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นางสาววิมลพรรณ รุ่งพรหม

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BIOACTIVE COMPOUNDS FROM MARINE SPONGE-ASSOCIATED BACTERIA

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งานวิจัยนี้เป็นส่วนหนึ่งของการเสาะหาสารออกฤทธิ์ทางชีวภาพจากสิ่งมีชีวิตในทะเล ซึ่งได้ศึกษาองค์ประกอบทางเคมีของฟองน้ำที่เก็บในประเทศออสเตรเลียและแบคทีเรียที่อยู่ร่วมกับฟองน้ำทะเลของไทย พบว่า จากฟองน้ำออสเตรเลีย *Aplysilla sulphurea* สามารถแยก chromodorolides A, B (2a-2b) และ chromodorolide C (2c) ซึ่งเป็นสารใหม่โดยทำการหาสูตรโครงสร้างด้วยเทคนิค 1D และ 2D นิวเคลียร์แมกเนติกเรโซแนนซ์ สารในกลุ่มนี้แสดงฤทธิ์ต้านเซลล์ P388 (Leukemia carcinoma cell lines) ที่สูงและยังแสดงฤทธิ์ฆ่าหนอนตัวกลม *Haemonchus contortus* และ *Trichostrongylus colubriformis* ในระยะตัวอ่อนซึ่งเป็นตัวก่อโรคที่สำคัญในแกะและปศุสัตว์

ในการศึกษาองค์ประกอบทางเคมีของจุลชีพในทะเลจากประเทศไทย สามารถแยกสารในกลุ่มเปปไทด์ ได้ 6 ตัว ได้แก่ cyclo [phenylalanyl-leucyl]$_2$ (3a), cyclo [leucyl-isoleucyl]$_2$ (3b), cyclo [leucyl-leucyl]$_2$ (3c), cyclo [leucyl-prolyl-leucyl-prolyl] (4a), cyclo [phenylalanyl-prolyl-phenylalanyl-prolyl] (4a), และ cyclo [phenylalanyl-prolyl-leucyl-prolyl](4c) และสารที่มีหมู่เฮทเทอร์โรแอโรมาติกที่ไม่สามารถหาสูตรโครงสร้างได้ การวิเคราะห์สูตรโครงสร้างหาได้จากวิธีทางสเปกโทรสโกปีและสเทอริโอเคมีที่สมบูรณ์ของกรดแอมิโนในสายเปปไทด์ ด้วยการวิเคราะห์โดย Marfey และเปปไทด์สี่ชนิดตัว (3a-3c และ 4c) เป็นสารใหม่ที่ไม่เคยมีรายงานมาก่อนในแบคทีเรียน้ำเค็ม ฤทธิ์ทางชีวภาพของเปปไทด์กำลังอยู่ระหว่างการทดสอบ

ภาควิชา.....เคมี..... ลายมือชื่อนิสิต.....
สาขาวิชา.....เคมี.....ลายมือชื่ออาจารย์ที่ปรึกษา.....
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WIMOLPUN RUNGPROM : BIOACTIVE COMPOUNDS FROM MARINE SPONGE-ASSOCIATED BACTERIA
THESIS ADVISOR : PROFESSOR UDOM KOKPOL, Ph.D., THESIS COADVISOR: ASSISTANT PROFESSOR WARINTHORN CHAVASIRI, Ph.D. AND ASSOCIATE PROFESSOR MARY J. GARSON, Ph.D., 102 pp. ISBN 974-17-6189-9.

As a part of searching for bioactive compounds from marine organisms, this research describes the chemical constituents of an Australia sponge *Aplysilla sulphurea* and marine bacteria associated with tropical Thai sponge. Two known chromodorolides A (**2a**), B (**2b**) and a new derivative chromodorolide C (**2c**) have been isolated from an Australian sponge and characterized by 1D and 2D NMR experiments. Chromodorolides exhibited the significant cytotoxicity against P388 mouse leukemia cell lines and also showed nematocidal activity against the larval stages of *Haemonchus contortus* and *Trichostrongylus colubriformis*, two important pathogens of sheep and other ruminants.

In the chemical investigation of marine microorganism from Thailand, six peptides : cyclo [phenylalanyl-leucyl]$_2$ (**3a**), cyclo [leucyl-isoleucyl]$_2$ (**3b**), cyclo [leucyl-leucyl]$_2$ (**3c**), cyclo [leucyl-prolyl-leucyl-prolyl] (**4a**), cyclo [phenylalanyl-prolyl-phenylalanyl-prolyl] (**4b**), and cyclo [phenylalanyl-prolyl-leucyl-prolyl](**4c**), including unidentified heteroaromatic units were isolated from bacteria associate with tropical Thai sponge. The peptide structures were determined using spectroscopic methods and the absolute configurations were deduced using Marfey's analysis. Four peptides **3a-3c** and **4c** were identified as new metabolites from marine bacteria. The bioassay for peptide will be undertaken.

Department.....Chemistry.....Student's signature.....W. Rungprom.....
Field of study.....Chemistry.....Advisor's signature.....U. Kokpol.....
Academic year ..... 2004.....Co-advisor's signature.....W. Chavasiri.....
Co-advisor's signature.....Mary J. Garson.....

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LIST OF ABBREVIATIONS

br s	broad singlet (NMR)
δ	chemical shift
$CDCl_3$	deuterated chloroform
CD_3OD	deuterated methanol
d	doublet
dd	doublet of doublet
DEPT	distortionless enhancement by polarization transfer
ESI	electrospray ionisation
EtOAc	Ethyl acetate
FDAA	1-fluoro-2,4-dinitrophenyl -5-L-alaninamide
g	gram
HMBC	heteronuclear multiple-bond coherence spectroscopy
HSQC	heteronuclear single quantum coherance
HPLC	high performance liquid chromatography
HR ESIMS	high resolution electrospray ionization mass spectroscopy
Hz	hertz
J	coupling constant (NMR)
mL	milliliter (s)
MeOH	methanol
mmol	millimole (s)
m	multiplet (NMR)
min	minute
MS	mass spectroscopy
m/z	mass per charge
NMR	nuclear magnetic resonance
ppm	part per million
Pro	proline

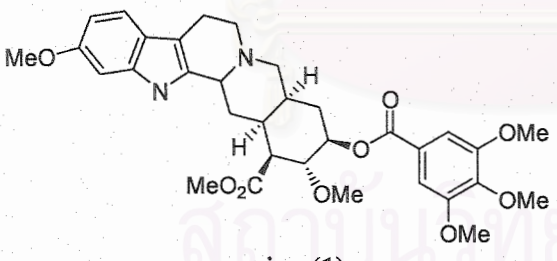
LIST OF ABBREVIATIONS (cont.)

RP-HPLC	reverse phase high performance liquid chromatography
R_f	retardation factor
s	singlet (NMR)
t	triplet (NMR)
TLC	thin layer chromatography
t_R	retention time
TOCSY	total correlated spectroscopy
UV	ultraviolet spectroscopy

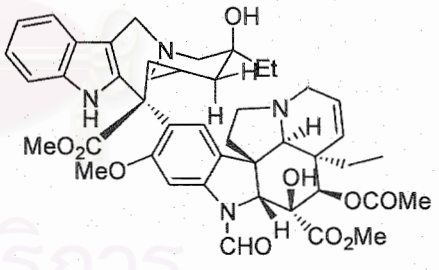
CHAPTER I

INTRODUCTION

Natural products are the secondary or non-primary metabolites produced by living organisms, and have been utilized by humans for many purposes including as food, pesticides, insecticides and medicines. The research scrutinized into the pharmacological properties of natural products has led to the discovery of many potently active agents for many years. Plants have served as a major source of novel chemical structures with potent biological activities, which have subsequently been developed as drugs for a long time.[1] For example, the yohimbine alkaloid, reserpine (**1**) has attracted attention from chemists for both its medicinal properties and its intriguing molecular structure. The bisindole alkaloid, vincristine (**2**) inhibits mitosis by binding to tubulin, thus allowing a broad spectrum of activity in the treatment of various carcinoma cell lines.[2,3]



reserpine (**1**)



vincristine (**2**)

In recent years, some untreatable diseases causing human death worldwide are found such as AIDS, malaria and cancers. Accordingly, the scientists have tried to search for novel pharmacological agents from other sources such as microorganisms and marine organisms. Over the last decade, the marine environment has been recognized as a rich source of biologically active natural products. Each year, an increasing number of novel marine metabolites are reported in the literature, strongly indicating that the marine

environment is likely to continue to be a prolific source of new natural product for many years to come.[4]

1.1 The Marine Natural Product Chemistry

There are an estimated 150,000 species of marine organisms; these can be divided into three main groups, namely marine invertebrates, marine plants (algae) and marine microorganisms. The distribution of marine natural products by phylum is shown in Figure 1.1.[5]

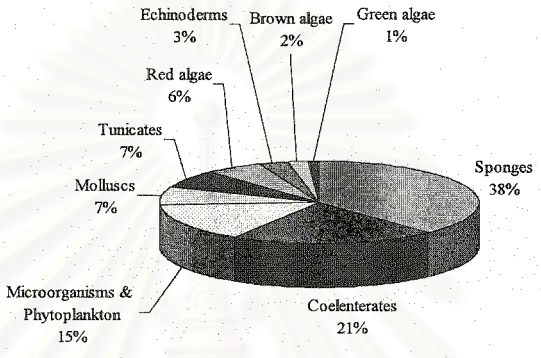


Figure 1.1 The distribution of marine natural products by phylum[5]

Secondary metabolites are produced for many purposes, but a key role may be to facilitate the survival of the host species in the highly competitive marine environment. The use of secondary metabolites as a means to attract and deter is documented for both marine plants and invertebrate animals in response to predation, spatial competition and reproduction.[6] In addition, the role of microbial symbionts in the production of biologically active compounds by marine invertebrates has recently been explored. Many metabolites from marine organisms have never previously been found in terrestrial organisms. In addition to terpenoids, flavonoids, alkaloids and glycosides which are documented from the plant literature, marine organisms also contain novel halogenated compounds, polypeptides, polyketides and desipeptides that have no terrestrial counterparts owing to the physical and chemical properties of marine environments that are quite different from terrestrial conditions. These differences depending on the various biology between the ocean and terrestrial resource are (i) high quantity of elements in the sea such as salt content and halogen element (Cl^-, 19,000 mg/L; Br^-, 65 mg/ L; and I^-/IO_3^-, $5x\ 10^{-4}$ mg/ L)[7], (ii) hydrostatic pressures that increase 1 atm at every ten meters in the depth of the ocean (iii) unusually high or low temperatures and light in the sea water. These properties make the food chains in ocean environment become complex.

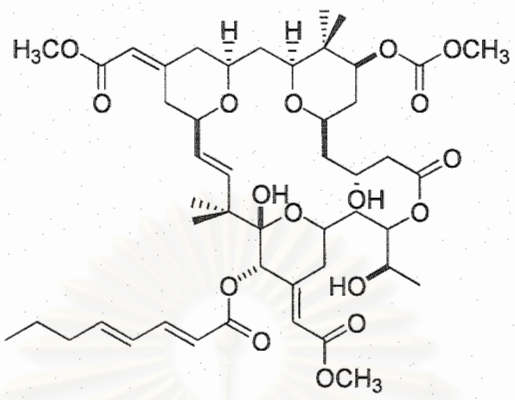
The discoveries of marine natural products throughout the past quarter century have led to pharmaceutical products that have entered preclinical and clinical development. Marine natural products and their analogues that have entered into clinical trials are presented in Table 1.1. [8,9]

Table 1.1 Marine natural products and their analogues currently in clinical trials[9]

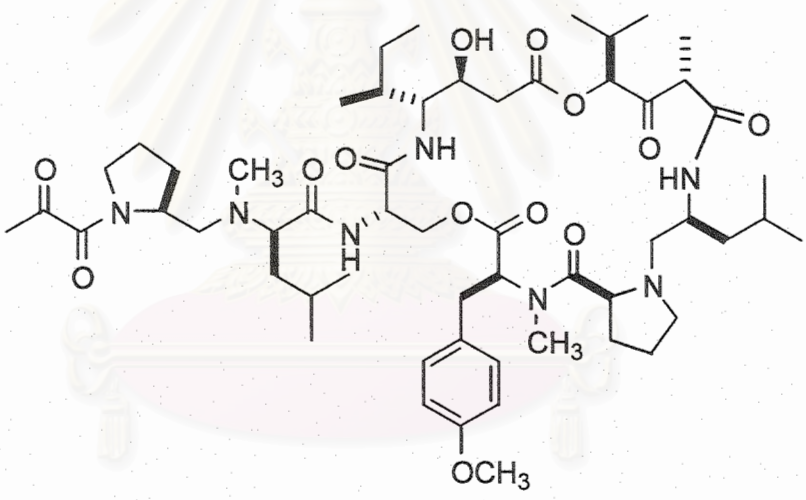
Compound	Disease area	Phase
Agelasphin analogue (KRN 7000) (Sponge: *Agelas mauritianus*)	Cancer	I
Contignasterol (IPL-567) (Sponge: *Petrosia contignata*)	Inflammation	I
Methopterosin (Soft coral: *Pseudopterogorgia elisabethae*)	Inflammation/ wound	I
Anabacine analogue (GST-21) (Marine worm: *Amphiporus lactifloreus*)	Alzheimer/schizophernia	I
Dolastatin 10 (Sea hare: *Dolabella auricularia*)	Cancer	II
Dolastatin 15 analogue (Lu-103793) (Sea hare: *Dolabella auricularia*)	Cancer	I
Ziconitide (Cone snail: *Conus magnus*)	Pain	III
Bryostatin 1 (Bryozoan: *Bugula neritina*)	Cancer	II
Didemnin B (Tunicate: *Tridemnum solidum*)	Cancer	II
Dehydrodidemnin B (Tunicate: *Aplidium albicans*)	Cancer	II
Ecteinascidin 743 (Tunicate : *Ecteinascidia turbinata*)	Cancer	II
Squalamine (Shark: *Squalus acanthias*)	Cancer	I

An anticancer compound currently under investigation in phase II clinical trials, bryostatin 1 (**3**) was isolated primarily from the bryozoan *Bugula neritina.* This compound shows potent antineoplastic activity and continues to be of interest as an aquaculture product.[10] Dehydrodidemnin B (**4**) is a cytotoxic compound, showing a broad spectrum of cytotoxicity against several carcinoma cell lines. This desipeptide was first isolated from the tunicate *Aplidium albicans.*[11] Dehydrodidemnin B (**4**) which shows improved hepatotoxicity over the related compound didemnin B, the parent compound was first discovered from the tunicate *Tridemnum solidum.*[12] Ecteinascidin 743 (**5**) is a tetrahydroisoquinoline alkaloid which was first isolated from *Ecteinascidia turbinata.*[13] It is a quite remarkable natural product because of the selective cytotoxicity against various

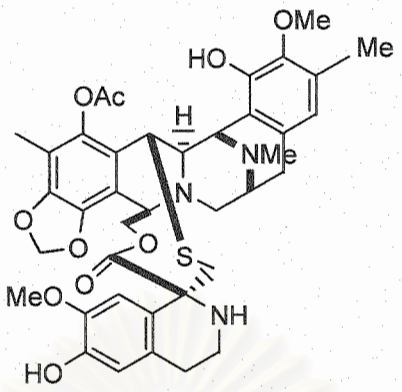
carcinoma cell lines *in vivo*. Among these novel drug candidates, Ecteinascidin 743 (**5**) has been found to be the most promising new drug.[14,15]



bryostatin 1 (**3**)



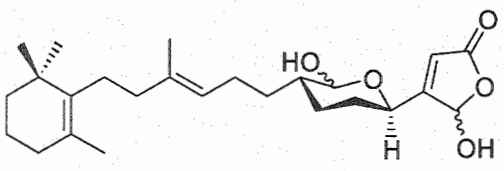
dehydrodidemnin B (**4**)



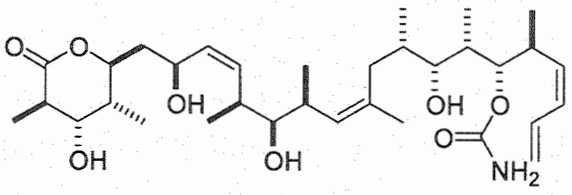
ecteinascidin 743 (**5**)

1.2 Natural Product Chemistry of Marine Sponge.

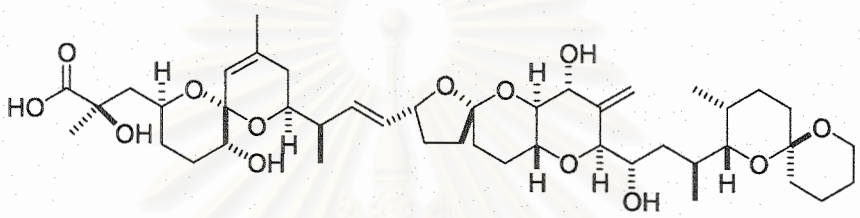
Marine sponges are one of the richest sources of interesting bioactive compounds among the marine invertebrates. Sponges are soft-bodied sessile invertebrates, which lack the physical defense afforded by a shell or thick exoskeleton. Instead, they have developed chemical defenses to prevent predators or larval settlement by other organisms.[16] Novel compounds discovered from sponges have shown potential bioactivity, with several novel sponge compounds showing promising as candidates for new pharmaceuticals. Manoalide (**6**), an antimicrobial sesterterpene originally isolated from *Luffariella variabilis*, inhibits phospholipase A_2 (PLA_2 from bee venom: IC_{50} μM) and is being developed as an anti-inflammatory drug.[17] Discodermolide (**7**) first isolated from *Discodermia disoluta* has been found to be a promising anticancer lead, with a mechanism of action involving the stabilization of microtubules, similar to taxol.[18] Okadaic acid (**8**) and calyculin A (**9**), both cytotoxic polyketides from *Halichondria okadai* and *Discodermia calyx*, respectively, are selective inhibitor of protein phosphatase 1 and 2A.[19,20] Contignasterol (**10**), a highly oxygenated sterol isolated from *Petrosia contignata*, shows anti inflammatory activity.[21] All the above are lead compounds from sponges which are potential candidates to be a new drug.



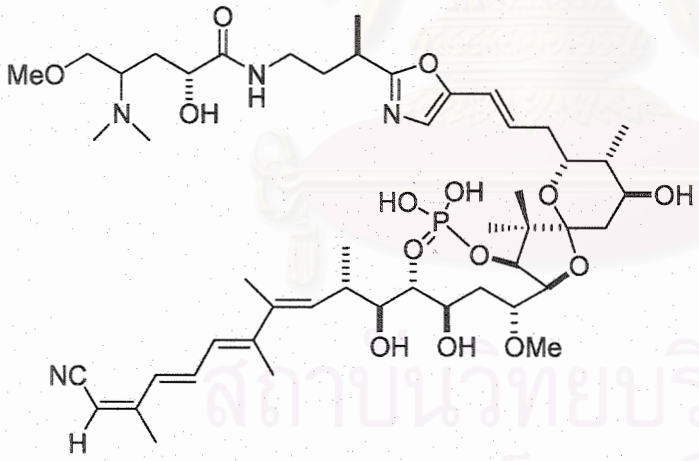
manoalide (**6**)



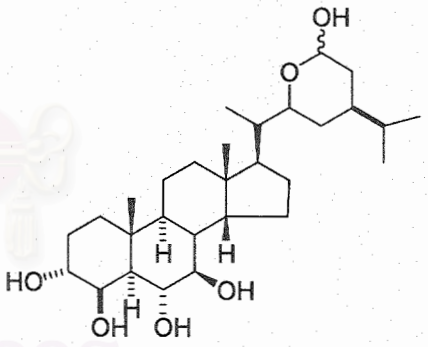
discodermolide (**7**)



okadaic acid (**8**)



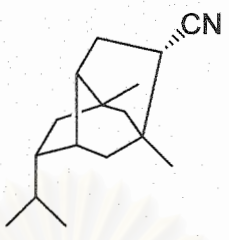
calyculin A (**9**)



contignasterol (**10**)

A second sources of novel marine chemicals are nudibranchs, which are shell-less mollusks acting as predators on sponges. Nudibranchs feed on sponges and concentrate the sponge compounds as a potential chemical defensive. 9-Isocyanopupukeneane (**11**) is a metabolite isolated from the nudibranch *Phyllidia varicosa* and also from its prey a sponge *Hymeniacidon* sp. [22] This metabolite is excreted by the mollusks and protects the delicate shell-less opisthobranch mollusk from its predators. Thus the component is the

allomone of the browser-prey relationship. This piece of research not only demonstrates the finding of a bioactive metabolite, but also shows the important role of the ecological interactions between two organisms in the marine ecosystem.[23]



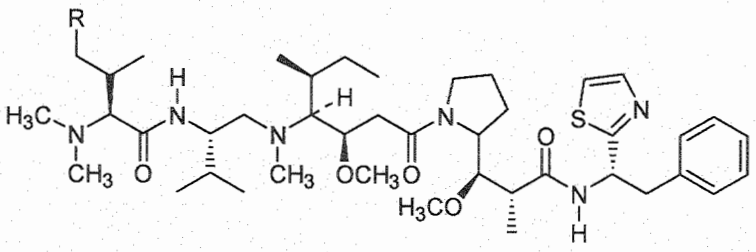
. 9-isocyanopupukeneane (**11**)

.

1.3 Natural Product Chemistry of Marine Microorganisms

Marine microorganisms are considered to be a new promising source of bioactive substance. Recently, marine microorganisms have proven to produce several bioactive metabolites and some of them are expected to serve as lead compounds for drug development.[24] The relevant investigations have also suggested that some bioactives isolated from marine invertebrates such as sponges, coelenterates, mollusks or protochordates are truly originating from symbiotic microorganisms such as bacteria, blue green algae, fungi and dinoflagellates. Since it is quite impossible to collect unlimited amount of marine animals and plants, isolation and cultivation of symbiotic microorganism are of great importance for sufficient and constant supply of bioactive compounds. Through this research, many novel compounds from marine microorganisms with potent biological activity have been discovered.

Regarding the true origin of compounds discovered in marine invertebrate, some evidence suggests that dietary or symbiotic algae have participated in the production of metabolites. For example, the blue green algae, *Lyngbya majuscula* was determined to be a source of aplysiatoxin found in the sea slug *Aplysia* sp.[25,26] Similarly, highly active anticancer compounds found in other sea hares have their origin in the dietary blue green algae. Symplostatin (**12**)[27], a close analogue of dolastatin 10 (**13**)[28], has been found in a marine cyanobacteria *Symploca hydnoides*. The aforementioned compounds are probably introduced into the invertebrates by the food chain.



dolastatin 10 (R=H) (**12**)

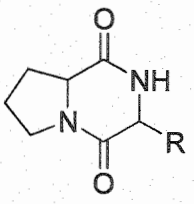
symplostatin 1 (R= CH_3) (**13**)

Marine bacteria are most generally defined by their requirement for sea water or more specifically sodium ion for growth. Differences in the chemical composition of the bacterial cell wall can be determined by gram-staining which shows the differences between gram-positive and gram-negative bacteria. From early study of marine bacteria, it was determined that most sea water marine bacteria are gram negative because the cell wall of gram negative bacteria are better adapted for survival in the marine environment. Unlike, cyanobacteria, marine bacteria do not have chlorophyll for photosynthesis[6] and also usually associated everywhere in marine environment. Marine bacteria have been isolated from three sources, namely sea water, sediments and surface of marine invertebrates.[30]

1.4 Natural Products of Marine Bacteria Associated with Sponge

Bioactive compounds of marine sponge have been extensively studied by marine natural product chemists. The isolated compounds from sponges are usually found in small quantities and also discovered in marine microorganism which form symbioses in sponge. The structural characteristics of the compounds strongly suggested that microorganisms living on or in sponge may be responsible for the production of many bioactive compounds found in sponges. Some compounds have been isolated from extracts of sponge and also found in culture media of marine bacteria isolated from sponges.[30]

The gram positive bacteria *Micrococcus* sp. was isolated from the sponge *Tedania ignis*. Three diketopiperazines (**14-16**) were obtained from the culture media of *Micrococcus* sp.[31] and these compounds were identical with those previously isolated from the extracts of *Tedania ignis*.[32] All of them show antimicrobial activity against various bacteria.

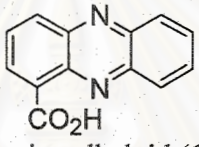


R= $CH_2CH(CH_3)_2$ (**14**)

R= $CH(CH_3)_2$ (**15**)

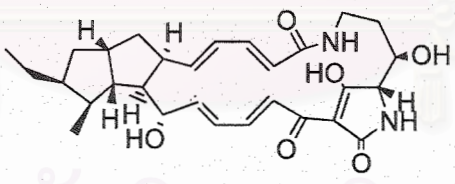
R= CH_3 (**16**)

In 1996 Jayatilake *et al*. reported the isolation of the diketopiperazine (DKP) (**14-16**) which were dipeptide containing proline unit and the phenazine alkaloid (**17**) were separated from a strain of *Pseudomonas aeruginosa* associated with the Antarctic sponge *Isodictya setifera*.[33] Therefore, this evidence indicated that the same metabolites could be produced from different bacterial strains.



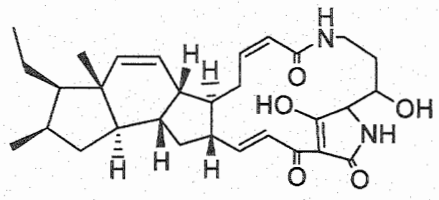
phenazine alkaloid (**17**)

Alteramide A (**18**) is the cytotoxic alkaloid which was isolated from the culture media of *Alteromonas* sp. obtained from the Japanese sponge *Halichondria okadai*. Alteramide A displays cytotoxicity against the P388 leukemia cell line.[34]

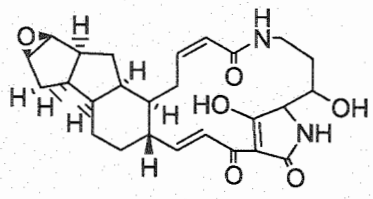


alteramide A (**18**)

The structure of alteramide A is similar to those of the antibiotic compound, ikarugamysin (**19**) and discodermolide (**20**). The former was produced by a terrestrial *Streptomyces* sp. while the latter was isolated from the marine sponge *Discodermia dissoluta*. Both metabolites exhibited antifungal and cytotoxic activity. Since alteramide A was obtained from bacteria isolated from a sponge, it can be suggested that discodermolide (**20**) is of microbial origin.[35]



ikarugamysin (**19**)



discodermide (**20**)

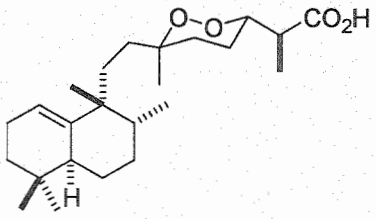
1.5 Marine Natural Products in Thailand

Approximately, a half part of Thailand located on the coastlines between the Gulf of Thailand in the east and the Andaman sea in the west, both of which the part of Indian ocean. The marine environments have been explored and found to be highly biodiversity environment. The earlier research on marine in Thailand was mainly aimed to explore its environment and ecology.

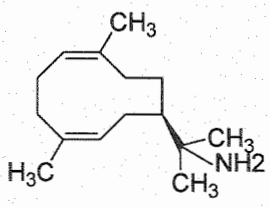
Figure 1.2 The map of Thailand

During the last decade, the research on marine natural products has been initiated. Many bioactive compounds have been reported. For instance, mycaperoxide H (**21**),[36] a new cytotoxic norsesterterpene peroxide, was isolated from the Thai marine sponge *Mycale* sp. This sponge was collected from Srichung island in the Gulf of Thailand. In 2004,

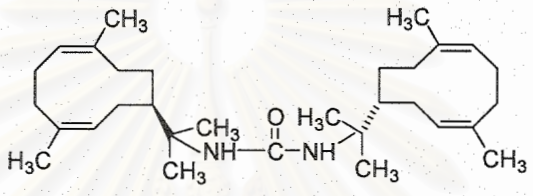
Suwanborirux *et al.* reported the isolation of two new germacranes (**22-23**) from marine sponge *Axinyssa* sp. collected from the Andaman sea.[37] Accordingly, sponges have also served as a major source of marine natural product.



mycaperoxide H (**21**)



(1*Z*,4*Z*)7α*H*-11-aminogermacra-1(10), 4-diene (**22**)



N-*N*-11-bis[(1*Z*,4*Z*)-7α*H*-germacra-1(10), 4-dienyl]urea (**23**)

At present, the environmental resources in Thailand are currently inadequate to conduct large scale searches for bioactive marine organisms. Therefore, marine bacteria associated with Thai sponges became an alternative source for challenging examination, because that natural product resource could be cultured in the laboratory. Additionally, searching for bioactive compound from marine bacteria in Thailand is later a new research topic. All the above mentioned suggested marine microorganisms be of interest for studying the chemical constituents along with their biological activities. The objective and expectation of the research described in the thesis are as follow:

The objective of this research

To find bioactive compounds from the culture of marine bacteria which are derived from the marine bacteria associated with tropical Thai sponges

To find bioactive compounds from an Australian sponge *Aplysilla sulfurea.*

An ultimate expectation of this research is to look forward to newly discovered bioactive compounds for the use in basic research or medical applications.

CHAPTER II

Chromodorane Diterpenes from an Australian Sponge *Aplysilla sulphurea*

2.1 Introduction

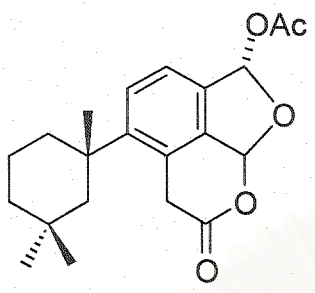
Encrusting sponges of the family Aplysillidae (order Dendroceratida) have been shown to contain a large array of diterpenoids of the spongian class.[38] Two species of yellow sponge *Aplysilla tango* and *Aplysilla sulphurea* can be distinguished by their yellow color. Several degraded spongian diterpenes were isolated. Aplysulphurin (**24**) was the major constituent of *A. sulphurea*, and was later also found in *A. tango*.

Figure 2.1 The tropical marine sponge *Aplysilla sulphurea*

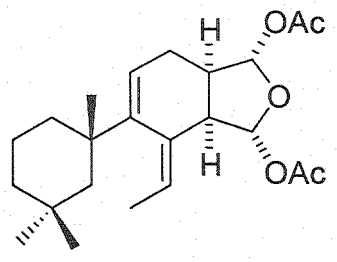
As a part of the research program towards searching for bioactive compounds from an Australian sponges. Sponges were collected at Mooloolaba, Queensland.

The yellow sponge *Aplysilla sulphurea* (Figure 2.1) was close to *Aplysilla var. sulphurea*. The sponge color is changed from yellow to blue on death. The yellow color of *Aplysilla sulphurea* gradually changes to blue within an hour, whereas *Aplysilla var. sulphurea* has changed only a small portion. *Aplysilla tango* is an encrusting sponge very similar in appearance to *Aplysilla sulphurea*. Five degraded spongian diterpenes were previously isolated as gracilin A (**25**), aplytandiene-1 (**26**), aplytandiene-2 (**27**), aplytangene-1 (**28**), aplytangene-2. (**29**) There has been only are report on the chemical constituent of *Aplysilla var. sulphurea* in which two spongian diterpene

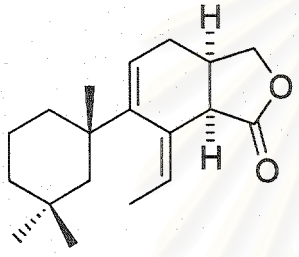
were isolated and identified to be 15,16-diacetoxyspongian (**30**) and 16-oxospongian (**21**). [39,40]



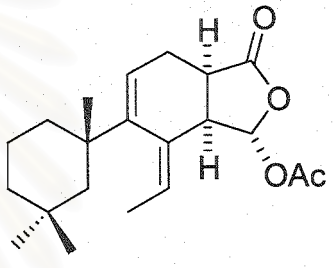
aplysulphurin (**24**)



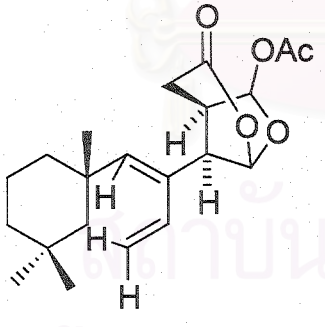
gracilin A (**25**)



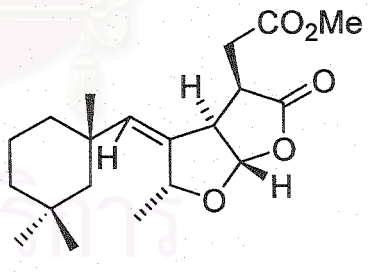
aplytadiene-1 (**26**)



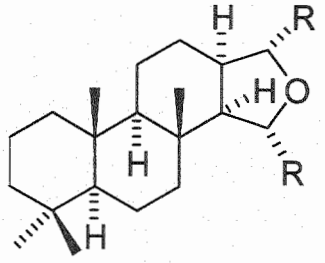
aplytandiene-2 (**27**)



aplytangene-1 (**28**)



aplytangene-2 (**29**)



R= $OCOCH_3$ (**30**) R=H (**31**)

2.2 Experimentals

2.2.1 Biological Material

An Aplysillid sponge *Aplysilla sulphurea* was collected from Fish hole dive site, Gneering reef, Mooloolaba, Queensland Australia, in December 2002. The sample was preserved in the freezer at –20 C^{O} until extraction. A voucher specimen is available from Dr. Mary Garson, the sponge deposited at chemistry department, the University of Queensland

2.2.2 Equipments

Nuclear magnetic resonance (NMR) spectra were recorded on Bruker DRX 500 NMR spectrometer operating at 500.1325 MHz for ^{1}H NMR and at 125.7685 MHz for ^{13}C NMR. ^{1}H NMR and ^{13}C NMR chemical shifts were referenced to residual solvent peaks: δ_H 7.25 and δ_C 77.0 for $CHCl_3$. The HSQC experiments were optimized for $^{1}J_{CH}$ = 135 MHz and the HMBC experiments for $^{n}J_{CH}$ = 8 Hz. ESI MS and HRESIMS was recorded on a Finnigan MAT 900 XL double focusing magnetic sector mass spectrometer. Optical rotations were measured on a Perkin Elmer 241 MC polarimeter. The isolation of compound **2a** to **2c** was performed by high performance liquid chromatography carried out on a Waters μ-porasil semi preparative column (300 mm x 7.8 mm) connected to a Waters 515 HPLC pump and a Gilson 132 refractive index detector. All solvents were freshly distilled or were of HPLC grade.

