Studies on inhibitors of enzymes involved in tumor metastasis from marine invertebrates

（海洋無脊椎動物からのがん転移関連酵素阻害物質に関する研究）

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By
Shuhei Murayama

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Introduction

1. Cancer is the Major Cause of Deaths

With the change of the living environment of human, the cause of deaths has been changing. Before 1947, tuberculosis was the 1st place of the cause of deaths, in Japan. Isolation of streptomycin made tuberculosis to be non-fatal disease, and deaths caused by tuberculosis suddenly decreased.[1] Then cerebrovasucular disease took the 1st place until 1980. From 1980, the cancer has been the 1st place of the cause of deaths in Japan, and the number increases year by year (Figure 1).[2] In 2004, over 300,000 people died of cancer. These data suggest that, with extension of the life of the human, malignant neoplasm has come to the surface. Therefore, the quest for anticancer drugs is in urgent need.

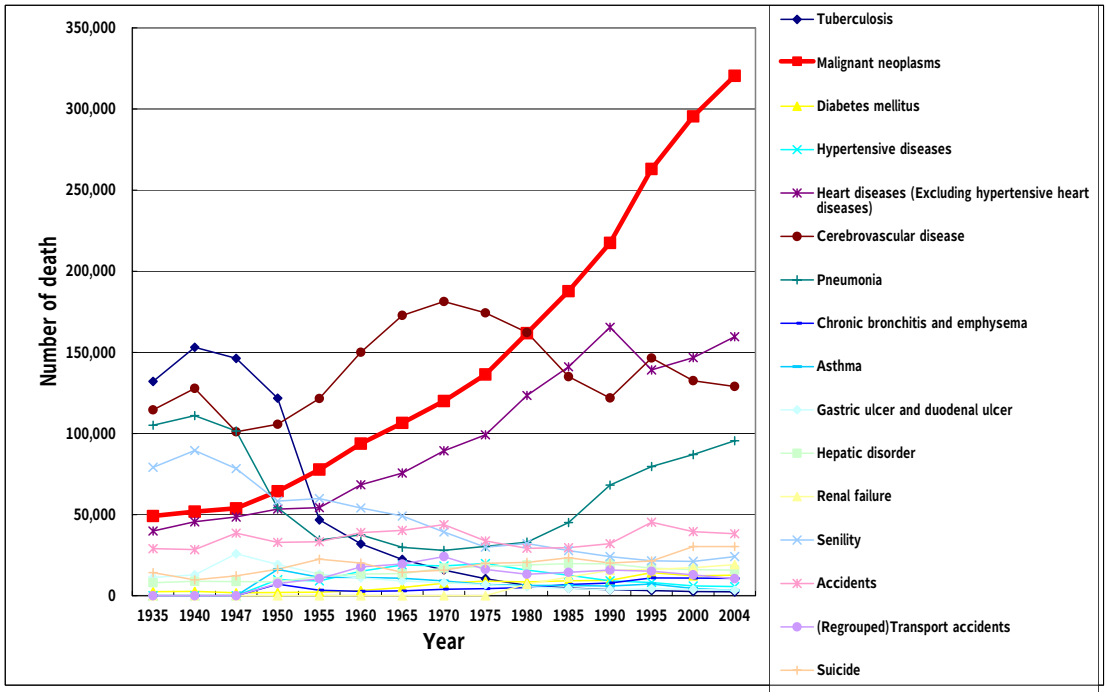


Figure 1. Change of the cause of deaths in Japan

2. Mechanism of Tumor Metastasis

Acquisition of invasion and metastatic ability is one of the main characteristic features of malignant tumor cells. The occurrence of tumor metastasis bodes poorly for the patients and lead to death in more than 90 % of the cases.[3] So tumor metastasis is the most serious problem in cancer, and considered as an important target in cancer chemotherapy. Invasive malignant tumor cells from metastatic tumors follow the steps as follows (Figure 2):[4]

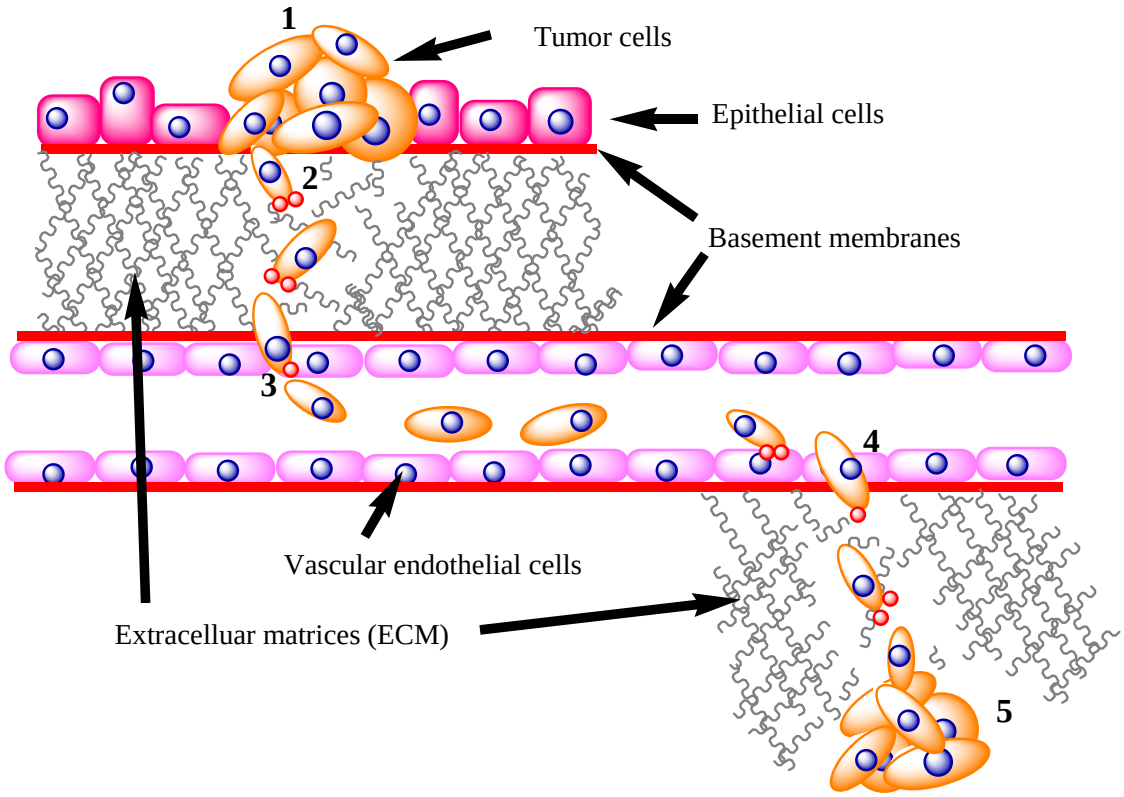


Figure 2. Processes of the tumor invasion

1. The primary tumor cells have a tendency to invade the surrounding tissue, but extracellular matrix (ECM) prevents invasion by physical attack.
2. Tumor cells produce a variety of enzymes to degrade the basement membrane and ECM to migrate into the tissue toward the blood vessels which are produced by angiogenesis.
3. After reaching the blood vessel, tumor cells produce enzymes, degrade basement membrane, and enter the blood stream. Then, cells are transported to remote sites by the blood stream.
4. Tumor cells adhere to the vascular endothelial cells and again degrade the basement membrane to escape from the vessel.
5. Migrated tumor cells restart invasion into the tissue by degrading the ECM proteins, and proliferate to form the metastatic tumors.

For these processes of tumor invasion and metastasis, degradation of adhesion molecules, basement membrane, and ECM proteins comprised the most important part. It has been revealed that the expression levels of the proteases responsible for the basement membrane and ECM degradation are closely related to the malignancy of the cancer cells. All ECM degrading proteases are considered to play different roles in malignant tumors; matrix metalloproteases (MMPs) degrade ECM proteins and serine and cysteine cathepsins support MMPs by activating them or degrading the

other components of ECM proteins. Among others cathepsin B of cysteine protease family, has been shown to possess specific physiological activities including cancer progression.[5] Cathepsin B, not only directly degrades surrounding tissues, but also activates pro-uPA, which in turn can convert plasminogen to plasmin. Plasmin is capable of degrading several components of ECM (e.g., fibrin, fibronectin, proteoglycans, and laminin) and may activate MMPs such as interstitial collagenase (MMP-1), stromelysin-1 (MMP-3), gelatinase B (MMP-9), metalloelastase (MMP-12), and collagenase-3 (MMP-13). These MMPs can degrade many different components of the ECM, including collagen I and IV, which are seldom degraded by other proteases such as MMP, gelatins, and proteoglycans (Figure 3).[6]

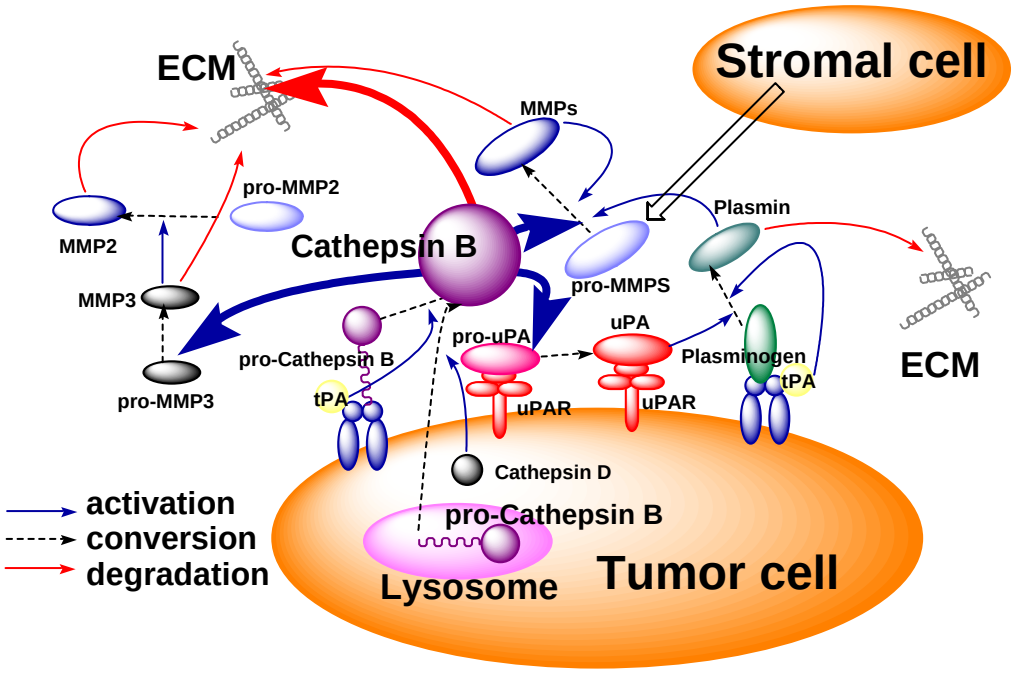


Figure 3. Enzymes related tumor invasion

Therefore, small-molecule inhibitors against cathepsin B from natural sources are regarded as potential anticancer agents, which prevent tumor metastasis or angiogenesis.[7] Representative inhibitors against cysteine protease enzymes including cathepsin B are briefly introduced in the follower sections.

3. Cysteine Protease Inhibitors from Natural Sources

A large number of small-molecule compounds were isolated from the nature, and some of them were useful for drugs like streptomycin, and also used to elucidate functions of enzymes. Many marine invertebrates are known to be a rich source of unusual small-molecule natural products, which are structurally diverse and have potent bioactivity.[8] As shown in the following part,

numbers of protease inhibitors have been reported from marine invertebrates and other sources.

3.1. Peptidyl Aldehyde

Development of peptidyl aldehydes as inhibitors of cysteine proteases is based on two independent research strategies: (1) The assumption that a tetrahedral intermediate is involved in enzymatic hydrolysis has led to investigation of the effect of carbonyl compounds on these enzymes, with the intention of developing analogs of this transition state; (2) During screening of culture filtrates of different *Streptomyces* strains, many peptidyl aldehydes were isolated as cysteine protease inhibitors. NMR studies on the interaction between these compounds and cysteine protease, showed the mechanism of the inhibition of cysteine proteases by peptidyl aldehydes (Figure 4). The aldehyde is essential for inhibition. Many dipeptidyl and tripeptidyl aldehydes have been isolated and synthesized with the aim of improving selectivity.

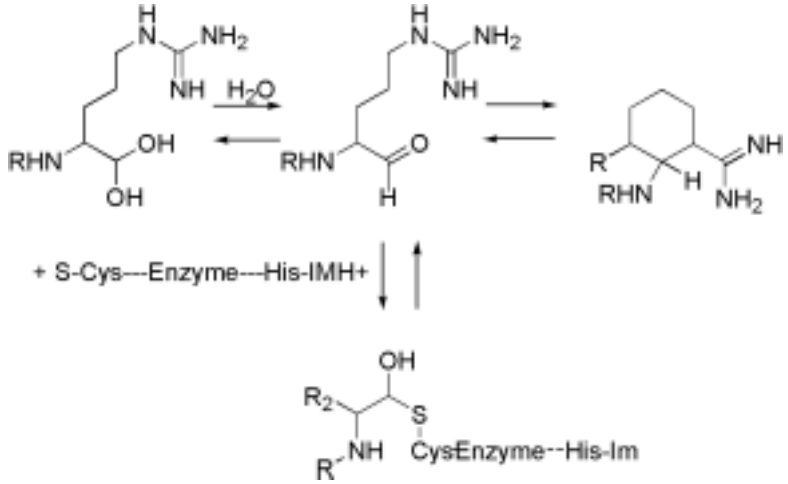
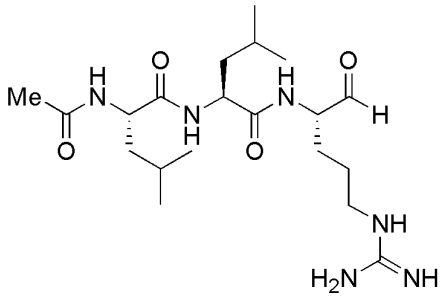


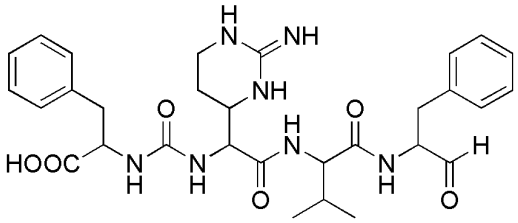
Figure 4. Inhibition of cysteine proteases by peptidyl aldehydes

Leupeptin (**1**) isolated from *Streptomyces* sp, is a representative peptidyl aldehyde inhibitor of cysteine proteases.[10] Leupeptin suppressed the formation of metastatic foci of Yoshida ascites hepatoma AH100B cells.[11] Leupeptin inhibits cathepsin B with an IC_{50} value of 0.44 μg/mL.



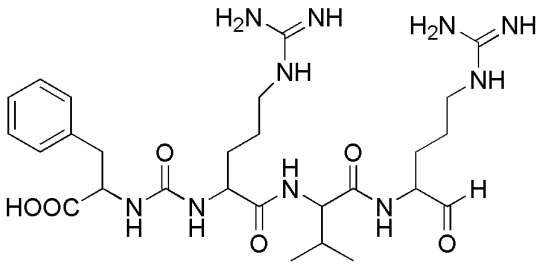
Leupeptin (**1**)

A microbial product chymostatin (**2**), papain and cathepsin B inhibitor, was found from *Streptomyces* sp. Chymostatin inhibits papain and cathepsin B with an IC_{50} value of 7.5 μg/mL and 2.6 μg/mL.[12]



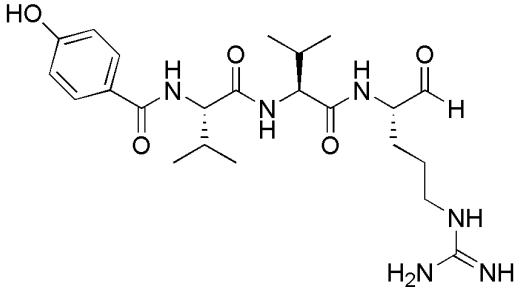
Chymostatin (**2**)

Antipain (**3**), protease inhibitory peptidyl aldehyde, was found from *Actinomycetes violascens*. Antipain inhibits papain with an IC_{50} value of 0.16 μg/mL.[13]



Antipain (**3**)

Not only from terrestrial bacteria, bot also from marine invertebrate were isolated peptidyl aldehyde inhibitors possessing structural features related to those of leupeptin. An example is tokaramide A (**4**), isolated from the marine sponge *Theonella* aff. *mirabilis.* Tokaramide A inhibits cathepsin B with an IC_{50} value of 29.0 ng/mL.[14]



Tokaramide A (**4**)

3.2. Peptidyl Epoxysuccinyl Derivatives

Since E-64 were isolated, systematic studies were carried out to investigate the role of the

different structural components of the inhibitor in enzyme inhibition and the *trans*-L-(S,S)-epoxysuccinic acid was discovered to be the reactive group essential for inhibition. Many peptidyl epoxysuccinyl derivatives were found from natural sources or synthesized, NMR spectroscopic investigations showed that the active site thiolate attacks at C-3 of the oxirane ring (Figure 5), and the epoxide ring is opened with inversion of the configuration at C-3.

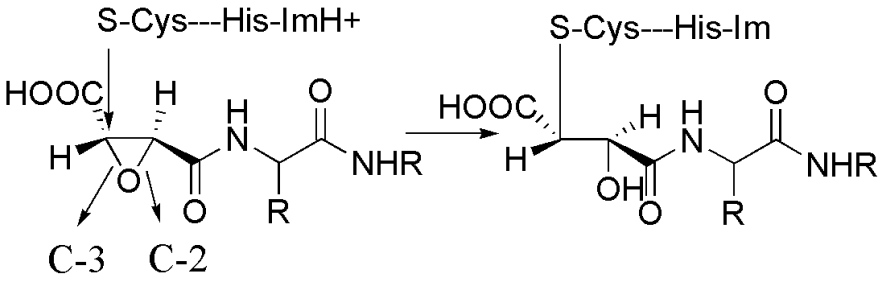
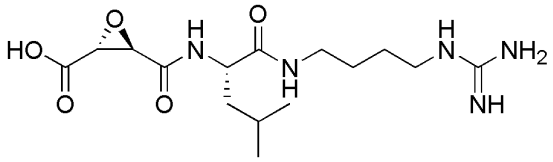


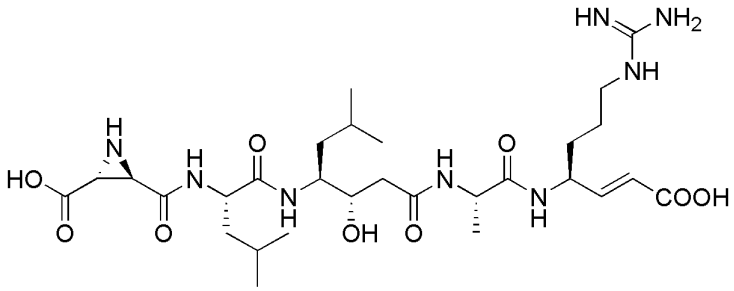
Figure 5. Inhibition by epoxysuccinyl derivatives

E-64 (**5**) is an epoxysuccinyl peptide type of inhibitor isolated from the fungus *Aspergillus japonicus*.[15] E-64 blocked the invasion of H-59 carcinoma cells into a reconstituted basement membrane (Matrigel), and decreased the number of spontaneous metastasis in M5076 tumor bearing mice.[16] E-64 also inhibits cathepsin B with an IC_{50} value of 4.9 ng/mL.



E-64 (**5**)

Miraziridine A (**6**) is a distantly related aziridine dicarboxylate derivative. Aziridine dicarboxylates inhibit cysteine proteases as do epoxysuccinyl derivatives. Miraziridin A was isolated from the sponge *Theonella* aff. *mirabilis*, and showed inhibition against cathepsin B with an IC_{50} value of 1.4 μg/mL.[17]

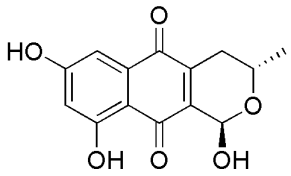


Miraziridine A (**6**)

3.3. Non-peptide Inhibitors

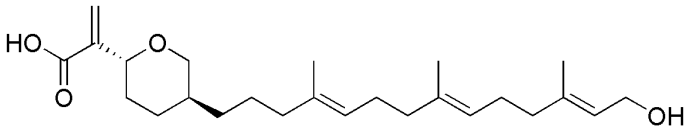
Peptide structures are not always required for cysteine proteases inhibitory activity; various other structures are isolated as cysteine proteases inhibitors from natural sources.

The naphthoquinone thysanone (**7**), isolated from fungus *Thysanophora penicilloides*, is a novel human rhinovirus 3C-protease inhibitor. Thysanone shows inhibition against HRV 3C protease with an IC_{50} value of 13 μg/mL.[18]

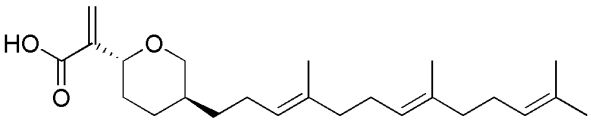


Thysanone (**7**)

Non-peptide cysteine protease inhibitors have also been discovered from marine invertebrates. Terpenoids, barangcadoic acid A (**8**) and rehopaloic acids A (**9**) to G were isolated from Indonesian marine sponge *Hippospongia* sp. as human Ras-converting enzyme (hRCE1) inhibitors. Barangcadoic acid A and rhopaloic acid A to E all had IC_{50} values of ~ 10 μg/mL against RCE-proteases.[19]

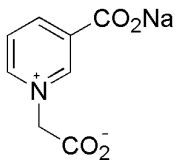


Barangcadoic acid A (**8**)



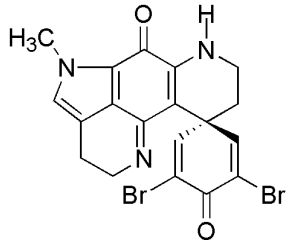
Rehopaloic acids A (**9**)

1-Carboxymethylnicotinic acid (**10**) was described in 1991 as synthetic product and isolated in 1998 from a marine sponge *Anthosigmella* cf. *raromicrosclera* as a cysteine protease inhibitor. 1-Carboxymethylnicotinic acid inhibits papain with an IC_{50} value of 80 μg/mL.[20]



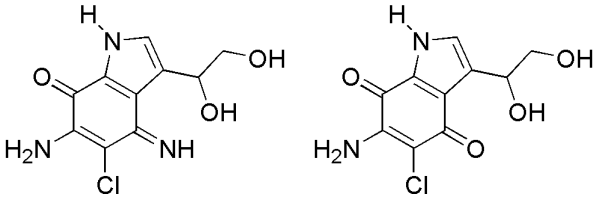
1-Carboxymethylnicotinic acid (**10**)

Discorhabdin P (**11**) was isolated from deep-water Caribbean sponge of the genus *Batzella*. Discorhabdin P inhibits the phosphatase activity of calcineurin and peptidase activity of CPP32 with IC_{50} values of 0.55 and 0.37 μg/mL. Discorhabdin P also showed *in vitro* cytotoxicity against P-388 and A-549 cell lines with IC_{50} values of 0.025 and 0.41 μg/mL.[21]



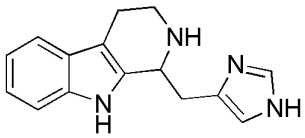
Discorhabdin P (**11**)

Secobatzellin A (**12**) was isolated from deep-water marine sponge of the genus *Batzella*. Secobatzelin A inhibits CPP32 with IC_{50} value of 0.02 μg/mL, but its analog secobatzelin B (**13**) dose not inhibit CPP32.[22] Both compounds showed *in vitro* cytotoxicity against P-388 and A-514 cell lines.



