BIOACTIVE SECONDARY METABOLITES FROM TROPICAL AND SUB-TROPICAL MARINE INVERTEBRATES

DISSERTATION

by
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DEPARTMENT OF NATURAL PRODUCT CHEMISTRY

GRADUATE SCHOOL

TOHOKU PHARMACEUTICAL UNIVERSITY

MARCH 2013

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Abstract

Bioactive Secondary Metabolites from Tropical and Sub-tropical Marine Invertebrates

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Marine invertebrates have proven to be an important resource for bioactive natural product discovery. Secondary metabolites isolated from marine invertebrates often possess unique structural features and show potent biological activities. During our ongoing search for bioactive secondary metabolites, we collected marine invertebrates, such as sponges and ascidians, from tropical and sub-tropical coral reefs and screened for antimicrobial and cytotoxic activities.

Three new unique sesquiterpenes, eurysponginsA–C (**1**–**3**), were isolated from a marine sponge *Euryspongia* sp. collected at Iriomote Island, Okinawa, Japan. Compound **1** possessed a bicyclic furanosesquiterpene structure with six- and eight-membered rings, whereas compounds **2** and **3** had an α,β-unsaturated-γ-lactone ring instead of the furan ring in **1**. Only five natural products (**5**–**9**) in this class have been reported, and, therefore, compounds **1**–**3** are the sixth–eighth examples of natural products. Compounds **1**–**3** had no inhibition effect against protein tyrosine phosphatase 1B (PTP1B), an important target enzyme for the treatment of type II diabetes, while the dehydro derivative of **1** [dehydroeurysponginA (**4**)] inhibited the activity of PTP1B (IC_{50} = 3.6 μM).

In our screening program to search for PTP1B inhibitors, we found that the ethanol extract of an Indonesian marine sponge *Lamellodysidea herbacea* exhibited a significant inhibitory activity against PTP1B. Bioassay-guided isolation yielded a polybromodiphenyl ether (**10**) as a sole bioactive component. The structure of **10** was identified by spectroscopic data for **10** and its methyl ether derivative (**11**) and by

comparing data for **10** with the reported values for 2-(3',5'-dibromo-2'-methoxyphenoxy)-3,5-dibromophenl. Compound **10** remarkably inhibited the PTP1B activity (IC_{50}=0.85 μM) and showed a moderate cytotoxicity against two human cancer cell lines, HCT-15 (colon) and Jurkat (T-cell lymphoma), with IC_{50} values of 12 and 9.5 μM, respectively. Compound **11** maintained potent inhibitory activity against PTP1B (IC_{50} = 1.7 μM) but did not show apparent cytotoxicty at 18 μM against these cancer cells. Four ester derivatives, acetyl (**12**), butyryl (**13**), hexanoyl (**14**), and benzoyl (**15**), were prepared from **10** and their activities evaluated against PTP1B and two cancer cell lines to investigate the structure-activity relationships. Although compounds **12-15** exhibited potent inhibitory effect against PTP1B activity, cytotoxicity against HCT-15 and Jurkat cells was observed as a similar efficacy to that of **10.** From these results, compound **11** was found to be the supreme inhibitor of PTP1B with no apparent cytotoxicity among them. Therefore, **11** may be a lead compound for making a new type of PTP1B inhibitor. Moreover, compound **11** did not inhibit the cell growth of Huh-7 cells (hepatoma). Hepatocytes are one of the locations of PTP1B, and Huh-7 cells are used to study the mechanism of action of compound **11**.

Two known pyridoacridine alkaloids, shermilamine B (**16**) and kuanoniamine D (**17**), were obtained from an Indonesian marine ascidian *Cystodytes* sp. Compound **17** showed cytotoxicity against HCT-15 and Jurkat cells (IC_{50} = 19 and 4.1 μM, respectively), while the IC_{50} values of **16** was >26 and 6.7 μM, respectively.

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Chapter 1. Introduction

1.1. Marine Natural Products

Natural products have been the major sources of chemical diversity for the pharmaceutical discovery over the past century, that is, natural products and their derivatives have played an important role in the drug discovery and creation of therapeutic agents (Newman and Cragg, 2007; Bugni, *et al*., 2008). Values of natural products can be assessed using three criteria: (1) the rate of introduction of new chemical entities of wide structural diversity, including serving as templates for semisynthetic and total synthetic modifications, (2) the number of diseases treated or prevented by these substances, and (3) their frequency of use in the treatments of diseases (Chin, *et al*., 2006). Many natural products and their derivatives have successfully developed for clinical use to treat human diseases. The role of natural products in the drug history has undergone many changes during the past 30 years. Newman and Cragg (2012) reviewed the role of natural products in the drug discovery and development process from 1981 to 2010. During this period of 30 years, the total number of 1355 compounds has been approved as new drugs. Figure 1-1 shows the sources of drugs approved between 1981 and 2010 including peptides/proteins (B, 15%) and Vaccines (V, 6%). Figure 1-2 shows the sources of small-molecules (N-1073) in which 36% approved drugs were synthetic (S), and, therefore, 64% were natural products and related compounds (Cragg, *et al*., 2012). The large proportion of natural products in drugs discovery making them good candidates for further drug development.

Marine natural products often possess unique structures, which, in turn, lead to new biological and pharmacological activities.

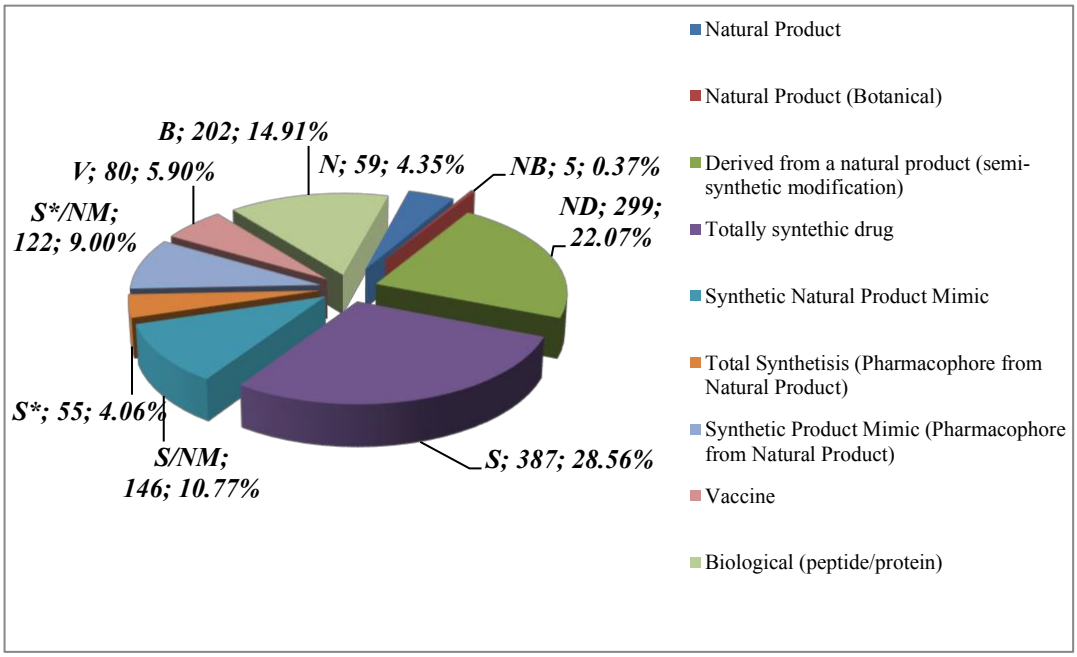


Figure 1-1. Source of all drugs approved from 1981 to 2010 (Adapted from Newman and Cragg, 2012).

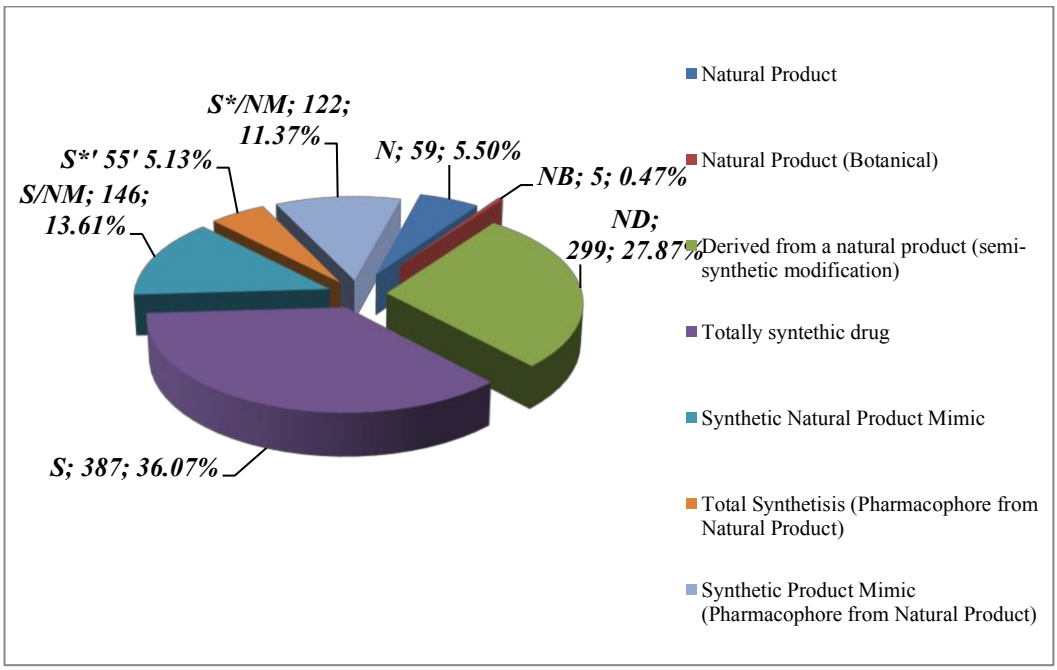


Figure 1-2. Source of small-molecule drugs approved from 1981 to 2010 (Newman and Cragg, 2012).

It has been widely recognized that marine natural products exhibit promising bioactivities including antiparasitic, anti-infective, anticancer, cytotoxicity, antifungal, antibacterial, antiviral, anti-inflammatory, and enzyme-inhibitory activities (Jiang, *et al*., 2012; Blunt, *et al*., 2011; Kornprobst, 2010; Newman and Cragg, 2009; Diyabalanage, *et al*., 2012).

The organisms producing marine natural products are divided into three major biological classes: marine microorganisms (including phytoplanktons), marine algae, and marine invertebrates. In particular, marine invertebrates have demonstrated exceptional potency as a prolific source of novel and structurally diverse natural products (Hildebrand, *et al*., 2004).

The specialty of living environments in the oceans including high concentration of salt, high pressure, low concentration of oxygen, and dark conditions, has made marine invertebrates evolved unique metabolic pathways. Most marine invertebrates lack a physical protection in the form of an exoskeleton, and, therefore, it is clear to understand that the most benthic marine organisms produce an array of secondary metabolites as unique chemical protection strategy against large and small predators and competitors for space and resources to provide them successful survival and evolution (Jiang, *et al*., 2012). Many of marine organisms perform features of chemical diversities that are not found in terrestrial organisms. It is well known that the concept of drugs from the sea had attracted high interest to discover new candidates of medicines, and that marine invertebrates such as sponges and ascidians are rich sources of a variety of secondary metabolites (Jiang, *et al*., 2012). Marine sponges are incredibly attractive targets because of their high biodiversity, and ascidians are also very unique among marine invertebrates since

they produce a large numbers of nitrogen-containing metabolites (Wang and Namikoshi, 2007).

Within the three decades, thousands of new compounds have been reported from a variety of marine organisms. Some of these compounds have unique structures, and various interesting bioactivities have successfully been described. The overview of bioactive compounds isolated from 1989 to 2010 are shown in Figures 1-3 and 1-4, which indicate that sponges and ascidians are important sources of bioactive marine natural products (Blunt, *et al*., 2012; Faulkner, 2002).

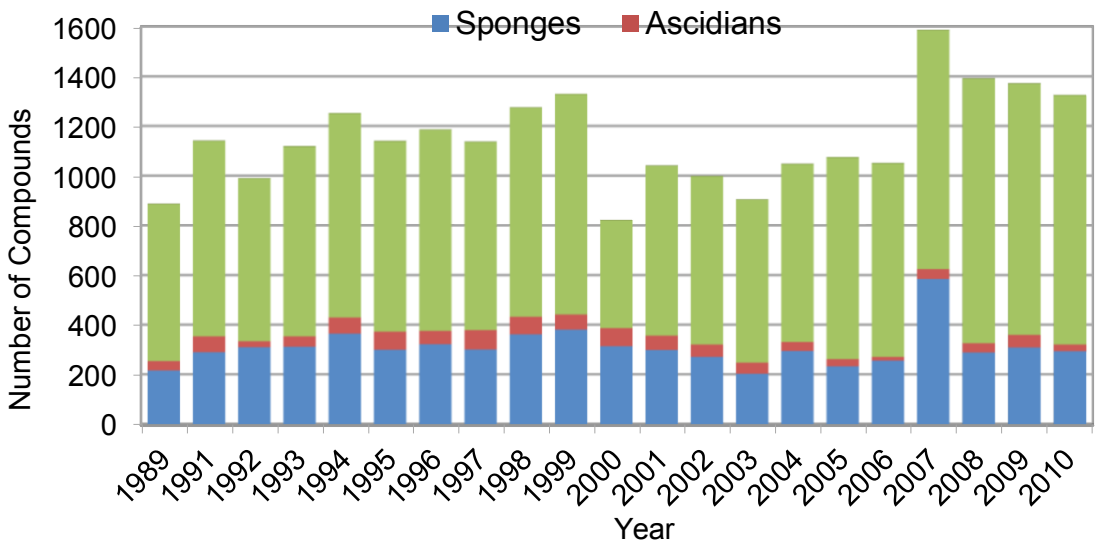


Figure 1-3. New compounds from marine organisms during 1992–2012 (N = 16,514). (Adapted from Blunt *et al*. 2012 and Faulkner 2002).

1.2. Bioactive Secondary Metabolites From Sponges

Sponges (Phyllum Porifera) are the oldest living group of the multicellular organisms (metazoans), but they are very successfully and highly evolved organisms to adapt and survive longer than any other multicellular animals. They lack a differentiation of organs including gonad, and mostly living in the marine environments. Three main

subdivisions have been generally recognized depending on the nature of skeletons that they secrete: calcareous, siliceous, and skeletal.

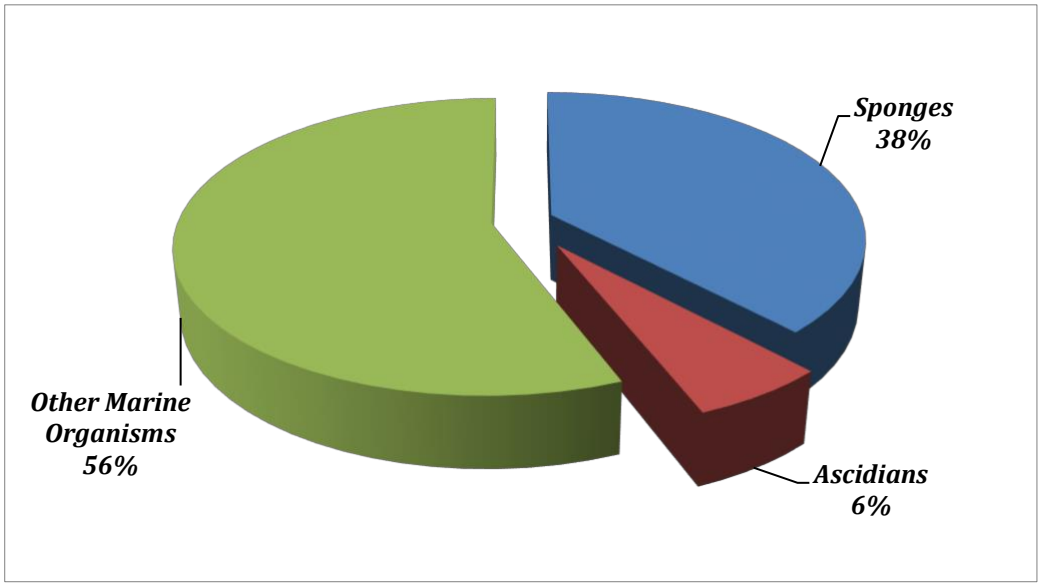


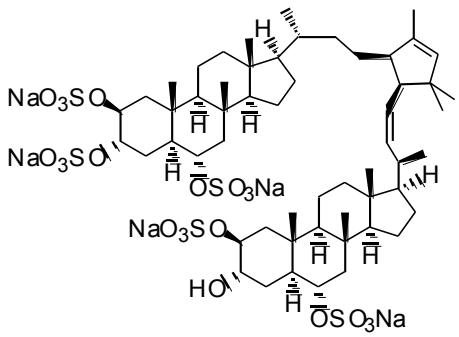
Figure 1-4. Sources of marine natural products obtained during 1992–2012 (N = 16,514).
(Adapted from Blunt *et al*. 2012 and Faulkner 2002).

The phylum Porifera is traditionally divided into two subphyla, Silicispongia and Calcispongiae. The Silicispongia is further devided into Hexactinellida and Demospongia, and Calcispongiae contains only Calcarea (calcareous sponges). Calcareous and hexactinellid sponges are exclusively marine. Sponges live at all depths from shore level to the deep. In the adult stage, sponges permanently attach (sessile), filter feed, and live in association with many organisms. Even though the most of them comprise a silica-based skeleton composed of spicules, their survivals are challenged by a huge number of microorganisms. Sponges provide homes for animals and a variety of microorganisms, and a steady stream of nutrients. As a phylum, sponges are an incredibly attractive research target because of their high biodiversity, widespread distribution and unique biological activity. To date, there are

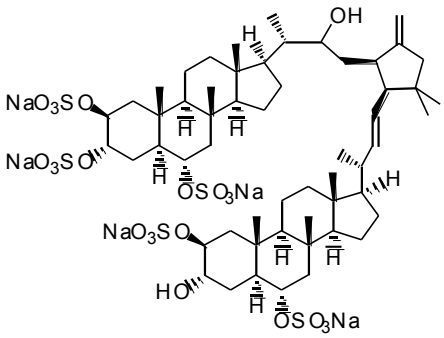
over 500 of the approximately 10,000 known species have been studied with respect to the biological activities of the sponges (Kornprobst, 2010). Furthermore, the numbers of novel chemical entities from marine natural products research (1992 to 2012) (Blunt, *et al*., 2012; Faulkner, 2002) have exceeded about 6283 secondary metabolites, which have determined from sponges (N = 16,514).

Secondary metabolites play the important roles in defending sponges from predation, biofouling, and microbial attack, and they apparently provide a selective advantage in the competition for space and other resources for their sponge hosts. Most of these compounds have powerful bioactivities including anticancer, antifungal, antibacterial, antibiotic, antiviral, anti-inflammatory, and enzyme-inhibitory activities (Jiang, *et al*., 2012; Blunt, *et al*., 2011; Kornprobst, 2010; Newman and Cragg, 2009). Since sponge species are abundant and still attract much interests to both chemists and pharmacologists, and sponges will continue to provide new secondary metabolites, which could be used as candidates for new drugs.

Most recently, compounds from marine sponges have been reported in several papers. Sterols, manadosterols A and B, were obtained from *Lissodendryx fibrosa* collected from Manado, North Sulawesi, Indonesia, and two dimeric sterols showed an inhibitory activity against ubiquitin Ubc13-Uev1A interaction with IC_{50} values of 0.09 and 0.13 μM, respectively. The inhibition of both compounds against Ubc13-Uev1A was more potent than leucettamol A (IC_{50} = 106 μM) (Ushiyama, *et al*., 2012).

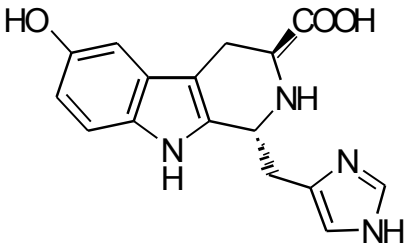


Manadosterol A

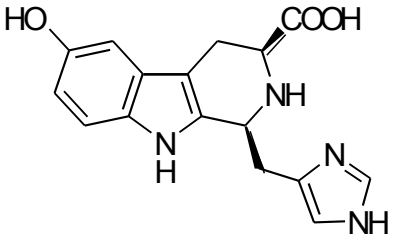


Manadosterol B

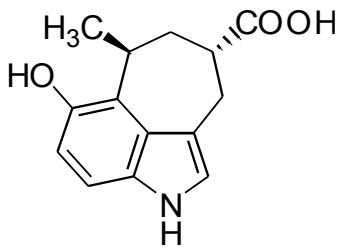
Another Indonesian marine sponge *Hyrtios reticulatus* afforded five hyrtioreticulins A-E (including three tetrahydro-ß-carboline alkaloids and two azepinoindole-type alkaloids) along with a known alkaloid hyrtioerectine B. These compounds showed an inhibitory activity against ubiquitin (E1) with IC_{50} values between 0.75 and 11 µg/mL. Hyrtioreticulins A was the most potent E1 inhibitor. All compounds did not show apparent cytotoxicity against HeLa cells even at 50 µg/mL (Yamanokuchi, *et al*., 2012).



