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# Marine Pharmacognosy

Trends and Applications

Edited by **Se-Kwon Kim**



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# Preface

Marine-derived bioactive compounds offer an abundant source of pharmacologically active agents with great chemical diversity and complexity and the potential to produce valuable therapeutic entities. The growing need for novel bioactives for the treatment of severe human diseases, such as cancer, microbial infections, and inflammatory processes, combined with the recognition that marine organisms provide a rich potential source of such substances, demands intensive research for new pharmaceutically active substances from marine organisms. Marine macroalgae, microalgae, blue-green algae, invertebrates, vertebrates, and marine-derived microorganisms are rich sources of pharmaceutically active compounds and have been recognized in traditional Chinese medicine since ancient times. Secondary metabolites like phlorotannins, phenolics, alkaloids, and other bioactive compounds such as fucoxanthin, sulfated polysaccharides, bioactive peptides, chitooligosaccharides from chitin and chitosan, and so on are well recognized for their pharmaceutical benefits.

With valued contributions from world-leading experts in Korea, Japan, China, UK, Germany, Egypt, Turkey, Sri Lanka, India, Vietnam, and Indonesia, this book provides a comprehensive account of marine-derived bioactive pharmaceuticals and their potential beneficial effects such as antioxidant, anticancer, antiviral, anticoagulant, antidiabetic, antiallergy, anti-inflammatory, anti-hypertensive, antibacterial, and radioprotective activities. Moreover, it discusses the sources, isolation and purification, chemistry, functionality interactions, applications, and industrial perspectives of a variety of marine-derived pharmaceuticals. The book may be used as a text or reference for fellows in medicinal chemistry, pharmacognosy, food chemistry, and health sciences at the senior undergraduate and graduate levels. Scientists in academia, research laboratories, marine biochemistry, natural products sciences, and industry will also find it of interest.

I am grateful to the experts who have provided state-of-the-art valued contributions to this book, and I am also grateful to CRC Press for the successful completion of this book.

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# Editor

**Prof. Se-Kwon Kim, PhD**, is currently working as a senior professor of marine biochemistry in the Department of Chemistry and is the director of the Marine Bioprocess Research Center at Pukyong National University in Republic of Korea. He has received his BSc, MSc, and PhD degrees from the Pukyong National University and joined as a faculty member. He served as a scientist in the University of Illinois at Urbana-Champaign, Illinois (1988–1989) and became a visiting scientist at the Memorial University of Newfoundland in Newfoundland, Canada (1999–2000).

He served as president for the “Korean Society of Chitin and Chitosan” (1986–1990) and for the “Korean Society of Marine Biotechnology” (2006–2007). He was also the chairman for the Seventh Asia-Pacific Chitin and Chitosan Symposium, which was held in South Korea in 2006. He is one of the board members of the “International Society of Marine Biotechnology” and “International Society for Nutraceuticals and Functional Foods.” Moreover, he was the editor-in-chief of the *Korean Journal of Life Sciences* (1995–1997), the *Korean Journal of Fisheries Science and Technology* (2006–2007), and the *Korean Journal of Marine Bioscience and Biotechnology* (2006–present). He won the best paper award from the American Oil Chemist’s Society and the Korean Society of Fisheries Science and Technology in 2002.

His major research interests are investigation and development of bioactive substances derived from marine organisms and their application in Asian medicine, nutraceuticals, and cosmeceuticals through marine bioprocessing and mass production technologies. Furthermore, he expanded his research into the development of bioactive materials from marine organisms for applications in Asian medicine, cosmeceuticals, and nutraceuticals. To date, he has authored over 500 research papers and holds 110 patents. In addition, he has written or edited more than 40 books.



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# 1 Past, Present, and Future of Marine Pharmacognosy

*Se-Kwon Kim*

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### 1.1 INTRODUCTION TO MARINE PHARMACOGNOSY

Marine organisms are rich sources of structurally diverse bioactive compounds with various biological activities, and their importance as a source of novel bioactive substances is growing rapidly. Marine natural products offer an abundant source of pharmacologically active agents with great chemical diversity and complexity and the potential to produce valuable therapeutic entities. The realization of this potential through the recent approval of two marine natural products has taken many decades (Glaser and Mayer 2009). With marine species comprising approximately half the total global biodiversity, the sea offers an enormous resource for novel pharmaceuticals. Recently, their value as a source of novel bioactive substances has grown rapidly and researchers have revealed that marine-originated compounds exhibit various biological activities. Although marine organisms are exposed to adverse environmental conditions such as light and high-oxygen concentrations that lead to the formation of free radicals, and other strong oxidizing agents, they do not undergo any serious photodynamic damage. Thus, it is known that marine organisms are able to generate bioactive compounds to protect themselves from external factors such as ultraviolet (UV) radiation, stress, and herbivores. Recently, scientists have explored various health-beneficial pharmaceutical bioactives from marine bioresources such as macroalgae, microalgae, fungi, bacteria, actinomycetes, invertebrates, and vertebrates. Among marine organisms, marine algae are rich sources of bioactive compounds with various biological activities. Moreover, marine-derived fungi have become an important source of novel pharmacologically active secondary metabolites and are considered as a rich source of bioactive compounds with medicinal and pharmaceutical benefits. This chapter discusses the recent trends and findings associated with novel pharmaceutical bioactives from marine organisms.

## 1.2 SOME POTENTIAL MARINE-DERIVED PHARMACEUTICAL INGREDIENTS

### 1.2.1 SECONDARY METABOLITES

Phlorotannins are phenolic compounds formed by the polymerization of phloroglucinol or defined as 1,3,5-trihydroxybenzene monomer units and biosynthesized through the acetate–malonate pathway. They are highly hydrophilic components with a wide range of molecular sizes ranging 126–650,000 Da. Marine brown algae accumulate a variety of phloroglucinol-based polyphenols, as phlorotannins could be used as functional ingredients in nutraceuticals with potential health effects (Wijesekara, Yoon, and Kim 2010). Among marine algae, *Ecklonia cava*, an edible brown algae, is a rich source of phlorotannins compared to others. Phlorotannins have several biological activities that are beneficial to health including antioxidant, anti-HIV, antiproliferative, anti-inflammatory, radioprotective, antidiabetic, anti-Alzheimer's disease, antimicrobial, and anti-hypertensive activities.

Marine organisms produce a wide array of fascinating terpenoid structures distinguished by characteristic structural features. Certain structural classes, for example, cembrane, chamigrene, amphilectane skeletons, and unusual functional groups such as isonitrile, isothiocyanate, isocyanate, dichloroimine, and halogenated functionalities, occur predominantly in marine metabolites. In the previous two decades, natural-product bioprospecting from the marine environment has resulted in hundreds of terpenoids with novel structures and interesting bioactivities, with more to be discovered in the future. These terpenoids display a wide range of biological activities against cancer, malaria, inflammation, and a variety of infectious diseases (viral and bacterial). Furthermore, marine fungi contain a pronounced degree of structurally diversified terpenoids such as monoterpenes, sesquiterpenes, diterpenes, sesterterpenes, triterpenes, steroids, and tetraterpenes. Meroterpenoids are most often isolated from fungi and marine organisms, but bacteria and higher plants produce such mixed biosynthesized products as well.

### 1.2.2 BIOACTIVE PEPTIDES

Bioactive peptides usually contain 3–20 amino acid residues, and their activities are based on their amino acid composition and sequence. These short chains of amino acids are inactive within the sequence of the parent protein but can be released during gastrointestinal digestion, food processing, or fermentation. Marine-derived bioactive peptides have been obtained widely from enzymatic hydrolysis of marine proteins (Kim and Wijesekara 2010), and they have been shown to possess many physiological functions, including antioxidant, antihypertensive or angiotensin-converting enzyme (ACE) inhibition, anticoagulant, and antimicrobial activities. In fermented marine food sauces such as blue mussel sauce and oyster sauce, enzymatic hydrolysis has already been done by microorganisms, and bioactive peptides can be purified without further hydrolysis. In addition, by-products of marine processing contain bioactive peptides with valuable functional properties (Kim and Mendis 2006). Marine-derived bioactive peptides have been shown to possess many physiological functions, including antihypertensive or angiotensin-I-converting enzyme inhibition, antioxidant, anticoagulant, and antimicrobial activities. Moreover, some of these bioactive peptides have been identified to posses nutraceutical potential for human health promotion and disease risk reduction (Shahidi and Zhong 2008) and, recently, the possible roles of food-derived bioactive peptides in reducing the risk of cardiovascular disease have been demonstrated (Erdmann, Cheung, and Schroder 2008).

### 1.2.3 SULFATED POLYSACCHARIDES

In recent years, various sulfated polysaccharides (SPs) isolated from marine algae have attracted much attention in food, pharmaceutical, and cosmetic industries. The SPs comprise a complex group

of macromolecules with a wide range of important biological activities. These polymers are chemically anionic and are widespread not only in marine algae but also in animals such as mammals and invertebrates. Marine algae are the most important source of nonanimal SPs, and the chemical structures of SPs vary according to the species of algae such as fucoidan in brown algae (Phaeophyceae), carrageenan in red algae (Rhodophyceae), and ulvan in green algae (Chlorophyceae) (Costa et al. 2010). These SPs exhibit various biological activities that are beneficial to health such as anti-HIV-1, anticoagulant, immunomodulating, and anticancer activities.

#### **1.2.4 CHITIN, CHITOSAN, AND CHITOOLIGOSACCHARIDE DERIVATIVES**

Chitin is the second most abundant biopolymer on Earth after cellulose and one of the most abundant polysaccharides. It is a glycan of  $\beta$  (1→4)-linked *N*-acetylglucosamine units and it is widely distributed in crustaceans and insects as the protective exoskeleton and cell walls of most fungi. Chitin is usually prepared from the shells of crabs and shrimp. Chitosan, a partially deacetylated polymer of *N*-acetylglucosamine, is prepared by the alkaline deacetylation of chitin (Kim, Nghiep, and Rajapakse 2006). It is noted that COSs are chitosan derivatives (polycationic polymers principally comprising glucosamine units) and can be generated via either chemical or enzymatic hydrolysis of chitosan. Recently, COSs have been the subject of increased attention in terms of their pharmaceutical and medicinal applications (Kim and Rajapakse 2005), due to their missing toxicity and high solubility as well as their positive physiological effects such as antioxidant, ACE inhibition, antimicrobial, anticancer, antidiabetic, hypcholesterolemic, hypoglycemic, anti-Alzheimer's, anticoagulant, and adipogenesis inhibition properties.

#### **1.2.5 CAROTENOIDS AND OTHER PIGMENTS**

Carotenoids are a family of pigmented compounds that are synthesized by plants, algae, fungi, and microorganisms, but not animals. They are the most important pigments in nature and are responsible for the various colors of different photosynthetic organisms. Carotenoids are thought to be responsible for beneficial properties in preventing human diseases including cardiovascular diseases, cancer, and other chronic diseases (Agarwal and Rao 2000).

#### **1.2.6 STEROLS**

All eukaryotes universally contain large amounts (20%–30%) of higher sterols in their plasma membranes. Different eukaryotic kingdoms have different higher sterols for membrane reinforcement, such as cholesterol in animals, ergosterol in fungi, and phytosterols in plants. Phytosterols (plant sterols) are triterpenes, and most of them contain 28 or 29 carbons and 1 or 2 carbon–carbon double bonds, typically one in the sterol nucleus and sometimes a second one in the alkyl side chain. Phytosterols have received much attention in the last few years because of their cholesterol-lowering properties.

### **1.3 PHARMACOLOGICAL ACTIVITIES OF MARINE-DERIVED PHARMACEUTICALS**

#### **1.3.1 ANTIOXIDANT ACTIVITY**

Antioxidants may have a positive effect on human health as they can protect the human body against damage by reactive oxygen species (ROSSs), which attack macromolecules such as membrane lipids, proteins, and DNA and lead to many health disorders such as cancer, diabetes mellitus, and neurodegenerative and inflammatory diseases with severe tissue injuries. Marine-derived pharmaceutically active compounds show promising antioxidative effects.

### 1.3.2 ANTICANCER ACTIVITY

Cancer is a leading cause of mortality around the world. Every year, over 7 million people die of cancer, which is 12.5% of total deaths worldwide. More than 70% of all cancer deaths occur in low- and middle-income countries, where health-care resources for prevention, diagnosis, and management are very limited. Over 40% of cancer is probably prevented by eating a healthy diet, exercising regularly, and quitting (or not starting) smoking. Marine-derived bioactives show excellent effect against cancer cell proliferation.

Cancer chemoprevention refers to the use of agents to block, inhibit, or reverse development of cancer in normal or preneoplastic tissue. Most chemical carcinogens require metabolic activation by phase I enzymes in order to induce a biological response. Induction of phase II drug-metabolizing enzymes such as GST, QR, or mEH is considered a major mechanism of protection against chemical stress and initiation of carcinogenesis. Critical DNA sequences are frequently found singly or multiply in the promoters of these genes, including antioxidant response elements (AREs) and xenobiotic-responsive elements (XREs). Two underlying mechanisms, the aryl hydrocarbon receptor (AhR)-XRE and nuclear factor erythroid 2-related factor (Nrf2)-ARE signaling pathways, are involved in the induction of Phase II enzymes. Numerous phytochemicals derived from marine organisms have been reported to interfere with a specific stage of the carcinogenic process. Many mechanisms have been shown to account for the anticarcinogenic actions of dietary constituents, but attention was recently focused on intracellular-signaling cascades as common molecular targets for various chemopreventive phytochemicals.

Apoptosis is programmed cell death in response to a variety of stimuli, and it is usually characterized by a distinct set of morphological and biochemical progress. However, deregulation of apoptosis can disrupt the delicate balance between cell proliferation and cell death, which can lead to diseases such as cancer. Most cancer cells block apoptosis via antiapoptotic signaling pathways in order to survive despite undergoing genetic and morphological transformations. Therefore, drugs that promote apoptosis may be effective against many cancers and should become an important strategy to counteract cancer. Apoptotic cells are characterized by certain morphologic features, including condensation of the cytoplasm and the nucleus, cell surface expression of phosphatidylserine, and internucleosomal cleavage of DNA. It has been shown that marine-derived pharmaceuticals are effective apoptosis enhancers in numerous human cancer cell lines.

### 1.3.3 ANTIVIRAL ACTIVITY

The potential antiviral activity of marine algal polysaccharides was first shown by Gerber et al. (1958), who observed that the polysaccharides extracted from *Gelidium cartilaginem* (Rhodophyceae) protected embryonic eggs against influenza B or mumps virus. The polysaccharides with antiviral activity were shown to be highly sulfated (Huheihel et al. 2002). Many species of marine algae contain significant quantities of complex structural SPs that have been shown to inhibit the replication of enveloped viruses including members of the flavivirus, togavirus, arenavirus, rhabdovirus, orthopoxvirus, and herpesvirus families (Witvrouw and De Clercq 1997). The chemical structure including degree of sulfation, molecular weight, constituent sugars, conformation, and dynamic stereochemistry of algal sulfated polysaccharides is used to determine their antiviral activity. In addition, both the degree of sulfation and the distribution of sulfate groups on the constituent polysaccharides play an important role in the antiviral activity of these SPs. Algal polysaccharides with low degrees of sulfation are generally inactive against viruses.

### 1.3.4 ANTICOAGULANT ACTIVITY

After the investigation of blood anticoagulant properties of marine brown algae (Killing 1913), it has been reported that SPs derived from marine algae are alternative sources for the manufacture

of novel anticoagulant drugs (Church et al. 1989; Matsubara 2004). Anticoagulant activity is among the most widely studied properties of SPs (Costa et al. 2010), and anticoagulants from marine algae have previously been reviewed in the literature (McLellan and Jurd 1992). Various anticoagulant SPs from marine algae have been isolated and characterized.

## 1.4 FUTURE TRENDS AND PROSPECTS

Marine resources are well recognized for their biologically active substances, which have great potential for use as pharmaceuticals. Moreover, much attention has been paid recently by consumers toward a healthy lifestyle with natural bioactive ingredients. Recent studies have provided evidence that marine-derived bioactive pharmaceuticals play a vital role in human health maintenance and disease prevention. The possibilities of designing new pharmaceutical drugs to, reduce, or regulate chronic malfunctions are promising. Therefore, the manufacture of safe and cheap natural bioactive pharmaceuticals from marine resources is promising and, due to valuable biological functions with beneficial effects on health, marine-derived bioactive pharmaceuticals have the potential to be used as active ingredients for preparing various drugs or nutraceutical and pharmaceutical products. Up until now, most of the biological activities of marine-derived bioactive compounds have been observed *in vitro* or in mouse-model systems. Therefore, further research studies are needed in order to investigate the activity of such compounds in human beings. However, marine-derived bioactive peptides are a gift from the sea and have promising capabilities for the development of novel nutraceuticals and pharmaceuticals. Since the ocean is where life first started, it is no surprise perhaps that the biggest potential source for new, bioactive ingredients beneficial to health originates from the same place. With so many new species of marine resources yet to be discovered, the potential for new marine-derived bioactive pharmaceuticals with beneficial effects on human health is immense and the pharmaceutical industry is poised for accelerated development in the near future. This contribution discusses the recent trends, findings, and prospects of marine-derived potential pharmaceuticals.

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# 2 Antidiabetic Compounds from Marine Organisms and Their Properties

*Miyuki Shiroasaki and Tomoyuki Koyama*

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## 2.1 INTRODUCTION

Since ancient times, diabetes has been treated orally with terrestrial herbal medicines. Although many plants have been proposed to have antidiabetic potential, only a few of them have been shown to be effective based on scientific and medical evidence. As shown in this chapter, many natural marine products have been recognized as sources of promising novel chemical leads for global studies to identify therapeutic agents with various mechanisms for the treatment of diabetes.

### 2.1.1 SYMPTOMS OF DIABETES

Diabetes is a lifestyle-related metabolic disease that is characterized by high blood glucose levels resulting from defects in insulin secretion, insulin action, or both. Insulin is required for cells to absorb glucose from the blood for use as fuel or for storage. In patients with diabetes mellitus, glucose metabolism is altered. On the basis of its etiology and clinical presentation, diabetes mellitus is classified into two types: (1) Type 1, known as insulin-dependent diabetes mellitus, is caused by the immunological destruction of pancreatic  $\beta$  cells resulting in insulin deficiency (Notkins 2002). Its pathogenesis involves environmental triggers that may activate autoimmune mechanisms in genetically susceptible individuals, leading to the progressive loss of pancreatic islet  $\beta$  cells (Harrison and Honeyman 1999). Many of the acute effects of this type of diabetes can be controlled by insulin

replacement therapy. (2) Type 2, also known as non-insulin-dependent diabetes mellitus, is characterized by both impaired insulin secretion and insulin resistance, a condition in which cells in insulin-targeted tissues (skeletal muscle, adipose tissue, and liver) fail to use insulin properly. The number of patients with type 2 diabetes is increasing rapidly and it is fast becoming a significant global health problem (Suriyaprom et al. 2009). Type 2 diabetes can lead to cardiovascular damage through several mechanisms, each of which in turn may accelerate or worsen the others (Ahmed, Muniandy, and Ismail 2009). Hyperglycemia plays an important role in the development of type 2 diabetes and complications associated with diseases such as microvascular and macrovascular diseases (Baron 1998). Persistent hyperglycemia, the common characteristic of diabetes, can lead to various complications, including diabetic retinopathy (Wong and Aiello 2000), diabetic nephropathy (Tesfaye et al. 2005), and diabetic neuropathy (Gross 2005). Therefore, effective control of blood glucose levels is the key for preventing or reversing diabetic complications and improving the quality of life in diabetic patients (DeFronzo 1999).

### 2.1.2 PHARMACOLOGICAL APPROACHES FOR THE PREVENTION AND TREATMENT OF DIABETES

Type 2 diabetes is controlled through dietary therapy, appropriate exercise, and the use of medicinal chemicals. Stabilization of blood glucose levels is known to be effective in the prevention and treatment of diabetes by increasing the exocytosis of insulin from  $\beta$  cells with insulin secretagogues (sulfonylureas) (Levetan 2007); increasing the sensitivity of peripheral insulin-responsive tissues to insulin and improving glycemic metabolism (insulin sensitizers) (Steppel and Horton 2004); delaying the digestion and intestinal absorption of monosaccharides as well as reducing blood glucose levels, which results in the suppression of postprandial hyperglycemia (inhibitors of  $\alpha$ -amylase and  $\alpha$ -glucosidase) (Lebovitz 1992); and the use of insulin-like peptides. In addition, the regulation of gastrointestinal hormones has also been examined as an effective approach for the treatment of type 2 diabetes by enhancing incretin action. Glucagon-like peptide-1 (GLP-1) is an incretin hormone that enhances glucose-stimulated insulin secretion and exerts direct and indirect effects on the cardiovascular system (Drucker 2003). It is noted that GLP-1 and its related incretin hormone, glucose-dependent insulinotropic polypeptide, are rapidly inactivated by the enzyme dipeptidyl peptidase IV (DPP-IV, EC 3.4.14.5), a key determinant of incretin bioactivity (Young et al. 1999). Therefore, GLP-1 receptor agonist and DPP-IV inhibitor are used for the treatment of type 2 diabetes. Furthermore, protein tyrosine phosphatase 1B (PTP1B) negatively regulates insulin signaling and is also being examined as another approach to treatment (Zhang and Zhang 2007). It is noted that PTP1B on the cytoplasmic surface of the endoplasmic reticulum in classical insulin-targeted tissues plays a key role in the development of insulin resistance by dephosphorylating the insulin receptor (IR) or the IR substrate (IRS), which has been strongly implicated in the metabolic syndrome. The PTP1B inhibitors have been viewed as promising therapeutic agents for treating obesity and type 2 diabetes.

In this chapter, we focus on bioactive compounds from marine organisms that have antidiabetic effects. Further, studies on their pharmacological activities through various mechanisms are introduced.

## 2.2 MARINE ALGAE

Marine algae have been established as healthy food materials that are rich in minerals and dietary fibers; they are commonly consumed as an important foodstuff worldwide. Over the past few decades, several studies have focused on this foodstuff as a source of potential bioactive materials. In particular, marine algae have attracted worldwide attention due to their various functions that could contribute to human health. Most studies on the antidiabetic effects of marine algae have considered edible brown algae in the orders Laminariales and Fucales. Sections 2.2.1 through 2.2.3 introduce antidiabetic compounds in several marine algae and their effects.

### 2.2.1 LAMINARIA

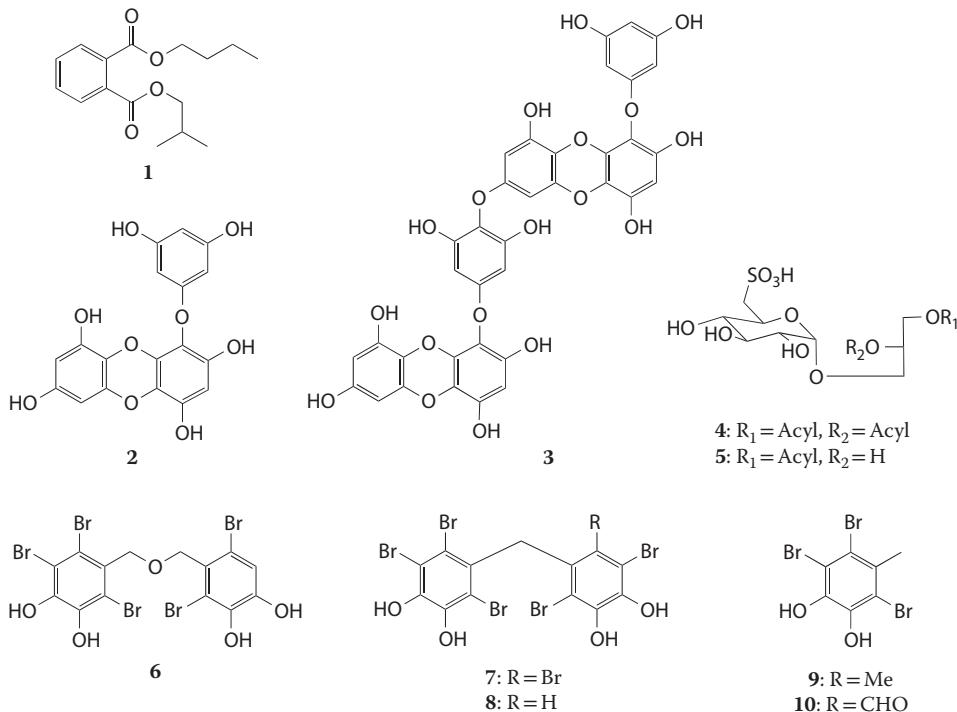
*Laminaria* sp. is a representative edible and medicinal brown alga found in East Asia. This alga is rich in nutrients and minerals and is used to promote human health. The preventive effects of an aqueous extract of *Laminaria japonica* on oxidative stress in streptozotocin (STZ)-induced diabetic rats were investigated. Pretreatment with this extract at 100 mg/kg orally for 5 days significantly reduced blood glucose levels in diabetic rats due to the antioxidant activity of the extract (Jin et al. 2004). The rhizoid of *L. japonica* also contains a useful bioactive component. Butyl-isobutyl-phthalate (BIP) (**1**) was isolated from the extract of rhizoid as a potent  $\alpha$ -glucosidase inhibitor, with a half maximal inhibitory concentration ( $IC_{50}$ ) of 38  $\mu$ m in *in vitro* experiments. *In vivo*, purified BIP had a significant hypoglycemic effect in STZ-induced diabetic mice (Bu et al. 2010). The inhibition pattern of BIP against  $\alpha$ -glucosidase was also studied. The interaction between BIP and  $\alpha$ -glucosidase was driven by both hydrophobic forces and hydrogen bonding (Liu et al. 2011). The hot water extract of *L. japonica* also showed antihyperglycemic effects in normal mice that had been loaded with carbohydrates. The unidentified active component was thought to inhibit glucose absorption by intestinal epithelial cells based on *in vivo* and *in vitro* experiments (Shirosaki and Koyama 2011).

*Ecklonia cava*, a kind of brown alga, is also popular in Korea and Japan as a food ingredient and traditional medicine. Pioneering studies indicated that dietary fiber was negatively associated with the onset of diabetes (Trowell 1973; Mochizuki et al. 1986). Mochizuki, Takahashi, and Yamamoto (1995) reported that oral treatment with dried algal powder (filtered with a 150- $\mu$ m mesh) suppressed postprandial elevation of the blood glucose level accompanied by an increase in the amount of endogastric glucose, and they concluded that the delay in gastric emptying associated with dietary fiber is one of the main factors in the antidiabetic activity of *E. cava*. In addition, several studies have demonstrated that the crude extract of *E. cava* and/or its ingredients have various biological activities, including radical-scavenging (Kang et al. 2005; Athukorala, Kim, and Jeon 2006), antiallergic (Kim et al. 2008), and anti-HIV-1 activities (Ahn et al. 2004). Recently, studies of *E. cava* have revealed that phlorotannins, oligomeric polyphenols consisting of the phloroglucinol unit with unique linkages, are the main constituents responsible for the biological activity of *E. cava*. Phlorotannins, typically eckol (**2**) and dieckol (**3**), have been shown to have potent antioxidative activity and inhibitory activity against carbohydrate digestive enzymes. Among phlorotannin derivatives, dieckol shows the highest inhibitory activity against  $\alpha$ -glucosidase and  $\alpha$ -amylase, with  $IC_{50}$  values of 10.97 and 124.98  $\mu$ mol/L, respectively. Furthermore, it was determined to be a noncompetitive  $\alpha$ -glucosidase inhibitor based on enzymatic analysis (Lee et al. 2009). These antidiabetic activities of phlorotannins were confirmed in animal experiments on STZ-induced diabetic mice (Lee et al. 2010) and C57BL/KsJ-db/db (db/db) mice (Lee et al. 2011). These results suggest that dieckol has an antidiabetic effect in type 2 diabetic mice by improving glucose and lipid metabolism and antioxidant enzymes.

A recent study demonstrated new activities in the extract of *E. cava* to prevent or improve diabetes (Kang et al. 2010). The antidiabetic effect of the methanol extract of *E. cava* was investigated using STZ-induced type 1 diabetic rats and C2C12 myoblasts. The methanol extract of *E. cava*, which has strong radical-scavenging activity, significantly reduced the blood glucose level and increased the insulin concentration in type 1 diabetic rats. The characteristic indications of diabetes, such as polyphagia and polydipsia, were also greatly improved by treatment with *E. cava*. The mechanism of action of *E. cava* appears to be mediated, at least partially, by the activation of both adenosine monophosphate (AMP)-activated protein kinase/ACC and PI-3 kinase/Akt signal pathways. These results indicate that *E. cava* has antidiabetic effects both *in vivo* and *in vitro*. *Ecklonia cava* could be a potential candidate for the development of medicinal preparations and nutraceutical or functional foods for diabetes and related symptoms.

Phlorotannins have also been isolated from other species of brown algae, *Ecklonia stolonifera* and *Eisenia bicyclis*, which have  $\alpha$ -glucosidase-inhibitory and PTP1B-inhibitory effects (Moon et al.

2011). The extract of *E. bicyclis* showed antidiabetic effects by inhibiting digestion by  $\alpha$ -glucosidase in diabetic KK-Ay mice (Iwai 2008). More detailed results and discussions regarding algal phlorotannins can be found in other reviews and comprehensive studies (Kim and Himaya 2011; Lee, Jeon, and Kim, Chapter 26).



### 2.2.2 FUCOID

*Hizikia fusiformis*, an edible brown alga that is found at the bottom of shallow water in East Asia, is used as a food in a variety of forms in littoral countries. Sulfoquinovosyldiacylglycerol (SQDG) (**4**) has been obtained from *H. fusiformis* as an inhibitor of yeast  $\alpha$ -glucosidase. SQDG showed competitive inhibition and had an inhibition constant ( $K_i$ ) value of 2.9  $\mu\text{M}$  (Kurihara, Ando, and Hatano 1995). The related compounds have been isolated from *H. fusiformis*. Digalactocycladiacylglycerol (DGDG) has shown a suppressing effect on the inhibitory activity of coexisting SQDG against  $\alpha$ -glucosidase, whereas DGDG alone showed no inhibition (Kurihara et al. 1996). Its deacylated derivative, sulfoquinovosylmonoacylglycerol (SQMG) (**5**), which was obtained from *H. fusiformis*, inhibited the reaction more potently than the deacylated derivatives of SQDG. However, the dideacylated derivatives of SQDG, sulfoquinovosylglycerol and sulfoquinovose, showed no inhibitory activity against  $\alpha$ -glucosidase. Therefore, they concluded that the expression of inhibition by these compounds would require hydrophobic acyl groups to interact with the hydrophobic site in the enzyme (Kurihara et al. 1997).

*Ascophyllum nodosum*, a brown alga in the rocky intertidal zone, is commonly found on the northeastern coast of North America and the northwestern coast of Europe. This alga contains many antioxidants, fucoxanthin, carotenoids, aromatic carboxylic acids, phlorotannins, and other phenolic compounds (Yan et al. 1996; Yoshie et al. 2000; Marais and Joseleau 2001) and is widely used as food, cosmetics, fertilizer and, recently, a functional food ingredient (Chan, Ho, and Phang 2006). The antidiabetic potential of the extract from *A. nodosum* has been described in some reports (Kwon, Vattem, and Shetty 2006). Zhang et al. (2007) reported that the  $\alpha$ -glucosidase-inhibitory effects of *A. nodosum* extract were associated with polyphenolic components in the extract.

An enriched polyphenolic fraction (PPE-F1) was shown to suppress the elevation of blood glucose in sucrose-loaded diabetic mice. Mice treated with PPE-F1 had decreased total blood cholesterol and glycated serum protein level compared with untreated diabetic mice. Apostolidis and Lee et al. (2010) investigated the potential of *A. nodosum* for the management of type 2 diabetes through the antioxidant-mediated inhibition of  $\alpha$ -glucosidase and  $\alpha$ -amylase. They showed that these inhibitory activities against yeast  $\alpha$ -glucosidase (EC 3.2.1.20) and porcine pancreatic  $\alpha$ -amylase (EC 3.2.1.1) increased with an increase in the phenolic content, and the best inhibitory potential was observed with water extract at 80°C, which contained the highest amount of phenolic phytochemicals. Their results supported previous reports on phlorotannin and its derivatives (average molecular weight = 8300) or polyphenol-rich fractions from *A. nodosum*.

In addition, a 70% MeOH extract of *Pelvetia babingtonii*, “ezoishige” in Japanese, showed inhibitory activity against rat-intestinal  $\alpha$ -glucosidase activities against maltose and sucrose, with IC<sub>50</sub> values of 2.24 and 2.84 mg/mL, respectively. The extract suppressed postprandial elevation of blood glucose levels in sucrose-loaded rats (Ohta et al. 2002). The inhibitory agent within the extract has not yet been elucidated.

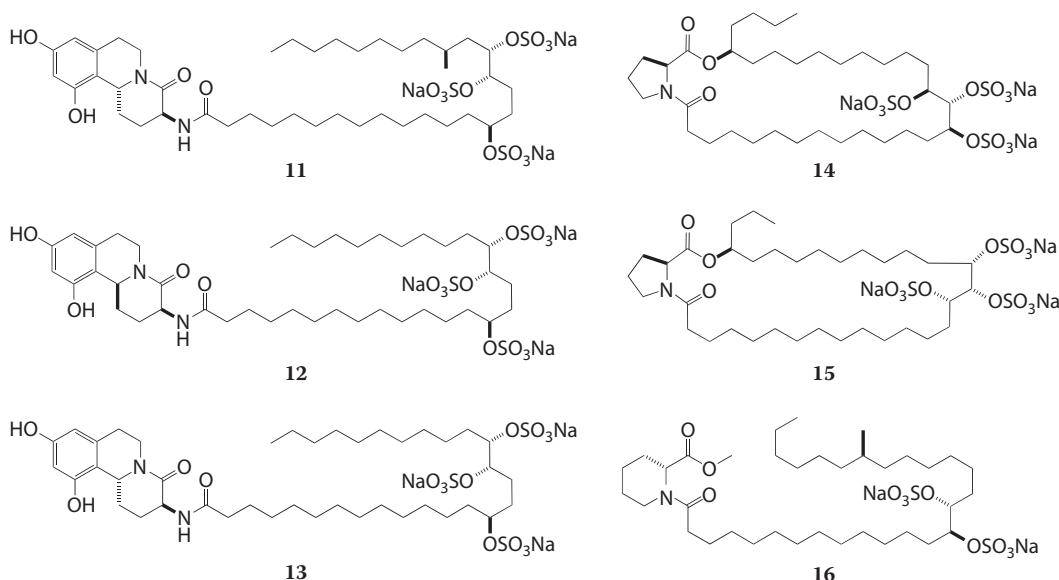
### 2.2.3 OTHER MARINE ALGAE

This section introduces other marine algae with antidiabetic activities that are not mentioned in Sections 2.2.1 and 2.2.2:

1. “Mekabu” is a brown alga (Phaeophyceae), the sporophyll of “wakame” (*Undaria pinnatifida*). In Japanese cooking, this alga is used for its glutinous properties in the preparation of soups and vegetable dishes. The stickiness of mekabu is due to the elution of indigestible polysaccharides, such as alginic acid, which are contained intracellularly. It has been reported (Yamanaka and Ogawa 1998; Yamanaka et al. 2000) that the viscous exudates of mekabu have an antidiabetic effect in glucose-loaded rats. These results suggest that the ingestion of mekabu viscous exudates together with glucose suppressed the maximum blood glucose level. Movement of the contents from the stomach to the small intestine was delayed, which is presumed to be the cause of the suppressed elevation of the blood glucose level. The retention time of the digestive tract contents was also longer, and fecal weight and amount of glucose in feces tended to be greater.
2. *Monostroma nitidum*, in the phylum Chlorophyta, is an edible green alga with thin layers of cells that grows off the coast. It is noted that *M. nitidum* is distributed in rocky areas in the upper intertidal zone of calm inlets. The oral administration of 0.2 g/kg *M. nitidum* (powder filtered with an 18.5- $\mu\text{m}$  mesh) significantly inhibited the postprandial elevation of blood glucose levels in carbohydrate-loaded rats (Saka et al. 2010). An  $\alpha$ -glucosidase-inhibitory activity was not observed. Administration of the hot water-soluble fraction (crude rhamnan sulfate extract) to rats was associated with a significantly lower blood glucose level than that in a control group. This antihyperglycemic effect may occur through a mechanism in which dietary fiber delays the movement and digestive absorption of carbohydrates in the small intestine. The active component in *M. nitidum* was considered to be rhamnan sulfate based on the aforementioned experimental results.
3. Red algae contain bromophenols (Li et al. 2008), which have been reported to exhibit a wide range of biological and pharmacological activities including antibacterial (Xu et al. 2003; Oh et al. 2008) and cytotoxic activities (Sun et al. 2005). Recently, bromophenols isolated from marine algae have been reported to be potential antidiabetic agents and act as both PTP1B inhibitors and  $\alpha$ -glucosidase inhibitors. Bromophenol derivatives from the red alga *Rhodomela confervoides*, which contain one or two 2,3-dibromo-4,5-dihydroxybenzyl units and are highly brominated, inhibit PTP1B activity (IC<sub>50</sub> values of 2.4, 1.7, 1.5, and 0.84  $\mu\text{M}$ , respectively), and *R. confervoides* extracts decrease blood glucose levels in diabetic rats.

The antihyperglycemic effects of ethanol extracts from *R. confervoides* in STZ-induced diabetic rats fed a high-fat diet were investigated. The STZ-induced diabetic rats treated with medium- and high-dose alga extracts showed remarkable reductions in fasting blood glucose (FBG) compared with the STZ-induced diabetic control rats. In addition to their inhibitory effects against PTP1B and  $\alpha$ -glucosidase, some bromophenols also inhibit aldose reductase (AR). It is noted that AR is the first enzyme of the polyol pathway, which is responsible for the formation of fructose from glucose and plays an important role in the development of degenerative complications of diabetes. The red alga *Symplocladia latiuscula* contains several bromophenols, including 2,2',3,6,6'-pentabromo-3',4,4',5-tetrahydroxydibenzyl ether (**6**); 2,3,6-tribromo-4,5-dihydroxymethylbenzene (**7**); 2,2',3,5',6-pentabromo-3',4,4',5-tetrahydroxydiphenylmethane (**8**); bis(2,3,6-tribromo-4,5-dihydroxyphenyl)methane (**9**); and 2,3,6-tribromo-4,5-dihydroxybenzaldehyde (**10**) (Wang et al. 2005). These bromophenols exhibited significant AR-inhibitory activity. The concentrations of test compounds that inhibited AR by 50% ( $IC_{50}$ ) were estimated from the least-squares regression lines in plots of logarithmic concentrations versus remaining activity. The results suggested that bromophenols might be promising candidates for the development of antidiabetic agents.

4. *Posidonia oceanica* (POE; Posidoniaceae) is not an alga; rather, it is a widely distributed phanerogam in the Mediterranean and Aegean seas that plays an important role in stabilizing the sea floor and the coastal ecosystems (Diaz-Almela, Marbá, and Duarte 2007). On the coast of western Anatolia, leaves of this seagrass are used as a remedy for diabetes and hypertension and also as a vitalizer. The antidiabetic effects of the extract of POE were investigated *in vivo*. Oral administration of POE for 15 days resulted in a dose-dependent decrease in blood glucose (Gokce and Haznedaroglu 2008). At concentrations of 150 and 250 mg/kg, POE exerted a protective effect on significantly decreased levels of antioxidants, that is, glutathione, superoxide dismutase, glutathione peroxidase, catalase, and nitric oxide. It is noted that POE (50 mg/kg) did not affect alloxan-induced alterations in the antioxidant status, although it did exhibit glucose-lowering and vasoprotective activities. These results suggest that the antidiabetic and vasoprotective effects of POE may be unrelated to its antioxidant properties. Further studies on the effects of POE on endothelial nitric oxide synthase (eNOS) activity via a PI3K pathway may provide new insights into diabetes and its vascular complications.

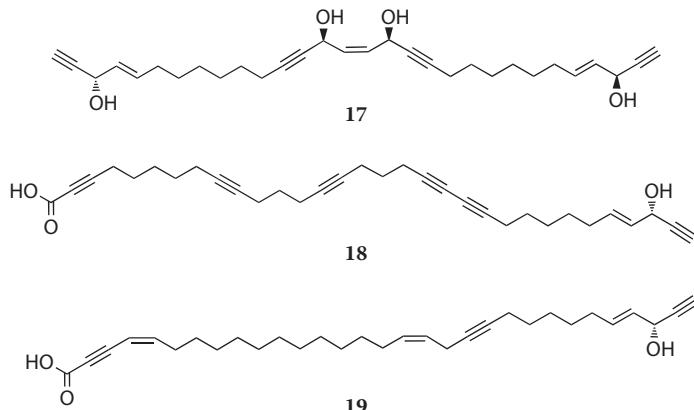


## 2.3 MARINE ANIMALS

Most natural marine products and their derivatives are produced by animals such as sponges, soft corals, and tunicates. These marine animals inhabit characteristic marine environments, and their metabolic systems have evolved to adapt to the environment. Various compounds with antidiabetic activity have been found in these marine animals.

### 2.3.1 SPONGES

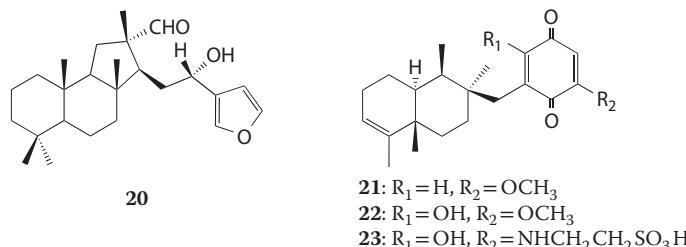
The marine sponge *Penares schulzei* collected from Hachijo Island, Japan, has been shown to contain the  $\alpha$ -glucosidase inhibitors schulzeines A (**11**), B (**12**), and C (**13**). These sulfated isoquinoline alkaloids inhibit  $\alpha$ -glucosidase with  $IC_{50}$  values of 48–170 nM (Takada et al. 2004). Further screening studies of  $\alpha$ -glucosidase inhibitors from metabolites of marine animals led to the development of new compounds with more potent inhibitory activities. Three other sulfated alkaloids, penarolide sulfate A<sub>1</sub> (**14**), penarolide sulfate A<sub>2</sub> (**15**), and penasulfate A (**16**), which were isolated from the sponge *Penares* sp., inhibited yeast  $\alpha$ -glucosidase activity with  $IC_{50}$  values of 1.2, 1.5, and 3.5  $\mu$ g/mL, respectively (Nakao et al. 2000, 2004). Another series of  $\alpha$ -glucosidase inhibitors, polyacetylenic compounds, that is, petrosynol (**17**), callyspongynic acid (**18**), and corticatic acid A (**19**) with  $IC_{50}$  values of 4.08, 0.16, and 0.25  $\mu$ g/mL, respectively, were isolated from the sponge *Callyspongia truncata* found on the coast of Japan (Nakao et al. 2002, MP\_39-3).



Screening for DPP-IV-inhibitory activity is performed in aqueous crude extracts. Inhibitory activity was found in extracts of the sponge *Xetospongia muta* and two species belonging to *Cnidaria* collected on the northern coast of Cuba near Havana (Pascual et al. 2007). Denaturing treatment with trichloroacetic acid (TCA) or heating at 60°C increased the inhibitory activity in the crude extract of *X. muta*. The molecule responsible for DPP-IV inhibition has a low molecular weight. The detected species are promising sources of natural DPP-IV inhibitors with potential therapeutic applications.

Another research group screened PTP1B inhibitors from marine animal sources as promising therapeutic agents against obesity and type 2 diabetes. The marine natural product hyrtiosal (**20**), from the marine sponge *Hyrtios erectus* (Igushi, Shimada, and Yamada 1992), was found to act as a PTP1B inhibitor and have extensive cellular effects on PI3K/AKT activation, glucose transport, and TGF $\beta$ /Smad2 signaling. This compound inhibited PTP1B activity in a dose-dependent fashion, with an  $IC_{50}$  value of 42  $\mu$ M in a noncompetitive inhibition mode (Sun et al. 2007). The sesquiterpene quinone 21-dehydroxybolinaquinone (**21**), together with two known related analogs, bolinaquinone (**22**) and dysidine (**23**), was isolated from the Hainan sponge *Dysidea villosa* (Li et al. 2009). Evaluation of its inhibitory activity against human PTP1B showed that dysidine had the strongest

PTP1B-inhibitory activity, with an  $IC_{50}$  value of 6.70  $\mu\text{M}$ . Due to its potent PTP1B-inhibitory activity and moderate specificity, **23** is expected to be developed as a potential lead compound for anti-diabetes agents.



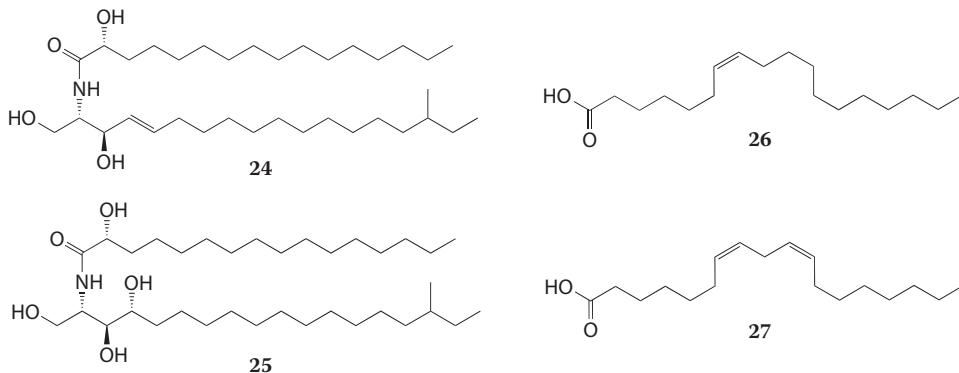
### 2.3.2 ECHINODERM

Echinoderms are found at all ocean depth, from the intertidal zone to the abyssal zone. They can be classified into five extant classes: (1) Crinoidea, (2) Asteroidea, (3) Ophiuroidea, (4) Echinoidea, and (5) Holothuroidea. Echinodermata species have been found to contain bioactive components, which have been documented to exhibit antibacterial, antifungal, antiviral, neurite outgrowth, and antitumor activities. In this section, the antidiabetic effects of extracts from Echinoderms are described.

Asteroidea (sea star) is known to be a rich source of saponins and ceramides. From the starfish *Luidia maculata*, sphingoglycolipids (Kawatake et al. 2002) were isolated and their structures elucidated. Further studies in the same sea star found two ceramides, LMCer-1-1 (**24**) and LMCer-2-1 (**25**), with antihyperglycemic effects (Higuchi et al. 2004; Inagaki et al. 2006). The chemical structures of **24** and **25** were assigned to be (2S, 3R, 4E, 2'R)-2-(2-hydroxyhexadecanoylamino)-16-methyl-4-octadecene-1,3-diol and (2S, 3R, 4E, 2'R)-2-(2-hydroxyhexadecanoylamino)-16-methyl-octadecane-1,3,4-triol, respectively. These ceramides suppressed hemoglobin A<sub>1c</sub> levels in diabetic db/db mice for 27 days (250  $\mu\text{g}/\text{mice}$ ). On the other hand, the collagen peptide that results from protease treatment is also of interest as a bioactive component in sea star. Oral treatment with the collagen peptide of sea star (CPS) suppresses postprandial blood glucose levels in rats and mice (Aso et al. 2009). It is noted that  $\alpha$ -glucosidase-inhibitory activity was not observed. The amount of glucose remaining in the stomach was significantly higher than that in the control group. The antihyperglycemic effect was thought to involve a mechanism in which dietary fiber delays the movement of carbohydrates from the stomach to the small intestine, and this interferes with or delays the digestive absorption of carbohydrates in the small intestine. This report suggests that CPS isolated from sea star may be useful for the treatment of diabetes.

Holothuroidea (sea cucumber) has long been used as a traditional medicine in East Asia due to its high nutraceutical value. *Stichopus japonicus*, a sea cucumber species that is found throughout East Asia, including Korea, China, and Japan (Kanno, Li, and Kijima 2005), contains antifungal triterpene glycosides named holotoxins A, B, and C (Kitagawa, Sugawara, and Yosioka 1976). Recently, two unsaturated fatty acids with strong  $\alpha$ -glucosidase-inhibitory activity, 7(Z)-octadecenoic acid (**26**) and 7(Z),10(Z)-octadecadienoic acid (**27**), were purified from the body wall of *S. japonicus* (Nguyen, Um, and Kim 2011). The  $IC_{50}$  values of compounds **26** and **27** against *Saccharomyces cerevisiae*  $\alpha$ -glucosidase were 0.51 and 0.67  $\mu\text{g}/\text{mL}$ , respectively, and those against *Bacillus stearothermophilus*  $\alpha$ -glucosidase were 0.49 and 0.60  $\mu\text{g}/\text{mL}$ , respectively. These compounds slightly inhibited rat-intestinal sucrase and maltase. In addition, both compounds showed a mixed type of inhibition against *S. cerevisiae*  $\alpha$ -glucosidase and were very stable under thermal and acidic conditions for up to 60 minutes. The inhibition constants for the inhibitor binding with free enzyme,  $K_I$  and with enzyme-substrate complex,  $K_{IS}$  values of compounds **26** and **27** were 0.44 and 0.22  $\mu\text{g}/\text{mL}$ , and 0.39 and 0.13  $\mu\text{g}/\text{mL}$ , respectively. Therefore, sea cucumber fatty acids may potentially be developed as a novel natural nutraceutical for the management of type 2 diabetes.

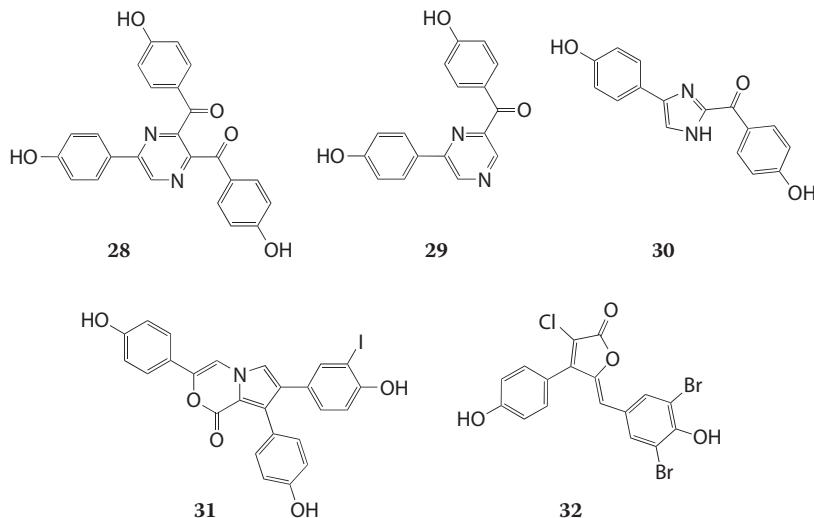
Echinoidea (sea urchin) is considered to be an important fishery resource rather than a pharmacognostical resource. The gonads of both male and female sea urchins are considered culinary delicacies, especially in Japan, but the remaining tissue is not used commercially. Recently, we found that the extract prepared from a kind of sea urchin suppressed the postprandial elevation of blood glucose levels in starch-loaded mice (our unpublished results). Further studies may enable the effective use of underutilized marine resources.



### 2.3.3 ASCIDIAN

*Halocynthia roretzi*, or sea squirt, is an edible marine animal with an orange-colored mantle covered by a hard tunic that is eaten in East Asia. The effects of *H. roretzi* lipids on white adipose tissue (WAT) weight and blood glucose in diabetic/obese KK-Ay mice were evaluated (Mikami, Hosokawa, and Miyashita 2010). Male diabetic/obese KK-Ay mice were fed a diet containing 5% *H. roretzi* lipids + 5% soybean oil for 5 weeks. In mice treated with *H. roretzi* lipids, WAT weight was reduced, blood glucose levels and leptin mRNA expression in epididymal adipose tissue were significantly decreased, blood leptin level tended to decrease, and blood adiponectin level tended to increase compared to the results in control mice. These results suggest that *H. roretzi* lipids are effective in preventing or reducing hyperglycemia through the attenuation of an increase in adipose tissue weight in KK-Ay mice. Adiponectin upregulates insulin signaling by activating PPAR  $\alpha$  and AMP kinase (Kadowaki and Yamaguchi 2005). In hypertrophied adipose tissue, dysregulation of adipocytokine secretion occurs and induces insulin resistance. Enhancement of adiponectin secretion will reduce in obese states and reduce the related symptoms (Kadowaki et al. 2006). Lipids extracted from *H. roretzi* contain n-3 polyunsaturated fatty acids (n-3 PUFAs) such as eicosapentaenoic acid and docosahexaenoic acid, as well as carotenoids. It is possible that n-3 PUFAs and other compounds have a combined effect.

Other ascidians contain AR inhibitors, which are characterized by a heterocyclic system with phenolic groups. Manzanaro, Salvá, and De la Fuente (2006) found inhibitory activity against human AR in several types of known marine natural compounds isolated from ascidians. Two pyrazine alkaloids, botryllazine A (**28**) and B (**29**), and an imidazole alkaloid (**30**) have been isolated from red ascidian *Botryllus leachi* (Durán et al. 1999). Lukianol B (**31**) has been isolated from the unidentified tunicate (Yoshida et al. 1992), and rubrolides A–H have been isolated from the colonial tunicate *Ritterella rubra* (Miao and Andersen 1991). These compounds showed inhibitory activity that was fivefold to sixfold more potent than that of the known AR inhibitor sorbinil (Beyer-Mears, Ku, and Cohen 1984). Among all the compounds tested, the potent inhibitory activity of **31** and rubrolide E (**32**) with IC<sub>50</sub> values of 0.6 and 0.8  $\mu$ M, respectively, must be stressed. A polyol-phosphate pathway is known to play an important role in reducing the complications of diabetes, and an AR inhibitor may be useful for suppressing serious complications in diabetic patients.



### 2.3.4 OTHER COMPOUNDS

This section will introduce compounds with antidiabetic activity from marine animals:

1. The antihyperglycemic activity in the soft corals *Sinularia firma* and *S. erecta* was investigated (Tamrakar et al. 2008). Methanolic extracts of *S. firma* and *S. erecta* reduce the blood glucose level by 14.5% and 16.1%, respectively, in STZ-induced diabetic rats at an oral dose of 250 mg/kg. These extracts were also found to inhibit the postprandial increase in hyperglycemia by 13.0% and 12.7%, respectively, in normal sucrose-loaded rats. To elucidate the probable mechanism of action of these extracts, their inhibitory effects against key target enzymes of the insulin/glucose/glycogen cascade, that is, PTP1B, glucose-6-phosphatase, and glycogen phosphorylase, respectively, were investigated. However, none of them showed a promising inhibitory effect on the tested enzymes. The mechanism of the antihyperglycemic effects of *Sinularia* extracts has not yet been determined.
2. The meat of some kinds of fish has been shown to have antidiabetic effects. The effects of a boiled fish meat paste, called “kamaboko” in Japanese, on lifestyle-related diseases have been investigated in mice. A suspension of kamaboko made from codfish was found to suppress the postprandial elevation of blood glucose levels after the oral administration of sucrose, maltose, starch, or glucose in mice (Honma, Koyama, and Yazawa 2008). However, Kamaboko did not inhibit  $\alpha$ -amylase or  $\alpha$ -glucosidase activities *in vitro*. The raw fish meat suspension also did not show these effects. Although the active components have not yet been identified, they are believed to be peptides, since the antihyperglycemic activity was inactivated by pretreatment with trypsin.

## 2.4 CONCLUSION

Natural medicines from marine sources include  $\alpha$ -glucosidase inhibitor, GLP-1 receptor agonist, insulin sensitizer, DPP-IV inhibitor, and PTP1B inhibitor. Some of these compounds have been shown to have the potential for preventing and treating diabetes, with excellent results in animal models and clinical trials. Therefore, further pharmacological studies *in vivo* are needed to prove that marine algae and marine animals are important sources of alternative antidiabetic medicines.

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# 3 Bioactive Compounds from Okinawan Marine Cyanobacteria

*Toshiaki Teruya, Osamu Ohno, and Kiyotake Suenaga*

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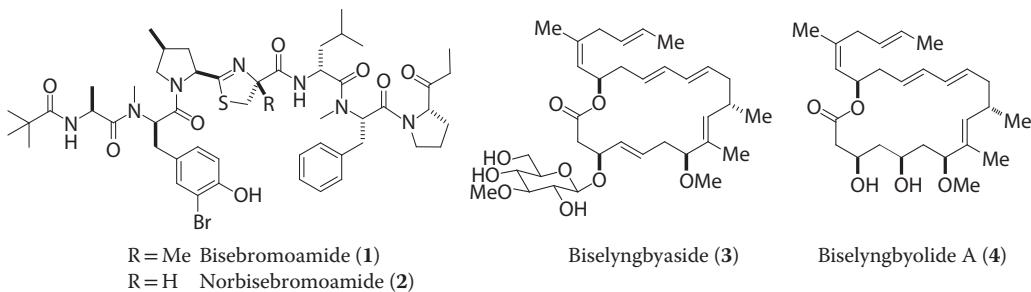
### 3.1 INTRODUCTION

Natural products, especially those from terrestrial plants and microbes, have been the most productive source of drug molecules over the years, and pharmacologically active compounds from plants and microbes continue to play an important role in developing new investigational drugs (Butler 2008; Newman and Cragg 2007). However, much attention has recently been given to marine organisms due to their remarkable biological activities (Molinski et al. 2009). In particular, cyanobacteria are prolific producers of biologically active compounds (Gerwick, Tan, and Sitachitta 2001). Cyanobacteria have been recognized as a source of pharmaceutical lead compounds (Tan 2007), for example, TZT-1027, a synthetic dolastatin 10 analog, is currently being evaluated in phase II clinical trials in the United States (Yamamoto et al. 2009). Dolastatin 10 was originally isolated from the sea hare *Dolabella auricularia* and has been subsequently isolated from marine cyanobacterium (Luesch et al. 2001; Pettit et al. 1987). Cryptophycin-309 and cryptophycin-249, which are derivatives of the terrestrial cyanobacterial peptide cryptophycin-1, have undergone preclinical efficacy studies (Liang et al. 2005).

Bisebromoamide (**1**), norbisebromoamide (**2**), and biselyngbyaside (**3**) were isolated from marine cyanobacteria *Lyngbya* sp., which was collected at Okinawa Prefecture, Japan, and biselyngbylide A (**4**), a congener of biselyngbyaside (**3**), was isolated from marine cyanobacteria *Lyngbya* sp., which was collected at Kagoshima Prefecture, Japan (Morita, Ohno, and Suenaga 2012; Sasaki et al. 2011; Teruya, Sasaki, Fukazawa, et al. 2009; Teruya, Sasaki, Kitamura, et al. 2009). All of the four compounds were isolated as cytotoxic compounds, and their structures were established by spectroscopic analyses including two-dimensional nuclear magnetic resonance (2D-NMR) techniques and by synthetic studies. This chapter presents the novel structures and intriguing biological activities of bisebromoamide (**1**), biselyngbyaside (**3**), and their analogs (**2** and **4**).

### 3.2 BISEBROMOAMIDE AND ITS ANALOGS

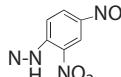
The marine filamentous cyanobacterium *Lyngbya* sp. was collected at Bise, Okinawa Prefecture. A crude organic extract of this material was subjected to fractionation guided by growth inhibitory activity against HeLa S<sub>3</sub> cells with solvent partition, column chromatography (octadecylsilyl silica gel,



methanol–water), and reversed-phase high-performance liquid chromatography (HPLC) to afford bisebromoamide (**1**) as a colorless oil (Teruya, Sasaki, Fukazawa, et al. 2009). The molecular formula of **1** was determined to be  $\text{C}_{51}\text{H}_{72}\text{BrN}_7\text{O}_8\text{S}$  on the basis of high-resolution electrospray ionization mass spectrometry (HR-ESIMS) and NMR data. The gross structure of **1** was established on the basis of spectroscopic data. To determine the absolute configuration of the eight chiral centers, we conducted chiral HPLC analysis of the acid hydrolysate and the ozonolysis–acid hydrolysis sequence and reduction with  $\text{NaBH}_4$  followed by acid hydrolysis. The absolute stereostructure of bisebromoamide was determined to be as shown in formula **1**. Bisebromoamide (**1**) contains a high degree of D-amino acids and *N*-methylated amino acids along with several other modified amino acid residues, suggesting a nonribosomal origin of biosynthesis. Furthermore, **1** possesses some unusual structural features, including a substituted methyl thiazoline (Me-Tzn) connected to a methylproline (Me-Pro). In addition, **1** contains the 2-(1-oxopropyl)pyrrolidine (Opp), *N*-methyl-3-bromotyrosine (*N*-Me-Br-Tyr), and *N*-pivalamide moieties. Bisebromoamide (**1**) is the first example of a natural product bearing the Opp unit. Bisebromoamide (**1**) showed cytotoxicity against HeLa S<sub>3</sub> cells with a half maximal inhibitory concentration ( $\text{IC}_{50}$ ) value of 0.04  $\mu\text{g}/\text{mL}$ . Bisebromoamide (**1**) was evaluated for anticancer activity using a panel of 39 human cancer cell lines (termed JFCR39) at the Japanese Foundation for Cancer Research, Tokyo, Japan. The average concentration required for 50% growth inhibition ( $\text{GI}_{50}$ ) against the panel of 39 cell lines was 40 nM. In addition, **1** exhibited significant antitumor activity in the *in vivo* human tumor xenograft tests. Furthermore, **1** was revealed to inhibit the phosphorylation of extracellular signal-regulated protein kinase (ERK) selectively in NRK cells by platelet-derived growth factor (PDGF) stimulation at 10–0.1  $\mu\text{M}$  of **1** and had no effect on the phosphorylation of AKT, PKD, PLC $\gamma$ 1, or S6 ribosomal protein at the same concentration. It is known that some tubulin modulators have an effect on the phosphorylation of ERK. The pattern of differential cytotoxicity of **1** against human tumor cell lines was evaluated by the Compare Program and was revealed not to be correlated with that shown by tubulin modulators. This result was supported by immunoblotting analysis using an antiacetylated lysine antibody. The level of acetylated tubulin, a marker for microtubule stability, is affected after treatment with some tubulin modulators. The total levels of acetylated tubulin remained unchanged after treatment with **1**. According to these results, ERK signaling pathways may be one of the intracellular targets of **1**. Aberrant activation of the Ras/Raf/MEK/ERK pathway is commonly observed in cancer cells (Roberts and Der 2007). Thus, the Ras/Raf/MEK/ERK pathway is an attractive target for cancer therapies. Recently, some inhibitors that target the components of the Ras/Raf/MEK/ERK pathway were developed, and RAF265, BAY 43-9006, and AZD6244 have reached the clinical-trial stage (Montagut and Settleman 2009). Furthermore, the structure–activity relationships of **1** have been investigated through the use of natural bisebromoamides and synthetic analogs (Sasaki et al. 2011). Bisebromoamide analogs **5**, **6**, **7**, and **8** were prepared from **1** and evaluated with regard to their cytotoxicities against HeLa S<sub>3</sub> cells. The data shown in Table 3.1 indicate that norbisebromoamide (**2**) and all its derivatives exhibit potent cytotoxicity, which is comparable to that of **1**. These results suggest that the ketone, bromine atom, and phenolic hydroxyl group of **1** are not important for its cytotoxicity.

In addition, the mode of action of bisebromoamide (**1**) has been studied and it has been revealed that bisebromoamide stabilizes actin filaments (Sumiya et al. 2011). Recently, Uesugi et al. discovered that

**TABLE 3.1**  
**Cytotoxicities of Natural Bisebromoamides and Synthetic Analogs**

Sample	$IC_{50}$ (ng/mL) <sup>a</sup>
1: R <sup>1</sup> = Me, R <sup>2</sup> = Br, R <sup>3</sup> = H, R <sup>4</sup> = O (bisebromoamide)	40
2: R <sup>1</sup> = H, R <sup>2</sup> = Br, R <sup>3</sup> = H, R <sup>4</sup> = O (norbisebromoamide)	45
3: R <sup>1</sup> = Me, R <sup>2</sup> = Br, R <sup>3</sup> = H, R <sup>4</sup> = H, OH	77
	
6: R <sup>1</sup> = Me, R <sup>2</sup> = Br, R <sup>3</sup> = H, R <sup>4</sup> =	91
7: R <sup>1</sup> = Me, R <sup>2</sup> = Br, R <sup>3</sup> = Me, R <sup>4</sup> = O	72
8: R <sup>1</sup> = Me, R <sup>2</sup> = H, R <sup>3</sup> = H, R <sup>4</sup> = O	82

<sup>a</sup> Cytotoxicities against HeLa S<sub>3</sub> cells.

nuclear protrusion in HeLa cells is selectively induced by actin-targeting compounds. Within 1 hour of treatment with natural products that are known to target actin, HeLa cells exhibited a marked morphological alteration characterized by protrusion of the nucleus. The HeLa cells treated with bisebromoamide also exhibited nuclear protrusion. Although both actin filament destabilizers and actin filament stabilizers induced nuclear protrusion, *in vitro* actin polymerization and depolymerization experiments were conducted to determine that bisebromoamide is, in fact, an actin stabilizer. In the presence of bisebromoamide, polymerization of pyrene-labeled G-actin was enhanced in a concentration-dependent manner. To further confirm the actin specificity of bisebromoamide, a fluorescent conjugate of bisebromoamide (Bise–Flu) was synthesized and its subcellular localization in HeLa cells was observed. At high concentrations (10 μM), Bise–Flu showed cytostatic activity and induced nuclear protrusion, forming aggregations of actin. At concentrations less than 1 μM, morphological alterations or cytostatic effects were not observed and Bise–Flu localized in a filamentous pattern similar to that of rhodamine–phalloidin, a marker of actin filaments. These results suggest that bisebromoamide targets actin filaments, which is consistent with the results of the *in vitro* experiments. The relationship between bisebromoamide (**1**)-induced actin stabilization and the antitumor activity of bisebromoamide has not been fully investigated. Because of the potential of **1** as a preclinical candidate in cancer chemotherapy, further structural and functional analyses might reveal the relationship between actin and other target biomacromolecules of **1**, which should offer better perspectives for the design and development of new antitumor drugs.

### 3.3 BISELYNGBYASIDE AND ITS ANALOGS

Biselyngbyaside (**3**), a new 18-membered macrolide glycoside, was isolated from a *Lyngbya* sp., which was also collected at Bise (Teruya, Sasaki, Kitamura, et al. 2009). The molecular formula of **3** was determined to be C<sub>34</sub>H<sub>52</sub>O<sub>9</sub> on the basis of HR-ESIMS. The gross structure of **3** was

established on the basis of spectroscopic data. The relative stereostructure of **3** was confirmed by analyses of coupling constants and the nuclear Overhauser effect spectroscopy (NOESY) spectrum. Furthermore, the absolute stereostructure was determined by the modified Mosher's method and also by synthetic means. Based on these findings, the complete stereostructure of biselyngbyaside was determined to be as shown in formula **3**. Biselyngbyaside (**3**) exhibited cytotoxicity against HeLa S<sub>3</sub> cells with an IC<sub>50</sub> value of 0.1 µg/mL. Biselyngbyaside (**3**) was evaluated against a panel of 39 human cancer cell lines (HCC panel) at the Japanese Foundation for Cancer Research (Yamori et al. 1999). The average 50% growth inhibition (GI<sub>50</sub>) value across all the cell lines tested was 0.60 µM, and **3** exhibited differential cytotoxicities: The central nervous system cancer cell line, SNB-78 (GI<sub>50</sub> 0.036 µM), and lung cancer cell line, NCI H522 (GI<sub>50</sub> 0.067 µM), were especially sensitive. Considering the pattern of growth inhibition against 39 cancer cell lines, it is likely that biselyngbyaside (**3**) inhibits cancer cell proliferation through a novel mechanism (Yamori et al. 1999). On the other hand, biselyngbyolide A (**4**) was recently isolated as a structural analog of biselyngbyaside (**3**) from another source of marine cyanobacterium (Morita, Ohno, and Suenaga 2012). Biselyngbyolide A (**4**) also exhibited remarkable growth inhibitory activity against HeLa S<sub>3</sub> cells with an IC<sub>50</sub> value of 0.14 µM. Furthermore, biselyngbyolide A (**4**) was shown to exhibit strong apoptosis-inducing activity against HeLa S<sub>3</sub> cells and HL60 cells. Since biselyngbyolide A (**4**) clearly induced apoptosis in two types of cell lines, biselyngbyaside (**3**) may also have the same potential and, thus, cancer cells might generally contain a specific target molecule of these compounds.

In addition to cytotoxic evaluation of biselyngbyaside (**3**), Woo et al. showed that **3** inhibited the receptor activator of nuclear factor-κB ligand (RANKL)-induced osteoclastogenesis in mouse monocytic RAW264 cells and primary bone marrow-derived macrophages (BMMs) (Yonezawa et al. 2012). Biselyngbyaside (**3**) was revealed to inhibit RANKL-induced increases of TRAP activity and formation of multinucleated osteoclasts in RAW264 cells and BMMs. Similar inhibitory effects of **3** on osteoclastogenesis were observed in cocultures of primary bone marrow cells (BMCs) and osteoblastic UAMS-32 cells. These inhibitory effects of biselyngbyaside were highly specific to the differentiation of osteoclasts and more effective than the growth inhibition of cancer cell lines, since IC<sub>50</sub> values for osteoclastogenesis were lower than the average IC<sub>50</sub> values for growth inhibition against 39 human cancer cell lines. Then, **3** was revealed to inhibit RANKL-induced expression of c-Fos and NFATc1, which are important transcription factors in osteoclast differentiation, without affecting early signaling events such as the phosphorylation of mitogen-activated protein kinases (MAPKs) and IκB. It was also found that **3** specifically decreased cell survival in differentiated osteoclasts, accompanied by caspase-3 activation and nuclear condensation, and attenuated osteoclastic resorption pit formation. These findings indicated that **3** decreased bone resorption via inhibition of osteoclastogenesis and induction of apoptosis. Thus, biselyngbyaside (**3**) was shown to possess the potential to become a new class of agents for bone lytic disorders mediated by osteoclastic bone resorption, including osteoporosis, periodontitis, and tumor metastasis into bone.

### 3.4 CONCLUSION

Marine cyanobacteria are prolific producers of novel biologically active compounds and have been recognized as a source of pharmaceutical lead compounds. Bisebromoamide (**1**) and biselyngbyaside (**3**) were isolated as novel metabolites of the marine cyanobacteria *Lyngbya* sp., which were collected at Okinawa. Their structures were successfully determined by spectroscopic analyses and synthetic studies. Analyses of biological functions revealed that bisebromoamide (**1**) targeted actin filaments and exhibited significant antitumor activity. Biselyngbyaside (**3**) suppressed RANKL-induced osteoclastogenesis and induced apoptosis of osteoclasts. Both of them were shown to possess therapeutic potential for treating diseases such as cancer and osteoporosis. Thus, the search for novel compounds from marine cyanobacteria will contribute to the discovery of new pharmaceuticals.

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# 4 Bioactive Secondary Metabolites from Marine-Derived Fungi

*Sherif S. Ebada and Peter Proksch*

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## 4.1 INTRODUCTION

Marine ecosystems are a prolific source of structurally unique metabolites that have encouraged natural product chemists over several decades to explore and recognize novel chemical entities possessing potential pharmacological activities (Molinski et al. 2009; Mayer et al. 2010). Several bioactive marine metabolites have already been developed into pharmaceuticals for treatment of serious human ailments ranging from pain and microbial infections to immune diseases and cancer (Ebada and Proksch 2011). In addition, numerous new compounds with potential application as drugs or drug leads have been reported from marine invertebrates such as sponges, ascidians, and soft corals. In the past, the development of such promising substances was often hindered by scale-up problems regarding the sustainable production of these substances in sufficient quantities required for clinical trials and future market needs. Alternative production strategies overcame these problems only in some cases (Duckworth et al. 2004). Therefore, only a few marine natural products have so far entered preclinical or clinical trials compared to the huge number of compounds reported from marine organisms that feature promising pharmacological activities (Mayer et al. 2010; Ebada and Proksch 2011).

The discovery of biologically active unusual marine nucleosides by Bergman and Feeney in the late 1950s (Bergman and Feeny 1950, 1951) provided the basis for the first marine-derived pharmaceuticals, cytarabine (Ara-C, Cytosar®) and vidarabine (Ara-A, Vira-A®). The compounds cytarabine and vidarabine have been approved by the U.S. Food and Drug Administration (FDA) as anticancer and antiviral drugs, respectively (Zhang et al. 2005).

After its discovery, ziconotide (Prialt®), a synthetic product that is identical to the naturally occurring  $\omega$ -conotoxin MVIIA, took more than two decades of research and development to get FDA approval in December 2004 as the first marine-derived pharmaceutical for the treatment of severe chronic pain (Terlau and Olivera 2004).

In July 2007, trabectedin (ET-743, Yondelis®), a marine natural product first isolated from the ascidian *Ecteinascidia turbinata*, was approved by the European Agency for the Evaluation of Medicinal Products (EMEA) for the treatment of refractory soft tissue sarcoma and ovarian cancer (Ebada et al. 2008). To provide sufficient amounts of ET-743 for market needs, different strategies were evaluated including aquaculture and chemical synthesis (Mendola 2000, 2003). Unfortunately, neither of the two strategies was promising as both gave only variable or very low yields. To overcome the supply problem, a breakthrough was achieved by PharmaMar, Madrid, Spain, the licensee of natural trabectedin (ET-743), who succeeded in developing a large-scale semisynthetic protocol starting with cyanoafracin B, an antibiotic that can be produced in multikilogram scale by fermentation of *Pseudomonas fluorescens* (Cuevas et al. 2000). Today, trabectedin (ET-743) is licensed by PharmaMar to Johnson & Johnson/OrthoBiotech for drug development in the United States (Aune, Furuta, and Pommier 2002).

Another class of powerful antitumor substances, the bryostatins, was first reported from the bryozoan *Bugula neritina* in early 1980s (Pettit et al. 1982). However, attempts for economically feasible total synthesis or for large-scale production by aquaculture were generally unsatisfactory. It was the discovery that genes included in the biosynthesis of this compound family are found within a bacterium associated with *B. neritina* (but not in the bryozoan) that opened up new opportunities for the biotechnological production of the hypothetical compound bryostatin-0, which is a plausible common basis for the 20 known bryostatins and contains all proposed pharmacophore elements (Sudek et al. 2007). The endosymbiotic  $\gamma$ -proteobacterium *Candidatus Endobugula sertula* has not yet been cultivated, but in the future molecular techniques may enable heterologous expression and thereby further development of bryostatins as drugs. Currently, bryostatin-1 is in several phase I and II trials and is being assessed as an anti-cancer drug (both in combination therapy and as a single drug) and an anti-Alzheimer's drug (<http://clinicaltrials.gov>).

Recently, a halichondrin B derivative, eribulin mesylate, was approved by FDA for treatment of breast cancer metastases and is currently marketed under the trade name Halaven® (Imhoff, Labes, and Wiese 2011).

The current pipeline of marine natural products, which contains approximately 20 different metabolites that are in preclinical and clinical trials, was recently reviewed (Mayer et al. 2010; Ebada and Proksch 2011; <http://clinicaltrials.gov>).

The aforementioned examples illustrate not only the success of marine drug discovery but also the serious problems accompanying the development of products from marine macroorganisms for pharmaceutical applications. They also highlight that in most cases aquaculture does not provide sufficient supplies of compounds that may fulfill market needs at reasonable prices. Therefore, in spite of the impressive number of marine natural products or natural product analogs that are already in the market or undergoing clinical trials, the supply problem remains unresolved in many cases.

Microorganisms that live in association with sponges and other marine invertebrates have been claimed to be the true sources of several metabolites recovered from their hosts (Proksch, Edrada, and Ebel 2002). As in the case of bryostatins, it is already proved that metabolites initially assigned to a host organism are in fact of microbial origin (Jensen and Fenical 1994; Dobretsov, Dahms, and Qian 2006; König et al. 2006; Egan, Thomas, and Kjelleberg 2008; Rungprom et al. 2008). Thus, an increasing number of compounds originally thought to be biosynthesized by sponges or other marine macroorganisms are now considered to be produced by their associated microorganisms (Hentschel, Usher, and Taylor 2006).

## 4.2 MARINE-DERIVED FUNGI AS IMPORTANT SOURCES OF NEW BIOACTIVE NATURAL PRODUCTS

Marine-derived fungi are an ecologically defined group of microorganisms distinguished into indigenous and nonindigenous species. Indigenous marine-derived fungi can be further divided into “obligate” fungi (exclusively growing and sporulating in the marine habitat) and “facultative” fungi (freshwater or terrestrial fungi, which retain their ability to grow and/or sporulate in the marine habitat). However, nonindigenous species are also referred to as “contaminants” or “transients” and comprise terrestrial or freshwater fungal species that stay dormant and are thus unable to grow or multiply in the marine habitat (Kohlmeyer and Kohlmeyer 1979).

Based on the specific physical, chemical, and biological characteristics of marine ecosystems, marine-derived fungi have given rise to metabolic pathways producing novel chemical scaffolds. Bioactive marine natural products with unique chemical structures form the subject of an excellent series of reviews entitled “Marine Natural Products” by Blunt et al. (2011), which have been published on a yearly basis from 2003 to 2011. In addition to demonstrating the vast variety of chemical scaffolds provided by marine-derived fungal products, the authors also demonstrate a wide spectrum of pharmacological activities ranging from antimicrobial (Donia and Hamann 2003), antituberculosis (El Sayed et al. 2000), antiviral (El Sayed 2000), antiparasitic (Kayser, Kiderlen, and Croft 2002), antihelminthic, antimalarial, antiprotozoal, anticoagulant, antiplatelet, anti-inflammatory, and antidiabetic effects or antitumor effects, whereas some of these compounds may also affect the cardiovascular, immune, and nervous systems (Mayer et al. 2007, 2010; Waters et al. 2010).

During the last decade, there has been significant research interest in the exploration of marine-derived fungi as sources of new natural products. Numerically, this interest has been transformed into a sharp increase in the number of natural products reported from marine microorganisms (rise of 62%) in 2007 compared to the number reported in 2006 (Blunt et al. 2009). This ratio is even more spectacular when comparing the number of identified microbial metabolites in 2007 to the average from 1965 to 2005, which revealed a 6 fold increase in the number of reported microbial metabolites (Imhoff, Labes, and Wiese 2011). The research interest on microbial secondary metabolites from marine-derived fungi has been further escalated by the introduction of promising new lead compounds as potential anti-infectives and antitumor leads for drug discovery (Gulder and Moore 2009; Olano, Mendez, and Salas 2009; Rahman et al. 2010; Waters et al. 2010). In addition to recently published reviews (Ebel 2010; Rateb and Ebel 2011), in this chapter we survey the most recent reports regarding major chemical classes of natural products from marine-derived fungi with particular attention on their pharmacological activities.

## 4.3 NITROGENOUS COMPOUNDS

### 4.3.1 ALKALOIDS

Shearinines represent a group of janthiterm-type indole triterpenoid alkaloids. Eight new shearinines (D–K) together with their parent compound, shearinine A (**1**), and the known ergot alkaloids paspaliterm A (Springer and Clardy 1980) and paspaline A (Dorner et al. 1984) were obtained from the acetone extract of an endophytic *Penicillium* sp. isolated from the Chinese mangrove plant *Aegiceras corniculatum* (Aegicerataceae) (Xu et al. 2007). Biosynthetically, shearinines are suggested to be prenylated derivatives of paspaline A and paspaliterm A followed by oxidative cyclization. Shearinines D (**2**), E (**3**) and, to a lesser extent, G (**4**) exhibited significant *in vitro* blocking activity on large-conductance calcium-activated potassium channels (Xu et al. 2007). Simultaneously, three further janthiterm-type alkaloids were detected, and they were unintentionally given the names shearinines D–F (Smetanina et al. 2007). “Shearinine F” of the latter three congeners was identical to shearinine K (**5**), whereas the other two congeners were new and thus should be renamed. Shearinine A, together with the latter two congeners, induced apoptosis in human leukemia (HL)-60 cells (Smetanina et al. 2007).

Aspochalasins (**6** and **7**) are fungal secondary metabolites belonging to cytochalasans, which are structurally characterized by an isoindolone moiety bearing a 2-methylpropyl group at the C-3 position and a macrocyclic ring connecting the C-8 and C-9 positions. Aspochalasins were reported from different fungal species of the genus *Aspergillus* isolated from terrestrial plants and soil (Zhou et al. 2004; Rochfort et al. 2005) and from the fungus *Spicaria elegans* isolated from marine sediments collected in Jiaozhou Bay, China, and cultivated following the “one strain, many compounds” (OSMAC) approach (Lin, Zhu, et al. 2009). Following this approach, a 7-day-old culture of *S. elegans* produced five new aspochalasins (M–Q) in addition to the known congeners aspochalasin B (**6**) and D (**7**) together with a novel spicochalasin A (**8**) (Lin, Zhu, et al. 2009). Going over all the aspochalasin structures, it was noticed that the diversity of these structures results from different oxidation–reduction effects at C-17–C-19; so different culture conditions, particularly longer culture times, were supposed to change the oxidation–reduction effect and induce more analogs. This notion was supported by the report that a 14-day-old culture of the same fungus led to the production of three new analogs, aspochalasins R–T (Lin, Zhu, et al. 2010). Among the previously mentioned aspochalasins, aspochalasin B (**6**) inhibited the growth of BEL-7402 cells ( $IC_{50} = 2.3 \mu\text{M}$ ), whereas aspochalasin D (**7**) moderately inhibited the growth of HL-60 cells ( $IC_{50} = 11.6 \mu\text{M}$ ) (Lin, Zhu, et al. 2009). Apart from aspochalasins B (**6**) and D (**7**), only spicochalasin A (**8**) and aspochalasin M (**9**) exhibited modest activity against HL-60 cells, with  $IC_{50}$  values of 19.9 and 20.0  $\mu\text{M}$ , respectively (Lin, Zhu, et al. 2009, 2010). These results altogether strongly implied that the  $\alpha,\beta$ -unsaturated ketone moiety may be an essential part of the pharmacophore (Lin, Zhu, et al. 2009). Moreover, aspochalasin L (**10**), which is isolated from a soil-derived fungus, *Aspergillus flavipes*, demonstrated HIV-1 integrase inhibitory activity with an  $IC_{50}$  value of 71.7  $\mu\text{M}$  (Rochfort et al. 2005).

The fungus *S. elegans* was also the source for cytochalasins (Liu et al. 2006, 2008), another type of cytochalasans characterized by the presence of a benzyl group at the C-3 position of the isoindolone moiety instead of the 2-methylpropyl group. These compounds were isolated from *A. flavipes* associated with the mangrove plant *Acanthus ilicifolius* (Lin, Zhang, et al. 2009). Instead of an 11- to 14-membered macrocyclic ring, cytochalasins Z<sub>10</sub>–Z<sub>15</sub> (Liu et al. 2008) and Z<sub>18</sub>–Z<sub>20</sub> (Lin, Zhang, et al. 2009) reveal an open 8- to 12-carbon side chain.

In the cytotoxicity assay against four different cell lines, cytochalasins showed inhibitory activities against the A549 cell line with  $IC_{50}$  values ranging from 0.0062 to 19.5  $\mu\text{M}$  (Liu et al. 2006, 2008; Lin, Zhang, et al. 2009). Interestingly, despite its slight structural difference from cytochalasin K (**11**), cytochalasin E (**12**) displayed almost three orders of magnitude higher cytotoxic activity against A549 and P388 cells with  $IC_{50}$  values of 0.0062 and 0.093  $\mu\text{M}$  for **12** compared to 8.4 and 89  $\mu\text{M}$  for **11**, respectively (Liu et al. 2006). This increase in activity might be due to the presence of an epoxide moiety in **12**.

Chaetoglobosins are cytochalasans featuring an isoindolone moiety bearing an (indol-3-yl)-methyl group at the C-3 position with a macrocyclic ring connecting the C-8 and C-9 positions. The fungal genus *Chaetomium*, which includes both marine- and terrestrial-derived species, was revealed to be a rich source of more than 45 chaetoglobosins and analogs. From *Chaetomium globosum* QEN-14, an endophytic fungus derived from the Chinese marine green alga *Ulva pertusa*, seven new chaetoglobosin analogs, cytoglobosins A–G, were obtained together with two structurally related known compounds, isochaetoglobosin D and chaetoglobosin F<sub>ex</sub> (Cui, Li, Li, et al. 2010). Cytoglobosins A–E and G were evaluated for their cytotoxic activities against the P388, A549, and KB cell lines. Results revealed that only cytoglobosins C (**13**) and D (**14**) display activity toward the A549 cell line with  $IC_{50}$  values of 2.3 and 2.6  $\mu\text{M}$ , respectively (Cui, Li, Li, et al. 2010).

Trichodermamides represent a group of alkaloids featuring the rare cyclic O-alkyl-oxime functionality. Trichodermamides A (**15**) and B (**16**) were purified from the fungal culture of *Trichoderma virens* isolated from the marine ascidian *Didemnum molle* collected near Madang, Papua New Guinea (Garo et al. 2003), and they were reisolated together with trichodermamide C (**17**) from the endophytic fungus *Eupenicillium* sp. isolated from the outer bark of the rainforest

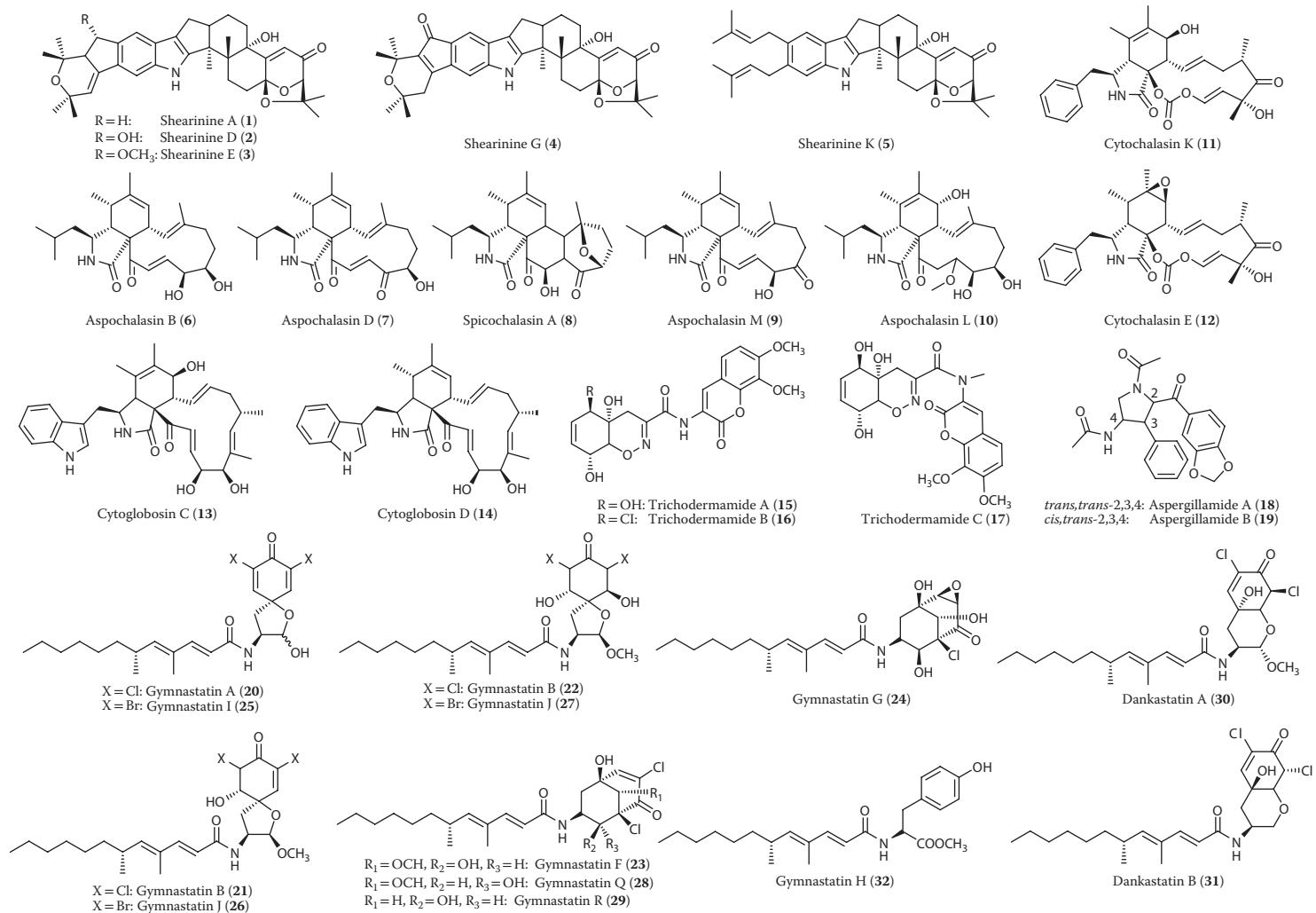
tree *Glochidion ferdinandi* (Davis et al. 2008). The structures of **16** and **17** were established by x-ray diffraction analysis, whereas the structure assignment and the absolute stereochemistry of **15** were obtained by spectral and chemical methods. In the cytotoxicity assay, only **16** and **17** exhibited antiproliferative activity against the HCT-116 cell line with IC<sub>50</sub> values of 0.7 (Garo et al. 2003) and 1.5 (Davis et al. 2008) μM, respectively.

Aspergillamide A (**18**) and its C-2 epimer, aspergillamide B (**19**), are pyrrolidine alkaloids obtained from the marine fungus *Aspergillus ustus* isolated from the Mediterranean sponge *Suberites domuncula* (Liu, Edrada-Ebel, et al. 2011). Aspergillamides A and B are structurally related to other pyrrolidine alkaloids reported from fungal strains of the genus *Aspergillus* such as *Aspergillus ochraceus* (Schwartz et al. 1988). Whereas related pyrrolidine alkaloids revealed a broad spectrum of antifungal activity, neither **18** nor **19** exhibited cytotoxic activity against the L5178Y cell line (Liu, Edrada-Ebel, et al. 2011).

Gymnastatins are halogenated polyketide alkaloids including 13 congeners, A–C (**20–22**), D, E, F (**23**), G (**24**), H, I–K (**25–27**), Q (**28**), and R (**29**) (Amagata, Minoura, and Numata 2006; Amagata et al. 2008, 2010), together with the related derivatives dankastatins A (**30**) and B (**31**) (Amagata et al. 2008). All were isolated as cytostatic metabolites from the fungus *Gymnascella dankaliensis* obtained from the marine sponge *Halichondria japonica*, collected in the Osaka Bay of Japan. Gymnastatins I–K (**25–27**) feature bromine atoms whereas gymnastatins A–C (**20–22**) are chlorinated. Gymnastatins F (**23**), G (**24**), Q (**28**), and R (**29**) featured a unique bicyclo[3.3.1] nonane ring system (Amagata et al. 2008, 2010). All gymnastatins and dankastatins were subjected to cytotoxicity assay against P388 cancer cells. Apart from gymnastatin H (**32**), all other gymnastatins and dankastatins revealed potent antiproliferative activities with IC<sub>50</sub> values in the low to sub-micromolar range (Amagata, Minoura, and Numata 2006; Amagata et al. 2008, 2010). The notion that the inhibitory activity of **24** is stronger than that of **23** implies that an α,β-epoxyketone system is more important than an α,β-unsaturated ketone for the activity of gymnastatin analogs (Amagata, Minoura, and Numata 2006). Whereas gymnastatins Q (**28**) and R (**29**), and dankastatins A (**30**) and B (**31**) share a conjugated ketone system in their structures, the inhibitory activities of **30** and **31** were more potent than those of **28** and **29** suggesting that a tetrahydropyran system is an important structural feature for enhancement of activity of these derivatives (Amagata et al. 2008). Moreover, gymnastatins I (**25**), J (**26**), and Q (**28**) were further investigated for their cytotoxic activity against a panel of 39 human cancer cell lines and found to exhibit pronounced activities (Amagata et al. 2008, 2010).

The fungus *Penicillium paneum* SD-44, which is isolated from a deep sea sediment collected in the South China Sea at a depth of 201 m, yielded a novel triazole carboxylic acid, penipanoid A (**33**), and two new quinazoline alkaloids, penipanoids B (**34**) and C (**35**) (Li, An, et al. 2011). In addition, a structurally related quinazolinone derivative (**36**), which was recently reported from the *Cordyceps*-colonizing fungus *Isaria farinosa* (Ma et al. 2011), was also isolated (Li, An, et al. 2011). Penipanoid A (**33**) was identified as the first example of a triazole derivative from a marine sediment-derived fungus, whereas penipanoid B (**34**) was recognized as a rare quinazolinone derivative possessing a dihydroimidazole ring system. In the antimicrobial activity assay, compounds **33–36** demonstrated activity against two different bacteria and five plant-pathogenic fungi. However, in the cytotoxicity assay, only **36** inhibited the growth of A549 and BEL-7402 cancer cells with IC<sub>50</sub> values of 17.5 and 19.8 μM, respectively (Li, An, et al. 2011).

Indoloditerpenes are a group of fungal secondary metabolites generally possessing an indole nucleus connected to a partially or fully cyclized diterpene unit. From the fungus *Aspergillus oryzae* obtained from the marine red alga *Heterosiphonia japonica*, two new indoloditerpene derivatives designated asporyzins A (**37**) and B (**38**) and one new indoloditerpene named asporyzin C (**39**) together with three known indoloditerpenes, JBIR-03 (**40**), emindole SB, and emeniveol, were isolated (Qiao et al. 2010). All isolated indoloditerpenoidal derivatives from *A. oryzae* were examined for their insecticidal and antimicrobial activities. Compound **40** revealed higher insecticidal activity compared to its oxidized derivatives, compounds **37** and **38**, in the brine



**FIGURE 4.1** Chemical structures of compounds **1–32**.

shrimp assay (*Artemia salina*), which was suggested to be due to the presence of indole and tetrahydrofuran moieties (Qiao et al. 2010). Moreover, in the antimicrobial activity assay, only asporyzin C (**39**) exhibited potent antibacterial activity; however, none of the tested compounds displayed any antifungal activity implying the importance of 4-hydroxy-4-methylpent-2-enyl moiety in **39** for its antibacterial activity. These findings implied that the endophytic fungus *A. oryzae* might play an important role in defending the host organism against herbivores and bacteria (Qiao et al. 2010). In order to further investigate the insecticidal mechanism, the isolated indoloditerpenes from *A. oryzae* were evaluated for acetylcholinesterase (AChE) inhibitory activity. Results showed that all tested compounds possessed low activity as modulators of AChE, indicating that their insecticidal activity may be due to targeting other key receptors and ion channels, possibly through modulating AChE (Qiao et al. 2010).

The fungus *Aspergillus fumigatus*, isolated from the Japanese zoanthid *Zoanthus* sp., was identified as a source of two new indole alkaloids, 2-(3,3-dimethyl-prop-1-ene)-costaclavine (**41**) and its epimer (**42**), together with the known compounds costaclavine (**43**), fumgaclavine A (**44**), and fumgaclavine C (**45**) (Zhang et al. 2012). In the cytotoxicity assay against the P388 cell line, compounds **41–43** and **45** exhibited only weak activity, whereas **44** was inactive (Zhang et al. 2012).

Asperginin (**46**) is a new alkaloid obtained from the mixed culture mycelia of two marine-derived epiphytic *Aspergillus* fungi, which were isolated from a rotten fruit of mangrove, *Avicennia marina*, collected in the South China Sea (Zhu et al. 2011). In addition to **46**, two known antibacterial compounds, neoaspagillic acid and kojic acid, were obtained as major metabolites from the same extract (Zhu et al. 2011). In the antimicrobial assay, asperginin (**46**) revealed moderate activity against three gram-positive bacteria and three gram-negative bacteria with minimum inhibition concentration (MIC) values ranging from 40 to 170 µM (Zhu et al. 2011).

Protuboxepins A (**47**) and B (**48**) are two new members of a rarely observed class of oxepin-containing alkaloids, which were obtained from the marine-derived fungus *Aspergillus* sp. SF-5044 isolated from the intertidal sediment of Dadaepo Beach, Korea (Lee et al. 2011). Previously described fungal metabolites that share the same structural features of **47** and **48** include oxepin-amides (Belofsky et al. 2000) and janoxepin (Sprogøe et al. 2005). Various biological activities such as anti-inflammatory (Belofsky et al. 2000) and antiplasmoidal (Sprogøe et al. 2005) activities have been reported for these compounds. In the cytotoxicity assay against a panel of five different cancer cell lines, only **47** showed weak growth inhibitory activity with IC<sub>50</sub> values between 75 and 250 µM (Lee et al. 2011).

#### 4.3.2 DIKETOPIPERAZINES

In addition to protuboxepins A (**47**) and B (**48**), two new diketopiperazine-type alkaloids have been isolated from the same extract of the marine-derived fungus *Aspergillus* sp. SF-5044, and they were designated as protubonines A (**49**) and B (**50**) (Lee et al. 2011). Neither **49** nor **50** revealed growth inhibitory activity in the cytotoxicity assay against a tested panel of five cancer cell lines at the 250 µM level (Lee et al. 2011).

Amauromine (**51**), a diketopiperazine alkaloid first isolated from the fungus *Amauroascus* sp. (Takase et al. 1985), was reisolated from a marine-derived fungus *Auxarthron reticulatum* obtained from the marine sponge *Ircinia variabilis* (Elsebai, Rempel, et al. 2011). Compound **51** is a vasodilatory alkaloid, which acts by blocking calcium channels (Takase et al. 1985). It showed a potent and selective cannabinoid CB<sub>1</sub> receptor antagonistic activity with a K<sub>i</sub> value of 178 nM, which may suggest **51** as a drug and/or a lead structure for drug development (Elsebai, Rempel, et al. 2011). Furthermore, two new related diketopiperazine alkaloids, novoamauromine (**52**) and *ent*-cycloechinulin (**53**), were isolated together with amauromine (**51**) and cycloechinulin (**54**) from the fungus *Aspergillus novofumigatus* CBS117520 (Ishikawa et al. 2010). In antifungal and cytotoxic activity assays, novoamauromine (**52**) exhibited modest activity (Ishikawa et al. 2010).

It is noted that 12-demethyl-12-oxo-eurotechninulin B (**55**) is a new dioxopiperazine alkaloid purified from the endophytic fungus *Eurotium rubrum*, which is isolated from the inner tissue of the semi-mangrove plant *Hibiscus tiliaceus* (Yan et al. 2012). Furthermore, 11 additional constituents, including one new anthraquinone derivative, were obtained from the same extract. Interestingly, compound **55** revealed mild selective cytotoxicity only toward the SMMC-7721 cancer cell line ( $IC_{50} = 65.4 \mu M$ ) when tested against a panel of seven tumor cell lines (Yan et al. 2012).

Variecolorins M–O (**56–58**), which are three new indole-containing diketopiperazine alkaloids, were isolated together with eight known analogs from a deep ocean sediment-derived fungus, *Penicillium griseofulvum* (Zhou et al. 2010).

Compounds **56–58** revealed weak radical-scavenging activities when investigated in the 2,2-diphenyl-1-picrylhydrazinyl (DPPH) assay with  $IC_{50}$  values of 135, 120, and 91  $\mu M$  compared to ascorbic acid as a positive control ( $IC_{50} = 26 \mu M$ ). None of the compounds showed activity in the cytotoxicity assay (Zhou et al. 2010).

Meleagrins and roquefortines are biogenetically related diketopiperazine-type alkaloids, which were mostly isolated from *Penicillium* sp. (Lin, Li, et al. 2009). Recently, from a deep ocean sediment-derived fungus *Penicillium* sp., four new diketopiperazine-type alkaloids, two new meleagrins (D [**59**] and E [**60**]), and two new roquefortines (H [**61**] and I [**62**]) were isolated (Lin, Feng, et al. 2010), together with the known meleagrin B (**63**) and meleagrin (**64**) (Lin, Li, et al. 2009).

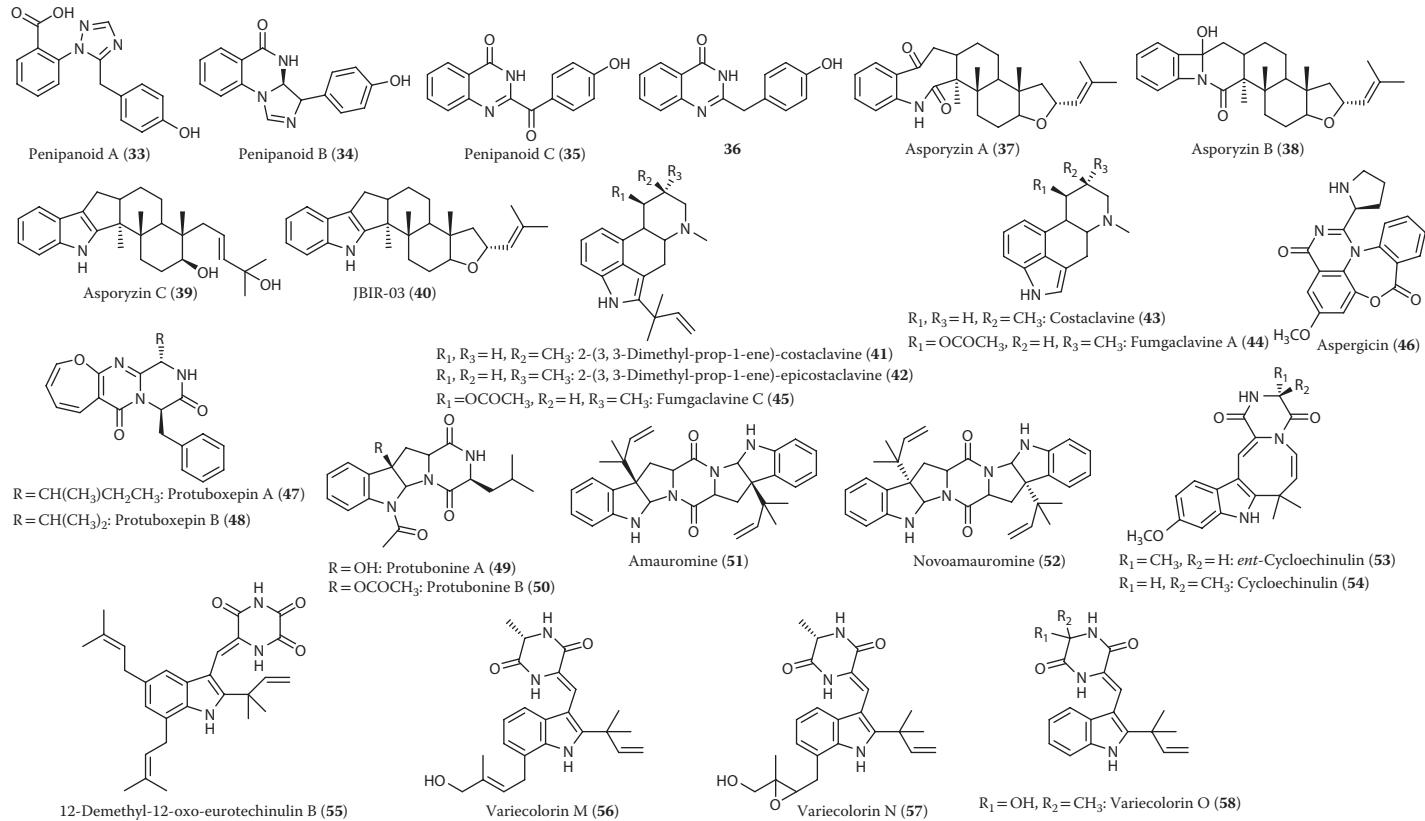
All new compounds (**59–62**) and meleagrin (**64**) were evaluated for their cytotoxic activity. Among the tested compounds, only meleagrin (**64**) exhibited moderate activity against the HL-60 and A549 cell lines with  $IC_{50}$  values of 7.4 and 19.9  $\mu M$ , respectively (Lin, Feng, et al. 2010). These results together with the previous evaluation of the antitumor activity of meleagrins (Lin, Li, et al. 2009) indicate that the addition of the acetate-mevalonate-derived C-5 or C-9 side chains on N-17 decreases the activity of meleagrin alkaloids, whereas diterpene substitution on the imidazole ring enhances the cytotoxic activity. Furthermore, on exploring the potential cytotoxic mechanisms of **63** and **64**, it was found that **64** induced HL-60 cell apoptosis, whereas **63** arrested the cell cycle through G<sub>2</sub>/M phase at 5 and 10  $\mu M$  concentrations (Lin, Feng, et al. 2010).

Talathermophilins represent a group of prenylated indole alkaloids featuring a diketopiperazine moiety in their structures. From the thermophilic fungal strain *Talaromyces thermophilus* YM3-4, collected from Tengchong hot springs, China, three new talathermophilins (C–E; **65–67**) were isolated together with the known congeners **69** and **70**, in addition to cyclo(glycyltryptophyl) (**68**), which was unprecedently reported as a natural product (Guo et al. 2011). Based on a postulated biosynthetic pathway, talathermophilins are considered to be precursors of other indole alkaloids such as echinulins and notoamides, which are fungal metabolites from terrestrial and marine isolates (Guo et al. 2011). This finding validates thermophilic fungi as a potential source of novel natural products with interesting structures and potential pharmacological activities, which could complement the metabolite libraries of fungi living at ambient temperatures.

Azonazine (**71**), a unique hexacyclic dipeptide featuring a diketopiperazine moiety, was isolated from a Hawaiian marine sediment-derived fungus *Aspergillus insulicola* (Wu et al. 2010). In the cytotoxicity assay, azonazine (**71**) was inactive when tested against the PC3, MCF-7, and RAW 264.7 cancer cell lines at concentrations up to 100  $\mu M$ . However, **71** exhibited anti-inflammatory activity through the inhibition of NF- $\kappa$ B luciferase ( $IC_{50} = 8.37 \mu M$ ) and the production of nitrile ( $IC_{50} = 13.70 \mu M$ ) compared to celastrol ( $IC_{50} = 0.3 \mu M$ ) as a positive standard (Wu et al. 2010).

#### 4.3.3 PEPTIDES

From the marine-derived fungal strain ZLN-60, which was identified as *Aspergillus versicolor*, two new cyclic pentapeptides, versicotides A (**72**) and B (**73**), were isolated (Zhou, Gao, et al. 2011). Structurally, **72** and **73** were recognized as new cyclic pentapeptides possessing one L-alanine residue, two anthranilic acid (2-aminobenzoic acid) residues, and two *N*-methyl-L-alanine residues (Zhou, Gao, et al. 2011). Among the cyclic pentapeptides, versicotides A (**72**) and B (**73**) are the first examples featuring

**FIGURE 4.2** Chemical structures of compounds 33–58.

2-aminobenzoic acid residues in their structures. Both **72** and **73** were subjected to *in vitro* cytotoxicity assay against the P388, BEL-7402, HL-60, and A549 cancer cell lines; however, no activity was observed (Zhou, Gao, et al. 2011).

A new cyclodepsipeptide designated EGM-556 (**74**) was isolated from a marine sediment-derived fungus *Microascus* sp. from Florida. Biosynthetically, the compound is of hybrid origin and the biosynthesis was turned on using the histone deacetylase (HDAC) inhibitor suberoylanilide hydroxamic acid (SAHA) (Vervoort, Drašković, and Crews 2011). The discovery of **74** represents an interesting outcome of expressing the hitherto silent HPN, hybrid polyketide synthase (PKS)/nonribosomal peptide synthase (NRPS), biosynthetic genes that can be implemented in the biosynthesis of structurally similar marine natural products such as the potent actin inhibitor jaspamide (jasplakinolide, **75**) (Vervoort, Drašković, and Crews 2011).

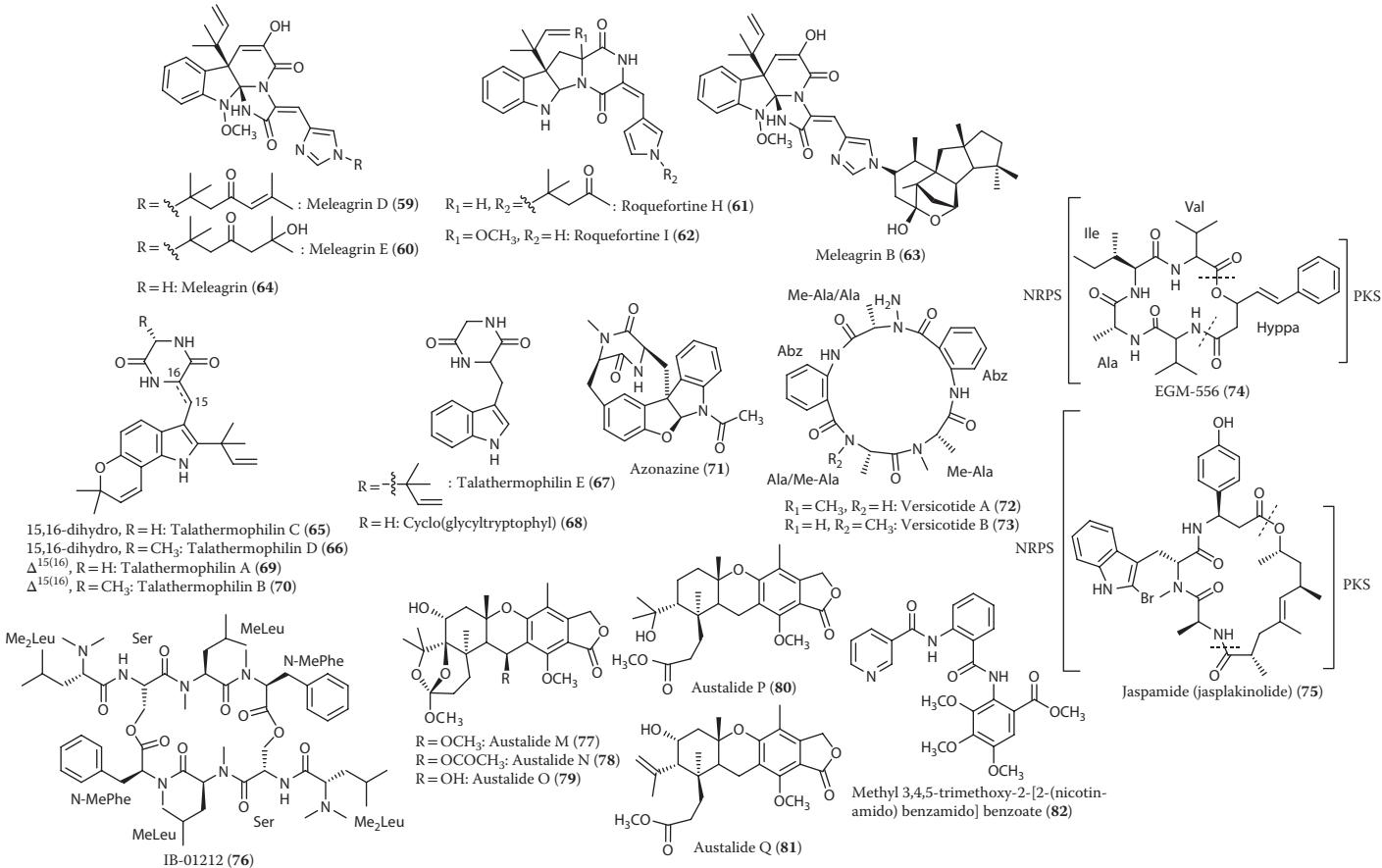
The cyclodepsipeptide IB-01212 (**76**) was isolated from the marine fungus *Clonostachys* sp. ESNA-A009 and was identified as a cytotoxic metabolite when evaluated using a panel of 14 different human tumor cell lines; it showed particular activity against the LN-caP, SK-BR3, HT29, and HELA cell lines with IC<sub>50</sub> values on the order of 10<sup>-8</sup> M (Cruz et al. 2006). Recently, IB-01212 (**76**) was successfully synthesized using a solid-phase synthesis protocol and assessed for its leishmanicidal activity at a low micromolar range of concentrations on two forms of the parasite (Luque-Ortega et al. 2010). Mechanistically, it was found that **76** acts through an apoptotic-like process by inducing a mitochondrial dysfunction, finally causing the death of the parasite (Luque-Ortega et al. 2010). Based on a comparative study with other IB-01212 surrogates, it was found that cycle size, preservation of the C-2 symmetry, and the nature of the bond between the two tetrapeptide halves may influence the leishmanicidal activity of this compound (Luque-Ortega et al. 2010).

#### 4.4 POLYKETIDES AND PRENYLATED POLYKETIDES (MERO TERPENOID)

From the Mediterranean sponge *Tethya aurantium* collected near the shores of Italy, a fungal isolate of the genus *Aspergillus* was obtained, and from its extract five new prenylated polyketides (meroterpenoids), designated as austalides M–Q (**77–81**), were isolated (Zhou, Mádi, et al. 2011). In addition to the austalides, eight further known compounds were reported from the same extract (Zhou, Mádi, et al. 2011). All the isolated fungal metabolites were assessed for their antiproliferative activity against murine cancer cell line L5178Y. However, none of the isolated austalides (**77–81**) revealed potential activity in this assay; only methyl 3,4,5-trimethoxy-2-[2-(nicotinamido)benzamido]benzoate (**82**) exhibited pronounced activity with an IC<sub>50</sub> value of 0.2 μM (Zhou, Mádi, et al. 2011).

The endophytic fungus *Aspergillus tubingensis* (GX1-5E) isolated from the radix of the Chinese mangrove plant *Pongamia pinnata* produced four new dimeric naphtha-γ-pyrone, which were designated as rubasperones D–G (**83–86**), together with four known monomeric naphtha-γ-pyrone, TMC 256 A1 (**87**), rubrofusarin B, fonescin, and flavasperone (Huang, Xiao, et al. 2011). Rubrasperone G (**86**) was identified as an atropisomer of **85**. The structural relationship between the isolated dimeric naphtha-γ-pyrone and comaparvins (Ghaly, Melek, and Mabry 2009; Chovolou et al. 2011) and comantherins (Francesconi 1980), which were reported from marine echinoderms, lends support to the hypothesis that marine-derived fungi may be the actual source of these compounds. In an *in vitro* cytotoxicity assay, only TMC 256 A1 (**87**) exhibited moderate activity (IC<sub>50</sub> values between 20 and 48 μM) when evaluated against six different human tumor cell lines (Huang, Xiao, et al. 2011).

Chloctanspirones A (**88**) and B (**89**), two novel chlorinated sorbicillinoids, together with their quasi precursors, terrestrols K (**90**) and L (**91**), were isolated from the marine sediment-derived fungus *Penicillium terrestre* (Li, Li, et al. 2011). Compounds **88** and **89** featured an unprecedented bicyclo[2.2.2]octane-2-spirocyclohexane skeleton. The cytotoxic activity of **88–91** was investigated using HL-60 and A549 cells. Chloctanspirone A (**88**) exhibited antiproliferative activity against the HL-60 and A549 cell lines with IC<sub>50</sub> values of 9.2 and 39.7 μM, respectively, whereas **89** was active only against HL-60 cells (IC<sub>50</sub> = 37.8 μM) (Li, Li, et al. 2011).



**FIGURE 4.3** Chemical structures of compounds 59–82.

The marine endophytic fungus *Coniothyrium cereale* produces the structurally rare polyketide-type alkaloids (–)-cereolactam (**92**) and (–)-cereoaldomine (**94**), possessing a lactam and an imine functionality, respectively, as well as the related metabolite (–)-tryptethelone (**93**) (Elsebai, Natesan, et al. 2011). Compounds **92** and **94** exhibited selective inhibitory activity of human leukocyte elastase with IC<sub>50</sub> values of 9.3 and 3.0 μM, respectively, whereas **93** was found to be inhibitory toward *Mycobacterium phlei*, *Staphylococcus aureus*, and *Escherichia coli* and cytotoxic against mouse fibroblast cells (IC<sub>50</sub> = 7.5 μM) (Elsebai, Natesan, et al. 2011).

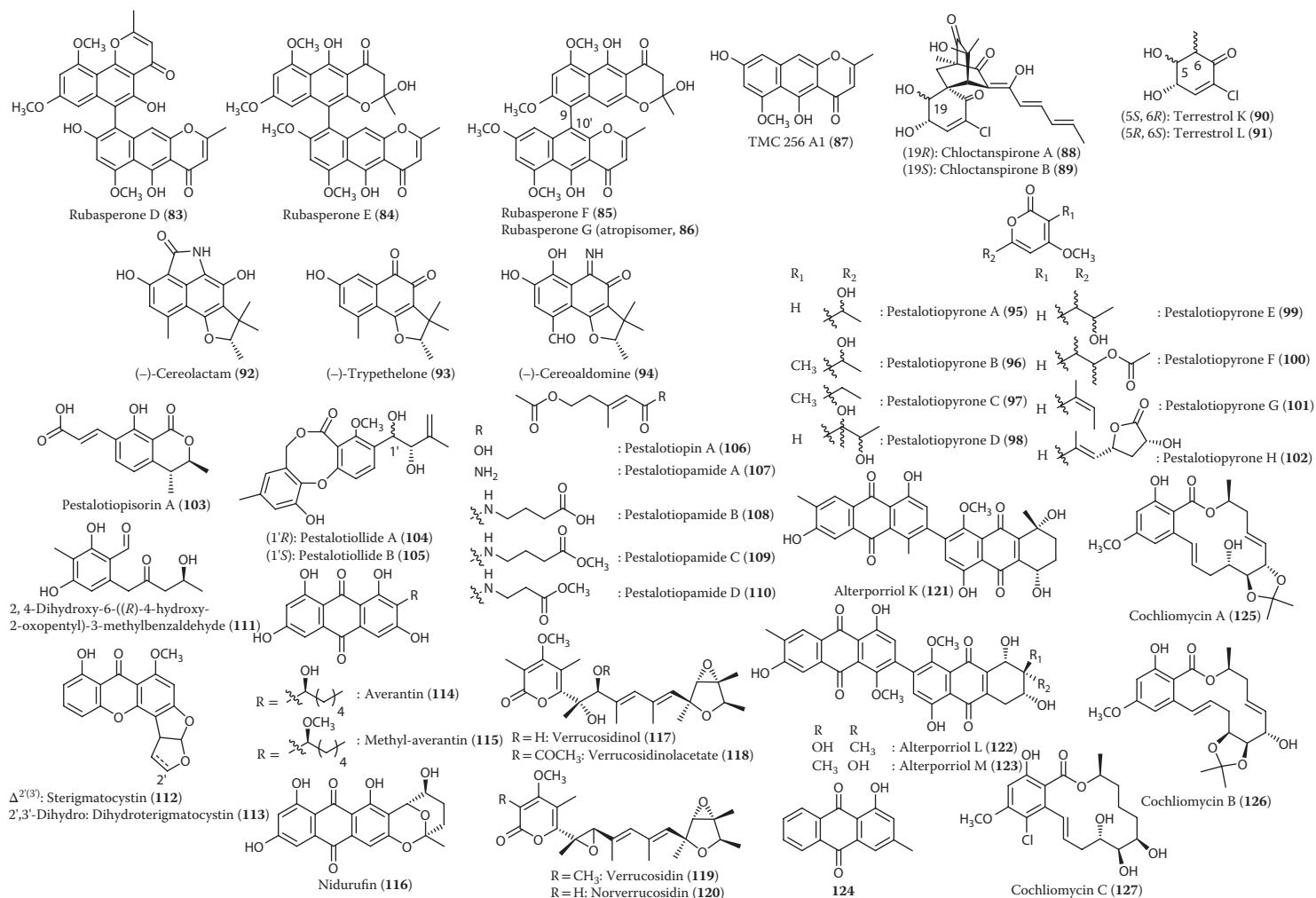
The fungus species *Pestalotiopsis* JCM2A4, isolated from the Chinese mangrove plant *Rhizophora mucronata*, proved to be particularly productive and yielded over 20 different compounds with 17 of them being new natural products including chromones, cytosporones, and coumarins (Xu et al. 2009a, 2009b; Xu, Ebada, and Proksch 2010). Recently, a detailed chemical investigation of the minor metabolites of the same fungal strain afforded 16 new compounds of polyketide origin, including pestalotiopyrones A–H (**95–102**), pestalotiopisoriin A (**103**), pestalotiollides A (**104**) and B (**105**), pestalotiopin A (**106**), and four amides (pestalotiopamides A–D, **107–110**), along with three known compounds, that is, nigrosporapyrone D, 2-anhydromevalonic acid, and *p*-hydroxybenzaldehyde (Xu et al. 2011). All compounds isolated were evaluated for their antimicrobial and antiproliferative activities; however, none of the tested compounds showed significant activity in the assays conducted (Xu et al. 2011).

The fungus *A. versicolor*, which is derived from the marine sponge *Petrosia* sp. collected off the coast of Jeju Island, Korea, produced an aromatic polyketide derivative, 2,4-dihydroxy-6-((R)-4-hydroxy-2-oxopentyl)-3-methylbenzaldehyde (**111**); two xanthones, sterigmatocystin (**112**) and its dihydro derivative (**113**); and five anthraquinones including averantin (**114**), methyl-averantin (**115**), and nidurufin (**116**) (Lee et al. 2010). Compounds **112** and **114–116** exhibited potent antiproliferative activity against five human solid tumor cell lines (A549, SK-OV-3, SK-MEL-2, XF-498, and HCT-15) with IC<sub>50</sub> values ranging between 1.06 and 14.2 μM. Furthermore, averantin (**114**) and nidurufin (**116**) displayed antibacterial activity against gram-positive clinical isolates with MIC values of 2.1–16.1 μM (Lee et al. 2010).

Two pyrone-type polyketides, verrucosidinol (**117**) and its acetate derivative (**118**), were isolated together with potent neurotoxins, verrucosidin (**119**) and its demethylated derivative (**120**), from the marine mud-derived fungus *Penicillium aurantiogriseum* (Yu et al. 2010). None of the compounds (**117–120**) showed significant antimicrobial activity when assessed against methicillin-resistant *Staphylococcus aureus* (MRSA), *Pseudomonas aeruginosa*, *Candida albicans* SC5314 or synergistic antifungal activity with ketoconazole as a positive standard (Yu et al. 2010).

Three new bisanthraquinone derivatives, alterporriols K (**121**), L (**122**), and M (**123**), along with six known compounds were obtained from extracts of the endophytic fungus *Alternaria* sp. ZJ9-6B, which is isolated from the mangrove plant *Aegiceras corniculatum* collected in the South China Sea (Huang, Pan, et al. 2011). Compounds **121–123** were the first isolated alterporriols featuring a C-2-C-2' linkage. In addition, crystallographic data of tetrahydroaltersolanol B were reported for the first time. In the cytotoxicity assay toward MDA-MB-435 and MCF-7 cell lines, alterporriol K (**121**) and alterporriol L (**122**) exhibited moderate activity with IC<sub>50</sub> values between 13.1 and 29.1 μM (Huang, Pan, et al. 2011). Recently, an in-depth study was directed toward investigating the cytotoxic and anticancer mechanisms of alterporriol L (**122**); results revealed that it could effectively inhibit cellular growth and proliferation in a dose-dependent manner (Huang et al. 2012). Moreover, alterporriol L was able to change reactive oxygen species, mitochondrial membrane potential, and cytosolic free calcium levels, leading to mitochondrial destruction, thereby inducing cancer cell apoptosis or necrosis (Huang et al. 2012).

The mangrove endophytic fungi *Halorosellinia* sp. (No. 1403) and *Guignardia* sp. (No. 4382) produced 14 anthracenedione derivatives; they were evaluated for their cytotoxic activity toward KB and KBv200 cells (Zhang et al. 2010). Some of them potently suppressed the growth of the investigated cell lines. Compound **124** displayed the strongest cytotoxicity with IC<sub>50</sub> values of 3.17 and 3.21 μM to KB and KBv200 cells, respectively (Zhang et al. 2010).

**FIGURE 4.4** Chemical structures of compounds 83–127.

The fungus *Cochliobolus lunatus*, which is obtained from the Chinese gorgonian *Dichotella gemmacea*, produced three new 14-membered resorcylic acid lactones, cochliomycins A–C (**125–127**). Two of them possess a rare natural acetonide group, whereas one features a 5-chloro-substituted lactone moiety (Shao et al. 2011). Antifouling activity was assessed for the first time for this class of metabolites using the larvae of the barnacle *Balanus amphitrite*. The results revealed that the resorcylic acid lactones with acetonide groups exhibited three- to fourfold higher activity than other derivatives lacking such a functionality. Moreover, the acetylated derivatives revealed lower activity compared to their nonacetylated congeners; this implied that the hydroxyl groups probably have an influence on antifouling activity (Shao et al. 2011). Interestingly, in the antimicrobial and cytotoxicity assays, none of the isolated resorcylic acid lactone derivatives with antifouling activity displayed significant activity. Thus, these compounds may deserve further investigation as lead structures for the discovery of new antifouling molecules (Shao et al. 2011).

Eight new  $\alpha$ -pyrone derivatives, that is, nigerapyrones A–H (**128–135**), along with two known congeners, asnipyrone A (**136**) and B (**137**), were isolated from *Aspergillus niger* MA-132, an endophytic fungus obtained from the fresh tissue of the marine mangrove plant *Avicennia marina* (Liu, Li, Meng, et al. 2011). The geometries and structures of **136** and **137** were explicitly determined. In cytotoxicity assays against eight tumor cell lines, compounds **129**, **131**, **132**, and **136** showed weak cytotoxicity against some of the tested cell lines; however, in the antimicrobial activity assay against two bacteria and four plant-pathogenic fungi, no obvious activity could be recognized for these compounds (Liu, Li, Meng, et al. 2011).

The root soil fungus *Aspergillus taichungensis* ZHN-7-07, which is isolated from the mangrove plant *Acrostichum aureum*, produced six new prenylated polyhydroxy-*p*-terphenyl metabolites, that is, prenylterphenyllins A–C (**138–140**), along with their parent compound (**145**); prenylcandidusins A–C (**142–144**); and one new polyhydroxy-*p*-terphenyl featuring a simple tricyclic C-18 skeleton, designated as 4''-dehydro-3-hydroxyterphenyllin (**141**), together with seven other known analogs (Cai et al. 2011). In the cytotoxicity assay using the HL-60, A549, and P-388 cell lines, only **138** and **145** exhibited moderate activity against all three cell lines ( $IC_{50}$  = 1.53–10.90  $\mu$ M), whereas **141** and **143** displayed moderate activity against the P-388 cell line only ( $IC_{50}$  of 2.70 and 1.57  $\mu$ M, respectively) (Cai et al. 2011).

A detailed chemical investigation of the marine sediment-derived fungus *Penicillium commune* QSD-17 afforded for the first time six new azaphilone derivatives, that is, comazaphilones A–F (**146–151**) (Gao, Li, Zhang, et al. 2011). The antimicrobial and cytotoxic activities of the six azaphilones against four bacteria, one pathogenic fungus, and seven tumor cell lines were evaluated, and the results revealed that comazaphilones C–E (**148–150**) displayed potent antimicrobial activity, whereas comazaphilones D–F (**149–151**) exhibited cytotoxic activity against the human pancreatic tumor cell line SW1990 (Gao, Li, Zhang, et al. 2011). Based on these results, the preliminary structure–activity relationships (SARs) indicated that the double bond at C-10 and the location of the orsellinic acid unit at C-6 in these azaphilones are important for their antimicrobial and cytotoxic activities, respectively (Gao, Li, Zhang, et al. 2011).

Five new polyketides, that is, fusaranthraquinone (**152**), fusarnaphthoquinones A–C (**153–155**), and fusarone (**156**), along with 18 known compounds were isolated from the sea-fan fungi *Fusarium* sp. PSU-F14 and PSU-F135 (Trisawan et al. 2010). The isolated compounds including anthraquinone, cyclopentanone, and naphthoquinone derivatives were investigated for their antibacterial, antifungal, antimycobacterial, and antimalarial activities; however, only some compounds showed mild-to-moderate activities in these assays (Trisawan et al. 2010). Interestingly, in the cytotoxic activity assay toward KB, MCF-7, and Vero cells, bostrycin (**157**) exhibited strong activity to all the tested cell lines with  $IC_{50}$  values of 0.9, 2.7, and 4.2  $\mu$ M, respectively (Trisawan et al. 2010). Furthermore, anhydrofusarubin (**158**) showed selective cytotoxicity to the MCF-7 and KB cell lines with  $IC_{50}$  values of 0.9 and 2.0  $\mu$ M, respectively. Interestingly, both **157** and **158** feature a common 1,4-naphthoquinone skeleton, which may be responsible for their cytotoxic activities, whereas the hydropyran unit in **158** might contribute to its selectivity (Trisawan et al. 2010).

Together with the dioxopiperazine alkaloid, 12-demethyl-12-oxo-eurotechinulin B (**55**), isolated from the endophytic fungus *Eurotium rubrum* derived from the semi-mangrove plant *Hibiscus tiliaceus*, the new anthraquinone derivative 9-dehydroxyeurotinone (**159**) was obtained (Yan et al. 2012). In the antimicrobial activity assay, compound **159** showed weak antibacterial activity against *E. coli* with an inhibition zone of 7.0 mm at 100 µg/disk compared to amphotericin B with an inhibition zone of 11.0 mm at 20 µg/disk; but in the cytotoxicity assay, it displayed a weak selective cytotoxic activity toward the SW1990 ( $IC_{50} = 92 \mu\text{M}$ ) cell line when evaluated against seven different cancer cell lines (Yan et al. 2012).

The fungus *Tritirachium* sp. SpB081112MEf2, which is isolated from the Okinawan marine sponge *Pseudoceratina purpurea*, produced three new xanthoquinodin-like compounds, that is, JBIR-97, JBIR-98, and JBIR-99 (**160–162**) (Ueda, Takagi, and Shin-ya 2010). Based on the slight differences in nuclear magnetic resonance (NMR) spectral data of compounds **160** and **161**, particularly at H-4', H-11, H-5', C-1', and C-10a', they were suggested to be diastereomers at C-10' and/or bridge sites at C-2' and C-4' (Ueda, Takagi, and Shin-ya 2010). Cytotoxic activities of JBIR-97, JBIR-98, and JBIR-99 (**160–162**) against HeLa and ACC-MESO-1 human cancer cell lines were evaluated, and they revealed cytotoxic effects against HeLa cells ( $IC_{50} = 11, 17$ , and  $17 \mu\text{M}$ , respectively) and against ACC-MESO-1 cells ( $IC_{50} = 31, 63$ , and  $59 \mu\text{M}$ , respectively) (Ueda, Takagi, and Shin-ya 2010).

Four new polyketides, that is, paecilocins A–D (**163–166**), were purified from the fungus *Paecilomyces variotii*, which is associated with the jellyfish *Nemopilema nomurai* (Liu, Li, Kim, et al. 2011). When evaluated for their antibacterial activity against pathogenic isolates including MRSA 3089, and multidrug-resistant *Vibrio parahemolyticus* 7001, paecilocins B (**164**) and C (**165**) displayed mild-to-moderate activity against both isolates with MIC values in the range 16–129 µM (Liu, Li, Kim, et al. 2011).

Asperdemin (**167**), a new meroterpenoid, was isolated together with two known compounds, diocrinol and viridicatol, from the marine fungus *A. versicolor* (Yurchenko et al. 2010). The absolute stereochemistry of asperdemin was determined by the modified Mosher's method. In addition, **167** exhibited weak cytostatic and membranolytic activities toward the development of embryos of the sea urchin *Stegylocentrotus nudus* at a concentration of 6.4 mM. It further induced the hemolysis of human erythrocytes at  $EC_{50} = 1.15 \text{ mM}$  (Yurchenko et al. 2010).

The fungus *Aspergillus insuetus* (OY-207), which is derived from the Mediterranean sponge *Psammocinia* sp., produced three novel meroterpenoids, that is, insuetolides A–C (**168–170**), together with four drimane sesquiterpenes including one new derivative (Cohen et al. 2011). Insuetolides feature a new carbon skeleton derived from the cyclization of a farnesyl unit and 3,5-dimethylorsellinic acid and are thought to arise from the same drimane-type precursor leading to andibenin, via an additional oxidation step prior to the last step of condensation to the pentacyclic intermediate (Cohen et al. 2011). Insuetolide A (**168**) exhibited weak antifungal activity toward *Neurospora crassa* with an MIC value of 140 µM, whereas insuetolide C (**170**) displayed mild cytotoxicity toward MOLT-4 human leukemia cells ( $IC_{50} = 117.4 \text{ mM}$ ) (Cohen et al. 2011).

Aspergillusones A (**171**) and B (**172**), two new hydrogenated xanthone derivatives, together with 13 other compounds were isolated from the sea fan–derived fungus *Aspergillus sydowii* PSU-F154 including three new sesquiterpenes (Trisuwant et al. 2011). All isolated compounds were assessed for their radical-scavenging activity using DPPH assay. Results revealed that only the dihydroxanthone derivative (**173**) showed antioxidant activity, with an  $IC_{50}$  value of  $17 \mu\text{M}$ , compared to the standard butylated hydroxyanisole ( $IC_{50} = 0.13 \mu\text{M}$ ), whereas the other tested derivatives remained inactive (Trisuwant et al. 2011). Compounds **171–173** are structurally similar. This may point to the importance of these functionalities for activity (Trisuwant et al. 2011). However, **173** differs from the isolated xanthone derivatives in the absence of a double bond at the C7-C8 position, implying that the planar structure of xanthones might diminish their antioxidant properties (Trisuwant et al. 2011).

