Isolation and Structure Elucidation of Secondary Metabolites from Marine Sponges and a Marine-derived Fungus

Isolierung und Strukturaufklärung von Naturstoffen aus marinen Schwämmen und einem marinen Pilz

Inaugural-Dissertation

zur

Erlangung des Doktorgrades

der Mathematisch-Naturwissenschaftlichen Fakultät

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vorgelegt von

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Erklärung

Hiermit erkläre ich ehrenwörtlich, daβ ich die vorliegende Dissertation „Isolierung und Strukturaufklärung von Naturstoffen aus marinen Schwämmen und einem marinen Pilz“ selbständig angefertigt und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt habe. Ich habe diese Dissertation in gleicher order ähnlicher Form in keinem anderen Prüfungsverfahren vorgelegt. Auβerdem erkläre ich, daβ ich bisher noch keine weiteren akademischen Grade erworben oder zu erwerben versucht habe.

Düsseldorf, 2005

Suwigarn Pedpradab

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Mr. Suwigarn Pedpradab

Duesseldorf, Germany

"Any enterprise that is not achieved through perseverance,
is fruitless; obstacles will occur.
When any enterprise undertaken with
such misdirected effort results
in the Death showing his face,
what is the use of such enterprise and misdirected effort."

(Phra Mahachanok, by King Bhumibol Adulyadej : The great Rama IX)

I did the endeavor in this work for my teachers

Table of contents

1. Introduction

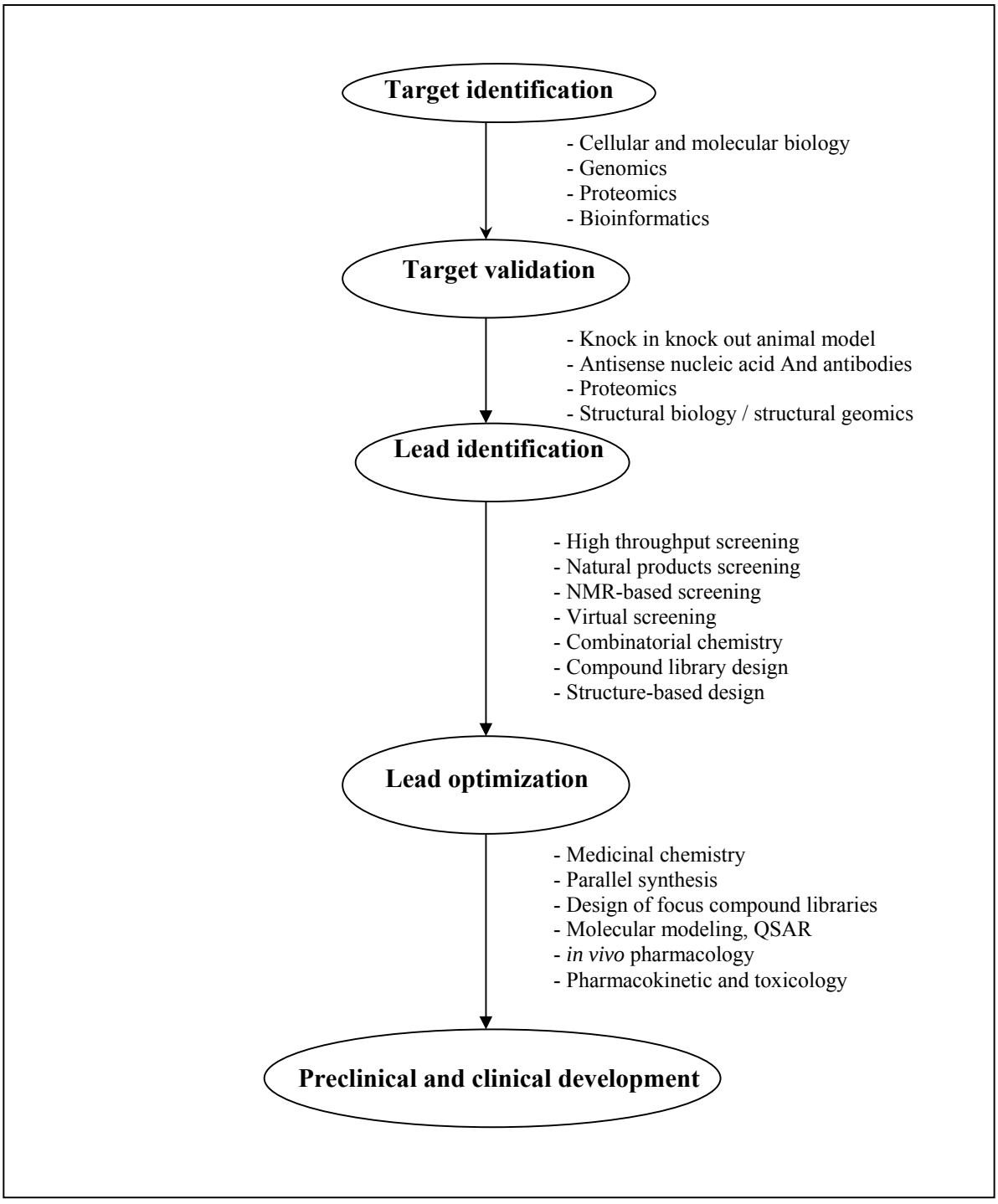
1.1. The drug discovery process

The drug discovery process can be divided into 4 steps namely: drug target identification, target validation, lead compounds identification, and optimization (**Scheme 1**). The aim of the target identification is to discover new genes and proteins. These are quantified and their expressions in diseased and normal cells are analyzed. The target validation process involves demonstrating the relevance of the target protein in a disease. This is accomplished primarily with highly specialized knock-in and knock-out animal models that are capable of mimicking the target disease state. In these models small molecules are used as inhibitors and antagonists. The lead identification phase focuses on identifying the compounds interaction with target proteins and modulating their activities. These compounds are either randomly identified or identified by using specific approaches. In lead optimization, small organic molecules are chemically modified and subsequently characterized pharmacologically in order to obtain compounds with suitable pharmacodynamic and pharmacokinetic properties for becoming a drug [Giersiefeld, *et al.,* 2003].

1.1.1. Natural products for lead identification

Approximately 30 % of the drugs in the market worldwide are natural products or their derivatives. Natural products show a diversity of chemical structures that are not accessible even by the most sophisticated synthetic concepts. Moreover, natural products have often opened up completely new therapeutic approaches. They have contributed to identifying and understanding novel biochemical pathways and proved to make not only valuable drugs available but also essential tools in biochemistry and molecular cell biology [Grabley and Sattler, 2003].

Table 1 and **Figure 1** summarize selected natural products currently evaluated as drug candidates. Natural products sources are basically of two types. Firstly, the terrestrial source which includes plants, animals, and microorganisms, and secondly, the marine source which focuses mainly on invertebrates. From the taxonomic consideration, marine organisms are significantly more diverse than terrestrial organisms. Furthermore, in contrast to animals from terrestrial habitats, invertebrates from marine environment are rich sources of complex natural products derived from numerous biosynthetic pathways (Kijjoa and Sawangwong, 2004).



Scheme1 Phase of drug discovery process (Giersiefen *et al.*, 2003)

Table 1 Selected natural products evaluated as new drug candidates [Grabley and Sattler, 2003].

Natural products	Source	Target	Indication	Status
CC1065	Streptomyces	DNA	Anticancer	Clinical trial
Epothilone	Myxobacterium	Microtubuli	anticancer	-
Fumagillin	Fungi	Angiogenesis	Anticancer, solid tumors, Kaposi's sarcoma	TNP-470 clinical trail
Flavopirinol	Plant	Kinase	Anticancer	Clinical trial
Calanolide A and B	Plant	DNA polymerest action on reverse transcription	AIDS (HIV I)	Clinical and preclini-cal trial
Discoder-molide	Marine sponge	microtubuli		Advanced preclinical trial
Manoalide	Marine sponge	Phospholipase A_2, Ca^+-release	Anti-inflammatory	-
Huperzine A	Moss	Cholinesterase	Alzheimer disease	Advanced - clinical trials and clinical trial in china

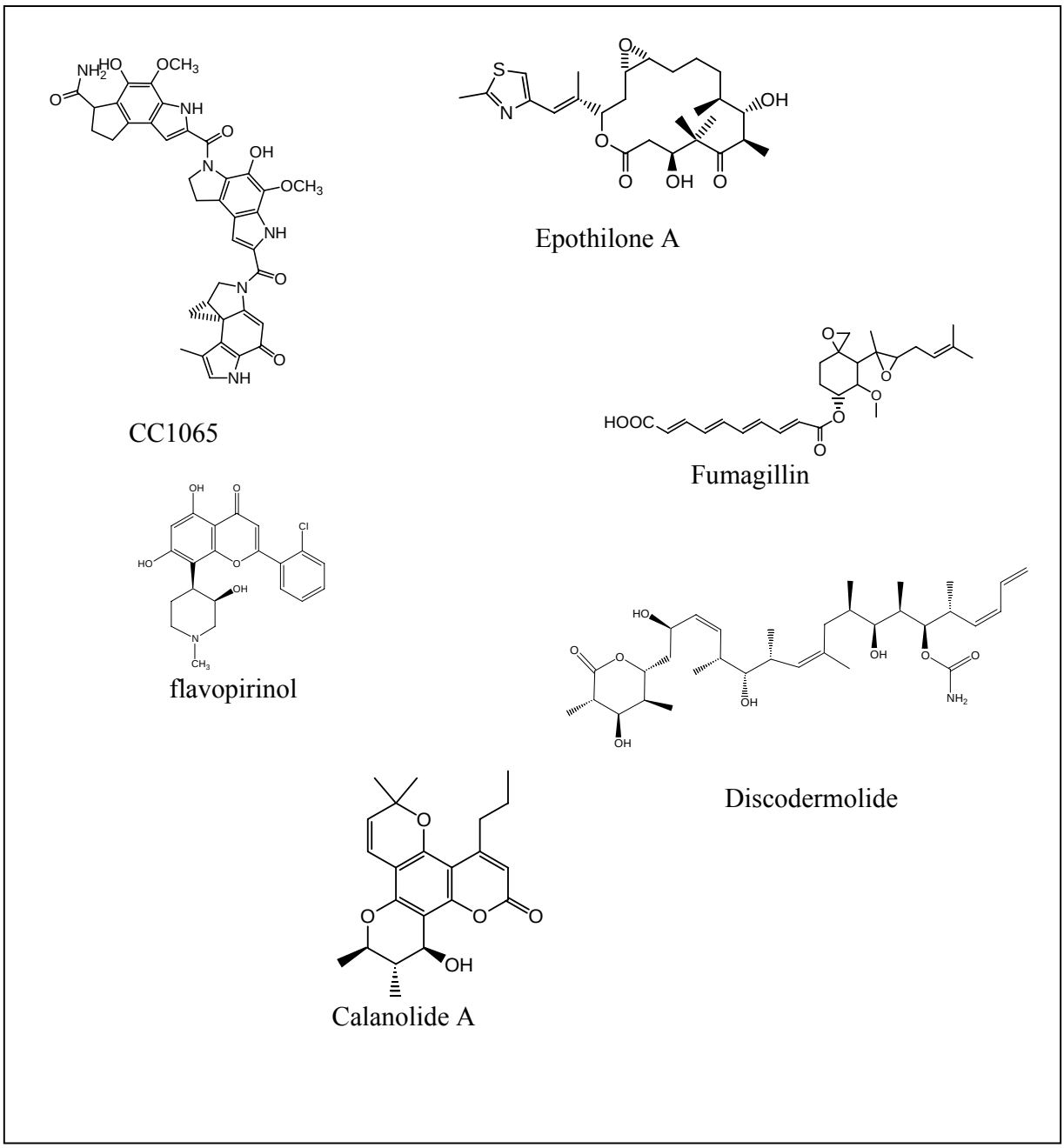


Figure 1 Structures of selected natural products evaluated as new drug candidates

1.2. Marine organisms as sources of drug discovery

1.2.1. Current status of marine natural products research

The ocean covers about 70 % of the earth surface providing a diverse living environment for invertebrates [Lalli and Parsons, 1993]. Therefore, marine natural products will play a major role in drug discovery in the future. The work on marine natural products started 54 years ago when Bergman discovered the novel bioactive arabino-nucleoside from the marine sponge *Cryptotethya crypta* [Bergman and Feeney, 1951]. This discovery encouraged natural products chemists to pay attention to marine natural products as important biomedical sources. Since 1997, there were 713 papers published on marine natural products chemistry [Munro *et al.,* 1999] and 677 new metabolites from marine organisms were reported in 2002 [Blunt *et al*., 2002]. The phylum Porifera has been the most well-studied (**Figure 2)**. In order to survive in a highly competitive environment, marine invertebrates produce a tremendous diversity of extreme toxic compounds. This has stimulated research groups to screen marine samples in various cytotoxicity assays.

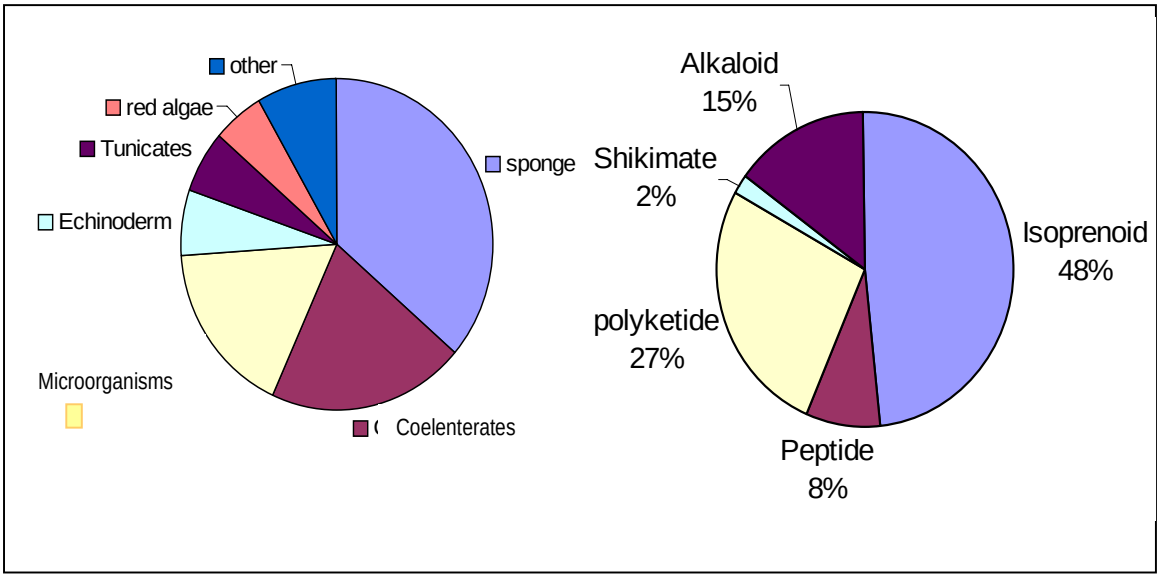
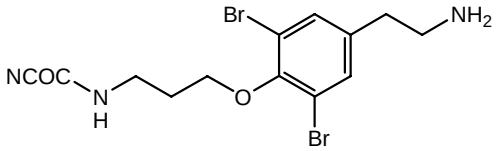


Figure 2 New marine natural products reported in 2002 divided according to phyla (1) and biosynthetic origin (2) (modified from Blunt *et al.*, 2002 and 2004)

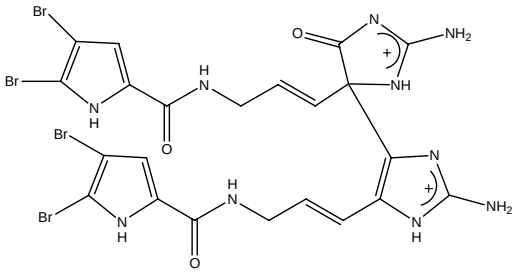
According to the numerous marine natural products reviews in the last 51 years such as those authored by Faulkner, Blunt, Gribble, Tolvanen, and Lounasmaa, marine natural products evaluation were mostly focused on anti-cancer and anti-inflammatory activity. Ecteinascidin 743, which was isolated from a tunicate, is one of the promising marine invertebrate metabolites and is presently in its Phase II clinical trial. This research is a cooperation between the Spanish pharmaceutical company PharmaMar and the American firm Johnson & Johnson [PharmaMar, 2003]. Other compounds such as aplidine (dehydrodinemnin B) and dolastatin 10 originated from a bryozoan. Didemnin B and a tunicate derived compound closely related to aplidine were also reported to be in clinical trial phase II [Munro *et al.*, 1999].

1.2.2. Other applications of marine natural products

Some of the compounds from marine invertebrates initially discovered were either too toxic or not effective in treating diseases for pharmaceutical purposes, but were found to be useful as biological tools or as cosmetic ingredients or as agrochemicals [Fenical, 1997]. The Caribbean gorgonian, *Pseudopterogorgonian elisabethae,* is an example of a source of marine natural product used in the cosmetic industry. The extract from this gorgonian shows anti-inflammatory activity, which nowadays is used as an ingredient in cosmetic skin care products [Proksch, *et al.,* 2002]. Biological tools or biochemical properties have contributed to the understanding of human diseases. Compounds (in case of pharmacological probes) that have high potential to reveal the biochemistry of diseases could be used as biological tools. This is exemplified by ziconotide, a peptide produced by *Conus* mollusk, which potentially blocks the calcium channel. This compound inhibits neurotransmitter release from incoming sensory fibers and spinal cord neurons further transmitting the signal to the brain [Olivera, 2002]. Furthermore, a lot of secondary metabolites are also reported to be anti-fouling and were presented in an excellent review by Fusetani in a natural products report 2004. Examples are (i) bromotyrosine derivative such as ceratinamine which was isolated from *Pseudoceratina purpurea*, and (ii) a bromo-pyrrole derivative, mauritiamine, is an oroidin dimer which was found in the marine sponge *Agelas mauritiana*.



Ceratinamine



Mauritiamine

Table 2 Selected promising marine natural products as drug candidates.

Compound name	Source	Chemical class	Company	Disease area	Status
Compounds targeting ion channel					
Ziconotide	Cone snail	Peptide	Neurex	Chronic pain	-
AM-336	Cone snail	Peptide	AMRAD	Chronic pain	PhaseI/II
GTS-21	Nemertine worm	Anabaseine derivative	Taiho	Alzheimer/ schizophrenia	PhaseI/II
Compounds targeting enzymes. A. Protein kinase inhibitors					
Bryostatin-1	Bryozoan	Polyketide	GPB Biotech	Cancer	Phase I
PLA_2 inhibitors					
OAS-389	Soft coral	Diterpene-pentoseglycoside	Osteroarthritis Science	Wound healing/ inflammation	PhaseI/II
B. Methionine aminopeptidase inhibitors					
LAF-389	Sponge	Amino acid derivative	Novartis	Cancer	Phase I
Manoalide	Sponge	Sesterterpene	Allergen Pharmaceutical	Inflammatory	Phase I
Microtubule interfering agents					
Dolastatin-10	Sea slug	Peptide	NCI/Knoll	Cancer	Phase II
ILX-651	Sea slug	Peptide	Ilex oncology	Cancer	Phase I
Cemadotin	Sea slug	Peptide	Knoll	Cancer	Phase II
Discodermolide	Sponge	Polyketide	Novartis	Cancer	Phase I
DND-interactive agent					
Yondelis™	Sea squirt	Isoquinolone	PharmaMar/ Johnson &Johnson	Cancer	Post clinical trial

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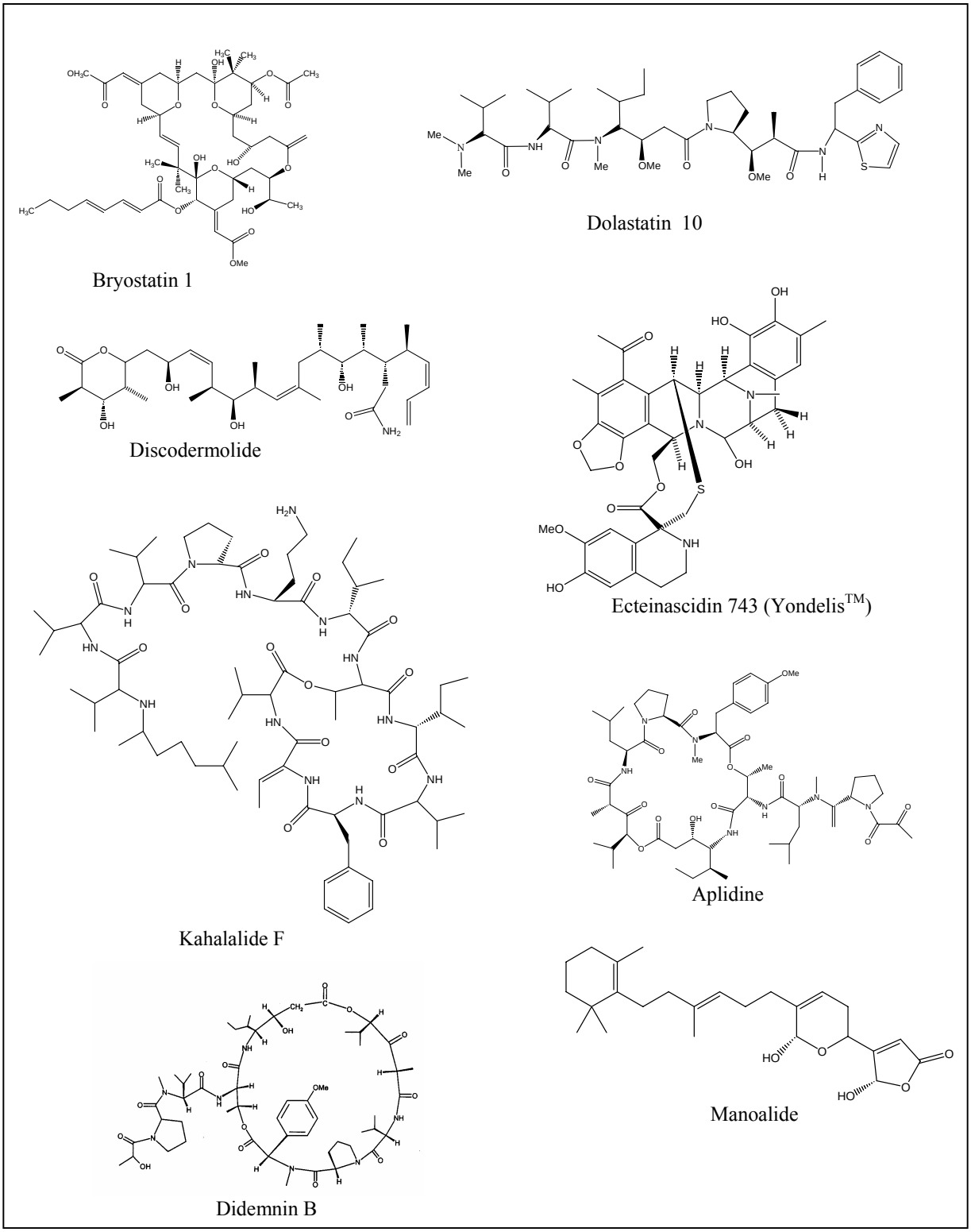


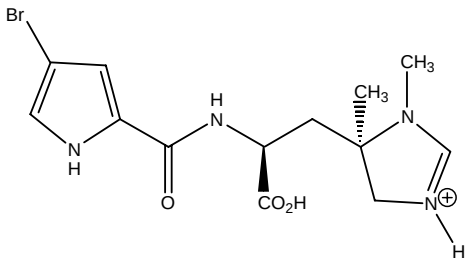
Figure 3 Some chemical structures of marine invertebrate metabolites as new drug candidates

1.3. Sponge chemistry

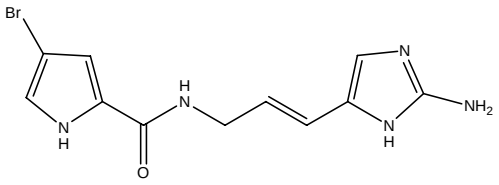
The largest groups of marine invertebrates as a source of secondary metabolites are the sponges. The structurally diverse varieties of metabolites have high therapeutic potential to treat human diseases and have made them worthy of research for marine natural product chemists [Ireland *et al.,* 1993]. Natural products isolated from the phylum Porifera account for 50 % of those reported from marine invertebrates. About 98 % of these metabolites are derived from amino acids, acetogenin, and the isoprenoid pathway [Hooper and Van Soest, 2002]. The phylum Porifera includes three classes which are primarily distinguished by their skeleton characteristics namely; Hexactinellida, Calcarea, and Demospongiae. Class Demospongia have been the largest reported source of secondary metabolites, 5,538 new natural products were described until 2004 [Marinlit, 2004]. 50 % of these metabolites are from the isoprenoid pathway, where 22 % and 25 % are from the acetogenin and amino acid biosynthetic pathways, respectively. The Demospongiae includes three subclasses (Homoscleromorpha, Tetractinomorpha, and Ceracinomorpha) and are composed of 12 orders [Hooper and Van Soest, 2002] of which Halichondria, Haplosclerida, and Dictyoceratida have been reported to contain the highest number of novel secondary metabolites. Secondary metabolites from the order Haplosclerida are evenly distributed among the isoprenoids (32%), acetogenin (39%), and amino acid (29 %) biosynthetic pathways. The acetogenic straight chain acetylenes are typical metabolites of this order [Van Soest *et al*., 1998]. However, the 3-alkylpiperidine amino acid derivatives, ranging from halitoxins to the highly modified manzamine and sararins, have received a great deal of attention from natural products chemists [Harper *et al.,* 2002].

Secondary metabolites produced by dictyoceratida sponges are predominantly of isoprenoid origin (84 %) and distributed within the dictyoceratid families e.g. linear furanosesterterpenes (Ircinidae), sesterterpenes with tetronic acid functional group (Thorectidae), meroterpenoids (Spongiidae) and sesquiterpenes (Dysideae) [Capon and Macleod, 1987; Holler *et al.*, 1997).

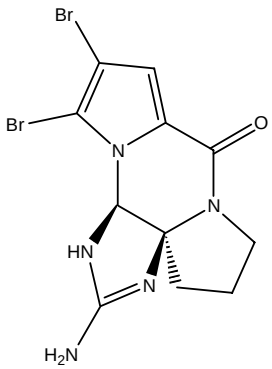
There are many classes of alkaloids which were isolated from marine sponges. However, one interesting group are the bromopyrrole-imidazole alkaloids due to its biological activities and structural diversity. About 90 compounds of this class of alkaloids were characterized [Hoffmann and Lindel, 2003]. These alkaloids included non-cyclized members e.g. oroidin, clathramide A, and hymenidin. The cyclized ones consist of hymenialdisine, cyclooroidin, and dibromophakellin as examples. These are mainly distributed in the families of Agelasidea, Axinellidae, and Halichondridae [Jin, 2005]. Some chemical structures of compounds mentioned above are shown below.



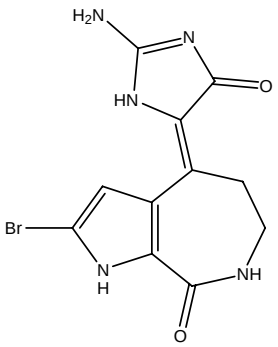
Clathramide A



Hymenidin



Dibromophakellin



Hymenialdisine

1.4. Marine fungi

Studies on marine fungi (mycology) are considerably less published than those of the protists and it is commonly assumed that they do not play a significant role in marine ecosystems [Munn, 2004]. However, the increasing number of secondary metabolites from marine-derived fungi [Faulkner, 2001 and 2002] prove that they are a rich source of bioactive substances of therapeutical potential. Some marine fungi grow and sporulate exclusively in a marine or estuarne habitat (obligate marine fungi) and some grow in fresh water or terrestrial milieu and are able to grow in a marine environment (facultative marine fungi) [Kohlmeyer, 1974]. Marine fungi distribution is mainly limited by dissolved oxygen and water temperature; they play a major role in the decomposition of marine plants [Munn, 2004].