2.2.3 Chemicals

Thin layer chromatography (TLC) was performed on aluminium sheets precoated with silica gel (Merck Kieselgel 60 PF 254). Adsorbents used for flash column chromatography were silica gel (Kieselgel 60, Merck) and Seppak silica gel cartridges. All solvent used in this research were commercial grade and distilled prior to use except for HPLC grade for HPLC analysis. TLC plates were visualized by UV at 254 and 365 nm or by spraying with the solution of vanillin in H_2SO_4.

2.2.4 Cytotoxicity assay against P388 Mouse Lymphoblast Cells

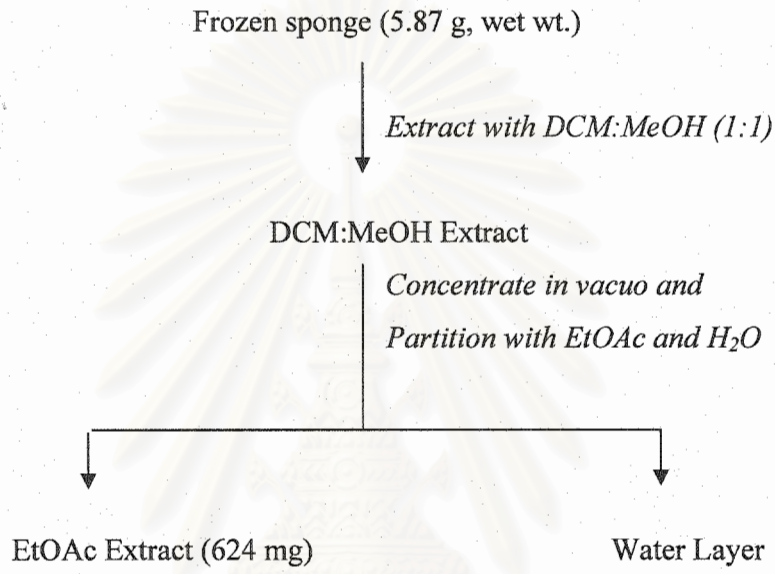
This assay was run by staff of the center for Phytochemistry, Southern Cross University, New South Wales. Mouse lymphoblast cells were obtained from ATCC. Cell culture media and sera were from in vitrogen and CSL. Cell cultures were routinely grown at 37° C, 5% CO_2 in DMEM containing 10% horse sera with 2 nM L-glutamate and 100 μg/mL penicillin and 100 μg/mL streptomycin. For the cytotoxicity assay, cell growing in log phase were diluted in the same media described above but without phenol red indicator, and then transferred (99 μL/well) to a 96-well tissue culture plate. Samples for testing were dissolved in DMSO at an initial concentration of 10 mg/mL and then diluted by serial dilution in DMSO to be 100, 10 and 1 μg/ml for crude extract and 10, 1 and 0.1 μg/ml for pure compounds. All concentrations were added in the well and placed in an incubator (37 C°, 5% CO_2) for 24 hr.

2.2.5 Anthelmintic assay

Nematocidal activity was determined using the method of Lacey *et al.* [41] Nematode (*Haemonchus contortus* and *Trichostrongylus colubriformis*) eggs were placed into wells of a mictotitre plate containing extracts or purified compounds in 2% agar. The eggs hatched overnight, a nutrient medium was added, and the larvae were held at 27°C for 6 days. The number of larvae that developed to the L3 stage was counted and compared to control wells. For each nematode species, the effect of chromodorolide C was examined in two separate experiment at concentrations of 100 and 10 μg/mL (duplicate or triplicate assays wells at each concentration for experiments one and two, respectively) and the percentage development was calculated for each assay relative to the mean development in twelve control assay wells. Data is therefore presented as Mean ± SE, n = 2 in separated experiments. This assay was run by Dr. Andrew Kotze of CSIRO Livestock Industries, Brisbane.

2.2.6 Extraction

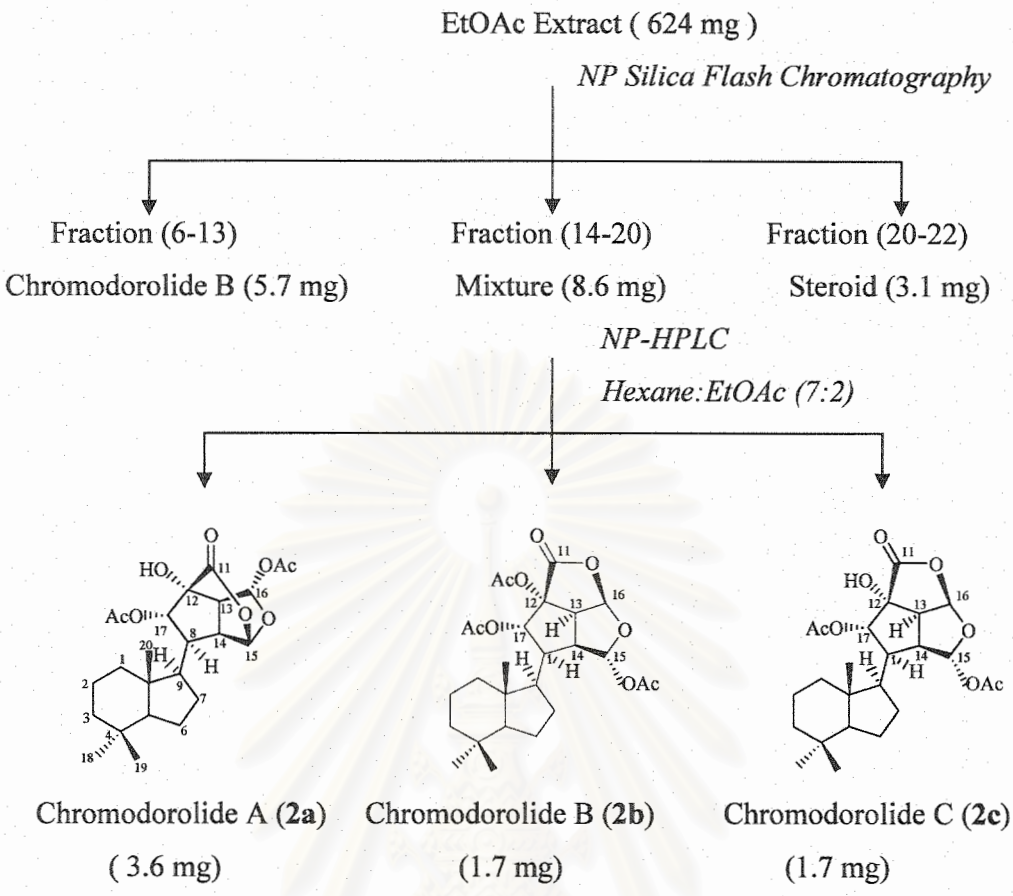
The frozen sponge (5.9 g) was cut into small pieces and extracted with 100 mL of DCM/MeOH (1:1) three times. The organic layer was filtered through a plug of cotton wool and the solvent was evaporated *in vacuo* to give the crude extract (624 mg), as shown in scheme 2.1



Scheme 2.1 The extraction procedure of *Aplysillid* sponge.

2.2.7 Isolation and Purification

The EtOAc extract (624 mg) was subjected to silica flash column chromatography using a step gradient from hexane to 100% EtOAc to give two major fractions. The first fraction was identified as chromodorolide B (**2b**) (5.7 mg) and the second fraction was purified by NPHPLC using hexane and EtOAc (7:2) as an eluent to furnish chromodorolide A (**2a**) (3.6 mg) and the new compound, chromodorolide C (**2c**) (1.7 mg). The summary of isolation and purification is shown in Scheme 2.2



Scheme 2.2 The summary of isolation and purification of EtOAc extract

2.2.8 Acetylation of Chromodorolide C (2c): To a solution of chromodorolide C (**2c**), 1.0 mg, 2.2×10^{-3} mmol) in dry pyridine (10 mL) was added acetic anhydride (1.0 mL). The mixture was stirred at room temperature overnight. The reaction was quenched by adding 1 mL of water, and extracted twice with 1 mL of EtOAc. The organic layer was blown down over N_2 gas. The acetate product (0.9 mg) was obtained as a colorless oil by purifying on silica-seppak using hexane:EtOAc (7:2) as an eluent, and identified as **2b** by comparison of their ^{1}H-NMR spectra.

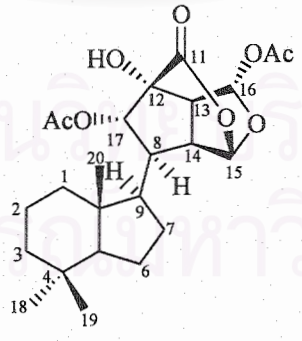
2.3 Results and Discussion

2.3.1 Structural Elucidation of Chromodorolide A (2a)

Compound **2a** (3.6 mg) was isolated as colorless oil; $[\alpha]_D$ -74° (*c* 0.1, CH_2Cl_2). Its mass spectra (Figure 2.3) showed the molecular peak at m/z 473 $[M+Na]^+$, corresponding with $C_{24}H_{34}O_8Na$ indicated by ESIMS.

The ^1H NMR spectrum (Figure 2.4) indicated the presence of methine protons signals at δ_H 6.35, 5.78, and 4.81 (1H each), and suggested that there were acetal functionalities. Additionally, there were methine protons that resonated at δ_H 3.00 (2H) and 2.45 (1H), and methyl protons at δ_H 2.11 and 2.07 which could be assigned to acetate moieties. The signals belonged to three methyl groups were detected at δ_H 0.83, 0.84 and 0.86. There were in addition several overlapping signals in the region δ_H 1.06-1.48.

The ^{13}C NMR spectrum (Figure 2.5) revealed 24 carbons, The presence of two carbonyl resonances at 168.8 and 170.0 ppm suggested the ester, while the deshielded carbon signals at 104.2, 95.5 suggested that there were two acetal functionalities which were also corresponded with δ_H 6.35 and 5.78. Moreover, the quaternary carbon signal at 79.9 ppm could be assigned for a tertiary alcohol. As the evidence above, compound **2a** could be diterpene possessing bisacetal oxalone ring fused. Based on the literature reported and molecular mass information, compound **2a** was identified by comparison of its spectroscopic data with those from the literature as chromodorolide A, which was previously reported from *Chromodoris cavae,* collected in Indian ocean.[42] The ^1H and ^{13}C NMR assignment of compound **2a** are shown in Table 2.1.



Chromodorolide A (**2a**)

Table 2.1 The ^{1}H and ^{13}C NMR spectral data for Compound **2a** in $CDCl_3$

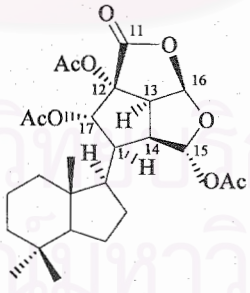
Atom #	$\delta^{1}H$ [a]	$\delta^{13}C$ [b]
1 ax	1.05, m	41.1
1eq	1.35, m	
2	1.50-1.52, m	20.8
3ax	1.08	40.4
3eq	1.69	-
4	-	33.1
5	1.11, m	57.8
6	1.31, m	19.8
6′	1.56, m	-
7	1.15, m	26.5
7′	1.80, m	-
8	2.45, m	44.6
9	1.55, m	51.6
10	-	42.5
11	-	172.4
12	-	79.9
13	3.00, brs	52.1
14	3.00, brs	46.3
15	5.78, brs	104.2
16	6.35, s	95.5
17	4.81, d, (J = 2.5 Hz)	78.8
Me-18	0.86, s	33.4
Me-19	0.84, s	20.8
Me-20	0.83, s	13.6
OCO$\underline{C}H_3$	2.07, s	20.8
O$\underline{C}OCH_3$	-	168.8
OCO$\underline{C}H_3$	2.11, s	20.8
O$\underline{C}OCH_3$	-	170.0

[a]500 MHz; sample in $CDCl_3$ reference at δ_H = 7.25 ppm
[b]125 MHz; sample in $CDCl_3$ reference at δ_C = 77.0 ppm.

2.3.2 Structural Elucidation of Chromodorolide B (2b)

Compound **2b** (7.4 mg) was isolated as colorless oil; $[\alpha]_D$ -93° (*c* 0.1 CH_2Cl_2). Its mass spectra (Figure 2.7) showed the molecular peak at m/z 493 $[M+H]^+$ and 515 $[M+Na]^+$corresponding with $C_{26}H_{36}O_9$.

The ^{13}C NMR (Figure 2.9) spectrum displayed four carbon signals at 170.2, 169.9, 169.2 and 169.1 ppm which could be assigned for three ester carbonyls and one lactone ring, respectively. The ^1H NMR spectrum (Figure 2.8) indicated the presence of acetal protons at δ_H 6.50 (1H) and 6.08 (1H), which were correlated in HSQC (Figure 2.11) to ^{13}C NMR at 97.7 and 103.4 ppm revealing that there were two acetal functionalities. From the COSY spectrum (Figure 2.10), the connection of acetal methine proton at δ_H 6.50 through the intervening methine proton resonated at δ_H 3.79 (1H), 2.93 (1H) to the second acetal at δ_H 6.08 was clearly observed. Additionally, the methine proton at δ_H 2.93 showed the correlation to a methine proton at δ_H 2.56 that was in turn correlated to a deshielded methine proton at δ_H 5.30. Additionally, the singlet protons at δ_H 0.84, 0.82 and 0.78 were assigned as three methyl groups and the signals at δ_H 2.19, 2.11and 2.04 were assigned as three protons of acetate moieties. By the literature survey, all NMR data as shown (Table 2.2) were compared with those of isolated compound. Compound **2b** was identified to be chromodorolide B, which was previously isolated from the nudibranch, *Chromodoris cavae*.[42]



chromodorolide B (**2b**)

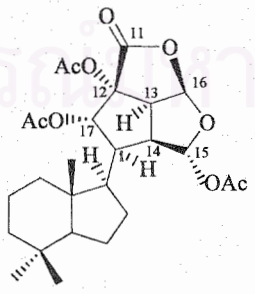
Table 2.2 The ^{1}H and ^{13}C NMR spectral data for Compound **2b** in $CDCl_3$

Atom #	$\delta^{1}H$ [a]	$\delta^{13}C$ [b]
1	1.03, dt (J = 3.6, 12.3 Hz)	40.9
	1.38, m	-
2	1.50-1.52, m	21.1
3	0.94, m	39.1
	1.69	-
4	-	33.0
5	1.09, dd (J = 6.6, 13.1 Hz)	56.9
6	1.55, m	19.9
	1.40, m	-
7	1.15,m	25.2
	1.80,m	-
8	2.56, ddd (J = 4.0, 7.5, 11.0 Hz)	48.0
9	1.70, dd (J = 9.5, 10.0 Hz)	50.3
10	-	43.8
11	-	169.1
12	-	81.3
13	3.79, dd (J = 6.0, 9.0)	50.4
14	2.93, t (J = 8.5 Hz)	45.6
15	6.50, bs	97.7
16	6.08, d (J = 6.0 Hz)	103.4
17	5.30, d (J = 12.5 Hz)	73.9
Me-18	0.78, s	33.4
Me-19	0.82, s	21.0
Me-20	0.84, s	13.7
OCO$\underline{C}H_3$	2.04, s	20.8
O$\underline{C}OCH_3$	-	169.2
OCO$\underline{C}H_3$	2.11, s	20.8
O$\underline{C}OCH_3$	-	169.9
OCO$\underline{C}H_3$	2.19, s	20.9
O$\underline{C}OCH_3$	-	170.0

[a] 500 MHz; sample in $CDCl_3$ reference at δ_H = 7.25 ppm
[b] 125 MHz; sample in $CDCl_3$ reference at δ_C = 77.0 ppm.

2.3.3 Structural Elucidation of Chromodorolide C (2c) (New compound)

The new chromodorane, compound **2c** was isolated as a colorless oil; $[\alpha]_D$ -78° (*c* 0.1 CH_2Cl_2) and had a molecular peak at *m/z* 473.2148 (Figure 2.12) corresponding to $C_{24}H_{34}O_8Na$ indicated by HRESIMS. The ^1H NMR spectrum (Figure 2.13) indicated the presence of three singlet methyls (δ_H 0.82, 0.83 and 0.76), and two additional singlet methyls (δ 2.05 and 2.13) that could be assigned to acetate moieties. Two methine protons (δ_H 6.51 and 6.00) supported the presence of acetal functionalities. Numerous complex signals in the region δ_H 1.06-1.48 were consistent with the compound having a terpene skeleton. In the ^{13}C NMR spectrum (Figure 2.14), signals at 174.8, 168.8 and 169.1 ppm were consistent with a lactone carbonyl and two acetate carbonyls respectively. The similarity of the ^1H and ^{13}C NMR spectra of **2c** to those of **2b** indicated that this new compound should be categorized in a chromodorane diterpene class, possessing a 3,4 ring fused bisacetal-oxalone. DQFCOSY (Figure 2.16) correlations connected the acetal methine proton H-15 at δ 6.51 through two intervening protons, H-14 at δ_H 2.98 and H-13 at δ 3.52, to the second methine H-16 at δ 6.00. However comparison of the spectral data showed that **2c** only differed from **2b** in its acetylation pattern. The HMBC spectrum (Figure 2.19) showed the connectivity from H-15 at δ_H 6.00 to an acetate carbonyl at δ 168.8 and from H-17 at δ 5.19 to the acetate carbonyl at 169.0 ppm, and from two methyl acetate signals at δ 2.05 and δ 2.13 to the acetate carbonyls at 168.8 and δ 169.1 ppm, respectively, confirming the position of the acetate substituents. Additionally, in **2c** a signal at 78.1ppm, assigned to C-12, was evident and this suggested a quaternary carbon bearing a hydroxy group, whereas in compound **2b,** the corresponding signal resonated at 79.9 ppm. These data manifestly identified **2c** as a new compound and allowed all resonances to be assigned (Table 2.3).



chromodorolide C (**2c**)

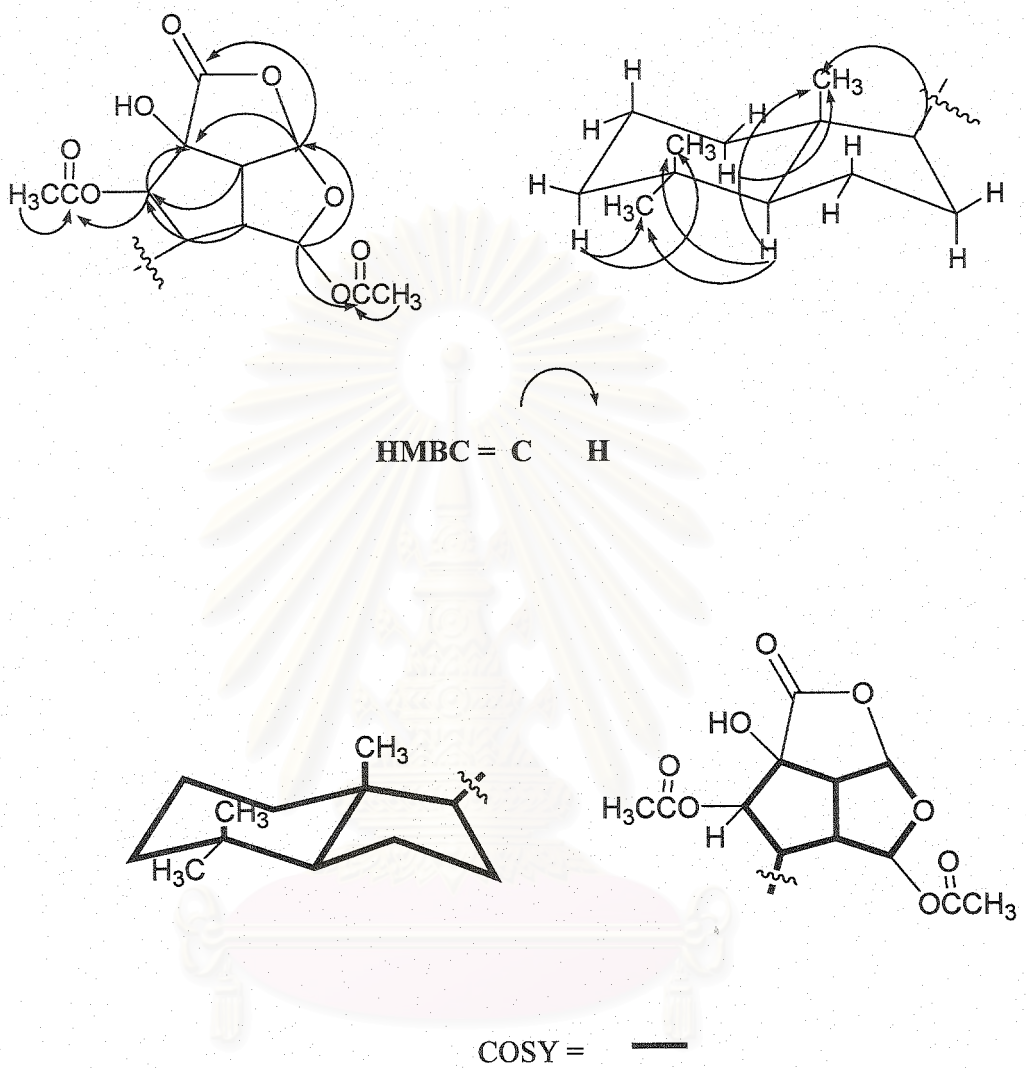


Figure 2.2 The ^{1}H-^{1}H COSY correlation and HMBC connectivity of compound **2c**

Table 2.3 The ^{1}H and ^{13}C NMR spectral data for Compound **2c** in $CDCl_3$

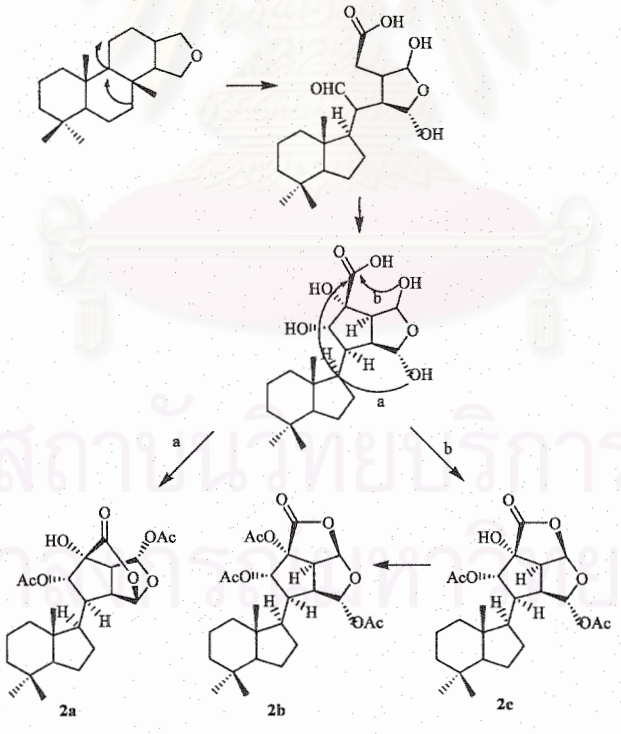
Atom #	$\delta^{13}C$[a]	$\delta^{1}H$	^{1}H-^{1}H COSY	HMBC[b]
1	39.0	ax 0.93, dt (J = 2.0, 12.0 Hz) eq 1.53, m	H-1eq H-1ax	Me-20
2	19.9	ax 1.38, m eq 1.55, m	H-2eq, H-3ax, H-3eq H-2ax, H-3ax	H-3ax
3	40.9	ax 1.03, dt (J = 3.0, 12.0 Hz) eq 1.38, m	H-2ax, H-2eq, H-3eq H-2ax, H-3ax	Me-18, Me-19
4	33.0	-	-	H-5, Me-18, Me-19
5	57.0	1.09, dd (J = 6.5, 13.0 Hz)	H-6ax, H-6eq	Me-18, Me-19, Me-20
6	21.10	ax 1.30, m eq 1.55, m	H-5, H-6eq, H-7 H-5, H-6ax, H-7	H-5
7	25.2	ax 1.42, m eq 1.55, m	H-6ax, H-6eq, H-9 H-6ax, H-6eq, H-9	-
8	47.5	2.58, ddd (J = 8.2, 12.0, 12.0 Hz)	H-9, H-14, H-17	H-15, H-17
9	50.2	1.71, dd (J = 9.8, 12.0)	H-7ax,H-7eq,H-8	H-8, H-17. Me-20
10	43.8	-	-	H-5, Me-20
11	174.8	-	-	H-16, H-17
12	78.1	-	-	H-13, H-14, H-16,
13	52.1	3.52, dd (J = 6.0, 8.2 Hz)	H-14, H-16	H-15, H-16
14	45.4	2.98, t (J = 8.2 Hz)	H-8, H-13	H-8, H-15
15	98.1	6.51, s	-	H-8, H-13, H-14
16	103.3	6.00, d (J = 6.0 Hz)	H-13	H-15
17	75.3	5.19, d (J = 12.0 Hz)	H-8	H-8, H-13, H-14
Me-18	33.4	0.82, s	-	Me-19
Me-19	21.0	0.83, s	-	H-5, Me-18,
Me-20	13.6	0.76, s	-	H-5, H-9
OAc	169.2	-	-	H-15, -OAc
	20.8	2.05	-	
OAc	168.8	-	-	H-17, -OAc
	20.9	2.13	-	

[a]500 MHz; sample in $CDCl_3$ reference at δ_H = 7.25

[b]125 MHz; sample in $CDCl_3$ reference at δ_C = 77.0 ppm

In particular, the bicyclic terpene framework of **2c** was carefully assigned by DQFCOSY and HMBC. The assignments for C-1 and C-3 were made on the basis of correlations to nearby methyl groups as shown in Table 2.3, while assignments for C-2 and C-6 were confirmed by HMBC correlations to H-3ax and to H-5, respectively. These data suggest that the NMR assignments published by Andersen *et al.* for chromodorolide B [43] may need the revision.

Small scale acetylation of **2c** with acetic anhydride in pyridine provided an acetate product whose ^{1}H NMR spectrum was identical to that of chromodorolide B **2b**. This is the first report of these chromodorane diterpenes from a sponge source. It has been proposed by Andersen *et al.*[43] that the biosynthesis of the chromodorane skeleton starts from a spongian diterpene followed by opening and contraction of the six membered ring to the five membered ring. Oxidative cleavage of a second six-membered ring, followed by lactonization of the C-11 carboxyl group with the hydroxy group at C-15 or C-16 then leads to two different bisacetal oxalone skeletons. The discovery of **2c** indicates that acetylation could be a stepwise process in the sponge.



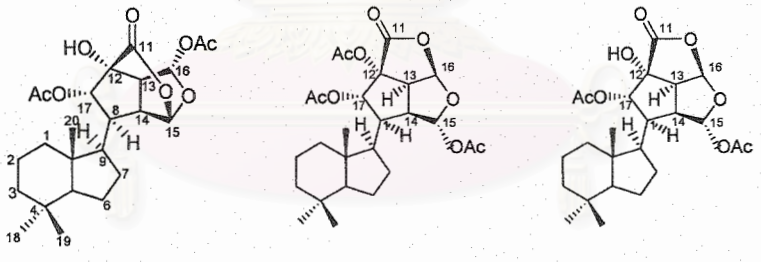
Scheme 2.3 The biosynthesis pathway of chromodorane diterpenes

2.4 The bioassay testing result

Chromodorolide A has been reported to exhibit cytotoxic and antimicrobial activity.[42] When tested individually against the P388 cell line, chromodorolides A, B and C displayed significant inhibition (66 (±3), 70 (± 2) and 42 (± 4) %, respectively) at concentrations of 10 μg/mL, but did not show useful activity at concentrations of 1 μg/mL. Chromodorolide A **2a** also showed nematocidal activity against the larval stages of *Haemonchus contortus* and *Trichostrongylus colubriformis*, two important pathogens of sheep and other ruminants. A concentration of 100 μg/mL caused 94 (± 3) % and 95 (± 4) % (Mean ± SE, n = 2 separate experiments) inhibition of the development of *H. contortus* and *T. colubriformis* larvae, respectively. A concentration of 10 μg/mL did not affect development for *H. contortus*, however *T. colubriformis* development was inhibited by 33 (± 1) %.

2.5 Conclusion

Two chromodorane diterpenes, chromodorolides A and B, were isolated together with the novel compound, chromodorolide C. This is the first report of the isolation of chromodorane diterpene from the *Aplysillid* sponge. Chromodorolides A and B have previously been found in *Chromodoris cavae*.[42]



chromodorolide A (**2a**) chromodorolide A (**2b**) chromodorolide A (**2c**)

Chromodorolide A and B (**2a-2b**) are identified as cytotoxic compounds, When tested against the P388 cell line, chromodorolides A, B displayed significant inhibition (66, 70 and 42%, respectively) at concentrations of 10 μg/mL Additionally, Chromodorolide A (**2a**) also showed considerable nematocidal activity against the larval stages of *Haemonchus contortus* and *Trichostrongylus colubriformis*.