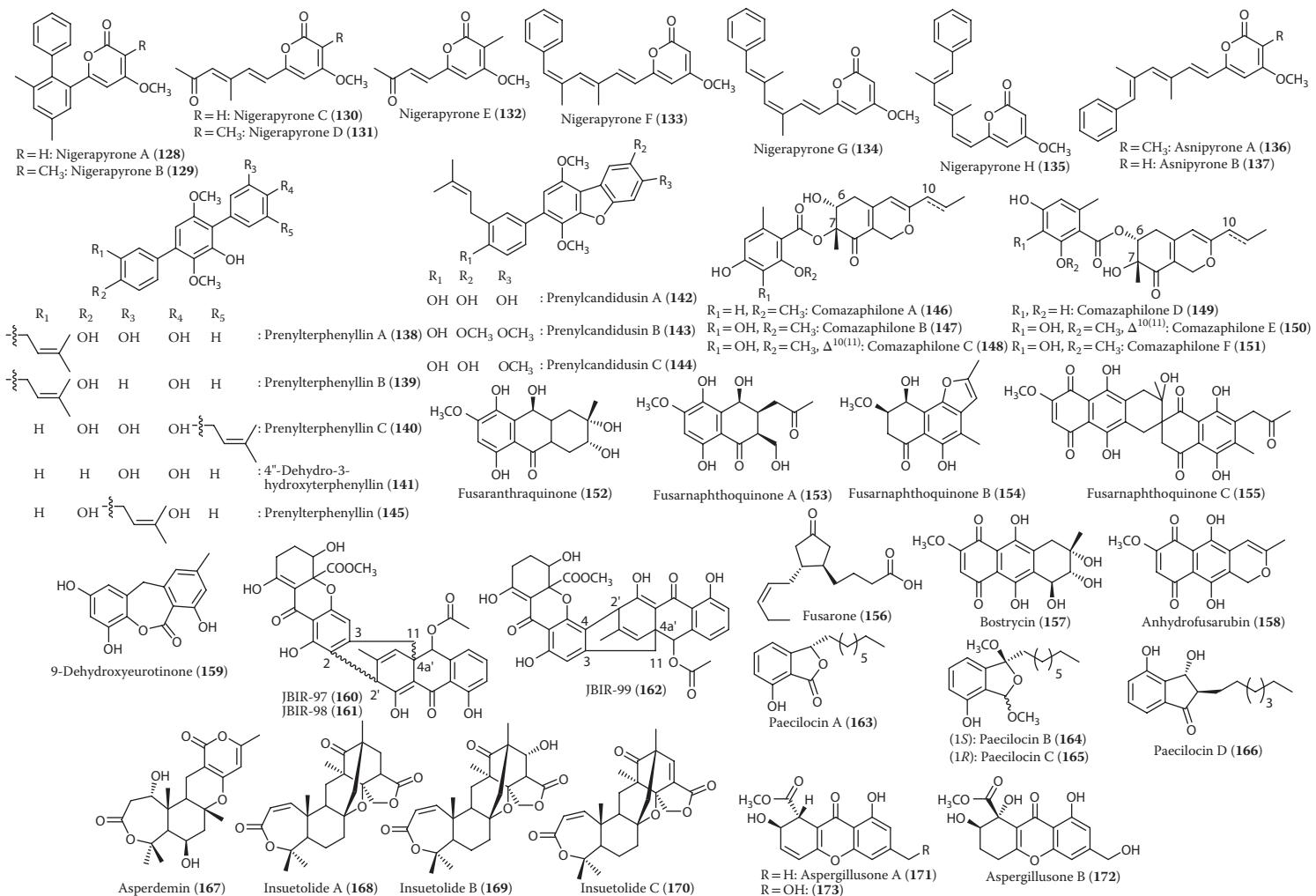


FIGURE 4.5 Chemical structures of compounds 128–173.

#### 4.5 TERPENOIDS AND LIPIDS

In addition to aspergillusones A (**171**) and B (**172**) and other compounds isolated from the sea fan–derived fungus *A. sydowii* PSU-F154, three new sesquiterpenes, that is, aspergillusenes A (**174**) and B (**175**) and (+)-(7S)-7-O-methylsydonic acid (**176**), together with the known compound (+)-(7S)-sydonic acid were isolated from the same extract (Trisuwan et al. 2011). In the antioxidant activity assay, neither the newly isolated sesquiterpenes (**174–176**) nor (+)-(7S)-sydonic acid revealed obvious activity (Trisuwan et al. 2011).

Along with the novel meroterpenoids, insuetolides A–C (**168–170**), which are isolated from a Mediterranean sponge–derived fungus *A. insuetus* (OY-207), the new drimane sesquiterpene (*E*)-6-(4'-hydroxy-2'-butenoyl)-strobilactone A (**177**) that was obtained from the same EtOAc extract exhibited mild cytotoxicity toward MOLT-4 human leukemia cells ( $IC_{50} = 143$  mM) (Cohen et al. 2011).

Five new ophiobolin-type sesterterpenoids (**178–182**) were isolated from *A. ustus*, which was obtained from the Mediterranean sponge *S. domuncula* (Liu, Edrada-Ebel, et al. 2011). The isolated ophiobolin-type sesterterpenoids were evaluated for their cytotoxic activity against the murine lymphoma L5178Y cell line at a concentration of 10 µg/mL; however, none of them inhibited cellular growth by more than 10%–20% at that concentration (Liu, Edrada-Ebel, et al. 2011).

The first naturally occurring 7-nor-ergosteroid with an unusual pentalactone B-ring moiety, that is, 7-nor-ergosterolide (**183**), was purified from the endophytic fungus *A. ochraceus* EN-31, which is isolated from the marine brown alga *Sargassum kjellmanianum*, together with two new steroid derivatives, that is, 3 $\beta$ -hydroxyergosta-8,24(28)-dien-7-one (**184**) and its 11 $\alpha$ -hydroxy derivative (**185**) (Cui, Li, Meng, et al. 2010). In addition, nine known related steroids were characterized from the same extract. The absolute stereochemistry of the new steroids (**183–185**) was determined by implementing the modified Mosher's method. In the cytotoxicity assay toward the NCI-H460, SMMC-7721, and SW1990 cancer cell lines, 7-nor-ergosterolide (**183**) exhibited antiproliferative activity with  $IC_{50}$  values of 12.1, 17.0, and 67.6 µM, respectively, whereas **185** showed cytotoxicity against the SMMC-7721 cell line with an  $IC_{50}$  value of 65.4 µM (Cui, Li, Meng, et al. 2010).

Acremostrictin (**186**), a novel highly oxygenated tricyclic lactone metabolite, was isolated from *Acremonium strictum*, a marine fungus collected from an unidentified Choristida sponge off the coast of Korea (Julianti et al. 2011). Acremostrictin features an unprecedented skeleton whose structure was unambiguously elucidated based on combined spectroscopic and x-ray crystallographic analyses. In the antimicrobial and antioxidant activity assays, **186** revealed weak and moderate activities, respectively, whereas it was inactive in the antiproliferative activity assay against the K562 cell line (Julianti et al. 2011).

A detailed chemical investigation of the methanolic extract of the marine fungus *Arthrinium* sp. derived from the Mediterranean sponge *Geodia cydonium* afforded four novel diterpenoids, arthrinins A–D (**187–190**), and one new diterpenoid, myrocin D (**191**), in addition to five known compounds including myrocin A and two xanthone derivatives, norlichexanthone and anomalin A (Ebada et al. 2011). The structures of arthrinins A–D (**187–190**) were recognized as being of hybrid origin and being derived from cleistanthane and pimarane diterpenes. The absolute configuration of arthrinins A–D (**187–190**) was established by the modified Mosher's method and by Rotating frame Overhauser enhancement spectroscopy (ROESY) spectra. The antiproliferative activity of the isolated compounds was evaluated toward four different tumor cell lines, that is, the L5178Y, K562, A2780, and A2780CisR cell lines. Results revealed that norlichexanthone and anomalin showed the strongest activities ( $IC_{50}$  values of 0.40–74.0 µM) (Ebada et al. 2011). These findings are in accordance with the results from protein kinase activity assays that included aurora-B, PIM-1, and VEGF-R2 kinases, which were inhibited by norlichexanthone and anomalin A with  $IC_{50}$  values between 0.3 and 11.7 µM (Ebada et al. 2011). Furthermore, in the *in vitro* angiogenesis assay against HUVECs sprouting induced by VEGF-A, myrocins D (**191**) and A and anomalin A inhibited endothelial cell sprouting with  $IC_{50}$  values of 2.6, 3.7, and 1.8 µM, respectively (Ebada et al. 2011).

In a parallel study, four new diterpenes (192–194), were isolated from the marine sponge-derived fungus *Arthrinium sacchari* (Tsukada et al. 2011). In addition, one new isocoumarin, that is, decarboxyhydroxycitrinone (195), and three known compounds were isolated from the same extract (Tsukada et al. 2011). Antiangiogenic activities of compounds **192–195** were assessed toward HUVECs and HUAECs using MTT assay. Decarboxyhydroxycitrinone (195), myrocin A, and libertellenone C showed weak antiproliferative activities against both cell lines (Tsukada et al. 2011). In contrast, cytochalasin E (12) markedly inhibited proliferation of HUVECs and HUAECs with higher activities than Ki8751 ( $IC_{50} = 1.0\text{--}2.0 \mu\text{M}$ ), which was used as a positive control (Tsukada et al. 2011).

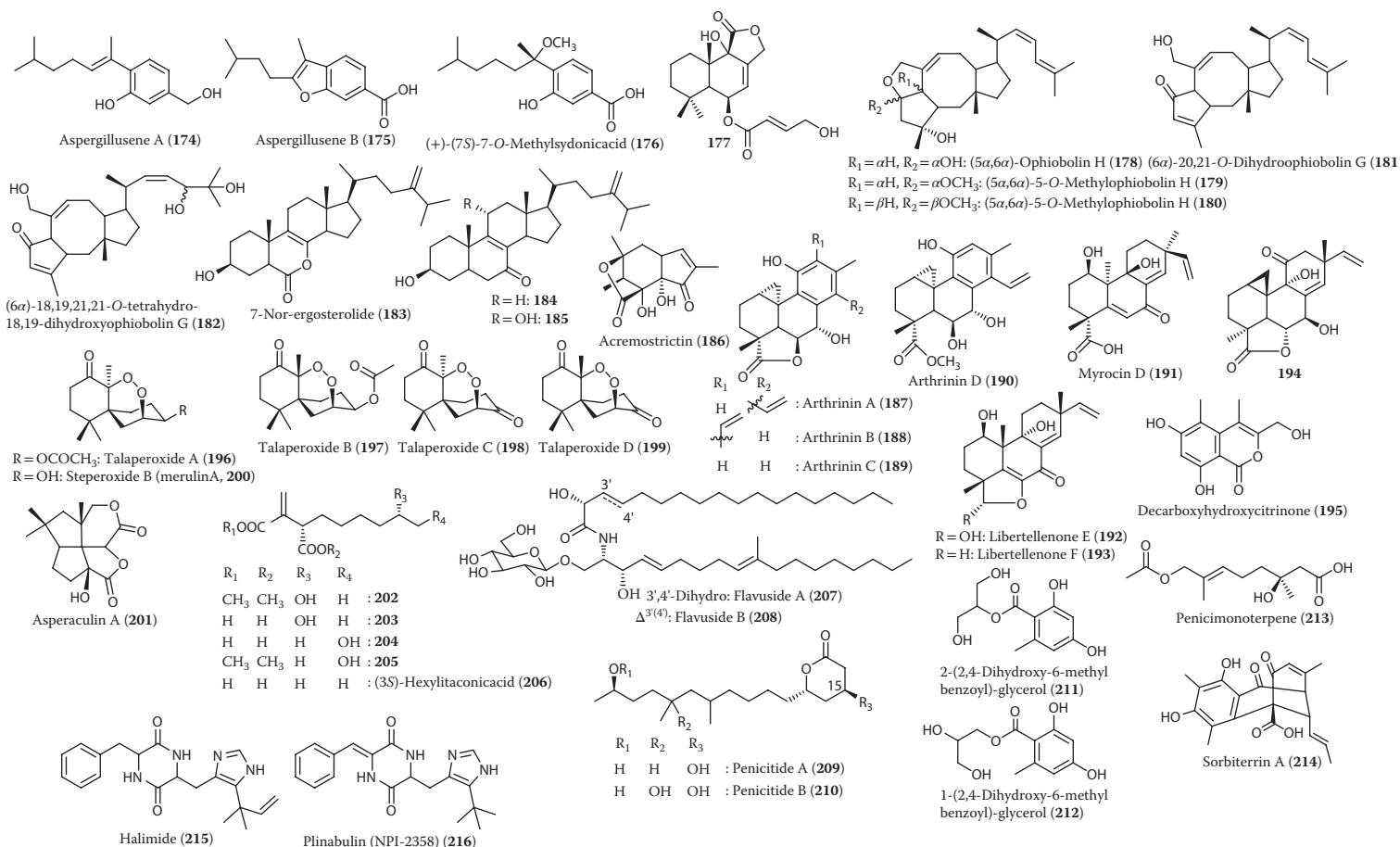
The endophytic fungus *Talaromyces flavus*, which is isolated from leaves of the Chinese mangrove plant *Sonneratia apetala*, produced four new norsesterpenoid peroxides, that is, talaperoxides A–D (**196–199**), together with the known analog steperoxide B (merulin A, **200**) (Li, Huang, et al. 2011). The absolute configurations of compounds **196**, **197**, and **200** were established by single-crystal x-ray crystallography (Li, Huang, et al. 2011). Cytotoxic activities of compounds **196–200** were evaluated *in vitro* against five human cancer cell lines, that is, MCF-7, MDA-MB-435, HepG2, HeLa, and PC-3, and results revealed that only talaperoxides B (**197**) and D (**199**) were cytotoxic against all the tested cancer cell lines with  $IC_{50}$  values between 2.8 and 6.4  $\mu\text{M}$  (Li, Huang, et al. 2011).

A novel sesquiterpenoid featuring a [5,5,5,6]fenestrane ring system, asperaculin A (**201**), was isolated from the fungus *Aspergillus aculeatus* CRI323-04, which is derived from the marine sponge *Xestospongia testudinaria* collected from Phi Phi Island, Thailand (Ingavat et al. 2011). In a cytotoxicity assay, asperaculin A (**201**) did not show obvious activity at concentrations up to 180  $\mu\text{M}$  against the HepG2, MOLT-3, A549, and HuCCA-1 cancer cell lines (Ingavat et al. 2011).

From the fungus *Penicillium* sp. (J05B-3-F-1), which is isolated from the marine sponge *Stelletta* sp. collected from the coast of Jeju island, Korea, four new hexylitaconic acid derivatives (**202–205**), along with (3S)-hexylitaconic acid (**206**) were identified (Li, Zhang, et al. 2011). Compounds **202–206** were evaluated for their anti-inflammatory activity using their inhibitory effects on the production of major proinflammatory mediators (nitric oxide [NO], interleukin [IL]-6, tumor necrosis factor [TNF]- $\alpha$ , and IL-1 $\beta$ ) in murine macrophage cells. Compounds **202** and **205** showed weak inhibition of IL-1 $\beta$  production at a concentration of 200  $\mu\text{M}$ . The ester forms of **202** and **205** showed higher inhibition of IL-6 and IL-1 $\beta$  expression than the corresponding free acids (**203**, **204**, and **206**), which might be attributed to the higher lipophilicity of the ester form enhancing their cellular permeability (Li, Zhang, et al. 2011).

The epiphytic fungus *Aspergillus flavus*, which is isolated from the green alga *Codium fragile* collected in GeoMun Island, Korea, produced two new antibacterial cerebrosides, that is, flavusides A (**207**) and B (**208**), in addition to four other known metabolites (Yang et al. 2011). Compounds **207** and **208** displayed mild-to-moderate antibacterial activity against *S. aureus* ( $MIC = 20.7 \mu\text{M}$ ), methicillin-resistant *S. aureus*, and multidrug-resistant *S. aureus* ( $MIC = 41.3 \mu\text{M}$ ) (Yang et al. 2011).

Chemical investigation of the endophytic fungus *Penicillium chrysogenum* QEN-24S, which is isolated from an unidentified Chinese marine red algal species of the genus *Laurencia* and which exhibits inhibitory activity against the pathogen *Alternaria brassicae* in dual culture test, afforded four new (**209–211** and **213**) and one known (**212**) secondary metabolites (Gao, Li, Du, et al. 2011). The new metabolites were identified as two polyketide sesquiterpenes, penicitides A (**209**) and B (**210**); two glycerol derivatives, 2-(2,4-dihydroxy-6-methylbenzoyl)-glycerol (**211**) and 1-(2,4-dihydroxy-6-methylbenzoyl)-glycerol (**212**); and one new monoterpene derivative, penicimonoterpenone (**213**) (Gao, Li, Du, et al. 2011). Interestingly, penicitides A (**209**) and B (**210**) featured a unique 10-hydroxy- or 7,10-dihydroxy-5,7-dimethylundecyl moiety substituting at C-5 of the  $\alpha$ -tetrahydropyrone ring, which is hitherto unprecedented among natural products (Gao, Li, Du, et al. 2011). The absolute configuration of C-15 in penicitide A (**209**) was determined to be *R* using the modified Mosher's method. In antifungal screening, penicimonoterpenone (**213**) displayed potent activity against the pathogen *A. brassicae* with an inhibition zone of 17 mm at a concentration of 20  $\mu\text{g}/\text{disk}$ , whereas **209** showed moderate activity with an inhibition zone of 6 mm at the same

**FIGURE 4.6** Chemical structures of compounds 174–216.

concentration. Furthermore, in the cytotoxic activity assay, penicitide A (**209**) exhibited weak activity against the HepG2 cell line with an IC<sub>50</sub> value of 102 µM, whereas the other compounds were inactive (Gao, Li, Du et al. 2011).

The marine sediment-derived fungus *Penicillium terrestris* produced a sorbicillin derivative, that is, sorbiterrin A (**214**), which featured a novel skeleton (Chen et al. 2012). The structure of **214**, including absolute configurations, was elucidated by analysis of NMR, MS data, and TDDFT CD calculations. Sorbiterrin A (**214**) displayed moderate AChE inhibitory effect with an IC<sub>50</sub> value of 70.2 µM (Chen et al. 2012).

## 4.6 CONCLUSIONS

Marine-derived fungi isolated from marine macroorganisms or from sediment as well as endophytic fungi from mangrove plants continue to be a prolific source of a plethora of natural products featuring unprecedented chemical skeletons and pharmacological activities. So far, marine-derived fungi have provided more than 1200 new natural products; some of them exhibit clinically relevant bioactivities. Probably the most important example is the tubulin-depolymerizing agent halimide (**215**), which chemically possesses a diketopiperazine moiety and was first isolated from the marine fungus *Aspergillus* sp. CNC-139 derived from the alga *Halimeda lacrimosa* collected in the Bahamas (Mayer et al. 2010). Halimide (**215**) was the lead structure for developing the closely related synthetic analog plinabulin (NPI-2358) (**216**), which is currently undergoing phase II clinical trials for patients with advanced non-small-cell lung cancer (Ebada and Proksch 2011). In the last few decades, natural products from marine-derived and endophytic fungi from mangrove plants have gained considerable attention, which have induced a tremendous increase in the number of isolated novel fungal metabolites with pronounced pharmacological activities. These outcomes together with the prominent discrepancy between the actual number of cultivated strains and the estimated biodiversity of marine-derived fungi have encouraged natural product chemists and pharmacologists alike to enthusiastically continue their efforts to search for novel metabolites and to extend the biological assays over the commonly assessed antimicrobial and cytotoxic activities in order to escalate the chance of discovering bioactive drug leads with pharmaceutical potential to treat, control, and/or relieve the global disease burden.

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# 5 Cytotoxic Briarane-Type Diterpenoids

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Xuefeng Zhou, and Yan Peng*

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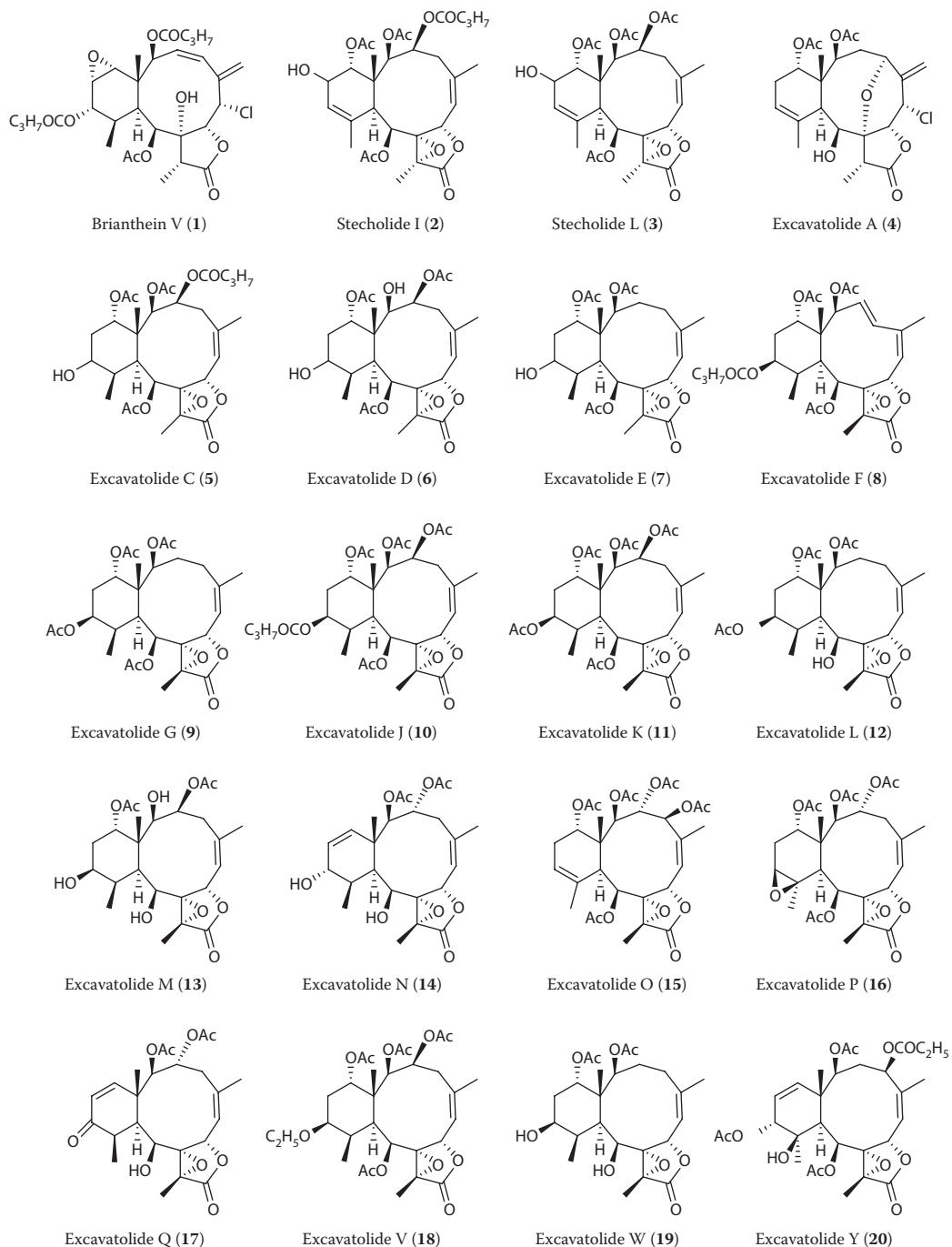
## 5.1 INTRODUCTION

Briarane diterpenoids are characterized by a bicyclic [8.4.0] ring system fused by a  $\gamma$ -lactone group, with high oxidization and esterification by all kinds of acyls, that is, acetyl, isovalerate, etc. These compounds exhibit a variety of biological activities such as cytotoxicity, anti-inflammatory activity, antivirus activity, insecticidal activity, immunomodulation, and antifouling activity. Because of their unique structures and interesting biological activities, significant efforts have gone into the discovery of new briarane diterpenoids. These efforts have culminated in the discovery of more than 600 briarane family members (Sung et al. 2005, 2011; Sung, Sheu, and Xu 2002; Sung, Sheu, et al. 2008) since the first briarane-type diterpenoid, briarane A, was isolated from the West Indian gorgonian *Briareum asbestinum* by Burks et al. in 1977 (Burks et al. 1977). Most of these briaranes possess a chlorine atom, an epoxy group, and a double bond as substituents.

## 5.2 CYTOTOXICITY

### 5.2.1 TOXICITY TO TUMOR CELL LINES

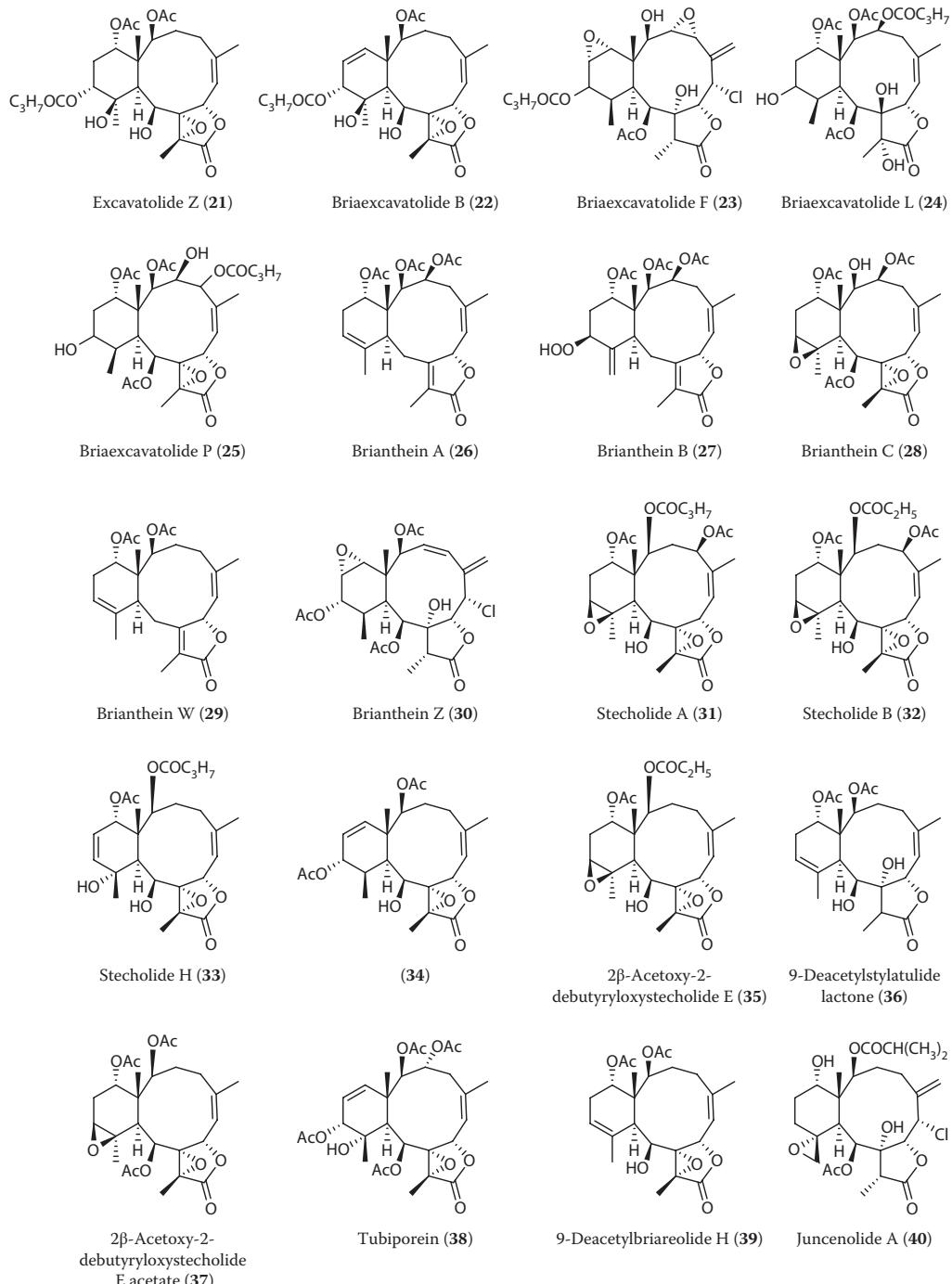
Brianthein V (**1**) (Figure 5.1) is a cytotoxic briarane from *Briareum asbestinum*, which was collected near Sandy Cay, Bahamas. The structure and absolute configuration of (**1**) were established by spectroscopic methods (infrared [IR], mass spectrometry [MS], and,  $^1\text{H}$  and  $^{13}\text{C}$  nuclear magnetic resonance [NMR]) and x-ray analyses. Brianthein V (**1**) showed *in vitro* cytotoxicity in the P-388 assay at 13  $\mu\text{g}/\text{mL}$  (Coval et al. 1988). Stecholides I (**2**) and L (**3**) were isolated from the Papua New Guinea gorgonian coral *B. excavatum*. The structures of these metabolites were established by interpretations of spectral analyses and conformational energy calculations. The structure, including the relative configuration of (**2**), was further confirmed by x-ray diffraction analyses. Stecholide I (**2**) showed *in vitro* cytotoxicity in the P-388 assay at 23  $\mu\text{g}/\text{mL}$  (Schmitz et al. 1993). Stecholide L (**3**) showed cytotoxicity against four tumor cell lines (P-388, A-549, HT-29, and MEL-28) at the  $\text{IC}_{50}$  levels of 10, 2.5, 5, and 5  $\mu\text{g}/\text{mL}$  (Schmitz et al. 1993; Rodriguez, Nieto, and Jimenez 1998). A series of briarane-type metabolites have been isolated from *B. excavatum*, which were collected along the coast of southern Taiwan; the Great Barrier Reef, Australia; and Sulawesi Island, Indonesia. Excavatolide A (**4**)



**FIGURE 5.1** Chemical structures of compounds (1–20).

showed *in vitro* cytotoxicity in KB and A-549 assays at 2.5 and 21.9 µg/mL, respectively (Sheu et al. 1998); excavatolide C (5) showed cytotoxicity against tumor cell lines (P-388, KB, A-549, HT-29) at the ED<sub>50</sub> levels of 0.3, 1.9, 1.9, and 1.9 µg/mL (Sheu et al. 1998); excavatolide D (6) showed cytotoxicity against tumor cell lines (P-388, KB, HT-29) at the ED<sub>50</sub> levels of 1.8, 4.2, and 1.3 µg/mL (Sheu et

al. 1998); excavatolide E (**7**) showed cytotoxicity against tumor cell lines (P-388, KB, A-549, HT-29) at the ED<sub>50</sub> levels of 1.6, 0.8, 1.2, and 1.6 µg/mL (Sheu et al. 1998); excavatolide F (**8**) showed cytotoxicity against tumor cell lines (P-388, KB, A-549, HT-29) at the ED<sub>50</sub> levels of 6.2, 7.0, 5.2, and 5.5 µg/mL (Sung et al. 1999); excavatolide G (**9**) showed cytotoxicity against two cell lines (P-388, A-549) at the ED<sub>50</sub> levels of 15.7 and 22.8 µg/mL (Sung et al. 1999); excavatolide J (**10**) showed cytotoxicity against tumor cell lines (P-388, KB, A-549, HT-29) at the ED<sub>50</sub> levels of 3.8, 6.5, 5.2, and 5.2 µg/mL (Sung et al. 1999); excavatolide K (**11**) showed cytotoxicity against tumor cell lines (P-388, KB, A-549, HT-29) at the ED<sub>50</sub> levels of 0.9, 3.3, 3.0, and 1.3 µg/mL (Sung et al. 1999); excavatolide L (**12**) showed cytotoxicity against tumor cell lines (P-388, A-549, HT-29) at the ED<sub>50</sub> levels of 5.8, 37.2, and 4.4 µg/mL (Sung et al. 1999); excavatolide M (**13**) showed cytotoxicity against tumor cell lines (P-388, KB, A-549, HT-29) at the ED<sub>50</sub> levels of 0.001, 1.0, 0.1, and 2.2 µg/mL (Sung et al. 1999); excavatolide N (**14**) showed cytotoxicity against tumor cell lines (P-388, KB, A-549, HT-29) at the ED<sub>50</sub> levels of 5, >10, >10, and >10 µg/mL (Neve, McCool, and Bowden 1999); excavatolide O (**15**) showed cytotoxicity against tumor cell lines (P-388, KB, A-549, HT-29) at the ED<sub>50</sub> levels of 5, 5, 5, and 10 µg/mL (Neve, McCool, and Bowden 1999); excavatolide P (**16**) showed cytotoxicity against tumor cell lines (P-388, KB, A-549, HT-29) at the ED<sub>50</sub> levels of 5, >10, >10, >10 µg/mL (Neve, McCool, and Bowden 1999); excavatolide Q (**17**) showed cytotoxicity against four tumor cell lines (P-388, KB, A-549, HT-29) at the ED<sub>50</sub> levels of 5, 10, 10, and 10 µg/mL (Neve, McCool, and Bowden 1999); excavatolide V (**18**) showed cytotoxicity against tumor cell lines (P-388, KB, A-549, HT-29) at the ED<sub>50</sub> levels of 3.9, 7.0, 19.1, and 20.4 µg/mL (Sheu et al. 1999b); excavatolide W (**19**) showed cytotoxicity against a tumor cell line (P-388) at the ED<sub>50</sub> level of 19.4 µg/mL (Sheu et al. 1999b); excavatolide Y (**20**) showed cytotoxicity against tumor cell lines (P-388, HT-29) at the ED<sub>50</sub> levels of 9.5 and 15.1 µg/mL (Sheu et al. 1999b); and excavatolide Z (**21**) (Figure 5.2) showed cytotoxicity against four tumor cell lines (P-388, KB, A-549, HT-29) at the ED<sub>50</sub> levels of 1.3, 6.5, 11.2, and 2.8 µg/mL (Sheu et al. 1999b). Briaexcavatolide B (**22**) showed cytotoxicity against tumor cell lines (P-388 and KB) at the ED<sub>50</sub> levels of 1.3 and 1.5 µg/mL (Sheu et al. 1999a); briaexcavatolide F (**23**) showed cytotoxicity against a tumor cell line (A-549) at the ED<sub>50</sub> level of 1.3 µg/mL (Sheu et al. 1999a); briaexcavatolide L (**24**) showed cytotoxicity against a tumor cell line (P-388) at the ED<sub>50</sub> level of 0.5 µg/mL (Sung et al. 2001); and briaexcavatolide P (**25**) showed cytotoxicity against tumor cell lines (P-388, A-549, HT-29) at the ED<sub>50</sub> levels of 0.9, 4.8, and 3.1 µg/mL (Wu et al. 2001). Brianthein A (**26**) is cytotoxic toward the human epidermoid carcinoma KB 3-1 cell line and the multidrug resistance cell line KB-C2 (Aoki et al. 2001). Further, briantheins B (**27**) and C (**28**) are cytotoxic toward the human epidermoid carcinoma KB 3-1 cells (Aoki et al. 2001). Brianthein W (**29**) was obtained from a Taiwanese gorgonian, *Briareum* sp., whereas brianthein Z (**30**) was isolated from *B. asbestinum*, which was collected from the Caribbean sea. Brianthein W (**29**) showed cytotoxicity against a tumor cell line (P-388) at the ED<sub>50</sub> level of 0.76 µg/mL (Sheu et al. 1996; Cardellina et al. 1984), and brianthein Z (**30**) showed *in vitro* cytotoxicity in the P-388 assay at 10 µg/mL (Grode, James, and Cardellina 1983; Coval et al. 1988). Stecholides A, B, and H (**31–33**), featuring the briarane carbon skeleton, were isolated from the gorgonian coral *Briareum stechei*, which was collected from the Dalton Reef area of the Australian Great Barrier Reef. Stecholide A (**31**) showed cytotoxicity toward P-388 tumor cells at 4.5 µg/mL (Bloor et al. 1992), stecholide B (**32**) showed cytotoxicity against a tumor cell line (P-388) at the ED<sub>50</sub> level of 5.4 µg/mL (Bloor et al. 1992), and stecholide H (**33**) showed cytotoxicity against a tumor cell line (P-388) at the ED<sub>50</sub> level of 10 µg/mL (Bloor et al. 1992). Briarane (**34**) was also obtained from the Taiwanese gorgonian *Briareum excavatum*; it is noted that (**34**) showed cytotoxicity against tumor cell lines (P-388, HT-29) at the ED<sub>50</sub> levels of 0.4 and 1.1 µg/mL, respectively (Bowden, Coll, and Vasilescu 1989; Sung et al. 2001). Collections of the gorgonian *Briareum* sp. from the coast of Taiwan yielded 2β-acetoxy-2-debutyryloxystecholide E (**35**) and 9-deacetylstylatulide lactone (**36**). In 1998, an Indonesian gorgonian, *Briareum* sp., afforded 2β-acetoxy-2-debutyryloxystecholide E acetate (**37**). It is noted that 2β-acetoxy-2-debutyryloxystecholide E (**35**) showed cytotoxicity against tumor cell lines (P-388, HT-29) at the ED<sub>50</sub> levels of 0.61 and 6.96 µg/mL (Sheu et al. 1996); 9-deacetylstylatulide lactone

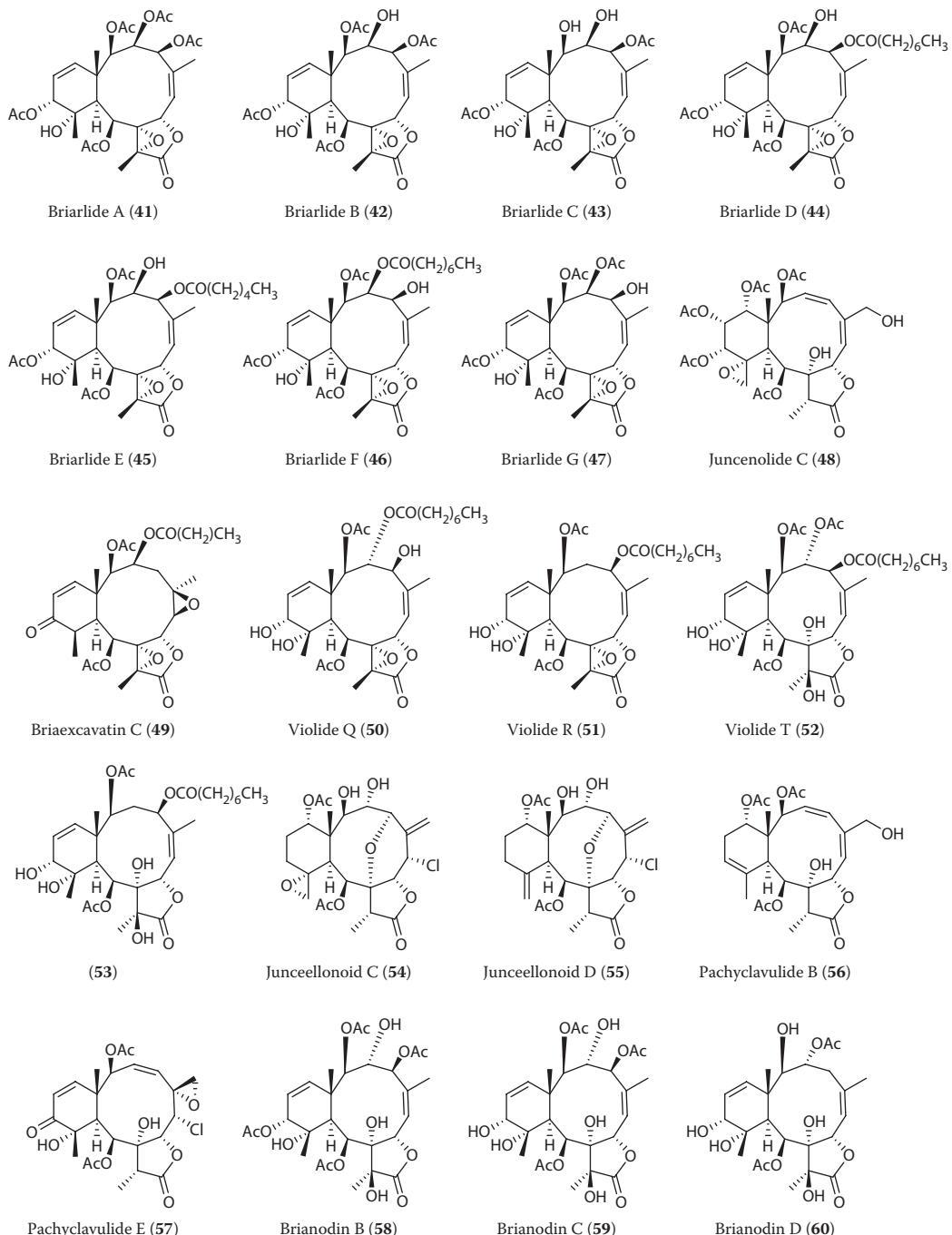


**FIGURE 5.2** Chemical structures of compounds (21–40).

(36) showed cytotoxicity against tumor cell lines (P-388, HT-29) at the  $\text{ED}_{50}$  levels of 1.12 and 1.79  $\mu\text{g}/\text{mL}$  (Sheu et al. 1996); and 2 $\beta$ -acetoxy-2-debutyryloxystecholide E acetate (37) showed cytotoxicity against tumor cell lines (P-388, KB, A-549, HT-29) at the  $\text{ED}_{50}$  levels of 1.59, 24.45, 17.39, and 10.07  $\mu\text{g}/\text{mL}$  (Sheu et al. 1996; Rodriguez, Nieto, and Jimenez 1998). In 1990, a soft coral of the genus *Tubipora* collected from Kuchinoshima Island of the Satsunan archipelago, Japan, whose

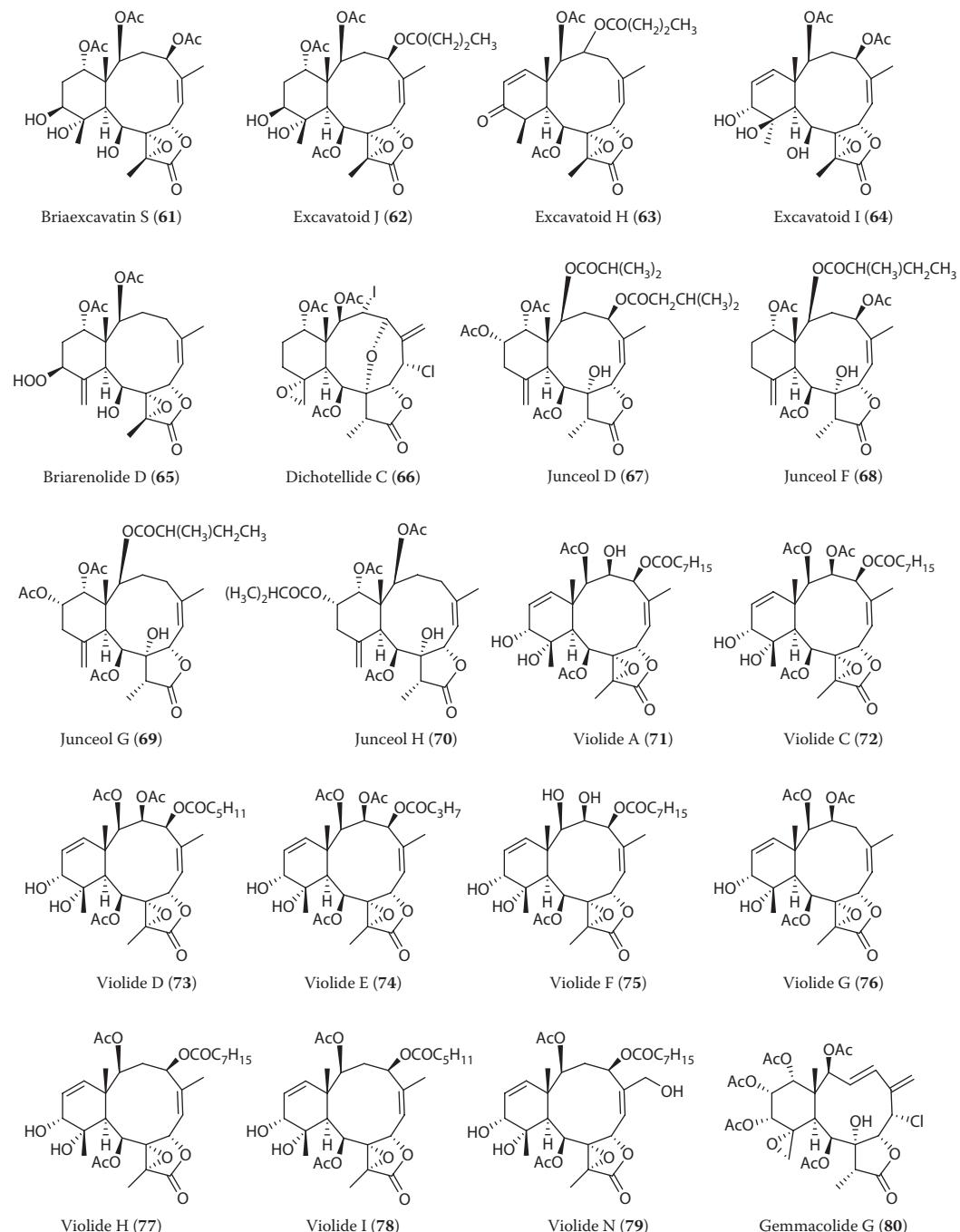
organic extract exhibited cytotoxicity toward the B-16 mouse melanoma tumor cells, was studied for discovering potential antitumor agents. A new diterpenoid of the briarane ring system, tubiporein (**38**), was isolated from this organism. The gross structure was determined by <sup>1</sup>H and <sup>13</sup>C NMR-based spectral analyses. Moreover, the relative configuration was elucidated by the interpretations of nuclear Overhauser effect (NOE) experiments and the coupling constant calculations. Tubiporein (**38**) exhibited cytotoxicity toward the B-16 tumor cells at the IC<sub>50</sub> level of 2.0 µg/mL (Natori, Kawai, and Fusetani 1990). It is noted that 9-deacetyl briareolide H (**39**) was also isolated from a Taiwanese gorgonian coral, *Briareum* sp., and exhibited cytotoxicity toward the P-388, KB, A-549, and HT-29 tumor cells at the IC<sub>50</sub> levels of 0.28, 0.27, 10.35, and 8.27 µg/mL, respectively (Bowden, Coll, and Konig 1990; Sheu et al. 1996). Juncenolide A (**40**) was isolated from the Taiwanese gorgonian coral *Junceella juncea*. The structure of (**40**) was established by two-dimensional NMR studies and was further confirmed by x-ray crystallographic analyses. Juncenolide A (**40**) was cytotoxic toward the human colon adenocarcinoma (DLD) and oral epidermoid carcinoma (KB-16) cells at concentrations of 3.4 and 5.9 µg/mL, respectively (Shen, Lin, and Chiang 2002).

The chemical constituents of a gorgonian coral belonging to the genus *Briareum*, which was collected from Amami Island, Kagoshima Prefecture, Japan, were studied. Briarlides A–G (**41–47**) (Figure 5.3) were obtained from this organism. The relative stereostructures of briarlides A–G (**41–47**) were determined by the interpretation of spectral data analysis (IR, MS, and <sup>1</sup>H and <sup>13</sup>C NMR spectrum). Briarlide A (**41**) exhibited cytotoxicity toward African green monkey kidney (Vero) and Madin–Darby canine kidney (MDCK) cells at the CC<sub>50</sub> levels of 2.07 and 4.74 µg/mL, respectively (Iwagawa et al. 2003). Briarlide B (**42**) exhibited cytotoxicity toward Vero and MDCK cells at the CC<sub>50</sub> levels of 18.9 and 15.4 µg/mL, respectively (Iwagawa et al. 2003); briarlide C (**43**) exhibited cytotoxicity toward Vero and MDCK cells at the CC<sub>50</sub> levels of 62.1 and 38.6 µg/mL, respectively (Iwagawa et al. 2003); briarlide D (**44**) exhibited cytotoxicity toward Vero and MDCK cells at the CC<sub>50</sub> levels of 2.26 and 2.49 µg/mL, respectively (Iwagawa et al. 2003); briarlide E (**45**) exhibited cytotoxicity toward Vero and MDCK cells at the CC<sub>50</sub> levels of 4.24 and 4.91 µg/mL, respectively (Iwagawa et al. 2003); briarlide F (**46**) exhibited cytotoxicity toward Vero and MDCK cells at the CC<sub>50</sub> levels of 4.26 and 3.49 µg/mL, respectively (Iwagawa et al. 2003); and briarlide G (**47**) exhibited cytotoxicity toward Vero and MDCK cells at the CC<sub>50</sub> levels of 22.2 and 67.1 µg/mL, respectively (Iwagawa et al. 2003). Juncenolide C (**48**) was obtained from the gorgonian coral *J. juncea*, which was collected off the Indian Ocean. Juncenolide C (**48**) exhibited mild cytotoxicity against the growth of human hepa adenocarcinoma (HEPA 59T/VGH) and oral epidermoid carcinoma (KB-16) cells at the CC<sub>50</sub> levels of 6.6 and 7.8 µg/mL, respectively (Shen et al. 2003). Briaexcavatin C (**49**) was isolated from the Taiwanese gorgonian coral *Briareum excavatum*. Briaexcavatin C (**49**) exhibited mild cytotoxicity toward MDA-MB-231 human breast tumor cells at the CC<sub>50</sub> levels of 17.50 µg/mL (Sung et al. 2006). In addition, violide Q (**50**), violide R (**51**), violide T (**52**), and (**53**) were isolated from the gorgonian *Briareum* sp., which was collected in the area of Bonotsu, Kagoshima Prefecture, Japan. Violide Q (**50**) exhibited cytotoxicity toward Vero and MDCK cells at the CC<sub>50</sub> levels of 5.09 and 4.88 µg/mL, respectively (Iwagawa et al. 2005); violide R (**51**) exhibited cytotoxicity toward Vero and MDCK cells at the CC<sub>50</sub> levels of 2.57 and 3.96 µg/mL, respectively (Iwagawa et al. 2005); violide T (**52**) exhibited cytotoxicity toward Vero and MDCK cells at the CC<sub>50</sub> levels of 39.5 and 55.3 µg/mL, respectively (Iwagawa et al. 2005); and (**53**) exhibited cytotoxicity toward Vero and MDCK cells at the CC<sub>50</sub> levels of 32.5 and 19.1 µg/mL, respectively (Iwagawa et al. 2005). The South China Sea gorgonian *J. fragilis* was found to contain junceellonoids C (**54**) and D (**55**). Junceellonoid C (**54**) showed mild cytotoxicity against the MDA-MB-231 and MCF cell lines at a concentration of 100 µM, but it was not active at a concentration of 33.3 µM (IC<sub>50</sub> values for this metabolite were not calculated) (Qi et al. 2005); junceellonoid D (**55**) showed mild cytotoxicity against the MDA-MB-231 and MCF cell lines at a concentration of 100 µM, but it was not active at a concentration of 33.3 µM (Qi et al. 2005). Study on the octocoral *Pachyclavularia violacea*, collected off Ishigaki Island, Okinawa Prefecture, Japan, has afforded pachyclavulide B (**56**) and pachyclavulide E (**57**). Pachyclavulide B (**56**) showed cytotoxicity toward SNB-75 (human central nervous system [CNS])



**FIGURE 5.3** Chemical structures of compounds (41–60).

at the  $GI_{50}$  level of 5.2  $\mu\text{M}$  (Iwasaki, Ito, Aoyagi, et al. 2006; Iwasaki, Ito, Nakamura, et al. 2006; Ito et al. 2007); pachyclavulide E (57) showed cytotoxicity toward SNB-75 (human CNS) at the  $GI_{50}$  level of 5.1  $\mu\text{M}$  (Ito et al. 2007). In 2008, Ishiyama et al. reported the occurrence of new briaranes, brianodins B–D (58–60), from an Okinawan octocoral *Pachyclavularia* sp. Brianodins B–D (58–60) were found to show modest activity toward L1210 (murine leukemia) and KB (human oral epidermoid carcinoma) tumor cells at  $IC_{50}$  levels over 10  $\mu\text{g}/\text{mL}$  (Ishiyama et al. 2008). Briaexcavatin



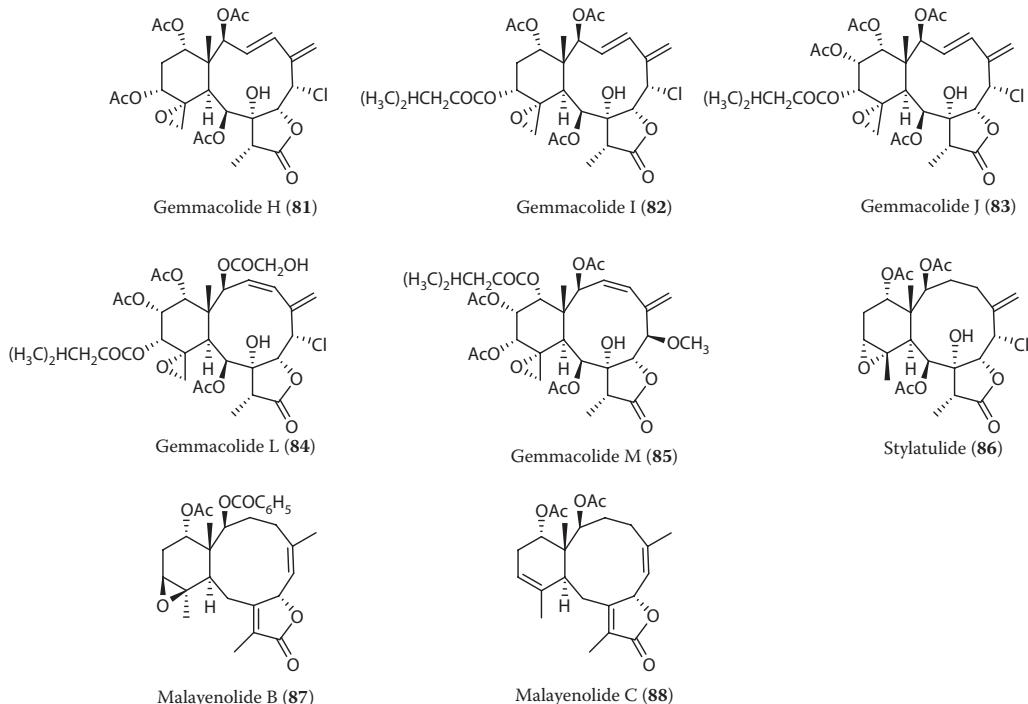
**FIGURE 5.4** Chemical structures of compounds (61–80).

S (61) was isolated from Formosan octocorals, the gorgonian *B. excavatum*; briaexcavatin S (61) (Figure 5.4) exhibited weak cytotoxicity toward various tumor cells at the IC<sub>50</sub> level of 37.8 µg/mL (Hwang et al. 2008). Excavatoids J, H, and I (62–64) were isolated from a cultured gorgonian species *Briareum excavatum*. Excavatoid J (62) showed cytotoxicity toward CCRF-CEM, human leukemia (HL)-60, DLD-1, and IMR-32 at the IC<sub>50</sub> levels of >40.0, 38.4, 25.1, and >40.0 µg/mL, respectively (Sung, Chen, et al. 2010); excavatoid H (63) showed cytotoxicity toward CCRF-CEM,

HL-60, DLD-1, and IMR-32 at the IC<sub>50</sub> levels of 13.1, >40.0, 21.4, and >40.0 µg/mL, respectively (Sung, Chen, et al. 2010); and excavatoid I (**64**) showed cytotoxicity toward CCRF-CEM, HL-60, DLD-1, and IMR-32 at the IC<sub>50</sub> levels of >40.0, >40.0, >40.0, and 31.1 µg/mL, respectively (Sung, Chen, et al. 2010). In continuing studies on the chemical constituents of a gorgonian coral identified as *Briareum* sp., which was collected from a tank equipped with a flow-through water system located in the National Museum of Marine Biology and Aquarium, Taiwan, a new hydroperoxybriarane, briarenolide D (**65**), was isolated. Briarenolide D (**65**) exhibited moderate cytotoxicity toward DLD-1 and CCRF-CEM cells at the IC<sub>50</sub> levels of 9.6 and 6.9 µg/mL, respectively (Sung, Lin, et al. 2010). The South China Sea gorgonian coral *Dichotella gemmacea* was found to contain dichotellide C (**66**); dichotellide C (**66**) showed marginal cytotoxicity against SW1990 cells (IC<sub>50</sub> = 45.0 µM) (Sun et al. 2011). Junceols D, F, G, and H (**67–70**) were isolated from a Formosan gorgonian coral, *Junceella juncea*; junceol D (**67**) exhibited cytotoxicity toward CCRF-CEM and DLD-1 cells at the IC<sub>50</sub> levels of 1.3 and 1.0 µg/mL, respectively (Sung, Pai, et al. 2008); junceol F (**68**) exhibited cytotoxicity toward CCRF-CEM cells at the IC<sub>50</sub> level of 4.9 µg/mL (Sung, Pai, et al. 2008); junceol G (**69**) exhibited cytotoxicity toward CCRF-CEM cells at the IC<sub>50</sub> level of 4.4 µg/mL (Sung, Pai, et al. 2008); and junceol H (**70**) exhibited cytotoxicity toward CCRF-CEM and DLD-1 cells at the IC<sub>50</sub> levels of 7.2 and 17.0 µg/mL, respectively (Sung, Pai, et al. 2008). Violides A, C, D, E, F, G, H, I, and N (**71–79**) were obtained from a Japanese gorgonian, *Briareum* sp., collected from the sea near the Satsuma Peninsula, Japan. The structures of compounds (**71–79**) were elucidated by the interpretations of spectral analyses (IR, MS, <sup>1</sup>H, and <sup>13</sup>C NMR) and chemical methods. The structures and relative configurations of violide A (**71**) were further confirmed by x-ray diffraction analyses. Violide A (**71**) exhibited cytotoxicity toward Vero and MDCK cells at the CC<sub>50</sub> levels of 1.90 and 1.90 µg/mL, respectively (Iwagawa et al. 1998, 1999a, 1999b); violide C (**72**) exhibited cytotoxicity toward Vero and MDCK cells at the CC<sub>50</sub> levels of 1.69 and 1.67 µg/mL, respectively (Iwagawa et al. 1999a, 1999b); violide D (**73**) exhibited cytotoxicity toward Vero and MDCK cells at the CC<sub>50</sub> levels of 2.53 and 3.57 µg/mL, respectively (Iwagawa et al. 1999a, 1999b); violide E (**74**) exhibited cytotoxicity toward Vero and MDCK cells at the CC<sub>50</sub> levels of 3.65 and 4.69 µg/mL, respectively (Iwagawa et al. 1999a, 1999b); violide F (**75**) exhibited cytotoxicity toward Vero and MDCK cells at the CC<sub>50</sub> levels of 3.93 and 4.03 µg/mL, respectively (Iwagawa et al. 1999a, 1999b); violide G (**76**) exhibited cytotoxicity toward Vero and MDCK cells at the CC<sub>50</sub> levels of 9.37 and 11.7 µg/mL, respectively; violide H (**77**) exhibited cytotoxicity toward Vero and MDCK cells at the CC<sub>50</sub> levels of 0.85 and 0.85 µg/mL, respectively; violide I (**78**) exhibited cytotoxicity toward Vero and MDCK cells at the CC<sub>50</sub> levels of 1.41 and 1.30 µg/mL; and violide N (**79**) exhibited cytotoxicity toward Vero and MDCK cells at the CC<sub>50</sub> levels of 3.3 and 3.2 µg/mL, respectively (Iwagawa et al. 2000). Gemmacolides G, H, I, J, K, K, and M (**80–85**), were isolated from the South China Sea gorgonian *Dichotella gemmacea*. The tumor cell growth inhibition activities of the new compounds were evaluated. In *in vitro* bioassays, gemmacolides (**80–84**) exhibited potential growth inhibition against tumor cell lines with IC<sub>50</sub> values of 8.4, 47.3, 20.6, <1.4, and 38.2 µM for A549 cells; and 38.4, 54.0, 25.0, 79.8, and 45.9 µM for MG63 cells, respectively. Compound (**85**) showed some activity only against A549 (IC<sub>50</sub> = 27.4 µM). It is very interesting to observe that compound (**84**) displayed stronger activity than those of analogs (**81–83**) and the positive control (adriamycin, IC<sub>50</sub> = 2.8 µM) (Li et al. 2011).

### 5.2.2 TOXICITY TO LARVAE

Stylatulide (**86**) (Figure 5.5) is the first briarane-type metabolite with toxicity originally isolated from the sea pen coral, *Stylatula* sp., collected in the intertidal zone at Isla Partida, Gulf of California. Stylatulide (**86**) was found to be toxic to the larvae of the copepod *Tisbe furcata johnsonii* at concentrations greater than 0.5 ppm (LD100) (Wratten et al. 1977; Wratten and Faulkner 1979). The malayenolides B–D (**87–89**) have been isolated from the sea pen coral *Veretillum malayense*, which was collected near Monado, Sulawesi, Indonesia. Malayenolide C (**88**) showed



**FIGURE 5.5** Chemical structures of compounds (81–88).

toxicity in the brine shrimp assay at the LC<sub>50</sub> level of 20 µg/mL (Fu, Schmitz, and Williams 1999); malayenolide B (**87**) showed toxicity in the brine shrimp assay at LC<sub>50</sub> levels less than 2 µg/mL (Fu, Schmitz, and Williams 1999); and malayenolide D (**89**) showed toxicity in the brine shrimp assay at the LC<sub>50</sub> level of 20 µg/mL (Fu, Schmitz, and Williams 1999).

### 5.3 CONCLUSION

All briarane-type compounds have been proved to be of marine origin. To date, over 600 briarane-type natural diterpenoids have been isolated from various marine organisms, particularly from the soft corals belonging to the subclass Octocorallia. Among them, 89 compounds were found to be cytotoxic. The following structural requirements were clarified: (1) The lactone group is essential for cytotoxicity. (2) The numbers of the substitutes on the 10-membered ring influence cytotoxicity. (3) The number and location of double bonds on the 10-membered ring affect the potency of cytotoxicity; exocyclic double bonds increase cytotoxicity. (4) Esterification of the hydroxyl on the cyclohexane ring increases cytotoxicity; the number of substitutes on the cyclohexane ring influence cytotoxicity. (5) The iodinated and chlorinated derivatives do not affect the potency of cytotoxicity.

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# 6 Pharmacoanalytical Procedures for Chondroitin Present in Raw Materials and Biological Fluids

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## 6.1 INTRODUCTION

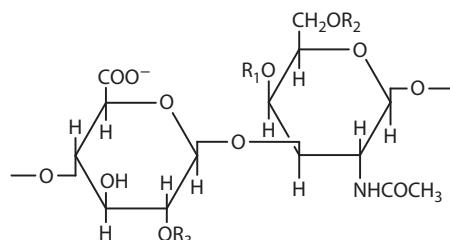
Chondroitin has been widely used over the last 20 years as a nutraceutical and pharmaceutical supplement, predominantly for the treatment of osteoarthritis and rheumatoid arthritis, although new therapeutic applications are being investigated, for example, in the field of allergy. In response to this demand, new commercial sources are being developed and new analytical procedures devised for monitoring raw materials, formulated products, and biological fluids.

Chondroitin sulfate (CS) is a linear, anionic glycosaminoglycan (GAG) widely distributed in human beings, other mammals, and invertebrates (Lamari and Karamanos 2006); it is usually found covalently linked to proteins forming proteoglycans (Imberty, Lortat-Jacob, and Perez 2007; Sisu et al. 2010). It is noted that CS along with other GAGs is an important component of the extracellular matrix of connective tissue, which may play pivotal roles in modulating many cellular events and physiological processes (Lauder 2009). The GAGs are present on all animal cell surfaces and may be classified into four main groups: (1) CS/dermatan sulfate (CS/DS), (2) hyaluronic acid (HA), (3) heparin sulfate/heparan/heparosan (HS/HP/HN), and (4) the keratan type. In fact, all GAGs are complex, anionic linear heteropolysaccharides comprising repeating disaccharide units, and it is the individual monosaccharide type and glycosidic bonds between the units that determine their classification (Figure 6.1).

Chondroitin occurs in cartilage of a wide variety of both marine and land animals. It is extracted from a number of marine organisms, preferably shark (various genera, including blue shark [*Prionace glauca*]), species of trout (*Oncorhynchus* spp.), skate (*Raja flavirostkis*), lesser spotted dogfish (*Scyliorhinus canicula*), salmon (*Salmo* and *Oncorhynchus* sp.), giant squid (*Architeuthis* spp.), flatfish (numerous genera), sturgeon (20 species from 4 genera in the family Acipenseridae), sea cucumber (numerous genera), and seafood waste, including fish scales. Yellow goosefish bone could also be a promising source as a substitute for shark cartilage, which is currently the major marine commercial source of chondroitin.

The worldwide availability of shark cartilage is rather limited, and much of the claimed shark chondroitin on the market today is actually derived from bovine trachea (see Section 6.2). Table 6.1 shows a comparison of different compositions of chondroitin of marine origin with samples of bovine and avian material.

The significance of the data shown in Table 6.1 is that marine samples can be clearly distinguished from the samples of both bovine and avian origin on the basis of the differences in charge density, the presence of disulfated disaccharides, and the 4s/6s ratios. Charge densities range from 1.1 to 1.2 for materials of marine origin and from 0.9 to 1.0 for others; disulfated disaccharides are



**FIGURE 6.1** Structures of chondroitin sulfate disaccharides:

- $R_1 = R_2 = R_3 = H$ : nonsulfated chondroitin
- $R_1 = SO_3^-$  and  $R_2 = R_3 = H$ : chondroitin-4-sulfate, CS-A
- $R_2 = SO_3^-$  and  $R_1 = R_3 = H$ : chondroitin-6-sulfate, CS-C
- $R_2 = R_3 = SO_3^-$  and  $R_1 = H$ : chondroitin-2,6-disulfate, CS-D
- $R_1 = R_2 = SO_3^-$  and  $R_3 = H$ : chondroitin-4, 6-disulfate, CS-E
- $R_1 = R_3 = SO_3^-$  and  $R_2 = H$ : chondroitin-2,4-disulfate, CS-A
- $R_1 = R_2 = R_3 = SO_3^-$ : trisulfated chondroitin

**TABLE 6.1**  
**Molecular Weights and Disaccharide Compositions of Marine Chondroitin Samples, with Comparable Data for Bovine Chondroitin**

Parameters	Shark	Raja	Bovine	Chicken
Molecular weight (average; kDa)	64.2–70.2	50.0–60.0	20.0–26.0	15.6–20.6
Disaccharides				
ΔDi-0S	3.0	3.0	6.0	8.0
ΔDi-6S	50.0	39	33.0	20.0
ΔDi-4S	29.0	43	61.0	72.0
ΔDi-2,6dis	15.0	13.0	N.D.	N.D.
ΔDi-4,6dis	2.0	1.0	N.D.	N.D.
ΔDi-2,4dis	1.0	1.0	N.D.	N.D.
Charge density	1.10–1.20	1.08–1.16	0.90–0.96	0.90–0.94
4s/6s ratio	0.45–0.70	1.00–1.40	1.50–2.00	3.0–4.0

Source: Volpi, N., *J. Pharm. Sci.*, 96, 3168–80, 2007.

Note: N.D. = not detected.

present only in materials of marine origin, and the 4s/6s ratios in marine materials are low at levels of 0.5–1.4 in marine samples as against 1.5–4.0 in other materials (up to 7.0 in porcine materials).

## 6.2 MANUFACTURE OF CHONDROITIN

There are few published accounts on the commercial manufacture of chondroitin, although a number of patents exist in the area.

The conventional extraction procedure is carried out via alkaline hydrolysis of crushed raw cartilage material with the addition of sodium chloride at 60°C for 3 hours. Next, the solution is acidified to pH 6.0 with hydrochloric acid and centrifuged (5000 rpm) at room temperature for 10 minutes to remove the insoluble part. The supernatant is then made alkaline to pH 8.5 with sodium hydroxide and hydrolyzed with the addition of pancreatic enzyme (5%) at 53°C for 3 hours; the enzymolysis is terminated by acidification to pH 6.0 at 65°C. The solution is subsequently centrifuged (5000 rpm) at room temperature for 10 minutes to remove the insoluble part, the supernatant is precipitated with two volumes of ethanol for 30 minutes, and the deposit is dehydrated by washing with ethanol at room temperature for 6 hours. The CS sodium salt is finally obtained by drying (Wang and Tang 2009).

A solvent-free mechanochemical extraction has been reported for the extraction of CS from shark cartilage. This process was carried out via mechanical pretreatment and mechanochemical treatment in an AGO-2 high-intensity planetary activator at room temperature under solvent-free conditions, and sodium hydroxide and silicon dioxide were mixed and finely ground using a stainless iron ball. The yield of CS was increased from 8.50% to 9.33% and the purity of the product was improved from 86.4% to 95.1% by using this technique (Wang and Tang 2009).

Stereocontrolled syntheses of related tetra- and hexasaccharide methyl glycosides of shark cartilage CS-D have been reported. The compounds were obtained from a common key disaccharide donor, and the 2-deoxy-2-trichloroacetamido group was used as an efficient stereocontrolling auxiliary. The D-glucuronyl donor was prepared from D-glucose, whereas the D-galactosaminyl acceptor was synthesized starting from D-glucosamine precursors (Karst and Jacquinet 2002).

Investigations of a number of alternative marine sources have been reported. The CS from the cartilage of lesser spotted dogfish (*S. canicula*) has been isolated and investigated. The CS in this

case appears very similar to that isolated from shark, which is a taxonomically related fish, with the percentage of CS-A being slightly higher (Gargiulo et al. 2009).

Skate cartilage is a fisheries by-product that is used for chondroitin manufacture. The simple process involves cartilage hydrolysis using Lypaine for 3 hours at pH 6.5 and 65°C, followed by centrifugation, tangential filtration using a membrane of pore size 0.1 µm, concentration, and desalting (Lignot, Lahogue, and Bourseau 2003). The by-products of giant squid, salmon, flatfish, and yellow goosefish were also investigated for manufacturing CS. Depolymerization with chondroitinase showed that purified CS did not contain other GAGs using agarose gel electrophoresis. The structure and purity of CS were confirmed by nuclear magnetic resonance (<sup>1</sup>H-NMR) spectroscopy, and the average molecular weights ranged from 22 to 116 kDa when analyzed by size-exclusion high-performance chromatography (SE-HPLC). Strong anion-exchange (SAX)-HPLC was performed to obtain disaccharide compositions. The purity (81.7% ± 1.3% to 114.2% ± 2.5%) and the yield (1.3% to 21.6%) varied depending on sources used. From the data, CS from giant squid cartilage, salmon cartilage, and yellow goosefish bone are promising marine sources to substitute for shark cartilage CS (Park et al. 2010).

Treatment of fish scales with proteases, followed by removal of polypeptide by-products, and fractionally precipitating the sulfates has been employed. Carp scales were treated with actinase E (mycolysin) and centrifuged to give a supernatant, which was evaporated, dialyzed to remove polypeptides, neutralized, and freeze-dried. The dried product was dissolved in 0.5 M sodium acetate and precipitated by ethanol to give CS-A and CS-C (Sumi et al. 2001).

Salmon or trout heads have been autoclaved at 120°C for 60 minutes to give crude CS residue, which was washed with hot water to give 0.8 wt% CS in 0.50 wt% yield. Purity above 90% was obtained by drying the crude CS and subjecting the dried powder to alkali treatment, enzyme treatment, ethanol precipitation, cation exchange, neutralization with NaOH, and drying (Katahira 2005).

Manufacture of stable dosage forms has also been researched. The physical properties of CS have been investigated to enable development of stable solid dosage forms. Polarized light microscopy and x-ray diffraction patterns show that CS is amorphous. Particle sizes of CS vary widely, depending on their source or manufacturing technique (e.g., granulation). The studied samples of shark-derived CS had a small median particle size (4 µm) compared to particles derived from bovine cartilage (17 µm). It is noted that CS is extremely hygroscopic and deliquescent. The Carr's indices were 25.2 and 53.6, and compression analysis showed that all CS samples exhibited plastic deformation behavior, with shark-derived CS forming superior compacts when compared with bovine samples (Ebube, Mark, and Hahm 2002).

A number of laboratory-scale preparative schemes for milligram levels have been published using anion exchange, cesium chloride density gradient centrifugation, SE-HPLC, and hydrophobic chromatography (Matsui and Oohira 2006; Silva 2006).

Published analyses of a range of commercially available chondroitin products showed variable content compliance (Lockwood 2011); this is probably caused by use of a range of animal sources for starting materials and the different chemical forms present. Analysis of chondroitin demonstrates a range of problems that are not seen in the majority of nutraceuticals; the disaccharide nature of the molecule is variable depending on biological origin, varying from 14–70 kDa (shark or skate, bovine, porcine, chicken). Within the chondroitin samples, the specific ratio of different disaccharides also varies and is further complicated by admixtures and other sources such as avian material (Volpi 2007). Disaccharide compositional analysis of 12 Japanese chondroitin supplements showed 2 products falsely labeled as being from shark, as opposed to their actual bovine origin (Sakai et al. 2007).

### 6.3 ANALYTICAL PROCEDURES USED FOR THE ANALYSIS OF CHONDROITIN IN RAW MATERIALS

It is noted that CS analysis causes several problems, as CS is a polymer of wide molecular weight range, has a small or an absent chromophore, and contains sulfate groups that render it hydrophilic. Its structure is closely related to other naturally occurring GAG polymers, which may be present

as impurities or adulterants resulting in confounding results (Jaksh, Wang, and Roman 2005). The *Handbook of Analytical Methods for Dietary Supplements* contains four techniques: (1) carbazole reaction, (2) cetyl pyridinium chloride titration, (3) size-exclusion chromatography, and (4) enzymatic hydrolysis followed by HPLC. The updated versions of these techniques have been published and are widely used today.

Other analytical techniques have been developed for the analysis of CS contained in raw materials and in nutraceutical and pharmaceutical preparations. The method of analysis employed depends on the information required, which may be for quantitative and qualitative determination or for structural characterization and origin determination.

Simple assays have been developed for analytical determinations, with or without prior separation or degradation, and they include dye-forming colorimetric methods, the cetylpyridinium chloride (CPC) titration, and the carbazole–uronic acid reaction technique. Chromatographic procedures such as SE-HPLC have also been widely utilized for the analysis of intact CS polymers.

In recent years, more sophisticated techniques have been proposed for structural characterization, which may give valuable information regarding the origin of CS. These techniques require the use of specific enzymes that hydrolyze the CS polymer into base disaccharide units ( $\Delta$ -disaccharides), which are then separated and analyzed as low-molecular-weight, negatively charged molecules. The main analytical techniques used for the analysis of CS disaccharides include various electrophoretic and chromatographic methods. Strong ion exchange, ion-pair, and amine column chromatographic methods are the most widely used procedures. The most recent innovation in the area of chondroitin analysis is the application of  $^1\text{H-NMR}$  and Fourier transform infrared (FTIR) spectroscopy.

#### **6.4 CHROMOGENIC QUANTITATIVE METHOD: URONIC ACIDS WITH CARBAZOLE**

The reaction of carbazole with uronic acid or complex polymers containing hexuronic acid with concentrated sulfuric acid has been well documented. This chromogenic method of analyzing carbohydrate content was first described by Dische (Bitter and Muir 1962; Stylianou, Triantaphyllidou, and Vynios 2006) and, since then, it has been successfully applied for the quantitative determination of CS. The original method proposed by Dische has been modified by various authors to achieve increased sensitivity and reproducibility (Galambos 1967). Bitter and Muir (1962) devised an adapted uronic acid–carbazole reaction based on the original method by Dische with the addition of 0.025 M borax to stabilize chromogen formation, which is then analyzed to quantify CS content.

All the aforementioned methods require the hydrolysis of GAGs in sulfuric acid at elevated temperatures (Galambos 1967) followed by reaction with carbazole, either at room temperature or 100°C (Bitter and Muir 1962; Gregory 1960). The carbazole reaction produces a color that can be detected by absorbance using a spectrophotometer at 530 nm, providing quantitative data in relation to uronic acid content.

The advantages of modifying Dische's carbazole reaction have been identified by Bitter and Muir. The chromogens produced by both glucuronic acid and iduronic acid under the conditions described by Dische are sensitive to light and are inherently unstable products. The introduction of borax during the hydrolysis stage produced maximum and immediate development of color, which was stable for at least 16 hours, and also decreased hydrolysis time to 10 minutes. The sensitivity was also found to be increased by a factor of two and optical density was found to be directly proportional to concentrations between 4 and 40  $\mu\text{g/mL}$ , with a detection limit at approximately 500 ng with greater overall reproducibility (Bitter and Muir 1962).

The main disadvantages of the carbazole reaction for the quality control of CS nutraceuticals and raw materials are its lack of specificity and ability to only evaluate total GAG content with no differentiation between individual GAGs, not to mention the reaction's inability to determine the origin of CS. If contaminants or adulterants such as carbohydrates and/or metals are present,

inaccurate and overestimated values of CS content may be recorded. Moreover, CS is commonly coformulated with glucosamine or prepared as a multicomponent preparation, which, as previously suggested, may interfere with this assay and, in such cases, complicated sample pretreatments such as solid-phase extraction or liquid–liquid extraction may be required. Although this technique is the most commonly used method for the quantitative determination of total GAG content, it requires highly skilled technicians to obtain consistent chromophore production (Okamoto et al. 2004).

Cesaretti et al. (2003) raised concern regarding the routine application of this process due to the time-consuming nature of the method and extensive number of samples required for processing with excessive consumption of resources including reagents and materials. To overcome these problems, they devised a 96-well assay for uronic acid–carbazole reaction, which reduced the amount of waste and allowed extensive reduction in processing time and greater availability for experimental repetition, highlighting the significance of the coefficient of variation (Cesaretti et al. 2003).

## 6.5 PHOTOMETRIC TITRATION: CETYL PYRIDINIUM CHLORIDE

Another method that is widely used for the determination of CS content in pharmaceutical raw materials and finished products is a photometric titration technique using CPC. Liang et al. (2002) developed and validated a photometric titration method for the quantitation of sodium CS (SCS) in a chewable tablet formulation.

The advantages of this photometric titration method are that it is fast, taking approximately 4 minutes to form the CS-CPC complex, and no complicated pretreatment is required. Although this assay is simple to perform with high precision, accuracy, robustness, and excellent percentage recovery, there are fundamental problems associated with this technique (Liang et al. 2002). Other polyanions, such as nucleic acids and complex polysaccharides, can react with CPC; therefore, this method lacks specificity for CS and false positive results may be obtained if any of these materials are contaminating the product. This method suffers from further limitations including inability to detect unsulfated CS (Volpi 2007) and incapacity to determine biological origin.

## 6.6 CHROMATOGRAPHIC METHODS

Due to the problems associated with the spectrophotometric assays discussed thus far, various chromatographic procedures have been introduced, for example, SE-HPLC.

### 6.6.1 SIZE-EXCLUSION HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

Choi et al. (2003) devised an SE-HPLC method for the quantitative determination of SCS contained in various pharmaceutical formulations, including tablets and capsules. Samples were extracted with water, filtered, and diluted to a final concentration equivalent to 0.1 mg/mL SCS. Samples standard stock solutions were directly separated by HPLC on a TSK gel HW-40F column with a photodiode array ultraviolet (UV) detection system set at 210 nm. Elution with acetonitrile–phosphate buffer (pH 6) at a flow rate of 1.0 mL/min was employed.

Choi et al. (2003) compared this method to three classical spectrophotometric techniques, including the carbazole–uronic acid assay, an acriflavine reagent assay contained in the *Korean Pharmaceutical Codex*, and a further technique described in the *Japanese Pharmaceutical Codex*. The advantages of this technique were primarily its simplicity and there being no requirement for complicated sample pretreatment. It showed good linearity between concentrations 0.05 and 0.5 mg/mL with improved reproducibility and precision compared with previous spectrophotometric techniques (Choi et al. 2003). The method showed no excipient interference, and it is therefore suitable for routine quality control of formulated preparations.

In another method, a range of commercially available GAGs were analyzed by SE-HPLC. Detection was carried out at 240 nm to monitor the formation of copper(II) complex in copper

sulfate solution at pH 4.5. The separation column used was manually wet packed in copper(II) sulfate solution (1 mM), and it was adjusted to pH 4.5 with 0.01 M sulfuric acid. A flow rate of 0.5 mL/min was used in each experiment and copper(II)-GAG complexes were detected online by UV absorbance at 240 nm. It is noted that SE-HPLC was used in this study to avoid potential interference of copper complex formation, which may occur with anion-exchange HPLC or reverse-phased (RP) ion-pairing HPLC due to the fact that these methods rely on the use of salt gradients or ion-pairing reagents. Toida et al. (1997) selected this method as it allowed the analysis of GAGs that cannot be depolymerized by enzymes such as chondroitin lyase. The limit of detection was approximately  $10^{-7}$  g for CS. The method was used for the analysis of de-*N*-acetylated and/or oversulfated CS that cannot be depolymerized by enzymes such as chondroitin lyases (Toida et al. 1997).

Disadvantages of HPLC exclusion modes include difficulties encountered in the initial setup, and column conditioning with mobile phase, which may be necessary for several hours prior to sample analysis (Stuart and Walker 2006). The main disadvantage of SE-HPLC is the inability to distinguish between polymers with similar molecular weights as chondroitin. Chondroitin analyte is excluded from the column based on its ability to permeate a sieve-like stationary phase; therefore, its position and retention time are not governed by conventional chromatographic parameters, such as composition of the mobile phase, column type, or column temperature (Stuart and Walker 2006). The main disadvantage of SE-HPLC, therefore, is its inability to analyze samples containing molecules of similar size to chondroitin. This drawback was recognized by Ji et al. (2007) who suggested that this analytical technique may in fact suffer from interference from both pharmaceutical excipients and other related GAGs. Other limitations are the inability to evaluate isomeric forms of CS and the incapacity to elucidate origin.

### 6.6.2 HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AFTER ENZYMATIC HYDROLYSIS

Specific enzyme degradation of CS followed by HPLC is used widely to quantitatively characterize CS contained in raw materials and biological fluids. Various HPLC methods, including ion-exchange chromatography and RP chromatography, with conductivity detection, UV detection, or precolumn derivatization with fluorescence detection have been used to separate and quantify unsaturated disaccharides produced by the enzymatic hydrolysis of CS. These methods have been used for the quantification of CS in biological samples and raw materials; however, until 2007 none of these methods had been used for the analysis of CS in formulated nutraceuticals (Ji et al. 2007).

Ji et al. (2007) developed the first HPLC method with UV detection after enzymatic hydrolysis for the quantification of CS contained in nutraceuticals. This technique involves aqueous extraction and subsequent unsaturated disaccharide production using an enzyme, chondroitinase AC II, to hydrolyze CS chains. This digestion method introduces a double bond between carbon-4 and carbon-5 of uronic acid, which absorbs UV light, allowing their specific quantification by UV detection at 240 nm after separation by ion-pairing RP HPLC. This study involved the preparation of sample test solutions, including raw materials from various sources, such as shark cartilage, porcine skin, and bovine trachea. Various dietary supplements were also obtained, and they included hard capsules, chewable formulations, softgels, tablets, and liquids from various pharmaceutical suppliers.

Following aqueous extraction and filtration, tris(hydroxymethyl)aminomethane buffer (tris buffer; adjusted to pH 7.3 with the addition of 6 M hydrochloric acid) and chondroitinase AC II enzyme solution were added and the mixture was heated at 37°C for 3 hours. Analytes were eluted with a linear gradient mobile phase; tetrabutylammonium bisulfate in water and tetrabutylammonium-acetonitrile in water buffer at a flow rate of 1.1 mL/min, using a polar RP column and analyzed using a 168 diode array UV detector, monitoring at 240 nm. This HPLC-UV detection method after enzymatic hydrolysis has many advantages over the traditionally used spectrophotometric assays. This technique is highly selective for CS and does not suffer from interference, which is known to adversely affect CPC titration methods and the carbazole–uronic acid reaction assay, nor does it require any special waste disposal. Due to the fact that adulterants and possible contaminants do

not jeopardize the validity of results obtained, this method is well suited for the analysis of finished products and multicomponent pharmaceuticals. Unlike previously reported spectrophotometric assays and size-exclusion chromatographic methods, this recently developed technique can be used for the compositional  $\Delta$ -disaccharide analysis of GAGs, which gives valuable information in relation to the origin of CS material. Although this technique has many overwhelming advantages as described here, it also has certain drawbacks. The costs of reference standards and enzymes are high, and this method of quantifying CS content requires much longer analysis times than certain spectrophotometric methods, including CPC titration (Ji et al. 2007).

In another method, GAG was released from 50 mg of raw material using successive digestion with protease and endo- $\beta$ -xylosidase. The GAG was then labeled with 2-aminopyridine by reductive amination. The pyridylaminoglycosaminoglycan product was chromatographed by ion-exchange HPLC using a TSK gel SAX analytical column. This method produced rapid separation and analysis of GAGs, chondroitin, dermatan, heparin, heparin, and HA (Takagaki et al. 1994).

Test samples of chondroitin raw material have been depolymerized using chondroitinase ABC. The chondroitin disaccharides produced were analyzed by SAX-HPLC and their contents quantified. Chondroitin was extracted with hexane followed by extraction with phenol–chloroform to remove oil and protein ingredients in the situation with soft capsules. Quantitative analysis of the disaccharides derived from raw materials and an ophthalmic formulation showed contents (in percentage) ranging from 39.5 to 105.6 and 103.3 (Sim et al. 2005).

Anionic exchange chromatography using a Dionex CarboPac PA-1 column with NaOH/NaCl eluents and absorbance detection at 232 nm was used for the analysis of a chondroitin/DS chain in order to determine the concentrations of CS, DS, and hyaluronan. Both commercially available and freshly prepared shark, whale, bovine, and human cartilage CSs were examined by this method, and it was found that freshly isolated shark cartilage CS contains significant amounts of GlcA2S $\beta$ (1-3)GalNAc6SA. It was revealed that for hyaluronan oligosaccharides, chain length controlled elution position; but for chondroitin and DS oligosaccharides, elution times during gradient elution primarily depended on the level of sulfation, although chain length and, hence, charge density also were involved. The sulfation position of GalNAc residues within an oligosaccharide was also important in determining chromatographic mobility. A reducing terminal 6-sulfate retards elution when compared with 4-sulfate; however, when present on an internal GalNAc residue it is the 4-sulfate-containing oligosaccharide that elutes later. These effects allow discrimination between oligosaccharides differing only in the position of GalNAc sulfation (Lauder et al. 2009).

A simple and reliable RP HPLC method was developed by Gatti et al. (2010) for simultaneous measurement of glucosamine and CS in dietary products. The procedure is based on the reaction of *o*-phthalodialdehyde with glucosamine and galactosamine coming from the galactosaminoglycan hydrolysis. Extraction and hydrolysis of the two GAGs was carried out with hydrochloric acid (7.5 N) at 80°C for 8 hours, and the precolumn derivatization reaction was carried out in alkaline conditions for 1 minute at room temperature. Resolution was carried out using a Phenomenex Synergi 4 $\mu$  fusion-RP 80 A (250 mm × 3.0 mm) column with a mobile phase of sodium acetate buffer (pH 5.9; 0.05 M) and methanol (85:15, v/v), and UV-DAD detection at  $\lambda$  = 340 nm. Linear responses were obtained and the limit of quantitation for both GAGs was about 60 pmol (Gatti et al. 2010).

### 6.6.3 HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY ANALYSIS OF CHONDROITIN DISACCHARIDES PRODUCED BY SOLVOLYSIS

Other studies that have utilized HPLC include the study by Qiu et al. (1996). They used an ion-exchange HPLC procedure to separate various disaccharides produced from GAGs by solvolysis with fluorometric detection by postcolumn derivatization. Qiu et al. (1996) selected this solvolysis method to produce disaccharides as it offered greater discrimination between DS and CS than previous techniques (Sections 6.6.1 and 6.6.2), which used various chondroitinases to produce unsaturated disaccharides for GAG quantitation. The method was developed for analyzing

combinations of *N*-acetyldermosine, *N*-acetylchondrosine, and *N*-acetylhyalobiuronic acid, which had been prepared from chondroitin. These disaccharides were eluted from a TSK gel SAX column using 0.1 M acetic acid containing potassium chloride, and they were detected fluorometrically by postcolumn derivatization. The method was later used to detect and quantify chondroitin and other GAGs in raw materials (Qiu et al. 1996).

## 6.7 CAPILLARY ELECTROPHORESIS

To address the problems associated with the use of expensive enzymes and the limited availability of such enzymes, Okamoto et al. (2004) developed a novel technique for the determination of CS based on conversion to desulfated chondro-disaccharides via an in-capillary enzyme reaction. This technique allows increased sensitivity and excellent resolving power and significantly reduces the consumption of samples and reagents in comparison with other analytical techniques employing off-line operation (Volpi 2004). The study involved the analysis of a commercially available ophthalmic solution by dilution with 10 mM tris-acetate buffer set at the optimal pH of 7.

Test solution and enzyme solution containing chondroitinase ABC and chondrosulfatase were introduced into the cathode inlet of the capillary by hydrodynamic injection (5 kPa), which was maintained at 37°C. After hydrolysis, a voltage of -30 kV was applied. Nonsulfated chondro-dissacharides were separated by capillary electrophoresis (CE) and detected using a diode-array detection system at 232 nm (Okamoto et al. 2004). The ability of this in-capillary enzyme technique to analyze large amounts of test samples without complex pretreatments, along with an approximate analysis time of 60 minutes and a superior safety profile, gives this procedure several advantages over certain spectrophotometric methods for the routine quality control of CS contained in raw materials and nutraceuticals. The reduction in the quantity of expensive enzymes necessary for analysis in comparison with HPLC methods, simple operation, and the ability to fully automate this system provide yet more advantages. Okamoto et al. (2004) also suggested further ways of decreasing cost by utilizing a microchip enzyme reactor.

It is noted that CE with diode array detection has been employed for the quantification of chondroitin levels in raw materials and formulated products. The method was also used in screening for other GAG or DNA impurities and determination of hyaluronan impurities in these entities. Analysis was performed with uncoated fused-silica capillaries using reversed polarity and an operating phosphate buffer (50 mM) of pH 3.0. The method has high sensitivity (lower limit of quantitation values of 30.0 µg/mL for CS and 5.0 µg/mL for hyaluronan), precision, and accuracy. Analysis of 11 formulated products revealed the presence of up to 1.4% hyaluronan impurities, with only 2 products being free from impurity. Further analysis of the samples after treatment with chondroitinase ABC and AC II, in order to depolymerize the chains to yield disaccharides, showed the compositions of the 11 samples and raw shark cartilage to be very different (Malavaki et al. 2008).

The perceived disadvantages of CE for the analysis of chondroitin-containing nutraceuticals include the requirement that sample solutions be prepared in microliter amounts due to the nature of the instrumentation. Another limitation is the necessity for precise temperature control of the whole system, including buffer vessels, column, and sample, which is required for good experimental reproducibility. In addition, to achieve run-to-run reproducibility, stable instrumental control is necessary in terms of electric current and voltage. Commercially available high-quality buffers are generally required for CE as quality and composition are also pivotal to achieve accurate results. This is even more significant given that CE uses very small quantities of buffers (Okamoto et al. 2004).

## 6.8 GAS CHROMATOGRAPHY/MASS SPECTROSCOPY

A major problem in the analysis of GAGs is the difficulty in quantitatively cleaving glycosidic bonds because of the stabilization of the glycosidic bonds and the relative instability of the liberated constituents. Methanolysis in the presence of barium acetate reduces the degradation of uronic acids and

increases the cleavage yield. The reaction products of chondroitin could be identified and quantified by gas chromatography (GC) and GC/mass spectroscopy (MS) of their heptafluorobutyrate derivatives of O-Me glycosides of monosaccharides. Temperature-programmed BP-1 capillary columns running up to 260°C resolved 35 different derivatives (Zanetta, Timmerman, and Leroy 1999).

## 6.9 FOURIER TRANSFORM INFRARED SPECTROSCOPY

The enzymic release of CS followed by the sulfate GAGs assay was employed to quantify CS in cartilage of shark fin, ray, crocodile, and chicken keel, and it was possible to identify the types of CSs using FTIR spectroscopy with potassium bromide pellets. The values ranged from 11.55 to 14.84 g per 100 g of dried cartilage, calculated as chondroitin-4-sulfate. Identification of dried CS extracts from samples revealed the presence of both chondroitin-4-sulfate and chondroitin-6-sulfate (Garnjanagoonchorn, Wongkalak, and Engkagul 2007).

## 6.10 NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY

The origin of chondroitin was investigated in 12 health-food products of Japanese origin, by carrying out disaccharide compositional analysis following enzymic depolymerization and <sup>1</sup>H-NMR spectroscopy. It was noted that 9 of the 12 products had labels indicating their origin to be shark cartilage. However, 2 of them were found to contain mammalian chondroitin. Sakai et al. (2007) also compared the ratio of the sulfate group to the galactosamine residue following the acid hydrolysis of chondroitin. The results suggested that the chondroitin from sharks had a ratio of more than 1.0, whereas that from mammals had a ratio of less than 1.0. This is an extremely cheap and simple technique for most laboratories (Sakai et al. 2007).

## 6.11 PROCEDURES USED FOR THE ANALYSIS OF BIOLOGICAL SAMPLES

Analysis of biological samples requires the use of more sensitive analytical techniques compared with those used for the analysis of pharmaceuticals, nutraceuticals, and raw materials in order to detect the much lower levels of CS present in biological samples. The analytical techniques used need to be sufficiently suitable for the detection of hydrophilic, polar compounds. Techniques that have been previously used to quantify chondroitin in tissue samples and various biological fluids include chromogenic quantitative methods, for example, colorimetric assays based on either uronic acids (Bitter and Muir 1962) or galactosamine (Stylianou, Triantaphyllidou, and Vynios 2006), and the application of cationic dyes, such as alcian blue and 1,9-dimethylmethylene blue (DMB). Other previously used methods include chromatographic methods (Imanari et al. 1996), such as HPLC with intact or enzymatically digested molecules and electromigration techniques, for example, cellulose acetate electrophoresis, gel (polyacrylamide or agarose) electrophoresis and, more recently, fluorophore-assisted carbohydrate electrophoresis and CE (Volpi and Macari 2006). Mass spectrometry (MS) techniques, including matrix-assisted laser desorption/ionization (MALDI) and electrospray ionization (ESI), have been used in isolation or conjugated with chromatographic and electrophoretic methods, such as HPLC and CE, respectively. Other methods include solid-phase assays, enzyme-linked immunosorbent assay (ELISA), and ELISA-based procedures (Stylianou, Triantaphyllidou, and Vynios 2006).

## 6.12 APPLICATION OF CATIONIC DYES

Various cationic dyes have been used for the detection and analysis of CS in biological samples. The most commonly used dyes include alcian blue; toluidine blue; DMB; and stains-all (Stylianou, Triantaphyllidou, and Vynios 2006). DMB is widely used in the analysis of GAGs due to its increased sensitivity (Farndale, Sayers, and Barrett 1982) in contrast to the alcian blue dye-binding assay and its markedly simplistic procedure when compared with other spectrophotometric approaches,

including the carbazole for uronic acid reaction (Bitter and Muir 1962). Farndale, Sayers, and Barrett (1982) devised a rapid spectrophotometric procedure that was an improvement on previous attempts to estimate sulfated GAGs using DMB (Taylor and Jeffree 1969) and alcian blue. The methods of dye binding prior to the procedure devised by Farndale et al. suffered from the disadvantage of unstable dye–GAG complex formation. The modified technique involved the use of a formate buffer, enabling the stabilization of the reagent and the complex, in place of the dibasic citrate/phosphate buffer that was previously recommended by Humble and Etringer (Farndale, Sayers, and Barrett 1982).

A cysteine protease (Berdowska 2004), papain (type III), was used to cleave GAGs from cartilage culture samples before being mixed with 2.5 mL DMB reagent. A metachromatic color was subsequently obtained and the absorbance was read at 595 nm. The resultant complex was stable and linear using GAG concentrations up to 60 µg/mL. The recorded experimental sensitivity was 1 µg, which could be further improved with the use of microcuvettes (Farndale, Sayers, and Barrett 1982).

The positive interaction of other polyanions such as DNA, RNA, and HA with DMB rendered this assay nonspecific for sulfate GAGs. Furthermore, the assay was unable to differentiate and permit the quantitation of individually sulfated GAGs and, therefore, accurate determination of CS and DS content was not possible (Farndale, Buttle, and Barrett 1986). Farndale, Buttle, and Barrett (1986) proposed further improvements to overcome the identified problems. Two significant modifications were the use of specific polysaccharidases to selectively degrade the GAGs and the adoption of new reagent conditions. Chondroitin AC and ABC lyases were used to produce unsaturated disaccharides (Schiller et al. 1999) prior to being treated with papain, which allowed the quantitative determination of individual GAGs including CS and DS. In contrast to the previous results, the selectivity of the assay for sulfated GAGs was drastically improved due to the modified reagent conditions. A lower pH of 3 and higher salt concentrations were achieved by preparing the color reagent in a glycine–hydrochloric acid buffer. This adjustment suppressed the previous interactions that occurred between DMB and other polyanions such as DNA and HA, thus achieving greater specificity (Farndale, Buttle, and Barrett 1986).

Overall, the main advantages of using dye-binding assays for the analysis of biological fluids are simplicity, rapidity, and convenience. The DMB has a recorded detection range between 2 and 50 µg/mL with a significantly improved detection limit of approximately 200 ng in comparison with alcian blue dye-binding assays (detection range: 12.5–400 µg/mL) (Bjornsson 1993). Dye-binding assays, therefore, offer good sensitivity and are ideally suited for rapid screening of biological samples.

The main disadvantage of the application of cationic dyes for the analysis of biological fluids is the lack of specificity associated with these assays. Cationic dyes contain hydrophobic side chains, which have the potential to interact with proteins and other molecules possessing negatively charged carboxyl groups and/or hydrophobic moieties. Although the use of certain proteases and carefully controlled pH conditions can reduce interference from these molecules, any interaction from contaminants will negatively affect CS quantification. Furthermore, it is only after the use of specific polysaccharidases such as chondroitin AC and ABC lyases that individually sulfated GAGs, including CS and DS, can be quantified.

### 6.13 ELECTROMIGRATION METHODS

Several electromigration techniques have been developed to separate, determine the origin, and quantify GAGs from various biological samples. Conventional methods employed to differentiate and examine GAGs in complex mixtures include electrophoresis on cellulose acetate, electrophoresis on nitrocellulose membrane, agarose gel electrophoresis, and electrophoresis on polyacrylamide gel (PAGE). Two additional methods, which were developed in the last decade, include high-performance CE (HPCE) and fluorophore-assisted carbohydrate electrophoresis (FACE) (Volpi and Macari 2006).

## 6.14 FLUOROPHORE-ASSISTED CARBOHYDRATE ELECTROPHORESIS

Volpi et al. (Volpi and Macari 2005) developed a simple technique to quantitatively and qualitatively evaluate CS from blood plasma samples collected from healthy males aged 30–63 years using FACE. Samples containing 100  $\mu$ L blood plasma were treated with proteinase K, and CS was subsequently extracted or purified using a filter membrane or an anion-exchange resin. The resultant CS recovered from each sample was enzymatically hydrolyzed by the action of chondroitin ABC lyase and later derivatized with 2-aminoacridone and separated by FACE. Electrophoretic separation of  $\Delta$ -disaccharides was performed on PAGEs containing 50% acrylamide/7.5% methylenebisacrylamide at 400 V for 60 minutes at 4°C. After separation, UV light was used to illuminate the gels and they were imaged using a charge-coupled device, and quantitative analysis of CS was achieved using a densitometer (Volpi and Macari 2006). The detection limit for CS was found to be 50 ng, with a linear response range of 50–2000 ng with excellent recovery. Volpi et al. reported several advantages of this technique in comparison with other electrophoretic and chromatographic techniques. The main advantages of FACE over agarose gel electrophoresis and HPLC separation equipped with postcolumn derivatization and fluorescence detection of CS disaccharides are lower cost, greater simplicity, and the fact that large sample volumes are not required. Although HPLC-UV detection offers greater sensitivity compared to other techniques for the quantitative evaluation of CS in biological fluids, FACE does not require complex and expensive equipment. In fact, the linearity, sensitivity, and reproducibility of FACE were found to be comparable to those of HPLC-UV detection using 2-cyanoacetamide as a fluorogenic reagent (Volpi and Macari 2005, 2006).

In another method, the levels of chondroitin in shark cartilage and its products were calculated by analyzing unsaturated disaccharides after treatment with chondroitinase ABC. The average molecular weights were evaluated by agarose gel electrophoresis, and the origins of chondroitin and formulated products determined by using pattern of disaccharide composition. Quantitative and compositional analysis of disaccharides after enzymatic depolymerization showed that the levels of chondroitin ranged from 0% to 28.92%  $\pm$  0.03%. All but one of the samples had  $\Delta$ Di-2,6diS and had more  $\Delta$ Di-6S than  $\Delta$ Di-4S, indicating that they originated from shark cartilage. In formulated products, this varied from 0.58%  $\pm$  0.01% to 21.30%  $\pm$  0.08%. (Sim et al. 2007).

The main advantages of FACE are as follows: Both quantitative and qualitative evaluation of complex polysaccharides can be achieved and analysis is not restricted to purified samples; instead, investigations can also be applied to complex mixtures, such as those found in biological specimens. In addition, multiple samples can be analyzed per analysis (Calabro et al. 2001), and procedures are usually simple and require only small volumes of samples (Calabro et al. 2000). Volumes as minute as 20  $\mu$ L were reported in one study (Volpi and Macari 2005). Application of FACE also allows determination of the fine structural characteristics and properties of GAGs. Furthermore, when electrophoretic approaches are combined with various staining procedures, derivatization techniques, and detectors, they become increasingly suitable for the analysis of biological samples with wide applications in pathology and pharmacology.

The main disadvantages of electrophoretic techniques are the potential for comigrating material (Hague, Masada, and Brandly 2002) and long analysis times; however, progress is being made in optimizing procedural rapidity to achieve greater viability for application in routine clinical analyses (Volpi and Macari 2006).

## 6.15 CHROMATOGRAPHIC METHODS

Chromatographic techniques have been widely used to separate and characterize CS in biological fluids and various biological samples. Numerous techniques have been developed including ion-exchange chromatography, gel permeation (SE) chromatography, and thin-layer or paper chromatography. An assortment of HPLC methods of normal-phase, RP, ion-exchange, and ion-pairing chromatography is also used, providing information regarding charge density, polydispersity, and

oligosaccharide size (Imanari et al. 1996). Various detection methods, some with comparable sensitivities, have been employed, such as conductivity detection, UV detection, derivatization with fluorescence detection, and  $^3\text{H}$ -labeling with radiochemical detection (Gioldassi and Karamanos 1999).

### 6.15.1 HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY ANALYSIS

Huang et al. (1995) used a simple HPLC method for the determination of CS in human whole blood, plasma, and blood cells. The technique they developed required the preparation of blood plasma, leukocytes, erythrocytes, and platelets from human blood samples obtained from healthy volunteers using ethylenediaminetetraacetic acid (EDTA), disodium salt, as an anticoagulant. Blood plasma was obtained by centrifuging each sample at 1500g for 15 minutes. Leukocytes were isolated by centrifugation and various purification processes according to the method of Olsson and Gardell (1967). Erythrocytes were isolated by density-gradient centrifugation and subsequent gel filtration in order to remove leukocytes and platelets from the homogeneous erythrocyte fraction. Platelets were also isolated by centrifugation of blood samples as described by Imanari et al. (1991).

The GAGs were extracted from isolated blood cells or whole blood cells by the addition of sodium dodecyl sulfate and further treatment as described by Emura and Mukuda (1973). The GAG samples were then digested with 0.05 units of chondroitinase ABC and/or AC II in 0.1 M tris-acetate buffer for 3 hours at 37°C and analyzed by postcolumn HPLC. The conditions under which HPLC was performed are as follows: It is noted that 10  $\mu\text{L}$  samples were loaded via a sample injector with a 20  $\mu\text{L}$  loop. A TSK gel amino-100 column was eluted at 30°C with 20 mM ammonium formate buffer (pH 5.0), containing 10 mM sodium sulfate in 4% acetonitrile, at a flow rate of 0.5 mL/min. For the separation of GAGs, a mobile phase consisting of acetonitrile (60%)–0.1 M tris–HCl buffer containing 0.1 M boric acid and 10 mM sodium sulfate pH 7.0 was found to be optimal (Huang et al. 1995).

The strong fluorescence of unsaturated disaccharides after reaction with 2-cyanoacetamide in sodium hydroxide was used for postcolumn fluorometric detection, with excitation and emission wavelengths set at 346 and 410 nm, respectively. The detection limit was at the nanogram level, suggesting that this technique may have useful applications in the qualitative and quantitative determination of CS in blood plasma and urine (Huang et al. 1995).

Another study used HPLC with an amine-bound silica column using a linear salt gradient to separate unsaturated CS disaccharides labeled precolumn with a fluorophore, 2-aminobenzamide (Kinoshita and Sugahara 1999). Kinoshita and Sugahara selected to conjugate CS disaccharides with 2-aminobenzamide as it offered superior sensitivity (by a factor of approximately 50) with a detection limit in the low picomole range, in contrast to other studies that had used 2-aminoacridone (Kitagawa, Kinoshita, and Sugahara 1995) as a tagging agent, and as it is 500–1000 times more sensitive than conventional HPLC methods using UV absorbance detection. Other studies have used 2-aminopyridine (Plaas, Hascall, and Midura 1996) to increase the sensitivity of detection; however, in terms of simplicity and rapidity, 2-aminobenzamide is superior to all others (Kinoshita and Sugahara 1999).

Overall, the main advantage of using HPLC for the analysis of biological fluids and tissue samples is that it is suitable for routine use, providing a simple platform and requiring relatively small quantities of materials. After depolymerization of CS, information regarding chain composition and CS content can be quantitatively obtained rapidly and reliably with excellent specificity and little interference from other molecules. The sensitivity of detection may be improved by derivatization with various fluorophores allowing microanalysis in the picomolar range. The equipment required is relatively inexpensive in comparison with other techniques, and HPLC can easily be hyphenated with various MS platforms including MALDI and ESI to facilitate fine-structure determination (Sisu et al. 2010; Stylianou, Triantaphyllidou, and Vynios 2006).

The perceived disadvantages of HPLC include relatively short column life and chemically unstable packing material. These drawbacks are particularly a problem with amino-bonded silica columns, which are extensively used for the separation of oligosaccharides derived from GAGs, and their subsequent compositional analysis (Imanari et al. 1996).

Another disadvantage of HPLC used for the analysis of biological samples includes long analysis times; in some studies, HPLC takes up to 90 minutes. However, in one study, Fluharty et al. (1982) developed an HPLC technique to analyze urine GAGs from patients using a Partisil-10 SAX column, which decreased the analysis time to around 17 minutes.

### 6.15.2 MASS SPECTROMETRY AND HYPHENATED TECHNIQUES

MS based on either MALDI or ESI has been developed and used widely for the structural elucidation of numerous GAGs including CS and DS. Furthermore, these MS techniques have been used in isolation or have been conjugated with previously described analytical methods, such as HPLC and CE. Such hyphenated techniques were introduced in the last decade to facilitate fine-structure determination (Sisu et al. 2010).

### 6.15.3 ELECTROSPRAY IONIZATION-MASS SPECTROMETRY— HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

Chromatographic separation of CS has been previously reported utilizing either porous graphitized carbon columns (Barroso, Didraga, and Bischoff 2005; Estrella et al. 2007) or RP HPLC as derivatized disaccharide (Volpi 2010) combined with MS spectroscopy.

Barroso et al. (2005) used an HPLC-ESI-MS technique with a porous graphitized column to study various biological samples obtained from patients suffering from chronic inflammatory lung disease. The primary purpose of their investigation was to identify any compositional or fundamental structural changes that may be present in lung proteoglycans and how these alterations may relate to disease state. The procedure involved homogenizing 20 g of excised human lung tissue and successive extraction with a suitable buffer (pH 6) containing a protease inhibitor for 20 hours at 4°C. After centrifuging the extract, the supernatant was filtered and separated using a fast protein liquid chromatography system. The bound fraction was eluted, subjected to buffer exchange, and enzymatically cleaved by the action of chondroitinase ABC. Biological fluids including bronchoalveolar lavage fluid, sputum, and urine were also subjected to pretreatments, including buffer exchange to 10 mM tris or pH adjustment, and later incubated for 12 hours with excess chondroitin ABC at 37°C. The conditions under which this study was performed are as follows: Mobile phase A comprised formic acid in ultrapure water, and mobile phase B consisted of 0.1% formic acid in acetonitrile. Chromatographic separation of the disaccharides was performed with an increasing gradient of mobile phase B, from 5% to 90%. A flow rate of 5 µL/min with a column temperature of 28°C was maintained over a period of 1 hour. The HPLC column was a porous graphitized carbon column. Analytes were ionized using an electrospray ionizing source operating in the negative ion mode and detected by an ion-trap mass spectrometer (Barroso, Didraga, and Bischoff 2005).

The main advantages of this technique when compared to other methods of analyzing CS are high sensitivity, simple methodology, increased versatility, and the ability to rapidly analyze different types of tissue extracts and biological fluids without prior derivatization or isolation of the disaccharide products. Barroso et al. recorded a limit of detection as low as 0.1 ng, which can be further improved to 0.01 ng using multiple reaction monitoring; thus, significant enhancement in sensitivity is provided. It has been documented that GAGs are subject to specific modifications (Barroso, Didraga, and Bischoff 2005; Bartold 1992) in certain disease states and, thus, the ability of the HPLC-ESI-MS technique to identify and quantify CS and DS isomers gives it a significant advantage other methods used to determine GAG structure.

Hyphenating CE and LC to ESI-MS circumvents certain problems associated with the mass spectroscopic analysis of CS in complex mixtures without prior chromatographic separation. The advantages include reduction in isobaric peak overlapping, reduced spectrum complexity, and less

ion suppression (Volpi 2010). The main limitation of LC-ESI-MS is poor separation of monosulfated and disulfated isomeric forms of CS and DS disaccharides; however, this drawback was recently overcome in a study by Volpi (2010), in which he used HPLC separation of disaccharide species derivatized with 2-aminoacridone with MS detection.

#### **6.15.4 MATRIX-ASSISTED LASER DESORPTION/IONIZATION-MASS SPECTROMETRY-HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY**

Nimptsch et al. (2009) were the first to use MALDI-time of flight (TOF)-MS to quantify CS. The aim of their research was to build on previous studies that had already successfully implemented MALDI-TOF-MS for the qualitative investigation of chondroitin disaccharides. Their paper describes a convenient technique having significant relevance to the study of pathology to quantify the amount of CS in biological samples by using the signal-to-noise (S/N) ratio subsequent to enzymatic digestion (Sisu et al. 2010). The method involved the preparation of native CS by dissolving and diluting CS with distilled water in a stepwise fashion to yield concentrations from  $0.1 \text{ mg}\cdot\text{mL}^{-1}$  down to  $0.1 \text{ }\mu\text{g}\cdot\text{mL}^{-1}$ . The CS solutions were then treated with 0.2 units of chondroitin ABC lyase for 2 hours at  $37^\circ\text{C}$  to produce disaccharides for MALDI-TOF-MS analysis required for system calibration. Test samples were ionized using a 337 nm nitrogen laser, with an average of 60 random single laser shots per analyte. The extraction voltage was set to 20 kV; both positive and negative ion spectra were recorded and acquired using an Autoflex mass spectrometer. Depending on the MALDI-TOF mass spectrum required, different matrix compositions were used. It is noted that 0.5 M 2,5-dihydroxybenzoic acid solution in methanol, and trihydroxyacetophenone were used for positive ion production and negative ion production, respectively. It is noted that 1.25  $\mu\text{L}$  of the analyte/matrix (1:1 v/v) mixture was spotted onto a MALDI plate and cocrystallized in air. The S/N ratio was used to quantitatively determine CS levels. Although isotopically labeled internal standards would have achieved greater quantification, they were not used in this study due to their expense and limited availability. The main advantages of this technique over other methods used for the analysis of CS are high sensitivity, requirement of low sampling volumes, and wide applicability in the analysis of complex mixtures due to high specificity. These advantages hold true especially when comparing this procedure with that of the carbazole and alcian blue dye-binding assay, which inherently requires large volumes of the analyte (sometimes up to several hundred microliters as opposed to approximately 1  $\mu\text{L}$  for MALDI). Unlike spectrophotometric assays, which can give false positive results due to their lack of specificity, MALDI exclusively detects CS and is also superior in terms of sensitivity with a detection limit of at least 5 pmol, which is comparable to the detection limits of ESI-MS techniques. It is noted that MALDI has several advantages over the more recently used ESI-MS methods, including easier interpretation of spectral data, enhanced applicability to high-throughput investigations, and higher tolerance to contaminating species (Nimptsch et al. 2009; Sisu et al. 2010).

The disadvantages of MALDI techniques in general are that they can be quite laborious and that time-consuming sample pretreatment is usually required, although in the aforementioned study these problems were somewhat circumvented.

#### **6.16 SOLID-PHASE ASSAYS, ENZYME-LINKED IMMUNOSORBENT ASSAYS, AND ELISA-LIKE TECHNIQUES**

Solid-phase assays (Hardingham et al. 1994), ELISAs (Vynios et al. 2001), and ELISA-like techniques (Vynios et al. 1999) have been applied in a few studies for the detection and quantification of low levels of CS in various biological samples.

One study (Vynios et al. 2001) used a solid-phase assay to quantify CS in human laryngeal cartilage from normal and cancerous subjects after laryngectomy to identify proteoglycan and GAG alterations that may provide evidence for tissue status.

Polystyrene ELISA plate wells were coated with 100 µL of glutaraldehyde solution and reacted with 100 µL of spermine in order to activate the surface. The CS chains were labeled with biotin as described by Vynios et al. (1999) to produce a biotin–CS complex, which would compete with GAGs contained in each sample for electrostatic binding sites on spermine. Avidin–peroxidase diluted with *o*-phenylenediamine was used to quantify the levels of biotin bound to the ELISA plates by measuring the absorbance at 492 nm, which, after calculation using calibration curves, allowed the quantification of CS contained in each sample.

Vynios et al. (2001) found that when heparin and CS were both contained in biological samples, accurate determination of GAGs was not achievable. To overcome this problem, cartilage samples were digested with papain and centrifuged at 6000 rpm for 5 minutes to remove any precipitates and undigested tissue. The supernatants were then hydrolyzed by the action of chondroitinase ABC lyase for 8 hours at 37°C to produce Δ-disaccharides. The resultant Δ-disaccharides were not able to compete with heparin for binding to spermine. After measuring total heparin-CS levels by direct analysis, followed by enzymatic cleavage of CS and further solid-phase analysis, determination of each GAG could be achieved by simple calculation.

A previous study by Vynios (Vynios et al. 1999) based on a similar solid-phase assay allowed the quantitative analysis of sulfated GAGs at the nanogram level without any degradative steps. The limit of detection was 9 ng, and over 90 samples could be simultaneously analyzed. This technique has similar sensitivity to the technique described by Vynios et al. (2001); the main advantage of this technique is that no degradation steps are required, in contrast to certain HPLC or HPCE techniques, which suggests that this procedure has greater applicability for routine analysis (Vynios et al. 1999). Overall, the main advantages of ELISA-like methodologies compared to other techniques are increased simplicity, excellent sensitivity, and the ability to analyze numerous samples in a relatively short period of time. The competitive binding assay described previously could determine levels of chondroitin as low as 9 ng, making this technique ideally suited for analyzing biological samples possessing low levels of CS for diagnostic purposes. The use of ELISA plates permitted the rapid analysis of a large number of samples with minimal sample volumes and proved to be an inexpensive method, with the ability to be automated, and was viable for routine analysis (Vynios et al. 1999, 2001). The main disadvantages of using the ELISA-based methodology are that it is able to determine only single analytes and that much time is taken to produce anti-analyte antibodies.

## 6.17 CONCLUSIONS

To evaluate CS content in biological specimens and/or pharmaceuticals, several analytical techniques have been developed. A number of techniques have been used specifically for the analysis of chondroitin of marine origin (see [Table 6.2](#)).

Other techniques discussed, which evolved for analysis of terrestrial and avian sources of chondroitin, have also been applied to marine chondroitin.

The analytical methodology employed depends on a number of features, including the type of analysis required, which may be for quantitative purposes or for CS structural characterization. Other factors such as the presence of contaminants or other GAGs may favor the use of one technique over another.

In recent years, with advances in technology, the analytical techniques used for the identification and quantification of CS have become progressively more sophisticated, and they are spurred on by an ever-increasing demand for more information regarding the structural characteristics of chondroitin and how these properties may impact biological systems. This has resulted in many studies evaluating the potential of CS as a therapeutic agent for many disease states and, more

**TABLE 6.2**  
**Selected Analytical Data Derived from Marine Chondroitin (CS) Sources**

Marine Source	Technique	Results	Reference
Shark	Size-exclusion HPLC-UV	Quantification of CS in formulated products	Choi et al. (2003)
Shark	Anion-exchange HPLC-UV	Quantification of CS in raw materials	Takagaki et al. (1994)
Shark, whale	Anion-exchange HPLC	Separation of 22 CS oligosaccharides	Lauder, Huckerby, and Nieduszynski (2000)
Whale	Ion-pair RP-HPLC-radiochem	Separation of 4 CS oligosaccharides	Gioldassi and Karamanos (1999)
Shark	Anion-exchange HPLC-UV	Separation of 4S, 6S disaccharides 22,000 Da	Sim et al. (2005)
Shark	Anion-exchange HPLC-UV	2,6 disaccharides identified, Di6S > Di4S concentration	Sim et al. (2007)
Shark	Ion-pair HPLC-UV	Separation and quantification of 0S, 4S, 6S CS	Ji et al. (2007)
Shark	HP-gel filtration	Identification of 4S CS 50,000 Da	Toida et al. (1997)
Shark	Anion-exchange HPLC-fluorimeter	Separation and quantification of raw material and CS formulations	Qui et al. (1996)
Shark, whale	CE	Separation of 4S, 6S, 2,6S disaccharides	Okamoto et al. (2004)
Shark	CE	Separation and quantification of raw material and CS formulations	Malavaki et al. (2008)
Shark, ray	FTIR	Quantification of 4S levels in 4 CS raw materials	Garnjanagoonchorn et al. (2007)
Shark, whale	GC-MS	Separation and quantification of oligosaccharides	Zanetta et al. (1999)

recently, exciting research has implicated CS motifs as potential biomarkers for pathological processes including cancers. If further technological advancements are made, and techniques with increased resolution and sensitivity are developed as a result, earlier diagnosis of specific diseases may be achieved, which could lead to significant and dramatic improvements in patient outcomes. As a consequence, there has been a surge in the development of techniques to evaluate CS in biological fluids and biopsy specimens, not to mention the quantification and structural analysis of chondroitin as a supplement and of the raw materials it is derived from.

In conclusion, it is of paramount importance that satisfactory techniques are continually developed and used to evaluate new therapeutic applications, including monitoring *in vitro* and *in vivo* experiments, and detail developments in clinical trials. On another level, these techniques can be used to protect consumers from potentially unsafe, ineffective, and low-quality chondroitin preparations and be enforced to confirm origin, purity, and label claims of finished nutraceutical products and raw materials.

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# 7 Marine Natural Products Targeting Nuclear Factor κB

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## 7.1 INTRODUCTION

The nuclear factor κB (NF-κB) transcription factor, which was first discovered in 1986, is a family of structurally related proteins that promote expression of over 150 genes involved in a variety of cellular processes (Kumar et al. 2004). It is a dimeric complex of various subunits that belongs to the Rel family, which includes RelA (p65), RelB, c-Rel, p50, and p52. In most cell types, inactive NF-κB complexes are kept in the cytoplasm via their noncovalent interaction with inhibitory proteins known as IκBs (Delhalle et al. 2004). In response to multiple stimuli, including cytokines, viral and bacterial pathogens, and stress-inducing agents, the latent cytoplasmic NF-κB/IκB $\alpha$  complex is activated by phosphorylation, ubiquitination, and proteosome-mediated degradation of IκB. Free and activated NF-κB translocates in the nucleus, and binds to its target DNA-binding site to initiate gene transcription. NF-κB is involved in the transcription of many proinflammatory and antiapoptotic genes, and it is now considered as a key element in the progression of carcinogenesis and inflammatory diseases (Heras and Hortelano 2009).

So far, three NF-κB activation pathways have been described: the first is the classical or canonical pathway, the second is the alternative pathway, and the third has been described recently and it can be activated by DNA-damaging drugs or ultraviolet light, without apparent IκB kinase (IKK) activation (Dolcet et al. 2005; Gilmore 2006).

The classical NF-κB activation pathway is the major pathway involved in proinflammatory signaling. It is triggered by a wide range of proinflammatory stimuli, which include cytokines, viral infections, and antigen receptors. During activation, the IKK $\beta$  subunit is the most responsible for IκB phosphorylation in serine residues. After phosphorylation, IκB proteins go through ubiquitin-dependent degradation by the proteasome, and NF-κB is translocated to the nucleus, where it acts as a nuclear transcription factor.

The alternative pathway is activated by members of the tumor necrosis factor (TNF) family. After stimulation of the receptor, an NF-κB signaling cascade is activated, leading to the formation of a p52–Rel-B complex, which translocates to the nucleus. This pathway is important for lymphocyte development and activation and provides a link between the innate and adaptive immune response (Bokulich 2005; Lawrence 2011).

Atherosclerosis, asthma, AIDS, cancer, diabetes, heart disease, muscular dystrophy, incontinencia pigmenti, rheumatoid arthritis, Alzheimer's disease, inflammatory bowel disease, and multiple sclerosis are known as NF- $\kappa$ B-related diseases (Tak and Frestein 2001; Courtois and Smahi 2006; Lawrence 2009).

## 7.2 NF- $\kappa$ B AND CANCER

NF- $\kappa$ B regulates expression of genes involved in the development and progression of cancer, including cell growth, differentiation, proliferation, migration, apoptosis, and metastasis (Suh and Rabson 2004). Cyclin D1 and *c-myc*, which have important roles in cell growth and proliferation, are NF- $\kappa$ B target genes. NF- $\kappa$ B also contributes to tumor development by regulating expression of genes (*VEGF*, *IL-8*, *uPA*, and *MMP9*) involved in angiogenesis, tumor invasion, and metastasis. Not only alterations of NF- $\kappa$ B/Rel genes associated with a series of leukemias and lymphomas, but also NF- $\kappa$ B/Rel gene products have been shown to have important proliferative and antiapoptotic activities that could contribute to the development, progression, and resistance to therapy of nonlymphoid tumor cells (Suh and Rabson 2004).

Different carcinogens also activate NF- $\kappa$ B during their damage in the body. It is well known that cigarette smoke contains several carcinogens that initiate and promote tumorigenesis and metastasis. Activation of NF- $\kappa$ B by cigarette smoke in a wide variety of cells may play a role in cigarette-smoke-induced carcinogenesis. Another example is *Helicobacter pylori*, which is a causative factor in 60–90% of cases of gastric cancer (Bokulich 2005). *H. pylori* proteins cause gastritis and carcinoma induced by IL-8 in a dose- and time-dependent manner via the NF- $\kappa$ B signaling pathway. On the other hand, it is well documented that NF- $\kappa$ B is one of the important growth factors for tumor cells (Agarwal et al. 2006). For example, constitutive activation of NF- $\kappa$ B in human cutaneous T cell lymphoma cells is mediated by the production of TNF and the proliferation of these lymphoma cells. Extensive research in the last few years suggests that NF- $\kappa$ B activation mediates resistance to cytokines, chemotherapeutic agents, and gamma irradiation, whereas suppression of NF- $\kappa$ B can sensitize tumor cells to these agents (Agarwall et al. 2006). Chemotherapy resistance is one of the important challenges in the cancer therapy. It has been suggested that NF- $\kappa$ B may be responsible for blocking the efficacy of chemotherapy and radiation in some types of tumor cells. NF- $\kappa$ B may induce expression of the multidrug-resistant P-glycoprotein. In some tumors, cells exposed to radiation or some chemotherapeutic drugs show increased activation of NF- $\kappa$ B. On the other hand, inhibition of NF- $\kappa$ B improves the apoptotic response to radiation therapy (Delhalle et al. 2004; Dolcet et al. 2005).

## 7.3 NF- $\kappa$ B AND INFLAMMATION

NF- $\kappa$ B has long been considered in the proinflammatory signaling pathway, mainly based on the activation of NF- $\kappa$ B by proinflammatory cytokines such as IL-1 and TNF- $\alpha$ . NF- $\kappa$ B promotes expression of cell adhesion molecules (ICAM-1, VCAM-1, E selectin, and tenascin C), vascular endothelial growth factor, and matrix metalloprotease-2 and -9. It is also involved in the activation of enzymes such as inducible nitric oxide synthase (iNOS), COX-2, 5/12-lipoxygenase, chemokines, and cytokines (IL-1 and TNF- $\alpha$ ). Relations of NF- $\kappa$ B with these molecules indicate its role in chronic inflammatory diseases such as rheumatoid arthritis, Crohn's disease, inflammatory bowel disease, ulcerative colitis, and asthma (Delhalle et al. 2004). NF- $\kappa$ B activation is observed in mucosal biopsy samples from patients with Crohn's disease and ulcerative colitis. Treatment of patients with inflammatory bowel diseases with steroids decreases the NF- $\kappa$ B activity in biopsy samples and reduces clinical symptoms. These results suggest that stimulation of the NF- $\kappa$ B pathway may be involved in the enhanced inflammatory response associated with these diseases. Atherosclerosis and its consequences, heart attack, stroke, and peripheral vascular insufficiency, are an important cause of morbidity and mortality among old people (Collinsi and Cybulsky 2001). Different growth factors,

cytokines, and chemokines released from endothelial cells, smooth muscle, macrophages, and lymphocytes are involved in this chronic inflammatory process. Physiological and pathological activation of the NF-κB system may contribute to the changes in gene expression that occur during atherosclerosis. Activated NF-κB has been identified in human atherosclerotic plaques, but is almost absent in nonatherogenic vessels. The effects of NF-κB regulation on the inflammatory response and on the control of cellular proliferation may play an important role in the initiation and progression of atherosclerosis (Yamamoto and Gaynor 2001).

On the other hand, the definition of NF-κB activation in chronic inflammation may take part in the promotion of tumor, since the antiapoptotic genes activated by NF-κB may contribute to the survival of damaged cells and allow the formation of precancerous tissues. The role of NF-κB as an important mediator in apoptosis resistance and inflammatory diseases reveals the potential benefits of its inhibition. Several steps of the NF-κB pathway, such as IKK activation, IκB phosphorylation and degradation, NF-κB nuclear translocation, and transcriptional activity, can be triggered by various natural derivative or synthetic inhibitors (Delhalle et al. 2004).

There are a lot of different structures, such as glucocorticoids, salisylates, and immunosupriments modulating NF-κB expression in different mechanisms, in the therapy of important diseases (Tak and Frestein 2001). For example, glucocorticoids have profound effects on the development and homeostasis of the immune system. They inhibit the NF-κB pathway in different mechanisms. Similarly, salisylates and nonsteroidal anti-inflammatory drugs are also important agents for the treatment of chronic inflammatory diseases. Their molecular target is known as, at least in part, inhibition of NF-κB activation. These agents suppress TNF-α-induced mRNA expression of VCAM-1 and ICAM-1 in endothelial cells. This inhibition of the NF-κB pathway in endothelial cells prevents transendothelial migration of neutrophils, supporting their anti-inflammatory action (Kopp and Ghosh 1994). Immunosuppressive agents are another important suppressor of the NF-κB pathway. For example, cyclosporin A and tacrolimus are used in organ transplantation to prevent the patient from acquiring host diseases. Both compounds inhibit the activation of calcineurin, which is required for the activation of NF-κB. To find new compounds from natural sources that selectively inhibit or modulate NF-κB may be a crucial step toward the development of anti-inflammatory and/or anticancer agents with fewer side effects (Bharti and Aggarwal 2002).

## 7.4 MARINE ORGANISMS AS PROMISING ACTIVE MOLECULE SOURCES

Marine organisms constitute an important source of novel molecules for new drug discovery and drug development researches. According to the data until 2004, researchers have isolated approximately 7000 marine natural products, 25% of which are from algae, 33% from sponges, 18% from coelenterates (sea whips, sea fans, and soft corals), and 24% from representatives of other invertebrate phyla, such as ascidians (also called tunicates), opisthobranch molluscs (nudibranchs, sea hares, etc.), echinoderms (starfish, sea cucumbers, etc.), and bryozoans (moss animals) (Kijjoa and Sawangwong 2004). The long evolutionary history of marine organisms makes them very diverse in secondary metabolite production. A great number of these compounds from marine organisms have been extensively investigated for their bioactive properties and demonstrated interesting anti-inflammatory, anticancer, cytotoxic, immunomodulating, antimicrobial, antiviral, neurosuppressive, or analgesic activities (Folmer et al. 2008; Erwin, Lopez-Legentil, and Schuhmann 2010; Schumacher et al. 2011). There are currently three U.S. Food and Drug Administration-approved drugs in the U.S. Pharmacopeia, namely, cytarabine (Cytosar-U1, Depocyt<sup>®</sup>), vidarabine (Vira-A<sup>®</sup>), and ziconotide (Prialt<sup>®</sup>). Currently, trabectedin (Yondelis<sup>®</sup>) has been approved by the European Agency for the Evaluation of Medicinal Products (EMEA), and is completing Phase III studies in the United States for approval (Hill and Fenical 2010; Mayer et al. 2010). In this study, marine natural compounds will be discussed for their effect on NF-κB and their potential benefits for medical research.

## 7.5 COMPOUNDS THAT INHIBIT NF-κB IN DIFFERENT MECHANISMS

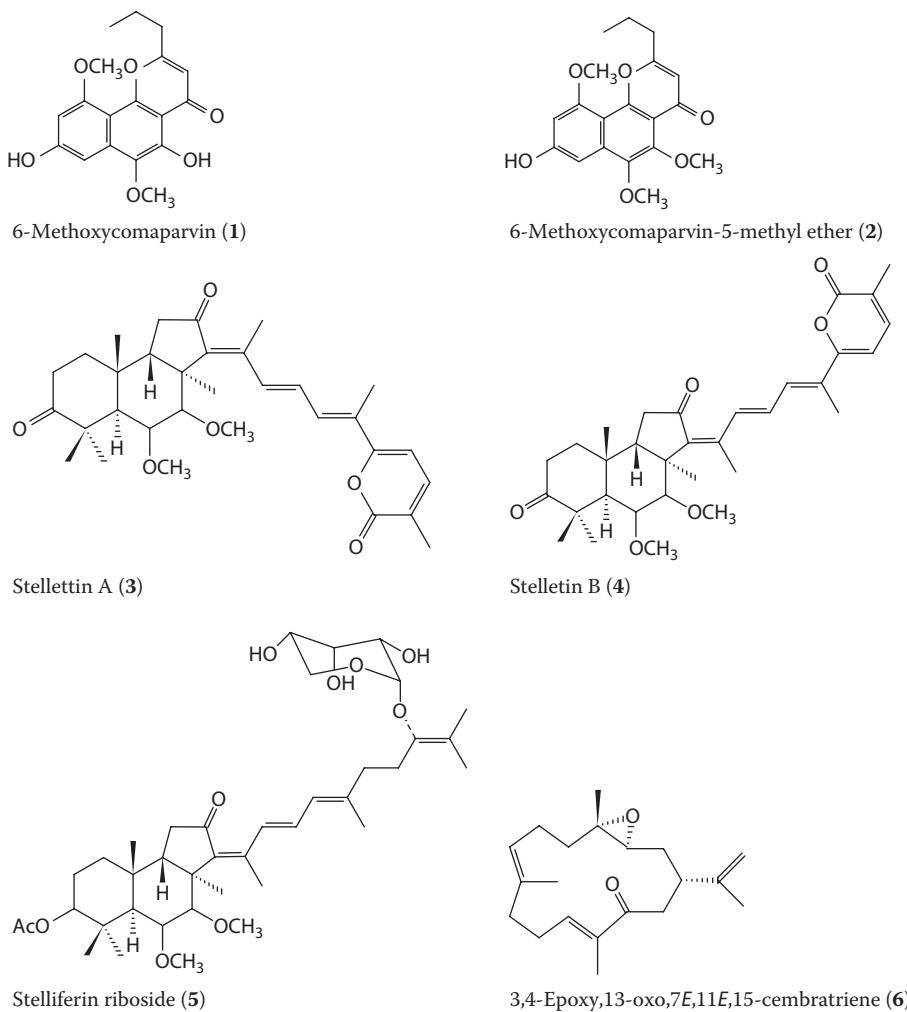
Despite the importance of NF-κB regulation in inflammatory and cancerous diseases, relatively few marine sources have been reported to date as NF-κB inhibitors. The marine natural products reported here as NF-κB inhibitors are given according to their structural features.

