STUDIES ON SERINE PROTEASE INHIBITORS FROM MARINE INVERTEBRATES

(海産無脊椎動物のセリンプロテアーゼ阻害物質に関する研究)

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STUDIES ON SERINE PROTEASE INHIBITORS FROM MARINE INVERTEBRATES

A Dissertation Submitted to the Graduate Division of the University of Tokyo in Partial Fulfilment of the Requirements for the Degree of Doctor of Philosophy in Fisheries Science

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By

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ABSTRACT

A total of 450 marine invertebrates consisting of 363 sponges, 48 coelenterates, 3 bryozoans, and 36 tunicates, were tested for inhibitory activities against thrombin and trypsin. Four species of sponges have been chosen from the viewpoints of activity and availability of samples. Eleven active compounds, of which ten were new, have been isolated and identified.

In Chapter I, the screening of 450 Japanese marine invertebrates is described. Marine sponges, of which 13.2 % inhibited thrombin and 9.1 % inhibited trypsin, and tunicates, of which 13.9 % inhibited trypsin, were considered to be good sources for anti-thrombin or anti-trypsin inhibitors.

Chapter II deals with the isolation and identification of oroidine from a sponge *Agelas* sp. collected off Hachijo-jima Island. It was the first report of the protease inhibitory activity for oroidine.

The isolation and structure elucidation of five new peptides, nazumamide A and cyclotheonamides C-F, from the marine sponge *Theonella swinhoei* collected off Hachijo-jima Island are described in Chapter III. The structure of nazumamide A was determined as 2,5-dihydroxybenzoyl-L-arginyl-L-prolyl-L-isoleucyl-L-α-aminobutyric acid. Nazumamide A inhibited thrombin with an IC_{50} of 2.8 μg/mL. Cyclotheonamides C-F were anologues of cyclotheonamide A, and their structures were determined by comparison of spectral data with those of cyclotheonamide A. They inhibited thrombin and trypsin with IC_{50}'s of 0.27-0.52 and 0.32-0.48 μg/mL, respectively.

Chapter IV deals with the isolation and structure elucidation of a thrombin and trypsin inhibitor, cyclotheonamide G from a morphologically different *Theonella swinhoei* which did not contain cyclotheonamide A. In cyclotheonamide G, a D-Phe residue in cyclotheonamide A was replaced by a D-Ile residue, and a formyl group by an L-Ala-Pha (phenyl acetyl) unit. Cyclotheonamide G inhibited thrombin and trypsin with IC_{50}'s of 0.24 and 0.31 μg/mL, respectively.

In Chapter V, the isolations and structure elucidations of toxadocials A-C and toxadocic acid A, novel tetra sulfated metabolites, from the sponge *Toxadocia cylindrica* collected in Hachijo-jima Island are described. It was emphasized that the FAB-MS/MS analysis was effective in the structure elucidation. Toxadocials A-C and toxadocic acid A inhibited thrombin with IC_{50}'s of 2.9-6.5 μg/mL.

INTRODUCTION

The ocean covers 71 % of the earth, and provides various circumstances; temperature, pressure, light, and salinity. Naturally, there is a diversity of living spaces. However, they have not been explored by humans until recent days; only a small part of them were used for food. In late 1960's, a new research field, marine natural products chemistry was born as results of such driving forces as popularization of SCUBA diving, the discovery of large amounts of prostaglandins from a Caribbean gorgonian[1], development of Ara-A and Ara-C from spongouridine and other novel sponge nucleosides. Therefore, research activity in this field have increased, and over 4000 of new compounds have been discovered from marine organisms.[2] Some of them are structurally novel and highly bioactive. Now, metabolites of marine organisms are believed to be useful leads.

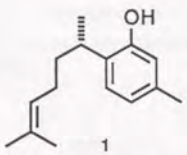
Holotoxins A and B isolated from the sea cucumber *Stichopus japonicus* are clinically used for dermatophytosis.[3] Cytotoxic substances have been actively exploited from marine organisms. And several promising substances have been discovered; didemnin B[4], bryostatin 1[5], halichondrin B[6], and dolastatin 10[7] are the most hopeful antitumor substances. Holotoxins were the rare sample of a drug of marine origin.

In addition to cytotoxic substances, isolation of inhibitors of such enzymes as H^+,K^+-ATPase, Na^+,K^+-ATPase, aldose reductase, and phospholipase A_2 have been attempted from marine organisms, and several inhibitors were obtained. These enzymes are related to human diseases, and their inhibitors are believed to be useful leads. Inhibitors of these enzymes discovered from marine organisms are described below.

H^+,K^+-ATPase inhibitors

H^+,K^+-ATPase of the intracellular secretory canalicule is activated by histamine and releases H^+ into gastral cavity. The excess activation of this ATPase leads to hyper acidity and causes digestion of mucosa. To suppress hyper acidity, control of secretion is more efficient than to neutralize gastric juice. H^+,K^+-ATPase inhibitors is expected to be antiulcer drugs.

Fusetani *et al.* isolated (+)-curcuphenol (**1**) and dehydrocurcuphenol (**2**) from a marine sponge *Epipolasis* sp.[8] **1** and **2** inhibited H^+,K^+-ATPase with IC_{50}'s of 8.3 and 23 μM, respectively. Hexaprenylhydroquinone sulfate (**3**)(IC_{50} 4.6 μM)[9] was obtained from a marine sponge *Dysidea* sp. collected off Hachijo-jima Island. A spermine derivative (**4**)(IC_{50} 5.5 μM) was isolated from a soft coral *Sinularia* sp. collected in Kochi Prefecture. The sponge *Siphonochalina truncata* collected at Izu Peninsula yielded three polyacetylenes, siphonodiol (**5**), dihydrosiphnodiol (**6**) and tetrahydrosiphonodiol (**7**)[10]. **5**-**7** inhibited H^+,K^+-ATPase with equal IC_{50}'s of 10 μM.



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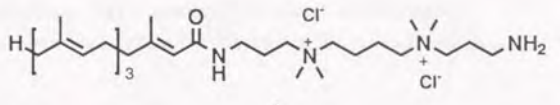

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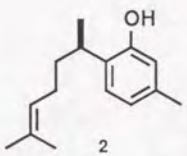

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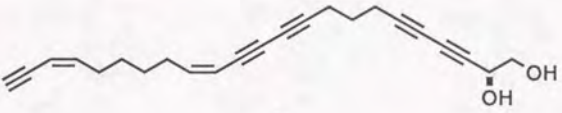

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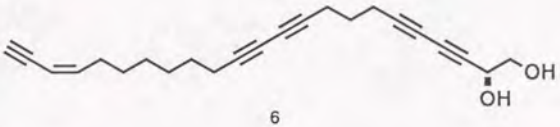

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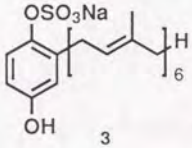

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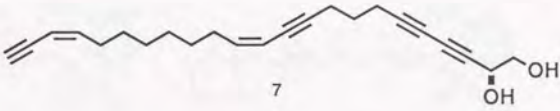

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Na⁺,K⁺-ATPase Inhibitors

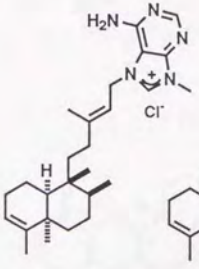
Na⁺,K⁺-ATPase regulates Na⁺ transportation in cell membranes and is directly related to contraction and relaxation of smooth muscles. Therefore, inhibitors of the enzyme will be useful for cardiovascular diseases. Agelasines A-F (**8**)-(**13**), 9-methyladenine derivatives of diterpenes, obtained from the Okinawan sponge *Agelas nakamurai*,[11,12] showed anti-Na⁺,K⁺-ATPase activity and antispasmodic properties. Two diterpene derivatives of hypotaurocyamine, agelasidines B (**14**) and C (**15**),[13] from the Okinawan sponge *Agelas nakamurai*, inhibited not only Na⁺,K⁺-ATPase with the IC_{50}'s of 10-50 μM, but also contraction of smooth muscle .

Dibromotyrosine derivatives, purealin (**16**) and lipopurealines A-C (**17**)-(**19**), from the Okinawan marine sponge *Psammaphysilla purea*[14,15] were Na⁺,K⁺-ATPase and myosin Ca⁺-ATPase inhibitors. Purealin was the first natural product which activates myosin K⁺, (EDTA)-ATPase. Apaplysillins I (**20**) and II (**21**), dibromotyrosine derivatives from *P. arabica*[16], possessed antimicrobial activity and inhibited Na⁺,K⁺-ATPase.

A pentacyclic quinone, xestquinone (**22**) was isolated from the Okinawan sponge *Xestospongia sapra* together with the known antimicrobial substance halenaquinone.[17] Xestoquinone was the first example of marine natural products having parallelism between the inotropic action and Na⁺/K⁺-ATPase inhibition.

Ptilodene (**23**) was obtained as an unstable minor constituent from the Oregon red alga *Ptilota filicina*.[18] Ptilodene showed the inhibition activity against dog renal Na⁺,K⁺-ATPase (57 % at 10^{-5} M).

Xestspongic acid ethyl ester (**24**), a bromopolyacetylene, from the sponge *Xestospongia testudinaria* collected at Mayotte[19] inhibited Na^+,K^+-ATPase with ID_{50} of 10^{-4}-10^{-3} M.



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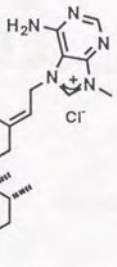

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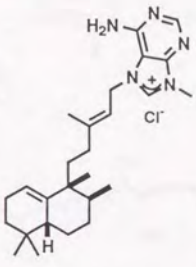

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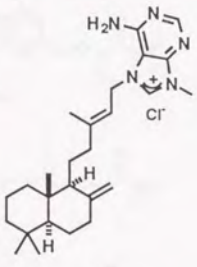

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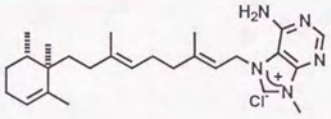

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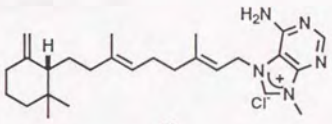

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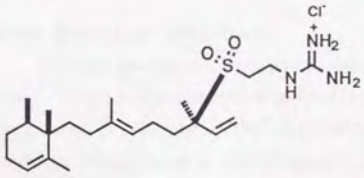

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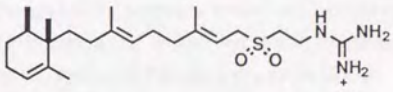

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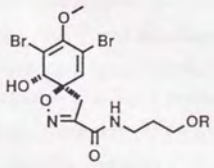

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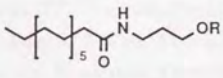

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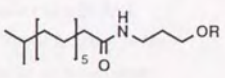

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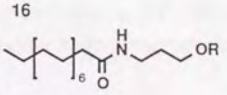

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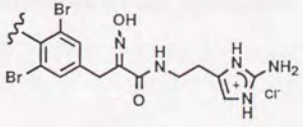

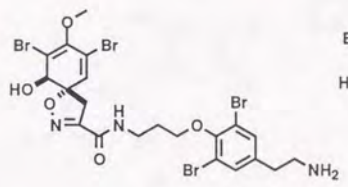

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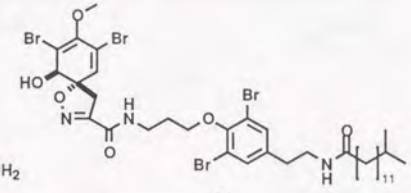

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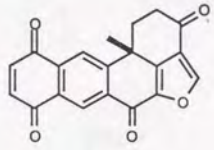

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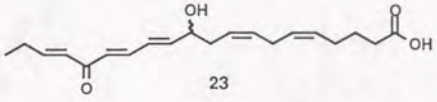

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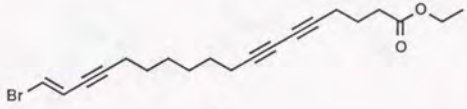

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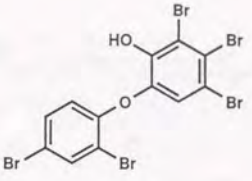
Aldose Reductase Inhibitors

Aldose reductase is known as an enzyme which catalyzes the reduction of glucose to sorbitol. Unusual accumulation of sorbitol in the eye lens or peripheral nerves was thought to cause cataract or neuropathy. The inhibition of aldose reductase will prevent the accumulation of sorbitol. Nakagawa *et al.* who screened over 100 species of Palauan sponges for aldose reductase inhibitory activity and obtained five inhibitors[20]; pentabromophenol (**25**) originally obtained from *Dysidea* sp.[21] inhibited 62.8 % activity at the concentration of 2 μM. While palinurin (**26**)[22] and secomanoalide (**27**)[23], metabolites of *Ircinia ramosa* and *Dactylospongia metachromia*, inhibited 37.7 % and 81.9 % of enzyme activity at the concentration of 2-3 μM, respectively. In addition, a new inhibitory sesterterpene, dysideapalaunic acid (**28**) was isolated from *Dysidea* sp. Dysideapalaunic acid was synthesized by Hagiwara *et al.* and was established its absolute configuration.[24] Two bovine lens aldose reductase inhibitors (**29**)-(**30**) were isolated from the red alga *Asparagopsis taxiformis* collected in Kagoshima Prefecture.[25]



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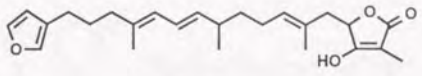

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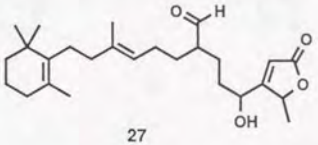

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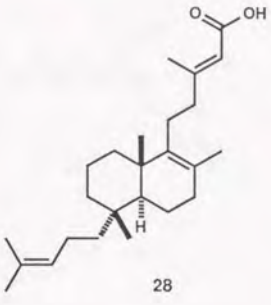

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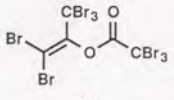

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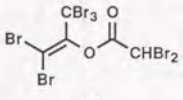

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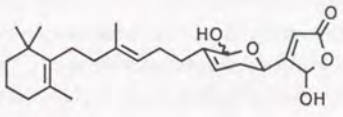
Phospholipase A_2 (PLA_2) Inhibitors

PLA_2 hydrolyzes cellular phospholipids and releases arachidonic acid, from which prostaglandins are synthesized. Prostaglandins are implicated in various forms of pain and inflammation. In biogenesis of prostaglandins, arachidonic acid formation is the rate determing step, therefore PLA_2 inhibitors are expected to be antiinflammatory drugs. Manoalide (**31**) was first reported by Scheuer *et al.* as an antibiotic sesterterpenoid from the marine sponge *Luffariella variabilis* collected at the Marshall Islands[26] and later found to be a potent PLA_2 inhibitor.[27] Three derivatives, secomanoalide (**27**), Z-neomanoalide (**32**) and *E*-neomanoalide (**33**) have been also obtained from the same sponge.[23] Luffariellolide (**34**) was reported by Faulkner *et al.* from a Palauan sponge *Luffariella* sp.[28] Luffariellolide inhibited the bee venom PLA_2 with IC_{50} of 0.23 μM. The maximum inhibition obtainable with luffariellolide was only 80 % as compared to complete inactivation of PLA_2 by manoalide. Inhibition by luffariellolide was partially (approx. 30 %) reversed by dialysis whereas manoalide inhibition is completely irreversible under dialysis condition. Luffolide (**35**) was also obtained from *Luffariella* sp. as a PLA_2 inhibitor.[29]

Matsunaga *et al.* isolated discodermins A-D (**36**)-(**39**) from the sponge *Discodermia kiiensis* collected at Shikine-jima Island.[30,31] Discodermins A-D inhibited PLA_2 with IC_{50}'s of 3.5-7.0 μM.

Palinurin (**26**) and hexaprenylhydroquinone sulfate (**3**) also showed PLA_2 inhibitory activity.



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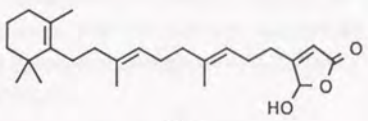

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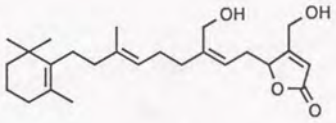

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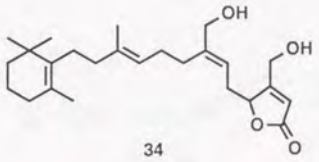

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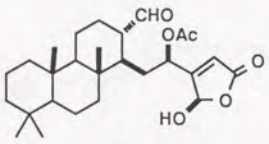

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HCO-D-Ala-L-Phe-X-D-Trp-L-Arg-D-Cys(O_3H)-L-Thr-L-MeGln-D-Leu-L-Asn-L-Thr-Sar

36; X = D-*t*-Leu-L-*t*-Leu
37; X = D-Val-L-*t*-Leu
38; X = D-*t*-Leu-L-Val
39; X = D-Val-L-Val

Protease Inhibitors

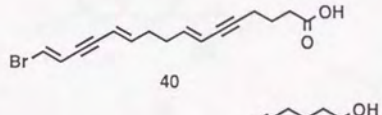
Many proteases are existing in the organisms and regulating various biological functions. Dysfunctions of these proteases may lead to diseases: excess activity of angiotensin converting enzyme (ACE) may result in hypertension; unusual activation of trypsin in pancreas causes acute pancreatitis; disorder of thrombin leads to arteriosclerosis or thrombosis; excessive cysteine protease activity is involved in muscular dystrophy[32] and bone resorption[33].

Anti HIV protease inhibitors have been vigorously investigated; seven bromoacetylenic acids (**40**)-(**46**) were obtained as inhibitors from *Xestospongia muta*.[34]

ACE plays an important role in regulation of blood pressure. It forms the vasoconstrictor angiotensin II from the inactive angiotensin I to inactivate the vasodilator bradykinin. Again, palinurin (**26**) showed inhibitory activity against ACE.

From a marine sponge of *Theonella* sp. , cyclotheonamides A (**47**) and B (**48**) which inhibited thrombin, trypsin and plasmin were obtained.[35] Cyclotheonamides A and B were

synthesized by some groups[36,37] and studied its mechanism of binding to thrombin.[38] Except for these two compounds, only tannins such as eckol (**49**)[39-41] and its derivatives (**50**)-(**51**)[42] from the brown alga *Ecklonia arborea* and phlorofucofuroeckol A (**52**)[43] from *Ecklonia kurome* were obtained for plasmin inhibitors.



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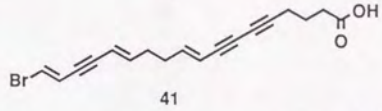

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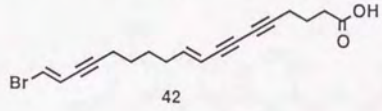

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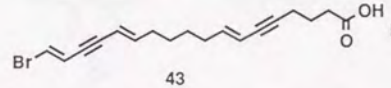

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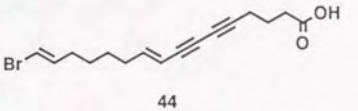

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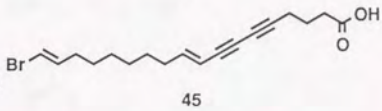

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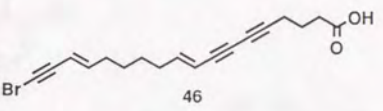
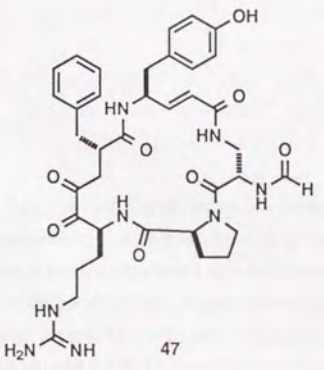
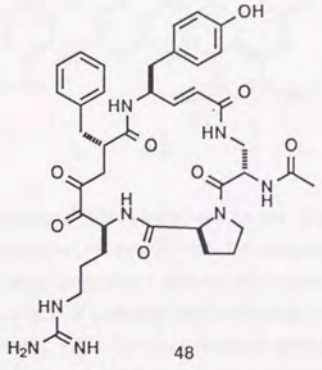


46



47



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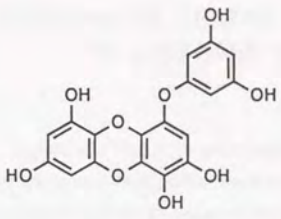

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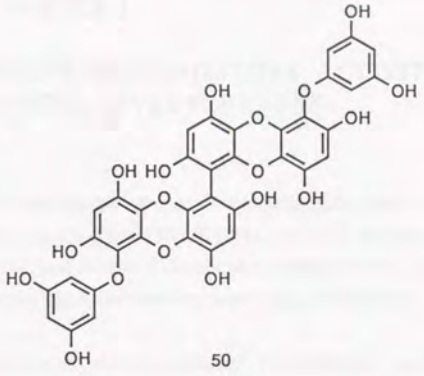
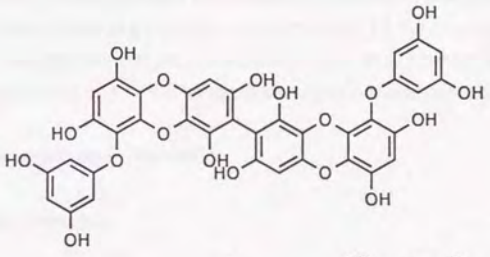
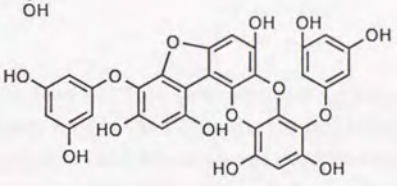


50



51



52

As mentioned above, only a few serine protease inhibitors have been known from marine organisms. Large-scale screenings for protease inhibitors will show the richness of the inhibitors in marine organisms, as was the case of antimicrobial or cytotoxic substances.

In the present study, extracts from 450 specimens of Japanese marine invertebrates were tested for anti-thrombin and anti-trypsin activities. About ten percent specimens (11.8 % for thrombin and 9.1 % for trypsin) were active in the assay. Then four species of sponge which showed activities were extracted. The active principles were isolated and their structures were elucidated. The details are described in the following chapters.

CHAPTER I

SCREENING OF SERINE PROTEASE INHIBITORY ACTIVITY IN JAPANESE MARINE INVERTEBRATES

A total of 450 marine invertebrates consisting of sponges, coelenterates, bryozoans, and tunicates collected from the Izu Peninsula, Hachijo-jima Island, Kyushu, and Shikoku were tested for the inhibitory activities of thrombin and trypsin. Collections were emphasized mainly on sponges, coelenterates, bryozoans, and tunicates, because they were easily collected in relatively large amounts.

Freshly-collected specimens were extracted with hot methanol. The hydrophilic and lipophilic layers of extract were screened for thrombin and trypsin inhibitory activities. Thrombin inhibitory activity was observed in 13.2 % of sponges, 8.3 % of coelenterates, and 2.7 % of tunicates, whereas trypsin inhibition was observed in 9.1 % of sponges, 6.3 % of coelenterates, and 13.9 % of tunicates. Activities were not observed in bryozoan samples.

1. Results and Discussion

1. 1. Samples

Invertebrates samples tested are listed in Table I-1. They were collected at various part of Japanese coasts, viz., the Izu peninsula (Atami, Kumomi, and Toji), Hachijo-jima Island, Shikoku (Kamaki, Nihei-jima Island, and Okino-shima Island), and Kyushu (Kuchinoerabu Island, Mage-jima Island, Nichinan-oshima Island, Shimano-urashima Island, Tanega-shima Island, and Yaku-shima Island). Deep-sea sponges were collected around Amami Islands.

Table I-1. A List of Animals Tested

Sample No.	Genus or Species	Collection Site
Sponges		
S5001-5016	not identified	Toji, Izu Pen.
S5101-5105	not identified	Hachijo-jima Is.
S5106	*Xestospongia* sp.	Hachijo-jima Is.
S5107-5122	not identified	Hachijo-jima Is.
S5123	*Psammaplysilla purpurea*	Hachijo-jima Is.
S5124-5130	not identified	Hachijo-jima Is.
S5131	*Jaspis* sp.	Hachijo-jima Is.
S5132-5139	not identified	Hachijo-jima Is.

Sample No.	Genus or Species	Collection Site
S5140	*Erylus placenta*	Hachijo-jima Is.
S5141-5142	not identified	Hachijo-jima Is.
S5143	*Theonella swinhoei*	Hachijo-jima Is.
S5144-5147	not identified	Hachijo-jima Is.
S5201-5203	not identified	Hachijo-jima Is.
S5204	*Theonella* sp.	Atami, Izu Pen.
S5205-5208	not identified	Atami, Izu Pen.
S5209	*Mycale* sp.	Atami, Izu Pen.
S5301-5307	not identified	Kumomi, Izu Pen.
S5308	*Discodermia* sp.	Kumomi, Izu Pen.
S5309-5310	not identified	Kumomi, Izu Pen.
S6001-6009	not identified	Nichinan-oshima Is.
S6010	*Jaspis* sp.	Nichinan-oshima Is.
S6011	not identified	Nichinan-oshima Is.
S6012	*Theonella* sp.	Nichinan-oshima Is.
S6013-6014	not identified	Nichinan-oshima Is.
S6015	*Mycale* sp.	Kamaki, Shikoku
S6016-6027	not identified	Kamaki, Shikoku
S6028	*Theonella* sp.	Kamaki, Shikoku
S6029	*Mycale* sp.	Kamaki, Shikoku
S6030-6036	not identified	Kamaki, Shikoku
S6101-6109	not identified	Hachijo-jima Is.
S6110	*Acanthella* sp.	Hachijo-jima Is.
S6111-6114	not identified	Hachijo-jima Is.
S6115	*Cinachyra* sp.	Hachijo-jima Is.
S6116-6120	not identified	Hachijo-jima Is.
S6121	*Epipolasis* sp.	Hachijo-jima Is.
S6122	not identified	Hachijo-jima Is.
S6123	*Cribrochalina* sp.	Hachijo-jima Is.
S6124-6127	not identified	Hachijo-jima Is.
S6128	*Acanthella* sp.	Hachijo-jima Is.
S6201-6214	not identified	Hachijo-jima Is.
S6215	*Theonella swinhoei*	Hachijo-jima Is.
S6216-6218	not identified	Hachijo-jima Is.
S7001	*Discodermia calyx*	Nichinan-oshima Is.
S7002	*Theonella* sp.	Nichinan-oshima Is.
S7003-7007	not identified	Nichinan-oshima Is.

Sample No.	Genus or Species	Collection Site
S7008	*Theonella* sp.	Nichinan-oshima Is.
S7009	not identified	Nichinan-oshima Is.
S7010	*Acanthella* sp.	Nichinan-oshima Is.
S7011-7012	not identified	Nichinan-oshima Is.
S7013	*Erylus placenta*	Nichinan-oshima Is.
S7014-7018	not identified	Nichinan-oshima Is.
S7020	not identified	Kamaki, Shikoku
S7021	*Theonella* sp.	Kamaki, Shikoku
S7022-7039	not identified	Kamaki, Shikoku
S7040	*Mycale* sp.	Kamaki, Shikoku
S7041-7046	not identified	Kamaki, Shikoku
S7101-7136	not identified	Hachijo-jima Is.
S7137	*Toxadocia cylindrica*	Hachijo-jima Is.
S7138	*Theonella swinhoei*	Hachijo-jima Is.
S7139	not identified	Hachijo-jima Is.
S8001-8004	not identified	Nichinan-oshima Is.
S8005	*Theonella* sp.	Nichinan-oshima Is.
S8006	*Theonella* sp.	Nichinan-oshima Is.
S8007-8011	not identified	Nichinan-oshima Is.
S8012-8014	not identified	Okino-shima Is.
S8015-8018	not identified	Nihei-jima Is.
S8101-8108	not identified	Hachijo-jima Is.
S8109	*Theonella swinhoei*	Hachijo-jima Is.
S8110	*Theonella swinhoei*	Hachijo-jima Is.
S8111	not identified	Hachijo-jima Is.
S8112	*Theonella swinhoei*	Hachijo-jima Is.
S8113-8115	not identified	Hachijo-jima Is.
S8201-8211	not identified	Hachijo-jima Is.
S8301	*Mycale* sp.	Toji, Izu Pen.
ADSS-1-3	not identified	Amami Is. deep sea
S8401	*Mycale* sp.	Atami, Izu Pen.
S8402-8404	not identified	Atami, Izu Pen.
S8405	*Mycale* sp.	Atami, Izu Pen.
S8406-8414	not identified	Atami, Izu Pen.
S9001	*Xestospongia* sp.	Yaku-shima Is.
S9002-9019	not identified	Yaku-shma Is.
S9020	*Theonella* sp.	Kuchinoerabu Is.

Sample No.	Genus or Species	Collection Site
S9021-9025	not identified	Kuchinoerabu Is.
S9026-9033	not identified	Magejima Is.
S9034-9057	not identified	Tanega-shima Is.
Coelenterates		
C5201-5204	not identified	Atami, Izu Pen.
C6001-6003	not identified	Nichinan-oshima Is.
C6004	not identified	Shimano-urashima Is.
C6005	not identified	Kamaki, Shikoku
C6101	not identified	Hachijo-jima Is.
C7001	not identified	Nichinan-oshima Is.
C7002	not identified	Kamaki, Shikoku
C7101-7102	not identified	Hachijo-jima Is.
C8001-8006	not identified	Nichinan-oshima Is.
C8007-8016	not identified	Okino-shima Is.
C8017-8018	not identified	Nihei-jima Is.
C8201-8202	not identified	Hachijo-jima Is.
C8401-8403	not identified	Atami, Izu Pen.
C9002-9005	not identified	Yaku-shima Is.
C9006-9008	not identified	Kuchinoerabu Is.
C9009	not identified	Mage-jima Is.
C9010-9012	not identified	Tanega-shima Is.
Bryozoan		
B5201-5202	not identified	Atami, Izu Pen.
B6101	not identified	Hachijo-jima Is.
Tunicates		
T5201	not identified	Atami, Izu Pen.
T6001-6005	not identified	Nichinan-oshima Is.
T6006-6015	not identified	Shimano-urashima Is.
T6016-6018	not identified	Kamaki, Shikoku
T7001-7002	not identified	Kamaki, Shikoku
T8001-8004	not identified	Okino-shima Is.
T8005-8007	not identified	Nihei-jima Is.
T8201	not identified	Hachijo-jima Is.
T8401-8402	not identified	Atami, Izu Pen.
T9001	not identified	Yaku-shima Is.
T9002-9005	not identified	Tanega-shima Is.

1. 2. Inhibitory Activity of the Extracts

A 10 g portion of the animal was extracted with MeOH. The MeOH extract was evaporated to dryness and partitioned between H_2O and $CHCl_3$. The water layer was dried and dissolved in 5 mL of H_2O to afford the hydrophilic extract (W), while the $CHCl_3$ layer was dissolved in 5 mL of EtOH to give the lipophilic extract (E). Two concentrations (2 and 20 μL) of the extracts were tested for thrombin and trypsin inhibitory activities.

Active samples and their activities are shown in Table I-2. Inhibitory activity was scored as follows: ++ 50-100 %; + 20-50 %, and - <20 %.