Secobatzellin A (**12**) Secobatzellin B (**13**)

Haplosclerodamine (**14**), a tryptamin derived alkaloid, was isolated from a sponge of the order *Haplosclerida* as an inhibitor of cysteine proteases cathepsin K. Haploscleridamine inhibits cathepsin K with an IC_{50} value of 6.58 μg/mL.[23]



Haplosclerodamine (**14**)

These discoveries suggest that the marine invertebrates are a rich source of the small-molecule cysteine protease inhibitors.

In this study, a search for inhibitors against cathepsin B from Japanese marine invertebrate was carried out.

Chapter I deals with the screening of the extracts of marine invertebrates for the inhibitory activity against cathepsin B. A total of 606 extracts prepared from 303 marine invertebrates, containing sponge, tunicate, coelenterate, mollusk, and bryzoan, were tested, and 25.1 % of organic and 15.5 % of aqueous extracts exhibited activity.

Chapter II describes the isolation, structure elucidation, and biological activity of a novel pteridine type cathepsin B inhibitor asteropterin from the marine sponge *Asteropus simplex*. The structure of asteropterin was determined as a conjugate of lumazine and *N*-methylhistamine. This compound inhibits cathepsin B with an IC_{50} value of 1.4 μg/mL.

Chapter III describes the isolation, structure elucidation, and biological activity of two new sterol dimers, shishicrellastatins A and B from the marine sponge *Crella (Yvesia) spinulata*. The isolation was achieved by HPLC in the presence of a high concentration of sodium perchlorate. They are sterol dimers connected through the side chains. Shishicrellastatins A and B inhibit cathepsin B with IC_{50} value of 7.0 μg/mL and 7.8 μg/mL, respectively.

Chapter IV deals with the preparation of a cathepsin B inhibitory substance mostly composed of inorganic material. The substance contains 26 % carbon and includes Mg, Na, and Ni.

Chapter V describes the isolation, structure elucidation, and biological activity of a compound from an unidentified marine sponge. The compound was related to shulzeines, sponge metabolites.

Chapter I
Screening of Japanese Marine Invertebrates for Inhibitory Activity against Cathepsin B

1. Introduction

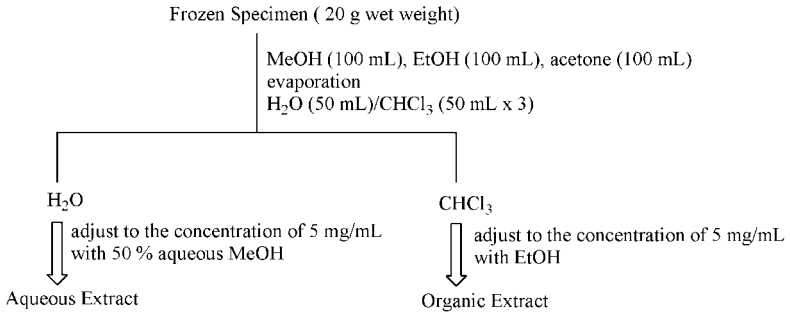
Over 20,000 compounds have been found from marine organisms. It suggests that marine samples are rich source of new compounds. Most marine natural products were isolated from organisms inhabiting shallow-water. However, more than 80 % of the sea on the earth are deeper than 100 m. To date only about 400 compounds have been discovered from deep-water samples.[24] Therefore, invertebrates collected from deep-water are especially good source of new small molecular compounds. Even though, deep-water is a hard place to collect samples, collecting there should be rewarding.

Against such a back ground, the extracts of 303 marine invertebrates including 90 deep-water samples, were screened for their inhibitory activities against cathepsin B.

2. Results and Discussion

2.1. Preparation of the Screening Samples

A total of 303 marine invertebrates, consisting of 203 sponges, 49 coelenterates, 5 mollusks, 3 bryozoans, and 43 tunicates were collected along the Japanese coasts during the period of 2002 - 2004. Among these samples, 72 sponges and 18 coelenterates were collected from the deep-water. Specimens were homogenized, extracted, combined, and partitioned between H_2O and $CHCl_3$, to afford aqueous and organic extracts, respectively (Scheme 1-1).



Scheme 1-1: Preparation of the screening samples

2.2. Results and Discussion

Both aqueous and organic extracts were subjected to the screening for inhibitory activity against cathepsin B. The activity was measured in duplicate at a concentration of 50 μg/mL. The results were categorized into three grades; double plus (++) represents more than 50 % inhibition, one plus (+) represents inhibition between 30-50 %, (-) represents inhibition lower than 30 % inhibitory activity. Results of the screening of inhibitory activity against cathepsin B is summarized in Table 1-1.

		Organic Extracts			Aqueous Extracts		
	Samples	++	+	++&+	++	+	++&+
Sponge	203[a]	15	42	57	15	19	34
(%)		7.4[b]	20.7	28.1	7.4	9.4	16.7
from shallow-water	131	11	21	32	11	14	25
(%)		8.4	16.0	24.4	8.4	10.7	19.1
from deep-water	72	4	21	25	4	5	9
(%)		5.6	29.2	34.7	5.6	6.9	12.5
Coelenterate	49	0	11	11	2	5	7
(%)		0	22.4	22.4	4.1	10.2	14.3
from shallow-water	31	0	11	11	1	4	5
(%)		0	35.5	35.5	3.2	12.9	16.1
from deep-water	18	0	0	0	1	1	2
(%)		0	0	0	5.6	5.6	11.1
Mollusk	5	0	0	0	0	0	0
(%)		0	0	0	0	0	0
Bryzoan	3	0	0	0	0	0	0
(%)		0	0	0	0	0	0
Tunicate	43	5	3	8	2	4	6
(%)		11.6	7.0	18.6	4.7	9.3	14.0
Total	303	20	56	76	19	28	47
(%)		6.6	18.5	25.1	6.3	9.2	15.5

Table 1-1. Result of the screening for cathepsin B inhibition

[a] number of samples [b] percentage

As shown in Tables 1-1, sponges, coelenterates and tunicates frequently exhibited potent inhibitory activity against cathepsin B. Organic extracts of sponges from deep-water showed cathepsin B inhibition activity more frequently than those from shallow-water. On the contrary,

organic extracts of coelenterates from shallow-water showed activity more frequently than those from deep-water. In this screening, mollusks and bryzoans did not show any inhibitory activity against cathepsin B.

3. Experimental Section

3.1. Collection of Marine Invertebrates

A total of 303 samples consisted of 203 sponges, 49 coelenterates, 5 mollusks, 3 bryozoans, and 43 tunicates were collected during 2002-2004. Shallow-water samples were from Shishi Island, Yaku Island, Shikine Island, Tokara Islands, and Hachijyo Island and collected by SCUBA. Deep-water samples were collected from the gulf of Suruga, Amami Ōshima, and Ōshima Shinsone by dredge. Samples were frozen immediately after collection and kept at - 20 ˚C until extraction.

3.2. Preparation of Test Solutions

Frozen samples (20 g wet weight) were homogenized and extracted with MeOH, EtOH, and acetone (100 mL, each). The combined extracts were concentrated and dissolved in 50 mL of H_2O and extracted with $CHCl_3$ (50 mL x 3). Each organic and aqueous layer was dried and adjusted to a concentration of 5 mg/mL with EtOH and 50 % aqueous MeOH, respectively.

3.3. Cathepsin B Inhibition Assay

Cathepsin B inhibition assay was carried out by following the modified method of Hiwasa *et al.*[25] Test samples (2 μL of 5 mg/mL solution) were added to the wells of 96 well microtiter plates; each well contained 100 μL of the buffer (0.05 M MES pH 6.0, 0.01% DTT, and 0.1 % Brij-35). Stock solution of bovine cathepsin B (Sigma Chemical Company Lot 61K7690; 1 unit/mL in 0.05 M MES pH 6.0 and 0.1 % Brij-35) was diluted by 100 times with the buffer. To each well, 50 μL of the protease solution and 50 μL of 25 μM substrate solution (Z-Arg-Arg-MCA; Peptide Institute, Inc. in DMSO) were added and incubated at 37 ˚C for 1 h. The fluorescence of the free AMC was measured on Molecular Devices SPECTRA MAX GEMINI fluorescence spectrometer with the excitation at 345 nm and the emission at 440 nm. The positive control was prepared by

addition of the buffer without enzyme, instead of the enzyme solution. The negative control was prepared by addition of MeOH instead of test solution. The fluorescence of the negative control was defined as 0 % inhibition and that of the positive control was defined as 100 % inhibition. The percent inhibition was calculated from the fluorescence of the wells containing test samples (Formula 1-1). All assays were performed in duplicate.

$$\text{inhibition (\%)} = \frac{[\text{negative control}] - [\text{sample}]}{[\text{negative control}] - [\text{positive control}]} \times 100$$

[ ] = fluorescence

Formula 1-1: Percent inhibition

Chapter II
Asteropterin, an Inhibitor of Cathepsin B, from the Marine Sponge *Asteropus simplex*

1.Introduction

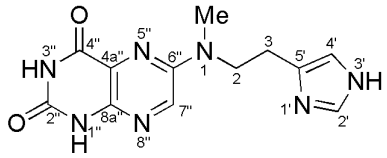
The extract of the marine sponge *Asteropus simplex* collected off Shikine Island (Figure 2-1) exhibited potent activity against cathepsin B in the screening. Extraction and fractionation of the extract following the inhibitory activity afforded a new pteridine type compound named asteropterin (**2-1**). This chapter deals with the isolation, structure elucidation, and the enzyme inhibitory activity of the new compound.

Figure 2-1. *Asteropus simplex*

2.Results and Discussion

2.1. Extraction and Isolation

The sponge (2 kg, wet weight) was extracted with MeOH, EtOH, and acetone. The extracts were combined, concentrated, and partitioned between MeOH-H_2O (9:1) and *n*-hexane. The aqueous MeOH fraction was separated by ODS flash column chromatography (MeOH-H_2O) and gel filtration. The active fractions were combined and repeatedly purified by reversed-phase HPLC to furnish 5.6 mg of asteropterin (**2-1**) as an yellowish powder (Figure 2-2).



2-1: Asteropterin

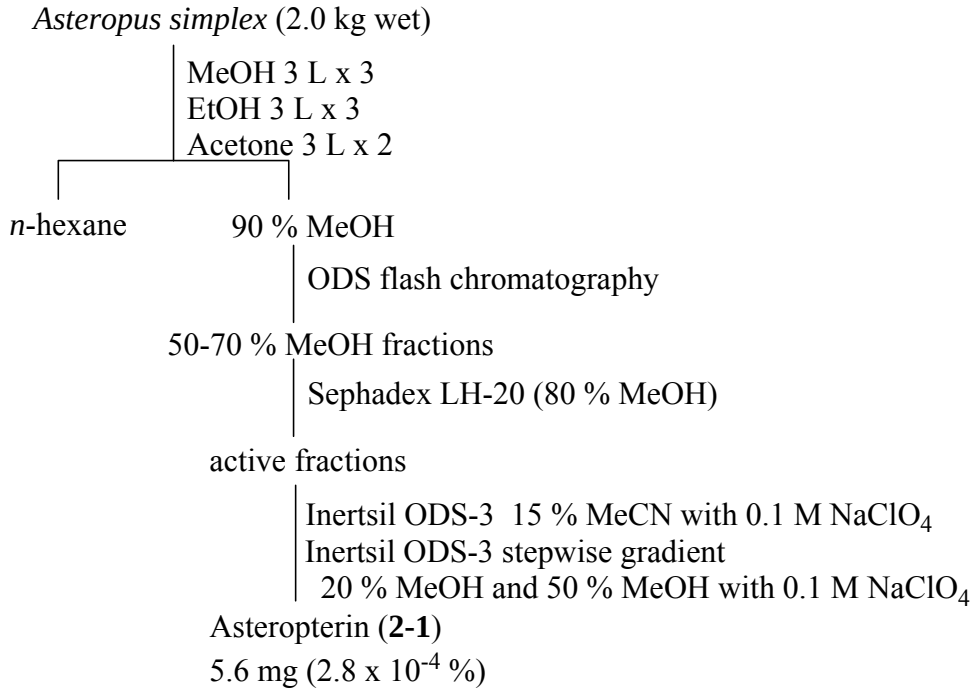


Figure 2-2. Isolation Scheme of Asteropterin.

2.2. Structure Elucidation

Asteropterin (**2-1**) had a molecular formula of $C_{12}H_{13}N_7O_2$ as determined by HR-ESIMS [*m*/*z* 288.12060 $(M + H)^+$, δ-0.30 mmu] and NMR data. UV spectrum in MeOH showed absorptions at λ_{max} 279 (ε 1360) and 416 nm (ε 290).

Analysis of the ^1H NMR spectrum of asteropterin (**2-1**) in conjunction with the HSQC data indicated the presence of two methylenes [δ_H/δ_C 2.92/22.4 (C-3) and 3.82/48.7 (C-2)], an *N*-methyl (3.06/36.0), three hydrogen-bearing sp^2 carbons [8.70/135.8 (C-2'), 8.30/134.0 (C-7"), and 7.35/116.6 (C-4')], and two NH [δ11.44 (H-3") and 11.48 (H-1")]. Six non-hydrogenated sp^2 carbon signals [δ122.7 (C-4a"), 131.4 (C-5"), 140.7 (C-8a"), 149.5 (C-2"), 151.0 (C-6"), and 161.6 (C-4")] were observed in the ^{13}C NMR spectrum (Table 2-1).

The COSY spectrum showed that the two methylene signals were mutually coupled and two aromatic protons (H-2' and H-4') were long-range coupled. The latter protons were attributed to a 5-substituted imidazole ring on the basis of $^1J_{CH}$ values [216 Hz (C-2') and 199 Hz (C-4')] and carbon chemical shift values.[26] An HMBC cross peak between the *N*-methyl protons and C-2 showed that C-2 was attached to a methylamino group. HMBC cross peaks from H_2-3 to C-4' and C-5' revealed that C-3 was connected to the imidazole ring at C-5'. From these data partial

structure **2-a** was deduced (Figure 2-3).

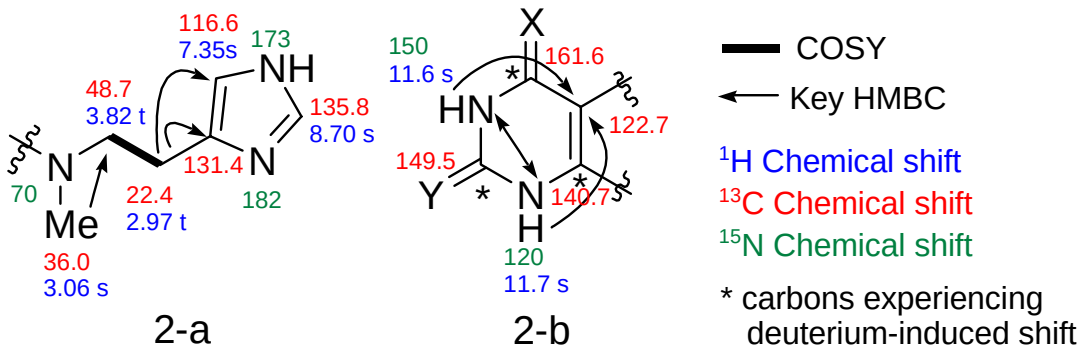


Figure 2-3. Partial Structures of Asteropterin

The remaining portion had a composition of $C_6H_3N_4O_2$ and contained six sp^2 carbons, only one of which was hydrogen-bearing and two NH. Deuterium-induced carbon chemical shifts were observed for C-2", C-4", and C-8a" indicating that these three carbons were adjacent to one or two of the NH group(s).[27] HMBC cross peaks were observed from both 1"-NH and 3"-NH to C-4a". Because C-4a" did not experience a deuterium-induced shift, these correlations were assigned as three-bond couplings, i.e. there must be a carbon atom between C-4a" and each NH group. Therefore, the remaining carbon that experienced a deuterium exchange shift should be placed between the two hydrogen-bearing nitrogen atoms to form partial structure **2-b** (Figure 2-3b), in which substituent X and Y were both assigned as an oxygen atom considering the carbon chemical shifts, leaving a unit with a composition of C_2HN_2 to link between partial structures **2-a** and **2-b**.

$^1J_{CH}$ value of 174 Hz for CH-7" (δ_H/δ_C 8.30/134.0) and the carbon chemical shift of C-7" suggested that this carbon was part of a six-membered ring and attached to a nitrogen atom.[26] An HMBC cross peak between H-7" and C-8a" confined the position of C-7" to be within two bonds from C-8a". HMBC cross peaks, *N*-CH_3/C-6" and H_2-2/C-6", indicated that partial structure **2-a** was connected to C-6". An HMBC cross peak between H-7" and C-6" and a ROESY cross peak between *N*-CH_3 and H-7" permitted to link C-6" and C-7". In order to satisfy these requirements, asteropterin (**2-1**) should be represented either by a pyridazine or pyrazine ring system (partial structures **2-c** and **2-d**, respectively) (Figure 2-4). The ^{15}N-HMBC spectrum of asteropterin (**2-1**) displayed a correlation of H-7" to a nitrogen at 300 ppm, which suggested the presence of a pyrazine ring rather than a pyridazine ring (δ_N 335 for pyrazine and δ_N 397 for pyridazine).[27-30] Therefore, asteropterin (**2-1**) has a lumazine skeleton. The NMR data of lumazine, xanthopterin, and isoxanthopterin supported this idea.[31]

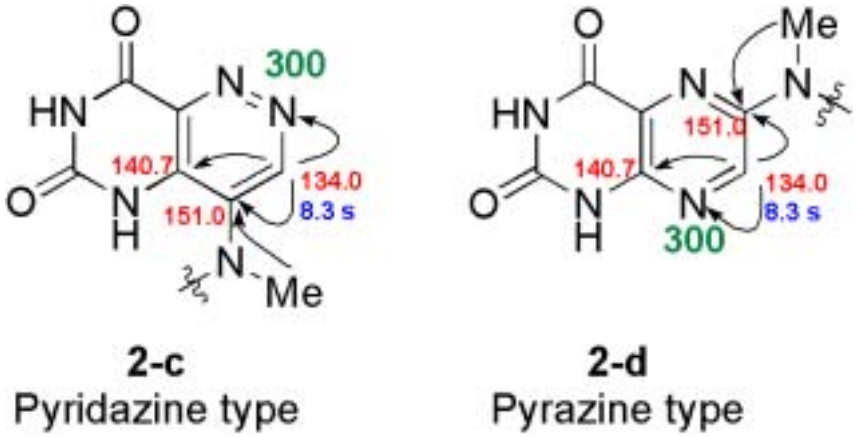
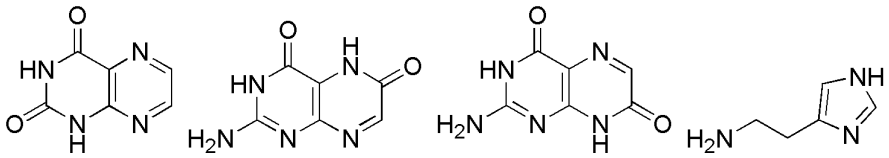


Figure 2-4. Proposal structures of asteropterin

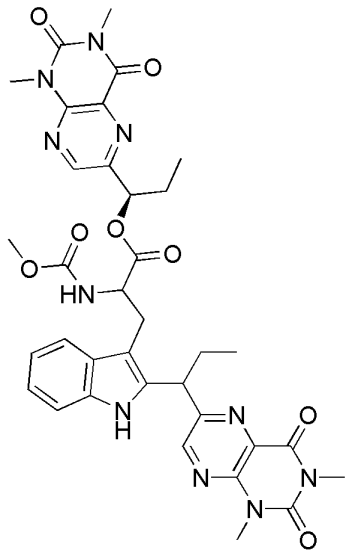
2.3. Biological Activity

Asteropterin (**2-1**) inhibited cathepsin B with an IC_{50} value of 1.4 μg/mL. We examined the activity of lumazine (**2-2**), xanthopterin (**2-3**), isoxanthopterin (**2-4**), histamine (**2-5**), and a mixture of lumazine and histamine (1:1), all of which were inactive against cathepsin B at concentration of 50 μg/mL. It suggests that linkage of these lumazine and histamine units is regard to display inhibition.



2-2:lumazine, **2-3:**xanthopterin, **2-4:**isoxanthopterin, **2-5:**histamine

Only a limited number of pteridines have been reported from sponges.[32-35] Most complex examples among them are pseudoanchynazines (**2-6**) from *Clathria* sp.. Several pteridine derivatives including lumazine and xanthopterin are known to inhibit xanthine oxidase.[36] Asteropterin (**2-1**) is a unique molecule with a combination of lumazine and *N*-methyl histamine with cathepsin B inhibitory activity.



2-6:pseudoanchynazine A

#	δC	δN	δH mult	COSY	HMBC
1		70			
2	48.7		3.82 t	3	3,5',6",1N-Me
3	22.4		2.92 t	2	1,2,1',4',5'
1N-Me	36.0		3.06 s		1,2,6"
1'		182	-		
2'	135.8		8.70 s	4’	1',3',4',5'
3'		173			
4'	116.6		7.35 s	2’	1',2',3',5'
5'	131.4				
1"		120	11.48 s		3", 4a"
2"	149.5				
3"		150	11.44 s		1", 4a"
4"	161.6				
4a"	122.7				
5"		-[b]			
6"	151.0				
7"	134.0		8.30s		4"[c],4a"[c],6", 8",8a"
8"		300			
8a"	140.7				

[a]measured in DMSO-d_6

[b]not observed

[c]very weak cross peak

Table 2-1. NMR Data for Asteropterin(**2-1**)[a]

3. Experimental Section

3.1 General Procedures

UV spectrum was recorded on a Hitachi 330 spectrophotometer. NMR spectra were recorded on a JEOL delta 600 NMR spectrometer at 600 MHz for ^{1}H, 150 MHz for ^{13}C, and 150 MHz for ^{15}N. ^{1}H and ^{13}C chemical shifts were referenced to the solvent peak (DMSO-d_6) at d 2.50 and 39.51 ppm respectively. ^{15}N chemical shift was referenced by calculated value of the solvent. Standard parameters were used for the 2D NMR spectra, which include gradient COSY, HSQC, HMBC. (+)-HRESI mass spectra were measured on a JEOL JMS-T100LC time-of-flight mass spectrometer. Fluorescence for enzyme inhibition assay was determined with a Molecular Devices SPECTRA MAX GEMINI fluorescence spectrometer. Reverse phase HPLC was performed on Inertsil ODS-3 (10-mm i.. x 250-mm) connected to a Shimadzu SPD-10AVP UV detector and two Shimadzu LC-10ADVP pumps. All solvent used for HPLC, UV, and MS were WAKO JIS special grade, and H_2O used was Millipore Elix 10. Sodium perchlorate used for HPLC was WAKO JIS special grade.