The effect of marine natural products, including 266 different extracts on TNF-α-induced NF-κB activation, has been investigated by Folmer et al (2009). This large-scale screening has covered 220 extracts from Fijian algae, sponges, cnidarians, ascidians, and echinoderms and 43 extracts from microalgae and cyanobacteria. Five extracts from Fijian marine organisms, a crinoid (*Comanthus parvicirrus*), a sponge (*Rhabdastrella globostellata*), two soft corals (*Sarcophyton* sp. nov. and *Sinularia* sp.), and a gorgonian (*Subergorgia* sp.), with strong NF-κB inhibitory activity have been selected based on the availability of supplementary biomass. They have been purified by bioassay-guided fractionation in order to identify their chemical contents and the mechanism of action of the compounds responsible for the bioactivity of the source extracts (Folmer et al. 2008, 2009). Fourteen percent of the tested extracts have been shown to have strong NF-κB inhibitory potential, and they have inhibited 65% or more of the NF-κB activity induced by TNF-α at the test concentration of 100 µg/mL. Bioactive extract of *C. parvicirrus* M. (Comasteridae), an echinoderm, has yielded two major naphthopyrones, 6-methoxycomaparvin (**1**) and 6-methoxycomaparvin-5-methyl ether (**2**) (Figure 7.1). Both compounds completely inhibit TNF-α-induced NF-κB activation and NF-κB–DNA binding at a minimum inhibitory concentration of 300 µM. Their results also show the kinase IKKβ to be the major target of 6-methoxycomaparvin and 6-methoxycomaparvin-5-methyl ether along the NF-κB activation pathway (Folmer et al. 2008). The extract from the sponge *R. globostellata* has yielded three bioactive triterpenoids—stellettin A (**3**), stellettin B (**4**), and stelliferin riboside (**5**). Two cembranoids, 3,4-epoxy,13-oxo,7E,11E,15-cembratriene (**6**) and 3,4-epoxy,13-oxo,7E,11Z,15-cembratriene (**7**), have been identified as the major bioactive compounds from the soft coral *Sarcophyton* sp. nov., and a carotenoid astaxanthin (**8**) has been isolated as the major bioactive compound from the extract of the gorgonian *Subergorgia* sp. The extract from the soft coral *Sinularia* sp. has yielded the cyclodepsipeptide jasplakinolide (**9**) as the major bioactive compound. The effects of the marine natural products **1–9** on the TNF-α-induced transcriptional activity of NF-κB have been examined using a luciferase reporter gene assay on pNF-κB-Luc K562 cells. All the bioactive compounds isolated from the above species, except the carotenoid astaxanthin (**8**) isolated from the gorgonian *Subergorgia* sp., have been shown to interfere directly with the binding of NF-κB to DNA (Figure 7.1). The carotenoid astaxanthin (**8**), as well as its synthetic counterpart, is the only NF-κB-inhibiting compound that no molecular target could be pointed. The antioxidative activity of astaxanthin may be responsible for the NF-κB inhibitory action of the compound (Takamatsu et al. 2003; Folmer et al. 2008, 2009).

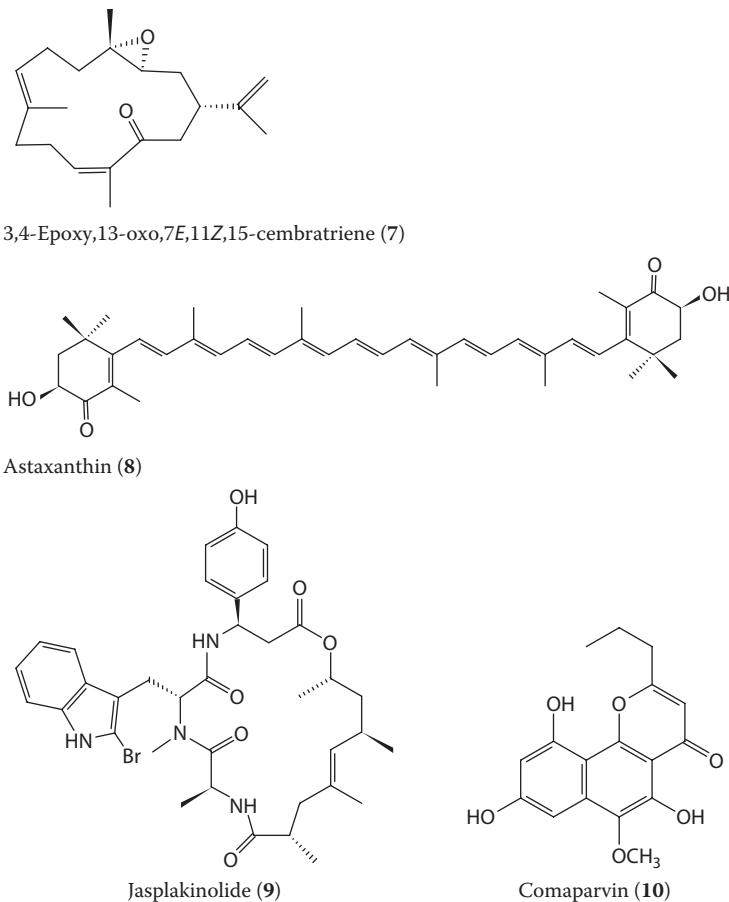
Astaxanthin (**8**) is a red-orange-colored carotenoid present in salmonid, shrimp, and crustacean aquaculture to provide their characteristic pink color. *Haematococcus pluvialis* is the richest source of natural astaxanthin. The antioxidant, anti-inflammatory, and anticancer properties of astaxanthin have been reported previously. In addition, its antitumoral properties have been described in several experimental animal models (Tanaka et al. 1995). Dietary intake of astaxanthin with those marine organisms may protect development of carcinogenesis. Yasui et al. have investigated possible inhibitory effects of astaxanthin against colitis-associated colon carcinogenesis using the azoxymethane (AOM)/dextran sulfate sodium (DSS) mouse model. In this model, a colon carcinogen, AOM, is used as an initiator at a low dose and a colitis-inducing agent, DSS, is used as a tumor promoter. The regimens can be applied to rats to induce colorectal carcinoma within a short period. Two different experiments have been performed in this model. In the first experiment, the effects of astaxanthin at three different doses, 50, 100, and 200 ppm in diet, have been evaluated on colitis-associated colon carcinogenesis induced by AOM/DSS in mice. In the second, the effects of the astaxanthin (100 and 200 ppm) in diet on DSS-induced colitis have been determined. As a result, dietary astaxanthin has significantly inhibited the occurrence of colonic mucosal ulcers, dysplastic crypts, and colonic

adenocarcinoma at week 20. Astaxanthin feeding has suppressed expression of inflammatory cytokines, including NF-κB, TNF-α, and IL-1β; has inhibited proliferation; and has induced apoptosis in the colonic adenocarcinomas. Feeding with 200-ppm astaxanthin, but not 100 ppm, has significantly inhibited the development of DSS-induced colitis. Astaxanthin feeding (200 ppm in diet) also has lowered protein expression of NF-κB and mRNA expression of inflammatory cytokines, including IL-1β, IL-6, and COX-2. These results indicate that the dietary astaxanthin suppresses the colitis and colitis-related colon carcinogenesis in mice through suppressing expression of inflammatory cytokines, including NF-κB. Taken together, astaxanthin is suggested as one of the candidates for prevention of colitis and inflamed colon carcinogenesis in humans (Yasui et al. 2011).

Similarly, Chovolou et al. have also studied the effect of *Comanthus* sp. on NF-κB inhibition by bioactivity-guided methods. Ten compounds, including anthraquinones and naphthopyrones, have been isolated from the active fractions. From these compounds, only comaparvin (**10**) and 6-methoxycomaparvin (**1**) have exhibited the TNF-α-induced NF-κB activity in rat hepatoma and human breast carcinoma cell lines (Figure 7.1). Comaparvin reduces chymotrypsin-like proteasomal activity, blocks nuclear translocation of NF-κB, and effectively inhibits TNF-α-induced IκB phosphorylation at between 50 and 100 μM concentrations, suggesting a role of this compound in



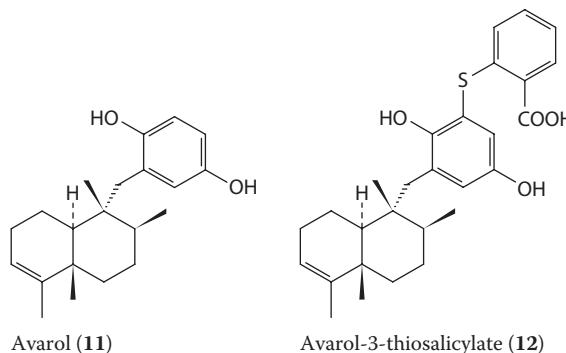
**FIGURE 7.1** Bioactive compounds from Fijian marine organisms (**1–10**).



**FIGURE 7.1** (Continued).

targeting IKK. Furthermore, comaparvin sensitizes cancer cells to apoptotic effects mediated by the proinflammatory cytokine TNF- $\alpha$ . As a result, comaparvin has been considered as a new inhibitor of the NF- $\kappa$ B signaling pathway targeting both proteasome function and I $\kappa$ B phosphorylation likely by a direct inhibitory effect on the IKK $\beta$  activity (Chovolou et al. 2011).

Another large-scale study including 50 extracts collected from the Twilight Zone (50–150 m), including sponges, gorgonians, and associated bacteria, together with 15 extracts from shallow water hard corals and 16 fractions from the methanol extract of the sponge *Subera* sp., assessed in a series of bioassays the chemopreventive and cytotoxic activities of the collected material. Induction of quinone reductase, inhibition of TNF- $\alpha$ -activated NF- $\kappa$ B, inhibition of aromatase, interaction with retinoid X receptor, inhibition of NOS, inhibition of DPPH, inhibition of HL-60, and MCF-7 cell proliferation have been determined. The sponge *Subera* sp. has been selected for further fractionation since it demonstrated significant activity in inhibition of aromatase, NO, and HL-60 proliferation. Ten extracts and 5 fractions have inhibited NF- $\kappa$ B by greater than 60%, 2 extracts and 2 fractions have inhibited DPPH more than 50%, 9 extracts and 2 fractions have affected the survival of HL-60 cells, 3 extracts and 6 fractions have affected quinone reductase, 3 extracts and 12 fractions have inhibited aromatase, 4 extracts and 5 fractions have inhibited NOS, and only 1 extract has inhibited the growth of MCF-7 cells by more than 95%. Two species of Callyspongiidae (74.0% and 73.8%), four species of *Pseudoalteromonas* (sp. 4–7; 76.1%, 74.3%, 72.9%, and 77.4%), and a species of Thorectidae (72.3%), Mycalidae (78.1%), Porifera (93.0%), Nephtheidae (77.2%), *Gordonia* sp3 (79.7%), and Poritidae (73.1%) have shown inhibition of NF- $\kappa$ B of more than 70%.



**FIGURE 7.2** Structures of bioactive sesquiterpenoids (**11–12**).

*Porifera* sp. has been found as the most promising sample among the tested extracts for NF-κB inhibition. This study is one of the first to document related unexplored habitat and organisms in Twilight Zone water around Guam (Schupp et al. 2009).

Avarol (**11**) is a marine sesquiterpenoid hydroquinone with interesting pharmacological properties, including anti-inflammatory and antipsoriatic effects (Figure 7.2). It is well known that activation of NF-κB induces the production of proteins, such as TNF-α, which also stimulate the activation pathway of NF-κB (Yamamoto and Gaynor 2001). In this way, a significant link between high levels of TNF-α and NF-κB activation has been found in the skin of psoriatic patients. Recently, anti-inflammatory therapies based on blocking TNF-α have been demonstrated to be effective in the treatment of psoriasis and could become a highly important option for the treatment of this kind of skin disease (Amigo 2007, 2008). The ability of avarol and its derivative avarol-3-thiosalicylate (**12**, TA) to inhibit NF-κB activation and TNF-α generation has been investigated *in vitro* and *in vivo* (Figure 7.2).

While TA has reduced LTB4, PGE2, and TNF-α production in activated leukocytes, oral and intrapouch administration of TA in the mouse air pouch model has reduced all these inflammatory mediators. TA has inhibited TNF-α-induced DNA binding of NF-κB in human HaCaT keratinocytes. Thus, by interfering with NF-κB activation, TA can act not only as an inhibitor of TNF-α-induced cellular functions, but also as an inhibitor of TNF-α production, because its transcription is, at least partly, dependent on the NF-κB pathway. Although activation of the NF-κB transcription factor system has been implicated in the induction of *COX-2* gene expression in many cell types (Yamamoto and Gaynor 2001), TA has not inhibited expression of this enzyme in stimulated human monocytes and similar results were obtained in RAW 264.7 macrophages. Finally, TA can also suppress NF-κB nuclear translocation *in vivo* (Amigo 2007, 2008).

Similarly, avarol (**11**) has also inhibited TNF-α generation in stimulated human monocytes ( $IC_{50}$  1 μM) and TNF-α-induced activation of NF-κB–DNA binding in keratinocytes. In the mouse air pouch model, administration of avarol has caused a dose-dependent reduction of TNF-α generation ( $ED_{50}$  9.2 nmol/pouch) as well as of IL-1β, PGE2, and LTB4 levels in pouch exudates. In the psoriasis-like model of 12-O-tetradecanoylphorbol-acetate–induced mouse epidermal hyperplasia, topical administration of avarol (0.6–1.2 μmol/site) has reduced edema, myeloperoxidase activity, and IL-1β, IL-2, and eicosanoid levels in skin. While epidermal hyperplasia and leukocyte infiltration have been observed in histopathological examination, suppressing *in vivo* NF-κB nuclear translocation by avarol has also been determined in mouse skin. According to the above results, antipsoriatic properties of avarol and TA have been attributed in part to the downregulation of several inflammatory biomarkers, such as TNF-α and NF-κB, in psoriatic skin (Amigo 2007, 2008).

Cycloamphilectenes (**13–18**) are potent marine diterpenes characterized with amphylecten skeleton. Six new cycloamphilectenes have been isolated from the Vanatu sponge *Axinella* sp. and tested for their anti-inflammatory properties on NO, PGE2, and TNF-α production in murine peritoneal macrophages (Ciasullo et al. 2002). These compounds have inhibited NO production with

$IC_{50}$  values in the submicromolar range through the inhibition of iNOS without affecting COX-2 expression. Among the 6 compounds, cycloamphilectene 2 (**14**) is able to reduce NO production without affecting TNF- $\alpha$  release (Figure 7.3). For the evaluation of the effect of cycloamphilectene 2 (**14**) on the NF- $\kappa$ B pathway, nuclear protein extracts from mouse peritoneal macrophages stimulated with zymosan have been tested either in the presence or in the absence of this compound, for NF- $\kappa$ B–DNA-binding activity using a radiolabeled NF- $\kappa$ B-specific oligonucleotide. Nuclear extracts of cells incubated with cycloamphilectene 2 and zymosan have shown a protein–DNA complex migrating at the same mobility, but the DNA-binding activity has been reduced compared to the zymosan control. Cycloamphilectene 2, which exhibits a topical anti-inflammatory activity, has been found to be an inhibitor of the NF- $\kappa$ B pathway offering a possible mechanism for the inhibition of iNOS expression (Lucas et al. 2003; Terraciano et al. 2006).

Cacospongiolide B (**19**), a sesterterpene from the sponge *Fasciospongia cavernosa*, is an inhibitor phospholipase A2 with anti-inflammatory properties (Figure 7.4). Its anti-inflammatory activity has been examined with regard to inflammatory response induced by zymosan in peritoneal macrophages and in the mouse air pouch. The compound has been found to inhibit zymosan-induced NF- $\kappa$ B–DNA-binding activity and the nuclear translocation of this transcription factor. Treatment of mouse peritoneal macrophages with cacospongionolide B has impaired phosphorylation of NF- $\kappa$ B inhibitory protein I $\kappa$ B- $\alpha$  and enhanced I $\kappa$ B- $\alpha$  expression. Downregulation of iNOS and COX-2 expression without catalytic activity has been determined for the suppression of zymosan-induced

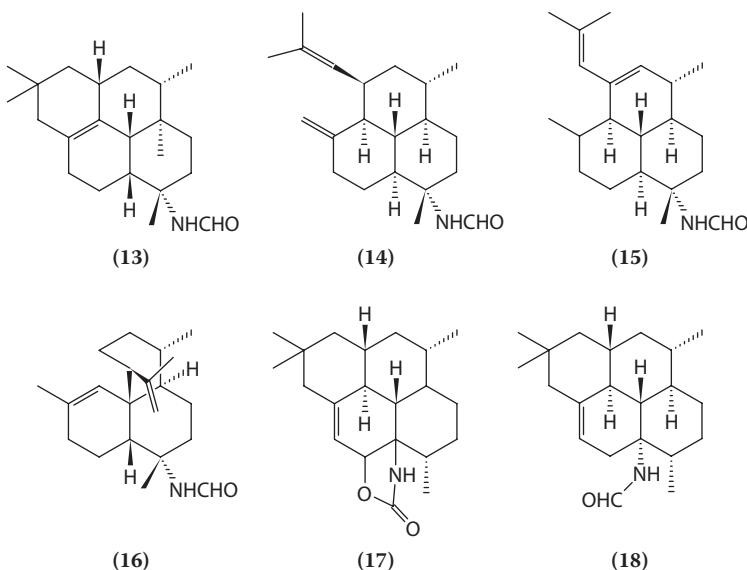


FIGURE 7.3 Cycloamphilectenes (13–18).

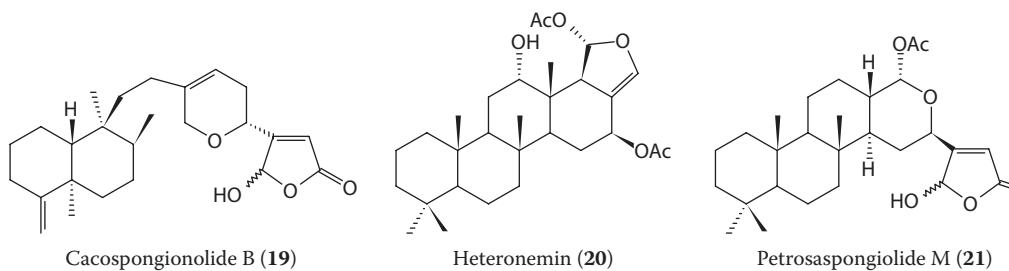


FIGURE 7.4 Nuclear factor  $\kappa$ B inhibitory marine sesterterpenes (19–21).

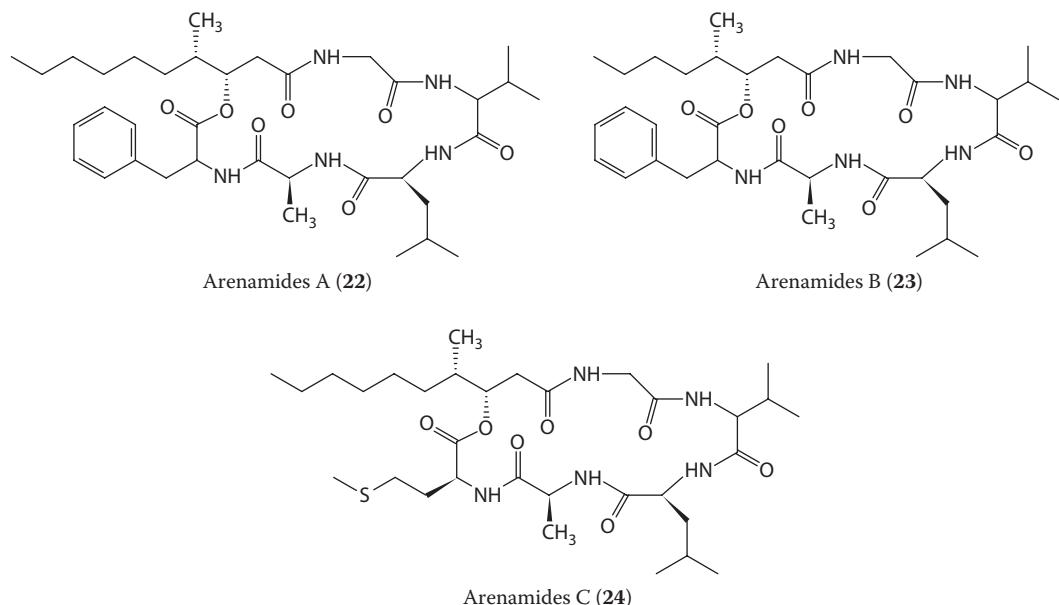
NO and PGE2 production in both macrophages and the mouse air pouch model. These results indicated that cacospongionolide B is able to control NO, PGE2, and TNF- $\alpha$  production both *in vitro* and *in vivo*, and this effect is mostly dependent on NF-κB inhibition (Pasadas et al. 2003).

A marine sesterterpene heteronemin (**20**), isolated from the sponge *Hyrtios* sp., has been investigated on chronic myelogenous leukemia cells (Figure 7.4). To understand the molecular mechanisms triggered by this compound, DNA microarray profiles have been performed and determined the genes that respond to heteronemin stimulation in TNF- $\alpha$ -treated cells and display an interaction effect between heteronemin and TNF- $\alpha$ . Heteronemin has been found to affect cellular processes, including cell cycle, apoptosis, mitogen-activated protein kinase (MAPK) pathway, and the NF-κB signaling cascade. To assess the underlying molecular mechanisms, inhibition of trypsin and the chymotrypsin-like proteasome activity by heteronemin have been determined at IC<sub>50</sub> of 0.4 mM. As a result of the inhibition of the NF-κB pathway, a reduction in cellular viability has been also observed. The apoptotic effect of heteronemin has been shown in different methods. These results have shown that this compound has potential as an anti-inflammatory and anticancer agent (Schumacher et al. 2010).

Petrosaspongiolide M (**21**) (Figure 7.4), isolated from the Caledonian marine sponge *Petrosaspongia nigra*, has been reported as a potent inhibitor of sPLA2 with anti-inflammatory properties in different experimental models. Its oral administration has significantly inhibited chronic inflammation induced by Freud's adjuvant in rats and acute inflammatory response in mice, with reduction in eicosanoids and TNF- $\alpha$  (Garcia Pastor et al. 1999). Petrosaspongiolide M reduces the production of NO, PGE2, and TNF- $\alpha$  in the mouse air pouch injected with zymosan. These effects have also been observed in mouse primary macrophages stimulated with zymosan. Inhibition of the above-mentioned inflammatory mediators has been related to the reductions in iNOS, COX-2, and TNF- $\alpha$  expression. Effects of petrosaspongiolide M on NF-κB inhibition have also been investigated, and it has been found that petrosaspongiolide M was a potent inhibitor of the NF-κB pathway since it strongly decreased NF-κB–DNA binding in response to zymosan in mouse peritoneal macrophages. In addition, petrosaspongiolide M can interfere with a key step in NF-κB activation, the phosphorylation of IκB $\alpha$ , resulting in the inhibition of IκB $\alpha$  degradation. Reduction of the release of a number of proinflammatory mediators, such as eicosanoids, NO, and cytokines, suggests the potential wide therapeutic spectrum of petrosaspongiolide M (Posadas et al. 2003).

Three new cyclohexadepsipeptides, arenamides A–C (**22–24**), have been isolated from the marine bacterial strain *Salinispora arenicola* (Figure 7.5). Chemopreventive and anti-inflammatory effects of arenamides have been tested by NF-κB, NO, and PGE2 inhibitory activity and cytotoxicity. Arenamides A and B, which are major compounds for *S. arenicola*, have been studied on NF-κB activity with stably transfected 293/NFκB-Luc human embryonic kidney cells induced by TNF- $\alpha$ . Arenamides A (**22**) and B (**23**) block TNF- $\alpha$ -induced activation in a dose- and time-dependent manner with IC<sub>50</sub> values of 3.7 and 1.7 μM, respectively. In addition, these compounds inhibit NO and PGE2 production in lipopolysaccharide (LPS)-induced RAW 264.7 macrophages. While moderate cytotoxicity has been observed with the human colon carcinoma cell line HCT-116, no cytotoxic effect has been observed against RAW 264.7 macrophages. These data suggest that the chemopreventive and anti-inflammatory characteristics of arenamides A and B need to be further investigated (Asolkar et al. 2009).

Kahalalides, cyclic peptide derivatives from a *Sacoglossan* mollusc, polypropenyl-1,4 hydroquinone derivatives from zoobenthos-inhabiting sponges, brominated pyrrole alkaloids from *Styliissa* sponges, and anthraquinones from marine crinoids have been investigated for their effect on intracellular signaling pathways, apoptosis, and oxidative stress in mammalian cell lines. The cytotoxic effect of these compounds has been assessed by the MTT method using different cell lines; the mode of cell death has been detected via caspase activity, nuclear fragmentation, and LDH assay. Distinct kahalalides show strong cytotoxicity against the C6 and MCF7 cell lines with nanomolar IC<sub>50</sub> values. Avarone and its derivatives show activation of the Nrf-2 signaling pathway as well as

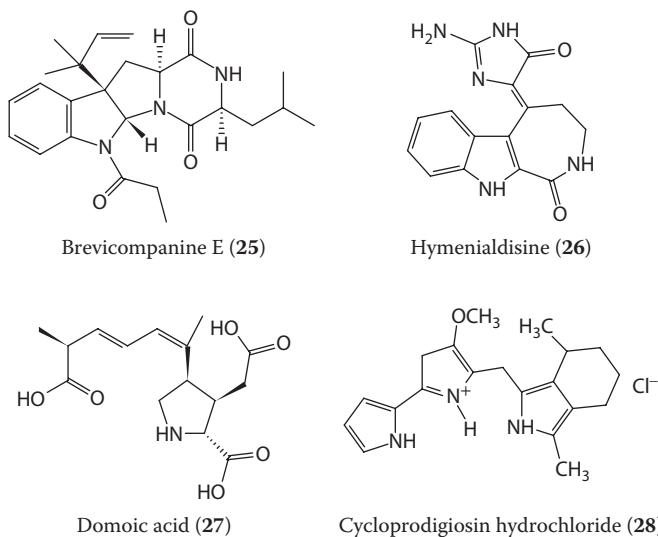


**FIGURE 7.5** Arenamides A–C (22–24).

inhibitory effects on activation of different protein kinases and NF- $\kappa$ B. This cytotoxicity is mediated at least in part by the induction of apoptotic cell death and disturbance of intracellular signaling pathways (Watjen et al. 2009).

*Chlamys farreri*, gonochoric Chinese scallop, is one of the important sources of polypeptide (polypeptide from *C. farreri* [PCF], Mr 879 kDa) that inhibit the human keratinocyte cell line, HaCaT cells, apoptosis after UVB exposure. Excessive exposure of solar ultraviolet radiation, particularly UVB, to humans causes many adverse effects because of the inducing effect of UVB on free radical formation and subsequent lipid peroxidation. The incidence of UVB-related skin problems and the interest in protecting the skin from the harmful effects of UVB enhance the research on the skin agents that protect cells against the harmful effects of UVB. PCF has been investigated for its mechanism for the inhibition of HaCaT cell apoptosis after UVB exposure. The result has indicated that PCF effectively inhibited UVB-induced HaCaT cell apoptosis. It has shown potential reactive oxygen species (ROS)-scavenging activities. While PCF increases expression of Cu, Zn-SOD, CAT, and GPx, it also decreases expression of pNF- $\kappa$ B/p65 and COX-2 in UVB-induced HaCaT cells. These data indicate that PCF suppressed UVB-induced COX-2 expression and the inhibitory effect may be via decreasing the activation of NF- $\kappa$ B. As a result, UVB causes apoptotic cell death by increasing ROS levels and decreasing expression of antioxidative enzymes, and induces NF- $\kappa$ B activation and COX-2 expression. It has been found that PCF could protect HaCaT cells from damage by UV irradiation via scavenging ROS and increasing expression of antioxidative enzymes as an antioxidant to block the signal pathway (Liu et al. 2009).

Rapid activation of microglia in inflammatory processes is occurred in aging and a number of age-related neurodegenerative diseases, including Parkinson's disease, Alzheimer's disease, amyotrophic lateral sclerosis, dementia resulting from infection with human immunodeficiency virus, and stroke. Brevicompanine E (25), an alkaloid that is isolated from a deep-ocean sediment-derived fungus, *Penicillium* sp., has been investigated for its effects on inflammatory mediators released from microglia (Figure 7.6). Brevicompanine E inhibits LPS-induced TNF- $\alpha$ , IL-1 $\beta$ , iNOS, and COX-2 in microglia. Electrophoretic mobility shift assay has demonstrated that brevicompanine E attenuated NF- $\kappa$ B and activator protein-1 DNA-binding activity in LPS-induced microglia. Compatible



**FIGURE 7.6** Nuclear factor  $\kappa$ B inhibitory marine alkaloids (25–28).

with the above results, brevicompanine E inhibits LPS-induced I $\kappa$ B $\alpha$  degradation, NF- $\kappa$ B nuclear translocation, and also Akt, c-Jun NH2-terminal kinase phosphorylation. These results indicate the potential effect of brevicompanine E for modulation of neuroinflammation (Yang et al. 2009).

Exposure of human rheumatoid synovial fibroblasts (RSFs) to IL-1 $\beta$  results in the upregulation of phospholipase A2 (PLA2) and COX-2 and subsequent biosynthesis of PGE2. Another marine alkaloid, hymenialdisine (26), from the sponges *Axinella verrucosa* and *Acanthella aurantiaca* (Cimino et al. 1982) has been characterized as an inhibitor of NF- $\kappa$ B activation and exposure of IL-1-stimulated RSF-inhibited PGE2 production in a concentration-dependent manner (Figure 7.6). Hymenialdisine has been determined to act specifically at the level of NF- $\kappa$ B inhibition and not as a general inhibitor of transcription. A direct effect of hymenialdisine on IL-1-induced NF- $\kappa$ B activation has been demonstrated by a significant reduction (80%) in NF- $\kappa$ B binding and inhibition of stimulated p65 migration from the cytosol of treated cells. Consistent with the role of NF- $\kappa$ B in the transcriptional regulation of COX-2 and 85-kDa PLA2, hymenialdisine-treated RSF has not transcribed the respective mRNAs in response to IL-1. The reductions in their respective protein levels and subsequent reductions in the ability to produce PGE2 have occurred. Specificity of action has suggested that IL-1-stimulated IL-8 production, which is known to be an NF- $\kappa$ B-regulated event, has also been inhibited by hymenialdisine, whereas IL-1-induced production of vascular endothelial growth factor, which is a non-NF- $\kappa$ B-regulated gene, has not been affected by exposure to hymenialdisine. Taken together, hymenialdisine inhibits IL-1-stimulated RSF PGE2 formation acting mostly through the modulation of NF- $\kappa$ B activation (Roshak et al. 1997).

A marine neurotoxin domoic acid (27) is an analog of the neurotransmitter glutamate and a potent agonist of kainate subtype glutamate receptors (Figure 7.6). Constant activation of these receptors causes rapid excitotoxicity, calcium-dependent cell death, and neuronal lesions in areas of the brain where kainate pathways are concentrated. To better comprehend responses to domoic acid-induced excitotoxicity, microarrays have been used to profile gene expression in the mouse brain after domoic acid exposure. Adult female mice have been subjected to domoic acid intraperitoneally at different doses. Total brain RNA from treated mice has been compared with time-matched controls. Real-time polymerase chain reaction has been performed on selected genes, and 3.96%, 3.94%, and 4.36% of the genes examined have been differentially expressed for the 30, 60, and 240 minute time points ( $p \leq 0.01$ ), respectively. Detailed examination of the data has resulted in a set of

56 genes used for trending analysis and K-medians and agglomerative clustering, and the inflammatory response element COX-2. Some later responding genes involve glucocorticoid responses (*Gilz* and *Sgk*), cold-inducible proteins (*Cirbp*, *Rbm3*), Map kinases (*Map3k6*), and NF- $\kappa$ B inhibition. The transcriptional profile induced by domoic acid (27) has shared similarity with expression profiles of brain ischemia and other excitotoxins, suggesting a common transcriptional response (Ryan et al. 2005).

Prodigiosins are a group of red pigments obtained from different strains of marine bacteria, such as *Serratia marcescens*, *Vibrio psychroerythrus*, and *Pseudoalteromonas denitrificans*. It has been reported that prodigiosin revealed a broad range of inhibitory activities against many bacterial, fungal, and protozoan species and induced apoptosis in cancer cell lines by the characteristic DNA laddering pattern and apoptotic bodies (Frustner 2003; Sundaramoorthy, Yogesh, and Dhandapani 2009). A stable analog of prodigiosin has been isolated from *P. denitrificans* and named cycloprodigiosin (28) (Figure 7.6). Its immunosuppressant potential has also been shown (Kawauchi et al. 1997). Later, the inhibitory effect of cycloprodigiosin on TNF- $\alpha$ -induced NF- $\kappa$ B activation was determined in luciferase gene reporter assay. Cycloprodigiosin has been found to inhibit NF- $\kappa$ B gene expression under different stimuli on HeLa, U373, and COS7 cells through the inhibition of transcriptional activation (Kim et al. 1999; Kamata et al. 2001; Terraciano et al. 2006).

*Gracilaria verrucosa* is a common marine red alga that has anticancer and antioxidant properties. Its polyunsaturated fatty acid composition, mainly arachidonic and eicosapentaenoic acids, has been determined in addition to the isolation of several glycolipids (Imbs et al. 2001). The anti-inflammatory constituents of *G. verrucosa* have been determined as two enone fatty acids (29–30) in different mechanisms (Figure 7.7). (E)-10-Oxoctadec-8-enoic acid (30) and (E)-9-Oxoctadec-10-enoic acid (30) inhibit the production of inflammatory biomarkers NO, TNF- $\alpha$ , and IL-6 by suppressing the nuclear translocation of NF- $\kappa$ B and phosphorylation of STAT1 in LPS-stimulated murine macrophages RAW 264.7 cells (Lee et al. 2009).

Semivioxanthin (31) has been evaluated for its immunoregulatory activity in mouse RAW 264.7 macrophages. It has been isolated from marine-derived fungi with the pyrone structure using different chromatographic systems. To identify the immunoregulatory activity, the effects of semivioxanthin on TNF- $\alpha$  and its mRNA expression and on expression of CD80, CD86, and MHC II, as well as the molecular mechanism underlying the immunologic enhancement properties of semivioxanthin, have been studied. Semivioxanthin treatment has resulted in the degradation of I $\kappa$ B $\alpha$ , which has been determined by immunoblotting, immunofluorescence, and electrophoretic mobility shift assay.

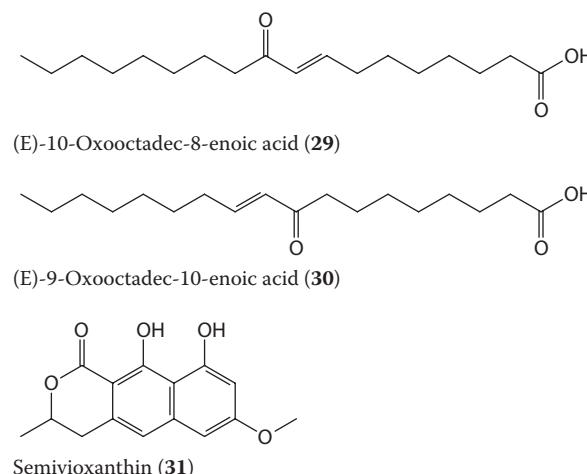


FIGURE 7.7 Other bioactive compounds (29–31).

In addition, it is also shown that TNF- $\alpha$  production has been prevented by NF-κB and MAPK inhibitors. Semivioxanthin has been found to regulate TNF- $\alpha$  production through NF-κB and MAPK signaling pathways. Activation of NF-κB and extracellular signal-regulated kinase (ERK1/2) is necessary for CD80, CD86, and MHC-II expression induced by semivioxanthin. These results suggest that semivioxanthin can regulate TNF- $\alpha$  production via NF-κB and MAPK signaling pathways and upregulate CD80, CD86, and MHC-II expression specifically through the NF-κB and ERK1/2 signal transduction pathways. These data suggest that semivioxanthin has immunoregulatory effects (Yang et al. 2008).

*Chlorella* is one of the important marine organisms and its free radical scavenging, anti-inflammatory, and antitumor activities have been described previously. Its main constituents, chlorophylls, phenolic compounds, and sterols, have been suggested as its active components. The effects of *Chlorella* dichloromethane extract on oxidative stress, NO production, iNOS expression, and NF-κB activation in LPS-stimulated macrophages have been investigated to clarify the bioactivity of *Chlorella*. Dichloromethane extract treatment of LPS-stimulated macrophages has reduced thiobarbituric acid reactive substance (TBARS) accumulation, enhancing the glutation level and activities of antioxidative enzymes, including SOD, catalase, glutathione peroxidase, and glutathione reductase. NO production is significantly suppressed dose dependently with an IC<sub>50</sub> of 30.5 μg/mL. About 50 μg/mL of extract also has suppressed NO production, levels of iNOS, and mRNA expression. In addition, a *Chlorella* extract also has suppressed specific DNA-binding activities of NF-κB with an IC<sub>50</sub> of 62.7 μg/mL. Inhibition of NO production with decreased iNOS protein and mRNA expression, and NF-κB activity by dichloromethane extract may contribute to the suppression of intracellular oxidative stress. Taken together, these results demonstrate that the extract might ameliorate inflammatory diseases by suppressing NO production through the inhibition of iNOS protein expression due to decreased mRNA transcription. Furthermore, the inhibition of mRNA transcription of iNOS by the *Chlorella* extract is, at least in part, due to the inhibition of NF-κB transactivation, which may be mediated by an antioxidative effect. Thus, the dichloromethane extract of *Chlorella* appears to be a potential therapeutic agent for treating LPS-induced inflammatory processes (Park et al. 2005).

## 7.6 CONCLUSION

In recent years, extensive phytochemical and bioactivity researches on marine natural products have led to the discovery of potential leading compounds and useful biological probes in marine sources. According to the research discussed in this chapter, marine sponges, algae, bacteria, and invertebrates are highly promising sources of NF-κB inhibitors with a wide range of molecular targets in the NF-κB pathway. Combined efforts focusing on the NF-κB pathway would definitely accelerate the development of new marine drugs. As a result, although many examples are stated in this review, there are a lot of marine crude extracts and natural compounds that require further research. Since marine natural compounds isolate an insufficient amount for *in vivo* and clinical research, modern marine culture techniques, *in vitro* tissue cultures, large-scale fermentations, and biotransformations studies should be combined with investigation of marine sources. In conclusion, the isolation or modification of novel marine products, as well as their analogs, and the extensive evaluation of their bioactivity with specific targets will promote the discovery of novel promising chemotherapeutic agents for improving human health.

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# 8 The Immunomodulatory Effect of Marine Algae on Allergic Response

Se-Kwon Kim, Thanh-Sang Vo, and Dai-Hung Ngo

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## 8.1 INTRODUCTION

Allergic response is known as an exaggerated reaction of the immune system to harmless environmental substances, such as animal dander, house dust mites, foods, pollen, insects, and chemical agents (Milián and Díaz 2004; Arshad 2010). The initial event responsible for the development of allergic reaction is the generation of allergen-specific CD4<sup>+</sup> T helper (Th)2 cells. Once generated, effector Th2 cells produce interleukin (IL)-4, IL-5, IL-9, and IL-13, which cause the production of allergen-specific immunoglobulin E (IgE) by B cells (Akdis, Blaser, and Akdis 2005). Subsequently, allergic reactions are induced upon binding of allergen to IgE, which is tethered to the high-affinity IgE receptor on the surface of mast cells and basophils. After the aggregation of cell-surface receptors is a cascade of intracellular events, including the increase of the intracellular Ca<sup>2+</sup> level, the release of preformed inflammatory mediators from secretary granules such as histamine and β-hexosaminidase, and the generation and secretion of the newly synthesized substances such as leukotrienes, prostaglandins, and cytokines. These mediators cause allergic inflammatory responses due to airway constriction, mucous production, and recruitment of inflammatory cells (Galli, Tsai, and Piliponsky 2008). Accordingly, the control of Th2-type cytokine expression, IgE levels, and inflammatory mediator production are especially important for the regulation of type I allergic reaction; thus, allergic diseases may be managed. Although successful immune modulation of allergic disease has been demonstrated *in vivo*, it often fails to translate into human clinical trials (Nguyen and Casale 2011). Thus, the search for potential drug candidates containing higher immunomodulatory activity is increasing in the pharmaceutical industry. In this regard, natural bioactive compounds and their derivatives are great sources for the development of new-generation antiallergic therapeutics.

Marine resources have been well recognized for their biologically active substances with great potential in drug discovery (Folmer et al. 2008). During the last decades, numerous novel compounds have been isolated from marine organisms and many of these substances have interesting biological activities (Blunden 2001; Blunt et al. 2006; Mayer et al. 2011; Ngo et al. 2011). Notably, marine algae are known to be one of the most important producers of biomass in the marine

environment. They produce a wide variety of chemically active metabolites in their surroundings as an aid to protect themselves against other settling organisms (Bhadury and Wright 2004). Furthermore, marine algae have been revealed to possess anticoagulant, antiviral, antioxidant, anti-allergic, anticancer, antiinflammatory, and antiobesity activities (Lincoln, Strupinski, and Walker 1991; El Gamal 2010; Wijesekara, Yoon, and Kim 2010). Therefore, marine algae are believed to be a promising source to provide not only novel biologically active substances for the development of pharmaceuticals but also essential compounds for human nutrition (El Gamal 2010; Gupta and Abu-Ghannam 2011). So far, brown algal *Sargassum hemiphyllum* and red algal *Carpopeltis affinis* have been used in Korean folk medicine as a therapeutic treatment of various allergic diseases (Na, Moon, Ko, et al. 2005; Na, Moon, Lee, et al. 2005). Recently, the role of marine algae as immuno-modulators of allergic response has been determined *in vitro* and *in vivo* by many researchers. This contribution, therefore, focuses specifically on the immunomodulatory effects of marine algae on allergic response and emphasizes their potential application as candidates of pharmaceuticals as well as nutraceuticals to prevent allergic disorders.

## 8.2 IMMUNOMODULATORY EFFECTS OF MARINE ALGAE ON ALLERGIC RESPONSE

### 8.2.1 REGULATIVE EFFECT ON TH1/TH2 CYTOKINE PRODUCTION

Type I allergy is characterized by an imbalance of Th1- and Th2-like immune responses with exaggerated production of IL-4, IL-5, IL-9, and IL-13 leading to production of IgE toward otherwise innocuous molecules. As is known, Th1 cells are characterized by the prevalent production of IL-2, interferon (IFN)- $\gamma$ , and tumor necrosis factor (TNF)- $\beta$ , without Th2-type cytokines. By contrast, Th2 cells are characterized by the prevalent production of IL-4, IL-5, IL-9, and IL-13 in the absence of Th1-type cytokines (Romagnani 2004). In general, Th1-polarized responses are highly protective against infections via eliciting phagocytes activation. Conversely, Th2-polarized responses induce differentiation, activation, and *in situ* survival of eosinophils (through IL-5); promotion of IgE production from B lymphocytes (through IL-4 or IL-13); and growth of mast cells (through IL-4, IL-9, and IL-10), as well as inhibition of several macrophage functions or development of Th1 cells (through IL-4, IL-10, and IL-13) (Romagnani 2000). Evidently, Th2 cytokines play a crucial role in allergic inflammatory responses. Thus, the immune modulation due to suppression of Th2 responses has been proposed as a promising concept for treatment of allergic diseases (Araujo et al. 2010; Nguyen and Casale 2011). Among various natural products, marine algae appear as potential modulators of allergic immune responses via blocking Th2 cytokine and IgE production, inhibiting important cells in Th2 responses, and stimulating Th1 responses. Sugiura and collaborators have demonstrated that a diet with dried *Eisenia arborea* powder (1–5 g/rat) resulted in a reduction of the serum IgE level and a shift in the Th1/Th2 balance due to suppressing the release of Th2 cytokines, IL-4 and IL-10, and enhancing expression of Th1 cytokine IFN- $\gamma$  from the rat spleen and mesenteric lymph nodes (Sugiura et al. 2008). Likewise, administration of *Ecklonia cava* and *Laurencia undulate* ethanol extracts in mice has decreased the number of eosinophils in the bronchoalveolar lavage fluid and blocked the influx of inflammatory cells into the lung around blood vessels and airway luminal narrowing (Kim et al. 2008; Jung et al. 2009). Furthermore, the production of Th2 cytokines, including IL-4 and IL-5, in the bronchoalveolar lavage fluid and the level of IgE in the serum have been significantly inhibited.

In addition, microalgae have also been known to possess immunomodulatory effects on allergic response. Indeed, *Spirulina* has been effective in decreasing the IgE antibody level and increasing IgG1 and IgA antibody production in the serum of the mice immunized with crude shrimp extract as an antigen (Hayashi et al. 1998). In a clinical trial, *Spirulina* consumption has resulted in significant amelioration in the symptoms and physical findings of allergic rhinitis patients that have been referred to be involved in inhibiting the production of IL-4 and suppressing the differentiation of

Th2 cells (Mao, Van de Water, and Gershwin 2005; Cingi et al. 2008). Similarly, oral administration of a hot-water extract of *Chlorella vulgaris* in mice has suppressed the production of IgE against casein antigen, which is accompanied by increasing mRNA expression of Th1 cytokines, including IFN- $\gamma$  and IL-12 (Hasegawa et al. 1999). Meanwhile, treating mice with *Chlorella pyrenoidosa* during the ovalbumin (OVA) sensitization process has significantly reduced eosinophil and neutrophil infiltration in the airways (Kralovec et al. 2005).

Recently, several active components in marine algae, such as polysaccharides, carotenoids, and phycocyanin, have been found to inhibit Th2 responses. According to Maruyama et al. (2005), fucoidan from *Undaria pinnatifida* has reduced the concentrations of Th2 cytokines, including IL-4 and IL-13, in the bronchoalveolar lavage fluid and has inhibited the increase of antigen-specific IgE in OVA-induced mouse airway hypersensitivity. In the same way, alginic acid oligosaccharides have been able to reduce IgE production and block Th2 development via enhancing IFN- $\gamma$  and IL-12 production and downregulating IL-4 production in splenocytes of mice (Yoshida et al. 2004; Uno, Hattori, and Yoshida 2006). Noticeably, feeding mice with  $\beta$ -carotene has led to inhibition of a wide range of Th2 cytokine production (IL-4, IL-5, IL-6, and IL-10) and enhancement of Th1 cytokine production (IFN- $\gamma$ , IL-12, and IL-2) (Sato et al. 2004). In a very recent study, Chang et al. (2011) have evaluated the therapeutic potential of R-phycocyanin (R-PC), one of the major pigment constituents of *Spirulina*, against allergic airway inflammation. R-PC can promote CD4 $^{+}$  T-cell stimulatory capacity and increase IFN- $\gamma$  expression in CD4 $^{+}$  T cells. Moreover, intraperitoneal administration of R-PC has suppressed OVA-induced airway hyperresponsiveness, serum levels of OVA-specific IgE, eosinophil infiltration, Th2 cytokine levels, and eotaxin in the bronchoalveolar lavage fluid of mice. These findings indicate that marine algae can enhance the immunological function toward Th1 activity, thus suppressing the Th2 activity in allergic responses.

### 8.2.2 SUPPRESSIVE EFFECT ON DEGRANULATION

Mast cell activation by IgE-dependent and IgE-independent stimuli brings about the process of degranulation that results in the fusion of the cytoplasmic granule membranes with the plasma membrane. This is accompanied by the fast external release of granule-associated stored mediators such as histamine,  $\beta$ -hexosaminidase, leukotrienes, prostaglandins, and cytokines (Church and Levi-Schaffer 1997; Nishida et al. 2005). These substances have been implicated in the majority of the acute symptoms in allergic responses, including mucus production, pruritus, vascular permeability, smooth-muscle constriction, and other symptoms of anaphylaxis (Rusznak and Peebles 2002). Thus, inhibition of allergic degranulation is a major target for potential antiallergic drugs. In this sense, a range of marine algae have been revealed to be able to suppress allergic degranulation via attenuating the release of histamine,  $\beta$ -hexosaminidase, and cytokines. Namely, the extracts of *Petalonia binghamiae*, *Chrysymenia wrightii*, *Scytopsiphon lomentaria*, *E. cava*, *U. pinnatifida*, *Codium fragile*, *Porphyra dentate*, and *Ulva japonica* have been shown to inhibit more than 50% of the  $\beta$ -hexosaminidase release from rat basophilic leukemia (RBL)-2H3 cells at concentrations of 100 and 200  $\mu$ g/ml. Among them, *P. binghamiae* appears to be most effective against degranulation of both RBL-2H3 cells and mouse eosinophils (Kimiya et al. 2008). Likewise, brown algae from the Ise-Shima region of Mie Prefecture, Japan, including *Ishige foliacea*, *Ishige okamurae*, *Sargassum micracanthum*, *Sargassum ringgoldianum*, *Spiraea thunbergii*, *E. arborea*, and *E. cava*, have exhibited significant inhibition on the histamine release from the rat mast cell line RBL-2H3 (Sugiura, Takeuchi, et al. 2006). Moreover, the brown alga *S. hemiphyllum* and the red alga *C. affinis* have been found to inhibit the atopic allergic reaction via regulation of inflammatory mediators in human mast cells (Na, Moon, Ko, et al. 2005; Na, Moon, Lee, et al. 2005). In particular, methanol extracts of *S. hemiphyllum* and *C. affinis* have effectively inhibited the release of histamine,  $\beta$ -hexosaminidase, IL-8, and TNF- $\alpha$  from the activated human mast cell line (HMC)-1 cells. Similarly, *Spirulina* has observed to suppress mast cell degranulation via attenuating the histamine release and TNF- $\alpha$  production from rat peritoneal mast cells (Yang, Lee, and Kim 1997; Kim et al. 1998).

In parallel, numerous bioactive components purified from marine algae have been isolated and identified as potential natural inhibitors against allergic degranulation. Conspicuously, phlorotannins derived from *E. cava*, such as fucodiphloroethol, phlorofucofuroeckol A, dieckol, and 6,6'-bieckol, have exhibited remarkable suppression on histamine and  $\beta$ -hexosaminidase releases from KU812 and RBL-2H3 cells induced by IgE with an IC<sub>50</sub> range of 27.8–65.8  $\mu$ M (Li et al. 2008; Le et al. 2009). In addition, several phlorotannins, such as eckol, 6,6'-bieckol, 6,8'-bieckol, 8,8'-bieckol, phlorofucofuroeckol (PFF)-A, and PFF-B, from the brown alga *Eisenia arborea* have caused a depression on the synthesis and release of leukotriene and prostaglandin from RBL cells (Sugiura et al. 2009). Specially, PFF-B has exposed strong activity against the histamine and  $\beta$ -hexosaminidase release with an IC<sub>50</sub> value of 7.8  $\mu$ M (Sugiura, Matsuda, et al. 2006, 2007). Besides, alginic acid, a naturally occurring hydrophilic colloidal polysaccharide obtained from various species of brown algae, has induced a decrease of 61% of the histamine release from rat peritoneal mast cells at a concentration of 0.01  $\mu$ g/ml. Moreover, its inhibitory activities are also observed due to diminishing expression of histidine decarboxylase and production of IL-1 $\beta$  and TNF- $\alpha$  in HMC-1 cells (Jeong et al. 2006). On the other hand, polyunsaturated fatty acids of 18:4n-3 and 16:4n-3 obtained from the marine algae *U. pinnatifida* and *Ulva pertusa* have been found to inhibit the production of leukotriene B4, leukotriene C4, and 5-hydroxyeicosatetraenoic acid in MC/9 mouse mast cells (Ishihara et al. 1998). Likewise, the histamine release is remarkably reduced in mast cells treated with  $\alpha$ -linolenic acid (Kawasaki et al. 1994; Gueck, Seidel, and Fuhrmann 2003),  $\gamma$ -linolenic acid, and docosahexaenoic acid (Gueck et al. 2004). Taken together, marine algae are indicated as promising candidates for the design of novel inhibitors of allergic degranulation.

### 8.3 CONCLUSION

The regulation of immune response is regarded as a promising therapeutic in the treatment of allergic diseases. Also, finding safe and efficient agents to decrease pathologic immune responses is an essential goal. Herein, a large number of potential agents derived from marine algae have been found to be effective against allergic reactions via suppression of Th2 cytokine production, inhibition of degranulation, and enhancement of Th1 cytokine production. The extensive studies exploring the immunomodulatory effects of marine algae will contribute to the development of novel antiallergic therapeutics. Thus, it can be suggested that marine algae will play a vital role in the pharmaceutical industry in the development of novel drugs against allergic disorders.

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# 9 Pharmacological Effects of Marine-Derived Bioactive Peptides

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### 9.1 INTRODUCTION

The world's oceans, which cover more than 70% of the Earth's surface, represent an enormous source of potential therapeutic agents. During the last few decades, numerous novel compounds have been found from marine organisms with interesting pharmaceutical activities. Therefore, marine organisms are believed to be a potential source of not only novel biologically active substances that aid in the development of pharmaceuticals but also compounds essential to human nutrition (Blunt et al. 2006; Vo and Kim 2010). In particular, marine peptides have attracted a great deal of attention due to their potential effects in promoting health and reducing disease risk.

These peptides have been obtained from algae; fish; mollusk; crustacean; and marine by-products including substandard muscles, viscera, skins, trimmings, and shellfish. Marine bioactive peptides based on their structural properties, amino acid composition, and amino acid sequences have been shown to display a wide range of biological functions including antihypertensive, antimicrobial, antioxidant, anticancer, anticoagulant, opioid agonistic, immunomodulatory, prebiotic, mineral binding, and hypocholesterolemic effects (Betoret et al. 2011; Rajanbabu and Chen 2011). In this regard, this chapter focuses on biological activities of peptides derived from marine resources and their potential health beneficial applications in the functional foods, nutraceutical, and pharmaceutical industries.

## 9.2 DEVELOPMENT OF BIOACTIVE PEPTIDES DERIVED FROM MARINE ORGANISMS

Components of proteins in marine foods contain sequences of bioactive peptides, which could exert a physiological effect in the body. Some of these bioactive peptides have been identified to possess nutraceutical potential that is beneficial in human health promotion. Moreover, the possible roles played by marine-derived bioactive peptides in reducing the risk of diseases have been reported. Bioactive peptides usually contain 3–20 amino acid residues, and their activities are based on amino acid composition and sequence. These short chains of amino acids are inactive within the sequence of the parent protein, but they can be released during gastrointestinal digestion, food processing, or fermentation (Pihlanto-Leppala 2001; Erdmann, Cheung, and Schroder 2008).

Bioactive peptides can be produced by *in vitro* enzymatic hydrolysis of different marine resources using appropriate proteolytic enzymes. Proteolytic enzymes from fish and aquatic invertebrates can be used for the hydrolysis of marine products to develop bioactive peptides that are applied in the food industry. The physicochemical conditions (temperature and pH) of the reaction media must be adjusted to optimize the activity of the used enzyme (Shahidi and Janak Kamil 2001). The crude proteinase was extracted from the pyloric ceca of tuna for enzymatic hydrolysis of cod frame protein under optimal pH and temperature conditions of the respective enzymes to obtain a high yield. Furthermore, the molecular weight of bioactive peptides is one of the most important factors in releasing peptides with desired functional properties (Deeslie and Cheryan 1981). Therefore, a suitable method for producing bioactive peptides with specific functional properties and desired molecular size characteristics involves the use of an ultrafiltration membrane reactor system. This system has the main advantage that the molecular weight distribution of the desired functional peptide can be controlled by adopting an appropriate ultrafiltration membrane. In order to obtain functionally active peptides, it is a suitable method to use a three-enzyme system for sequential enzymatic digestion. Moreover, it is possible to obtain serial enzymatic digestions in a system using a multistep recycling membrane reactor combined with an ultrafiltration membrane system to separate marine-derived bioactive peptides (Doyen et al. 2011). This membrane bioreactor technology equipped with ultrafiltration membranes is recently being used in the development of bioactive compounds and is considered a potential method of utilizing marine proteins as value-added nutraceuticals with beneficial health effects.

## 9.3 BIOLOGICAL PROPERTIES OF MARINE BIOACTIVE PEPTIDES AND THEIR POTENTIAL HEALTH BENEFITS

### 9.3.1 ANTIHYPERTENSIVE ACTIVITY

The peptides regulating blood pressure are potent inhibitors of angiotensin-I-converting enzyme (ACE). ACE plays a critical role in the regulation of blood pressure as it promotes the conversion of angiotensin-I to the potent vasoconstrictor angiotensin-II. It is noted that ACE belongs to a class of zinc proteases that require both zinc and chloride for their enzymatic activity. Therefore, in the development of drugs to control high blood pressure, inhibition of ACE is considered to be a useful therapeutic approach (Shahidi and Zhong 2008).

Currently, many natural ACE inhibitory peptides have been isolated from different food proteins such as cod frame, pollack skin, sea bream scales, yellowtail bone and scales, yellowfin sole frame, tuna frame and clam, krill, mussel, oyster, and shrimp (Table 9.1). Hence, there is great interest nowadays in obtaining bioactive peptides, which could be applied in the prevention of hypertension and in the initial treatment of mildly hypertensive patients (Guang and Phillips 2009).

The competitiveness of different antihypertensive peptides against ACE has been determined kinetically using Lineweaver–Burk plots. Generally, the mechanism of action of antihypertensive

**TABLE 9.1****Table Showing ACE Inhibitory Peptides Derived from Marine Organisms**

Source	Enzyme	Amino Acid Sequence	IC <sub>50</sub> (μM)	Reference
Alaska pollack	Alcalase + pronase + collagenase	LGP	0.72	Byun and Kim (2001)
Alaska pollack	Pepsin	FGASTRGA	14.7	Je et al. (2004)
Bonito protein		IKW	0.4	Hasan et al. (2007)
Hard clam	Protamex	YN	51.00	Tsai, Chen, and Pan (2008)
Rotifer	Alcalase	DDTGHDFFDTGEAM	9.64	Lee et al. (2009)
Tuna frame	Pepsin	GDLGKTTTVSNWSPPKYKDTP	11.28	Lee, Qian, and Kim (2010)
Sea bream	Protease	VIY	7.50	Fahmi et al. (2004)
Shrimp	Protease	IFVPAF	3.4	Lun et al. (2006)
Salmon muscle	Alcalase + papain	IW	1.20	Enari et al. (2008)
Sea cucumber	Bromelain + alcalase + protease	MEGAQEAQGD	15.90	Zhao et al. (2009)
Microalga	Pepsin	VECYGPNRPQF	29.60	Sheih, Fang et al. 2009
Wakame	Protease	IW	1.50	Sato et al. (2002)

peptides is different from that of synthetic drugs. Synthetic drugs basically indiscriminately block ACE by interfering with its action, whereas ACE inhibitory peptides interact much differently by competing with ACE. The ACE converts angiotensin-I to angiotensin-II by cleaving off a small peptide. Synthetic drugs work by directly blocking the action of ACE. The ACE actually reacts with the antihypertensive peptides instead of attacking angiotensin-I. Antihypertensive peptides relax the arterial walls and reduce fluid volume by inhibiting the formation of angiotensin-II. Therefore, antihypertensive peptides actually improve heart function and increase blood and oxygen flow to the heart, liver, and kidneys. Many studies have shown that tryptophan, tyrosine, phenylalanine, or proline at the C-terminal and branched-chain aliphatic amino acids at the N-terminal are suitable for a peptide binding to ACE as a competitive inhibitor (Li et al. 2004).

In addition, a noncompetitive mechanism has been observed in some peptides that were suggested to combine with an enzyme molecule to produce a dead-end complex, regardless of whether a substrate molecule is bound. For example, YLYEIAR (Nakagomi et al. 1998) and LIY (Nakagomi et al. 2000) have been found to act as noncompetitive inhibitors. The hydrophobicity of the N-terminus, which is one of the common features of ACE inhibitory peptides, may contribute to the inhibitory activity. The ACE inhibitory peptides are generally short-chain peptides, often carrying polar amino acid residues such as proline. Furthermore, structure–activity relationships among various peptide inhibitors of ACE indicate that binding to ACE is strongly influenced by the C-terminal tripeptide sequence of the substrate, and it is suggested that peptides containing hydrophobic amino acids at these positions are potent inhibitors (Qian, Je, and Kim 2007).

Numerous *in vivo* studies of marine-derived antihypertensive peptides in spontaneously hypertensive rats (SHRs) have shown potent ACE inhibitory activity (Zhao et al. 2009). In general, the reduction in systolic blood pressure (SBP) following oral administration (10 mg/kg of body weight) of peptides was on average 25 mmHg compared to controls (Je et al. 2005; Lee, Qian, and Kim 2010). This antihypertensive activity was similar to that of captopril, a commercial antihypertensive drug. Protein hydrolysates derived from oyster proteins and sea bream scale collagen have also exhibited antihypertensive activity in SHRs (Fahmi et al. 2004; Wang et al. 2008). However, variations in sample type, the dosage, and duration of administration make it difficult to compare these hydrolysates in terms of SBP reduction.

### 9.3.2 ANTIOXIDANT ACTIVITY

Reactive oxygen species (ROS), including singlet oxygen, hydrogen peroxide, superoxide anion, and hydroxyl radicals and other free radicals attack macromolecules such as DNA, proteins, and lipids, leading to many health disorders including cardiovascular diseases, aging, diabetes mellitus, neurodegenerative diseases, and cancer. Antioxidants may have a positive effect on human health as they can protect the human body against deterioration by free radicals and ROSSs (Butterfield et al. 2006).

To retard peroxidation processes in food, many synthetic antioxidants such as hydroxytoluene (BHT), butylated hydroxyanisole (BHA), *tert*-butylhydroquinone (TBHQ), and propyl gallate (PG) have been used. However, the use of these synthetic antioxidants must be strictly controlled due to potential health hazards. Hence, the search for natural antioxidants as safe alternatives to synthetic products is important in the food industry. Recently, the use of natural antioxidants available in food and other biological substances has attracted significant interest due to their presumed safety and nutritional and therapeutic values (Park et al. 2001; Ajila et al. 2007). A number of studies have shown that peptides derived from various marine protein hydrolysates such as fish (Slizyte et al. 2009), blue mussel (Jung, Rajapakse, and Kim 2005), conger eel (Ranathunga, Rajapakse, and Kim 2006), microalgae (Sheih, Wu et al. 2009), and squid (Rajapakse, Mendis et al. 2005) act as potential antioxidants. The antioxidant activity of marine-derived bioactive peptides has been determined by different *in vitro* methods, such as 2,2-diphenyl-1-picrylhydrazyl (DPPH), carbon-centered, hydroxyl, and superoxide anion radical scavenging activities, which have been detected by the electron spin resonance (ESR) spectroscopy method as well as intracellular free radical scavenging assays. The beneficial effects of antioxidant marine bioactive peptides are well known in scavenging ROSSs and free radicals or in preventing oxidative damage by interrupting the radical chain reaction of lipid peroxidation (Sampath Kumar, Nazeer, and Jaiganesh 2011). A bioactive peptide from jumbo squid inhibited lipid peroxidation in the linoleic acid model system, and its activity was found to be much higher than that of  $\alpha$ -tocopherol and was close to that of the highly active synthetic antioxidant, BHT (Mendis et al. 2005). Moreover, the bioactive antioxidant peptide from oyster (*Crassostrea gigas*) exhibited higher protective activity against polyunsaturated fatty acid peroxidation than the natural antioxidant,  $\alpha$ -tocopherol (Qian et al. 2008).

The antioxidant activity of marine peptides can be the results of specific scavenging of radicals formed during peroxidation, scavenging of oxygen containing compounds or metal-chelating ability. In addition, peptides isolated from marine fish proteins have greater antioxidant properties than  $\alpha$ -tocopherol in different oxidative systems (Jun et al. 2004). Antioxidant activities of bioactive peptides are mainly due to the presence of hydrophobic amino acids, some aromatic amino acids, and histidine. Gelatin peptides are rich in hydrophobic amino acids, and the abundance of these amino acids favors a higher emulsifying ability. Hence, marine gelatin peptides possess higher antioxidant effects than peptides derived from other proteins because of the high percentage of glycine and proline contained in them (Mendis et al. 2005). Therefore, antioxidant bioactive peptides derived from marine organisms may have great potential for use as pharmaceuticals, nutraceuticals, and a substitute for synthetic antioxidants. For example, Shahidi and colleagues (Shahidi, Han, and Synowiecki 1995) clearly demonstrated that addition of capelin fish protein hydrolysate to minced pork muscle at a level of 0.5%–3.0% reduced the formation of secondary oxidation products including thiobarbituric acid reactive substances (TBARSs) in the product by 17.7%–60.4%. However, the bitter taste of protein hydrolysates prevents the use of bioactive peptides as food additives and their bioactivity may be reduced through molecular alteration during food processing or interaction with other food ingredients. As a treatment to this bitterness, Shahidi and colleagues (Shahidi, Han, and Synowiecki 1995) treated fish protein hydrolysate with activated carbon, which removed the bitter peptides. The challenge for food technologists is to develop functional foods and nutraceuticals without the undesired side effects of the added peptides.

### 9.3.3 ANTI-HUMAN IMMUNODEFICIENCY VIRUS ACTIVITY

Human immunodeficiency virus type-1 (HIV-1) is the cause of acquired immune deficiency syndrome (AIDS), a major human viral disease with about 33.2 million people infected worldwide to date. Numerous studies have been reported that marine bioactive peptides can be used as anti-HIV components in functional foods or nutraceuticals and pharmaceuticals due to their therapeutic potential in the treatment or prevention of infectious diseases (Table 9.2).

Lee and Maruyama (1998) searched for HIV-1 protease-inhibiting substances from oyster (*Crassostrea gigas*). Two peptides inhibiting HIV-1 protease, LLEYSI and LLEYSL, were isolated from the hydrolysate of oyster proteins prepared with thermolysin. The peptides LLEYSI and LLEYSL exhibited strong inhibition of HIV-1 protease at 50% inhibitory concentration ( $IC_{50}$ ) values of 20 and 15 nM, respectively, and behaved as competitive inhibitors for HIV-1 protease with  $K_i$  values of 13 and 10 nM, respectively. Lee and Maruyama (1998) have found that the length of the amino acid sequence and the presence of C-, N-terminal hydrophobic amino acids in these peptides influence their inhibitory activity.

Besides, sponges have been traditionally known as a source of novel bioactive peptides. The novel structural features and diverse biological activities of these peptidic metabolites have generated considerable interest. Mirabamides from the marine sponge *Siliquariaspongia mirabilis* have been shown to potently inhibit HIV-1 fusion. Among mirabamides, mirabamide A was found to powerfully inhibit HIV-1 in neutralization and fusion assays with respective  $IC_{50}$  values of 40 and 140 nM, whereas mirabamide C and D were shown to be less effective ( $IC_{50}$  values between 140 nM and 1.3  $\mu$ M for mirabamide C, and 190 nM and 3.9  $\mu$ M for mirabamide D). Furthermore, mirabamides inhibited HIV-1 at the level of membrane fusion, presumably through interactions with HIV-1 envelope glycoproteins (Plaza et al. 2007). In addition, celebeside A and theopapuamide

**TABLE 9.2**  
**Table Showing HIV-1 Inhibitory Effects of Marine Peptides**

Sources	Peptide Name	Activity	Potency	Reference
Oyster <i>Crassostrea gigas</i>	LLEYSI LLEYSL	Inhibit HIV-1 protease	$IC_{50}$ : 20 nM 15 nM	Lee and Maruyama (1998)
Marine sponge <i>Siliquariaspongia mirabilis</i>	Mirabamide A Mirabamide C Mirabamide D	Inhibit HIV-1 neutralization and fusion	$IC_{50}$ : 0.04 and 0.14 $\mu$ M 0.14 and 1.3 $\mu$ M 0.19 and 3.9 $\mu$ M	Plaza et al. (2007)
Marine sponge <i>Homophymia</i> sp.	Homophymine A	Against HIV-1 infection	$IC_{50}$ : 75 nM	Zampella et al. (2008)
Marine sponge <i>Siliquariaspongia mirabilis</i>	Celebeside A Theopapuamide B	Block HIV-1 entry Neutralize HIV-1	$IC_{50}$ : 1.9 $\mu$ g/mL 0.8 $\mu$ g/mL	Plaza et al. (2009)
Marine sponge <i>Theonella mirabilis</i> <i>Theonella swinhonis</i>	Papuamide A Papuamide B	Inhibit HIV-1 infection	$EC_{50}$ : 4 ng/mL	Ford et al. (1999)
Marine sponge <i>Sidonops microspinosa</i>	Microspinosamide	Inhibit cytopathic effect of HIV-1 infection	$EC_{50}$ : 0.2 $\mu$ g/mL	Rashid et al. (2001)
Marine sponge <i>Neamphius huxleyi</i>	Neamphamide A	Against HIV-1 infection	$EC_{50}$ : 28 nM	Oku et al. (2004)
Marine sponge of the genus <i>Callipelta</i>	Callipeltin A	Inhibit cytopathic effects induced by HIV-1	$EC_{50}$ : 0.01 $\mu$ g/mL	Zampella et al. (1996)

B have been isolated from sponges of the same aforementioned species *Siliquariaspongia mirabilis*. Celebeside A is a cyclic depsipeptide incorporating a polyketide moiety and five amino acid residues, among which are the unusual amino acids phosphoserine and 3-carbamoyl threonine. Theopapuamide B is an undecapeptide comprising two previously unreported amino acids, 3-acetamido-2-aminopropanoic acid and 4-amino-2,3-dihydroxy-5-methylhexanoic acid. Theopapuamide B was active in the neutralization assay with an  $IC_{50}$  value of 0.8  $\mu\text{g}/\text{mL}$ , whereas celebeside A displayed inhibition of HIV-1 entry with an  $IC_{50}$  value of 1.9  $\mu\text{g}/\text{mL}$ . In addition, the anti-HIV activity of celebeside A correlates the presence of phosphoserine residue but absent in the inactive theopapuamide (Plaza et al. 2009). However, this hypothesis was ruled out by the evidence given in a study by Zampella and collaborators (2008). Homophymine A is a novel anti-HIV cyclodepsipeptide from the marine sponge *Homophymia* sp.; it contains an amide-linked 3-hydroxy-2,4,6-trimethyloctanoic acid moiety and 11 amino acid residues, including four unusual amino acid residues: (1) (2S,3S,4R)-3,4-diMe-Gln, (2) (2R,3R,4S)-4-amino-2,3-dihydroxy-1,7-heptandioic acid, (3) L-ThrOMe, and (4) (2R,3R,4R)-2-amino-3-hydroxy-4,5-dimethylhexanoic acid. Obviously, homophymine A lacks a  $\beta$ -methoxytyrosine residue, which is replaced by an *O*-methyl threonine residue; however, homophymine A was reported to potentially exhibit cytoprotective activity against HIV-1 infection with an  $IC_{50}$  value of 75 nM. The antiviral activity found in homophymine A ruled out the hypothesis that  $\beta$ -methoxytyrosine is essential for antiviral activity.

In a similar trend, depsipeptides isolated from a number of marine sponges have been identified to be active as HIV inhibitors. Neamphamide A, a novel HIV inhibitory depsipeptide obtained from the marine sponge *Neamphius huxleyi*, exhibited potent cytoprotective activity against HIV-1 infection with a 50% effective concentration ( $EC_{50}$ ) value of 28 nM (Oku et al. 2004). Similar to neamphamide A, callipeltin A, a novel antiviral and antifungal cyclodepsipeptide from a sponge of the genus *Callipelta*, exhibited the inhibition of cytopathic effects on CEM4 lymphocytic cell lines infected with HIV-1 at an  $EC_{50}$  value of 0.01  $\mu\text{g}/\text{mL}$  (Zampella et al. 1996). The general structure of callipeltin A with the N-terminus blocked and the C-terminus lactonized with a threonine residue is similar to that of didemnins, which possesses anti-HIV activity.

On the other hand, the novel cyclic depsipeptides papuamides A and B have been isolated from sponges *Theonella mirabilis* and *Theonella swinhoei* (Ford et al. 1999). They contain not only unusual amino acids including  $\beta$ -methoxytyrosine; 3-methoxyalanine; 3,4-dimethylglutamine; 2-amino-2-butenoic acid; and/or 2,3-diaminobutyric acid residues but also contain homoprolidine and 3-hydroxyleucine residues. They also contain a previously undescribed 2,3-dihydroxy-2,6,8-trimethyldeca-(4Z,6E)-dienoic acid moiety N-linked to a terminal glycine residue. They were reported to block the infection of human T-lymphoblastoid cells by HIV-1 sub(RF) *in vitro* with an  $EC_{50}$  of approximately 4 ng/mL. Papuamide A can block at the early stage of the viral life cycle, but not in HIV-1 envelope glycoprotein (Andjelic, Planelles, and Barrows 2008). Papuamide B also inhibits viral entry via interaction of this peptide with phospholipid present on the viral membrane at a concentration of 710 nM (Sagar, Kaur, and Minneman 2010). Another anti-HIV candidate is microspinamide, a new cyclic depsipeptide incorporating 13 amino acid residues isolated from the sponge *Sidonops microspinosa*. This peptide is the first naturally occurring peptide to contain a  $\beta$ -hydroxy-*p*-bromophenylalanine residue. Microspinamide inhibited the cytopathic effect of HIV-1 infection in an XTT-based *in vitro* assay with an  $EC_{50}$  value of approximately 0.2  $\mu\text{g}/\text{mL}$  (Rashid et al. 2001). Accordingly, sponges-derived peptides are indicated as promising candidates for the design of novel strong inhibitors of viral infection.

### 9.3.4 OTHER BIOLOGICAL ACTIVITIES

Marine peptides have been found to exhibit anticancer, anticoagulant, antidiabetic, antiobesity, and calcium-binding activities. According to recent studies, the anticancer activity of marine peptides has been evidenced by induction of apoptosis and inhibition of cell proliferation *in vitro*. These

peptides were obtained from anchovy sauce (Lee et al. 2004), sea slug (Wesson and Hamann 1996), sea hare (Madden et al. 2000), squid (Alemán et al. 2011), cod, plaice, salmon (Picot et al. 2006), tuna dark muscle (Hsu, Li-Chan, and Jao 2011), fish backbone (Naqash and Nazeer 2011), and shrimp shell (Kannan et al. 2011). Moreover, Wergedahl and colleagues (2004) have revealed that protein hydrolysate of salmon was able to reduce the risk of cardiovascular diseases by lowering plasma cholesterol levels and inhibiting the activity of Acyl-CoA cholesterol acyltransferase in Zucker rats.

Blood coagulation is processed by coagulation factors in order to stop the flow of blood though an injured vessel wall whenever an abnormal vascular condition and exposure to nonendothelial surfaces at sites of vascular injury occur. As endogenous or exogenous anticoagulants interfere with the coagulation factors, the blood coagulation can be stopped. These anticoagulants have been used for therapeutic purposes, for example, a cure for hemophilia (Kim and Wijesekara 2010). Although anticoagulant marine peptides have rarely been reported, they have been found from marine organisms such as marine echiuroid worm (Jo, Jung, and Kim 2008), starfish (Koyama et al. 1998), and blue mussel (Jung and Kim 2009). Moreover, marine anticoagulant proteins have also been purified from yellowfin sole (Rajapakse, Jung et al. 2005) and ark shell (Jung et al. 2007). These marine-derived anticoagulant peptides are noncytotoxic and can be potentially used as functional ingredients in nutraceuticals or pharmaceuticals.

Components that bind and solubilize minerals such as calcium can be considered to be beneficial in the prevention of dental caries, osteoporosis, hypertension, and anemia. Notably, some peptides derived from hoki and Alaska pollack frame proteins are known for their calcium-binding capability (Jung and Kim 2007). Moreover, improved calcium retention with hoki phospho-peptide intake was observed in osteoporosis-model rats to the same level as a commercially prepared casein oligophospho-peptide preparation (Jung, Lee, and Kim 2006). Calcium-binding peptides derived from marine organisms may have applications as dairy-free functional food or beverage ingredients for people with lactose intolerance, as anticarcinogenic ingredients, or as agents for reducing the risk of osteoporosis.

Obesity, or excessive body weight in the form of fat, has become a serious public health problem. Therefore, several lines of studies have provided due to finding the efficient agents and potential targets for antiobesity therapeutics. Herein, cholecystokinin, a biomarker associated with satiety, is identified as a promising target to reduce obesity (Szewczyk and Laudeman 2003). Meanwhile, low-molecular-weight peptides (1–1.5 kDa) from shrimp head protein hydrolysates have been found to be an effective agent for the stimulation of cholecystokinin release in secretin tumor cell (STC)-1 cell line (Cudennec et al. 2008). Thus, these peptides are suggested to be promising functional food ingredients, which reduce obesity via the regulation of cholecystokinin release.

## 9.4 CONCLUSION

It is assumed that much attention has been paid recently by researchers toward marine compounds as the safe and efficient agents in prevention or treatment of chronic diseases, such as heart disease, stroke, cancer, chronic respiratory diseases and diabetes. Consequently, a large number of bioactive agents from marine organisms have been identified based on specific assay systems or screening approaches. Interestingly, marine peptides have been found due to their various biological activities and health benefits. Moreover, the extensive studies performed on marine organism-derived peptides will contribute to the generation of novel functional foods as well as pharmaceutical products. Thus, marine peptides are believed to be a valuable source of bioactive compounds that could be used for the development of the food and pharmaceutical industries.

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# 10 Sea Cucumber Saponins

## *Realization of Their Anticancer Effects*

Se-Kwon Kim, S. W. A. Himaya, and Kyong-Hwa Kang

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### 10.1 INTRODUCTION

The search for natural products that can be used novel and effective pharmaceutical agents has gained much attention in natural product and pharmacology research. Among marine resources, marine animals have proven to be rich sources of interesting organic molecules, which have accumulated in them over years of evolution. Most of the marine invertebrates are sessile and are constantly targeted by the predators. To defend against these threats, these organisms are capable of releasing potent chemicals. A great number of compounds from marine invertebrates with diverse structural features and sound biological activities have been reported and reviewed in the literature. Among these, the compounds isolated from sea cucumbers are gaining more attention recently due to the presence of interesting compounds with potent biological activities. Sea cucumbers are soft-bodied wormlike echinoderms, which belong to the class Holothuroidea (De Moncerrat Iguez-Martinez et al. 2005). They have economic importance in Asian countries, specifically in China where several species are used in traditional medicine or eaten as delicacies. The taxonomical distribution of sea cucumbers consist of six main orders (Apodida, Elasipodida, Aspidochirotida, Molpadiida, Dendrochirotida, and Dactylochirotida), which includes 25 families, about 200 genera, and more than 1400 species. Sea cucumbers can be found in nearly every marine environment, but are most diverse on tropical shallow-water coral reefs.

Even though different types of natural products have been isolated from sea cucumbers, saponins (triterpenoid glycosides) are the major and most abundant type of compounds. Saponins are generally perceived as highly active natural products and sea cucumber saponins have been well characterized for their anticancer activities. Therefore, current work is focused on reviewing the structural features and the potential use of sea cucumber saponins as potential drug leads to be used in the pharmaceutical industry.

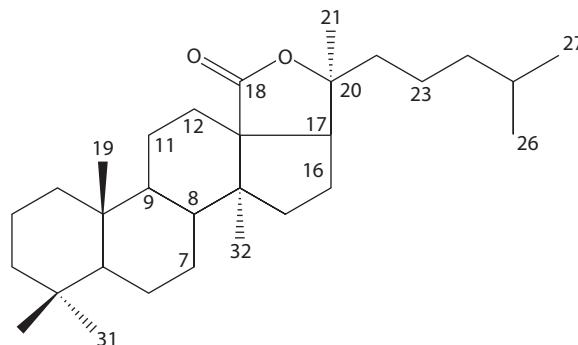
## 10.2 SAPONINS AND STRUCTURAL FEATURES OF SEA CUCUMBER SAPONINS

The term “saponin” is derived from the word “sapo,” a Latin word used for soap, due to their ability to form soap-like foam in aqueous solutions. Chemically, the foam formation ability of saponins corresponds to the presence of lipophilic aglycon moiety and hydrophilic glycon side chain (Augustin et al. 2011). The aglycon isoprenoids are mainly triterpenes or steroids derived and are connected covalently to one or more sugar moieties. Saponins are biosynthesized from mevalonate via farnesyl diphosphate and squalene. This pathway is specific for saponin biosynthesis and contrasts to the plastid-localized methylerythritol-phosphate (MEP) pathway, which is involved in the biosynthesis of monoterpenes, diterpenes, tetraterpenes (carotenoids), and polyphenols (Osbourne, Goss, and Field 2011).

Saponins are generally produced in plants as defense molecules against pathogens and herbivores. Hence, these are the most abundant category of secondary metabolites in terrestrial plants. Owing to this inherent defensive property, saponins have been used in traditional medicine formulations. Therefore, saponins have gained research attention for potential development as drug candidates by harvesting their pharmacological potential. Interestingly, it was found later that this group of compounds is the predominant secondary metabolites in sea cucumbers, which are presumed to be responsible for their general toxicity (Zhang, Li et al. 2006). More than 100 triterpene saponins have been isolated from many species of sea cucumbers belonging to different orders from the Pacific, Indian, and Atlantic oceans and the Mediterranean Sea (Antonov et al. 2008).

The triterpenoid moieties in the aglycon part are composed of lanostane derivatives (Zou et al. 2005) where the majority belongs to the holostane type (Dang et al. 2007). Holostane type triterpene glycosides include a  $3\beta,20S$ -dihydroxy- $5\alpha$ -lanostano-18,20-lactone (**1**) (Figure 10.1) structural feature. The holostane type saponins are classified into three groups: saponins with  $3\beta$ -hydroxyholost-9(11)-ene aglycon skeleton, saponins with  $3\beta$ -hydroxyholost-7-ene skeleton, and saponins that contain an aglycon moiety different to the above skeletons (Avilov et al. 2008). However, recent investigations have found sea cucumber saponins with novel aglycon structural features that have been identified as triterpene saponins with non-holostane aglycon structures. Non-holostane type aglycon moieties are a rare feature in sea cucumber triterpene glycosides. Only a few instances of non-holostane type glycosides have been reported.

The glycon part of the sea cucumber saponins is composed of two to six sugar units and it is covalently linked to the C-3 position of the aglycon unit (Chiludil et al. 2003; Kalinin et al. 2005). Quinovose, glucose, 3-O-methylglucose, xylose, and 3-O-methylxylose are the most prominent sugars present in the carbohydrate moieties of these glycosides (De Moncerrat Iiguez-Martinez et al. 2005). In the oligosaccharide chain, the first monosaccharide unit is always a xylose while 3-O-methylglucose and 3-O-methylxylose are always at the terminal. In some glycosides, sulfate groups are attached to the oligosaccharide chain. Most of them are mono-sulfated glycosides with a few occurrences of di- and tri-sulfated glycosides (Chiludil et al. 2003).

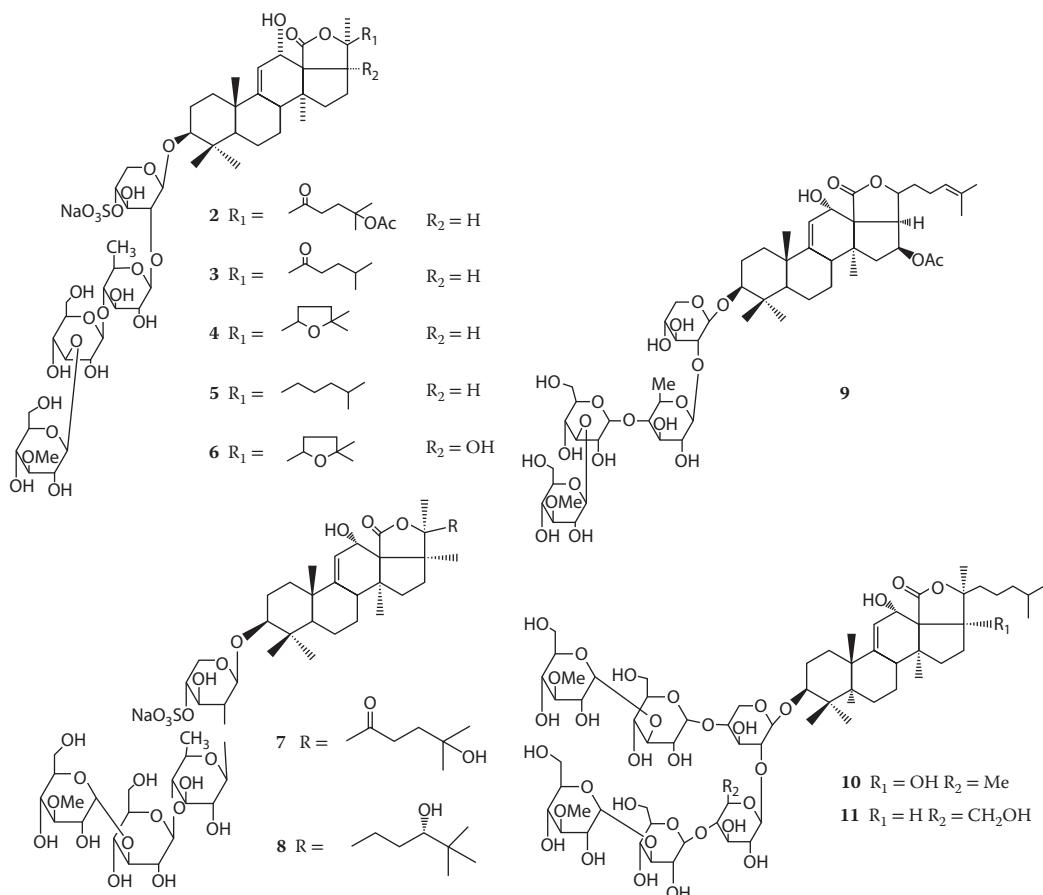


**FIGURE 10.1** Structure of the holostane group, which is the characteristic aglycon moiety in sea cucumber glycosides.

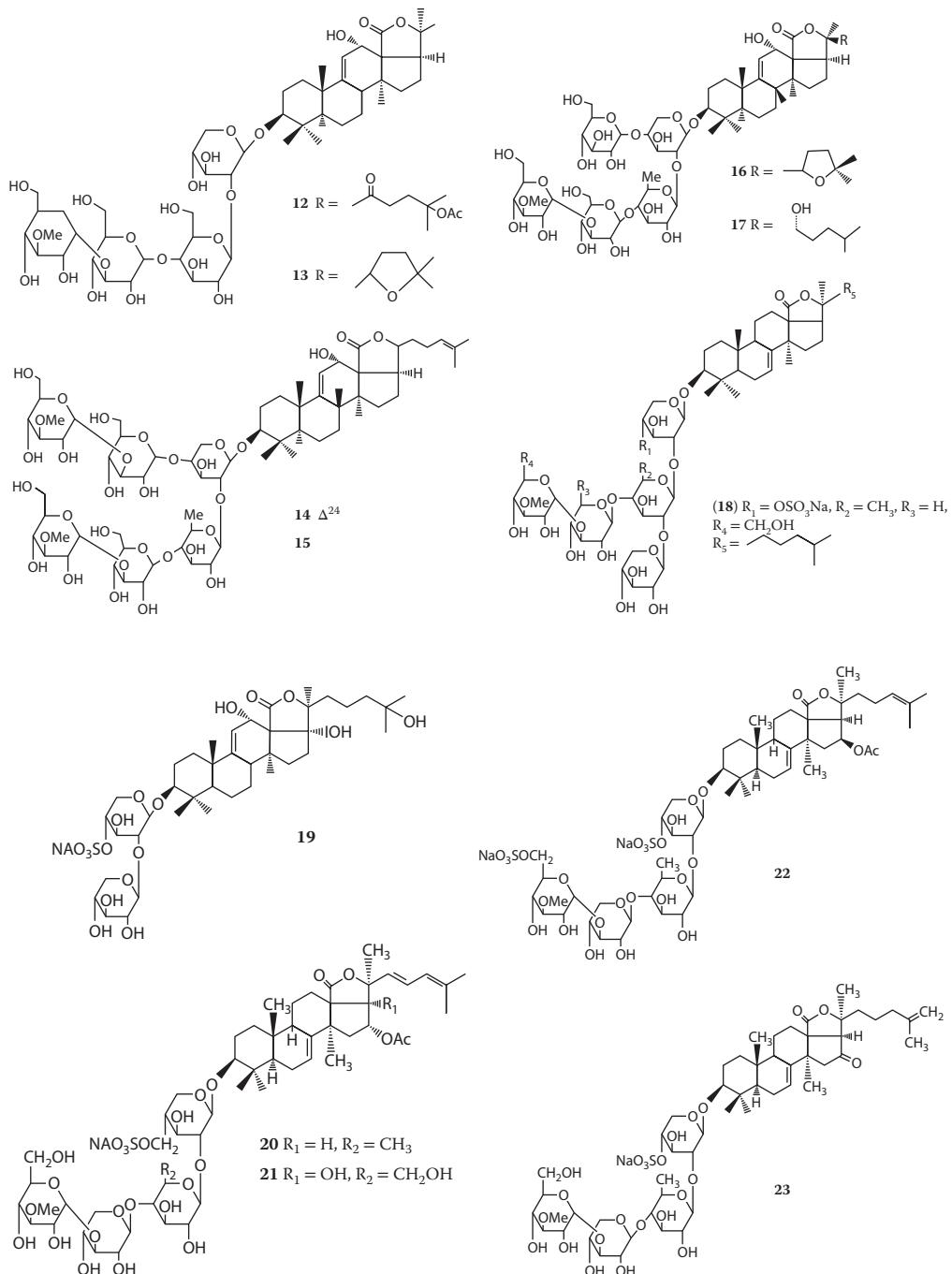
### 10.3 ANTICANCER ACTIVITIES OF TRITERPENE GLYCOSIDES OF SEA CUCUMBERS

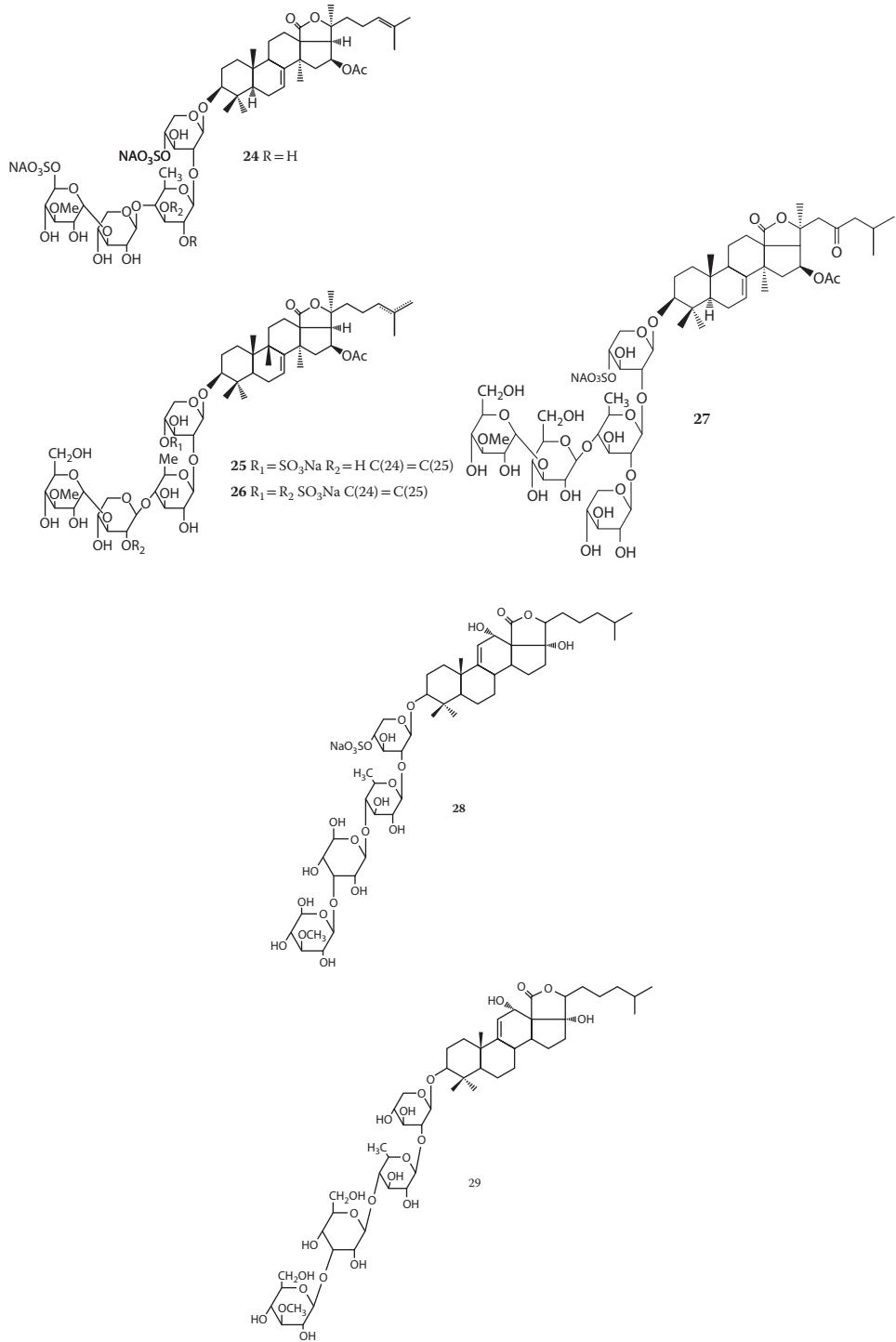
Sea cucumbers have long been known for their healing powers and thus have been used as an ingredient in Chinese traditional medicine rations. Extensive research on sea cucumbers has revealed that triterpene saponins isolated from sea cucumbers are primarily responsible for the inherent healing properties of sea cucumbers. Sea cucumber saponins have exhibited promising biological activities and much research has been conducted to explore their anticancer effect (Zhang, Yi et al. 2006; Dang et al. 2007; Han et al. 2008). *In vitro* studies have revealed that most of the triterpene glycosides of sea cucumbers are toxic toward cancer cells. In this section, the *in vitro* cytotoxic effects of identified saponins (Figure 10.2) toward cancer cells are discussed briefly.

The *in vitro* cytotoxicity of five triterpene glycosides, fuscocineroside A (2), B (3), and C (4), pervicoside C (5), and holothurin A (6) isolated from *Holothuria fuscocinerea* Jaeger on human leukemia HL-60 and human hepatoma BEL-7402 cells was analyzed and all compounds have shown a potent cytotoxicity toward both cell lines. However, compound 4 was found to be the most potent ( $IC_{50} = 0.88$ ,  $IC_{50} = 0.58 \mu\text{g/mL}$ ) in HL-60 and BEL-7402 cell lines, respectively (Zhang, Yi and Tang 2006). The triterpene glycosides from the sea cucumber *Holothuria scabra*, namely holothurin A3 (7) and A4 (8) are found to be strongly cytotoxic to cancer cell lines: human epidermoid carcinoma



**FIGURE 10.2** Structures of potent cytotoxic saponins from sea cucumbers.

**FIGURE 10.2** (Continued)

**FIGURE 10.2** (Continued)

(KB) and human hepatocellular carcinoma (Hep-G2), with  $IC_{50}$  values of 0.87 and 0.32  $\mu\text{g}/\text{mL}$  (for compound **7**) and 1.12 and 0.57  $\mu\text{g}/\text{mL}$  (for compound **8**), respectively (Dang et al. 2007). Arguside A (**9**) also exhibited significant cytotoxicity against different human tumor cell lines while showing the highest activity toward human colorectal carcinoma (HCT-116) cells ( $IC_{50} = 0.14 \mu\text{M}$ ) with more potency than the employed positive control, 10-hydroxycamptothecin (HCP) ( $IC_{50} = 0.84 \mu\text{M}$ ) (Liu et al. 2007). Argusides B (**10**) and C (**11**) have also shown potent cytotoxicity against human tumor cell lines, adeno carcinomic human alveolar basal epithelial cells (A549), HCT-116, HepG2, and human breast adenocarcinoma (MCF-7) cell lines. The cytotoxicity of the compounds on A549 (**10**- $IC_{50} = 0.48 \mu\text{g}/\text{mL}$ , **11**- $IC_{50} = 0.43 \mu\text{g}/\text{mL}$ ) and HCT-116 (**10**- $IC_{50} = 0.46 \mu\text{g}/\text{mL}$ , **11**- $IC_{50} = 0.38 \mu\text{g}/\text{mL}$ ) cells were more potent than the positive control V-16. However, there was no significant difference between the cytotoxicity of two compounds (Liu et al. 2008b). Besides, argusides D (**12**) and E (**13**) have also been tested for their anticancer activities in the above human cancer cell lines and revealed significant activity with  $IC_{50}$  values in the range of 3.36–7.77  $\mu\text{g}/\text{mL}$  (Liu et al. 2008a). This finding shows that compounds **10** and **11** are potent cytotoxic agents compared to compounds **12** and **13**. It has been reported that the length and type of sugar moieties of glycosides play an important role in terms of cytotoxic activity against tumor cells and this observation clearly indicates that. Moreover, the *in vitro* cytotoxicities of impatienside A (**14**) and bivittoside D (**15**) were evaluated extensively by employing seven human cancer cell lines and the results showed that both glycosides exhibited *in vitro* cytotoxicities similar to or better than that of the potent anticancer drug etoposide (V-16) in four human tumor cells: A549 (**14**- $IC_{50} = 0.35 \mu\text{g}/\text{mL}$ , **15**- $IC_{50} = 0.52 \mu\text{g}/\text{mL}$ ), HCT-116 (**14**- $IC_{50} = 0.45 \mu\text{g}/\text{mL}$ , **15**- $IC_{50} = 0.37 \mu\text{g}/\text{mL}$ ), DU-145 (**14**- $IC_{50} = 1.14 \mu\text{g}/\text{mL}$ , **15**- $IC_{50} = 0.937 \mu\text{g}/\text{mL}$ ), and KB (**14**- $IC_{50} = 1.6 \mu\text{g}/\text{mL}$ , **15**- $IC_{50} = 1.42 \mu\text{g}/\text{mL}$ ) (Sun et al. 2007). The structural differences between glycosides **14** and **15** were limited to their holostane skeleton, and there was no significant difference in the cytotoxicity of the two glycosides. However, pervicoside C (**5**), an analogue of **15** having the same aglycone but a different sugar chain, isolated from *H. fuscocinerea* Jaeger, exhibited weak activities against HCT-116 and A549 cancer cells, with  $IC_{50}$  values of 18.7 and 28.6  $\mu\text{g}/\text{mL}$ , respectively (Sun et al. 2007). According to these results, it is again confirmed that the length and type of sugar moieties of such glycosides play an important role in terms of cytotoxic activity against tumor cells.

17-Dehydroxyholothurinoside A (**16**) and griseaside A (**17**) are identified as promising anticancer agents due to their significantly higher cytotoxicity against four human tumor cell lines: A549 (**16**- $IC_{50} = 0.886 \mu\text{M}$ , **17**- $IC_{50} = 1.07 \mu\text{M}$ ), HL-60 (**16**- $IC_{50} = 0.245 \mu\text{M}$ , **17**- $IC_{50} = 0.427 \mu\text{M}$ ), BEL-7402 (**16**- $IC_{50} = 0.97 \mu\text{M}$ , **17**- $IC_{50} = 1.114 \mu\text{M}$ ), and human acute lymphoblastic leukemia cell line (Molt-4) (**16**- $IC_{50} = 0.34 \mu\text{M}$ , **17**- $IC_{50} = 0.521 \mu\text{M}$ ) compared to the positive control HCP (A549  $IC_{50} = 2.35 \mu\text{M}$ , BEL-7402  $IC_{50} = 2.6 \mu\text{M}$ , HL-60  $IC_{50} = 1.9 \mu\text{M}$ , Molt-4  $IC_{50} = 2.2 \mu\text{M}$ ) (Sun et al. 2007). Hillaside C (**19**) has also been tested for its anticancer potential against eight human tumor cell lines (A-549, MCF-7, human lung carcinoma cells—IA9, human clear cell carcinoma cells—CAKI-1, human prostate cancer cells—PC-3, KB, KB-VIN, and human colorectal adenocarcinoma cells—HCT-8) and has exhibited cytotoxicity with  $IC_{50}$  values in the range of 0.15–3.20  $\mu\text{g}/\text{mL}$  (Wu et al. 2006). Compared to the positive control HCP, compound **19** has shown more potent cytotoxicity toward CAKI-1 ( $IC_{50} = 0.15 \mu\text{g}/\text{mL}$ ) and KB-VIN ( $IC_{50} = 2.81 \mu\text{g}/\text{mL}$ ) cell lines. Three new triterpene glycosides, intercedensides A (**20**), B (**21**), and C (**22**) from *Mensamaria intercedens* Lampert, were widely studied for their anticancer activity employing 10 human tumor cell lines (A549, MCF-7, IA9, CAKI-1, human glioblastoma cells—U-87-MG, PC-3, KB, KB-VIN, human skin melanoma cells—SK-MEL-2, HCT-8). Interestingly, all compounds showed a significant cytotoxicity against all tumor cell lines within the  $IC_{50}$  value range of 0.7–4  $\mu\text{g}/\text{mL}$ , and the compounds **20** and **22** showed similar potencies, while compound **21** was generally more potent in all cell lines. Furthermore, compound **20** also exhibited significant *in vivo* antineoplastic activity against mouse Lewis lung cancer and mouse S180 sarcoma, with 48.39% and 57.48% tumor reduction levels (Zou et al. 2003).

New sulfated triterpene glycoside from *Pentacta quadrangularis*, philinopside E (**23**), showed a significant cytotoxicity ( $IC_{50} = 0.75\text{--}3.50 \mu\text{g/mL}$ ) against ten tumor cell lines (mouse lymphocytic leukemia cells—P388, HL60, A549, lung adenocarcinoma cells—SPC-A4, gastric carcinoma cells—MKN28, gastric carcinoma cells—SGC7901, BEL7402, human ovarian carcinoma—HO8901, human fetal lung fibroblasts—W138, human epithelial carcinoma cells—A431) (Zhang, Yi et al. 2006). Furthermore, sulfated triterpene glycoside intercedenside B (**24**) from *Pseudocolochirus violaceus* exhibited significant cytotoxicity against cancer cell lines MKN-45 (human gastric adenocarcinoma) and HCT-116 with  $IC_{50}$  values in the range of  $0.052\text{--}0.442 \mu\text{M}$  and both compounds showed significantly higher activity against HCT-116 compared to the positive control HCP (Zhang, Tang, and Yi 2007). Moreover, the sulfated triterpene glycosides, philinopsides A (**25**) and B (**26**), showed significant cytotoxicity ( $IC_{50} = 0.75\text{--}3.50 \mu\text{g/mL}$ ) against ten tumor cell lines (CAKI, HOS, KB-VIN, KB, SM-MEL-2, U87-MG, HCT-8, IA9, A549, and PC3) (Yi et al. 2006). Collectively, all these triterpene glycosides of sea cucumber are very potent cytotoxic agents toward a wide array of cancer types and the structural properties such as the composition of the sugar moiety and the sulfation in the glycon unit are directly affecting their cytotoxic potential.

Even though a number of saponin compounds are isolated and identified as potent cytotoxic agents, only a few of them have been studied to unravel the mode of their cytotoxicity. Among them, detailed cytotoxic mechanisms of frondoside A (**18**), cucumarioside A<sub>2</sub>-2 (**27**), echinoside A (**28**), and ds-echinoside A (**29**) have been reported against several cancer types *in vitro* and *in vivo*. All four compounds have shown their cytotoxicity toward cancer cells by arresting the cell cycle progression via activating the apoptosis pathways, which leads to the cell death. Frondoside A has shown potent apoptotic-inducing properties against breast cancer, pancreatic cancer, and leukemia (Marzouqi et al. 2011), cucumarioside A<sub>2</sub>-2 has studies against leukemia (Jin et al. 2009), and echinoside A and ds-echinoside A have been characterized against liver cancer (Zhao et al. 2012). These compounds activate the intrinsic apoptotic pathway via suppressing the tumor suppressor gene p53. With the suppression of p53, apoptosis pathways are induced and the caspases 3, 7, 8, and 9, which regulate the cell death process are activated. Interestingly, *in vivo* studies have confirmed that frondoside A (100  $\mu\text{g/kg/day}$ ) effectively decreased the growth of breast cancer xenografts in athymic mice without exerting any side effects (Marzouqi et al. 2011). Moreover, frondoside A is also capable of inhibiting cancer cell migration and invasion, which will ultimately reduce the progression of cancer to other parts of the body. Similarly, echinoside A and ds-echinoside A treatment (2.5 mg/kg) to the mice bearing H22 hepatocarcinoma tumors has reduced tumor weight by 49.8% and 55%, respectively (Zhao et al. 2012). These studies evidently prove the higher potential of these compounds as novel natural pharmacological agents against tumor growth and cancer progression.

## 10.4 STRUCTURE ACTIVITY RELATIONSHIPS

Even though there has not been a lot of research in the area, the anticancer activity of the sea cucumber saponins is believed to be directly correlated to their structural features. As suggested by many authors, the bioactivity of the triterpene glycosides is a result of its strong membranolytic activity. This membranolytic activity is a function of the structural feature of the glycoside (Kalinin 2000). The presence of an 18(20)-lactone as the aglycon with at least one oxygen group near it has critical significance for the biological activity of glycosides bearing a 9(11) double bond. Glycosides with a 7(8)-double bond in their aglycon structure with the absence of a 16-ketogroup are more active than those with the presence of a 16-ketogroup (Kalinin et al. 1996). The characteristics of the attached glycon structure are also critical for the bioactivities of the sea cucumber triterpene glycosides. It has been found that for the actions leading to modification of the cellular membrane, the presence of a linear tetrasaccharide chain is significant (Kalinin et al. 1992). In addition, Maltsev et al. (1985) reported that glycosides having quinovose as a second monosaccharide unit are more active over others. The sulfation of the sugar chain is also a significant factor related to bioactivity. A sulfate group at C-4 of the first xylose residue increases the effect against membranes. The absence of a sulfate

group at C-4 of the xylose residue in biosides decreases its activity more than one-fold in magnitude. On the other hand, the presence of a sulfate at C-4 of the first xylose in branched pentanosides with the 3-O-methyl group as a terminal monosaccharide increases activity. However, the same sulfate can decrease the activity of branched pentanosides, which have glucose as the terminal residue. Besides, sulfate groups attached to a C-6 position of terminal glucose and 3-O-methylglucose residues impart a great reduction in activity (Kalinin 2000).

## 10.5 FUTURE PROSPECTS: TOWARD ANTICANCER DRUG LEADS

Holothurians have been used as ingredients for traditional Chinese medicine for years. In addition, holothurin A is marketed in Japan as an ingredient in an antifungal medicine. Even though there are many lead compounds with promising potential to be used as drugs for cancer therapy, the cytotoxicity itself would be a constraint for this purpose, because most of the compounds could be cytotoxic toward normal cells in addition to the cancerous cells. However, in finding therapeutics from natural products, the preference is always given to the compounds having high specificity toward cancer cells in their cytotoxic action while minimizing the damage to normal cells. Therefore, considerable clinical studies should be conducted employing the lead compounds before introducing them to the drug development phase. Moreover, the possibility of continuous supply of the product and the ecological importance of the sea cucumber are factors of importance before entering the drug development phase. Being a product of a natural resource, the continuous supply of saponins is leading to environmental and economic concerns due to the complex technologies required for the purification process. The structural complexities have challenged the chemical synthesis, which could limit the entering of these compounds into the drug development phase. However, with the advances in synthetic chemistry and with the understanding of saponin biosynthetic processes, new opportunities for exploitation of these compounds as drug leads are opening up.

## 10.6 CONCLUDING REMARKS

To date, the vast diversity of sea cucumbers has paved the way for natural product chemists to mine for new bioactive compounds. The survival demand has resulted in the evolution of sophisticated compounds and among them sea cucumber triterpene glycosides are the most studied. The proven anticancer effects of several triterpenoid glycosides reveal their potential use in drug development studies. However, the mechanisms of the activity should be studied more as there is a considerable gap in this area compared with the isolation rate of new compounds.

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# 11 Phlorotannins as Potential Antibacterial Agents from Marine Brown Algae

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### 11.1 INTRODUCTION

Since the 1970s, more than 15,000 structurally diverse bioactive natural products with an astounding array of biological activities have been discovered from marine microbes, algae, and invertebrates. Although more than 70% of the Earth's surface is covered by oceans, we use only less than 10% of the total ocean area (Schultes 1978). Notably, many marine organisms live in complex habitats exposed to extreme conditions and in adapting to a new environment, they produce a wide variety of secondary metabolites, which cannot be found in other organisms. Moreover, considering its great taxonomic diversity, investigations related to the search of new bioactive compounds from the marine environment can be seen as an almost unlimited field. In addition, the biological productivity of terrestrial ecosystems has also simply reached what it can achieve; the marine biodiversity of the ocean can be expected to have new therapeutic agents (Bugni and Ireland 2004).

Increasing resistance to antibiotics of clinically important bacteria is a major concern throughout the world (Kaplan and Mason 1998). Over the past 20 years, investigators from virtually every corner of the world have documented that increasing proportions of *Staphylococcus aureus* are resistant to penicillin and other antibiotics. As a result, these days, the majority of *S. aureus* are swamped with methicillin-resistant *S. aureus* (MRSA). In spite of the available effective treatments against the serious infections due to MRSA, the high mortality rates still are a major concern. There are a few new agents in development that can be expected to benefit the situation in the next decade (Gould et al. 2009). Over the past 50 years, *S. aureus* has become resistant to most antibiotics except vancomycin and other glycopeptides. Recently, these antibiotics are the mainstay of treatment for the multidrug-resistant *S. aureus*, and therefore the possibility that vancomycin resistance might transfer from vancomycin-resistant enterococci to multidrug-resistant *S. aureus* is extremely worrying (Weigel et al. 2003). The emergence of MRSA resistant to the "last resort" antibiotics (vancomycin and teicoplanin) has created an urgent need in the discovery of alternative antibiotics (Alim et al. 2009).

One of the ways of preventing antibiotic resistance is by using new compounds that are not based on the existing synthetic antimicrobial agents. In particular, the search for novel natural sources from marine ecosystems could lead to the isolation of new antibiotics (Tan and Zou 2001). Many

organisms produce marine natural products that possess unique structural features as compared to terrestrial metabolites (Larsen et al. 2005). In the marine environment, where all surfaces are constantly exposed to the threat of surface colonization, sessile organisms remain relatively free from biofouling (Rhimou et al. 2010). Furthermore, the chemical compounds produced by marine organisms are less well known than those of their terrestrial counterparts. Among marine organisms, edible seaweeds have been identified as an underexploited plant resource and a source of functional foods. It is believed that the physiological and genetic characteristics of seaweeds differ compared to terrestrial plants. These are extensively used in food and medicine (Lee et al. 2008). The ability of seaweeds to produce secondary metabolites of antimicrobial value, such as volatile components (phenols and terpenes) (Cox et al. 2010; Demirel et al. 2009; Gressler et al. 2011; Gupta and Abu-Ghannam 2011; Kotnala, Grag, and Chatterji 2009; Patra et al. 2008), steroids (Shanmughapriya et al. 2008), phlorotannins (Wang et al. 2009), and lipids (Shanmughapriya et al. 2008), has been already studied. Among these, phlorotannins as polyphenolic secondary metabolites are found only in brown algae (Heo and Jeon 2005).

Thus, the screen for antimicrobial agents as safe alternatives and secondary metabolites from marine algae is attracting attention in the food industry. This chapter focuses on phlorotannins derived from marine algae and presents their potential application as antimicrobial agents.

## 11.2 PHLOROTANNINS FROM MARINE BROWN ALGAE

Marine algae have become an important source of pharmacologically active metabolites. They are widely distributed and abundant throughout the coastal areas of many countries. In addition, they are a source of useful secondary metabolites such as agar, carrageenan, and alginic acid with interesting pharmaceutical properties (Taskin, Ozturk, and Kurt 2001). Among marine algae, brown algae have been reported to contain higher phlorotannin contents as marine phenolic compounds (Heo and Jeon 2005). Phlorotannins consist of polymers of phloroglucinol (1,3,5-trihydroxybenzene) units and are formed in the acetate-malonate pathway in marine algae. Furthermore, these phlorotannins are highly hydrophilic components with a wide range of molecular sizes (126 Da–650 kDa) (Ragan and Glombitsa 1986; Wijesekara and Kim 2010).

Furthermore, several phlorotannins purified from brown seaweeds such as *Ecklonia cava*, *E. kurome*, *E. stolonifera*, *Eisenia aborea*, *E. bicyclis*, *Ishige okamurae*, and *Pelvetia siliquosa* contain medicinal and pharmaceutical benefits and have shown strong antioxidant, anti-inflammatory, antiviral, antitumor, antidiabetes, and anticancer properties (Cha, Je, and Kim 2011; Eom et al. 2011; Gupta and Abu-Ghannam 2011; Kim et al. 2009).

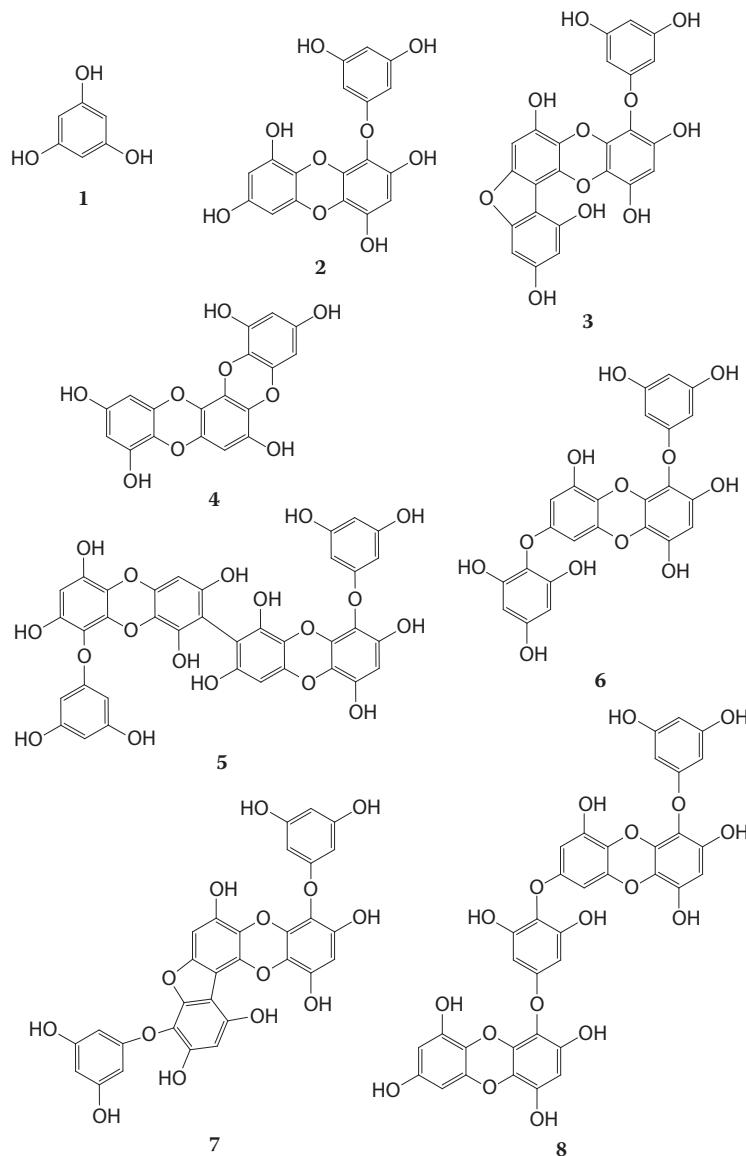
Eckol, dieckol, and phloroglucinol from *E. cava* have shown potential skin whitening effect (Heo et al. 2009) and antihypertensive effect (Wijesinghe, Ko, and Jeon 2011). In addition, *E. cava* contains other phlorotannins including 6,6'-bieckol, 8,8'-bieckol, 8,4''-dieckol, dioxinodehydroeckol, fucodiphlorellol G, phlorofucofuroeckol-A, and triphlorellol-A (Ahn et al. 2004; Li et al. 2009). *E. kurome* and *E. bicyclis* have been reported to contain phlorotannin compounds such as eckol, phlorofucofuroeckol A, and dieckol, and 8,8'-bieckol was isolated (Nagayama et al. 2002). Phlorotannins in *E. arborea* possess a strong antiallergic effect and their structures are elucidated as eckol, 6,6'-bieckol, 6,8'-bieckol, 8,8'-bieckol, phlorofucofuroeckol-A, and phlorofucofuroeckol-B (Sugiura et al. 2006). Moreover, 6,6'-bieckol diphlorellohydroxycarmalol and phloroglucinol have been isolated from the brown algae *I. okamurae* (Zou et al. 2008). Collectively, phlorotannins can be used as functional ingredients in the food and pharmaceutical industries.

## 11.3 ANTIBACTERIAL EFFECT OF PHLOROTANNINS

Some synthetic preservatives and additives used in the food industry have been evaluated to function as toxic to various cells and organs, mutagens, and tumor promoters over long-term use (Kahl and Kahl 1983; Sasaki et al. 2005). Therefore, recently, there has been a great deal of interest in

the search for novel natural antibiotics and these studies have shown that phlorotannins in brown algae can act as a potential antimicrobial agent in the food and pharmaceutical industries (Choi et al. 2010; Eom 2012; Lee et al. 2008).

The isolated and characterized phlorotannins (**1–8**) from brown algae with antimicrobial activity are presented in Figure 11.1, such as phloroglucinol (**1**), eckol (**2**), fucofuroeckol-A (**3**), dioxinodehydroeckol (**4**), 8,8'-bieckol (**5**), 7-phloroeckol (**6**), phlorofucofuroeckol-A (**7**), and dieckol (**8**). In addition, triphloroethol A, 6,6'-bieckol, and 8,4''-dieckol have also been reported. These isolated phlorotannins have shown antimicrobial effect against food-borne pathogenic bacteria, antibiotic resistance bacteria, and human tinea pedis fungus (Table 11.1).



**FIGURE 11.1** Structures of phlorotannins: phloroglucinol (**1**), eckol (**2**), fucofuroeckol-A (**3**), dioxinodehydroeckol (**4**), 8,8'-bieckol (**5**), 7-phloroeckol (**6**), phlorofucofuroeckol-A (**7**), dieckol (**8**).

**TABLE 11.1**

Phlorotannin Compounds with Antibacterial Effect

Source	Phlorotannin	Antimicrobial Activity	IC <sub>50</sub> <sup>a</sup>	References
<i>Eisenia bicyclis</i>	Eckol (2)	Inhibition of <i>Staphylococcus aureus</i>	32–64 µg/mL <sup>b</sup>	Eom (2012)
	Dieckol (8)	and methicillin-resistant <i>S. aureus</i> (MRSA)		
	Dioxinodehydroeckol (4)			
	Fucofuroeckol-A (3)			
	7-phloroeckol (6)			
<i>Ecklonia cava</i>	Phlorofucofuroeckol (7)			Choi et al. (2010)
	Eckol (2)	Inhibition of <i>S. aureus</i> , MRSA, <i>Salmonella</i> sp.	125–250 µg/mL <sup>b</sup>	
	Dieckol (8)	Inhibition of <i>Trichophyton rubrum</i>	148 mg/mL <sup>b</sup>	
<i>Ecklonia kurome</i>	8,8'-Bieckol (5)	Inhibition of MRSA and <i>Bacillus cereus</i>	96.5–>800.8 µg/ mL <sup>c</sup>	Nagayama et al. (2002)
	Eckol (2)			
	Dieckol (8)	Inhibition of <i>Campylobacter jejuni</i> , <i>Escherichia coli</i> ,	22.3–>800.8 µg/ mL <sup>c</sup>	
	Phlorofucofuroeckol A (7)	<i>Salmonella enteritidis</i> ,		
<i>Ecklonia stolonifera</i>	Phloroglucinol (1)	<i>S. typhimurium</i> , <i>Vibrio parahaemolyticus</i>		Lee et al. (2008)
	Dieckol (8)	Inhibition of <i>S. aureus</i> and MRSA, <i>Bacillus subtilis</i>	32–64 µg/mL <sup>b</sup>	
		Inhibition of <i>Acinetobacter</i> sp., <i>Klebsiella pneumonia</i> , <i>Legionella birminghamensis</i> , <i>Salmonella typhimurium</i> , <i>Shigella flexneri</i>	128–256 µg/mL <sup>b</sup>	

<sup>a</sup> IC<sub>50</sub>: concentration of a compound required for 50% inhibition *in vitro*.<sup>b</sup> MIC: minimum inhibitory concentration.<sup>c</sup> MBC: minimum bactericidal concentration.

Lee et al. (2010) revealed that dieckol purified from *E. cava* was responsible for fungicidal activity. Dieckol has shown a potent antifungal activity against *Trichophyton rubrum* associated with dermatophytic nail infections in humans. In addition, it has shown a potent inhibition of cell membrane integrity as well as cell metabolism against *T. rubrum*. According to Choi et al. (2010), minimum inhibitory concentration (MIC) values for eckol of *E. cava* with potent antimicrobial activity against MRSA is in the range of 125–250 µg/mL. Dieckol isolated from *E. stolonifera* might possess stronger anti-MRSA activity than eckol and the MICs of dieckol were in the range of 32–64 µg/mL (Lee et al. 2008). Although the current knowledge on the relationship between the structure and activity of the active phlorotannins is limited, the physiological activity may depend on the degree of polymerization of phlorotannin derivatives (Wijesekara and Kim 2010). In addition, according to the significant results of anti-MRSA activity in comparison to catechin derivatives as positive control, it has been reported that the MICs of (−)-epigallocatechin, (−)-EGCg, (+)-galloallocatechin, and (−)-galloallocatechin from green tea (*Camellia sinensis*) against MRSA were 64 µg/mL (Stapleton et al. 2004). Thus, the anti-MRSA activity of phlorotannins isolated from *E. bicyclis* was superior to or equal to those of catechins originated from green tea (Eom 2012).

Phlorotannins from *E. kurome* have been reported for bactericidal activity against food-borne pathogenic bacteria. Moreover, the oral administration of the phlorotannins did not show any effect in mice. The interactions between bacterial proteins and phlorotannins were supposed to play an important role in the bactericidal action of phlorotannins (Nagayama et al. 2002).

Therefore, it is thought that phlorotannins from brown algae would be very useful in the food and pharmaceutical industries as antibiotic agents. In addition to phlorotannins, brown algae include various health-enhancing compounds such as fucoxanthin, sulfated polysaccharides, sterols, polyunsaturated fatty acids, and soluble fibers (Kim et al. 2002).

## 11.4 CONCLUSION

Marine natural products provide a rich source of chemical diversity that can be used to develop novel, potential, and useful therapeutic agents. Certain marine products have been reported to exhibit antimicrobial effects against several pathogens. Furthermore, increasing consumer knowledge of the link between safety and health has raised the demand for novel health promotion and functional food ingredients. Hence, in an effort to discover an alternative antibiotic, marine organisms have attracted much attention. Thus, phlorotannins derived from brown algae are effective antibiotics against food-borne pathogenic bacteria. In conclusion, phlorotannins have the potential to expand their application in the food industry as potential antimicrobial agents.

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# 12 Bioactive Molecules from Symbiotic Marine Dinoflagellates

*Masaki Kita, Toshiyasu Inuzuka, Norihito Maru, and Daisuke Uemura*

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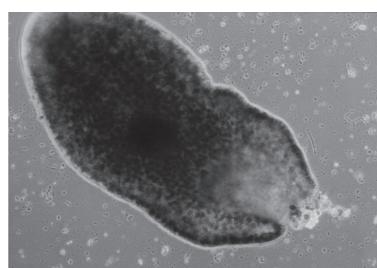
### 12.1 INTRODUCTION

A variety of biologically and physiologically active secondary metabolites have been isolated from marine sources. In particular, huge polyol and polyether compounds composed of a long carbon backbone functionalized by oxygen atoms, so-called “super-carbon-chain (SCC) compounds” (Uemura 1991, 2010) such as palytoxin, halichondrin, ciguatoxin, and maitotoxin, are some of the most attractive molecules in natural products chemistry (Yasumoto and Murata 1993; Murata and Yasumoto 2000; Kita and Uemura 2005, 2007; Uemura 2006; Uemura et al. 2009). It has been suggested that the primary producers of such secondary metabolites may be microalgae, bacteria, and fungi, and they are carried through symbiosis, association, a food chain, and other forms of nutrient-dependency (Shimizu 1993, 1996, 2003; Daranas, Norte, and Fernández 2001; Kita and Uemura 2006; Ueda and Uemura 2007; Nakamura, Kitamura, and Uemura 2009). Among them, symbiotic marine dinoflagellates have attracted the attention of natural products chemists, biologists, and ecologists, since they are rich sources of unique bioactive secondary metabolites.

There is great potential for future studies with respect to the material level of symbiosis. The symbiotic marine dinoflagellate *Symbiodinium* sp., which is the member of Zooxanthellae, is the dominant algal symbiont for both reef-building corals and many other invertebrates in the world's oceans (Trench 1981; Blank and Trench 1985; Rowan and Powers 1991). Corals require intracellular *Symbiodinium* to provide photosynthesis, which fuels the energetically expensive deposition of calcium carbonate (Bellwood et al. 2004). However, recent deterioration of the environment has led to catastrophic damage to corals, including coral bleaching and overgrowth by organisms that cover corals. For instance, outbreaks of the blackish encrusting sponge *Terpios hoshinota* have occurred on mostly Pacific reefs and have killed a wide range of corals (Plucer-Rosario 1987; Rützler and Muzik 1993). Recently, we observed that *T. hoshinota* in the Nakijin coral reef in Okinawa Prefecture, Japan, overgrew corals from the bottom and the symbiotic dinoflagellates moved out before they were completely overgrown (Uemura 2006). The cytotoxic compounds nakiterpiosin, nakiterpiosinon, and terpiodiene have been isolated from this encrusting sponge species (Teruya et al. 2002, 2003, 2004).

In addition, the Papuan jellyfish *Mastigias papua* has algae living inside and around its legs, which may be related to coral bleaching. Notably, this jellyfish can survive more than 10 days in a plastic bottle, as long as it is irradiated with light (Figure 12.1). Under light, the dinoflagellates (Zooxanthellae) within the jellyfish may produce oxygen and some nutrients to enable the jellyfish to survive (Sachs and Wilcox 2006; Reynolds et al. 2008; Uemura et al. 2012). We are especially interested in an ecological system in which algae are removed from the host and a different alga is transplanted onto the host. This would enable us to identify the substances that are essential for symbiosis and the substances that allow for the removal and transplantation of algae. Many questions must still be answered, such as how the symbiotic algae are taken into the host and whether the algae emit any substances that prevent the host from eating them. The answers to these questions should help us to better understand a presently unknown, but very interesting, ecological system.

We have focused on the identification of natural key compounds that control biologically and physiologically intriguing phenomena (Kuramoto, Arimoto, and Uemura 2003, 2004; Kita, Sakai, and Uemura 2006; Kita et al. 2010). Various bioactive secondary metabolites, including long carbon-chain polyol compounds, have been found in symbiotic dinoflagellates. Recently, a polyol macrolide (symbiodinolide) (Kita et al. 2007a), amphoteric iminium alkaloids (symbioimines) (Kita et al. 2004, 2005), and polyol spirocyclic compounds (symbiospirools) (Tsunematsu et al. 2009) have been isolated from extracts of *Symbiodinium* sp. Several unique polyol compounds, such as durinskiols (Kita et al. 2007b, c; Siwu et al. 2008) from *Durinskia* sp.,



An Okinawan flatworm  
*Amphiscolops* sp.



A jellyfish *Mastigias papua*



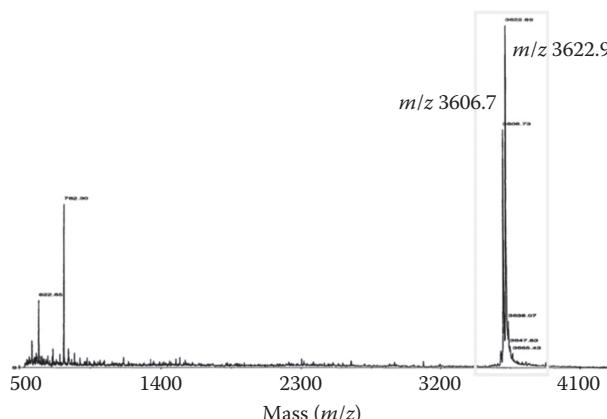
A sea slug *Chelidonura fulvipunctata*

**FIGURE 12.1** Host animals of symbiotic marine dinoflagellates.

symbiopolyol (Hanif et al. 2010) from an unidentified dinoflagellate species, and karatungiols (Washida et al. 2006) and amdigenol A (Inuzuka et al. 2012) from *Amphidinium* sp. have been isolated. Based on their structural, biological, and conformational diversity, these long carbon-chain polyol compounds may play various roles, such as in defense, in chemical communication with the host animals, or as nutrient sources. This review highlights the recent advances in structural and biological studies on these huge polyol compounds and related unique secondary metabolites.

## 12.2 ISOLATION OF BIOACTIVE SECONDARY METABOLITES FROM SYMBIOTIC DINOFLAGELLATES

As mentioned earlier, marine huge polyol and polyether compounds are remarkable molecules due to their extraordinary structures and significant biological activities. Palytoxin (2680 Da) and maitotoxin (3422 Da) (Murata et al. 1994) are currently believed to have the longest carbon chains in nature (more than 100 Å in length), except for biopolymers. Generally, marine huge molecules can be categorized as polyketide metabolites without a repeating unit, which makes it difficult to elucidate their structures. During our quest to identify SCC compounds from marine dinoflagellates, we found a group of huge molecules that were detected by matrix-assisted laser desorption/ionization with time-of-flight (MALDI-TOF) mass spectrometry. After several extensive attempts, the huge molecules were generally detected by a linear positive mode with a matrix  $\alpha$ -cyano-4-hydroxycinnamic acid, as the best conditions thus far for showing very unstable and low-intensity huge masses. For example, the dinoflagellate *Symbiodinium* sp. obtained from the marine acoel flatworm *Amphiscolops* sp. was found to produce huge polyol compounds (~3600 Da) (Figure 12.2). Furthermore, a marine dinoflagellate obtained from the zoanthid *Palythoa* sp. that possess palytoxin was shown to contain the largest molecule identified so far (8245 Da) (Uemura et al. 2012). Purification of this molecule as a minor compound was hampered by its instability during separation, and further advances in separation methods are needed to isolate this unusual molecule. Huge polyol and polyether compounds larger than 2000 Da are considered to be midsize molecules that fall between small (drug-like) natural products and biopolymers, and further studies on their three-dimensional structures and dynamics should contribute to the creation of new scientific fields (Kita and Uemura 2010).



**FIGURE 12.2** MALDI-TOF mass spectrum of 80% aqueous EtOH extracts of the dinoflagellate *Symbiodinium* sp.

### 12.3 SYMBIODINOLIDE, A NOVEL POLYOL MACROLIDE THAT ACTIVATES N-TYPE CA<sup>2+</sup> CHANNEL

Symbiodinolide was isolated from extracts of symbiotic dinoflagellates *Symbiodinium* sp., which were collected from the Okinawan flatworm *Amphiscolops* sp. A large-scale cultivation (~400 L) of the dinoflagellate using a seawater medium with Provasoli's Ert-Schreiber (ES) supplement was carried out. After 2 or 3 months of cultivation, dinoflagellates were collected and 9.3 mg of symbiodinolide was purified from 88 g of cultured dinoflagellates (Kita et al. 2007a).

#### 12.3.1 STRUCTURE OF SYMBIODINOLIDE

The molecular formula of symbiodinolide was elucidated to be C<sub>137</sub>H<sub>232</sub>NNaO<sub>57</sub>S, with an overall C<sub>129</sub> carbon-chain skeleton and 43 hydroxyl groups. It contained a 62-membered macrolactone, a bis-epoxide moiety, and 6,6-spiroacetal and hemiacetal rings. The entire planar structure of symbiodinolide was confirmed by detailed analyses of the degradation products obtained by alkaline hydrolysis. Furthermore, to obtain the degraded fragments in as simple a form as possible, we examined the ethenolysis (olefin cross-metathesis with ethylene) reaction using an olefin metathesis catalyst, but encountered a difficulty with regard to solubility: symbiodinolide did not dissolve in CH<sub>2</sub>Cl<sub>2</sub> or toluene, whereas the catalyst did not dissolve in MeOH. We overcame this problem by choosing MeOH–CH<sub>2</sub>Cl<sub>2</sub> or MeOH–pyridine mixed solvent systems. With the use of an excess amount of the second-generation Hoveyda–Grubbs catalyst, three fragments of symbiodinolide with a terminal olefin were obtained in a reproducible fashion: C14–C23, C24–33, and C34–41 (Kita et al. 2007a; Han and Uemura 2008). Meanwhile, the lactone ring in symbiodinolide was opened by methanolysis, and subsequent ethenolysis of a seco ester using the second-generation Grubbs catalyst gave the C1–C13 fragment and the C14–C25' fragment. Unexpectedly, the allylic position of the 13,14-diol underwent cleavage to give two α,β-unsaturated aldehydes in both cases. These results suggest that a *vic*-diol adjacent to a C = C bond plays a crucial role in the oxidative cleavage reaction.

(symbiodinolide)

The relative stereochemistry of symbiodinolide was determined by a detailed analysis of nuclear Overhauser effects (NOEs) and coupling constants. The stereochemistries between spiroacetal and hemiacetal moieties, a six-membered ether ring part, a bis-epoxide fragment, C5–C7 and C64–C66 triol moieties, and the C1'–C25' side-chain have been determined. The stereochemistry of the C44–C51 tetraol moiety was determined by Kishi's Universal NMR Database method. With the help of synthetic studies on degraded fragments and partial structures of symbiodinolide (Takamura et al. 2008, 2009a, b, c, 2010a, b; Han et al. 2009; Murata et al. 2009), the absolute configurations of C14–C40, C69–C73, C83–C103, and C3'–C18' in symbiodinolide have been confirmed. Importantly, symbiodinolide possesses carboxyl and amino groups on each side of linear carbon chains (C<sub>115</sub>/C<sub>104</sub>) and thus is considered to be a huge ω-amino acid that is biosynthesized by polyketide synthases from glycine-like precursors, as with palytoxin.