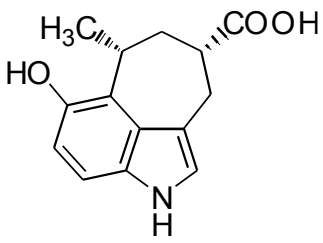
Hyrtioreticulin A



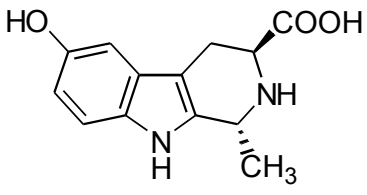
Hyrtioreticulin B



Hyrtioreticulin C

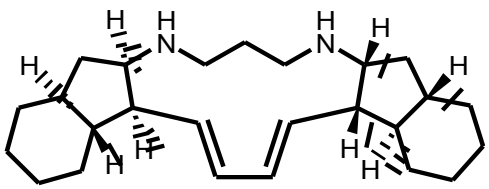


Hyrtioreticulin D

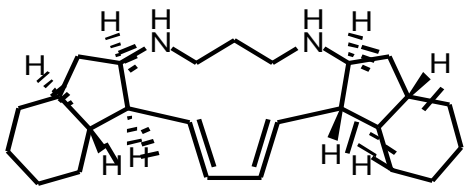


Hyrtioreticulin E

A potent cytotoxicity against human solid cancer cell lines MCF-7 (breast), LnCap (prostate), Caco-2 (colon), and HCT-15 (colon) have shown by the extract of an Indonesian marine sponge *Haliclona* sp. A study on nuclear morphological changes and flow cytometric analysis suggested that the components in the extract would induce an apoptosis to these cancer cells. Bioassay-guided isolation yielded two pentacyclic alkaloids, papuamine and haliclonadiamine which inhibited cell proliferation of six human cancer cell lines with IC_{50} values of 0.93–1.50 and 1.00–4.44 μM, respectively, and both compounds accumulated lymphoma U937 cells at the sub-G1 phase and induced a condensation of chromatin and fragmentation of nucleus (Yamazaki, *et al*., 2012)

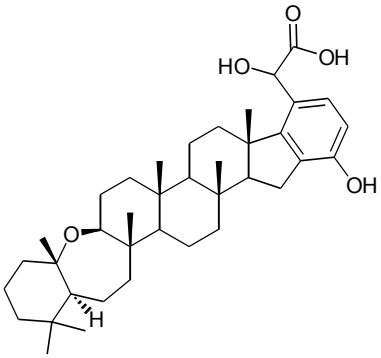


Papuamine

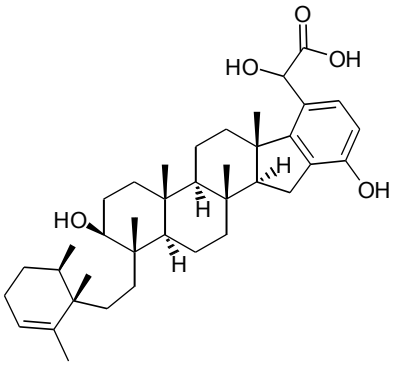


Haliclonadiamine

Two new glycolic acid merohexaprenoids, halicloic acids A and B, were obtained from *Haliclona* (*Halichoclona*) sp. collected in the Philippines. These compounds showed cytotoxicity against the human IDO (plays a central role in tumor cell-evasion) with IC_{50} values of 10 and 11 μM, respectively (Williams, *et al*., 2012).

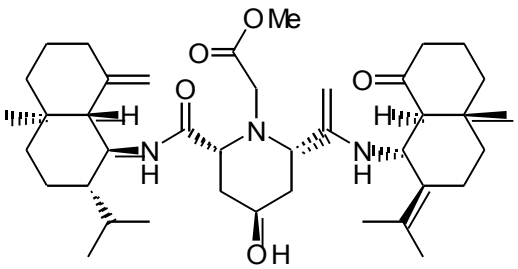


Halicloic acid A

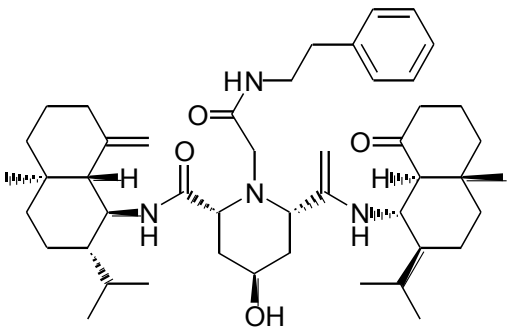


Halicloic acid B

Kobayashi and co-workers (2012) reported two new dimeric sesquiterpenoids, halichonadins K and L, from *Halichondria* sp. collected in Okinawa, Japan. Halichonadin K showed a moderate cytotoxicity against human epidermoid carcinoma KB cells (IC_{50} = 10.6 μg/mL).



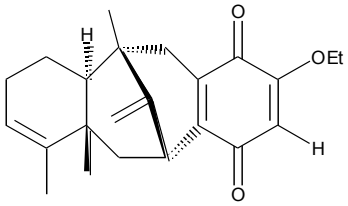
Halichonadin K



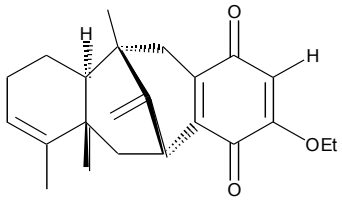
Halichonadin L

Cytotoxicity against four human cancer cell lines, cervix (HeLa), lung (A549), breast (MDA231), and hepatoma (QGY7703) was reported for four new sesquiterpene quinones, dysidavarones A-D, obtained from *Dysedea avara* collected

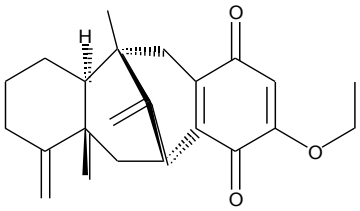
at Xisha Island, South China Sea. Dysidavarone A showed a growth inhibitory effected against HeLa cells with an IC_{50} value of 39.9 μM, and dysidavarone D was active against four cell lines with IC_{50} values of 28.8, 21.4, 11.6, and 28.1 μM, respectively. In addition, dysidavarones A and D also showed an inhibitory activity against protein tyrosine phosphatase 1B (PTP1B) with IC_{50} values of 9.98 and 21.6 μM, respectively, using oleanolic acid as a positive control (IC_{50} = 1.90 μM) (Jiao, *et al*., 2011).



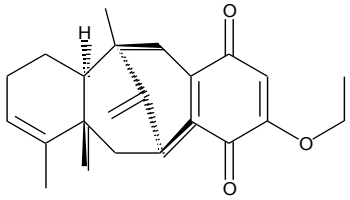
Dysidavarone A



Dysidavarone B



Dysidavarone C



Dysidavarone D

1.3. Bioactive Secondary Metabolites From Ascidians

Ascidians belong to the phylum Chordata, subphylum Urochordata, which includes three classes, Ascidiacea, Thaliacea, and Appendicularia. Members belonging to this taxon are often referred to as tunicates or sea squirts, because their bodies are covered with a sack or tunic (Davidson, 1993; Pawlik and Pisut 2002; Kornprobst, 2010; Jiang, *et al*., 2012). Ascidians are present in the entire world of the sea and at all depths, but most species have been harvested within the upper 500 meters.

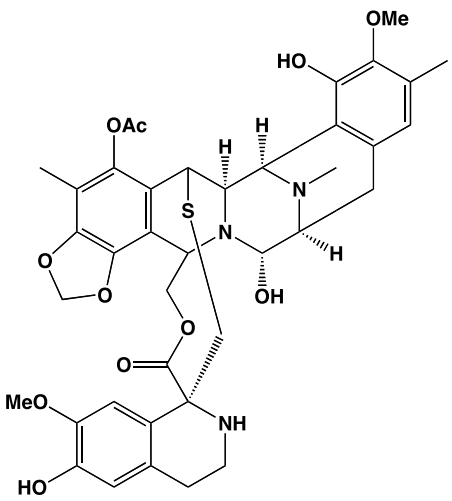
Ascidians are conspicuous members of marine fouling and benthic communities, filter feeders, microphagous or suspension feeders. Seawater charged with food particles enters through the oral siphon and circulates in the body through the gill. They have the ability to attach themselves to rocks and shells. Ascidiacea is the largest and most diverse class in Urchordata and takes many different forms, which divided into two types, solitary and colonial species. Solitary species generally live as isolated individuals, and many small individuals, called zooids, are living together in a common tunic in colonial species. Ascidians produce secondary metabolites that significantly reduce predation on both larvae and adults. It has been demonstrated that some of metabolites are made by symbiotic bacteria (Schmidt, *et al*., 2012). Ascidians are unique among sessile invertebrates in possessing a well-developed circulatory system. This unique feature may allow the encapsulation of bioactive compounds within blood cells, and their circulation throughout the animal may allow the chemical compounds to fulfill their ecological role while avoiding autotoxity (Lee, *et al*., 2012).

The chemistry of ascidians is characterized by the abundance of nitrogenous compounds (Wang and Namikoshi, 2004), and generally divided into two major groups, cyclic peptides and alkaloids, of which there are many families, often characteristic of an order or a genus. These cyclic peptides contain few of the amino acids but are powerful chelators of heavy metal cations. This abundance of nitrogen-containing derivatives is also often associated with aromatic rings among the alkaloids, such as indole, carbazole, pyridoacridine, and isoquinoline rings, and with heteroaromatic rings among the cyclic peptides (Kornprobst, 2010).

Over the past decades, ascidians have been shown to be a prolific source of natural products with promising biomedical potentials (Blunt, *et al*., 2012). Ascidians

are sources of structurally interesting and biologically active natural products. Most of these products fall within the area of cancer therapy, and several ascidian-derived compounds have entered into preclinical and clinical trials as antitumor agents (Blunt, *et al*., 2012, Faulkner, 2002).

The first ascidian secondary metabolite, geranyl hydroquine, was isolated from *Aplidium* sp. in 1974 (Wang and Namikoshi, 2004). Meanwhile, in 1990, structures of alkaloids named ecteinascidins, were reported from the Caribbean ascidian *Ecteinascidia turbinata*. After 17 years of investigation, one of the alkaloids, ecteinacscidin-743 (ET-743), became the first ascidian-derived anticancer drug for the treatment to refractory soft-tissue sarcomas, approved on October 2007 by European Commission (marketed under the trade name Yondelis by PharmaMar/Johnson & Johnson/OrthoBiotech) (Molinski, *et al*., 2009).



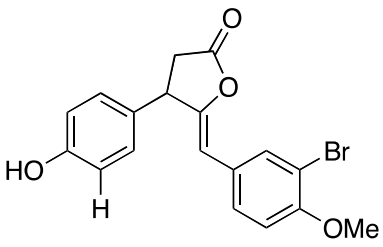
Ecteinascidin-743

Beside the successful application of ET-743, two cyclic depsipeptides, didemnim B from tropical *Tridemnum solidum* and aplidine from *Aplidium albicans* collected from Mediterranean, have been extensively investigated for more than 20

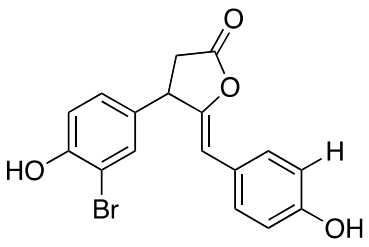
years. Aplidine (dehydrodidemnim B) is a dehydro derivative of didemnin B. Didemnin B was suspended because of its side effects, but aplidine does not have such side effects. Aplidine was first reported in 1991 in a patent by Rinehart and is expected to be approved as an anticancer drug (Molinski, *et al*., 2009).

Chemical studies on ascidians have grown considerably from 1992, and 1007 new secondary metabolites have been isolated up to 2010 (Figure 1-4). These new compounds exhibited remarkable bioactivities, such as cytotoxicity, antimicrobial, antiviral, and anti-inflammatory activities, and novel chemical structures are the origin of the various mechanisms of pharmacological activities (Blunt, *et al*., 2012; Faulkner, 2002).

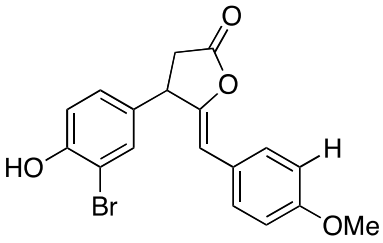
Synoicum globosum collected from South African led to the isolation of four new compounds, 3”-bromorubrolide F, 3’-bromorubrolide E, 3’-bromorubrolide F, and 3”-dibromorubrolide E, together with two known compounds, rubrolides E and F. These compounds were tested for their antimicrobial activities, and 3’-bromorubrolide E, 3”-dibromorubrolide E, and rubrolides F showed moderate antimicrobial activities against methicillin-resistant *Staphylococcus aureus* (MRSA) and *S. epidermidis* (Sikorska, *et al*., 2012).



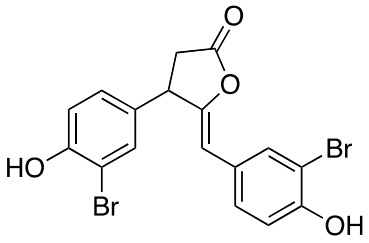
3’’-Bromorubrolide F



3’-Bromorubrolide E

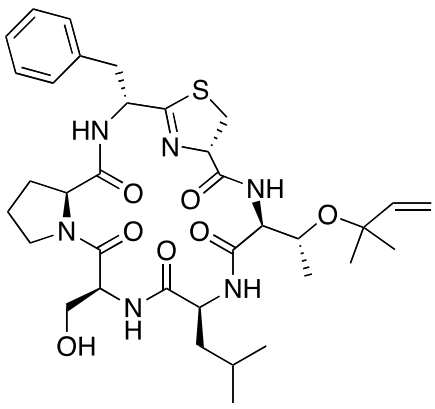


3'-Bromorubrolide F

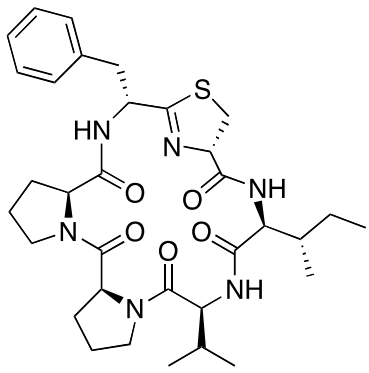


3''-Bromorubrolide F

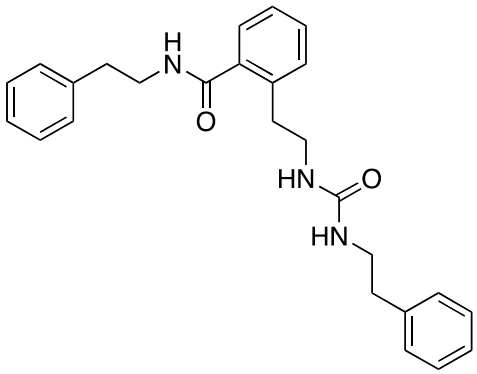
Screening for anti-HIV compounds from *Didemnum molle* collected in New Britain, Papua New Guinea was reported, and three new compounds, mollamides E and F and molleurea A, were evaluated by the anti-HIV integrase inhibition assay and cytoprotective cell-based assay. Mollamide F was active in both assays with IC_{50} values of 39 and 78 μM, respectively, and molleura A showed activity only in the cytoprotective cell-based assay with an IC_{50} value of 60 μM (Lu, *et al*., 2012).



Mollamide E

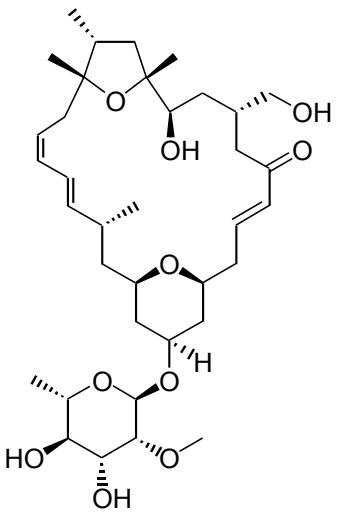


Mollamide F

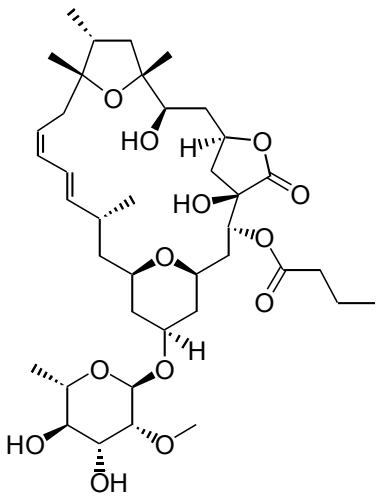


Molleurea A

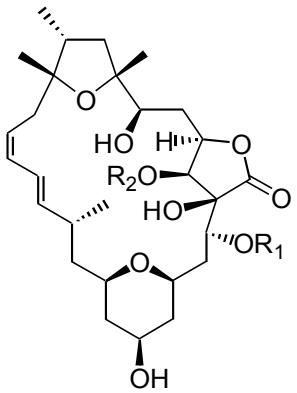
Mandelalides A-D were isolated from *Lissoclinum* sp. collected in the Algo Bay, South Africa. Mandelalides A and B showed a potent cytotoxicity against human NCI-H460 lung cancer cells (IC_{50} = 12 and 44 nM, respectively) and mouse Neuro-2A neuroblastoma cells (IC_{50} = 29 and 84 nM, respectively) (Sikorska, *et al.,* 2012).



Mandelalide A



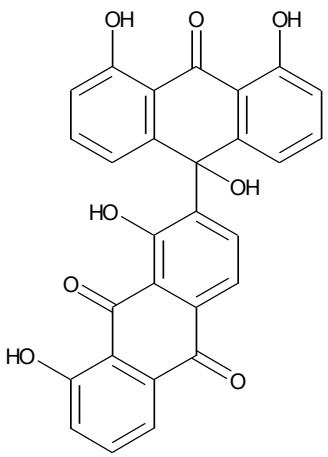
Mandelalide B



Mandelalides C $R_1 = COCH_2CH_2CH_3$ $R_2 = OH$

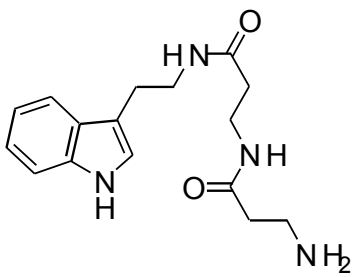
Mandelalides D $R_1 = COCH_2CH_2CH_3$ $R_2 = COCH_2CH_2CH_3$

An antiplasmodial anthrone-anthraquinone, albopuctatone, was afforded from *Didemnum albopunctatum* collected in the Great Barrier Reef, Australia. Albopuctatone showed activity against chloroquine-resistant and sensitive strains of malaria parasite *Plasmodium falciparum* with IC_{50} values of 5.3 and 4.4 ± 0.5 μM, respectively (Carrol, *et al*., 2012).

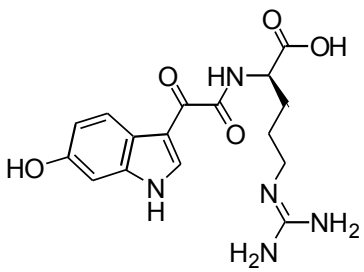


Albopunctatone

An Indonesian ascidian *Leptoclinides dubius* collected at Lembeh Strait, North Sulawesi gave two new tryptamine derivates, leptoclinidamide and (-)-leptoclinidamine B. Both compounds were tested for their cytotoxicity against two human cancer cell lines (colon adenocarcinoma HCT-15 and T-cell leukemia Jurkat cells), however, both compounds had no activity against these cell lines at 30 μM (Yamazaki, *et al*., 2012).



Leptoclinidamide



(-)-Leptoclinidamine B

1.4. Aims of This Study

As part of our studies on the bioactive components from marine invertebrates, 90 samples of marine ascidians and sponges were collected on September 2010 at North Sulawesi, Indonesia. The ethanol extracts were tested for the antimicrobial activity and cytotoxicity. The data of the screening are listed in Table 1-1.

The main objectives of this study were to investigate bioactive secondary metabolites from Tropical and Sub-tropical marine invertebrates.

This Ph.D. thesis consists of the following four sections:

1. Three New Unique Sesquiterpenes, Eurysponginsin A - C, from an Okinawan Marine Sponge *Euryspongia* sp.
2. A Polybromodiphenyl Ether from an Indonesian Marine Sponge *Lamellodysidea herbacea* and its Chemical Derivatives Inhibit Protein Tyrosine Phosphatase IB, an Important Target for Diabetes Treatment
3. Two pyridoacridine alkaloids, shermilamine B and kuanoniamine D, from an Indonesian ascidian *Cystodytes* sp.
4. Isolation and bioactivity of compounds from an Indonesian ascidian *Herdmania* sp.