Table I-2. Inhibition Activity of the Extracts against Thrombin and Trypsin

		Thrombin		Trypsin	
Sample No.		20 μL	2 μL	20 μL	2 μL
Sponges					
S5104	W	+	-	+	-
	E	-	-	+	-
S5107	W	-	-	-	-
	E	+	-	-	-
S5108	W	-	-	-	-
	E	+	-	-	-
S5117	W	+	-	-	-
	E	-	-	-	-
S5118	W	+	-	-	-
	E	-	-	-	-
S5124	W	+	-	-	-
	E	-	-	-	-
S5143	W	++	+	-	-
	E	-	-	-	-
S6009	W	-	-	-	-
	E	-	-	+	-
S6012	W	++	+	-	-
	E	-	-	-	-
S6104	W	+	-	-	-
	E	-	-	-	-
S6120	W	-	-	+	-
	E	-	-	-	-

Sample No.		Thrombin		Trypsin	
		20 µL	2 µL	20 µL	2 µL
S6121	W	+	-	++	+
	E	-	-	-	-
S6126	W	+	-	-	-
	E	-	-	-	-
S6206	W	+	-	-	-
	E	-	-	+	-
S6207	W	+	-	+	-
	E	+	-	+	-
S6208	W	+	-	-	-
	E	+	-	-	-
S6214	W	+	-	-	-
	E	-	-	-	-
S6215	W	++	-	+	-
	E	++	+	-	-
S7004	W	-	-	-	-
	E	+	-	+	-
S7007	W	+	-	++	+
	E	-	-	-	-
S7008	W	++	+	-	-
	E	-	-	-	-
S7009	W	+	-	-	-
	E	-	-	-	-
S7015	W	+	-	-	-
	E	+	-	-	-
S7027	W	+	-	-	-
	E	-	-	-	-
S7101	W	+	-	-	-
	E	-	-	-	-
S7103	W	-	-	-	-
	E	+	-	-	-
S7105	W	+	-	-	-
	E	-	-	-	-
S7107	W	+	-	-	-
	E	-	-	-	-
S7125	W	-	-	-	-
	E	+	-	+	-

Sample No.		Thrombin		Trypsin	
		20 µL	2 µL	20 µL	2 µL
S8001	W	+	-	+	-
	E	-	-	-	-
S8003	W	+	-	-	-
	E	-	-	-	-
S8004	W	-	-	-	-
	E	+	-	-	-
S8005	W	++	++	++	++
	E	++	+	-	-
S8006	W	++	++	++	++
	E	+	-	-	-
S8009	W	-	-	-	-
	E	+	-	-	-
S8017	W	-	-	-	-
	E	+	-	-	-
S8018	W	+	-	+	-
	E	+	-	+	-
S8104	W	+	-	-	-
	E	-	-	+	-
S8107	W	+	-	-	-
	E	+	-	-	-
S8108	W	-	-	-	-
	E	+	-	-	-
S8109	W	+	-	-	-
	E	-	-	-	-
S8110	W	++	++	++	++
	E	++	+	-	-
S8112	W	++	++	++	+
	E	+	-	-	-
S8113	W	-	-	-	-
	E	+	-	+	-
S8114	W	-	-	-	-
	E	+	-	-	-
S8402	W	-	-	+	-
	E	-	-	-	-
S9012	W	++	+	-	-
	E	-	-	-	-

Sample No.		Thrombin		Trypsin	
		20 µL	2 µL	20 µL	2 µL
S9015	W	-	-	-	-
	E	-	-	++	-
S9018	W	-	-	-	-
	E	-	-	+	-
S9019	W	-	-	-	-
	E	-	-	+	-
S9024	W	++	+	-	-
	E	-	-	++	+
S9026	W	-	-	++	+
	E	-	-	-	-
S9027	W	-	-	+	-
	E	-	-	-	-
S9031	W	-	-	+	-
	E	-	-	-	-
S9033	W	-	-	+	-
	E	-	-	-	-
S9036	W	-	-	+	-
	E	-	-	-	-
S9038	W	-	-	-	-
	E	-	-	+	-
S9039	W	-	-	-	-
	E	+	-	++	+
S9041	W	-	-	+	-
	E	-	-	-	-
S9044	W	-	-	-	-
	E	-	-	+	-
S9047	W	-	-	+	-
	E	++	+	+	-
S9049	W	-	-	-	-
	E	+	-	+	-
Coelenterares					
C8004	W	-	-	++	+
	E	-	-	-	-
C8008	W	-	-	++	+
	E	+	-	-	-

Sample No.		Thrombin		Trypsin	
		20 µL	2 µL	20 µL	2 µL
C8010	W	-	-	-	-
	E	+	-	-	-
C8011	W	-	-	-	-
	E	+	-	-	-
C8018	W	-	-	-	-
	E	+	-	-	-
C8403	W	-	-	+	-
	E	-	-	-	-
Tunicates					
T6019	W	-	-	+	-
	E	-	-	-	-
T8002	W	-	-	++	++
	E	+	-	-	-
T8003	W	-	-	++	+
	E	-	-	-	-
T8006	W	-	-	++	+
	E	-	-	-	-
T8007	W	-	-	+	-
	E	-	-	-	-

The incidence of anti-thrombin and anti-trypsin activities is summarized in Table I-3. Thrombin inhibitory activity was found in 13.2 % of sponge samples tested, 8.3 % of coelenterates, and 2.7 % of tunicates. No bryozoans showed activity, though only three species were tested. Sponges showed the highest incidence of active samples in anti-thrombin test. In the coelenterates and tunicates, anti-thrombin activity was found only in lipophilic extracts, while the higher incidence of activity was found in the hydrophilic extracts of sponges. Anti-trypsin activity was found in 9.1 % of sponges, 6.3 % of coelenterates, and 13.9 % of tunicates. Again, no bryozoans showed activity. Coelenterates and tunicates showed activities only in the hydrophilic fractions, while about the same percentages of lipophilic and hydrophilic extracts were active in sponges.

Table I-3. Incidence of Activity in the Phyla (%)

Animal	Number of samples tested	Thrombin			Trypsin		
		Active samples	E	W	Active samples	E	W
Sponges	363	13.2	6.6	9.4	9.1	4.7	5.5
Coelenterates	48	8.3	8.3	0	6.3	0	6.3
Bryozoans	3	0	0	0	0	0	0
Tunicates	36	2.7	2.7	0	13.9	0	13.9

The incidence of anti-thrombin and anti-trypsin activities in this screening was far behind those of antimicrobial, cytotoxic, and antiviral activities in marine invertebrates. As a recent example reported in 1981, Rinehart *et al.* found that 70 % of sponges, 28 % of coelenterates, and 22 % of tunicates collected in the Caribbean water showed antimicrobial activities[44]. Another screening for antimicrobial, cytotoxic and antiviral activities in New Zealand marine organisms revealed that 28 % of sponges, 41 % of tunicates, 19 % of bryozoans, and 38 % of echinoderms were anti-microbials; more than 20 % of sponges, tunicates, bryozoans, echinoderms, and annelids were antiviral[45]. The incidence of antimicrobial activity in sponges is five times as much as that of anti-thrombin. This means that the screening for protease inhibitory activity might be more selective than that for antimicrobial or cytotoxic activities.

Strong anti-thrombin and anti-trypsin activities were observed in sponges of the genus *Theonella* collected at Hachijo-jima Island. From these samples, nazumamide A, cyclotheonamide A-F were obtained. From the sponge *Agelas* sp. (S7125), oroidine was obtained as an inhibitor, while tetrasulfated compounds were isolated from *Toxadocia cylindrica* (S7137). The sponge S9024 appeared identical with S7125. Specimens S9012, S9026, C8004, C8008, T8002, T8003, and T8006 are promising sources of new thrombin or trypsin inhibitors.

2. Experimental Section

2. 1. Collection

Invertebrates samples were collected mainly by SCUBA at various part of Japanese coast : the Izu Peninsula, Hachijo-jima Island, Shikoku, and Kyushu (Table I-1).

2. 2. Preparation of Test Solutions

A 10 g portion of the animal was extracted under reflux with MeOH (100 mL x 2). The combined extract was evaporated to dryness and partitioned between H_2O and $CHCl_3$. The

water layer was dried and dissolved in 5 mL of H_2O to afford the hydrophilic extract, while the $CHCl_3$ layer was dissolved in 5 mL of EtOH to give the lipophilic extract.

2. 3. Thrombin Inhibition Assay

Thrombin inhibition assay was performed by a modification of the method of Sevendsen *et. al.*[46] Two concentrations of test solutions (2 and 20 μL) were tested. The 2 μL test solution was diluted to 20 μL with MeOH. To each test solution in microtiter plates 90 μL of thrombin solution was added and the mixture preincubated at 37 °C for 10 min. Then, 90 μL of substrate solution was added to start the reaction. The absorbance at 405 nm was measured after the 10-min incubation at 37 °C.

Thrombin was purchased from Sigma Chemical Co. Substrate was obtained from Bachem Feinchemikalien AG. Thrombin (1.1 U/mL) was dissolved in Tris-imidazole buffer (pH 8.2, Ip 0.15) which had been prepared by mixing a 1:1 mixture of 0.1 M imidazole-HCl and 0.1 M Tris-HCl with a 1:1 mixture of 0.1 M imidazole and 0.1 M Tris both in 0.1 M NaCl. Ion strength of the buffer was adjusted by addition of equal volume of 0.2 M NaCl. Benzoyl-phenylalanyl-valyl-arginine-*p*-nitroanilide was used as the substrate for thrombin: 5 mg of the substrate dissolved in 1 mL of DMSO and 10 μL of this solution was diluted with 990 μL of the Tris-imidazole buffer.

2. 4. Trypsin Inhibition Assay

Trypsin inhibition assay was performed by a modification of the method of Cannell *et al.*[47] Similarly, two concentrations of test solutions (2 and 20 μL) were assayed. To the test solution in microtiter plates 45 μL of trypsin solution and 45 μL of 0.4 M Tris-HCl buffer (pH 7.6) were added and then preincubated at 37 °C for 10 min. After the preincubation, 90 μL of substrate solution was added to begin the reaction. The absorbance at 405 nm was measured after 10 min incubation at 37 °C.

Trypsin and substrate were purchased from Sigma Chemical Co. Trypsin was dissolved in 50 mM Tris-HCl (pH 7.6) to prepare 150 U/mL solution. *N*-Benzoyl-D, L-arginine-*p*-nitroanilide (BAPNA) was used as substrate of trypsin: 43.3 mg of BAPNA in 1 mL of DMSO was diluted with 99 mL of 50 mM Tris-HCl (pH 7.6).

CHAPTER II

ISOLATION AND IDENTIFICATION OF OROIDINE AS AN INHIBITOR OF THROMBIN AND TRYPSIN FROM THE MARINE SPONGE, *AGELAS* SP.

The aqueous layer of the EtOH extract of a marine sponge, *Agelas* sp. collected off Hachijo-jima Island inhibited both thrombin and trypsin. The active principle was isolated and identified as oroidine (**53**) by means of spectroscopic analysis.

1. Results and Discussion

1. 1. Isolation and Structure Elucidation

The EtOH extract of wet sponge was partitioned between H_2O and $CHCl_3$. Considerable amounts of insoluble material appeared between the two phases contained most of the activity. This was fractionated by ODS flash chromatography followed by ODS HPLC to yield an active compound (4.3×10^{-3} % based on wet weight).

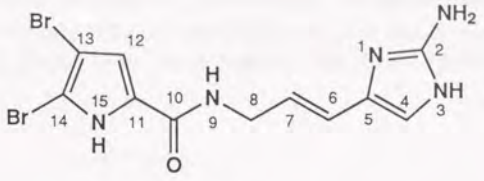
It inhibited not only thrombin with an IC_{50} of 12.5 μg/mL but also trypsin.

The FABMS (Fig II-1) showed the 1:2:1 ion at *m/z* 390, indicating the presence of two bromine atoms. Literature search revealed that it had the same molecular weight as oroidine[48]. Comparison of ^1H NMR (Fig II-2) and UV data (Fig II-3) with those for oroidine disclosed their identity. Thus the active compound was identified as oroidine (**53**).

Table II-1. ^1H NMR Data of **53**[a]

#	δ (ppm)	*J* (Hz)
4	6.75 s	
6	6.30 dt	15.0, 1.5
7	6.09 dt	15.0, 5.3
8	4.04 dd	5.3, 1.5
15	6.84 s	

a; in CD_3OD



53

Oroidine (**53**) was first reported as a metabolite of the Mediterranean sponge *Agelas oroides* in 1971. Since then, many related compounds have been isolated. A variety of bioactivities have been reported for these metabolites: hymenialdicin from Okinawan sponge *Hymeniacidon aldis* be cytotoxic[49]; keramadine from Okinawan sponge *Agelas* sp. as an antagonist of serotonergic receptors[50]; hymenidin from Okinawan sponge *Hymeniacidon* sp. was antiserotonergic[51]. However, no report has been concerned with inhibitory activity against serine proteases. Thrombin and trypsin recognize Arg or Lys residues in the substrates: the guanidine moiety of oroidine is likely to mimic Arg. It is interesting to examine whether other related compounds inhibit thrombin or not.

2. Experimental Section

2. 1. General Methods

The ^{1}H NMR spectrum was recorded on a Bruker AC 300 NMR spectrometer in CD_3OD. The FAB-MS was measured on a JEOL JMX SX102/SX102 tandem mass spectrometer. Glycerol was used as a matrix.

2. 2. Biological Material

The sponge sample was collected by SCUBA at a depth of 15 m at Nazumado in Hachijo-jima Island in October 1991, immediately frozen after collection, and preserved at -20 °C until extraction.

2. 3. Extraction and Isolation

A 148 g portion of the sponge was homogenized and extracted with EtOH. The EtOH extract was filtered, evaporated, and partitioned between H_2O and $CHCl_3$. This partitioning procedure produced a considerable amount of material between two layers, which inhibited thrombin and trypsin. The insoluble material was collected by filtration and subjected to ODS

flash chromatography (aqueous MeCN; stepwise elution). The fraction eluted with 30 % MeCN was purified by ODS HPLC (Cosmosil $5C_{18}$-AR; 25 % MeCN) to yield 6.3 mg of **53**.
Oroidine (53): UV (MeOH) λmax 275 nm (ε 6800), FAB-MS *m/z* 390$(M+H)^+$, ^1H NMR see Table II-1.

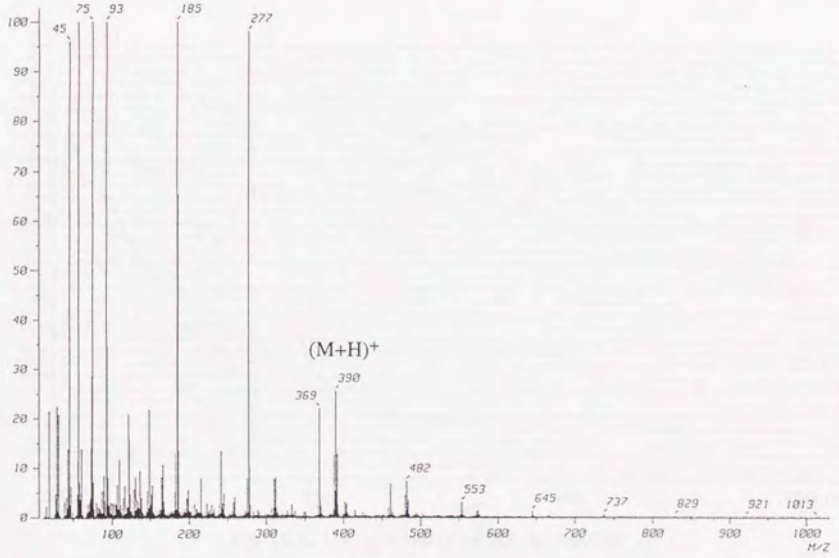


Fig II-1. FAB-MS of **53** (matrix; glycerol)

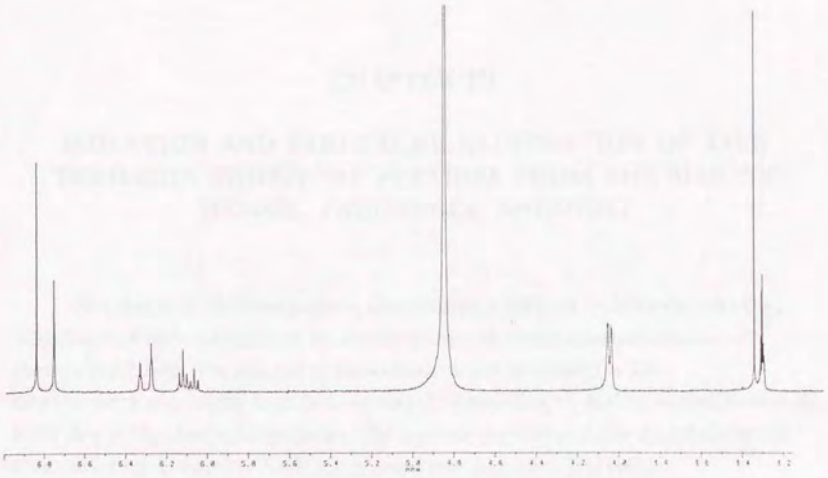


Fig II-2. ^1H NMR spectrum of **53** in CD_3OD

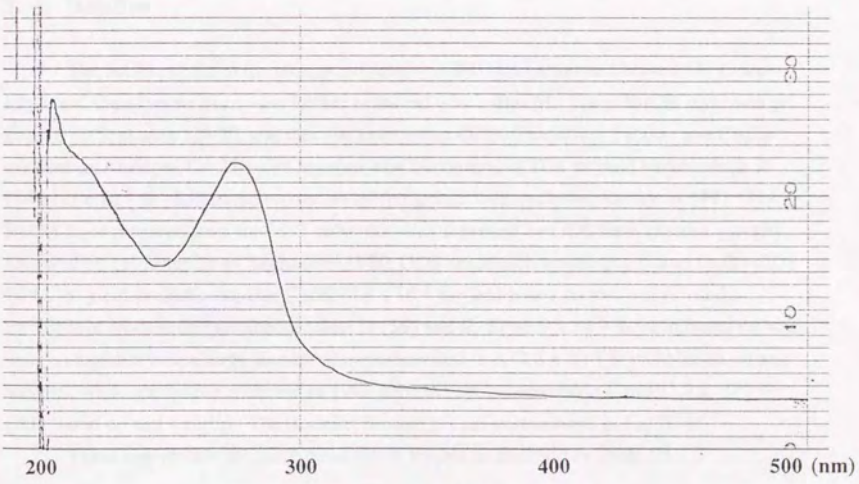


Fig II-3. UV spectrum of **53** in MeOH

CHAPTER III

ISOLATION AND STRUCTURE ELUCIDATION OF FIVE THROMBIN INHIBITORY PEPTIDES FROM THE MARINE SPONGE, *THEONELLA SWINHOEI*

Five thrombin inhibitory peptides, nazumamide A (**54**) and cyclotheonamides C-F, (**55**)-(**58**) have been isolated from the marine sponge, *Theonella swinhoei* collected off Hachijo-jima Island. The structure of nazumamide A was determined as 2,5-dihydroxybenzoyl-L-arginyl-L-prolyl-L-isoleucyl-L-α-aminobutyric acid by interpretation of 2D NMR data and by chemical degradation. The structure elucidation of new cyclotheonamides were carried out by means of NMR spectroscopy and chemical degradation.

1. Results and Discussion

1. 1. Isolation

The EtOH extract of the sponge collected in 1990 was partitioned between H_2O and Et_2O, and the aqueous phase was further extracted with *n*-BuOH. The *n*-BuOH layer was gel-filtered on Sephadex LH-20, and then fractionated on ODS. The active fraction was finally purified on Asahipak GS 320 with aqueous MeCN containing TFA to yield nazumamide A (**54**)(1.0×10^{-5} % yield based on wet weight) together with cyclotheonamide A (**47**). The *n*-BuOH layer prepared from the 1991 collection was separated by ODS flash chromatography, followed by gel-filtration on Sephadex LH-20, ODS column chromatography and finally ODS HPLC to yield cyclotheonamide C (**55**)(3.8×10^{-5} % yield based on wet weight) and a inseparable mixture of cyclotheonamides D (**56**) and E (**57**)(8.9×10^{-5} % yield based on wet weight) together with a large amount of cyclotheonamide A (2.8×10^{-4} % yield based on wet weight), while specimens collected in 1992 afforded cyclotheonamide F (**58**)(1.7×10^{-4} % yield based on wet weight). The isolation procedures are summerized in Fig III-1.

These compounds inhibited thrombin or trypsin as depicted in Table III-1.

Table III-1. Inhibitory Activities of 54-58

	Inhibitory activities (IC_{50} μg/mL)	
Compounds	Thrombin	Trypsin
nazumamide A	2.8	-
cyclotheonamide C	0.30	0.48
cyclotheonamides D+E	0.27	0.33
cyclotheonamide F	0.52	0.32

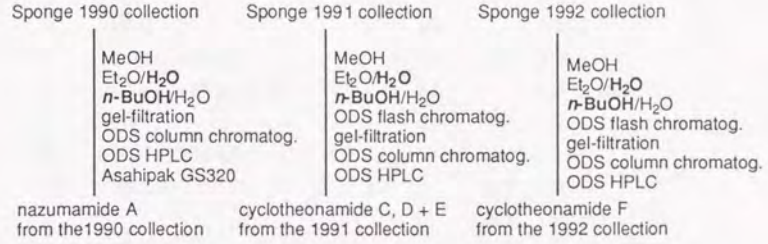


Fig III-1. Isolation of nazumamide A, cyclotheonamides C, D+E, and F

1. 2. Structure Elucidation

1. 2. 1. Nazumamide A

Nazumamide A (**54**) had a molecular formula of $C_{28}H_{43}N_7O_8$ as determined by FAB-MS [*m/z* 606 $(M+H)^+$](Fig III-7) and NMR data (Table III-2, Fig III-8-13). Though negative to ninhydrin reagent, NMR spectra indicated **54** to be a peptide. Interpretation of the COSY (Fig III-9) and HOHAHA (Fig III-10)[52] spectra in CD_3OH disclosed the presence of Pro, Ile, Arg, and α-aminobutyric acid (Aba) residues, which was supported by standard amino acid analysis of the acid hydrolysate (Fig III-14). Beside these amino acid residues, the ^1H NMR spectrum included signals for 1,2,4-trisubstituted benzene (δ 6.75, 6.84, 7.29) which was easily identified as a 2,5-dihydroxybenzoyl (DHB) group by HMQC (Fig III-11)[53] and HMBC (Fig III-12)[54] spectra.

The sequencing of the above-mentioned units was done by a combination of HMBC and ROESY (Fig III-13)[55] data. There were three sequential HMBC crosspeaks, viz., Aba NH/Ile CO; Ile NH/Pro CO; Arg NH/DHB CO. Furthermore, the sequence of Arg and Pro was established by a ROESY crosspeak between Arg α-H and Pro δ-H_2, thereby completing the sequence of DHB-Arg-Pro-Ile-Aba (Fig III-2).

Table III-2. NMR Data of Nazumamide A (54)[a]

		^{13}C	^{1}H			^{13}C	^{1}H
DHB	1	118.7			γ-1	26.6	2.03 m
	2	152.9			γ-2		2.08 m
	3	119.1	6.75 d 8.7		δ-1	49.5	3.71 m
	4	122.6	6.84 dd 3.0, 8.7		δ-2		3.87 dt 10.0, 7.1
	5	151.4			CO	174.8	
	6	116.3	7.29 d 3.0				
	CO	169.3		Ile	α	61.5	4.20 t 7.6
					β	38.8	1.88 m
Arg	α	52.8	4.96 ddd 6.2, 6.8, 7.4		γ-1	26.5	1.21 qdd 7.4, 9.2, 13.6
	β-1	30.3	1.81 m		γ-2		1.60 m
	β-2		1.99 m		δ	12.1	0.92 t 7.4
	γ	26.1	1.73 m		γ'	16.5	0.97 d 6.8
	δ	42.9	3.22 m		CO	173.3	
	N=C	159.2			NH		8.07 d 7.9
	CO	172.5					
	α-NH		8.91 d 7.4	Aba	α	56.6	4.21 dt 5.4, 7.2
	δ-NH		7.39 m		β-1	26.9	1.72 m
					β-2		1.87 m
Pro	α	62.2	4.53 dd 4.6, 8.4		γ	10.9	0.91 t 7.4
	β-1	31.4	2.01 m		CO	176.6	
	β-2		2.25 m		NH		7.83 d 7.4

a; solvent CD_3OH

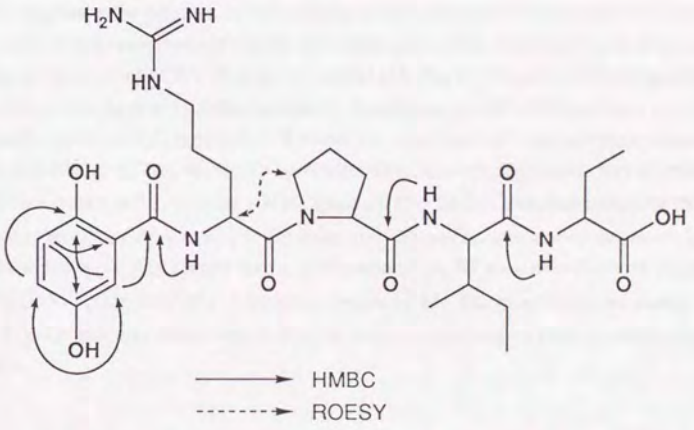


Fig III-2. HMBC and ROESY correlations in 54

Absolute configuration of all amino acid residues was determined as L by HPLC analysis of the acid hydrolysate after derivatization with Marfey's reagent (Fig III-3, 15)[56].

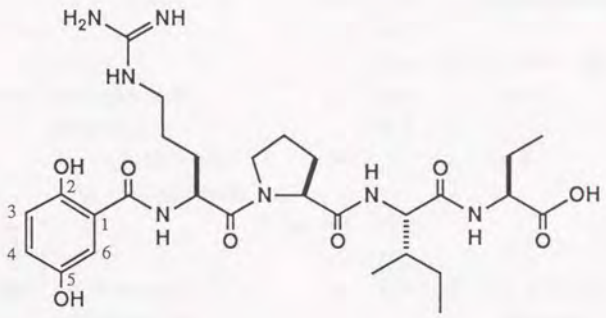


Fig III-3. The structure of nazumamide A (54)

1. 2. 2. Cyclotheonamide C

Cyclotheonamide C (**55**) had a molecular formula of $C_{38}H_{49}N_9O_9$ as determined by HR-FABMS [*m/z* 776.3724 $(M+H)^+$, Δ -0.7 mmu]. The NMR spectra were quite similar to those of cyclotheonamide A (**47**)(Fig III-17-22). The amino acids analysis of acid hydrolysate revealed the presence of 1 mol each of Pro, Phe, 2,3-diaminopropionic acid (Dpr) and α-keto arginine (K-Arg)(Fig III-23). The ^1H NMR spectrum (Fig III-17) exhibited two sets of signals, suggesting the presence of two conformers or a mixture of two compounds (Table III-3). In spite of this complexity of signals, the assignment of Pro, Phe, Dpr and K-Arg could be done by comparison of COSY (Fig III-18), HOHAHA (Fig III-19) and HMQC (Fig III-20) spectral data with those of cyclotheonamide A. In addition, the ^1H NMR spectrum revealed a remarkable triplet methyl signal at δ 1.18 which was correlated with oxymethylene proton signals (δ 3.49 and 3.37) in the COSY spectrum. This oxymethylene was in turn coupled to an oxymethine carbon at δ 82.6 in the HMBC spectrum (Fig III-21), therefore indicating the presence of an ethoxy ether moiety in the molecule. Taking consideration of the molecular formula which was C_2H_2O larger than cyclotheonamide A, **55** must have δ-ethoxy vinylogous tyrosine (E-V-Tyr) in stead of a vinylogous tyrosine in **47**. The geometry of the double bond of the E-V-Tyr unit was determined as *E* on the basis of a geminal coupling constant value of 16.1 Hz.

Table III-3. NMR Data[a] of Cyclotheonamide C (55)

	#	^{13}C	^{1}H		#	^{13}C	^{1}H
formyl	CO	162.8	8.04 bs	Phe	β-1	40.1	3.04 dd (13.6, 5.3)
					β-2		2.88 dd (13.6, 4.7)
Dpr	CO	171.5			1'	137.6	
	α	49.7	4.72 m		2'	131.0, 130.8[b]	6.77 d (6.5), 7.03 d (6.5)[b]
	β-1	40.6	4.22 dt (5.3, 10.7)		3'	129.0	7.12 m
	β-2		2.78 bt (11.4)		4'	127.7	7.14 m
	αNH		8.39 d (8.8), 8.42 d (5.2)*b*		NH		7.24 m
	βNH		8.45 d (10.4), 8.72 d (9.6)*b*				
				K-Arg	CO	170.9	
E-V-Tyr	CO	167.1			α	99.3[c]	
	α	126.0	6.09 dd (16.1, 2.7),		β	55.0, 55.5[b]	4.03 dt (2.6, 10.6),
			6.08 dd (16.1, 2.7)*b*				3.96 bt (10.6)[b]
	β	142.3	6.74 dd (16.1, 3.2),		γ-1	24.6	1.93 m
			6.77 dd (16.1, 3.2)*b*		γ-2		1.51 m
	γ	57.3	4.68 m		δ-1	25.6	1.65 m
	δ	82.6	4.52 d (5.3)		δ-2		1.53 m
	1-1	65.3	3.49 m		ε	41.7	3.09 q (6.0)
	1-2		3.37 m		N=C	158.4	
	2	15.0	1.18 t (6.7)		αNH		7.96 d (9.3), 7.92 d (9.3)[b]
	3	131.0			εNH		7.23 m
	4	129.5	7.21 d (8.3), 7.12 m*b*				
	5	116.3	6.80 d (8.3), 6.81 d (8.3)*b*	Pro	CO	173.4	
	6	158.1			α	61.3	4.48 m
	NH		8.36 d (8.4)		β-1	30.8	2.24 m
					β-2		1.98 m
Phe	CO	171.7			γ	25.6	1.95 m
	α	55.0	4.70 m		δ-1	49.2	3.78 m
					δ-2		3.53 m

a; in CD_3OH

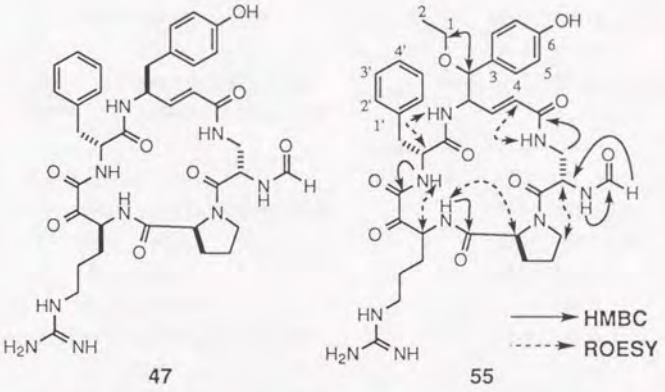
b; 3:1 signal pair was observed

c; the α-ketone was observed to make hemiketal with solvent CD_3OH

The sequencing of these units was carried out by HMBC and ROESY (Fig III-22) experiments in CD_3OH. HMBC correlations between an amide proton and a carbonyl carbon in the adjacent residue were observed between Dpr/formyl carbon, Phe/K-Arg and K-Arg/Pro. A HMBC correlation was also observed between β-proton of Dpr (δ 2.78) and carbonyl carbon of E-V-Tyr. The ROESY spectrum exhibited a crosspeak between the amide proton of E-V-Tyr and the α-proton of Phe. Inter-residual ROESY crosspeaks observed between α and β

protons of Dpr residue and δ protons of Pro residue confirmed their connectivity. Thus, the sequence of cyclotheonamide C was determined as shown (Fig III-4).

The stereochemistries were determined by Marfey's method to be L-Pro, D-Phe and L-Dpr, respectively, which are identical with those in cyclotheonamide A (**47**) (Fig III-24).



47 **55**

Fig III-4. HMBC and ROESY correlations in 55

1. 2. 3. Cyclotheonamides D and E

The NMR data indicated these compounds were analogs of cyclotheonamide A (Fig III-27-32). The FAB-MS (Fig III-33) of an inseparable mixture of cyclotheonamides D (**56**) and E (**57**) revealed an intense ion peak at *m/z* 780 $(M+H+MeOH)^+$, which led to misunderstanding of purity of this fraction. HR-FABMS established the molecular formula of $C_{36}H_{45}N_9O_9$; one more oxygen atom than cyclotheonamide A. Amino acid analysis showed the presence of 1 mol each of Pro, Phe, Dpr and K-Arg (Fig III-34). Considering the molecular formula, vinylogous tyrosine must possess one more oxygen atom. Interpretation of NMR data was not straightforward, since δ-proton and NH proton of hydroxy vinylogous tyrosine residue (H-V-Tyr), aromatic protons of Phe residue, and β-NH proton of Dpr residue appeared double for each isomer. The stereochemistries of Pro, Phe and Dpr were determined to be L, D and L, respectively, by the Marfey's procedure (Fig III-35). Therefore, the mixture contained either isomeric K-Arg or H-V-Tyr unit. Since ^1H NMR signals (Fig III-27) around H-V-Tyr unit were very complicated, it is likely that the two compounds differred in the stereochemistry at δ-position of H-V-Tyr. The geometry of the double bond in H-V-Tyr unit was shown to be *E* judging from a vicinal coupling constant of 15.8 Hz.