3.2. Animal Material

Sponge samples were collected using SCUBA at a depth of 10-15 m off Shikine Island, 200 km south of Tokyo (34º 19' N; 139º 11' E) in 2001. The specimens were frozen after collection and preserved at -20 ºC until extraction. The sponge was identified as *Asteropus simplex* (order Astrophorida, class Geodiidae). A voucher specimen was deposited at the Zoological Museum, University of Amsterdam, (ZMA POR 16718).

3.3 Cathepsin B Inhibitory Assay

Inhibitory activity against cathepsin B was determined essentially according to the method described in Chapter I. The IC_{50} values were determined by plotting the percent inhibitions obtained for each sample concentration on semi logarithmic graph paper.

3.4. Extraction and Isolation

The sponge (2 kg, wet weight) was extracted with MeOH (3 L x 3), EtOH (3 L x 3), and acetone (3 L x 2). The combined extracts were concentrated and partitioned between 90% MeOH and *n*-hexane. The aqueous MeOH fraction was separated by ODS flash column chromatography using stepwise elution of aqueous MeOH (0 - 100%). The factions eluted with 50-70% MeOH were combined and further separated by gel filtration on Sephadex LH-20 column with 80%

MeOH/20% H_2O. The active fractions were further combined and purified by reversed-phase HPLC on an Inertsil ODS-3 column with 15% MeCN/85% H_2O containing 0.1 M $NaClO_4$. The active fraction was purified by stepwise gradient HPLC on an Inertsil ODS-3 column with 20% MeOH/80% H_2O and 50% MeOH/50% H_2O containing 0.1 M $NaClO_4$ to afford asteropterin (**2-1:** 5.6 mg, 2.8 x 10^{-4} % yield based on weight).

Asteropterin(2-1): yellowish powder; UV (MeOH) λ_{max} 279 (ε 1360) and 416 nm (ε 290); ^{1}H, ^{13}C and ^{15}N NMRdata, see Table 2-1; HR-ESIMS *m/z* 288.12060 (calcd for $C_{12}H_{14}N_7O_2$ 288.12090).

4. Supportiong Information

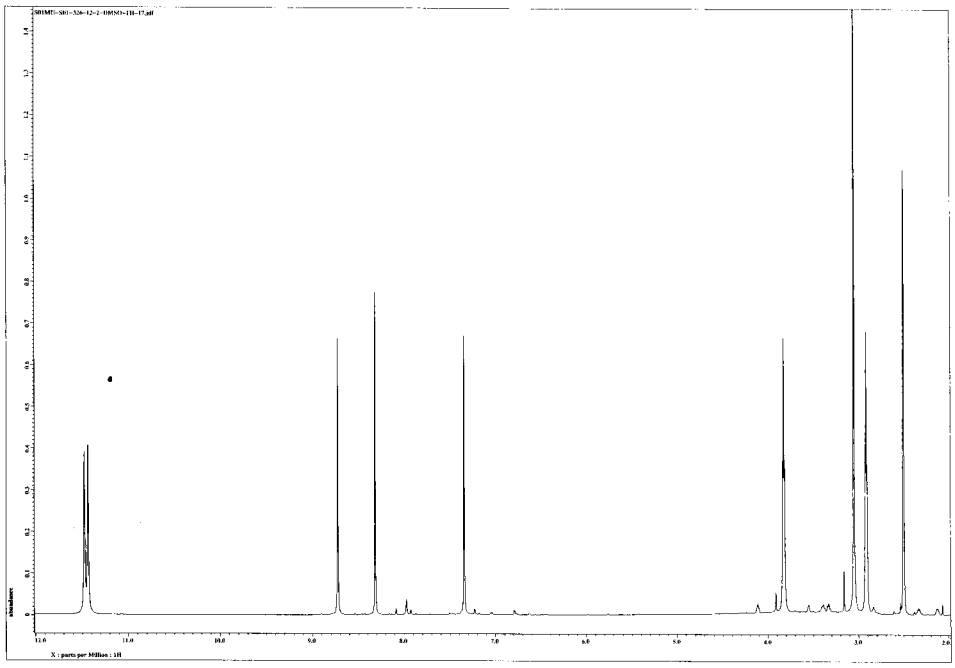


Figure S2-1. ^{1}H NMR spectrum of asteropterin in DMSO-d_6

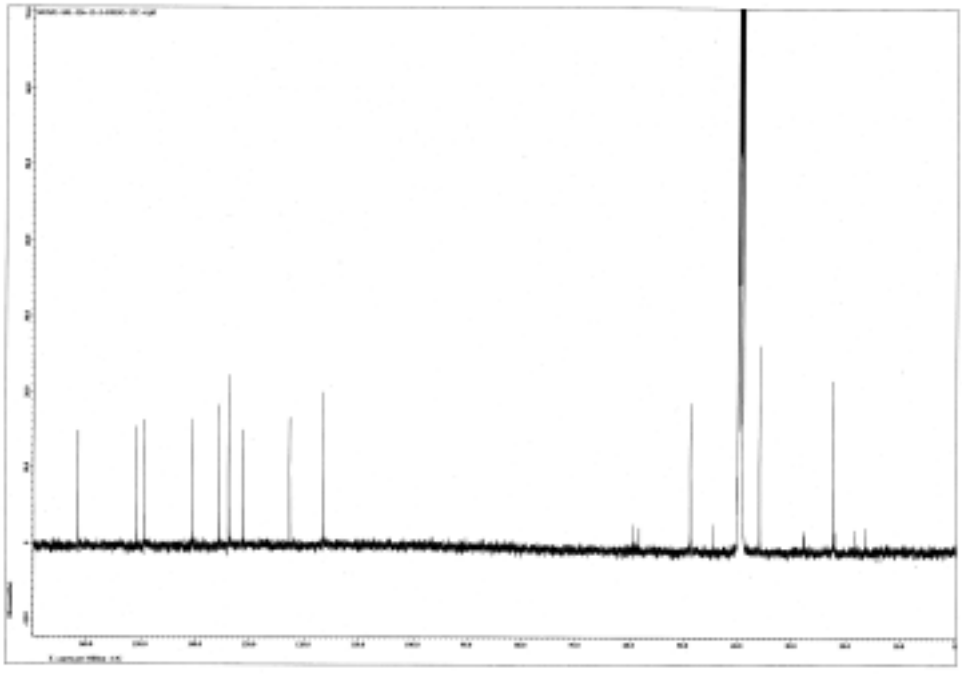


Figure S2-2. ^{13}C NMR spectrum of asteropterin in DMSO-d_6

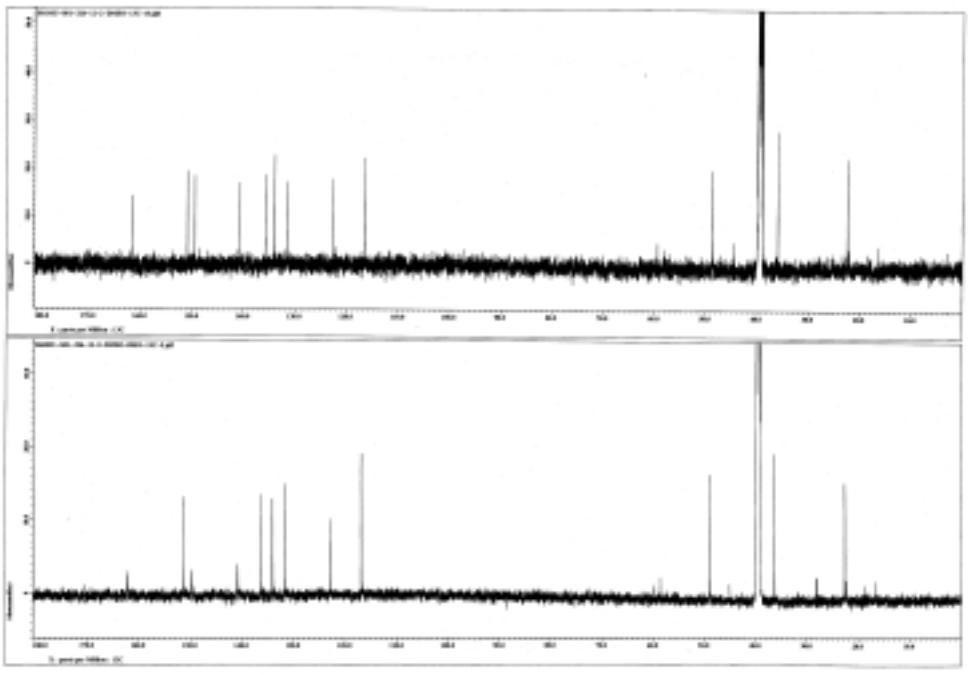


Figure S2-3. ^{13}C NMR spectra of asteropterin in DMSO-d_6 and in DMSO-d_6 with DOH

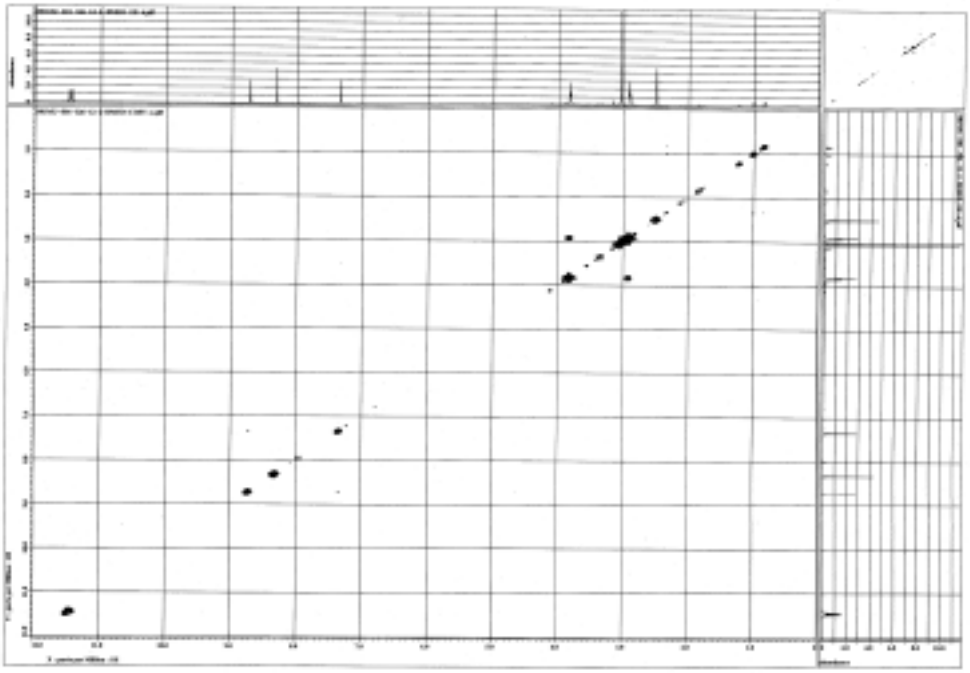


Figure S2-4. COSY spectrum of asteropterin in DMSO-d_6

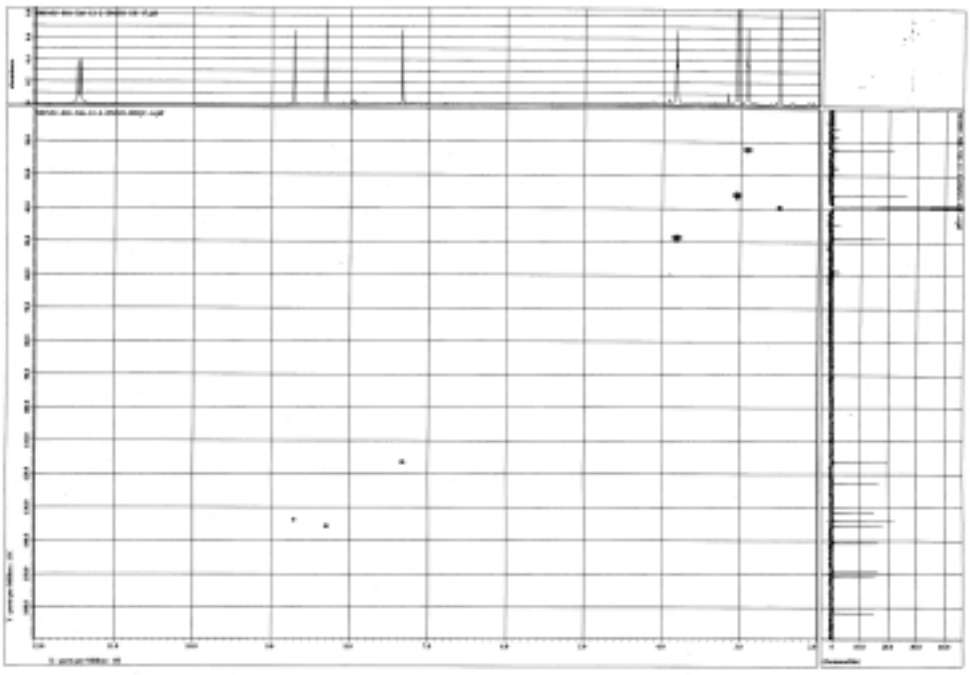


Figure S2-5. HSQC spectrum of asteropterin in DMSO-d_6

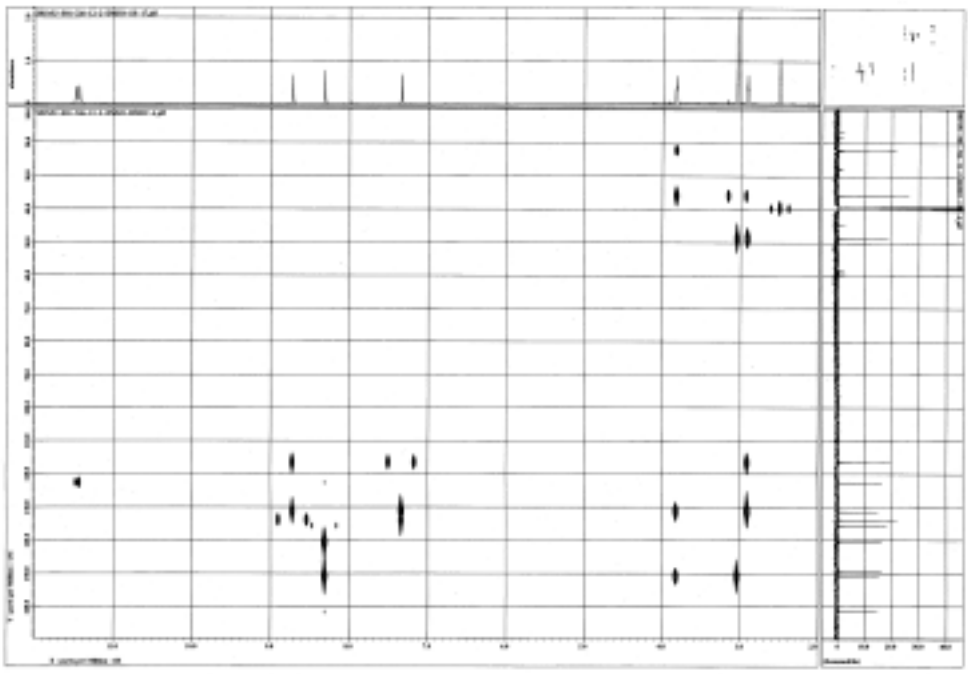


Figure S2-6. HMBC spectrum of asteropterin in DMSO-d_6

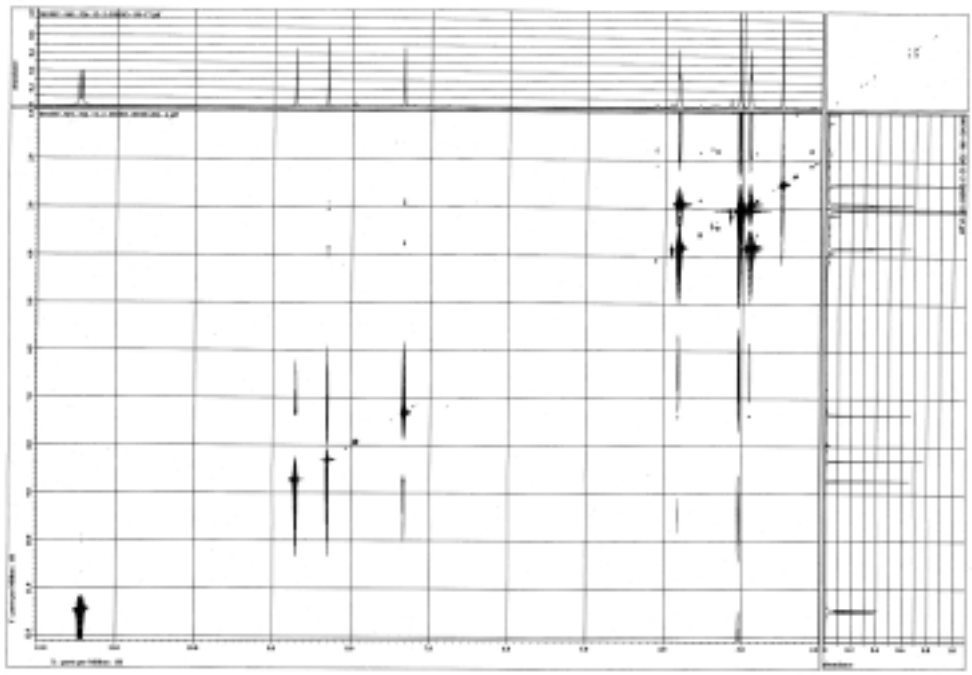


Figure S2-7. TOCSY spectrum of asteropterin in DMSO-d_6

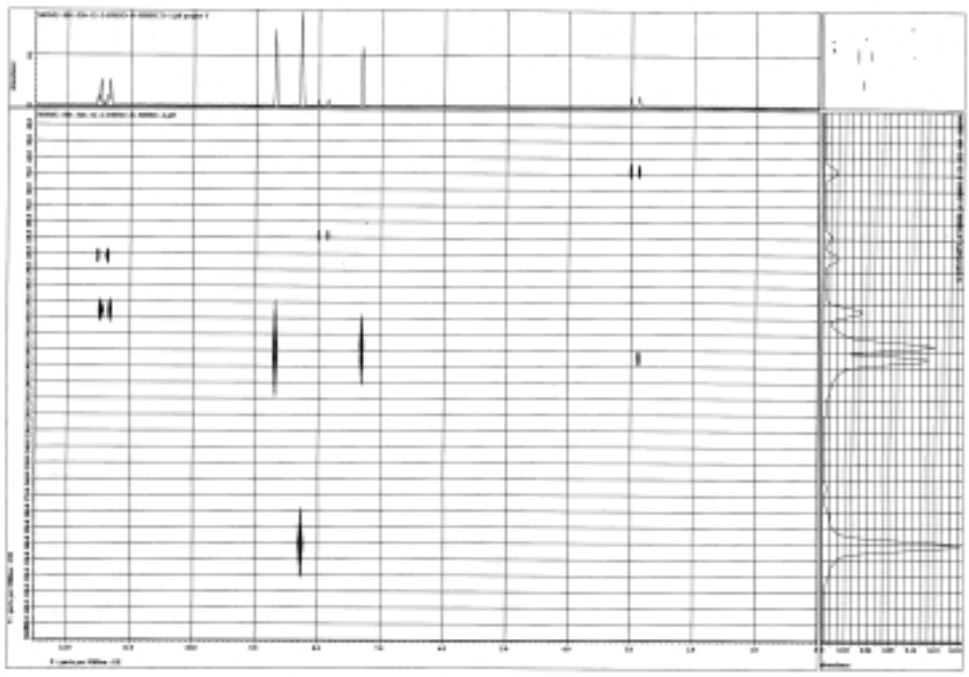


Figure S2-8. ^{15}N-HMBC spectrum of asteropterin in DMSO-d_6

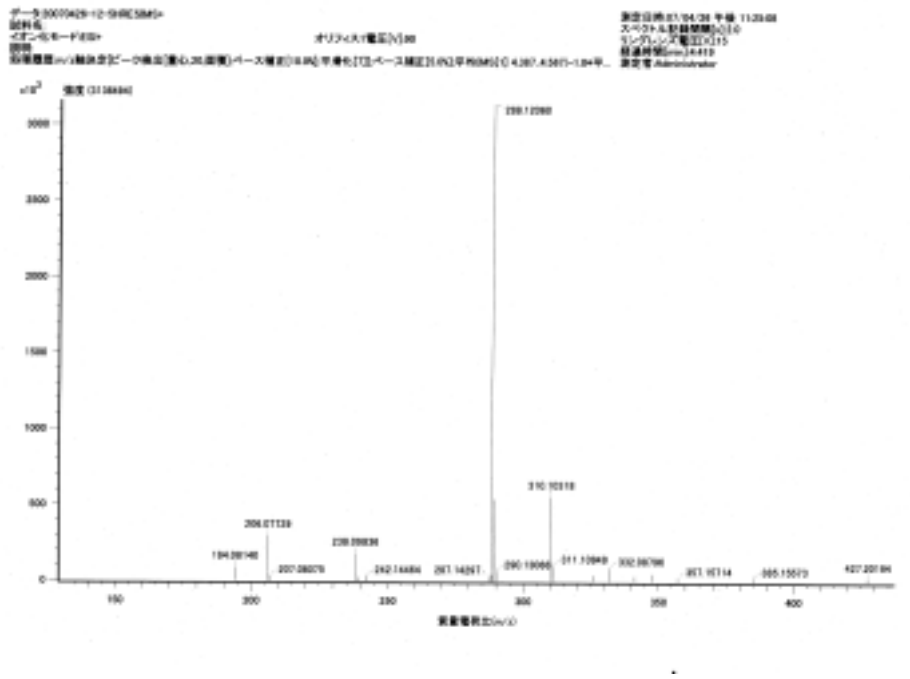


Figure S2-9. HR-ESIMS spectrum of asteropterin (positive mode)

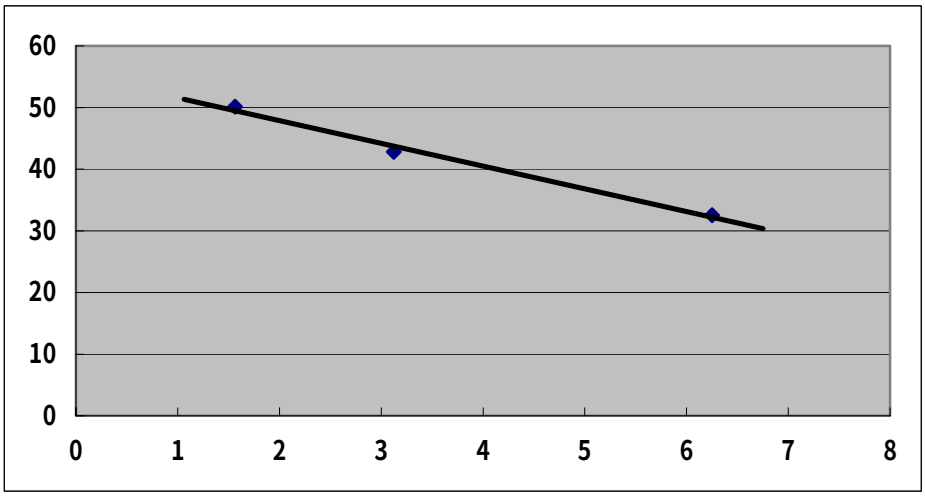


Figure S2-10. Cathepsin B inhibitory activity of asteropterin

Chapter III
Shishicrellastatins, Inhibitors of Cathepsin B, from the Marine Sponge *Crella (Yvesia) spinulata*

1.Introduction

The extract of the marine sponge *Crella (Yvesia) spinulata* collected off Shishi Island (Figure 3-1) exhibited activity against Cathepsin B in the screening. Extraction and fractionation of the extract by monitoring the inhibitory activity afforded two new dimeric steroid named shishicrellastatins A and B (**3-1**). This chapter deals with the isolation, structure elucidation, and the enzyme inhibitory activity of these new compounds.