2.6 Spectroscopic information

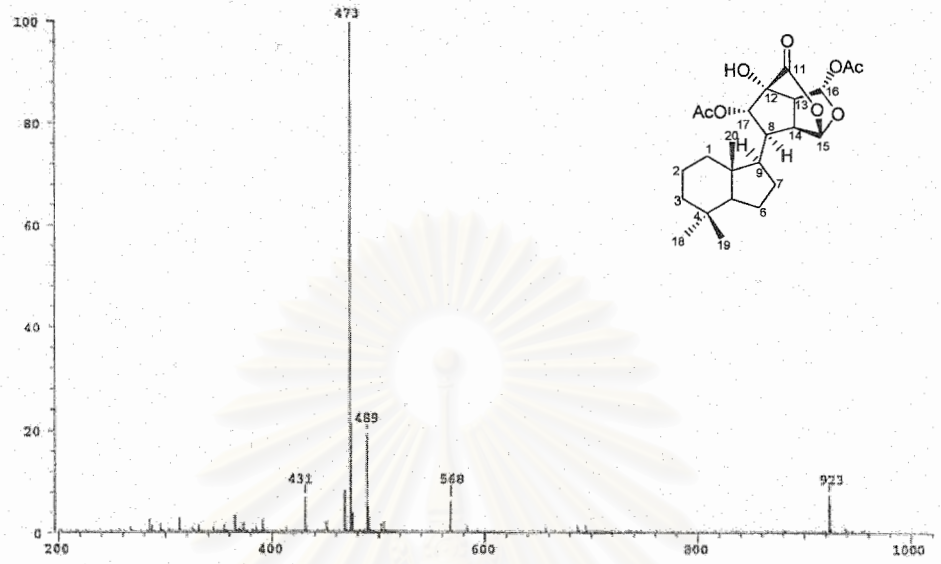


Figure 2.3 The ESIMS spectrum of Chromodorolide A (**2a**)

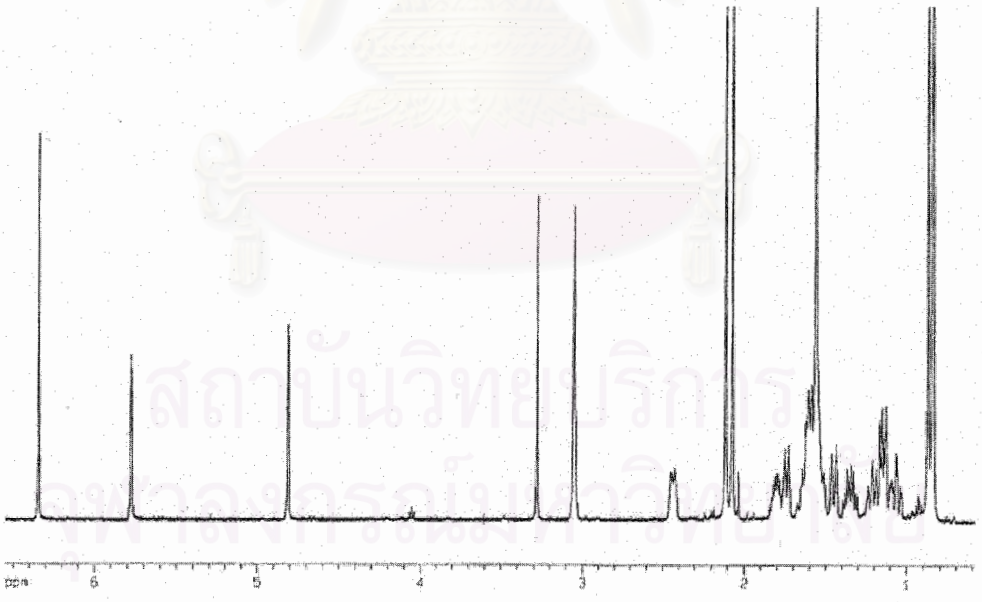


Figure 2.4 The ^{1}H NMR spectrum of Chromodorolide A(**2a**)

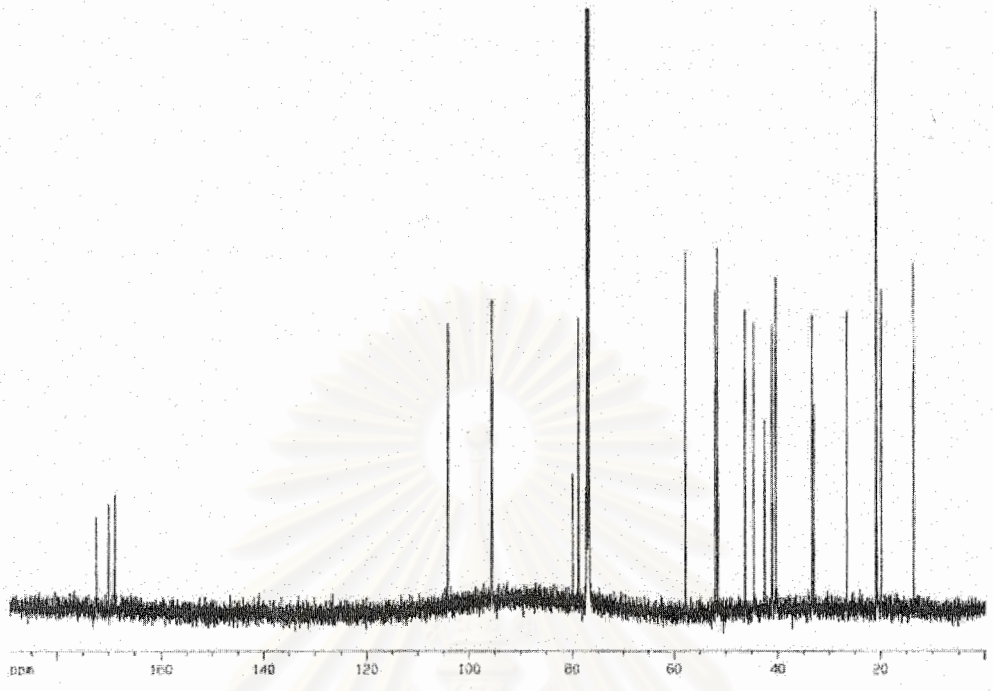


Figure 2.5 The ^{13}C NMR spectrum of Chromodorolide A(**2a**)

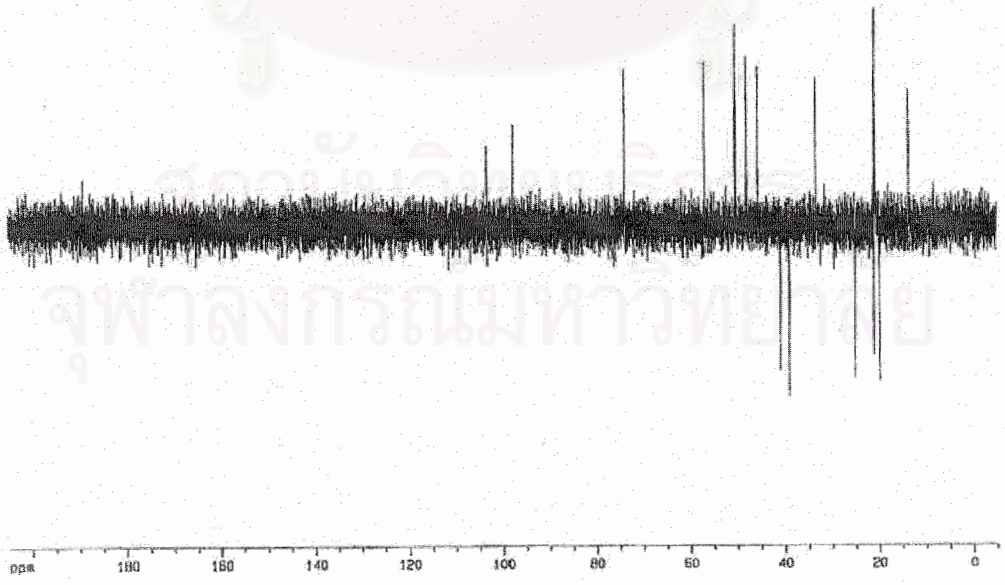


Figure 2.6 The DEPT 135 spectrum of Chromodorolide A (**2a**)

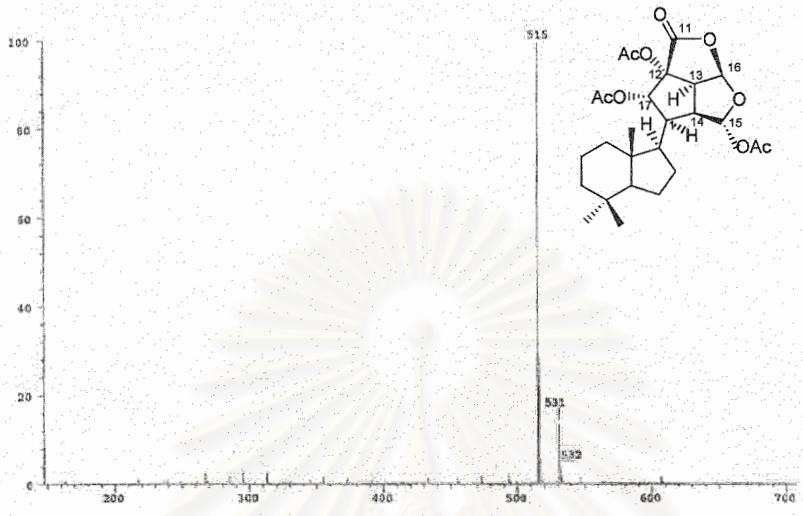


Figure 2.7 The ESIMS spectrum of Chromodorolide B (**2b**)

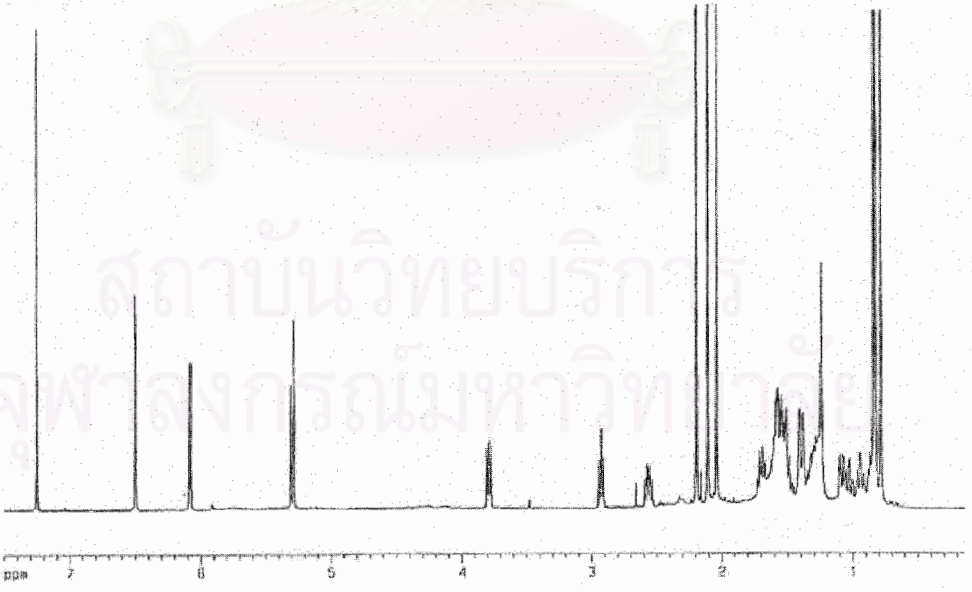


Figure 2.8 The ^{1}H NMR spectrum of Chromodorolide B (**2b**)

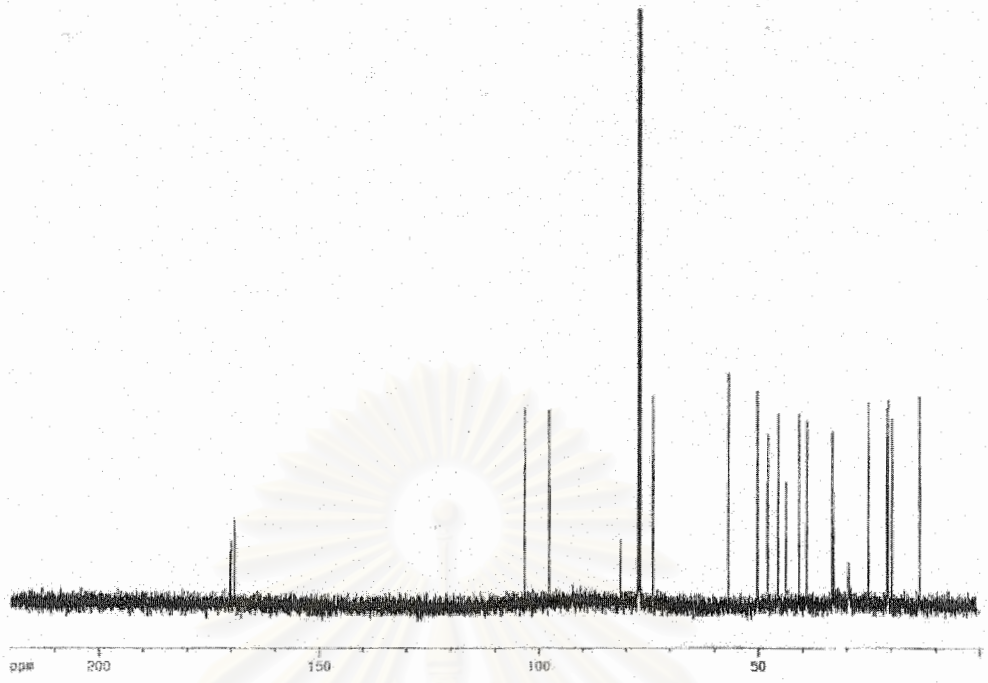


Figure 2.9 The ^{13}C NMR spectrum of Chromodorolide B (**2b**)

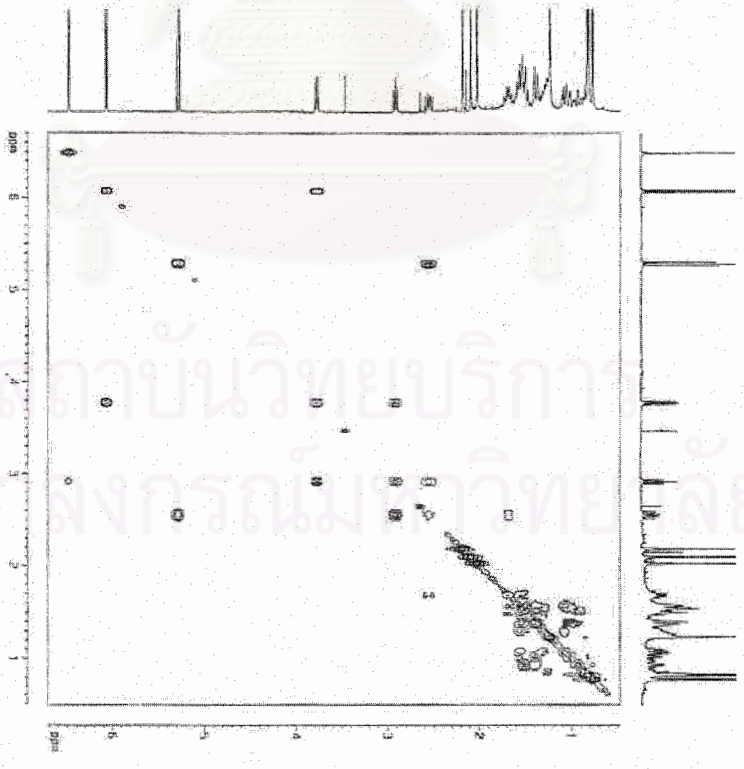


Figure 2.10 The ge ^{1}H-^{1}HCOSY spectrum of chromodorolide B (**2b**)

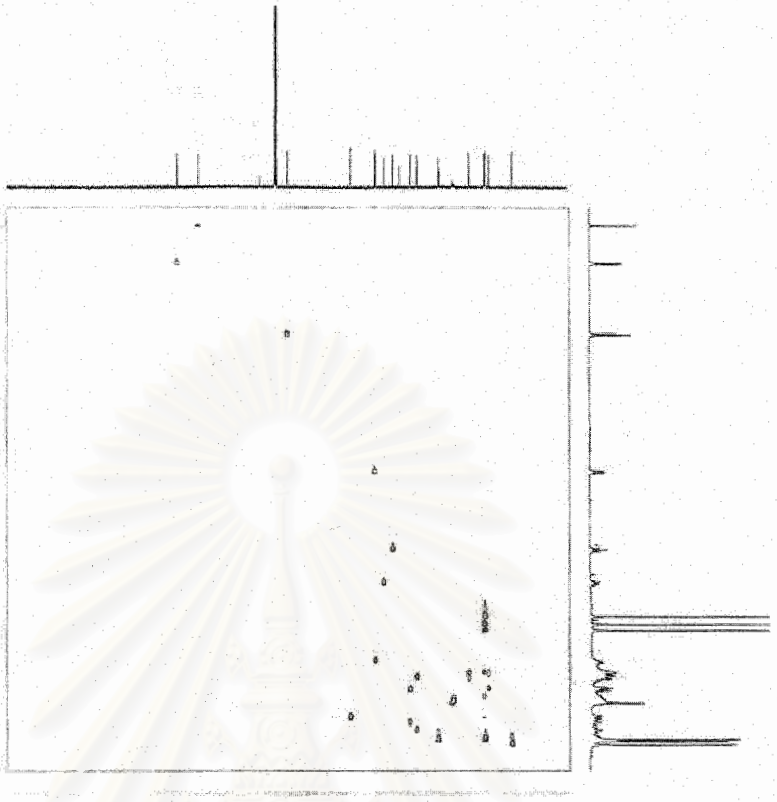


Figure 2.11 The HSQC spectrum of chromodorolide B (**2b**)

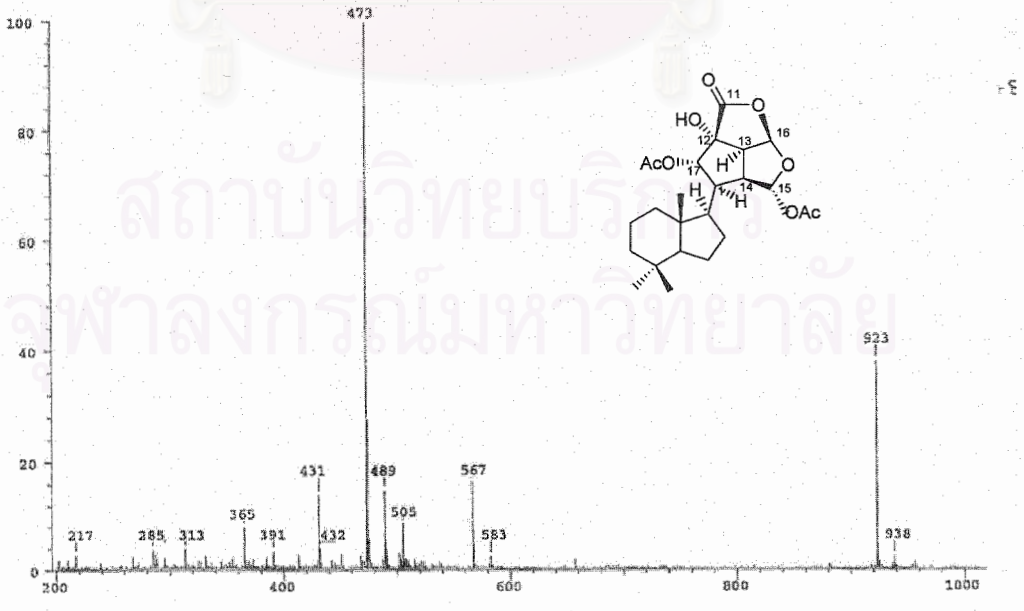


Figure 2.12 The ESIMS spectrum of chromodorolide C (**2c**)

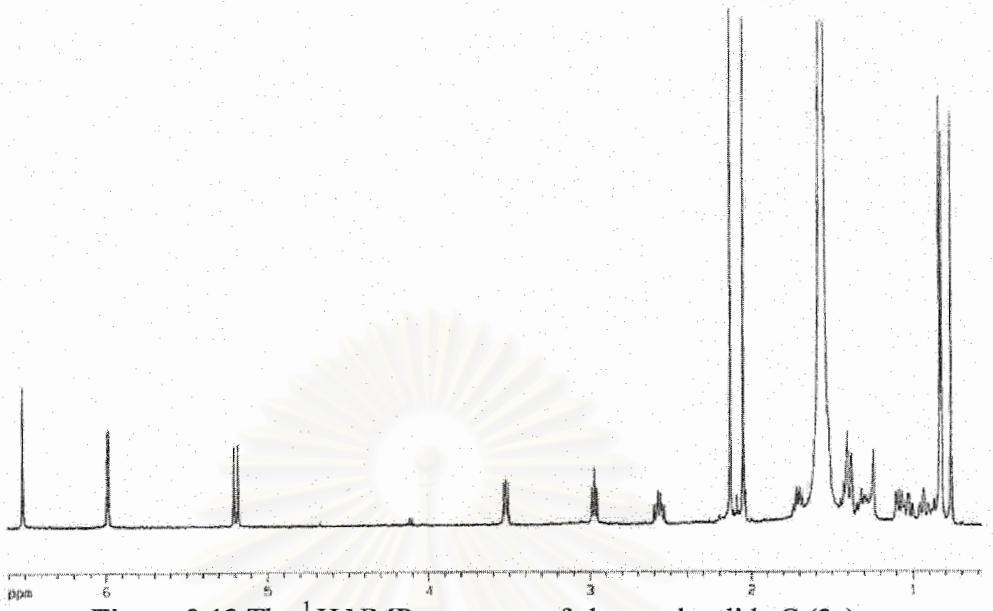


Figure 2.13 The ^{1}H NMR spectrum of chromodorolide C (**2c**)

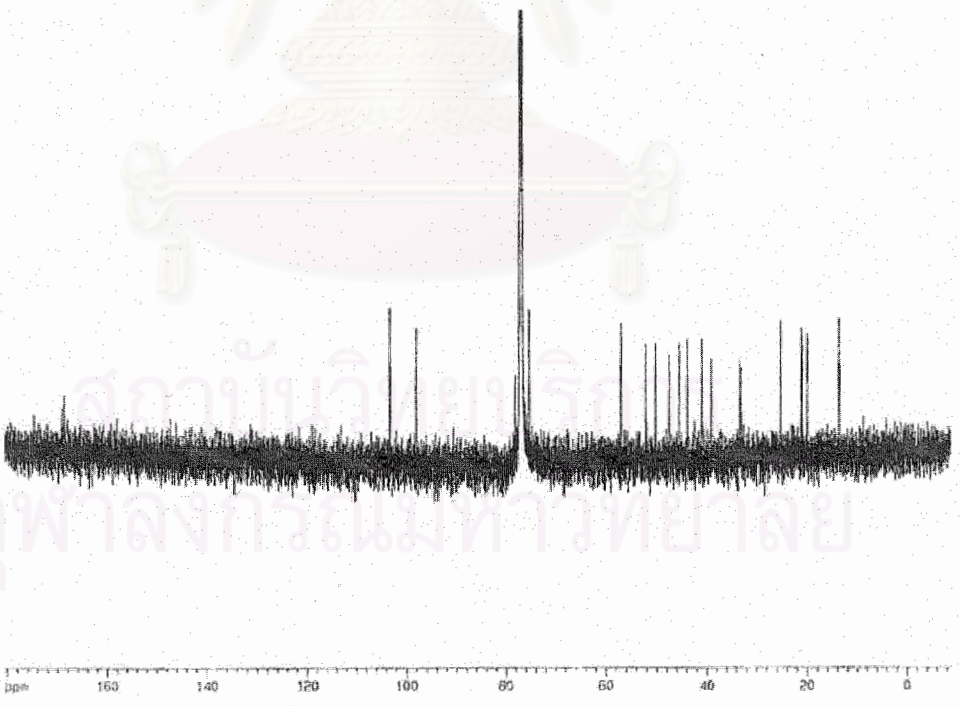


Figure 2.14 The ^{13}C NMR spectrum of chromodorolide C (**2c**)

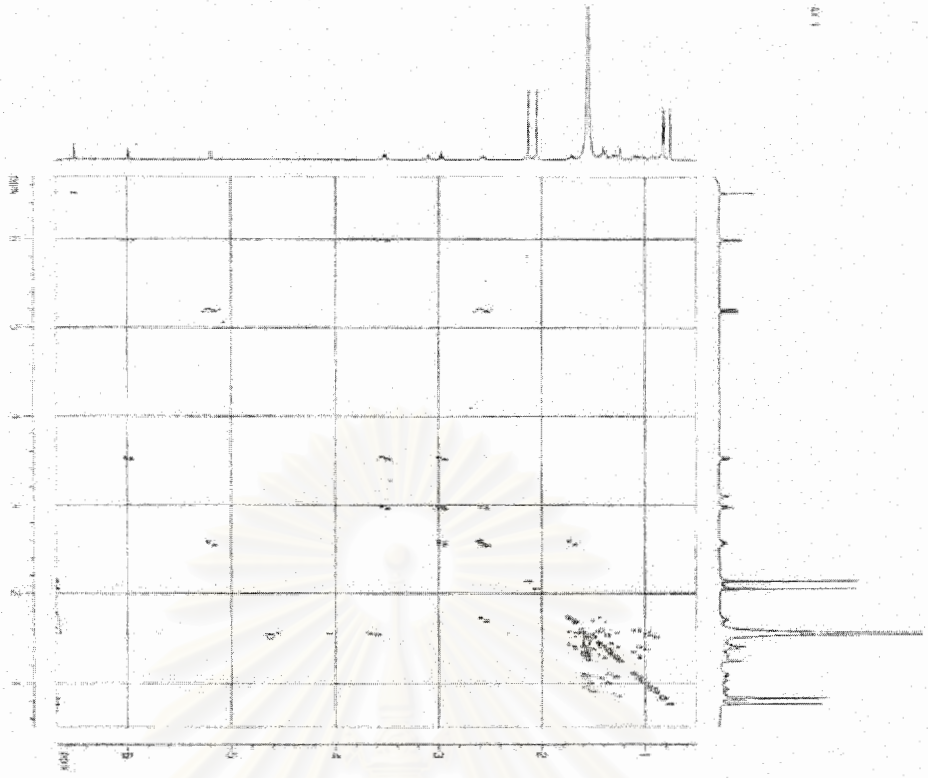


Figure 2.15 The DQFCOSY spectrum of chromodorolide C (**2c**)

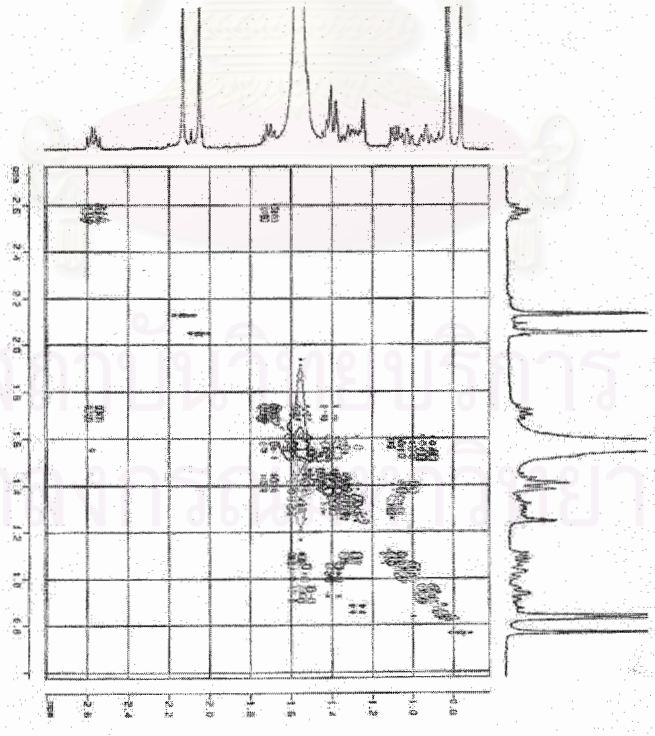


Figure 2.16 The DQFCOSY spectrum of chromodorolide C (**2c**)(Expand)

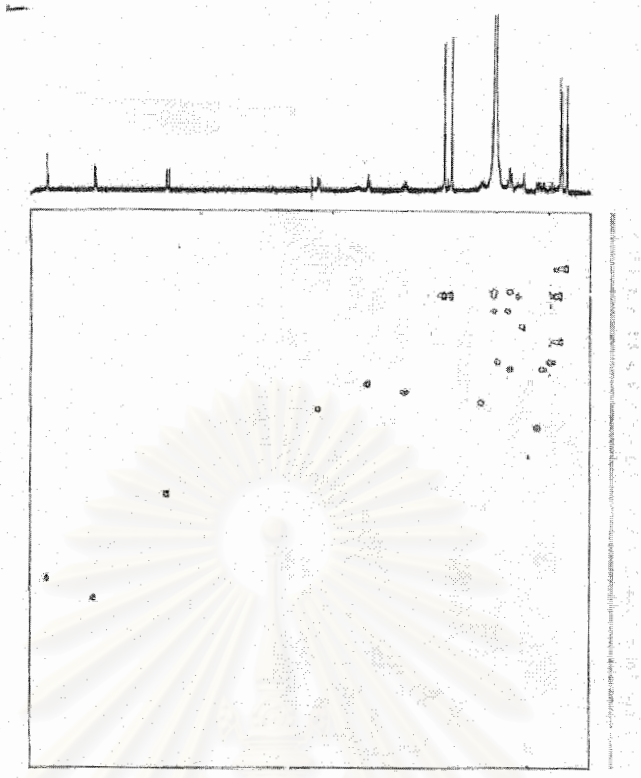


Figure 2.17 The HSQC spectrum of chromodorolide C (**2c**)

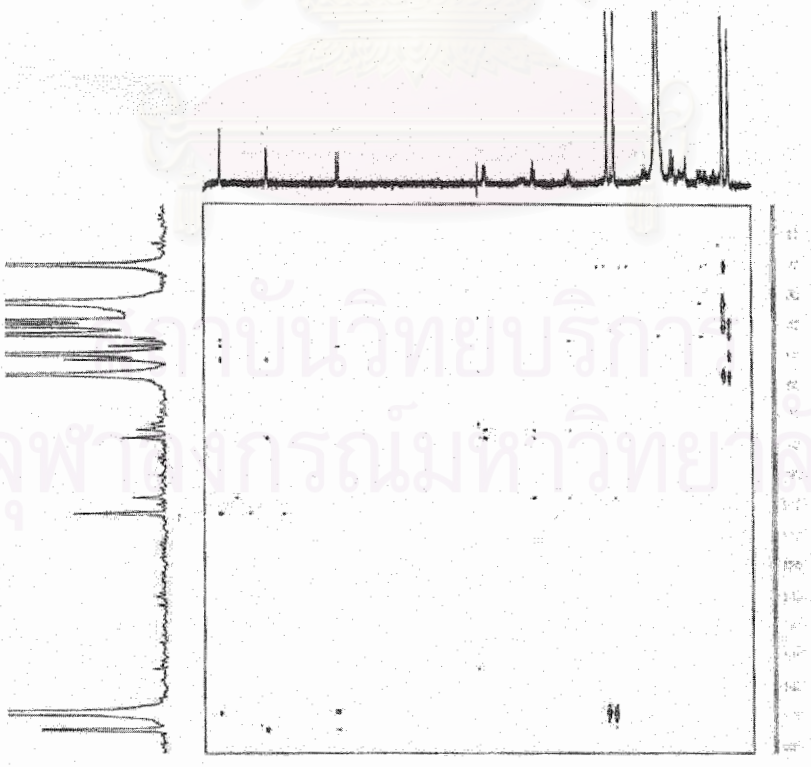


Figure 2.18 The HMBC spectrum of chromodorolide C (**2c**)

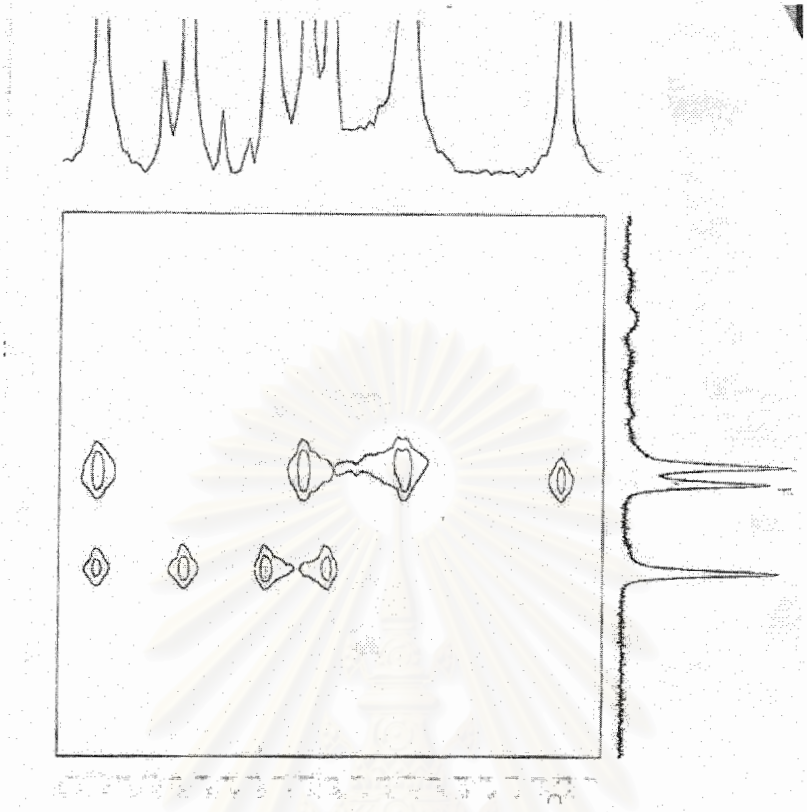


Figure 2.19 The HMBC spectrum of chromodorolide C (**2c**) (Expand)

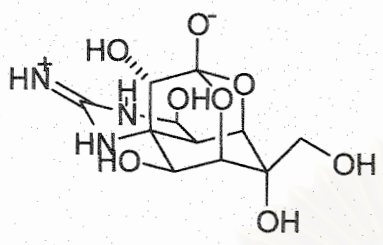
CHAPTER III

Part I : Peptides from a Marine Bacterium Associated with a Tropical Thai sponge.