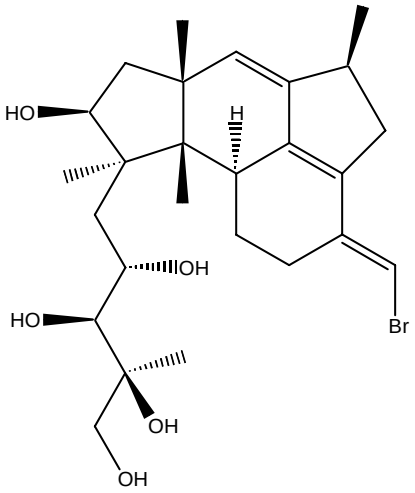
1.4.1. Mangrove fungi

Approximately one fourth of the world's coastline is dominated by mangroves with a high diversity of thriving microorganisms [Sridhar, 2004]. Fungi in mangrove areas consist of genera and species that are common in terrestrial habitats which contain a large number of *Aspergillus* and *Penicillium* spp. The higher mycota or manglicolous fungi on submerged parts of mangrove include 42 species [Kohlmeyer and Kohlmeyer, 1979]. Nevertheless, isolation and purification those fungi need special techniques that are still limited.

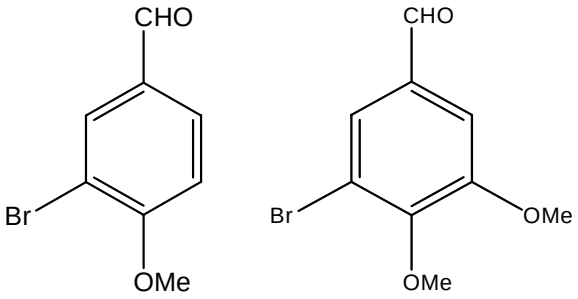
1.4.2. Secondary metabolites from marine-derived fungi

Until 1990 when the first marine-derived fungi antibiotic, siccayne, was reported [Bugni and Ireland, 2004], chemical studies of marine-derived fungi were rare. Afterwards, 272 new secondary metabolites isolated from marine-derived fungi has led to the discovery of novel carbon

skeletons providing important evidence that marine-derived fungi are a potential rich source of pharmaceutical leads [Bugni and Ireland, 2004]. The secondary metabolites from fungi were reviewed by Faulkner 2002-2004, and then Bugni and Ireland reported the distribution of marine-derived fungi as shown in **Figure 4**. Furthermore, several marine-derived fungi produce organo-metallic compounds such as the first bromophenols reported in marine sediment [Gordon, 1999] and the novel sesterterpene neomangicol B from the marine fungus, *Fusarium* sp. [Renner *et al.,* 1998].



Neomangicol B



Bromophenol compounds

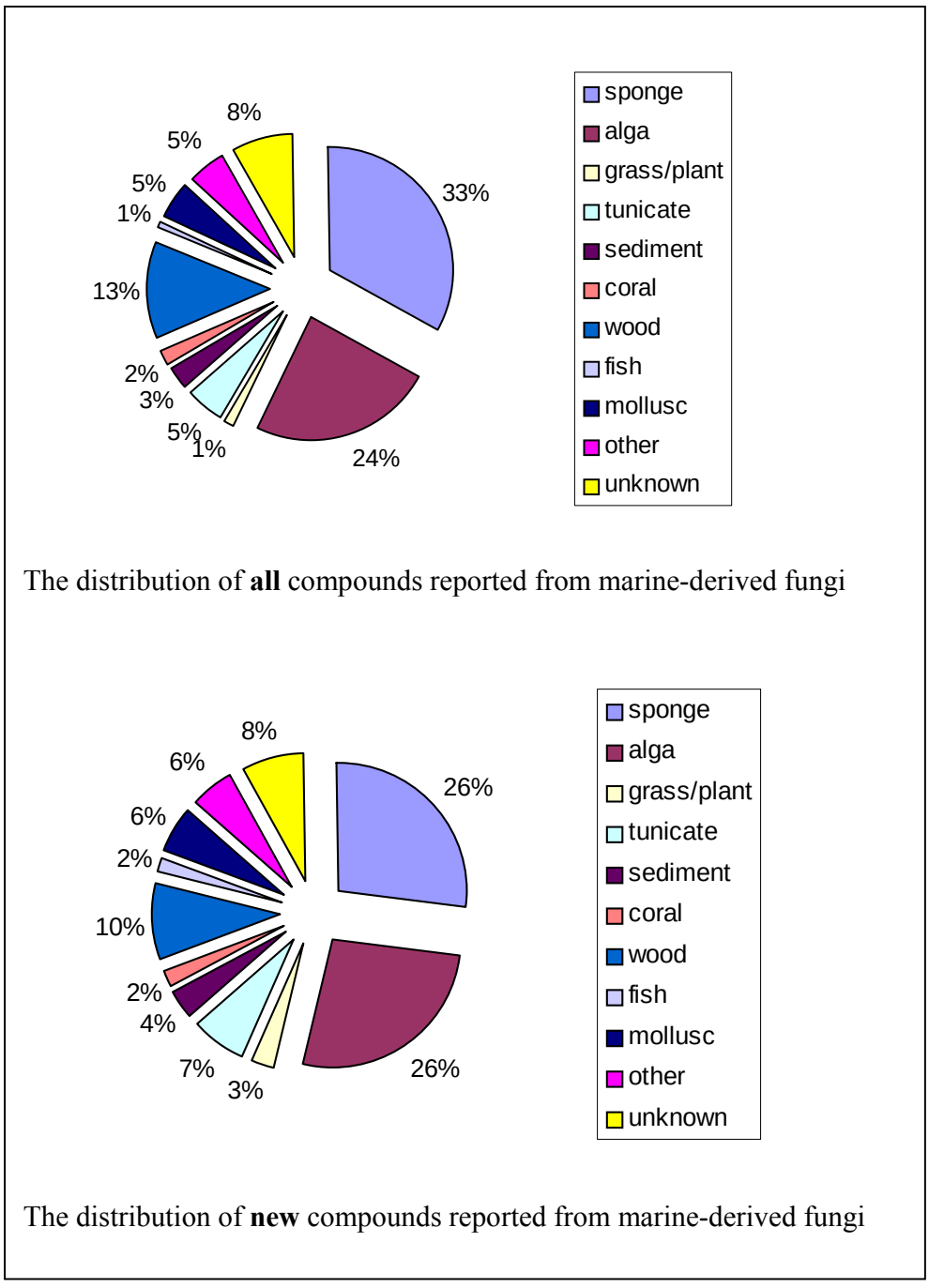


Figure 4 Distribution of marine-derived fungi. A comparison between all and new natural products produced by marine-derived fungi (Bugni and Ireland, 2004).

1.5. Significance of the study

1. Lead identification is an important part of the drug discovery process. This increases the diversity of active prototype molecules which can be candidates for new drugs. Terrestrial and marine environments are challenging sources of a variety of novel biologically and pharmacologically active compounds.

2. Marine invertebrates are a rich source of biologically active secondary metabolites. The biosynthesis of secondary metabolites by these invertebrates has been speculated as a result of their physical and biochemical adaptation to their environment. In the last two decades, many new compounds were isolated from these organisms and have been promoted as candidates for the development of new drugs, especially as anti-cancer drugs. There is therefore, a need to continue research for discovery of novel secondary metabolites from marine invertebrates.

3. Marine metabolites have a wide application not only for pharmaceutical purposes but also for biological tools. Hence, there is a need for a continuous intensive research in marine natural products chemistry.

1.6. Aim of the study

The aim of this research is to isolate and structurally elucidate biologically active secondary metabolites from Indopacific marine sponges and mangrove fungi.

1.7. Scope of the study

1. Bioactivity-guided fractionation using antimicrobial and cytotoxic activities using different cell lines.
2. The isolation strategies supported by biological activities and spectral analysis.
3. Characterization of isolated compounds using NMR and MS analysis.

2. Materials and methods

2.1. Animal materials

Marine sponges were collected in the Andaman Sea, Thailand and Bali Island, Indonesia by SCUBA diving at the dept of 40-70 feet. The samples were kept in sealed plastic bags then preserved in a freezer at a temperature of -20 °C. Mangrove fungus was collected from SiKao mangrove forest, Trang Province, Thailand. Mangrove sediments were collected at 10 centimeters below the soil surface by core sampling and kept in a sealed sterile plastic bag at a temperature of -4 °C prior to isolation and purification of the marine fungus. Six sponges and one mangrove fungus were investigated in this study. All sponge samples were identified by Dr.Rob W.M. van Soest, Zoologisch Museum, Amsterdam, the Netherlands.

2.1.1. Sponges collected from the Andaman Sea, Thailand

1 *Dragmacidon* sp.

Taxonomic data

Phylum : Porifera

Class : Demospongiae

Order : Hadromerida

Family : Axinellida

Genus : *Dragmacidon*

Species : undescribed

Dragmacidon sponges have a lumorecticulate choanosomal skeleton and an undifferentiated axial and extra-regions. The surface is more or less smooth with a short conule or tubercle. It has multi-spicular spongin fiber. Voucher specimen number ZMA.POR.16782 is deposited at the Zoologisch Museum, Amsterdam, the Netherlands.

2. *Stylissa flabelliformis*

Taxonomic data

Phylum : Porifera

Class : Demospongiae

Order : Hadromerida

Family : Dictyonellidae

Genus : *Stylissa*

Species : *Flabelliformis*

The spongin fiber skeleton of *Stylissa Flabelliformis* consists of styles arranged in a confused plumose reticulation with many single spicules in confusion. The sponge is erect, flabellate, or compressed-lobate with irregularly conulose and/or a ridged surface. The surface is also smooth between the conules. Choanosomal skeletons have a slightly condensed axis and differ between axial and extra-axial skeletons. Voucher specimen (ZMA.POR.17287) is deposited at the Zoologisch Museum, Amsterdam, the Netherlands.

3. *Dysidea granulosa* Bergquist

Taxonomic data

Phylum : Porifera

Class : Demospongiae

Order : Dictyoceratida

Family : Dysideae

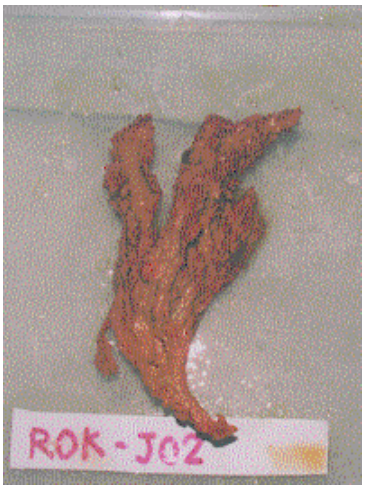
Genus : *Dysidea*

Species : *granulosa*

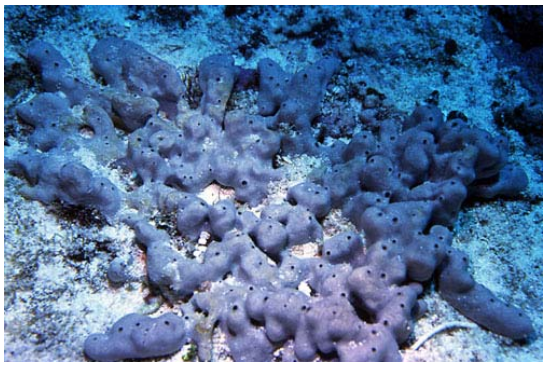
The *Dysidea* sponge has a thick encrusting, massive, or a branching growth form, often with a marked conulose. The skeleton consists of a regular, usually rectangular, arrangement of concentrically laminated primary and secondary fibers. Textures are usually soft and compressible. Voucher specimen (ZMA.POR.17286) is deposited at the Zoologisch Museum, Amsterdam, the Netherlands.

Figure 5 Sample collection sites in Thailand.

Dragmacidon sp.



Stylissa flabelliformis



Dysidea granulosa

Figure 6 Sponge materials collected in Thailand.

2.1.2. Sponges collected in Indonesia

1 *Aaptos suberitoides*

Taxonomic data

Phylum : Porifera

Class : Demospongiae

Order : Hadromerida

Family : Tethyidae

Genus : *Aaptos*

Species : *suberitoides*

The sponge has radiated skeleton of strongyloxeas with three size categories such as the small forms, dense ectosomal palisade, or no microscleres. It shows encrusting or massive growth forms. The basal surface has root-like papillae. They are asexual reproduction by budding. The texture is smooth and mucous. Voucher specimen (ZMA.POR.17716) is deposited at the Zoologisch Museum, Amsterdam, the Netherlands.

2. *Agelas nemoecinata*

Phylum : Porifera

Class : Demospongiae

Order : Agelasida

Family : Agelasidae

Genus : *Agelas*

Species : *nemoecinata*

The sponge skeletons are made up of fibers cored and ectinated by a verticilliate megascleres. These sponges have large spongin fibers with unique style of spicules. They are four growth forms including ramose, lamellate, tubular, and massive growth form. Internal textures are extremely tough. Normal color of these sponges are orange or red. Voucher specimen (ZMA.POR.17715) is deposited at the Zoologisch Museum, Amsterdam, the Netherlands.

3. *Pseudoceratina purpurea*

Phylum : Porifera

Class : Demospongiae

Order : Verongida

Family : Pseudoceratinidae

Genus : *Pseudoceratina*

Species : *purpurea*

The sponge fiber skeleton is organized on a dendritic plan. Pith elements are only present in the fibers. The matrix of the sponge is extremely dense and heavily reinforced by collagen. Sponge surface is smooth and firm. Voucher specimen (ZMA.POR.17800) is deposited at the Zoologisch Museum, Amsterdam, the Netherlands.

Figure 7 Sample collection sites in Indonesia.

2.1.3. Mangrove fungus, *Eurotium clevalieri*

The mangrove sediment was spread on the surface of a malt agar plate and incubated at 27 ºC. In order to get a pure mono-culture of the fungus, purification through several sub-cultures onto fresh malt agar plates were repeatedly carried out. The collected fungi were maintained on malt agar plates using the Wickman medium. For getting rid of bacterial contaminants, chloramphenicol (2 g/l), streptomycin sulphate (0.1 g/l) and penicillin G (0.1 g/l) were added to the medium. When a pure mono-culture of the fungus is obtained, the fungus was then stored in the refrigerator at a temperature of 4 ºC for long-term storage. In order to keep the fungi collection alive, they were transferred periodically (every three months) to a fresh media. Prior to screening of biological activity, the fungi were cultured in a liquid media (300 ml). After a certain period of incubation, particularly when the rapid growth had ended, the fungi were then harvested.

Mycelium and broth were primarily extracted with ethyl acetate. The raw extracts were then screened for antimicrobial activity. Along with biological screening assay, an aliquot of the raw extract was also subjected to HPLC-DAD and LC-MS analysis to gain an overview of the chemical constituents present in the samples. The fungus was sent to Centraalbureau Voor Schmimmelcultures, Baarn, the Netherlands for identification. The fungus was identified are described below.

Phylum : Eumycota

Subphylum : Ascomycotina

Class : Plectomycetidae

Order : Eurotiales

Family : Eurotiaceae

Genus : *Eurotium*

Species : *clevalieri*

Aaptos suberitoides

Pseudoceratina purpurea

Agelas nemoecinata

Figure 8 Sponge materials collected in Indonesia.

2.2. Chemical used

2.2.1. General laboratory chemicals

Agar-Agar	Merck
Anisaldehyde (4-methoxybenzaldehyde)	Merck
(-)-2-butanol	Merck
Dimethylsulfoxide	Merck
Formaldehyde	Merck
Hydrochloric acid	Merck
Potassium hydroxide	Merck
Potassium iodide	Merck
Concentrated sulfuric acid	Merck
Trifluoroacetic acid (TFA)	Merck
Glacial acetic acid	Merck
Bismuth (III) nitrate	Merck
Sulfuric acid	Merck
Ethanol	Merck
Propanol	Merck
Ninhydrin G.R.	Merck

2.2.2. Chemical reagents

2.3.2.1 Dragendorff reagent: for detection of alkaloids and other nitrogen containing compounds.

Preparation method

Solution A: 0.85 g bismuth (III) nitrate was dissolved in 10 ml of glacial acetic acid and 40 ml water.

Solution B: 8 g potassium iodide was dissolved in 20 ml water.

Stock solution: equal parts of A and B were mixed. The mixture can be stored in a amber-colored bottle for longer period time.

Spray solution: before use, 1 ml of the stock solution was mixed with 2 ml of glacial acetic acid and 10 ml water.

2.2.2.2. Anisaldehyde - sulfuric acid: detection reagent for sugar, steroids, and other terpene compounds.

Preparation method

Spray solution: 0.5 ml anisaldehyde was mixed with 50 ml of glacial acetic acid and 1 ml of 95 % sulfuric acid was added.

Treatment: The plates were sprayed then heated at 100 - 105 ºC until maximal intensity of spots were observed. The background color may be brightened by water vapor. Lichen constituents, phenols, terpenes, sugars, and steroids turn violet, blue, red, grey, and green.

Modified spray solution: For visualization of sugars; 0.5 ml anisaldehyde was mixed with 9 ml of ethanol, 0.5 ml of 97 % sulfuric acid, and 0.1 ml acetic acid was added.

Treatment : The sprayed chromatogram was heated for 5-10 minutes at 90-100 °C

2.2.2.3. Ninhydrind (II) chloride: for detection of amino acids

Preparation method

2 g ninhydrin was dissolved in 40 ml water under heating and a solution of 0.08 g tin (II) chloride in 50 ml water was added. The precipitate was filtered off and the stock solution was preserved at 8 ºC.

Spray solution: 50 ml water and 450 ml 2-propanol were added to 25 ml of the stock solution.

2.3. Solvents

Acetone

Acetonitrile

Cyclohexane

Dichoromethane

Ethanol

Ethyl acetate

Hexane

Methanol

All solvents were distilled prior to use and spectroscopic grades were used for spectroscopic experiment.

2.4. Equipment used

Balance	: Mettler 200
	: Mettler At 250
Centrifuge	: Kendro D-37520 osterde
Fraction collector	: Retriener III SCO
Freeze dryer	: LYOVAC GT2
	Pump TRIVAC D10E
Hot plate	: Camag
Syringe	: Hamiltom 1701 RSN
Mill	: Molinex 354
PH-Electrode	: Inolab
	Behrotest PH 10-Set
Rotary Evaporator	: Buchi Rotavapor R-200
	: Heating Bath B-490
Pump	: Vaccubrand CVC2II
Drying Oven	: Heraeus T5050
Sonicator	: Bandelin Sonorex PK 102
Speed Vac	: Savant SPD111V
UV-Lamp	: Camag (254 and 366 nm)
Nitrogen generator	: Nitrox UHPN3001

2.4.1. HPLC equipment

2.4.1.1. Analytical HPLC

Pump : Dionex P580A LPG

Detector : Dionex, photo diode array detector UVD 340S

Column : Thermostat STH 585

Auto sampler : ASI-100T

Software : Chromeleon Ver 6.3

2.4.1.1. Semi-preparative HPLC

Pump : Merck-Hitachi L-7100

UV detector : L-7400

Column : Eurospher-100, 8mm

Recorder : Flatbed Recorder Kipp & Zonnen

: Merck-Hitachi, D-200 Chromato integrator

2.4.1.2. Preparative HPLC

Pump : Varian Prestar 218

Detector : UV-VIS 320, Photodiode array

2.4.1.3. Liquid Chromatography/Mass Spectrometry (LC/MS)

LC/MS: High liquid pressure chromatography (HPLC) is a powerful tool for separation of complex mixtures. When a mass spectrum of each component can be recorded as it elutes from the LC column, quick characterization of the components is greatly facilitated. Usually, ESIMS is interfaced with LC to make an effective online LC/MS. HPLC/ESI-MS was carried out using a Finnigan LCQ DECA-7000 mass spectrometer connected to a UV detector. The sample was dissolved in a water/MeOH mixture and injected to a HPLC/MS set up. HPLC was run on a Eurospher C-18 reversed phase column. The measurements were done at the Institute of Pharmaceutical Biology and Biotechnology, HHU Duesseldorf, Germany. For standard measurements, a linear gradient of 10% to 90% acetonitrile in combination with nanopure water with 0.1% formic acid in 35 minus was used.

2.5. Chromatography Methods

2.5.1. Thin Layer Chromatography (TLC)

TCL was performed on a precoated plates with Si-gel F_{254} (layer thickness 0.2 mm, Merck, Darmstadt, Germany) as stationary phase. Liquid mobile phases were either semi-polar (CH_2Cl_2 : MeOH; 9:1,v/v) or non polar (Hexane : EtOAc; 8 : 2 ,v/v). Reversed phase (RP) was used for polar fractions. The TLCs were performed on precoated plates of C18 F_{254} (layer thickness 0.25 mm, E. Merck, Darmstadt, Germany) as stationary phase. The mobile phase systems were MeOH : H_2O; 3:7, 8:2 and 1:1 (v/v).

A one-dimensional ascending development technique was used to detect the constituents of an extract on TLC plate. Visual detection was done in daylight and under UV

light at a wave length of 254 and 344 nm depending on the group of compounds investigated. The separated compounds were also detected by spraying with a variety of chemical reagents previously described.

2.5.2. Column Chromatography

In this study, three different kinds of columns were used. The columns differed in their packing material used as stationary phase.

- Normal phased Column Chromatography: Si-gel F_{254} with a particle size of 0.004 – 0.063 mm or 230-400 mesh (Merck) was used as stationary phase. Combinations of organic solvents such as hexane, dichloromethane, and methanol were used by step gradient or isocratic elution.

- Reversed Phase Column Chromatography: RP-18 column was eluted step gradient starting with 60% MeOH in water which was increased to 100 % MeOH. For separation of fractions of low quantities a prepacked Lobar column was used.

-Gel Permeation Chomatography on Sephadex LH-20 material: this technique was applied for separations of mixtures containing different sizes of molecules. The gel material was suspended in an appropriate solvent and packed into a glass column. The column height was 80-90 cm had to be equilibrated for 12-24 hrs before sample was loaded.

2.5.3. Vacuum Liquid Chromatography (VLC)

Because it is fast and has a efficient separation power, VLC is a useful method for fractionations of complex extracts in large amounts. VLC can be used in routine work-up as an initial separation procedure. General setup of the technique is described as follows:

Adsorbent : Si-gel 60 (230-400 mesh ASTM, Merck)

Packing : The adsorbent was suspended in hexane in a sintered glass filter column (diameters 6 cm) at a height of 10-15 cm.

Loading the sample: The sample was dissolved in an appropriate solvent and mixed with the solid phase (Si-60) material. The solvent is evaporated and the dry sample mixture was loaded on top of the adsorbent. The mobile phase eluted through the column under vacuum. The separated fractions were collected in round bottom flasks and routinely checked by TLC.

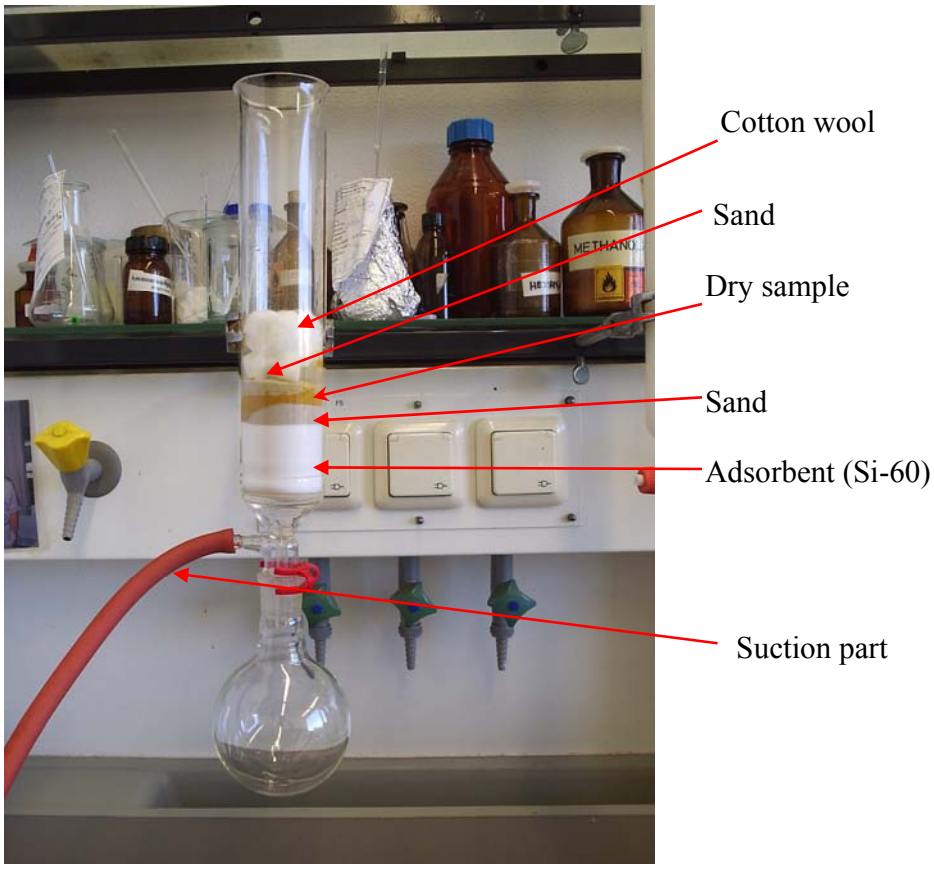


Figure 9 Vacuum Liquid Chromatography

2.5.4. Semi-preparative HPLC

Semi-preparative HPLC was used for the purification of the isolated compounds from complex and nearly pure fractions. Each injection was concentrated to 3 mg/ml and the maximum injection volume was 1 ml. The flow rate was set to 5 ml/min.

2.5.5. Preparative HPLC

Preparative HPLC was applied for mixtures of large quantities. The maximum injection concentration was set to 30 mg/ml and 5 ml were injected. The separation efficiency was monitored in a semi-preparative HPLC prior to use of a preparative column. The flow rate was calculated from the preliminary tests on a semi-preparative column then multiplied with a constant factor.

2.5.6. Medium Pressure Liquid Chromatography (MPLC)

Medium Pressure Liquid Chromatography (MPLC) was introduced in 1979 for separation of diateromethic oxasoline (Top, 1979). The technique inquires a pressure of 50-105 bar, which properly separates larger amount of samples (100 mg – 100 g). The separation efficiency depends on the mixture complexicity, solvent combination, and the pressure introduced.

2.5.7. Analytical HPLC

Analytical HPLC was used for identification of the constituents in the fractions and for checking the degree of purity of isolated compounds. Different gradient programs were used as follows:

- Starting with 100 % H_2O and increased to 100 % methanol in 35 minutes
- Starting with 90 % H_2O and increased to100 % methanol in 35 minutes.
- Detection was performed under four UV wavelengths, 235, 254, 280, and 340 nm.