#### 12.3.2 BIOLOGICAL ACTIVITIES OF SYMBIODINOLIDE

Symbiodinolide produced a significant increase in the intracellular free Ca<sup>2+</sup> concentration at 7 nM against differentiated IMR-32 neuroblastoma cells in the presence of nifedipine (L-type Ca<sup>2+</sup> channel blocker) (Kita et al. 2007a). This result revealed that symbiodinolide possessed significant voltage-dependent N-type Ca<sup>2+</sup> channel-opening activity. Furthermore, symbiodinolide significantly facilitated a neurogenic twitch in a guinea pig ileum specimen (EC<sub>50</sub> 0.27 μM) and inhibited 30 nM ω-conotoxin GVIA-induced reduction of the neurogenic twitch (IC<sub>50</sub> 0.37 μM), which established that this molecule was a specific agonist at Ca<sup>2+</sup> channel N-type receptor (Hong, Roan, and Chang 1996). In contrast, it showed relatively weak acute toxicity against mice (LD<sub>90</sub> ~5 mg kg, i.p. injection). Of the various enzymatic profiling screening assays considered, symbiodinolide showed a significant cyclooxygenase-1 (COX-1)-inhibitory effect at 2 μM (65% inhibition) (Kita and Uemura 2007).

Furthermore, to consider the role of long carbon-chain polyol compounds in symbiotic relationships, these compounds were added to the host animals. Notably, symbiodinolide caused immediate rupture of the tissue surface of the host animal (acoel flatworm *Amphiscolops* sp.) at 2.5  $\mu\text{M}$  (Kita et al. 2007a; Uemura et al. 2009). It is largely unknown how many polyol compounds, such as symbiodinolide, are accumulated in a flatworm. Still, our preliminary results suggest that symbiodinolide may act as a defense substance that prevents predation of the host animal.

### 12.3.3 STRUCTURAL COMPARISON WITH RELATED COMPOUNDS

Symbiodinolide has been shown to be a structural congener of zooxanthellatoxins (ZTs) and zooxanthellamides (ZADs) and has a similar 62-membered monosulfated macrolactone moiety and bis-epoxide moiety (Figure 12.3). The molecular weight of symbiodinolide was 36 mass units (mu) smaller than that of ZT-A (Nakamura et al. 1995) and 6 mu larger than that of ZT-B (Nakamura

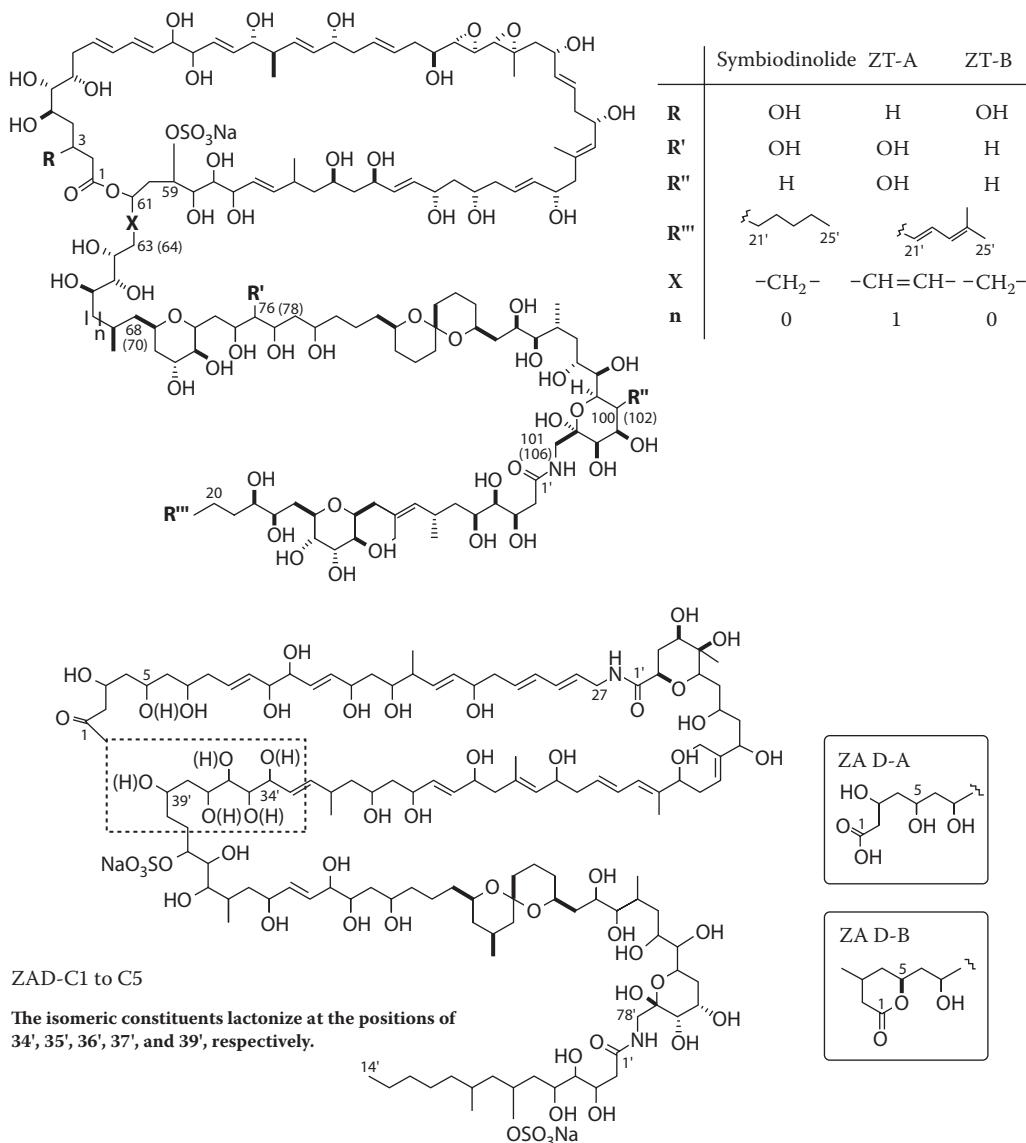


FIGURE 12.3 Comparison of the structures of symbiodinolide, ZTs, and ZADs.

et al. 1996). Other differences between symbiodinolide and ZTs included the presence or absence of three hydroxyl groups on C3, C76, and C100. In the case of ZT-A, the absolute stereochemistries of the six-membered ether part (C71–C75), the spiroacetal moiety (C81–C94), and the side chain part (C3'–C7' and C11'–C18') were determined by chemical synthesis of its degraded fragments (Nakamura, Fujimaki, and Murai 1996; Nakamura, Sato, and Murai 1996; Nakamura, Takahashi, and Murai 1998; Nakamura et al. 2000), which were identical to those of symbiodinolide. Recently, two seco acid congeners of ZTs, ZAD-A and ZAD-B, and 63- to 66-membered macrolides, ZAD-Cs, have also been isolated from the same dinoflagellate species (Onodera et al. 2003, 2004, 2005).

ZTs and ZADs have been reported to have vasoconstrictive activity in rat blood vessels. The EC<sub>50</sub> values for ZT-A and ZADs were 1.2, >30, >3, and 0.39 μM, respectively (Moriya et al. 2001; Onodera et al. 2003). Vasoconstrictive activity was only seen with the macrolactone congeners ZTs and ZAD-Cs, which indicated that the huge macrolactone structures were important for their activity. Furthermore, ZT-A caused aggregation in rabbit washed platelets, accompanied by an increase in the cytosolic Ca<sup>2+</sup> concentration (Rho et al. 1995). Thus, this potent voltage-dependent Ca<sup>2+</sup> channel-opening activity may be a common feature of symbiodinolide and ZTs and may be intimately involved in such constrictive activity. Further structural and biological studies of symbiodinolide are currently underway.

## 12.4 SYMBIOIMINE, A POTENTIAL ANTI-OSTEOCLAST DIFFERENTIATION DRUG

In our continuing search for biologically active compounds, unique amphoteric iminium compounds, symbioimine and neosymbioimine, were also isolated from this dinoflagellate *Symbiodinium* sp. (Kita et al. 2004, 2005).

Symbioimine is a novel tricyclic iminium alkaloid that occurs in nature as an inner salt of an imine and an aryl sulfuric acid. The IR spectrum of symbioimine showed absorption bands for hydroxy (3450 cm<sup>-1</sup>), iminium (1690 cm<sup>-1</sup>), and sulfate (1240, 1140, 1050 cm<sup>-1</sup>) groups. The characteristic <sup>13</sup>C NMR signal at 188.0 (C-5) implied the presence of an iminium functionality in this water-soluble amphoteric compound. Crystallization of symbioimine from water gave well-formed, monocyclic colorless crystals as a monohydrate. Its structure, which consists of a characteristic 6,6,6-tricyclic iminium ring, was deduced based on the results of spectroscopic and X-ray crystallographic analysis. Finally, the absolute stereochemistry of symbioimine was confirmed to be 2*R*, 3*R*, 4*S*, 9*R*, 12*S*, based on the Flack parameter. Neosymbioimine is a congener of symbioimine, which possesses a 6,6,6-tricyclic iminium ring, an aryl sulfate moiety, and three methyl groups. Recently, the total synthesis of symbioimine and neosymbioimine was achieved and their structures were established (Varseev and Maier 2006, 2007; Zou, Che, and Snider 2006; Kim and Thomson 2007).

Symbioimine inhibited osteoclastogenesis of the murine monocytic cell line RAW264, which can differentiate into osteoclasts following treatment with receptor activator of nuclear factor-κB ligand (RANKL) (EC<sub>50</sub> 44 μg/mL) (Kita et al. 2004). RANKL induces the formation of osteoclast-like multinucleated cells in cultures of bone marrow cells. Meanwhile, it did not affect cell viability even at 100 μg/mL. Thus, symbioimine is a potential antiresorptive drug for the prevention and treatment of osteoporosis in postmenopausal women.

Symbioimine also significantly inhibited cyclooxygenase 2 (COX-2) activity at 10 μM, while it had only a weak inhibitory effect toward COX-1. The overexpression of COX-2 has been observed in many kinds of tumors, and its role in carcinogenesis and angiogenesis has been extensively investigated (Warner et al. 1999; Reddy et al. 2000). Several COX-2-selective inhibitors, such as rofecoxib, celecoxib, and sulindac, have been developed. Because of its moderate subtype specificity, symbioimine may be useful for the development of new nonsteroid anti-inflammatory drugs to treat COX-associated diseases, such as inflammatory diseases and cancer (Kita and Uemura 2006).

## 12.5 SYMBIOSPIROLS, POLYOL COMPOUNDS POSSESSING TWO 5,5-BISPIROACETAL UNITS

Symbiospirols A, B, and C, long carbon-chain compounds with a molecular formula of  $C_{70}H_{126}O_{15}$ , were isolated from the cultured symbiotic dinoflagellate *Symbiodinium* sp., which is the same strain that produced symbiodinolide and symbioimines (Tsunematsu et al. 2009). From the cultured dinoflagellate (129 g wet wt), 117 mg of symbiospirol A was isolated, along with two minor stereoisomers, symbiospirols B and C (9.4 and 3.4 mg, respectively). On the basis of spectroscopic analyses and degradation reactions, their planar structures and partial relative stereochemistries were elucidated. Symbiospirols consisted of a  $C_{67}$ -linear chain with two 1,6-dioxaspiro[4,4]nonane rings, a  $\beta,\beta'$ -dihydroxyl ketone moiety, eight hydroxyl groups, and a tetrahydropyran ring. In addition, symbiospirols B and C were determined to be 27- and 41-*epi*-symbiospirol A, respectively.

Symbiospirol A had an inhibitory effect against L-phosphatidylserine-induced protein kinase C (PKC) activation ( $IC_{50} = 19.7 \mu M$ ). Symbiospirol A may bind to the phospholipid binding site of classical PKC as an antagonist-like compound and thus may be useful as a reagent to suppress inflammation-related diseases.

## 12.6 DURINSKIOLS, LONG CARBON-CHAIN POLYOL COMPOUNDS FROM DURINSKIA SP.

Durinskiol A was isolated from the symbiotic dinoflagellate *Durinskia* sp., which was collected from the Okinawan nudibranch *Chelidonura fulvipunctata*. From 400 L of cultured *Durinskia* sp. (191 g wet wt), 20 mg of durinskiol A was isolated (Kita et al. 2007b). The molecular formula was deduced to be  $C_{110}H_{198}O_{38}$  based on a high resolution electrospray ionization mass spectrometry (HR-ESIMS) analysis. An extensive 2D-NMR analysis in  $CD_3OD$  allowed us to establish a  $C_{93}$  carbon-chain poly-oxygenated skeleton, which included a 6,5,6-bis-spiroacetal ring, five six- or seven-membered rings, and two sugar moieties.

### 12.6.1 STRUCTURE DETERMINATION OF DURINSKIOLS

MS/MS analysis is a useful tool for elucidating the internal structures of natural products, even on a nanomolar scale or less. Unfortunately, however, MS/MS analysis of durinskiol A itself was not successful, since charge-remote fragmentation was not observed effectively. To confirm the entire planar structure of durinskiol A, we developed another fluorescent-label method to enhance the sensitivities of degraded fragments. We found that ozonolysis of the terminal alkene followed by direct reductive amination using sodium cyanoborohydride can introduce 7-methyl-4-aminocoumarin (AMC) even with complex long carbon-chain polyol compounds (Kita et al. 2007b). In fact, durinskiol A was converted to the bis-AMC derivative (C2–C92 fragment) quantitatively. Furthermore, cleavage of the 1,2-diol moiety by sodium periodate followed by reduction gave the C2–C14 and C15–C92 fragments, and thus it was confirmed that a 1,2-diol moiety in the long carbon-chain in durinskiol A was located at C14. Furthermore, based on the results of tandem FAB MS/MS analyses of the two AMC derivatives, an ionic charge located at both terminals of the amino group facilitated typical charge-remote fragmentations derived from the ether rings and polyol moieties. Thus, this AMC derivatization method was shown to be quite useful for capturing and analyzing the degraded fragments even on a minute scale.

The relative stereochemistry of durinskiol A was determined by spectroscopic analysis, including NOE correlated spectroscopy (NOESY), rotating Overhauser enhancement and exchange spectroscopy (ROESY), nuclear Overhauser enhanced differential spectroscopy (NOEDF), and homonuclear *J*-resolved NMR spectra (Kita et al. 2007c). A molecular modeling study for the bis-spiroacetal ABC-ring model compound showed that the calculated distances of all protons for

which NOEs were observed in rings ABC in durinskiol A were less than 2.7 Å. Similarly, the seven-membered D-ring was confirmed to be a trans-fused ether based on the molecular modeling study. All of the observed NOEs were applicable, and the *J* values of vicinal protons (H34 to H38) estimated from their dihedral angles in the calculated seven-membered ether model compound mostly coincided with those of natural durinskiol A.

Recently, a new durinskiol congener, durinskiol B, was isolated from *Durinsksia* sp., and its molecular formula was identical to that of durinskiol A (Siwu et al. 2008). The planar structure of durinskiol B, including its partial relative stereochemistry, was almost identical to that of durinskiol A, except for the presence of one methyl excess and a shorter length of the carbon chain. Regarding the biosynthesis of durinskiols, it is assumed that they are derived from a common linear unsaturated fatty acid precursor. Thus, the formation of a cyclopropane ring on the double bond would arise from a carbocation intermediate provided by S-adenosyl methionine, followed by C–C bond cleavage to construct a methylene moiety in durinskiol A. Meanwhile, a methylation mechanism from a carbocation intermediate can be postulated by accepting hydride from a reducing agent, such as NADPH, to give durinskiol B.

### 12.6.2 BIOLOGICAL ACTIVITIES OF DURINSKIOLS

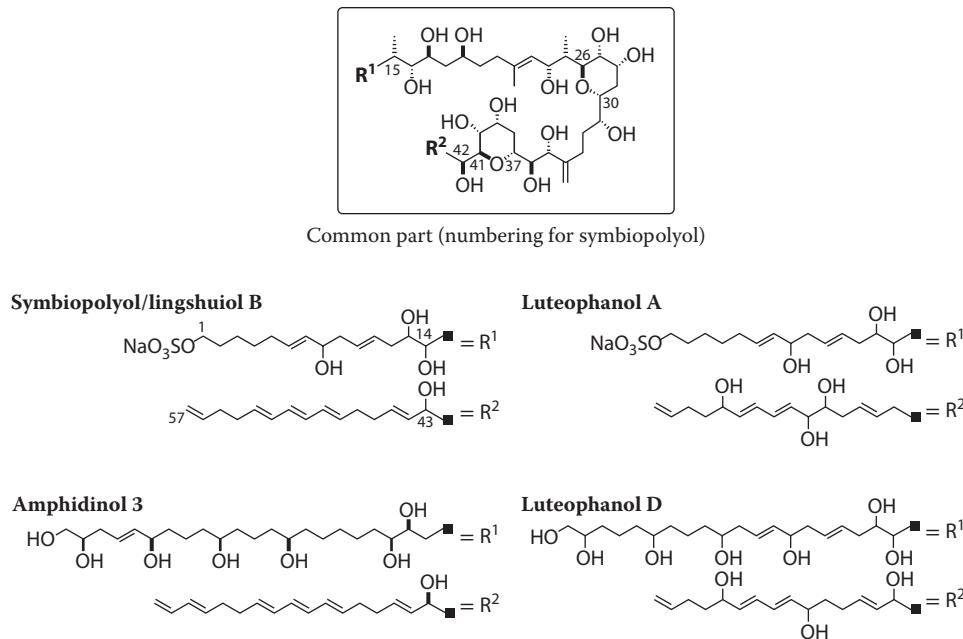
Durinskiol A has been shown to cause a short body length, abnormal pigment pattern and pericardiac and yolk-sac edema in zebrafish at 188 µM (Kita et al. 2007b). Meanwhile, this compound did not show significant vasoconstrictive activity against rat aortic rings even at 100 µM. Unlike palytoxin or ZTs, which are known to be vasoconstrictive polyol compounds, durinskiol A lacks a terminal amino group, which may explain its lower activities.

## 12.7 KARATUNGIOLS AND SYMBIOPOLYOL

Karatungiols A and B, two novel antimicrobial polyol compounds, were isolated from the cultivated symbiotic marine dinoflagellate *Amphidinium* sp., which was obtained from an unidentified marine acoel flatworm collected at Karatung Island, Indonesia (Washida et al. 2006). Karatungiols consist of a C<sub>69</sub>-linear chain with a ketone moiety, 24 or 25 hydroxyl groups, and two tetrahydropyran rings. The relative stereochemistries of the two tetrahydropyrans of karatungiols A have been established. Karatungiol A exhibited antifungal activity against NBRC4407 *Aspergillus niger* at 12 µg/disc and antiprotozoan activity against *Trichomonas foetus* at 1 µg/mL. Karatungiol B was a dehydrated analog of karatungiol A and possessed an α,β-unsaturated ketone moiety.

Recently, during functional screening to identify inhibitors of vascular cell adhesion molecule-1 (VCAM-1) from our symbiotic marine dinoflagellate library (Kuramoto et al. 1996; Arimoto et al. 1998), we encountered an active water-soluble fraction from a symbiotic marine dinoflagellate (unidentified) obtained from the jellyfish *M. papua* (see above). From this cultured dinoflagellate, symbiopolyol was isolated (Hanif et al. 2010), and its planar structure was identical to that of lingshuiol B (Huang et al. 2004b), which was previously isolated from *Amphidinium* sp. Although the stereochemical assignments of both compounds are not yet complete, it was suggested that symbiopolyol is the enantiomer of lingshuiol B, because of the opposite signs of their optical rotations.

Symbiopolyol significantly inhibited the expression of VCAM-1 in human umbilical vein endothelial cells (HUVECs) and in TNF-α/IL-4-induced cell adhesion between HUVEC and Ramos cells at 10 µg/mL. Since Ramos cells highly express VLA-4, which is the ligand of VCAM-1, the adhesion between Ramos cells and HUVEC is largely mediated by VCAM-1 (Vonderheide et al. 1994). On the other hand, symbiopolyol did not have any cytotoxic effect against HUVEC up to 15 µg/mL. Thus, symbiopolyol may be a potential anti-inflammatory agent. To the best of our knowledge, this is the first report of a high-molecular-weight polyol compound (MW > 1000) with strong reducing activity against the expression of VCAM-1 in HUVEC.



**FIGURE 12.4** Comparison of the structures of symbiopolyol and related polyol compounds.

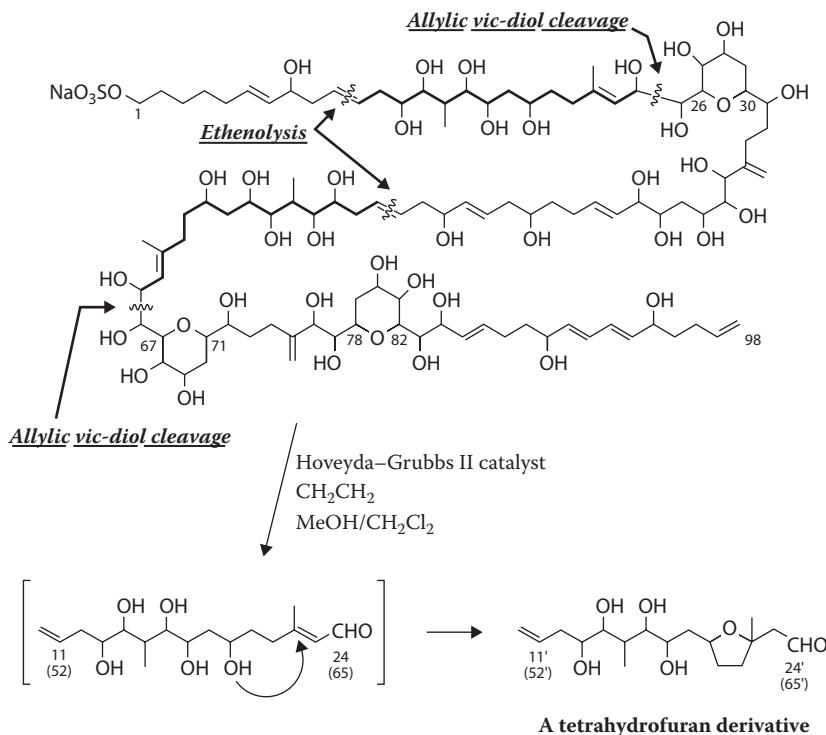
Various poly-hydroxy-polyene antifungal and hemolytic compounds have been isolated from members of the dinoflagellate *Amphidinium* sp. and *Karlodinium* sp., including amphidinols (Paul et al. 1995, 1997; Murata et al. 1999; Morsy et al. 2005, 2006; Oishi et al. 2008), luteophanols (Doi et al. 1997; Kubota et al. 1998, 1999, 2005), colopsinols (Kobayashi et al. 1999; Kubota et al. 1999), lingshuols (Huang et al. 2004a, b), amphezonol (Kubota et al. 2006), karlotoxins (Van Wagoner et al. 2008; Peng et al. 2010), and carteraols (Huanga et al. 2009). Among them, the absolute stereochemistries of amphidinol 3 and karlotoxin 2 have been established. Karatungiols possess a saturated chain without conjugated trienes, whereas symbiopolyol/lingshuol B, amphidinol 3, and carteraols have a conjugated triene and a terminal carbon–carbon double bond at one end of the molecule (Figure 12.4) (Uemura et al. 2012). Further studies on the structure–activity relationship of these polyol compounds and their target molecules in living organisms are in progress.

## 12.8 AMDIGENOL A, LONG CARBON-CHAIN POLYOL COMPOUNDS FROM AMPHIDINIUM SP.

Many species of microalgae adhere to the surface of the marine red alga *Digenea simplex*. This alga is well-known to produce kainic acid, which may serve as the first line of defense in *D. simplex* to prevent possible predation by herbivorous animals (Sakai et al. 2005). Recently, we isolated and cultured some species of dinoflagellates from Okinawan *D. simplex*, and one of them, *Amphidinium* sp., was found to produce some huge compounds of more than 2000 mu, by MALDI or ESI-MS analyses in seawater medium after culture of the dinoflagellate. Accordingly, we separated the medium and isolated a novel polyol compound with a molecular weight of 2169 mu, amdigenol A (Inuzuka et al. 2012; Uemura et al. 2012).

### 12.8.1 STRUCTURE DETERMINATION OF AMDIGENOL A

The dinoflagellate *Amphidinium* sp. was cultured in seawater medium. After 2 months of culture, the dinoflagellate was removed from the seawater medium by filtration. The seawater medium (120 L)



**FIGURE 12.5** Degradation of amdigenol A by olefin metathesis catalyst.

was separated by column chromatography with TSK G-3000S polystyrene gel, DEAE-Sephadex, and Sephadex LH-20. Finally, purification by continuous reversed-phase HPLC produced amdigenol A (5.0 mg). Interestingly, the same procedure produced only 0.4 mg of amdigenol A from the ethanol extract of the dinoflagellate.

The molecular formula of amdigenol A was found to be C<sub>104</sub>H<sub>177</sub>NaO<sub>43</sub>S by positive and negative ESI-MS analysis. The [M – SO<sub>3</sub>Na + Na]<sup>+</sup> ion peak (*m/z* 2089) observed in MALDI-TOF-MS suggested the presence of one sulfate group. However, <sup>13</sup>C-NMR spectra contained only 78 carbon signals, and some signals had a relatively high intensity. Therefore, we estimated that some of the same partial structures were present in this compound. The carbon–carbon connectivities of amdigenol A were determined by an extensive 2D-NMR analysis and by degradation reactions. Treatment of amdigenol A with the second-generation Hoveyda–Grubbs catalyst gave many segments, including a tetrahydrofuran derivative (Figure 12.5). From the carbon skeleton and the oxidized positions, the C11’–C24’ or C52’–C65’ part of the segment was estimated to correspond to the C11–C24 or C52–C65 part of amdigenol A. Thus, the C24–C25 and/or C65–C66 bonds in amdigenol A were cleaved by ethenolysis to give a conjugated aldehyde intermediate, and cyclization of the hydroxyl group at C19 or C60 to the β-position of the aldehyde then gave the tetrahydrofuran derivative. On the basis of these results, the C98–linear carbon skeleton of amdigenol A was established. The position of the sulfate group was estimated to be C1 by a tandem mass spectrometric (MS/MS) analysis. Finally, the remaining oxymethine carbons were suggested to bear hydroxyl groups, and thus the planar structure of amdigenol A was determined.

### 12.8.2 STRUCTURAL COMPARISON WITH RELATED COMPOUNDS

Amdigenol A has partial structures similar to those seen in amphidinols. Amphidinol analogs consist of a core part that includes a trisubstituted olefin, an exo-olefin and bis-tetrahydropyrans,

and two linear side chains. In amdigeneol A, the C63–C82 part corresponds to a core part and the C22–C41 would also correspond to the situation if the C37 hydroxyl group attacks the C41 carbon to produce a ring ether. Therefore, amdigeneol A is likely formed linearly by two amphidinol analogs.

On the basis of the results of a detailed 2D NMR analysis, the relative stereochemistries of the tetrahydropyran parts in amdigeneol A were found to coincide with those in the amphidinol analogs. The sulfate ester terminal side chain of amdigeneol A, C1–C14, is the same as that of lingshuol B (Huang et al. 2004b), luteophanol A (Kubota et al. 1998), and symbiopolyol (Hanif et al. 2010), whereas the olefin terminal side chain, C84–C98, is the same as that of luteophanol D (Kubota et al. 2005). For these reasons, amdigeneol A is likely formed linearly by two amphidinol analogs, although the two amphidinol analogs are not simply dimerized. The core structure of the amphidinol analogs is important for the formation of their overall shape and thus for their biological activity (Houdai et al. 2005; Houdai, Matsumori, and Murata 2008). Therefore, a double-core structure of amdigeneol A would give much information about the biosynthetic pathway and the mechanisms of the biological and physiological activities of the long carbon-chain compounds, including the relevance to those of their amphidinol analogs.

### 12.8.3 BIOLOGICAL ACTIVITIES OF AMDIGENOL A

Amdigeneol A showed weak cytotoxicity against 3T3-L1 murine adipocytes ( $IC_{50} = 59 \mu\text{g/mL}$ ), while its antimicrobial activity was not examined. Further studies on the stereochemical analyses, the biosynthetic pathway, and the mechanism of their biological activities, including the relevance to those of their amphidinol analogs, are underway.

## 12.9 CONCLUSION

This review highlighted the recent advances in structural and biological studies on long carbon-chain polyol compounds. The discovery of new bioactive molecules, facilitated by a deeper understanding of nature, will advance our knowledge of biological processes and lead to new strategies to treat disease. Recent technological advancements including spectroscopic analyses and genetic approaches have provided outstanding opportunities for new discoveries, even in the case of scarce, unstable, and composite compounds. Furthermore, cleavage reactions of allylic 1,2-diol using an olefin metathesis catalyst and fluorescent-labeling methods for MS/MS analysis have been developed to achieve the structural elucidation of huge polyol compounds larger than 2000 mu.

There are many unanswered questions regarding marine huge polyol and polyether compounds, such as whether this type of compound has any limitations with regard to the molecular weight or length of the carbon chain and the need for and/or physiological roles of these unique metabolites in marine ecosystems. Further studies on the conformation, mode of action, and interaction of these unique marine secondary metabolites with biomacromolecules are essential and may lead to the creation of a new field in bioscience.

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# 13 Pharmacological Potential of Phlorotannins from Marine Brown Algae

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### 13.1 INTRODUCTION

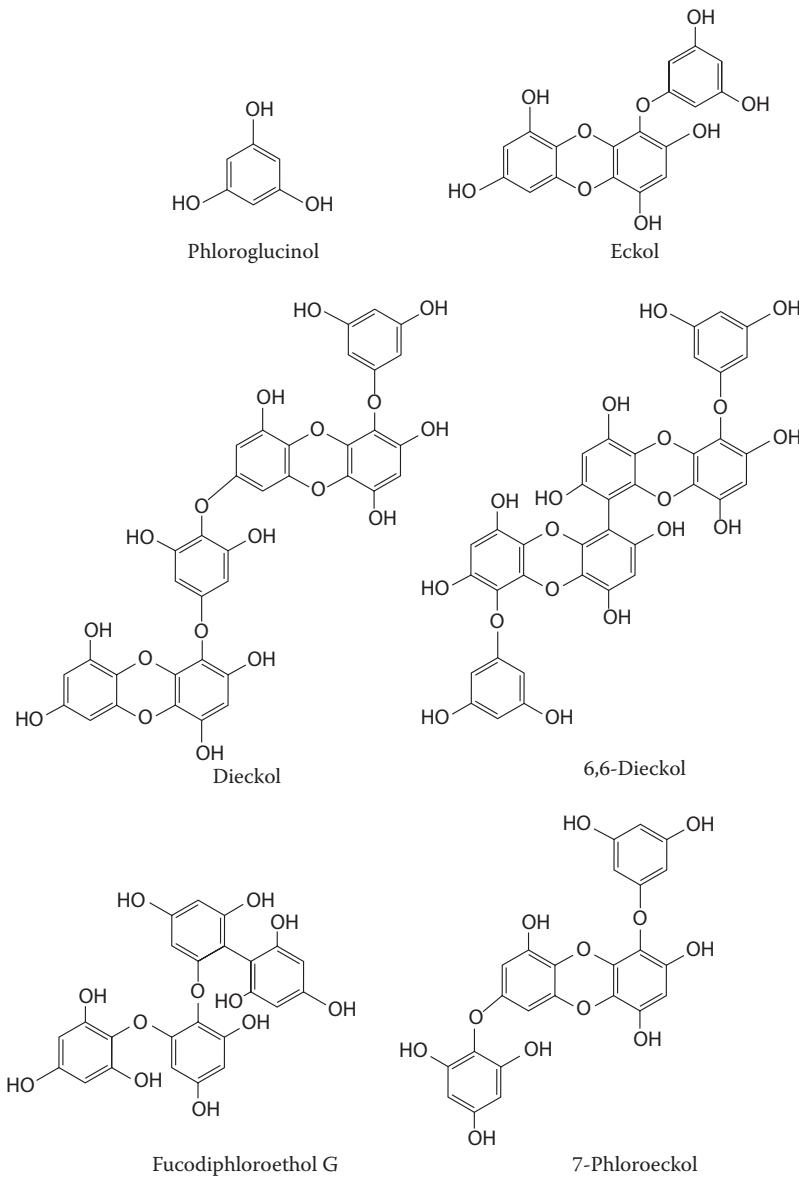
Two-thirds of the world's biomass are found in the ocean with marine species comprising around half of the total global biodiversity. Among marine organisms, marine algae have been reported to possess many health benefits as pharmacological food, specifically reducing the risk of some diseases. Presently, marine algae are under the limelight as a food ingredient, and their nutritional value is found to be far better than a food because of their low content of lipids, high concentration of polysaccharides, rich minerals, polyunsaturated fatty acids and vitamins, and the presence of a vast array of bioactive metabolites (Gupta and Abu-Ghannam 2011).

Marine algae are classified into brown, green, and red algae based on their photosynthetic pigments. Compared to other classes, brown algae, which comprise the class Phaeophyceae, are unique in being phylogenetically very far removed from all other eukaryotic macrophytes (Van den Hoeck, Mann, and Jahns 1995). So, intensive efforts are being made by marine scientists to identify and characterize bioactive compounds from brown algae to exploit them as medicinal ingredients. Despite having found a large number of compounds from marine brown algae with medicinal properties, few of those compounds have shown real potency to be used as a nutraceutical or pharmaceutical. Among them, phlorotannins are the most significant group of biologically active substances that determine pharmacological value of brown algae. The amount of phlorotannins contained in brown algae is found to be higher than red and green algae (Holdt and Kraan 2011). These brown algal phlorotannins have been extensively characterized for their potential biological activities. Hence, this chapter focuses on the medicinal potential of phlorotannins isolated from marine brown algae.

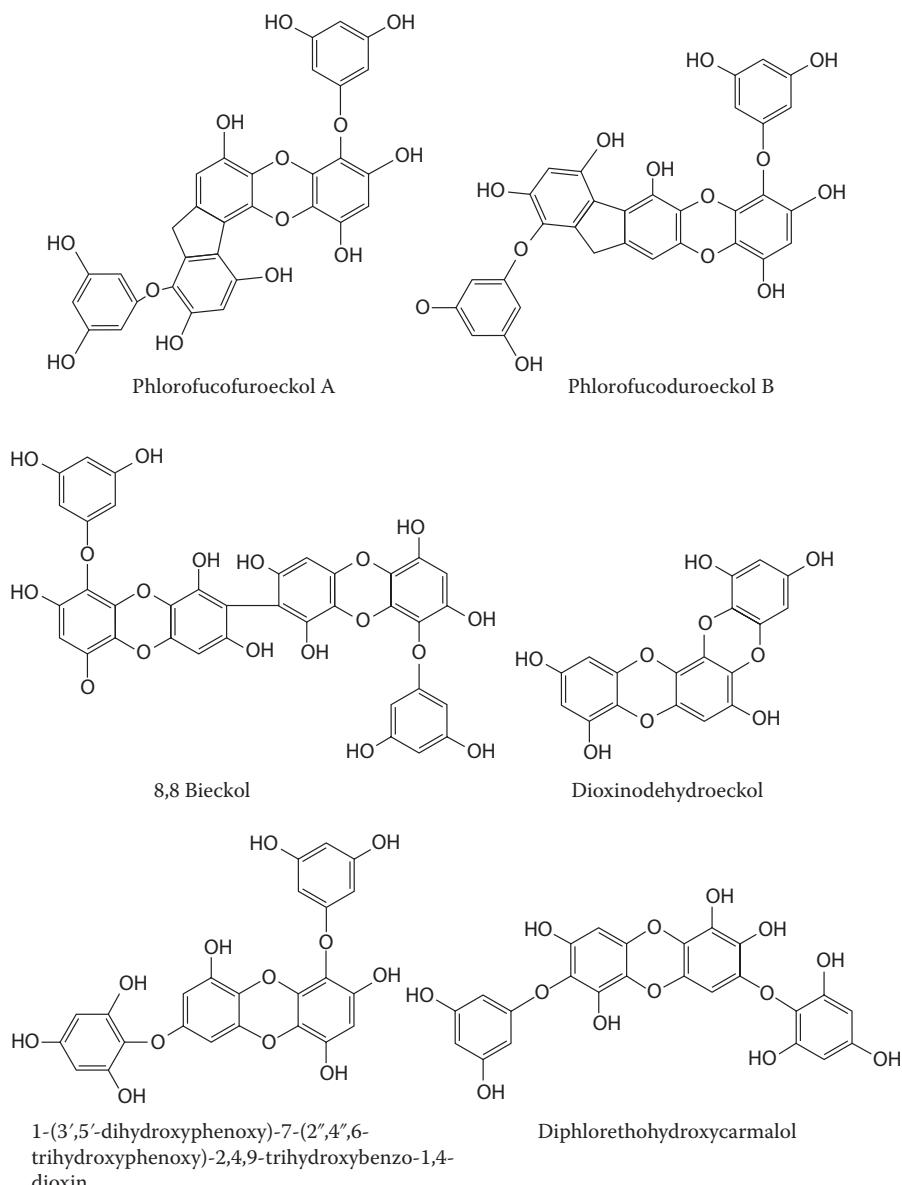
### 13.2 PHLOROTANNINS

In marine brown algae, phlorotannins, the only group of tannins, which is a large and diverse group of phenolic secondary metabolites, are biosynthesized via the acetate malonate pathway (Arnold and Targett 2002). Polyphenols from terrestrial plants are derived from gallic and ellagic acids, whereas the algal polyphenols are derived from polymerized phloroglucinol units (Figure 13.1). The monomeric units are linked through aryl–aryl bonds and diaryl ether bonds are forming different subgroups of phlorotannins (Glombitza and Pauli 2003). Their molecular size ranges between 162 Da and 650 kDa (Breton, Cérantola, and Gall 2011).

The phlorotannins are localized in physodes of the algae, which are membrane-bound cytoplasmic vesicles, and the fusion of physodes with cell membranes results in a secretion of



**FIGURE 13.1** Structures of phlorotannins isolated from marine brown algae.

**FIGURE 13.1** (Continued)

phlorotannins, complexing finally with alginic acid (Li et al. 2009). One possibility is that phlorotannins are bound with cell wall and hemiacetal bonds, both of them are covalent bonds, and thus increase the strength (Appel 1993). This binding ability of phlorotannins has been suggested to promote prooxidant activity as polyphenols are prone to oxidation in causing oxidative stress (Appel 1993; Barbehenn et al. 2005). Therefore, pharmacological values of the brown algae are also related to the presence of phlorotannins. Pharmacological values of phlorotannins are related to their structure and especially to the degree of polymerization, where oligophenols generally are considered to be more active than highly polymerized compounds (Toth and Pavia 2001).

### 13.3 PHARMACOLOGICAL EFFECTS OF PHLOTANNINS

Brown algal phlorotannins have been extensively studied for their potential health benefits and they have shown promising effects against radical mediated oxidative stress, photodamage, cancer, allergy, diabetes, inflammation, and viral and microbial infections. They are generally investigated to identify the valuable phlorotannins, which are different between species and among algae genotypes and populations due to environmental factors. With a vast range of biological activities, phlorotannins are believed to be the most promising candidates to be developed as nutraceuticals and pharmaceuticals. This section covers the major biological activities of phlorotannins isolated from brown algae.

#### 13.3.1 ANTIOXIDANT EFFECTS

Antioxidants are intimately involved in the prevention of cellular damage, which is the common pathway for cancer, aging, and a variety of diseases. Therefore, a wide range of capacity metabolites have been assessed against oxygen-induced stress and thus decreases the risk of human chronic diseases.

Many researchers have shown that marine brown algae serve as an important bioresource of antioxidative phlorotannins with significant pharmaceutical potential. Phlorotannins are electron-rich compounds, which are prone to efficient electron-donation reactions and produce phenoxy radical species as intermediates in the presence of oxidizing agents thus can expect the antioxidant activity. The phlorotannins from brown algae have been shown to overcome the sensitivity problem inherent in the detection of endogenous radicals in biological systems (Yan et al. 1996). Therefore, they have received great attention and have been investigated extensively since they are highly free radical scavengers in nature and less toxic than synthetic antioxidants (Jung, Heo, and Wang 2008).

##### 13.3.1.1 Free Radical Scavenging Ability

Oxidative stress is the result of an imbalance between prooxidant and antioxidant homeostasis that leads to lipid peroxidation, and DNA and protein damage. It has been demonstrated that oxidative stress is involved in inflammation and apoptosis, the two main causes of cellular death. Therefore, antioxidative therapeutics are in great demand to act against free radicals. In this aspect, phlorotannins derived from marine brown algae are promising as one of the most valuable natural antioxidants. A number of studies have shown the antioxidant potential of phlorotannins on scavenging of radicals formed during peroxidation, scavenging of oxygen-containing compounds, and metal-chelating ability. According to research, phlorotannins have shown significant scavenging ability toward hydroxyl, superoxide, alkyl, and 1,1-diphenyl-2-picryl hydrazyl (DPPH) radicals *in vitro*, and more potent antioxidant activities than commercially available antioxidants such as  $\alpha$ -tocopherol, butylated hydroxyl-anisole (BHA), and butylated hydroxytoluene (BHT). Furthermore, Zou et al. (2008) and Li et al. (2009) showed that phlorotannins are isolated from *Ishige okamurae* and *Ecklonia cava*; diphlorethohydroxycarmalol/6,6-bieckol and fucodiphloroethol G/dieckol are potent reactive oxygen species (ROS) scavengers in  $H_2O_2$ -induced microglial cells. Most of the phlorotannins that are purified from brown algae are responsible for marine algal antioxidant activities and protective effects against free radical-induced cell damage.

##### 13.3.1.2 Radioprotective Ability

Ultraviolet (UV) radiation of the sun is strongly oxidative and directly linked with photodamage of the skin cells. UVB (280–320 nm) radiation especially induces the overproduction of ROS, which interacts with cellular DNA, proteins, and lipids to alter their cellular functions (Heo et al. 2010). Besides the occurrence of well-known radioprotective brown algae mentioned previously, some other radiation absorbing/screening phlorotannins have also been studied. Following this research, phlorotannins have been reported as a reducer of ROS generated by UVB (Yan et al. 1997;

Hupel et al. 2011). Regular intake of antioxidants would be a useful strategy to resist photodamage. Therefore, phlorotannins have been isolated from brown algae and researched for their application against the radiation-induced cellular oxidative damage and membrane lipid peroxidation of the skin. Heo et al. (2009) and Ko et al. (2011) have found that dieckol (100 µM) isolated from *E. cava* increases cell survival up to 77.1% and 88.42% in 50 mJ cm<sup>-2</sup> of UVB-irradiated human dermal fibroblasts and epithelial keratinocytes, respectively. Dieckol exhibits a high antioxidant activity scavenging superoxide anions and inhibits lipid peroxidation resulting from UV-induced production of ROS. On JeJu Island (Korea), the leaves of *E. cava* have been traditionally used to heal sunburn (Hwang 2010). Furthermore, diphloroethoxyhydroxycarmalol isolated from *I. okamurae* has also been studied for its photoprotective ability that has shown 45.57% ROS scavenging ability and 49.33% inhibition of DNA damage at 250 µM concentrations in UVB-irradiated human dermal fibroblasts (Heo et al. 2010). Phlorotannins derived from marine brown algae have been recognized to counteract several types oxidative-physiological damage caused by UV irradiation. The protective effect of phlorotannins is mainly due to their antioxidant and damage inhibition activities. Taken together, it may be assumed that phlorotannins have the potential to be used as radioprotective agents in pharmaceutical areas.

### 13.3.2 ANTI-INFLAMMATORY EFFECTS

Inflammation has been found to be a pathophysiological condition underlying various diseases such as arthritis, cancer, diabetes, and neurodegenerative and cardiovascular diseases. Many researchers have demonstrated the anti-inflammatory potential of phlorotannins derived from marine brown algae. *E. cava* also has been reported to possess anti-inflammatory effect and their derived tannin compounds, dieckol and 1-(3',5'-dihydroxyphenoxy)-7-(2'',4'',6-trihydroxyphenoxy)-2,4,9-trihydroxybenzo-1,4-dioxin, were able to suppress arthritis by inhibiting the expression of proinflammatory enzymes, such as inducible NO synthase (iNOS) and cyclooxygenase-2 (COX-2), which accounted for the large production of nitric oxide (NO) and prostaglandin E<sub>2</sub> (PGE<sub>2</sub>), respectively (Ryu et al. 2009). Furthermore, phloroglucinol has also decreased inflammation by inhibiting inflammatory mediators; tumor necrosis factor α (TNF-α), interleukin 1β (IL-1β), interleukin 6 (IL-6), NO, and PGE<sub>2</sub> in lipopolysaccharide (LPS) stimulated microglial cells (Jung et al. 2009; Kim and Kim 2010). Interestingly, in all these results, the signaling pathway of the phlorotannin-mediated anti-inflammatory effect was found to be mediated through phosphorylation of nuclear factor-κB (NF-κB), the transcription factor that regulates gene expression.

As a result, phlorotannins can be considered potential candidates for the treatment of inflammatory diseases by the inhibition of the production of proinflammatory mediators. Phlorotannins have a great potential as anti-inflammatory agents in the pharmaceuticals area; however, further clinical trials are needed.

### 13.3.3 ANTICANCER EFFECTS

#### 13.3.3.1 Antiproliferative Activity

Cancer is a serious disease with a complex pathogenesis, which threatens human life greatly. Hence, many recent studies have been carried out to find cancer chemopreventive and/or chemotherapeutic agents from edible and natural resources. Marine brown algae have been known to have various useful compounds such as flavonoids and other phenolic compounds. Phlorotannins isolated from edible marine alga *E. cava*, dioxinodehydroeckol and 1-(3',5'-dihydroxyphenoxy)-7-(2'',4'',6-trihydroxyphenoxy)-2,4,9-trihydroxybenzo-1,4-dioxin, have shown antiproliferative effects on human breast cancer cells (Kong et al. 2009). Among them, dioxinodehydroeckol has shown stronger ability in inducing apoptosis, accounting for 55% cell death at 100 µM treatment. Moreover, phlorotannin extracts from *Laminaria japonica* show antiproliferative effects on human hepatocellular carcinoma cells ( $IC_{50}$ -200 µg/mL) and murine leukemic cells ( $IC_{50}$ -120 µg/mL) (Yang et al. 2010).

### 13.3.3.2 Inhibition of Cancer Metastasis

The invasion and metastasis of cancer depend on the angiogenesis of tumorous stroma and the degradation of extracellular matrix (ECM). Therefore, tumor angiogenesis is the principal mechanism involved in cancer mortality, leading to the spread of cancer from its originated place to another site. Proteolytic enzymes such as matrix metalloproteinases (MMPs) mediate the degradation of environmental barriers, such as the ECM, metastasis, and angiogenesis of cancer. Therefore, inhibition of the proteolytic activity by phlorotannins may be a therapeutic target to prevent cancer metastasis. Zhang et al. (2010) reported that 6'6'bieckol (100  $\mu$ M) derived from *E. cava* decreased the expression of MMP 2, 9 in PMA-activated human fibrosarcoma cells. Moreover, 6'6'-bieckol inhibited the phosphorylation of NF- $\kappa$ B signaling, which regulates the expression of MMP 2, 9. Another study carried out by Lee, Kang et al. (2011) demonstrated that polyphenolic extracts of brown algae have a potential inhibitory effect on metastasis in human cancer cell at the signaling levels such as the Akt signaling pathway. The inhibitory effect is even greater than that of doxycycline (10  $\mu$ g/mL), a commercially available MMP inhibitor. Angiogenesis is the process by which new blood vessels are made to facilitate the invasion of cancers and fucodiphloroethol G from *E. cava* has inhibited this process in an angiogenesis-induced cellular model (Li et al. 2011). These findings show that phlorotannins derived from edible brown algae could be used as a chemotherapeutic agent for cancer treatment.

### 13.3.4 ANTIALLERGIC EFFECTS

Allergic diseases are hypersensitivity disorders of the immune system and affect approximately one-third of the general population in the world. Allergic reactions occur due to environmental and diet changes. Besides, the prevalence and incidence of allergies are increasing. Recently, some studies have reported that brown algae extract and/or a single phlorotannin such as dieckol could block the release of histamine from anti-DNP IgE-sensitized rat basophile leukemia cells, RBL-2H3 cells (Shim et al. 2009). Hyaluronidase enzymes are also known to play an important role in allergic reaction. Samee et al. (2009) found that phlorotannin of *Sargassum tenerimum* is a strong inhibitor of hyaluronidase ( $IC_{50}$ -21  $\mu$ g/mL). The  $IC_{50}$  value of *S. tenerimum* was found to be similar to that of a natural inhibitor of hyaluronidase catachin ( $IC_{50}$ -20  $\mu$ g/mL) and lower than or almost similar to that of the commercially antiallergic drug disodium, cromoglycate ( $IC_{50}$ -39  $\mu$ g/mL). 6'6'-Bieckol (Le et al. 2009), fucodiphloroethol G, and phlorofucofuroeckol A isolated from *E. cava* also showed significant antiallergic activity by inhibiting histamine release by modulating the binding between IgE and Fc $\epsilon$ RI receptor, which is a high-affinity receptor for IgE, on the cell surface and mediates as effector cells in allergic reactions in human basophilic leukemia (KU812) and RBL-2H3 (Li et al. 2008). In addition, phlorofucofuroeckol-B ( $IC_{50}$ -7.8  $\mu$ M) from brown alga, *Eisenia arborea*, has antiallergic activity by reducing the  $\beta$ -hexosaminidase enzyme (equivalent to histamine) more than the antiallergic drug tranilast ( $IC_{50}$ -46.6  $\mu$ M) and epicatachin gallate ( $IC_{50}$ -22  $\mu$ M) (Sugiura et al. 2007). According to these results, phlorotannins from brown algae may serve as potential functional agents in antiallergic therapy.

### 13.3.5 ANTIDIABETIC EFFECTS

Diabetes mellitus is a complex disorder characterized by hyperglycemia, such as blood vessels and nerves. Effective control of blood glucose level is the key to prevent or reverse diabetic complications and improve the quality of life in diabetic patients. Therefore, there has been a growing interest in alternative therapies and in the therapeutic use of natural products for diabetes, especially those derived from marine brown algae (Lee, Min et al. 2011).

Marine brown algae, phlorotannins in particular, are known to provide an abundance of bioactivities with great pharmaceutical foods and biomedical potential. Fucodiphloroethol G ( $IC_{50}$ -19.52  $\mu$ M), dieckol ( $IC_{50}$ -10.79  $\mu$ M), 6,6'-bieckol ( $IC_{50}$ -22.22  $\mu$ M), 7-phloreckol ( $IC_{50}$ -49.49  $\mu$ M), and phlorofucofuroeckol A ( $IC_{50}$ -19.71  $\mu$ M) from *E. cava* have shown significant inhibition on  $\alpha$ -glucosidase activity (Lee et al. 2009). Several other studies have revealed *in vivo* antidiabetic effects by treating

phlorotannins to diabetic mouse models, C57BL/KsJ-db/db mice (Iwai 2008; Lee, Min, et al. 2011). Furthermore, treating with brown algae extracts such as *E. cava* (Kang et al. 2010) and *I. okamurae* (Min et al. 2011) has resulted in the reduction of plasma glucose level and improved insulin resistance *in vivo*, respectively. Diabetes is closely related to diet and incorporation of these phlorotannins from brown algae without the side effects associated with synthetic drug treatments as medicinal dietary supplements would be a promising prevalence strategy for diabetes. Therefore, it seems that phlorotannins are promising antidiabetic agents or pharmaceutical sources that will be helpful for the management of diabetes.

### 13.4 CONCLUSIONS

Marine algal polyphenols, known as phlorotannins, have only been found to exist in brown algae. These phlorotannins are a diverse group depending on their structure and composition. Much attention has been paid to the strong activities of phlorotannin oligomers against oxidative stress, inflammation, cancer, allergy, and diabetes *in vitro* and *in vivo*. Phlorotannins from brown algae have the potential to expand to adverse body conditions by modulating as pharmaceutical agents. Taken together, phlorotannins are valuable sources of biological activities and could be introduced for the preparation of novel functional ingredients in food and also as a good approach for the treatment or prevention of chronic diseases.

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# 14 Microalgae as Sources of Biomaterials and Pharmaceuticals

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## 14.1 INTRODUCTION

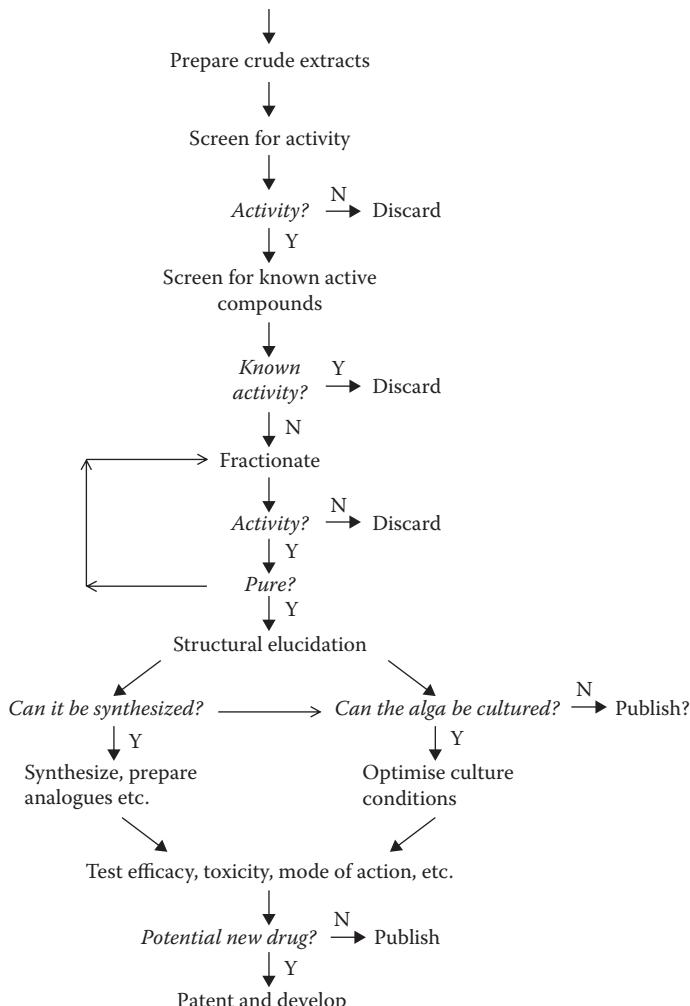
The use of algae for therapeutic purposes has a long history (Glombitzka and Koch 1989), and the systematic examination of algae for biologically active substances, especially antibiotics began in the 1950s. Early studies were concerned mainly with *in vitro* studies of the action of the compounds and it was only in the 1970s, at the Roche Research Institute of Marine Pharmacology (RRIMP) in Australia, that the focus shifted to emphasize *in vivo* examination of activities using a very extensive range of screens (Baker 1984; Reichelt and Borowitzka 1984). RRIMP also pioneered the screening of crude extracts rather than pure compounds, and the use of bioactivity in the screens to direct the isolation and identification of the active compound (Baker 1984). This approach is now the most commonly used and is illustrated in [Figure 14.1](#).

In the last decade, the screening of microalgae, especially the cyanobacteria (blue-green algae), for antibiotics and pharmacologically active compounds has received ever increasing interest. Most of the past works have focused on macrophytes and the microalgae only started to be studied widely in the 1980s (Kellam and Walker 1989; Patterson et al. 1991). Not only are the microalgae proving to be valuable sources of novel biologically active molecules, but since many can be cultured they also have the potential advantage over macroalgae (and invertebrates) of being easier to culture commercially.

They, therefore, have the potential to be used to produce those chemically complex molecules that are difficult to synthesize.

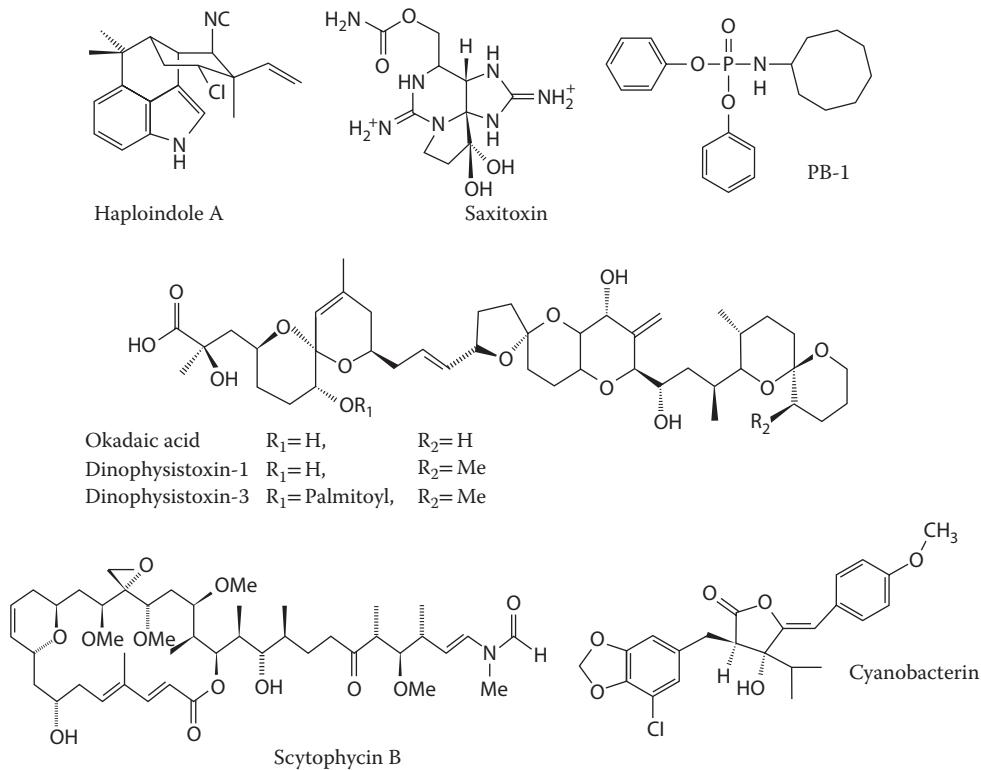
## 14.2 ANTIMICROBIALS

A large number of microalgal extracts and/or extracellular products have been found to have anti-microbial activity, although for many of these the structure and identity of the active constituent is not yet known (Pesando 1990). There are several identified antibacterial and antifungal substances



**FIGURE 14.1** Flow diagram showing the process followed in the search for bioactive molecules from microalgae.

including fatty acids (Findlay and Patil 1984), glycolipids (Duff, Bruce, and Antia 1966), acrylic acid (Sieburth 1959), phenolics (De Cano et al. 1990), bromophenols (Pedersen and DaSilva 1973), terpenoids, carbohydrates (Ramamurthy 1970), N-glycosides (Bonjouklian et al. 1991), peptides (Berland, Bonin, and Cornu 1972), polysaccharides (Pesando and Gnassia-Garelli 1979), acrolyl-choline (Taylor et al. 1974), acrolyl-diketone (Trick, Andersen, and Harrison 1984), isonitrile-containing indole alkaloids such as haploindole A (Figure 14.2) (Moore et al. 1987), and various toxins such as nodularin, goniautoxin, saxitoxin, okadaic acid (Figure 14.2), and ciguatoxin (Nagai, Satake, and Yasumoto 1990; Carmichael 1992). Unfortunately, most of the studies have only used *in vitro* assays and, in analogy with macroalgae, it is likely that most of these compounds will have little or no application in medicine as they are either too toxic or inactive *in vivo* (Table 14.1 and Reichelt and Borowitzka 1984). They may, however, serve as useful leads to new synthetic antibiotics or may find application in agriculture. For example, the tjipanazoles, isolated from the cyanobacterium, *Tolyphothrix tjipanensis*, are indolo [2,3-a] carbazoles, similar to those found in actinomycetes and slime moulds, but without a pyrrolo[3,4-c] ring (Bonjouklian et al. 1991). They show little cytotoxicity and no *in vivo* activity against *Candida albicans*, however, tjipanazole Al

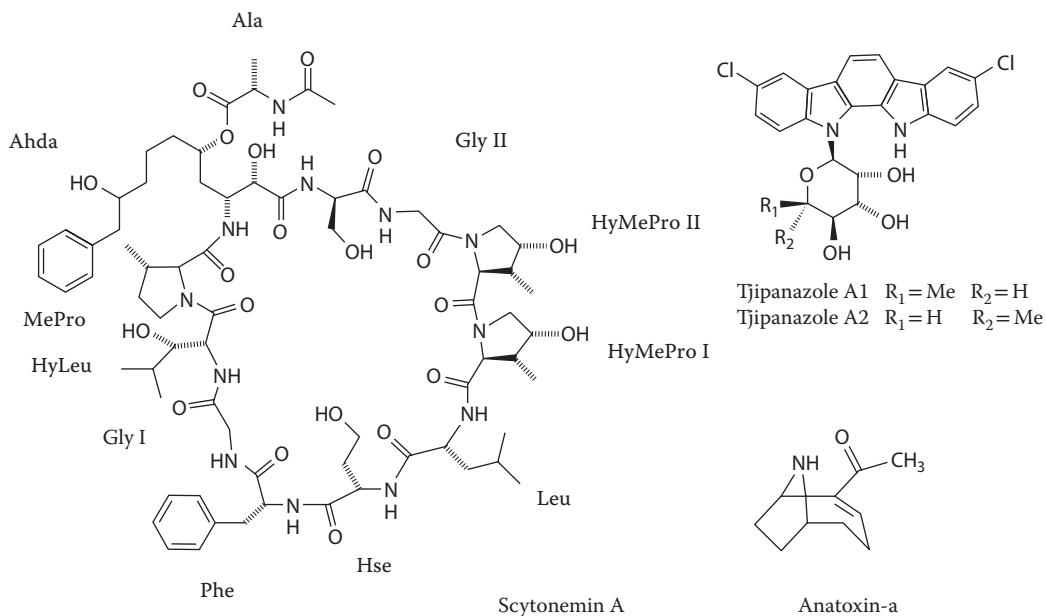


**FIGURE 14.2** Structure of bioactive compounds from dinoflagellates (saxitoxin [several dinoflagellates]); okadaic acid and dinophysistoxin (*Prorocentrum lima*); PB-I (*Ptychodiscus brevis*) and cyanobacteria (haploindole A [*Hapalosiphon fontinalis*])); cyanobacterin (*Scytonema hofmanni*); and scytophyycin B (*Scytonema pseudohofmanni*).

**TABLE 14.1**  
Results of Antibiotic Activity Screening of Marine Algal Extracts against *Staphylococcus aureus* (Sa), *Streptococcus pyogenes* (Spy), *Streptococcus pneumoniae* (Spn), *Escherichia coli* (Ec), and *Pseudomonas aeruginosa* (Pa)

	Sa	Spy	Spn	Ec	Pa
Total number of extracts tested	435	352	353	438	439
Total <i>in vitro</i> active	86	124	138	18	19
Total number tested <i>in vivo</i>	44	43	76	10	16
Total <i>in vivo</i> active	5	4	2	0	0
% <i>in vivo</i> actives (as % of total extracts tested)	1.15	1.14	0.57	0	0

and A2 (Figure 14.3) show appreciable fungicidal activity against rice blast and leaf rust wheat infections. Other algal toxins may also be of interest in environmental management. For example, the algaecides produced by some cyanobacteria, such as the γ-lactone, cyanobacterin (Figure 14.2) produced by *Scytonema hofmanni* (Gleason et al. 1986), fischerellin from *Fischerella muscicola* (Gross, Wolk, and Juttner 1991), and an unidentified extracellular product of an *Oscillatoria* sp. (Bagchi, Palod, and Chauhan 1990; Chauhan, Marwah, and Bagchi 1992) may find use in the control of algal blooms. Cyanobacterin has also been patented as a herbicide (Gleason 1986).



**FIGURE 14.3** Structure of bioactive compounds from cyanobacteria scytonemin a (*Scytonema* sp.); tjipanazole A1 (*Tolyphothrix tjipanensis*); and anatoxin-a (*Anabaena flos-aquae*).

### 14.3 ANTIVIRAL ACTIVITY

A number of cyanobacteria and very few other microalgae have been screened for antiviral activity so far, but the limited results available are promising (Patterson, Baker, et al. 1993). For example, Rinehart et al. (1981) have found that over 5% of the extracts of cultured cyanobacteria screened by them showed antiviral activity against *Herpes simplex* virus type II, and >5% had activity against respiratory syncytial virus. Lau et al. (1993) have also screened extracts of over 900 strains of cyanobacteria for inhibition of reverse transcriptases of avian myeloblastosis virus and human immunodeficiency virus type 1, and they found that over 2% of these algae showed promising activities. The active compounds have, however, not been identified as yet with the exception of an anti-AIDS sulfolipid (Gustafson et al. 1989).

### 14.4 TOXINS AND PHARMACOLOGICALLY ACTIVE COMPOUNDS

Many microalgae, especially the cyanobacteria and the dinoflagellates, produce toxins, some of which have gained some importance as pharmacological tools, although they have generally not found use in therapy (Ikawa and Sasner 1990). Of particular interest is saxitoxin (Figure 14.2) found in a number of dinoflagellates. Its effect is similar to tetrodotoxin. These toxins have been shown to block the influx of sodium through excitable nerve membranes, thus preventing the formation of action potentials (Hille 1975). Saxitoxin has been used as an aid in certain microsurgical procedures and as an experimental treatment for short sightedness. The gonyautoxins show a similar mode of action. The anatoxins (Figure 14.3) produced by *Anabaena flos-aquae* are potent postsynaptic depolarizing neuromuscular blocking agents (Carmichael 1992). Another type of toxin produced by dinoflagellates is the ichthyotoxic phosphorous substance, PB-1 (Figure 14.2) (DiNovi et al. 1983). Although these toxins themselves are not likely to be useful as pharmaceuticals, they can serve as models for the rational design of useful compounds.

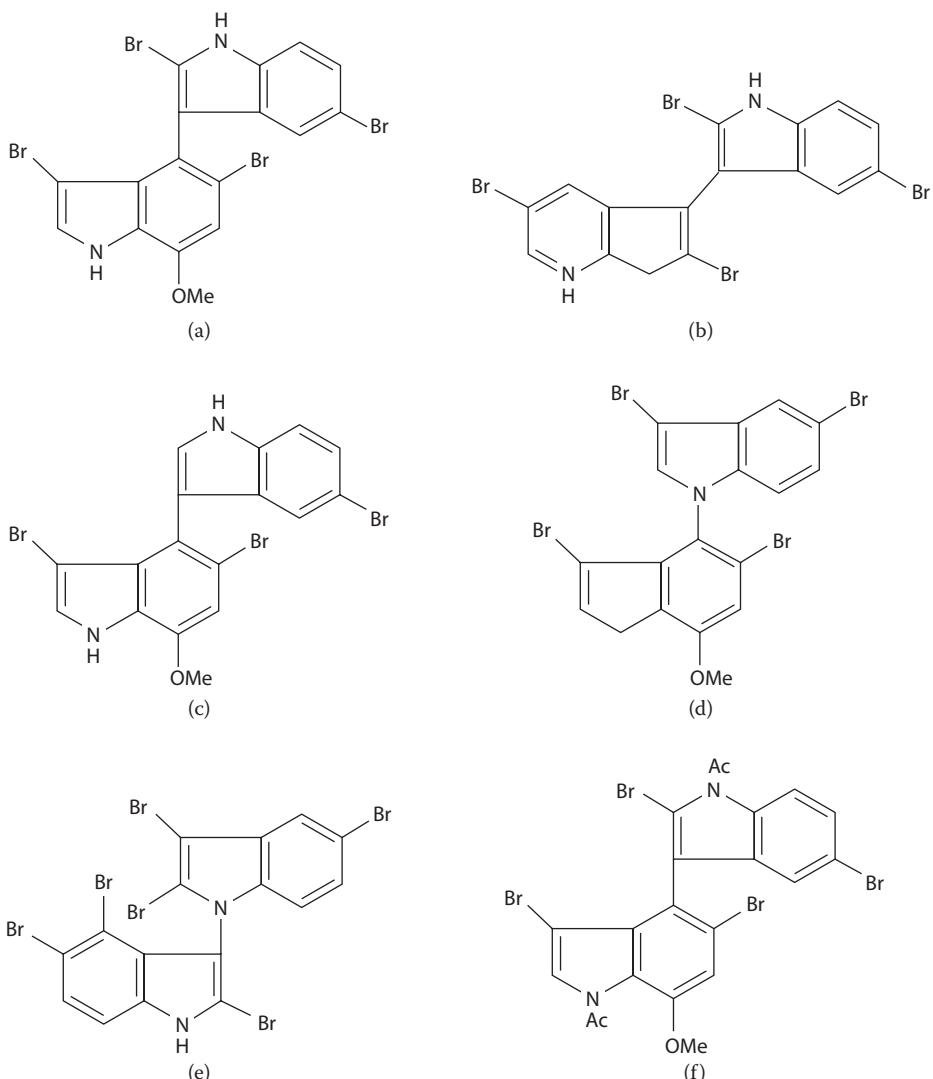
Another group of toxins of interest to the study of cellular regulation are okadaic acid and 35-methyl okadaic acid (dinophysistoxin-1) (Figure 14.2), produced by dinoflagellates, and the peptide hepatotoxins, microcystin and nodularin, produced by cyanobacteria.

These toxins are protein phosphatase inhibitors (Chen et al. 1993; Luu et al. 1993). Reversible phosphorylation of proteins on serine, threonine, and tyrosine residues by protein kinases and phosphatases is a principal mechanism by which eukaryotic cells respond to extracellular signals (Holmes and Borland 1993). These algal toxins are, therefore, useful tools in the study of cellular regulation. A very large number of microcystins have now been characterized and these provide an excellent opportunity for structure–activity studies (Rinehart, Namikoshi, and Choi 1994). Among the cyanobacteria, numerous cytotoxic compounds, some of which have potential as anticancer drugs have been characterized (Patterson et al. 1991). These compounds include tubericidin and toyocamycin, both of which have also been found in *Streptomyces* (Barchi et al. 1983; Patterson et al. 1991) and new unique macrolides such as scytophyycin B (Figure 14.2) isolated from *Scytonema pseudohofmanni*. The scytophyycins show cytotoxicity against the KB (a human nasopharyngeal carcinoma) cell line at 1 ng/mL, as well as moderate activity against murine, intraperitoneally implanted P388 lymphocytic leukemia and Lewis lung carcinoma (Ishibashi et al. 1986; Moore et al. 1986). Similar activities have been reported for the scytophyycin, tolytoxin, from *T. conglutinata* var. *colorata* and *S. mirabile* (Stewart et al. 1988; Carmeli, Moore, and Patterson 1990), and for indocarbazoles isolated from *Nostoc* (Knubel et al. 1990). The cytostatic effect of tolytoxin apparently results from an inhibition of actin polymerization, thus disrupting microfilament organization in eukaryotic cells (Patterson and Carmeli 1992; Patterson, Smith, et al. 1993).

Macrolides with antitumor action have also been isolated from dinoflagellates, that is, amphidinolide-A from an *Amphidinium* sp. (Kobayashi et al. 1986). An alternative screen for potential anticancer activity, including protein kinase C, protein tyrosine kinase, and inosine monophosphate dehydrogenase has also resulted in a range of compounds from cyanobacteria, cryptophytes, and chrysophytes (Gerwick et al. 1994). Several hydrophilic extracts of cyanobacteria also have shown cardiotonic activity in isolated mouse atria. Although, in many cases, this activity can be attributed to tyramine, positive ionotropic activity in several species of Scytonemataceae has been attributed to compounds called the tolypophycins (Moore, Patterson, and Carmichael 1988) and, in the case of an *Anabaena* sp., to an unusual chlorine-containing cyclic peptide, puwainaphycin C (Gregson 1986). The cyclic peptide, scytonemin A (Figure 14.3), from a *Scytonema* sp. has also been shown to be a moderately strong calcium agonist (Helms et al. 1988). The diatom, *Nitzschia pungens* var. *multiseries*, is also a potential source of the glutamate agonist, domoic acid (Laycock, de Freitas, and Wright 1989). Domoic acid is structurally similar to glutamic and aspartic acid and acts as a potent excitant (agonist) of glutamate receptors; as such it could be an important tool in the study of neurodegenerative disease. *N. pungens* has been shown to contain >1% of dry weight of domoic acid in culture and could therefore be an excellent source of this compound.

Another interesting group of bioactive compounds isolated from microalgae is the brominated biindoles isolated from field-collected material of the intertidal cyanobacterium, *Rivularia firma* (Norton and Wells 1982). The major compound isolated from this alga, (+)-7'-methoxy-2,3,5,5'-tetrabromo-3,4'-bi-1Hindole (Figure 14.4a) showed both anti-inflammatory and anti-amphetamine activities. However, it also produced tremors in rats and mice. Two other less abundant biindoles (Figure 14.4b and c) isolated from *R. firma* retained the anti-inflammatory activity without showing significant tremorigenic effects, and the remaining two (Figure 14.4d and e) showed no activity (Baker 1984). The synthetic diacetate (Figure 14.4f) of Figure 14.4a had only a very slight tremorigenic effect while maintaining the anti-amphetamine activity as well as being active in the acetic acid writhing test. This study shows that the preparation of analogues can be useful in producing compounds with desired activities without undesirable side effects. However, because of the inability to culture this species as well as difficulties in synthesis, these promising activities were not pursued.

There are many other interesting leads to pharmacologically active compounds in cultured microalgae. These include angiotensin-converting enzyme inhibitory activity in several species of cyanobacteria, Chlorophyceae, Dinophyceae, and Raphidophyceae (Yamaguchi, Murakami, and Okino 1989); these may lead to new antihypertensive agents. Similarly, Lincoln, Strupinski, and



**FIGURE 14.4** Structure of brominated biindoles isolated from *Rivularia firma*. The major compound isolated is (a), and compounds (b) to (e) were minor constituents. Compound (f) is a synthetic analogue of (a).

Walker (1990) found that extracts from a wide range of cultured microalgae affected electrically evoked muscle contraction and resting muscle tone in isolated guinea pig ileum. Similarly, aqueous extracts of *Phaeodactylum tricornutum* and *Dunaliella tertiolecta* showed activity as a central nervous system (CNS) depressant and a potential muscle relaxant (Villar et al. 1992) and *Chlorella stigmatophora* extracts showed anti-dopaminergic activity (Laguna, Villar, Calleja, et al. 1993). Aqueous extracts of *Tetraselmis suecica* and *Isochrysis galbana* also showed activities in CNS screens, however, these activities have been difficult to characterize by standard pharmacological criteria (Laguna, Villar, Cadavid, et al. 1993).

The active principles in these extracts have not been characterized further in any of these studies, nor have the active compounds been isolated. However, the high proportion of pharmacologically active extracts indicates that microalgae cultures are very promising sources of bioactive molecules, some of which may eventually find application in medicine or veterinary science.

## 14.5 OTHER ACTIVITIES

Microalgae are also sources of known bioactive compounds such as vitamins and fatty acids. Many microalgae synthesize vitamins such as pro-vitamin A ( $\beta$ -carotene), vitamin B12, B6, biotin, and so on (Borowitzka 1988). Of these, the green halophilic alga *Dunaliella salina* is the best natural source of  $\beta$ -carotene and is grown commercially as a source of  $\beta$ -carotene for use as a dietary supplement and a natural food coloring in Australia, the United States, and Israel (Borowitzka and Borowitzka 1989). Carotenoids such as carotene and fucoxanthin also have antitumor and cancer preventative activity (Okuzumi et al. 1990; Davison, Rousseau, and Dunn 1993). Microalgae, especially marine microalgae, are also excellent sources of polyunsaturated fatty acids such as  $\gamma$ -linolenic acid, arachidonic acid, eicosapentaenoic acid, and docosahexaenoic acid (Cohen and Cohen 1991). These essential fatty acids are important for the treatment and prevention of a range of diseases, and are also important in human nutrition (Okuyama 1992; Zevenbergen and Rudrum 1993). Algal species can be selected for the preponderance of a particular fatty acid, and the content of these fatty acids can be manipulated by changing culture conditions (Chrismadha and Borowitzka 1994).

## 14.6 PRODUCTION OF BIOLOGICALLY ACTIVE SUBSTANCES BY ALGAL CULTURE

Several studies have shown that the production of the active compounds depended on the growth phase and/or culture conditions (Armstrong et al. 1991; Patterson et al. 1991; Morton and Bomber 1994) and this means that culture conditions for the production of bioactive compounds must be optimized.

Several studies, and our own experience, have also shown that the desired activity may decline or be lost in culture. For example, Carmichael (1986) reports that anatoxin-a toxicity of *Anabaena flos-aquae* NRC-44-1 disappeared when the medium was changed from ASM-1 to BG-11. The reasons for this loss need to be understood before a reliable culture system for production can be developed.

Nutrient limitation, especially N and P limitation, has been shown to be necessary for the production of high levels of acutiphycin in *Oscillatoria acutissima* and maximum concentrations are achieved in early stationary-phase cultures. Addition of N, P, or organic carbon greatly reduces the formation of acutiphycin (Moore, Patterson, and Carmichael 1988). Carmichael (1986) and Rapala et al. (1993) made similar observations on biotoxin production in planktonic cyanobacteria. On the other hand, antibiotic production in *Nostoc muscorum* and *Scytonema* sp. was most affected by the nitrogen and iron content of the medium and was enhanced in actively growing cultures (Bloor and England 1991; Chetsumon, Fujieda, et al. 1993; Chetsumon, Miyamoto, et al. 1993). The production of tolytoxin in *Scytonema ocellatum* is also unusual for a secondary metabolite in that it is produced throughout the cell cycle (Patterson and Bolis 1993).

Other environmental factors may also be important; for example, the antibiotic cyanobacterin LU1 from *Nostoc linckia* is synthesized throughout the growth cycle, but is favored by low temperatures (Gromov et al. 1991). Similarly, lipid production in diatoms is enhanced by silicon starvation, and low temperatures generally enhance the content of long-chain polyunsaturated fatty acids such as eicosapentaenoic acid in many algae. Microcystin production in *Microcystis aeruginosa* is also enhanced under red or green light compared to white light (Utkilen and Gjølme 1992). At this time, our understanding of the physiological control of secondary metabolite formation in algae is extremely limited, nor do we know much about the biosynthetic pathways. Such studies are necessary if we are to fully exploit the potential of the microalgae and if we wish to use genetic engineering and recombinant DNA technology to increase the production of bioactive compounds by developing either overproducing strains or strains in which the compound of interest is synthesized throughout the cell cycle. Such work also requires the development of suitable probes and methods for transforming many algae. Such systems are reasonably well developed for the cyanobacteria, but not yet for the eukaryotic algae (Craig, Reichelt, and Reichelt 1988; Dunahay et al. 1992; Kindle

and Sodeinde 1994). Classical mutagenic methods can also be applied and have already been used to produce  $\beta$ -carotene overproducing strains (Shaish, Ben-Amotz, and Avron 1991).

Some of the genes for bioactive molecules also appear to be carried on plasmids, for example, the toxin-producing gene in *Microcystis* (Hauman 1981). In contrast, the toxin genes of *Anabaena* do not appear to be carried on a plasmid (Kumar and Gorham 1975). Plasmid-borne genes may be easier to manipulate and could possibly be introduced into other species that are more easily cultured.

## 14.7 CONCLUSIONS

The microalgae represent a very large, untapped reservoir of novel compounds, many of which are likely to show biological activity. The cyanobacteria are the most intensively studied so far; however, there is no good reason that other algal groups also do not contain active compounds of interest. For example, diatoms show a wide range of antibacterial and antifungal activities (Pesando and Gnassia-Barelli 1979; Viso, Pesando, and Baby 1987), but little work has been done to identify the active compounds, nor have many diatoms been screened for other activities. One obvious limitation to the degree of screening of algae other than the cyanobacteria, especially the dinoflagellates, has been the greater difficulty in culturing many of these. However, the recent increase in the screening of microalgae for potentially useful natural products should provide an impetus for the development of improved media and culture conditions. The ability to culture microalgae and their great biochemical diversity makes them a valuable potential renewable source of new drugs, growth regulators, and other useful chemicals. Although some algal products are already available, continued isolation and screening of microalgae is required as well as studies of algal physiology and biochemistry.

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# 15 Marine Ascidiants

## *A Promising Resource for Bioactive Compounds*

*H. Abdul Jaffar Ali and M. Tamilselvi*

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### 15.1 INTRODUCTION

The need for new life-saving therapeutic compounds has been expanding greatly because of the evolving resistance of microorganisms to existing antibiotics, emergence of new viral diseases, and appearance of drug-resistant tumors. Terrestrial resources have been exhaustively explored and, thus, academic and industry researchers are striving to get lead molecules from the inner space of oceans.

The ocean environment is massively complex, consisting of extreme variations in pressure, salinity, temperature, and biological habitats. The rationale for searching drugs from the marine

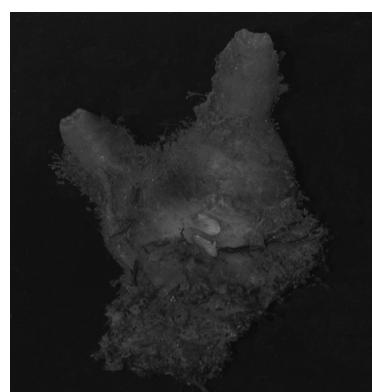
environment is that marine plants and animals have adapted to all sorts of marine environments and drive them to produce a variety of molecules with unique structural features. These marine molecules exhibit various types of biological activities, with compounds of high economic interest having potential applications in the pharmaceutical and medical sectors. Despite the fact that the biodiversity in the marine environment far exceeds that in the terrestrial environment, research into the use of marine natural products as pharmaceutical agents is still in its infancy. This could be attributed to the lack of ethnomedical history and the difficulties involved in the collection of marine organisms. But with the development of new diving techniques, remotely operated machines, etc., it is possible to collect marine samples.

In recent years, over 20,000 bioactive compounds have been extracted from various marine animals such as tunicates, sponges, soft corals, sea hares, nudibranchs, bryozoans, sea slugs, and other marine organisms. In this scenario, marine ascidians are gaining paramount importance in remaining a vast, untapped source for medicines with enormous therapeutic potential that has attracted the interest of both chemists and pharmacologists.

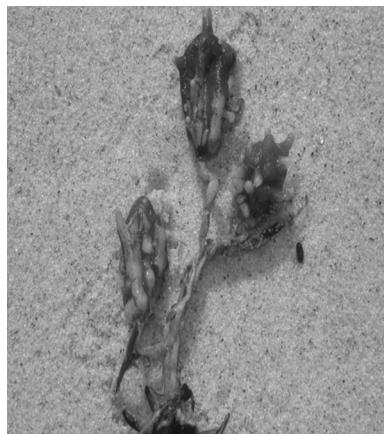
### 15.1.1 MARINE ASCIDIANS

Ascidians belong to the class Ascidiacea in subphylum Tunicata or Urochordata comprise approximately 3000 described species and found in all marine habitats from shallow water to deep sea. They are cosmopolitan and exclusively marine. Ascidians go by several names. They are called tunicates because their bodies are enclosed within a tunic made up of a cellulose-like material, tunicin, which is a very rare occurrence within the animal kingdom. Another common name is sea squirt; they are so called because they pump water through their branchial (or) feed sac once the animals are removed from the substratum or disturbed. Ascidians are the most diverse class of the subphylum Tunicata, and they are distinguished from the other classes in the subphylum by their sessile habits. They occur in three forms: (1) simple or solitary, (2) social and (3) colonial (Figures 15.1 through 15.3).

The first clear explanation on ascidians was made by Schlosser in 1756 in a letter entitled “An Account of Interested, Fleshy, Coral-Like Substance” regarding the widely distributed colonial ascidian *Botryllus schlosseri* collected along the British Islands. The term “tunicate” originates from the polysaccharide-containing tunic that envelops the animal and forms a flexible skeleton. Ascidian remains sessile following larval settlement throughout their adult life and inhabits a wide variety of habitats such as soft sediments, coral reefs, and rocky substrates. The rigidity of the outer layer and toxicity of the tissues aid in keeping the tunicate clean and unfouled by other organisms (Goodbody 1962). They are a key ecological group because of their invasive potential and ability to thrive in eutrophic (nutrient-rich) environments. Introduction of nonindigenous ascidians



**FIGURE 15.1** Solitary ascidian.



**FIGURE 15.2** Social ascidian.



**FIGURE 15.3** Colonial ascidian.

into harbors in both tropical and temperate waters is now common with the rate of introductions increasing steadily, although they sometimes cause severe damage to natural fauna by overgrowth (Lambert and Lambert 1998; Lambert 2001; Abdul Jaffar Ali, Sivakumar, and Tamilselvi 2011; Tamilselvi et al. 2011). In contrast, several species of ascidians are cultured for food primarily in Japan, Korea, and France. The solitary ascidian *Halocynthia roretzi* has long been popular as seafood in Japan and Korea with a market value of US\$18 million in 2006.

Most recently, ascidians have increasingly become the target of natural products research. A large number of natural products has been isolated from ascidians and tested for various biological activities, especially from colonial ascidians.

### 15.1.2 METHODOLOGY

The most relevant peer-reviewed literature published during the last two decades covering marine natural products was surveyed for this chapter. During this period alone, over 2500 molecules from ascidians were described. In this chapter, only those compounds displaying a high potential for industrial applications are addressed.

All the ascidians producing the compounds selected for inclusion in this chapter were grouped into orders and families of the class Ascidiacea of the subphylum Urochordata (Table 15.1).

This approach is helpful in identifying which taxonomic groups of ascidians screened so far display the highest potential of yielding new drugs or pharmacological products derived from marine bioactive compounds. The class Ascidiacea currently includes 2 orders, 25 families, and over 3000 valid species.

**TABLE 15.1**  
**Percentage of Pharmaceutical Compounds Contributed by Marine Ascidians of the Class Ascidiacea**

Class	Order	Suborder	Family	Percentage (%)
Asciidae	Enterogona	Aplousobranchia	Cionidae	1.5
			Diazonidae	1.5
			Stomozoidae	—
			Holozoidae	—
			Pycnoclavellidae	1.5
			Clavelinidae	6.5
			Polycitoridae	31
			Placentelidae	2.9
			Euherdmaniidae	—
			Pseudodistomidae	—
			Protopolyclinidae	—
			Ritterellidae	1.5
			Polyclinidae	8
			Didemnidae	23
Pleurogona	Stolidobranchia	Phlebobranchia	Plurellidae	—
			Perophoridae	2.2
			Rhodosomatidae	—
			Asciidiidae	1.5
			Agnesiidae	—
			Styelidae	18
			<b>Suborders</b>	3
			Styelinae	
			Botryllinae	4
			Polyzoinae	13
			Pyuridae	5
			Molgulidae	0.7

## 15.2 BIOMEDICAL COMPOUNDS FROM ASCIDIANS

Interest in the chemistry of ascidiants was kindled in as early as 1847 when a German physiologist discovered in the blood cells of these invertebrates the presence of large amounts of vanadium and sulfuric acid, along with an uncharacterized nitrogenous metabolite. It has been amply demonstrated that ascidiants are prolific producers of novel bioactive metabolites, which include a diverse array of alkaloids and a small number of preclinical and clinical trials as antitumor agents. Research efforts from 1977 to 1987 were mostly focused on algae with the production of 883 new compounds, followed by sponges with 736 new compounds, and then coelenterates with 560 new metabolites. In stark contrast, the ascidiants were the source of only 65 metabolites during this period (Ireland et al. 1992). Attention has been focused more recently on ascidiants because of their biologically active metabolites. Although the birth of the field of marine natural products is generally credited to Bergmann and Feeney (1950), it was not until 1974 that Fenical isolated the first ascidian metabolite geranyl hydroquinone from *Aplidium* sp. Even then, research on ascidiants was

initiated more recently than that on other marine invertebrates; it is significant that the first marine natural product to enter human clinical trials, didemnin B, is an ascidian metabolite. Members of Aplousobranchia, particularly Polycitorids and Didemnids, are the best represented group, with more than 80% of ascidian species to produce natural products.

Didemnum ascidians are excellent sources of novel biologically active compounds of varied biosynthetic origin (Waslyk et al. 1983). Many researchers have isolated the different secondary metabolites and observed the biological activities of such compounds from colonial ascidians (Hamamoto et al. 1983; Faulkner 1984; Schmitz et al. 1989; Hawkin et al. 1990; Carroll et al. 1996; Fukuzawa, Matsunaga, and Fusetani 1997).

Solitary ascidians appear to be a less reliable source of natural products and, indeed, only 10% of ascidians producing natural products are solitary. In addition, Munro et al. (1989) reported that only 1 of the 12 solitaries examined in New Zealand waters showed activity. Nevertheless, several solitary species belong to family Styelidae are noteworthy for the presence of natural products. Azumi, Yokosawa, and Ishii (1990) isolated a novel antimicrobial tetrapeptide-like substance from the solitary ascidian *H. roretzi*. Further, Lindquist and Fenical (1997) reported that five novel benzoids from *Polycarpa aurata* exhibited significant antifungal activity against *Saccharomyces cerevisiae* and *Candida albicans*.

### 15.3 WHY SHOULD AN ASCIDIAN PRODUCE BIOACTIVE COMPOUNDS?

Ascidians use chemical weapons by synthesizing novel compounds for defense purposes. By virtue of their sedentary and filter feeding as well as mucous feeding habits, ascidians accumulate a high concentration of bacteria from endostyle to atrial siphon. In order to get rid of microcosms, ascidians synthesize various kinds of antimicrobial compounds. It has been proposed that antimicrobial substances function as humoral factors in the defense mechanisms of invertebrates, which lack humoral immunoglobulin. Among invertebrates, ascidians are noticeable animals from the viewpoint of the evolution of the immune system because they are prochordates, which occupy the phylogenetic position between vertebrates and true invertebrates.

Ascidians, as sedentary and good biofoulers, compete for space and food, which triggers the ascidians to produce chemicals most effective to kill rapidly dividing cells of microfoulers. The ability of chemicals to kill rapidly dividing cells is the hallmark of chemotherapy. Anticancer drugs often act by killing the rapidly dividing cells of a tumor, but they generally do not harm “normal” healthy cells. These ideas provide a connection between marine chemical warfare and the possible application of marine natural products in medicine.

The fouling of an ascidian’s surface may significantly reduce its fitness. The potential to become fouled should therefore cause a potent selection pressure for the evolution of antifouling defenses, although this may depend on whether any such defense has multiple functions. A growing body of evidence indicates that marine ascidians have developed a wide variety of defensive mechanisms against foulers. For instance, motile invertebrates may avoid fouling through a range of behavioral responses. Such responses are not available to ascidians; they must rely on either chemical or physical deterrents to maintain their surfaces relatively free of foulers.

Many benthic ascidians are relatively free from predation because of mechanical defenses such as tough tunics or spicules and chemicals defenses that may deter predators. Although a species may be chemically defended against most generalized predators, it may be vulnerable to specialized predators. These specialists could, in fact, be attracted to the very chemicals that deter other predators, and these chemicals may be incorporated into the specialists’ own defense.

### 15.4 ASCIDIAN-DERIVED COMPOUNDS

There are a few examples of marine-derived compounds that have successfully reached the market as therapeutic drugs.

#### 15.4.1 DIDEMNIN B

Didemnin B was originally isolated from the Caribbean tunicate *Trididemnum solidum* and was the first marine compound to enter human cancer clinical trials as a purified natural product in 1981. Early investigation into the bioactivity of this compound revealed marked antiviral and cytotoxic activity in *in vitro* tests using standard mouse leukemia cell lines. Mechanistically, didemnin B interrupts protein synthesis in target cells by binding noncompetitively to palmitoyl protein thioesterase. Didemnin B was the first defined marine natural product to enter clinical trials as a potential anticancer drug.

#### 15.4.2 APLIDINE (APLIDIN®)

Aplidine was isolated from the Mediterranean tunicate *Aplidium albicans*. It was first reported in 1991 patent application. In preclinical animal tests, aplidine exhibited anticancer properties. The molecule has been described as a multifactorial apoptosis inducer, and it has other beneficial attributes such as low toxicity and a high specificity for tumor cells. The compound also inhibits the expression of receptor proteins (ornithine decarboxylase) and the secretion of proteins (vascular endothelial growth factor) involved in growth and vascularization of certain tumor types.

#### 15.4.3 DIAZONAMIDE A

The marine natural product diazonamide A was first reported in 1991. It was extracted from the Philippine ascidian *Diazona angulata* by the William Fenical Chemistry Lab at the Scripp's Institution of Oceanography, La Jolla, California. Both analogs possess potent microtubulin interactive activity. Diazonamide A is an inhibitor of microtubule assembly, arresting the process of cell division in cultures exposed to treatment. Examination of treated cells reveals a loss of spindle microtubule assemblies and also microtubules associated with the interphase stage of the cell cycle.

#### 15.4.4 ECTEINASCIDIN 743 (YONDELIS®)

Ecteinascidin 743 was isolated from the Caribbean sea squirt (*Ascidia*) *Ecteinascidia turbinata*. It is classified as a tetrahydroisoquinoline alkaloid. Preclinical trials showed ET-743 was active against a range of tumor types in standard animal models. Subsequent human trials showed efficacy against advanced soft-tissue sarcoma, osteosarcoma, and metastatic breast cancers. It is noted that ET-743 has been codeveloped under the trade name Yondelis by the Spanish marine pharmaceutical company PharmaMar, Madrid, Spain, and the Johnson & Johnson subsidiary Ortho Biotech, USA. The cancer drug Yondelis has been approved by National Institute for Health and Clinical Excellence (NICE), London for use on the National Health Service (NHS), United Kingdom, but it was only after the manufacturer offered to meet the cost of anyone needing more than five cycles.

#### 15.4.5 VITILEVUAMIDE

Vitilevuamide is a bioactive cyclic peptide isolated from the ascidians *Didemnum cuculiferum* and *Polysyncraton lithostrotum* (the same animal is the source of the antimicrobial/antitumor compound namanamicin). Vitilevuamide is one of the several novel tubulin interactive agents recently discovered from marine invertebrate sources. Research on the mechanism of action of this two-ringed marine peptide reveals that vitilevuamide inhibits tubulin polymerization and can arrest the cell cycle of target cells in the G2/M phase.

## 15.5 ALKALOIDS FROM ASCIDIANS

Ascidians' chemistry is dominated by the presence of nitrogenous metabolites that could be basically divided into two structural type-based groups: (1) peptides and (2) polycyclic aromatic alkaloids. More than 300 alkaloid compounds have been isolated from marine ascidians and they have reported pharmacological activity. The class of alkaloids from ascidians indeed includes a large variety of structures, ranging from complex pyridoacridines followed by carboline-based alkaloids, indole-based alkaloids, and tyrosine-derived alkaloids to simple protoalkaloids.

Pyridoacridine alkaloids isolated from ascidians are typically tetra- or pentacyclic aromatic alkaloids usually possessing a functionalized alkylamine side chain. Many of these compounds have generated interest both as challenging problems for structure elucidation and synthesis and due to their bioactivities. In general, pyridoacridines are cytotoxic and some of them possess potent anti-viral, antifungal, antibacterial, antitumor, and antiparasitic activities. For the majority of this class, cytotoxicity has been shown to be due to DNA-binding properties, topoisomerase (TOPO) inhibition, or production of reactive oxygen species (ROS). The known tetracyclic pyridoacridine alkaloids from marine sources are dominated by those isolated from ascidians. They show an oxygen function at C8, which can be a carbonyl group, a hydroxyl group, or an ether moiety (Viracaoundin et al. 2001; Appleton et al. 2002; Nilar, Carté, and Butler 2002; Torres et al. 2002; López-Legentil et al. 2005; Clement et al. 2008).

Polysubstituted  $\beta$ -carbolines, as well as dihydro-, and tetrahydro- $\beta$ -carbolines form a large group of tryptophan-derived ascidian metabolites. The majority of these alkaloids have been isolated from tunicates belonging to the genus *Eudistoma*; other sources are the genera *Ritterella*, *Pseudodistoma*, *Didemnum*, *Synoicum*, and *Lissoclinum*. Almost all the reported compounds are hydroxylated and/or brominated at C5, C6, C7, and C8; apart from a few members of this group, which are unsubstituted at C1, they show different substituents at C1, such as pyrrole, pyrrolidine, or indole rings, as well as oxygenated, aminated, or thiomethylated alkyl residues (Rashid et al. 2001; Schupp et al. 2003; Oku, Matsunaga, and Fusetani 2003; Ravinder et al. 2005; Wang et al. 2008; Kearns and Rideout 2008; Takahashi et al. 2010; Lake et al. 2011).

The structures of indole-based alkaloids isolated from ascidians span a wide range of complexity, spreading from the simple 6-bromoindole-3-carbaldehyde, which was isolated from *Pyura sacciformis* and previously found in a marine pseudomonad, to the complex indolocarbazoles of the staurosporine type isolated from ascidians belonging to the family Polycitoridae (Rudi et al. 2000; Schupp et al. 2001; Schupp, Proksch, and Wray 2002; Aiello et al. 2003; Garrido et al. 2003; Gompel et al. 2004; Trardy et al. 2004; Seldes et al. 2007; Reyes et al. 2008; Henrich et al. 2009; Aiello et al. 2010; Takada et al. 2010).

Tyrosine is the precursor of a large number of alkaloids whose structures are characterized by the Ar-C2-N subunit derived from Tyr, commonly via dopamine; often, additional Ar-C1 and Ar-C2 moieties are present, which are derived from a partial degradation of the amino acids Phe or Tyr. The aromatic ring of all these subunits is usually oxygenated at '4, 3,4- or 3, 4, 5 positions. The amino acid 2-amino,3-(3',4'-dihydroxyphenyl) propionic acid (dopa), in particular, appears to play an important role in the metabolism of ascidians, serving as the apparent precursor of not only peptide products but also unique alkaloid structures, such as those of lamellarins and ecteinascidins. Simple aromatic alkaloids, derived directly or indirectly from phenylalanine, tyrosine, phenylethylamine, tyramine, or dopamine, as well as complex, highly condensed structures fall into the group of tyrosine- and phenylalanine-derived alkaloids (Faulkner 2002).

Alkaloids based on a 2-amino-3-hydroxyoctadecane moiety, generally referred to as lysine-derived metabolites, have been found in several genera of ascidians. Ascidians have also been the source of protoalkaloids, which are simple amines in which the nitrogen is not in a heterocyclic ring. *Clavelina* and *Pseudodistoma* genera have been prolific in the production of linear 2-aminoalkanols and their unsaturated and/or acetylated derivatives. Structurally, these compounds are related to

sphingosine derivatives, which are central structural elements of sphingolipids and important constituents of the lipid portion of cell membranes in living organisms. The carbon chain length of these sphingolipid derivatives vary from C12 to C18 amino alcohols (Kossuga et al. 2001; Garrido et al. 2001; Aiello et al., 2007, 2009).

## 15.6 NONNITROGENOUS COMPOUNDS

More than 20% of natural products derived from ascidians fall into the category of nonnitrogenous compounds. These compounds are mostly terpenoid or steroid in origin and are well represented across most ascidian families. The sterol mixtures isolated from tunicates, analogously to other marine invertebrates, are very complex, contrary to those in vertebrates and to most of the evolutionary higher invertebrates. Only sterols from the class Ascidiacea, sub-order Stolidobranchiata, have been investigated so far. Recently, the sterol compositions of tunicates were compared and a chemotaxonomic scheme of tunicates was proposed (Slantchev et al. 2002). It appeared that sterol compositions of tunicates have some common characteristics such as presence of significant amounts of stanols, including in some cases coprostanol-type stanols. Cholesterol and cholestanol were assumed to be the main sterols, followed by brassicasterol (Ballantine, Lavis, and Roberts 1977). Ergosterol-type sterols and 4-methyl sterols were also found (Zollo et al. 1986). According to their sterol composition, tunicates were proposed to be divided into three groups: (1) the first group included tunicates from the families Tethyidae and Pyuridae; (2) the second group included the families Ascidiidae, Botryllidae, Cionidae, and Molgulidae; and (3) the third group included the family Styelidae. These conclusions were based on a limited number of investigated species, and future investigations are necessary to confirm or reject the proposed classification. Sterols, volatiles, and lipids were isolated and identified from lipophilic extracts from two tunicates, *Styela* sp. and *Phallusia* sp., occurring in the Eastern Mediterranean (Slantcheva et al. 2002).

Marine organisms produce a wide array of fascinating terpenoid structures distinguished by characteristic structural features. Yezoquinolide and sargachromenol were originally reported to be from marine brown algae such as *Sargassum sagamianum* var. *yezoense* and *Sargassum serratifolium*. However, the tuberatolides yezoquinolide and sargachromenol have now also been found in the Korean marine tunicate *Botryllus tuberatus*. Therefore, it is possible that these terpenoids are synthesized by symbiotic marine microorganisms in brown algae and tunicates. Alternatively, it is also possible that the source organisms may have similar biosynthetic genes that dictate the biosynthesis of yezoquinolide and sargaquinoic acid, the precursor of sargachromenol. The isoprenoid tuberatolide A; a pair of diastereomeric meroterpenoids, tuberatolide B and 2'-*epi*-tuberatolide B; and farnesoid X receptor (FXR) were isolated from the Korean marine tunicate *B. tuberatus*, along with some known eroterpenoids. In recent studies, FXR has been reported to be a promising drug target in the treatment of atherosclerosis (Choi et al. 2011).

## 15.7 BIOACTIVITY

Most recently, ascidians have increasingly become the target of natural products research. A large number of natural products has been isolated from ascidians and tested for various biological activities, especially from colonial ascidians. In relation to natural products, members of Aplousobranchia, particularly Polycitorids and Didemnidids, are the best represented group with more than 80% of ascidian species from which natural products have been isolated. Didemnum ascidians are excellent sources of novel biologically active compounds of varied biosynthetic origin. Cytotoxicity of the ascidian metabolite is the most frequently listed agent against a variety of tumor cell lines, followed by antimicrobial and anti-inflammatory activities.

### 15.7.1 ANTIBACTERIAL ACTIVITY

Antibacterial resistance is a threat of global magnitude having considerable impact on mortality and health care-associated costs. The problem has recently been worsened by the steady increase in multiresistant strains and the restriction of antibiotic discovery and development programs. The common pathogenic bacteria, which include *Escherichia coli*, *Klebsilla pneumoniae*, *Haemophilus influenzae*, *Streptococcus pneumoniae*, and *Proteus vulgaris*, are the major causative agents of nosocomial infections (Saonuam et al. 2008). Generally, nosocomial infections develop in the respiratory tract (Nicholls, Pease, and Gree 1975) and the urinary tract (Blunt et al. 2007). Since the early days of marine natural product discovery, Porifera (sponges) and Chordata (including ascidians) have dominated the scene as the major contributing phyla of novel bioactive compounds (Rinehart et al. 1984). A number of bioactive compounds have also been isolated from ascidians, exhibiting activities such as antiviral (Moquin-Pattee and Guyot 1989), cytotoxic (Azumi et al. 1990), antibacterial, and enzyme inhibitory activities (Sato et al. 1995). These compounds mainly comprise various derivatives of alkaloids and peptides.

The well-known microbial antibiotic enterocin was isolated from *Didemnum* sp. Polycarpamine B from the solitary ascidian *P. aurata* was found to be a significant inhibitor *in vitro* of the fungi *S. cerevisiae* and *C. albicans* (Lindquist and Fenical 1990). A much smaller tetrapeptide, possessing both dehydrotryptophan and dopa units, has been isolated and characterized from the hemocytes of the solitary ascidian *H. roretzi* (Azumi et al. 1990). Morulin PM was isolated as an unusual posttranslationally modified peptide possessing both topa (3,4,5-trihydroxyphenylalanine) and 6-bromotryptophan residues from the morula cells of the vanadium-accumulating ascidian *Phallusia mammillata* by Taylor et al. (1997). Structural studies revealed that the C-terminal residue of the peptide possesses a dehydrotopamine residue. The presence of both dehydrotopamine unit and 6-bromotryptophan in the same peptide indicates the likelihood of antibacterial activity for this compound. However, test results have not supported this hypothesis so far, although more systematic studies may resolve this issue. In this regard, it is interesting to note that the octapeptide called plicatamide, possessing the structure Phe-Phe-His-Leu-His-Phe-His-dehydrodopamine, was isolated from the tunicate *Styela plicata* and shown to possess powerful antibiotic activity against *Staphylococcus aureus* (Tincu et al. 2003). Both wild-type and methicillin-resistant *S. aureus* strains exhibited massive efflux of potassium ions on exposure to plicatamide. Within seconds, bacterial strains exposed to plicatamide ceased to consume molecular oxygen and became nonviable. Plicatamide was also shown to be a potent hemolytic agent against human erythrocytes, but it had no effect on ovine erythrocytes. Its relatively small size, combined with its rapid impact on ion channels, makes plicatamide a powerful lead compound to develop potential antibiotic compounds in the future. The hemocytes of *Styela clava* possess a 32-amino acid antimicrobial peptide, called styelin D, having multiple dopa units (but no dehydrodopa units) (Tincu and Taylor 2004). Styelin D inhibited the growth of both gram-positive and gram-negative bacteria and exhibited hemolytic and cytotoxic properties against eukaryotic cells. The remaining group of stylins (comprising A, B, C, and E, all isolated from *S. clava*) forms pores in bacteria causing leaching of nutrients and eventual cell death. Two other dopa- and topa-containing peptides, halocyamines and ferreascidin, have also been isolated from tunicates.

### 15.7.2 ANTINEOPLASTIC AND ANTITUMOR ACTIVITY

The marine biosphere has long held great promise as a source of anticancer compounds. Although a number of screening efforts have indicated a much higher percentage of antineoplastic/antitumor activity in marine organisms than in terrestrial plants, only recently have marine natural products made their first appearance in clinical trials at the National Cancer Institute, Maryland—first, the didemnins, and then the bryostatins from ascidians (John 1986).

The first group of marine compound possessing dehydrotyrosine unit is botryllamides isolated from *Botryllus* sp. (McDonald et al. 1995). They were isolated from two species of ascidians, *Botryllus* sp. from the Philippines and *B. schlosseri* from the Great Barrier Reef of Australia. They showed mild cytotoxicity against the human colon cancer cell line HCT-116. In a bioassay-directed survey, Barenbrock and Kock (2005) identified tubastrine as the active compound from several ascidians that showed inhibition of epidermal growth factor receptor using a protein tyrosine kinase assay. Tubastrine was isolated as the principal inhibitory compound from the ascidian *Dendrodoa grossularia*. Pearce et al. (2008) identified tubastrine and a number of its dimeric products, called orthidines, from the ascidian *Aplidium orthium*. Henrich et al. (2009) used a different species of *Botryllus* (*B. tyreus*) and isolated a number of botryllamides, all possessing dehydrotyramine units. These compounds are apparently derived from a tyrosyl tyrosine dipeptide by modification. Botryllamides proved to be effective inhibitors of the ATP-binding cassette (ABC) transporter called ABCG2, which is associated with multidrug resistance. They were selective in inhibiting ABCG2 (also known as breast cancer-resistant protein [BCRP]) and not the two other types of transporters (ABCB1-encoding P-glycoprotein [P-gp] and ABCC1-encoding multidrug-associated protein 1 [MDR-1]) (Takada et al. 2010).

Lissoclimides, cytotoxic diterpenes from *Lissoclinum voeltzkowi*, were isolated by Biard et al. (1994). Hemocytes from the ascidian *Ciona intestinalis* are able to lyse rabbit, human, guinea pig, and sheep erythrocytes *in vitro* (Parrinello et al. 1995). Polycarpine dihydrochloride, a new cytotoxic dimeric disulfide alkaloid, and four related compounds have been isolated from extracts of the ascidian *Polycarpa clavata* (Kang and Fenical 1996). The *in vivo* antitumor activity of the dimeric disulfide alkaloid polycarpine isolated from the ascidians *P. clavata* (Kang and Fenical 1996) and *P. aurata* (Abas et al. 1996) and related synthetic analogs has been investigated (Popov et al. 2002).

Tramandarins A and B, new cytotoxic depsipeptides from Brazilian ascidians of Didemnidae, were evaluated against human cancer cell lines (Vervoort and Fenical 2000). Sebastianines A and B isolated as biologically active pyridoacridine metabolites, which show cytotoxic activities toward colon cancer cells, have been extracted from a Brazilian collection of the ascidian *Cystodytes dellechiaiei* (Torres et al. 2002). The identified ecteinascidins exhibited potent cytotoxicity toward tumor cell lines and growth inhibition of *Mycobacterium tuberculosis* H37Ra. The sulfated steroid was found to be responsible for sperm activation and attraction in Japanese collections of the ascidians *Ciona intestinalis* and *C. savignyi* (Yoshida et al. 2002). A study of the Thai ascidian *Ecteinascidia thurstoni*, using a potassium cyanide–pretreatment isolation procedure, identified two known alkaloid ecteinascidins and two novel analog ecteinascidins (Suwanborirux et al. 2002). Pearce et al. (2007) reported that Ascidiathiazones A from New Zealand ascidians *Aplidium* sp. inhibited the *in vitro* production of superoxide by PMA-stimulated human neutrophils in a dose-dependent manner. Martinez-García et al. (2007) described the *in vitro* antiproliferative activity against different tumor cell lines of ascidian extracts and provided some insights into the role of the microbial community associated with tunicate in the production of these compounds. It is noted that *C. dellechiaiei* extracts showed remarkably high antiproliferative activity in human lung carcinoma A-549, colon adenocarcinoma H-116, pancreatic adenocarcinoma PSN-1, and breast carcinoma SKBR3 cell lines.

### 15.7.3 PLANT GROWTH REGULATORY ACTIVITY

There have been a number of reports on the discovery of growth regulators in marine algae, but most of these studies comprised qualitative analyses for known growth regulators. More recently, a number of studies have indicated the presence of growth-promoting compounds of unknown structure in some algae. In contrast, little attention has been focused on growth regulators in sessile invertebrates. When one considers the intense competition for space on rocky and coralline substrates, the presence of growth regulators in invertebrates certainly seems plausible. Some organisms overgrow others, whereas some resist overgrowth. Chemical constituents are probably responsible for these competitive advantages. The tunicate (BT-II) extract is a potent inhibitor of root growth in seedlings (John 1986).

#### 15.7.4 INSECT CONTROL

There are a number of examples of chemical defense against predation or herbivores in the literature on marine natural products, and similar patterns have been found in a number of terrestrial plant–insect relationships. If a compound were found to be toxic to or inhibit feeding by marine invertebrates, might that compound not exert the same effects or similar effects on invertebrates in the terrestrial biosphere? To explore this possibility, an examination of the response of insects to marine natural products with suspected or demonstrated antifeedant behavior was initiated. Zeti et al. (2001) reported that methanol extract of seven species of Malaysian tunicates such as *Clavelina picta*, *Eudistoma obscuratum*, *Didemnum molle*, *Atrilolum robustum*, *Phallusia* sp., *Didemnum* sp., and *Aplidium* sp. showed insecticidal activity against *Anopheles maculatus* and *Aedes aegypti* comparable to that of DDT, indicating that the tunicates were as effective as the synthetic insecticide in killing the mosquitoes.

#### 15.7.5 ANTI-INFLAMMATORY ACTIVITY

Inflammation is a common characteristic of many debilitating human diseases including gout, rheumatoid arthritis, asthma, and chronic obstructive pulmonary diseases. Many of the current treatments involve the use of nonsteroidal anti-inflammatory drugs (NSAIDs) that target the cyclooxygenase and phospholipase enzymes in the inflammatory cascade. Although these therapies can be effective, some individuals fail to respond to treatment or experience cardiovascular and gastrointestinal side effects. Refractory disease and poor tolerance to side effects highlight a need for alternative anti-inflammatory treatments. The genus *Aplidium* is one of the largest genera of ascidians and is found worldwide (Tan and Berridge 2000). While screening for anti-inflammatory natural products from New Zealand biota, an extract of the ascidian *Aplidium* sp. exhibited strong inhibition of superoxide production by human neutrophils stimulated with phorbol myristate acetate (PMA) (Pearce et al. 2007). A wide variety of biologically active compounds have been reported from *Aplidium* sp. including simple prenylated quinones and hydroquinones that exhibit antiproliferative activities. Lepadin D with an unidentified counterion and lepadins E and F were isolated as antiplasmodial and antitrypanosomal alkaloid constituents of a *Didemnum* spp. ascidian collected from Stanley Reef, the Great Barrier Reef. Coproverdine is a cytotoxic alkaloid isolated by bioassay-directed fractionation of an unidentified ascidian collected from the Three Kings Islands, New Zealand. Many inflammatory diseases, including gouty, arthritis and nonatopic asthma, are associated with the infiltration of neutrophils and the subsequent production of damage-causing superoxides. Therefore, inhibition of superoxide production by human neutrophils represents an alternate target of acute inflammatory response (Levesque, Brophy, and Zhang 2005).

#### 15.7.6 ANTIFOULING ACTIVITY

The control of foulers on artificial surfaces, such as the hulls of vessels, intake pipes, and drilling structures, is expensive. Organotin-based antifouling paints are demonstrably damaging to the environment and may include impacts on commercially harvested species. There is a clear need to develop new, more environmentally benign solutions to the problem of fouling.

Natural products or extracts derived from a range of marine invertebrates have been tested for their ability to dissuade the settlement of marine invertebrates. Only a small number of natural products derived from ascidians have demonstrated antifouling activity. The deterrent effects of eudistomins G and H, two closely related β-carbolines isolated from *Eudistoma olivaceum*, have been examined. Another compound, homarine, has been isolated from *Aplidium nordmanni* and *Botryllus leachii*. Although these are the only natural products for which antifoulant activity has been demonstrated, numerous studies with crude extracts from ascidians indicate that there may be considerable potential for the discovery of further active natural products. Among the 52 members of genera of Aplousobranchia, about 20 members showed potential antifoulant activity; they are followed by Stolidobranchia (18%) and Phlebobranchia (12%).

### 15.7.7 ANTIOXIDANT ACTIVITY

Antioxidants play an important role in the prevention of chronic ailments such as heart disease, cancer, diabetes, hypertension, stroke, and Alzheimer's disease by protecting the cells from damage caused by "free radicals," which are highly reactive oxygen compounds. In healthy individuals, the production of free radicals is balanced by the antioxidative defense system; however, oxidative stress is generated when equilibrium favors free radical generation as a result of depletion of antioxidant levels.

Many antioxidant compounds as candidates or synthetic models were isolated for the development of drugs applied to treatment of the aforementioned diseases. Sato et al. (1989) isolated one novel chromene and two novel hydroquinones from the extract of the colonial tunicate *Amaroucium multiplicatum* and reported that these compounds were more potent than two standard antioxidants on the inhibitory effects on lipid peroxide formation in rat liver microsomes and on soybean 15-lipoxygenase. Antioxidants, prenylated hydroquinones and nonactive chromene or chroman, extracted from the marine colonial tunicate, *Aplidium californicum* have been found to inhibit superoxide anion production in rat alveolar macrophages and in the xanthine/xanthine oxidase system (Cotelle et al. 1991). Three hydroquinone compounds have been isolated from the Indian Ocean tunicate *Aplidium savignyi* in addition to geranylhydroquinone and the 2-(3-droxy-3,7-dimethyloct-6-enyl)-1,4-benzenediol (Aknin et al. 1999).

### 15.7.8 DETERRENT ACTIVITY

Many benthic ascidians are relatively free from predation as they are exhibiting mechanical. Mechanical defenses such as tough tunics or spicules and chemical defense may deter predators. Although these species may be chemically defended against most generalized predators, it may be vulnerable to specialized predators. These specialists could, in fact, be attracted to the very chemicals that deter other predators, and these chemicals may be incorporated into the specialists' own defense.

An exhaustive survey of the literature indicated that the ascidians have been targeted for new pharmacological drugs all over the world. Only a meager number of studies have been reported from the Indian coast, although more than 400 species including 65 new species have been recorded from this area (Jaffar Ali and Sivakumar 2007; Abdul Jaffar Ali, Sivakumar, and Tamilselvi 2009, 2010, 2011). Abdul Jaffar Ali, Sivakumar, and Tamilselvi (2008) isolated a tyrosine-derived antibacterial compound from *Phallusia nigra* at Tuticorin coast, India. Ananthan et al. (2011) reported the presence of novel drugs acting against human urinary tract infectious pathogens.

## 15.8 CONCLUSIONS

An intensive research effort during the past two decades has generated an impressive number of bioactive compounds isolated from marine ascidians, which remain unique among marine invertebrates. Many of these compounds exhibit biomedically important activities; among them, cytotoxicity is the most frequently listed activity. The intense pressure to find and develop more profitable molecules for all sorts of industries continues to fuel the bioprospecting of marine invertebrates. This chapter shows that many ascidian species are promising sources of marine bioactive compounds of medical, economic, and scientific interest. Aplidin, didemnin B, and Yondelis are good examples of current biotechnological metabolites employed as anticancer drugs. In the present study, only 10% of extant ascidian species are represented, displaying by far the highest number of promising MNPs. Antitumor drugs are the main area of interest in the screening of MNPs from ascidians. There is considerable potential for the development of new pharmacologically active compounds from ascidians. There is much evidence that underscores this potential.

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# 16 Chitosan and Its Derivatives for Treatment of Diabetic Complications

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## 16.1 INTRODUCTION

### 16.1.1 CHITOSAN AND DERIVATIVES

Chitosan is a functional and basic linear polysaccharide prepared by N-deacetylation of chitin in the presence of alkaline. In general, deacetylation cannot be completely achieved even under harsh treatments. The degree of deacetylation usually ranges from 70% to 95%, depending on the method used. Thus, chitosan is available with various molecular weights and deacetylation degrees. Chitosan is insoluble in water, alkali, and organic solvents, but is soluble in most solutions of organic acids when the pH of the solution is below 6. The industrial production and application fields of chitosan have been steadily increasing since 1970s. Early applications of chitosan have been centered on the treatment of wastewater, heavy metal adsorption, food processing, immobilization of cells and enzymes, resin for chromatography, functional membrane in biotechnology, animal feed, and so on. The recent trend is toward producing high-valuable industrial products such as cosmetics, drug carriers, and pharmaceuticals. Chitin and chitosan are known to exhibit antitumor, antibacterial, hypcholesterolemic, and antihypertensive activity (Kim and Rajapakse 2005). The main motive for the development of new applications for chitosan lies in the fact that it is a very abundant polysaccharide, as well as nontoxic and biodegradable. Despite its functions and importance as a biomaterial, the applications of chitosan in food and biomedical industries are narrowed owing to its poor solubility, high molecular weight, and viscosity. There is evidence about the nonabsorption or indigent absorption of chitin and chitosan in the human intestine due to lack of enzymes to cleave the  $\beta$ -glucosidic linkage in chitosan. Since chitosan is a large water-insoluble biopolymer, it is difficult for the mammalian body to absorb it. In this respect, enzymatic hydrolysis of chitosan to obtain oligomers is of great interest recently (Jeon and Kim 2000).

Chitosan oligosaccharides (COS) are hydrolyzed derivatives of chitosan composed of  $\beta$ -(1-4) D-glucosamine units. They have better properties such as lower viscosity, relatively smaller molecular size than chitosan, and short chain length with free amino groups, which makes COS highly soluble in aqueous solutions. COS are effective agents for lowering blood cholesterol and pressure, controlling arthritis, and enhancing antitumor properties (Kim and Rajapakse 2005). Since COS are biodegradable, water-soluble, and nontoxic compounds (Qin et al. 2006), they might be beneficial biomaterials for diseases with increasing morbidity and mortality rates, such as diabetes and obesity.

Since diabetes is a chronic disease, it must be kept under control by improving impaired insulin secretion from  $\beta$ -islet cells (pancreas) or elevating insulin efficiency on several tissues. Diabetic disorders, especially hyperglycemia, can lead to serious damage to many parts of the body—in particular, the nerves and blood vessels (Vinik et al. 2003). The cause of diabetes is not fully known, although it is clearly shown that both genetic and environmental factors, notably obesity, play important roles. Differentiated adipocytes secrete obesity-related factors called adipokines. Plasma leptin, tumor necrosis factor- $\alpha$ , and nonesterified fatty acid levels are all elevated in obesity and play a role in causing insulin resistance (Leong and Wilding 1999). Therefore, suppression and regulation of obesity can be achieved by inhibiting adipocyte differentiation and forcing adipocytes to lipolysis to reduce accumulated white adipose tissue (Yamauchi et al. 2001; Langin 2006). Thus, the increased control of the harmful effects of the accumulation of adipose tissue and its metabolism contributes to the search for a better understanding of the prevention of diabetes.

### 16.1.2 DIABETES

It is well known that most of the diabetic complications and impaired cell function in type 2 diabetes are mediated by hyperglycemia (Williamson et al. 1993; Ceriello 2005). Increasing levels of reducing sugars in the blood under hyperglycemic conditions trigger sets of reactions resulting in formation of reactive oxygen species (ROS), which promote oxidative-stress-induced tissue damage (Poitout and Robertson 2002; Robertson and Harmon 2006). Glucose, as a primary energy source and regulator of cell function, especially induces such reactions. In type 2 diabetes, although patients can retain healthy pancreatic  $\beta$ -cells for many years after the disease onset, chronic exposure to high glucose will impair  $\beta$ -cell function in later stages. Impaired  $\beta$ -cell function leads to cellular damage in patients with type 2 diabetes (Ihara et al. 1999; Robertson et al. 2003). Research into glucose-dependent reactions in pancreatic cells indicates that glucose can harm  $\beta$ -cell function by producing ROS through insulin secretion and glycation pathways (Robertson 2004). In addition, pancreatic  $\beta$ -cells are already known to be more susceptible to damage from oxidative stress than other tissues, due to low expression of antioxidative enzymes (Lenzen, Drinkgern, and Tiedge 1996). Therefore, due to glucose-related oxidative stress,  $\beta$ -cells can lose their ability to synthesize insulin and enter an apoptotic stage upon exposure to hyperglycemic conditions (Donath et al. 1999). As a result, this type of oxidative stress is liable for deterioration of pancreatic  $\beta$ -cells in the later stages of type 2 diabetes. It is also shown that  $\beta$ -cells can be protected from tissue damage or apoptosis by treatment with chemical antioxidants such as NAC and cysteine (Tanaka et al. 1999).

With a long onset and serious complications, which usually result in a high morbidity rate, the treatment of diabetes is a major concern in all countries. Up to now, many kinds of antidiabetic medicines from natural resources have been developed for patients (Ivorra, Paya, and Villar 1989; Grover, Yadav, and Vats 2002; Koski 2004; Li et al. 2004), but most of these biochemical agents are not suited for mass production to meet to be a pharmaceutical agent. The natural compounds demonstrate a significant practice and show bright potential in the treatment of diabetes and its complications with their naturally occurring structure and relatively fewer side effects. In this respect,

chitin, chitosan, and its derivatives with available large numbers of different chemical structures and bioactivities offer a great potential to recover from and/or to prevent obesity and diabetes.

## 16.2 DERIVATIZATION

Chitosan and its monomer glucosamine are highly derived recently in order to find new natural compounds with higher bioactivity than their predecessors (Fenton et al. 2000; Jiang et al. 2007; Prabaharan 2008). The main derivation of chitosan is forming soluble forms of chitin, which makes it more biofriendly and easily absorbed by the body after oral administration (Kuroiwa et al. 2002; Hai et al. 2003; Il'ina and Varlamov 2004; Mao et al. 2004). Bioefficiency of chitin mainly depends on its absorption in human body. Therefore, derivation of water soluble forms of chitin which can easily be absorbed through body opened up new angles for chitin derivation toward novel bioactive compounds. In this respect, chitosan is the main derivative of chitin. Rather than chitosan, COS are highly bioactive derivatives of chitosan with significantly higher absorption rates and water solubility (Qin et al. 2002).

Besides oligomerization, another main derivation for chitin and its monomer glucosamine is adding negatively and/or positively charged side chains. In this manner, glucosamine, chitin, chitosan, and COS are reformed under chemical conditions to give sulfated, phosphorylated, carboxymethyl, deoxymethyl derivatives, and so on (Kochkina and Chirkov 2000; Fei Liu et al. 2001; Huang, Khor, and Lim 2005; Kim, Park, et al. 2005; Je and Kim 2006; Cho et al. 2011; Kim et al. 2010). This diversity of derivatives comes with a high variety of bioactivities, including improvement in the effectiveness of the compound in the case of already reported activities.

## 16.3 ANTIDIABETIC ACTIVITY

Chitosan-based products are known to have many biological activities, such as antitumor, anti-HIV, antifungal, and antibiotic, as well as activities against oxidative stress (Kendra and Hadwiger 1984; Nishimura et al. 1998; Xie et al. 2001; Kim et al. 2008; Artan et al. 2010). The activities can be grouped into two according to the use of chitin-based products. These products are highly used as indirect helping agents to enhance the effectiveness of other active compounds through chemical modification or nonchemical linkage against diabetes and obesity. On the other hand, the main role of chitin-based products is that they act as therapeutic nutraceutical agents directly against diabetes and obesity. In both cases, derivatives of these natural products express significant potential in the search for bioactive pharmaceuticals against obesity and obesity-related diabetes.

### 16.3.1 INDIRECT ACTIVITY

The preferred route of drug administration for patients is mostly the oral route for chronic therapy of diseases and complications. However, delivery of many therapeutic peptides and proteins through the digestive system is still an unsolved problem basically because of the size, hydrophilicity, and unstable conditions of these molecules. Thus, several chitosan derivatives have been developed over the years with improved properties for enhanced applicability (Fernandez-Urrusuno et al. 1999; Thanou, Verhoef, and Junginger 2001). Therefore, recent studies have focused on carrier products for administration of insulin efficiently in pre- or postdiabetic patients, and lately one of these products is a chitosan derivative. Portero et al. (2007) have reported that chitosan sponges are quite successful in buccal administration of insulin. Moreover, up-to-date studies presumed that chitosan-derived particles are intensely usable for insulin administration orally with their high protective effect and harmless structure (Hari, Chandy, and Sharma 1996; Krauland, Guggi, and Bernkop-Schnurch 2004; Krauland, Guggi, and Bernkop-Schnurch 2006). Results of some related studies have suggested that the observed drug delivery activity of chitosan is highly promising in the case of insulin. For example, studies have shown that chitosan–insulin nanoparticles have a

strong affinity to rat intestinal epithelium 3 h after oral administration (Ma, Lim, and Lim 2005). This suggests that chitosan as a cofactor for drug delivery makes insulin absorption safe and rapid. Carboxymethyl-hexanoyl chitosan is an amphiphilic chitosan derivative with important swelling ability and water solubility under natural conditions, and studies have shown that these hydrogels can be used for encapsulating the poorly water-soluble drugs for effective drug delivery (Liu and Lin 2010), which opens the way for efficient insulin delivery by chitosan derivatives. Furthermore, Mao et al. (2005) have shown that polyethylene glycol (PEG)-trimethyl chitosan complexes are efficiently coupled with insulin and easily taken up by Caco-2 cells.

Besides the drug delivery activity for insulin, studies have shown that chitosan complexes can be efficiently used for gene delivery for gene therapy (Koping-Hoggard et al. 2001). Therefore, it can be easily adduced that chitosan complex derivatives are potent gene delivery targets for prevalent diseases such as diabetes. Furthermore, it has been reported that these chitosan complexes possess higher uptake and transfection efficiency than other polysaccharide complexes used for both drug and gene delivery (Huang, Khor, and Lim 2004). Several studies have been conducted to prove chitosan can be a nontoxic alternative to other cationic polymers, and results have demonstrated a prominent potential for further studies of chitosan-based gene delivery systems (Sato, Ishii, and Okahata 2001). All these results suggest that chitosan and chitosan-based derivatives are notable steps towards to invention of a harmless agent for drug and gene delivery, which is extremely crucial for diabetic patients' improved life standards.

Moreover, studies on streptozotocin (STZ)-induced diabetic rats have expressed that chitosan-based sponges are highly effective at healing diabetic wounds in addition to treatment of diabetic patients. Wang et al. (2008) suggest that application of chitosan–collagen complex is an ideal wound-healing cover to enhance recovery of healing of wounds such as diabetic skin wounds, which provides great potential for chitosan and its derivatives to be used clinically for diabetic patients.

To conclude, chitosan-based polymers show great potential for treatment of diabetes therapeutically with their efficient drug and gene delivery properties as well as effectiveness on diabetic wound healing.

### 16.3.2 DIRECT ACTIVITY

Overweight and obesity, two common health-threatening conditions, are considered to result in diabetes worldwide, but there are not enough treatments. Therefore, studies of chitosan focus on its fat-lowering and fat-preventing activities. Several researchers have demonstrated that chitosan tends to bond with the ingested dietary fat and carry it out in the stool while preventing its absorption through the gut (Kanauchi et al. 1995). Relevant research about the fat-lowering activity of chitosan also has shown that chitosan is capable of absorbing fat up to five times its weight. In respect to these results, there are several studies showing that chitosan derivatives lower the levels of low-density lipoproteins (LDL) while increasing those of high-density lipoproteins (HDL). Studies of chitosan and its fat-lowering activity have expressed that chitosan and its derivatives are highly effective hypocholesterolemic agents with the ability of decreasing blood cholesterol level up to as much as 50% (Maezake et al. 1993; Jameela, Misra, and Jayakrishnan 1994). Moreover, studies on patients with diabetes have clearly shown that daily administration of chitosan could drop blood cholesterol levels by 6% with an increased level of HDL. Additionally, COS, an oligomerized derivative of chitosan, show high activity in regulating blood cholesterol levels. Studies have reported that COS are capable of regulating cholesterol levels even in the liver. COS prevent the development of fatty liver caused by the action of hepatotropic poisons. A few studies have been carried out to investigate the action mechanism of COS in regulating the serum cholesterol level, and several of them have suggested a possible mechanism of COS lowering the LDL levels. As Remunan-Lopez et al. (1998) suggest, the ionic structure of COS binds bile salts and acid, which inhibit lipid digestion through micelle formation. However, Tanaka et al. (1997) suggest

a different mechanism of chitosan and COS where lipids and fatty acids are directly bonded by chitosan.

In addition to the fat-lowering mechanisms of chitosan and its derivatives, studies have also proven that chitosan administration can increase the insulin sensitivity of animal models (Neyrinck et al. 2009). It has been shown that 3-month administration of chitosan significantly increased the insulin sensitivity in obese patients and expressed a highly notable decrease in body weight and triglyceride levels (Hernandez-Gonzalez et al. 2010).

On the other hand, glucosamine and its derivatives are reported to be highly effective at inhibiting adipogenesis *in vitro*. Recent studies have shown that phosphorylated derivatives of glucosamine inhibited the adipogenesis of 3T3-L1 cells as well as fat accumulation (Kim et al. 2010). Several studies have suggested that the acetylated chitin treatment causes adipocytes to break down fats and lower their triglyceride accumulation as much as half of control cells (Kong et al. 2011). Kong, Kim, and Kim (2009) have demonstrated clearly that sulfated derivatives of glucosamine inhibited the proliferation and adipogenesis mechanism through AMPK pathways in 3T3-L1 cells. Glucosamine and acetylated-, sulfated-, and phosphorylated-glucosamine derivatives are reported as successful adipogenic inhibitors with an intense potential to prevent weight gain by adipogenesis in patients who are at risk for diabetes. Furthermore, it has been reported that COS inhibit the fat accumulation and adipogenesis in the 3T3-L1 cell line (Cho et al. 2008). In addition, studies have shown that treatment with glucosamines reduced the triglyceride content of adipocytes and enhanced glycerol secretion as a lipid-lowering effect. Most of these studies have expressed the better activity of chitosan-based compounds such as COS and glucosamines, after derivation by adding a charged side chain by phosphorylation and sulfation. Therefore, it can be suggested that the cationic power of glucosamine and COS plays the main role in their antiobesity effect. Further, a selective synthesis of phosphorylated or sulfated derivatives of chitosan and glucosamine will open up the way to a better understanding of the structure–mechanism relation. However, current research has presented strong evidence that chitosan shows its antiobesity effect through the PPAR- $\gamma$  pathway of adipogenic differentiation and results in fewer adipocytes and lower lipid accumulation. Collectively, chitosan and its derivatives, such as glucosamine and COS, successfully inhibit the differentiation of cells into adipocytes as well as enhance adipocytes to hydrolyze the triglycerides that show a significant effect against lipid accumulation of the body. This effect of chitosan and its derivatives demonstrates an important impact against obesity in the way of diabetes progression. Hence, they show a great amount of potential to be used as pharmaceutical agents.

Furthermore, chitosan and its oligosaccharides act as antidiabetic agents for treatment of diabetes by protecting pancreatic  $\beta$ -cells. In type 2 diabetes, although patients can retain healthy pancreatic  $\beta$ -cells for many years after the disease onset, chronic exposure to high glucose will impair  $\beta$ -cell function in later stages. Impaired  $\beta$ -cell functionality leads to cellular damage in patients with type 2 diabetes (Ihara et al. 1999). Therefore, protection of  $\beta$ -cells is quite important for elevated insulin secretion as a part of diabetes treatment. Recent studies have reported COS as a protective agent for pancreatic  $\beta$ -cells against high-glucose-dependent cell deterioration (Karadeniz et al. 2010). It is suggested that, at the same time, COS could effectively accelerate the proliferation of pancreatic islet cells with elevated insulin secretion to aid in the lowering of blood glucose levels. Liu et al. (2007) have reported that COS treatment could improve the general situation and diabetic symptoms of rats, decrease blood glucose levels, and normalize the impaired insulin sensitivity. Moreover, COS have been reported as a preventive agent against type 1 diabetes in nonobese diabetic mice, which might be related to several bioactivities of COS (Cao et al. 2004). These results support the hypothesis that COS can prevent pancreatic  $\beta$ -cells of diabetic patients and normalize crucial insulin secretion. The mechanism behind this protection has been studied and is related to the immunopotentiation and antioxidation activity of COS.