Table III-4. NMR Data[a] of Cyclotheonamide D (56) and E (57)

	#	^{13}C	^{1}H		#	^{13}C	^{1}H
formyl	CO	163.0	8.04 bs	Phe	1'	136.6, 137.1[b]	
Dpr	CO	171.0			2'	131.1, 131.0[c]	6.67 m, 6.64 d (6.8), 6.92 d (6.8), 6.96 d (6.8)[c]
	α	49.9	4.72 m		3'	129.3	7.07 m, 7.15 m[b]
	β-1	40.9	4.22 dt (6.5, 11.8)		4'	128.0	7.13 m
	β-2		2.80 bt (11.1)		NH		7.29 m, 7.22 d (6.8)[b]
	αNH		8.47 d (5.6), 8.44 d (5.6)[b]				
	βNH		8.49 d (11.3), 8.77 d (11.3)[b]				
				K-Arg	CO	171.2	
H-V-Tyr	CO	167.6			α	*d*	
	α	126.1	6.12 d (15.8), 6.09 d (15.8)[b]		β	55.0, 55.6[b]	4.05 q (9.4), 3.97 t (9.8)[b]
	β	142.0, 143.0, 142.3[b]	7.10 m, 6.85 m, 7.17 m[b]		γ-1	24.5	1.93 m
					γ-2		1.52 m
	γ	57.5	4.61 bt (7.9), 4.65, 4.67, 4.69[c]		δ	25.7	1.67 m
					ε		1.54 m
	δ	75.5, 75.8[b]	4.42 d (8.5), 4.44 d (8.5)[b]			41.9	3.10 quint. (6.5)
					N=C	158.4	
	1	134.0, 133.7[b]			αNH		7.96 d (10.1), 7.91 d (10.1)[b]
	4	129.3	7.27 d (7.9)		εNH		7.27 m
	5	116.4	6.80 d (7.9), 6.77 d (7.9)[b]				
	6	158.2		Pro	CO	173.7	
	NH		8.28 d (7.9), 8.25 d (7.9), 8.33 d (9.6), 8.31 d (9.6)[c]		α	61.6	4.48 bdd (3.9, 4.6)
					β-1	30.9, 30.4[b]	2.23 m
Phe	CO	171.6			β-2		1.97 m
	α	55.3	4.55 q (6.5)		γ	25.6	1.95 m
	β-1	40.4	2.84 dd (6.5 13.1), 2.98 dd (6.5, 13.1)[b]		δ-1	49.1	3.78 m
					δ-2		3.51 m
	β-2		2.70 dd (4.6, 13.1), 2.82 m[b]				

a: in CD_3OH

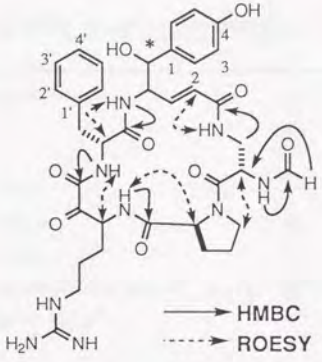
b: 3:1 signal pair was observed

c; mixtures of 3:1 signal pairs

d; not observed

The amino acid sequence was determined by interpretation of HMBC (Fig III-31) and ROESY (Fig III-32) data. HMBC crosspeaks were observed between NH protons and the carbonyl carbons of the adjacent amino acids two bonds away, viz. Dpr NH/formyl carbon, H-

V-Tyr NH/Phe C-1, Phe NH/K-Arg C-1, and K-Arg NH/Pro C-1. An HMBC crosspeak was also observed between the β-carbon protons of Dpr residue and the carbonyl carbons of the adjacent H-V-Tyr three bond away, while a ROESY crosspeak was observed between the α-proton of Dpr residue and the δ-protons of Pro residue. Therefore, the structures of **56** and **57** were as shown.



56, **57**; epimeric at *

Fig III-5. HMBC and ROESY correlations in 56 and 57

1. 2. 4. Cyclotheonamide F

The NMR data (Fig III-37-42) and FAB-MS [$(M+H)^+$ *m/z* 730] (Fig III-43) indicated cyclotheonamide F (**58**) was a dehydro derivative of cyclotheonamide A. Two sets of ^1H NMR signals suggested the presence of two major conformers (Table. III-5). The assignment of signals for Pro, Phe, Dpr and K-Arg residues were done by comparison of COSY (Fig III-39), HOHAHA (Fig III-39) and HMQC (Fig III-40) spectra with those of **47**. In the ^1H NMR spectrum (Fig III-37), α- and β-protons of V-Tyr in **47** were missing. A singlet olefinic proton was, instead, observed at δ 6.89, suggesting the presence of a dehydro vinylogous tyrosine unit (D-V-Tyr), which was supported by UV absorption at 332 nm (ε 12000)(Fig III-44). The geometry of the disubstituted double bond was *E* on the basis of a vicinal coupling constant of 15.8 Hz. The geometry of the trisubstituted double bond was determined to be Z, because a ROESY cross peak was observed between α-proton and δ-proton in the D-V-Tyr unit (Fig III-42).

Sequencing of cyclotheonamide F was carried out by HMBC (Fig III-41) and ROESY data in CD_3OH. The HMBC spectrum revealed crosspeaks between the NH protons and the carbonyl carbons of the adjacent amino acids two bond away: Dpr NH and formyl carbon, α-proton of Phe/K-Arg C-1 and K-Arg NH/Pro C-1. An HMBC crosspeak was also observed

between β-proton of Dpr (δ 2.78) and carbonyl carbon of D-V-Tyr. As for Pro and Dpr, contours were observed between α protons of Dpr and δ protons of Pro in the ROESY spectrum. Because of the weakness of NH proton signal of D-V-Tyr, the connectivity between D-V-Tyr and Phe was not confirmed in HMBC spectra. But there was no way but the connecting between D-V-Tyr and Phe.

Table III-5. NMR Data[a] of Cyclotheonamide F (58)

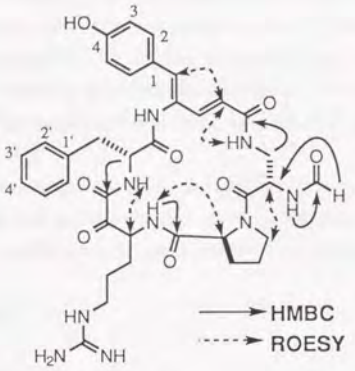
	#	^{13}C	^{1}H		#	^{13}C	^{1}H
formyl	CO	163.2	8.06 bs, 8.05 bs[b]	Phe	1'	137.2	
					2'	129.7	7.16 m
Dpr	CO	171.0			3'	128.3	7.21 m
	α	49.5	4.72 m		4'	126.9	7.18 m
	β-1	40.6	4.29 m		NH		7.63 bd (7.4)
	β-2		2.82 m)				
	αNH		8.44 bd (5.3), 8.42 bd (6.3)[b]	K-Arg	CO	172.5	
	βNH		8.85 m, 8.87 m[b]		α	*c*	
					β	54.5, 55.3[b]	4.13 q (8.2),
D-V-Tyr	CO	168.2					4.03 q (8.2)[b]
	α	121.0,120.6	6.21 d (15.8),		γ-1	24.3	1.88 m
			6.17 d (15.8)[b]		γ-2		1.54 m
	β	129.7	7.26 m,		δ-1	25.8	1.68 m
			7.31 m[b]		δ-2		1.55 m
	γ	139.5			ε	41.5	3.12 m
	δ	136.3	6.89 s		N=C	158.5	
	1	126.0			αNH		8.02 m, 7.98 d (8.9)[b]
	2	131.2, 130.2	7.39 d (8.4), 7.44 d (8.4)[b]		εNH		7.27 m
	3	115.5	6.75 d (8.9), 6.80 d (8.9)[b]				
	4	159.7		Pro	CO	173.5	
	NH		7.11 m		α	61.2	4.51 m
					β-1	30.7	2.22 m
Phe	CO	172.0			β-2		2.00 m
	α	54.2	5.03m		γ	25.4	1.93 m
	β-1	40.0	3.04 dd (13.9, 8.2)		δ-1	49.0	3.74 m
	β-2		3.27 dd (13.9, 4.1)		δ-2		3.54 m

a; in CD_3OH

b; 3:1 signal pair was observed

c; not observed

The stereochemistries of Pro, Phe and Dpr residues were determined by the Marfey's method to be L, D and L, respectively, which are identical with those in cyclotheonamide A (Fig III-45).



58

Fig III-6. HMBC and ROESY correlations in 58

1. 3. Discussion

The thrombin-inhibitory tetrapeptide named nazumamide A (**54**) was isolated from the marine sponge *Theonella swinhoei*, from which cyclotheonamides A and B were obtained. The name, nazumamide A, was coined after the collection site, Nazumado in Hachijo-jima Island. Nazumamide A is the first naturally-occurring peptide with *N*-terminus blocked by the *N*-2,5-dihydroxybenzoate group. The acid is known as, *inter alia*, a constituent of *Penicillium griseofalvum*.[57] This peptide was succeedingly synthesized by Shioiri *et al.*[58] The synthetic nazumamide A inhibited thrombin by 30 % at a concentration of 100 μg/mL. The discrepancy of the inhibitory activities between the natural and synthetic samples was probably due to the *ca.* 0.1 % contamination of cyclotheonamide A in the natural preparation, because the latter inhibited thrombin with an IC_{50} of 0.076 μg/mL. It is difficult to detect a 0.1 % contamination of cyclotheonamide A by either NMR or FAB-MS. Interestingly, **54** was isolated only from the specimens collected in 1990. Similarly, cyclotheonamide B was isolated only from the specimens collected in 1989. They may be good examples of the variability in the composition of secondary metabolites of marine sponges.

Cyclotheonamides C, D, E and F were also isolated from the same sponge *Theonella swinhoei*. The 1991 collection did not contain cyclotheonamide F, while the 1992 collection contained cyclotheonamide F along with cyclotheonamides C, D and E as minor components. This suggested that cyclotheonamides C, D and E may be artifacts produced by addition of H_2O or EtOH to cyclotheonamide F during the extraction and isolation procedure.

Recently, the structure of cyclotheonamide A-human α-thrombin complex was studied by means of X-ray crystallography.[38] In this study, α-keto amide of cyclotheonamide A proved to play an important role when it binds to thrombin, in addition to the interactions by the Pro-Arg substructure. The carbonyl carbon of α-keto amide of cyclotheonamide A and the hydroxy group of Ser^{195}, part of the thrombin catalytic triad His^{57}-Asp^{102}-Ser^{195}, form a tetrahedral intermediate (hemiketal) that resembled a transition state for peptide hydrolysis. Cyclotheonamides C-F, also having α-keto amide, must bind to thrombin in the similar way and inhibit thrombin. It is interesting to elucidate on the mode of inhibition of trypsin by cyclotheonamide A.

Cyclotheonamides are cyclic, and cyclic peptides are known to resistant to hydrolytic enzymes. The study of structure-activity correlations of cyclotheonamides will offer good information for design and synthesis of thrombin inhibitors as potent antithrombotic drugs.

2. Experimental Section

2. 1. General Methods

NMR spectra were recorded on a Bruker AM 600 NMR spectrometer operating at 600 MHz for ^1H and 150 MHz for ^{13}C. ^1H and ^{13}C NMR chemical shifts were referenced to solvent peaks: δ_H 3.3 and δ_C 49 ppm for CD_3OH. FAB-MS were measured on a JEOL JMX-SX102/SX102 tandem mass spectrometer using glycerol as a matrix. Amino acid analyses were carried out with a Hitachi 835 amino acid analyzer. Optical rotations were determined on a JASCO DIP-371 digital polarimeter in CH_3OH. UV spetra were recorded on a Hitachi 330 spectrometer.

2. 2. Biological Materials

The sponge samples were collected by SCUBA at depths of 10-20 m at Nazumado in Hachijo-jima Island in October 1990, October 1991, and November 1992. They were identified by Professor Patricia R. Bergquist, University of Auckland.

2. 3. Extraction and Isolation

The EtOH extract of the sponge samples collected in 1990 was partitioned between H_2O and Et_2O, and the aqueous phase was further extracted with *n*-BuOH. The *n*-BuOH layer was gel-filtered on Sephadex LH-20 with MeOH. Fractions of 10 mL were collected and monitored by bioassay and TLC. The active fractions were combined and separated by ODS flash chromatography [H_2O-MeOH (step gradient)] followed by ODS column chromatography [H_2O-MeCN-TFA (77:23:0.5)]. An active peak, whose major constituent was cyclotheonamide A was separated by ODS HPLC [H_2O-MeCN-TFA (77:23:0.5)] followed by

HPLC on Asahipak GS 320 [H_2O-MeCN-TFA (90:10:0.5)] to yield nazumamide A (**54**; 1.0 x 10^{-5} % yield based on wet weight).

The EtOH extract of the sponge collected in 1991 was partitioned between Et_2O and H_2O. The aqueous layer was further extracted with *n*-BuOH. The *n*-BuOH extract was separated by ODS flash chromatography with aqueous MeCN containing 0.05 % TFA. The 30 % MeCN-0.05 % TFA fractions were subsequently separated by gel-filtration on Sephadex LH-20 (MeOH), ODS column chromatography (23 % MeCN containing 0.05 % TFA), and finally ODS HPLC (the same solvent system) to yield cyclotheonamide C (**55**; 3.8 x 10^{-5} % based on wet weight) and inseparable mixtures of cyclotheonamides D and E (**56** and **57**; 8.9 x 10^{-5} % based on wet weight) together with a large amount of cyclotheonamide A.

The specimens collected in 1992 afforded cyclotheonamide F (**58**; 1.7 x 10^{-4} % based on wet weight) by the same isolation procedures.

Nazumamide A: colorless amorphous solid; $[\alpha]^{23}_D$ -87.1° (*c* 0.075, MeOH); UV (MeOH) λmax 212 nm (ε 36000), 240 (11300), 325 (4400)(Fig III-16); $C_{28}H_{43}N_7O_8$; FAB-MS *m/z* 606 $(M+H)^+$; ^1H and ^{13}C NMR (CD_3OH) see Table III-2.

Cyclotheonamide C: colorless amorphous solid; $[\alpha]^{23}_D$ -5.04° (*c* 0.5, MeOH); UV (MeOH) λmax 270 nm (ε 4100)(Fig III-25); $C_{38}H_{49}N_9O_9$; HR-FABMS *m/z* 776.3724 $(M+H)^+$, (Δ -0.7 mmu); FAB-MS *m/z* 776 $(M+H)^+$, 794, 808, 822 (Fig III-26); ^1H and ^{13}C NMR (CD_3OH) see Table III-3.

Cyclotheonamide D+E: colorless amorphous solid; $[\alpha]^{23}_D$ +0.16° (*c* 0.5, MeOH); UV (MeOH) λmax 273 nm (ε 2000), 328 (300)(Fig III-36); $C_{36}H_{45}N_9O_9$; HR-FABMS *m/z* 748.3450 $(M+H)^+$ (Δ +3.2 mmu); ^1H and ^{13}C NMR (CD_3OH) see Table III-4.

Cyclotheonamide F: colorless amorphous solid; $[\alpha]^{23}_D$ +42.4° (*c* 1.0, MeOH); UV (MeOH) λmax 271 nm (ε 7000), 332 (12000); $C_{36}H_{43}N_9O_8$; FAB-MS *m/z* 730 $(M+H)^+$; ^1H and ^{13}C NMR (CD_3OH) see Table III-5.

2. 4. Derivatization with Marfey's Reagent

A 0.1 mg portion of peptide was hydrolyzed with 6 N HCl at 110 °C for 17 h. The hydrolysate was lyophilized and mixed with 50 μL of 0.1 % Marfey's reagent in acetone and 100 μL of 0.1 N $NaHCO_3$, and the mixture was kept at 80 °C for 3 minutes. To the reaction mixture 50 μL of 0.2 N HCl was added and submitted to HPLC analysis [column, Cosmosil $5C_{18}$-MS; mobile phase, linear gradient from 0.1 N TFA to MeCN-50 mM KH_2PO_4 (1:1)].

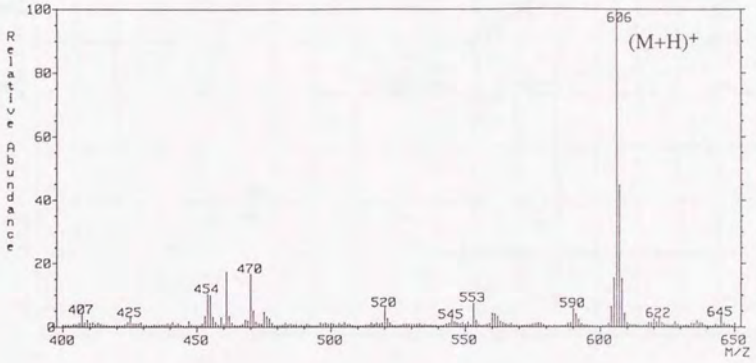


Fig III-7. FAB-MS of **54** (matrix; glycerol)