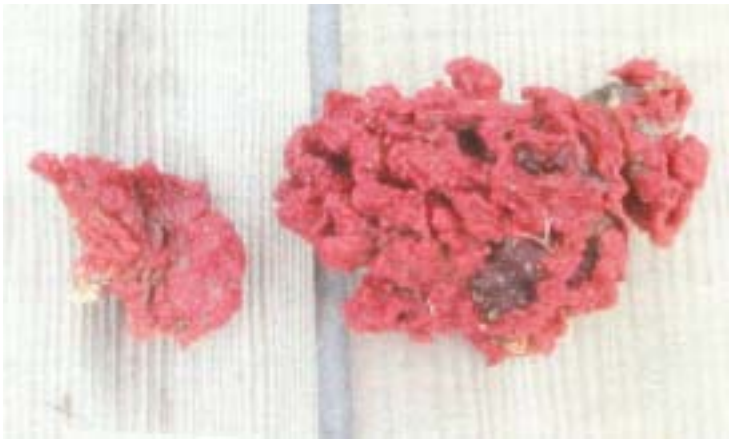


Figure 3-1. *Crella (Yvesia) spinulata*

2.Results and Discussion

2.1. Extraction and Isolation

The sponge *Crella (Yvesia) spinulata* was extracted with MeOH, and the extract was partitioned between H_2O and $CHCl_3$. The aqueous phase was then partitioned between H_2O and *n*-BuOH. All the organic layers were combined and separated by SiO_2 gel flash column chromatography followed by SiO_2 gel column chromatography. The active fractions were repeatedly purified by reversed-phase HPLC in the presence of a high concentration of $NaClO_4$ to furnish 1.3 mg of shishicrellastatin A (**3-1**) and 2.7 mg of shishicrellastatin B (**3-2**) each as colorless solid (Figure 3-2).

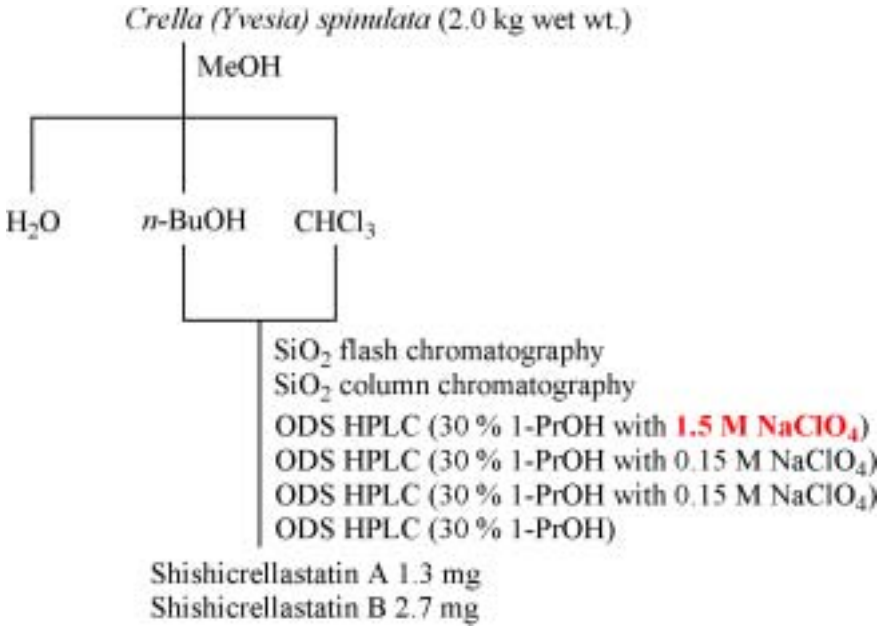
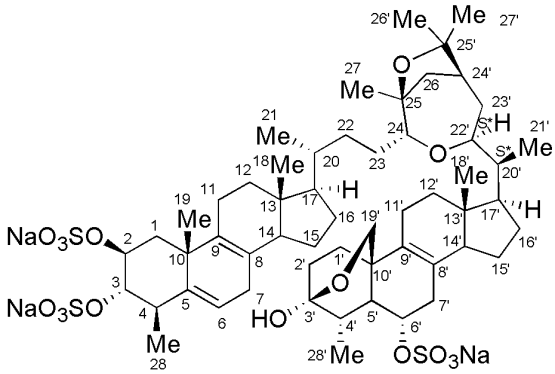
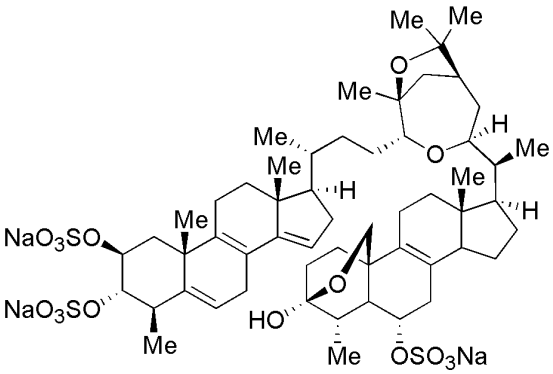


Figure 3-2. Isolation scheme of shishicrellastatins



3-1: Shishicrellastatin A



3-2 Shishicrellastatin B

2.2. Structure Elucidation

Shishicrellastatin A (**3-1**) was optically active and had the molecular formula of $C_{56}H_{83}Na_3O_{16}S_3$ as determined by high-resolution positive ion electro spray ionization mass spectrometry [(+)-HRESIMS] in conjunction with the 1D and 2D NMR data. The molecular formula indicated a high degree of unsaturation in the molecular and three sulfate units. UV spectrum in MeOH showed an absorption at λ_{max} 205 nm (ε 4000).[37] The ^{1}H NMR spectrum exhibited six singlet and four doublet methyl signals. The edited-HSQC spectrum showed the presence of 19 methylenes and 12 methines including 5 oxymethines. One olefinic proton signal was observed. The presence of 7 non-protonated sp^3 carbons, including one acetal, and three non-protonated sp^2 carbons was demonstrated by the ^{13}C NMR spectrum. The presence of three

sulfur atoms and many oxygen atoms together with the deshielded oxymethine carbons suggested that shishicrellastatin A had three sulfate groups. 14 degrees of unsaturation for the rest of the molecule accounted for by three double bonds and 11 rings (Table 3-1).

Interpretation of the high-field region of the ^{1}H NMR data by COSY and TOCSY spectra was hampered by the presence of many overlapping signals. Therefore, it was necessary to sort our signals by using HMBC cross peaks from methyl signals and use them as the starting points to assemble partial structures. The ^{1}H and ^{13}C NMR data, in particular, the number of methyl groups, suggested the presence of two steroid units. Unit **a** comprised A and B rings of in steroid I in which C2 and C3 were oxygenated, C4 was methylated, and C5-C6 and C8-C9 double bonds were introduced. Unit **b** constituted the rest of the steroid skeleton with oxygenation at C24 and C25. Importantly $H_2$26 were coupled to H24' in unit **d** demonstrating the formation of a covalent bond between the side chains of two steroid units. Unit **c** composed A and B rings of steroid II , in which C19' was oxidized to alcohol and linked to C3' through an oxygen atom, C4' was methylated, C6' was oxygenated and C8'-C9' double bond was introduced. Unit **d** comprised the rest of steroid II, in which C22' and C25' were oxidized and C24' was covalently linked to C26. At this moment, shishicrellastatin A was shown to be a congener of crellastatins (Figure3-3).

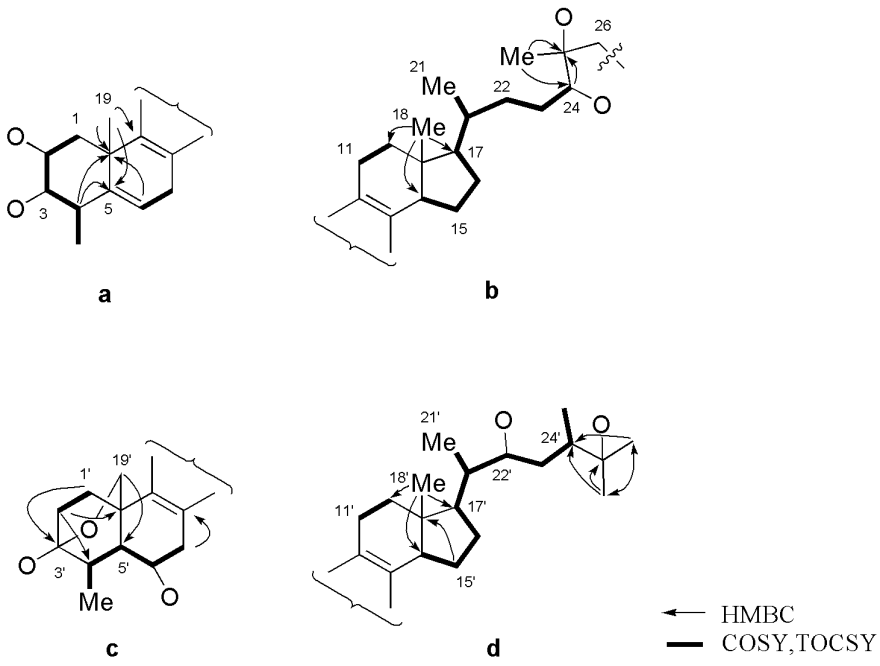


Figure 3-3. Partial structures of shishicrellastatins

ROESY cross peaks, H19/H11β and H7β/H15α suggested that steroid I was composed of partial structures **a** and **b**, and the cross peaks H19'β/H11'β and H7'β/H15'α suggested that steroid

II was comprised of partial structures **c** and **d** (Table 3-2). Deuterium exchange experiment in the ^{13}C NMR displayed that the exchangeable hydrogen atom was present only on the oxygen atom at C3'. The linkage of steroid I and II was determined by HMBC correlation. An HMBC cross peak between H24 and C22' revealed an ether linkage between C22' and C24. Because shishicrellastatin A had one more unsaturation and C2, C3, and C6' appeared to be sulfated on the basis of ^{1}H and ^{13}C NMR data, C25 and C25' were connected by an ether bond.

The relative stereochemistry of each steroid skeleton was assigned on the basis of ^{1}H-^{1}H coupling constants, ROESY data, and comparison of chemical shift values with those in the literature (Table 3-2). H2 was equatrial (α-oriented), because it exhibited a large coupling with neither H1α, H1β, nor H3. H3 was assigned as axial (α-oriented) on the basis of an intense ROESY cross peak with H1α. A small coupling and intense ROESY cross peak between H3 and H4 showed that H4 was equatorial and α-oriented. There was a ROESY cross peak between Me-18 and Me-19, demonstrating that the two methyls were on the same face of the tetracyclic skeleton. The absence of ROESY cross peak between Me-18 and H-14 indicated that the CD ring junction was *trans*, which was supported by the ^{13}C chemical shift of C-18 (11.2 ppm in Δ^{8}-14α-cholesterole; 20.2 ppm in Δ^{8}-14α-cholesterole acetate). A ROESY cross peak between Me-18 and H-20 suggested that H-17 was α-oriented. An intense COSY cross peak between H17 and H20 suggested that the two protons were in anti-periplanar relationship. A distinct ROESY cross peak between Me-21 and H12β suggested the 20R* sterochemistry.[38] The relative stereochemistry of steroid II was similarly assigned. A ROESY cross peak between H-19'α and H-6' suggested that those protons were on the same face of the ring system. H5' and H6' were in the anti-periplanar relationship on the basis of a large coupling constant (9.8 Hz). A ROESY cross peak between Me-28' and H-5' showed that they were syn. ROESY cross peaks between Me-18' and CH_2-19', between Me-18' and H-20' and between Me-21' and H-12'β as well as a large coupling between H-17' and H-20' and carbon chemical shift of C18' (δ_H 11.5 ppm) suggested that steroid II was a conventional Δ^{8}-steroid with 4α-methyl substitution. (Figure 3-4)

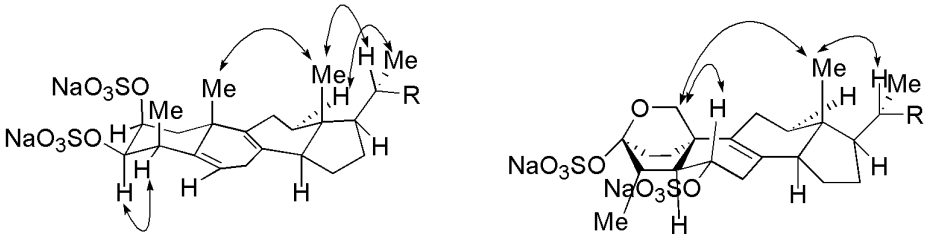


Figure 3-4. Related structures of shishicrellastatin A's steroid units

The stereochemistry of the bicyclic system in the side chain was also analyzed on the basis of the ROESY data. H-22' was correlated with H-26β and H-23β, suggesting that H22', H26 and C23 were all axially oriented in the same face of the oxepane ring. Because Me-27' was correlated to H26α, C25' and 25-O were placed on the other face of the oxepane ring.

Then we attempted to correlate the stereochemistry of C22' with that of C20'. H20' and H22' were gauche because the coupling between H20' and H22' was small. We considered the possibility of two local enantiomeric structures separately (Figure 3-5).

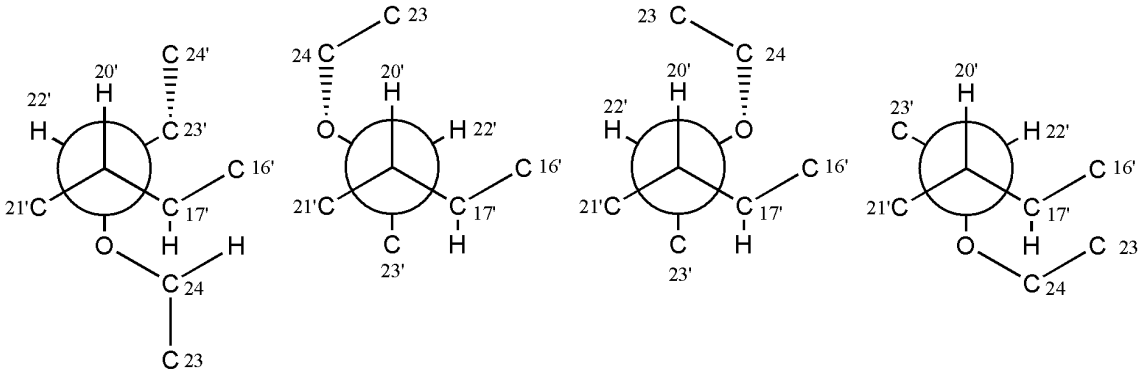


Figure 3-5. Newman projection of proposal related stereochemistry of shishicrellastatin A

In the 22'R* isomer, there are two stable conformers with H20'-H22' gauche conformation (Figure 3-5, A1 and A2). Similarly, two stable conformations (Figure 3-5, B1 and B2) are possible for the 22' S* isomer. In the ROESY spectrum, two diagnostic cross peaks implicating the conformation of the relevant portion were observed: H16'β/H22' (H16' is syn to H17' on the basis of the ROESY cross peak between H16'β and H17') and H23α/H22'. In both conformers A1 and B1, H16'β and H22 are too much separated to give a ROESY cross peak. Therefore, conformers A1 and B1 were rejected. On the other hand in conformers A2 and B2, H16'β and H22' as well as H23β and H22' are both with in the distance to give ROESY cross peaks. Conformers A2 and B2 could be differentiated by the distance between H17' and H_2-23. However ROESY cross peak between these protons were not distinguishable due to overlapped signals. The other feature to discriminate between conformers A2 and B2 is the distance between H17' and C22'-oxygen atom. In conformer B2, H17' is close to the oxygen atom, whereas they are separated in conformer A2. ^{1}H chemical shift of H17' suggests that H17' is significantly deshielded compared to H17, indicating that H17' is a spatially close to C22-O. Therefore, we concluded that shishicrellastatin A had conformer B2 with 20'-S*, 22'-S* stereochemistry. Because all of our attempts to hydrolyse the sulfate esters and to prepare MTPA esters were unsuccessful, we were not

able to determine the absolute stereochemistry of shishicrellastatin A.

Shishicrellastatin B had the molecular formula with two less hydrogen atoms than that of shishicrellastatin A as determined by (+)-HRESIMS in conjunction with the 1D and 2D NMR data (Table 3-3). The NMR data showed the presence of one additional double bond (δ_H 5.31; δ_C 117.2 and 151.4) in shishicrellastatin B. A comparison of the HSQC spectra of the two compounds suggested that the structural units **a**, **c**, and **d** were conserved, but signals in ring D in steroid I were perturbed. The sp^3 carbons at C14 and C15 were replaced by sp^2 carbons, indicating the introduction of $\Delta^{14,15}$-olefin. This was in agreement with the UV absorption at λ_{max} 205 (ε 2000) and 250 nm (ε 1600).[37]

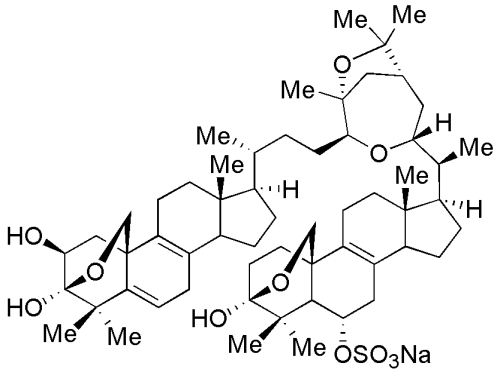
The relative stereochemistry of the two steroid units were assigned on the basis of ROESY data (Table 3-4). The relative stereochemistry of the side chain portion was assigned to be identical with that of shishicrellastatin A, because NMR data for this portion were almost superimposable for the two compounds.

2.3. Biological Activity

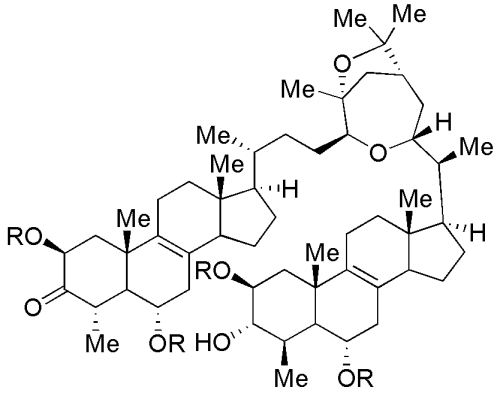
Shishicrellastatin A (**3-1**) and B (**3-2**) inhibited cathepsin B with an IC_{50} value of 7.0 μg/mL and 7.8 μg/mL.