Preparative HPLC (Varian)

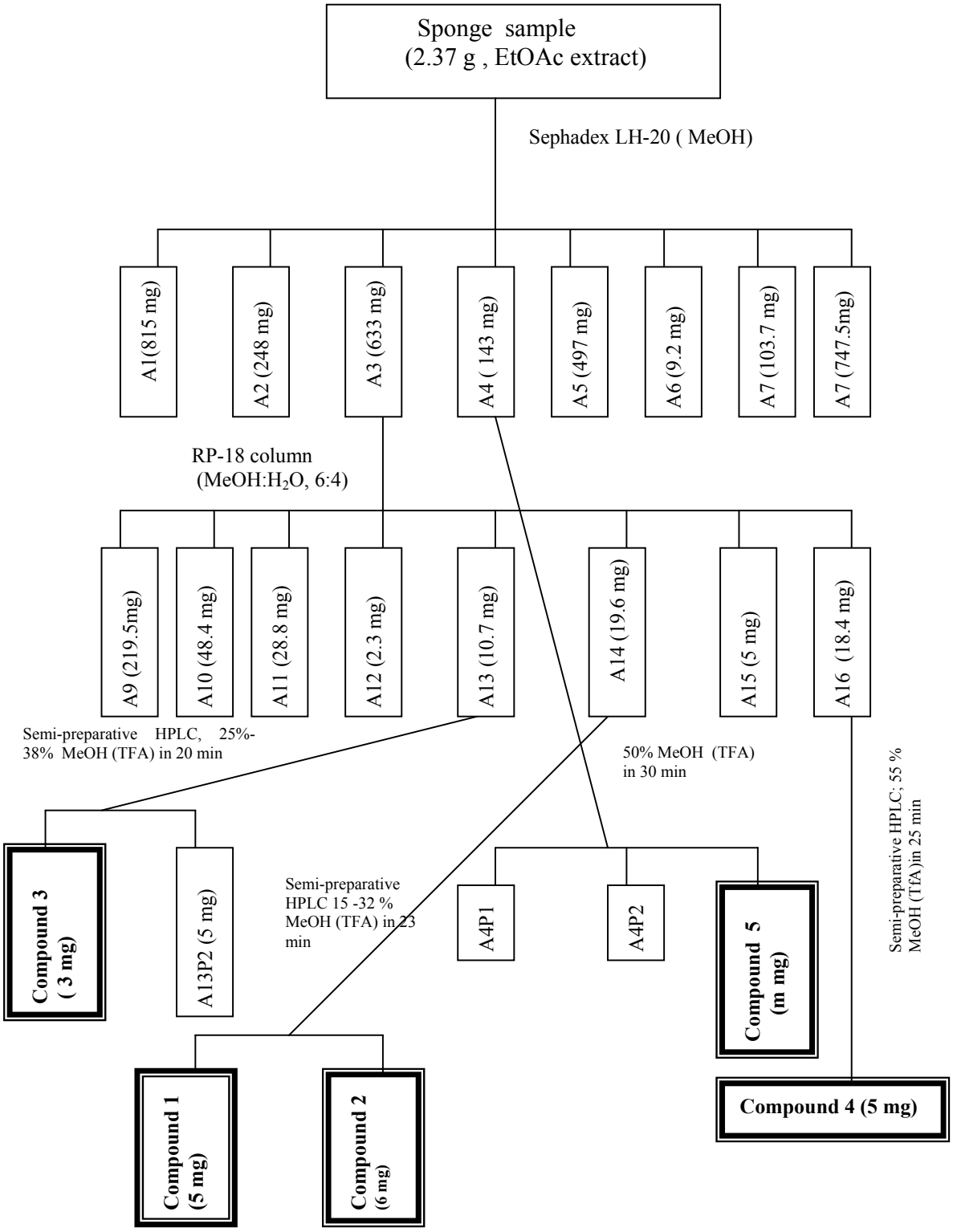
Preparative Column

Semi-preparative HPLC columns

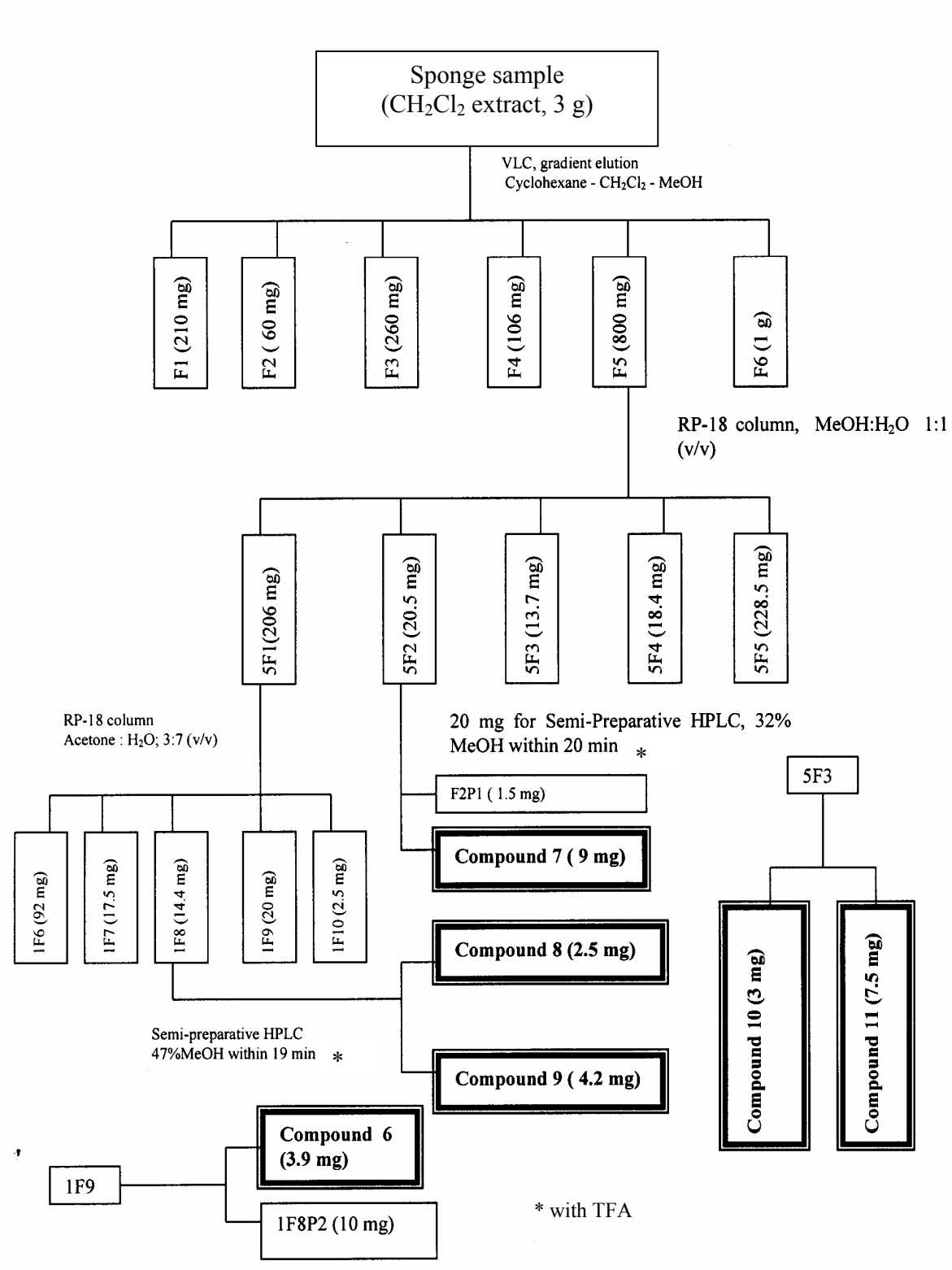
Medium Pressure Liquid Chromatography

Figure10 Chromatography equipment used for natural products isolation

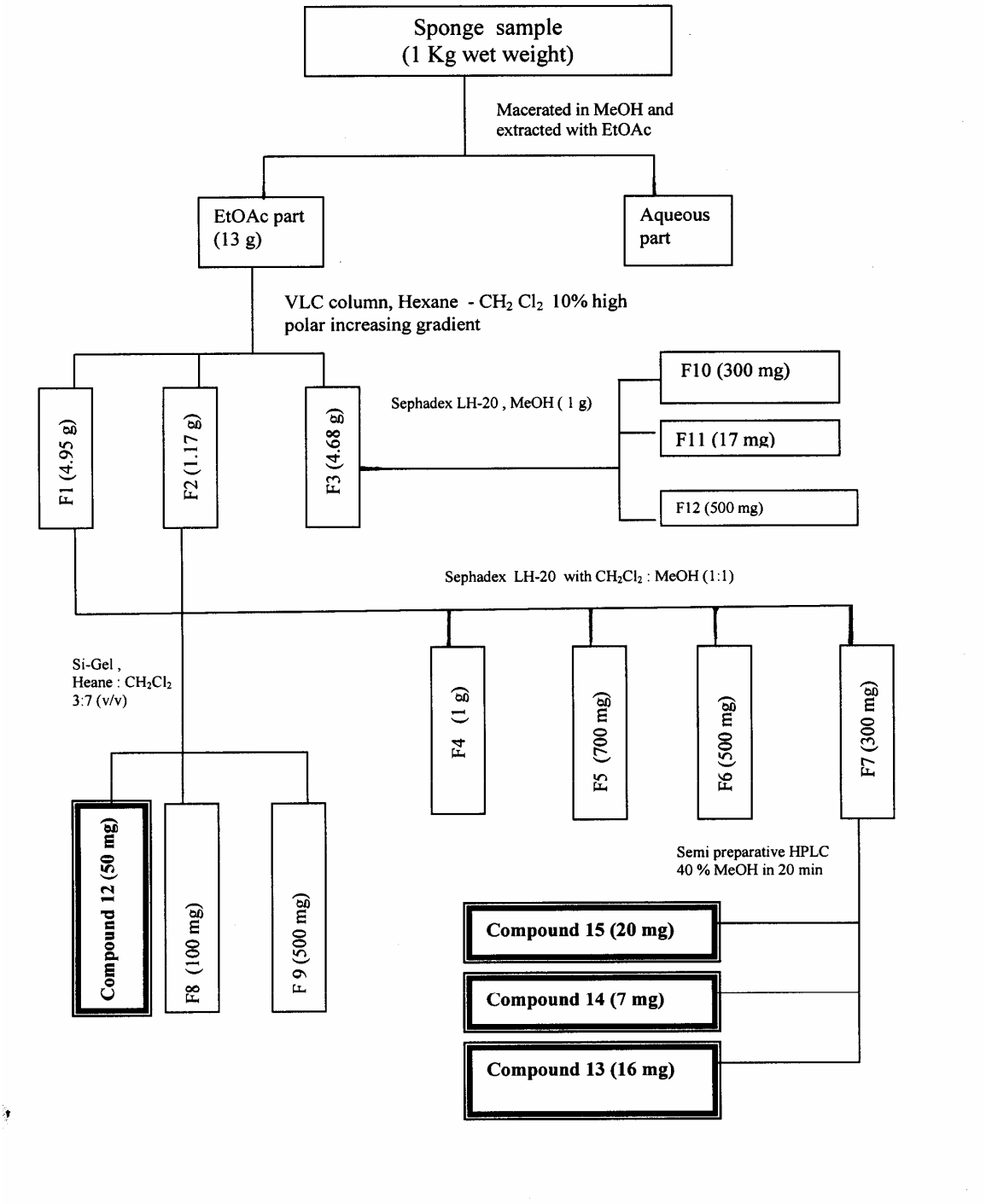
2.6. Isolation procedure of secondary metabolites.



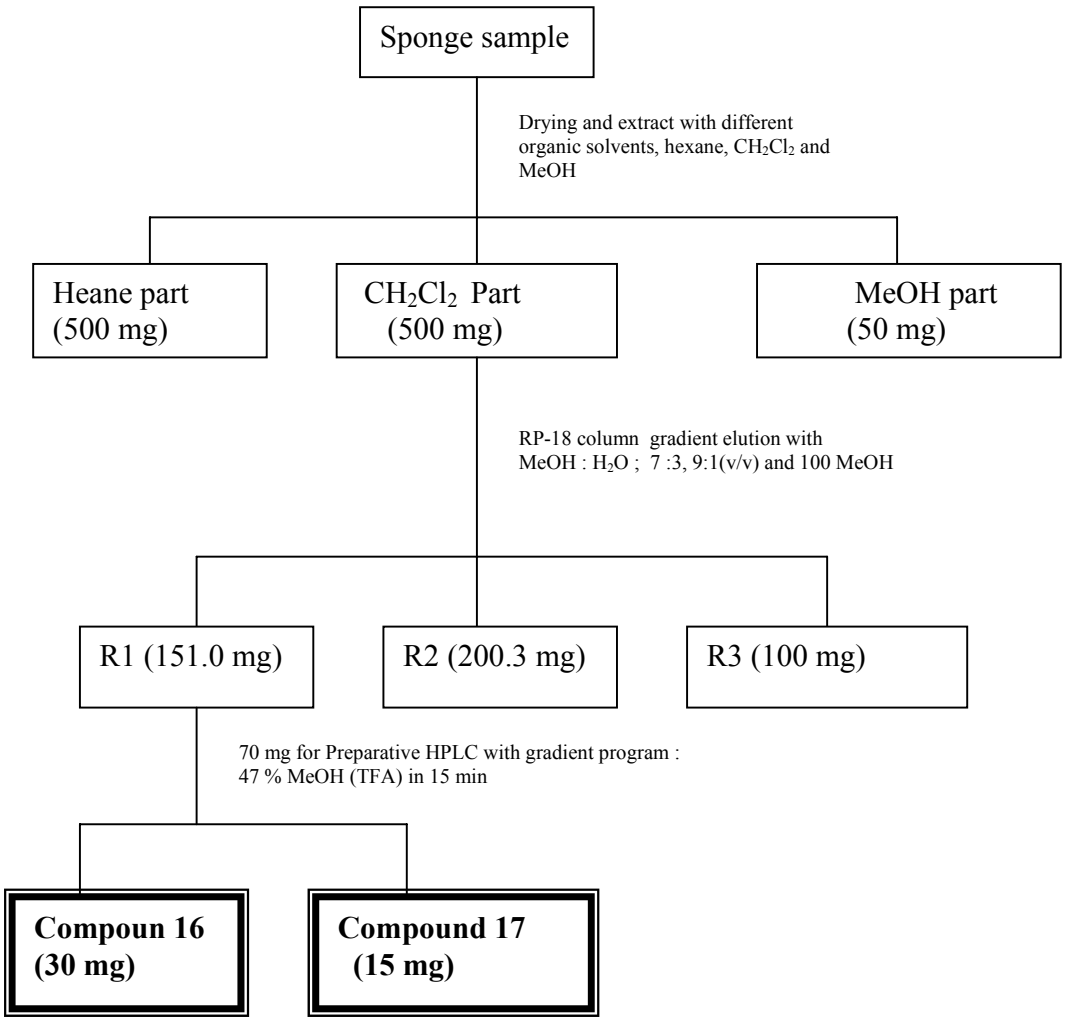
Scheme 2 Isolation procedure of compounds from marine sponge *Dragmacidon* sp.



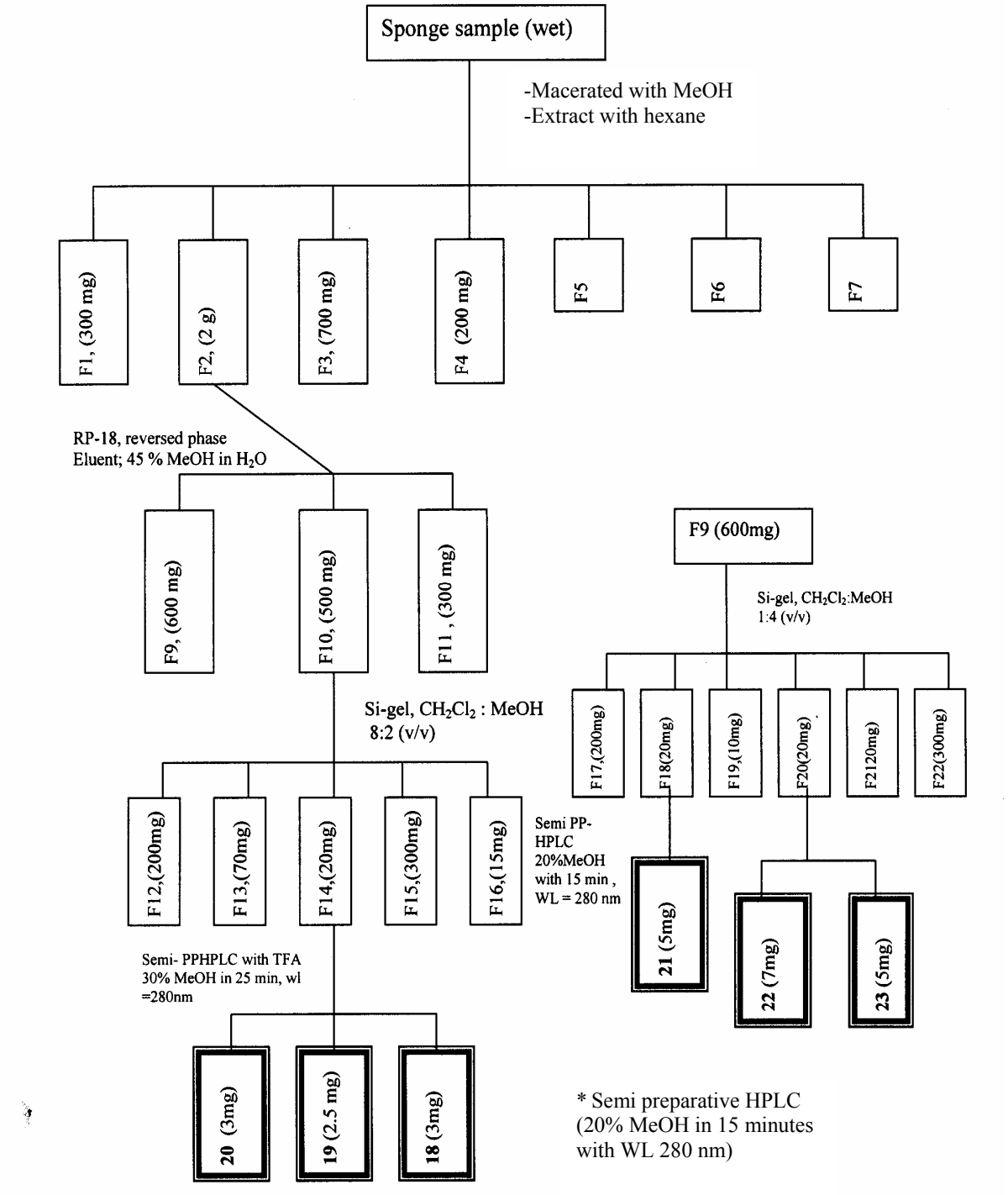
Scheme 3 Isolation procedure of compounds from marine sponge *Stylissa flabelliformis*



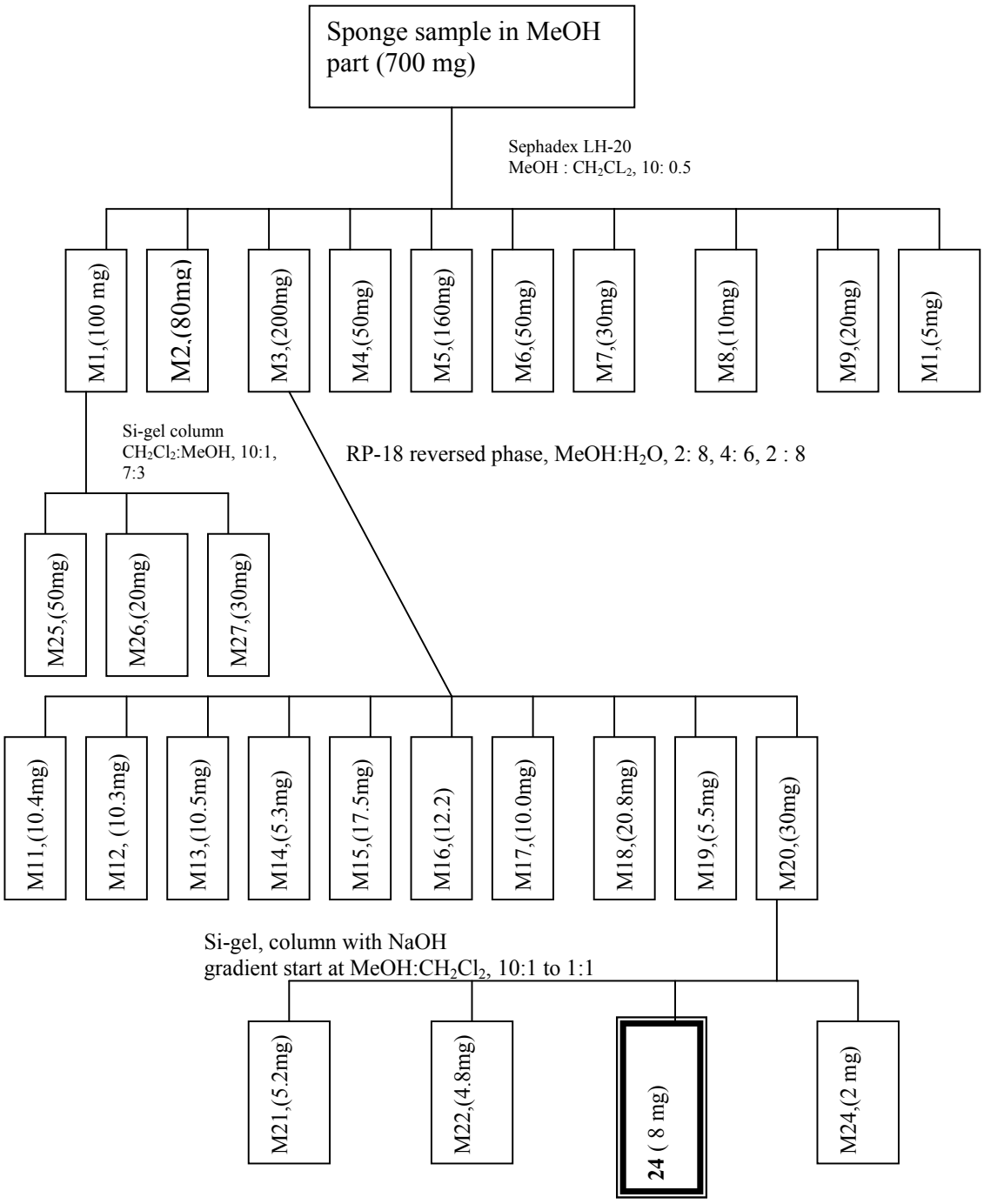
Scheme 4 Isolation procedure of compounds from marine sponge *Dysidea granulosa*



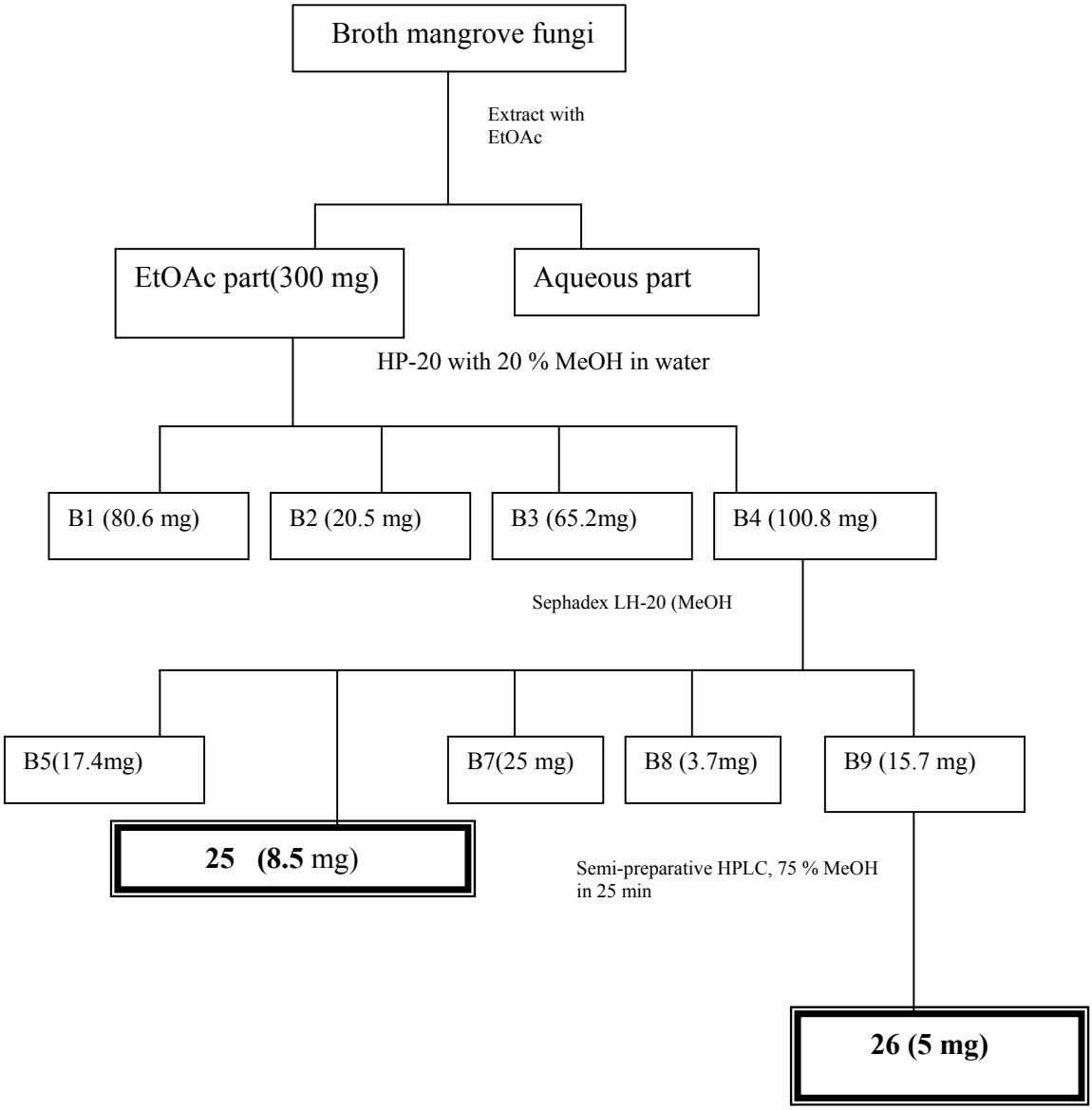
Scheme 5 Isolation procedure of compounds from marine sponge *Aaptos suberitoides*



Scheme 6 Isolation procedure of compounds from marine sponge *Agelas nemoecinata*



Scheme 7 Isolation procedure of compounds from marine sponge *Pseudoceratina purpurea*



Scheme 8 Isolation procedure of compounds from mangrove fungus, *Eurotium chevalieri*

2.7. Structure elucidation of isolated compounds

2.7.1. Mass spectrometry (MS)

As low resolution MS,ESI, EI, and FAB-MS were used.

EIMS (Electron Impact Mass Spectrometry): The compound is vaporized in an evacuated chamber (ca 10^{-6}-10^{-5} torr) and then bombarded with electrons with an energy of 25-80 eV (2.4-7.6 MJ/mol). The high energy electron current causes a valence electron to be ejected from the compound, which generates a cation-radical molecular ion $[RH]^{+\cdot}$. The high energy electron current do not only ionizes the organic molecule but also cause extensive fragmentation. It gives a fragmentation pattern which is used for characterization of compounds. The disadvantage of this experiment is the frequent absence of a molecular ion. The experiment was performed on Finnigan MAT 8430 by Dr. Peter Thommes, Institue of Anorganische and Struktur Chemie, Heinrich Heine University Duesseldorf, Germany.

ESIMS (Electron Spray Ionization): This is a method for ejecting ionized molecules from a solution by creating a fine spray of highly charged droplets in the presence of a strong electronic field. This type of ionization is highly conducted to form of multiple-charged molecules, for example $[M+H]^+$, $[M-H]^-$, and $[M+Na^+]$. The experiment was performed on Finnigan LCQ. This instrument is an ion trap mass spectrometer capable of both single stage MS/MS and multiple stage MS^n analyses having an APCI or ESI source. The masses that can be measured ranges from m/z 50 to 2000. The LCQ DECA was coupled to a Hewlett Packard 1100 HPLC for tandem LC/MS/MS applications. Xcalibur software integrated all instrument operations and could assist acquisition by performing data dependent scans for an optimum MS/MS spectra collection.

FABMS (Fast Atom Bombardment Mass Spectrometry): This is a useful method for polar molecules up to 20 KDa. It enables both non-volatile and high molecular weight compound to be analyzed. In this technique, a solution of the sample placed in a low-viscosity matrix is bombed with neutral fast heavy atoms (Xe, Ar, 7 keV), and both positive and negative can be obtained from a FABMS spectra.

High Resolution MS (HRMS): The HRMS is achieved by passing an ion beam through an electrostatic analyzer before it enters to magnetic sector. By using double focusing, the mass of an ion can be determined to an accuracy of approximately 0.0001 mass units (around 1 ppm). HRMS was determined at the GBF, Braunschweig, Germany.

2.8. Nuclear Magnetic Resonance Spectroscopy (NMR)

NMR measurements were done at the Institute for Anorganische und Makromoleculare Chemie at the HHU Duesseldorf and GBF, Braunschweig. ^{1}H and ^{13}C spectra were recorded at 300K° on Bruker DPX 300, ARX 400, 500 and DMX 600 NMR spectrometer. TMS was used as internal standard and all 1-D and 2-D spectra were obtained using the standard Brucker software. The samples were dissolved in a suitable solvent such as DMSO-d_6, CD_3OD and $CDCl_3$. Chemical shift values, δ, were given in ppm and the coupling constant, *J*, in Hz.