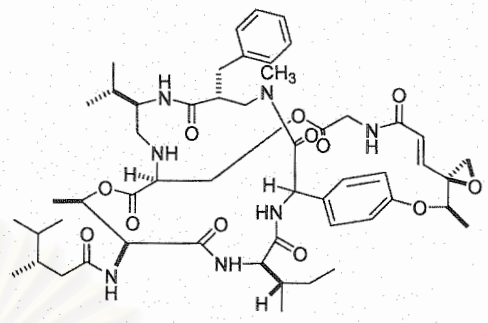
3.1 Introduction

The marine environment is a rich source of biologically active natural products many of which have not been found in terrestrial sources. This is due to the physical and chemical properties of marine environment that are different from terrestrial environments.[43] Marine microorganisms are widely recognized as the newly emerging source of secondary metabolites. These organisms flourishing in diverse marine environments have produced a variety of structurally unique and biologically active compounds that have attracted considerable attention for biomedical studies.[44]

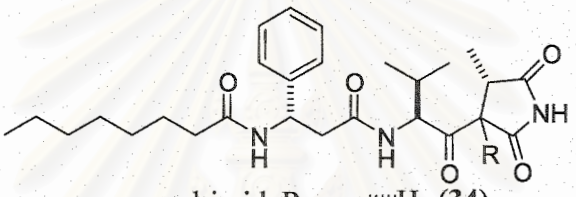
Marine bacteria are defined as those bacteria needing sodium ions for growth. Among them, chemoheterotrophic eubacteria are the most thoroughly studied of the bacteria, because many strains can be easily cultured in nutrient-rich media. Therefore, marine bacteria are a renewable resource. One such marine source are the marine bacteria associated with marine invertebrates. These are considered to be of particular importance since metabolites previously thought to arise from the invertebrates may in fact be biosynthesized by their endobionts.[45] Numerous bioactive compounds of marine invertebrates have later been found to be of bacterial origin. For example, tetrodotoxin (TTX) (**32**) causes illness and death following the consumption of raw puffer fish, but lately it has been found that *Pseudomonas* sp. from the skin of puffer fish is the true producer of TTX.[46] The potent anti-inflammatory, salinamide A (**33**) and its derivatives are produced by *Streptomyces* sp. strain CNB-09 which was first isolated from the jellyfish *Cassiopeia xamachana*.[47] Andrimid (**34**) and moramide A-C (**35-37**) are produced by *Pseudomonas fluorescens* isolated from tissue of an unidentified Alaskan tunicate and show antimicrobial activity against methicillin resistant *Staphylococcus aureus*.[48]These example show that the metabolites from marine bacteria have a high biodiversity of their structures and show useful bioactivities.



tetrodotoxin (**32**)

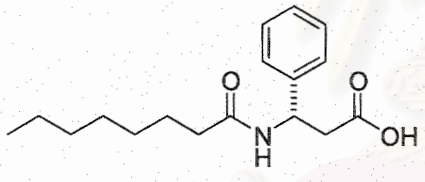


salinamide A (**33**)

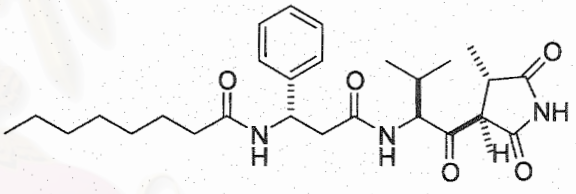


andrimid R = ''''H (**34**)

moramide C R = —OH (**35**)

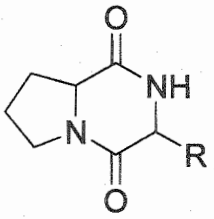


moramide A (**36**)

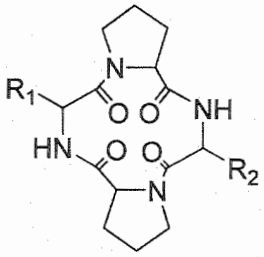


moramide B (**37**)

Many bioactive components from marine bacteria are nitrogen-containing compounds and are the combination and integration of peptide, polyketide and alkaloid fragments which utilize amino acids as a starter unit in their biosynthesis partway. Mostly peptide from marine bacteria are small peptides such as diketopiperazine (**38**). DKP's are cyclic dipeptide which have previously been isolated from terrestrial organism. For marine bacterium-derived, DKP's have been isolated from strain of *Pseudomonas aeruginosa* associated with the Antarctic sponge *Isodictya setifera*.[49] In 2003 Shin *at el* reported the isolation of a new cyclic peptide *cyclo* (L-isoleucyl-L-prolyl-L-leucyl-L-prolyl) (**39**)[50] from a marine bacterium derived from sediment. This compound is related to three cyclic tetrapeptides (**40-42**)[51] that have been previously reported from the marine ascidian *Cystodytes delle chiajei* which are cyclic tetra peptide containing- proline unit

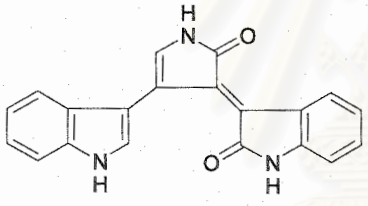


Diketopiperazine (**38**)

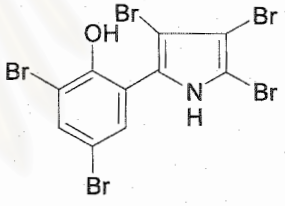


R1 = Leu, R2 = lLeu (**39**)
R1 = Leu, R2 = Leu (**40**)
R1 = Val, R2 = Val (**41**)
R1= Phe, R2 = Phe (**42**)

In continuing research on marine bacteria in Thailand, a strain of marine bacterium associated with tropical Thai sponges has been investigated and has been reported the isolation of violacein (**43**) and pentabromopseudiline (**44**). Both compounds were previously the first bacterial natural products from *Pseudomonas bromoutilis* and also exhibited autoinhibitory acivity to the producing strain *Chromobacterium*.[49]



(**43**)



(**44**)

The discovery of bioactive compounds from marine bacteria associated with tropical Thai sponges provided an alternative source of natural product. As the mentioned above, marine bacteria are of interest for further studying of their novel metabolites. As a part of a research program at marine research institute, the preliminary bioassay screening of marine bacterial extract was carried out. One gram-negative strain of marine bacterium derived from tropical Thai sponge later identified as Demospongae, family Halisarcidae Schmidt was of interest. The methanolic extract of this strain exhibited strong antibacterial activity against several bacterial testing stains, thus it was selected to further study. In this chapter describes the isolation of a mixture of peptides from this marine bacterial strain, while the topic of the next chapter of this thesis is the isolation and elucidation of some additional cyclic tetrapeptides that contain proline.

3.2 Experimental

3.2.1 Equipments

Nuclear magnetic resonance (NMR) spectra were recorded on Bruker DRX 500 and DMX 750 MHz spectrometers. ESI mass spectra were recorded on a Finnigan MAT 900 XL double focusing magnetic sector mass spectrometer. LC-ESIMS; Agilent, column, Zorbax (2.1X50 mm, 5 μm, SB-C18) and HPLC analysis were performed on Agilent series 1100 instrument with a Waters reversed phase analytical column Ultrasphere (ODS dp 5 micrometer; 4.6 x 250 mm) and semi-preparative column μ bondapak C-18 (7.8 x 300 mm) using a step gradient starting from 50% MeOH in water to 100% MeOH in 20 minutes. The detector was set at 250 nm.

3.2.2 Chemicals

Thin layer chromatography (TLC) was performed on aluminum sheets precoated with silica gel (Merck, Kieselgel 60 PF_{254}). Adsorbents used for open column chromatography were Silica gel (Merck Kieselgel 60) and sep-pak cartridges (Water ™). Most solvents used in this research were of commercial grade and were distilled prior to use except for those of HPLC grade quality, which were used directly for HPLC experiment.

3.2.3 NMR experiment

All 1D and 2D NMR spectra were recorded on Bruker DRX 500 MHz and DMX 750 MHz spectrometers. The sample was placed in a shigami NMR tube and NMR data sets were collected in both CD_3OD and CD_3OH. The HMBC and the phase sensitive geHSQC spectra were acquired with 64 and 24 transients, respectively. The evolution delay was set for $^{n}J_{CH}$ of 8Hz (geHMBC) and $^{n}J_{CH}$ 135 Hz (geHSQC). The DQFCOSY spectra were acquired with 16 and 24 transients per increment while NOESY were acquired with 32 transients per increment. A mixing time of 800 ms was used in the NOESY experiment and 0.5 s for the TOCSY experiment.

3.2.4 Collection and Taxonomic Identification

Tropical marine sponges were collected in the Gulf of Thailand by the staff of Marine Research Institute Burapa University, Chonburi, Thailand and the bacterial strains were isolated and purified by Dr. Chutiwan Detchsakulwattana of this university.

The taxonomic identification of the sponge was carried out by Mr. Sumet Puthchakarn. This sponge is Demospongae, family Halisarcidae Schmidt, and is

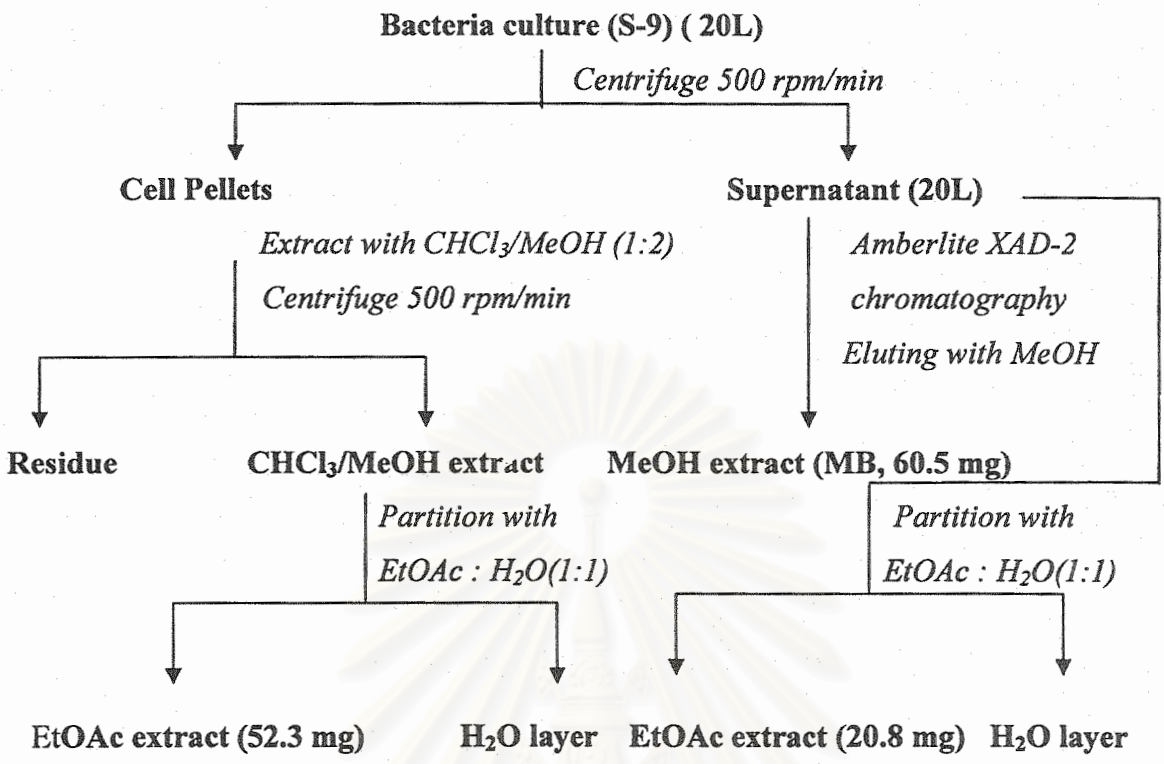
characterized by the presence of irregular tubular chambers and the lack of any fibrous or mineral elements in the skeleton. The order contains one family and one genus (Halisarca) with 24 nominal species, however many are so poorly described that they are unrecognizable, and the number of valid species is probably around ten. The genus has a wide distribution in temperate and tropical shallow waters. The small size and fragile texture of these sponges render it unlikely that they would be detected in fixed or deep water dredge sample, and consequently they may be more widespread than present data indicates.[50]

3.2.5 Fermentation

The bacterial strain (S-9) was isolated from a Thai sponge culture in ORI medium (Ocean Research Institute). The ingredients of ORI are 0.1% proteose peptone, 0.1% yeast extract, 0.05% phyton, 0.02% $Na_2S_2O_3$, 0.005% Na_2SO_3, Fe^{2+}. The condition of the fermentation was maintained at pH 7.6, 25 °C, 3 days.

3.2.6 Extraction and Isolation

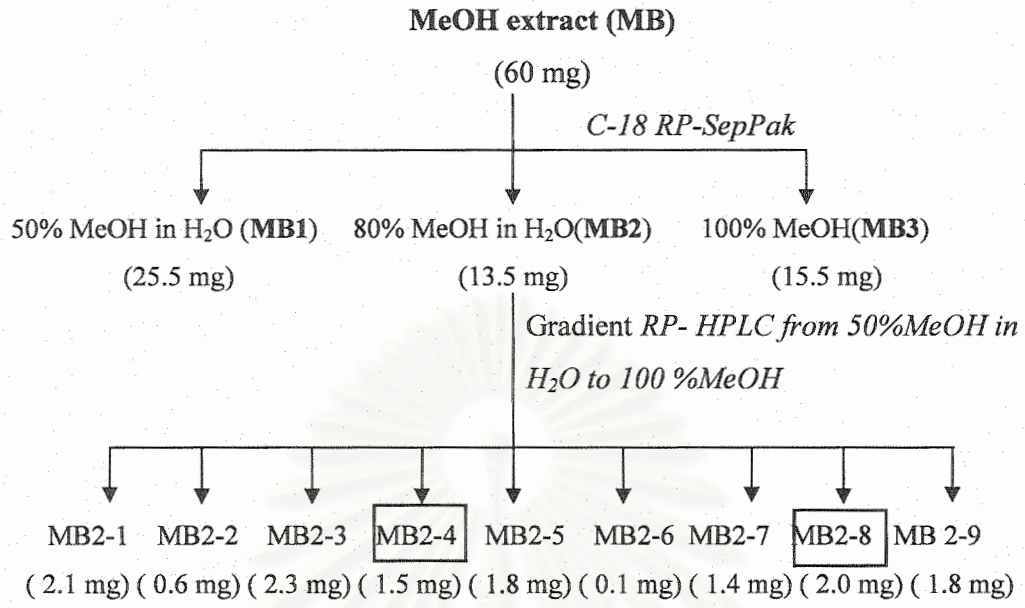
The combined culture broth (20 L) was centrifuged to separate bacterial cells from supernatant. Bacterial cells were extracted with 500 mL $CHCl_3$: MeOH (1:2) three times and filtered. The combined filtrate was evaporated *in vacuo* and further extracted by partitioning between EtOAc and water (1:1) (500 mL). The organic layer was evaporated to afford the EtOAc extract (52.3 mg). The supernatant was divided into two parts, the first part (5.0 L) was passed through an Amberlite XAD-2 column chromatography and eluted with 100% MeOH to give MeOH extract (60.5 mg). The second part (8.0 L) was further partitioned with EtOAc, then concentrated by evaporation *in vacuo* to achieve EtOAc extract (20.8 g). All crude extracts were assayed for antimicrobial activity. The methanolic extract displayed antimicrobial activity against various bacterial cell lines. This fraction was selected for further separation. The summary of extraction is shown in Scheme 3.1.



Scheme 3.1 The extraction for bacterial strain

3.2.7 Fractionation and Purification

The active fraction (MB) 60 mg was fractionated by C-18, reverse phase sep pak eluting with step gradient, starting from 50% then 80% methanol in water and then 100% methanol to give three fractions (MB1-MB3). The second fraction was selected for further separation as a result of the presence of peptide signals, suggested by ^{1}H NMR. Accordingly, this fraction was subjected to RP-HPLC by step gradient starting from 50% MeOH in H_2O to 100% MeOH. The HPLC chromatogram for separation of MB fraction is shown in figure 3.1. Two major fractions MB2-4 and MB2-8 with R_t 10.5 and 13.3 min respectively were obtained. The summary of isolation is shown in scheme 3.2



Scheme 3.2 The fractionation and purification procedure of **MB** fraction

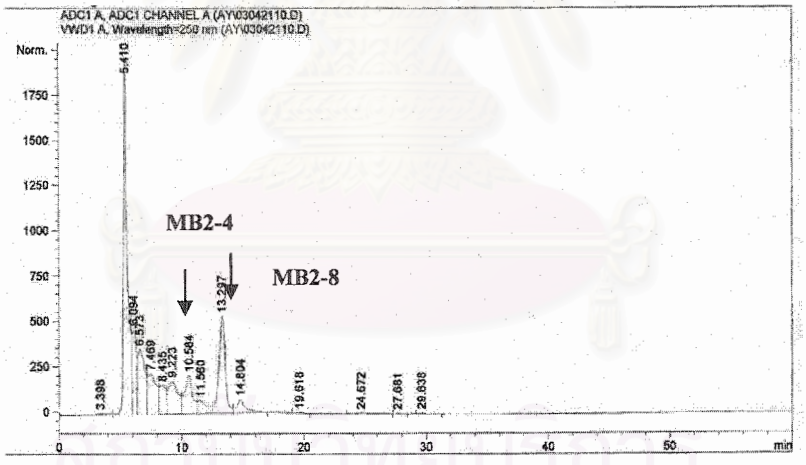
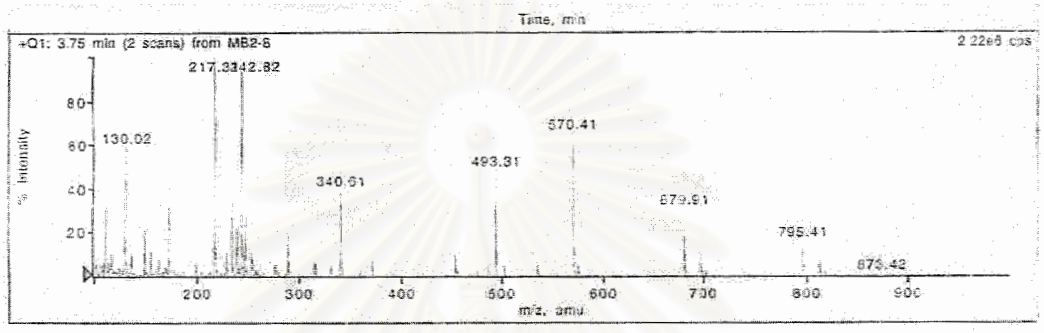


Figure 3.1 The HPLC chromatogram of **MB2** fraction

3.3 Result and Discussion

3.3.1 The Structural Elucidation of Peptide

Fraction **MB2-8** (1.5 mg) was isolated as a yellow amorphous solid and was found to be a mixture of three metabolites by the analysis of LC-ESIMS (Figure 3.2) also displayed the molecular peak in range of m/z 200-900.



e NMR spectra of the fraction were complex, and showed two type of signals, including many that could be matched up to peptide signal as discuss in detail below. However, there were also ^{1}H and ^{13}C NMR spectra that could not be assigned to peptide component. These signals were subsequently assigned to be heteroaromatic component of fraction MB2-8, as described in section 3.3.2 of this thesis.

Returning to the peptide components, ^{1}H NMR spectra (Figure 3.7) ran in CD_3OD indicated the presence of methine α protons of five different amino acids at δ_H 3.94, 3.90, 3.84, 3.65 and 4.31. When the sample ran in CD_3OH (Figure 3.8), five additional signals assigned to NH protons could be detected at δ_H 8.30,8.23, 8.20, 8.14 and 8.05. Nevertheless, the spectrum also revealed signals, each integrating for one proton, with unusual chemical shifts corresponding to aromatic protons at δ_H 7.75, 7.89 , indicative of heteroaromatic. The HMBC spectrum (Figure) suggested five $\underline{C}ONH_2$ bonds at 168.8, 170.5, 169.5, 171.0 and 171.1 ppm and aromatic carbons were found at 136.6, 131.5, 129.3 and 128.2 ppm. The amino acid residues was determined by the combination of ^{1}H NMR , TOCSY and COSY spectra which revealed the presence of phenylalanine (Phe), isoleucine (Ileu) and three different leucine (Leu1, Leu2 and Leu3) fragments.

The α proton at δ_H 4.31 (dd, J = 5.0, 4.4 Hz) was identified as the α proton of phenylalanine since it was coupled to the two β protons at δ_H 2.95 (dd, J= 14.0, 5.0 Hz) and 3.28 (dd, J = 14.0, 4.4 Hz). The NOESY spectrum also showed an NH proton at δ_H

8.20 correlated to the signal at δ_H 4.31, 2.95 and an aromatic proton at δ_H 7.20 (d, J = 8.4 Hz). This last signal was coupled to a proton at δ_H 7.26 (d, J = 7.5 Hz) and also coupled to the signal at δ_H 7.31 (dd, J = 7.5, 8.4 Hz).

The proton at δ_H 3.84 (dd, J = 4.2, 1.12 Hz) was defined as the α proton of isoleucine residue as a result of the intervening coupling of protons between αH (δ_H 3.84)/βHa (δ_H 1.93), βHb(δ_H 1.93)/γCH_3(δ_H1.02, 3H), γH(δ_H 1.25, 1.54) and γH (δ_H 1.25, 1.54)/δCH_3 (0.95) which were observed in the DQFCOSY spectrum. The correlations between the βH and the γ methyl and γ methylene protons suggested that the residue was isoleucine. Additionally the NH signal of this unit was confirmed by TOCSY which showed the linking from a NH signal (δ_H 8.05) to the signal at δ_H 3.84, 1.93, 1.25 1.02 and 0.95.

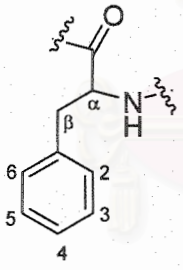
The first Leucine unit (Leu-1) had an α proton (αH3) at δ_H 3.94. The DQFCOSY revealed the cross peaks between αH (δ_H 3.94)/βHa,b (δ_H 1.75, 1.61), βHa,b/ γH(δ_H 1.86) and γH /δaCH_3 , δbCH_3 (δ_H 0.96, 0.97). The correlation between γ protons and both δ methyls suggested this unit was leucine. The TOCSY spectrum also showed the linking from an NH at δ_H 8.23 to α, β, γ and δ protons at δ_H 3.94, 1.86, 1.75 and 1.61 respectively.

The second Leucine unit (Leu-2) showed a similar coupling pattern to Leucine-1. Starting from an α proton at δ_H 3.90, the DQFCOSY showed the links between αH/βHa,b (δ_H1.71, 1.62). βHa,b/ γH(δ_H 1.85) and γH /δaCH_3 , δCH_3 (δ_H 0.96, 0.98). The NH proton was assigned by TOCSY which revealed an NH at δ_H 8.30 linked to the other proton within this Leucine unit.

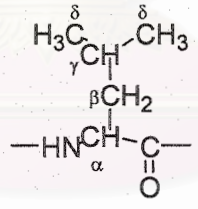
The remaining methine proton δ_H3.65 is assigned as the αH of Leucine-3 TOCSY showed the correlation between αH at δ_H3.65 and an unusual signal at δ_H 0.06, methine proton at δ_H 1.42 and two methyl signals at δ_H 0.73 and 0.69. Hence, the signals at δ_H 1.42 could be assigned as γH which was also coupled to the signal at δ_H 0.87. The signals at δ_H 0.73 and 0.69 were assigned to the δaCH_3, δbCH_3 respectively. Interestingly, the αH signal coupled to a single β proton at δ_H 0.06. while in the other leucine units in this peptide were observed the coupling between αH to both adjacent protons. However, an unusual signal also showed the geminal coupling to δ_H 0.87 (d, J= 14 .0Hz). TOCSY spectra also gave the same correlation as showing in DQFCOSY when the experiment ran

at 298 K°. After reduce the temperature from 298 to 278 K° the linking from NH signal at δ_H 8.14 to 0.06, 0.87, 1.42, 0.73 and 0.69 were observed in the tocsy spectrum. Thus, the unusual down field signals at δ_H 0.06 (1H, m, J = 4, 10, 14 Hz) and δ_H 0.87 (1H, m, J= 14 Hz) could be assigned as β proton of leucine-3. Both of them were attached to the same carbon at 44.9 ppm as indicated by HSQC spectrum.

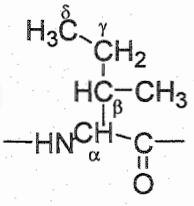
The attachment of protons with their corresponding carbon were well-assigned by HSQC, while quaternary carbons could be assigned by HMBC techniques. The amide carbon of each amino acid residues were assigned by the ^3J CH correlation from α protons to amide carbon. The amide carbons at 168.8, 169.5, 171.0 and 171.1 were assigned as amide carbon of phenylalanine, isoleucine, leucine-1 and 2, respectively. However, the unusual leucine, Leu-3 showed the correlations from β proton signals at δ_H 0.06 and 0.87 to an amide carbon at 170.5 ppm. ^{1}H and ^{13}C assignment of all amino acid residues in the same peptide are shown in Table 3.1. Now that the amino acid constituent had been identified, the connectivity of the amino acids needed to be deduced. This was done using data from the HMBC, DQFCOSY and NOESY spectra.



Phenylalanine

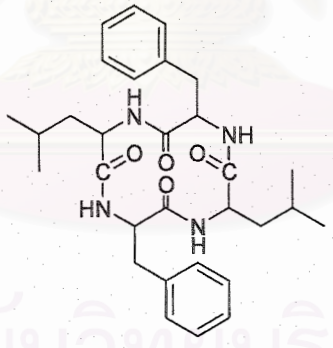


Leucine

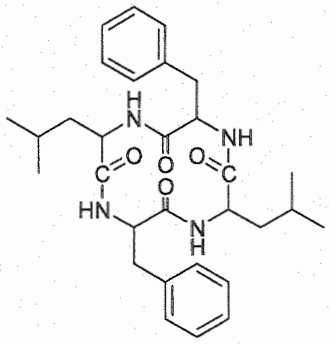


Isoleucine

In the TOCSY spectra, the α methine proton at δ_H 3.65 of leucine-3 correlates to the α methine proton of phenylalanine at δ_H 4.32, which must represent a five bond coupling. In the HMBC spectra a long- range interaction was observed between the carbonyl of leucine-3 at 170.5 ppm to the α methine proton of Phe at δ_H 4.31 and from the carbonyl of phenylalanine at 168.8 ppm to the α methine proton of leucine-3 at δ_H 3.65. The NOESY spectrum showed the coupling through space from the NH of leucine-3 to the α proton of phenylalanine. Also the higher shielded leucine methyl signal at δ_H 0.69 showed correlations to the 4- and 3/5-aromatic protons of the phenylalanine ring in the NOESY spectrum. Hence, leucine-3 is next to phenylalanine (Phe-Leu-3) which now explains the unusual upfield chemical shift at δ_H 0.06 of β proton of leucine, which is shielded by aromatic ring of phenylalanine. The NOESY data also suggested that one leucine-3 methyl is sitting over the benzene ring, while the other may be tilted back towards the alpha proton of its own chain. The LC–ESIMS was found m/z 261[(M+2H)/2]$^+$ and also found m/z 521 [M+H]$^+$ which corresponding with [Phe-Leu] and [Phe-Leu-Phe-Leu], respectively. Thus, one component of MB2-8 is cyclic peptide (Phenylalanyl-Leucyl-Phenylalanyl-Leucyl) (**3a**).



Cyclo(Phenylalanyl -Leucyl- Phenylalanyl-Leucyl) (**3a**)



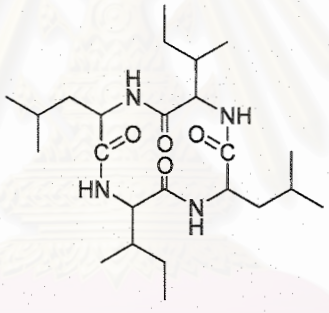
Cyclo(Phenyl-Leucyl-Phenyl-Leucyl) (**3a**)

Table 3.1a ^{1}H and ^{13}C NMR for peptide component of fraction MB2-8

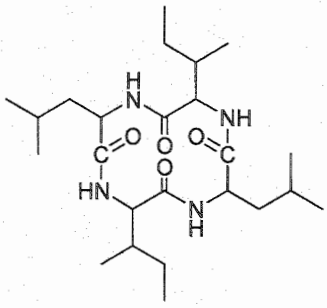
N0.	Residue	Position	$\delta^{1}H$ [a]	$\delta^{13}C$ [b]	DQFCOSY	HMBC ($^{n}J_{CH}$ = 8Hz)
1	Phenylalanine	α	4.31, dd (*J* = 5.0,4.4 Hz)	57.2 (d)	βH1a, b	βH1a, b
		β	2.95, m (a)	39.9 (t)	αH1, βH1b	αH1, H1(2/6)
			3.28, m (b)			
		1	-	136.6 (s)	δ2 CH_3	H1(3/5)
		2/6	7.20, d (*J* = 8.4 Hz)	131.5 (d)	H1(3/5)	H1(2/6), H1(4)
		3/5	7.31, t (*J* = 7.5, 8.4 Hz)	129.3 (d)	H1(2/6), H1(4)	H1(3/5), H1(4)
		4	7.26, t (*J* = 7.5 Hz)	128.2 (d)	H1(3/5)	H1(3/5)
		CO	-	168.8 (s)		βH1a, αH2
		NH	8.20, s		-	-
2	Leucine	α	3.65, ddd (*J* = 8.1, 4.4, 1.0 Hz)	53.8 (d)	βH2a, βH2b, αH1	αH2, βH2a, βH2b
		β	0.06, m (a)	44.9 (t)	αH2, βH2b	δH2a, δH2b
			0.87, m (b)		γH2, βH2a	
		γ	1.42, m	24.4(d)	βH2a, βH2b δH2a	δH2a, δH2b
					δH2b	
		δ(CH_3)	0.69, d (*J* = 6.6Hz) a	23.1 (q)	γH2	δH2b, βH2a, βH2b
		δ(CH_3)	0.73, d (*J* = 6.6 Hz) b	21.1 (q)	γH2	δH2b, βH2a, βH2b
		CO	-	170.5(s)	-	H2α, βH2a, βH2b, αH1
		NH	8.14, s	-	-	

[a] obtained in both CD_3OD and CD_3OH recorded on 750 MHz
[b] Assignments made from HSQC correlation and HMBC

For the next component, the TOCSY spectrum revealed the intercorrelation between the α methine proton at δ_H 3.94 of Leucine-1 to α methine proton of isoleucine at δ_H 3.84, which must represent a five bond coupling ($^5J_{C\text{-}H}$ = 1.1 Hz). Additionally, the NOESY spectrum showed the coupling through space between α proton of isoleucine at δ_H 3.84 to NH proton at δ_H 8.23 of leucine-1. The intensities of the NH proton signal in CD_3OH of leucine-1and isoleucine were of similar size and indicated that both residue could be part of the same peptide. Hence, leucine was placed next to isoleucine. The Leu-Ileu fragment corresponded to m/z 226 $[(M+2H)/2]^+$ and m/z 452 $[M+H]^+$ which were observed in LC ESIMS. Thus, cyclo (Leucyl-Isoleucyl-Leucyl-Isoleucyl) (**3b**) was another component of MB2-8.



cyclo (Leucyl-Ileucyl-Leucyl-Ileucyl) (**3b**)



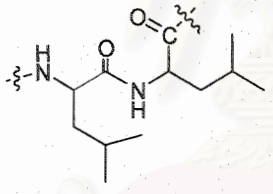
cyclo (Leucyl-Ileucyl-Leucyl-Ileucyl) (**3b**)

Table 3.1a ^{1}H and ^{13}C NMR for peptide component of fraction MB2-8

N0.	Residue	Position	$\delta^{1}H$ [a]	$\delta^{13}C$ [b]	DQFCOSY [a]	HMBC [b] ($^{n}J_{CH}$ = 8Hz)
1	Isoleucine	α	3.84, dd (J = 4.1,1.1 Hz)	60.7 (d)	βH1, αH2, NH2	γH1(CH_3)
		β	1.93, m	40.3 (d)	γH1a, γH1b, γH1(CH_3), αH1	αH1, γH1(CH_3), δ1(CH_3)
		γ	1.25, m (a) 1.54, m (b)	25.6 (t)	βH1, γH1a, δ (CH_3) βH1, γH1b,	αH1,γH1(CH_3), δ1(CH_3)
		γ (CH_3)	1.02, d (J = 7.1 Hz)	15.4 (q)	βH1	-
		δ (CH_3)	0.95, d (J = 7.6 Hz)	11.9 (q)	γH1a,γH1b	
		CO	-	169.5	-	αH2
		NH	8.05, s	-	-	-
2	Leucine-1	α	3.94, d (J = 4.2 Hz)	54.0 (d)	βH2a, βH2b, αH1	αH2, βH2a, βH2b
		β	1.61, m (a) 1.75, m (b)	44.5 (t)	αH2, βH2b, γH2 αH2, βH2a, γH2	γH2, δH2a, δH2b
		γ	1.86, m	25.0 (t)	βH2a, βH2b, δH2a, δH2b	βH2a, βH2b, δH2b
		δ_a(CH_3)	0.96, d (J = 6.8 Hz)	21.6 (q)	γH2	βH2a, βH2b, δH2b
		δ_b(CH_3)	0.97, d (J = 6.9 Hz)	23.4 (q)	γH2	βH2a, βH2b, δH2a
		CO	-	171.8	–	αH2, βH2a,b
		NH	8.23, s	-	αH1	

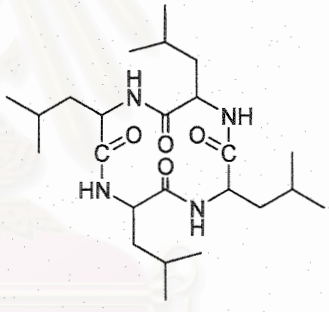
[a] obtained in both CD_3OD and CD_3OH recorded on 750 MHz
[b] Assignments made from HSQC correlation and HMBC

The last unit, leucine -2 fragment have no correlation to link peptide as shown in **3a** and **3b**. Both compounds revealed 5 bond coupling from the α proton to the α proton of the next amino acid in TOCSY and NOESY spectra including, 5 bond coupling from the α proton to carbonyl carbon in HMBC. However, the intensities of the α proton signal of leucine-2 was of similar size as the others leucine units. Hence, 2 units of leucine-2 were placed next to each others because ^{1}H NMR showed only one set of ^{1}H NMR. The LC-ESIMS spectra display numerous fragments which was containing leucine residue, the fragment at m/z 113 was one leucine unit, and the fragment at m/z 226 (2xLeu), 452 (4xleu), 678 (6xLeu) and 904 (8XLeu) were observed. Among them, the fragment m/z 226, 452 were corresponding with [leucine-leucine] and [leucine-leucine]$_2$. Thus, this compound was proposed as *cyclo* [Leucyl-Leucyl]$_2$. The structure was evident by the one set of NMR suggested it was cyclic peptide of the same amino acid unit.