2.4. Conclusion

Crellastatins (**3-3**) were isolated from a Vanuatu Island marine sponge *Crella* sp.,[39] whereas shishicrellastatins were obtained from the Japanese marine sponge *Crella (Yvesia) spinulata*. The two sponges are of the same genus and crellastatins and shishicrellastatins share the common feature of steroids dimerized through side chains. We analyzed relative stereochemistry of the bicyclic system in the side chain, as reverse of the previously reported compounds. The two classes of compounds are significantly different in the functionalization of the sterol skeltons, e.g. the number of the sulfate esters, the number of methyl groups at C4. Another related compounds hamigerols (**3-4**) have been recently reported from the medditeranean sponge *Hamigera hamigera*.[40]



Crellastatin A (**3-3**)



Hamigerol A (**3-4** R=SO_3Na)

Steroid I				
#	δC	δH mult	COSY	HMBC
1α	40.0	1.59 overlapped	2	
1β		2.17 br	2	
2	77.9	4.97 overlapped	1α,1β,3	
3	78.2	4.39 br	2,4	
4	43.9	3.05 br	3,28	2,3,5,6,10,28
5	145.3			
6	123.2	5.61 br	7β	10
7α	30.2	1.95 br		
7β		2.56 br	6	
8	127.2			
9	135.0			
10	38.5			
11α	23.6	1.92 br		
11β		2.15 overlapped	12α	9
12α	38.4	1.46 overlapped		
12β		2.05 br		9
13	43.4			
14	53.2	2.17 overlapped	15α	
15α	24.0	1.68 br	14,16β	
15β				
16α	31.9	1.48 overlapped	17	
16β		2.23 br	15α	
17	55.6	1.26 br	16α,20	
18	11.9	0.71 s		12,13,14,17
19	28.5	1.48 overlapped		1,5,9,10
20	36.6	1.51 br	17,21	
21	19.3	1.00 d	20	17,20,22
22α	34.6	1.21 br		24
22β		1.62 overlapped	23α	
23α	24.6	1.71 br	22β,24	
23β			23a	
24	84.3	3.29 overlapped		25,22'
25	88.1			
26α	36.7	2.07 overlapped	26β,24'	25
26β		2.18 overlapped	26α,24'	
27	27.4	1.20 s		24,25,26,25'
28	19.4	1.45 overlapped	4	3,4,5

Steroid II				
#	δC	δH mult	COSY	HMBC
1'α	34.0	2.02 overlapped	2'α,2'β	3'
1'β				
2α'	28.2	1.68 br	1'α,2'β	4',10',28'
2β'		2.03 overlapped	1'α,2'α	
3'	99.1			
4'	42.3	2.21 br	5',28'	
5'	51.7	1.48 overlapped	4',6'	6'
6'	78.3	4.59 br	5',7'α,7'β	
7'α	35.9	2.08 overlapped	6',7'β	
7'β		2.75 br	6',7'α	8',9'
8'	128.6			
9'	131.9			
10'	40.2			
11'α	23.7	1.37 overlapped	12'β	
11'β		1.99 overlapped		9'
12'α	38.2	1.42 overlapped	11'α	
12'β		2.00 overlapped		8'
13'	43.0			
14'	53.7	2.14 overlapped	15'α	9'
15'α	25.3	1.66 br	14',16'α	13'
15'β		1.38 overlapper		
16'α	30.3	1.38 overlapper	15',17'	
16'β		1.44 overlapped		15'
17'	52.9	1.61 overlapped	16'α,20'	
18'	11.5	0.62 s		12',13',14',17'
19'α	71.1	3.91 d		
19'β		3.94 d		5'
20'	44.0	1.39 br	17',21',22'	
21'	13.9	1.02 d	20'	17',20',22'
22'	70.8	3.74 br	20',23'α,23'β	
23'α	30.6	1.30 br	22'β	
23'β		2.07 overlapped	22'α	
24'	45.8	2.13 overlapped	23α,26β	25,26,22'
25'	85.6			
26'	32.5	1.24 s		24',25',27'
27'	25.8	1.35 s		24',25',26'
28'	18.8	1.18 d	4'	3',4',5'

Table 3-1. NMR data of shishicrellastatin A

Steroid I			Steroid II	
#	ROESY		#	ROESY
1α	2,3		1'α	
1β			1'β	
2	1α,3		2α	2'β
			2β	4',2'α
3	1α,2,4		3'	
4	3,6,28		4'	2'α,5',6'
5			5'	4',7'α,28'
6	4,7β		6'	4',7'β,19'α
7α			7'α	5',7'β
7β	6,15α		7'β	6',7'α,15'α
8			8'	
9			9'	
10			10'	
11α			11'α	
11β	19		11'β	19'β
12α			12'α	
12β	18,21		12'β	16'β,21'
13			13'	
14	15α,16β,18		14'	
15α	7β,14,16β		15'α	7'β,16'α,18'
15β			15'β	
16α	18		16'α	15'α
16β	12β,14,15α		16'β	17',22'
17			17'	16'β
18	14,16α,19,20,21		18'	12'β,15'α,19'α,19'β,21'
19	11β,18		19'α	6',18'
			19'β	11'β,18'
20	18		20'	21',22'
21	12β,18		21'	12'β, 18',20'27'
22α	22β,23α,24		22'	23α,26β,16'β,20',23'α,23'β
22β	22α			
23α	22α,22'		23'α	22'
23β			23'β	22'
24	22α		24'	
25			25'	
26α	27’		26'	22'
26β				
27			27'	26α,21'
28	4		28'	5'

Table 3-2. ROESY data of shishicrellastatin A

Steroid I				
#	δC	δH mult	COSY	HMBC
1α	38.5	1.66	1β,2	
1β		2.69	1α,2	
2	76.5	5.00	1α,1β,3	
3	77.0	4.53	2,4	
4	42.8	3.00	3,5,28	C3,C5,C6,C10,C28
5	145.3			
6	121.2	5.63	7β	
7α	26.9	2.52		C8
7β		2.54	6	
8	128.2			
9	140.2			
10	38.5			
11α	22.5	1.89	12α,12β	
11β		2.09		
12α	36.8	1.40	11α	C9
12β			11α	
13	46.2			
14	151.4			
15	117.2	5.31	16α,16β	C13,C14,C17
16α	35.8	2.11	15,16β,17	C13,C14,C15
16β		2.36	15,16α,17	
17	56.1	1.55	16α,16β,20	
18	15.9	0.85 s		C12,C13,C14,C17
19	27.5	1.46		C5',C9',C10'
20	33.1	1.78	17,21,22β	
21	18.2	0.98	20	C17,C20,C22
22α	34.5	1.35		
22β		1.58	20,23α	
23α	23.8	1.32	22,24	C24
23β		1.59	24	
24	84.0	3.20	23α,23β	
25	88.1			
26α	35.5	2.01		
26β		2.18	24'	C25,C22'
27	26.0	1.15		C24,C25
28	18.0	1.41	4	C3,C4,C5

Steroid II				
#	δC	δH mult	COSY	HMBC
1'α	35.5	2.01	2'	C3'
1'β		2.18		
2'α	26.8	1.67	1'α	C3'
2'β				
3'	99.1			
4'	40.8	2.12	5',28'	
5'	50.0	1.43	4',6'	
6'	77.1	4.47	5',7'α,7'β	
7'α	34.2	1.97	6',7'β	
7'β		2.62	6',7'α	C8'
8'	132.3			
9'	122.1			
10'	40.2			
11'α	26.7	1.99	12'α	C8'
11'β				
12'α	36.5	1.36	11'α	
12'β		1.97		
13'	42.7			
14'	51.6	2.11	15'β	
15'α	29.2	1.07	14'	
15'β		1.20	16'α	
16'α	30.6	1.44	15'β,17'	
16'β				C13'
17'	51.2	1.51	16'α,20'	
18'	9.9	0.58		C12',C13',C14',C17'
19'α	70.2	3.84		C3'
19'β		3.90		C5',C10'
20'	42.4	1.33	17',21',22'	
21'	12.2	0.97	20'	C17',C20',C22'
22'	70.0	3.67	20',23'α	
	3.0			
23'α	32.5	1.99	22',24'	
23'β		2.09		
24'	44.2	2.11	26β,23'α	C25,C26,C22'
25'	85.6			
26'	31.2	1.21		C24',C25',C27'
27'	24.2	1.30		C24',C25',C26'
28'	17.5	1.17	4'	C3',C4',C5'

Table 3-3. NMR data of shishicrellastatin B

Steroid I			Steroid II	
#	ROESY		#	ROESY
1α	1β,2,3		1'α	2'α,28'
1β	1α		1'β	28'
2	1α,3		2α	1'α
			2β	
3	1α,2,4		3'	
4	3,6,28		4'	6',28'
5			5'	28'
6	4,7α,7β		6'	4',7'α,19'α,19'β
7α	6,7β		7'α	6',7'β
7β	6,7α		7'β	7'α
8			8'	
9			9'	
10			10'	
11α			11'α	
11β			11'β	
12α			12'α	
12β			12'β	
13			13'	
14			14'	
15			15'α	
			15'β	
16α	16β,21		16'α	
16β	16α		16'β	
17			17'	
18	20,21		18'	20',21'
19			19'α	6',19'β
			19'β	6',19'α
20	18		20'	18',21',22'
21	16α,18,22β		21'	18',20',23'β
22α	24		22'	23β,20',23'β,24'
22β	21			
23α			23'α	
23β	22'		23'β	21'.22'
24	22α,27		24'	22'
25			25'	
26α			26'	
26β				
27	24		27'	
28	4		28'	1'α,1'β,4',5'

Table 3-4. ROESY correlation of shishicrellastatin B

3. Experimental Section

3.1 General Procedures

NMR spectra were recorded on a JEOL delta 600 NMR spectrometer at 600 MHz for ^{1}H and 150 MHz for ^{13}C. ^{1}H and ^{13}C chemical shifts were referenced to the solvent peak (MeOD-d_4) at δ 3.31 and 49.15 ppm respectively. Standard parameters were used for the 2D NMR spectra, which included gradient COSY, edited HSQC, HMBC, and ROESY. (+)-HRESI mass spectra were measured on a JEOL JMS-T100LC time-of-flight mass spectrometer. Fluorescence for enzyme inhibition assay was determined with a Molecular Devices SPECTRA MAX GEMINI fluorescence spectrometer. UV spectra were recorded on a Shimadzu BioSpec-1600 spectrophotometer Optical rotations were recorded on a JASCO DIP-1000 digital parameter. Reverse phase HPLC was performed on Cosmosil AR-II (10-mm i.d. x 250-mm) connected to a Shimadzu SPD-6AV UV detector and Shimadzu LC-6AD pump. All solvents used for HPLC, UV, $[\alpha]_D$, and MS were WAKO JIS special grade, and the H_2O used was Millipore Elix 10. Sodium perchlorate used for HPLC was Nacalai Tesque extra pure reagent.

3.2. Animal Material

A specimen of *Crella (Yvesia) spinulata* was collected in July 2002 by scuba diving (12-15 m) off Shishi Island (32-15.94N; 130-15.75E), Kagoshima Prefecture. The specimen was frozen after collection and kept at –20 ˚C until extraction.

3.3 Cathepsin B Inhibitory Assay

Inhibitory activity against cathepsin B was determined essentially according to the method described in Chapter I. The IC_{50} values were determined by plotting the percent inhibitions obtained for each sample concentration on semi logarithmic graph paper.

3.4. Extraction and Isolation

The sponge (2.0 kg wet) was extracted with MeOH (3L x3), and the extract was concentrated under vacuum to yield a dark brown oil. This oil was partitioned between H_2O (500 mL) and $CHCl_3$ (500 mL x 3). The aqueous fraction was partitioned between H_2O (500 mL) and *n*-BuOH (500 mL x 2). All organic fractions were combined and separated by SiO_2 gel flash column chromatography with $CHCl_3$: MeOH: H_2O (10:0:0-6:4:1). $CHCl_3$: MeOH: H_2O (6:4:1) fraction was separated by SiO_2 gel column chromatography with $CHCl_3$: MeOH: H_2O (10:0:0-6:4:1). $CHCl_3$: MeOH: H_2O (6:4:1) fraction (497.3 mg) was chromatographed using C_{18} HPLC at flow late

of 4 mL/min with isocratic conditions of 30% 1-PrOH/70% H_2O containing 1.5 M $NaClO_4$ to yield fractions containing compound 3A and 3B. Fraction 3A was purified using C_{18} HPLC at flow late of 4 mL/min with isocratic condition of 30% 1-PrOH/70% H_2O containing 0.15 M $NaClO_4$ twice and isocratic condition of 30% 1-PrOH/70% H_2O to yield shishicrellastatin A (**3-1**, 1.3 mg). Fraction 3B was purified using C_{18} HPLC at a flow late of 4 mL/min with isocratic condition of 30% 1-PrOH/70% H_2O containing 0.15 M $NaClO_4$ twice and isocratic condition of 30% 1-PrOH/70% H_2O to yield shishicrellastatin B (**3-2**, 2.7 mg).

Shishicrellastatin A (3-1): isolated as a white solid; $[\alpha]_D$+10.88° (c 0.05 MeOH); UV (MeOH) λ_{max} 204.5 nm (ε 3978); ^1H and ^{13}C NMR data, see Table 1; (+)-HRESIMS *m*/*z* 1199.44290 (calculated for $C_{56}H_{83}Na_3O_{16}S_3$ $[M+Na]^+$ 1199.44340, Δ-0.50 mmu)

Shishicrellastatin B (3-2): isolated as a white solid; $[\alpha]_D$+11.40° (c 0.10 MeOH); UV (MeOH) λ_{max} 204.5 nm (ε 2021), 249.5 nm (ε 1639); ^1H and ^{13}C NMR data, see Table 3; (+)-HRESIMS *m*/*z* 1197.43039 (calculated for $C_{56}H_{81}Na_3O_{16}S_3$ $[M+Na]^+$ 1199.42775, Δ-2.64 mmu)

4. Supportiong Information

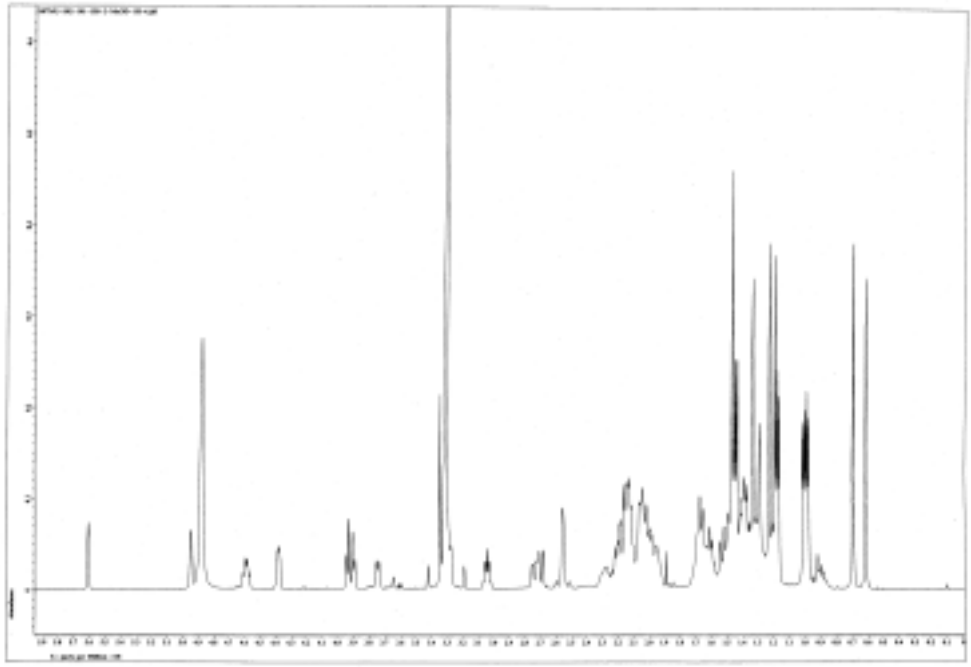


Figure S3-1. ^{1}H NMR spectrum of shishicrellastatin A in MeOD-d_4

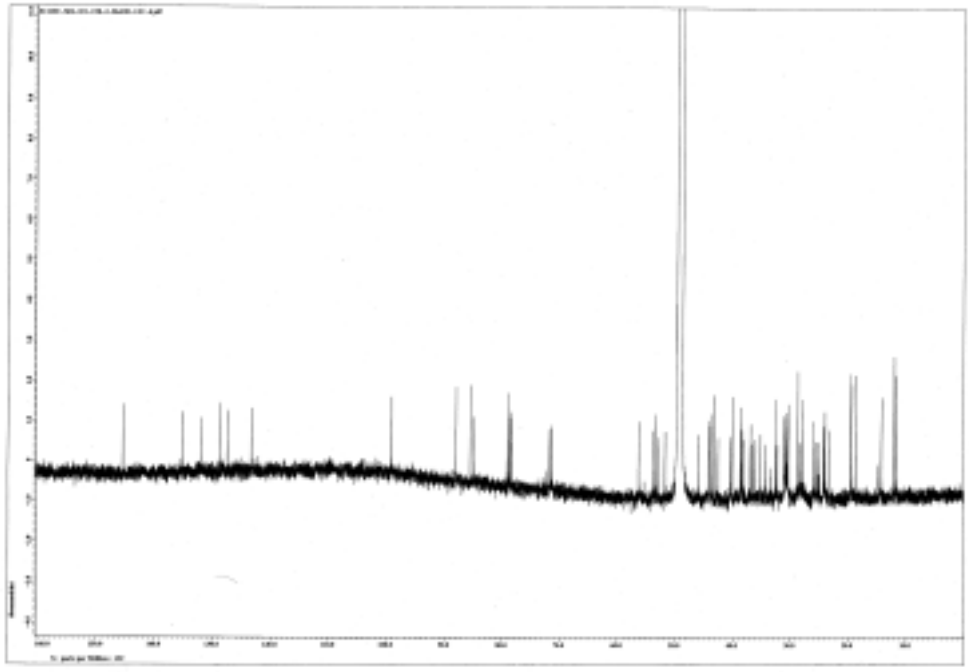


Figure S3-2. ^{13}C NMR spectrum of shishicrellastatin A in MeOD-d_4

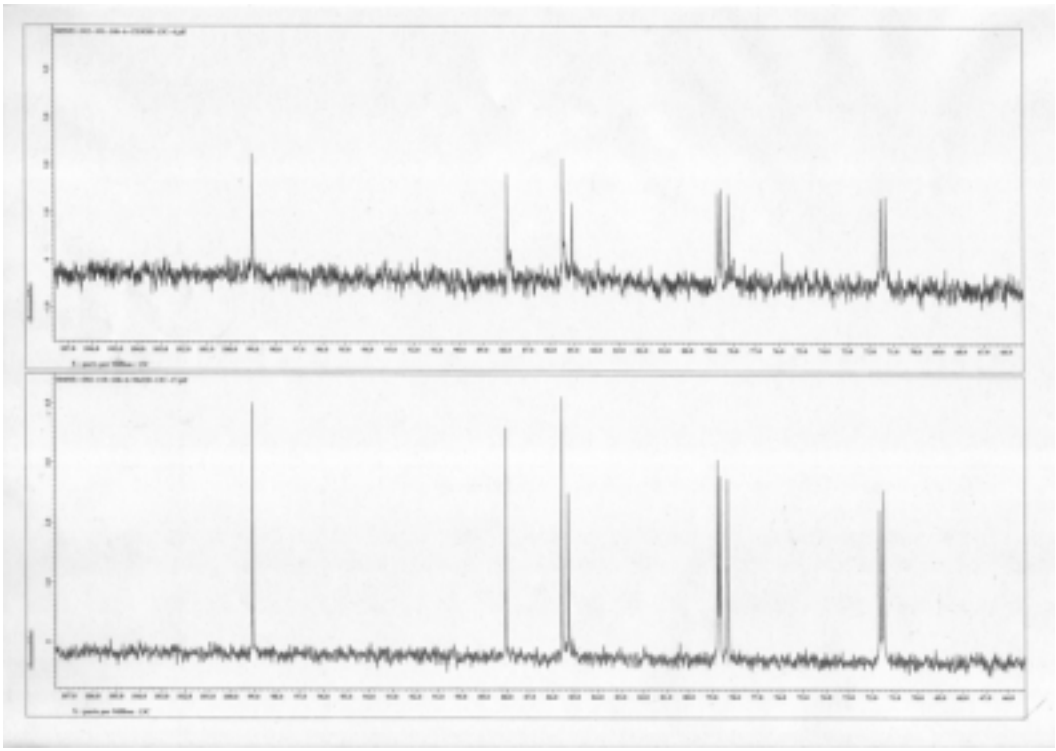


Figure S3-3. ^{13}C NMR spectra of shishicrellastatin A in MeOD-d_4 and MeOD-d_4 with D_2O