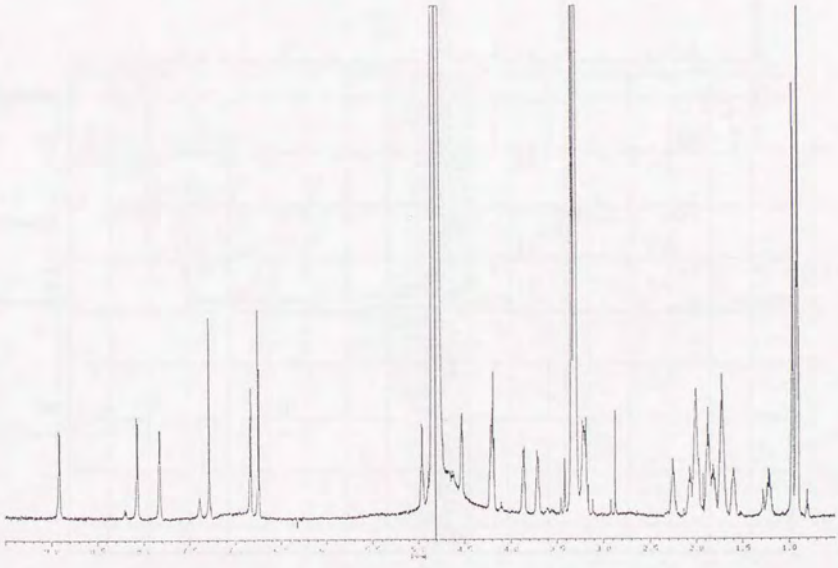


Fig III-8. ^{1}H NMR spectrum of **54** in CD_3OH

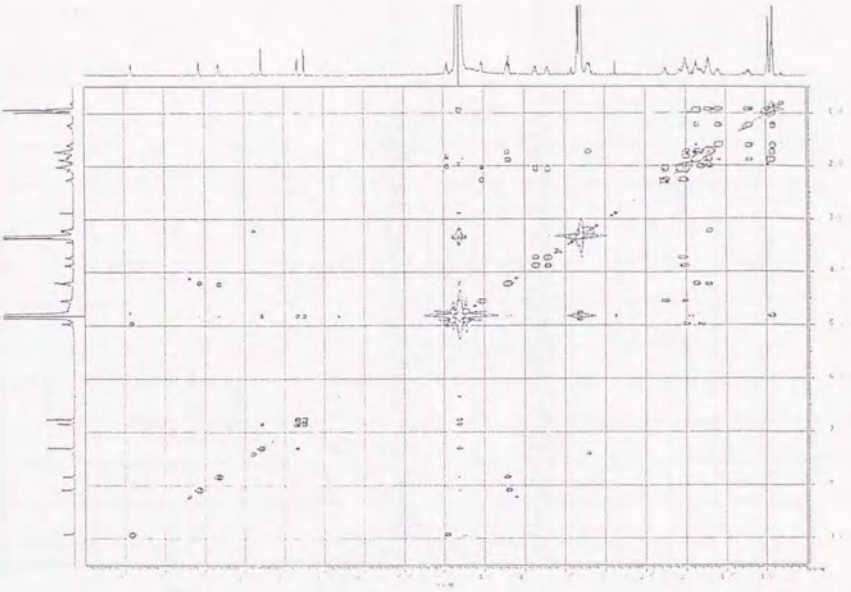


Fig III-9. COSY spectrum of 54 in CD_3OH

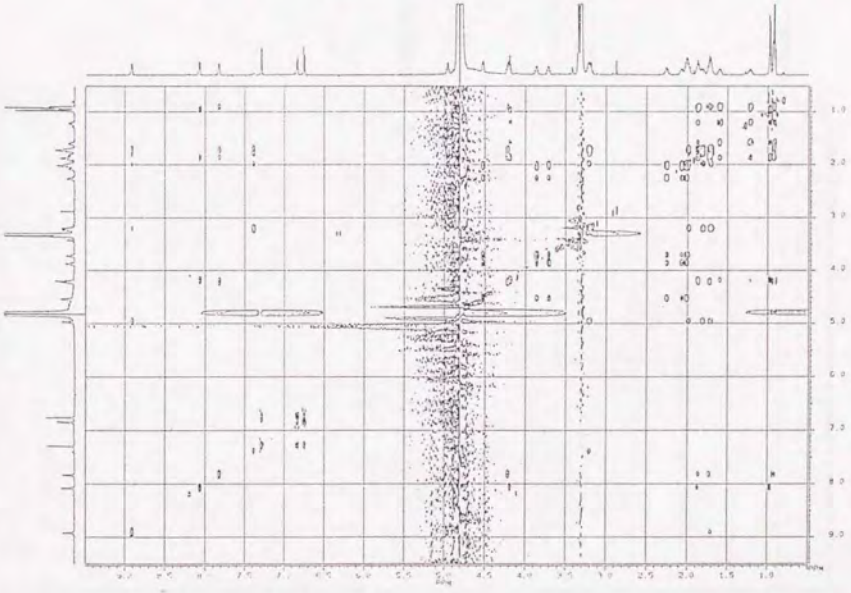


Fig III-10. HOHAHA spectrum of 54 in CD_3OH

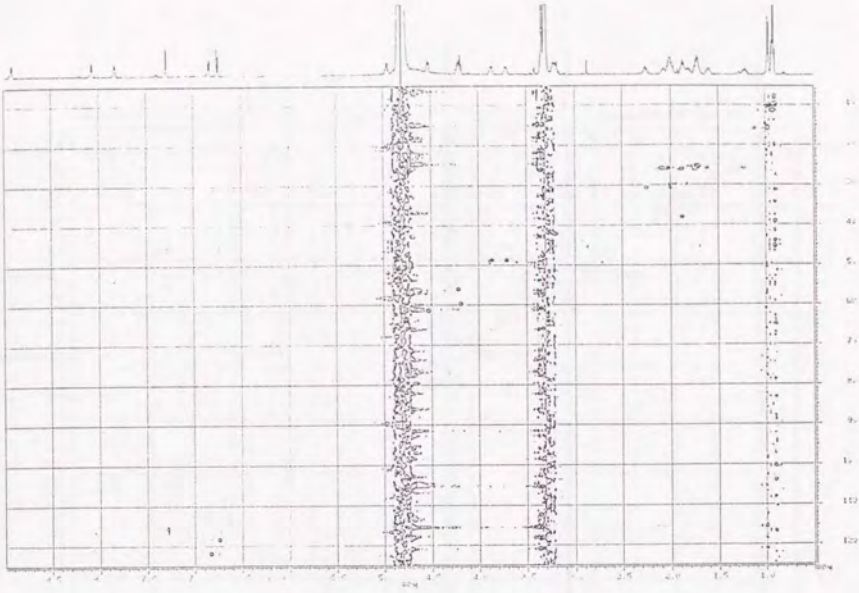


Fig III-11. HMQC spectrum of **54** in CD_3OH

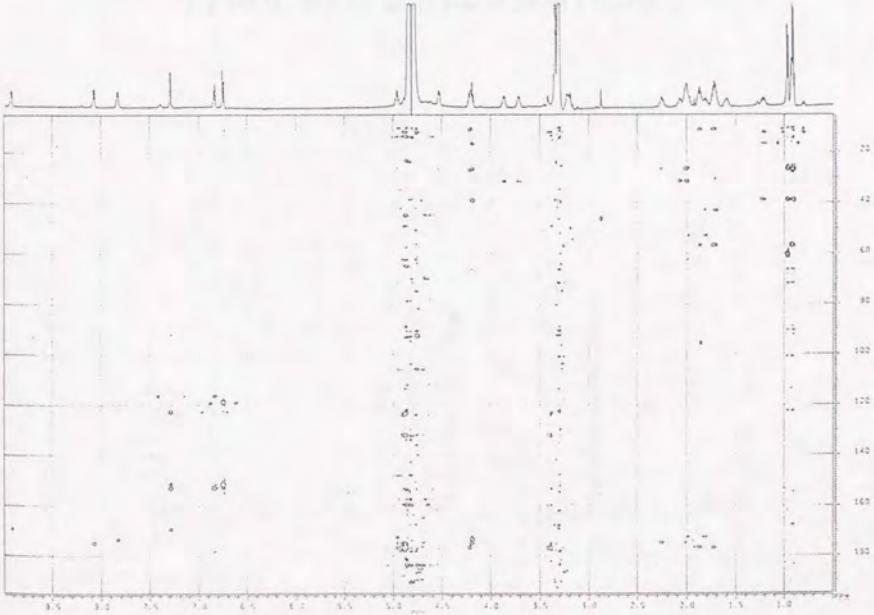


Fig III-12. HMBC spectrum of **54** in CD_3OH

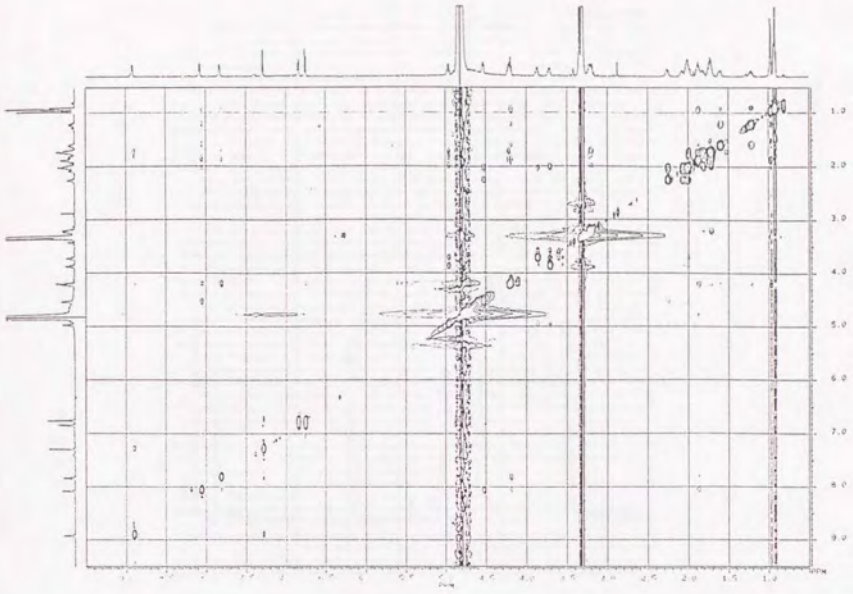


Fig III-13. ROESY spectrum of **54** in CD_3OH

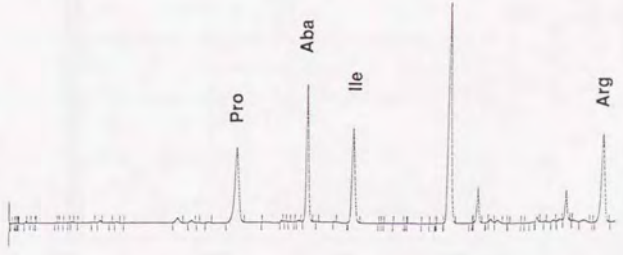


Fig III-14. Amino acids analysis of **54**

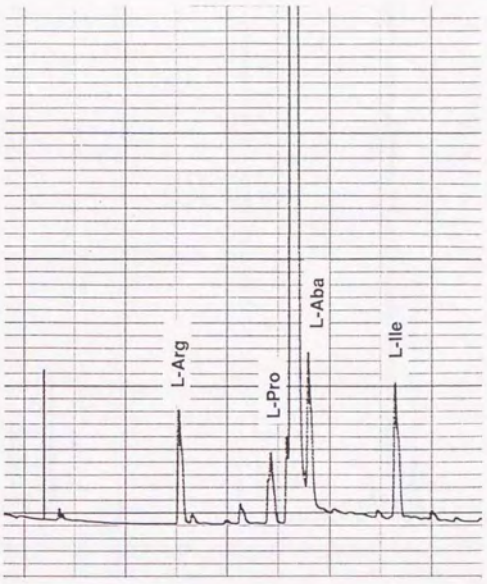


Fig III-15. HPLC analysis of acid hydrolysate by Marfey's method

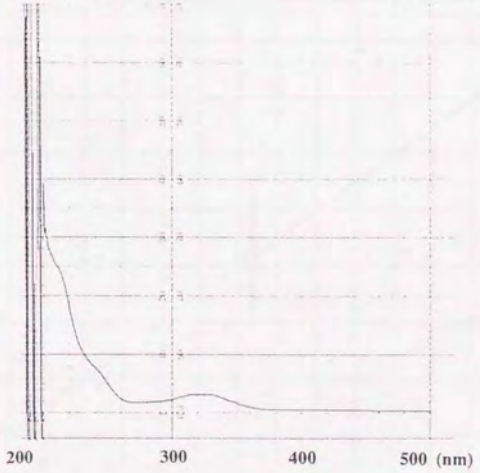


Fig III-16. UV spectrum of 54 in MeOH

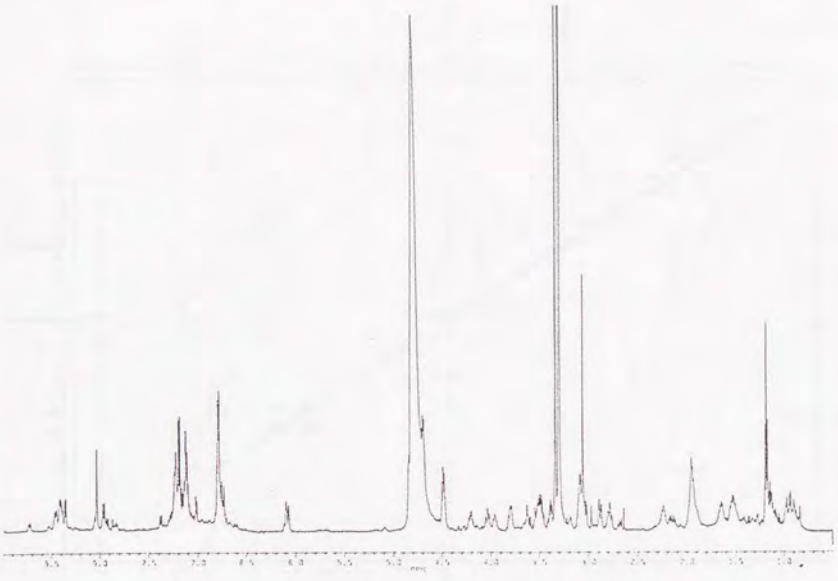


Fig III-17. ^{1}H NMR spectrum of **55** in CD_3OH

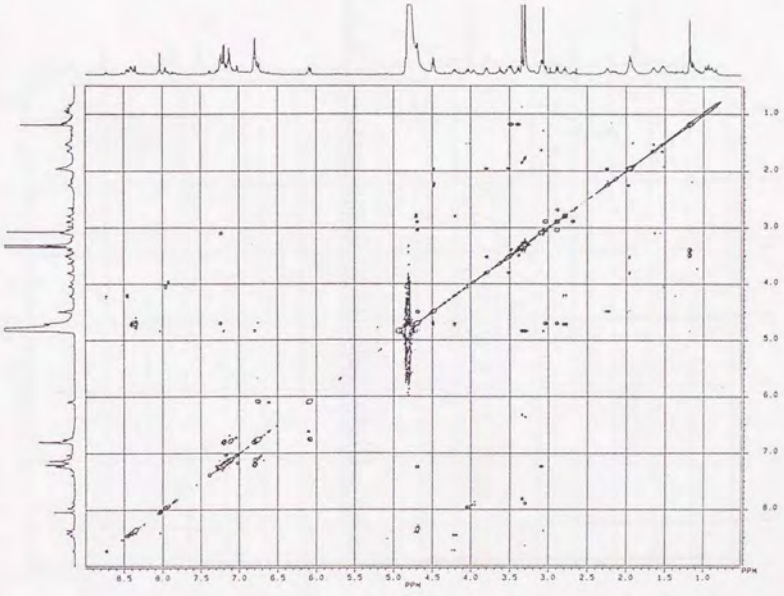


Fig III-18. COSY spectrum of **55** in CD_3OH

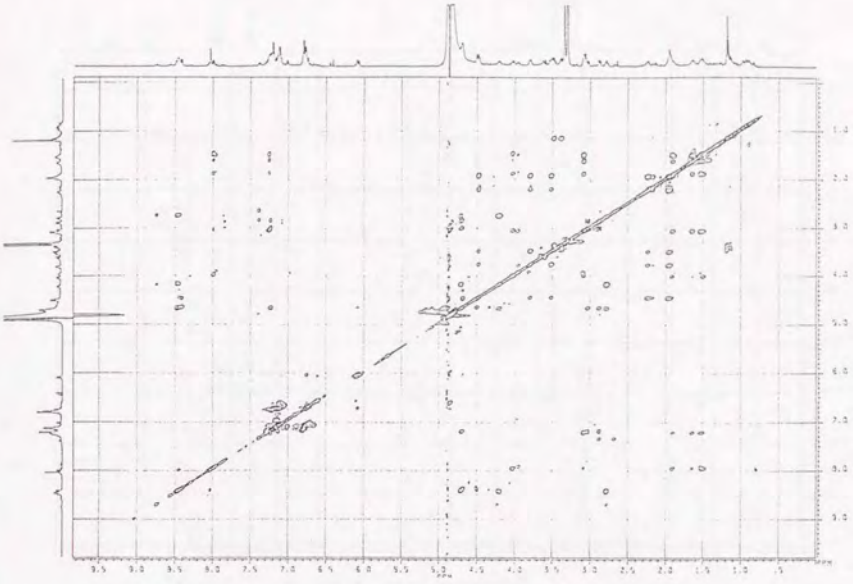


Fig III-19. HOHAHA spectrum of **55** in CD_3OH

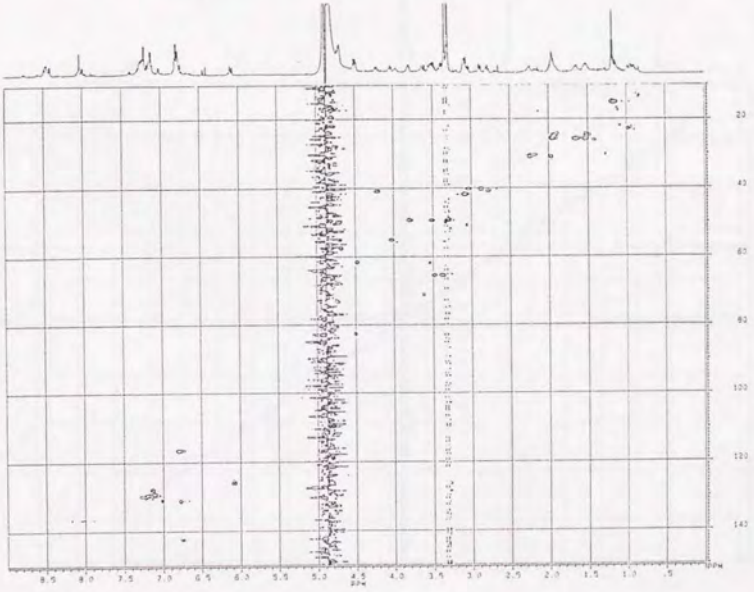


Fig III-20. HMQC spectrum of **55** in CD_3OH

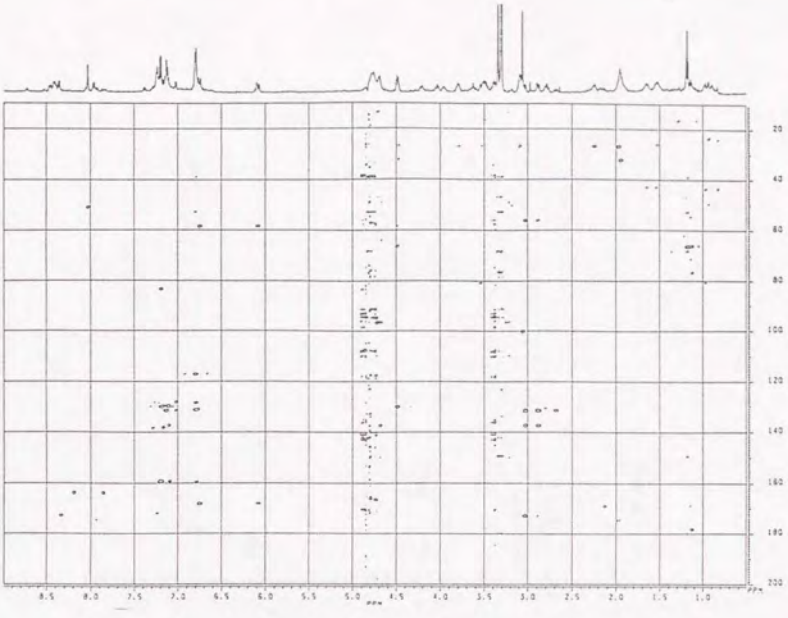


Fig III-21. HMBC spectrum of **55** in CD_3OH

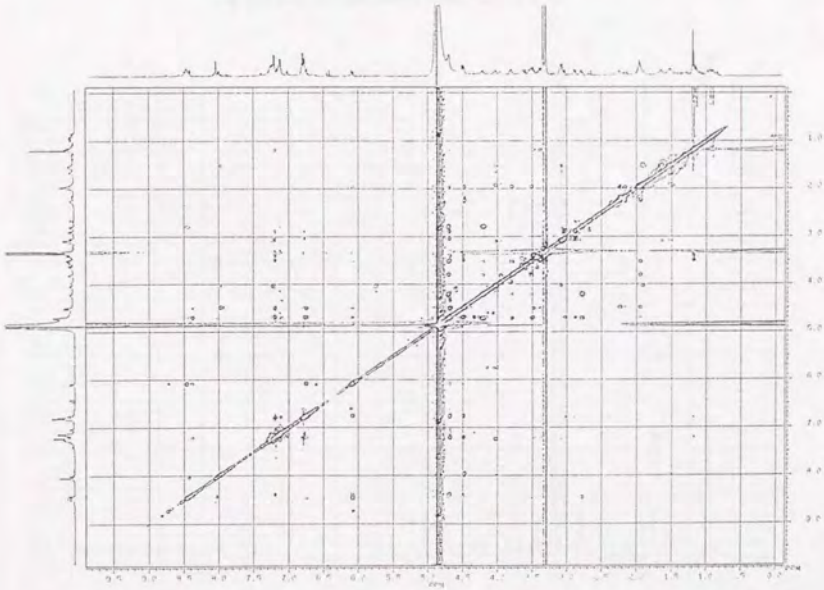


Fig III-22. ROESY spectrum of **55** in CD_3OH

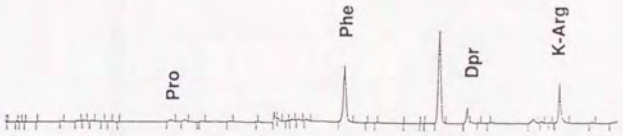


Fig III-23. Amino acids analysis of 5 5

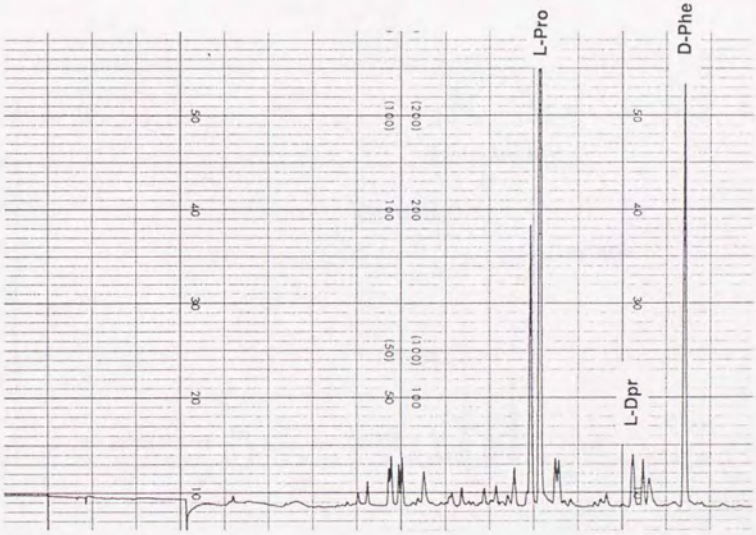


Fig III-24. HPLC analysis of acid hydrolysate by Marfey's method

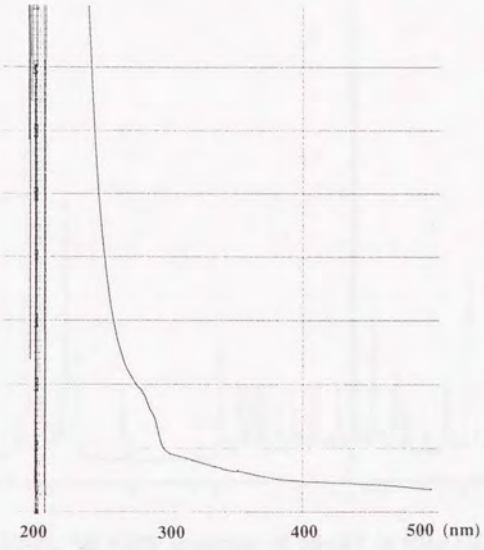


Fig III-25. UV spectrum of 55 in MeOH

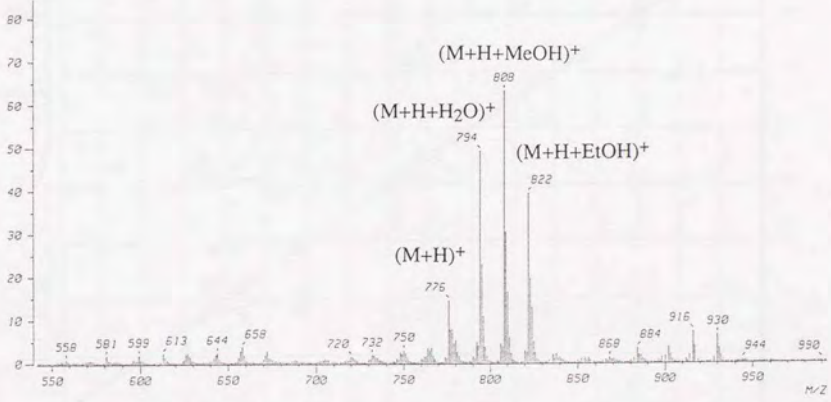


Fig III-26. FAB-MS of 55 (matrix; glycerol)

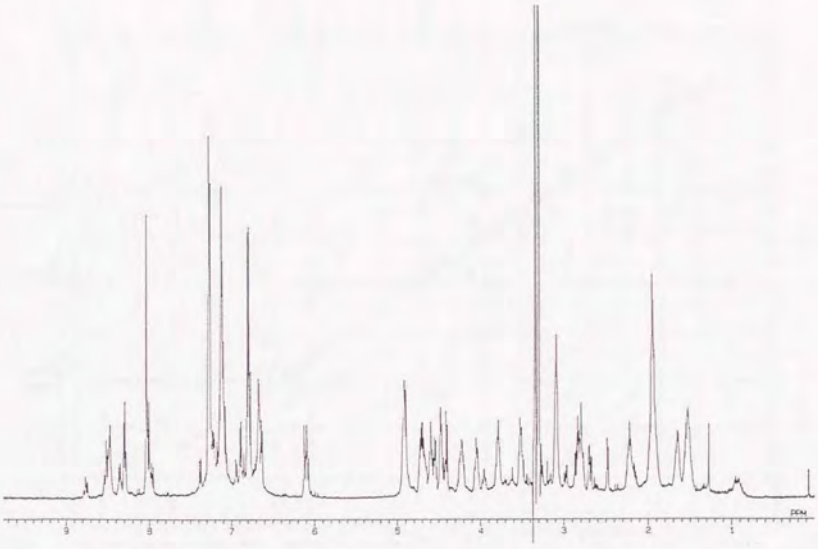


Fig III-27. ^{1}H NMR spectrum of **56+57** in CD_3OH

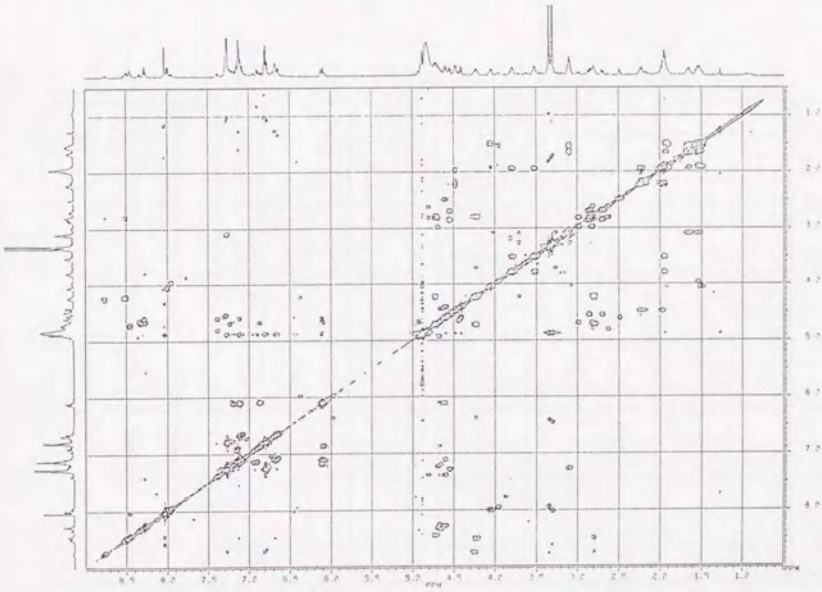


Fig III-28. COSY spectrum of **56+57** in CD_3OH

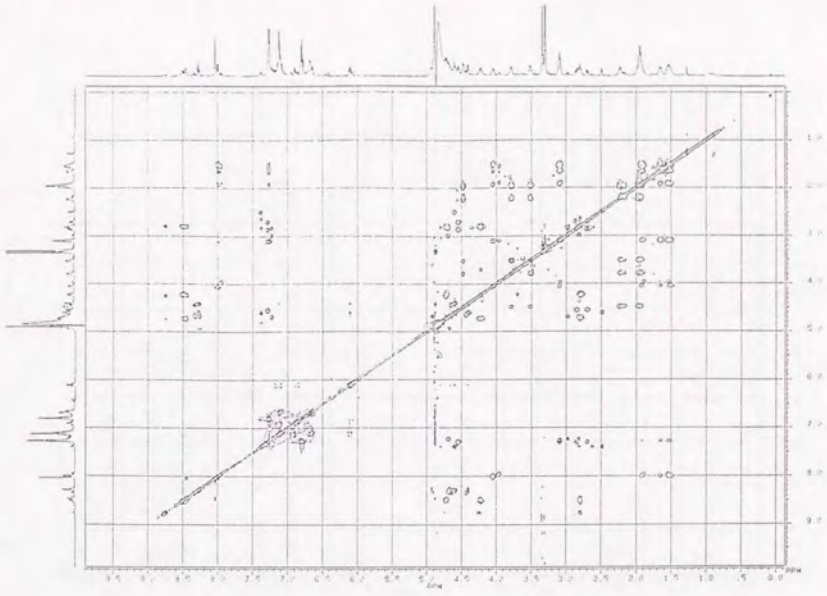


Fig III-29. HOHAHA spectrum of **56+57** in CD_3OH

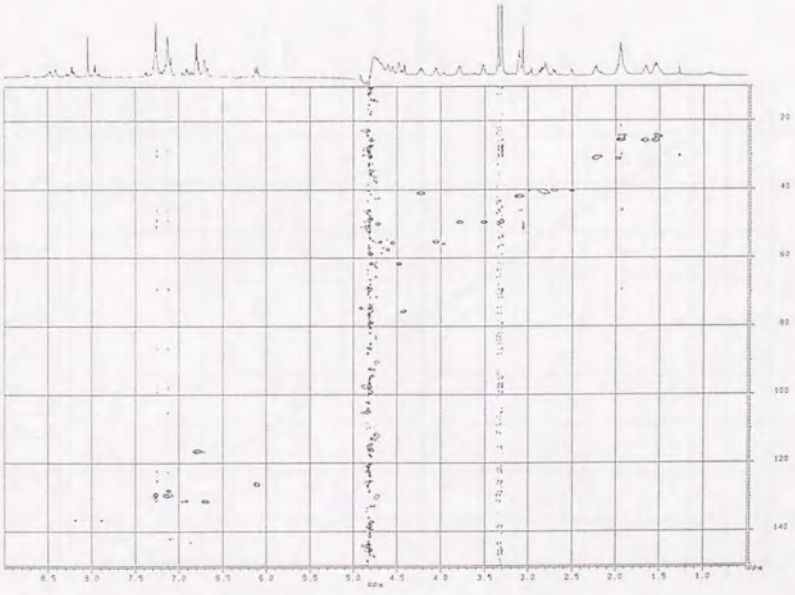


Fig III-30. HMQC spectrum of **56+57** in CD_3OH

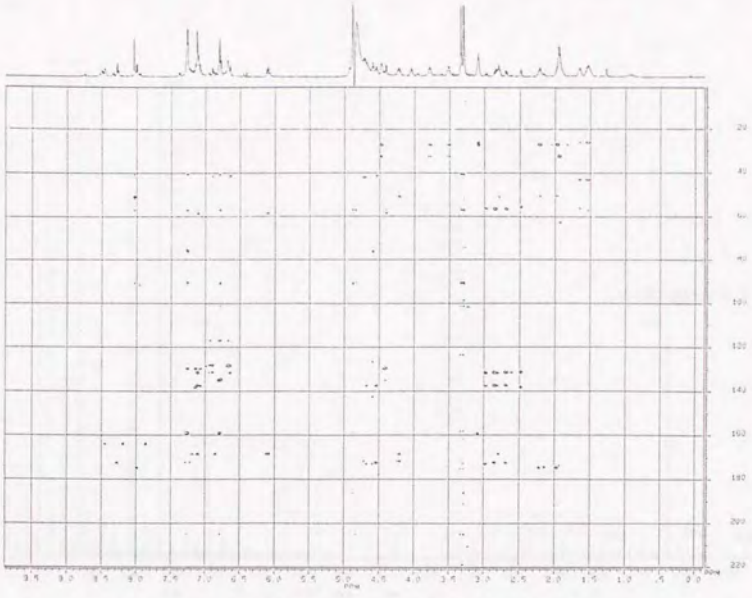


Fig III-31. HMBC spectrum of **56+57** in CD_3OH

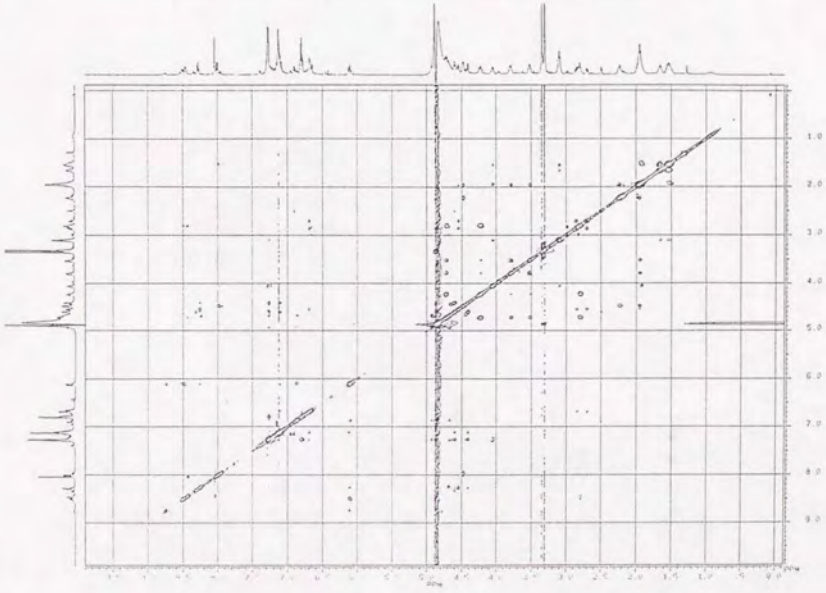


Fig III-32. ROESY spectrum of **56+57** in CD_3OH

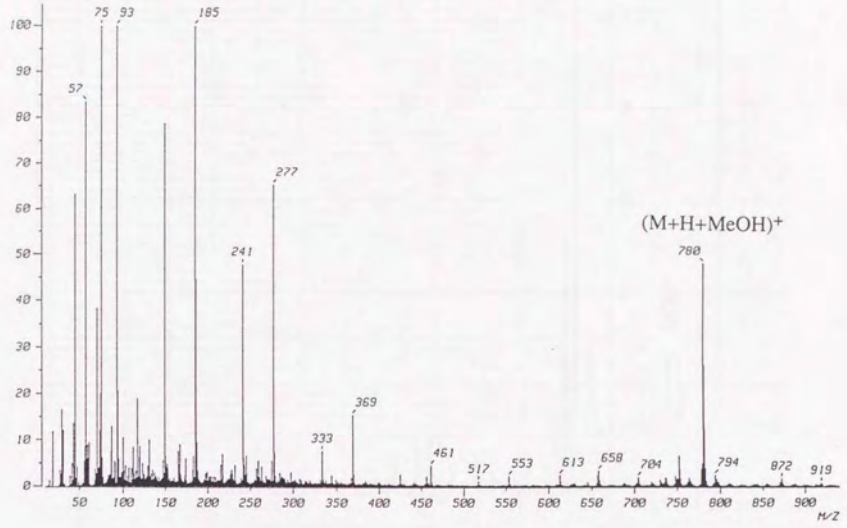


Fig III-33. FAB-MS of 56+57 (matrix; glycerol)

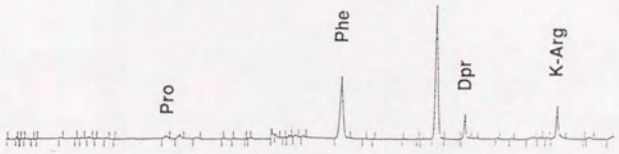


Fig III-34. Amino acids analysis of 56+57

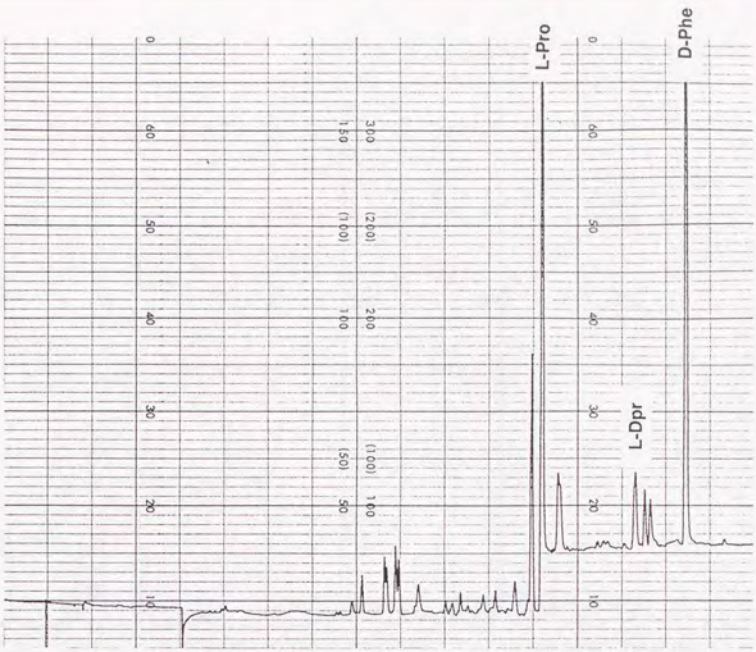


Fig III-35. HPLC analysis of acid hydrolysate by Marfey's method

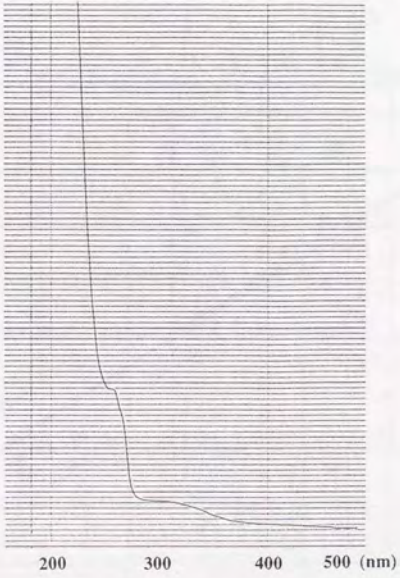


Fig III-36. UV spectrum of 56+57 in MeOH

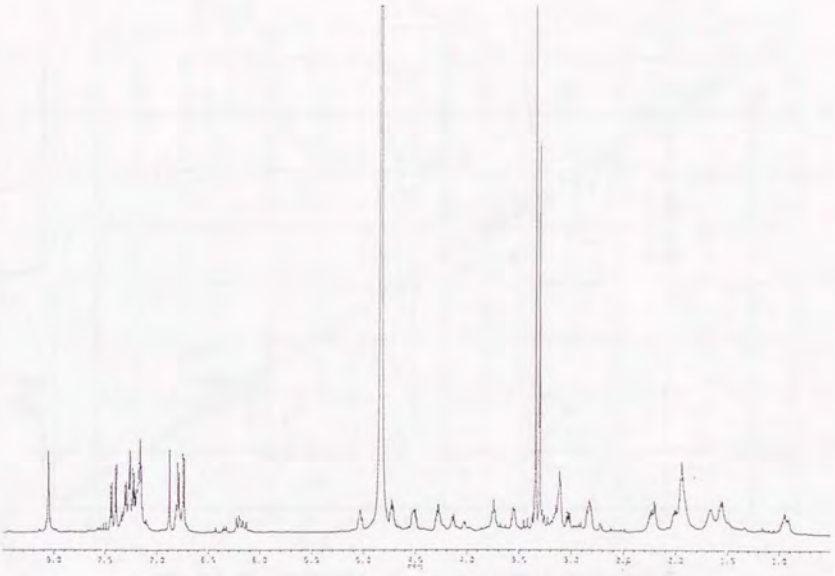


Fig III-37. ^{1}H NMR spectrum of **58** in CD_3OH

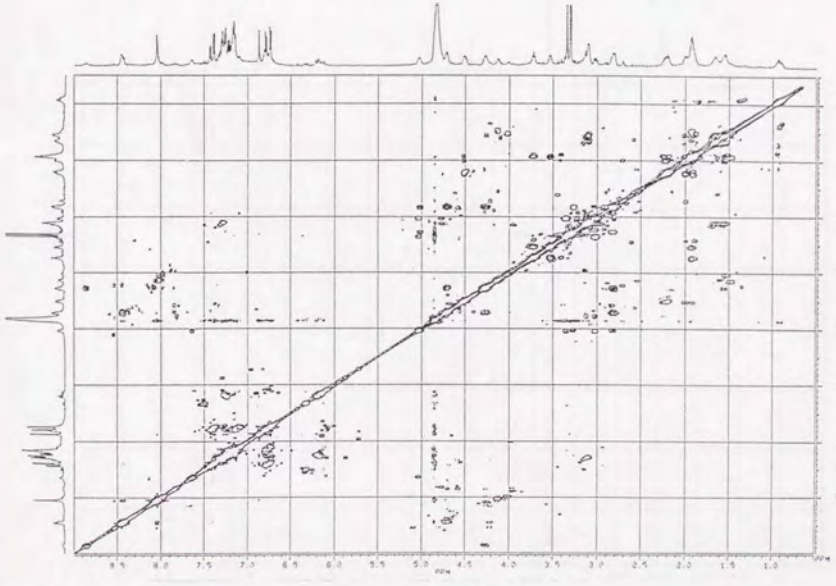


Fig III-38. COSY spectrum of **58** in CD_3OH

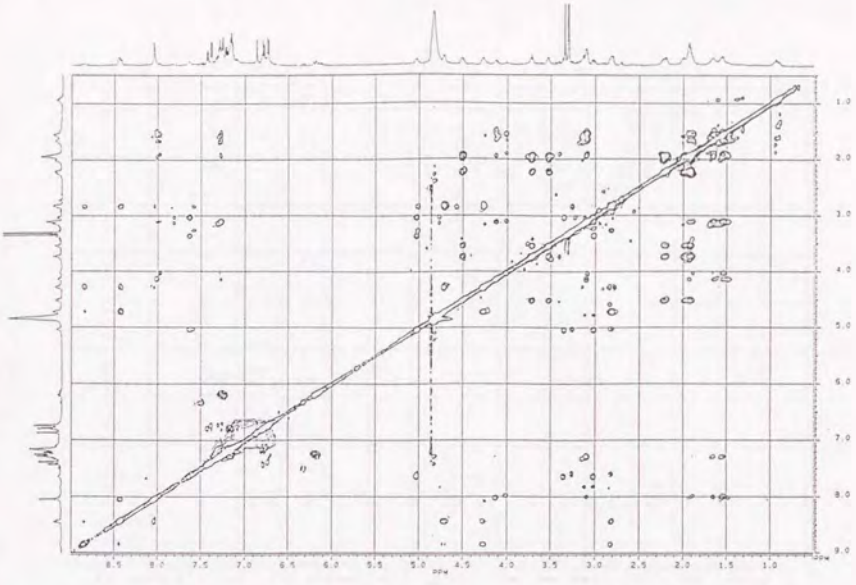


Fig III-39. HOHAHA spectrum of **58** in CD_3OH

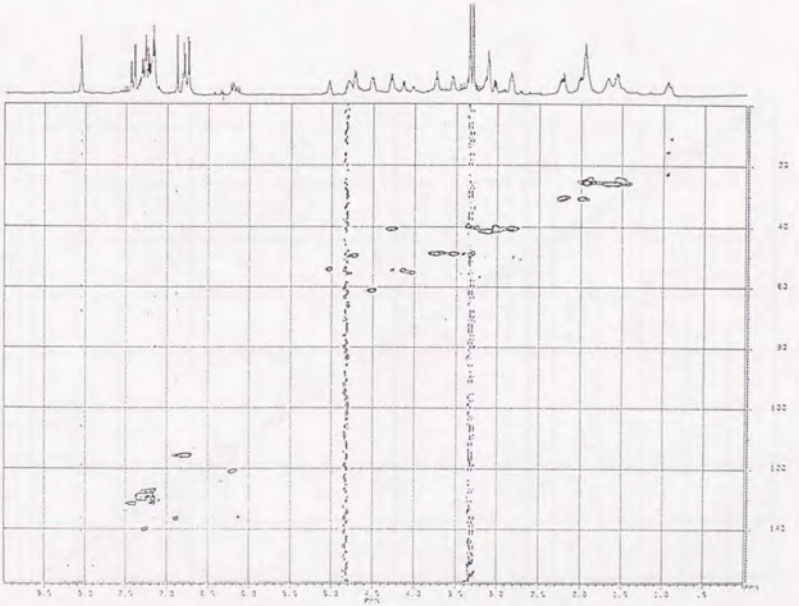


Fig III-40. HMQC spectrum of **58** in CD_3OD

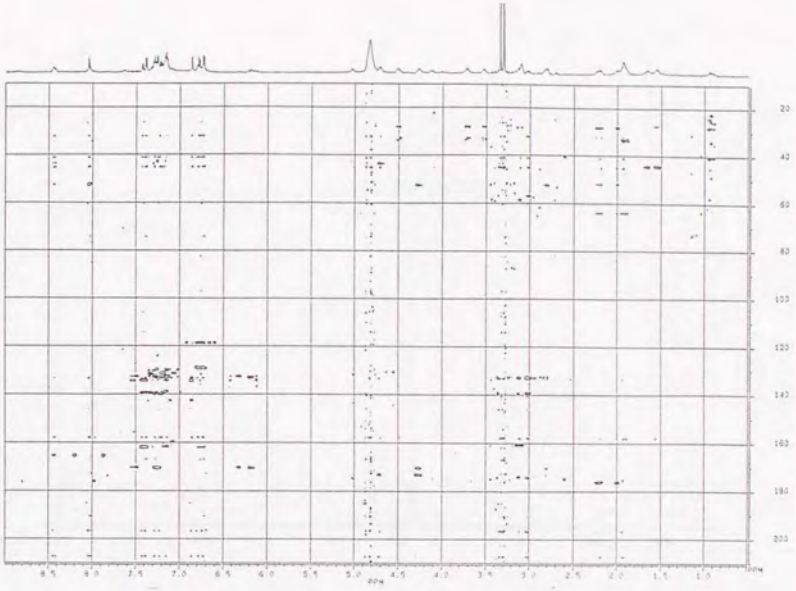


Fig III-41. HMBC spectrum of **58** in CD_3OH

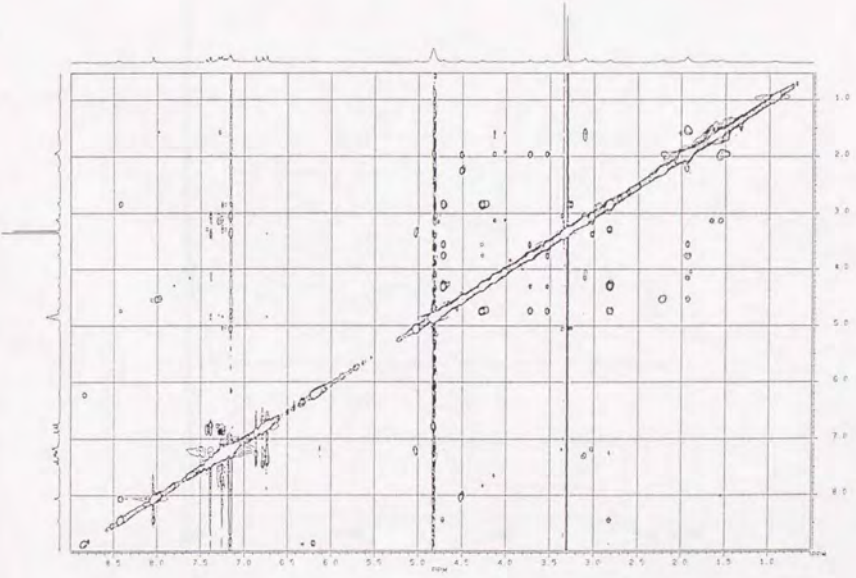


Fig III-42. ROESY spectrum of **58** in CD_3OH

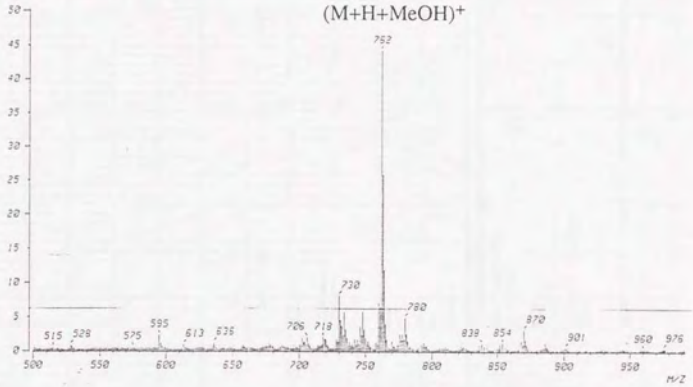


Fig III-43. FAB-MS of 58 (matrix; glycerol)

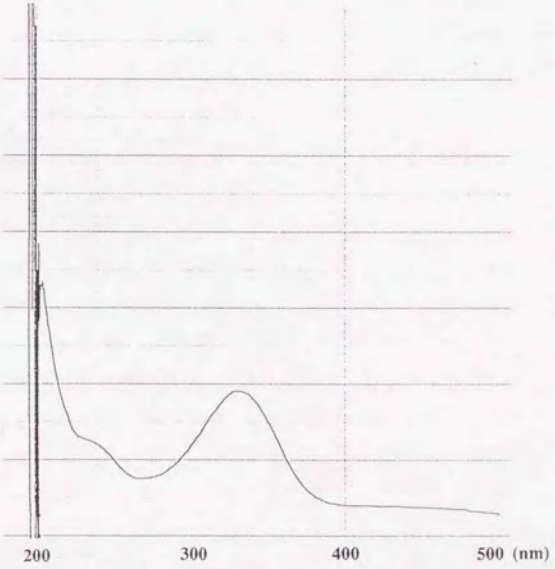


Fig III-44. UV spectrum of 58 in MeOH

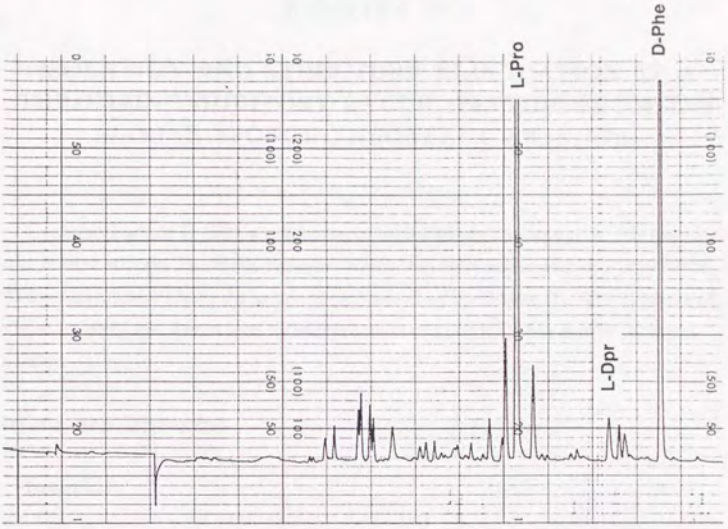


Fig III-45. HPLC analysis of acid hydrolysate by Marfey's method

CHAPTER IV

ISOLATION AND STRUCTURE ELUCIDATION OF A THROMBIN INHIBITORY CYCLIC PEPTIDE FROM THE MARINE SPONGE *THEONELLA SWINHOEI*

Cyclotheonamide G (**59**), a potent thrombin inhibitory cyclicpeptide, has been isolated from the marine sponge, *Theonella swinhoei* which was collected in Hachijo-jima Island, but morphologically distinct from the same species described in Chapter III. The structure of cyclotheonamide G was determined by interpretation of 2D NMR data and by chemical degradation.