Table 1-1. Antimicrobial and cytotoxic activity of ascidians and sponges collected in 2010 at Manado, Indonesia.

Sample number	Weight		Remarks (Identification) (note)	Assay data				
	organism (g)	extract (mg)		Antimicrobial (mm) *S. aureus*	*E. coli*	*C. albicans*	*M. hiemalis*	Cytotoxicity V79
2010 Sept. 11 (Pt. 1)	Bunaken South (Tawara Point 1)							
10-09-11=1-1	55.9	98.30	colonial	-	-	-	-	20.2
10-09-11=1-2	33.2	45.40	colonial	-	-	-	-	17.9
2010 Sept. 11 (Pt. 2)	Bunaken South (Tawara 2)							
10-09-11=2-1	129	908.12	sponge	-	-	-	-	58.3
10-09-11=2-2	94	770.54	colonial	13	12	26	-	100/100
10-09-11=2-3	5,183	33374.90	*Herdmania momus*	-	-	-	-	45.4
10-09-11=2-4	386	189.20	solitary	-	-	-	-	93.1/79.1
10-09-11=2-5	30.8	909.10	colonial	-	-	-	-	60.1/57.1
10-09-11=2-6	14	586.53	solitary	-	-	-	-	26.1/57.8
10-09-11=2-7	411	2504.78	solitary	-	-	-	-	100/100
10-09-11=2-8	12.2	107.21	colonial	-	-	-	-	14.7
10-09-11=2-9	205	524.53	solitary	-	-	-	-	97.7/90.3
2010 Sept. 12 (Pt. 1)	Manterawu (Mantehage)							
10-09-12=1-1	69	301.11	solitary	-	-	-	-	39
10-09-12=1-2	26.4	318.69	colonial	-	-	-	-	18.8
10-09-12=1-3	26.6	524.94	colonial	-	-	-	-	26.6
10-09-12=1-4	16.2	333.14	colonial	-	-	-	16	99.1/99.5
10-09-12=1-5	199	1532.43	colonial	-	-	-	-	100/98.4
10-09-12=1-6	135	1106.88	colonial	-	-	-	-	27.1
10-09-12=1-7	187	1365.13	*Eudistoma reginum*	-	-	-	-	100/99.5
10-09-12=1-8	239	2437.21	*Herdmania momus*	-	-	-	-	26.6
10-09-12=1-9	39.2	717.44	sponge	-	-	-	-	12.4
10-09-12=1-10	63	863.78	colonial	-	-	-	-	53.2
10-09-12=1-11	47.7	1311.26	colonial	-	-	-	-	31.7
10-09-12=1-12	56	764.85	colonial	-	-	-	-	98.6/96.2

Table 1-1. (cont.)

Sample number	Weight organism (g)	Weight extract (mg)	Remarks (Identification) (note)	Assay data Antimicrobial (mm) *S. aureus*	*E. coli*	*C. albicans*	*M. hiemalis*	Cytotoxicity V79
2010 Sept. 12 (Pt. 2) Bunaken North (Sachiko Point)								
10-09-12=2-1	163	1343.92	sponge	-	-	-	-	24.3
10-09-12=2-2	36.2	200.11	colonial	-	-	-	-	100/100
10-09-12=2-3	118	1277.25	colonial	-	-	-	-	7.3
10-09-12=2-4	7.5	111.72	colonial	-	-	-	-	33.9
10-09-12=2-5	685	5910.84	*Herdmania momus*	-	-	-	-	16.1
10-09-12=2-6	41.5	192.95	colonial	-	-	-	-	58.7
10-09-12=2-7	14.9	323.36	colonial	-	-	-	-	25.2
10-09-12=2-8	45.2	391.70	colonial	-	-	-	-	33.5/43.8
10-09-12=2-9	24.9	392.36	colonial	-	-	-	-	56.9
10-09-12=2-10	136	126.79	colonial	-	-	-	-	17.4
10-09-12=2-11	276	2129.06	solitary	-	-	-	-	14.7
10-09-12=2-12	136	1296.05	colonial	-	-	-	-	5.5
10-09-12=2-13	15	335.87	colonial	-	-	-	-	66.1/67.2
2010 Sept. 13 (Pt.1) Lembeh (Pulau Putus)								
10-09-13=1-1	79	631.80	colonial	-	-	-	-	84.9/82.4
10-09-13=1-2	2.5	70.63	colonial	9	-	18	-	100/100
10-09-13=1-3	34	861.62	solitary	-	-	-	-	26.1
10-09-13=1-4	123	2110.27	solitary	-	-	-	-	25.2
10-09-13=1-5	12	694.66	solitary	-	-	-	-	25.2/39.1
2010 Sept. 13 (Pt. 2) Lembeh (Batu Merah)								
10-09-13=2-1	359	2986.76	solitary	-	-	-	-	21.6
10-09-13=2-2	487	1650.65	colonial	-	-	-	-	15.1
10-09-13=2-3	13	760.35	solitary	-	-	-	-	31.2
10-09-13=2-4	3	54.74	colonial	-	-	-	-	22.5
10-09-13=2-5	28.8	494.40	colonial	-	-	-	-	10.6

Table 1-1. (cont.)

Sample number	Weight organism (g)	Weight extract (mg)	Remarks (Identification) (note)	Assay data Antimicrobial (mm) *S. aureus*	*E. coli*	*C. albicans*	*M. hiemalis*	Cytotoxicity V79
2010 Sept. 15 (Pt. 1) Manado Tua South (Bualo)								
10-09-15=1-1	2,944	15418.40	*Herdmania momus* ?	-	-	-	-	23.4
10-09-15=1-2	34.2	2181.48	solitary	-	-	-	-	16.5
10-09-15=1-3	17.9	459.02	colonial	-	-	-	-	29.8/18.9
10-09-15=1-4	5.7	73.70	colonial	-	-	-	-	100/97.3
10-09-15=1-5	1.7	16.01	colonial	8	-	22	18	100/100
10-09-15=1-6	3.1	45.61	colonial	-	-	-	15	30.3
10-09-15=1-7	17.8	503.13	colonial	-	-	-	-	57.3
10-09-15=1-8	27	499.22	colonial	-	-	-	18	67/81.3
10-09-15=1-9	4.2	68.37	colonial	-	-	-	-	69.3
10-09-15=1-10	15.5	607.92	solitary	-	-	-	-	36.7
10-09-15=1-11	209	1228.82	colonial	-	-	-	-	39.4
10-09-15=1-12	60.4	587.79	colonial	-	-	-	-	29.4
2010 Sept. 15 (Pt. 2) Manado Tua East (Negeri)								
10-09-15=2-1	26.1	534.95	colonial	-	-	-	-	50.9
10-09-15=2-2	11.8	116.14	colonial	-	-	-	22	56
10-09-15=2-3	30.9	566.48	colonial	-	-	-	-	78.4/77.3
10-09-15=2-4	0.6	17.24	colonial	9	-	22	26	100/100
10-09-15=2-5	53	1304.01	colonial	-	-	-	-	60.6
10-09-15=2-6	38	428.31	colonial	-	-	-	22	32.6/30.5
10-09-15=2-7	1.2	29.49	colonial	-	-	-	32	32.6
10-09-15=2-8	9.2	97.35	colonial	-	-	-	-	37.2
10-09-15=2-9	350	2513.84	colonial	-	-	-	-	40.8
10-09-15=2-10	51.1	763.16	colonial	-	-	-	-	21.1
10-09-15=2-11	347	1694.14	*Herdmania momus*	-	-	-	-	29.4
10-09-15=2-12	191	1101.20	colonial	-	-	-	-	17
10-09-15=2-13	37.3	449.40	colonial	-	-	-	-	11.9
10-09-15=2-14	2.5	402.11	colonial	-	-	-	-	55
10-09-15=2-15	36.2	576.64	colonial	-	-	-	-	40/38.4
10-09-15=2-16	30.3	447.16	colonial	-	-	-	-	22.5

Table 1-1. (cont.)

Sample number	Weight organism (g)	Weight extract (mg)	Remarks (Identification) (note)	Assay data Antimicrobial (mm) *S. aureus*	*E. coli*	*C. albicans*	*M. hiemalis*	Cytotoxicity V79
2010 Sept. 16 (Pt. 1) Malalayang (Kolongan Beach)								
10-09-16=1-1	2	21.59	colonial	-	-	-	-	6.9
10-09-16=1-2	18.4	251.51	solitary	-	-	-	-	20.2
10-09-16=1-3	2.5	32.00	colonial	-	-	-	-	37.6
10-09-16=1-4	81	1551.94	solitary	-	-	-	-	33
10-09-16=1-5	78.8	536.07	colonial	-	-	-	-	53.7
10-09-16=1-6	8.1	125.60	colonial	-	-	-	-	38.5/27.2
10-09-16=1-7	1.6	29.97	colonial	-	-	-	-	71.1/68.3
10-09-16=1-8	29.5	165.17	colonial	-	-	-	-	15.1
10-09-16=1-9	12.1	125.73	colonial	-	-	-	-	35.3
10-09-16=1-10	72.3	513.01	colonial	-	-	-	-	50.9
10-09-16=1-11	11.9	70.71	colonial	-	-	-	-	27.1
10-09-16=1-12	11.4	147.21	colonial	-	-	-	-	32.1
10-09-16=1-13	1.4	22.26	colonial	9	-	-	-	100/98.9
10-09-16=1-14	9	91.82	colonial	-	-	-	-	61/69.7
10-09-16=1-15	35.3	685.89	colonial	-	-	-	-	2.8
10-09-16=1-16	8.8	79.10	colonial	-	-	-	-	100/100

Table 1-1. (cont.)

Sample number	Weight organism (g)	Weight extract (mg)	Remarks (Identification) (note)	Assay data Antimicrobial (mm) *S. aureus*	*E. coli*	*C. albicans*	*M. hiemalis*	Cytotoxicity V79
2010 Sept. 16 (Pt. 2)	Malalayang (Mutiara)							
10-09-16=2-1	29.7	150.23	colonial	-	-	-	-	99.5/100
10-09-16=2-2	4.1	68.04	colonial	15	-	15	-	100/100
10-09-16=2-3	19.4	302.34	colonial	-	-	-	-	99.5/100
10-09-16=2-4	27.4	271.83	colonial	-	-	-	15	63.3/53.8
10-09-16=2-5	9.6	130.52	colonial	-	-	-	-	22.9
10-09-16=2-6	17.7	284.33	colonial	19	-	-	-	100/100
10-09-16=2-7	48.8	422.82	colonial	-	-	-	-	50.5
10-09-16=2-8	122	662.78	colonial	-	-	-	-	2.3

Positive Control	amphotericin B	chloramphenicol	amphotericin B	amphotericin B
	10 mg/disc			
inhibition zone (nn)	18	13	28	10
Negative Control	-	-	-	-

Antimicrobial activity: inhibition zone (mm) at 250 mg/disc (disc diameter, 6 mm)

Staphylococcus aureus IAM 12544T (Gram-positive bacterium), *Escherichia coli* IAM 12119T (Gram-negative bacterium)

Candida albicans IFM 4954 (yeast), *Mucor hiemalis* IAM 6088 (filamentous fungus)

V97: inhibitory activity (%) against the colony formation of Chinese hamster V79 cells at 50 mg/mL (90-100%, 60-90%, 50-60%)

Chapter 2. Three New Unique Sesquiterpenes, Eurysponginsins A–C, from an Okinawan Marine Sponge *Euryspongia* sp.

2.1. Introduction

As described in **Chapter 1**, marine sponges have been proven to be one of the important resources for the discovery of bioactive natural products. Many metabolites isolated from marine sponges possess unique structural features and potent biological activities (Blunt, *et al*., 2012; Faulkner, 2002). Sponges of the genus *Euryspongia* have been shown to contain various types of secondary metabolites, including steroidal sulfates (Boonlarppradab, *et al*., 2007), secosteroids (Dopeso, *et al*., 1994; van Altena, *et al*., 1999), hydroquinones (Hallock, *et al*., 1998), sesquiterpene quinones (Urban and Capon, 1996), and furanoterpenoids (Clark, *et al*., 1999; van Altena, 1989; Hochlowsky, *et al*., 1982). During our search program for novel and useful metabolites from marine organisms, three new unique sesquiterpenes, named eurysponginsins A–C **(1**–**3**), were isolated from a marine sponge *Euryspongia* sp. collected at Iriomote Island, Okinawa, Japan (Figure 2-1).

Figure 2-1. Marine sponge *Euryspongia* sp.

The structures of **1**–**3** were assigned on the basis of their spectroscopic data as unique sesquiterpenes possessing a six- and eight-membered bicyclic skeleton (Figure 2-2). Only five compounds, pallescensin B (**5**) (Cimino, *et al*., 1975), nakafuran-8 (**6**) (Schulte, *et al*., 1980), 5-hydroxynakafuran-8 (**7**) and 5-acetoxynakafuran-8 (**8**) (Cardellina and Barnekow, 1988), and *O*-methyl nakafuran-8 lactone (**9**) (Shao, *et al*., 2006), have thus far been reported as natural products in this class (Figure 2-2). The natural products **1**–**3** did not show inhibitory activity against protein tyrosine phosphatase 1B (PTPIB), an important target for the treatment of type II diabetes, while the dehydrated product of **1**, dehydroeuryspongin A (**4**), inhibited the activity of this enzyme (IC_{50} = 3.6 μM). Isolation, structure elucidation including stereochemistry, and biological activity of compounds **1**–**4** are described in the following sections.

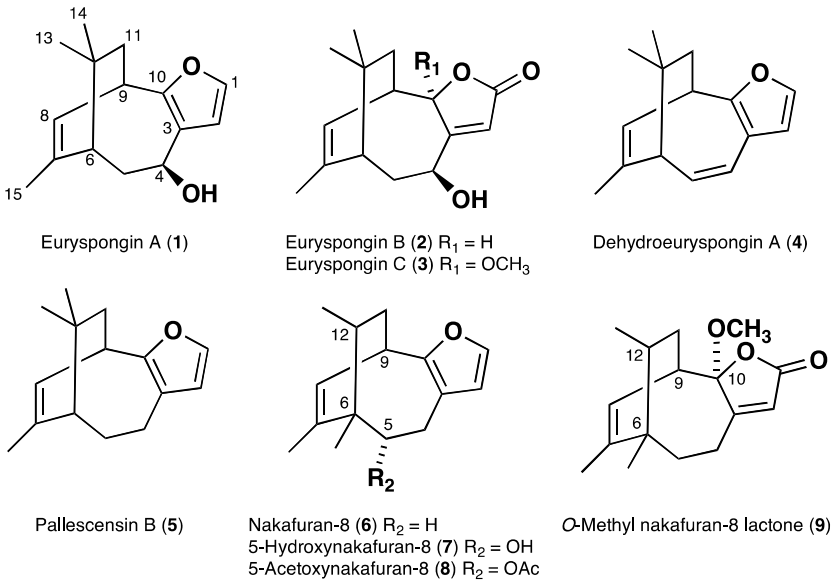


Figure 2-2. Structures of compounds **1**–**9**

2.2. Extraction and Isolation

The marine sponge *Euryspongia* sp. (306.6 g, wet weight) was collected in

Iriomote Island, and extracted with ethanol. The ethanol extract (1.1 g) was separated by an ODS column (100 g) followed by repeated preparative HPLC to give eurysp ongins A (**1**, 12.7 mg), B (**2**, 0.90 mg), and C (**3**, 1.55 mg) as colorless oils.

2.3. Structure Elucidation

2.3.1. Planar Structure of Eurysp ongin A (1)

The molecular formula, $C_{15}H_{20}O_2$, of eurysp ongin A (**1**) was assigned from HREIMS (*m/z* 232.1462 $[M]^+$, Δ –0.1 mmu) and NMR data (Table 2-1). The ^1H and ^{13}C NMR spectra of **1** showed 19 proton and 15 carbon signals, which were classified into three methyls, two methylenes, two sp^3 methines, one sp^3 oxygenated methine, one sp^3 quatenary carbon, two sp^2 methines, one sp^3 oxygenated methine, two sp^2 quaternary carbons, and one sp^2 oxygenated quaternary carbon from HMQC data. The presence of an OH group was revealed from an IR absorption at 3402 cm^{-1} and the molecular formula of **1**. Three partial structures (I–III), shown as bold lines in Figure 2-3, were elucidated from the ^1H-^1H COSY spectrum of **1.** An α,ß-disubstituted furan ring in the partial structure I was assigned from HMBC correlations from H-1 (δ 7.15) and H-2 (6.37) to C-3 (122.6) and C-10 (150.1) (Figure 2-3) and UV absorption at 220 nm (Schulte, *et al*., 1980). HMBC correlations from H_3-13 (δ 0.78) to C-11 (44.2), C-12 (33.4), and C-14 (30.1), from H_3-14 (0.90) to C-6 (48.1), C-12, and C-13 (36.3), and from H_3-15 (1.87) to C-6, C-7 (141.5), and C-8 (120.2) connected partial structures II and III by forming a six-membered ring. The connections of a furan ring and, consequently, an eight-membered ring were elucidated from HMBC correlations from H-4 (δ 4.59) to C-3 and C-10, from H_2-5 (2.16 and 2.27) to C-3, from H-9 (3.45) to C-10, and from H_2-11 (1.56 and 1.64) to C-10 (Figure 2-3). Thus, the skeletal structure of **1** was assigned as shown in Figure 2-3.

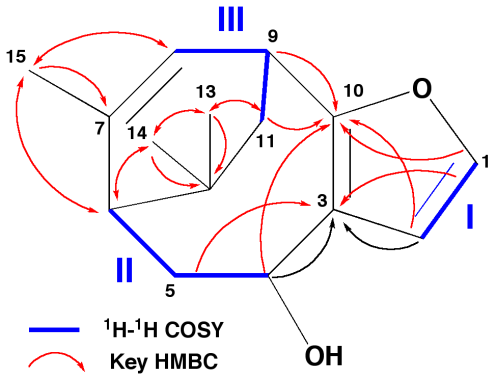


Figure 2-3. ^{1}H-^{1}H COSY and HMBC correlations of **1**

2.3.2. Stereochemistry of Euryspongin A (1)

The relative stereochemistry of **1** was elucidated by the analysis of NOESY data ($CDCl_3$) and 1D NOE difference experiments in C_6D_6 (Figure 2-4). Correlations between H-4 (δ 4.59)/H_3-15 (1.87), H-5b (2.16)/H_3-13 (0.78), H-6 (2.08)/H_3-14 (0.90), H_3-15/H-8 (5.74), and H-8/H-9 (3.45) were observed in the NOESY spectrum of **1** measured in $CDC1_3$ (Figure 2-4). Assignment of the stereochemistry at C-4 to C-6 was also confirmed by NOE difference experiments in C_6D_6 (Figure 2-4) because ^{1}H signals were observed closely to each other in $CDC1_3$. NOE difference spectra in C_6D_6 exhibited strong enhancements between H-4 (δ 4.45)/H_3-15 (1.47), H-6 (1.80)/H_3-14 (0.78), and H-6/H_3-15, Considering these NOE data, the Monte Carlo conformational analysis was performed with an MMFF94 force field utilizing Spartan'04 as shown in Figure 2-4. The modified Mosher's method (Ohtani, *et al.*, 1991) was applied to investigate the absolute stereochemistry of **1**, but the reaction of **1** with S-(+)-α-methoxy-α-(trifluoromethyl)phenylacetyl chloride (MTPA-Cl) resulted in the decomposition of **1**. Thus, the absolute stereochemistry of **1** was tentatively assigned by comparing the specific rotation with that of the related compound, 5-hydroxynakafuran-8 (**7**) (Cardellina and Barnekow, 1988), since **1** and **7** will possibly be produced via the identical biosynthetic pathway. The absolute configuration of 5-

hydroxynakafuran-8 (**7**) was reported to be (5*S*, 6*S*, 9*R*, 12*R*), and, therefore, the absolute stereochemistry of **1** was suggested to be depicted as shown in Figure 2-2. However, further study should be necessary to define the assignment.