Renal failure is one of the most common diseases caused by diabetes mellitus. The metal cross-linked complex of chitosan, chitosan–iron (III), has been recently reported to be highly active in reducing phosphorus serum levels to treat chronic renal failure (Schoninger et al. 2010). This

relatively new derivative of chitosan is significantly capable of adsorbing serum phosphorus in alloxan diabetes-induced rats with symptoms of renal failure progression.

Moreover, recent studies indicate that patients with diabetes may be at a higher risk for blood coagulation than nondiabetic persons. This life-threatening condition urges to be treated for patients with diabetes. Therefore, the sulfated derivative of chitosan has been shown to possess an anticoagulant potency (Vongchan et al. 2002). Furthermore, studies have reported that sulfated chitosan does not show antiplatelet activity unlike heparin, which is an effective anticoagulant agent. Collectively, results prove that sulfated chitosan is a more efficient agent than heparin, although heparin has been used for a long time for blood coagulation treatment.

In addition to COS, chitosan has also been reported to prevent the development and symptoms of non-insulin-dependent diabetes in rats as well as the complications of STZ induction (Kondo et al. 2000). Briefly, reports suggest that chitosan products protect pancreatic cells and insulin secretion mechanism in diabetic conditions. Furthermore, these compounds can decrease the progression and complication rate of diabetes onset in animal models, demonstrating great potential for chitosan products to be used as a nutraceutical for the treatment of diabetes.

## 16.4 CONCLUSIONS

High mortality and morbidity rates of diabetes make the diagnosis, prevention, and treatment more important as more and more patients have been diagnosed with diabetes in the world in recent years. Besides diabetes, factors relating to diabetes, such as obesity and damaged pancreatic cells, must be kept under control in order to prevent diabetes onset. In this manner, chitosan and its derivatives possess various biological activities and have a remarkable potential to be used in several therapeutic applications. Thus, many of the studies carried out to search for antidiabetic activities of chitosan-based compounds provide detailed acting mechanisms and activity for prevention and/or treatment of diabetes-based complications. Chitosan and its derivatives such as COS and glucosamines as monomers express high activity in a manner of lowering lipid accumulation and cholesterol as well as pancreatic  $\beta$ -cell prevention. In addition, studies have proved that chemical modification of these compounds could express better activity and enhance understanding of the mechanism lying behind antidiabetic effects. Therefore, future research should be directed to enhance the effectiveness of chitosan-based compounds in order to gain more active and fewer harmful agents. Collectively, this evidence suggests that chitosan-based agents are highly potent nutraceuticals for the treatment and prevention of diabetes and diabetes-related complications.

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# 17 Alkaloids as Pharmaceutical Agents from Marine Fungi

Se-Kwon Kim and Yong-Xin Li

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## 17.1 INTRODUCTION

More than 70% of the Earth's surface is covered by seas and oceans. Their microorganism resources are abundant and partly comprise fungi, actinomycete, bacteria, and so on. These natural resources have only recently been explored for natural products; many of them are biologically active and potentially useful (Bugni and Ireland 2004; Bhakuni and Rawat 2005). Marine organisms live in high salt, high pressure, low temperature, and hypoxia, which are different environments compared with those of terrestrial organisms; hence, marine organisms form a number of secondary metabolites unique in structure (Menna et al. 2011). Alkaloids are a group of naturally occurring, nitrogen-containing, biologically active heterocyclic compounds. In addition to carbon, hydrogen, and nitrogen, alkaloids may also contain oxygen, sulfur, and, more rarely, other elements such as chlorine, bromine, and phosphorus (Knölker 2011). During the last few years, a large number of biologically important alkaloids with antiviral, antibacterial, anti-inflammatory, antibiotic, antioxidant, antitumor, anticancer, and cytotoxic activities have been isolated from marine sources (Kumar and Rawat 2011). More specifically, marine-derived fungi from the marine environment have shown great potential as suggested by the diversity of secondary metabolites. In recent years, numerous marine-derived fungi have been identified, and from them a variety of novel alkaloid compounds have been isolated. In general, more than 1200 new compounds have been isolated and identified from the broth of marine fungi, and they have shown different bioactivity. This chapter summarizes the development of alkaloids isolated from marine fungi, which includes indole, quinazoline, pyridoacridine, diketopiperazine, diterpenes, triazole, dihydroimidazole, and pyrrole alkaloids. These compounds are interesting areas of research for their potential anticancer and antitumor bioactivity.

## 17.2 BIOACTIVE ALKALOIDS FROM MARINE-DERIVED FUNGI

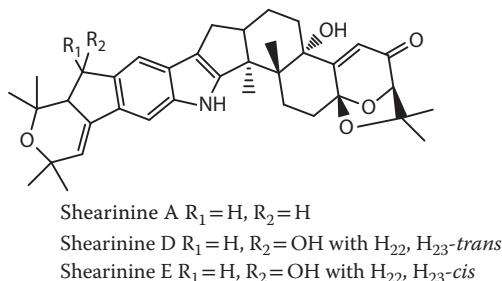
Cytotoxic agents, as anticancer drugs, exert their antitumor activity by interfering with some mechanisms. Therefore, cancer chemotherapy is typically associated with severe side effects. Indole alkaloids are a class of alkaloids containing a structural moiety of indole, including more than 4100 known compounds, and it is one of the largest classes of alkaloids. Many of them possess significant physiological activity and some of them are used in medicine. Therefore, in order to extend the previous studies of these biologically active compounds, this chapter describes the alkaloid isolated from marine-derived fungi against a number of tumor lines. Shearinine A, an indole diterpenoid alkaloid, has been isolated from organic extracts of the sclerotoid ascostromata of *Eupenicillium*

*shearii* (NRRL 3324) (Belofsky and Gloer 1995), and shearinines D and E have been isolated from an endophytic *Penicillium* sp. (strain HKI0459) (Smetanina et al. 2007; Xu et al. 2007). Shearinines A, D, and E (Figure 17.1) induce apoptosis in human leukemia HL-60 cells, and shearinine E inhibits EGF-induced malignant transformation of JB6 P<sup>+</sup> Cl 41 cells in a soft agar.

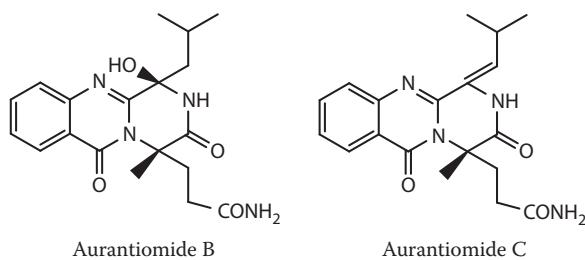
Two quinazoline alkaloids, aurantiomides B and C, have been isolated from the sponge-derived fungus strain *Penicillium aurantiogriseum* SP0-19 (Xin et al. 2007). Aurantiomides B and C (Figure 17.2) have shown moderate cytotoxicities against the HL-60, BEL-7402, and P388 cell lines. 18-Oxotryprostatin A, 14-hydroxyterezine D, and 6-methoxyspirotryprostatin B (Figure 17.3) have been isolated from *Aspergillus sydowii* and have shown weak cytotoxicity against human alveolar basal carcinoma A-549 cells. In addition, 6-methoxyspirotryprostatin B has been active against HL-60 cells (Zhang et al. 2008).

Polyketide-type alkaloids (-)-cereoalactam and (-)-cereoaldomine have been isolated from marine-derived fungus *Coniothyrium cereale*. The protease human leukocyte elastase (HLE) is involved in the pathology of chronic obstructive pulmonary disease, pulmonary emphysema, rheumatoid arthritis, and cystic fibrosis. (-)-Cereoalactam and (-)-cereoaldomine (Figure 17.4) have shown selective inhibition of HLE with IC<sub>50</sub> values of 9.28 and 3.01 μM, respectively (Elsebai et al. 2011).

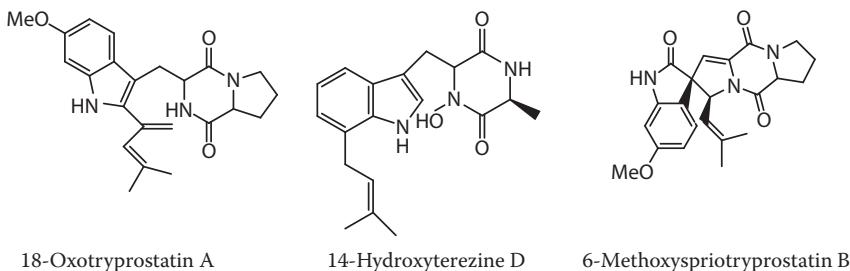
Sorbicillactone A (**Figure 17.5**) is a sorbicillin-derived alkaloid from a saltwater culture of a *Penicillium chrysogenum* strain isolated from a specimen of the Mediterranean sponge *Ircinia*



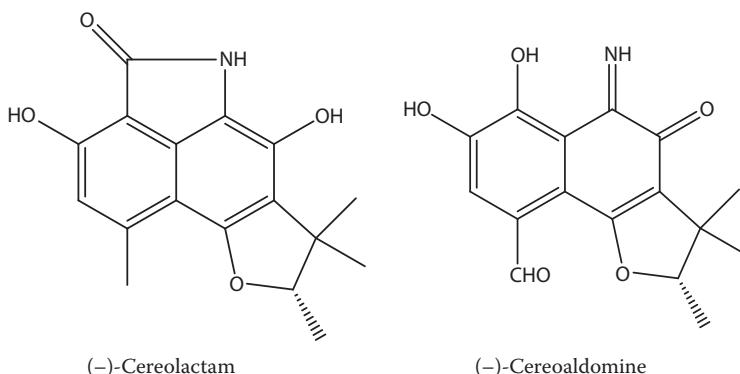
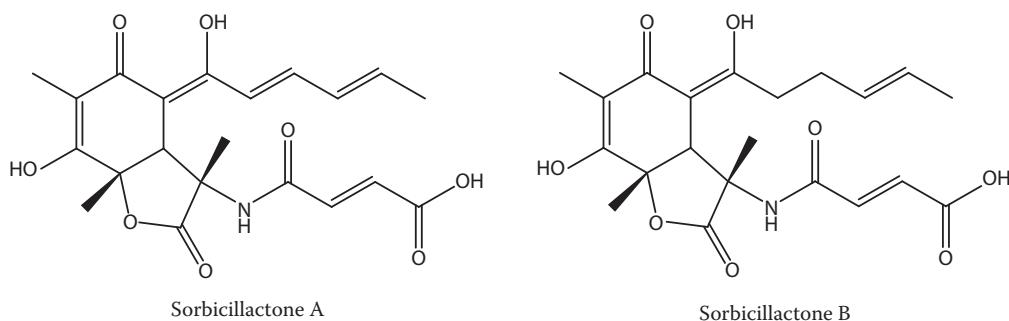
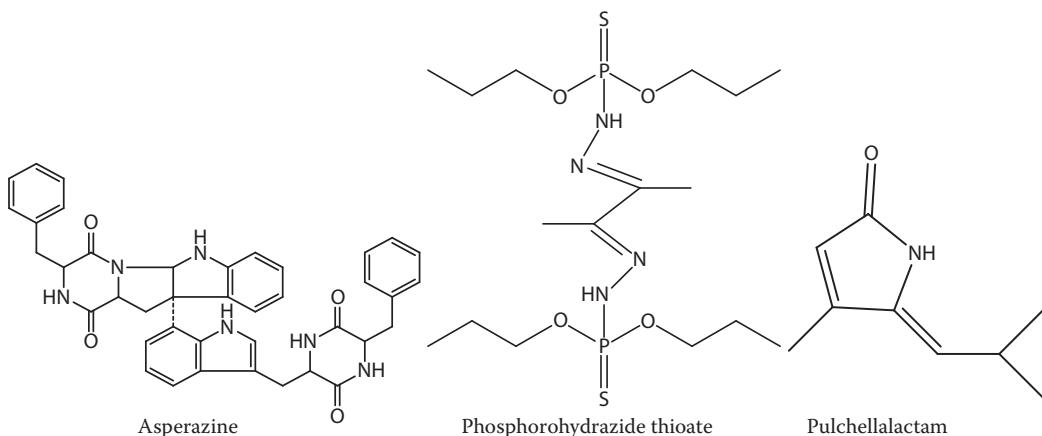
**FIGURE 17.1** Structures of shearinine A, D, and E.



**FIGURE 17.2** Structures of aurantiomides B and C.



**FIGURE 17.3** Structures of 18-oxotryprostatin A, 14-hydroxyterezine D, and 6-methoxy spirotryprostatin B.

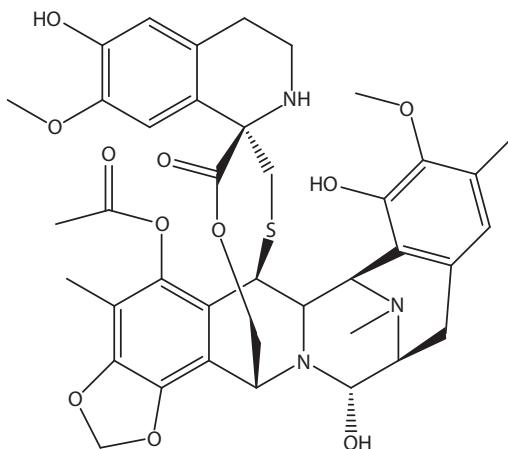
**FIGURE 17.4** Structures of (-)-cereolactam and (-)-cereoaldomine.**FIGURE 17.5** Structures of sorbicillactone A and sorbicillactone B.**FIGURE 17.6** Structures of asperazine, phosphorohydrazide thioate, and pulchellalactam.

*fasciculate*. Sorbicillactone A is active against leukemia cells without showing notable cytotoxicity (Bringmann et al. 2007).

Asperazine (Figure 17.6), isolated from a *Hyrtios proteus* sponge-derived *Aspergillus niger*, has shown selective cytotoxicity against leukemia cells. Another obligate marine fungus, *Lignincola laevis*, known from marsh grass has been shown to produce phosphorohydrazide thioate (Figure 17.6), which is cytotoxic against L1210 cells at  $0.25 \mu\text{g mL}^{-1}$ . Pulchellalactam (Figure 17.6) has been isolated from cultures of the driftwood marine fungus *Corollospora pulchella* and has exhibited inhibitory activity against the CD45 phosphatase (Bugni and Ireland 2004).

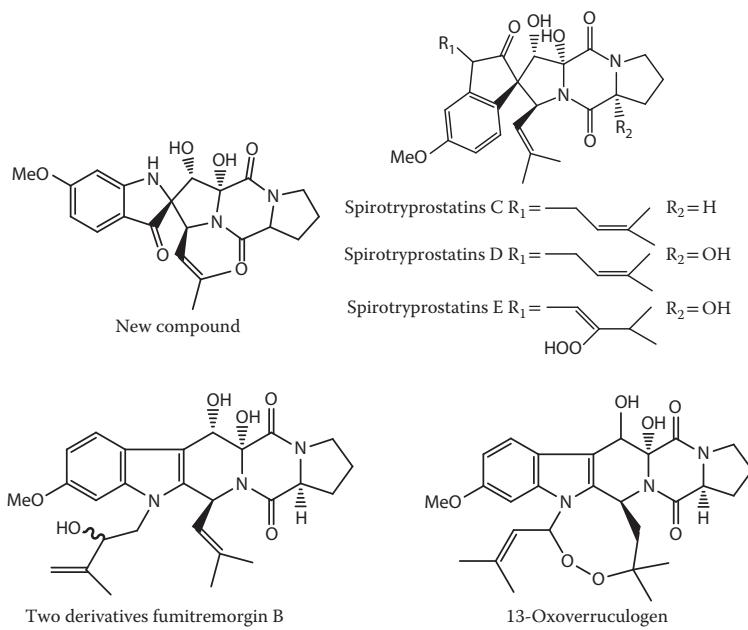
Trabectedin (Yondelis) (Figure 17.7) is a novel antitumor agent originally isolated from the Caribbean marine tunicate, *Ecteinascidia turbinata*, which has been selected for clinical investigation due to its potent cytotoxic activity against a variety of tumor cell lines *in vitro* and human tumor xenografts *in vivo* (Herrero et al. 2006).

Seven new prenylated indole diketopiperazine alkaloids, including a new compound, spirotryprostins C–E, 2 derivatives of fumitremorgin B, and 13-oxoverruculogen (Figure 17.8), have been isolated from the holothurian-derived fungus *Aspergillus fumigatus*. The structures of the new compounds have been determined on the basis of extensive spectroscopic data and amino acid analysis. All new compounds have been evaluated for their cytotoxic activities on the MOLT-4, A549, HL-60, and BEL-7420 cell lines by the 3-(4,5-cimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and Sulforhodamine B (SRB) methods (Wang et al. 2008).



Trabectedin

**FIGURE 17.7** Structure of trabectedin.

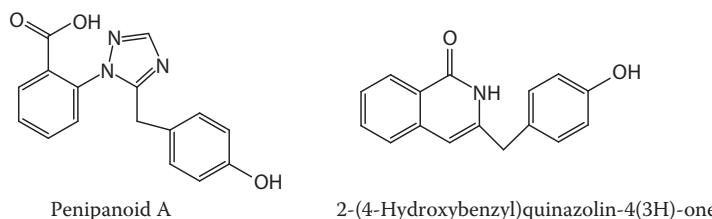


**FIGURE 17.8** Structures of seven prenylated indole diketopiperazine alkaloids.

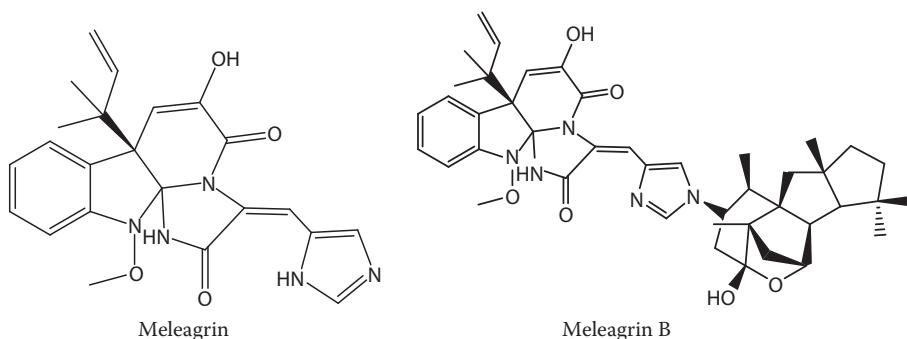
A triazole carboxylic acid, penipanoid A, and a very recently reported quinazolinone derivative have been isolated from the marine sediment-derived fungus *Penicillium paneum* SD-44. The cytotoxicity of penipanoid A and a quinazolinone, 2-(4-hydroxybenzyl)quinazolin-4(3H)-one, has been evaluated (Li et al. 2011) (Figure 17.9).

Meleagrin alkaloids have been isolated from a deep-ocean sediment-derived fungus *Penicillium* sp.: meleagrin B and meleagrin (Figure 17.10), which have induced HL-60 cell apoptosis or have arrested the cell cycle through the G2/M phase, respectively. Du et al. (2010) have proposed that the distinct substitutions on the imidazole ring could have a significant influence on the cytotoxicity of these alkaloids.

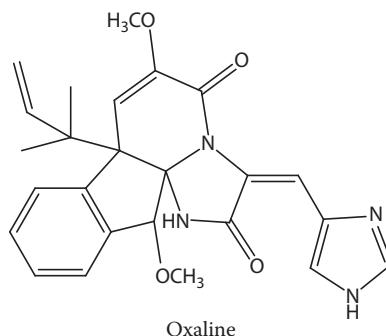
Oxaline is a fungal alkaloid isolated from the culture broth of *Penicillium oxalicum* and *Aspergillus japonicus*. Oxaline (Figure 17.11) is found to inhibit cell proliferation and to induce cell cycle arrest at the G2/M phase in Jurkat cells. Furthermore, oxaline inhibits polymerization of microtubule protein and purifies tubulin dose-dependently *in vitro* (Koizumi et al. 2004).



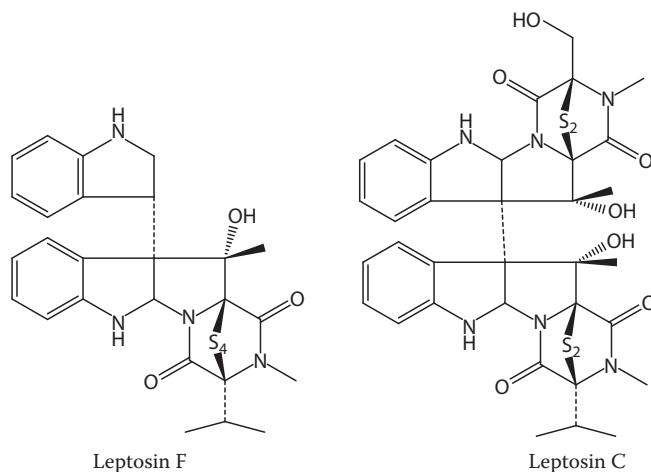
**FIGURE 17.9** Structures of penipanoid A and 2-(4-hydroxybenzyl)quinazolin-4(3H)-one.



**FIGURE 17.10** Structures of meleagrin and meleagrin B.



**FIGURE 17.11** Structure of oxaline.



**FIGURE 17.12** Structures of leptosin C and leptosin F.

Leptosin C and leptosin F (Figure 17.12), sulfur-containing indole derivatives, have been isolated from marine fungus *Leptosphaeria* sp. as cytotoxic substances. Leptosin F inhibits the activity of topois I and II, whereas Leptosin C inhibits topo I *in vitro*. Interestingly, both of the compounds have been found to be catalytic inhibitors of topo I (Yanagihara et al. 2005).

### 17.3 CONCLUSION

The possibility of medicinal breakthrough discoveries from marine-derived fungi has radically increased in a few years. Because the environments of marine fungi differ from those of terrestrial fungi, the secondary metabolites of marine-derived fungi show structural diversity that is significantly different from that of terrestrial fungi. In general, marine-derived fungi are one of the least studied groups of fungi and therefore represent a great opportunity for the discovery of new pharmacologically active agents. Alkaloids from marine-derived fungi have various anticancer and antitumor activities, including antiproliferative and apoptosis-inducing activities. In this chapter, we have focused on the anticancer and antitumor alkaloids isolated from marine-derived fungi *Penicillium* sp., *Aspergillus* sp., *Eupenicillium* sp., and *Lignincola* sp., and have shown their anticancer and antitumor bioactivity. Therefore, the alkaloids from marine-derived fungi have great prospects for application as marine pharmaceutical agents.

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# 18 Pharmacological Effects and Prospects of Marine Algae in Promoting Women's Health and Longevity

*Se-Kwon Kim, Ratih Pangestuti, and A. B. Susanto*

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### 18.1 INTRODUCTION

Ninety percent of the world's living biomass is found in the oceans, with marine species comprising approximately half of the total global biodiversity (Pangestuti and Kim 2011). Therefore, marine organisms with their wide diversity are being recognized as rich sources of pharmacological materials (Shahidi 2008; Shahidi and Alasalvar 2011; Shahidi and Janak Kamil 2001). Among marine organisms, marine algae (sometimes referred as seaweeds) have long been used as traditional remedies in the Eastern hemisphere (Heo et al. 2009). The term "marine algae," as used herein, generally refers to marine macroalgae or sometimes referred as seaweeds.

Marine algae are mainly classified into three major classes based on their pigmentation, namely, brown, red, and green algae, which are referred to as Phaeophyceae, Rhodophyceae, and Chlorophyceae, respectively (Khan et al. 2010). The amount and type of pigments present is found to differ according to the algae classes. Three basic classes of pigments found in marine algae are chlorophylls, carotenoids, and phycobiliproteins.

Up to now, there are a number of reviews available on the pharmaceuticals and medicinal bioactive compounds derived from marine algae. In spite of extensive studies and reviews on nutritional value and on potential health benefits of marine algae for humans, there is little available literature focusing on the pharmacological role of marine algae for women. Therefore, this chapter focuses on the pharmacological roles and prospects of marine algae and presents an overview of their potential benefits for women's health and longevity.

## 18.2 PHARMACOLOGICAL EFFECTS OF MARINE ALGAE ON WOMEN'S HEALTH AND LONGEVITY

### 18.2.1 THERAPEUTIC EFFECTS OF MARINE ALGAE ON CANCERS THAT OCCUR MAINLY IN WOMEN

Breast cancer is the leading cause of cancer-related deaths among women worldwide (Parkin 2001; Geyer et al. 2006). Globally, more than 1.1 million women are diagnosed each year, representing around 10% of all newly diagnosed cancer cases (Anderson et al. 2006). The mortality rate for premenopausal breast cancer is almost four times greater in the Western world than in the East Asian nations. In breast cancer etiology, genetics are thought to play a smaller role than environmental factors such as food diets. One important difference in the diet and remedies of East Asian populations compared with Western populations is that the amount of fish and marine algae consumption is higher in the former.

Many authors have investigated the effect of *Laminaria angustata* consumption and development of breast cancer in female Sprague-Dawley rats induced with the carcinogen 7,12-dimethylbenz(a)anthracene (DMBA), a widely used rat mammary cancer model (Huggins, Grand, and Brillantes 1961; Teas, Harbison, and Gelman 1984). Diet containing 5% *L. angustata* has been found to be effective in delaying the time of DMBA-induced tumor developments. Although the mechanism for *L. angustata* activity is not elucidated yet, the authors argue that the bioactivity of *L. angustata* may be due to their nutrient content such as polyphenols, sulfated polysaccharides, vitamins, minerals, and carotenoids. In accordance, wakame (*Undaria pinnatifida*) and mekabu (sporophyll of wakame) have been demonstrated to reduce the incidence, multiplicity, and size of breast tumors in female Sprague-Dawley rats induced with DMBA (Funahashi et al. 1999, 2001). Considering that wakame and mekabu are particularly rich in iodine, the investigators have suggested that the cancer inhibition was brought about by the iodine. More recently, statistical correlations between dietary intake of iodine and breast cancers have been carried out; however, their exact mechanisms of action are not yet completely understood (Ellerker 1955; Majem et al. 1988).

Apoptosis or programmed cell death is a key process in cancer development and progression, which can be characterized through a distinct set of morphological and biochemical progresses. Inactivation of apoptosis has been considered to be one of six fundamental hallmarks of cancer; therefore, apoptosis is a major target of cancer therapy development up to the present (Brown and Attardi 2005). Dioxinodehydroeckol, a phloroglucinol derivative from *Ecklonia cava*, has a potential inhibitory effect against growth of human breast cancer cells (MCF-7) via induction of apoptosis (Kong et al. 2009). Furthermore, 1 µg/ml of mekabu has strongly induced apoptosis in three human breast cancer cell lines (MCF-7, T-47D, and MDA-MB-231)—the induction of apoptosis was even greater than 5-fluorouracil, a chemotherapeutic agent frequently used in human breast cancer clinics. Hence, developing novel molecules derived from marine algae that promote apoptosis in breast cancer cells by targeting both the intrinsic and extrinsic apoptotic pathways may lead to the development of effective breast cancer therapies.

Estrogen-dependent cancers, such as breast, endometrial, and ovarian cancer, are among the leading causes of morbidity and mortality in American women (Kramer and Wells 1996). Increased incidence of these cancers is predicted in the future, and the need for primary prevention is clear. Epidemiological studies have demonstrated that the incidence rates of estrogen-dependent cancers are among the highest in Western, industrialized countries, while the rates are much lower in China and Japan (Parkin, Pisani, and Ferlay 1999; Parkin et al. 2005). Due to some research, low estrogen-dependent cancer rates have been attributed to the soy-rich and marine algae diets inherent among Asian populations (Teas et al. 2009). For example, dietary intake of *Alaria esculenta* and soy protein has been reported to modify the estrogen and phytoestrogen metabolism in healthy postmenopausal women (Teas et al. 2009). In another pilot study on women, Skibola (2004) has demonstrated that intake of *Fucus vesiculosus* (bladderwrack) significantly increased the total number of days of the

menstrual cycle, reduced circulating 17 $\beta$ -estradiol levels, and elevated serum progesterone levels in premenopausal women with abnormal menstrual cycling histories (Skibola 2004). Moreover, *F. vesiculosus* has been demonstrated to modulate endocrine hormones in female Sprague-Dawley rats and human luteinized granulose cells (Skibola et al. 2005). Hence, it may be assumed that intake of marine algae may contribute to the lower estrogen circulating level, which may correlate to the lower incidence of hormone-dependent cancers in women.

Cervical cancer is the second most common cancer in women worldwide, and more women die annually from cervical cancer than from AIDS (Munoz et al. 2003; ElHage 2005; Kaplan-Myrth and Dollin 2007). It is the principal cancer of women in most developing countries, where 80% of cases occur (Munoz et al. 2003). Recent reports have demonstrated that extracts of several marine algal species—*Palmaria palmate* (dulse), *Laminaria setchellii*, *Macrocystis integrifolia*, *Nereocystis leutkeana*, *Udotea flabellum*, and *Udotea conglutinata*—were able to inhibit cervical cancer cell proliferations *in vitro* (Yuan, Carrington, and Walsh 2005; Yuan and Walsh 2006; Moo-Puc, Robledo, and Freile-Pelegrin 2009). The goal of most current cancer therapy is to reduce the number of tumor cells and to prevent their further accumulation. Hence, the antiproliferative activity of marine algae in cervical cancer cells demonstrates the potential of marine algae as a therapeutic agent for cervical cancer treatment.

In addition, formation of cancer cells in the human body can be directly induced by free radicals, and natural anticancer drugs as chemopreventive agents have gained positive popularity in the treatment of cancer. Therefore, marine algal radical-scavenging compounds such as phlorotannins, sulfated polysaccharides, carotenoids, and carmamol derivatives can be used indirectly to reduce cancer formation in the female body.

Taken together, marine algae and their secondary metabolites have shown promising anticancer activities; hence, marine algae have great potential to improve women's health and longevity by being a part of anticancer pharmaceuticals, medicinal foods, and nutraceuticals. However, future studies are needed focusing on the synergistic benefits of consuming different marine algal species, recommended doses and timing of intake, and preparation methods for marine algae in order to maximize the desired effect in the prevention of cancer, particularly cancers that occur mainly in women.

### **18.2.2 MARINE ALGAE INHIBIT THE INFECTION OF HUMAN PAPILLOMA VIRUS IN FEMALE GENITALS**

Infection by certain human papilloma virus (HPV) types in female genitals has been associated with cervical cancer; hence, HPV prevention has received great attention from scientific studies (Lehtinen and Dillner 2002). The first generation of HPV vaccine is currently available on the market to prevent HPV infection (Paczos, Liu, and Chen 2010). However, the high cost of the vaccine has been a cause for concern and the vaccine will be too expensive for use in the developing world. Therefore, the search for potential anti-HPV candidates having higher inhibitory activity and lower price has generated great interest in pharmaceutical industries. In this regard, natural bioactive compounds and their derivatives are potential sources for the development of pharmaceuticals as new-generation anti-HPV therapeutics that are more effective, have fewer side effects, and are less expensive.

A large number of marine algae contain significant quantities of complex structural sulfated polysaccharides that have been demonstrated as potent inhibitors of a wide variety of viruses, such as HPV (Witvrouw and De Clercq 1997; Pujol et al. 2007; Campo et al. 2009). Carrageenan, a sulfated polysaccharide of d-galactose and 3,6-anhydro-d-galactose extracted from the Rhodophyceae, has been used in food products for centuries. Recently, carrageenan has been shown to bear anti-HPV activity *in vitro* (Campo et al. 2009). Buck et al. have noted that carrageenan, particularly *t*-carrageenan, inhibits HPV three orders of magnitude more potently than heparin does, a highly effective model for

HPV inhibitor (Buck et al. 2006). Carrageenan acts primarily by preventing the binding of HPV virions to cells and blocks HPV infection through a second, postattachment heparin sulfate-independent effect. This mechanism is consistent with the fact that carrageenan resembles heparin sulfate, which is known as a HPV cell-attachment factor. Furthermore, some of the milk-based products that contain carrageenan block HPV infectivity *in vitro*, even when diluted million-fold (Buck et al. 2006). In another study, carrageenan has been reported to inhibit genital transmission of HPV in a female mouse model of cervicovaginal (Schiller and Davies 2004; Roberts et al. 2007). In addition, carrageenan has been able to generate antigen-specific immune responses and antitumor effects in female (C57BL/6) mice vaccinated with HPV-16 E7 peptide vaccine (Zhang et al. 2010).

On the basis of these findings, carrageenan can be an alternative source of a novel therapeutic candidate for HPV by being a part of drugs. There are numerous advantages of carrageenan over other classes of antiviral agents, such as relatively low production costs, broad spectrum of antiviral properties, low cytotoxicity, safety, wide acceptability, and novel modes of action. This suggests that carrageenans are promising candidates in the near future. However, further studies with clinical trials are needed for their anti-HPV activity in women.

### 18.2.3 EFFECTS OF MARINE ALGAE ON OBESITY

Obesity may occur in any gender; however, it is more likely to occur in women (Popkin and Doak 1998; Rennie and Jebb 2005). Obesity among women (from teen and seniors) continues to increase in many industrialized and developing countries, which causes a worrying health trend (Kelishadi 2007). A detrimental effect of obesity on female reproductive system has been demonstrated consistently (Pettigrew and Hamilton-Fairley 1997). Furthermore, it has been continuously reported that media and sociocultural factors continue to pressurize young women to be thin, which promotes body dissatisfaction, eating disturbance, depression, and negative effects in young women (Stice, Maxfield, and Wells 2003). Therefore, women may pay a higher health price for obesity than men. Accordingly, many categories of natural and synthetic compounds demonstrated as antiobesity drugs have been used by women to reduce their weight. However, synthetic antiobesity agents are believed to have certain side effects, such as unacceptable tachycardia, hypertension, improved lipid blood levels, improved glucose metabolism, and disturbance of female reproductive system (Bays 2004). Hence, more scientific efforts have been dedicated to study pharmaceuticals that can act as antiobesity agents.

In the last four decades, researchers have found that soluble dietary fibers are negatively associated with obesity. Marine algae are particularly rich in two different types of fiber: soluble and insoluble (Table 18.1) (Lahaye 1991). *Eisenia bicyclis*, sometimes referred as arame, contains more than 50% soluble fiber of its dry weight; the other brown algal species, *F. vesiculosus*, contains around 40% insoluble fiber per dry weight (Lahaye 1991; Ruperez and Saura-Calixto 2001). In the human body, soluble and insoluble fibers act in a very different way. Consumption of marine algal soluble fibers such as carrageenan, agar, and alginate are primarily associated with hypcholesterolemic and hypoglycemic effects (Panlasigui et al. 2003). For example, alginates have been shown to modulate appetite and energy intake in models of acute feeding. Upon reaction with gastric acid (acid-soluble calcium source), alginates undergo ionic gelation to form an alginate gel that can slow gastric emptying, stimulate gastric stretch receptors, reduce intestinal nutrient uptake, and influence the glycemic response (Dettmar, Strugala, and Craig Richardson 2011). In accordance, ingesting calcium-gelled alginate-pectin twice per day has been reported to reduce spontaneous food intake in overweight and obese women (Pelkman et al. 2007). Furthermore, insoluble fibers such as cellulose, xylans, and mannans are associated with excretion of bile acids, increased fecal bulk, and decreased intestinal transit time (Moore, Park, and Tsuda 1998; Burton 2003).

More recently, Maeda et al. have reported that dietary intake of fucoxanthin significantly attenuates the weight gain of white adipose tissue (WAT) and expressed uncoupling protein 1 (UCP1) in diabetic/obese KKA<sup>y</sup> female mice (Maeda et al. 2005, 2007). The potential involvement of

**TABLE 18.1**  
**Soluble, Insoluble, and Total Fiber (% Dry Weight) in Some Edible Marine Algae**

Margin Algae	Soluble Fiber	Insoluble Fiber	Total Fiber	References
<b>Chlorophyceae</b>				
<i>Ulva lactuca</i> (sea lettuces)	21.3	16.8	38.1	Burtin (2003)
<i>Enteromorpha</i> sp (ao nori)	17.2	16.2	33.4	Burtin (2003)
<b>Rhodophyceae</b>				
<i>Porphyra teneri</i> (nori)	14.56	19.22	33.78	Ruperez and Saura-Calixto (2001)
<i>Chondrus crispus</i> (Irish moss)	22.25	12.04	34.29	Ruperez and Saura-Calixto (2001)
<b>Phaeophyceae</b>				
<i>Hijiki fusiformis</i> (hijiki)	16.3	32.9	49.2	Lahaye (1991)
<i>Himanthalia elongate</i> (sea spaghetti)	25.7	7.0	32.7	Lahaye (1991)
<i>Eisenia bicyclis</i> (arame)	59.7	14.9	74.6	Lahaye (1991)
<i>Undaria pinnatifida</i> (wakame)	17.31	16.26	33.58	Ruperez and Saura-Calixto (2001)
<i>Laminaria digitata</i> (kombu)	9.15	26.98	36.12	Ruperez and Saura-Calixto (2001)
<i>Fucus vesiculosus</i> (bladderwrack)	9.80	40.29	50.09	Ruperez and Saura-Calixto (2001)
<i>Durvillaea antarctica</i>	27.7	43.7	71.4	Ortiz et al. (2006)

fucoxanthin to attenuate the weight gain of WAT may correlate to the presence of unusual double-allenic bonds at the C-7' position (Miyashita and Hosokawa 2009). WAT is the predominant type of adipose tissue and commonly called “fat” in mammals (Trayhurn and Wood 2005). Besides its role in energy storage, WAT is now recognized as an endocrine and active secretory organ through its production of biologically active mediators termed “adipokines” (Curat et al. 2006). Excess production of adipokines includes proinflammatory factors and chemokines, has been linked with obesity, and plays an important role in the development of obesity-related disease (Trayhurn and Wood 2005). Therefore, fucoxanthin activity to attenuate the weight gain of WAT in female mice demonstrates the potential of fucoxanthin for the prevention and treatment of obesity and diabetes particularly in women. Dioxinodehydroeckol and 1-(3',5'-dihydroxyphenoxy)-7-(2",4",6-trihydroxyphenoxy)-2,4,9-trihydroxydibenzo-1,4-dioxin, two phloroglucinol derivatives isolated from *E. cava*, have significantly inhibited adipocyte differentiation in 3T3-L1 cells, suggesting its potential use as a functional ingredient in obesity management.

According to those findings, marine algae may serve as a potential candidate for pharmaceutical and functional foodstuffs with health benefits, especially for obesity management. Hence, negative effect in women—particularly in young women—caused by pressures to be thin can be minimized by the application of marine algae in foods, pharmaceuticals, and so forth. Additionally, marine algae will develop a new approach for the treatment of obesity in addition to currently available antiobesity agents. Therefore, marine algae are a potent natural source for the development of foods and pharmaceuticals for the management of obesity.

#### 18.2.4 POTENT EFFECTS OF MARINE ALGAE ON OSTEOPOROSIS

Osteoporosis is a skeletal condition characterized by decreased bone mineral density (BMD) (mass/volume unit) that leads to an increased risk of fractures (Beikler and Flemmig 2003).

A number of studies have identified that osteoporosis occurs much more frequently in women than in men (Cadarette et al. 2000; Hannan et al. 2000; Schuit et al. 2004). There are many reasons for the high prevalence of osteoporosis in women. First, at skeletal maturity, men have 30–50% bone mass compared with women (Christiansen 1993; Nieves et al. 2005). Second, although decreased BMD occurs in both men and women with age, the decrease in BMD is substantially greater in women after menopause (Kanis et al. 1997; Riggs et al. 2004; Riggs et al. 2008). Therefore, it is very important to help postmenopausal women to prevent them from progressing to osteoporosis.

Fujita et al. (1996) have indicated that active absorbable algal calcium (AAA Ca) is effective for improving BMD in elderly subjects. AAA Ca is a mixture of active absorbable calcium (AA Ca) and heated algal ingredients prepared by heating cleaned oyster and marine algae (*Cystophyllum fusiforme*) submaximally under reduced pressure (Fujita et al. 1996). Furthermore, a mineral-rich extract from red marine algae *Lithothamnion calcaerum* has been demonstrated to increase mineral content and bone strength in female mice on a Western-style diet (Aslam et al. 2010). However, it is not clear yet which mineral in the algal extracts preserves bone structure and function in female mice. The algal extract is currently available as a food supplement under the name Aquamin (GRAS 000028), which is currently used in various products for human consumption in Europe, Asia, Australia, and North America.

In addition, Das et al. have demonstrated the effects of fucoxanthin on osteoclastogenesis. Treatment with 2.5  $\mu\text{M}$  fucoxanthin has also induced apoptosis accompanied by activation of caspase-3 in osteoclast-like cells. Those *in vitro* studies suggest that fucoxanthin suppresses osteoclastogenesis via the inhibition of osteoclast differentiation and the induction of apoptosis in osteoclasts (Das et al. 2010). Hence, dietary fucoxanthin may be useful for the prevention of bone diseases such as osteoporosis and rheumatoid arthritis, which are known to be related to bone resorption.

Collectively, marine algae may be a potent natural source for the development of functional foods and pharmaceuticals to prevent osteoporosis. Moreover, it is important to evaluate other marine algal species that may have a great potential as antiosteoporosis agent.

### 18.3 PROSPECTS OF MARINE ALGAE IN PROMOTING WOMEN'S HEALTH

In Asian culture, marine algae have always been of particular interest as marine food sources (Khan et al. 2010). Edible marine algae (sometimes referred as seaweeds or sea vegetables) have accounted for more than 10% of Japanese diet with an average consumption of 1.4 kg/person/year (Burton 2003). In Korea, 37 days after delivering their babies, new mothers are served with miyeok-guk, which is a hot and spicy marine algae soup (Dennis et al. 2007). Korean believes that miyeok-guk provides nutrition and helps the new mother to regain her energy. Marine algae have been demonstrated as rich sources of structurally diverse biologically active compounds with a great pharmaceutical and biomedical potential; therefore, it represents one of the most nutritious plant foods. Several epidemiologic studies provided evidence that marine algae consumption correlates with low breast cancer rates in East Asia. As an example, 1-year prevalence case of breast cancer incidence per 100,000 persons in Japan and China are 42.2 and 13.1, respectively, versus 125.9 and 106.2 cases in North America and Europe, respectively (Pisani, Bray, and Parkin 2002; Yuan and Walsh 2006). More recently, there has been growing interest in marine algae and their constituents as pharmaceuticals, functional foods, and nutraceuticals with potential health benefit effects as sources of antioxidants to reduce the risk of diseases. Marine algae are an important source of bioactive ingredients that can be applied to many aspects of processing pharmaceuticals, healthier foods, and developing functional foods.

In addition, the wide diversity of marine algae and numerous undiscovered unique metabolites present in marine algae are interesting sources to increase the number of novel drugs to improve women's health and longevity. However, large-scale human studies are required to identify the prophylactic and therapeutic effects of marine algae on diseases that occur mainly in women.

## 18.4 CONCLUSIONS

The wide range of biological activities associated with natural compounds derived from marine algae such as phlorotannins, alginates, sulfated polysaccharides, and carotenoids have the potential to expand the nutritional and health beneficial value of marine algae in pharmaceuticals and food industries. Furthermore, the wide diversity of marine algae and numerous undiscovered unique metabolites present in marine algae are interesting sources to increase the number of novel pharmaceuticals that are beneficial for women's health, beauty, and longevity. Accordingly, the possibility of designing new medicinal foods or nutraceuticals and pharmaceuticals derived from marine algae is promising. On the other hand, clinical trials are needed to confirm anticancer, antiviral, antosteoporosis, and antiobesity activity of marine algae. In addition, further research studies are needed in order to investigate marine algae activities in women.

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# 19 Marine Sponge- Associated Microbes

## *A Source of Biologically Active Metabolites*

*Se-Kwon Kim, Pradeep Dewapriya, and Yong-Xin Li*

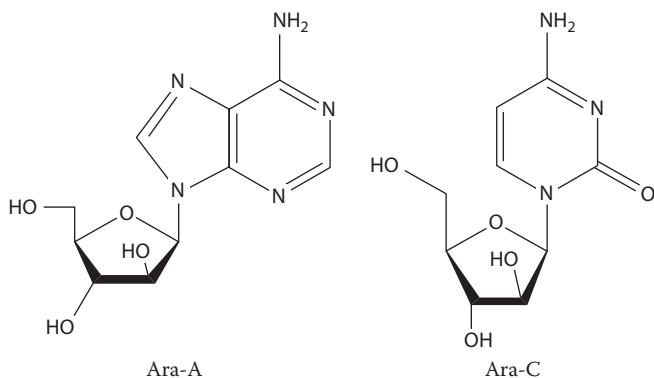
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### 19.1 INTRODUCTION

Since the first exploration of the marine environment for natural compounds in 1945 by Werner Bergmann, marine sponges have been considered the most prolific and important source of new bioactive compounds in the marine environment. Because of their immense production of new compounds, sponges are considered a chemical factory in the marine environment and a gold mine to chemists. Thus, marine sponges have gained much attention in various scientific disciplines (Bergmann and Feeney 1951; Baby and Sujatha 2010). The pioneering work of Werner Bergmann has led to the development of chemical derivatives Ara-A (vidarabine) and Ara-C (cytarabine), two nucleosides with significant anticancer and antiviral activity that have been approved for clinical use as the first marine-derived natural products. Since then, marine sponges have been a good candidate for pharmaceutically active metabolites and thousands of research articles have been published to reveal their potential (Molinski et al. 2009; Mayer et al. 2010).

Medicinal applications of sponges go back to ancient times. Sponges saturated with different solutions, such as plant extracts, iodine, pure wine, and urine, have been used for various diseases, and physicians have recommended sponges against all kinds of wounds, bone fractures, dropsy, stomachaches, infectious diseases, and testicular tumors (Sipkema et al. 2004). By 1974 two compounds, Ara-A and Ara-C (Figure 19.1), derived from marine sponge metabolites took part in clinical treatments, but from then until 2004 no other marine compounds have been approved for clinical use. Although thousands of new biologically active compounds, such as halichondrin B



**FIGURE 19.1** Sponge-derived compounds that have been approved for clinical use.

(*Halichondria okadai*), manoalide (*Luffariella variabilis*), contignasterol (*Petrosia contignata*), and okadaic acid (*H. okadai*), have been discovered from marine sponges, only a few of these compounds have been able to enter clinical trials. All these findings have been hampered by a major problem—supply of adequate active metabolites, as the concentration of desired metabolites is generally low and thousands of tons of sponge biomass is required to harvest the compound. Therefore, wild harvest of sponges for bioactive compounds is not an economically feasible and environmental-friendly practice. To overcome this constraint, various kinds of remedies, such as chemical synthesis of active metabolites, sponge aquaculture, and sponge cell cultures, are in progress to obtain active compounds isolated from marine sponges (Thakur and Muller 2004). With the finding of a marine *Micrococcus* sp. associated with sponge *Tedania ignis* that produces metabolites ascribed to the sponge, an argument has been raised on the true producer of active metabolites that have been extracted from marine sponges. If those compounds were derived from a symbiotic microorganism, culturing the microorganism would provide an improved source of the bioactive compound (Stierle, Cardellina, and Singleton 1988). In this chapter, we will discuss pharmacologically active metabolites of marine sponge-associated microorganisms and their prospects in drug development.

## 19.2 MARINE SPONGES

Sponges (phylum *Porifera*) are the most primitive form of the multicellular animals that have existed for 700–800 million years. Over 6000 different sponge species inhabit almost all the different environments in the sea. Basically, marine sponges consist of three sublineages: Calcarea (5 orders and 24 families), Demospongiae (15 orders and 92 families), and Hexactinellida (6 orders and 20 families). Approximately 95% sponges are classified as Demospongiae (Lafi 2005; Thomas et al. 2010). They show fascinating variation in shapes, size, and color. While giant barrel sponges grow up to 70 cm in height, tiny encrusting sponges have only a half-inch long body (Thakur and Muller 2004). A sponge's body is a collection of different types of cell layers. The gelatinous matrix “mesophyl” fills the space between the external cell layer, pinacoderm, and the internal choanoderm layer. The body is reinforced by skeletal elements, spicules (a needle-like silica or calcium structure), and spongia (collagen fibers) (Uriz et al. 2003).

Sponges are efficient filter feeders and have an ability to filter over 2000 L of water per day in order to obtain nutrients such as organic particles and microorganisms in the water. Seawater is pumped through pores called ostia on the surface of the sponge and circulates along the channels in the body. Specialized flagellated cells, choanocytes, that exist on channels filter food particles from water. Filtered food particles are transferred to mesophyl and digested via phagocytosis with another group of sponge cells, the archaeocytes. Despite the fact that sponges feed on microorganisms, the body harbors dense and diverse microbial communities (Taylor et al. 2007). As with all the sessile organisms and invertebrates, sponges follow a unique defense mechanism against intensive

evolutionary pressure from competitors that threaten by overgrowth, poisoning, infection, or predation. Instead of using tissue and skeletal components as a physical defense, sponges highly rely on the production of various kinds of secondary metabolites as a form of defense, and it is believed that marine sponges are rich in secondary metabolites due to the ability to biosynthesis or accumulate these defense chemicals (Thakur and Muller 2004).

### 19.3 MICROBIAL COMMUNITIES OF MARINE SPONGES

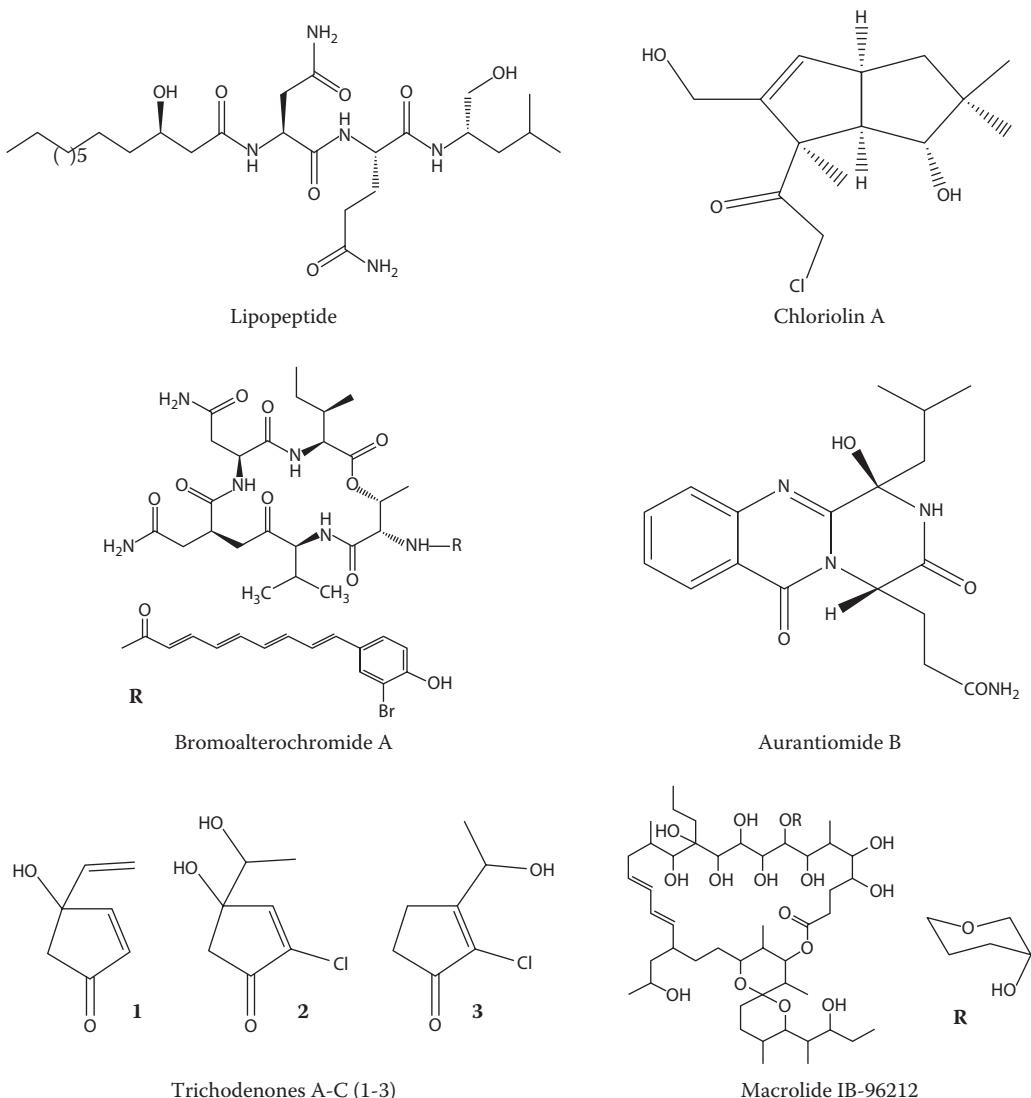
Sponges have gained much attention mainly because microbial communities associated with sponges play an important role. In fact, some sponge species (bacteriosponges) comprise up to 40% of sponge tissue volume with microbial densities in excess of  $10^9$  cells/mL of sponge tissue (Hentschel, Usher, and Taylor 2006; Taylor et al. 2007). Interestingly, many researchers have proved that all three domains of microbial life—bacteria, archaea, and eukarya (fungi and microalgae)—reside within marine sponges (Garson et al. 1998; Friedrich et al. 1999; Webster, Watts, and Hill 2001). While the diversity of microbial communities of sponges is incredibly high, distantly related sponges from geographically different regions hosting the same microbial symbiont reveal that many of these microorganisms are specific to marine sponges (Webster and Taylor 2012). Even though the association of microbes with sponges and its benefits to either partner have not been fully described, it shows characteristics of symbiosis.

Bacteria are the prominent sponge-associated microbes and the majority belong to the group of  $\alpha$ -bacterium. In addition to 14 recognized bacterial phyla, phyla *Candidate* and *Chlorobi* have been identified in marine sponges with the aid of novel techniques such as 16s RNA and fluorescence in situ hybridization (Friedrich et al. 1999; Webster et al. 2001). Almost all the archaea that have been found in sponges are members of phylum *Crenarchaeota* (a few exceptions, which belong to *Euryarchaeota*, have been reported) (Lee et al. 2003). In the group of eukaryotic microbes, diatom and dinoflagellate serve as primary producers of marine sponges in addition to the major primary producer, cyanobacteria. In addition to direct ingestion of microorganisms as a source of energy, these photosynthetic microbes provide a considerable amount of the total energy requirement of sponges. Fungi that occur in sponges have gained much attention because of their biotechnological potential. It has been proved that sponge-associated fungal species are rich sources of biologically active secondary metabolites. Moreover, there are several lines of evidence on sponge-associated yeasts and viruses and their biodiversity (Taylor et al. 2007). It is clear from the literature that these microbes have a profound effect on host metabolic processes. While photosynthetic microbes provide the energy requirement, others contribute to the defense systems of sponges (Schmidt et al. 2000). Further, sponge-associated microbes produce various kinds of metabolites as a part of their metabolic process and make sponges a rich source of diverse chemical compounds and a potential source of pharmaceutical agents (Schmidt et al. 2000; Hochmuth and Piel 2009). From this point forward, we will focus on biologically active metabolites of sponge-associated microbes and their potential as pharmaceutical agents.

### 19.4 PHARMACOLOGICAL POTENTIAL OF MICROBIAL METABOLITES

#### 19.4.1 ANTICANCER AND ANTITUMOR COMPOUNDS

The term “cancer” refers to more than 100 forms of diseases. When cells violate the general rule of cell proliferation and follow their own regulation mechanism in reproduction, a cancer triggers. Some of these types of cells possess an ability to migrate from the site where they began to another organ or a tissue. These types of malignant tumor cells in particular become more aggressive and lethal over time (Iiizumi et al. 2008). Over the past century, scientists have struggled to identify suitable agents, natural or synthetic, to combat cancer. In this regard, microbial metabolites have been a top-rated natural source that has the potential to prevent and cure cancer. In this part, the



**FIGURE 19.2** Examples of sponge-derived anticancer metabolites.

focus is given to the potential of microbial metabolites (Figure 19.2; Table 19.1) in cancer therapy, while cancer preventive metabolites are discussed in latter part of the chapter.

Sponge-derived fungi are a prolific source of anticancer metabolites. Coriolin B and three other new chlorinated cyclic sesquiterpenes have been discovered from a fungus strain derived from a *Juspis* marine sponge. Biological studies have revealed that these metabolites strongly inhibit human breast and central nervous system cancer cell lines with IC<sub>50</sub> values of 0.7 µg (breast) and 0.5 µg (neuroblastoma) (Thomas et al. 2010; Blunt et al. 2012). Ethanol extracts of a static culture of *Aspergillus niger* isolated from the Mediterranean sponge *Axinella damicornis* have yielded a new secondary metabolite, bicoumanigrin A, that has an *in vitro* antiproliferative activities on human cancer cell lines. Addition of 1–20 µg/mL of bicoumanigrin A has resulted in a mean growth inhibition up to 50% (Hiort et al. 2004). Moderately cytotoxic new quinazoline alkaloids have been discovered from *Penicillium aurantiogriseum*, which has been obtained from sponge *Mycale plumose* collected in Jiaozhou Bay, Qingdao, China. The cytotoxicity assay has exhibited that these alkaloids

**TABLE 19.1**  
**Examples of Anticancer Metabolites from Sponge Microbes**

Compound	Biological Activity	Microorganism	Reference
Lipopeptide	Cytotoxic	Fungi	Lee et al. (2010)
Cyclic sesquiterpene	Anticancer	Fungi	Thomas et al. (2010) Cheng et al. (1994)
Bicoumanigrin A	Antiproliferative	Fungi	Hiort et al. (2004)
Quinazoline alkaloids	Cytotoxic	Fungi	Xin et al. (2007)
Trichodenones	Cytotoxic	Fungi	Amagata et al. (1997)
Cyclodepsipeptides	Antitumor	Fungi	Yu et al. (2008)
Indolocarbazole alkaloids	Antitumor	Actinobacteria	Hernandez et al. (2000)
Alkaloids, polyketides, and macrolide	Cytotoxic	Actinobacteria	Schneemann et al. (2010)
Cyclopropane and hexadecanoic acid	Topoisomerase I	Actinobacteria	Lee (1998)
Crude extract	Cytotoxic	Surface bacteria	Thakur et al. (2005)

are toxic against the P388, BEL-7402, A-549, and HL-60 cell lines with  $IC_{50}$  values ranging from 52 to 54  $\mu\text{g}/\text{mL}$  (Xin et al. 2007). Among the novel metabolites isolated from marine sponge-derived fungus strains, trichodenones A–C, which have been isolated from *Trichoderma harzianum*, originally separated from the sponge *H. okadai*, have shown a significant cytotoxicity against P388 lymphocytic leukemia cells (Thomas et al. 2010). Moreover, *Aspergillus versicolor* isolated from a marine sponge *Petrosia* sp. produces a cytotoxic lipopeptide that has been tested against five human tumor cell lines (A549, human lung cancer; SK-OV-3, human ovarian cancer; SK-MEL-2, human skin cancer; XF 498, human central nervous system (CNS) cancer; and HCT15, human colon cancer). The lipopeptide significantly acts against XF498 and HCT15 cell lines compared with the commercial drug Doxorubicin (Lee et al. 2010). Two other new cyclodepsipeptides have been found in a marine sponge-derived fungus, *Scopulariopsis brevicaudata*. At a final concentration of 10  $\mu\text{g}/\text{mL}$ , compounds have exhibited strong antiproliferative activity against pancreatic tumor cells. Because of this antiproliferative activity, these compounds have been patented as a potential antitumor drug candidate (Yu et al. 2008).

Interestingly, marine bacteria have also been proved as potent anticancer metabolite producers. The actinobacterial species *Micromonospora* isolated from the marine sponge *Clathrina coriacea* has produced strong antitumor indolocarbazole alkaloids. Investigations of biological activity suggest that the sugar moiety of compounds has resulted in their strongest activity (Hernandez et al. 2000). Schneemann et al. (2010) have conducted a comprehensive analysis of marine actinobacteria associated with the sponge *Halichondria panacea*. In this study, 46 actinobacterial strains have been investigated and 122 different substances with 88 unidentified compounds have been reported. Among the identified metabolites, alkaloids, polyketides, and macrolide possess a potent cytotoxic activity. Further, 30 strains out of 46 have been confirmed for the presence of biosynthesis genes encoding polyketide synthases and nonribosomal peptide synthetases, which provide a good indication of the pharmaceutical potential of these strains. The topoisomerase I inhibitor cyclopropane and 14-methylhexadecanoic fatty acids produced by *Streptomyces* sp. strain KM86-913 have been isolated from marine sponges collected under the seashore of Keomun Island, Korea. Since this type of inhibitor blocks the ligation step of the cell cycle and subsequently leads to apoptosis via breaking the integrity of the genome, these metabolites have a potential to develop as antitumor drugs (Olano, Mendez, and Salas 2009). Thakur et al. (2005) have investigated the bioactive potential of sponge surface-associated bacteria and sponge primmorph-associated bacteria and have revealed that the *n*-butanol extract of a sponge surface bacterium is toxic against PC12 cells, while the extracts from

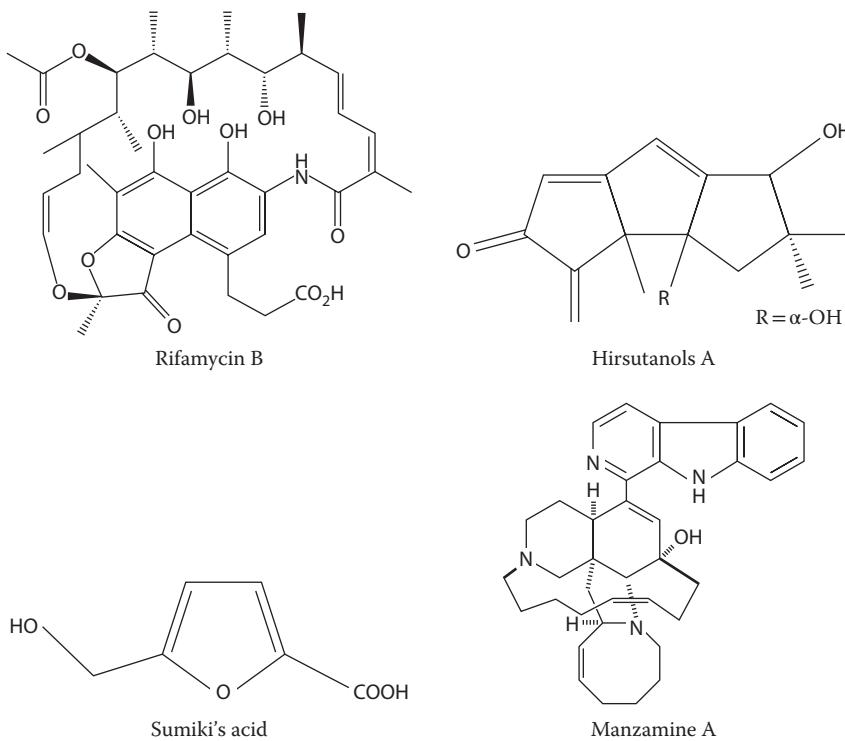
the primmorph-associated bacteria exhibit potent cytotoxicity against HeLa cells. All these explorations highlight that sponge-associated microbes have the potential to develop as anticancer agents. However, further investigations on these potent anticancer metabolites are required to confirm their mode of action.

### 19.4.2 ANTIMICROBIAL METABOLITES

Microbial infections are one of the major causes of mortality around the world, especially in developing countries, and the problems have been steepened by the development of drug resistance in disease-causing organisms. The emergence of drug resistance continuously demands new metabolites to treat infectious diseases, and marine microbes are ideal candidates to explore novel antibacterial metabolites, because marine microorganisms are different from those of terrestrial counterparts. Dozens of studies have proved that sponges produce potent antimicrobial metabolites. Here we provide an overview of antimicrobial substances (Figure 19.3) that have been produced by sponge-associated microbial communities.

#### 19.4.2.1 Antibiotics

In the global search for new antibiotics, sponge-associated microbes have been rated as a promising source, because sponges harbor a number of phylogenetically unidentified microbial species. Kim et al. (2006) have discovered that clinically used antibiotics rifamycin B and rifamycin SV are produced by marine sponge-derived actinobacterial *Salinispora* strains. Indeed, this finding has revealed that new actinobacterial species other than the known soil actinobacterial species *Amycolatopsis mediterranei* have the ability to produce rifamycin and it would be useful to explore new rifamycin sources to overcome developing resistance for commercial sources. Several novel and known metabolites that possess strong antibacterial activity against clinically important



**FIGURE 19.3** Sponge-derived antimicrobial metabolites.

**TABLE 19.2**  
**Examples of Antimicrobial Metabolites from Sponge-Derived Microbes**

Compound	Sponge Species	Microbial Species	Action Spectrum	Reference
Rifamycin B and rifamycin SV	<i>Pseudoceratina clavata</i>	<i>Salinispora</i> sp.	Antibiotic	Kim et al. (2006)
Hirsutanols A	<i>Haliclona</i> sp.	NA	Antibiotic	Wang et al. (1998)
Sumiki's acid	<i>Callyspongia aerizusa</i>	<i>Cladosporium herbarum</i>	Antibiotic	Jadulco et al. (2001)
NA	<i>Dendrilla nigra</i>	<i>Nocardiopsis dassonvillei</i>	Antibiotic	Selvin et al. (2009)
NA	<i>Doriopsis granulosa</i>	<i>Enterobacter</i> sp.	Antibiotic	Gopi et al. (2012)
NA	<i>Sigmadocia fibulatus</i>	<i>Bacillus</i> sp.	Antibiotic	Anand et al. (2006)
NA	NA	NA	Antibiotics	Papaleo et al. (2012)
YM-202204	<i>Halichondria japonica</i>	<i>Phoma</i> sp.	Antifungal	Nagai et al. (2002)
NA	NA	<i>Streptomyces</i> sp.	Antifungal	Abdelmohsen et al. (2010)
Manzamines	<i>Haliclona</i> sp.	<i>Micromonospora</i> sp.	Antimalarial	Ang et al. (2000)
NA	<i>Clathria indica</i>	NA	Antimalarial	Ravikumar and Inbaneson (2012)

NA - Not Available

pathogens have been discovered from sponge-derived microbes (Table 19.2). While hirsutanols A, a new cyclic sesquiterpene isolated from the fungus separated from an Indo-Pacific sponge *Haliclona* sp., significantly acts against *Bacillus subtilis*, furan carboxylic Sumiki's acid isolated from the sponge-derived fungus *Cladosporium herbarum* acid is active against both *B. subtilis* and *Staphylococcus aureus* (Wang et al. 1998; Jadulco et al. 2001). Selvin et al. (2009) have found both organic-solvent and water-soluble antimicrobial compounds from a sponge-derived actinomycete, *Nocardiopsis dassonvillei* MAD08. Because of nonhemolytic, bactericidal, and surface-active properties, these compounds act against bacterial growth by inhibiting adhesion of pathogenic bacteria to host tissues, and thus compounds are more potent even at the early stages of pathogenesis. Although the mode of action is not clear, numerous crude extracts obtained from the fermentation broth of sponge-derived microbial species that were evaluated against most common pathogenic bacteria have shown that these extracts potently inhibit the pathogen *Escherichia coli* while significantly acting against several others. These results show that detailed studies on extracts will pave the way for the discovery of new antimicrobial drugs (Gopi et al. 2012; Anand et al. 2006). A comprehensive study on Antarctic sponge-associated microbial communities has been performed by Papaleo et al. (2012) with the aim of identifying new antimicrobial agents. Among the identified 140 bacterial strains, most of the strains have completely inhibited the growth of bacteria belonging to the *Burkholderia cepacia* complex. The final conclusion of the study is that sponge-associated bacteria represent an untapped source for the identification of new antimicrobial compounds.

#### 19.4.2.2 Antifungal Metabolites

Even though many antifungal medications are currently used, side effects such as liver damage, effects on estrogen level, and some allergic reactions lower their effectiveness and popularity. Likewise, treatments with immunosuppressive drugs and diseases like AIDS continually emphasize the search for new antifungal agents (Munoz et al. 2006). In this regard, sponge-derived microbial metabolites have been shown to have potential as new antifungal agents. A recent study on actinobacterial species isolated from several sponges collected from the Mediterranean Sea and the Red Sea has reported that putatively new *Streptomyces* sp. produce potent antifungal agents

against *Candida albicans* with significant inhibition of some clinically important bacterial species (Abdelmohsen et al. 2010). A similar discovery was reported a few years ago from the metabolites of sponge-derived fungus *Phoma* sp. In addition to the inhibition of *C. albicans*, the above-mentioned compounds act against *Cryptococcus neoformans* and *Aspergillus fumigatus* (Nagai et al. 2002). These fungal species are virulent in immunocompromised patients, and the most commonly used clinical drug amphotericin B shows some acute and chronic toxicities. In this case, the above findings have increased interest in exploring sponge-associated microbes for a broad spectrum of fungicidal activity with fewer side effects.

#### 19.4.2.3 Antimalarial Metabolites

Natural products play a significant role in drug discoveries for parasites, and quinine, which has been initially isolated from the barks of the cinchona tree, is one of the best examples. Until the development of drug resistance by some parasites, these natural products and their chemical derivatives have cured millions of people. Due to the constant emergence of resistant strains and the absence of effective vaccines, there is a pressing need for novel metabolites. Manzamines are sponge-derived novel antimalarial agents that have been initially perceived as sponge metabolites. Recent research has proved that manzamines are true microbial metabolites and that sponge-derived actinomycetes of the genus *Micromonospora* produce manzamines. Large-scale fermentation of sponge-associated microbes to produce manzamines with proved antimalarial activity is under research level (Ang et al. 2000; Taylor et al. 2007). Recently, Ravikumar and Inbaneson (2012) have studied the sponge *Clathria indica*-derived bacterial extract against the malaria parasite *Plasmodium falciparum*. *In vitro* screening of antiplasmodial activity have shown that one bacterial strain produces potent antimalarial metabolites with an  $IC_{50}$  value of 19.59  $\mu\text{g}/\text{mL}$  and the activity is highly comparable with that of chloroquine ( $IC_{50}$  28.80  $\mu\text{g}/\text{mL}$ ). Since marine sponges have several novel and potent antimalarial metabolites, profound explorations of those sponge species might reveal the true producers of those metabolites similarly to the case of manzamines.

#### 19.4.3 ANTIOXIDANT AND ANTI-INFLAMMATORY METABOLITES

Inflammation, free radical formation, and oxidative stress are normal cellular processes in a healthy body, and there are several means to control these processes. However, these phenomena raise the first step of many diseases, such as cancer, heart disease, stroke, and Alzheimer's. Anti-inflammation treatments and antioxidants are the front-line defense strategies of chronic diseases (Hussain, Hofseth, and Harri 2003). Thus, there is great interest in searching for sources of antioxidants from natural sources to combat emerging lifestyle-related diseases, and marine sponges are a more popular source of novel metabolites than conventional metabolites. Without limiting to sponges, studies have taken interest in sponge-associated microbes to realize the dream of marine-derived novel antioxidants. A novel antioxidant aromatic polyketide has been discovered from the fungus *A. versicolor* isolated from the sponge *Petrosia* sp. The chemical investigations reveal that the antioxidant activity of this compound is comparable to that of butylated hydroxyanisole and significantly higher than that of butylated hydroxytoluene (BHT) (Li et al. 2011). Two other novel indole derivatives have been isolated from the sponge-derived yeast *Pichia membranifaciens*. DPPH (1,1-diphenyl-2-picrylhydrazyl) radical-scavenging capacity of the compounds has been less than that of BHT (Sugiyama et al. 2009). A fermentation study of marine actinomycetes, originally separated from the sponge *Mycale mytilorum*, has shown that this strain was able to produce carotenoids under white fluorescent light. Thin-layer chromatography analysis and high-performance liquid chromatography analysis have confirmed that the carotenoid extract consists of phytoene, which is a precursor of antioxidative pigments, and that this phytoene could be used for mass production of antioxidative substances (Dharmaraj, Ashokkumar, and Dhevendran 2009).

Hundreds of research articles have been published to prove that marine sponges are a potential source of novel anti-inflammatory agents. Keyzers and Coleman (2005) have comprehensively discussed

84 anti-inflammatory compounds derived from marine sponges. However, none of them was able to enter clinical trials. An interesting finding was reported by Stirele, Cardellina, and Singleton (1991) two decades ago. A *Micrococcus* sp. isolated from the fire sponge yielded benzothiazoles that have previously been reported as sponge metabolites, and these benzothiazoles are potent anti-inflammatory agents (Piel 2004; Chaudhary et al. 2010). The result waves a hint that the same phenomenon would be able to apply for other anti-inflammatory metabolites, and detailed exploration may reveal the truth.

## 19.5 PROSPECTS OF MICROBIAL METABOLITES

On the basis of the above details, two facts have emerged clearly: the diversity of sponge–microbial association is huge, and these symbionts are capable of producing metabolites that have been initially isolated from sponges. Thus, it seems that the microbiological approach is a more economically viable step to produce sponge-derived bioactive metabolites than other alternatives such as aquaculture and sponge cell culture. However, to realize the mass production of biologically active metabolites for drug development, there are a few more steps to be completed. The available knowledge on the biological activity of microbial metabolites is preliminary since most of them are *in vitro* experiments, and detailed *in vitro*, *in vivo*, and clinical studies should be conducted to identify molecular targets of metabolites and their probable side effects. The bottleneck of the cultivation approach of sponge symbionts is accomplishing successful cultivation of associated microorganisms by standard techniques while maintaining the production of bioactive compounds of interest. Even though the isolation and characterization of associated microbial strains sounds easier, many difficulties have been reported in mass culturing of symbiotic microbes, as there is a mutual relationship between the host sponge and the symbiont. Therefore, new culturing techniques for symbiotic microorganisms should be implemented, as some authors have reported that improved cultivability of sponge-associated microorganisms is possible by supplementing media with sponge extracts (Taylor et al. 2007). Another possible approach is genetic engineering, by which biosynthetic genes of microbes can be cloned, sequenced, and expressed in suitable hosts that can be easily cultured, since many biosynthetic gene clusters, such as polyketide synthase, have been isolated from sponge-associated microbes (Thakur and Muller 2004; Schirmer et al. 2005; Hochmuth and Piel 2009). Indeed, successful search of new drug candidates from sponge-associated microorganisms is a multidisciplinary research goal.

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# 20 Marine Actinobacteria

## *A Potential Source of Antifungal Compounds*

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### 20.1 INTRODUCTION

Antibiotic research from the discovery of Fleming to our days has been a fascinating, exciting, continuously changing, and developing adventure. As a result of the frenzied research of the past 50+ years, in our days, tens of thousands of natural products derived from microbial sources are known. Interest in the field has been generally increasing, although sometimes there has been decline; interest and the whole story shows some cyclic features with successes and failures and evolved around changing clinical needs and new enabling technology. After the revolution in the “heroic” or “golden” era, in the 1940s and early 1950s, when almost all groups of important antibacterial antibiotics (tetracyclines, cephalosporins, aminoglycosides, and macrolides) were discovered, the success story continued. It seemed in the 1950s and 1960s that the main problems of chemotherapy had been solved. Antibiotics discovered in this period were mainly isolated from *Streptomyces* species, representing approximately 70–80% of all the isolated compounds. They were primarily active against bacteria and fungi. In this period, the discovery of antitumor, antiviral, and nonantibiotic—enzyme inhibitory—metabolites had just started (Bérdy 1985).

In the next period, between the 1970s and 1990s, the efficiency of research had decreased. The costs of research had increased, and although the number of discovered new compounds still increased, they were mainly analogues of known compounds. The scope of search for various bioactive microbial products had, however, broadened. The exploration and wide utilization of the antitumor (doxorubicin) and agricultural antibiotics (antiparasitic avermectin, feed additive monensin, and herbicide glufosinate) and the early discoveries of utilization of microbial metabolites in the pharmacological fields (cyclosporine and statins), were important new features. The problems of chemotherapy (emerging new pathogens and escalation of multiresistant strains) had become serious. In this period, besides the leading role of actinobacterial products (65–70%), the considerable increase of the discovery of “rare actinobacterial” products (up to 30%) was also noticeable. The chemical structures of almost all discovered compounds had been elucidated (Bérdy 1988).

After these years, from the 1990s forward, the exponential increase of the number of new metabolites (mainly nonantibiotic compounds, analogous, and minor compounds) still continued, but the occurrence of new chemical types had diminished. Due to urgent clinical needs, the increasing serious problems of chemotherapy (multiresistant strains, reappearing mycobacteria, HIV, etc.), new challenges in the therapy of physiological diseases and in agriculture, and the renovation of the classical screening methods, allowed by the new technologies, were highly required. Methodological innovation accompanied by changes in conception. Cost-effective high-throughput screening methods (robotics and instrumentation) based on mainly biochemical rationale and the wide application of diverse genetic manipulations became more and more widespread. The rapid progress of the human genome project provided access to a wide range of new molecular targets implicated in diverse noninfectious diseases. The screenings have become more efficient than ever. The dramatic increase of the isolation of nonantibiotic compounds with pharmacological and agricultural activities (up to 60–65% of all isolated compounds), the increasing share of various fungal metabolites (up to 50%), and the chemical synthesis of more and more complicated structures are the most characteristic features of this present period. It seems, with the opening of the twenty-first century, a new era of antibiotic research has opened (Bérdy 1989).

Notwithstanding the failures, the almost exponential increase of the total number of discovered compounds in past decades surprisingly became constant. In 1940, there were only 10–20 discovered compounds; in 1950, 300–400; in 1960, approximately 800–1000; and in 1970, already 2500 antibiotics were known. From that time, the total number of known bioactive microbial metabolites has doubled every 10 years. In 1980, there were about 5000; in 1990, 10,000; and in 2000, already almost 20,000 antibiotic compounds were known. By the end of 2002, over 22,000 bioactive secondary metabolites (including antibiotics) were published in the scientific and patent literature. Unfortunately, these quantitative improvements do not mean similar qualitative, practical results. The expected corresponding spectacular successes, in spite of the great scientific and technical developments, are still waiting to be fulfilled (Bérdy 1995).

Because of the fascinating technical improvements in the separation and isolation techniques, in recent days, it is likely that—over the antibiotics—close to one million naturally occurring compounds are known; however, it is supposed that today (at the end of 2003), due to the intensive use of genetic methods and high-throughput screening techniques, the number of existing and detected compounds may be definitely higher. The majority of natural products are derived—besides the microbial products isolated from prokaryotic bacteria and eukaryotic microorganisms, where almost all of the antibiotic producing microbes (except the animal protozoa) are belonging to from higher plants and various animal organisms. Higher plant metabolites represent at least 5,00,000–6,00,000 compounds, covering a great number of common plant compounds such as alkaloids, flavonoids, terpenoids, steroids, and carbohydrates. The approximate number of known natural products derived from the main types of plant and animal organisms is summarized in Table 20.1.

**TABLE 20.1**  
**Approximate Number of Known Natural Products**

Source	All Known Compounds	Bioactives	Antibiotics
Natural products	Over one million	200,000–250,000	25,000–30,000
Plant kingdom	600,000–700,000	150,000–200,000	~25,000
Microbes	Over 50,000	22,000–23,000	~17,000
Algae, lichens	3,000–5,000	1,500–2,000	~1,000
Higher plants	500,000–600,000	~100,000	10,000–12,000
Animal kingdom	300,000–400,000	50,000–100,000	~5,000
Protozoa	Several hundreds	100–200	~50
Invertebrates	~100,000	NA	~500
Marine animals	20,000–25,000	7,000–8,000	3,000–4,000
Insects/worms/etc.	8,000–10,000	800–1,000	150–200
Vertebrates (mammals, fishes, amphibians, etc.)	200,000–250,000	50,000–70,000	~1,000

Source: Bérdy, J., *J. Antibiot.*, 58, 1, 2005.

Note: NA, Data not available.

An important part of the natural products, the group of small molecular secondary metabolites of microorganisms, usually exhibits some kind of biological activity, and these compounds, the bioactive secondary microbial metabolites, represent the main topic of the recent review. Secondary metabolites are known from the ancient times, and they were mainly botanicals. The first crystalline fungal product from *Penicillium glaucum* considered a microbial secondary metabolite was mycophenolic acid, discovered in 1896 by Gosio (Demain and Fang 2000).

## 20.2 DISTRIBUTION OF BIOACTIVE NATURAL PRODUCTS

Antibiotics and similar natural products, being secondary metabolites, can be produced by almost all types of living things. They are produced by prokaryotic and eukaryotic organisms belonging to the plant and the animal kingdoms, alike. The secondary metabolite-producing ability, however, is very uneven in the species of living world. In the prokaryotae and plant kingdom, there are distinct groups of organisms, namely, unicellular bacteria, eukaryotic fungi, and first of all filamentous actinobacteria, that are the most frequent and most versatile producers.

In the group of prokaryotic, unicellular bacteria, the *Bacillus* and the *Pseudomonas* species are the most frequent producers. In recent years, myxobacteria and cyanobacteria species seem to have joined these distinguished organisms as prolific species. Mycobacteria, mycoplasmales, and spirotheces are far less frequent producers. The total number of known bioactive compounds in this group is about 3800, 17% of all microbial metabolites. The filamentous actinobacterial species produce over 10,000 bioactive compounds, 7600 derived from *Streptomyces* and 2500 from the so-called rare actinobacteria (rare-actino) species, and represent the largest group (45%) of bioactive microbial metabolites. From the known (altogether 22,500) antibiotics and similar bioactive microbial compounds, less than 1%, or only about 150 compounds, are in direct use in human and veterinary medicine and agriculture. In human therapy, about 100 compounds, most of them derived from actinobacterial species, are in direct practical use (Bérdy 2005).

**TABLE 20.2****Approximate Number of Bioactive Microbial Natural Products According to Their Producers**

Source	Antibiotics	Other Bioactive Metabolites	Total Bioactive Metabolites	Practically Used (in Human Therapy)	Inactive Metabolites
Bacteria	2,900	900	3,800	10–12 (810)	3,000–5,000
Actinobacteria	8,700	1,400	10,100	100–120 (70–75)	5,000–10,000
Fungi	4,900	3,700	8,600	30–35 (13–15)	2,000–15,000
Total	16,500	6,000	22,500	140–160 (~100)	20,000–25,000

Source: Bérdy, J., *J. Antibiot.*, 58, 1, 2005.

As a best approximation, the total number of additional “inactive” microbial products is about 20,000–25,000; therefore, today, close to 50,000 microbial metabolites may be known. According to the main types of microbial producers, the numbers of compounds, including both antibiotics and “other bioactive” metabolites, practically used compounds, and the approximate numbers of the inactive microbial metabolites are summarized in the Table 20.2.

Over the bioactive microbial compounds from higher species of the plant kingdom such as algae and lichens and mainly vascular plants, more than 13,000 antimicrobial antitumor/antiviral compounds were isolated. Additionally, from species of the animal kingdom, some 7000 bioactive compounds, derived from various marine and terrestrial animals, are also described. It is important to note that the overlapping between products of the main groups of microbial producers (e.g., fungi and actinobacteria) is also very rare, less than 1%. The overlapping between *Streptomyces* and the taxonomically similar rare-actino products is more frequent, but it is still only about 10%.

Obviously, various actinobacteria, first of all the *Streptomyces* species and filamentous fungi and to a lesser extent several bacterial species, are the most noteworthy producers with respect to numbers, versatility, and diversity of structures of the produced metabolites. The significance and frequency of these main types of microbes as producers of bioactive metabolites has varied significantly in past decades. In the beginning of the antibiotic era, the fungal (penicillin and griseofulvin) and the bacterial (gramicidin) species were in the foreground of interest, but after the discovery of streptomycin and later chloramphenicol, tetracyclines, and macrolides, the attention turned to the *Streptomyces* species. In the 1950s and 1960s, the majority (~70%) of antibiotics were discovered from these species. In the next two decades, the significance of the non-*Streptomyces* actinobacterial species (rare-actinos) was increased, up to a 25–30% share of all antibiotics. From the early 1990s, the number of bioactive compounds isolated from various filamentous and other microscopic and higher fungal species continuously increased up to more than 50% by the turn of the millennium (2000). The interest in bacteria in recent years has only slightly increased. Simultaneously, the ratio of actinobacterial compounds naturally has definitely decreased.

The most characteristic and a little bit surprising feature of recent years is the declining representation of the formerly exhaustively investigated actinobacteria. Their share among all microbial products presently is only 30–35%, in contrast to the 75–80% share from the 1960s to the 1980s. The shift in their apparent participation is rather the result of the favored fashion of fungal screening in some laboratories. Presently, the claim for new microbial pharmacophores led to the shift of efforts in most places toward the discovery of fungal products from the large pool of the untapped fungal world. However, the present slight overestimation of the capability of fungi is rather a periodic phenomenon. It is very likely that the changes in interest toward the favorite microbes (as happened with actinobacteria in earlier years) depends on new expectations, the changing needs in the human therapy, and probably sometimes on fashion. It is also likely that for numerous reasons,

the actinobacteria, besides the fungi, will remain equally important and promising producers in the future.

The serious problems of chemotherapy, the again and again increasing resistance of bacteria and fungi, the newly emerging old and new pathogens (mycobacteria, anaerobes, virus, etc.), the high mortality of some common bacterial diseases, the problems of viral infections and neoplastic diseases, and so on all require new agents, which, we strongly believe, will be greatly based on sophisticated study of the until now less-known new, rare-actino as producers. In Table 20.3, the numbers of all antibiotics (without other activities), the numbers of the antibiotics exhibiting additional "other" bioactivities (in parenthesis), and the "other bioactive" metabolites as well as the total numbers are summarized according to the main producer types and several specific producer species.

Notwithstanding the recent drop, 45% of the presently known bioactive microbial metabolites, over 10,000 compounds, were still isolated from various actinobacterial species, 34% from *Streptomyces*, and 11% from the rare-actinos. The most frequent producers, the *Streptomyces* species, produce 7600 compounds (74% of all actinobacteria), while the rare-actino represent 26%, altogether 2500 compounds. The representation of rare-actino products in 1970 was only 5%. In this group, *Micromonospora*, *Actinomadura*, *Streptoverticillium*, *Actinoplanes*, *Nocardia*, *Saccharopolyspora*, and *Streptosporangium* species are the most frequent producers, each producing several hundreds of antibiotics (Donadio, Carrano et al. 2002). In Table 20.4, the numbers of actinobacterial species, including all rare-actinos, known to produce bioactive metabolites are summarized.

TABLE 20.3

## Approximate Number of Bioactive Microbial Metabolites Producers and Bioactivities

Source	Bioactive Secondary Microbial Metabolites				
	Antibiotics		Bioactive Metabolites		
	Total	With Other Activities	No Antibiotic Activity	Antibiotics Plus "Other Bioactives"	Total Bioactive Metabolites
Bacteria	2,900	780	900	1,680	3,800
Eubacteriales	2,170	570	580	1,150	2,750
<i>Bacillus</i> sp.	795	235	235	300	860
<i>Pseudomonas</i> sp.	610	185	185	370	795
Myxobacteria	400	130	10	140	410
Cyanobacteria	300	80	340	420	640
Actinobacteria	8,700	2,400	1,400	3,800	10,100
<i>Streptomyces</i> sp.	6,550	1,920	1,080	3,000	7,630
Rare-actinos	2,250	580	220	800	2,470
Fungi	4,900	2,300	3,700	6,000	8,600
Microscopic fungi	3,770	2,070	2,680	4,750	6,450
<i>Penicillium/Aspergillus</i>	1,000	450	950	1,400	1,950
Basidiomycetes	1,050	200	950	1,150	2,000
Yeasts	105	35	35	70	140
Slime moulds	30	5	20	25	60
Total microbial	16,500	5,500	6,000	11,500	22,500
Protozoa	35	10	5	45	50

Source: Bérdy, J., *J. Antibiot.*, 58, 1, 2005.

**TABLE 20.4**  
**Number of Actinobacterial Species Producing Bioactive Microbial Metabolites**

Name of the Actinobacteria	No. of Bioactive Metabolites	Name of the Actinobacteria	No. of Bioactive Metabolites
<b><i>Streptomycetaceae</i></b>		<b><i>Thermomonosporaceae</i></b>	
<i>Streptomyces</i>	~8,000	<i>Actinomadura</i>	345
<i>Streptoverticillium</i>	258	<i>Saccharothrix</i>	68
<i>Kitasatospora</i>	37	<i>Microbispora</i>	54
<i>Chainia</i>	30	<i>Actinosynnema</i>	51
<i>Microellobosporia</i>	11	<i>Nocardiopsis</i>	41
<i>Nocardiooides</i>	9	<i>Microtetraspora/nonomuria</i>	26/21
<b><i>Micromonosporaceae (actinoplanetes)</i></b>		<b><i>Thermoactinomyces</i></b>	14
<i>Micromonospora</i>	740	<i>Thermopolyspora</i>	1
<i>Actinoplanes</i>	248	<i>Thermoactinopolyspora</i>	1
<i>Dactylosporangium</i>	58	<b><i>Mycobacteriaceae (actinobacteria)</i></b>	
<i>Ampullariella</i>	9	<i>Nocardia</i>	(357)
<i>Glycomyces</i>	2	<i>Mycobacterium</i>	57
<i>Catenuloplanes</i>	3	<i>Arthrobacter</i>	25
<i>Catellatospora</i>	1	<i>Brevibacterium</i>	17
<b><i>Pseudonocardiaceae</i></b>		<i>Proactinomyces</i>	14
<i>Saccharopolyspora</i>	131	<i>Rhodococcus</i>	13
<i>Amycalotopsis/nocardia</i>	120/357	<b><i>Other (unclassified) species</i></b>	
<i>Kibdellosporangium</i>	34	<i>Actinosporangium</i>	30
<i>Pseudonocardia</i>	27	<i>Microellobosporia</i>	11
<i>Amycolata</i>	12	<i>Frankia</i>	7
<i>Saccharomonospora</i>	2	<i>Westerdykella</i>	6
<i>Actinopolyspora</i>	1	<i>Kitasatoa</i>	5
<b><i>Streptosporangiaceae (maduromycetes)</i></b>		<i>Synnenomyces</i>	4
<i>Streptosporangium</i>	79	<i>Sebekia</i>	3
<i>Streptoalloteichus</i>	48	<i>Elaktomyces</i>	3
<i>Spirillospora</i>	11	<i>Excelsospora</i>	3
<i>Planobispora</i>	10	<i>Waksmania</i>	3
<i>Kutzneria</i>	4	<i>Alkalomyces</i>	1
<i>Planomonospora</i>	2	<i>Erythrosporangium</i>	1
		<i>Streptoplanospora</i>	1
		<i>Microechinospora</i>	1
		<i>Salinospora</i>	1

Source: Bérdy, J., *J. Antibiot.*, 58, 1, 2005.

These fastidious organisms, the rare-actinos, produce perhaps the most diverse and most unique, unprecedented, sometimes very complicated compounds exhibiting excellent antibacterial potency and usually low toxicity. It is interesting that several chemical types, such as simple terpenoids or benzoids, are almost completely absent from these compounds. In this group of metabolites, there are numerous practically very important compounds such as gentamicins, erythromycins, vancomycin, or rifamycin. Numerous recently introduced chemotherapeutic and agricultural agents (ziracin, dalbavancin, and spynosin) are also rare-actino products. It is noteworthy that the vancomycin-ristocetin type complicated glycopeptides are produced almost exclusively by various rare-actino species. Currently, more than 50 rare-actinos are known as producers of the 2500 bioactive compounds, but in 1970, only 11 rare-actino species, producing altogether 50 compounds, were known. The number of all taxonomically described rare-actinos today is close to 100, but this number, due to the recently developed genetic and isolation techniques, will be quickly increasing. The present relatively low occurrence of rare-actinos, in contrast to *Streptomyces* species, is derived from the fact that they are hard to isolate from the environment and difficult to cultivate and maintain under conventional conditions. These are reasons why these species are still regarded to be rare. Recently, however, advanced isolation techniques have been developed, and with these techniques from an environmental sample, the overwhelming majority of these rare species could be isolated (Donadio, Carrano et al. 2002; Donadio, Monciardini et al. 2002). In light of our accumulated knowledge and the statistical data, however, the potency of the *Streptomyces* species should not be underestimated. Their capacity to produce promising new compounds will certainly be unsurpassed for a long time and still they have been producing the majority of the chemotherapeutically applied antibiotics.

### 20.3 BIOACTIVITIES OF SECONDARY METABOLITES

The presently known secondary microbial metabolites exhibit a great number of diverse and versatile biological effects, first of all antimicrobial activities. In the scientific literature, already hundreds of different pathogenic and other microbes (Gram-positive and Gram-negative bacteria, fungi, yeasts, etc.) are described as test microorganisms in the direct activity-based screenings. The most frequent test organisms were *Bacillus subtilis*, *Staphylococcus aureus*, *Micrococcus (Sarcina) lutea*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Saccharomyces cerevisiae*, *Candida albicans*, and others. Antiviral tests, inhibition of viral enzymes, activities connecting with neoplastic diseases from simple cytotoxicity assay methods (P-388, KB, L-1210 cell lines) to angiogenesis inhibition, and so on are used most frequently for detection of other, nonantimicrobial activities of metabolites.

However, the infectious diseases are mainly treated with natural antibiotics and their derivatives, while still the majority of drugs applied in the so-called physiological diseases are synthetic products. The challenge for natural medical products to treat these diseases is huge. There is an urgent need in this area to identify totally new active chemotypes at least as lead compounds for effective drug development. The list of nonantibiotic biological activities used in the new screening projects presently covers more than 1000 different types of bioactivities, cell-based receptor binding, or enzymatic assay methods and many other specific tests and targets. In our days, more and more newer, sophisticated, and diverse assays are used worldwide in various screening protocols. The main types of bioactivities and the frequency of their occurrence in the scientific literature, according to historical and practical point of views, are summarized in [Table 20.5](#).

We have to note that in Table 20.5, and in most cases, the statistical numbers indicate the discovered and published bioactivities or other characteristics and not all existing ones, and therefore, some statistical data (percentage of bioactivities) may be misleading due to the frequent exclusive use of the specific tests, assays, and models in various screening projects. Missing data in the publications, for example, do not exclude the possible existence of any activity or characteristics. From a practical point of view, most of the presently known nonantibiotic bioactivities may be classified as pharmacological-biochemical or medical activity, agricultural activity, and regulatory, biophysical, and other activities.

The first type of “other” bioactive compounds with possible medical activity in the largest number covers the potentially very important and promising metabolites with enzyme inhibitory activities. Presently, over 3000 compounds are known to possess inhibitory activity against about 300–350 various enzyme systems. The agriculturally active compounds, of course, may also include enzyme inhibitory compounds. There are about 800 immunoactive compounds (immune suppressive and immune stimulatory) and hundreds of compounds with the most diverse regulatory, inhibitory, agonist, and antagonist activity, including anti-inflammatory/antioxidative, hypcholesterolemic, antimetabolite, and various toxic (mycotoxic, etc.) actions. Biochemical activities such as tubulin (microtubule) assembly inhibition, interferon-inducing activity, antimitotic/antimitogenic activity, DNA damaging activity, antimutagenic effects, apoptosis-inducing activity, angiogenesis inhibition, and so on also frequently occur. The assays detecting these activities show extremely large variations, and the range of the final physiological, biological, biochemical, phytochemical, and microbiological effects include close to 1000 bioactivity categories. The compounds discovered by these specific methods may be called (distinguishing them from the antibiotics) microbial medicinal products or “biopharmaceutics.”

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**TABLE 20.5**  
**Bioactivity Types of Microbial Metabolites and Number of Discovered Bioactivities**

Type of Activity	Number of Discovered Bioactivities
<b>Antibiotic activities (16,500 compounds)</b>	
<i>Antimicrobial activity</i>	
Antibacterial: Gram positive	11,000~12,000
Gram negative	5,000~5,500
<i>Mycobacteria</i>	800~1,000
Antifungal: Yeasts	3,000~3,500
Phytopathogenic fungi	1,600~1,800
Other fungi	3,800~4,000
Antiprotozoal:	~1,000
<i>Chemotherapeutic activity</i>	
Antitumor (cytotoxic)	5,000~5,500
Antiviral	1,500~1,600
<b>Other bioactivities (11,500 compounds)</b>	
<i>Pharmacological activity</i>	
Enzyme inhibitor	3,000~3,200
Immunological activity (suppressive, modulatory)	~800
Biochemical activity (DNS, tubulin, mitotic, etc.)	~1,000
Other (antagonistic, modulatory, anti-inflammatory, etc.) activities	2,000~2,500
<i>Agricultural activity</i>	
Pesticide (antiparasitic, algicide, amoebicide, etc.)	900~1,000
Herbicide (phytotoxic, plant growth regulatory, etc.)	1,800~1,900
Insecticide/miticide/larvicide/deterrent	1,100~1,200
Feed additive, preservative	300~400
Other activities	~1,000
Microbial regulators (growth factors, microbial hormones, morphogens)	~500
Biophysical effects (surfactants, etc.)	~300

Source: Bérdy, J., *J. Antibiot.*, 58, 1, 2005.

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## 20.4 ANTIBIOTIC ACTIVITIES

Sixty percent of the presently known bioactive microbial metabolites, about 14,000 compounds, exhibit antimicrobial (antibacterial, antifungal, and antiprotozoal) activity, approximately 5000 compounds exhibit antitumor activities, and about 1500 compounds exhibits antiviral activity. Of course, these observed activities may be significantly overlapping. In addition to antibiotic activities, some 11,500 microbial metabolites, half of the compounds, possess some kind of additional (or exclusive) “other” nonantibiotic bioactivities. Among them, there are about 6000 metabolites without any recognized antimicrobial activity exhibiting exclusively some kind of “other” biological activity. They are the so-called other bioactive metabolites. The numbers of antibiotics (“classical” antibiotics) and the “other bioactive” metabolites according to their origin are summarized in Tables 20.2 and 20.3. As derived from the numbers given in the tables, in total 16,500 compounds, 73% of all bioactive metabolites, may be considered strictly as antibiotics.

In the group of antibiotics, the inhibitory activity against Gram-positive, Gram-negative, and mycobacteria exist in 66%, 30% and 5% (10,900, ~5,000, and 350) of the compounds respectively. Altogether, 5600 compounds (34%) show antifungal activity, for which 21% (3500) are active against yeasts, 11% (1800) are active against phytopathogenic fungi, and 24% (4000) are active against other fungal species. About 2000 compounds, for example, the polyene antibiotics, exhibit exclusively antifungal/antiyeast activity (Donadio, Monciardini et al. 2002).

The combination of various antimicrobial and other activities shows a wide variation. A great part of antibiotic compounds exhibit exclusive activity against Gram-positive bacteria (~30%), but there are “broad antibacterial spectrum” compounds with activity against Gram-positive, Gram-negative, and mycobacteria (15%), and there are the broadest spectrum compounds with additional antifungal activity (12%). Only 1.5% of the compounds (250 metabolites, e.g., the polymyxins) exhibits activity only against Gram-negative bacteria. The number of antimicrobial compounds with additional antitumor and/or antiviral activities is about 3000. There are about 500 compounds showing exclusively antitumor activity, and approximately 10–100 compounds are active solely against viruses or protozoa.

Several significant differences occur in the frequency of various antibiotic activities according to their microbial origin. The actinobacterial and bacterial products exhibit primarily antimicrobial activities. About 74% of all actinobacterial products, over 80% of the rare-action products, and similarly 70~75% of various bacterial products exhibit antibacterial and/or antifungal activities. In contrast, only 40~45% of all the fungal products have some kind of antimicrobial, frequently antifungal, activities. The antitumor activity shows less significant differences in its distribution, namely, 30%, 24%, and 27% for actinobacterial, bacterial, and fungal products, respectively, have antitumor activity.

Based on the abovementioned information, among the microbial sources of antimicrobial compounds, actinobacteria, especially marine actinobacteria, are potential sources of novel antibiotics with pharmaceutical interest. Hence, the exploitation of marine actinobacteria for screening and antimicrobial compound production has been covered in Section 20.5.

## 20.5 ACTINOBACTERIA

Actinobacteria are well known as secondary metabolite producers and hence they receive high pharmacological and commercial interest. In 1940, Selman Waksman discovered actinomycin (streptomycin) from soil (terrestrial) actinobacteria, which won him a Nobel Prize. Subsequently, hundreds of naturally occurring antibiotics have been discovered in these terrestrial microorganisms, especially from the genus *Streptomyces*. Despite the long list of currently available antibiotics in the market, there are very few antifungal antibiotics, but they are a significant group of drugs and have an important role in the control of mycotic diseases. Only a limited number of antifungal agents are currently available for the treatment of life-threatening yeast and mold infections. However,

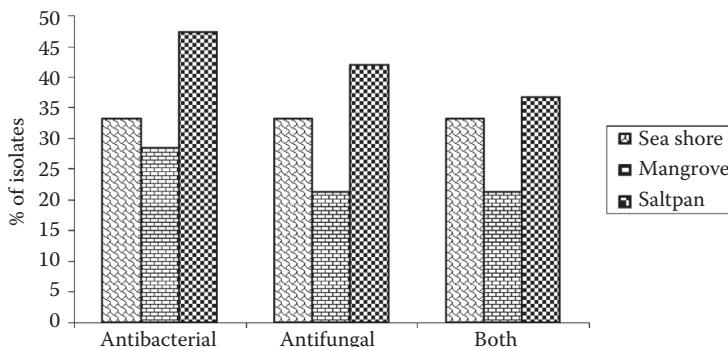
many compounds, polyenes in particular, cannot be used because of their toxicity, while they are of interest in agriculture, animal therapy, and industry. These antifungal agents show some limitations such as the significant nephrotoxicity of amphotericin B and emerging resistance to azoles, despite several recent improvements such as lipid formulations of polyenes with lower toxicity and new triazoles (voriconazole, ravuconazole, and pasaconazole) with a wider spectrum of action, including activity against some azole-resistant isolates. The search for a new, safer, broad spectrum antifungal antibiotic has been progressing slowly. The development of new antifungal agents, preferably natural ones with novel mechanisms of action, is an urgent clinical need.

## 20.6 MARINE ACTINOBACTERIA

Marine environments, namely, seashore, mangrove, and salt pan soils, have an enormous biodiversity potential. Unfortunately, only in recent decades have marine environments been explored for novel actinobacteria. Furthermore, only very little work has been done on the antifungal effect of actinobacteria on pathogenic fungi from coastal environments. There is a growing demand and need for new antifungal compounds to control many emerging fungal diseases of human beings, plants, and animals. For example, the opportunistic human pathogens *Candida albicans* and other non-*albicans* species have acquired considerable significance in the recent past due to the enhanced susceptibility of immunocompromised patients. These pathogenic species of *Candida* derive their importance not only from the severity of their infections but also from their ability to develop resistance against antifungals. Widespread and prolonged use of azoles has led to the rapid development of the phenomenon multidrug resistance (MDR), which poses a major hurdle in antifungal therapy. Various mechanisms that contribute to the development of MDR have been implicated in *Candida* as well as in other human fungal pathogens, and some of these include overexpression of mutations in the target enzyme of azoles, lanosterol 14 alpha-demethylase, and transcriptional activation of genes encoding drug efflux pump proteins belonging to ATP-binding cassette (ABC) as well as to major facilitator superfamilies (MFS) of transporters. The ABC transporters, CDR1, CDR2, and an MFS pump CaMDR1, play a key role in azole resistance as deduced from their high level of expression found in several azole-resistant clinical isolates (Prasad and Kapoor 2005). Since there is a constant development of resistance toward existing antifungal antibiotics, it is more essential to identify new, safe, and more effective antifungal agents to eradicate human fungal diseases. Identification of new novel antibiotics is a major challenge to the pharmaceutical industry, especially with the increase in opportunistic infections in the immunocompromised host. Thus, there is a vast potential for new and efficient antifungal antibiotics to make a major impact on healthcare.

## 20.7 ANTIFUNGAL COMPOUNDS FROM MARINE ACTINOBACTERIA

The biodiversity of actinobacteria from marine and hypersaline environments has been carried out by several workers from different countries, and they have published their research findings on antifungal compounds as follows: Okazaki and Okami (1972); Goodfellow and Haynes (1984); Pisano et al. (1989); Jensen, Dwight, and Fencial (1991); Jensen et al. (2005); Imamura et al. (1993); Takizawa, Colwell, and Hill (1993); Hayakawa et al. (1995); Ivanova et al. (1998); Zheng et al. (2000); and Gomes et al. (2000) demonstrated chitinolytic activity of acinobacteria on fungal mycelium; methyl-substituted  $\beta$ -lactam compounds from *Streptomyces noursei* (DPTD21) and 4'-phenyl-1-naphthyl-phenyl acetamide that was produced by *Streptomyces* sp. by Mincer et al. (2002); Maskey et al. (2003); Oskay, Tamer, and Azeri (2004); Kokare et al. (2004); Magarvey et al. (2004); Kim, Garson, and Furest (2005); Maldonado et al. (2005); Ilić, Kontantinovic, and Todorović (2005); and You et al. (2005); DPTB16 by Dhanasekaran, Thajuddin, and Panneerselvam 2008; Bull and Stach (2007); Lam (2006); Parungao, Maceda, and Villano (2007); and Olano, Méndez, and Salas (2009); highly oxygenated and derivatives of carbohydrates antimicrobial compounds by marine



**FIGURE 20.1** Antifungal activity of marine actinobacterial isolates.

streptomycetes by Vijayakumar (2006) and Vijayakumar et al. (2011a, b); oxohexaene and cephalaxine were produced by marine actinobacteria, namely, *Streptomyces* sp. RM17 and *Streptomyces* sp. RM42 respectively, in a study by Remya and Vijayakumar (2008); and nonpolyene antifungal antibiotic by Augustine and Kapadnis (2005) and Augustine, Bhavsar, and Kapadnis (2005).

In a study, a total of 68 isolates of actinobacteria isolated from the marine soils of Palk Strait regions, Bay of Bengal, South India, were screened for their antimicrobial activity against pathogenic bacteria and fungi by the cross streak method. Among the 68 isolates, 25 isolates showed antimicrobial activity. Among them, 24, 21, and 20 isolates showed antibacterial, antifungal, and both antibacterial and antifungal activity respectively (Figure 20.1). Among the 24 antibacterial antagonistic actinobacteria, 20 isolates inhibited the growth of Gram-positive bacteria, 17 isolates inhibited the growth of Gram-negative bacteria, and 13 isolates inhibited the growth of both Gram-positive and Gram-negative bacteria. The maximum percentage (47.4%) of antibacterial actinobacteria was found in salt pan soil followed by seashore soil (33.33%) and mangrove soil (28.57%). The maximum percentage of the isolates of actinobacteria, which showed antifungal antagonistic activity, was found in salt pan soil (42.1%) followed by seashore soil (33.33%) and mangrove soil (21.43%). The percentage contribution of both antibacterial and antifungal activities of actinobacteria was highest in salt pan soils (36.84%) (Vijayakumar, 2006). A similar type of work has been conducted by various workers in the Indian coastal area (Dhanasekaran, Panneerselvam, and Thajuddin 2005; Dhanasekaran, Sivamani et al. 2005; Dhanasekaran, Rajkumar et al. 2005; Vijayakumar et al. 2007, 2008, 2009, 2010, 2011, 2012a, b; Dhanasekaran, Thajuddin, and Panneerselvam 2008; Dhanasekaran et al. 2009; Baskaran, Vijayakumar, and Mohan 2011). The fungicidal potential of the marine actinobacteria was also studied and reported by Dhanasekaran, Thajuddin, and Panneerselvam (2011). Based on this report, it is clear that marine actinobacteria are a potential source of antifungal drugs. Many reports on antifungal compound producing actinobacteria are given in Table 20.6.

## 20.8 ISOLATION OF MARINE ACTINOBACTERIA

### 20.8.1 SELECTION OF MATERIALS FOR THE ISOLATION OF MARINE ACTINOBACTERIA

Many thousands of actinobacteria have been isolated from the environment until now, but we have little information about the geographical or ecological distribution of these microbes. Therefore, it is generally impossible to predict the sites in which a particular actinobacterial taxon or strain will occur. Thus, the selection of macro-environment or micro-environment as a source of useful isolates remains largely a matter of chance and hopeful initiative. According to the results of Takahashi et al. (1993), most of the actinobacteria occur within 1 m below the ground. Compared to terrestrial

**TABLE 20.6**  
**Antifungal Compound Production Screening by Various Studies**

Organism	Activity Against	References
Actinobacteria	<i>Fusarium oxysporum</i> , <i>Fusarium cubense</i> , and <i>Penicillium graminicolum</i>	Meredith (1946)
<i>Streptomyces</i> sp.	<i>Trichophyton</i> sp., <i>Fusarium</i> sp., <i>Penicillium</i> sp., and certain bacteria	Leben, Stessel, and Keitt (1952)
Actinobacteria	Yeast	Takahashi et al. (1993)
<i>Streptomyces antibioticus</i>	<i>Helminthosporium sativum</i>	Stevenson (1956)
<i>Streptomyces</i> , <i>Micromonospora</i> , and chitinolytic actinobacteria	Yeast and filamentous fungi	Pisano et al. (1989); Pisano, Sommer, and Taras (1992)
<i>Microbispora</i> sp.	<i>Aspergillus niger</i>	Hayakawa et al. (1995)
Actinobacteria	<i>Candida albicans</i>	Mukhopadhyay et al. (1999); Hwang et al. (2001)
<i>Streptomyces aureus</i>	<i>H. oryzae</i> , <i>Curvularia lunata</i> , and <i>Trichophyton mentagrophytes</i>	Chakrabarty and Chandra (1979)
<i>Streptomyces hygroscopicus</i>	<i>Trichophyton mentagrophytes</i> and <i>Calbicans</i>	Gurusiddaiah et al. (1979)
<i>Streptomyces violaceoniger</i>	<i>Macrophomina phaseolina</i> and <i>Calbicans</i>	Hussain and El-Gammal (1980); Ivanova et al. (1998)
<i>Streptomyces globus</i>	<i>Alternaria solani</i> , <i>Aspergillus niger</i> , <i>Curvularia pallescens</i> , <i>Trichophyton rubrum</i> , <i>T. mentagrophytes</i> , <i>Candida albicans</i> , and <i>Phytophthora</i> sp.	Nair et al. (1994); Hwang et al. (1996)
<i>Streptomyces arabicus</i>	<i>Alternaria brassicae</i>	Sharma, Gupta, and Singh (1985); El-Shahed (1994)
<i>Streptomyces</i> sp.	<i>Botrytis</i> sp., <i>Helminthosporium</i> sp., <i>Fusarium</i> sp., and <i>Pyricularia</i> sp.	Wang and Sehn (1992)
<i>Streptomyces</i> sp.	<i>Phytophthora</i> sp.	Hwang, Ahn, and Moon (1994); Tang et al. (2000)
<i>Streptomyces aerocolonigenes</i>	<i>Candida albicans</i>	Nishio et al. (1989)
<i>Streptomyces griseochromogenes</i>	Phytopathogenic fungi, <i>Streptomyces scabies</i> , and <i>Botrytis</i> sp.	Eckwall and Schottel (1997); Xiao, Kinkel, and Samac (2002)
<i>Streptomyces parasinopilosus</i>	<i>Trichophyton</i> sp., <i>Candida</i> sp.	Masanito et al. (1989)
<i>Streptomyces</i> sp.	<i>Trichophyton</i> and phytopathogenic fungi	Nair et al. (1989)
<i>Streptomyces violaceus</i>	<i>Candida albicans</i>	Hwang et al. (1996); Siu et al. (1997)
<i>Streptomyces roseiscleroticus</i> (sultricin) and <i>S. hygroscopicus</i> (yatakemycin)	<i>C. albicans</i> , <i>Cryptococcus neoformans</i> , <i>Aspergillus fumigatus</i> , <i>Fusarium moniliforme</i> , <i>Trichophyton mentagrophytes</i> , <i>Blastomyces dermatitidis</i> , and <i>Petriellidium boydii</i>	Atalan (1997); Zheng et al. (2000); Datta et al. (2001)
<i>Streptomyces</i> sp. (nonpolyene)	<i>Candida albicans</i> , <i>Candida tropicalis</i> , <i>Botrytis cinerea</i> , <i>Aspergillus fumigatus</i> , <i>Fusarium solani</i> , <i>Fusarium oxysporum</i> , <i>Pythium irregularare</i> , and <i>Trichophyton mentahrophytes</i>	Yon et al. (1995); Ouhdouch et al. (1996)
<i>S. violaceusniger</i> (new macrolide)	<i>Cryptococcus neoformans</i> , <i>Candida albicans</i> , <i>Candida tropicalis</i> , <i>Candida parapsioli</i> , <i>Candida glabrata</i> , <i>Aspergillus fumigatus</i> , <i>Aspergillus flavus</i> , <i>Trichophyton mentagrophytes</i> , <i>Trichophyton rubrum</i> , <i>Microsporum canis</i> , <i>Microsporum gypseum</i> , <i>Phytophthora capsici</i> , <i>Magnaporthe grisea</i> , and <i>Colletotrichum gloeosporides</i>	Woo and Kamei (2003); Fulgueira, Amigot, and Madni (2004)

**TABLE 20.6 (Continued)**  
**Antifungal Compound Production Screening by Various Studies**

Organism	Activity Against	References
<i>Streptomyces</i> sp.	<i>Saccharomyces cerevisiae</i> , <i>Fusarium moniliforme</i> , <i>Candida albicans</i> , <i>Botrytis pumilus</i> , <i>Fusarium oxysporum</i> , and <i>Aphanocladium macrosporum</i>	Saadoun et al. (1999)
Actinoplanets	Fungi	Takizawa, Colwell, and Hill (1993)
<i>Streptomyces</i> sp.	Fungi	Biabani et al. (1997)
<i>Streptomyces albidoflavus</i> var. <i>marina</i>	<i>C. albicans</i> , <i>Cryptococcus</i> sp., and <i>A. fumigatus</i>	Zhou and Zheng 1998
<i>Streptomyces bottropensis</i> and <i>Streptomyces griseoruber</i>	<i>Agrobacterium tumefaciens</i> and <i>C. albicans</i>	Saadoun and Al-Momani (2000)
<i>Actinomadura</i> sp.	<i>C. albicans</i> and fungi	Srivibool (2000)
<i>Streptomyces</i> sp.	Fungi	Ellaiah et al. (2002)
<i>Streptomyces</i> sp.	<i>C. albicans</i> , <i>Cryptococcus humiculus</i> , <i>S. cerevisiae</i> , <i>Phytophthora cinnamomi</i> , <i>Pestalotiopsis sydowiana</i> , <i>Sclerotinia homoeocarpa</i> , <i>Alternaria alternata</i> , <i>Mucor circinelloides</i> , <i>Pythium aphanidermatum</i> , <i>Pythium oligandrum</i> , <i>Pythium porphyrae</i> , <i>T. rubrum</i> , and <i>Ustilago maydis</i>	Narayana, Ravikiran, and Vijayalakshmi 2004; Augustine, Bhavsar, and Kapadnis (2005); Dhanasekaran, Panneerselvam, and Thajuddin (2005)
<i>S. antibioticus</i> and <i>Streptomyces rimosus</i>	Yeast	Sahin and Ugur (2003)
<i>Streptomyctetes</i>	Phytopathogenic and human pathogenic fungi	Oskay, Tamer, and Azeri (2004)
Endophytic <i>Streptomyces</i> sp.	<i>Rhizoctonia solani</i>	Cao et al. (2004)
<i>Streptomyces plicatus</i> and <i>Frankia</i> sp.	<i>A. solani</i> , <i>A. alternata</i> , <i>F. solani</i> , <i>Phytophthora megasperma</i> , <i>Verticillium dahliae</i> , and <i>S. cerevisiae</i>	Aghighi et al. (2004)
Marine actinobacteria	<i>R. solani</i> , <i>Pyricularia oryzae</i> , <i>H. oryzae</i> , and <i>Colletotrichum falcatum</i>	Kathiresan, Balagurunathan, and Masilamani Selvam (2005); Dhanasekaran, Sivamani et al. (2005); Dhanasekaran, Rajakumar et al. (2005)

soils, marine sediments and soils have proven to be the one of the best sources for the isolation of antagonistic actinobacteria (Okami 1986; Vijayakumar et al. 2007; 2011a, b; 2012; Olano, Mendez, and Salas 2009), along with salt mine samples (Yang et al. 2008); mangrove environment (Remya and Vijayakumar 2008; Dhanasekaran, Thajuddin, and Panneerselvam 2008; Dhanasekaran et al. 2009); estuary, sand dune, and industrially polluted coast soil, salt marsh soil (Al-Zarban et al. 2002; Kathiresan, Balagurunathan, and Masilamani Selvam 2005); coral reefs (Lam 2006); salt pan environment (Lakshminipathy and Kannabiran 2009; Vijayakumar et al. 2011b); sea anemone (Chen et al., 2009); marine sponge (Gandhimathi et al. 2009); beach soil (Ogunmwonyi et al. 2010); endophytic actinobacteria (Ravikumar et al. 2011); seawater (Reddy, Ramakrishna, and Raja Gopal 2010); salt-ern (Chun et al. 2000); and so on.

## 20.8.2 PRETREATMENT OF THE SAMPLES

A wide range of pretreatment has been applied to enhance the isolation of actinobacteria. These include chemical enrichment and physical/chemical treatments of the samples. Many of these exert a clear selectivity for the isolation of particular actinobacterial taxa, but the theoretical basis of their

**TABLE 20.7**  
**Antibiotics Used in the Selective Media**

Selective Agent	Actinobacteria Selected
Polymyxin + penicillin	Actinobacteria
Penicillin + NaCl	<i>Streptomyces</i>
Streptomycin, rifampicin	<i>Actinomadura</i>
Gentamycin	<i>Micromonospora</i>
Kanamycin	<i>Actinomadura</i>
Tetracycline	<i>Nocardia</i>
Nitrofurazone	<i>Streptomyces</i>

action is not always known. In the physical treatment, samples dried at 40°C for 2–16 hours or 55–110°C for 10 minutes greatly increased the recovery of actinobacteria, as reported by Hayakawa and Nonamura (1989). This pretreatment has been proved effective even when combined with agar other than humic acid-vitamin (HV) agar such as starch-casein agar and actinomycetes isolation agar. Additionally, chitin agar, nutrient agar, and so on were also providing necessary nutrients for the cultivation/isolation of actinobacteria. Many antibiotics are also used in selective media for the isolation of actinobacteria (Table 20.7).

### 20.8.3 SELECTION OF INCUBATION CONDITIONS AND PERIOD

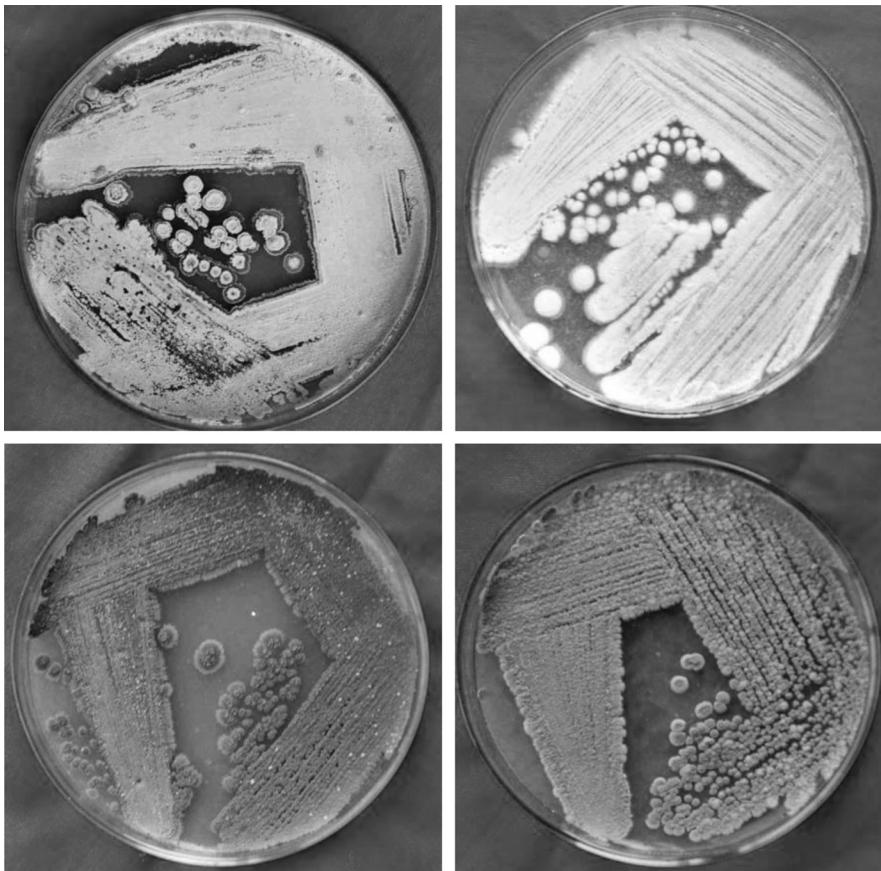
Unless thermophilic isolates are being sought, the incubation temperature is not a major variable as most actinobacteria grow optimally between 25°C and 30°C. Several novel taxa have been detected after prolonged incubation. Normally, incubation periods for isolation are from 4 to 7 days. But, slow growers can grow only after 15–20 days at the same temperature. Furthermore, a single collection does not give a complete picture of actinobacterial diversity. It needs frequent visits to the field, isolation from different substrates collected from the habitat, and usage of different media.

### 20.8.4 SELECTION OF COLONIES

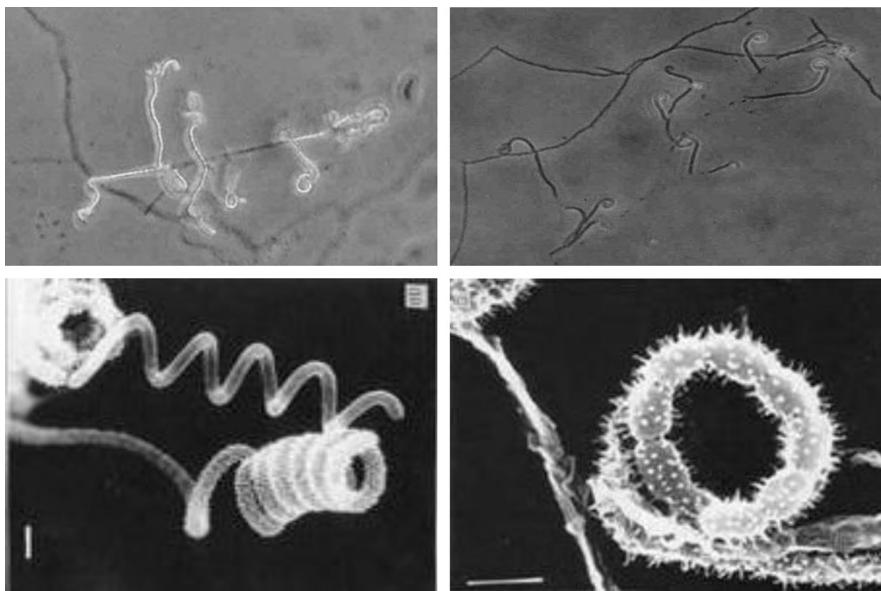
This final step in the isolation procedure is often the most frustrating and time-consuming. The main morphological characters to be observed are spore chain morphology, color of aerial mycelium, pigmentation of the substrate mycelium (Figures 20.2 and 20.3), diffusible pigments, and melanin pigment production, and also physiological characters such as H<sub>2</sub>S production, hydrolyses, enzyme activity, cell wall amino acids, whole cell sugars, growth in inhibitory compounds, utilization of various carbon and nitrogen sources, and so on.

### 20.8.5 IDENTIFICATION OF ACTINOBACTERIA

Once the actinobacteria are isolated from the samples, their antibiotic activity against different pathogens is tested by either cross streak plate technique or agar overlay assay. Only antagonistic isolates can be used for identification since identification and classification are very difficult in actinobacterial research. To overcome the constraints in the traditional method of identification, Ugawa et al. (1989) discovered a method of identification through Actinobase. Actinobase, a computer program, consists of a file of scanning electron microscope (SEM) images and a table of the characteristics in laser diskette. The image file stores 7117 SEM images of 1199 actinobacteria strains. The data cover over 34 characteristics (Table 20.8) for 1321 actinobacteria strains from 43 genera, which had been extracted from the descriptions of the International Streptomyces Project (ISP) and other descriptions. The system supports genus level identification, except *Streptomyces*, which includes additional data



**FIGURE 20.2** Various colonial morphology of actinobacteria on culture media.



**FIGURE 20.3** Microscopic nature of some actinobacteria: top—light microscopy; bottom—SEM.

**TABLE 20.8**  
**Characteristics Used for Identification**

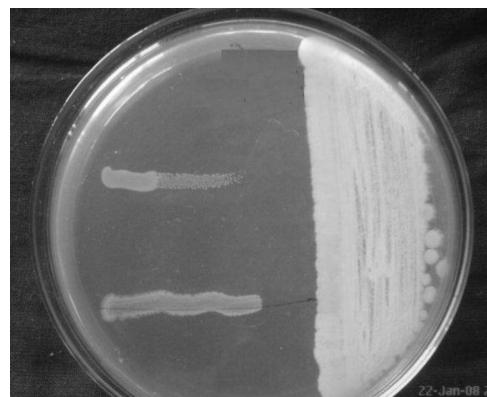
Chemical Characteristics	Morphological Characteristics	Cultural, Physiological, and Other Characteristics
Cell wall type	True mycelium	Acid fastness
Isomers of diaminopimelic acid	Fragmentation of vegetative mycelium	Aerial mass formation and its color
Cell wall type	Motile elements production	Color of reverse side of colonies
Whole cell sugar pattern	Acroetal budding	Melanoid pigment
Phospholipid	Spore formed on aerial mycelia	Color of the soluble pigments
Menaquinone	Spores on substrate mycelium	Facultative anaerobe
Fatty acid	Sporangia on SEM	Growth at 45°C
	Synnema formation	Halophilic nature
	Spore motility	Utilization of carbon compounds
	Number of spore	
	Shape of sporangium	
	Shape of motile sporangiospore	
	Spore chain morphology	
	Spore surface	
	Special morphology	

for species level identification. To help direct comparison with an unknown strain, the system displays SEM images in accordance with the result of retrieval (Balagurunathan and Subramanian, 1993).

Nowadays, the most powerful approach to solve taxonomic problems of actinobacteria is the study of nucleic acids. Comparison of nucleic acids yields considerable information on the true relatedness. Molecular systematics, which includes both classification and identification, has its origin in the early nucleic acid hybridization studies but has achieved a new status following the identification of nucleic acid sequences through sequencing techniques (O'Donnell, Embley, and Goodfellow 1993). The sequence analysis of the gene coding for the ribosomal subunits (16S, 23S, and 53S rRNA), in particular, the 16S rRNA gene, has become an important tool in bacterial identification since it provides information about the phylogenetic placement of the species (Woese 1987; Brenner, Staley, and Kreig 2001). DNA-based molecular methods have been used for species differentiation and the identification of actinobacteria. The significance of phylogenetic studies based on 16S rDNA sequences is increasing in the systematics of bacteria and actinobacteria (Yokota 1997). Sequences of 16S rDNA have provided actinobacteriologists with phylogenetic trees that allow the investigation of the evolution of actinobacteria and also provide the basis for identification.

## 20.9 SCREENING OF THE ANTIMICROBIAL ACTIVITY

The search for novel metabolites especially from actinobacteria requires a large number of isolates (over thousands) in order to discover an actinobacterial population with a novel compound of pharmaceutical interest. Because of this, the research will be more promising if diverse and more actinobacteria are sampled and screened. Such attempts need to be continued both in the sample collection area and from the adjoining places during various climatic conditions so as to screen more isolates for novel therapeutics. The antimicrobial-producing property of actinobacteria was screened by the cross streak method (Egorov 1985). A single streak of the actinobacteria was made on the surface of the modified nutrient agar medium and incubated at  $28 \pm 2^\circ\text{C}$ . After observing a good ribbon-like growth of the actinobacteria on the Petri plates, the fungal pathogens were streaked at right angles to the original streak of actinobacteria and incubated at  $27^\circ\text{C}$ . The inhibition zone was measured after 24 and 48 hours. Based on the presence and absence of an inhibition zone, the antifungal compounds producing actinobacteria were selected ([Figure 20.4](#)).



**FIGURE 20.4** Screening of antimicrobial activity of actinobacteria by the cross streak plate method.

## 20.10 FERMENTATION AND STRUCTURE DETERMINATION OF METABOLITES

After identification, the antagonistic actinobacteria can be studied for the isolation of compounds through fermentation parameters by the shake flask technique. Fermentation parameters can be manipulated to encourage the production of diverse secondary metabolites. The most intense antifungal activity of the actinobacteria was selected and its antifungal spectrum was tested against the pathogenic fungi. The selected actinobacterial isolates were inoculated separately into a 500-mL conical flask containing starch casein broth and shaken at  $28 \pm 2^\circ\text{C}$  and 250 rpm for 7 days. After incubation, the staling substances were filtered through filter paper (Whatman No. 1) and then through a Seitz filter (G5). The filtrate was transferred aseptically into the conical flasks and an equal volume of solvent was added to the cell-free culture filtrates and shaken for 2 hours, and the antifungal compounds were extracted (Sambamurthy and Ellaiah 1974). The fungal pathogens spread over on the surface of Sabouraud's dextrose agar medium, wells were made using sterile cork borer, and then the cell-free filtrates were added separately in the wells and incubated at  $27^\circ\text{C}$  for 24–48 hours. After incubation, the diameter of the zone of inhibition around the wells was measured to evaluate the antifungal activity of actinobacterial isolates. In an antibiotic production process, selection of solvent for extraction is an important step since the cost and availability of the solvents used in the extraction of an antibiotic will greatly influence the overall process.

It is generally easier to optimize metabolite production and to obtain more reproducible conditions in shake flasks. When a crude extract is found to be active in a screening assay, isolation and purification of the active principle is arrived at by solvent partitioning, thin layer chromatography, and high performance liquid chromatography with diode assay spectral analysis. It is important that screening "hit" due to previously discovered structures is identified quickly so that resources are not wasted. This process is referred to as dereplication.

The following data are needed to characterize and determine the structure of a metabolite: melting point (if possible); elemental analysis; optical rotation data; infrared spectrum; UV spectrum, mass spectrum; one-dimensional  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectrum; and two-dimensional  $^1\text{H}$ - $^1\text{H}$  cosy and  $^{13}\text{C}$  NMR spectrum. The identification of previously discovered compounds that are not known to be active in a particular assay can often be accomplished quickly by taking advantage of the available database of natural product structures. For example, after obtaining the absorbance maxima and molecular weight for a bioactive metabolite, this information can be combined with the taxonomy of the process organisms. This information can also be used to generate a list of potential structures from a database (Fiedler 1993). Based on this, a series of further experiments (determination of melting point pH and temperature stability, solubility qualitative, and quantitative analyses of functional groups) can be designed to confirm that the bioactivity results obtained are either from a previously described structure or from a chemical entity.

## 20.11 CONCLUSION

Preliminarily, studies on diversity of actinobacteria requires regular visits to the sampling stations, isolation of actinobacteria from different substrates collected from various habitats using various culture media. Such attempts need to be continued both in the same area and from the adjoining places during various climatic conditions so as to screen new isolates for novel therapeutics. Ultimately, the success of studies will also depend on the development of appropriate fermentation conditions and downstream processing technologies so as to bring out new classes of antibiotics from marine actinobacteria.

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# 21 Marine Drugs

## *Treatment for Osteoporosis and Related Bone Diseases*

Jayachandran Venkatesan and Se-Kwon Kim

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### 21.1 INTRODUCTION

Bone is made up of seven hierarchical structures and consists of hydroxyapatite and collagen as major constituents (Venkatesan and Kim 2010a, 2010b; Venkatesan et al. 2011a, 2011b). Defects in bone can occur due to many reasons such as motor accidents, birth defects, osteoporosis, arthritis, bone gangrene, and low calcium levels. The mass and function of bones depend on the maintenance of a complicated balance between osteoclast-mediated bone resorption and osteoblast-mediated bone formation. Calcium and phosphate are the two minerals that are essential for normal bone formation. Osteoblasts secrete a calcifiable matrix that contains minerals; collagen; and a small amount of noncollagenous proteins including osteopontin, osteonectin, bone sialoprotein, and osteocalcin (Gay, Gilman, and Sugiyama 2000). The function of osteoclasts is to remove bone tissue by removing its mineralized matrix and breaking up the organic bone (90% collagen). An increase in the number of osteoclast cells and their function normally induces bone osteoporosis, indicating that osteoclasts play a pivotal role in bone homeostasis (Manolagas 2000; Miyamoto and Suda 2003).

Osteoporosis typically reflects an imbalance in skeletal turnover such that bone resorption exceeds bone formation. An inhibitor of osteoclast differentiation and/or function is expected to be useful for treatment of bone lytic diseases such as osteoporosis, rheumatoid arthritis, and tumor metastasis into bone.

### 21.2 TREATMENT FOR OSTEOPOROSIS AND RELATED BONE DISEASES

The goals of osteoporosis treatment are to control pain from the disease, reduce bone loss, and prevent bone fractures with medicines or hormone therapies. There are several types of treatments for osteoporosis among which the most famous ones include bisphosphonates, estrogen agonists/antagonists, parathyroid hormone, hormone therapy, and the recently developed receptor activator

of nuclear factor- $\kappa$ B ligand (RANKL) inhibition. Estrogen agonists/antagonists in combination with estrogen for prevention and treatment of osteoporosis have also been studied (Stovall and Pinkerton 2008). Bazedoxifene for the prevention of postmenopausal osteoporosis (Gennari et al. 2008), parathyroid hormone (Black et al. 2003; Finkelstein et al. 2003; Horwitz et al. 2010; Neer et al. 2001), estrogen therapy (Eskridge et al. 2010; Genant et al. 1997; Lindsay 1987; Lindsay and Tohme 1990), hormone therapy (Engel et al. 2011; Penti et al. 2009), and the recently developed RANKL inhibitor (McClung 2006, 2007) treatment options are currently available for osteoporosis treatment.

