine more substantially than unlabeled lysine decreased the incorporation of radioactive pipercolate. These results provided evidence that pipercolic acid is a more direct precursor of the heterocyclic system of sirolimus than is lysine. A similar conclusion was reached regarding the formation of the heterocyclic moiety of ascomycin using an analogous set of competitive incorporation studies with labeled and unlabeled lysine and pipercolic acid [24–26]. The incorporation of DL[1- $^{13}$ C] lysine into ascomycin led to a single 80-fold enrichment at the resonance corresponding to C1, confirming the radioactive incorporation results.

On the basis of studies of pipercolate formation in other systems, a minimum of two alternate biosynthetic pathways for the conversion of lysine to pipercolate can be envisioned: (i) cyclization of lysine to form 1-piperidine-6-carboxylate occurring with retention of the  $\alpha$ -amino nitrogen atom and  $\epsilon$ -deamination (pathway a) and (ii) cyclization of lysine to 1-piperidine-2-carboxylate proceeding with retention of the  $\epsilon$ -amino nitrogen atom and elimination of the  $\alpha$ -deamination (pathway b). To distinguish between these possibilities, incorporation studies with ascomycin using DL-lysine- $\alpha$ - $^{15}$ N and DL-lysine- $\epsilon$ - $^{15}$ N were carried out [24–26]. Mass spectrometric analysis of the resulting ascomycin revealed a high level of  $^{15}$ N enrichment (49%) but only from the experiment utilizing DL-lysine- $\epsilon$ - $^{15}$ N. The level of enrichment was sufficiently high to give a measurable coupling to the natural abundance  $^{13}$ C signals for C2 and C6 of ascomycin, clearly indicating the location of the  $^{15}$ N. These results are consistent with a pathway from lysine to pipercolate involving loss of the  $\alpha$ -nitrogen atom.

The pipercolate is incorporated into the immunosuppressants by formation of an amide linkage with the acyl group of the growing polyketide chain. It has been suggested that pipercolate is activated in a manner described for non-ribosomally synthesized peptide antibiotics [83]. In these processes, the amino acid is generally activated as an acyl adenylate using ATP and subsequently transferred to a cysteine residue of the enzyme to form an amino acid thioester [84]. Peptide bond formation occurs by transfer of the activated amino acid on a phosphopantetheine arm to a second component of the enzyme. An enzyme likely responsible for activation of L-pipercolic acid as the adenylate derivative during the biosynthesis

of ascomycin was isolated, purified, and characterized from *S. hygroscopicus* subsp. *Ascomyceticus* [83]. This enzyme was active in an ATP-pyrophosphate exchange assay in the presence of pipecolate or pipecolate adenylate. In the presence of ATP and  $Mg^{2+}$ , the enzyme was able to bind pipecolate in a form that could be precipitated by trichloroacetic acid. This binding was inhibited by sulfhydryl group inhibitors. All of these observed properties are consistent with the activation of the carboxylate group of pipecolate, first as an adenylate derivative and subsequently, as an enzyme-bound thioester derivative. Antibody raised to the purified enzyme was used to follow the antigen during the course of fermentation. Maximal levels of antigen were observed when synthesis of ascomycin was maximal. A surprising 10 of 12 ascomycin-nonproducing mutants were shown to lack a detectable pipecolate-activating enzyme in Western blots. It was suggested that a genetic analysis of ascomycin biosynthesis might explain this unusually high number [83]. These data are strongly indicative of a role of this enzyme in activating pipecolic acid for ascomycin biosynthesis. The substrate specificity of the enzyme with a variety of amino acids was tested using the pyrophosphate-ATP exchange assay. Of the amino acids found in proteins, only L-proline was active. A variety of proline and pipecolate acid derivatives were found to be active with the enzyme. In other systems, it has been shown that reaction at the thiol site is often more specific than the exchange reaction [85]. Furthermore, many of the compounds active in the exchange reaction are poor inhibitors (with  $IC_{50}$  values in the 0.1–3 mM range) of the binding of pipecolate ( $K_m$  of 0.4  $\mu M$ ) to the enzyme. Accordingly, very high intracellular concentrations of these amino acids would be needed in a fermentation to produce an ascomycin analog. However, even with such caveats, a prolyl derivative of tacrolimus was reported to be a minor component in fermentation broths [86]. Furthermore, with whole-cell-directed biosynthesis experiments, high levels of proline have resulted in the formation of prolylascomycin (prolylimmunomycin) [83]. These results would suggest that there are further opportunities for obtaining novel immunosuppressants by precursor-directed biosynthesis experiments with proline and pipecolate analogs. Such an approach might be most productive either in a chemically defined medium supplied with minimal lysine or a mutant deficient in pipecolate production. Whether such an approach will generate an analog with greater activity is a separate issue. It has been noted that the prolyl analog of ascomycin is considerably less potent in immunosuppression than ascomycin [86]. Addition of proline to the ascomycin producer, *S. hygroscopicus* var. *ascomyceticus*, resulted in replacement of the pipecolate moiety by proline [87].

Proline was also effectively incorporated to yield prolyl derivatives of sirolimus and tacrolimus. Growth of a frameshift mutant (in *rapL*, yielding a nonproducing culture lacking L-lysine cyclodeaminase) of the sirolimus producer with L-*trans*-4-hydroxyproline yielded 4-hydroxypropyl-26-demethoxy-sirolimus and 4-hydroxyprolylsirolimus [88]. L-*cis*-4-Hydroxyproline and L-*cis*-3-hydroxyproline were also incorporated into new compounds. No new compounds were

produced from 3,4-dehydroproline and picolinic acid, whereas pyrrole-2-carboxylic acid inhibited growth. Addition of L-pipecolate brought sirolimus production to near wild-type levels. Activation of pipecolate analogs by the pipecolate-incorporating enzyme (PIE) is necessary, but not sufficient, for producing sirolimus analogs. The thiol site also exerts specificity. Furthermore, the analogs may not inhibit the binding of pipecolate to the enzyme.

Tacrolimus contains a six-membered nitrogen-containing heterocyclic ring. Pyridine and pyrimidine derivatives were tested for their effect on production because they contain such a structure [89]. Both picolinic acid (pyridine-2-carboxylic acid) and pipecolic acid stimulated production by *S. tsukubaensis* three- to sevenfold.

#### 4.4.3

#### The Final Step in Biosynthesis of Ascomycins and Tacrolimus

In sirolimus, interestingly, the cyclohexane ring of the starting unit is reduced during the transfer to module 1. The starting unit is then modified by a series of Claisen condensations with ACP-bound malonyl or methylmalonyl substrates, extending the polyketide by two carbons per each condensation. After each successive condensation, the growing polyketide is further modified according to enzymatic domains which are present to reduce and dehydrate the newly formed dicarbonyl compound, thereby introducing the diversity of functionalities observed in sirolimus (Figure 4.3). The biosynthesis of the sirolimus core is accomplished by a type I PKS in conjunction with a nonribosomal peptide synthetase (NRPS). The domains responsible for the biosynthesis of the linear polyketide of sirolimus are organized into three multienzymes, RapA, RapB, and RapC, which contain a total of 14 modules (Figure 4.3). The three multienzymes are organized such that the first four modules of polyketide chain elongation are in RapA, the following six modules for continued elongation are in RapB, and the final four modules to complete the biosynthesis of the linear polyketide are in RapC [90]. Once the linear polyketide is complete, L-pipecolic acid, synthesized by a lysine cycloamidase from L-lysine (see the preceding), is added to the terminal end of the polyketide by an NRPS, RapP, and then cyclized, yielding the unbound product, pre-sirolimus [91].

The core macrocycle, pre-sirolimus, is then modified (Figure 4.4) by an additional five enzymes, RapI, RapJ, RapM, RapN, and RapQ which lead to the final product, sirolimus. The core macrocycle is first modified by RapI, an S-adenosylmethionine (SAM)-dependent O-methyltransferase (MTase), which O-methylates at C39. Next, a carbonyl is installed at C9 by RapJ, a cytochrome P-450 monooxygenase (P-450), and RapM, another MTase, O-methylates at the C16 position. Finally, RapN, another P-450, installs a hydroxyl at C27 and O-methylation by RapQ, a distinct MTase, at C27 immediately follows yielding sirolimus [92].

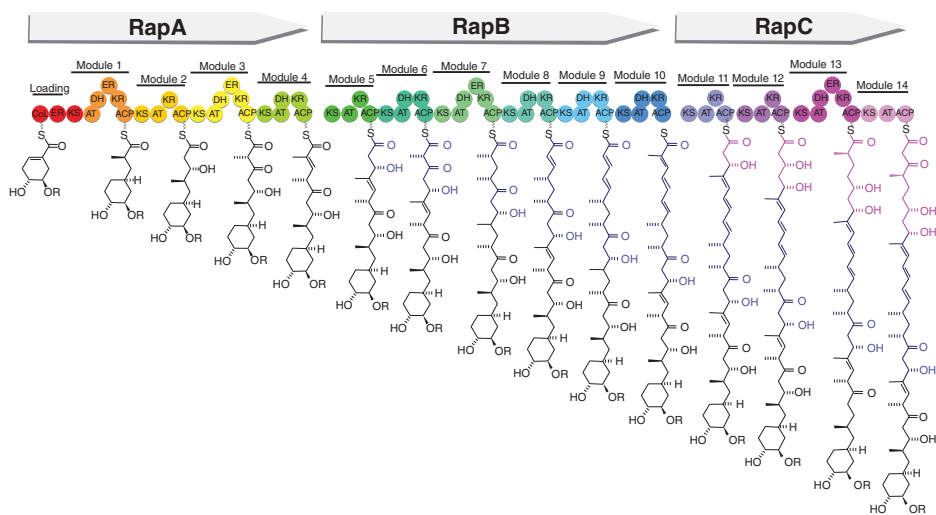
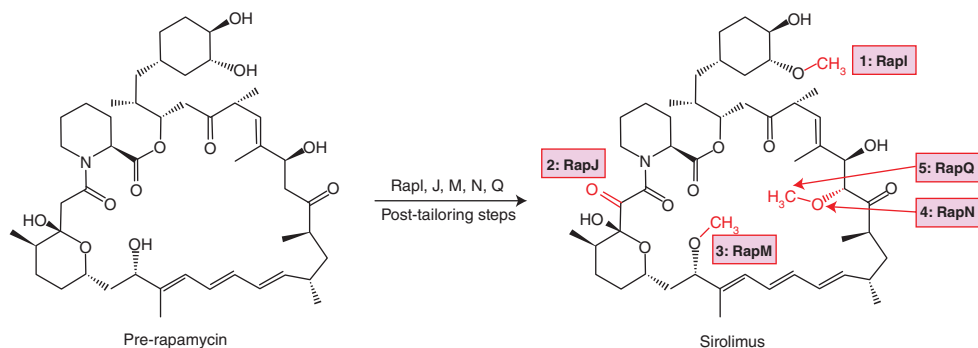


Figure 4.3 Domain organization of the PKS of sirolimus and biosynthetic intermediates.



**Figure 4.4** Pre-rapamycin, unbound product of PKS and NRPS, and the sequence of "tailoring" steps which convert unbound pre-rapamycin into sirolimus.

A methyl transferase (FKMT) catalyzing the conversion of 31-*O*-desmethyltacrolimus to tacrolimus has been purified 600-fold with an overall 3% purification from *Streptomyces* sp. MA6858 (ATCC No. 55098) [93]. The 31-*O*-desmethyltacrolimus was obtained by microbial transformation [94]. Similarly, a methyl transferase (DIMIT) capable of converting desmethylascomycin to ascomycin was purified 140-fold in 2.5% yield from *S. hygroscopicus* subsp. *Ascomyceticus* [24–26]. Both of these enzymes have an absolute requirement for magnesium ions and SAM. The enzymes have very similar physical and kinetic properties. The FKMT is able to convert 31-*O*-desmethylascomycin as effectively as it does for 31-*O*-desmethyltacrolimus. By comparison, only 10–20% efficiency has been reported for C31 methylation of the following compounds by either DIMIT or FKMT: 15,31-*O*-bis-desmethylascomycin, 13,31-*O*-bis-desmethylascomycin, and 13,15,31-*O*-tris-desmethylascomycin. A variety of 32-substituted-31-*O*-desmethylated ascomycin analogs were not substrates for either methyl transferase. Furthermore, the efficient conversion of 31-*O*-desmethylascomycin, in comparison to the bis- and tris-desmethylated compounds, would indicate that the methylation at C-31 is the last step in both ascomycin and tacrolimus biosyntheses. Finally, the disruption of the FKMT gene in a tacrolimus-producing strain has purportedly given rise to a system that produces only 31-*O*-desmethyltacrolimus, thus providing further evidence that this enzyme is the one involved in the final C31 methylation step of tacrolimus biosynthesis. Utilization of DIMIT and microbial desmethylation of both tacrolimus and ascomycin has allowed investigators to produce a whole series of desmethylated products [94, 95]. These compounds have all been tested for immunosuppressive activity in an *in vitro* proliferation assay. This work has clearly demonstrated that the methylation at C15 is critical for full biological activity of ascomycin.

The post-PKS modification steps of tacrolimus biosynthesis include C9 oxidation catalyzed by the cytochrome P450 hydroxylase FkbD and 31-*O*-methylation, but the sequence of these reactions and the exact route have remained unclear.

Recently, the substrate flexibility of two enzymes involved and the existence of two parallel biosynthetic routes to tacrolimus have been demonstrated through the identification of all intermediates and *in vitro* enzymatic reactions (Figure 4.5) [96].

#### 4.4.4

#### Formation of the Substituted Cyclohexyl Moiety of Sirolimus, Tacrolimus, and Ascomycins

Biosynthesis of the 31-membered macrocycle of tacrolimus, ascomycin, and sirolimus begins as the loading domain is primed with the starter unit, (1*R*,3*R*,4*R*)-dihydroxy-cyclohexanecarboxylic acid (DHCHC), which is derived from the shikimate pathway. The role of shikimic acid as a biosynthetic precursor to this unit has been investigated in both the sirolimus and ascomycin systems [24–26, 97].

The conclusion that methylation at C31 is the last step in tacrolimus and ascomycin biosynthesis implicates DHCHC as the putative primer unit for formation of these immunosuppressants including sirolimus. The role of shikimic acid as a biosynthetic precursor to this unit has been investigated in both the sirolimus and ascomycin processes [24–26, 97]. For the biosynthetic studies with sirolimus, shikimate with a 5- to 10-fold  $^{13}\text{C}$  enrichment at all carbons except C1 was obtained from [1- $^{13}\text{C}$ ] glucose feeding to a fermentation of an amino acid–auxotrophic mutant of *Klebsiella pneumoniae* [98]. When this compound was added to producing cultures of *S. hygroscopicus*, sirolimus enriched with  $^{13}\text{C}$  at C39 and C41–C45 was obtained. No other enrichments were observed, clearly indicating the role of shikimate in the formation of the DHCHC unit. Unfortunately, this experiment did not allow for the orientation of shikimic acid during incorporation into sirolimus to be clearly determined. However, on the basis of the stereochemical configuration of the sirolimus C42-hydroxy and C43-methoxy substituents, it was suggested that C5 of shikimate gives rise to C42 of sirolimus (Figure 4.6) [98].

The likely shikimate-derived origin of the substituted cyclohexyl ring of ascomycin has also been demonstrated. In this case, incorporation of D-[1- $^{13}\text{C}$ ] erythrose with *S. hygroscopicus* subsp. *ascomyceticus* produced ascomycin with a single major  $^{13}\text{C}$  enrichment at C31. This result is consistent with the pathway (Figure 4.6) in which erythrose is used to provide shikimate enriched with  $^{13}\text{C}$  at C5 which is subsequently utilized to provide the substituted cyclohexane ring of ascomycin. These incorporation studies demonstrate the shikimate origin of the cyclohexane moiety of the immunosuppressants but do not determine whether shikimate is incorporated directly or is modified first [98].

Evidence that shikimic acid is converted to DHCHC before incorporation into ascomycin has been obtained [99]. Two putative intermediates in this conversion to DHCHC, *trans*-4,5-[2- $^2\text{H}$ ]dihydroxycyclohex-1-enecarboxylic acid and *trans*-3,4-dihydroxy-[2,3,4,5,6- $^2\text{H}_5$ ]cyclohexa-1,5-dienecarboxylic

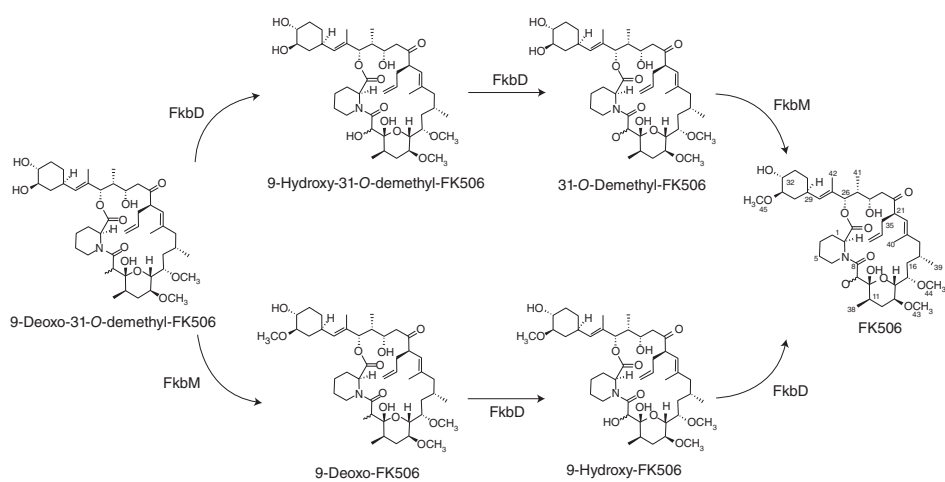
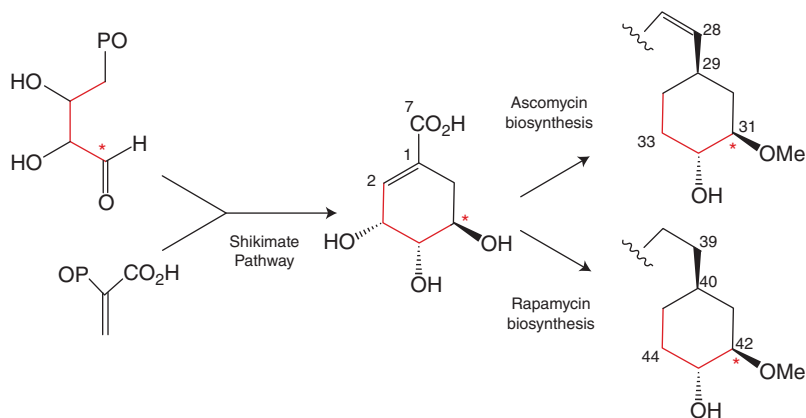


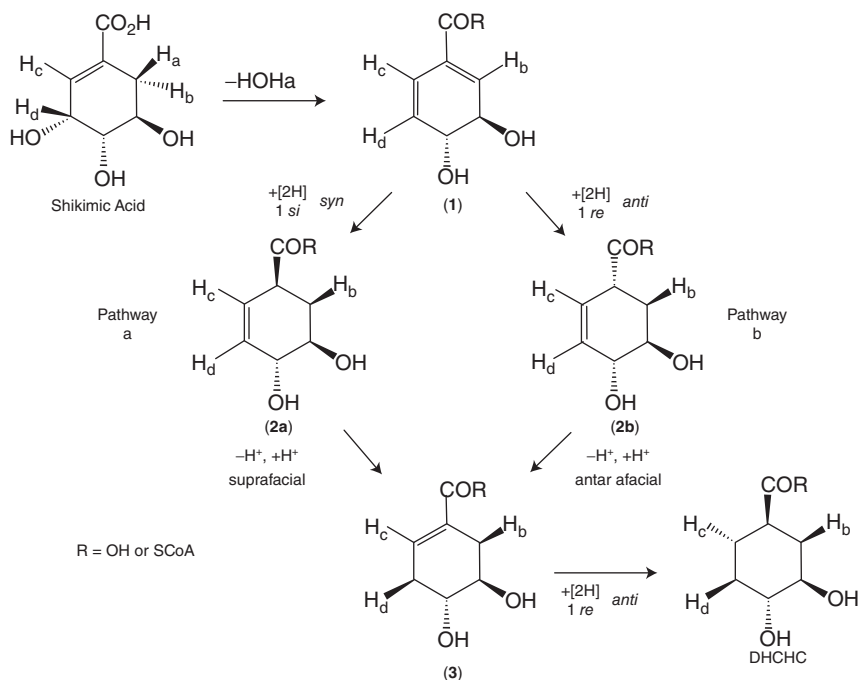
Figure 4.5 Post-PKS modification pathway for tacrolimus (FK506) biosynthesis.



**Figure 4.6** The shikimate origin of the substituted cyclohexyl moiety of sirolimus and ascomycin.

acid, were successfully incorporated into ascomycin by producing cultures of *S. hygroscopicus*. Furthermore, the fate of the hydrogen atoms attached to the ring of shikimate during this transformation was monitored by incorporation studies with regiospecifically deuterated shikimates. The regio- and stereospecific location of deuterium labels on the cyclohexyl ring of ascomycin produced in each of these studies was obtained by  $^2\text{H}$  NMR analysis. The results obtained were consistent with a proposed pathway (Figure 4.7) which commences with either a *syn*- or *anti*-1,4-conjugate elimination of the C3 hydroxyl group and a C6 hydrogen of shikimic acid to produce (3*R*,4*R*)-3,4-dihydroxycyclohexa-1,5-dienecarboxylic acid (compound **1** of Figure 4.7). A reduction of the  $\Delta^1$  double bond of this compound produces (4*R*,5*R*)-4,5-dihydroxycyclohex-2-enecarboxylic acid (**2a** or **2b**) which is subsequently converted to (4*R*,5*R*)-4,5-dihydroxycyclohex-1-enecarboxylic acid (compound **3**) by an isomerization of the remaining double bond from the  $\Delta^2$  to the  $\Delta^1$  position. Two alternate stereochemical pathways for the conversion of compounds **1**–**3** are consistent with the results from the incorporation studies: a *syn* reduction of the  $\Delta^1$  double bond with hydrogen addition to the *si* face of C1 to produce compound **2a**, followed by subsequent suprafacial 1,3-allylic rearrangement of compound **2a** to produce compound **3** (Figure 4.7, pathway a); an *anti* reduction of the  $\Delta^1$  double bond of compound **1** with hydrogen addition to the *re* face of C1 (*anti* addition) to produce **2b**, and a subsequent antarafacial 1,3-allylic rearrangement of **2b** to produce compound **3** (Figure 4.7, pathway b). All previously studied rearrangements of this type [100, 101], including the interconversion of 2-cyclohexenylcarbonyl-CoA and 1-cyclohexenylcarbonyl-CoA [102–105], have been shown to be suprafacial. In many of these cases, evidence that a single catalytic residue acts as a general acid–base catalyst for the isomerization has been presented. On the basis of these precedents, a *syn* reduction of compound **1** to produce compound **2a** seems to be





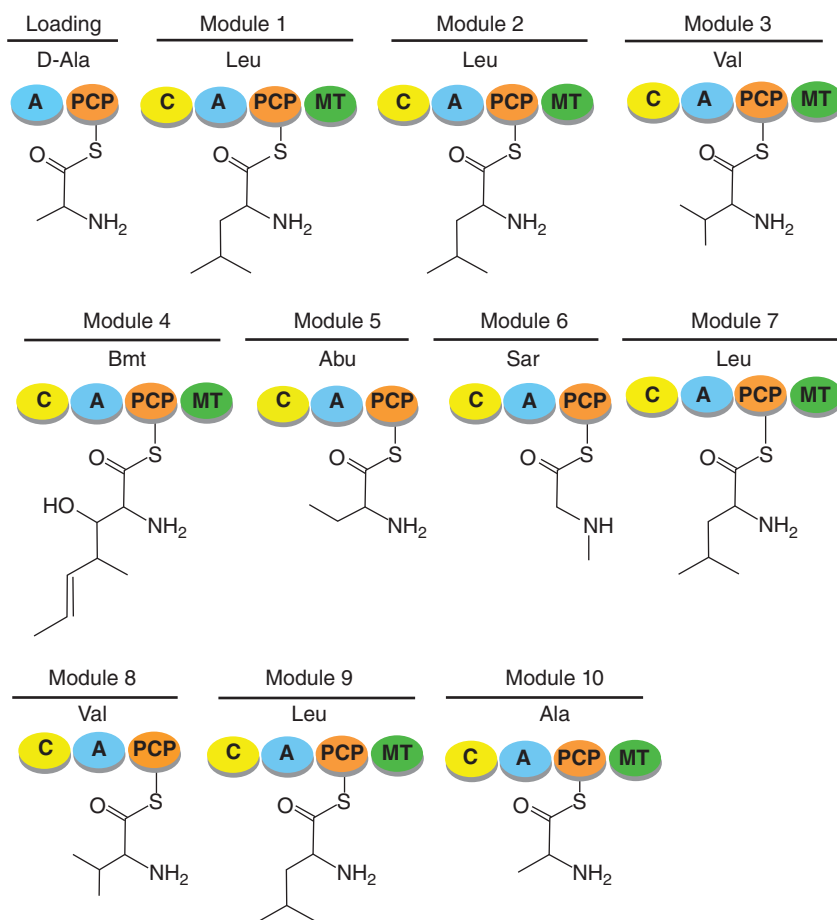
**Figure 4.7** Proposed pathways to DHCHC from shikimic acid in *Streptomyces hygroscopicus*.

the more likely pathway (Figure 4.7, pathway a). In the final step of the proposed pathway, compound **3** is reduced to DHCHC by an anti addition of hydrogen. Recently, *fkbO* in the tacrolimus/ascomycin gene cluster and its homolog *rapK* in the sirolimus gene cluster have been shown to encode a chorismatase that is responsible for the biosynthesis of (3*R*,4*R*)-3,4-dihydroxycyclohexa-1,5-dienecarboxylic acid (compound **1** of Figure 4.7) from chorismate [106], suggesting that compound **1** of Figure 4.7 originates from chorismate rather than from shikimate.

#### 4.4.5

##### Biosynthesis of Cyclosporin

The biosynthesis of cyclosporin involves an NRPS, cyclosporin synthetase, which is a multienzyme polypeptide (Figure 4.8). The open reading frame (ORF) encoding cyclosporin synthetase was the largest genomic ORF known in biology [107]. Its size is 45.8 kb and it encodes a protein of  $M_r$  1 689 243. It activates all constituent amino acids of cyclosporin A to thioesters via amino acyladenylates, and carries out specific *N*-methylation reactions. Included in its structure are 11 similar amino acid-activating domains, seven of which possess *N*-methyl transferase activities. Seven of the 11 amino acid residues of cyclosporin A are methylated.



**Figure 4.8** Cyclosporin biosynthesis. Bmt = butenyl-methyl-threonine, Abu = L-alpha-aminobutyric acid, and Sar = sarcosine.

The enzyme carries out at least 40 reactions in an assembly belt-like mechanism. Formation of three nonprotein amino acids, D-alanine,  $\alpha$ -aminobutyrate, and (4-*R*)-4-[(*E*)-2-butenyl]-4-methyl-L-threonine, occurs prior to the incorporation by separate enzymes of secondary metabolism. During elongation, the activated amino acids are linked by peptide bonds leading to enzyme-bound nascent peptide chains. Cyclosporin synthetase contains four domains, one each for an adenylation, thiolation, condensation, and a *N*-methyltransferase domain. The adenylation domain is responsible for substrate recognition and activation, whereas the thiolation domain covalently binds the adenylated amino acids to phosphopantetheine, and the condensation domain elongates the peptide chain.

Cyclosporin synthetase substrates include L-valine, L-leucine, L-alanine, L-glycine, 2-aminobutyric acid, 4-methylthreonine, and D-alanine. D-Alanine is the starting amino acid in the biosynthetic process [108]. With the adenylation domain, cyclosporin synthetase generates the acyl-adenylated amino acids, then covalently binds the amino acid to phosphopantetheine through a thioester linkage. Some of the amino acid substrates become N-methylated by SAM. The cyclization step releases cyclosporin from the enzyme [109]. The need for an amino acid, such as D-Ala, indicates that cyclosporin synthetase requires the action of other enzymes such as a D-alanine racemase. The racemization of L-Ala to D-Ala is pyridoxal phosphate dependent. The formation of butenyl-methyl-L-threonine is performed by a butenyl-methyl-L-threonine PKS that uses acetate or malonate as its starting material [110].

#### 4.5

#### Genetics and Strain Improvement

Cyclosporin A is synthesized from its precursor amino acids by cyclosporin synthetase, a single multifunctional enzyme. Cloning of the corresponding coding region of this synthetase shows that it contains an ORF of 45.8 kb which encodes a peptide with a calculated  $M_r$  of 1 689 243. The predicted gene product contains 11 amino acid-activating domains that are very similar to one another and to the domains of other peptide synthetases. Seven of these domains harbor N-methyltransferase functions. This is the largest genomic ORF described so far.

The sirolimus gene cluster in *S. hygroscopicus* contains three extremely large ORFs encoding the modular PKS [90]. Raps A, B, and C contain 70 active sites which carry out 70 catalytic functions and represent one of the largest multienzyme systems known. The integration of six or even four modules in a single polypeptide is unprecedented. The DNA fragment which was sequenced comprised 107.3 kbp and contained over 20 additional ORFs. The three clustered type I PKS genes together encode 14 homologous modules (sets of enzyme activities), each catalyzing a specific round of chain elongation. Between two of these is a fourth gene (*rapP*), encoding the PIE, which probably catalyzes closure of the macrolide ring. On either side of the PKS genes are 22 ORFs [111]. These include *rapJ* and *rapN*, apparently encoding two cytochrome P-450 monooxygenases, *rapO* encoding a ferredoxin, *rapI*, *rapM*, and *rapQ* potentially encoding three SAM-dependent O-methyltransferases, and *rapL*, encoding the enzyme which cyclodeaminates L-lysine to L-pipecolate. Adjacent genes probably are involved in regulation and export.

The lysine cyclodeaminase gene *rapL*, encoding the enzyme converting lysine to pipecolate by  $\alpha$ -deamination, is to the right of *rapC*. However, in the tacrolimus gene cluster, the analogous gene *fkbl* is on the left side of the gene cluster [72, 90]. The two cyclodeaminase genes show 72% identity to each other. The *rapP* gene,

encoding the enzyme responsible for activation and incorporation of pipercolate into sirolimus, is 55% identical to *fkpP*. Both are found to lie between two of the three PKS genes, *rapP* between *rapA* and *rapC*, and *fkpP* between *fkpB* and *fkpA*. Gene *fkpP* encodes an NRPS. Two genes of the sirolimus biosynthetic cluster in the sirolimus-producing *S. hygroscopicus*, that is, *rapG* and *rapH*, encode positive regulatory proteins for sirolimus production [112]. Overexpression of either gene increases sirolimus formation, whereas their deletions eliminate sirolimus biosynthesis. They act by affecting the promoter of the operon. The sirolimus-producing *S. hygroscopicus* ATCC 29253 culture also produces nigericin and another polyketide [63] which is likely elaiophyllin. This second type-I PKS gene cluster was isolated and found not to be involved in production of sirolimus or nigericin [63, 64].

Cheng *et al.* [113] reported that treatment of protoplasts or spores with gentamicin yielded improved sirolimus-producing mutants. Further work on the use of protoplasts to genetically improve sirolimus production was described by Chen *et al.* [114]. The combination of classical mutagenesis and rational metabolic engineering was utilized by Jung *et al.* [115] to improve sirolimus production and to show the importance of the methylmalonyl-CoA precursor supply pathway for sirolimus formation.

A large transcript of 22 kb appears to be involved in the production of tacrolimus [72]. The cluster contains an operon of three genes. *FkbA* contains 19 FAS-like domains which include a  $\beta$ -ketoacyl ACP synthase (KS), acyltransferase (AT), dehydratase (DH), enoylreductase (ER),  $\beta$ -keto-reductase (KR), and ACP in the order: 5'-KS-AT-DH-ER-KR-ACP-KS-AT-KR-ACP-KS-AT-DH-ER-KR-ACP-KS-AT-ACP-3'. These incorporate carbons 8 through 15 into the macrolactone ring. Biosynthesis of the macrolactone ring of tacrolimus involves 10 elongation cycles that mechanistically resemble the steps in fatty acid synthesis [72]. Sequencing of a 40-kb DNA segment of the tacrolimus gene cluster from *Streptomyces* sp. MA6548 revealed two additional PKS genes, *fkpB* and *fkpC*, which lie upstream of *fkpA*, the PKS gene recently shown to be responsible for the last four condensation steps of the tacrolimus biosynthesis [72]. Genes *fkpB* and *fkpC* are contiguous and encode, respectively, the first (790 129 Da) and the second (374 438 Da) components of the tacrolimus PKS, a complex of three multidomain polypeptides. The predicted domain structures of *FkpB* and *FkpC* are analogous to that of *FkpA* and comprise 30 FAS-like domains arranged in six modules. Each module performs a specific extension cycle in the assembly of the carbon skeleton of the tacrolimus macrolactone ring. The component activities for the initiation of the polyketide chain consist of a dihydrocyclohexenylcarbonyl-CoA synthetase and a dihydrocyclohexenylcarbonyl-CoA reductase, required for the formation of the dihydrocyclohexylcarbonyl-CoA starter unit, and an acyl-carrier-protein to which the starter unit is anchored and translocated to the appropriate site on the PKS multienzyme; these are located at the N-terminal region of the *FkpB* polypeptide. A third gene, *fkpL*, lies at one end of the cluster and encodes lysine cyclodeaminase which catalyzes  $\alpha$ -deamination and cyclization of lysine into

pipecolate. A fourth gene *fkpP*, located at the other end of the sequence reported here, encodes a peptide synthetase required for the activation and incorporation of the pipecolate moiety into the completed acyl chain. Finally, the cluster carries a gene, *fkpO*, whose product is involved in the biosynthesis of the starter unit DHCHC. Recently, it has been revealed that four contiguous genes, *tcsA*, *tcsB*, *tcsC*, and *tcsD*, involved in the biosynthesis of the unique allyl side chain of tacrolimus, are commonly found upstream of the genes for methoxymalonyl-ACP biosynthesis (*fkpG*, *fkpH*, *fkpI*, *fkpJ*, *fkpK*, and *fkpL*) in all sequenced tacrolimus gene clusters [67].

Targeted gene disruption in *Streptomyces* sp. MA6548, a tacrolimus producer, yielded 9-deoxy-31-*O*-demethyl tacrolimus, and *O*-demethyl tacrolimus [116]. The former was 70-fold less active and the latter 5-fold less active than tacrolimus in the *in vitro* assay for proliferation of mouse T cells stimulated with ionomycin. The former showed one-fifth the activity of tacrolimus against *A. niger* ATCC 6275 (zone test on agar). The latter also had antifungal activity.

To improve tacrolimus titers, UV irradiation [117] and sequential adaptation [118] have been used. Mo *et al.* [119] found methylmalonyl-CoA to be the limiting factor in the tacrolimus producer *Streptomyces clavuligerus* CKD1119. It also is important for sirolimus production. Scientists increased titers by genetically engineering the methylmalonyl mutase (MCM) pathway and the propionylmalonyl-CoA carboxylase (PCC) pathway into *S. clavuligerus* CKD1119 and *S. hygroscopicus* UV2-2 [67, 115]. They supplemented the medium with propionate for the PCC pathway. Mo *et al.* [119] combined classical random mutagenesis with metabolic engineering to increase tacrolimus production. They did this by analyzing production-limiting precursors and then adding vinyl propionate as precursor. In addition, overexpression of the positive regulatory gene *fkpN* in the strain in which the negative regulatory gene *tcs7* had been deleted resulted in a fourfold increase in tacrolimus production compared to that by the wild-type strain in *Streptomyces* sp. KCTC 1140BP [120].

Classical techniques have also been used to improve sirolimus production [114, 121, 122]. For example, Zhu *et al.* [122] increased sirolimus production threefold in *S. hygroscopicus* HD-04-5 with UV irradiation.

#### 4.6 Fermentation and Nutritional Studies

After the isolation of strain AY B-994 of *S. hygroscopicus* (NRRL 5491; ATCC 29253), it was first grown on yeast–starch agar [9]. The best solid medium for its growth was tomato paste–oatmeal agar. Good carbon sources for growth on agar were D-glucose, D-fructose, D-mannitol, myo-inositol, starch, and glycerol. Those supporting moderate growth were D-xylose, L-arabinose, L-rhamnose, raffinose, lactose, and D-maltose. The optimum pH and temperature for growth were 6–8

and 25–27 °C, respectively. Spores were produced on tomato paste–oatmeal agar after 14 days. Initial studies on sirolimus production were done using a liquid medium containing glucose, oatmeal, enzymatic digest of casein, blackstrap molasses, NaCl, and tap water for inoculum preparation and fermentation. Later studies [13] employed a seed medium containing glucose, soybean meal,  $(\text{NH}_4)_2\text{SO}_4$ ,  $\text{CaCO}_3$ , and tap water and a large-scale production medium containing glucose, soybean meal,  $(\text{NH}_4)_2\text{SO}_4$ ,  $\text{KH}_2\text{PO}_4$ , and antifoam.

Biosynthetic studies [69–71] were done with a second natural isolate, strain AY B-1206, which produced more sirolimus and less demethoxysirolimus. Sporulation was carried out on a glucose– $\text{KNO}_3$ –inorganic salts agar. Fermentation was done in a liquid medium containing glycerol, leucine, glutamic acid, lysine, yeast extract, and inorganic salts. These media had been developed by Sehgal and Vezina at Ayerst Canada. In a later biosynthetic study [80], lysine was replaced by monobasic ammonium phosphate with no negative effects being observed.

Up until 1994, no chemically defined medium had been reported. In a study on carbon source nutrition, Kojima *et al.* [123] began with the yeast extract-containing medium used by Paiva *et al.* [68] as aforementioned. Removal of yeast extract markedly reduced growth and sirolimus production. Addition of glucose, aspartic acid, arginine, and histidine replaced the effect of yeast extract and thus medium 1 (Table 4.1) was developed. It was used to determine the effect of carbon sources as replacements for glucose plus glycerol on growth and sirolimus production. In medium 1, the insoluble  $\text{CaCO}_3$  of earlier media had been replaced by 100 mM MES buffer to facilitate observation of growth. The support of growth and sirolimus production by 34 carbon sources was then examined. Of these carbon sources, 19 supported growth and sirolimus production. Eight carbon sources failed to support growth. Seven compounds exerted catabolite repression or inhibition, that is, they supported growth but not production of sirolimus. After further studies, it was deemed that a mixture of  $20 \text{ g l}^{-1}$  fructose plus  $5 \text{ g l}^{-1}$  mannose was the best combination of two carbon sources and these were chosen for medium 2 (Table 4.1).

Minerals exert different types of regulatory control of sirolimus biosynthesis. Cheng *et al.* [124] reported that limitation of phosphate, ammonium, and magnesium was required for optimal production in medium 2. While total growth was maximal at 100 mM  $\text{K}_2\text{HPO}_4$ , production peaked at 5–10 mM. Ammonium chloride supported maximum growth at 25 mM but a level as low as 5 mM inhibited sirolimus formation. Magnesium sulfate showed its maximum effect on growth at 1 mM but inhibited sirolimus production at levels higher than 0.01 mM. Although it is not uncommon for production of macrolide antibiotics to be under control of nitrogen and phosphate sources, regulation by  $\text{Mg}^{2+}$  has been only rarely described. In contrast to the negative effects of the aforementioned minerals,  $\text{FeSO}_4$  increasingly stimulated sirolimus production at concentrations up to 0.36 mM with virtually no effect on growth. Medium 3 (Table 4.1) was devised by virtue of the information obtained from these studies [124].

**Table 4.1** Chemically defined media sequentially developed for growth of *Streptomyces hygroscopicus* and production of sirolimus [1, 78, 79, 100–102].

Component	Medium 1	Medium 2	Medium 3	Medium 4
Glycerol (g)	20.0	—	—	—
D(+)-Glucose (g)	10.0	—	—	—
D-Fructose (g)	—	20.0	20.0	20.0
D(+)-Mannose (g)	—	5.0	5.0	5.0
Na-L-Aspartate (g)	1.5	1.5	1.5	1.5
L-Arginine (g)	0.5	0.5	0.5	0.5
L-Histidine-HCl (g)	0.5	0.5	0.5	0.5
L-Lysine (g)	—	—	—	10.0
Shikimic acid (g)	—	—	—	10.0
K <sub>2</sub> KHPO <sub>4</sub> (g)	2.0	2.0	1.7	1.7
KH <sub>2</sub> PO <sub>4</sub> (g)	2.0	2.0	—	—
NaCl (g)	5.0	5.0	5.0	5.0
ZnSO <sub>4</sub> ·7H <sub>2</sub> O (mg)	60	60	60	60
MgSO <sub>4</sub> ·7H <sub>2</sub> O (mg)	256	256	2.5	2.5
MgCl <sub>2</sub> ·6H <sub>2</sub> O (mg)	510	510	—	—
MnSO <sub>4</sub> ·H <sub>2</sub> O (mg)	12	12	12	12
FeSO <sub>4</sub> ·7H <sub>2</sub> O (mg)	100	100	100	100
(NH <sub>4</sub> ) <sub>6</sub> Mo <sub>7</sub> O <sub>24</sub> ·4H <sub>2</sub> O (mg)	18	18	18	18
Na <sub>2</sub> B <sub>4</sub> O <sub>7</sub> ·10H <sub>2</sub> O (mg)	10	10	10	10
CoCl <sub>2</sub> ·6H <sub>2</sub> O (mg)	10	10	10	10
CuCl <sub>2</sub> ·2H <sub>2</sub> O (mg)	1.3	1.3	1.3	1.3
Na <sub>2</sub> SO <sub>4</sub> (mg)	360	360	360	360
MES buffer (g)	21.3	21.3	21.3	21.3
pH (initial)	6.0	6.0	6.0	6.0

All weights are per liter.

The effect of amino acid addition to medium 3 (which already contained aspartate, arginine, and histidine as nitrogen sources) was studied by Cheng *et al.* [125]. Of 18 amino acids tested at 1 g l<sup>-1</sup>, only L-lysine showed a marked stimulation of both volumetric and specific sirolimus production. Its effect was maximal at 10 g l<sup>-1</sup>, the highest concentration examined. Lysine stimulation is probably due to its conversion to pipercolic acid, a precursor of sirolimus [80]. Suppression of sirolimus production was observed with L-methionine and L-phenylalanine. Since methionine is a sirolimus precursor [69–71], it is peculiar that it interferes with sirolimus formation. However, for some unknown reason, methionine often suppresses formation of secondary metabolites for which it acts as precursor [126–129]. One possibility for the phenylalanine suppression of sirolimus formation was considered to be feedback inhibition of shikimic acid formation, as shikimate is a precursor of sirolimus [69–71]. However in a later study demonstrating the stimulation of sirolimus biosynthesis by exogenous shikimate (57 mM), it was also shown that shikimate does not

reverse phenylalanine interference [97]. Shikimate stimulation had not been observed earlier when tested in complex medium [98]. Only upon development of the effective chemically defined medium 3 plus lysine was a shikimate response observed [97]. Medium 4 (Table 4.1) was the most effective chemically defined medium available for sirolimus biosynthesis.

The importance of various factors such as carbon source regulation [123], nitrogen source regulation [130, 131], and the precursor effects of acetate, propionate, methionine [69–71], pipercolic acid [80], and shikimic acid [98] for sirolimus production were all utilized in the strain improvement of *S. hygroscopicus* by Zhu and coworkers [122]. Starting with a strain producing  $150 \text{ mg l}^{-1}$ , a mutant resistant to lysine inhibition was obtained which produced  $260 \text{ mg l}^{-1}$  of sirolimus. Further mutation to high glucose resistance yielded a strain making  $350 \text{ mg l}^{-1}$ . Mutation to resistance to high shikimate concentrations and then to auxotrophy to tryptophan and phenylalanine resulted in production of  $450 \text{ mg l}^{-1}$ . Fed-batch fermentation with glycerol and potassium phosphate supplementation yielded  $812 \text{ mg l}^{-1}$ . A review on the biosynthesis, regulation, and mutagenic improvement of sirolimus production was written by Park *et al.* [131].

Only little is known about the nutrition of *S. tsukubaensis* strain 1993, the producer of tacrolimus [10]. The seed medium for its production contained glycerol, corn starch, glucose, cotton seed meal, corn steep liquor, and calcium carbonate. The production medium contained soluble starch, corn steep liquor, dried yeast, calcium carbonate, and Adekanol (a defoaming agent). High ammonium and phosphate levels, but not glucose, interfere with tacrolimus production in *Streptomyces* sp. [132]. Similarly to sirolimus (see the preceding), its formation is inhibited by methionine. Methyltransferases have been purified from the producers of tacrolimus and immunomycin. They require  $\text{Mg}^{2+}$  and SAM and are specific for a particular site of methylation. Mutants developed by increasing resistance to tacrolimus produced  $0.9 \text{ g l}^{-1}$  in 7 days [118].

Ascomycin production by *S. hygroscopicus* var. *yakushimaensis* strain No. 7238 showed an optimum temperature of  $28^\circ\text{C}$  and utilized D-glucose, sucrose, lactose, maltose, D-trehalose, inositol, inulin, and salicin [22, 23]. For inoculua, a seed medium (pH 6.5) containing glycerol, corn starch, glucose, cotton seed meal, dried yeast, corn steep liquor, and calcium carbonate was used. The production medium was made up of glucose, corn steep liquor, dried yeast, gluten meal, wheat germ, calcium carbonate, and Adekanol [22, 23]. Byrne *et al.* [25] grew ATCC 14891 on slants of ISP medium, in a seed medium containing glucose, yeast extract, Hy-Case SF, and mineral salts, and a fermentation medium of glucose, glycerol, corn steep liquor, yeast extract, L-tyrosine, lactic acid, and MOPS buffer. Only little is known about the nutrition of the ascomycin producers.

The effects of nitrogen sources on red pigment production by *Monascus* are of interest [133, 134]. Certain amino acids like glutamate markedly stimulate the process because they are incorporated during biosynthesis to form a side chain which does not exist in the conventional cell-bound, water-insoluble red pigment [135]



As a result, the product is shifted to an extracellular, water-soluble product which is markedly overproduced. On the other hand, other amino acids are very poor for pigment formation as nitrogen sources in the fermentation. One of these, leucine, was found to negatively affect production in the presence of glutamate by causing destabilization of pigment synthase(s); it did not repress synthase formation nor did it inhibit synthase action [136]. Formation of these polyketide pigments is under phosphate control, the mechanism involving enzyme inhibition rather than repression. Pigment formation is stimulated by  $Zn^{2+}$ ,  $Mn^{2+}$ , and  $Fe^{2+}$  via stimulation of synthase action, rather than an effect on induction or enzyme stabilization. On the other hand,  $Mg^{2+}$  inhibits pigment synthase action.

Oil was found to stimulate polyketide antibiotic and tacrolimus titers [117]. Mo *et al.* [119] used vinyl propionate as a precursor to convert propionyl-CoA to methylmalonyl-CoA by the PCC pathway in a strain containing lipase activity. Tween 80 was found to be the best inducer of lipase [119]. The lipase is used to catalyze cleavage of ester bonds of long-chain acylglycerols to glycerol and free fatty acids. The free fatty acids are converted to acetyl-CoA or propionyl-CoA which are polyketide precursors.

#### 4.7

#### Other Activities of Immunosuppressants

More than any other group of microbial secondary metabolites, the immunosuppressants possess the greatest diversity of biological activities [137–141]. For instance, sirolimus has antifungal, antitumor, neuroprotective, autoimmune, and antiangiogenic properties in addition to its immunosuppressive ability.

With regard to the antitumor activity of sirolimus, it acts as an inducer of apoptosis and interferes with angiogenesis [142]. On the other hand, cyclosporin A promotes tumor growth, and transplant patients have been killed by tumors. Sirolimus is more active as an antitumor agent than tacrolimus [19]. Sirolimus and its derivatives specifically inhibit the mTOR protein kinase, involved in both immunosuppression and antitumor activities. Sirolimus also shows synergy with protein tyrosine kinase (PTK) inhibitors such as Gleevec® (Imatinib) which is used for leukemia, but for which resistance is developing [143]. The sirolimus analogs temsirolimus (Torisel™), everolimus, and ridaforolimus are used for renal cell carcinoma (advanced kidney cancer). They are also employed for refractory mantle cell lymphoma, and advanced pancreatic neuroendocrine tumors. Mycophenolic acid also appears to have antiangiogenic activity [34]. It has also been used to prevent the kidney problems associated with lupus erythematosus.

Sirolimus, cyclosporin A, and tacrolimus also reverse multidrug resistance to antitumor agents in mammalian cells [144, 145]. The best for reversal in tumor cells is the non-immunosuppressive cyclosporin A derivative valspodar (PSC-833) [146].

Yeasts and filamentous fungi are inhibited by cyclosporin A, tacrolimus, and sirolimus. Sirolimus and its less immunosuppressive analogs are effective against *C. albicans* and *C. neoformans* via FKBP12-dependent inhibition of TOR kinases [50]. These enzymes are necessary for stationary phase entry, expression of ribosomal protein genes, nitrogen catabolite repression, and translation. The sensitivity of yeasts to cyclosporin A and tacrolimus is due to their need for calcineurin to promote yeast survival during cation stress. Susceptible fungi include *C. albicans*, *C. neoformans*, *C. immitis*, *A. niger*, *A. fumigatus*, and *N. crassa* [43, 50]. In *C. neoformans*, calcineurin is needed for virulence. In mice, sirolimus and tacrolimus are more active as antifungal agents than cyclosporin A [11]. It appears that the antifungal and immunosuppressive effects are unrelated because two non-immunosuppressive analogs of cyclosporin A and the non-immunosuppressive tacrolimus derivative, a C18-hydroxy C21-ethyl analog (L-685,818) are active against *C. neoformans* [3, 54, 55]. They act by binding to cyclophilin A and inhibiting the action of the fungal calcineurin. L-685,818 is non-immunosuppressive because its combination with human immunophilin (human FKBP12) does not inhibit vertebrate calcineurin, but when in combination with fungal FKBP12, it does [54, 55]. Thus, it is possible to exploit subtle differences in the structures of human and fungal FKBP12 [43]. Since TOR proteins are involved negatively in nutrient repression, addition of sirolimus induces certain yeast genes whose transcription is repressed by nitrogen abundance, glucose abundance, and also genes involved in the diauxic shift [51–53].

Cyclosporin A and tacrolimus convert the normally fungistatic activity of azoles (e.g., fluconazole) against *C. albicans*, *Candida glabrata*, and *Candida krusei* into fungicidal activity by inhibiting the protein phosphatase calcineurin [147]. Even non-immunosuppressive analogs of tacrolimus have this ability. Non-azole drugs that inhibit other steps of ergosterol biosynthesis (terbinafine, fenpropimorph) are also improved in activity by immunosuppressants and their non-immunosuppressive analogs.

Cyclosporin A was originally developed as an antifungal agent in 1970 by Sandoz Pharmaceutical Co. This cyclic undecapeptide immunosuppressant also has antiparasitic and anti-inflammatory activities. Cyclosporin A analogs are being clinically tested against the inflammatory disease asthma and are showing promising results [148]. They exhibit decreased nephrotoxicity and have different pharmacology and metabolism. Cyclosporin A is being considered as a drug to correct mitochondrial dysfunction and muscle apoptosis in patients with collagen VI myopathies, which include muscular dystrophy.

Cyclosporin A is active against HIV [149]. Non-immunosuppressive analogs also have antiviral activity against HIV and hepatitis C virus (HCV) [150]. One such compound, called *Debio-025*, is showing beneficial results in clinical trials. Mycophenolic acid has activity against yellow fever, dengue, and Japanese encephalitis virus [151]. Cyclosporin A has activity against the malaria parasite *Plasmodium falciparum* in agreement with its genome-containing sequences encoding cyclophilin and calcineurin [152, 153].

Another activity of tacrolimus and sirolimus is stimulation of nerve cells [154, 155]. Thus, they might find use for combating neurological disorders [156]. Immunosuppressants have neuroprotective activity, that is, they promote neurite outgrowth and protect neurons. Non-immunosuppressive analogs of sirolimus have been produced which retain neuroprotective activity. The tacrolimus analog 36-methyl-FK506 possesses improved neurite outgrowth activity [67]. Certain ascomycin derivatives, made by combinatorial biosynthesis, are being studied for nerve regeneration [157].

Sirolimus has found a novel use in cardiology because sirolimus-impregnated stents are less prone to proliferation and restenosis, which usually occur after treatment of coronary artery disease. The traditional treatment for coronary artery disease was the coronary artery bypass graft in which a vein from the leg is spliced around the heart blockage. The number of such surgical procedures has dropped over the years. This drop was due to the use of cardiac stents (small metallic scaffolds) inserted into blocked arteries by threading them through the body from a small thigh opening, thus obviating heart surgery (balloon angioplasty). The stents are impregnated with sirolimus in a thin polymeric coating over the steel mesh. The drug is continuously eluted and prevents vascular smooth muscle cell proliferation; thus, the cells cannot grow over the mesh. As a result, stent restenosis is prevented. Such restenosis occurs in 15–60% of patients receiving bare stents.

Tacrolimus is also being studied for skin diseases. Recently, a topical preparation has been shown to be very active against atopic dermatitis (eczema), a widespread skin disease. The ascomycins, although structurally related to rapamycin, were never commercialized as immunosuppressants, but have anti-inflammatory activity, and are being used for topical treatment of skin diseases such as atopic dermatitis, allergic contact dermatitis, and psoriasis [158]. Novartis has modified the structure of ascomycin and produced Elidel<sup>®</sup>, an agent for inflammatory skin diseases [159]. It is used as a topical drug to relieve atopic dermatitis in children and also has use in Netherton syndrome. Launched in 2002, it already had sales of \$349 million in 2004.

Sirolimus extends life span in mice and is being considered for possible use against progeria (Hutchinson–Gilford progeria syndrome) in children. This is a rare disease which resembles accelerated aging and kills children in their teens. When tested on cells from children suffering from progeria, sirolimus promoted cleavage of progerin, the mutant protein that accumulates in cells of afflicted children and extended the survival of the cells [160].

## 4.8

### Concluding Remarks

The antifungal peptides and antibiotics cyclosporin, tacrolimus, sirolimus, mycophenolic acid, and ascomycin were ignored for a long time, until they reached their medical niche as immunosuppressants. These immunosuppressants

act by inactivating the body's T cells, binding enzymes called *immunophilins*, and triggering a set of reactions which eventually inactivate the immune system. They share their structure with macrolide antibiotics. The large 23-membered ring of the ascomycin family is similar to the 21-membered ring of sirolimus. The biosynthesis of these involves a series of complex reactions carried out by multienzyme polypeptides that catalyze reactions in a belt-like manner, first forming a chain, then undergoing elongation and cyclization. Genes encoding these enzymes have been cloned and studied. Immunosuppressants are finding applications not only in organ transplantation, but also in the treatment of various life-crippling disorders such as autoimmune disorders, cancer, AIDS, asthma, skin diseases, respiratory ailments, and malaria, to name a few. Research focusing on unraveling the mysteries regarding the genetics, biosynthesis, and molecular mode of action of these drugs will open more doors for their further applications in the world of medicine.

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## 5

# Activators and Inhibitors of ADAM-10 for Management of Cancer and Alzheimer's Disease

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### 5.1

#### Introduction to ADAM Family of Enzymes

A disintegrin and metalloproteinase (ADAM) is a family of transmembrane and secreted proteins contains ~750 amino acids; ADAMs are involved in various functions of the cells, including cell adhesion, signaling, migration, and proteolysis. ADAM proteins contain a disintegrin and a metalloproteinase domain, combining features of cell surface adhesion molecules and proteases [1]. They play a vital role in cell adhesion and proteolytic processing of the ectodomains for various cell surface receptors and signaling molecules. The human genome has been identified with 25 ADAM genes, out of which, 4 appear to be pseudogenes [2]. Although ADAMs are known for their proteolytic effect, only 13 of the 21 genes in the family encode functional proteases in humans. ADAM metalloproteinases are involved in “ectodomain shedding” of diverse growth factors, cytokines, receptors, and adhesion molecules, making it a key player in the class of enzymes executing “sheddase” activity. Enzymes ADAM-17 and ADAM-10 are widely studied for their role in pathological and physiological conditions. Involvement of these enzymes in diverse biological processes, such as, cell fate determination in the nervous system, cell migration, sperm–egg interactions, axon guidance, muscle development, and immunity have been identified. Dysregulation of these proteolytic enzymes contributes to various pathological states including cardiovascular disease, asthma, inflammatory diseases, cancer, and Alzheimer's disease (AD) [3, 4].

ADAM is also a member of zinc protease family. Zinc proteases are classified on the basis of the primary structure of their catalytic sites, and are categorized as gluzincin, metzincin, inuzincin, carboxypeptidase, and DD carboxypeptidases [5]. The ADAM proteases fall within the adamalysin subfamily of the metzincin superfamily of Zn-dependent metalloproteinases with catalytic site amino acid sequence, HEXGHXXGXXHD, indicating proteolytic functionality [6]. Adamalysins are identified by a unique integrin receptor-binding disintegrin

domain. The domain structure of the ADAMs consists of a prodomain, a metalloprotease domain, a disintegrin domain, a cysteine-rich domain, an epidermal growth factor (EGF)-like domain, a transmembrane domain, and a cytoplasmic tail [7]. The adamalysins subfamily also includes the class III snake venom metalloproteases (SVMPs) and the ADAM-TS family, which show close structural resemblance with ADAMs. Although ADAMs share substrates for proteolytic activity with matrix metalloproteinases (MMPs), domain structures of these enzymes are highly distinguishable. ADAMs are proposed to function as proteases for matrix degradation, cell migration, and localized shedding of various proteins, including cytokines and growth factors, from membrane-anchored proteins. Structurally, proteases from the ADAM family closely resemble structures of proteolytic enzymes from the SVMP and MMP families. Structural similarity and differences with respect to domain organization of the proteases is compared, and their membrane or secreted location is illustrated in Figure 5.1 [8].

Our current understanding about proteolytic activities of ADAMs and ADAM-mediated shedding is largely based on mechanistic studies conducted with enzymes ADAM-10 and ADAM-17 [7]. Other proteolytic enzymes from the ADAM class, ADAM 2, 9, 12, and 19 are also observed to elicit important biological roles. However, in this review we focus on the enzyme ADAM-10 for its involvement in cancer and AD. We will discuss the involvement of ADAM-10 in cancer and AD, and the potential of activators and inhibitors of ADAM-10 as therapy for these diseases.

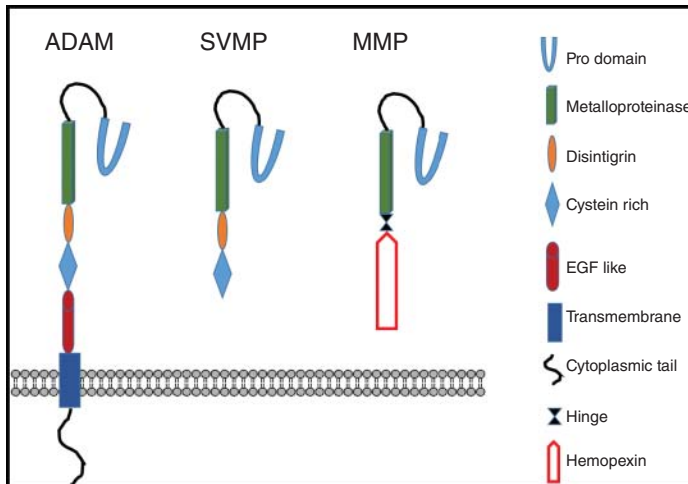


Figure 5.1 Comparison of the domain structures of ADAM, SVMP, and MMP.



## 5.2

### ADAM-10 Structure and Physiological Roles

The enzyme ADAM-10 (also known as *kuzbanian*) contains the metalloproteinase consensus sequence “HExxH” as observed in almost all enzymes in the ADAM family [9]. It catalytically mediates proteolytic cleavage of cell surface integral membrane proteins within their juxta-membrane region, and releases a soluble ectodomain protein in the extracellular space. This process of ectodomain shedding is commonly referred to as *shedase* activity [7]. The human form of ADAM-10 is synthesized as a 748 amino acid precursor, which matures into a type I transmembrane glycoprotein having characteristic domain structure of the ADAM family. The N-terminal signal sequence of a 19 amino acid is followed by a prodomain, a metalloproteinase and a disintegrin domain, a cysteine-rich region, a transmembrane domain, and an SH3-enriched cytoplasmic tail (Figure 5.1) [10]. ADAM-10 is known to mediate the ectodomain shedding of a large number of cell surface proteins including the cell adhesion molecule L1, epidermal growth factor receptor, notch, ephrin-A2, receptor for advanced glycation end products (RAGE), N-cadherin [11], and the amyloid precursor protein (APP) [10, 12–15].

Although the role of ADAM-10 is mostly studied in pathogenesis, high incidences of death in ADAM-10 knockout mice suggested its physiological importance. ADAM-10 has been now identified as an essential enzyme for development of brain cortex, and for its involvement in various biological processes [16]. Deviation from the normal levels of the enzyme has been marked in the pathological conditions.

## 5.3

### Pathological Significance

ADAM-10-mediated ectodomain shedding modulates the function of cell adhesion molecules, and regulates paracrine, juxtacrine, and autocrine signaling. ADAM-10 activates several signaling molecules, receptors, and chemokines by its “shedase” activity. ADAM-10 itself is also subject to regulated intramembrane proteolysis. Overexpression of ADAM-10 assists in tumor progression and metastasis, and is also known to cause resistance to doxorubicin in hepatocellular carcinoma [17]. Downregulation of ADAM-10 has been observed in neurodegenerative diseases. Dysregulation of this enzyme and associated pathological conditions are listed in Table 5.1.

### 5.3.1

#### Modulating ADAM Activity in Neurodegeneration

Deposition of neurotoxic plaques of amyloid beta peptides ( $A\beta$ ) is considered as hallmark of the neurodegenerative AD. Both ADAM-10 and APP are expressed

**Table 5.1** Pathological conditions with dysregulated levels of ADAM-10.

Dysregulated ADAM-10 levels and associated diseases	References
Atherosclerosis	[18]
Colon carcinoma	[19]
Ectopic dermatitis	[18]
Gastric cancer	[20]
Prostate cancer	[21]
Ovarian cancer	[22]
Oral squamous cell carcinoma	[23]
Neurodegenerative diseases	[24]

in human cortical neurons during different developmental stages. Mutations in ADAM-10 have been associated with a reduction of  $\alpha$ -secretase activity which is observed in late onset of AD [25]. Upregulation of ADAM-10 mRNA expression is also observed in severe cases of AD [26]. Mice overexpressing ADAM-10/APP were observed with increased synaptogenesis, and increased capability of learning and long-term memory [27]. In addition to the potent neurotrophic activities of soluble APP, the increased ADAM-10 expression might contribute to neuroprotective effects due to the shedding of several additional substrates in the brain, such as, L1 and N-cadherin. Neural cell adhesion molecule L1 (which is a substrate of ADAM-10) regulates neuronal migration, neurite outgrowth, and myelination. The ADAM-10 substrate N-cadherin is known to play a crucial role in synaptic development and neuronal survival. It is speculated that shedding of these molecules modulates neuronal cell contact and migration influencing cell-signaling pathways. ADAM-10 inhibition could lead to harmful side effects in the nervous system. This speculation is supported through an animal experiment in which cell-penetrating peptides interfered with ADAM-10 function resulting in AD-like neuropathology in mice within a very short period [28].

### 5.3.2

#### **ADAM-10 in Cancer Pathology**

When ADAM-10 activity is upregulated, an increase in soluble ligands and receptors could be generated that promote tumor cell proliferation and migration. A variety of mechanisms exists whereby released proteins can cause or exacerbate cancer. Various substrates of ADAM-10 have been observed to assist tumor progression. Table 5.2 provides a list of ADAM-10 substrates and their roles in cancer progression.

**Table 5.2** ADAM-10 substrates and their roles in cancer.

Substrates for ADAM-10 involved in cancer progression	Role in cancer progression	References
Betacellulin	Angiogenesis, proliferation, migration, and differentiation	[29]
EGF	Proliferation, invasion, metastasis, angiogenesis, and inhibition of apoptosis	[30]
IL-6	Proliferation and invasion	[31]
CD-30	Apoptosis and gene regulation	[32]
HER-2	Proliferation, invasion, metastasis, angiogenesis, and inhibition of apoptosis	[33]
L-1	Adhesion, migration, invasion, metastasis, inhibition of apoptosis, and gene regulation	[12]
CD44	Adhesion, migration, invasion, metastasis, inhibition of apoptosis, and gene regulation	[34, 35]
E-cadherin	Adhesion, migration, invasion, metastasis, and gene regulation	[36, 37]
N-cadherin	Adhesion and migration	[11, 36]
Notch	Tumorigenesis, angiogenesis, and inhibition of apoptosis	[38]
Delta	Tumor growth and progression and inhibition of apoptosis	[39]
Ephrin	Migration	[40]

## 5.4

### ADAM-10 as Potential Drug Target

The enzyme ADAM-10 has been studied in various disease conditions such as atherosclerosis, ectopic dermatitis, colon carcinoma, gastric cancer, leukemia, prostate cancer, ovarian cancer, and AD [18]. Given that ADAM 10 is an  $\alpha$ -secretase for APP processing, it can be considered to be a protective factor in the etiology of AD [41, 42]. The amyloidogenic and possible pathological pathway involves cleavage of APP by both  $\beta$ -secretase (BACE) and a  $\gamma$ -secretase–presenilin-1 complex that results in the formation of A-beta, which might then aggregate and deposit as extracellular amyloid plaques in the AD [43]. Overexpression of ADAM-10 has been observed to assist tumor progression, and has been recently observed to develop resistance to chemotherapy [17, 44]. Hence, inhibitors and activators of ADAM-10 can serve as potential drug targets for treatment of AD and cancer. The following sections will elaborate on the current advancement toward developing ADAM-10-modulating drugs by employing synthetic and natural products.

## 5.5

## Synthetic Inhibitors of ADAM-10

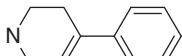
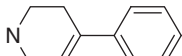
In pursuit of developing potent, selective, and orally bioavailable ADAM-10 inhibitors, a group of scientists from InCyte Corporation have published a series of papers on inhibitors with different structural moieties. The first report in 2007 extensively varied the substituents, and generated two potent and selective inhibitors (Table 5.3) [45].

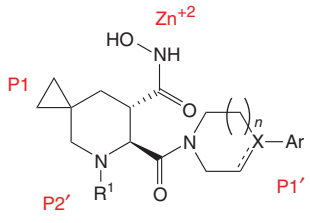
Another report from the same group revealed that the same scaffold can be successfully manipulated to generate a series of selective ADAM-10 inhibitors (Table 5.4) [45]. These inhibitors demonstrated improved selectivity toward MMP-2 (19 times) and MMP-9 (16-fold) compared to the previous report. The newly discovered inhibitors exhibited slightly lower plasma exposure than the other one in discrete oral pharmacokinetic murine studies.

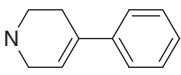
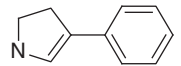
Considering the synthetic difficulty posed by the chiral spiro-cyclopropyl ring, another library of selective ADAM-10 inhibitors with a different scaffold was reported by the same group [45]. The general structure of the inhibitors is depicted in Table 5.5.

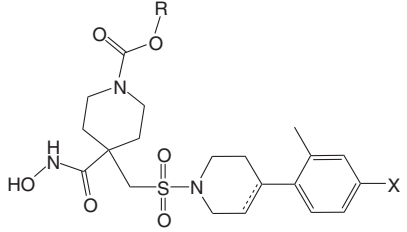
For the design of inhibitors for metalloenzymes, optimization of the P' pocket is believed to bring specificity and selectivity. The P1 region is solvent exposed, and it does not contribute significantly to the selectivity. Burns and coworkers applied the nonconventional approach to design new inhibitors. They successfully designed and synthesized a set of very potent and selective inhibitors, and demonstrated that the role that absolute configuration of C5 plays is critical in determining inhibitory potency (Table 5.6) [46].

Table 5.3 Selective inhibitors for ADAM-10.

Enzymatic binding IC <sub>50</sub> (nM)		HER-2	ADAM-10	MMP-1	MMP-2	MMP-3	MMP-9
R <sup>1</sup>	R <sup>2</sup>						
H		18	33	3900	5	1200	113
COOMe		68	59	>5000	39	4000	428

**Table 5.4** Modified, improved inhibitors for ADAM-10.


Enzymatic binding IC <sub>50</sub> (nM)		HER-2	ADAM-10	MMP-1	MMP-2	MMP-3	MMP-9
R <sup>1</sup>	R <sup>2</sup>						
COOMe		68	59	>5000	39	4000	428
COOMe		34	26	>5000	488	>5000	3048

**Table 5.5** Selective ADAM-10 inhibitors lacking the spiro-cyclopropyl group.


Enzymatic binding IC <sub>50</sub> (nM)		HER-2	ADAM-10	MMP-1	MMP-2
R	X				
(2 <i>S</i> )-Pyrrolidin-2-ylmethyl	CN	6.5	9.1	>5000	1272
(2 <i>R</i> )-Pyrrolidin-2-ylmethyl	CN	23	11	>5000	1380
Pyrrolidin-3-yl	CN	13	7.8	>5000	>2000

As an extension of the aforementioned approach (optimization of P1 moiety), Li *et al.* [46] reported a series of carbamate-based selective and potent ADAM-10 inhibitors (Table 5.7).

There are a few patent applications related to ADAM-10 inhibitors. David and coworkers, in their invention, claimed amino acid-based hydroxamate derivatives

**Table 5.6** Selective ADAM-10 inhibitors based on trisubstituted cyclohexanes.

R <sup>1</sup>	R <sup>2</sup>	C5 Enzymatic binding IC <sub>50</sub> (nM)				
			ADAM-10	MMP-1	MMP-2	MMP-3
	CH <sub>2</sub> C(O)piperidin-1 yl <i>S</i>	15	>5000	100	>5000	683
	CH <sub>2</sub> C(O)piperidin-1 yl <i>R</i>	165	—	1000	—	—
	CH <sub>2</sub> C(O)piperidin-1 yl <i>S</i>	21	>5000	>5000	>5000	>5000
	CH <sub>2</sub> C(O)piperidin-1 yl <i>R</i>	915	—	>5000	—	—

as inhibitors of ADAM-10 with a representative compound (**PT1**) having IC<sub>50</sub> < 50 nM (Figure 5.2). This invention also claimed that these derivatives will be useful to elucidate the role of ADAM-10, and will be a treatment option for cancer, arthritis, asthma, atopic dermatitis, and allergic rhinitis [47].

The same group was awarded another patent for a different class of compounds (representative structure **PT2** and **PT3**, Figure 5.2) as the first selective ADAM-10 inhibitor. These compounds were tested against eight metalloproteases, and the representative compound was found to be selective for ADAM-10 over MMP-1 [48]. As with the earlier patent, this patent also claimed that it would provide a treatment option for different diseased conditions where ADAM-10 plays a critical role.

In another patent application, a set of hydroxamates and their Mg<sup>2+</sup> salts were claimed to be ADAM-10 inhibitors (**PT4**, Figure 5.2). This is the first claim to use the salt form as the inhibitor [49]. These compounds/salts were also applied with an acceptable pharmaceutical carrier. It was claimed that the salts and the

**Table 5.7** Reported carbamate-based selective ADAM-10 inhibitors.

X	HER-2 IC <sub>50</sub> (nM)	ADAM-10 IC <sub>50</sub> (nM)	MMP-1 IC <sub>50</sub> (nM)	MMP-2 IC <sub>50</sub> (nM)	MMP-3 IC <sub>50</sub> (nM)	MMP-9 IC <sub>50</sub> (nM)
	7.3	9.6	>5000	344	>5000	>5000
	4.4	35	>5000	843	>5000	>5000
	27.5	26	>5000	1475	>5000	>5000

composition with the pharmaceutical carrier would be beneficial in treating a wide array of diseases such as cancer, arthritis, diseases related to angiogenesis, renal diseases, heart diseases, atherosclerosis, stroke, inflammation, ulcer, infertility, scleroderma, endometriosis, mesothelioma, and diabetes.

In another invention, Wood claimed a different use of the inhibitor **PT5** (Figure 5.2). This compound is an inhibitor of ADAM-10 and MMP-2, and can be used to treat aneurysmal dilatation or blood vessel wall weakness [50]. Different formulations of **PT5** with other MMP inhibitors in acceptable pharmaceutical carrier were also reported. It was also claimed that, in conjunction with angiotensin II inhibitors, angiotensin II receptor blocker, angiotensin-converting enzyme (ACE) inhibitor, or cyclophilin inhibitors, this compound or the formulations could treat aneurysmal dilatation, blood vessel wall weakness, specifically abdominal aortic aneurysm and thoracic aneurysm.

In another patent, Bird and coinventors have reported a few peptides capable of inhibiting ADAM-10. This was the first report of inhibition of ADAM-10 by peptides or peptide-like molecules. The sequence of three representative peptides are as follows: [2-(7-methoxy-2-oxo-2H-chromen-4-yl)-acetyl-Arg-Ala-Glu-Gln-Gln-Arg-Leu-Lys-Ser-Gln-Asp-Leu-Glu-Ser(2,4-dinitrophenylamine)-COOH], 2-(7-methoxy-2-oxo-2H-chromen-4-yl)-acetyl-Pro-Leu-Ala-Gln-Ala-Val-Ser(2,4-dinitrophenylamine)-Arg-Ser-Ser-Ser-Arg-CONH<sub>2</sub>, and AC-NH-Arg-Ala-Glu-Arg-Leu-Lys-Ser-Gln-Asp-Leu-CONH<sub>2</sub>. According to the claim, these peptides

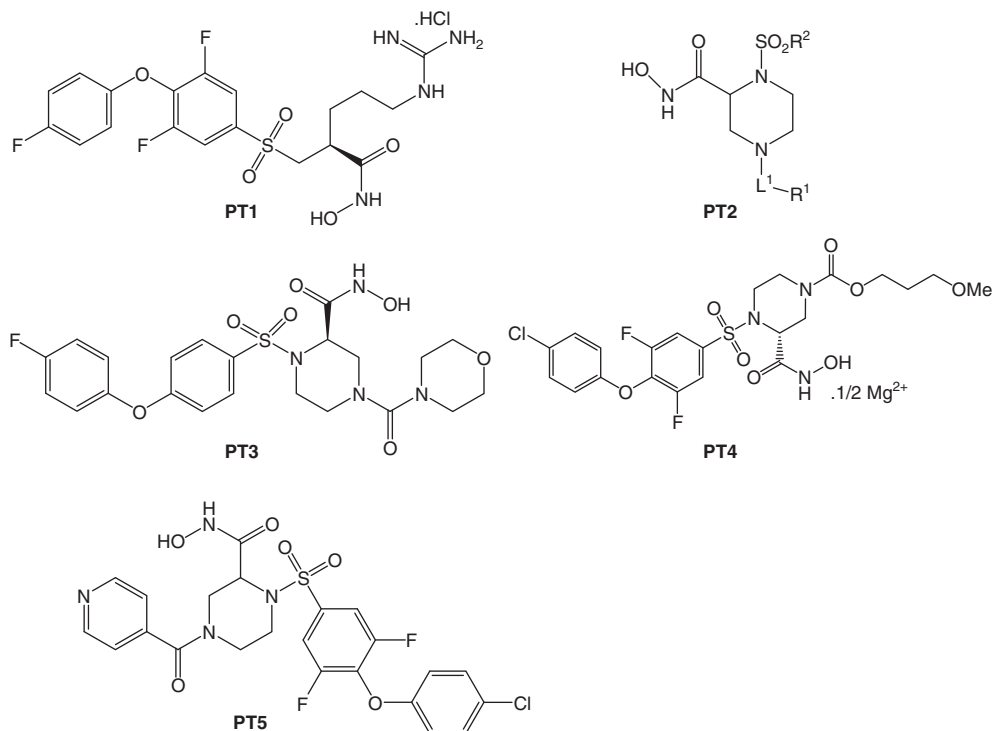


Figure 5.2 ADAM-10 inhibitors reported in patent applications.

can be used for the treatment of allergic diseases such as allergic rhinitis and asthma [51].

## 5.6

### Natural Products as Activators and Inhibitors for ADAM-10

Natural products offer molecular diversity and biological functionality, which is indispensable for drug discovery. Exploitation of these diverse structural databases for their potential targets can reveal vast numbers of potential candidates for drug discovery [52]. Various natural product activators and inhibitors of ADAM-10 have been identified as potential leads for treatment of diseases [53]. Natural products have a great potential for generating leads that can be further exploited for drug discovery [54]. Vincristine, irinotecan, etoposide, and paclitaxel are examples of plant-derived compounds that are being employed clinically for cancer treatment. Drugs from marine sources and microbial sources have also impacted various aspects of disease treatment and prevention [55–57]. However, the lack of an extract library poses some difficulty



in advancement of these products to the clinical stages. For treatment of AD, numerous natural products have been used traditionally. Some of those have been recognized as inhibitors of the enzyme acetylcholinesterase, while some of them are activators of ADAM-10 [58]. In this review, we will view activators of ADAM-10 as treatment options for AD and inhibitors as potential anticancer therapy.

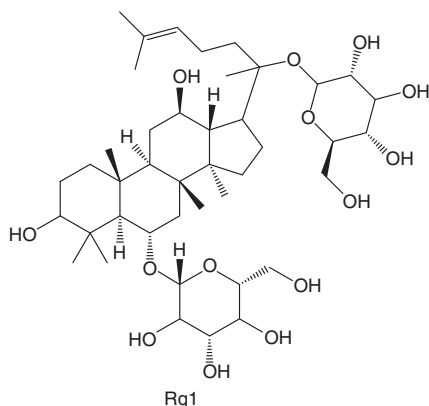
## 5.7

### Natural Products as ADAM-10 Activators

More than 20 plant species have been identified for their roles in disease prevention that include *Ginseng*, *Curcuma longa*, *Ginkgo biloba*, *Lycoris radiate*, *Galanthus nivalis*, *Magnolia officinalis*, *Polygala tenuifolia*, *Salvia lavandulaefolia*, *Salvia miltiorrhiza*, *Coptis chinensis*, *Crocus sativus*, *Evodia rutaecarpa*, *Sanguisorba officinalis*, *Veratrum grandiflorum*, *Picrorhiza kurvoa*, *Acorus calamus*, and *Marlierea racemosa*. The list of these species will keep on growing as terpenoids and alkaloids show great potential as cognition enhancers for the treatment of AD [59, 60]. Although many chemical entities from this pool of natural products can be ADAM-10 activators, only a few have been studied as potential ADAM-10 activators.

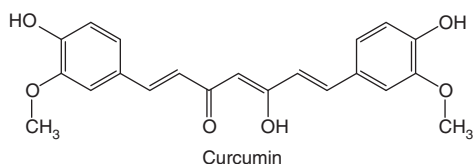
ADAM-10 expression is also known to be regulated by nicotinamide adenine dinucleotide-dependent deacetylase sirtuin-1 (SIRT1). Enzymatic activation of SIRT1 can be achieved by the red wine compound resveratrol. This compound has successfully reduced plaque pathology in an APP-transgenic AD mouse model, suggesting that resveratrol and other polyphenolic stilbenoid compounds may also have neuroprotective properties. Cleavage of RAGE mediated by ADAM-10 and matrix metalloproteinase-9 (MMP-9) is also observed in AD [61]. Hence, strategies to increase  $\alpha$ -secretase-mediated processing of APP and RAGE may have therapeutic values for the treatment of AD. Today, it is known that ADAM-10 conducts the major  $\alpha$ -secretase activity, which mediates APP and RAGE processing. The  $\alpha$ -secretase-mediated APP cleavage can be activated via several G protein-coupled receptors and receptor tyrosine kinases; protein kinase C, mitogen-activated protein kinases, phosphatidylinositol 3-kinase, camp cyclic adenosine monophosphate (cAMP), and calcium. As discussed in the previous section, ADAM-10 acts as  $\alpha$ -secretase on APP, and hence the expression of ADAM-10 and non-amyloidogenic APP processing can be enhanced as a treatment option for AD. However, excessive activation should be avoided as ADAM-10 also mediates shedding of diverse signaling molecules, and overexpression of ADAM-10 has been associated with tumorigenesis and tumor progression [62].

## 5.7.1

**Ginsenoside R**

Ginsenoside Rg1 is a pharmacologically active ingredient of ginseng extracts. It has been observed to have neuroprotective activity. It has been also observed to be a protective agent against neurodegenerative diseases induced by mitochondrial toxins [63]. Rg1 improves spatial learning and memory, and is a useful agent for prevention and treatment of cognitive impairment associated with AD [64]. An animal study on D-galactose-injected rat model of AD exhibited increase in ADAM-10 levels, and decrease in BACE1 expression when treated with ginsenoside Rg1 [65].

## 5.7.2

***Curcuma longa***

Curcumin (diferuloylmethane) is the main active ingredient from the extracts of plant *C. longa* (turmeric). It was speculated that this compound will successfully cross the blood–brain barrier owing to its hydrophobic nature [66]. Curcumin was suggested as a promising therapy for AD as it displays anti-inflammatory, antioxidant properties along with its involvement in regulation of  $\beta$ -APP biology [67]. It can impair A $\beta$ 42 production, reduce  $\beta$ APP protein levels, suppress A $\beta$ -induced BACE1 upregulation, and attenuate  $\beta$ APP maturation in the secretory pathway, thereby decreasing A $\beta$  [68, 69]. Although this molecule is highly hydrophobic in nature, clinical studies showed its low absorption,

rapid metabolism, and inherent instability. Clinical trials carried out with curcumin did not show any improvement of cognitive functions in humans with dementia [70]. To increase its bioavailability, its metabolite tetrahydrocurcumin, and three amino acid-conjugated curcumin derivatives, curcumin–isoleucine, curcumin–phenylalanine, and curcumin–valine were tested for their cognition-enhancing properties. It was observed that the amino acid conjugates induced the constitutive  $\alpha$ -secretase cleavage of  $\beta$ APP through an increase in ADAM-10 immunoreactivity. Curcumin conjugates can certainly serve as new lead to natural compounds for improvement in cognitive functions in AD patients [71].

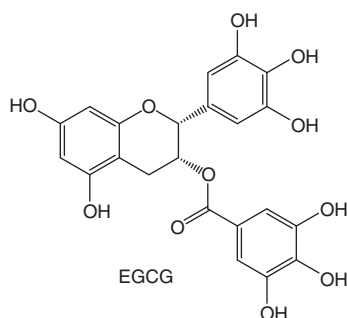
### 5.7.3

#### *Ginkgo biloba*

*G. biloba* extract is another example of a well-studied natural product for its applications in cognitive dysfunction associated with AD. Clinical trials report that *G. biloba* extracts represent reduction in age-associated memory impairment and dementia, including AD [72]. However, the mechanisms behind their neuroprotective ability remain to be fully established. The effect of EGb761 (standardized extract of *G. biloba* leaves) on the APP metabolism has been investigated by both *in vitro* and *in vivo* models. Neuroprotective effect and enhanced learning and memory in those brain regions was observed along with increase in  $\alpha$ -secretase activity [73].

### 5.7.4

#### Green Tea



Green tea flavonoids are gaining popularity for their antioxidant properties, and for enhancing cognitive properties. However, not all the flavonoids help in enhancing cognitive functions. Epigallocatechin-3-gallate (EGCG) has been identified as cognition enhancer [74, 75]. Anti-amyloidogenic properties of green tea have been evaluated *in vivo* in mice [76, 77]. The mechanism of EGCG has been postulated as a promoter of  $\alpha$ -secretase cleavage [78], however, it needs to be further investigated as a therapeutic target for AD.

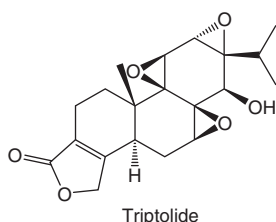
## 5.8

### Natural Products as ADAM-10 Inhibitors

Overexpression of ADAM-10 in cancer and inflammatory diseases increases the potential of its inhibitors as therapeutic entities. Inhibitors of ADAM-10 have been observed to exhibit anti-inflammatory and anticancer activity *in vitro* [79]. However, very few natural products have been tested as ADAM-10 inhibitors. Triptolide and its derivative minnelide have been used clinically for treatment of pancreatic cancer. The drugs are still in clinical trials, but are worth a mention here as triptolide has been found to inhibit ADAM-10 *in vivo*.

#### 5.8.1

##### Triptolide



Triptolide, a diterpene triepoxide, was first isolated and structurally characterized in 1972 from the medicinal plant *Tripterygium wilfordii*. It has been used for centuries in traditional Chinese medicine to treat inflammatory and autoimmune diseases. It has also demonstrated its antitumor effect, and now the triptolide derivatives are in clinical trials for treatment of pancreatic cancer. Multiple studies have been published describing molecular mechanism underlying the antitumor effect of triptolide [80]. Multiple molecular mechanisms have been postulated for the antitumor effect of triptolide which will not be discussed in detail in this chapter. One of the mechanisms relevant to this discussion is inhibition of ADAM-10. ADAM-10 is a novel target of triptolide.

The multiple modes of action for triptolide have been attributed to its functional groups. The anti-inflammatory function of triptolide has been linked to its 12,13-epoxide group, whereas the C-14 hydroxyl group was associated with immunosuppressive functions [81]. The disruption of the 9,11-epoxide group was shown to affect the antitumor activity of triptolide. Moreover, in a recent study, triptolide-binding activity in cells was shown to be both saturable and reversible, as well as calcium dependent with localization to the plasma membrane. It was shown that triptolide-induced cell death in HeLa cells was affected by calcium levels, and partially by C-14 hydroxyl group, whereas NF $\kappa$ B transcriptional inhibition was calcium independent and affected by disruption of the 12,13-epoxide group [82].

Despite these advances and intense investigations by numerous groups, the mechanisms responsible for its anticancer activities are not fully understood. One of the putative targets of triptolide is ADAM-10. The overexpression of ADAM-10 in tumors including leukemia led the researchers to investigate the effect of triptolide treatment on ADAM-10 expression in U937 and MCF-7 cells. The effects of siRNA mediated knockdown of ADAM-10 expression and triptolide treatment on the proliferation of MCF-7 cells were also investigated. ADAM-10 has been identified as a potential target for the triptolide.

The mechanisms that are involved in triptolide-mediated apoptosis or growth inhibition in cancer cells are not fully understood. However the interaction between triptolide and ADAM-10 is observed using affinity chromatography, mass spectrometry, and Western blot analysis using an anti-ADAM-10 antibody. Triptolide, at concentrations in the nanomolar range, exhibited significant decrease in ADAM-10 expression followed by the appearance of an ADAM-10-cleaved product which allowed to confirm this interaction. Furthermore, triptolide treatment of MCF-7 breast cancer cells expressing ectopic ADAM-10 or dominant negative ADAM-10 (DN ADAM-10) resulted in a decreased expression of ADAM-10 with a concomitant increase in ADAM-10 cleaved products. Hence, ADAM-10 is a target of triptolide and this presents a novel strategy to inhibit ADAM-10 activity in tumorigenesis [83].

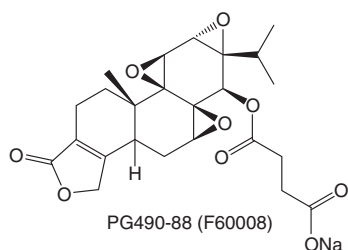
Natural compounds may have multiple cellular targets in order to achieve their beneficial biological effects such as tumor growth inhibition. Clinical trials in China revealed that triptolide achieved a total remission rate of 71% in mononucleocytic leukemia and 87% in granulocytic leukemia, which is more effective than any chemotherapeutic agents currently available [84]. A Phase I clinical trial of the effect of a water-soluble derivative of triptolide on solid tumors is ongoing in Europe [85]. It is possible that triptolide has multiple cellular targets for inducing cancer-cell death. It is expected that this small-molecule natural product may prove to be a candidate for the systemic therapy of cancer, and has a great potential either as a single agent or in combination with conventional therapies. Modulating the structure, and therefore function, of triptolide may lead to the development of a promising strategy applied to a broad spectrum of cancer treatments [84].

Triptolide was also observed to increase cytotoxicity of chemotherapeutic agents in a synergistic manner. A combination therapy of 5-fluorouracil and triptolide showed better cytotoxicity in colon carcinoma and KB cancer cells. Triptolide combined with 5-FU exhibited synergistic effects at lower concentrations, and promoted apoptosis, but did not increase the side effects of chemotherapy for colon cancer [86]. Triptolide was also observed to decrease the expression of multidrug-resistant protein and multidrug resistant (MDR) in KB-7D and KB-tax cells. For pancreatic cancer, compared to single treatment, combination of triptolide with ionizing radiation (IR) reduced cell survival to 21%, enhancing tumor cell apoptosis. Tumor growth of human pancreatic cancer

cells AsPC-1 xenografts was further reduced in the group treated with triptolide combined with IR in comparison with single treatment *in vivo* [87]. Triptolide was also able to enhance the activities of dexamethasone in multiple myeloma cell lines. Triptolide has been observed to induce apoptosis in dexamethasone-sensitive and dexamethasone-resistant cells [87]. Enhancement of the cytotoxic effect was also observed in combination therapy of doxorubicin with triptolide. Triptolide has shown antitumor activity against a broad spectrum of tumors.

#### 5.8.1.1 Novel Derivatives and Carriers of Triptolide

Although triptolide has emerged as potent anticancer product of natural origin, some synthetic and semisynthetic derivatives of triptolide have been studied *in vivo* and *in vitro*. One of such derivatives is PG490-88, a semisynthetic derivative of triptolide (also known as *F60008*), which gets converted into triptolide *in vivo* executing apoptosis in human tumor cells [80]. This compound has been also observed to enhance sensitivity of the cancer cells to chemotherapy [88].



Although triptolide possesses potent antitumor activity, it also shows immunosuppressive activities which are disadvantageous for treatment of cancer [89]. In order to avoid these untoward side effects, a novel polymeric micellar system containing triptolide (TP-PM) was constructed by the solvent evaporation method using methoxypolyethylene glycol-poly (D,L-lactic acid)-block copolymer as the carrier [90]. These nanocarriers showed long-term stability and potential to inhibit tumor growth significantly when administered via intravenous injections. Simultaneously, TP-PM had no effect on the thymus index, spleen index, spleen lymphocyte proliferation, and tumor necrosis factor (TNF)- $\alpha$  and interleukin (IL)-2 levels in serum as compared to triptolide. Triptolide encapsulated in polymeric micelles does not demonstrate immunosuppressive activity but still exhibits its antitumor effect [91]. However, the clinical use of triptolide is often limited by its severe toxicity and water-insolubility. New water-soluble triptolide derivatives have been designed and synthesized. Minnelide is a water-soluble phosphate salt of triptolide, which is currently in clinical trials for treatment of pancreatic cancer [92]. Other water-soluble triptolide analogs such as PG490-88 or F60008 are also in clinical trials for treatment of prostate cancer [80, 93].

## 5.9

**Concluding Remarks**

Natural products offer various opportunities for treatment of chronic ailments such as cancer and AD. Involvement of ADAM-10 in the progression of cancer and AD is well known, and various synthetic activators and inhibitors of ADAM-10 have been synthesized and tested as potential therapy options. Natural products from plant, marine, and microbial sources have been used for anticancer therapy, and also for managing the symptoms of AD. However, very few studies indicate interactions between natural products and ADAM-10. ADAM-10 overexpressed in cancer and is downregulated in AD, but many natural products show neuroprotective as well as anticancer properties. Our understanding of the natural products as activators and inhibitors of ADAM-10 is currently in the infantile stage, and further studies are required in the field for exploring safe and effective treatment options. In conclusion, natural product activators and inhibitors of ADAM-10 can certainly serve as lead compounds for drug discovery and development for treatment of cancer and AD.

**Abbreviations**

A $\beta$	amyloid $\beta$ -peptide
ADAM	a disintegrin and a metalloproteinase
AD	Alzheimer's disease
APP	amyloid precursor protein
EGCG	epigallocatechin-3-gallate
EGF	epidermal growth factor
HER	human epidermal growth factor receptor
IL	interleukin
MDR	multidrug resistant
MMP	matrix metalloproteinase
RAGE	receptor for advanced glycation endproducts
SVMP	snake venom metalloproteases
TNF	tumor necrosis factor
ADAM-TS	A disintegrin and a metalloproteinase with thrombospondin motifs
IC50	Half maximal inhibitory concentration

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## 6

# Structure and Biological Activity of Polyether Ionophores and Their Semisynthetic Derivatives

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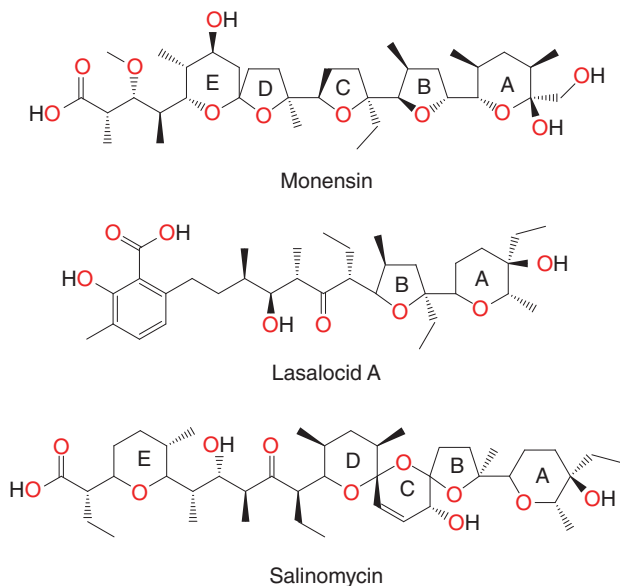
### 6.1

#### Introduction

Natural carboxylic polyether ionophores such as monensin, salinomycin, and lasalocid have been objects of great interest because of their antibacterial, antifungal, antiparasitic, and antiviral biological activities. Recently, it has been found that polyether ionophores might be also important chemotherapeutic agents in the treatment of cancer. These compounds have shown potent activity against the proliferation of various cancer cells – including those that display multidrug resistance (MDR) – and cancer stem cells (CSCs).

Polyether ionophores represent a very large and important group of naturally occurring compounds produced by *Streptomyces* spp. Increased interest in compounds of this type has been observed in recent years. There are over 120 naturally occurring ionophores known so far [1]. Major commercial use of ionophores is to control coccidiosis. They are also used as growth promoters in ruminants. These compounds specifically target the ruminal bacteria populations and their use permits increasing production efficiency. In 2003, the antimicrobials most commonly used in beef cattle production were ionophores. Lasalocid (Avatec<sup>®</sup>, Bovatec<sup>®</sup>), monensin (Coban<sup>®</sup>, Rumensin<sup>®</sup>, Coxidin<sup>®</sup>), salinomycin (Bio-cox<sup>®</sup>, Sacox<sup>®</sup>), narasin (Monteban<sup>®</sup>, Maxiban<sup>®</sup>), maduramycin (Cygro<sup>®</sup>), and laidlomycin propionate (Cattlyst<sup>®</sup>) were the ionophores whose combined annual sales were more than \$150 million [2, 3]. Ionophores can also be used in the production of ion-selective electrodes [4, 5]. Polyether skeletons of the pseudocyclic structure of polyether ionophores are able to form complexes with metal cations and facilitate their transport across cellular membranes. It has been shown that the chemical modification of polyether antibiotics can change the ability and the selectivity of metal cations binding and modifying the mechanism of cation transport, thereby leading to new antibacterial and anticancer active compounds.

All the aforementioned applications of ionophores are closely related to their structure and ability to form complexes with metal cations (host–guest complexes) and transport these complexes across lipid bilayers and cell membranes. This chapter deals with the structure and chemical properties of ionophores and their derivatives. Monensin, salinomycin, and lasalocid acid are three of the best-known polyether ionophores, and hence detailed discussion on each of them is taken care of (Scheme 6.1). Basic information about other polyether ionophores is given in Table 6.1.



**Scheme 6.1** Structures of the most important polyether ionophores.

## 6.2

### Structures of Polyether Ionophores and Their Derivatives

Polyether ionophores are also known as *nigericin antibiotics*. They make a large class of related compounds consisting of similar building units. Polyether ionophores all contain tetrahydrofuran and tetrahydropyran rings, sometimes with spiro junctions. The chemical formulae, normally written in linear form, hide the fact that almost all of these compounds occur in cyclic form, with head-to-tail hydrogen bonds formed between the carboxyl group present on one side of the molecule and the hydroxyl group on the other. The cyclic conformation is found in uncomplexed molecules as well as in cation complexes [59]. The interior part of these molecules is polar because of the presence of ether, hydroxyl, and carbonyl



Table 6.1 Basic information about polyether ionophores.

Compound	Year and source of isolation	Biological activity	Crystal structure	References
Nigericin (185) (Figure 6.28)	1951, <i>Streptomyces hygroscopicus</i>	Gram-positive bacteria	Crystal salt with Ag <sup>+</sup>	[6, 7]
Grisorixin (186) (Figure 6.28)	1975, <i>Streptomyces griseus</i>	Gram-positive bacteria and antimalarial ( <i>Plasmodium falciparum</i> )	Crystal salt with Ag <sup>+</sup>	[8–10]
Lonomycin (187) (Figure 6.28)	1975, <i>Streptomyces ribosidificus</i>	Gram-positive bacteria and <i>Plasmodium falciparum</i>	Crystal salt with Tl <sup>+</sup>	[11, 12]
Mutalomycin (188) (Figure 6.28)	1977, <i>Streptomyces mutabilis</i>	Gram-positive bacteria and anticoccidial ( <i>Eimeria tenella</i> )	Crystal salt of its epimer 28-epimutalomycin with K <sup>+</sup> (Figure 6.29)	[13, 14]
A-204A (189) (Figure 6.28)	1973, <i>Streptomyces albus</i>	Gram-positive bacteria, fungi, and anticoccidial ( <i>Eimeria tenella</i> )	Crystal salt with Na <sup>+</sup>	[15]
Carrimycin (190) (Figure 6.28)	1977, <i>Streptomyces hygroscopicus</i>	Gram-positive bacteria and fungi ( <i>Candida albicans</i> ) anticoccidial ( <i>Eimeria tenella</i> , <i>Eimeria acervulina</i> , and <i>Eimeria maxima</i> )	Crystal salt with Tl <sup>+</sup>	[16, 17]
Septamycin (191) (Figure 6.28)	1975, <i>Streptomyces hygroscopicus</i>	Gram-positive bacteria and anticoccidial ( <i>Eimeria tenella</i> , <i>Eimeria acervulina</i> , and <i>Eimeria maxima</i> )	<i>p</i> -Bromophenacyl ester derivative	[18, 19]
K-41 (192) (Figure 6.28)	1976, <i>Streptomyces hygroscopicus</i>	Gram-positive bacteria and antimalarial ( <i>Plasmodium falciparum</i> )	Crystal salt of its <i>p</i> -bromobenzoate with Na <sup>+</sup>	[20–22]
Antibiotic 6016 (193) (Figure 6.28)	1978, <i>Streptomyces albus</i>	Gram-positive bacteria, mycobacteria, and anticoccidial ( <i>Eimeria tenella</i> )	Crystal salt with Tl <sup>+</sup> (Figure 6.30)	[23, 24]

(continued overleaf)

Table 6.1 (Continued)

Compound	Year and source of isolation	Biological activity	Crystal structure	References
Dianemycin (194) (Figure 6.31)	1978, <i>Streptomyces hygroscopicus</i>	Gram-positive bacteria, fungi, and mycobacteria, anticoccidial ( <i>Eimeria tenella</i> )	Crystal salt with Na <sup>+</sup>	[25, 26]
Lenoremycin (195) (Figure 6.31)	1975, <i>Streptomyces hygroscopicus</i>	Gram-positive bacteria and fungi	Crystal salt with Ag <sup>+</sup>	[27, 28]
Endusamycin (196) (Figure 6.31)	1989, <i>Streptomyces endus</i>	Gram-positive bacteria, anaerobic bacteria ( <i>Treponema hyodysenteriae</i> ), and anticoccidial ( <i>Eimeria tenella</i> , and <i>Eimeria acervulina</i> )	Crystal salt with Rb <sup>+</sup>	[29]
CP-80219 (197) (Figure 6.31)	1975, <i>Streptomyces hygroscopicus</i>	Gram-positive bacteria and spirochete ( <i>Treponema hyodysenteriae</i> )	Crystal salt with Rb <sup>+</sup>	[30]
X-206 (198) (Figure 6.32)	1971, <i>Actinomyces</i> strain K99-0413	Gram-positive bacteria and anti malarial ( <i>Plasmodium falciparum</i> )	Crystal salt with Ag <sup>+</sup>	[31, 32]
Alborxin (199) (Figure 6.32)	1975, <i>Streptomyces albus</i>	Gram-positive bacteria and anticoccidial ( <i>Eimeria necatrix</i> )	Crystal salt with K <sup>+</sup>	[33, 34]
Calcimycin (200) (Figure 6.33)	1976, <i>Streptomyces chartreusensis</i>	Gram-positive bacteria and fungi	Crystal salts with Mg <sup>2+</sup> (Figure 6.34), Ni <sup>2+</sup> , Zn <sup>2+</sup>	[35–42]
Cezomycin (201) (Figure 6.33)	1982, <i>Streptomyces chartreusensis</i>	Gram-positive bacteria	—	[43]
X-14885A (202) (Figure 6.33)	1983, <i>Streptomyces chartreusensis</i>	Gram-positive bacteria: <i>Staphylococcus aureus</i> , <i>Bacillus subtilis</i> , spirochete <i>Treponema hyodysenteriae</i>	—	[44]

Cationomycin (203) (Figure 6.35)	1982, <i>Actinomadura azurea</i>	Gram-positive bacteria	Crystal salt with Tl <sup>+</sup> (Figure 6.36)	[45–48]
Ionomycin (204) (Figure 6.37)	1978, <i>Streptomyces conglobatus</i>	Gram-positive bacteria	Crystal salt with Ca <sup>2+</sup> (Figure 6.38)	[49–51]
X-14868A (205)X-14868B (206)X-14868C (207)X-14868D (208) (Figure 6.39)	1983, <i>Nocardia</i> (strains: X-14868A, X-14868B, X-14868C and X-14868D)	Active: Gram-positive bacteria and spirochete ( <i>Treponema hyodysenteriae</i> )	—	[52, 53]
Ferensimycin A (209)Ferensimycin B (210) (Figure 6.40)	1982, <i>Streptomyces</i> <i>sp.</i> No 5057	Active: Gram-positive bacteria	—	[54]
Zincophorin (211) (Figure 6.41)	1984, <i>Streptomyces griseus</i>	Active: Gram-positive bacteria and anticoccidial ( <i>Eimeria tenella</i> )	Crystal salt with Mg <sup>2+</sup>	[55]
CP-78545 (212) (Figure 6.41)	1988, <i>Streptomyces griseus</i>	Active: Gram-positive bacteria, anticoccidial ( <i>Eimeria tenella</i> ), and anaerobic bacteria ( <i>Treponema hyodysenteriae</i> )	Crystal salt with Cd <sup>2+</sup>	[56]
Indianomycin (213) (Figure 6.42)	1979, <i>Streptomyces antibioticus</i>	Active: Gram-positive bacteria	Crystal structure of its bromophenetylamine salt	[57, 58]

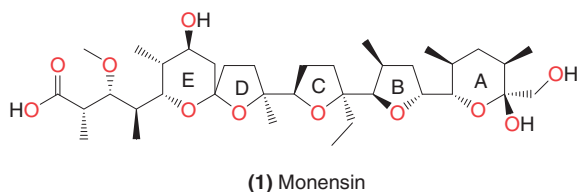
oxygen atoms, which facilitate the complexation of cations, whereas the exterior part of the molecule is hydrophobic, facilitating diffusion of complexes through a lipid bilayer [59, 60]. The carboxyl group is mostly deprotonated at physiological pH, leading to the formation of neutral complexes of the type  $[L^-M^+]$ . The normally neutral nature of cation complexes has an important consequence for the biological action of polyether ionophores. According to Westley, the polyether ionophores are classified as monovalent polyethers if they are not able to transport divalent cations, and as divalent polyethers if besides monovalent cations, they also transport divalent ones [61].

### 6.2.1

#### Monensin and Its Derivatives

Monensin (see Figure 6.1, compound **1**) was first isolated from *Streptomyces cinnamomensis* [62]. Crystal structures of monensin hydrate and its salts with sodium, lithium (see Figure 6.2), and rubidium have been resolved [63–66]. Recently, Pantcheva *et al.* have shown that monensin can also form two types of salt complex species with divalent metal cations. In the first type, monensin sodium salt forms complexes with metal dichloride of  $[M(MON-Na)_2]Cl_2 \cdot H_2O$  stoichiometry, where  $MON^-$  = monensin anion and  $M = Co^{2+}$ ,  $Mn^{2+}$ , or  $Cu^{2+}$ , so that the divalent metal cation is tetrahedrally coordinated by oxygen atoms of two carboxylic groups of two monensin sodium salt molecules and by two chloride anions. The sodium cation remains in the hydrophilic cavity of the ligand and cannot be replaced by the transition metal cation. The second type of monensin complexes with the divalent metal cations is the neutral salt of the formula  $[M(MON)_2 \cdot (H_2O)_2]$  ( $M = Mg^{2+}$ ,  $Ca^{2+}$ ,  $Zn^{2+}$ ,  $Ni^{2+}$ ,  $Cd^{2+}$ , and  $Hg^{2+}$ ). These salts consist of two monoanionic ligands (monensinates) bound in a bidentate coordination mode to the metal cation. These types of monensin salt complexes with divalent metal cation are untypical, because the etheric oxygen atoms do not play any role in the complexation of the cations. In contrast, in the typical complexes of monensin with monovalent metal cations, the etheric oxygen atoms of the monensin ligand are always involved in the complexation process.

The investigation has proved, for the first time, that monensin is able to form stable complexes with divalent metal cations [67–72]. The structure of monensin complex with  $Co^{2+}$  cation is shown in Figure 6.3.



**Figure 6.1** Structure of monensin.

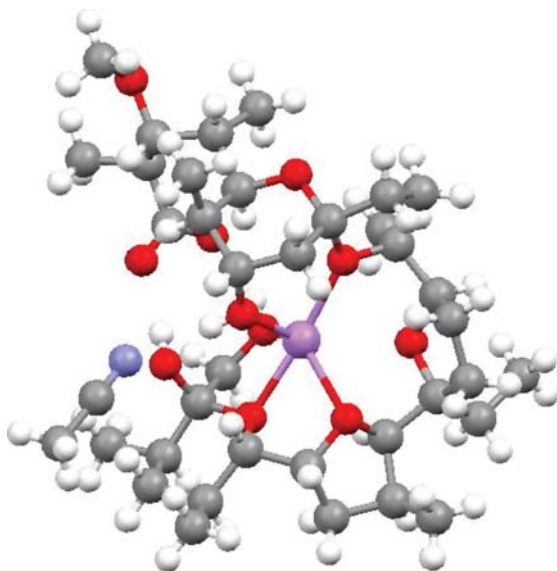


Figure 6.2 Crystal structure of monensin lithium salt.

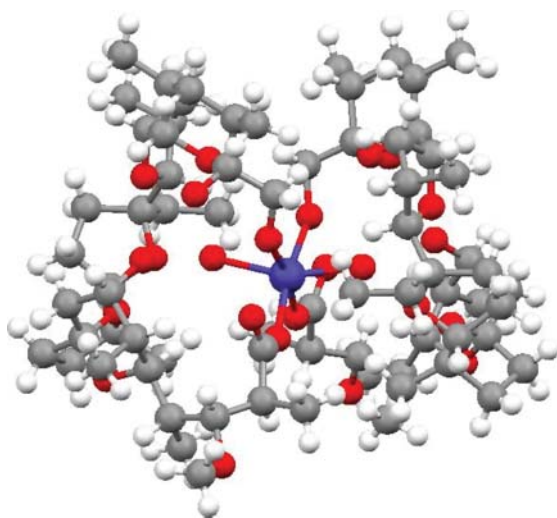


Figure 6.3 Crystal structure of monensin complex with  $\text{Co}^{2+}$  cation.

A series of monensin urethanes (see Figures 6.4 and 6.5, compounds **2–16**) esters, ethers, amides, and bioconjugates with aminoacids has been described [73–99]. These derivatives were obtained by chemical modification of O(IV)H (see Figure 6.6, compounds **17–32**), O(XI)H (see Figure 6.7, compounds **33–37**), hydroxyl groups and carboxyl group (see Figures 6.8 and 6.9, compounds **38–60**).

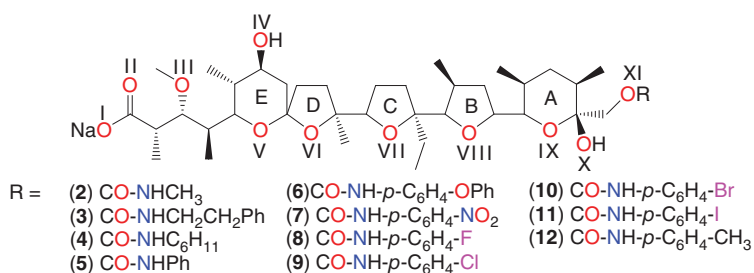


Figure 6.4 Structures of selected monensin urethanes.