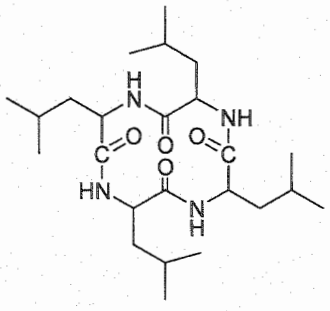
[Leu-Leu]

m/z 226



[Leu-Leu]$_2$

m/z 452



cyclo[Leucyl-Leucyl]$_2$ (**3c**)

Table 3.3a ^{1}H and ^{13}C NMR for peptide component of fraction MB2-8

N0.	Residue	Position	δ^1H [a]	δ^{13}C [b]	DQFCOSY [a]	HMBC ($^nJ_{CH}$ = 8Hz)
1	Leucine-2	α	3.90, dd, (J = 9.1, 4.7)	54.4(d)	βH, βHb	αH, βHa, βHb
		β	1.71, m (a)	45.6 (t)	αH, βHb, γH	γH, δHa, δHb
			1.62, m (b)		αH, βHa, γH	
		γ	1.85, m	25.0(d)	βHa, βHb	βHa, βHb,
					δHa, δHb	δHb (CH_3)
		δa (CH_3)	0.96, d (J= 6.5 Hz)	21.6 (q)	γH	βHa, βHb, δHb
		δb (CH_3)	0.98, d (J= 7.2 Hz)	23.3 (q)	γH	δHa , βHb, δHb
		CO	-	171.1 (s)	-	αH, βHa, βHb
		NH	8.30, s	-	-	-

[a] obtained in both CD_3OD and CD_3OH recorded on 750 MHz
[b] Assignments made from HSQC correlation and HMBC

Thus, MB2-8 fraction was shown to consist of three cyclic tetrapeptides namely, cyclo [phenyl-leucyl]$_2$ (**3a**), cyclo[Leucyl-Ileucyl]$_2$ (**3b**) and cyclo[Leucyl-Leucyl]$_2$ (**3c**). All of peptide are new natural occurring cyclic tetrapeptide from marine bacterium. The limitation for the elucidation their structure were the purity and the quantity, the small amount of MB2-8 was a mixture. Accordingly, MB2-8 need for separation and purification of peptide by HPLC. The pure peptide were subjected to the LC-ESIMS and HRESIMS to confirm their molecular weight. To syntheses all peptide and compare with those spectrum to confirm the structural identity.

3.3.2 The Structure Elucidation of Heterocyclic Units

In the earlier section of this chapter, it was deduced that fraction MB2-8 contained three cyclic peptides. One of the features of the NMR spectra that complicated analysis of this fraction was that there were some additional NMR signals that clearly did not belong to the three cyclic peptide compounds. These signals were two downfield singlets, each integrating for ^1H, at δ_H 7.89 and 7.75 accompanied by two singlet methyls at δ_H 2.52 and 2.55. The HSQC spectrum revealed that these four different proton signals were linked to carbon at 127.4, 129.4, 19.9 and 20.5 ppm. The NOESY spectra also showed the coupling through space between δ_H 2.55 and 7.75, and between δ_H 2.51 and 7.86. The HMBC spectra revealed the correlation between aromatic proton at δ_H 7.75 signals at to 140.7, 141.3 and 20.5, while the aromatic proton at δ_H 7.89 correlated to 144.3, 146.9 and 19.9 ppm. The chemical shift values indicated that all these extra signals belonged to hetero aromatic units. Additionals correlations were between the methyl proton at δ_H 2.55 and 127.4, 141.3 and 146.9 ppm and methyl proton δ_H 2.52 and the carbon at 129.4, 141.3 and 146.9 ppm. This evidence indicated that there were two individual aromatic units. However, the methyl protons at δ_H 2.55, 2.52 correlated to the same carbons at 141.3 and 146.9 ppm. Conversely, neither methyl proton correlated to the carbons at 140.7 and 144.3 ppm while the aromatic protons correlated to different carbons (δ_H 7.75 to 140.65 and 141.27 ppm) and (δ_H 7.89 to 144.31 and 146.90 ppm).

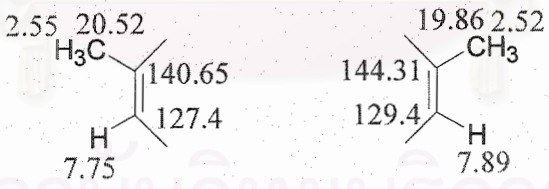
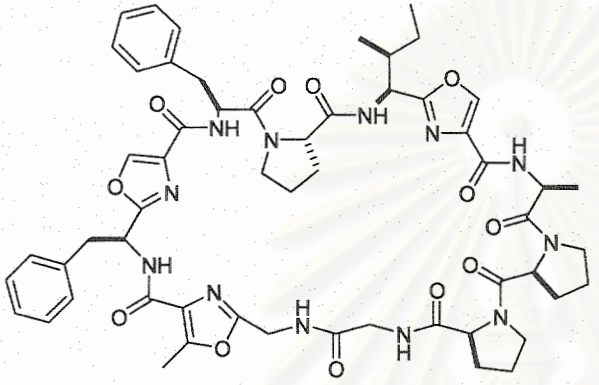


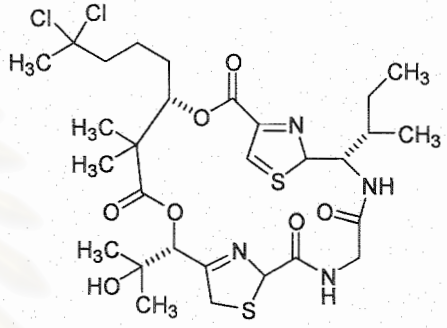
Figure 3.2 The carbon and proton assignment for heteroaromatic unit

Figure 3.3 The HMBC and NOESY correlations for heteroaromatic unit

The natural product of purely amino acid are rare from marine organism. The oxazoles and thiazole moieties usually found in the peptide chain. The heterocyclization of cysteine residues to form thiazole while serine and threonine counterparts to form oxazole. A wide range of marine derived peptide contain oxazole ring example, wewakazole,[50] lyngbyabellin A.[51] which have been isolated from cyanobacteria. Cyanobacteria are best recognized for its production of peptide.

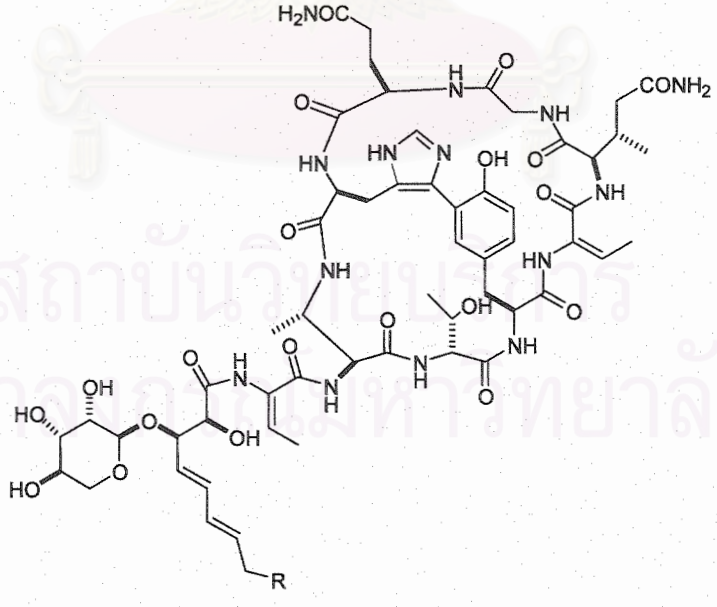


wewakazole (**43**)

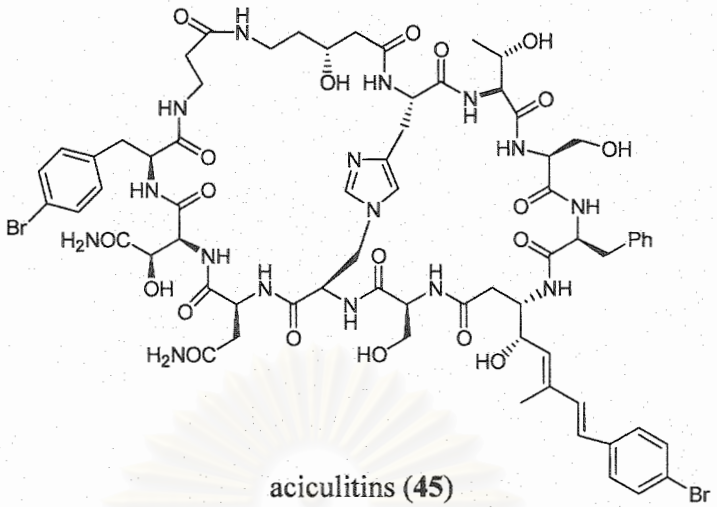


lyngbyabellin (**44**)

Additionally, an attractive feature of the peptides from lithistid sponges in the presence of histidine that joined two chain end of peptide in theonellamide F[52] and aciculitins.[53]



theonellamide F (**45**)



aciculitins (**45**)

However, the HMBC correlation of these heteroaromatic unit of MB2-8 seem like the correlation that usually observed in oxazole, thiazole or imidazole units. Accordingly, the chemical shift of this unit were compared with literature values of some heteroaromatic, oxazole, thiazole or imidazoles.[54,55]

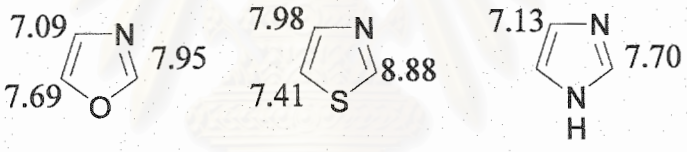


Figure 3.4 The proton chemical shifts of oxazole, thiazole and imidazole[54,55]

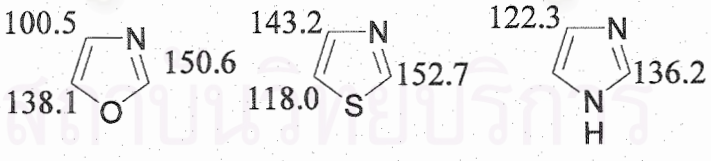


Figure 3.5 The carbon chemical shifts of oxazole, thiazole and imidazole[54,55]

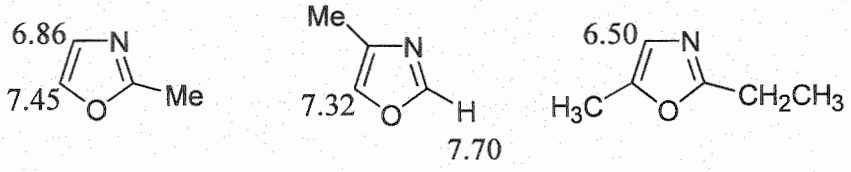
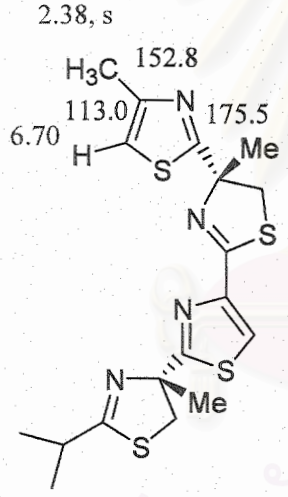
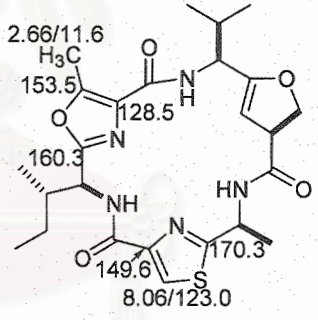


Figure 3.6 The carbon chemical shift of methyl oxazole[56,57]

When compare with chemical shift values in literature,[58] the proton chemical shift values seem to fit with thiazole unit but the carbon chemical shifts are different. In all the examples above, the methyl carbon signals resonated at the range between 11-13 ppm for oxazoles, whereas, in the compound from MB2-8, the carbon chemical shift value for the methyl is close to 20.0 ppm. The ^{13}C chemical shift of O=C-X (X=heteroatom) occurs at 150-160 ppm for oxazoles and 160-170 ppm for thiazoles. While in compound MB2-8, signal were observed in the range 144-150 ppm. However, that was a comparison with the isolated system of heteroaromatic. In fact, a heteroaromatic unit incorporated into peptide structure usually demonstrates the wide range of the unusual chemical shift values which caused by the influence of the other residues within its chain. For example, dihydromirabazole (**47**),[57] dolastatin(**48**)[58] were complicated compound containing thiazoles and oxazoles unit.



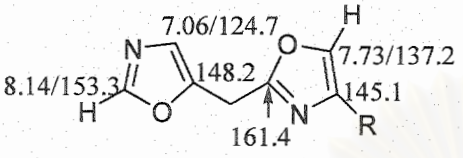
dihydromirabazole (**47**)



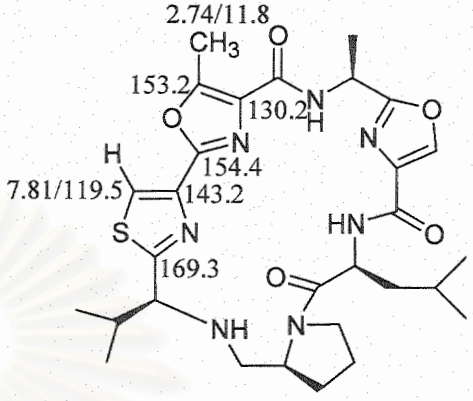
dolastatin 1 (**48**)

The HMBC experiment is a practical method for assigning the peptide chain. Spectral data for peptides containing oxazole and methyl oxazole residues have often noted a lack of HMBC correlation between the carbonyl equivalent and the α proton of adjacent amino acid. This non peptidic component of MB2-8 also have no HMBC connectivity. Noticeably, the compound might be contained two similar heteroaromatic units each possessing methyl moiety and both of them were placed next to each other. For instance in, bengazole (**49**),[58] leucamide (**50**),[59] muscoride (**51**)[60] were all alkaloids containing two heteroaromatic units. In all of these, the carbon carbonyl equivalent

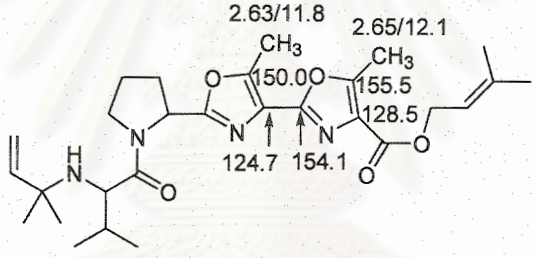
resonated in the range of 150-170 ppm while in the MB2-8 component the signals were found in the range of 140-149 ppm.



bengazole(**49**)



leucamide (**50**)



muscoride (**51**)

From the above mentioned, information, it was concluded that the heteroaromatic component do not involved oxazole thiazole or imidazole residues. The quaternary carbons in the range of 140-149 ppm suggest the compound must contain substituted aromatic or ring fused with heteroaromatic ring. Based on the NMR information the carbon and proton signal were certainly assigned for two unit of heteroaromatics. The four carbon at 20.5, 127.4 and 140.7 are in the first unit and 19.9, 129.4 and 144.3 are in the second unit. In HMBC spectra, both methyl aromatic signals at 19.9 and 20.5 ppm correlated to the same two quaternary carbon at 141.3 and 146.9 ppm which were assigned **a** and **b**. The aromatic proton also showed correlations to these same carbon a and b. Hence a possible structure(), in which the carbon assigned as **a** and **b** appear as ring fused aromatic carbon. The example of ring fused heteroaromtic unit and their NMR data are shown in the (figure)

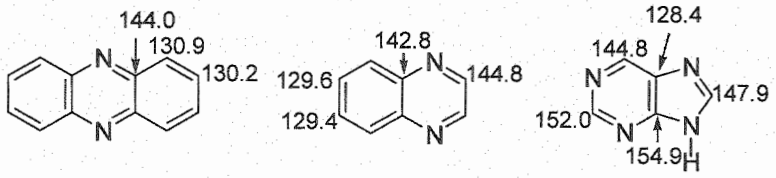


Figure 3.7 The ^{13}C chemical shift of some condense heteroaromatic RingsRef

X = heteroatom

Figure 3.8 The possible structure of heteroaromatic unit

As a result of NMR data, suggested the possible structure as figure The appearance of carbon chemical shift from HSQC and HMBC experiment were good agree with two five membered ring. when X atom was nitrogen atom which was pyrrolic ring fused which can not be found in the nature and the proton chemical shift of pyrrolic ring resonance in the range 6.8-7 while heteroaromatic component resonance at 7.5-7.9. Additionally, the pyrrolic ring fuse have never been found in the nature thus no NMR information for the comparison with these unit and the biosynthesis possibility. Notably ^1H NMR spectra suggested hetero aromatic units were not stable fragment for the reason that the decreasing of integration values of methyl protons (δ_H2.55 and 2.52). While the peptide are more stable indicated by the invariable integration of α proton signal. The problem of the identification these unit were the limitation of sample quantity and no information about their molecular weight. Consequently, to confirm structure of heteroaromatic unit, MB2-8 by the separation with analytical HPLC and then collected the trace amount for HRESIMS which was the useful method for elucidated theirs structure at the moment.

3.4 Conclusion

The fraction MB2-8 were successively separated by step gradient HPLC, starting from 50% MeOH in water to 100% MeOH. This fraction identified as mixture of threes cyclic tetrapeptides, Cyclo (Phenylalanyl-Leucyl-Phenylalanyl-Leucyl) (**3a**) Cyclo (Phenylalanyl-Leucyl-Phenylalanyl-Isoleucyl) (**3b**) Cyclo(Leucyl-Leucyl-Leucyl-Leucyl) (**3c**) and an unidentified heteroaromatic components. The strategy for investigation of MB2-8, the sample was first investigated by NMR experiments, using CD_3OD (d_4) as solvent spectra ran on a DMX 400 NMR instrument gave a good ^{1}H-NMR spectra but there was insufficient sensitivity for acquisition of ^{13}C NMR. Subsequently, the sample was ran using DRX 500 NMR spectrometer, and HMBC and HSQC spectra were observed. The sample was transfered to shigemi tube and ran on DMX 500 and 750, respectively that gave some more detailed structure information. The peptide structure was appeared after the sample was ran in CD_3OH (d_3) since these techniques allowed the presence of amide protons to be observed. TOCSY and DQFCOSY were useful methods for elucidating amino acid residue. Detailed interpretation of HMBC and TOCSY experiment revealed the intercorrelations between amide carbonyl and the proton of adjacent amino acid units. As a result of the limitation of the structure elucidation were small quantity of sample, the complexity of the sample and no mass spectral data exceptionally for high resolution mass spectroscopy. Thus, MB2-8 need to be separated into the individual peptide components, Molecular weight information on each individual peptide could then be obtained by high resolution electron impact (EI) or electrospray ionization (ESI) spectra. Additionally, the structure elucidation of the heteroaromatic component can be completed by additional quantities of MB2-8 are made available through large scale culture of the bacterial strain. A second approach to this structure elucidation problem would be to explore synthesis of the individual peptide components, so as to compare their NMR characteristics with those of the peptide mixture present in fraction MB2-8. synthesis of the peptide would also allow detailed biological evolution of the peptides.

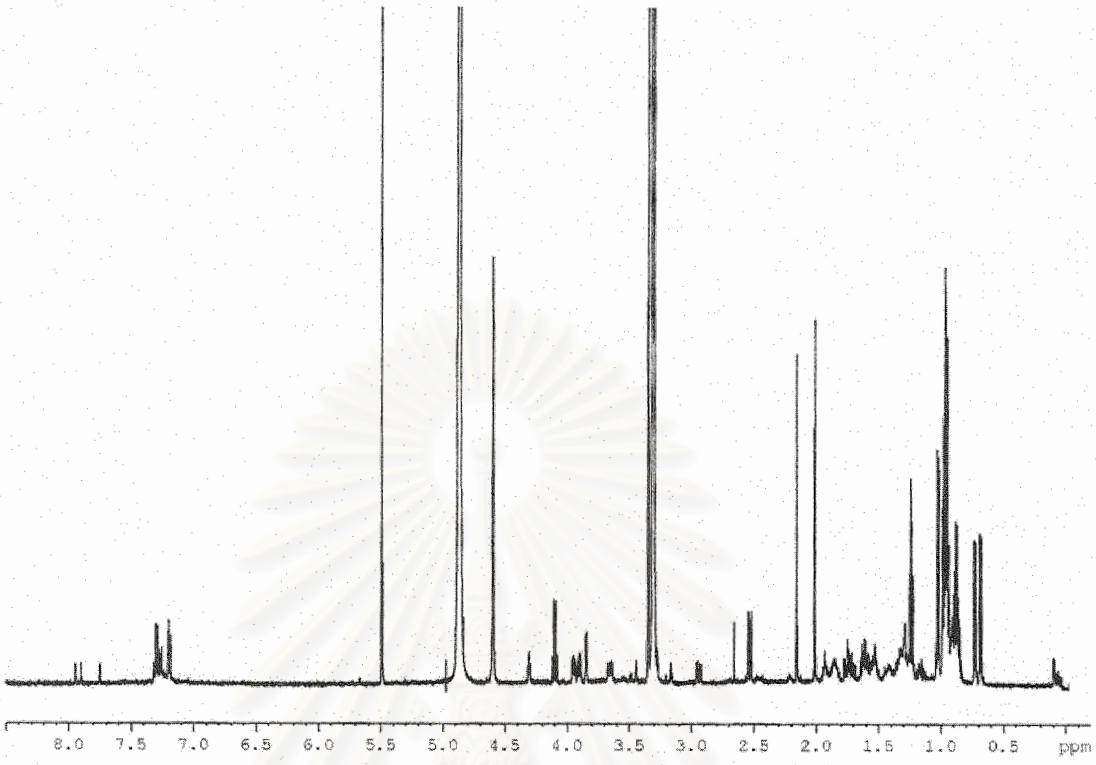


Figure 3.9 The ^{1}H NMR spectra recored on 500 NMR spectrometer

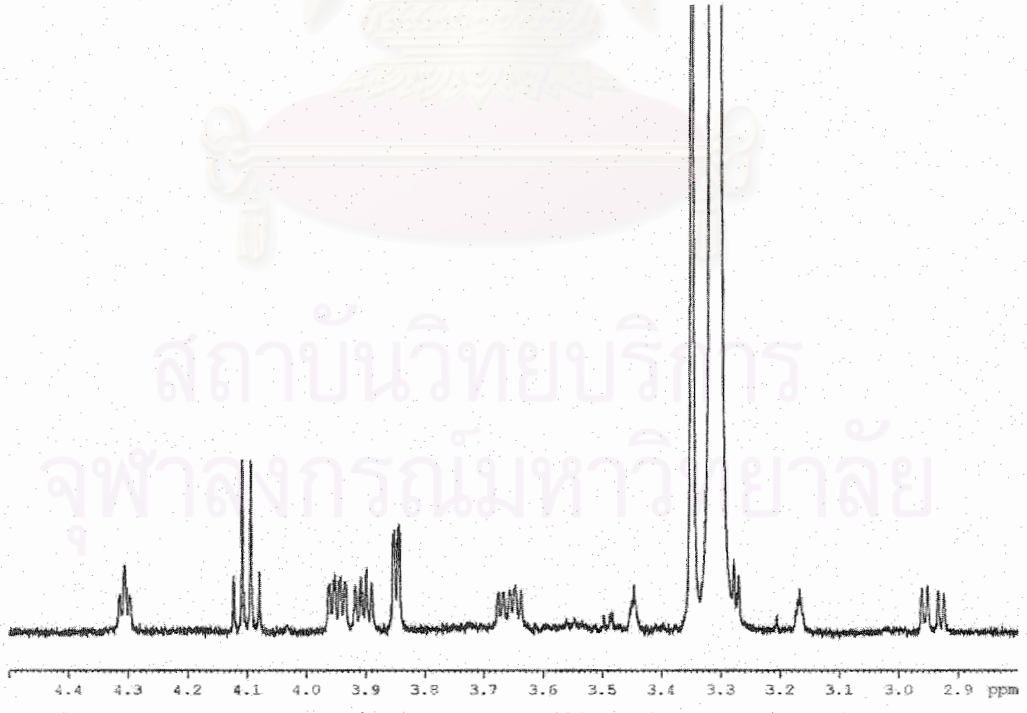


Figure 3.10 The ^{1}H NMR spectra recored on 500 NMR spectrometer

(α proton of amino acid)

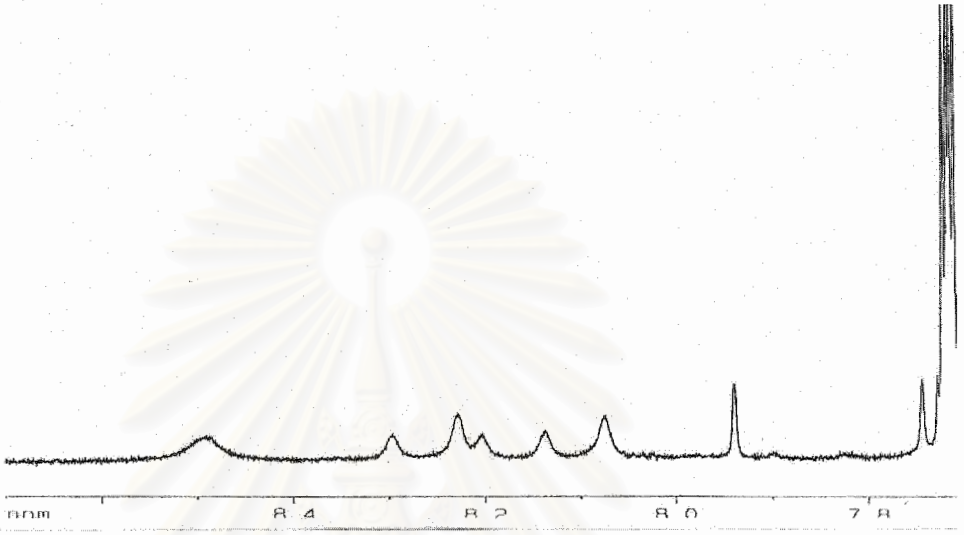


Figure 3.11 The ^{1}H NMR spectra in CD_3OH (NH proton of amino acid)