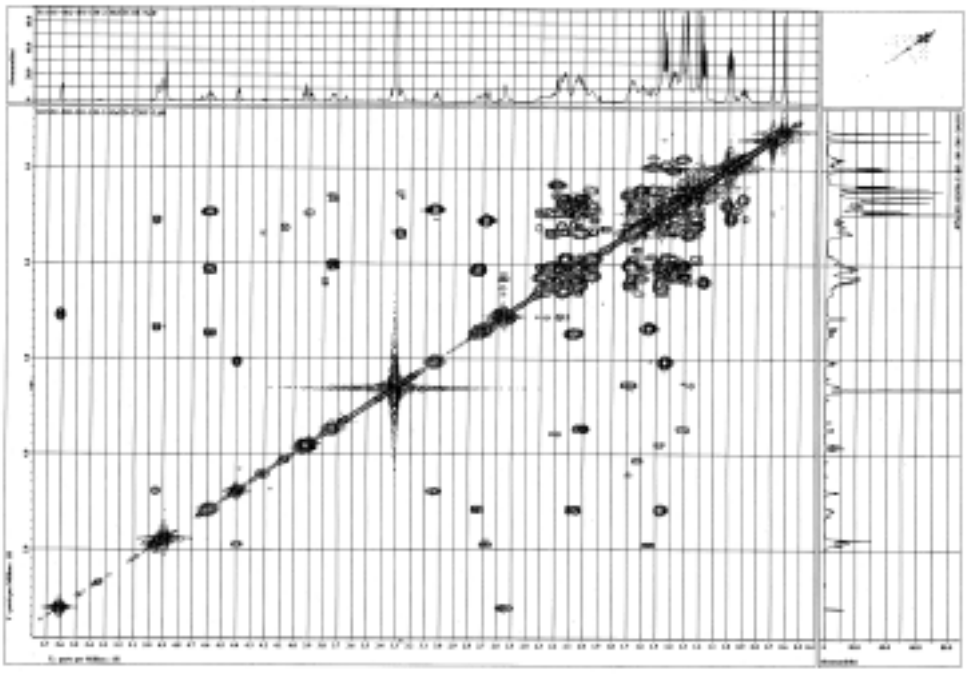


Figure S3-4. COSY NMR spectrum of shishicrellastatin A in MeOD-d_4

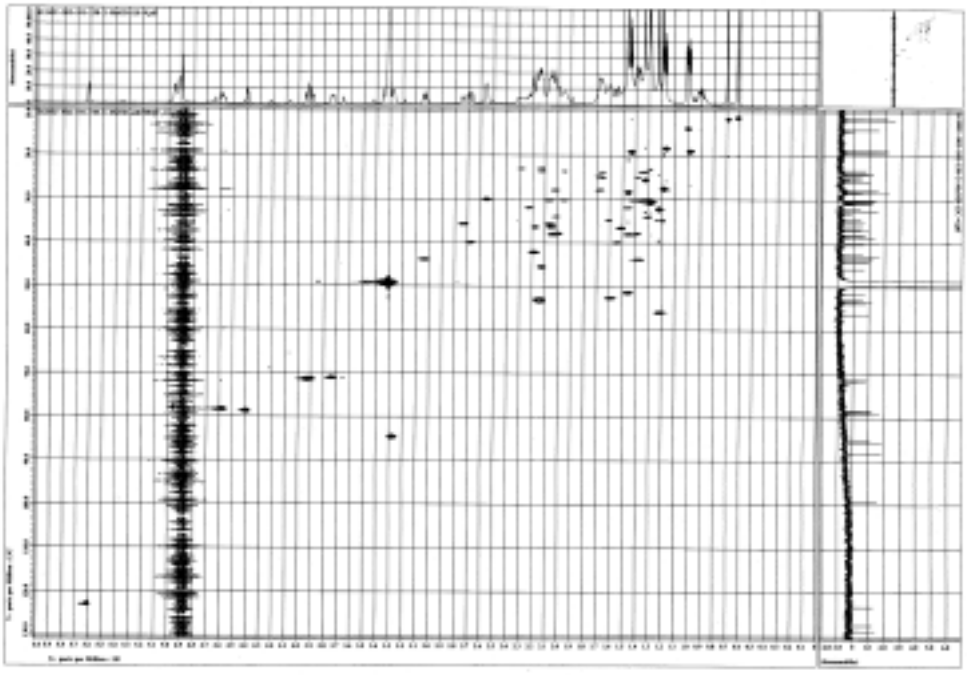


Figure S3-5. HSQC NMR spectrum of shishicrellastatin A in MeOD-d_4

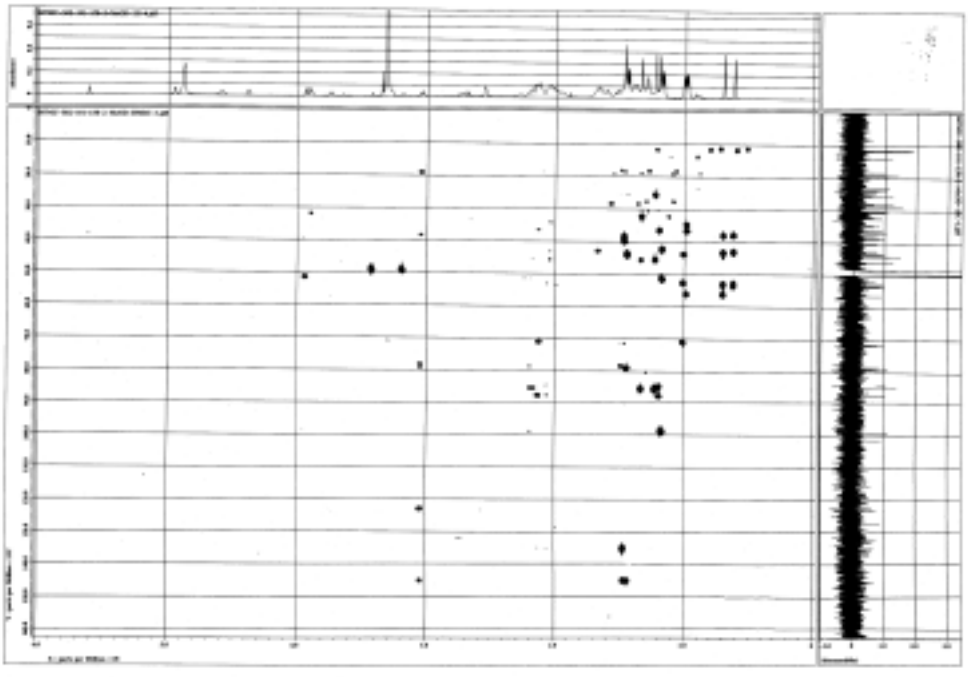


Figure S3-6. HMBC NMR spectrum of shishicrellastatin A in MeOD-d_4

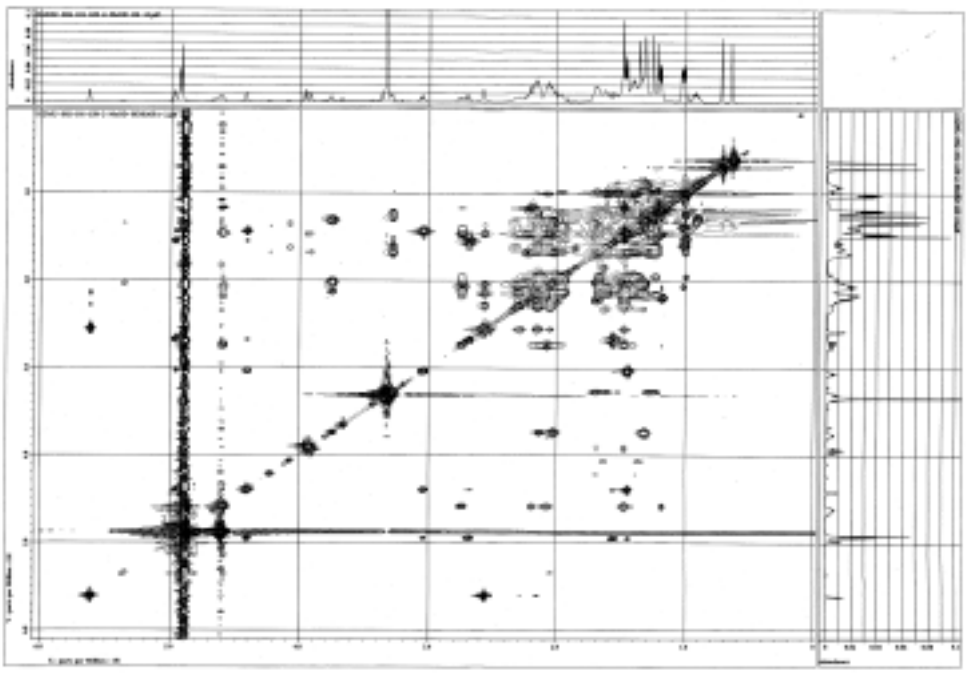


Figure S3-7. TOCSY NMR spectrum of shishicrellastatin A in MeOD-d_4

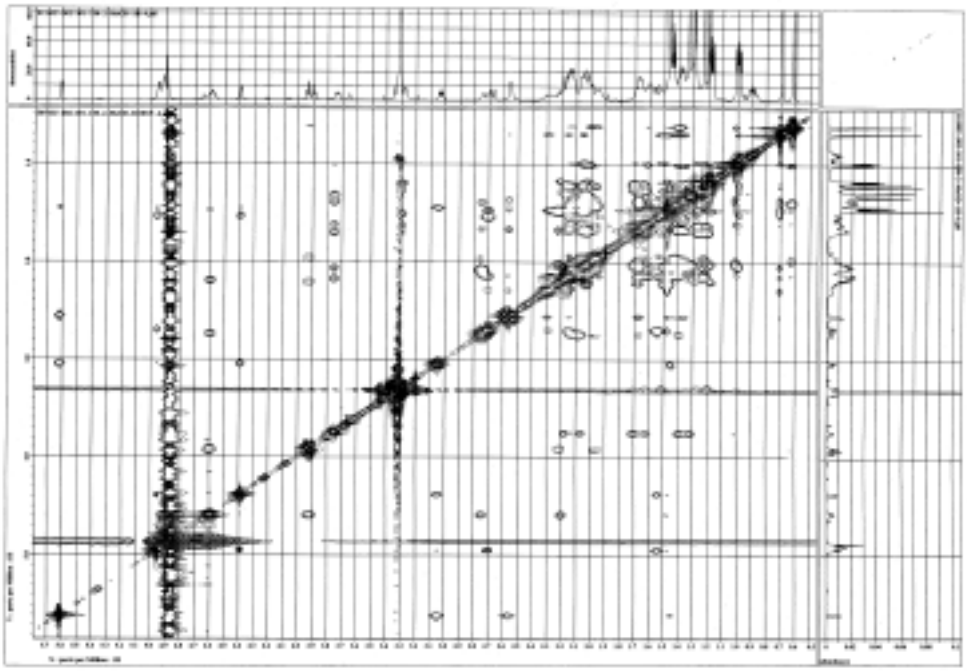


Figure S3-8. ROESY NMR spectrum of shishicrellastatin A in MeOD-d_4

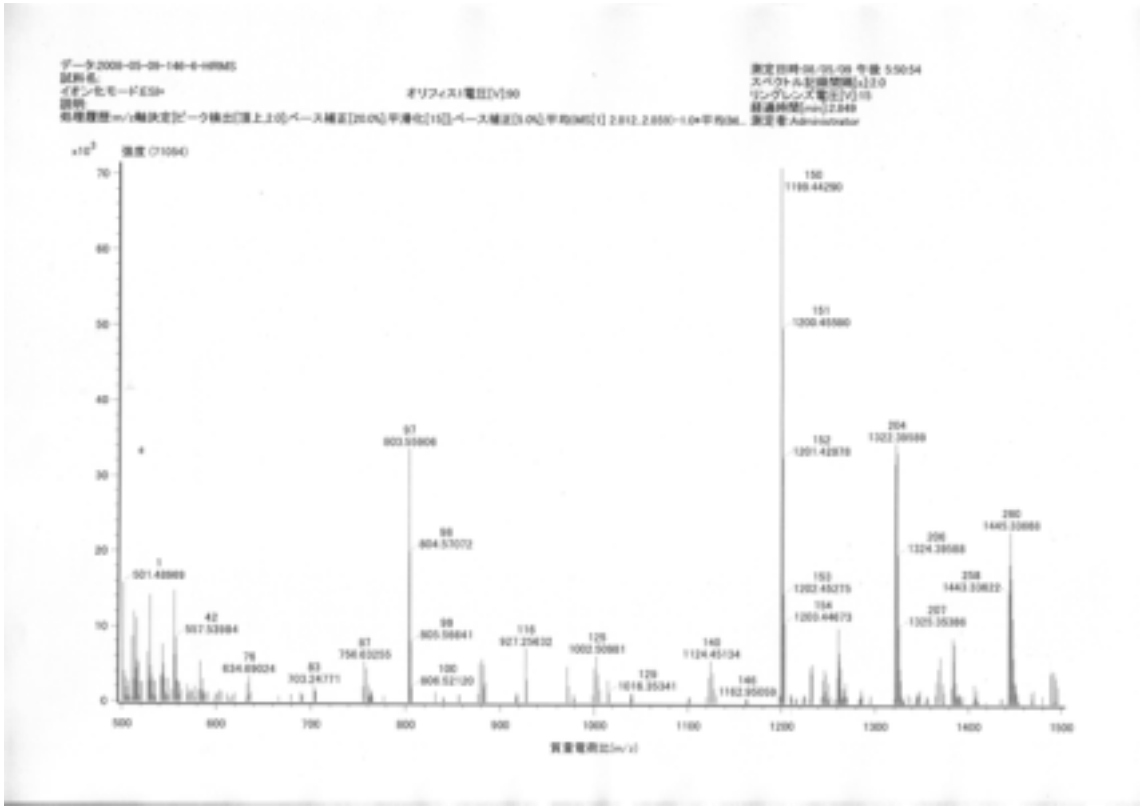


Figure S3-9. HR-ESIMS spectrum of shishicrellastatin A (positive mode)

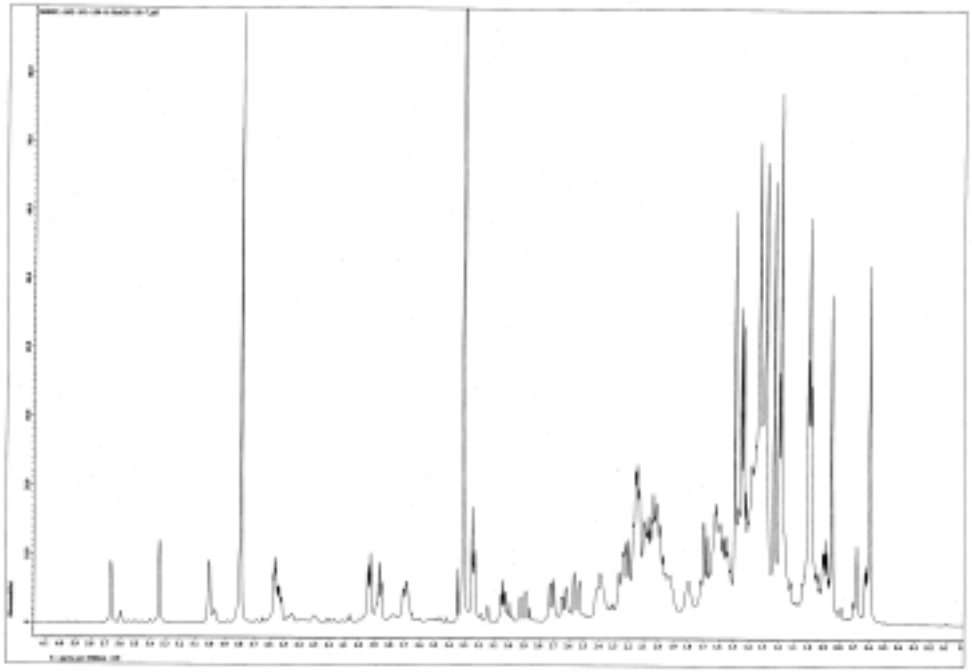


Figure S3-10. ^{1}H NMR spectrum of shishicrellastatin B in MeOD-d_4

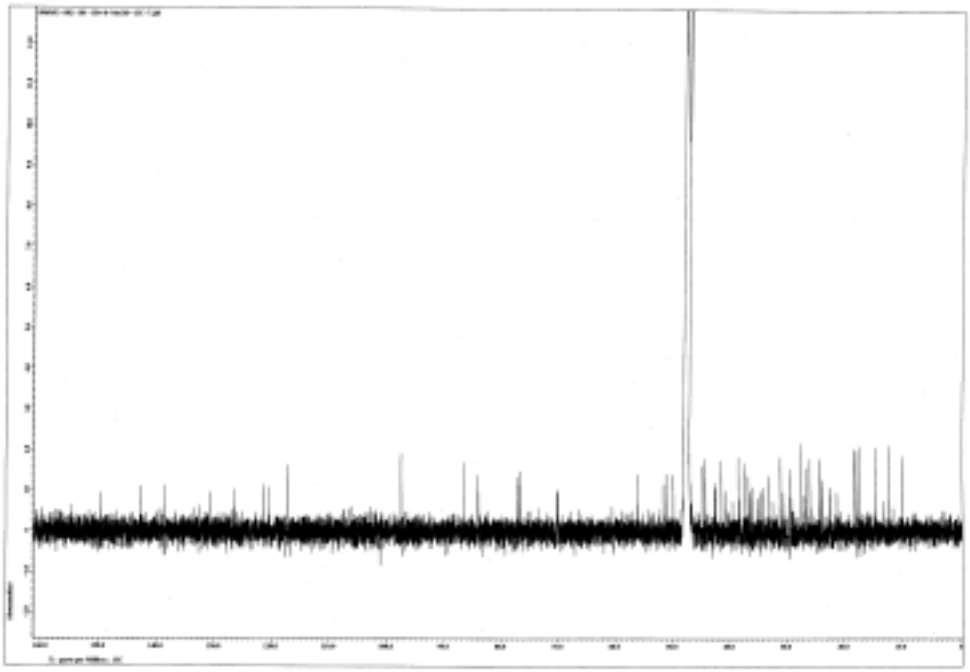


Figure S3-11. ^{13}C NMR spectrum of shishicrellastatin B in MeOD-d_4

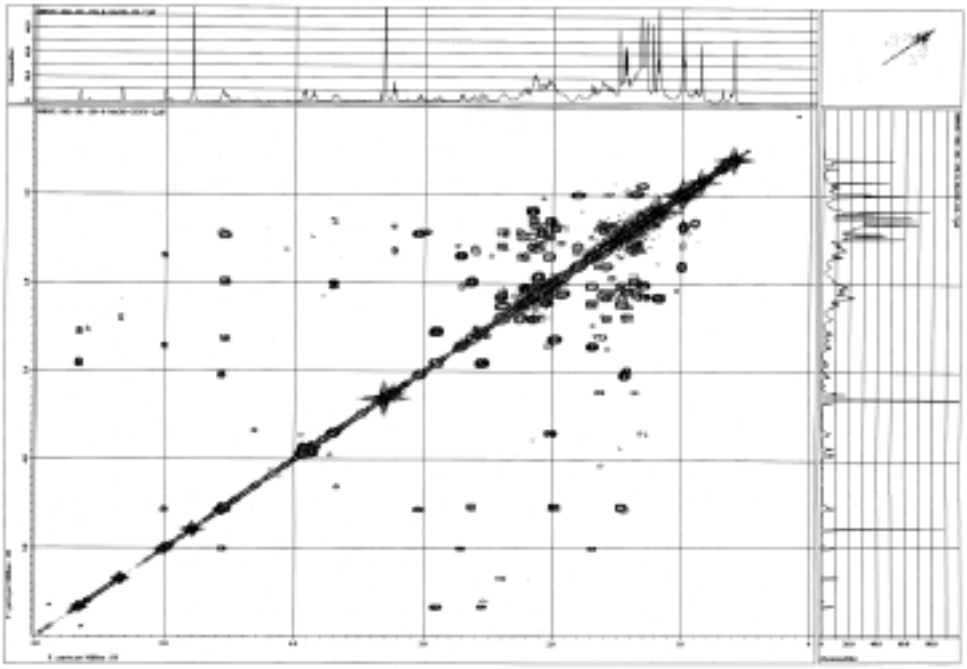


Figure S3-12. COSY NMR spectrum of shishicrellastatin B in MeOD-d_4

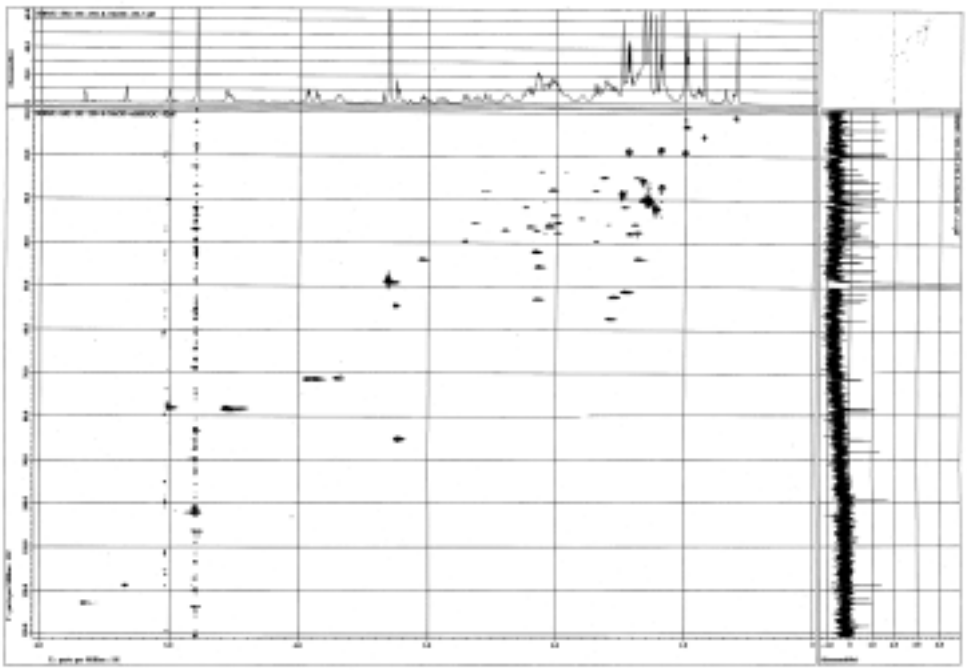


Figure S3-13. HSQC NMR spectrum of shishicrellastatin B in MeOD-d_4

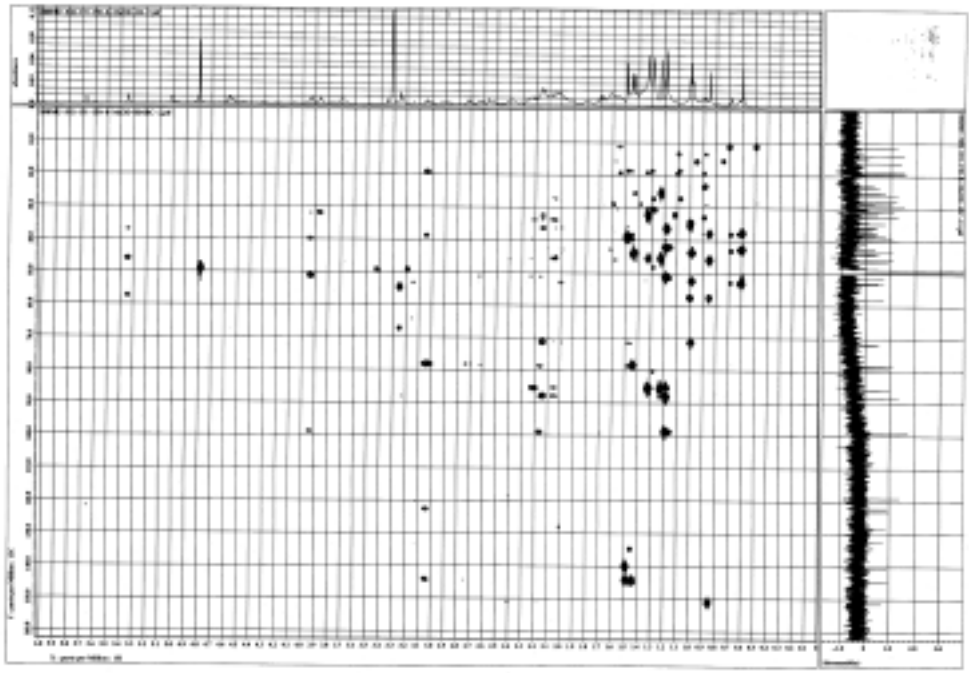


Figure S3-14. HMBC NMR spectrum of shishicrellastatin B in MeOD-d_4

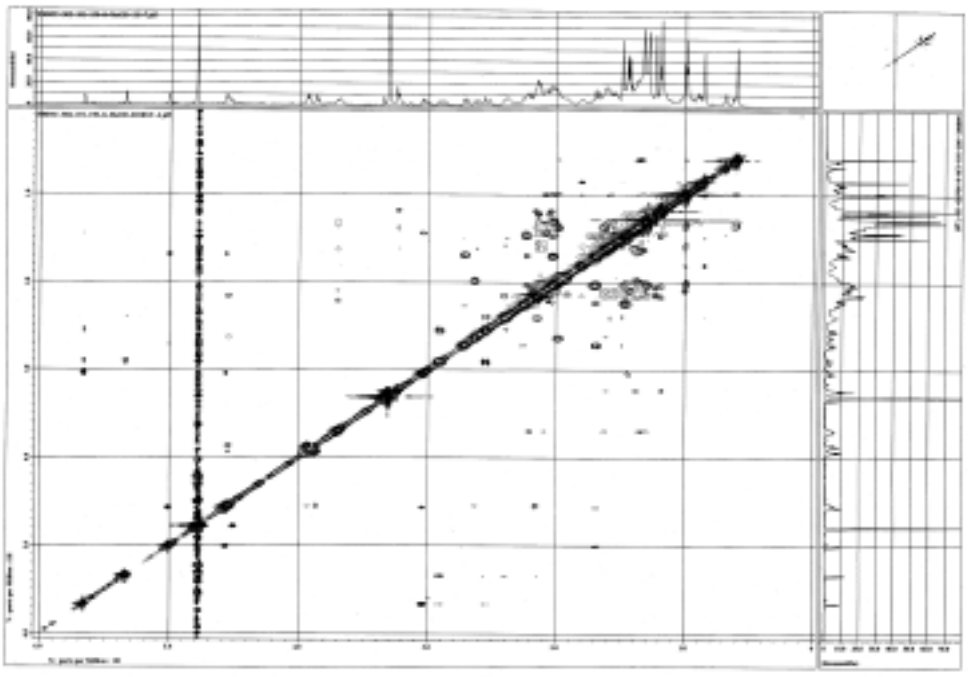


Figure S3-15. ROESY NMR spectrum of shishicrellastatin B in MeOD-d_4

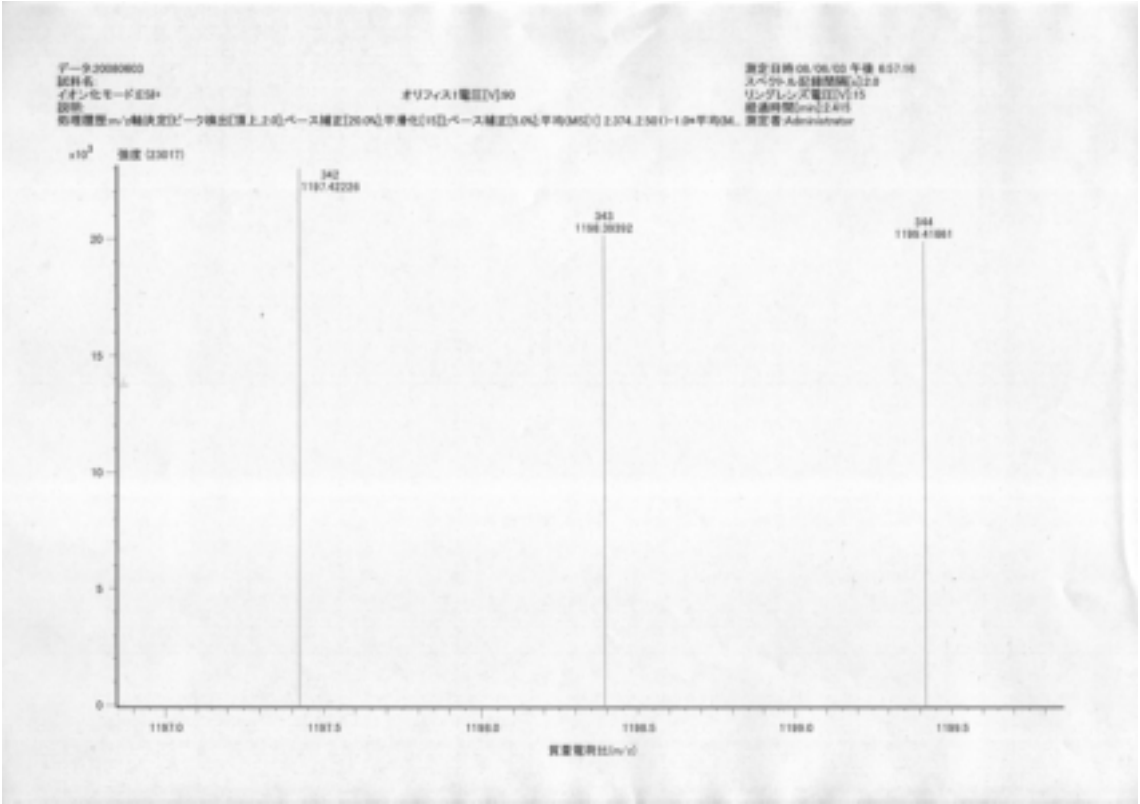


Figure S3-16. HR-ESIMS spectrum of shishicrellastatin B (positive mode)