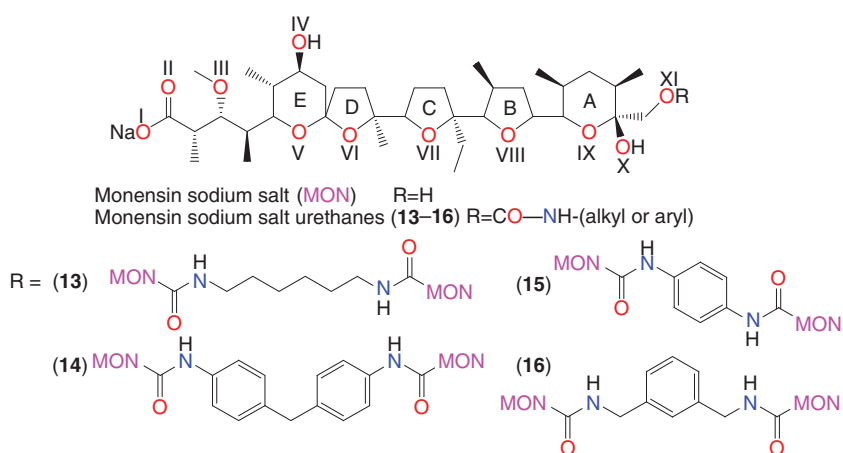


Figure 6.5 Structure of monensin diurethanes.

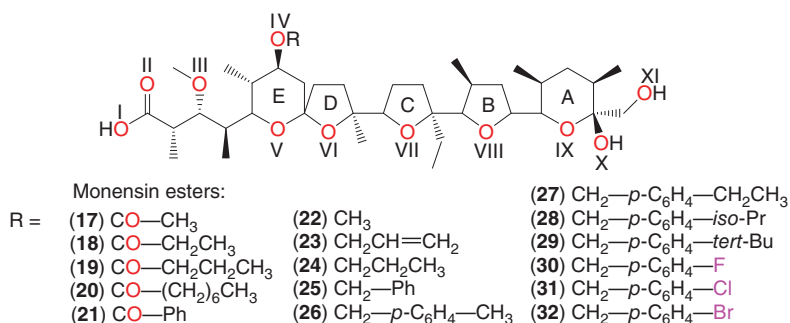


Figure 6.6 Structures of monensin O(IV)H group derivatives.

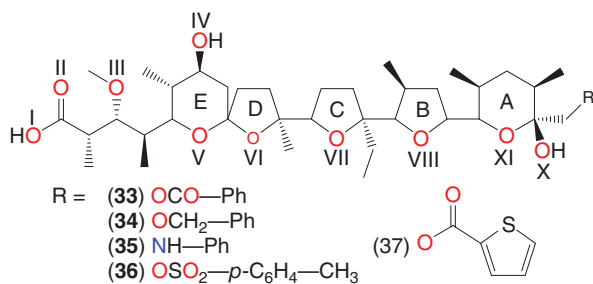


Figure 6.7 Structures of monensin O(XI)H group derivatives.

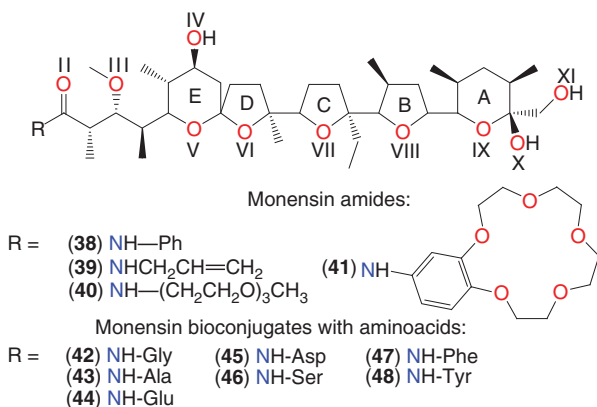


Figure 6.8 Structures of monensin amides and its bioconjugates with amino acids.

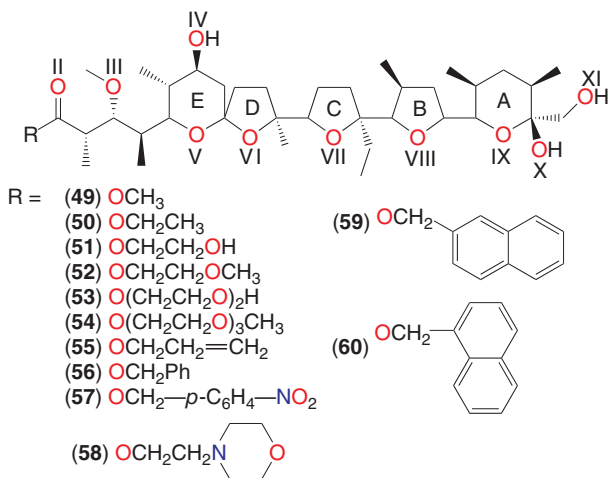


Figure 6.9 Structures of monensin esters.

Lactonization (see Figure 6.10, compounds **61–66**) of monensin has also been described [100].

Crystal structures of several of them have been reported and the structure of monensin 1-naphtylmethyl ester [101] is shown in Figure 6.11. In 2011, Huczyński *et al.* have shown for the first time that a monensin derivative was able to form complexes with divalent cations. They obtained a complex of *N*-allylamide of monensin with strontium perchlorate. In this complex,  $\text{Sr}^{2+}$  is coordinated by six atoms of the monensin derivative molecule, one oxygen atom of the perchlorate anion, and the nitrogen atom of the acetonitrile molecule (Figure 6.12) [99].

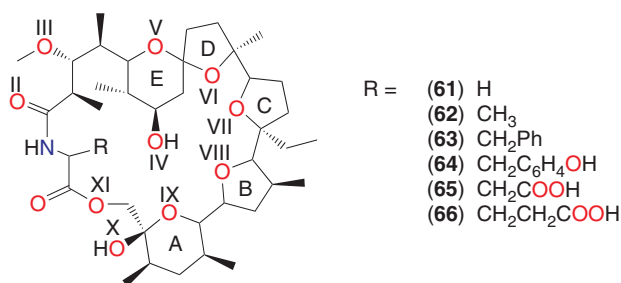


Figure 6.10 Structures of monensin lactones.

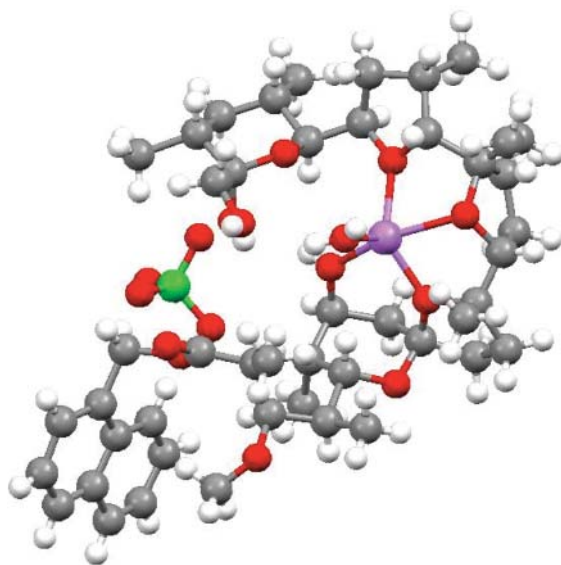
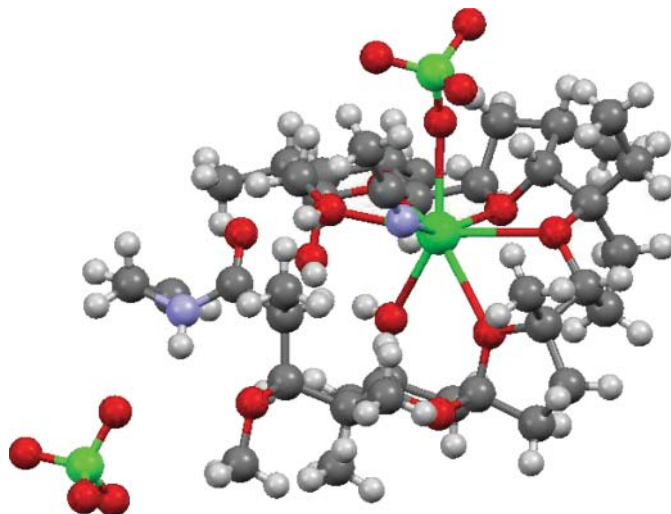


Figure 6.11 Crystal structure of monensin 1-naphtylmethyl ester with the lithium perchlorate.





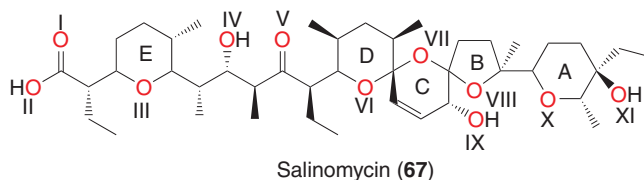
**Figure 6.12** Crystal structure of monensin *N*-allylamide complex with strontium perchlorate.

### 6.2.2

#### Salinomycin and Its Derivatives

Salinomycin (see Figure 6.13, compound **67**) was isolated from *Streptomyces albus*. Its crystal structure has been established by X-ray analysis of its *p*-iodo-phenacyl ester [102]. Compounds structurally related to salinomycin have also been obtained and described [103–105]. The relative ability of salinomycin to form complexes with various cations decreases in the order  $K^+ > Na^+ > Cs^+ > Sr^{2+} > Ca^{2+}$ ,  $Mg^{2+}$ . Salinomycin has been shown to transport monovalent cations from an aqueous buffer into an organic solvent more effectively than divalent ones [106].

Salinomycin is active against Gram-positive bacteria including mycobacteria and some filamentous fungi. No activity has been observed against Gram-negative bacteria and yeast. The acute toxicity of salinomycin in mice was examined and its median lethal dose ( $LD_{50}$ ) was  $18 \text{ mg kg}^{-1}$  intraperitoneally and  $50 \text{ mg kg}^{-1}$  orally. The anticoccidial salinomycin activity was estimated on chicken infected with



**Figure 6.13** Structure of salinomycin.

*Eimeria tenella*. Salinomycin was effective in reducing mortality of the chicken and caused an increase in average body weight of treated infected chicken compared to those of untreated infected controls [107].

A series of amide (see Figure 6.14, compounds **68–86**) and ester (see Figure 6.15, compounds **87–99**) as well as O-acylated (see Figure 6.16, compounds **100–121**) derivatives of salinomycin have been described [108–113].

### 6.2.3

#### Lasalocid Acid A and Its Derivatives

Lasalocid acid A (compound **122**), known as *lasalocid acid*, was first isolated from *Streptomyces lasaliensis* [114]. Four homologs of lasalocid acid, lasalocid acid B (compound **123**), lasalocid acid C (compound **124**), lasalocid acid D

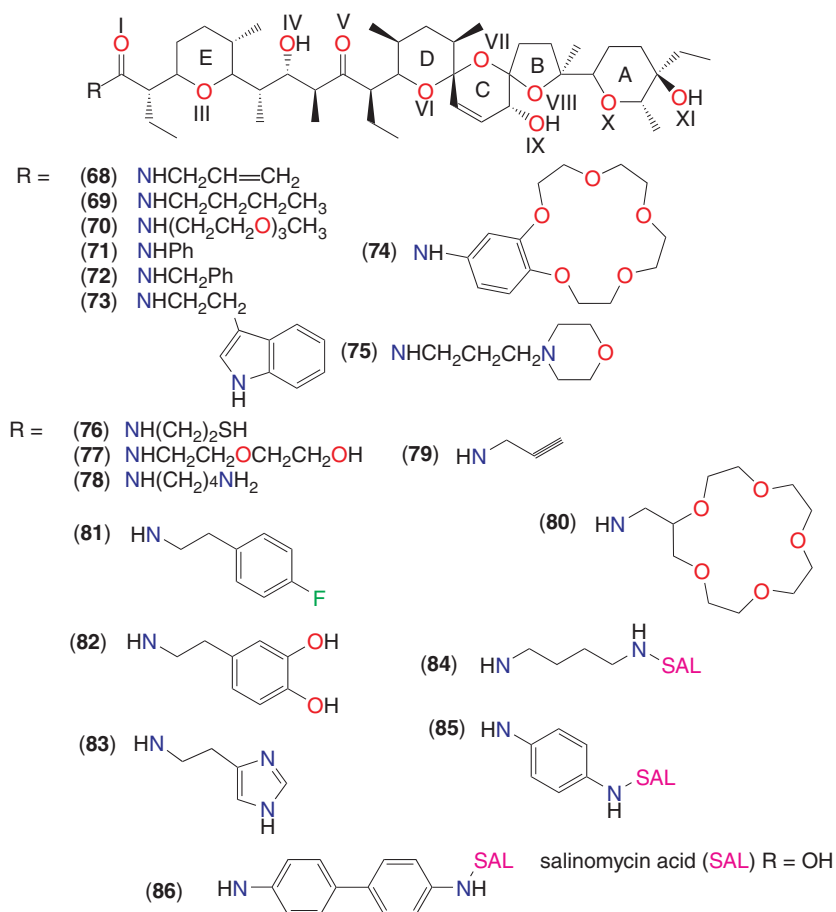


Figure 6.14 Structures of salinomycin amide derivatives.

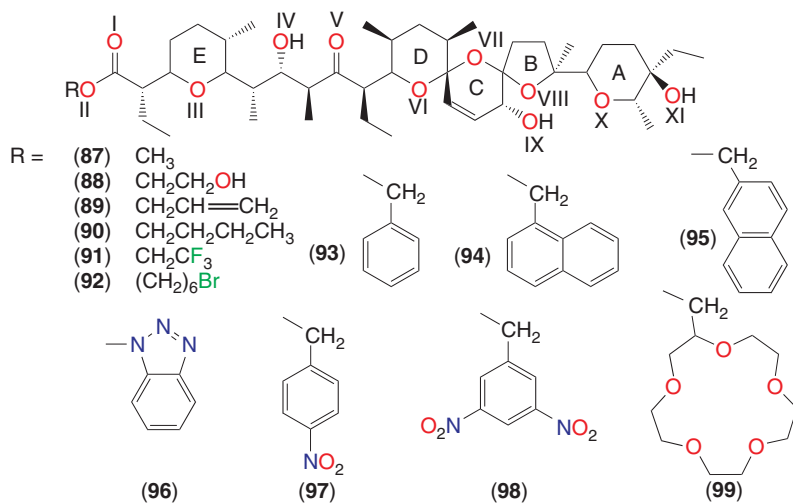


Figure 6.15 Structures of salinomycin ester derivatives.

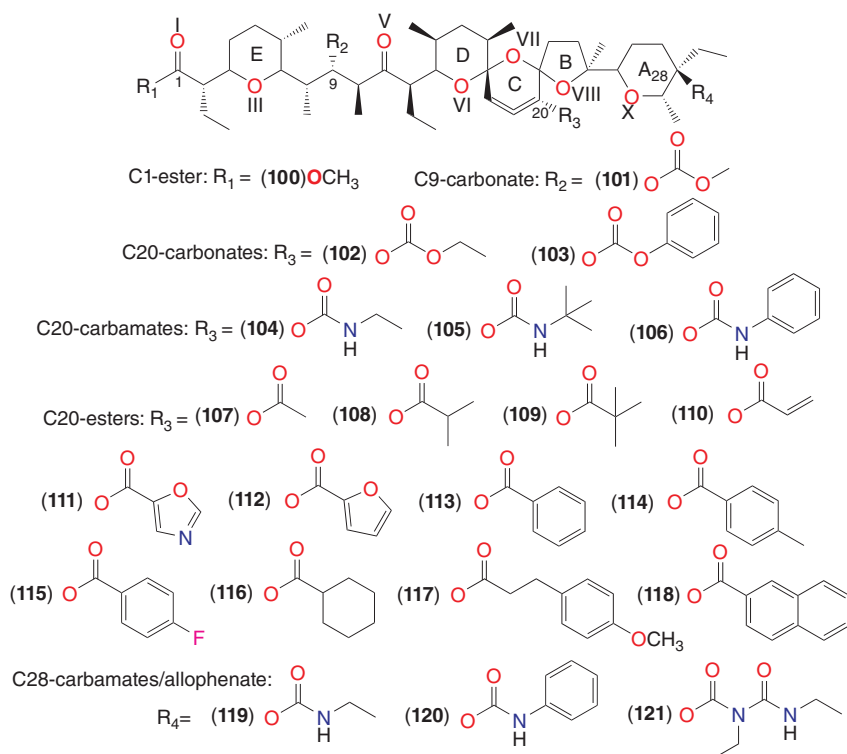


Figure 6.16 Structure of O-acylated derivatives of salinomycin R<sub>1</sub> = Na and R<sub>2-4</sub> = H unless otherwise stated.

(compound **125**), and lasalocid E (compound **126**), have been reported [115]. The structures of lasalocid acid and its homologs are presented in Figure 6.17.

Crystal structures of lasalocid acid sodium, silver, and barium salts have been reported in [116, 117]. Interestingly, lasalocid forms a 2:2 dimeric complex with silver (see Figure 6.18) [116].

Lasalocid is able to form complexes with divalent cations, and the crystal structures of complexes with strontium and barium (see Figures 6.19 and 6.20) have been resolved. In both cases, lasalocid forms dimeric complexes with cations of 2:1 stoichiometry [117]. The lasalocid acid complex with thallium cation is quite unusual, because the monomeric unit of this complex is stabilized by strong intramolecular aryl-Tl type-metal half sandwich bonding interactions [118].

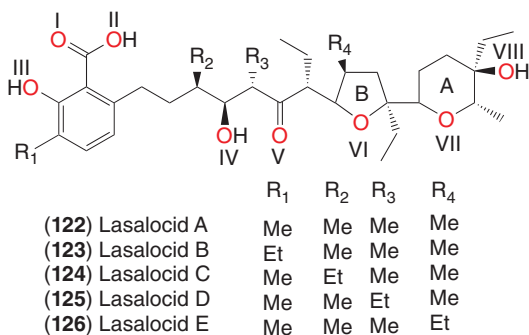


Figure 6.17 Structure of lasalocid acid and its homologs.

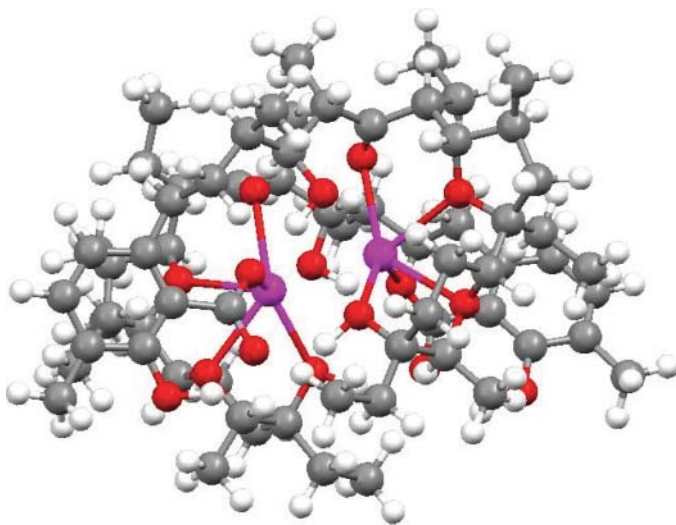
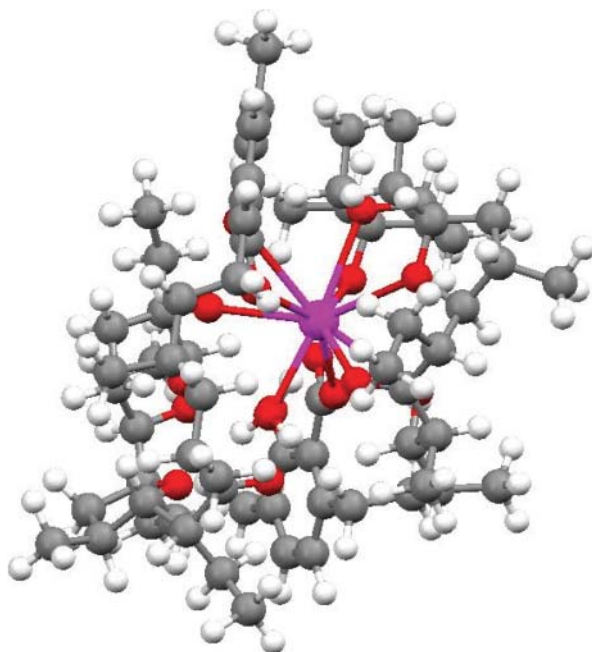


Figure 6.18 Crystal structure of dimeric complex of lasalocid with silver cation on 2:2 stoichiometry.

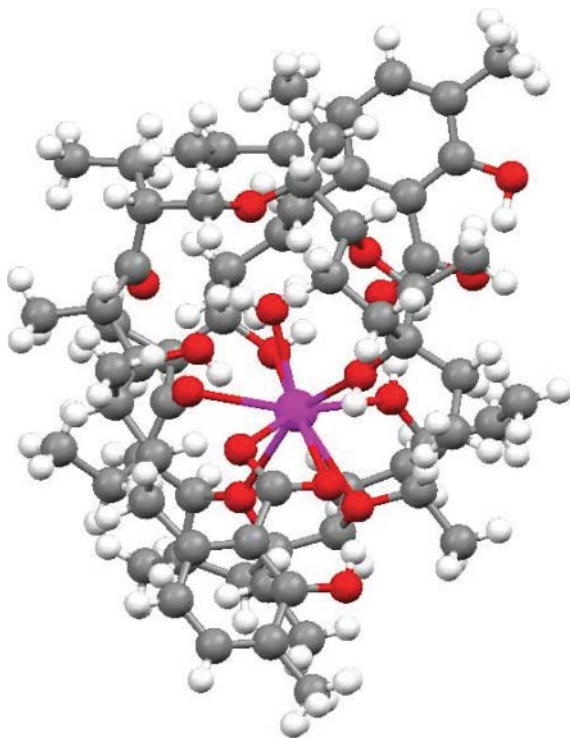


**Figure 6.19** Crystal structure of dimeric complex of lasalocid with barium cation on 2:1 stoichiometry.

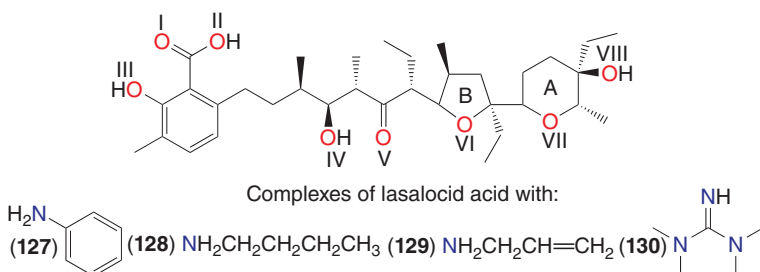
Besides mono- and divalent cations, lasalocid acid is also able to form complexes with amines and *N*-organic bases (see Figure 6.21, compounds **127–130**) [119–122]. The crystal structure of lasalocid acid complex with 1,1,3,3-tetramethylguanidine is shown in Figure 6.22. The ability of lasalocid acid to form complexes with amines was also used to resolve racemic amines by fractional crystallization. It was shown that after crystallization of racemic amines the *R* isomer predominated by between 6- and 10-fold over the *S* isomer [123]. A series of lasalocid acid sodium salts (see Figure 6.23, compounds **131–147**) and lasalocid acid derivatives (see Figure 6.24, compounds **148–161**) have been described by Westley *et al.* [124], in which carboxylic, phenolic, and ketone groups, as well as aromatic ring, are substituted.

A series of lasalocid acid ester (see Figure 6.25, compounds **162–177**) and Mannich base (see Figure 6.26, compounds **178–184**) derivatives has been studied in [125–142]. The crystal structure of lasalocid *o*-nitrobenzyl ester is shown in Figure 6.27.

Lasalocid acid is active against Gram-positive bacteria and its sodium salt (Bovatec, Avatec) is used in veterinary practice to prevent coccidiosis in poultry and to improve nutrient absorption and feed efficiency in ruminants. Recently, four complexes of lasalocid with amines have been obtained. Two of them i.e.



**Figure 6.20** Crystal structure of dimeric complex of lasalocid with strontium cation on 2 : 1 stoichiometry.



**Figure 6.21** Structure of lasalocid acid complexes with amines.

complexes with phenylamine and butylamine have been tested *in vitro* for cytotoxic activity against human cancer cell lines: A-549 (lung), MCF-7 (breast), HT-29 (colon), and mouse cancer cell line P-388 (murine leukemia cell line) (leukemia). Lasalocid and its complexes have been found to be strong cytotoxic agents toward cancer cell lines. The cytostatic activity of the compounds studied

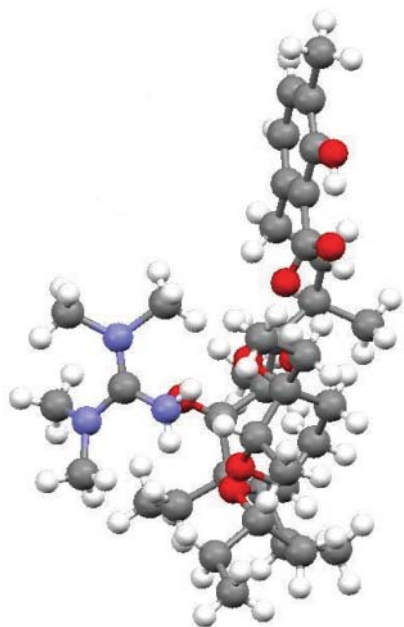


Figure 6.22 Crystal structure of lasalocid acid complex with 1,1,3,3-tetramethylguanidine.

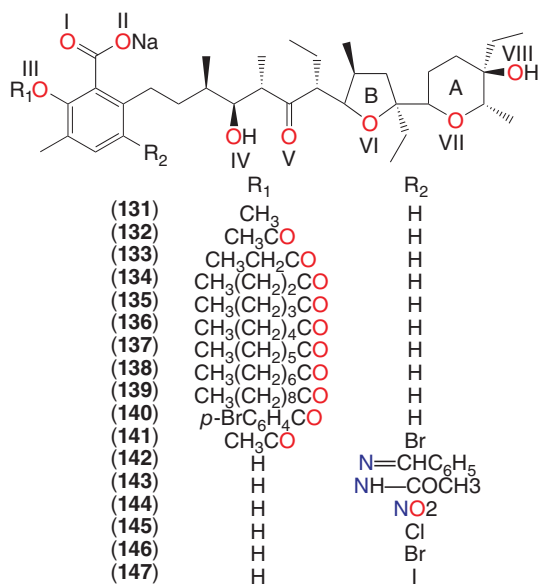


Figure 6.23 Structures of lasalocid acid sodium salt derivatives.

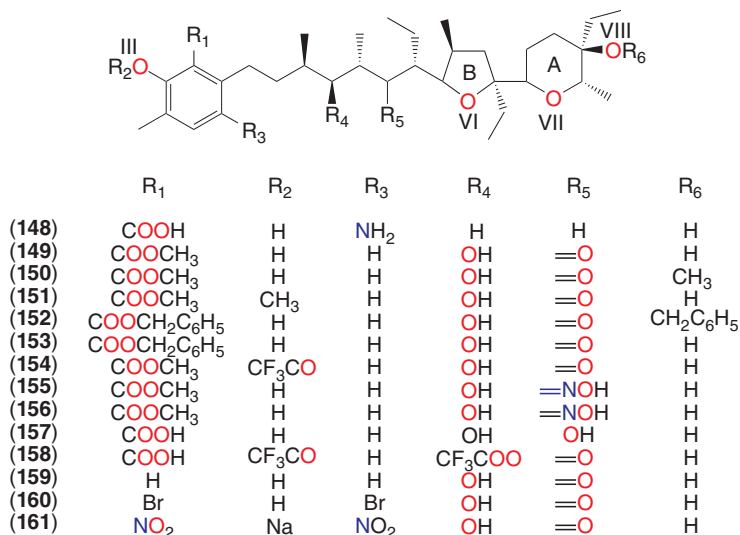


Figure 6.24 Structures of lasalocid acid obtained by Westley.

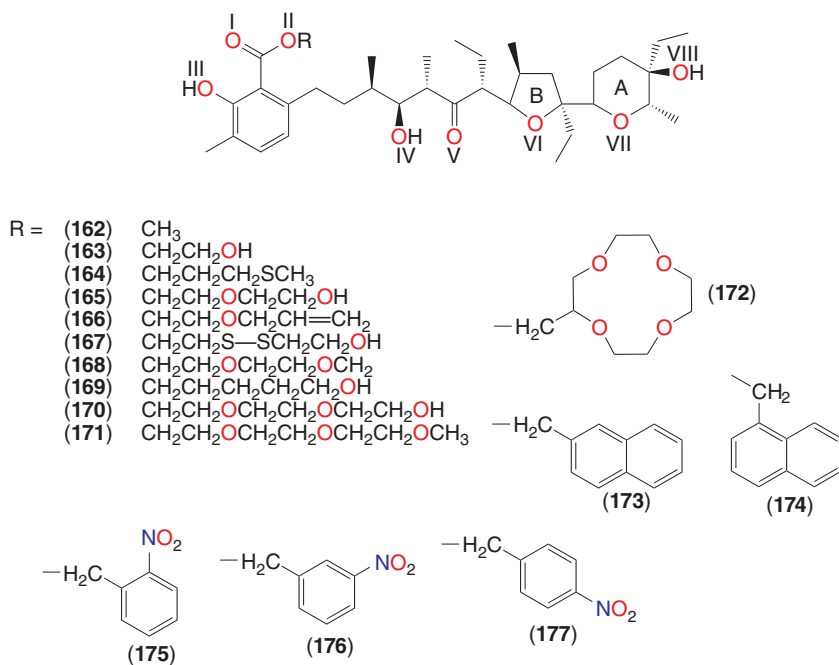


Figure 6.25 Structures of lasalocid ester derivatives.



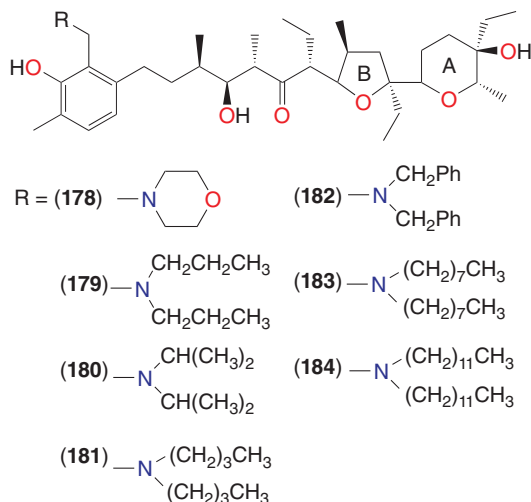


Figure 6.26 Structure of lasalocid acid Mannich bases.

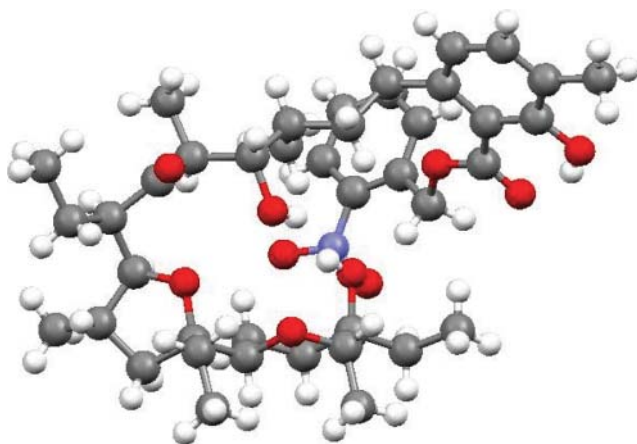


Figure 6.27 Crystal structure of lasalocid *ortho*-nitrobenzyl ester.

is greater than that of cisplatin, indicating that lasalocid and its complexes are promising candidates for new anticancer drugs [122].

#### 6.2.4

#### Other Polyether Ionophores

##### 6.2.4.1 Ionophores with Monensin Skeleton

The largest group of carboxylic ionophores are compounds with monensin (compound **1**) skeleton consisting of rings A–E. Nigericin (compound **185**), grisorexin

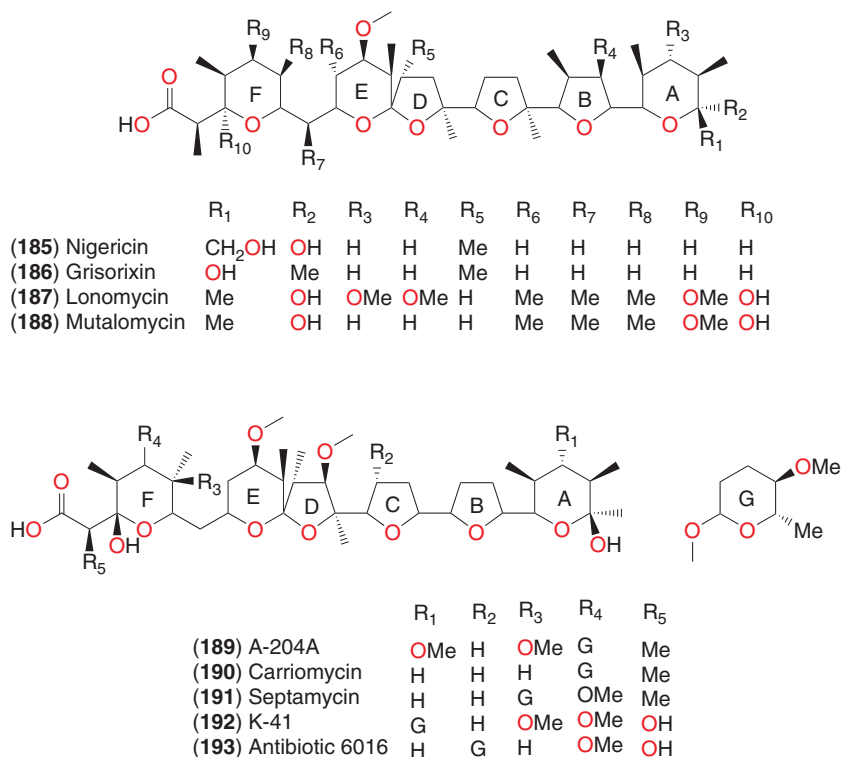


Figure 6.28 Structures of antibiotics with monensin skeleton.

(compound **186**), lonomycin (compound **187**), and mutalomycin (compound **188**) have an additional tetrahydropyran ring E, while A204-A (compound **189**), carriomycin (compound **190**), septamycin (compound **191**), K41 (compound **192**), and antibiotic 6016 (compound **193**) have a hexopyranose ring G attached to the ligand as an  $\alpha$ -glycoside in A-204A and as a  $\beta$ -glycoside in other antibiotics (see Figures 6.28–6.30).

#### 6.2.4.2 Polyether Ionophores with Dianemycin Skeleton

The compounds dianemycin (compound **194**), lenoremycin (compound **195**), endusamycin (compound **196**), and CP-80219 (compound **197**) have skeletons that are somewhat different from those of the aforementioned antibiotics with monensin skeletons. In the compounds of this group there is an additional F sugar moiety (see Figure 6.31) attached to ring C in dianemycin (compound **194**), ring E in lenoremycin (compound **195**), ring D in endusamycin (compound **196**), and ring E in CP-80219 (compound **197**).

Basic information about the aforementioned ionophores and several other compounds are collected in Table 6.1 (Figures 6.32–6.42).

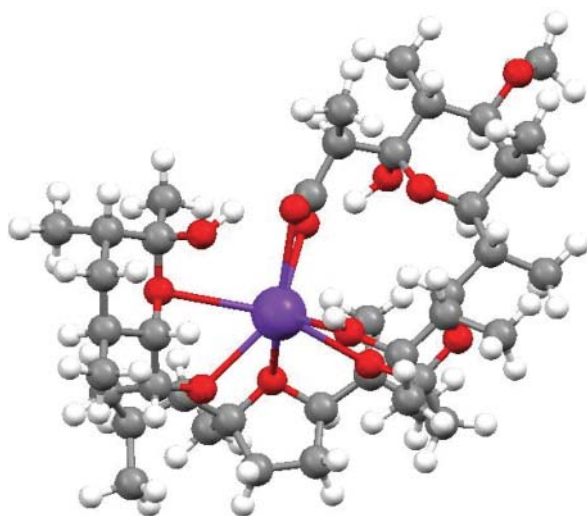


Figure 6.29 Crystal structure of 28-epimutalomycin potassium salt.

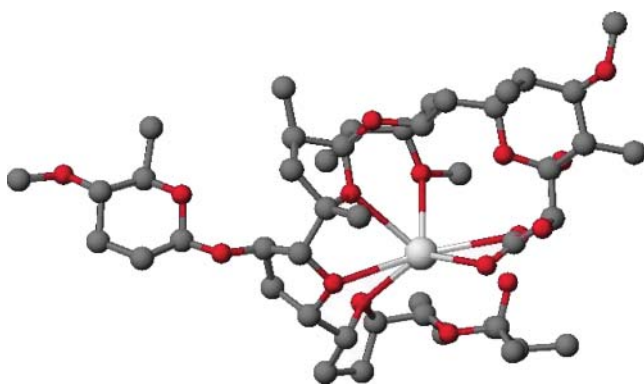


Figure 6.30 Crystal structure of antibiotic 6016 thallium salt.

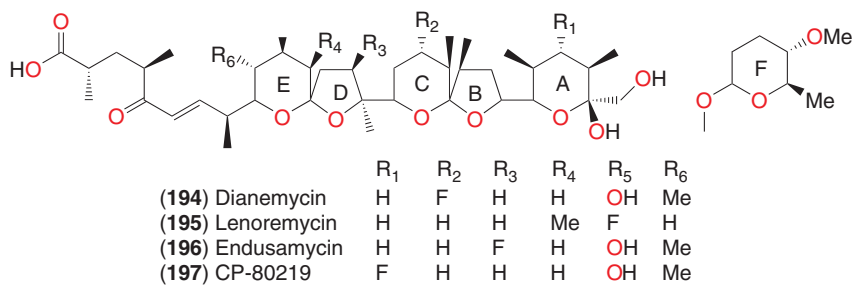


Figure 6.31 Structures of antibiotics with dianemycin skeleton.

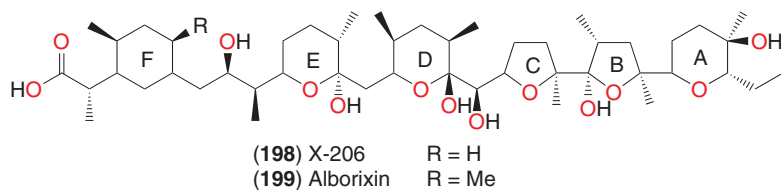


Figure 6.32 Structure of alborixin and X-206.

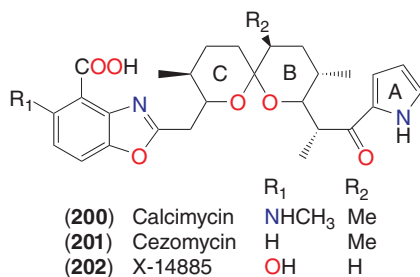


Figure 6.33 Structure of calcimycin, cezomycin, and X-14885.

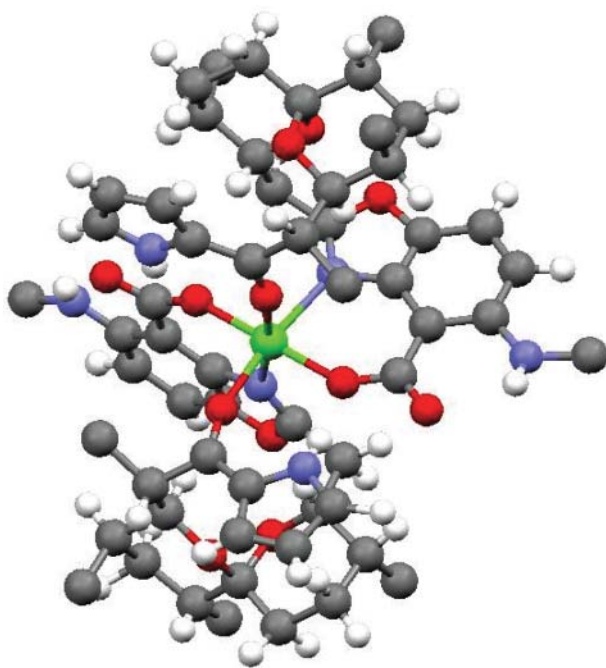


Figure 6.34 Crystal structure of 2:1 complex of calcimycin with the magnesium cation.

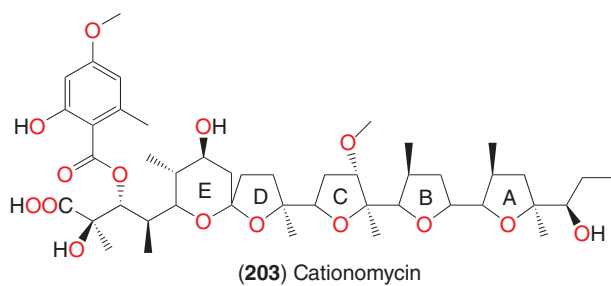


Figure 6.35 Structure of cationomycin.

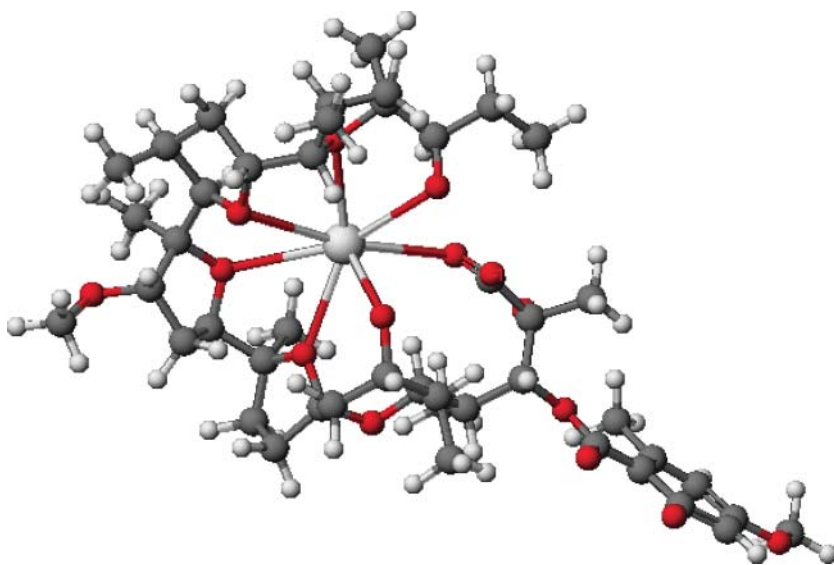


Figure 6.36 Crystal structure of cationomycin thallium salt.

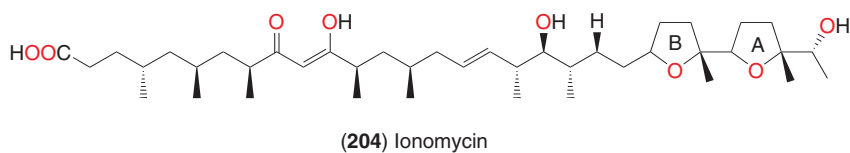


Figure 6.37 Structure of ionomycin.

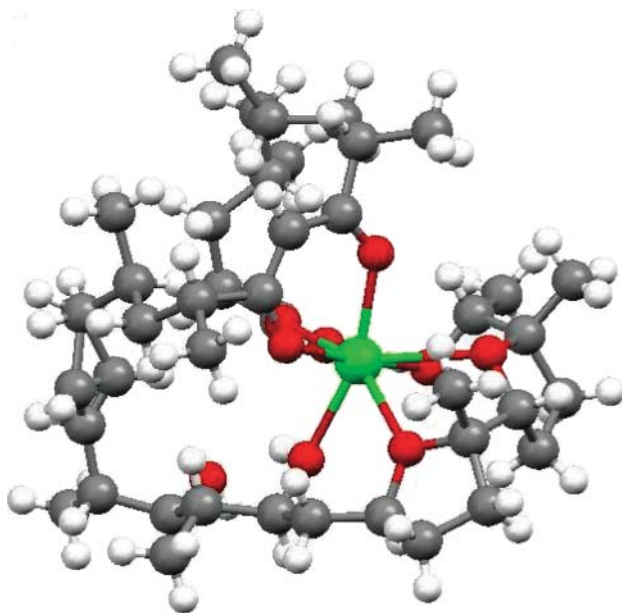


Figure 6.38 Crystal structure of ionomycin complex with the calcium cation.

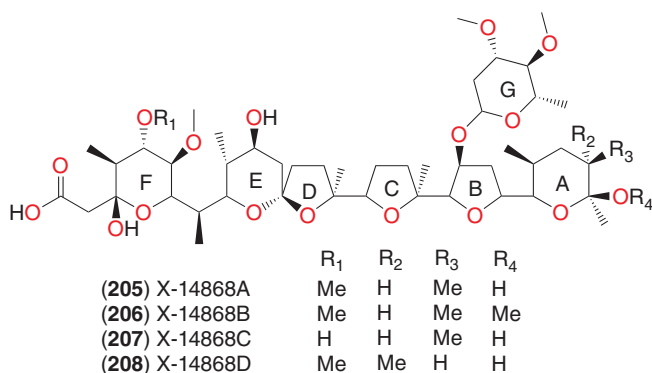


Figure 6.39 Structures of X-14868A, X-14868B, X-14868C, and X-14868D.

### 6.3

#### Chemical Properties of Polyether Ionophores and Their Derivatives

##### 6.3.1

##### Complexes of Ionophores with Metal Cations

The most important chemical property of polyether ionophores is their ability to form complexes with metal cations and transport them across natural and artificial

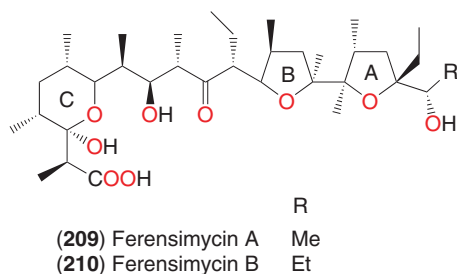


Figure 6.40 Structures of ferensimycin A and B.

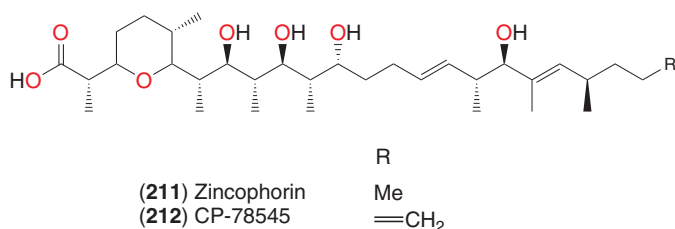


Figure 6.41 Structures of zincophorin and CP-78545.

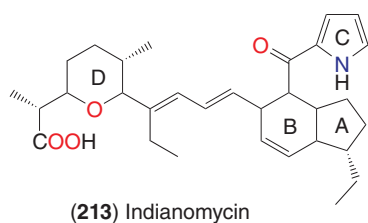


Figure 6.42 Structure of indianomycin.

lipid bilayers. Polyether ionophores can be divided into those that are able to form complexes only with monovalent cations (i.e., monensin) and those that are able to form complexes with both mono- and divalent cations (i.e., lasalocid). Moreover lasalocid acid is able to form complexes with amines and N-organic bases.

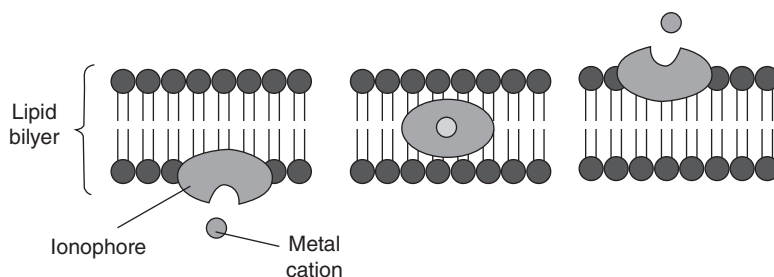
According to the X-ray studies, the complexed cation resides in a cage formed by oxygen atoms of the ionophore. The hydrophobic skeleton of the ionophore is wrapped around this cage rendering the whole complex lipophilic. It has been called a *tennis ball seam*. Complexation of metal cation by a polyether ionophore is always accompanied by the formation of a pseudocyclic structure stabilized by “head-to-tail” intramolecular hydrogen bonds between the carboxylic group on one side of the molecule and hydroxyl groups on the other. The formation of the pseudocyclic structure facilitates transport of the cation across a lipid bilayer [59]. Ionophore captures the cation on one side of the lipid bilayer in a stepwise process,

replacing the solvate molecules one by one with their polar groups. The complex then moves across the membrane and releases the cation on the other side (see Figure 6.43). The ionophore then diffuses back to the opposite side of the membrane, where the whole process can be repeated [59]. The remarkable selectivity of some ionophores is attributed to their structure and to the size of the cage formed by the oxygen atoms. Only cations of just the right radius fit the cavity perfectly, whereas larger ones deform it and smaller ones assume a nonoptimal coordination geometry [59]. The data on selectivity of several ionophores are collected in Table 6.2.

### 6.3.2

#### Mechanism of Cation Transport

Recognition of different mechanisms of ion transport by an ionophore antibiotic is needed to fully understand the mechanism of action of these compounds,



**Figure 6.43** Transport of cations across lipid bilayer by ionophores.

**Table 6.2** Selectivity of several polyether ionophores [6, 25, 31, 35, 49, 52, 55, 59].

Antibiotic	Cation binding sequence	
	Monovalent cations	Divalent cations
Monensin	$\text{Na}^+ \gg \text{K}^+ > \text{Rb}^+ > \text{Li}^+ > \text{Cs}^+$	—
Nigericin	$\text{K}^+ > \text{Rb}^+ > \text{Na}^+ > \text{Cs}^+ > \text{Li}^+$	—
Antibiotic X-206		
Dianemycin	$\text{Na}^+ > \text{K}^+ > \text{Rb}^+ \approx \text{Cs}^+ > \text{Li}^+$	—
Lasalocid	$\text{Cs}^+ > \text{Rb}^+ \approx \text{K}^+ > \text{Na}^+ > \text{Li}^+$	$\text{Ba}^{2+} > \text{Ca}^{2+} > \text{Mg}^{2+}$
Calcimycin	$\text{Li}^+ > \text{Na}^+ > \text{K}^+$	$\text{Ca}^{2+} > \text{Mg}^{2+} > \text{Ba}^{2+}$
Salinomycin	$\text{K}^+ > \text{Na}^+ > \text{Cs}^+$	$\text{Sr}^{2+} > \text{Ca}^{2+}, \text{Mg}^{2+}$
X-14868A	$\text{K}^+ > \text{Rb}^+ > \text{Na}^+ > \text{Li}^+ > \text{Cs}^+$	—
X-14868B	—	$\text{Mg}^{2+} > \text{Ca}^{2+} > \text{Ba}^{2+} \approx \text{Sr}^{2+}$
Zincophorin	—	$\text{Zn}^{2+} \approx \text{Cd}^{2+} > \text{Mg}^{2+} > \text{Sr}^{2+} \approx \text{Ba}^{2+} \approx \text{Ca}^{2+}$
Ionomycin	—	$\text{Ca}^{2+} > \text{Mg}^{2+} \gg \text{Sr}^{2+} = \text{Ba}^{2+}$



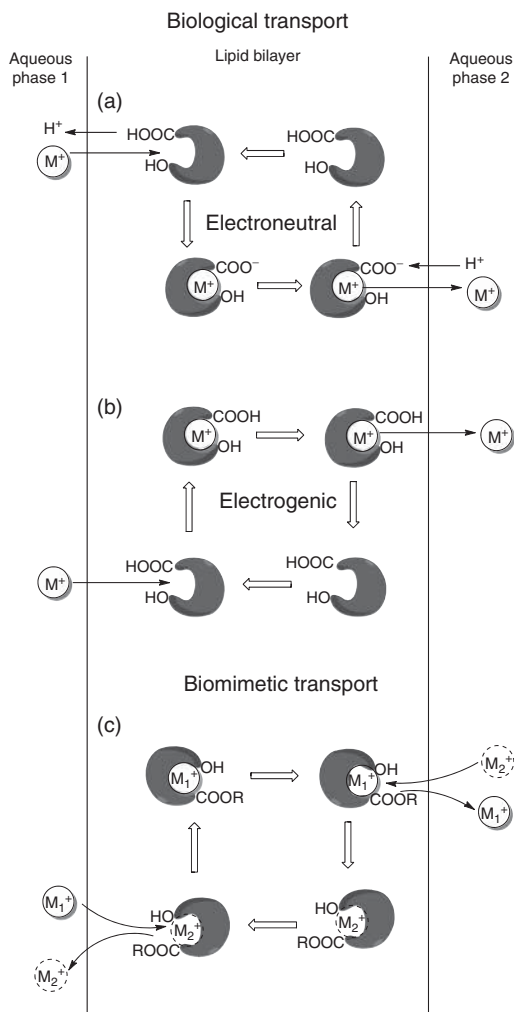
including their biological activity. The mechanism of cation transport by an ionophore is strongly dependent on the environment in which it occurs. In a neutral or slightly alkaline environment, the carboxyl group of ionophores is deprotonated ( $I-COO^-$ ) and the cation is transported by the electroneutral process (see Figure 6.44a). In this type of cation ( $M^+$ ) transportation, the polyether ionophore anion ( $I-COO^-$ ) binds the metal cation or proton ( $H^+$ ) to give a neutral salt ( $I-COO^-M^+$ ) or a neutral ionophore in the acidic form ( $I-COOH$ ), and only one uncharged molecule containing either a metal cation or proton can move through the cell membrane [143]. In 2012, Huczyński *et al.* showed that the process of complexation occurs in a non-alkaline environment (see Figure 6.44b). The complex is formed with the polyether ionophore in its acidic form ( $I-COOH-M^+$ ) instead of the polyether ionophore anion ( $I-COO^-M^+$ ) and the transport of cations is an electrogenic process [143].

The third type, termed *biomimetic* (see Figure 6.44c) is realized by derivatives of polyether ionophores with block carboxylic function such as amides or esters [143]. The ability to form complexes with cations and transport them through a lipid bilayer is behind the ionophores biological activity which will be described in the next section.

## 6.4 Biological Activity

Intracellular concentrations of potassium cations are higher than those of sodium cations and extracellular concentrations are reversed. Cation concentration gradients are essential for normal cell functioning [144]. Knowledge of various mechanisms of cation transport (see Figure 6.44) implemented by polyether antibiotics is essential for full understanding of the principles of operation of this class of compounds and to explain their broad spectrum of biological properties. These biological properties are related to their natural ability to complex metal cations (mainly  $Na^+$  and  $K^+$ ) and transport them across the lipid membranes from the external environment into the cell. There, the coordinated ions are released, which results in a disturbance of the natural  $Na^+/K^+$  concentration gradient and intracellular pH change, leading to mitochondrial injury, cell swelling, vacuolization and, finally, programmed cell death (apoptosis) [145–147].

In the subsequent sections, the antimicrobial and antiproliferative properties of the most widely used, most often chemically modified and most biologically effective ionophores, namely, monensin (**1**), salinomycin (**67**), and lasalocid acid (**122**) will be discussed in detail. Chemical structures of the biologically active polyether antibiotics and their derivatives have been discussed in the preceding. The most important types of biological activity of polyether antibiotics include antibacterial, especially against Gram-positive bacteria, antifungal, antiparasitic, antimalarial, antiviral, coccidiostatic as well as anticancer activity.



**Figure 6.44** Electroneutral (a), electrogenic (b), and biomimetic (c) transport of cations by polyether ionophores.

## 6.4.1

**Antibacterial Activity of Polyether Antibiotics and Their Derivatives**

Polyether antibiotics exhibit activity both against drug-sensitive as well as MDR bacteria strains and the activity against Gram-positive bacteria is much stronger than against Gram-negative bacteria. This has been explained by much greater complexity of the structure of the Gram-negative bacteria cell wall [148]. The outer membrane of these bacteria is impermeable to hydrophobic compounds, such as ionophores, and their complexes.

Biological activity of polyether antibiotics was mentioned for the first time by Westley [145, 149]. He reported that lasalocid acid, monensin, salinomycin, and other ionophores show moderate *in vitro* activity against several Gram-positive bacteria, such as *Sarcina lutea*, *Mycobacterium phlei*, *Streptomyces cellulosa*, *Paecilomyces varioti*, standard strains of *Staphylococcus aureus*, and especially *Bacillus* sp. (see Table 6.3). Additionally, it has been shown that lasalocid acid, monensin, and a few other polyether antibiotics are active against some of the anaerobic bacteria, such as *Clostridium* sp., *Eubacterium* sp., *Peptococcus* sp., and *Propionibacterium* sp., but generally, the vast majority of these types of bacteria are resistant to the action of polyether antibiotics, which are moreover not effective against Gram-negative bacteria. The discovery of polyether antibiotics has stimulated further studies on their antimicrobial properties.

*S. aureus* is a species of bacteria commonly encountered on the skin and/or mucous membranes of the nose of healthy people, which is harmless, but when it gets into the human body can cause serious blood, bone, or joints infections. MRSA (methicillin-resistant *S. aureus*) is the strain of *S. aureus* resistant to methicillin and other penicillin-type antibiotics [150, 151]. It has been demonstrated that monensin and salinomycin are strongly effective against MRSA, much more than the reference compound used in tests, that is, methicillin. Moreover, it has been shown that these antibiotics are active against vancomycin-resistant *Enterococci* (VRE) as well as Gram-positive bacteria, such as *Enterococcus faecalis*,

**Table 6.3** Antibacterial *in vitro* activity of monensin (1), salinomycin (67), and lasalocid acid (122) against different strains of Gram-positive bacteria [149].

Compound	Minimal inhibitory concentration ( $\mu\text{g ml}^{-1}$ )					
	<i>S. aureus</i>	<i>S. lutea</i>	<i>Bacillus</i> sp.	<i>M. phlei</i>	<i>S. cellulosa</i>	<i>P. varioti</i>
<b>1</b>	3.1	12.5	0.4–3.1	12.5	6.3	ia <sup>a</sup> )
<b>67</b>	3.1	3.1	0.2–0.8	6.3	3.1	3.1
<b>122</b>	1.6	3.1	0.2–3.1	12.5	6.3	ia <sup>a</sup> )

Data are given as MIC (minimal inhibitory concentration,  $\mu\text{g ml}^{-1}$ ).

a) ia – inactive compound.

*Bacillus subtilis*, *S. aureus*, *Micrococcus luteus*, and *Mycobacterium smegmatis*. These ionophores, in accordance with previous observations, are not active against Gram-negative bacteria, such as *Salmonella typhimurium*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Alcaligenes faecalis* [152].

These results have been confirmed by the documented evidence of high activity of monensin against methicillin-sensitive *S. aureus* (MSSA) and MRSA as well as against new Gram-positive bacteria, such as *Bacillus cereus* and *Enterococcus hirae* [92]. Additionally, the same research group has reported new antibacterial properties of lasalocid acid and salinomycin against various standard strains of *S. aureus* and *Staphylococcus epidermidis* (see Table 6.4), and other Gram-positive bacteria, such as *E. faecalis*, *E. hirae*, *B. subtilis*, *B. cereus*, and *M. luteus* [119, 120, 122] as well as MSSA and MRSA [108, 109]. Tests have clearly shown that all three antibiotics are inactive against Gram-negative bacteria.

On the other hand, it has been shown that lasalocid acid and iso-lasalocid acid are active against nine Gram-positive bacteria, although iso-lasalocid is inactive against *E. tenella* even at high concentration [149]. Recent studies have indicated that monensin can be active against *Mycobacterium tuberculosis* (99% inhibition at the appropriate concentration), which is a causative agent of most cases of tuberculosis as well as against anaerobic *Clostridium perfringens* bacterial strains. Lasalocid is inactive in these tests [153, 154].

An extremely interesting direction of research is the chemical modification of polyether antibiotics leading to the formation of compounds that differ in complexation selectivity, structure of complexes formed, ion transport mechanism, toxicity, and biological properties. The best, in this respect, seems to be monensin. The synthesis and antimicrobial properties of a series of monensin urethanes, obtained by modification of O(XI)H group, have been reported (see Figure 6.4, compounds 2–12) [73]. These derivatives are very interesting, because they

**Table 6.4** *In vitro* activity of monensin (1), salinomycin (67), and lasalocid acid (122) against standard strains of *Staphylococcus aureus* as well as *Staphylococcus epidermidis* [92, 108, 109, 119, 120, 122].

	Minimal inhibitory concentration ( $\mu\text{g ml}^{-1}$ )		
	1	67	122
<i>S. aureus</i> NCTC 4163	2	2	4–12.5
<i>S. aureus</i> ATCC 25923	1	2	4–12.5
<i>S. aureus</i> ATCC 6538	2	2	4–12.5
<i>S. aureus</i> ATCC 29213	1	2	4–12.5
<i>S. epidermidis</i> ATCC 12228	2	2	4–12.5
<i>S. epidermidis</i> ATCC 35984	ia <sup>a)</sup>	2	ia <sup>a)</sup>

Data are given as MIC (minimal inhibitory concentration,  $\mu\text{g/ml}$ ).

a) ia – inactive compound.

are able to transport monovalent cations about 10-times more effectively than the chemically unmodified antibiotic. In addition, it was found that monensin urethanes, especially phenylurethanes, show up to 10-fold higher activity against Gram-positive bacteria than the parental compound, for example, for *S. aureus* (MIC = 0.4–1.6  $\mu\text{g ml}^{-1}$ ), *Streptococcus faecium* (MIC = 0.05–0.4  $\mu\text{g ml}^{-1}$ ), *M. phlei* (MIC = 0.8–1.6  $\mu\text{g ml}^{-1}$ ), *Bacillus* sp. (MIC = 0.02–1.6  $\mu\text{g ml}^{-1}$ ), and others. Furthermore, monensin urethanes are coccidiostatic effective. In addition, four phenylurethanes (compounds **6–7**, **11–12**) are active against three anaerobes, *Bacteroides fragilis*, *Clostridium histolyticum*, and *Clostridium septicum*.

The synthesis and antibacterial activity of monensin diurethanes have been also studied (see Figure 6.5, compounds **13–16**) [75]. The results have indicated that all diurethanes obtained show, to a greater or lesser extent, biological activity against Gram-positive bacteria, MSSA as well as MRSA; the most effective proves to be 1,4-phenylene diurethane (compound **15**) with minimal inhibitory concentration (MIC) values of 20.7–41.4  $\mu\text{g ml}^{-1}$ . Since then, more reports about chemical modification of monensin have been published. Monensin derivatives with modified O(IV)H group, such as ethers and esters, have been also synthesized and biologically characterized (see Figure 6.6, compounds **17–32**) [76, 77]. All monensin esters (compounds **17–21**) reveal lower antibacterial activity against aerobic and anaerobic bacteria than the parental compound. On the other hand, results of the tests have clearly shown that the most effective are benzyl ethers (compounds **25–32**) whose antimicrobial activity is much higher than that of the starting material. It is associated with the highly hydrophobic nature of benzyl substituents, which increases the solubility of monensin derivatives in the bacterial cell membrane [77].

The chemical modification of O(XI)H group leading to various derivatives of monensin, including amines, ethers, esters, and sulfonates has been analyzed (see Figure 6.7, compounds **33–37**) [78]. Among the derivatives tested, fenylaminomonensin (compound **35**) exhibits the strongest antimicrobial activity against Gram-positive bacteria with MIC values of 0.2–6.25  $\mu\text{g ml}^{-1}$ . This activity is not only higher than that of unmodified monensin, but also higher than that of a very active derivative – monensin phenylurethane (compound **5**). Moreover, monensin sulfonate (compound **36**) demonstrates high activity against *B. cereus* bacteria. Biological activity of these compounds is linked with complexation selectivity altered by chemical modification. Ester and sulfonate derivatives (compounds **36–37**) were established to cause an increase in the effective transport of potassium cations through the biological membranes, while there is a decrease in the transport of sodium cations. Thus, these monensin derivatives preferentially complex and transport potassium cations rather than sodium cations [79].

Chemical modifications of the carboxyl group of monensin have been also considered. A series of monensin amides as well as bioconjugates with amino acids has been synthesized (see Figure 6.8, compounds **38–48**) [80, 94–98]. In addition, the lactonization reaction of monensylamino acids, in which the

carboxyl group of the amino acids moiety is connected with O(XI)H group of monensin, has been carried out (see Figure 6.10, compounds **61–66**) [100]. All monensin amides (compounds **38–41**) show comparable, but a slightly smaller activity against Gram-positive bacteria than the starting material (MIC = 1–12.5 and 6.25–50  $\mu\text{g ml}^{-1}$  against some standard strains of *S. aureus* for unmodified monensin and its amides, respectively). The most active among them is monensin phenylamide (compound **38**), with MIC = 6.25–12.5  $\mu\text{g ml}^{-1}$  (except the activity against *E. hirae* with MIC > 400  $\mu\text{g ml}^{-1}$ ) [94–98]. On the other hand, macrocyclic lactones show lower activity against anaerobic bacteria than the parental ionophore, for example, MIC = 25–50  $\mu\text{g ml}^{-1}$  against *Peptostreptococcus anaerobius* (for compounds **61–66**), whereas for monensin it is 1.56  $\mu\text{g ml}^{-1}$  [100]. In the last decade, syntheses of series of monensin esters have been presented (see Figure 6.9, compounds **49–60**) and their ability to form complexes with the monovalent and divalent metal cations has been investigated [82–85, 87–91, 93, 101, 155, 156]. Results of antimicrobial activity of monensin esters show that three of them (compounds **54**, **55**, and **58**) are active against some strains of Gram-positive bacteria with MIC = 6.25–100  $\mu\text{g ml}^{-1}$  and against some standard strains of *S. aureus*. Furthermore, morpholinoethyl ester (compound **58**) shows high activity against methicillin-sensitive and MRSA. The same esters (compounds **54**, **55**, and **58**) show moderate activity against MSSA as well as MRSA [92]. The synthesis of macromolar dimers and trimers of monensin has been carried out. These derivatives show moderate activity against Gram-positive bacteria, but they are completely inactive against Gram-negative bacteria [157].

Antibacterial activity of some series of monensin complexes has been also reported (see Figure 6.3) [67, 68, 70, 72, 154, 69]. Efficacy of monensin complexes with sodium, manganese(II) and cobalt(II) against three Gram-positive (*B. subtilis*, *B. cereus*, and *S. lutea*) and two Gram-negative (*Salmonella enteritidis* and *E. coli*) microorganisms has been characterized. All complexes tested are effective against *B. subtilis*, *Bacillus mycoides*, and *S. lutea* with the same MIC values. In addition, it has been shown that alkaline-earth monensin complexes with magnesium and calcium are effective against three aerobic Gram-positive bacteria (*B. subtilis*, *B. mycoides*, and *S. lutea*). Monensin complexes with zinc(II) and nickel(II) are active against the earlier mentioned Gram-positive bacteria, especially against *S. lutea*. In this case, activity of monensin complexes is higher than that exhibited by the chemically unmodified ionophore. Recent studies have indicated that all complexes (with Mg(II), Ca(II), Mn(II), Co(II), Ni(II), and Zn(II)) are active against Gram-positive anaerobic bacterium *C. perfringens* spp. in varying concentration range. However, Gram-positive *B. cereus* as well as Gram-negative *S. enteritidis* and *E. coli* bacteria are resistant to the effect of the complexes tested.

Additionally, it has been proved that monensin complex with thallium(I) shows significant potency to inhibit the growth of *M. tuberculosis* (97% inhibition at the optimum concentration) [153], which indicates that this compound can

be effectively used in the fight against tuberculosis. This activity is comparable to that of the starting material. Lasalocid acid complexes with thallium(I), lanthanum(III), and gadolinium(III) are inactive toward this bacteria species. The lasalocid acid derivatives with demonstrated biological activity include also its complexes with amines (see Figure 6.21, compounds **127–130**). The complex with allylamine (compound **129**) shows higher antibacterial activity than that of the parent compound [119]. On the other hand, the complex with 1,1,3,3-tetramethylguanidine (compound **130**) has antibacterial activity comparable to that of lasalocid acid [120]. Biological properties of the complexes with phenylamine and butylamine (compounds **127** and **128**, respectively) have been also studied [122]. The first complex shows slightly higher antibacterial activity in comparison to that of lasalocid acid (MIC = 2–8 and 2–4  $\mu\text{g ml}^{-1}$  against tested Gram-positive bacteria). The antibacterial activity of pure acid and complex with butylamine is similar (MIC = 2–8  $\mu\text{g ml}^{-1}$  in both cases). All complexes are inactive toward Gram-negative bacteria. Other interesting lasalocid acid derivatives are phenolic hydroxyl group esters, para to the phenol ring-substituted derivatives, anhydro, and epoxide ones (see Figures 6.23 and 6.24, compounds **131–148**) [124]. The vast majority of these derivatives are active against *Bacillus* sp., except for three (compounds **133**, **136**, and **137**). In addition, all these derivatives, except the methyl ether (compound **131**) and the four nitrogen-substituted derivatives (compounds **142–144** and **148**), are active against *E. tenella* at 300 ppm in fodder. However, this activity is lower than that of the starting compound.

The first publications about biological activity of salinomycin derivatives appeared only in 2012, when the synthesis and antimicrobial properties of one ester (compound **96**) [108] and eight amides (compounds **68–75**) [109] have been presented (see Figures 6.14 and 6.15). In 2014, a new series of salinomycin amides and esters have been synthesized (see Figures 6.14 and 6.15, compounds **76–99**, not including compound **96**, which has been previously synthesized) [111, 112]. The tests have clearly shown that four esters (compounds **89**, **91**, **96**, and **98**) and seven amides (compounds **71**, **74–78**, and **86**) are active against standard strains of *S. aureus* (see Table 6.5), MSSA, and MRSA with activity comparable to that of unmodified antibiotic. Among salinomycin amide derivatives, phenyl amide (compound **71**) with an aromatic substituent shows a considerably stronger activity against hospital strains of MSSA and MRSA than the corresponding active amides, indicating that the aromatic substituent in the amide moiety has a strong influence on the antibacterial activity. On the other hand, trifluoroethyl ester (compound **91**) shows higher antibacterial activity than unmodified salinomycin, its other esters, and the antibacterial drug – ciprofloxacin. As with the other ionophores, salinomycin derivatives are inactive against tested Gram-negative bacteria [108, 109, 111, 112].

**Table 6.5** Antibacterial *in vitro* activity of three selected amides (71, 75, and 76) and three esters (89, 91, and 98) of salinomycin against different strains of Gram-positive bacteria in comparison to the activities of unmodified salinomycin (67) [108, 109, 111, 112].

	Minimal inhibitory concentration ( $\mu\text{g ml}^{-1}$ )						
	67	71	75	76	89	91	98
<i>S. aureus</i> NCTC 4163	2	2	8	16	32	1	4
<i>S. aureus</i> ATCC 25923	2	2	16	16	32	1	4
<i>S. aureus</i> ATCC 6538	2	2	16	16	32	1	4
<i>S. aureus</i> NCTC 29213	2	2	16	32	64	2	8
<i>S. epidermidis</i> ATCC 12228	2	2	8	32	64	1	8
<i>S. epidermidis</i> ATCC 35984	2	4	16	32	64	2	4

Data are given as MIC (minimal inhibitory concentration,  $\mu\text{g ml}^{-1}$ ).

#### 6.4.2

##### Antifungal Activity of Polyether Antibiotics and Their Derivatives

In general, the antifungal activity of polyether antibiotics is lower than their antibacterial activity. In many cases, MIC values are greater than the highest concentration of the tested compounds [7, 10, 108, 109, 122, 145, 158, 159]. However, there are some exceptions to this rule.

Several fungi species are moderately sensitive to the action of ionophores, including monensin, lasalocid acid, and salinomycin [145]. Recently, it has been shown that *Saccharomyces cerevisiae* and *Fusarium oxysporum* f.sp. *albedinis* are sensitive to monensin and lasalocid acid. Results of these studies have clearly shown that monensin and lasalocid acid are effective inhibitors (with 100% and 90% inhibition at a suitable concentration, respectively) against the growth of *Saccharomyces* [153]. *F. oxysporum* is a highly pleomorphic microorganism, whose diverse forms can behave both as saprophytes and parasites in various plants. Some of them can infect plants belonging to different families, for example, celery or peas. *F. oxysporum* f.sp. *albedinis*, responsible for the vascular fusariosis of the date palm, is a serious problem for agriculture. It has been proved that monensin shows significant potency to inhibit the growth of this kind of fungus (100% inhibition at the optimum concentration). Lasalocid acid is inactive against *F. oxysporum* f.sp. *albedinis* [153].

*Candida albicans* is a species of fungi that cause opportunistic infections in immunocompromised patients. It has been demonstrated that monensin induces at *C. albicans* an inhibition of germination and modifications of the morphogenesis, due to the accumulation of chitin involving thickening at the parietal level [160]. As far as the filamentous fungi are concerned, it has been reported



that monensin inhibits the secretion of the *Fusarium solani* and *Colletotrichum gloeosporioides* cutinases [161].

*Pneumocystis jirovecii*, formerly classified as a *Pneumocystis carinii* [162], is a cosmopolitan fungus causing human *P. carinii* pneumonia (PCP) [163]. The pathogenicity of this microorganism is directly related with the condition of the immune system. In patients with efficient immune system, the infection is asymptomatic and such people easily master this disease. However, in people with impaired immune system, *P. jirovecii* causes interstitial pneumonia. This applies to patients with AIDS (acquired immunodeficiency syndrome, a disease caused by human immunodeficiency virus, HIV) or patients with other severe immunodeficiency syndromes. Pneumonia caused by this fungus is a common cause of death in patients with AIDS [164, 165]. It has been shown that lasalocid acid demonstrates *P. jirovecii* growth-inhibitory effect and at the optimum concentration, it induces 91.3% cyst and 92.0% trophozoite reduction [164]. The potential of lasalocid acid for protecting rats against PCP was also evaluated. Lasalocid acid is effective in a dose-dependent manner; a dose of  $10 \text{ mg kg}^{-1} \text{ day}^{-1}$  is protective for 80% of infected rats. Simultaneously, it has been observed that a dose of  $20 \text{ mg kg}^{-1} \text{ day}^{-1}$  is safe and nontoxic for the subjects tested [165].

Some of monensin urethanes, besides the unmodified antibiotic, are active against *C. albicans* and *Penicillium digitatum* fungus. The most active in these studies appear to be phenylurethanes (see Figure 6.4, compounds 5–12), especially *p*-fluorophenyl (compound 8) and *p*-chlorophenyl (compound 9) urethanes with MIC values in the range of 6.3–12.5 and 0.08–6.3  $\mu\text{g ml}^{-1}$ , respectively [73]. On the other hand, some of the monensin and lasalocid acid complexes are active against *F. oxysporum* f.sp. *albedinis* and *S. cerevisiae* fungi. Lasalocid acid complex with lanthanum(III) shows a greater inhibiting activity against *F. oxysporum* strain than its complexes with thallium(I) and gadolinium(III) (29%, 17%, and 23% inhibition at the optimum concentration of La(III), Tl(I) and Gd(III), respectively), but free lasalocid acid shows the highest activity (38% inhibition at the optimum concentration). Among the tested complexes the most active is that of monensin with thallium(I). It is highly active against *F. oxysporum* f.sp. *albedinis* (87% inhibition at the optimum concentration) and against *S. cerevisiae* (100% inhibition at the optimum concentration). In both cases, their activity is similar to that of the parent compound [153].

### 6.4.3

#### Antiparasitic Activity of Polyether Antibiotics and Their Derivatives

A very serious recent problem is the emergence and spread of parasitic protozoan strains which are resistant to several currently marketed antimalarial drugs, such as chloroquine. It is a serious public health threat, especially in poor countries [166–168]. Therefore, an important issue is to discover new antimalarial agents and polyether antibiotics belong to this group. Many of these compounds exhibit

high antimalarial activity with  $IC_{50}$  (half maximal inhibitory concentration) values in the nano- as well as picomolar range [169].

It has been shown that lasalocid acid, salinomycin, and especially monensin are effective against *Plasmodium* sp. [169]. *In vivo* antimalarial activity of monensin against *Plasmodium chabaudi* and *Plasmodium vinckei petteri* with  $ED_{50}$  (half-maximal effective dose) values between 0.4 and 4.1 mg kg<sup>-1</sup> has been also reported [170]. In addition, the activity against *Plasmodium falciparum in vitro* and *P. vinckei petteri in vivo* with  $ED_{50} = 1.1$  mg kg<sup>-1</sup> and  $ED_{90} = 3.5$  mg kg<sup>-1</sup> has been proved (100% of infected mice have been cured at the optimum concentration). What is interesting is that this activity is about 25-fold more potent than that of the commonly used antimalarial agent – chloroquine. Antimalarial action of monensin can be explained by impaired function of the nutrient and other vacuolar organelles of the parasite as well as intracellular acidification, which lead to the cell death [171]. It is worth noting that some of the polyether antibiotics show antimalarial activity against chloroquine-resistant strains. The effects of several ionophores against chloroquine-sensitive as well as chloroquine-resistant strains of *P. falciparum* have been demonstrated. Results of these studies have clearly shown that monensin and salinomycin are more active against chloroquine-resistant strains than the commonly used drugs, such as amodiaquine, artemether, artemisinin, artesunate, pyrimethamine, trimethoprim, or quinine. The cytotoxicity and selectivity against normal mammalian cells has been also demonstrated [33, 172].

The difference in the *in vitro* activity of polyether antibiotics between normal mammalian cells and *P. falciparum* has been analyzed. It turned out that some of these compounds, such as monensin, exhibit significantly higher (approximately 35-fold) activity toward the malarial parasite cells than to normal cells [169]. The *in vivo* toxicity of monensin, lasalocid acid, and other ionophores has been studied in rats infected with *P. chabaudi* and *P. vinckei petteri* [170, 172]. Coccidiosis, a parasitic disease caused by *Coccidia* protozoa (of the genus *Eimeria*), is a serious problem in cattle, poultry and rabbit breeding, which significantly affects the economic results of breeding. It has been shown that polyether antibiotics are effective against *E. tenella* [149]. Therefore, the first approved application of this class of compounds was their use in the prophylactic and therapeutic treatment of coccidiosis in poultry [173–175] and as growth-promoting agents [176, 177], among which lasalocid acid and salinomycin are the most prevalent [145].

It has been reported that monensin and salinomycin are anticoccidial agents active against *E. tenella*, *Eimeria necatrix*, *Eimeria acervulina*, *Eimeria maxima*, and *Eimeria brunette*. These compounds are more effective than many commonly used drugs with anticoccidial indexes (ACIs) of 144.1 and 127.1 for monensin sodium salt and salinomycin sodium salt, respectively [53, 178, 179]. *Cryptosporidium parvum* is a species of protozoan pathogen affecting the alimentary tract in humans and animals. In humans, it causes a disease called *cryptosporidiosis*, whose symptoms are nausea, vomiting, diarrhea, shortness of

breath, cough, and raised temperature. In people with immunodeficiency (for example, people living with AIDS), this infection can lead to death [180–183].

The *in vitro* and *in vivo* tests have demonstrated that monensin, lasalocid acid, salinomycin, and a few other ionophores are active against *C. parvum*. For example, monensin reduces growth of this species of protozoan by more than 90% [184–191]. *Toxoplasma gondii* is a species of pathogenic protozoa of the genus *Toxoplasma* causing toxoplasmosis in animals, while it causes zoonosis in humans. It occurs in about 200 species of birds and mammals, but the final hosts are cats in which the oocysts are formed. Toxoplasmosis infection in humans takes place directly by ingestion of dirty vegetables containing cysts or indirectly by eating undercooked meat. Infection with this parasite causes fatal central nervous system lesions and is particularly dangerous, especially for patients with AIDS [192–198].

Usually, treatment for toxoplasmic encephalitis is a combination therapy of pyrimethamine and sulfadiazine. Unfortunately, this type of chemotherapy is not effective against the cystic stage of *T. gondii* [199, 200]. It has been proved that some of the polyether antibiotics are active against the tachyzoite stage of *T. gondii* [195, 197, 201]. *In vitro* and *in vivo* studies have proved that very low dosages of monensin considerably change the swollen cysts and numbers of *T. gondii* vacuoles. Low-dose treatment with this compound (about  $0.1 \mu\text{g ml}^{-1}$ ) eliminates abnormal cells [197].

Neosporosis is a disease caused by the protozoan *Neospora caninum*, found in dogs and domestic cattle. This leads to profound defects in the central nervous system and death in young dogs. In cows, the parasite causes miscarriages and a high mortality rate among young calves. It has been shown that 43 chemotherapeutics, including 8 miscellaneous antiprotozoal agents, dihydrofolate reductase/thymidylate synthase inhibitors, lincosamides, macrolides, pentamidine analogs, sulfonamides as well as 6 polyether antibiotics, such as monensin, lasalocid acid, and salinomycin are effective against *N. caninum* [202].

Among all tested derivatives, only monensin urethanes show antimalarial activity (see Table 6.6). Chemical modification of monensin has led to improvement in its *in vivo* antimalarial activity against *P. falciparum* [79]. Additionally, it has been shown that phenylurethane and chlorophenylurethane (see Figure 6.4, compounds **5** and **9**, respectively) are active against *Plasmodium berghei*. This activity is as much as 40 times higher in comparison with that of the starting material. The toxicity of monensin alkylurethanes (compounds **2–4**) is lower than that of the parental compound and phenylurethanes (compounds **5–12**) are similar, except bromophenylurethane (compound **10**), which was three times less toxic than unmodified monensin [73].

On the other hand, it has been shown that lasalocid acid derivatives, such as phenolic hydroxyl group esters, para to the phenol ring-substituted derivatives, and anhydro and epoxide derivatives (see Figure 6.23, compounds **132–141** and **145–147**) are active against *E. tenella*; the exception is methyl ether

**Table 6.6** Antimalarial and coccidiostatic *in vivo* activity in mice of selected monensin urethanes (5–7 and 9–12) in comparison to monensin sodium salt (1-Na<sup>+</sup>) [73].

	Antimalarial activity against <i>P. berghei</i>	Coccidiostatic activity against <i>E. tenella</i>	
	(mg kg <sup>-1</sup> )	(ppm)	ADI <sup>a)</sup>
1-Na <sup>+</sup>	80	120	0.8
5	4	120	0.5
6	8	120	2.1
7	5	70	0.5
9	2	70	0.9
10	16	70	0.5
11	5	120	0.1
12	5	70	1.1

a) ADI is the average degree of infections and values of <1.5 indicate high coccidiostatic activity.

(compound **131**) and the nitrogen-substituted derivatives (compounds **142–144** and **148**). However, this activity is lower than that of the parental compound [124].

#### 6.4.4

##### Antiviral Activity of Polyether Antibiotics

HIV attacks and weakens the immune system. In the final stages of infection, it leads to AIDS, which is characterized by a very low level of CD-4 lymphocytes. This results in a high morbidity from such diseases as some forms of cancer, fungal infections, or atypical pneumonia.

Interestingly, polyether antibiotics, including lasalocid acid, monensin, and salinomycin have been found effective as anti-HIV agents, against both the acute and chronic infection [203–207]. Salinomycin has been the most potent in these studies (see Table 6.7) [206].

Polyether antibiotics have a slightly different mechanism of action against human immunodeficiency virus, because they target different stages of the HIV infection cycle, that is, pre- and post-absorption steps [203–207]. Generally, these compounds are divided into two main groups on the basis of the exposure of the H9 cells to ionophores before, during, and after viral absorption. The first group, including lasalocid acid, inhibits the absorption of the virus and the second group, including monensin and salinomycin, interferes with the virus at the post-absorption stage.

Moreover, some of the polyether antibiotics are effective against several other DNA and RNA viruses. Lasalocid acid, monensin, and other ionophores have been tested against transmissible gastroenteritis coronavirus (TGEV), Newcastle disease virus (NDV), Angara disease virus (ADV), infectious canine hepatitis virus

**Table 6.7** Anti-HIV *in vitro* activity of monensin (1), salinomycin (67), and lasalocid acid (122) in acute and chronic infection.

	Acute infection (H9)			Chronic infection (U937)		
	EC <sub>90</sub> (μg ml <sup>-1</sup> )	IC <sub>50</sub> (μg ml <sup>-1</sup> )	IC <sub>50</sub> /EC <sub>90</sub>	EC <sub>90</sub> (μg ml <sup>-1</sup> )	IC <sub>50</sub> (μg ml <sup>-1</sup> )	IC <sub>50</sub> /EC <sub>90</sub>
<b>1</b>	4.22 ± 2.60	13.44 ± 2.80	3.18	0.50 ± 0.25	0.59 ± 0.19	1.18
<b>67</b>	0.40 ± 0.04	6.98 ± 2.90	17.63	0.09 ± 0.02	0.28 ± 0.05	3.14
<b>122</b>	2.37 ± 2.90	48.80 ± 4.70	20.63	0.20 ± 0.20	1.35 ± 0.35	6.92

Data are given as MIC ± SD (minimal inhibitory concentration, μg ml<sup>-1</sup>) as well as EC<sub>90</sub> ± SD (effective concentration, μg ml<sup>-1</sup>) [206].

(ICH) as well as infectious bovine rhinotracheitis virus (IBRV), which are major issues in stockbreeding [145, 177]. It has been shown that lasalocid acid, monensin, and salinomycin can be regarded as anti-NDV, anti-ADV, and anti-TGEV agents with significant activity against these types of animal viruses.

#### 6.4.5

##### Anticancer Activity of Polyether Antibiotics and Their Derivatives

One of the most important and the most difficult challenges of modern science is the fight against neoplastic diseases. According to the World Health Organization (WHO), cancer is the major cause of death worldwide. In 2012, the number of people who died from cancer amounted to 8.2 million. It is expected that annual cancer cases will rise from 14 million in 2012 to 22 million within the next two decades [208].

One of the reasons why cancer is so difficult to treat is the presence of CSCs. They are not numerous, but well hidden in the tumor, which significantly impede their location and destruction. What is worse, if not removed during therapy, they often cause recurrence of the disease. In addition, CSCs exhibit very strong chemo- and radioresistance [209]. For this reason, all over the world, intense studies to find the effective agents against CSCs have been undertaken.

Over the last decades, various types of natural compounds have been used as antitumor agents, both in their original, chemically unmodified forms as well as in the synthetically transformed forms [143]. Therefore, it seems that the easiest way to discover new effective anticancer drugs is chemical modification of naturally occurring substances with proven high biological activity, such as polyether antibiotics. The antiproliferative activity of lasalocid acid has been proved in 2013. Its activity was tested against six different cell lines: human breast adenocarcinoma cell line (MCF-7), human colon adenocarcinoma cell line (HT-29), human lung adenocarcinoma cell line (A-549), human lung microvascular endothelial cell line (HLMCEC), P-388, and murine embryonic fibroblast cell line (BALB/3T3).

In some cases, the activity is higher and the cytotoxicity to normal cells is lower than those of the commonly used cytostatic drug – cisplatin [122, 142].

In the same research group, the anticancer activity of a series of lasalocid acid Mannich base derivatives (see Figure 6.26, compounds **178–184**) as well as lasalocid acid salts with amines (see Figure 6.21, compounds **127–130**) against the same cancer cell lines has been found (see Table 6.8) [122, 142]. All lasalocid acid complexes are moderately to highly active against all tested cancer cell lines. All the compounds are very promising, because they are more effective than the cisplatin positive control. Lasalocid acid and its salts show almost equipotent activity as cisplatin only against MCF-7 and P-388 cell lines. The cytotoxicity of all tested compounds is about threefold stronger than that of cisplatin against HT-29, A-549, and HLMEC cell lines, which may suggest their potential antiangiogenic activity. In addition, moderate activity of the complexes against normal fibroblast cell lines may predict their lower toxicity in further *in vivo* studies. The structure–activity relationship (SAR) study revealed that the lasalocid acid complexes holding the aromatic amine species (compound **127**) show similar cytotoxicity to that of aliphatic amine species (compounds **128–130**) and slightly lower than that of pure lasalocid acid [122].

On the other hand, Mannich bases of lasalocid acid, except for three derivatives (compounds **182–184**), show superior activity against MCF-7, HT-29, and

**Table 6.8** Antiproliferative activity of lasalocid acid (**122**) and its complexes with amines (**127** and **128**) and the most effective Mannich bases (**178–181**) against various cancer cell lines [122, 142].

	IC <sub>50</sub> ± SD (μM)					
	A-549 <sup>a)</sup>	HT-29 <sup>b)</sup>	MCF-7 <sup>c)</sup>	P-388 <sup>d)</sup>	HLMEC <sup>e)</sup>	BALB/3T3 <sup>f)</sup>
<b>122</b>	1.80 ± 0.61	1.95 ± 0.78	3.12 ± 1.21	4.54 ± 1.00	1.75 ± 0.54	7.35 ± 0.89
<b>127</b>	1.99 ± 0.18	4.56 ± 0.79	5.96 ± 0.94	3.98 ± 0.67	0.57 ± 0.09	7.49 ± 1.49
<b>128</b>	1.99 ± 0.15	4.61 ± 0.83	6.28 ± 0.92	3.22 ± 1.30	0.56 ± 0.03	6.88 ± 1.76
<b>178</b>	6.81 ± 0.52	5.74 ± 1.35	2.08 ± 0.57	3.23 ± 0.89	4.86 ± 0.31	14.33 ± 5.45
<b>179</b>	5.30 ± 0.24	7.03 ± 2.67	5.62 ± 1.06	3.58 ± 0.82	4.12 ± 0.52	22.72 ± 16.33
<b>180</b>	5.62 ± 0.45	5.33 ± 1.77	5.50 ± 0.92	4.79 ± 0.55	4.63 ± 0.42	17.05 ± 7.03
<b>181</b>	5.27 ± 0.81	7.02 ± 2.04	6.11 ± 0.51	6.35 ± 2.07	4.43 ± 0.78	ia <sup>g)</sup>

Data are given as IC<sub>50</sub> ± SD (μM).

- a) Human lung adenocarcinoma cell line (A-549).
- b) Human colon adenocarcinoma cell line (HT-29).
- c) Human breast adenocarcinoma cell line (MCF-7).
- d) Murine leukemia cell line (P-388).
- e) Human lung microvascular endothelial cell line (HLMEC).
- f) Murine embryonic fibroblast cell line (BALB/3T3).
- g) ia – inactive compound.

A-549 than that of the standard anticancer drug – cisplatin. From among all these compounds, three Mannich bases (compounds **178–179** and **182**) show the most potent antiproliferative activity against P-388 cell line – this activity is higher than that of unmodified lasalocid acid. Lower activity of derivatives with alkyl-aryl (compound **182**) groups or long alkyl groups (compounds **183–184**) in view of the high molecular weight and volume suggests that these types of substituents are unfavorable. In addition, most of the Mannich bases are less toxic against normal cell lines than the reference compound – cisplatin [142]. The anticancer properties of monensin were discovered earlier than those of lasalocid acid. It has been demonstrated that this ionophore significantly inhibits the proliferation of human colon cancer cell line (SNU-C1), lymphoma cell line, and myeloma cell line (NCI-H929) as well as inducing G1 and/or G2-M phase arrest in these cells. Detailed analysis indicated that monensin inhibits the lymphoma cell line proliferation not only by inducing cell cycle arrest but also by triggering apoptosis through the loss of mitochondrial transmembrane potential [210–212].

In addition, potential *in vitro* cytotoxicity of monensin toward immunotoxins and its beneficial role in overcoming the MDR of cancer cells has been proved. These studies have demonstrated that the cytotoxic activity of immunotoxin SWA11 ricin A-chain is 100-fold increased in the presence of monensin (99.9% of clonogenic tumor cells were eliminated at  $10^{-7}$  M concentration). Kinetic studies revealed that monensin enhances the rate of protein synthesis inhibition twofold and eliminates the lag phase. Moreover, it has been shown that monensin is a potent proliferation inhibitor of the KB parent and KB/MDR cells and also markedly reduces doxorubicin efflux from KB/MDR cells. In a 2 h experiment, the presence of monensin causes a nearly threefold increase in the intracellular accumulation of doxorubicin in KB/MDR cells. The  $ID_{50}$  of doxorubicin against KB/MDR cells in the presence of monensin after a 72 h drug exposure is reduced about fivefold, while doxorubicin cytotoxicity against KB parent cells is not significantly altered [213–218].

In view of the high lipophilicity and short half-life of monensin, suitable drug delivery systems are needed to obtain the desired *in vivo* effects. In several studies interesting delivery systems, including long-circulating liposomes as well as nanoparticles have been presented. It has been shown that long-circulating monensin liposomes overcome the human doxorubicin-resistant breast adenocarcinoma cells (MCF-7/DX). MCF-7/DX cells were treated with various anticancer agents (doxorubicin, etoposide, and *Paclitaxel*) alone and in combination with long-circulating monensin liposomes. It has been proved that long-circulating monensin liposomes overcome the drug resistance in MCF-7/DX cells to doxorubicin, etoposide, and *Paclitaxel* by 16.5, 5.6, and 2.8-times, respectively. The separate use of doxorubicin and long-circulating monensin liposomes induce minimal apoptosis (<10%) in MCF-7/DX cells, whereas a combination of doxorubicin with

long-circulating monensin liposomes induce apoptosis in ~40% of MCF-7/DX cells [219–222].

Furthermore, monensin has been incorporated into liposomes and has been used in combination with specific immunotoxins against human cancer cell lines *in vitro* (malignant mesothelioma H-MESO-1 (malignant mesothelioma cell line), colorectal carcinoma LS174T (human colorectal carcinoma cell line) as well as human glioblastomas MG-1, U87, and U373) and *in vivo* (in mice). These studies have demonstrated that the combination of immunotoxin (especially ricin A-chain) with liposomal monensin is fivefold more effective against H-MESO-1 cells, 1000-fold more effective against U373 cells, and 2200-fold more effective against U87 cells than immunotoxin and monensin acid in buffer. *In vivo* studies have clearly shown that liposomal monensin in combination with immunotoxin substantially prolongs survival in about 21% of mice bearing H-MESO-1 cells after 160 days of treatment [223].

Recent studies have also revealed that monensin is one of the most potent and cancer-specific inhibitors from among the well-known drugs and druglike compounds, in screening of prostate cancer. It has been shown that only four, including monensin, from 4910 tested compounds selectively inhibit prostate cancer cell growth at nanomolar concentrations. The anticancer activity of monensin has been proved to be linked with induction of apoptosis, potent reduction of mRNA androgen receptors and proteins as well as elevated intracellular oxidative stress in prostate cancer cells. On the other hand, the antiproliferative effects of monensin have been found to be potentiated by combinational treatment with antiandrogens [224].

Salinomycin caught the attention of physicians and scientists from around the world in 2009, when it was announced that the antibiotic is nearly 100-fold more effective toward the breast CSCs than the commonly used cytostatic drug – *Taxol* (*Paclitaxel*) [225]. The tests were conducted on about 16 000 substances, of which only 32 were destroying programmed CSCs and the most effective proved to be salinomycin. Since then, extensive research work has been undertaken to explain the unusual antitumor properties of this compound.

It is noted that salinomycin forces apoptosis of dangerous tumor cells. Blood samples were collected from patients with leukemia, from which lymphocytes, which play a key role in protecting the body's immune system, were isolated. Then, the obtained cells were treated with different doses of salinomycin. The application of the highest dose of ionophore resulted in apoptosis of almost all leukemic cells, with no damage to the normal cells [226].

Salinomycin is capable of inducing programmed cell death of human tumor cells of various tissues exhibiting MDR, for example, leukemic CSCs exhibiting resistance by expression of ATP-binding cassette transporters (ABC) [227–229]. These transporters belong to a family of transmembrane proteins capable of removing various cytostatic drugs from the cytosol of cells. This leads to the development of MDR in these cells, which is a major obstacle in the fight against neoplastic



diseases [230–232]. It has been discovered that salinomycin is able to overcome drug resistance in human leukemic CSC cells

It has been shown that salinomycin inhibits the Wnt signaling pathway and induces tumor cell apoptosis in chronic lymphocytic leukemia [233]. This ionophore has been found to strongly inhibit a proximal Wnt/ $\beta$ -catenin signaling and block phosphorylation of the Wnt-LRP6 (low-density lipoprotein receptor-related protein 6) coreceptor which leads to its degradation. The Wnt/ $\beta$ -catenin signaling pathway plays a role in stem cell renewal and is involved in the pathogenesis of different types of cancer. Aberrant activation of the Wnt signaling pathway in normal stem cells may result in their transformation into CSCs [234]. Thus, the compounds affecting the Wnt/ $\beta$ -catenin signaling pathway are potential drugs against CSCs.

Additionally, *in vitro* tests have also confirmed the potent antitumor activity of salinomycin against human lung adenocarcinoma A-549 cells [235]. It has been demonstrated that this compound causes concentration- and time-dependent reduction in viability of LNM35 (another human lung cancer cell line) and A-549 cells through a caspase 3/7-associated cell death pathway. Similarly, it significantly decreases the growth of LNM35 and A-549 colonies in soft agar. Development of numerous metastases is the main problem in the combat of lung cancer. What is interesting is that this ionophore induces a time- and concentration-dependent inhibition of cell migration and invasion. It has been also proved that salinomycin induces a marked increase in the expression of the proapoptotic protein NAG-1 leading to the inhibition of lung cancer cell invasion, but not cell survival [236].

The ability of this ionophore to reduce the subpopulation of colon adenocarcinoma CSCs and the considerable activity against human colon cancer cells have been observed. Moreover, these cells are more sensitive to the effects of salinomycin than to oxaliplatin, a cytostatic drug commonly used in antitumor chemotherapy of colorectal cancer. The use of salinomycin causes a decrease in mobility as well as colon tumor cell clonogenicity [237]. Salinomycin blocks the growth and migration of chemoresistant prostate cancer cells and also causes accumulation of reactive oxygen species (ROSs), which leads to the depolarization of the mitochondrial membrane and cell apoptosis. The androgen-sensitive (LNCaP) and classical (PC-3 and DU-145) human prostatic cancer cells have been proved to have reduced viability upon exposure to salinomycin, depending on the dose and duration of exposure. Nonmalignant prostate cells (RWPE-1) were found less sensitive to this antibiotic. Salinomycin induces apoptosis in prostate cancer cells (PC-3) by increasing the intracellular level of ROS, which is accompanied by a reduction in mitochondrial membrane potential, Bax protein translocation to mitochondria, release of cytochrome *c* into the cytoplasm, activation of caspase-3, and cleavage of the key enzyme in many nuclear physiological processes, poly[ADP-ribose]polymerase 1 (PARP-1) [238].

It has been demonstrated that this antibiotic can substantially sensitize the tumor cells treated by irradiation which can lead to DNA damage by two

pathways and to decrease in p21 protein levels. These damages, by inhibiting CSCs division, support the antiapoptotic effect of tumor cells [239–241]. In addition, the sensitizing effect of salinomycin has been evidenced upon treatment with cytostatic agents [242]. The role of salinomycin as a P-glycoprotein (P-gp) inhibitor, which supports the flow of anticancer drugs from cells, has been also tested [228]. Salinomycin is a stronger sensitizer than the well-known inhibitor of P-gp – *Verapamil* [239]. Moreover, it has been proven that the CSCs resistant to *Taxol* (*Paclitaxel*), 5-fluorouracil, and cisplatin are susceptible to the effects of salinomycin [239, 242–244]. It also sensitizes tumor cells treated with the commonly used cytostatic drugs – *Taxol* (*Paclitaxel*), *Docetaxel*, vinblastine, and colchicine [243, 244]. Synergistic antitumor effect of salinomycin in combination with gemcitabine against human pancreatic cancer cells has been also evidenced; salinomycin strongly inhibits the growth of CSCs and gemcitabine effectively dampens its viability [245].

Salinomycin inhibits proliferation and induces apoptosis of human nasopharyngeal carcinoma cell *in vitro* and suppresses tumor growth *in vivo* [246]. The inhibitory effect of salinomycin on the proliferation, migration as well as invasion of human endometrial CSCs has also been documented [247]. The tests performed on three human breast cancer cell lines MCF-7, T47D, and MDA-MB-231 have clearly shown that salinomycin is able to destroy MCF-7 and MDA-MB-231 cells, by the upregulation of p21, downregulation of surviving as well as histone H3 and H4 hyperacetylation [248]. The influence of salinomycin on growth and migration in pancreatic and human hepatocellular carcinoma cell lines *in vitro* and *in vivo* has been studied [249, 250].

Salinomycin induces apoptosis in cisplatin-resistant colorectal as well as cisplatin-resistant ovarian cancer cells by accumulation of ROS and inhibition of cell signaling molecules, such as Akt and NF- $\kappa$ B [251, 252]. Growth inhibitory effects of salinomycin in the ovarian OV2008, C13, A2780, A2780-cp, SKOV3, and OVCAR3 cancer cell lines which are potentially associated with the p38 mitogen-activated protein kinase (MAPK) activation [253], have been observed *in vitro* and *in vivo* tests.

The exact mechanism of anticancer action of salinomycin is not yet understood, but it may be related to the ability of this ionophore to lower intracellular pH, resulting in subsequent inhibition of DNA synthesis (*in vitro* around 100% DNA synthesis inhibition was reported by 0.5 mM of salinomycin) [254]. It seems that salinomycin, demonstrating a strong affinity to potassium cations, promotes the outflow of these ions from the mitochondria and cytoplasm. Results obtained for other polyether antibiotics suggest that a decrease in potassium concentration is necessary for apoptosis of human lymphoma and human lung cancer cells [227]. This suggests that salinomycin, causing an unconventional way of cancer cell death, is involved, at least partly, in exhausting the cytoplasmic and mitochondrial potassium concentration and/or interferes with the potassium membrane potential. Excessive potassium channel expressivity in mitochondrial membrane and in

cytoplasm, which is noted in many human cancer cells, plays an important role in the cell cycle progression, proliferation, and apoptosis of tumor cells. The effect of salinomycin on the overexpression of these channels is much promising for development of new cancer therapies [225]. Salinomycin induces cell death with autophagy through activation of endoplasmic reticulum stress in human cancer cells [255].

Induction of apoptosis by the commonly used cytostatic drugs mostly depends on the expression of a functional p53 protein, but salinomycin causes programmed cell death irrespective of the level of this protein in the cell. Salinomycin is involved in the activation of a separate apoptotic pathway, which is not accompanied by a change in the cell cycle and which is independent of the cancerous p53 protein suppressor, proteasomes, and caspase activity [227]. In 2010, salinomycin was approved for testing on humans. The tests were made on a group of patients with invasive carcinoma of the head, neck, breast, and ovary in the screening studies. Patients were administered 200–250  $\mu\text{g kg}^{-1}$  of salinomycin intravenously every second day for 3 weeks. Two cases are described in the literature in detail. In both cases, the administration of salinomycin resulted in inhibition of disease progress over an extended period. Acute side effects were rare and serious long-term adverse side effects were not observed [256]. An interesting direction of research is the chemical modification of salinomycin, which leads to obtain various derivatives with significantly lower toxicity than unmodified ionophore and better biological activity. These derivatives could be used not only in veterinary medicine but also in medicine.

Until now, the synthesis, chemical structure, and antiproliferative properties of a series of amides (see Figure 6.14, compounds **68–86**) and esters (see Figure 6.15, compounds **87–99**) as well as O-acylated derivatives (see Figure 6.16, compounds **100–121**) of salinomycin have been described [108–113]. Cytostatic activity of salinomycin amides (compounds **68–86**) and esters (compounds **87–99**) were determined *in vitro* against human leukemia cells sensitive and resistant to vincristine (human vincristine-sensitive leukemia cell line (HL-60) and human vincristine-resistant leukemia cell line (HL-60/vinc)), human colon cancer cells sensitive and resistant to doxorubicin (LoVo and LoVo/DX), and against normal murine embryonic fibroblasts (BALB/3T3). The tests were made for concentrations from 100 to 0.1 mM. Doxorubicin and cisplatin were the reference compounds. The salinomycin amides were tested in concentration ranges from 100 to 0.1 mM.

All the aforementioned and tested compounds are more or less active in the specified concentration range. Their activities were different and depended on the tested cell line. Salinomycin derivatives break (strongly or moderately) the MDR of used cancer cells and this process depends on the chemical nature of salinomycin derivatives [110]. The results indicate that salinomycin and its dopamine (compound **82**) and especially 4-fluorophenethyl (compound **81**) amides exhibit the highest ability to inhibit proliferation of different cancer cell

**Table 6.9** Antiproliferative activity of salinomycin (**67**) and its selected amides (**69**, **71–74**, **81–82**) and esters (**90–91**, **94**, **96**) against various cancer cell lines [110–112].

	IC <sub>50</sub> ± SD (μM)				
	HL-60 <sup>a)</sup>	HL-60/vinc <sup>a)</sup>	LoVo <sup>b)</sup>	LoVo/DX <sup>b)</sup>	BALB/3T3 <sup>c)</sup>
<b>67</b>	0.44 ± 0.16	3.44 ± 0.32	1.11 ± 0.15	6.23 ± 1.72	28.08 ± 4.63
<b>69</b>	3.88 ± 0.04	5.31 ± 0.68	4.04 ± 0.17	3.26 ± 0.61	8.21 ± 1.14
<b>71</b>	3.79 ± 0.07	4.31 ± 0.53	4.11 ± 0.15	3.21 ± 0.49	7.08 ± 1.40
<b>72</b>	3.63 ± 0.25	6.02 ± 0.72	4.02 ± 0.17	3.31 ± 0.76	7.26 ± 1.02
<b>73</b>	3.52 ± 0.13	4.31 ± 0.52	3.45 ± 0.26	2.78 ± 0.47	9.98 ± 4.71
<b>74</b>	3.08 ± 0.25	6.87 ± 0.27	6.24 ± 1.08	5.65 ± 1.12	25.47 ± 4.24
<b>81</b>	2.26 ± 0.24	6.74 ± 1.15	4.09 ± 0.14	2.34 ± 0.49	45.80 ± 20.94
<b>82</b>	2.77 ± 1.11	6.88 ± 1.08	3.88 ± 0.24	4.26 ± 0.74	5.69 ± 2.17
<b>90</b>	3.58 ± 0.45	4.15 ± 1.50	4.04 ± 0.09	3.99 ± 0.06	24.32 ± 7.27
<b>91</b>	0.47 ± 0.22	3.05 ± 0.38	0.78 ± 0.24	0.80 ± 0.07	23.82 ± 6.49
<b>94</b>	3.73 ± 0.21	9.33 ± 1.47	7.34 ± 0.35	4.70 ± 0.28	35.80 ± 1.66
<b>96</b>	1.84 ± 0.37	5.25 ± 0.55	4.11 ± 0.20	6.61 ± 1.12	31.90 ± 5.33

Data are given as IC<sub>50</sub> ± SD (μM).

- a) Human leukemia cell line sensitive and resistant to vincristine (HL-60 and HL-60/vinc).  
 b) Human colon cancer cell line sensitive and resistant to doxorubicin (LoVo and LoVo/DX).  
 c) Normal murine embryonic fibroblasts (BALB/3T3).

lines (see Table 6.9) [111]. Among the obtained ester derivatives of salinomycin, trifluoroethyl ester (compound **91**) has revealed the strongest antiproliferative activity. This activity is higher than that of unmodified salinomycin, its other esters, as well as cytotoxic drugs such as cisplatin or doxorubicin (see Table 6.9). In addition, it has been shown that a given ester moiety to some extent determines the bioactivity of these derivatives. Generally, the preliminary SAR studies have demonstrated that the most potent anticancer compounds among salinomycin esters are those which contain trifluoroethyl ester group (compound **91**) or are accompanied by short aliphatic chain (compounds **87** and **90**),  $\alpha$ -naphthylmethyl (compound **94**), or polar di-*o*-nitrobenzyl (compound **98**) ester substituents [112].

Moreover, the effect of the obtained compounds on normal murine fibroblasts has been tested. Some of the tested derivatives are characterized by a weaker toxicity against BALB/3T3 cell line (IC<sub>50</sub> > 20 μM). Salinomycin and most of its analogs are less toxic against normal embryonic murine fibroblasts than the commonly used cytostatic drugs – doxorubicin and cisplatin [108, 110–112].

The antiproliferative activity of the O-acylated derivatives (compounds **100–121**) against two human breast adenocarcinoma cell lines (JIMT-1 and MCF-7) has been evaluated. The C20-acylated analogs (compounds **102–118**) display IC<sub>50</sub> values lower (to one-fifth) or similar to that of salinomycin sodium salt toward both cancer cell lines (IC<sub>50</sub> = 0.52–0.59 μM and IC<sub>50</sub> = 0.09–0.81 μM

for salinomycin sodium salt and its C20-acylated analogs, respectively). The most active C20 derivatives are those with the least bulky substituents in each series – ethyl carbonate (compound **102**,  $IC_{50} = 0.09–0.13 \mu M$ ), ethyl carbamate (compound **104**,  $IC_{50} = 0.16–0.26 \mu M$ ) as well as acetate (compound **107**,  $IC_{50} = 0.11 \mu M$ ). The analogs deprived of stabilizing interaction between the carboxylate and the C9-hydroxyl group (compound **101**) exhibit reduced activity ( $IC_{50} = 1.67–1.85 \mu M$ ). The preserved anticancer activity of the C28 carbamates (compounds **119–121**,  $IC_{50} = 0.62–5.56 \mu M$ ) suggest that in biological membranes, C28-hydroxyl group does not contribute to the ion binding of salinomycin sodium salt by hydrogen bonding to the carboxylate [113].

Recent studies indicate that divalent metal complexes of salinomycin with manganese, cobalt, and nickel are more active than salinomycin salts with sodium or potassium. In addition, the liposomal encapsulation of these complexes is not detrimental to their antiproliferative activities. It has been proved that the liposomal formulations exert comparable cytotoxic effects to that of free divalent metal complexes and in some cases they outclass the nonencapsulated agents [257].

## 6.5

### Concluding Remarks

Natural products are the original source of well known and commonly used drugs. Polyether ionophores, which belong to a large group of antibiotics, are unique natural compounds because they exhibit a broad spectrum of biological activities, including antibacterial, antiviral, and anticancer activities as described in the preceding.