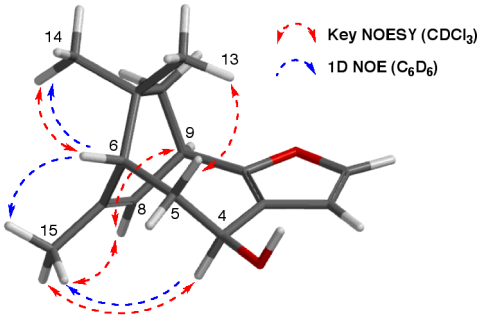


Figure 2-4. Relative stereochemistry of **1**

2.3.3. Planar Structure of Euryspongin B (2)

Euryspongin B (**2**) showed the $[M]^+$ ion at *m/z* 248.1401 (Δ –1.1 mmu) in HREIMS, and the molecular formula was determined to be $C_{15}H_{20}O_3$ using NMR data (Table 2-1). IR absorptions at 3545 and 1748 cm^{-1} indicated the presence of an OH and carbonyl groups. The ^1H and ^{13}C NMR spectra of **2** were similar to those of **1,** except for signals due to the furan ring in **1**. An oxygenated sp^3 methine (δ_H 4.85, δ_C 83.5) and carbonyl (δ_C 172.5) signals were detected in the ^1H and ^{13}C NMR spectra of **2** instead of two oxygenated sp^2 methine signals due to the C-1 (δ_H 7.15, δ_C 138,8) and C-10 (δ_H 150.1) positions in those of **1** as the most remarkable differences between **2** and **1**. The presence of an α,β-unsaturated-γ-lactone in **2** was elucidated by the analysis of HMBC data (Figure 2-5) together with IR and UV data (Shao, et al, 2006). Consequently, the structure of **2** was assigned as an α,β-unsaturated-γ-lactone derivative of **1** at the furan ring.

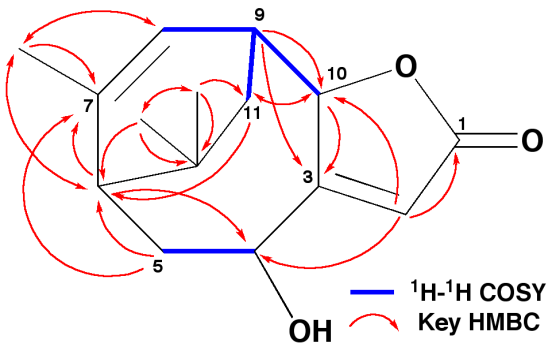


Figure 2-5. ^{1}H-^{1}H COSY and HMBC correlations of **2**

2.3.4. Planar Structure of Eurypongin C (3)

HREIMS (*m/z* 278.1509 $[M]^+$, Δ –0.9 mmu) and NMR data (Table 2-1) for **3** deduced the molecular formula of eurypongin C (**3**) as $C_{16}H_{22}O_4$. The physico-chemical properties of **3** were similar to those of **2**, indicating that they share a similar skeleton. Differences in the molecular weight and formula between **2** and **3** (30, CH_2O) were detected in the NMR spectra of **2** and **3**. The ^{1}H and ^{13}C NMR spectra of **3** showed an extra OMe signal at δ_H 3.23 (δ_C 51.1) and an acetal carbon signal at δ_C 109.4 instead of an oxygenated methine (δ_H 4.85, δ_C 83.5) at the C-10 position in **2**. An HMBC correlation was observed from OMe signal at δ_H 3.23 to δ_C 109.4 (C-10). Thus, the structure of eurypongin C (**3**) was assigned as a 10-methoxy derivative of eurypongin B (**2**).

2.3.5. Stereochemistry of Eurypongins B (2) and C (3)

The stereochemistry of compounds **2** and **3** was determined to be the same as that of **1** from the analysis of ^{1}H coupling constants ($CDC1_3$) and NOE data (C_6D_6). ^{1}H coupling constants measured in $CDC1_3$ and NOE correlations in C_6D_6 of **1-3** were very similar to each other. The configuration at C-4 and C-10 in **2** and **3** was determined from NOE data. The NOE difference spectra of **2** and **3** in C_6D_6 showed

enhancements between H-4 (δ 3.57)/H-10 (4.25) in **2** and between H-4 (3.96)/10-OMe (2.89) in **3** (Figures 2-6 and 2-7). Considering these NOE data, the Monte Carlo conformational analyses were performed with an MMFF94 force field utilizing Spartan'04 (Wavefunction, Irvine, CA) as shown in Figures 2-6 and 2-7.

Table 2-1. ^{1}H and ^{13}C NMR data for eurysp ongins A–C (**1**–**3**) in $CDCl_3$

Position	Eurysp ongin A (**1**)		Eurysp ongin B (**2**)		Eurysp ongin C (**3**)	
	δ_c	δ_H (*J* in Hz)	δ_c	δ_H (*J* in Hz)	δ_c	δ_H (*J* in Hz)
1	138.8	7.15 d (1.9)	172.5	-	166.8	-
2	109.3	6.37 d (1.9)	118.0	6.26 s	119.9	6.30 d (1.0)
3	122.6	-	176.1	-	170.1	-
4	65.9	4.59 dd	67.0	4.32 dd	65.7	4.32 ddd
		(11.1, 4.8)		(10.6, 5.5)		(10.0, 5.6, 1.0)
5a	40.8	2.27 ddd	34.9	2.22 ddd	35.6	2.22 ddd
		(14.0, 9.2, 4.8)		(14.1, 8.6, 5.7)		(14.0, 8.2, 5.8)
5b		2.16 ddd		1.95 ddd		1.98 ddd
		(13.9, 11.0, 1.1)		(13.9, 10.6)		(14.0, 10.6)
6	48.1	2.08 d (8.7)	47.2	1.89 d (8.8)	47.5	1.90 d (8.7)
7	141.5	-	142.5	-	140.4	-
8	120.2	5.74 dq	118.8	5.61 d (6.6)	119.4	5.60 dq
		(7.3, 1.4)				(7.3, 1.0)
9	33.8	3.45 ddd	33.9	2.96 brt	37.2	2.97 brt
		(7.3, 6.0, 2.2)				
10	150.1	-	83.5	4.85 s	109.4	-
11	44.2	1.56 dd	30.2	1.25 m	33.8	1.25 m
		(13.3, 6.0)				
		1.64 dd		1.35 dd		1.53 dd
		(13.3, 2.2)		(15.0, 9.5)		(15.5, 10.1)
12	33.4	-	34.0	-	33.1	-
13	36.3	0.78 s	29.3	0.93 s	29.8	0.92 s
14	30.1	0.90 s	33.7	0.95 s	34.7	0.96 s
15	23.5	1.87 d (1.4)	24.0	1.81 s	24.0	1.82 d (1.0)
10-OCH_3					51.1	3.23 s

Accordingly, the relative stereochemistry of **2** and **3** were assigned as shown in Figure 2-2. These configurations were supported by the relative stereochemistry of a related lactone derivative, *O*-methyl nakafuran-8 lactone (**9**), determined by X-ray crystallography (Shao, *et al*., 2006), which has the same configuration as **3**.

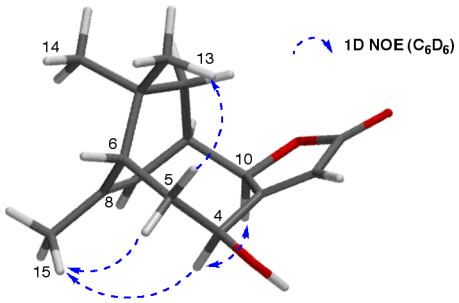


Figure 2-6. Relative stereochemistry of **2**

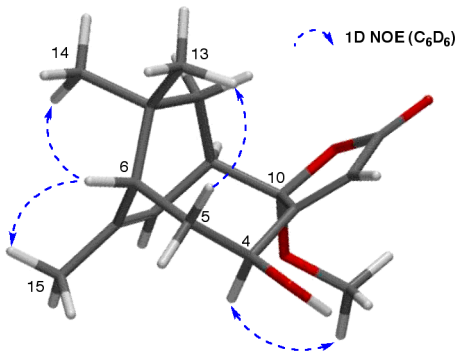


Figure 2-7. Relative stereochemistry of **3**

2.4. Biogenetic Pathway to Eurypongins A–C (1–3)

Eurypongins A–C are unique sesquiterpenes possessing a six- and eight-

membered bicyclic skeleton with a furan or γ-lactone ring. Only five natural products in this class, pallescensin B (**5**), nakafuran-8 (**6**), 5-hydroxynakafuran-8 (**7**), 5-acetoxy-nakafuran-8 (**8**), and *O*-methyl nakafuran-8 lactone (**9**), have thus far been reported (Cimino, *et al*., 1975; Schulte, *et al*., 1980; Cardellina and Barnekow, 1988; Shao, *et al*., 2006). Therefore, we added three more examples (**1**–**3**) to this class of natural products. Based on the structures of all analogues, possible biogenetic routes to eurypongins A–C (**1**–**3**) is shown in Figure 2-8. Generally, sesquiterpene structures from marine sponges are believed to come from the farnesyl precursor via cyclization and/or rearrangements. Two cyclization reactions in FPP lead to an intermediate having a five- and six-membered rings, which convert to pallescensin B (**5**) by the linkage between 9 and 10 positions. After formation of six- and eight-membered bicyclic skeleton, a hydroxylation at C-4 position results in production of eurypongin A (**1**), and, then, eurypongins B (**2**) and C (**3**) are produced by oxidation of a furan ring followed by addition of a methoxy group. Regarding nakafuran-8 analogues (**6**–**9**), migration of a methyl group from C-12 to C-6 is occurred. However, nakafuran-8 type congeners were not detected in the ethanol extract of *Eurypongin* sp. Therefore, it is suggested that this marine sponge (or symbiotic bacteria) do not have an ability of methyl migration from C-12 to C-6, such as methyltransferases.

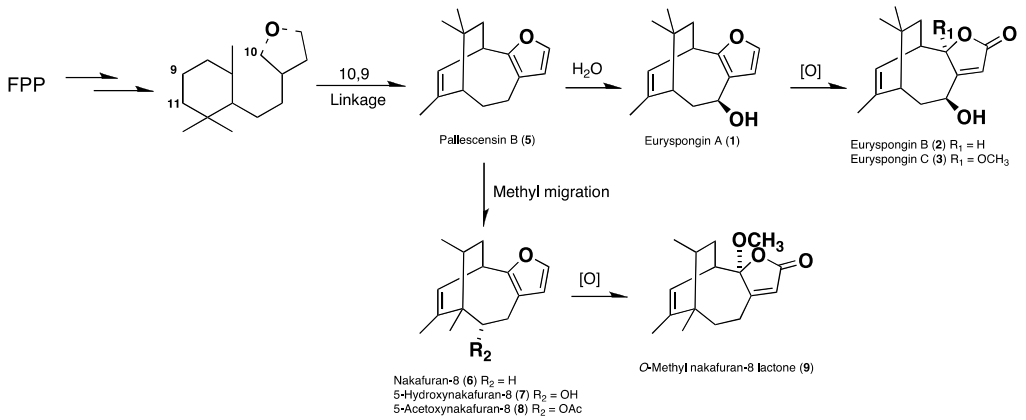


Figure 2-8. Proposed biogenetic pathway to eurypongins A–C (**1**–**3**)

2.5. Biological Activity

Nakafuran-8 (**6**) (Huang, et al., 2008) and *O*-methyl nakafuran-8 lactone (**9**) (Shao*, et al*., 2006) were reported to have inhibitory activity against protein tyrosine phosphatase (PTP) 1B. This enzyme plays a major role in dephosphorylation of the insulin receptor and is regarded as a key target for the treatment of type-II diabetes and obesity (Popov, 2011). Therefore, we established the high-throughput bioassay method according to the published method with a slight modification (Cui, *et al*., 2006, Yamazaki, *et al*., 2012), and eurypongins A–C (**1**–**3**) were tested for their effect against PTP1B activity. Compounds **1**–**3** did not show apparent activity even at 35–40 μM. In the same bioassay, oleanolic acid (Liu, 1995), a positive control, inhibited PTPIB activity with an IC_{50} value of 1.1 μM (Figure 2-9).

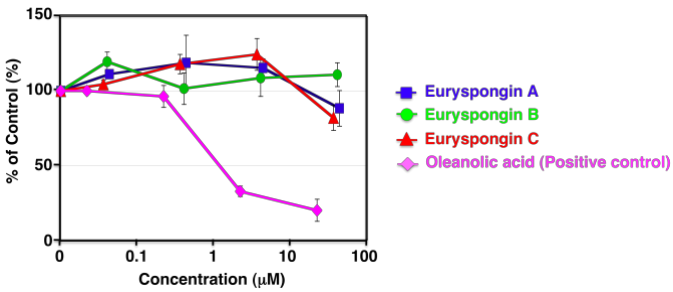


Figure 2-9. Inhibitory activity of eurypongins A–C (**1**–**3**) against PTP1B

It was presumed that differences in bioactivity between compounds **1**–**3** and two active compounds (**6** and **9**) were attributed to an OH group at C-4 in **1**–**3**, and, then, the activity of a dehydro derivative of **1,** dehydroeurypongin A (**4**) was examined. As expected, compound **4** showed inhibitory activity against PTP1B with an IC_{50} value of 3.6 μM (Figure 2-10). Interestingly, compounds **1**–**4** did not inhibit cell proliferation of two human cancer cell lines, HCT-15 (colon) and Jurkat (T-cell lymphoma) cells, at 45 μM. Furthermore, the growth inhibition of **4** on cell viability of human hepatoma Huh-7 cells was examined. Since PTP1B is located in the insulin-

targeted tissues such as liver, muscle, and fat cells, Huh-7 cells are used for cell-based experiments to investigate the mechanism of action of PTP1B inhibitors. Compound **4** showed no apparent cytotoxicity (Figure 2-10).

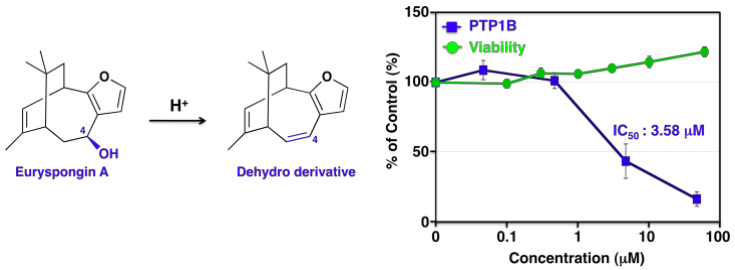


Figure 2-10. Inhibitory activity of dehydroeuryspongin A (**4**) against PTP1B and viability to Huh-7 cells

2.6. Conclusion

Three new unique sesquiterpenes, eurypongins A–C (**1**–**3**) were obtained from the EtOH extract of an Okinawan marine sponge *Euryspongia* sp. The structure of **1** was assigned on the basis of spectroscopic data including various NMR experiments. Compound **1** had a unique bicyclic furanosesquiterpene structure with six- and eight-membered rings. The ^{1}H-NMR spectra of **2** and **3** were very similar to that of **1**. Comparison of NMR data among compounds **1**–**3** indicated that a furan ring in **1** was replaced by an α,β-unsaturated-γ-lactone ring in **2** and **3**. The relative stereochemistry of compounds **1**–**3** was deduced by the analysis of ^{1}H-^{1}H coupling constants and NOE experiments, and further investigation on the absolute stereochemistry of **1**–**3** is now in progress. Regarding the biological activity, the dehydrated product (**4**) of **1** (dehydroeuryspongin A) was found to inhibit the activity of PTP1B with an IC_{50} value of 3.6 μM, although compounds **1**–**3** had no inhibition effect against PTP1B. Interestingly, compound **4** did not inhibit cell proliferation of human hepatoma Huh-7 cells even at 100 μM.

2.7. Experimental Section

2.7.1. General

EI-MS was performed by a JMS-MS 700 mass spectrometer (JEOL, Tokyo, Japan). ^{1}H- and ^{13}C-NMR spectra were recorded on a JNM-AL-400 NMR spectrometer (JEOL) at 400 MHz for ^{1}H and 100 MHz for ^{13}C in $CDCl_3$ (δ_H 7.26, δ_C 77.0). UV spectra were measured on a Hitach U-3310 UV-Visible spectrophotometer (Tokyo, Japan) and IR spectra on a PerkinElmer Spectrum One Fourier transform infrared spectrometer (Waltham, MA, USA). Specific rotations were measured with a digital polarimeter (P-2300; JASCO, Tokyo, Japan). Preparative HPLC was carried out using the L-6200 system (Hitachi Ltd., Tokyo, Japan).

2.7.2. Materials

Fetal bovine serum (FBS) and other culture materials were purchased from Invitrogen (Carlsbad, CA, USA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals including organic solvent were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan).

2.7.3. Marine Sponge

The marine sponge was collected by scuba diving in the coral reef at Iriomote Island, Okinawa, Japan, in 2010 and identified as *Euryspongia* sp. The voucher specimen was deposited at the Faculty of Pharmaceutical Sciences, Tohoku Pharmaceutical University as 10-10-11 = 2-2-9.

2.7.4. Extraction and Isolation

The ethanol extract was evaporated to dryness (1.1 g) and applied on an ODS column (100 g). The column was eluted stepwise with 0, 50, 60, 70, 80, 90, and 100% CH_3OH in H_2O (each 400 mL). The fraction eluted with 80% CH_3OH (140.8 mg) was purified by preparative HPLC (column, PEGASIL ODS (Senshu Sci. i.d. 10 x 250 mm); solvent, 70% CH_3OH; flow rate, 2.0 mL/min; detection, UV 220 nm) to give eryspongin A (**1**) (t_R = 17.1 min) as a colorless oil (12.7 mg). Eryspongin B (**2**) was isolated as a colorless oil (0.90 mg) by preparative HPLC with 65% CH_3OH (t_R = 23.1 min) from the fraction eluted with 60% CH_3OH (29.5 mg). The fraction eluted with 70% CH_3OH (84.6 mg) was separated by preparative HPLC (70% CH_3OH, (t_R = 24.2 min) to yield eryspongin C (**3**) as a colorless oil (1.55 mg).

Eryspongin A (1): a colorless oil; $[\alpha]^{20}_D$ –33.9 (*c* 0.10, $CHCl_3$); IR (KBr) ν_{max} 3402, 2357, 1630, 1385 cm^{-1} ; UV (MeOH) λ_{max} 220 (ε 12850), 269 (ε 8630); EIMS *m/z* 232 $[M]^+$; HREIMS *m/z* 232.1462 $[M]^+$; calcd for $C_{15}H_{20}O_2$, 232.1463); ^1H NMR (C_6D_6) δ 7.05 (1H, d, *J* = 1.9 Hz), 6.51 (1H, d, *J* = 1.5 Hz), 5.51 (1H, d, *J* = 7.6 Hz), 4.45 (1H, dd, *J* = 10.7, 4.9 Hz), 3.45 (1H, brt, *J* = 7.8 Hz), 2.08 (1H, ddd, *J* = 13.2, 10.7, 1.5 Hz), 1.99 (1H, ddd, *J* = 14.2, 8.8, 4.9 Hz), 1.80 (1H, d, *J* = 8.8 Hz), 1.69 (1H, dd, *J* =13.4, 1.7 Hz), 1.63 (3H, d, *J* = 1.5 Hz), 1.47 (1H, dd, *J* = 13.2, 6 3 Hz), 0.78 (3H, s), 0.72 (3H, s); ^1H and ^{13}C NMR ($CDCl_3$), see Table 2-1.

Eryspongin B (2): a colorless oil; $[\alpha]^{20}_D$ +90.4 (*c* 0.10, $CHCI_3$); IR (KBr) ν_{max} 3545, 1748, 1648, 1424 cm^{-1}; UV (MeOH) λ_{max} 201 (ε 20510), 220 (ε 11310); EIMS *m/z* 248 $[M]^+$; HREIMS *m/z* 248.1401 ($[M]^+$; calcd for $C_{15}H_{20}O_3$, 248.1412); ^1H NMR (C_6D_6) δ 6.02 (1H, s), 5.02 (1H, d, *J* = 5.3 Hz), 4.25 (1H, s), 3.57 (1H, dd, *J* = 10.1,

5.8 Hz), 2.60 (1H, brt), 1.74 (1H, ddd, *J* = 14.5, 8.7, 5.8 Hz), 1.56 (1H, dd, *J* = 12.6, 10.6 Hz), 1.37 (3H, s), 1.33 (1H, d, *J* = 8.7 Hz), 1.03 (1H, dd, *J* = 15.0, 9.4 Hz), 0.86 (1H d, J *J* = 15.5 Hz), 0.66 (3H, s), 0.65 (3H, s); ^{1}H and ^{13}C NMR ($CDCl_3$), see Table 2-1.

Eurypongin C (3): a colorless oil; $[\alpha]^{20}_{D}$ +52.2 (*c* 0.16, $CHC1_3$); IR (KBr) ν_{max} 3468, 1760, 1647, 1427 cm^{-1}; UV (MeOH) λ_{max} 201 (ε 20600), 220 (ε 11410); EMS *m/z* 278 $[M]^+$; HREIMS *m/z* 278.1509 ($[M]^+$; calcd for $C_{16}H_{22}O_4$, 278.1518); ^{1}H NMR (C_6D_6) δ 6.06 (1H, s), 5.54 (1H, d, *J* = 6.8 Hz), 3.96 (1H, dd, *J* =10.6, 5.8 Hz), 2.90 (1H, brt), 2.89 (3H, s), 1.72 (1H, m), 1.59 (1H, dd, *J* = 14.1, 10.4 Hz), 1.48 (3H, s), 1.40 (1H, d, *J* = 8.2 Hz), 1.23 (1H, dd, *J* = 15.9, 8.0 Hz), 0.93 (1H, d, *J* = 15.5 Hz), 0.68 (6H, s); ^{1}H and ^{13}C NMR ($CDCl_3$), see Table 2-1.