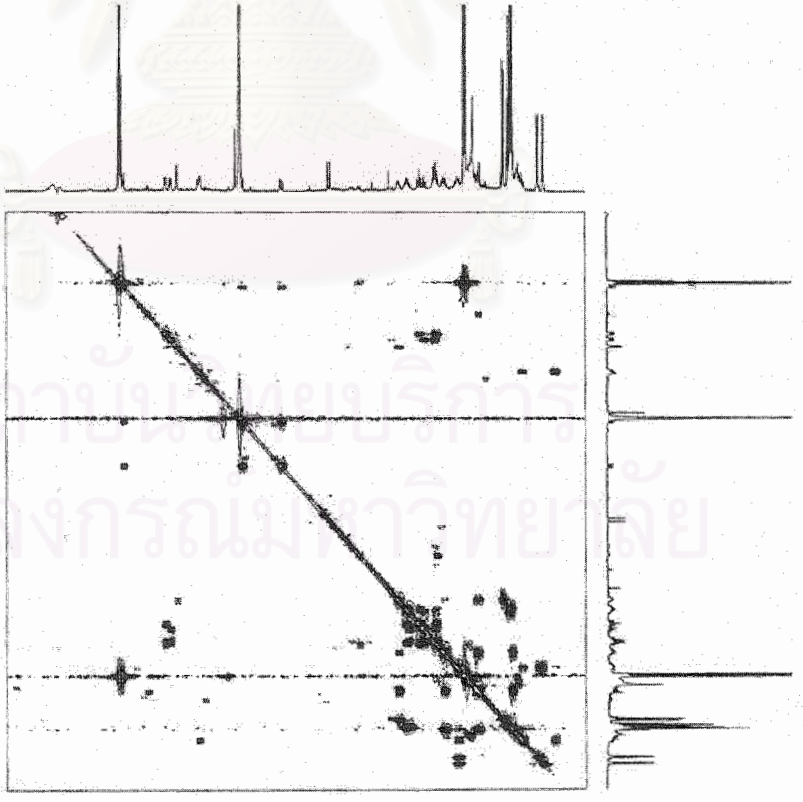


Figure 3.12 The DQFCOSY spectrum of MB2-8

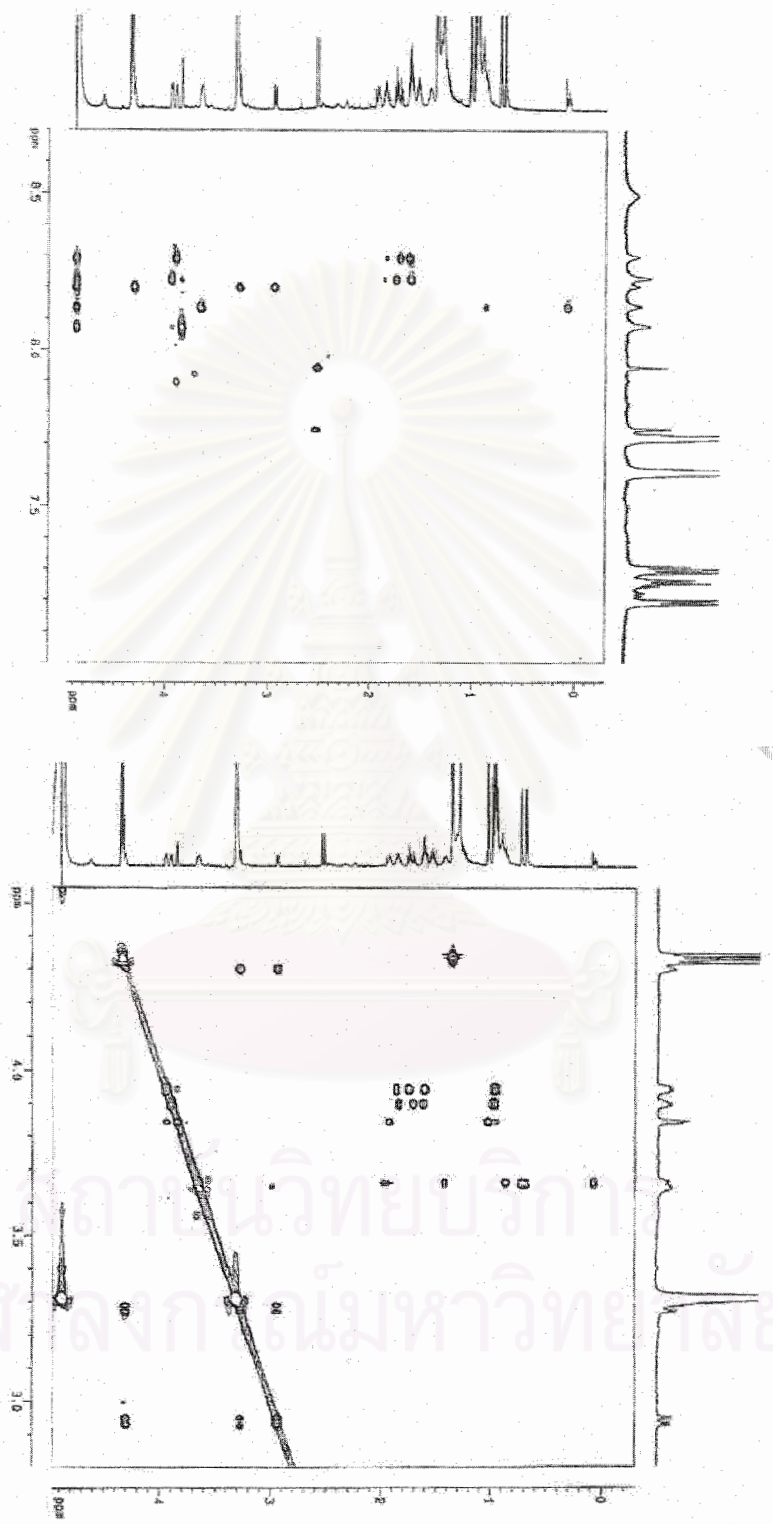


Figure 3.13 TOCSY spectra of MB2-8

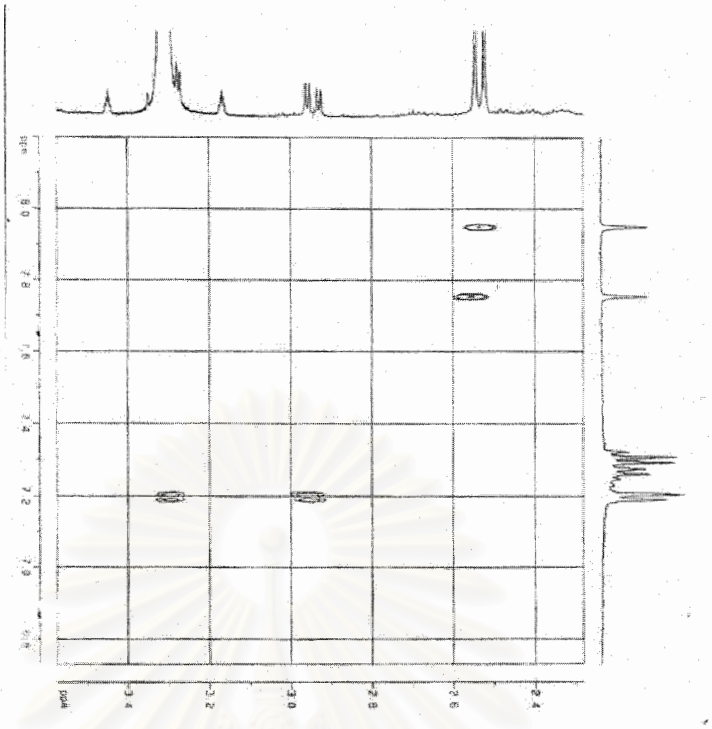


Figure 3.14 The NOESY spectra of MB2-8

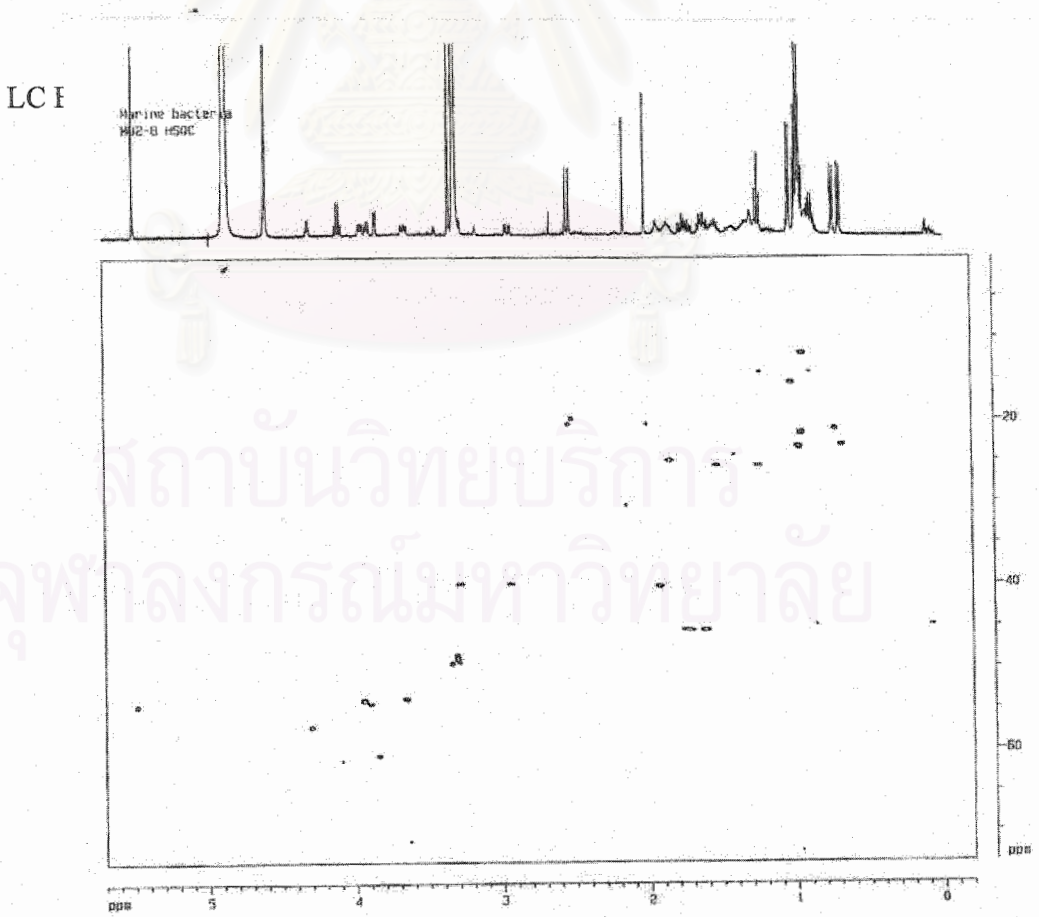


Figure 3.15 HSQC spectrum of MB2-8

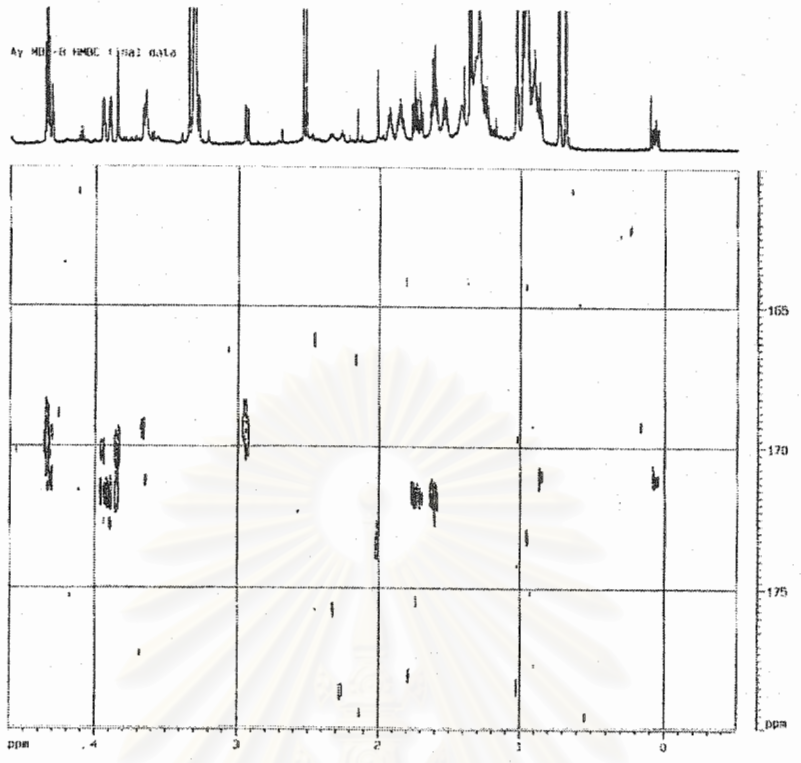


Figure 3.17 The HMBC spectrum of MB2-8

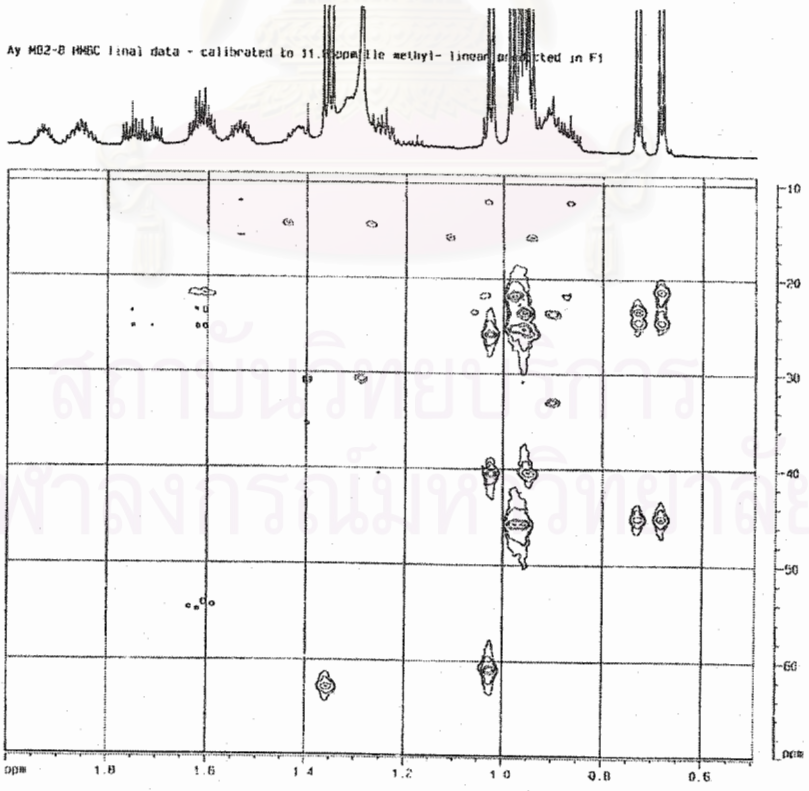


Figure 3.18 The HMBC spectrum of MB2-8 (up field)

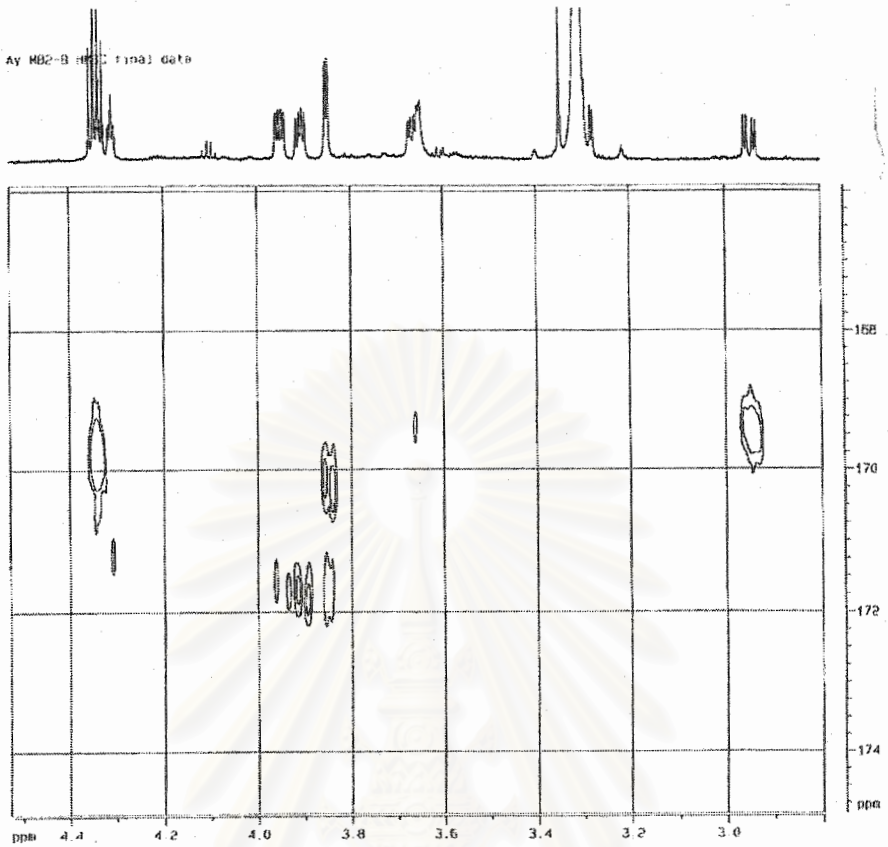


Figure 3.19 The HMBC spectrum of MB2-8 (down field)

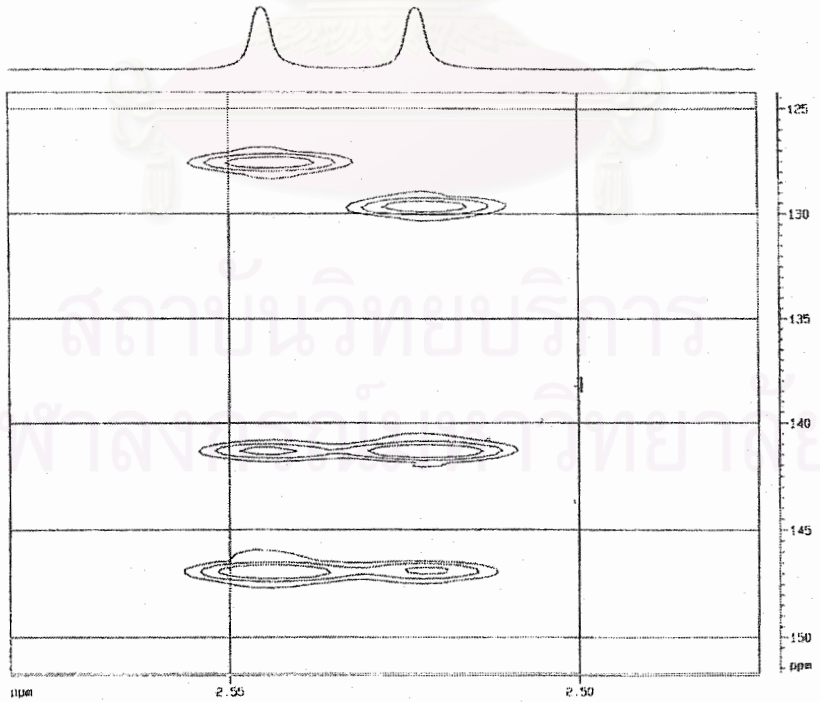
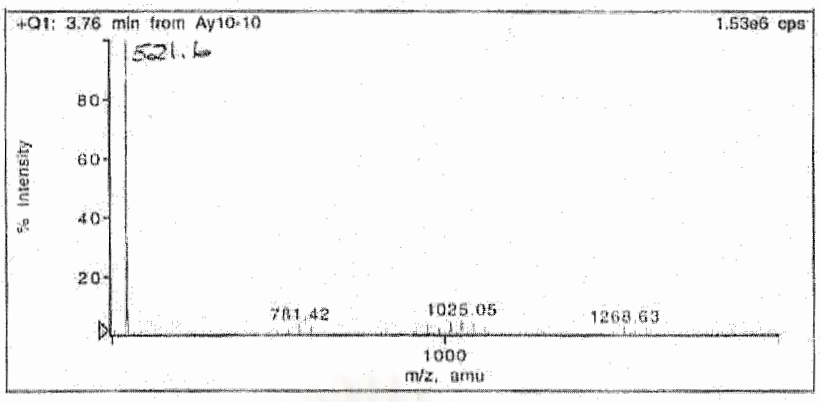
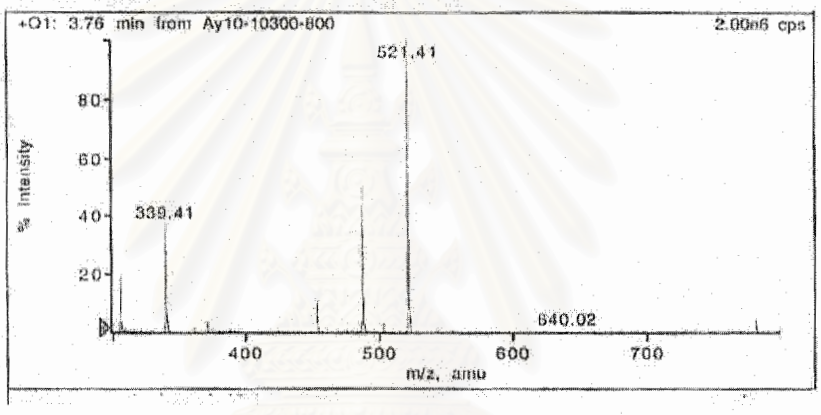


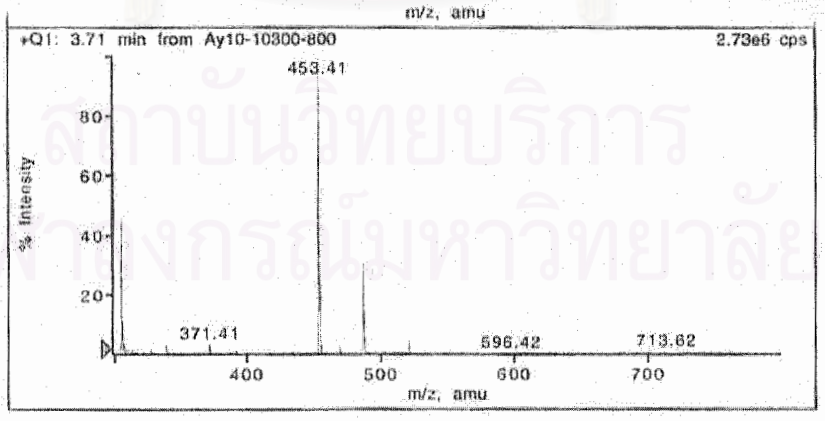
Figure 3.20 The HMBC spectra of MB2-8 (heteroaromatic unit)



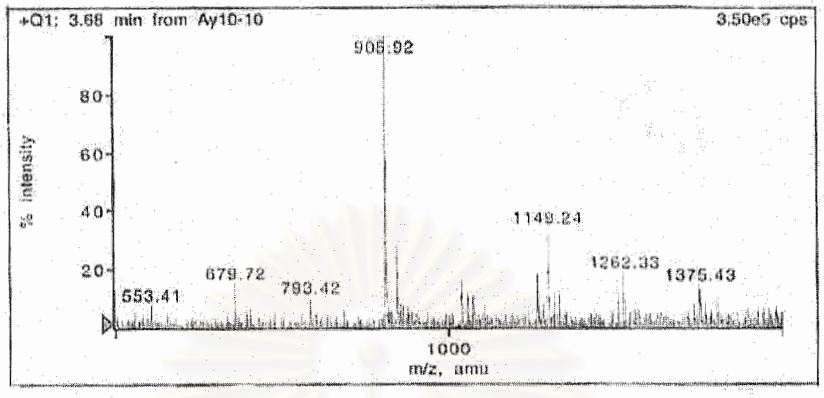
LC ESIMS spectrum



LC ESIMS spectrum



LC ESIMS spectrum



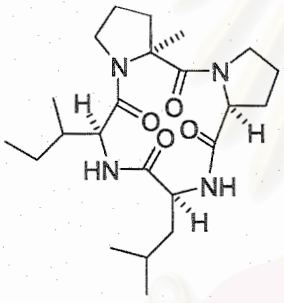
LC ESIMS spectrum

CHAPTER IV

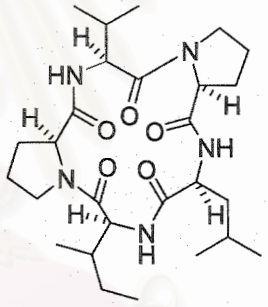
Part II : Cyclic Tetrapeptides from a Marine Bacterium Associated with a Tropical Thai Sponge.

4.1 Introduction

As mentioned in chapter III, cyclic peptides have been isolated from a range of marine organism, including sponges, tunicates and their associated marine bacteria. Fenestin A and B (**53**, **54**) represent cyclic tetra- and pentapeptides isolated from Fijian sponge *Leucophloeus fenestra*.[63]

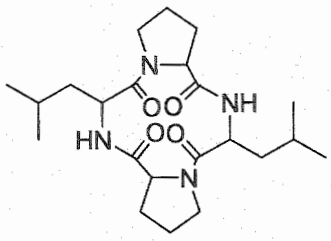


fenestin A (**53**)

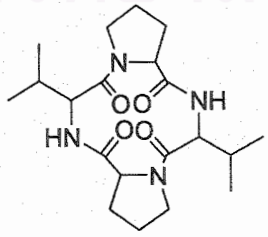


fenestin B (**54**)

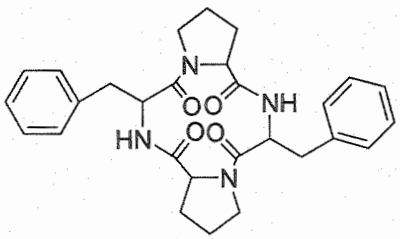
In 1993, Francisco *et al*. reported the isolation and partial characterization of three cyclic tetrapeptides (**55-57**) from the New Caledonian tunicates *Cystdytes delle chiajei*.[48]



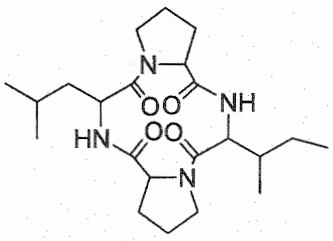
(**55**)



(**56**)

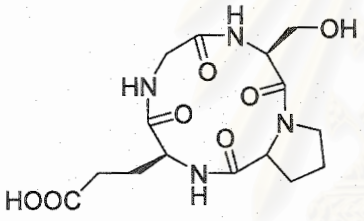


(57)

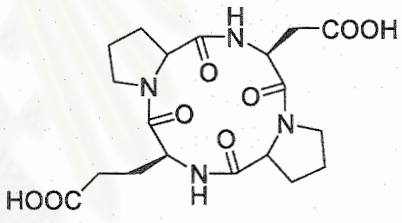


(58)

More recently, Shin *et al.* described the cyclic peptide (L-isoleucyl-L-prolyl-L-leucyl-L-prolyl) (**58**) from actinomycete *Nocardiopsis* sp. isolated from a Pacific deep sea sediment.[47] The cyclic peptides cyclo-(glycyl-L-seryl-L-prolyl-L-glutamyl) (**59**) and cyclo-(glycyl-L-glutamyl -L-prolyl-L-glutamyl) (**60**) have been reported from a Ruegeria strain of bacteria associated with the sponge *Suberites domuncula*.[64]



(59)



(60)

All of these medium ring peptides are characterized by the presence of proline, which may facilitate cyclisation of the linear peptide to cyclic product. A second interesting feature of these cyclic peptides is that some of them (*eg* **55-57**) are symmetric in structure (ABAB), while others contain an asymmetric arrangement (AABB) of amino acid building blocks, for example in fenestin A (**53**). The previous chapter in this dissertation explored the leucine-containing cyclic peptides from a Thai marine bacterial culture. The second fraction (MB2-4) of the isolation also contained cyclic peptide components, and a discussion of their isolation and structural elucidation forms the basis of this chapter.

4.2 Experimental

All experimental procedures are as described in chapter III, but an additional experiment was amino acid analysis by Marfey's method.

4.2.1 Equipments

The products from amino acid analysis were carried out on HPLC Water™ model 662 model 486MS, Altech ™ Econosil C18, variable-wavelength UV detector at λ 340 nm and model 600s controller.

4.2.2 Chemicals

(FDAA, Marfey's reagent) 1-Fluoro-2,4-dinitrophenyl-5-L-alaninamide, authentic L-, D-proline, L-,D leucine and L-,D-phenylalanine.

4.2.3 NMR experiment

All 1D and 2D NMR spectra were recorded on Bruker DRX 500 MHz and DMX 750 MHz spectrometers, the sample was placed in shigami NMR tube and NMR data sets were collected in CD_3OD.

4.2.4 Amino Acid Analysis

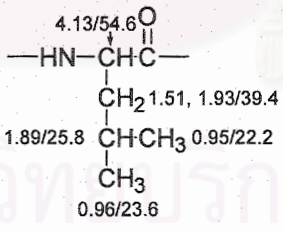
Amino acid composition of MB2-4 was accomplished following the general guide line of Marfey's analysis.[69] MB2-4 (300 μg) was hydrolyzed with 0.5 mL of 6 N HCl placed in sealed ampoule at 105°C for 12 hours. Trace HCl was removed under N_2 steam. The resulting of hydrolysate was resuspended in 50 μL of 0.1% of 1-fluoro-2,4-dinitrophenyl-5-L-alaninamide (FDAA, Marfey's reagent) solution in acetone and 100 μL of 0.1 N $NaHCO_3$ were added. The reaction mixture was heated at 80°C for 3 min. The solution was cooled to room temperature, neutralized with 50 μL of 0.2 N HCl and diluted with 100 μL of MeCN:H_2O:TFA(50:50:0.05). This solution was analyzed by reversed phase HPLC with isocratic solvent system (Altech ™ Econosil C18; solvent 40% MeCN + 0.05% TFA,) in 20 min at 1.0 mL/min (UV detector at λ 340 nm) The retention times of authentic L-, D-proline were 5.56 and 6.06 min, L-, D-leucine were 18.7 and 28.6 min, and L-,D-phenylalanine were 20.0 and 32.9 min. The retention times of hydrolysate products at 5.54, 18.7 and 20.0 min, respectively suggested the presence of L-proline, leucine and phenylalanine.

4.3 The Results and Discussion

4.3.1 Structural Elucidation of MB2-4

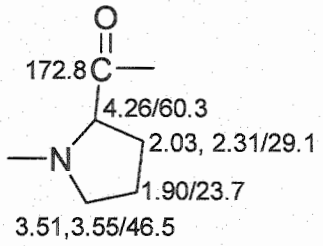
The fraction MB2-4 fraction from HPLC (1.5 mg) was isolated as a pale yellow solid. The ^{1}H NMR spectrum (Figure 4.5) clearly suggested that the fraction contained peptides, since the signals for α proton of amino acids were visible at δ_H 4.07, 4.13, 4.21, 4.26, and 4.45. Additionally, the ^{13}C NMR spectral data (Figure 3.6) showed corresponding six carbonyl signals at 172.8, 171.3, 170.8, 168.9, 167.4 and 166.8 ppm, and suggested six amino acid residues were contained in the peptide mixture. However, the LC-ESIMS data indicated that this fraction was a mixture of three compounds which had the molecular peak $[M+H]^+$ at m/z 489, 455 and 421, respectively. Each amino acid component was then identified by extensive 1D and 2D NMR experiments.

The structural elucidation of the peptide of MB2-4 started with methine proton at δ_H 4.13 (t, *J*=5.0 Hz) linked to adjacent protons by ^{1}H-^{1}H COSY. Cross peak between α proton at δ_H 4.13 and methylene β protons at δ_H 1.51 and 1.93 were observed. Both methylene protons were also correlated to a methine γ proton at δ_H 1.89 as were two doublet methyl groups at δ_H 0.95 and 0.96. All the above proton assignment suggested that this amino acid unit was a leucine unit. Following from these proton assignments, attached carbon chemical shifts were determined using an HSQC experiment. The methine carbon at 54.6 ppm was identified as α carbon and the methylene carbon at 39.4 ppm was assigned to be the β carbon, with two methyl carbons at 23.6 and 22.2 ppm.



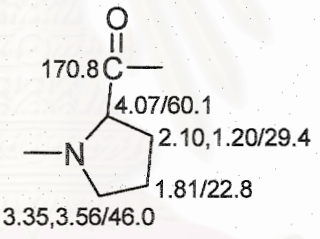
Leucine

The second amino acid residue was identified as proline (ProA). A methine α proton at δ_H 4.26 (t, *J*= 7.5 Hz) showed ^{1}H-^{1}H COSY correlations to two β protons (δ_H 2.31, 2.03), and then each β proton showed correlations to the γ proton (δ_H 1.90, 2.03), which were further coupled to δ_H protons 3.51 and 3.55. The ^{13}C NMR spectra revealed the presence of methine α carbon at 60.3ppm, and three methylene carbons at 29.1, 23.7 and 46.5 ppm were assigned as β, γ and δ carbons, respectively.



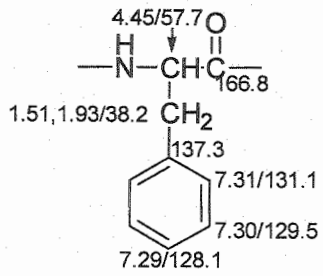
proline A

The signal at 4.07 (t, *J*=5.33 Hz) correlated to methylene β proton at 2.10 and 1.20 which were then correlated to methylene γ proton in the ^{1}H-^{1}H COSY (Figure 4.9). Both the signals of β and δ protons also correlated to methylene γ proton at 1.81(2H). All above proton signals suggested a second proline unit (ProB). The ^{13}C NMR, Dept 135 and HSQC spectra (Figure) allowed the identification of the carbon signals of this proline residue as methine α carbon at 60.1 ppm with the methylene carbons of β, γ and δ position resonating at 29.4, 22.8 and 46.0 ppm, respectively.



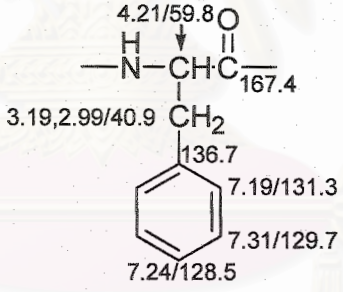
proline B

The next amino acid that was identified as a phenylalanine residue. The up field methine proton at 4.45 coupled to β methylene proton at 1.51 and 1.93 which correlated to aromatic carbons at 131.1 (C-2) and 137.3 (C-1) ppm indicated by HMBC. The ^{1}H NMR spectrum revealed the presence of overlapping aromatic signal but clearly separated from data set of phenylalanine I. The carbons and attached protons were assigned by HSQC, the aromatic protons at 7.31 (H-2), 7.30 (H-3) and 7.29 (H-4) were corresponding with the carbons at 131.1 (C-2), 129.5 (C-3) and 128.1 (C-4) ppm. The assignment of amide was confirmed by the HMBC cross peak between the signal at 166.8 ppm to α and β protons. Thus, this unit could be assigned as phenylalanine A (PheA).



phenylalanine A

Additional signals in DQFCOSY spectrum, the chemical shift at 4.21 (t, *J*= 4.8 Hz) was assigned as α proton of phenylalanine (Phe B) coupled to the double doublet which could be assigned to the β proton at 3.19 (dd, *J* = 4.8, 13.8 Hz) and 2.99 (dd, *J*= 4.8, 13.8 Hz) by in ^{1}H-^{1}H COSY. In the HMBC spectrum, both β protons displayed long range correlations to aromatic carbons at 131.3 (C-2) and 136.7 (C-1) ppm. This evidence clearly suggested the presence of the phenylalanine unit. The aromatic protons at 7.19, 7.31 and 7.24 were linked to aromatic carbons at 131.3 (C-1), 129.7 (C-3) and 128.5 (C-4) ppm, and the carbons at 59.8 and 40.9 were assigned as α and β carbons of phenylalanine by HSQC.

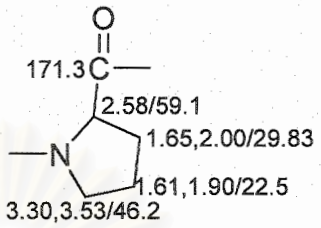


phenylalanine B

Phenylalanine A was different from phenylalanine B by the integration intensity of the methine α protons, integrating for 2.5 protons for Phe-A and 0.5 proton for Phe-B. Hence, Phe-A and Phe-B were not in the same peptide chain.

Inspection of the ^{1}H NMR spectrum showed a remaining set of amino acid signals. The signal at 2.58 (dd, *J* = 6.3 Hz) linked to a methine carbon at 59.1 ppm, seemed like an α methine carbon of one amino acid even though the proton signal was higher field than the common α methine protons. This was an unusual chemical shift for an α methine proton. Cross peaks were observed in ^{1}H-^{1}H COSY between this α proton

at 2.58 and methylene protons at 1.65, 2.00 (β protons) and to methylene protons at 3.30, 3.53 (δ proton) which also correlated to γ protons at 1.61and 1.90. This evidence strongly suggested this unusual unit was a third proline unit (ProC).



proline C

The carbonyl carbons within each amino acid residue were assigned from HMBC correlations between the C=O and their respective α protons. Cross peaks were observed in the HMBC spectrum between Leu-CO (168.9 ppm)/Leu–αH (δ_H 4.13), ProA-CO (172.8 ppm)/ProA–αH (δ_H 4.26), ProB-CO(171.8 ppm)/ProB–αH (δ_H 4.07), PheA-CO(166.8 ppm)/PheA–αH (δ_H 4.45), PheB-CO/PheB–αH and ProC-CO (171.3 ppm)/ProC–αH (δ_H 2.58) as presented in Figure 4.1

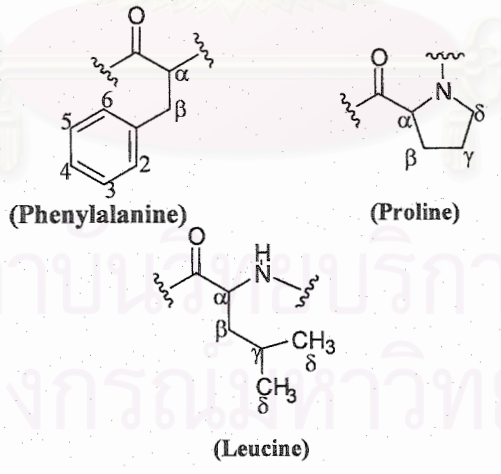


Figure 4.1 The assignment of amino acid residue

Table 4.1 The proton and carbon assignment of MB2-4 component

N0.	Residue	Position	δ^1H [a]	$\delta^{13}C$ [b]	DQFCOSY	HMBC $^nJ_{C-H}$ = 8 Hz
1	Leu	α	4.13, t (J = 5.0 Hz)	54.6 (d)	αH2, H1βa, H1βb	αH2
		β	1.51, m 1.93, m	39.4 (t)	αH1	δ1 CH_3, δ2 CH_3
		γ	1.89, m	25.8(d)	δ2 CH_3 δ1 CH_3	αH1
		δ1 CH_3	0.96, d (J = 6.5Hz)	23.6 (q)	Hlγ, δ2 CH_3	δ2 CH_3 H2βa, H2βb
		δ2 CH_3	0.95, d (J = 6.4 Hz)	22.2 (q)	Hlγ, δ1 CH_3	δ1 CH_3, H2βa, H2βb
		CO	-	168.9	-	H2α, H2βa, H2βb
2	Pro A	α	4.26, t (J = 7.5 Hz)	60.3 (d)	αH1, βH2a, βH2b	γH2a, γH2b
		β	2.31, m 2.03, m	29.1 (t)	αH2, γH2a, γH2b	δH3a, δH3b
		γ	1.90, m 2.03, m	23.7 (t)	βH32, βH2b, δH2a, δH2b	δH2a, δH2b
		δ	3.51, m 3.50, m	46.5 (t)	γH2a, γH2b	βH32, βH2b
		CO	-	172.8	-	βH3a, βH3b
3	Pro B	α	4.07, t (J = 5.3 Hz)	60.1(d)	αH4, αH5, βH3a, βH3b	γH3a, γH3b
		β	2.10, m 1.20, m	29.4 (t)	αH3, γH3a, γH3b	δH3a, δH3b
		γ	1.81(2H), m	22.8 (t)	βH3a, βH3b, δH3a, δH3b	δH3a, δH3b
		δ	3.35, m 3.56, m	46.0 (t)	γH3a, γH3b	βH3a, βH3b
		CO	-	171.8	-	αH4
4	Phe A	α	4.45, t (J = 4.8 Hz)	57.7 (d)	αH4, βH4a, βH4b,αH4	αH3
		β	1.51, m 1.93, m	38.2 (t)	αH4	δ1 CH_3, δ2 CH_3
		1	-	137.3 (s)	-	H2/6, H3/5
		2/6	7.31, d (J= 7.3 Hz)	131.1 (d)	H3,5	H3/5
		3/5	7.30, d (J = 7.2 Hz)	129.5 (d)	H2,6	H2/6
		4	7.29, d (J = 7.9 Hz)	128.1(d)	H2, 5	H2/6, H3/5
		CO	-	166.8	-	αH4, βH4a, βH4b

Cont.