1. Results and Discussion

1. 1. Isolation

The water soluble portion of the MeOH extract was extracted with *n*-BuOH, whose layer was fractionated by gel-filtrations, and chromatography on an NH_2 column. The active fraction was finally purified on ODS HPLC (30 % MeCN, 0.1 % TFA) to afford cyclotheonamide G as a colorless amorphous solid (2.25 x 10^{-4} % yield based on wet weight).

This compound inhibited not only thrombin with an IC_{50} of 0.24 μg/mL, but also trypsin with an IC_{50} of 0.31 μg/mL.

Sponge
|
MeOH
Et_2O/H_2O
n-BuOH/H_2O
Sephadex LH-20 (MeOH)
TOYOPEARL HW40SF (MeOH with 2 % AcOH)
amino-phase column chromatography ($CHCl_3$-MeOH)
amino-phase column chromatography (H_2O-MeOH)
ODS HPLC (30 % MeCN with 0.1 % TFA)
|
cyclotheonamide G

Fig IV-1. Isolation of cyclotheonamide G

1. 2. Structure Elucidation

Cyclotheonamide G (**59**) {$[\alpha]^{23}_D$ -17.6° (*c* 0.075, MeOH)} had a molecular formula of $C_{43}H_{59}N_{10}O_9$ as determined by HR-FABMS [$(M+H)^+$ *m/z* 859.4521, Δ +5.4 mmu]. Amino acid analysis of acid hydrolysate revealed the presence of 1 mol each of Ala, Pro, Ile, Dpr, and K-Arg (Fig IV-3).

Table IV-1. NMR Data of Cyclotheonamide G[a]

		^1H	^{13}C[b]			^1H	^{13}C[b]
Pha	CO	-	173.5	Ile	CO	-	171.8, 172.3
	α	3.52 bs	43.0		α	4.29 dd (5.9, 8.5),	58.2, 57.8
	1	-	136.3			4.24 m[c]	
	2	7.25 m	129.6		β	1.36 m, 1.39 m[c]	40.5, 41.2
	3	7.29 m	129.0		γ	1.3 m	26.9, 27.0
	4	7.22 m	127.5		δ	0.80 d (4.5),	13.2
						0.82 d (3.5)[c]	
Ala	CO	-	174.3		γ'	0.60 d (6.8),	14.4, 14.2
	α	4.32 quint. (7.2)	49.7			0.54 d (6.8)[c]	
	β	1.30 d (7.2)	17.3		NH	7.32 d (8.5)	-
	NH	8.36 d (7.2), 8.35 d (7.2)[c]	-				
				K-Arg	CO	-	170.9
Dpr	CO	-	170.9		α	-	[d]
	α	4.59 m	51.2		β	4.05 m, 4.10 m[c]	54.6
	β-1	4.19 m	40.7		γ-1	1.97 m	24.7
	β-2	2.72 dt (12.0, 10.7)	-		γ-2	1.50 m	-
	αNH	8.12 d (8.5), 8.16 d (8.5)[c]	-		δ-1	1.59 m	26.0
	βNH	8.78 d (10.3), 8.53 d (10.3)[c]	-		δ-2	1.70 m	-
					ε	3.10 q (7.0)	41.7
V-Tyr	CO	-	167.3		C=N	-	158.2
	α	6.13 d (15.3)	124.2		αNH	8.04 d (10.1),	-
	β	6.80 dd (15.3, 2.4),	143.2			8.00 d (10.1)[c]	
		6.77 dd (15.3, 2.4)[c]			εNH	7.20 m	-
	γ	4.7 m	53.3				
	δ-1	3.05 m	39.3	Pro	CO	-	173.7
	δ-2	2.59 dd (11.8, 13.9),	-		α	4.49 t (6.2),	61.4
		2.55 dd (11.6, 14.0)[c]				4.48 t (6.2)[c]	
	1	-	129.4		β-1	2.22 m	30.8
	2	7.05 d (8.5)	130.5		β-2	1.96 m	-
	3	6.69 dd (8.5, 2.4)	115.8		γ	1.95 m	25.7
	4	-	157.0		δ-1	3.8 m	48.9
	δ-NH	8.29 d (8.5), 8.21 d (8.5)[c]	-		δ-2	3.5 m	-

a; in CD_3OH.

b; ^{13}C chemical shifts were reduced from HMQC and HMBC spectra.

c; 3:2 signal pairs were observed in CD_3OH.

d; not observed

The ^1H NMR spectrum exhibited signals for a 3:2 mixture of two conformers (Fig IV-4). However, all NMR signals were unambiguously assigned by COSY, HOHAHA, HMQC and HMBC experiments (Fig IV-5-8), which confirmed the presence of Ala, Pro, Ile, Dpr and K-Arg residues.

In addition, signals for four aliphatic protons (δ 3.52, 3.05 and 2.55) and nine aromatic protons (δ 6.69 x 2, 7.05 x 2, 7.22, 7.29 x 2 and 7.25 x 2) could be observed in the ^1H NMR spectrum. The aromatic protons were assigned for a mono- and di-substituted benzene rings by interpretation of the HMQC and HMBC spectra. The disubstituted benzene ring was a part of a vinylogous tyrosine unit (V-Tyr) which was deduced from the HMBC spectrum. The *E* geometry of the double bond in V-Tyr unit was inferred from a vicinal coupling constant of 15.3 Hz. The singlet methylene signal at δ 3.52 was correlated with a carbonyl carbon (δ 173.5) and C-1 and -2 carbons (δ 136.3 and 129.6) of a mono-substituted benzene ring in the HMBC spectrum, thereby revealing the presence of a phenyl acetyl group (Pha). Connection of all these units through an amide bond fulfilled the molecular formula obtained by HR-FABMS.

The sequencing of these units was done by analysis of the HMBC and ROESY (Fig. IV-9) spectra measured in CD_3OH (Fig IV-2). Sequencial correlations were observed in the HMBC spectrum: K-Arg NH/Pro C-1; Ile NH/K-Arg C-1; V-Tyr NH/Ile C-1; Dpr β-proton/V-Tyr C-1; Dpr α-NH/Ala C-1; Ala NH/Pha CO. Connectivity between Dpr and Pro residues was established by ROESY crosspeaks between δ-protons of Pro and α-proton of Dpr. Therefore, the gross structure was determined as shown.

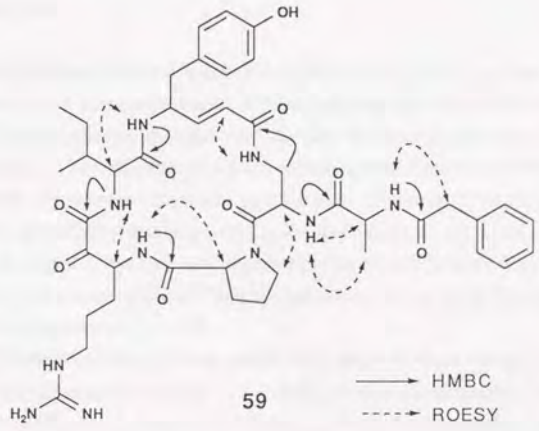


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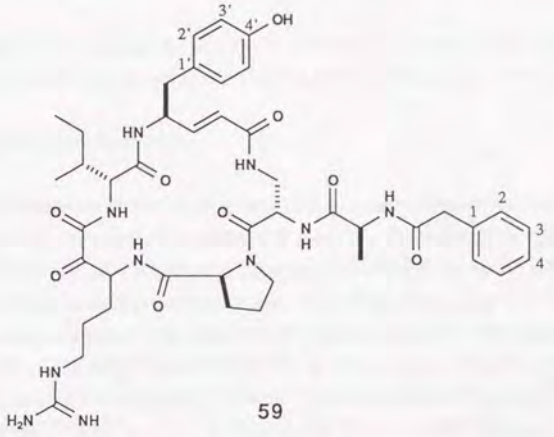
Fig IV-2. HMBC and ROESY correlations between the residues

The stereochemistry of amino acid residues was determined by HPLC analysis of the acid hydrolysate after derivatization with Marfey's reagent; L-Pro, L-Ala, L-Dpr, and D-Ile (Fig IV-10). The stereochemistry of V-Tyr was determined by chemical degradation as follows. Cyclotheonamide G was ozonized followed by hydrolysis with 6 N HCl. The hydrolyzate was derivatized with Marfey's reagent and analyzed by HPLC, which showed the presence of L-Asp (Fig IV-11). The stereochemistry of K-Arg remained to be determined.



59

1. 3. Discussion

Theonella swinhoei collected at Kaminato, Hachijo-jima Island contained cyclotheonamide G as a minor constituent. Although this sponge was morphologically distinct from the same species collected at Sokodo or Nazumado in Hachijo-jima Island, it fell in the same classification. In fact, this sponge did not contain cyclotheonamide A. The further insight into relationship between chemistry and taxonomy of these different morphs will be interesting. The phenyl acetyl group present in cyclotheonamide G was a rare example in marine natural products. It has been only reported in valdivone B isolated from the South African soft coral *Alcyonium valdivae*[59] and monodontamide A obtained from the marine gastropod mollusk *Monodonta labio*.[60]

Cyclotheonamide G has an α-keto amide which plays an important role in cyclotheonamide A binding to thrombin. It is also known that a hydrophobic interaction is formed between D-Phe of cyclotheonamide A and Leu^{40}-Leu^{41} of thrombin. Substitution of D-Phe by D-Ile in cyclotheonamide G did not alter the potent anti-thrombin activity; the side chain of Ile seems to function similar to the benzene ring of D-Phe residue.

2. Experimental Section

2. 1. General Methods

See chapter III.

2. 2. Biological Material

The sponge was collected by SCUBA at a depth of 10 m at Kaminato, Hachijo-jima Island in 1986, immediately frozen after collection, and preserved at -20 °C until extraction.

2. 3. Extraction and Isolation

The MeOH extract of the frozen sponge (800 g) was partitioned between H_2O and Et_2O, and the aqueous layer was further extracted with *n*-BuOH. The *n*-BuOH extract was gel-filtered on Sephadex LH-20 (MeOH) and Toyopearl HW-40SF (2 % AcOH in MeOH). The active fractions were combined and fractionated on an NH_2-column, first with $CHCl_3$-MeOH (stepwise gradient) and second with H_2O-MeOH (stepwise gradient). The active fraction was finally purified by ODS HPLC (30 % MeCN, 0.1 % TFA) to give 1.8 mg of cyclotheonamide G as colorless amorphous solids (**59**, 2.25 x 10^{-4} % yield based on wet weight).
Cyclotheonamide G: $[\alpha]^{23}_D$ -17.6° (*c* = 0.075, MeOH); UV (MeOH) λ_{max} 273 nm (ε 2700), 323 (ε 400)(Fig IV-12); $C_{43}H_{59}N_{10}O_9$; HR-FABMS *m/z* 859.4521 $(M+H)^+$ (Δ +5.4 mmu); FAB-MS (pos, glycerol) *m/z* 859 $(M+H)^+$, 891 $(M+H+MeOH)^+$ (Fig IV-13)

2. 4. Derivatization with Marfey's Reagent

See Chapter III.

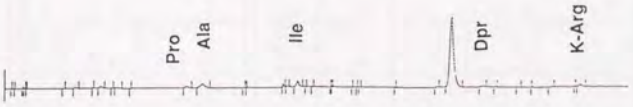


Fig IV-3. Amino acid analysis of **59**

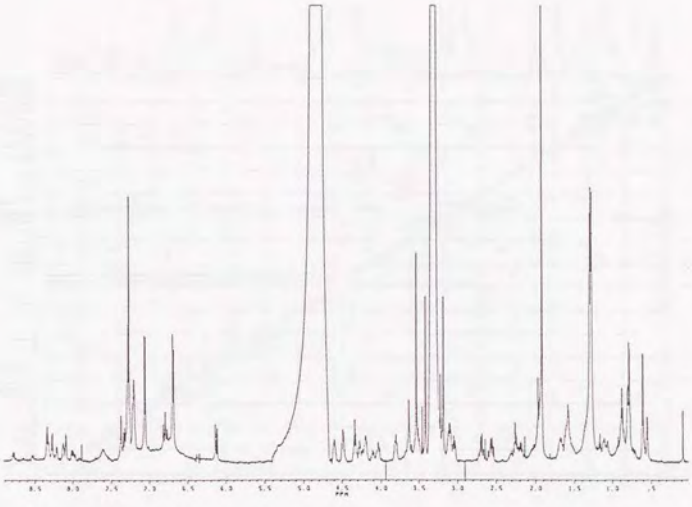


Fig IV-4. ^{1}H NMR spectrum of **59** in CD_3OH

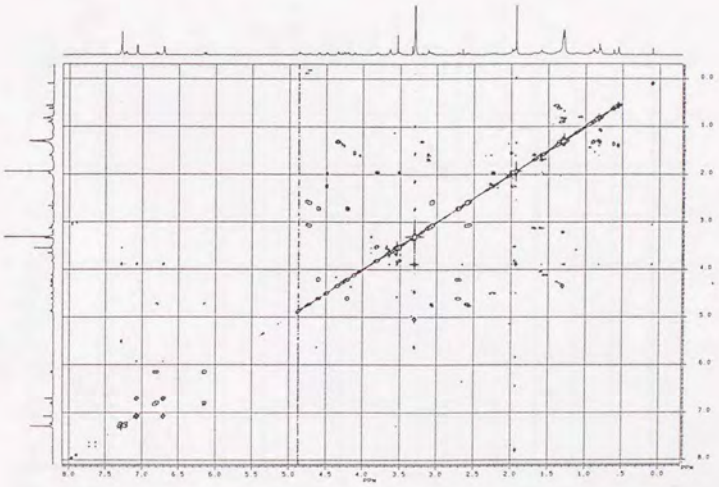


Fig IV-5. COSY spectrum of **59** in CD_3OD

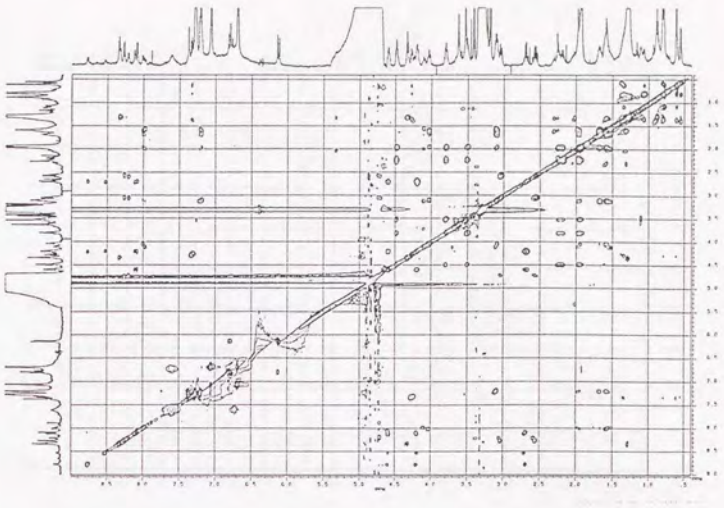


Fig IV-6. HOHAHA spectrum of **59** in CD_3OH

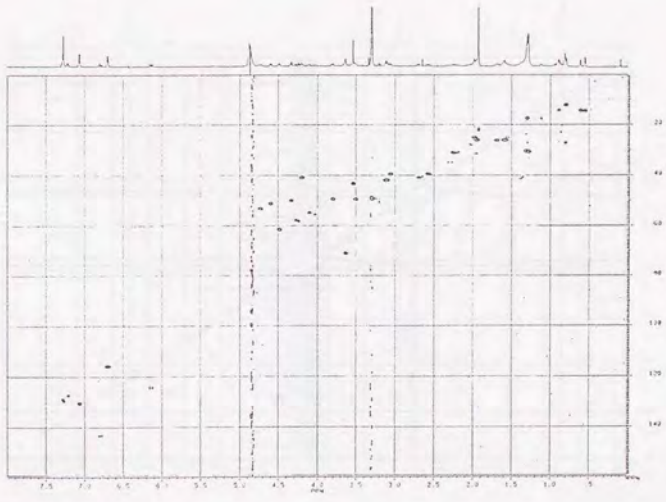


Fig IV-7. HMQC spectrum of **59** in CD_3OD

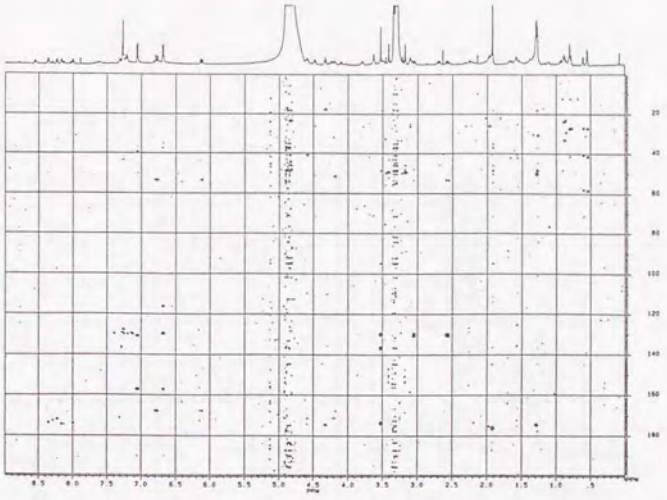


Fig IV-8. HMBC spectrum of 59 in CD_3OH

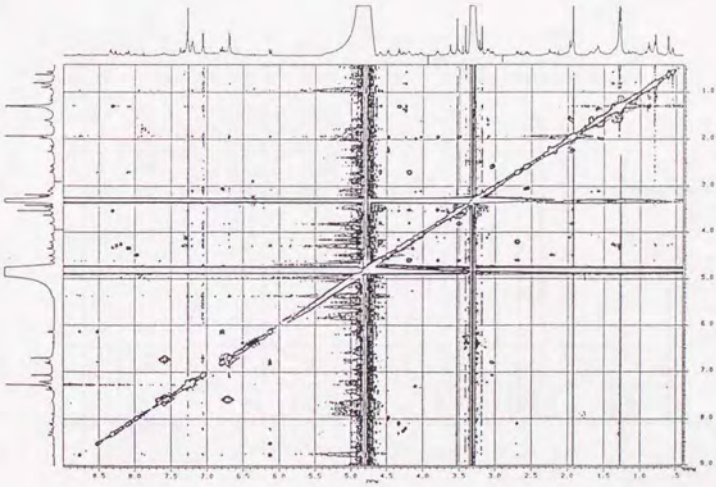


Fig IV-9. ROESY spectrum of 59 in CD_3OH

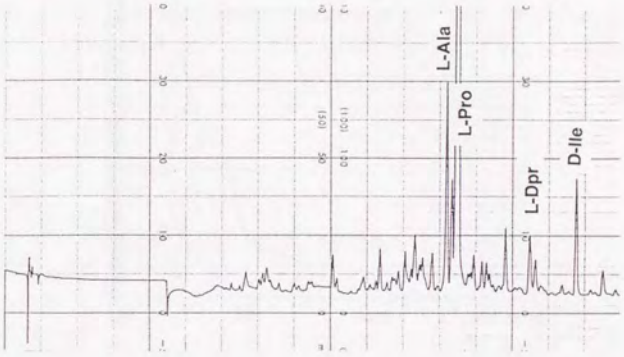


Fig IV-10. HPLC analysis of acid hydrolysate by Marfey's method

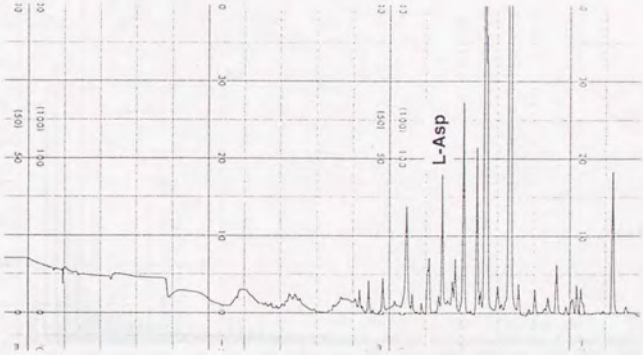


Fig IV-11. HPLC analysis of acid hydrolysate after ozonolysis by Marfey's method

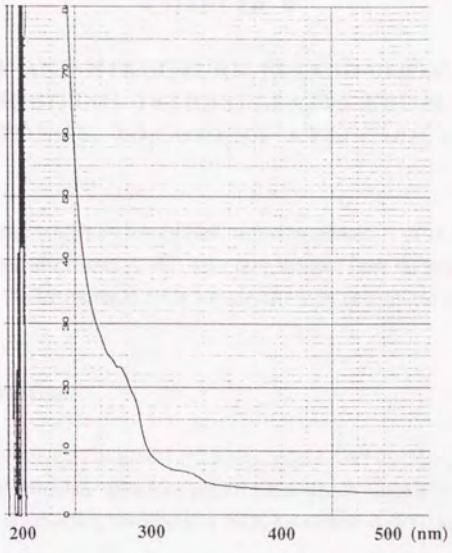


Fig IV-12. UV spectrum of **59** in MeOH

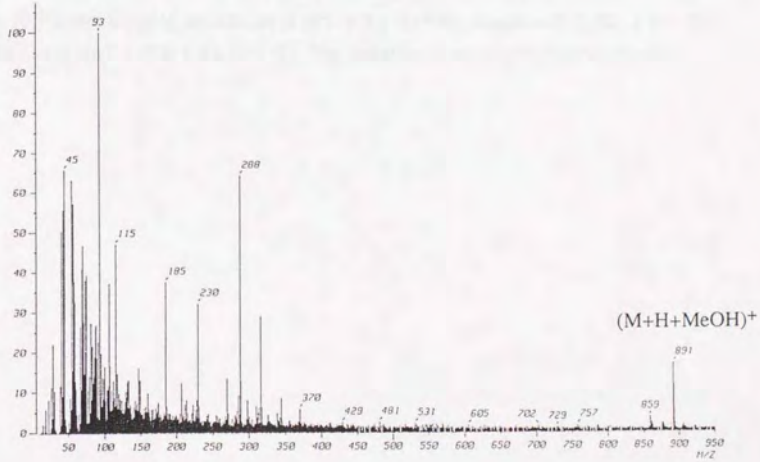


Fig IV-13. FAB-MS of **59** (matrix; glycerol)

CHAPTER V

ISOLATION AND STRUCTURE ELUCIDATION OF FOUR THROMBIN INHIBITING TETRASULFATES FROM THE MARINE SPONGE, *TOXADOCIA CYLINDRICA*

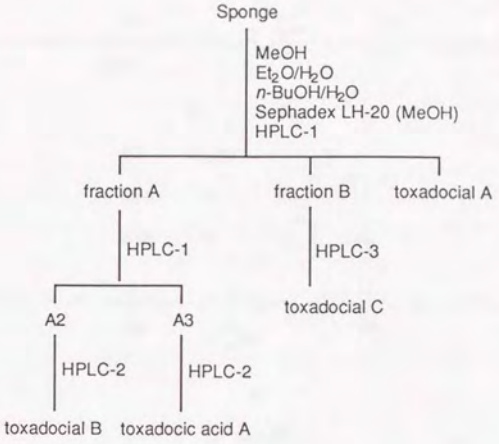
Four new thrombin-inhibitory metabolites named toxadocial A (**60**), toxadocial B (**61**), toxadocial C (**62**), and toxadocic acid A (**63**) have been isolated from the marine sponge *Toxadocia cylindrica*. The structures of these compounds were determined by spectroscopic and chemical methods.

1. Results and Discussion

The extract of the sponge *Toxadocia cylindrica* collected off Hachijo-jima Island was highly inhibitory against thrombin. Bioassay-guided fractionation of the MeOH extract afforded several active substances including toxadocial A (**60**), toxadocial B (**61**), toxadocial C (**62**), and toxadocic acid A (**63**).

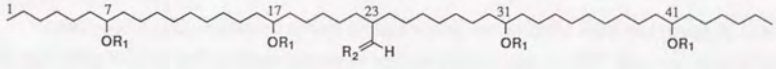
1. 1. Isolation

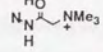
The water soluble portion of the MeOH extract of the frozen sponge (1.9 kg) was gel-filtered on Sephadex LH-20 followed by repetitive reverse-phase HPLC to yield toxadocial A (**60**, 2.9 x 10^{-3}% wet weight), toxadocial B (**61**, 4.4 x 10^{-4} %), toxadocial C (**62**, 1.9 x 10^{-3} %), and toxadocic acid A (**63**, 5.8 x 10^{-4} %). The isolation scheme is depicted in Fig V-1.

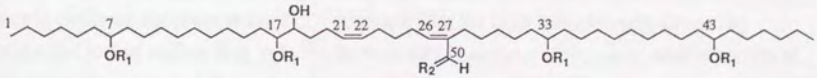


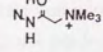
HPLC-1; on Cosmosil $5C_{18}$-AR with 52 % MeCN containing 0.25 M $NaClO_4$
HPLC-2; on *L*-column with 45.5 % MeCN containing 0.1 M $NaClO_4$
HPLC-3; on *L*-column with 46.5 % MeCN containing 0.1 M $NaClO_4$

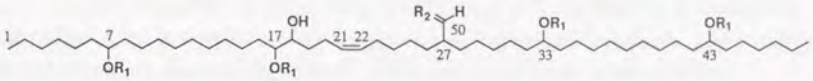
Fig V-1. Isolation of toxadocials and toxadocic acid A



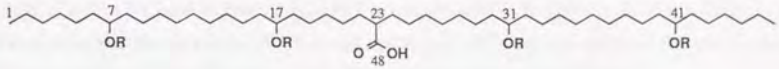
	R_1	R_2
60	SO_3Na	O
60a	H	O
60b	H	
60c	H	



	R_1	R_2
61	SO_3Na	O
61a	H	
61b	H	



	R_1	R_2
62	SO_3Na	
62a	H	
62b	H	



	R
63	SO_3Na
6a	H

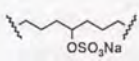
1. 2. Structure Elucidation

1. 2. 1 Toxadocial A

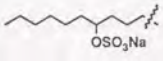
Toxadocial A (**60**) contained sulfate groups which was inferred from an intense IR band at 1250 cm^{-1} (Fig V-3) as well as from a positive sodium rhodizonate test.[61] This was supported by a deshielded methine signal (δH 4.31 quint, δC 81.0 d) equivalent to four methines in the NMR spectra (Fig V-4, 5). In addition to four sulfated methines, the ^1H and ^{13}C NMR spectra demonstrated two terminal methyls (δH 0.89 t, δC 14.4 q), a methine (δH 2.22 m, δC 53.2 d), an aldehyde (δH 9.51 d, δC 207.6 d), and alkyl chains. The aldehyde group was equilibrated with a hemiacetal (δH 4.38, δC 101.4) in CD_3OD.

Determination of the molecular formula for **60** was a key step in the structure determination. The negative ion FAB-MS using *m*-nitrobenzyl alcohol (NBA) or glycerol as a matrix exhibited ion peaks at *m/z* 1137, 1035 and 933; the latter two ions were formed by sequential loss of $NaSO_3$ (Fig V-6).[62] Further, HPLC analysis of the water soluble portion of the hydrolyzate revealed the presence of 4 sulfate groups in **60**.[63] Therefore, the ion at *m/z* 1137 was an $(M-Na)^-$ ion, which together with NMR data led to a molecular formula of $C_{48}H_{92}O_{17}S_4Na_4$ (molecular weight, 1160). When diethanolamine was used as a matrix, ion peaks were shifted to *m/z* 1224, 1122, and 1020, respectively, which were 87 mu higher than those observed when *m*-nitrobenzyl alcohol or glycerol was used as a matrix (Fig V-7). This can be rationalized by the reaction of diethanolamine with the aldehyde group in the molecule to generate a Schiff's base, which rearranged to an enamine during ionization in a mass spectrometer.

Interpretation of NMR data including the COSY (Fig V-8) and HMQC (Fig V-9) spectra was hampered due to severely overlapping signals, e.g., four oxymethine signals. Fortunately, an HMQC-HOHAHA spectrum (Fig V-10),[64] which gave signals good enough to deduce correlations, led to units **A**, **B**, and **C**; **A** and **B** were duplicated. The oxymethine protons correlated with methylene carbons at δ 35.3, 30.8, and 26.0 in the HMQC-HOHAHA spectrum, thus placing all oxymethines in the middle of alkyl chains of similar environment to construct unit **A**. Besides a large methylene envelope at δ 30.8, the ^{13}C NMR spectrum exhibited two-carbon methylene signals at δ 35.3, 33.0, 30.5, 26.1, and 23.7, all of which were correlated with terminal methyl protons in the HMQC-HOHAHA spectrum, thereby defining unit **B**. An aldehyde was attached to a methine carbon flanked by methylene carbons at δ 29.9 and 28.1 (unit **C**). Because of repetitive units in the molecule, elucidation of the gross structure was impossible by NMR experiments. Therefore, some chemical transformations were attempted.



A



B

C

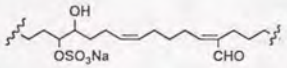
Toxadocial A was hydrolyzed with 1 N HCl (100 °C, 30 min) to afford a tetraol **60a** as a colorless amorphous solid. A molecular formula of $C_{48}H_{96}O_5$ was established by HR-FABMS [*m/z* 753.7359 $(M+H)^+$], which confirmed the molecular formula of the parent compound. Again, NMR data led to structural units **A**-**C**, which were also supported by a FAB-MS/MS experiment. However, no further information was obtained. A tetraacetate provided no useful information. Then, another target was picked up: the aldehyde group with which a Schiff's base could be generated. Girard's reagents T and P[65] were employed to facilitate analysis of fragment ions; ions arising from cleavage at the same site would differ by 20 mu for the two derivatives.[66] The FAB-MS/MS spectrum of the Girard's reagent P derivative **60b** showed intense ions at *m/z* 800, 770, 664, 614, 558, and 530 (Fig V-11), while the Girard's reagent T derivative **60c** led to corresponding ions 20 mu smaller, thus allowing assignment of the positions of four hydroxyl groups and an aldehyde (Fig V-12). To confirm this assignment FAB-MS/MS/MS experiments were carried out with daughter ions of **60b** at *m/z* 558 and 530; ion at *m/z* 558 yielded fragment ions at *m/z* 472 (C22-C41) and 442 (C22-C40), whereas ion at *m/z* 530 led to ions at *m/z* 444 (C8-C24) and 414. Thus, structures proposed for **60** and **60a** were correct.