## 21.3 MARINE-DERIVED COMPOUNDS

Current strategies for bone repair have accepted limitations, and the search for natural compounds from marine sources, for synthetic graft materials, or for scaffolds that support ex vivo bone tissue engineering continues. However, biomimetic strategies have led to the investigation of naturally occurring porous structures as templates for bone growth. The marine environment is rich in mineralizing organisms with porous structures, some of which are currently being used as bone graft materials, whereas some others are in early stages of development (Clarke et al. 2011).

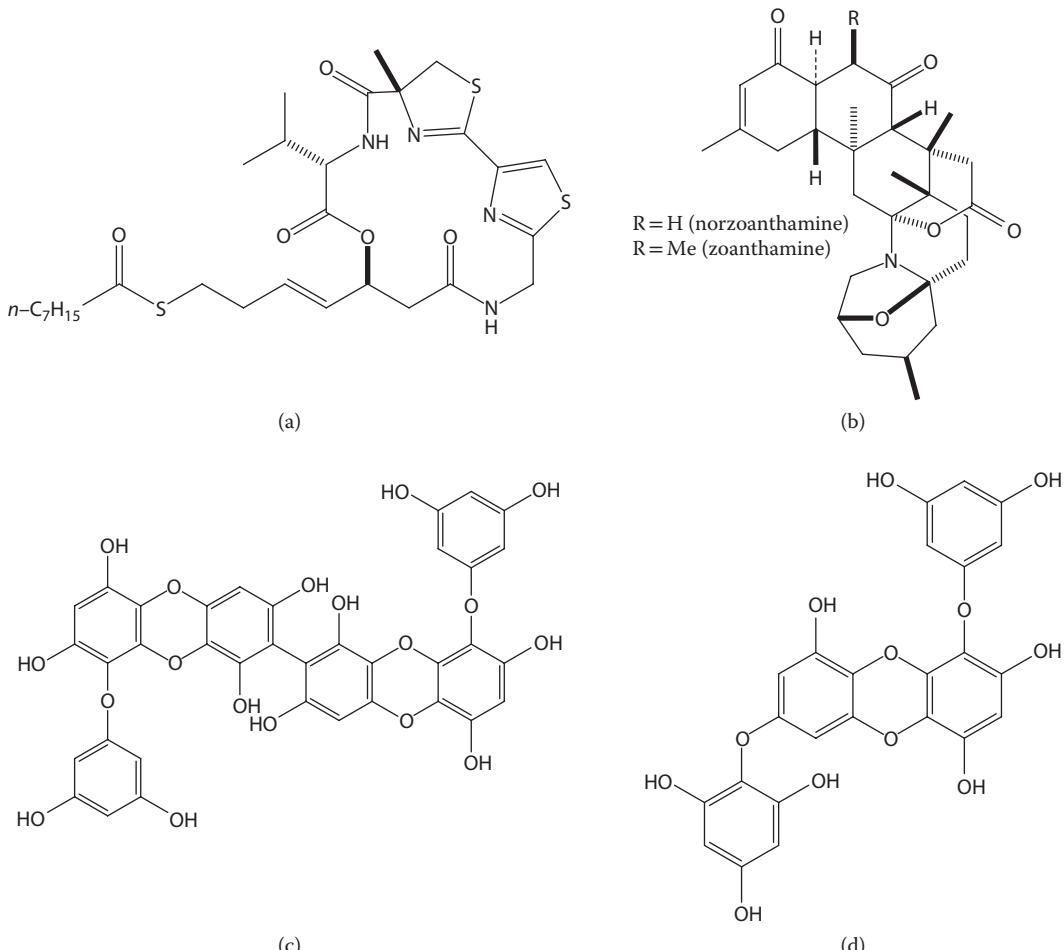
In addition, marine-derived compounds are sources of numerous therapeutic agents. Recent progress in discovering drugs from natural product sources has resulted in compounds that are being developed to treat cancer, resistant bacteria and viruses, and immunosuppressive disorders. Many of these compounds were discovered by applying recent advances in understanding the genetics of secondary metabolism in microorganisms, exploring the marine environment, and applying new screening technologies. Several bioactive molecules have been isolated from marine sources, which are used for treatment of osteoporosis and other human related diseases (Balunas et al. 2008; Beutler and McKee 2003; Bourguet-Kondracki and Kornprobst 2005; Kinugawa, Fukuzawa, and Tachibana 2009; Newman, Cragg, and Battershill 2009; Nguyen, Jung, and Kim 2011; Yamaguchi et al. 2001).

### 21.3.1 MARINE ALGAE-DERIVED COMPOUNDS

Marine algae are generally known as seaweeds; they contain abundant active compounds. They are commonly found in seashores in all shapes and are classified into three different kinds, that is, red, green, and brown algae or protists, chromists, and plantae, respectively (Hedgpeth 1957). Several extracts and purified compounds of marine algae have been reported for the suppression of osteoclast differentiation. Marine collagen peptides (MCPs) derived from chum salmon (*Oncorhynchus keta*) skin have been investigated for the development of femurs in growing rats of both sexes (Xu, Han, and Li 2010).

The modification of chromatin structure and thereby regulation of gene transcription through histone deacetylases (HDACs) plays an important role in osteogenesis and is considered to be a promising potential therapeutic target for bone diseases. Largazole (Figure 21.1a) exhibited *in vitro* and *in vivo* osteogenic activity by HDAC inhibition and significantly induced the expression of alkaline phosphatases, induced the expression of osteopontin, and increased the expression of Runx2 and BMPs. Largazole showed *in vivo* bone-forming efficacy in the mouse calvarial bone formation assay and the rabbit calvarial bone fracture-healing model (Lee et al. 2011).

Norzoanthamine (Figure 21.1b) is a nontoxic marine alkaloid, and its collagen protection activity indicates that it provides significant therapeutic benefits. Norzoanthamine accelerates the formation of a collagen-hydroxyapatite composite and enhances collagen release from an immobilized matrix vesicle model. Norzoanthamine recognizes a peptide chain nonspecifically and stabilizes its secondary structure, and collagen has polyvalent binding sites for norzoanthamine. Collagen-norzoanthamine supramolecular association is considered to be one of the most significant modes of action for enhancement of bone formation. Norzoanthamine suppressed the proteolysis of not only



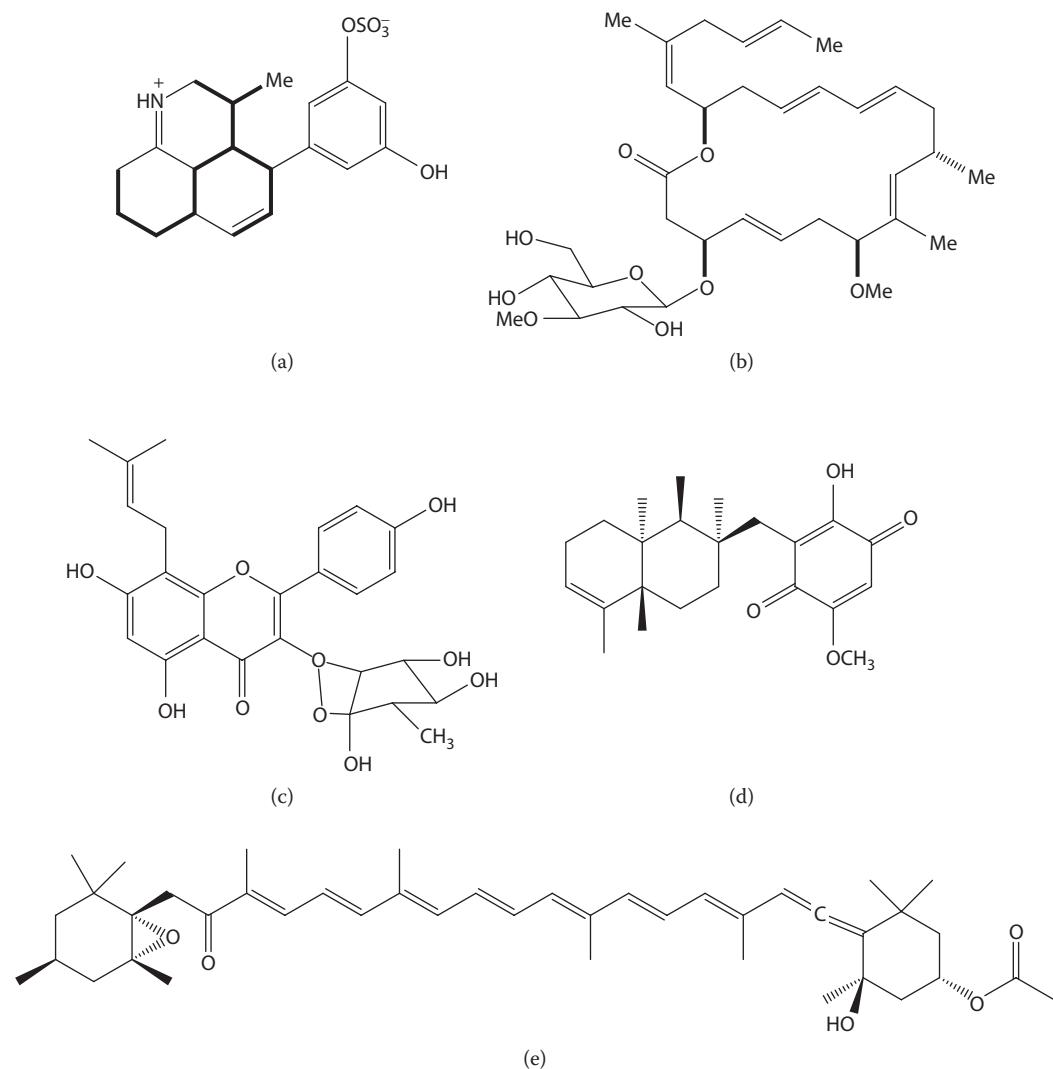
**FIGURE 21.1** Structures of (a) largazole, (b) norzoanthamine, (c) dieckol, and (d) 1-(3',5'-dihydroxyphenoxy)-7-(2'',4'',6''-trihydroxyphenoxy) 2,4,9-trihydroxydibenzo-1,4,-dioxin.

collagen but also elastin and bovine serum albumin; so it apparently has a universal protective effect of guarding extracellular matrix (ECM) proteins from degradation (Hikage et al. 1998; Kinugawa, Fukuzawa, and Tachibana 2009). Norzoanthamine has also been isolated from the zoanthid *Zoanthus* sp., which suppresses the decrease in bone weight and strength in ovariectomized mice indicating that it could be a good candidate as an osteoporotic drug (Kuramoto et al. 1998; Kuramoto, Chou, and Uemura 1999). Norzoanthamine, which was isolated from the colonial zoanthid *Zoanthus* sp., was shown to have antiosteoporosis activity in ovariectomized mice. Regarding marine extracts, the fucoxanthin-rich component from brown algae has been shown to have suppressive effects against osteoclast differentiation. It also showed antiosteoporosis activity in ovariectomized mice by regulating the balance between bone resorption and bone formation (Koyama 2011).

Arthritis is one of the most prevalent chronic inflammatory diseases, and it is characterized by structural and biochemical changes in major tissues of the joint, including degradation of the cartilage matrix and insufficient synthesis of the ECM. *Ecklonia cava* (EC) is a member of the family Laminariaceae, which is an edible marine brown alga with various bioactivities. The methanol extracts of brown alga EC, dieckol (Figure 21.1c), and 1-(3',5'-dihydroxyphenoxy)-7-(2'',4'',6''-trihydroxyphenoxy) 2,4,9-trihydroxydibenzo-1,4,-dioxin (Figure 21.1d) have been used for arthritis treatment at the *in vitro* level (Ryu et al. 2009).

The effect of the fractionated extracts obtained from *Sargassum horneri* (*S. horneri*) on bone calcium content and osteoclast-like cell formation *in vitro* has also been investigated. The effects of *S. horneri* on bone components in the femoral diaphyseal and metaphyseal tissues of young and aged rats were studied. The oral intake of the water-solubilized *S. horneri* extract significantly altered the bone components of young rats *in vivo* (Uchiyama et al. 2004; Uchiyama and Yamaguchi 2002).

Paenol inhibits RANKL-induced osteoclastogenesis by inhibiting ERK, p38, and NF-KB pathways (Tsai et al. 2008). Symbioimine (Figure 21.2a) from the symbiotic marine dinoflagellate *Symbiodinium* sp. exhibits inhibitory effect on osteoclast differentiation (Kita et al. 2004). Biselyngbyaside (Figure 21.2b) was isolated from the marine cyanobacterium *Lyngbya* sp. and subjected to osteoclast differentiation study. Biselyngbyaside also inhibited RANKL-induced osteoclastogenesis in mouse monocytic RAW264 cells and primary bone marrow-derived macrophages at a low concentration. The effects of *Spirulina* algae on bone metabolism in ovariectomized estrogen-deficient rats and hind limb-unloaded mice have also been examined (Ishimi et al. 2006). In the RANKL-induced signaling pathways, biselyngbyaside inhibited the expression



**FIGURE 21.2** Structures of (a) symbioimine, (b) biselyngbyaside, (c) ikarisoside A, (d) bolinaquinone, and (e) fucoxanthin.

of c-Fos and NFATc1, which are important transcription factors in osteoclast differentiation. In mature osteoclasts, biselyngbyaside decreased resorption pit formation. Biselyngbyaside also induced apoptosis accompanied by the induction of caspase-3 activation and nuclear condensation, and these effects were negated by the pancaspase inhibitor z-VAD-FMK (Yonezawa et al. 2012).

Inhibition of osteoclastogenic differentiation by ikarisoside A (Figure 21.2c) in RAW 264.7 cells via JNK and NF- $\kappa$ B signaling pathways was recently reported (Choi et al. 2010). Lucas et al. (2003) studied the modulatory effect of bolinaquinone (Figure 21.2d), a marine sesquiterpenoid, on acute and chronic inflammatory processes (Lucas et al. 2003). Fucoxanthin (Figure 21.2e), which induces apoptosis, also induced osteoclast differentiation in a study conducted by Das and colleagues (Das et al. 2010).

### 21.3.2 MARINE-DERIVED MICROBIAL COMPOUNDS

Marine microbes have made a unique contribution to the health and well-being of people throughout the world. Marine microbes have the capability to produce several primary and secondary metabolites, such as amino acids, vitamins, and nucleotides, which constitute half of the pharmaceuticals on the market today (Beutler and McKee 2003; Brakhage and Schroeckh 2011; Lam 2006; Zotchev 2012). A growing number of marine microorganisms are the sources of novel and potentially life-saving bioactive secondary metabolites (Vignesh, Raja, and James 2011). In addition, an efficient method of total synthesis of novel bioactive microbial metabolites has been achieved by Sunazuka and others (Sunazuka, Hirose, and Omura 2008).

An extensive review for marine microbe's metabolites and their biological activities has been written. Bacteria, fungi, actinomycetes, microalgae, cyanobacteria and diatoms were mentioned as the important resources for the production of the metabolites. In addition, the biological activities such as antitumor, anticancer, anti-HIV, antimicrotubule, antiproliferative and photo protective have been reviewed (Bhatnagar and Kim 2010). Moreover, only a few compounds of marine microbes were found to be active in terms of bone-related diseases as shown in [Figures 21.3](#) and 21.4.

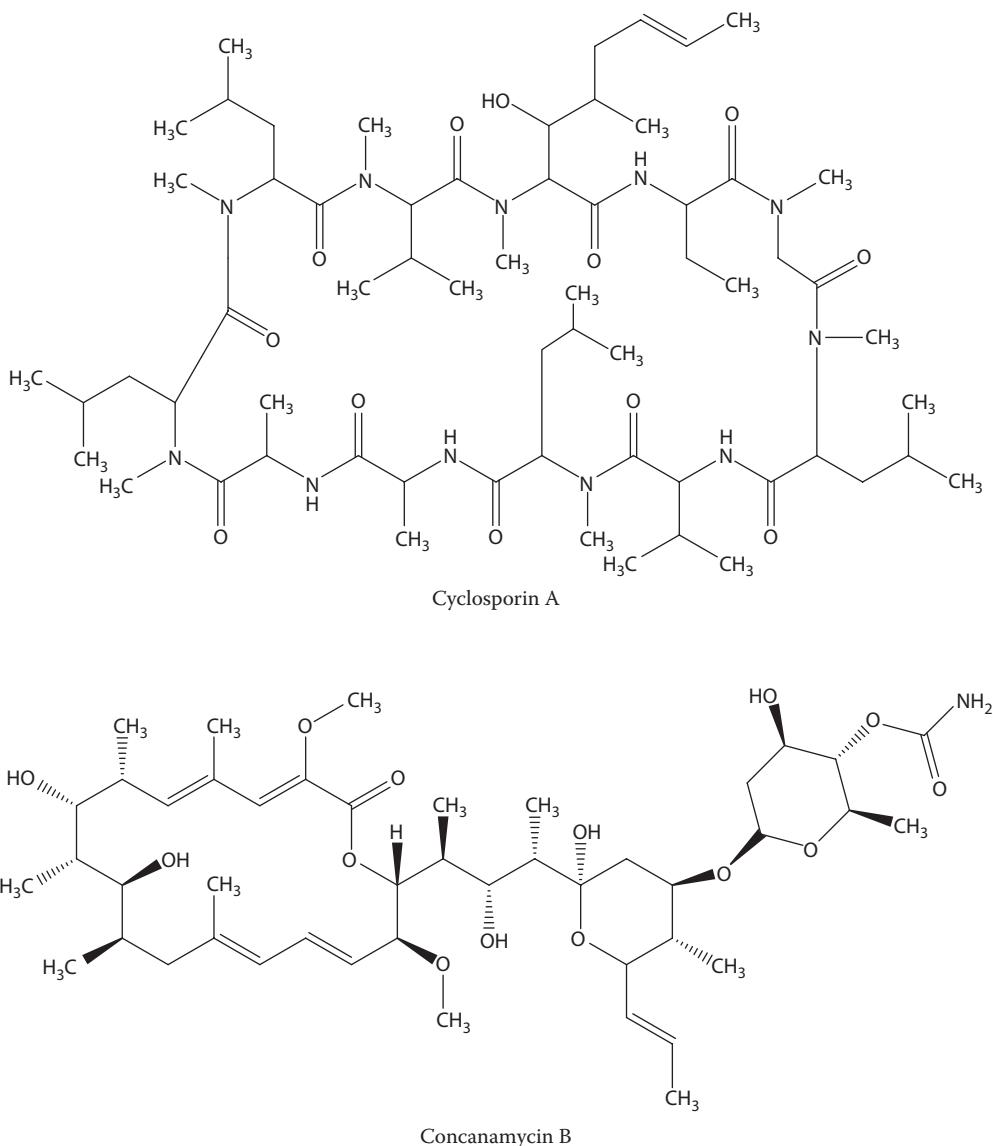
### 21.3.3 MARINE SPONGE-DERIVED COMPOUNDS

Sponges are sessile animals that filter water through their porous bodies and ingest food particles and dissolved materials. There are more than 7000 species of sponges alive today living in both freshwater and marine environments and are the oldest known multicellular animals. Sponges live in all types of regions all over the world. It is noted that 99% of all sponges live in the marine environment. Several bioactive compounds have been isolated from marine sponges as potential sources and checked against antimicrobial tests (Fusetani, Matsunaga, and Konosu 1981). The osteoclast differentiation have been inhibited by symbioimine and haterumalide compounds. Haterumalides ([Figure 21.5](#)), the 14-membered cytotoxic macrolides from the Okinawan sponge *Ircinia* sp., show potent cytotoxicity (Kita, Sakai, and Uemura 2006).

### 21.3.4 ROLE OF MARINE SPONGE MATERIALS IN BONE TISSUE ENGINEERING

For the past two decades, biominerallisation has become an exciting source of inspiration for the development of novel bionic and biomimetic approaches for tissue engineering applications. Recent advancements in bone substitute material such as coral, marine sponges and synthetic bisphosphonates are expended for osteoporosis treatment (Wang et al. 2011).

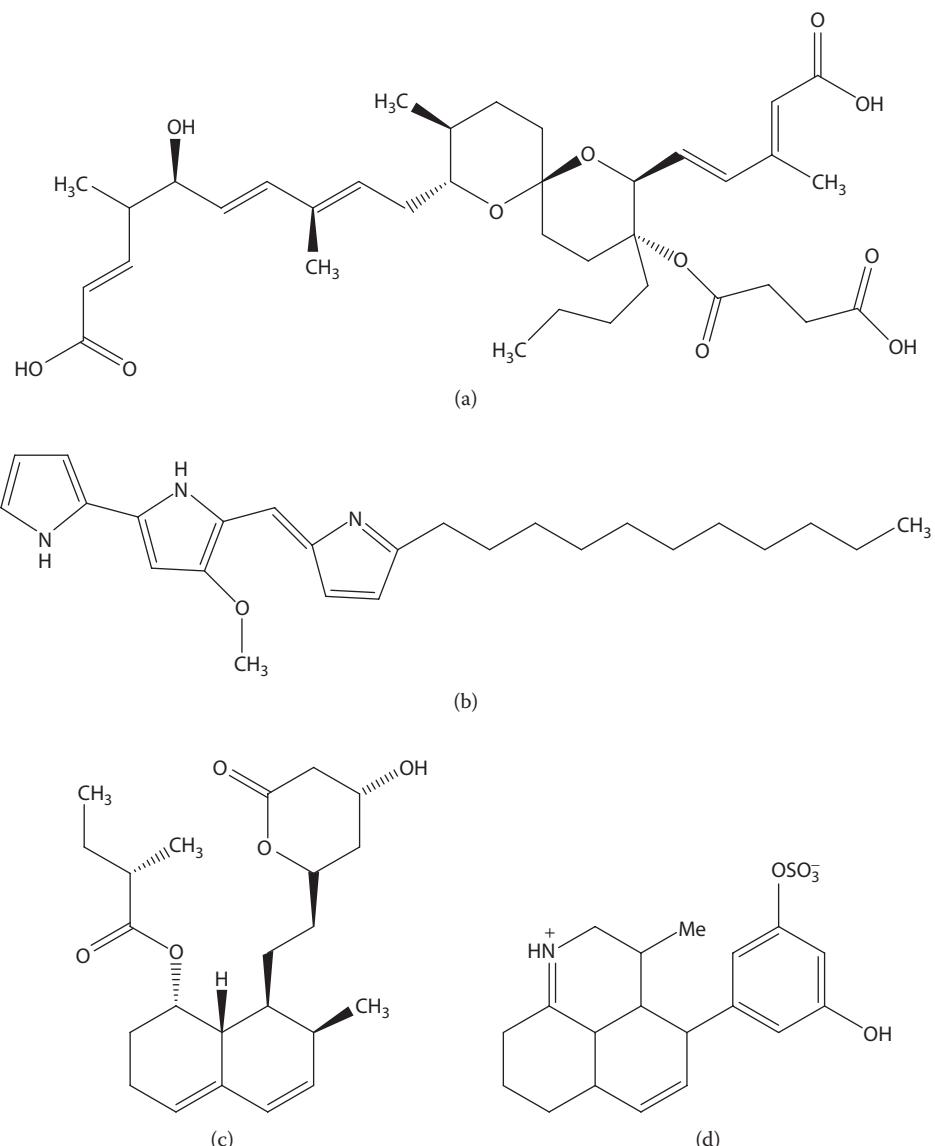
A natural marine sponge skeleton is used as a potential scaffold on the basis of the hydration potential of the fiber, the presence of open interconnected channels created by the fiber network, the collagenous composition of the fiber, and the structural diversity of fiber architecture. The abundance and structural diversity of natural marine sponge skeletons and their potential as multifunctional, cell conductive, and cell inductive frameworks indicate a promising new source of scaffolds for tissue regeneration (Green et al. 2003).



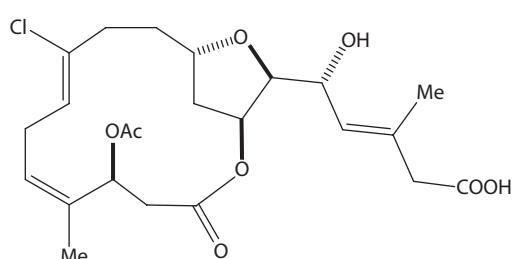
**FIGURE 21.3** Marine-derived microbial compound in the inhibition of osteoclast differentiation.

A marine sponge (*Ircinia fusca*)-derived collagen with chitosan/hydroxyapatite has been studied *in vitro* for bone tissue engineering. Cell proliferation in chitosan/hydroxyapatite/marine sponge collagen scaffolds was relatively higher than that in pure chitosan, which was observed by MTT assay and Hoechst staining using the MG-63 cell line (Pallela et al. 2012). In another study, the marine sponge *Callyspongiidae* was used as the scaffold and it was suggested that natural marine sponges are promising as new scaffolds for use in bone tissue engineering (Lin et al. 2011).

The scaffolds with sponges, Dalmata Fina (*Spongia officinalis* Linnaeus, Adriatic Sea), Fina Silk (*Spongia zimocca*, Mediterranean), and Elephant Ear (*Spongia agaricina*, Caribbean), with high viscous hydroxyapatite solution have been studied for tissue engineering purposes. The most promising one among the ceramic tissue-engineered bone scaffolds developed, *S. agaricina* replicas, demonstrated an overall porosity of 56%–61% with 83% of the pores ranging between 100 and 500  $\mu$ m and an interconnectivity of 99.92%; further, they can be used for bone tissue engineering



**FIGURE 21.4** Inhibition of osteoclast differentiation by marine microbial compounds: (a) reveromycin A, (b) prodigiosin 25C, (c) mevastatin, and (d) symbioimine.



**FIGURE 21.5** Structure of haterumalides.

(Cunningham et al. 2010). Marine sponges, namely, *Verongula gigantea* and octocorals (*Isodella sp.*) has been suggested as a novel source for bone and cartilage replacement (Born et al. 2010).

The collagen-derived biomaterials for matrix-induced and -assisted bone and cartilage tissue regeneration include the small intestine submucosa (SIS) Restor™, ACI-Maix® collagen membrane, Chondro-Gide collagen membrane, Permacol collagen Ossix, lycoll collagen membrane, and five types of collagen-based marine sponge skeletons. Certain characteristics of different scaffold materials with comparable chemical compositions may vary significantly. This variation may have a relevant impact on the suitability of the scaffolds for bone and cartilage regeneration. It suggests that the ACI-Maix membrane is the best available collagen-derived material for an MACI®/MACT® application. The collagenous fiber skeleton of marine sponges provides a suitable bio-scaffold for bone regeneration due to its superior cell adhesion and cell proliferation (Zheng et al. 2007).

The chondrocyte seeded sponge sulfated poly-N-acetyl glucosamine provided the best healing of cartilages compared to the sponges alone (Kang et al. 2005).

## 21.4 CONCLUSIONS

Limited sources and compounds have been explored from marine sources for treatment of bone-related diseases, specifically osteoporosis treatment. More research work is needed for further implications. Although synthetic bisphosphonate compounds are more promising for osteoporosis treatment, we believe that marine algae-, microbes-, and sponge-derived compounds are excellent in terms of biocompatibility without side effects both *in vitro* and *in vivo*. Further clinical trials for marine active compounds are considered necessary for their further commercialized implications.

## ACKNOWLEDGMENTS

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# 22 Bioactive Compounds from Marine Sources for Gastrointestinal Cancer Treatment

*Se-Kwon Kim and Mustafa Zafer Karagozlu*

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## 22.1 INTRODUCTION

The sea is the origin of the life and it covers over 70% of the Earth's surface. In addition, it is particularly rich in biodiversity. Marine organisms synthesize biologically active secondary metabolites because they lack immune system defense mechanisms. These secondary metabolites play a major role in the defense mechanism of host organisms. Secondary metabolites also support adaptation of host organisms to extreme environmental challenges. These natural products have historically been an invaluable source of therapeutic agents. Compounds from biological sources continue to play an extremely important role in the development of therapeutics because of their enormous structural diversity. The variety of marine organisms discovered to date suggests a dramatic potential for drug discovery, and much remains to be discovered in the depths of oceans. Surprisingly, approximately 22,000 natural products of marine origin have been discovered so far, whereas 131,000 terrestrial natural products exist (Blunt et al. 2011). The marine environment has a much richer biodiversity than that of terrestrial areas. In the area of marine research, a recent census of marine life that involved the participation of 2700 scientists from over 80 nations assessed the diversity, distribution, and abundance of marine life, which resulted in the discovery of over 6000 potentially novel species (Butler et al. 2010; Fautin et al. 2010; Miloslavich et al. 2010). This research shows that marine organisms are an important source of unknown natural compounds whose pharmacological importance must be evaluated. However, almost half of the drugs introduced between 1940 and 2006 were of natural origin or inspired by natural products, and they clearly have the most dramatic impact in the area of cancer treatment (Newman and Cragg 2007; Koehn and Carter 2005). Despite the considerable progress achieved in medical research, cancer is still one of the highest-ranking causes of death in the world. It is the second most common cause of death due to disease after heart disease, and according to the World Health Organization (WHO) it will be the cause of death of more than 10 million people in 2020. Hence, the potential of marine natural bioactive compounds is promising to be a rich source of therapeutic agents for fighting cancer.

## 22.2 WHAT IS GASTROINTESTINAL TRACT CANCER?

Gastrointestinal tract cancer is the malignant condition of the gastrointestinal tract and is a major health problem worldwide. The prognosis for patients with gastrointestinal tract cancer is grim. Most of the gastrointestinal cancer cases are reported as metastatic cancer. Surgery is an option for gastrointestinal cancer, but results vary depending on the affected organ. For example, it is a curative option in 50% of colorectal cancers, whereas it is less effective in gastric cancers where the overall 5-year survival rate is less than 10% (Hasegawa et al. 2003).

Gastrointestinal cancer is divided into two main groups according to the affected organ. In the first group, cancer affects the organs in the upper side of the body. This group is called upper gastrointestinal cancer. Stomach cancer, esophagus cancer, and liver cancer are in this group. The second group is called lower gastrointestinal cancer. In this group, the organs in the lower side of the body are affected by cancer. This group includes small intestine, appendix, colon/rectum (colorectal), and anus cancers.

## 22.3 MARINE COMPOUNDS FOR UPPER GASTROINTESTINAL CANCER TREATMENT

Gastric or stomach cancer is the second most frequent death cause of cancer, the first is lung cancer, around the world. Almost two-thirds of cases occur in Eastern Europe, South America, and Asia with 42% occurring in China alone (Jemal et al. 2009). Moreover, it is one of the most common cancers in Europe ranking fifth after lung, prostate, colorectal, and bladder cancers in men and breast cancer, colorectal cancer, lung cancer, and cancer of the corpus uteri in women. The sex-dependent ratio (the male-to-female ratio in incidence rates) is about 1.6:1 (Boyle and Ferlay 2005).

There are geographic and ethnic differences in gastric cancer incidence around the world and in its trends for each population with time. The incidence patterns observed among immigrants change according to where they live. These factors indicate the close association of gastric cancer with modifiable factors such as diet. Substantial evidence from ecological, case-control, and cohort studies strongly suggest that the risk of cancer increases with a high intake of various traditional salt-preserved foods as well as salt per se and that this risk could be decreased with a high intake of fruits and vegetables (Kono and Hirohata 1996). The risk of gastric cancer is also highly related to inheritance; approximately 10% of cases show a genetic component. Other established nondietary factors include cigarette smoking (Anton-Culver, Lee-Feldstein, and Taylor 1993) and infection with *Helicobacter pylori*. Although *H. pylori* live between the mucosal and epithelial cells of the human stomach for many decades without adverse consequences, the presence of *H. pylori* is associated with an increased risk of gastric adenocarcinoma (Linz et al. 2007).

The potential use of marine natural compounds in the treatment of cancer has been a driving force for researchers to focus on the benefits of marine organisms such as marine algae (Puglisi et al. 2004; Barros et al. 2005). The prevention of gastric cancer therefore represents one of the most important aspects of cancer control strategies around the world. Radical-scavenging compounds such as polysaccharides from marine algae can be used indirectly to reduce cancer formation in the human body.

Porphyran is a sulfated polysaccharide from marine algae. Kwon et al. (Kwon and Nam 2006) purified porphyran from *Porphyra haitanensis* and evaluated its anticancer activity on AGS human adenocarcinoma cells. It has been known that specific IGF-IR inhibition with a neutralizing antibody, an antagonistic peptide, or a selective kinase inhibitor has activity against diverse tumor cell types and is one of the causes of antiproliferative/proapoptotic molecular induction (Li et al. 2004; Saxena and Moorthy 2007). In the study, the effect of IGF-I on porphyran-treated AGS cells was determined and, as a result, the authors declared that porphyran-induced apoptosis is involved in the IGF-IR-mediated signaling pathway in AGS gastric cancer cells. Another porphyran was purified from *Porphyra yezoensis* and its apoptotic activity on the AGS human adenocarcinoma cell

line was confirmed by Kwon et al. (Kwon and Nam 2007). The porphyran isolated from red alga, *P. yezoensis*, also shows apoptotic activity on the AGS cell line. Furthermore, Kwon and Nam (2007) declared that porphyran from different marine algae showed the same apoptotic activity on the AGS cell line by following the mitochondrial pathway.

Moreover, bryostatin-1, a macrocyclic lactone derived from the marine bryozoan *Bugulaneritina*, is receiving considerable attention in view of its demonstrated antitumor activity *in vitro* and *in vivo*. De Lorenzo et al. assessed the induction of cyclooxygenase-2 (COX-2) in liver adenocarcinoma cell lines (De Lorenzo et al. 2003). The observation that bryostatin-1-induced COX-2 mRNA, COX-2 protein, and prostaglandin synthesis in the nanometer range via a protein kinase C, mitogen-activated protein kinase, activator protein-1 pathway suggests that the addition of selective COX-2 inhibitors might increase the antitumor efficacy of bryostatin-1 as an antitumor agent on stomach cancer.

Hwang et al. (2008) declared that polysaccharides extracted from *Capsosiphon fulvescens* inhibit alcohol-induced cell death. In addition, they reduce the expression of COX-2 and iNOS enzymes which plays a role in the healing of gastric ulcers. They proved that polysaccharides from marine algae can be used as cancer protection agents.

Didemnin B is a cyclic depsipeptide produced by ascidians of the family Didemnidae. Beasley et al. studied the excretion and tissue concentrations of [<sup>3</sup>H] didemnin B in mice after intraperitoneal administration (Beasley et al. 2005). Interestingly, they found that the pancreas had the greatest concentration of the radiolabel at both high and low doses 7 days after administration, which suggested possible efficacy in animal models for the treatment of pancreatic cancer.

Moreover, fucoidan is a fucan sulfate occurring in brown marine algae. Shibata et al. (1999) studied the inhibitory effect of *Cladosiphon* fucoidan on *H. pylori* adhesion to human stomach. Their research proved that the fucoidan inhibited bacterial binding to human gastric cells. It was also shown that this fucoidan blocks both Leb- and sulfatide-mediated attachment of *H. pylori* to gastric cells.

Park et al. examined the pharmacology of dideoxypetrosynol A, a polyacetylene from the marine sponge *Petrosia* sp. They declared that the anti-proliferative effects of dideoxypetrosynol A on cancer cells were involved in cell cycle arrest at the G1 to the S phase transition in human adenocarcinoma cell lines.

Go et al. reported that the glycoprotein extracted from the brown alga Go et al. reported that the glycoprotein extracted from the brown alga *Laminaria japonica* induces apoptosis on HT-29 human colon cancer cells. They declared that the glycoprotein extracted from *L. japonica* inhibited AGS cell growth by following multiple apoptotic (extinct and instinct) pathways (Go et al. 2010). Treatment of glycolipids caused some changes in the Fas receptor pathway and the mitochondrial pathway (Go, Hwang, and Nam 2010).

## 22.4 NATURAL MARINE COMPOUNDS FOR LOWER GASTROINTESTINAL CANCER TREATMENT

The colon is a muscular organ and the last part of the digestive system in human beings; the rectum is the final portion of the colon. Colorectal cancer is the third most common type of cancer worldwide (Boyle and Ferlay 2005; Parkin 2004) after lung and stomach cancers. Among them, cancer of the colon is more frequent than rectal cancer. Especially in developed countries, the ratio of colon to rectum cases can rise to 2:1 or more. But in nonindustrialized countries, the rates are almost similar. On the other hand, comparison of the incidence rates of colon cancer in developed and undeveloped countries shows that colorectal cancer is more common in industrialized countries. Only 50% of the colorectal cancer patients survive in developed countries (Tyczynski et al. 2003). It remains relatively uncommon in Africa and much of Asia. Further, incidence rates of this cancer increase with industrialization and urbanization. It has been much more common in high-income countries, but it is now increasing in middle- and low-income countries. The incidence rate remains

relatively higher in North America, Europe, and Australia rather than in South America, Asia, and Africa (Parkin 2004).

The development of colorectal cancer in human beings involves both genetic and environmental factors. A major environmental factor appears to be diet. Even if it has not been proved beyond doubt, it is suggested that too much intake of red meat, processed meat, and alcoholic drinks increases the risk of colorectal cancer (Chao et al. 2005). The evidence is stronger for colon cancer than rectum cancer. On the other hand, dietary calcium and vitamin D are inversely related to the incidence of colon cancer (Kwak and Chung 2006). Another environmental risk factor for colorectal cancer is smoking. Smoking has consistently been positively associated with large colorectal adenomas (Giovannucci 2001). There is strong evidence to suggest that alcohol and smoking have a greater relative effect together than alone. In addition, inheritance is also important factor for the risk of colorectal cancer, especially because most of the colorectal cancer patients who are affected in early ages have a family history of cancer (Strate and Syngal 2005).

Konishi et al. investigated the carotenoids fucoxanthinol and halocynthiaxanthin isolated from the sea squirt *Halocynthia roretzi* (Konishi et al. 2006). Both carotenoids inhibited the growth of human leukemia, breast cancer, and colon cancer cells *in vitro* in a dose- and time-dependent manner by a mechanism that required the induction of apoptosis and the concomitant reduction of the apoptosis-suppressing protein Bcl-2.

Go et al. reported that the glycoprotein extracted from the marine alga Go et al. reported that the glycoprotein extracted from the brown alga *Laminaria japonica* induces apoptosis on HT-29 human colon cancer cells. They declared that the glycoprotein extracted from *L. japonica* inhibited growth in a dose- and time-dependent manner (Go, Hwang, and Nam 2010). The inhibition of glycolipid growth is associated with multiple apoptotic (extinct and instinct) pathways. Treatment of glycolipids caused some changes in the Fas signaling pathway and the mitochondrial pathway. The Fas signaling pathway is a major apoptosis-related extinct signaling pathway. In this pathway mechanism, the Fas receptor signaling pathway is initiated by a binding of the ligand on the cell surface, which then forms the death-inducing signaling complex (DISC) and activates caspase-8. Activation of caspase-8 initiates a cascade of caspases and leads to apoptotic cell death. Moreover, they also observed decreased levels of Bcl-2 expression and increased levels of Bad expression after treatment by *L. japonica* glycoprotein. It is noted that Bcl-2 and Bad are members of the Bcl apoptotic protein family, and they play a vital role in mitochondrial apoptotic pathway (Go, Hwang, and Nam 2010).

Rangel et al. declared new mechanistic information on the cyclic peptides geodiamolides A, B, H, and I isolated from the marine sponge *Geodia corticostylifera*. The researchers noted that peptides A and H had potent antiproliferative activity against two human cancer cell lines and disorganized F-actin filaments in a dose-dependent manner (Rangel et al. 2006). Interestingly, normal cell lines did not show cytoskeleton alterations after treatment with the geodiamolides, suggesting a putative biomedical potential for these novel compounds.

Richardson and Ireland continued the characterization of the antitumor activity of the small non-nitrogenous lactone lissoclinolide isolated from the marine ascidian *Lissoclinum patella* (Richardson and Ireland 2004). Lissoclinolide was able to particularly inhibit the growth of cell lines in the NCI colon tumor panel. Although the ultimate molecular target of lissoclinolide remained undetermined, the most notable observation was that 2.4  $\mu$ M of lissoclinolide strongly arrested the G/M phase of the cell cycle in both p53 competent and null human colon carcinoma HCT 116 cell lines after 24 or 48 hours of exposure.

In addition, brown algal extract itself can exhibit an anticancer effect on colon cancer. Mei et al. have published a study to this end. In their study, they collected the brown algae *Lethariella zahlbruckneri* and extracted with methane and acetone. They declared that both extracts showed dose- and time-dependent antiproliferative activity on HT-29 cells (Ren et al. 2009). Furthermore, the apoptotic activities of the extracts on HT-29 cells were evaluated. Finally, the study showed that the acetone extract induced apoptosis via caspase-dependent and caspase-independent pathways (Ren et al. 2009).

Tong et al. discovered a novel sulfated saponin, philinopside A, isolated from the sea cucumber *Pentacta quadrangularis*, which possesses dual antiangiogenic and antitumor effects. Philinopside A inhibited angiogenesis in human adenocarcinoma cells as well as tumor growth both *in vitro* and *in vivo* by a synergistic mechanism that appeared to involve inhibition of receptor tyrosine kinases.

Besides, many active compounds from marine organisms have been characterized by researchers. Sulfur-containing polybromoindoles were isolated from the red alga *Laurenda bringniartii* (El Gamal 2010); aromatic sesquiterpenes, dimeric sesquiterpenes of cyclolaurane type, and sesquiterpene alcohols of bisabolene type were isolated from organic extracts of *Laurencia microcladua* (Kladi et al. 2007); terpenoid was isolated from the tropical brown algae *Stylopodium zonale* (Dorta et al. 2002); furoplocamoid, perfuroplocamoid, pirene, and tetrachlorinated cyclohexane were isolated from the red alga *Plocamium cartilagineum* (Argandoña et al. 2002); and tetrahydro- $\beta$ -carboline was isolated from the red alga *Callophytus oppositifolius* (Ovenden et al. 2011). These were active against HT-29 and SW480 cells.

## 22.4 CONCLUSION

Although more natural products were identified from terrestrial organisms than from marine organisms, the biodiversity of marine organisms has potential for identifying new valuable compounds. The potential of designing new functional foods and pharmaceuticals from marine organisms make them one of the most interesting research areas for scientists. Many natural products have been purified from marine organisms, but until now most of the anti-gastrointestinal cancer activities of marine-derived extracts or compounds have been observed *in vitro*. Therefore, further research studies are needed in order to investigate their activity in human subjects.

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# 23 Marine Algae

## *Pharmacological Values and Anti-Inflammatory Effects*

Se-Kwon Kim, Thanh-Sang Vo, and Dai-Hung Ngo

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### 23.1 MARINE ALGAE

Marine organisms represent an enormous resource of natural products. In particular, marine algae are one of the most important producers of biomass in the marine environment. They include a wide variety of plants that range from diatoms, which are microscopic, unicellular organisms, to seaweeds extending over 30 m. Therefore, two major types of algae can be identified: macroalgae (seaweeds) and microalgae. Microalgae are found in both benthic and littoral habitats and also throughout the ocean waters as phytoplankton (Garson 1989). Phytoplankton comprises organisms such as diatoms (Bacillariophyta), dinoflagellates (Dinophyta), green and yellow-brown flagellates (Chlorophyta, Prasinophyta, Prymnesiophyta, Cryptophyta, Chrysophyta, and Rhaphidophyta), and blue-green algae (Cyanophyta). As photosynthetic organisms, this group plays a key role in the productivity of oceans and constitutes the basis of the marine food chain (Bold and Wynne 1985). Meanwhile, the macroalgae (seaweeds) are classified as green algae (division Chlorophyta), red algae (division Rhodophyta), and brown algae (division Phaeophyta). Seaweeds are widely distributed in the ocean, occurring from the tide level to considerable depths, free-floating or anchored, and include kelp, dulse, rockweed, and sea lettuce. Many are of economic importance as food, fertilizer, agar, potash, or a source of iodine (Schaeffer and Krylov 2000). Interestingly, marine algae are considered a source of bioactive compounds as they are able to produce a great variety of secondary metabolites characterized by a broad spectrum of biological activities.

### 23.2 PHARMACOLOGICAL VALUE OF MARINE ALGAE

Before the 1950s, the medicinal properties of seaweeds were restricted to traditional and folk medicines as remedies of various physical ailments in Asian countries, mainly in China, Japan, and Korea (Lincoln, Struponski, and Walker 1991). In particular, *Sargassum fulvellum* and *Sargassum thunbergii* have long been used as anti-helminthic agents and recorded many uses in the treatment of lump, dropsy, swollen, and painful scrotum and urination problems (Kang et al. 2008).

In Hawaii and Indonesia, the red seaweeds *Hypnea nidifica* J. Agardh and *Hypnea musciformis* (Wulffen) J. V. Lamouroux are used as a vermifuge remedy for stomach troubles caused by parasitic infections (Zaneveld 1959). Moreover, consumption of brown marine algae is believed to ameliorate some inflammatory disorders, breast cancer, and high cholesterol levels (Fitton 2003). In particular, brown marine algae such as *Undaria* and *Laminaria* species are recommended for treating some types of cancers in China (Ji and Zhang 1998). In Korea, the frequency of seaweed soup intake in lactating mothers is believed to provide many health benefits for mothers and their children. It is assumed that the level of iodine content in the breast milk of Korean lactating mothers is much higher than in other countries (Moon and Kim 1999). Meanwhile, the iodine content in seaweeds is considered as a detoxifying agent that inhibits absorption of similar radioactive elements by the body (Gong et al. 1991). In addition, the descriptions of seaweeds from Oriental Materia Medica indicate that *Haizao* (*Sargassum*) is a strong agent in transforming phlegm and dissipating nodules, and it is suitable for treating goiter and scrofula. *Kunbu* (*Laminaria* and *Ecklonia*) is an efficient agent in reducing congealed blood and treating liver–spleen enlargement, liver cirrhosis, and tumors (Hsu et al. 1986). According to Ajit Kandale and colleagues, *Fucus* species of brown seaweeds have been used for the treatment of thyroid disorders. The therapeutic effects of using powdered *Fucus* resemble the therapeutic effects of thyroxine medications, such as shrinking of goiters, weight loss, resolution of symptomatic non-autoimmune hypothyroidism, return of vim and vigor, lessening of psychiatric disruptions, and resolution of eczemas. Furthermore, the consumption of *Kombu* (*Laminaria*) can regulate physiological processes such as resolution of coronary artery disease, healthier liver function, higher metabolic rate, faster food transit time, lower low-density lipoprotein (LDL) cholesterol, and higher high-density lipoprotein (HDL) cholesterol blood levels (Kandale et al. 2011). Notably, *Sargassum hemiphyllum* and *Carpopeltis affinis* are identified in Korean folk medicine as a therapeutic treatment of various allergic diseases (Na, Moon, Ko et al. 2005; Na, Moon, Lee et al. 2005). Besides, red algae containing carrageenan have been used for millennia to treat respiratory ailments, especially intractable sinus infections and lingering pneumonias (Kandale et al. 2011).

Although the use of algae for therapeutic purposes has a long history, the pharmacological properties of marine algae have been studied widely only since the 1980s. The major advances in the design of *in vitro* screens such as enzyme activities or action of compounds on cultured cell lines allow a much greater number of samples to be screened faster (Borowitzka 1995). Presently, various compounds from marine algae have been discovered due to their biological activities such as anticoagulant, antivirus, antioxidant, antiallergy, anticancer, anti-inflammation, and antiobesity (Lincoln, Struponski, and Walker 1991; El Gamal 2010; Wijesekara, Yoon, and Kim 2010). Thus, numerous marine algae have been believed to be effective against diseases and they can be applied to the development of novel pharmaceuticals as well as nutraceuticals.

### 23.3 ANTI-INFLAMMATORY THERAPEUTIC EFFECTS OF MARINE ALGAE

Inflammation is a critically important aspect of host responses to various stimuli including physical damage, ultraviolet irradiation, microbial invasion, and immune reactions (Gordon 1998; Gautam and Jachak 2009). The classical key features of inflammation are redness, warmth, swelling, and pain. Inflammation cascades can lead to the development of diseases such as chronic asthma, rheumatoid arthritis, multiple sclerosis, inflammatory bowel diseases, and psoriasis. Many of these diseases are debilitating and are becoming increasingly common in our aging society. Currently, several classes of drugs such as corticosteroids, non-steroidal anti-inflammatory drugs (NSAIDs), and biologics are used to treat the inflammatory disorders. However, these drugs possess several adverse effects, and biologics are expensive. Therefore, there is still a vital need for the development of new anti-inflammatory drugs with satisfactory tolerability for long-term use. Since ancient times, several natural products such as curcumin, serrapeptase, bromelain, and ginger have been used in the treatment of inflammation. Meanwhile, marine algae, such as *S. fulvellum* and

*S. thunbergii*, have recorded many uses in the treatment of dropsy, swollen, and painful scrotum in the Asian medical textbook Donguibogam (1999). Furthermore, *Porphyra dentate* has also long been used worldwide in folk medicine for the treatment of inflammatory diseases such as hypersensitivity, lymphadenitis, and bronchitis (Kazłowska et al. 2010). Recently, marine algae have been found as great sources of anti-inflammatory agents (Singh, Kate, and Banerjee 2005; Cumashi et al. 2007; Abad, Bedoya, and Bermejo 2008). Several marine algae have been shown to be effective against inflammation *in vivo* and *in vitro*.

### 23.3.1 *IN VIVO ANTI-INFLAMMATORY ACTIVITIES*

According to Boonchum et al. (2011), aqueous extracts of brown alga *Turbinaria conoides* have been known for their anti-inflammatory effect against ethyl phenylpropionate-induced ear edema and carrageenan-induced hind paw edema in rats. The anti-inflammatory activity of *T. conoides* is comparable to phenylbutazol and acetylsalicylic acid, which were used as the reference control drugs. Moreover, brown algae *S. fulvellum*, *S. thunbergii*, and *Sargassum wightii* have been determined to inhibit edema in the rat and mouse. The butanol extract (100 mg/kg) of *S. wightii* collected during winter season was effective (87%) in reducing carrageenan-induced edema in rats compared to reference drugs aspirin (79%) and ibuprofen (57%) (Dar et al. 2007). Meanwhile, the dichloromethane extract (0.4 mg/ear) of *S. fulvellum* and the ethanol extract (0.4 mg/ear) of *S. thunbergii* inhibited an inflammatory symptom of mouse ear edema by 79% and 72% respectively (Kang et al. 2008). Similarly, the anti-inflammatory effect of microalga *Spirulina* has been shown to reduce the levels of  $\beta$ -glucuronidase in zymosan-induced arthritis in mice (Remirez et al. 2002).

On the contrary, phycocyanin extracted from the microalga *Arthrospira maxima* exerted anti-inflammatory activity by reducing arachidonic acid (AA)-induced mouse ear edema, carrageenan-induced rat paw edema, acetic acid-induced rat colitis, and cotton pellet granuloma in rats (Romay, Ledon, and Gonzalez 1998; González et al. 1999). The anti-inflammatory action was suggested to be due to its antioxidative and oxygen-free radical scavenger properties. Furthermore, polysaccharide obtained from red microalgae *Porphyridium* inhibited immune cell recruitment by blocking the migration and adhesion of polymorphonuclear leukocytes (Matsui et al. 2003). Oral administration of water-soluble crude polysaccharide from brown alga *Turbinaria ornata* evoked a considerable inhibition on carrageenan-induced paw edema in rats and on acetic acid-induced vascular permeability in mice (Ananthi et al. 2010). Some lectins isolated from *Pterocladiella capillacea*, *Caulerpa cupressoides*, and *Hypnea cervicornis* also exhibited anti-inflammatory effects. *P. capillacea* lectin (72.9 mg/kg) and *C. cupressoides* lectin (27 mg/kg) reduced the number of writhes in rats by 52% and 86% respectively. Additionally, *P. capillacea* lectin (8.1 mg/kg) and *C. cupressoides* lectin (9 mg/kg) significantly reduced neutrophil migration by 84% and 66% respectively (Silva et al. 2010; Vanderlei et al. 2010). Meanwhile, lectin agglutinin isolated from the *H. cervicornis* has been shown to inhibit hypernociception induced by carrageenan and ovalbumin in rats. The inhibition of inflammatory hypernociception was associated with the prevention of neutrophil recruitment to the plantar tissue (Figueiredo et al. 2010). Besides, the anti-inflammatory  $\Omega$ -3 polyunsaturated fatty acids including stearidonic acid and eicosapentaenoic acid obtained from brown alga *Undaria pinnatifida* were determined to be active against mouse ear edema induced by phorbol myristate acetate with  $IC_{50}$  values of 160 and 230  $\mu$ g/ear respectively (Khan et al. 2007). Taken together, it suggests that marine algae could be a good approach for the treatment or prevention of inflammatory diseases.

### 23.3.2 *IN VITRO ANTI-INFLAMMATORY ACTIVITIES*

Obviously, the inflammatory mediators such as prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) and nitric oxide (NO) play a critical role in virtually every step of inflammation and implicate in the pathogenesis of various inflammatory diseases. Substantially, PGE<sub>2</sub> and NO are products of the inducible isoforms

of cyclooxygenase (COX)-2 and inducible nitric oxide synthase (iNOS) enzymes. Besides, phospholipases A<sub>2</sub> (PLA<sub>2</sub>s) are known as a family of enzymes catalyzing the hydrolysis of membrane phospholipids into AA, which is the major precursor of pro-inflammatory eicosanoids (Vane et al. 1994; Yedgar, Cohen, and Shoseyov 2006). Therefore, these enzymes are regarded as key therapeutic targets for regulation of inflammatory diseases. In this sense, marine algae have been recognized as a promising candidate to modulate the inflammatory mediator production through the inactivation of COX-2, iNOS, and phospholipases A<sub>2</sub> (PLA<sub>2</sub>s) activity. Indeed, brown algae *Dictyota dichotoma*, *Sargassum micracanthum*, and *Petalonia binghamiae* exhibited potent inhibition on NO and PGE<sub>2</sub> productions by suppressing lipopolysaccharide (LPS)-induced protein and mRNA expression of iNOS and COX-2 in RAW 264.7 macrophages (Yoon et al. 2009a,b; Yang et al. 2010). Likewise, iNOS and COX-2 expressions in LPS-stimulated BV2 microglia were suppressed by *Ecklonia cava* treatment (Jung, Ahn et al. 2009). Notably, several active compounds derived from brown algae have been identified to be responsible for inhibitory effects on inflammation-mediating enzymes. In particular, polysaccharides such as fucoidan obtained from *Fucus vesiculosus*, *E. cava*, *Laminaria japonica*, and *S. hemiphyllum* significantly diminished the production of inflammatory mediators NO as well as PGE<sub>2</sub> due to the reduction in the expression of iNOS and COX-2 in macrophage and microglia cells (Yang et al., 2006; Cui et al. 2010; Hwang et al. 2011; Kang et al. 2011; Park et al. 2011). In parallel, phlorotannins of eckol, phlorofucofuroeckol A, dieckol, and 8,8'-bieckol isolated from brown alga *Eisenia bicyclis* had pronounced inhibitory effects on sPLA<sub>2</sub> from porcine pancreas and bee venom with an IC<sub>50</sub> range of 100–200 µM. Among them, 8,8'-bieckol exposed the strongest inhibition on soybean lipoxygenases (LO) and 5-LO with IC<sub>50</sub> values of 38 and 24 µM respectively. Conversely, dieckol and eckol are strong inhibitors of COX-1 (75%) and COX-2 (43%) at 100 µM respectively (Shibata et al. 2003). These inhibitory effects may cause a reduction in the synthesis and release of leukotriene and prostaglandin (Sugiura et al. 2009). Further, dieckol from *E. cava* was shown as a neuroprotective agent due to alleviation of iNOS and COX-2 expression in BV2 microglia (Jung, Heo, et al. 2009). Similarly, dieckol from *E. cava*, diphlorethohydroxycarmalol from *Ishige okamurae* and phlorofucofuroeckol A from *Ecklonia stolonifera* also displayed potent downregulation on iNOS and COX-2 expression in human umbilical vein endothelial cells and RAW 264.7 macrophage cells (Kim et al. 2009; Heo, Hwang, et al. 2010; Lee et al. 2010). In addition, fucoxanthin, a specific carotenoid found in common brown algae such as *Myagropsis myagroides* and *I. okamurae*, has been studied for its anti-inflammatory function. Herein, fucoxanthin induced suppressive effects in the level of iNOS and COX-2 expressions and concomitant reductions in the production of NO and PGE<sub>2</sub> in RAW 264.7 macrophage cells (Heo, Yoon et al. 2010; Kim et al. 2010). Recently, the meroditerpene epitaondiol from *Stylopodium flabelliforme* and the methoxylated fatty acid 7-methoxy-9-methylhexadeca-4,8-dienoic acid from *I. okamurae* were considered as efficient PLA<sub>2</sub> inhibitors with IC<sub>50</sub> values of 3.8 and 2 µM respectively (Terracciano et al. 2006; Cho et al. 2008).

Besides brown algae, several green algae and red algae also provide useful additional therapy for controlling the activities of inflammatory enzymes. Namely, green algae *Codium fragile*, *Capsosiphon fulvescens*, and *Ulva conglobata* caused significant inhibitory effect on iNOS and COX-2 expressions in RAW 264.7 macrophage cells, AGS cells, and BV2 microglia cells respectively (Jin et al. 2006; Hwang et al. 2008; Han et al. 2010). Notably, the compound rhipocephalin from the green alga *Rhipocephalus phoenix* and the compound caulerpenyne from green alga *Caulerpa prolifera* have been shown to inhibit bee venom sPLA<sub>2</sub> with an IC<sub>100</sub> value of 4.1 µM and IC<sub>92</sub> value of 4.2 µM respectively (Mayer et al. 1993). Similarly, the bromohydroquinones cymopol and cyclocymopol isolated from the green alga *Cymoplia barbata* inhibit bee venom sPLA<sub>2</sub> activity with IC<sub>98</sub> values of 4.7 and 3.4 µM respectively (Mayer et al. 1993). Meanwhile, the bioactive peptide *trans*, *trans*-ceratospongamide purified from red alga *Ceratodictyon spongiosum* exhibited potent inhibition to sPLA<sub>2</sub> expression in a cell-based model for anti-inflammation with an ED<sub>50</sub> value of 32 nM. *trans*, *trans*-Ceratospongamide was also shown to inhibit the expression of a human-sPLA<sub>2</sub> promoter-based reporter by 90% (Tan et al. 2000). Finally, the inactivation of bee venom

sPLA<sub>2</sub> activity by the bromophenols vidalol A and B isolated from red alga *Vidalia obtusaloba* was observed with an IC<sub>50</sub> value of 1.6 µg/mL (Potts, Faulkner, and Jacobs 1992). Accordingly, this evidence suggests that marine algae could provide interesting lead agents for the design of inhibitors of COX-2, iNOS, and sPLA<sub>2</sub> enzymes, which contribute to attenuation of inflammatory mediator productions in inflammatory response.

### 23.4 CONCLUSION

Currently, many of chronic diseases are becoming common in our aging society throughout the world. Clinically used drugs suffer from the disadvantage of side effects and high cost of treatment. Therefore, finding alternative drugs that are safe and efficient against diseases are necessary goals. Herein, natural products are recognized as a rich source of leads for the pharmaceutical industry. In particular, marine algae are widely known as a potential source of bioactive compounds with numerous health benefit effects. Notably, a large number of marine algae have been found to be effective against inflammatory responses *in vitro* and *in vivo*. Thus, marine algae are believed to be an alternative source of novel drugs for treatment of inflammatory diseases as well as other chronic diseases.

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# 24 Marine Microbial Pharmacognosy *Aspects and Prospects*

*Se-Kwon Kim and Ira Bhatnagar*

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## 24.1 INTRODUCTION

Nature is an excellent source of chemically diverse compounds with great therapeutic potential (Bhatnagar and Kim 2010a). Moreover, the natural compounds have a high affinity to the target, which makes them more efficient as drugs. Little loss of entropy when they bind to a protein and their bioavailability further enhance their suitability as successful pharmaceutical agents. Their flexibility to acquire different conformations in aqueous and lipophilic environments makes them the compounds of choice over their synthetic counterparts. The hydrosphere harbors a vast variety of marine organisms that are assorted in their physiology and adaptations giving rise to a diversification in natural compounds derived from them. These natural molecules are not only used by themselves but also serve as lead molecules for manipulation by chemical or genetic means (Demain 2009). It is noteworthy that marine sources have also demonstrated tremendous abilities as producers of anti-cancer compounds and secondary metabolites, which act against infectious diseases and inflammation. Blunt et al. (2009) listed that in the marine environment, sponges (37%), coelenterates (21%), and microorganisms (18%) are major sources of biomedical compounds, followed by algae (9%), echinoderms (6%), tunicates (6%), molluscs (2%), bryozoans (1%), and so on (Blunt et al. 2009). However, marine microorganisms have not been given the attention they deserve, and a very limited insight into the capabilities and bioactive potential of marine microorganisms is available in literature to date. Extensive reviews on marine pharmacology have been published in the past encompassing the antihelminthic, antibacterial, anticoagulant, antifungal, anti-inflammatory, antimalarial, antiprotozoan, antituberculosis, antiviral, and other miscellaneous mechanisms of action of marine natural products (Mayer et al. 2009).

Microorganisms, including certain bacteria, fungi, and algae, produce secondary metabolites that may have some degree of bioactivity, either against another microorganism or acting against certain physiological states of a diseased body. These metabolites, otherwise known as bioactive substances, are profoundly used as antibiotics and may be effective against infectious diseases such as HIV-1 (Cragg and

Newman 2001), conditions of multiple bacterial infections (penicillin, cephalosporins, streptomycin, and vancomycin), or neural tube defects and neuropsychiatric sequelae (Finglas et al. 2003; Berdy 2005).

Some drugs have also been found to be useful against carcinomas (bleomycin, dactinomycin, doxorubicin, and staurosporine), risk of coronary heart disease, or may act as immunosuppressants (cyclosporin) to aid in organ transplantation (Ruiz et al. 2010), thus making the microbial secondary metabolites an enormous source of pharmaceutical importance. Since the 1920s when the first antibiotic penicillin was discovered, it was believed that soil microorganisms are the largest source of novel drugs. It is their biological and chemical diversity (Table 24.1) coupled with the underlying competence to produce novel secondary metabolites with antimicrobial and nutritive effects that has led to the widespread use of microorganisms in the economic, industrial-scale production of drugs.

## 24.2 ANTICANCER METABOLITES OF MARINE MICROBIAL ORIGIN

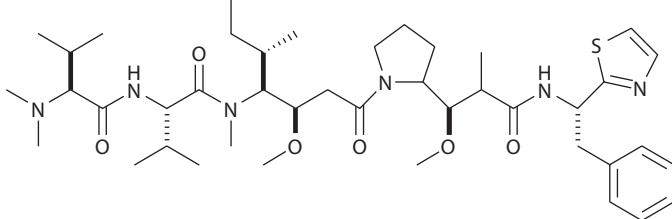
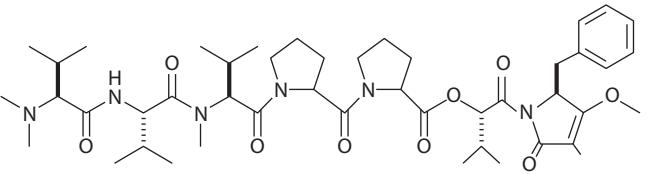
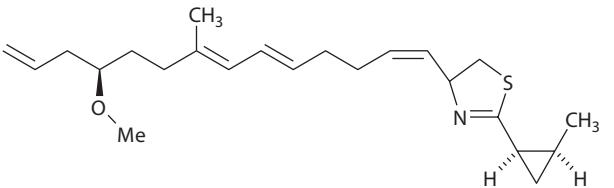
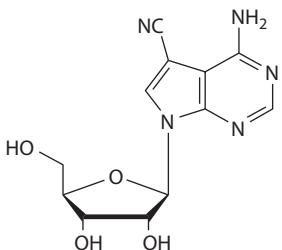
One of the deadliest diseases in the medical field is considered to be cancer. Apart from the preventive therapies, it is important to find a curative measure that holds no loopholes and acts accurately and precisely to curb cancer. Marine chemotherapy is well recognized nowadays, and profound development has been achieved by researchers to deal with the different molecular pathways of tumors (Bhatnagar and Kim 2010b). Some such pharmacotherapeutic metabolites are mentioned here.

Nuclear factor- $\kappa$ B (NF- $\kappa$ B) is a ubiquitous transcription factor, a dimer of proteins of the Rel family whose deregulated expression may lead to cancer. NF- $\kappa$ B is activated by various stimuli, including tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), interleukin-1, and lipopolysaccharide (Baldwin Jr 1996). Certain fungal metabolites show promising potential as novel anticancer agents as they modulate the activity of NF- $\kappa$ B. A fungal symbiont, *Penicillium chrysogenum* of the marine sponge *Ircinia fasciculata*, produces an antileukemic agent, sorbicillactone-A, which has been now qualified for human trials, owing to its amazing anticancer properties (Thakur and Thakur 2006). Yet another compound of marine microbial origin is a diketopiperazine known as plinabulin (NPI-2358), isolated from a marine alga-associated *Aspergillus* sp. CNC-139 (Kanoh et al. 1997). This compound also inhibits tubulin assembly and acts as a vasculature disrupting agent that destabilizes the tumor vascular endothelial architecture and leads to cell damage (Nicholson et al. 2006).

A study published a couple of years back reported three new cyclohexadepsipeptides, arenamides (A–C), from the fermentation broth of a marine bacterial strain *Salinispora arenicola*. They studied the effect of arenamides A and B on NF- $\kappa$ B activity with stably transfected 293/NF- $\kappa$ B-Luc human embryonic kidney cells induced by treatment with TNF. It was observed that arenamides A and B blocked TNF-induced activation of NF- $\kappa$ B in a dose- and time-dependent manner (Asolkar et al. 2008). Inspired by the previous studies, Nam et al. (2010) have recently isolated fijiolide A, a potent inhibitor of TNF- $\alpha$ -induced NF- $\kappa$ B activation, from a marine-derived bacterium of the genus *Nocardiopsis*. It was observed to reduce TNF- $\alpha$ -induced NF- $\kappa$ B activation by 70.3%, with an IC<sub>50</sub> value of 0.57  $\mu$ M. Their data proposes fijiolide A as a promising lead for more advanced anticancer testing (Nam et al. 2010).

Other major players in oncogenesis are the members of the protein kinase C (PKC) family of serine/threonine kinases. One of the isoforms, PKC $\epsilon$ , has been demonstrated to increase proliferation, motility, and invasion of fibroblasts or immortalized epithelial cells (Michael and Quintin 2009). It is worth mentioning here that the marine environment has provided a very efficient class of PKC inhibitors, known as bryostatins. These are highly oxygenated marine macrocyclic lactones/macrolides with a unique polyacetate backbone that binds to the regulatory domain of PKC. Short-term exposure to bryostatin-1 promotes activation of PKC, whereas prolonged exposure promotes significant downregulation of PKC. It has been reported that bryostatin-1 inhibits proliferation, induces differentiation, and promotes apoptosis in numerous hematological and solid tumor cell lines (Kortmansky and Schwartz 2003). Since their discovery from the marine

**TABLE 24.1**  
**Chemical Structure and Biological Activity of Some of the Marine Microbial Metabolites**

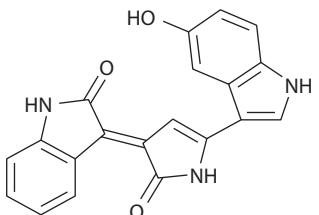
Compound Name	Compound Structure	Biological Activity	Organism
Dolastatin 10		Antimicrotubule and the synthetic analogue TZT-1027 as antitumor	
Dolastatin 15		Antimicrotubule and the synthetic analogue ILX-651 as antitumor	Cyanobacteria
Curacin A		Antimicrotubule	
Toyocamycin		Antifungal	

(Continued)

**TABLE 24.1 (Continued)****Chemical Structure and Biological Activity of Some of the Marine Microbial Metabolites**

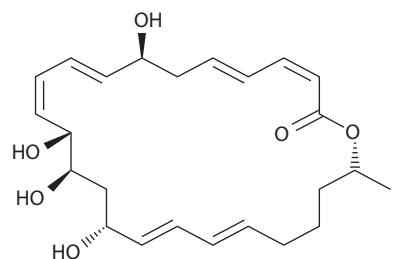
Compound Name	Compound Structure	Biological Activity	Organism
Resistoflavine		Anticancerous and antibacterial	Actinomycetes
Marinomycin A		Antitumor and antibiotic	
Daryamide C		Antitumor	

Violacein



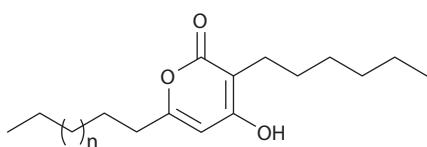
Antiprotozoal

Macrolactin S



Antibacterial

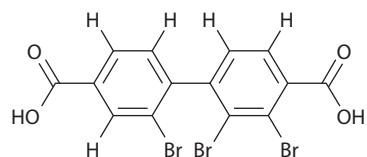
Pyrone I and II



Antibacterial

Bacteria

MC21-B

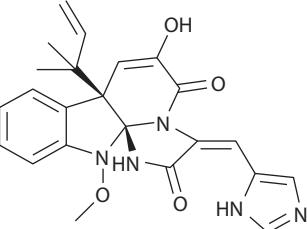
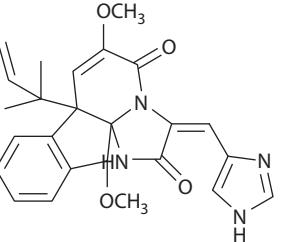
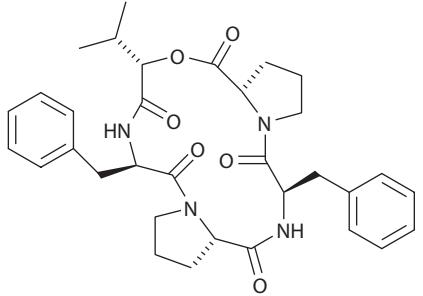


Antibacterial

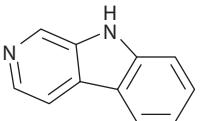
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TABLE 24.1 (Continued)

## Chemical Structure and Biological Activity of Some of the Marine Microbial Metabolites

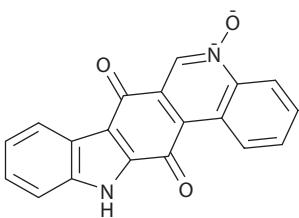
Compound Name	Compound Structure	Biological Activity	Organism
Meleagrin		Antitumor	
Oxaline		Antitumor	Fungi
Alternaramide		Antibacterial	

Norharman



Enzyme inhibitor

Calothrixin-A

Antimalarial and  
anticancerous

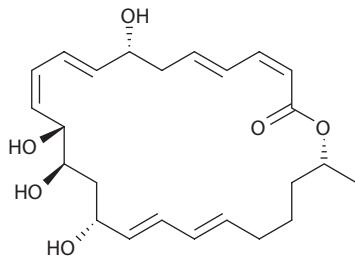
Algae

Eicosapentaenoic acid (EPA)

Treats heart disease,  
anti-inflammatory agent  
(rheumatoid arthritis and  
immunodeficiency diseases)

Antibacterial and antilarval

Macrolactin V

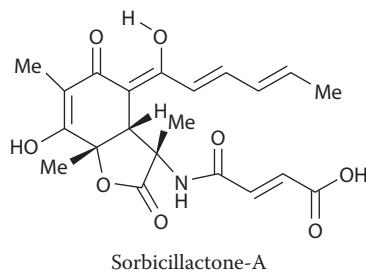


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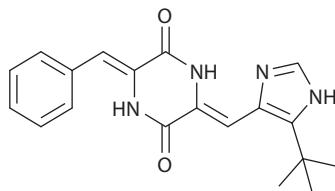
**TABLE 24.1 (Continued)****Chemical Structure and Biological Activity of Some of the Marine Microbial Metabolites**

Compound Name	Compound Structure	Biological Activity	Organism
DAPG		Antibacterial (anti-MRSA, anti-VRSA, and anti-VRE)	Symbiotic microorganisms
BE-43472B		Antibacterial (anti-MRSA and anti-VRE)	

Source: Modified from Bhatnagar, I. and S. K. Kim, *Mar. Drugs*, 8, 2673–2701, 2010a and Bhatnagar, I. and S. K. Kim., *Mar. Drugs*, 8, 2702–2720, 2010b.

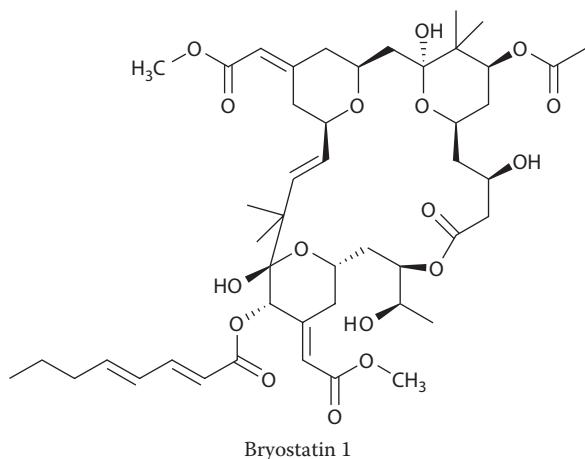


**STRUCTURE 24.1** Scorbicillactone-A.



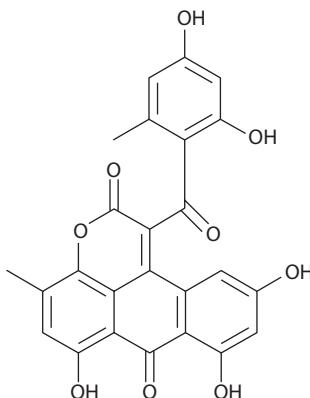
**STRUCTURE 24.2** Plinabulin.

bryozoa *Bugula neritina* in 1982 (Pettit et al. 1982) and showing high activity against the murine P388 lymphocytic leukemia, 20 natural bryostatins are a part of our knowledge today (Mutter and Wills 2000). Bryostatin 1 is now known to be produced by the bryozoan's bacterial symbiont, *Candidatus Endobugula sertula* (Davidson et al. 2001). Their low toxicity and antineoplastic nature makes them promising candidates for cancer chemotherapeutics. It has proven to be such a promising candidate that it is being evaluated as an antitumor agent against myeloma, acute myeloid leukemia, chronic lymphocytic leukemia (CCL), AIDS-related lymphoma, non-Hodgkin's lymphoma; and colorectal, renal, prostate, head and neck, cervix, ovarian, breast, peritoneal, stomach, esophagus, anus, and non-small cell lung cancer (Mutter and Wills 2000). It is currently under phase I clinical trial under the aegis of the National Cancer Institute (United States) (Mayer et al. 2010). Studies are now under way to understand the chemical basis of the biological activity of bryostatins.



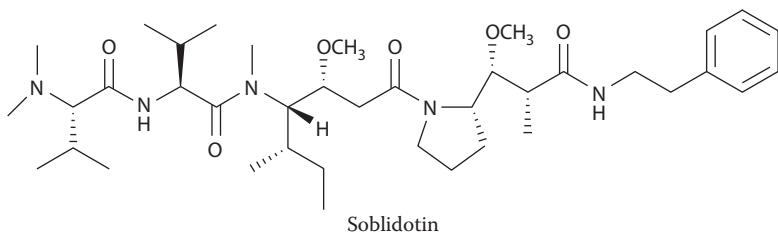
**STRUCTURE 24.3** Bryostatin 1.

A couple of years ago, Du et al. (2007) isolated a novel anthraquinone derivative with naphtho[1,2,3-de]chromene-2,7-dione skeleton and named it aspergiolide A. It was isolated from a marine filamentous fungus, *Aspergillus glaucus*, in the Fujian province of China and was found to exhibit cytotoxicity against the K562 and P388 cell lines (Du et al. 2007). The same group has recently worked on the antitumor activities of alkaloids isolated from a *Penicillium* sp. derived from deep ocean sediment. They isolated two new meleagrin analogues, meleagrin D and E, and two new diketopiperazines, roquefortine H and I, which showed weak cytotoxicity, compared to the previously reported Meleagrins that induced Human Promyelocytic Leukemia (HL-60) cell apoptosis or arrested the cell cycle through G2/M phase respectively. They proposed that the distinct substitutions on the imidazole ring could have a significant influence on the cytotoxicity of these alkaloids (Du et al. 2010).

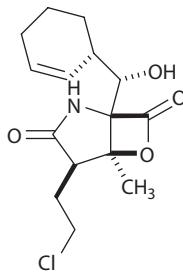


**STRUCTURE 24.4** Aspergiolide A.

The other marine microbial compound under phase III clinical trials is a peptide soblidotin (TZT-1027), derived from a marine bacterium (Mayer et al. 2010). It acts by inhibiting tubulin assembly and disrupting the vasculature of tumor cells, causing them to collapse (Watanabe, Natsume, and Kobayashi 2007). Marizomib (salinosporamide A; NPI-0052), a proteasome inhibitor isolated from a marine bacterium *Salinospira tropica* (Feling et al. 2003), is also undergoing phase I clinical trials under the auspices of Nereus Pharmaceuticals (San Diego, California). Interesting properties such as a broader and longer-lasting proteasome inhibition, efficacy against a wider range of hematologic malignancies and many solid tumor models, less cytotoxicity to normal cells, higher *in vivo* potency, and potential for both oral and intravenous administration make salinosporamide A a very promising anticancer agent (Bhatnagar and Kim 2010b).



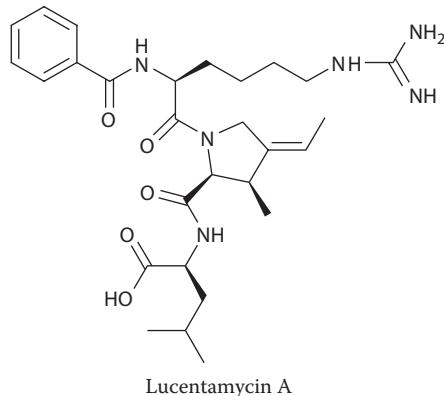
**STRUCTURE 24.5** Soblidotin.



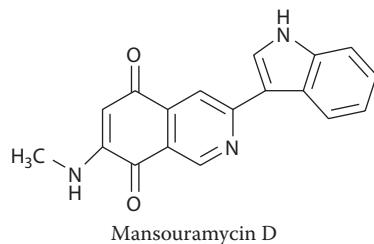
Salinosporamide A (NPI-0052)

**STRUCTURE 24.6** Salinosporamide A.

In the exploration of marine-derived actinomycetes as a source of antitumor compounds, Cho et al. (2007) isolated four new 3-methyl-4-ethylidene proline-containing peptides, lucentamycins A–D, from the fermentation broth of a marine-derived actinomycete, *Nocardiopsis lucentensis* (strain CNR-712). Out of the four compounds, lucentamycins A and B were observed to have significant *in vitro* cytotoxicity against human colon carcinoma (HCT-116) cells (Cho et al. 2007). In a report published a couple of years ago, five isoquinoline quinones, four new derivatives (mansouramycin A–D), and the known 3-methyl-7-(methylamino)-5,8-isoquinolinedione were isolated from the ethyl acetate extract from the marine-derived Mei37 isolate of *Streptomyces* sp. These isolated compounds, when subjected to cytotoxicity analysis against 36 tumor cell lines, indicated significant cytotoxicity with great degree of selectivity for non-small cell lung cancer, breast cancer, melanoma, and prostate cancer cells (Hawas et al. 2009) suggesting their potential as anticancer drugs.



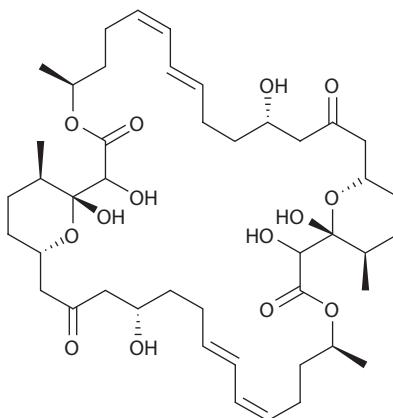
Lucentamycin A

**STRUCTURE 24.7** Lucentamycin A.

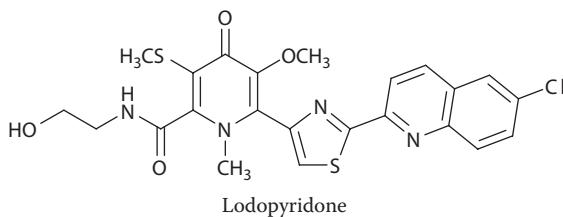
Mansouramycin D

**STRUCTURE 24.8** Mansouramycin D.

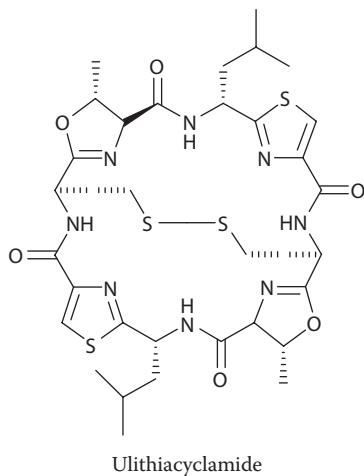
In the same year, Perez and coworkers isolated a macrodiolide tartrolon D from the fermentation broths of *Streptomyces* sp. MDG-04-17-069. The tartrolons are a class of compounds that have attracted a great deal of attention owing to their interesting biological properties. The isolated tartrolon in this study was found to display strong cytotoxic activity against three human tumor cell lines, namely, lung (A549), colon (HT29), and breast (MDA-MB-231) (Pérez et al. 2009). Yet another study reported the secondary metabolites of a marine *Saccharomonospora* sp. collected at the La Jolla Submarine Canyon. Chemical examination yielded a novel alkaloid lodopyridone, which was found to be cytotoxic ( $IC_{50} = 3.6 \mu M$ ) to HCT-116 human colon cancer cells (Maloney et al. 2009).



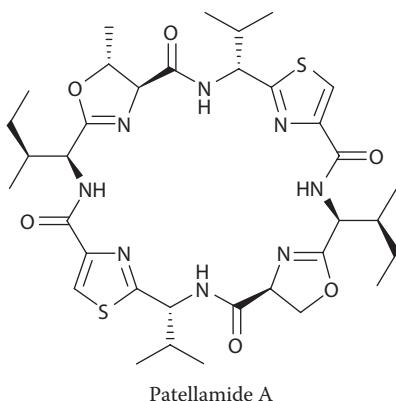
Tartrolon D

**STRUCTURE 24.9** Tartrolon D.**STRUCTURE 24.10** Lodopyridone.

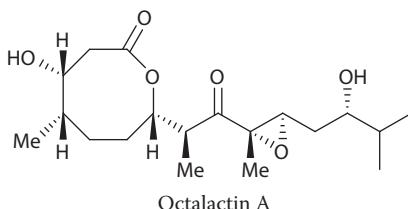
Certain anticancer compounds, which were initially thought to be obtained from marine sources, are now known to be produced by cyanobacteria (Luesch et al. 2002). Ulithiacyclamide and patellamide A belong to cyanobactins, produced by cyanobacteria, which have potent antimalarial, antitumor, and multidrug reversing activities (Sivonen et al. 2010). About 10 years ago, Tapiolas et al. (1991) isolated and characterized two closely related novel compounds, cctalactins A and B, from a marine-derived *Streptomyces* sp. isolated from the surface of an unidentified gorgonian of the genus *Pacifigorgia*. They reported that octalactin A exhibited strong cytotoxic activity toward B-16-FLO murine melanoma and HCT-116 human colon tumor cell lines with  $IC_{50}$  values of 0.0072 and 0.5 pg/mL respectively (Tapiolas et al. 1991). Cyanobacteria produce a family of antitumor agents known as cryptophycins, which interfere with the tubulin assembly. A synthetic cryptophycin derivative (LY355703, CRYPTO 52) is in the early stages of clinical evaluation. Depsi peptide (NSC 630176) is a bicyclic peptide isolated from *Chromobacterium violaceum*. It decreases mRNA expression of the *c-MYC* oncogene, causes cell-cycle arrest at G0-G1, and acts as an inhibitor of a histone deacetylase. These properties make it a promising anticancer agent for which the phase I clinical trials are soon to begin (Schwartzmann et al. 2001).



**STRUCTURE 24.11** Ulithiacyclamide.



**STRUCTURE 24.12** Patellamide.

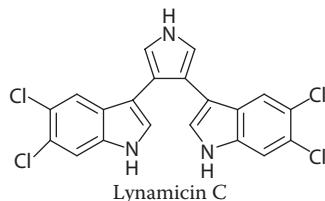


**STRUCTURE 24.13** Octalactin A.

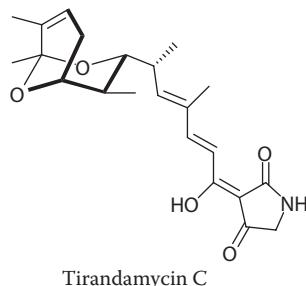
### 24.3 ANTIMICROBIAL METABOLITES OF MARINE MICROBIAL ORIGIN

Microorganisms produce secondary metabolites that may act against other microbes. Such metabolites are termed as antimicrobials. Marine microbes are now being considered as a rich source of antimicrobial compounds based on the studies mentioned here and many more. Actinomycetes from natural sources are widely recognized to produce secondary metabolites, including many antimicrobials such as streptomycin, erythromycin, and tetracycline, with original and ingenious structures and potent biological activities (Takahashi and Omura 2003). Therefore, actinomycetes are considered to

be a potent resource for new lead compounds in drug development. Many marine isolates of actinomycetes have been reported to be producers of novel antimalarial (Prudhomme et al. 2008) and antimicrobial agents. A California-based study was successful in isolating a series of chlorinated bisindole pyrroles, lynamicins A–E, from a novel strain of a marine actinomycete, *Marinispora*. This isolate from marine sediment collected off the coast of San Diego (California) demonstrated broad-spectrum activity against both Gram-positive and Gram-negative organisms. When tested for their antimicrobial spectrum against a panel of 11 pathogens, these compounds showed activity against drug-resistant pathogens such as methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant *Enterococcus faecium* (McArthur et al. 2008). Carlson et al. (2009) have reported the isolation of novel dienoyl tetramic acids tirandamycin C and tirandamycin D with activity against vancomycin-resistant *Enterococcus faecalis*, from the marine environmental isolate *Streptomyces* sp. 307-9.

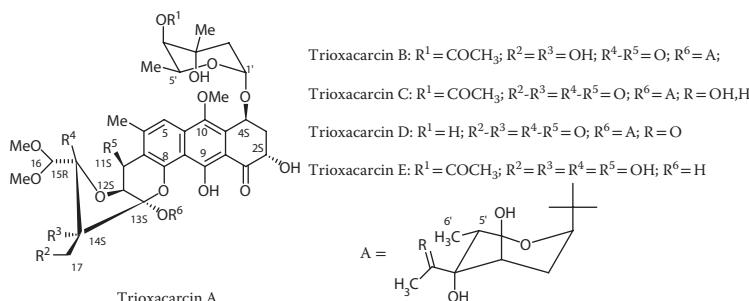


**STRUCTURE 24.14** Lynamicin C.



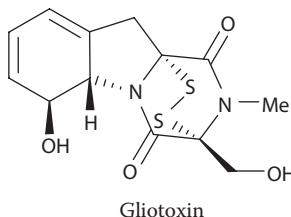
**STRUCTURE 24.15** Tirandamycin C.

Maskey et al. (2004) isolated trioxacarcins A, B, and C along with three new derivatives designated as trioxacarcins D, E, and F, from the ethyl acetate extract of *Streptomyces* sp. isolate B8652. All trioxacarcins showed high antibacterial activity, whereas some of them showed high antitumor and antimalarial activity (Maskey et al. 2004).



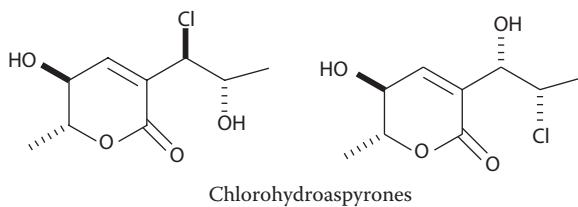
**STRUCTURE 24.16** Trioxacarcins.

Fungi have long been explored for antimicrobial metabolite production. Further, marine-derived fungi have been widely studied for their bioactive metabolites and have proven to be a rich and promising source of novel anticancer, antibacterial, antiplasmodial, anti-inflammatory, and antiviral agents (Bhadury, Mohammad, and Wright 2006; Newman and Hill 2006). Some marine fungi have unique new carbon frameworks, which are exceptional in nature. Compounds produced by such fungi are of interest as new lead structures for medicine as well as for plant protection. A Korea-based study resulted in the isolation of a novel antibacterial dioxopiperazine, dehydroxybisdethiobis-methylthio-gliotoxin, and the previously reported bisdethiobis-methylthio-gliotoxin and gliotoxin, from the broth of a marine-derived fungus of the genus *Pseudallescheria*. All three compounds exhibited potent antibacterial activity against the methicillin-resistant and multidrug resistant *S. aureus*, whereas gliotoxin showed a significant radical scavenging activity against 1,1-diphenyl-2-picrylhydrazyl (DPPH) with an  $IC_{50}$  value of 5.2  $\mu\text{M}$  (Li et al. 2006).



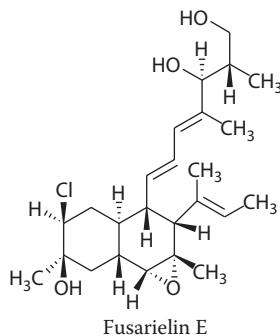
**STRUCTURE 24.17** Gliotoxin.

Another study from the same source reported two novel antibacterial aspyrone derivatives, namely, chlorohydroaspynes A and B, and the previously described aspyrone, asperlactone, and penicillic acid from the broth of a marine isolate of the fungus *Exophiala* and were found to have mild antibacterial activity against *S. aureus* (Zhang et al. 2008).

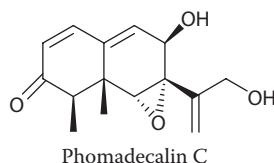


**STRUCTURE 24.18** Chlorohydroaspynes.

In the search for novel antimitotic and antifungal substances from marine-derived fungi, Gai et al. (2007) reported that low concentrations of the ethanolic (EtOH) extracts of the culture broth of a *Fusarium* sp. (strain 05JANF165) were bioactive. Their search for the basis of this bioactivity led to the identification and purification of a new antifungal antibiotic and the chemical structure was elucidated as fusarielin E (Gai et al. 2007). A study conducted in the United States in 2002 reported five new natural products, phomadecalins A, B, C, and D, and phomapentenone A, from cultures of *Phoma* sp. (NRRL 25697), a mitosporic fungal colonist isolated from the stromata of *Hypoxylon* sp. These compounds were characterized structurally, and four compounds (phomadecalins A–D) were found to be active against Gram-positive bacteria, *Bacillus subtilis* (ATCC 6051) and *S. aureus* (ATCC 29213) (Che, Gloer, and Wicklow 2002).

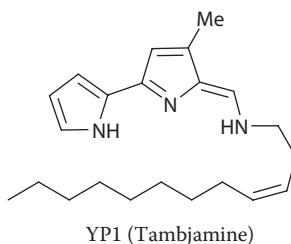


**STRUCTURE 24.19** Fusarielin E.



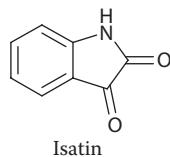
**STRUCTURE 24.20** Phomadecalin C.

A couple of years ago, the seawater species *P. phenolica* was reported to inhibit methicillin-resistant *S. aureus* strains due to a brominated biphenyl compound, 3,3',5,5'-tetrabromo-2,2'-diphenyldiol (Isnansetyo and Kamei 2003). Some strains of *P. luteoviolacea* also seem to have activity against protists (Kamei et al. 1986). Egan et al. (2002) reported that the yellow pigment of *Pseudoalteromonas tunicata* has antifungal activity, which was later identified as a tambjamine (4-methoxypyrrrole-containing bioactive compounds) like alkaloid and designated as YP1 (Franks et al. 2005). These tambjamines have been isolated from marine invertebrates and have been previously reported to possess antimicrobial, antitumorigenic, immunosuppressive, antiproliferative, and ichthyodeterrent activities (Lindquist and Fenical 1991).



**STRUCTURE 24.21** Tambjamine YP1.

A study in 2004 reported hemolysis and inhibition of *Candida albicans* by employing the butanolic extracts of the algal-associated species *Pseudoalteromonas issachenkoni* cultures. Further analysis of the ethyl acetate extracts revealed that the basis of this antifungal activity was isatin (indole-2,3-dione) (Byun et al. 2003). On similar lines, a recent study reported antibacterial and anti-larval compounds from marine gorgonian-associated bacterium *Bacillus amyloliquefaciens* SCSIO 00856 isolated from the South China Sea gorgonian, *Junceella juncea*. The broth of this strain showed strong antibacterial activity toward *Escherichia coli*, *B. subtilis*, and *S. aureus* and anti-larval activity toward the larvae of bryozoan *B. neritina*. When subjected to isolation procedures, a new 24-membered ring lactone, macrolactin V, was obtained from the culture broth (Gao et al. 2010).



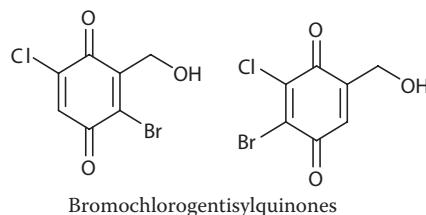
**STRUCTURE 24.22** Isatin.

Cyanobacteria are a diverse group of Gram-negative bacteria, also known as blue-green algae, that produce an array of secondary compounds with selective bioactivity against vertebrates, invertebrates, plants, microalgae, fungi, bacteria, viruses, and cell lines (Lopes et al. 2010). Some reviews in the past have proved that they produce a wide variety of secondary metabolites with antifungal, antiviral, antibiotic, and other activities, which make them an interesting candidate of potential pharmaceutical importance (Patterson, Larsen, and Moore 1994; Falch 1996). Desbois, Mearns-Spragg, and Smith (2009) isolated an antibacterial polyunsaturated fatty acid, eicosapentaenoic acid (EPA), from the marine diatom, *Phaeodactylum tricornutum* Bohlin, which showed activity against a range of both Gram-positive and Gram-negative bacteria, including Methicillin-resistant *Staphylococcus aureus* (MRSA) (Desbois, Mearns-Spragg, and Smith 2009). Despite the number of antimicrobial compounds isolated so far, the search is still on for novel metabolites of antibiotic potential from marine microbes.

#### 24.4 PHOTOPROTECTIVE METABOLITES OF MARINE MICROBIAL ORIGIN

Photoprotection is a group of mechanisms that minimize the damage that an organism suffers when exposed to ultraviolet (UV) radiation. These mechanisms can be controlled or organized by certain organic and inorganic compounds or substances (e.g., melanin) produced by different terrestrial and aquatic sources (Pallela, Na-Young, and Kim, 2010). A number of photoprotective compounds such as scytonemins (exclusively in cyanobacteria), mycosporines (in fungi), mycosporine-like amino acids (MAAs, in cyanobacteria, algae, and animals), phenylpropanoids and flavonoids (higher plants), melanins (humans and other animals and even some bacteria), and several other UV-absorbing substances of unknown chemical structures from different organisms have been developed to counteract the photodamage (Sinha, Singh, and Häder 1998; Sinha et al. 2007). Photoprotection is a major biological concern with respect to the source of natural bioactive molecules that have the antiphotoaging effect and especially the safer marine sources have been identified in the past few decades. As presented in this chapter, various compounds that potentially support the photoprotective mechanisms of strong cosmeceutical and pharmaceutical value have already been isolated from different marine microbial sources like algae, fungi, bacteria, and phytoplankton.

Nenkep et al. (2010) recently reported the isolation of halogenated benzoquinones (bromochlorogentisylyquinones A and B), with significant radical scavenging activity against DPPH, from a marine-derived *Phoma herbarum* strain.



**STRUCTURE 24.23** Bromochlorogentisylyquinones.

Many sunscreen/cosmetic compositions have been discovered from bacteria, which have been adopted for the photoprotection of human skin and/or hair, because of their underlying anti-photoaging principle. More interestingly, bacterial melanin, an active photoprotecting pigment, protects against DNA damage under full UV-B irradiation (Wan et al. 2007). The bacterial melanin exhibited excellent protection of bioinsecticide against UV-C and natural solar irradiation, thus raising a question as to whether the pigment also has a protective effect on DNA against the full UV spectrum (Wan et al. 2007). Holmes et al. (1995) recently described the bacterial (*Klebsiella aerogenes*) photoprotection through extracellular cadmium sulfide crystallites (CdS), where these semiconductor particles absorb radiation in the UV spectral region. Hence, when *K. aerogenes* produces extracellular CdS material in response to the stressed environments containing cadmium ions, a photoprotective layer is formed. Bacteria, especially the ones like archaea and other extremophiles species have tremendous implications for survival in extraterrestrial habitats and are very advantageous in present-day astrobiological research for the detection of the protectant biomolecules (Edwards et al. 2006).

UV-B-absorbing mycosporines with photoprotective activity are present-day targets of fungal species, for example, lichenized ascomycete *Collema cristatum* (Torres et al. 2004). The pure compound from this source prevented UV-B-induced cell destruction in a dose-dependent manner and partially prevented pyrimidine dimer formation. When applied to the skin prior to irradiation, it completely prevented UV-B-induced erythema. Kogej et al. (2006) later identified two different mycosporines and three unidentified UV-absorbing compounds in fungal isolates from hypersaline waters and polar glacial ice.

Cyanobacteria, a primitive group of Gram-negative prokaryotes, are known to have a wide range of habitat and thus they are supposed to have developed mechanisms leading to adaptations to survive in extreme climates and withstand critical processes such as heat, cold, drought, salinity, nitrogen starvation, photo-oxidation, anaerobiosis, and osmotic and UV stress (Tandeau de Marsac and Hounard 1993). There are a number of adaptation strategies by which cyanobacteria try to avoid high white light and UV radiation stress. These adaptations range from 1) migration into habitats of reduced light exposure through phototactic, photokinetic, and photophobic responses and vertical migration (Bebout and Garcia-Pichel 1995); 2) production of quenching agents such as carotenoids (Gw and Ku 1984); or 3) photoprotective systems of superoxide dismutase that neutralize the highly toxic reactive oxygen species produced by UV-B radiation (Vincent and Quesada 1994). However, the most common protective mechanism is the production of UV-absorbing substances such as MAAs and scytonemin (Garcia-Pichel, Wingard, and Castenholz 1993; Sinha, Singh, and Häder 1998). Brenowitz and Castenholz (1997) established the correlation between UV protection and scytonemin presence under solar irradiance in monospecific population of *Calothrix* sp., a naturally occurring cyanobacterium. It was shown that high scytonemin content is required for uninhibited photosynthesis under high UV flux. Such photoprotective compounds can be well utilized in the pharmaceutical and cosmeceutical industries as fairness solutions, after-burn ointments, and UV-protective sunscreen lotions.

## 24.5 PROSPECTS AND ASPECTS

The field of marine pharmacognosy is vast and deep. Despite a number of pharmaceutical/nutraceutical and cosmeceutical metabolites being isolated from marine microbes, only a few reach the clinical stage. Further, the difficult level to culture and the lack of literature on the isolation procedures and standardized culture conditions of marine microbes make the situation even worse with a small number of academics undertaking such studies. These problems get lofty with the lack of funding and infrastructure resources and experience in the biotechnology firms to perform the extensive, late-stage clinical development programs that are needed for regulatory approval of a drug. However, the advances in molecular biology and culturing technologies are bridging the

gap between the challenges pertaining to the exploitation of the marine environment as a potential source of natural protective compounds.

The reluctance of large pharmaceutical companies to invest in early-stage research raises another financial setback for marine natural product research. The huge structural diversity of marine natural compounds makes the isolation and purification of these compounds difficult, and the development of novel pharmaceutical drugs from these natural sources possesses problems that are not usually met when one deals with synthetic compounds. Prevailing threats to global marine biodiversity including overfishing, habitat loss, invasive species and pollution, rising water temperatures, and ocean acidification are further making marine microbial drug research more and more difficult (Bhatnagar and Kim 2010a).

The vast biodiversity of the marine environment can serve as a rich source of such natural compounds. However, keeping the abovementioned challenges in view, it becomes mandatory to design specific strategies for improvement of their therapeutic potential. A semisynthetic approach coupled with the bioinformatic, metagenomic, and proteomic studies could be one of the alternatives to enhance the yield of lead natural compounds. It can be achieved by modifying the functional groups of existing natural compounds. This would lead to the generation of structural analogues with greater pharmacological activity and fewer side effects. Increasing use of genomics and implication of combinatorial biosynthesis can be applied for discovery and modification of natural marine microbial products.

A more intrinsic insight into the biology and interspecies relationship of marine microbes would help for better understanding of the kind of compounds that may be isolated from them. As per Raghukumar (2008), a more systematic research should be initiated for fungi from deep-sea, hypoxic zones (with low oxygen levels) and hydrothermal vents for enzymes, degradation of xenobiotics, and bioremediation applications. He further suggested that genomic and proteomic studies with novel organisms such as *Corallochytrium limacisporum* as a model of animal fungal allies will hopefully help in basic research on evolutionary biology (Raghukumar 2008). An evolutionary approach coupled with an ecological perspective can do wonders in the field of marine pharmacognosy, and a careful approach in this field can lead to increased production of bioactive compounds with high efficiency. Not only this, there are newer fields of biology, such as pharmacogenomics and pharmacogenetics, which are proving to be very helpful for understanding patient susceptibility to specific pharmacological agents. There is still scope for a higher magnitude of research and investigation to explore the potential of marine microorganisms to outcast their terrestrial counterparts in the pharmacognosial arena.

## ACKNOWLEDGMENT

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# 25 Pharmacognosy Prospects of Marine Algal Derivatives in the Management of Skin Inflammation

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## 25.1 INTRODUCTION

### 25.1.1 PRURITIC SKIN INFLAMMATION

Human skin is the anatomical barrier that acts as an imperative screen between internal and external environment and renders defense against pathogens and damage. However, many environmental factors and lifestyle habits in this modernized world contribute to several allergic diseases, which are estimated to be evident in one-third of the general population. These allergic diseases are initiated by an imbalance in the activation of mast cells, which leads to the inflammatory mediators (Le et al. 2009). Overproduction of inflammatory mediators increases the risk of numerous pathological conditions that include chronic inflammatory skin diseases. One skin inflammatory disease that needs an effective medicinal approach to be managed is atopic dermatitis (AD). AD is a chronic inflammatory skin disease associated with a personal or family history of allergy. AD is a common, often chronic (long-lasting) skin disease that affects a large percentage of the world's population and involves susceptibility genes, the environment, defective skin barrier function, and immunologic responses (Hamid, Boguniewicz, and Leung 1994). AD can occur at any age, and this pruritic inflammation's lifetime prevalence is 10–20% in children and 1–3% in adults (Leung et al. 2004). The fundamental lesion in AD is a defective skin barrier that results in dry itchy skin and is aggravated by mechanical injury inflicted by scratching. This allows entry of antigens via the skin and creates a milieu that shapes the immune response to these antigens (Oyoshi et al. 2009). AD is categorized into two types based on molecular responses. One is termed "extrinsic" and involves immunoglobulin E (IgE)-dependent sensitization that makes up to 70–80% of the patients, and the other form is known as "intrinsic" and involves IgE-mediated sensitization that makes up to 20–30% of the patients worldwide (Novak and Bieber 2003). Both forms of AD have associated eosinophilia. In extrinsic AD, memory T cells expressing the skin-homing receptor,

cutaneous lymphocyte-associated antigen (Patankar et al. 1993), produce increased levels of T-helper (Th)2 cytokines. These include interleukin (IL)-4 and IL-13, which are known to induce isotype switching to IgE synthesis, as well as IL-5, which plays an important role in eosinophil development and survival. These Cutaneous Lymphocyte-associated Antigen (CLA)<sup>+</sup> T cells also produce abnormally low levels of interferon (IFN)- $\gamma$ , a Th1 cytokine known to inhibit Th2 cell function. Intrinsic AD is associated with less IL-4 and IL-13 production than extrinsic AD (Leung et al. 2004). Hence, in order to control the hypersensitive reactions in skin, it is understood that the excessive production of IgE should be regulated, which can be considered as the best approach to cure AD.

### 25.1.2 PHARMACOGNOSY AND IMPORTANCE OF MARINE ALGAE

Pharmacognosy is a field that evaluates the medicinal ability of a naturally originated substance. The definition and practice of pharmacognosy have been evolving since the term was first introduced about 200 years ago (Kinghorn et al. 2004) as making use of drugs from medicinal plants has experienced significant development from the formulation of crude drugs to the isolation of biologically active compounds in drug discovery. The American Society of Pharmacognosy terms pharmacognosy as “the study of the physical, chemical, biochemical and biological properties of drugs, drug substances, or potential drugs or drug substances of natural origin as well as the exploration of new drugs, drug substances from natural sources.” Pharmacognosy involves the extensive study of natural products from various sources including plants, bacteria, fungi, and marine organisms (Balunas and Kinghorn 2005).

The role of pharmacognosy has seen an ever-demanding acceleration in the wider context of pharmaceutical research as there is an increased interest in the study of natural products as potential drugs and revolutionary approaches in the research fields (Bruhn and Bohlin 1997). Several terrestrial organisms have been exploited in the search for natural medicines and resulted in a good number of bioactive components that are useful as drugs. The other fact is 79% of the globe is filled with seawater that gives a researcher a wider choice of natural sources for the isolation of bioactive compounds. Moreover, the extremely vulnerable environment deep in the sea facilitates the production of unique bioactive compounds by marine organisms for their survival. It is a known fact that the marine environment has been the source of diverse life forms that produce different biologically active compounds. Sea organisms have been consistently contributing unparalleled bioactive compounds that have profound applications in nutraceuticals, cosmeceuticals, and drug industries. In this process, screening of natural products from marine organisms that could be potentially used as drug leads has gained much popularity and has became a hot field of research in life sciences (Thomas and Kim 2010). Among marine organisms, recently, the isolation and characterization of the biologically active components from seaweeds have gained much attention from various research groups across the world (Thomas and Kim 2011). Marine macroalgae are considered as dietary components and also as an alternative medicine in Asian countries like Japan, Korea, and China (Ali et al. 2000). Out of the four major classes of macroalgae, red and brown macroalgae are currently considered as potential sources of bioactive compounds that have a profound effect on human health systems (Renn 1993; Wijesekara, Yoon, and Kim 2010). Moreover, scientific investigations strongly suggest that the metabolites isolated from marine algae are known to exhibit beneficial biological effects (Kim et al. 2005). Marine brown algae like *Ecklonia cava* and *Eisenia bicyclis* have been intensively investigated for their human beneficial bioactive components including phlorotannins, polysaccharides, pyropheophytin, tripeptides, and oxylipin and also the beneficial bioactivities that include anti-inflammation, inhibition of hyaluronidase activity, and antidiabetic activity (Shibata et al. 2002; Okada et al. 2004). With the extensively available new strategies of pharmacognosy, it is possible to evaluate the marine algal polysaccharides' and phlorotannins' ability in reducing the risk of pruritic inflammation and controlling AD. In this chapter, an attempt has been made to discuss the potential of marine algal polysaccharides and phlorotannins

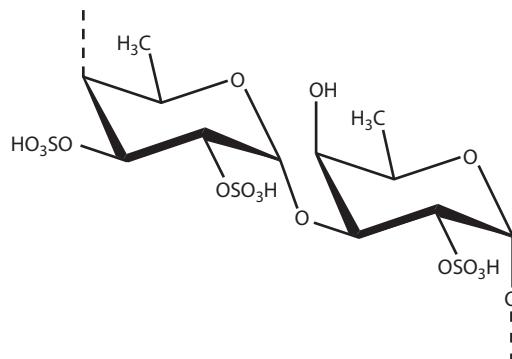
in inhibiting the overexpression of IgE and their possible pharmacognosy applicability as drug leads in the treatment of AD.

## 25.2 PHARMACOGNOSY PROSPECTS OF MARINE ALGAL POLYSACCHARIDES IN THE TREATMENT OF AD

Marine algae are known to produce different polysaccharides including alginates, laminarans, and fucoidans. They usually contain large proportions of L-fucose and sulfate, together with minor amounts of other sugars such as xylose, galactose, mannose, and glucuronic acid. These algal polysaccharides have been attributed with many biological activities such as anticoagulant, antithrombotic, antitumoral, and antiviral activities. In particular, fucoidans (Figure 25.1) from marine algae have been reported to exhibit outstanding biological activities that aid human health. Fucoidans are sulfated polysaccharides that are exclusively found in the cell wall of seaweeds. This polysaccharide ingredient is composed a polymer of  $\alpha$ -D-fucose with sulfate groups on some of the fucose residues at the four positions (Patankar et al. 1993). Scientific reports clearly suggest that marine algal-derived fucoidans inhibit the low-density lipoprotein uptake by macrophage through the prevention of their binding to the scavenger receptor (Nishikawa, Arai, and Inoue 1990).

Based on the scientific fact that inhibition of IgE production could serve as a major therapeutic breakthrough for treating AD, a research group reports that fucoidan inhibited the production of IgE and *Ce* germline transcription in murine B cells induced by IL-4 (100 ng/mL) and anti-CD40 antibodies (10  $\mu$ g/mL). They also suggested that there was a significant effect on the production of IgE. Their results suggest that fucoidan inhibited IgE production by preventing the nuclear factor (NF)- $\kappa$ B p52-mediated pathways activated by CD40 (Oomizu et al. 2006). An enormous scientific endeavor has been invested for the screening of natural anti-inflammatory compounds from terrestrial resources. Interestingly, many terrestrial plant sources have served as antiallergic compounds that could cure skin-related inflammations. One such discovery was Konjac glucomannan, which is a highly viscous, water-soluble, and high-molecular-weight polysaccharide, consisting of a single chain of D-Glucose and D-Mannose joined by  $\beta$ -1, 4-linkage with some branches (Kato et al. 1998). This polysaccharide possesses high quantities of Konjac and is obtained from the tubers of the potato-like plant *Amorphophallus konjac*. When NC/Nga mice (commonly used experiment model for AD) were administered orally with pulverized Konjac glucomannan, it not only reduced the overproduction of IgE but also prevented the elevation of plasma IgE as well as the development of eczematous lesions and scratching behavior (Onishi et al. 2005).

The potential of fucoidans in reducing the concentration of IL-4 and IL-13 in bronchoalveolar lavage fluid and also the inhibition of IgE in ovalbumin (OVA)-induced mouse airway hypersensitivity was reported by Maruyama et al. They have extracted mekabu fucoidan from marine alga *Undaria pinnatifida* and demonstrated its role in augmentation of type 1 Th1 cell response



**FIGURE 25.1** Chemical structure of fucoidan unit.

in normal BALB/c mice and the suppression of production of Th2 cytokines that have relieved pulmonary inflammation in the experimental animals. This suggest the possible candidature of marine-derived fucoidan as a potential lead in the treatment of inflammatory skin disorders like AD (Maruyama et al. 2005).

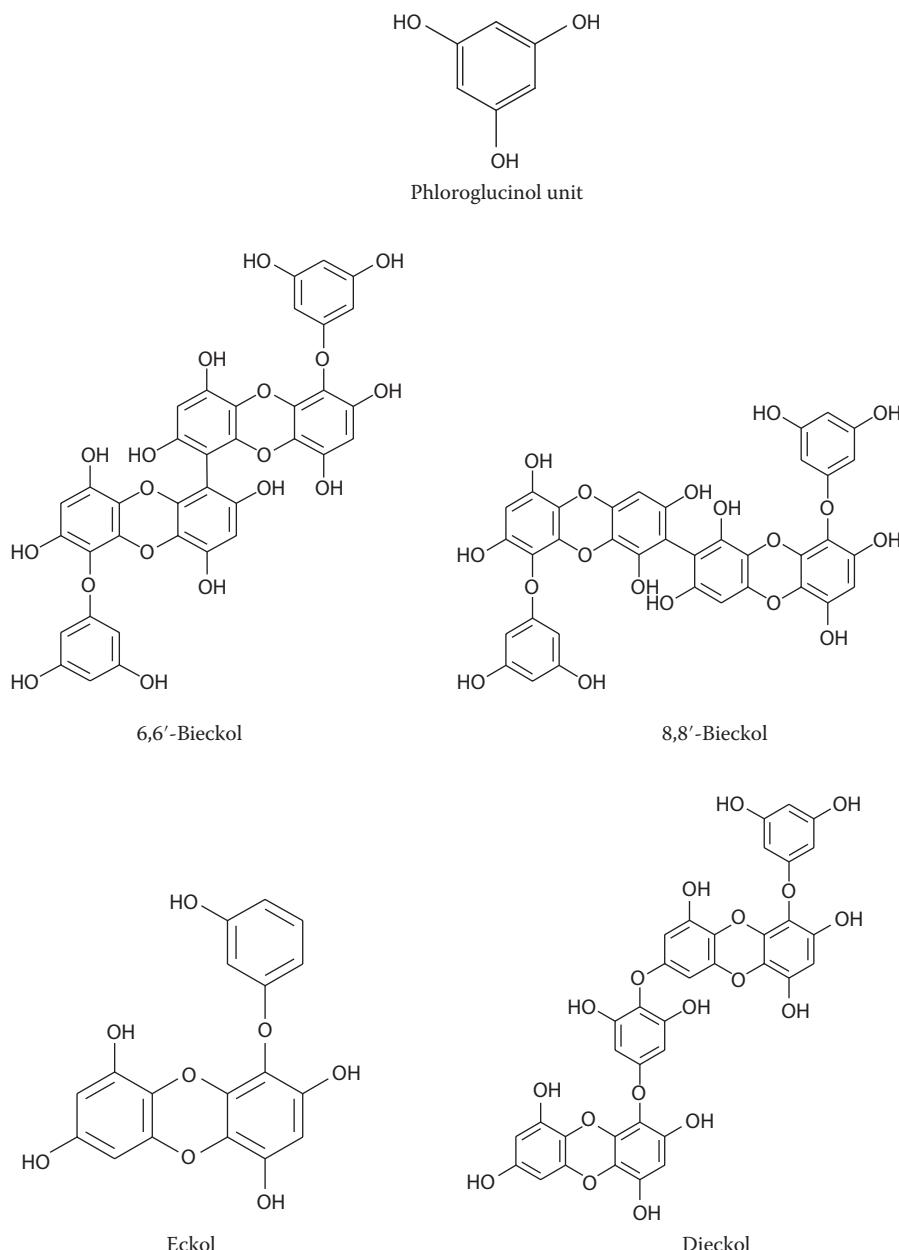
Fucoidan is reported to have the ability to cure the skin burns caused by ultraviolet (UV) radiations. In one investigation, the inhibition of UV-B-induced matrix metalloproteinase-1 (MMP-1) expression in pretreated human skin fibroblast (HS68) cells with fucoidan is reported. Their results suggest that the fucoidans have effectively inhibited the extracellular signal-regulated kinase (ERK) activation. Interestingly, the suppression of the MMP-1 mRNA expression was also reported, thus suggesting it as a potential agent for the prevention and treatment of skin photoaging (Moon et al. 2008). Hence proving the application of sulfated polysaccharides in the field of pharmacognosy to design natural drugs for the treatment of AD.

Apart from fucoidans, alginic acid derived from marine algae has been reported to exhibit several biological effects. They are also reported to reduce skin inflammation by modulating the immune responses. As it is understood that IgE and Th2 immune responses are interlinked that majorly contribute for AD, inhibition of these responses could be a better approach for treating skin inflammations. The ability and mechanism of alginic acid oligosaccharide (ALGO), an oligosaccharide obtained from natural edible polysaccharide, for suppressing Th2 responses was examined by Yoshida et al. The treatment of ALGO in the lymph node cells obtained from  $\beta$ -lactoglobulin ( $\beta$ -LG)-primed BALB/c mice has resulted in significant suppression of antigen-induced Th2 development by inducing IL-12 production and also IgE suppression (Yoshida et al. 2004).

### 25.3 PHARMACOGNOSY PROSPECTS OF PHLOROGLUCINOL DERIVATIVES IN THE TREATMENT OF AD

Marine macroalgae render enormous biologically important and beneficial ingredients that aid in the betterment of human health. In recent days, the isolation and characterization of the biologically active components from sea algae has gained a lot of attention from various research groups across the world. The epidemiological and clinical studies have revealed that consumption of plant-derived foods and drinks could reduce the risk of oxidative damage-related diseases such as inflammation, aging, and other lifestyle diseases (Thomas and Kim 2011). The biological effects of the marine algae are thought to be possible because of the abundant presence of polyphenolic compounds. The marine algal phloroglucinol derivatives otherwise known as phlorotannins are exclusively confined to marine brown algae. A few marine algal-derived phlorotannins are shown in [Figure 25.2](#). These phlorotannins are suggested to be formed by the polymerization of phloroglucinol (1,3,5-trihydroxybenzene) monomer units and biosynthesized through the acetate-malonate pathway, also known as the polyketide pathway. The phlorotannins are highly hydrophilic components with a wide range of molecular sizes ranging between 126 kDa and 650 kDa and are mostly found in brown algae (Ragan and Glombitza 1986; Singh and Bharate 2006). Phlorotannins that have possible potential as natural drugs for treatment of AD are listed in [Table 25.1](#).

Because of the rich content of polyphenolic compounds in *E. cava*, it is used as a food ingredient and folk medicine against allergic diseases in Asian countries, especially in Korea. Crude extract from *E. cava* was investigated for its antiallergic activity by Le et al. In their investigation, isolation of the two main bioactive phlorotannin derivatives 6,6'-bieckol and 1-(3',5'-dihydroxyphenoxy)-7-(2'',4'',6-trihydroxyphenoxy)-2,4,9-trihydroxydibenzo-1,4-dioxin for the first time from this genus, together with phloroglucinol and dieckol, resulted. These derivatives were assessed by histamine release assay on human basophilic leukemia (KU812) and Rat basophilic leukemia (RBL-2H3) cultured cell lines respectively. Furthermore, flow cytometric analysis indicated that the potential antiallergic mechanism is due to the suppression of binding activity between IgE and Fc $\epsilon$ RI. This investigation suggests phloroglucinol derivatives as potential drug leads for cosmetic and pharmacognosy fields (Le et al. 2009).



**FIGURE 25.2** Marine algal phloroglucinol and its derivatives.

Normally, the allergic effects result due to the depolymerization of hyaluronic acid (HA) in the extracellular matrix of the connective tissues by hyaluronidase. Hence, hyaluronidase is a potential target for inhibiting allergic responses in human. The anti-hyaluronidase effects of phlorotannins from brown algae *E. bicyclis* and *Ecklonia kurome* were tested *in vitro*. The phlorotannins, namely, eckol (a trimer), phlorofucoxanthin A (a pentamer), dieckol, and 8,8'-bieckol (hexamers), have exhibited effective anti-hyaluronidase effects. Moreover, it was noticed that crude phlorotannins had a stronger inhibitory effect on hyaluronidase than well-known inhibitors such as catechins and sodium cromoglycate. In particular, 8,8'-bieckol was reported to exhibit a stronger hyaluronidase

**TABLE 25.1**  
**Phloroglucinol Derivatives and Their Antiskin Allergic Effects**

Pharmacognosy Application	Phlorotannin and Brown Algal Species	References	
Inhibitory effect on histamine release	6,6'-bieckol, <i>E. cava</i> Methanolic extracts, <i>E. arborea</i>	Le et al. (2009) Sugiura, Matsuda, et al. (2006)	
	Eckol 6,6'-bieckol 6,8-bieckol 8,8-bieckol Phlorofucofuroeckol A Phlorofucofuroeckol B	Sugiura et al. (2007)	
Inhibitory effect on hyaluronidase	Eckol Phlorofucofuroeckol A and dieckol 8,8'-bieckol	<i>E. cava</i> <i>E. arborea</i> <i>E. bicyclis</i> and <i>E. kurome</i>	Shibata et al. (2002)
Inhibitory effect on FceRI overexpression	Methanolic extracts, <i>E. cava</i>	Shim et al. (2009)	
Inhibitory effect on overexpression of IgE	Phlorofucofuroeckol A, <i>E. arborea</i>	Sugiura, Matsuda, et al. (2006)	
Inhibitory effect on MMP-1 expression	Eckol Dieckol	<i>E. stolonifera</i>	Joe et al. (2006)

inhibitory effects with an  $IC_{50}$  value of 40  $\mu\text{M}$ , which was about seven times stronger than that of DSCG (a major and active component of antiallergic drugs) (Shibata et al. 2002).

Atopy and skin allergies are often caused due to the increased systemic exposure to histamine. Systemic exposure to histamine is determined by a balance between the processes of release from mast cells and basophils, which are considered as precursor cells, and inactivation via endogenous metabolic pathways. A key pathogenic determinant of AD may reside with a disruption in the balance among those physiologic processes determining systemic exposure. Hence, inhibition of excess production of histamine might be a better strategy for the effective control of AD (Kennedy et al. 2008). The methanolic extracts from marine brown alga *Eisenia arborea* have shown  $74.3 \pm 33.8\%$  inhibition of histamine release from rat basophile leukemia cells (RBL-2H3) sensitized with anti-dinitrophenyl (anti-DNP) IgE and stimulated with Dinitrophenylated-bovine serum albumin (DNP-BSA). These observations clearly suggest that methanol extracts that are usually rich in phlorotannins exhibit the capability to reduce the histamine release and treat histamine-related inflammatory diseases that include AD (Sugiura, Matsuda, et al. 2006). This suggests the importance of polyphenolic derivatives from marine algae as potential anti-inflammatory substances that could be useful leads for drug development in the pharmacognosy field.

FceRI, a high-affinity receptor for IgE, is the target for basophils and mast cells on the cell surface, and they actively elicit allergic-related reactions. Methanolic extracts from *E. cava* were assessed for their inhibitory effects on the expression levels of FceRI in human basophilic KU812F cells. In this investigation, it was reported that *E. cava* methanolic extracts have lowered the cell surface expression of FceRI in a dose-dependent manner. The extract was also capable of reducing the binding between IgE or serum IgE and cell surface FceRI. Reverse transcription polymerase chain reaction (RT-PCR) analysis revealed that Ecklonia cava (EC) extract reduced the mRNA expression of total cellular FceRI  $\alpha$ -chain. FceRI-mediated release of histamine was

also greatly reduced by these extracts in a dose-dependent manner. Therefore, these results suggest that methanolic fractions of brown algae might exert their antiallergic activity through negative regulation of FceRI expression and a decrease in histamine release (Shim et al. 2009). On the contrary, the histamine release inhibitory effects in rat basophile leukemia cells (RBL-2H3) sensitized with anti-DNP IgE and stimulated with DNP-BSA by 80% methanolic extracts from 41 sea algae were assessed along with one seagrass methanolic extract. Out of them, methanolic extracts from seven brown algae alone have exhibited histamine release inhibitory activity from RBL cells. The cytotoxic effects of the seven brown algal extracts were investigated by Trypan blue staining; only *E. arborea* and *Sargassum thunbergii* did not show cytotoxic effects. This suggests *E. arborea* and *S. thunbergii* may contain compounds that have antiallergic effects without inducing cell death (Sugiura, Takeuchi, et al. 2006). Eckol, 6,6'-bieckol, 6,8'-bieckol, phlorofucofuroeckol-A, and phlorofucofuroeckol-B obtained from *E. arborea* have been reported to exhibit activities similar to or greater than the typical inhibitor for allergies, epigallocatechin gallate. Phlorofucofuroeckol-B showed the greatest activity among the tested phlorotannins at 2.8 times greater than epigallocatechin gallate (Sugiura et al. 2007).

*In vitro* investigations of phlorotannin named phlorofucofuroeckol-B isolated from *E. arborea* studies on rat basophile leukemia cells (RBL)-2H3 cells confirmed that this phlorotannin is capable of inhibiting histamine release assuring the antiallergic property (Sugiura, Matsuda, et al. 2006). According to these investigations, it is understood that phloroglucinol derivatives possess the ability to reduce the production of IgE and other inflammatory responses. Thus, it is suggested that in more advanced molecular studies, animal model studies are highly recommended to establish phlorotannins as potential pharmacognosy leads for the treatment of AD. Moreover, there are several reports confirming the ability of phlorotannins in skin disease treatments. For example, phlorotannins eckol and dieckol isolated from *Ecklonia stolonifera* have attenuated the expression of (MMP-1) expression in human dermal fibroblasts. These findings reveal that the inhibition of MMP-1 (which is an interstitial collagenase and mainly responsible for the degradation of dermal collagen in human skin aging process) expression by *E. stolonifera*-derived phlorotannins was in correlation with the inhibition of both NF-κB and activator protein-1 (AP-1) reporter activity (Joe et al. 2006).

## 25.4 CONCLUSIONS AND FURTHER PROSPECTS

The synthetic medical practices available for the treatment of AD are not only expensive but also proven to develop few undesirable side effects. The need for naturally occurring medicinal substances for the treatment of AD is currently a popular area of research. As there is an outstanding development in the fields of biotechnology and cheminformatics, there seems to be a bright chance for the pharmacognosy to be developed more for the screening of natural drugs or drug-like substances from natural sources. As a matter of fact, to date, there have been many formulations from terrestrial organisms for the treatment of AD. However, the vast bioecological diversity deep below the sea offers a wide range of organisms for the screening of drug-like substances. The proper application and utilization of pharmacognosy principles on the exploitation of marine algal components would assure the establishment of effective drug leads for the cure of pruritic skin inflammation, AD. Moreover, with the availability of biotechnology and bioinformatics, soon the field of marine pharmacognosy will unravel many more potential natural drugs from marine algae for the complete management of skin inflammations.

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# 26 Antidiabetic Effect of Dieckol, a Marine Polyphenol, and Its Mechanisms of Blood Glucose Regulation

Seung-Hong Lee and You-Jin Jeon

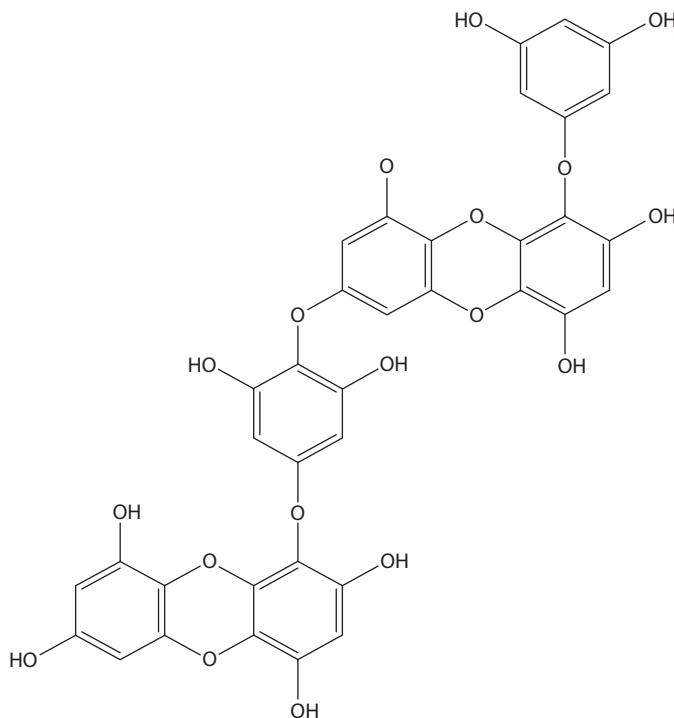
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## 26.1 INTRODUCTION

Polyphenolic compounds are one of the most common classes of secondary metabolites found in terrestrial plants and marine algae. There are fundamental differences in the chemical structures of polyphenols in both terrestrial and marine plants (Shibata et al. 2002). Phlorotannins, algal polyphenols consisting of phloroglucinol units linked to one another in various ways, occur broadly among the brown and red algae (Singh and Bharate 2006). Several studies have demonstrated the various biological benefits associated with phlorotannins, including antioxidant, anticoagulant, antibacterial, anti-inflammatory, and anticancer activities (Shibata et al. 2002; Mayer and Hamann 2005; Heo et al. 2008; Kong et al. 2009).

The brown alga *Ecklonia cava* is plentifully produced on Jeju Island in Korea. It is popular in Korea and Japan as a food ingredient, as a supplement of animal feed and fertilizers, and as a medicine. The total polyphenolic compounds (phlorotannins) in *E. cava* are richer than in other brown algae (Heo et al. 2005). Phlorotannin components of *E. cava* include phenolic secondary metabolites such as eckol (a closed-chain trimer of phloroglucinol), 6,6'-bieckol (a hexamer), dieckol (a hexamer) (Figure 26.1), phlorofucofuroeckol (a pentamer), and triphlorethol-A that are influential for biological activities (Kang et al. 2005a,b; Ahn et al. 2007). Among these phlorotannins, dieckol is one of the major and active compounds. Its attributes include antioxidant activity, antiallergic activity, inhibition of human immunodeficiency virus-1 reverse transcriptase, and inhibition of expression of matrix metalloproteinase-1 (MMP-1) (Ahn et al. 2004; Joe et al. 2006; Ahn et al. 2007; Le et al. 2008).



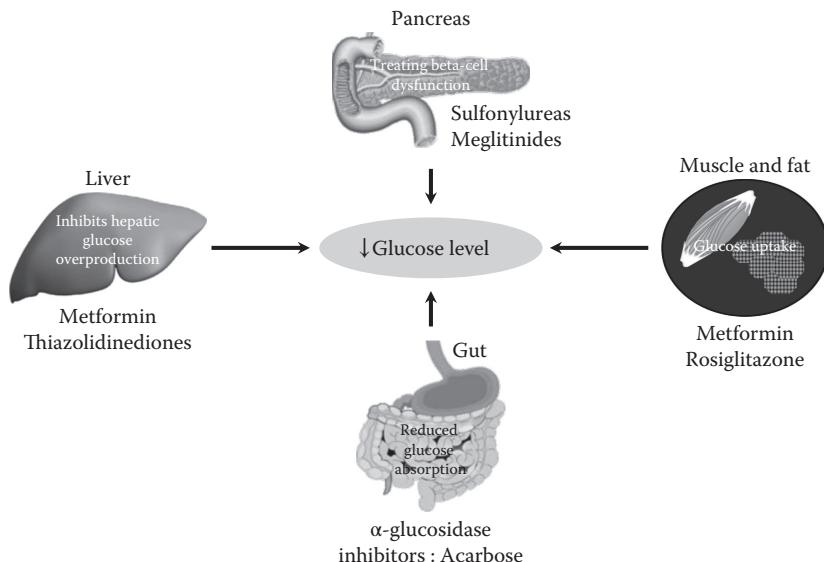
**FIGURE 26.1** Chemical structure of dieckol isolated from *E. cava*.

In this chapter, the antidiabetic effect of dieckol isolated from *E. cava* and its mechanisms of blood glucose regulation have been reviewed.

## 26.2 OVERVIEW OF DIABETES

Diabetes mellitus is characterized by abnormal metabolism of glucose, due in part to resistance to the action of insulin in peripheral tissues. The characteristic symptoms are polyuria, polydip-sia, and polyphagia. Diabetes mellitus is a serious chronic disease that is developing prominence amidst an increasingly obese and aging world population. Diabetes mellitus is a complex disorder that is characterized by hyperglycemia. It is largely classified into insulin-dependent diabetes mellitus (type 1 diabetes) and non-insulin-dependent diabetes mellitus (type 2 diabetes). In particular, type 2 diabetes is an increasing worldwide health problem and is the most prevalent form of diabetes (Zimmet, Alberti, and Shaw 2001). Hyperglycemia plays an important role in the development of type 2 diabetes and complications associated with the disease such as microvascular and macrovascular diseases (Baron 1998). Therefore, effective control of blood glucose levels is the key to preventing or reversing diabetic complications and improving the quality of life for diabetic patients (DeFronzo 1999).

Currently available therapies for type 2 diabetes include insulin and various oral antidiabetic drugs such as sulfonylureas, metformin, rosiglitazone (RG),  $\alpha$ -glucosidase inhibitors, and thiazolidinediones (Figure 26.2). However, these therapies have either limited efficacy or significant mechanism-based side effects like hypoglycemia, flatulence, and body weight gain of enhancement of gastrointestinal problems. Therefore, recently, there has been a growing interest in alternative therapies and in the therapeutic use of natural products for diabetes, especially those derived from herbs (Chang et al. 2006; Jung et al. 2007). This is because plant sources are usually considered to be less toxic with fewer side effects than synthetic ones.



**FIGURE 26.2** Major targeted sites of oral antidiabetic drugs.

### 26.3 POSTPRANDIAL HYPERGLYCEMIA-LOWERING EFFECT

A sudden increase in blood glucose levels, which causes hyperglycemia in type 2 diabetes patients, occurs as the result of the hydrolysis of starch by pancreatic  $\alpha$ -amylase and glucose uptake due to intestinal  $\alpha$ -glucosidases (Gray 1995). An effective strategy for the management of type 2 diabetes patients involved the profound inhibition of intestinal  $\alpha$ -glucosidases and the mild inhibition of pancreatic  $\alpha$ -amylase (Krentz and Bailey 2005). Several natural resources have been evaluated for their ability to suppress the production of glucose from carbohydrates in the gut or glucose absorption from the intestine (Matsui et al. 2007).  $\alpha$ -Glucosidase is one of the glucosidases located within the brush-border surface membranes of intestinal cells and is a key enzyme in carbohydrate digestion (Lebovitz 1997). Similarly,  $\alpha$ -amylase catalyzes the hydrolysis of  $\alpha$ -1,4-glucosidic linkages of starch, glycogen, and a variety of oligosaccharides, and  $\alpha$ -glucosidase further degrades the disaccharides into simpler sugars, which are readily available for intestinal absorption. The inhibition of their activity in the human digestive tract is regarded as an effective method for the control of diabetes by diminishing the absorption of glucose decomposed from starch by these enzymes (Hara and Honda 1990). Therefore, effective and nontoxic inhibitors of  $\alpha$ -glucosidase and  $\alpha$ -amylase have long been sought.