N0.	Residue	Position	δ^1H [a]	$\delta^{13}C$ [b]	DQFCOSY [a]	HMBC [b]
5	Phe B	α	4.21, t (J = 4.8 Hz)	59.8(d)	βH5a, βH5b,	αH3
		β	3.19, dd (J = 4.8, 13.8 Hz)	40.9 (t)	αH5	
			2.99, dd (J= 4.8, 13.8 Hz)			
		1	-	136.7 (s)	-	$\beta H4_{a,b}$
		2/6	7.19, d (J= 7.2 Hz)	131.3 (d)	H3,5	βH4a,b, H4, H5
		3/5	7.31, d (J = 7.2 Hz)	129.7 (d)	H2,6	
		4	7.24, d (J = 7..9 Hz)	128.5 (d)	H2,5	
		CO	-	167.4	-	αH4,βH4a, βH4b
6	Pro C	α	2.58 ,dd (J =6.4 Hz)	59.1(d)	βH6a, βH6b , αH4	αH5
		β	1.65	29.8 (t)	αH6, γH6a, γH6b	δH1a, δH1b
			2.00			
		γ	1.61	22.5 (t)	βH6a, βH6b,	γH1a, γH1b
			1.90		δH1a, δH1b	
		δ	3.30, m	46.2 (t)	γH1a, γH1b	-
			3.53, m			
		CO	-	171.3	-	αH4

[a] obtained in CD_3OD recorded on 500 MHz
[b] HMBC correlation for at $^nJ_{C-H}$ = 8 Hz

Consequently, six individual amino acids, one leucine, two phenylalanines and three different prolines were readily identified from NMR data. All six amino acids could be constructed from the 2D NMR data. The carbon and proton assignments are shown in table 4.1.

The amino acid sequence of component MB2-4 was established by HMBC, COSY and NOESY data. An 8 Hz optimized HMBC experiment was used to initiate amino acid sequencing. Firstly, the $^3J_{C-H}$ correlation was observed in HMBC spectra between the carbonyl carbon of phenylalanine B at 167.4 ppm and the α proton of proline C at δ_H 2.58. ^1H-^1H $^5J_{H-H}$ COSY cross peaks were observed between ProC-αH (2.58) and PheB-αH (4.21), and between ProA-αH (4.26) and Leu-αH (4.13) . Interestingly, ProB-αH (δ_H 4.07) showed COSY interactions to both PheA-αH (δ_H 4.45) and PheB-αH (4.21). The HMBC and COSY correlation clearly suggested that proline C was located adjacent to phenylalanine B (fragment I) and proline B was linked to both phenylalanines A and B (fragment II). The last amino acid, leucine was placed next to proline A (fragment III)

corresponding with their integration. The correlations within partial fragments are shown in Figure 3.2. These data suggested the key molecular fragments of the peptide component of MB2-4 shown in Figure 4.2 .

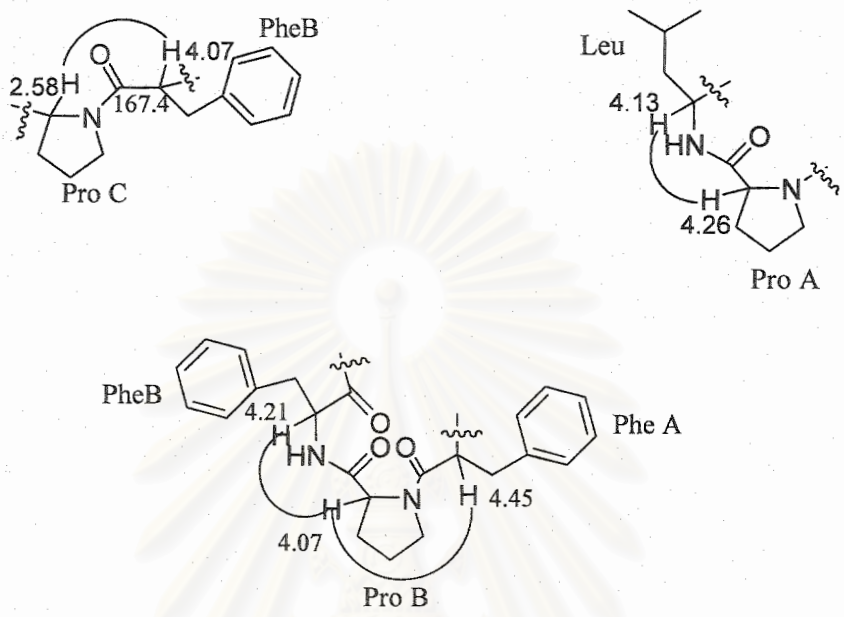


Figure 4.2 The DQFCOSY correlation of compound **MB2-4**

However, the mass spectral data was an alternative method used to resolve this sequencing issue. LCESI-MS revealed significant molecular peaks for $[M+H]^+$ at *m/z* 489, 455, 421 and also showed $[(M+2H)/2]^+$ ions at *m/z* 245 and 211.

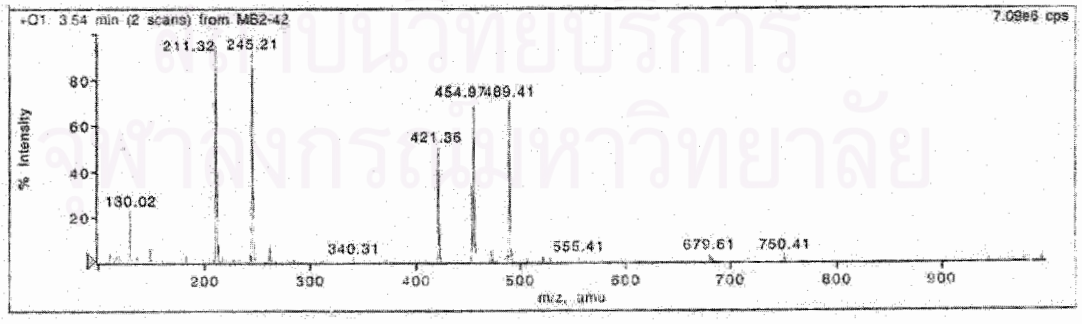
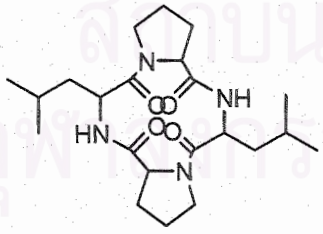
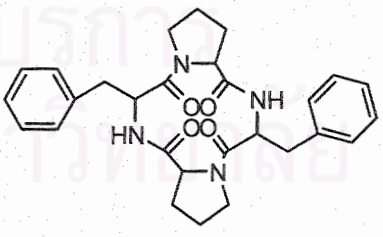


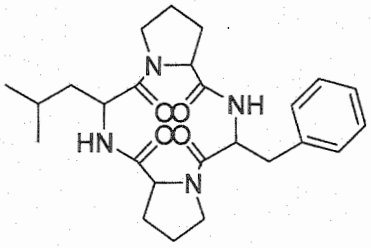
Figure 4.3 The LC-ESIMS spectrum of MB2-4

At this point, it was recognized a similarity of this data to that of Shin *et al.*, who described the cyclic peptide cyclo-[L-isoleucyl-L-prolyl-L-leucyl-L-prolyl] (**58**) with $[M+H]^+$of 421. Therefore a peptide component of MB2-4 seemed to be cyclo-[L-leucyl-L-prolyl-L-leucyl-L-prolyl](**55** or **4a**) consisting of two units each of leucine and proline. This symmetrical cyclopeptide would give an $[M+2H]^+$ion at 211, as was observed in the LC-MS data. The peptide differs from Shin's compound by the replacement of an isoleucine by leucine. We then considered the pair of ions at 489 and 245 in the LC-MS spectrum, and recognized that these corresponded to $[M+H]^+$ and $[(M+2H)/2]^+$ respectively for a compound of molecular weight 488. If the two leucine's of 4x were replaced by phenylalanine, then the product *cyclo* (phenylalanyl-prolyl- phenylalanyl-prolyl)(**57** or **4b**) has a molecular weight.of 488. This cyclic peptide has been reported from an ascidian *Cystodytes delle chiajei*[48] but was not well characterized by these authors. The NMR data for this compound is presented in $CDCl_3$ and so could not easily be compared with the data for our peptide **4b**, which was acquired in d_4 MeOH. The remaining component of the peptide mixture of MB2-4 had m/z of 455, which could now be seen to fit the cyclic peptide *cyclo*-(phenylalanyl-prolyl-leucyl-prolyl)(**4c**). This peptide had an asymmetric structure and so no $[(M+2H)/2]^+$ ion was observed. This is a new peptide structure, and has not been reported previously from marine organisms. Therefore, three peptides had been identified. These were *cyclo*-(L-leucyl-L-prolyl-L-leucyl-L-prolyl)(**4a**), *cyclo*-(phenylalanyl-prolyl-phenylalanyl-prolyl)(**4b**) *cyclo*-(phenylalanyl-prolyl-leucyl-prolyl)(**4c**).





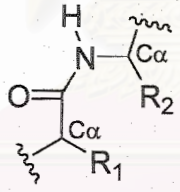
cyclo(leucyl-prolyl-leucyl-prolyl)(**4x**) *cyclo*(phenyl-prolyl-phenyl-prolyl)(**4y**)



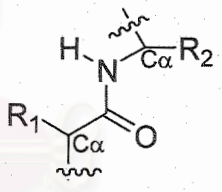
cyclo (leucyl-prolyl-phenyl-prolyl) (**4c**)

The next consideration was to assign the six amino acid components, one leucine, two phenylalanines and three prolines, to these three individual peptides. However, this NMR analysis was complicated because proline residue can show two conformations of the amide bond (*cis* or *trans*) in peptide chains, and these two conformational possibilities lead to proline-containing peptides showing more than one set of signals. Therefore it was difficult to assign individual proline NMR signals to the suggested peptide structures.

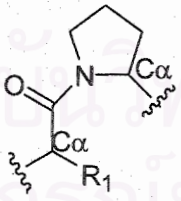
Peptide units can adopt two different conformations, *cis* and *trans*. In the *trans*-form, the C=O and the N-H groups were pointed in opposite directions whereas in the *cis* –form they were pointed in the same direction. For the most peptide, the *trans*-form is about 1000 times more stable than *cis*-form.[66]



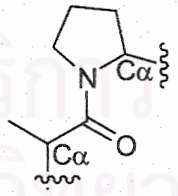
cis



trans



cis-proline



trans-proline

In the molecule of peptide-containing proline ring, the *trans* form is only about four times more stable than the *cis*-form. The geometry of the peptidic linkage was assigned on the basis of the difference in ^{13}C chemical shift values of the β carbon and γ carbon of proline residues ($\Delta\delta_{C\beta\text{-}\gamma}$) [67]. The use of values ($\Delta\delta_{C\beta\text{-}\gamma}$) in *cyclo*(Leucyl-Prolyl-Leucyl-Prolyl allowed to deduce the *cis* conformation ($\Delta\delta_{C\beta\text{-}\gamma}$ = 9.4 ppm) for peptidic

bond between Leu1-CO-N-Pro1 and *trans* conformation ($\Delta\delta_{C\beta-\gamma}$ = 3.5 ppm) for Leu2-CO-N-Pro 2.

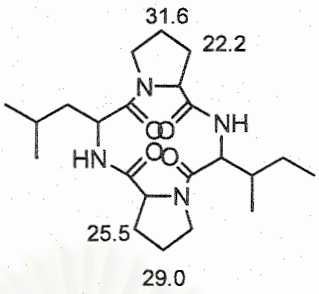
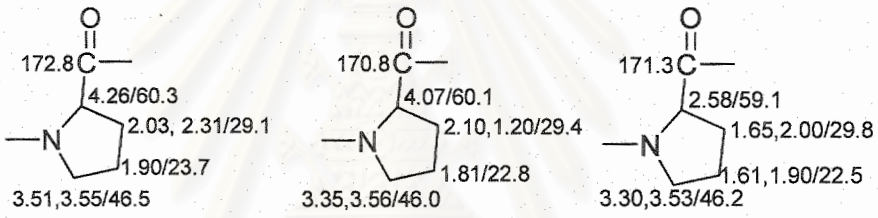


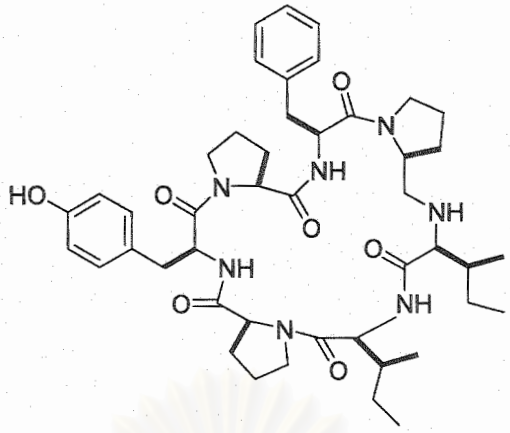
Figure 4.4 δC_β and δC_γ assignment for cyclo(leucyl-prolyl)$_2$ (**58**) by Francisco *et al* [48]

The difference in ^{13}C chemical shift of ($\Delta\delta_{C\beta-\gamma}$) for proline residue in MB2-4 for proline A, B and C were 5.4, 6.6 and 6.7 ppm, respectively.



proline A prolineB proline C

The values ($\Delta\delta_{C\beta-C\gamma}$) of prolines A, B and C are in the range between *cis* and *trans* conformation. The role of proline in these molecules has been linked to control of conformation of the molecule in solution because restricted of dihedral angel (ϕ) of proline.[67] Generally, cyclic peptide or cyclic tetrapeptide could be dissolved in chloroform. The NMR experiment of MB2-4 was run in CD_3OD. The NMR data of **4b** differed significantly from that obtained in CD_3OD. This is suggested to be due to conformational change taking place within the molecule. The most significant change was evident in βC and γC of proline indicated that the conformation are switching from *cis* to *trans*. These evidences have previous been observed in Phakellistatin 2(**61**)[68] which gave the different conformation, less polar (in $CDCl_3$) and more polar conformation (in CD_3OD).

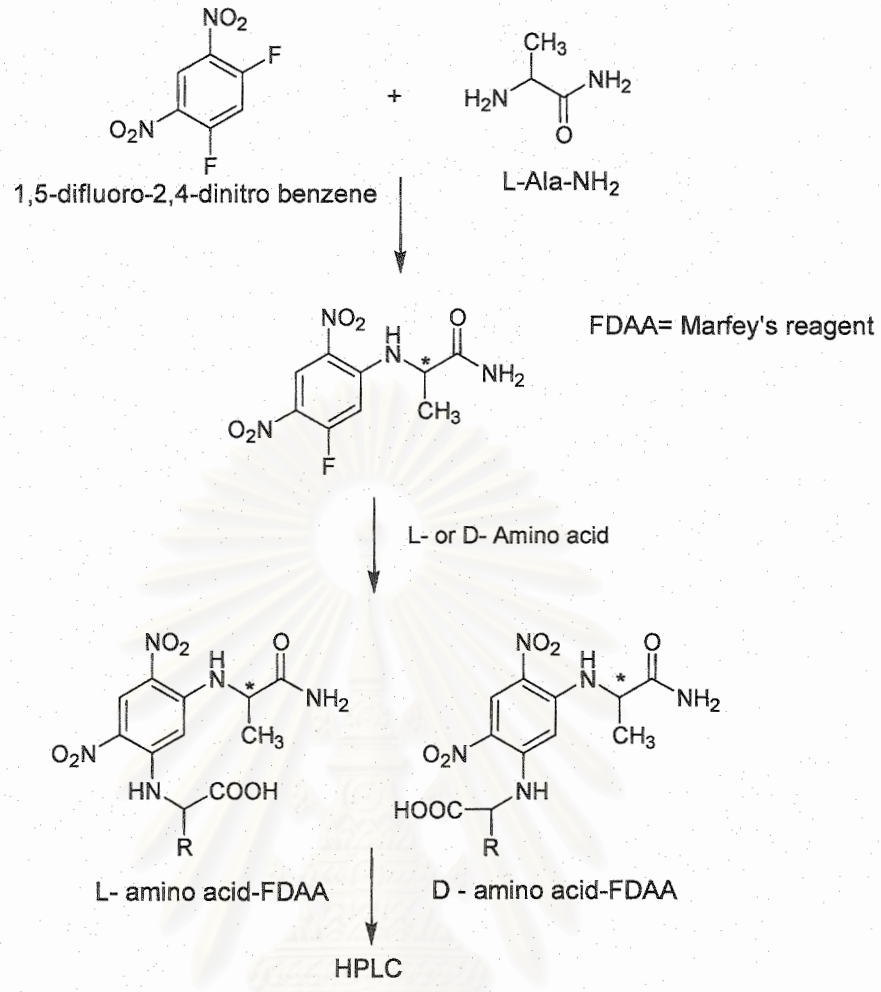


Phakellistatin 2 (**61**)

The difference in conformation observed in CD_3OD is similarly large and is due mainly to the disruption of one hydrogen bond between NH and C=O. However, MB2-4 was a mixture, their conformation are certainly unambiguous.

4.4 The Stereochemistry of the Cyclic Peptides

The stereochemistry of the peptide mixture MB2-4 was explored by the acid hydrolysis and derivatisation of peptide with Marfey's reagent followed by HPLC analysis.[69] The mechanism of Marfey's method is shown in Scheme 4.1. The amino acid components of MB2-4 were demonstrated to have the L- stereochemistry of phenylalanine, leucine and proline in the peptide mixture.



Scheme.4.1 The reaction scheme for Marfey's method

4.5 Conclusion

The chemical investigation of peptide fraction MB2-4 led to isolation of two known cyclic peptide (**4a** and **4b**) and a new cyclic peptide (**4c**). The peptide structure was elucidated by the assistance of NMR and LCESIMS experiments. The stereochemistry of individual amino acids was deduced by Marfey's method. Since MB2-4 was a mixture, thus there was a need for separation by HPLC. If the compounds can be separated by HPLC, the individually purified peptide should be further studied by NMR (in both $CDCl_3$ and in d_4-MeOH) and by LC-MS. High resolution mass spectral studies (HRESIMS) would also confirm the proposed structures. Finally the stereochemistry conformation of each of these proline containing peptides could be explored in much more details than is possible with the mixture MB2-4. Individual peptides could also be subjected to biological assay.

4.6 The spectroscopic data

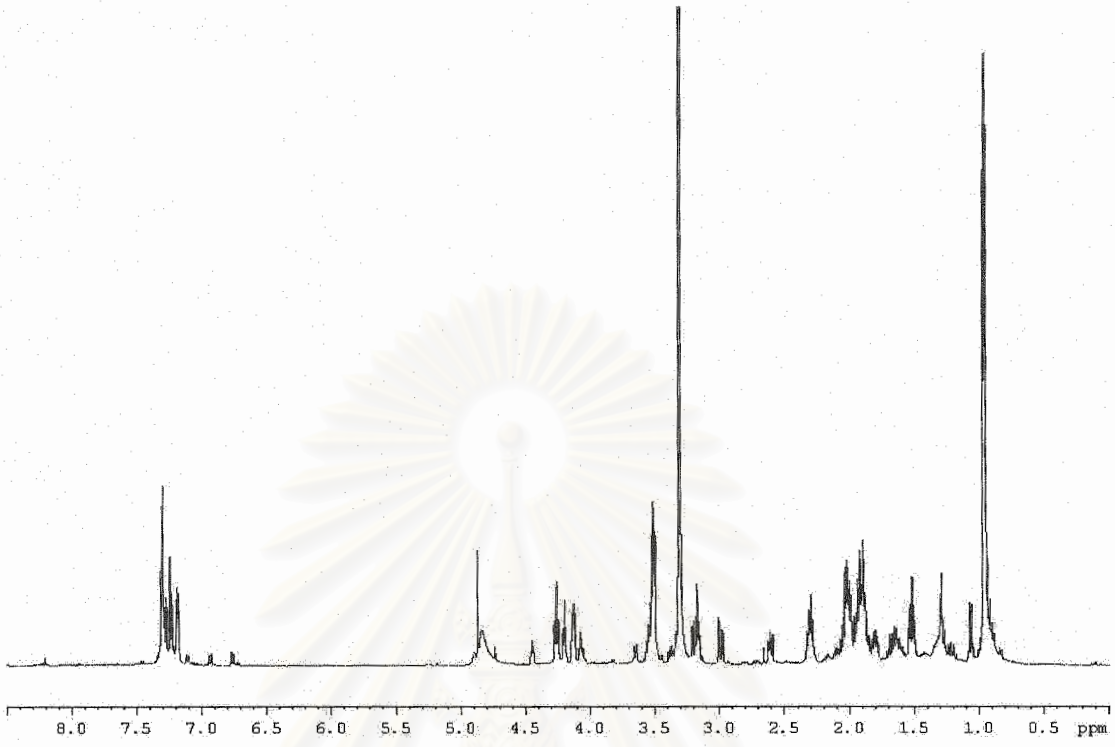


Figure 4.5 The ^{1}H NMR spectrum of MB2-4

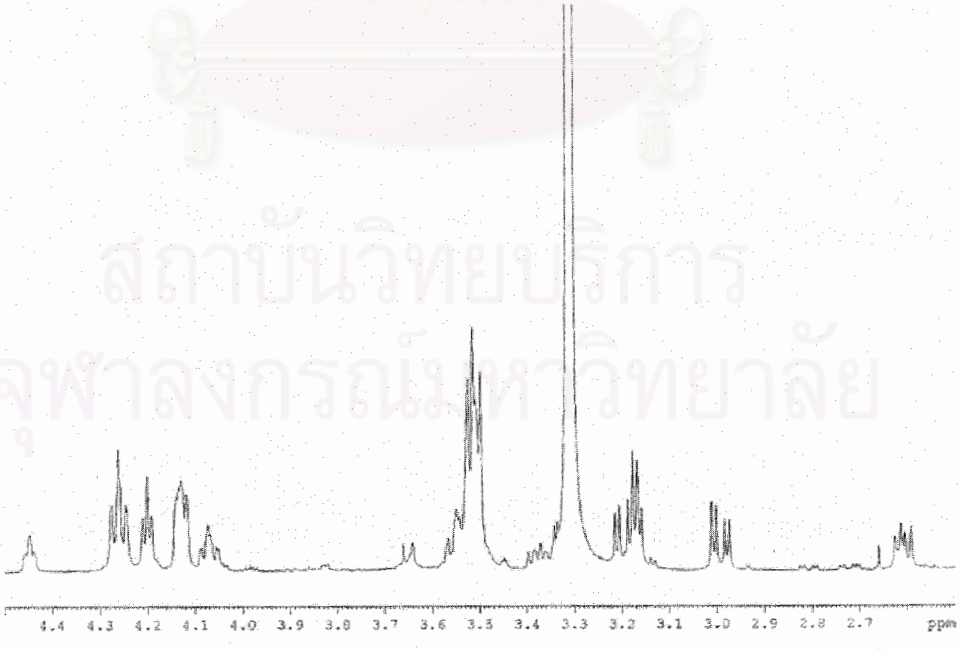


Figure 4.6 The ^{1}H NMR spectrum of MB2-4 (expand)

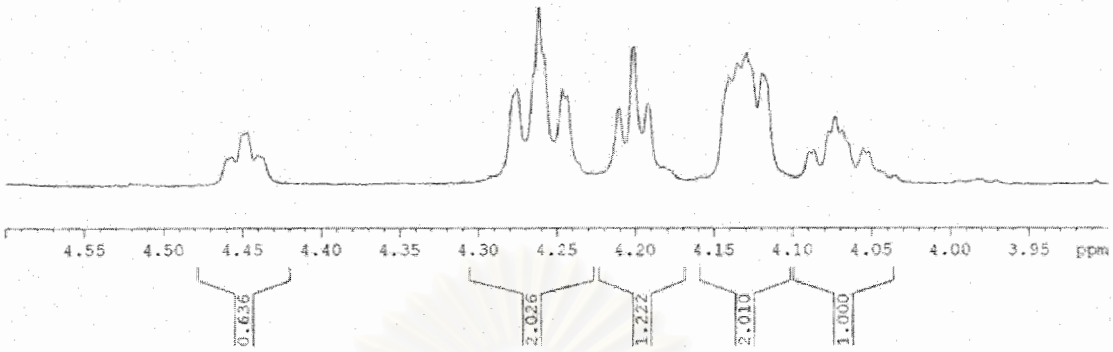


Figure 4.7 The integration ^{1}H NMR spectrum of MB2-4

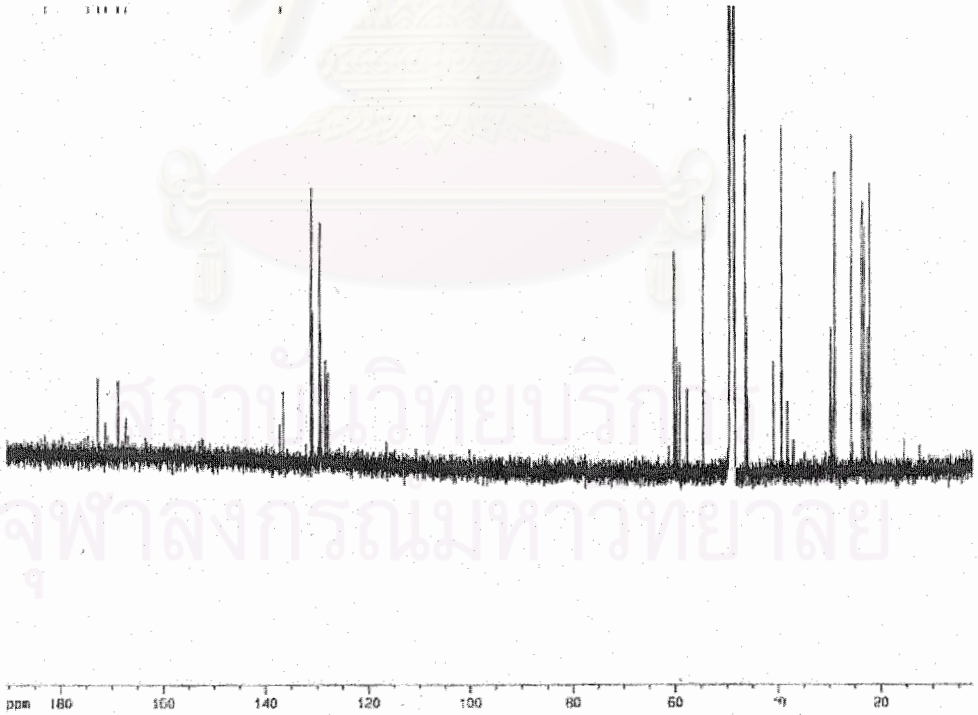


Figure 4.8 The ^{13}C NMR spectrum for MB2-4

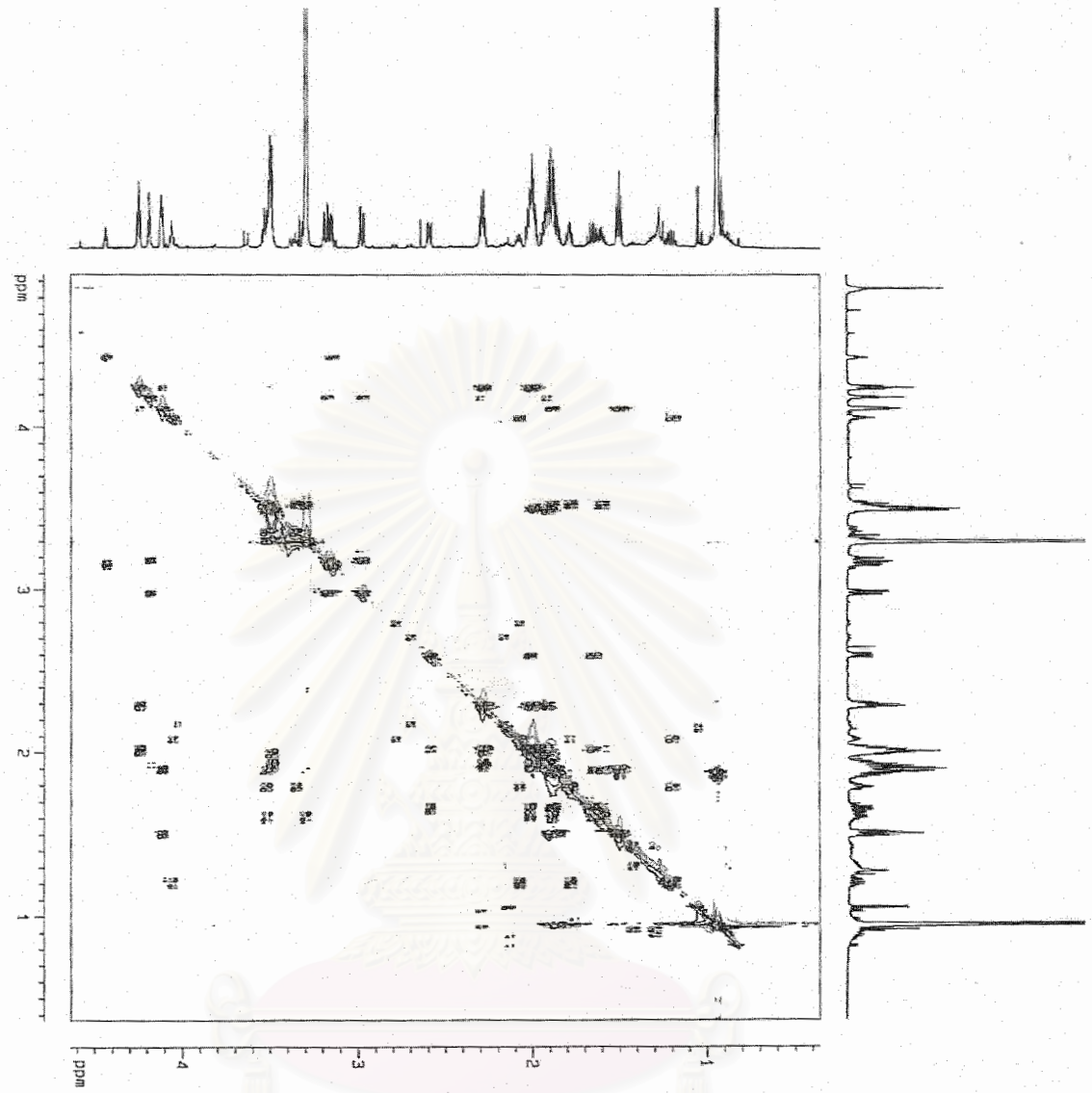


Figure 4.9 The DOFCOSY spectrum of MB2-4

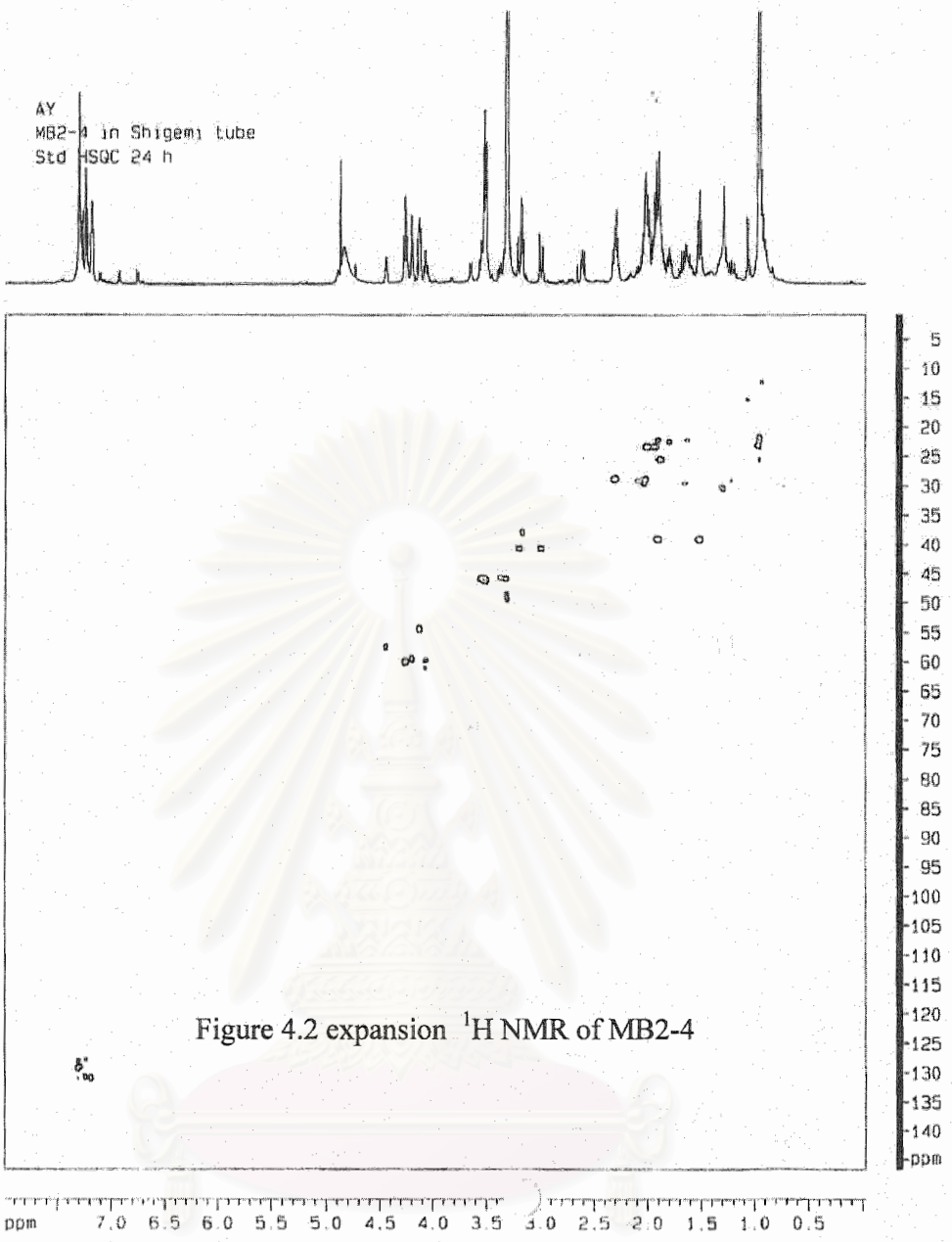


Figure 4.2 expansion ^{1}H NMR of MB2-4

Figure 4.10 The HSQC spectrum of MB2-4 (expand)