2.9. The optical activity

Optical rotation was conducted on Perkin-Elmer-241 MC Polarimeter by measuring the angle of rotation at the wavelengths of 546 and 579 nm. A mercury vapor lamp was used and the sample was run at an ambient temperature of 25 °C in a 0.5 ml cuvette with 0.1 dm length. The specific optical rotation was calculated by following equation;

$$[\alpha]_D^{20} = \frac{[\alpha]_{579} \times 3.199}{4.199 - \frac{[\alpha]_{579}}{[\alpha]_{546}}}$$

Where $[\alpha]_D^{20}$ = the specific rotation at the wave length of sodium D-line, 589 nm, at the temperature of 20 °C

$[\alpha]_{579}$ and $[\alpha]_{546}$ = the optical rotation at the wave length 579 and 546 nm, were calculated respectively by using following formula

$$[\alpha]_\lambda = \frac{100 \times \alpha}{1 \times c}$$

Where α = the measurement angle of the rotation in degrees

I = the length in dm of the polarimeter tube

c = the concentration of the substance expressed in g/100 ml

2.10. Bioactivity studies

2.10.1. Anti-microbial activity

The crude extracts and pure compounds were tested for anti-microbial activity against bacteria; *Bacillus subtilis* and *Escherichia coli*, yeast, *Sacharomyces cerevisiae* and phytopathogenic fungi; *Cladosporium herbarum* and *C. cucumerinum*.

Culture preparation: The agar diffusion assay was performed according to the Aauer Kirby-test (DIN 58940, Bauer *et al.*, 1996). Prior to testing, few colonies (3-10) of the microorganism used in the bioassay, were sub-cultured in 4 ml of tryptose-soy broth medium (Sigma, FRG) and incubated for 2 -5 hours. This was done to produce a bacterial suspension of moderate cloudiness. The suspension was diluted with a saline solution to a density visually equivalent to a $BaSO_4$ standard.

Agar diffusion assay: Aliquots of the test solution were applied to sterile filter-paper discs (5 mm diameter, Oxoid Ltd.) using a final disc loading concentration of 500 µg for the crude extract and 50 and 100 µg for the pure compounds. The impregnated discs were placed on agar plates previously seeded with the selected test organisms along with a disc containing solvent blanks. The plates were incubated at 37 °C for 24 hrs and antimicrobial activity could be detected as clear zone of inhibition surrounding the disc. The inhibition zone was determined in millimeters.

2.10.2. Cytotoxicity test: Cytotoxicities were carried out by Prof. Dr. Müller (Mainz University, Germany). Cytotoxicity against L5178Y mouse lymphoma cells, hela cervix carcinoma cells, and PC12 rat brain tumor cells was performed using the microtubule tetrazolium (MTT) assay [Carmichael *et al.,* 1978]. Stock solutions in ethanol 96 % (v/v) were prepared. Exponentially growing cells were harvested, counted and diluted appropriately. 50 µl cell suspensions with approximately 3,750 cells were pipetted into 96 well plates. Subsequently, 50 µl of sample solution (concentration varying from 3- 10 µg/ml) was added to each well. The small amount of ethanol present in the wells did not affect the experiments. The test plates were incubated at 37 °C with 5% CO_2 for 71 hrs. A solution of 3-(4,5-dimethydiazol-2-yl)-2,5-diphenyltetrasolium bromide (MTT) was prepared at 5 mg/ml in saline phosphase buffer

(PBS;1.5 mM KH_2PO_2, 6.5 mM Na_2HPO_4, 137 mM NaCl, 2.7 mM KCl; pH 7.4). From this solution, 20 µl was pipetted into each well. The yellow MTT penetrates healthy living cells and in the presence of mitochondrial dehydrogenases, it transforms to a blue formazan complex. After the incubation periods of 3 hrs and 45 minus at 37 °C in an incubator humidified with 5% CO_2, the medium was centrifuged (15 min, 20 °C, 210 x g). The cells were lysed with 200 µl of DMSO to liberate the formed formazan product. After thorough mixing, the absorbance was measured at 520 nm by using a scanning micro-well spectrophotometer. The color intensity is correlated with the number of healthy living cells. Cell survival was calculated using the formula:

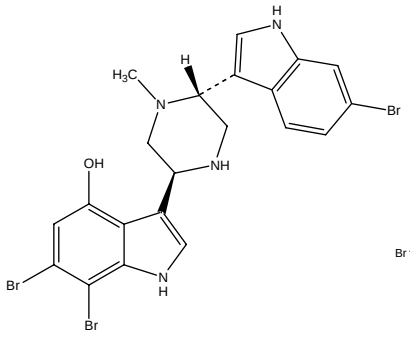
$$\text{Survival (\%)} = 100 \times \frac{\text{absorbance of treated cells} - \text{absorbance of culture medium}}{\text{absorbance of untreated cells} - \text{absorbence of culture medium}}$$

All experiments were carried out in triplicates and repeat three times. As control, media with 0.1 % EGMME/DMSO was used.

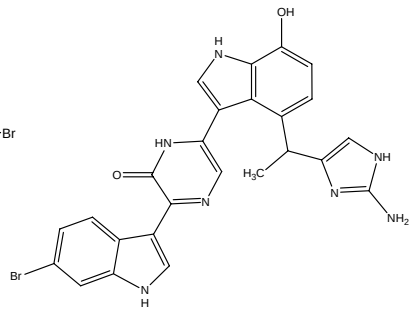
3. Results

3.1. Secondary metabolites from the sponge *Dragmacidon* sp.

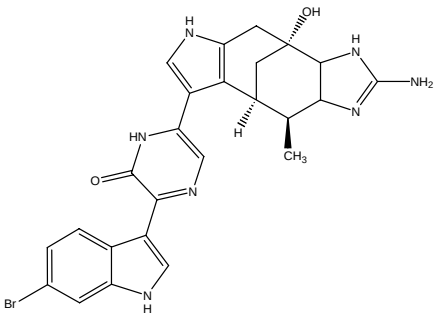
Sponges of the genus *Dragmacidon* are known to produce antiviral indol alkaloids ,however, there has been no report for the presence of compounds with a β-carboline moiety from this genus [Cutignano *et al.,* 2002]. β-Carboline compounds have been isolated from several sponges, examples are keramamines [Nakamura *et al.,* 1987], isolated from *Pellina* sp. and manzamine from *Xestospongia* [Ichiba *et al.,* 1988]. Other sponge genera include *Amphimedon* [Tsuda *et al*., 1994], *Haliclona* [Sakai and Higa, 1986, Kobayashi *et al.,* 1995, Sakai *et al*., 1987], *Hytios* [Kondracki *et al.,* 1996], and *Pachyllina* [Ichiba *et al.,* 1994], *Ircinia* [Kondo *et al.,* 1992]. β-Carboline alkaloids were also found to occur in the soft coral *Lignopsis spongiosum* [Cabrera and Seldes, 1999] and in the marine ascidian *Dinemnum* sp. [Schumacher and Davidson, 1995]. Some reported compounds which were isolated from the genus *Dragmacidon* are shown below.



Dragmacidin



Dragmacidin D

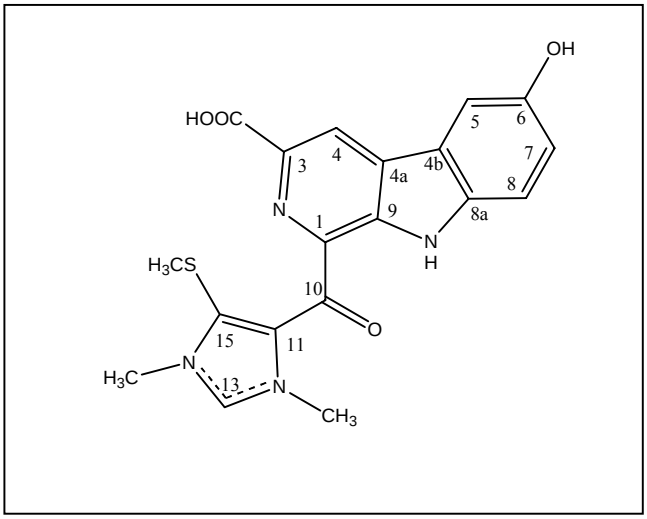


Dragmacidin F

Based on the HPLC and LC-MS chromatograms of the methanol sponge extract this led to the isolation of five indole alkaloids, comprising of three known indole carboxylic acid derivatives and two new β-carboline alkaloids.

3.1.1. Structure elucidation of Dragmacidonamine A and B

3.1.1.1. Dragmacidonamine A (1, new compound)



Dragmacidonamine A (**1**) was obtained as an orange oil and showed a molecular ion peak at *m/z* 397 $[M]^+$ in positive ESIMS experiments. The $[M + H]^+$ pseudo molecular ion peak was not observable in the positive mode in ESI (**Figure 11**). This suggested the occurrence of a protonated nitrogen function in the compound which gave the molecule a positive charge. The observed molecular ion peak in the positive mode was consistent with that perceived in the negative ESIMS spectrum of the compound **1** which was a pseudo-molecular ion peak at *m/z* *395*.8 $[M-H]^-$. The EI spectrum also showed a molecular ion peak at *m/z* 397 $[M]^+$. Both the (+) ESI-MS/MS and EI spectra also gave a significant base peak at *m/z* 353 $[M - CO_2]^+$ which was attributable to the presence of a carboxylic function in the molecule. The HREIMS corresponded to the molecular formula $C_{19}H_{17}N_4O_4S.^+$

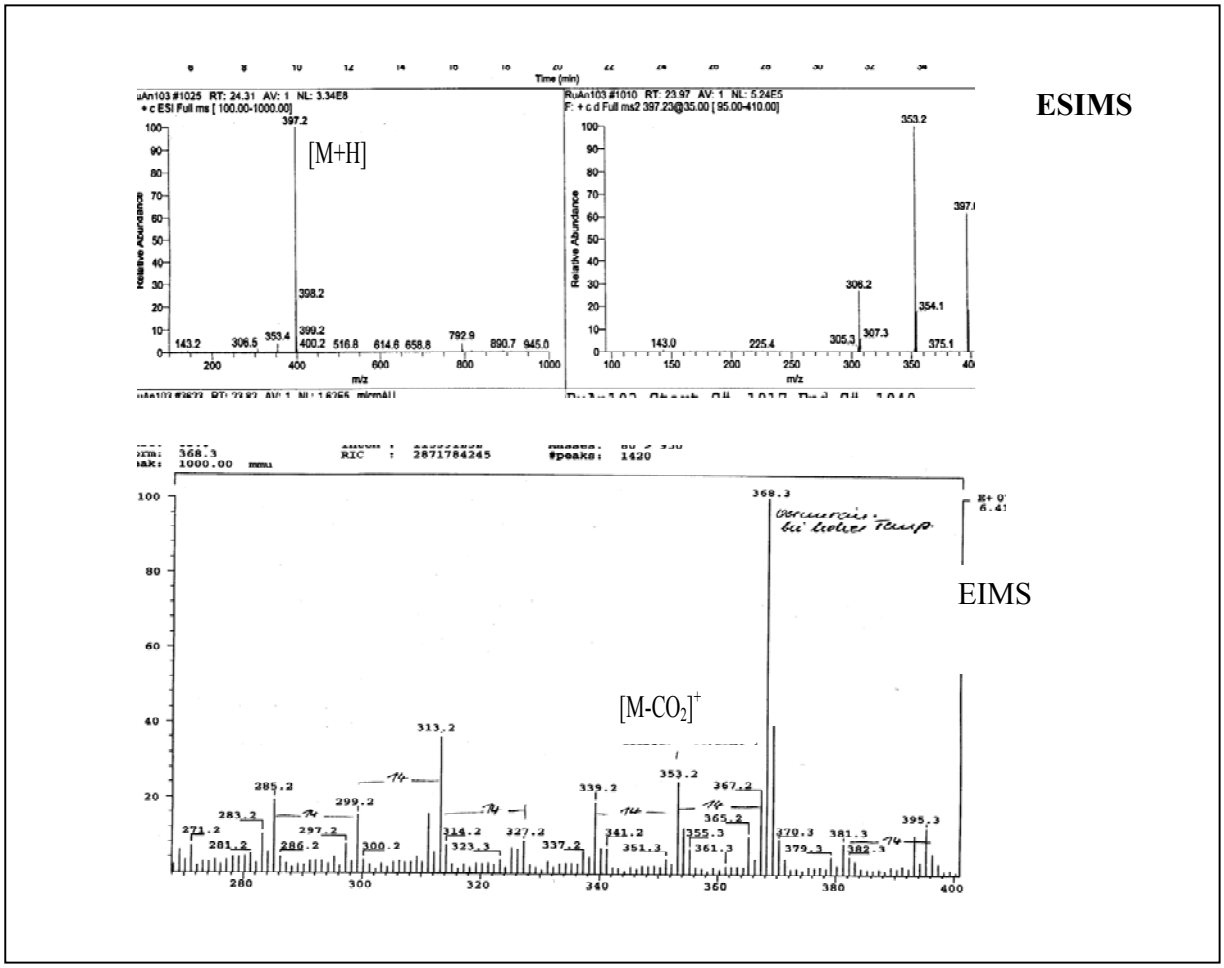
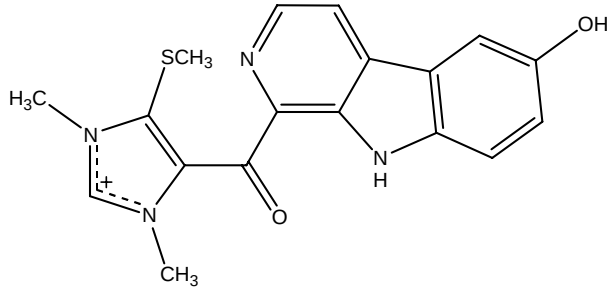


Figure 11 ESIMS and EIMS of **dragmacidonamine A** (**1**)

The ^{1}H- and ^{13}C-NMR spectra (**Figure 12** and summarized on **Table 3**) were comparable to those of hyrtiomanzamine [Kobayashi *et al*., 1990]. Compound **1** had a 44 mass unit difference from hyrtiomanzamine. The ^{1}H-NMR spectrum of **1** revealed the occurrence of an ABX spin system which exemplified a 1,3,4-trisubstituted benzene as demonstrated by the doublet signals at 7.78 (br d, J = 1.9 Hz), 7.20 (dd, J = 1.9, 8.2 Hz) and 7.69 ppm (d, J = 8.2 Hz).



Hyrtiomanzamine

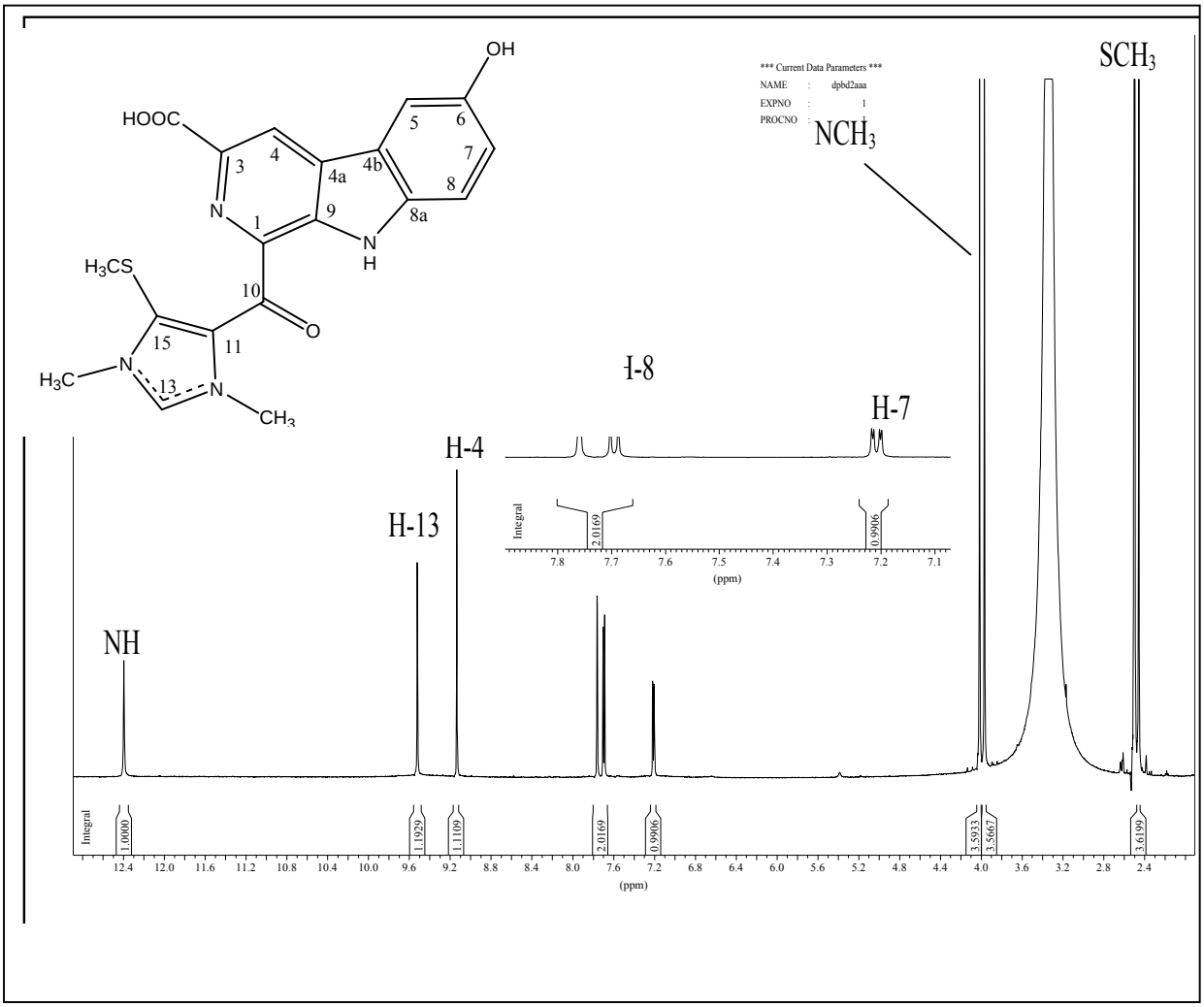
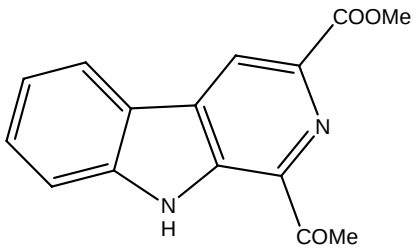


Figure 12 ^{1}H – NMR of the **dragmacidonamine A** (DMSO-d_6)

The β-carboline unit was established through the observed long-range HMBC correlations (**Figure 14**) of the broad NH singlet at δH 12.40 with carbons at δC 121.1 (C-4b), 132.1 (C-4a) and 134.1 ppm (C-1). The substitution pattern on the pyridine part of the compound was verified through the HMBC correlations of the methine singlet at δH 9.15 with the quaternary carbons at δC 121.1 (C-4b) and 133.4 ppm (C-9a) which suggested the C-4 position of the methine singlet. This implied the occurrence of a 1,3,6-trisubstituted-β-carboline moiety in **1**. Furthermore, the presence of a carboxylic acid substituent at C-3 was proved by the HMBC cross peak of H-4 with a carbonyl resonance at δC 166.2 ppm, primarily not detectable from its ^{13}C NMR spectrum due to the broadness of the signal. The ^{13}C NMR spectrum (**Figure 13)** of compound **1** revealed only one carbonyl signal at δC 183.9 ppm which corresponded to the keto unit at C-10**.** The ^{13}C NMR data for the pyridine ring were comparable to those of the 2-methyl-9H-pyrido[3,4*b*]-indole-3-carboxylic acid, a β-carboline isolated from the soft coral, *Lignopsis spongiosum*. In addition, the proton chemical shift of H-4 at δH 9.15 ppm was also compatible with that observed in related compounds, as in the latter alkaloid (δH 9.21, in DMSO-TFA) and in 1-acetyl-3-carboxymethoxy-β-carboline (δH 9.05 ppm, in $CDCl_3$), which was isolated from a Chilean Solanaceae, *Vestia lycioides* [Faini, Castillo and Torres, 1978]



1-acetyl-3- carboxymethoxy-β–carboline

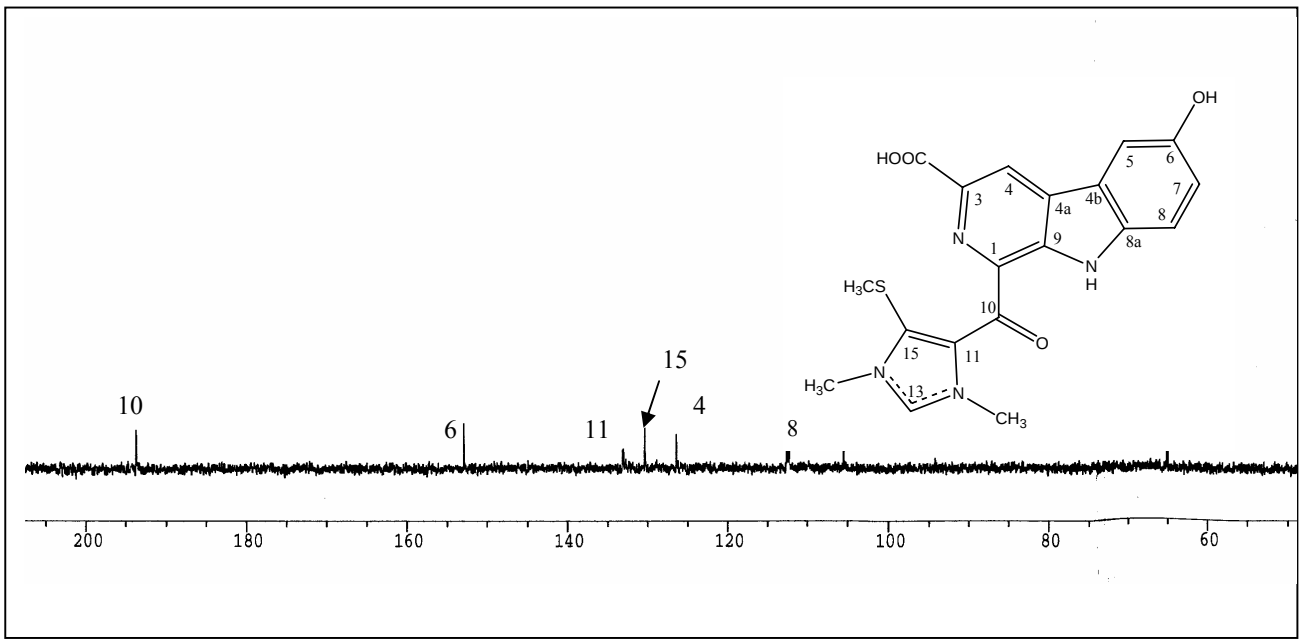


Figure 13 ^{13}C-NMR spectra of **dragmacidonamine A (**DMSO-d_6**)**

The proton and carbon chemical shifts of the methyl signals were very similar to those found in the imidazolium nucleus of hyrtiomanzamine. N-methyl singlets at δH 4.10 and 3.91 ppm were detectable with their corresponding carbon resonances at δC 34.2 and 37.8 ppm, respectively. The ^{1}H-NMR data of the N-methyl functions were also closely related to those in norzooanemonin (δH 3.97 and 3.87 ppm, in D_2O), an alkaloid isolated from the Carribean gorgonian, *Pseudopterogorgia americana* [Weinerimer, *et al.*, 1973]. Observed resonances at δH 2.50 and δC 18.6 ppm were assigned to a thiomethyl group. The presence of the thiomethyl function was deduced from the carbon-proton coupling constant value of 140.0 Hz as detected in hyrtiomanzamine ($^{1}J_{C\text{-}H}$ = 141.7 Hz), didemnoline A ($^{1}J_{C\text{-}H}$ = 142.0 Hz) [Kobayashi *et al.*, 1995], and varamin B ($^{1}J_{C\text{-}H}$ = 142.0 Hz) [Schumacher and Davidson, 1995]. The N,N-dimethyl imidazole ring system has been verified from the long range HMBC correlations of the proton at

δH 9.55 (H-13) with the N-methyl carbons at δC 34.2 and 37.8 as well as with the quaternary carbons at δC 134.8 (C-11) and 131.9 ppm (C-15). The imidazolium proton H-13 showed a direct HMBC correlation with the carbon resonating at δC 140.4 ppm. Accordingly, dragmacidonamine A was unambiguously elucidated to be the 3-carboxylate derivative of hyrtiomanzamine.

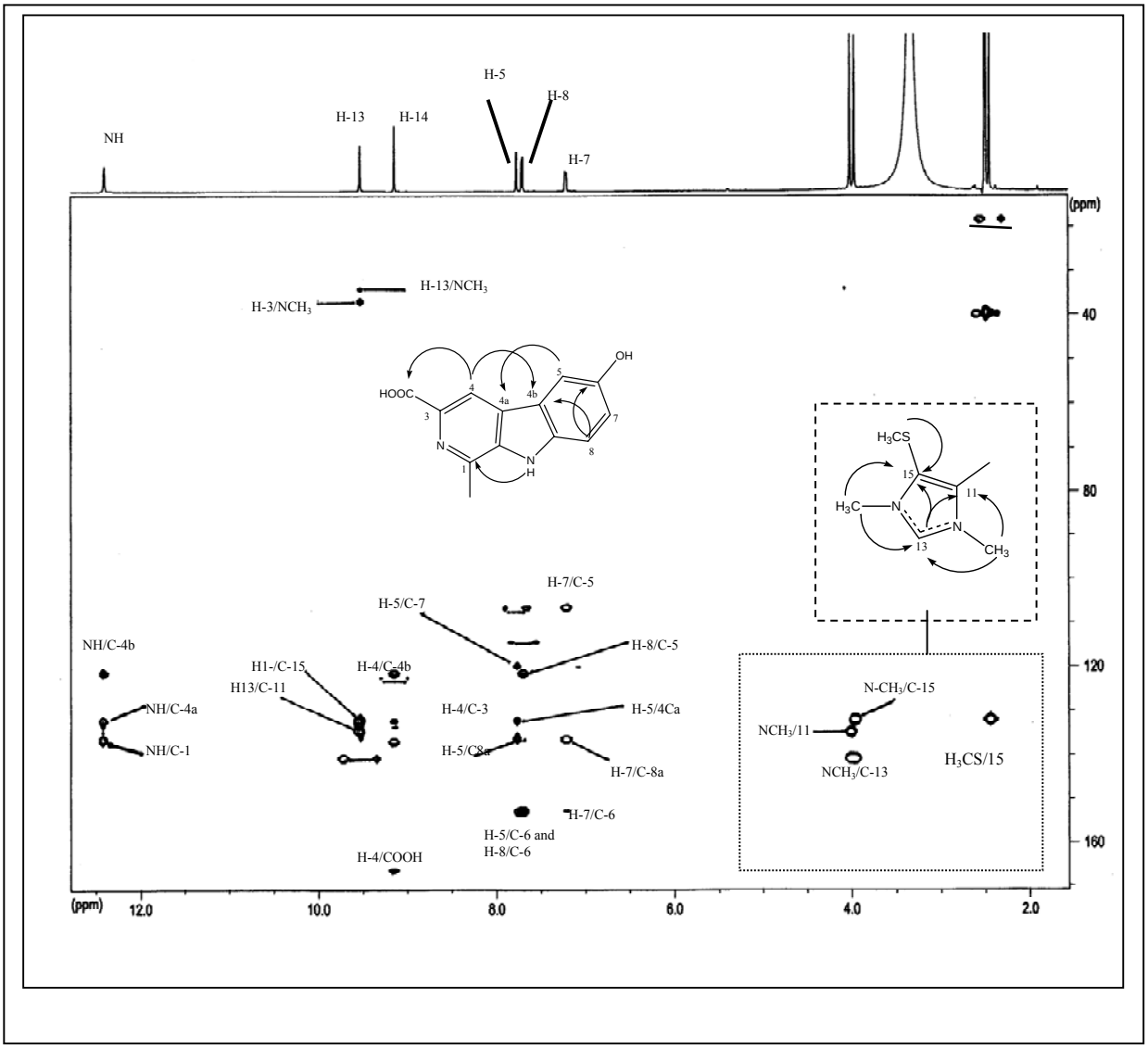
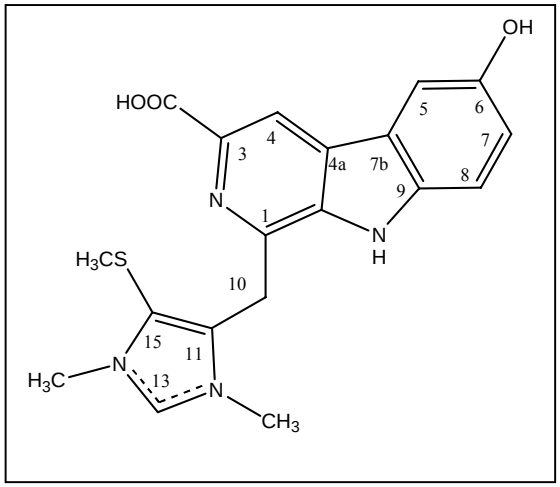


Figure 14 Important C-H long range correlation of **dragmacidonamine A**

3.1.1.2. Dragmacidonamine B (2, new compound)



Dragmacidonamine B (**2**) was obtained as a slightly brown-colored oil which showed a molecular ion peak at *m/z* 383 $[M]^+$ in both the EIMS and positive ESIMS experiments while its negative ESIMS spectrum showed a pseudo-molecular ion peak at *m/z* 382.2 $[M - H]^-$. The molecular formula $C_{19}H_{19}N_4O_3S^+$ was established by HREIMS. Compound **2** had a 14 mass unit difference from dragmacidonamine A. Both the (+) ESI-MS/MS and EI spectra (**Figure 15)** gave a significant base peak ion at *m*/*z* 339 $[M - CO_2]^+$, which also ascribed the occurrence of a carboxylic unit in the molecule as in compound 4. Additional evidence was the observable (–) ESI-MS/MS fragment ion at *m*/*z* 338.4 $[(M - CO_2) - H]^-$. The ^{1}H- and spectra (**16 Table 3**) were comparable to those of compound **1**.