High biological activity of polyether ionophores is related to their unique chemical structure, as well as their ability to form complexes with mono- and divalent metal cations and transport them across lipid membranes. It results in a disturbance of the natural cation concentration gradient and intracellular pH change, leading to mitochondrial injury, cell swelling, vacuolization and, as a consequence, programmed cell death (apoptosis).

On the other hand, the easiest method for preparing biologically effective compounds is the chemical modification of substances with proven high biological activity. Tests clearly demonstrated that these derivatives very often are more antimicrobial and anticancerous compared to their parent compounds.

All the aforementioned studies prompt scientists to search for a new group of polyether ionophore derivatives, which will be more effective in coordination of the metal cations and less toxic especially for humans. Only then can such compounds potentially become drugs in the near future.

**Abbreviations**

A2780 and A2780-cp	Ovarian cancer cell lines
A549	human lung cancer cell line
ACI	anticoccidial index
ADI	average degree of infections
AIDS	acquired immunodeficiency syndrome
BALB/3T3	normal embryonic murine fibroblasts
C13	ovarian cancer cell line
CSCs	cancer stem cells
DU-145	prostate cancer cell line
ED <sub>90</sub>	effective dose required for desired effect in 90% of the population exposed to it
H-MESO-1	malignant mesothelioma cell line
HIV	human immunodeficiency virus
HL-60	human vincristine-sensitive leukemia cell line
HL-60/vinc	human vincristine-resistant leukemia cell line
HLMEC	human lung microvascular endothelial cell line
HT-29	human colon adenocarcinoma cell line
IC <sub>50</sub>	half-maximal inhibitory concentration
JIMT-1	human breast adenocarcinoma cell line
LD <sub>50</sub>	median lethal dose
LNCaP	prostate cancer cell line
LN35	human lung cancer cell line
LoVo	human doxorubicin-sensitive cell line
LoVo/DX	human doxorubicin-resistant cell line
LRP6	low-density lipoprotein receptor-related protein 6
LS174T	human colorectal carcinoma cell line
MCF-7	human breast adenocarcinoma cell line
MCF-7/DX	doxorubicin-resistant breast adenocarcinoma cell line
MDA-MB-231	breast cancer cell line
MDR	multidrug resistance
MG-1	human glioblastomas
MIC	minimal inhibitory concentration
MRSA	methicillin-resistant <i>S. aureus</i>
MSSA	methicillin-sensitive <i>S. aureus</i>
NCI-H929	lymphoma and myeloma cell line
OV2008 and OVCAR3	ovarian cancer cell lines
P-388	murine leukemia cell line
PARP-1	poly[ADP-ribose]polymerase 1
PC-3	prostate cancer cell line
ROS	reactive oxygen species
RWPE-1	nonmalignant prostate cell line

SKOV3	ovarian cancer cell line
SAR	structure–activity relationship
SNU-C1	human colon cancer cell line
T47D	breast cancer cell line
U87 and U373	human glioblastomas

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## 7

### Bioactive Flavaglines: Synthesis and Pharmacology

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#### 7.1

##### Introduction

A recent survey of the first-in-class medicines approved over the last decades indicates that a quarter of them are derivatives of natural products [1]. This report clearly demonstrates the importance of these compounds in the development of new drugs. Indeed, natural compounds display certain notable advantages compared to synthetic drugs [2]. Firstly, natural products are secondary metabolites that were selected through evolution to act as chemical weapons or signaling molecules, with the ability to reach their receptor in the targeted organism. As such, they often have the ability to cross biological membranes. Many of these are suspected to be substrates of membrane transporters. This is an important issue, because natural compounds that are identified on the basis of *in vitro* pharmacological assays are often also active *in vivo*. Secondly, natural products, in general, have more chiral centers, more varied ring systems, a higher ratio of  $Csp^3/Csp^2$ , less nitrogen, and more oxygen atoms than synthetic drugs. This structural complexity provides excellent opportunities to explore new areas of chemical space and to generate original and therefore patentable compounds.

Although natural products had traditionally been invaluable as a source of medicines, the development of new natural compounds in oncology was interrupted for over 10 years with the advent of targeted therapies [2]. After the approval of Topotecan in 1996, the development of other natural products essentially came to a stop because of the nearly exclusive focus on targeted therapies by the pharmaceutical industries. However, because targeted therapies did not fulfill all their expectations, natural product derivatives returned to front stage. This is evident from the approval of 14 of these compounds between 2007

and 2013 for use in oncology [2]. Since the initial report of rocaglamide (**1**) by King and collaborators in 1992 [3], there has been growing interest from chemists and biologists in this unique class of cyclopenta[*b*]benzofurans called *flavaglines* (or sometimes, *rocaglamides* or *rocaglates*). Figure 7.1 offers a glimpse of certain significant natural and synthetic flavaglines.

The flavaglines are a family of more than 100 cyclopenta[*b*]benzofurans found in Asian plants of the genus *Aglaia* (Meliaceae). These compounds display potent insecticidal, antifungal, anti-inflammatory, neuroprotective, cardioprotective, and above all, anticancer activities. Their most intriguing feature is the selectivity of their cytotoxicity toward cancer cells. Indeed, as far as we know, all cancer cell lines and transformed cell lines are sensitive to this cytotoxicity, while primary cell cultures of noncancerous cells are not affected. This selective cytotoxicity was first described by Marian and collaborators [4]. It has also been observed that flavaglines promote the survival of neurons and cardiac cells toward many types of stresses. This unique feature is not rationalized with our current state of knowledge; it seems that these compounds target a feature that is consubstantial to the nature of the cancer itself.

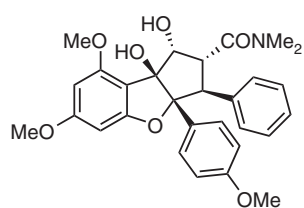
The chemistry and pharmacology of flavaglines has been the object of several reviews [5–9]. Over the last year, we observed acceleration in the pharmacological investigation of the flavaglines, marked in particular by the identification of their molecular targets. The purpose of this article is to highlight the most recent advances on these exciting anticancer agents, with a special emphasis on their mode of action.

## 7.2

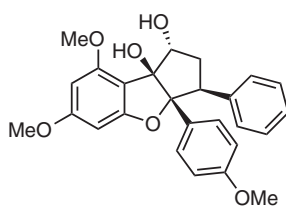
### Biosynthetic Aspects

Along with flavaglines, aglaforbesins **18** and aglains **19** are also characteristic metabolites of the genus *Aglaia* (Scheme 7.1). The term *flavagline* proposed by Harald Greger from the University of Vienna, originally covered these three groups of compounds [10] but over time, it has tended to solely refer to cyclopenta[*b*]benzofurans because of their distinctive pharmacological activities. These three families of secondary metabolites display the same patterns of substitution and stereochemical relationships. Proksch was the first to propose a biosynthetic pathway that begins with the condensation of hydroxyflavone (**14**) with a cinnamic amide (**15**) to afford an aglain (**16**) that may undergo an  $\alpha$ -ketol rearrangement to yield a flavagline **17** [11]. Reduction of intermediary ketones, possibly by NADPH, would generate the diols **18**, **19**, and **20**. Aglaforbesins are also probably generated by the same process, but with an addition of the cinnamic amide on the hydroxyflavone that occurs with the opposite orientation.

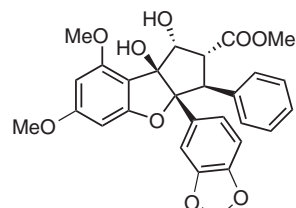




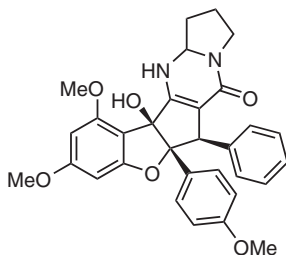
1: Rocaglamide



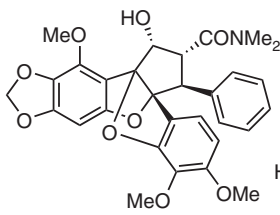
2: Rocaglaol



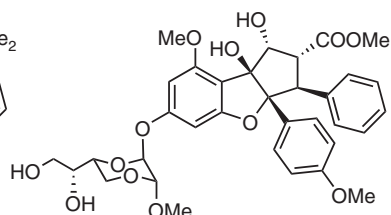
3: Methyl 4'-demethoxy-3',4'-methyleneedioxyrocaglate



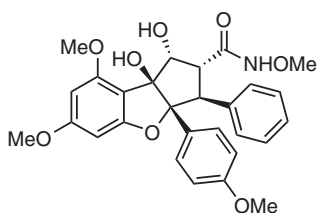
4: Aglaiastatine



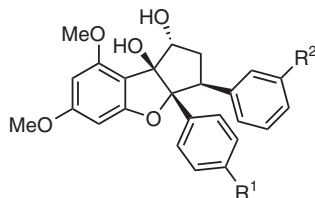
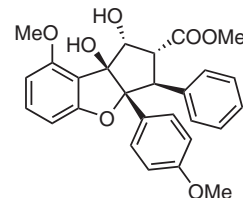
5: Cyclorocaglamide



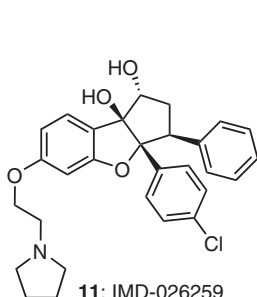
6: Silvestrol



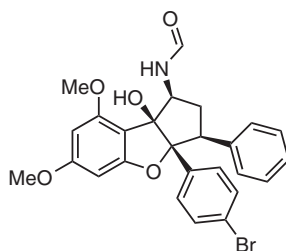
7: Rohinitib

8: IMD-019064, R<sup>1</sup> = OMe, R<sup>2</sup> = F  
9: FL3, R<sup>1</sup> = Br, R<sup>2</sup> = H

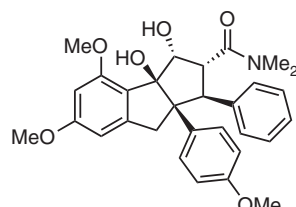
10: Demethoxy-methylester rocaglate



11: IMD-026259

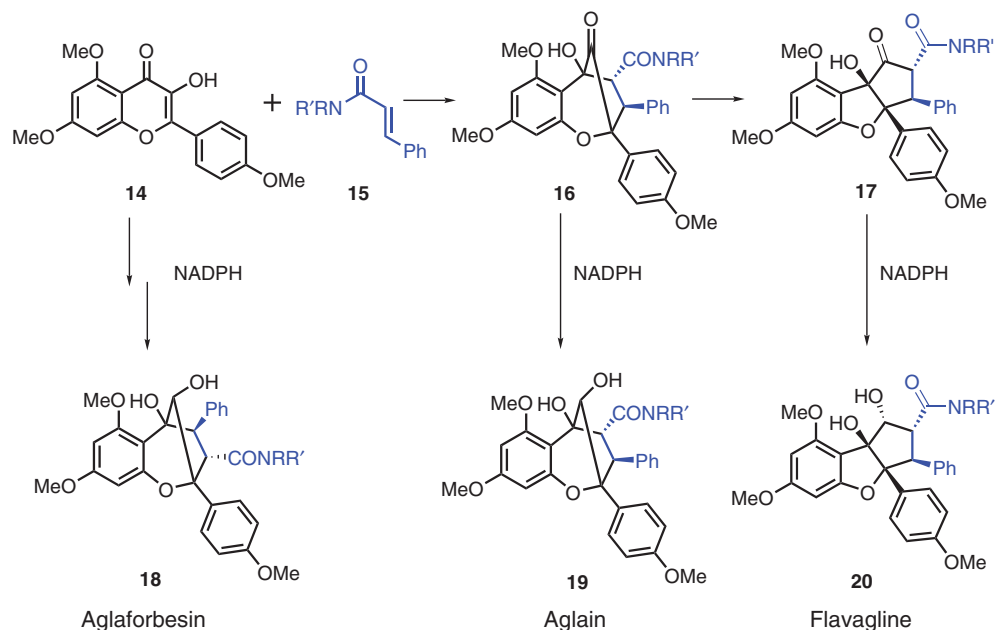


12: FL23



13

Figure 7.1 Representative natural (1–6) and synthetic (7–13) flavaglines.



**Scheme 7.1** Hypothetical biosynthesis of flavaglines and related aglaforbesins and aglains.

### 7.3

#### Synthesis of Flavaglines

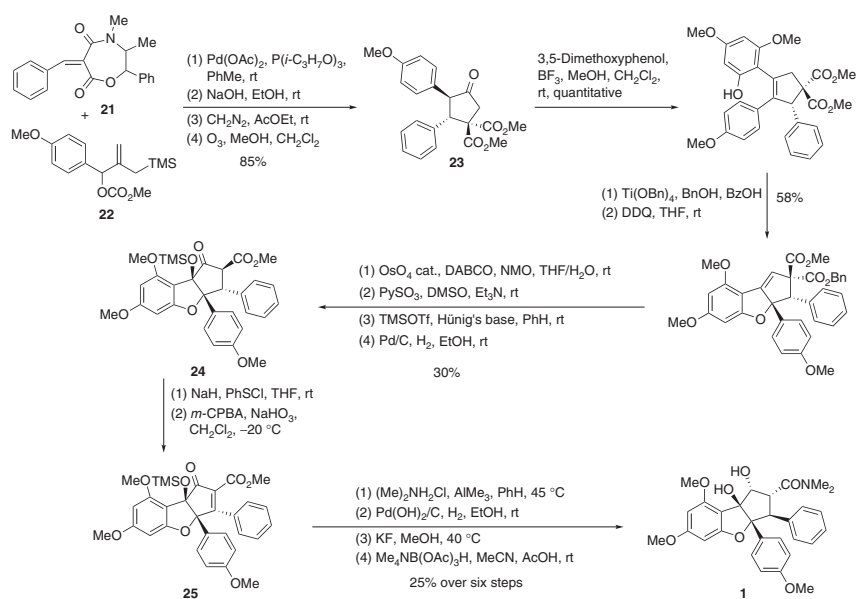
##### 7.3.1

##### Chemical Syntheses

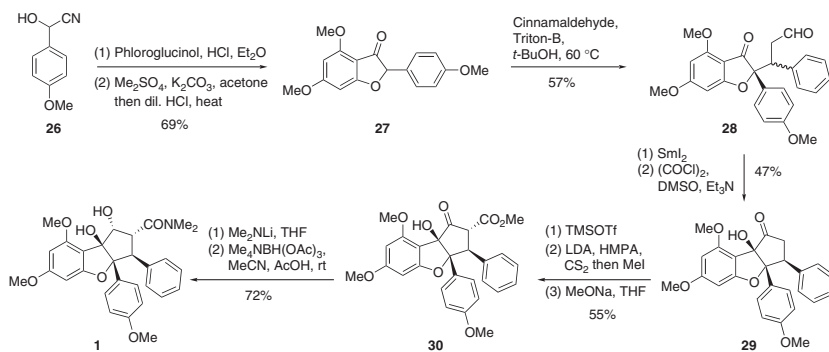
The first total synthesis of a flavagline, rocaglamide (**1**), was described in 1990 by Trost *et al.* [12]. Since then, multiple laboratories were attracted by this challenge due to the complexity of this structure characterized by two contiguous quaternary chiral centers and two adjacent aryl groups in *cis*-orientation on the cyclopentane ring (Scheme 7.2).

Trost's approach relies on the enantioselective [3+2]-cycloaddition of a trimethylenemethane derivatives generated from **22** with the oxazepinedione **21** (Scheme 7.2). Subsequent transformations, including the condensation of 3,5-dimethoxyphenol to cyclopentanone **23**, gave access to the flavaglines' skeleton but with an incorrect configuration compared to the natural product. Six additional steps afforded rocaglamide (**1**) with the desired stereochemistry via a dehydro-intermediate **25**. Despite these long and multiple steps, this synthesis remains the only enantiospecific one to date.

Since Trost's synthesis, more than 10 syntheses of flavaglines have been described. In 1992, Taylor *et al.* developed a racemic synthesis of rocaglamide



Scheme 7.2 Trost's enantioselective synthesis of rocaglamide [12].



Scheme 7.3 Taylor's synthesis of rocaglamide (1) [13].

which was later improved by Dobler's group in 2001 [13, 14]. Taylor's strategy begins with a Hoesch reaction between cyanohydrin **26** and phloroglucinol to afford benzofuranone **27** (Scheme 7.3) [13]. Aldehyde **28**, obtained by a Michael addition, was converted to the cyclopentanone **29** after cyclization and Swern oxidation. Silylation was followed by enolate formation, sequential addition of carbon disulfide and iodomethane, and treatment with sodium methoxide gave  $\beta$ -keto ester **30**, which was converted to rocaglamide (**1**) in the next two steps.

Dobler and colleagues [14] modified the previous strategy using cyanohydrin **31** in an umpolung reaction to generate the cyclopentanone **29** (Scheme 7.4) after deprotection. This ketone was treated with Stiles' reagent to give the ester **30**, which was then transformed into rocaglamide (**1**) in three steps.

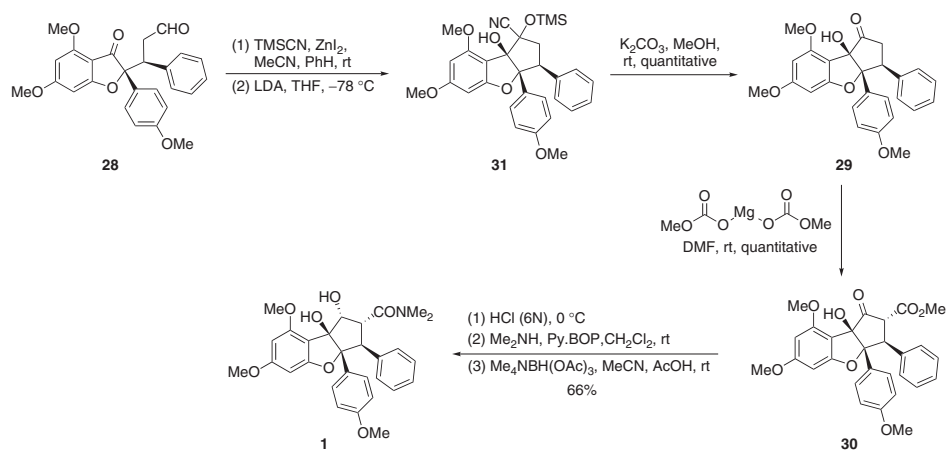
In 2008, Qin and his group [15] further modified Taylor's synthesis by introducing a methoxycarbonyl to the Michael acceptor, therefore circumventing Stiles carboxylation. Condensation of benzofuranone **27** with the dimethyl 2-benzylidenemalonate afforded the adduct **32**, which underwent a pinacol coupling promoted by  $\text{SmI}_2$  giving access to **30** (Scheme 7.5).

In 2004, Ragot's group [16] published a synthesis of flavaglines based on an intramolecular hydroxyepoxide opening. Cyclopentenone **35** was obtained in two steps from bromoketone **33** and triphenylphosphorane **34**. Heating  $\beta$ -ketoester **35** in dimethyl sulfoxide (DMSO), followed by  $\alpha$ -bromination and elimination of HBr afforded the  $\alpha$ -bromo enone **36** (Scheme 7.6). Suzuki reaction with boronate **37** provided didemethoxyrocaglaol **40** upon diastereoselective reduction, epoxydation, and hydrogenation with spontaneous cyclization via intermediate **39**.

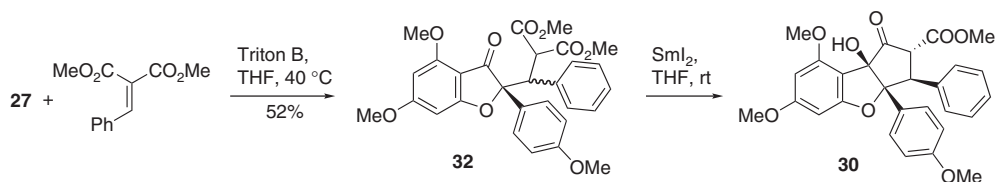
In 2009, Frontier and coworkers [17] established a new synthetic route to flavaglines based on a Nazarov-type reaction. The investigators used the benzofuranone **27** as a starting material, similarly to Taylor and Dobler. Alkylation with vinyl magnesium bromide and cleavage of the resulting alcohol provided the aldehyde **41** (Scheme 7.7). After introduction of the phenylacetylene moiety and protection of the propargylic alcohol, compound **42** was deprotonated with *tert*-butyllithium and quenched with *n*- $\text{Bu}_3\text{SnCl}$  to afford the key intermediate **43**. The tricyclic skeleton of flavaglines was obtained by a Nazarov-type cyclization from highly reactive allenyl oxide **45** generated *in situ* with *m*-CPBA. The tributylstannyl group was cleaved off during this oxidation-ring closure reaction. The resulting intermediate **46** gave access to ketoester **30** using palladium-mediated carbonylation.

Magnus and his group developed another synthesis of flavaglines also based on a Nazarov reaction. Intermediate **50** was prepared in six steps from the alkyne **48** (Scheme 7.8) [18]. Treatment of compound **50** with  $\text{SnCl}_4$  induced its cyclization to afford the cyclopentenone **51**. Subsequent hydrosilylation, palladium-mediated introduction of a carboxymethyl group, and hydroxylation afforded the methyl rocaglate **54**.

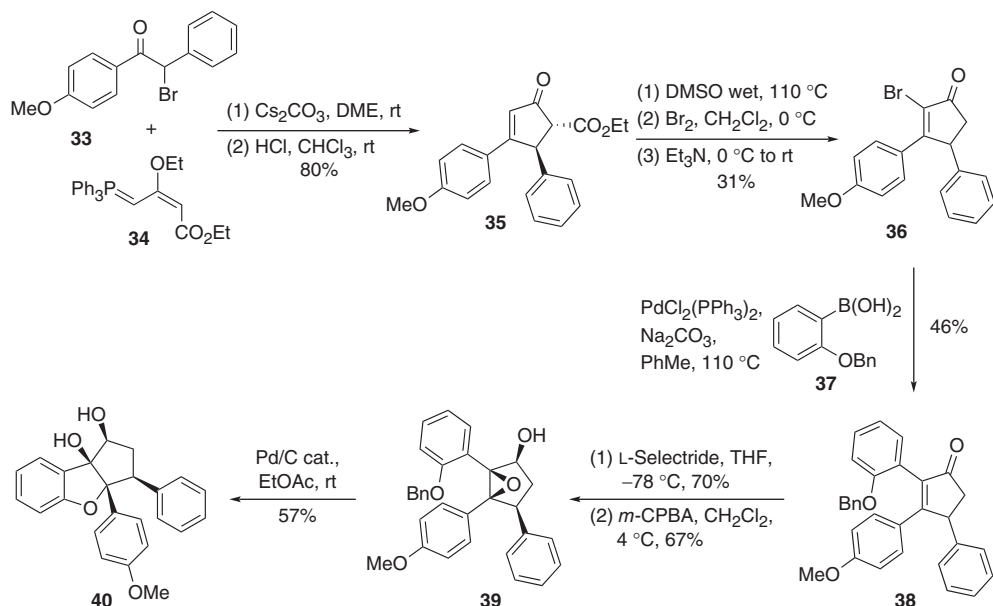
In 2012, Magnus' group [19] improved their synthesis of methyl rocaglate with an unprecedented Nazarov reaction promoted by acetyl bromide. The dienone **56**



Scheme 7.4 Dobler's racemic synthesis of rocaglamide [14].



Scheme 7.5 Qin's synthesis of rocaglamide [15].



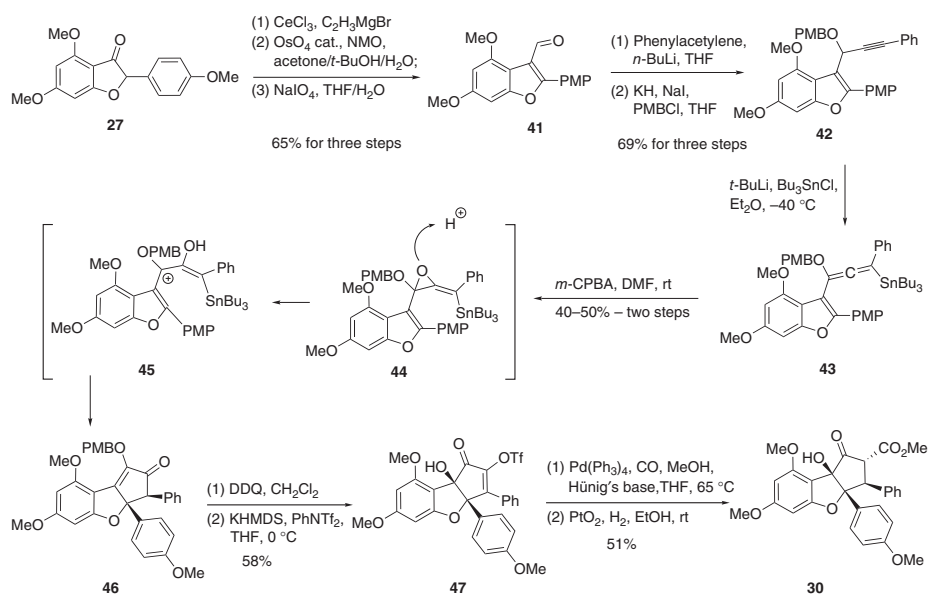
Scheme 7.6 Ragot's synthesis of flavagline skeleton [16].

was converted into the intermediate **57** with 81% yield (Scheme 7.9). Cyclopentenone **58** was then transformed into dehydroflavagline **59** in six steps, which afforded methyl rocaglate in two steps following Trost's strategy.

### 7.3.2

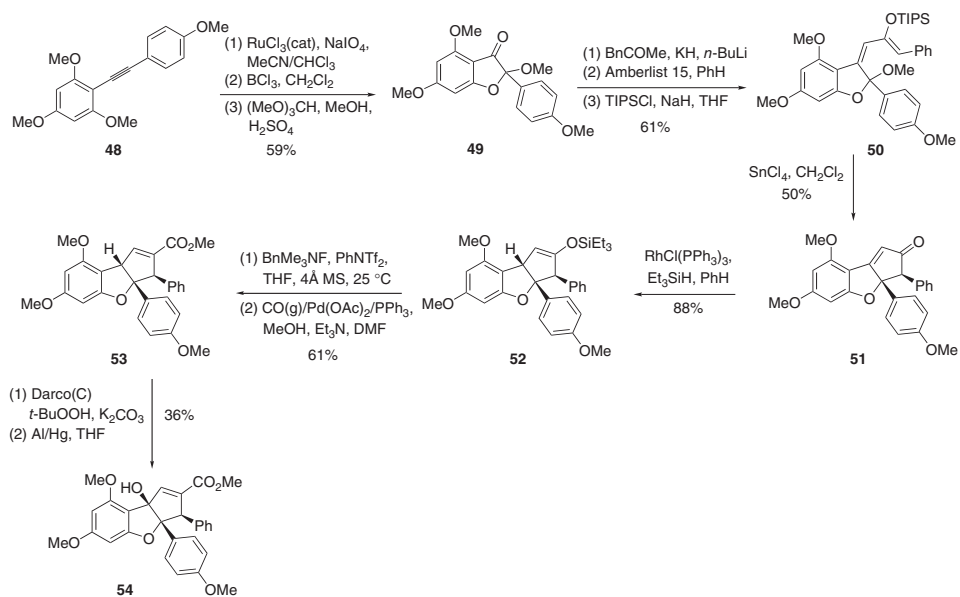
#### Biomimetic Synthesis of Flavaglines

On the basis of Proksch's [6] proposal for biosynthesis (Scheme 7.1), in 2004, Porco reported biomimetic synthesis of flavaglines from 3-hydroxyflavones and cinnamic esters using photochemistry (Scheme 7.10) [20]. Irradiation of **14** with **60** gave the adduct **61** through a [3+2]-cycloaddition reaction. A  $\beta$ -acyloin rearrangement of this intermediate in basic conditions gave the cyclopentanone **62** which upon diastereoselective reduction gave methyl rocaglate **63**. In 2012,

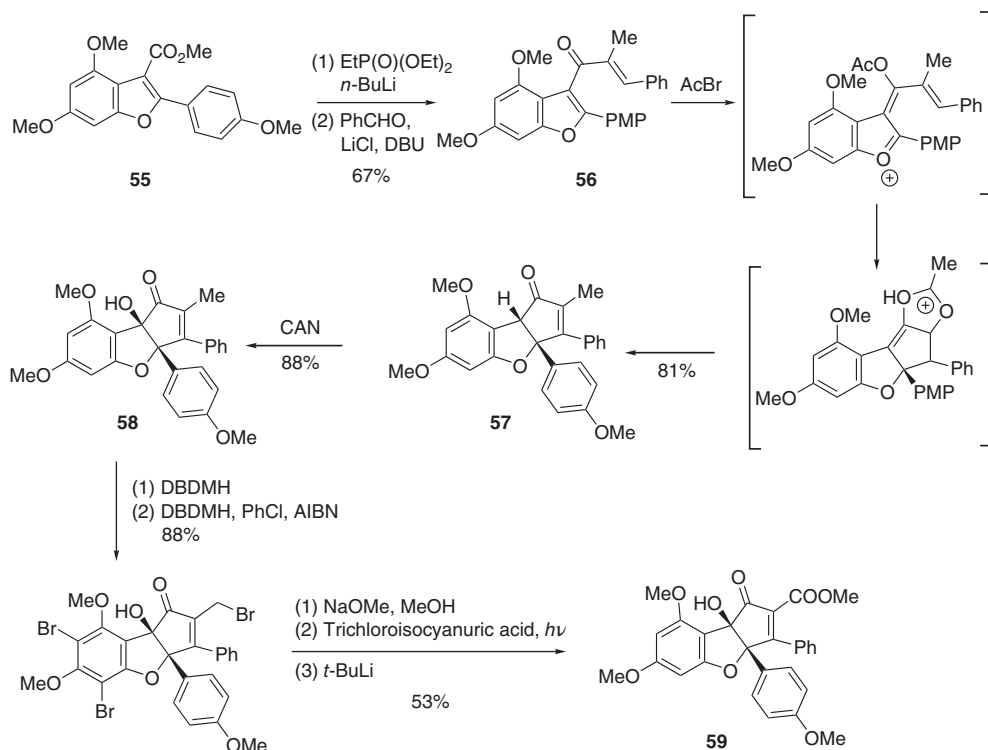


Scheme 7.7 Frontier's synthesis of rocaglamide based on a Nazarov reaction [17].





Scheme 7.8 Magnus' synthesis to access flavaglines [18].



**Scheme 7.9** Magnus' synthesis of methyl rocaglate by acetyl bromide-mediated Nazarov reaction [19].

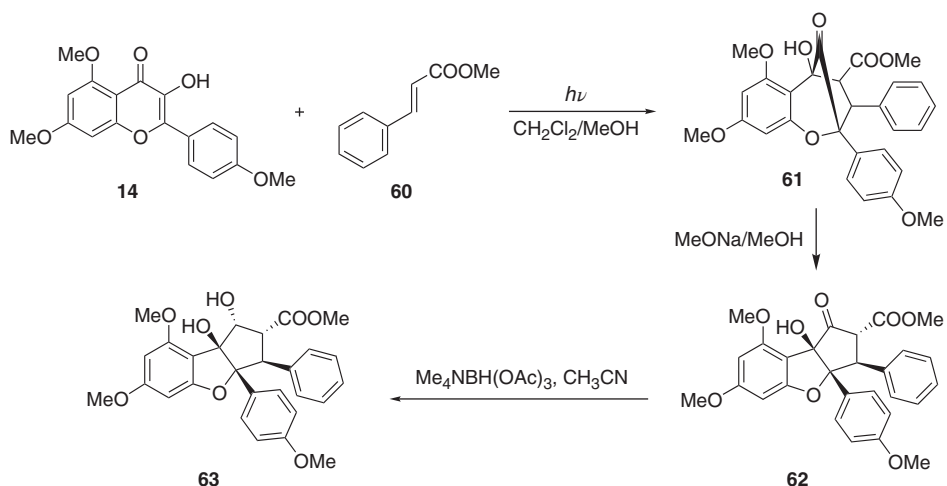
the same group of investigators developed a chiral version of this approach using the TADDOL ( $\alpha,\alpha,\alpha,\alpha$ -tetraaryl-1,3-dioxolane-4,5-dimethanol) derivative to prepare **61** in 69% yield and 85.5 : 14.5 er [21].

### 7.3.3

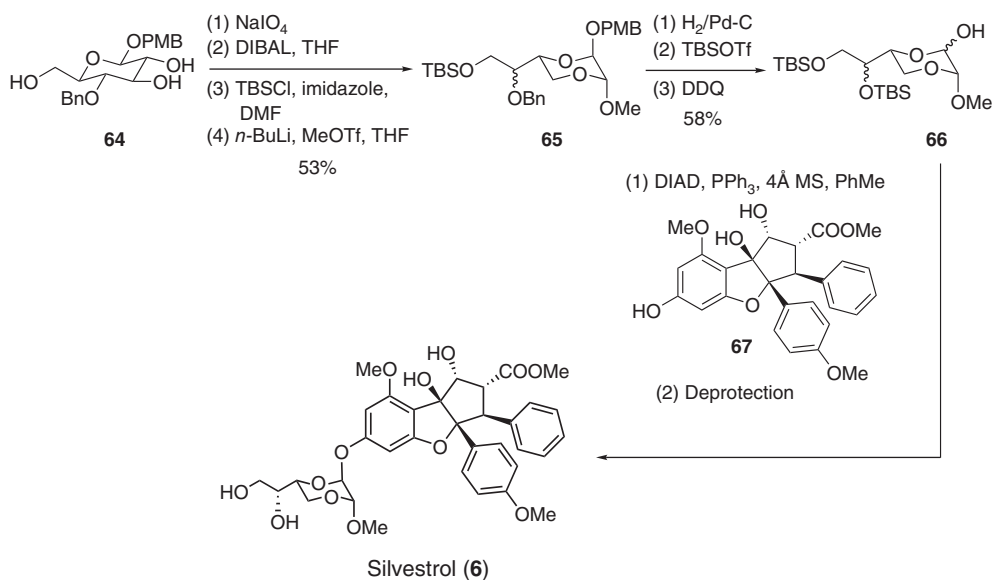
#### Synthesis of Silvestrol (6)

In 2007, in the same issue of *Angewandte Chemie* Porco's and Rizzacasa's groups published a total synthesis of silvestrol (**6**), a flavagline substituted by a pseudo-sugar. Both teams used the [3+2]-photocycloaddition to prepare the cyclopenta[*b*]benzofuran core of the molecules [22, 23]. The main difference between Porco's and Rizzacasa's approaches lies in the synthesis of the 1,4-dioxanyloxy intermediate.

Rizzacasa and collaborators [22] conceived their approach on the basis of the periodic cleavage of D-glucose derivative **64** (Scheme 7.11). Reduction of this intermediate with DIBAL, followed by protection with TBSCl, and O-methylation



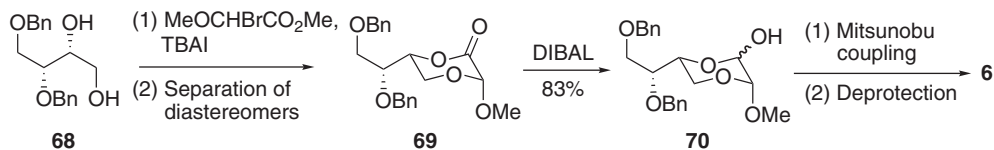
**Scheme 7.10** Porco's biomimetic synthesis of rocaglamide [20].



**Scheme 7.11** Rizzacasa's synthesis of silvestrol 6 [22].

afforded **65**, which was transformed into lactol **66**. A Mitsunobu reaction with compound **67** and deprotection yielded silvestrol **6**.

On the other hand, Porco and his group [23] condensed diol **68** with 2-bromo-2-methoxy acetate to obtain lactone **69**, which after reduction with DIBAL, yielded the lactol **70** (Scheme 7.12).

Scheme 7.12 Porco's synthesis of silvestrol **6** [23].

## 7.4

### Pharmacological Properties of Flavaglines

#### 7.4.1

##### Anticancer Activity

Rocaglamide (**1**) was shown to display a potent *in vivo* antileukemic activity right from its discovery [3]. Thereafter, a similar compound, methyl 4'-demethoxy-3',4'-methylenedioxyrocaglate (**3**) was found to delay for 3 weeks the growth of BC1 breast tumor implanted in athymic mice [24]. Because the tumors were not eradicated, flavaglines remained aloof from mainstream research works, which were mainly dedicated at that time to cytotoxic agents. The emergence of targeted therapies in the following decade modified the evaluation of the therapeutic potential of cytostatic agents, which prompted flavaglines to exit from limbo [25]. In many murine cancer models, flavaglines increased lifetime by one to several weeks and potentiated the efficacy of other anticancer agents (Table 7.1). Probably, the most impressive result was observed by Pelletier and colleagues who showed that silvestrol suppresses the growth of xenografted breast tumors addicted to eIF4A (eukaryotic initiation factor-4A) signaling [26]. Additional studies indicated that flavaglines might be particularly beneficial to alleviate the resistance to other chemotherapeutic agents (Table 7.1).

The scaffold proteins prohibitins-1 and 2 (PHB1, PHB2) were identified as molecular targets of flavaglines by Li-Weber and colleagues [39] at the German Cancer Research Center (DKFZ). PHB1 and PHB2 form heterodimers and oligomers with each other and also with numerous other signaling proteins [40]. PHB functions are regulated by the signaling of insulin, IGF1 (insulin-like growth factor), EGF (endothelial growth factor), TGF- $\beta$  (transforming growth factor), and IgM (immunoglobulin M) receptors and also by the kinases Akt, CamK (calmodulin-dependent protein kinase) IV, and PKC- $\delta$ . These posttranslational modifications impact intracellular localization of PHBs by affecting their affinity for specific lipids. PHBs are essential to maintain the structural and functional integrity of mitochondria. In the nucleus, PHBs control DNA synthesis and transcription by interacting with many transcription factors, histone deacetylases, histone methyltransferases, transcriptional corepressors, and minichromosome maintenance (MCM) proteins. In addition, PHBs regulate many cytoplasmic

**Table 7.1** *In vivo* activity of flavaglines in murine cancer models.

Compound	Murine model of cancer/ observed effects/(doses)	References
Rocaglamide (1)	P388 lymphocytic leukemia/increase in lifetime: T/C <sup>a</sup> of 156%/(1 mg kg <sup>-1</sup> )	[3]
Rocaglamide (1)	AsPC-1metastatic pancreatic cancer/suppression of tumor growth (T/C of 37%) and significant increase in lifetime/(5 mg kg <sup>-1</sup> )	[27]
<i>N</i> -Desmethyl-rocaglamide	RMA T lymphoma/potential of the effects of concanavalin A (otherwise inactive) to significantly inhibit tumor progression/(2.5 mg kg <sup>-1</sup> <i>i.p.</i> three times a week for 2 weeks)	[28]
Rohinitib (7)	M091 human myeloid leukemia cells/inhibition of tumor growth and suppression of glucose uptake/(1 mg kg <sup>-1</sup> <i>i.p.</i> 4 consecutive days a week for 3 weeks)	[29]
<b>10</b>	E $\mu$ -Myc/(myr)Akt lymphomacells/potential of the effects of doxorubicin (otherwise inactive): increase in lifetime by 9 days/(0.2 mg kg <sup>-1</sup> <i>i.p.</i> daily for 5 days)	[30]
Silvestrol (6)	L3.6pl pancreatic cancer and RPMI-8226 myeloma/absence of significant effect/(0.2 mg kg <sup>-1</sup> <i>i.p.</i> , 5 days/week)	[31]
Silvestrol (6)	E $\mu$ -Myc/(myr)Akt lymphoma/potential of the effects of doxorubicin (otherwise inactive): increase in lifetime by 7 days (used in monotherapy, silvestrol did not display any significant effect)/(0.2 mg kg <sup>-1</sup> <i>i.p.</i> daily for 5 days)	[30]
Silvestrol (6)	P388 leukemia/increase in lifespan corresponding to a T/C of 150%/(2.5 mg kg <sup>-1</sup> <i>i.p.</i> for 5 days)	[32]
Silvestrol (6)	PC3 prostate cancer/reduction of the mean tumor weight by 60%/(3 mg kg <sup>-1</sup> <i>i.p.</i> three times per week for 3 weeks)	[33]
Silvestrol (6)	Pten <sup>+</sup> /E $\mu$ -Myc lymphoma/potential of the effects of doxorubicin: increase in lifetime by 5 days compared to doxorubicin alone (silvestrol alone was inactive)/(0.2 mg kg <sup>-1</sup> <i>i.p.</i> daily for 5 days)	[34]
Silvestrol (6)	E $\mu$ -Myc/eIF4E lymphoma/potential of the effects of doxorubicin: increase in lifetime by 16 days compared to doxorubicin alone (silvestrol alone was inactive)/(0.2 mg kg <sup>-1</sup> <i>i.p.</i> daily for 5 days)	[34]
Silvestrol (6)	E $\mu$ -Tcl-1 chronic lymphocytic leukemia Significant reduction in B-cell number (1.5 mg kg <sup>-1</sup> <i>i.p.</i> per day for 5 days)	[35]

(continued overleaf)

Table 7.1 (Continued)

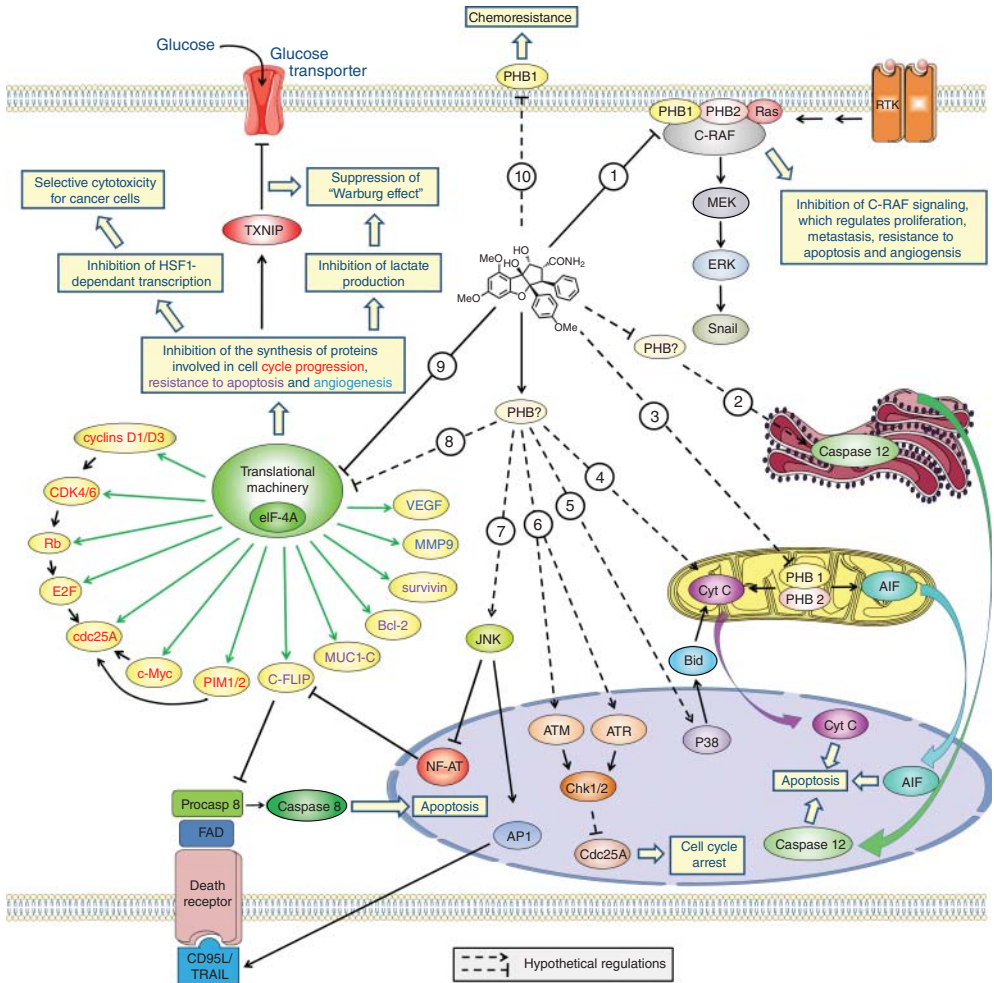
Silvestrol (6)	697 B-ALL acute lymphoblastic leukemia Increase in lifetime by more than 2 weeks (1.5 mg kg <sup>-1</sup> <i>i.p.</i> every other day)	[35]
Silvestrol (6)	MDA-MB-231 breast cancer/considerable suppression of tumor growth after more than 75 days/(0.5 mg kg <sup>-1</sup> <i>i.p.</i> daily for 8 consecutive days)	[26]
Silvestrol (6)	PC-3 prostate cancer/considerable suppression of tumor growth after more than 50 days/(0.5 mg kg <sup>-1</sup> <i>i.p.</i> daily for 8 consecutive days)	[26]
Silvestrol (6)	MV4-11 acute myeloid leukemia/increase in lifetime by more than 1 month impressively, 30% were still alive 74 days after engraftment (all vehicle-treated mice were dead 31 days after engraftment)/(1.5 mg kg <sup>-1</sup> <i>i.p.</i> every other day for 3 weeks)	[34]
Silvestrol (6)	E $\mu$ -Myc/Tsc2 <sup>-/-</sup> /PIM2 lymphoma/potential of the effects of rapamycin: increase in lifetime by 6 days compared to rapamycin alone/(0.2 mg kg <sup>-1</sup> <i>i.p.</i> daily for 7 days)	[36]
Silvestrol (6)	Mantle cell lymphoma/increase in lifetime by 2 weeks/(1.5 mg kg <sup>-1</sup> <i>i.p.</i> every other day)	[37]
FL23 (12)	3LL lung carcinoma/significant suppression of tumor growth/(25 mg kg <sup>-1</sup> <i>i.p.</i> twice a week for 17 days)	[38]

a) T/C: Treated versus control.

Source: Adapted from Ref. [6] with permission of Future Science Ltd.

proteins such as the kinases C-Raf, Akt, and MLK2, the phosphatase Shp1/2, the chaperones Hsp70, and mortalin/Grp75 or the phospholipase C $\gamma$ 2 [40].

Activation of the kinase C-Raf by Ras requires a direct interaction with PHBs, which can be disrupted by flavaglines (Figure 7.2) [39]. Their anticancer activities could thus be partially explained by the inhibition of Ras-C-Raf-MEK-ERK (extracellular-signal-regulated kinase) signaling, which is constitutively activated in many human cancers [41]. This observation that binding of flavaglines to PHBs prevents their interaction with C-Raf has recently been confirmed by Chen, He, and collaborators in the context of pancreatic ductal adenocarcinoma (PDAC) [27]. In PDAC, constitutive activation of C-Raf is the result of constitutive mKRAS activity, the most common oncogenic mutation in PDAC. The investigators also found that in highly malignant AsPC-1 human PDAC cell lines, PHB1 was over-expressed and mainly localized in the plasma membrane and cytosol, whereas in poorly malignant Capan-2 PDAC cells, PHB1 was poorly expressed and uniformly distributed within the cells. Rocaglamide was found to exert a strong inhibitory effect against ERK1/2 activities in AsPC-1 cells, reducing phosphorylation of the



**Figure 7.2** Anticancer mechanisms of flavaglines. 1. Inhibition of the Ras-dependent C-Raf activation. 2, 3. Translocation of caspase-12 and AIF to the nucleus to induce apoptosis. 4. Translocation of cytochrome C to the nucleus to induce the intrinsic apoptotic pathway. 5. Activation of p38-mediated transcription of the pro-apoptotic Bcl-2 family. 6. Induction of the ATM/ATR-Chk1/2-Cdc25A pathway leading to cell cycle arrest. 7. Activation of JNK-dependent transcription of the pro-apoptotic proteins CD95 ligand and c-FLIP. 8. Hypothetical PHB-dependent inhibition of the

translational machinery. 9. Inhibition of eIF4A leading to a downregulation of expression of proteins involved in cell cycle progression, resistance to apoptosis, and angiogenesis. This inhibition of protein synthesis leads to suppression of the activity of the transcription factor HSF1 and an upregulation of the tumor suppressor TXNIP. TXNIP blocks glucose uptake and consequently prevents the "Warburg effect." 10. Hypothetical inhibition of cell surface PHB1-mediated chemoresistance. (Adapted from Ref. [5] with permission from Elsevier).

transcriptional factor Snail, one of the main promoters of epithelial-mesenchymal transition (EMT). Reversal of the EMT phenotype in these cells was characterized by upregulation of *E*-cadherin and  $\beta$ -catenin and downregulation of vimentin. These *in vitro* effects were also observed *in vivo*. Indeed, rocaglamide prevented the dissemination of cancer cells in the liver and lungs in PDAC-xenografted mice, in addition to significantly inhibiting tumor growth.

The initiation step of protein synthesis is a highly regulated and rate-limiting process that is emerging as a promising target in oncology. Indeed, the synthesis of many factors controlling oncogenesis, angiogenesis, and chemoresistance necessitates the helicase eIF4A. Using affinity chromatography, Rizzacasa and colleagues [42] demonstrated that some flavaglines directly bind to eIF4A. Sadlish and colleagues [43] substantiated this discovery using chemogenomic profiling to validate eIF4A as the main target of flavaglines in yeast. By means of mutagenesis and *in silico* modeling, they identified the binding site of flavaglines and proposed a model to account for the enhancement of eIF4A binding to mRNAs promoted by flavaglines. This action inhibits the recycling of eIF4A leading to an inhibition of cap-dependent translation, which had already been observed by Pelletier and colleagues [26]. Indeed, flavaglines have been reported for several years to inhibit protein synthesis, and in particular the translation of the mRNAs encoding CDK4 (cyclin-dependent kinase), CDK6, cyclins D1 and D3, *cdc25A*, Bcl-2 (B-cell lymphoma 2), survivin, Mcl-1 (myeloid cell leukemia protein 1), the PIM1/2 kinases, c-Myc, VEGF (vascular endothelial growth factor), matrix metalloproteinase 9 (MMP9), and MUC1-C [26, 36, 39, 44–46]. It is noteworthy that depletion of the anti-apoptotic proteins Mcl-1 and Bcl-2 was not necessary for the induction of apoptosis by flavaglines in lymphoma cells [47], B-cell malignancy mantle cell lymphoma [37], chronic lymphocytic leukemia, or acute lymphoblastic leukemia [35].

In 2013, Whitesell, Lindquist, and colleagues [29] have reported in *Science* about how the inhibition of protein synthesis selectively impedes the proliferation and survival of cancer cells without affecting normal cells. These authors screened more than 300 000 compounds for the inhibition of the transcriptional factor heat shock factor 1 (HSF1), which is deeply involved in metabolic reprogramming, survival, and proliferation of cancer cells in addition to heat-shock response. Among these compounds, rocaglamide (**1**) was found to be the most potent and selective inhibitor of HSF1 signaling ( $IC_{50} \approx 50$  nM). Additional tests with other compounds showed that another flavagline called *rohinitib* (**7**, Figure 7.1) was even more active ( $IC_{50} \approx 20$  nM). This molecule inhibited HSF1-dependant transcriptional activity much more effectively in cancer cell lines, than in proliferating nontumorigenic cells. The cascade of events that links the inhibition of protein synthesis to HSF1 signaling remains unknown for the moment. In addition, flavaglines were shown to overturn a metabolic feature in cancer cells called the *Warburg effect*, manifested by an elevated rate of glycolysis and lactic acid production. Rohinitib upregulated the thioredoxin-interacting protein (TXNIP), a tumor suppressor that regulates cellular redox potential and glucose uptake.



This effect on TXNIP leads to a spectacular inhibition of glucose uptake and lactate production in several cancer cell lines.

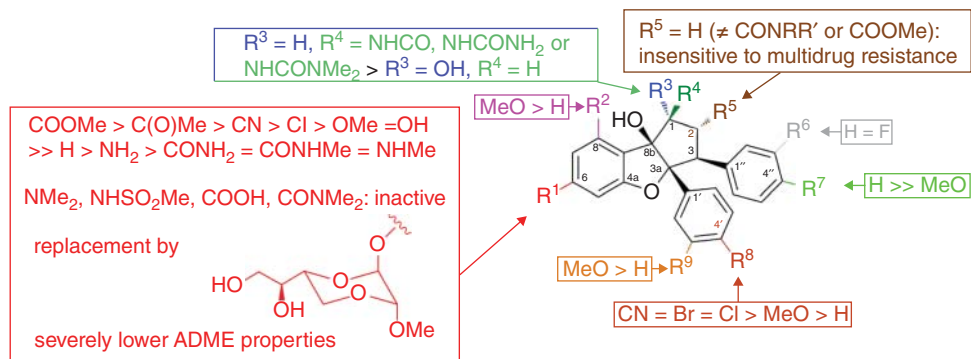
These authors also showed that wild-type mouse embryonic fibroblasts (MEFs) are less sensitive to rohinib cytotoxicity than mutant MEFs overexpressing HSF1, which are used as a model of premalignant cells with early-stage oncogenic lesions. Cancer cells carry an abnormal number of chromosomes, causing a proteotoxic stress that is attenuated by HSF1 activity. The same authors showed that rohinib is more cytotoxic to trisomic than to wild-type MEFs. Human cancer cell lines with high chromosomal instability were extremely responsive to rohinib. Altogether, these data provide some clues as to decipher why flavaglines are selectively cytotoxic to cancer and immortalized cells without affecting normal cells viability.

It is quite uncommon for structurally complex natural products to target two classes of proteins, even though there are some precedents [2]. Remarkably, both PHBs and eIF4A are over-activated in many malignant tumors and are regarded as promising targets to treat cancers [40, 48].

As far as we know, apoptosis is the only type of cellular death induced by flavaglines in cancer cells. It may occur through the classical intrinsic and extrinsic pathways [22–24] or independently of caspase-3 via the apoptosis-inducing factor (AIF) or caspase-12 [49]. Whether these effects involve PHB1, PHB2, or eIF4A remains unknown. Interestingly, flavagline **10**, developed by Tremblay and coworkers [31] at Infinity Pharmaceuticals, is highly cytotoxic to cancer cells but does not inhibit protein synthesis. This observation indicates that in some cell types, a sole action on PHBs is sufficient to induce apoptosis without involving inhibition of translation.

Several flavaglines have been examined in murine models of cancers. Silvestrol (**6**), which has been the most widely studied, was unfortunately shown to be very susceptible to P-glycoprotein-mediated multidrug resistance [34]. In contrast, compounds that are not substituted at position 2 with an ester or an amide are highly cytotoxic to cancer cells with acquired multidrug resistance (Figure 7.3) [38, 49, 50]. In few murine models of cancer, flavaglines did not display any significant effect [31] but in most instances, they significantly increased lifetime [3, 26, 30, 32, 34–37]. It is noteworthy that in all of these *in vivo* experiments, flavaglines did not show any overt indication of toxicity.

Recently, Li-Weber and collaborators demonstrated that rocaglamide activates the DNA repair pathway (or DNA damage checkpoint) without inducing any significant DNA damage or modifying the redox status of cancer cells. The authors hypothesized that rocaglamide blocks DNA replication promoting the transcription of genes responsive to DNA replication stress. Activation of ATM (ataxia telangiectasia mutated) and ATR (ataxia telangiectasia and Rad3-related) ATM induces ATR Chk1/Chk2 (Chk, checkpoint kinase) phosphorylation, and consequently Cdc25A degradation, which stops the cell cycle at the G1-S transition [50]. Importantly, activation of the ATM/ATR-Chk1/2-Cdc25A pathway that led



**Figure 7.3** Key structure–activity relationship findings related to flavagline antiproliferative activity. (Adapted from Ref. [3] with permission from Elsevier).

to leukemic cell growth inhibition which was not observed in normal proliferating T cells [51]. These findings confirmed the selective cytotoxicity of flavaglines toward malignant cells.

#### 7.4.2

##### Anti-inflammatory and Immunosuppressant Activities

In addition to their anticancer effect, flavaglines exhibit potent anti-inflammatory properties, which are mediated by activation of the MAP (mitogen-activated protein) kinases, JNK (c-Jun N-terminal kinase), and p38-inhibition of nuclear factor of activated T-cells (NFATs) signaling [52]. At higher doses, flavaglines also block pro-inflammatory NF- $\kappa$ B (nuclear factor kappa-light-chain-enhancer of activated B cells) signaling, probably through an action on PHBs [53]. Indeed, PHBs are known to be involved in the activation of NF- $\kappa$ B [54]. Nevertheless, the exact mechanism of action and the molecular target involved in these effects remain undefined.

#### 7.4.3

##### Cytoprotective Activity

IMD-019064, a flavagline originally developed at Bayer, inhibits the release of pro-inflammatory factors from microglia, astrocytes, and endothelial cells [55]. On the basis of these anti-inflammatory activities, Bayer scientists examined its neuroprotective effects and showed that it protects mice in models of Parkinson's disease and traumatic brain injury. Optimization of this compound yielded IMD-026259 (**11**, Figure 7.1) which is currently undergoing a preclinical trial for the treatment of Parkinson's disease by the company IMD Natural Solutions [56]. Many analogs were patented, but structure–activity relationships (SARs)

data were not revealed. Our own lab also has investigated the neuroprotective activity of flavaglines and identified several derivatives such as FL3 (9) and FL23 (12) that protect neurons from several stresses more efficiently than IMD-019064 [49]. In the course of our research, we established the first SAR data for neuroprotection. We also showed that flavaglines offer some sort of protection against the neurotoxicity induced by cisplatin, an anticancer medicine [50]. On the basis of these observations, we studied whether flavaglines could protect other tissues from the side effects of anticancer treatments. We focused our attention on anthracyclines such as doxorubicin, which are commonly used anticancer medicines, even though they are highly cardiotoxic [57]. We found that flavaglines protect cardiomyocytes from two totally unrelated types of stress: doxorubicin-induced cardiotoxicity and serum starvation [49]. We also demonstrated that the synthetic flavagline FL3 (9, Figure 7.1) greatly lowered mortality induced by doxorubicin [58]. It also reduced cardiac apoptosis and fibrosis. These observations suggest that flavaglines may enhance the efficacy of cancer treatments and at the same time alleviate the cardiac side effects of these treatments. FL3 was shown to induce cytoprotection through a phosphorylative activation of the small heat-shock protein Hsp27, which is a key factor in the resistance of cardiomyocytes to many types of stresses [59]. This observation represents a small piece of the puzzle. Deciphering the mechanisms underlying both cytoprotection in normal cells and cytotoxicity in cancer cells is a fascinating challenge that deserves further in-depth investigation. It is still unclear whether cytoprotection is mediated through PHBs or eIF4A. On one hand, PHBs represent an attractive hypothesis because they display different functions and intracellular localizations in cancer and normal cells [40]. In addition, some flavaglines that do not inhibit protein synthesis display potent neuro- and cardioprotective activities (unpublished data). On the other hand, Jerry Pelletier and colleagues showed that a pretreatment with rohitinib protects *in vitro* hair follicle cells against the apoptosis induced by paclitaxel in a model of chemotherapy-induced alopecia (hair loss) [60]. This protection against paclitaxel-induced apoptosis was shown to be due to a transient suppression of translation initiation. Unfortunately, this effect was not translated *in vivo*: rohitinib failed to protect mice against cyclophosphamide-induced alopecia.

#### 7.4.4

##### **Antimalarial Activities**

Recently Julia Walochnik and collaborators [61] showed that two flavaglines, rocaglamide and aglafoline, display some antimalarial activities that are intermediate between those of artemisinin and quinine. However, these promising results remain to be validated *in vivo* before their antimalarial potential can be examined in detail.

## 7.5

### Structure–Activity Relationships (SARs)

In addition to data that were obtained with natural compounds, a few laboratories have synthesized flavaglines to explore the structural requirements for their anticancer properties. Considering that extensive description of SAR data has recently been reviewed (Figure 7.3), we will disclose only its most striking features [8]. Martin Tremblay and colleagues from Infinity Pharmaceuticals synthesized many derivatives of silvestrol and confirmed that flavaglines substituted by a pseudo-sugar suffer poor ADME (absorption, distribution, metabolism, and excretion) properties that preclude their development as a medicine [31]. This group disclosed for the first time the SAR for both cytotoxicity and inhibition of translation. Interestingly, both of these activities followed the same trends, suggesting that inhibition of cap-dependent translation is a critical component of the mechanism involved in cytotoxicity. However, these authors identified demethoxy-methyl ester rocaglate **10** (Figure 7.1), which displays a strong cytotoxicity without inhibiting translation, suggesting another mechanism of action, probably involving PHBs.

Many flavaglines are sensitive to multidrug resistance mediated by efflux pumps. Our group showed that the deletion of the amide moiety in rocaglamide derivatives leads to rocaglaol analogs that are totally insensitive toward multidrug resistance [49].

The structural requirements for cytoprotection have been disclosed in only one article so far [50]. These SARs are close to those for cytotoxicity in cancer cells, even though some slight differences have been observed in the relative ranking of activity. Direct evaluation of the activity on PHB signaling and inhibition of eIF4A would greatly contribute to better characterize the structural requirements of flavaglines for their various pharmacological activities.

## 7.6

### Concluding Remarks

Flavaglines hold a great potential as a new class of therapeutics, but much basic research is still required before initiation of clinical trials. In oncology, it is crucial to identify why some tumors more responsive to flavagline treatments than others are. This knowledge would greatly optimize the design of clinical assays through the stratification of patients based on predictive biomarkers, such as the overexpression of PHBs, PHB-interacting proteins, or an addiction to eIF4A signaling.

Even though the mode of action of cytoprotection remains poorly understood, it is for the treatment of Parkinson's disease that flavaglines are the closest to reaching clinical trials. Indeed, the German Company IMD Natural Solution is, as far as

we know, the most advanced to initiate a clinical trial for this ailment with IMD-026259 [56].

PHBs are emerging as key regulators of cell metabolism, development, and survival that integrate multiple internal and external signals. More than 50 proteins have been shown to directly interact with PHBs, and this number is constantly growing. Identifying new physiological and pathophysiological functions of PHBs may provide novel therapeutic intervention for additional types of diseases.

Up to now, structures of the disclosed flavaglines analogs remain close to those of the natural compounds. Only the carbaisostere **13** developed by Novartis scientists (Figure 7.1) has been published. The absence of a corresponding patent suggests that it may not be pharmacologically active. However, the cyclopenta[*b*]benzofuran core would likely need to be modified to generate patentable molecules that could advance toward clinical assays.

### Abbreviations

ADME	absorption, distribution, metabolism, and excretion
AIF	apoptosis-inducing factor
ATM	ataxia telangiectasia mutated
ATR	ataxia telangiectasia and Rad3-related
Bcl-2	B-cell lymphoma 2
CamK	calmodulin-dependent protein kinase
CDK	cyclin-dependent kinase
Chk	checkpoint kinase
DBDMH	1,3-dibromo-5,5-dimethylhydantoin
eIf4A	eukaryotic initiation factor-4A
EMT	epithelial-mesenchymal transition
ERK	extracellular-signal-regulated kinase
HSF	heat shock factor
IGF	insulin-like growth factor
IgM	immunoglobulin M
JNK	c-Jun N-terminal kinases
MAP	mitogen-activated protein
MCM	minichromosome maintenance
Mcl-1	myeloid cell leukemia protein 1
MEFs	mouse embryonic fibroblasts
NFAT	nuclear factor of activated T-cells
NF- $\kappa$ B	nuclear factor kappa-light-chain-enhancer of activated B cells
PDAC	pancreatic ductal adenocarcinoma
PHB	prohibitin
SAR	structure–activity relationships
TADDOL	$\alpha,\alpha,\alpha,\alpha$ -tetraaryl-1,3-dioxolane-4,5-dimethanol

TGF	transforming growth factor
TXNIP	thioredoxin-interacting protein
VEGF	vascular endothelial growth factor

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## 8

# Beneficial Effect of Naturally Occurring Antioxidants against Oxidative Stress–Mediated Organ Dysfunctions

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### 8.1

#### Introduction

Imbalance between pro-oxidants and antioxidants in a living system or oxidative stress, as it is generally termed, is a comparatively new concept that has been extensively implicated in biomedical sciences for about two decades. This phenomenon plays significant role in the pathophysiology of highly prevalent diseases such as hypertension, diabetes, preeclampsia, atherosclerosis, acute renal failure, and Alzheimer's and Parkinson's diseases, among others. Cells are constantly exposed to oxidants from physiological processes, such as mitochondrial respiration [1] as well as pathophysiological conditions such as inflammation, foreign compound metabolism, radiation, and many others [2]. The cellular metabolism of oxygen generates potentially harmful reactive oxygen species (ROS). ROS consist of a family of highly reactive species and can be beneficial for some physiological functions, for example, they are used by the immune system to attack and kill pathogens. In normal physiological conditions, ROS are continuously generated in organisms and are effectively eliminated by several antioxidant defenses (e.g., vitamins, oligo elements, proteins, and enzymes). Increased ROS levels in the cell, however, have a substantial impact either leading to defective cellular functions, aging, and/or disease. Therefore, a better understanding of the roles of ROS-mediated signaling in normal cellular function as well as in various diseases is necessary for developing therapeutic tools for oxidative stress–related pathophysiology. This chapter aims to present a brief idea of the beneficial role of naturally occurring antioxidants in oxidative stress–mediated organ pathophysiology; it is believed that this will inspire readers and researchers in the field molecular toxicology, applied pharmacology, and other related fields of research. Here we discuss the beneficial efficacy of various antioxidants (e.g., mangiferin, arjunolic acid, silymarin, curcumin, and baicalein) in the light of a number of reported experimental studies. A few words regarding the use of antioxidants and preconditioning to protect an organism against ROS is also discussed [3].

## 8.2

## Oxidative Stress and Antioxidants

ROS possess a strong oxidizing effect and induce damage to biological molecules, including proteins, lipids, and DNA, with concomitant changes in their structure and function [4]. In a series of pathological conditions, an extensive generation of ROS appears to overwhelm natural defense mechanisms, dramatically reducing the levels of endogenous antioxidants, resulting in oxidative stress [5]. As epidemiological studies indicate that the major nutritional antioxidants, such as vitamin E, vitamin C, and  $\beta$ -carotene, may be beneficial in preventing several chronic disorders [6], considerable interest has arisen in the possible reinforcement of antioxidant defenses. Two fundamental conditions must be fulfilled for an antioxidant: (i) the compound must be present in low concentrations relative to the substrate to be oxidized and (ii) the species resulting from its oxidation must be stable through the formation of intramolecular hydrogen bonds [7].

## 8.2.1

## Mangiferin and Its Beneficial Properties

Mangiferin, 2-*C*- $\beta$ -D-glucopyranosyl-1,3,6,7-tetrahydroxyxanthone ( $C_{19}H_{18}O_{11}$ ; Mw, 422.35; melting point, anhydrous 271 °C) [8], a natural *C*-glucoside xanthone [9] from bark, leaves, roots, and fruits of *Mangifera indica* Linn., mango (Figure 8.1). *M. indica* Linn. belongs to the family Anacardiaceae and is the source of many natural xanthones, polyphenols, and so on. Various studies have reported that mangiferin has a broad range of therapeutic uses. It possesses antioxidant [10–12], antidiarrheal [13], dyslipidemic [14], antidiabetic [15], antiallergic [16], antibacterial [17], anti-HIV [18], and anticancer [19] properties. It is also used as an analgesic, and as an immunomodulatory [20] and immunostimulatory [21] agent.

## 8.2.1.1 Antioxidant Activity of Mangiferin

The protective antioxidant capabilities of a *M. indica* stem bark extract (Vimang<sup>®</sup>) and its main polyphenol mangiferin were investigated *in vivo* in OF1 mice [11]. Both Vimang and mangiferin were found to protect the animals from 12-*O*-tetradecanoylphorbol-13-acetate (TPA, a stimulator of ROS production)-induced

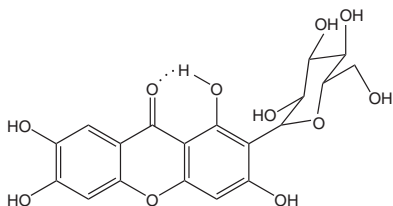
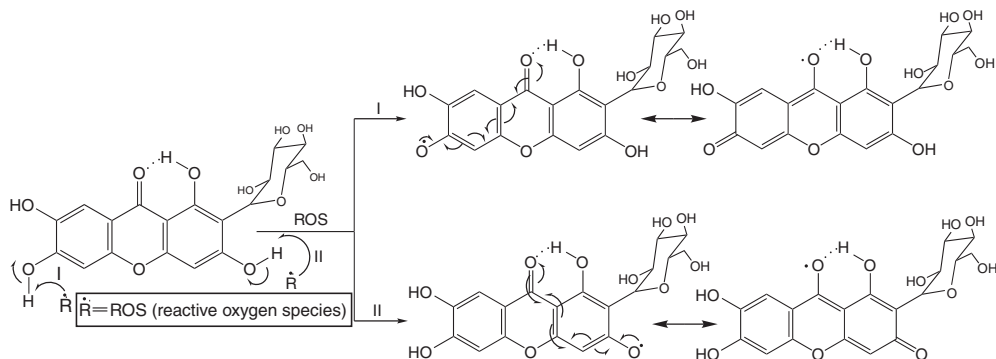


Figure 8.1 Chemical structure of mangiferin.



**Figure 8.2** Reaction of mangiferin with free radicals (ROS) and resonating canonical forms of two phenoxyl radicals.

oxidative damage. Considering the levels of a series of biomarkers, for example, (i) the activity of the major antioxidant enzymes superoxide dismutase (SOD) and glutathione peroxidase (GPx); (ii) total sulfhydryl group protein content (TSH), a marker for protein oxidation; (iii) markers for oxidative stress, lipid peroxidation, malondialdehyde (MDA), and 4-hydroxyalkenals (4-HA); (iv) fragmentation of nuclear DNA; and (v) cytochrome *c* reduction and  $H_2O_2$ ; mangiferin was either comparable or better (GPx, TSH, lipid peroxidation, DNA fragmentation, cytochrome *c* reduction, and  $H_2O_2$  levels) than the nutritional antioxidants in protecting mice from oxidative stress. Besides, mangiferin showed the same pattern of effect as Vimang except for GPx (no change in GPx levels) [11]. DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging properties of mangiferin suggests its strong antioxidant activity ( $EC_{50} 5.8 \pm 0.96 \text{ mg ml}^{-1}$  or 13.74 mM) comparable to that of rutin, a usual antioxidant used for medical purposes [22]. The free radical scavenging property of mangiferin can probably be understood from the structural point of view as it contains four phenolic H-atoms, two of which could easily be abstracted by suitable free radicals (e.g., ROS) to form two phenoxyl radicals that are stabilized by resonance (Figure 8.2) [23, 24].

#### 8.2.1.2 Anti-inflammatory Activity of Mangiferin

Inflammatory processes involve a broad range of chemical mediators; these include nitric oxide (NO) and prostanoids synthesized by inducible isoforms of NO synthase (iNOS) and cyclooxygenase (COX-2), respectively. Vascular events related to an inflammatory reaction include dilatation of the small arterioles resulting from increased blood flow and permeability [25–27].

Beltran *et al.* [28] investigated the effects of Vimang and mangiferin on COX-2 and iNOS expression of noradrenaline-induced vasoconstriction in vascular smooth muscle cells from mesenteric arteries of normotensive (WKY) and spontaneously hypertensive rats (SHRs) with and without stimulation by interleukin- $1\beta$  (IL) ( $1 \text{ ng ml}^{-1}$ ; 24 h). In both the strains, no effect was exerted by Vimang and

mangiferin themselves on iNOS or COX-2 vascular expression in the absence of IL-1 $\beta$ ; they could, however, prevent the two enzymes induction by IL-1 $\beta$ , showing their potent anti-inflammatory actions. Besides, mangiferin was found to decrease NO production and iNOS mRNA levels in activated macrophages [29, 30]. As NF- $\kappa$ B (nuclear factor-kappa B) plays an important role in the induction of the promoter for both COX-2 and iNOS genes, the inhibition of NF- $\kappa$ B activation appears to be involved in the anti-inflammatory mechanisms of action [31]. Both in WKY and SHR rats, Vimang but not mangiferin could induce a reduction of the contractions elicited by noradrenalin. Results suggest that the inhibitory effect of Vimang on vasoconstrictor responses and COX-2 and iNOS expression would be mediated by different compounds.

### 8.2.1.3 Immunomodulatory Effect

Most of the genes (such as those encoding pro-inflammatory cytokines, chemokines, adhesion molecules, and inflammatory enzymes) which are overexpressed in inflammation contain  $\kappa$ B sites within their promoter suggesting that these genes are controlled predominantly by the NF- $\kappa$ B [32]. The activation of NF- $\kappa$ B and its associated kinases, I $\kappa$ B $\alpha$  kinase (IKK) depends largely on the production of ROS [33].

Mangiferin mediates the downregulation of NF- $\kappa$ B, suppresses NF- $\kappa$ B activation induced by inflammatory agents, including tumor necrosis factor (TNF), increases the intracellular glutathione (GSH) levels, and potentiates chemotherapeutic agent-mediated cell death. This information suggests a possible role of mangiferin in combination therapy for cancer [34]. It is likely that these effects are mediated through its ROS-quenching and GSH-enhancing ability. Increased intracellular (GSH) levels are indeed known to inhibit the TNF-induced activation of NF- $\kappa$ B [35].

Leiro *et al.* [20] characterized *in vivo* immunomodulatory activity of mangiferin on thioglycollate-elicited mouse macrophages which were stimulated with lipopolysaccharide (LPS) and gamma interferon (IFN- $\gamma$ ). The synthesis and expression of cytokines involved in the NF- $\kappa$ B signal transduction pathway was investigated by microarray. Mangiferin significantly (i) hinders NF- $\kappa$ B activation induced by LPS, TNF, and IL-1 at the level of TNF receptor-associated factor 6; (ii) inhibits NF- $\kappa$ B-mediated signal transduction (inhibition of two genes of the Rel/NF- $\kappa$ B/I $\kappa$ B family, RelA and RelB); (iii) inhibits toll-like receptor proteins, including c-Jun N-terminal kinase 1 and 2 (JNK1 and JNK2); (iv) inhibits proteins involved in the response to TNF and in apoptotic pathways triggered by DNA damage; and (v) inhibits a series of pro-inflammatory cytokines (IL-1 $\alpha$ , IL-1, IL-6, IL-12, TNF- $\alpha$ , granulocyte, and macrophage colony-stimulating factors, A2) as well as various intracellular and vascular adhesion molecules (VCAM-1) [20]. These results indicate that, in addition to ROS-scavenging properties, mangiferin modulates the expression of a large number of genes critical for the regulation of apoptosis, viral replication, tumorigenesis, inflammation, and various autoimmune diseases. Results suggest that mangiferin protects cells against oxidative

damage and mutagenesis; and will probably be a valuable therapeutic in the prevention and treatment of inflammatory diseases and/or cancer.

#### 8.2.1.4 Antidiabetic Activity

Diabetic mellitus is one of the most recognizable endocrine metabolic disorders fundamentally characterized by hyperglycemia via disruption of carbohydrate, fat, and protein metabolism from insufficiency of secretion or action of endogenous insulin (WHO). It is associated with hyperglycemia caused by defects in insulin secretion or insulin action. In type 1 diabetes, pancreatic  $\beta$ -cells are destroyed, usually by autoimmune inflammatory mechanisms; type 2 diabetes is a complex metabolic disorder associated with  $\beta$ -cell dysfunction and with varying degrees of insulin resistance [36]. Hyperglycemia is a well-distinguished pathogenic factor of chronic complications in diabetic mellitus and not only generates excessive free radicals (ROS) but also attenuates antioxidative machineries through glycation of the antioxidant enzymes [37]. Streptozotocin (STZ) is commonly used as a diabetic inducer in experimental animals and its toxicity is generated by nitric oxide (NO) on pancreatic  $\beta$ -cells. The cellular toxicity of STZ is linked with the ROS formation resulting in oxidative damage of various organ tissues [38]. Hence, oxidative stress has been considered to be a general pathogenic factor of diabetic complications including nephropathy [39] and cardiopathy [40]. Diabetic nephropathy is the most serious microvascular complication of diabetes mellitus and the most common cause of end-stage renal disease (ESRD). This is usually found in both types 1 and 2 diabetes worldwide [41]. It is caused by the damage to small blood vessels in the kidneys that in turn become less efficient or ultimately fail to function [42]. Diabetic nephropathy has been characterized by glomerular hypertrophy, glomerular hyperfiltration, increased urinary albumin secretion, increased basement membrane thickness, and mesangial expansion with the accumulation of extracellular matrix proteins (ECM) [43, 44]. Hyperglycemia is strongly associated with increased production of ROS. The plausible major sources of ROS in diabetic nephropathy are the activation of polyol pathways, advanced glycation end products (AGEs), autooxidation of glucose, xanthine oxidase activity, mitochondrial respiratory chain deficiencies, NAD(P)H oxidase and nitric oxide synthase (NOS) [45, 39]. Therefore, a molecule possessing both hypoglycemic and antioxidant properties might be considered a protective agent against diabetic complications [46, 47].

**Effect on Type 1 Diabetes** Muruganandan *et al.* [8, 48] investigated the effects of mangiferin on hyperglycemia, atherogenicity, and oxidative damage to cardiac and renal tissues in STZ-induced diabetic rats. After 30 days, diabetic rats were administered mangiferin or insulin (positive control) daily for 28 days. STZ (i) reduced catalase (CAT) and SOD activities in kidney, increased in the heart (possibly through a compensatory mechanism) and did not affect erythrocytes and (ii) showed a significant increase of MDA, creatine phosphokinase (CPK),

glycosylated hemoglobin, glucose, triglycerides (TGs), total low density lipoprotein cholesterol (LDL-C), cholesterol and decrease of high-density lipoprotein cholesterol (HDL-C) in all tissues. In diabetes-induced rats, repeated i.p. injections of insulin or mangiferin for 28 days significantly reduced the tissue MDA levels, restored the changes in cardiac and renal antioxidant enzyme activities, and reduced the glycosylated hemoglobin and CPK levels. This antidiabetic activity of mangiferin could involve mechanisms other than pancreatic  $\beta$ -cell insulin release/secretion. The extra pancreatic actions [49] could consist of (i) a stimulation of peripheral glucose utilization; (ii) an enhancement of glycolytic and glycogenic processes [50]; and (iii) a glycemia reduction through the inhibition of glucose intake. The last hypothesis could be supported by the finding that mangiferin isolated from roots of *Salacia reticulata* inhibits  $\alpha$ -glucosidases (sucrase, isomaltase, and maltase;  $IC_{50}$  values of 87, 216, and  $1.4 \mu\text{g ml}^{-1}$ , respectively) [51]. Treatment with mangiferin resulted in a potent antihyperlipidemic and antiatherogenic activities in diabetic rats (strong and significant reduction in atherogenic index, total cholesterol, TG, LDL-C associated with concomitant significant increase in HDL-C) [48]. In glucose-loaded normal rats, mangiferin induces a significant improvement in oral glucose tolerance but without change of basal plasma glucose levels [48]. These studies show that mangiferin exhibits potent antidiabetic, antihyperlipidemic, antiatherogenic, and antioxidant properties without causing hypoglycemia and would offer a greater therapeutic benefit for the management of diabetes mellitus and diabetic complications.

Sellamuthu *et al.* [52] also investigated the antihyperglycemic effect of mangiferin (purified from methanolic root extract of *Salacia chinensis*) in STZ-induced diabetic rats. Mangiferin, administered orally for 30 days to diabetic rats, significantly decreased the levels of blood glucose, glycosylated hemoglobin as well as increased the levels of insulin and hemoglobin. The activities of hexokinase, pyruvate kinase, glucose-6-phosphate dehydrogenase, glycogen synthase, and glycogen content level increased to near normal in mangiferin-treated diabetic rats. Besides, activities of lactate dehydrogenase, glucose-6-phosphatase (G6Pase), fructose-1,6-diphosphatase and glycogen phosphorylase were also significantly decreased in liver tissue of the diabetic rats. These findings demonstrated that mangiferin possess antidiabetic activity.

**Effect on Type 2 Diabetes** Type 2 diabetes is mainly controlled by exercise and diet; however, therapeutic agents possessing stimulatory insulin secretion ability, have also been suggested for this purpose. Mangiferin, 7 h after oral administration in KK-Ay mice (an animal model of type 2 diabetes), diminished the baseline glucose level by 56% [53]. Using the same animal model Miura *et al.* [54] reported that mangiferin treatment followed 30 min exercise (120 min motorized treadmill) for 2 weeks decreased the blood cholesterol ( $\sim 40\%$ ) and triglyceride levels ( $\sim 70\%$ ). Mangiferin or exercise alone did not manipulate cholesterol but extensively decreased triglyceride levels. Therefore, mangiferin is definitely worthy of



further investigation for these beneficial effects on hyperglycemia and hyperlipidemia in type 2 diabetes.

#### 8.2.1.5 Iron Complexing Activity of Mangiferin

Andreu *et al.* [55] investigated the iron-complexing ability of mangiferin as a primary mechanism for protection of rat liver mitochondria against  $\text{Fe}^{2+}$ -citrate-induced lipid peroxidation. Thiobarbituric acid reactive substances (TBARSs) and antimycin A-insensitive oxygen consumption were used as quantitative measures of lipid peroxidation. Mangiferin (10  $\mu\text{M}$ ) showed superior protection against  $\text{Fe}^{2+}$ -citrate-induced (50  $\mu\text{M}$ ) mitochondrial swelling and loss of mitochondrial transmembrane potential. The  $\text{IC}_{50}$  value for mangiferin protection against  $\text{Fe}^{2+}$ -citrate-induced mitochondrial TBARS formation was around 10 times lower than that for *tert*-butylhydroperoxide mitochondrial induction of TBARS formation. The xanthone derivative also inhibited iron citrate induction of mitochondrial antimycin A-insensitive oxygen consumption, stimulated oxygen consumption due to  $\text{Fe}^{2+}$  autoxidation, and prevented  $\text{Fe}^{3+}$  ascorbate reduction. Absorption spectra of mangiferin- $\text{Fe}^{2+}/\text{Fe}^{3+}$  complexes also suggest the formation of a transient charge transfer complex between  $\text{Fe}^{2+}$  and mangiferin, accelerating  $\text{Fe}^{2+}$  oxidation, and the formation of a more stable  $\text{Fe}^{3+}$ -mangiferin complex that is unable to participate in a Fenton-type reaction and lipid peroxidation propagation phase. In conclusion, these results show that *in vitro* antioxidant activity of mangiferin is related to its iron-chelating properties and is not merely due to the scavenging activity of free radicals. These results are of pharmacological relevance and mangiferin along with its natural extracts could be potential candidates for chelation therapy in diseases which are related to abnormal intracellular iron distribution or iron overload.

#### 8.2.1.6 Mangiferin Protects against Mercury-Induced Toxicity

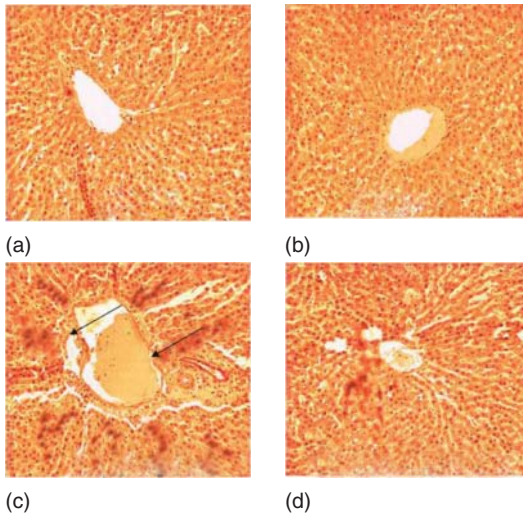
Mercury (Hg) is a broad environmental and industrial pollutant which induces various changes in the tissues of both humans and animals. In nature, mercury (Hg) exists in a wide variety of physical and chemical states, each of which has specific properties for target organs. Exposure of Hg vapor and organic Hg compounds specifically damages the central nervous system (CNS), whereas the kidney, liver, and gastrointestinal tract are the target organs for inorganic Hg compounds. A number of studies on mercury toxicity showed that mercuric chloride ( $\text{HgCl}_2$ ) generates oxygen free radicals.  $\text{Hg(II)}$  has a great affinity for sulfhydryl ( $-\text{SH}$ ) groups of biomolecules, thus depleting intracellular thiols including reduced GSH [56]. Its covalent binding to  $-\text{SH}$  changes the protein's conformation and generates adducts by side chain modification. Hg promotes free radical formation via thiol complexation and finally leads to change in the shape and activity of proteins [57]. It imposes a loss of antioxidant activity in GPx, glutathione reductase (GR), CAT, glutathione-S-transferase (GST), and SOD. Generation of ROS in the cellular cytoplasm may increase the mitochondrial hydrogen peroxide production and lipid peroxidation of mitochondrial

membrane, resulting in a loss of membrane potential and finally leading to cellular necrosis or apoptosis [58, 59]. Antioxidant property of mangiferin is also well established and therefore, this molecule may be considered as a candidate against mercury-induced oxidative stress. Agarwala *et al.* [60] investigated the cytoprotective effect of mangiferin on HgCl<sub>2</sub>-induced toxicity by colony formation assay, fluorescence microscopy, flow cytometric DNA analysis, and DNA fragmentation pattern assay. Further, the cytoprotective effect of mangiferin against HgCl<sub>2</sub> toxicity was also assessed by evaluating its effect on biochemical parameters such as GSH, GST, SOD, CAT, mitochondrial membrane potential, and ROS levels. A significant increase in the surviving fraction was observed with mangiferin administered 2 h prior to various concentrations of HgCl<sub>2</sub>. Furthermore, mangiferin treatment inhibited the percentage of HgCl<sub>2</sub>-induced apoptotic cells and the levels of ROS, along with decreased values (activity/content) of GSH, GST, SOD, and CAT. Studies demonstrated the cytoprotective potential of mangiferin, which may be attributed to quenching of the HgCl<sub>2</sub>-induced ROS in the cells because of oxidative stress, restoration of mitochondrial membrane potential, and normalization of various cellular antioxidant activities.

#### 8.2.1.7 Mangiferin Protects Murine Liver against Pb(II)-Induced Hepatic Damage

Lead (Pb) is a universal toxic metal that affects several organs (liver, kidneys, etc.) and the central nervous, endocrine as well as reproductive systems [61] of the human body. Lead also damages cellular substances and changes cellular genetics similarly to other naturally occurring heavy metals such as arsenic, mercury, and cadmium. The mechanism of lead toxicity, in general, involves oxidative damage that affects cell membrane and activates factors susceptible to transcription [62–64]. Several reports on lead nitrate [Pb(NO<sub>3</sub>)<sub>2</sub>] exposure demonstrated that it produces ROS and disturbs the prooxidant–antioxidant balance. Typically Pb(II) binds to sulfhydryl (–SH) groups of biomolecules, disrupts structural protein synthesis, changes calcium homeostasis, lowers the level of available sulfhydryl antioxidant, and reduces GSH stores in the body [65]. Lead-induced hepatic damage typically causes lipid peroxidation via the production of ROS [66]. It decreases the activities of several antioxidant enzymes such as CAT, SOD, GPx, and GR [67].

Pal *et al.* [23] investigated the molecular mechanisms of the protective action of mangiferin against lead-induced hepatic pathophysiology. Pb in the form of Pb(NO<sub>3</sub>)<sub>2</sub> induced oxidative stress, hepatic dysfunction, and cell death. Posttreatment with mangiferin diminished the formation of ROS and reduced the levels of serum marker enzymes such as alanine aminotransferase (ALT) and alkaline phosphatase (ALP). Mangiferin also reduced Pb(II)-induced alterations in antioxidant machineries (CAT, SOD, GPx, GR, and GSH) and restored the mitochondrial membrane potential in addition to mutual regulation of Bcl-2/Bax. Furthermore, mangiferin-inhibited Pb(II)-induced activation of mitogen-activated protein kinases (MAPKs) (phospho-ERK1/2 (extracellular



**Figure 8.3** Histopathological changes in liver tissue (stained with hematoxylin and eosin dye). (a) Normal, (b) mangiferin, (c) Pb(II)-exposed, and (d) Pb(II)+mangiferin.

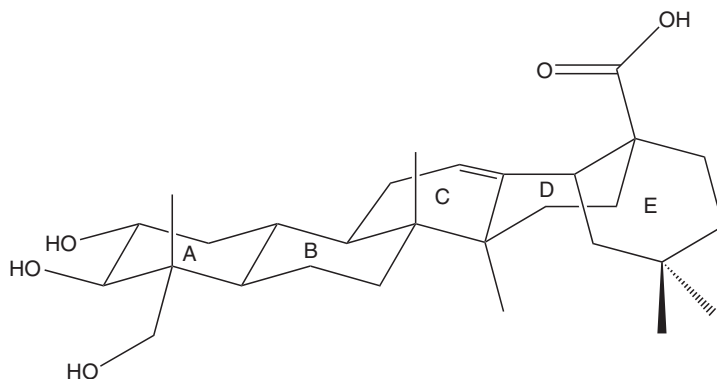
signal-regulated kinase), phosphor-JNK phospho-p38), nuclear translocation of NF- $\kappa$ B, and apoptotic cell death as was evidenced by DNA fragmentation, flow cytometry (FITC-labeled Annexin V and propidium iodide), and histological assessments (Figure 8.3). *In vitro* studies using hepatocytes as the working model also showed the protective effect of mangiferin in Pb(II)-induced cytotoxicity. All these beneficial effects of mangiferin contributes toward the considerable reduction of apoptotic hepatic cell death induced by Pb(II). In summary, mangiferin supplementation appears to be a promising approach for the hepatoprotection in Pb(II)-induced liver dysfunction and cell death.

Considering all these beneficial effects of mangiferin coupled with the absence of any noticeable toxicity, we are optimistic in arguing that this unique xanthone deserves further in-depth research to explore its potent pharmaceutical potential in hepatic and other organ pathophysiology as well as in diabetic complications.

### 8.2.2

#### Arjunolic Acid

Arjunolic acid, a triterpenoid saponin, first isolated from *Terminalia arjuna* [68], is considered to be a novel phytochemical that possesses multifunctional therapeutic activities. Later, it was also isolated from other medicinal plants such as *Combretum nelsonii* [69], *Leandra chaeton* [70], *Cochlospermum tinctorium* [71], and *Cornus capitata* [72]. Triterpenoids are an important class of plant secondary metabolites derived from C<sub>30</sub> precursors [73] and they possess a wide range of



**Figure 8.4** Chemical structure of arjunolic acid.

biological activities [74]. Chiral triterpene acid has a rigid pentacyclic backbone with two equatorial hydroxyl groups and one equatorial hydroxymethyl group attached to the “A” ring. The carboxyl group is attached at the ring junction of the cis-fused “D” and “E” rings (Figure 8.4). Being known for its cardioprotective effect over centuries, experimental studies also proved its inherent functions such as prevention of myocardial necrosis, platelet aggregation and coagulation, lowering of blood pressure, heart rate, and cholesterol levels, which lend support to the claim for its traditional usage. Apart from its cardioprotective effects, arjunolic acid protects the cells from metal-induced toxicity and it also possesses anti-inflammatory, antitumor, antimicrobial, and antidiabetic activity. It is a potent antioxidant and a free radical scavenger. The free radical-scavenging activity was determined using 2,2-diphenyl-1-picrylhydrazyl and its *in vivo* antioxidant potential was assessed by an ferric reducing/antioxidant power (FRAP) assay [75].

#### 8.2.2.1 Cardioprotective Effects of Arjunolic Acid

**Arjunolic Acid Protects Heart from Myocardial Necrosis** Sumitra *et al.* [76] investigated the cardioprotective actions of arjunolic acid by showing its effect on platelet aggregation, coagulation, and myocardial necrosis. Intraperitoneal arjunolic acid administration to the rats having isoproterenol-induced myocardial necrosis, exhibited a decrease in serum enzyme levels demonstrated a decrease in serum enzyme levels (such as lactate dehydrogenase, creatine kinase, and creatine kinase–MB) and the electrocardiographic changes got restored toward normalcy. Arjunolic acid treatment also restored the levels of SOD, CAT, GPx,  $\alpha$ -tocopherol, and reduced GSH, ceruloplasmin, ascorbic acid, myeloperoxidase, and lipid peroxide. Histological studies on the heart tissue reveal that arjunolic acid could inhibit isoproterenol-induced engorgement of coronary vessels. This study also demonstrates the antiplatelet and anticoagulant activity of

arjunolic acid. So arjunolic acid could possibly be recognized as an effective cardioprotective agent against tissue injury caused by myocardial necrosis.

**Arjunolic Acid Protects Heart from Doxorubicin Induced Cardiac Apoptosis** Ghosh *et al.* [77] demonstrated that arjunolic acid acts as a protective agent against the anticancer drug doxorubicin-induced (Dox) cardiotoxicity. Dox administration activated proapoptotic p53, p38, and JNK MAPKs, resulted in Bax translocation, disrupted mitochondrial membrane potential, and potentiated mitochondrion-mediated caspase-dependent apoptotic signaling in cardiomyocytes in association with increased dichlorofluorescein (DCF) intensity subsequent to intracellular  $H_2O_2$  production in myocytes. Treatment with CAT reduced this intensity and increased cell viability. Intracellular  $H_2O_2$  thus produced activated the p38-JNK and p53-mediated pathways. CAT treatment also distinctly decreased the Dox-mediated activation of p38 and JNK, suggesting that  $H_2O_2$  is involved in the activation of MAPKs. The pharmacological inhibitors of p53 and p38-JNK also suppressed the Dox-induced apoptosis with simultaneous inhibition of antiapoptotic Bcl-2 family of proteins. Arjunolic acid treatment ameliorates nearly all of these apoptotic actions of Dox and conserved cell viability. Besides, rats treated with Dox displayed retarded growth of body and heart as well as elevated apoptotic indices in heart tissue; arjunolic acid treatment, however, successfully suppressed all these Dox-induced cardiac abnormalities. In summary, we say that Dox induces cardiac apoptosis via the activation of JNK-p38 and p53-mediated signaling pathways, where  $H_2O_2$  acts as the mediators of these pathways. Arjunolic acid can effectively neutralize this action of Dox and could potentially protect the heart and cardiomyocytes from the severe Dox-induced cardiovascular complications.

**Arjunolic Acid Prevents Arsenic-Induced Myocardial Damage** Arsenic toxicity is one of the risk factors associated with cardiovascular diseases. Arsenic exposure is linked with direct myocardial damage, cardiac arrhythmias, and cardiomyopathy [78]. Manna *et al.* [75] demonstrated the protective effects of arjunolic acid in arsenic-induced cardiac oxidative damage. Oral administration of sodium arsenite ( $NaAsO_2$ ) causes significant accumulation of arsenic in cardiac tissues of the experimental mice in addition to decreasing cardiac antioxidant enzyme activities. Arsenic exposure also diminishes the levels of cardiac GSH and total thiol contents and increases the levels of oxidized GSH, lipid peroxidation end products (MDA), and protein carbonyl content. Pretreatment with arjunolic acid to sodium arsenite exposure protects cardiac tissue from arsenic-induced oxidative injury by preventing the changes in the levels of antioxidants. In addition to oxidative stress, arsenic intoxication increases total cholesterol and decreases in high-density lipoprotein (HDL) cholesterol level in the experimental mice. The same sodium arsenite intoxication causes disorganization of normal radiating pattern of cell plates in the heart. Arjunolic acid pretreatment, however, can suppress the hyperlipidemia and

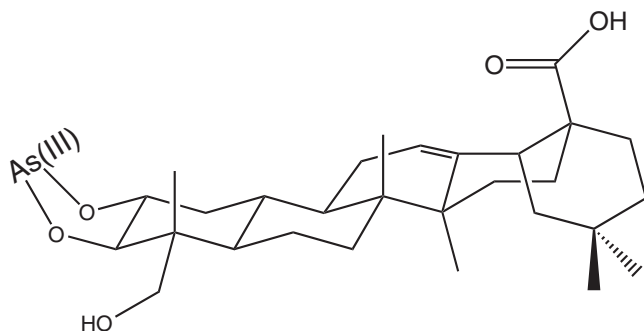


Figure 8.5 Arjunolic acid-As(III) complex.

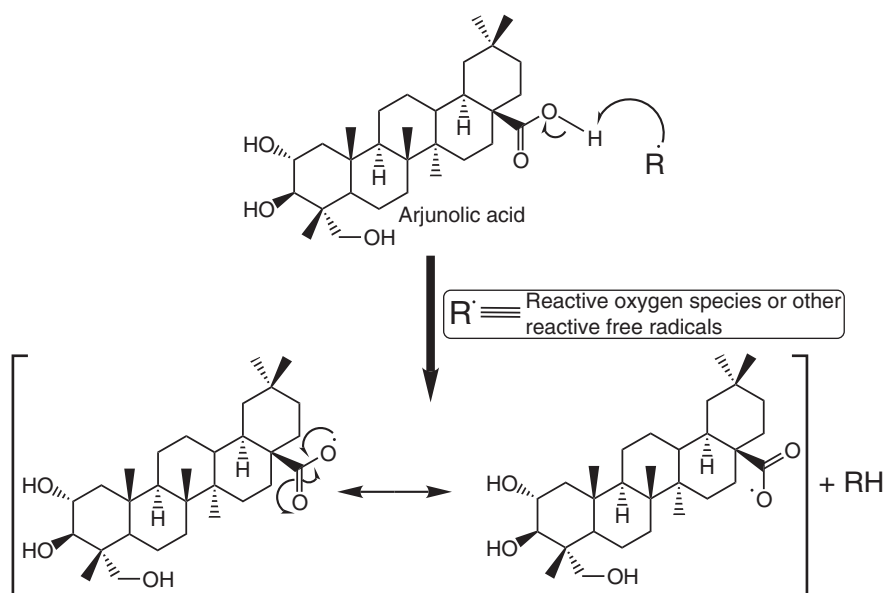


Figure 8.6 Free radical intermediate of arjunolic acid: 2 equiv resonating canonical forms.

help maintaining the normal radiating pattern of the heart. Arjunolic acid also reduces the extent of arsenic concentration as well as DNA fragmentation in the heart tissue and prevents toxin-induced reduction in the heart weight to body weight ratio. Results suggest that arjunolic acid could effectively ameliorate the reduction in the intracellular antioxidant potential and protect the myocardium from arsenic intoxication by chelating the free arsenic in the system. The cardio-protective effects of arjunolic acid could be attributed to its powerful antioxidant, free radical scavenging, and metal-chelating properties (Figures 8.5 and 8.6).

### 8.2.2.2 Antidiabetic Activity

Manna *et al.* [79] demonstrated the protective role of arjunolic acid in STZ-induced diabetes in the pancreatic tissue of rats. STZ administration causes an increase in the production of both ROS and reactive nitrogen species (RNS) in the pancreas of experimental animals. Formation of these reactive intermediates diminishes the intracellular antioxidant defense, increases the levels of lipid peroxidation, protein carbonylation, serum glucose, and TNF- $\alpha$ . Treatment of animals with arjunolic acid, both pre and post to STZ administration efficiently reduces these adverse effects by inhibiting the extreme ROS and RNS production as well as by downregulating the activation of phospho-ERK1/2, phospho-p38, NF- $\kappa$ B, and mitochondrial-dependent signal transduction pathways leading to apoptotic cell death.

Manna *et al.* [80] have also reported the efficacy of arjunolic acid against STZ-induced diabetic nephropathy in rats. Diabetic renal damage is associated with increased kidney weight to body weight ratio, glomerular area and volume, blood glucose level, blood urea nitrogen (BUN), and serum creatinine. This renal dysfunction increases the production of ROS and RNS, enhances lipid peroxidation and protein carbonylation, and decreases intracellular antioxidant defense in the kidney tissue. Treatment of the animals with arjunolic acid successfully ameliorates diabetic renal dysfunctions by reducing oxidative as well as nitrosative stress and deactivating the polyol pathways. Histological studies reveal multiple foci of hemorrhagic necrosis and cloudy swelling of tubules in the kidney of toxin control and this is reduced after treatment with arjunolic acid, suggesting that arjunolic acid could act as a protective agent against the renal dysfunctions developed in STZ-induced diabetes.

### 8.2.2.3 Arjunolic Acid Protects Organs from Acetaminophen (APAP)-Induced Toxicity

**Kidney Protection** Acetaminophen (APAP) is an extensively used analgesic and antipyretic drug and it is nontoxic at therapeutic doses. However, unintended or intentional overdose causes acute liver and kidney failure [81]. Rats exposed to a nephrotoxic dose of APAP show changes in the levels of a number of biomarkers (BUN, serum creatinine levels, etc.) related to renal oxidative stress. A decrease in antioxidant activity and elevation in renal TNF- $\alpha$  and nitric oxide levels was also observed in this pathophysiology. Arjunolic acid treatment both pre- and post-exposure to APAP protects the change in these biomarkers, compensates insufficiencies in the antioxidant defense mechanism, and suppresses lipid peroxidation in renal tissue. Experimental evidence suggests that APAP-induced nephrotoxicity is a caspase-dependent process that involves the activation of caspase-9 and caspase-3 in the absence of cytosolic cytochrome *c* release. These results provide evidence that inhibition of nitric oxide overproduction and preservation of intracellular antioxidant status may play an essential role in the protective effects of

arjunolic acid against APAP-induced renal damage. Arjunolic acid acts as a potential therapeutic agent to protect renal tissue from the harmful effects of acute APAP overdose [82].

**Liver Protection** In another study, Ghosh *et al.* [83] reported the protective role of arjunolic acid against APAP-induced acute hepatotoxicity. Exposure of rats with a hepatotoxic dose of APAP changed a number of biomarkers and induced necrotic cell death. Pretreatment with arjunolic acid affords significant protection from liver injury. Arjunolic acid also prevents APAP-induced hepatic GSH depletion and APAP-metabolite formation. The results of this study suggest that this preventive action of arjunolic acid can be due to the metabolic inhibition of the specific forms of cytochrome P450 that activates APAP to NAPQI. In addition, administration of arjunolic acid 4 h after APAP exposure reduces APAP-induced JNK and downstream Bcl-2 and Bcl-xL phosphorylation and thus, prevents mitochondrial permeabilization, loss in mitochondrial membrane potential, and cytochrome *c* release. Therefore, arjunolic acid could effectively protect the liver against APAP-induced hepatotoxicity through inhibition of P450-mediated APAP bioactivation and inhibition of JNK-mediated activation of mitochondrial permeabilization.

#### 8.2.2.4 Arjunolic Acid Protects Liver from Sodium Fluoride-Induced Toxicity

Fluoride is widely used in a number of industrial practices and is a ubiquitous ingredient of drinking water, foodstuffs, and dental products [84]. Excess intake of fluoride, however, causes fluorosis, a slow progressive degenerative disorder. Besides other toxic effects, fluoride accumulation induces oxidative stress. Ghosh *et al.* [85] have conducted a study to investigate the effect of arjunolic acid against sodium fluoride (NaF)-induced cytotoxicity and necrotic cell death in hepatocytes. Dose-dependent studies suggest that incubation of hepatocytes with sodium fluoride significantly decreases the cell viability as well as intracellular antioxidant potential. Incubation with arjunolic acid both before and in combination with sodium fluoride almost normalizes the altered activities of antioxidant indices. The increased cell viability and reduced cellular ROS in arjunolic acid-treated hepatocytes prove the anticytotoxic effect of arjunolic acid. Treatment with arjunolic acid enhanced the cellular antioxidant capability and protected hepatocytes against sodium fluoride-induced cytotoxicity and necrotic death.

#### 8.2.2.5 Protection against Arsenic-Induced Toxicity

Arsenic, one of the ubiquitous environmental pollutants, induces tissue damage, which is a major concern to human population. Its exposure occurs from inhalation, absorption through the skin, and primarily, by ingestion of contaminated food and drinking water. An impaired antioxidant defense mechanism followed by oxidative stress is the major cause of arsenic-induced toxicity. Arjunolic acid has been shown to protect arsenic-induced cytotoxicity probably due to its free radical scavenging as well as metal-chelating properties and thereby, diminishing the arsenic burden in the cells [75].



**Hepatic Protection** Manna *et al.* [86] investigated the hepatoprotective action of arjunolic acid against arsenic-induced oxidative damage in murine livers. Oral administration of sodium arsenite significantly reduced the activities of enzymatic and nonenzymatic antioxidants and in addition, it was also been shown to increase the activities of serum marker enzymes, namely, alanine transaminase and ALP, DNA fragmentation, protein carbonyl content, lipid peroxidation and end products, and the level of oxidized GSH. Treatment with arjunolic acid before arsenic administration prevents alterations in the activities of all antioxidant indices and levels of the other parameters studied. Histological studies reveal less centrilobular necrosis in the liver treated with arjunolic acid before arsenic intoxication when compared to the liver treated with the toxin alone. The ability to attenuate arsenic-induced oxidative stress in murine liver can probably be via the antioxidant mechanism of arjunolic acid.

Manna *et al.* [87] have further reported that the incubation of hepatocytes with sodium arsenite caused reduction in the cell viability and activities of the intracellular enzymatic as well as nonenzymatic antioxidants, enhanced lipid peroxidation, and increased the activities of alanine transferase and ALP. Administration of arjunolic acid before and also along with the toxin almost normalizes the altered activities of antioxidant indices. This study clearly reveals the ability of arjunolic acid to maintain the integrity of the cell membrane. Arjunolic acid has been demonstrated to possess free radical-scavenging activity and it can enhance the cellular antioxidant capability against sodium arsenite-induced cytotoxicity.

**Protection against Oxidative Insult in Brain** Sinha *et al.* [88] have demonstrated the ability of arjunolic acid to ameliorate arsenic-induced oxidative insult in the brain. Oral administration of arsenic in the form of sodium arsenite significantly decreases the levels of antioxidant enzymes, and cellular metabolites, reduces GSH and total thiols, and increases the level of oxidized GSH. In addition, it enhances the levels of lipid peroxidation end products and protein carbonyl content. Pretreatment with arjunolic acid almost normalizes the aforementioned indices. Histological findings reveal that arsenic-treated brain tissue shows more frequent nuclear pyknosis. Treatment with arjunolic acid prior to the arsenic intoxication reduces nuclear pyknosis and shows almost normal architecture, similar to that of the control. The aforementioned effects can be attributed to the antioxidant activity of arjunolic acid.

**Protection against Nephrotoxicity** Sinha *et al.* [89] have reported the efficacy of arjunolic acid against arsenic-induced nephrotoxicity in a mouse model. Oral administration of sodium arsenite causes a significant accumulation of arsenic in renal tissues and it alters the activities of serum markers, antioxidant enzymes, and the levels of lipid peroxidation end products. Treatment with arjunolic acid almost normalizes the aforementioned indices. Histological studies also

indicate the preventive role of arjunolic acid against sodium arsenite-induced nephrotoxicity.

**Testicular Protection** Manna *et al.* [90] have reported the preventive role of arjunolic acid against arsenic-induced testicular damage in mice. Administration of arsenic (in the form of sodium arsenite) significantly decreases the intracellular antioxidant activity, the activities of the antioxidant enzymes, and the levels of cellular metabolites. In addition, arsenic intoxication enhances testicular arsenic content, lipid peroxidation, protein carbonylation, and the level of GSH disulfide. Exposure to arsenic also causes significant degeneration of the seminiferous tubules with necrosis and defoliation of spermatocytes. Pretreatment with arjunolic acid prevents the arsenic-induced testicular oxidative stress and injury to the histological structures of the testes, which can be due to its intrinsic antioxidant property.

#### 8.2.2.6 Mechanism of Action of Arjunolic Acid

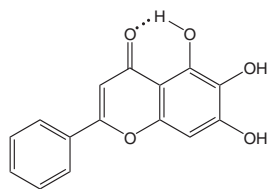
The preventive role of arjunolic acid against arsenic-induced cardiac oxidative stress could be due to formation of a five-membered chelate complex between arsenic and two equatorial hydroxyl groups of arjunolic acid (Figure 8.5). This chelate formation possibly eliminates the free toxin from the system and thus inhibits it from causing any further oxidative damage to the tissue.

In addition, the presence of one carboxylic hydrogen atom may be responsible for its free radical–scavenging activity [75]. Therefore, arjunolic acid acts as a good chelator against arsenic-induced toxicity. Manna *et al.* [87] established that arjunolic acid reveals preventive role similar to that of vitamin C. Therefore, it can be expected that arjunolic acid could also be oxidized by ROS to the analogous keto-derivative similarly to vitamin C, as it has one primary and two secondary hydroxyl groups. In addition, arjunolic acid also contains one carboxylic hydrogen atom which can easily be abstracted by any free radical (Figure 8.6). This property of arjunolic acid may explain its DPPH radical–scavenging activity.

### 8.2.3

#### Baicalein

Baicalein (5,6,7-trihydroxyflavone) (Figure 8.7) is a major flavonoid in the herb, *Scutellaria baicalensis* used extensively in traditional Asian medicine [91]. It is reported that baicalein exhibits a variety of biological effects, including anti-inflammatory, antiviral, and antitumor effects [92, 93]. In addition, baicalein has strong antioxidant activity against ROS and inhibits lipid peroxidation in mitochondria [94, 95]. Many studies have shown that baicalein inhibits H<sub>2</sub>O<sub>2</sub>-induced oxidative damage and apoptosis in several types of cells [96–98].



Baicalein

Figure 8.7 Chemical structure of baicalein.