Lee, Park et al. (2010) evaluated the inhibitory effects of dieckol against  $\alpha$ -glucosidase and  $\alpha$ -amylase to elucidate the possible use of dieckol as an anti-hyperglycemic agent. Dieckol exhibited stronger inhibitory activity against both  $\alpha$ -glucosidase and  $\alpha$ -amylase than that of the commercial carbohydrate digestive enzyme inhibitor, acarbose, which evidenced no cytotoxicity (Table 26.1). Polyphenolic compounds, such as tannins from terrestrial plants and phlorotannins from marine algae, associate with various proteins to form complexes. The results of several recent studies have demonstrated that the hydroxyl groups in polyphenolic compounds may, therefore, perform a crucial function in promoting inhibitory activity (Stern et al. 1996; Kim et al. 2008). Dieckol, a type of phlorotannin, was the marine algal polyphenolic compound isolated from *E. cava*. Thus, dieckol should bind to the active or binding sites of the enzymes, resulting in the inhibition of enzyme activity. Also, Lee, Park et al. (2010) investigated the anti-hyperglycemic effect of dieckol in streptozotocin-induced diabetic and normal mice after consuming starch. The increase in postprandial blood glucose levels was suppressed significantly in both streptozotocin-induced diabetic

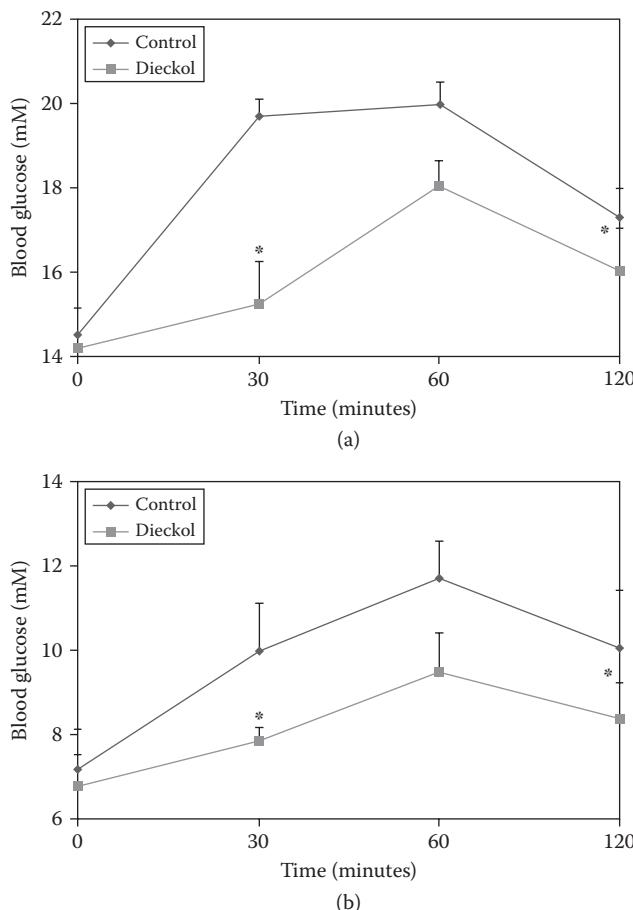
**TABLE 26.1****IC<sub>50</sub> Values of Inhibitory Effect of Dieckol on  $\alpha$ -Glucosidase and  $\alpha$ -Amylase**

Sample	IC <sub>50</sub> (mM) <sup>a</sup>	
	$\alpha$ -Glucosidase	$\alpha$ -Amylase
Acarbose	1.05 ± 0.03	1.09 ± 0.07
Dieckol	0.24 ± 0.05*	0.66 ± 0.06*

Source: From Lee et al., *Food Chem. Toxicol.*, 48, 2633–2637, 2010a. With permission.

<sup>a</sup> IC<sub>50</sub> value is the concentration of sample required for 50% inhibition. Each value is expressed as mean ± SD in triplicate experiments.

\* Significantly different from control at  $p < .05$ .



**FIGURE 26.3** Blood glucose levels after the administration of dieckol in streptozotocin-induced diabetic mice (a) and normal mice (b). Control (distilled water) and dieckol (100 mg/kg) were coadministered orally with starch (2 g/kg). Each value is expressed as mean ± SD for seven mice ( $n = 28$ ). A significant difference from the controls was identified at \* $p < .05$  as analyzed by Duncan's multiple range test. (From Lee et al., *Food Chem. Toxicol.*, 48, 2633–2637, 2010a.)

and normal mice that were treated with dieckol ([Figure 26.3](#)). This study described that dieckol may delay the absorption of dietary carbohydrates, resulting in the suppression of an increase in postprandial blood glucose level.

These results suggest that dieckol may prove useful as an effective natural antidiabetic compound.

## 26.4 EFFECT OF GLUCOSE UPTAKE IN SKELETAL MUSCLE

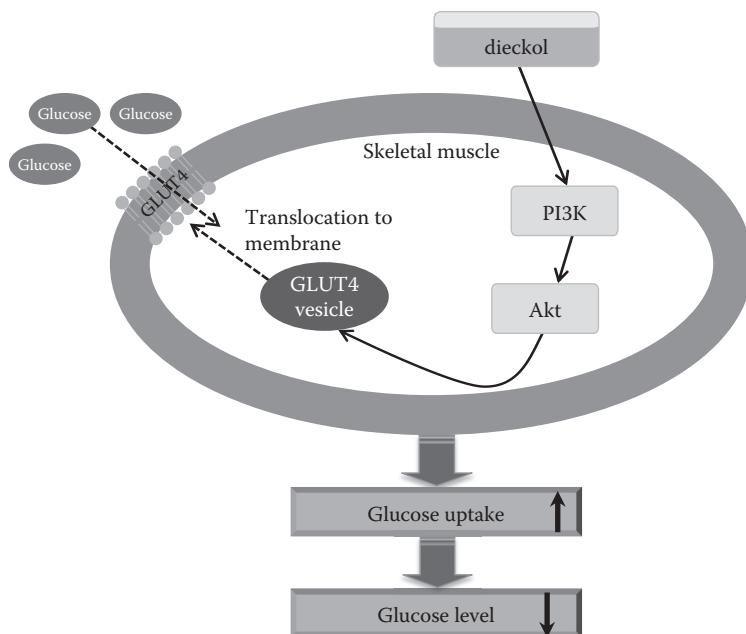
Skeletal muscle plays a major role in the regulation of energy balance (Ozcan et al. 2006) and is the primary tissue for glucose uptake and disposal. Indeed, glucose uptake by skeletal muscle accounts for >70% of the glucose removal from serum in humans (Cormont et al. 1993). Insulin-stimulated glucose uptake in skeletal muscle is critical for reducing blood glucose levels. It is therefore considered an important target tissue for type 2 diabetes (Sheetz and King 2002). Therefore, the discovery of novel compounds that stimulate glucose uptake in skeletal muscle could provide new options for the treatment of insulin resistance and type 2 diabetes.

Guan (2011) investigated the effect of dieckol on glucose uptake in muscle cells. Results of the glucose uptake assay revealed dieckol-induced increases of glucose uptake in differentiated muscle cells that were dose-dependent, demonstrating its metabolic effects on skeletal muscle cells.

In skeletal muscle, glucose uptake can be activated by at least two major mechanisms. The first is an insulin signaling pathway through phosphatidylinositol-3 kinase (PI3-K) and Akt activity. Activation of PI3-K and Akt promotes glucose transporter 4 (Glut4) translocation from an intracellular pool to the plasma membrane (Taniguchi, Emanuelli, and Kahn 2006; Dugani et al. 2008). The other mechanism involves the heterotrimeric metabolite-sensing protein kinase, 5' adenosine monophosphate (AMP)-activated protein kinase (AMPK). AMPK plays a major role in energy homeostasis in ATP-depleting metabolic states such as ischemia, hypoxia, heart shock, and oxidative stress (Harder et al. 2001; Raj and Dentino 2002). Once activated under such conditions, it accelerates the ATP-generating catabolic pathway involving glucose uptake and fatty acid oxidation through the direct regulation of key metabolic enzymes (Sheetz and King 2002). Recent studies report that AMPK serves as a key metabolic sensor for the cellular regulation of insulin-independent glucose uptake and glycogen metabolism (Ozcan et al. 2004; Hotamisligil, 2006). Skeletal muscle AMPK is activated by exercise and numerous compounds, including metformin (Zou et al. 2004) and thiazolidinediones (Konrad et al. 2005), resulting in increased glucose uptake.

In order to confirm that dieckol exerts its action on glucose uptake through the insulin signaling pathway or AMPK, further experiments were conducted to determine the involvement of insulin or the AMPK signaling pathway in this stimulatory effect (Guan, 2011). Dieckol stimulated Akt phosphorylation, and this was abolished by pretreatment with wortmannin, highly selective inhibitors. This study also tried to find out the association of dieckol with the AMPK pathway in the course of glucose uptake, but no relationship was found. This result suggests that Akt may play a crucial role in dieckol-stimulated glucose uptake.

Furthermore, Glut4 translocation to the plasma membrane of L6 myotubes was markedly increased by treatment with dieckol. In addition, the increased translocation of Glut4 to the plasma membrane of dieckol-treated L6 myotubes was almost completely abolished by wortmannin pretreatment. Therefore, these results suggest that the dieckol-stimulated increase in Glut4 translocation to the plasma membrane possibly occurs through the activation of the PI3-K/Akt signaling pathway ([Figure 26.4](#)). Hyperglycemic-hyperinsulinemic clamp analyses of human type 2 diabetic patients show that insulin resistance in muscle is caused by a defect in glucose transport. The principle glucose transporter in muscle is Glut4, which is the primary mediator of both basal and insulin-stimulated glucose transport. Thus, the effects of dieckol



**FIGURE 26.4** Glucose uptake mechanism of dieckol in skeletal muscle cells.

in activating Glut4 translocation are reflected in an increased ability of the muscle cells to transport glucose.

## 26.5 EFFECTS OF GLUCOSE METABOLISM IN *db/db* MICE, A MODEL OF TYPE 2 DIABETES MELLITUS

The antidiabetic effects of dieckol-rich extract (dieckol-RE) of *E. cava* for 6 weeks was investigated on C57BL/KsJ-*db/db* (*db/db*), type 2 diabetic mice, and the efficacy was compared with an oral antidiabetic agent, RG (Lee et al. 2012). The *db/db* mice were randomly divided into three groups. Thereafter, the control group of *db/db* mice were fed with a standard semisynthetic diet (AIN-93G), while the other two groups of *db/db* mice were fed with a standard semisynthetic diet containing RG (Avandia; GlaxoSmithKline, United Kingdom; 0.005 g/100 g diet) or dieckol-RE (0.5 g/100 g diet) for 6 weeks. This study observed that the supplementation of dieckol-RE can improve blood glucose levels and impaired glucose tolerance in type 2 diabetic mice following supplementation for 6 weeks. In addition, the dieckol-RE supplements significantly lowered the glycosylated hemoglobin level, a useful parameter in the monitoring of long-term blood glucose, compared to the control *db/db* mice (Table 26.2). Glycosylated hemoglobin is also widely used in screening for diabetes and glucose intolerance (Shafir 1992). These findings suggest that the supplementation of dieckol-RE improve glucose homeostasis in type 2 diabetic mice.

Abnormal hepatic glucose metabolism is a major symptom of type 2 diabetes, and it contributes to postprandial hyperglycemia (Basu et al. 2001). Hepatic glucokinase (GK) plays a major role in controlling blood glucose homeostasis and its activity is low in diabetes (Postic et al. 1999). Glucose-6-phosphatase (G6Pase) is a key enzyme controlling hepatic gluconeogenesis and glucose output in liver and is normally suppressed by the action of insulin (Nordlie, Bode, and

**TABLE 26.2**

**The Effects of the Supplementation of AG-Dieckol for 6 Weeks on the Levels of Blood Glucose, Glycosylated Hemoglobin ( $HbA_{1c}$ ), Plasma Insulin, and Insulin Resistance in C57BL/KsJ-*db/db* Mice<sup>d</sup>**

	<i>db/db</i> <sup>e</sup>	<i>db/db-RG</i> <sup>f</sup>	<i>db/db</i> -Dieckol-RE <sup>g</sup>
Glucose (mmol/L)			
Initial	8.64 ± 0.84 <sup>a</sup>	8.19 ± 1.73 <sup>a</sup>	8.4 ± 0.74 <sup>a</sup>
Final	18.09 ± 3.8 <sup>a</sup>	5.18 ± 0.49 <sup>c</sup>	10.50 ± 3.48 <sup>b</sup>
$HbA_{1c}$ (%)	13.37 ± 2.72 <sup>a</sup>	5.43 ± 0.88 <sup>c</sup>	7.51 ± 0.38 <sup>b</sup>
Plasma insulin (pmol/L)	115.56 ± 9.03 <sup>a</sup>	37.92 ± 3.61 <sup>c</sup>	52.36 ± 3.61 <sup>b</sup>
HOMA-IR	26.42 ± 0.05 <sup>a</sup>	5.08 ± 0.02 <sup>c</sup>	11.97 ± 0.02 <sup>b</sup>

*Source:* From Lee et al., *Food Chem. Toxicol.*, 50, 575–582, 2012. With permission.

*Note:* HOMA-IR, homeostatic index of insulin resistance.

<sup>a–c</sup> Means in the same row not sharing a common superscript are significantly different between groups ( $p < .05$ ).

<sup>d</sup> Means ± SE ( $n = 7$ ).

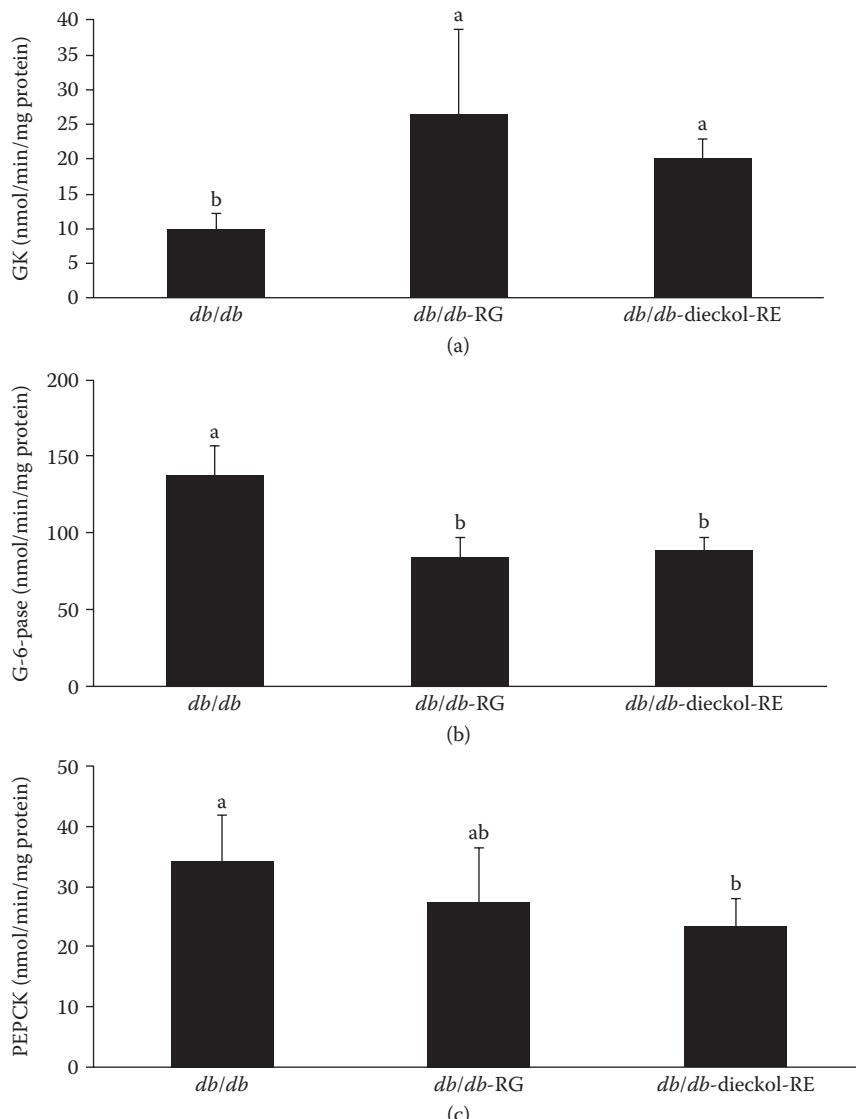
<sup>e</sup> C57BL/KsJ-*db/db* mice.

<sup>f</sup> C57BL/KsJ-*db/db* mice supplemented with rosiglitazone.

<sup>g</sup> C57BL/KsJ-*db/db* mice supplemented with dieckol-RE.

Foster 1993). Due to their strategic positions in liver glucose metabolism, both of these enzymes are supposed to be the target of important regulatory mechanisms of hepatic glucose production (Mithieux 1997). Lee et al. (2012) reported that hepatic GK activity was significantly higher in the dieckol-RE-supplemented *db/db* mice than in the control *db/db* mice. Furthermore, when the *db/db* mice were supplemented with dieckol-RE, hepatic G6Pase activity was significantly lowered, compared to the control *db/db* mice, thereby reducing hepatic glucose production. Among glucose-regulating genes, an enhanced expression of hepatic phosphoenolpyruvate carboxykinase (PEPCK) gene has been identified in most forms of diabetes and contributes to an increased hepatic glucose output (Davies et al. 2001). Dieckol-RE supplements lowered hepatic PEPCK activity compared to the control *db/db* mice. RG, an insulin sensitizer, also lowered G6Pase and PEPCK activity, whereas it increased the GK activity in the liver of the *db/db* mice (Lee et al. 2012), (Figure 26.5). Thus, the antidiabetic effects of the dieckol-RE supplements seemed to be mediated through stimulating GK activity and inhibiting G6Pase and PEPCK activity in the liver of *db/db* mice.

In general, the *db/db* mice exhibit an initial phase of hyperinsulinemia, and insulinopenia can be progressively developed with age, a feature commonly observed in the late stages of type 2 diabetes (Fujiwara, Wada, and Fukuda 1991). The study of Lee et al. (2012) also observed that the plasma insulin levels of the dieckol-RE-supplemented *db/db* mice were significantly lower than the control *db/db* mice and they were a little higher than RG-supplemented *db/db* mice (Table 26.2). RG has been reported to enhance insulin action, thereby improving glucose tolerance and reducing hyperinsulinemia in animals and humans with type 2 diabetes (Patel et al. 1997). Simple indexes of insulin resistance, namely, HOMA-IR, were calculated using fasting glucose and insulin levels. HOMA-IR is an index of insulin resistance whose value should increase with increasing insulin resistance. The HOMA-IR was significantly lowered in the dieckol-RE group mice as compared to the control group mice (Lee et al. 2012). Accordingly, the concomitant decrease in



**FIGURE 26.5** The effects of the supplementation of dieckol-RE for 6 weeks on the hepatic glucokinase (a), glucose-6-phosphatase (b), and phosphoenolpyruvate carboxykinase (c) activities in C57BL/KsJ-*db/db* mice. Values are means  $\pm$  SE,  $n = 7$ . <sup>a-b</sup>Means not sharing a common letter are significantly different between groups ( $p < .05$ ). *db/db-RG*, *db/db* mice supplemented with rosiglitazone; *db/db-AG-dieckol*, *db/db* mice supplemented with dieckol-RE. (From Lee et al., *Food Chem. Toxicol.*, 50, 575–582, 2012.)

plasma insulin and the improvement of glucose tolerance suggest that dieckol-RE may enhance insulin sensitivity.

These results suggest that supplementation of dieckol-RE exerts a beneficial effect on hepatic glucose metabolism partly through improvement of insulin sensitivity in type 2 diabetic *db/db* mice.

## 26.6 PROTECTIVE EFFECT OF DIABETES COMPLICATION

Several recent studies have demonstrated that hyperglycemia can cause glucose to undergo autooxidation to generate intermediates that lead to the formation of reactive oxygen species (ROS), nitric oxide (NO), peroxynitrite ( $\text{ONOO}^-$ ), and advanced glycation end products (AGE), which cause various complications of diabetes such as nephropathy, retinopathy, and neuropathy. Moreover, hyperglycemia is thought to be an important regulator of vascular lesion development. Hyperglycemia-induced endothelial cell dysfunctions accelerate the process of atherosclerotic complications. Vascular disorders are thought to participate in the pathogenesis of atherosclerosis. Lee, Han et al. (2010) investigated the protective effects of dieckol against high glucose-induced oxidative stress using human umbilical vein endothelial cells (HUVECs). In this study, HUVEC damage occurred as a result of high glucose-induced overproduction of ROS, lipid peroxidation products, and NO. In addition, high glucose levels induced the overexpression of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) and enhanced nuclear factor (NF)- $\kappa$ B activation in HUVECs. Dieckol protected cells against the oxidative stress by reducing ROS generation and inhibiting production of iNOS and COX-2 and by reducing NF- $\kappa$ B activation. Taken together, these results suggest that dieckol may contribute to the prevention of oxidative stress-related diseases including diabetes.

## 26.7 CONCLUSIONS

Dieckol isolated from *E. cava*, a brown alga, exhibited multiple antidiabetic effects. Firstly, dieckol inhibits  $\alpha$ -glucosidase and  $\alpha$ -amylase activities and alleviates postprandial hyperglycemia in streptozotocin-induced diabetic mice. Secondly, Akt activation was involved in mediating the effects of dieckol on glucose transport activation. Moreover, supplementation of dieckol-RE for 6 weeks exerts a beneficial effect on hepatic glucose metabolism partly through improvement of insulin sensitivity in type 2 diabetic *db/db* mice. Additionally, dieckol is a potential therapeutic agent that may ameliorate the damage induced by diabetes-associated hyperglycemia-induced oxidative stress. Therefore, it seems likely that dieckol is a promising antidiabetic agent or pharmaceutical source that will be helpful for the improvement of type 2 diabetes.

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# 27 Allergy and Its Remedies from Marine Sources

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### 27.1 INTRODUCTION

Allergy (allos = other, different, strange + ergon = activity) is a hypersensitivity disorder of the immune system (Michelle 2010). It is defined as the hypersensitivity to a substance that causes the body to react to any contact with an allergic substance. Allergy is one of the four forms of hypersensitivity and is formally called type I hypersensitivity, which in turn is classified into immediate and late-phase reaction. The immediate hypersensitivity reaction occurs minutes after exposure and includes release of vasoactive amines and lipid mediators, whereas the late-phase reaction occurs 2–4 hours after exposure and includes the release of cytokines. If these reactions are ignored, the entire body gets involved with allergic consequences and then anaphylaxis can take place, which is an acute, systemic reaction that can prove to be fatal. Allergy can be of many types depending on the etiological factor of the outside environment surrounding the human beings ([Table 27.1](#)). These factors affect an individual minimally as well as drastically depending on the individual's genetically triggered immunity level.

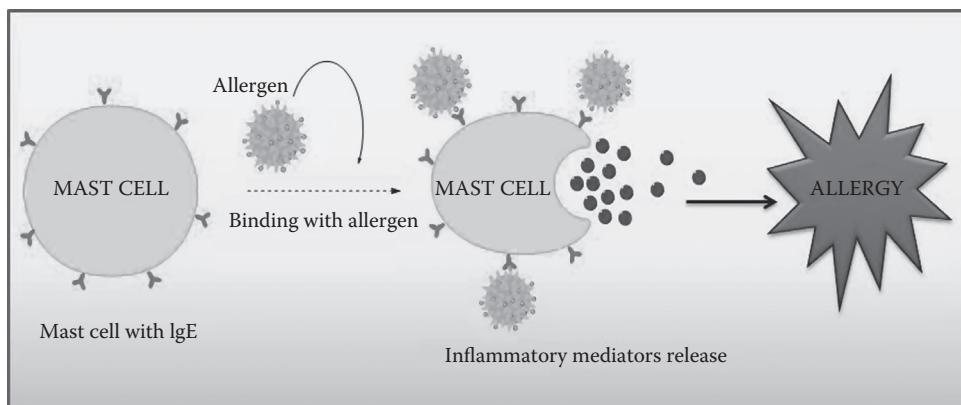
Environmental as well as genetic factors are involved in the triggering of allergy, which lead to various inflammatory responses in human body (Ayers et al. 2008). In general, following exposure to allergens, acute allergic sensitization involves the expansion of T-helper (Th)2 cells that secrete a multitude of cytokines interleukin (IL)-4, IL-5, IL-9, and IL-13 as well as chemokines such as thymus and activation-regulated chemokine and macrophage-derived chemokine, further leading to Th2 cell recruitment and activation of B cells (Mazzarelli 2010). B cells produce allergen-specific immunoglobulin E (IgE) that circulates and binds to mast cells and basophils; this consequence is influenced by IL-4. Further exposure to the allergens results in the crosslinking of IgE on mast cells and basophils causing degranulation of cells, release of several mediators like histamine, proteases, chemokines, prostaglandins, leukotrienes, and so on ([Figure 27.1](#)).

Inhalation of allergens trigger bronchoconstriction through the recruitment of activated eosinophils, neutrophils, lymphocytes, and macrophages that lead to asthma, characterized by bronchial wall remodeling, hyperresponsiveness, and chronic inflammation of airways (Larché, Robinson, and Kay 2003). Detailed information about the types of mediators and their roles is listed in [Table 27.2](#).

Although treatment of allergy usually involves various drugs containing epinephrine, antihistamines, and corticosteroids, there is great interest in searching for antiallergic compounds and

**TABLE 27.1**  
**Different Allergic Complications and Their Etiological Agents**

Type of Allergy	Etiological Agent
Atopic dermatitis	Primarily by various allergens, stress, or fatigue
Hay fever (allergic rhinitis)	Pollen from trees or other microscopic substances
Hives (urticaria)	Medications, food, and physical agents
Allergic asthma	Pollen from trees, mold spores, and mold fragments
Anaphylaxis	Animal dander (from hair, skin, or feathers) and saliva, dust, cockroach feces
Poison ivy/oak/sumac	Venom from insect bites and stings, foods, and medication
Bee sting allergy	A normal reaction to a bee sting is different from a bee sting allergy
Pet allergies	A cat or a dog or any other pets
Latex allergy	Direct contact with latex, for example, latex gloves, touching other latex-containing products
Mold allergy	Mold present in most indoor and outdoor spaces, many food items
Cosmetic allergies	Often by the cosmetics of skin and face
Drug allergies	Penicillin, aspirin, etc.
Eye allergies (allergic conjunctivitis)	Eye contact with allergens
Food allergies	Milk, egg, nuts, soy, fish, shellfish, wheat, etc.
Cigarette smoke allergy	Asthma and allergies due to cigarette smoke



**FIGURE 27.1** Diagrammatic representation of mechanism of IgE-mediated allergy.

their sources worldwide. This is because of many of the undesirable side effects like headache, dry mouth, and/or drowsiness, especially in children, that are potentially caused the current over-the-counter (OTC) prescription and herbal-based medications on the market for allergies. In this regard, naturally derived medicinal food and medicine are being targeted to combat the allergy that does not show any secondary complication on the physiological status of the body. Among the medicinally recommended foods, marine-derived foods are gaining tremendous pharmacognosial importance to deal with allergy (Vo, Ngo, and Kim 2012). Various marine flora and fauna have been traditionally used as potent antiallergic and antiasthmatic foods in most of the Asian countries. Because of the biomedical importance of the chemical as well as the protein molecules, marine-derived foods are explored and exploited for specific therapeutic applications. Although the value of these compounds or molecules is outstanding, pharmacokinetic analysis of the marine

**TABLE 27.2**  
**Overview of Mediators Released by Mast Cells in Type 1 Hypersensitivity and Their Actions**

Allergic Complication	Mediators
Vasodilation and increased permeability	Histamine Platelet-activating factor (PAF) Leukotriene C4, D4, and E4 Prostaglandin D2 Neutral proteases
Smooth muscle spasm	Histamine PAF Leukotriene C4, D4, and E4 Prostaglandin
Leukocyte extravasation	Cytokines (e.g., chemokines and TNF) Leukotriene B4 Chemotactic factors for neutrophils and eosinophils

*Source:* Mitchell, R. S., V. Kumar, S. L. Robbins, A. K. Abbas, and N. Fausto. 2007. *Robbins Basic Pathology* (8th ed.). Philadelphia: Saunders.

active components/extracts presents profound analytical challenges because of their chemical complexity, lack of standards for many compounds of medical and pharmacological interest, and detection limitations of the analytical methods since many marine bioactive molecules are available in low quantities.

The notion of consuming medicinally and pharmacologically important marine consumable foods is not to be restricted but also can be extended to other non-Asian countries (Kim and Pallela 2011). Hence, it is important to highlight the pharmacological significance of marine medicinal foods that deal with various allergic complications (Kim, Vo, and Ngo 2011). This chapter deals with the basics of allergy and its remedies from antiallergic agents of marine plants and animals, thereby presenting an overview of their pharmacodynamic potential in the treatment of allergic disorders.

## 27.2 ANTIALLERGIC REMEDIES FROM MARINE PLANTS

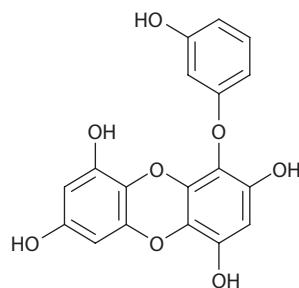
Although marine algae are believed to be safe and can be used as potential pharmacological agents for several diseases, they have not been as extensively studied as terrestrial plants. Marine algae compete well with the terrestrial medicinal plants for treating various diseases, and there is a great opportunity for marine researchers to explore these floral communities. Great interest has been shown in marine macroalgae and microalgae in the past decade because of their diversity and potential ability to produce various antiallergic moieties (Table 27.3).

Among the macroalgae, the brown alga *Ecklonia* species has proven to be the richest producer of medical and pharmacological leads. Despite its medicinal importance for various diseases, the phloroglucinol-containing phlorotannins from these species play a crucial role to fight with allergy and inflammation (Shim et al. 2009; Senevirathne and Kim 2011). The antiallergic activity of *Ecklonia cava* (EC) extract was previously evaluated in a murine asthma model, which showed that the fractions from EC extract resulted in 58% inhibition against histamine release (Kim et al. 2008). In addition to these studies, phloroglucinol derivatives from *E. cava* were reported for their inhibitory activity against histamine release in human (KU812) and rat (RBL-2H3) basophilic leukemia cell lines (Li et al. 2008). Other marine algae *Eisenia bicyclis* and *Eisenia arborea* have also been

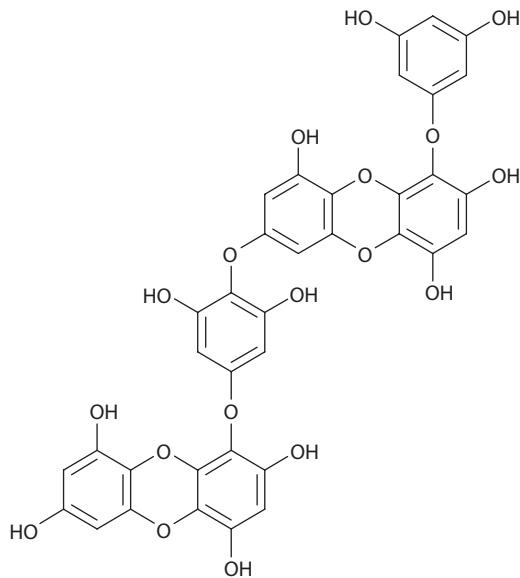
**TABLE 27.3**  
**Consumable Marine Algal Species That Aid in Combating Allergy**

Name of the Species	Consuming Part	Allergic Complication
<i>Carpopeltis affinis</i>	Whole plant	Allergy
<i>Chlorella vulgaris</i>	Whole plant	Allergy
<i>Chlorella pyrenoidosa</i>	Whole plant	Allergy
<i>Chrysomenia wrightii</i>	Whole plant	Asthma
<i>Codium fragile</i>	Whole plant	Allergy
<i>Dunaliella salina</i>	Whole plant	Asthma
<i>Ecklonia cava</i>	Whole plant	Allergy
<i>Eisenia bicyclis</i>	Whole plant	Allergy and asthma
<i>Eisenia arborea</i>	Whole plant	Allergy and asthma
<i>Ishige foliacea</i>	Whole plant	Allergy
<i>Ishige okamurae</i>	Whole plant	Allergy
<i>Laurencia undulata</i>	Whole plant	Asthma
<i>Laminaria japonica</i>	Whole plant	Allergy and asthma
<i>Petalonia binghamiae</i>	Whole plant	Allergy and asthma
<i>Porphyra dentate</i>	Whole plant	Allergy
<i>Porphyridium purpureum</i>	Whole plant	Asthma
<i>Sargassum tenerimum</i>	Whole plant	Asthma
<i>Sargassum cervicorne</i>	Whole plant	Allergy
<i>Sargassum hemiphyllum</i>	Whole plant	Asthma
<i>Sargassum thunbergii</i>	Whole plant	Asthma
<i>Sargassum micracanthum</i>	Whole plant	Allergy
<i>Sargassum ringgoldianum</i>	Whole plant	Allergy
<i>Sargassum graminifolium</i>	Whole plant	Asthma
<i>Scytoniphon lomentaria</i>	Whole plant	Asthma
<i>Spirulina platensis</i>	Whole plant	Allergy
<i>Ulva japonica</i>	Whole plant	Asthma
<i>Undaria pinnatifida</i>	Whole plant	Allergy

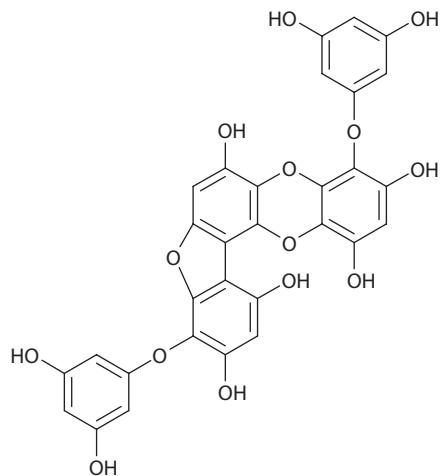
identified to contribute in antiallergic mechanisms by possessing various phlorotannins, namely, phloroglucinol, phlorofucofuroeckol A, dieckol, and so on (Shibata et al. 2002). More importantly, 8,8'-bieckol from *E. bicyclis* potentially inhibited the hyaluronidase enzyme. The effect of these phlorotannins against hyaluronidase enzyme is higher than the existing inhibitors such as catechins and sodium cromoglycate.



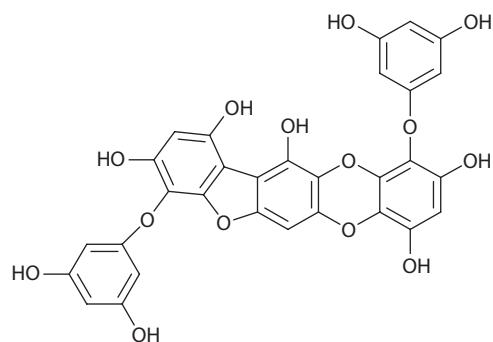
**STRUCTURE 27.1** Eckol.



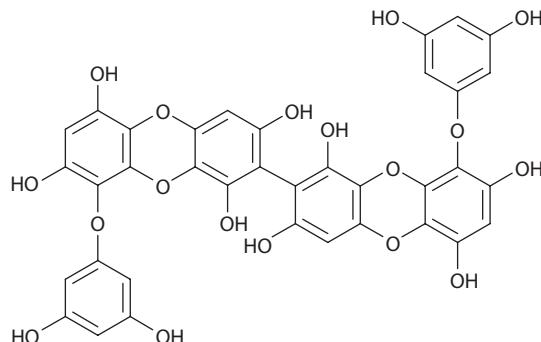
**STRUCTURE 27.2** Dieckol.



**STRUCTURE 27.3** Phlorofucofuroeckol-A.



**STRUCTURE 27.4** Phlorofucofuroeckol-B.



**STRUCTURE 27.5** 8,8'-Bieckol.

*E. arborea* was examined for its antiallergic effect *in vivo*, using Brown Norway rats as an allergy animal model (Sugiura et al. 2009). According to these studies, the rats were preliminarily immunized with oral administration of ovalbumin (OVA) and daily fed with *E. arborea* powder (1–5 g/rat). *E. arborea* induced a change in the Th1/Th2 balance through the suppression of the release of Th2 cytokines, IL-4 and IL-10, and by enhancing the expression of Th1 cytokine interferon (IFN)- $\gamma$  in spleen and mesenteric lymph nodes. The results clearly indicate that both the OVA-specific and the total serum IgE levels were suppressed in the rats fed with a diet of dried *E. arborea* powder.

Several other marine algal species such as *Sargassum tenerimum*, *Sargassum cervicorne*, *Sargassum graminifolium*, *Sargassum thunbergii*, *Ulva japonica*, and *Laminaria japonica* also exhibited tremendous antiallergic effects (Kimiya et al. 2008; Samee et al. 2009). *S. tenerimum* significantly suppressed passive cutaneous anaphylaxis (PCA) and active cutaneous anaphylaxis (ACA) in female Balb/c mice, which was comparable to the antiallergic effect of the drug disodium cromoglycate. In addition, ethanolic extracts of *S. tenerimum*, *S. cervicorne*, and *S. graminifolium* showed profound antiallergenic activities *in vivo* (Haider et al. 2009). In another study, a Korean folk medicine from brown alga *Sargassum hemiphyllum* has been used for the therapeutic treatment of various allergic diseases by the inhibition of atopic allergy through the regulation of inflammatory mediators in mast cells (Na et al. 2005a). In particular, methanolic extract of *S. hemiphyllum* effectively inhibited the release of histamine,  $\beta$ -hexosaminidase, IL-8, and tumor necrosis factor (TNF)- $\alpha$  from the activated mast cells. When *S. hemiphyllum* was orally administered at a dose of 0.1 g/kg for 1 hour, it showed a remarkable inhibitory effect on PCA reaction with an inhibition rate of 49.71%. These results infer that this species has great inhibitory effect on the allergic reactions and can be used in the treatment of allergic inflammatory diseases such as atopic dermatitis (Hwang et al. 2010). *S. thunbergii* exhibited inhibitory effect on the allergic consequences *in vitro* and *in vivo*, without showing any significant cytotoxicity. On the contrary, *Ishige foliacea*, *Ishige okamurae*, *Sargassum micracanthum*, and *Sargassum ringgoldianum* were also reported to be able to suppress the histamine release from rat basophilic leukemia (RBL-2H3) cells (Sugiura et al. 2006). Our recent explorations of *I. okamurae* to alter the levels of histamine and cytokine in the calcium ionophore A23187-induced human basophilic KU812F cells indicated the inhibition of allergic inflammation (Vo et al. 2011).

A red alga *Carpopeltis affinis* is also known to show potential antiallergic responses as it is effective against atopic allergic reaction *in vitro* (Na et al. 2005b). It was observed that *C. affinis* significantly alleviated the release of various mediators of allergic reaction, namely, histamine,  $\beta$ -hexosaminidase, IL-8, and TNF- $\alpha$  from mast cells. Moreover, the protective effect of an edible red alga *Laurencia undulata* against murine allergic airway reactions has also been elucidated recently (Jung et al. 2009).

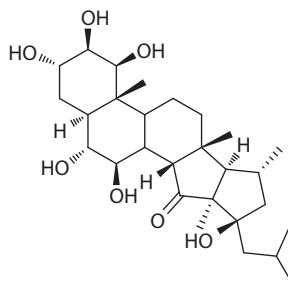
Similar to the macroalgae, various edible microalgae can also modulate both adaptive and innate aspects of immunity, leading to several antiallergic responses. The clinical effect of *Spirulina*

species on allergic rhinitis was also determined due to inhibition of the production of IL-4 and suggestive suppression of the differentiation of Th2 cells (Mao, Water, and Gershwin 2005).

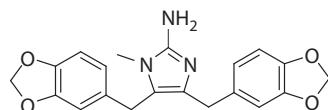
### 27.3 ANTIALLERGIC REMEDIES FROM MARINE ANIMALS

Medicinal, pharmacological, and nutraceutical applications from marine animals have to be brought forward in such a manner that the consumption of these commonly available faunal communities can be directed for best human diet practices (Kim and Pallela 2011). Marine animals, as a whole or a part, contribute crucially in triggering several biomedical mechanisms involved in allergic/inflammatory cascades (Alves et al. 2009) (Table 27.4). More importantly, a number of receptor antagonists with potential as biochemical tools or structural therapeutic leads have been isolated from marine animals.

Previously, several pharmacologically important antiallergic moieties have been isolated from various marine animals. Examples include xestobergsterol from *Xestospongia berguissta* that inhibits IgE-mediated histamine release from mast cells and is 5000 times more potent than the commercially available antiallergic drug disodium cromoglycate (Jha and Zi-rong 2004). Another molecule leucettamine A from *Leucetta microraphis* is a potent and selective antagonist for the receptor for leukotriene, a non-peptide metabolite of arachidonic acid produced mainly in inflammatory cells.



**STRUCTURE 27.6** Xestobergsterol B.



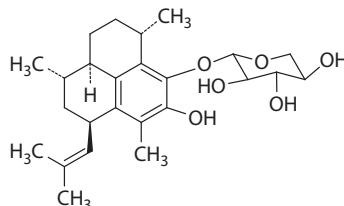
**STRUCTURE 27.7** Leucettamine A.

Immunosuppressants are valuable drugs that are produced by microorganisms and were first isolated as antibiotics (Hwang et al. 2010). For example, rapamycin (sirolimus), tacrolimus (FK506), and cyclosporin A were discovered as antifungal antibiotics (High 1994). However, their medical niche as immunosuppressants was later identified and it was proven that the antifungal and immunosuppressive activities may be unrelated since nonimmunosuppressive derivatives have also been obtained with good antifungal activity (Cruz et al. 2000). The cyclosporine are a group of over 25 closely related cyclic undecapeptides produced as secondary metabolites by certain species of filamentous fungi. Cyclosporin A analogues have been clinically tested against the inflammatory disease asthma and have shown promising results (Eckstein and Fung 2003). They exhibit decreased nephrotoxicity and have different pharmacology and metabolism. Although initially isolated from a terrestrial fungus *Tolyphocladium inflatum* (Gams) in 1972, cyclosporin A has also been isolated from marine fungal sp. *Microdochium nivale* associated with *Porteresia coarctata*, a marine salt marsh grass from mangrove environment distributed along the Central West Coast (CWC) of India (Bhosale et al. 2011).

**TABLE 27.4****Consumable Marine Animal Species That Aid in Combating Allergy**

Name of the Species	Consuming part	Allergic Complication
<i>Hippocampus reidi</i>	Whole animal	Asthma, epilepsy
<i>Rhincodon typus</i>	Liver, cartilage, fins, meat	Immunity disorders, cancer, arthritis, psoriasis, and other allergic reactions
<i>Thaleichthys pacificus</i>	Fat	Skin complications
<i>Echinaster brasiliensis</i>	Whole animal	Asthma
<i>Echinaster echinophorus</i>	Whole animal	Asthma
<i>Luidia senegalensis</i>	Whole animal	Asthma
<i>Mellita quinquiesperforata</i>	Whole animal	Asthma
<i>Oreaster reticulatus</i>	Whole animal	Asthma
<i>Echinometra lucunter</i>	Whole animal	Asthma
<i>Caiman crocodilus</i>	Skin	Asthma, allergies, and epilepsy
<i>Haliotis asinina</i>	Whole animal	Cough and immune disorders

The Caribbean gorgonian *Pseudopterogorgia elisabethae* is an example of another field of application of marine natural products, which is not directly concerned with drug discovery but nevertheless holds considerable economic potential. Extracts of *P. elisabethae* show anti-inflammatory activity owing to the presence of unusual diterpene glycosides, pseudopterosins, and are nowadays used as an ingredient for cosmetic skin care products (Mayer et al. 1998) to prevent allergic reactions.

**STRUCTURE 27.8** Pseudopterosin A.

It is interesting to note that few of the marine natural products currently in clinical trials or under preclinical evaluation against inflammation/allergy or asthma are produced by invertebrates such as sponges, tunicates, mollusks, or bryozoans (Proksch, Edrada, and Ebel 2002). In a search for nonallergic substances from sea, Pallela et al. (2010) have isolated the native marine collagens from sponge *Ircinia fusca* (Pallela, Bojja, and Janapala 2011). Another compound known as manolide has also been isolated from a marine sponge *Luffariella variabilis* and is under phase I clinical trial against inflammation/psoriasis (De Rosa et al. 1998). A synthetic analogue of contignasterol (IZP-94,005) known as IPL 576,092, isolated from sponge *Petrosia contignata*, is also under phase I clinical trial against asthma/inflammation (Coulson and O'Donnell 2000).

Not only marine animals as such but their products are also quite popular among the pharmaceutical aspirants. Chitosan and its derivatives like chitooligosaccharides (COS) from crustaceans play a major role in defending various dreadful diseases (Kim 2011). COS of three different molecular weight ranges (1–3 kDa, 3–5 kDa, and 5–10 kDa) were investigated for their abilities against allergic reactions in RBL-2H3 cells. Furthermore, the inhibitory activities of COS were evidenced on the calcium ionophore A23187 plus phorbol 12-myristate 13-acetate (PMA)-induced expression of TNF- $\alpha$ , IL-1 $\beta$ , IL-4, and IL-6. It was also confirmed that COS showed the suppressive effects on the phosphorylation of extracellular signal-regulated kinases (MEK/ERK) and p38 kinase, indicating

that COS could be used as an inhibitor in regulating mast cell-mediated allergic inflammatory responses (Vo, Kong, and Kim 2011).

Taking the described ecological importance of marine natural products into consideration, it is therefore a surprise that the majority of drug candidates from the sea has so far been isolated from invertebrates, which thrive in tropical (e.g., as inhabitants of coral reefs) or subtropical seas where the grazing pressure by predators such as fishes is higher than in any other ecosystem of the world (Proksch, Edrada, and Ebel 2002). Under these severe selective pressures, only those organisms that can rely on effective means of chemical defense will survive. It can be taken as a justification for marine invertebrates being a rich source of bioactive compounds.

## 27.4 CONCLUSION

Oceans have always been envisioned as a potential source for pharmacognosial leads to treat various diseases and are of great interest for researchers operating in marine natural product chemistry, phytochemistry, pharmacology, molecular biology, pharmacognosy, and so on. The pharmacognosial impact of these floral and faunal communities for targeting antiallergic molecules has been considered for the past few decades; however, further insights to increase knowledge in the field of anti-inflammatory and antiallergy agents from the marine kingdom are necessary to combat recently emerging immunoallergic diseases.

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# 28 Anticancer Mechanisms of Actin Depolymerization Agent Pectenotoxin-2

*Gi-Young Kim and Yung Hyun Choi*

## CONTENTS

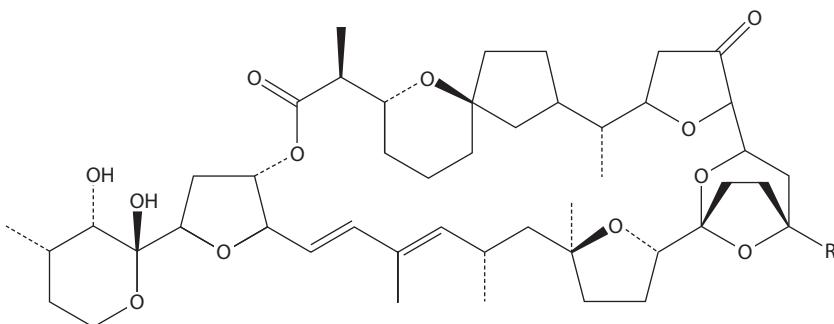
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## 28.1 INTRODUCTION

Pectenotoxins (PTXs) are macrolactones with multiple polyether ring units that have been shown to contaminate shellfish in various parts of the world including Australia, Japan, New Zealand, and a number of European countries (Draisci et al. 1996; James et al. 1999; Suzuki et al. 2001). Therefore, the PTX group is considered as a group of polyether-lactone toxins that induce diarrhea. It is noted that 15 PTX analogs, which are related to their structures, have been discovered, with different toxicological potencies (Espina and Rubiolo 2008). They have been detected in microalgae and bivalve mollusks where they were first isolated from the Japanese scallop *Patinopecten yessoensis* (Yasumoto et al. 1985).

It is noted that PTX-2 (Figure 28.1), which is a cause of diarrheic shellfish poisoning (DSP), is a family of cyclic polyether macrolide toxins isolated from many species of the dinoflagellate genus *Dinophysis*, and it was initially detected in *Dinophysis fortii* (Draisci et al. 1996). The PTX-group toxins were later isolated from *Dinophysis acuminata*, *Dinophysis norvegica*, *Dinophysis rotundata*, and *Dinophysis acuta* (Lee et al. 1987; Suzuki et al. 1998; MacKenzie et al. 2002). Earlier studies indicate that PTX-2 produces severe diarrhea in mice (Ishige, Satoh, and Yasumoto 1988), it is toxic after oral administration, and PTX-2 toxins are more toxic to the liver when administered intraperitoneally in mice because PTX-2 produces congestion under the liver capsule as a result of circulatory disorder (Ogino, Kumangai, and Yasumoto 1997; Ito et al. 2008). Recently, it was reported that PTX-2 has potent cytotoxic activities against several cancer cell lines; moreover, its biological and functional mechanisms have been intensively investigated.

It is found that many natural PTXs known macrocyclin polyethers show toxicity against the actin cytoskeleton (Spector et al. 1999; Leira et al. 2002; Espina et al. 2008). The ability of these



**FIGURE 28.1** Chemical structure of pectenotoxin-2.

PTXs to interfere with actin cytoskeleton dynamics allows them to play important roles as precursor molecules in chemotherapy; moreover, their potential utility in the treatment of cancer has been recognized (Allingham, Miles, and Rayment 2007). Recent studies have demonstrated that PTX-1, PTX-2, PTX-6, and PTX-11 disrupt the organization of filamentous actin (F-actin) cytoskeleton in different cell lines such as neuroblastoma cells and rabbit enterocytes (Spector et al. 1999; Ares et al. 2007). Therefore, it is proposed that some PTXs exert their toxicity by mechanisms of interrupting the actin cytoskeleton via its depolymerization. Among these PTXs, PTX-2 has been shown to have a major effect on actin depolymerization. Particularly, many *in vitro* and biochemical studies show that PTX-2 inhibits actin polymerization in a concentration-dependent manner but not tubulin, which is another molecule to regulate mitotic separation and cytokinesis (Spector et al. 1999). The first study experimentally found that the actin stress fiber was disrupted by PTX-2. Further, it inhibited the velocity and degree of pyrene–actin polymerization as well as viscosity of F-actin in a concentration-dependent manner (Hori et al. 1999; Karaki et al. 1999). These results suggest that PTX-2 is a potent actin-depolymerizing agent and a potent anticancer agent possessing a unique mode of action.

As PTX-2 toxins have an effect on actin polymerization, many studies have been performed regarding cell cycle arrest, endoreduplication, apoptosis, as well as anti-inflammatory effects because actin is one of the most abundant and common cytoskeletal proteins for cell growth, motility, signaling, and maintenance of cell shape. Therefore, it affects cell cycle progression into G<sub>1</sub>, S, and G<sub>2</sub>/M phases. In the rest of the chapter, we discuss the effect of PTX-2 on the aforementioned phases by relating different cellular signaling cascades.

## 28.2 EFFECT OF PECTENOTOXIN-2 ON CELL CYCLE PROGRESS

In this section, the effect of PTX-2 on cell cycle arrest in different stages as well as endoreduplication is explained. It is well established that PTX-2 strongly induces cell cycle arrest at the G<sub>2</sub>/M phase as well as the G<sub>1</sub> phase in different cancer cells and synovial fibroblasts. It is further shown that this potent agent induces cell cycle arrest and endoreduplication in a cell type–independent manner.

### 28.2.1 G<sub>2</sub>/M PHASE CELL CYCLE ARREST BY PECTENOTOXIN-2 IN CANCER CELLS

Cell cycle arrest is one of the key factors of an effective chemopreventive agent. Many studies demonstrate that PTX-2 toxins possess strong antiproliferative properties in various cancer cell lines. In particular, PTX-2 prevented the proliferation of cancer cells by interfering of various stages of cell cycle distribution. In the first part of this section, we discuss the effect of PTX-2 interfering G<sub>2</sub>/M phase arrest. Recently, we reported that PTX-2 caused significant G<sub>2</sub>/M phase arrest in human leukemia cells (Moon et al. 2008). Further experimental results indicated that not only leukemia cells but also human hepatoma as well as breast cancer cells suffered cell cycle arrest at the same stages.

In these studies, PTX-2 increased the phosphorylation of Cdc25C and decreased the protein levels of Cdc2 (cyclin-dependent kinase 1 [CDK1]) and cyclin B1, and the M phase-specific marker protein phospho-histone 3 was markedly increased by PTX-2 (Moon et al. 2008). Moreover, induction of G<sub>2</sub>/M phase arrest by PTX-2 was regulated by the extracellular signal-regulated kinase (ERK) and c-jun N-terminal kinase (JNK) pathways, and inhibitors of ERK and JNK further increased the phosphorylation of Cdc25c expression at G<sub>2</sub>/M arrest stages.

It is well known that Cdc25C directs dephosphorylation of cyclin B-bound Cdc2 and triggers entry into mitosis (Draetta and Eckstein 1997; Sanchez et al. 1997). It is also thought to suppress tumor suppressor p53-induced growth arrest by CDKs. However, cancer cells show abnormal growth patterns due to the deregulation of these proteins. Activated Cdc25C is dissociated from 14-3-3 at the G<sub>2</sub>/M transition, together with its hyperphosphorylation on several sites within its regulatory N-terminal domain, mediated by CDKs and polo-like kinase 1 (Plk1) (Myer, Bahassi, and Stambrook 2005). Sanchez et al. (1997) reported that increased phosphatase activity of Cdc25C could lead to the activation of Cdc2/cyclin B, which results from the dephosphorylation of Cdc2 by Cdc25C. On the other hand, phosphorylation at serine 216 induces the cytosolic retention of Cdc25C through 14-3-3 binding (Eymin et al. 2006). This phenomenon is a good strategy to block or delay mitotic entry. Therefore, as we expected, PTX-2-induced G<sub>2</sub>/M phase arrest is associated with the repression of Cdc2 and cyclin B1 expression and the induction of phosphorylation of Cdc25C at serine 216 (Moon et al. 2008).

In another experiment, we investigated how PTX-2 induces G<sub>2</sub>/M phase cell cycle arrest in human breast cancer cells via ataxia-telangiectasia-mutated (ATM)- and checkpoint kinases 1/2 (CHK1/2)-mediated phosphorylation of Cdc25C (Moon et al. 2010). As mentioned earlier, phosphorylation at serine 216 induces the cytosolic retention of Cdc25C through 14-3-3 binding in response to DNA damage (Eymin et al. 2006). Therefore, cell cycle checkpoints play an important role in safeguarding against genomic DNA errors that may occur during chromosome segregation and DNA replication (Sanchez et al. 1997). The activation of checkpoints that are responsive to DNA damage or incomplete DNA replication ultimately results in the inhibition of CDKs. It is noted that CHK1 and CHK2 can be activated in response to DNA damage through phosphorylation on ser-345/ser-317 and Thr-68, respectively. The Cdc25, when phosphorylated on serine 216 by activation of CHKs, thus blocks downstream Cdc activation and mitosis. According to the study by Singh (Singh et al. 2004), human cancer cell-derived CHK2(−/−) cells are significantly more resistant to G<sub>2</sub>/M arrest when compared with CHK2(+/+) cells. Our previous data shows that phosphorylation of CHKs was increased by PTX-2 in a concentration-dependent manner (Moon et al. 2010). It is well known that ATM phosphorylates contain several key proteins such as p53 and CHKs that initiate activation of DNA damage checkpoints, leading to cell cycle arrest or apoptosis. Therefore, our study shows that PTX-2-treated cells markedly increase the serine-216 phosphorylation of Cdc25C associated with ATM-dependent activation of CHKs. Furthermore, Cdc25 phosphatase may prevent entry into mitosis and stabilize the tumor suppressor protein p53, leading to cell cycle arrest. However, the PTX-2-induced G<sub>2</sub>/M arrest was not significantly different between MDA-MB-231 and MCF-7 cells, indicating that p53 is independent for G<sub>2</sub>/M arrest induced by PTX-2 (Moon et al. 2010).

## 28.2.2 G<sub>1</sub> PHASE CELL CYCLE ARREST BY PECTENOTOXIN-2 IN SYNOVIAL FIBROBLASTS

Regulation of the cell cycle involves in regulating the survival of a cell such as repairing genetic damage and prevention of uncontrolled cell division. Two key classes of regulatory molecules, cyclins and CDKs, cooperatively regulate a cell's progress through the cell cycle checkpoint (Li and Brooks 1999). It is noted that CDKs rely on a cyclin partner for enzymatic activity. These cyclin-CDK complexes phosphorylate their substrates under consumption of ATP and are thereby involved in the cell cycle. Once bound by its cyclin partner, subsequent activity is regulated by both activating phosphorylation and inhibitory phosphorylation on the CDK subunit.

Cyclin D is the first regulator produced in the cell cycle where it binds with CDK4, and this complex triggers the phosphorylation of retinoblastoma susceptibility protein (Rb) (Jeffrey, Tong, and

Pavletich 2000). Activation of Rb dissociates from the E2F complex and thus E2F gets activated and it results in transcription of various genes like cyclin E and cyclin A. Similarly, the complex between cyclin D/E and CDKs is an obvious candidate for the control of retinoblastoma protein (Rb) phosphorylation (Li and Brooks 1999). Therefore, any factors affecting the activity of these kinases could be potent targets to control the normal activation of pRB and, thereby, G<sub>1</sub> phase cell cycle arrest occurs.

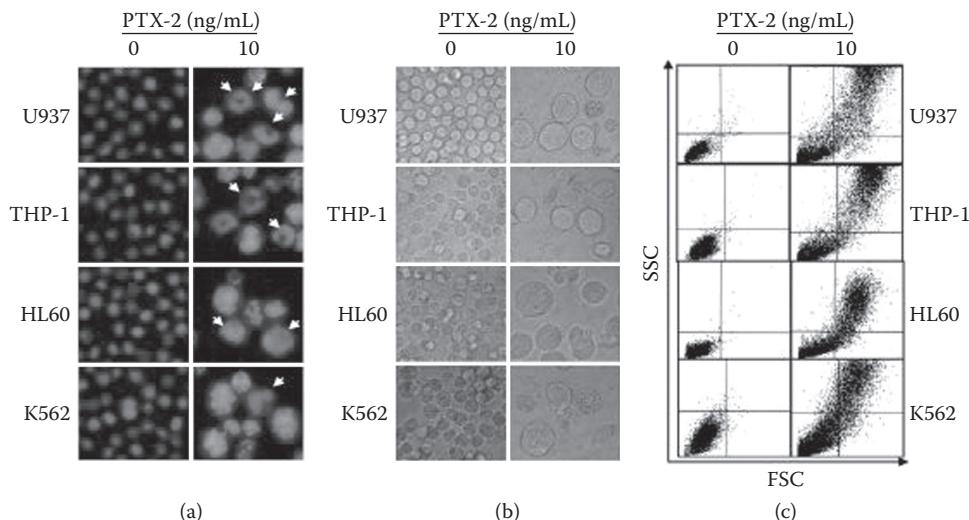
The effects of PTX-2 on the G<sub>1</sub> phase regulatory factors are well documented according to the study by Park et al. (2011). They reported that PTX-2 selectively induces the expression of the CDK inhibitor p21, along with the induction of the tumor suppressor p53, at both mRNA and protein levels in synovial fibroblasts. The PTX-2 also blocked pRB phosphorylation and decreased the levels of expression of E2F-1 and E2F-4. The p21 protein binds with CDKs and regulates the cell cycle at G<sub>1</sub> phase. The PTX-2 also resulted in a significant increase in the binding of CDKs (CDK2 and CDK4) with p21. Therefore, the inhibition activity of CDKs by PTX-2 through the upregulation of complex formation between p21 and CDKs causes cell cycle arrest at G<sub>1</sub> stage. Therefore, PTX-2 shows G<sub>1</sub> arrest in synovial fibroblasts by regulating the factors involved in G<sub>1</sub>/S phase cell cycle progression.

### 28.2.3 INDUCTION OF ENDOREDUPPLICATION BY PECTENOTOXIN-2

Based on the broad spectrum of cell types in which endoreduplication occurs, many hypotheses have been generated to explain the functional importance of this phenomenon. Endoreduplication can be understood simply as a variant form of the mitotic cell cycle in which mitosis is aborted prior to cytokinesis due in part to modulation of cyclin-dependent activity. However, many aspects of cell cycle control require negative regulation of CDKs. Negative regulation of CDK activity is achieved either by phosphorylation of the catalytic subunit or via the binding of CDK inhibitory proteins known as CKIs (Niculescu et al. 1998). The other way is that disruptive of actin cytoskeleton by extracellular agents can be effect the cell growth, motility, signaling, and maintenance of cell morphology (Spector et al. 1999). Recent studies have investigated whether the binding and stabilizing of actin microfilaments using actin polymerization inhibitors could inhibit the growth of several tumor cell lines (Landry and Huot 1995). Thus, cytotoxin agents have been shown to play important roles in anticancer therapy due to their ability to interfere with actin cytoskeleton dynamics and their potential utility in the treatment of cancer has been recognized.

In particular, PTX-2 has been shown to have a potent cytotoxic effect in human cancer cell lines. Recent studies have also demonstrated that the anticancer effect of PTX-2 is due to disruption of the actin cytoskeleton through the inhibition of actin polymerization, *in vitro* and *in vivo* (Espina and Rubiolo 2008). We experimentally found that PTX-2 treatment decreases the fluorescence intensity of phalloidin-FITC in a dose-dependent manner in human leukemia U937 cells (Moon et al. 2008). It is indicated that PTX-2 causes a decrease in cell proliferation through the depolymerization of actin. In addition, maintenance of constant cell size during cellular proliferation must be coordinated with the rate of cell division. The failure of mitotic cell division as caused by PTX-2 induces an increase in cell size, and this phenomenon was shown by PTX-2 when 10 ng/mL of PTX-2 treatment for 72 hours in leukemia cells contributed to an increase in cell size ([Figure 28.2](#)).

Microtubules are present in cell cytoskeletal networks, which play important roles in many aspects of the fundamental processes of cell growth and development, including cell division, cell expansion, intracellular organization, and cell motility (Takemoto and Hardham 2004). The disruption of dynamics of microtubule polymerization has been known to induce endoreduplication. However, we experimentally found that PTX-2 did not change tubulin polymerization *in vitro*, whereas induction of giant sizes by PTX-2 in leukemia cells could be promoted by actin depolymerization (Moon et al. 2008). A recent study also showed that the increased expression of CDK2 and p21 is associated with endoreduplication (Chang et al. 2000). Since CDK2 is essential for the G<sub>1</sub>/S transition, consistent or overexpressed CDK2 in G<sub>2</sub>/M arrested cells could induce endoreduplication by reentering the S phase. Interestingly, p21 and CDK2 protein levels increased significantly in response to PTX-2 (Moon et al. 2008). Thus, it induces G<sub>2</sub>/M phase arrest and endoreduplication



**FIGURE 28.2** Pectenotoxin-2 (PTX-2) inhibits mitosis and induces large cell sizes in human leukemia cells. Cells were seeded at  $5 \times 10^4$  cells/mL and then treated with PTX-2 (10 ng/mL) for 72 hours: (a) The cells were fixed and stained with 4',6-diamidino-2-phenylindole (DAPI) to visualize their DNA. Cells were analyzed by fluorescence microscopy (400 $\times$ ). White arrows represent the multinucleus. (b) The morphology of cells treated with or without PTX-2 was examined under light microscopy (400 $\times$ ). (c) Cell size (FSC) and intracellular granules (SSC) were detected by flow cytometric analysis.

via the modulation of cell cycle-regulating proteins. One of the signaling pathways that can control a cell cycle is the mitogen-activated protein kinase (MAPK) signaling cascade. Actin dysfunction accelerates the ERK and JNK signaling pathways, and delays entry into mitosis in mammalian cells (Reiterer and Yen 2006; Lee and Song 2007). PTX-2 was found to significantly induce the phosphorylation of ERK, JNK, and p38. Further data shows that the ERK and JNK signaling pathways are involved in PTX-2-induced actin dysfunction, suggesting the presence of an actin checkpoint at the G<sub>2</sub>/M transition in leukemia cells (Moon et al. 2008). Not only that endoreduplication of the cancer cell lines is found cell types independently by using several type of cancer cells *in vitro* by PTX-2. Thus, PTX-2 induces endoreduplication through different cell signaling pathways, promotes actin depolymerization, and disrupts the organization of the actin cytoskeleton.

## 28.3 INDUCTION OF APOPTOSIS BY PECTENOTOXIN-2 IN CANCER CELLS

### 28.3.1 EFFECT OF PECTENOTOXIN-2 ON NUCLEAR FACTOR- $\kappa$ B AND ITS RELATED GENE PRODUCT

Cell signaling has been most extensively studied in the context of human diseases where is related to proliferation, survival, and transformation of cells that deeply investigated in current cancer therapeutics (Hombach-Klonisch et al. 2008). The components of signaling networks include several kinases such as serine/threonine protein kinase, tyrosine-specific protein kinases, histidine-specific protein kinases, and phosphoinositide-3-kinase, which maintain the metabolic function of cells (Donat et al. 2009). Abnormal activation of these kinases or their downstream transcription factors may result in uncontrolled cell growth, which results in a malignant mass. The activation of distinct sets of transcription factors by numerous intracellular signaling pathways may cause numerous cell functions. Among them, nuclear factor  $\kappa$ B (NF- $\kappa$ B) plays a key role in cellular signal transduction systems by regulating the immune response to infection (Darnell 2002). However, improper regulation of NF- $\kappa$ B has been linked to inflammatory and autoimmune diseases, cancer, septic shock, and viral infection (Guzman et al. 2001). Therefore, over the past couple of decades, many chemopreventive agents have been investigated as inhibitors of NF- $\kappa$ B pathway.

It is noted that NF- $\kappa$ B can be activated by cytokines, bacterial toxins, viral products, oxidative stress, toxic metals, and ultraviolet light (Jeong et al. 2004). Activation of NF- $\kappa$ B by such extracellular stimuli leads to the nuclear translocation of the NF- $\kappa$ B complex, especially p65 and p50. The NF- $\kappa$ B binds to specific sequences of the DNA promoter region. This DNA/NF- $\kappa$ B complex then recruits other proteins and ends up with different translated proteins including cell proliferation proteins, cell cycle-regulating proteins, and proteins that regulate apoptosis (Guttridge et al. 1999). Chemopreventive agents that can block NF- $\kappa$ B pathways are frequently used to understand the mechanism of inhibiting activity in different cancer cell lines. Thus, we have recorded the effect of PTX-2 on constitutive NF- $\kappa$ B activation and NF- $\kappa$ B-regulated gene expression in human leukemia cancer cells (Kim, Moon, Heo, et al. 2008). We have found that PTX-2 inhibits constitutive and induced expression of NF- $\kappa$ B activation. A number of reports have demonstrated that NF- $\kappa$ B activation maintains tumor cell viability and that inhibition of NF- $\kappa$ B activity alone is sufficient to induce cell death. On the other hand, active NF- $\kappa$ B turns on the expression of genes regulate cell proliferation and protects the cell from conditions that would otherwise cause it to die via apoptosis (Jeong et al. 2004). It was significantly confirmed that several NF- $\kappa$ B inhibitors act as potent enhancers of chemotherapy inducing apoptosis in various cancer cells. One of our studies showed that PTX-2 significantly inhibits constitutive and induced NF- $\kappa$ B, leading to blockage of the I $\kappa$ B proteolytic pathway *in vitro* (Kim, Moon, Heo, et al. 2008). Therefore, NF- $\kappa$ B plays an important role in the cytotoxicity of PTX-2, because NF- $\kappa$ B regulates many genes to induce cell proliferation, cell survival, and apoptosis. As such, many different types of human tumors have misregulated NF- $\kappa$ B, which is constitutively active. Our studies further shows that NF- $\kappa$ B involved in proliferation (cyclooxygenase-2 [Cox-2]) and anti-apoptosis (IAP-1, IAP-2, and XIAP) was significantly inhibited by PTX-2 treatment. Therefore, PTX-2 has the ability to downregulate the NF- $\kappa$ B-dependent antiapoptotic gene products.

### 28.3.2 PROAPOPTOTIC EFFECT OF PECTENOTOXIN-2

Programmed cell death is a key process in cancer development and progression. The ability of cancer cells to avoid apoptosis and continue to proliferate is one of the fundamental characters of cancer and induction of apoptosis is a major target of development of cancer therapy (Debatin 2004). Therefore, apoptosis plays an important role in regulating the number of cells in both human embryonic development and adult tissue homeostasis. Apoptotic cells are characterized by several unique features including nuclear fragmentation, chromatin condensation, blebbing, loss of cell membrane asymmetry and attachment, cell shrinkage, and chromosomal DNA fragmentation (Debatin 2004; Jin and el-Deiry 2005). The process of apoptosis is controlled by a diverse range of cell signals, which may originate either extracellularly or intracellularly.

Extracellular signals may either cross the plasma membrane or transduce any process by which a biological cell converts one kind of signal or stimulus into another, and a cell initiates intracellular apoptotic signaling in response to a stress (Chowdhury, Tharakan, and Bhat 2006; Fulda and Debatin 2006). These signals may positively or negatively affect apoptosis. Inhibition of apoptosis can result in a number of cancers, autoimmune diseases, inflammatory diseases, and viral infections. Accumulating data indicate that many chemopreventive and/or chemotherapeutic agents can cause tumor cell death through the induction of apoptosis. Therefore, the induction of apoptotic cell death is an important mechanism of many anticancer drugs.

Many studies have reported correlations between drug responsiveness and tumor genotypes. As the p53 gene is inactivated in the majority of human cancers, it plays a major role in preventing tumorigenesis by responding to both cellular stress and DNA damage. Much effort has therefore been expended on determining the effects of p53 inactivation on cancer cell response to therapeutic agents (Song, Ouyand, and Bao 2005; Halaby and Yang 2007). Another important aspect is that NF- $\kappa$ B has been shown to play a role in tumorigenesis, which involves its constitutive activation within a wide variety of tumor types. Many researchers indicate that NF- $\kappa$ B activation can maintain

tumor cell viability and that inhibiting NF- $\kappa$ B activation alone can be sufficient to induce cell death (Guttridge et al. 1999). Additionally, Akt is known to activate IKK, which immediately leads to NF- $\kappa$ B activation and cell survival. It is noted that Akt could phosphorylate Bcl-2 antagonist of cell death (BAD) on ser-136, which causes BAD to dissociate from the Bcl-2/Bcl-X complex and lose the proapoptotic function (Prasad et al. 2009). Therefore, Akt inhibitors also have been considered as good anticancer candidates. Several reports have shown that NF- $\kappa$ B inhibitors act as potent enhancers of chemotherapy-induced apoptosis. Similarly, PTX-2 is reported as an apoptotic agent related to many cancer cell lines by inhibiting many antiapoptotic genes.

It is noted that PTX-2 inhibits the expression of antiapoptotic and antiproliferative genes known to be regulated by NF- $\kappa$ B activity. One of our studies shows that PTX-2 significantly inhibits constitutive NF- $\kappa$ B, leading to blockage of the I $\kappa$ B proteolytic pathway in leukemia cells (Kim, Moon, Heo et al. 2008). Suppression of nuclear translocation of NF- $\kappa$ B by the presence of the NF- $\kappa$ B inhibitor pyrrolidine dithiocarbamate (PDTC) enhanced PTX-2-induced apoptosis. It further shows that PTX-2-induced apoptosis may be related to the down-regulation of the antiapoptotic protein Bcl-xL as well as the upregulation of the proapoptotic protein Bax, and antiapoptosis (AP-1, IAP-2, and XIAP) was significantly inhibited by PTX-2 treatment. Therefore, PTX-2 sensitizes apoptosis by suppressing NF- $\kappa$ B activity via inactivation of Akt.

Shin et al. (2008) reported that PTX-2 caused the proteolytic activation of caspases such as caspase-3, -8, and -9 in Hep3B cells but not in HepG2 cells. This compound also downregulated the expression of proteins of the IAP family, inhibitors of caspases, such as XIAP, cIAP-1, and cIAP-2. These data indicated that caspase-3 plays an important role in PTX-2-induced apoptosis in p53-deficient Hep3B cells. It is noted that PTX-2 also increased the levels of DR4 and DR5 and the increase in DR4/5 and Bax levels probably contributed to the activation of caspase-8 in Hep3B cells.

Many studies have demonstrated that p53 directly activates the transcription of a number of genes including cyclin-dependent kinase inhibitor p21 (WAF1/CIP1), the major mediator of p53 cell cycle inhibitory capacity and the apoptotic genes (Chae et al. 2005). We found that PTX-2 activates an intrinsic pathway of apoptosis in p53-deficient tumor cells compared to those cells with functional p53 both *in vitro* and *in vivo* (Shin et al. 2008). Another experiment showed that apoptosis induced by PTX-2 in Hep3B cells was associated with the modulation of the Bcl-2 family, activation of caspases, and loss of mitochondrial membrane potential (Bunch et al. 2005). Although PTX-2 induced apoptosis in p53-deficient Hep3B cells, HepG2 cells were much more resistant to PTX-2, which suggests that PTX-2 acts by a cytotoxic mechanism that seems to be p53 independent. Another study shows the same phenomenon by mitochondria integrates death signals through Bcl-2 family proteins (Kim, Moon, Heo et al. 2008). Release of cytochrome *c* and smac causes caspase activation through the loss of membrane potential, increasing the permeability of the outer membrane. This mitochondrial apoptosis pathway is involved in the apoptosis induced by abnormality of actin dynamics. All these data indicate that Bcl-2 family proteins are differentially regulated in p53<sup>-/-</sup> cells. Therefore, the aforementioned data show that PTX-2 induces apoptosis in diverse ranged by effecting NF- $\kappa$ B protein, caspases and changing the mitochondrial potential through suppression of actin polymerization.

## 28.4 EFFECT OF PECTENOTOXIN-2 ON TELOMERASE ACTIVITY

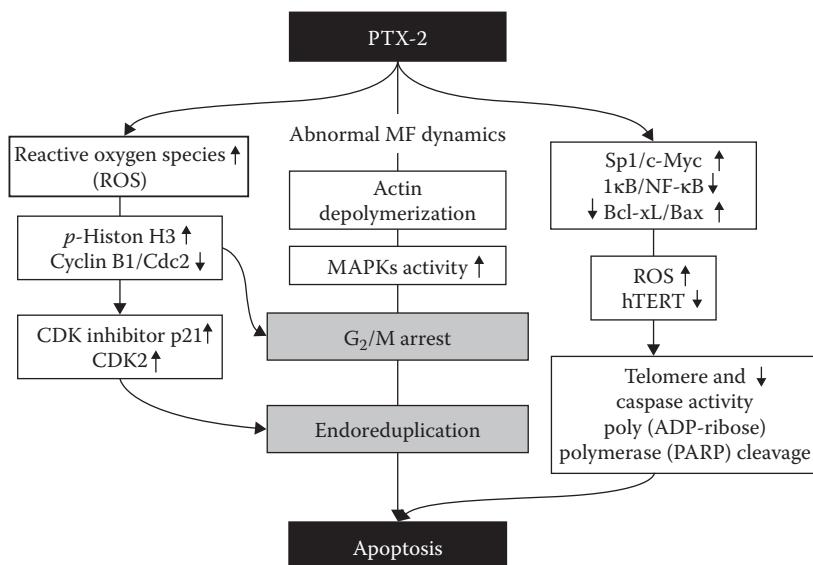
Telomeres are repetitive DNA sequences located at the termini of linear chromosomes, which are responsible for maintaining chromosomal stability (Philippi et al. 2010). As human telomeres grow shorter, cells eventually reach the limit of their replicative capacity and progress into senescence. However, when cells begin to become cancerous, they divide more often but their telomeres maintain not to be short (Nakamura et al. 1997). The enzyme known as telomerase can prevent this activity by elongating the telomeres. Therefore, this enzyme offers an attractive target for chemoprevention and other anticancer strategies.

The telomerase ribonucleoprotein complex consists of two essential components: (1) a catalytic protein subunit, human telomerase reverse transcriptase (hTERT), and (2) a template RNA (hTR) (Cong, Wen, and Bacchetti 1999). The hTR is present in all cell types, whereas hTERT mRNA is not detected in telomerase-negative cells where it is present in germinal and cancer cells. The promoter region of hTERT contains E-boxes and GC-boxes, the consensus binding sequences for Myc and Sp1, respectively (Kim, Moon, Kang et al. 2008). It is noted that Myc and Sp1 activate *hTERT* transcription by binding with the promoter region and subsequent cell proliferation. It is also known that posttranscriptional and postranslational modifications tightly control the activity of hTERT. Therefore, hTERT has received considerable attention for its role in regulating telomerase activity.

Thus, many researches have been done by focusing on the telomerase activity regarding hTERT with different anticancer applications. According to our findings, PTX-2 suppressed telomerase activity in human leukemia cells via the transcriptional downregulation of hTERT through a reduction of c-Myc and Sp1 activities (Kang et al. 1999). Akt1 is involved in cellular survival pathways, by inhibiting apoptotic processes. It is noted that Akt phosphorylation could also be a potent inducer of telomerase activation via hTERT phosphorylation linked to nuclear localization. PTX-2 was observed to downregulate p-Akt and the phosphorylation and translocation of hTERT. Thus, PTX-2 treatment regulates hTERT at the posttranslational level by downregulating its phosphorylation by the Akt pathway (Kang et al. 1999). Finally, PTX-2 can be used as an inhibitor of telomerase activity via the transcriptional and posttranslational suppression of hTERT.

## 28.5 CONCLUSION

Cancer is the uncontrolled growth of cells coupled with their malignant behavior, invasion, and metastasis. Most commonly, chemotherapy was applied for cancer treatment in past decades. As a result of the treatment of chemotherapeutic drugs, the effect on any particular stage of cell functions led to cell death. This chapter focuses on refreshing the anticancer effect of PTX-2 on different cancer cells (Figure 28.3). It is noted that PTX-2 regulates the normal behavior of cancer cells through different cell signaling pathways. Therefore, it is obvious that PTX-2 has good potential to be used as an anticancer drug in the future has good potential to be used as an anticancer drug in



**FIGURE 28.3** Schemes of pectenotoxin-2-induced G<sub>2</sub>/M arrest, endoreduplication, and apoptosis in human cancer cells: in the figure, MF denotes microfilament.

the future. So it is necessary to conduct further research to investigate moreover anticancer properties of these chemicals and finally used as cancer therapeutics.

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# 29 Marine Mucin

## *A Prospective Cosmetic and Pharmaceutical*

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### 29.1 BACKGROUND

Recently, interest in mucins has been increasing in a steady fashion, particularly in pharmaceutical and cosmeceutical studies. A large number of secondary metabolites produced by microorganisms, plants, and marine organisms have attracted much attention as bioactive substances for complementary treatment of diseases. In addition, various secondary metabolites have been widely used as preventive agents for skin related diseases. In this context, marine-based sources such as animals, plants, bacteria, and fungi have been thoroughly studied and used as ways to find new agents with certain bioactivities (Haefner 2003; Hill and Fenical 2010). To date, several cosmeceutical compounds have been extracted and characterized from marine sources (Kim et al. 2008). Marine mucin which is a common cosmeceutical, has been isolated from marine molluscs in different forms with various bioactivities. (Kimura, Gohda, and Sakurai 2003).

Mucins belong to a family of large extracellular high-molecular-weight O-glycosylated proteins, which are formed by tandem repeats of amino acid chains rich in cysteine, serine, and threonine coated with oligosaccharide side chains (Sheehan et al. 1991; Gerken 1993). Mucin serves biosystems as an efficient lubricant, as a mediator in cell signaling and, most importantly, by forming chemical barriers in mainly epithelial and connective tissues as well as several tissues with mucose layers. These crucial roles of mucin are a result of its gel-like structure (Linden et al. 2008). Mucins are reported to present in two different forms in biological systems. They are secreted or found in membrane-bound forms. As expected, mucins in membrane-bound forms are responsible for cell-cell and cell-extracellular matrix signaling, whereas secreted mucins are characterized as being produced by epithelia with the ability of forming viscoelastic gels, which is responsible for the elasticity and liquid state of mucus. Moreover, possible biological role of the membrane-bound mucins

in cancer development leaded to several studies which focused on clarifying the action mechanism of mucin in detail. (McGuckin et al. 1995; Ajioka, Allison, and Jass 1996).

Mucins are able to bind high amounts of water during the process of protecting a compound from proteolysis owing to its tandem amino acid repeats with linked sugar chains. Therefore, mucins can easily keep their mucosal state, enabling the utilization of mucins as protective or drug delivery agents (Ahuja, Khar, and Ali 1997). Mucins are considered to be a significant lead for further development and discovery of novel bioactive agents. Antibacterial effect of mucin has been reported and improved by derivatization which added value to potential utilization of mucin as an important cosmeceutical (Slomiany et al. 1996).

In addition to the aforementioned properties of human mucins, mucins with several other properties have been isolated from marine sources. It has been suggested that these mucins are of higher efficiency in terms of skin protection and moisturizing. In this manner, further detailed research along these lines is being carried out to improve the utilization of mucin as a cosmeceutical agent.

## 29.2 MUCINS

### 29.2.1 MUCIN STRUCTURE

Mucins possess distinct properties regarding the structural components of several mucin and mucin derivatives. The unique biophysical and biochemical properties of mucins are due to their large sizes and densely glycosylated regions. Mucins are quite large glycoproteins with molecular weights ranging from 0.5 to 20 MDa. These extracellular, highly glycosylated proteins are found in secreted and membrane-bound forms, which share many common features. It is noted that 50%–80% of mucin molecular mass consists of a highly glycosylated part, largely *N*-acetylglicosamine, *N*-acetylgalactosamine, *N*-acetylneuraminic acid, fucose, and galactose (Offner and Troxler 2000). The high hydrophobicity of the carbohydrate part of mucin forms the transmembrane domain of the membrane-bound mucins. The carbohydrate chains are linked to the protein core of the mucins by O-linked glycosylation of hydroxyl side chains of threonine and serine, which primarily make up the protein core (Perez-Vilar and Hill 1999). The rest of the molecular mass (20%–50%) is formed by the protein core. The protein structure of mucin includes distinct regions. Extensive tandem repeats of serine, threonine, and proline comprise the main glycosylation region through their hydroxyl side chains, which enable O-linked glycosylation of the carbohydrate chain of mucin. Other regions are amino and carboxyl ends of the protein core, and they have high amounts of cysteine. Large amounts of cysteine in these regions are suggested to be responsible for globular formation and the subsequent polymerization of multimers formed from dimmers via disulfide bond formation (Carlstedt, Lindgren, and Sheehan 1983).

Mucins are a family having a huge number of members as all animals including marine-originated ones produce mucin and mucin-like glycoproteins. Although all mucins share some common features, mucins from different origins show distinct properties as the contents of carbohydrate chains and protein cores and the fashion of *O*-glycans vary and result in various efficiencies in terms of biophysical properties (Park 2007).

Characterization of mucins is rather difficult biochemically due to their complex O-glycosylation and the large sizes of molecules. However, as a consequence of ongoing and increasing interest on mucins, several organisms are subjected to extraction process of mucin, which results in improved characterization and screening results. Hence, mucins are able to be tested for their unique bioactivity in detail.

### 29.2.2 MUCIN RESOURCES

Mucin exerts its beneficial effect when it is applied topically in a similar manner with other agents of extracellular matrix and mucosae. Hence, the large-scale production of these compounds in related

industries exhibits a great advantage when placed in the market as pharmaceuticals, cosmetics, and food additives. However, in the case of mucin, dietary supply is on a basic level and mass production has not been introduced yet. Therefore, mucin is considered to be extracted and purified from raw materials as of the other components of mucosa and extracellular matrix which show distinct bioactivity, to be provided into market.

Mucin can be easily found abundantly in various organisms because of its primal duty as the primary component of mucus layers in animals. Recently, human mucins were thoroughly identified in both gene expression and glycoprotein secretion levels (Moniaux et al. 2001; Thornton, Rousseau, and McGuckin 2008). The close relation between mucin gene and cancer development urges the detailed studies which focus on identification and elucidation of the mucin pathway, from production to mechanism of action. (Wakatsuki, Yamada, and Narikiyo 2008). In this context, the biochemistry of mucins has been studied heavily in recent years, which enables researchers to study the unique properties and therapeutic actions of mucins from different resources.

Mammal saliva and intestinal epithelium have become the main sources for mucin isolation over the years. Several mucin genes in human beings have been identified and studied in detail (Moniaux et al. 2001). However, characterization of human mucin is aimed at identifying the role of mucin in diseases rather than the therapeutic activity of mucin. Reports claim that numerous diseases are in close relation with the systems in within mucus layer which also includes mucin. In order to treat mucin-related diseases or find novel bioactive mucins, several organisms are of great interest for mucin isolation such as slugs and mollusks, which are rich in mucus layers consisting of high amounts of unique mucins.

### 29.2.3 MARINE MUCINS

Several natural products from marine organisms have been reported to express bioactivity against prevalent diseases such as cancer, diabetes, and obesity. With marine species comprising approximately one-half of the total global biodiversity, the sea offers an enormous resource for compounds to be extracted and purified for therapeutic purposes (Aneiros and Garateix 2004). Moreover, many compounds have been identified from marine-based organisms with cosmeceutical effects and have been reported to protect skin and tissues from harmful outside effects. Furthermore, various kinds of substances have been acquired from marine organisms on the grounds that they are living in a quite oppressive, competitive, and aggressive environment, which is very different in many aspects from the terrestrial environment, a situation that enables the production of unique and potent active molecules. In this context, on account of the need for isolation of novel mucin-like substances to aid the potential pharmaceutical utilization of mucin, marine resources are considered to be important.

Marine species comprise organisms that produce diverse mucins to work as protective barriers and lubricants in their systems (Kimura et al. 2004; Ohta, Sato, and Ushida 2009; Urai, Nakamura, and Uzawa 2009). To date, mollusks have been the main source of extracellular matrix and mucosa-based substances because they exhibit relatively large amounts of mucus layers to protect themselves in the marine environment. Recent studies have shown that jellyfish has the potential to be a significant resource of mucin and mucin-like glycoproteins (Masuda et al. 2007). Several researchers have isolated and characterized distinctive mucin components from various jellyfish species. On the other hand, for therapeutic purposes, some species of squids are reported to produce bioactive mucins; they offer the ability to extract large amounts of mucins with a designed protocol (Kimura 2005). Mucosa layer-based components of the mollusks are reported to be diverse in chemical structures and they present in relatively high amounts. Mucins from marine resources will become the center of attention and be investigated in detail once their importance in therapeutic applications and further insights on novel cosmeceuticals become obvious.

### 29.3 THERAPEUTIC EFFECTS AND APPLICATIONS OF MUCINS

The concentration of secreted mucin is the key aspect of the physical state of mucus and its dependence on environmental factors such as ionic strength and pH is reported to be involved in several diseases (Lee et al. 2005). Mucus acts as a physical barrier to extracellular toxins, mainly bacteria, and consists of high amounts of mucin as the main component. However, some bacteria possess adhesins, which make bacteria able to reside in the mucus by binding to mucins. Several bacterial infections are reported to be caused by this interaction between bacteria and mucins (Dharmani et al. 2008). Ulcers, for instance, are a result of *Helicobacter pylori*, which resides in the mucus layer of the stomach (Slomiany and Slomiany 1991). Considering the lack of a barrier ability against bacteria leads the infections, keeping or improving the physiochemical properties of mucin in order to form a strong barrier against any bacterial infection is a promising way to obtain a bacteria-free mucus layer. Several studies have shown that mucins from different tissues and organisms are able to suppress bacterial adhesion (Shi et al. 2000). Snail mucus mucin is known to possess strong antibacterial activity against both the gram-positive bacteria *Bacillus subtilis* and *Staphylococcus aureus* and the gram-negative bacteria *Escherichia coli* and *Pseudomonas aeruginosa* (Iguchi, Aikawa, and Matsumoto 1982). Surprisingly, mucins have the ability of not only showing antibacterial activity but also acting as a protective surfactant to cover biomaterials in order to suppress immunological response (Sandberg et al. 2009). Moreover, the lubrication action of mucin leads the way in producing oral surface covering substitutes for, among others, saliva (Berg, Lindh, and Arnebrant 2004). Additionally, these properties of mucin must be investigated and improved to prevent postsurgical adhesions, to present easiness for clinical usage of stents, and to exhibit protective treatment for contact lenses (Svensson and Arnebrant 2010). Mucus layers have an important role as the first step of nutrient and drug diffusion. Further, optimizing the mucus adhesion of nutrients and drugs is gaining more interest for improved absorption of nutrients and, more importantly, directed drug delivery. In this context, forming protecting mucins in gel form in order to keep a molecule away from acidic proteolysis promotes a great opportunity for designing pH-specific drug delivery. Besides, among bioadhesive drug delivery methods, mucoadhesive drug delivery systems are gaining importance in the pharmaceutical field due to their therapeutic advantage on controlling the rate and amount of drug release (Shaikh et al. 2011). This activity of mucins in drug delivery makes mucin studies a promising novel area in the medicine industry, especially in drug delivery applications.

### 29.4 MARINE MUCINS AS COSMECEUTICALS

Marine organisms are an extremely heterogeneous group and are excellent reservoirs for identifying and extracting biologically active substances having the potential to act as cosmeceuticals (Kim et al. 2008). Increasing interest for mucin from several fields pave the way to investigate the potential of mucin in detail. In this context, ability to provide diverse mucin and mucin-like glycoproteins makes marine resources center of attention regarding mucin studies.

The unique physical properties of mucins and mucin-like glycoproteins are considered to depend on their high sugar coating and protein core which varies between different tissues or organisms. The repeating structure of carbohydrate chains of mucin, as well as tandem repeats of amino acid chains, promotes mucin as a useful compound in various areas of interest. However, as a secretory large protein, the *in vitro* utilization of mucin is limited. Novel marine mucin-like glycoproteins are purified and characterized in order to present new applications for mucin rather than *in vitro* utilization. On the other hand, as the interest on mucin is growing, mucin is on the way to be utilized orally in addition to topical application. Several other materials such as gum, honey, disinfectants, gelatins, and preservatives are suggested to broaden or improve the effects of mucin when used as a composition or mixture (Adikwu and Alozie 2007; Momoh, Adikwu, and Eraga 2008).

Mucins from different organisms possess numerous effects and uses such as retaining the moisture of mucosa layers and epithelia, lubrication, and tissue protection. Mucin is also used as a substitute for original mucus-based layers such as saliva; a drug delivery carrier for effective absorption; and a barrier for protection against bacteria, viruses, and infectious components like tissue particles (Svensson and Arnebrant 2010). Considering these effects, it can be easily suggested that mucin holds much potential as a highly effective cosmeceutical. Cosmeceutical use of marine mucin, however, has not been studied widely, yet. Nonetheless, there are reports that are presenting valuable information regarding how significant further research in order to improve mucin efficiency which results in broad range of application in pharmaceutical and cosmetic industries.

As of the previous decade, several products including snail mucin are available in the market that claim to be effective cosmeceuticals with moisturizing, wound-healing, and antibacterial effects; the wound-healing properties of snail mucin have already been reported (Adikwu and Ikejiuba 2005). Snail mucin significantly reduced wound size on the fourth day of its application in comparison with nontreated wounds. Moreover, administration of snail mucin with honey exerted a higher wound-healing effect than that exerted when mucin acted alone. In addition to the wound-healing effect, snail mucin showed antibacterial effect against both gram-positive and gram-negative bacteria strains (Iguchi, Aikawa, and Matsumoto 1982). Recent studies also reported mucin as an enhancer for insulin absorption and a potential agent for drug delivery (Adikwu 2005). In light of detailed studies of the aforementioned properties of snail mucin, marine equivalents or derivatives of mucin-like glycoproteins have also attracted the attention of researchers in developing novel products of complementary treatment and food additives.

Qniumucin, a novel mucin derivative, has been isolated from several species of jellyfish and its structure thoroughly characterized (Masuda et al. 2007). This mucin is reported to be similar to the human-type mucin MUC5AC, which also consists of eight residues. The study has showed that this mucin has a repeating tandem of Val-Val(Ile)-Glu-Thr(-GalNAc)-Thr(-GalNAc)-Ala-Ala-Pro. Interestingly, it has been reported that qniumucin comprises some different sugars in its glycosylation parts along the threonine-*N*-acetyl-D-galactosamine linkage. Unlike vertebrate mucins, this marine mucin does not include sialic acid. As a result of these unique properties, mucin becomes closer to be a highly efficient therapeutic agent. Mucin from moon jelly (*Aurelia aurita*) was investigated for hygroscopic and moisturizing properties in comparison with a strong moisturizing agent, hyaluronic acid, which is used widely in cosmetics today. The results clearly showed that this marine mucin from jellyfish exhibits three times more moisturizing and hygroscopic activities compared to hyaluronic acid (Ushida et al. 2008). Furthermore, the chemical and biophysical properties of this marine mucin lead the way for the utilization and application of this compound as a protective biomaterial with lubricating and moisturizing effects as well as a starting material for the large-scale industrial synthesis of bioactive mucin-like glycoproteins. In addition, mucin extraction yield from jellyfish was quite high compared to that of terrestrial sources which offers more profitable utilization in industrial approaches.

Recently, a marine mucin was isolated from the nidamental gland mucilage of a marine mollusk, *Todarodes pacificus*, at the factory production level by solubilization with mild alkali followed by precipitation with alcohol containing NaCl (Aso and Kimura 2006). The protocol that was used for the isolation of this marine mucin is considered to strengthen the possibility of extraction of large-scale mucins to be used by the cosmeceutical industry. Similar to qniumucin, this mucin also does not include sialic acid and shows unique properties that are different from vertebrate mucins. The protein core of this mucin consists of threonine, proline, and isoleucine, and the weight ratio of protein: sugar: sulfate in this mucin is 16.4 : 80.3 : 3.3. It has been reported that the aqueous solution is very viscous and transparent. Considering the reported properties of this mucin from squid, it is highly expected that mucin from different species of squids can serve as a new efficient cosmeceutical with high moisturizing and skin-protecting functions because of the expected similarity in structure but possible diversity in consistence and content (Aso, Kimura, and Miyata 2003).

## 29.5 CONCLUSION

To date, several mucin-like glycoproteins have been isolated and characterized from jellyfish, squid, and marine worms, which has resulted in increased attention and interest on marine mucin studies. Novel marine mucins have been reported to have unique properties and contents different from those of terrestrial mucin. The already studied properties of terrestrial mucin show the significance of mucin in wound healing; moisturizing; and skin protection from bacteria, viruses, and inflammatory infectants. Unfortunately, studies on the possible cosmeceutical effects of purified marine mucins are not yet enough to understand the true potential of mucins in this domain. Nevertheless, reported data on the moisturizing and skin-protecting effects of marine mucin are quite promising, and further studies into the detailed effects of marine mucin in terms of cosmeceutical efficiency are expected to produce significant results and insights that will lead to the wide application of marine mucin in the cosmeceutical domain.

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# 30 Neuropharmacological Properties of Marine Plants

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## 30.1 INTRODUCTION

Plants have formed the basis of traditional medicine systems that have been in existence for thousands of years, and plant-based traditional medicines continue to play an essential role in health care (Gurib-Fakim 2006). Although modern medicine is available in most countries, traditional herbal medicines have maintained popularity for historical and cultural reasons. In addition, they have been used as alternative or complementary therapies, including medicinal herbs (WHO 1999). In the pharmaceutical industry, natural products isolated from medicinal plants have served as models for the creation of synthetic analogues (Borowitzka 1995). Natural products or drugs derived from them represent more than 50% of all the drugs in clinical use, such as many of those used for the treatment of cancer (Alonso, Castro, and Martinez 2005; Gurib-Fakim 2006).

Pharmacognosy of medicinal plants and their natural products has been restricted to terrestrial plants. In historical view, due to easy accessibility of terrestrial plants, they have served as the major source of traditional medicines and modern pharmaceuticals (Borowitzka 1995; Jha and Zi-rong 2004). Such classic drugs as aspirin, vincristine, and morphine were derived from natural products isolated from terrestrial plants (Villa and Gerwick 2010). Pharmacologically active compounds from terrestrial plants still play a role as an important pipeline for development of new drugs (Newman and Cragg 2007; Molinski et al. 2009).

Marine plants (macroalgae or seaweeds) are as important as terrestrial plants in the pharmaceutical and nutraceutical industries. The use of marine plants in the treatment of human ailments has a long history (Hoppe 1979; Glombitza and Koch 1989). In China, early records of medicinal marine plants appeared in Chinese literature about 2000 years ago (Chengkui et al. 1984). In Asian countries such as Korea, Japan, and China, several brown seaweeds have been used for centuries as traditional medicines for the treatment of hyperthyroidism and hypotension (de Almeida et al. 2011). Prior to the 1960s, the medicinal properties of seaweeds were restricted to traditional and folk medicines (Lincoln, Strupinski, and Walker 1991).

Marine plants have been promising sources of bioactive primary and secondary metabolites (Faulkner 2002; de Almeida et al. 2011), and their discovery has significantly expanded in the past three decades (Cardozo et al. 2007; O'Sullivan et al. 2010). However, modern pharmacognosy of marine plants is still in its infancy. This may be due to the lack of ethnopharmacological history and the difficulty of collection (Faulkner 1992; Jha and Zi-rong 2004). In addition, pharmacognosy of marine plants has been performed mainly in Asian countries, where such plants are frequently cultivated and consumed.

Recent trends in drug research from natural sources suggest that marine plants are a promising group to furnish novel bioactive substances (Ioannou and Roussis 2009), and pharmaceutical firms have started looking toward marine organisms, including seaweeds, in their search for new drugs from natural products (Smit 2004).

After the isolation of neuropsychoactive constituents, such as morphine from opium poppies, that affect the central nervous system (CNS), scientific understanding on neuropharmacological effects of terrestrial plants has significantly advanced over the past two centuries (Pengelly 1997). Furthermore, during the past few decades, research in the area of herbal neuropharmacology has increased markedly, with an abundance of *in vitro* and *in vivo* studies validating neurological effects (Kumar 2006; García-García et al. 2008; Sarris et al. 2011). Several terrestrial plants provide a high level of evidence, such as St John's wort (*Hypericum perforatum*) for depression and valerian (*Valeriana officinalis*) for insomnia (Bent et al. 2006; Sarris et al. 2011). Presently, a number of medicinal plants are available as over-the-counter herbal medicines.

Although a large number of studies on neuropharmacological effects of terrestrial plants and their constituents have been performed, marine plants have undergone successive periods of neglect in neuropharmacology. There is very limited information on the potential application of natural products from marine plants for neuropsychiatric disorders. Recently, in the area of marine pharmacognosy, the development of marine natural products is emerging as an important field for neuropharmacology (Martinez 2007). Several marine drugs derived from marine organisms (worm and snail) are currently in clinical trials or have been launched for the treatment of neuropathic pain, schizophrenia, and Alzheimer's disease (AD) (Martinez 2007; Alicino et al. 2012; Zawieja, Kornprobst, and Métais 2012). When considering numerous pharmacologically active metabolites and previous reports on neuropharmacological effects of marine plants (Smit 2004; Cho, Yang et al. 2012), they can be considered as a promising source for development of novel neuropsychological drugs like terrestrial plants and other marine organisms.

Until now, a comprehensive look at the neuropharmacological properties of marine plants has been absent in reviews and in books for marine pharmacognosy. Therefore, this chapter focuses on neuropharmacological effects and the potential of marine plants as a source for novel drugs to treat neuropsychological disorders.

## 30.2 SEDATIVE-HYPNOTIC EFFECTS

Sleep is a complex neurological process that is important in mammalian homeostasis and required for survival (Lorton et al. 2006). In humans, sleep is vital to maintain health and well-being due to its primary function of providing rest and restoring the body's energy levels (Krueger et al. 2008). However, insomnia is currently a widespread health complaint and has become a more prevalent and disruptive problem in the modern society (Borja and Daniel 2006; Doghramji 2006; Erman 2008). According to the numerous surveys conducted in countries around the world, over 30% of the adult population suffers from chronic or occasional insomnia (NIH 2005).

Herbal sleep aids, which contain specific constituents or extracts of medicinal plants, have recently become popular as alternative medications to prescription sleep drugs to improve sleep quality and avoid side effects (Meletis and Zabriskie 2008). In Western countries, herbal medicines, such as valerian (*V. officinalis*), St John's wort (*H. perforatum*), passionflower (*Passiflora incarnata*), and kava-kava (*Piper methysticum*), are readily available (Attele et al. 2000; Meolie et al. 2005). In particular, the valerian root preparation is the world's top-selling natural sleep aid, and its hypnotic effects have been recognized since the eighteenth century in Europe (Bent et al. 2006; Fernández-San-Martín et al. 2011). It has been reported by numerous clinical trials that valerian might improve sleep quality without producing side effects (Bent et al. 2006).

A large number of studies on sedative-hypnotic terrestrial plants have been performed, whereas marine plants have not been recognized as a potential source of natural hypnotics. Until now, there

**TABLE 30.1**  
**Sedative-Hypnotic Marine Plants**

Marine Plant	Extract	Activity	Active Compounds	Mechanism	Reference
<i>Cystophora moniliformis</i> (Phaeophyta)	Water-soluble	Sedative Anticonvulsant	Farnesylacetone derivatives	—	Baker (1984) and Ravi et al. (1982)
<i>Plocamium mertensii</i> (Rhodophyta)	Lipid-soluble	Sedative Anticonvulsant	—	—	Baker (1984)
<i>Plocamium costatum</i> (Rhodophyta)	Lipid-soluble	Sedative	—	—	Baker (1984)
<i>Himanthalia elongate</i> (Phaeophyta)	Protein-rich	Sedative-hypnotic Anticonvulsant Myorelaxant	—	—	Anca et al. (1990), Anca, Lamela, and Calleja (1993), and Anca et al. (1993)
<i>Sargassum tenerimum</i> (Chlorophyta)	Methanol	Sedative-hypnotic	—	—	Kamat et al. (1994)
<i>Caulerpa sertularioides</i> (Chlorophyta)	Methanol	Sedative-hypnotic	Caulerpin	—	Kamat et al. (1994)
<i>Cystoseira usneoides</i> (Phaeophyta)	Methanol	Sedative-hypnotic Anticonvulsant	—	—	Vázquez-Freire et al. (1995)
<i>Ecklonia cava</i> (Phaeophyta)	Ethanol and enzymatic hydrolysis	Sedative-hypnotic Anticonvulsant	Phlorotannins (eckstolonol, triphlorethol A, eckol, fucodiphlorethol G, 6,6'-bieckol, and dieckol)	Positive allosteric modulation of GABA <sub>A</sub> -BZD receptors	Cho, Yang et al. (2012) and Cho, Han et al. (2012)

are few reports on the sedative-hypnotic effects of marine plants. Table 30.1 shows sedative-hypnotic marine plants and their active compounds and mechanism.

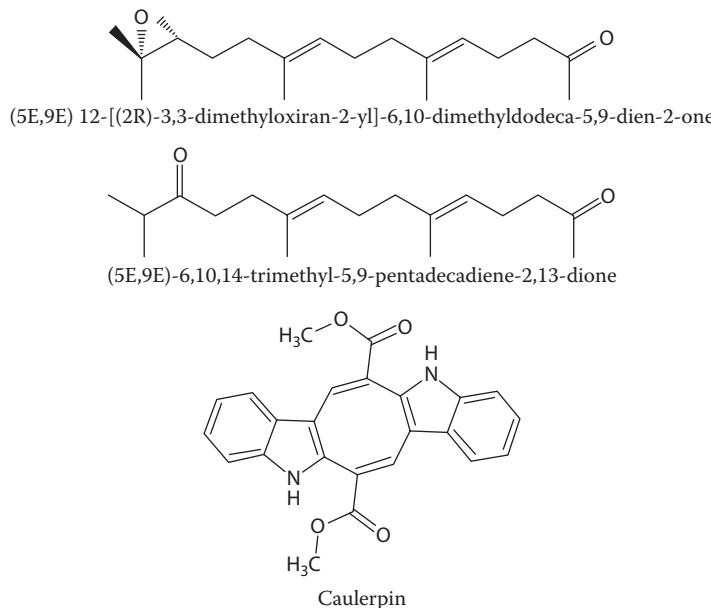
Early studies on neuropharmacological properties of marine plants were performed by the initiative that was manifested in the Roche Research Institute of Marine Pharmacology (RRIMP) from 1974 to 1981 (Baker 1984). This was the first major commercial attempt to mount an integrated multidisciplinary approach to the challenges of pharmaceutical investigation and development of marine plants (Baker 1984). At this level of preliminary investigation, 159 Australian marine plants, covering Chlorophyta (green seaweed), Rhodophyta (red seaweed), and Phaeophyta (brown seaweed), were investigated for various pharmacological activities including neurological effects. In the investigation of RRIMP, the water-soluble extract of *Cystophora moniliformis* (Phaeophyta) and the lipid-soluble extracts of *Plocamium mertensii* and *Plocamium costatum* (Rhodophyta) showed sedative or anticonvulsant activities. Two farnesylacetone derivatives (Figure 30.1) with anticonvulsant activity were isolated from the extract of *C. moniliformis* (Kazlauskas, Murphy, and Wells 1978; Ravi et al. 1982); however, precise mechanism of anticonvulsant activity of these compounds was not demonstrated.

According to the report of Anca et al. (1990), the *Himanthalia elongata* extract showed CNS depressant effects in mice. *H. elongata*, which is known by the common names sea thong and sea spaghetti, is a brown alga in the order Fucales and is found in the northeast Atlantic Ocean and the North Sea (The Seaweed Site 2012). The *H. elongata* extract (300 mg/kg, i.p.) significantly prolonged sleep duration in mice treated with barbiturate (55 mg/kg, i.p.) and postponed pentylenetetrazol-induced death. It also showed significant reductions in spontaneous motor activity and weak myorelaxant. Fractions F1 (Anca, Lamela, and Calleja 1993) and F2 (Anca et al. 1993) from *H. elongata* showed sedative-hypnotic effects like their extracts. In their studies, active constituents and mechanism were not demonstrated.

Kamat et al. (1994) screened 69 marine plants distributed in Indian coastal area for researching on CNS activity. Among the marine plant extracts, Chlorophyta *Sargassum tenerrimum* and *Caulerpa sertularioides* exerted CNS depressant activity. The methanol (90%) extracts of *S. tenerrimum* and *C. sertularioides* potentiated pentobarbital-induced sleep and showed depression of both spontaneous and locomotor activities. According to the report of Kamat et al. (1994), caulerpin (Figure 30.1), a constituent of *C. sertularioides*, could be responsible for its CNS depressant effects as caulerpin has been reported to produce sedation (Doty and Santos 1966).

The sedative-hypnotic effect of the brown alga *Cystoseira usneoides* was reported by Vázquez-Freire et al. (1995). Administration of the *C. usneoides* extract at 25 mg/kg significantly potentiated the pentobarbital-induced sleep and delayed pentylenetetrazole-induced convulsions. They suggested that the hypnotic effect of *C. usneoides* may be mediated by the  $\gamma$ -aminobutyric acid (GABA)ergic system; however, there was no experimental *in vitro* and *vivo* evidence supporting its GABAergic mechanism.

Recently, studies on hypnotic effect and GABAergic mechanism of the edible brown seaweed have been reported (Cho, Han et al. 2012; Cho, Yang et al. 2012). The extracts of 30 Korean seaweeds, including 8 green seaweeds, 11 red seaweeds, and 11 brown seaweeds, were screened for binding activity to the GABA type A-benzodiazepine ( $\text{GABA}_A$ -BZD) receptor (Cho, Yang et al. 2012). The  $\text{GABA}_A$ -BZD receptor has been considered a most important target for development of



**FIGURE 30.1** Two farnesylacetone derivatives isolated from *Cystophora moniliformis* (Phaeophyta) with anticonvulsant activity (Ravi et al. 1982), and caulerpin, a constituent of *Caulerpa sertularioides* (Chlorophyta). (From Kamat, S. et al., *Indian J. Exp. Biol.*, 32, 418–422, 1994.)

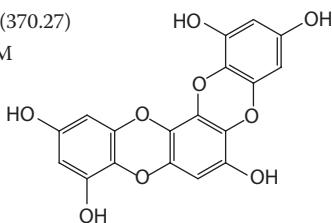
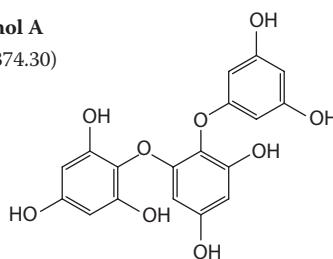
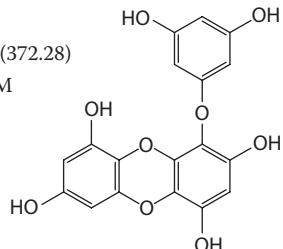
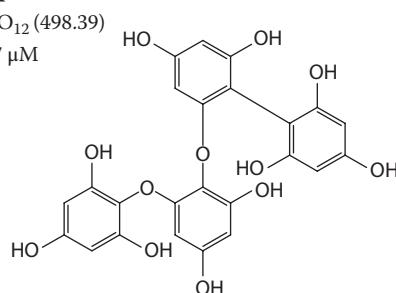
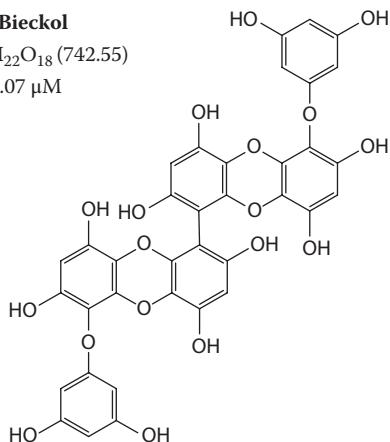
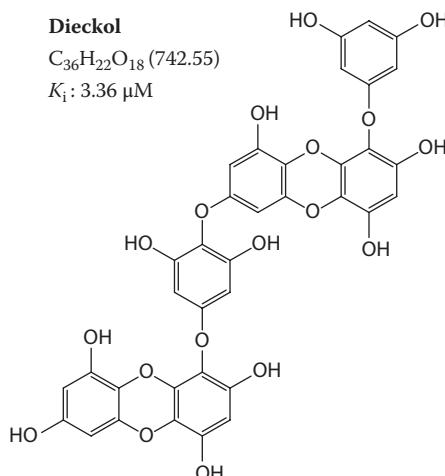
sedative-hypnotic drugs (Bateson 2006; Ebert, Wafford, and Deacon 2006). GABA is the major inhibitory neurotransmitter in CNS, and GABAergic neurotransmission plays a key role in sleep regulation (Smith and Simpson 2003). Both BZDs (i.e., diazepam) and non-BZDs (i.e., zolpidem) stimulate the ability of GABA to cause hyperpolarization of membrane by allowing a chloride anion ( $\text{Cl}^-$ ) influx (Trevor and Way 2007). As a result, the inhibition of neurotransmission is achieved, and subsequently, these agents produce sedative-hypnotic, anxiolytic, and anticonvulsant activities (Stephenson 1995; Erman 2005).

Among the screened Korean seaweeds, the most active seaweed was the brown alga *Ecklonia cava* (EC), and half-maximal inhibitory concentration ( $\text{IC}_{50}$ ) value of the EC ethanol extract (ECE) was 0.1269 mg/mL. In the hypnotic dose (45 mg/kg) of pentobarbital-induced sleep test, ECE (100–1000 mg/kg) resulted in a dose-dependent decrease in sleep latency and an increase in sleep duration. With a sub-hypnotic dose (30 mg/kg) of pentobarbital, ECE at 1000 mg/kg significantly increased the sleep duration ( $38.7 \pm 6.3$  minutes) and the rate of sleep onset (92%). This result indicates that ECE acts in a decisive role with regard to sleep induction in mice. An EC enzymatic extract at >500 mg/kg also showed significant anticonvulsant and hypnotic effects on the respective mice seizure induced by picrotoxin and on the mice sleep induced by pentobarbital (Cho, Han et al. 2012).

The ethyl acetate (EtOAc) fraction among the EC solvent fractions was found to have the lowest  $\text{IC}_{50}$  value (0.0185 mg/mL) for [ $^3\text{H}$ ]-flumazenil binding to the  $\text{GABA}_A$ -BZD receptor and to show the highest hypnotic effect at the concentration of 100 mg/kg in an animal assay. According to the previous reports on EC phlorotannins (Shibata et al. 2004; Kim et al. 2009; Lee et al. 2010), the EtOAc fraction of EC has the characteristics of a phlorotannin-rich fraction. Phlorotannins, which are oligomers and polymers of phloroglucinol (1,3,5-trihydroxybenzene), are an extremely heterogeneous group of compounds that are structurally different from polyphenols of terrestrial plants based on gallic acids or flavones (Shibata et al. 2002). Phlorotannins have only been found to exist within brown seaweeds (Shibata et al. 2004), and EC contains more phlorotannins than do other brown seaweeds (Heo et al. 2003). Six active phlorotannins (eckstolonol, triphlorellol A, eckol, fucodiphlorellol G, 6,6'-bieckol, and dieckol) were isolated from the EtOAc fraction of ECE using the  $\text{GABA}_A$ -BZD receptor binding activity-guided fractionation (Figure 30.2). Major phlorotannins of the EtOAc fraction were eckol, eckstolonol, dieckol, and triphlorellol-A, and their  $K_i$  (binding affinity,  $\mu\text{M}$ ) values for [ $^3\text{H}$ ]-flumazenil binding were 1.070, 1.491, 3.072, and 4.419 respectively. In the report of Cho, Yang et al. (2012), the marine natural product phlorotannins were for the first time characterized as a  $\text{GABA}_A$ -BZD receptor ligand. A number of flavonoids with affinity to the  $\text{GABA}_A$ -BZD receptors have been isolated from terrestrial plants (Jäger and Saaby 2011). For example, apigenin, which was isolated from chamomile (*Matricaria recutita*), was found to have a  $K_i$  value of 4  $\mu\text{M}$  (Viola et al. 1995). 6-Methylapigenin (*Valeriana wallichii*) (Wasowski et al. 2002) and hispidulin (*Artemisia herba-alba*) (Salah and Jäger 2005) were found to have  $K_i$  values of 0.5 and 8  $\mu\text{M}$  respectively. Wogonin with a  $K_i$  value of 0.92  $\mu\text{M}$  was isolated from *Scutellaria baicalensis* Georgi through the  $\text{GABA}_A$ -BZD receptor-binding assay (Hui et al. 2002). Binding affinities of phlorotannins were found to be similar to those of flavonoids isolated from terrestrial sedative plants.

The hypnotic effects of ECE and the EtOAc fraction were significantly inhibited by a specific  $\text{GABA}_A$ -BZD antagonist flumazenil (Figure 30.3), indicating that active compounds in ECE and the EtOAc fraction act directly at the BZD-binding site of  $\text{GABA}_A$  receptors. These findings support that EC induces sleep through positive allosteric modulation of the  $\text{GABA}_A$ -BZD receptors like the  $\text{GABA}_A$ -BZD receptor agonist diazepam (Figure 30.4). According to the following results of Cho and coworkers, six phlorotannins showed characteristics of the  $\text{GABA}_A$ -BZD receptor agonist with sedative-hypnotic activity through GABAergic mechanism. These results are the first report on the GABAergic mechanism of natural products from marine plants with hypnotic activity.

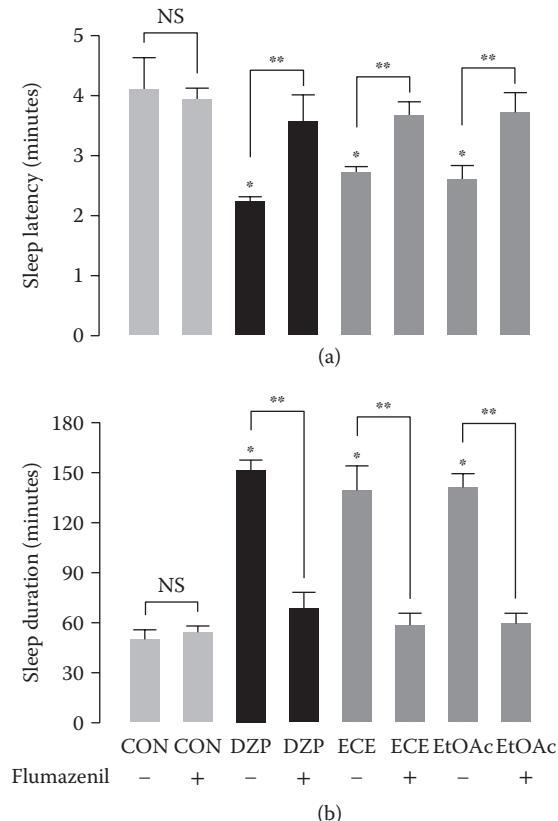
Studies on the sedative-hypnotic effects of marine plants have been not widely investigated relative to those of terrestrial plants. However, the above mentioned evidence shows the potential of

**Eckstolonol** $C_{18}H_{10}O_9$  (370.27) $K_i$ : 1.49  $\mu M$ **Triphlorethol A** $C_{18}H_{14}O_9$  (374.30) $K_i$ : 4.42  $\mu M$ **Eckol** $C_{18}H_{12}O_9$  (372.28) $K_i$ : 1.07  $\mu M$ **Fucodiphloretol G** $C_{24}H_{18}O_{12}$  (498.39) $K_i$ : 2.97  $\mu M$ **6,6'-Bieckol** $C_{36}H_{22}O_{18}$  (742.55) $K_i$ : 3.07  $\mu M$ **Dieckol** $C_{36}H_{22}O_{18}$  (742.55) $K_i$ : 3.36  $\mu M$ 

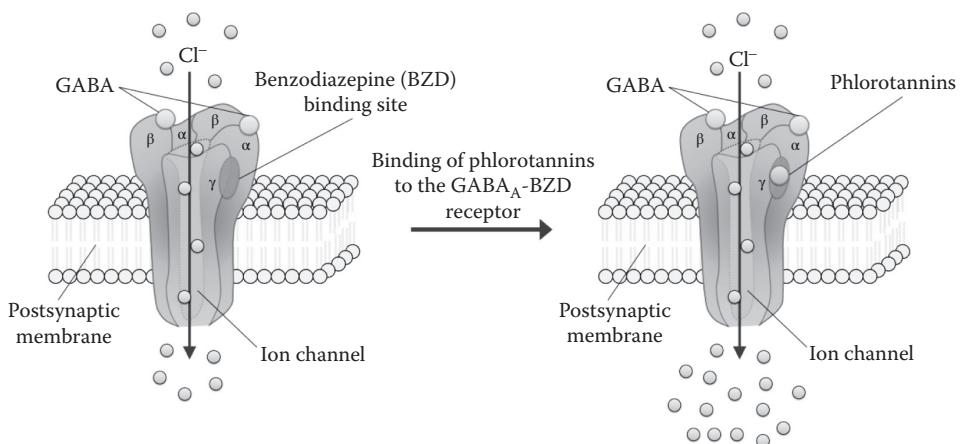
**FIGURE 30.2** Molecular structure, chemical formula (molecular weight), and binding affinity ( $K_i$ ) to the GABA<sub>A</sub>-BZD receptors of the active phlorotannins isolated from the ethyl acetate fraction of *Ecklonia cava* ethanol extract. (From Cho, S. et al., *Food Chem.*, 132, 1133–1142, 2012.)

marine plants as a sedative-hypnotic source. Depression of CNS is mediated by agonistic or antagonistic modulation of various receptors of neurotransmitters, such as serotonin, histamine, and adenosine as well as GABA (Sigel and Buhr 1997). Therefore, the binding and functional activity screening of the marine plant extracts for various neurotransmitter receptors related to sleep regulation gives significant information on research for the sedative-hypnotic marine plants.

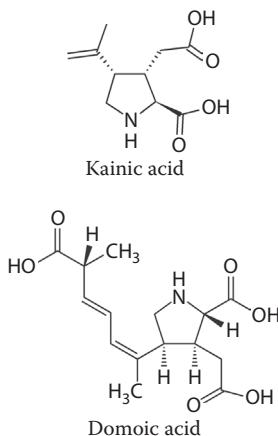
Neurotransmission in CNS is of two types, inhibitory and excitatory. As mentioned above, GABA is the major inhibitory neurotransmitter, and phlorotannins induce sleep by acting as a positive allosteric modulator of the GABA<sub>A</sub> receptors. In contrast, excitatory (CNS stimulant) activity of marine plants has also been reported. Chlorophyta (*Ulva fasciata* and *Chnoospora implexa*) and Rhodophyta (*Centroceras clavulatum*, *Gelidiella acerosa*, *Hypnea cervicornis*, and *Hypnea musciformis*) showed CNS stimulant activity as evidenced by a marked increase in spontaneous



**FIGURE 30.3** Effects of flumazenil on the changes in sleep latency (a) and sleep duration (b) in mice treated with ECE and its EtOAc fraction. Mice received pentobarbital 45 minutes after oral administration (p.o.) of CON (0.5% CMC-saline, 10 mL/kg), DZP (2 mg/kg), ECE (1000 mg/kg), and EtOAc fraction (200 mg/kg). Flumazenil (8 mg/kg, i.p.) was administered 15 minutes before oral administration. Each column represents mean  $\pm$  SEM ( $n = 10$ ). \* $p < .01$ , significant as compared to the control group (Dunnett's test). \*\* $p < .01$ , significant between flumazenil treatment and no flumazenil treatment (unpaired Student's  $t$ -test). CON, control group; DZP, diazepam; ECE, *Ecklonia cava* ethanol extract; EtOAc, ethyl acetate; NS, not significant. (From Cho, S. et al., *Food Chem.*, 132, 1133–1142, 2012.)



**FIGURE 30.4** Sleep-inducing mechanism of phlorotannins, known as brown seaweed polyphenol. Phlorotannins stimulate (allosteric positive modulation) the ability of GABA that induces Cl<sup>-</sup> influx. Hyperpolarization of neurons by Cl<sup>-</sup> influx inhibits the excitatory transmission and induces sleep.



**FIGURE 30.5** Neuroexcitatory marine natural products kainic acid and domoic acid isolated from marine plants.

and locomotor activities (Kamat et al. 1994). Kainic acid and domoic acid, a marine amino acid, with excitatory activity have been isolated from marine plants (Figure 30.5) (Laycock, Freitas, and Wright 1989; Moloney 1998). They have been demonstrated to act as a glutamate receptor agonist (Laycock, Freitas, and Wright 1989). The amino acid glutamate is responsible for most of the excitatory neurotransmission in CNS (Vogensen et al. 2011). These CNS stimulant marine plants and natural products have been known as a neurotoxin. *U. fasciata* showed 46.4 mg/kg of LD<sub>50</sub> (lethal dose 50%) (Kamat et al. 1994), and kainic acid and domoic acid induce seizure (Sawant et al. 2008). In particular, kainic acid has been used as a traditional medicine of anthelmintics in Japan (Shinozaki and Shibuya 1976).

### 30.3 NEUROPROTECTIVE EFFECTS

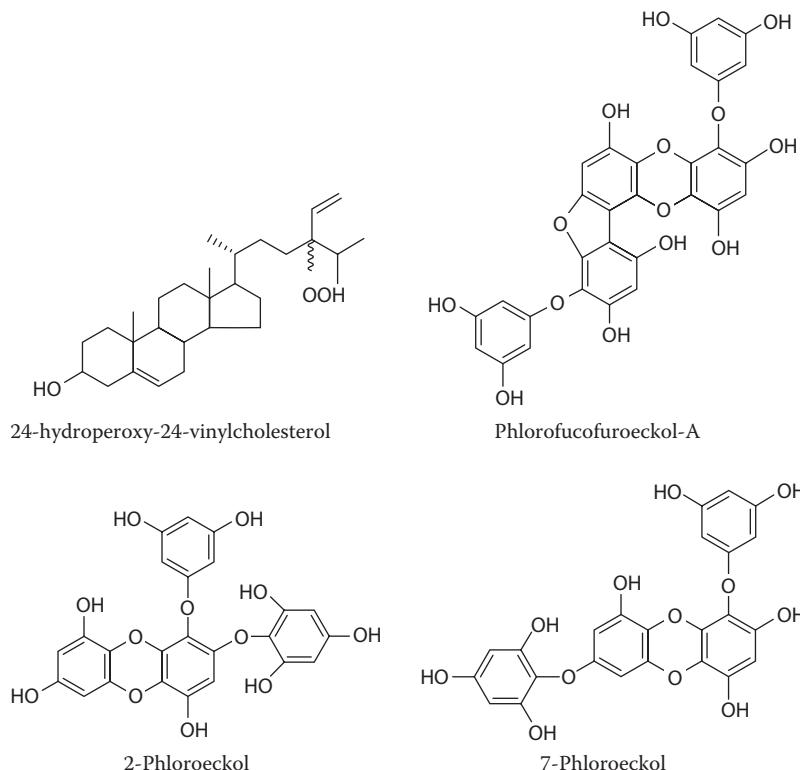
During the past decade, the neuroprotective effects of marine plants have been intensively investigated in the neuropharmacological research field of marine plants. In particular, many research groups have focused on a potential therapeutic agent from marine plants for the treatment of neurodegenerative disorders such as AD. Neurodegenerative diseases are estimated to surpass cancer as the second most common cause of death among elderly by the 2040s (Ansari, Siraj, and Inamdar 2010; Bjarkam et al. 2001). It has been reported that cholinergic function and neuroinflammation are deeply related to the neurodegenerative disorders (Tabet 2006; Tsartsalis, Panagopoulos, and Mironidou-Tzouveleki 2011).

AD, the most common form of dementia, is a progressive neurodegenerative disease, which results in memory loss, behavior disturbances, personality changes, and a decline in cognitive abilities (Stoppe et al. 1996; Pietrini et al. 2000). AD is uniquely characterized by the selective deposition in the cortical and hippocampal regions of aggregates of the  $\beta$ -amyloid (A $\beta$ , derived from amyloid precursor protein [APP]) (Francis et al. 1999; Hicks et al. 2011). In AD, there is an inexplicable failure of the cholinergic innervation of the cerebral cortex that arises from basal forebrain cholinergic neurons (Schliebs and Arendt 2011). According to this cholinergic hypothesis, deficiency in the neurotransmitter acetylcholine (ACh) is deeply associated with AD (Tabet 2006). Consequently, the inhibition of acetylcholinesterase (AChE) enzyme, which catalyzes the breakdown of ACh, has become one of the most prescribed treatment strategies for AD (Pangestuti and Kim 2010; Erdogan et al. 2011).

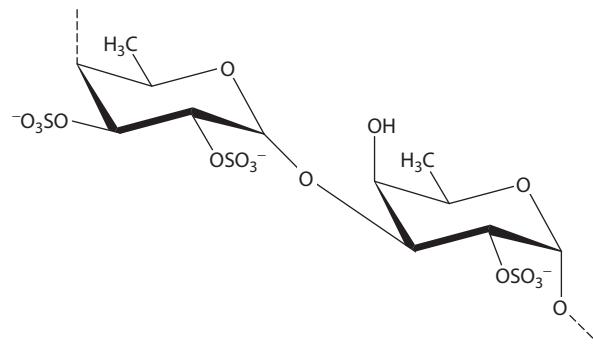
Recently, various marine plants and their constituents have been investigated for AChE inhibitory activity (Stirk, Reinecke, and van Staden 2007; Yoon et al. 2008, 2009; Suganthy, Karutha

Pandian, and Pandima Devi 2010). The AChE inhibitory activity of 27 Korean seaweeds was screened by Yoon et al. (2009). In their screening investigation, *Ecklonia stolonifera* showed significant inhibitory activity, and one sterol and eight phlorotannins were isolated as active compounds. The compounds ( $IC_{50}$ ,  $\mu M$ ) were 24-hydroperoxy-24-vinylcholesterol (389.1), eckstolonol (42.66), eckol (20.56), phlorofucoxanthin-A (4.89), dieckol (17.11), 2-phloroeckol (38.13), and 7-phloroeckol (21.11) (Figure 30.6). Moreover, eckstolonol and phlorofucoxanthin-A exhibited inhibitory activities toward both AChE and butyrylcholinesterase (BChE). Both AChE and BChE are important in pathogenesis of AD (Erdogan et al. 2011). However, phloroglucinol, which is a monomer, and triphlorellol-A, the opened-chain trimer of phloroglucinol, did not inhibit ACh at the concentrations tested. The results of the inhibitory activity of different phlorotannin compounds suggest that the degree of polymerization and the closed-ring structure play key roles in the inhibitory potential of phlorotannins (Yoon et al. 2009). Suganthy et al. (2010) screened several seaweeds distributed in South Indian coastal area. *Hypnea valentiae*, *Padina gymnospora*, *Ulva reticulata*, and *Gracilaria edulis* exhibited inhibitory activity to AChE with  $IC_{50}$  values of 2.6, 3.5, 10, and 3 mg/mL respectively, while *H. valentiae*, *Enteromorpha intestinalis*, *Dictyota dichotoma*, and *U. reticulata* showed 50% inhibition to BChE at concentrations 3.9, 7, 6.5, and 10 mg/mL respectively.

Most of the marine algal compounds with ChE inhibitory activity were phenolic compounds such as phlorotannins. However, it has been recently reported that fucoidan, a complex sulfated polysaccharide, derived from brown seaweed, improves cognitive impairment induced by infusion of A $\beta$  (Gao et al. 2012). Fucoidan (Figure 30.7) ameliorated A $\beta$ -induced learning and memory impairment in animal behavioral tests (Morris water maze, single-trial passive avoidance test, and eight-arm radial maze task). Furthermore, Gao et al. (2012) reported that fucoidan reversed the



**FIGURE 30.6** 24-hydroperoxy-24-vinylcholesterol and phlorotannins with acetylcholinesterase (AChE) inhibitory activity isolated from *Ecklonia stolonifera*. (From Yoon, N. Y. et al., *Fish. Sci.*, 74, 200–207, 2008.)



**FIGURE 30.7** Structure of fucoidans isolated from *Fucus vesiculosus*.

decreased activity of choline acetyltransferase, superoxide dismutase, glutathione peroxidase, and content of ACh as well as the increased activity of AchE and content of malondialdehyde in hippocampus of A $\beta$ -injected rats. Regulating the cholinergic system, reducing oxidative stress, and inhibiting the cell apoptosis were proposed as the mechanisms of its cognitive improvement.

Several synthetic drugs with ChE inhibitory activity are available; however, the side effects and the relatively low bioavailability limit their uses in medicine and there is still great demand to discover novel ChE inhibitors (Orhan, Orthan, and Gürkaynak 2011). Studies on the ChE inhibitory effects of marine algal compounds is in infancy stage, therefore marine plants have great potential as future drug candidates with better ChE inhibitory activity.

In AD, the cholinergic hypothesis is the dominant one; however, the neuroinflammation hypothesis also contributes to the pathogenesis of AD (Tsartsalis, Panagopoulos, and Mironidou-Tzouveleki 2011). Neuroinflammatory responses in the CNS have been known to play a critical role in the onset of neurodegenerative disorders (Liu et al. 2002; Li et al. 2011). The involvement of microglia and astrocytes in the onset and progress of neurodegenerative process in AD is becoming increasingly recognized (Agostinho, Cunha, and Oliveira 2010). Numerous studies show the presence of a number of markers of inflammation in the AD brain: elevated inflammatory cytokines and chemokines and accumulation of activated microglia in the damaged regions (Lee et al. 2010). McGeer and Rogers (1992) reported that long-term use of anti-inflammatory drugs suppresses the progression of AD and delays its onset, suggesting that there is a close correlation between neuroinflammation and AD pathogenesis.

*In vitro* and *vivo* anti-inflammatory activities of marine plants have been demonstrated by a number of investigations; however, their scientific approach of anti-neuroinflammatory activity has been poorly carried out, and until now, only a few studies have been reported (Pangestuti and Kim 2011). Jung and coworkers reported that the ethanol extract (Jung, Ahn et al. 2009) of EC and its active constituent dieckol (Jung, Heo et al. 2009) inhibit the levels of proinflammatory mediators such as nitric oxide (NO), prostaglandin-E<sub>2</sub> (PGE<sub>2</sub>), proinflammatory cytokines (tumor necrosis factor- $\alpha$  [TNF- $\alpha$ ]), interleukin-6 (IL-6) and IL-1 $\beta$  in lipopolysaccharides (LPS)-stimulated BV2 cells by blocking nuclear factor- $\kappa$ B (NF- $\kappa$ B), and mitogen-activated protein kinases (MAPKs). According to the report by Jin et al. (2006), *Ulva conglobata* methanol extract significantly attenuated the neurotoxicity induced by glutamate in HT22 cells and inhibited NO production induced by IFN- $\gamma$  in BV2 cells and completely suppressed the expression of the proinflammatory enzyme cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS). In their investigation, active constituents were not identified. Recently, the first evidence on anti-neuroinflammatory activity of fucoidans has been reported by Cui et al. (2010). Fucodian, which is 7000 Da, consisting of 48% total sugar (including 28% fucose) exerted a potent inhibitory effect against LPS-induced NO production by microglia. It also suppressed phosphorylation of p38 and extracellular signal-regulated

kinase (ERK) at a concentration of 125 µg/mL. The results suggest that this inhibitory action of fucoidan involves suppression of p38 and ERK phosphorylation.