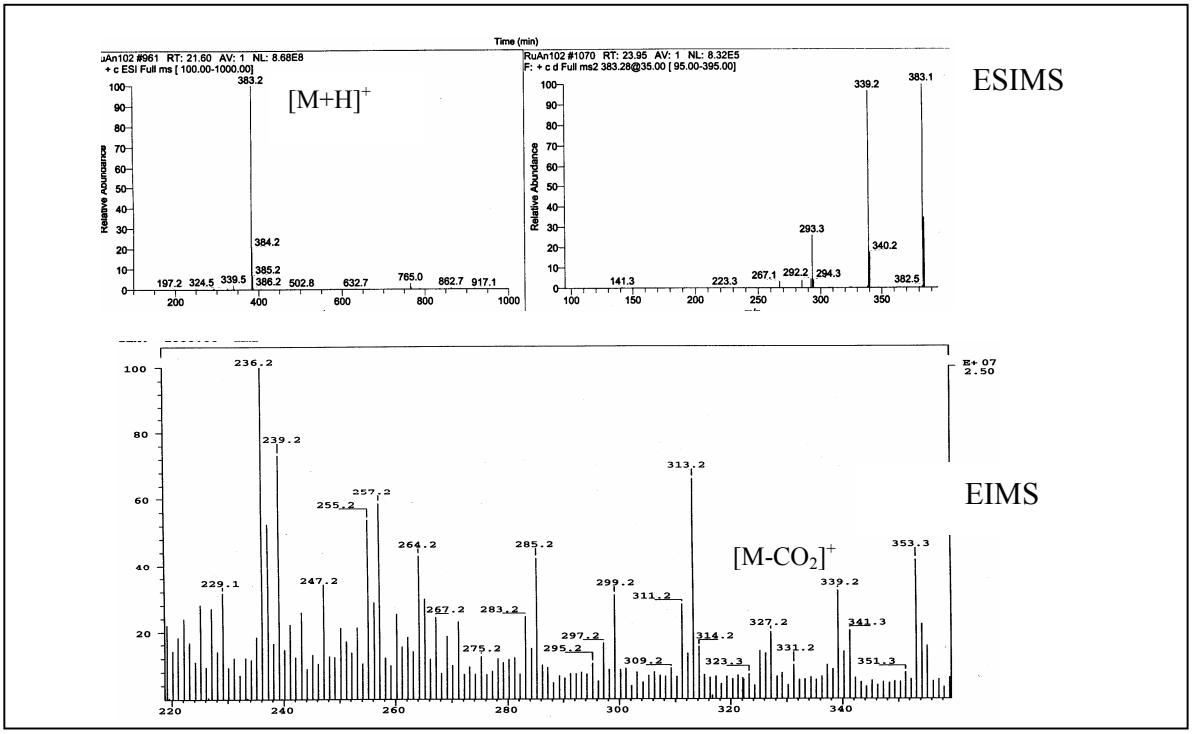


Figure 15 ESIMS and EIMS of **dragmacidonamine B**

The ^{1}H-NMR data of **2** indicated the presence of a similar 1,3,6-trisubstituted-β-carboline moiety and an identical thiomethylated N,N-dimethyl imidazole ring system as found in the latter derivative [Aiello, *et al.,* 1987]. However, the proton resonances for compound **2** were rather shielded when compared to those in compound **1**. Notable differences in ^{13}C-NMR chemical shifts between the two compounds were also discernible and the most significant difference was observed for C-1, which was deshielded to δC 142.6 (Δ 8.5 ppm) while C-11 was shielded to δC 127.5 (Δ 9.1 ppm).

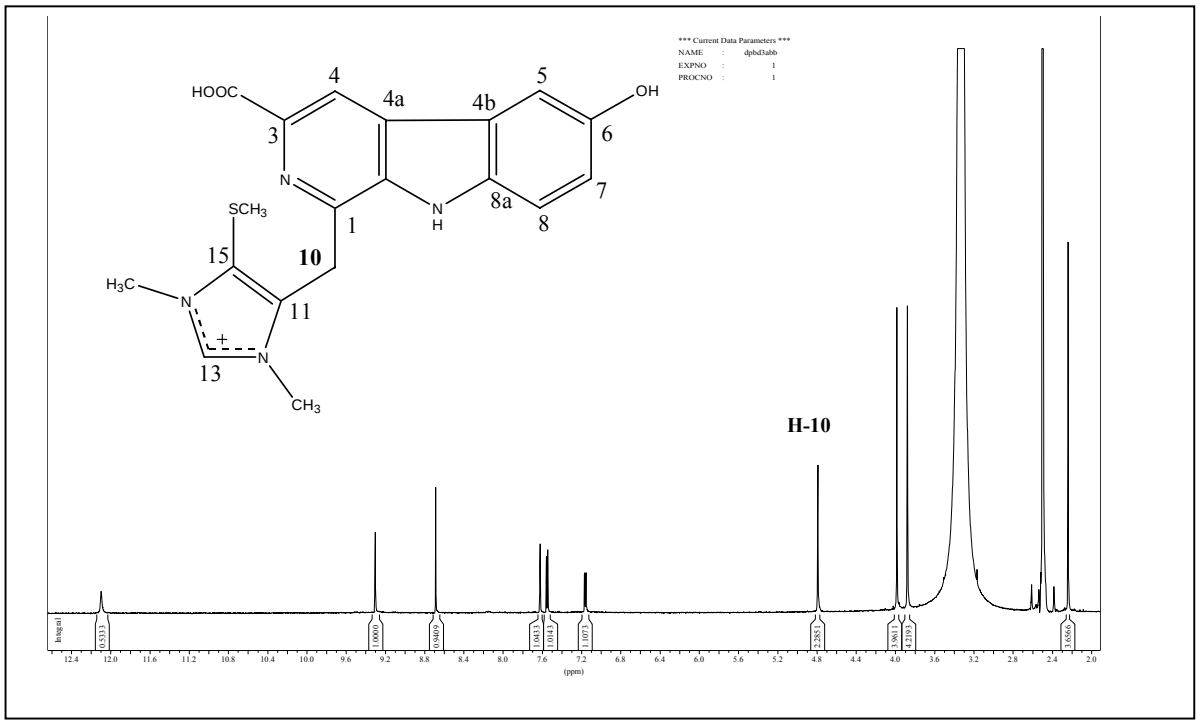
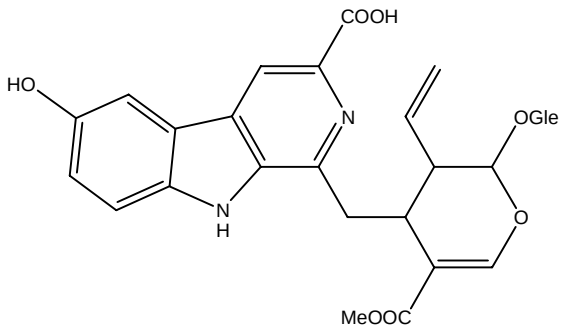


Figure 16 ^{1}H – NMR of the **dragmacidonamine B** (DMSO-d_6)

The appearance of a methylene signal was detected at δC 28.0 ppm which then exhibited a direct correlation with a methylene singlet at δH 4.80 ppm as revealed by its HMBC spectrum (**Figure 17)**. Furthermore, the methylene singlet at δH 4.80 showed long range HMBC correlations with carbon signals belonging to both the β-carboline unit (C-1 and C-9a) and the imidazole moiety (C-11, C-13, C-15). In addition, the absence of a carbonyl signal at δC 183.9 ppm as previously found in compound **1** implied the disappearance of the keto function at C-10 which consequently accounted for the 14 mass unit difference of this congener from compound **1**. This likewise explained the deshielding and shielding effect on C-1 and C-11, respectively, on which both moieties were connected together through a sp^3 bridge. The ^{1}H and ^{13}C-NMR data of the β-carboline unit, particularly the pyridine portion of compound **2**, were compatible to those of cordifoline and desoxycordifoline (δH-4/C-4 = 8.69/114.2, δC-1= 142.9, $\delta_{C\text{-}3/C\text{-}9a}$ 135.6, and δC-4a 128.4 ppm). Cordifolines are β-carboline 3-carboxylate glucoalkaloids isolated from the heartwood of *Adina* and *Nauclea* species, both from the family Naucleaceae and also from an endemic East African species, *Strychnos mellodora*. Compound **2** was identified as dragmacidonamine B.



Cordifoline

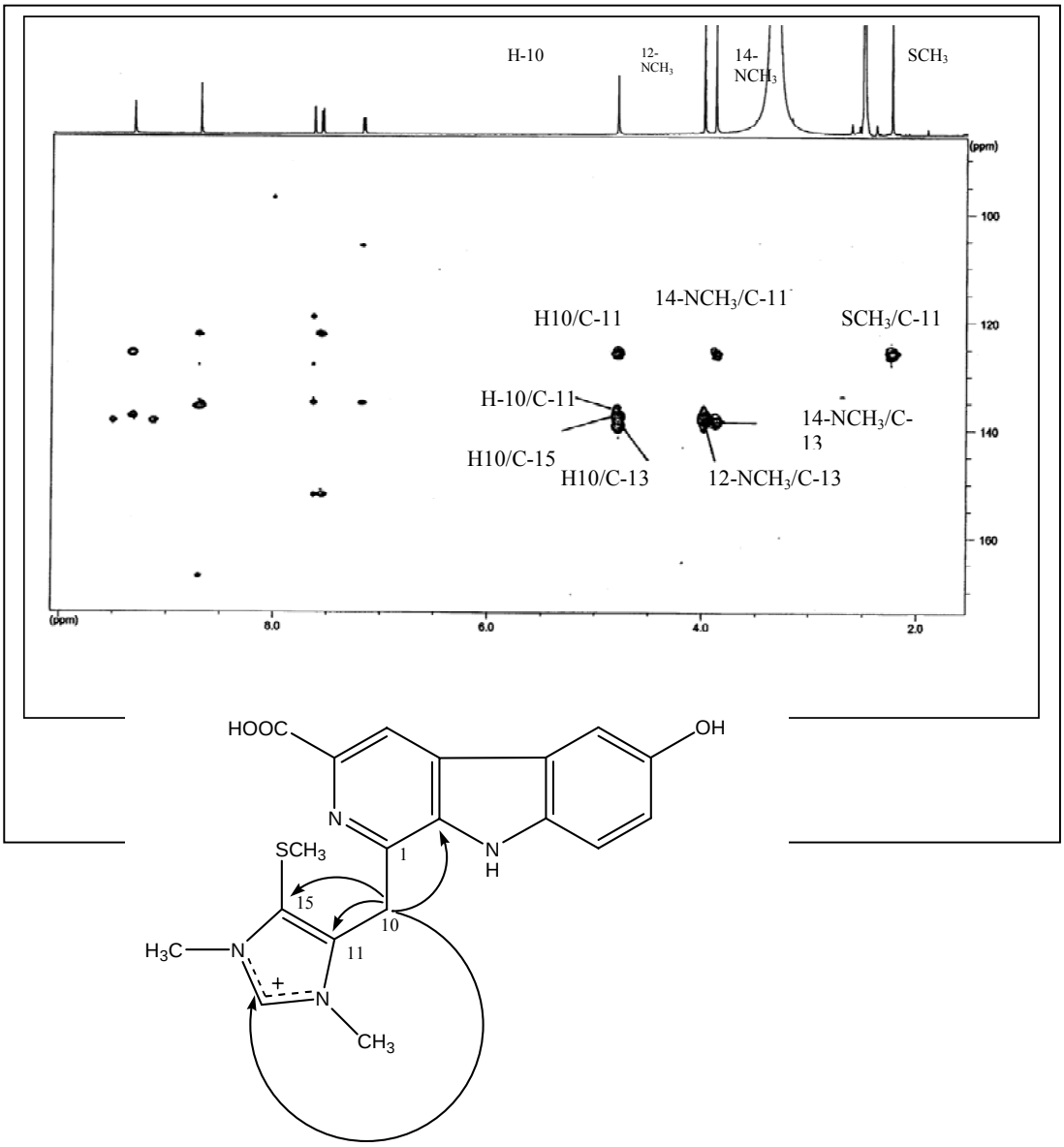


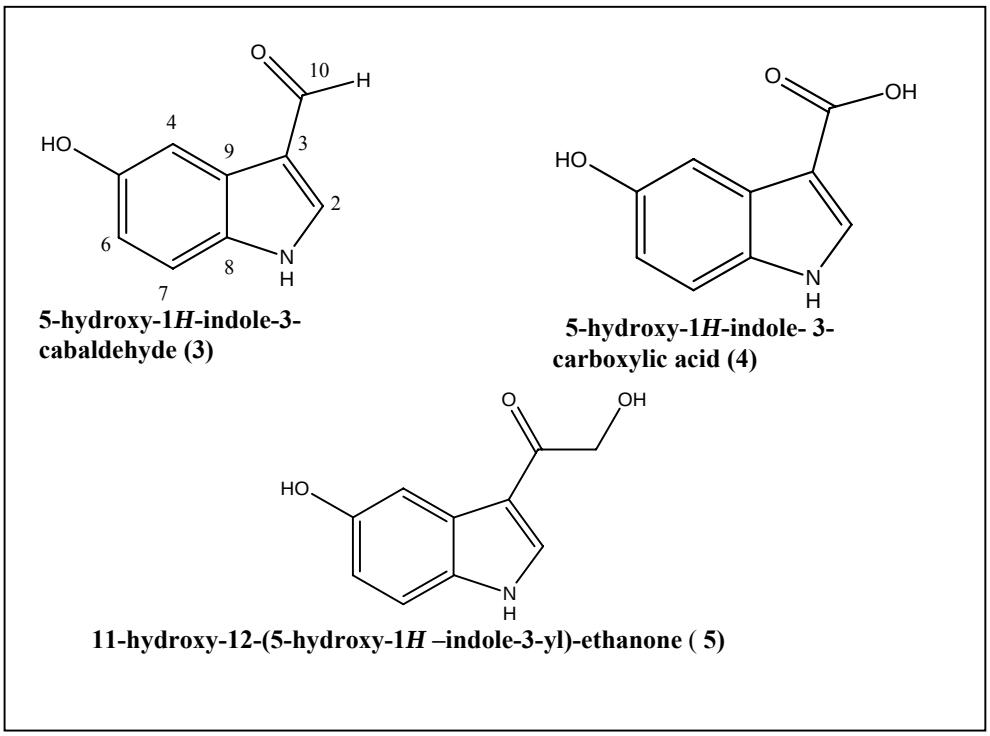
Figure 17 Important C-H long range correlation of **dragmacidonamide B**

Table 3 NMR data of compounds **1** and **2** (DMSO-d_6)

Position	**Compound 1**		HMBC	**Compound 2**		HMBC
	^{1}H (multiplicities, J in Hz)	^{13}C		^{1}H (multiplicities, J in Hz)	^{13}C	
1	-	134.1 (s)	-	-	142.6 (s)	-
2	-	-	-	-	-	-
3		132.3 (s)			135.7 (s)	
4	9.15 (brs)	122.3 (d)	C-1, C-3, C-4b, C-9a, COOH	8.69 (brs)	119.0 (s)	C-4a, C-4a, C-9a, COOH
4a	-	132.1 (s)	-	-	127.5 (s)	-
4b	-	121.1 (s)	-	-	122.2 (s)	-
5	7.78 (brd,1.9)	106.5 (d)	C-4a, c-6, C-7, C-7a	7.61(brd, 2.2)	106.0 (d)	C-4a, C-6, C-7, C-8a
6	-	152.6 (s)	-	-	151.9 (s)	-
7	7.20 (dd, 1.9, 8.2)	119.0 (d)	C-5, C-6, C-8a	7.10 (dd,2.2,9.5)	119.1 (d)	C-5, C-8a
8	7.69 (d, 8.2)	114.0 (d)	C-4b, C-6, C-8a	7.69 (d, 9.5)	116.0 (d)	C-4b, C-6
8a	-	136.2 (s)	-	-	135.1 (s)	-
9a	-	133.4 (s)	-	-	135.7 (s)	-
10	-	183.9 (s)	-	4.80 (s)	28.0 (t)	C-1, C-9a, -11, C-13, C-15
11	-	134.8 (s)	-	-	125.7 (s)	-
13	9.55 9s)	140.4 (d)	C-11, C-15, NCH_3-12, NCH_3	9.30 (s)	138.1 (d)	C-11, C-15, NCH_3-12
15	-	131.9 (s)	-	-	137.3 (s)	-
COOH	-	166.2 (s)	-	-	166.8 (s)	-
NCH_3	4.10 (q)	34.2 (s)	C-11, C-13	3.87 (s)	35.0 (q)	C-11, C-13
NCH_3	3.91 (s)	37.8 (q)	C-13, C-15	3.97 (s)	36.0 (q)	C-13, C-15
SCH_3	2.50 (s)	18.6 (q)	C-15	2.24 (s)	18.1 (q)	C-15
NH	12.40 (brs)	-	C-1,C- 4a, C-4b	-	12.0 (brs)	-

3.1.2. Structure elucidation of compounds 3 -5

3.1.2.1. 5-hydroxy-1*H*-indole-3-cabaldehyde (3, known compound), 5-hydroxy-1*H*-indole-3- carboxylic acid (4,known compound) and 11-hyhroxy-12-(5-hydroxy-1*H* –indole-3-yl)-ethanone (5, known compound)



5-hydroxy-1*H*-indole-3-cabaldehyde (3)

5-hydroxy-1*H*-indole- 3-carboxylic acid (4)

11-hydroxy-12-(5-hydroxy-1*H* –indole-3-yl)-ethanone (5)

Compounds **3, 4** and **5** were isolated as white amorphous powders by Sephadex LH-20 and RP-18 column chromatography followed by semi preparative HPLC (see **Scheme 2**). These compounds revealed their pseudomolecular ion peaks as shown in **Figure 18**. Compound **3** exhibited $[M+H]^+$ at *m/z* 161. For compound **4**, a peak at *m/z* 177 $[M+H]^+$ was observed which was 16 mass unit larger than that of compound **3**. Compound **5** gave a

pseudomolecular ion peak at *m/z* 191 $[M+H]^+$ which had a 30 mass units difference to that of **3**. From the comparison of the Ms spectra, it was indicated that compound **4** contains one oxygen atom more than compound **3** while compound **5** contains a CH_2O fragment. ^{1}H-NMR of compounds **3, 4**, and **5** (**Figure 19**) exhibited 1,2,4-trisubstituted aromatic rings and a typical singlet of an pyrrolic proton at the position two. The three compounds differed on their substituents at C-3. ^{13}C-NMR of compound **3**, diagnosed through its HMBC spectra revealed an aldehyde functional group at δC 184.3. Compound **4** showed a carboxylic acid functional group at δC 193.8 while compound **5** showed a ketone carbonyl at δC 196.0 and a *CH_2OH* observed at δC 65.0. Therefore, compound **3** was identified as hyrtiosin A which was previously isolated from the Okinawan marine sponge *Hyrtios erecta* [Kobayashi *et al.,* 1990]. Compounds **4** and **5** were identified as **5-**hydroxy-1*H*-indole-3-carboxylic acid and 11-hydroxy-12-(5-hydroxy-1*H*–indole-3-yl)-ethanone, respectively. Chemical structure analysis was completed by means of HMBC and comparison with data from the literature.

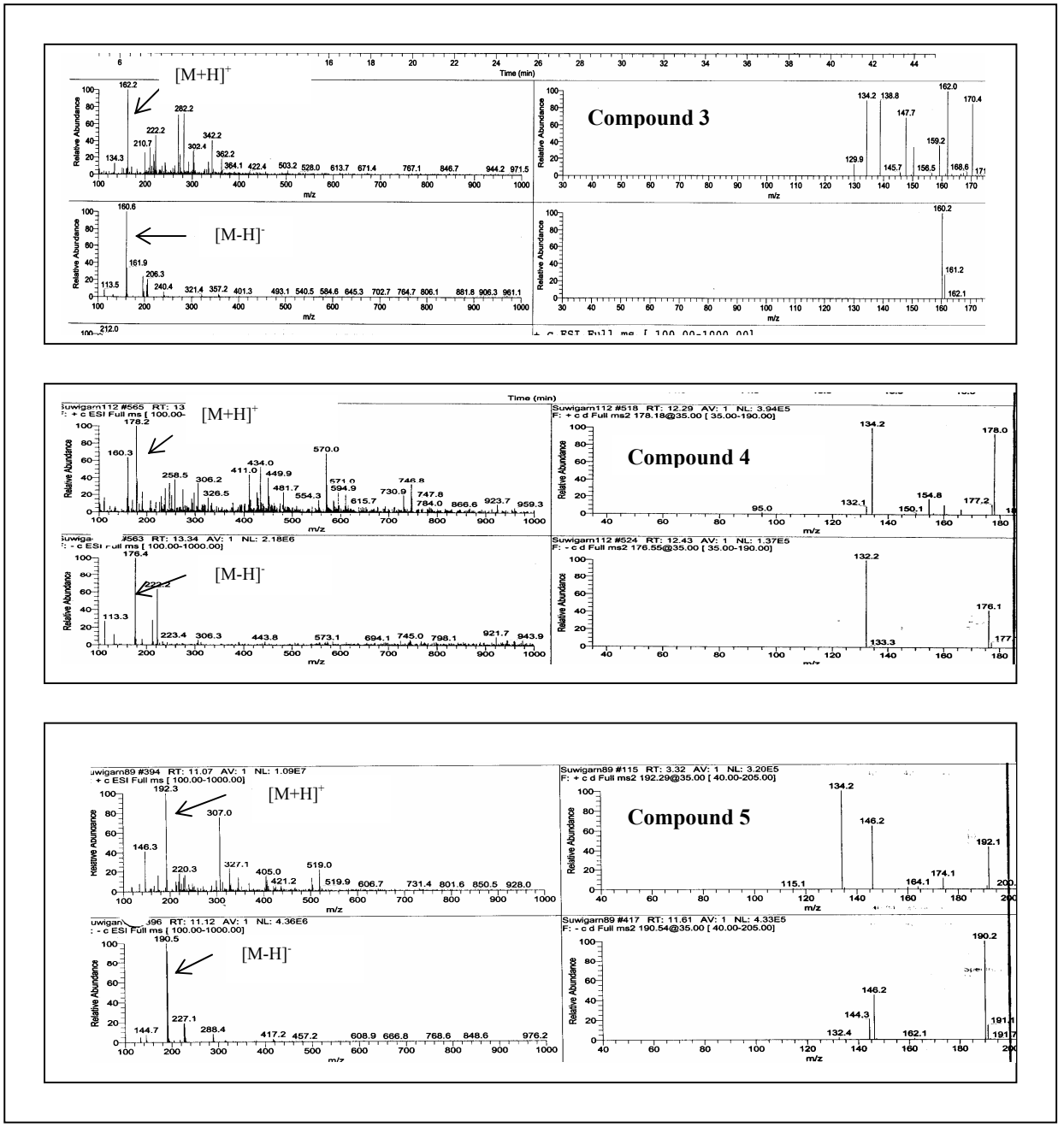


Figure 18 ESIMS of compounds **3, 4,** and **5**.

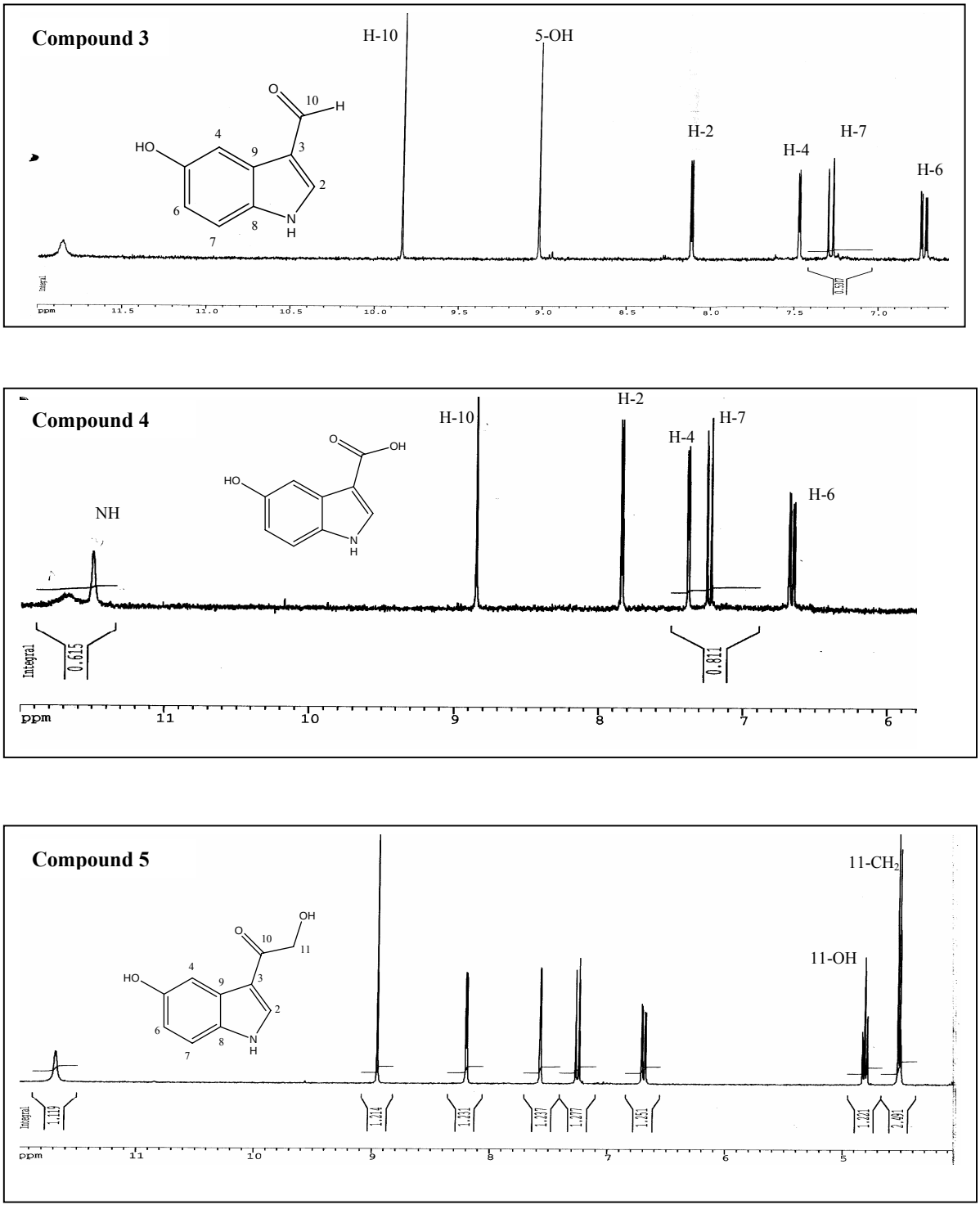


Figure 19 ^{1}H-NMR of the compounds **3, 4** and **5** (DMSO-d_6)

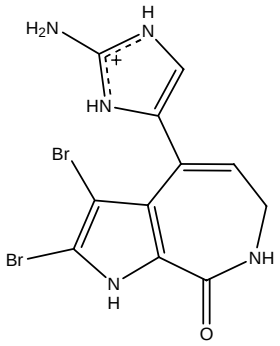
Table 4 ^{1}H and ^{13}C – NMR data of the compounds **1**, **2,** and **3** (DMSO-d_6)

Position	Compound **3**		Compound **4**		Compound **5**	
	δ ^{1}H (multiplicity, *J* in Hz)	δ ^{13}C* (multiplicity *J* in Hz)	δ ^{1}H (multiplicity *J* in Hz)	δ ^{13}C* (multiplicity *J* in Hz)	δ ^{1}H (multiplicity *J* in Hz)	δ ^{13}C* (multiplicity *J* in Hz)
1 NH	11.90 (brs)	-	11.82 (brs)	-	11.70 (brs)	-
2	8.11(brd, 3.0)	138.0, d	7.91 (brd, 3.0)	138.0, d	8.01 (brd, 3.0)	130.3, d
3	-	117.2, s	-	128.0, s	-	114.0, s
4	7.4 2 (d, 2.5)	104.5, d	7.47 (d, 2.5)	104.2, d	7.55 (brd, 1.8)	105.4, d
5 , OH	-	152.0, s	-	152.2, s	-	152.0, s
6	6.73 (dd, 2.5, 8.8)	113.0, t	6.73 (dd, 2.50, 8.8)	113.3, t	6.72 (dd, 1.8, 8.8)	112.3, t
7	7.25 (d, 8.8)	113.5, d	7.25 (d, 8.8)	111.5, d	7.31 (d, 8.8)	112.6, d
8	-	133.0, s	-	130.2, s	-	133.0, s
9	-	125.5, s	-	125.5, s	-	126.4, s
10	9.89 (s)	184.3, d	-	193.8, d	-	196.0, d
11	-	-	-	-	4.54 brd	65.0, t

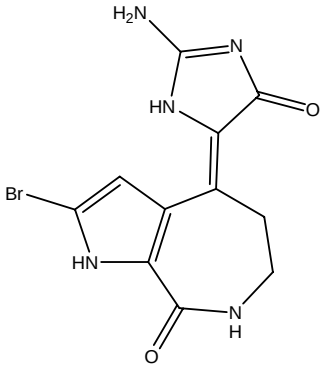
* ^{13}C-NMR data deduced from HMBC spectra

3.2. Secondary metabolites from the marine sponge, *Stylissa flabelliformis*

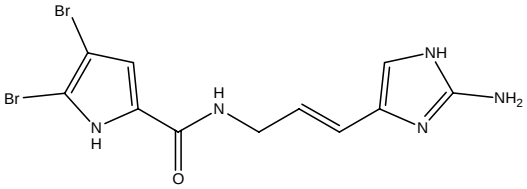
Sponges of the genus *Stylissa* (syn *Axinella*) produce several types of secondary metabolites especially, bromopyrrole alkaloids which have been commonly occurring in this genus. Some of them exhibit inhibitory activities toward C-erbB-kinase enzyme, cyclin-dependtent kinase 4, have α - adrenoreceptor blocking activity and have also shown cytotoxicity towards human tumor LoVo cells [Bergquist, 1978; Inaba, Sato *et al.,* 1998; Tasdemir *et al*., 2002]. Some reported compounds which were isolated from the genus *Stylissa* are shown below.