2.7.5. Preparation of Dehydroeurypongin A (4)

Dehydroeurypongin A (**4**) was formed in an NMR tube. Three days after measuring 2D NMR spectra of **1** in $CDC1_3$, the ^{1}H NMR spectrum showed signals due to a dehydro product (**4**). The ^{1}H and ^{13}C NMR spectra of this product revealed that signals due to **1** had disappeared and the purity of **4** was quite high. Compound **4** was recovered by evaporating the solvent.

Dehydroeurypongin A (4): a pale yellow oil; $[\alpha]^{20}_{D}$ +55.9 (*c* 0.46, $CHC1_3$); UV (MeOH) λ_{max} 200 (ε 17900), 280 (ε 5480); EIMS *m/z* 214 $[M]^+$; HREIMS *m/z* 214.1365 ($[M]^+$; calcd for $C_{15}H_{18}O$, 214.1358); ^{1}H NMR ($CDCl_3$) δ 7.02 (1H, d, *J* = 1.5 Hz), 6.20 (1H, d, *J* = 1.9 I-lz), 6.06 (1H, d, *J* = 12.3 Hz), 5.71 (1H, dd, *J* = 12.1, 9.7 Hz), 5.67 (1H, dd, *J* = 7.3, 1.5 Hz), 3.30 (1H, t, *J* = 8.0 Hz), 2.40 (1H, d, *J* = 9.7

IIz), 1.90 (1H, d, *J* = 14.0 Hz), 1.81 (3H, d, *J* = 1.4 Hz), 1.64 (1H, dd, *J* =14.3, 9.4 Hz), 1.13 (3H, s), 0.97 (3H, s); ^{13}C NMR ($CDCl_3$) δ 161.9, 137.7, 133.0, 130.8, 122.1, 118.2, 114.4, 113.7, 51.7, 41.5, 39.6, 33.2, 32.4, 29.1, 23.4.

2.7.6. PTP1B Inhibitory Assay

Protein tyrosine phosphatase 1B (PTP1B) inhibitory activity was determined by measuring the rate of hydrolysis of a substrate, *p*-nitrophenyl phosphate (pNPP, Sigma, St. Louis, MO), according to the published method with a slight modification (Cui, *et al*., 2006). Briefly, PTP1B (100 μL of 0.5 μg/mL stock solution; Enzo Life Sciences, Farmingdale, NY) in 50 mM citrate buffer (pH 6.0) containing 0.1 M NaCI, 1 mM dithiothreitol (DTT), and 1 mM *N,N,N′,N′*-ethylenediamine tetraacetate (EDTA) were added to each well of a 96-well plastic plate (Corning Inc., Corning, NY, USA). Each sample (2.0 μL in MeOH) was added to each well to make the final concentration from 0 to 4.7-5.6 μM and incubated for 10 min at 37 °C. The reaction was initiated by the addition of pNPP (100 μL of 4.0 mM stock solution) in the citrate buffer, incubated at 37 °C for 30 min, and terminated with the addition of 10 μL of a stop solution (10 M NaOH). The optical density of each well was measured at 405 nm using an MTP-500 microplate reader (Corona Electric Co., LTD., Ibaraki, Japan). PTP1B inhibitory activity (%) is defined as $[1-(ABS_{sample} - ABS_{blank})/-(ABS_{control} - ABS_{blank})] \times 100$. ABS_{blank} was the absorbance of wells containing only the buffer and pNPP. $ABS_{control}$ was the absorbance of *p*-nitrophenol liberated by the enzyme in the assay system without a test sample, whereas ABS_{sample} was that with a test sample. The assays were performed in two indipendent experiments for all each sample. Oleanolic acid (Tokyo Chemical Industry, Tokyo, Japan), a known phosphatase inhibitor (Liu, *et al*., 1995), was used as a positive control.

2.7.7. Cytotoxicity Assay Against HCT-15 and Jurkat Cells

HCT-15 and Jurkat cells were obtained from the Center for Biomedical Research, Institute of Development, Aging, and Cancer, Tohoku University (Miyagi, Japan). The cell lines were cultured in RPMI-1640 medium. The medium was supplemented with 10% fetal bovine serum, 100 units/mL penicillin, and 100 μg/mL streptomycin. Exponentially growing cells, cultured in a humidified chamber at 37 ^{o}C containing 5.0% CO_2, were used for the experiments.

Cytotoxic activity was evaluated using the colorimetric MTT assay (Mosmann,

1983). HCT-15 (1.0 x 10^4 cells in 100 μL) or Jurkat cells (2.0 x 10^4 cells in 100 μL) were added to each well of a 96-well plastic plate. Each sample (1.0 μL in MeOH) was added to each well to make the final concentration from 0 to 39-47 μM, and the cells were incubated for 48 h at 37 ^{o}C. MTT (10 μL of 5.5 mg/mL stock solution) and a cell lysate solution (90 μL, 40% *N,N*-dimethylformamide, 20% sodium dodecyl sulfate, 2.0% CH_3COOH and 0.03% HC1) were added to each well, and the plate was shaken thoroughly by agitation at room temperature for overnight. The optical density of each well was measured at 570 nm using an MTP-500 micro plate reader.

2.7.8. Cytotoxicity Assay Against Huh-7 Cells

Cytotoxic activity against Huh-7 cells was assessed by the MTT assay, a modification of our previously described method. Following the treatment of cells with test samples, 10 μL of MTT (5.0 mg/mL in saline) was added to each well, incubated for 90 min at 37 ˚C, and centrifuged (300 *g* for 5 min), and the supernatant was aspirated off. The cells were lysed and solubilized by the addition of 100 μL of

0.04 M HCl in 2-propanol. The absorbance of each well was determined at 590 nm using an Inter-med model NJ-2300 Microplate Reader (Cosmo Bio Co., Ltd., Tokyo, Japan). Survival (%) was calculated relative to the control.

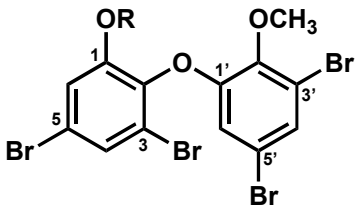
Chapter 3. A Polybromodiphenyl Ether from an Indonesian Marine Sponge *Lamellodysidea herbacea* and its Chemical Derivatives Inhibit Protein Tyrosine Phosphatase 1B, an Important Target for Diabetes Treatment

3.1. Introduction

Type-II diabetes mellitus (T2DM) is a chronic metabolic disorder characterized by ß-cell dysfunction and insulin resistance, and has emerged as major health care burden around the world (Bugianesi*, et al*., 2010; Romero, *et al*., 2010; Hummasti, *et al*., 2010). Protein tyrosine phosphatase 1B (PTP1B) is an enzyme found in the important insulin-targeted tissues such as liver, muscle, and fat cells. PTP1B plays a key role as a negative regulator in insulin signal transduction (Byon, *et al*., 1998) by dephosphorylating activated insulin receptors (IR) or insulin receptor substrates (IRS) (Tonks, *et al*., 1988; Gonzalez-Rodriguez, *et al*., 2010). An excess of PTP1B will impair insulin down-regulation (Ahmad, *et al*., 1995; Elchebly, *et al*., 1999; Haj, *et al*., 2005) leading to type-II diabetes mellitus. Based on the above research results, the inhibition of PTP1B has been expected as a novel therapeutic strategy, and much attention has been paid to PTP1B inhibition using small molecules for the treatment of type-II diabetes (Barr, *et al*., 2010; Zhang, *et al*., 2007; Lee, *et al*., 2007).

As described in **Chapter 3**, we established a bioassay system against activity of PTP1B, and found dehydroeuryspongin A (**4**) as a potent inhibitor from Okinawan marine sponge *Euryspongia* sp. To further search for more potent PTP1B inhibitors, we started to screen the ethanol extracts of Indonesian marine organisms such as marine sponges and ascidians. In the course of our screening program, the extract of an Indonesian marine sponge *Lamellodysidea herbacea* exhibited significant

inhibitory activity against PTP1B. Bioassay-guided separation of the extract lead to the isolation of a bioactive component, and the structure was assigned as 2-(3',5'-dibromo-2'-methoxyphenoxy)-3,5-dibromophenol (**10**) (Norton, *et al*., 1981). Fortunately, compound **10** was more potent inhibitor (IC_{50} = 0.85 μM) than compound **4** (IC_{50} = 3.6 μM). Furthermore, to investigate structure-activity relationships of **10**, five derivatives were prepared from **10**. In this chapter, the PTP1B inhibitory activity and cytotoxicity against two human cancer cell lines, HCT-15 (colon) and Jurkat (T-cell lymphoma), of compound **10** and its methyl ether **11** and ester derivatives (**12-15**) are described.



10 R = H
11 R = CH_3

Figure 3-1. Structures of compounds **10** and **11**

3.2. Screening

Among the ethanol extracts of 90 samples of marine sponges and ascidians (Table 1-1) collected in the coral reef at North Sulawesi, Indonesia, the ethanol extract of a marine sponge *Lamellodysidea herbacea* (Figure 3-2) showed a potent inhibitory activity (IC_{50} = 0.58 μg/mL) against PTP1B in the screening bioassay. Bioassay-guided isolation by preparative HPLC yielded compound **10** as an inhibitor of PTP1B. The other fractions obtained after separation of compound **10** did not show an inhibitory activity against PTP1B.

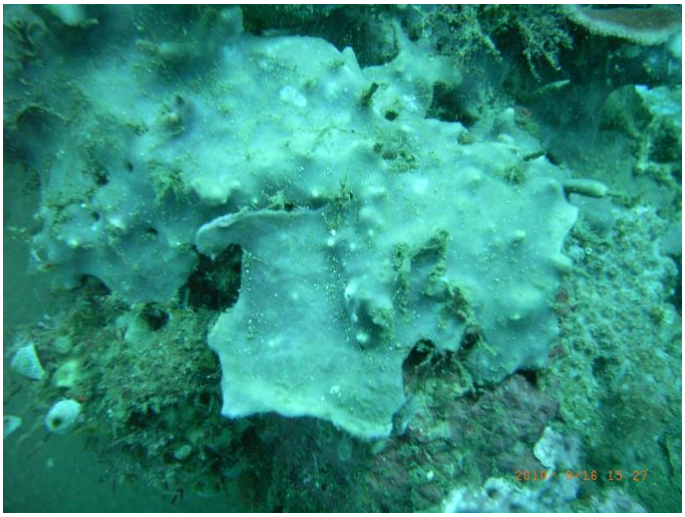


Figure 3.2. Marine sponge *Lamellodysidea herbacea*

3.3. Structure Identification

The EI-MS spectrum of **10** showed the presence of four Br atoms, and the molecular formula $C_{13}H_8Br_4O_3$ was deduced from HREI-MS data. The ^{13}C NMR spectrum of **10** revealed 13 carbon signals, and the signals due to two sets of meta-coupled aromatic protons (δ 6.80, 7.18, 7.35, and 7.45) and OMe protons (δ 4.03) were detected in the ^1H NMR spectrum. The positions of an OMe, OH, and four Br atoms were assigned by the analysis of 2D NMR (^1H-^1H COSY, HMQC, and HMBC) data for **10** and confirmed by the NOE experiments of the methyl derivative (**11**). NMR data for **10** were identical with those of the reported values for 2-(3',5'-dibromo-2'-methoxyphenoxy)-3,5-dibromophenol (Figure 3-1) (Norton, *et al.*, 1981).

3.4. Biological Activity

Compounds **10** and **11** inhibited the PTP1B activity (Figure 3-3) with the IC_{50} values of 0.85 and 1.7 μM, respectively, which were almost the same efficacy as that of oleanolic acid (1.1 μM), a positive control (Table 3-1). Oleanolic acid is a ubiquitous triterpene detected in various plants, most of which are used as crude

Asian drugs for the treatments of inflammations, cancers, hepatitis, and diabetes (Liu, *et al*., 2006; Nishino, *et al*., 1998; Sato, *et al*., 2007) and has recently been reported to have a significant inhibitory activity against PTP1B (Zhang, *et al*., 2008). Oleanolic acid derivatives were demonstrated to promote cellular insulin signaling by increasing the level of insulin receptor phosphorylation (Zhang, *et al*., 2008). The highest concentration of compound **11** did not show a dose dependent effect (Figure 3-3). This will be due to a solubility problem of **11** at higher concentration in this bioassay system. Interestingly, the methylation of a phenol in **10** reduced the cytotoxicity against HCT-15 and Jurkat cells (Figure 3-3 and Table 3-1). Compound **10** had a moderate cytotoxicity against HCT-15 and Jurkat cells with the IC_{50} values of 12 and 9.5 μM, respectively. On the other hand, **11** did not show an apparent cytotoxicity at 18 μM.

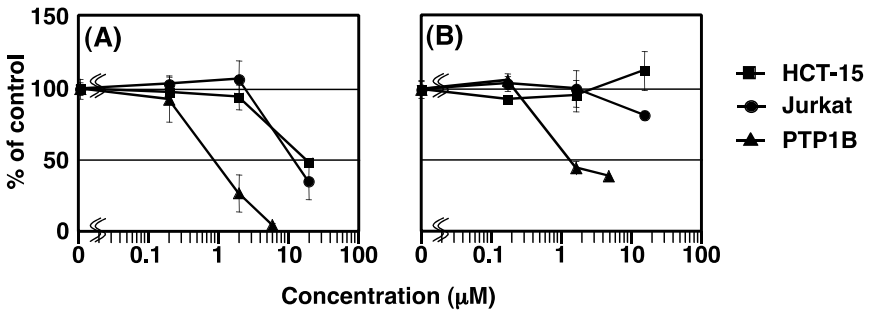
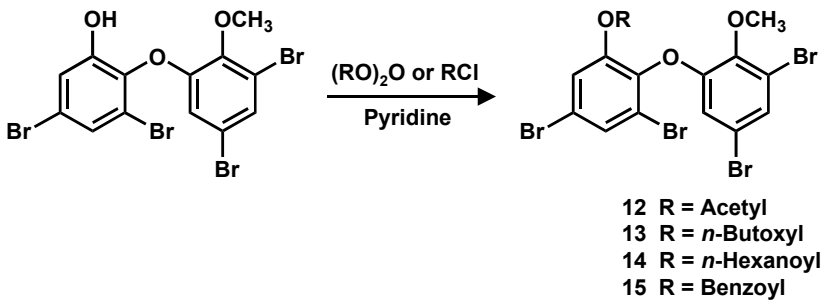


Figure 3.3. Inhibitory ativity of **10** (A) and **11** (B) against PTP1B and two human cancer (HCT-15 and Jurkat) cells

Therefore, the ester derivatives (**12**–**15**) were prepared from **10** (Figure 3-2) and tested for their activity against PTP1B and two cancer cell lines (Table 3-1). Compounds **12**–**15** exhibited the comparable to stronger inhibitory activity against PTP1B than that of **10,** but cytotoxicity against HCT-15 and Jurkat cells were observed. From these results, **11** is found to be the most interesting compound among

these compounds, as **11** possessed a potent inhibitory activity against PTP1B and showed much reduced cytotoxicity. Thus, the inhibitory activity of **10** and **11** on cell proliferation of human hepatoma Huh-7 cells was examined. Since PTP1B is located in the insulin-targeted tissues such as liver, muscle, and fat cells, Huh-7 cells are used for cell-based experiments to investigate the mechanism of action of PTP1B inhibitors. Compound **11** showed the weaker cytotoxicity (IC_{50} = 48 μM) than **10** (32 μM) (Table 3-1). The cell-based experiments are now in progress using Huh-7 cells and compound **11**.



Scheme 3-1. Preparation of compounds **12**–**15**

Polybrominated diphenyl ethers have been isolated from marine organisms, such as sponges, ascidians, and algae, and reported to exhibit a variety of biological activities: antibacterial and antifungal activities (Sharma, *et al*., 1972; Salva and Faulkner, 1990; Handayani, *et al*., 1997; Sionov, *et al*., 2005); brine shrimp toxicity (Handayani, *et al*., 1997); antimicroalgal activity (Hattori, *et al*., 2001); anti-inflammatory activity (Kuniyoshi, *et al*., 1985); maturation of starfish oocytes (Liu, *et al*., 2004); and inhibitory activities to several enzymes (Liu, *et al*., 2004; Fu, *et al*., 1995; Xu, *et al*., 2005). In this study, we demonstrated that a known bromodiphenyl ether **(10)** was a potent inhibitor of PTPlB, an important target enzyme for the treatment of type-II diabetes, and the methoxy derivative (**11**) is more useful than the

original phenol and the ester derivatives. Compound **11** will be a new lead compound for PTP1B inhibitors.

Table 3-1. Inhibitory activity of compounds **10-15** against PTP1B and three human cancer cell lines

Compound	IC_{50} (μM)			
	PTP1B	Cytotoxicity		
		Huh-7	HCT-15	Jurkat
10	0.85	32	12	9.5
11	1.7	48	> 46	> 46
12	0.62	NT	10.3	6.0
13	0.68	NT	14.3	9.6
14	0.69	NT	7.1	8.1
15	0.97	NT	4.3	20
Oleanolic acid	1.1	NT	NT	NT

NT: Not test

3.5. Conclusion

As described above, a known polybrominated ether (**10**) obtained from the ethanol extract of marine sponge *Lamellodysidea herbacea* collected in Indonesia was found to show a potent inhibitory activity against PTP1B during our screening program. The structure of **10** was confirmed by spectroscopic data for **10** and its methyl ether derivative (**11**). Both compounds (**10** and **11)** have more potent inhibitory activity against PTP1B with IC_{50} values of 0.85 and 1.7 μM, respectively, than that of dehydroeuryspongin A (**4**, IC_{50} = 3.6 μM). Interestingly, compound **11** maintained potent inhibitory activity against PTP1B without a cytotoxicty at 18 μM against two human cancer cell lines, HCT-15 (colon) and Jurkat (T-cell lymphoma) cells, although compound **10** showed a moderate cytotoxicity against these cancer cells. Because it is possible that cytotoxic effect due to phenolic hydroxyl group is reduced by *O*-methylation, five ester derivatives (**12**–**15**) were prepared from **10** and their inhibitory effects were evaluated to investigate structure-activity relationships.

Compounds **12**–**15** exhibited potent inhibitory effects against PTP1B activity, but also showed cytotoxicity against HCT-15 and Jurkat cells similar to that of **10**. These results suggested that these ester derivatives were hydrolyzed by a variety of esterases in the cells, and exhibited growth inhibition against these cell lines as same as **10**. Based on these studies, compound **11** was found to be the supreme inhibitor of PTP1B with no apparent cytotoxicity among them. Therefore, **11** may be a lead compound for making a new type of PTP1B inhibitor. Moreover, compound **11** did not inhibit the cell growth of Huh-7 cells (hepatoma). Hepatocytes are one of the locations of PTP1B, and study on the mechanism of action of compounds **4** and **11** remains to be identified using Huh-7 cells.

3.6. Materials and Methods

3.6.1. General

EI-MS was performed by a JMS-MS 700 mass spectrometer (JEOL, Tokyo, Japan). The ^{1}H and ^{13}C NMR spectra were recorded on a JNM-AL-400 NMR spectrometer (JEOL) at 400 MHz for ^{1}H and 100 MHz for ^{13}C in $CDCl_3$ (δ_H 7.26, δ_C 77.0). Preparative HPLC was carried out using the L-6200 system (Hitachi Ltd., Tokyo, Japan).

3.6.2. Materials

Fetal bovine serum (FBS) and other culture materials were purchased from Invitrogen (Carlsbad, CA, USA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) was purchased from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals including organic solvent were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan).

3.6.3. Marine Sponge

The marine sponge was collected by scuba diving in the coral reef at Manado, Indonesia, in 2010 and identified as *Lamellodysidea herbacea* (Figure 3-2). The voucher specimen was deposited at the Faculty of Fisheries and Marine Science, Sam Ratulangi University and the Faculty of Pharmaceutical Sciences, Tohoku Pharmaceutical University as 10-09-16=2-6.

3.6.4. Extraction and Isolation

The frozen marine sponge (94 g, wet weight) was thawed, cut into small pieces, and extracted three times with ethanol. The ethanol extract was evaporated to dryness (284.3 mg) and 20 mg of the crude extract was subjected to HPLC separation (90% MeOH, detection, UV 210 nm; flow rate, 2.0 mL/min) using an ODS column (PFGASIL ODS, 10 mm x 250 mm, Senshu Scientific Co., Tokyo, Japan) to give 5.4 mg of 2-(3',5'-dibromo-2'-methoxyphenoxy)-3,5-dibromophenol (**10**).