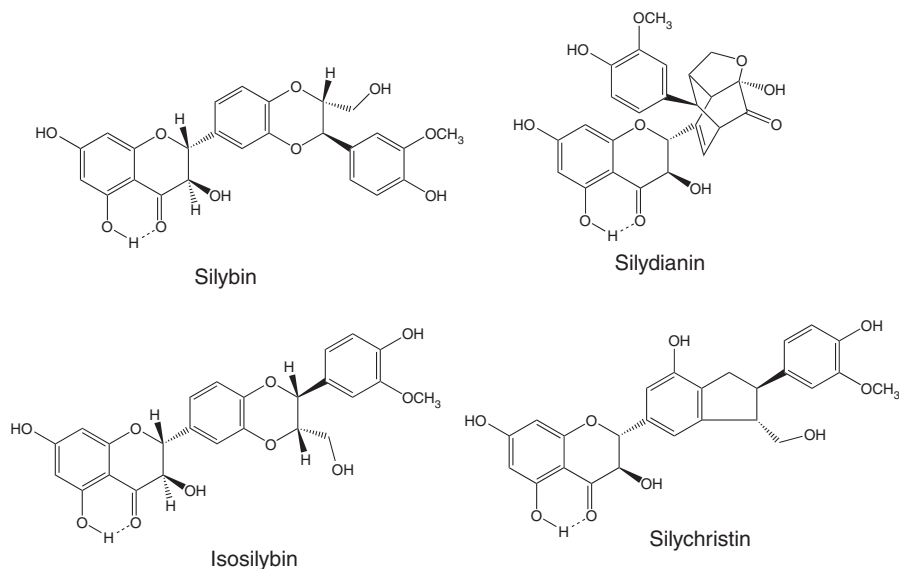
Accumulating evidence suggests that baicalein can act selectively within MAPK-signaling cascades [96, 98]. However, the role of MAPK in the action of baicalein on human melanocytes and the ability of baicalein to protect these cells against  $H_2O_2$ -induced oxidative damage and apoptosis are still unknown.

#### 8.2.3.1 Baicalein Protects Human Melanocytes from $H_2O_2$ -Induced Apoptosis

Removal of  $H_2O_2$  by antioxidants has been proven to be beneficial to patients with vitiligo. Baicalein has, therefore, been used in vitiligo therapy in Chinese traditional medicine because of its antioxidant activity. Liu *et al.* [99] investigated the potential protective effect and mechanisms of baicalein against  $H_2O_2$ -induced apoptosis in human melanocytes and found that baicalein significantly inhibited  $H_2O_2$ -induced apoptosis, intracellular ROS generation, and changes in the mitochondrial membrane potential. It also reduced the Bax/Bcl-2 ratio, the release of cytochrome *c*, the activation of caspase-3, and the phosphorylation of p38MAPK in a concentration-dependent manner. The results demonstrate that baicalein exerts a cytoprotective role in  $H_2O_2$ -induced apoptosis by inhibiting the mitochondria-dependent caspase activation and p38 MAPK pathway.

#### 8.2.3.2 Protection against Doxorubicin-Induced Cardiotoxicity

Dox presents a dose-dependent and additive cardiotoxicity as a serious side effect [100]. Adriamycin administration in rats causes cardiomyopathy and congestive heart failure [101]. Cardiotoxic side effects represent a serious complication of anticancer therapy with Dox and various mechanisms have been proposed to explain Dox-induced cardiotoxicity [102]. Dox (10  $\mu$ M) increased cell death, DNA fragmentation, and the phosphorylation of ROS-mediated proapoptotic kinase, JNK in chicken cardiomyocytes. Treatment with baicalein (25  $\mu$ M) significantly reduced Dox-induced free radical formation and cell death by inhibiting apoptosis. Dox also weakened the dissipated mitochondrial potential and increased DNA fragmentation. On the other hand, baicalein treatment successfully restored the mitochondrial membrane potential and repaired the DNA. In addition, simultaneous baicalein treatment did not affect the antiproliferative effect of Dox in human breast cancer MCF-7 cells [103]. This study established that baicalein protects cardiomyocytes from Dox toxicity by scavenging free radicals and preventing apoptosis.



**Figure 8.8** Structures of bioactive compounds of silymarin.

## 8.2.4

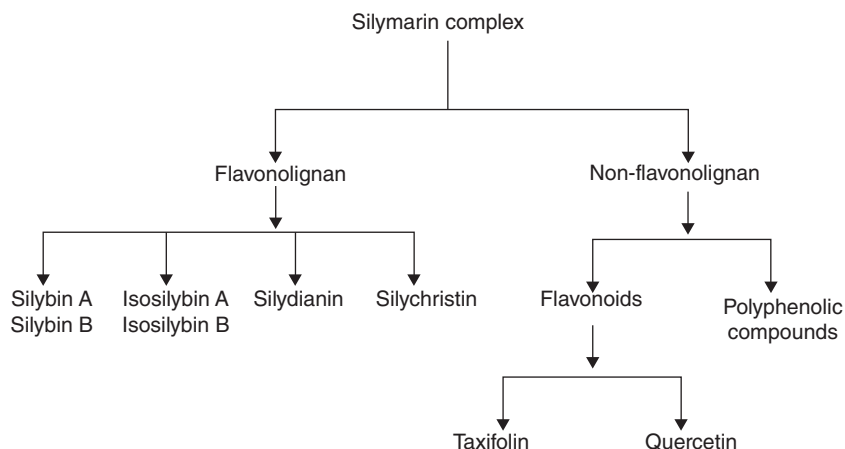
### Silymarin

*Silybum marianum*, commonly known as *milk thistle*, has been used for the treatment of liver and gallbladder disorders, including hepatitis, cirrhosis, and jaundice, for long time. It is proven to be useful for the protection against *Amanita phalloides* mushroom and other toxin poisoning [104] (Figure 8.8).

Silymarin, the active component of this plant, is a mixture consisting of ~70–80% silymarin flavonolignans (silybin A and B, isosilybin A and B, silydianin, and silychristin) and flavonoids (taxifolin and quercetin), and the remaining 20–30% consisting mainly of polyphenolic compounds (Figure 8.9) [105]. Silymarin has been shown to possess a wide range of biological and pharmacological effects, including antioxidant activity [106], stimulation of protein synthesis and cell regeneration [107, 108], as well as impressive anticancer effects against several human carcinoma cell lines [109, 110]. Moreover, antidiabetic activity [111], cardioprotection [112], anti-inflammatory, hypolipidemic, neuroprotective, and immune modulative effects of this molecule have also been well documented.

#### 8.2.4.1 Physicochemical and Pharmacokinetic Properties of Silymarin

Silymarin has low water solubility ( $0.04 \text{ mg ml}^{-1}$ ) and no lipophilic properties [113]. Literature reveals that silymarin can be administered safely and with efficacy



**Figure 8.9** Constituents of silymarin complexes.

at doses of 1200–1500 mg day<sup>-1</sup>. The typical adult dose of silymarin (orally administered) is 240–800 mg day<sup>-1</sup> [114].

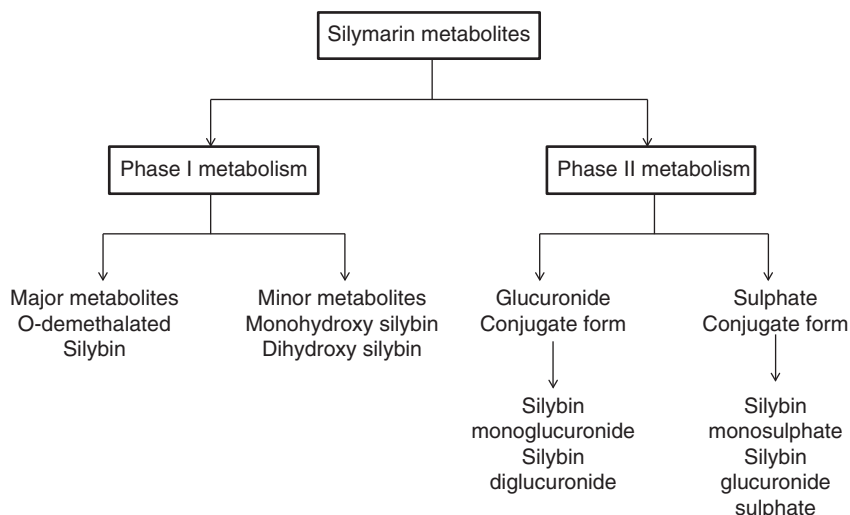
#### 8.2.4.2 Metabolism of Silymarin

Silymarin (standardized as silybin) undergoes phase I and phase II biotransformation (Figure 8.10) in the liver after delivery. It is metabolized by CYP450-2C8 enzyme system *in vitro* into *o*-demethylated silybin (major) and mono- as well as dihydroxy-silybin (minor) metabolites [115]. During phase II, multiple conjugation reactions occur including the formation of silybin monoglucuronide, silybin diglucuronide, silybin monosulfate, and silybin diglucuronide sulfate [116].

#### 8.2.4.3 Antioxidant Activity of Silymarin

Silybin, the major active constituent of silymarin, exhibits hepatoprotective properties under a number of clinical and experimental conditions [117]. Two main mechanisms of silybin action have been proposed so far: a cell-regenerating function and a cytoprotective effect. The cell-regenerating mechanism is based on the increase of ribosomal synthesis of cellular proteins through stimulation of DNA-dependent RNA-polymerase I and subsequent rRNA transcription established in liver and kidney cells [118]. Cytoprotection is mediated by two factors: (i) direct interaction of silybin with cell membrane components [119] and (ii) silybin's general antioxidant properties [120]. Inhibition of lipoperoxidation in erythrocytes, isolated and cultured hepatocytes [121], and human mesangial cells [122] is accepted by most investigators as one of silybin's major protective mechanisms.

Free radicals, including the superoxide radical, hydroxyl radical (OH<sup>•</sup>), hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), and lipid peroxide radicals have been shown to directly



**Figure 8.10** Different metabolites of silymarin.

participate in liver diseases [123]. The antioxidant properties of silymarin were evaluated by studying its capability to react with the relevant biological ROS such as superoxide anion radical ( $O_2^-$ ), hydrogen peroxide ( $H_2O_2$ ), hydroxyl radical ( $HO^\bullet$ ), and hypochlorous acid ( $HOCl$ ) [124] and thereby neutralizing them. Several studies reported that silymarin is not a good scavenger of  $O_2^-$  and no reaction of this molecule with  $H_2O_2$  has been detected ( $IC_{50}$   $200 \mu\text{mol l}^{-1}$ ). However, it does react rapidly with  $HO$ -radicals in free solution at approximately diffusion-controlled rate ( $IC_{50}$   $1.2-7 \mu\text{mol l}^{-1}$ ).

In some *in vitro* studies, silymarin, at a concentration equivalent to the usual therapeutic dosage, has been shown to markedly increase the expression of SOD in lymphocytes in patients with alcoholic cirrhosis [104]. As there is no evidence available yet whether silymarin has any direct effect on ethanol metabolism and/or reaction with cytochrome P450-2E1, it can be said that the antitoxic effects exerted by silymarin is exclusively due to its antioxidant and free radical-scavenging properties [125]. In a study, silymarin has been shown in experimental rats to inhibit the alterations of reduced GSH level and SOD activity induced by APAP at toxic doses [126]. Moreover, in some other studies, silymarin has been shown to inhibit cell lysis as indicated by changes in the ALT levels when hepatocytes were exposed to carbon tetrachloride and galactosamine [127]. Besides, some *in vitro* experiments with simian kidney cells demonstrated that silymarin administration before or after the drug (paracetamol, cisplatin, and vincristin)-induced injury could avoid or ameliorate the toxicity [128].

#### 8.2.4.4 Protective Effect of Silydianin against Reactive Oxygen Species

The inhibitory effect of silydianin on the *in vitro* production and release of oxidative products has been examined. Polymorphonuclear neutrophils (PMNs) play an important role in the initiation and propagation of inflammatory responses and their apoptosis is a major mechanism associated with the resolution of inflammatory reactions. When cells were cultured with silydianin, caspase-3 was activated. This observation was an indication of the execution phase of apoptosis. Initiation of apoptosis was accompanied by a decrease in luminol-enhanced chemiluminescence and superoxide radical ( $O_2^{\bullet-}$ ) release in freshly isolated cells and lipid peroxidation in mouse spleen microsomes. No significant effect of silydianin on PMN hydrogen peroxide production was found. Results suggest a possible anti-inflammatory activity of silydianin, which regulates caspase-3 activation, affects cell membranes, and probably acts as a direct free radical scavenger [129].

#### 8.2.4.5 Diabetes and Silymarin

Apart from its antioxidant properties silybin and its derivative dehydrosilybin, the most important bioactive compound of silymarin, could inhibit glucose uptake in several model systems. Both flavonoids reduce basal and insulin-dependent glucose uptake by 3T3-L1 adipocyte cells in a dose-dependent manner; particularly, dehydrosilybin showed significant inhibition. However, insulin signaling itself was not impaired and immunofluorescence and subcellular fractionation showed that insulin-induced translocation of GLUT4 (glucose transporter) to the plasma membrane also remained unchanged. Hexokinase activity also was not affected, indicating that silybin and dehydrosilybin interfere directly with glucose transport across the plasma membrane. Expression of GLUT4 in *Chinese hamster ovary* cells (CHO cells) was shown to counteract the inhibition of glucose uptake by both flavonoids. Besides, treatment of CHO cells with silybin and dehydrosilybin reduced cell viability which was partially rescued by GLUT4 expression. Kinetic analysis also confirmed the fact that silybin and dehydrosilybin inhibit GLUT4-mediated glucose transport in a competitive manner. In short, this study concludes that silybin and dehydrosilybin inhibit cellular glucose uptake by directly interacting with GLUT transporters [130].

#### 8.2.4.6 Silibinin Protects H9c2 Cardiac Cells from Oxidative Stress

Cardiac hypertrophy is the primary response of the heart to various extrinsic and intrinsic stimuli, and it is characterized by specific molecular and phenotypic changes. Recent studies, both *in vitro* and *in vivo*, suggest the involvement of ROS in the hypertrophic response. Silibinin, the most important flavonolignan of the silymarin mixture, was evaluated for its effects in (i) preventing hydrogen peroxide ( $H_2O_2$ )-induced cellular damage and (ii) blocking the phenylephrine-induced hypertrophic response using the *in vitro* model of embryonic rat heart-derived H9c2 cells. Results showed that silibinin supplement is safe up to 200  $\mu$ M and that did not affect cell viability. Pretreatment of the cells with silibinin resulted in their protection under experimental conditions compared to untreated cells as

evidenced by cell viability and DNA fragmentation assays. Furthermore, silibinin attenuated the phenylephrine-induced hypertrophic response as showed by the measurement of cell surface area, upregulation of atrial natriuretic peptide, and increase of cellular protein levels. Moreover, silibinin repressed the phenylephrine-induced phosphorylation of ERK1/2 kinases. This study also suggested that silibinin might attenuate the phenylephrine-induced hypertrophic response of H9c2 cells mainly by virtue of its antioxidant properties involving the inhibition of the intracellular signaling pathways mediated by ERK1/2 MAPKs and Akt [131].

#### 8.2.4.7 Silymarin Protects Liver from Doxorubicin-Induced Oxidative Damage

Silymarin is well known for its ability to interfere with apoptotic signaling while acting as an antioxidant [132]. This *in vivo* study was designed to explore the hepatotoxic potential of the well-known anticancer drug, Dox, and in particular whether pretreatment with silymarin can prevent hepatotoxicity by reducing Dox-induced oxidative stress and regulating the expression of apoptotic signaling proteins such as Bcl-xL, and by minimizing liver cell death via either apoptosis or necrosis. Male ICR mice were taken for experimental purpose. It was shown that Dox alone induced frank liver injury (N50-fold increase in serum ALT) and oxidative stress (N20-fold increase in MDA) as well as direct damage to DNA (N15-fold increase in DNA fragmentation) compared to normal animals. Silymarin has been shown to exert its ameliorative properties by giving protection against all of the aforementioned disorders. In short, these results demonstrated that silymarin (i) reduced delayed onset, or prevented toxic effects of Dox which are primarily mediated by hydroxyl radical production, (ii) performed as an antioxidant molecule, (iii) protected the genome integrity, and (iv) antagonized apoptotic and necrotic cell death while increasing antiapoptotic protein levels and minimizing the release of cytochrome *c* from mitochondria of liver. These observations demonstrate the protective actions of silymarin in liver and indicate the possibility of such protection to other organs as well [133].

#### 8.2.4.8 Silymarin and Hepatoprotection

One of the major causes of hepatotoxicity is oxidative stress-mediated tissue damage which can be prevented by the use of silymarin. It has been [134] shown that silibinin preserves the functional and structural integrity of hepatocyte membranes by preventing alterations of their phospholipid structure resulted from the use of carbon tetrachloride and by restoring ALP activity. An earlier report suggests the mechanisms of action of silymarin that provide protection against lipid peroxidation and the hepatotoxicity of carbon tetrachloride in mice [132]. The authors conclude that silymarin provides the protective action by reducing carbon tetrachloride-induced metabolic activation and by acting as an antioxidant that prevents chain rupture. Other investigators have shown that silymarin gives



hepatoprotection against specific injury induced by microcystin (a hepatotoxin), paracetamol, halothane, and alloxan in several experimental models [135–137].

#### 8.2.4.9 Stimulation of Liver Regeneration

Some *in vivo* and *in vitro* experiments were performed on rat liver to find out whether silymarin has any capacity in the process of liver regeneration. Results show that silibinin, the most active component of silymarin, produced a significant increase in the formation of ribosomes and DNA synthesis, as well as an increase in protein synthesis [138]. Interestingly, the increase in protein synthesis was induced particularly in injured livers, not in healthy controls [139] and the mechanism of this unique function is still unclear. Some recent data suggest that the physiological regulation of RNA polymerase I at specific binding sites stimulates the formation of ribosomes [140]. In a rat model of experimental hepatitis caused by galactosamine, treatment with intraperitoneal silymarin almost prevented the inhibitory effect of galactosamine on the biosynthesis of liver proteins and glycoproteins [141]. These data again support the results of previous experiments in a similar model of acute hepatitis in the rat, in which silymarin protected hepatic structures, liver glucose stores, and enzyme activity from galactosamine induced injury [142]. The capacity of silymarin to stimulate protein synthesis has also been confirmed in neoplastic cell lines [139].

#### 8.2.5

##### Curcumin

Curcumin [1,7-bis (4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione] is a well-known polyphenolic compound isolated from the rhizomes of *Curcuma longa* (turmeric). Turmeric has been used for at least 2500 years, mostly in Asian countries, as an anti-inflammatory agent to treat gas, colic, toothaches, chest pain, and menstrual difficulties [143]. Turmeric has also been shown to heal pain, stomachache, liver diseases, jaundice, and so on, and is considered as an important ingredient in both Ayurvedic and Chinese herbal medicine [144]. It is a good antibacterial agent for those chronically weak or ill, with a name in Sanskrit that means “germicide.”

Curcumin has been shown to exert a wide range of pharmacological activities including antibacterial [145] anti-inflammatory, anticancer, antioxidant [146], hypoglycemic [147], anti-atherosclerotic, antimicrobial [148], and wound healing [144, 149] activities. These activities of curcumin have been predicted to be responsible for its complex molecular structure and chemical features, as well as its ability to interact with multiple signaling molecules. How a single agent can show such diverse effects has been a closed book over the years both in the fields of basic sciences and clinical practice. However, increasing evidence with human participants has indicated curcumin’s ability to modulate multiple cell signaling

molecules such as apoptotic proteins, COX-2, NF- $\kappa$ B [150], STAT3 (signal transducers and activators of transcription), IKK $\beta$  (inhibitor of nuclear factor kappa-B kinase subunit beta), IL-1 $\beta$ , IL-6 [151], endothelin-1, C reactive protein (CRP) [152], GST [153], PSA, pro-inflammatory cytokines (TNF- $\alpha$ ) [154], VCAM, prostaglandin E2, MDA, GSH [155], pepsinogen, phosphorylase kinase (PhK) [156], creatinine, transferrin receptor, total cholesterol, transforming growth factor (TGF)- $\beta$ , triglyceride, HO-1 (heme oxygenase 1) [157], antioxidants, AST (aspartate transaminase), and ALT.

#### 8.2.5.1 Chemical Composition of Turmeric

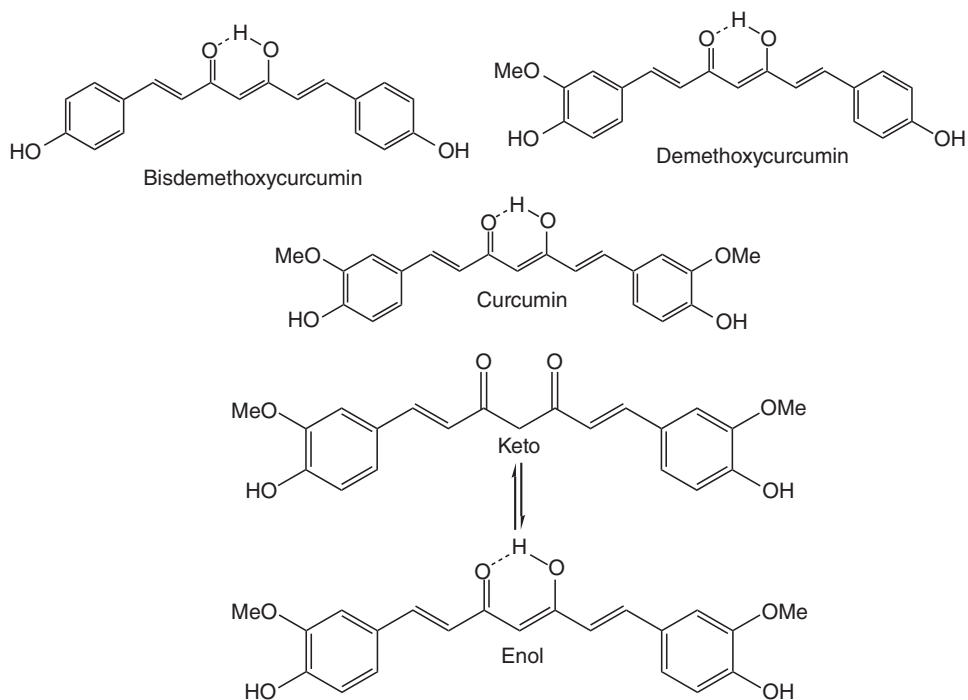
Although commercial curcumin is a mixture of three curcuminoids: curcumin (71.5%), demethoxycurcumin (19.4%), and bisdemethoxycurcumin (9.1%) [158] turmeric itself is a complex mixture of more than almost 200 compounds such as diarylheptanoids and diarylpentanoids, phenolic compounds, different types of terpenes, steroids, and some alkaloids. Among these compounds, curcumin, which is the main bioactive compound, belongs to the group diarylheptanoids.

#### 8.2.5.2 Metabolism of Curcumin

Numerous studies suggested that curcumin undergoes metabolism upon oral administration in animals and almost every metabolite produced during metabolism has some beneficial effect apart from its antioxidant property. Curcumin firstly undergoes metabolic O-conjugation into curcumin glucuronide and curcumin sulfate and bioreduction to tetrahydrocurcumin (THC), hexahydrocurcumin, octahydrocurcumin, and hexahydrocurcuminol in rats and mice *in vivo* [152, 157, 158] and in human and rat hepatocytes in culture. Reduced curcumin is subjected to glucuronidation into curcumin glucuronide, dihydro-curcumin-glucuronide, THC-glucuronide, and curcumin sulfate [159]. THC prevented radiation induced lipid peroxidation [160] and induced antioxidant enzymes activity *in vitro* [161]. In a rat model, dietary administration of THC reduced polyps formation in azoxymethane-induced colon carcinogenesis [162]. Hexahydrocurcumin, another metabolite, has been shown to reduce the ability to inhibit COX-2 expression compared to curcumin [157] and induce cell cycle arrest in human colorectal cancer SW480 cells [163]. In another study, it has been shown that the free radical-scavenging activity of octahydrocurcumin is higher than that of curcumin [164]. However, curcumin metabolite and curcumin sulfate have been shown to have less biological activity compared to curcumin [157]. Therefore, these studies indicate that during and after metabolism of curcumin, different metabolites are produced which show biological activities that are mostly more advantageous than those of curcumin itself.

#### 8.2.5.3 Antioxidant Activity of Curcumin

The way curcumin exerts its antioxidant mechanisms has been well established by laser flash photolysis and pulse radiolysis [165]. It has been demonstrated that the keto-enol-enolate equilibrium of the heptadienone moiety of curcumin is mainly



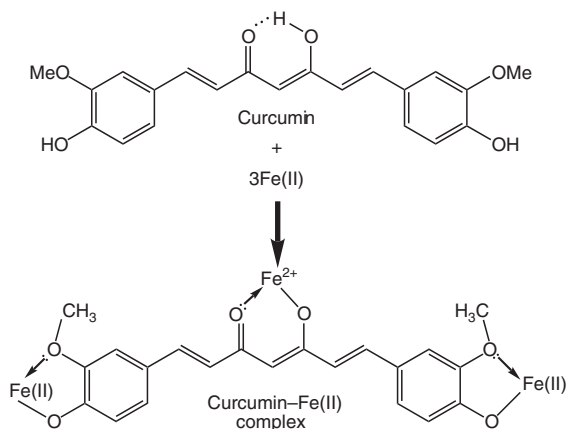
**Figure 8.11** Plausible key regions of curcumin for free radical scavenging and antioxidant activities.

responsible for its physicochemical and antioxidant properties. In neutral and acidic pH, the keto form dominates, and curcumin behaves as an extraordinarily potent H-atom donor (Figure 8.11). Curcumin, as with other phenolic antioxidants, usually scavenges free radicals by an electron-transfer mechanism which is determined by the one-electron oxidation potential of the parent antioxidants, [165]. In another study, the antioxidant activity of curcumin was demonstrated by inhibition of controlled initiation of styrene oxidation [166].

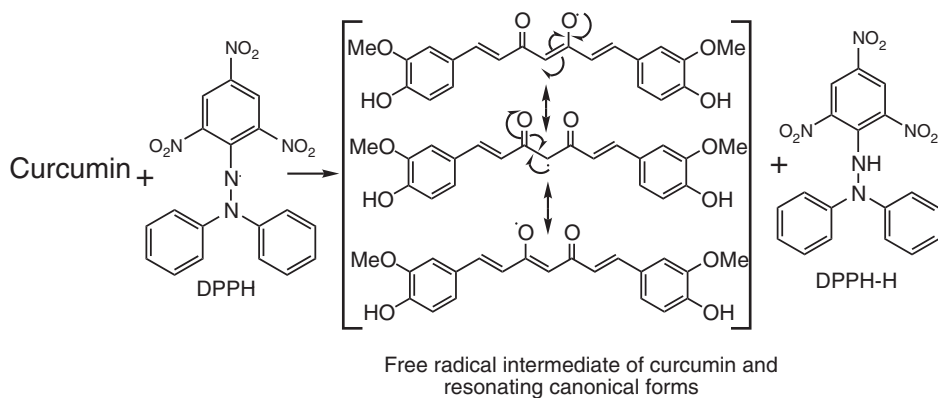
The data reveal that curcumin has a marked capacity for iron binding, indicating that its main action as a peroxidation inhibitor may be related to its iron-binding capacity [167] (Figure 8.12).

Biological systems can produce hydrogen peroxide [168]. It can cross membranes and may slowly oxidize a number of compounds. It is known that  $\text{H}_2\text{O}_2$  is toxic and induces cell death *in vitro* [169]. It is used in the respiratory burst of activated phagocytes. Curcumin however, has effective hydrogen peroxide-scavenging activity.

The free radical chain reaction is mainly instrumental in lipid peroxidation. Free radical scavengers may directly react with and thereby quench peroxide radicals to terminate the peroxidation chain reaction [170]. Curcumin has also



**Figure 8.12** Fe(II)-chelating property of curcumin.



**Figure 8.13** Free radical-scavenging property of curcumin.

been shown to possess such radical scavenging property in several experiments. Curcumin was found to be an extremely effective antioxidant in *in vitro* assays including DPPH• (Figure 8.13), 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS<sup>•+</sup>), *N,N*-dimethyl-*p*-phenylenediamine dihydrochloride (DMPD<sup>•+</sup>) [171], and hydrogen peroxide-scavenging and metal-chelating activities when compared to standard antioxidant compounds such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT),  $\alpha$ -tocopherol, and trolox [172].

**Curcumin Effectively Lowered Oxidative Stress Markers** For this purpose, [173], U937 monocytes were cultured with control (7 mM) and high glucose (35 mM) in the absence or presence of curcumin (0.01–1  $\mu$ M) at 37 °C for 24 h. In addition, diabetes was induced in Sprague-Dawley rats by injection of STZ (i.p.,

65 mg kg<sup>-1</sup> BW). Control buffer, olive oil, or curcumin (100 mg kg<sup>-1</sup> W) supplementation was given by gavage daily for 7 weeks. At the end of the experiment, blood was collected by heart puncture with light anesthesia. Results show that the effect of diabetes on lipid peroxidation, IL-6, IL-8, MCP-1, and TNF- $\alpha$  secretion was reduced effectively by curcumin treatment in cultured monocytes. Results were also the same in the case of an *in vivo* study. In the rat model, diabetes caused a significant increase in blood levels of IL-6, MCP-1, TNF- $\alpha$ , glucose, HbA<sub>1C</sub>, and oxidative stress. All of these effects were significantly decreased by curcumin supplementation. Thus, the authors claimed that curcumin could decrease markers of vascular inflammation and oxidative stress levels in both *in vitro* and *in vivo* studies and suggested that curcumin supplementation could reduce glycemia and the risk of vascular inflammation effectively in diabetes. HbA<sub>1C</sub>

#### 8.2.5.4 Diabetes and Curcumin

Oxidative stress is currently suggested as one of the most critical underlying causes in diabetes and diabetic complications [174]. During diabetes, hyperglycemia causes increased production of free radicals, especially ROS, almost in all tissues from glucose auto-oxidation and protein glycosylation [175]. The increase in the level of oxygen free radicals in diabetic pathogenesis could be due to their increased production and/or decreased destruction by nonenzymic and enzymic antioxidants such as reduced GSH, CAT, SOD, peroxidase, and so on. The level of these antioxidant molecules and the availability of glucose in various types of cells together influence the susceptibility of various tissues to oxidative stress [176]. In order to fight oxidative stress, interest has been focused mainly on easily available, inexpensive antioxidant molecules like curcumin. It has been shown that curcumin could protect against many types of diabetes-related disorders.

**Effect of Curcumin on Glycemia in Animal Model of Diabetes** The most commonly used animal in studying the effect of curcumin is the rat. Various diabetic rat models were employed to investigate the effect of curcumin on diabetes. In alloxan-induced diabetic rats, STZ-induced rat models, and STZ-nicotinamide-induced rat models [35], oral supplementation of various dosages of curcumin [177–180] were able to inhibit body weight loss and reduce the levels of glucose, hemoglobin (Hb), and glycosylated hemoglobin (HbA<sub>1C</sub>) in blood [177]. Insulin sensitivity was also improved. Moreover, oral delivery of turmeric aqueous extract or curcumin [181] showed a marked reduction in blood glucose in STZ-induced diabetes model in rats. In high fat diet (HFD)-induced insulin resistance and type 2 diabetic rat models, oral delivery of curcumin resulted in an antihyperglycemic effect and improved insulin sensitivity [182]. Dietary curcumin also showed the same type of hypoglycemic effect [183]. Diabetic mice models were also employed to investigate the plausible effect of curcumin on hyperglycemia. In STZ-induced Swiss

diabetic mice, intraperitoneal injection of curcumin markedly prevented hyperglycemia, glucose intolerance, and hypoinsulinemia [184]. The result was also as expected in case of HFD-induced diabetic mouse model [185].

The possible mechanism behind the antidiabetic effects of curcumin was explained as follows. Firstly, curcumin has been shown to attenuate TNF- $\alpha$  levels [184] and plasma free fatty acids (FFAs) [182]. It can also inhibit NF- $\kappa$ B activation and activate the function of Nuclear factor (erythroid-derived 2)-like 2 (Nrf2) as well [185]. Curcumin can also inhibit protein carbonyl [186], lipid peroxidation, and lysosomal enzyme activities (such as *N*-acetyl- $\beta$ -D-glucosaminidase,  $\beta$ -D-glucuronidase,  $\beta$ -D-galactosidase) [183]. Moreover, curcumin can decrease the levels of TBARSs and the activity of sorbitol dehydrogenase (SDH) [187]. Secondly, curcumin can also elevate plasma insulin level and increase lipoprotein lipase (LPL) activity [188]. Last but not the least, curcumin is involved in the process of activation of liver enzymes, and directly deals with glycolysis, gluconeogenic, and lipid metabolic processes [188].

**Curcumin and Diabetes-Associated Liver Disorders** Diabetic patients often suffer from various liver disorders [189] mainly because of increasing oxidative stress and curcumin has been shown to prevent those disorders [190]. Improved lipidemia by curcumin is attributed to the induction of PPAR- $\gamma$  activity [147]. Curcumin is also involved in normalization of enzymatic activities [188] related to lipid peroxidation [181], glucose metabolism, and antioxidant enzymes (superoxide dismutase and catalase (SODC) and GPx). Moreover, many other enzymes such as hepatic glucose-regulating enzymes (G6Pase, phosphoenolpyruvate carboxykinase (PEPCK), hepatic lipid-regulating enzymes (fatty acid synthase, 3-hydroxy-3-methylglutaryl coenzyme reductase, and acyl-CoA, cholesterol acyltransferase) [191] are acted upon by curcumin and mostly all the effects are beneficial. AMP-activated protein kinase (AMPK) acts as an energy regulator that controls whole-body glucose homeostasis in the liver and other key tissues in type 2 diabetes [192]. G6Pase and PEPCK are two key enzymes involved in hepatic gluconeogenesis. Increased expression of G6Pase and PEPCK lead to deleterious effects in type 2 diabetes [193]. Earlier, studies showed that curcumin inhibited PEPCK and G6Pase activities in H4IIE rat hepatoma and Hep3B human hepatoma cells [194]. Curcumin could also increase phosphorylation of AMPK [195] and its downstream target acetyl-CoA carboxylase (ACC) in H4IIE and Hep3B cells.

#### 8.2.5.5 Efficacy of Biodegradable Curcumin Nanoparticles in Delaying Cataract in Diabetic Rat Model

Earlier reports suggested that feeding of dietary antioxidant curcumin was effective in delaying STZ-induced diabetic cataract in rats mainly through its antioxidant property [196]. Moreover, they also found that curcumin inhibited diabetes-induced expression of vascular endothelial growth factor (VEGF) in

rat retina [197] and the enzyme aldose reductase (AR) in the eye lens of rat [198]. The dose used in the study [196] was equivalent to or slightly higher than the amount of average daily intake (ADI) of turmeric [199] in food. Although dietary supplementation of curcumin offered some benefits in preclinical studies, the translation has been very poor and the doses used in clinical trials are not feasible for practical purpose mainly because of the lack of its extensive intestinal and hepatic metabolic biotransformation resulting in poor oral bioavailability [200–203]. Therefore, recent focus is on its delivery methods with a view to improve the therapeutic efficacy [204]. Use of polymeric nanoparticles as oral delivery vehicles is being explored extensively now for pharmaceutically challenging compounds [205, 206]. It has been shown that encapsulating curcumin in polymer nanoparticles could improve peroral bioavailability of curcumin at least nine times compared to that of plain curcumin [207].

### 8.3

#### Concluding Remarks

In this chapter, we discussed a variety of bioactivities, multifunctional therapeutic applications, and signaling properties of naturally occurring antioxidants. It is clear that these antioxidants play a number of beneficial roles in oxidative stress-induced organ dysfunctions. This chapter also unravels the potential use of naturally occurring antioxidants as novel promising therapeutic strategies.

#### Abbreviations

AGEs	advanced glycation end products
ALP	alkaline phosphatase
ALT	alanine aminotransferase
AMPK	AMP-activated protein kinase
APAP	Acetaminophen
AST	aspartate transaminase
BUN	blood urea nitrogen
CAT	catalase
COX-2	cyclooxygenase-2
DPPH	1,1-diphenyl-2-picrylhydrazyl
ERK	extracellular signal-regulated kinase
GLUT	glucose transporter
GPx	glutathione peroxidase
GSH	Glutathione
4-HA	4-Hydroxyalkenals
GST	glutathione S-transferase

HDL	high-density lipoprotein
HO-1	heme oxygenase 1
IKK $\beta$	inhibitor of nuclear factor kappa-B kinase subunit beta
IL-1	interleukin 1
LPS	lipopolysaccharide
JNK	c-jun N-terminal kinase
MAPK	mitogen-activated protein kinase
MCP-1	monocyte chemotactic protein-1
MDA	malondialdehyde
NF- $\kappa$ B	nuclear factor $\kappa$ B
Nrf2	Nuclear factor (erythroid-derived 2)-like 2
O <sub>2</sub> <sup>•-</sup>	superoxide anion
•OH	hydroxyl radical
RNS	reactive nitrogen species
ROS	reactive oxygen species
SOD	superoxide dismutase
STAT3	signal transducers and activators of transcription
STZ	streptozotocin
TNF $\alpha$	tumor necrosis factor $\alpha$
TPA	12-O-tetradecanoylphorbol-13-acetate
VEGF	vascular endothelial growth factor

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## 9

# Isoquinoline Alkaloids and Their Analogs: Nucleic Acid and Protein Binding Aspects, and Therapeutic Potential for Drug Design

Gopinatha S. Kumar

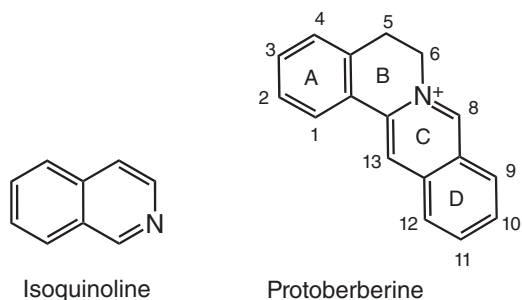
### 9.1

#### Introduction

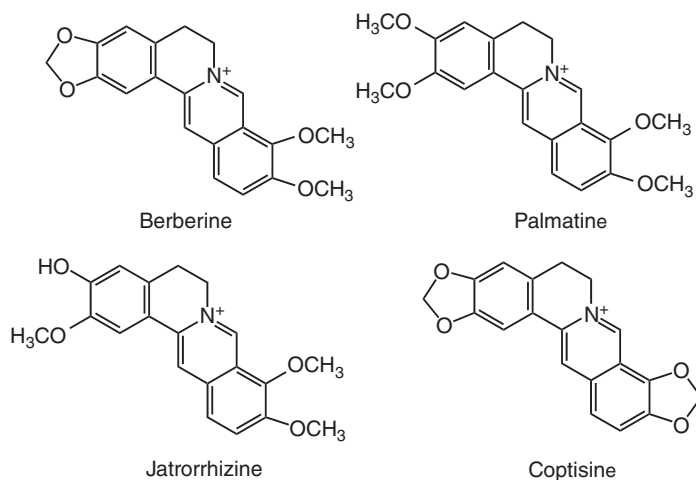
Alkaloids are an extensively distributed important group of natural products mostly of plant origin. The precise role of alkaloids in plants is still controversial, but it is generally believed that in addition to being secondary metabolites they help to protect the plants in their defense mechanism. It is also suggested that alkaloids may serve as a storage form of nitrogen and act as protective agents against harmful effects of ultraviolet light. Most alkaloids are bitter and have very unpleasant taste.

Alkaloids have been of remarkable interest and attraction to human life for a long time both in terms of their high medicinal value and cultural utility. They have been extensively used in herbal folk medicines in many parts of the world, such as in traditional Chinese and native North American medicine, and Indian ayurvedic systems. Of the many alkaloid groups, isoquinolines form a large and abundant one in the plant kingdom. Isoquinolines are derived biosynthetically from the amino acid tyrosine generating the fundamental intermediate 3,4-dihydroxyphenylethylamine (dopamine) and carbonyl units of different origin. It is estimated that more than 4000 compounds of this structure are known so far.

The isoquinoline moiety (Scheme 9.1) is the basic building block for many subgroups of alkaloids such as protoberberines, benzophenanthridines, and benzylisoquinolines. Structurally, the protoberberines are built on 5,6-dihydrodibenzo [*a,g*]quinolizinium with a positively charged nitrogen atom. Protoberberines are nonplanar due to an unsaturated B ring. Ring A is tilted by about 22° to the plane of the C–D rings (Scheme 9.1). The protoberberine alkaloids are one of the most extensively distributed groups of the isoquinoline alkaloids. They are present in at least 10 plant families such as Alangiaceae, Annonaceae, Berberidaceae, Fabaceae, Fumariaceae, Lauraceae, Menispermaceae, Papaveraceae, Ranunculaceae, and Rutaceae.



**Scheme 9.1** Structure of isoquinoline and protoberberine moieties.



**Scheme 9.2** Few important natural protoberberine alkaloids.

Berberine, palmatine, jatrorrhizine, and coptisine (Scheme 9.2) are the main members of the 2,3,9,10-tetra substituted quaternary protoberberine alkaloids. Many other representatives of the isoquinoline and benzylisoquinoline alkaloids such as the antitussive and anticancer drug noscapine, and the antimicrobial agent sanguinarine, are also distributed in many botanical families. Others are more restricted to certain plant families such as pelletine in the Cactaceae, cularine in the Fumariaceae, and morphine in the Papaveraceae families. Morphine is still the most extensively used painkiller in medicine. Heroin is the diacetyl derivative of morphine. Other isoquinoline alkaloids such as codeine or papaverine are also considered useful as medicinal drugs. The use of traditional medicine of natural origin has tremendous potential for the treatment of chronic disorders, as synthetic drugs may cause undesirable and unknown side effects that may lead to more complications. Some general aspects of the quaternary iminium protoberberine alkaloids have been reviewed recently [1].

In this chapter, the current knowledge on the *in vitro* interactive aspects of a few important isoquinoline alkaloids, namely, berberine, palmatine, jatrorrhizine, and coptisine, and their many recently known synthetic analogs with various nucleic acid structures and proteins is reviewed to understand their therapeutic potential and efficacy for drug design.

## 9.2

### Isoquinoline Alkaloids and Their Analogs

#### 9.2.1

##### Berberine

Berberine ((5,6-dihydro-9,10-dimethoxybenzo[*g*]-1,3-benzodioxole 5,6-aquino-lizum) (Scheme 9.2)) is naturally found in rhizomes of *Berberis* and *Coptis*, and has been used in clinical antibacterial treatments for a long time. Berberine has a polycondensate system with partial saturation in the ring B (Scheme 9.1) that renders it buckled. This structure is highly stable and is resistant to pH and temperature changes. Berberine displays a wide range of biochemical and pharmacological activities. A large number of papers on its biological activity have been published [1, 2]. Berberine was used in China as a nonprescription drug for diarrhea for decades [3, 4]. It was also used as an antibiotic and its antibacterial action was demonstrated against a variety of bacterial species several years ago [5, 6]. Berberine possesses various other biological activities such as antimicrobial, [7, 8] antifungal [9], antimalarial [10, 11], antileishmanial [12], antimutagenic [13], and anticancer activities [14–16]. More recently, its anti-Alzheimer's disease [17] and cholesterol lowering effects [18] have been revealed. It is noteworthy that berberine has been suggested to be a novel cholesterol-lowering drug working through a unique mechanism distinct from statins. The hypoglycemic effect of berberine has also been revealed in many clinical studies; the data and probable mechanisms have been reviewed [19–21]. Berberine is of particular interest and may provide extra advantage in cases of diabetic cardiovascular complications because of its cholesterol-lowering property. The alkaloid is also characterized by its antioxidant and aldose reductase inhibitory activities, through which it alleviates diabetic nephropathy in animal models. The important advantage of berberine is its safety at therapeutically active concentrations;  $IC_{50}$  values of 228.93 and 15.88  $mg\ kg^{-1}$  were observed in rabbits and guinea pigs, respectively [22]. Another report suggests the  $LD_{50}$  values of berberine to be 9.03 and 57.6  $mg\ kg^{-1}$ , respectively, in mice models when administered by intravenous and intraperitoneal methods [23]. A recent review has highlighted the various biological activities of berberine [24].

Nucleic acids, particularly deoxyribonucleic acid (DNA), have been thought to be one of the potential druggable biotargets for the high anticancer activity of

berberine. Studies on the binding of berberine to DNA have been known since late 1960. DNA interaction aspects of protoberberine alkaloids have been reviewed in great detail recently [25–28]. Studies on the interaction with ribonucleic acids have been initiated only very recently [29–31]. Many berberine analogs have recently been synthesized and studies of nucleic acid–binding with these analogs have just begun.

#### 9.2.1.1 Interaction of Berberine with Deoxyribonucleic Acids

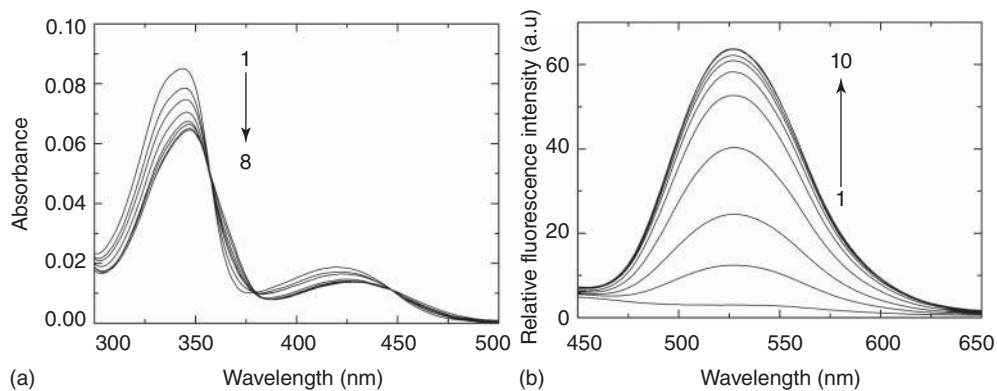
The interaction of berberine with DNA was first reported by Krey and Hahn in 1969. They had suggested the formation of an intercalation complex between berberine and calf thymus DNA from a variety of physicochemical experiments [32]. Subsequently, Wilson and coworkers [33] investigated the interaction using natural and closed superhelical DNAs, the results of which predicted a partial intercalation mode for berberine due to its buckled geometry.

A detailed investigation on the interaction of berberine with various DNAs was undertaken by Maiti and colleagues [34–38]. From extensive spectroscopic and hydrodynamic studies, using a variety of natural and synthetic DNAs of varying base composition and sequence, and closed plasmid DNAs, it was postulated that berberine binds to DNA by partial intercalation.

The conclusion of intercalation was proposed, essentially based on hypochromic and bathochromic effects in absorption, large enhancement of the rather weak berberine fluorescence, strong viscosity changes of linear DNA, and the unwinding–rewinding effects in the closed superhelical DNA. The affinity of berberine was established to be of the order of  $10^5 \text{ M}^{-1}$ . The affinity of berberine to poly(dA-dT)·poly(dA-dT) was found to be the highest among the many natural and synthetic DNAs investigated. Furthermore, the studies of Maiti and Kumar spanning a period of three decades established the base specificity and sequence selectivity of berberine, and the thermodynamics of its interaction [35–38]. Berberine, although optically inactive, on binding to DNA acquired induced optical activity in its visible absorption spectral region [36, 37]. The studies of Maiti and colleagues were also supported by nuclear magnetic resonance (NMR) results [39, 40]. The competition dialysis experiments of Ren and Chaires [41] also supported the adenine-thymine (AT) specificity of berberine originally proposed by Maiti and Kumar.

A typical absorption and fluorescence spectral titration of berberine with DNA is presented in Figure 9.1. Berberine has thus been established to be an AT (adenine-thymine) base pair–specific DNA binder. However, the proposed intercalation model of Wilson, Maiti, and their colleagues was not supported by the NMR study of Mazzini *et al.* [42], who instead proposed a groove-binding model for the berberine–DNA complexation. The controversy was, however, put to rest by the recent X-ray studies [43] on oligonucleotide complexes that underscored the intercalative binding originally proposed by the groups of Wilson and Maiti.



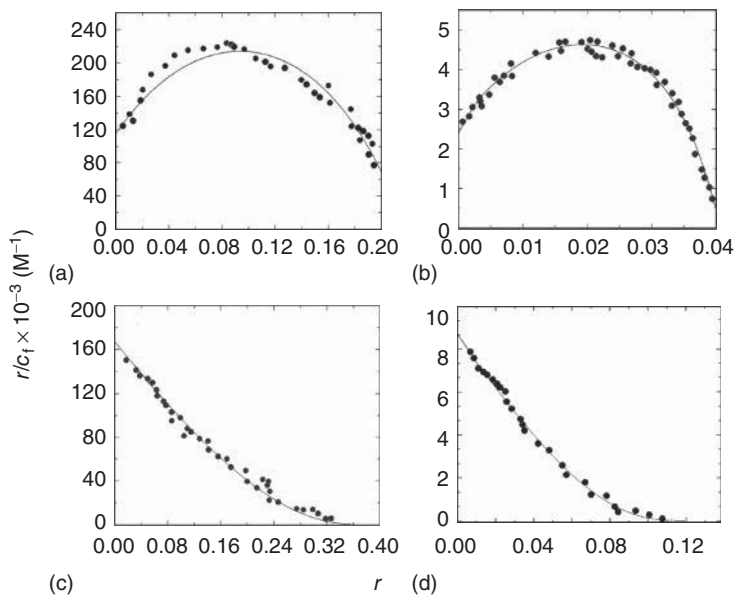


**Figure 9.1** (a) Representative absorption spectral changes of berberine on complexation with calf thymus DNA. Curves 1–8 denote berberine ( $3.8 \mu\text{M}$ ) treated with 0, 3.2, 15.5, 30, 56.7, 78.2, 97.2, and  $121 \mu\text{M}$  of DNA. (b) Spectrofluorimetric titration of berberine treated with increasing concentration of calf thymus DNA. Curves 1–10 denote berberine ( $3.0 \mu\text{M}$ ) treated with 0, 2.9, 26.8, 35.8, 50, 85.1, 100, 118, 144.6, and  $162.6 \mu\text{M}$  of DNA.

New aspects on berberine–DNA complexation were subsequently elucidated. Bhadra *et al.* [44] reported that berberine binds to B-form DNA cooperatively. From extensive studies with several synthetic DNAs, it was shown that the cooperativity of binding was also dependent on the sequence of base pairs. Berberine exhibited cooperative binding to alternating polypurine–polypyrimidine sequences while homopurine–homopyrimidine sequences bound noncooperatively. The cooperative and noncooperative Scatchard plots of berberine binding to these sequences are presented in Figure 9.2. The binding parameters of berberine to various DNAs reported are collated in Table 9.1. The thermodynamics of the interaction was also extensively studied employing microcalorimetric techniques. It was suggested that berberine exhibited entropy-driven binding to polynucleotides with AT sequences and hydrophobic forces were proposed to be involved significantly in these interactions. Table 9.2 presents the thermodynamic parameters of the complexation of berberine with various DNAs. The studies on berberine–DNA complexation thus present intercalative binding with moderate affinity and high specificity to the AT base pair sequences with the B-form structure. The binding was found to induce significant conformational changes in the DNA and the bound alkaloid molecules acquired induced optical activity, reiterating the strong interaction of the dipole moments of the DNA and the alkaloid molecules.

#### 9.2.1.2 DNA Binding of Berberine Analogs

The bioavailability of berberine is quite low, the DNA binding affinity is moderate, and therefore, efforts to improve the bioavailability and enhance the binding affinity were undertaken through the use of novel delivering systems and synthesizing



**Figure 9.2** Cooperative and noncooperative Scatchard plots for the binding of berberine to (a) poly(dA-dT)·poly(dA-dT), (b) poly(dG-dC)·poly(dG-dC), (c) poly(dA)·poly(dT), and (d) poly(dG)·poly(dC). (Reprinted from Bhadra *et al.*, 2008 [44] with permission from Elsevier.)

**Table 9.1** Binding parameters of berberine–DNA complexation from spectroscopy and calorimetry data at 20 °C.

DNA/polynucleotide	% AT content	Mode of binding	Binding constant ( $10^5 \text{ M}^{-1}$ )	
			Spectroscopy	Calorimetry
<i>Clostridium perfringens</i>	73	Noncooperative	$0.86 \pm 0.15$	$0.90 \pm 0.05$
Calf thymus	58	Cooperative	$0.13 \pm 0.06$	$0.12 \pm 0.02$
<i>Escherichia coli</i>	50	Cooperative	$0.12 \pm 0.03$	$0.11 \pm 0.03$
<i>Micrococcus lysodeikticus</i>	28	Noncooperative	$0.09 \pm 0.06$	$0.09 \pm 0.01$
Poly(dA-dT)·poly(dA-dT)	100	Noncooperative	$1.70 \pm 0.40$	$1.11 \pm 0.15$
Poly(dA)·poly(dT)	100	Cooperative	$1.15 \pm 0.16$	$0.08 \pm 0.01$
Poly(dG-dC)·poly(dG-dC)	0	Noncooperative	$0.08 \pm 0.01$	$0.08 \pm 0.01$
Poly(dG)·poly(dC)	0	Cooperative	$0.02 \pm 0.01$	nd

nd: no data.

new derivatives. Berberine solubility could also be significantly enhanced for improved drug bioavailability by forming inclusion complexes [45–47]. A recent review highlights the importance of structural modifications in berberine for enhanced biological activities [48]. Berberrubine is an analog of berberine with an OH group attached at the 9-position (Scheme 9.3). The interaction of this

**Table 9.2** Thermodynamic parameters of the complexation of berberine with various DNAs at 20 °C.

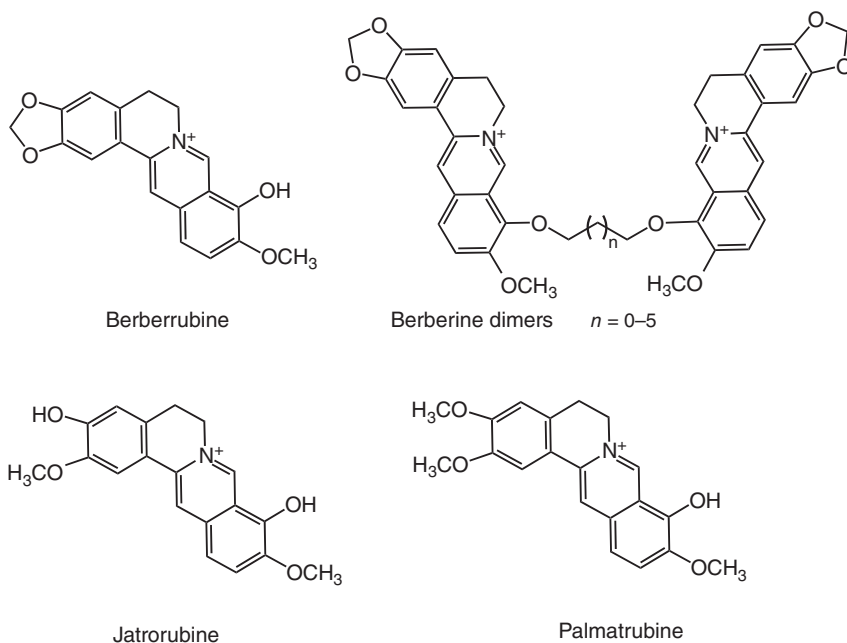
DNA/polynucleotide	$\Delta G^\circ$	$\Delta H^\circ$	$T\Delta S^\circ$
<i>Clostridium perfringens</i>	-6.23	-1.80	4.43
Calf thymus	-5.50	-2.01	3.34
<i>Micrococcus lysodeikticus</i>	-5.33	-4.92	0.80
Poly(dA-dT)·poly(dA-dT)	-6.81	-1.02	5.71
Poly(dA)·poly(dT)	-6.16	+1.56	7.71
Poly(dG-dC)·poly(dG-dC)	-4.91	-9.52	4.61

$\Delta G^\circ$  is the standard molar Gibbs energy change,  $\Delta H^\circ$ , the standard molar enthalpy changes, and  $T\Delta S^\circ$  is the entropy contribution to the binding.

compound in comparison with berberine and other analogs with many double stranded oligodeoxyribonucleotides was studied by electrospray ionization mass spectral technique [49, 50]. A stronger binding of berberrubine compared to berberine along with the AT base specificity was also reported [50]. Chen *et al.* synthesized a number of berberine dimers with alkyl chains of varying lengths (Scheme 9.3). They studied the binding to DNA in comparison with berberine [51]. It was reported that these dimers bound to DNA and oligonucleotides with 100-fold higher affinity compared to berberine, and the highest association constant was obtained with berberine dimer with a propyl alkyl chain spacer. Formation of strong intercalation complexes of these dimers with DNA have also been suggested from ethidium displacement studies.

As early as in the year 2000, Krishnan and Bastov [52] had suggested that 9-substitution of the isoquinoline chromophore has a major influence in the DNA topoisomerase II inhibition mechanism presumably by facilitating better interaction with enzyme and/or enzyme–DNA complexes. Structure–activity relationship of protoberberines also suggested the 9-position to be an important determinant in expressing enhanced biological activities [53]. Another important position on the isoquinoline moiety is the 13-position. Long carbon-chain substituents at the C-13 and 9-positions of berberine were suggested to have better antitumor activity and less toxic effects [54, 55]. A number of berberine analogs with various substitutions at the 9 and 13 positions of the chromophore have been synthesized and studied for enhanced DNA-binding activity [56–60]. The structures of some of these analogs are presented in Scheme 9.4.

Characterization of many quaternary berberines modified at the C-9 and C-13 positions showed that they bind to DNA with higher affinity. The notable achievements among these studies are in the interaction of the analogs with 9-amino alkyl ether and those of different alkyl side chain lengths with phenyl and diphenyl groups [58–60]. These analogs exhibited entropy-driven binding compared to the enthalpy-driven binding of berberine, revealing the critical role of the side chains in the intercalation process. It was found that for amino alkyl analogs,



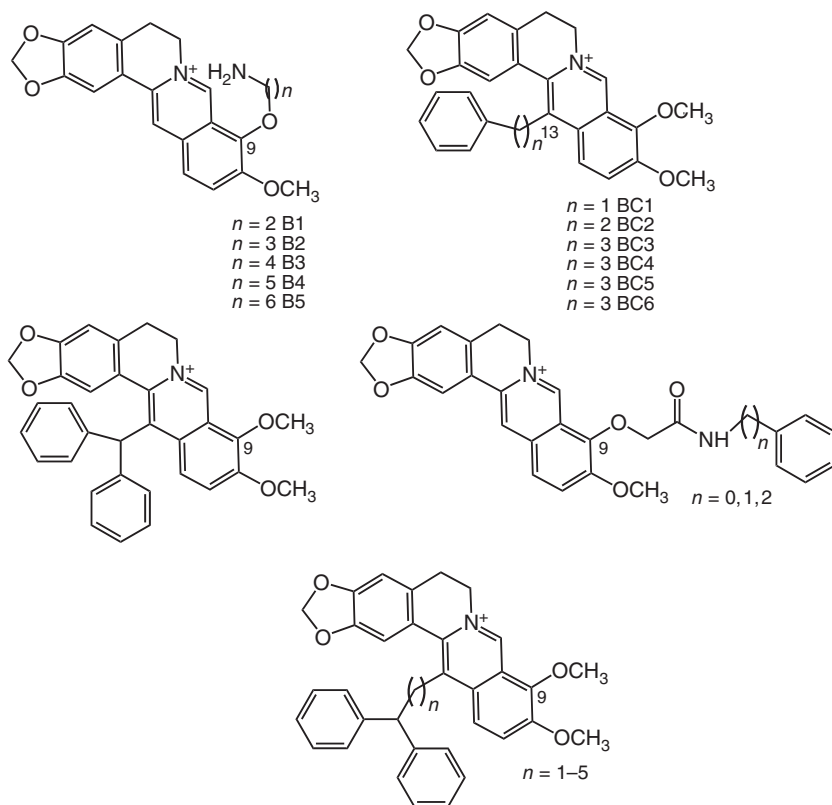
**Scheme 9.3** Structure of berberrubine, berberine dimers, jatrorubine, and palmatrubine.

the binding affinity increased as the alkyl chain length enhanced (BC1-3), while a critical length of the alkyl chain with  $(\text{CH}_2)_3$  effected maximum affinity for phenyl alkyl and biphenyl compounds. Variation in the binding affinity and thermodynamic parameters with the alkyl group for the 13-substituted phenyl alkyl berberines are shown in Figure 9.3. Furthermore, chain-length enhancements beyond  $(\text{CH}_2)_3$  (BC4-6) led to decreased affinity in the latter cases. Significant features of the interaction profile of these analogs is their noncooperative binding, higher stabilization of the DNA, stronger AT specificity, stronger conformational perturbations and overall better intercalation efficacy [59, 60].

Basu *et al.* synthesized 9-*O-N*-aryl/aryl-alkyl amino carbonyl methyl-substituted analogs (Scheme 9.4) of berberine and studied the DNA-binding activity and observed remarkable amplification in the binding affinities compared to berberine [61]. These analogs exhibited noncooperative binding that was entropy driven as in the case of the phenyl and biphenyl analogs (see the preceding). This study also suggested the binding affinity to be dependent critically on the length of the alkyl chain.

### 9.2.1.3 Binding of Berberine and Analogs to Polymorphic DNA Conformations

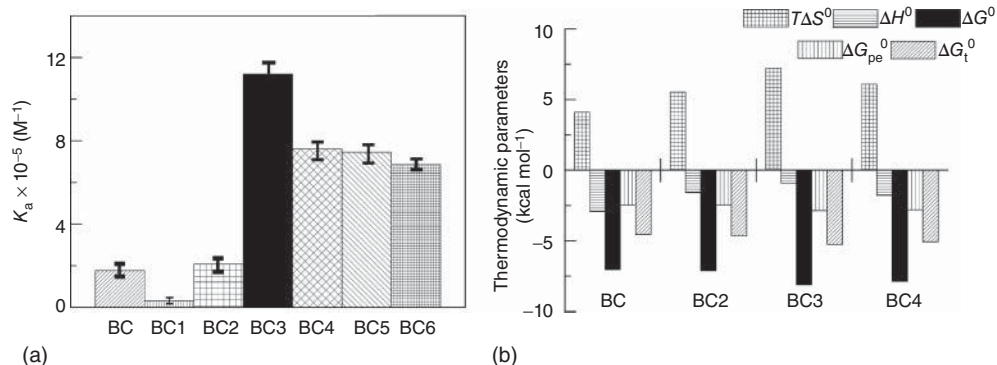
Another important aspect of berberine DNA interaction was the studies with different polymorphic conformations. Berberine was reported to bind to the protonated  $\text{H}^{\text{L}}$ -form of natural and synthetic DNAs [62, 63]. The binding of



**Scheme 9.4** Structure of the 9- and 13-substituted analogs of berberine.

berberine was found to switch its AT base pair specificity of B-form to GC (guanine-cytosine) specificity when the DNA structure adopted the protonated left-handed Hoogsteen base paired  $H^L$ -form. This was revealed from the large circular dichroism (CD) change as well as the large induced CD spectra acquired by the alkaloid in the presence of natural DNAs and the GC polynucleotides in the protonated  $H^L$ -form. The intrinsic CD of poly(dG-dC)·poly(dG-dC)–berberine interaction in B and  $H^L$ -form and the induced CD spectra for berberine in the presence of B and  $H^L$ -form of poly(dG-dC)·poly(dG-dC) are presented in Figure 9.4. Furthermore, berberine was found not to bind to the left-handed Z-DNA structure [64].

More recent studies have established the strong interaction of berberine with various other DNA structures. Studies on the interaction of berberine with DNA triplexes have been reported by various groups. Ren and Chaires [41] have first shown from competition dialysis assay and optical melting studies that berberine binds more strongly to poly(dA)·2poly(dT) compared to the parent duplex poly(dT)·poly(dA). Das *et al.* [65] subsequently showed that the Hoogsteen base

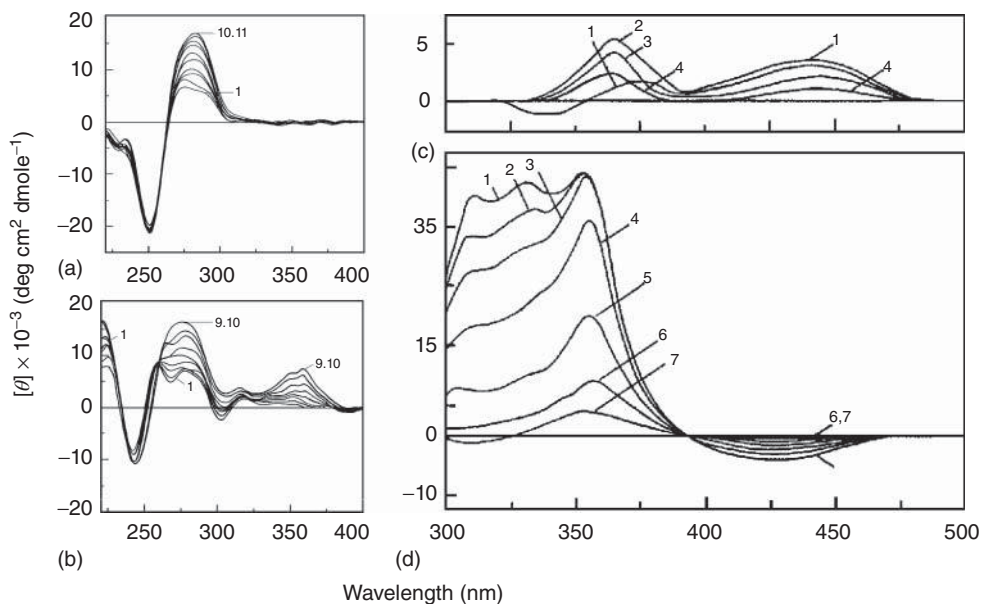


**Figure 9.3** (a) Bar chart showing comparative binding affinities of berberine and six 13-phenylalkyl-substituted berberines (BC1-6) with calf thymus DNA and (b) comparative thermodynamic profiles of interaction of BC, BC2, BC3, and BC4 (Scheme 9.4) with calf thymus DNA. (Reprinted from [59] with permission from the American Chemical Society.)

paired third strand of poly(dA)·2poly(dT) was stabilized selectively by berberine without affecting the duplex stability. Detailed spectroscopic and thermodynamic studies on the binding of berberine to triple helical DNA was reported by Sinha *et al.* [66], thereby suggesting that the binding of berberine to triplex DNA is intercalative and through cooperative interaction.

Sinha *et al.* found that the binding of berberine to the DNA triplex, poly(dA)·2poly(dT) involved strong stabilization of the third strand structure leading to strong energy transfer from the base triplets to the bound alkaloid. The thermodynamics of the binding as revealed from isothermal titration calorimetry (ITC) studies was found to be exothermic and favored by both enthalpy and entropy contributions leading to perturbation of the triplex conformation. Typical absorption spectral changes, thermal melting profiles, CD spectral changes, and ITC profile of berberine-poly(dA)·2poly(dT) triplex complexation are presented in Figure 9.5.

The 9-*O*-( $\omega$ -amino alkyl) ether berberines and 13-phenyl alkyl analogs (Scheme 9.4) also bound to poly(dA)·2poly(dT) triplex strongly [67, 68]. In the case of the 9-*O* analogs, the binding was noncooperative, while with the 13-phenyl alkyl analogs it was cooperative. The affinity of the 9-*O*-( $\omega$ -amino alkyl) ether berberines was also found to increase as the side chain length was enhanced and the highest affinity was of the order of  $10^6 \text{ M}^{-1}$  for the analog with six  $\text{CH}_2$  groups (B5, Scheme 9.4) while for the 13-phenyl alkyl analogs the affinity of  $10^6 \text{ M}^{-1}$  was observed for analog (BC4, Scheme 9.4) with side chain having four  $\text{CH}_2$  groups [68]. Beyond the threshold of four  $\text{CH}_2$  groups, the affinity decreased. In both cases, intercalative binding, stronger than those of berberine, was observed. Strong stabilization of the triplex structure without affecting the duplex thermal stability confirmed the specificity of these analogs to the triplex over the duplex. Energetically, the binding was reported to be more entropy driven in these

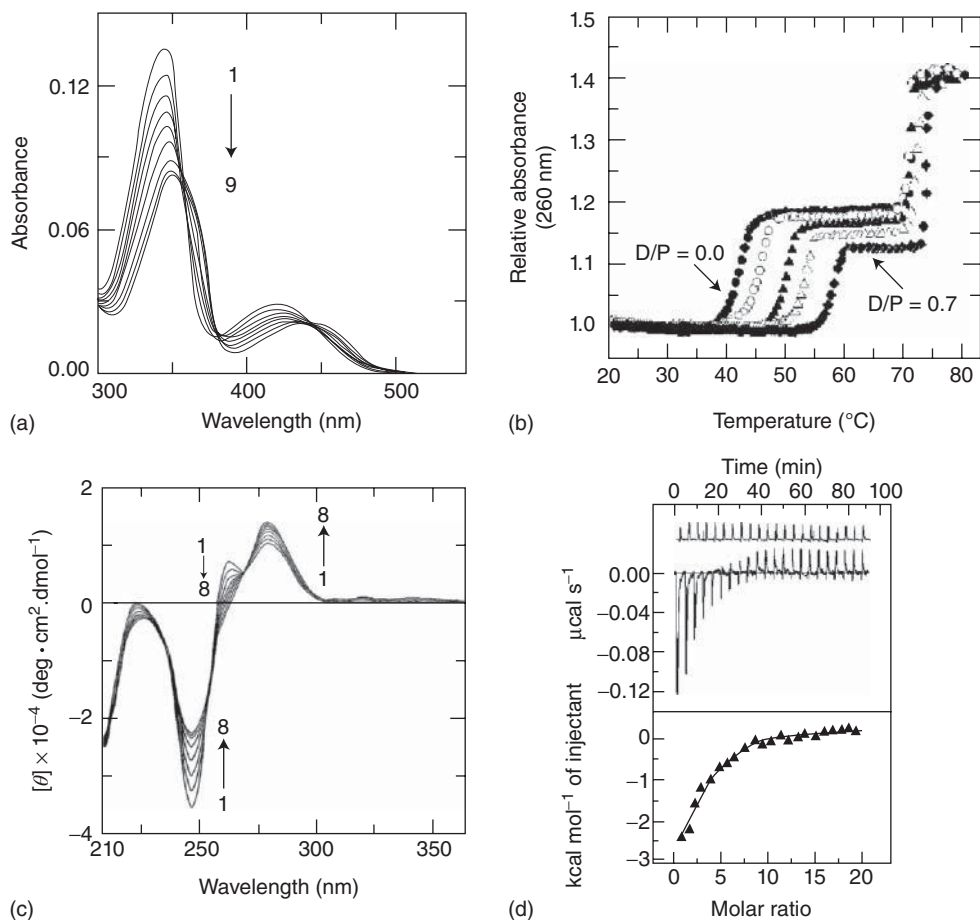


**Figure 9.4** Circular dichroism spectral changes of (a) B-form poly(dG-dC)·poly(dG-dC) (40  $\mu\text{M}$ ) treated with 0, 1.63, 3.67, 6.11, 9.40, 12.60, 15.81, 19.04, 22.24, 25.43, and 28.62  $\mu\text{M}$  of berberine represented by curves (1–11) at pH 7.0 at  $10 \pm 1^\circ\text{C}$  and (b) H<sup>L</sup>-form poly(dG-dC)·poly(dG-dC) (40  $\mu\text{M}$ ) treated with 0, 6.52, 12.99, 16.22, 19.44, 22.64, 25.83, 29.02, 32.20, and 35.35  $\mu\text{M}$  of berberine

represented by curves (1–10) at pH 3.4 at  $10 \pm 1^\circ\text{C}$ . Induced CD spectra of berberine (50  $\mu\text{M}$ ) treated with (c) 500, 143, 100, and 50 mM of B-form poly(dG-dC)·poly(dG-dC) represented by curves (1–4) at pH 7.0 and (d) 500, 400, 300, 100, 50, and 25  $\mu\text{M}$  of H<sup>L</sup>-form poly(dG-dC)·poly(dG-dC) represented by curves (1–7) at pH 3.4. (Reprinted from [62] with permission from Elsevier.)

analogs as the chain length of the side chain increased. This study also revealed the critical threshold length of the substituents to 13-phenyl alkyl analogs to be important in conferring the stability and specificity of binding to the triplexes similar to that observed with duplex DNA.

The ability and selectivity of berberines to bind to quadruplex structures over duplexes and triplexes may be advantageous for exerting therapeutic applications. Telomeres in rapidly dividing cells tend to form quadruplex structures that may be a potential anticancer target. The bound small molecules can trap DNA in a quadruplex conformation and, in this manner, may either inhibit telomere extension by telomerase or perturb telomere-capping mechanisms. Neidle, Maiti, Kumar, and their group [69–71] studied the binding of berberine to G-quadruplex structures formed by the human telomeric, the *c-myc* oncogene, and other biologically important G-rich sequences. The binding affinity was found to be of the order of  $10^6 \text{ M}^{-1}$ , one order higher than those to duplexes and triplexes. A stacking intercalative interaction with 1 : 1 binding stoichiometry has



**Figure 9.5** (a) Absorption spectral changes of berberine (5.0  $\mu\text{M}$ ) treated in the presence of (poly(dA)·2poly(dT)). Curves 1–9 denote 0, 5.0, 15.0, 25.0, 35.0, 50.0, 75.0, 100.0, and 125.0  $\mu\text{M}$  of triplex, respectively. (b) Thermal-melting profiles of poly(dA)·2poly(dT) (40.0  $\mu\text{M}$ ) (●) and its complexes with berberine at D/P ratio of 0.1 (○), 0.30 (▲), 0.50 (Δ), and 0.70 (●). (c) CD spectra of poly(dA)·2poly(dT) (26.7 mm) treated with 0.0, 3.0, 6.0, 9.1, 12.54, 15.79, 19.20, and 22.95  $\mu\text{M}$  of berberine (curves 1–8). (d) ITC profile for the titration of poly(dA)·2poly(dT) with berberine. (Reproduced from [66] with permission from John Wiley and Sons.)

been observed by Arora and Bhadra. The complete thermodynamics of the interaction also have been worked out in these studies of Maiti and Kumar's group. It was shown that the binding was favored by negative enthalpy and positive entropy contributions and an overall favorable Gibbs energy to the binding. Arora *et al.* suggested that berberine stacking at the external G-quartet is mainly aided by the  $\pi-\pi^*$  interaction and the stabilization of the high negative charge density of O6 of



guanines by the positively charged N7 of berberine. Bhadra *et al.* also suggested that the binding to the quadruplex structure was noncooperative and entropy driven with large hydrophobic contribution to the binding Gibbs energy. Recently, from NMR experiments, Sharma *et al.* [72] commented that berberine could bind to DNA sequence 5'-G3-C5-G3-3' that may form both i-motif and G-quadruplex types of secondary structures. More recently, the binding of berberine and analogs to a G-quadruplex sequence has been validated by molecular dynamics simulation studies [73]. The first crystal structure of the berberine-G-quadruplex complex was solved recently [74]; it showed an interesting case of two stacked berberine molecules bound side by side into G-tetrad leading to a 2:1 ligand to G-tetrad molar ratio in the complex. This appears to be different from the 1:1 geometry proposed from spectroscopic and calorimetric data in dilute solutions [70, 71]. Structural studies and molecular modeling approaches now have, however, revealed binding stoichiometries >1:1 and self-association of berberine induced by human telomeric G-quadruplex DNA [75].

A number of berberine analogs, particularly with substitutions at the 9- and 13-positions, with higher binding affinity over berberine to G-quadruplex structures, have also been reported suggesting that they might become lead compounds for inhibitory effect on telomerase activity and for the development as new anticancer drugs [55, 76–80]. Using electrospray ionization mass spectrometry, a berberine derivative substituted at its 13-position with a 5-nitro-2-phenylindolylmethyl group was shown to have modest binding affinity for a tetrameric quadruplex DNA, and significant selectivity over duplex DNA [76]. Many 9-O and 9-N-substituted analogs stabilized the quadruplex structure over the duplex stronger than berberine as revealed from a variety of physicochemical experiments [55, 77–80]. Compared to berberine, these substituted analogs exhibited stronger binding affinity with G-quadruplex and higher inhibitory activity for telomerase. Introduction of a side chain with proper length at the 9-position of berberine would thus significantly strengthen the binding affinity with G-quadruplex, resulting in the amplification of telomerase activity.

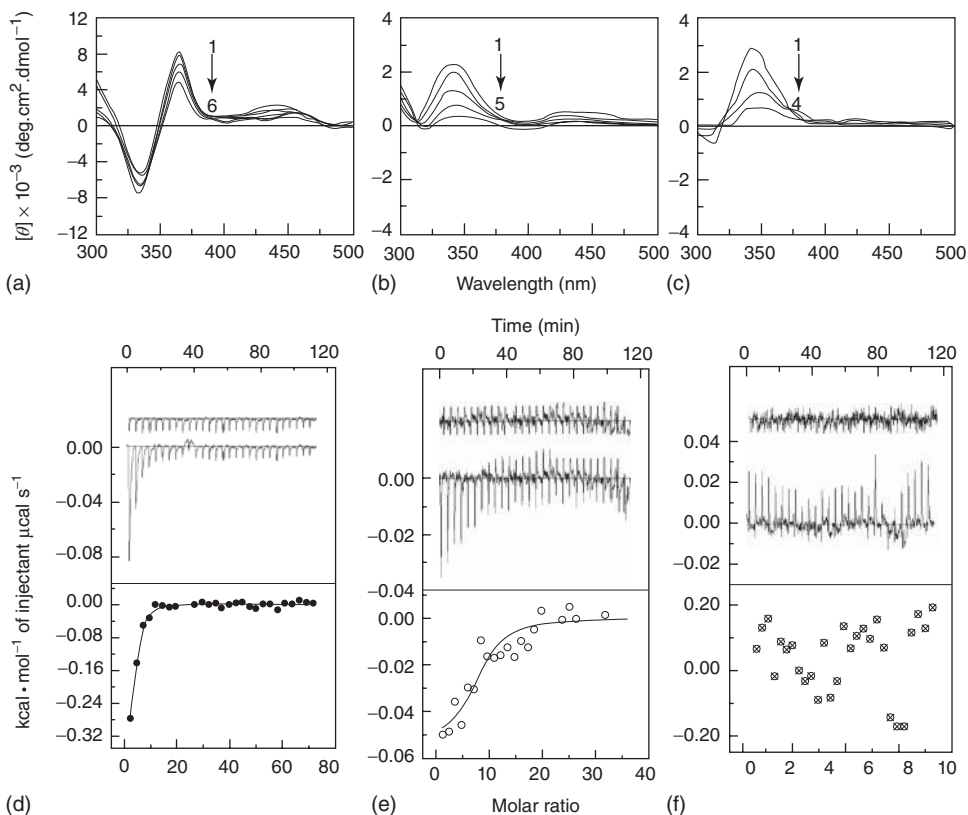
#### 9.2.1.4 Interaction of Berberine and Analogs with Ribonucleic Acids

RNA molecules have attracted recent interest for therapeutic targeting after the realization of their critical roles in many cellular activities and also the discovery of a number of new RNA molecules such as micro RNA, si RNA, and so on. The binding of berberine and related compounds to various ribonucleic acids has been initiated only very recently. The research in this area has been initiated first in the author's laboratory. Sinha *et al.* [81] studied the interaction of berberine along with a number of other small molecules with A-form and protonated forms of the RNA, poly(C)·poly(G). Hypochromic and bathochromic changes were effected in the berberine absorption spectrum on binding by both forms of the RNA, but the changes were more pronounced with the protonated form. The binding affinity of berberine to the protonated form was evaluated to be many folds higher than that with the A-form. The results from a number of studies involving spectroscopy and

viscometry substantiated the high preference of berberine to the protonated form of this RNA. It was suggested that berberine intercalated to the protonated form of the RNA and did not intercalate to the native A-form structure. Subsequently, Islam *et al.* [82, 83], studied the binding of berberine to tRNA (transfer ribonucleic acid) using a series of spectroscopic and calorimetric techniques. The cooperative binding of berberine induced moderate conformational changes in tRNA<sup>Phe</sup>. This study suggested that the binding was favored by both enthalpy and entropy changes. The results of the effect of [Na<sup>+</sup>] ion on the binding and the detailed parsing of the standard molar Gibbs energy suggested that although the alkaloid is positively charged, non-polyelectrolytic forces have a major role in the binding phenomenon. Differential scanning calorimetry studies revealed that three transitions in the melting of the tRNA were stabilized by the alkaloid binding. The partial intercalative binding and the site of intercalation was confirmed by molecular-docking studies [83]. More recently, the binding of a number of 9-O-N-aryl/arylalkyl amino carbonyl methyl-substituted berberine analogs to tRNA<sup>Phe</sup> reported almost 10-fold higher affinity over berberine [84]. These analogs changed the enthalpy-driven binding of berberine to entropy-driven interaction. Furthermore, the length of the side chain at the 9-position was reported to be very critical for effecting high binding affinity.

The structural and thermodynamic aspects of the binding of berberine to double stranded (ds) RNAs, poly(A)·poly(U) (AU), poly(C)·poly(G) (CG), and poly(I)·poly(C) (IC) were studied in details by Islam *et al.* using a battery of biophysical and thermochemical experiments [85]. The binding of berberine was found to proceed with positive cooperativity and the mode was suggested to be by partial intercalation. Berberine induced conformational changes in the polyribonucleotides and the bound molecules acquired induced CD. The changes were higher with poly(A)·poly(U). The detailed thermodynamic aspects of the interaction were also revealed from this study. The binding affinity was of the order of 10<sup>6</sup> M<sup>-1</sup> to poly(A)·poly(U) and one order lower for the IC sequence and very weak for the GC sequence. This study revealed that the binding of berberine to the ds RNAs was favored by large favorable entropy changes and small negative enthalpy and a predominant role for the non-polyelectrolytic forces. An enthalpy-entropy compensation was also reported. Furthermore, the heat capacity values evaluated from the temperature dependence of the enthalpy values suggested the involvement of significant hydrophobic forces in the interaction profile. The induced CD and calorimetric profiles of the binding of berberine with the three ds RNA sequences are presented in Figure 9.6. It was reported that berberine binds strongly with the double-stranded RNA structures and the binding was higher for the AU sequences compared to IC and CG sequences. The thermodynamic parameters of berberine to the three ds RNAs are presented in Table 9.3.

Binding of berberine to many single-stranded ribonucleic acids has also been studied [86]. With single-stranded RNAs, poly(G), and poly(I), berberine



**Figure 9.6** Induced circular dichroism spectra of berberine (50  $\mu$ M) in the presence of (a) 500, 400, 300, 200, 100, and 50  $\mu$ M poly(A)-poly(U) (curves 1–6), (b) 500, 400, 300, 200, and 100  $\mu$ M poly(I)-poly(C) (curves 1–5), and (c) 500, 300, 200, and 100  $\mu$ M poly(C)-poly(G) (curves 1–4); (d) ITC profiles for the titration of poly(A)-poly(U) (e), poly(I)-poly(C) (f), and poly(C)-poly(G) (f) with berberine, at 20  $^{\circ}$ C. (Reprinted from [85] with permission from the American Chemical Society.)

exhibited strong noncooperative binding with affinity of the order of  $10^5$  M<sup>-1</sup>. Much weaker binding was observed to poly(U) and poly(C). The thermodynamics of the interaction suggested exothermic binding with favorable enthalpy and entropy changes. The 9-*O-N*-aryl/arylalkyl amino carbonyl methyl analogs (Scheme 9.4) also bound poly(G) and poly(I) much more strongly than poly(U) and poly(C). This study also suggested the chain length at the 9-position to be critical for manifesting strong interaction [87].

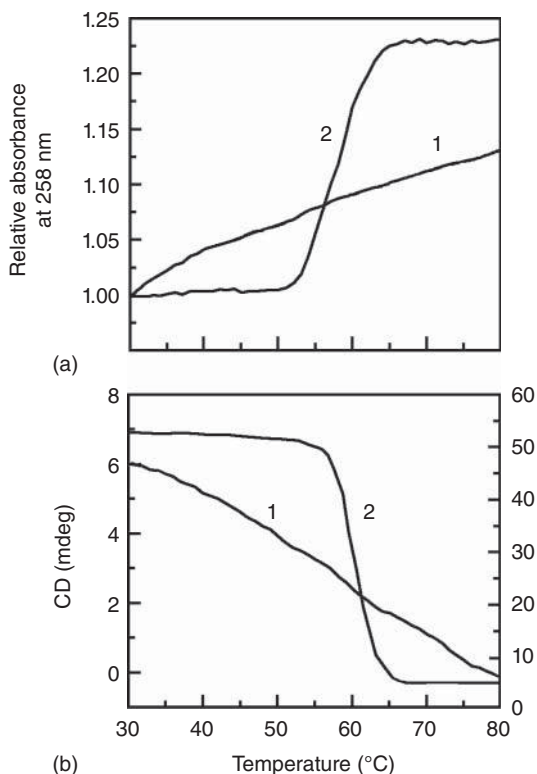
Berberine showed remarkably strong binding to poly(A). Several years ago, Maiti and colleagues [88] reported that berberine binds to poly(A) with high affinity, much higher than its affinity to tRNA and double-stranded DNA. Maiti and Kumar [89] later studied, in detail, the interaction of berberine with

**Table 9.3** Isothermal calorimetric data for the binding of berberine, palmatine, and coralyne to ds RNAs 20 °C.

RNAs	Alkaloid	$K \times 10^{-6}$ ( $M^{-1}$ )	$\Delta G^\circ$ (kcal mol $^{-1}$ )	$\Delta H^\circ$ (kcal mol $^{-1}$ )	$T\Delta S^\circ$ (kcal mol $^{-1}$ )
Poly(A)·poly(U)	Berberine	0.81 ± 0.07	-7.97	-2.15	5.82
	Palmatine	1.23 ± 0.07	-8.22	-2.62	5.60
Poly(I)·poly(C)	Berberine	0.61 ± 0.08	-7.80	-0.86	6.94
	Palmatine	0.94 ± 0.07	-8.06	-3.54	4.52
Poly(C)·poly(G)	Berberine	nd	nd	nd	nd
	Palmatine	0.78 ± 0.07	-7.95	-2.01	5.94

nd: no data.

single- and double-stranded poly(A). It was suggested that berberine bound noncooperatively with single-stranded poly(A) with affinity of the order of  $10^6 M^{-1}$ . The binding was shown to result in the formation of a conservative pair of induced CD bands in the alkaloid absorption region for the bound alkaloid molecules with unusually large ellipticity. Detailed studies confirmed intercalation of the alkaloid to the single-stranded stacked helical organization of poly(A). This study also reported that berberine did not show any binding affinity to the low-pH-induced ds form of poly(A). These results were augmented subsequently by the studies of Giri and Kumar [90] who reported the thermodynamics of the binding of berberine to poly(A) by calorimetry and suggested an enthalpy-driven binding of the alkaloid. In this study, high binding affinity of berberine to poly(A) was observed but the formation of self-structure (see the following) in poly(A) was not evident and suggested that buckled structural geometry of the alkaloid prevented self-structure formation in poly(A). Furthermore, Giri also hypothesized the lack of self-structure formation in poly(A) to be due to noncooperative binding of the alkaloid to the polynucleotide and the nonplanarity of the alkaloid structure. The molecular aspects of the binding of berberine to single-stranded poly(A) have been reviewed in detail subsequently [25–31]. Although self-structure formation, wherein two strands of poly(A) probably form a parallel-stranded structure with adenine–adenine base pairs, was not apparent from the spectroscopy data of Maiti and Giri [88–90], a new dilution method introduced by Hud suggested the possibility of such a structural reorganization in poly(A) in the presence of berberine [91]. This was later confirmed by experiments involving berberine, 9- $\omega$ -amino alkyl ether and 9-*O*-*N*-aryl/arylalkyl analogs [92, 93]. A typical absorbance and CD melting profile of self-structure induced by the 9-*O*-*N*-aryl/aryl alkyl analog of berberine (B'1, Scheme 9.4) is shown in Figure 9.7. The self-structure induction in single-stranded poly(A) since then has become an important structural reorganization of poly(A) and may have remarkable implication in cancer for the development of RNA-targeted drugs. A number of other alkaloid molecules also have been reported to induce this



**Figure 9.7** Absorbance (a) and circular dichroism (b) melting profiles of poly(A) (curve 1) and poly(A) complexed with 9-*O*-*N*-aryl/arylalkyl amino carbonyl methyl

berberine analog (B'1, Scheme 9.4) (curve 2). (Reproduced with modification from [93] with permission from The Royal Society of Chemistry.)

unique structural reorganization in poly(A) [25–31, 90, 94, 95]. The molecular aspects of the self-structure are now being studied in many laboratories.

The binding of berberine to triplex RNA was studied with the triplex poly(A)·2poly(U) where the alkaloid exhibited noncooperative binding with an affinity of the order of  $10^5 \text{ M}^{-1}$  [96]. Binding stabilized the third strand of the triplex and the mode of interaction was revealed to be by intercalation leading to conformational changes. The thermodynamics of the triplex binding suggested large negative enthalpy and small favorable entropy contributions.

Recently, the binding of 9-*O*-alkyl ether and 13-phenyl alkyl analogs (Scheme 9.4) to the RNA triplex poly(A)·2poly(U) was reported utilizing a series of biophysical and microcalorimetric methods [67, 97]. Both of these series of analogs bound triplex noncooperatively by intercalation with one order higher binding ( $10^6 \text{ M}^{-1}$ ) compared to berberine. In the case of the 9-*O*-alkyl ether analogs, the affinity was observed to be enhanced with the increase in chain

length but in with the 13-phenyl alkyl analogs, a threshold length of the alkyl chain with  $(\text{CH}_2)_4$  showed the highest binding, after which the affinity decreased [68, 97]. The binding with these analogs was shown to be clearly entropy driven, suggesting the role of the side chain in controlling the thermodynamics of the interaction. Overall, the 9-*O*-alkyl ether analogs showed higher binding compared to the 13-phenyl alkyl analogs. A similar study with 9-*O*-*N*-aryl alkyl amino carbonyl methyl analogs (Scheme 9.4) with poly(A)·2poly(U) was performed in comparison to the parent duplex poly(A)·poly(U) by Basu *et al.* [98]. These analogs also bound the triplex RNA noncooperatively by intercalation and exhibited higher affinity over the duplex RNA. The chain length of the substitution also was reported to have significant effect in the binding phenomenon.

#### 9.2.1.5 Interaction of Berberine and Analogs with Proteins

Although the interaction of berberine with nucleic acids has been extensively studied in many laboratories, only few studies describe its interaction with proteins. One of the earliest studies of berberine–protein interaction was by Kovar [99] who described the formation of a complex of horse liver alcohol dehydrogenase and berberine. In a more recent study [100], berberine was shown to bind and inhibit the assembly and GTPase activity of FtsZ protofilaments and inhibit the FtsZ GTPase activity. NMR and molecular-docking studies revealed that the binding ( $K_D$  0.023  $\mu\text{M}$ ) site of the alkaloid overlaps with that of GTP on FtsZ. These results suggested the inhibitory role of berberine on the assembly function of FtsZ, establishing it as a novel FtsZ inhibitor that halts the first stage in bacterial cell division. The efficacy of a drug depends on its ability to be delivered at the active site and hence its affinity to carrier proteins needs to be understood for effective sequestration and distribution in the blood stream. One of the earliest studies for berberine in this direction was by Tanaka and colleagues [101] who suggested binding of berberine to bovine serum albumin (BSA) to be nonspecific and hydrophilic, and with a binding affinity of the order of  $10^4 \text{ M}^{-1}$ . Li and colleagues investigated the effect of berberine on the secondary structure of human serum albumin (HSA) using fluorescence, Fourier transform infrared (FT-IR), and CD spectroscopic methods. The binding was found to be of the order of  $10^4 \text{ M}^{-1}$  and it was suggested that the binding involved mostly hydrophobic and some contributions from electrostatic interactions. From displacement experiments, berberine was suggested to bind to the subdomain IIA of the protein leading to conformational changes manifested by reduction of  $\alpha$ -helices and increase of turn structure [102].

Hu *et al.* [103] using spectroscopy experiments suggested that berberine formed a complex with HSA with moderate binding affinity ( $10^4 \text{ M}^{-1}$ ) and the alkaloid was close to Trp-214 of the protein. On the basis of thermodynamic parameters deduced from the spectral data at different temperatures, it was suggested that electrostatic interactions played a major role in berberine–HSA interaction. This study also proposed the site of binding of berberine on HSA to be in the subdomain II. A similar study and conclusion was reported for its binding to BSA

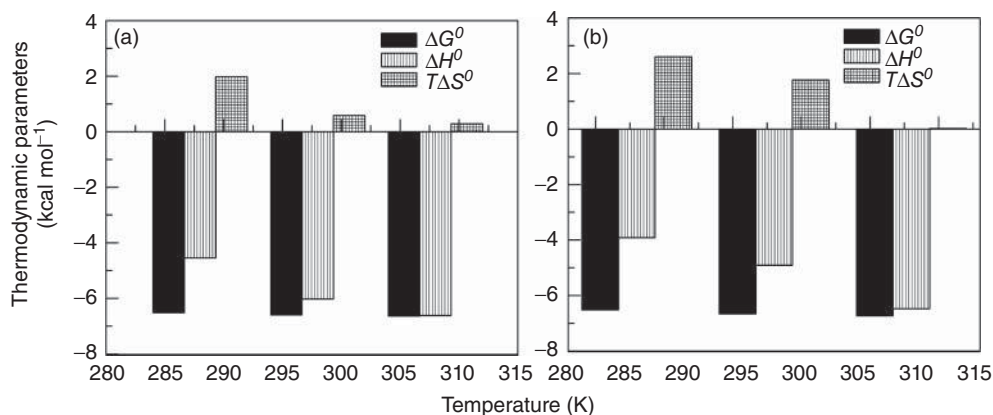
**Table 9.4** Binding affinity of berberine and palmatine to various proteins as elucidated from isothermal titration calorimetry studies at 25 °C.

	Berberine (M <sup>-1</sup> )	Palmatine (M <sup>-1</sup> )
Bovine serum albumin	6.97 ± 0.19 × 10 <sup>4</sup>	5.91 ± 0.29 × 10 <sup>4</sup>
Human serum albumin	7.75 ± 0.17 × 10 <sup>4</sup>	7.11 ± 0.12 × 10 <sup>4</sup>
Hemoglobin	6.49 ± 0.24 × 10 <sup>4</sup>	8.70 ± 0.40 × 10 <sup>4</sup>
Lysozyme	5.95 ± 0.24 × 10 <sup>4</sup>	4.30 ± 0.40 × 10 <sup>4</sup>

also [104]. The proposal of binding of berberine to Sudlow site I in subdomain IIA of the proteins was, however, contradicted by a very recent report by Marszałek *et al.* [105] by optical spectroscopy and pulse radiolysis techniques. It was suggested that the bound alkaloid molecules are located at the protein/water interface rather than bound inside the Sudlow site I in subdomain IIA as proposed in many earlier studies.

Detailed thermodynamic aspects of the binding of berberine to BSA and HSA were described by Khan *et al.* [106], through calorimetric techniques. It was found that the affinity of the alkaloid to the serum proteins was of the order of 10<sup>4</sup> M<sup>-1</sup> (Table 9.4), in agreement with the previous reports based on spectroscopic experiments. This detailed study suggested that the binding of the alkaloid to serum proteins was enthalpy dominated and entropy favored. Furthermore, it was also suggested that polyelectrolytic forces make a significant contribution to the binding forces. The heat capacity data revealed significant burial of nonpolar surface area during the complexation. A pictorial description of the variation of the thermodynamic parameters of the binding of berberine to BSA and HSA is presented in Figure 9.8. From three-dimensional and synchronous fluorescence results, and CD data, this study has showed change in the helical structure of the protein on binding of berberine to both HSA and BSA. The interaction of some analogs of berberine have also been studied with serum proteins [107, 108]. Thus, the exact binding site of berberine on the serum proteins still remains controversial.

Hemoglobin is the protein of the red blood cells that carries oxygen to the tissues. The binding studies of berberine to hemoglobin (Hb) using biophysical tools was undertaken recently in the author's laboratory [109]. It was shown that berberine binds closer to the β-Trp-37 residue of the protein molecule at the α1-β2 interface. Evidence for this has been advanced from absorbance, fluorescence, CD experiments, and calculation of FRET (fluorescence resonance energy transfer) distance between the Trp residue and the alkaloid. Furthermore, the binding of the alkaloid away from the central cavity of the protein was suggested in this study. Secondary structural changes in the protein conformation were also evident from CD and fluorescence data. Calorimetry results have suggested that the binding was favored by enthalpy and entropy changes. Hydrophobic and electrostatic interactions were proposed to be major forces that dictate the



**Figure 9.8** Plots of variation of thermodynamic parameters against temperature for the binding of berberine to (a) BSA and (b) HSA. (Reproduced partially from [106] with permission from Springer.)

binding process. The affinity of the alkaloid to hemoglobin was moderate and of the order of  $10^4 \text{ M}^{-1}$ .

Investigations on the interaction of the alkaloid to the antimicrobial enzyme lysozyme were reported very recently, by Cheng *et al.* [110] and Jash and Kumar [111]. Hydrophobic interaction was suggested to be the type of binding force involved between lysozyme and berberine. Berberine was shown to selectively oxidize the Trp residues of Lys under photoexcitation and the reaction mechanism was determined to be an electron transfer process. Jash and Kumar studied in depth the interaction through spectroscopic and calorimetric techniques, and reported the affinity of berberine to lysozyme to the order of  $10^4 \text{ M}^{-1}$ , significant conformational changes in the structure of the protein, endothermic and entropy-driven binding, with hydrophobic and van der Waals forces playing key role in the binding interaction of the alkaloid in the  $\beta$ -domain of the protein.

Thus, these studies with various proteins revealed the binding affinity of the alkaloid to be of the order of  $10^4 \text{ M}^{-1}$  and lower than those reported with nucleic acids.

### 9.2.2

#### Palmatine

Palmatine is a close analog of berberine distributed in plants that are also the sources of berberine. Palmatine bears the same tetracyclic buckled structure as berberine (Scheme 9.1) but differs in the nature of the substituents at positions 2,3 on the benzo ring. The physical and chemical properties of palmatine are more or less close to those of berberine, but the biological properties differ significantly. This alkaloid finds use in folk medicine in the treatment of jaundice, dysentery,

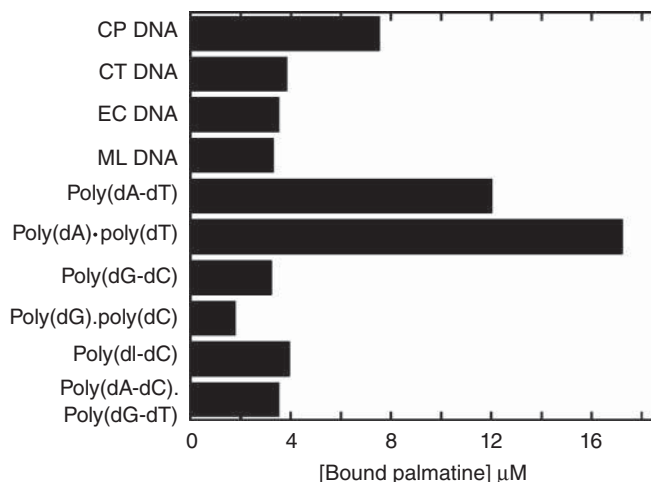


hypertension, inflammation, and liver-related diseases and viral infections [112]. It has been demonstrated to have antiarrhythmic, analgesic, antimalarial, and antiseedative effects. The bioactivity of palmatine presents as sedative and antioxidant activities [113, 114]. It has also been shown to be an inhibitor of  $\beta$ -site amyloid precursor protein-cleaving enzyme 1 (BACE 1), acetyl-, and butyryl-cholinesterases [113]. Palmatine was found to inhibit West Nile virus without significant cytotoxicity. It also suppressed dengue virus and yellow fever virus in a dose-dependent manner [115]. A number of studies revealed the anticancer properties of this alkaloid [116, 54]. The cytotoxic activities and the DNA binding have been studied by Bailly and colleagues [117].

#### 9.2.2.1 Interaction of Palmatine and Analogs to Deoxyribonucleic Acids

The DNA-binding properties of palmatine was reported to be consistent with a “mixed-mode” DNA-binding model for protoberberines in which a portion of the ligand molecule intercalates into the double helix, while the non-intercalated portion of the ligand molecule protrudes into the minor groove of the host duplex, where it is thereby available for interactions with atoms lining the floor and/or walls of the minor groove [118]. Pilch and coworkers [119] in a later study involving palmatine and three other protoberberine analogs suggested that palmatine stabilizes DNA against thermal-strand separation and unwinds DNA by  $11^\circ$ ; this is consistent with the intercalation of the alkaloid to DNA. Furthermore, their study suggested enthalpy-favored interaction resulting from stacking interaction between the alkaloid and the base pairs, contributing 50% of the thermodynamic driving force for the complexation. Intercalation of only C and D rings with A and B rings of the protoberberine moiety (Scheme 9.1) protruding to the interior of the minor groove of the DNA has been proposed. Detailed interaction of palmatine with DNA was reported by Bailly and coworkers who suggested DNA as a potential bio-target for this alkaloid [117]. It was suggested that for the topoisomerase I-mediated DNA cleavage, both DNA binding and enzyme interactions are important. In a related spectroscopic study, Hirakawa and colleagues [120] reported that photoexcited alkaloid generated singlet oxygen when DNA-palmatine complexes are formed, thereby enumerating its use as a functional photosensitizer on by interaction with DNA.

The noncovalent complexes of palmatine along with many other alkaloids including berberine, jatrorrhizine, and copticine were investigated by using electrospray ionization mass spectrometry by Chen *et al.* [49]. Higher binding of palmatine over berberine was observed in this study with oligonucleotides. The results indicated that palmatine exhibited the highest binding affinity with the double-stranded DNA compared to berberine and many other alkaloids. The first exclusive and detailed study on the DNA binding of palmatine was presented by Bhadra *et al.* who, using spectroscopic, viscometric, and calorimetry techniques, elucidated the mode of binding, base pair specificity, and thermodynamics of the binding [121, 122]. These studies proposed the intercalation model and AT base



**Figure 9.9** The results of a competition dialysis experiment of palmatine to various DNAs represented in terms of the concentration of the alkaloid bound. (Reprinted from Bhadra and Kumar [27] with permission from John Wiley and Sons.)

pair specificity for the binding of palmatine. The binding affinity of palmatine to the AT polynucleotides was found to be of the order of  $10^5 \text{ M}^{-1}$ , while that to the GC sequences was one order of magnitude lower. ITC experiments showed  $K_b$  of palmatine-poly(dA)·poly(dT) at  $20^\circ\text{C}$  equal to  $2.30 \times 10^5 \text{ M}^{-1}$  and to poly(dA-dT)·poly(dA-dT) as  $1.63 \times 10^5 \text{ M}^{-1}$  [123]. Palmatine exhibited cooperative binding to homo sequences of AT and GC runs and exhibited noncooperative binding to mixed sequences and heterosequences [121]. A representative result depicting the base pair specificity [27] from a competition dialysis assay is presented in Figure 9.9. The energetics of the interaction suggested that the complex formation was exothermic and favored by both enthalpy and entropy contributions except in the case of homo AT sequences where it was entropy driven. The binding of palmatine was proposed to be involving mostly non-polyelectrolytic forces [123]. However, from luminescence measurements, UV–vis spectroscopy experiments, cyclic voltametry, and quenching techniques, palmatine binding to calf thymus DNA was suggested to involve a mixed mode with prevalent groove-binding interactions [124]. A number of analogs with carbon-chain substituents at the C-13-position of palmatine were also synthesized and their antitumor activity elucidated [54].

#### 9.2.2.2 Interaction of Palmatine with RNA

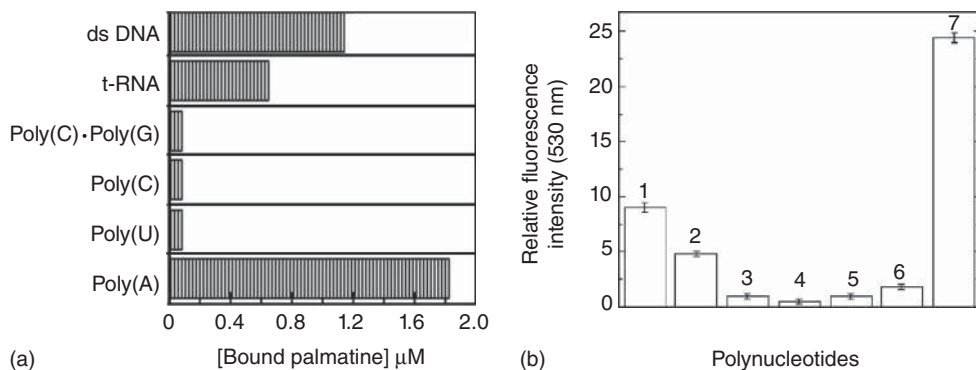
Palmatine binding has been studied, in comparison with berberine with many RNA structures. The binding study of palmatine to tRNA was reported by Islam *et al.* [82, 83] using various biophysical and calorimetric tools in comparison to berberine. It was revealed that palmatine binds noncooperatively to tRNA with

an affinity of the order of  $10^5 \text{ M}^{-1}$  close to that of berberine. The binding features were almost similar to those of berberine, exothermic binding, driven by enthalpy and entropy, strong thermal stabilization of the structure and non-electrostatic component becoming the dominant contributor to the Gibbs free energy of binding. Further detailed study revealed cooperative binding of palmatine on tRNA with significant structural changes on binding. Partial intercalation of palmatine to tRNA was supported by drug displacement and molecular modeling studies. It was suggested that like berberine, the binding of palmatine was near the acceptor stem of the tRNA molecule.

Similarly to berberine, palmatine also exhibited stronger interaction to single stranded sequences of poly(G) and poly(I) compared to poly(C) and poly(U). The binding was exothermic and the Gibbs energy contribution was from both favorable enthalpy and entropy changes. With poly(U) and poly(C) palmatine showed very weak affinity [86]. Giri *et al.* studied the binding aspects of palmatine to poly(A) and showed that the alkaloid has very high binding affinity. From spectroscopy and calorimetry, it was deduced that the binding affinity of palmatine to poly(A) was  $(8.36 \pm 0.26) \times 10^5 \text{ M}^{-1}$  [125]. The alkaloid was revealed to bind non-cooperatively. A comparative fluorescence study revealed very high preference of palmatine to poly(A) over a number of single- and double-stranded RNAs and DNAs. The high affinity was also exemplified by high structural change in CD with the bound alkaloids assuming induced CD and enthalpy-driven binding in calorimetry. This was further supplemented by data from a competition dialysis assay [126]. But this study suggested no self-structure formation in poly(A) by palmatine. Data showing the high binding of palmatine to poly(A) over other nucleic acids is presented in Figure 9.10.

The double-stranded RNA binding aspects of palmatine in comparison to berberine was studied by Islam [85]. The study engaged three ds RNAs, namely, poly(A)·poly(U), poly(C)·poly(G), and poly(I)·poly(C). The binding of palmatine, similarly to berberine was found to be cooperative to these ds RNA polynucleotides. The affinity of palmatine was as strong as that of berberine with equilibrium constant of the order of  $10^6 \text{ M}^{-1}$  with poly(A)·poly(U) and one order lesser at  $10^5 \text{ M}^{-1}$  for poly(I)·poly(C) and poly(C)·poly(G). Thermodynamically, the binding to these ds RNAs was favored by negative enthalpy and positive entropy changes. As in the case of berberine, palmatine binding also was dominated by non-electrostatic interactions and characterized by negative heat capacity changes and exhibited enthalpy–entropy compensation phenomena. On the other hand, the binding of palmatine to the low-pH-induced double-stranded poly(A) was found to be very weak [126].

Binding of palmatine to RNA triplex was also investigated in comparison to berberine using the model triplex poly(A)·2poly(U) [96]. The alkaloid was found to bind noncooperatively and stabilized the Hoogsteen base paired third strand of the triplex as revealed from enhanced thermal stabilization of the triplex denaturation profile. Compared to berberine, palmatine binding to the triplex was much



**Figure 9.10** (a) Results of a competition dialysis experiment showing preferential binding of palmatine to ss poly(A) over other DNAs and RNAs. (b) Relative steady-state fluorescence intensity of palmatine (10  $\mu\text{M}$ ) at 530 nm in the presence different

polynucleotides at polynucleotide phosphate/palmatine molar ratio 12. Here 1–7 indicates data for ds calf thymus DNA, tRNA, poly(C)·poly(G), poly(C), poly(U), ds poly(A), and ss poly(A), respectively. (Reprinted from [126] with permission from Elsevier.)

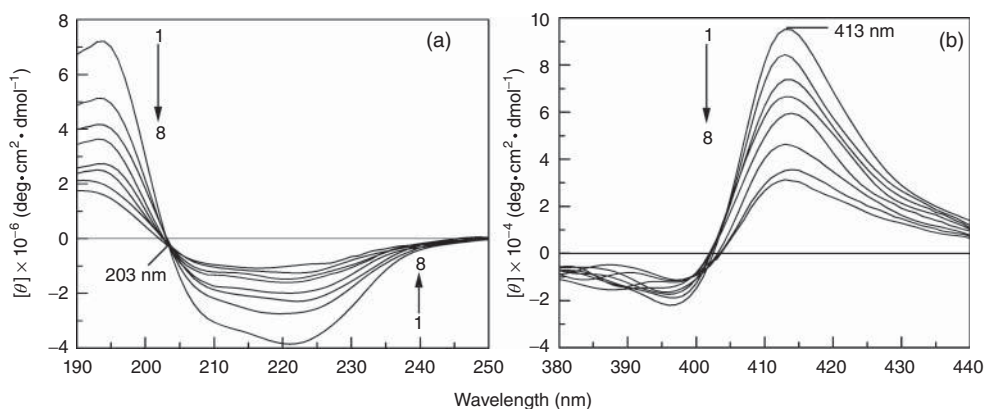
stronger as revealed from spectroscopic and calorimetric results. Intercalation was proposed from polarization of the emission spectra of the complex, energy transfer from the triplet bases to the bound alkaloid and viscometry data. The binding was shown to perturb the conformation of the triplex structure, leading to the alkaloid acquiring optical activity as indicated by the CD data. Thermodynamic data showed both enthalpy and entropy favored the intercalation of the alkaloid to the RNA triplex similar to berberine.