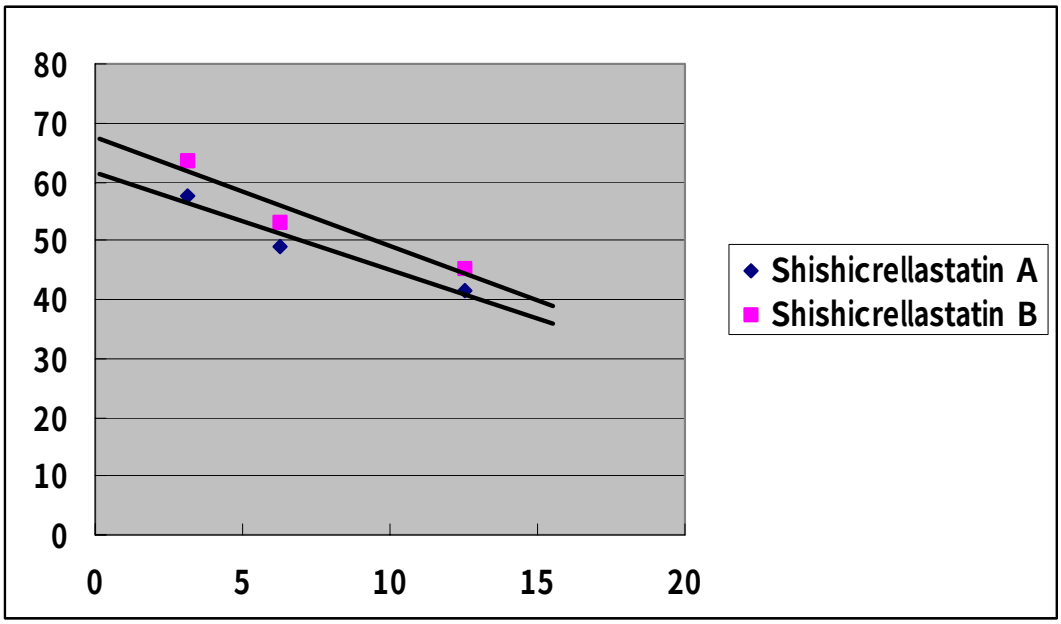


Figure S3-17. Cathepsin B inhibitory activity of shishicrellastatins

Chapter IV
Purification of a Potent Inhibitory Fraction of Cathepsin B, from an Unidentified Marine Sponge

1.Introduction

The extract of an unidentified marine sponge collected off Shishi Island (Figure 4-1) exhibited potent activity against Cathepsin B. Extraction and fractionation of the extracts following the inhibitory activity afforded a potently active fraction. This chapter deals with the purification of the active fraction and its preliminary characterization.

Figure 4-1. Unidentified sponge

2.Results and Discussion

2.1. Extraction and Isolation

The sponge (1 kg, wet weight) was extracted with MeOH three times. The extracts in blue color were concentrated and partitioned between H_2O and $CHCl_3$. The aqueous phase was separated by ODS flash column chromatography (MeOH-H_2O). The fraction eluted with H_2O showed potent activity against cathepsin B. The fraction only dissolved in H_2O, and an addition of MeOH to the aqueous solution precipitates. The active fraction was separated by gel filtration with water, in which the activity eluted at the solvent front. This material was repeatedly purified by reversed-phase HPLC with H_2O to furnish 10 mg of active fraction as a bluish powder. The active fraction was very polar fraction and never retained in reverse phase columns.

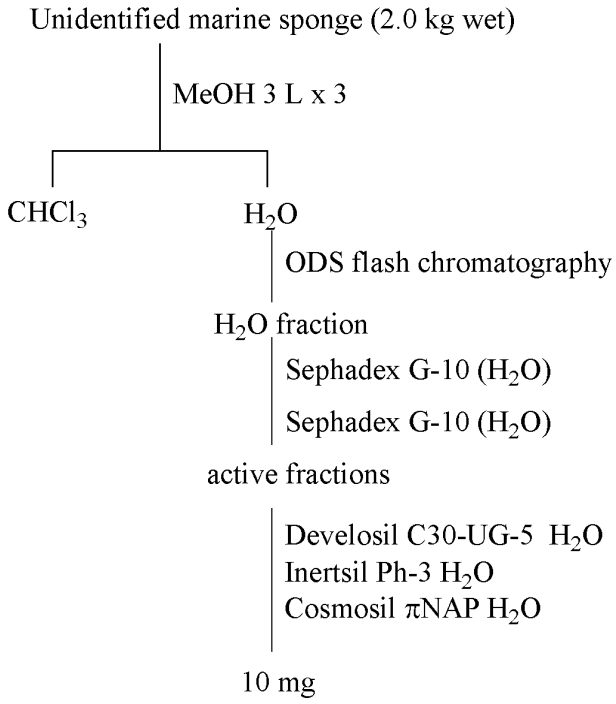


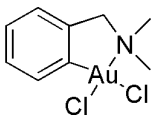
Figure 4-2. Isolation Scheme

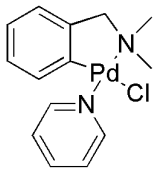
2.2. Elemental Analysis

^{1}H NMR spectrum of the fraction gave broad signals, and ^{13}C NMR spectra did not give signals even after prolonged measurement. These feature implied whether the presence of paramagnetic elements or the absence of organic components. A combustion analysis suggested that the fraction contained 25.94 % carbon, 3.49 % hydrogen, and 4.30 % nitrogen. If the purity of the fraction was high enough, the carbon content was too little, for organic compounds. Therefore, it occurred to us that the rest of the fraction should be composed of inorganic elements. Then ICP - mass spectrometry showed the presence of magnesium, sodium, and nickel in an approximate ratio of 2:1:1. It should be noted that the fraction did not contain halogen or other anionic species. Even if there were carbon and hydrogen atoms in the molecule, they might be broadened by magnesium ion.

2.3. Discussion

Metal-containing organic compounds are well-known, e.g. chlorophylls and vitamin B_{12}.[41,42] Some metal-containing organic molecules inhibit enzymes. Cyclometallated complex containing platinum group metals, as Au (III) (**4-1**), and Pd (II) (**4-2**) are known to inhibit cathepsin B, by coordination with the active site thiol.[43]





4-1 **4-2**

Nickel ion is known as a competitive slow-binding inhibitor of the jack bean urease which is a cysteine protease. Interestingly, the urease is a nickel metalloenzyme, and the nickel ion has an essential role in catalysis.[44] However a high concentration of heavy metal ions including nickel ion, disturbs the active center of urease.[45]

We cannot say for sure, but the nickel ion in our material is may be responsible for inhibitory activity.

2.4. Biological Activity

The fraction inhibits cathepsin B with an IC_{50} value of less than 1.56 μg/mL. The fraction is in pale, blue color, and when dissolved in the buffer, for enzymatic reaction the fraction turned to brown. The color changing may be linked to the cathepsin B inhibitory activity.

3. Experimental Section

3.1. General Procedures

NMR spectra were recorded on a JEOL delta 600 NMR spectrometer at 600 MHz for ^{1}H, and 150 MHz for ^{13}C. ^{1}H chemical shifts was referenced to the solvent peak (DHO) at δ4.80 ppm respectively. Fluorescence for enzyme inhibition assay was determined with a Molecular Devices SPECTRA MAX GEMINI fluorescence spectrometer. Reversed phase HPLC were performed on Develosil C30-UG-5 (10-mm i.d. x 250-mm x 2), Inertsil Ph-3 (10-mm i.d. x 250 mm x 2), and Cosmosil πNAP (10-mm i.d. x 250-mm x 2). Each column was connected to a Shimadzu SPD-10AVP UV detector and two Shimadzu LC-10ADVP pump. H_2O used for HPLC was Milipore Elix 10.

3.2. Animal Material

Sponge samples were collected using SCUBA at a depth of 10-15 m off Shishi Island, (32º 16' N; 130º 14' E) in 2002. The specimens were frozen and kept at -20 ºC until extraction.

3.3 Cathepsin B Inhibitory Assay

Inhibitory activity against cathepsin B was determined essentially according to the method described in Chapter I. The IC_{50} values were determined by plotting the percent inhibition obtained for each sample concentration on semi logarithmic graph paper.

3.4. Extraction and Isolation

The sponge (1 kg, wet weight) was extracted with MeOH (3 L x 3). The combined extracts were concentrated and partitioned between water and $CHCl_3$. The water fraction was separated by ODS flash column chromatography using stepwise elution of aqueous MeOH (0 - 100 %). The faction eluted with water was separated twice by gel filtration on Sephadex G-10 column with water. The active fractions were combined and purified by reversed-phase HPLC on a Develosil C30-UG-5 column with water. The active fraction was purified by HPLC on an Inertsil Ph-3 column with water. The active fraction was purified by HPLC on a Cosmosil πNAP column with water.

4. Supportiong Information

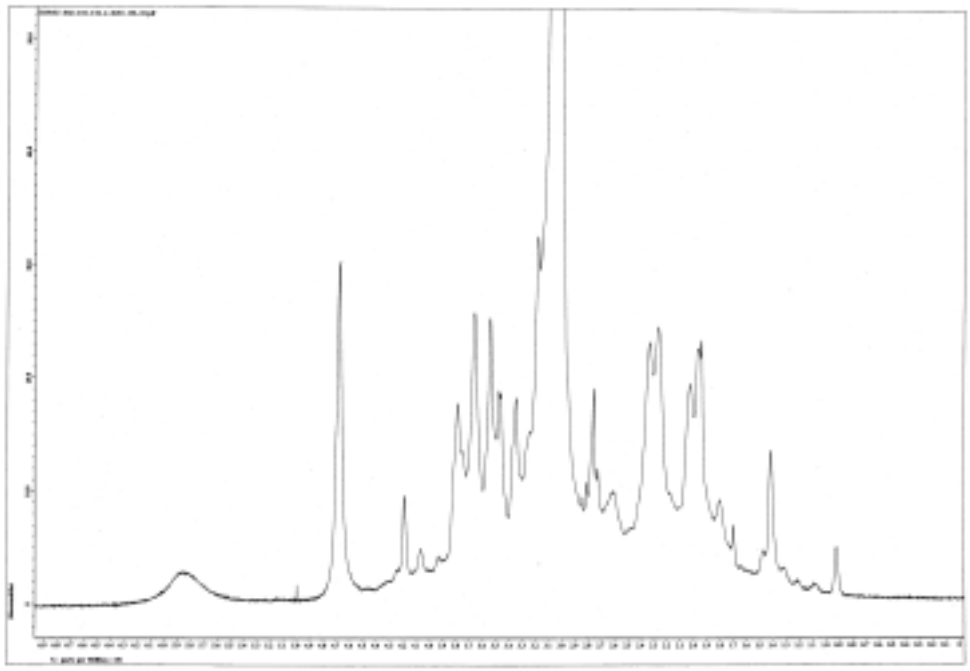


Figure S4-1. ^{1}H NMR spectrum of the fraction in D_2O

有機元素分析結果

138-3	C 25.94 %	H 3.49 %	N 4.30 %

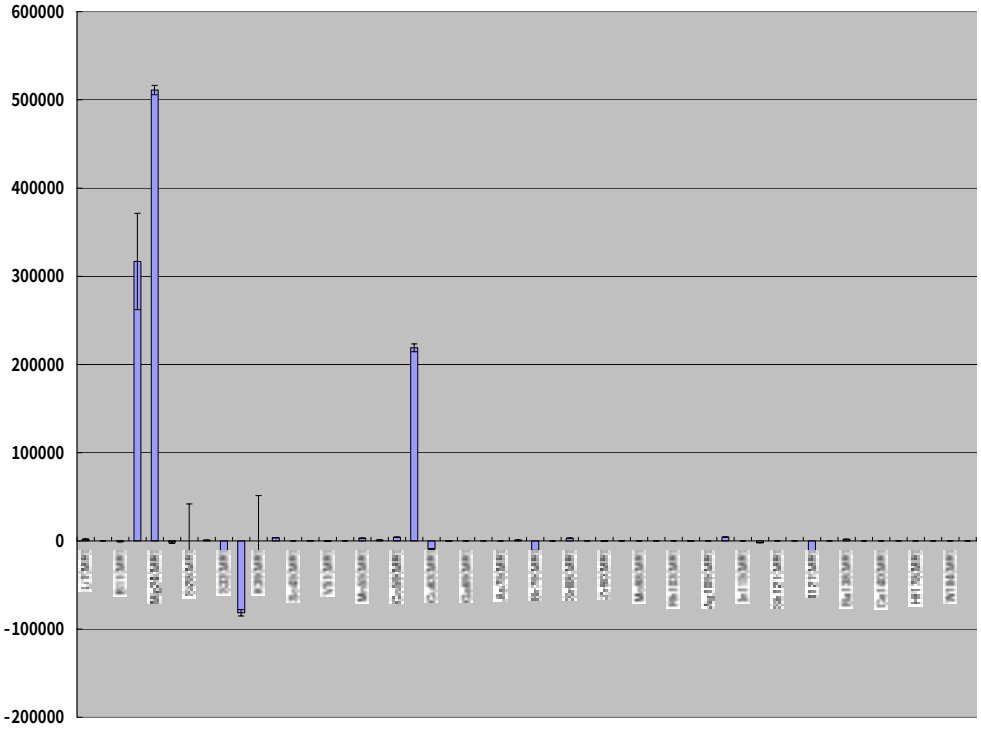
Figure S4-2. Combustion analysis data of the fraction

Isotope	Error	Intensity B cor AVG [cps]	Intensity B cor STD [cps]	Intensity B cor RSD [%]	
Li7(MR)	-	1800.2	601.6	33.42	
Be9(MR)	-	0	0		not def.
B11(MR)	-	-547.2	768.5	140.45	
Na23(MR)	-	316769.8	54632.2	17.25	
Mg24(MR)	-	511109.7	5317	1.04	
Al27(MR)	-	-1480.4	1421	95.99	
Si28(MR)	-	-515553.8	41968.1	8.14	
P31(MR)	-	754.5	348.8	46.23	
S32(MR)	-	-27811.8	3293.1	11.84	
Cl35(MR)	-	-81486.6	3663.1	4.5	
K39(MR)	-	-274974.2	51276.1	18.65	
Ca44(MR)	-	3705	123.8	3.34	
Sc45(MR)	-	-9	1.5	16.97	
Ti48(MR)	-	285.5	61.3	21.48	
V51(MR)	-	-328.5	80.3	24.43	
Cr52(MR)	-	96.8	22.2	22.97	
Mn55(MR)	-	3049.5	244.3	8.01	
Fe56(MR)	-	878.5	402.2	45.79	
Co59(MR)	-	4222.3	92	2.18	
Ni60(MR)	-	218942.4	4548.3	2.08	
Cu63(MR)	-	-9184.3	267	2.91	
Zn66(MR)	-	208.6	27.6	13.24	
Ga69(MR)	-	0	6.9	1.17E+18	
Ge74(MR)	-	22.3	19.6	87.91	
As75(MR)	-	-30	10.7	35.69	
Se78(MR)	-	742.3	470.6	63.4	
Br79(MR)	-	-10898.7	255.1	2.34	
Rb85(MR)	-	-20	21.7	108.32	
Sr88(MR)	-	3085.2	40.1	1.3	
Y89(MR)	-	0	5.1	3.44E+18	
Zr90(MR)	-	-379.3	27	7.11	
Nb93(MR)	-	15.6	10.2	65.4	
Mo98(MR)	-	20	1.9	9.62	
Ru102(MR)	-	1.1	3.3	300	
Rh103(MR)	-	-1.1	6.7	600	
Pd106(MR)	-	159	30.3	19.05	
Ag109(MR)	-	-24.5	11.7	47.93	
Cd114(MR)	-	4302.1	196.9	4.58	
In115(MR)	-	-23.3	9.6	41.24	
Sn120(MR)	-	-1945.2	139.9	7.19	
Sb121(MR)	-	-3.3	5.1	152.75	

Te130(MR)	-	8.9	24.6	276.71
I127(MR)	-	-17957.9	2854.1	15.89
Cs133(MR)	-	-4.4	3.8	86.6
Ba138(MR)	-	1774.6	156.3	8.81
La139(MR)	-	-7.8	10.2	130.93
Ce140(MR)	-	-7.8	7.7	98.97
Gd158(MR)	-	-1.1	0	0
Hf178(MR)	-	-5.6	0	0
Ta181(MR)	-	6.7	10.2	152.75
W184(MR)	-	-4.4	1.9	43.3
Re185(MR)	-	-6.7	0	0
Os189(MR)	-	1.1	1.9	173.21
Ir193(MR)	-	0	0	not def.
Pt195(MR)	-	-40	8.4	20.95
Au197(MR)	-	-1.1	3.8	346.41
Hg202(MR)	-	52.3	13.5	25.85
Tl205(MR)	-	-28.9	20	69.23
Pb208(MR)	-	-205.8	18.6	9.02
Th232(MR)	-	-22.2	3.8	17.31
U238(MR)	-	-418.7	49.2	11.76

Errorcodes : S=Amplifier Skipped D=Intensity Defocussed
O=Overflow

Table S4-1. ICP mass srectorometry of the fraction



Graph S4-1. ICP mass srectorometry of the fraction

Chapter V
An Inhibitory Fraction of Cathepsin B, from an Unidentified Deep-water Sponge

1.Introduction

The extract of an unidentified deep-water sponge collected at Ōshima Shinsone (Figure 5-1) exhibited activity against cathepsin B. Extraction of the sponge and fractionation by following the inhibitory activity afforded an active fraction containing a new schulzeine type compound. This chapter deals with the isolation, partial structure elucidation, and the activity of the fraction.

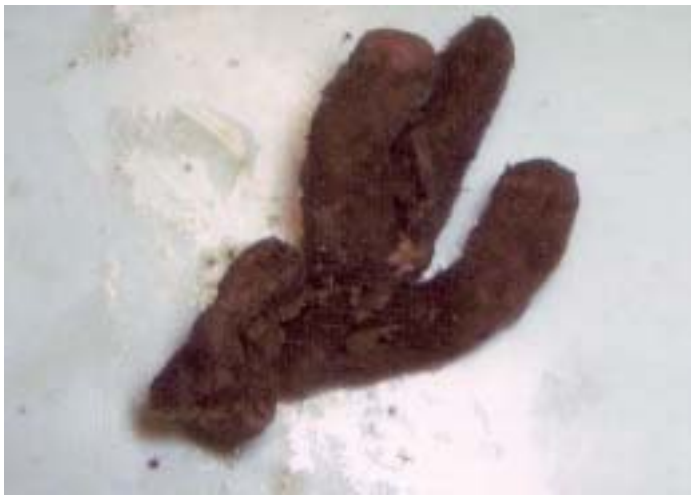


Figure 5-1. Unidentified deep-water sponge

2.Results and Discussion

2.1. Extraction and Isolation

The sponge (400 g, wet weight) was extracted with MeOH, and EtOH. The extracts were combined, concentrated, and partitioned between H_2O and $CHCl_3$, and the aqueous layer was partitioned between H_2O and *n*-BuOH. The $CHCl_3$ layer was partitioned between 90 % MeOH and *n*-hexane, and the 90 % MeOH layer was partitioned between 60 % MeOH and $CHCl_3$. The *n*-BuOH layer and the 60 % MeOH layer showed cathepsin B inhibitory activity. These fractions were combined and separated by silica gel flash column chromatography and reversed phase flash chromatography. Streaked TLC spots and broad HPLC peaks suggested that the active fraction contained the sulfate esters. Therefore, the active fraction was purified by C18 HPLC using aqueous MeCN in the presence of a high concentration of $NaClO_4$ to afford an active fraction

(Figure 5-2). By adding $NaClO_4$ in the mobile phase the HPLC pattern was significantly improved.

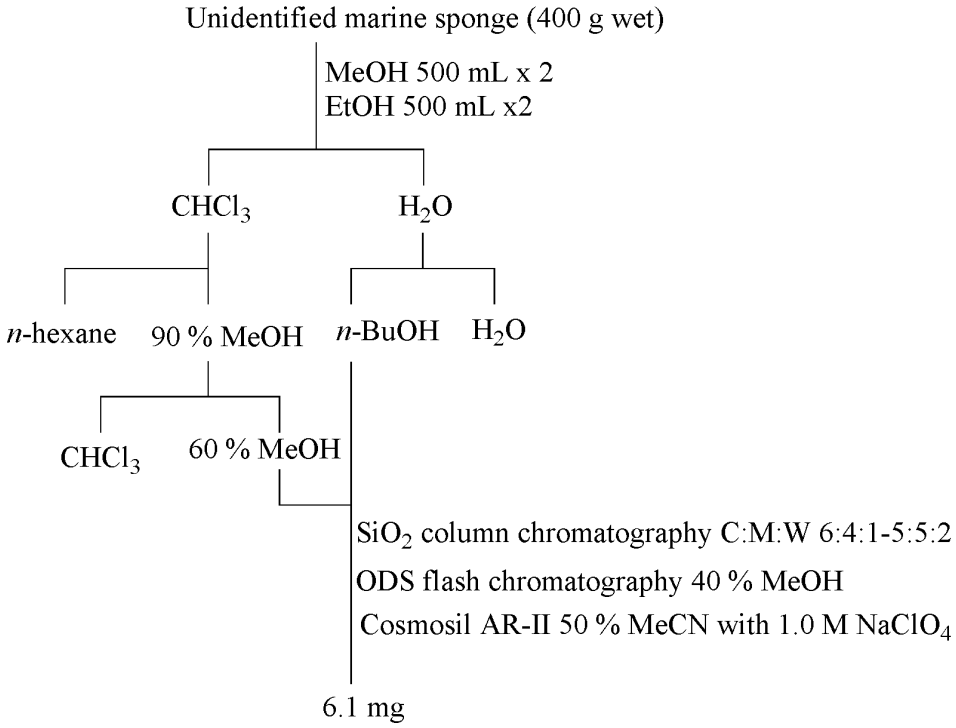


Figure 5-2. Isolation scheme of the active fraction

2.2. Structure Elucidation

Analysis of the ^{1}H NMR spectrum of the fraction in conjunction with the edited HSQC data indicated the presence of two CH_3, 23 or more CH_2, and nine CH. ^{13}C NMR spectrum of the compound further suggested the presence of 12 non-protonated carbons.