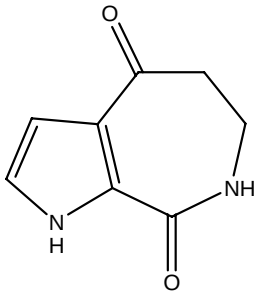
Stevensine



Hymenialdisine



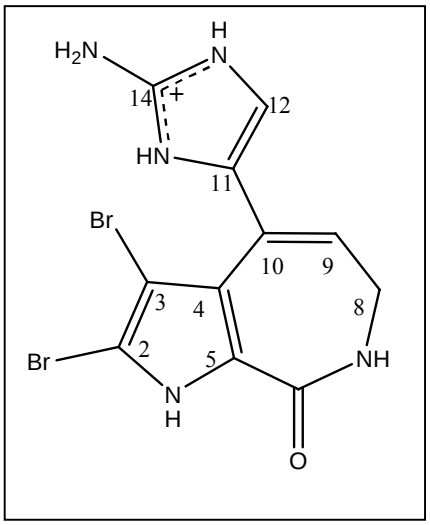
Hymenidin



Aldisine

3.2.1. Structure elucidation of the isolated compounds 6-9

3.2.1.1. Stevensine (6, known compound)



Compound **6** was isolated as an orange amorphous solid which showed quasimolecular ion peaks at *m/z* 386, 388, and 390 by ESIMS (**Figure 20**). The Ms indicated the presence of two bromine substituents in the molecule. It exhibited UV absorbance at λ_{max} 224 and 235 nm. ^{1}H-NMR data (**Figure 21** and summarized in **Table 5**), showed two important groups of signals, first a methylene doublet for an azepine ring system at δH 3.57 ppm (C*H*$_2$-8) correlating with a triplet proton in the aromatic region at δH 6.25 (C*H*-9) with a coupling constant of 7.0 Hz. Second, a singlet proton for an imidazole ring at proton δH 6.80 (C*H*-12) was also observed. This was confirmed through an HMBC experiment which is summarized in **Table 5**. Two key correlations from H-8 (δH 3.57) to C-10 (δC 120.9) and to the amine carbonyl C-6 (δC 161.0) were observed in the HMBC spectrum. This information was used to assign the protons in the azepine ring. The proton resonance observed at δH 6.80 (C*H*-12) is typical for an imidazole system which is compatible with the one reported in the literature (δH 6.81 recorded in CD_3OD)

[Albizati and Faulkner, 1985]. Thus, compound **6** was identified as stevensine which was previously isolated from Micronesian marine sponge, *Phakellia flabellata* [Albizati and Faulkner, 1985]. The ^{1}H-NMR data of compound **6** were also found to be identical to those previously reported.

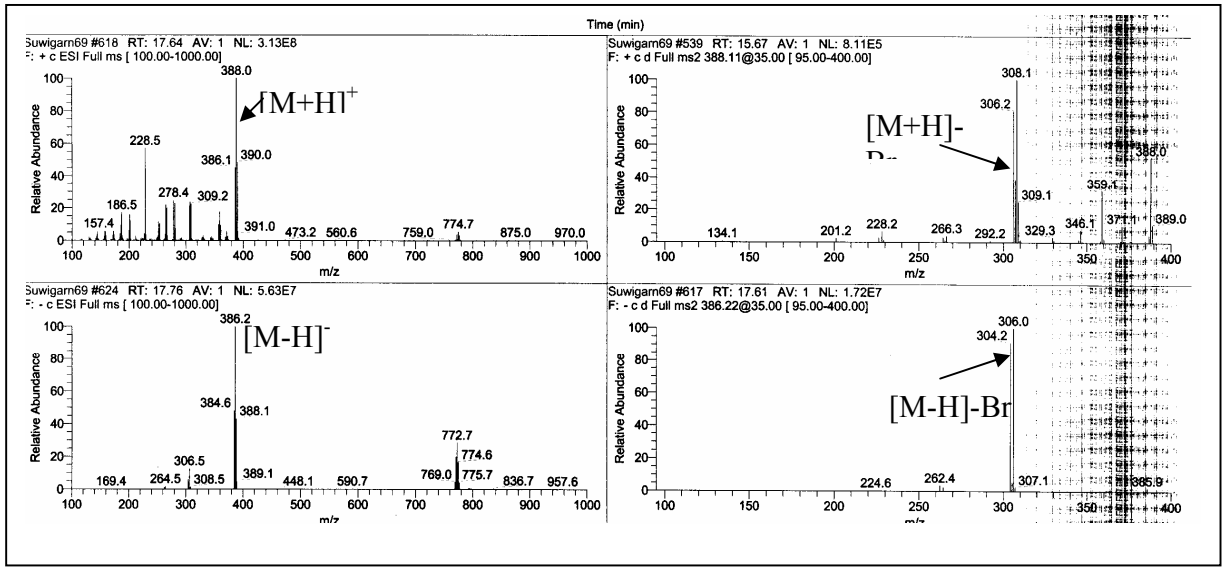


Figure 20 ESIMS spectra of compound **6**

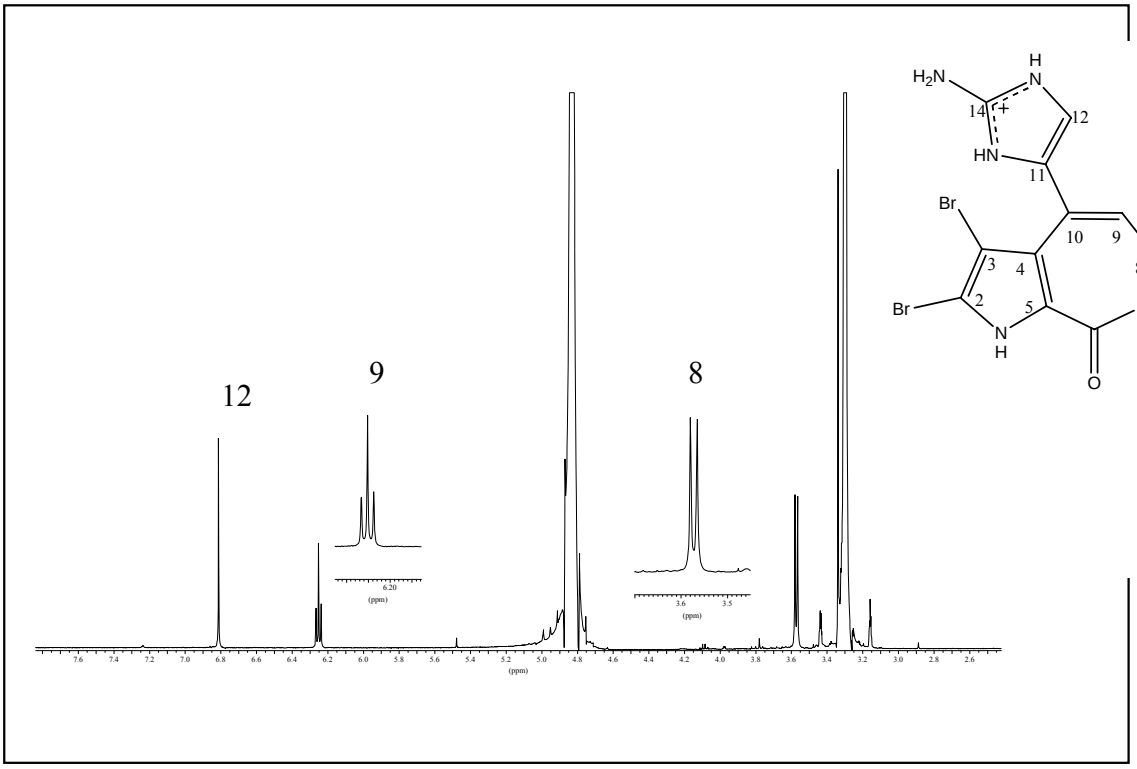
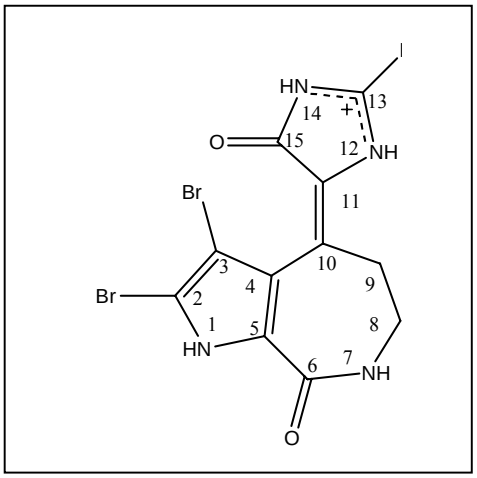


Figure 21 ^{1}H-NMR of compounds **6** (MeOH-d_4)

3.2.1.2. Spongiacidin A (7, known compound)



Compound **7** was purified as an amorphous yellow solid by semi-preparative HPLC (see **Scheme 4**). It showed UV absorbances at λ_{max} 272 and 332 nm, respectively. ESIMS (**Figure 22**) showed $[M+H]^+$ ion peaks at *m/z* 402, 404, and 406 which revealed the occurrence of two bromine substituents in the molecule as found in compound **6**. The ^{1}H-NMR **(Figure 23)** exhibited only one broad singlet signal at δH 3.42 for two methylene protons of an azepine ring system which was comparable to spongiacidin A [Inaba *et al.*,1998] with a broad singlet resonance at δH 3.26 in DMSO-d_6 for both CH_2-8 and CH_2-9. Spongiacidin A was shown to have the *cis*-configuration on the double bond at C-10 and C-11 while a previously structurally related compound, 3-debrominated spongiacidin A, was reported to exhibit the *trans*-configuration. The structure of the latter compound was confirmed by X-ray analysis [Cimino *et al.*,1982]. Spongiacidin A is a secondary metabolite found in a *Hymeniacidon* sponge while 3-debrominated spongiacidin A was isolated from both *Axinella verrucosa* and *Acanthella aurantiaca*. In the case of compound **7**, the stereochemistry on the double bond could not be

determined due to the very small yield, it was not possible to record a 2D NMR experiment. Comparison of the spectral data with the literature led to the identification of compound **7** as spongiacidin A. Like spongiacidin A, compound **7** exhibited identical uv maxima at 270 and 332 nm. It was found to be active against c-erbB-2 kinase and cyclin-dependent kinase 4 with IC_{50}s of 8.5 and 32 µg/ml, respectively [Inaba *et al*., 1988].

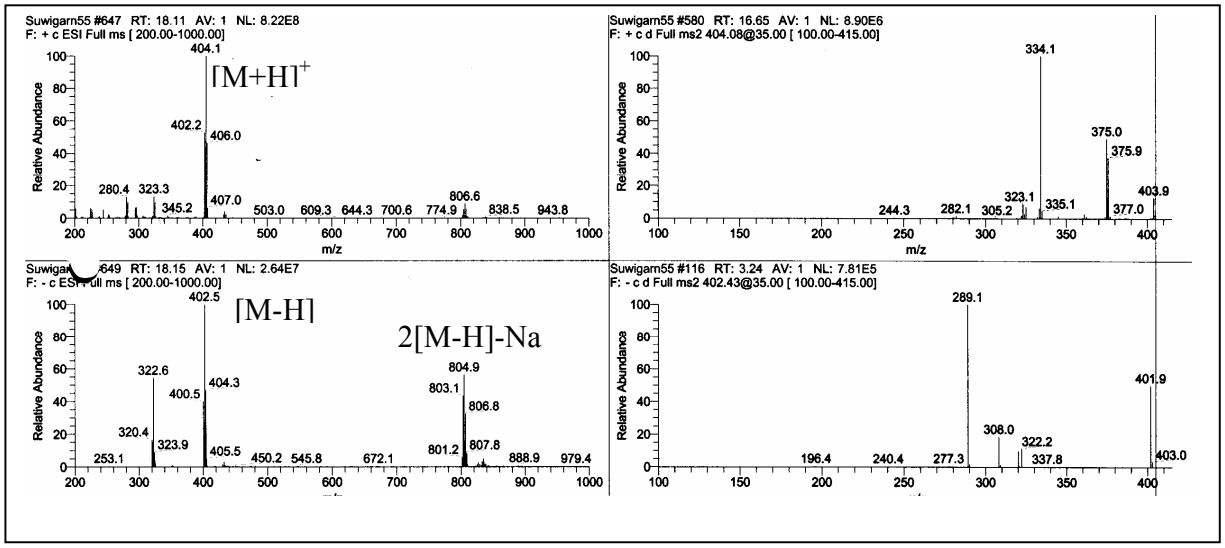


Figure 22 ESIMS spectra of compounds **7**

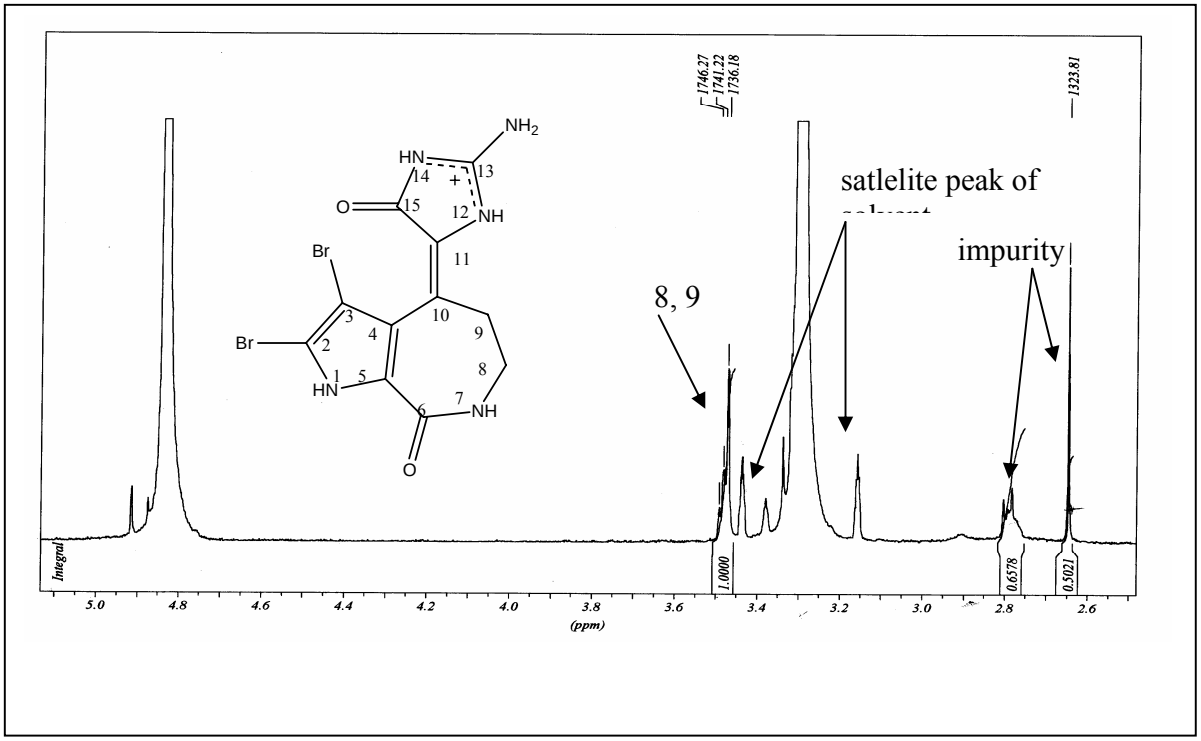
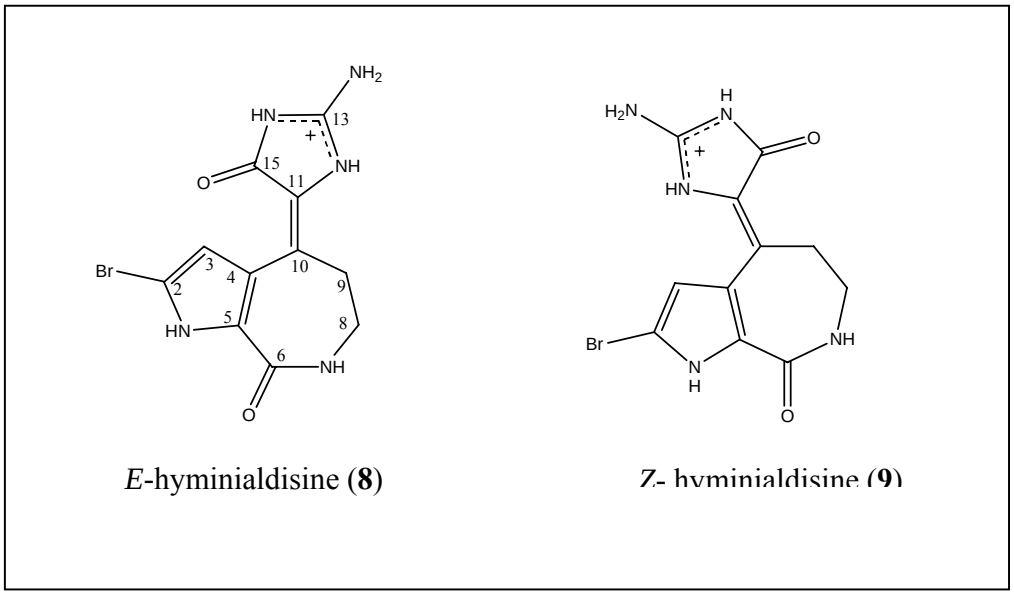


Figure 23 [1] H-NMR spectra of compounds **7** (MeOH-d_4)

3.2.1.4. *E*-hymenialdisine and *Z*- hymenialdisine (8 and 9, known compounds)



E-hyminialdisine (**8**) *Z*- hyminialdisine (**9**)

Compounds **8** and **9** were purified and isolated as a yellow amorphous solid, respectively. They showed UV absorbances at λ_{max} 212, 262 and 354 nm, respectively. Their ESIMS spectra again revealed the occurrence of a bromine substituent in both of the molecules as indicated by their pseudomolecular ion peaks at *m/z* 322 and 324 (**Figure 24 and 26)** which was compatible with hyminialdisine [Cimino *et al.,* 1982]. ^{1}H-NMR spectrum of compound **8** (**Figure 25)** exhibited a broad singlet at δH 6.60 (H-3) which was assigned to a pyrrole ring proton and another broad singlet at δH 3.34 attributed to the methylene protons (H_2-8 and H_2-9) for the azepine ring as found in the previous compound spongiacidin A (summarized **Table 5).** The ^{1}H-NMR spectrum of compound **9** (**Figure 27**) exhibited almost identical resonances with compound **8** except for the upfield singlet of pyrrole proton at δH 5.41. The ^{1}H-NMR of this mixture spectra as mentioned above is in accordance with the *Z* and *E* configurations of hyminialdisine [Cimino *et al*., 1982, Francis *et al*., 1985].

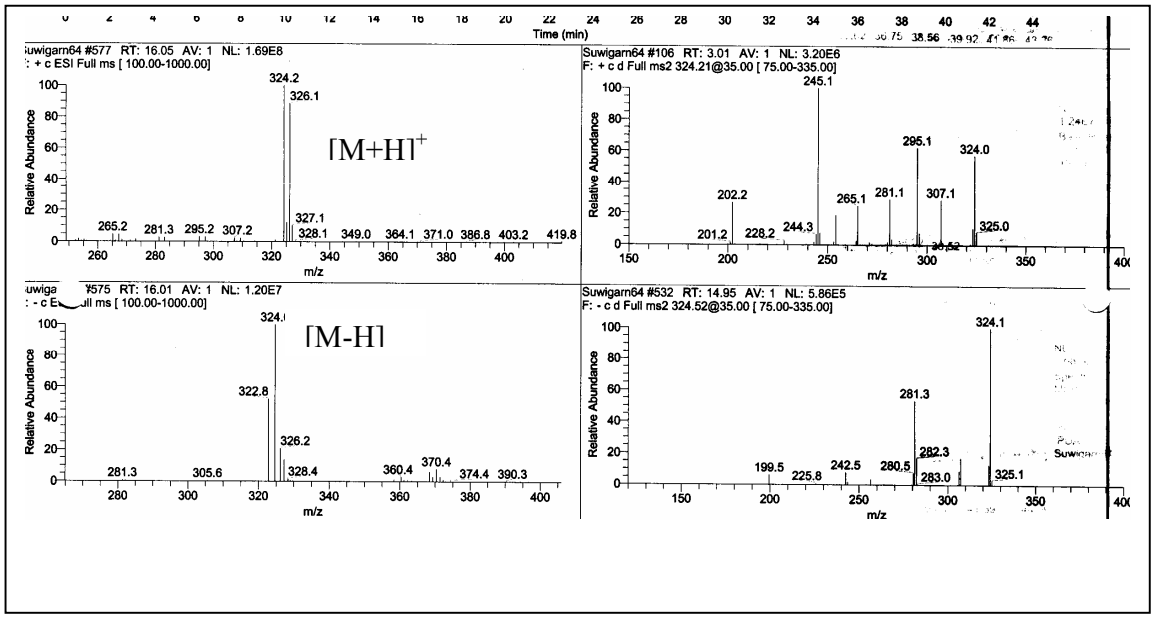


Figure 24 ESIMS spectra of compound **8**

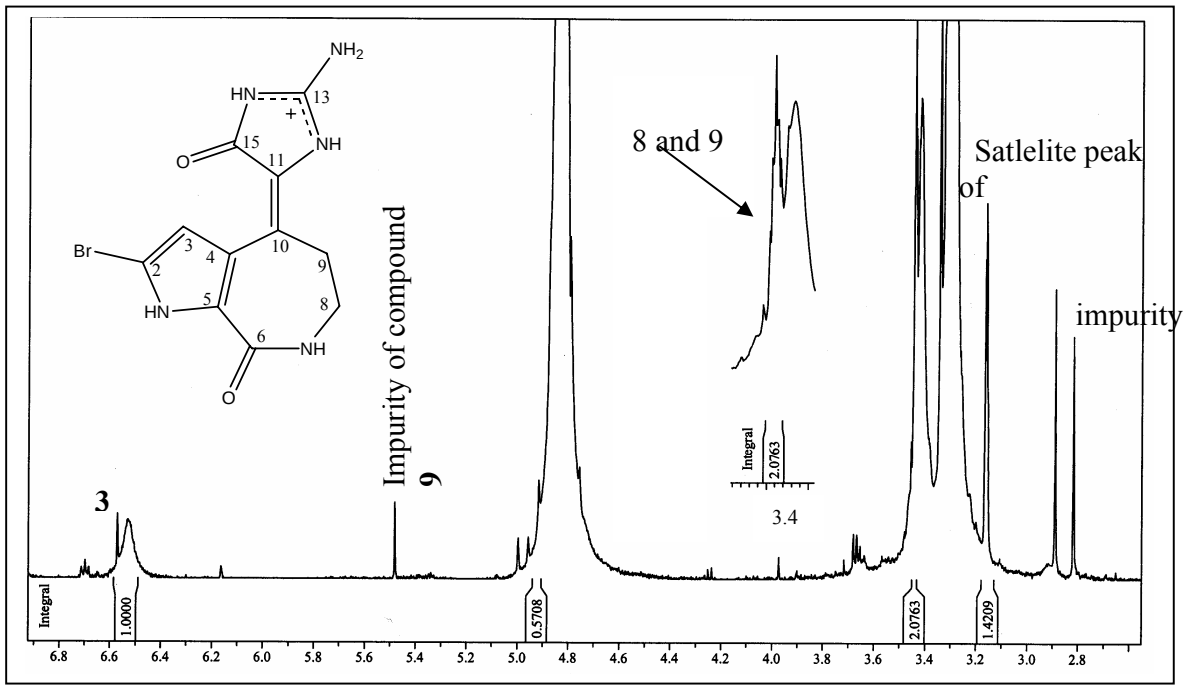


Figure 25 [1] H-NMR spectra of compound **8** (MeOH-d_4)

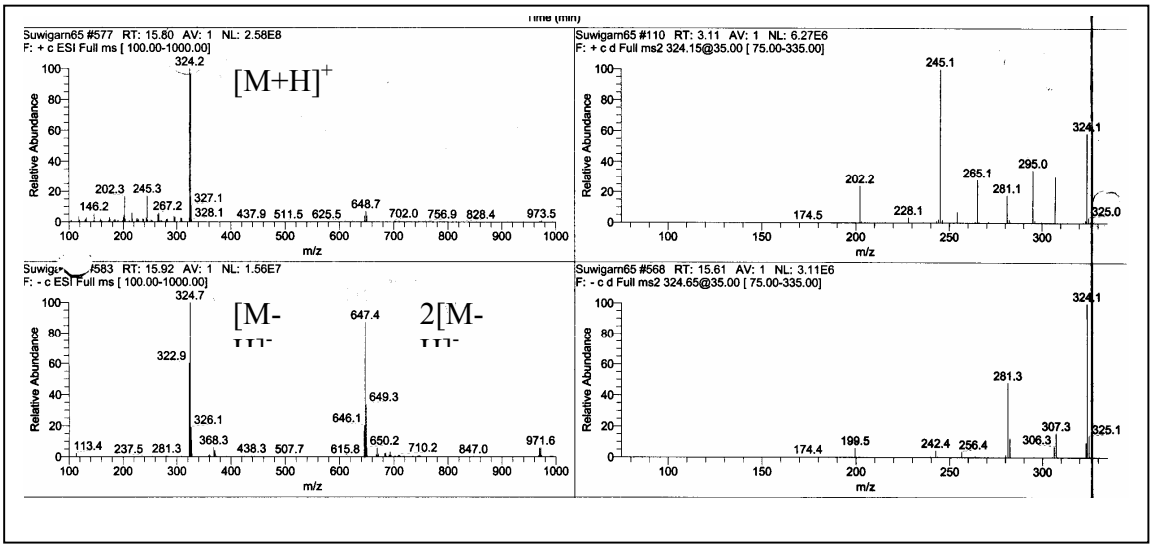


Figure 26 ESIMS spectra of compound **9**

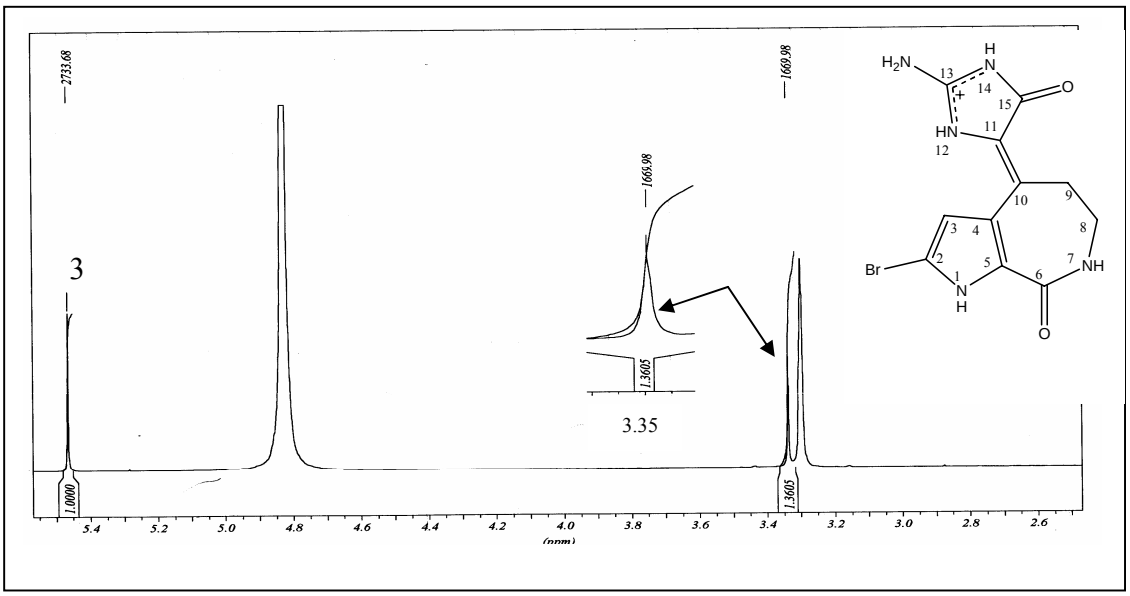


Figure 27 [1] H-NMR spectra of compound **9** (MeOH-d_4)

Compound **8** was considered to be the *E* – isomer because of the deshielding effect of the amide carbonyl group at C-15 on the methine proton at C-3 which was more downfield (δH 6.60) than in the *Z* isomer (δ 5.41 ppm) (compound **9**). The structure of both compounds differ cite regard to the geometrical isomerism of the double bond at positions C-10 and C-11. Therefore, compounds **8** and **9** were elucidated as *E* and *Z* –hymenialdisine, respectively.