### 9.2.2.3 Interactions of Palmatine with Proteins

The interactions of palmatine with some functional proteins have been studied mostly in comparison with berberine. The interaction of palmatine with HSA was investigated by spectroscopic techniques by Wang and coworkers [127]. The binding was found to be moderate with an affinity value of  $8.84 \times 10^4 \text{ M}^{-1}$  and leading to some conformational changes. Temperature-dependent studies and the evaluated thermodynamic parameters led to the suggestion that electrostatic interaction played a major role in the interaction that was mostly near the tryptophan residues of the protein. A study of the interaction between palmatine and BSA was carried out by a spectroscopic approach [128]. The results of synchronous fluorescence spectra and UV–visible absorption spectra revealed that the conformation of BSA changed on binding. Determination of the specific interaction between palmatine and BSA revealed a distance of 3.36 nm between the binding sites. Palmatine was suggested to bind to site II (subdomain IIIA) of BSA. The thermodynamics of the interaction of palmatine in comparison with berberine with BSA and HSA was investigated using calorimetric techniques [106]. Thermodynamic results revealed that there was only one class of binding sites for palmatine

on BSA and HSA. The equilibrium constant was of the order of  $10^4 \text{ M}^{-1}$  with both proteins but slightly higher with HSA. Palmatine affinity to both proteins was close to that of berberine. The binding was enthalpy dominated and entropy favored and salt-dependent studies suggested that electrostatic interaction had a significant role in the binding process. The binding of palmatine changed the conformation of proteins by reducing their helicity. It was concluded that electrostatic interactions played the major role in the palmatine–BSA association reaction. The interaction between palmatine and bovine hemoglobin was investigated by multi-spectroscopy techniques [129]. Results suggested that electrostatic forces played an important role in the binding process. The order of magnitude of the binding constant was  $10^4 \text{ M}^{-1}$  and palmatine was suggested to bind at  $\beta$ -37 tryptophan in the hydrophobic cavity of hemoglobin. The interaction was suggested to lead to change in the microenvironment and the conformation of the protein [128, 129].

The binding of palmatine to human hemoglobin and lysozyme was studied in comparison with berberine using spectroscopic and calorimetric techniques [109, 111]. Palmatine was proposed to be bound at the  $\alpha 1\beta 2$  interface of hemoglobin close to the binding site of berberine and lead to conformational changes in the protein as revealed from CD changes in the near UV and Soret band region (Figure 9.11). A closer position to Trp-37 for palmatine compared to berberine was revealed from FRET studies. The affinity of palmatine to the protein of  $(8.70 \pm 0.40) \times 10^4 \text{ M}^{-1}$  was, however, much lower than that of berberine  $(6.49 \pm 0.24) \times 10^5 \text{ M}^{-1}$ . Detailed thermodynamic studies suggested the involvement of strong hydrophobic interactions together with electrostatic interactions in the complexation.



**Figure 9.11** (a) Intrinsic circular dichroism (far UV CD) spectral changes of hemoglobin (5  $\mu\text{M}$ ) on interaction with 0, 2.67, 5.35, 8.02, 10.67, 13.33, 15.98, and 18.63  $\mu\text{M}$  of palmatine. (Reproduced from [109] with permission from The Royal Society of Chemistry.) (b) Soret band CD spectral changes of Hb

Similarly, the binding of palmatine to lysozyme [111] also revealed an affinity slightly lower than that of berberine. The binding was endothermic and enthalpy driven and the stabilizing forces were van der Waals force and hydrogen bonds along with electrostatic interactions. The binding data of palmatine to proteins reported in these investigations have been collated in Table 9.4.

### 9.2.3

#### Other Isoquinoline Alkaloids: Jatrorrhizine, Copticine, and Analogs – DNA/RNA and Protein Interactions

Jatrorrhizine and copticine (Scheme 9.2) are other alkaloids found to be medicinally important. They are suggested to be the potential new antimelanoma drug candidates [129]. Like berberine, they have promising antimutagenic/anticarcinogenic [13], hypoglycemic, and neuroprotective effects [130]. Jatrorrhizine was also found to have antimalarial potency [10, 131]. Study of the comparative behavior of the DNA complexation of jatrorrhizine with berberine and palmatine suggested the same binding site on DNA [132]. An intercalative binding mode for this alkaloid by electrospray ionization mass spectrometry was suggested [133]. The binding of jatrorrhizine to HSA was studied by several groups. Li *et al.* [134] observed the presence of a single class of binding site on HSA and its binding constants to be  $7.278 \times 10^4 \text{ M}^{-1}$  at room temperature. On the basis of CD and FT-IR spectral data, a change in the protein secondary structure was suggested in the presence of jatrorrhizine. From the thermodynamic functions calculated from the van't Hoff equation, it was suggested that hydrophobic and electrostatic interactions played a major role in the binding. This study, based on displacement and molecular modeling data, predicted jatrorrhizine to be binding to site I of HSA. Mi *et al.* [135] studied the interaction of jatrorrhizine with BSA using biophysical studies. The thermodynamic parameters indicated that electrostatic interactions and hydrogen-bonding interactions played a major role in the association. Site-marker-competitive displacement experiments and molecular modeling demonstrated that the alkaloid is mainly located within the hydrophobic pocket of subdomain IIIA of BSA and this was also supported by FRET distances. The binding of jatrorrhizine to HSA was recently studied by two groups [136, 137]. The binding affinity was of the order of  $10^4 \text{ M}^{-1}$  and from thermodynamic data, electrostatic interaction has been suggested to be a major force in the binding. Jatrorrhizine was suggested to be located in the vicinity of the tryptophan (Trp) residue. Molecular modeling results showed that the jatrorrhizine–HSA complex was formed not only through electrostatic forces but also through  $\pi$ – $\pi$  stacking and hydrogen bonding interactions. The site-selective binding of jatrorrhizine to HSA was performed using fluorescence spectroscopy, UV–vis spectroscopy, and molecular modeling [137]. The results indicated the formation of a complex with binding affinities of the order  $10^4 \text{ M}^{-1}$ . The thermodynamic parameters studied revealed that the binding was characterized by negative enthalpy and positive

entropy changes and that electrostatic interactions played a major role in the association within the hydrophobic pocket of the subdomain IIIA of HSA leading to changed molecular conformation for the protein.

Chen *et al.* [49] studied the binding of coptisine to many oligonucleotides along with berberine, palmatine, jatrorrhizine, and berberubine, using electrospray ionization mass and fluorescence spectroscopy. Coptisine was found to have higher affinity than palmatine and close to berberine to some oligonucleotides. In another recent study, Ma *et al.* used electrospray ionization mass spectrometry in the negative ion mode to screen the noncovalent complexes between a number of alkaloids including coptisine with double-helical oligonucleotides at different molar ratios [138]. Coptisine was found have an affinity close to that of berberine. Detailed nucleic acid- and protein-binding studies on coptisine are still awaited.

Berberrubine, jatrorubine, and palmatrubine (Scheme 9.3) have been studied with calf thymus DNA, poly(dA-dT)·poly(dA-dT), poly(dG-dC)·poly(dG-dC), and eight AT-rich 12-mer double-stranded DNAs. The DNA intercalating binding affinities of these protoberberine alkaloids have been reported to be significantly higher over their parent compounds and that all of them exhibited AT specificity [139].

Overall, the important features of the isoquinoline alkaloids that are revealed to be important for nucleic acid binding are the quaternary nitrogen atom, aromaticity of ring C, the type of *O*-alkyl substituents on rings A,D, and the size of substituent at 9 and 13 positions.

### 9.3

#### Concluding Remarks

Natural products, especially, alkaloids, have exhibited great potential as effective bioactive compounds for drug discovery. Protoberberine alkaloids represent an interesting group of natural alkaloids with remarkable biological activities, the most important being their antitumoral properties. The important advantage of these molecules is their large natural abundance, low toxicities, and rich background knowledge of their utility in folk medicines. Therefore, it is to our advantage to understand and exploit them as effective therapeutic agents. This is possible only through understanding their structure–activity relationships. One of the most important routes to the bioactivity of these alkaloids appears to be due to their DNA and RNA binding. In this chapter, up-to-date knowledge on the binding aspects of some of the most important isoquinoline alkaloids and their analogs are presented. Most of the studies on these alkaloids are based on spectroscopy and calorimetry results. A large volume of published works suggests that berberine and palmatine form strong intercalation complexes with both DNA and RNA. The advancement of the analytical techniques in the last decade has enabled establishment of the precise mode, mechanism, and base

specificity of binding of these alkaloids to DNA and to some extent to RNA without any ambiguity. Isoquinolines are by and large AT base pair-specific intercalative agents, which is clearly of remarkable significance as most of the so far known classical intercalating agents are GC specific. It has been now identified that suitable substitutions at 9 and 13 positions of the isoquinoline ring can significantly enhance the binding affinity. Furthermore, the fact that berberine switches its binding specificity from AT base pairs to GC base pairs when the DNA structure changes from the B-form to H<sup>I</sup>-form indicates its ability to recognize different conformational states of the DNA under different conditions. An important aspect of the isoquinolines is their ability to bind to triplex and quadruplexes that can be used for potential targeting of specific structures as anticancer therapeutics. Another important and versatile aspect of their interaction is the induction of self-structure in polyadenylic acid that can be developed for targeting the mRNA tail as a therapeutic target. Furthermore, the isoquinolines have great binding affinity to single- and double-stranded RNA structures and medium binding affinity to serum proteins. The elucidation of the recognition mechanism and the availability of a large volume of results in the last few years may provide useful guidance for their development as potential therapeutic agents in the near future.

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#### Abbreviations

LD <sub>50</sub>	lethal dose, 50%
DNA	deoxyribonucleic acid
AT	adenine-thymine
NMR	nuclear magnetic resonance
GC	guanine-cytosine
CD	circular dichroism
tRNA	transfer ribonucleic acid
BSA	bovine serum albumin
Hb	hemoglobin
HSA	human serum albumin
FRET	fluorescence resonance energy transfer
ITC	isothermal titration calorimetry



FT-IR      Fourier transform infrared  
mRNA      messenger ribonucleic acid

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## 10

# The Potential of Peptides and Depsipeptides from Terrestrial and Marine Organisms in the Fight against Human Protozoan Diseases

Jean Fotie

### 10.1

#### Introduction

Protozoan diseases such as malaria, trypanosomiasis, leishmaniasis, amebiasis, toxoplasmosis, cryptosporidiosis, sarcocystis, coccidiosis, babesiosis, and giardiasis are among the 17 tropical diseases listed as “neglected” by the World Health Organization (WHO). These diseases are caused by parasites of the genus *Plasmodium*, *Trypanosoma*, *Leishmania*, *Entamoeba*, *Toxoplasma*, *Cryptosporidium*, *Sarcocystis*, *Eimeria*, *Babesia*, and *Giardia*, respectively. Since these diseases usually occur in the same area of the world, coinfection of two or more of these parasites in a single individual are routine. This translates into a much more severe hardship than it is often indicated by the statistics. In the less wealthy parts of the world, their incidence is not only physical but it is also economical and even sociocultural. It is estimated that about 3.3 billion people (more than half of the world’s population) are at risk of these diseases [1–3] which are responsible for more than 500 million cases and nearly 1 million deaths each year. On a global scale, these protozoan diseases are believed to account for more than 10% of world global disease burden, and yet less than 1% of new drugs marketed between 1975 and 2010 were developed for their treatment [1–3].

Some of these diseases such as sarcocystis (caused by *Sarcocystis* sp.) and coccidiosis (caused by *Eimeria* sp.) are predominant primarily in animals, and are transmitted to human only through ingestion of contaminated feces or undercooked meats [4]. Other protozoan diseases such as amebiasis (caused by *Entamoeba* sp.), cryptosporidiosis (caused by *Cryptosporidium* sp.), and giardiasis (caused by *Giardia* sp.) are more prevalent in humans, with outbreaks usually associated with opportunistic infections spread through contaminated water supplies. Since this latter series of diseases are caused by free-living protozoa usually found in wastewaters and sewage, their incidence can easily be curtailed through strict hygiene guidelines.

More difficult to control are malaria, trypanosomiasis, leishmaniasis, toxoplasmosis, and babesiosis which are vector-borne diseases. They are transmitted to humans predominantly through the bites of infected mosquitoes, flies, bugs, and ticks. Among these vector-borne protozoan diseases, toxoplasmosis and babesiosis are often so mild that they can go unnoticed in an infected individual, but they can be life-threatening to people with no spleen, the elderly, and people with weak immune systems [5–8]. Babesiosis is a malaria-like disease caused by parasites of the genus *Babesia*, and transmitted to humans through tick bites or blood transfusion, while cats are believed to be the primary source of toxoplasmosis for humans, although ingestion of raw or undercooked meat and fecal contamination of hands are also considered significant risk factors [6, 8, 9].

Unlike toxoplasmosis and babesiosis, malaria, trypanosomiasis, and leishmania always result in much more serious conditions, with malaria being the most devastating of all. Malaria is a life-threatening disease caused by parasites of the genus of *Plasmodium* that are transmitted to people through the bites of infected *Anopheles* mosquitoes. According to the latest estimates, released in December 2013, there were about 207 million cases of malaria in 2012 and an estimated 627 000 deaths [1, 2]. Most of these deaths are attributable primarily to *Plasmodium falciparum* which causes most of the severe cases of malaria including cerebral malaria, believed to be more than 90% lethal regardless of the method of treatment used. However, *Plasmodium vivax* can also cause severe cases of malaria, but its incidence is relatively low when compared to *P. falciparum*. Artemisinin-based therapies are the only effective methods currently available for curing multidrug-resistant strains of *Plasmodium* around the world. However, in recent years, parasite resistance to artemisinins has been detected in Cambodia, Myanmar, Thailand, and Vietnam, probably due to the use of oral artemisinins as monotherapy [2, 10]. Although this resistance to artemisinin is still very mild and limited, its emergence would be disastrous because of the lack of alternative treatments. In fact, no new antimalarial drug is foreseen to be introduced in the market in the next decade. The worst is that there are currently no licensed vaccines against malaria, although RTS,S/AS01 (Mosquirix) vaccine against *P. falciparum* is currently being evaluated in a large clinical trial in seven countries in Africa with final results expected by the end of this year (2014) [2].

Leishmaniasis is another major protozoan disease transmitted by the bite of sand flies, and can affect different part of the body including skin (cutaneous leishmaniasis), spleen (visceral leishmaniasis), liver, and other organs [11, 12]. The disease is caused by a parasite of genus *Leishmania*, with *Leishmania major* and *tropica* which cause cutaneous leishmaniasis, and *Leishmania infantum* and *donovani* which cause visceral leishmaniasis [11, 12]. The incidence of the disease is not known with certainty, but it is estimated that half a million to one million people around the world suffer from cutaneous leishmaniasis, while about half a million suffer from visceral leishmaniasis [1, 12, 13].

As for trypanosomiasis, there are two major forms: human African trypanosomiasis (HAT) also known as *sleeping sickness* which is more prevalent in Africa and Chagas disease which is more prevalent in South America [14, 15]. HAT is transmitted through tsetse fly bites, and according to the WHO, 90% of African sleeping sickness cases are due to *Trypanosoma brucei gambiense* parasite, while the remaining 10% are attributed to *T. brucei rhodesiense* parasite [1, 14, 15]. The incidence of HAT has been decreasing; going down from 17 616 new cases reported 2004 to only 7197 in 2012 [3]. On the other hand, Chagas disease or American trypanosomiasis is caused by *Trypanosoma cruzi* transmitted to animals and people by insect vectors known as the *triatomine bug* (or *kissing bug*) [14–17]. Chagas disease is endemic throughout much of Mexico, Central America, and South America where an estimated eight million people are infected [16, 17], and the Centers for Disease Control and Prevention CDC estimates that more than 300 000 people with *T. cruzi* infection live in the United States [15–17].

In many respects, treatments currently available for curing these diseases are far from been ideal. Severe side effects and the development of resistance to many of these treatments are among the major hurdles. So, the search for a new generation of therapies for the treatment, control, and eradication of these diseases is crucial and urgent. In this chapter, we explore the possible participation of peptides and depsipeptides in the fight against human protozoan diseases.

## 10.2

### Antiprotozoan Peptides and Depsipeptides of Natural Origin and Their Synthetic Analogs

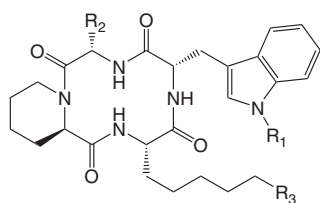
#### 10.2.1

##### Apicidins

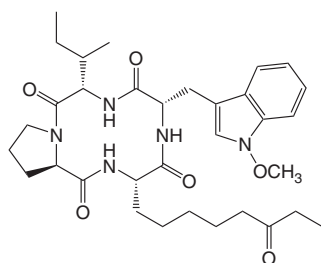
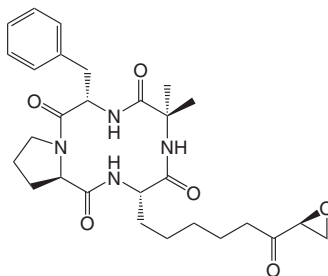
Apicidins are a small group of fungal cyclic tetrapeptides that were first isolated in 1996 from *Fusarium pallidoroseum*, an endophytic fungus from Costa Rica [18]. Several synthetic and naturally occurring apicidins have been reported, including apicidin (**1**), apicidins A (**2**), B (**3**), C (**4**), D<sub>1</sub> (**5**), D<sub>2</sub> (**6**), D<sub>3</sub> (**7**), E (**8**), and F (**9**) which are all structurally related to chlamydocin (**10**) [18–26]. They structurally consist of four amino acids, namely, *N*-methoxy-*L*-tryptophan, *L*-isoleucine, *D*-pipercolic acid, and *L*-2-amino-8-oxodecanoic acid (Aoda). However, in contrast to apicidins, chlamydocin (**10**) possess an  $\alpha$ -ketoepoxide moiety in their Aoda side chain [26].

Protozoan parasites of the family of apicomplexan are known to possess several homologs of histone deacetylases (HDACs), a family of Zn-dependent enzymes that play crucial roles in modulating mammalian cell chromatin structure, transcription, and gene expression via a dynamic process of acetylation and deacetylation of lysine residues of histones [23, 27]. HDAC inhibitors can suppress the

deacetylation process in living organisms that ultimately results in growth arrest and apoptotic cell death. Naturally occurring and synthetic apicidins have been shown to display a strong and reversible inhibitory activity on HDACs, with  $IC_{50}$  in the nanomolar range [18, 23, 24]. When tested in a whole-cell assay on *Eimeria tenella*, apicidin D1 (**5**,  $IC_{50}$  0.013  $\mu$ M) was the most active, followed by apicidin A (**2**,  $IC_{50}$  0.052  $\mu$ M), apicidin (**1**,  $IC_{50}$  0.093  $\mu$ M), apicidin C (**4**,  $IC_{50}$  0.10  $\mu$ M), apicidin D3 (**7**,  $IC_{50}$  0.40  $\mu$ M), apicidin B (**3**,  $IC_{50}$  0.41  $\mu$ M), and apicidin D2 (**6**, 0.80  $\mu$ M) [24]. Furthermore, when evaluated in a similar whole-cell assay on *P. falciparum*, apicidin D1 (**5**,  $IC_{50}$  0.034  $\mu$ M) was the most active, followed by apicidin A (**2**,  $IC_{50}$  0.059  $\mu$ M), apicidin C (**4**,  $IC_{50}$  0.069  $\mu$ M), apicidin (**1**,  $IC_{50}$  0.15  $\mu$ M), apicidin D3 (**7**,  $IC_{50}$  0.18  $\mu$ M), apicidin B (**3**,  $IC_{50}$  0.19  $\mu$ M), apicidin F (**9**,  $IC_{50}$  0.67  $\mu$ M), and apicidin D2 (**6**, 1.60  $\mu$ M). Although apicidin E (**8**) was also mentioned to display some antimalarial activity, no  $IC_{50}$  was provided [23, 24, 26].



- 1:  $R_1 = OCH_3$ ;  $R_2 = CH_3CHCH_2CH_3$ ;  $R_3 = COCH_2CH_3$  (Apicidin)
- 2:  $R_1 = H$ ;  $R_2 = CH_3CHCH_2CH_3$ ;  $R_3 = COCH_2CH_3$  (Apicidin A)
- 4:  $R_1 = OCH_3$ ;  $R_2 = CH_3CHCH_3$ ;  $R_3 = COCH_2CH_3$  (Apicidin C)
- 5:  $R_1 = OCH_3$ ;  $R_2 = CH_3CHCH_2CH_3$ ;  $R_3 = COCH(OH)CH_3$  (Apicidin D1)
- 6:  $R_1 = OCH_3$ ;  $R_2 = CH_3CHCH_2CH_3$ ;  $R_3 = COCH(S-OH)CH_2CH_3$  (Apicidin D2)
- 7:  $R_1 = OCH_3$ ;  $R_2 = CH_3CHCH_2CH_3$ ;  $R_3 = CH_2CH(OH)CH_3$  (Apicidin D3)
- 8:  $R_1 = OCH_3$ ;  $R_2 = CH_3CHCH_2CH_3$ ;  $R_3 = CH_2CH_2CH_3$  (Apicidin E)
- 9:  $R_1 = OCH_3$ ;  $R_2 = CH_2Ph$ ;  $R_3 = COOH$  (Apicidin F)

Apicidin B (**3**)Chlamydocin (**10**)

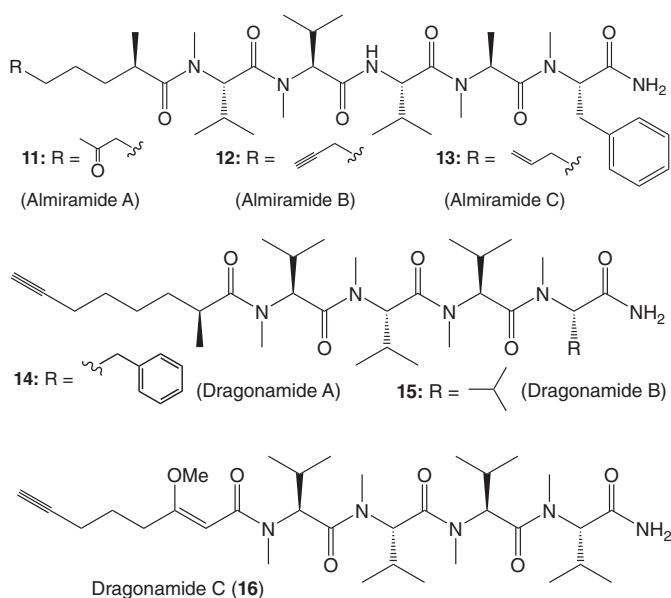
Structure–activity relationship studies of apicidins and derivatives show that slight structural changes can result in a significant improvement of the antimalarial activity. Therefore, this family of compounds represents potential leads for the development of new antimalarial drugs. It is important to mention that the total synthesis of cyclic tetrapeptoid derivatives (apicidin, apicidin A, trapoxin A and B, and chlamydocin) was achieved in 2001 by Murray *et al.* [28].

### 10.2.2

#### Almiramides and Dragonamides

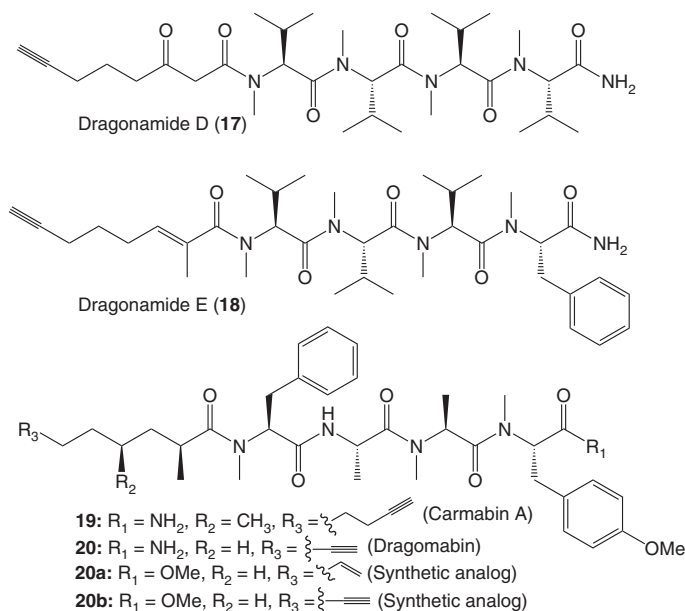
Just like apicidins, almiramides are a family of unusual acyclic *N*-methylated lipopeptides originally isolated from a Panamanian collection of marine

cyanobacterium *Lyngbya majuscula* [29, 30]. Since the tetrapeptide portion of these molecules is essentially made of *N*-methyl-L-phenylalanine, *N*-methyl-L-valine, and *N*-methyl-L-alanine, they are structurally related to another family of lipopentapeptides, namely, dragonamides, with the major difference that the (2*S*)-methyloct-7-ynoic acid end in dragonamides is replaced by (2*R*)-methyloct-7-ynoic acid in almiramides [29–31]. Although several synthetic derivatives are currently reported in the chemical literature, only almiramides A–C (**11–13**) and dragonamides A–E (**14–18**) were actually isolated from natural sources, namely, *Lyngbya sp.* [29–34]. Furthermore, carmabin A (**19**) and dragomabin (**20**) were also isolated from the same natural source, but unlike almiramides and dragonamides, these latter compounds contain, in addition to *N*-methyl-L-phenylalanine and *N*-methyl-L-alanine, L-alanine, and *N,O*-dimethyl-L-tyrosine in their tetrapeptide portion [29–31].



All these compounds and their synthetic analogs have been evaluated for their antimalarial, antileishmanial, and antitrypanosomal activities [29–31]. In fact, when testing these compounds against the W2 chloroquine-resistant *Plasmodium* strain, carmabin A (**19**), dragomabin (**20**), and dragonamide A (**14**) exhibited  $IC_{50}$  values of 4.3, 6.0, and 7.7  $\mu\text{M}$ , respectively [31], while almiramides A–C were found to be inactive up to the highest tested concentration of 13.5  $\mu\text{M}$  [29]. However, when evaluated against Vero cells, carmabin A was the most toxic with an  $IC_{50}$  value of 9.8  $\mu\text{M}$ , followed by dragonamide (**13**;  $IC_{50}$  67.8  $\mu\text{M}$ ) and dragomabin ( $IC_{50}$  182.3  $\mu\text{M}$ ) [31].

Against *Leishmania donovani*, almiramide A (**11**) appeared to be inactive at a concentration up to 13.5  $\mu\text{M}$ , while almiramides B and C (**12** and **13**) displayed a better activity, with  $\text{IC}_{50}$  values of 2.4 and 1.9  $\mu\text{M}$ , respectively [29, 30]. Against Vero cells, almiramides A–C displayed a very good safety profile, with  $\text{IC}_{50}$  values of 113.1, 52.3, and 33.1  $\mu\text{M}$ , respectively [29, 30]. Structure–activity-relationship studies indicated that this activity could be improved through the preparation of analogs [29, 30]. However, the best synthetic analog so far displayed an  $\text{IC}_{50}$  of 1.6  $\mu\text{M}$ , an activity in the same magnitude as almiramide C ( $\text{IC}_{50}$  1.9  $\mu\text{M}$ ) [29, 30].



Against *T. brucei*, almiramide A was also inactive, while almiramides B and C displayed only weak activity, with  $\text{IC}_{50}$  values of 6.0 and 3.0  $\mu\text{M}$ , respectively [30]. However, through structure–activity relationship studies, a series of more active analogs were prepared. In the most active derivative (**20a**,  $\text{IC}_{50}$  of 0.4  $\mu\text{M}$ , selective index of 47), the free amide was replaced by a methyl ester, and the terminal alkyne by a terminal alkene of the same number of carbons [30], while, in the most selective synthetic analog (**20b**,  $\text{IC}_{50}$  of 0.6  $\mu\text{M}$ , selective index of 270), only the free amide was replaced by a methyl ester, the terminal alkyne was maintained [30]. Using a series of activity-based synthetic probes to explore both the molecular target of these compounds in *T. brucei* lysates and the site localization through epifluorescence microscopy, it was shown that almiramides exert their activity likely through the perturbation of glycosomal function through the disruption of membrane assembly machinery [30]. It is important to mention that glycosomes are organelles specific to kinetoplastid parasites, and is the house of the first seven



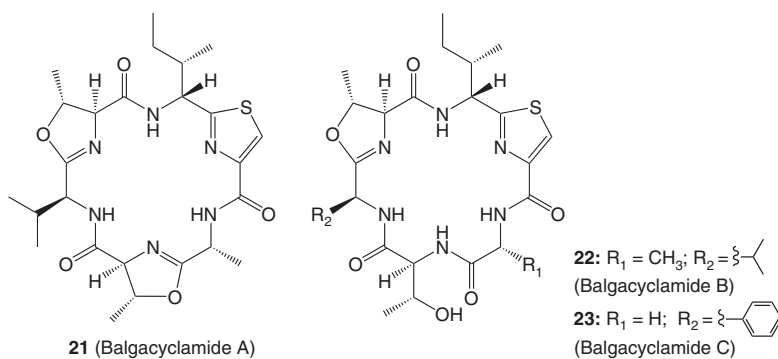
steps of glycolysis which have been shown to be essential for parasite survival in the bloodstream stage [35, 36].

### 10.2.3

#### Balgacyclamides

Balgacyclamides A–C (**21**–**23**) are hetero-macrocylic peptides isolated from *Microcystis aeruginosa* EAWAG 251 [37]. Their core structure is made of alanine, valine, and isoleucine, with hydrophobic residues containing heterocyclic rings such as oxazol(ine) or thiazol(ine) [37]. In fact, balgacyclamide A (**21**) is a tetracyclic peptide containing methyloxazoline as well as a thiazole moieties [37], while balgacyclamide B (**22**) is rather a tricyclic peptide, but structurally similar to balgacyclamide A, with only one methyloxazoline instead of the two as observed in balgacyclamide A, with the other methyloxazoline residue replaced by threonine [37]. Finally, balgacyclamide C (**23**) is very similar to its congeners, with its core structure made of methyloxazoline, phenylalanine, threonine, glycine, thiazoline, and isoleucine sequence [37]. The key feature of balgacyclamide C is the replacement of a valine and alanine from other congeners by a phenylalanine and a glycine, respectively [37].

Balgacyclamides A (**21**) and B (**22**) were shown to display micromolar-level activity when evaluated against the chloroquine-resistant strain K1 of *P. falciparum*, with  $IC_{50}$  values of 9.0 and 8.2  $\mu$ M, respectively [37]. However, these two compounds were inactive against *T. brucei*, with  $IC_{50}$  values of 59 and 51  $\mu$ M, respectively (against *T. brucei rhodesiense* STIB 900) [37] and balgacyclamide B was only marginally active against *L. donovani* MHOM-ET-67/L82, with  $IC_{50}$  value of 28  $\mu$ M. Nevertheless, both compounds displayed good selectivity with respect to their cytotoxicity to the L6 rat myoblast cell line, for which no activity was detected up to 150  $\mu$ M [37].

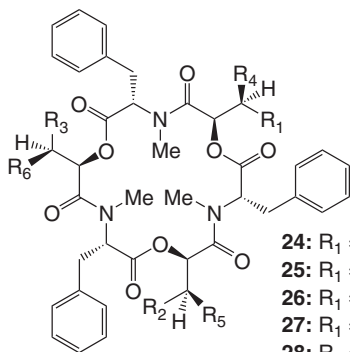


## 10.2.4

**Beauvericins and Allobeauvericins**

Beauvericins and allobeauvericins are a class of cyclohexadepsipeptides with a core structure made of three *L-N*-methylphenylalanine units connected alternately with three *D*-2-hydroxyisovaleric acid residues. They are primarily isolated from *Beauveria* sp., a family of arthropod pathogenic fungi [38–41], although they have been induced through precursor-directed biosynthesis in several other fungi including *Paecilomyces tenuipes* BCC1614 and *Acremonium* sp. BCC 28424 [42, 43]. These compounds are known to act as insecticides and are toxic to mosquito larvae [44], blowfly [45], and Colorado potato beetles [44]. Amongst the analogs reported so far, beauvericins A and B isolated from *Beauveria bassiana*, have in their structure one and two 2-hydroxy-3-methylpentanoic acid residues, respectively, instead of *D*-2-hydroxyisovaleric acid [46–48].

On evaluating some of these compounds for their antiplasmodial activity against the K1 strain of *P. falciparum* [42, 47], they displayed moderate antimalarial activity: beauvericin (**24**,  $IC_{50}$  1.7  $\mu$ M), beauvericins A (**25**,  $IC_{50}$  2.3  $\mu$ M) and B (**26**,  $IC_{50}$  2.8  $\mu$ M), allobeauvericins A (**28**,  $IC_{50}$  2.5  $\mu$ M), B (**29**,  $IC_{50}$  3.0  $\mu$ M), and C (**30**,  $IC_{50}$  1.9  $\mu$ M), while beauvericin C (**27**) was inactive [42, 47]. However, these compounds were also very toxic to the Vero cell suggesting that their antimalarial activity might be related to their cytotoxic behavior.



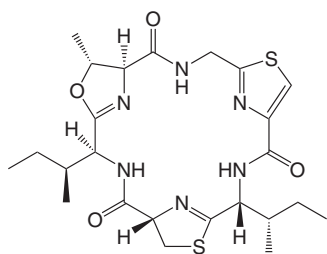
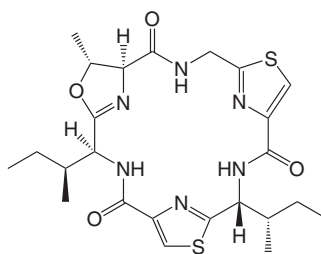
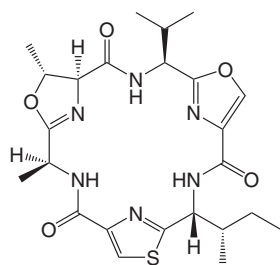
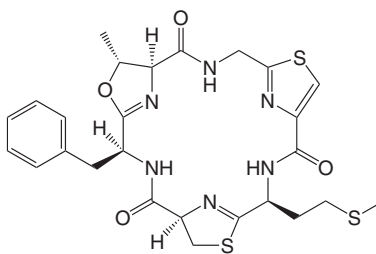
- 24:**  $R_1 = R_2 = R_3 = R_4 = R_5 = R_6 = \text{Me}$  (Beauvericin)  
**25:**  $R_1 = \text{Et}$ ,  $R_2 = R_3 = R_4 = R_5 = R_6 = \text{Me}$  (Beauvericin A)  
**26:**  $R_1 = R_2 = \text{Et}$ ,  $R_3 = R_4 = R_5 = R_6 = \text{Me}$  (Beauvericin B)  
**27:**  $R_1 = R_2 = R_3 = \text{Et}$ ,  $R_4 = R_5 = R_6 = \text{Me}$  (Beauvericin C)  
**28:**  $R_1 = R_2 = R_3 = R_5 = R_6 = \text{Me}$ ,  $R_4 = \text{Et}$  (Allobeauvericin A)  
**29:**  $R_1 = R_2 = R_3 = R_6 = \text{Me}$ ,  $R_4 = R_5 = \text{Et}$  (Allobeauvericin B)  
**30:**  $R_1 = R_2 = R_3 = \text{Me}$ ,  $R_4 = R_5 = R_6 = \text{Et}$  (Allobeauvericin C)

## 10.2.5

**Aerucyclamides**

Aerucyclamides A–D (**31–34**) are a class of hexacyclopeptides isolated from *M. aeruginosa* PCC 7806 [49, 50]. The core structure of these compounds comprises an alternation of hydrophobic and hydrophilic serine, threonine, and cysteine amino acids, with the side chains of these polar amino acids heterocyclized to

form an oxazole or thiazole ring or their reductive derivatives [49, 50]. Aerucyclamide A (**31**) is a rare example of a cyclamide that features oxazoline, thiazoline, and thiazole moieties in a single molecule, while aerucyclamide B (**32**) is an oxidative derivative of **31** [49, 50]. Actually, the treatment of aerucyclamide A with  $\text{MnO}_2$  in benzene at  $50^\circ\text{C}$  for 90 min led to the formation of aerucyclamide B [49]. It is important to mention that the structure of aerucyclamide C (**33**) turned out to be identical to that previously reported for microcyclamide 7806A [51], prompting the revision of the structure of microcyclamide 7806A [50]. In this revised structure, microcyclamide 7806A appeared to possess an ester involving the threonine group, and an ammonium residue, instead of the oxazoline ring [50]. More importantly, microcyclamide 7806A and aerucyclamide B were obtained from aerucyclamide C under acidic conditions ( $\text{CF}_3\text{CO}_2\text{H}$  in  $\text{H}_2\text{O}$ ) through a HPLC separation. This observation is in agreement with the hypothesis that all these compounds are actual metabolites produced via ribosomal synthesis in *M. aeruginosa* PCC 7806 [50]. Aerucyclamides A–D were all evaluated for their antimalarial activity against the K1 strain of *P. falciparum* ( $\text{IC}_{50}$  values of 5.0, 0.7, 2.3, and  $6.3\ \mu\text{M}$ , respectively) and for their antitrypanosomal activity against *T. brucei rhodesiense* STIB 900 ( $\text{IC}_{50}$  values Aerucyclamide D of 56.3, 15.9, 9.2, and  $50.1\ \mu\text{M}$ , respectively), with their toxicity evaluated against rat myoblast L6 cells ( $\text{IC}_{50} > 168, 120, 106,$  and  $>153\ \mu\text{M}$ , respectively) [50].

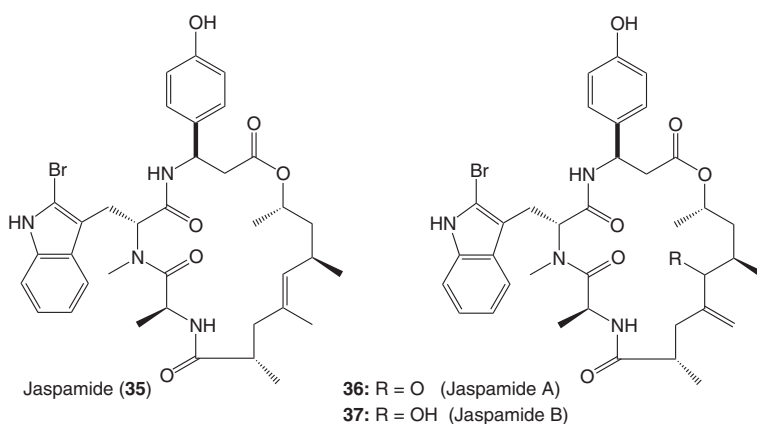
Aerucyclamide A (**31**)Aerucyclamide B (**32**)Aerucyclamide C (**33**)Aerucyclamide D (**34**)

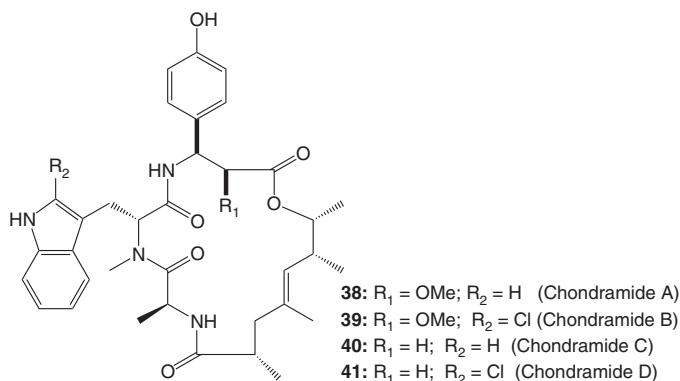
## 10.2.6

**Chondramides and Jaspamides**

Chondramides and jaspamides are a class of depsipeptides structurally made of two main moieties, the tripeptide portion comprising alanine, *N*-methyltryptophan and a  $\beta$ -tyrosine or  $\alpha$ -methoxy- $\beta$ -tyrosine residue, and the aliphatic carboxylic portion consisting of the hydroxyl-methylated-enoic acid unit [52–55]. In this respect, they are also related to geodiamolides [56–58] and neosiphoniamolide A [59]. However, there are significant differences between chondramides and jaspamides. The first difference is the presence of an acetyl in chondramides versus lactoyl starter unit for the polyketide moiety of jaspamides [52–55, 59]. Furthermore, the aliphatic carboxylic portion in chondramides is made up of (*E*)-7-hydroxy-2,4,6-trimethyloct-4-enoic acid, while in jaspamides the chain consists of a 8-hydroxynonenoic acid unit [52–55, 59]. Jaspamide (35), also known as *jaspakinolide*, and jaspamides B (36) and C (37) are widely distributed in *Jaspis* sp. [53–55, 59, 60], but they have also been found in other sponge genera, including *Auleta constricta* [61] and *Hemiassterella minor* [56], while chondramides A–D (38–41) have usually been reported from *Chondromyces* sp. [62–65].

This family of compounds is well known to stabilize actin filaments [7, 9, 66, 67]. Actin filaments are involved in cell motility, cell invasion, and phagocytosis among other processes, and apicomplexan parasites such as *Toxoplasma gondii* and *Plasmodium* sp. rely on them to cross biological barriers and invade host cells [7, 9, 66, 67]. Experiments have shown that this family of compounds inhibits cell invasion and causes the formation of an apical protrusion by redistribution of actin Jaspamide (Jaspamide A) (Jaspamide B) filaments [7, 66–69].





In whole-cell assays, the effect of jasplakinolide on the growth, invasion, and actin cytoskeleton of *P. falciparum* was examined. Jasplakinolide appeared to decrease the parasitemia in a synchronized culture of *P. falciparum* strain FCR-3 in a time- and concentration-dependent manner [69]. The decrease became evident at day 2 at concentrations of 0.32  $\mu\text{M}$  and above, and parasites are cleared at day 4. However, further studies using Giemsa-stained smears of *P. falciparum*-infected erythrocytes showed that this compound has no effect on the development of schizonts from ring forms [69]. However, there were no ring form-infected erythrocytes when jasplakinolide was administered, even after the release of merozoites, indicating that merozoites exposed to jasplakinolide failed to invade erythrocytes [69]. In addition, investigations involving *P. berghei* ookinetes also showed an uncoordinated motility induced by jasplakinolide at low concentrations, with a total absence of motility at high concentrations [70]. Similar results were observed when treating *T. gondii* [7, 9, 71], *E. histolytica*, and *E. invadens* [72] with jasplakinolide in whole-cell assays.

Although chondramides have not been tested in a whole-cell assay against any strain of *Plasmodium* for their antimalarial activity, they were shown to disrupt growth of *T. gondii* *in vitro* [66]. Furthermore, testing a series of synthetic derivatives of chondramide with substitutions in the  $\beta$ -tyrosine moiety indicated that these compounds block parasite growth on host cell monolayers, with  $\text{EC}_{50}$  values that ranged from 0.3 to 1.3  $\mu\text{M}$  [66]. It is important to mention that the total synthesis of jaspamides and chondramides has been achieved by several research groups [66, 73–78].

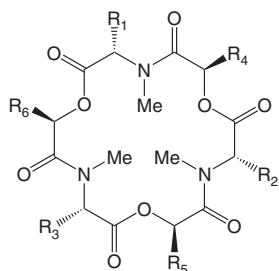
### 10.2.7

#### Enniatins and Beauverniatins

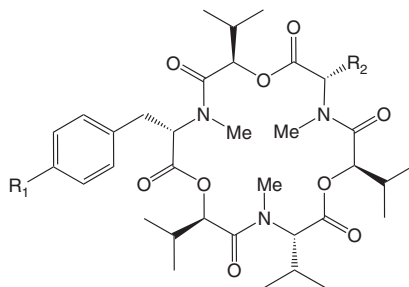
Enniatins are a class of antibiotic cyclohexadepsipeptides predominantly isolated from *Fusarium* species [79–81], but they have also been found in filamentous fungus *Acremonium* sp. [43, 82] as well as in insect pathogenic fungus

*Verticillium hemipterigenum* [83, 84]. Their core structure is made of three units of *N*-methyl-*L*-amino acid which are alternately linked to three units of (*R*)-2-hydroxyisovaleric acid resulting in an 18-membered cyclodepsipeptide structure. The major difference between enniatins and beauvenniatins is that the latter class of compounds possesses scrambled aromatic or aliphatic *N*-methyl-*L*-amino acid side chains [43].

The antimalarial activity of many compounds from this family including enniatins B (42), B4 (43), C (44), G (45), H (46), and I (47) as well as beauvenniatins A–E (48–52) were evaluated against the K1 strain of *P. falciparum* [43, 81]. Many of them displayed decent antimalarial activities, with  $IC_{50}$  values in the micromolar range: enniatins B ( $IC_{50}$  0.42  $\mu$ M), B4 ( $IC_{50}$  0.31  $\mu$ M), C ( $IC_{50}$  1.6  $\mu$ M), G ( $IC_{50}$  0.67  $\mu$ M), H ( $IC_{50}$  2.9  $\mu$ M), and I ( $IC_{50}$  0.36  $\mu$ M) [81], beauvenniatins A (3.0  $\mu$ g ml<sup>-1</sup>), B (3.0  $\mu$ g ml<sup>-1</sup>), C (3.4  $\mu$ g ml<sup>-1</sup>), D (>10  $\mu$ g ml<sup>-1</sup>), and E (2.9  $\mu$ g ml<sup>-1</sup>) [43]. These compounds also exhibited slight toxicity against Vero cells. It should be mentioned that the total synthesis of these compounds has also been achieved by several research groups [85–90].



- 42:  $R_1 = R_2 = R_3 = R_4 = R_5 = R_6 = \text{Me}$  (Enniantins B)  
 43:  $R_1 = i\text{-Bu}$ ,  $R_2 = R_3 = R_4 = R_5 = R_6 = i\text{-Pr}$  (Enniantins B<sub>4</sub>)  
 44:  $R_1 = R_2 = i\text{-Bu}$ ,  $R_3 = R_4 = R_5 = R_6 = i\text{-Pr}$  (Enniantins C)  
 45:  $R_1 = R_2 = R_3 = i\text{-Bu}$ ,  $R_4 = R_5 = R_6 = i\text{-Pr}$  (Enniantins G)  
 46:  $R_1 = R_2 = R_3 = R_5 = R_6 = i\text{-Pr}$ ,  $R_4 = s\text{-Bu}$  (Enniantins H)  
 47:  $R_1 = R_2 = R_3 = R_6 = i\text{-Pr}$ ,  $R_4 = R_5 = s\text{-Bu}$  (Enniantins I)



- 48:  $R_1 = \text{H}$ ,  $R_2 = \text{Bz}$  (Beauvenniatins A)  
 49:  $R_1 = \text{H}$ ,  $R_2 = i\text{-Pr}$  (Beauvenniatins B)  
 50:  $R_1 = \text{OH}$ ,  $R_2 = i\text{-Pr}$  (Beauvenniatins C)  
 51:  $R_1 = \text{H}$ ,  $R_2 = p\text{-Bz-OH}$  (Beauvenniatins D)  
 52:  $R_1 = \text{OH}$ ,  $R_2 = \text{Bz}$  (Beauvenniatins E)

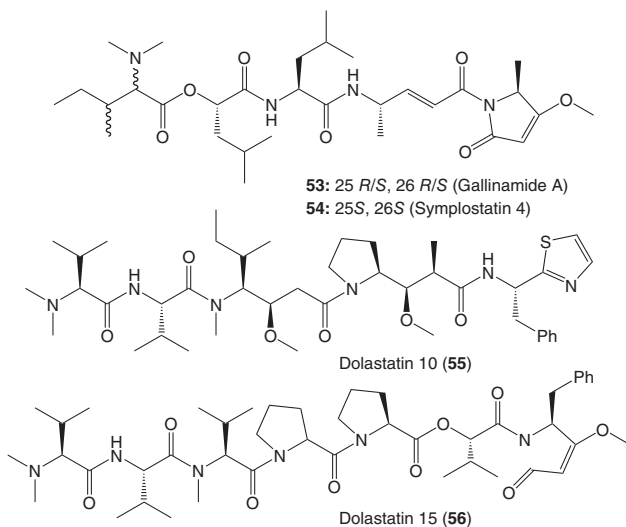
### 10.2.8

#### Gallinamide A, Dolastatins 10 and 15, and Symplostatin 4

Gallinamide A (53), a linear depsipeptide isolated from *Schizothrix* species [91], and symplostatin 4 (54) originally isolated from *Symplococa* species [92–95] share structural features with dolastatins 10 (55) and 15 (56) also isolated from *Symplococa* species [92–95]. Their core structure is comprised of an aliphatic backbone, a dimethylated *N*-terminal amino acid, an unusual 4(*S*)-amino-2(*E*)-pentenoic acid moiety, and a *C*-terminal *N*-acyl pyrroline unit. However, unlike symplostatin 4, the absolute configuration of the *N,N*-dimethylisoleucyl (C25 and C26) stereocenters of gallinamide A are not elucidated, although spectroscopic data

from symplostatins 4 have suggested that these two compounds are diastereomers [96].

Gallinamide A (**53**) was shown to have a broad but moderate antiprotozoan activity, with  $IC_{50}$  values of  $8.4 \mu\text{M}$  against the chloroquine-resistant W2 strain of *P. falciparum*,  $9.3 \mu\text{M}$  against *L. donovani*, and  $16.9 \mu\text{M}$  against *T. cruzi*, with only a marginal toxicity against mammalian Vero cells [91]. Symplostatins 4 (**54**), dolastatins 10 (**55**) and 15 (**56**) were more potent against 3D7 strain of *P. falciparum*, with  $IC_{50}$  values of 74, 0.1, and 200 nM, respectively [97, 98].



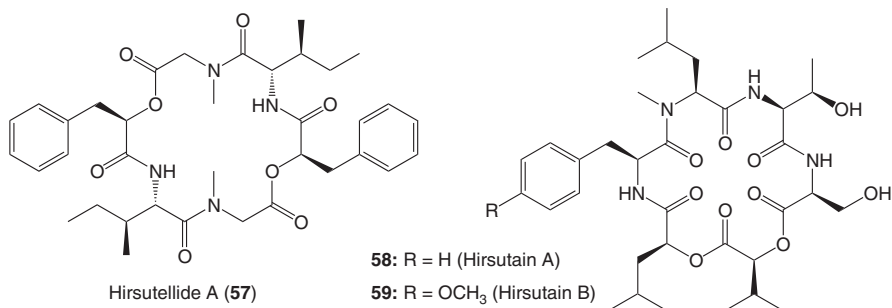
### 10.2.9

#### Hirsutatins and Hirsutellides

Hirsutellide A (**57**), and hirsutatins A (**58**) and B (**59**) are cyclohexadepsipeptides isolated from insect pathogenic fungi of the *Hirsutella* family, with hirsutellide A from *Hirsutella kobayassi* BCC 1660 [82] and hirsutatins A and B from *Hirsutella nivea* [99]. Hirsutellide A consists of two identical tridepsipeptides comprised of three basic subunits, 2-hydroxy-3-phenylpropanoic acid, *N*-methylglycine, and isoleucine, identically disposed around a twofold rotational plane of symmetry [82], while hirsutatins A and B are made of three amino acid residues, namely, *N*-methyl-*L*-leucine, *L*-threonine, and *L*-serine, with two 2-hydroxycarboxylic acid residues consisting of *L*-2-hydroxyisovaleric acid and *L*-2-hydroxyisocaproic acid [99]. The only major difference between these two latter compounds is the fact that the phenylalanine of hirsutatin A is replaced in hirsutatin B by a 4'-methoxytyrosine residue [99].

When evaluated for its antimalarial activity against the K1 strain of *P. falciparum*, hirsutellide A displayed a moderate activity, with an  $IC_{50}$  value of

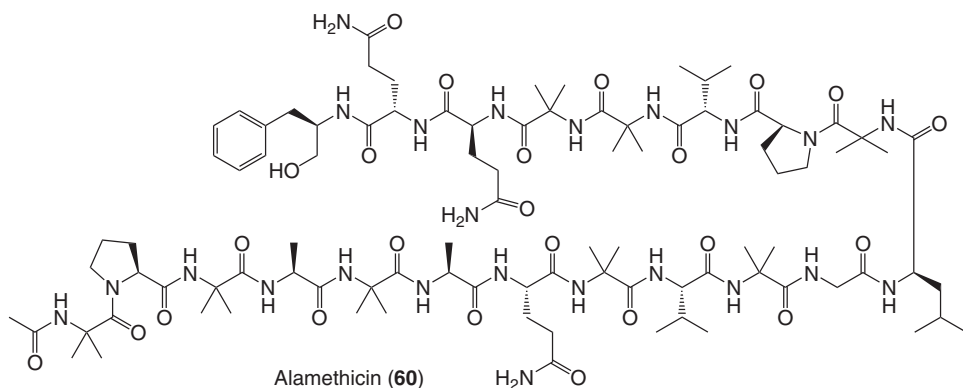
4.2  $\mu\text{M}$  [82], while amongst hirsutatins A and B, only hirsutatin B exhibited any antimalarial activity, with  $\text{IC}_{50}$  value of 5.8  $\mu\text{g ml}^{-1}$  [99]. Hirsutatin A was inactive even at a concentration of 20  $\mu\text{g ml}^{-1}$ , and none of the three compounds was toxic to Vero cells. It is important to mention that the total synthesis of hirsutellide A has been achieved by Xu *et al.* [100], rendering possible a structure–activity relationship study and optimization of its antimalarial activity.



#### 10.2.10

#### Alamethicin

Alamethicin (**60**) is a well-known linear peptide of fungal origin primarily isolated from *Paecilomyces* species [101–104]. It is rich in  $\alpha$ -aminobutyric acid, with a total of 20 residues consisting primarily of alanine, proline, valine, glutamine, and glycine [102, 103]. This compound has been shown to possess a wide window of biological activities [101–107]. It was also shown to possess a moderate activity against a Nigerian strain of *P. falciparum*, with an  $\text{IC}_{50}$  value of 2  $\mu\text{g ml}^{-1}$  [108]. This compound was more active when tested *in vitro* against *T. b. brucei* strain GUTat 3.1 and *T. b. rhodesiense* strain STIB900, with  $\text{IC}_{50}$  values of 0.17 and 0.38  $\mu\text{g ml}^{-1}$ , respectively [101].

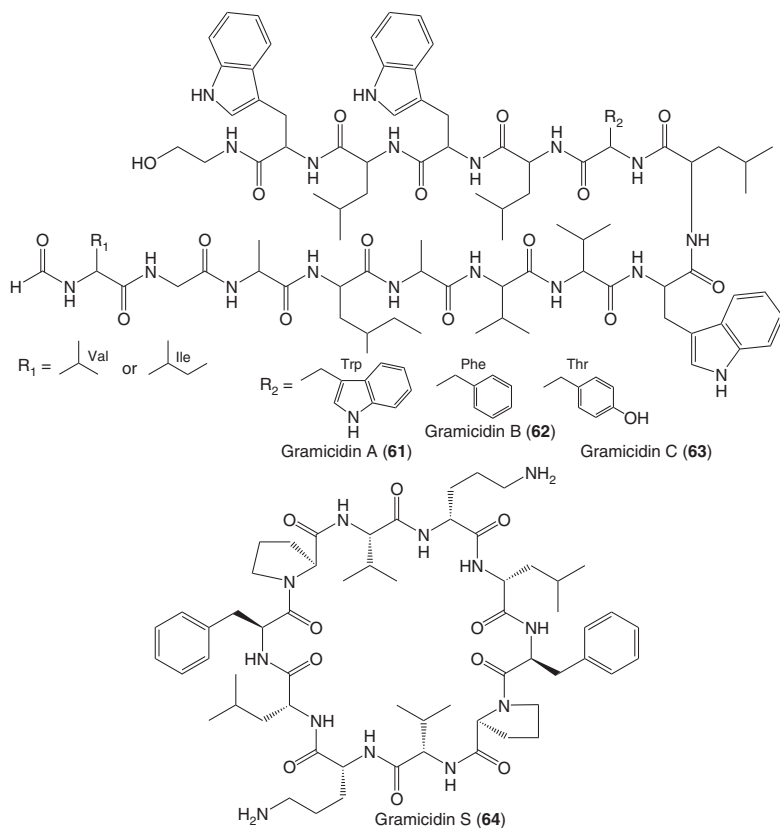




## 10.2.11

**Gramicidins**

Gramicidins are a family of peptides produced by *Bacillus brevis*, and well known for their wide window of biological activities including a strong antibiotic activity against Gram(+ve) bacteria [108–117]. Amongst them, gramicidins D, which is a mixture of six pentadecapeptides including gramicidin A (**61**), B (**62**), and C (**63**), and gramicidin S (**64**); a cyclodecapeptide also known as *gramicidin soviet* was investigated for the antimalarial activity of its components [108, 118]. Structurally, the components of gramicidin D are made up of alternating L- and D-glycine, valine, leucine, alanine, tryptophan, and tryptophan-ethanolamine, with some not well characterized variations between valine and isoleucine in these gramicidin constituents as their respective structures are not fully elucidated [108–117]. Gramicidin S on the other hand is Gramicidin A Gramicidin B Gramicidin C composed of two identical pentapeptides joined head to tail, made up of valine, ornithine, leucine, phenylalanine, and proline [108–117].

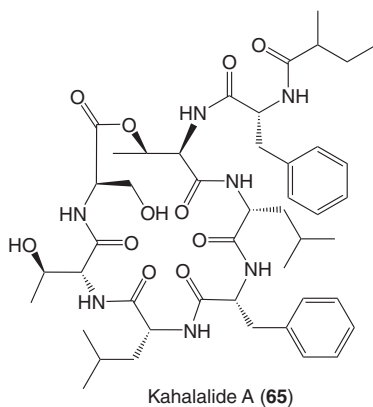


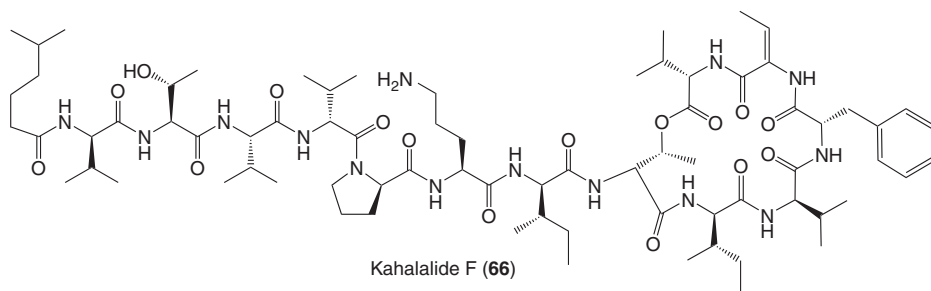
When evaluated for their antimalarial activity against *P. falciparum*, these compounds displayed different levels of activity, with gramicidin D ( $IC_{50}$   $0.035 \text{ ng ml}^{-1}$ ) being about 20 000 times more potent than gramicidin S ( $IC_{50}$   $667 \text{ ng ml}^{-1}$ ) [108]. Gramicidin D also appeared to be very potent when evaluated *in vivo* in a 4-day suppressive test with *P. chabaudi* and *P. vinckei-petteri* infected mice [118]. This compound was also shown to display usable activity against *Leishmania amazonensis* [119].

### 10.2.12

#### Kahalalides

Kahalalides are a family of marine depsipeptides primarily found in the Hawaiian herbivorous sacoglossan marine mollusk *Elysia rufescens* [120]. However, the green alga (*Bryopsis* sp.) on which the mollusk feeds is also a source of kahalalide F, but at a much lower concentration than that found in the mollusk [120–122]. Structurally, kahalalide D, the smallest of this class of depsipeptides, consists of three amino acids (L-arginine, L-proline, and D-tryptophan) and a  $\beta$ -hydroxy group as its fatty acid portion (3-hydroxy-7-methyloctanoic acid), which furnishes the ester linkage of the depsipeptide cycle. Next in size are kahalalides A, B, C, and E, with either six (C and E) or seven (A and B) amino acid residues. In kahalalide A (**65**), the ester linkage arises from the carboxyl of serine and the hydroxyl of the second threonine, with 2-methylbutyric acid, which form an amide with one of the phenylalanines, constituting its fatty acid portion. Kahalalide G is the only acyclic compound of the series, with the ester linkage between the carboxyl of the terminal valine and the hydroxyl of terminal threonine missing. Kahalalide A (**65**) is reported to possess modest antimalarial activity against *P. falciparum* ( $IC_{50}$   $11 \text{ } \mu\text{M}$ ) [120], while kahalalide F (**66**) was found to display moderate *in vitro* antileishmanial activity against *L. donovani* and *Leishmania pifanoi* promastigote as well as against *L. pifanoi* amastigotes, Kahalalide F with  $IC_{50}$  values of 6.13, 8.31, and  $29.53 \text{ } \mu\text{M}$ , respectively [123].

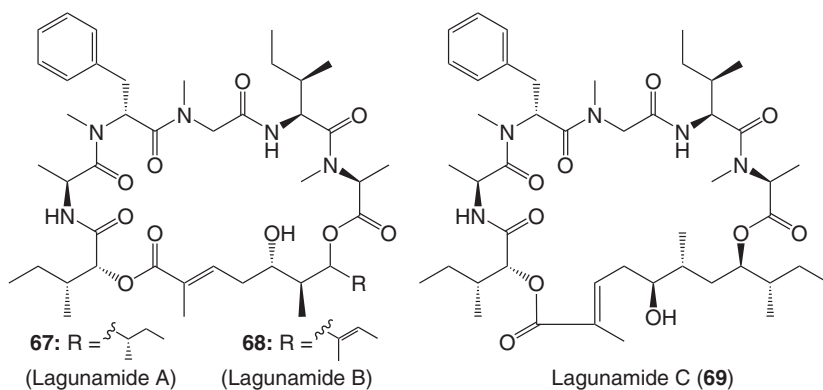




## 10.2.13

**Lagunamides**

Lagunamides are a class of cyclic depsipeptides from the marine cyanobacterium *Lyngbya majuscula* [124–126]. They are structurally made up of five proteinogenic amino acids, namely, *N*-methylalanine, isoleucine, *N*-methylglycine, *N*-methylphenylalanine, and alanine, with the hydroxyl acid portion made up of 2-hydroxyisoleucic acid [124–126]. Lagunamides A–C (67–69) were found to exhibit a pronounced antimalarial activity when tested against the chloroquine-sensitive NF54 strain of *P. falciparum*, with  $IC_{50}$  values of (Lagunamide A) (Lagunamide B) (Lagunamide C) 0.19, 0.91, and 0.20  $\mu$ M, respectively [124–126].



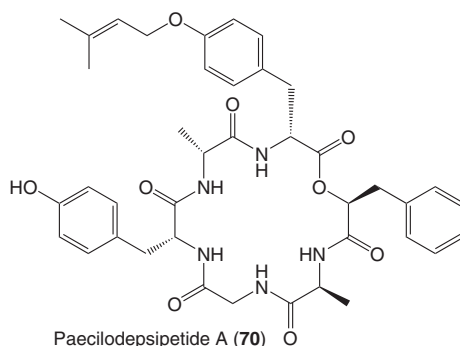
## 10.2.14

**Paecilodepsipeptides**

Paecilodepsipeptide A (70) is a cyclic depsipeptide first reported from the insect's pathogenic fungus *Paecilomyces cinnamomeus* BCC 9616, together with its linear

analogs paecilodepsipeptides B and C [127]. Structurally, paecilodepsipeptide A consists of five amino acid residues including a glycine, two alanines, a tyrosine, and an unusual *O*-prenyl-*D*-tyrosine. The ester linkage of the depsipeptides cycle arises through a coupling between the hydroxyl group of the 2-hydroxycarboxylic acid residue (3-phenyllactic acid) and the carboxyl of the *O*-prenyltyrosine. The linear analogs (paecilodepsipeptides B and C) are the hydrolysis and methanolysis derivatives of paecilodepsipeptide A, respectively [127].

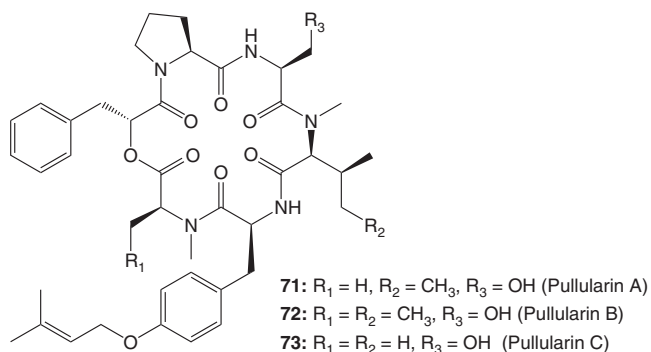
Amongst these compounds, only paecilodepsipeptide A displayed antimalarial activity against *P. falciparum* K1 strain, with an  $IC_{50}$  value of 4.9  $\mu M$ , with no toxicity to Vero cells even at a concentration of 67  $\mu M$  [127]. Linear analogs were shown to be inactive in this assay, suggesting the importance of the cyclic depsipeptide structure for the antimalarial activity [127].



### 10.2.15

#### Pullularins

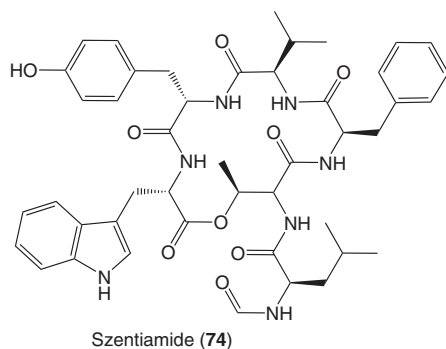
Pullularins are a class of cyclohexadepsipeptides isolated from endophytic fungus *Pullularia* sp. BCC 8613. Structurally, these compounds vary not only in the substitution patterns on the amino acids, but also by the nature of amino acids themselves. However two structural features are consistent across them: one (*S*)-*O*-prenyltyrosine unit and a combination of (*S*)-proline and (*R*)-2-hydroxy-3-phenylpropionic acid [128, 129]. For example, in pullularin B, the *N*-methylalanine found in pullularin A is replaced by 2-methylaminobutyric acid, while in pullularin C, the same *N*-methylalanine found in pullularin A (pullularin A) is replaced by *N*-methylisoleucine. Pullularins A (71), B (72), and C (73) were (pullularin B) found to possess moderate (pullularin C) antimalarial activity, with  $IC_{50}$  values of 4.6, 4.2, and 13  $\mu M$ , respectively, with little or no toxicity to Vero cells and KB, BC, and NCI-H187 cancer cell lines [129].



## 10.2.16

**Szentiamide**

Szentiamide (**74**) is a cyclohexadepsipeptide isolated from the entomopathogenic bacterium *Xenorhabdus szentirmaii* DSM 16338T, and structurally made of D-leucine, L-threonine, D-phenylalanine, D-valine, L-tyrosine, and L-tryptophane [130, 131]. This compound was tested against several protozoa parasites including *T. b. rhodesiense*, *L. donovani*, and *P. falciparum* [131]. Szentiamide was found to be more active against *P. falciparum* ( $IC_{50} = 0.995 \mu\text{g ml}^{-1}$ ) and only marginally active against *T. b. rhodesiense* and *L. donovani*, with  $IC_{50}$  values of 10.0 and  $11.0 \mu\text{g ml}^{-1}$ , respectively, but with a 50- to 80-fold weaker cytotoxicity (L6 cells,  $IC_{50} = 57.4 \mu\text{g ml}^{-1}$  and HeLa cells,  $IC_{50} > 80 \mu\text{g ml}^{-1}$ ) [131].

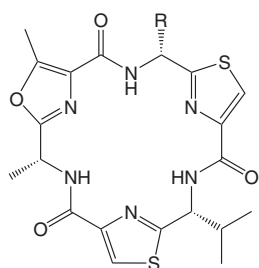


## 10.2.17

**Venturamides**

Similar to aerucyclamides, venturamides is a family of thiazole and oxazole cyclodepsipeptides isolated from the marine cyanobacterium *Oscillatoria* sp.

[132, 133]. In addition to oxazole and thiazole, their structure comprise amino acids such D-alanine and D-valine, with the major difference between venturamides A (75) and B (76) being the fact that the alanine adjacent to the thiazole ring in venturamide A is replaced by a threonine residue in venturamide B [132, 133]. When testing venturamides A and B for their antiprotozoa activities against the W2 chloroquine-resistant strain of *P. falciparum*, *T. cruzi*, and *L. donovani*, both compounds displayed a moderate antimalarial activity with  $IC_{50}$  values of 8.2 and 5.2  $\mu\text{M}$ , weak antitrypanosomal activity with  $IC_{50}$  values of 14.6 and 15.8  $\mu\text{M}$ , and only a marginal antileishmanial activity ( $IC_{50} > 19 \mu\text{M}$ ) for both compounds [132]. It should be mentioned that both compounds displayed only mild cytotoxicity to mammalian Vero cells ( $IC_{50}$  86 and 56  $\mu\text{M}$ , respectively) [132].



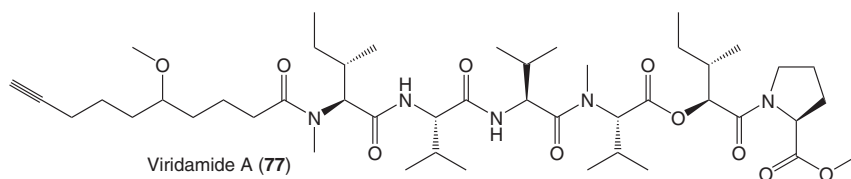
75: R = CH<sub>3</sub> (Venturamide A)

76: CHOHCH<sub>3</sub> (Venturamide B)

### 10.2.18

#### Viridamides

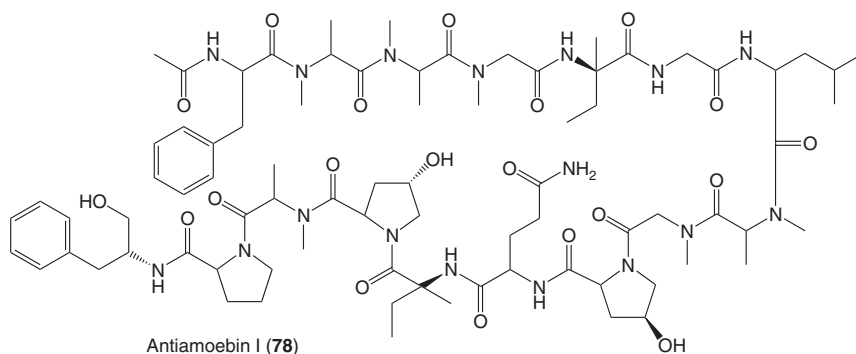
Viridamides are a class of lipodepsipeptides from the marine cyanobacterium *Oscillatoria nigro-viridis*, and structurally comprised of an *N*-methylisoleucine, two aniline residues, an *N*-methylvaline, a 2-hydroxy-3-methylpentanoic acid residue, a 5-methoxydec-9-ynoic acid moiety, and an unusual proline methyl ester terminus [134, 135]. Viridamide A (77) was shown to possess broad antiprotozoa activities, with an  $IC_{50}$  value of 5.8  $\mu\text{M}$  against *P. falciparum*, 1.5  $\mu\text{M}$  against *Leishmania mexicana*, and 1.1  $\mu\text{M}$  against *T. cruzi*, with no cytotoxicity against cancer lines HCT-116 ( $IC_{50}$  250  $\mu\text{M}$ ) and H-125 ( $IC_{50}$  200  $\mu\text{M}$ ) [135].



## 10.2.19

**Antiamoebin I**

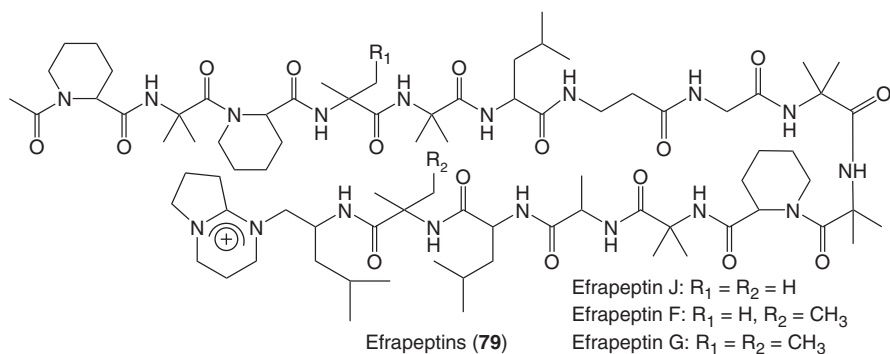
Antiamoebin I (78) belongs to the family of peptaibol antibiotics isolated from fungi of the species of *Cephalosporium*, *Emericellopsis*, *Gliocladium*, and *Stilbella* [136–138]. This peptide is comprised of five  $\alpha$ -aminoisobutyric acid and two isovaline residues, out of a total of 16 residues that constitute its structure, with the remaining amino acids been glycine, leucine, phenylalanine, hydroxyproline, glutamine, and proline [136–138]. Antiamoebin I was shown to possess a weak antimalarial activity ( $IC_{50}$  6.16  $\mu$ M) when evaluated against *P. falciparum* clone T9/106 [139]. When evaluated *in vivo* in mice affected with *Trypanosoma evansi*, this compound was able to display some positive effect, but all the infected mice eventually died by day 7 [140].



## 10.2.20

**Efrapeptins**

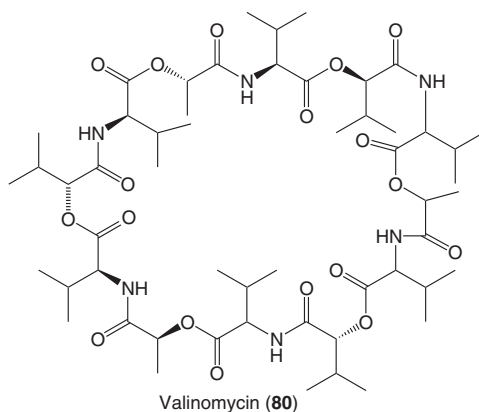
Efrapeptins (79) are a mixture of closely related antibiotic peptides found in *Tolypocladium* sp. [141–146]. Their structure consists primarily of the nonproteinogenic amino acids  $\alpha$ -aminoisobutyric acid, isovaline,  $\beta$ -alanine, and pipercolic acid, with a characteristic acetylated N-terminus, and an unusual cationic C-terminal head group, namely, 1-isobutyl-2-[1-pyrrolo-(1,2-a)pyrimidinium-2,3,4,6,7,8-hexhydrolethyl]ethylamine. The structure of only some of the peptides from efrapeptins' mixtures has been fully established [141–146]. Efrapeptins mixture Efrapeptins Efrapeptins J: Efrapeptins F: was found to Efrapeptins G: display potent antimalarial activity when tested against *P. falciparum* clone T9/106, with an  $IC_{50}$  value of 1.37  $\mu$ M [139].



## 10.2.21

**Valinomycin**

Valinomycin (80) is an antibiotic cyclododecadepsipeptide first reported from *Streptomyces fulvissimus* in 1955 [147], but latter found in other *Streptomyces* sp. [148–151]. Its structure consists of a sequence of a triple repeating unit of D- $\alpha$ -hydroxyisovaleryl-D-valyl-L-lactyl-L-valyl [148–151]. This compound was found to possess a broad window of antiprotozoan activities. In fact, it displayed strong activity against the 3D7 strain of *P. falciparum* ( $IC_{50}$  7.1 nM) [108, 152], *L. major* ( $IC_{50}$  3.2 nM) [149], and *T. brucei* ( $IC_{50}$  0.11  $\mu$ M) [149]. This compound was also very active against *Babesia* sp. with only a marginal effect on Vero cells ( $IC_{50}$  10  $\mu$ M) [148, 153].



## 10.2.22

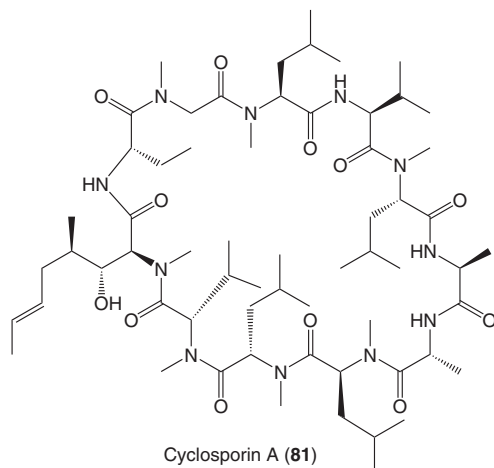
**Cyclosporins**

Cyclosporins are a large class of closely related cyclic undecapeptides initially found in *Trichoderma polysporum*, which was initially known as *Tolypocladium*



*niveum* and also referred to as *Tolypocladium inflatum* or *Beauveria nivea* (for a comprehensive review on the history of this class of compounds, cf. [154]). However, this family of compounds has been found in many other fungal species [154]. Cyclosporin A (**81**) is the most abundant and most studied family. Structurally, it consists of L-aminobutyric acid, sarcosine, four units of methyl leucine, L-valine, L-alanine, D-alanine, and methylvaline [154].

Cyclosporin A (**81**) is well known for its immunosuppressive activity, but has also displayed a wide range of biological activities including anti-inflammatory, antifungal, and antiparasitic properties [155–165]. In fact, cyclosporin A displayed a pronounced antimalarial activity *in vitro* and *in vivo*, with an  $IC_{50}$  value in the lower micromolar range against several *P. falciparum* strains *in vitro*, and a potential to clear infection in mice with five daily oral doses of 25 mg  $kg^{-1}$  [156, 162, 166–169]. It was also shown to display strong activity against a wide range of protozoa parasite including *Trypanosoma* sp. [158, 159, 161, 170], *Leishmania* sp. [155, 171–173], *Toxoplasma* sp. [174–177], *Cryptosporidium* sp. [157, 178], *Eimeria* sp. [160, 179, 180], and *Giardia* sp. [163, 181].

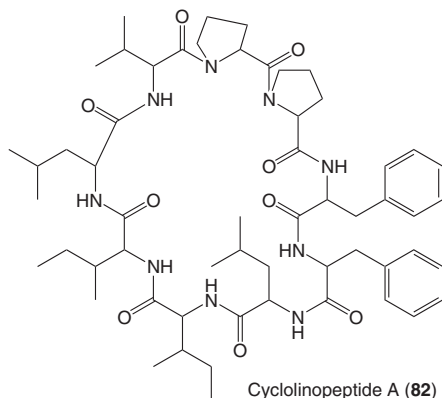


### 10.2.23

#### Cyclolinopeptides

Cyclolinopeptides are a group of natural hydrophobic cyclic peptides comprising eight or nine amino acid residues, and routinely isolated from *Linum usitatissimum* [182–185]. Cyclolinopeptide A (**82**), the first of the group to be discovered, is structurally made of two units of proline, two units of phenylalanine, two units of leucine, two units of isoleucine, and one unit of valine. Eight other derivatives were then isolated from flaxseed, and two are synthetic derivatives [182–185]. Among them, only cyclolinopeptide A and synthetic analogs have displayed some

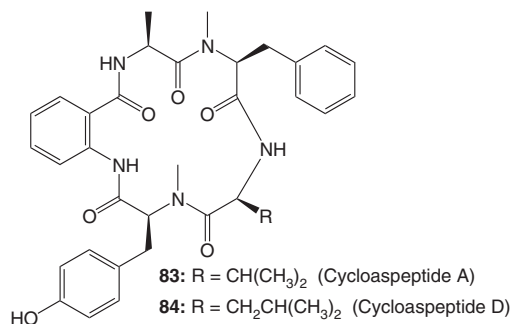
antimalarial activity against *P. falciparum*, with  $IC_{50}$  values between 0.8 and  $16 \mu\text{g ml}^{-1}$  [169].



#### 10.2.24

#### Cycloaspeptides

Cycloaspeptides A–E Cyclolinopeptide A are a group of pentacyclopeptides regularly found in *Penicillium* sp. [186–190], although some have been reported in *Isaria farinosa* [191] and *Aspergillus* sp. [192, 193]. They are structurally made of L-alanine or L-N-methylalanine, L-N-methylphenylalanine, L-leucine, L-methyltyrosine residues, and an amino benzoyl, with some major differences in their sequencing from one compound to another. Among them, only cycloaspeptides A (83) and D (84) have exhibited (Cycloaspeptide A) (Cycloaspeptide D) some activity ( $IC_{50}$  3.5 and  $4.7 \mu\text{g ml}^{-1}$ , respectively) against *P. falciparum* [190].

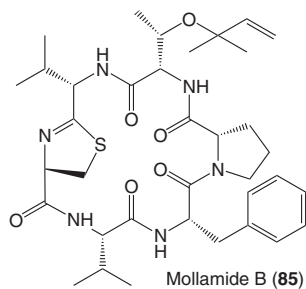


#### 10.2.25

#### Mollamides

Mollamides A–F are a class of thiazoline hexapeptides found in Indonesian tunicate *Didemnum molle* [194–196]. Although many compounds from this family possess the very characteristic isoprene side chain, they are different not

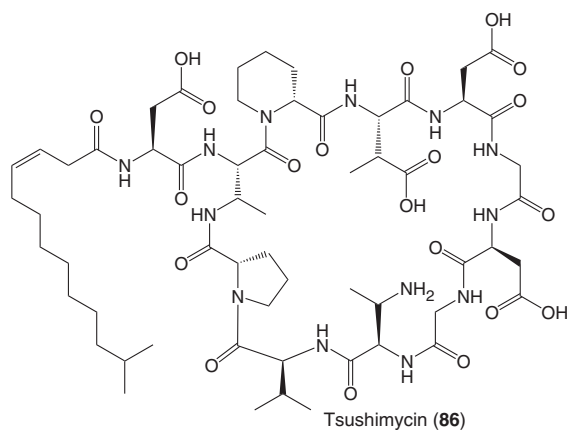
only by the nature of amino acid residues present, but also by their sequencing in each molecule. For example, the sequence in mollamide B consists of proline-phenylalanine-valine-thiazoline-valine-threonine, while in mollamide C, it consists of glycine-proline-isoleucine-thiazole-leucine-serine [195]. Only mollamide B (**85**) was shown to exhibit moderate antimalarial activity against D6 clone and W2 clone strains of *P. falciparum*, with  $IC_{50}$  values of 2.0 and  $2.1 \mu\text{g ml}^{-1}$ , respectively [195]. Against *L. donovani*, this compound displayed only a marginal activity, with an  $IC_{50}$  value of  $18 \mu\text{g ml}^{-1}$  [195].



#### 10.2.26

#### Tsushimycin

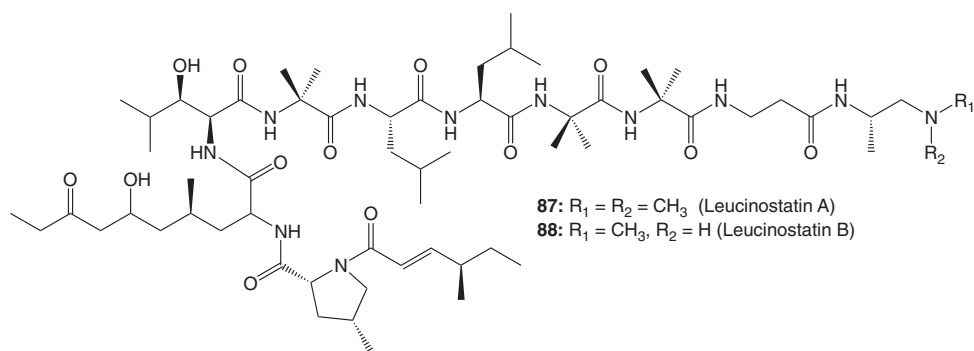
Tsushimycin (**86**) is a cyclic peptide antibiotic that was first reported from the culture filtrate of a *Streptomyces* strain Z-237 [197, 198]. Structurally, this compound is related to the amphotycin and glutamycin groups, but differs in the amino acid and fatty acid moieties. This compound is comprised of aspartic acid, glycine, proline, pipercolic acid, valine,  $\beta$ -methylaspartic acid, and  $\alpha,\beta$ -diaminobutyric acid, with the fatty acid moiety made of *cis*-3-isotetradecenoic acid [197–199]. When tested *in vitro* against *T. b. brucei* strain GUTat 3.1 and *T. b. rhodesiense* strain STIB900, this compound exhibited potent antitrypanosomal activity, with  $IC_{50}$  values of 1.09 and  $2.49 \mu\text{g ml}^{-1}$ , respectively [101].



## 10.2.27

**Leucinostatins**

Leucinostatins are a group of linear peptides isolated from a common hyphomycetes *Paecilomyces lilacinus* (Thom) Samson [200–203]. These compounds are essentially made of  $\beta$ -alaninamide, *cis*-4-methyl-1-(4-methyl-1-oxo-2-hexenyl), L-proline, 3-hydroxy-L-leucine, 2-methylalanine, 6-hydroxy-4-methyl-8-oxo-L-2-aminodecanoic acid, and L-leucine residues. The difference between the members of this group reside in the number of individual amino acid residues present, the substitution pattern on these residues, as well as the length of the fatty acid chain [200–203]. Leucinostatins A (**87**) and B (**88**) were shown to display a strong *in vitro* activity against *T. b. brucei* strain GUTat 3.1 and *T. b. rhodesiense* strain STIB900, with  $IC_{50}$  values of 7.8 and 3.4  $ng\ ml^{-1}$  for leucinostatin A, and 8.3 and 4.4  $ng\ ml^{-1}$  for leucinostatin B, respectively [101].

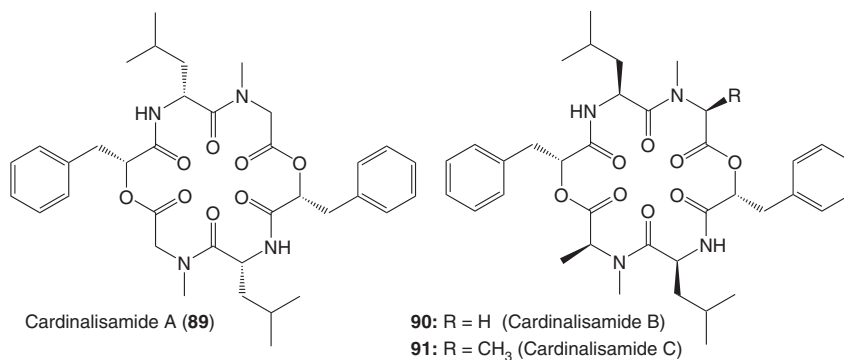


## 10.2.28

**Cardinalisamides**

Cardinalisamides A–C (**89–91**) are a group of cyclodepsipeptides isolated from the insect pathogenic fungus *Cordyceps cardinalis* NBRC 103832 [204]. These are hexacyclodepsipeptides made of three different amino acid residues and a phenyllactic acid moiety. While cardinalisamide A (**89**) is made only of two units of *N*-methylglycine, two units of leucine, and two units of phenyllactic acid, cardinalisamide B (**90**) is comprised of one unit of *N*-methylglycine, one unit of *N*-methylalanine, two units of leucine, and two units of phenyllactic acid [204]. The only difference between cardinalisamides B and C (**91**) is the replacement of the *N*-methylglycine units in cardinalisamide B with a unit of *N*-methylalanine in cardinalisamide C [204]. These three compounds were shown to display moderate *in vitro* antitrypanosomal activity against *T. b. brucei*, with  $IC_{50}$  values of 8.6, 8.7, and 8.6  $\mu g\ ml^{-1}$ , respectively, but were also toxic against normal human

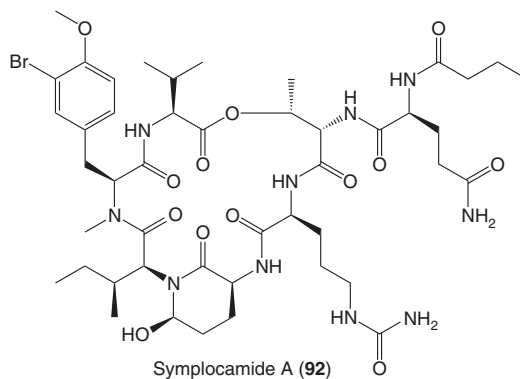
diploid fibroblasts (MRC-5 cells) with  $IC_{50}$  values of 18.5, 14.0, and 23.8  $\mu\text{g ml}^{-1}$ , respectively [204].



### 10.2.29

#### Symplocamide A

Symplocamide A (92) is a cyclodepsipeptide isolated from marine *Cyanobacterium symploca* sp. [205, 206]. Structurally, this compound is comprised of four aminoacid residues (L-valine, L-isoleucine, L-glutamic acid, and L-threonine), a 3-amino-6-hydroxypiperidone unit, an *N*, *O*-dimethyl-3-bromotyrosine unit, and a butamide unit [205, 206]. This compound displayed a moderate antimalarial activity against W2 strain of *P. falciparum* ( $IC_{50}$  0.95  $\mu\text{M}$ ), and was inactive against *T. cruzi* ( $IC_{50}$  > 9.5  $\mu\text{M}$ ) and *L. donovani* ( $IC_{50}$  > 9.5  $\mu\text{M}$ ) [206].

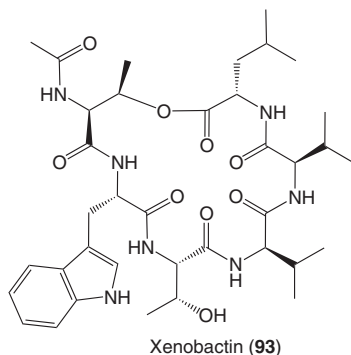


### 10.2.30

#### Xenobactin

Xenobactin (93) is a hexacyclodepsipeptide reported from the entomopathogenic bacteria of the genus *Xenorhabdus* [207]. Structurally, this compound is

comprised of six amino acid residues, namely, valine, leucine, isoleucine, threonine, and tryptophan, with the depsi moiety made of acetic acid and the threonine residue incorporated twice. [207]. This compound was shown to display a weak *in vitro* activity ( $IC_{50}$   $12.5 \mu\text{g}\cdot\text{ml}^{-1}$ ) against the erythrocytic stage of *P. falciparum* NF54, and only a marginal activity against the bloodstream form of *T. brucei rhodesiense* STIB900 ( $IC_{50}$   $31.6 \mu\text{g}\cdot\text{ml}^{-1}$ ) and the trypomastigote Xenobactin forms of *T. cruzi* Tulahuen C4 ( $IC_{50}$   $67.0 \mu\text{g}\cdot\text{ml}^{-1}$ ) [207].



### 10.3

#### Concluding Remarks

As discussed in this chapter through selected examples, peptides and depsipeptides are a wide and very diverse family of compounds, with a broad window of biological and pharmacological activities. As a result, some of these compounds are currently in clinical use or have entered human clinical trials as antibiotic or anticancer agents. Although many of them have been shown to display broad and usable antiprotozoan activities, none of them, to the best of our knowledge, has advanced in clinical use or clinical trials for that purpose. Furthermore, as many of these infections are listed as neglected diseases, and are endemic mainly in the less wealthy parts of the world, the limited quantities of peptides and depsipeptides often isolated from natural sources, coupled to their tedious synthetic accessibility are potential drawbacks to this family of compounds as potential antiprotozoan drug candidates. However, as the total synthesis of many of these families of compounds has been achieved, this should enable structure–activity relationship studies that could lead to more potent analogs. In fact, the main goal of structural optimization is to generate analogs with superior activity while removing the unnecessary complexity of the molecule. It is then possible that the most potent analog could also be easily accessible. It is then worthwhile for this family of compounds to be given serious and careful consideration for their antiprotozoan activity. It is anticipated that the present chapter would act as a stimulus in this direction.

## Abbreviations

Aoda	L-2-amino-8-oxodecanoic acid
CDC	Centers for Disease Control and Prevention (in USA)
HAT	human African trypanosomiasis
HDAC	histone deacetylases
WHO	World Health Organization

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## 11

# Sesquiterpene Lactones: A Versatile Class of Structurally Diverse Natural Products and Their Semisynthetic Analogs as Potential Anticancer Agents

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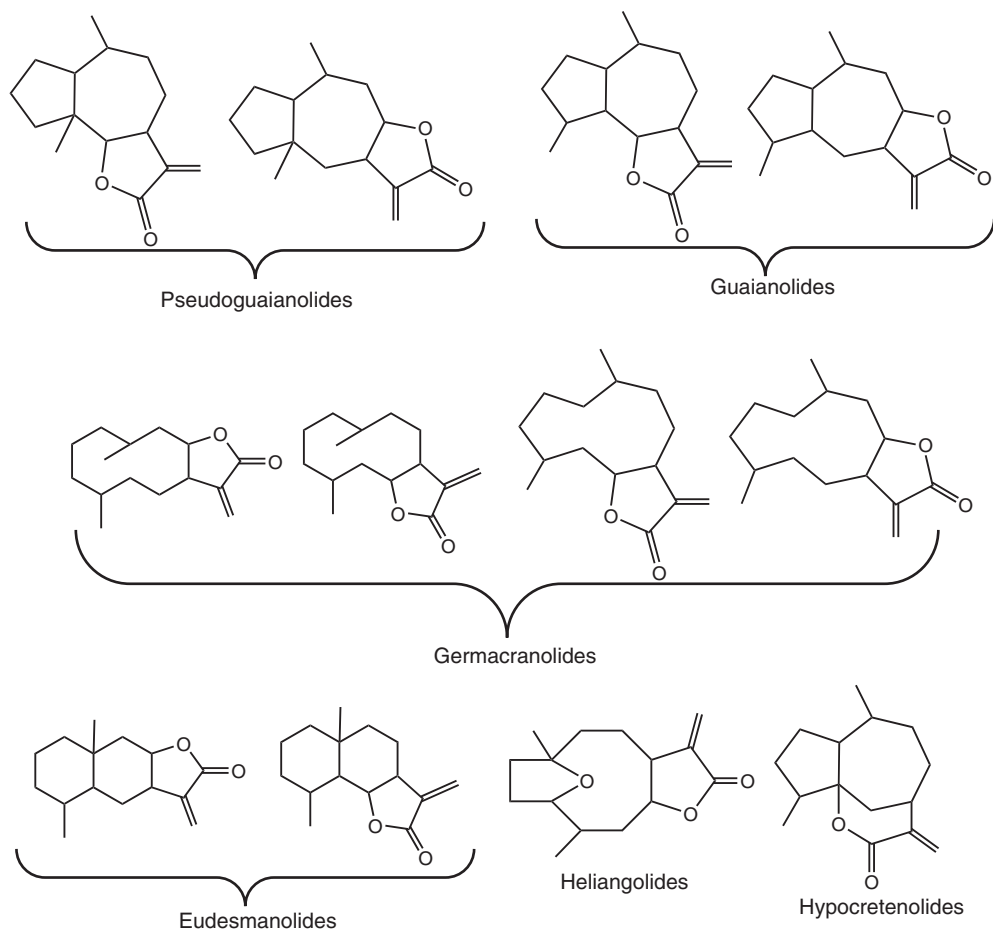
### 11.1

#### Introduction: Structural Features and Natural Distribution

Sesquiterpene lactones (SLs) constitute a large and diverse group of biologically active plant secondary metabolites that have been identified in several plant families such as Acanthaceae, Anacardiaceae, Apiaceae, Euphorbiaceae, Lauraceae, Magnoliaceae, Menispermaceae, Rutaceae, Winteraceae, and Hepatideae. [1]. However, the greatest numbers are found in the Compositae (Asteraceae) family with over 3000 different structures being reported [2]. SLs are a group of secondary metabolites that represent a diverse and unique class of natural products and are an important constituent of essential oils, which are formed from head-to-tail condensation of three isoprene units and subsequent cyclization and oxidative transformation to produce a cis or trans fused lactone. These phytochemicals are primarily classified on the basis of their carbocyclic skeletons into pseudoguaianolides, guaianolides, germacranolides, eudesmanolides, heliangolides, hryptocretenolides, and so on (Figure 11.1). The suffix “olide” refers to the lactone function and is based on costunolide, a germacranolide which is related to the 10-membered carbocyclic sesquiterpene, germacrone. However, SLs exhibit a variety of other skeletal arrangements.

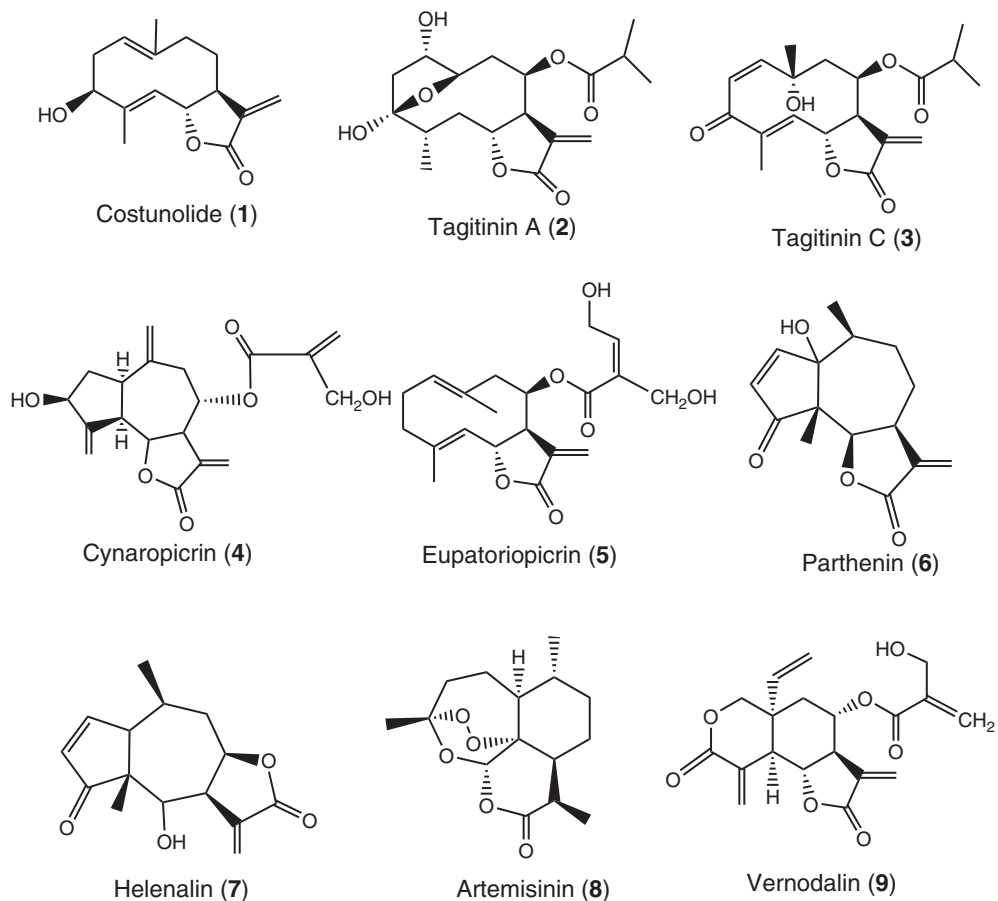
An individual plant species generally produces one skeletal type of SLs concentrated primarily in the leaves and flower heads. The percentage of SLs per dry weight may vary from 0.01% to 8%. Losses of livestock intoxicated by plants containing SLs are well known. In fact, they have been shown to exhibit a wide range of biological activities.

An important feature of the SLs is the presence of a  $\gamma$ -lactone ring (closed toward either C-6 or C-8) containing in many cases, an  $\alpha$ -methylene group which is also the feature most responsible for the biological effects and sometimes essential for the cytotoxic activity. Among other modifications, the incorporation of hydroxyls or esterified hydroxyls and epoxide ring are common. A few SLs occur in glycoside form and some contain halogen or sulfur atoms [3]. A majority



**Figure 11.1** Basic skeletons of sesquiterpene lactones.

of SLs have shown cytotoxic activity (KB and P388 leukemia *in vitro*) and activity against *in vivo* P388 leukemia. Structure–activity relationship (SAR) studies showed that various cytotoxic SLs react with thiols, such as cysteine residues in the protein, by rapid Michael type of addition. These reactions are mediated chemically by the  $\alpha,\beta$ -unsaturated carbonyl system present in the SLs. These studies support the view that SLs inhibit tumor growth by selective alkylation of growth regulatory biological macromolecules such as key enzymes, which controls cell division, thereby inhibiting a variety of cellular functions, which directs the cell into apoptosis. Differences in activity between individual SLs may be explained by different numbers of alkylating structural elements. However, other factors such as lipophilicity, molecular geometry, and chemical



**Figure 11.2** Structurally diverse sesquiterpenes lactones (SLs 1–9).

environment or the target sulfhydryl may also influence the activity of SLs. Some structurally diverse SLs are shown in Figures 11.2 and 11.3. Distribution of the different structural classes of SLs is depicted in Table 11.1.

## 11.2

### Anticancer Activity of Sesquiterpenes Lactones

In recent years, many researchers over the world have reported that SLs possess potential anticancer activity. Some of the important compounds of this class are screened herein in regard to their anticancer activity.

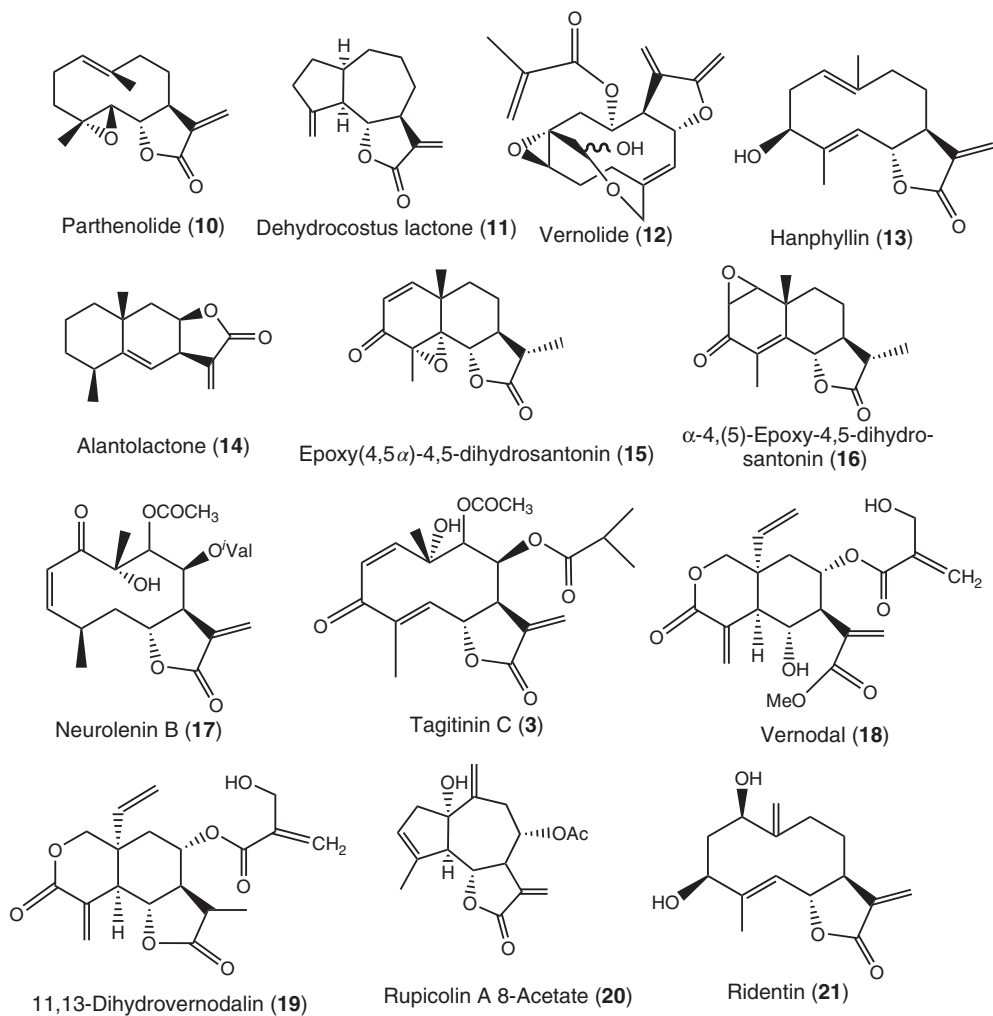


Figure 11.3 Structurally diverse sesquiterpene lactones (10–21).

### 11.2.1

#### Costunolide and Analogs

Costunolide (1, Figure 11.2) is an active component from the crude extract of the root of *Saussurea lappa clarks*, a traditional Chinese medicinal herb [3]. The anticancer property of costunolide was first reported in a rat intestinal carcinogenesis model induced by azoxymethane and supported by a subsequent study using a 7,12-Dimethylbenz(a)anthracene (DMBA)-induced hamster buccal pouch carcinogenesis model [4]. Following these two *in vivo* experiments,



**Table 11.1** Distribution of different structural classes of sesquiterpene lactones in *Compositae* plants.

Tribes (number of genera)	Number of genera with sesquiterpene lactones	Type of lactones present
Eupatorieae (50)	4	Germacranolides Elemnolides Guaianolides Ambrosanolides <i>Seco</i> -Ambrosanolides
Vernonieae (50)	4	Germacranolides Elemnolides Guaianolides
Astereceae (100)	1	Germacranolides Guaianolides Elemnolides
Inuleae (100)	5	Guaianolides Xanthanolides Ambrosanolides Helenanolides <i>Seco</i> -Eudesmanolides <i>Seco</i> -Ambrosanolides
Heliantheae (250)	24	Germacranolides Elemnolides Guaianolides Eudesmanolides Xanthanolides Ambrosanolides Helenanolides <i>Seco</i> -Eudesmanolides <i>Seco</i> -Ambrosanolides <i>Seco</i> -Helenanolides
Senecioneae (50)	4	Germacranolides Xanthanolides Eremophilanolides Helenanolides Bakkenolides
Anthemideae (50)	10	Germacranolides Elemnolides Guaianolides Helenanolides Cadinanolides Chrymoranolides

*(continued overleaf)*

Table 11.1 (Continued)

Tribes (number of genera)	Number of genera with sesquiterpene lactones	Type of lactones present
Arcototeae-Calenduleae (50)	1	Guaianolides
Cynareae (50)	8	Germacranolides Elemnolides Guaianolides Eudesmanolides
Mutisieae (55)	1	Eudesmanolides
Lactucaae (75)	7	Germanocranolides Eudesmanolides Guaianolides

considerable efforts have been devoted to understand the mechanism responsible for the anticancer activity of costunolide. First, costunolide is a potent apoptotic inducer in cancer cells, via multiple pathways. It has been reported that costunolide readily depletes intracellular glutathione (GSH) and disrupts the cellular redox balance [5]. It triggers an intracellular reactive oxygen species (ROS) burst which leads to mitochondrial dysfunction: loss of mitochondrial membrane potential, onset of mitochondrial membrane transition, and release of mitochondrial pro-apoptotic proteins [6]. The apoptosis-inducing activity of costunolide was found to be closely associated with Bcl-2, based on observations that costunolide treatment decreased the anti-apoptotic Bcl-2 protein expression [7] while overexpression of Bcl-2 protein attenuated costunolide-induced apoptosis [8]. Secondly, costunolide suppresses NF- $\kappa$ B (nuclear transcription factor- $\kappa$ B) activation via prevention of I $\kappa$ B phosphorylation [9], a process also responsible for the strong anti-inflammatory activity of costunolide [10]. Third, costunolide is capable of promoting leukemia cell differentiation [11], inhibiting endothelial cells angiogenesis [12] and disrupting nuclear microtubule architecture in cancer cells [8]. The anticancer and chemopreventive properties of various costunolide analogs and potential role of costunolide with some endogenous stimulator of cell differentiation have attracted attention as a new lead for the development of anticancer drugs. Leukemia cell differentiation is increased when combined with 1,25-dihydroxyvitamin D<sub>3</sub>, which can be an alternative approach for the treatment of cancer [13]. Antiproliferative activity of costunolide against human breast cancer cells has been demonstrated. The results also showed the microtubular-interacting activity, suggesting this could be a new target for costunolide [8]. In recent years, there has been much interest among the researchers around the globe to look for more potent compounds

derived from costunolide. Synthesis of some amino derivatives of costunolide involving Michael-type addition has been carried out. The synthesized compounds (**22a–22d**, Figure 11.4) exhibited better cytotoxicity and selectivity along with improved safety index compared with the parent compound [14]. Isocostunolide (**23**, Figure 11.4) isolated from the roots of *Inula helenium* also showed inhibitory effect on proliferation of embryonic fibroblast and cancer cells [15]. 7-Hydroxycostunolide (**24**, Figure 11.4) isolated from *Podachaenium eminens* along with other SL-containing  $\alpha,\beta$ -unsaturated cyclopentenone were evaluated for cell viability, cell membrane integrity/cell death, and detection of apoptotic features. In the assessment of cytotoxicity along with cell death characteristic, 7-hydroxycostunolide (**24**) containing  $\alpha$ -methylene- $\gamma$ -lactone showed very rapid apoptotic morphology, strong rapid phosphatidylserine exposure, and macrophage response with approximately threefold caspase activity [16]. The induction mechanism of apoptosis by costunolide involves the receptor-mediated pathway preceding the mitochondria-dependent pathway [17]. Besides costunolide and other known sesquiterpenes, a new compound isodihydrocostunolide (**25**, Figure 11.4) isolated from dried roots of *Saussurea lappa* exhibited potent cytotoxic activity. The aforementioned compound was evaluated against various cancer cell lines where it exhibited good activity against Colo 205 (Inhibitory concentration ( $IC_{50}$ ),  $27.03 \pm 0.67 \mu\text{g ml}^{-1}$ ), A431 ( $IC_{50}$ ,  $107 \pm 7.46 \mu\text{g ml}^{-1}$ ), and MCF ( $IC_{50}$ ,  $35.05 \pm 9.37 \mu\text{g ml}^{-1}$ ) cell lines [18]. Combination strategy of plant lactones including costunolide enhanced the all-*trans* retinoic acid (ATRA)-mediated HL-60 (human leukemia) cell differentiation through a distinct pathway without elevation of basal intracellular calcium level [19]. 9 $\beta$ -Acetoxycostunolide (**26**, Figure 11.4) isolated from *Cyathocline purpurea* inhibited the growth of L1210 ( $IC_{50}$ ,  $0.89 \pm 0.06 \mu\text{g ml}^{-1}$ ),

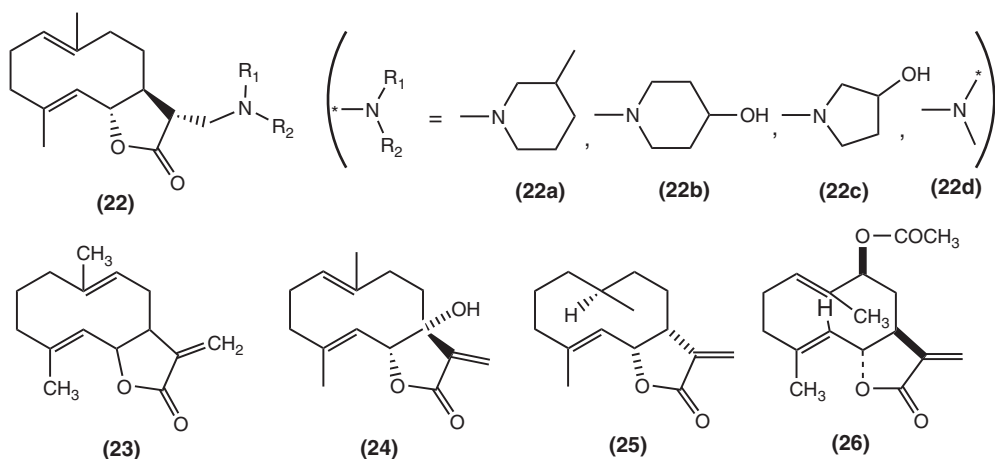


Figure 11.4 Various anticancer semisynthetic analogs of costunolide (22–26).