Marine plants and their active constituents have shown potential as anti-neuroinflammatory agents. Considering the merits of marine plants, such as relatively low production costs, low cytotoxicity, safety, and wide acceptability, the possibility for industrial applications is great. However, clinical trials and evaluation in various animal model assays for marine algal anti-neuroinflammatory activity should be widely instigated.

Phenolics, carotenoids, and polysaccharides isolated from marine plants have been well known as marine antioxidants. Phenolic compounds act as free radical scavengers, reducing agents, and metal chelators and thus effectively inhibit lipid oxidation (Pangestuti and Kim 2011). Phlorotannin dieckol has been shown to scavenge reactive oxygen species (ROS) production in murine microglia (BV2) cells (Jung, Ahn et al. 2009). In addition, according to the report of Wijesekara, Yoon, and Kin (2010), most phenolics isolated from marine plants are responsible for antioxidant activities and protective effects against oxidative stress-induced cell damage. Carotenoids are one of the major antioxidants with a strong radical scavenging activity in marine algae (Nomura et al. 1997; Yan et al. 1999). Fucoxanthin obtained from *Padina tetrastromatica* has shown higher potential to be used as an antioxidant than β-carotene in modulating antioxidant enzyme in plasma and liver of retinol-deficient rats (Ravi Kumar, Narayan, and Vallikannan 2008; Sangeetha, Bhaskar, and Baskaran 2009). It has also been reported that marine algal sulfated polysaccharides, such as fucoidan, can be used as potent antioxidants (Wijesekara, Pangestuti, and Kim 2010; Jiao et al. 2011). Marine plants are a good source of antioxidants; however, the various antioxidant effects of phenolics, carotenoids, and sulfated polysaccharides in CNS have been not widely investigated.

In addition, seaweed compounds have been reported as a protective agent against neurotoxin materials. As an example, Aβ peptides have been demonstrated to possess neurotoxic effect on neuron and glial cells although the precise mechanisms by which this occurs have yet to be elucidated (Butterfield 2002). Excessive accumulation of Aβ in the brain has been characterized as a major pathological hallmark of AD, and recently, fucoidan has been reported to block Aβ neurotoxicity in neuronal cell by abolishing the inhibitory effect of Aβ on the phosphorylation of protein kinase C (PKC) (Garrido et al. 2002). In addition, Luo et al. (2009) showed that fucoidan isolated from *Laminaria japonica* was able to protect against 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced neurotoxicity in an animal model of Parkinsonism (C57/BL mice) and dopamnergic (MN9D) cells by antioxidative stress activity. Moreover, alginates from *Halimeda incrassata* and *Bryothamnion triquetrum* have been shown to protect methylmercury-induced neurotoxicity in GT1-7 cells (Fallarero et al. 2003).

Furthermore, a common pathological hallmark of various neurodegenerative diseases is the loss of particular subsets of neurons that may be a consequence of various forms of neural cell death, including necrosis and apoptosis (Bains and Shaw 1997). A study carried out by Jhamandas et al. (2005) successfully showed that fucoidan isolated from *Fucus vesiculosus* was able to protect rat cholinergic neuronal death induced by Aβ<sub>1-42</sub>. Fucoidan pretreatment inhibited the activation of caspase-9 and caspase-3, which have been suggested to mediate the terminal stages of neuronal apoptosis (Cowan et al. 2001). In addition, aqueous extracts of *B. triquetrum* have been demonstrated to protect GT1-7 cells' death produced by severe chemical hypoxia/aglycemia insult, which further reduced the cytotoxicity and early production of free radicals. The authors suggest that the protective effects of *B. triquetrum* extract are partially related to the presence of ferulic acid (Fallarero et al. 2006).

It was reported that neurite outgrowth is a fundamental neuronal feature and plays an important role in neuronal development during embryogenesis and in the adult brain (Khodosevich and Monyer 2010). *Sargassum macrocarpum* and its two active components, sargaquinoic acid and sargachromanol, have been shown to promote neurite outgrowth in rat pheochromocytoma (PC12) cells (Kamei and Sagara 2002; Tsang and Kamei 2004; Tsang et al. 2005).

In addition, phlorotannins derived from *Eisenia bicyclis* have been demonstrated to inhibit A $\beta$  cleavage enzyme (BACE-1) activity, which represents candidate biomarkers of AD (Tang et al. 2006; Jung, Oh, and Choi 2010). When considering that almost all currently available medications for AD are AChE inhibitors, the suppression of BACE-1 by phlorotannins will enhance the medications and/or therapy for AD patients. Furthermore, Lee et al. (2007) demonstrated that fucoidan treatment resulted in an increase in cell proliferation of human neuroblastoma (SH-SY5Y) cell induced by A $\beta$ . Hence, it may suggest that fucoidan has potential neuroprotective effects.

### 30.4 CONCLUSION AND FUTURE PROSPECTS

In neuropharmacognosy of marine plants, neuroprotective effects have been the most widely investigated activity, and sedative-hypnotic, anticonvulsant, and CNS stimulant activities have also been reported. This scientific evidence shows that marine plants have potential as neuropharmacological agents. However, neuropharmacognosy of marine plants is still in the infancy stage compared to that of terrestrial plants. Activities of marine plants on depression, stress, anxiety, appetite control, and pain relief have been not yet explored. In other words, this means that marine plants could be an attractive and promising source for finding novel neuropharmacological agents. There are over 10,000 species of marine plants worldwide (McHugh 2003). Most of the marine plants are underutilized or unused and are currently unexplored in the studies on CNS.

In CNS, there are numerous neurotransmitters such as ACh, dopamine, serotonin, histamine, norepinephrine, epinephrine, glutamate, and GABA. They exert their effects by binding to specific receptors on the neuronal membrane. Neuropsychological disorders are associated with impairments of the neurotransmitter system, and neuropharmacological drugs produce their therapeutic activity by acting as agonists and antagonists of neurotransmitter receptors. Therefore, in the research field of neuropharmacological effects of marine plants, screening of binding or functional activity of marine plant extracts and compounds on various neurotransmitter receptors is the most important step. It can give basic information to the researchers and the interested parties for research of neuropharmacological effects of marine plants. For example, marine plants contain various alkaloids such as phenylethylamine alkaloids and indole alkaloids (Güven, Percot, and Sezik 2010). Since alkaloid compounds are structurally similar to amines, such as serotonin, dopamine, and histamine, they may have potential affinity to the receptors of such neurotransmitters. Indeed, the brominated indole alkaloid 5,6-dibromo-N,N-dimethyltryptamine, which is isolated from marine sponge, showed antidepressant effect in animal assay (Kochanowska et al. 2008). Hu et al. (2002) reported that indole alkaloids from marine sponge showed high affinity for serotonin type 2 receptors.

With the aging world population, neuropsychological disorders are set to become a major public health challenge (Hobson, Meara, and Taylor 2007). The global burden of disease study conducted by the World Health Organization, the World Bank, and the Harvard School of Public Health has produced evidence that pinpoints neuropsychological disorders as one of the greatest threats to public health (WHO 2006). Therefore, the pharmaceutical industry is currently focusing on drug development to treat neuropharmacological disorders. Neuropharmacological study on marine plants is being a more and more important research area in marine pharmacognosy. Marine algal natural products will become one of the major pipelines for developing novel neuropsychotherapeutic drugs in the future.

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# 31 Antiparasitic Secondary Metabolites from Marine Actinobacteria

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## 31.1 INTRODUCTION

### 31.1.1 ACTINOBACTERIA SOURCE OF DRUGS

The order Actinobacteria is composed of approximately 80 genera. Actinobacteria are Gram-positive bacteria showing a filamentous growth. They are a group of organisms widespread in nature and play a significant role in the future of biotechnology, because of their importance as producers of vitamins, enzymes, antitumor agents, immunomodifying agents, and mainly, antibiotic compounds (Goodfellow, Williams, and Mordarski 1988). According to Sanglier et al. (1993), between 1988 and 1992, more than a hundred new molecules from actinobacteria were discovered.

The diversity of actinobacteria secondary metabolites is unrivaled and unmatched in medical significance, accounting for more than 50% of the antibiotics identified to date (Magarvey et al. 2004; Prudhomme et al. 2008). Approximately 75% of these originated from the *Streptomyces* genus and at least 5000 documented bioactive compounds are known as being produced by this

genus. In screening for microorganisms able to produce bioactive compounds, the exploration of new soils and habitats has been recommended. Secondary metabolites (natural products) have provided a fundamental source of drugs for fighting infection, inflammation, and cancer in humans.

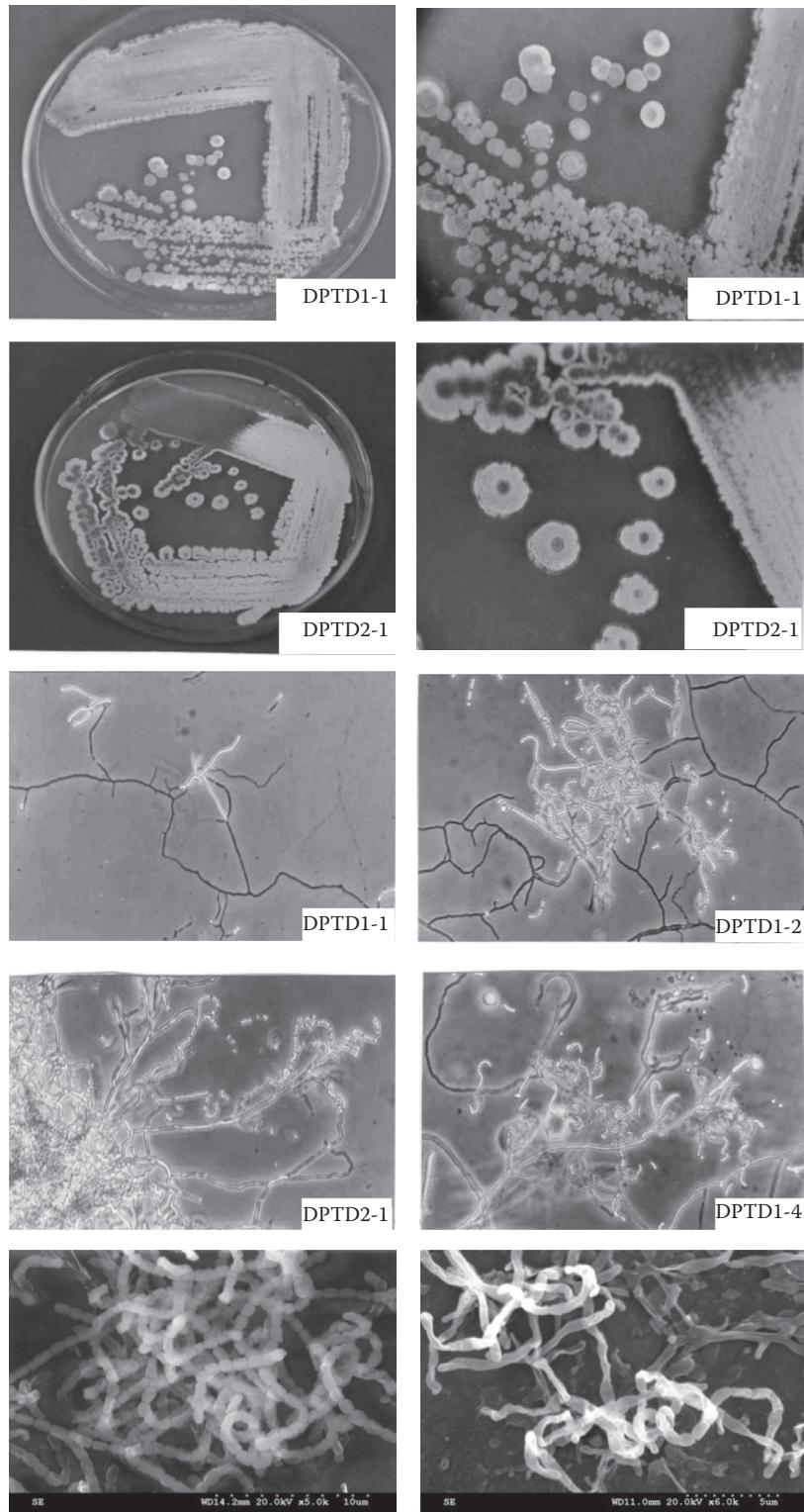
Extensive screening of terrestrial actinobacteria, started in the early 1950s, has yielded many important drug leads, later developed into antimicrobial (amphotericin B, erythromycin, vancomycin), anticancer (daunorubicin, bleomycin, mitomycin), and immunosuppressive (rapamycin) drugs (Zotchev 2011). Despite this apparent success, most of the actinobacteria-based screening programs at big pharmaceutical companies have been abandoned in recent years due to several reasons. One of the reasons was high cost of the internal screening programs, combined with the low number of new drug leads and relatively low profit on drugs such as new anti-infective (Donadio et al. 2010), anti-fungal, antibacterial, antihelminthic compounds (Dhanasekaran, Panneerselvam, and Thajuddin 2008b; Dhanasekaran 2010; Vijayakumar et al. 2012), fungicidal agents (Dhanasekaran, Thajuddin, and Panneerselvam 2011) anticandidial compound (Dhanasekaran, Panneerselvam, and Thajuddin 2008b; Saha et al. 2012), and herbicidal agents (Dhanasekaran, Thajuddin, and Panneerselvam 2010, Dhanasekaran et al. 2012). Another reason has been frequent rediscovery of the same compounds, mostly because of the redundancy of the samples, as well as strain isolation and screening technologies. In recent years, actinobacteria isolated from the marine environment sediments, sponges, tunicates, neuston, saltpan (Dhanasekaran et al. 2005; Dhanasekaran, Panneerselvam, and Thajuddin 2008a), estuary (Dhanasekaran et al. 2009), and mangroves (Dhanasekaran et al. 2010) have attracted considerable attention (Liu et al. 2010; Lane and Moore 2011). However, development of both sampling and cultivation techniques allowed isolation of representatives of several true marine actinobacterial genera producing novel compounds with interesting biological activities (Jensen et al. 2005).

Substantially increased interest in natural products, especially those originating from marine organisms, and the proven potential of marine bacteria to synthesize diverse compounds will inevitably make marine actinobacteria the focus of intensive research. Its major advantage is that, if successful, it will result in the production of a compound whose structure, novelty, and biological activity can be assessed straight away. This present chapter briefly describes actinobacterial antiparasitic compounds that may be useful for control and management of parasitic diseases (Figure 31.1).

### 31.1.2 ANTIPARASITIC COMPOUNDS

Ecological disturbances exert an influence on the emergence and proliferation of parasitic diseases including malaria, amoebiasis, leishmaniasis, trichomoniasis, cryptosporidiosis, giardiasis, trypanosomiasis, ascariasis, schistosomiasis, filariasis, onchocerciasis, and loiasis. Each environmental change, whether occurring as a natural phenomenon or through human intervention, changes the ecological balance and context within which disease hosts or vectors and parasites breed, develop, and transmit disease. Each species occupies a particular ecological niche, and vector species subpopulations are distinct behaviorally and genetically as they adapt to man-made environments. Deforestation and ensuing changes in land use, human settlement, commercial development, road construction, water control systems (dams, canals, irrigation systems, reservoirs) and climate, singly and in combination, have been accompanied by global increases in morbidity and mortality from emergent parasitic disease. The combined effects of environmentally detrimental changes in local land use and alterations in global climate disrupt the natural ecosystem and can increase the risk of transmission of parasitic diseases to the human population.

Nonetheless, infectious and parasitic diseases rank second among the “top killers” of the world, according to a WHO report on the global burden of disease. In low-income countries, especially in Africa, these diseases are even the dominant causes of death. In the developed world, the incidence of bacterial, viral, and parasitic infections was dramatically reduced throughout the twentieth century, mainly as a result of improved hygiene, sanitation the development of vaccines and antibiotics.



**FIGURE 31.1** Cultural and morphological characteristics of *Streptomyces* sp.

**TABLE 31.1**  
**Parasitic Diseases in Human, Plants, and Animals**

S. No.	Name of the Parasite	Mode of Transmission	Human/Plant/Animal Diseases
1.	<b>Protozoans</b>		
	<i>Plasmodium falciparum</i>	Mosquito born transmission— <i>Anopheles</i> mosquito	Malaria
	<i>Leishmania major</i>	Sandfly- <i>Phlebotomus</i>	Leishmaniasis, kala-azar, black fever, and Dumdum fever
	<i>Trypanosoma brucei</i>	Tsetse fly	African trypanosomiasis/Sleeping sickness
2.	<b>Helminths</b>		
	<i>Ascaris lumbricoides</i>	Contaminated vegetables, soil, and water	Ascariasis
	<i>Wuchereria bancrofti</i>	<i>Culex</i> mosquito	Lymphatic filariasis
	<i>Loa loa</i>	Deer fly/Mango fly— <i>Chrysops</i> spp.	Loa loa filariasis
	<i>Brugia malayi</i>	<i>Mansonia</i> , <i>Anopheles</i> , and <i>Aedes</i> mosquitoes	Lymphatic filariasis
	<i>Mansonella ozzardi</i>	Arthropods transmission— <i>Culicoides</i> sp. and <i>Simulium</i> sp.	Serous Cavity filariasis
	<i>Meloidogyne incognita</i>	Soil-borne transmission	Root knot—Vegetables, cereals, ornamentals, pasture, trees and shrubs, sugarcane, tobacco, cotton, potatoes
	<i>Enterobius vermicularis</i>	Human to human transmission by ingestion of infectious eggs	Enterobiasis
	<i>Trichuris trichiura</i>	Soil-transmitted helminths	Trichuriasis
	<i>Haemonchus contortus</i>	Feed and water borne transmission	Haemonchosis in sheep and goats
	<i>Caenorhabditis elegans</i>	Soil borne transmission	—

Diseases caused by tropical parasites affect hundreds of millions of people worldwide, but have been largely neglected for drug development because they affect poor people in poor regions of the world. Most of the current drugs used to treat these diseases are decades old and have many limitations, including the emergence of drug resistance. The organisms responsible for these diseases have a fascinating biology, and many potential biochemical targets are now apparent. These neglected diseases present unique challenges to drug development (Table 31.1).

## 31.2 SCREENING OF ANTI PARASITIC SECONDARY METABOLITES

### 31.2.1 ANTIPLASMODIAL ACTIVITY ASSAY

The antiparasitic compound was serially diluted and administered in quadruplicate to parasite cultures in 96-well plates to achieve 0.2% parasitemia with a 2% hematocrit. The plates were then incubated for 72 h at 37°C. Following incubation, 100 µL of lysis buffer containing 0.2 µL/mL SYBR Green I was added to each well. The plates were incubated for 1 h in the dark, and a 96-well fluorescence plate reader was used to measure relative fluorescence. The 50% inhibitory concentration ( $IC_{50}$ ) was determined using a nonlinear regression analysis of the logistic dose response curves using the software GraphPad Prism.

### 31.2.2 PARASITE PROLIFERATION ASSAYS IN *P. FALCIPARUM*

Parasite proliferation assays were done in 96-well plates using the DNA intercalating fluorescent dye, SYBR Green. The increase of parasite DNA contained in human red blood cells after 72 hours of incubation with actinobacterial extracts. The extracts were tested at three initial concentrations: 1, 10, and 100 mg/mL. Five crude extracts showed a 50 to 100% inhibition effect at 1 mg/mL. The most active fraction was further selected and identified to be the marine actinobacteria, *Salinispora tropica*.

### 31.2.3 NEMATICIDAL ACTIVITY

Culture broth, crude extract, and pure compounds from the nematicidal actinobacteria isolates were tested on eggs and second-stage nematode (*Meloidogyne incognita*). The samples were dissolved in dimethyl sulfoxide (DMSO) before adding water (final concentration: 0.5% DMSO), while the culture broth was tested directly. The concentration of samples was adjusted to 250 µg/mL. Active samples were further tested at 120, 60, and 30 g/mL (Nitao et al. 2002). Eggs were surface-disinfected with 1% sodium hypochlorite (NaOCl) and washed three times with sterile distilled water to remove residual NaOCl. They were incubated at room temperature (25 ± 3°C) for 48 h and the newly hatched nematode suspension was transferred into each well of a 24-well tissue culture plate containing 1mL of sample solution. As negative control, 0.5% DMSO in water was used. The plate was incubated at room temperature (25 ± 3°C) for 96 h and daily examined for dead juveniles (Sun et al. 2006). Three replicates were done for each sample. Living and dead nematodes were counted under the microscope; mortality was calculated. The immobile, malformed, or motionless nematodes when probed with a fine needle were considered to be dead.

$$\text{Juvenile mortality} = 100 \times \frac{\text{dead juveniles}}{\text{total juveniles}}$$

### 31.2.4 HATCHING INHIBITION ANALYSIS

The root-knot nematode *M. incognita* eggs, cultured in a greenhouse on chili were surface-disinfected with 1% NaOCl and washed three times with sterile distilled water to remove residual NaOCl. A 50 µL egg suspension (150–200 eggs/50 µL) was pipetted into each well of a 24-well tissue culture plate containing 1 mL of test solution. The plate was incubated at room temperature (25 ± 3°C) for 7 days and daily examined for egg hatch rate (Sun et al. 2006). Three replicates were done for each sample. Percentage of egg hatch was determined by counting all eggs and nematodes under the microscope and calculated.

$$\text{Percentage of egg hatch} = 100 \times \frac{\text{juveniles}}{(\text{eggs} + \text{juveniles})}$$

### 31.2.5 TOXICITY ASSAY

The effective nematicidal compound was tested for brine shrimp (*Artemia salina*) toxicity. The compound was dissolved in DMSO at a concentration of 1 mg/mL for this experiment (Jump Thong et al. 2010). The eggs (*M. incognita*) were suspended in artificial seawater and kept for hatching in a separation funnel under aeration for 3 days. A 990 µL larvae suspension (50 larvae) was pipetted into each well of a 24-well tissue culture plate. 10 µL of the DMSO solution was added into each

well as control. The plate was incubated at room temperature ( $25 \pm 3^\circ\text{C}$ ) for 24 h. The mortality of *A. salina* was determined by counting living and dead larvae were calculated.

$$\text{Mortality} = 100 \times \frac{\text{dead larvae}}{\text{total number of larvae}}$$

### 31.3 ACTINOBACTERIAL ANTIPARASITIC SECONDARY METABOLITES

#### 31.3.1 IVERMECTIN

Marine microorganisms produce several antiparasitic secondary metabolites. The more important ones are ivermectins produced by *Streptomyces avermitilis*. Ivermectin (Dihydro avermectin BI) is used in practice. It is a potent antiparasitic compound active against a broad spectrum of nematode and arthropode parasites (Ikeda and Omura 1995). Ivermectin is believed to act mainly through interactions with invertebrate glutamategated chloride channel, but other targets such as spleen cells and 2-aminobutyric acid receptors may also play important roles in the antiparasitic activity of ivermectin (Burkhard 2000). It is used to control internal and external parasites in animals and is also used in human medicine. More than 18 million people are treated with ivermectin each year. Delivery modes include oral, topical, and injections. Ivermectin is applied against *Onchocerca volvulus*, which evokes onchocerciasis (river blindness). It shows potent microfilaricidal activity against the major filarial parasites of humans: *Wuchereria bancroftii*, *Brugia malayi*, *Loa loa*, and *Mansonella ozzardi*. Ivermectin also has excellent efficacy against both human strongyloidiasis and cutaneous larva migrants for which good alternative treatments have not been available, and it is as effective as currently available drugs against the intestinal nematodes *Ascaris lumbricoides*, *Trichuris trichiura*, and *Enterobius vermicularis*; against the human hookworms, it shows only partial efficacy (Ottesen and Campbell 1994).

#### 31.3.2 ANTIMALARIAL $\beta$ -CARBOLINE AND INDOLACTAM ALKALOIDS

Four new  $\beta$ -carboline alkaloids, designated marinacarbolines A–D, two new indolactam alkaloids, 13-*N*-demethyl-methylpendolmycin and methylpendolmycin-14-*O*- $\alpha$ -glucoside, and the three known compounds, 1-acetyl- $\beta$ -carboline, methylpendolmycin, and pendolmycin, were obtained from the fermentation broth of *Marinactinospora thermotolerans* SCSIO 00652 a new actinobacteria belonging to the family *Nocardiopsaceae* (Huang et al. 2011). The new compounds were inactive against a panel of eight tumor cell lines ( $\text{IC}_{50} > 50 \mu\text{M}$ ) but exhibited antiplasmodial activities against *Plasmodium falciparum* lines 3D7 and Dd2, with  $\text{IC}_{50}$  values ranging from 1.92 to 36.03  $\mu\text{M}$ .

#### 31.3.3 MILBEMYCIN

Milbemycins are a large family of related 10-membered macrolide antibiotics with anthelmintic activity. First reported to have been isolated from *Streptomyces hygroscopicus* subsp. *aureolacrimosus* as part of an insecticidal screening program further milbemycins have since been isolated from the original, mutant strains and a number of other Streptomycetes. These include *S. cyanogriseus*, *S. thermoarchaensis*, and *S. hygroscopicus* E225. Milbemycins have also been isolated from a hybrid organism obtained by protoplast fusion of *S. avermitilis* and *S. hygroscopicus*. A closely related group of compounds, the avermectins, was discovered as a result of screening for compounds with anti-helminthic activity, and the structure of a number of different avermectins has now been elucidated.

### 31.3.4 ANTIMYCIN A9

A new antimycin group antibiotic, antimycin A9, was isolated from a cultured broth of *Streptomyces* sp. K01-0031 together with antimycins A3a, A3b, A4, and A7 and flazin methyl ester. Antimycin A9 is the first antimycin having an aromatic 8-acyl residue. It showed potent nematocidal and insecticidal activities against *Caenorhabditis elegans* and *A. salina*, respectively (Shiomi et al. 2005). It inhibited bovine heart NADH oxidase at nanomolar level like other known antimycins. Nematocidal and insecticidal activities of these isolated compounds were studied by a microplate assay using free-living nematode *C. elegans* and brine shrimp *A. salina*. Antimycins were active against both organisms.

### 31.3.5 FERVENULIN

The actinobacteria *Streptomyces* sp. CMU-MH021 produced secondary metabolites fervenulin that inhibited egg hatching and increased juvenile mortality of the root-knot nematode *M. incognita* *in vitro* (Lumyong et al. 2011). The chemical structure of fervenulin (6,8-Dimethylpyrimido(5,4- e)-1,2,4-triazine-5,7(6H,8H)-dione) 16S rDNA gene sequencing showed that the isolate sequence was 99% identical to that of *Streptomyces roseoverticillatus*. The culture filtrates from different culture media were tested for nematocidal activity. The maximal activity against *M. incognita* was obtained by using modified basal medium. The nematicidal assay-directed fractionation of the culture broth delivered fervenulin and isocoumarin. Fervenulin, a low molecular weight compound, shows a broad range of biological activities. However, nematicidal activity of fervenulin was not previously reported. The nematicidal activity of fervenulin was assessed using the broth microdilution technique. The lowest minimum inhibitory concentrations of the compound against egg hatching of *M. incognita* was 30 µg/mL and increase in juvenile mortality of *M. incognita* was observed at 120 µg/mL. Moreover, at the concentration of 250 µg/mL, fervenulin showed killing effect on second-stage nematode juveniles of *M. incognita* up to 100% after incubation for 96 h. Isocoumarin, another bioactive compound produced by *Streptomyces* sp. CMU-MH021, showed weak nematicidal activity with *M. incognita*.

### 31.3.6 NANCHANGMYCIN AND MEILINGMYCIN

*Streptomyces nanchangensis* NS3226 is a wild type producer of nanchangmycin and meilingmycin. *S. nanchangensis* was isolated from the soil in Nanchang, China. *S. nanchangensis* produces at least two kinds of insecticidal antibiotics (Sun et al. 2002). The polyether nanchangmycin structurally and biologically resembles dianemycin (Czerwinski and Steinrauf 1971), which is used in poultry farming. The 16-membered macrolide meilingmycin resembles milbemycin and has a similar aglycone and antiparasitic activity as ivermectin. In addition, *S. nanchangensis* produces at least two other antibiotics of unknown structure but with clearly different biological activities. Both meilingmycin and nanchangmycin are very active against a broad spectrum of harmful nematodes, insects and are nontoxic for mammals and plants (Ouyang et al. 1993).

### 31.3.7 BAFILOLIDES

During an evaluation of Australian actinobacteria for potential nematicides, three *Streptomyces* isolates, A233, A239 and A240, were identified as producing metabolites that strongly inhibited development in the free-living stages of the parasitic nematode *Haemonchus contortus*. Fermentation of the *Streptomyces* in liquid and on solid media led to the isolation of bafilolides A, B, C, and D from *Streptomyces* culture A239 and leucanicidin from cultures A233 and A240. The nematicidal activity of the bafilolides are restricted to the free-living nematode

*Caenorhabditis elegans*, where bafilomycin C was noted as a weak inhibitor, and to the pine wood nematode *Bursaphelenchus lignicolus*, where bafilomycin B and leucanicidin were also weakly active. The bafilolides are closely related to another group of macrolides, the hygrolidms (Lacey et al. 1994).

### 31.3.8 VALINOMYCIN, STAUROSPORINE, AND BUTENOLIDE

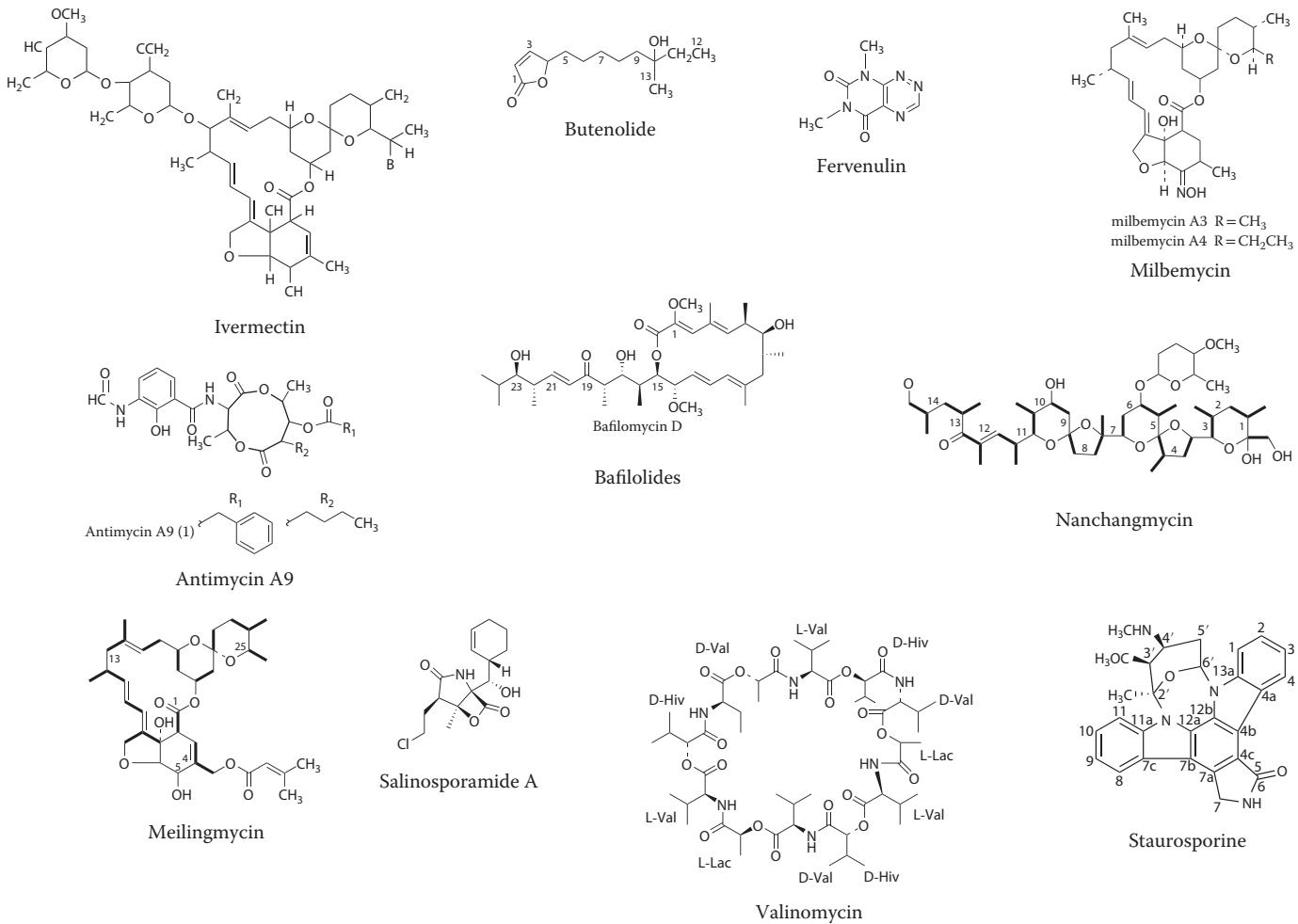
*Streptomyces* sp. from Mediterranean sponges were screened for antiparasitic activities. Bioassay-guided isolation and purification yielded three previously known compounds namely, cyclic depsipeptide valinomycin, indolocarbazole alkaloid staurosporine, and butenolide. This is the first report on the isolation of valinomycin from a marine source. These compounds exhibited novel antiparasitic activities specifically against *Leishmania major* (valinomycin  $IC_{50} < 0.11 \mu M$ ; staurosporine  $IC_{50} 5.30 \mu M$ ) and *Trypanosoma brucei brucei* (valinomycin  $IC_{50} 0.0032 \mu M$ ; staurosporine  $IC_{50} 0.022 \mu M$ ; butenolide  $IC_{50} 31.77 \mu M$ ).

### 31.3.9 SALINOSPORAMIDE A

Salinosporamide A, produced by the marine actinobacteria *Salinispora tropica*, shows strong inhibitory activity against the erythrocytic stages of the parasite cycle. The potential of secondary metabolites derived from marine microorganisms is used to inhibit *Plasmodium* growth. Currently, salinosporamide A is used in phase I trials for the treatment of refractory multiple myeloma and malaria (Prudhomme et al. 2008). *In vitro* effect of salinosporamide A showed an  $IC_{50}$  value against parasite growth of 11.461 nM. The  $IC_{50}$  range of chloroquine, mefloquine, and artemisinin were similar to that of the salinosporamide A under the culture conditions (Figure 31.2 and Tables 31.2 and 31.3).

## 31.4 CONCLUSION

Substantially increased interest in natural products, especially those originating from marine organisms, and proven potential of marine bacteria to synthesize diverse compounds will inevitably make marine actinobacteria the focus of intensive research. Because of the fact that actinobacteria are very diverse and each species might have special requirements not only for growth, but also for the production of secondary metabolites, this approach will require considerable effort. Its major advantage is that, if successful, it will result in the production of a compound whose structure, novelty, and biological activity can be assessed straight away. However, this cultivation-dependent technology is unlikely to reveal the full biosynthetic potential. The second approach, genome-based bioprospecting, will be developed in parallel to address this question. Its success will depend on such factors as development of efficient tools for bioinformatic analysis of the genomes, allowing identification of unique biosynthetic gene clusters. It seems likely that combination of these approaches may result in establishment of robust pipelines for drug discovery from marine actinobacteria in the near future. The search for innovative antiparasitics must consider, among other things, the following problems at present: there are only a few drugs in the market for the treatment of many different parasitic diseases. Target specificity, making better use of the biochemical and biological characteristics of individual parasite species, should enhance drug efficacy. Screening of marine actinobacterial natural products provides the chance to discover new molecules of unique structure with high activity and selectivity, which can be further optimized by semi or fully synthetic procedures for the control and management of parasitic diseases.

**FIGURE 31.2** Chemical structure of antiparasitic compounds.

**TABLE 31.2**  
**Antiparasitic Activities of the Compounds ( $IC_{50}$ )**

Compound	<i>L. Major</i>	<i>T. Brucei</i> (48h)	<i>T. Brucei</i> (72h)
Valinomycin	<0.11	0.0032	0.0036
Sturosporamide	5.30	0.022	0.035
Butenolide	>100	31.77	33.08

**TABLE 31.3**  
**Antiparasitic Compounds from Actinobacterial Isolates**

S. No.	Name of the Actinobacteria	Name of the Compound	Antiparasitic Activity	Reference
1.	<i>Streptomyces avermitilis</i>	Ivermectin	Parasites— <i>Wuchereria bancroftii</i> , etc. Intestinal nematodes— <i>Ascaris lumbricoides</i> etc.	Ikeda and Omura (1995)
2.	<i>Marinactinospora thermotolerans</i> SCSIO 00652	Marinacarbolines A–D	<i>Plasmodium falciparum</i>	Huang et al. (2011)
3.	<i>Streptomyces hygroscopicus</i> sub sp. <i>aureolacrimosus</i>	Milbemycin	<i>Enterobius vermicularis</i>	Warr et al. (1994)
4.	<i>Streptomyces</i> sp. K01-0031	Antimycin A9	<i>Caenorhabditis elegans</i>	Shiomi et al. (2005)
5.	<i>Streptomyces</i> sp. CMU-MH021	Fervenulin	Root knot nematode— <i>Meloidogyne incognita</i>	Lumyong et al. (2011)
6.	<i>Streptomyces nanchangensis</i> strain NS3226	Nanchangmycin and Meilingmycin	Antinematode	Ouyang et al. (1993)
7.	<i>Streptomyces</i> isolates A233, A239, and A240	Bafilolides	<i>Caenorhabditis elegans</i> and <i>Burasaphelencus ligiculus</i>	Lacey et al. (1994)
8.	<i>Streptomyces</i> sp.	Valinomycin, Staurosporine, and Butenolide	<i>Leishmania major</i> and <i>Trypanosoma brucei</i>	Sheila et al. (2010)
9.	<i>Salinispora tropica</i>	Salinosporamide A	<i>Plasmodium</i> sp.	Prudhomme et al. (2008)
10.	<i>Streptomyces nanchangensis</i>	Meilingmycin	Antiparasitic	Sun et al. (2002)
11.	<i>Streptomyces avermitilis</i>	Avermectin	Anthelmintic	Ikeda et al. (1999)
12.	<i>Streptomyces tanashiensis</i> strain Kala UC5063	Kalafungin	Antifungal, antibacterial, antiprotozoal	Johnson and Dietz (1968)
13.	<i>Streptomyces michiganensis</i> var. <i>amyloyticus</i> var. nova	Thiamycins	Anthelmintic, antiprotozoal	Cassinelli et al. (1970)
14.	<i>Streptomyces lisandri</i> nov. sp.	Axenomycins	Anthelmintic, antiprotozoal, antifungal	Bruna, Ricciardi, and Sanfilippo (1973)

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# 32 Pharmaceutical Aspect of Metabolites from Marine Algae on Skin Health

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### 32.1 INTRODUCTION

Skin plays an important role in body protection. The primary function of skin is to protect body from damage and provide a barrier between the internal and external environments. The skin also as physiological barrier against infection by pathogens as an innate immune defense (Madison 2003; Proksch, Brandner, and Jensen 2008). The skin is the largest and most exposed organ of the body, which greatly increases its risk of getting disease or complications. For this reason, skin diseases occur more frequently than any other diseases.

Skin diseases can be categorized as specific types. The common types of skin diseases involve inflammatory disorder and viral, bacterial, and fungal diseases that are associated with various and unique symptoms (Hur et al. 2008).

Skin diseases can also involve more serious medical problems. Moreover, skin diseases can be caused by cancers, hormonal imbalances, nutritional deficiencies, and a host of other problems. There are several medicines for the treatment of skin diseases. However, skin drugs are still the underlying medical problems because of their serious side effects.

Accordingly, cosmeceuticals have attracted increased attention because of their beneficial effects on human health. Therefore, numerous studies have been conducted on the general aspects of the chemical structures, biochemical properties, and biotechnological applications of bioactive substances. Marine organisms are extremely diverse and different from land organisms, which are excellent reservoirs for identifying and extracting biologically active substances (Kim et al. 2008).

Marine algae and their extracts have been studied as potential antioxidants in recent years (Heo et al. 2005; Athukorala et al. 2006). Most photosynthesizing plants including seaweeds are exposed to high oxygen concentrations and combination of light, which lead to the formation of reactive radical species and other strong oxidizing agents. For these reasons, they commonly suffer from serious photodynamic damage *in vivo* (Park et al. 2005). Therefore, their cells may have protective

mechanisms with the potential to act as pharmaceuticals, nutraceuticals, cosmeceuticals, and natural antioxidative compounds (Dykens et al. 1992; Sukenik et al. 1993; Matsukawa et al. 1997).

Biological activities of algal-derived metabolites are closely related to various skin-related symptoms that make these organisms attractive targets for the development of novel cosmetics and skin agents (Hur et al. 2008). Indeed, algae extracts have been used as cosmeceuticals such as moisturizing agents, thickening agents, hyperpigmentation agent, and antioxidants in many countries. Recognizing the potential of these compounds as active ingredients of cosmetics or skin drugs, leading countries have launched the commercial development of algal-derived skin agents based on natural products. In this chapter, recent progress in the application of bioactive compounds derived from marine algae as cosmeceuticals are described.

## 32.2 METABOLITES FROM MARINE ALGAE

Algae are important source to produce various second metabolites and more than 20,000 different species are identified up to date. A number of species have been used as anticoagulants, antibiotics, antihypertensive agents, blood cholesterol reducers, dilatory agents, insecticides, and anti-tumorigenic agents (Fitton, Irhimeh, and Falk 2007). In cosmetic fields, algae have been used as thickening agents, water-binding agents, antioxidants, and potential skin irritants. For example, the phycocyanin present in blue-green algae has been used as a treatment for allergenicity, which causes dermatitis on the basis of patch tests (Bermejo et al. 2000). Moreover, algae are a valuable source of nutrition; they contain proteins, vitamin A, sugar, starch, vitamin B1, iron, sodium, phosphorus, magnesium, copper, and calcium. Most of these are beneficial for skin, either as emollients or as antioxidants (Rupérez, Ahrazem, and Leal 2002).

### 32.2.1 TOCOPHEROLS (VITAMIN E)

Naturally,  $\alpha$ -tocopherol can be produced only by photosynthetic plants, which protect cell membranes against oxidative damage caused by UV exposure. It may play a vital role in the prevention of UV-induced photo-damage of skin and eyes, degenerative diseases such as atherosclerosis, cardiovascular diseases, and cancer (Regina and Traber 1999).

Tocopherols are composed of a chromanol ring and a lipophilic side chain of an isoprene molecule. They present in eight different forms based on the position of the methyl group substitution in the chromanol ring and unsaturation of the hydrophobic side chain. The antioxidative action of tocopherols is identified owing to the hydroxyl group in the chromanol ring, which donate a hydrogen atom to inactivate free radicals.

It has been reported that  $\alpha$ -tocopherol stimulates glutathione synthesis in keratinocytes through the modulation of cellular glutamylcysteine synthetase mRNA expression (Masaki et al. 2002).

Cosmeceutical application of tocopherols to the skin inhibits the suppression of  $H_2O_2$  production, xanthine oxidase activity, myeloperoxidase activity, and lipid peroxidation (Rahman et al. 2008). In addition,  $\alpha$ -tocopherol acetate suppresses UV-B-induced lipid peroxidation, expression of IL-8 mRNA, and AP-1 DNA binding activity in a dose-dependent manner (Wu et al. 2008). The  $\alpha$ -tocopherol is expected to downregulate MMP-1 through its suppressive effects on AP-1 DNA binding. Dermal fibroblasts isolated from aged donors produce higher levels of MMP-1 than those from young donors. The  $\alpha$ -tocopherol inhibits the increased level of collagenase gene transcription, whereas it increases the level of tissue inhibitor of metalloproteinase (TIMP) through the inhibition of protein kinase C activity in aged fibroblasts (Ricciarelli et al. 1999).

### 32.2.2 MERODITERPENOIDS

Meroditerpenoids consist of polyprenyl chain attached to hydroquinone ring moiety, and they are found in animals, plants, and microorganisms (Luckner 1984). In the marine environment, these

compounds are especially abundant in brown algae such as *Cystoseira* and *Sargassum* genera (Blunt et al. 2007). These metabolites exhibit various biological activities that may act as antioxidants, antimicrobial and skin protectants (Sunassee and Davies-Coleman 2012). Brown algae, *Sargassum* species, are reported to produce metabolites such as steroids (Tang et al. 2002a), glycerides, plastoquinones (PQ), chromanols, and chromenes (Reddy and Urban 2009).

### 32.2.3 PLASTOQUINONES

PQ from the *Sargassum* genus generally adopts the same structural skeleton and differs primarily in the linear terpene chain moiety (Blunt et al. 2007).

PQ is a component of the electron transport chain in light-dependent reactions of photosynthesis that exhibits in vitro antioxidant potency similar to  $\alpha$ -tocopherols (Kruk, Jemiołrzemska, and Strzalka 2003). It has been shown that  $\text{PQH}_2$  can inhibit membrane lipid peroxidation and scavenge of radical species both in vitro and in vivo (Maciejewska et al. 2002). Numerous studies also indicated the regulatory function of the  $\text{PQH}_2$  in many physiological and molecular signaling processes (Strzalka et al. 2009). Many of the PQ and their derivatives displayed significant antioxidant, potent antiviral, or moderate antitumor activities (Iwashima et al. 2005).

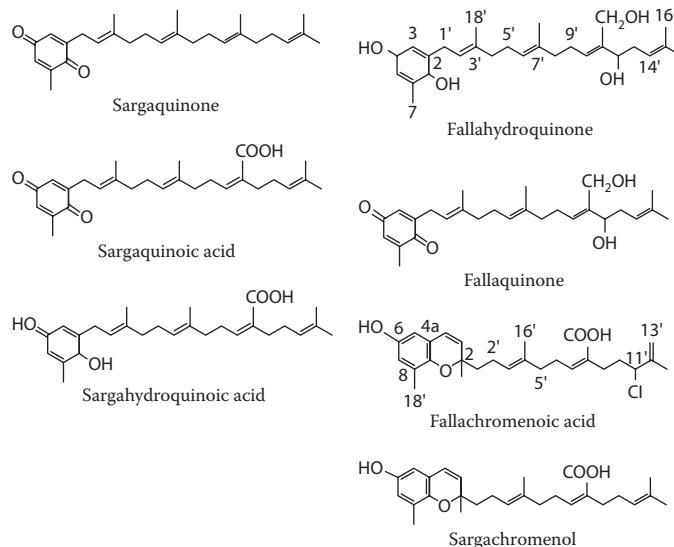
### 32.2.4 CHROMENES

The chromenes related to tocotrienol are frequently found in the brown algae sp., and are particularly abundant within the *Sargassum* genus (Seo et al. 2006). Studies on biological activities of tocotrienols revealed that they inhibit the proliferation of several tumor cells. Also, they are effective against muscle cell growth, which plays a central role in atherosclerosis (Silva et al. 2001). Brown algal-derived chromene metabolites exhibit anticancer and antimutagenic activities as well as inhibitory activities against various enzymes (Stonik, Makarieva, and Dimitrenok 1992; Yamamoto et al. 1999).

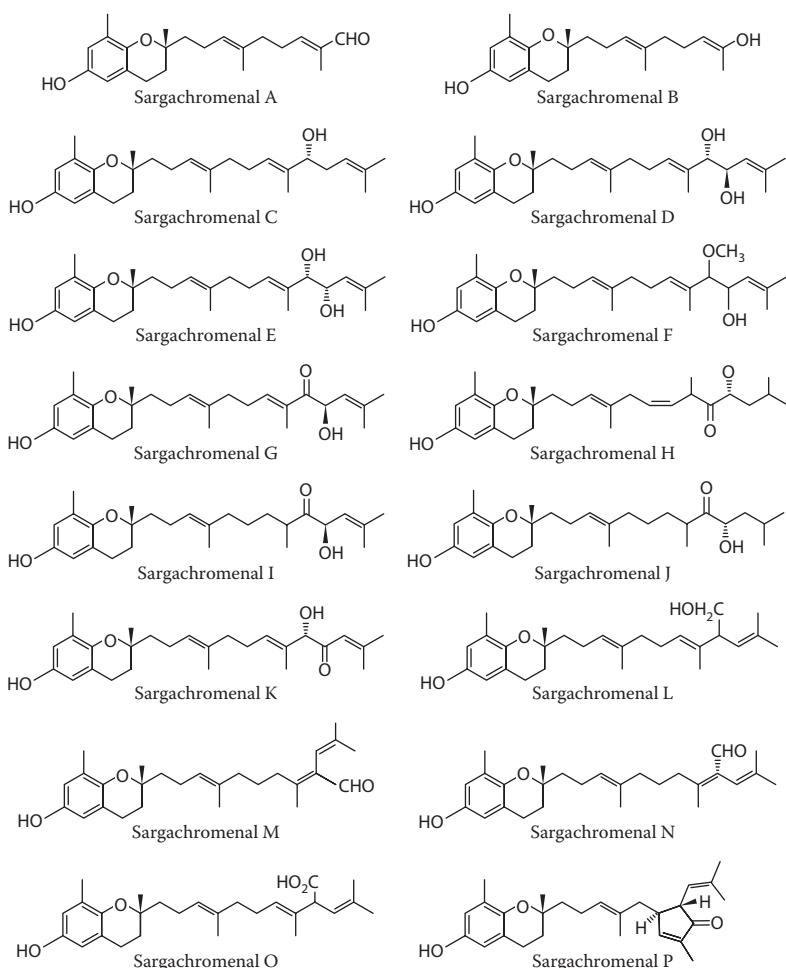
Chromenes related to tocotrienol are frequently found among the brown algae, particularly abundant within the *Sargassum* genus (Seo et al. 2006). Tocotrienols are fat-soluble molecules related to the family of tocopherols. Structurally, tocopherols and tocotrienols share some resemblance consisting of a common chromanol head and a side chain at the C-2 position. However, they are distinguished by their side chains. While tocopherol has a saturated phytol tail, tocotrienol possesses an unsaturated isoprenoid side chain. Tocotrienols have been shown to have unique functional properties. Interestingly, tocotrienols have been shown to reduce plasma cholesterol levels and other lipid- and nonlipid-related risk factors for cardiovascular disease.

The hydroxyl group (phenolic) is necessary for chromenes to function as antioxidants. The phenolic hydroxyl group on the chromanol ring of chromenes reacts with free radicals, causing termination of the auto-oxidation chain reaction. This action is called free-radical scavenging and involves donation of the phenolic hydrogen to a fatty acid free radical as well as free-radical quenching. In this process, an inactive tocopheroxyl free radical is formed, which is converted to tocopherylquinone. The quinone form is reduced back to the tocopherol form by a reducing agent such as ascorbic acid; thus, chromene molecules are recycled and might repeatedly function as antioxidants. Reddy and Urban (2009) have described the isolation and structure determination of three new meroditerpenoids, fallahydroquinone, fallaquinone, and fallachromenoic acid, from *Sargassum fallax* together with the known meroditerpenoids, sargaquinone, sargaquinic acid, sargahydroquinic acid, and sargachromenol ([Figure 32.1](#)).

In addition, several meroditerpenoids of the chromene class (sargachromenals A-P) were isolated from the brown alga *Sargassum siliquastrum* ([Figure 32.2](#)). These compounds included diverse side chains and exhibited significant antioxidative activity and inhibition of butylcholine esterase (Jung et al. 2008).



**FIGURE 32.1** Meroditerpenoids from the *Sargassum fallax*.



**FIGURE 32.2** Chromenes from the *Sargassum siliquastrum*.

### 32.3 MERODITERPENOIDS ON SKIN HEALTH

Sargaquinone, sargaquinic acid, and sargahydroquinic acid are frequently found in *sargassum* sp., one of the representative classes of secondary metabolites of meroditerpenoids (Jang et al. 2005). Among the meroditerpenoids, Sargachromenol isolated from *Sargassum sagamianum* is reported to have nerve growth factor (NGF)-dependent nerve cell growth-promoting activity (Tsang et al. 2005).

A recent study reported that Sargachromenol prevents or improves skin disease caused by hyperproliferation through the control of keratinocytes apoptosis.

Skin diseases may be induced by several factors including inflammations, benign or malignant tumors, hormones, injuries, or pathological changes such as retroplasia. In particular, damage to the stratum corneum may be a direct cause of skin disease.

Typically, skin diseases are caused by the hyperproliferation of keratinocytes including skin aging, photoaging, and pigmentary diseases. Skin aging and photoaging are induced by environmental factors such as chronic exposure to UV associated with generation of oxygen free radicals in the skin. UV exposure may result in peroxidation of the lipid components of the cell membrane and hardening of the cell membrane, thereby interfering with the supply of oxygen and nutrients into the cells. As skin metabolism and replacement of skin cells are interrupted because of these reasons, the stratum corneum of the skin becomes thicker and the skin experiences a lot of changes in structure and function.

Sargachromenol induces cytotoxicity and apoptosis of keratinocytes, thereby destroying and/or lysing keratinocytes. It can be effectively used as a keratinocyte lysing agent at the skin area where keratinocytes are hyperproliferating because of aging, photoaging, and pigmentation (Hur et al. 2008).

### 32.4 CONCLUSIONS

The biological activities of marine algal-derived metabolites are closely related to various skin-related symptoms. In addition, algae extracts are being used as cosmetics such as thickening agents, moisturizing agents, and antioxidants. The meroditerpenoids are major components of brown alga *Sargassum* sp. and are known to be involved in the enhancement of skin functions. Since a combination of meroditerpenoids with topical reagents has been clinically applied to treat hyperproliferative skin disease, meroditerpenoids could be effective therapeutic agents for skin disease. While *sargassum* genera remain, meroditerpenoids-rich extracts have a growing role to play and strong potential for commercial success as cosmetics or skin drugs.

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# 33 Clues for Cancer from Ocean-Derived Molecules and Role of In Silico Techniques in Anticancer Drug Discovery

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## 33.1 INTRODUCTION

Cancer is the leading cause of death in economically developed countries and the second leading cause of death in developing countries, exceeded only by heart disease. The global burden of cancer continues to increase largely because of the aging and growth of the world population along with the increasing adoption of cancer-causing behaviors, particularly smoking, in economically developing countries. Based on the GLOBOCAN 2008 estimates, about 12.7 million cancer cases and 7.6 million cancer deaths are estimated to have occurred in 2008; of these, 56% of the cases and 64% of the deaths occurred in the economically developing world. Breast cancer is the most frequently diagnosed cancer and the leading cause of cancer death among females, accounting for 23% of the total cancer cases and 14% of the cancer deaths. Lung cancer is the leading cancer in

males, comprising 17% of the total new cancer cases and 23% of the total cancer deaths. Deaths from cancer worldwide are projected to continue to rise, with an estimated 13.1 million deaths in 2030 (Ferlay et al. 2010).

It is well known that genetic changes progressively convert normal cells into cancer cells. Although more than 100 distinct types of cancer exist, only six essential alterations in cell physiology cause malignant cell growth: (1) self-sufficiency in growth signals, (2) insensitivity to antigrowth signals, (3) evasion of apoptosis, (4) limitless replicative potential, (5) sustained angiogenesis, and (6) metastasis. Hanahan and Weinberg (2011) reported that these six “hallmarks of cancer” are present in almost every type of human tumor.

Chemotherapeutic agents currently approved for the treatment of invasive disease may exhibit initial efficacy; however, the development of resistance to therapy and concerns over tolerability are common and generally limit the treatment options available to physicians and patients. Novel chemotherapeutic agents are, therefore, necessary to increase survival, delay disease progression, and improve tolerability. In cancer chemotherapy, drugs administered injure rapidly dividing normal cells and, therefore, have substantial side effects when administered to patients. Anticancer agents that lack side effects and target cancer-specific molecules to eliminate cancer cells while sparing normal cells are the focus of present research (Sawyers 2004). Although advances in the field of chemopreventive and therapeutic medicine have been made regularly over the last several years, the search for novel anticancer treatments continues. New insights into mechanisms responsible for neoplastic disease are significantly changing the general philosophical approach toward cancer treatment.

Drugs derived from natural products have a giant impact on the present-day antitumor drug discovery regime. The importance of natural products in the field of therapeutics may be attributed to their high affinity to the target, little loss of entropy when binding to a protein, and bioavailability. Moreover, natural compounds are quite flexible in conformational acquisition in aqueous and lipophilic environments (Bhatnagar and Kim 2010). For many years, research has essentially focused on plants and terrestrial microorganisms, mainly because these specimens are easily available and folk traditions have described beneficial effects from their use. Recent studies in the field of cancer research have revealed promising compounds, which are isolated from natural sources, with proven anticancer activity. Three examples of such compounds are trabectedin (Yondelis®; PharmaMar, Madrid, Spain), cytarabine (Cytosar-U®; Bedford Laboratories, Bedford, OH), and eribulin mesylate (Halaven®; Eisai, Inc., New Jersey), which represent the first three described marine anticancer drugs (Schumacher et al. 2011). Indeed, almost 50% of the antitumor agents approved in the last 50 years of the twentieth century are either compounds derived from natural sources or (semi-) synthetic analogs of these products (Newman and Cragg 2007). Natural compounds remain a high-output source of promising chemotherapeutic or chemopreventive agents in current cancer research (Villa and Gerwick 2010). Currently, many pharmaceutical companies have therapeutic compounds of marine origin under development.

The rich variety of organisms in the marine environment must adapt to extreme marine environmental conditions such as high pressure; high salt concentration; low nutrient concentration; low, but steady, temperatures (except the high temperatures near underwater volcanoes and the extremely low temperatures in polar regions); limited sunlight; and low oxygen content. To acclimate to these conditions, marine organisms possess unique characteristics that differentiate them from terrestrial organisms in many aspects, such as metabolism, behavior, information transfer, and adaptation strategies (Hu et al. 2011). The majority of marine invertebrates lack natural defense systems (e.g., innate immune systems) necessary to survive in the competitive environment; hence, they synthesize biologically active secondary metabolites. These metabolites play a role in the defense of host habitats and the adaptation of organisms to extreme environmental challenges (Schumacher et al. 2011).

Marine organisms comprise approximately half of the total biodiversity, thus offering a vast source to discover useful therapeutics. A recent census of marine life that involved the participation

of 2700 scientists from over 80 nations and assessed the diversity, distribution, and abundance of marine life resulted in the discovery of over 6000 potentially novel species (Butler et al. 2010; Fautin et al. 2010; Miloslavich et al. 2010). As a consequence of these research efforts, it is clear that the marine environment represents an important source of unknown natural compounds whose medicinal potential must be evaluated. Efforts to exploit this biodiversity through the identification of new chemical compounds have discovered approximately 22,000 natural products of marine origin so far, whereas 131,000 terrestrial natural products exist. The major sources of biomedical compounds are sponges (37%), coelenterates (21%), and microorganisms (18%) followed by algae (9%), echinoderms (6%), tunicates (6%), mollusks (2%), bryozoans (1%), etc. (Blunt et al. 2011).

Marine algae became an industrial resource much earlier than marine invertebrates and marine microorganisms (including phytoplankton). This is mainly based on the farming of edible species or the production of agar, carrageenan, and alginate. Marine macroalgae are used as foods in many places; particularly in Japan, Korea, and China, sea vegetable has been used as a crude drug for treatment of iron deficiency and diseases such as goiter, Basedow's disease, and hyperthyroidism. Also, considering their great taxonomic diversity, investigations related to the search of new biologically active compounds from algae comprise an almost unlimited field. Most species of red, brown, and green algae have been utilized on an industrial scale for nearly 100 years, which indicates that novel compounds from marine algae are more suitable as potential drugs than those from marine invertebrates (Hu et al. 2011).

Seaweeds or macroalgae belong to the lower plants, which means that they do not have roots, stems, and leaves. Instead they are composed of a thallus (leaflike structure) and sometimes a stem and a foot. Some species have gas-filled structures to provide buoyancy. The majority of the large conspicuous forms of attached marine plants are seaweeds, predominantly those in the three plant divisions Chlorophyta (green algae), Phaeophyta (brown algae), and Rhodophyta (red algae). Each group is characterized by specific combinations of photosynthetic pigments. Seaweeds are extremely abundant in intertidal zones, and in clear tropical waters they can extend to depths of up to 200 m. Where they are abundant, seaweeds greatly influence environmental conditions for other types of marine life by providing food, protection from waves, shade, and a substrate on which to attach. Due to their abundance in shallow waters and ease of collection, seaweeds were one of the first groups of marine organisms whose natural products chemistry was studied extensively.

In the classical Indian, Ayurvedic, and Siddha systems of medicine, little information has been reported regarding the medicinal use of seaweeds. Seaweeds have been employed as dressings, as ointments, and in gynecology (Trease and Evans 1996). The extracts and active constituents of various algae have been shown to exhibit antibacterial activity *in vitro* against gram-positive and gram-negative bacteria. The production of antimicrobial metabolites was considered to be an indicator of the capability of seaweeds to synthesize bioactive secondary metabolites (Del Val et al. 2001). There are numerous reports of compounds derived from macroalgae with a broad range of biological activities, such as antibacterial (Nair, Chabhadiya, and Chanda 2007), antiviral (Richards et al. 1978), anticoagulant (Athukorala et al. 2007), and antifouling activities (Hellio et al. 2004).

Seaweeds are important sources of proteins, iodine, vitamins, and minerals and, hence, their metabolites have shown promising activities against cancer incidence. Seaweeds also contain high amounts of polyphenols such as catechin, epicatechin, epigallocatechin gallate, and gallic acid, as reported in *Halimeda* sp. (Chlorophyceae), which exhibits cutting-edge anticancer potential (Boopathy and Kathiresan 2010). Hence, drug discovery from seaweeds for the treatment of various types of cancers is highly warranted.

### 33.2 ANTICANCER PHARMACOLOGY OF MARINE ALGAE

Carcinogenesis is a complex process controlled by various signal transduction pathways linked to processes such as inflammation, cell differentiation and survival, and metastasis. Most of the players of these pathways are interrelated, and irregularities in their cross talk result in impairment

of cellular functions leading to tumor generation and progression (Bhatnagar and Kim 2010). To design effective drugs against cancer, it is mandatory to understand the underlying tumor physiology and the changes occurring in the tumor microenvironment.

### 33.2.1 MATRIX METALLOPROTEINASE INHIBITORS

Zinc-dependant endopeptidases such as matrix metalloproteinases (MMPs) have been extensively studied due to their evident role in carcinogenesis and cellular invasion by catabolizing the extracellular matrix (Gill and Parks 2008). Apart from playing a major role in invasion, angiogenesis, and metastasis during tumor progression, MMPs are also important for cancer cell transformation, growth, apoptosis, signal transduction, and immune regulation. The MMP inhibitory effects of phlorotannins from the marine brown alga *Ecklonia cava* have revealed that its extract could specifically inhibit both MMP-2 and MMP-9 activities significantly ( $p < 0.001$ ) at a concentration of 10 µg/mL in human dermal fibroblasts and HT1080 cells by fluorometric assay. In addition, the *E. cava* extract did not exert any cytotoxic effect even at 100 µg/mL, proposing its potential use as a safe MMP inhibitor (Kim et al. 2006). Expression of MMP-1 was dramatically attenuated by treatment with eckol or dieckol, which were purely isolated from *Ecklonia stolonifera*, indicating that these compounds are active principles to inhibit MMP-1 expression in human dermal fibroblasts (Joe et al. 2006).

### 33.2.2 NUCLEAR FACTOR-κB INHIBITORS

Nuclear factor-κB (NF-κB) is a ubiquitous transcription factor, a dimer of proteins of the Rel family including NF-κB1 (p50), NF-κB2 (p52), RelA (p65), RelB, and c-Rel, whose deregulated expression may lead to cancer (Keutgens et al. 2006). It is noted that NF-κB is activated by various stimuli, including tumor necrosis factor-α (TNF-α), interleukin-1, and lipopolysaccharides (LPSs). Extracts from three species of Alariaceae, *Eisenia bicyclis*, *E. cava*, and *E. stolonifera*, show strong inhibition of both NF-κB and activator protein 1 (AP-1) reporter activities (Joe et al. 2006). Phlorofucofuroeckol A isolated from the edible brown alga *E. stolonifera* inhibited the activation of Akt and p38 mitogen-activated protein kinase (MAPK) in LPS-treated RAW 264.7 cells; it also regulates inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) expressions through the NF-κB-dependent transcriptional control associated with inhibition of multiple signalling proteins, suggesting potential candidates of phloroglucinol derivatives for treatments of inflammatory diseases (Kim, Lee, and Shin 2011).

### 33.2.3 HYPOXIA-INDUCIBLE FACTOR INHIBITORS

Hypoxia-inducible factor (HIF) is a heterodimeric transcription factor that is composed of a hypoxia-inducible α subunit (HIF-1α and HIF-2α) and a constitutively expressed β subunit (HIF-1β). The HIF mediates the adaptation of cells and tissues to low oxygen concentrations. Tumor progression is associated with not only increased microvascular density but also intratumoral hypoxia (Hockel and Vaupel 2001). Loss of HIF-1 activity has been shown to have immense negative effects on tumor growth, vascularisation, and energy metabolism in xenograft assays (Semenza 2001; Kung et al. 2000). Thus, a number of HIF inhibitors have been designed with the aim of finding a new direction for tumor therapy. Laurenditerpenol, which was isolated from bioassay-guided fractionation of the lipid extract of the red alga *Laurencia intricata* Lamouroux (Rhodomelaceae), yielded the first marine natural product that inhibited HIF-1 activation (Mohammed et al. 2004). It was shown to inhibit HIF-1 activation by blocking hypoxia-induced HIF-1α protein accumulation and suppressed mitochondrial oxygen consumption at electron transport chain (ETC) complex I at a 50% inhibitory concentration ( $IC_{50}$ ) value of 0.8 µM.

### 33.2.4 DEOXYRIBONUCLEIC ACID METHYLTRANSFERASE-1 INHIBITORS

The compound halomon [6(*R*)-bromo-3(*S*)-bromomethyl)-7-methyl-2,3,7-trichloro-1-octene] was first isolated from the red alga *Portieria hornemannii* (Lynbye) collected in the Philippines in 1992. Halomon exhibited strong differential cytotoxicity to brain-, renal-, and colon-derived cell lines in the *in vitro* human tumor cell line screen of the National Cancer Institute (NCI), Maryland. On the basis of its unprecedented cytotoxicity profile, halomon was selected by the NCI for preclinical drug development (Fuller et al. 1992). However, research and development of halomon as an anticancer lead has been limited by the lack of a reliable natural source and the failure to show *in vivo* effects. Andrianasolo et al. (2006) rediscovered the red alga *P. hornemannii* at Madagascar. The organic extract possessed a potent inhibitory activity to the DNA methyltransferase-1 (DNMT-1) isoform. The DNMT-1 causes methylation of cytosine phosphodiester-linked guanine dinucleotide (CpG) by catalyzing the transfer of a methyl group from *S*-adenosylmethionine to the 5' position on cytosine residues residing at CpG sites. In many cancers, promoters of tumor suppressor genes are silenced by hypermethylation at CpG sites and, thus, the inhibition of DNMT-1 could potentially reverse tumor growth. Halomon and (3 $Z$ )-6-bromo-3-(bromomethylidene)-2-chloro-7-methylocta-1,6-diene were tested for DNMT-1 enzyme inhibition assay and were found to have activities of 1.25 and 1.65  $\mu$ M, respectively (Andrianasolo et al. 2006).

### 33.2.5 DEOXYRIBONUCLEIC ACID POLYMERASE INHIBITORS

At least 19 different DNA polymerases have been identified in eukaryotic cells. Ohta et al. (1998) found that the sulfolipid metabolite sulfoquinovosyldiacylglycerol (KM-043) isolated from a marine red alga, *Gigartina tenella*, inhibited eukaryotic DNA polymerases  $\alpha$  and  $\beta$  ( $IC_{50}$  values of 0.25 and 3.6  $\mu$ M, respectively) and HIV-reverse transcriptase type 1 but did not influence the activities of prokaryotic DNA polymerases. 2,3,6-tribromo-4,5-dihydroxybenzyl alcohol and its methyl ether were isolated from the marine red alga *Sympyocladia latiuscula*, which completely inhibited 1.5 units of Taq DNA polymerase at 0.5  $\mu$ g and 5  $\mu$ g respectively (Jin et al. 2008).

### 33.2.6 TELOMERASE INHIBITORS

Telomerase is a ribonuclear protein that is detected in more than 90% of primary cancer tissues using a telomeric repeat amplification protocol, which is an early marker for cancer detection. Moreover, telomerase is upregulated in 95% of breast carcinomas but not in adjacent normal tissues by reducing the telomere length, which makes it an ideal target for anticancer drug development. Kanegawa et al. (2000) screened 304 marine algae samples that were collected from various coasts of Japan. In particular, the MeOH extract from the green alga *Caulerpa sertularioides* strongly inhibited telomerase activity when added to a MOLT-4 cell culture. Screening of specific secondary metabolites from marine algae by bioinformatic tools against telomerase enzyme would probably result in more evidence for telomerase inhibitors in the future.

### 33.2.7 INOSINE 5'-PHOSPHATE DEHYDROGENASE

The enzyme inosine 5'-phosphate dehydrogenase (IMPDH) catalyzes the NAD-dependent oxidation of inosine 5'-phosphate (IMP) to xanthosine 5'-monophosphate and is the key enzyme in de novo guanosine 5'-triphosphate (GTP) biosynthesis (Carr et al. 1993). The two substrates of IMPDH bind in an obligate order: the IMP precedes NAD, and the products also dissociate in an obligate fashion, with NADH preceding xanthosine 5'-monophosphate. The activity of IMPDH is tightly linked with cell proliferation and the inhibition of IMPDH has anticancer, antiviral, and immunosuppressive effects (Jackson et al. 1975). Gerwick and coworkers (1994) at Oregon State University, Corvallis, Oregon, evaluated over 500 extracts of marine microalgae (primarily cyanobacteria) and

macroalgae for their ability to inhibit IMPDH. This assay yielded 24 active extracts and resulted in the isolation of the bromophenolic compound isorawsonol ( $IC_{50} = 18 \mu M$ ) from the tropical marine green alga *Avrainvillea rawsonii* (Chen et al. 1994).

### 33.2.8 APOPTOSIS

Apoptosis represents a universal and efficient form of cell death that is executed through a highly ordered intrinsic cellular suicide program. Mutations that cause uncontrolled cell growth and those that lead to insufficient cell death occur commonly in neoplasia and contribute to the etiology of cancer. Elucidation of apoptotic pathways and an increased understanding of the importance of apoptosis in the development and progression of cancer have provided impetus for the development of apoptosis-targeted therapies (Nagle et al. 2004). Thrysiferyl 23-acetate is a cyclic ether that contains a squalene carbon skeleton. Thrysiferyl 23-acetate was isolated as a potent cytotoxin (Effective dose ( $ED_{50}$ ) of 0.3 ng/mL against P388 cells) from the marine red alga *Laurencia obtusa* collected in Japan (Suzukia et al. 1985). In serum-deprived Jurkat cells, thrysiferyl 23-acetate (10  $\mu M$ ) induced chromatin condensation and DNA fragmentation, which are the hallmarks of apoptosis. Although thrysiferyl 23-acetate has been shown to selectively inhibit serine/threonine phosphoprotein phosphatase 2A (PP2A) (Matsuzawa et al. 1994), its apoptotic activity is not dependent on the inhibition of PP2A (Matsuzawa et al. 1999).

A glycoprotein from the brown alga *Laminaria japonica* displayed several apoptotic features, such as DNA fragmentation, sub-G1 arrest, caspase-3 activation, and poly ADP ribose polymerase (PARP) degradation, in HT-29 colon cancer cells. The mechanism of apoptosis may be mediated via multiple pathways, including the Fas signaling pathway and the mitochondrial pathway, and cell cycle arrest (Go, Hwang, and Nam 2010). Similarly, porphyran, a sulfated polysaccharide from marine red algae, *Porphyra haitanensis*, showed apoptotic activity by following the mitochondrial pathway on AGS human adenocarcinoma cell line (Kwon and Nam 2006). Fucoxanthin, a carotenoid from the edible seaweed *Undaria pinnatifida* induces apoptosis and enhances the antiproliferative effect of the PPAR $\gamma$  ligand, troglitazone, on human colon cancer cells lines, Caco-2, HT-29 and DLD-1 (Hosokawa et al. 2004).

### 33.2.9 ANTIMITOTIC AGENTS

Antimitotic agents are classified as tubulin-interactive agents, that interfere with the polymerization or depolymerization of tubulin. Actin inhibitors are those that interfere with the polymerization or depolymerization of actin, and kinesin inhibitors are those that disrupt the function of kinesin motor proteins. The compound 14-ketostypodiol diacetate from brown algae, *Stylopodium flabelliforme*, inhibited microtubules by delaying the lag period associated with nucleation events during assembly and significantly decreased the extent of microtubule polymerization in DU-145 human prostatic cells. It also inhibited cell proliferation by affecting the protease secretion and the in vitro invasive capacity, both properties of cell for metastases (Depix et al. 1998).

### 33.2.10 MULTIDRUG RESISTANCE

Multidrug resistance (MDR) is one of the main causes for the failure of chemotherapeutic cancer treatments. It is noted that MDR was first described by Biedler and Riehm (1970), based on investigations in resistant cell lines derived from a Chinese hamster lung tissue-derived cell line (DC-3F) and a Chinese hamster fibroblastic cell line (CLM-7). The 170 kDa surface glycoprotein P-glycoprotein (P-gp) membrane transporter acts as an ATP-dependent drug efflux pump that actively removes a variety of structurally diverse xenobiotics and natural product-based drugs with

different cellular targets and mechanisms of action (Juliano and Ling 1976). A novel marine terpenoid, dehydrothrysiferol, isolated from a Canarian red alga, *Laurencia viridis*, showed growth inhibition in oral squamous carcinoma cells with S-phase arrest but no apoptosis (Pec et al. 1998). The IC<sub>50</sub> values of dehydrothrysiferol against the P-gp overexpressing multidrug-resistant KB-8-5 cells were about 2.6 times greater in the nonresistant KB-3-1 cells relative to the resistant KB-8-5 cells. Studies conducted in a fluorescence-based efflux system measuring the interference of a test compound with MRP1 (multidrug resistance-associated protein 1)-mediated drug extrusion suggested that dehydrothrysiferol did not inhibit MRP1-mediated drug transport (Pec et al. 2002).

Hormone-unresponsive breast cancer is associated with poorer prognosis than hormone-receptor expressing malignant mammary tumors. Estrogen-negative breast cancer cells were more sensitive to dehydrothrysiferol than their receptor-positive counterparts and induction of apoptosis might be transduced through more than one effector pathway. Initial studies suggested that dehydrothrysiferol may modulate MDR, but modulation of these proteins has subsequently shown to be false. (Pec et al. 1998). Also, dehydrothrysiferol significantly reduced the adhesion of breast cancer cells through the very late activation antigen (VLA) integrins  $\alpha 2\beta 1$  and  $\alpha 5\beta 1$  by an apoptosis, when studied on low amounts of the extracellular matrix. Since the activation state of integrins is recognized as an essential factor in metastasis formation, the action of dehydrothrysiferol in regulating integrin affinity may be a potential therapeutic strategy in cancer therapy (Pec et al. 2007).

### 33.3 INTERNATIONAL SCENARIO

As mentioned earlier there are numerous secondary metabolites of marine origin wherein marine algae contribute to about 9% (Blunt et al. 2011). Moreover, the role of marine macroalgae specifically in cancer pharmacology around the globe is promising. Adding to the excitement, Parish and associates (Coombe et al. 1987; Parish et al. 1987; Parish and Snowden 1988) discovered that sulfated polysaccharides, including fucoidin and carrageenan, inhibit tumor cell-derived heparanases. Nagumo (1983) discovered that sulfated polysaccharides from *Sargassum kellmanianum* inhibited mouse S 180 tumor growth, and Matsumoto et al. (1984) reported that carrageenan, which is not active alone, significantly potentiates the effect of mitomycin against leukemia L-1210 ascites tumor in mice. Red algae of the genus *Laurencia* (Rhodomelaceae) are cosmopolitan species with a wide distribution throughout the world. Their secondary metabolites include sesquiterpenes, diterpenes, triterpenes, and acetogenins, which are usually characterized by the presence of one or more halogen atoms in their structures. Due to their relatively high degree of halogenation, many of these molecules either are biologically active or play an ecological role in their ecosystems, often exhibiting antibacterial, antifungal, antiviral, anti-inflammatory, antiproliferative, cytotoxic, antifouling, antifeedant, ichthyotoxic, and insecticidal activities (Lhullier et al. 2010). All three cuparene sesquiterpenes isolated from *Laurencia microcladia* were found to exhibit significant cytotoxic activity against two lung cancer cell lines (Kladia et al. 2005). Although cytotoxic activity cannot be correlated with the presence or absence of specific functional groups, it was probably influenced by a combination of factors, including the overall three-dimensional (3D) structure of the molecules and the spatial orientation of their substituents (Lhullier et al. 2010).

Oxygenated desmosterols of the red alga *Galaxaura marginata* exhibited significant cytotoxicity toward several cancer cell lines such as P388, KB, A549, and HT-29 (Sheu et al. 1996). Bromoditerpenes from the red alga *Sphaerococcus coronopifolius* exhibited cytotoxic activity on NSCLC-N6-L16 and A549 human lung cancer cell lines (Smyrniotopoulos et al. 2008). Bromophycolide A was cytotoxic against several human tumor cell lines by specific induction of apoptosis (Kubanek et al. 2005). Bromophycolides C–I from the Fijian red alga *Callophyicus serratus* displayed modest antineoplastic activity against a range of human tumor cell lines. The most selective of these was bromophycolide H, and its strongest activity was against the breast tumor cell line DU4475 (IC<sub>50</sub> = 3.88  $\mu$ M) (Kubanek et al. 2006).

### 33.4 INDIAN SCENARIO

Considerable work has been done on the chemical aspects of seaweeds of the Indian coast of which those up to 1970 have been reviewed by Umamaheshwara Rao (1970). Kesava Rao (1992) has compiled the elemental composition of Indian marine algae. Utilization of agar and algin by many industries has led to research on cultures of agarophytes and alginophytes (Oza et al. 1994; Sivakumar and Rengaswamy, 2000; Kaladharan et al. 2001; Oza et al. 2001). Although the interest in marine pharmacology of institutions and laboratories around the world has been increasing over the last three decades, in India only sporadic work has been done in this field comprising mainly preliminary studies done by institutions in coastal areas on the marine organisms they come across (Agshikar et al. 1979; Naik et al. 1980). A systemic effort toward pharmacological exploration of the marine wealth of India began in the 1980s with the collaboration of a number of universities. Their study involved the broad pharmacological screening of approximately 500 marine samples in which various activities, such as antifertility, antiviral, hypotensive, and CNS-stimulant activities, were detected (Bhakuni 1991). The leads were so promising that it was decided to have a broad based project and, thus, a national multicenter project, "Development of Potential Drugs from the Ocean," funded by the Department of Ocean Development, Government of India, New Delhi, was started in 1990, which continues to this day. Keeping in mind the number of cytotoxic chemicals of marine origin, a few seaweeds, *Acanthaphora spicifera*, *Ulva reticulate*, *Gracilaria folifera*, and *Padina boergesenii*, from the Gulf of Mannar region were screened for their cell viability. It was identified that the alcoholic extracts of the seaweeds exhibited cytotoxic activity (Vasantha 2002). Further to the screening studies, Hannah R. Vasantha and coworkers tested the alcoholic extracts of the red alga *Acanthaphora spicifera* from the vicinity of the Mandapam coast, Tamil Nadu, India, for its tumoricidal effect in Ehrlich's ascites carcinoma cells developed in mice. The species *A. spicifera* exhibited tumoricidal activity at a dose of 20 mg/kg comparable to the standard drug 5-flurouracil. This was evidenced by an increase in mean survival time, a decrease in tumor volume, and viable cell count (Vasantha, Rajamanickam, and Saraswathy 2004). Turbinaria acid from brown algae, *Turbinaria ornata*, was reported to have cytotoxic property against tumor cells (Asari, Kusumi, and Kakisawa 1989). Similarly, monoterpenoids, sargol, sargol-I, and sargol-II, isolated from the brown alga *Sargassum tortile* exhibited cytotoxic activity (Numata et al. 1991). Simultaneously, a linear cytotoxic diterpene bifurcadiol, which was isolated from the brown alga *Bifurcaria bifurcate* by Guardia et al. (1999), was found to exhibit cytotoxicity against various cultured human tumor cell lines, such as A549, SK-OV-3, SKL-2, XF-498, and HCT. It has been identified that heparin and other proteoglycans have a role in neoplasia in regulating the growth of endothelial cells and controlling the proliferation of other cells through its interaction with growth factors.

An extract of *Laurencia brandenii* from the southwest coast of India was evaluated for brine shrimp cytotoxicity and hatchability assay using *Artemia salina*, where the petroleum:chloroform (6:4) fraction showed an LD<sub>50</sub> value of 93 µg/mL; at 200 µg/mL, 100% hatching inhibition was achieved. Recently, methanolic extracts of seven brown seaweeds occurring in the Indian coastal waters were screened and reported for their cytotoxic and antioxidant properties (Vinayak, Sabu, and Chatterji 2011).

### 33.5 IN SILICO METHODS IN CANCER DRUG DISCOVERY

Computational screening (also known as virtual screening) refers to the use of a computer-based method to select compounds from a library or database of compounds in order to identify the ones that are likely to possess a given activity, such as the ability to inhibit the action of a particular therapeutic target. Virtual screening has an inherent advantage over traditional, and even experimental, high-throughput screening (HTS) due to its massive parallel-processing ability; using virtual screening, millions of compounds per week can be tested.

Compound–target interactions validated experimentally are available in the scientific literature, and this information is in varied interests of biological, physical, or pharmacological research. Rapid development of information and communication technologies during the previous few decades has dramatically changed our capabilities of collecting, analyzing, storing, and disseminating all types of data. Databases containing millions of chemical compounds tested in various biological assays are increasingly becoming available as online collections. Online compound resources can be classified into two groups: (1) clinically oriented drug “encyclopedias” and (2) chemically oriented small molecule databases. Clinically oriented drug resources include the Pharmacogenomics Knowledge Base (PharmGKB) and the RxList, which is a drug index resource on the Internet (Hatfield, May, and Markoff 1999; Hodge, Altman, and Klein 2007). These knowledge bases tend to offer very detailed clinical information about selected drugs (their pharmacology, metabolism, and indications), with their data content being targeted toward pharmacists, physicians, and consumers.

Chemically oriented small molecule databases include PubChem, ChEBI, ChemSpider, TTD (Therapeutic Target Database), and KEGG (Kyoto Encyclopedia of Genes and Genomes) (Chen, Ji, and Chen 2002; Kanehisa et al. 2006; Degtyarenko et al. 2008; Wang et al. 2009; Pence 2010). These excellent databases provide information about the nomenclature, structure, and/or physical properties of large numbers of small-molecule drugs and, in some cases, their drug targets. These databases are typically oriented toward medicinal chemists, biochemists, and molecular biologists.

It is noted that PubChem (<http://pubchem.ncbi.nlm.nih.gov>) is a public repository for biological properties of small molecules hosted by the U.S. National Institutes of Health (NIH). The website PubChem is primarily intended to serve as a repository for HTS data from federally funded screening centers and academic research laboratories; the major advantage of PubChem resides in its ability to serve as a chemical gateway to biomedical databases such as PubMed (Wang et al. 2009). The PubChem BioAssay database currently contains biological test results for more than 700,000 compounds (Wang et al. 2012).

The DrugBank (<http://www.drugbank.ca>) is a richly annotated resource that combines detailed drug data with comprehensive drug target and drug action information (Wishart et al. 2008). Each DrugCard entry now contains more than 100 data fields with half of the information being devoted to drug/chemical data and the other half being devoted to pharmacological, pharmacogenomic, and molecular biological data. Recently, the Seaweed Metabolite Database (<http://www.swmd.co.in>) was developed to share organized information about marine algal compounds and their biological activity available in the literature. Apart from this prior information, the database also contains the geographical origin of a seaweed, its method of compound extraction, and a chemical description of its compounds (Davis and Vasanthi 2011).

The study of protein–small molecule interactions is vital for understanding protein function and for practical applications in drug discovery. Protein crystal structures, nearly 80,000 in the RCSB Protein Data Bank currently, provide crucial insights into protein function and enable researchers to study interactions in atomic detail (Berman et al. 2007). CancerResource addresses the complexity of cancer by not only covering a large, but specific, set of compound–target interactions, experimental data, and supporting information but also by allowing individual data to be processed for advanced analyses (<http://bioinformatics.charite.de/cancerresource/>; Ahmed et al. 2011). Homologous proteins have similar functions and often interact with their small molecules in a similar manner. Thus, it is possible to infer protein–small molecule interactions even if there are no crystal structures available for a particular protein of interest as long as there are structures of sufficiently close homologs. The resource BindingDB (<http://www.bindingdb.org>) has experimentally determined the binding affinities of protein–ligand complexes, for protein targets including isoforms and mutational variants, and small-molecule ligands (Liu et al. 2007). Potential applications of these databases are if a naturally occurring compound inhibits cellular proliferation, a search of the database for chemically similar compounds may reveal that a similar compound binds a protein known to be involved in regulation of the cell cycle thus elucidation of the mechanism of a biological effector molecule.

Virtual screening has been widely used to discover new leads by computationally identifying compounds with higher probability of strong binding affinity to a target protein. Screening methods can be classified into structure-based and ligand-based approaches based on the amount of structural and bioactivity data available. In the structure-based approach, where the 3D structure of the receptor is known, high-throughput docking is employed (Sousa et al. 2006). Docking involves a complex optimization task of finding the most favourable 3D binding conformation of the ligand to the receptor molecule. Being computationally intensive, docking is not suitable for very large virtual screening experiments. If the information on the receptor is scanty, the ligand-based method is used, which is efficient and robust in screening chemical databases and virtual libraries against molecules with known activities or properties (Geppert, Vogt, and Bajorath 2010). On the assumption that structurally similar molecules exhibit more similar biological activity than dissimilar or less similar molecules, quantitative structure–activity relationship (QSAR) modeling provides an effective means for both exploring and exploiting the relationship between chemical structure and its biological action toward the development of novel drug candidates (Tropsha 2010).

The concept of QSAR was introduced by Corwin Hansch and coworkers in the 1960s. The QSAR approach can be generally described as an application of data analysis methods and statistics to develop models that can accurately predict biological activities or properties of compounds based on their structures. The most fundamental goal is to predict whether a given molecule will bind to a target and, if so, how strongly it will bind to the target. It is noted that QSAR attempts to find a consistent relationship between biological activity and molecular properties, so that these “rules” can be used to evaluate the activity of new compounds.

The general form of a QSAR equation is  $P(i) = f(\text{SD}_i)$ , where  $P(i)$  is a physical, chemical, or biological property of the compound  $i$ ;  $\text{SD}_i$  is a vector of structural descriptors of  $i$ ; and  $f$  is a mathematical function such as linear regression, partial least squares (PLS), artificial neural networks, or support vector machines. The two main objectives for the development of a QSAR are as follows: (1) A predictive and robust QSAR with a specified chemical domain for prediction of activity of untested molecules is developed. (2) The QSAR acts as an informative tool by extracting a significant pattern in the descriptor related to the measured biological activity and allows one to understand the mechanism of the given biological activity, which could help in designing novel molecules with improved activity profiles.

Cheminformatics study entails the calculation of chemical descriptors that are expected to accurately reflect intricate details of underlying chemical structures (Tropsha 2010). A molecular descriptor can be defined as a numerical representation of chemical information encoded within a molecular structure via a mathematical procedure. Types of QSAR are based on the dimensionality of molecular descriptor used. In 0D-QSAR, the descriptors are derived from the molecular formula, for example, molecular weight, number, and type of the atoms. A substructure list representation of a molecule can be considered a one-dimensional (1D) molecule representation, and it consists of a list of molecule fragments.

A molecular graph contains topological or two-dimensional (2D) QSAR information of how the atoms are bounded in a molecule, as well as information on both the type of bonding and the interaction of particular atoms. Molecular hydrophobicity (lipophilicity) is normally quantified as  $\log P$  where  $P$  is the partition coefficient, a measure of differential solubility of a compound in two immiscible solvents. The octanol/water coefficient,  $P$ , is the ratio of concentration of a neutral molecule in 1-octanol to its concentration in water when the phases are at equilibrium (Kujawski et al. 2012). In toxicology, partitioning is critical to understanding the tendency of chemicals to cross biological membranes, and the properties of 1-octanol are similar to those of natural membranes. Other descriptors are the ones related to steric effects, such as the molar refraction (MR) index, various parameters accounting for the shape of a compound, and descriptors indicating the presence or absence of certain structural features.

The 3D-QSAR descriptors include molecular surface, molecular volume, and other geometrical properties. Popular 3D-QSAR methods are comparative molecular field analysis (CoMFA), comparative

molecular similarity indices analysis (CoMSIA), and GRID (Bordas, Komives, and Lopata 2003). The basic idea behind CoMFA is that the biological activity of molecules is related to their electrostatic and steric interactions. The molecules (ligands) being studied are aligned structurally on a 3D grid. Using a probe atom, electrostatic and steric fields are determined at every point in the grid. It is noted that CoMSIA, on the other hand, also takes into account hydrophobic parameters. The GRID is similar to CoMFA and can also be used to determine the interaction energies between the probe and the ligand. In addition, GRID can be used to calculate hydrogen-bonding energies (Duch et al. 2007).

In four-dimensional QSAR (4D-QSAR), the fourth dimension represents an ensemble of conformations, orientations, or protonation states for each molecule (Vedani et al. 2000). This reduces the bias that may come from the ligand alignment, but it requires identification of the most likely bioactive conformation and orientation (or protonation state), which is frequently obtained using evolutionary algorithms. Five-dimensional QSAR (5D-QSAR) carries this one step further, allowing for changes in receptor-binding pocket and ligand topology (Vedani and Dobler 2002). Adding solvation effects to 5D-QSAR results in six-dimensional QSAR (6D-QSAR), which allows, in combination with flexible docking, relatively accurate identification of the endocrine-disrupting potential associated with a drug candidate (Vedani, Dobler, and Lill 2005). Software tools are used for calculation of molecular descriptors; most of them are publicly available and free for academic use (from ChemAxon, OpenEye, and OpenBabel), but some are commercial tools (from VLifeMDS).

Success of QSAR modeling depends on the appropriate selection of a data set for QSAR studies. The number of compounds in the data set for QSAR studies should not be too small or, for practical reasons, too large. In model validation schemes, the data set is divided into three subsets: (1) training, (2) test, and (3) external evaluation sets. Training sets are used in model development, and if they are too small chance correlation and overfitting become major problems not allowing one to build truly predictive models. In the case of continuous response activity, the number of compounds in the training set should be at least 20 and about 10 compounds should be in each of the test and external evaluation sets; so, the total minimum number of compounds should not be less than 40. In the case of classification or category response activity, the training set should contain at least about 10 compounds of each class, and test and external evaluation sets should contain no less than 5 compounds for each class. Outliers in a data set can be errors due to structure representation or biological activity, and they should be removed before proceeding with model development (Tropsha 2010). Statistical and machine learning techniques, such as multiple linear regression (MLR), principal component analysis (PCA), and PLS, are then used to solve the problem. It should be mentioned that MLR is still one of the most widely used artificial intelligence techniques in QSAR studies.

### 33.6 CONCLUSION

Drug discovery and development in this millennium is armed with not only new and efficient techniques for producing and screening new entities but also computational techniques, both hardware and software, that were unimaginable a decade ago. It is now possible to design algorithms and empirical screens to predict *a priori* absorption and distribution properties of lead molecules in silico. Although computational methods are well established in drug discovery and molecular design, their application in the field of natural products is still in its infancy and more specifically to marine-derived drugs. Computer-assisted approaches, such as docking, pharmacophore modeling, and virtual screening, have to be carried out in the field of bioactive natural products to assess their druggability. This can potentially save research from pursuing wrong leads. The investment of time and resources that can be directed to more promising novel agents will allow the lead-to-market time to shorten considerably in the near future. Combined with experimentation and informatics, computer modeling is expected to accelerate drug discovery, more specifically drug discovery from marine-derived products, to find solutions to many problems such as cancer in the near future.

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# 34 Anticancer Compounds from Marine Microorganisms

Hee Jae Shin

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## 34.1 INTRODUCTION

It is widely accepted that new drugs, especially anticancer drugs and antibiotics, are urgently required. Despite the recent de-emphasis on natural product research by the pharmaceutical industry, no other drug discovery platform has proved to be as effective in yielding unique chemical structures with either direct application in the treatment of disease or the capacity to serve as chemical scaffolds from which molecules with enhanced efficacy can be derived. There is little doubt that myriad structural motifs remain undiscovered from natural sources and that these molecules will be an important source of new medicines in the future (Jensen et al. 2005). Due to a recent deceleration in natural product research in terrestrial habitats, there is increasing interest in the exploration of marine microorganisms for novel metabolites such as anticancer and antimicrobial compounds. In exploring new sources of bioactive natural products, the marine environment warrants particular attention in view of its remarkable diversity of microorganisms and metabolic products. The oceans are highly complex environments and house a diverse assemblage of microorganisms that occur in environments with extreme variations in pressure, salinity, and temperature. The oceans cover around 70% of the Earth's surface and present themselves as an unexplored area of opportunity. Marine microorganisms encompass a complex and diverse assemblage of microscopic life forms, of which it is estimated that only 1% has been cultured or identified to date (Bernan, Greenstein, and Carter 2004). It is estimated that marine environments, including the subsurface, harbor approximately  $3.67 \times 10^{30}$  microorganisms (Whitman, Coleman, and Wiebe 1998) that represent an extraordinary and dynamic gene pool of biodiversity. The majority of these microbes have never been cultured, identified, or classified, and their enormous chemical richness remains untapped (DeLong 1997). However, in the past two decades this situation has changed as a result of the rapid progress achieved in related fields. The search for bioactive compounds is presently reaching a new dimension with such diverse approaches as genomics, proteomics, bioinformatics, combinatorial biosynthesis, combinatorial chemistry, targeted drug development, directed evolution of key enzymes, phage-display libraries, automation, and high-throughput screening (Wagner-Dobler et al. 2002).

Natural products are the most consistently successful source of drug leads. Of the 175 anti-cancer drugs approved between 1940 and 2006, 42% were either natural products or compounds derived from natural products (Newman and Cragg 2007). Many antitumor drugs are natural

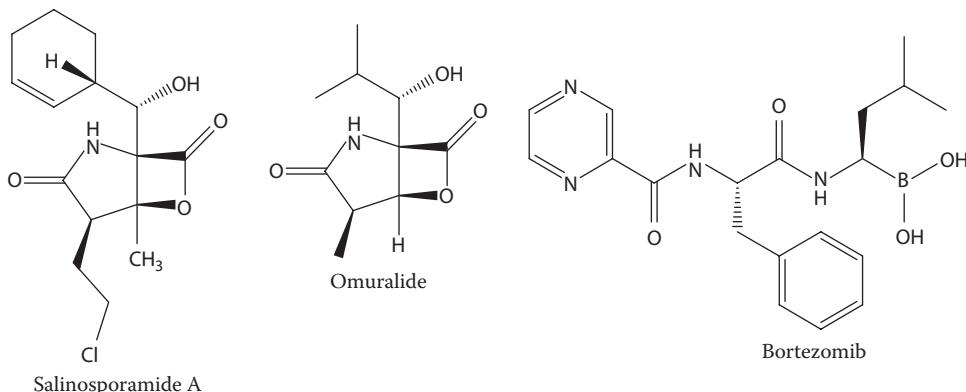
products isolated from microorganisms or plants growing in environmental niches (Gullo et al. 2006; Itokawa et al. 2008). In the last few decades, marine niches have also been explored and new bioactive anticancer compounds have been isolated (Blunt et al. 2008). Marine organisms provide a seemingly endless parade of novel structures. New carbon skeletons were described with a frequency that exceeded all expectations. Several structural features are uniquely or predominantly marine (Faulkner et al. 2000a). The most striking change in the direction of marine natural product chemistry since 1993 was reflected in a sudden increase in reports of new metabolites from marine microorganisms (Wagner-Dobler et al. 2002).

In this chapter, we focus on novel anticancer compounds isolated from marine microorganisms and classify them by their origins.

## 34.2 MARINE ACTINOMYCETES

Actinomycetes are filamentous gram-positive bacteria belonging to the phylum Actinobacteria, which represents one of the largest taxonomic units among the 18 major lineages currently recognized within the domain Bacteria (Ventura et al. 2007). Around 23,000 bioactive secondary metabolites produced by microorganisms have been reported and over 10,000 of these compounds are produced by actinomycetes, representing 45% of all bioactive microbial metabolites discovered to date (Berdy 2005). Progress has been made recently on drug discovery from actinomycetes by using high-throughput screening and fermentation, mining genomes for cryptic pathways, and combinatorial biosynthesis to generate new secondary metabolites related to existing pharmacophores (Baltz 2008). An intriguing picture of the diversity of marine actinomycetes is beginning to emerge. Once largely considered to originate from dormant spores that washed in from land (Goodfellow and Haynes 1984), it is now clear that specific populations of marine-adapted actinomycetes not only exist but add significant new diversity within a broad range of actinomycete taxa (Mincer et al. 2002; Stach et al. 2003). In addition, in the past few years marine actinomycetes have been a huge source of new compounds, and their isolation all around the globe, from shallow costal sediments to the deepest sediments from the Mariana Trench, Pacific Ocean, demonstrates that actinomycetes are ubiquitous in marine sediments, but at lower numbers than in soil (Ghanem et al. 2000; Zheng et al. 2000; Fiedler et al. 2005; Fenical and Jensen 2006; Panthom-Aree et al. 2006; Ward and Bora 2006; Bredholdt et al. 2007; Bull and Stach 2007; Gontang, Fenical and Jensen 2007; Jensen and Lauro 2008; Anzai et al. 2008; Hong et al. 2009; Maldonado et al. 2009). Marine actinomycetes have attracted much attention since they have unique metabolic and physiological capabilities that not only ensure their survival in extreme habitats but also offer the potential to produce compounds with antitumor activity and other interesting pharmacological activities that are not observed in terrestrial microorganisms (Fenical, Sethna, and Lloyd 2002; Blunt et al. 2006; Mayer et al. 2007; Blunt et al. 2009; Williams 2009). Even with the limited screening efforts that have been dedicated to the discovery of new compounds to date, the discovery rate of novel bioactive metabolites from marine actinomycetes has recently surpassed that of their terrestrial counterparts (Lam et al. 2010).

One successful example of bioprospecting of marine actinomycetes is the isolation of salinosporamide A (named NPI-0052 by Nereus Pharmaceuticals, Inc., San Diego, California) from the novel marine genus *Salinispora* by Fenical's group at the Scripps Institution of Oceanography, La Jolla, California (Feling et al. 2003). Salinosporamide A was first isolated from the culture broth of *Salinispora tropica* strain CNB392, an obligate marine bacterium isolated from a marine sediment sample, as a potent proteasome inhibitor ([Figure 34.1](#)). Salinosporamide A displayed potent in vitro cytotoxicity against HCT-116 human colon carcinoma cells with the half maximal inhibitory concentration ( $IC_{50}$ ) values of less than 2 ng/mL. Salinosporamide A also showed potent and highly selective activity in the 60-cell-line panel of the National Cancer Institute (NCI), Bethesda, Maryland, with a mean the concentration required to achieve 50% growth inhibition ( $GI_{50}$ ) value of less than 10 nM (Fenical et al. 2009). The greatest activity was observed against NCI-H226 non-small cell lung cancer, SF-539 CNS cancer, SK-MEL-28 melanoma, and MDA-MB-435 breast cancer (all with the amount of

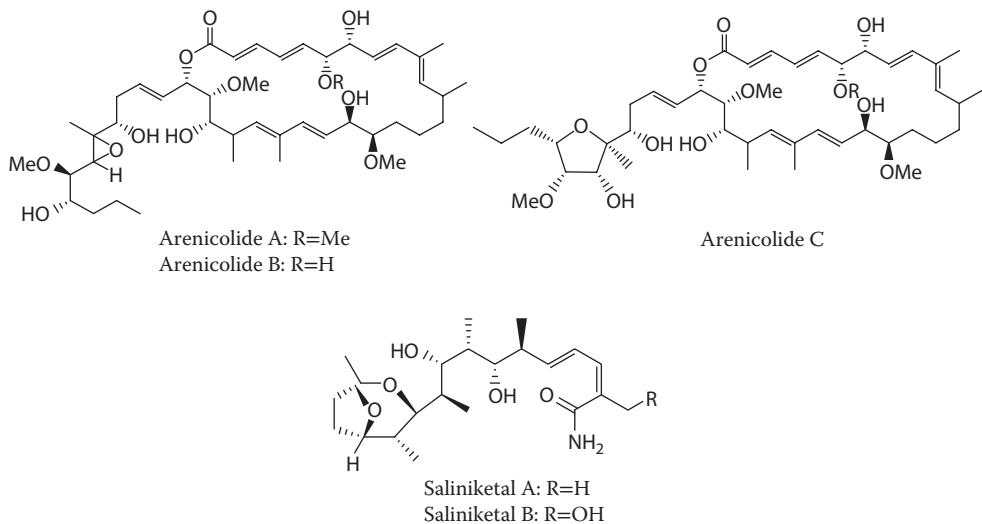


**FIGURE 34.1** Proteasome inhibitors.

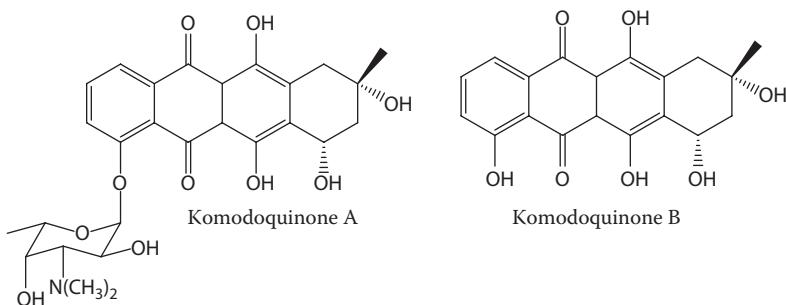
a material which causes the death of 50% ( $LC_{50}$ ) values less than 10 nM). In addition to bortezomib (Figure 34.1), several small-molecule 20S proteasome inhibitors are already well established as research tools (Kisselev and Goldberg 2001), including the  $\beta$ -lactone- $\gamma$ -lactam inhibitor omuralide (Figure 34.1). Based on the structural relationship of salinosporamide A to the previously described  $\beta$ -lactone omuralide, salinosporamide A was tested for its effects on proteasome function. When tested against purified 20S proteasome, salinosporamide A inhibited proteasomal chymotrypsin-like proteolytic activity with an  $IC_{50}$  value of 1.3 nM (Feling et al. 2003). Salinosporamide A is approximately 35 times more potent than omuralide, which was tested as a positive control in the same assay. Salinosporamide A recently entered phase II human clinical trials for the treatment of multiple myeloma.

Marine actinomycetes are also well-known producers of polyketides, which are a large family of natural products produced by a sequential set of reactions catalyzed by polyketide synthases (PKSs). The carbon skeletons of polyketides are synthesized by stepwise decarboxylative Claisen-type condensation of acyl-CoA precursors that are further reduced and modified based on the programming encoded by different domains present in PKSs with ketoreductase, dehydratase, and enoylreductase activities. A large number of polyketides with antitumor activity have been isolated from marine actinomycetes. Arenicolides, which are 26-membered polyunsaturated macrolactones, produced by the obligate marine actinomycete *Salinispora arenicola* strain CNR-005 were isolated from a marine sediment sample collected at a depth of 20 m from the coastal water around the island of Guam (Figure 34.2) (Williams et al. 2007). From the same strain of *S. arenicola*, two bicyclic polyketides, saliniketals A and B, were also isolated. Saliniketals A and B (Figure 34.2) were found to inhibit ornithine decarboxylase (ODC) induction, an important target for the chemoprevention of cancer, with  $IC_{50}$  values of 1.95 and 7.83  $\mu$ g/mL, respectively (Williams et al. 2007). The ODC is a directed transcriptional target of the oncogene *myc* and is overexpressed in various tumor cells (Gerner and Meyskens 2004). Anthracyclines are an important group of antitumor compounds with a basic 7,8,9,10-tetrahydro-5,12-naphthacenequinone structure that is glycosylated at C7. Two anthracyclines, komodoquinone A and its aglycone, komodoquinone B (Figure 34.3), were isolated from solid-state fermentation of the marine *Streptomyces* sp. KS3, which was isolated from marine sediments. Komodoquinone A is a unique anthracycline, in which a new amino sugar is connected to the D-ring of the anthracyclinone skeleton, and was found to induce neuronal cell differentiation in the neuroblastoma cell line, Neuro 2A (Itoh et al. 2003).

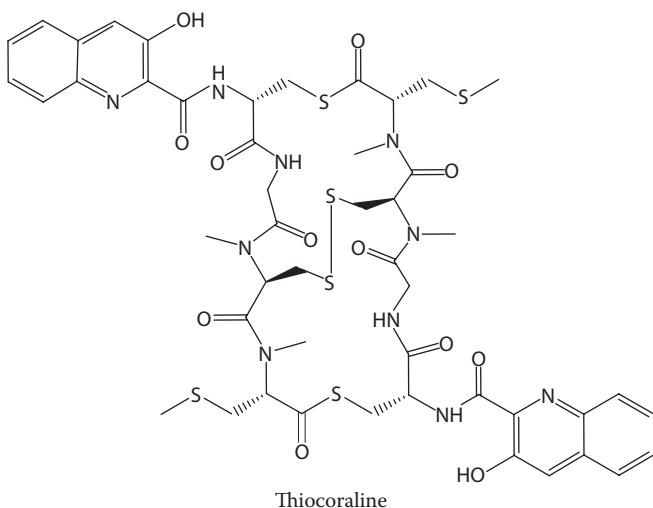
The peptides synthesized by nonribosomal peptide synthetases (NRPSs), which are also quite open, have some unique structural features and exhibit potent anticancer activity. Thiocoraline (Figure 34.4) is an antitumor cyclic thiodepsipeptide isolated from the mycelium of a marine actinomycete, *Micromonospora* sp. L-13-ACM2-092 (Pérez Baz et al. 1997; Romero et al. 1997), that has been shown to exhibit exceptionally potent activity in the L1210 mouse leukemia cytotoxic assay ( $IC_{50} = 200$  pM) (Boger et al. 2001) and to inhibit the elongation activity of DNA polymerase



**FIGURE 34.2** Arenicolides and saliniketals from the obligate marine actinomycete *Salinispora arenicola* strain CNR-005.



**FIGURE 34.3** Structures of komodoquinones A and B isolated from the marine *Streptomyces* sp. KS3.

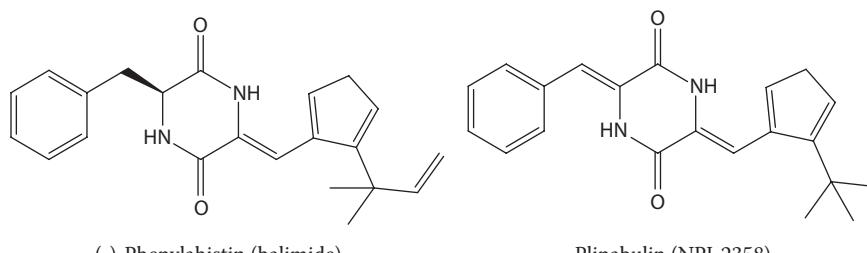


**FIGURE 34.4** Structure of thiocoraline, an antitumor cyclic thiodepsipeptide produced by two actinomycete strains *Micromonospora* sp. ML1 and *Micromonospora* sp. ACM2-092 isolated from a marine mollusk and a soft coral, respectively.

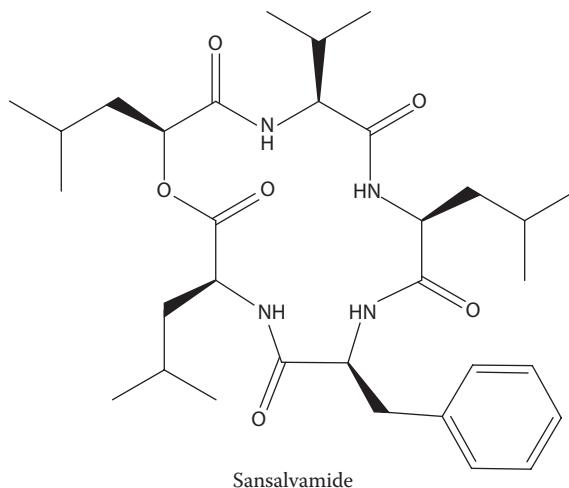
$\alpha$  in both LoVo and SW620 human colon cancer cells (Erba et al. 1999). The latter mechanism was invoked to account for the arrest observed in the G1 phase of the cell cycle and the decrease in the rate of S phase progression toward G2/M phases, given that this agent neither inhibits DNA topoisomerase II nor induces DNA strand breaks. The Spanish marine drug company PharmaMar, Madrid, has reported that thiocoraline shows activity against several standard drug screens, including breast cancer, colon cancer, renal cancer, and melanoma. Target cells appear to be inhibited through the inhibition of DNA polymerase  $\alpha$  enzyme. The most recent published literature suggests that thiocoraline is about to enter phase I clinical trials (Newman and Cragg 2004). Thiocoraline represents a model of an anticancer agent acquired from marine microorganisms and illustrates how the problems of drug supply can be overcome by microbial cultures.

### 34.3 MARINE FUNGI

Marine fungi are prolific producers of bioactive compounds including antitumor drugs (Belofsky et al. 1999; Bhadury, Mohammad, and Wright, 2006; Newman and Hill 2006). In 2011, a 3-million-Euro program to discover anticancer drugs from marine fungi was started. Hypha Discovery, Ltd., Uxbridge, United Kingdom, attended the inaugural meeting for the Marine Fungi project in Kiel, Germany, which aims to identify natural products having the potential to be developed as anticancer agents. It is noted that 11 institutions from 7 countries have set up a consortium, receiving a funding of 3 million Euro over a 3-year period from the European Commission, under the Seventh Framework Program (FP7). A rich profile of biologically active metabolites is described from filamentous fungi of terrestrial origin, especially from just three genera: (1) *Penicillium*, (2) *Aspergillus*, and (3) *Fusarium* (Lene 1996). However, marine fungi are less studied compared to their terrestrial counterparts and other ecological groups. Obligate marine fungi still remain an unexplored resource, although marine facultative fungi have been studied due to their production of new metabolites that are not found in terrestrial fungi. Recently, there is more interest on studying biologically active metabolites from higher fungi (Basidiomycota), endophytic fungi, and filamentous fungi from marine habitats, the symbiotic lichens (Boopathy and Kathiresan 2010). Because of their particular living conditions, including salinity, nutrition, high pressure, and temperature variations, and competition with bacteria, viruses, and other fungi, they may have developed specific secondary metabolic pathways different from terrestrial fungi (Liberra and Lindequist 1995). Recent investigations on marine filamentous fungi looking for biologically active secondary metabolites indicate their tremendous potential as a source of new medicines (Namikoshi et al. 2002). Plinabulin (NPI-2358) (Figure 34.5) is based on the diketopiperazine phenylahistin (also known as halimide), which was isolated from cultures of the marine-derived fungus *Aspergillus ustus* isolated from a marine alga, *Halimeda lacrimosa* (Kanoh et al. 1997). Plinabulin is a potent, selective tumor vascular disrupting agent (VDA) and one of over 200 synthetic analogs in the series prepared following the discovery of the parent compound, which was isolated from a marine fungus, as opposed to terrestrial sources for other VDAs. Plinabulin has a dual effect on tumors: it selectively attacks existing tumor blood vessels, leading to tumor necrosis



**FIGURE 34.5** Structure of phenylahistin: phenylahistin was isolated from cultures of a marine-derived fungus, *Aspergillus ustus*. Its synthetic analog, plinabulin, is under clinical trials.



**FIGURE 34.6** Structure of sansalvamide, a cyclic pentadepsipeptide isolated from a marine fungus, *Fusarium* sp.

without affecting normal vasculature, and it has a direct apoptotic effect on tumor cells. Plinabulin binds to the colchicine-binding site of  $\beta$ -tubulin preventing polymerization and disrupting the cytoplasmic microtubule network. Preclinical studies indicated plinabulin had a favorable safety and activity profile when added to standard cancer therapies. These findings, together with the fact that plinabulin can synergize with or complement chemotherapeutics and antiangiogenesis agents, led to the initiation of the Assessment of Docetaxel and Vascular Disruption in Non–Small Cell Lung Cancer (ADVANCE) phase I/II trial in 2009. An update on clinical trials with plinabulin, which has shown a favorable safety profile accompanied by positive therapeutic effects and its use in combination with other anticancer drugs, has been published (Mita et al. 2010). Plinabulin is being developed by Nereus Pharmaceuticals, Inc., for cancer treatment (<http://www.nereuspharm.com/>).

Sansalvamide (Figure 34.6) is a cyclic pentadepsipeptide isolated from organic extracts of the mycelium of a marine fungus of the genus *Fusarium* collected from the surface of the seagrass *Halodule wrightii* (Belofsky et al. 1999). Sansalvamide exhibited selective in vitro cytotoxicity toward COLO 205 colon and SK-MEL-2 melanoma cancer cell lines.

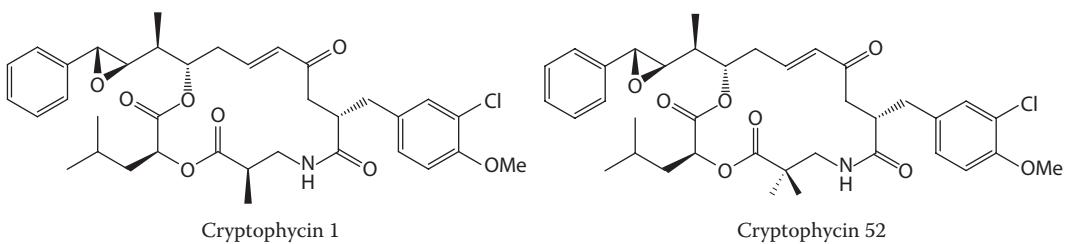
#### 34.4 MARINE CYANOBACTERIA

More than 50% of marine cyanobacteria are potentially exploitable bioactive substances that either are effective in killing cancer cells by inducing apoptotic death or affect cell signaling by activating the members of the protein kinase C family of signaling enzymes. A number of these cytotoxic compounds and their synthetic derivatives are in clinical trials as treatment against various cancer forms. It has been predicted that based on structural similarities with cyanobacterial compounds, at least 35% of the 20 marine-derived anticancer agents in clinical trials could have marine cyanobacteria as the biogenic source (Simmons and Gerwick 2008). This high proportion of marine natural products potentially deriving from marine cyanobacteria underlines the importance of these ancient microbes as sources of novel therapeutic drugs. The pioneering studies of Richard E. Moore (Cardellina and Moore 2010) at the University of Hawaii conducted in 1970s through early 2000s revealed marine cyanobacteria to be an extremely rich source of secondary metabolites. Over the ensuing 20 years, the Moore group pioneered marine cyanobacteria in antitumor and antifungal drug discovery, leading to the discovery of a large number of novel products, including hapalindoles (Moore, Cheuk and Patterson 1984), scytophyccins (Ishibashi et al. 1986), anatoxins (Matsunaga et al. 1989), tolytoxins (Carmeli, Moore, and Patterson 1999), tantazoles (Carmeli et al. 1990), and cyclindrocyclophanes

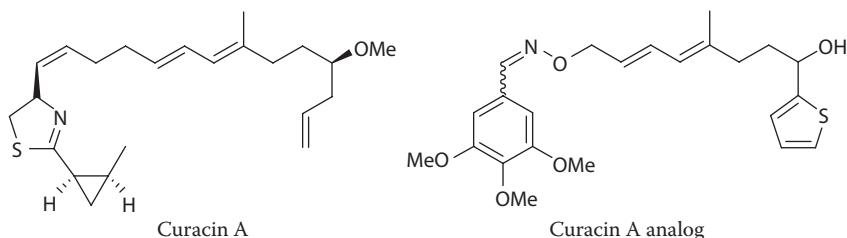
(Moore et al. 1990). When cryptophycins, a group of more than 25 cyanobacterial metabolites with strong tubulin-destabilizing activities (Smith et al. 1994; Corbett et al. 1997; Panda et al. 1997), were discovered, expectations were high that one of these natural products could be developed into a useful anticancer drug. In fact, the prototype cryptophycin 1 (Figure 34.7) (Golakoti et al. 1996), the major representative of this class of natural anticancer drugs from the cyanobacterial symbiont *Nostoc* sp. ATCC 53789, is one of the most potent tubulin-destabilizing agents ever found (Smith et al. 1994). Cryptophycin 52 (Figure 34.7), a synthetic analog, was developed, and it reached phase II clinical trials in collaboration with Eli Lilly (Sessa et al. 2002; Edelman et al. 2003; D'Agostino et al. 2006). The synthetic analog cryptophycin 52 was chosen because no large-scale biotechnological production method existed for cryptophycins. Eventually, the high production costs and toxic side effects of cryptophycin 52 caused the development of cryptophycin 52 and all other analogs of the cryptophycin family to be stopped. Several groups have studied in a very comprehensive way for the biosynthesis of the cryptophycins. The studies cover a wide range of topics, including incorporation experiments, cloning of gene clusters, important biosynthetic enzymes, and the production of new cryptophycin analogs by precursor-directed biosynthesis. Cryptophycin 52 is now biotechnologically producible, which may make this drug easier to access and less costly to produce if further pursued.

Curacin A (Figure 34.8) is a unique thiazoline-containing lipopeptide possessing exquisite anti-mitotic activities, and it was isolated as a major component (8%–10%) from the organic extract of the marine cyanobacterium *Lyngbya majuscula* from Curaçao (Gerwick et al. 1994). Subsequent biological screening of this molecule in the NCI 60 cell line revealed curacin A as a potent anti-tubulin drug with an  $IC_{50}$  value of 0.72  $\mu\text{M}$  in inhibiting tubulin polymerization. Due to its unique structural features and high potency as an antitubulin agent, curacin A was a target of several total syntheses, and numerous synthetic analogs were generated for structure–activity relationship (SAR) studies (Wipf et al. 2004). The curacin A analog (Figure 34.8) is the simplified compound which is three times more potent as compared to curacin A in inhibiting GTP/glutamate-induced polymerization of tubulin with an  $IC_{50}$  value of 0.17  $\mu\text{M}$  (Wipf et al. 2002).

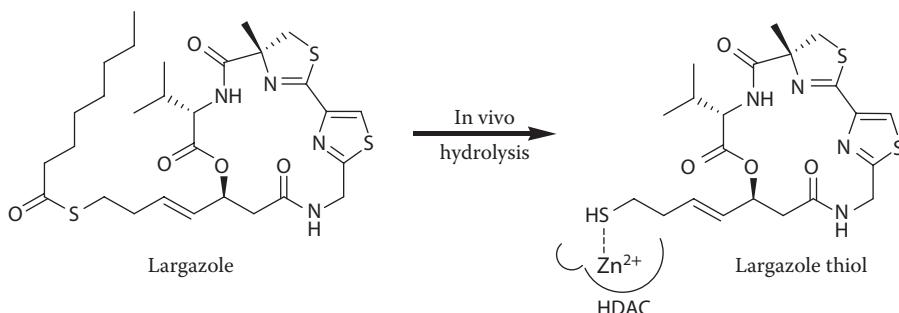
Largazole (Figure 34.9) is a macrocyclic depsipeptide originally isolated from the marine cyanobacterium *Symploca* sp., which is indigenous to the warm, blue-green waters of Key Largo, Florida (Taori, Paul, and Luesch 2008). Largazole possesses highly differential growth-inhibitory activity and preferentially targets transformed cells over nontransformed cells. The intriguing structure and biological activity of largazole have attracted strong interest from the synthetic chemistry



**FIGURE 34.7** Structures of cryptophycin 1 and its synthetic analog, cryptophycin 52, which show strong tubulin-destabilizing activities.



**FIGURE 34.8** Structures of the potent antitubulin agents curacin A and its analog.

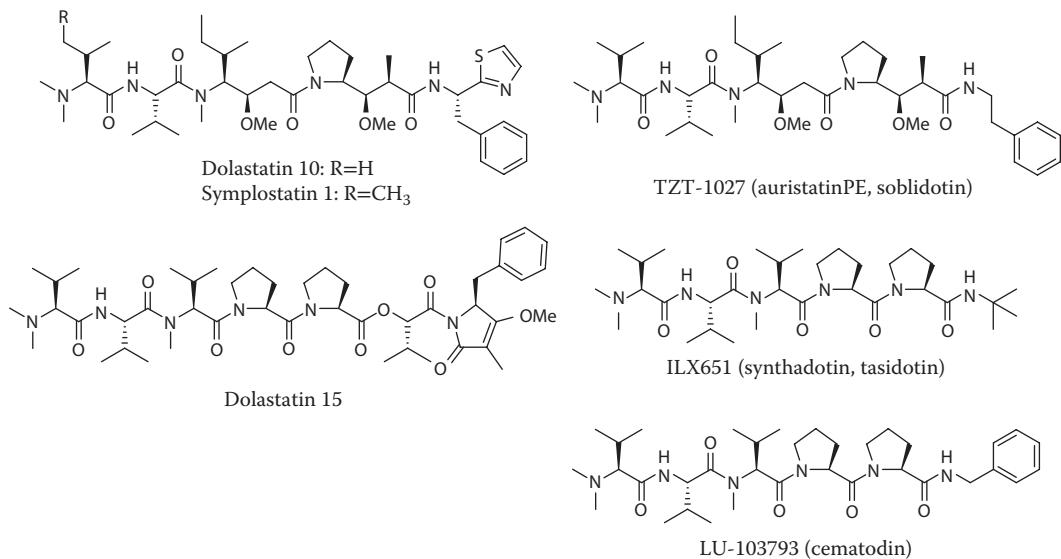


**FIGURE 34.9** Structure of largazole, a potent antiproliferative agent from the Floridian marine cyanobacterium *Symploca* sp.: largazole possesses potent HDAC inhibitory activity. Hydrolysis of its thioester side chain in vivo yields a free thiol group capable of coordinating to the catalytic  $Zn^{2+}$  ion of HDAC enzymes.

community to establish synthetic routes to largazole and to investigate its potential as a cancer therapeutic. Similar to romidepsin, largazole is a prodrug; hydrolysis of its thioester side chain in vivo yields a free thiol group capable of coordinating to the catalytic  $Zn^{2+}$  ion of histone deacetylase (HDAC) (Figure 34.9) enzymes. Indeed, largazole thiol (Figure 34.9) is believed to be the most potent inhibitor of HDAC enzymes (Bowers et al. 2009); it exhibits low nanomolar inhibitory activity against several HDAC enzymes (Ying et al. 2008; Bowers et al. 2009) and remarkable antiproliferative effects. Largazole was recently hailed in *Newsweek* as the latest victory in bioprospecting the vast gold mine of marine natural products for new disease therapies (Behar 2010).

### 34.5 MICROORGANISMS ASSOCIATED WITH INVERTEBRATES (SYMBIOTICS)

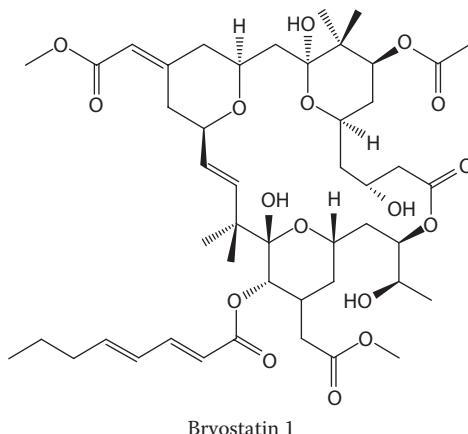
Over the last few decades, circumstantial evidence has led to the hypothesis that many of the compounds isolated from marine macroorganisms, such as sponges, ascidians, and other invertebrates, in fact have a microbial origin (Bewley and Faulkner 1998). Marine macroorganisms harbor commensal microorganisms that include prokaryotic bacteria, cyanobacteria (blue-green algae), eukaryotic microalgae, and fungi within host tissues where they reside as extra- and intracellular symbionts. More generally, marine invertebrate species are remarkable for how extensively they associate with marine microorganisms. For instance, marine sponges consist of 40% bacteria by weight. These and other marine invertebrates harbor diverse microorganisms, sometimes benefitting through rich suites of microbially produced chemicals that protect these sponges or other hosts against predators or pathogens. Increasing evidence implicates microbial symbionts as the true source of many marine organism-derived compounds, which makes marine microbial symbionts a hot spot in the field of marine microbiology and marine natural products because of their potential for solving the bottleneck problem of marine natural product supply. There is now abundant evidence to suggest that a significant portion of the bioactive metabolites thought originally to be products of the source animal are often synthesized by their symbiotic microorganisms. Several anticancer metabolites from marine sponges that have progressed to preclinical or clinical-trial phases, such as discodermolide, halichondrin B, and bryostatin 1, are thought to be products derived from their microbiotic consortia. Of 20 compounds deriving from (or inspired by) marine natural products that are currently or were recently in clinical trials for the treatment of cancer, 15 were isolated from sponges, tunicates, and mollusks and only 5 came directly from a microorganism (Simmons et al. 2008). However, based on biosynthetic parallels, distribution in taxonomically diverse organisms and, in a few cases, subsequent direct isolation from a producing microorganism, 16 of these anticancer molecules actually derive from microbial sources and only 4 derive from macroorganisms. For example, dolastatin 10 (Figure 34.10), which was originally isolated from the sea hare *Dolabella auricularia*, was later shown to originate from cyanobacteria of the genera *Symploca* and *Lyngbya* on which sea hares or



**FIGURE 34.10** Anticancer marine cyanobacterial metabolites and their synthetic analogs.

other marine organisms feed (Pettit et al. 1987; Pettit et al. 1989; Luesch et al. 2001). The exquisite anticancer activity of dolastatin 10 was subsequently attributed to its binding to tubulin at a distinct site near the vinca alkaloid site. This results in the disruption of normal tubulin function as well as the exchangeable guanosine triphosphate site, leading to cell cycle arrest in the metaphase. In the 1990s, dolastatin 10 entered phase I clinical trials through the NCI and subsequently progressed to phase II trials. However, further clinical trials were discontinued due to disappointing results, including the development of peripheral neuropathy in 40% of patients (Pitot et al. 1999) and insignificant activity in patients with hormone refractory metastatic adenocarcinoma (Vaishampayan et al. 2000) and recurrent platinum-sensitive ovarian carcinoma (Hoffman, Blessing, and Lentz 2003). Dolastatin 15 (Figure 34.10), a seven-subunit depsipeptide derived from the sea hare *D. auricularia* (Pettit et al. 1989), is a potent antimitotic agent structurally related to the antitubulin agent dolastatin 10. This compound was isolated from extracts of the Indian Ocean sea hare *D. auricularia* in trace amounts (6.2 mg from 1600 kg of wet sea hare [ $4 \times 10^{-7}\%$ ]), which again strongly implicates a cyanobacterial source for this metabolite. Dolastatin 15 was also isolated from a Palauan cyanobacterium, *Lyngbya* sp. (Luesch et al. 2002). In addition, dolastatins 10 and 15 served as drug leads for the development of synthetic analogs, TZT-1027, ILX-651, and LU-103793 (Figure 34.10), usually with improved pharmacological and pharmacokinetic properties (Mita et al. 2006; Watanabe, Minami, and Kobayashi 2006). The antitumor activity of TZT-1027 (soblidotin), a synthetic derivative of dolastatin 10, was found to be superior to existing anticancer drugs, such as paclitaxel and vincristine, and TZT-1027 is currently undergoing phase I testing for treating solid tumors (Watanabe, Minami, and Kobayashi 2006). The third-generation dolastatin 15 analog ILX-651 (or tasidotin) is another antitumor agent currently undergoing phase II trials after its successful run in phase I trials (Mita et al. 2006).

Bryostatins are a group of macrolide lactones; they were first isolated in the 1960s by George Pettit from extracts of a species of bryozoan, *Bugula neritina*. The structure of bryostatin 1 (Figure 34.11) was determined in 1982 by Pettit and coworkers (Pettit et al. 1982). Since then, other members of this family have been isolated, and some 20 bryostatins are known today (Hale and Manaviazar 2010). It has also been established that the true source of bryostatins is not actually *B. neritina* but rather a bacterial symbiont, *Candidatus Endobugula sertula* (Sudek et al. 2007). Originally isolated in the 1960s, bryostatin 1 has been evaluated in several phase I and II clinical trials for the treatment of a variety of leukemias, ovarian cancers, and prostate cancers and in combination with other anticancer drugs such as paclitaxel (Schwartz and Shah 2005; Wang et al. 2003). There have been



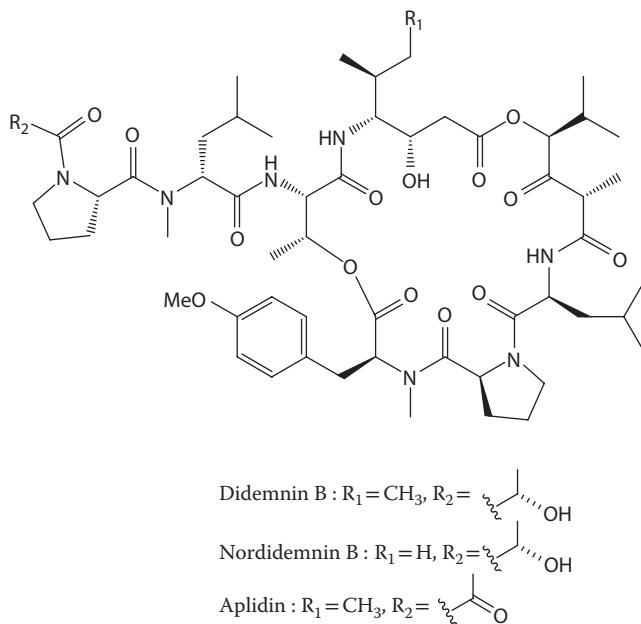
**FIGURE 34.11** Structure of bryostatin 1, a macrolide lactone that was first isolated from a species of bryozoan, *Bugula neritina*: the true producer of bryostatin 1 is a bacterial symbiont, *Candidatus Endobugula sertula*.

more than 80 anticancer clinical trials of bryostatin 1, and current trials have been shifted to focus on combination therapy (Newman and Cragg 2004). In addition, bryostatin 1 has shown promising activity relevant to a number of other diseases and conditions, including diabetes (Way, Katai, and King 2001), stroke (Sun et al. 2008), and Alzheimer's disease (Etcheberrigaray et al. 2004; Alkon, Sun, and Nelson 2007). Obtaining an adequate supply of bryostatin through aquaculture is difficult, which impedes its development as a therapeutic. This supply issue will become a serious problem if bryostatins are approved for use in human beings and large quantities of the material are required. Therefore, the identification of the gene cluster opens the way for potential production of this promising anticancer candidate in a more suitable bacterial host, such as *Escherichia coli*.

Didemnin B (Figure 34.12) was first isolated from Caribbean tunicates of the family Didemnidae (Rinehart et al. 1981). The authors suggested that didemnins are products of symbiosis between tunicates and cyanobacteria because of their occurrence in taxonomically distant species of tunicates and the presence of *N,O*-dimethyltyrosine residue, which was also found in metabolites of the cyanobacterium *Lyngbya majuscule* (Sings and Rinehart 1996). Even though a wide variety of peptides have been isolated from cyanobacteria, none of them are structurally related to didemnins. Therefore, no direct evidence to determine the primary producer of didemnins is available. A Japanese group has discovered a didemnin-producing bacterium,  $\alpha$ -proteobacterium *Tistrella mobilis*, obtained from marine sediments; they isolated didemnin B and nordidemnin B (Figure 34.12) from the extract of culture broth of *T. mobilis*. Didemnin B demonstrated the most potent antitumor activity, and its development was initiated by the NCI (Simmons et al. 2005). Didemnin B was the first marine natural product to enter clinical trials as an anticancer agent. Phase II clinical trials revealed the anticancer activity of didemnin B against various types of cancer. However, unfortunately, didemnin B exhibited high toxicity through a high incidence of anaphylactic reactions in patients and the NCI discontinued its developmental efforts. Aplidin, which has a pyruvic acid unit in place of the lactic acid unit in didemnin B, is now being developed by PharmaMar and clinical trials are ongoing (Mateos et al. 2010).

## 34.6 CONCLUSION

Marine microorganisms have tremendous potential in providing therapeutic leads with unique chemical structures and biological activities, as illustrated by compounds such as salinosporamide A, bryostatins, and aplidin. The aforementioned examples illustrate the intense excitement that surrounds the achievements of the past decade in the area of new anticancer leads from marine



**FIGURE 34.12** The didemnin family of depsipeptides, which exhibits potent anticancer activity.

microorganisms. Discovery of marine microorganisms producing potent anticancer compounds has ushered in a new era in marine pharmacology. Understanding the optimum ecological conditions that drive the sustainable production of bioactive compounds from marine microorganisms and invertebrate-associated microorganisms helps in formulating various production strategies. Bacterial origin could have important implications for drug development, since it may ultimately permit the creation of fermentation-based production systems that are superior to current methods relying on mass harvestings or multistep total syntheses. To reach this goal, there are several potential strategies to hurdle the challenges posed by the difficulty in culturing most endosymbionts and the complexity of many metagenomes. Recent studies have shown that by careful experimental design, even members of bacterial groups that were previously regarded as unculturable can be readily cultivated. A better ability to predict the genes necessary for a metabolic pathway will in the future allow a researcher in an increasing number of cases to focus on biosynthetic steps for characteristic and rare structural moieties instead of common pathways leading to polyketide- or peptide-core skeletons. Adoption of different cultivation strategies and metagenomic approaches is the need of the hour in discovering new genes, enzymes, and natural products and in enhancing the commercial production of marine drugs from marine microorganisms.

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