1. 2. 2. Toxadocial B

Toxadocial B (**61**) was obtained as a colorless amorphous solid, $[\alpha]^{23}_D$ +3.7 ° (*c* 0.2, MeOH) and had a molecular formula of $C_{50}H_{92}S_4O_{18}Na_4$ which was determined by negative ion HR-FABMS $[(M-Na)^-$ *m/z* 1177.4861, Δ +0.1 mmu]. The ^1H NMR spectrum (Fig V-14) contained signals for two primary methyls [δ 0.77 (6H, t, J = 6.8 Hz)], four allylic methylenes [δ

2.16 (2H, q, J = 7.3 Hz), 2.29 (2H, q, J = 7.4 Hz), 2.30 (2H, q, J = 7.4 Hz), 2.39 (1H, m), and 2.55 (1H, dq, J = 14.3, 7.3 Hz)], five oxygenated methines [δ 4.28 (1H, dt, J = 10.0, 2.5 Hz), 4.86 (1H, quint, J = 5.9 Hz), 4.88 (2H, quint, J = 5.9 Hz), and 5.08 (1H, buried under solvent signal)], three olefinic protons [δ 5.44 (1H, dt, J = 10.5, 7.3 Hz), 5.57 (1H, dt, 10.5, J = 7.3 Hz), and 6.43 (1H, t, J = 7.4 Hz)], and an aldehyde [δH 9.50 (1H,s)], while the ^{13}C NMR spectrum (Fig V-15) revealed signals for five oxygenated methines [δC 72.60, 78.78 (2C), 78.88, and 82.90], four sp^2 carbons (δC 129.43, 131.23, 143.97, and 155.33), and an aldehyde (δC 195.31) together with methyl [δC 14.2 (2C)] and numerous methylene signals. A characteristic C23 methine in **60** (δC 53.2) was missing, suggesting the presence of an α, β-unsaturated aldehyde group in **61**, which was supported by spectral data [δC 195.31, ν_{max} 1670 cm^{-1}, λ_{max} 232 nm (ε 9900)](Fig V-16, 17).

Interpretation of the COSY spectrum (Fig V-18) starting from an olefinic proton on a trisubstituted double bond (δ 6.43) revealed that the two double bonds were connected through three methylene carbons. The Z-geometry of the disubstituted double bond was deduced from a vicinal coupling constant of 10.5 Hz. The proton at δ 5.57 was coupled to allylic methylene protons at δ 2.39 and 2.55, which were in turn correlated with methylene protons at 1.77 and 2.01. The latter methylene protons were further coupled to an oxy-methine at δ 4.28 which was correlated with another oxygenated methine at δ 5.08. This methine proton was also coupled to methylene protons at δ 1.71 and 2.02, both of which gave crosspeaks with a methylene protons at δ 1.73 and 1.55. Incidentally, *E*-geometry of the trisubstituted double bond was secured by NOESY[65] (Fig V-19) crosspeaks; δ 6.43/9.50 and 9.50/ 2.29. The methylene protons at δ 2.29 showed a COSY crosspeak with methylene protons at δ 1.39, which were correlated with a methylene in the envelope at δ 1.16. Considering HMQC crosspeaks (Fig V-20), δH 4.28/δC 72.6 and 5.08/82.9, partial structure **D** could be assigned, which comprises the central moiety of the molecule. The HMBC (Fig V-21) and HMQC-HOHAHA (Fig V-22) spectra supported the existence of partial structure **D**.



D

The two remaining segments contained three sulfated methines, two terminal methyls, and 28 methylenes. Since further structural information could not be obtained by NMR experiments, MS/MS techniques were applied as the case of toxadocial A. Toxadocial B was treated with dilute HCl to give rise to a pentaol which was treated consecutively with Girard's reagents P and T, yielding compounds **61a** and **61b**. The FAB-MS/MS of **61a** and **61b** gave ions with 20 mu difference, revealing that fragment ions included the cationic portion in the center of the molecule (Fig V-23). Three sets of intense ion peaks 30 mu apart in the FAB-MS/MS of **61b** at *m/z* 820/790, 664/634, and 634/604 as depicted in Fig V-24 led to determination of the position of the

five hydroxyl groups, whereas ions at *m/z* 578, 536 and 508 supported the location of the α, β-unsaturated aldehyde group. FAB-MS/MS data of the corresponding derivatives (**61c** and **61d**) prepared from tetrahydrotoxadocial B were consistent with the assigned structure (Fig V-34, 36).

Table V-1. NMR Data of Toxadocial B (61)[a]

#C	^{13}C	^{1}H	#C	^{13}C	^{1}H
1	14.20	0.77 (t, 6.8)	26	155.33	6.43 (t, 7.4)
2	22.84	1.15 (m)	27	143.97	-
3	32.04	1.15 (m)	28	24.12	2.29 (t, 7.4)
4	29.81	1.16 (m)	29	28.99	1.39 (quint, 7.4)
5	25.47	1.53 (m)	30	~30	1.25 (m)
6	35.02	1.91 (m), 1.81 (m)	31	25.20	1.53 (m)
7	78.78	4.88 (quint, 5.9)	32	34.82	1.91 (m), 1.81 (m)
8	34.93	1.91 (m), 1.81 (m)	33	78.88	4.86 (quint, 5.9)
9	25.47	1.53 (m)	34	34.93	1.91 (m), 1.81 (m)
10	29.76	1.25 (m)	35	25.47	1.53 (m)
11	~30	1.16 (m)	36	~30	1.25 (m)
12	~30	1.16 (m)	37	~30	1.16 (m)
13	~30	1.16 (m)	38	~30	1.16 (m)
14	29.76	1.25 (m)	39	~30	1.16 (m)
15	26.07	1.73 (m), 1.55 (m)	40	29.76	1.25 (m)
16	31.56	2.02 (m), 1.71 (m)	41	25.47	1.53 (m)
17	82.90	5.08	42	34.93	1.91 (m), 1.81 (m)
18	72.60	4.28 (dt, 10.0, 2.5)	43	78.78	4.88 (quint, 5.9)
19	32.21	2.01 (m), 1.77 (m)	44	35.02	1.91 (m), 1.81 (m)
20	24.56	2.55 (dq, 14.3, 7.3), 2.39 (m)	45	25.47	1.53 (m)
21	131.23	5.57 (dt, 10.5, 7.3)	46	29.81	1.16 (m)
22	129.43	5.44 (dt, 10.5, 7.3)	47	32.04	1.15 (m)
23	27.19	2.16 (q, 7.3)	48	22.84	1.15 (m)
24	28.92	1.50 (quint, 7.3)	49	14.20	0.77 (t, 6.8)
25	28.70	2.30 (q, 7.4)	50	195.31	9.50 (s)

a; in C_5D_5N

1. 2. 3. Toxadocial C

Toxadocial C (**62**) had a molecular formula of $C_{50}H_{94}S_4O_{18}Na_4$ as determined for an $(M-Na)^-$ ion by HR-FABMS. Toxadocial C had two more hydrogen than **61**, suggesting that one double bond in **61** was reduced. The IR absorbtion at 1720 cm^{-1} (Fig V-26) suggested that the aldehyde (δ_C 205.65, δ_H 9.64) was linked to an sp^3 carbon instead of an α, β-unsaturated aldehyde as in **61** (Fig V-27, 28). Interpretation of the COSY (Fig V-29), HMQC (Fig V-30),

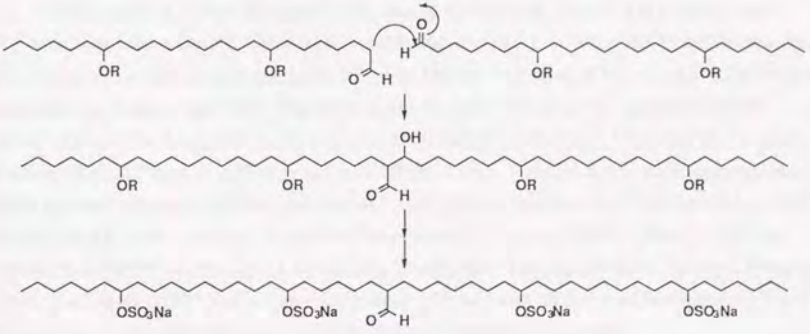
and HMQC-HOHAHA (Fig V-31) spectra led to a partial structure **D** lacking the C26 olefin. The position of the remaining three sulfate esters was similarly determined by FAB-MS/MS to obtain gross structure **62** (Fig V-32, 33). FAB-MS/MS data of the corresponding derivatives (**62c** and **62d**) prepared from dihydrotoxadocial C were identical with those of **61c** and **61d** (Fig V-34-37).

1. 2. 4. Toxadocic Acid A

The molecular formula of toxadocic acid A (**63**) was determined as $C_{48}H_{92}S_4O_{18}Na_4$ by the negative ion HR-FABMS. The NMR spectra (Fig V-39-43) were almost identical with those of **60** except for a ^{13}C signal (Fig V-40) at δ 179.15, revealing the presence of a carboxylic acid instead of an aldehyde in toxadocial A (**60**). Acid hydrolysis of **63** yielded a tetraol **63a**, whose structure was elucidated by negative ion FAB-MS/MS measurements. As shown in Fig V-44 prominent ions at *m/z* 681/651 and 525/495 led to location of hydroxyl groups, while ions at *m/z* 439 and 411 indicated the position of the carboxyl group at C23.

1. 3. Discussion

Toxadocials are an unprecedented class of natural products with one carbaldehyde in the middle of a long alkyl chain. Some remotely related compounds have been isolated from terrestrial microbes and echinoderms; izumenolide, a sulfated lactone from *Micromonospora chalcea* subsp. *izumensis*[68] and a bis-sulfate from the starfish *Asterias forbesi*.[69] Toxadocials are likely to be biosynthesized through aldol condensation of two units of hydroxylated (or sulfated) aldehydes followed by dehydration and reduction (Fig V-2).



toxadocial A

Fig V-2. Proposed biogenesis of toxadocial A

Toxadiocials A-C and toxadocic acid A had similar activities against thrombin with IC_{50}'s of 6.5, 4.6, 3.2, and 2.7 μg/mL, respectively. This may indicate that their activity is mainly attributable to sulfate esters. In fact, halistanol sulfate and halistanol sulfates A-E, trisulfated steroids, isolated from a marine sponge *Epipolasis* sp. inhibited thrombin with IC_{50} values of 17, 17, 23, 16, 47 and 90 μg/mL.[62]

2. Experimental Section

2. 1. General Methods

^1H and ^{13}C NMR spectra were recorded on a Bruker AM 600 NMR spectrometer in C_5D_5N at 300 K. FAB-MS and FAB-MS/MS were measured on a JEOL JMX-SX102/SX102 tandem mass spectrometer using *m*-nitrobenzyl alcohol (positive ion mode) and triethanolamine (negative ion mode) as matrices. Infrared spectra were recorded on a JASCO IR-G infrared spectrometer. Optical rotations were determined on a JASCO DIP-371 digital polarimeter in methanol. UV spectrum was recorded on a Hitachi 330 spectro photometer.

2. 2. Biological Material

The sponge *Toxadocia cylindrica* was collected off Hachijo-jima Island by SCUBA at depths of 5-20 m in November. 1991, immediately frozen after collection, and preserved at -20 °C until extraction.

2. 3. Extraction and Isolation

The concentrated MeOH extract of the sponge (1.9 kg, wet weight) was extracted with Et_2O and then with *n*-BuOH. The MeOH soluble portion (16.7 g) of the *n*-BuOH extract was gel-filtered through a column of Sephadex LH-20 with MeOH. Fractions of 10 mL were collected and monitored by bioassay and TLC. The major active fractions (1.4 g) were separated by ODS HPLC on Cosmosil $5C_{18}$-AR (2 x 25 cm) with 52 % MeCN containing 0.25 M $NaClO_4$ to yield fractions A (220.7 mg), B (113 mg) and pure **60** (55.2 mg). Fraction A was rechromatographed under the same procedure yielding fractions A1 - A6. Further purification of fractions A2 and A3 by ODS HPLC on an *L*-column (Chemicals Inspection and Testing Institute, Japan, 1 x 25 cm) with 45.5 % MeCN containing 0.1 M $NaClO_4$ afforded **61** (8.4 mg) and **63** (11.1 mg). Fraction B was purified by ODS HPLC on an *L*-column (1 x 25 cm) with 46.5 % MeCN containing 0.1 M $NaClO_4$ and desalted through an ODS short column to give 36.6 mg of **62**.

Toxadocial A (60): $[\alpha]^{23}_D$ -2.2° (*c* 1.0, MeOH); FAB-MS (neg, NBA) *m/z* 1137 $(M-Na)^-$, 1035, 933; IR (film) 3450, 2940, 2860, 1720, 1250, 1070, 940 cm^{-1}; ^1H NMR (CD_3OD) δ 9.51 (d, *J*=3.0 Hz, H-48), 4.31 (quint, *J*=4.9 Hz, H-7, H-17, H-31, H-41), 2.22 (m, H-23), ~1.65 (m,H-6, H-8, H-16, H-18, H-30, H-32, H-40, H-42), 1.63~1.46 (m), ~1.39 (m, H-5, H-

9, H-15, H-19, H-29, H-33, H-39, H-43), ~1.3 (m, H-2~4, H-10~14, H-20, H-24~28, H-34~38, H-44~46), 0.89 (t, *J*=6.7 Hz, H-1, H-47); ^{13}C NMR (CD_3OD) 207.6 (d, C-48), 81.0 (d, C-7, C-17, C-31, C-41), 53.2 (d, C-23), 35.3 (t, C-6, C-8, C-16, C-18, C-30, C-32, C-40, C-42), 33.0 (t, C-3, C-45), 30.8 (t, C-10~14, C-20, 21, C-25~28, C-34~38), 30.5 (t, C-4, C-44), 29.9, 28.1 (t, C-22, C-24), 26.0 (t, C-5, C-9, C-15, C-19, C-29, C-33, C-39, C-43), 23.7 (t, C-2, C-46), 14.4 (q, C-1, C-47).

Toxadocial B (61): colorless amorphous solid; $[\alpha]^{23}D$ +3.7 ° (*c* 0.2, MeOH); $C_{50}H_{92}S_4O_{18}Na_3$; HR-FABMS *m/z* 1177.4861 $(M-Na)^-$ (Δ +0.1 mmu); FAB-MS (neg, NBA) *m/z* 1179 $(M-Na)^-$ 1077, 975 (Fig V-38); IR (film) ν_{max} 3470, 2925, 2850, 1670, 1640, 1460, 1380, 1350, 1220, 1060, 930, 820, 770 cm^{-1}; UV (MeOH) λ_{max} 232 nm (ε 9900); ^1H and ^{13}C NMR (C_5D_5N) see Table V-1.

Toxadocial C (62): colorless amorphous solid; $[\alpha]^{23}D$ +2.2 ° (*c* 0.2, MeOH); $C_{50}H_{94}S_4O_{18}Na_3$; HR-FABMS *m/z* 1179.5026 $(M-Na)^-$ (Δ +1.0 mmu); FAB-MS (neg, NBA) *m/z* 1177 $(M-Na)^-$ 1075, 973 (Fig V-25); FAB-MS (neg, NBA) *m/z* 1153 $(M-Na)^-$ 1051, 949 (Fig V-45); IR (film) ν_{max} 3470, 2925, 2850, 1720, 1640, 1460, 1380, 1350, 1220, 1060, 930, 820, 770 cm^{-1}; ^1H NMR (C_5D_5N) δ 9.64 (bs), 5.50 (dt, *J* = 10.5, 7.3 Hz), 5.40 (dt, *J* = 10.4, 7.3, Hz), 5.07 (m), 4.84 (quint, *J* = 4.2 Hz), 4.25 (bd, *J* = 10.1 Hz), 2.52 (m), 2.34 (m), 2.20 (m), 2.06 (q, *J* = 7.0 Hz), 2.01 (m), 1.97 (m), 1.87 (m), 1.77 (m), 1.76 (m), 1.72 (m), 1.71 (m), 1.56 (m), 1.54 (m), 1.50 (m), 1.34 (m), 1.25 (m), 1.22 (m), ~1.2 (m), 1.15 (m), 0.77 (t, *J* = 6.9 Hz); ^{13}C NMR (C_5D_5N) δ 205.65, 130.48, 130.00, 83.03, 78.94 (3C), 72.62, 51.99, 34.89 (3C), 34.82 (2C), 34.74, 32.24, 32.02 (2C), 31.45, ~30 (12C), 29.71 (2C), 28.94, 28.77, 27.26, 27.17, 26.83, 26.04, 25.40 (5C), 25.19, 24.51, 22.83 (2C), 14.20 (2C).

Toxadocic acid A (63): colorless amorphous solid; $[\alpha]^{23}D$ +0.6 ° (*c* 0.36, MeOH); $C_{48}H_{92}S_4O_{18}Na_3$; HR-FABMS *m/z* 1153.4917 $(M-Na)^-$ (Δ +5.7 mmu); FAB-MS (neg, NBA) *m/z* 1153 $(M-Na)^-$ 1051, 949 (Fig V-45); IR (film) ν_{max} 3470, 2925, 2850, 1710, 1640, 1460, 1380, 1350, 1220, 1060, 1040, 930, 770 cm^{-1} (Fig V-46); ^1H NMR (C_5D_5N) δ 4.88 (quint, *J* = 5.9 Hz), 4.87 (quint, *J* = 5.9 Hz), 2.58 (tt, *J* = 9.2, 4.4 Hz), 1.91 (m), 1.83 (m), 1.81 (m), 1.57 (m), 1.54 (m), 1.45 (m), ~1.2 (m), 1.16 (m, H-2), 1.14 (m), 0.77 (t, *J* = 7.3 Hz); ^{13}C NMR (C_5D_5N) δ 179.15, 78.83, 78.79 (3C), 46.49, 35.00 (2C), 34.95 (2C), 34.92 (3C), 34.86, ~33.0 (2C), 32.04 (2C), ~30 (14C), 29.77 (2C), 27.91 (2C), 25.46 (6C), 25.43, 25.33, 22.84 (2C), 14.20 (2C).

Tetraol (60a): $[\alpha]D$ -3.7° (*c* 0.175, $CHCl_3$); $C_{48}H_{97}O_5$; HR-FABMS *m/z* 753.7359 $(M+H)^+$ (Δ +2.3 mmu); FAB-MS (neg, TEA) *m/z* 751 $(M-H)^-$ (Fig V-13); ^1H NMR (CD_3OD) 9.53 (d, *J*=3.0 Hz, H-48), 3.57 (4H, m, H-7, H-17, H-31, H-41), 2.22 (m, H-23), ~1.41 (m,H-6, H-8, H-16, H-18, H-30, H-32, H-40, H-42), ~1.3 (m, H-2~5, H-9~15, H-18~22, H-24~29, H-33~39, H-43~46), δ 0.89 (6H, t, *J*=6.7 Hz, H-1, H-47).

2. 4. Preparations of Girard's Reagent P and T Derivatives

A 0.1 mg portion of either toxadocial A, B or C was refluxed with 1 N HCl (1 mL) for 30 min, and the reaction mixture was extracted with Et_2O. To the residue of the organic layer was added 20 μL of Girard's reagents P or T in AcOH (3.3 μmol/mL) and the mixture was heated at 70 °C for 30 min.

2. 5. Hydrogenation

To a solution of toxadocial B (1.0 mg) in MeOH was added 15 mg of 5 % Pd on activated carbon and stirred under 1 atm of H_2 at room temperature for 10 h. The reaction mixture was filtered through a membrane filter which was further washed with $CHCl_3$/MeOH/H_2O (6:4:1).

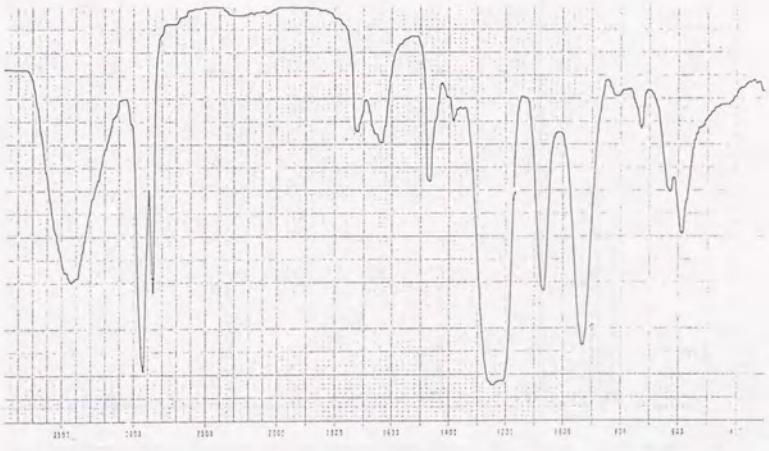


Fig V-3. IR spectrum of toxadocial A (60) (film)

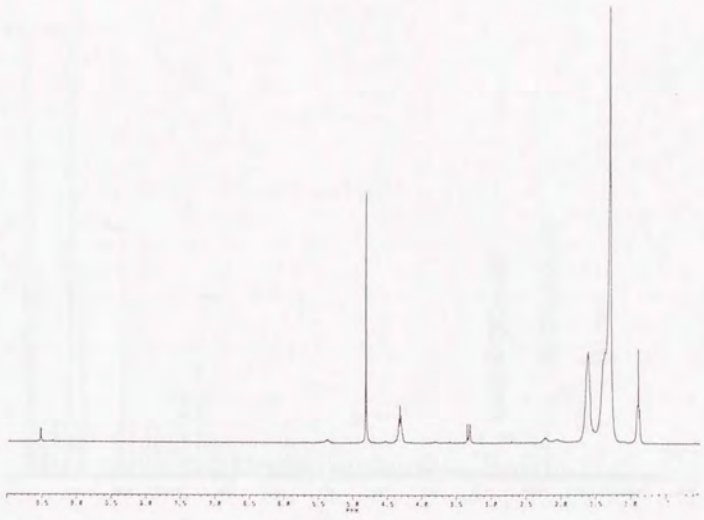


Fig V-4. ^{1}H NMR spectrum of toxadocial A (60) in CD_3OD

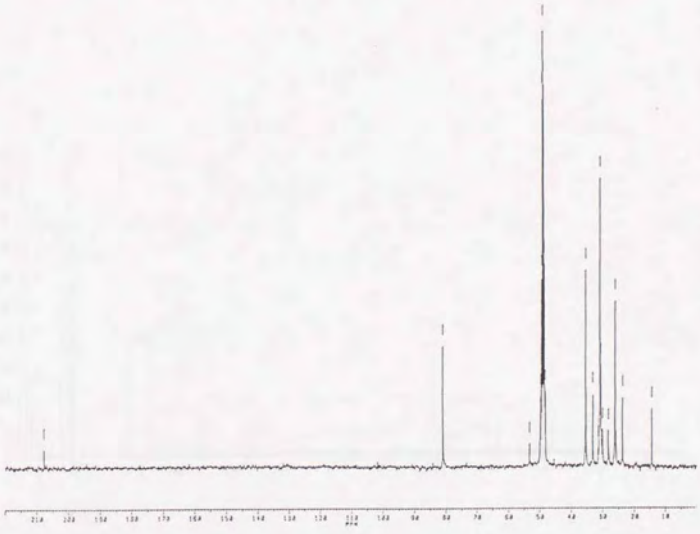


Fig V-5. ^{13}C NMR spectrum of toxadocial A (60) in CD_3OD

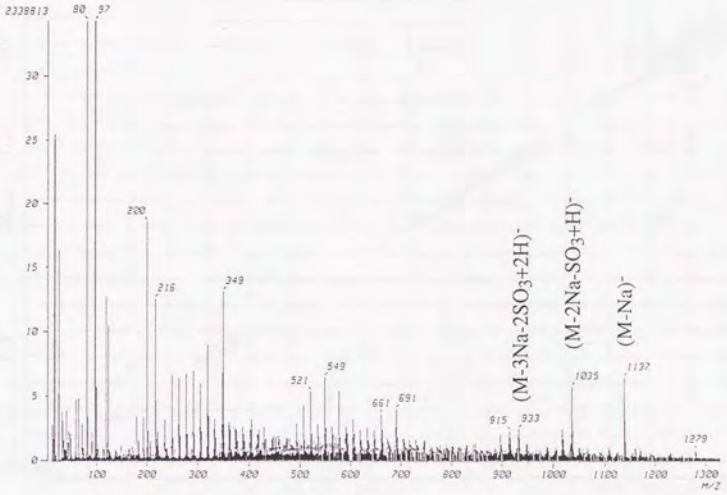


Fig V-6. Negative ion FAB-MS of toxadocial A (60) (matrix; NBA)

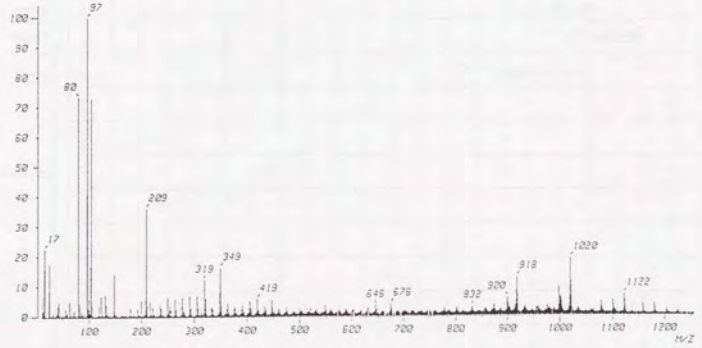


Fig V-7. Negative ion FAB-MS of toxadocial A (60) (matrix; DEA)

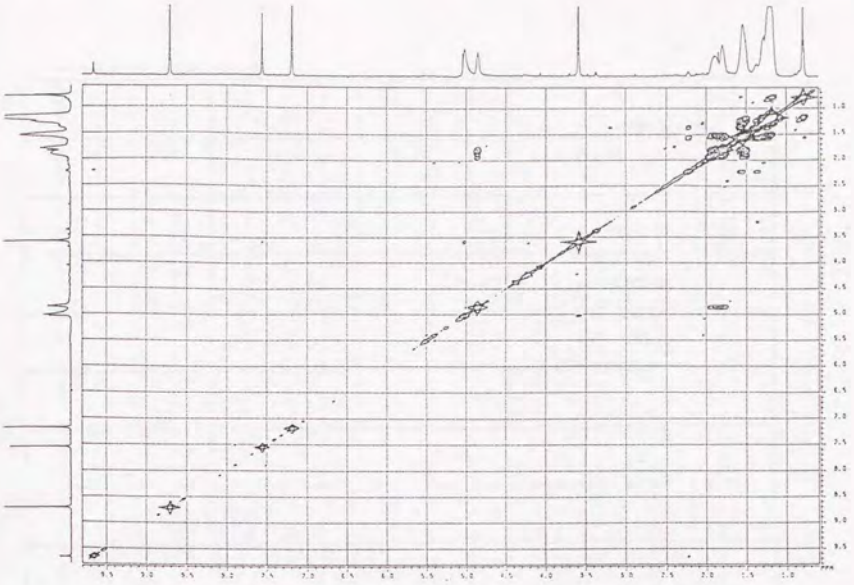


Fig V-8. COSY spectrum of toxadocial A (60) in CD_3OD

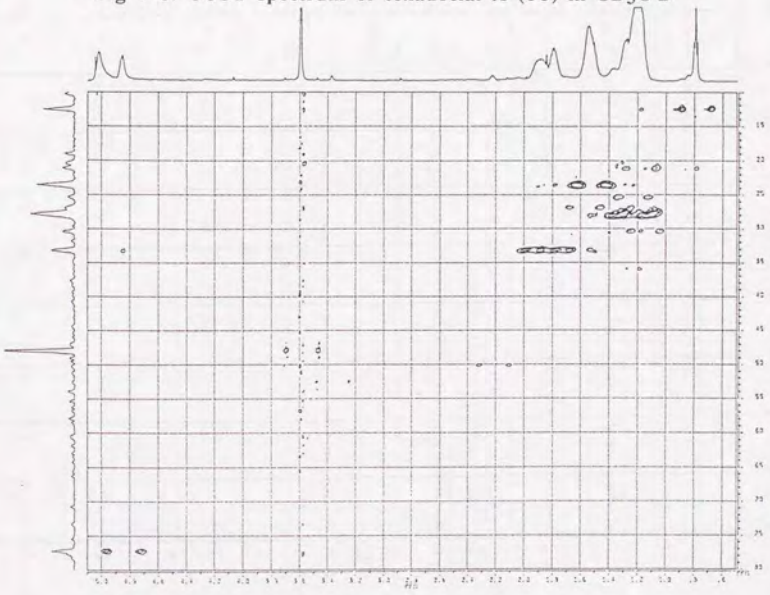


Fig V-9. HMQC spectrum of toxadocial A (60) in CD_3OD

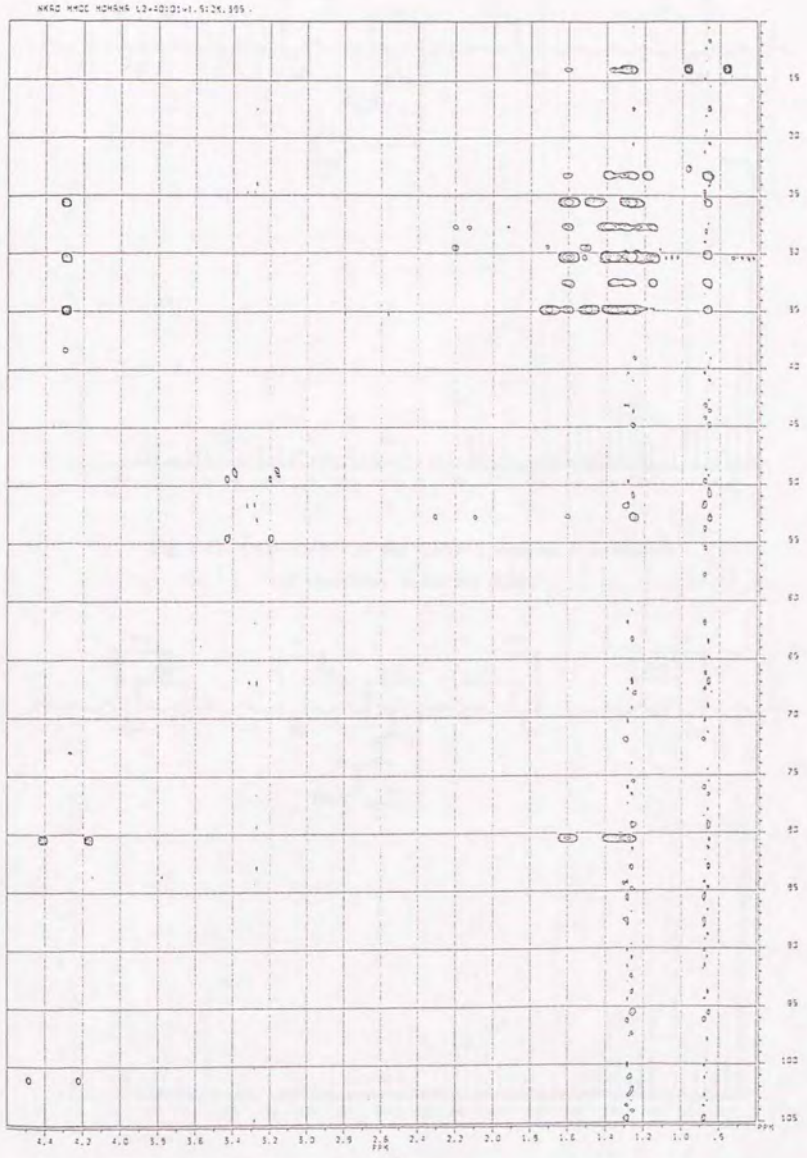


Fig V-10. HMQC-HOHAHA spectrum of toxadocial A (60) in CD_3OD

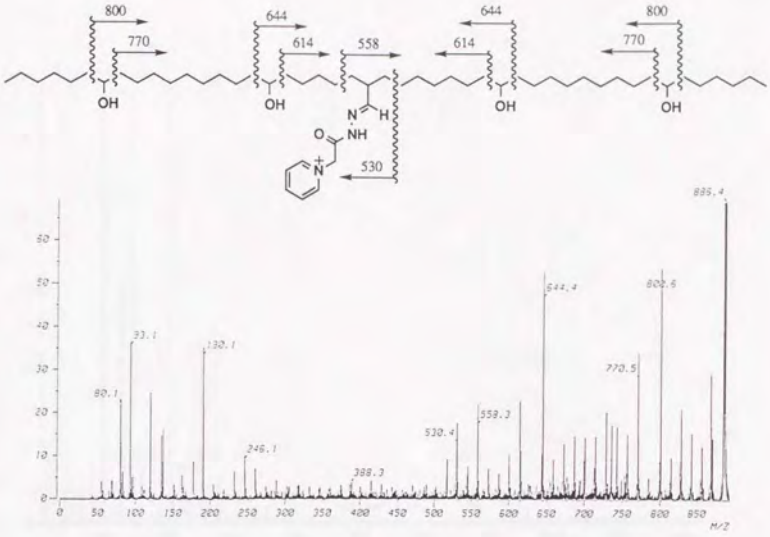


Fig V-11. FAB-MS/MS of the Girard's reagent P derivative of toxadocial A tetraol (60b)