2-(3',5'-Dibromo-2'-methoxyphenoxy)-3,5-dibromopheno1 (10)

Obtained as a viscous oil; ^{1}H NMR ($CDC1_3$) δ 4.03 (3H, s), 6.80 (1H, d, J = 4.0), 7.18 (1H, d, J = 4.0), 7.35 (1H, d, J =4.0), 7.45 (1H, d, J = 4.0); ^{13}C NMR ($CDC1_3$) δ 61.5, 117.3, 117.3, 118.7, 119.0, 119.9, 120.1, 127.4, 130.5, 139.0, 145.9, 150.5, 150.7; EI-MS *m/z* 528, 530, 532, 534, and 526 [M^+]; HREI-MS *m/z* 527.7180 (Δ –2.7 mmu, calcd for $C_{13}H_8{}^{79}Br_4O_3$. 527.7207), 529.7203 (Δ +1.7 mmu, calcd for $C_{13}H_8{}^{79}Br_3{}^{81}Br_1O_3$: 529.7186), 531.7159 (Δ –0.7 mmu, calcd for $C_{13}H_8{}^{79}Br_2{}^{81}Br_2O_3$: 531.7166), 533.7137 (Δ –0.9 mmu, calcd for $C_{13}H_8{}^{79}Br_1{}^{81}Br_3O_3$: 533.7146), 535.7103 (Δ –2.2 mmu, calcd for $C_{13}H_8{}^{81}Br_4O_3$: 535.7125).

3.6.5. Preparation of Methyl Derivative (**11**)

TMS-diazomethane (73 μL, 0.064 mmol) was added to a MeOH solution of **10** (3.8 mg, 0.0071 mmol in 300 μL) and stirred at room temperature for 14 h. The reaction mixture was concentrated *in vacuo* to give a brown material, and a product was purified by preparative HPLC (90% MeOH) using ODS column (PFGASIL ODS) to give 2.0 mg (0.0037 mmol, 52 %) of 3,5-dibromo-2-(3',5'-dibromo-2'-methoxyphcnoxy)-1-methoxybenzene (**11**).

3,5-Dibromo-2-(3',5'-dibromo-2'-methoxyphenoxy)-1-methoxybenzene (11)

Obtained as a viscous oil; ^{1}H NMR (CDC1$_3$) δ 3.76 (3H, s), 4.00 (3H, s), 6.46 (1H, d, J = 4.0), 7.09 (1H, d, J = 4.0), 7.36 (1H, d, J = 4.0), 7.42 (1H, d, J = 4.0); ^{13}C NMR (CDC1$_3$) δ 57.2, 61.7, 116.4, 117.2, 119.3, 119.4, 119.6, 128.2, 129.5, 137.2, 140.1, 146.2, 152.1, 154.2; EI-MS *m/z* 542, 544 546, 548, and 550 [M^+]; HREI-MS *m/z* 541.7386 (Δ +2.2 mmu, calcd for $C_{14}H_{10}{}^{79}Br_4O_3$: 541.7364), 543.7319 (Δ –2.4 mmu, calcd for $C_{14}H_{10}{}^{79}Br_3{}^{81}Br_1O_3$: 543.7343), 545.7318 (Δ –0.5 mmu, calcd for $C_{14}H_{10}{}^{79}Br_2{}^{81}Br_2O_3$: 545.7323), 547.7288 (Δ –1.4 mmu, calcd for $C_{14}H_{10}{}^{79}Br_1{}^{81}Br_3O_3$: 547.7302), 549.7262 (Δ –2.0 mmu, calcd for $C_{14}H_{10}{}^{81}Br_4O_3$: 549.7282).

3.6.6. Preparation of Derivatives 12–15

Acetic anhydride (100 μL, 1.1 mmol) and 4-(dimethylamino) pyridine (1.0 mg, 0.0080 mmol) were added to a solution of **10** (3.0 mg, 0.056 mmol) in pyridine (100 μL), and the resulting solution was stirred at room temperature for 12 h. The reaction mixture was concentrated *in vacuo* to dryness, and a product was purified by preparative HPLC (column; PEGASIL ODS, 10 mm x 250 mm; solvent, 90% MeOH;

detection, UV at 220 nm; flow rate, 2.0 mL/min) to give1.2 mg (0.0022 mmol, 30%) of 3,5-dibromo-2-(3',5'-dibromo-2'-rnethoxyphenoxy)phenyl ethanoate (**12**). The other derivatives (**13**–**15**) were prepared using the following regents instead of acetic anhydride: *n*-butyric anhydride (**13**, 1.4 mg, 0.0023 mmol, 32%), *n*-hexanoic anhydride (**14**, 1.1 mg, 0.0018 mmol, 25%), and benzoyl chloride (**15**, 1.5 mg, 0.0023 mmol, 33%)

3,5-Dibromo-2-(3',5'-dibromo-2'-methoxyphenoxy)phenyl ethanoate (12)

Obtained as viscous oil; ^{1}H NMR (CDC1$_3$) δ 2.08 (3H, s), 3.95 (3H, s), 6.78 (1H, d, J = 2.4), 7.18 (1H, d, J = 2 4), 7.34 (1H, d, J = 2.4), 7.44 (1H, d, J = 2.4); EI-MS *m/z* 570, 572 574, 576, and 578 [M^+]; HREI-MS *m/z* 569.7316 (Δ +0.3 mmu, calcd for $C_{15}H_{10}{}^{79}Br_4O_4$: 569.7313), 571.7296 (Δ +0.4 mmu, calcd for $C_{15}H_{10}{}^{79}Br_3{}^{81}Br_1O_4$: 571.7292), 573.7283 (Δ +1.1 mmu, calcd for $C_{15}H_{10}{}^{79}Br_2{}^{81}Br_2O_4$: 573.7272), 575.7247 (Δ –0.4 mmu, calcd for $C_{15}H_{10}{}^{79}Br_1{}^{81}Br_3O_4$: 575.72S1), 577.7216 (Δ –1.5 mmu, calcd for $C_{15}H_{10}{}^{81}Br_4O_4$: 577.7231).

3,5-Dibromo-2-(3',5'-dibromo-2'-methoxyphenoxy)phenyl butanoate (13)

Obtained as a viscous oil; ^{1}H NMR (CDC1$_3$) δ 0.89 (3H, t, J = 7.2), 1.57 (2H, m), 2.29 (2H, t, J = 7.2), 3.95 (3H, s), 6.58 (1H, d, J = 1.9), 7.36 (1H, d, J = 2.4), 7.40 (1H, d, J = 1.9), 7.71 (1H, d, J = 2.4); EI-MS *m/z* 598, 600 602, 604, and 606 [M^+]; HREI-MS *m/z* 597.7625 (Δ –0.1 mmu, calcd for $C_{17}H_{14}{}^{79}Br_4O_4$: 597.7626), 599.7580 (Δ –2.5 mmu, calcd for $C_{17}H_{14}{}^{79}Br_3{}^{81}Br_1O_4$: 599.7605) 601.7569 (Δ –1.6 mmu, calcd for $C_{17}H_{14}{}^{79}Br_2{}^{81}Br_2O_4$: 601.7585), 603.7591 (Δ +2.7 mmu, calcd for $C_{17}H_{14}{}^{79}Br_1{}^{81}Br_3O_4$: 603.7564), 605.7518 (Δ –2.6 mmu, calcd for $C_{17}H_{14}{}^{81}Br_4O_4$: 605.7544).

3,5-Dibromo-2-(3',5'-dibromo-2'-methoxyphenoxy)phenyl hexanoate (14)

Obtained as a viscous oil; ^{1}H NMR ($CDC1_3$) δ 0.87 (3H, t, *J* = 6.8), 1.25 (4H, m), 1.51 (2H, m), 2.30 (2H, t, *J* = 7.8), 3.95 (3H, s), 6.57 (1H, d, *J* = 2.0), 7.36 (1H, d, *J* = 2.0), 7.40 (1H, d, *J* = 2.0), 7.71 (1H, d, *J* = 2.4); EI-MS *m/z* 626, 628 630, 632, and 634 [M^+]; HREI-MS *m/z* 625.7952 (Δ +1.3 mmu, calcd for $C_{19}H_{18}{}^{79}Br_4O_4$: 625.7939), 627.7924 (Δ +0.6 mmu, calcd for $C_{19}H_{18}{}^{79}Br_3{}^{81}Br_1O_4$: 627.7918), 629.7881 (Δ –1.7 mmu, calcd for $C_{19}H_{18}{}^{79}Br_2{}^{81}Br_2O_4$: 629.7898), 631.7874 (Δ –0.4 mmu, calcd for $C_{19}H_{18}{}^{79}Br_1{}^{81}Br_3O_4$: 631.7878), 633.7856 (calcd for $C_{19}H_{18}{}^{81}Br_4O_4$: 633.7856).

3,5-Dibromo-2-(3',5'-dibromo-2'-mcthoxyphenoxy)phenyl benzoate (15)

Obtained as a viscous oil; ^{1}H NMR ($CDC1_3$) δ 3.80 (3H, s), 6.68 (1H, d, *J* = 2.4), 7.27 (1H, d, *J* =1.9), 7.41 (2H, t, *J* = 7.7), 7.52 (1H, d, *J* =1.9), 7.59 (1H, t, *J* = 7.3), 7.76 (1H, d, *J* = 2.4), 7.83 (2H, d, *J* = 7.2); EI-MS *m/z* 632, 634, 636, 638, and 640 [M^+]; HREI-MS *m/z* 631.7455 (Δ –1.4 mmu, calcd for $C_{20}H_{12}{}^{79}Br_4O_4$: 631.7469), 633.7468 (Δ +1.9 mmu, calcd for $C_{20}H_{12}{}^{79}Br_3{}^{81}Br_1O_4$: 633.7449), 635.7433 (Δ +0.5 mmu, calcd for $C_{20}H_{12}{}^{79}Br_2{}^{81}Br_2O_4$: 635.7428), 637.7430 (Δ +2.3 mmu, calcd for $C_{20}H_{12}{}^{79}Br_1{}^{81}Br_3O_4$: 637. 7407), 639.7379 (Δ –0.9 mmu, calcd for $C_{20}H_{12}{}^{81}Br_4O_4$: 639.7388).

3.6.7. PTP1B Inhibitory Assay

Protein tyrosine phosphatase 1B (PTP1B) inhibitory activity was determined by measuring the rate of hydrolysis of a substrate, *p*-nitrophenyl phosphate (pNPP, Sigma, St. Louis, MO) according to the published method with a slight modification

(Cui, *et al*., 2006) as described in **Chapter 2** (**2.7.6**).

3.6.8. Cytotoxicity Assay Against HCT-15 and Jurkat Cells

The cytotoxicity assay was performed as described in **Chapter 2** (**2.7.7**).

3.6.9. Cytotoxicity Assay Against Huh-7 Cells

This bioassay was performed as described in **Chapter 2** (**2.7.8**).

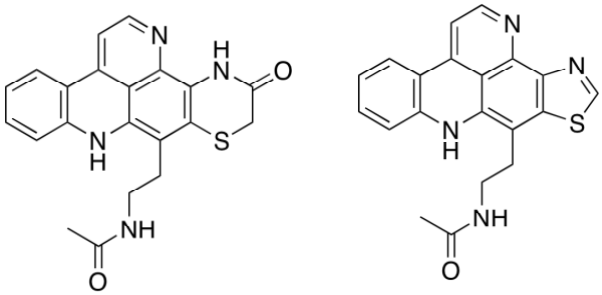
Chapter 4. Isolation and Bioactivity of Two Pyridoacridine Alkaloids, Shermilamine B and Kuanoniamine D, from an Indonesian Ascidian *Cystodytes* sp.

4.1. Introduction

Marine invertebrates, such as marine sponges and ascidians, algae, and marine-derived microorganisms, are living in highly competitive environments, and, therefore, produce a wide variety of toxic chemicals to prevent parasitism, predation, biofouling, and so on (Marshall and Barrows, 2004). Therefore, the screening program to search for marine cytotoxic components against mammalian cells was simultaneously carried out in addition to the screening of PTP1B inhibitors.

Among marine organisms, ascidians are a rich source of biologically active nitrogenous substances with high chemical diversity. More than 80% of new compounds from ascidians contain nitrogen, and about 70% of nitrogenous compounds are alkaloids. The colonial ascidian of the genus *Cystodytes* sp. (class Ascidiacea, Aplousobranchia, Polycitoridae) inhabits benthic rock environments and widely distributed in the Atlantic, Pacific, and Indian Oceans. Morphology of *Cystodytes* varies greatly in the color, specular composition, and shape. Moreover, *Cystodytes* have been reported to contain several alkaloids, including ascididemin, 11-hydroxyascididemin, cystodytins A-I, sebastianines A and B, and so on (Wang and Namikoshi, 2007). These marine natural products are classified into the pyrodoacridine alkaloids, which are known to exhibit various biological activities including cytotoxicity, inhibition of topoisomerase II, anti-HIV activity, Ca^{2+} releasing activity, and intercalation with DNA (Skyler, 2002; Wang and Namikoshi, 2007).

In the course of our study on bioactive marine natural products, the ethanol extract of an Indonesian colonial ascidian *Cystodytes* sp. showed a potent cytotoxicity against HCT-15, Jurkat, and V79 cells. Bioassay-guided separation of the ethanol extract by preparative HPLC led to the isolation of two known pyridoacridine alkaloids, shermilamine B (**16**) and kuanoniamine D (**17**) (Figure 4-1). Shermilamine B (**16**) has originally been isolated from a colonial ascidian *Trididemnum* sp. collected at Guam (Carroll, *et al*., 1989), and kuanoniamine D (**17**) was afforded from a Micronesian tunicate and its predatory mollusk *Chelynotus semperi* as a cytotoxic compound against KB cells (Caroll, *et al*., 1990). Compounds **16** and **17** have also been isolated from a Mediterranean ascidian *Cystodytes dellechiajei* and tested against two bacterial strains, *Escherichia coli* and *Micrococcus luteus* (Bontemps, *et al*., 2010). In this chapter, isolation, structure elucidation, and biological activity of shermilamine B (**16**) and kuanoniamine D (**17**) from an Indonesian *Cystodytes* sp. are described.



Shermilamine B (**16**) Kuanoniamine D (**17**)

Figure 4-1. Structures of shermilamine B (**16**) and kuanoniamine D (**17**)

4.2. Screening

The ethanol extracts of 90 marine sponges and ascidians collected in the coral reef at North sulawesi, Indonesia were tested for their effects on the colony formation of Chinese hamster V79 cells (Table 1-1), and the extract of a colonial ascidian

Cystodytes sp. (Figure 4-2) inhibited the colony formation of V79 cells (84% inhibition at 50 μg/mL) in the screening bioassay. Influence on V79 cells reflects the direct action of compounds on the cells, and, therefore, is used to select active compounds for the bioassay of inflammatory cytokine production. In addition, the ethanol extract of this ascidian showed weak inhibitory activity on the cell proliferation of two human cancer cell lines, HCT-15 and Jurkat (44% and 29% inhibition at 50 μg/mL).

Figure 4-2. Colonial ascidian *Cystodytes* sp.

4.3. Extraction and Isolation

The colonial ascidian (Figure 4-2) was extracted immediately after collection with ethanol on a boat. The extract was evaporated, and purified by preparative HPLC using an ODS column to give compounds **16** (6.3 mg) and **17** (8.5 mg) as dark purple gums (Figure 4-3).

4.4. Structure Identification

Compound **16** showed a molecular ion at *m/z* 390 in the EI-MS. The ^{1}H NMR spectrum of **16** revealed the presence of a heterocyclic aromatic moiety by the

aromatic proton signals at the lower field and two methylenes as a side chain. Compound **16** had a similar UV spectrum to those of pyridoacridine alkaloids. Based on these information, a literature search yielded the structure of shermilamine B as a candidate, and the structure was confirmed by comparing ^{1}H NMR and physico-chemical data (appearance and UV spectrum) for **16** with those for the reported values (Carroll, *et al*., 1989).

Compound **17** showed similar ^{1}H NMR and UV spectra to those of **16**, but the EI mass spectrum of **17** gave a molecular ion peak at *m/z* 360, which was 30 mu smaller than that of **16**. From these spectroscopic data, compound **17** was supposed to be a pyridoacridine alkaloid. A literature search provided the structure of kuanoniamine D as a candidate, and ^{1}H NMR and physico-chemical data (appearance and UV spectrum) for **17** were identical to those for kuanoniamine D (Caroll, *et al*., 1990).

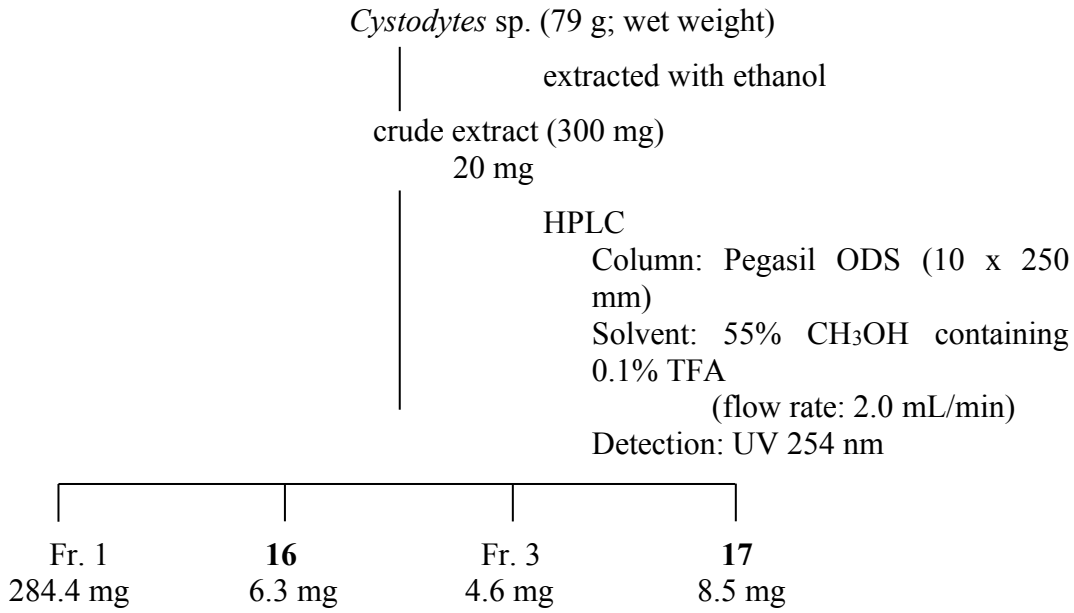


Figure 4-3. Separation procedure of *Cystodytes* sp.

4.5. Biological Activity

Influence on the colony formation of Chinese hamster V79 was tested as described in the Experimental section. Kuanoniamine D (**17**) inhibited the colony formtion with an EC_{50} value of 6.2 μM, but shermilamine B (**16**) was not active up to 20 μM (Table 4-1). Cytotoxicity of shermilamine B (**16**) and kuanoniamine D (**17**) against HCT-15 and Jurkat cells was evaluated after 48 h by the MTT method. The 50% inhibitory concentration (IC_{50}) values of compound **17** against HCT-15 and Jurkat cells were 4.1 and 19 μM, respectively, whereas compound **16** was less effective with IC_{50} values of 6.7 and >26 μM, respectively (Table 4-1). The inhibitory activity of **17** was more potent than that of **16**. Therefore, the thiazole moiety in **17** will be more favorable for the cytotoxicity than the thiazinone moiety in **16**.

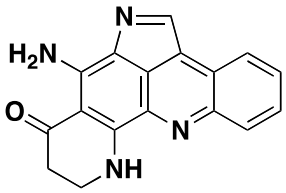
Table 4-1. Effect of shermilamine B (**16**) and kuanoniamine D (**17**) against V79, Jurkat, and HCT-15 cells

Compound	V79	HCT-15	Jurkat
	EC_{50} (μM)	IC_{50} (μM)	
Shermilamine B (**16**)	> 20	6.7	> 26
Kuanoniamine D (**17**)	6.2	4.1	19

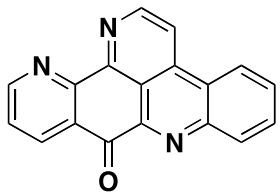
4.6. Conclusion

In our continuous screening program for cytotoxic marine natural products, two known alkaloids, shermilamine B (**16**) and kuanoniamine D (**17**), were obtained from an Indonesian marine ascidian *Cystodytes* sp. by solvent extraction and preparative HPLC. Structures of compounds **16** and **17** were identified by spectroscopic studies. These compounds belong to pyridoacridine alkaloids, which contain a pentacyclic aromatic skeleton. Cytotoxicity of compounds **16** and **17** against two human cancer cell lines, HCT-15 and Jurkat cells, were evaluated, and

kuanoniamine D (**17**) showed more potent growth inhibition against two cancer cells than that of shermilamine B (**16**). This relationship is comparable with the results of inhibition effects of **16** and **17** on the colony formation of Chinese hamster V79 cells. Since the influence on V79 cells reflects the direct action of compounds on the cells, this assay system is used to select active compounds for the bioassay of inflammatory cytokine production. In fact, we have isolated two known pyridoacridine alkaloids, plakinidine D (**18**) and ascididemin (**19**) (Figure 4-4), using the bioassay with Chinese hamster V79 cells in our previous study (EC_{50} = 2.2 μM and 49 nM, respectively). Compound **19** exhibited a strong inhibitory activity on the TNF-α production in lipopolysaccharide (LPS)-stimulated murine macrophage-like RAW264.7 cells at concentration of 0.3 μM. Therefore, an inhibitory activity of kuanoniamine D (**17**) on the production of inflammatory cytokines such as TNF-α, IL-1, and IL-6 will be an interesting future study. The inhibitory effect on the cytokine production by pyridoacridine alkaloids has not been reported, and, therefore, this is the first to show the inhibition of the TNF-α production by this class of alkaloids.