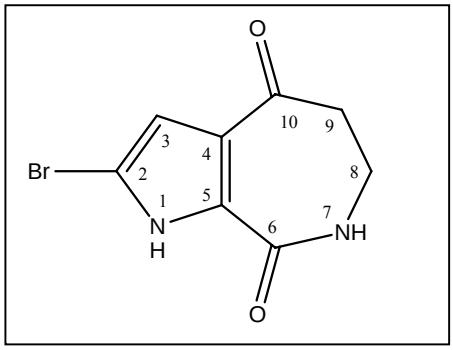
Table 5 NMR data of compounds **6-9** (DMSO-d_6)

Position	Stevensine **(6)**			3-bromohymenialdisin **(7)**	*E*-hyminialdisine **(8)**	*Z*-hyminialdisine **(9)**
	^{1}H-NMR (multiplicity,*J in Hz*)	^{13}C-NMR* , multiplicities	HMBC	^{1}H-NMR (multiplicity,*J in Hz*)	^{1}H-NMR (multiplicity,*J in Hz*)	^{1}H-NMR (multiplicity,*J in Hz*)
3	-	-	-	-	6.60 (brs)	5.41 (brs)
6	-	161.0, s	-	-	-	-
8	3.57 (d,7.0)	-	C-10, C-6, C-9	3.42 (brs)	3.34 (brs)	3.34 (brs)
9	6.25 (t, 6.9,)	126.0, s	-	3.42 (brs)	3.34 (brs)	3.34 (brs)
10	-	120.9, s	-	-	-	-
12	6.80 (s)	-	-	-	-	-

* ^{13}C-NMR data determined from HMBC spectrum

3.2.2. Structure elucidation of the isolated compounds 10 and 11

3.2.2.1. 2-bromoaldisine (10, known compound)



Compound **10** was isolated as a white powder. It showed UV absorbances at λ_{max} 224 and 311 nm. Compound **10** showed [M+H] $^{+}$ quasimolecular ion peaks at *m/z* 224 and 310 in its ESIMS spectra (**Figure 28)** which indicated the presence of one bromine substituent in the molecule. The ^{1}H-NMR spectrum of compound **10** (**Figure 29**) exhibited two methylene proton signals at δH 2.80 (H_2-9) and at δH 3.50 (H_2-8) together with a methine proton in the aromatic region at δH 6.60 as found in a pyrrole ring. The HMBC data of compound **10** (**Figure 30** and summarized in **Table 6**) showed the correlation of proton at δH 3.50 (H-8) to the carbonyl carbon at δC 161.0 (C-10), and 43.3 ppm (C-9) proton at δH 2.80 (H-9) to δC 194.2 (C-10) and 36.02 (C-8). These correlations confirmed the assignment of the methylene protons at positions 8 and 9. Furthermore, the proton resonance at δH 6.60 gave correlations to quaternary carbons at position 2 (δC 105.0), 4 (δC 124.5), and 5 (δC 129.5) which suggested that this proton belongs to C-3 of the pyrrole ring. Based on the ESIMS, ^{1}H-NMR, HMBC data and in comparison with

the literature data, compound **10** was identified as 2-bromoaldisine which was previously isolated from *Hymeniacidon aldis* collected from Guam Island [Schmitz *et al*., 1985].

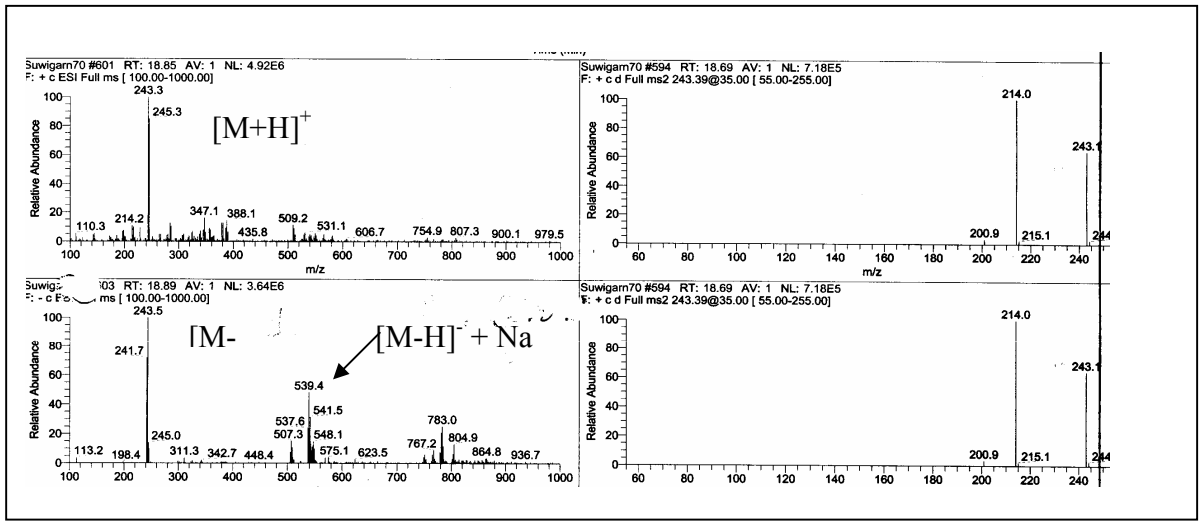


Figure 28 ESIMS of compound **10**

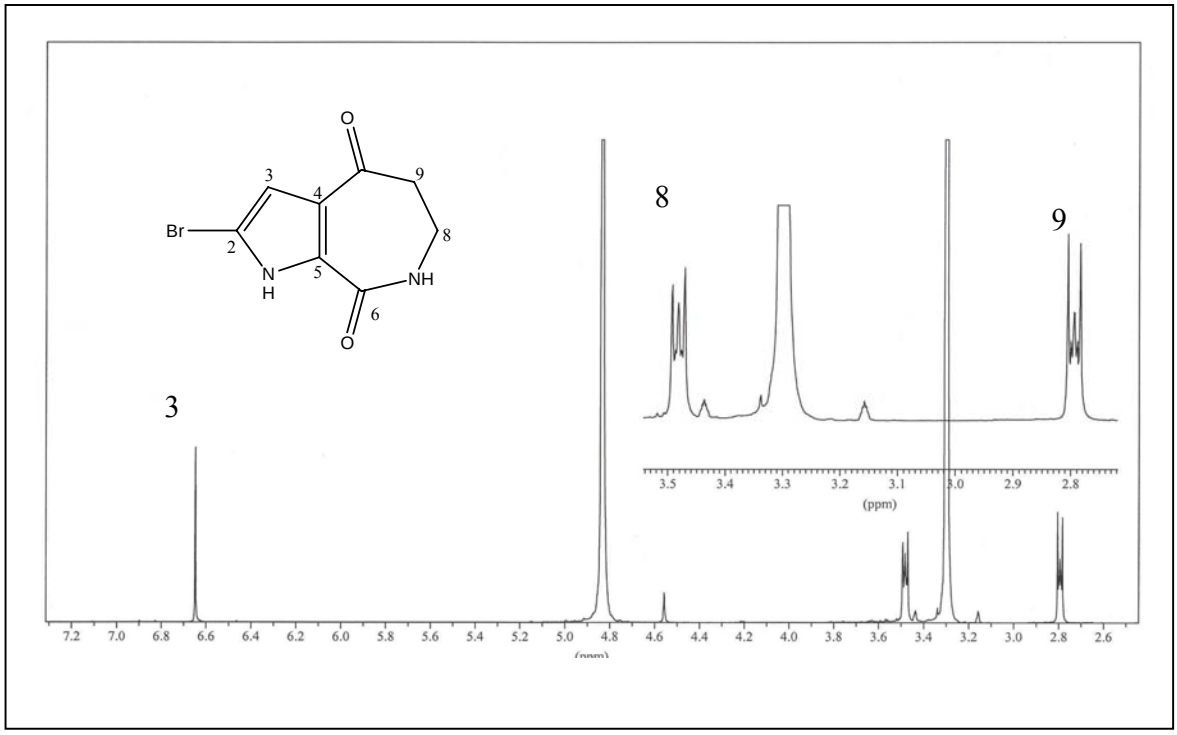


Figure 29 ^{1}H-NMR spectra of compounds **10** (MeOH-d_4)

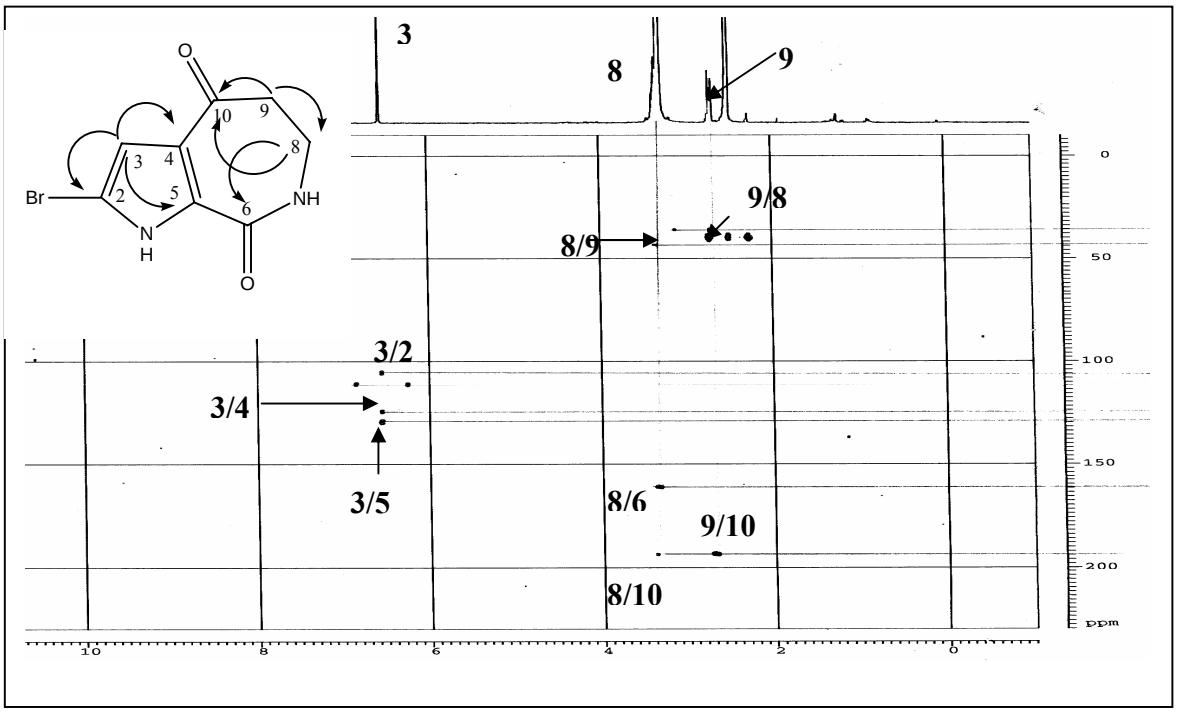
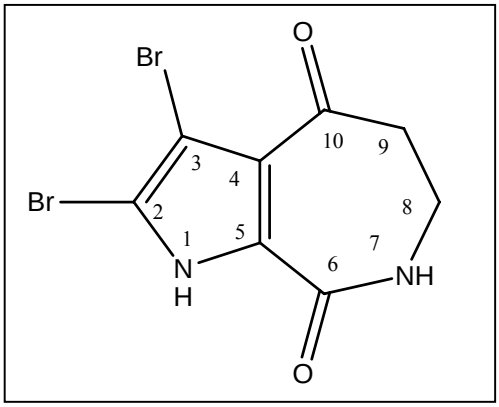


Figure 30 Important C-H long range correlations of compound **10**

3.2.2.2. 2,3-dibromoaldisine (11, new compound)



Compound **11** was purified as white powder with UV absorbance at λ_{max} 224 and 311 nm. The ^{1}H-NMR spectrum of compound **11** (**Figure 32**) showed only two methylene signals in the aliphatic region at δH 4.10 and 2.90 similar to compound **10**. However, a singlet proton at δH 6.60 as present in the spectrum 10 was absent. Therefore, compound **11** should be fully substituted in the pyrrole ring. According to the negative ESIMS spectrum (**Figure 31),** it showed quasimolecular ion peaks at *m/z* 319, 321, and 323 with intensities of 1:2:1 which supported the presence of two bromine substituents in the molecule. To confirmed the structure of **11**, compound **10** was brominated and its ^{1}H-NMR and Ms spectra were formed to be identical to those of **11**. Therefore, compound **11** was identified as 2,3-dibromoaldisine.

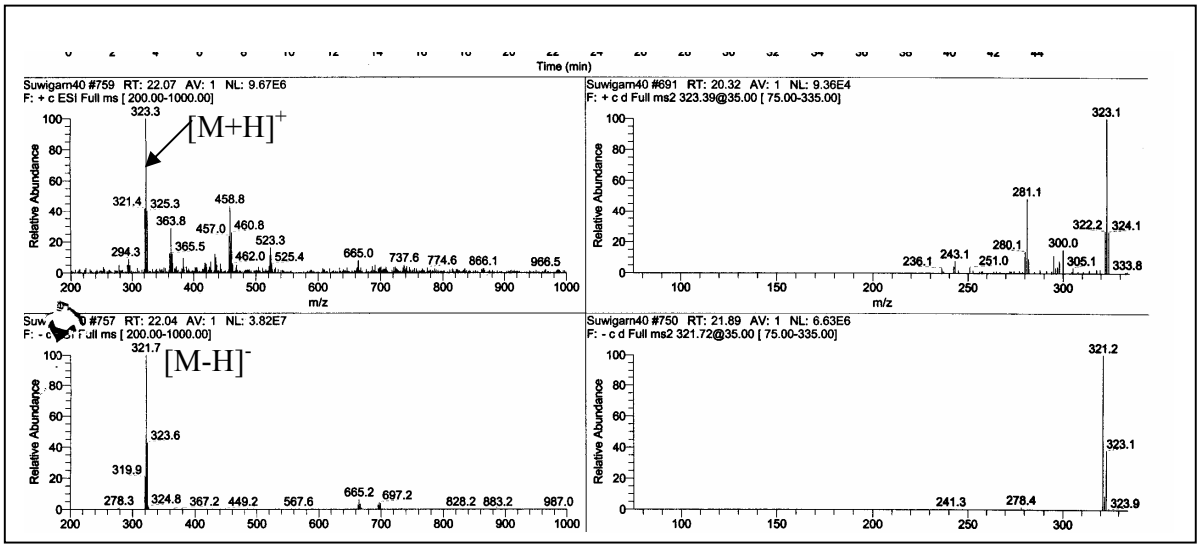


Figure 31 ESIMS of compound **11**

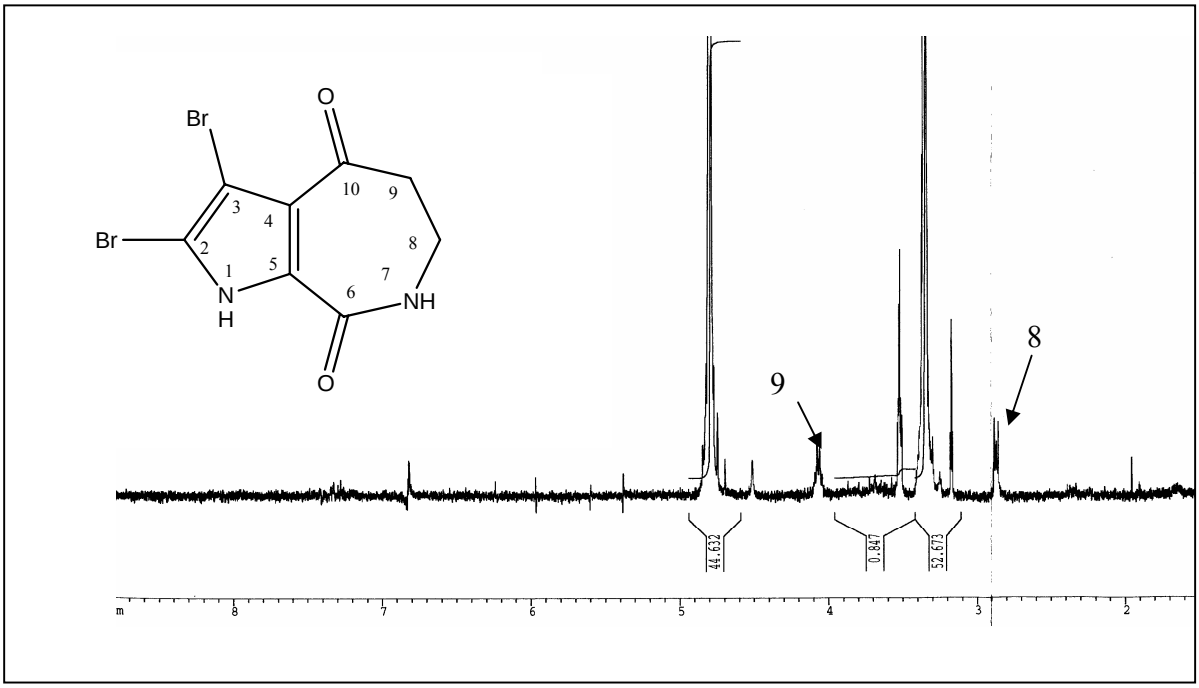


Figure 32 ^{1}H-NMR of compound **11** (MeOH-d_4)

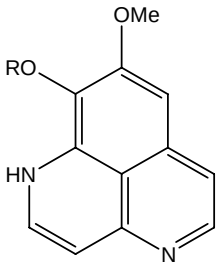
Table 6 ^{1}H-NMR data of compounds **10** and **11** (MeOH-d_4)

Position	2-bromoaldisine **(10)**			2,3-dibromoaldisine **(11)**
	^{1}H-NMR (multiplicity,*J in Hz*)	^{13}C-NMR	HMBC	^{1}H-NMR (multiplicity,*J in Hz*)
1	-	-	-	-
2	-	105.0, s	-	-
3	6.60 (s)	110.8, d	C-2, C-4, C-5	-
4	-	124.5, s	-	-
5	-	129.5, s	-	-
6	-	161.0, s	-	-
7	-	-	-	-
8	3.50 (dd,2.5, 3.15)	36.02 , t	C-6,C-9, C-10	4.10 (brs)
9	2.80(dd,2.53,3.15)	43.3, t	C-8, C-10	2.90 (brs)
10	-	194.2, s	-	-

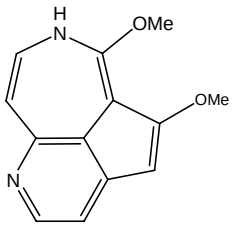
3.4. Secondary metabolites from the sponge *Aaptos suberitoides*

3.4.1. Chemical constituents from the genus *Aaptos*

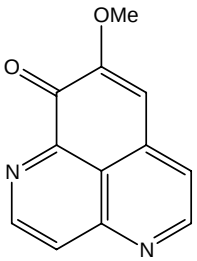
Sponges of the genus *Aaptos* were reported to produce interesting alkaloids containing the 1H- benzo [de]-[1,6]-naphthyridine skeleton [Nakamura, Kobayashi and Ohizumi, 1987] and other aaptosin skeletons as shown below;



Aaptamine : R= Me
Demethylaaptamine : R = H



Aaptosin



Dehydroaaptamine

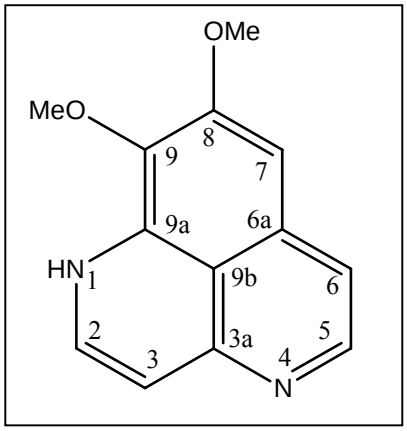
Aaptamine alkaloids are known for their pharmacological activities. They have been reported to exhibit activities including high potent α-adrenoreceptor blocking activity on vascular smooth muscle [Nakamura, Kobayashi and Ohizumi, 1987], and were also shown to be cytotoxic toward murine leukemia cell P388 and other human tumor cell lines. Their anti-tumor activity is depended upon the oxidation of the hydroxyl function at the C-9 to give a carbonyl

function which result to an increase in activity. Aaptosin on the other hand was found to be inactive [Shen, Lin and Duh, 1999].

3.4.2. Structure elucidation of compounds 16 and 17

Compounds **16** and **17** were purified as an amorphous dark solid by preparative HPLC (see **scheme 5**). Structure elucidations of both compounds were performed by means of ESIMS and by 1D and 2D NMR experiments.

3.4.2.1. Aaptamine (16, known compound)



Compound **16** showed a pseudomolecular ion peak at *m/z* 229 by positive ESIMS (**Figure 44**). It showed UV absorbance λ_{max} 240 and 254 nm. Its ^{1}H-NMR (**Figure 45**) showed five resonances in the aromatic region which indicated the presence of a 1,6-napthpyridine skeleton [Walz and Sundberg; Sugino, *et al*., 1999; Into, 1998]. Two methoxy singlets (δH3.82 and 4.10), and two sets of *ortho* protons (δH 7.81, 6.35, and δH 7.42, 6.93) together with an

additional singlet at δH 7.42 were observed. According to its ^{13}C-NMR spectrum data (**Figure 46**), it has eleven aromatic corbons and two methoxy groups in the molecule.