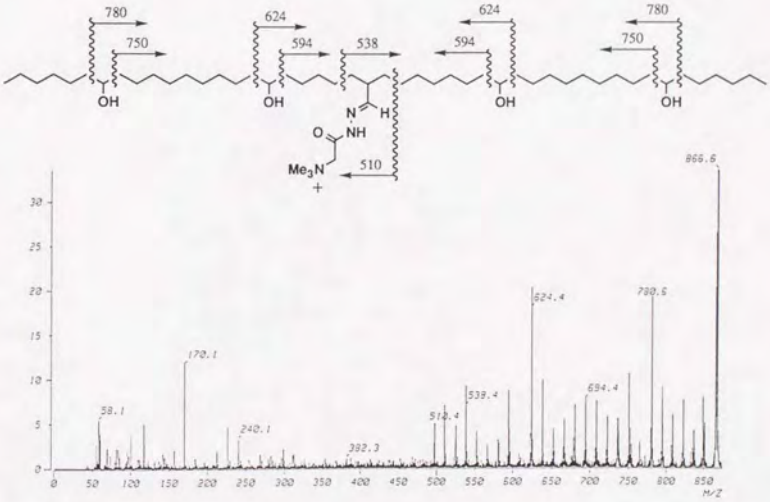


Fig V-12. FAB-MS/MS of the Girard's reagent T derivative of toxadocial A tetraol (60c)

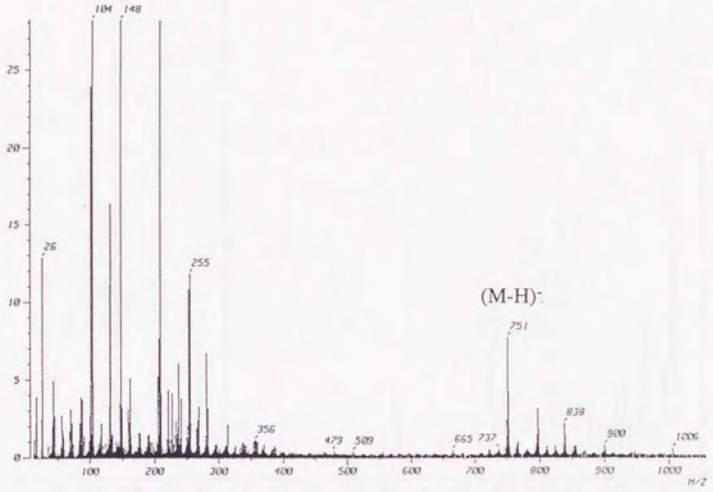


Fig V-13. Negative ion FAB-MS of a tetraol (60a)(matrix; TEA)

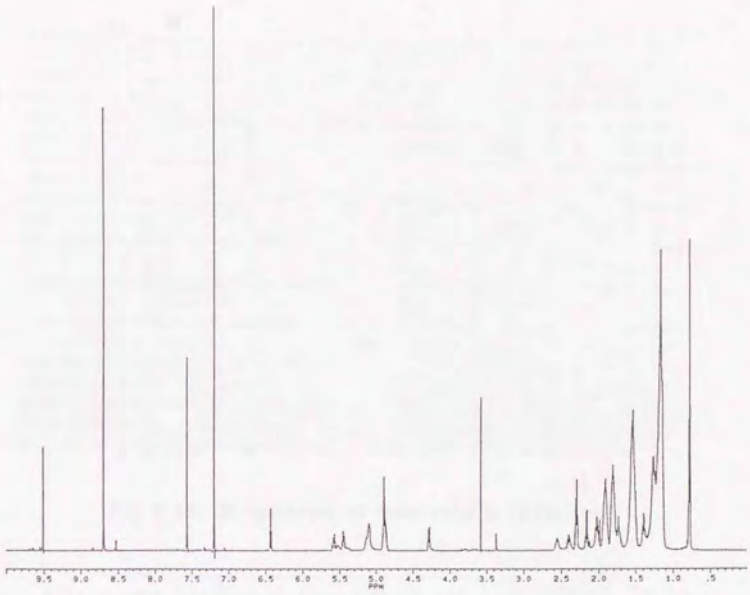


Fig V-14. ^{1}H NMR spectrum of toxadocial B (61) in C_5D_5N

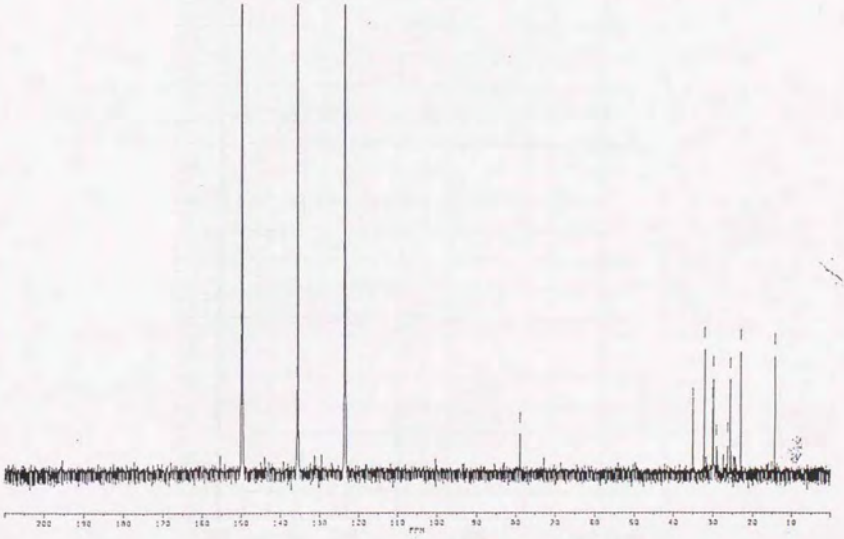


Fig V-15. ^{13}C NMR spectrum of toxadocial B (61) in C_5D_5N

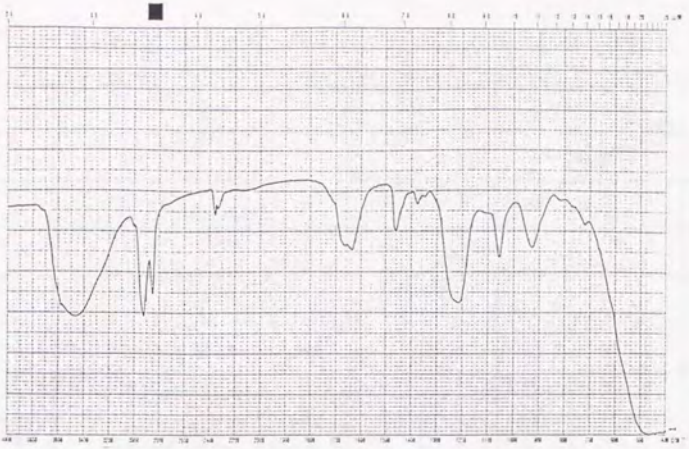


Fig V-16. IR spectrum of toxadocial B (61)(film)

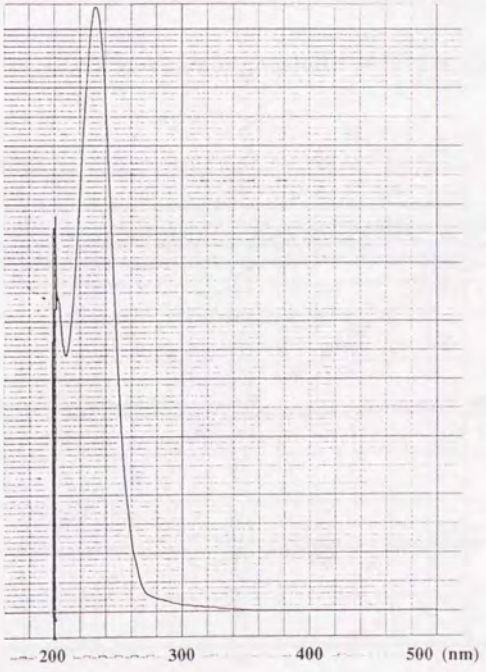


Fig V-17. UV spectrum of toxadocial B (61) in MeOH

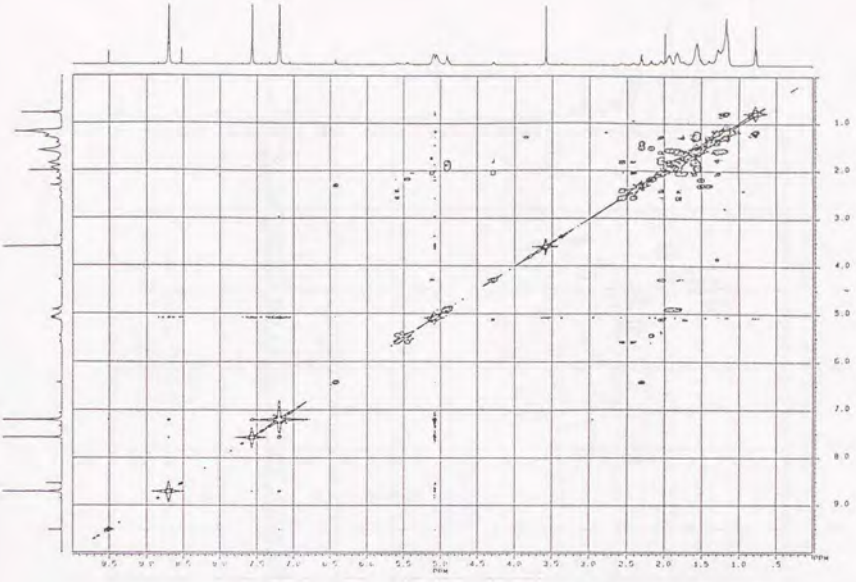


Fig V-18. COSY spectrum of toxadocial B (61) in C_5D_5N

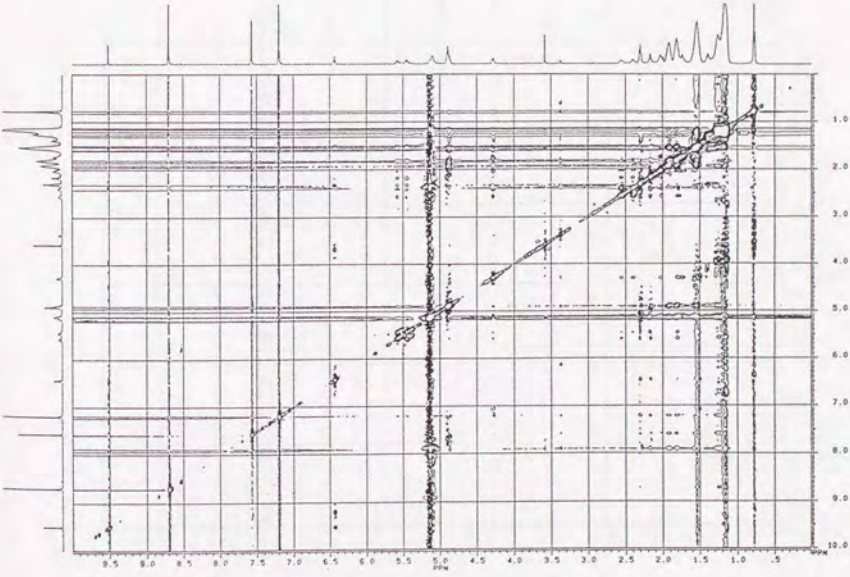


Fig V-19. NOESY spectrum of toxadocial B (61) in C_5D_5N

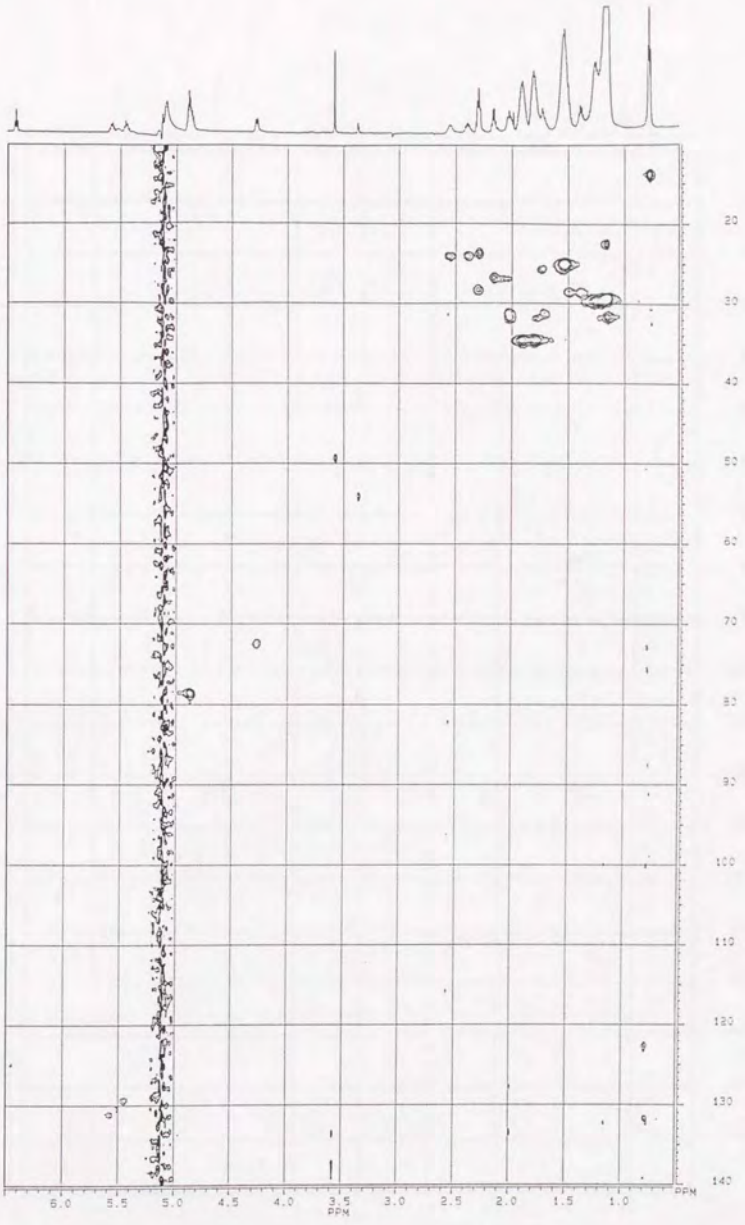


Fig V-20. HMQC spectrum of toxadocial B (61) in C_5D_5N

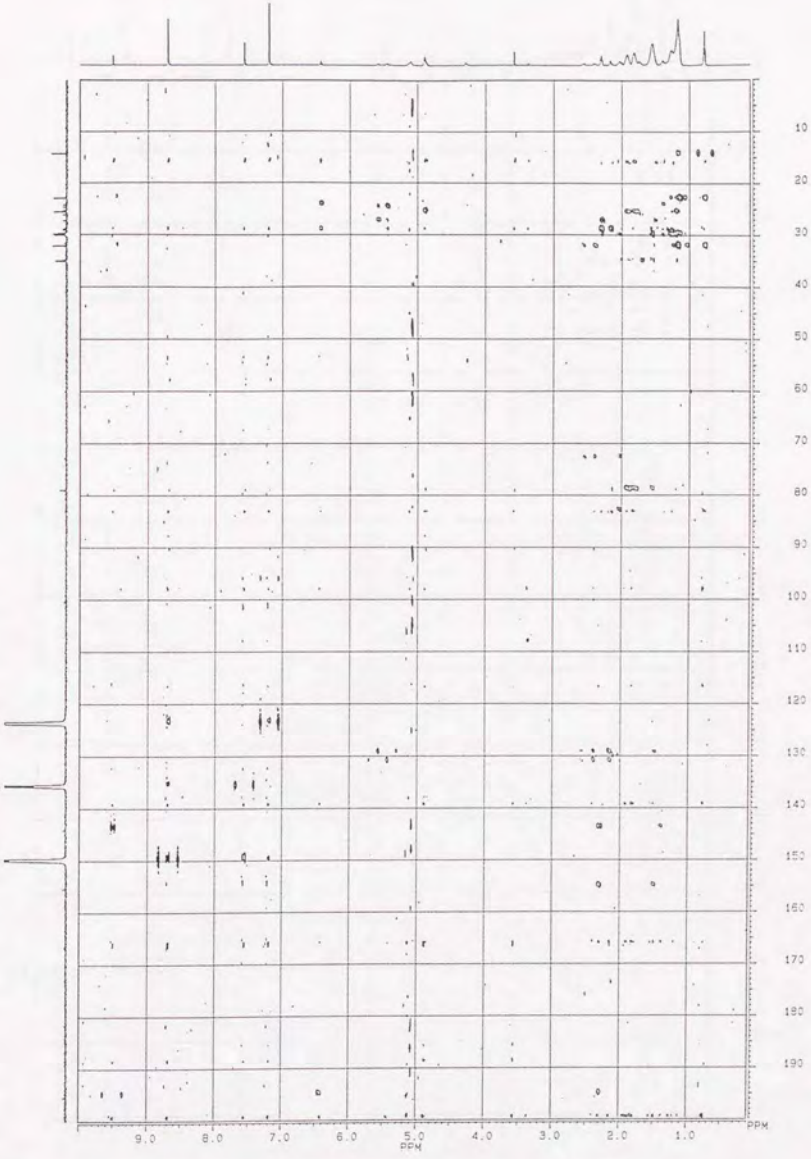


Fig V-21. HMBC spectrum of toxadocial B (61) in C_5D_5N

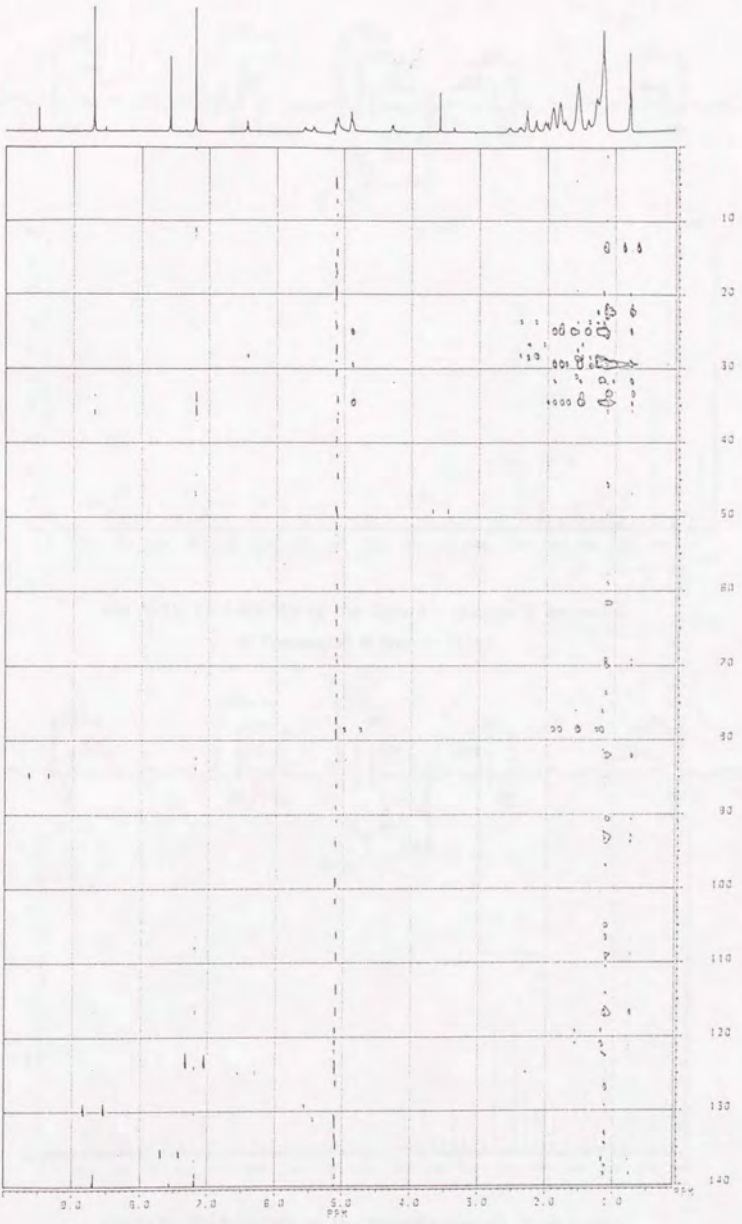


Fig V-22. HMQC-HOHAHA spectrum of toxadocial B (61) in C_5D_5N

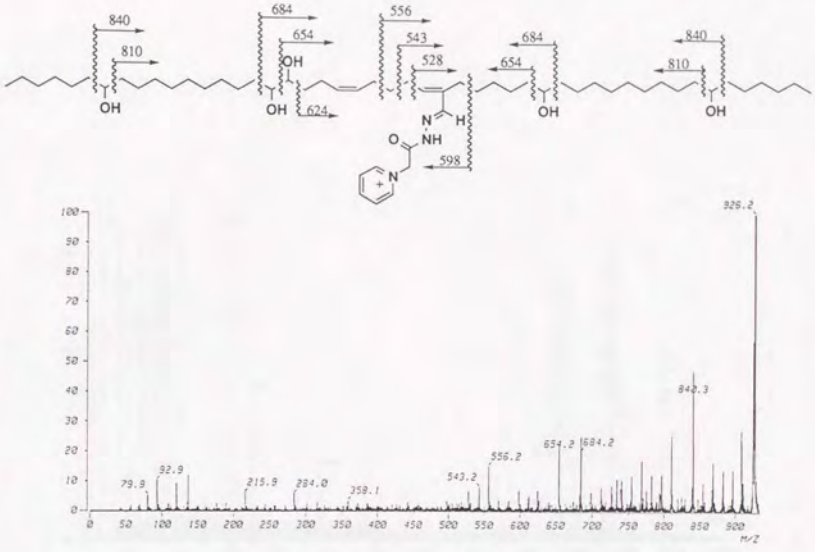


Fig V-23. FAB-MS/MS of the Girard's reagent P derivative of Toxadocial B tetraol (61a)

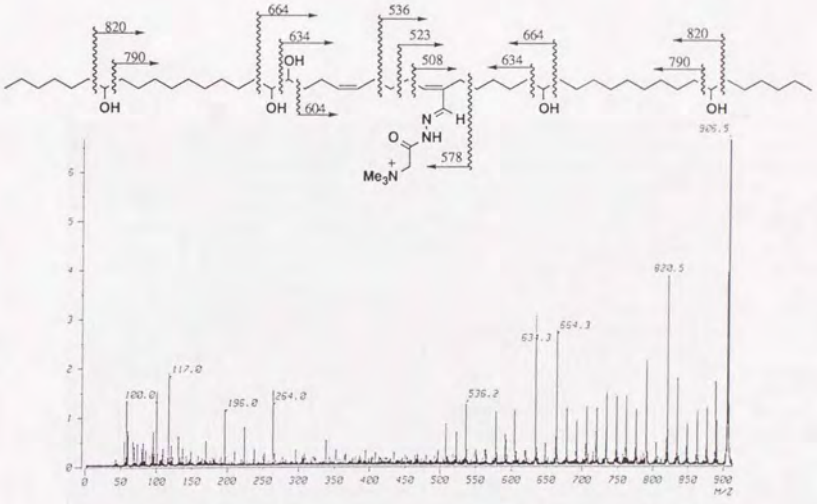


Fig V-24. FAB-MS/MS of the Girard's reagent T derivative of toxadocial B tetraol (61b)

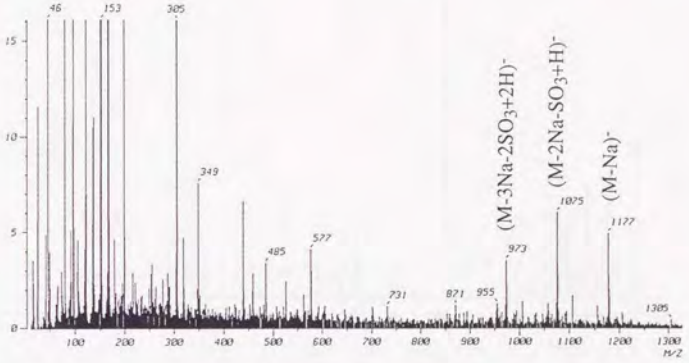


Fig V-25. Negative ion FAB-MS of toxadocial B (61) (matrix; NBA)

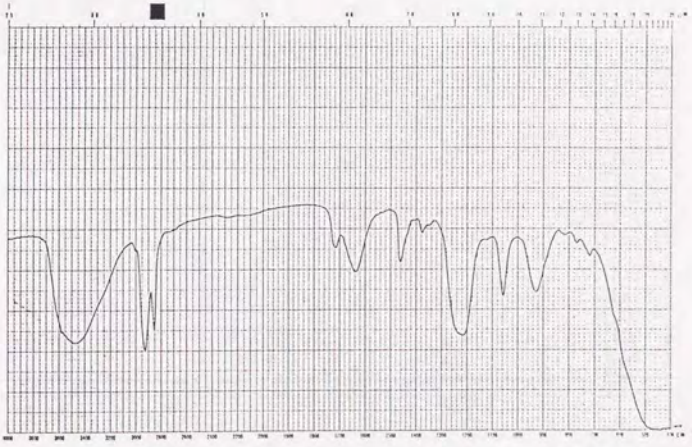


Fig V-26. IR spectrum of toxadocial C (62)(film)

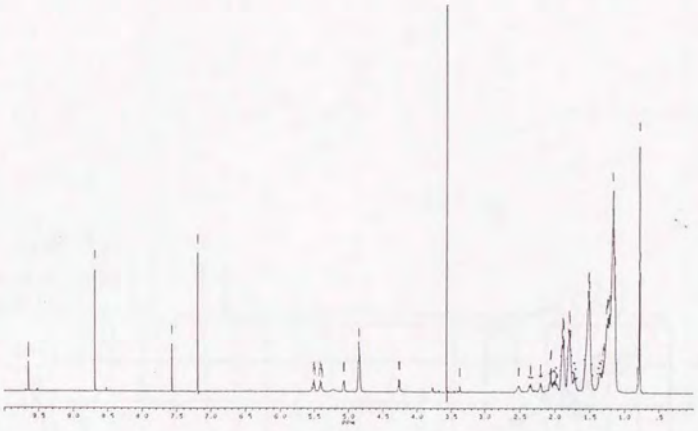


Fig V-27. ^{1}H NMR spectrum of toxadocial C (62) in C_5D_5N

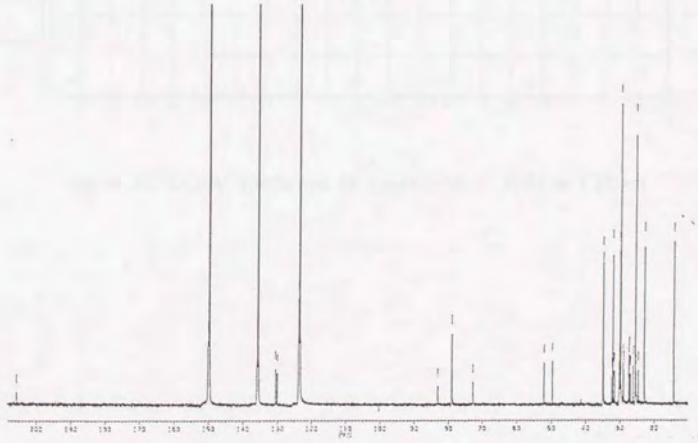


Fig V-28. ^{13}C NMR spectrum of toxadocial C (62) in C_5D_5N

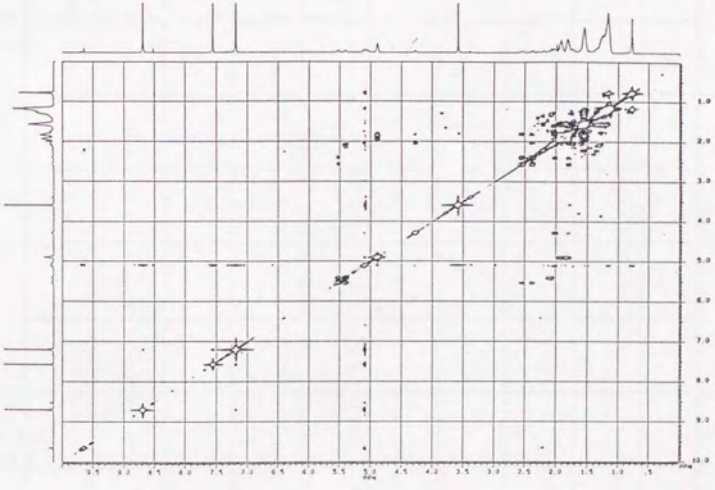


Fig V-29. COSY spectrum of toxadocial C (62) in C_5D_5N

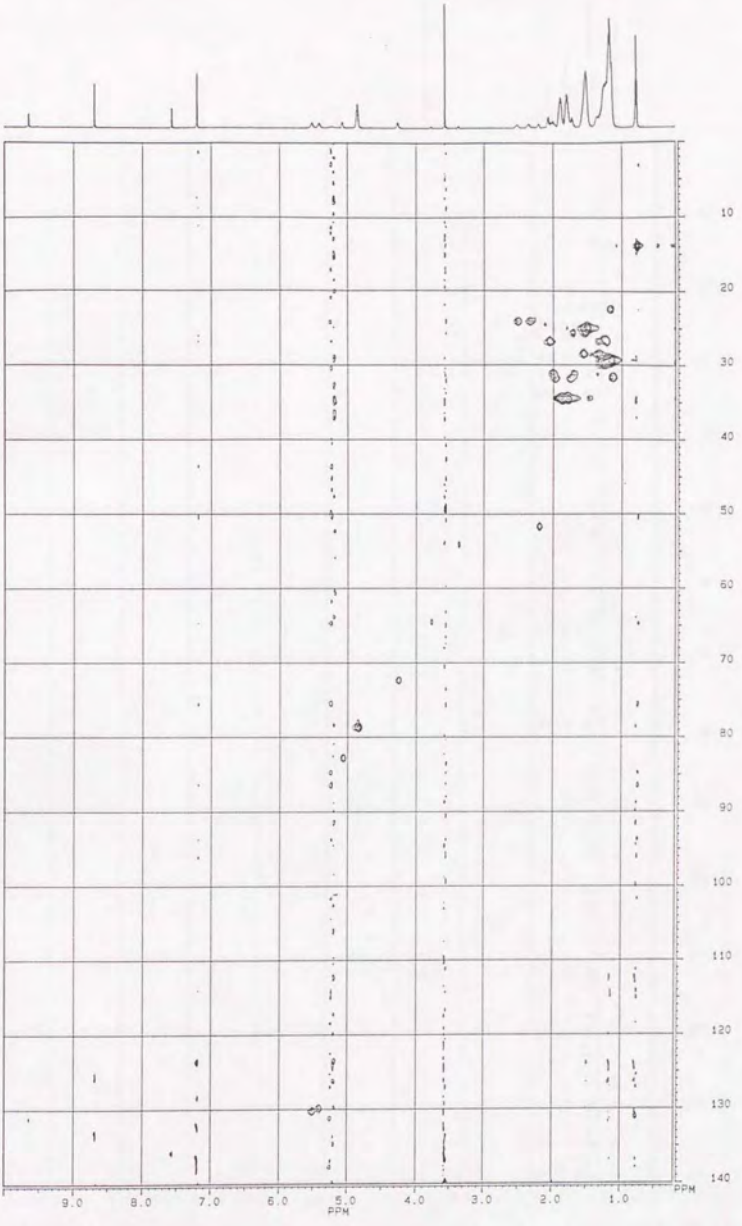


Fig V-30. HMQC spectrum of toxadocial C (62) in C_5D_5N

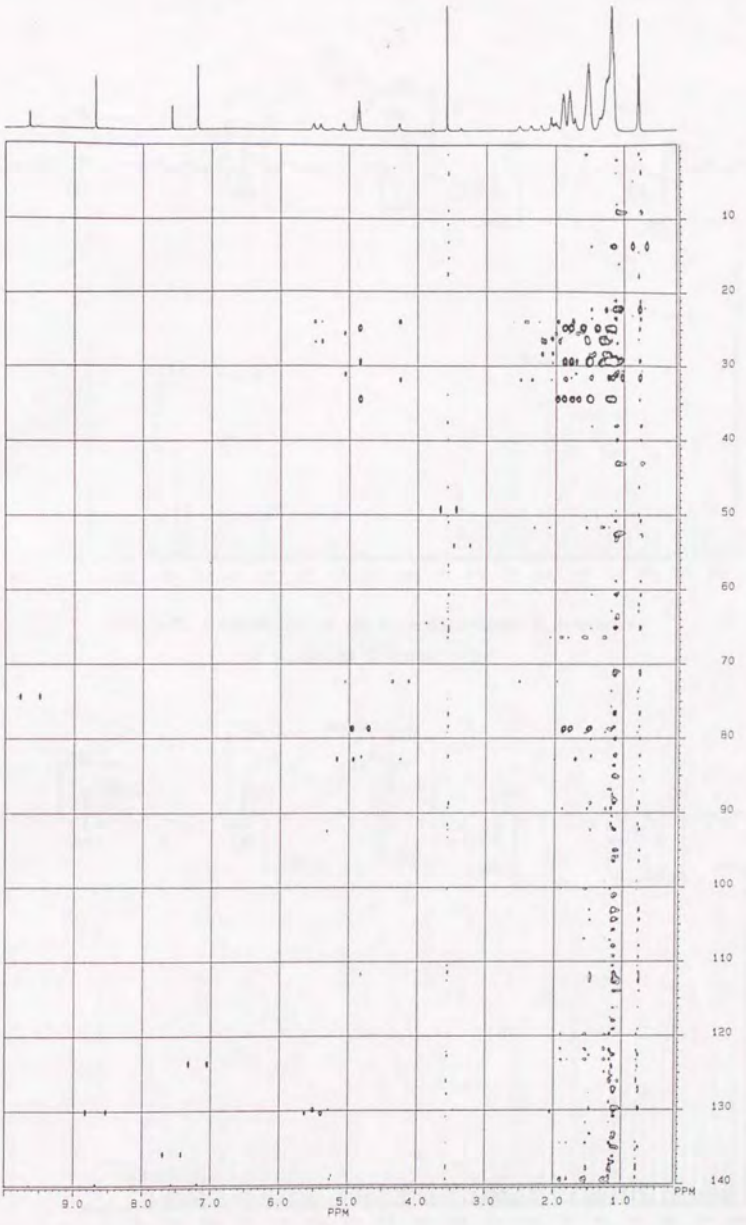


Fig V-31. HMQC-HOHAHA spectrum of toxadocial C (62) in C_5D_5N

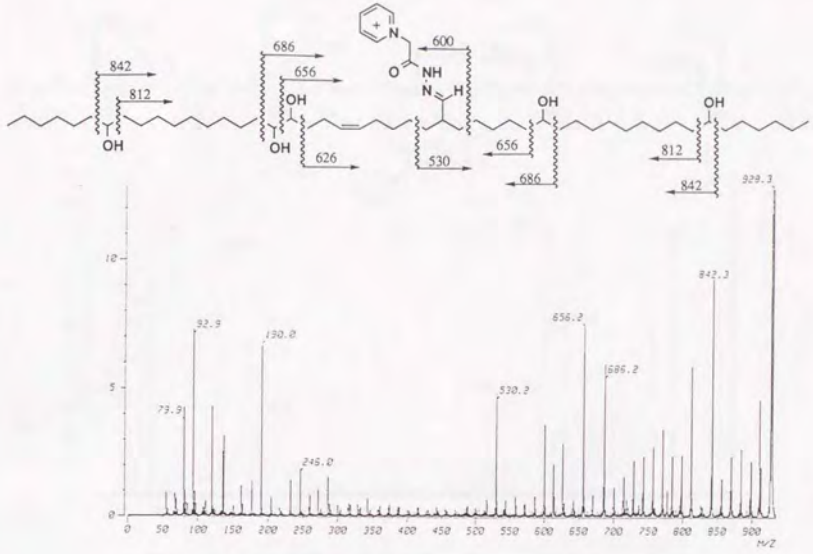


Fig V-32. FAB-MS/MS of the Girard's reagent P derivative of toxadocial C tetraol (**62a**)