CCRF-CEM ( $IC_{50}$ ,  $0.41 \pm 0.03 \mu\text{g ml}^{-1}$ ), KB ( $IC_{50}$ ,  $0.25 \pm 0.02 \mu\text{g ml}^{-1}$ ), LS174T ( $IC_{50}$ ,  $1.28 \pm 0.05 \mu\text{g ml}^{-1}$ ), and MCF-7 ( $IC_{50}$ ,  $0.63 \pm 0.07 \mu\text{g ml}^{-1}$ ) cells *in vitro* [20]. Costunolide inhibitory effect on cell viability of 11Z and 12Z human endometriotic epithelial cell demonstrated that it may be a promising candidate for the treatment of endometriosis [21]. Inhibition of TNF- $\alpha$  (tumor necrosis factor)-induced MDA-MB-231 cell migration and spreading in breast cancer has also been proved [22].

### 11.2.2

#### Parthenolide and Analogs

Parthenolide (**10**, Figure 11.3), the major SL responsible for bioactivity of feverfew (*Tanacetum parthenium*), is a traditional herb which has been used for the treatment of fever, migraine, and arthritis for centuries. One well-explored bioactivity of parthenolide is its potent anti-inflammatory effect, which is mainly achieved through its strong inhibitory effect on NF- $\kappa$ B activation. It has been well established that parthenolide acts in multiple steps along the NF- $\kappa$ B-signaling pathway [23]. By suppressing NF- $\kappa$ B, parthenolide inhibits a group of NF- $\kappa$ B-regulated pro-inflammatory cytokines, such as interleukins (ILs) and prostaglandins [24]. The anticancer activity of parthenolide has been pursued in a number of laboratories. A large number of studies have been undertaken to investigate the mechanism of action of parthenolide at molecular levels in the different phases of carcinogenesis. The data were obtained using different tumor cell systems. Parthenolide induced apoptosis in pre-B acute lymphoblastic leukemia lines, including cells carrying chromosomal translocations [25]. Parthenolide induced rapid apoptotic cell death distinguished by loss of nuclear DNA, externalization of cell membrane phosphatidyl-serine, and depolarization of mitochondrial membranes at concentrations ranging from 5 to 100  $\mu\text{M}$ . Steele *et al.* investigated the *in vitro* actions of parthenolide on cells isolated from patients with chronic lymphocytic leukemia. Brief exposure to the SL (1–3 h) was sufficient to induce caspase activation and commitment to cell death. The mechanism of cell killing was via parthenolide-induced generation of ROS, resulting in turn in a pro-apoptotic Bax conformational change, release of mitochondrial cytochrome C, and caspase activation. Other studies also demonstrated that parthenolide-mediated apoptosis correlated well with ROS generation. Parthenolide strongly induced apoptosis in four multiple myeloma cell lines, although there are considerable differences in susceptibility to the SL. KMM-1 and MM1S sensitive to parthenolide possess less catalase activity than the less-sensitive KMS-5 and NCI-H929 cells. These findings indicate that parthenolide-induced apoptosis in multiple myeloma cells depends on increased ROS and that intracellular catalase activity is a crucial determinant of their sensitivity to parthenolide. Bedoya *et al.* also reported the antiproliferative and apoptosis-inducing effects of parthenolide on human multiple myeloma cells, mediated by an enhancement of caspase-3

activity [26]. Rapid cellular death of targeted leukemia stem cells induced by parthenolide, sparing the normal hematopoietic cells, proves its usefulness in Acute myeloid leukemia (AML) therapy [27]. The cytotoxicity of parthenolide with  $IC_{50}$  value  $8 \mu\text{M}$  was measured using an MTT assay. In addition to tubulin carboxypeptidase (TCP) inhibition shown by parthenolide, it was also concluded that unsaturated  $\alpha$ - $\beta$  lactone is essential for TCP inhibition [28]. Histone deacetylase 1 (HDAC1) depletion observed in various cancer cell types, was specifically depleted by parthenolide without affecting other class I/II HDAC [29]. In recent years, in order to look for more potent molecules, various kinds of semisynthetic analogs of parthenolide have been synthesized. Primary and secondary amine addition products of parthenolide (**27**, Figure 11.5) have been synthesized and evaluated against human caucasian acute lymphoblastic leukemia (CCRF-CEM) cells and human anaplastic large T-cell lymphoma Sternberg-Reed (SR) cells. The analogs derived from tyramine (**27a**, Figure 11.5) showed the growth inhibition toward CCRF-CEM cells with Growth inhibition ( $GI$ )<sub>50</sub> and Total growth inhibition (TGI) values of less than 10 nM. Similarly, another analog (**27b**, Figure 11.5) exhibited the cytostatic activity toward human anaplastic large T-cell lymphoma (SR) cells with  $GI_{50}$  value  $<0.01 \mu\text{M}$  and full inhibition of growth at a concentration of  $0.041 \mu\text{M}$  [30]. The effects of SLs on melanin content and cell number in B16 melanoma cells were examined by treating the cells with 3-isobutyl-1-methylxanthine (IBMX) and SLs including parthenolide. The results demonstrated the antimelanotic activity of parthenolide [31]. Some chemical modifications have been made to develop the semisynthetic analogs (**28–32**, Figure 11.5) of parthenolide. Since the activation of NF- $\kappa$ B induces the gene expression involved in various pathological conditions, including cancer, the effect of these parthenolide derivatives on NF- $\kappa$ B-driven transcription was studied. Knowing for a fact that matrix metalloproteinase-9 (MMP-9) is overexpressed in various pathological conditions including cancer invasion and metastasis, the effect of compounds were also studied on MMP-9 gene transcription. The compounds (**30–32**, Figure 11.5) showed statistically different results with higher activity than the parthenolide [32]. Various derivatives of parthenolide with primary and secondary amines have been synthesized. The antileukemic activity of synthesized amino parthenolide analogs against acute myeloid leukemia (AML) cells in culture was studied. Compounds **33–35** (Figure 11.5) were the most potent compounds in the series with Lethal dose ( $LD$ )<sub>50</sub> values of 1.7, 1.8, and  $1.6 \mu\text{M}$ , respectively. It was also found that the activity displayed by aminoparthenolide analogs from secondary amines was more significant than that from primary amines [33]. 9 $\beta$ -Acetoxyparthenolide (**36**, Figure 11.5), which is one of the main constituents isolated from *C. purpurea*, exhibited anticancer activities *in vitro*. Its inhibitory effects on the growth of a panel of murine and human tumor cell lines have been estimated with  $IC_{50}$  values ranging from 0.29 to  $1.08 \mu\text{M}$  [20]. Modulation of intracellular redox status in prostate cancer (PC) and normal prostate cells by parthenolide has

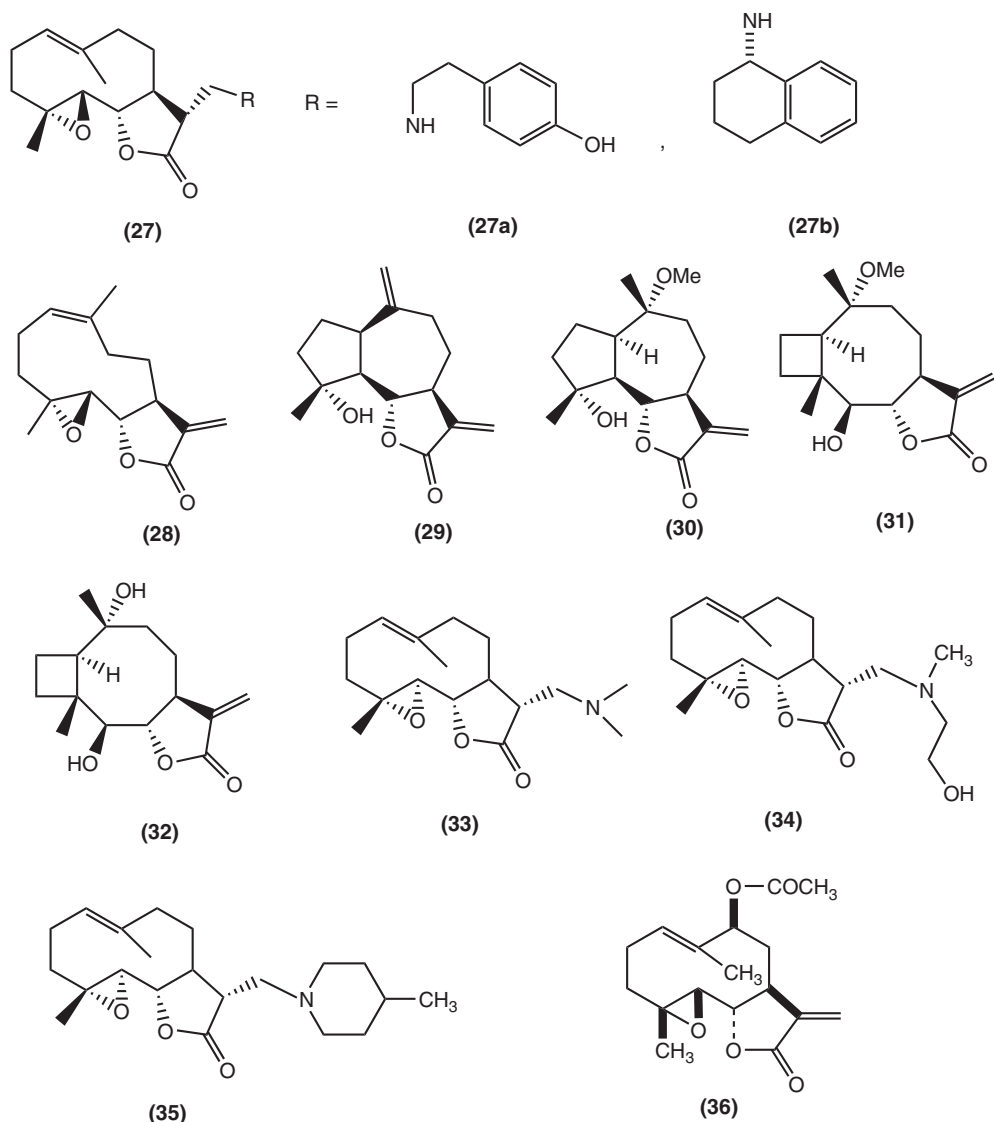


Figure 11.5 Various anticancer parthenolide analogs (27–36).

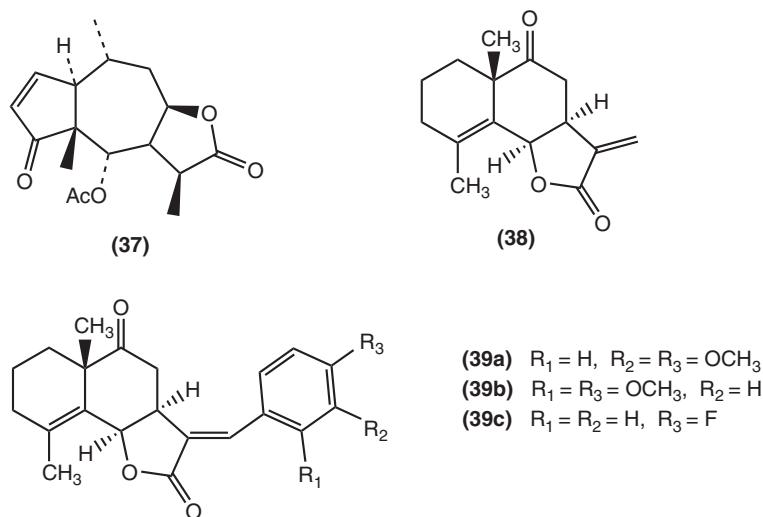
been demonstrated. The studies showed the selective radiosensitization effect of parthenolide on PC cells without affecting the normal prostate epithelial cells (PrECs). The results were confirmed by demonstrating the parthenolide induction of oxidative stress in PC3 cells, activation of NADPH oxidase in PC3 cells, induction of FOXO3a phosphorylation in PC3 cells, and increased GSH in normal PrECs [34]. *In vitro* cytotoxicity against 5637 bladder cancer cells

by parthenolide in a dose- and time-dependent manner has been determined by the MTT assay. These findings have suggested it is a potent molecule for the treatment of bladder cancer. Most of the synthesized fluorinated amino derivatives of the parthenolide showed antiproliferative activity very similar to that of the parent compound [35].

### 11.2.3

#### Helenalin and Analogs

Helenalin (7, Figure 11.2), another SL from the *Arnica* species, has been reported to possess cytotoxicity and anticancer activity. Earlier studies demonstrated its potent activity in inhibiting nucleic acid and protein synthesis [36]. Similar to other anticancer SLs, its mechanism of action mainly involves (i) thiol depletion, (ii) inhibition of NF- $\kappa$ B, and (iii) induction of apoptosis [37]. These prominent bioactivities make helenalin another potential anticancer agent. Inhibition of the NF- $\kappa$ B by helenalin-mono-and-bis-glutathionyl adducts has been demonstrated [38]. The influence of different L-cysteine and GSH concentrations on the cytotoxicity of the 11 $\alpha$ ,13-dihydrohelenalin acetate (DHA) (37, Figure 11.6) against KB cells was demonstrated. The results showed unchanged cytotoxicity ( $IC_{50}$ ,  $6.3 \pm 0.31$  to  $6.5 \pm 1 \mu\text{M}$ ) on addition of different molar concentrations of L-cysteine [39]. Differential downregulation of PMA-induced mRNA levels in Jurkat T cells and human peripheral blood mononuclear cells (PBMCs) by DHA, a type of SLs, has also been investigated [40]. DHA, isolated from *Arnica*



**Figure 11.6** Dihydrohelenalin acetate (37) and tourneforin analogs (38–39) displaying cytotoxic activity.

*montana*, was evaluated with other SL-containing  $\alpha$ -methylene- $\gamma$ -lactone for cell death characteristics along with cytotoxicity. DHA with  $IC_{50}$  45.8  $\mu$ M (95% confidence intervals (CI): 36.2–57.9  $\mu$ M,  $n = 3$ ) showed progressive apoptotic cell morphology with approximately twofold caspase activity [16]. Inhibition of NF- $\kappa$ B resulting in an increase of autophagy markers and caspase activation by parthenolide can be therapeutically useful in conditions such as acute myeloid leukemia (AML) [41]. Demonstration of cytotoxic effect of  $\beta$ -cyclodextrin-helenalin complexes on T47D cells suggests a kind of drug delivery for helanin using cyclic oligosaccharide [42].

#### 11.2.4

##### Artemisinin and Its Derivatives

Given the high accumulation of iron in cancer cells, researchers Henry Lai and Narendra Singh became interested in possible artemisinin (ART) (**8**, Figure 11.2) activity against malignant cells and have used ART against numerous cancer cells *in vitro* [43]. The aza-Michael addition reaction has been employed to synthesize some derivatives of dihydroartemisinin with high selectivity index (SI) against HeLa cells ( $IC_{50}$ , 0.37  $\mu$ M) [44]. Antitumor effect with conformations along with drug-receptor interactions of deoxoartemisinins and carboxypropyldeoxoartemisinins have been studied [45]. There are a number of properties shared by cancer cells that favor the selective toxicity of ART against cancer cell lines and against cancer *in vivo*. In addition to their high rates of iron flux via transferrin receptors when compared to normal cells, cancer cells are also particularly sensitive to oxygen radicals. ART becomes cytotoxic in the presence of ferrous ion. Since iron influx is naturally high in cancer cells, ART and its analogs can selectively kill cancer cells *in vivo* [46].

It has been established through the SAR studies of ART and its various kinds of C-12/C-13 ether/ester derivatives that only peroxide linkage affects the antimalarial and anticancer activity. Furthermore, several drawbacks associated with these compounds, namely, solubility, thermal and hydrolytic stability, bioavailability, short half-life, and so on, have led to development of second-generation C-12/C-13 trioxane derivatives. Furthermore, it was thought worthwhile that the extent of antimalarial activity depends upon the extent of the number of peroxide units, which can be increased by adding of additional ART moiety through careful chemical manipulations. Thus, researchers have directed their efforts toward the synthesis of various kinds of ART dimers, trimers, and tetramers of various lengths and flexibility. ART dimers reported till date have displayed structural diversity separated through ART monomer units with or without linkers of various lengths and flexibility with diverse stereochemistry. Several of these C-12/C-13 carbon ART dimers have shown outstanding antimalarial and anticancer activity and are better than C-12 ether/ester dimers. ART trimers and tetramers of C-12/C-13 nonacetal derivatives have also been reported in



recent years, wherein ART units are connected through linkers of various kinds with diverse lengths and stereochemistry. However, the number of ART dimers synthesized so far is far greater than the number of ART trimers and tetramers. Many of these dimers, trimers, and tetramers have shown outstanding antimalarial and anticancer activity compared to ART and related compounds and are in various phases of clinical trials. In recent years, much progress has been made by scientists around the world in the synthesis of ART derivatives including the dimers and trimers which we have recently reviewed [47, 48]. Furthermore, it is possible to increase or enhance iron flux in cancer cells by introducing conditions that lead to increased intracellular iron concentrations. However, intact *in vivo* systems do not need holotransferrin, as the body provides all the necessary iron transport proteins. In recent years, in order to search for potential anticancer agents many researchers have directed their efforts toward synthesizing various kinds of ART dimers, trimers, and tetramers wherein several have shown potential anticancer activity and are in various phases of clinical trials [49]. A series of ART-related endoperoxides derivatives exhibited greater cytotoxicity and chemical stability than artesunate [50]. Triazolyl-substituted semisynthetic derivatives of ART showed good inhibitory effect against various cancer cell lines [51]. A series of dimer phosphate ester analogs of ART were screened against the Jurkat T-cell acute lymphoblastic leukemia cell line. Their anticancer activities without affecting normal cells have been reported [52a]. Adducts of ART and its analogs such as artesunate and dihydroartesunate with transferrin have shown more promising effects against cancer cells when compared to ART alone [52b].

### 11.2.5

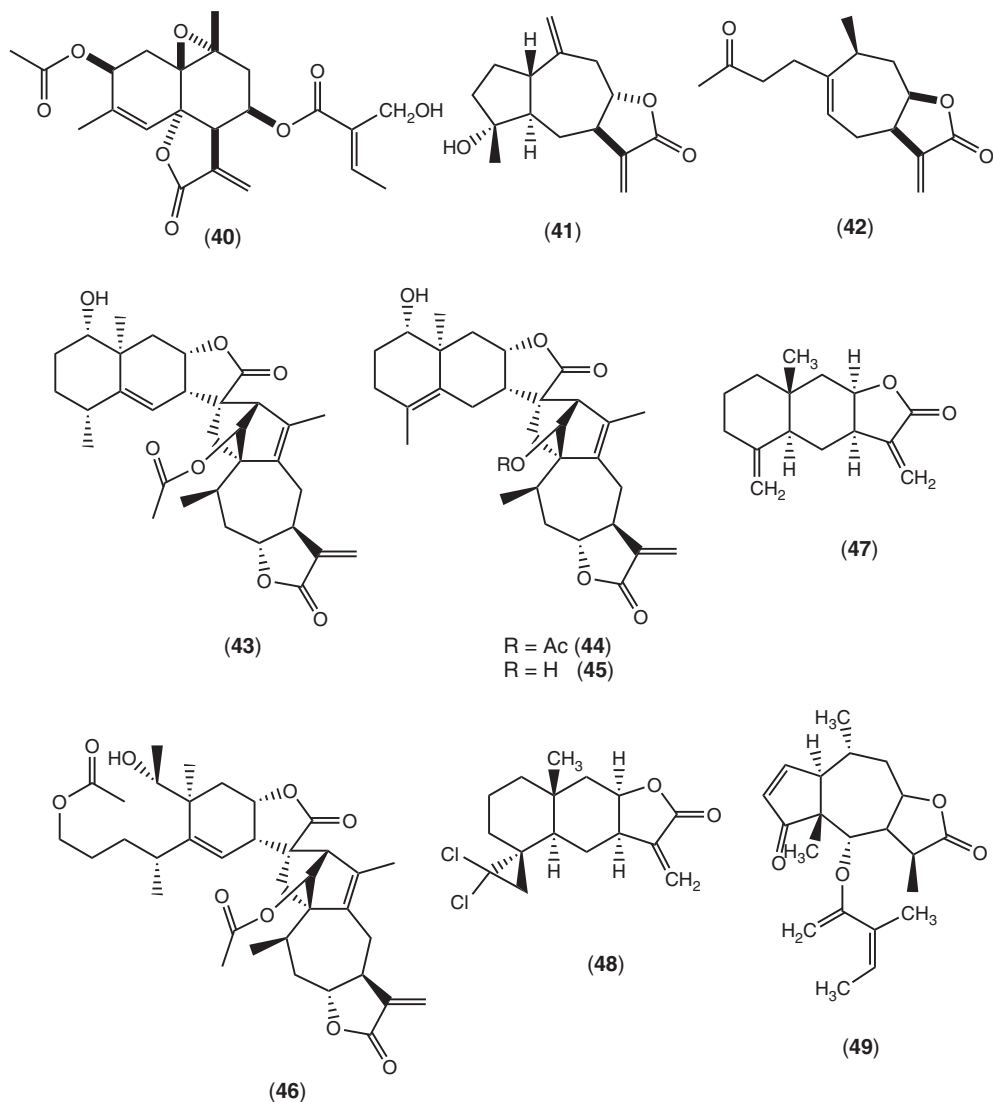
#### Tourneforin and Its Derivatives

Tourneforin (**38**, Figure 11.6), an SL of *Artemisia tournefortiana*, is an important compound containing  $\alpha$ -methylene- $\gamma$ -lactone. The 13-arylated derivatives (**39a–39c**, Figure 11.6) of tourneforin were synthesized and tested against the lymphoid tumor cells. Arylidene-substituted derivatives displayed better cytotoxicity than the parent tourneforin [53].

### 11.2.6

#### Eupalinin

Eupalinin A (**40**, Figure 11.7), isolated from *Eupatorium chinense L.*, induces autophagic cell death (ACD) in HL60 cells. GSH-regulated conversion of autophagy to apoptosis in HL 60 cells by eupalinin A was also demonstrated [54]. In addition to this, the induction of autophagy in cancer cells has been shown by eupalinin [55].



**Figure 11.7** Eupalinin A (40), inviscolide, and related compounds (41–42); japonicones (43–46), isoalantolactone, and related compounds (47–48); and 6-*O*-angeloylenolin (49) displaying anticancer activity.

### 11.2.7

#### Inviscolide and Related Compounds

Inviscolide (41, Figure 11.7) was isolated from the leaves of *Inula viscosa* along with other SL (42, Figure 11.7) and studied for their anticancer potential against

human melanoma cell lines. Inhibition of the proliferation of melanoma cell lines was measured by an MTT assay in which the  $IC_{50}$  values of inuviscolide was observed from  $37 \pm 2.1$  to  $41.1 \pm 3.8$  mM. Profound  $G_2/M$  arrest along with induction of apoptosis was investigated by these SL [56]. The decreased production of IL-2 and IL-1 required for *in vivo* angiogenesis and invasiveness of tumor cells has been demonstrated by the aforementioned compound (**41**, Figure 11.7) [57].

#### 11.2.8

##### Japonicones

Japonicones, comprising eudesmane and guaiane sesquiterpenes, are dimeric SLs. Four new sesquiterpenes japonicones A–D (**43–46**, Figure 11.7) were isolated from the dried aerial parts of *Inula japonica*; among them japonicone A (**43**, Figure 11.7) exhibited the most potent antitumor activity. The compounds were tested against four tumor cell lines, A549, LOVO, CEM, and MDA-MB-435 in which the aforementioned compound showed cytotoxicity with  $IC_{50}$  values 1.620, 0.256, 0.001, and  $0.198 \mu\text{g ml}^{-1}$  respectively [58].  $G_2-M$  phase arrest, apoptosis, and inhibition of TNF- $\alpha$ -stimulated NF- $\kappa$ B activity and nuclear translocation shown by japonicone A prove it to be a potential chemotherapeutic agent for treatment of lymphomas [59].

#### 11.2.9

##### Isoalantolactone and Related Compounds

Isoalantolactone (**47**, Figure 11.7), a positional isomer of alantolactone, belongs to the category of SLs. In recent years, various synthetic modifications have been carried out to assess its anticancer potential. Synthesis and cytotoxicity evaluation of chloro derivatives of isoalantolactone have been carried out. Cytotoxicity of isoalantolactone (**47**, Figure 11.7) and its chloro derivatives were examined against CEM-13 tumor cells, U-937 tumor cells, and Metallothionein (MT)-4 tumor cells. For compound **48** (Figure 11.7), the 50% inhibition of the tumor cells viability for U-937 tumor cells and MT-4 tumor cells were 3.2 and 11.1  $\mu\text{M}$ , respectively [60a]. Dose-dependent inhibition of androgen-sensitive (LNCaP) as well as androgen-independent (PC3 and DU-145) PC cells by isoalantolactone is mediated by ROS mechanism [61]. Testes-specific protease 50 (TSP50) expression has been investigated as a potential target for the treatment of cancer. Inhibition of the promoter activity of TSP50 gene at mRNA and protein levels also has been shown by alantolactone [60b].

## 11.2.10

**6-O-Angeloylenolin**

6-O-Angeloylenolin (**49**, Figure 11.7), a plant-derived SL was first isolated from *Centipeda minima*. The isolated compound was found to have potent activity on the inhibition of proliferation of Human nasopharyngeal cancer (CNE) cells. The results were demonstrated as cell-cycle arrest and induction of apoptosis in CNE cells by the aforementioned compound [62].

## 11.2.11

**Miscellaneous STLs Under Different Classes**11.2.11.1 **Guaianolides**

Guaianolides are derived from germacrene A–D with majority of compounds belonging to family Apiaceae. In recent years, these compounds have attracted the interest of researchers around the world because of their anticancer properties. Various guaianolides along with their structures are discussed here to summarize their anticancer potential (Figures 11.8 and 11.9). Aguerin B (**50**), 8 $\alpha$ -acetoxylaluzanin C (**51**), cynaropicrin (**4**), and deacylcynaropicrin (**52**) were isolated from the flowers of *Hemisteptia lyrata* Bunge. The *in vitro* cytotoxicity of the compounds was tested against six types of human tumor cell lines by the sulforhodamine B (SRB) assay method. The highest cytotoxic activity was observed in case of aguerin B and cynaropicrin against the MCF-7 (IC<sub>50</sub>: 1.1  $\pm$  0.3 and 1.1  $\pm$  0.5 mg ml<sup>-1</sup>, respectively) and HCT-15 cells (IC<sub>50</sub>: 1.4  $\pm$  0.5 and 0.9  $\pm$  0.4 mg ml<sup>-1</sup>, respectively) [63]. Two new dimeric guaianolide-type SLs (**53**, **54**) were isolated from the leaves of *Warionia saharae*. The IC<sub>50</sub> values of the investigated SLs ranged from 1.0  $\pm$  0.14 to 2.2  $\pm$  0.33  $\mu$ M against the HeLa, Jurkat T, and PBM cells [64]. The inhibitory effect of cynaropicrin on activation of major adhesion molecules (CD29 ( $\beta$ 1 integrins), CD43, and CD98) on the cells assessed by U937 (promonocytic cells) homotypic aggregation has been examined. The results suggest its application in CD29- and CD98-mediated diseases such as metastasis of leukocyte cancer cells [65]. Arguerin B and cynaropicrin with other SLs (**55**–**57**) were isolated from a perennial herb, *Saussurea calciccola*. The cytotoxicity was evaluated against the five cultured human tumor cells including A549 (non-small cell lung adenocarcinoma), SK-OV-3 (ovarian), SK-MEL-2 (skin melanoma), XF498 (Central nervous system, CNS), and HCT15 (colon) using SRB bioassay. Aguerin B and cynaropicrin exhibited significant cytotoxicity against five human tumor cell lines with Effective dose (ED)<sub>50</sub> of 0.29–1.37 and 0.23–1.72  $\mu$ g ml<sup>-1</sup>, respectively [66]. Ixerochinolide (**58**), a guaianolide-type SL and some known guaianolides were isolated from the medicinal Chinese herb *Ixeris chinensis*. The compound was tested for cytotoxic activity against the human PC-3 (prostate) cancer cell line where it exhibited the significant growth inhibition with IC<sub>50</sub> value 1.6  $\mu$ g ml<sup>-1</sup> [67]. From the methanolic root and

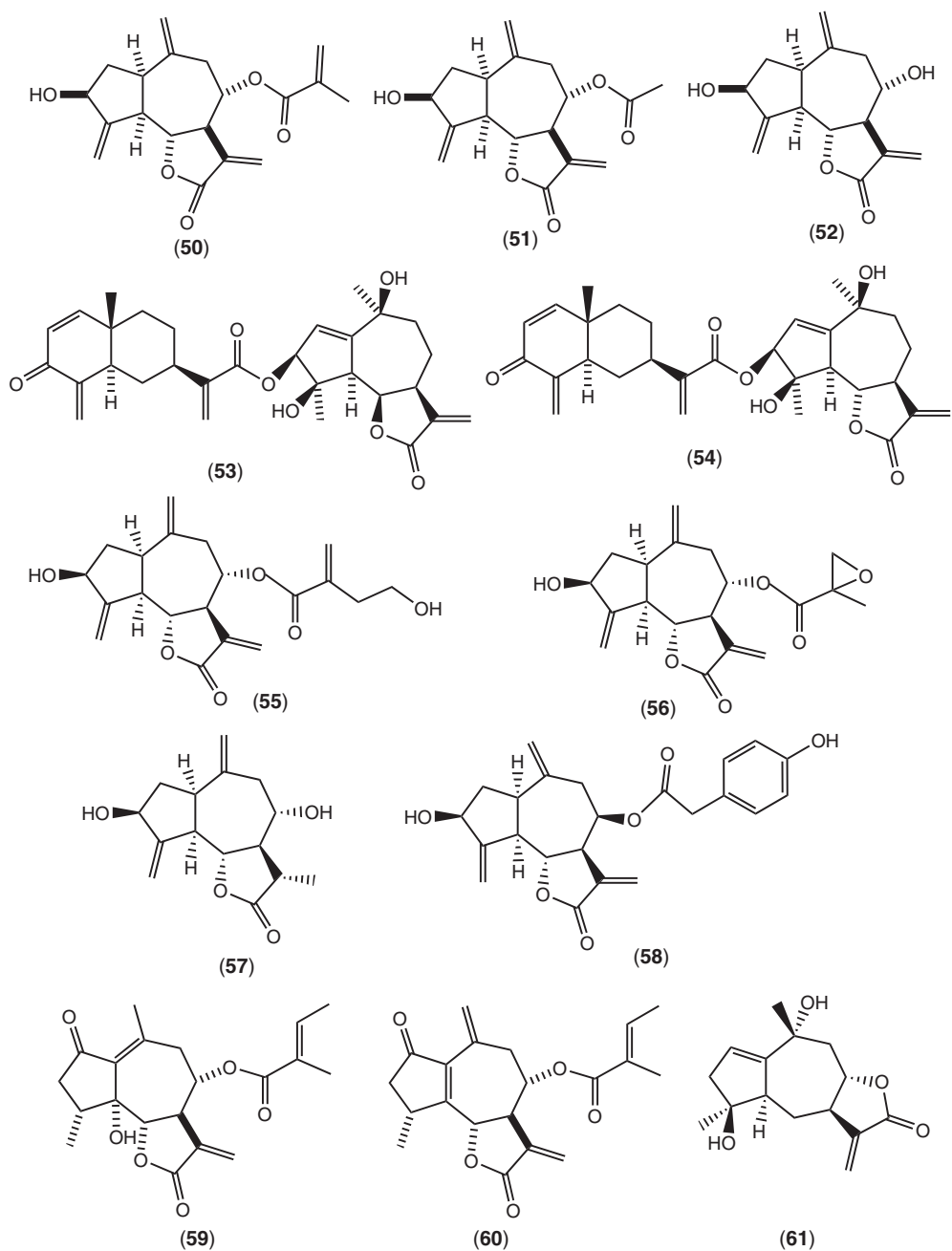
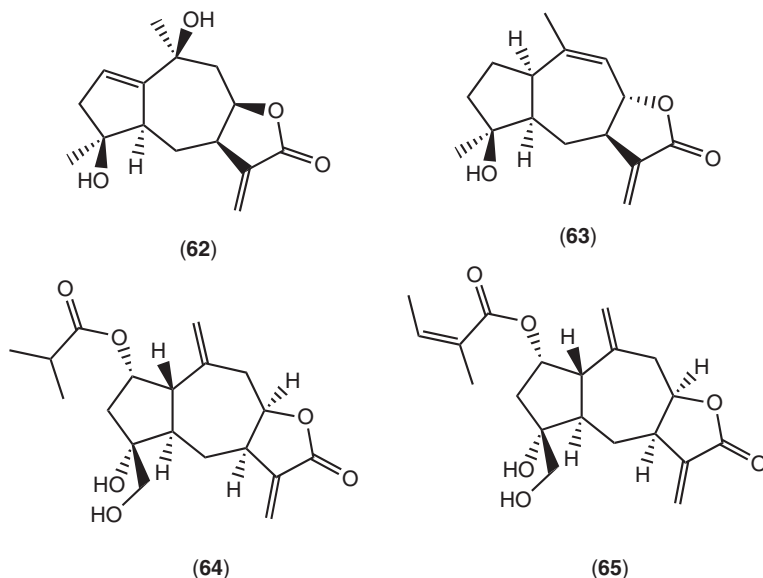
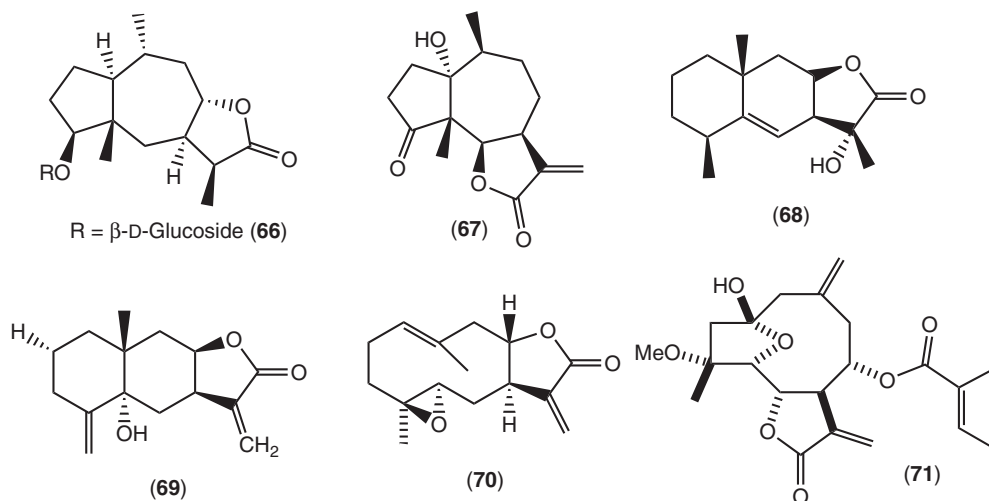


Figure 11.8 Various guaianolide sesquiterpene lactones (I).



**Figure 11.9** Various anticancer guaianolide sesquiterpene lactones (II).

stem extract of *Elephantopus mollis*, some new SLs having structural moiety of guaiadien-olide (**59**) and guaiatrien-olide (**60**) were obtained. Cytotoxic assessment of these compounds against neuroblastoma B104 displayed the significant activity with  $IC_{50}$  values 1.93 and 2.13  $\mu\text{M}$ , respectively [68]. Five guainolides with two new compounds (**61**, **62**) were isolated from *Carpesium faberi*. These compounds were evaluated for their antiproliferative activities against MCF-7 human breast cancer cells. Among them, the compound **63** exhibited antiproliferative activity with  $IC_{50}$  value 16.3  $\mu\text{g ml}^{-1}$  [69]. Minimolide G (4 $\beta$ ,15 $\beta$ -dihydroxy-2 $\alpha$ -isobutyryloxy-guaia-10(14),11(13)-dien-8 $\beta$ ,12-olide) and minimolide H (4 $\beta$ ,15 $\beta$ -dihydroxy-2 $\alpha$ -angeloxloxy-guaia-10(14),11(13)-dien-8 $\beta$ ,12-olide) were isolated from *C. minima*. The assessment of their antiproliferative activity against the human nasopharyngeal cancer cell line (CNE) was carried out. The results indicated the inhibitory activity with  $IC_{50}$  values of 61.4 and 28.7 mM, for minimolide G and minimolide H (**64**, **65**), respectively [70a]. A series of guaianolide-type SLs derivatives with arylation of  $\alpha$ -methylene- $\gamma$ -lactone moiety were tested against doxorubicin-resistant cell line HL-60/A. Out of these synthesized compounds, some exhibited good activity against the doxorubicin-resistant cell line HL-60/A ( $IC_{50}$  = 6.2–19  $\mu\text{M}$ ) [70b]. Various triazolyl analogs of ludratin (belonging to guaianolide STLs) were screened against A549, HCT-116, PC-3, MCF-7, T98G human cancerous cell lines. Few compounds displayed good cytotoxic activity against these cancer cell lines [70c].



**Figure 11.10** Various anticancer pseudoguaianolides (66–67), eudesmanolides (68–69), and germacranolides (70–71).

#### 11.2.11.2 Pseudoguaianolides

This class of compounds is normally characterized by migration of the carbon-4 (C-4) methyl group to carbon-5 (C-5) in the guaianolide ring system. One new pseudoguaianolide (66, Figure 11.10) and some known compounds were obtained from *C. faberi*. These compounds were evaluated for their antiproliferative activities against MCF-7 human breast cancer cells. One of the compound displayed significant activity with an  $IC_{50}$  value of  $3.9 \mu\text{g ml}^{-1}$  [69]. Coronopilin (67, Figure 11.10), one of the pseudoguaianolides has been obtained from *Ambrosia arborescens*. Two leukemia-derived cells, Jurkat and U937, were taken for the study of this compound. The isolated compound was evaluated on leukemia cells for population expansion. The  $IC_{50}$  values for inhibition of leukemia cell growth were  $5 \pm 0.2$  and  $11 \pm 0.32 \mu\text{M}$  after incubation of 48 h for Jurkat and U937 cells, respectively [71].

#### 11.2.11.3 Eudesmanolides

Eudesmanolides, classified into two subfamilies 12,6-eudesmanolides and 12,8-eudesmanolides, are the important class of molecules having good antitumoral properties. 11 $\alpha$ -Hydroxy-eudesm-5-en-8 $\beta$ ,12-olide (68, Figure 11.10), an eudesmane-type SL was isolated from the roots of the traditional Chinese plant *Inula racemosa*. The compound showed cytotoxic activity against BEL-7402 (human liver cancer) and HCT-8 (human colon cancer) cell lines [72]. Three eudesmenolides were isolated from the whole plant of *C. faberi*. Among these

isolated SLs, compound **69** (Figure 11.10) showed the most potent antiproliferative activity with  $IC_{50}$  value  $3.0 \mu\text{g ml}^{-1}$  against MCF-7 human breast cancer cells using the MTT assay [69].

#### 11.2.11.4 Germacranolide

These germacrane skeleton-based compounds are exemplified by various anticancer natural products such as tulipinolide and costunolide. In recent years, some new germacranolides having anticancer potential have been isolated. Their structures along with antiproliferative and cytotoxic properties are discussed here.  $4\beta,5\alpha$ -Epoxy-1(10),11(13)-germacradiene-8,12-olide (**70**, Figure 11.10) isolated from the methanolic extract of dried roots of *I. helenium* exhibited antiproliferative activity against MK-1, HeLa, and B16F10 cell lines *in vitro* with  $GI_{50}$  ranging from 12 to  $33 \mu\text{M}$  [73]. A new germacratrien-olide (**71**, Figure 11.10) was isolated from *E. mollis*. The isolated compound was found to be an active molecule after cytotoxicity evaluation against the mouse neuroblastoma B104 cells with  $IC_{50}$  value of  $1.58 \mu\text{M}$  [68].

#### 11.2.11.5 Other Anticancer Sesquiterpene Lactones

Overexpression of survivin, belonging to apoptosis inhibitor gene family has been observed in human cancers. A new drimane SL, SF002-96-1, isolated from fermentation of an *Aspergillus* species, inhibited survivin promoter activity in a dose-dependent manner with  $IC_{50}$  values of  $3.42 \mu\text{M}$  ( $1.3 \mu\text{g ml}^{-1}$ ) [74]. Antrocin, a secondary metabolite isolated from *Antrodia camphorata* showed the inhibition of cell proliferation in two nonsmall-cell lung cancer cells, namely, H441 and H1975, with  $IC_{50}$  values 0.75 and  $0.83 \mu\text{M}$ , respectively [75]. Various hirsutinolides isolated from combined leaves and stems of *Vernonia cinerea* displayed inhibitory effects against the viability of U251MG glioblastoma and MDA-MB-231 breast cancer cells [76].

### 11.3

#### Structure–Activity Relationships (SARs) of Sesquiterpenes Lactones

It is generally believed that the bioactivity of SLs is mediated by alkylation of nucleophiles through their  $\alpha$ ,  $\beta$ - or  $\alpha$ ,  $\beta$ ,  $\gamma$ -unsaturated carbonyl structures, such as  $\alpha$ -methylene- $\gamma$ -lactones or  $\alpha,\beta$ -unsaturated cyclopentenones. These structural elements react with nucleophiles, especially the cysteine sulfhydryl groups by Michael-type addition. Therefore, it is widely accepted that thiol groups such as cysteine residues in proteins, as well as the free intracellular GSH, serve as the major targets of SLs. In essence, the interaction between SLs and protein thiol groups or GSH leads to reduction of enzyme activity or causes the disruption of GSH metabolism and vitally important intracellular cell redox balance (Figure 11.11).



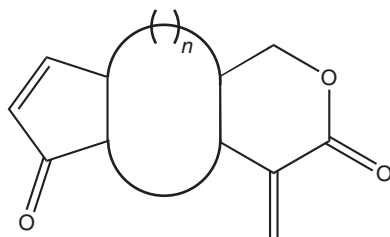


Figure 11.11 General structure of sesquiterpene lactones.

The relationship between chemical structure and bioactivity of SLs has been studied in several systems, especially with regard to cytotoxicity, anti-inflammatory, and antitumor activity. It is believed that the exo-methylene group on the lactone is essential for cytotoxicity because structural modifications such as saturation or addition to the methylene group resulted in the loss of cytotoxicity and tumor inhibition. However, it has also been shown that the factor responsible for the cytotoxicity of SLs might be the presence of the  $O=C-C=CH_2$  system, regardless of whether it is a lactone or cyclopentenone. It was later demonstrated that the presence of additional alkylating groups greatly enhanced the cytotoxicity of SLs. Furthermore, It was established that the  $\alpha$ -methylene- $\gamma$ -lactones and  $\alpha,\beta$ -unsaturated cyclopentenone ring (or  $\alpha$ -epoxycyclopentenone) present in SLs is essential for their *in vivo* antitumor activity. It has been confirmed through various published reports that the versatile biological activities displayed by SLs is due to presence of either  $\alpha$ -methylene- $\gamma$ -lactones and  $\alpha,\beta$ -unsaturated cyclopentenone ring. In summary, the differences in activity among individual SLs may be explained by differences in the number of alkylating elements, lipophilicity, molecular geometry, and the chemical environment of the target sulfhydryl group.

## 11.4

### Concluding Remarks

SLs are an important group of natural products obtained from many species of medicinal plants. Their structural diversity and versatile potential biological activities such as anticancer, anti-inflammatory, antitumor, antimalarial, antiviral, antibacterial, and antifungal activities have been of interest to chemists involved in drug discovery research. Although the exact mechanism of action of SLs is not well known, it has been documented through various published reports that the biological activity displayed by majority of SLs is due to the presence of  $\alpha$ -methylene- $\gamma$ -lactones and  $\alpha,\beta$ -unsaturated cyclopentenone ring. This chapter offers an overview of various kinds of structurally diverse anticancer SLs which may be useful for the chemists/pharmacologists working in the area of drug discovery.

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### Abbreviations

AML	acute myelogenous leukemia
ACD	autophagic cell death
ART	artemisinin
DHA	dihydrohelenalin acetate
HDAC	histone deacetylase
HL	human leukemia
MMP-9	matrix metalloproteinase-9
NF- $\kappa$ B	nuclear transcription factor- $\kappa$ B
PBMCs	peripheral blood mononuclear cells
PrEC	prostate epithelial cell
SLs	sesquiterpene lactones
SRB	sulforhodamine B
SI	selectivity index
TCP	tubulin carboxypeptidase
TNF	tumor necrosis factor
TSP	testes-specific protease

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## 12

# Naturally Occurring Calanolides: Chemistry and Biology<sup>1</sup>

Goutam Brahmachari

### 12.1

#### Introduction

Calanolides are naturally occurring pyranocoumarin derivatives found in *Calophyllum* species such as *Calophyllum lanigerum* Miq. var. *austrororiaceum* (T.C. Whitmore) P.F. Stevens, *Calophyllum teysmannii* var. *inophylloide*, and *Calophyllum brasiliense* (leaves) – tropical rainforest trees belonging to the family Clusiaceae/Guttiferae [1–4]. These natural pyranocoumarins are well known for their potent antihuman immunodeficiency virus (anti-HIV) activity. Among the series, (+)-calanolide A and (–)-calanolide B (also known as *costatolide*) have been found very promising. (+)-Calanolide A has been reported to exhibit potent antituberculosis (anti-TB) activity as well [5]. A good number of chemical libraries of calanolides have been synthesized and screened for their so-called efficacies. Owing to low availability of naturally occurring calanolides, studies on the total syntheses of these polycyclic coumarins appeared to be very fascinating to the organic chemists in providing material for preclinical and clinical research. This has ultimately led to the development of a number of synthetic schemes for the total synthesis of natural calanolides. The National Cancer Institute (NCI) has been playing an active and supportive role in the development of the calanolide class of compounds, both natural and synthetic, for unraveling their clinical possibilities. Intensive research on calanolides is apparent from the number of related patents filed and approved in this field – about 38 approved patents on calanolide compounds are available so far, which deal with the isolation technique, synthesis of various calanolides and their analogs, and evaluation of their anti-HIV and other pharmaceutical potentials [6]. This chapter is aimed to focus on naturally occurring calanolides, with regard to their anti-HIV and anti-TB potential, their chemical analogs, and total syntheses.

<sup>1</sup>This chapter is dedicated to the loving memory of Santosh K. Brahmachari.

## 12.2

### Naturally Occurring Calanolides: Structures and Physical Properties

About nine calanolide derivatives **1–9** are known so far from natural sources, and they were isolated from the *Calophyllum* species (tropical rain forest trees; family: Clusiaceae/Guttiferae); structures and physical properties of these isolates are presented in Figure 12.1 and Table 12.1 [1, 7, 8].

## 12.3

### Anti-HIV and Antituberculosis Potential of Calanolides

#### 12.3.1

##### Anti-HIV Potential of Calanolides

Naturally occurring calanolides have already been demonstrated to possess potent anti-HIV activity [6]. (+)-Calanolide A (**1**), a novel non-nucleoside specific reverse-transcriptase inhibitor (NNRTI), is the most promising member among the series and is under development as an AIDS (acquired immunodeficiency syndrome) chemotherapeutic. NRTIs (nucleotide reverse transcriptase inhibitors) (both nucleoside and nucleotide RTIs) were the first class of antiretrovirals (ARVs) used in the HIV/AIDS therapy. After entry into the cells and undergoing intracellular phosphorylation, they can be incorporated into the evolving deoxyribonucleic acid (DNA) chain under the action of reverse-transcriptase (RT) [9, 10]. NRTIs act by competing with the natural nucleoside triphosphates for binding of the nucleotide site of the RT active site [11, 12]. Inside cells, nucleoside analogs or nucleotides need to be phosphorylated into triphosphate forms to act as alternative substrates of HIV-1 (human immunodeficiency virus type 1) RT. Compared to natural nucleoside triphosphates, nucleoside RT inhibitors lack the 3'-hydroxyl group of the deoxyribose, allowing them to act as chain terminators blocking DNA synthesis and resulting in the abortion of the following virus life cycle [13, 14].

Kashman *et al.* [1] demonstrated that (+)-calanolide A protects HIV-1 replication and cytopathicity efficiently with  $EC_{50}$  (half maximal effective concentration) and  $IC_{50}$  (half maximal inhibitory concentration) values of 0.1 and 20  $\mu\text{M}$ , respectively; however, it was found to be essentially inactive against strains of the less common HIV-2 [1, 2]. Interestingly, the test compound was found to be active not only against the AZT-resistant (azidothymidine) G-9106 strain of HIV-1 (at an  $EC_{50} \leq 0.03 \mu\text{M}$ ) but also against the pyridinone-resistant A17 strain (developed by Merck; at an  $EC_{50} \leq 0.60 \mu\text{M}$ ); it also remained fully active against virus isolates with zidovudine (AZT) and 3TC resistance-engendering mutations [3, 15–17]. It was revealed from the works of Quan *et al.* [15] that (+)-calanolide A (**1**) could exert activity against HIV with the two most common NNRTI-related

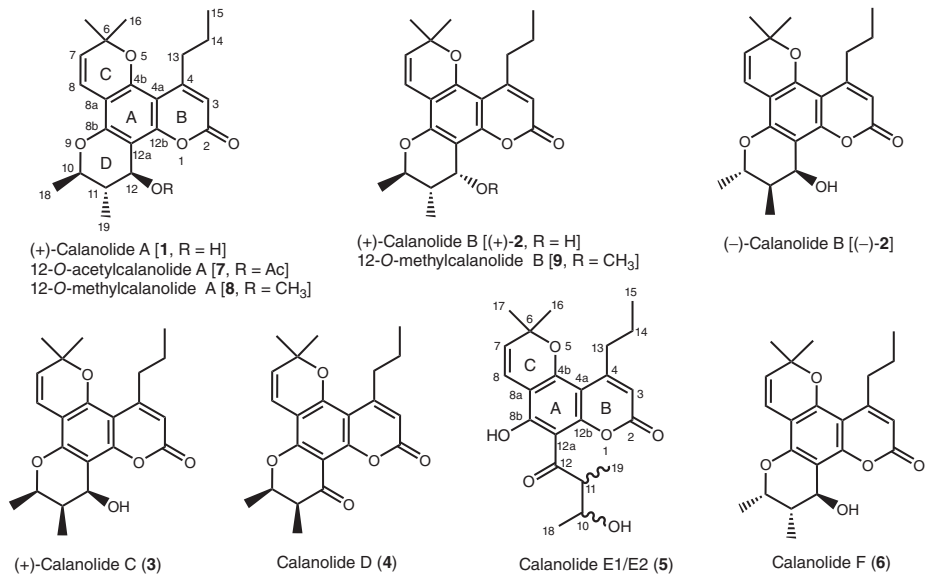


Figure 12.1 Naturally occurring calanolides [1, 7, 8].

**Table 12.1** Natural abundance and physical properties of calanolides [1, 7, 8].

Compounds	Source <i>Calophyllum</i> sp.	Molecular formula	Molecular weight	Optical rotation
(+)-Calanolide A (1)	<i>C. lanigerum</i> <i>C. brasiliense</i>	C <sub>22</sub> H <sub>26</sub> O <sub>5</sub>	370.18	[α] <sub>D</sub> = +60° (CHCl <sub>3</sub> , c 0.7)
(+)-Calanolide B ((+)-2)	<i>C. lanigerum</i>	C <sub>22</sub> H <sub>26</sub> O <sub>5</sub>	370.18	[α] <sub>D</sub> = +10° (acetone, c 1.0)
(-)-Calanolide B ((-)-2)	<i>C. lanigerum</i> <i>C. brasiliense</i>	C <sub>22</sub> H <sub>26</sub> O <sub>5</sub>	370.18	[α] <sub>D</sub> = -38.6° (acetone, c 0.5)
(+)-Calanolide C (3)	<i>C. lanigerum</i> <i>C. brasiliense</i>	C <sub>22</sub> H <sub>26</sub> O <sub>5</sub>	370.18	[α] <sub>D</sub> = +96.3° (CHCl <sub>3</sub> , c 0.1)
Calanolide D (4)	<i>C. lanigerum</i>	C <sub>22</sub> H <sub>24</sub> O <sub>5</sub>	368.16	[α] <sub>D</sub> = +60° (CHCl <sub>3</sub> , c 0.5)
Calanolide E1 (5)	<i>C. lanigerum</i>	C <sub>22</sub> H <sub>28</sub> O <sub>6</sub>	388.19	[α] <sub>D</sub> = +28.4° (MeOH, c 0.64)
Calanolide E2 (5)	<i>C. lanigerum</i>	C <sub>22</sub> H <sub>28</sub> O <sub>6</sub>	388.19	[α] <sub>D</sub> = +79.1° (MeOH, c 0.64)
Calanolide F (6)	<i>C. teysmannii</i>	C <sub>22</sub> H <sub>26</sub> O <sub>5</sub>	370.18	[α] <sub>D</sub> = -51.5° (CHCl <sub>3</sub> , c 0.35)
12-O-acetyl calanolide A (7)	<i>C. lanigerum</i>	C <sub>24</sub> H <sub>28</sub> O <sub>6</sub>	412.19	[α] <sub>D</sub> = +20° (CHCl <sub>3</sub> , c 0.5)
12-O-methyl calanolide A (8)	<i>C. lanigerum</i>	C <sub>23</sub> H <sub>28</sub> O <sub>5</sub>	384.19	[α] <sub>D</sub> = +32° (CHCl <sub>3</sub> , c 0.8)
12-O-methyl calanolide B (9)	<i>C. lanigerum</i>	C <sub>23</sub> H <sub>28</sub> O <sub>5</sub>	384.19	[α] <sub>D</sub> = +34° (CHCl <sub>3</sub> , c 0.5)

mutations, K103N and Y181C – a substitution at codon Y188H of RT was found to be associated with 30-fold resistance to the drug *in vitro*. The compound enhanced the activity against virus isolates with the Y181C mutation, which confers resistance to other NNRTIs and against viruses that have both AZT resistance and the Y181C mutation. Even though (+)-calanolide A (1) exhibited reduced activity against HIV-1 with the K103N mutation, it remained fully active against virus isolates that express both the K103N and the Y181C mutations. This resistance profile is a unique feature of the compound as the Y181C and K103N mutations are two of the most commonly observed mutations in laboratory and clinical virus isolates from patients receiving other NNRTIs, including nevirapine, delavirdine, and efavirenz [18].

Further characterization of the selective antiviral activity and mechanism of action of (+)-calanolide A (1) were carried out by Currens *et al.* [19]; the drug was found to inhibit a wide variety of laboratory strains of HIV-1, with EC<sub>50</sub> values ranging from 0.10 to 0.17 μM. At the same time, it inhibited promonocytotropic and lymphocytotropic isolates from patients in various stages of HIV disease and drug-resistant strains as well. From their detailed studies, it was evidenced

that the drug acted early in the infection process, similar to the known HIV RT inhibitor 2',3'-dideoxycytidine. In enzyme inhibition assays, (+)-calanolide A (**1**) potently and selectively inhibited recombinant HIV-1 RT, but not cellular DNA polymerases or HIV-2 RT within the concentration range tested [16, 19]. Quan *et al.* [15] also inferred that the drug is metabolized by cytochrome P450 3A (CYP3A) and the drug levels may be enhanced if coadministered with ritonavir (*Norvir*). Synergistic effect was observed *in vitro* for the combination of (+)-calanolide A (**1**) with a number of other ARV agents, including NRTIs, NNRTIs, and protease inhibitors [3, 8, 15, 17, 20].

Currens *et al.* [19] studied the biochemical mechanism of inhibition of HIV-1 RT by (+)-calanolide A (**1**) using two template/primer systems – ribosomal ribonucleic acid (RNA) and homopolymeric rA-dT 12–18. Kinetic analyses indicated that (+)-calanolide A (**1**) inhibits HIV-1 RT by a complex mechanism involving two possible binding sites, a property that has not been observed for any other NNRTI [3]. The experimental data indicated that the compound binds to the active site of the enzyme and interferes with dNTP (deoxynucleotide triphosphate) binding. It was also evidenced that the test compound shares some binding domains with both phosphonoformic acid and 1-ethoxymethyl-5-ethyl-6-phenylthio-2-thiouracil, presumably reflecting that it interacts with RT near both the pyrophosphate-binding site and the active site of the enzyme [19]. In addition, the *in vivo* anti-HIV efficacy of (+)-calanolide A (**1**) was evaluated in the hollow fiber-based mouse model; following oral or parenteral administration on a once- or twice-daily treatment schedule, (+)-calanolide A (**1**) was capable of completely suppressing virus replication in two distinct and separate physiologic compartments (peritoneal cavity and subcutaneous site). Furthermore, a synergistic effect was observed for the combination of (+)-calanolide A (**1**) and AZT in this animal model. The *in vivo* efficacy demonstrated here, along with a variety of *in vitro* studies, suggests that (+)-calanolide A possesses features favorable for its development as a clinical candidate against AIDS [8].

A number of *in vitro* studies demonstrated that (+)-calanolide A (**1**) displays protective effect on established cell lines as well as primary human cells against a wide range of laboratory and clinical isolates of HIV-1, including syncytium-inducing and non-syncytium-inducing viruses, T-tropic and monocyte-macrophage tropic viruses, with  $EC_{50}$  values ranging from 0.02 to 0.5  $\mu$ M [1, 3, 16, 17, 19]. The compound showed less cytotoxicity toward the host cells – its 50% infective concentration was found to be ~100–200 times greater than the anti-HIV-1-activity concentration in all cell lines tested [3, 19, 21]. (+)-Calanolide A (**1**) was well tolerated at oral doses of up to 150 mg  $kg^{-1}$  in rats and 100 mg  $kg^{-1}$  in dogs; toxicities associated with the oral administration of (+)-calanolide A (**1**) for up to 28 days in animals were gastric irritation and subsequent gastric hyperplasia and edema in the rat, and salivation in the dog. *In vitro* and *in vivo* assays for mutagenicity have been negative, and the compound did not produce teratologic effects when administered to rats during gestation [8]. *In vitro*

studies indicated that the metabolism is qualitatively similar in rats, dogs, monkeys, and humans, with four to seven main metabolites produced [8]. CYP3A4 is the primary isoform of P450 that metabolizes (+)-calanolide A (**1**), although CYP2C may be involved as a minor isoform [8]. Animal studies have shown that compound-related radioactivity distributes into both the brain and the lymph after oral administration, while after intravenous administration, the radioactivity accumulates in the brain [8]. These studies indicated that (+)-calanolide A (**1**) crosses the blood–brain barrier and may be preferentially distributed in the lymphatic system [22]. Indeed, in rat studies, the oral administration of radio-labeled (+)-calanolide A resulted in a mean ratio of lymph to serum radioactivity of 2.8:1 after 6 h. (+)-Calanolide A (**1**) binds extensively (>97%) to human and animal plasma proteins and to human  $\alpha_1$ -acid glycoprotein [6, 20].

The NCI has played an active and supportive role in the development of the calanolide class of compounds including aspects of preclinical development, such as the synthesis of analogs, *in vivo* animal assays, serum-binding studies, formulation, pharmacology, and toxicology [23]. Sarawak MediChem Pharmaceuticals [24, 25], a 50/50 joint venture between the State Government of Sarawak (Malaysia) and Advanced Life Sciences (Woodridge, IL), has the exclusive worldwide license to the calanolide class of compounds from the NCI. They have successfully completed early Phase I/II 48-subject clinical trial of calanolide A in combination therapy for HIV, which evaluated the effect of therapy on pharmacokinetic enhancement and safety [26]. The clinical trials were carried out in both healthy and HIV-infected volunteers; 47 HIV-negative, healthy volunteers were treated with (+)-calanolide A (**1**); the toxicity of the drug after oral administration was minimal, and the most reported adverse effects were dizziness, oily aftertaste, headache, and nausea [8, 20]. Results of the trials also confirmed that the combination therapy was effective in increasing the blood levels of calanolide in human volunteers. No drug accumulation was seen over the entire dosing period, although (+)-calanolide A did have a relatively long elimination half-life (15–20 h); it must be emphasized that with respect to the pharmacokinetic parameters, the intrasubject variability was high [20]. Another experimental study [27] enrolled 43 HIV-infected subjects in a randomized, double-blind, placebo-controlled, dose-ranging study of (+)-calanolide A (**1**); twice daily doses of 200, 400, and 600 mg of placebo were taken for 14 days. It was observed that the viral load reduction in the 600 mg arm at day 14 was  $-0.81$  l, which was greater than placebo ( $p < 0.027$ ). Recently, multicenter Phase II clinical trials were initiated on patients with HIV infection in the United States and in Malaysia, focusing on the assessment of its long-term anti-HIV activity in combination with other anti-HIV agents and an assessment of the long-term durability of such drug combinations [24].

(–)-Calanolide B (**2**) is also a novel non-nucleoside HIV-1-specific reverse-transcriptase inhibitor [2, 28]; the isolate was found to exhibit promising protective effect against HIV-1 replication with  $EC_{50}$  and  $IC_{50}$  values of  $0.2 \pm 0.1$

and  $5.9 \pm 1.9 \mu\text{M}$ , respectively [29]. Huerta-Reyes *et al.* [4] also evaluated that it shows  $\sim 76\%$  inhibition against the HIV-1 RTase with  $\text{IC}_{50}$  value of  $0.5 \mu\text{M}$ . Interestingly, its enantiomers (i.e., (+)-calanolide B (**2**)) were observed to be inactive against the virus [30]. (–)-7,8-Dihydrocalanolide B (i.e., dihydrocostatolide) was also reported to possess almost identical anti-HIV potency with that of (–)-calanolide B (**2**) [17]. Hence, the potent antiviral properties of (–)-calanolide B (**2**) and its dihydro-derivative might be good anti-HIV drug candidates in the near future.

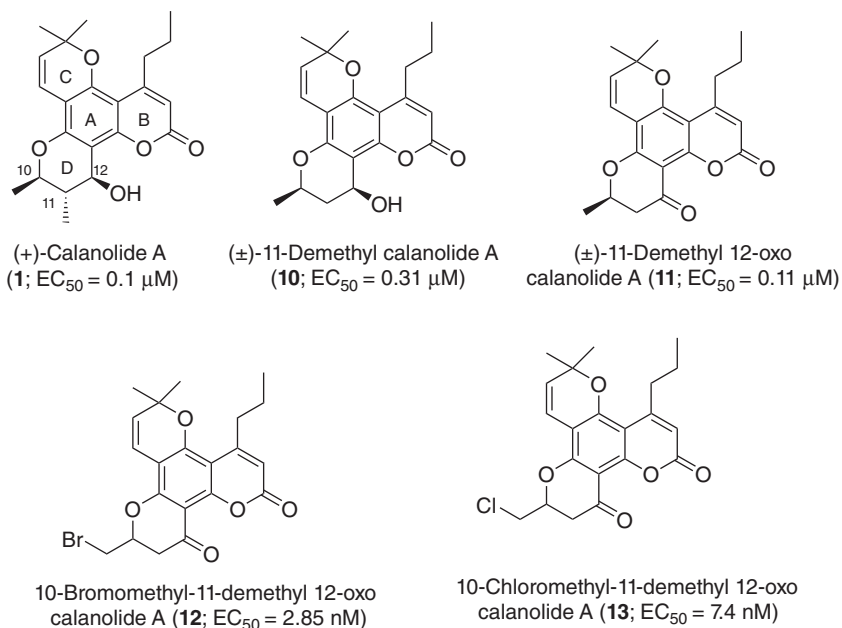
Calanolides E2 (**5**) and F (**6**) were found to exhibit moderate anti-HIV activity with  $\text{IC}_{50}$  values of 2.5 and  $12.7 \pm 1.0 \mu\text{M}$ , respectively ((–)-calanolide B (**2**) was used as positive control;  $\text{IC}_{50} = 9.8 \pm 0.8 \mu\text{M}$ ;  $\text{EC}_{50} = 0.22 \pm 0.03 \mu\text{M}$ ) [7].

### 12.3.2

#### Studies on Structure–Activity Relationships (SARs) of Calanolides

From the studies of structure–activity relationships (SARs), the importance of the presence of methyl groups at C-10 and C-11 and a hydrogen bond acceptor at C-12 in exhibiting the anti-HIV-1 activity by the compound was established [30–32]. It was also worked out that in the case of calanolides, the C-12 hydroxyl group should be *S* configured, while the C-12 hydroxyl of inophyllums can be either *S* or *R* configured for better activity [33]. (+)-Calanolide A (**1**) was about 50 times more active on HIV-1 RT inhibition compared to cordatolides A and B, indicating the importance of its *n*-propyl substituent at C-4 [34].

Studies on SARs were investigated with calanolide A (**1**) by some other groups and libraries of derivatives were also synthesized followed by a study of their comparative anti-HIV efficacy. It was demonstrated that ( $\pm$ )-11-demethyl calanolide A (**10**) also has inhibitory activity against HIV-1, with an  $\text{EC}_{50}$  value of  $0.31 \mu\text{M}$  and a range of therapeutic index (TI) from 21 to 169; however, this compound was found to be toxic for cells tested both *in vitro* and in mice [35]. Fortunately, 11-demethyl-12-oxo calanolide A (**11**), which was the precursor of ( $\pm$ )-11-demethyl calanolide A synthesis and having two fewer chiral carbon centers at the C-11 and C-12 positions than (+)-calanolide A, also found to exhibit similar inhibitory activity against HIV-1, with an  $\text{EC}_{50}$  value of  $0.11 \mu\text{M}$  and a better TI of 818 [36]. Because there is only one chiral carbon center remaining in the structure, this result encouraged the investigators to pursue further studies of its SARs by using combinatorial chemistry technology. A chemical library based on tetracyclic dipyrano coumarin (**11**) (Figure 12.2) was constructed by the same group of investigators (Liu and coworkers) who then carried out their anti-HIV efficacy evaluation *in vitro* [37]. From their detailed investigation and through a systematic analysis of SARs, 10-bromomethyl-11-demethyl-12-oxo calanolide A (**12**) was identified as a novel compound from the library to have a much higher inhibitory potency ( $\text{EC}_{50} = 2.85 \text{ nM}$ ) and TI ( $>10\,526$ ) than that of (+)-calanolide A against HIV-1 through the assays *in vitro* [37]. This finding provided a very



**Figure 12.2** Calanolide-based chemical library in search of anti-HIV agents [37, 38].

important clue that modifications of the D ring at the C-10 position may be conducted to obtain drug candidates with activity against HIV-1 [37]. To address the concern that compound **12** may confer toxicity, later on, the same group of investigators [38] modified the ring D by replacing the bromine atom at position C-10, with the aim of finding alternative antiviral drug candidate presenting similar or higher anti-HIV activity against both wild-type and drug mutants with tolerated toxicity, and acceptable oral bioavailability. Hence, they prepared a new anti-HIV-1 compound, 10-chloromethyl-11-demethyl-12-oxo-calanolide A (F18; **13**), with low  $EC_{50}$  value (7.4 nM) and high selectivity index ( $SI = 1417$ ); they demonstrated a druggable profile with 32.7% oral bioavailability in rat, and tolerated oral single dose toxicity in mice. It especially exhibited the feature of highly efficient suppression of both the wild-type HIV-1 ( $EC_{50} = 7.4 \text{ nM}$ ) and Y181C mutant HIV-1 ( $EC_{50} = 0.46 \text{ nM}$ ) [38]. As a result, F18 (**13**) has been considered as an attractive antiretroviral (ARV) candidate for treating HIV-infected patients. Recently, Li and his group [39] developed a simple and accurate liquid chromatography–tandem mass spectrometry/mass spectrometry (LC–MS/MS) to quantify F18 in rat plasma in order to apply the method to study the pharmacokinetics of F18 in rats. F18 was monitored by positive electrospray ionization in the selected reaction-monitoring mode; the standard curve was linear over the range of 2–1000  $\text{ng ml}^{-1}$ . The method was used to determine the plasma concentration of F18 after a single oral dose of 50  $\text{mg kg}^{-1}$  in rats [39].



## 12.3.3

**Antituberculosis Potential of Calanolides and Related Derivatives**

Tuberculosis (TB), caused by *Mycobacterium tuberculosis* (*Mtb*), is the world's number one infectious pathogen that kills 2–3 million people annually [40, 41]. Currently, one third of the world's population is infected with *Mtb* and 8.9–9.9 million new and relapse cases of TB are reported every year [42]. The emergence of new cases, the increased incidence of multidrug resistant (MDR) strains of *Mtb*, the adverse effects of first- and second-line anti-TB drugs, and the increased incidence of TB associated with viral infections (HIV) have led to renewed research interest in natural products and their analogs in the hope of discovering new antitubercular leads [43, 44]. At present, there is no standard optimal anti-TB therapy in AIDS patients, and no single agent that is active against infections caused by both HIV and *Mtb*. It is clear that there is an urgent need for anti-TB drugs with improved properties such as enhanced activity against MDR strains, reduced toxicity, shortened duration of therapy, rapid mycobactericidal mechanism of action, and the ability to penetrate host cells and exert antimycobacterial effects in the intracellular environment. In particular, new agents with activity against both TB and HIV infections are in great demand [5]. Xu *et al.* [5] reported for the first time that the naturally occurring anti-HIV-1 agent (+)-calanolide A is capable of showing activity against all of the strains of *Mtb* tested, including those resistant to the standard antitubercular drugs; the natural product exhibited minimum inhibitory concentration (MIC) of  $3.1 \mu\text{g ml}^{-1}$  against replicating *M. tuberculosis* (R-*Mtb*). Efficacy evaluations in macrophages revealed that (+)-calanolide A significantly inhibited intracellular replication of *Mtb* H37Rv at concentrations below the MIC observed *in vitro*. On the basis of preliminary mechanistic studies, the investigators indicated that the test compound rapidly inhibits RNA and DNA synthesis followed by an inhibition of protein synthesis. Compared with known inhibitors, this scenario is more similar to effects observed with rifampin, an inhibitor of RNA synthesis. Since (+)-calanolide A was active against a rifampin-resistant strain, it is believed that these two agents may involve different targets. (+)-Calanolide A and its related pyranocoumarins were the first class of compounds identified to possess antimycobacterial and ARV activities, representing a new pharmacophore for anti-TB activity. The pharmacokinetic data indicate that the (+)-calanolide A concentrations in plasma may be comparable to the observed *in vitro* MICs against *Mtb*. Therefore, the dual activities may render (+)-calanolide A as a possible chemoprophylactic agent for TB in HIV-infected people or vice versa if clinical benefits can be demonstrated. However, the compound was almost equally toxic to Vero cells ( $\text{LD}_{50} = 7.6 \mu\text{g ml}^{-1}$ ) (lethal dose, 50%), with an unacceptable selectivity index (SI) of 2.4 [5]. Hence, interest has been initiated to explore calanolides as antimycobacterial agents, particularly using high-throughput screening of synthesized chemical libraries analogous to calanolides with modified rings C and D.

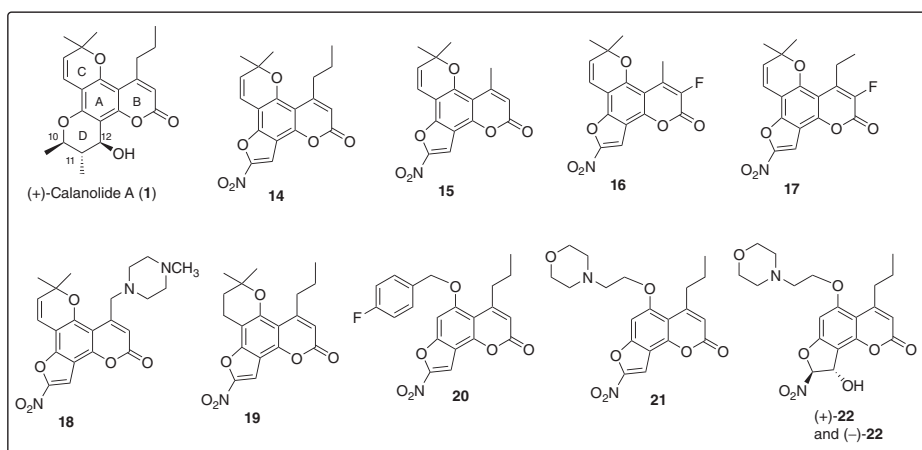


Figure 12.3 Calanolide-based chemical library in search of antitubercular agents [46].

In 2013, Guo and Liu devised a scaffold-hopping strategy of structure modification of (+)-calanolide A by replacing ring D with various five- or six-membered nitrogen-containing heterocycles in anticipation of obtaining further understanding of the SAR. The newly designed and synthesized analogs bear a novel backbone, which may lead to a better understanding of SAR and anti-HIV and anti-TB potency [45]. In the following year, Liu and his group [46] initiated a high-throughput screening of a synthesized chemical library (more than 70 compounds) enriched for calanolides (modified structures at ring C and D) and identified several derivatives with selective activity against replicating and/or nonreplicating *Mtb*. Further SAR studies gave information about more potent synthetic calanolides with decreased toxicity, including three compounds with activity against *Mtb*-infecting human macrophages. The prominent synthetic calanolide analogs are shown in Figure 12.3, and their anti-TB (against both replicating and nonreplicating WT *Mtb* H37Rv bacteria) efficacies are presented in Table 12.2.

The investigators observed that analogs, particularly those bearing 2-nitrofurans at the ring D position, had markedly improved *in vitro* efficacy and reduced mammalian cell toxicity compared with the parent compound, (+)-calanolide A. For example, compound **20** had MIC values of 0.6 and 3  $\mu\text{g ml}^{-1}$  against R-*Mtb* and NR-*Mtb*, respectively, with LD<sub>50</sub> values >100  $\mu\text{g ml}^{-1}$  to HepG2 cells. Similarly, compound **21** was improved over (+)-calanolide A both in potency (39-fold against R-*Mtb*; MIC<sub>90</sub> = 0.08  $\mu\text{g ml}^{-1}$ ) and SI (60-fold). Compound **16** had an MIC of 0.08  $\mu\text{g ml}^{-1}$  (R-*Mtb*) (a 40-fold improvement over (+)-calanolide A) and was nontoxic to HepG2 cells, with an LD<sub>50</sub> value of >50  $\mu\text{g ml}^{-1}$ . Several of the active analogs (**14**, **15**, **17–19**, (+)-**22**, and (–)-**22**) had potent bactericidal activity

**Table 12.2** Anti-TB efficacies of some selected synthetic calanolide analogs [46].

Compounds	MIC <sub>90</sub> ( $\mu\text{g ml}^{-1}$ ) (against R- <i>Mtb</i> H37Rv)	MIC <sub>90</sub> ( $\mu\text{g ml}^{-1}$ ) (against NR- <i>Mtb</i> H37Rv; 7 days exposure)	LD <sub>50</sub> (for HepG2 cells)	Selectivity index (SI) SI = LD <sub>50</sub> /MIC <sub>90</sub> for R- <i>Mtb</i>
<b>1</b>	3.1	—	—	2.4
<b>14</b>	0.3	0.6	3	8
<b>15</b>	0.08	0.2	1	16
<b>16</b>	<0.1	0.2	>50	>510
<b>17</b>	0.6	1	>25	>40
<b>18</b>	0.08	0.3	3	31
<b>19</b>	0.6	1	>50	>80
<b>20</b>	0.6	3	>100	>160
<b>21</b>	0.08	0.2	10	125
(+)- <b>22</b>	0.08	0.08	0.8	10
(–)- <b>22</b>	0.08	0.08	2	20

against *Mtb* H37Rv residing in primary human macrophages. However, two of the nitrobenzofuran-containing lead compounds were found to be genotoxic to mammalian cells. Although genotoxicity precluded clinical progression, the profound, selective mycobactericidal activity of these calanolides should enable their use as tool compounds in identifying pathways essential for *Mtb* during replication, nonreplication, and infection of human macrophages. In addition, these potent calanolides will aid further structure-based design of more effective and drug-like antimycobacterial agents [46].

## 12.4

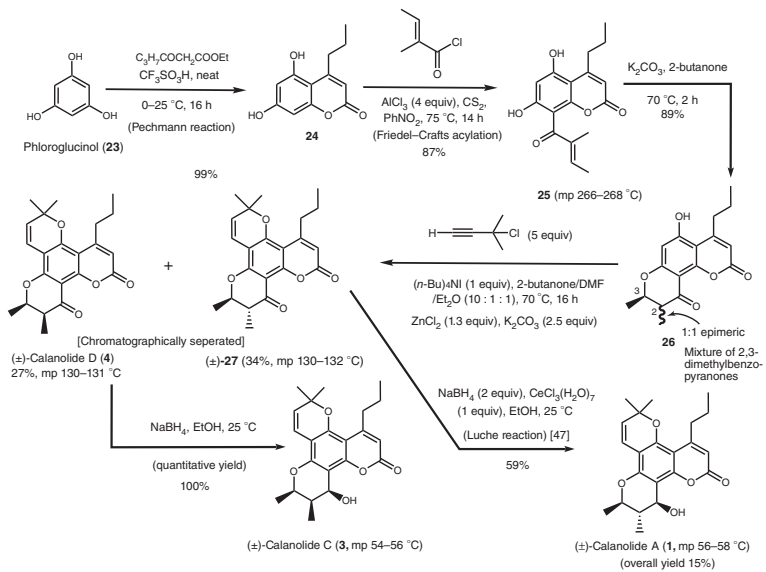
### Total Syntheses of Calanolides

Owing to low availability of naturally occurring calanolides, various workers developed total synthetic approaches for these polycyclic coumarins in order to provide materials including related analogs for preclinical and clinical research. Supplies of such compounds from plant material are not only extremely limited to certain *Calophyllum* species and difficult to obtain but also the other nearby members of the same species did not contain the same material. Hence, practical total synthesis of calanolides, particularly calanolides A and B is of great importance [6]. This section is devoted to summing up such synthetic developments.

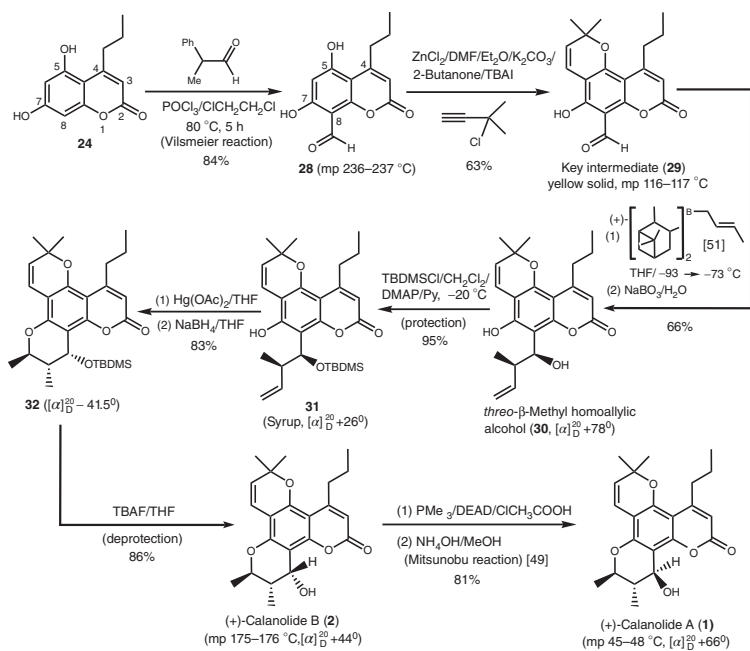
In 1993, Dreyer and his group [47] first reported total syntheses of ( $\pm$ )-calanolides A (**1**), C (**3**), and D (**4**) (Scheme 12.1). The investigators utilized the Pechmann reaction to construct coumarin ring **24** from phloroglucinol (**23**) in the first step followed by Friedel–Crafts acylation with tigloyl chloride to obtain compound **25**. Compound **26**, formed on base treatment of **25**, thereafter resulted in a clean formation of chromenes ( $\pm$ )-**27** and ( $\pm$ )-**4** in 1.3:1 ratio (61% combined yield after chromatographic separation). The spectral evidences for compound ( $\pm$ )-**4** were identical with reported natural calanolide D [1]. Sodium borohydride reduction of ( $\pm$ )-**4** proceeded quantitatively to ( $\pm$ )-**3** whose spectral data matched with calanolide C [1]. Luche reduction [48] of ketone ( $\pm$ )-**27** afforded ( $\pm$ )-calanolide A [( $\pm$ )-**1**] in a highly stereoselective manner with 59% yield (overall yield of 15%).

Later on in 1995, Baker and coworkers [49] reported the first enantioselective total synthesis of (+)-calanolide A (**1**) and (+)-calanolide B (**2**); the key intermediate **29** underwent a series of reactions to afford **2** in good yield. Thereafter, the investigators efficiently converted (+)-calanolide B (**2**) into (+)-calanolide A (**1**) via a modified Mitsunobu reaction [50] (Scheme 12.2).

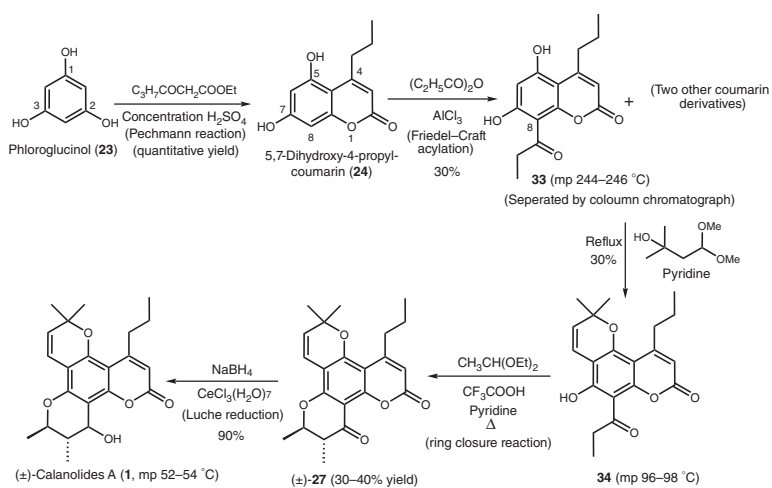
Xu and his group [52] synthesized racemic calanolide A from chromene **34**, the key intermediate in their approach. Compound **34** was obtained from phloroglucinol **23** by means of a series of reactions as depicted in Scheme 12.3. Chromene **34** then underwent reaction with acetaldehyde diethyl acetal in the presence of trifluoroacetic acid and pyridine to furnish racemic chromanone **27** with the desired



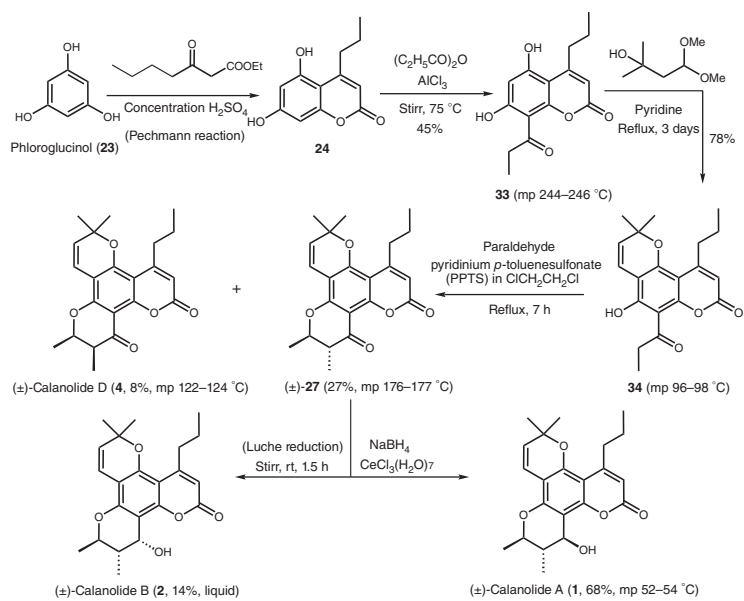
Scheme 12.1 Dreyer's total synthesis of (±)-calanolides A, C, and D [47].



Scheme 12.2 Baker's synthesis of (+)-calanolides A and B [49].

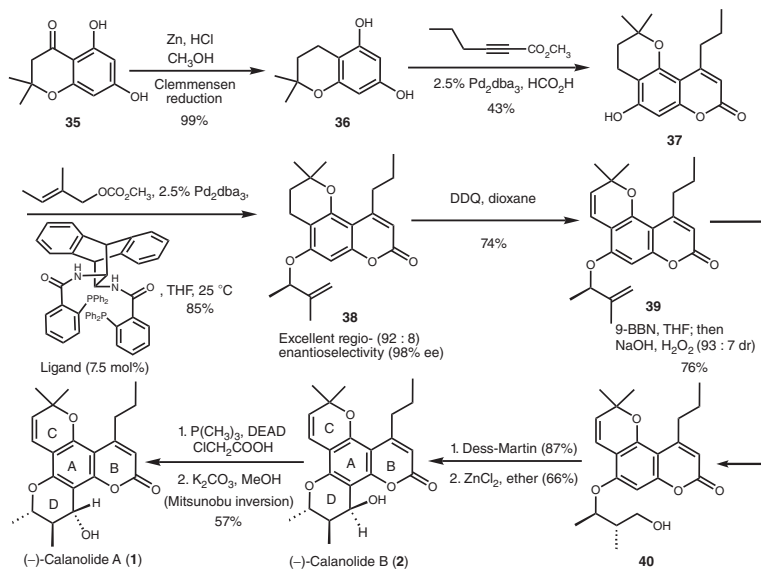


Scheme 12.3 Xu's total synthesis of (±)-calanolide A [52].

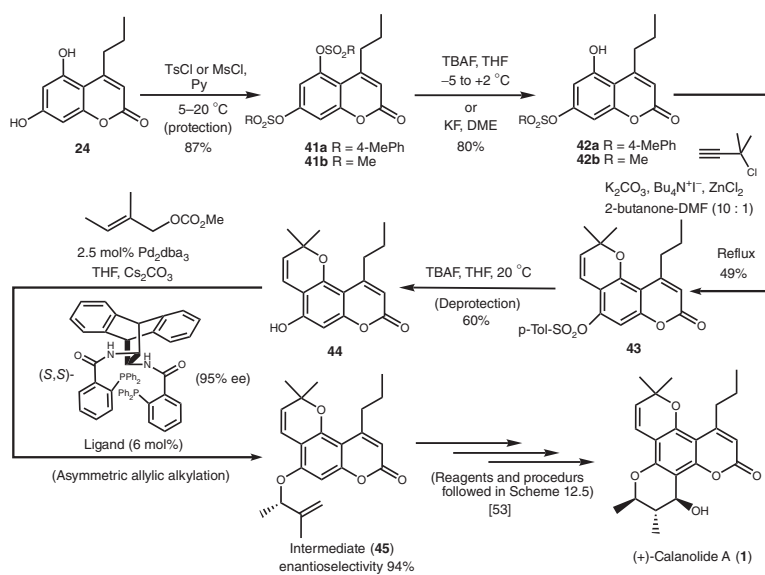


**Scheme 12.4** Xu's total synthesis of (±)-calanolides A, B, and D [53].

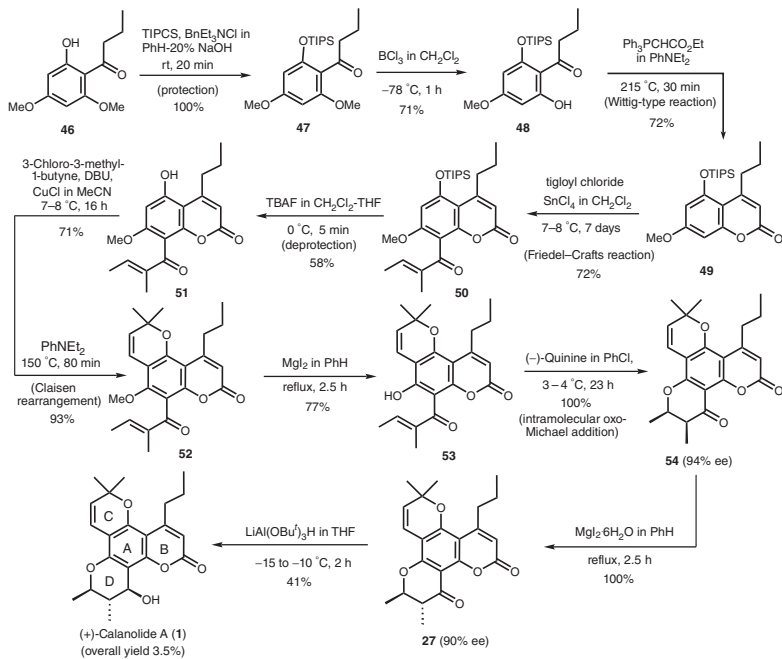




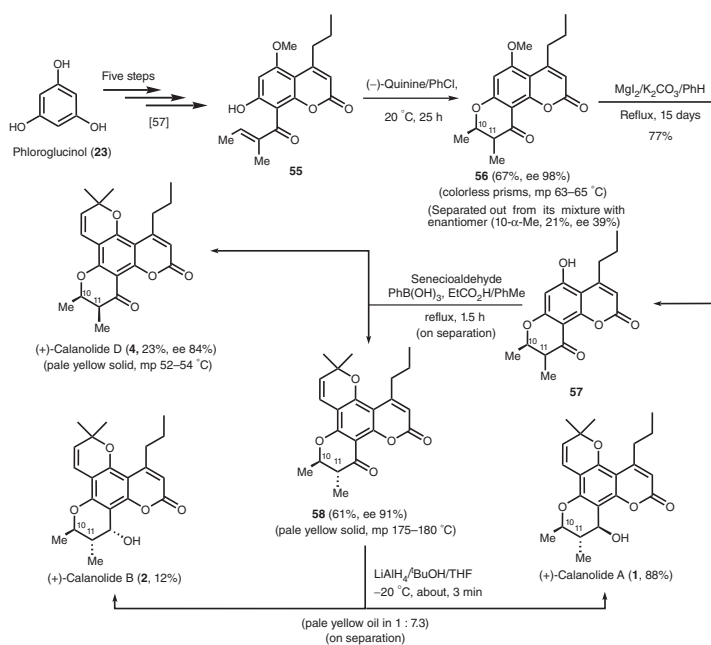
Scheme 12.5 Trost's total synthesis of (-)-calanolides A and B [54].



Scheme 12.6 Fox's synthesis of key intermediate of (+)-calanolide A [56].



Scheme 12.7 Ishikawa's total asymmetric synthesis of (+)-calanolide A [57].



**Scheme 12.8** Ishikawa's synthesis of (+)-calanolides A, B, and D [58].

stereochemical arrangement. Luche [48] reduction of ( $\pm$ )-**27** using  $\text{NaBH}_4/\text{CeCl}_3$  at  $-10^\circ\text{C}$  was found to be highly selective in affording ( $\pm$ )-calanolide A (**1**) in 90% yield with 90% purity. The same group [53] also accomplished the synthesis of ( $\pm$ )-calanolides A (**1**), B (**2**), and D (**4**) from phloroglucinol (Scheme 12.4).

In 1998, Trost and Toste [54] developed a concise method to prepare (–)-calanolides A (**1**) and B (**2**) in good yields (Scheme 12.5) – the key intermediate chromene **39** on chemoselective hydroboration with 9-BBN (9-borabicyclo[3.3.1]nonane) afforded alcohol **40** with 93:7 diastereomeric ratio for the newly formed stereocenter within the molecule. Compound **40** was then readily oxidized with the Dess–Martin periodinane, and the resulting aldehyde diastereoselectively cyclized with  $\text{ZnCl}_2$  to afford the thermodynamically less stable ent-calanolide B (**2**) (dr 10:1) that eventually was converted to (–)-calanolide A (**1**) via Mitsunobu inversion [50, 55]. Later on, Fox *et al.* [56] developed an improved asymmetric synthesis of the key intermediate **45** (ee 94%) (Scheme 12.6) by using the Trost palladium(0)-catalyzed asymmetric allylation route [6] and compound **45** could then be successfully transformed into (+)-calanolide A (**1**) [54].

A 13 step enantioselective total synthesis of (+)-calanolide A (**1**) was achieved by Ishikawa and his group [57] using (–)-quinine-catalyzed asymmetric intramolecular oxo-Michael addition (IMA) of *o*-tigloylphenol **53** as the key step followed by  $\text{MgI}_2$ -mediated isomerization of the *cis*-chromanone **54** into its *trans*-isomer **27** (ee 90%). Reduction of this enantiomerically rich precursor **27** with lithium tri(*tert*-butoxy)aluminum hydride afforded (+)-calanolide A (**1**) with an overall yield of 3.5% (Scheme 12.7). However, this synthetic route was modified later on by the same group of investigators in terms of enhancing product yields with reduced steps (Scheme 12.8) [58].

## 12.5

### Concluding Remarks

Natural calanolides, well known for their anti-HIV potential, occupy a significant position in the pyranocoumarin class of compounds. In addition, these pyranocoumarins have also been found to exhibit anti-TB activity. Such promising pharmaceutical activity coupled with low availability of natural calanolides has evoked tremendous interest among the organic chemists to undertake systematic chemical studies toward achieving the total synthesis of this class of compounds. Preclinical and clinical results of both natural and synthetic calanolides have been found to be much encouraging and eventually are regarded as the potential “leads” in the development of future anti-HIV and anti-TB drugs. A considerable number of related patents have also been filed so far. However, more in-depth and extensive studies on calanolides in all relevant aspects are still warranted.

We do anticipate that the present overview would boost the on-going development in this direction.

### Acknowledgment and Disclosure

This chapter is based upon the published review article entitled *Naturally Occurring Calanolides: An Update on Their Anti-HIV Potential and Total Syntheses* by Goutam Brahmachari and his group, which appeared in *Recent Patents on Biotechnology*, 2014, **8**, 3–16 (Bentham Science Publishers). *Materials used in this book chapter are reproduced with permission from the Publisher.*

### Abbreviations

AIDS	acquired immunodeficiency syndrome
ARV	antiretroviral
AZT	azidothymidine (or zidovudine)
9-BBN	9-borabicyclo[3.3.1]nonane
CYP3A	cytochrome P450 3A
DBU	1,8-diazabicyclo[5.4.0]undec-7-ene
DDQ	2,3-dichloro-5,6-dicyano-1,4-benzoquinone
DEAD	diethyl azodicarboxylate
DMAP	4-dimethylaminopyridine
DME	dimethyl ether
DMF	dimethylformamide
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
EC <sub>50</sub>	half maximal effective concentration
HIV	human immunodeficiency virus
HIV-1	human immunodeficiency virus type 1
IC <sub>50</sub>	half maximal inhibitory concentration
IMA	intramolecular oxo-Michael addition
LD <sub>50</sub>	lethal dose, 50% (the lethal dose at which 50% of the population if killed in a given period of time)
MDR	multidrug resistant
MsCl	methanesulfonyl chloride
MIC	minimum inhibitory concentration
<i>Mtb</i>	<i>Mycobacterium tuberculosis</i>
NCI	National Cancer Institute
NNRTIs	non-nucleoside reverse-transcriptase inhibitors
NRTI	nucleotide Reverse Transcriptase Inhibitor
NSI	non-syncytium-inducing
PPTS	pyridinium <i>p</i> -toluenesulfonate
RNA	ribonucleic acid

RT	reverse transcriptase
SI	selectivity index
TBAF	tetra- <i>n</i> -butylammonium fluoride
TBAI	tetrabutylammonium iodide
TBDMSCl	<i>tert</i> -butyldimethylsilyl chloride
THF	tetrahydrofuran
TIPCS	triisopropylchlorosilane
TsCl	<i>p</i> -toluenesulfonyl chloride
WT	wild type

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## 13

# Selective Estrogen Receptor Modulators (SERMs) from Plants

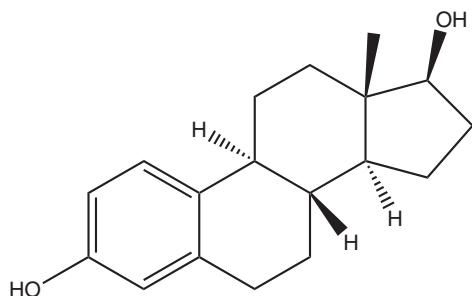
*Divya Lakshmanan Mangalath and Chittalakkottu Sadasivan*

### 13.1

#### Introduction

The physiological role played by phytoestrogens has received considerable attention because of their ability to mimic the functions of estrogen. Phytoestrogens include several groups of steroidal compounds and are widely distributed within the plant kingdom. The most potent estrogen produced in the body is 17- $\beta$ -estradiol (E2) (Figure 13.1). The estrogenic responses within the body are mediated by estrogen receptors (ERs), either ER $\alpha$  (estrogen receptor alpha) or ER $\beta$  (estrogen receptor beta) which are members of the nuclear receptor (NR) superfamily of ligand-dependent nuclear transcription factors (TFs). Estrogen can also exert its effect through membrane-bound estrogen receptors (mER $\alpha$  or mER $\beta$ ) or G-protein-coupled estrogen receptor 30 (GPR30).

Estrogen plays a prominent role in female development and reproduction. It has beneficial effects on the cardiovascular, musculoskeletal, immune, and central nervous systems of both males and females [1]. In contrast, estrogen has been linked to the increase in the occurrence of breast cancer and different uterine lesions and tumors. Phytoestrogens are strikingly similar to the mammalian estrogen, E2 in their chemical structures. They bind to ERs, with a preference for ER $\beta$ . The compounds that selectively inhibit or stimulate ERs in various tissues are known as *selective estrogen receptor modulators (SERMs)* and the process is known as *selective estrogen receptor modulation*. In other words, the SERMs act like estrogens in some tissues but block estrogen action in other tissues. This property of phytoestrogens is currently taken into consideration for the development of plant-based therapeutics against various kinds of hormone-responsive cancers. In this chapter, we do intend to highlight certain plant-derived SERMs.



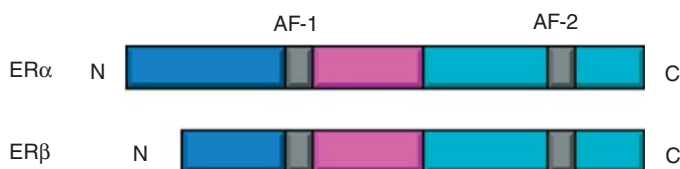
**Figure 13.1** Structure of 17- $\beta$ -estradiol.

### 13.2

#### Structure of Estrogen Receptor

ERs are homologous to other members of ligand-modulated nuclear TFs. ERs contain evolutionarily conserved, structurally and functionally distinct domains: the N-terminal domain, central DNA-binding domain (DBD), and C-terminal ligand-binding domain (LBD). DBD is the most conserved among the domains, whereas the N-terminal domain is the most variable in sequence and length. Two distinctive, conformationally dynamic regions responsible for the ligand-dependent transcriptional activation functions of ER are designated as activation function 1 (AF-1) and activation function 2 (AF-2) (Figure 13.2). AF-1 is located in the N-terminal domain of the receptor and the ligand-dependent AF-2 is located in the C-terminal domain. Both AF (activation function) domains recruit a range of co-regulatory protein complexes to the DNA-bound receptor.

The crystal structures of the LBD of ER in complex with the endogenous estrogen, E2, and a selective antagonist raloxifene have been solved by Brzozowski *et al.*, in 1997 [2]. These structures provide an instructive model for the members of this family. The overall architecture of the ER LBD (helices H3–H12) is similar to that seen in the crystal structures of other NR LBDs. The LBD is folded into a three-layered antiparallel  $\alpha$ -helical sandwich comprising a central core layer of three helices (H5/6, H9, and H10) sandwiched between two additional layers of



**Figure 13.2** (a, b) Schematic representation of ER $\alpha$  and ER $\beta$ . Dark blue: N-terminal domain; pink: central DNA-binding domain (DBD); cyan: C-terminal ligand-binding domain (LBD); and gray: AF regions located in the N- and C-terminals.

helices (H1–4 and H7, H8, H11). This helical arrangement maintains a sizeable ligand-binding cavity at the narrower end of the domain.

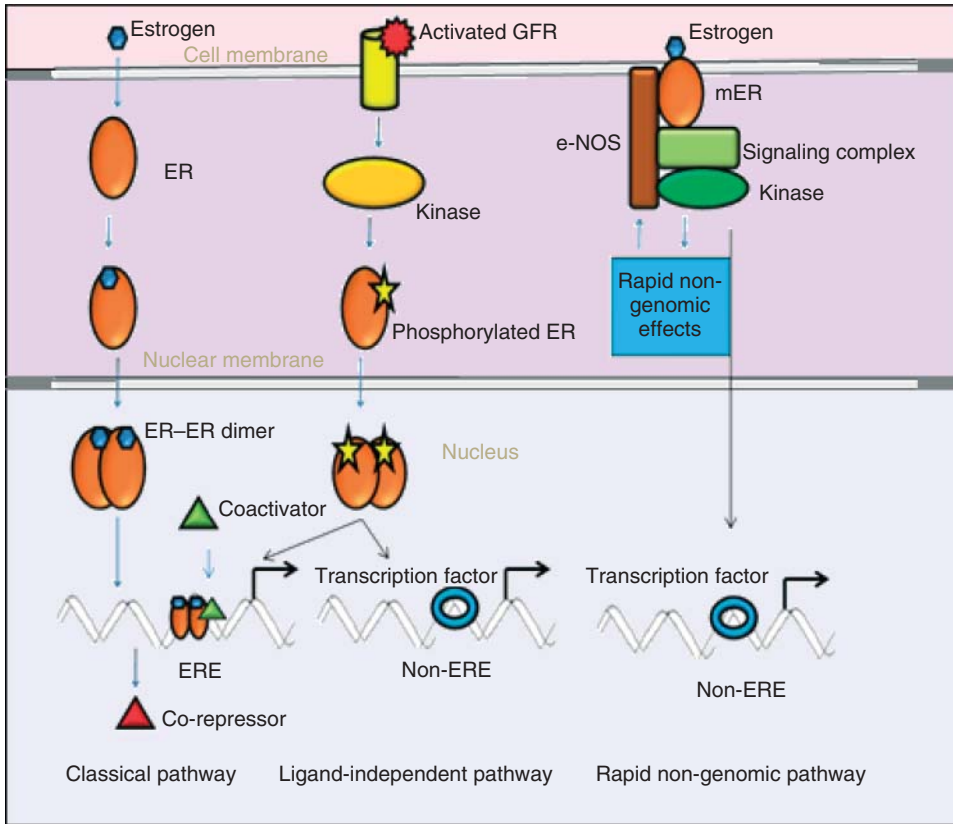
The E2-binding cavity is completely partitioned from the external environment and occupies a relatively large portion of the ER LBDs hydrophobic core. It is located at one end of the molecule and is formed by parts of H3 (Met 342 to Leu 354), H6 (Trp 383 to Arg 394), H8 and the preceding loop (Val 418 to Leu 428), H11 (Met 517 to Met 528), H12 (Leu 539 to His 547), and the S1/S2 hairpin (Leu 402 to Leu 410). E2 binds diagonally across the cavity between H11, H3, and H6. The phenolic hydroxyl of the A-ring of E2 makes direct hydrogen bonds to the carboxylate of Glu 353, the guanidinium group of Arg 394 and a water molecule. The 17- $\beta$  hydroxyl of the D ring makes a single hydrogen bond with His 524 in H11. The molecule also makes a number of hydrophobic contacts. The combination of the specific polar and nonpolar interactions account for the ability of ER to selectively recognize and bind E2 with subnanomolar affinity over a large and varied range of endogenous steroids. The ability of ER to bind different nonsteroidal compounds can be attributed to the size of the cavity, which has a volume nearly twice that of E2's molecular volume.

### 13.3

#### Estrogen Receptor Signaling

Ligand-dependent ER signaling begins with the binding of ligand to ER. The two ERs, ER $\alpha$  and ER $\beta$ , exert similar affinities for E2 and bind to the same regulatory regions of estrogen-responsive genes designated as estrogen-responsive elements (EREs), a 13 bp oligonucleotide sequence with 10 nucleotides forming an inverted repeat (GGTCAnnnTGACC) [3]. ER binds to the EREs as a dimer, with one ER molecule interacting with 5 bp of the inverted repeat. Upon binding of an agonist, the LBD adopts a conformation which facilitates the binding of coactivators (CoAs) (Figure 13.3). If the ligand is an antagonist, the binding of CoAs is prevented [2]. In the case of the binding of E2, the cell-specific transcriptional response depends on multiple factors, the most immediate being the composition of co-regulatory proteins and characteristics of the promoters of estrogen-responsive genes. The most-described ER CoAs include the p160 SRC (steroid receptor coactivator) family members and p300/CBP.

CoAs generally function by remodeling the chromatin structure of the target gene promoter and by facilitating transcription initiation through TFs and RNA polymerase-II by means of protein–protein interactions [4]. Although most studies were focused on the induction of AF-2 – CoA interactions by estradiol and their inhibition by antiestrogens, some studies have shown that the AF-1 can physically and functionally interact with CoAs in a hormone-independent manner. Besides, there is a third mechanism of action of ligand-activated ERs occurring



**Figure 13.3** ER signaling pathways: in the classical pathway, the hormone-bound ER dimerizes and binds to the estrogen-responsive elements (ERE) and displaces co-repressors (CoRs) from the DNA, recruits coactivator (CoA) proteins, and activates the expression of target gene. In ligand-independent pathway the activation of growth factor receptor (GFR) leads to the activation of specific kinases that directly phosphorylate the ER, resulting in altered

gene expression, either directly by the ER or through ER interactions with other transcription factors (TFs). In rapid nongenomic signaling pathway, estrogen induces a subpopulation of cell membrane-associated ERs to form a signaling complex resulting in rapid activation of specific kinases leading to rapid nongenomic effects resulting in phosphorylation and enzymatic activation of e-NOS.

within seconds or minutes after the addition of E2. This rapid nongenomic signaling events of estrogen is mediated through membrane-bound forms of ERs designated as GPR30, mER $\alpha$ , and mER $\beta$ , resulting in the downstream activation of specific kinases and their effector molecules.

Although both the ERs, ER $\alpha$  and ER $\beta$ , bind to the same DNA-responsive elements and their target genes are also the same, there exists a basic difference in

their expression/distribution profile and eventual outcome of the transcription [5]. The E2–ER $\alpha$  complex rapidly activates multiple signaling pathways committed to both cell cycle progression and prevention of apoptotic cascades supporting the survival of cells. In contrast, the E2–ER $\beta$  complex induces rapid and constant phosphorylation of p38 MAPK, which in turn is involved in caspase-3 activation and cleavage of poly-(ADP-ribose) polymerase, driving cells into the apoptotic cycle and cell death [2].

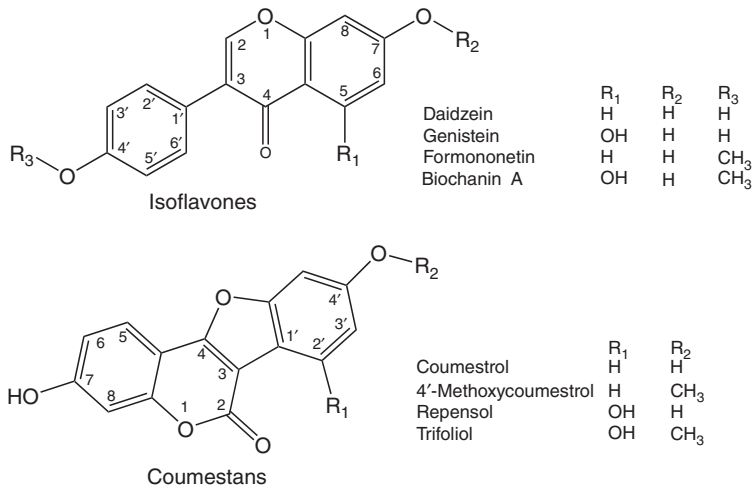
It is observed that in normal ovaries, both the ERs are present but their distribution differs with ER $\beta$  predominantly found in the granulose cells of follicles and ER $\alpha$  in the interstitial regions of the ovaries. Studies have indicated that ER $\alpha$  is required for normal mammary gland maturation and development. In normal adult mammary tissues, ER $\beta$  predominates. Many of the growth-stimulatory effects of estrogens in breast cancer have been linked to ER $\alpha$ . The balance between the expressions of ER $\alpha$  and ER $\beta$  are delicate and any slight interference can lead to metabolic disorders and cancers.