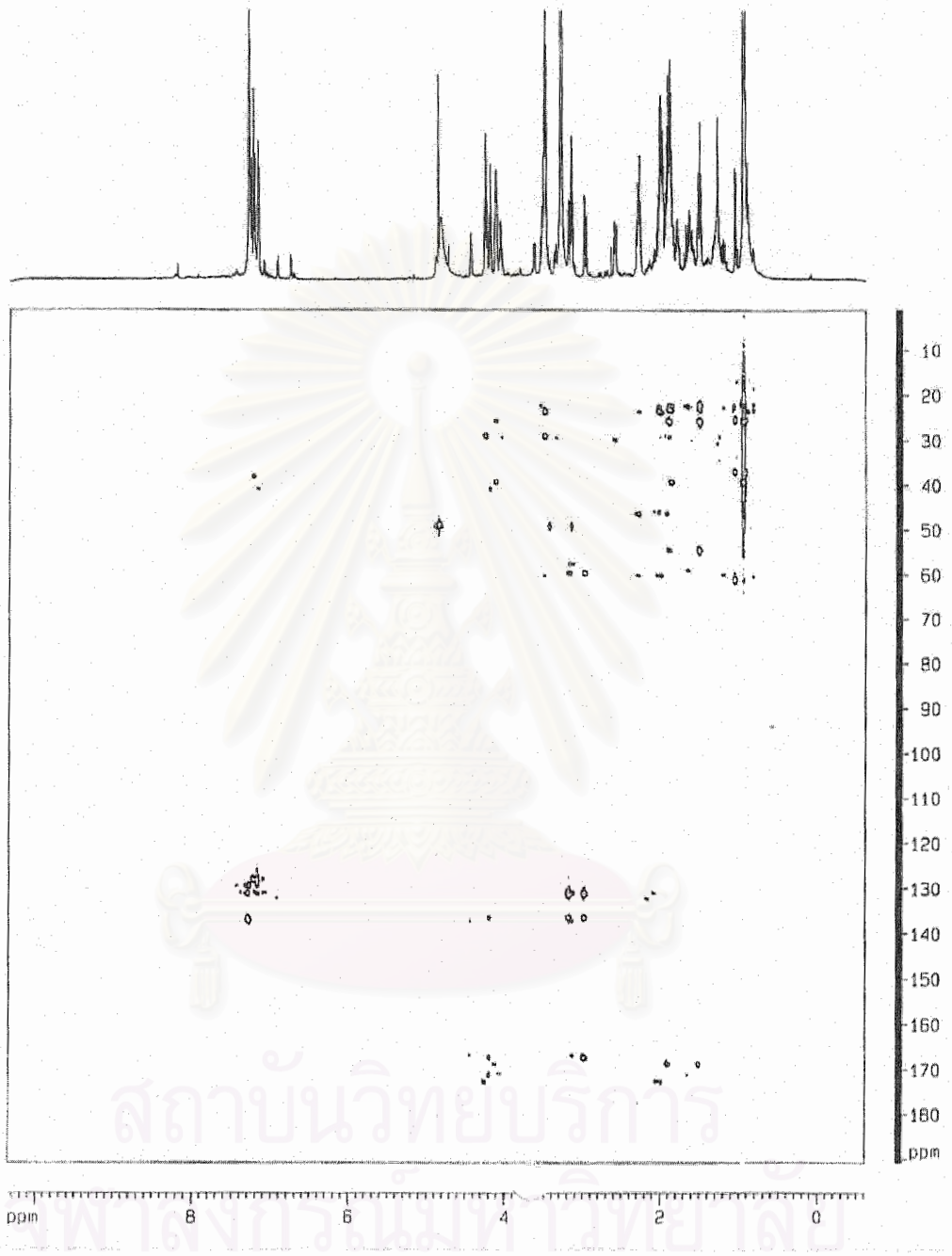


Figure 4.11 The HMBC spectrum of MB2-4

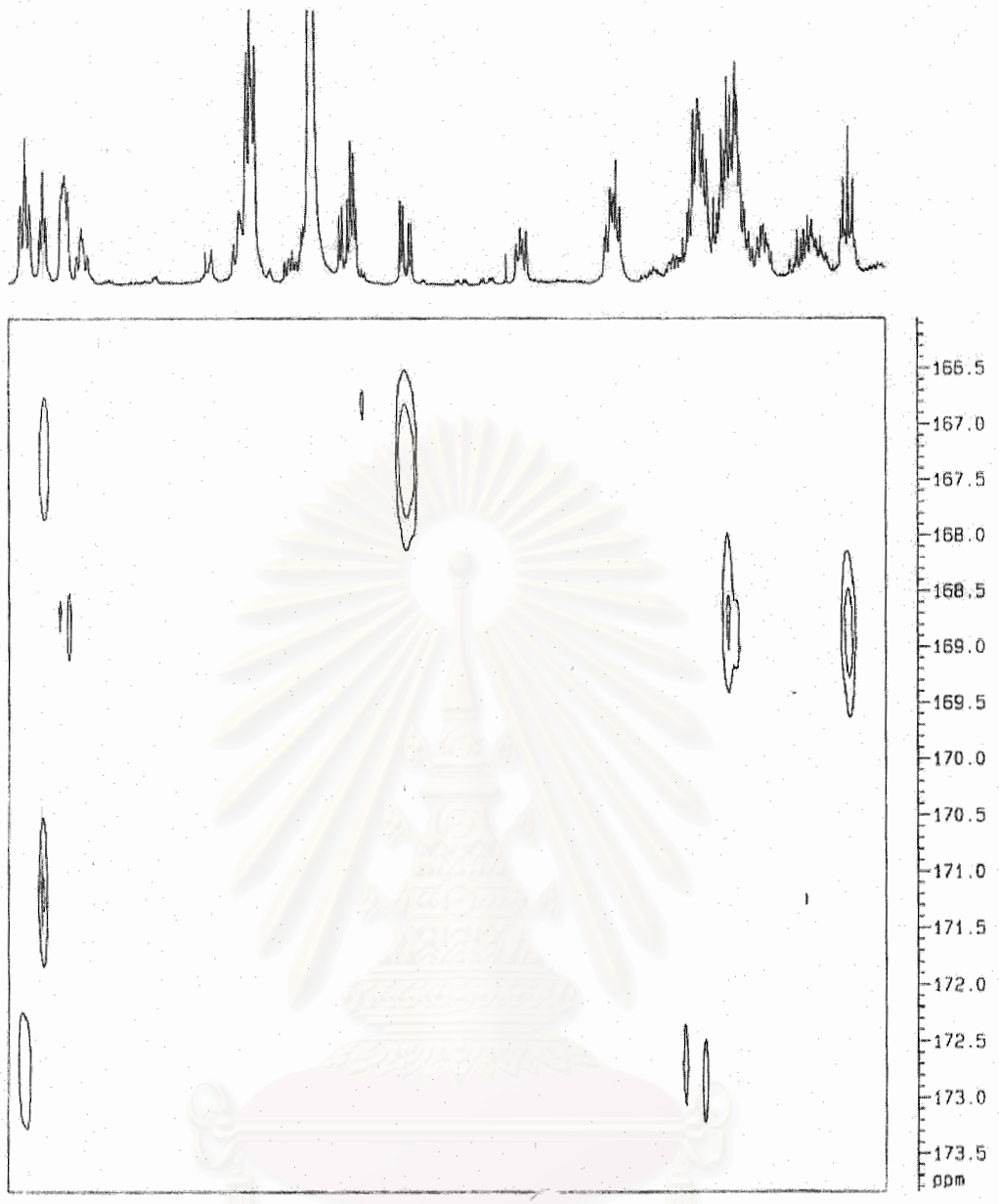


Figure 4.12 The HMBC spectrum of MB2-4 (expand)

CHAPTER V
CONCLUSION

The search for bioactive compounds from marine organism, the chemical investigation on an Australian sponge *Aplysilla sulphurea* provided three chromodorane diterpenes. chromodorolides A (**2a**), B (**2b**) were isolated together with a new compound chromodorolide C (**2c**). This is the first report of the isolation of chromodorane diterpene from the *Aplysillid* sponge. As a part of the research on marine bacterium associated with tropical Thai sponge led to the isolation of six cyclic peptide : cyclo-(phenylalanyl - leucyl-)$_2$ (**3a**), cyclo-(leucyl-isoleucyl)$_2$ (**3b**), cyclo-(leucyl-leucyl)$_2$ (**3c**), cyclo-(leucyl-prolyl-leucyl-prolyl) (**4a**), cyclo-(phenylalanyl-prolyl-phenylalanyl-prolyl)(**4b**) and cyclo(phenylalanyl-prolyl-leucyl-prolyl)(**4c**) and including unidentified heteroaromatic unit.

All isolated substances from marine sponge and marine bacteria associated with tropical Thai sponge including their bioactivity were summarized in Table 5.1

Table 5.1 The structure of isolated compound from marine organism and their bioactivities.

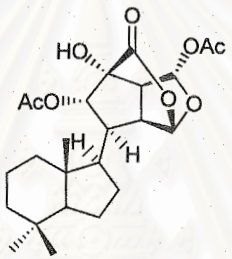
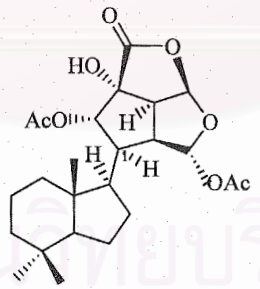
Marine organism	Compounds	Structure	Molecular formula	Bioactivity
Aplysilla sulphurea	chromodorolide A **(2a)**		$C_{24}H_{34}O_8$	antimicrobial activity cytotoxicity nematocidal activity
	chromodorolide B **(2b)**		$C_{26}H_{36}O_9$	antimicrobial activity cytotoxicity

Table 5.2 (Cont.)

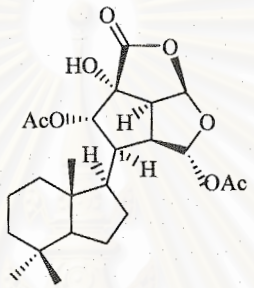
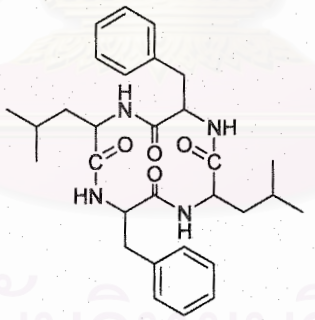
Marine organism	Compounds	Structure	Molecular formula	Bioactivity
Aplysilla sulphurea	chromodorolide C (**2c**) **(New compound)**		$C_{24}H_{34}O_8$	cytotoxicity
Marine bacterium	cyclo(Phenylalanyl - Leucyl- Phenylalanyl- Leucyl) (**3a**) **(New compound)**		$C_{30}H_{40}N_4O_4$	-

Table 5.2 (Cont.)

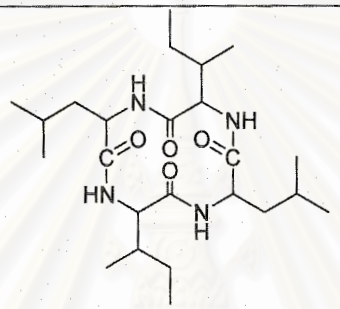
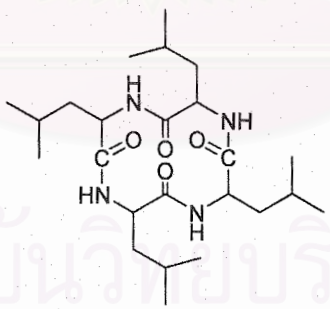
Marine organism	Compounds	Structure	Molecular formula	Bioactivity
Marine bacterium	*cyclo* [leucyl-isoleucyl]$_2$ **(3b)** **(new compound)**		$C_{24}H_{44}N_4O_4$	-
	cyclo[Leucyl-Leucyl]$_2$ **(3c)** **(New compound)**		$C_{24}H_{44}N_4O_4$	-

Table 5.2 (Cont.)

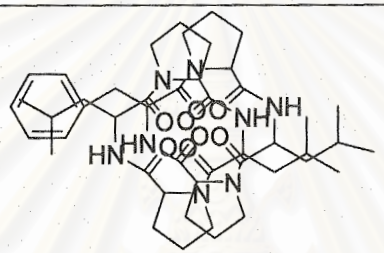
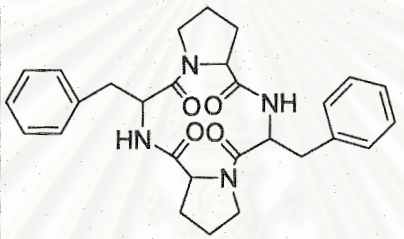
Marine organism	Compounds	Structure	Molecular formula	Bioactivity
Marine bacterium	*Cyclo*(Leucyl-Prolyl-leucyl-Prolyl)(**4a**)		$C_{24}H_{36}N_4O_4$	-
	Cyclo(Phenylalanyl - Prolyl-leucyl-Prolyl)(**4b**) **(new compound)**		$C_{25}H_{34}N_4O_4$	-

Table 5.2 (Cont.)

Marine organism	Compounds	Structure	Molecular formula	Bioactivity
Marine bacterium	Cyclo-(Phenylalanyl - Prolyl- Phenylalanyl - Prolyl)(**4c**)		$C_{28}H_{32}N_4O_4$	**n.d.**

***n.d. no detected**

It can be seen from this research work that the isolated compounds from sponge were the bioactive compounds which showed the wide range of bioactivity such as cytotoxic activity against P388 carcinoma cell lines, nematocidal activity and antimicrobial activity. Chromodorolide A (**2a**) showed higher activity than chromodorolides B (**2b**) and C (**2c**) in cytotoxic activity against P388 carcinoma cell lines and nematocidal activity. Thus, chromodorolide A is the bioactive compound which is more potent and selected to further study for its bioactivities.

From the isolation of compounds from marine bacteria associated with Thai sponge, all of them were identified as a cyclic terapeptide, six cyclic peptides had the striking structure with complicated NMR. Compounds **3a-3c** and **4y** were identified as new naturally occurring compounds. This is the first report on the peptide from marine bacteria in Thailand, indicating that marine bacteria is the new challenging topic for continue studying on natural product chemistry. However, some known compounds have been reported about their activity for example, both **4x** and **4z** both of them revealed the antibacterial activity. All of peptides isolated were detected in the fraction that showed antibacterial activity. However, the peptides were not testing bioassay due to the limited supply of the compound.

Proposal for future work

MB2-4 and MB2-8 were a mixture of known cyclic peptides including some new peptides. Additionally the peptides were also detected in the others fraction such as MB2-2. The mixture of peptide components was elucidated by the assistance of high performance instruments. The NMR experiments were recorded on 500, 750 MHz and samples were prepared on Shigami tube and trace analysis of peptide molecular mass were recorded on LC ESIMS. To complete theirs structural elucidation, MB2-4 and MB2-8 need to be separated to individual compound and then measured theirs molecular mass by HRESIMS. However, it can be seen in this research, this bacterial culture was found to be the rich source of peptides. Therefore, this bacterial strain could be cultured in the large scale and investigated their chemical constituents and bioactivities including the absolute stereochemistry.

REFERENCES

1. Carte, B. K., Biomedical Potential of Marine Natural Products, *Bioscience*, **1996**, 46, 271-286.
2. Woodson, R. E., Youngken, H. W., Schittler, E. and Schneider, J. A., Rouwolfia : Botany, Pharmacognosy, Chemistry and Pharmacology, Little Brown and Co : Boston, **1957**.
3. Mann, J., Davidson, R. S., Hobbs, J. B., Banthorpe, D. V. and Harborne, J. B., Natural products : their chemistry and biological significance, 1st edition, Longman : New York **1994**, 2.
4. Faulkner, D. J., Highlights of marine natural product chemistry, *Nat. Prod. Rep*., **2002**, *17*, 1-6.
5. Blunt, J.W., Copp, B. R., Munro, M. H. G., Northcote, P.T. and Prinsep, M. R., Marine natural product, *Nat. Prod. Rep*., **2003**, *20*, 1-48.
6. Attaway, D. H. and Zaborsky, O.R., *Marine biotechnology Volume I : Pharmaceutical and Bioactive Natural product*., Plenum Press, **1993**, 2.
7. Fenical, W., Natural Products Chemistry in the marine environment, *Science*, **1982**, *215*, 923-928.
8. Fusetani, N., Drugs from the Sea., Reinhardt Druck, Basel, **2000**.
9. Shu, Y., Recent Natural Products Based Drug Development: A Pharmaceutical Industry Perspective, *J. Nat. Prod*., **1998**, *61*, 1053-1071.
10. Pettit, G. R., Herald, C. L., Doubek, D. L., Herald, D. L., Arnold, E. and Clardy, J. Isolation and structure of Bryostatin1, *J. Am. Chem. Soc*., **1982**, *104*, 6846-6848.
11. Rinehart, K. L., Antitumor compounds from tunicates, *Med. Res. Rev.*, **2000**, *20*,1-27.
12. Sakai, R., Rinehart, K. L., Kishore, V., Kundu, B., Faircloth, G., Gloer, J. B. and Carney, J.R., Structure-activity relationships of the didemnin, *J. Med. Chem*., **1996**, *39*, 2819-2834.
13. Rinehart, K. L., Holt, T. G., Fregeau, N .L., Stroh, J. G., Kiefer, P. A., Sun, F., Ki, L. H. and Martin, D. G., Antitumor agents from the Caribbean tunicate Ecteinascidia turbinata, *J. Org. Chem*., **1990**, *55*, 4512-4515.

14. Valoti, G., Nicoletti, M. I., Faircloth, G., Jimeno, J. J. and Giavazzi, R., Antitumor effect of Ecteinascidin-743 (ET743) on Human Ovarian Carcinoma Xenografts, *Proc Annu Meet Am Assoc Cancer Res*, **1997**, *A*, 1477.

15. Carbonero, R. G., Harmon, D., Seiden, M., Jimeno, J., Merriam, P., Waxman, A., Sukpo, J., Quigley, M.T. and Ryan, D., Ecteinascidin-743 (ET-743) induces objective responses and disease control in patients with advanced non-osseous sarcomas: result from phase II trial, *Annals of Oncology*, **2000**,11,126.

16. Konig, G. M. and Wright, A., Marine Natural products research : Current directions and future potential, *Planta. Med*., **1996**, *62*, 193-211.

17. De Silva, E. D. and Scheuer, P. J., Manoalide, an antibiotic sesterterpenoid from the marine sponge *Luffarariella variabilis*: *Tetrahedron Lett*. **1980**, *21*, 1611-1614.

18. Gunasekera, S. P., Gunasekera, M., Longley, R. E. and Schulte, A. K., Discodermolide: A new bioactive polyhydroxylated lactone from the marine sponge Discodermia dissoluta, *J. Org. Chem*., **1990**, *55*, 4912-4915.

19. Tachibana, K., Scheuer, P. J., Tsukitani, Y., Kikuchi, H., Van, D., Clardy, J., Gopichand, Y. and Schmizt, F. J., Okadaic acid, a cytotoxic polyether from two marine sponge of the genus Halichondria. *J. Am. Chem. Soc*., **1981**, *103*, 2469-2471.

20. Kato, Y., Fusetani, N., Matsunaga, S., Hashimoto, K., Fujita, S. and Furuya, T., Calyculin A, a novel antitumor metabolite from the marine sponge Discodermia calyx. *J. Am. Chem. Soc*, **1986**, *108*, 2780-2781.

21. Burgoyne, D. L., Andersen, R. and Allen, T. M., Contignasterol a highly oxygenated steroid with the ‘unnatural’ 14b configuration from the marine sponge *Petrosia contignata*, *J. Org. Chem*., **1992**, *57*, 525-528.

22. Burreson, B. J. and Scheuer, P. J., 9-Isocyanopupukeanane, a marine invertibrate allomone with a new sesquiterpene skeleton, *J. Am. Chem. Soc*., **1975**, *97*, 4763-4764.

23. Cimino, G., De Rosa, S., De Stefano, S., Morrone, R. and Sodano, G., The chemical defense of nudibranch mollusks, *Tetrahedron*, **1985**, *41*, 1093-1100.

24. Fenical, W., Chemical studies of marine Bacteria: Developing a new resource, *Chem. Rev*., **1993**, *93*, 1673-1683.

25. Mynderse, J., Moore, R. E., Kashiwaki, M. and Morton, T., Antileukemia activity in the oscillatoriaceae: Isolation of debromoaplysiatoxin from Lyngbya, *Science*, **1997**, *196*, 538-540.

26. Luesch, H., Yoshida, W. Y., Moore, R. E., Paul, V. J. and Corbett T. H., Total structure., Total structure determination of aplatoxin A, a new potent novel cytotoxin from the marine cyanobacterium *Lyngbya majuscula*, *J. Am. Chem Soc*., **2001**, *123*, 5418-5423.

27. Harrigan, G. G., Luesch, H., Yoshida, W. Y., Moore, R. E., Nagel, D. G., Paul, V. J., Mooberry, S. L., Corbett T. H. and Valeriote, F. A., Symplostatin 1: A dolastatin 10 analogue from marine cyanobacterium *Symploca hydnoides*, *J. Nat. Prod*., **1998**, *61*, 1075-1077.

28. Pettit, G. R., Kamano, Y., Herald, C. L., Tuinman, A. A., Boettber, F. E., Kizu, H., Schmidt, J. M., Baczynskyj, L., The isolation and structure of a remarkable marine animal antineoplatic constituent, dolastatin 10, *J. Am. Chem. Soc*., **1987**, *109*, 6883-6885.

29. Wood, E. J. F., *Marine Microbial Ecology*, Chapman Hall, **1965**.

30. Kobayashi, J. and Ishibashi, M., Bioactive metabolites of symbioses marine microorganism, *Chem. Rev*., **1993**, *93*, 1753-1769.

31. Octarit, J. M., Ohta, S., Kamimura, N., Yamaoka, Y. and Shimizu, T. A., A novel antimicrobial substance from a strain of the bacterium *Vibrio* sp., *J. Nat. Prod.*, **1994**, *4*, 309-312.

32. Gerwick, W. H. and Sitachitta, N., Nitrogen-Containing metabolite from marine bacteria, The Alkaloids VOL.53, Academic Press, 239-281.**2000**

33. Jayatilake, G. S., Thornton, M. P., Leonard, A. C., Grimwade, J. E. and Baker, B. J., Metabolites from an Antarctic Sponge–Associated Bacterium *Pseudomonas aeruginosa*, *J. Nat. Prod*., **1996**, *59*, 293-295.

34. Shigamori, H., Bae, M. A., Yazawa, K., Sasaki, T. and Kobayashi, J., Alteramide A, a new tetracyclic alkaloid from bacterium Alteromonas sp. associated with the marine sponge *Halichondria okadai*, *J. Org. Chem*., **1992**, *57*, 4317-4320.

35. Andersen, R. J., Wolfe, M. S. and Faulkner, D. J., Autotoxin antibiotic production by a marine chromobacterium, *Marine Biology*, **1974**, *27*, 281-285.

36. Phuwapraisirisan, P., Matsunaga, S., Fusetani, N., Chitanawisuti and Kritsanapuntu, S and Menasveta, P., Mycaperoxy H, a new cytotoxic

norsesterterpene peroxide from Thai marine sponge *Mycale* sp., *J. Nat. Prod*., **2003**, *66*, 289-291.

37. Satitpatipan, V. and Suwanborirux, K., New Germacranes from a Thai Marine sponge, *Axinyss*a n. sp., *J. Nat. Prod.*, **2004**, **67**, 503-505.
38. Molinski, T. F. and Faulkner, D. J., A new diterpene lactone from an Australian *Aplysilla* species., *J. Org. Chem*. **1986**, *51*, 1144-1146.
39. Tischler, M., Andersen, R. J., Choudhary, M. I. and Clardy. J., Terpenoids from the sponge *Aplysilla glacialis* and specimens of the nudibranch *Cadlina luteomarginata* found on the sponge., *J. Org*. *Chem.* **1991**, *56*, 42-47.
40. Karuso, P., Taylor, W. C. and White, A.H. The constituents of marine sponges. 11. Isolation of 8 diterpenes from *Aplysilla rosea*. *Aust. J. Chem,* **1986**, *39*, 1629-1641.
41. Lacey, E, Redwin, J. M., Gill, J. H., Demargheriti, V. M. and Waller, P. J. In *Resistance of Parasites to Antiparasitic Drugs*; Boray, J.C.; Martin, P.J.; Roush, R.P., Eds; MSD AGVET, Rahway, NJ, **1990**, 177-184.
42. Dumdei, E. J., de Silva, E. D. and Andersen, R. J. Chromodorolide A, a rearranged diterpene with a new carbon skeleton from the Indian ocean nudibranch *Chromodoris cavae*. *J. Am. Chem Soc*. **1989**, *111***,** 2712-2713.
43. Morris S. A., Dumdei, E. J., de Silva, E. D. and Andersen, R. J., Chromodorane diterpenes from the tropical dorid nudibranch *Chromodoris cavae*. *Can. J. Chem*. **1991**, *69*, 768-771.
44. Kobayashi, J. and Ishibashi, M., Bioactive metabolites of symbiotic marine microorganisms, *Chem. Rev.*, **1993**, *93*, 1753-1769.
45. Yasumoto, T., Yasumura, D., Yotsu, M., Michishita, T., Endo, A., and Kotaki, Y., Bacterial production of tetrodotoxin and anhydrotetrotoxin, *Agric. Biol. Chem*., **1986**, *50*, 793-795
46. Moore, B. S., Trischman, J. A., Seng, Dieter, Kho, D., Jensen, P. R. and Fenical, W., A Salinamides, Anti-inflammatory depsipeptides from a marne *streptomycete*, *J. Org. Chem*., **1999**, *54*, 1145-1150.
47. Needham, J., Kelly, M. T., Ishige, M. and Andersen, R. J., Andrimid and moiramides A-C, metabolites produce in culture by a marine isolation of the bacterium *Pseudomonas fluorescens*: structure elucidation and biosynthesis, *J. Org. Chem*., **1994**, *59*, 2058-2063.

47. Shin, J., Seo, Y., Lee, HS., Rho, JR. and Mo, S. J., A new cyclic peptide from a marine derived bacterium of the genus Nocardiopsis, *J. Nat. Prod*., **2003**, *66* 883-884.

48. Aracil J. M., Fadli, M., Jeanty, G., Banaigs, B., Francisco, C., Lafeeergue, F., Heitz, A. and Aumelas, A., Nouveaux cyclo tetrapeptides isoles de I' Ascidic Cytodytes Delle Chiajei, *Tetrahedron Lett*., **1991**, *32*, 2609-2612.

49. Bergquist, P. R. and Cook, S. de C., Family Halisarcidae Schmide, Systema porifera: *A guide to the classification of sponge, Edited by John N. A.Hooper and Rob, W.M. Van Soest, Kluwer Acadmic, Plenum Publisher*, **2002**

50. Nogel, L. M., Marquez, B. L. and Gerwick, W.H., Wewakazole, a novel cyclic dodecapeptide from a Papua New Guinea *Lyngbya majuscula*, *Org. Lett.*, **2003**, *5*, 3-6.

51. Cardellina, J. H., Marner, F. J. and Moore, R. E., Seaweed Dermatitis: Structure of Lyngbyatoxin A, *Science*, **1979**, *204*, 193-195.

52. Matsunaga, S., and Fusetani, N., Theonellamides A-E, cytotoxic bicyclic peptides, from marine sponge Theonella sp., *J. Org. Chem.*, **1995**, *60*, 1177-1181.

53. Bewley, C. A., He, H., Williams, D. H. and Faulkner, D. J., Aciculitins A-C : Cytotoxic and Antifungal cyclic peptides from the Lithistid sponge *Aciculites orientalis*., *J. Am, Chem, Soc*., **1996**, *118*, 4314-4321.

54. Jin, Z., Muscarine, imidazoles, oxazole and Thiazole alkaloid, *Nat. Pro. Rev*., **2003**, *20*, 584-605.

55. Videnov, G., Kaiser, D., Kempter, C. and Gunther, J., Synthesis of naturally occurring conformationally restricted oxazole- and thiazole containing di- and tripeptide mimetics, ***Angrew***. *Chem. Int. Ed. Eng*. **1996**, *35*, 1503-1506.

56. Deavenport, D. L., Harrison, C. H. and Rathburn, D. W., Nuclear magnetic resonance spectra of substituted oxazoles and oxazolidinones, 1973, 5, 285-289.

57. Boyce, R. J. and Pattenden, G., and Naturally occurring 4-methylthiazoles, a total snthesis of (-)-[4R, 4S']- Didehydromirabazole A, *Tetrahedron*, **1995**, *51*, 7313-7320.

58. Mitchell, S. S., Faulkner, D. J., Rubins, K. and Bushman, F. D., Dolastatin 3 and two novel cyclic peptides from a Palauan collection of *Lyngbya majuscule., J. Nat. Prod.*, **2000**, *63*, 279-282

59. Thale, Z., Kinder, F. R., Bair, K. W., Bontempo, J., Czuchta A. M., Versace, R. W., Phillips, P. E., Sanders, M. L., Wattanasin, S., and Crews, P., Bengamides Revisited: New Structures and Antitumor Studies., *J. Org. Chem.* **2001,** *66*, 1733-1741.

60. Kehraus, S., Konig, G. M. and Wright, A. D., Leucamide A: a new cytotoxic heptapeptide fom the Australian sponge *Leucetta microraphis*, *J. Org. Chem*., **2002**, *67*, 4989-4992.

61. Nagatsu, A., Kajitani, H. and Sakakibara, J., Muscuride A : new oxazole peptide alkaloid from fresh water cyanobacterium *Nostoc muscorum*, **1997**,

62. Pretsch, E., Bühlmann, P. and Affolter, C., *Structure Determination of Organic Compound*, Springer, Heidelberg, **2000**, 109-110.

63. Omar, S., Tenembaum, L., Manes, L. V. and Crew, P., Novel marine sponge derived amino acid, the fenestins, *Tetrahedron Lett*., **1988**, *43*, 5489-5492.

64. Mitova, M., Popov, S. and Rosa, S. D., Cyclic peptide from a Ruegeria strain of bacteria associated with the sponge *Suberites domuncula*, *J. Nat. Prod*., **2004**, *67*, 1178-1181.

65. Marfey, P., Determination of D-aminoacid, II Use of a bifunctional reagent, 1,5-difluoro-2,4 dinitrobenzene, *Carlsberg Res. Commun*., **1984**, *49*, 591-596.

66. Loiseau, N., Gomis, J. M., Santolini, J., Marcel, D. and Andre', F., Predicting comformational states of cyclic tetrapeptides, *Biopolymer*, **2003**, *69*, 363-385.

67. Tabudravu, J., Morris, L. D. and Jaspar, M., Wainunuamide, a histidine-containing proline rich cyclic heptapeptide isolated from the Fijian marine sponge *Stylotella aurantium*., *Tetrahedron Lett*., **2001**, *42*, 9723-9726

68. Tabudarvu, J., Morris, L. D., Van den Bosch, J. and Smith, N., Two distinct conformers of the cyclic heptapeptide phakellistatin 2 isolated from the Fijian Marine sponge Stylotella aurantium, *J. Org. Chem*., **2002**, *67*, 8593-8601.

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