The COSY spectrum showed three partial structures (Figure 5-3). Partial structure **a** consisted of three proton spin systems, C11b-C1-C2-C3, C6-C7, and C2'-C3'. HMBC correlations, H3/C1', H2'/C1, and H3'/C1', suggested that C11b-C1-C2-C3 spin system and C2'-C3' spin system were connected through an amide or an ester bond. ^{1}H and ^{13}C chemical shift values suggested that C6 and C11b were linked to a nitrogen atom, and that C7 was flanked by an aromatic carbon. HMBC correlations, H3/C4, H6/C4, H6/C11a, H7/C11a, and H11b/C11a, suggested that C11b-C1-C2-C3 and C6-C7 spin systems formed an octahydro-quinolizin-4-one unit. The chemical shift values for C9 (δ_C 157.0) and C11 (δ_C 158.1) and HMBC cross peaks, H7/C7a, H8/C7, C10, C11, C11a, and H10/C9, C11, C11a, suggested the presence of a tetrasubstituted benzene ring which was fused with the octahydroquinolizin-4-one unit. The chemical shifts for these portion

resembled those with schulzene A (Figure 5-4).[46] Partial structure **b** was made of two proton spin systems connected through a ketone. HMBC correlation, H1.58 ppm/C200.2 ppm and H2.82 ppm/C200.2 ppm, supported this idea. Partial structure **c** was a terminal *n*-propyl group. These partial structures should be connected through methylene chain. There were minor signals which accounted for the same tricyclic system in unit **a**. This finding clearly displayed that the fraction required further purification.

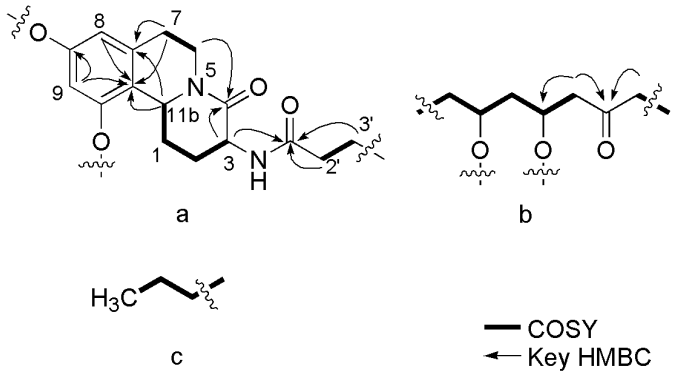
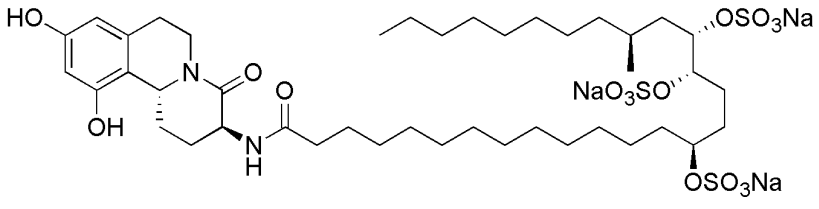
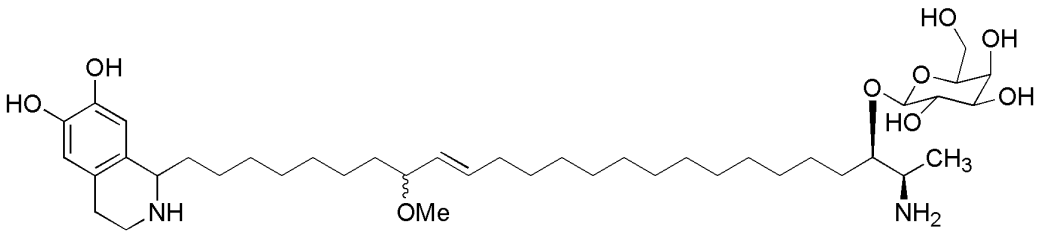


Figure 5-3. Partial structures of the active fraction

Isoquinolines are rare among marine natural products. The schulzeines, isolated from *Penares schulzei* as α-glucosidase inhibitors are characterized by 9,11-dihydroxytetrahydro-isoquinoline with a long alkyl chain whose central portion was functionalized with three sulfate groups. Oceanalin A isolated from a marine sponge *Oceanapia* sp., has dihyroxytetrahydro-isoquinolin and a long alkyl chain with a methoxyl group.[47] (Figure 5-4)



Schulzeine A



Oceanalin A

Figure 5-4. Related compounds

2.3. Biological Activity

The fraction inhibits cathepsin B with an IC_{50} value of 6.25 μg/mL.

3. Experimental Section

3.1. General Procedures

NMR spectra were recorded on a JEOL delta 600 NMR spectrometer at 600 MHz for ^{1}H, and 150 MHz for ^{13}C. Standard parameters were used for the 2D NMR spectra obtained, which included gradient COSY, edited HSQC, HMBC, and TOCSY. Chemical shits of ^{1}H and ^{13}C NMR were referenced to the solvent peaks; δ_H 3.31 and δ_C 49.15 for CD_3OD. Fluorescence for enzyme inhibition assay was determined with a Molecular Devices SPECTRA MAX GEMINI fluorescence spectrometer. Reverse phase HPLC was performed on Cosmosil AR-II (10- mm i.d. x 250-mm) connected to a Shimadzu SPD-6AV UV detector and Shimadzu LC-6AD pump. Acetonitrile used for HPLC were Nacalai Tesque JIS special grade and the H_2O used was Millipore Elix 10. Sodium perchlorate used for HPLC was nacalai tesque extra pure reagent.

3.2. Animal Material

Sponge sample was collected by dredging at a depth of 150 m at Ōshima Shinsone, (28º 80' N; 129º 30' E) in 2004. The specimen was frozen after collection and preserved at -20 ºC until extraction.

3.3. Cathepsin B Inhibitory Assay

Inhibitory activity against cathepsin B was determined essentially according to the method described in Chapter I. The IC_{50} values were determined by plotting the percent inhibition obtained for each sample concentration on semi logarithmic graph paper.

3.4. Extraction and Isolation

The sponge (400 g, wet weight) was extracted with MeOH (500 mL x2) and EtOH (500 mL x2). The combined extracts were concentrated and partitioned between water (500 mL) and $CHCl_3$ (500 mL x2), the aqueous layer was partitioned between water (500 mL) and *n*-BuOH (500 mL). Organic layer was separated between 90 % MeOH (400 mL) and *n*-hexane (600 mL), and the 90 % MeOH layer was separated between 60 % MeOH (600 mL) and $CHCl_3$ (300 mL). The aqueous MeOH and *n*-BuOH fractions were combined and separated by silica gel column

chromatography (C:M:W 10:0:0-5:5:2 500 mL each). Fraction eluted with C:M:W 6:4:1 and 5:5:2, showed cathepsin B inhibitory activity, these fraction were combined, evaporated and separated by ODS flash column chromatography using stepwise elution of aqueous MeOH (0 - 100 % 300 mL each) and aqueous MeCN (70, 80 % 300 mL each). The faction eluted with 40 % MeOH was active against cathepsin B and chromatographed using C_{18} HPLC at flow late of 2 mL/min with isocratic conditions of 50 % MeCN/50 % H_2O containing 1 M $NaClO_4$. The active fraction was desalted by ODS column to get active fraction (6.1 mg, 1.5 x 10^{-3} % yield based on weight).

4. Supportiong Information

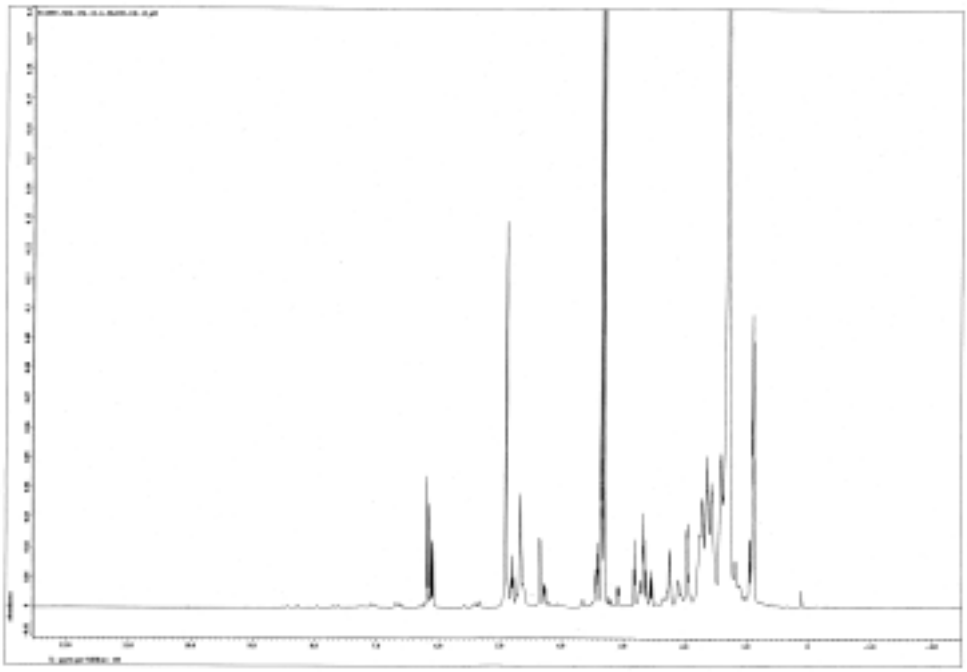


Figure S5-1. ^{1}H NMR spectrum of the fraction in MeOD-d_4

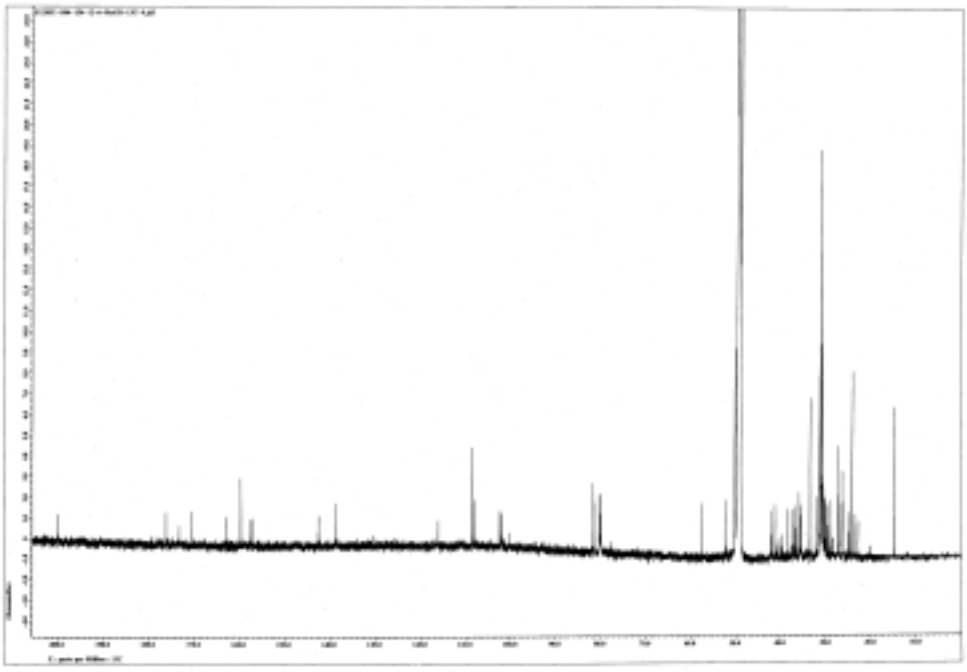


Figure S5-2. ^{13}C NMR spectrum of the fraction in MeOD-d_4

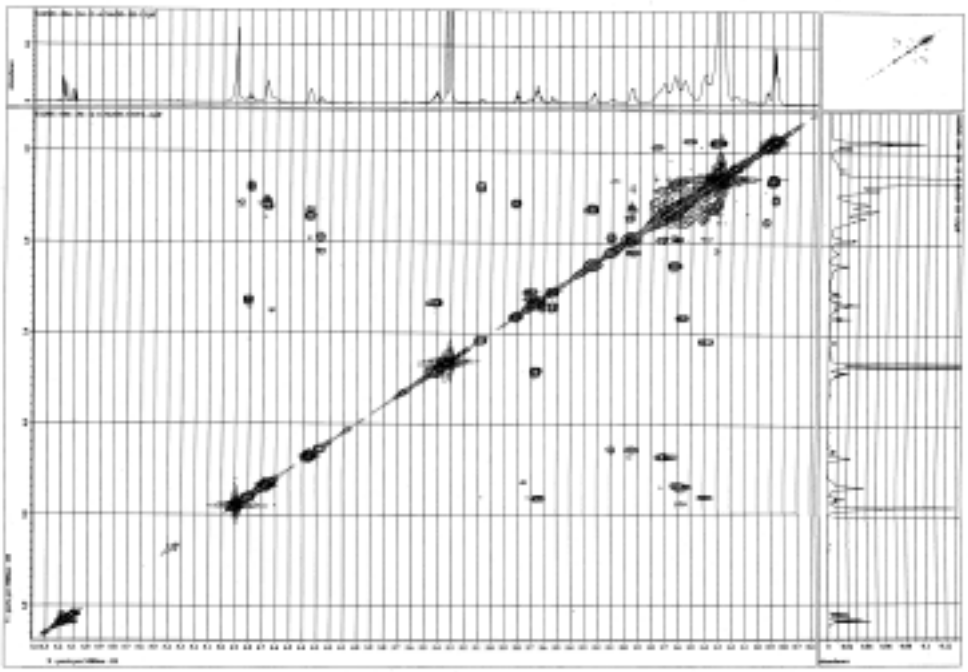


Figure S5-3. COSY spectrum of the fraction in MeOD-d_4

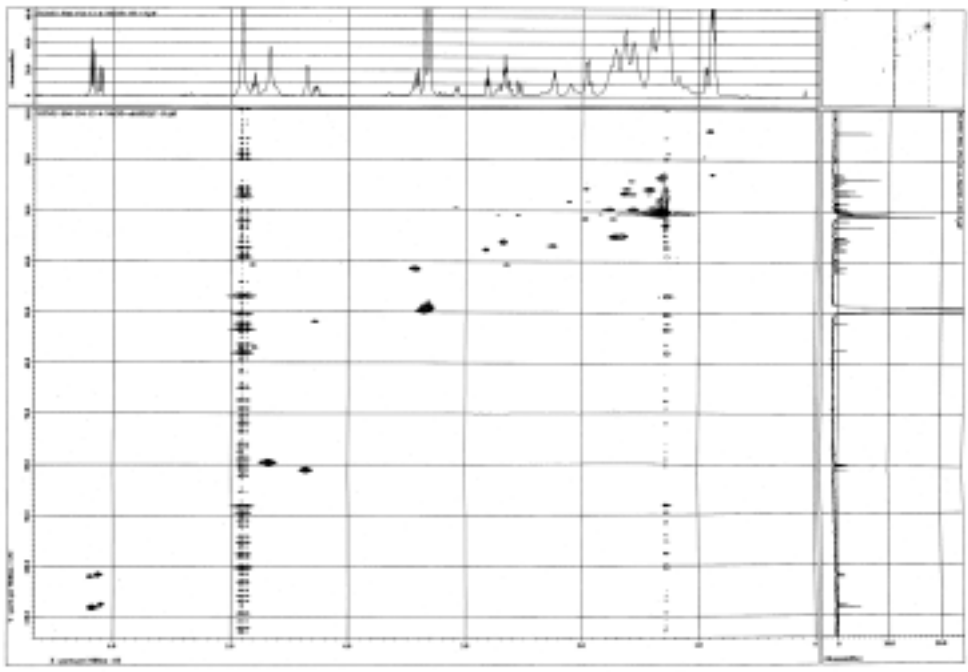


Figure S5-4. HSQC spectrum of the fraction in MeOD-d_4

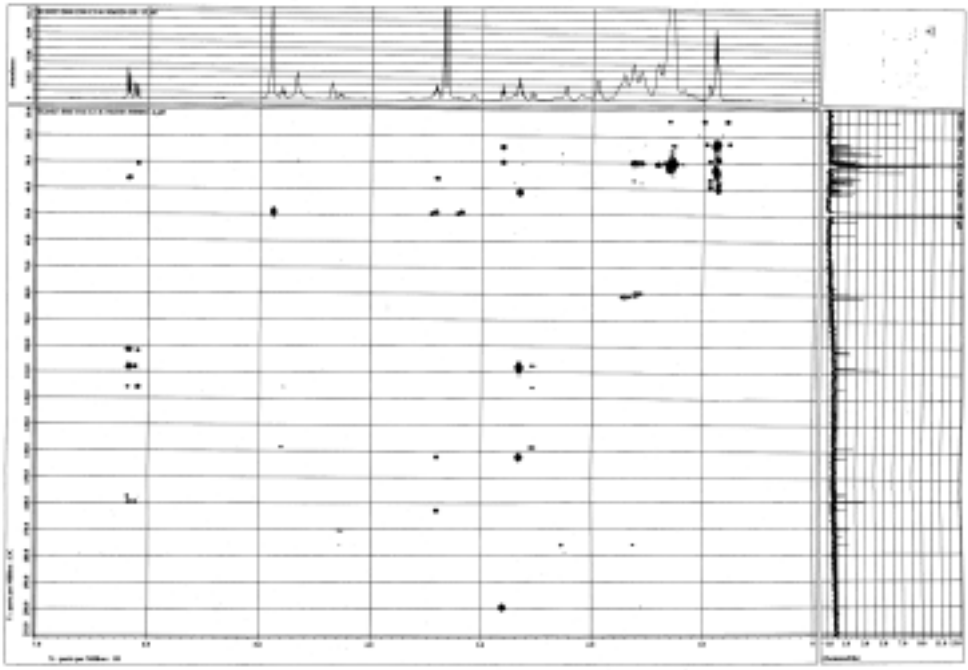


Figure S5-5. HMBCspectrum of the fraction in MeOD-d_4

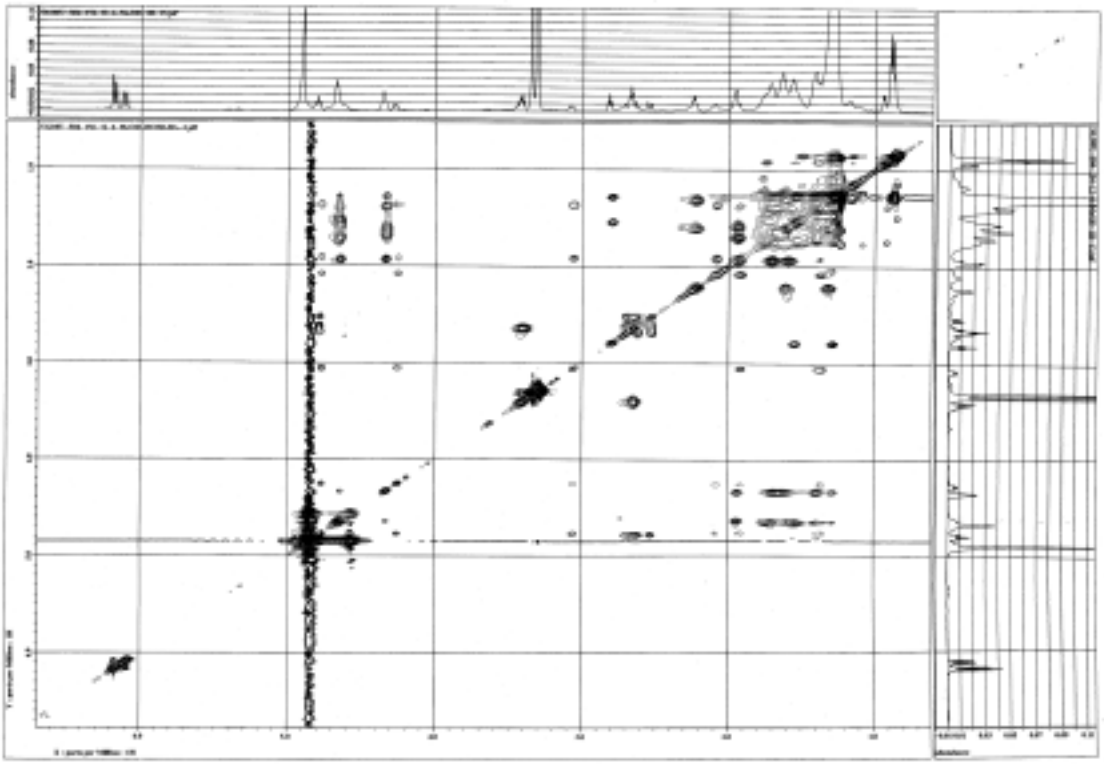


Figure S5-6. TOCSY spectrum of the fraction in MeOD-d_4

Conclusions

In order to discover novel anti-tumor agents from untapped marine organisms, inhibitors of cathepsin B were searched from Japanese marine invertebrates. The extracts of 308 marine invertebrate were examined, and 25% of organic extract and 15% aqueous extract showed activity.

Through this study, three new cathepsin B inhibitors were isolated and two cathepsin B inhibitory fractions were found. Structures of these compounds were elucidated by spectroscopic methods.

Asteropterin (**2-1**) from the marine sponge *Asteropus simplex* was a new pteridine derivative, connected to *N*-methyl histamine. Interestingly, asteropterin inhibits cathepsin B, but its components such as, lumazine, histamine and their mixture do not inhibit cathepsin B. It suggests that the combination of the lumazine and histamine units is important for the inhibition.

Shishicrellastatins A (**3-1**) and B (**3-2**) from the marine sponge *Crella (Yvesia) spinulata* were new dimeric steroid. Previously, crellastatins were isolated from the sponge of the same genus. Shishicrellastatins and crellastatins share the common feature of steroids dimerized through side chains. The origin of these highly unusual molecules is an interesting question.

The active fraction described in chapter IV contained a large proportion of metal ions. It shows potent inhibitory activity against cathepsin B. Although the material we obtained was not pure, isolation and structure elucidation of the active constituent in the fraction, is a challenging problem.

The compound discussed in chapter V was isolated from a deep-water sponge, and related to schulzeines from the shallow-water sponge *Penares schulzei*. The observation that this class of compounds inhibits cathepsin B is noteworthy.

In this study it was demonstrated that marine invertebrate including those from the deep-water are rich source of enzymes inhibitors.

It is hoped that inhibitors obtained in this study will be useful in the welfare mankind.

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