#### 13.4

##### Selective Estrogen Receptor Modulators from Plants

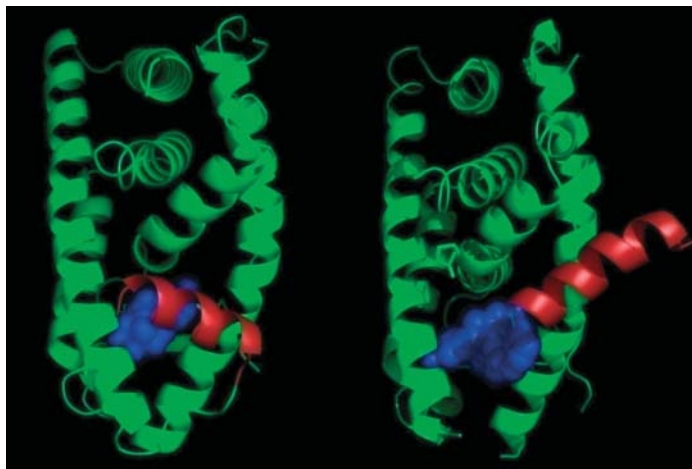
Certain plant-derived compounds are capable of exerting effects similar to the female sex hormone, estrogen. As they are structurally similar to estrogen, they have the ability to cause estrogenic or/and antiestrogenic effects [6] and are hence termed as *phytoestrogens* or *plant-derived xenoestrogens*. Most of the phytoestrogens identified so far belong to steroid compounds or substituted phenolic compounds. At present, phytoestrogens are not considered as nutrients, as their absence in the diet does not produce any characteristic deficiency syndrome. Many phytoestrogens show somewhat higher affinity for ER $\beta$  compared to ER $\alpha$ . They act like estrogens in some tissues but block the estrogens in others and hence they are called *SERMs*. SERMs may exhibit an agonistic or antagonistic effect depending upon the context in which their activity is tested [7]. For example, the synthetic SERMs, tamoxifen and raloxifene show ER antagonistic activity in breast and agonistic activity in bone, while only tamoxifen shows agonistic activity in the uterus. Structures of some of the plant-based phytoestrogens are shown in Figure 13.4. Most of the phytoestrogens discovered so far can act as SERMs and hence has found use in the treatment of estrogen-mediated cancers [8].

Phytoestrogens were previously thought to compete with E2 for the ligand-binding site thus arresting ER in its inactive conformations that are unfavorable for CoA interactions. However recent studies put forth that cell type-specific occurrence of co-regulators decide the transcriptional activities of ligand-bound ERs. The bound ligands bring some specific conformational changes in ERs especially with respect to helix 12 (Figure 13.5), facilitating the binding of co-regulator proteins and interactions with the ERE. In the presence of phytoestrogens, it appears that ER $\beta$  is more efficient than ER $\alpha$  in recruiting CoAs such as TIF2



**Figure 13.4** Structures of isoflavones and coumestans, examples of phytoestrogens.

and SRC-1. Once bound to ERs, phytoestrogens can initiate transcription either classically through interactions with EREs or by binding early immediate genes, such as Jun and Fos. Thus SERMs induce active ER conformation and signaling but the recruitment of co-regulators decides whether the ultimate transcriptional event is estrogenic or antiestrogenic.



**Figure 13.5** Crystal structures of ER $\alpha$  in complex with (a) E2 (PDB ID: 1ERE) and (b) raloxifene, an antagonist (PDB ID: 1ERR). The bound ligands are shown in blue color. The helix 12 (shown in red) is oriented differently in different structures.



## 13.5

### Molecular Basis of the Distinct SERM Action

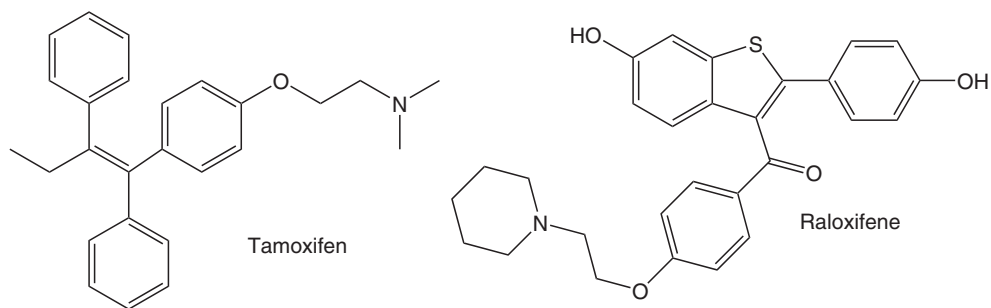
The factors that influence tissue-specific effects of SERMs include co-regulators (CoAs and co-repressors (CoRs)), TFs, and the DNA sequence which provides the organizational framework for these interactions. ER activity is influenced by the nature of chromatin structure at the target gene promoter and it varies depending on the cell types. The posttranslational modifications of ER and co-regulators, also affect their interactions and activities.

As mentioned earlier in the text, a conformationally dynamic region of the LBD designated as AF-2 is the region mainly responsible for ER's ligand-dependent transcriptional activation. The key element of the conformational switch is a short helical region, the helix 12 (H12), located at the C-terminal of the LBD (Figure 13.5).

The orientation of H12 with respect to the rest of the LBD is affected by the bound ligand. Ligands regulate NR-mediated recruitment of CoAs by binding to the LBD of the receptor and inducing a conformational change, thus allowing the recognition of a specific motif within the coactivator protein known as the *NR box* or *LXXLL domain* (where L is leucine and X is any amino acid). An agonist stabilizes the conformation of ER that is advantageous to its efficient interaction with CoAs and thus facilitates transcriptional activation. In such a "transcriptionally active" conformation, the helices that comprise the AF-2 (helices H3–H5 and H12) form a shallow hydrophobic pocket for the binding of leucine-rich LXXLL motifs of NR CoAs. In this conformation, H12 is oriented transversely to the entrance of the binding pocket and constitutes a key part of the CoA binding surface. The orientation adopted by H12 in the antagonist-bound form does not facilitate the transcriptional activation. A general view was that the H12 itself binds in the hydrophobic pocket instead of CoAs, by mimicking LXXLL motif. But, recent analysis has suggested an alternative and more convincing explanation: the H12 region encompasses an extended CoR (CoRNR) box sequence that occludes the AF-2 site and prevents the interaction of coactivators.

Tamoxifen and raloxifene (Figure 13.6) are synthetic SERMs widely used in the treatment of many estrogen-dependent disorders. Tamoxifen acts as an antagonist of the ER in breast tissue via its active metabolite, hydroxytamoxifen. It acts as an agonist in other tissues such as the endometrium. Raloxifene has estrogenic activities in bone and antiestrogenic activities in uterus and breast. The molecules of tamoxifen and raloxifene contain bulky side chains that cannot be accommodated in the ligand-binding pocket. As a consequence, H12 is sterically hindered from adopting the proper agonist-bound conformation. Thus, the differences in the positioning of H12 may be the cause of the tissue-specific actions of SERMs.

The fact that ER $\alpha$  and ER $\beta$  are differentially expressed in various tissues and can respond differentially to a given SERM should also be considered in the context of tissue selectivity of SERMs [9]. Tamoxifen is a partial agonist of ER $\alpha$  and



**Figure 13.6** Structures of tamoxifen and raloxifene, two synthetic S ERMs.

a pure antagonist of ER $\beta$ . Similarly, tamoxifen and raloxifene exhibited different activity on an activator protein 1 (AP-1) depending on whether ER $\alpha$  or ER $\beta$  is expressed. Moreover, when both ERs are expressed in the same cell, their combined response to a ligand was different from the response of either one alone. SERMs tamoxifen, raloxifene, and GW5638 show different biological activities on the complement 3 promoter (which contains imperfect EREs) in a hepatoma cell line expressing exogenous ER $\alpha$ . Thus, in addition to receptor-ligand affinity, there should be many other factors that contribute to the selective activity of SERMs. Hence, the molecular mechanisms causing the differences between ER subtypes with respect to SERM activity largely remain elusive.

The mode of activity of SERM may also depend on the nature of the target promoter. This has been illustrated for both ER $\alpha$  and ER $\beta$ . For example, in a uterine cell lines transfected with ER $\alpha$ , tamoxifen increases the activity of an AP-1-based promoter, acts as a partial agonist on the TGF $\alpha$  (transforming growth factor alpha) promoter, and acts as a full antagonist on an ERE-containing promoter. The sequence of the ERE may also influence SERM activity, demonstrated by a synthetic, variant ERE, which promotes higher agonistic activity of tamoxifen compared to the consensus ERE in transient transfection experiments.

The tissue-specific effects of SERMs may depend on the intracellular signaling pathways that are stimulated by extracellular factors that crosstalk to ERs [10]. The activation of camp cyclic adenosine monophosphate (cAMP)/protein kinase-A pathway increases the partial agonist activity of tamoxifen and decreases its antagonist activity. Similarly, in HeLa (uterine cervical adenocarcinoma) cells, both cAMP and dopamine increase the partial agonist activity of tamoxifen. The cAMP apparently increases the agonist activity of tamoxifen by altering the recruitment of co-regulators. Also cAMP and EGFs (epidermal growth factors) inhibit the tamoxifen-induced recruitment of the CoR SMRT to ER $\alpha$ . Furthermore, SRC-1 enhances ER activity induced by cAMP and tamoxifen. Thus extracellular signals are capable of modulating the activity of SERMs by regulating the expression, activity, and interactions of ERs and their co-regulators.

### 13.6

#### SERMs in the Treatment of Estrogen-Mediated Cancers

Whether phytoestrogens increase or decrease the risk of breast cancer is a difficult task to deal with. It has been established that estrogens advance breast tumorigenesis. The factors which lead to the increase in the “lifetime estrogen exposure” (such as early menarche, short duration breastfeeding, and low parity) are linked to the increased level of breast cancer risk [6]. Most of the growth-stimulatory effects of estrogens in breast cancer have been linked to the overexpression of ER $\alpha$ . While ER $\beta$  is the one which is predominantly expressed in the normal breast cells, its level was found decreased in breast tumors [8]. In addition to the breast, ER $\beta$  is largely expressed in bone, cardiovascular system, uterus, bladder, prostate, lung, ovarian granulosa cells, and testicular Sertoli and germ cells. This level can change during the lifetime and is sexually dimorphic, predominantly in the brain. Thus the ER subtypes control different facets of reproduction, behavior, and neuroendocrine function and probably have differential roles across the lifespan.

Genistein is 7- to 48-fold more selective to ER $\beta$  than to ER $\alpha$ . The relative estrogenic potency of genistein for ER $\beta$  is ~30-fold higher than that for ER $\alpha$ . Once bound, isoflavones act like tamoxifen which is an ER agonist in uterus and bone but an antagonist in breast. The fact that most phytoestrogens bind ER $\beta$  with higher affinity than ER $\alpha$  is functionally significant as ER $\alpha$  and ER $\beta$  are differentially distributed throughout the body and appear to upregulate different gene families [7]. In breast tumor cells, the suite of genes upregulated by ER $\beta$  activation enhances apoptosis and generally suppresses proliferation, while activation of ER $\alpha$  does largely the opposite. Thus the SERMs might be useful as lead compounds in the development of drugs for the treatment of estrogen-mediated cancers.

### 13.7

#### Concluding Remarks

Different physiological functions of the body such as reproduction, behavior, and neuroendocrine function are regulated by estrogen through ER subtypes. These receptors have tissue-specific functions with respect to each other. For example, ER $\alpha$  induces cell proliferation, whereas ER $\beta$  antagonizes this action. Thus their differential expression and activation in a balanced manner is necessary for the normal functioning of the body and any imbalance in this expression leads to oncogenesis and several autoimmune diseases. Under such circumstances, phytoestrogens which mimic the function of endogenous estrogen can be judiciously used for regulating this imbalance and reverting back the normal functions of the body. Thus, SERMs may be considered as reliable lead compounds for the development of drugs in the treatment of estrogen-mediated cancers and autoimmune diseases.

### Abbreviations

ADP	Adenosine diphosphate
AF-1	activation function 1
AF-2	activation function 2
AP-1	activator protein 1
CBP	CREB binding protein
CREB	cAMP response element-binding protein
CoA	coactivator
CoR	co-repressor
CoRNR	corepressors that associate with nuclear hormone receptors (NRs)
DBD	DNA binding domain
E2	17- $\beta$ -estradiol
ER	estrogen receptor
ERE	estrogen responsive element
ER $\alpha$	estrogen receptor alpha
ER $\beta$	estrogen receptor beta
GFR	growth factor receptor
GPR30	G-protein coupled estrogen receptor 30
H12	Helix 12
LBD	ligand-binding domain
mER	membrane bound estrogen receptors
MAPK	Mitogen-activated protein kinases
NOS	nitric oxide synthase
NR	nuclear receptor
PARP	poly-(ADP-ribose) polymerase
PDB ID	Protein Data Bank ID
SERMs	selective estrogen receptor modulators
SMRT	Silencing mediator for retinoid or thyroid-hormone receptors
Tfs	transcription factors

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## 14

# Introduction to the Biosynthesis and Biological Activities of Phenylpropanoids

*Luzia V. Modolo, Cristiane J. da Silva, Fernanda G. da Silva, Leonardo da Silva Neto, and Ângelo de Fátima*

### 14.1

#### Introduction

Secondary metabolites are implicated in several traits in plants such as quality, yield, disease resistance, stress tolerance, color, and fragrance. The properties of plant secondary metabolites as nutraceuticals and phytomedicines are equally well known. The biosynthesis of three main classes of secondary metabolites is reported to occur in the plant kingdom, namely, phenolics (or phenylpropanoids), terpenoids, and nitrogen-containing compounds (e.g., alkaloids, nonprotein amino acids, amines, cyanogenic glycosides, and glucosinolates). Much of the huge variety of plant secondary metabolites comes from a limited number of chemical scaffolds. As for phenylpropanoid molecules, the scaffolds vary from simple hydroxycinnamic acid derivatives to complex polyphenols (flavonoids) that are modified by a broad range of enzymes.

In this chapter, we illustrate the key enzymes involved in the biosynthesis of flavonoid, coumarin, and stilbene scaffolds and also provide a brief description of the role of these subclasses of phenylpropanoid compounds in some plant species. Last but not least, we provide an overview of the biological activities of some remarkable phenylpropanoids.

### 14.2

#### Biosynthesis of Phenylpropanoids

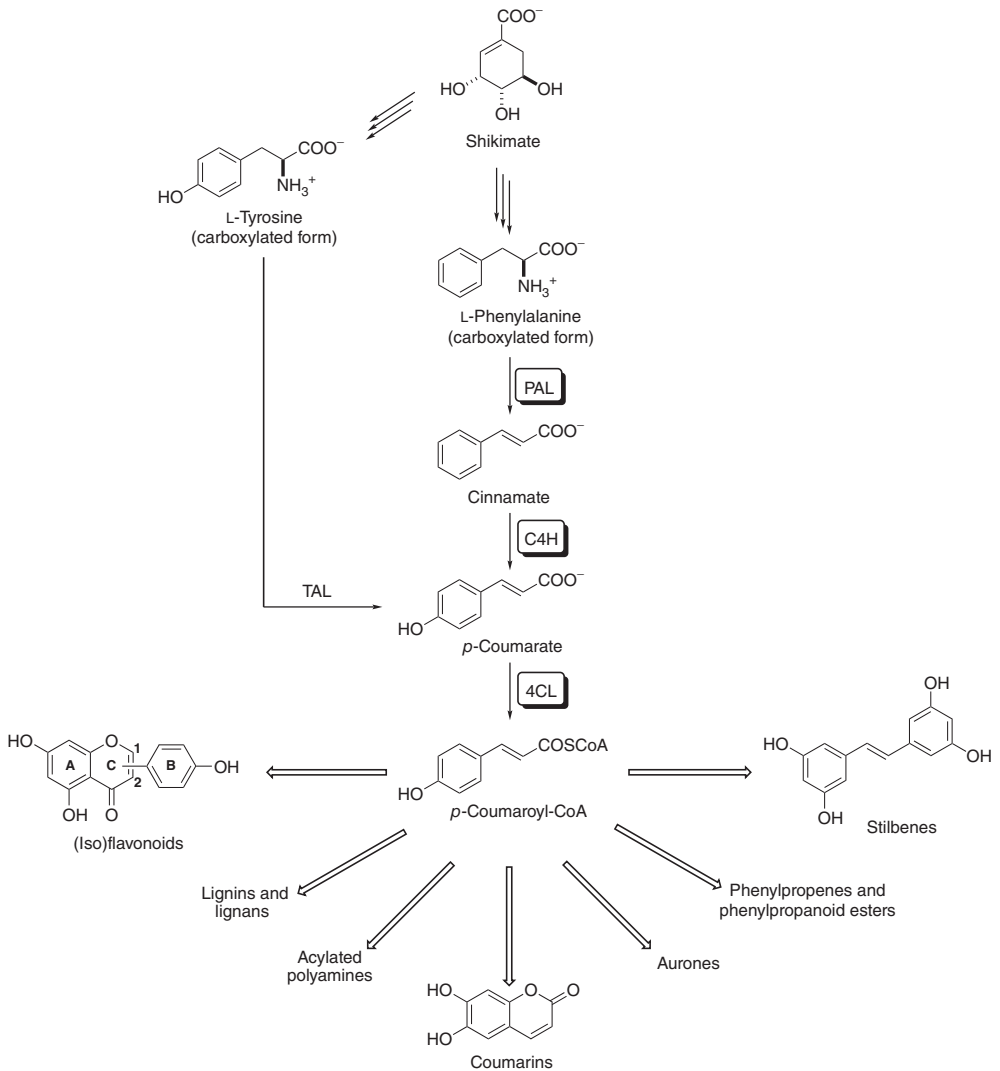
Phenylpropanoids are plant-derived natural products whose backbone is constituted of a six-carbon aromatic ring bound to a  $C_3$  skeleton ( $C_6-C_3$ ). Besides contributing to plant responses to abiotic and biotic stimuli, phenylpropanoids exert structural roles and influence plant reproduction and colonization of new habitats [1–3]. The first committed step of the phenylpropanoid pathway is

the non-oxidative deamination of the carboxylated form of L-phenylalanine or L-tyrosine to *p*-cinnamate or *p*-coumarate (C<sub>6</sub>-C<sub>3</sub>), catalyzed by phenylalanine ammonia lyase (PAL) and tyrosine ammonia lyase (TAL), respectively (Figure 14.1). Both amino acids originate from the shikimate pathway. Taking as an example the pathway initiated with PAL activity, cinnamate 4-hydroxylase (C4H) promotes the conversion of *p*-cinnamate to *p*-coumarate that is further condensed to coenzyme A to yield *p*-coumaroyl-CoA [4]. An array of enzymes can act on *p*-coumarate-CoA to furnish a plethora of phenylpropanoids classified as (iso)flavonoids, coumarins, stilbenes, aurones, lignans, lignins, phenylpropenes, phenylpropanoids esters, and acylated polyamines (Figure 14.1). This chapter will give emphasis to flavonoids, coumarins, and stilbenes, bringing an overview of their main functions in plants and how this knowledge has allowed humankind to benefit from these interesting classes of phenylpropanoids. More information on the phenylpropanoids not covered in this chapter can be found in a recent review by Vogt [3].

Flavonoids are plant metabolites that constitute one of the largest and most-studied groups of phenylpropanoids [5]. From the structural point of view, they consist of an aromatic ring A bound to a six-membered ring named C (aromatic or not) bound to the C1 of an aromatic ring B (Figure 14.1). This class of natural products comprises approximately 10 000 substances divided into two main subclasses: the 2-phenylchromans (flavanones, flavones, flavonols, flavan-3-ols, and anthocyanidins) and the 3-phenylchromans (isoflavones, isoflavans, and pterocarpans) [5]. The flavonoid biosynthesis begins with the condensation of 1 mol of *p*-coumaroyl-CoA to 3 mol of malonyl-CoA in a reaction assisted by chalcone synthase (CHS) to form naringenin chalcone, an organic compound that bears a C<sub>6</sub>-C<sub>3</sub>-C<sub>6</sub> skeleton (Figure 14.2). The first flavonoid (naringenin; flavanone) is then synthesized from the action of chalcone isomerase (CHI) on naringenin chalcone. Naringenin is the key molecule for the biosynthesis of the other flavonoids such as anthocyanidins, flavan-3-ol, flavones, flavonols, isoflavans, isoflavones, and pterocarpans (Figure 14.2).

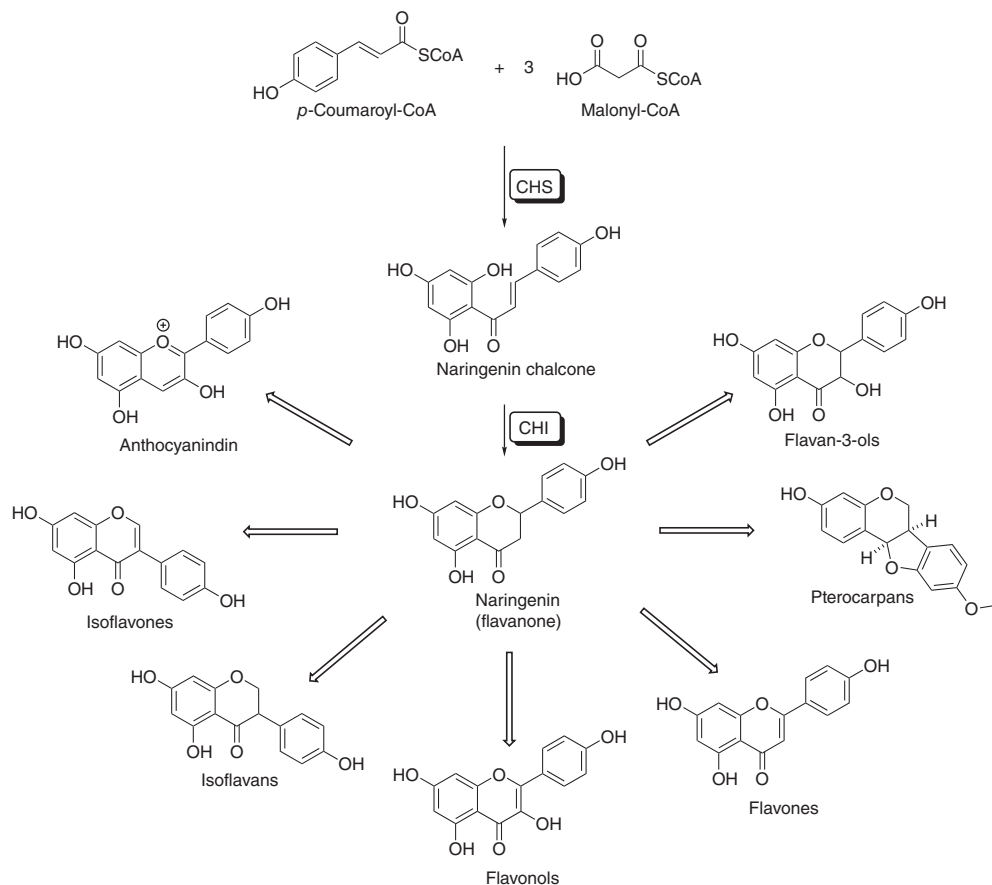
The basic structure of coumarins is formed by a pyrone-phenyl system [6] (Figure 14.1). The full biosynthetic pathway that leads to the production of coumarins in plants remains to be disclosed [7–11]. There is evidence that some coumarins can be derived solely from the shikimic acid pathway, while others appear to come from mixed routes (shikimic acid and acetate pathways). Regardless of the biosynthetic route, *p*-coumaric acid was determined to be the main precursor of coumarins [12]. Figure 14.3 describes the reactions that take place in plant cells to produce the coumarin scopoletin. This type of unequivocal route was recently disclosed and discussed by Bourgaud and coworkers [7]. After being formed, *p*-coumaroyl-CoA is condensed to shikimic acid in the presence of hydroxycinnamoyl transferase (HCT) furnishing *p*-coumaroylshikimic acid. The hydroxylation of this product at C3', catalyzed by a cytochrome P450 monooxygenase (C3'H), yields caffeoylshikimic acid. By the action of HCT





**Figure 14.1** Schematic representation of the steps of the phenylpropanoid pathway that lead to the formation of *p*-coumaroyl-CoA. The phenylpropanoid classes synthesized in plant tissues are listed. Flavonoids are phenylpropanoids constituted of a C<sub>6</sub>-C<sub>3</sub>-C<sub>6</sub> skeleton in which ring B is bound to C2 of ring C. The isoflavonoid designation refers to a flavonoid whose chemical structure presents the ring B bound to C3 of

ring C. Stilbenes, on the other hand, bear a C<sub>6</sub>-C<sub>2</sub>-C<sub>6</sub> skeleton, while the basic structure of coumarins are constituted of a phenylpyrone system. The enzymes involved in the initial steps of phenylpropanoids pathways include 4-coumarate:CoA ligase (4CL), cinnamate 4-hydroxylase (C4H), phenylalanine ammonia lyase (PAL), and tyrosine ammonia lyase (TAL). Triple arrows indicate the occurrence of multiple steps.

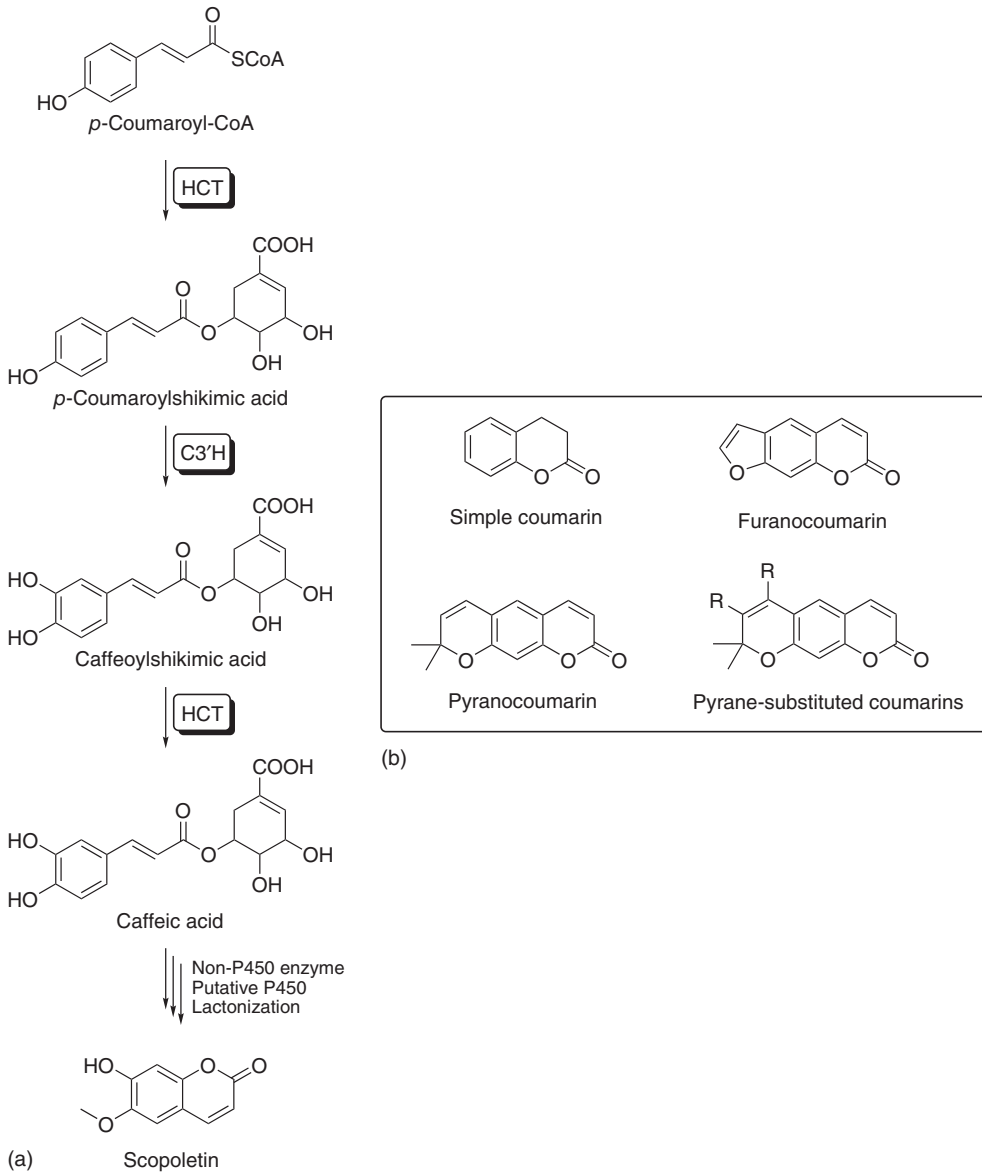


**Figure 14.2** Schematic representation of the formation of naringenin chalcone, a key molecule for the biosynthesis of flavonoids. CHI, chalcone isomerase and CHS, chalcone synthase.

enzyme, caffeoylshikimic acid is transformed to provide caffeic acid, which after few steps is converted to scopoletin (Figure 14.3).

The first coumarin reported was isolated by Vogel in 1820 from the seeds of *Dipteryx odorata* (tonka bean; Fabaceae) [13]. Over 1300 coumarins have been identified and isolated from different tissues of more than 150 plant species belonging to Apiaceae, Caprifoliaceae, Clusiaceae, Guttiferae, Nyctaginaceae, Oleaceae, Rutaceae, and Umbelliferae families [14–17].

The most accepted classification of coumarins includes four subclasses: simple coumarins (e.g., 7-hydroxycoumarin and 6,7-dihydroxycoumarin), furanocoumarins (e.g., psoralen and angelicin), pyranocoumarins (e.g., xanthyletins and seselins), and pyrone-substituted coumarins (e.g., 4-hydroxycoumarin) (Figure 14.3) [12, 18].



**Figure 14.3** Schematic representation of scopoletin biosynthesis (a) and general structure of coumarins (b). C3'H, cytochrome P450 monooxygenase and HCT,

hydroxycinnamoyl transferase. Triple arrows indicate the occurrence of multiple steps.

The letter R represents different substituents, such as hydrogen, alkyl, aryl, among others.

Stilbenes are polyphenolic compounds produced by some families of plants, such as Vitaceae, Dipterocarpaceae, Gnetaceae, Pinaceae, Fabaceae, and Cyperaceae [19]. The substances classified as stilbenoids have in their structure the carbon skeleton framework  $C_6-C_2-C_6$  (Figure 14.1) and exist as both monomers and complex oligomers. Monomeric stilbenes have a simple chemical structure comprised of two aromatic rings linked by an ethylene group with *E* or *Z* configuration.

As for stilbene biosynthesis, a series of malonyl-CoA-derived acetyl units are sequentially added to *p*-coumaroyl-CoA by means of the activity of stilbene synthase [20].

Glycosyltransferases, methyltransferases, acyltransferases, prenyltransferases, sulfotransferases, 2-oxoglutarate-dependent dioxygenases, P450 monooxygenases, and reductases are enzymes that can act on flavonoid, coumarin, and stilbene scaffolds to generate new molecules with improved water or lipid solubility and/or altered biological function. The knowledge of the function of phenylpropanoids in plant tissues has been used as inspiration for studies in which the effects of such secondary metabolites on human health are investigated. The following sections will present an overview of the roles of (iso)flavonoids, coumarins, and stilbenes in plants and some remarkable biological activities reported so far for these classes of phenylpropanoids.

### 14.3

#### Some Phenylpropanoid Subclasses

##### 14.3.1

##### Flavonoids

###### 14.3.1.1 Function in Plants

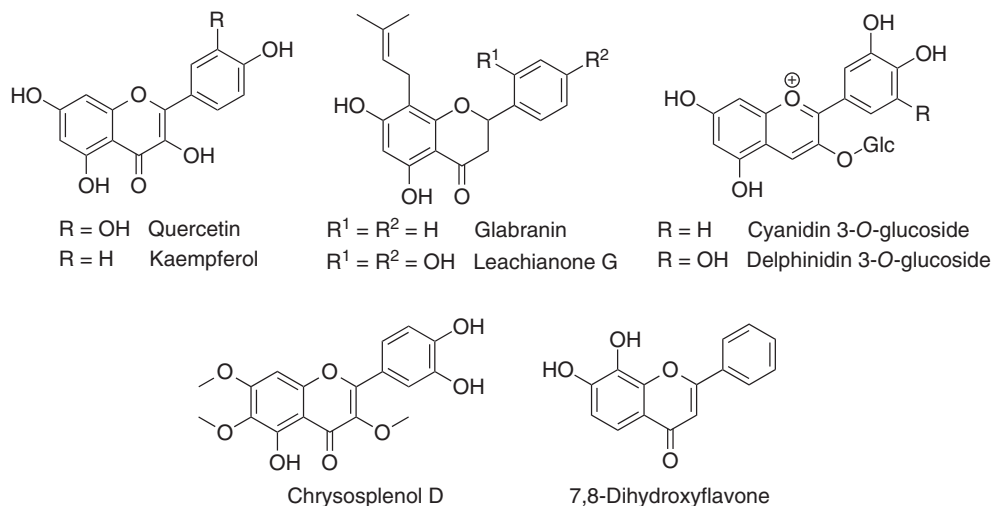
Flavonoids are found in all plant organs [21–23] in which their distribution, accumulation, and structural diversity can change with plant age [21], phenological stage [23], and environmental stimuli [24, 25]. Anthocyanins exhibit many functions which include attraction of pollinators and dispersers [26, 27]. The flavonol quercetin 3-*O*- $\beta$ -D-glucuronide and the flavones I3-II8-biapigenin are floral ultraviolet (UV)-absorbing pigments of *Hypericum calycinum* (Hypericaceae), also known to contribute to the attraction of pollinator insects [26, 27]. Anthocyanins-enriched fruits are attractive to certain birds that after benefiting from a high-energy diet containing antioxidant substances can contribute to seed dispersal, leading to reproductive success of plant species [27]. Another important role played by flavonoids in plants is the protection against environmental stresses. It is well known that one of the first events triggered in plant cells upon stress is the oxidative burst characterized by the overproduction of reactive oxygen species (superoxide anion, singlet oxygen, hydroxyl radical, and hydrogen peroxide, among others). Despite the presence of other flavonoids, anthocyanins

were found to be the molecules responsible for degrading hydrogen peroxide in *Pseudowintera colorata* (Winteraceae) under mechanical injury [28]. Anthocyanins were shown to form complexes with cadmium(II), which reduced the damage in *Azolla imbricata* (Azollaceae) plants caused by this heavy metal [29]. The ability of flavonoids to absorb UV radiation is also pivotal in leaves as it allows for the protection of the photosynthesis apparatus from UV-B rays [30–32]. As for the growth and development of plants, flavonoids function as endogenous inhibitors of auxin transport [33, 34]. *Arabidopsis thaliana* (Brassicaceae) plants (*tt4*) bearing a mutation in the gene *CHS* that encodes for CHS exhibited increased secondary root development due to the higher rate of auxin transport in comparison to wild-type plants. Further, wild-type plants supplemented with the flavonoid naringenin had decreased root growth and gravitropism as this occurs when plants are treated with auxin transport inhibitors [33]. The ability of flavonoids to mediate symbiotic interactions between plants and nitrogen-fixing microorganisms is documented [34]. Nodule formation in flavonoid-defective *Medicago truncatula* (Fabaceae) roots was impaired and application of the flavonoids naringenin or liquiritigenin rescued the phenotype typical of wild-type plants [34]. Some flavonoids are reported as phytoalexins, substances capable of inhibiting microbial growth. Among a series of examples, it was found that *Sorghum bicolor* (Poaceae) plants accumulate 3-deoxyanthocyanidins in response to anthracnose fungus [35]. *Glycine max* (Fabaceae) cotyledons challenged with elicitor molecules extracted from cell wall of the fungus *Diaporthe phaseolorum* f. sp. *meridionalis* accumulated the pterocarpan phytoalexins glyceollin I to III [36]. Maackiain and 2-hydroxy-4-methoxypterocarpin isolated from roots of *Ulex europaeus* (Fabaceae) inhibited the growth of the fungus *Cladosporium cladosporioides* [37]. The mortality of second instar gypsy moth (*Lymantria dispar*) increased when fed with quercetin-3-*O*-glucoside-enriched extracts of *Pinus* species (Pinaceae) [38].

#### 14.3.1.2 Pharmacological Properties

The structures of some flavonoids of pharmacological interest are shown in Figure 14.4. Cyanidin-3-*O*-glucoside produced in *Chrysophyllum cainito* (star apple; Sapotaceae), *Eugenia uniflora* (Surinam cherry; Myrtaceae), and *Myrciaria cauliflora* (jaboticaba; Myrtaceae) tissues and delphinidin-3-*O*-glucoside from *E. uniflora* efficiently scavenged the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical [39]. This indicates the potential of anthocyanins as antioxidant agents.

*In vitro* experiments showed that flavonoids (artemetin, chrysoplenetin, and chrysosplenol-D) extracted from cell suspension cultures of *Artemisia annua* (Asteraceae) inhibited the growth of *Plasmodium falciparum* by 50% when applied at the range of 24–65  $\mu$ M [40]. These results show the potential of artemetin, chrysoplenetin, and chrysosplenol-D as antimalarial agents. The intraperitoneal administration of flavonoid 7,8-dihydroxyflavone doubled the



**Figure 14.4** Examples of flavonoids of pharmacological interest. Glc, glucosyl group.

mean survival time of STIB795 mice challenged with *Trypanosoma brucei brucei* in experiments in which the flavone was used at a total dose of 200 mg kg<sup>-1</sup> [41].

The flavonoid glabranin and its derivative 7-O-methyl-glabranin efficiently inhibited the replication of dengue virus type 2. Maximum inhibition (75%) of virus replication was reached when the flavonoid was used at 5 μM [42]. Quercetin also presented significant inhibitory effect on this same virus serotype [43]. The human immunodeficiency virus (HIV) replication was affected by lawinal extracted from *Desmos* species (Annonaceae) and baicalin from *Scutellaria baicalensis* (Lamiaceae) [44, 45].

Anti-herpetic activity of leachianone G, 5,7-dimethoxyflavanone-4'-O-[2''-O-(5'''-O-trans-cinnamoyl)-β-D-apiofuranosyl]-β-D-glucopyranoside, and quercetin was also documented [46–48]. Quercetin also affected the multiplication rate of three strains of adenovirus [48]. The flavone chryso splenol C isolated from *Pterocaulon sphacelatum* (Asteraceae) inhibited the growth of poliovirus by 50% when applied at 0.27 μg ml<sup>-1</sup> [49]. The replication rate of parainfluenza virus type-3 was reduced in the presence of rutin, 5,7-dimethoxyflavanone-4'-O-β-D-glucopyranoside, and 5,7,3'-trihydroxyflavanone-4'-O-β-D-glucopyranoside [47].

Various flavonoids extracted from *Galium fissurense* (Rubiaceae), *Viscum album* (Loranthaceae), and *Cirsium hypoleucum* (Asteraceae) exhibited antimicrobial activity against strains of *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, *Staphylococcus aureus*, *Enterococcus faecalis*, and *Candida krusei* [47]. The flavonoids were more potent than the reference drug fluconazole against *C. krusei* [47].

The proliferation of human promyelocytic leukemia cell line (HL-60) was inhibited by 50% from incubations with 2',3',5,7-tetrahydroxyflavone from *S. baicalensis* at 9.5  $\mu\text{M}$  [50]. Quercetin induced apoptosis in HL-60 cells through the activation of caspase-3, inhibition of cyclooxygenase-2 gene and protein expression and regulation of Bcl-2 and Bax, components of apoptotic cascade [51]. Thus, the anti-inflammatory properties, ability to regulate estrogen levels and cell redox state, as well as the selectivity toward cancer cells make flavonoids interesting molecules for therapeutics and/or cancer prevention [52, 53].

Research studies demonstrating the benefits of flavonoids-rich diet for the prevention of diseases have been carried out. A diet based on supplementation with quercetin, cyanidin, pelargonidin, epicatechin, and proanthocyanidin was shown to reduce the risks of Parkinson's disease in men [54]. A flavonoid-enriched diet also reduced the risk of death from coronary heart disease in postmenopausal women [55].

A nutraceutical formulation based on soft gel capsules containing polymethoxylated flavones from *Citrus* species (Rutaceae) and tocotrienols from palm trees (Arecaceae) was patented [56]. This formulation was shown to be effective in treatment and prevention of cardiovascular diseases, atherosclerosis, and hypercholesterolemia [56]. The production of food with increased content of valuable flavonoids is also a reality. Plant phenotypes exhibiting higher levels of anthocyanins, specially cyanidin 3-*O*-glucoside, were obtained as a strategy to improve nutritional value of food plants for human and animal consumption [57]. Other examples of genetically engineered plants with increased levels of anthocyanins include purple tomato and blood orange [58, 59].

### 14.3.2

#### Coumarins

##### 14.3.2.1 Function in Plants

Coumarins are reported to play an array of functions in plants, which include the ability to scavenge free radicals [60–62], control pathogen dissemination [63–66], and act as allelochemical [67] in addition to protecting plants from abiotic stresses [68–71].

One of the best-known responses of coumarins in plants is the antifungal activity. From this knowledge, various fungicides were developed on the basis of the chemical structure of coumarins [64, 65, 72]. Although the mechanism by which coumarins kill fungi is still not fully understood, it is suggested that such class of phenylpropanoids induces morphological changes in the mitochondrial matrix of fungal cells, leading to decrease of energy production, inhibition of mitosis, and ultimately, prevention of fungi proliferation [73]. The role of coumarins as insecticide is also reported. For instance, the levels of scopoletin and ayapin in *Helianthus annuus* (sunflower; Asteraceae) leaves increased fivefold upon damage caused by beetles [74]. As a result, the outbreak of new beetle eggs

was prevented [74]. The coumarin murraxocin isolated from *Boenninghausenia albiflora* (Rutaceae) and chemically characterized by nuclear magnetic resonance (NMR) studies was shown to cause high percentage of mortality of the forest insect pests *Plecoptera reflexa*, *Clostera cupreata*, and *Crypsitya coclesalis* even when used as a component from the crude plant extract [75]. Coumarins also present nematicidal activity. The furocoumarins, psoralen 8-geranyloxy, imperatorin, and heraclenin extracted from roots of *Heracleum candicans* (Apiaceae) were shown to be very effective against the nematodes *Bursaphelenchus xylophilus* and *Panegrellus redivivus* at concentrations lower than  $200 \text{ mg l}^{-1}$  [76]. These nematodes promote lesions that lead to degeneration of plant tissues and organs as well as the induction of gall formation. Little is known about the effect of coumarins on phytopathogenic bacteria. It has been reported that isoarnottinin 4'-O-glycoside isolated from leaves of *Prangos uloptera* (Apiaceae) exhibited a minimal inhibitory concentration (MIC) of  $100 \text{ } \mu\text{g ml}^{-1}$  against *Erwinia carotovora*, a bacteria that causes soft rot disease in a number of economically important crops [77]. Coumarins present in several plant species can work as allelochemicals by inhibiting the growth of neighboring plants and then avoiding competition for resources such as light, water, and nutrients [73].

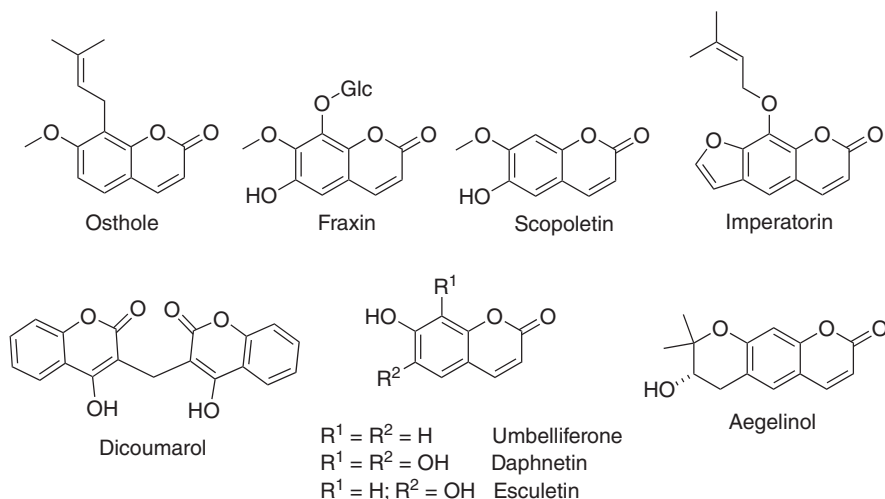
Intensification of coumarins biogenesis may also be induced in response to abiotic stresses, such as nutrient or water deficiency, presence of a xenobiotic compound, mechanical injuries, or high levels of radiation. Besides the insecticidal properties, scopoletin and ayapin were determined to protect sunflower plants from the toxic effects of high concentrations of copper(II) or short-wave UV irradiation [78, 79]. The levels of coumarins greatly increase in plant tissues when *Matricaria chamomilla* (Asteraceae) is exposed to aluminum(III) or copper(II), suggesting a protective function for coumarins against xenobiotics in general [71, 80].

#### 14.3.2.2 Pharmacological Properties

The structures of some coumarins of pharmacological interest are presented in Figure 14.5. The coumarin osthole was shown to be an efficient inhibitor of breast cancer cells (MCF-7 and MDA-MB-231 lines) proliferation [81]. Coumarins from *Zanthoxylum schinifolium* (Rutaceae) also induced apoptosis on HL-60 cells, PC-3 prostate cancer cells, SNUC5 colorectal cancer cells, Panc-1 human pancreatic carcinoma, epithelial-like cell line, and Hep2 human laryngeal carcinoma cells [82–84].

Calanolides were described as potent inhibitors of HIV proliferation [14]. The antiviral effect of (+)-calanolide A and (–)-calanolide B isolated from *Calophyllum lanigerum* (Clusiaceae) leaf was associated with the capacity of such coumarins to inhibit the viral reverse transcriptase [85, 86]. Other examples of anti-HIV coumarins include pseudocordatolide C, calanolide E, and imperatorin from *C. lanigerum* and *Calophyllum teysmannii* (Clusiaceae) [87].





**Figure 14.5** Examples of coumarins of pharmacological interest. Glc, glucosyl group.

The antioxidant property is believed to be linked to the presence of a benzopyrone moiety in the structure of coumarins [62]. The protective role of fraxin, a coumarin glucoside synthesized in *Weigela florida* var. *glabra* (Caprifoliaceae) leaves, was demonstrated in HUVECs human umbilical vein endothelial cells under hydrogen peroxide stress [88]. The ability of scopoletin, esculetin, fraxetin, umbelliferone, and daphnetin to scavenge free radicals and prevent lipid peroxidation is attributed to the presence of a catechol group in the structure of these plant natural products [61].

The anti-inflammatory potential of coumarins was also described from *in vitro* experiments in which it was demonstrated that esculetin and imperatorin act stimulating the production of macrophages and inhibiting lipoxygenase and cyclooxygenase activities [14, 89, 90]. It was also shown that coumarins are able to remove proteins and fluid from injured tissues by stimulating proteolytic enzymes and phagocytosis process [91].

Similarly to the action against phytopathogenic microorganisms, the coumarins anthogenol, imperatorin, aegelinol, and agasyllin were proven to strongly inhibit the growth of *Enterococcus* species, *Shigella dysenteriae*, *S. aureus*, *Salmonella typhi*, and *Enterobacter aerogenes* [92, 93]. Psoralen, imperatorin, and ostruthin are considered efficient against fungi of clinical interest [7, 14].

The best-known function of coumarin is related to anticoagulant activity. Dicoumarols, widely produced in *Melilotus alba* (Fabaceae), are examples of coumarin with anticoagulant activity [94]. The blood clot prevention by these coumarins are related to their capability of interfering in the conversion of vitamin K to the corresponding 2,3-epoxide. It is noteworthy that warfarin, a nonnatural coumarin, is widely used in anticoagulant therapies [95, 96].

A number of coumarins are described as repressors of hypertension [97], tuberculosis [98], multiple sclerosis [99], and hyperglycemia [100]. The potential use of coumarins as anti-adipogenic [101] and antiseizure agents [102] is also claimed.

Recently, Lin and Yan [103] determined that a non-P450 monooxygenase from *Escherichia coli*, named HpaBC, is able to *o*-hydroxylate the coumarin umbelliferone, yielding esculetin. By using a whole-cell biocatalysis approach, the production of esculetin was scaled up reaching 2.7 g l<sup>-1</sup> in almost 100% yield [103]. Thus, genetic engineering of plants and microorganisms has becoming an interesting tool for increasing the production of valuable natural products.

### 14.3.3

#### Stilbenes

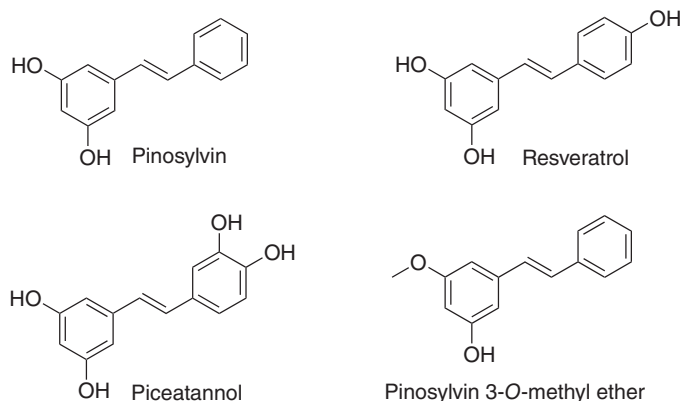
##### 14.3.3.1 Function in Plants

Important studies report that some stilbenoids may show allelochemical activity that culminates in growth inhibition of other nearby plants [104]. Stilbenes from *Carex distachya* (Cyperaceae) can inhibit root elongation and shoot growth of a series of plants species [105]. Piceatannol and resveratrol from *Scirpus maritimus* (Cyperaceae) were reported as efficient inhibitors of photosynthesis and growth of plants [104].

The antioxidant activities of stilbenes are notorious, likely due to the presence of multiple phenolic hydroxyl groups in the carbon backbone [106]. Recent studies with *Parthenocissus laetevirens* (Vitaceae) allowed the isolation of high amounts of oligomeric stilbenoids from plant roots and stems after exposure to UV irradiation [107]. This indicates that the increase in such stilbene biosynthesis provided the chemical protection necessary to *P. laetevirens* to cope with the oxidative burst generated in plant tissues by UV irradiation [107].

Accumulation of high amounts of pinosylvin and pinosylvin 3-*O*-methyl ether in the heartwood of Scots pine helped plants prevent decay caused by microorganism attack [108]. A possible mechanism of action for resveratrol against fungi was also described in studies performed with dormant conidia of *Botrytis cinerea*, a phytopathogenic fungus that cause gray mold in *Vitis vinifera* (grape; Vitaceae) particularly during the grape-ripening stage [109]. Resveratrol at 260 μM, concentration similar to that found in grape leaves and fruits, causes damage to *B. cinerea* conidia [109].

Pinosylvin 3-*O*-methyl ether, which accumulates in high amounts in *Alnus crispa* (Betulaceae), is believed to be a potent deterrent to *Lepus americanus* (snowshoe rabbit) feeding [110]. Stilbenes from the bark of *Yucca periculosa* (Asparagaceae) interfered with the insect *Spodoptera frugiperda* sclerotization and molting [111].



**Figure 14.6** Examples of stilbenes of pharmacological interest.

#### 14.3.3.2 Pharmacological Properties

The structures of some stilbenes of pharmacological interest are shown in Figure 14.6. Among the stilbenoids, resveratrol is the most studied concerning pharmacological activities [112]. The anticancer activity of resveratrol was first reported in 1997, in which it was demonstrated that this stilbene can inhibit tumor initiation and carcinogenesis progression [113]. Thereafter, various studies have shown that this stilbene compromises the proliferation of tumor cell lines of different histological origins, namely, breast [114], leukemia [115], prostate [116], colon [117], and bladder cancers [118]. The proliferation of intestinal tumor cell lines and induction of apoptosis on human melanoma cells was also observed from cell incubation in the presence of piceatannol [119, 120].

The potential use of resveratrol in the treatment of ischemic brain damage was also reported [121–123]. The neuroprotective function of resveratrol makes this compound eligible for further studies related to the treatment of Huntington's disease [124]. The therapeutic potential of resveratrol with respect to Alzheimer's disease was investigated, revealing that this phenylpropanoid induces the degradation of amyloid- $\beta$  peptides [125].

Resveratrol was also reported to inhibit the production of tumor necrosis factor alpha (TNF $\alpha$ ) and nitric oxide (NO) by lipopolysaccharide (LPS)-activated microglia, molecules associated with inflammatory processes [126, 127]. In addition, LPS-induced release of interleukin-1 beta (IL-1 $\beta$ ) from microglia during inflammation was suppressed by resveratrol [128]. These results indicate the potential of resveratrol for further studies on therapeutics of neuroinflammatory disorders.

Resveratrol is also able to protect human low-density lipoprotein (LDL) from oxidation catalyzed by copper [129]. Lipid peroxidation events were prevented in tumor cells under UV irradiation when the cultured cells were supplemented with resveratrol [130, 131].

The cardioprotective effect of resveratrol was revealed from studies of aortic ring relaxation in rats [132] and platelet aggregation in patients [133].

*E. coli* strain XL1-Blue overexpressing the gene *HpaBC* that encodes for a non-P450 monooxygenase produced piceatannol at  $1.2 \text{ g l}^{-1}$  (roughly 100% yield) when the bacterial growth medium was supplemented with resveratrol [103]. This approach was proven to be effective for the production of such natural products of pharmacological interest.

#### 14.4

##### Concluding Remarks

Plants are unequivocally great sources of molecules with a broad variety of biological activities. Indeed over 50% of therapeutic drugs released in the market are based on or derived from plant natural products. Among them, special attention is given to phenylpropanoid compounds for their pharmacological and nutraceutical potentials. Although organic synthesis has been contributing to the production of pharmaceuticals, the chemical synthesis of valuable, structurally complex natural products is still a challenge. In this sense, the use of genetic engineering and molecular biology approaches can become useful for improving the production of valuable phenylpropanoids in living systems once the biosynthesis pathway in plants is known [5, 19, 57–59, 103, 112, 134].

Much is known of the potential of phenylpropanoids for the promotion of human health. However, novel phenylpropanoids are still to be discovered as much of the huge diversity of plant species worldwide are not yet taxonomically classified. Then, new lead compounds may emerge that will improve the quality of life in humans and animals.

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##### Abbreviations

4CL	4-coumarate:CoA ligase
C3'H	cytochrome P450 monooxygenase
C4H	cinnamate 4-hydroxylase
CHI	chalcone isomerase
CHS	chalcone synthase
DPPH	1,1-diphenyl-2-picrylhydrazyl radical

HCT	hydroxycinnamoyl transferase
Hep2	human laryngeal carcinoma cell line
HIV	human immunodeficiency virus
HL-60	human promyelocytic leukemia cell line
HUVEC	human umbilical vein endothelial cells
IL-1 $\beta$	interleukin-1 $\beta$
LDL	low-density lipoprotein
LPS	lipopolysaccharide
MCF-7	human breast cancer cell line
MDA-MB	human breast cancer cell line
MIC	minimal concentration necessary to inhibit an event by 50%
NMR	nuclear magnetic resonance
NO	nitric oxide
PAL	phenylalanine ammonia lyase
Panc-1	human pancreatic carcinoma cell line
PC-3	human prostate cancer cell line
SNUC5	human colorectal cancer cell line
TAL	tyrosine ammonia lyase
TNF $\alpha$	tumor necrosis factor alpha
UV	ultraviolet

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## 15

# Neuropeptides: Active Neuromodulators Involved in the Pathophysiology of Suicidal Behavior and Major Affective Disorders

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### 15.1

#### Introduction

Suicide is among the leading causes of death in the general population. Men are more likely to commit suicide than women are, with elderly males being at especially high risk [1, 2]. Approximately 1 million persons take their lives by suicide each year, and it has been suggested that rates of completed suicides are underreported [3]. Rates of attempted suicides are 20–30 times higher compared to completed suicides and also indicate severe psychological suffering [4, 5]. Major affective disorders are chronic and disabling disorders associated with a significantly increased risk for suicidality [6]. Other risk factors for suicide are impulsivity, hopelessness, and loss of impulse control [7]. For example, a study by Corruble *et al.* [8] suggested that recent suicide attempts were associated with behavioral loss of control and cognitive impulsivity in severely depressed subjects. These dimensions should be carefully investigated when assessing suicide risk.

Although important advances have been made in the effort to understand suicidal behavior, the current knowledge regarding the neurobiological basis of this complex phenomenon is incomplete. As fundamental mediators of the stress response and environmental adaptation [9], it has been proposed that peptide hormones may play a crucial role in the central nervous system development as well as in the pathophysiology of many neuropsychiatric conditions [10, 11]. Neuropeptides may bind with high specificity to metabotropic or G-protein-coupled receptors as opposed to neurotransmitters that act as important mediators of neurotransmission through synapses. Neuropeptides are stored in large dense-core vesicles, conversely neurotransmitters are generally secreted in small presynaptic vesicles. Ludwig [12] suggested that peptide signals play a fundamental role in information processing; they possess neurotrophic properties and are able to mediate a more long-lasting neurotransmission compared to typical neurotransmitters [13]. As frequently released, neuropeptides may

support the activity of some neurotransmitters in order to integrate individual adaptive functions to homeostatic challenges [9]. Some neuropeptides have been explored as potential treatment targets in major psychiatric disorders. Cholecystokinin (CCK) and neuropeptide Y (NPY) have been tested for the development of antianxiety treatment [14]. Moreover, corticotropin-releasing factor (CRF), neurokinin 1 (NK1), and substance P systems have been explored as treatment targets and reported to mediate 5-hydroxytryptamine (5-HT) neuron activities in the dorsal raphe (DR) networks; a system that may be involved in the pathophysiology of mood disorders [15]. In addition, it has been suggested that major affective and anxiety disorders are associated with a hyperactivity of CRF and arginine vasopressin (AVP) systems [13, 16, 17].

At present, the potential interactions between psychoactive medications and neuropeptides systems are poorly understood. Some antidepressant medications like paroxetine have been associated with a reduction of vasopressinergic overexpression [18]. Importantly, postmortem studies suggest that suicidal behavior may be associated with neuropeptide dysregulation [19, 20]. Increased CCKB receptor gene expression has been found in the prefrontal, the cingulate cortex as well as in the cerebellum of suicide completers [19]. Moreover, increased CCK receptor binding was reported in the frontal and cingulate cortex of suicide victims [20]. Furthermore, Westrin *et al.* [21] reported lower plasma levels of NPY in individuals with recent suicide attempts, and Widdowson *et al.* [22] found lower NPY levels in the prefrontal cortex and caudate nucleus of suicide completers compared to age-matched control subjects with other causes of death than suicide. Depressed patients and suicide completers may also display reduced NPY levels throughout the brain compared to healthy controls or individuals deceased from nonsuicidal causes [23]. This present chapter is aimed to extensively investigate the current literature about the exact role of neuropeptides in suicidal behavior and major affective disorders.

## 15.2

### Methods

A detailed search strategy about the involvement of neuropeptides in suicidal behavior/major affective disorders has been conducted using the following databases: PubMed/Medline, Scopus, and ScienceDirect. The search used a combination of the following terms: *Neuropeptides* OR *Central Nervous System Peptides* AND *Suicid\** (including suicidal behaviors OR suicide ideation OR suicidal thoughts OR deliberate self-harm OR suicidal attempts). Relevant full-text articles published during the period between 1985 and 2014 have been carefully screened. The reference lists of the articles included in the present chapter were also manually checked for relevant studies. We included studies according to the following criteria: (i) being an original manuscript in a peer-reviewed journal and (ii) having investigated the involvement of neuropeptides in suicidal behavior.

## 15.3

## Involvement of Neuropeptides in the Pathophysiology of Suicidal Behavior and Major Affective Disorders

## 15.3.1

## Corticotropin-Releasing Factor

CRF has a specific molecular structure (see Figure 15.1) and binds the G-protein-coupled receptors CRF1 and CRF2. Several studies documented the involvement

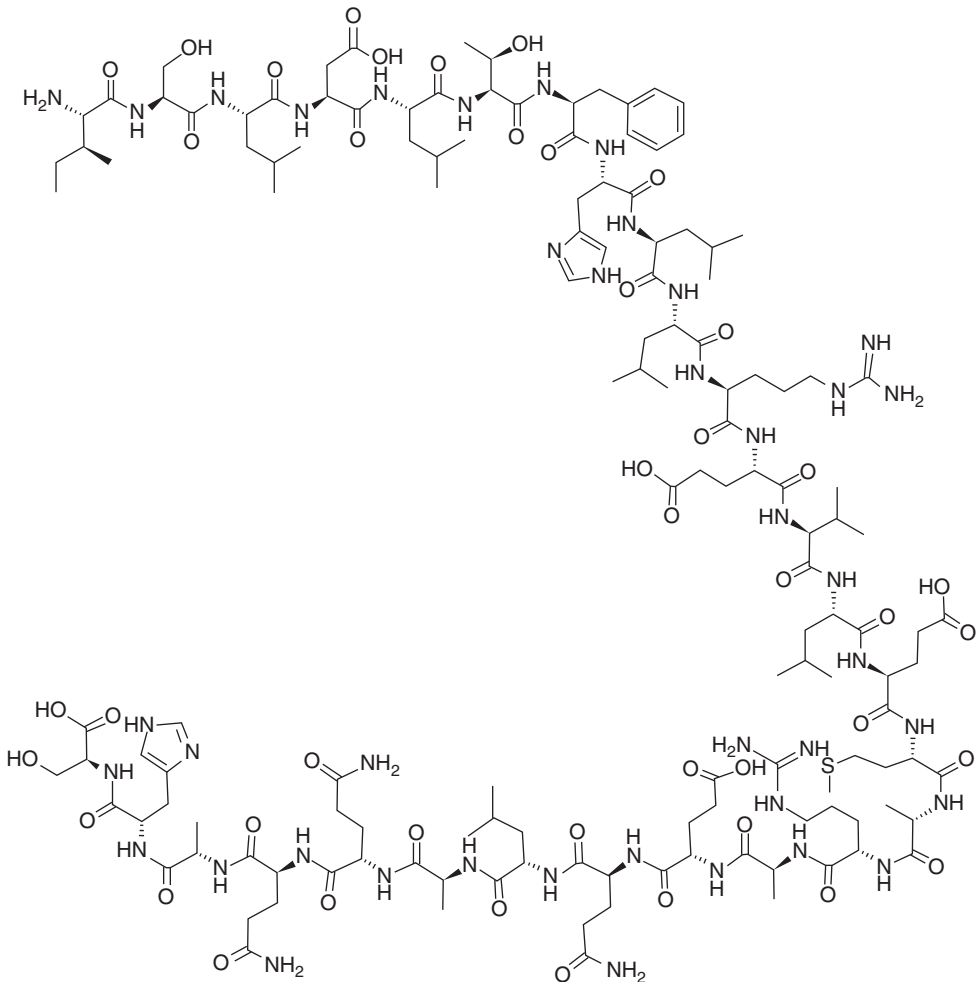


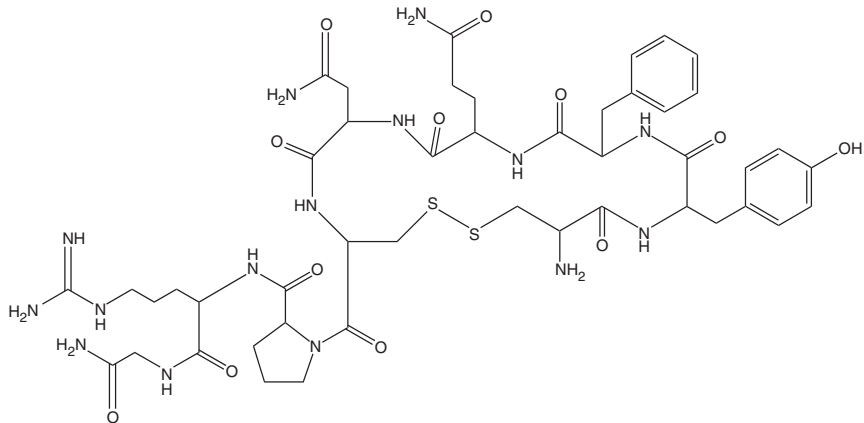
Figure 15.1 Chemical structure of corticotropin-releasing factor (CRF).

of CRF/CRF1 system in stress-related disorders [9, 24–26]. The activity of hypothalamic pituitary adrenal (HPA) axis may be closely regulated by CRF, and HPA-axis dysfunction is often observed in patients with major affective disorders [27]. Interestingly, antidepressant medications may restore HPA-axis alterations [28]. Postmortem studies have found evidence for CRF abnormalities in suicidal and depressed subjects [29, 30], and cerebrospinal fluid (CSF)-CRF levels may decrease after electroconvulsive treatment of depressed subjects [28]. These conditions are strongly related to the hyperactivity/dysregulation of CRF system. Also, CRF has been implicated in emotional regulation, learning and memory, and autonomic/monoaminergic modulation [24, 31]. Abnormalities in neuropeptide/hormone systems seem to be regulated by CRF antagonism [18]. However, some studies did not confirm the involvement of CRF in major affective disorders [32].

### 15.3.2

#### Arginine Vasopressin

AVP is released by the magnocellular terminals into the posterior pituitary and it has been reported to be involved in the reabsorption of water in the kidney (for its molecular structure, see Figure 15.2). The binding of AVP to V1a, V1b, and V3 G protein-coupled receptors determine vasoconstriction, ACTH (adrenocorticotrophic hormone) release, and antidiuresis. Importantly, the interaction between AVP and CRF in the parvocellular neurons is critical to maintain HPA-axis activity. Behavioral correlates of aggression and sociality, as well as learning and memory all seem to be closely dependent on AVP neurotransmission [33]. Increased AVP concentrations have been observed not only in the brain but also in plasma of depressed patients [34, 35]. AVP neurotransmission seems to



**Figure 15.2** Chemical structure of arginine vasopressin (AVP).



be critical in determining HPA axis hyperactivity in depressed individuals [36]. Childhood-onset affective disorders and anxiety-related behaviors are associated with AVP genetic variation or inactivation in animals [37, 38]. Recently, subjects with major depression and/or anxiety have been successfully treated using AVP antagonists [39, 40]. These compounds have indeed demonstrated promising antidepressant and anxiolytic properties, for example, by modulating the stress response [41].

### 15.3.3

#### Oxytocin

Oxytocin (Figure 15.3) usually binds to a single G-protein-coupled receptor, the effects of which are involved in important functions such as childbirth, lactation, sexual behaviors, social memory, and cognition. The synthesis of oxytocin occurs

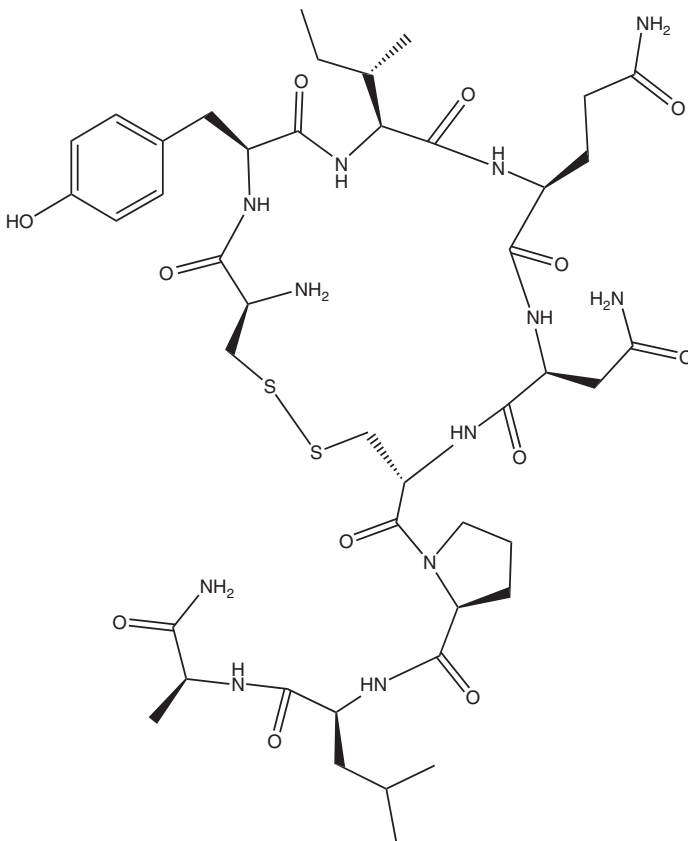


Figure 15.3 Chemical structure of oxytocin.

in the paraventricular and supraoptic nuclei of the hypothalamus, thereafter oxytocin is transported to the posterior pituitary and released into the following brain regions: amygdala, hypothalamus, hippocampus, and nucleus accumbens. Oxytocin infusion stimulates maternal behavior in animal studies [42] and affects social memory in both animals and humans. Specifically, social recognition may be inhibited by oxytocin antagonism in rats, whereas it may be experimentally stimulated using an oxytocin agonist injected into the lateral ventricles [43]. On the basis of animal studies, oxytocin is an important modulating neuropeptide involved in the regulation of social interaction. In humans, intranasal oxytocin administration is associated with recognition memory for faces but not for nonsocial stimuli [44]. Moreover, oxytocin may affect the stress response associated with HPA activity both in animals [45] and humans [46]. Oxytocin agonists also showed anxiolytic properties according to preclinical tests [47, 48].

Unfortunately, the clinical administration utility of oxytocin is limited by the short half-life and poor blood–brain barrier penetration inherent to the physiochemical properties of most neuropeptides. The activation of the amygdala in response to fearful or threatening stimuli in humans may be reduced by intranasal administration of oxytocin [49]. Few studies have shown that patients with major depression and schizophrenia exhibit lower plasma [50] and CSF concentrations of oxytocin [51], respectively. Oxytocin agonists have also been reported to be effective on the basis of preclinical tests of antidepressant activity [48]. CSF oxytocin has also been reported as a relevant modulator of suicidal intent/interpersonal violence in those who have attempted suicide. Jokinen *et al.* [52] reported that suicide attempters had lower CSF oxytocin levels than healthy volunteers. They also found that CSF oxytocin showed a significant negative correlation with suicide intent, whereas lifetime violent behavior demonstrated a trend to negative correlation with CSF oxytocin. In the regression analysis, only suicide intent remained a significant predictor of CSF oxytocin corrected for age and gender.

Importantly, oxytocin agonists may restore prepulse inhibition after disruption by *N*-methyl-*D*-aspartate (NMDA) antagonists or dopamine agonists in rats [48, 53]. Finally, as reduced plasma levels of oxytocin has been reported in autistic children [54], oxytocin has been associated with a significant improvement in speech comprehension, social recognition [55], and repetitive behaviors [56] that are known as *disabling symptoms* in autistic children. On the basis of a recent systematic review of randomized controlled trials [57], oxytocin was reported as well tolerated, inducing only mild side effects. However, restlessness, increased irritability, and increased energy occurred more often under oxytocin. Oxytocin may represent an interesting and promising treatment option in autism, especially in measures of emotion recognition and eye gaze, reported to be impaired in the early course of autism spectrum disorder.

## 15.3.4

**Galanin**

Galanin (GAL) has a specific molecular structure (Figure 15.4); its binding of galanin to receptor1 (GALR1), GALR2, and GALR3 is associated with the following biological functions: food and alcohol intake, reproduction, metabolism osmoregulation, and seizure threshold in human midbrain and limbic areas [58]. The activation of GALR1 is associated with depressive-like behavior, whereas the activation of GALR3 leads to a reduction of depressive-like behavior [59]. Whether GAL may modulate the HPA-axis activity has not yet been determined [60]. Serotonergic and noradrenergic fibers are modulated by GAL neurotransmission [61] and the hyperactivity of locus coeruleus neurons may inhibit activity of mesocorticolimbic dopamine neurons in the ventral tegmentum through the release of GAL from terminals on locus coeruleus axons in the ventral tegmentum. This is confirmed from the observation in animals that recovery to normal activity in the home cage is enhanced by infusion of a GAL receptor antagonist, galantide, into the ventral tegmentum [62]. Also, GALR3 antagonists are associated with anxiolytic and antidepressant effects according to animal models [63], whereas the activation of GALR2 protects the hippocampus from neuronal damage [64].

## 15.3.5

**Tachykinins**

Tachykinins (Tk)s include substance P (Figure 15.5), neurokinin A (NKA) (Figure 15.6), and neurokinin B (NKB) (Figure 15.7) that may be found in the cortex, spinal cord and nucleus accumbens, septum, and the amygdala (brain areas known to be involved in emotional processes) [65]. Importantly, substance P and NKA may be found in neurons and interneurons together with glutamate,  $\gamma$ -aminobutyric acid (GABA), monoamines, and acetylcholine and bind to G protein-coupled seven-transmembrane domain receptors: tachykinin receptor 1 (TACR1), TACR2, and TACR3 [66]. NK1 and NK3 receptors are distributed throughout the brain, conversely the NK2 receptor in the smooth muscle of the gastrointestinal, respiratory, and urinary tracts, and (based on animal models) in the prefrontal cortex and hippocampus of rats [67]. Species heterogeneity has been suggested for NK1 and NK3, but not for NK2. Beaujouan *et al.* [68] suggested that TACR (NK1 and NK3) antagonists may be more likely to have affinity for the guinea pig and human receptor isoforms than the rat receptor isoforms. Therefore, in order to characterize their effects, specific antagonists for NK (neurokinin) receptors have been developed using appropriate behavioral models in specific species [69–71].

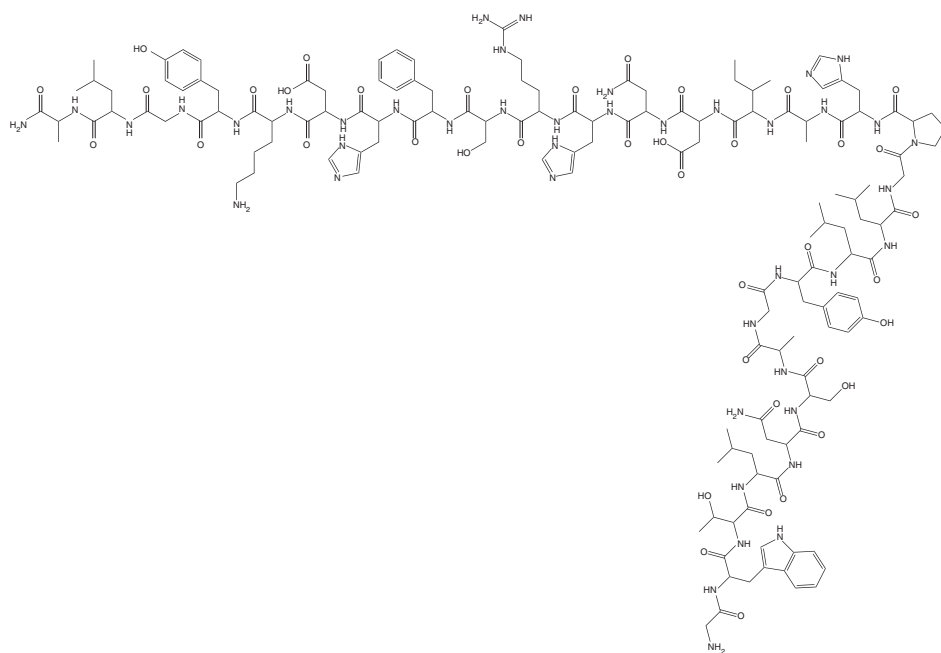


Figure 15.4 Chemical structure of GAL.

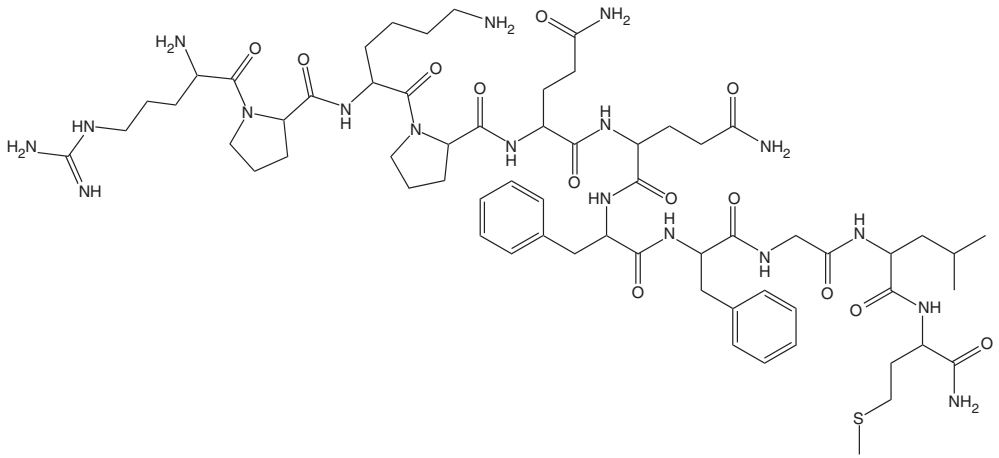


Figure 15.5 Chemical structure of substance P.

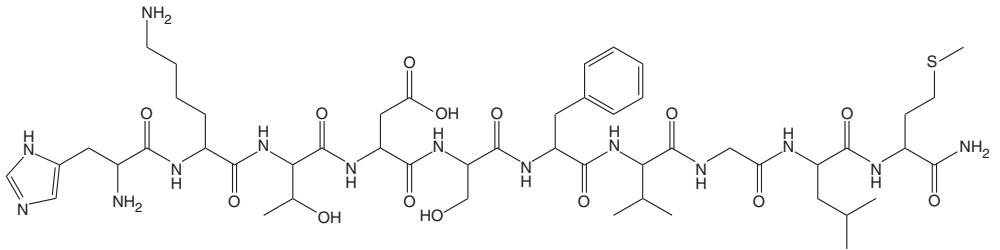


Figure 15.6 Molecular structure of neurokinin A.

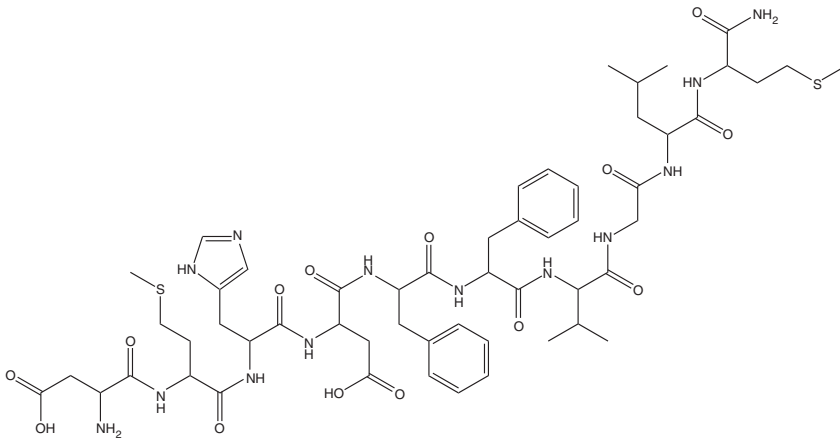


Figure 15.7 Molecular structure of neurokinin B.

The binding of substance P with G-protein-coupled TACRs – NK1, NK2, and NK3 are associated with nociception, respiration, cardiovascular, and thermoregulation, gut motility, and emetic response. A relevant involvement of substance P in stress-related disorders has also been reported [72]. Noradrenergic and serotonergic circuits are modulated by substance P/NK1 system; in particular, NK1 antagonism is associated with increased serotonergic activity in some brain areas (e.g., hippocampus and lateral septum) [73]. Whether substance P/NK1 system may modulate the activity of HPA axis and stress responsiveness is a matter of debate [72]. The release of substance P was usually enhanced by stressful situations [74] and NK1 antagonism exhibited anxiolytic properties as well as a reduction of social anxiety within the amygdala according to both animal [75, 76] and human models [77]. In addition, NK2 antagonists have been associated with antidepressant and anxiolytic effects based on preclinical evidence [78], whereas NK3 receptors have been proposed as valid targets for the development of medications with antipsychotic properties [79].

#### 15.3.6

##### **Neuropeptide Y**

NPY (Figure 15.8) is a neuropeptide involved in several biological functions such as circadian rhythms, neurogenesis, neuroprotection, nociception, feeding behavior, neuronal excitability, emotion and cognition, stress response, and resilience. NPY effects are closely associated with the activity of GABA, 5-HT, and catecholamines [80, 81]. Animal models revealed that NPY central neurotransmission is associated with anxiolytic/antidepressant effects [82]. Specifically, the activation of receptors Y1 is related to anxiolytic/antidepressant activity [83], the activation of Y2 with anxiogenic results [84], and Y5 antagonism with unclear effects [85].

Enhancing NPY neurotransmission may be associated with a control of stress response via the modulation of CRF and noradrenergic fibers. This is further confirmed by the fact that NPY is also associated with anxiolytic activity in the amygdala where CRF receptors and high NPY levels have been demonstrated [86]. However, there is also evidence suggesting no association between NPY levels and neuropsychiatric disorders (for more details see the comprehensive study of Eaton *et al.* [87]).

#### 15.3.7

##### **Cholecystokinin**

The binding of CCK (Figure 15.9) to CCKR1 and CCKR2 is associated with the following biological functions: gastric emptying, gallbladder contraction, pancreatic enzyme release, and suppression of appetite [88]. CCK peptides may significantly modify important behaviors that are mediated by dopamine. It seems that the binding of CCK with CCKR2 may inhibit dopamine release into the anterior

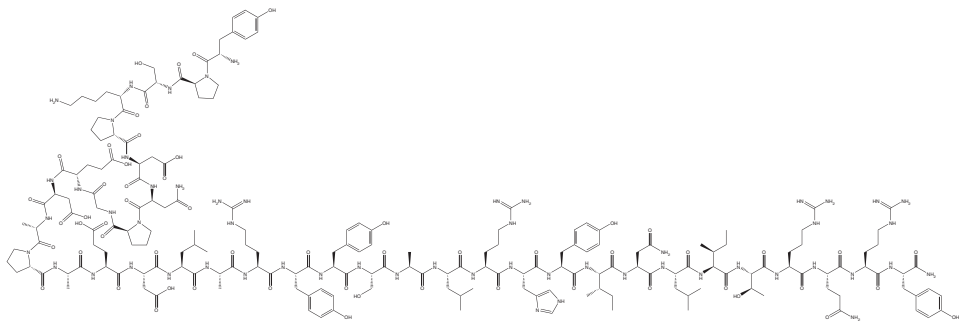
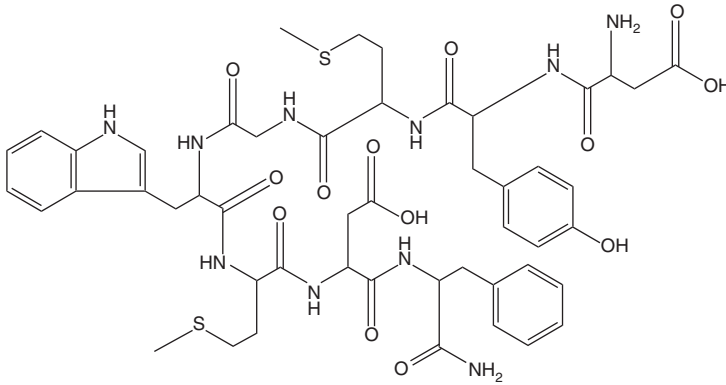


Figure 15.8 Chemical structure of neuropeptide Y.



**Figure 15.9** Chemical structure of cholecystokinin (CCK).

nucleus accumbens, whereas the binding with CCKR1 is related to opposite effects into the posterior nucleus accumbens [89, 90]. Some evidence suggested that CCK may be implicated in both stress- and anxiety-related disorders [91–94].

#### 15.3.8

##### **Dynorphins**

In 1981, Goldstein *et al.* [95] recognized for the first time the complete sequence of dynorphin-A (Dyn-A) (Figure 15.10). “Dyn” was borrowed from the Greek, whereas “orphin” refers to the opioid nature of these compounds. Findings in epilepsy and addiction are mostly consistent; therefore the functions of Dyn are widely accepted in these pathological conditions, whereas the data related to emotional control mechanisms are rather inconsistent [96]. Data related to the role of Dyn-A in the pathophysiology of major depression are derived from animal models [97], thus the investigation of the molecular mechanisms underlying dynorphins in humans requires caution.

#### 15.3.9

##### **Orexin**

Orexins/hypocretins (Figures 15.11 and 15.12) were identified for the first time in 1998 in the lateral hypothalamus [98], in particular in the perifornical region of the lateral hypothalamus and posterior hypothalamic area [99, 100]. These peptides stimulate food intake; in fact, their name is related to the Greek word “orexin” meaning appetite [101]. Multiple evidence suggests the role of orexins/hypocretins in the regulation of stress responses and many biological functions in humans. Nambu *et al.* [100] reported that hypocretins might be responsible for regulation of the stress response. The binding of hypocretins





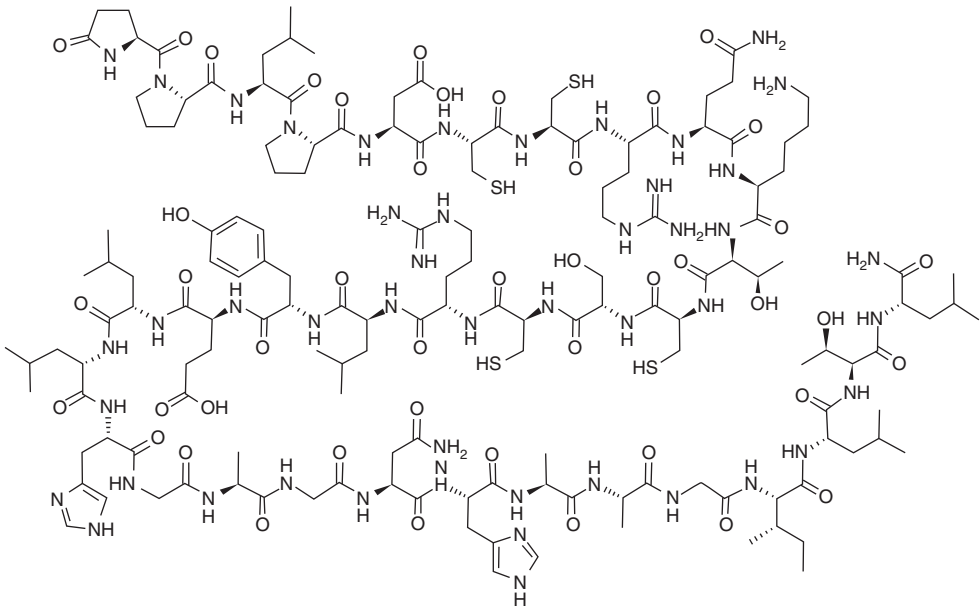


Figure 15.11 Chemical structure of orexin A.

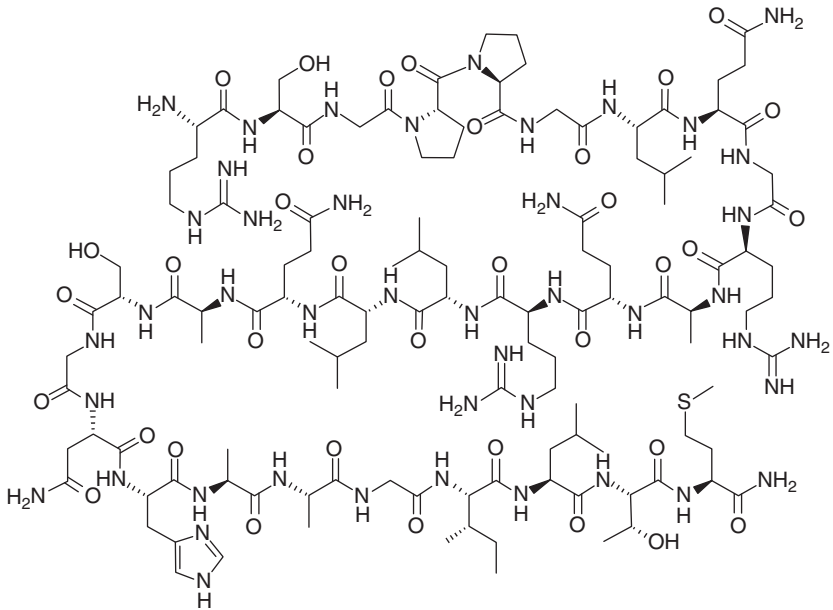


Figure 15.12 Chemical structure of orexin B.

to the hypocretin-1 receptor has been reported with a two to threefold higher affinity than that to hypocretin-2 receptor [101]. Humans with a lack of orexin develop the clinical condition narcolepsy, characterized by sudden and uncontrolled sleep attacks as well as cataplexia. Orexin neurons have been shown to be critically involved in the generation of panic anxiety in an animal model and in suicide attempters; high levels of orexin in the CSF are likewise associated with symptoms of anxiety [102]. In contrast, suicide attempters with depression have low levels of orexin in the CSF [103]. Thus, orexins seem to have an important role in regulating wakefulness and the state of vigilance.

### 15.3.10

#### Neurotensin

The neuropeptide neurotensin (NT) (Figure 15.13) was first discovered in 1973 [104] and postulated to act in the brain together with other neurotransmitters such as dopamine, 5-HT, GABA, glutamate, and cholinergic systems [105–107]. This neuropeptide binds G protein-coupled seven-transmembrane domain receptors (NTR1 and NTR2) [108, 109] and a type I amino acid receptor (NTR3) [109]. It has been suggested that NTR1 may play a critical role as a general modulator of neurotransmitter systems [110]. Interestingly, NTR1 is present in both neurons and glial cells and is widely distributed throughout the brain [111–114]. Dysfunction of NT neurotransmission and its ability to regulate dopaminergic function has been hypothesized to play a critical role in the pathophysiology of schizophrenia [105].

Importantly, some brain circuits involved in NT neurotransmission have been shown to regulate the mechanism of action of some antipsychotic medications together with the rewarding and/or sensitizing properties of drugs of abuse. Overall, NT receptors have been reported as interesting novel targets for the treatment of psychoses or drug addiction.

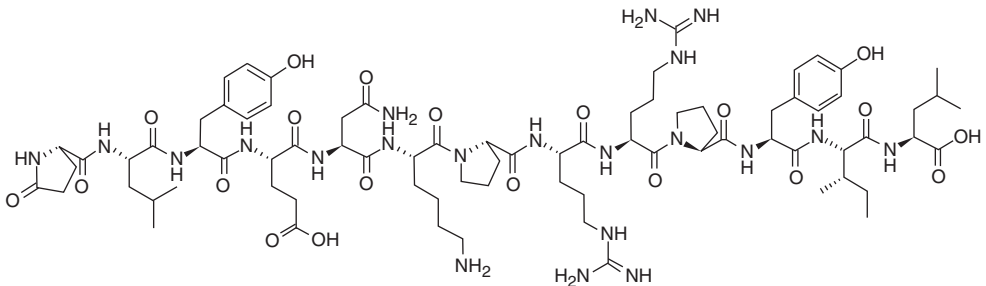


Figure 15.13 Chemical structure of neurotensin (NT).

## 15.3.11

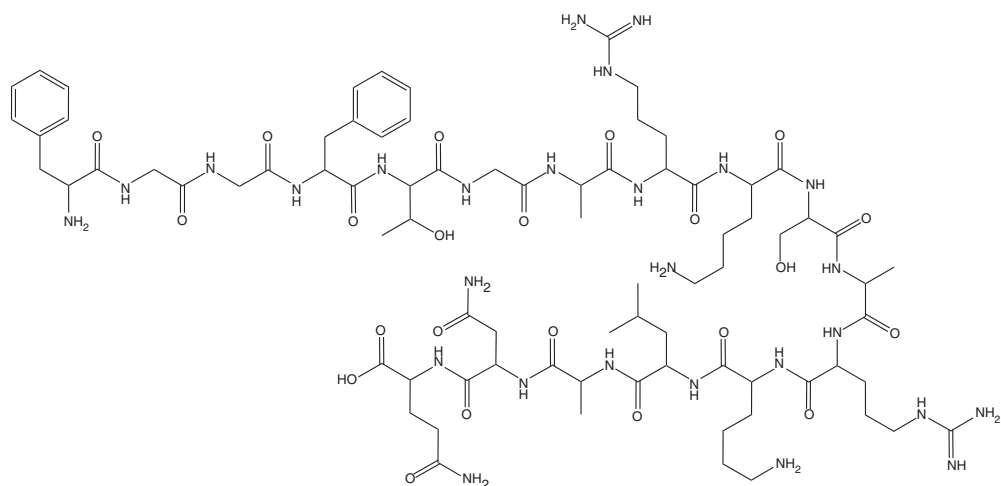
**Nociceptin**

The neuropeptide nociceptin (orphanin FQ) (Figure 15.14) binds the opioid-like G protein-coupled receptor, (ORL1 (opioid receptor-like)) [115]. This neuropeptide may be found in multiple brain regions such as the cortex, olfactory nucleus, amygdala, and hippocampus. According to animal models, it has been suggested that nociceptin agonists may be successfully used in anxiety disorders, whereas nociceptin antagonists seem to be useful in the treatment of major depression [115]. Although some non-peptide ORL1 ligands have been tested, none has been clinically developed to date. This may be mainly related to the potential of ORL1 agonists to induce amnesic effects when administered as anxiolytic-like medications.

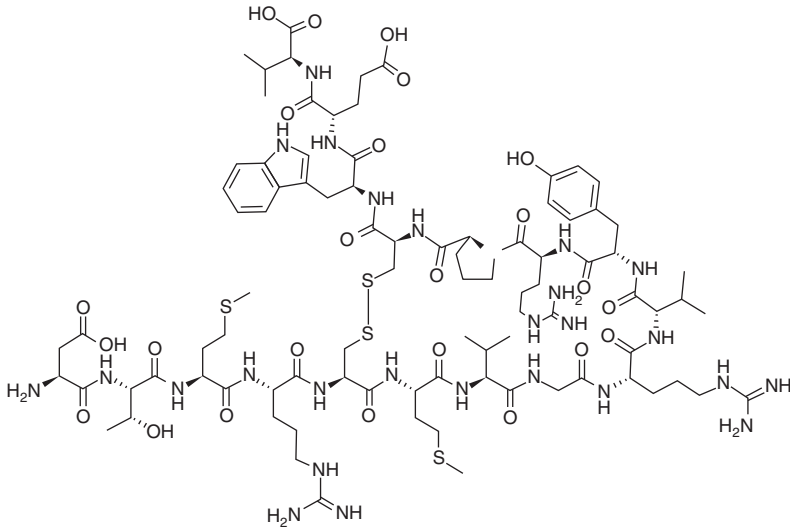
## 15.3.12

**Melanin-Concentrating Hormone**

The binding of melanin-concentrating hormone (MCH) (Figure 15.15) to its G protein-coupled receptors (MCHR1 and MCHR2) is evident predominantly in the hypothalamus. Given the wide distribution of MCHR1 throughout the brain and its preferential location in the cortex, hippocampus, amygdala, and nucleus accumbens, it has been hypothesized that MCH plays an important role in mood disorders and schizophrenia [116, 117]. Importantly, anxiolytic- and antidepressant-like effects have been observed after administration of MCHR1



**Figure 15.14** Chemical structure of nociceptin.



**Figure 15.15** Chemical structure of melanin-concentrating hormone (MCH).

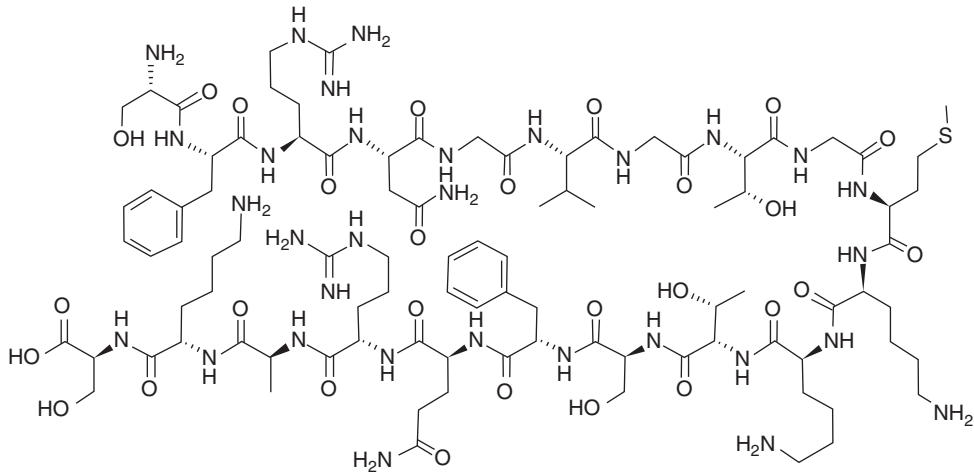
antagonists. Other evidence suggested a possible modulating MCH role of sensorimotor gating in subjects with schizophrenia. Although some non-peptide MCHR1 antagonists have been tested, none of them has to date been clinically developed in the treatment of major psychiatric disorders. This may be related to the possible adverse effects emerging when MCHR1 antagonists are used (MCHR1 are highly expressed in the following biological tissues: adipose tissue, intestines, lymphocytes, and pituitary).

### 15.3.13

#### Neuropeptide S

The neuropeptide S (NPS) (Figure 15.16) and its G protein–coupled receptor (NPSR) are reported to be involved in the following biological functions: arousal, anxiety, and memory [118]. The administration of NPS in animal models potently modulates wakefulness and could also regulate anxiety [119]. Specifically, central administration of NPS increases locomotor activity in mice and decreases paradoxical rapid eye movement (REM) sleep and slowwave sleep in animal models. NPS activates its cognate receptor and may induce mobilization of intracellular  $\text{Ca}^{2+}$ . According to animal models, NPS may constitute a potential novel treatment option for anxiety diseases such as panic and posttraumatic stress disorder.

As suggested by Ionescu *et al.* [118], NPS may exert its effects after intranasal administration. Specifically, NPS showed internalization and accumulation after intranasally administration of neuropeptide in single brain neurons. Therefore, the



**Figure 15.16** Molecular structure of neuropeptide S (NPS).

implementation of NPS may be a potential novel treatment option for anxiety disorders. Unfortunately, no NPSR ligands have been developed to date as possible drug candidates with anxiolytic or antidepressive-like effects.

#### 15.4

##### The Association between Neuropeptides, Suicidality, and Major Affective Disorders

In addition to modulating the stress response, accumulating evidence suggested that neuropeptides play a fundamental role in the pathophysiology of major affective disorders and suicidal behavior. Some studies, summarized as follows, analyzed the association between orexin dysfunctions and suicidal behavior. Lindqvist *et al.* [120] found that low levels of CSF orexin together with high levels of CSF interleukin-6 (IL-6) and monoamine metabolites 5-hydroxyindoleacetic acid (5-HIAA) and homovanillic acid (HVA) were associated with violent suicide method and risk for suicide completion. Also, as mentioned, individuals with major affective disorders display reduced orexin concentrations compared to subjects with adjustment disorder and dysthymia [103]. In addition, Brundin *et al.* [121] showed that lower orexin levels were associated with higher inertia/reduced motor activity in a sample of 101 suicide attempters. One year after the suicide attempt, the orexin levels had increased in the CSF at the same time as symptoms of suicidality had decreased significantly [122]. Inder *et al.* [123] found, in a sample of depressed subjects, significant positive correlations between plasma AVP, ACTH, and cortisol levels, and increased plasma AVP levels were found in subjects who had attempted suicide.

There are also studies investigating neuropeptides alterations in patients with nonsuicidal self-injury (NSSI). Stanley *et al.* [124] found that subjects with NSSI had lower CSF  $\beta$ -endorphin ( $\beta$ -end) and met-enkephalin (Met-e) levels than control subjects, but no difference was found in dynorphin levels. Some studies have also suggested associations between abnormal corticotropin-releasing hormone (CRH) neurotransmission and depressive disorders. Increase in CRH-IR levels in the locus coeruleus was associated with age of illness onset in 11 depressed subjects who had completed suicide compared to control subjects [125]. In a sample of suicide attempters, Träskman-Bendz *et al.* [126] found that, those who had made prior suicide attempts had significantly lower CRH levels compared to those who had not. Moreover, the suicide attempters with major depressive disorders had significantly lower somatostatin (SOM), CRH, and delta sleep-inducing peptide (DSIP) levels than subjects with other psychiatric diagnoses. Westrin *et al.* [127] reported that CSF-SOM, but not CSF-CRH concentrations, increased with clinical improvement in suicidal patients independently of psychiatric diagnoses. Depressed individuals with a history of suicide attempts displayed increased plasma DSIP levels [128]. Moreover, Scarone *et al.* found that decreased  $\beta$ -end concentrations have been reported in the left temporal and frontal cortex, as well as caudate nucleus of seven suicides when compared to seven healthy controls by [129]. The authors also reported an asymmetrical concentration of  $\beta$ -end (left less than right) levels in the frontal cortex and caudate nucleus of suicides.

Coccaro *et al.* [130] studied CSF NPY-LI in patients with aggression (endophenotype of suicide) and found it higher in personality disorder subjects compared with healthy volunteers and in subjects with intermittent explosive disorder compared with those without intermittent explosive disorder. In personality disorder subjects, CSF NPY-LI was directly correlated with composite measures of aggression and impulsivity and a composite measure of impulsive aggression.

To what extent psychoactive treatments influence neuropeptides concentrations is currently unclear. Antidepressant medications may affect the levels of CSF-NPY and SP according to Olsson *et al.* [131]. Alterations in CRH and NPY plasma levels in depressed suicide attempters have been documented by Westrin *et al.* [21]. In particular, cortisol levels were higher and CRH and NPY concentrations were lower in those who recently attempt suicide. NPY concentrations seem to positively correlate with some depressive symptoms such as psychasthenia, irritability, and stability and negatively with other symptoms such as muscular tension, psychasthenia, and verbal aggression in depressed individuals [128]. Conversely, cortisol levels have been reported to correlate positively with validity, extraversion, and verbal aggression in the same group of patients. Recently, Garakani *et al.* [132] found no significant changes in glutamate and CRH in 18 patients with major depressive disorder who have been treated with venlafaxine compared to 25 healthy controls, but only a posttreatment reduction in glutamine was reported.

There are also some postmortem studies in the current literature about the possible involvement of neuropeptides in suicidal behavior/major affective disorders. As suggested by Merali *et al.* [133], an association between suicides and site-specific abnormalities of CRH, neuromedin B (NMB), AVP, and gastrin-releasing peptide (GRP) levels has been found in a sample of 30 suicide completers and 37 healthy controls. Conversely, no significant alterations in Y1 or Y2 mRNA expression concentrations have been reported in suicide victims and controls by other authors [134]. Specifically, Caberlotto and Hurd [134] found that Y2 mRNA expression was increased in layer IV in subjects with suicide as the cause of death, and a negative correlation emerged between Y1 mRNA expression concentrations and increasing age in the prefrontal cortex. Region-specific decreases in neuropeptide levels in subjects with a history of depression have been postulated by Widdowson *et al.* [22] who showed, in another postmortem study, that NPY levels were significantly reduced in the frontal cortex and caudate nucleus of suicide completers when compared to age-matched controls.

Recently Guillaume *et al.* [135] showed that suicidal patients with a history of sexual abuse had significantly lower Iowa gambling task (IGT, a measure to determine decision-making) scores than nonsexually abused individuals. Polymorphisms within CRHR1 and CRHR2 genes interacted with both childhood sexual abuse and emotional neglect to influence IGT performance. This suggests that childhood sexual abuse and emotional neglect may have long-term effects on decision-making through an interaction with key HPA axis genes, which may have a major impact in the development of suicidal behavior.

However, there are also studies in literature that do not confirm the association between neuropeptide abnormalities and suicidal behavior/major affective disorders. Pitchot *et al.* [136] found no correlation between depression severity and AVP-neurophysins or post-DST cortisol concentrations in 28 depressed inpatients. The authors reported no significant difference between DST suppressors and non-suppressors in terms of AVP neurophysins. Also, Brunner *et al.* [137] found no difference in CSF AVP levels between 19 drug-free depressed psychiatric inpatients (9 suicide attempters) and 9 neurological control subjects; furthermore, there was no association between CSF AVP and monoamine metabolites. Moreover, Roy did not find any significant differences in mean CSF levels of NPY, SOM, diazepam-binding inhibitor (DBI), GABA, or CRH between suicidal and nonsuicidal depressed subjects [138]. In addition, there were no differences in neuropeptide concentrations between those who had attempted suicide during a 5-year follow-up and those who had not. The author suggested that neuropeptides are not major determinants of suicidal behavior or its repetition in depression [138]. In a postmortem study, depressed suicide victims with comorbid alcohol dependence and age-matched healthy controls did not differ significantly in mean NPY concentrations in the frontal cortex [139]. In addition, patients with suicidal ideation and those without did not differ regarding thyroid-stimulating hormone (TSH), free tyroxine (FT4), ACTH, and total L-tryptophan (L-TRP) concentrations [140].



In bipolar patients, levels of CRH have been studied in both suicidal and non-suicidal patients; however, the two groups of bipolar patients do not differ among themselves but show lower CRH compared to healthy controls [141].

In summary, many studies documented neuropeptide abnormalities in depressed subjects with suicidal behavior but there are also studies that found negative results.

## 15.5

### Discussion of the Main Findings

Neuropeptides have been suggested to play a key role in the pathophysiology of major affective disorders and suicidal behavior. Although many reports indicate an association between neuropeptidergic dysfunction and mood disorders and suicidal behavior, neuropeptides may be not considered as robust biomarkers of suicidal behavior in its whole complexity [142].

There are studies [21, 22, 120, 122–125, 127–129, 131–133] included in our chapter that found an association between neuropeptides and suicidal behavior, but there are also studies [126, 136–140] that did not confirm this association. In addition, there are studies reporting neuropeptides alterations in subjects with major depression [103, 121, 128]. When addressing the association between neuropeptide abnormalities and suicide/major affective disorders, it is important to understand that some reports indicated increases, others reductions, and others again no significant changes in terms of neuropeptide alterations in depressed suicide attempters or suicide completers. Therefore, it is fundamental to characterize the meaning and implications of each neuropeptide abnormality according to the design of the individual studies. Also, both the variability in terms of psychopathology in individuals exhibiting suicidal behavior and different response to psychoactive treatments should be taken adequately into account when interpreting the main findings of these studies. Importantly, some neuropeptide responses may be evoked by specific stressors: structural damage may stimulate the release of SP, oxytocin release may be evoked by social threats, altered energy homeostasis may be associated with MCH changes, and AVP release may be stimulated by blood volume depletion. Overall, orexin, CRE, VGF (nerve growth factor inducible), CCK, SP, and NPY may be considered important neuromodulators of emotional processing [143, 144].

According to the main findings of the selected studies, neuropeptides may be considered as crucial mediators of the stress response and major affective disorders. They have been hypothesized as critical molecules in the interaction between genes and environment and they are able to act as gene regulators. For example, GAL may modulate monoaminergic neurotransmission in the locus coeruleus

and the DR nuclei in stress-related disorders [145]. On the basis of recent evidence [146], GAL showed a relevant upregulated expression in response to stressful stimuli in both physiological and pathological conditions. Specifically, stressful events may epigenetically stimulate the expression of tyrosine hydroxylase (able to control catecholamine synthesis) [145, 147, 148], and GAL [145, 149], increasing noradrenergic neurotransmission or modifying postsynaptic neuronal functions involved in depression. In a placebo-controlled double-blind randomized study, Murck *et al.* [150] demonstrated that GAL has a critical role in stress, depression-like behaviors, and anxiety when administered to 10 patients with depression, who were on a stable dose of trimipramine. The authors suggested an acute antidepressive effect of GAL as assessed by the Hamilton Depression Rating Scale.

Recently, Juhasz *et al.* [146] analyzed a population of 2361 subjects and reported that variants in genes for GAL and GALR1, GALR2, GALR3 were associated with increased risk of depression and anxiety in those who experienced childhood adversity or recent negative life events. The authors found an increased expression of GAL system genes in individuals with high levels of life stress when compared to low/moderately stressed subjects. Surprisingly, they reported that effect of the GAL system genes was stronger than that of the well-known serotonin-transporter-linked polymorphic region (5-HTTLPR) polymorphism in the serotonin transporter gene. Importantly, GAL pathway seems to play a fundamental role in the pathophysiology of major depression, presumably strengthening the vulnerability to psychosocial stress.

Concerning the involvement of NPY in major affective disorders, there are studies in the literature reporting an important role of NPY in emotional responses and stress- or depression-related conditions [151]. Significantly, higher cortisol, CRH, and lower NPY concentrations have been reported in recent suicide attempters compared to healthy controls [21]. As mentioned by Westrin *et al.* [128], NPY levels are positively associated with depressive symptoms such as psychasthenia, irritability, and stability, and negatively with validity in depressed patients.

Another important shortcoming that requires attention when analyzing the association between neuropeptide alterations and suicide/major affective disorders is related to the fact that some studies do not report significant changes in neuropeptide concentrations in patients with suicidal behavior [124, 136, 138–140].

Some studies investigated heterogeneous groups of patients and reported no significant differences in means levels of neuropeptide concentrations between the diagnostic subgroups. Overall, no significant changes in neuropeptide concentrations may emerge among those exhibiting suicidal behavior.