Plakinidine D (**18**)



Ascididemin (**19**)

Figure 4-4. Structures of plakinidine D (**18**) and ascididemin (**19**)

4.7. Experimental Section

4.7.1. General

EI mass spectra were performed by JMS-MS 700 mass spectrometer (JEOL, Tokyo-Japan). ^{1}H NMR spectra were recorded on JNM-AL-400 NMR spectrometer (JEOL, Tokyo-Japan) at 400 MHz in DMSO-*d*$_6$ (δ_H 2.46, δ_C 39.5) or CD_3OD (δ_H 3.31, δ_C 49.0). Preparative HPLC was carried out using L-6200 system (Hitachi Ltd. Tokyo-Japan).

4.7.2. Ascidian

The ascidian was collected by scuba diving at the coral reef in Lembeh Strait, North Sulawesi, Indonesia in September 2010 and identified as *Cystodytes* sp. The voucher specimen is deposited at Tohoku Pharmaceutical University as 10-09-13=1-1.

4.7.3. Extraction and Isolation

The ascidian (79 g, wet weight) was cut into small pieces and soaked in EtOH on a boat immediately after collection. The organism was further extracted three times with EtOH to give the crude extract (300 mg), of which 20 mg were separated by preparative HPLC (column, Pegasil ODS (10 mm x 250 mm); solvent 55% MeOH containing 0.1% TFA; flow rate, 2 mL/min; detection, UV at 254 nm) to afford 6.3 mg of shermilamine B (**16**) and 8.5 mg of kuanoniamine D (**17**).

Shermilamine B (16)

Obtained as a dark purple gum; ^{1}H NMR spectrum (DMSO-*d*$_6$, Figure 4-4) δ 1.93 (3H), 2.99 (2H), 3.12 (2H), 3.16 (2H), 7.06 (1H), 7.24 (1H), 7.45 (1H), 7.53 (1H), 8.07 (1H), 8.54 (1H), 8.58 (NH), 9.33 (1H) 10.43 (1H); EI-MS *m/z* 390 [M^+] (Figure 4-5).

Kuanoniamine D (17)

Obtained as a dark purple gum; ^{1}H NMR (DMSO-d_6, Figure 4-6) δ 1.85-1.89 (3H, s), 3.09 (2H, t), 3.16 (2H, dt), 7.00 (1H, dt), 7.49 (1H, d), 7.49 (1H, d), 7.87 (1H, d), 7.99 (1H, d), 8.39 (1H), 8.57 (1H, d), 9.29 (1H, s), 10.94 (1H, s); EI-MS *m/z* 360 [M^+] (Figure 4-7).

4.7.4. Materials

Fetal bovine serum (FBS) and other culture materials were purchased from Invitrogen (Carlsbad, CA, USA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals and organic solvents were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan).

4.7.5. Influence on the Colony Formation of Chinese Hamster V79 Cells

Chinese hamster V79 cells were grown as a monolayer culture in Eagle's MEM with 10% heat-inactivated FBS. The relative plating efficiencies were determined as the ratio of the number of colonies in various concentrations of samples to that in the sample-free control. Two hundred cells were seeded on a 60/15-mm plastic plate with 4 mL culture medium and incubated overnight at 37 ^{o}C. After each sample in DMSO (4 mL) was added to the culture medium, cells were further cultured for four days. The numbers of colonies in the sample plates were counted and compared with those in control cultures.

4.7.6. Cytotoxicity Assay Against HCT-15 and Jurkat Cells

This bioassay was performed as described in **Chapter 2** (**2.7.7**).

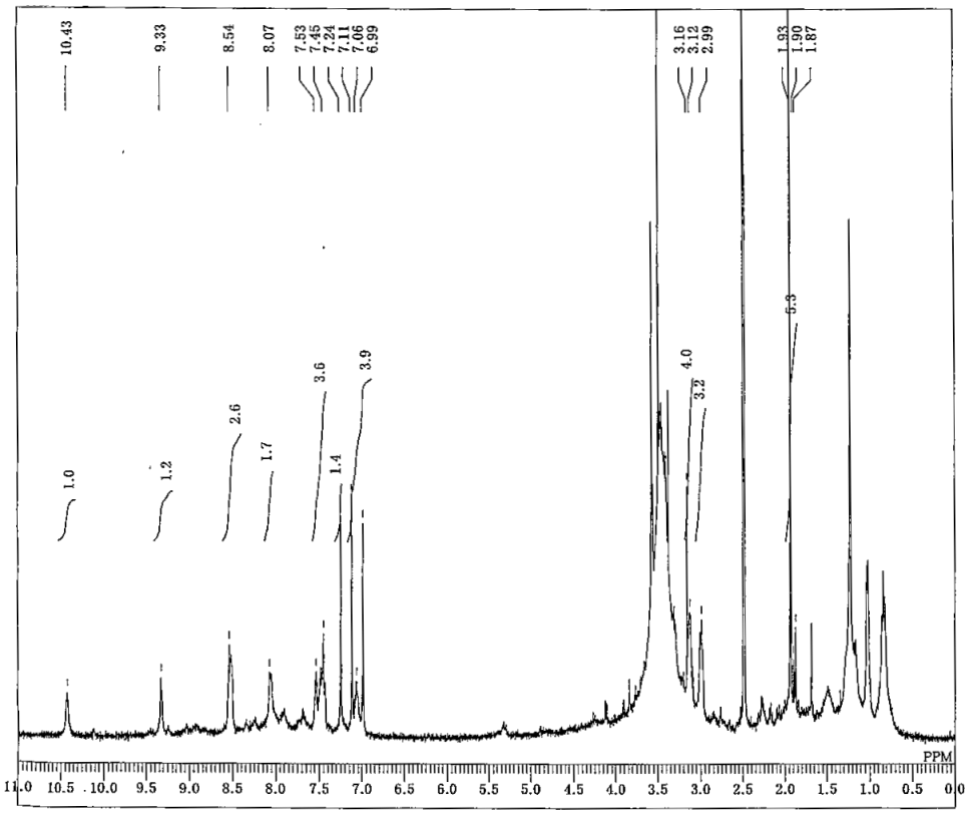


Figure 4-4. ^{1}H NMR spectrum of compound **16**

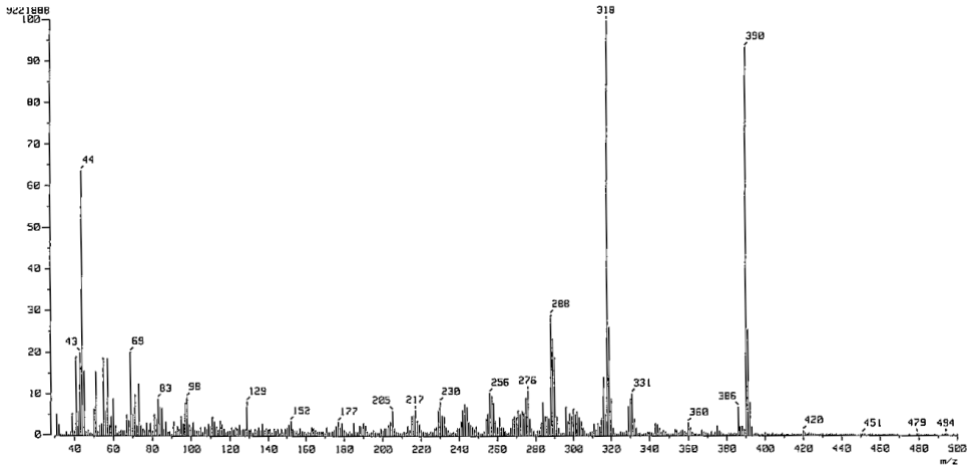


Figure 4-5. EI mass spectrum of compound **16**

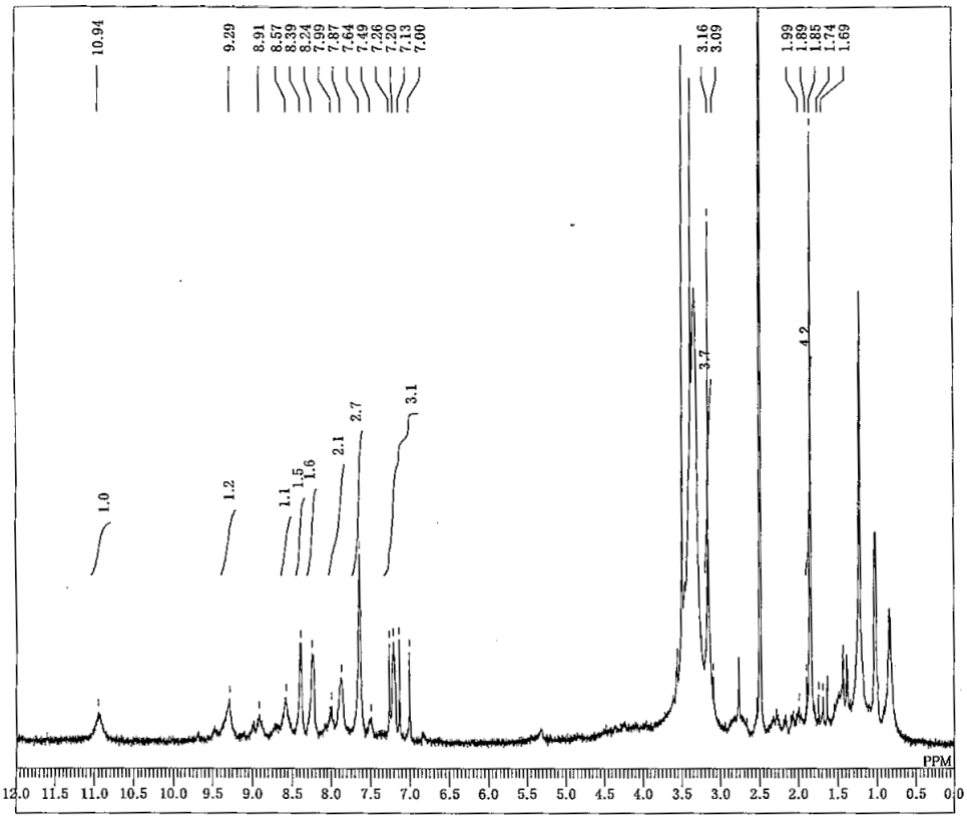


Figure 4-6. ^{1}H NMR spectrum of compound **17**

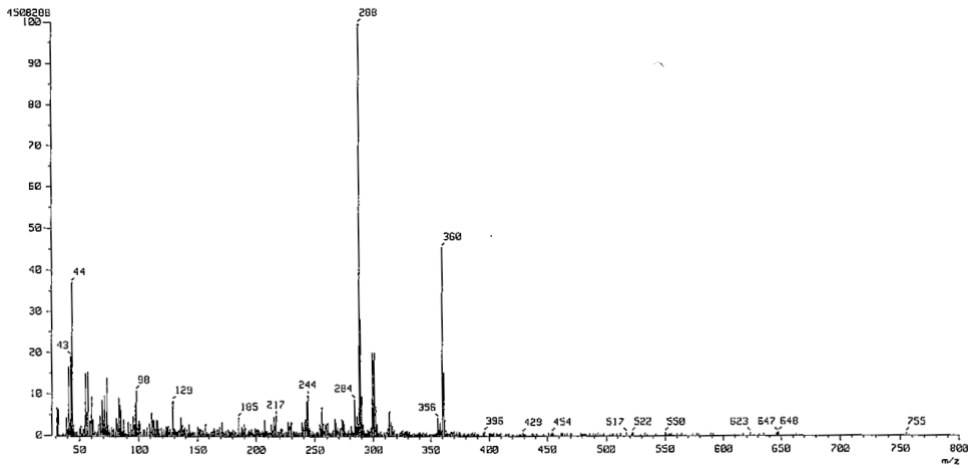


Figure 4-7. EI mass spectrum of compound **17**

Chapter 5. Isolation of Components from an Indonesian Solitary Ascidian *Herdmania momus*

5.1. Introduction

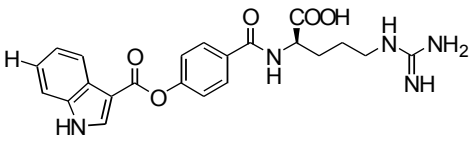
As described in the previous Chapters, marine organisms are a rich source of bioactive secondary metabolites. In fact, six useful compounds including three new euryspongins have been obtained from marine sponges and ascidian as described in Chapters 2–4.

Ascidians belong to a group of sessile marine filter feeders and are classified into colonial and solitary species. Colonial ascidians are characterized by many small individuals, called zooids, living together in a common tunic, whereas solitary species generally live as each isolated individual. Although many secondary metabolites such as cyclic peptides, pyridoacridines, and various alkaloids have been reported from colonial ascidians, reports on the chemical components of solitary ascidians have been limited. Only four solitary species, *Dendrodoa grossularia* (Loukaci, *et al.*, 1996), *Polycarpa aurata* (Lindquist, *et al.*, 1990; Abas, *et al.*, 1996; Wang, *et al.*, 2007), *P. clavata* (Kang, *et al.*, 1996), and *Herdmania momus* (Cheng, *et al.*, 1995; Li, *et al.*, 2011; Li, *et al.*, 2012), have thus far been investigated on their bioactive secondary metabolites.

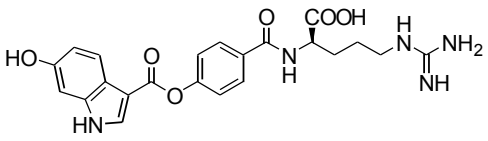
The solitary ascidians of the genus *Herdmania* (Subclass Pleurogona, Order Stolidobranchia, Family Pyuridae) are found in all warm waters. The first metabolites from *H. momus*, oleic acid, β-sitosterol, p-hydroxybenzaldehyde, ethyl-α-D-glucopyranoside, and thymidine, were reported in 1995 (Cheng, *et al.*, 1995). Most recently, Li and co-workers have reported eleven new amino acid derivatives, herdmanines A-K (Figure 5-1), from *H. momus* collected at Jeju Island, Korea.

Herdmanine D exhibited a moderate suppressive effect on the production of nitric oxide (NO) in LPS-activated murine macrophage cells, and herdmanines I and K showed significant PPAR-γ activation in Ac2F rat liver cells.

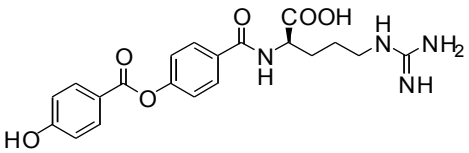
Thus, solitary ascidians are very attractive organisms as a source of new useful compounds. As a part of our search program for novel and useful metabolites from marine organisms, *H. momus* has been collected from several sites at North Sulawesi, Indonesia in 2009 and 2010. Chemical investigation on the Indonesian *H. momus* has thus far led to the isolation of compounds **20** and **21**. In this chapter, the isolation and structure analysis of compounds **20** and **21** are described.



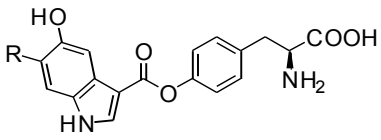
Herdmanine A



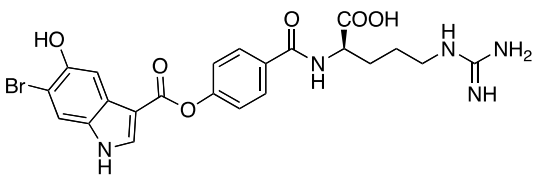
Herdmanine B



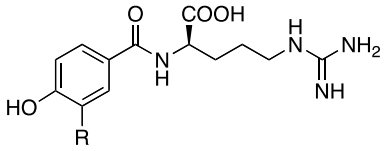
Herdmanine C



Herdmanine D



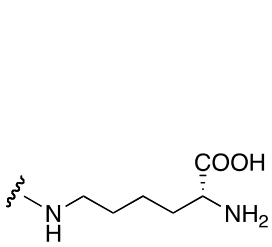
Herdmanine E



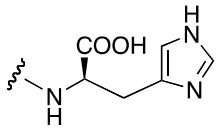
Herdmanine F : R = Br

Herdmanine G : R = OCH_3

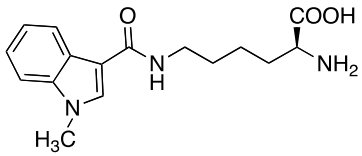
Herdmanine H : R = H



Herdmanine I



Herdmanine K



Herdmanine J

Figure 5-1. Structures of herdmanines A-K

5.2. Extraction and Isolation of Compounds 20 and 21

The solitary ascidian *H. monus* (Figure 5-2) was collected at Manado Tua Island, Indonesia, and extracted with ethanol to give the crude extract (14.7 g), which showed 23.4% inhibition against the colony formation of Chinese hamster V79 cells at 50 μg/mL. An ODS column chromatography followed by repeated preparative HPLC from 5 g of the extract afforded compounds **20** (1.9 mg) and **21** (1.7 mg) as

brown gums (Figure. 5-3).

Figure 5-2. Marine ascidian *Herdmania* sp.

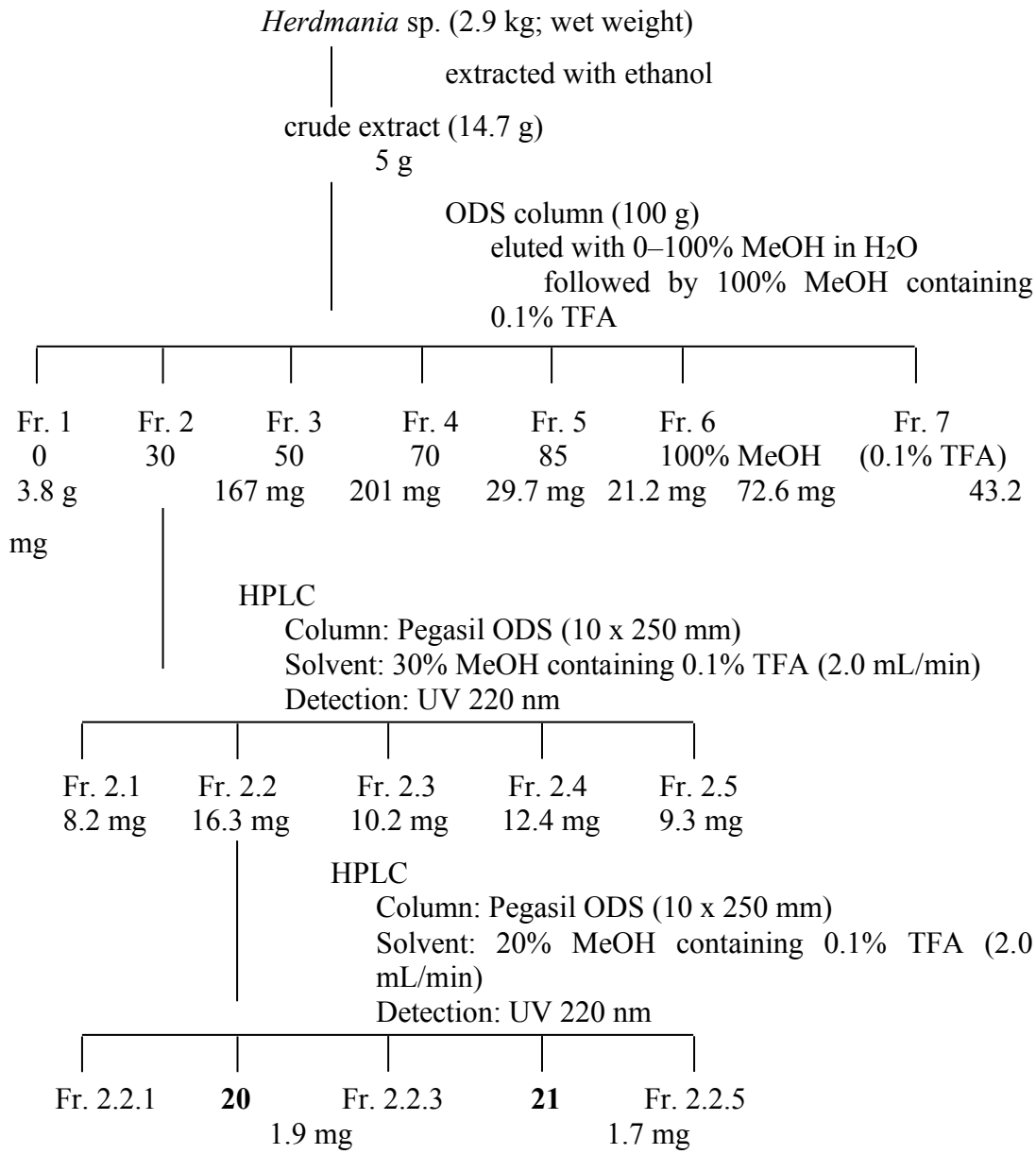


Figure 5-3. Separation procedure of compounds **20** and **21**.