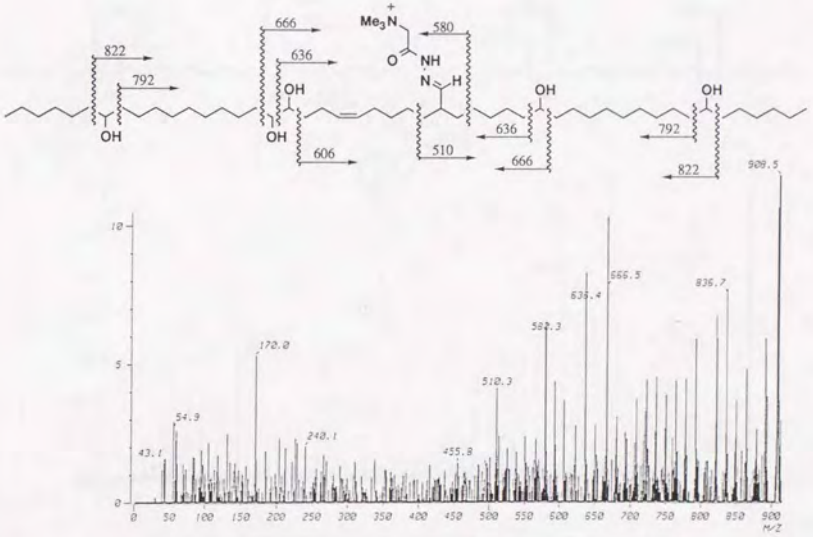


Fig V-33. FAB-MS/MS of the Girard's reagent T derivative of toxadocial C tetraol (**62b**)

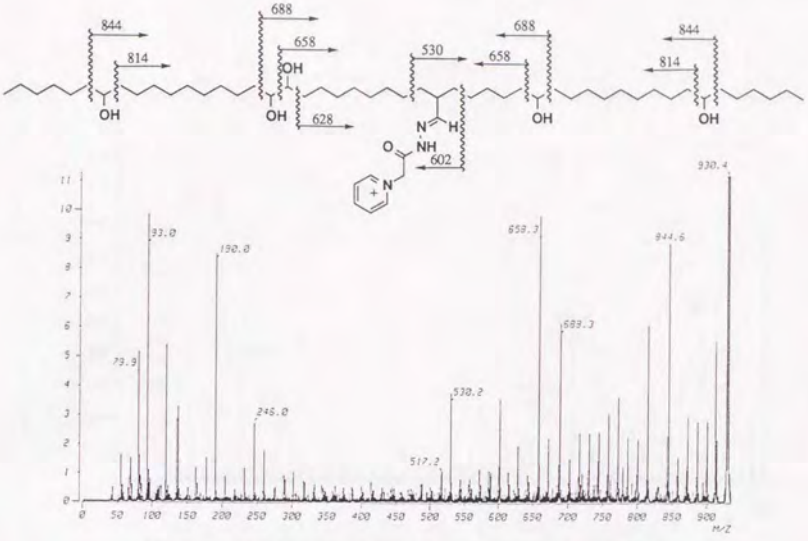


Fig V-34. FAB-MS/MS of the Girard's reagent P derivative of tetrahydrotoxadocial B tetraol (61c)

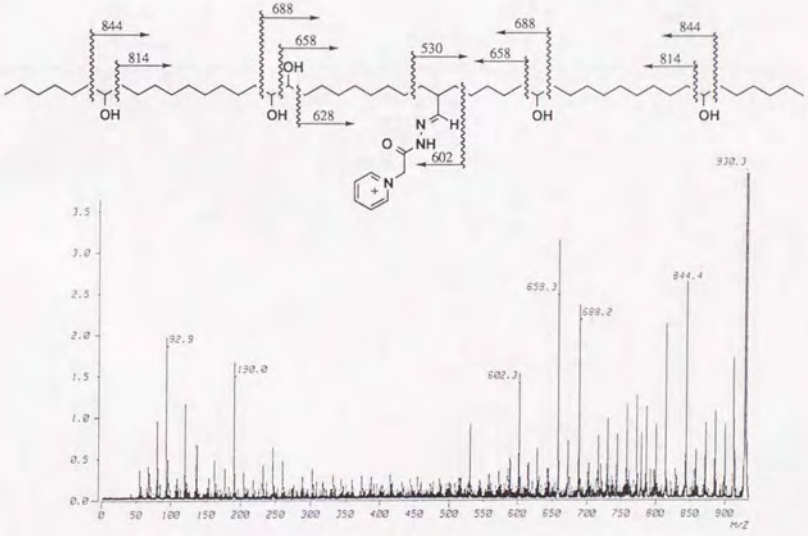


Fig V-35. FAB-MS/MS of the Girard's reagent P derivative of dihydrotoxadocial C tetraol (62c)

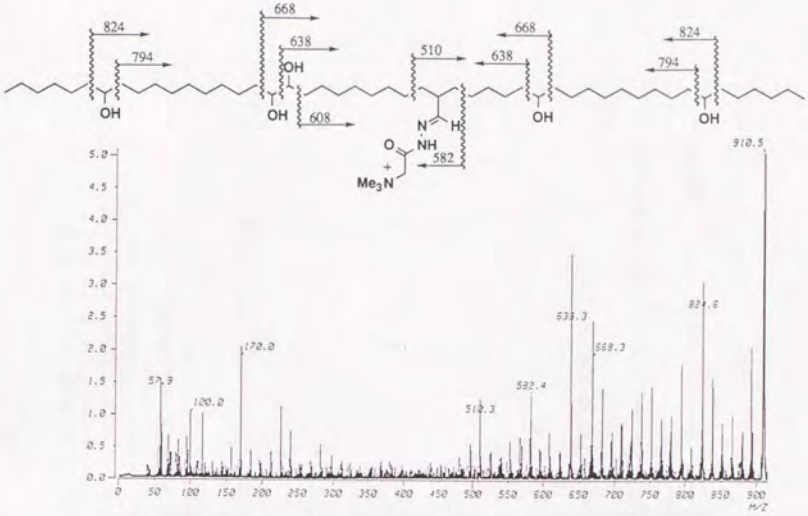


Fig V-36. FAB-MS/MS of the Girard's reagent T derivative of dihydrotoxadocial B tetraol (61c)

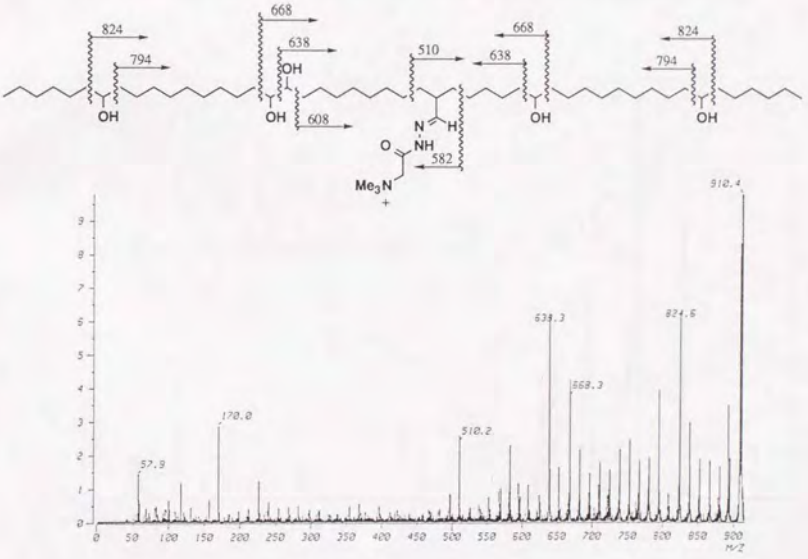


Fig V-37. FAB-MS/MS of the Girard's reagent T derivative of dihydrotoxadocial C tetraol (62c)

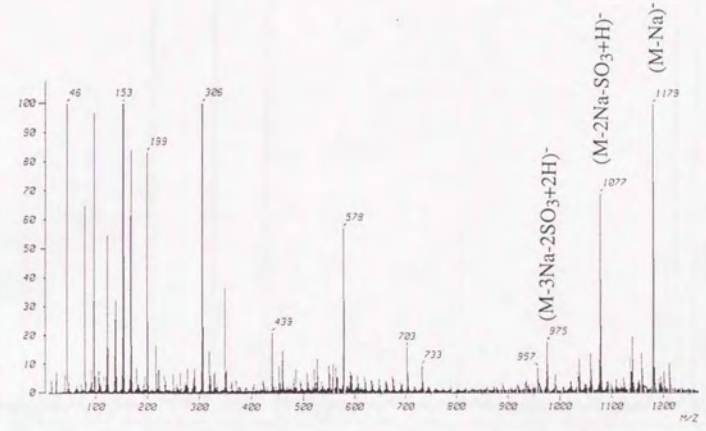


Fig V-38. Negative ion FAB-MS of toxadocial C (62)(matrix; NBA)

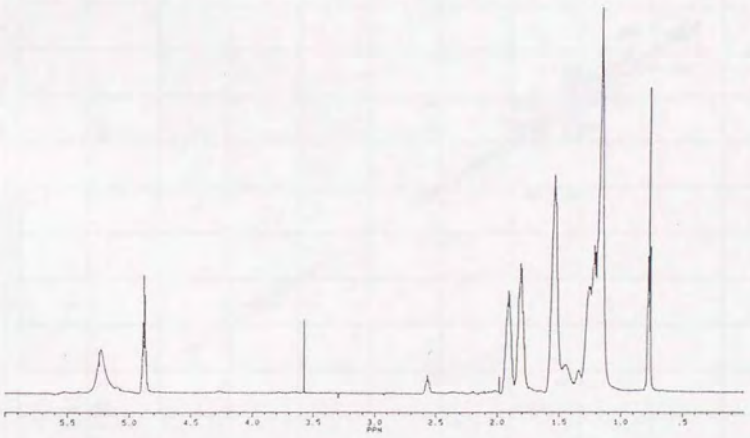


Fig V-39. ^{1}H NMR spectrum of toxadocic acid A (63) in C_5D_5N

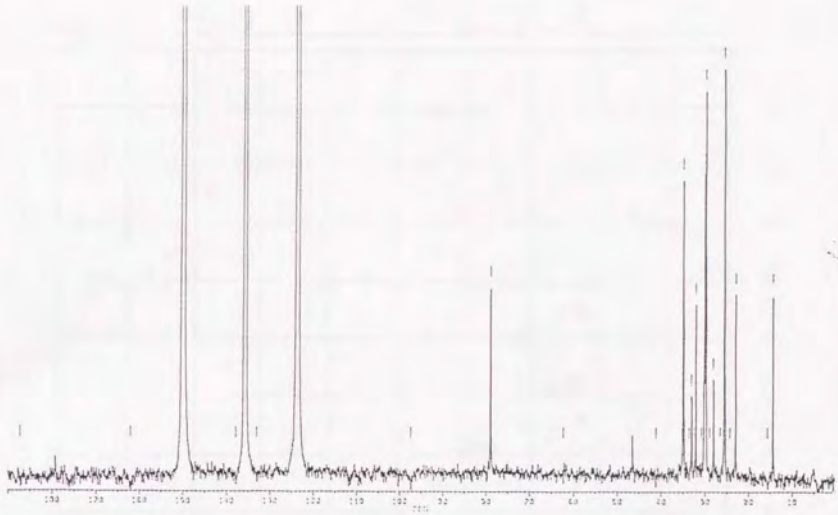


Fig V-40. ^{13}C NMR spectrum of toxadocic acid A (63) in C_5D_5N

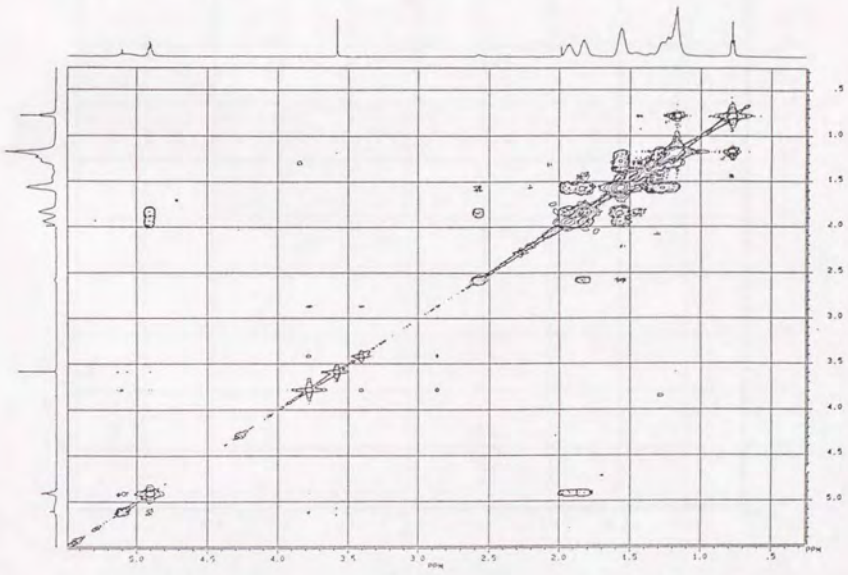


Fig V-41. COSY spectrum of toxadocic acid A (63) in C_5D_5N

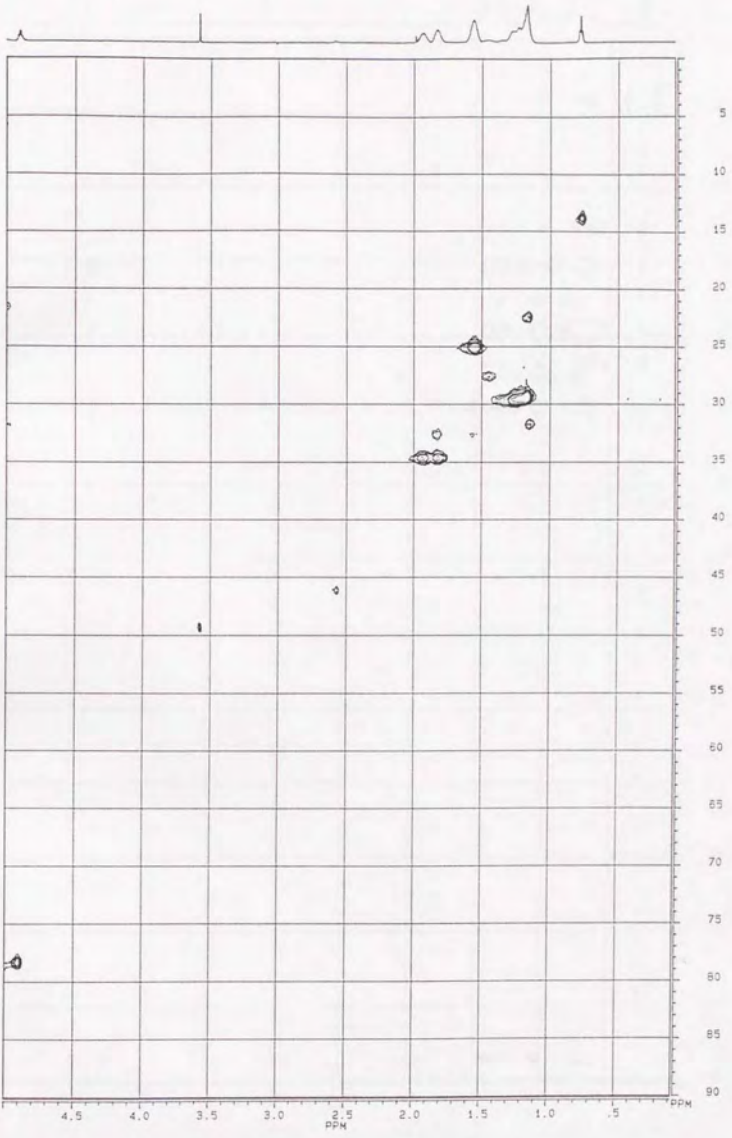


Fig V-42. HMQC spectrum of toxadocic acid A (63) in C_5D_5N

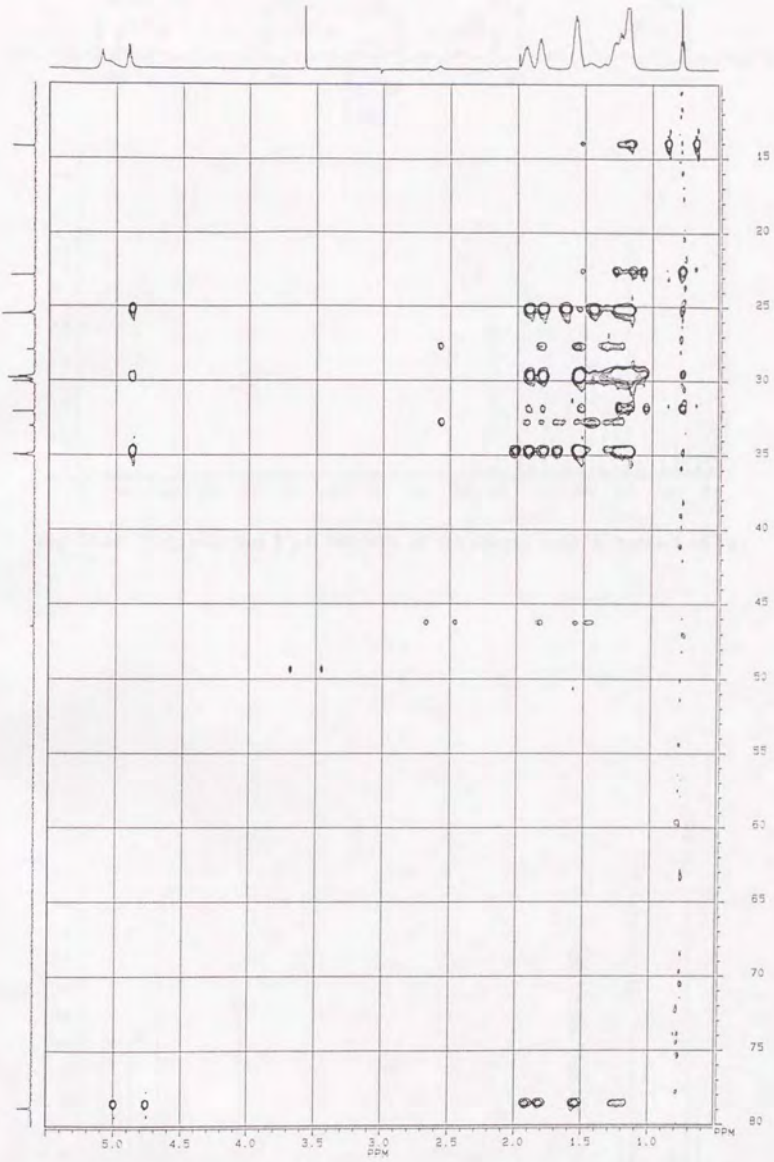


Fig V-43. HMQC-HOHAHA spectrum of toxadocic acid A (63) in C_5D_5N

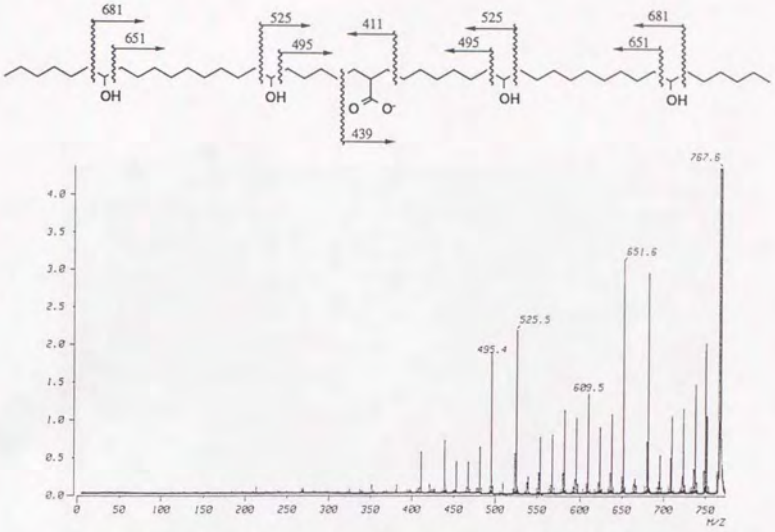


Fig V-44. Negative ion FAB-MS/MS of toxadocic acid A tetraol (63a)

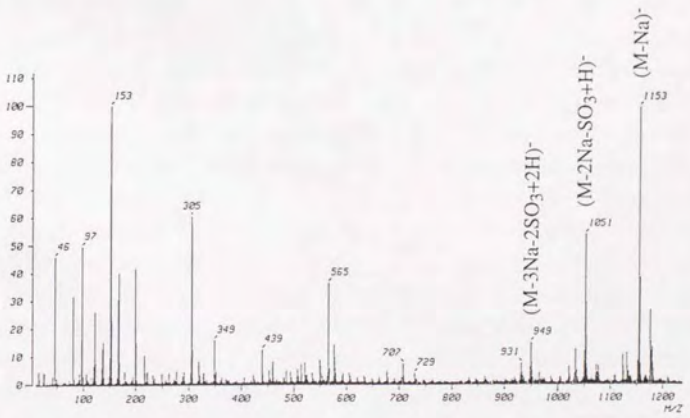


Fig V-45. Negative ion FAB-MS of toxadocic acid A (63)(matrix; NBA)

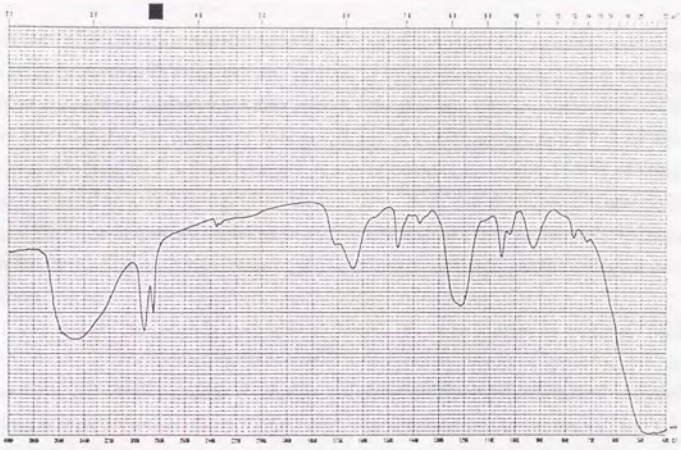


Fig V-46. IR spectrum of toxadocic acid A (63)(film)

CHAPTER VI

CONCLUSIONS

A total of 450 marine invertebrates consisting of sponges (363 samples), coelenterates (48 samples), bryozoans (3 samples), and tunicates (36 samples) collected along Japanese coasts were tested for inhibitory activities against thrombin and trypsin. Thrombin inhibitory activities were observed in 13.2 % of sponges, 8.3 % of coelenterates, and 2.7 % of tunicates. Trypsin was inhibited by 9.1 % of sponges, 6.3 % of coelenterates, and 13.9 % of tunicates. Bryozoans showed no activities against both enzymes. Both anti-thrombin and anti-trypsin activities were more frequently observed in hydrophilic extracts than in lipophilic extracts. Sponges are the most promising source of serine protease inhibitors, as the cases for other bioactivities.

Eleven active compounds have been isolated from three species of sponges. Their identification and structure determinations were carried out by means of spectroscopic and chemical methods. From a sponge *Agelas* sp. collected off Hachijo-jima Island, oroidine, a known guanidino compounds from *Agelas* spp., was obtained as a thrombin and trypsin inhibitor. This is the first report for this compound to be inhibitiory against serine proteases.

A linear tetrapeptide, nazumamide A and cyclic peptides, cyclotheonamides C, D, E, and F have been isolated as minor active constituents from the sponge *Theonalla swinhoei* collected off Hachijo-jima Island, whose major inhibitor was cyclotheonamide A. Interestingly, nazumamide A was found only in the sponge collected in 1989. The anti-thrombin activity of nazumamide A was first determined to be as an IC_{50} of 2.8 μg/mL. However, the synthetic nazumamide A showed weaker activity, i.e. 30 % inhibition against thrombin at a concentration of 100 μg/mL. It was suspected that a small amount of cyclotheonamide A was contaminated in the natural nazumamide A. Cyclotheonamides C-F exhibited equally potent inhibitory activity with cyclotheonamide A (IC_{50}'s of 0.27-0.52 μg/mL against thrombin; IC_{50}'s of 0.32-0.48 μg/mL against trypsin). Cyclotheonamides C-E are likely to be artifacts derived from cyclotheonamide F. A closely-related cyclotheonamide, cyclotheonamide G was isolated from a morphologically different *Theonella swinhoei* collected also in Hachijo-jima Island, which did not contain cyclotheonamide A. The major inhibitor against thrombin and trypsin in this sponge was cyclotheonamide G which possessed a similar potency as cyclotheonamide A.

Toxadocials A-C and toxadocic acid A, tetrasulfated alkyl aldehydes or an acid, were isolated from the marine sponge *Toxadocia cylindrica* from Hachijo-jima Island as thrombin inhibitors (IC_{50} 2.9-6.5 μg/mL). It should be noted that the FAB-MS/MS analysis was effective in structure determination of those compounds possessing long alkyl chains.

Inhibitors so far isolated from marine organisms can be grouped into two types. Toxadocials and halistanol sulfates comprise one group with several sulfate groups in the molecule. Their inhibitory activities are similar to each other (toxadocials; IC_{50} 2.9-6.5 μg/mL, halistanol sulfates; IC_{50} 16-90 μg/mL). Activity of these compounds may be attributable to sulfate groups.

Oroidine, nazumamide A and cyclotheonamides comprise the other group. A Pro-Arg partial structure or its mimic is common in this group. A Pro-Arg unit is known to interact with thrombin in case of synthetic inhibitors, D-Phe-Pro-Arg-CH_2Cl (PPACK)[70,71] and Me-D-Phe-Pro-Arg-H (GYKI-14766).[72,73]

Oroidine can be considered as a Pro-Arg mimic; its amine part with a cyclic guanidine moiety possesses a similar length and basicity as Arg, while the bromopyrrole carboxylic acid part contains a nitrogen in five-membered ring as in case of Pro.

Nazumamide A had a reverse partial structure, Arg-Pro. The lack of the Pro-Arg unit in nazumamide A might be the reason for the moderate activity of nazumamide A.

Cyclotheonamides had a Pro-K-Arg unit, which is a mimic of Pro-Arg unit. In addition to the interaction of the Pro-K-Arg unit to thrombin, the role of the α-keto amide when cyclotheonamide A binds to thrombin, characterizes cyclotheonamide A as a unique thrombin inhibitor. Cyclotheonamides C-G have the same moiety of Pro-K-Arg in their structures, therefore they must inhibit thrombin in the same manner. A hydrophobic interaction of the D-Phe residue with thrombin is also clarified. Substitution of D-Phe by D-Ile in cyclotheonamide G scarecely have influence on its activities; the side chain of Ile functions may be similar to the benzene ring of D-Phe.

Cyclotheonamides have two remarkable properties; the α-keto amide moiety and their cyclic nature. Cyclic peptides are known to be resistant to hydrolytic enzyme, therefore cyclotheonamides may become hopeful leads for the drugs.

In conclusion, sponges turned out to be a good source of serine protease inhibitors. Only a few examples of serine protease inhibitors from marine invertebrates have been reported before this study. Further studies will lead to discovery of new types of protease inhibitors.

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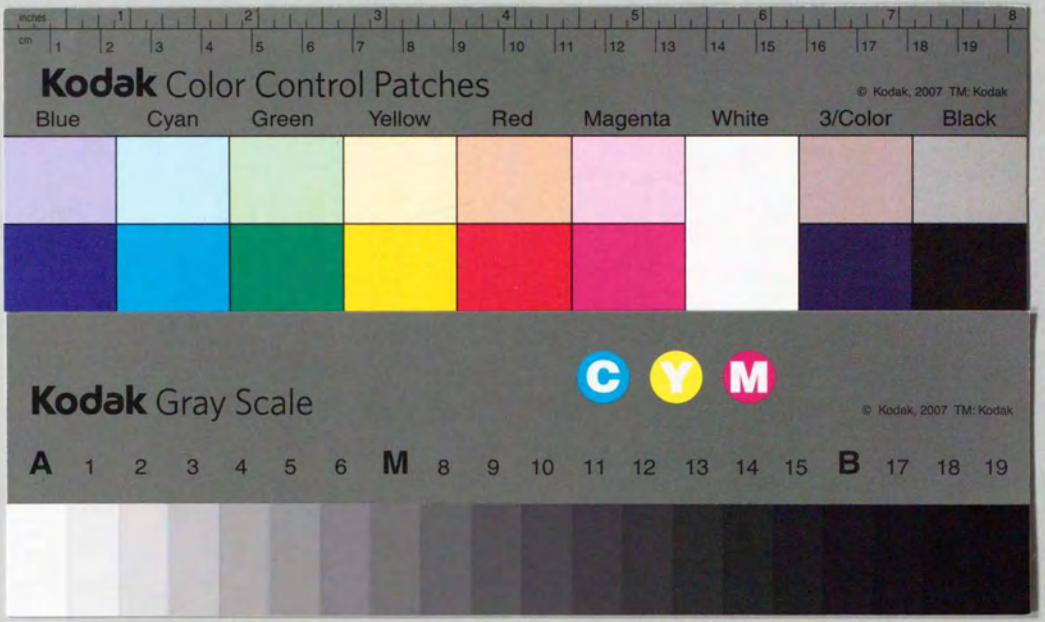
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