Some studies investigated plasma neuropeptide levels, which do not necessarily reflect central neuropeptide levels. Furthermore, there are reports that do not control for the medications used by the patients, which might be potential confounders [127]. Westrin *et al.* [127] found that SOM changes were associated with symptomatic recovery in both suicidal and depressed patients. Not all

studies were able to find any differences in neuropeptides between patients with major depression and those with suicidal behavior. As many of the neuropeptide abnormalities were reported in subjects with suicidal behavior who were also depressed, it is possible that the association between neuropeptides and suicidality is closely influenced by the underlying psychiatric condition (e.g., major depression). In addition, most studies addressing the association between neuropeptides alterations and suicidal behavior are cross-sectional in nature and did not assess patients who are using psychometric instruments, whereas other limitations include the lack of long-term follow-up evaluations. Importantly, the naturalistic design of most studies did not control for the effect of confounding factors such as duration of treatment, duration of disease, and the diagnostic subtypes of the illness. Samples of patients with major affective disorders may usually include mixed and heterogeneous patients, suicidal patients are frequently not screened for the underlying psychiatric conditions and suicide attempters might be not controlled concerning the lethality of suicide attempts. Relevantly, not all studies include a control group or, alternatively, healthy volunteers were not matched for age/other relevant variables.

Recently, neuropeptide receptors have been postulated to serve as interesting targets for the treatment of many psychiatric disorders. Compounds targeting neuropeptide receptors may represent intriguing molecules presumably able to be used in the treatment of neuropsychiatric disorders [152]. On the basis of preclinical and clinical evidence,  $5\text{-HT}_1\text{A}$ , CRE, vasopressin, and NT receptors have been suggested to be potential targets for future treatments of psychiatric disorders. Some neuropeptide receptor agonists/antagonists have been proposed to be candidate medications for the treatment of major affective disorders and suicidal behavior, specifically. Neuropeptides and their agonists/antagonists may be administered alone or in combination with standard antidepressant medications to ameliorate depressive symptoms.

Importantly, valid biomarkers should be available soon for clinicians in order to ascertain whether neuropeptide alterations are fundamental pathogenetic factors or exclusively the “tip of the iceberg” in terms of molecular changes involved in the pathophysiology of major depression/suicide. In future studies, it will be crucial to analyze whether personalized treatments are possible using drugs targeting neuropeptide receptors.

## 15.6

### Concluding Remarks

Neuropeptidergic circuits seem to act as fundamental mediators of human behaviors. These molecules may represent interesting mediators of stress-related disorders, major affective conditions, and suicidal behavior. They may act directly by inducing changes in monoaminergic systems or indirectly by modifying other

molecular mechanisms that are known to be strongly involved in the pathophysiology of major affective disorders. Correcting abnormal neuropeptidergic functioning in suicide/major depression could serve to identify novel targets for drug development. In addition, as pharmacological treatment options are currently not satisfactory, using neuropeptide receptor agonists/antagonists as new candidate medications might represent a promising strategy in the treatment of major affective disorders and suicidal behavior. Although many studies identified a positive association between neuropeptide alterations and major depressive disorders/suicidal behavior, it is, however, unlikely that neuropeptides may currently represent definitive biomarkers of suicidality/depression. Future studies are needed in order to elucidate the complex nature of neuropeptidergic abnormalities underlying suicidal behavior and major affective conditions.

### Abbreviations

5-HIAA	5-hydroxyindoleacetic acid
5-HT	5-hydroxytryptamine
ACTH	adrenocorticotrophic hormone
AVP	arginine vasopressin
CSF	cerebrospinal fluid
CCK	cholecystokinin
CRF	corticotropin-releasing factor
CRH	corticotropin-releasing hormone
DSIP	delta sleep-inducing peptide
DBI	diazepam-binding inhibitor
DR	dorsal raphe
DST	Dexamethasone suppression test
Dyn-A	dynorphin-A
GAL	galanin
GABA	gamma-aminobutyric acid
GRP	gastrin-releasing peptide
HVA	homovanillic acid
HPA	hypothalamic pituitary adrenal
IL-6	interleukin-6
L-TRP	L-tryptophan
MCH	melanin-concentrating hormone
MCHR	Melanin-concentrating hormone receptors
Met-e	met-enkephalin
VGf	nerve growth factor inducible
NPSR	Neuropeptide S receptor
NK	neurokinin
NK1	neurokinin 1

NKA	neurokinin A
NKB	neurokinin B
NMB	neuromedin B
N/OFFQ	Nociceptin/orphanin FQ
NPS	neuropeptide S
NPY	neuropeptide Y
NT	neurotensin
NTR	Neurotensin receptor
NMDA	<i>N</i> -methyl- <i>D</i> -aspartate
NSSI	non-suicidal self-injury
ORL	opioid receptor-like
REM	rapid eye movement
5-HTTLPR	serotonin-transporter-linked polymorphic region
SOM	somatostatin
SP	Substance P
TACR	tachykinin receptor
Tk	tachykinins
β-end	β-endorphin

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## 16

# From Marine Organism to Potential Drug: Using Innovative Techniques to Identify and Characterize Novel Compounds – a Bottom-Up Approach

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### 16.1

#### Introduction

Natural products remain an important source of chemical diversity, and as such are an invaluable source of lead structures for the development of new pharmaceuticals [1–6]. Even in the last 20 years, many newly approved compounds were derived from natural products [7]. During this same period, however, the pharmaceutical industry largely abandoned natural products research [8]. From a North American perspective, most large firms had natural products research divisions in the early 1990s, but by 2009 only that of Wyeth remained. With the takeover of Wyeth by Pfizer in 2009, only Novartis and several Japanese firms continue to have active natural products discovery programs.

This apparent divergence between discovery and licensed products can in part be explained by the long timelines in pharmaceutical development, but we believe that the overreliance on bioassay throughout the screening and isolation process has led to an incompatibility of natural product research with the modern demands of the pharmaceutical industry [9]. Breakthroughs in molecular biology and understanding of signal transduction pathways have led to single receptor proteins becoming popular targets. At the same time, huge advances in computing power and robotics have introduced the era of high-throughput screening (HTS) [2]. The combination of these two developments led to the need for large compound libraries to screen against a number of very specific assays. Combinatorial synthesis seemed to fill this need, but it was soon realized that this approach also led to large numbers of very similar compounds based on a set of simple templates, and that smaller libraries covering a greater chemical diversity were required. While it is recognized that natural products would be able to provide this chemical diversity, there was no obvious way to rapidly provide a large number of purified natural products unbiased by a specific biological

1) AJ Singh and JJ Field contributed equally to the chapter.

activity [10]. HTS of crude extracts proved unreliable in single receptor assays owing to the complex mixture of compounds (including primary metabolites) present. A similar problem existed in the early days of combinatorial chemistry screening in which pooled libraries also had a mixed sample composition because of pooling of beads in a single sample. Bioassay-guided isolation of compounds also proved too slow to keep up with the pace of assay development throughout the screening and isolation stages [10].

We believe this “top-down” approach of focusing on a single target from the outset has led to the paradigm of bioassay-guided isolation of natural product chemistry no longer being “fit for purpose.” This dilemma has led us to develop a different approach, which can be thought of as “bottom-up.” Here the focus is to concentrate and reveal the unusual secondary metabolites present in a source organism. A preselection strategy for molecules with drug-like solubility and early detection of structural features of the metabolites provides us with the ability to assess, at an early stage, the broad pharmaceutical potential present within source material. Our method can be divided into two steps: (i) separation of secondary metabolites from the primary metabolites and (ii) spectroscopic analysis of the secondary metabolite-enriched fraction. When we have found spectroscopic features of interest, we use these peaks to guide fraction selection through the subsequent stages of purification. Our techniques are constantly evolving and can be varied to suit the source organism or target molecule(s) of interest. What follows is our current general methodology as applied to marine invertebrates (usually sponges [11]) and macroalgae.

To concentrate a secondary metabolite fraction, we use a very crude form of reversed-phase chromatography that is in essence a preparative-scale, solid-phase extraction. Before our interest in the secondary metabolites approach, we devised a rugged method for partitioning crude extracts. Crude extracts of marine organisms are usually a complex mixture of compounds of diverse polarity, often in saturation in the crude extract. Almost any form of concentration of the extract results in precipitation of some of these components. In our experience, even the act of combining the first and second methanolic extracts of a marine sponge can cause a large precipitation of nonpolar compounds. Seeking a more broadly applicable partitioning method, we selected a polymeric reverse-polarity stationary phase using polystyrene-divinylbenzene copolymer (PSDVB). We found that while a single pass of the crude extracts through a bed volume of PSDVB beads did not retain many secondary metabolites, it did retain most of the very nonpolar components, allowing us to combine the eluents of the first and second extracts, and furthermore, dilute them with an equal volume of water. This combined mixture could then be passed through the same PSDVB column. Many more of the mid-polarity components adhere to the beads, allowing further water to be added to the eluent. We then continued this process of “cyclic loading” until the most polar compounds of interest were retained on the beads. Our final dilution is usually in 25% MeOH in H<sub>2</sub>O. Once the crude extract is cyclic loaded onto a PSDVB column, it can



be batch eluted with fractions of H<sub>2</sub>O with increasing proportions of an organic modifier such as MeOH or Me<sub>2</sub>CO in the usual reversed-phase manner.

Using this technique to purify known biologically active secondary metabolites (for example, mycalamide A from *Mycale hentscheli* [12]), we noticed that most primary metabolites eluted in either the early polar fraction (0–20% Me<sub>2</sub>CO/H<sub>2</sub>O) or the later very nonpolar fraction (80–100% Me<sub>2</sub>CO); whereas, most secondary metabolites eluted in the mid-polarity range (40–60% Me<sub>2</sub>CO/H<sub>2</sub>O). Thus, using this method, we enriched for the secondary metabolites that consisted of the most novel structures for lead compounds in medicinal chemistry. Moreover, most of the mass of the crude extract was associated with the unwanted primary metabolites (salts, sugars and amino acids, or nonpolar fats and steroids), leaving most of the novel secondary metabolites in the mid-polarity low mass fractions that were collected. We realized that these low mass, mid-polarity fractions could be concentrated and dissolved in a sufficiently small volume of solvent for spectroscopic analysis. A series of subsequent tests on a variety of known bioactive sponge metabolites allowed us to simplify the partitioning to three elutions (30%, 75%, and 100% Me<sub>2</sub>CO/H<sub>2</sub>O) with the secondary metabolites all concentrated in the middle fraction. Not all secondary metabolites, however, were found in the mid-polarity fractions – unusual sugars and amino acids were found in the early fractions, and many steroids and some other simple terpenes partitioned to the very nonpolar fractions, along with fats. The vast majority of potentially active metabolites, however, did partition to the mid-polarity fraction. This is not surprising, as it is well understood that potent bioactivity requires some hydrophilicity (bioavailability) and some hydrophobicity (enthalpic binding to neutral regions on a target protein). After partitioning of the crude extract, the resulting fractions were narrower in polarity range, with the mid-polarity fraction now fully soluble in many organic solvents.

## 16.2

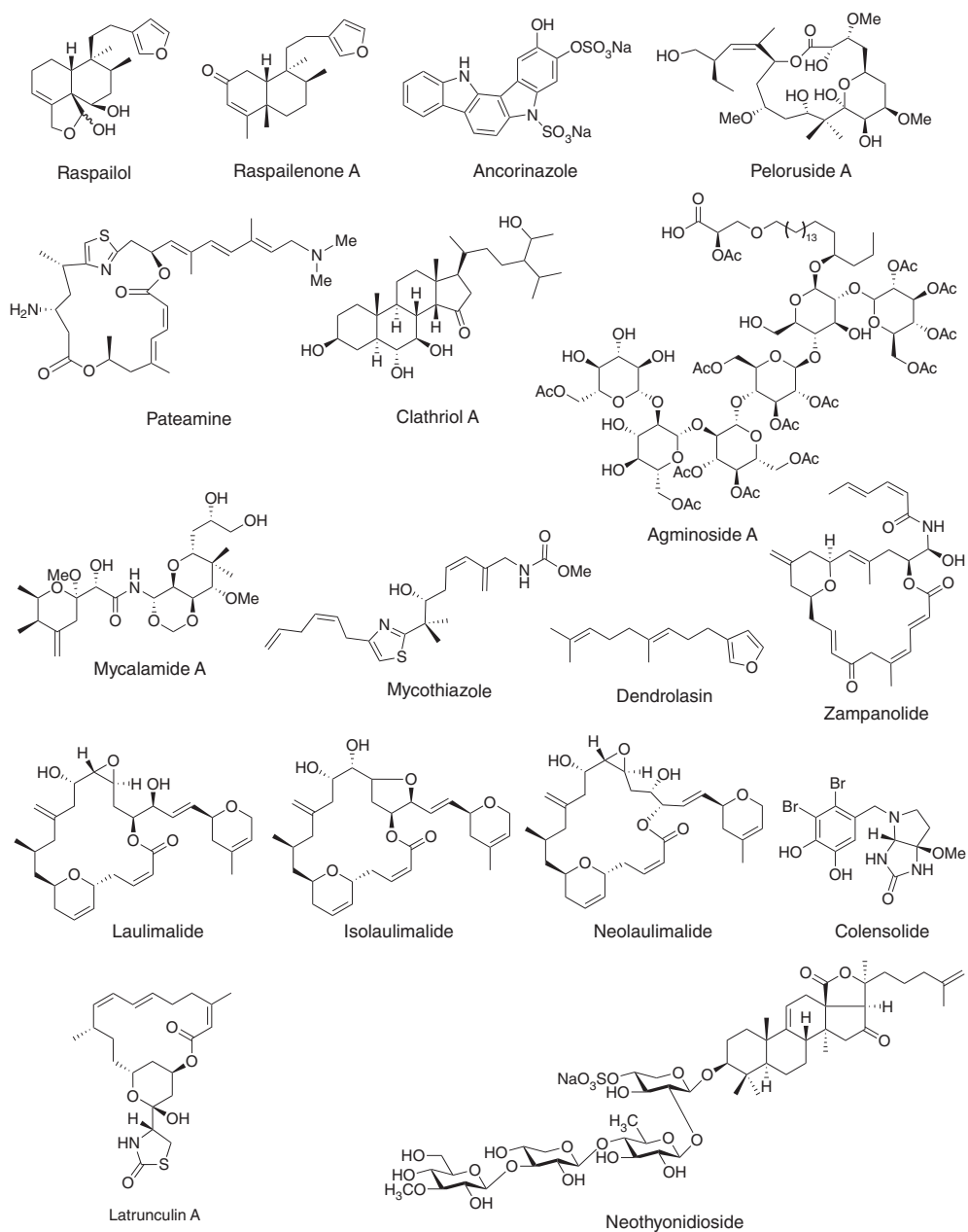
### Structural Screening Approach

To obtain maximum information about structure of the constituents of a fraction, we chose NMR analysis. While NMR spectroscopy is quite insensitive compared to mass spectrometry (MS) analysis, it is better suited to an unbiased analysis of a complex mixture because of its linear response and high general structural information content. MS analysis could be run on the sample and might be more useful with specific classes of compounds. Working with New Zealand-sourced (temperate-water) marine sponges, we found that the resulting mid-polarity fraction from the crude extracts of a 100 g (wet weight) sample, typically 40–50 mg of organic matter, will usually dissolve in 500 μl of a deuterated solvent, which is a useful volume for a 5 mm NMR tube. This represents a considerable level of concentration, making it possible for the minor metabolites to be examined spectroscopically. While the less expensive deuterated solvent CDCl<sub>3</sub> will usually

dissolve this fraction, we find a number of secondary metabolites give unsatisfactory, broadened NMR signals. We resolved this problem by using CD<sub>3</sub>OD. Marine sponges sourced from subtropical waters produce such large amounts of secondary metabolites that an extract of a 20 g sample is sufficient for NMR analysis.

Starting with simple 1D <sup>1</sup>H NMR spectra of the mid-polarity fraction on a collection of New Zealand sponges, all medium polarity extracts showed resonances associated with various fatty acids or triglycerides obscuring portions of the spectrum, typically with about one in every 10 screens showing unique <sup>1</sup>H NMR peaks of secondary metabolites. Early examples of novel secondary metabolites detected with the use of this technique were the clerodane diterpenes raspailol and raspailenone [13], the indolocarbazole ancorinazole [14], and the polyketide macrolide peloruside A [15]. The chemical structures of all natural products discussed in this chapter are presented in Figure 16.1. Peloruside A, in particular, was found amongst larger amounts of the previously described mycalamide A and pateamine, both of which are also potent cytotoxins, making it highly unlikely that peloruside A would have ever been found using bioassay-guided isolation techniques.

Despite these early successes, we were concerned that only compounds with olefinic resonances were detected because other regions of the 1D <sup>1</sup>H NMR spectrum were obscured by residual primary metabolites. Two-dimensional-NMR experiments offer greater spectral dispersion and structural information, but are typically much less sensitive. Gradient selection has improved their sensitivity, and we chose to use the COSY (correlation spectroscopy) and HSQC (heteronuclear single-quantum coherence) experiments for their ability to detect secondary metabolites in the mid-polarity fraction. While the COSY is inherently more sensitive than the HSQC experiment, we found that COSY did not offer a significant increase in definitive structural information over the simple 1D <sup>1</sup>H NMR spectrum. The HSQC, while much less sensitive, offered much greater spectral dispersion because of the greater range offered by the <sup>13</sup>C dimension and the very definitive functional group identification. Thus, a useful screen of a marine organism extract could be obtained in about 4 h of acquisition time. Dispersed over the second dimension, even the residual primary metabolites offer such a complex pattern that the human eye cannot reliably detect the occasional unusual peak or hit. We had to develop some way of removing the common peaks from the 2D spectrum of a single sponge mid-polarity extract, and this was accomplished by combining many HSQC spectra to obtain an “average sponge” mid-polarity HSQC spectrum. The peaks of primary metabolites are very high as they are present in all samples; whereas, the secondary metabolites, unique to each sponge sample, remain very weak by comparison. We then subtracted this “average sponge sample” or “mask” from each individual sponge HSQC spectrum to reveal those signals unique to that sponge sample. Among a number of terpenoids, the 14β sterol clathriol A and highly unusual glycolipids, represented



**Figure 16.1** Structures of natural products isolated by NMR-guided isolation.

by agminoside A, were revealed by this technique where no protonated alkene resonances were present [16, 17].

The HSQC screen has been successful in terms of selecting for secondary metabolites, as demonstrated by the diverse range of structures that have been isolated in our laboratory using the method described (Figure 16.1). However, this NMR experiment does have its limitations. While the experiment provides discrete  $^1\text{H}$ - $^{13}\text{C}$  correlations, there is no direct evidence that any combination of these correlations in a crude extract can be attributed to a single molecular entity. The next advance in our screening protocol would naturally involve a progression to screening for substructures within the crude extract. The HMBC (heteronuclear multiple-bond correlation) experiment provides correlation data between  $^1\text{H}$  and  $^{13}\text{C}$  nuclei separated by two to four bonds, and although this is a less sensitive test than the HSQC, it is a more powerful experiment with respect to the information that is available. A functional HMBC screen is potentially a powerful technique at the first fractionation step because it allows families of compounds to be identified at an early stage of purification. This helps determine if a sponge extract is worthy of further investigation without fully committing to the purification of a given extract. At the very least, an HMBC screen can be performed concurrently with the HSQC screen experiment, and thus interesting one-bond correlations in the HSQC screen can be extended to multiple-bond correlations from the HMBC screen. Similarly to the HSQC experiment, spectral resolution in the HMBC experiment is achieved by dispersion in the carbon dimension. However, as the proton and carbon are not directly attached, there is no general trend associated, and therefore full dispersion is available throughout the whole two-dimensional space of the spectrum. The pulse sequence for the HSQC experiment is more complex than that for the HMBC experiment and involves  $^{13}\text{C}$  decoupling during acquisition. This means that the HMBC experiment is more robust and less susceptible to changes in pulse-width calibration. While we find that we can obtain useful high-resolution HMBC data on the mid-polarity fraction after 8 h of acquisition, lower-resolution data after 4 h of acquisition could probably be used for the initial detection of structural motifs.

Interpreting HMBC spectra can be difficult when differentiating between two-bond and three-bond correlations; however, certain functional groups have very diagnostic correlation patterns. For example, aromatic protons tend to show much stronger  $^3J_{\text{CH}}$  correlations than  $^2J_{\text{CH}}$  correlations. Because of their higher relative intensity, correlations from methyl groups also give consistent patterns depending on the nature of their attachment points and adjacent centers, for example, *gem*-dimethyl, isopropyl(idene), and oxymethyl.

#### 16.2.1

##### Case Study 1: Colensoide from *Osmundaria colensoi*

On the basis of HSQC screening, a red algae collected from the Northland region of New Zealand was found to have a large number of related secondary

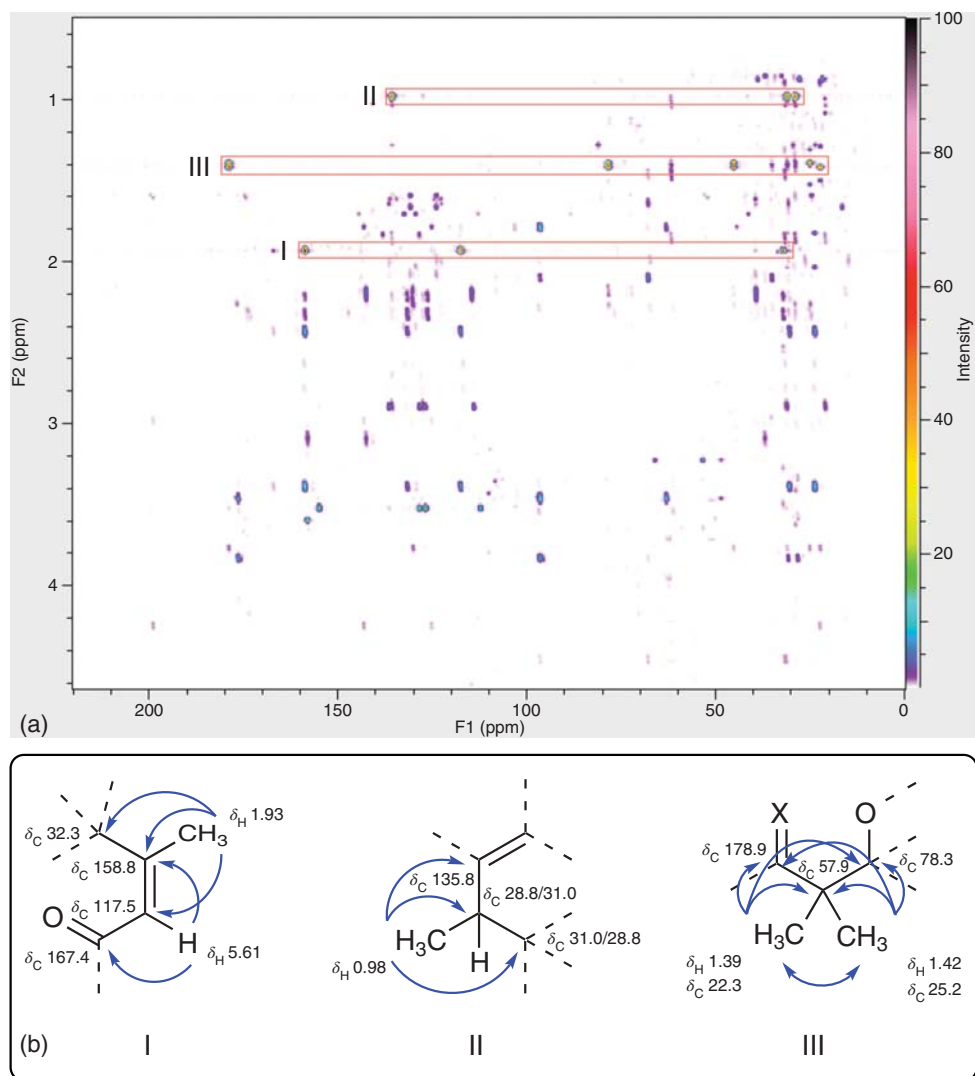
metabolites in the medium-polarity fraction. An HMBC spectrum was obtained on this fraction, and the combination of spectral data allowed the identification of these metabolites as bromophenols, a class of well-known red algal metabolites. The HMBC experiment was further used to detect substructures not previously ascribed to bromophenols. In particular, a diastereotopic methylene pair was identified with HMBC correlations to an additional diastereotopic methylene and a deshielded methine. Subsequent spectroscopic-guided isolation led to the purification of colensolide, a new bromophenol with an unusual bicyclic nitrogenous appendage [18].

### 16.2.2

#### Case Study 2: Zampanolide from *Cacospongia mycofijiensis*

A tan-colored sponge collected from 'Eua, Tonga, was subjected to an NMR-based screening protocol. The  $^1\text{H}$  NMR spectrum of the screen fraction was interesting because of the high concentration of material and the large range of chemical functionality present. Proton resonances in the olefinic ( $\delta_{\text{H}}$  5.00–7.00) and oxymethine ( $\delta_{\text{H}}$  3.00–5.00) regions were evident alongside very few methyl signals. This information led to the belief that these compounds were polyketide-derived. Because of the interesting  $^1\text{H}$  NMR spectrum, the mid-polarity screen fraction of this sponge was among the first to be analyzed with the HMBC screen. Substructures were generated from the HMBC screen spectrum (Figure 16.2a), with methyl-based substructures the first and most readily identified. Figure 16.2b shows three main subunits (I–III), the first of which was an olefinic methyl ( $\delta_{\text{H}}$  1.93) and olefinic methine ( $\delta_{\text{H}}$  5.61) as part of an  $\alpha,\beta$ -unsaturated carbonyl system ( $\delta_{\text{C}}$  167.4; 158.8, 117.5). Also apparent was a secondary methyl ( $\delta_{\text{H}}$  0.98,  $J = 6.5$  Hz) correlating to an olefinic methine ( $\delta_{\text{H}}$  135.8) and two  $\text{sp}^3$ -hybridized carbon centers ( $\delta_{\text{C}}$  31.0; 28.8). The third substructure was a *gem*-dimethyl moiety flanked by oxygenated ( $\delta_{\text{C}}$  78.3) and deshielded non-protonated ( $\delta_{\text{C}}$  178.9) centers. While establishing these substructures was informative, there was not enough evidence at this stage to determine the complete structure of these compounds without attempting further purification protocols.

A portion of the 75%  $\text{Me}_2\text{CO}/\text{H}_2\text{O}$  screening fraction was chromatographed further on PSDVB and silica gel to afford two major components. These now pure compounds were identified as latrunculin A and mycothiazole, respectively. Latrunculin A incorporated the olefinic and secondary methyls (subunits I and II), while mycothiazole accounted for the *gem*-dimethyl group (subunit III). The furanosesquiterpenoid dendrolasin was later identified from analysis of the 100%  $\text{Me}_2\text{CO}$  screen fraction. Thus, on the basis of its morphology and chemotaxonomy, this sponge was tentatively assigned as *C. mycofijiensis*. Interestingly, Crews and coworkers [19, 20] also noted latrunculin A and the furanosesquiterpenoid dendrolasin as the only constituents from a single Tongan collection of *C. mycofijiensis*; however, it was not stated exactly where this specimen was collected.



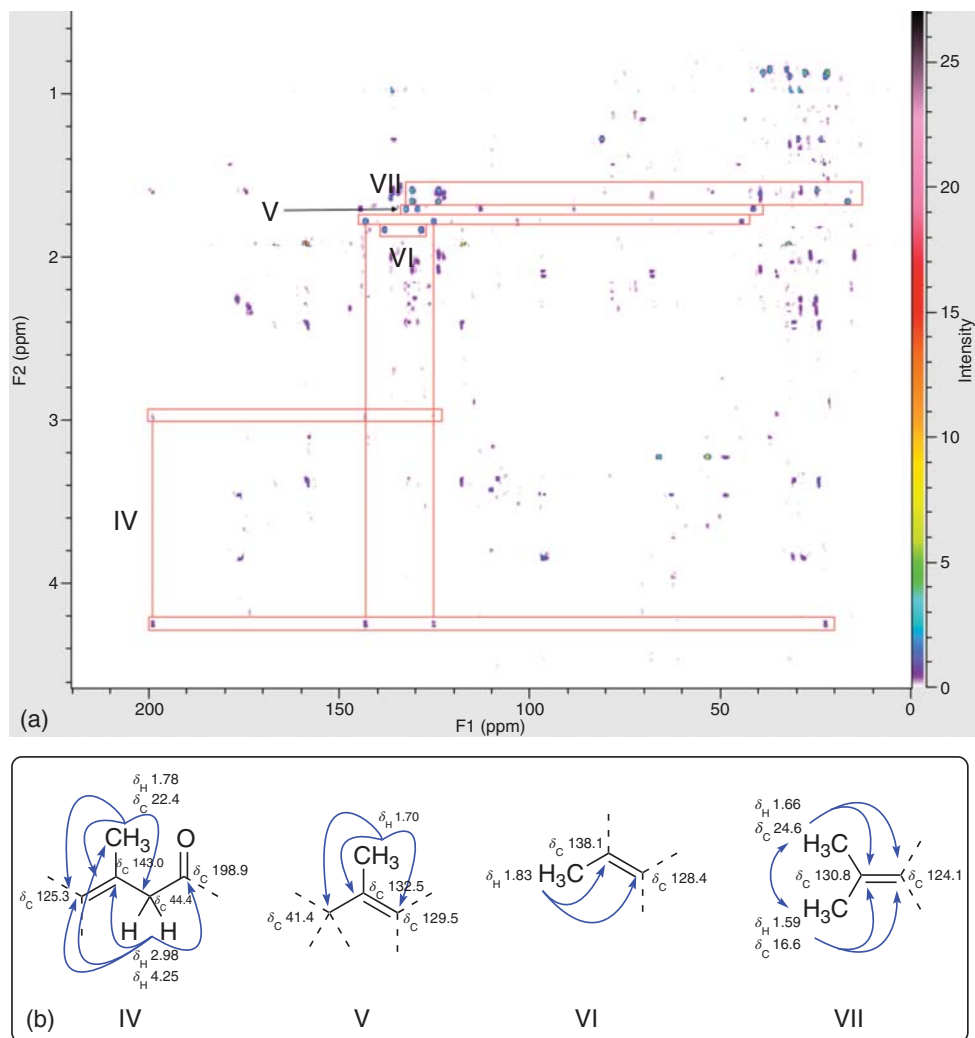
**Figure 16.2** (a) Expanded view of the screen HMBC spectrum of *Cacospongia mycofijiensis*. (b) Initial methyl-based substructures generated from the 75% Me<sub>2</sub>CO/H<sub>2</sub>O screen HMBC spectrum of *Cacospongia mycofijiensis*.

Using in-house software, the HMBC spectra of now purified latrunculin A and mycothiazole were mathematically subtracted from the HMBC screen in an attempt to observe the correlation data of any minor components that may have been obscured (Figure 16.3a). Another set of four methyl-based correlations (IV–VII) was identified (Figure 16.3b). The first set of correlations

was from a diastereotopic methylene pair ( $\delta_{\text{H}}$  4.25, 2.98) that corresponded to a methyl-substituted alkene ( $\delta_{\text{C}}$  143.0; 125.3;  $\delta_{\text{H}}$  1.78,  $\delta_{\text{C}}$  22.4) and a carbonyl residue at  $\delta_{\text{C}}$  198.9 – typical of an  $\alpha,\beta$ -unsaturated ketone. Next, an internal, trisubstituted alkene was constructed from a methyl resonance at  $\delta_{\text{H}}$  1.78 to three carbons ( $\delta_{\text{C}}$  132.5; 129.5; 41.4). Correlations from a methyl resonance at  $\delta_{\text{H}}$  1.83 to two olefinic carbons ( $\delta_{\text{C}}$  138.1; 128.4) formed the basis of a terminal, methyl-substituted alkene. Finally, an isopropylidene unit was inferred from correlations consistent with two methyl resonances ( $\delta_{\text{H}}$  1.66,  $\delta_{\text{C}}$  24.6;  $\delta_{\text{H}}$  1.59,  $\delta_{\text{C}}$  16.6) correlating to two  $\text{sp}^2$ -hybridized carbon centers ( $\delta_{\text{C}}$  130.8; 124.1) and to their complementary methyls. Partial structure **VII** was consistent with dendrolasin, which, although it was found in this screen fraction as a minor compound, was a major component of the nonpolar fraction. A search of the MarinLit database (<http://www.chem.canterbury.ac.nz/marinlit/marinlit.shtml>) of partial structures **IV**–**VI** produced a list featuring 198 results for **IV**, 6924 for **V**, and 7205 for **VI**. When the term *latrunculin* was included in the search query of partial structures **IV**–**VI**, the list reduced to two unique results: zampanolide and dactyloide. Further isolation protocols were required to determine whether these compounds were actually present as constituents of this sponge.

A bulk extraction of 341.0 g of *C. mycofijiensis* followed by purification of the resulting 80%  $\text{Me}_2\text{CO}/\text{H}_2\text{O}$  fraction using DIOL and silica gel ( $\text{EtOAc}/\text{CH}_2\text{Cl}_2$ ) yielded the three major compounds, dendrolasin (107 mg), *latrunculin A* (233 mg), and mycothiazole (225 mg), in large quantities. *Laulimalide* (38 mg), *isolaulimalide* (2 mg), and *neolaulimalide* (2 mg), all microtubule-stabilizing agents (MSAs), were also isolated, despite not having been previously identified in the screen. Importantly, smaller amounts of the target metabolite, zampanolide (5 mg) were isolated, confirming our proposition of its presence and further validating the use of the HMBC experiment as a screening tool. No isolation of this compound had been reported in the literature since its initial isolation in 1996 [21]. Thus, the re-isolation of zampanolide in this study provided an opportunity to further assess its biological profile [22].

In the preceding example, the potent MSA *laulimalide* would have masked a top-down screen for cytotoxicity and MSA activity, and no subsequent search of the extract for a second MSA activity would have been made. However, using our bottom-up method, we were able to identify and isolate zampanolide, which was present in the extracts at lower concentrations and turned out to be a potent and unusual new MSA [22, 23]. This illustrates how the bottom-up approach can identify new novel secondary metabolites in organisms rich in bioactive compounds where one activity of a major component of the extract can mask the activity of compounds present at lower concentrations or compounds with less potent activity.



**Figure 16.3** (a) Expanded view of the screen HMBC spectrum of *Cacospongia mycofijiensis*, following mathematical subtraction of HMBC data of latrunculin A and

mycothiazole. (b) Residual methyl-based substructures generated from the 75% Me<sub>2</sub>CO/H<sub>2</sub>O screen HMBC spectrum of *Cacospongia mycofijiensis*.

### 16.3

#### Testing for Bioactivity by Screening in Mammalian Cells

Once a novel compound is isolated, its biological activity needs to be characterized. While there are many different assays available that screen for biological



activity, it should be pointed out that the type of screen chosen will partly determine whether or not a compound comes up as a positive “hit” in that screen. Specific target assays will only establish activity against that particular target, and a potentially useful compound could be dropped from a screening program for this reason, despite having significant, but unrealized, activity against other target proteins not tested for in the screen.

In the case of general cell proliferation or cytotoxicity testing, it is important to consider the cell line to be used for screening as there can be huge variation in the resistance of cells toward different compounds. For example, mycothiazole exhibited low nanomolar cytotoxicity in adherent, cervical cancer-derived HeLa cells, but in the non-adherent, promyeloid leukemic HL-60 cell line, the activity was in the low micromolar range, giving a 34 000-fold difference in  $IC_{50}$  values [24]. In addition, the  $IC_{50}$  values for peloruside A in a panel of 25 nonsmall cell lung cancer cell lines ranged from 10 nM to greater than 250 nM (Professor J. Minna, University of Texas Southwestern Medical Center, Dallas, TX, personal communication). Whether cells are dividing or not is also an important factor if cell division machinery is the target of the compound. For example, primary macrophages isolated and cultured from a mouse are completely insensitive to the mitotic inhibitor peloruside A, as are unstimulated splenocytes (see Figure 2 of Crume *et al.* [25]).

Cell proliferation assays are essentially untargeted and are widely used to assess a number of factors, most importantly whether a new compound is cytotoxic (kills cells or disrupts their metabolism) or cytostatic (prevents growth and proliferation). Traditionally, these assays involved [ $^3H$ ]-thymidine or BrdU incorporation assays; however, more straightforward colorimetric assays that test for cell viability and cell metabolism are more commonly used now. These popular multifunctional assays can be used with cells in suspension or monolayer culture [26], and are ideally suited for testing general drug sensitivity and cytotoxicity and can be conducted in a high-throughput manner. For example, the tetrazolium salt MTT and second-generation tetrazolium dyes XTT or WST-1 are commonly used in cell proliferation assays [27]. Tetrazolium dye assays measure the active metabolism of cells, and therefore cells must be alive and metabolizing to give a positive absorbance signal for a colored formazan product. They do not, therefore, distinguish between the cytostatic and cytotoxic effects of a compound. Other assays such as the sulforhodamine B (SRB) assay [28] measure cell density on the basis of the amount of cellular protein. These assays are less susceptible to metabolic artifacts, effectively measuring total cell concentration directly by the amount of protein present, although even in the SRB assay, variations in cell size can lead to misinterpretation of the number of cells present.

A commonly used way of measuring cell death or viability is to use trypan blue dye exclusion. This dye is only permeable to dead cells, and stains them blue. Fluorescent probes are easier to quantify and also take advantage of membrane permeability changes in dying cells to distinguish between dead and live cells in a fluorescent microscope. For example, calcein-AM, stains live cells green but does

not stain dead cells; whereas, other dyes such as propidium iodide only stain the nuclear DNA of dead cells as they are impermeable to living cells in which the plasma membrane is intact.

One of the problems with screening for bioactivity in cultured cells is the possibility that the natural product is metabolized inside the cell, and therefore an unknown metabolite is the actual bioactive molecule. We came across an interesting variation on this theme with peloruside A when attempting to locate an appropriate site for attaching a fluorescent label. A chloroacetate group was attached to the C-24 hydroxyl group of peloruside A on the side chain (Figure 16.1), and this analog was found to have similar activity in cultured cells to the parent compound, suggesting that a fluorophore tag could be attached to this region of peloruside A without interfering with its activity. However, a subsequent study that investigated the ability of the chloroacetate analog to polymerize purified tubulin showed no activity at all [29]. The obvious conclusion from this work was that the chloroacetate group was being cleaved off inside the cell by endogenous esterases, leaving the parental peloruside A to interact with its target.

Once a compound is deemed cytotoxic or cytostatic, the next step is to determine its biological target, and to do this, a comprehensive evaluation of the compound using a number of pharmacological tools and focused protocols are required. We either use specific assays in mammalian cells or use yeast, either baker's yeast *Saccharomyces cerevisiae* or the fission yeast *Saccharomyces pombe* can be used as model organisms. One of the limitations of using yeast in bioactivity screens is a greatly reduced sensitivity of yeast to drugs. Compounds that are active in mammalian cells at nanomolar concentrations often require micromolar concentrations for growth inhibition in *S. cerevisiae*. For example, the  $IC_{50}$  for peloruside A in yeast is about 35  $\mu\text{M}$  [30] compared to 20–30 nM in mammalian cancer cell lines [31]. Hence, for natural products that are often produced in low amounts, supply of sufficient compound for testing in yeast becomes a major issue. The extent of this problem can be alleviated somewhat by using yeast strains in which the pleiotropic drug efflux pump system has been knocked down [32, 33]. For example, the  $IC_{50}$  for peloruside A in *S. cerevisiae* was approximately halved to 19  $\mu\text{M}$  by knockout of the main drug efflux systems through mutation of the transcription factor genes involved (mutant strain *pdr1 $\Delta$ pdr3 $\Delta$* ) [34], thus, reducing the amount of peloruside A required by about half. Another limitation of screening natural products in yeast is the possibility of contaminants at low levels contributing to the bioactivity when micromolar amounts of the test compound are required for growth inhibition. For example, in the purification of mycothiazole from *C. mycofijiensis* described in the preceding sections [24], an activity was identified in chemical genetic screening that indicated actin as the target [35]. This activity, however, was later shown to be due to a very low level of latrunculin A contamination (less than 0.05%) in one of the mycothiazole preparations. Latrunculin A is a well-characterized actin inhibitor [36] and is very active at low concentrations (<10 nM) in yeast; whereas, the  $IC_{50}$

of mycothiazole in yeast with deficient drug efflux pump activity is from 12 to 22  $\mu\text{M}$  [35]. Hence, it is important to synthesize the natural product to ensure that the synthetic form has the same effect as the natural product to rule out the possibility of a contaminating metabolite in the extract being the source of bioactivity and not the lead compound.

Once a novel compound has been identified as sufficiently biologically active to be a lead compound for its development as a pharmaceutical drug, its mode of action needs to be determined. Compounds with no known mode of action can make it into the clinic, but knowing their mechanism and target molecule aids in their further development, permitting finer tuning via analog design to reduce secondary target side effects and generate less complex structures that are easier to make and that retain parent compound potency, or even improve upon it.

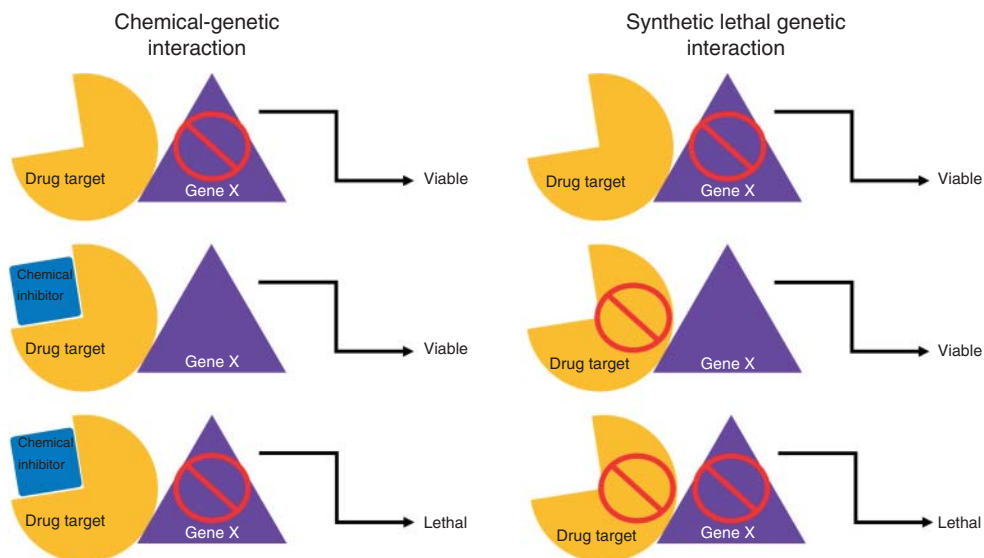
## 16.4

### Chemical Genetics and Network Pharmacology in Yeast for Target Identification

A good first approach to finding clues about the mode of action of a new natural product is to carry out DNA microarray screens in yeast. Yeast was the first eukaryote to have its genome sequenced and was found to have many genes homologous to higher eukaryotes, including unexpected ones such as proto-oncogenes. Many basic cell biology and biochemical pathways in yeast are very similar to mammalian cells, and these, along with its facile genetics and simple lifecycle, have made yeast a good model for eukaryotic cells [37]. The genetic tractability of yeast and development of a genome-wide set of knockout strains [38] has made possible HTS for bioactive small molecules (Figure 16.4 and see discussion on synthetic lethality in the following paragraphs).

Use has been made of the fact that certain small molecule inhibitors (SMIs) bind to their protein targets and ablate their function, thus, mimicking a deletion of the gene that codes for that protein [39–41]. Such gene function ablation by SMIs is the basis of what is now called *chemical genetics* or *chemical genomics*. SMIs can be used paired with a genetic knockout mutant creating a synthetic lethal combination of a gene knockout and a drug, just as with a genetic knockout pair of double mutants (reviewed in Parsons *et al.* [42]). Although the yeast model cannot directly reveal lead compounds for human therapeutics, the immense cost of a new therapeutic development (capitalized to ~\$US 1.8b; [43]) makes yeast a compelling, versatile, and cheap prescreening system for novel compounds before using more expensive cells and animal model testing.

Measuring bioactivity of new SMIs can be readily automated in yeast using gene knockout strains in hypersensitivity microchip assays (Figure 16.2) [44]. Such HTS is possible because each of the deleted coding open-reading frames (~6300) is systematically flanked by a 20 bp unique identifier called a *barcode* (Figure 16.5a) [38]. A portion of these gene deletions (17%) were found to be essential for viability [38], meaning that ~5000 genes were not essential for viability when deleted.



**Figure 16.4** Chemical-genetic interactions. The principle of synthetic lethality involves the mutation of two genes that are each nonessential when mutated on their own, but when both genes are mutated together, the double-mutant is lethal, and no growth occurs (see right side of figure). This often occurs when the genes are in two redundant pathways such that one gene can replace the other for an essential reaction in the cell. In chemical genetic interactions, the second

mutation is replaced by a drug that inhibits the activity of the gene product, effectively doing the same thing as the mutation by blocking the pathway that the gene product is involved in. The genes that cause lethality (or sickness) by making the yeast hypersensitive to the drug can provide clues to the pathways that are affected by the drug. (Adapted from Parsons *et al.* [42] from the Nature Publishing Group.)

This genome-wide set of nonessential deletion strains can be used to ask which nonessential gene knockouts confer hypersensitivity to a new bioactive compound in a pooled competitive growth assay [44] that quantifies the DNA “barcodes” flanking the deletions (Figure 16.5b) thereby measuring relative growth rates.

Utilizing this microchip assay in our laboratories, we probed the mode of action of neothyonidioside, a powerful eukaryote cell division inhibitor from a New Zealand sea cucumber. We found that neothyonidioside affects the ergosterol (yeast cholesterol equivalent) biosynthetic pathway [45]. A follow-up mutagenesis screen successfully selected for resistance mutants to neothyonidioside. The mutant lesion was located in the genome by a special linkage-disequilibrium mapping method [46] to NCP1, a NADPH-dependent reductase in the ergosterol pathway. However, NCP1 was not the actual target of the drug. A mutation in NCP1 caused lowering of cellular ergosterol and reduced the binding of neothyonidioside to membrane-resident ergosterol – the real target of the drug.

Endocytosis in wild-type cells in the presence of neothyonidioside was blocked, and thus formation of vacuolar multivesicular bodies in the cells was inhibited [45], possibly because of reduced membrane fluidity as a result of the bound drug. The actual downstream cause of neothyonidioside toxicity was therefore a cellular process, the result of many genes, and not a single gene product. In another recent study from our laboratory using barcode chips, we showed that the natural product MSA laulimalide affected specific microtubule motor functions and sister chromatid separation in cell division (Figure 16.6) that were not affected by MSAs such as benomyl [34], also demonstrating the utility of this type of screen for inferring mode of action of a new chemical entity.

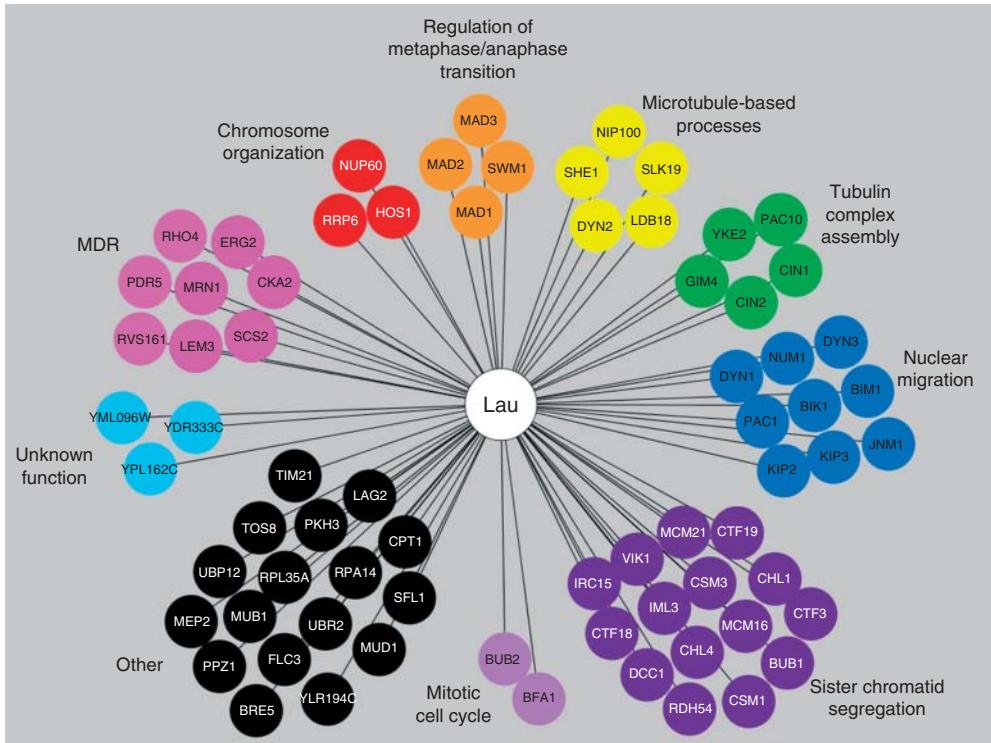
The assumption of multigene molecular contributions to phenotype is now the basis for “network pharmacology” [47, 48]. Polygenic contribution to phenotype is not new to classical genetics where quantitative traits are known to be the additive genetic effects of a few to multiple contributing genes (see for example, MacKay [49]) but is a concept that is relatively new in the pharmaceutical industry, because it implies that effective drugs in all likelihood will need to target the products of multiple genes. A good illustration is the centuries-old remedy salicylic acid extracted from willow bark. Salicylate regulates many genes, including COX-2 (cytokine), mitogen-activated protein kinase (MAPK)/38p, JNK-p, adenosine release, and prostaglandin-E2 (inflammatory effects), among others. An alternative to “promiscuous” drugs like salicylate is to use a combination of drugs targeting several different pathways [50].

Complex network contributions to phenotype may possibly explain individual differences in symptom severity, commonplace in many diseases, owing to small individual differences in contributing genes in an interaction network. For example, cystic fibrosis is a protein misfolding disease resulting in dysfunctional chloride channels. A mutation ( $\Delta 508F$ ) in the cystic fibrosis transmembrane receptor (CFTR) is the major cause of CFTR misfolding that prevents its normal

**Figure 16.5** The yeast deletion collection and parallel analysis. (a) Construction strategy for the yeast deletion-mutant collection. Each yeast open reading frame (ORF) is replaced with a “deletion cassette” that consists of an antibiotic-resistance marker, *kanR* (which confers resistance to kanamycin), and two unique 20 nt molecular barcodes (“up tag” (UP) and “down tag” (DN)). Each barcode is flanked by common primer sites (indicated by colored half-arrows). Incorporation of the cassette into the yeast genome is accomplished through homologous recombination of 45-bp regions of homology upstream and downstream of the yeast ORF. (b) Parallel analysis of large pools of deletion mutants. Populations of pooled mutant cells,

each marked with unique molecular barcodes, are grown in the presence or absence of a growth-inhibitory drug. Genomic DNA is extracted from the pool of mutants, and barcodes that represent each strain are amplified by PCR using common primers that are labeled with fluorescent markers Cy3 or Cy5. Drug-sensitive mutants are identified by competitive hybridization of the barcode PCR products to a microarray that contains oligonucleotides corresponding to each barcode, giving a quantitative readout of the proportion of each mutant in a mixed population. (Figure and legend from Boone *et al.* [44], with effects permission from the Nature Publishing Group.)



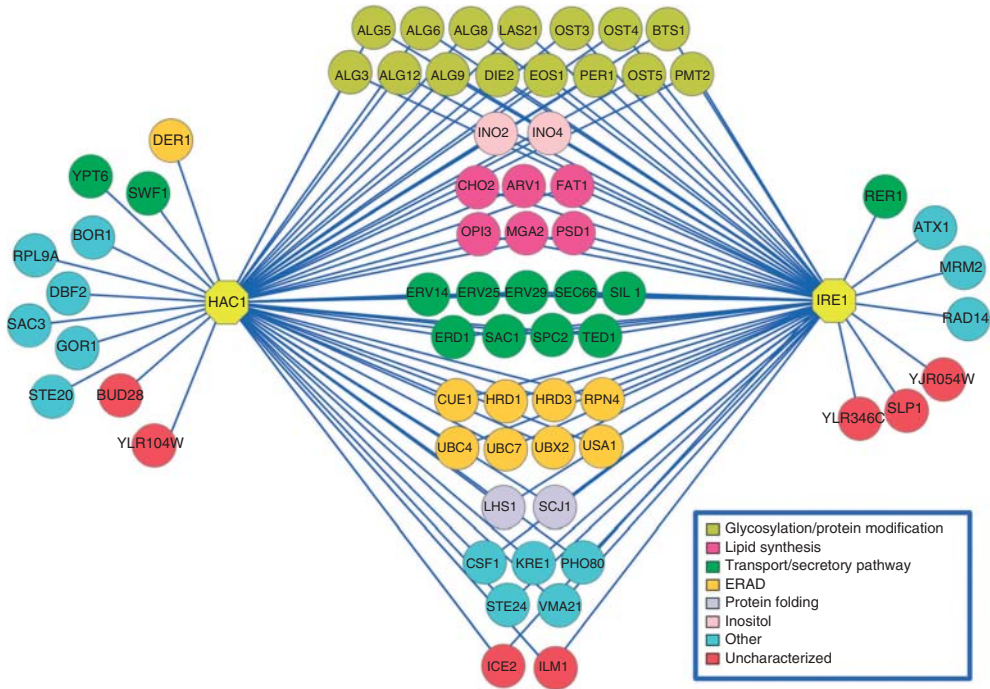


**Figure 16.6** Competitive growth assay showing gene deletions that are hypersensitive to the natural product small-molecule inhibitor, laulimalide. Gene hits included genes related to the microtubular motors

dynein (DYN) and kinesin (KAR and KIP) as well as chromosome instability and centromere genes (CEN) and kinetochore genes (CTF19 and MCM16).

trafficking to the plasma membrane and leads to degradation of the misfolded protein. The effect of the  $\Delta 508F$  mutation is actually modified by many other gene products [51] that in individuals might differently affect disease severity. As an example, one such gene, *AHA1*, that modifies CFTR folding, when suppressed in the network context is able to reverse the protein CFTR misfolding and restore the chloride channel wild type phenotype [51].

When two nonessential (viable) gene deletions are combined in a double mutant, and the cells become unviable, this epistatic interaction is called *synthetic lethality* (SL) (Figure 16.4). In yeast, SL genetic interactions are highly informative because they indicate related functionality in the genes making up the double-mutant deletion pairs (Figure 16.4). *IRE1* and *HAC1* are known to be involved in the unfolded protein response (UPR) [52], and Figure 16.7 shows a large overlap of SL epistatic gene deletions, confirming the closely related functionality of *HAC1* and *IRE1*.



**Figure 16.7** Synthetic lethality networks reveal functional relationships. Genetic interaction network depicting the overlap of interactions identified from SGA analysis of the query genes *ire1D* and *hac1D*. Genes

are represented as nodes with colour annotated to gene function while edges represent a negative genetic interaction. (From Bircham *et al.* [53] from the Royal Society of Chemistry.)

SL combinations may be created *en masse* by a technique called *synthetic genetic array analysis* [54] in which a “query” gene knockout is matrix-mated against the other 5000 nonessential gene knockouts of the deletion mutant array (DMA). A query gene deletion is usually one of known function about which one wishes to establish its genetic interaction networks.

In a number of key studies, SL genetic networks have been constructed of overlapping SL pairs from eight query genes related to cytoskeleton [54], 132 query genes covering many other functions [55], 5 query genes related to histone acetyltransferases [56], and 1712 genome-wide query genes [57], the latter generating 5.4 million pairs of double mutants involving 75% of the yeast genome. Of all double mutant combinations in yeast assessed to date, 3.1% (about 170 000 interactions) display epistatic genetic interactions in fitness; that is, interactions are significantly more or less than the sum of the constituent mutants’ individual genetic effects [57]. From such analyses it is possible to gain insight into the function of unknown genes that are linked from one to several orders of SL links. An example



is the function imputed to the unknown LGE1 and other genes by network linkage analysis in a study of pleiotropic drug resistance [58].

Using special conditional mutants of essential genes, it is also possible to measure genetic interactions of essential gene knockouts [57]. Genomic and genetic interaction information elucidated by these methodologies is readily accessible on an extraordinarily useful curate database called the *Saccharomyces Genome Database* maintained by Stanford University.

Using methods similar to chemical genetic SL screening techniques (Figures 16.4 and 16.5), pairs of SMIs can be used to create SL combinations in the absence of mutations. This allows investigation of pathways that have essential gene products operating. For SMI screening, a chemical genetic approach to gene function ablation can be achieved by printing glass slides with an SMI array (10,800 compounds per slide) to affinity-isolate-tagged proteins from cell lysates [41, 59]. This approach generates not only interesting biological probes but also potential new therapeutics.

A chemical genetic approach has a number of advantages over a purely genetic interaction approach. SMIs work rapidly, they are reversible, and can be titrated in their “mutational” knockout allele effects; whereas, null mutations in general are irreversible and cannot be titrated. In SL genetic analysis, reversibility of mutations is limited to conditional and temperature-sensitive mutations. As mentioned in the preceding sections, essential gene mutations are difficult to work with, and use of SMIs allows a more facile study of essential gene interactions.

Most of the chemical genetic analyses described in the preceding sections involve looking for growth/no-growth phenotypes related to systematic gene knockouts created by gene deletions in the DMA or by gene product ablation using SMIs. With the advent of a genome-wide set of GFP-labeled yeast fusion proteins [60] (now commercially available), much more subtle (morphological) phenotypes can be studied as a function of perturbation by an SMI, or through a set of gene deletions using high-content automated microscopy. Such automated microscopy (for example, an EvoTec Opera – automated confocal microscope) allows identification of “texture changes” (EvoTec Opera datasheet, PerkinElmer) in intracellular morphology of particular GFP-labeled proteins and can be interpreted as unique phenotypes (by special image analysis algorithms) as a result of the deletion mutants [53, 61].

Genetic network analysis can lead to discovery of new basic biological pathways. To illustrate, during normal growth of yeast, about 30% of cell protein synthesis results in unfolded proteins [62], giving rise to a basal level of endoplasmic reticulum (ER) stress. There is a system of about 300 genes, called the *UPR system* (recently reviewed by Walter and Ron [52]), that routinely corrects the basal misfolds [63, 64]. We asked what happens when one deliberately overstresses the UPR by overloading the cell with proteins that are unable to fold properly – as is seen in numerous human diseases, including some cancers [65]. To mimic this situation, we overexpressed a mutant of carboxypeptidase (CPY\*) that (CPY\*)cannot

properly fold, but nonetheless the yeast cells still functioned, probably by saturating the secretory pathway and degrading CPY\* [66]. We then questioned which nonessential genes were necessary for the cells to function normally under these conditions by placing CPY\*-overexpressing cells on the yeast DMA genetic background and following the GFP-labeled secretory pathway reporters. In a novel finding, we identified a set of nonessential kinetochore genes that became necessary for the maintenance/expansion of the ER that occurs in CPY\* overexpression [61]. These genes by themselves were “nonessential” but when combined with the CPY\* UPR overload, they became essential for the secretory pathway and cellular function. This opened a unique window on cancer cells which are constantly under excess UPR ER stress, whereas normal cells are not. The implication is that if nonessential kinetochore gene products can be targeted with drugs in cancer cells, one should have a relatively specific cancer cell therapy. Currently, there are no known kinetochore drugs to test this hypothesis; however, future research may allow us to evaluate this concept further.

Thus, the use of chemical genetic techniques in yeast opens up a whole new level and depth of investigation of natural products, providing clues to mode of action that can later be translated to mammalian cells. Techniques in yeast are better able to deal with multiple pathways and provide a framework for network pharmacology of bioactive compounds that would not be possible using conventional screening methods in cultured mammalian cells. Although a primitive eukaryote, yeast has been shown to be a good model for human disease [67] and continues to be an essential element of drug evaluation in the search for new pharmaceuticals.

## 16.5

### Identification of Protein Targets by Proteomic Analysis on 2D Gels

To support the results of the DNA microarray analyses in yeast, effects of a lead compound on the proteome of cells can be investigated using 2D difference gel electrophoresis (2D-DIGE) [68]. Proteins are labeled with fluorescent CyDyes, and the abundances of separated proteins are compared on the basis of their fluorescence intensities. The protein spots on the gel are then cut out and identified by MS and database searching of their peptide fragments after chymotrypsin digestion. The RIKEN Institute in Japan has a ChemProteoBase program that matches changes in proteomic profiles from 2D-DIGE to known drug effects [69].

## 16.6

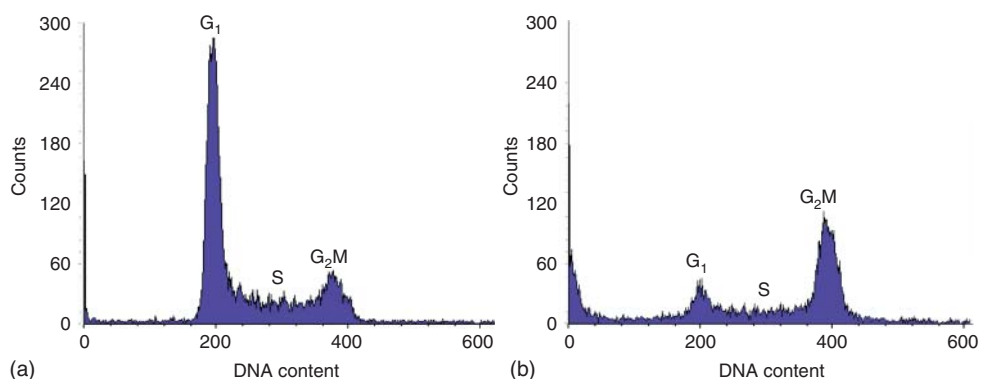
### Validation of Compound Targets by Biochemical Analysis

Even without chemical genetic analysis or proteomics to provide clues to the mode of action of a novel natural product, by examining the structure of novel compounds, a medicinal chemist can speculate on their mode of action. However,

caution needs to be taken as the skeletal chemical structure is not representative of the 3D conformation of the compound. For example, peloruside A has some structural similarities to the bryostatins, with both containing large ring structures in their core, a pyranose ring adjoining a *gem*-dimethyl moiety, and similar patterns of oxygenation [70]. The hemiketal center of the pyranose ring and the side chain of peloruside A were predicted to be part of a common pharmacophore also seen in the bryostatins, which activate protein kinase C. This was later found to be incorrect when further investigation revealed that peloruside A had no effect on the action of protein kinase C [70]. Tubulin was subsequently identified as the primary target of peloruside A [31].

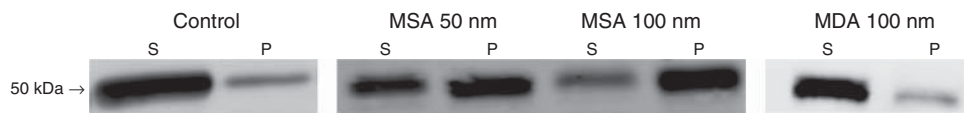
Multifunctional assays are carried out in whole animals, isolated organisms or tissues, and in cultured cells. These assays are not highly specific, and the observed effect cannot be directly linked to a single mechanism of action and generally include downstream responses. Historically, the use of panels of cell lines, such as the National Cancer Institute 60-cell line panel in the United States [71] or more recently the MorphoBase program at the RIKEN Institute in Japan [72] have shown some success in determining the nature of the target of a novel compound as different target dysfunctions lead to unique cell morphologies.

A large number of compounds target cell division machinery, and this can be detected by flow cytometry as a  $G_2M$  block (cells arrest in mitosis) (Figure 16.8). Arrest of the cell cycle in the  $G_1$  phase may indicate an inhibitor of cycle-dependent kinase proteins and arrest at the S phase a DNA-interfering agent such as 5-fluorouracil or doxorubicin. If a  $G_2M$  block occurs (Figure 16.9), then the compound of interest is most likely targeting either actin or tubulin. The dynamic polymerization/depolymerization of tubulin is necessary for chromosome separation, and actin polymerization is required for cytokinesis; however, it is clear



**Figure 16.8** HL-60 (human promyelocytic leukemia cells) cells stained with propidium iodide and analyzed by flow cytometry. (a) Control cells, majority of which appear

in the  $G_1$  resting phase. (b) Cells treated for 16 h with an MSA (8 nM); majority of cells are blocked in  $G_2M$  indicating a mitotic block.



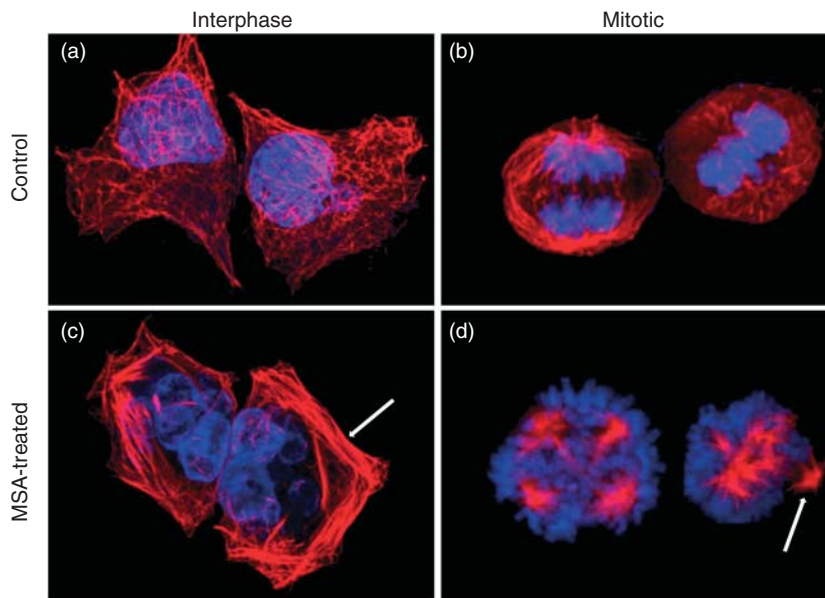
**Figure 16.9** *In situ* tubulin polymerization assay with 1A9 cells (human ovarian carcinoma). In control cells, the majority of the tubulin is in soluble form. Treatment with an MSA for 16 h results in a concentration-dependent shift of tubulin from the soluble fraction to the polymerization fraction, indicating stabilization of microtubules. Treatment with a microtubule-destabilizing agent (MDA) for 16 h results in a shift of tubulin into the soluble fraction, indicating tubulin depolymerization.

that drugs that target the microtubules can also target nonmitotic microtubule functions as well as mitotic function [73]. If a  $G_2M$  block is observed, there are two relatively straightforward assays to confirm if the target protein is tubulin or actin. *In situ* tubulin polymerization in cells quantifies the amount of soluble versus polymerized tubulin in control cells versus drug-treated cells using sodium dodecyl sulfate polyacrylamide gel electrophoresis and Western blotting. Depending on the cell line used, the control cells contain certain percentages of tubulin in either the soluble or pelleted fraction after cell lysis. For example, the myeloid leukemic cell line HL-60 contains about 50% soluble and 50% polymerized tubulin; whereas, the ovarian carcinoma cell line 1A9 contains about 90% soluble tubulin. If a compound polymerizes tubulin, then the tubulin will shift into the polymerized or pellet fraction. The opposite is true of a compound that destabilizes microtubules (MDA). Figure 16.9 shows the effect of zampanolide in this assay. We have used this assay to identify two new MSAs in our laboratory: peloruside A [31] and zampanolide [22]. Confocal microscopy can also be used to visually assess drug effects on microtubules and/or actin. By staining fixed cells with tubulin or actin antibodies or a fluorescent actin-binding compound such as phalloidin, the respective cytoskeleton of control or drug-treated cells can be observed. Paclitaxel an MSA and colchicine and vinblastine MDAs and latrunculin A (an inhibitor of actin polymerization) can be used as controls. Compounds that cause microtubule formation or stabilize microtubules may result in bundling or induce the formation of multiple mitotic asters (Figure 16.10), whereas compounds that depolymerize microtubules or actin will result in total loss of the respective protein networks (Figure 16.11).

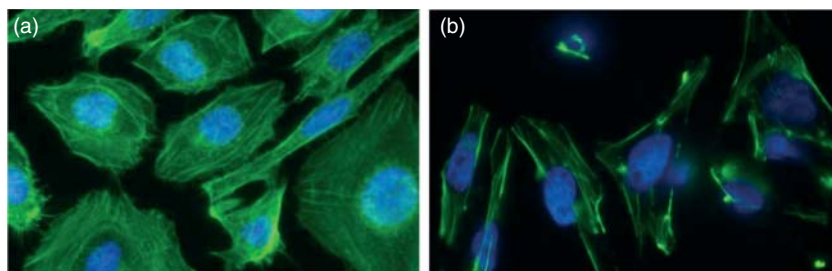
## 16.7

### Next Steps in Drug Development

Since natural products are made in small quantities by nature, supply becomes an important aspect in further development of the compound. Unless the compound can be easily synthesized, the only other way of obtaining a sufficient supply is by harvesting it from the natural source, and this is not a sustainable option.



**Figure 16.10** 1A9 human ovarian carcinoma cells (a–c) and HL-60 human promyelocytic leukemia cells (d) stained for anti- $\alpha$ -tubulin and labeled with anti-rabbit secondary antibody. MSA-treated cells (16 h) (c,d) show typical features of microtubule bundles in interphase cells (white arrow) and formation of multiple micronuclei (c). Mitotic cells show the formation of multiple asters (white arrow) (d).



**Figure 16.11** HeLa (human cervical cancer cells) stained with DAPI and phalloidin. Phalloidin binds F-actin filaments and prevents depolymerization. (a) Control cells and (b) cells treated with  $0.5\ \mu\text{M}$  latrunculin. Note the loss of actin staining. (Figure courtesy of Kirsten Meyer.)

For example, with compounds isolated from marine sponges, all the available sponges would need to be harvested in a particular collection area to isolate a clinically relevant amount of the compound; however, this has the potential to destroy the supply and thus is not a sustainable solution. One way around this is through aquaculture of the source organism to produce large enough

amounts of the compound, although this too has its drawbacks. Most sessile invertebrates are slow growing and unculturable; however, the pharmaceutical firm PharmaMar was able to aquaculture the tunicate, *Ecteinascidia turbinata*, to generate clinically relevant amounts of the tetrahydroisoquinoline trabectedin (ecteinascidin 743; Yondelis<sup>®</sup>), a potent anticancer drug [74]. In our case, a large-scale aquaculture of peloruside A was successfully set up, and large quantities of the producing sponge *M. hentscheli* were able to be grown. However, before the sponge was harvested, a population of nudibranchs destroyed most of the sponges being aquacultured [75]. This along with the fact that peloruside A is difficult to synthesize has meant that no further clinical research with peloruside A has begun despite promising primary *in vivo* results (unpublished data). If natural products prove useful and one wants to turn them into a drug, the best way to sustainably do this is to develop a scaled-up synthesis.

Although the synthesis of the parent compound is the initial step in further drug development, natural congeners or synthetic or semisynthetic analogs can also be studied and compared to the parent compound. Structure–activity relationships can help determine which regions of the compound are essential and which can be altered to make the analog more or less potent, more soluble, or less likely to cause unwanted side effects. For example, zampanolide is cytotoxic in the low nanomolar range; whereas, the closely related dactyloide, which lacks the side chain of zampanolide, is cytotoxic in the low micromolar range. This is due to the interaction of the zampanolide side chain with tubulin, causing more potent stabilization of the microtubule lattice [76].

After sufficient supply of a lead compound becomes available, the next step in development of anticancer compounds such as MSAs is to move into *in vivo* animal models, such as xenograft studies in mice, to compare the *in vivo* efficacy of the drug with current clinically used MSAs such as Taxol<sup>®</sup>, Taxotere<sup>®</sup>, and Ixempra<sup>®</sup>. In early studies, it is important to examine a compound's tolerability (maximum tolerated dose), its side effect profile, and its pharmacokinetics.

## 16.8

### Concluding Remarks

In conclusion, although targeted drug therapies and high-throughput, large library screens have taken over much of the bioactivity-based natural product screening of the past, the fact remains that the search for novel structures is still necessary to provide a database of unique compounds that may be used to develop new pharmaceutical leads for development of drugs. This is true whether targeting a general process such as mitosis, or targeting a specific pathway such as protein kinase or epidermal growth factor signaling.

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## Abbreviations

CFTR	cystic fibrosis transmembrane receptor
COSY	correlation spectroscopy
CPY*	carboxypeptidase mutant
2D-DIGE	2D difference gel electrophoresis
DAPI	4',6-diamidino-2-phenylindole
DMA	deletion mutant array
DIOL	2,3-dihydroxy-1-propoxypropyl derivatized silica
ER	endoplasmic reticulum
HMBC	heteronuclear multiple-bond correlation
GFP	green fluorescent protein
HTS	high-throughput screening
HSQC	heteronuclear single-quantum coherence
MSA	microtubule-stabilizing agent
MDA	microtubule-destabilizing agent
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenoyltetrazolium bromide
PSDVB	polystyrene-divinylbenzene copolymer
PCR	polymerase chain reaction
SL	synthetic lethality
SMI	small molecule inhibitor
UPR	unfolded protein response

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## 17

# Marine Natural Products: Biodiscovery, Biodiversity, and Bioproduction

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### 17.1

#### Introduction

Oceans represent a notable area of our planet and provide a range of valuable ecosystem services [1]. Their fundamental role is further noted by the substantial fraction of the Earth's biodiversity that oceans harbor. Current estimates of extant biodiversity in our planet point toward ~8.7 million species [2], with 2.2 million species inhabiting our oceans. Nevertheless, "only" 250 000 marine species are currently accepted [3], which suggests that nearly 90% of all extant marine species remain undiscovered. Indeed, while only one (phylum Onychophora) of all animal phyla presently known is not recorded in marine ecosystems, 15 animal phyla are exclusive to the world's oceans [4].

Marine biodiversity has been unanimously acknowledged as a valuable source of new chemical diversity [5]. Marine natural products (MNPs) have been continuously discovered throughout the world's oceans [6, 7] and providing an important source of new chemical entities that hold noteworthy leads for drug discovery and development [8]. These bioprospecting efforts have been driven by the urgent need for new chemical structures, as the diversity and abundance of MNPs displaying biological activity increases the chances of discovering new drugs [9]. It is also important to note that the number of drugs approved in the past decade is lower than that recorded in previous ones [10]. Furthermore, the number of infectious pathogens developing resistance toward antibiotics is increasing faster than the development of new drugs from bioactive natural products [11]. In this urgent quest for new drugs, it has been unanimously acknowledged that MNPs could be the "blue gold" that will even the odds [8, 12]. This statement is supported by the extremely rich marine biodiversity and primitive versions of human genetic systems present in marine organisms, which, unlike plants that have been the traditional source of antibiotics, hold particular promise for the development of new drugs [13].

The biodiscovery of MNPs has mostly been achieved through bioprospecting marine organisms in their local environment [14]. As taxonomical and geographical selection of bioprospected organisms is a critical step in MNP discovery [15, 16], different strategies have been used to efficiently and effectively survey chemical diversity [17]: (i) exploring untapped geographical regions, (ii) exploring different groups of marine organisms, or (iii) combining both of these sampling strategies. In this view, the assessment of chemical, taxonomical, and geographical trends on MNP discovery may provide important information for future bioprospecting efforts and maximize the success of finding new molecules that may eventually be used to feed the drug discovery pipeline. Beyond the importance of identifying the dominant taxa and regions being bioprospected for new MNPs, such information is also critical for nations worldwide willing to assess the potential that their marine environment may hold, particularly for the pharmaceutical industry.

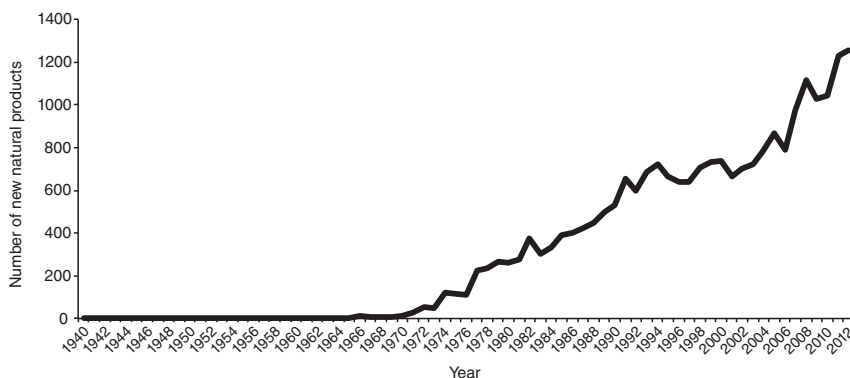
We herein address biodiscovery of MNPs, bioprospecting, and bioproduction processes of marine molecules potentially relevant for drug discovery and development. The biodiscovery of marine chemical diversity is assessed from an historical perspective, along with the trends of bioprospecting MNPs in relation to taxonomical groups and geographical regions. Such information is further used to estimate the potential of marine biodiversity to provide new chemical entities displaying bioactive features of great interest to the pharmaceutical industry. Particularly, the most-biodiverse and less-bioprospected taxonomical groups are noted, together with the biodiscovery of MNPs in extreme environments, such as the deep-sea and polar areas. Biogeography is defined here and its potential use as a tool for bioprospecting is summarized. Lastly, as the discovery of new MNPs is solely the first step towards the development of marine drugs, the production of biological material is also addressed. As the “supply issue” has been identified as a significant bottleneck for marine drug discovery [18], this topic is reviewed with emphasis on the loss of the source, replicability issues, and bioproduction of MNPs.

## 17.2

### **Biodiscovery: What and Where?**

The first MNP was reported in 1940 and concerned a pigment discovered in a sea anemone [19]. Since then, research on MNP chemistry has substantially been developed and the number of MNPs discovered from marine organisms has exponentially increased (Figure 17.1). By 1973, when the first review of MNPs was reported [20], a total of 191 new MNPs were already documented. This number has been rapidly increasing, and in 2013, a total of 23 570 MNPs have been reported from marine organisms [21].

It is important to note that although the first MNP was reported in 1940 [19], the initial increase of MNP biodiscovery was only recorded in the 1970s (Figure 17.1).



**Figure 17.1** Number of new marine natural products discovered between 1940 and 2012. (Data source: MarinLit [21].)

In the first 30 years (1940/70) only 50 MNPs were discovered, which is equivalent to 0.2% of all known MNPs. The biggest increase in the biodiscovery of new molecules (305%) occurred in the 1970s and 1980s. After the 1980s, the discovery of MNPs steadily progressed; the discovery of MNPs in the 1990s increased 177% as compared to the 1980s, and in the 2000s, the number of MNPs discovered was 144% higher than that of the 1990s. This trend is likely associated with the advent of modern snorkeling techniques and the introduction of SCUBA, which allowed sampling at deeper marine environments. While the first MNP originated from organisms sampled in intertidal areas, the advent of snorkeling equipment allowed researchers to sample up to 5 m deep. The subsequent generalized use of SCUBA diving gear made possible the collection of organisms at depths up to 30/40 m. The use of manned submersibles and, more recently, remotely operated vehicles (ROVs) further contributed to enhancing bioprospecting efforts in deeper marine environments [22]. Curiously, while deep-sea habitats are unanimously considered as a “premium” target for bioprospecting, the number of MNPs discovered in regions deeper than 50 m still represents a small proportion of all MNPs [23]. Technological developments in laboratory techniques and equipment, such as molecular biology and assays, high-throughput screening technology, combinatorial chemistry, nuclear magnetic resonance, and mass spectrometry, also prompted a notable increase in the discovery of MNPs [24].

### 17.2.1

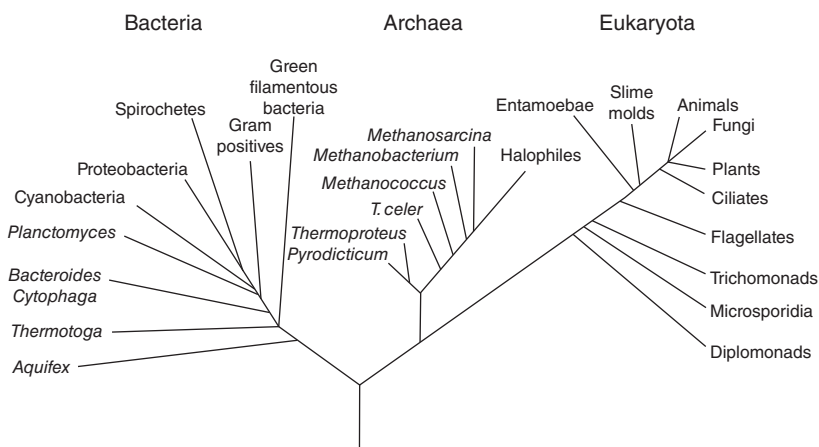
#### Taxonomic Trends

MNPs have been discovered in a large variety of organisms, ranging from bacteria to fish [25]. The biological taxonomy is first organized in three domains (Archaea, Bacteria, Eukaryota), as introduced by Woese and collaborators [26]. Living

organisms distribute along these three domains as depicted in Figure 17.2. Kingdom is the second highest taxonomic rank below domain, and the following kingdoms are currently recognized: Animalia, Archaea, Bacteria, Chromista, Fungi, Plantae, Protozoa, and Viruses [3]. The subsequent taxonomic ranks are Phylum, Class, Order, Family, Genus, and Species.

Natural product discovery from marine organisms have been focused on particular branches of the phylogenetic tree (Figure 17.2), particularly eukaryotic organisms, which account for over 90% of all MNPs discovered thus far. Furthermore, ~70% of all MNPs were discovered in animals [21], which notes the biased bioprospecting efforts toward macroorganisms, that is, organisms that can be observed without using equipment, such as dissecting or binocular microscopes. Indeed, 53% of all MNPs were discovered in sponges and cnidarians (Table 17.1), that is, phylum Porifera and Cnidaria, respectively. It is also important to note that although Chordata represent 4.5% of all MNPs, 1006 of such molecules are associated with ascidians (subphylum Tunicata), which are usually grouped together with marine invertebrates in this research field [6].

In spite of the dominance of organisms from particular phyla as source of new MNPs (Table 17.1), the trends in biodiscovery associated with each group have not been similar since 1940 (Figure 17.3). Sponges (phylum Porifera) notably contributed to the increase of MNPs discovered in the 1970s and 1980s. However, the yearly number of new MNPs associated with sponges in the 1990s and 2000s has been relatively similar and corresponds to  $278 \pm 36$  new MNPs year<sup>-1</sup> (average  $\pm$  standard deviation). In opposition to this trend, an increase in the number of MNPs discovered from cnidarians has been observed in the past decades [6]. Also notable is the increase of MNP biodiscovery in fungi, particularly from phylum Ascomycota, where the number of MNPs discovered since 2000 represent 91% of



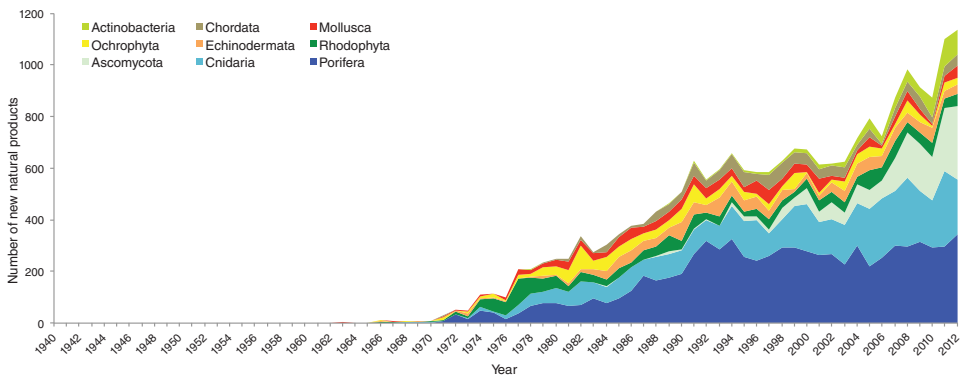
**Figure 17.2** Universal phylogenetic tree of life in rooted form showing the three domains and major branches.



**Table 17.1** Total number of marine natural products (MNPs) according to kingdom and phylum.

Kingdom	Phylum	Number of MNP
Animalia	Chordata	1 065
	Cnidaria	4 633
	Echinodermata	1 289
	Mollusca	1 080
	Porifera	7 874
Bacteria	Actinobacteria	624
	Cyanobacteria	474
Chromista	Myzozoa	286
	Ochrophyta	1 214
Fungi	Ascomycota	1 779
Plantae	Chlorophyta	258
	Rhodophyta	1 663
Other		1 214
Total		23 453

Only the phyla accounting for 95% of all MNPs are represented.  
Data source: MarinLit [21].



**Figure 17.3** Number of new marine natural products (MNPs) discovered from several phyla between 1940 and 2012. Only the phyla representing 90% of all known MNPs are represented. (Data source: MarinLit [21].)

all marine compounds isolated from this group. Similarly, 89% of all MNPs discovered from Actinobacteria were bioprospected after 2000. A contrasting trend was observed for marine algae, as only 36%, 27%, and 35% of the MNPs from phylum Rhodophyta, Ochrophyta, Chlorophyta, respectively, were discovered after 2000. These data indicate that bioprospecting efforts in the marine environment have been steadily shifting from algae to invertebrates and, lately, to microorganisms.

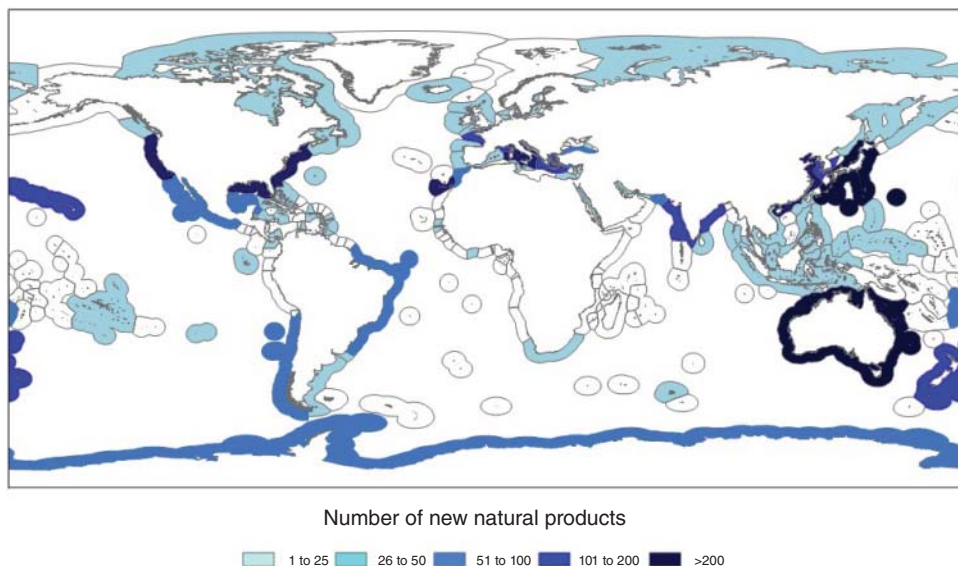
## 17.2.2

**Geographical Trends**

Geographical selection of sampling sites is a critical decision in MNP bioprospecting [9]. Collection sites must be carefully selected in order to combine high biological diversity and high density of target organisms. This will consequently maximize the number of species available to sample and minimize detrimental impacts to the collection site. The latter issue is further noted as impact assessment of the collection site is relevant when monitoring chemical diversity, as the loss of biodiversity associated with overexploitation and habitat degradation are primary marine conservation issues [27].

Despite the importance of a careful selection and consideration of the sampling site, a comprehensive dataset displaying chemical, taxonomic, and geographic information has been lacking. To the best of our knowledge, only three studies thoroughly assembled and analyzed geographical data associated with MNP biodiscovery [6, 7, 15]. One of these studies provided an in-depth analysis of biodiscovery hotspots for marine algae [7], while the other two assessed the geographical trends in the bioprospecting efforts of marine invertebrates during the 1990s and 2000s [6, 15]. Marine algae have been a historically important source of MNPs with relevant applications in cosmetic, nutraceutical, and pharmaceutical industries [28, 29]. The biodiscovery trends of MNPs from algae were thoroughly analyzed by Leal and collaborators [7]. This latter study grouped algal MNPs in different biogeographic classifications (marine ecoregions, biodiversity hotspots, and exclusive economic zones (EEZs)) in order to support biodiversity analysis and the understanding of underlying macroecological and phylogeographical trends. Such biological and ecological features may be noted by MNP data, as the biodiscovery of MNPs is likely to be associated with regions displaying high marine biodiversity or environmental properties that favor the evolutionary development of new secondary metabolites. Besides the chemical ecology of particular organisms or marine ecosystems, it is also important to consider the possibility that the geographic analysis of bioprospecting may reflect the preference of researchers toward particular species or sampling sites. For instance, the analysis of the biodiscovery of MNPs from algae associated with EEZs shows higher bioprospecting efforts in temperate areas surrounding developed countries, such as Japan, Australia, and China (Figure 17.4). These three countries, in addition to Italy, USA, and the EEZ of the Canary Islands (Spain) represented 44% of all algal MNPs discovered since 1965 [7].

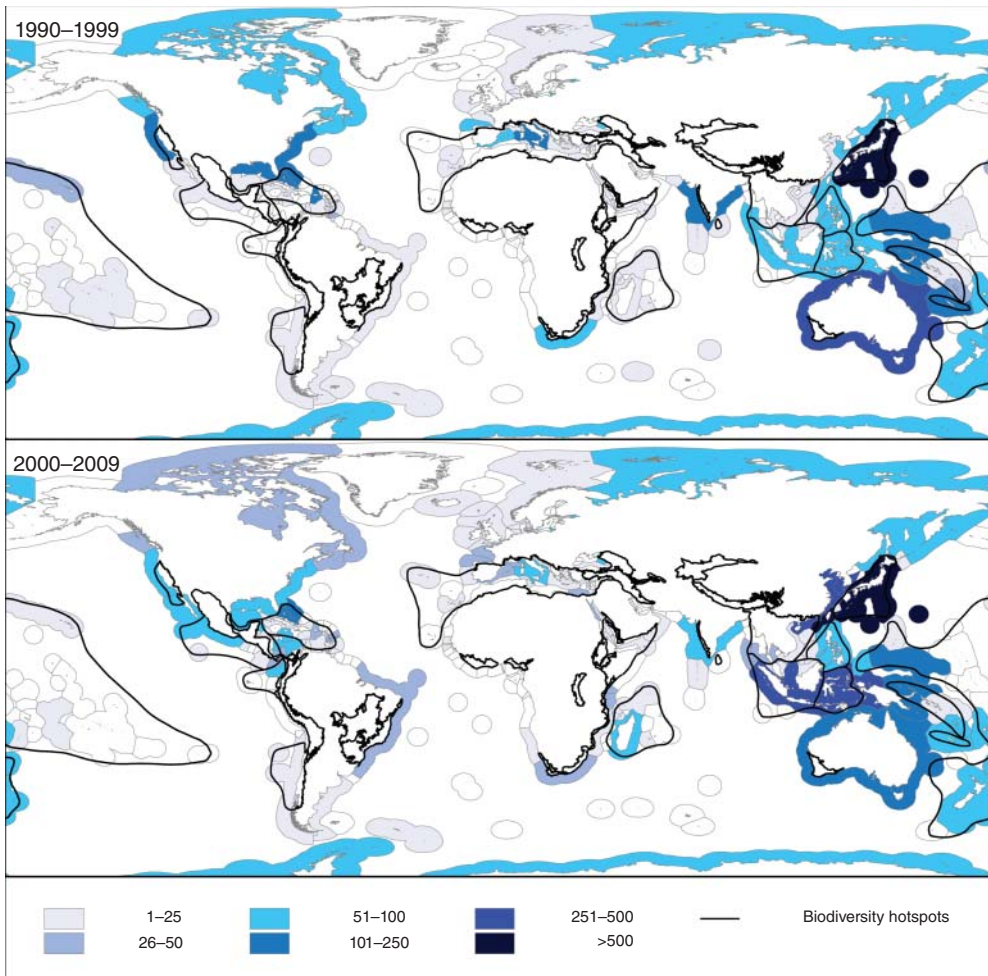
Distinct geographical trends are observed when comparing bioprospecting efforts associated with marine algae or invertebrates. While most algal MNPs were discovered in the United States, European countries enclosing the Mediterranean Sea, Australia, China, and Japan (Figure 17.4), the dominant countries in the discovery of MNPs from invertebrates were those in the Indo-Pacific, along with Japan and Australia (Figure 17.5). Indeed, Japan notably stands apart from



**Figure 17.4** New marine natural products discovered from algae between 1965 and 2012 for each exclusive economic zone. (Reproduced from Ref. [7] with permission from The Royal Society of Chemistry.)

all other countries, as it accounts for 17% of all MNPs discovered in marine invertebrates in the 1990s and 2000s [6]. In this same period, 40% of MNPs from invertebrates were discovered in Japanese, Taiwanese, Australian, South Korean, and Chinese EEZ (Figure 17.5). The largest increases over decades in MNPs from invertebrates were observed in the EEZ of China, Taiwan, Indonesia, and South Korea.

The overall picture of bioprospecting on marine invertebrates shows that most efforts have been focused on tropical coral reefs (Figure 17.5). These highly diverse, complex, and fragile ecosystems are the main habitat of a large number of invertebrate species, such as corals and sponges, which have been the main sources of new molecules [15]. Indeed, the high biodiscovery of MNPs from invertebrates in tropical regions contrasts with results observed from polar and temperate environments (Figure 17.5). A latitudinal theory suggesting an inverse relationship between latitude and chemical defense strategies in marine invertebrates has been hypothesized on the basis of geographical comparisons on early chemical ecology studies [30]. This theory follows the principle that chemical defense is primarily driven by predation pressure. As biological diversity is higher in the tropics, chemical diversity consequently increases in these regions. While intense bioprospecting efforts have not targeted extreme regions in polar areas, recent studies on marine organisms from Antarctica show that the levels of bioactivity in this particular region are comparable to those recorded in temperate and



**Figure 17.5** Number of new marine natural products from invertebrates for world exclusive economic zones during the 1990s and 2000s. Boundaries of biodiversity hotspots worldwide are also presented. (Reproduced from Ref. [6] with permission from the authors and PLOS.)

tropical marine environments [31]. Furthermore, recent sampling expeditions in the Southern Ocean revealed extremely high biodiversity levels across a wide range of taxa [32], with the number of new MNPs perspecies being similar in both tropical and polar regions [6]. This further supports the theory that polar regions, particularly Antarctica, may hold a remarkable potential for the biodiscovery of MNPs. The accessibility of sampling grounds may certainly explain some of the geographical trends currently recorded. It is easier to sample marine invertebrates in your coastal “backyard,” such as intertidal flats and shallow coral

reefs, as compared to remote areas with difficult access, such as polar regions and deep-sea habitats. The same principle can be applied to algae. The higher number of algal MNPs recorded in temperate regions (Figure 17.4) probably results from biased bioprospecting efforts by scientists screening this group of organisms in the coastal waters of temperate countries, such as Italy and Spain [7].

It is important to use biogeographic information to direct future bioprospecting efforts and maximize the chances of discovering new chemical diversity. This is particularly relevant because of our limited understanding on the dynamics of certain marine habitats, such as the open ocean and the deep sea. The findings provided by biogeographic analysis of chemical diversity may help researchers to focus or redirect their bioprospecting efforts toward less explored taxonomical groups or geographical regions, in order to maximize their chances of biodiscovery. Contrastingly, more conservative approaches are also possible, as researchers may want to use such information to concentrate their efforts in taxa and/or regions displaying high chemical diversity. Although the field of biogeography has been traditionally associated with biology and macroecology, applications to marine chemical ecology have been rare but successfully employed [6, 7] and are therefore encouraged.

## 17.3

### Biodiversity

#### 17.3.1

##### Exploring Marine Biodiversity

Biodiversity is the fundamental resource for discovering new chemical entities. While the importance of marine biodiversity for human society is already acknowledged at various levels, its true value as a source of new molecules with biotechnological applications (with emphasis to new pharmaceuticals) is likely underestimated. It will only be possible to merge bioprospecting interests and conservation efforts, along with social, ecological, and financial sustainability, by completely acknowledging the importance of marine biodiversity for industrial applications of any type.

While bioprospecting has been steadily increasing, thousands of species remain unscreened. Further, biodiscovery of MNPs has not been evenly distributed across marine taxa (Table 17.1). In fact, biodiscovery of MNPs has been extremely biased toward certain invertebrate groups, particularly sponges and cnidarians, probably due to the large number of species currently accepted within these phyla (Porifera: ~8300 species; Cnidaria: ~11 000 species) [3]. However, while researchers have been mainly focused on phylum Porifera and phylum Cnidaria, it is important to note that other groups of marine invertebrates are also specious, such as phylum Annelida (~12 500 species), Mollusca (~44 000 species), and Arthropoda (~56 000

species) [3]. This data highlights that the role of biodiversity in the discovery of new MNPs and as a driver of bioprospecting efforts still requires further research. It is also important to note that the low number of MNPs described from annelids, molluscs, and arthropods in relation to sponges and cnidarians is likely to be associated with their life traits. Several marine invertebrates, particularly from phylum Porifera and Cnidaria, are sessile and soft bodied, and therefore must rely on chemical defences tuned through their evolutionary history to deter predators, keep competitors away, or paralyze their prey [9, 33]. This is particularly relevant in tropical reefs, where large numbers of sponge and cnidarian species occur, and chemical interactions play a key role in interspecific competition. Organisms inhabiting highly biodiverse tropical areas, particularly coral reefs, developed an array of chemical compounds that have been the focus of recent bioprospecting efforts [8]. Contrastingly, while Arthropod species such as crustaceans are extremely diverse, they often display high mobility and do not rely on chemical “weapons” to paralyze prey and deter predators. Phylum Mollusca, for instance, is also an extremely diverse group that includes bivalves, cephalopods, and gastropods, among others. While cephalopods are extremely mobile, bivalves and most gastropods rely on hard shells to gain protection from predators. However, sea snails within genus *Conus* are known to produce powerful toxins and sea slugs, particularly from order Nudibranchia, are soft-bodied species with potent chemical weapons that have been an important source of new MNPs [34].

Marine bioprospecting efforts have been focused on less than 1% of all invertebrate species [6] and less than 3% of all macroalgae [7]. The extant biodiversity yet to be screened does not necessarily correspond to broadcast bioprospecting efforts to other groups of marine organisms. Researchers have actually shifted their focus toward microorganisms and specific invertebrate groups [6, 35]. For instance, bioprospecting efforts during the 2000s showed an increasing preference for cnidarians instead of sponges [6]. Nearly 3000 new MNPs have been isolated from corals in the past two decades and promising leads for drug discovery were identified [16]. However, MNP discovery has been uneven among coral species, as only about 3% of MNPs from anthozoans were yielded from stony corals (Scleractinia) [21]. Although the number of species within the Alcyonacea (soft corals) is twice that of stony corals [3], it is likely that the larger number of MNPs currently known from soft corals is rather a consequence of biased bioprospecting efforts: ~30 versus ~300 species of stony and soft corals, respectively [21]. Contrastingly, the percentage of bioactive bacterial isolates associated with stony corals is relatively higher [36]. The tissue, skeleton, and mucus of these corals contain dense and diverse populations of microorganisms that provide diverse benefits to their cnidarian host, such as support to coral nutrition and, most importantly, protection against pathogens. These symbiotic microorganisms display a diverse bioactivity and have provided promising leads for drug discovery (e.g., cytotoxicity, antibiotic activity, and activity against human tumor cell lines) [21]. Such findings

are in line with the growing awareness that MNPs discovered in several invertebrate species may in fact be produced by their symbiotic microbiota [10].

### 17.3.2

#### Protecting Marine Biodiversity

As previously noted, marine biodiversity is a critical driver of chemical diversity. Therefore, conservation efforts to protect marine biodiversity are extremely valuable from a pharmaceutical point of view in order to protect resources with great potential for future biotechnological applications. It is also important to preserve species with potential for drug discovery that are yet to be discovered, as marine organisms currently face an unprecedented rate of extinction due to anthropogenic activities impacting marine habitats [37].

Another important topic associated with marine biodiversity and its bioprospecting for chemical diversity is biopiracy. Worldwide marine regions are politically divided through EEZs, which are areas over which a state has jurisdiction concerning the exploration and use of marine resources present therein. However, the discovery of a particular molecule in a given EEZ does not necessarily mean that the country that has jurisdiction over that area performed the bioprospecting and screening efforts. This issue has raised a growing awareness in recent years due to biopiracy and benefit-sharing issues [38, 39]. Indeed, a large number of scientific publications can be found where the researchers describing a new MNP are not affiliated with the country where the sampling was performed. Tropical countries, where most bioprospecting efforts have been performed (Figures 17.4 and 17.5), are often developing countries without technical expertise and resources available for major breakthroughs in marine chemistry and pharmacology. Nonetheless, a decreasing trend in the discovery of new MNPs has been observed in some tropical countries within high-biodiversity regions, particularly in Oceania [40, 41]. Two hypotheses can be discussed to interpret such results. The first is associated with growing restrictions that block external researchers from accessing biodiversity within many countries. For instance, the state of Queensland, Australia, which encompasses the Great Barrier Reef and contains the country's highest levels of biodiversity, implemented the *Biodiscovery Act 2004* to encourage the “development in the State of value added biodiscovery” and “ensure that the State obtains a fair and equitable share in the benefits of the biodiscovery” [42]. All projects related to bioprospecting in this region currently have local partners, at least to provide access to local biodiversity. This legislation decreases the chances of foreign countries to sample biological material within the Australian EEZ, particularly in Queensland, without sharing their findings and potential profits with the Australian government. Secondly, the restriction of trawling activities in many habitats may have also contributed to a decrease in bioprospecting efforts in tropical areas beyond shallow coral reefs. Benthic trawling is a relatively easy method to collect invertebrates from the sea floor,

as biological material is easily captured and brought to the surface. However, increasing limitations have been applied to trawling activities, particularly when bottom trawling is employed in areas known for their high biodiversity [43]. Thus, sampling in deeper habitats became more difficult as the access to specialized technology to explore the deep sea, such as submersibles and ROVs, is still an expensive and somewhat limited option.

The analysis of biodiversity and chemical diversity explored in marine regions is therefore a potential tool to alert nations worldwide of the potential value of their marine resources. The world's oceans have been shown to be the last stronghold of global biodiversity, a feature commonly overlooked by human society. By recognizing the importance of marine biodiversity to bioprospecting, developing nations may find an extra motivation to advocate and enforce marine conservation policies.

#### 17.4

##### **From Biodiscovery to Bioproduction**

While the number of new MNPs discovered has been exponentially increasing in the past decades, the number of marine-derived molecules that reach the drug discovery pipeline and are ultimately commercialized is dramatically smaller. The global marine pharmaceutical pipeline currently includes only 8 approved drugs, 12 MNPs in different chemical phases, and a large number of molecules from marine organisms in preclinical trials [24]. One of the bottlenecks acknowledged to affect the success of marine drug discovery is the availability of sufficient material for complete biological and chemical evaluation and eventual production [13, 14].

To a certain degree, the search for new MNPs still relies on the harvest of wild specimens. This is a major constraint for the development of new marine drugs [8, 13] as it commonly involves two major bottlenecks: sustainability and replicability [44]. Large amounts of biomass of the source organism can be required to provide milligrams to grams of pure compound to fuel the drug discovery process [14]. Fortunately, the unsustainable harvest of marine organisms for drug discovery is no longer taking place [13, 45]. The intensive and unregulated collection of target organisms to deliver the amount of metabolite required for drug development is likely to severely impact benthic communities and rapidly become an unsustainable practice. The same rationale can be applied when bioprospecting targets are naturally rare in the collection site and/or are already endangered. Replicability issues are associated to the effect of environmental variability and changes at the community level that may affect the chemical ecology of these organisms [46]. Individuals of the same species collected in different regions and/or periods may not display the same chemical composition [47] and consequently fail to assure the supply of a target compound (a pitfall



commonly termed as *loss of the source*), which causes a replicability issue. This is also a potential caveat for the original detection of bioactive metabolites, as environmental and individual variability in the chemical composition of target organisms may affect screening results [44]. Another constraint associated with replicability issues is the potential loss of the source associated with the extinction of the target species. This is certainly relevant in the oceans of today and tomorrow, as vulnerability to extinction in marine ecosystems is predicted to be higher in tropical coral reefs [40, 48], which are important bioprospecting hotspots [6].

The relatively low natural abundance of bioactive metabolites that is often recorded in marine organisms [49] is usually not a drawback for the initial steps of the drug discovery pipeline, as only small amounts of biomass are commonly required. Nevertheless, while the amount of pure compound required for screening an isolate is usually lower than 1 mg, an increase in several orders of magnitude of target compound quantity (e.g., a few grams) is needed to progress toward preclinical trials [14]. As the metabolite progresses through clinical trials and reaches commercialization, kilograms of the pure compound are required to supply drug production, which likely corresponds to an annual capture or production of several tons of the target organism [14, 50]. The challenges associated with the harvest of target organisms to yield such quantities of a given pure compound unsurprisingly triggers supply issues that increase once the compound progresses into later development stages of the drug discovery pipeline [51]. The production of these molecules at a scale large enough to fulfill the needs of drug discovery and potential commercial applications is, therefore, a major issue [41, 51].

Current R&D strategies of the pharmaceutical industry to overcome this supply problem are largely associated with the development of synthetic or semisynthetic analogs, as well as with the design of molecules displaying a lower complexity and a similar bioactive function that are possible to synthesize using standardized techniques [50]. This alternative, however, has several constraints, particularly the noteworthy complexity of certain marine molecules (most are chiral and display intricate structures) that is very difficult, and sometimes impossible, to replicate in the laboratory [51, 52]. Furthermore, the large number of steps often required to produce such synthetic analogs, together with the notable number of misassigned products, commonly represent an extreme financial burden that most drug discovery companies are not willing to support [53]. It is therefore undeniable that the “supply problem” is at the center of the main constraints impairing drug discovery from the marine environment [41, 44, 51]. However, the interest in the remarkable properties of MNPs remains appealing enough to inspire innovative solutions to the “supply problem” [41]. Among the potential solutions for such bottlenecks, the use of alternative approaches, such as *in toto* aquaculture of the holobiont [45] and the culture of symbiotic microorganisms present in the microbiome of invertebrates [54], are certainly promising approaches. For instance, coral aquaculture offers a strong potential

for both the discovery of highly diverse metabolites and the production of large amounts of bacterial biomass [45]. The latter study shows that the calculated costs for metabolite biomass production through coral aquaculture are relatively low, representing only 3.5–15.0% of total preclinical development costs. Ultimately, the aquaculture of marine organisms, particularly invertebrate species, may hold an untapped potential for drug discovery.

Aquaculture may indeed overcome two key bottlenecks: replicability and sustainability. This approach may continuously provide metabolite biomass from organisms exposed to the same environmental conditions [55]. Aquaculture may also be of particular importance to promote the continuous presence of bioactive metabolite-producing symbiotic microorganisms associated with invertebrates through the maintenance of consistent environmental conditions. This recognition led to various efforts targeting sponge aquaculture, but with limited success [56]. Contrastingly, coral aquaculture is well established and allows the large-scale production of monoclonal organisms [57, 58]. Curiously, to date, coral aquaculture has hardly been applied for drug discovery research.

Current aquaculture methods available are mariculture (*in situ*) and captive breeding (*ex situ*) [59]. Micropropagation through tissue explants is also a versatile *ex situ* approach [60]. Both *in situ* and *ex situ* culture methods have advantages and disadvantages [61]. While *in situ* aquaculture completely relies on the marine environment to supply important ingredients that promote biomass development, human resources are only needed to build and maintain infrastructures, to stock marine organisms in the beginning of the culture process, and harvest them at the end of the production cycle. However, the ability to manipulate culture conditions *in situ* is limited to species selection and location of the cultivation site. In contrast, while *ex situ* methods have higher economic costs associated with equipment and maintenance, they allow the manipulation of abiotic and biotic factors in order to maximize productivity and biomass production, as well as the yield of the target metabolite [57, 62]. Furthermore, *ex situ* culture allows for a better control of the cultured genotypes as it allows genetic selection of specimens yielding a higher production of the target metabolite.

## 17.5

### Concluding Remarks

Overall, the global biodiversity of the world's oceans is yet to be properly valued by human society. It is critical to preserve areas that harbor great biodiversity potential, as well-regulated and conscientious bioprospecting contributes to merge goals of ecosystem conservation, socioeconomic development through partnerships, and benefit sharing. The true potential of several groups of marine organisms, particularly invertebrates, is still largely overlooked, as several of them

are suitable targets for ongoing efforts to develop new products from the sea, particularly new drugs. It is therefore important that nations holding these genetic resources benefit from potential economic revenues associated with bioprospecting activities, and advance toward social and ecological sustainability.

The analysis of biodiscovery, biodiversity, and bioproduction of MNPs allow us to conclude that although new technologies have promoted significant advances in the collection, screening, and identification of a whole new range of molecules, marine chemical ecology is still several decades behind its terrestrial counterpart [31]. New analytical spectroscopy technologies have pushed the limits of observation, so that discovery of new molecules requires only a few micrograms of biomass, a threshold remarkably lower than that required only 10 years ago to perform such analysis [41]. These and additional technological breakthroughs are likely to enhance the biodiscovery of MNPs, as in the years to come, a small amount of animal biomass is expected to allow the screening of even more molecules than those we are able to record at present.

Advances in aquaculture techniques may also play a key role in the solution of some of the critical challenges faced by the pharmaceutical industry, as they can secure a constant and reliable supply of target biological material. Aquaculture may not be the definitive solution to solve the supply issue in marine drug discovery from bioprospecting to drug commercialization; however, it might be a suitable approach for the initial steps of the drug discovery pipeline, that is before clinical trials, when the use of synthetic or semisynthetic alternatives may not be technically and/or financially suitable.

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