5.3. Structure Analysis of Compounds 20 and 21.

Compound **20** showed the molecular ion peaks at *m/z* 267 $[M+H]^+$ (positive mode) and 265 $[M–H]^–$ (negative mode) in the FAB-MS (Figures 5-6 and 5-7). The ^1H and ^{13}C NMR spectra of **20** indicated 15 proton and 8 carbon signals (Figures 5-4 and 5-5), which were classified into two methyls, four methylenes, one sp^3 methines, and one carbonyl carbon.

Compound **21** exhibited the molecular ion peaks at *m/z* 323 $[M+H]^+$ (positive mode) and 321 $[M–H]^–$ (negative mode) in the FAB-MS (Figures 5-10 and 5-11). The ^1H NMR spectrum of **21** revealed two sets of methylene and aromatic protons (Figure 5-8). The ^{13}C NMR spectrum of **21** (Figures 5-9) also showed aromatic and aliphatic carbon signals comparable with the ^1H NMR spectrum of **21**.

A literature search with the spectroscopic data for compounds **20** and **21** revealed that these compounds have not been reported from any solitary ascidians. Therefore, compounds **20** and **21** have most likely new structures. Elucidation of the structures of compounds **20** and **21** are now in progress.

5.4. Conclusion

As described in **5.1**, metabolites from solitary ascidians are very interesting. Therefore, investigation on an Indnesian solitary ascidian *H. monus* has been carried out. Compounds **20** and **21** have, thus far, been isolated by ODS column and HPLC separation from the ethanol extract. The NMR spectra of **20** and **21** did not match to any compounds reported from solitary ascidians. Consequently, elucidation of the structures of compounds **20** and **21** is an interesting object.

5.5. Experimental Section

5.5.1. General

FAB-MS was performed by a JMS-MS 700 mass spectrometer (JEOL, Tokyo, Japan). The ^{1}H and ^{13}C NMR spectra were recorded on JNM-AL-400 NMR spectrometer (JEOL, Tokyo-Japan) at 400 MHz in CD_3OD (δ_H 3.31, δ_C 49.0). Preparative HPLC was carried out using L-6200 system (Hitachi Ltd., Tokyo, Japan).

5.5.2. Ascidian

The solitary ascidian was collected by scuba diving at Manado Tua Island, North Sulawesi, Indonesia in September 2010 and identified as *Herdmania monus*. The voucher specimen is deposited at Tohoku Pharmaceutical University as 10-09-15=1-1.

5.5.3. Extraction and Isolation

The ascidian (2.9 kg, wet weight) was cut into small pieces and soaked in ethanol on a boat immediately after collection. The organism was further extracted three times with ethanol. From the crude extract (14.7 g), 5 g were suspended in H_2O and adsorbed on an ODS column (100 g). The ODS column was eluted stepwise with 0, 30, 50, 70, 85, and 100% MeOH in H_2O followed by MeOH containing 0.1% TFA to give seven fractions (Fr.1–Fr.7). Fr. 2 (167.2 mg), eluted with 30% MeOH, was separated by preparative HPLC (column, Pegasil ODS (10 mm × 250 mm); solvent 30% MeOH containing 0.1% TFA; flow rate, 2 mL/min; detection, UV at 220 nm) to afford five fractions (Fr. 2-1–2-5). Preparative HPLC separation (column, Pegasil ODS (10 mm × 250 mm); solvent 20% MeOH containing 0.1% TFA; flowrate, 2

mL/min; detection, UV at 220 nm) of Fr.2-2 yielded 1.9 mg of compound **20 (**eluted at 37.3 min) and 1.7 mg of compound **21** (eluted at 47.6 min).

Compound 20

Obtained as a brown gum; ^{1}H NMR spectrum (CD_3OD) δ 0.92 (6H, d), 1.43 (2H, q), 1.61 (2H, m), 3.00 (2H, t), 3.29 (2H, m), 3.66 (2H, t) (Figure 5-4); ^{13}C NMR spectrum (CD_3OD) δ 22.8, 27.0, 36.7, 38.9, 39.1, 51.0, 161.5 (Figure 5-5); FAB-MS *m/z* 267 $[M+H]^+$ (Figure 5-6).

Compound 21

Obtained as a brown gum; ^{1}H NMR spectrum (CD_3OD) δ 2.83 (2H, t), 2.99 (2H, t), 3.47 (2H, t), 3.66 (2H, t), 7.22 (8H, m) (Figure 5-8); ^{13}C NMR spectrum (CD_3OD) δ 36.3, 36.7, 42.1, 51.0, 127.5, 129.6, 129.8, 140.1, 161.5 (Figure 5-9); FAB-MS *m/z* 323 $[M+H]^+$ (Figure 5-10).

5.5.4. Antimicrobial Assay

The growth inhibitory activity was examined by the paper disc method against *Mucor hiemalis* IAM 6088 (fungus), *Saccharomyces cerevisiae* IAM 1438T (yeast), *Staphyloccoccus aureus* IAM 12544T (Gram-positive bacterium), and *Escherichia coli* IAM 12119T (Gram-negative bacterium) as test microorganisms.

5.5.5. Influence on the Colony Formation of Chinese Hamster V79 Cells

This bioassay was performed as described in **Chapter 4** (**4.7.5**).

5.5.6. Cytotoxicity Assay Against HCT-15 and Jurkat Cells

Cytotoxicity assay was performed as described in **Chapter 2** (**2.7.7**).

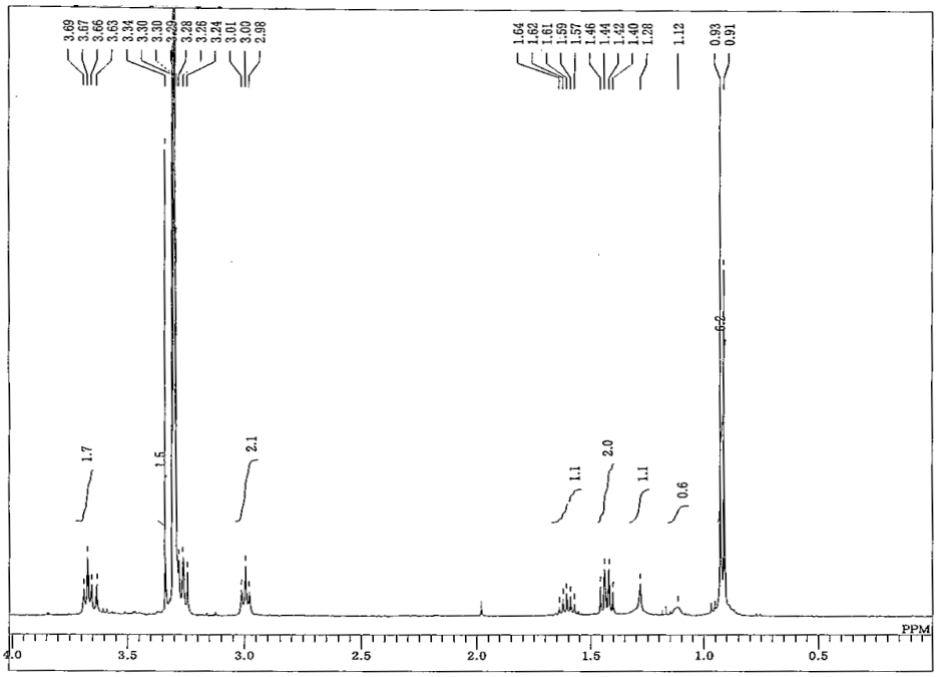


Figure 5-4. ^{1}H NMR spectrum of compound **20**

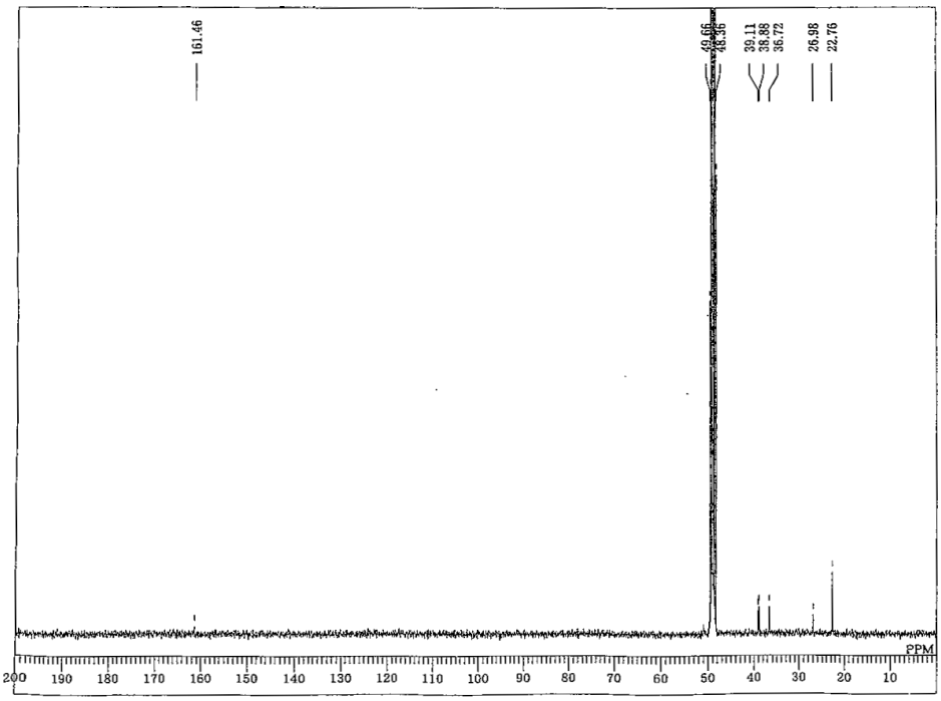


Figure 5-5. ^{13}C NMR spectrum of compound **20**

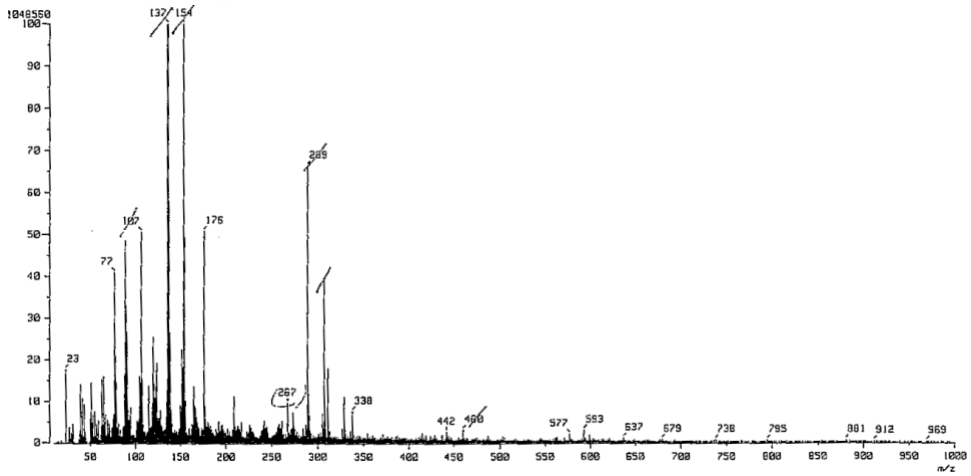


Figure 5-6. FAB-MS spectrum (positive mode) of compound **20**

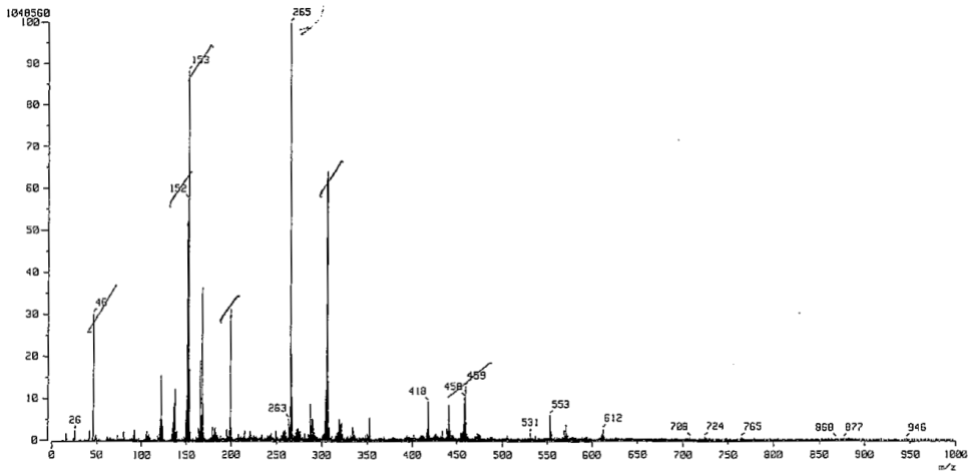


Figure 5-7. FAB-MS spectrum (negative mode) of compound **20**

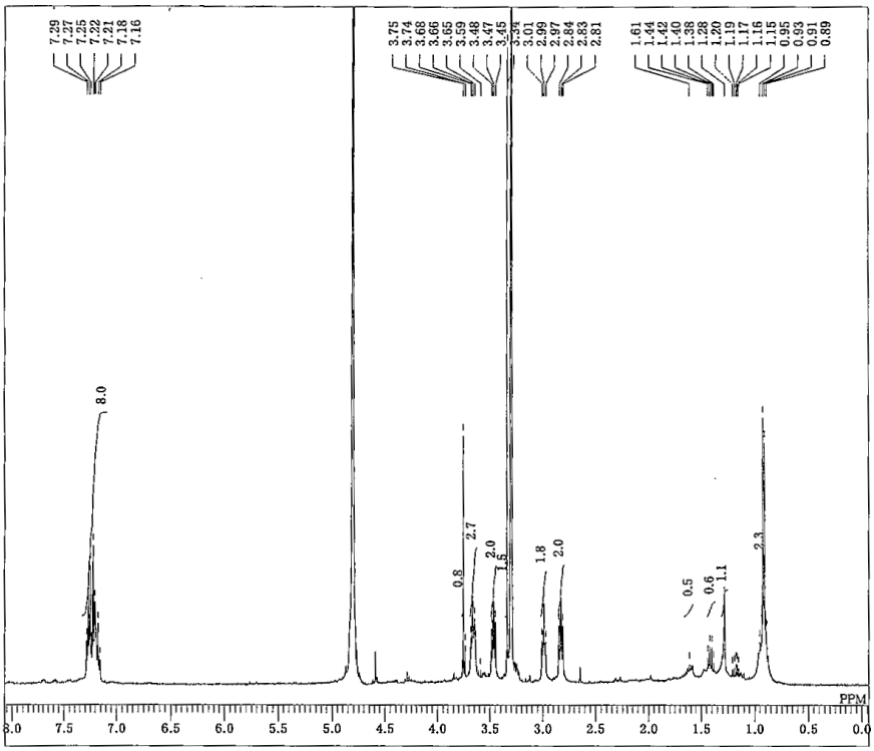


Figure 5-8. ^{1}H NMR spectrum of compound **21**

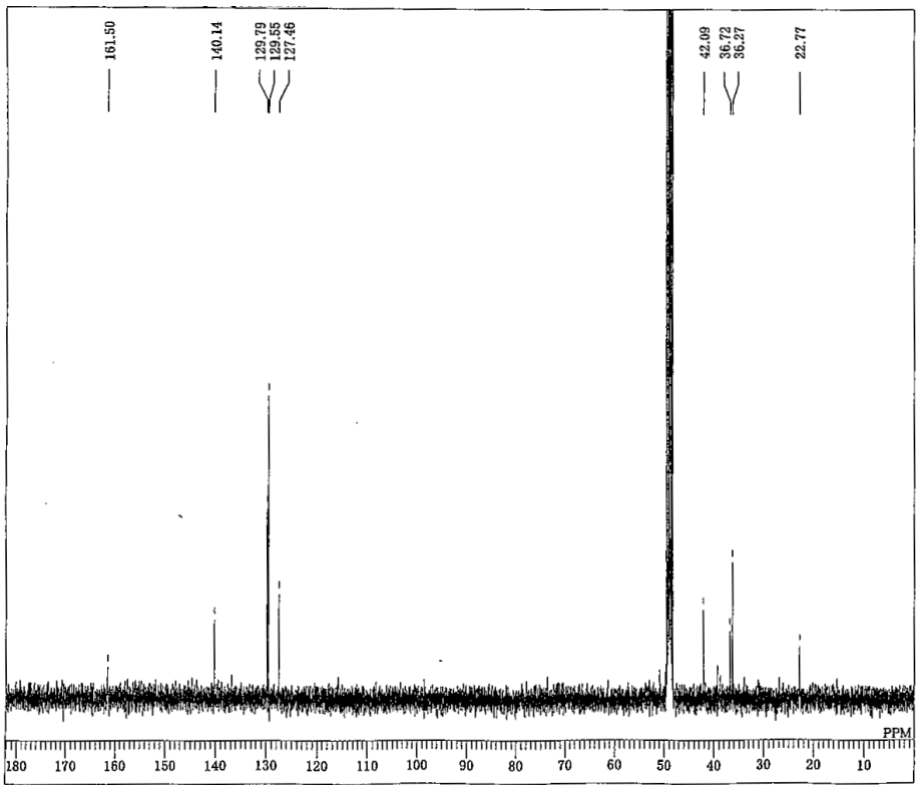


Figure 5-9.^{13}C NMR spectrum of compound **21**

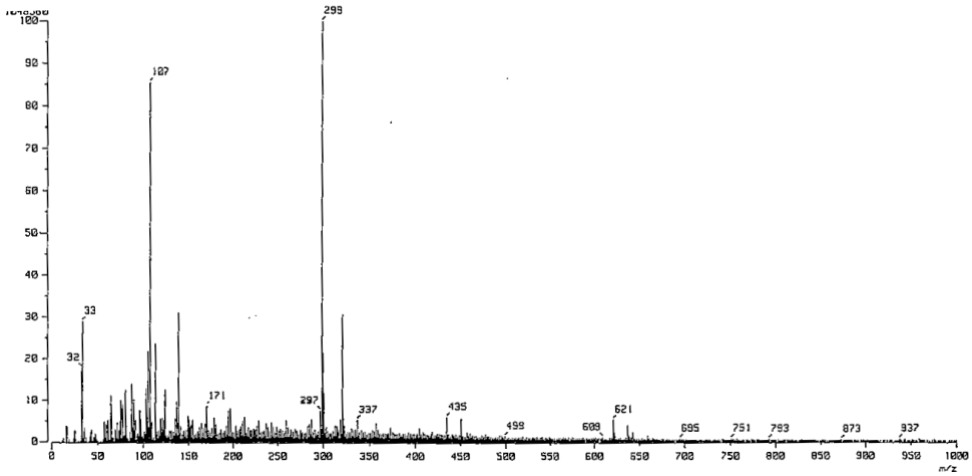


Figure 5-10. FAB-MS spectrum (positive mode) of compound **21**

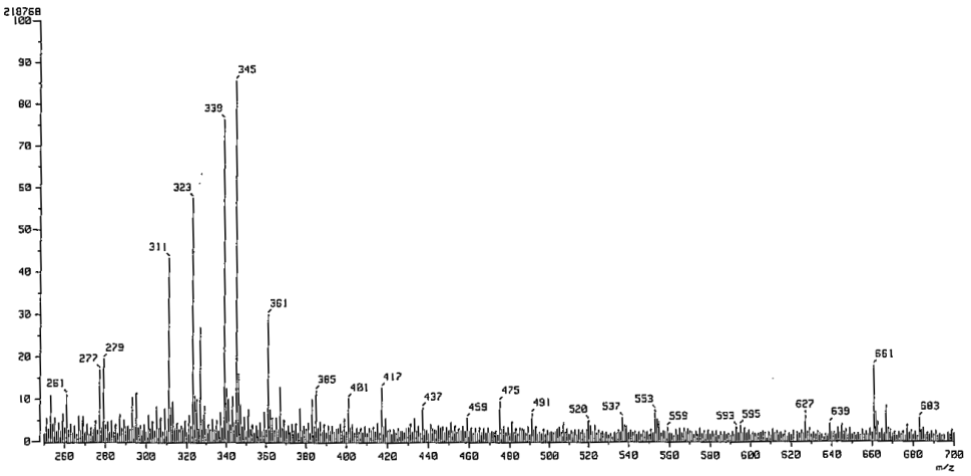


Figure 5-11. FAB-MS spectrum (negative mode) of compound **21**

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