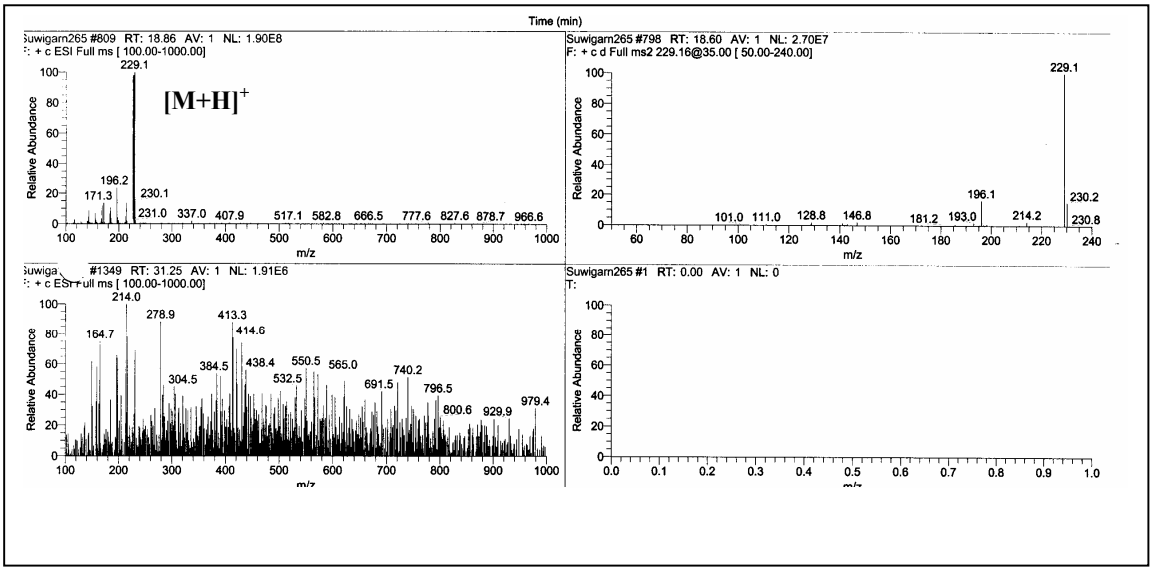


Figure 44 ESIMS of compound **16**

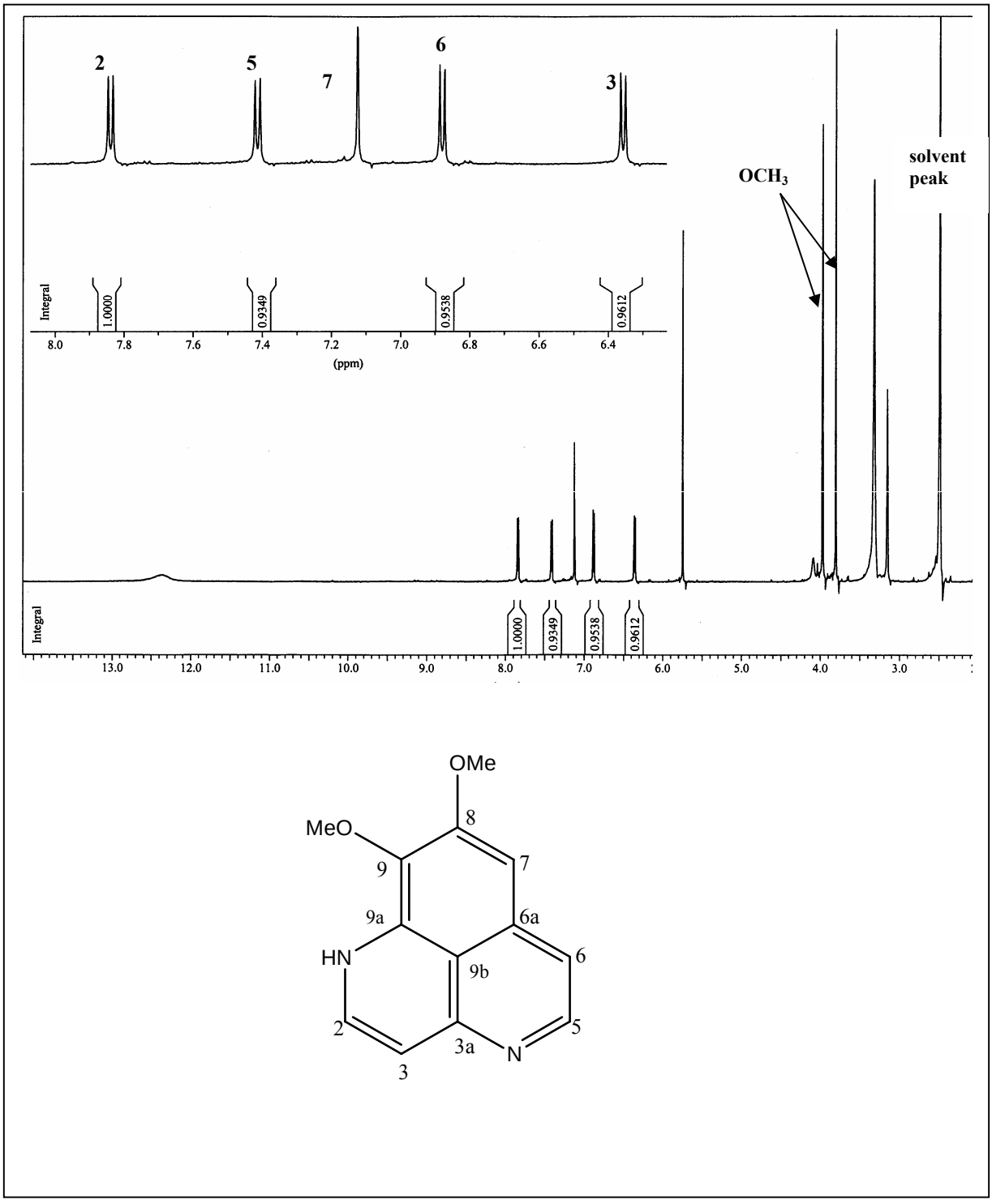


Figure 45 ^{1}H-NMR of compound **16** (DMSO-d_6)

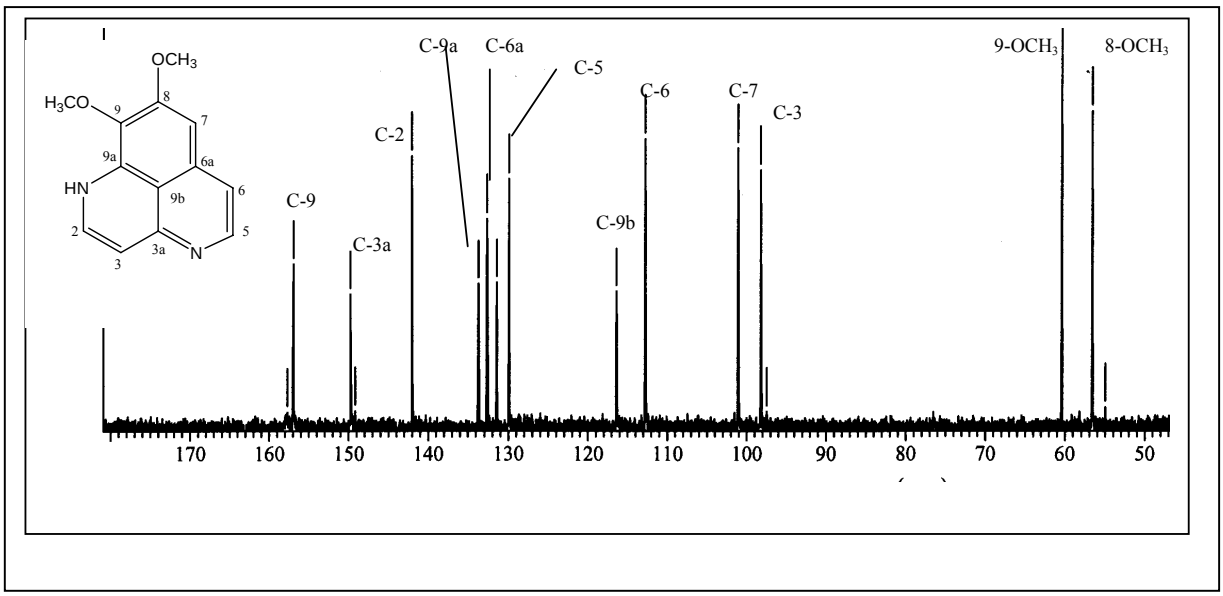


Figure 46 ^{13}C-NMR of compound **16** (DMSO-d_6)

Structure elucidation was performed by intensive analysis of C-H long range correlation data through its HMBC spectrum (**Figure 47**). The methoxy proton at δH 3.82 was positioned on C-9 because it showed long range correlation with δC 156.9 while the methoxy resonance at δH 4.10 was aligned to C-8 as it correlated with δC 131.3. Furthermore, a singlet proton at δH 7.10 ppm was proved to be at C-7 as it revealed ^3J correlations with C-9 (δC 156.9), C-9a (δC 133.7) and C-6 (δC 112.6). Two bond correlations were also observed from H-7 (δH 7.10) with C-6a (δC 132.6) and C-8 (δC 131.3). The HMBC data were also used to confirm the placement of two sets of *ortho* paired protons in compound **16**. The doublet proton at δH 7.81 gave cross peaks with C-9a (δC 132.6) and C-3a (δC 149.7) while its doublet pair at δH 6.35 correlated with C-9b (δC 116.3) which suggested that this set of *ortho* protons was located at C-2 (δC 142.0) and C-3 (δC 98.1), respectively. The doublet proton at δH 7.42 showed correlations

to C-6a (δC 132.6) and C-3a (δC 149.7), and consequently its corresponding doublet proton at δH 6.93 correlated with C-9b (δC 116.3) and C-7 (δC 100.9) which indicated that the second pair of *otho* protons was located at C-5 (δC 129.8) and C-6 (δC 112.6), respectively. Thus, compound **16** was identified as aaptamine.

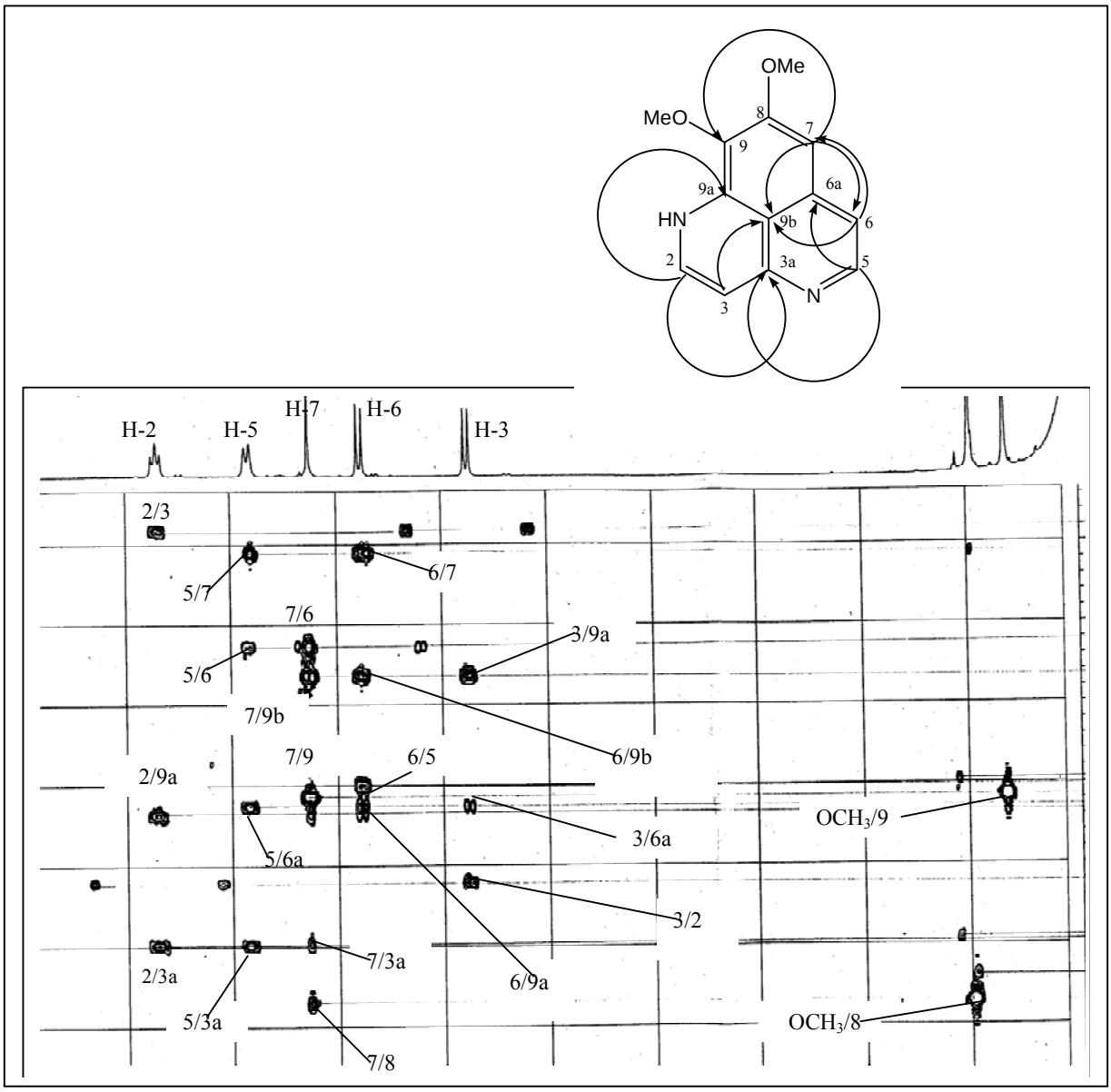
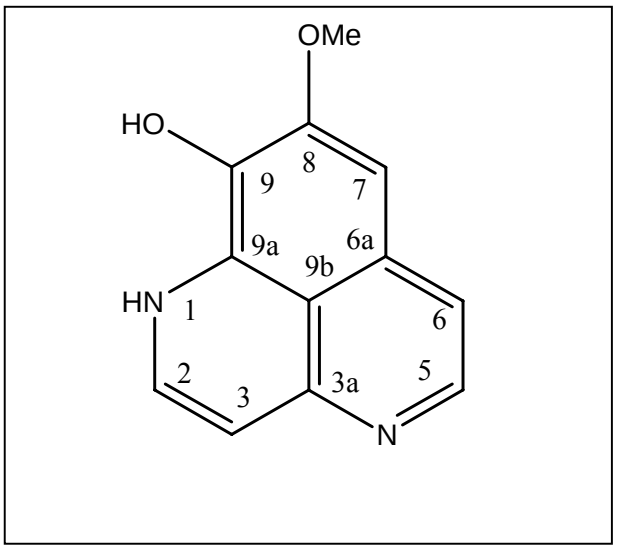


Figure 47 The important C-H long range correlation of compound **16**

3.4.2.2. Demethylaaptamine (17, known compound)



^{1}H-NMR spectrum of compound **17 (Figure 49)** exhibited only one methoxy unit resonating at δH 3.92 and an hydroxyl function was observed at δH 10.10. This was in accordance to its ^{13}C-NMR spectrum data **(Figure 50)**, which showed also one methoxy carbon at δC 53.6. Its ESIMS data (**Figure 48**) showed a pseudomolecular ion peak at *m/z* 215, which is 15 mass units less than that of compound **16** and additionally suggested the presence of only one methoxy group in the molecule.

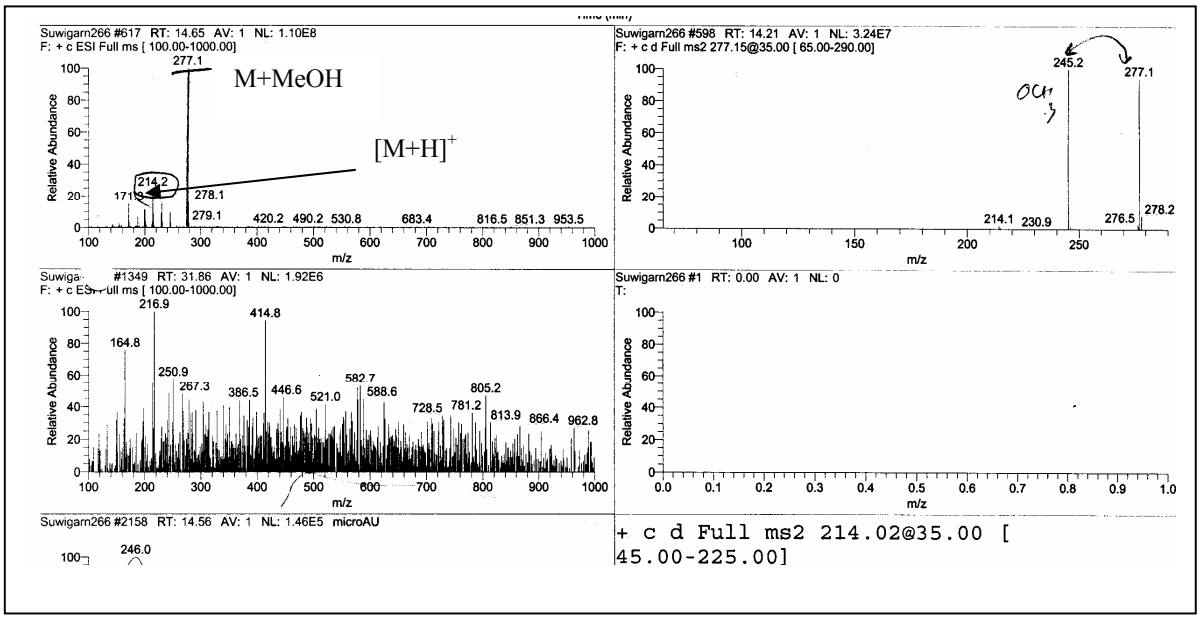


Figure 48 ESIMS of compound **17**

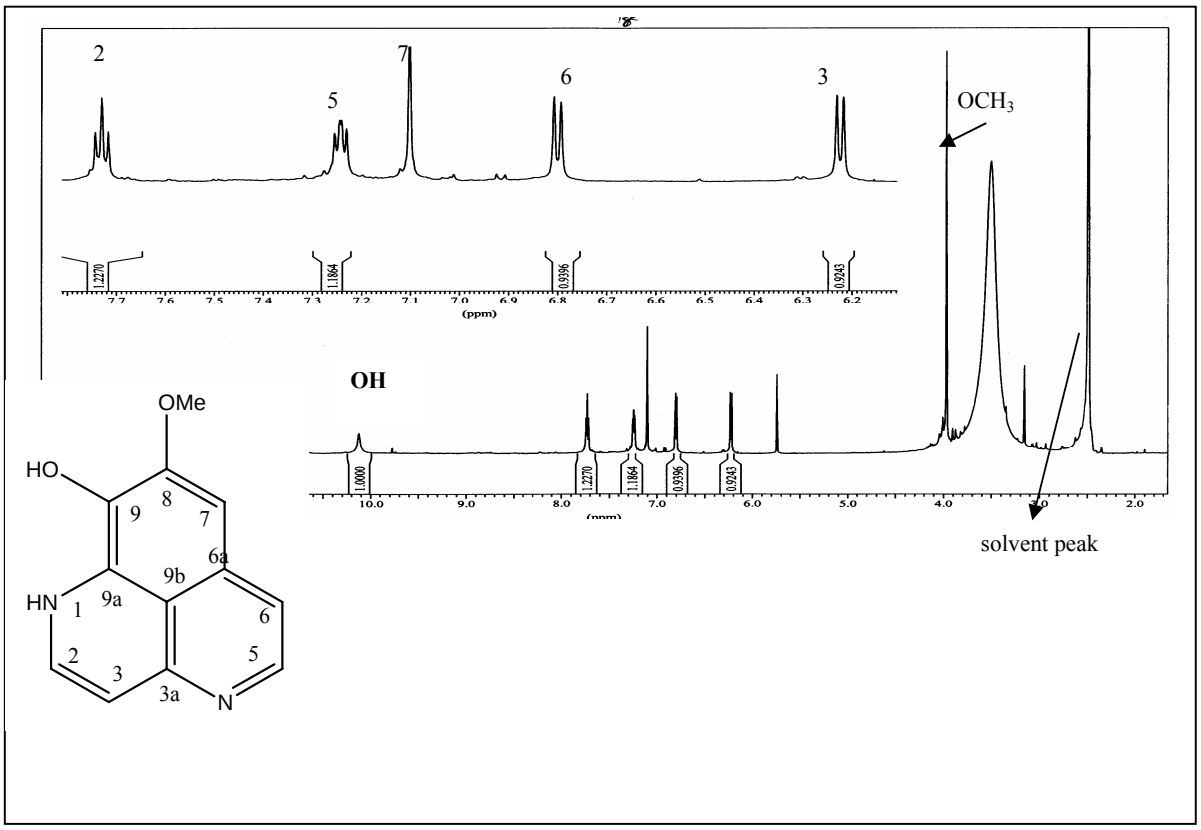


Figure 49 ^{1}H-NMR of compound **17** (DMSO-d_6)

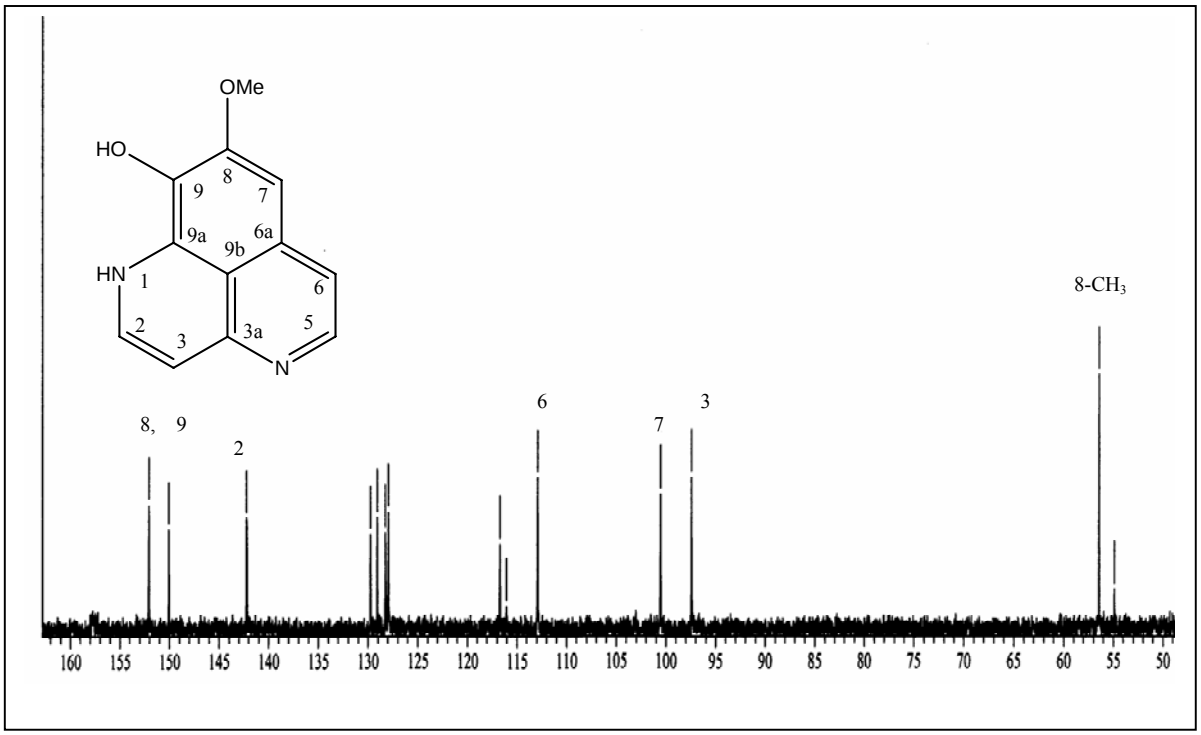


Figure 50 ^{13}C-NMR spectra of compound **17** (DMSO-d_6)

HMBC spectral data **(Figure 51)** were also used to assign the position of the methoxy and hydroxy groups in compound **17**. The cross peak of δH 3.92 (OMe) with δC 151.8 proved the assignment of the methoxy function at C-8. The three important cross peaks of the methine proton at δH 7.10 (H-7) with δC 152.0, 112.7 (C-6) and 127.7 (C-6a) confirmed the attachment of the OH at C-9 (δC 152.0). For the two *otho* doublet pairs and other assignments, they were almost identical as found in compound **16.** Based on the above discussed data, compound **17** was identified as demethylaaptamine. Both compounds **16** and **17** were previously isolated from the Okinawan marine sponge, *Aaptos aaptos* [Nakamura, Kobayashi and Ohzumi, 1982 and 1987]. They exhibited antimicrobial activity against *B.subtilis*, cytotoxic activity

towards three different oncogenic cell lines, and showed also antioxidative activities [Tsukamato *et al.,* 2003; Batczewski *et al.,* 1990; Shen *et al.,* 1999].

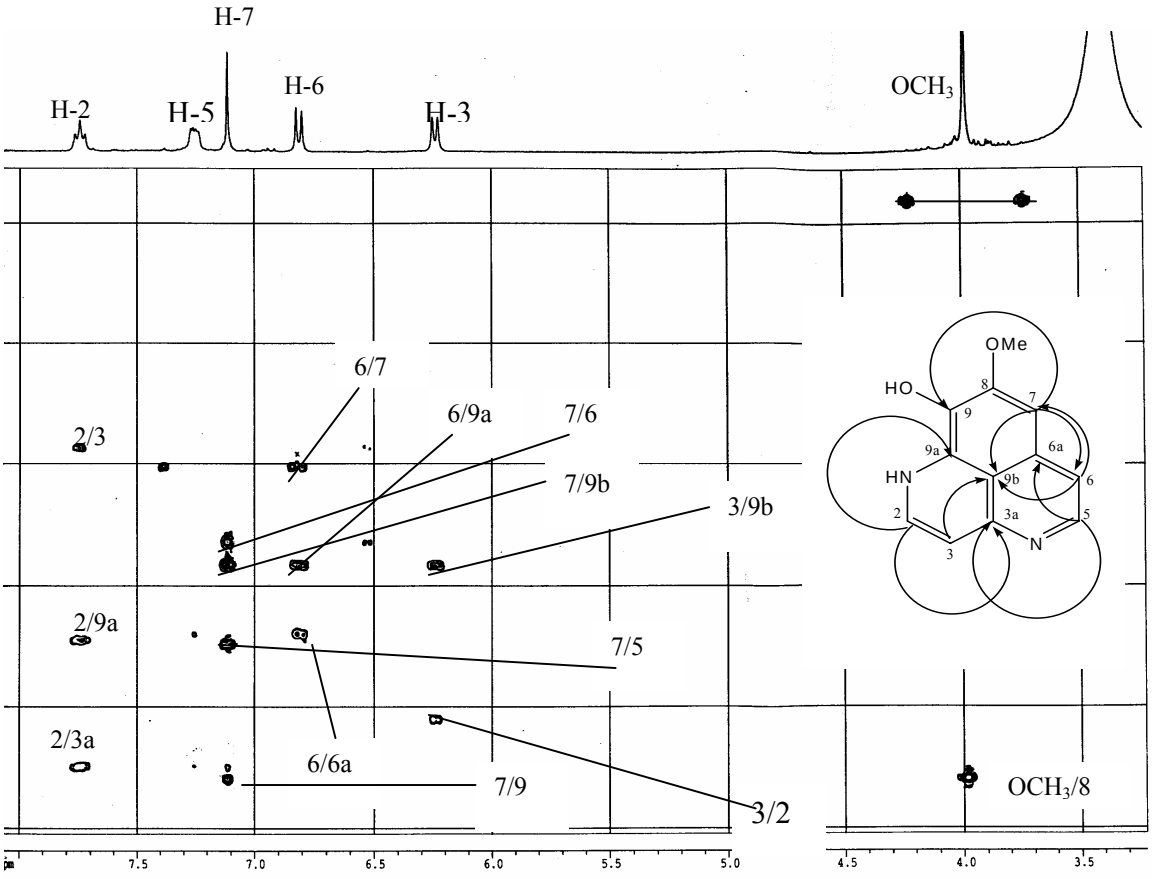


Figure 51 Important C-H long range correlation of compound **17**

3.4.3. Bioactivities study

Both compounds **16** and **17** showed interesting bioactivities comprising anti-microbial activity and cytoxicity as shown in table below.

Table 10 Bioactivities result of compounds **16** and **17**

Biological activities	Type of cell	ED_{50} µg/ml	
Cytotoxicity		**17**	**18**
	L - cell	0.5	0.6
	Hela - cell	10	7.3
	PC-12 - cell	4.1	1.9
Antimicrobial activity against *B. subtilis*		Inhibition zone (mm)	
	10 µg/ml*	-	8
	20 µg/ml**	8	-

* The sample was applies for 5 µg/disc and

** 10 µg/disc

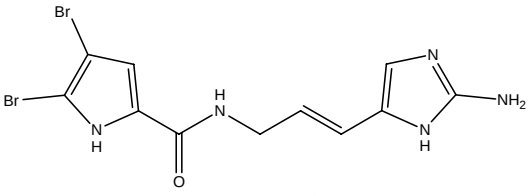
Table 11 NMR data of isolated compounds from *Aaptos suberitoides* (DMSO-d_6)

Position	**Compound 16**			**Compound 17**	
	δH (ppm), multiplicities, J in Hz	δC* (ppm), multiplicities	HMBC	δH (ppm), multiplicities, J in Hz	δC* (ppm), multiplicities
1, NH	12.65 (brs)	-	-	12.64 (brs)	-
2	7.81(t, 6.2, 6.2)	142.0, d	3a, 3, 9a	7.81(t, 6.6, 7.1)	141.8, d
3	6.35(d, 6.2)	98.1, d	2, 6a, 9b	6.35 (d, 7.1)	97.1, d
3a	-	149.7, s	-		149.8, s
5	7.42 (brt, 6.9, 4.1)	129.8, d	3a, 6, 6a, 7	7.25 (brt, 5.1, 7.2)	129.5, d
6	6.93 (d, 6.9)	112.6, d	6a, 7, 9b	6.82 (d, 7.2)	112.7, d
6a	-	132.6, s	-	-	127.7, s
7	7.10 (s)	100.9, d	3a, 6, 6a, 8, 9, 9b	7.10 (s)	100.3, d
8	-	131.3, s	-	-	151.8, s
9	-	156.9, s	-	-	152.0, s
9a	-	133.7, s	-	-	128.8, s
9b	-	116.3, s	-	-	116.9, s
OCH_3	3.82 (s)	56.4, q	8	3.92 (s)	56.3, q
OCH_3	4.10 (s)	60.3, q	9	-	-
OH				10.10 (brs)	

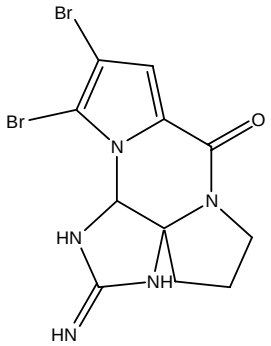
* multiplicities determined by DEPT 135 spectrum. TMS was used as internal reference

3.5. Secondary metabolites from the sponge *Agelas nemoecinata*

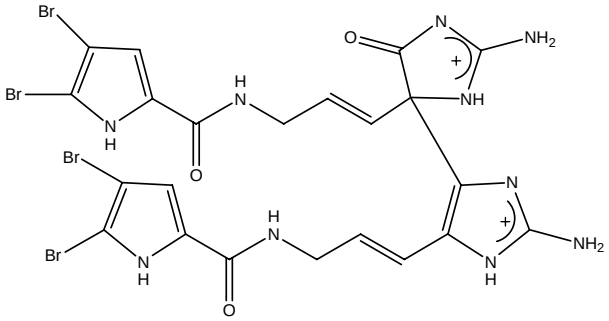
Bromopyrrole-imidazole alkaloids have been interesting secondary metabolites for marine natural products chemists because of their structural diversity and important biological activities. These molecules are exclusively elaborated by the secondary metabolisms of marine sponges belonging to the order Agelasida, Axinellida and Halichondrida. More than 50 bromopyrrole-imidazole alkaloids have been isolated [Forenza, *et al.*, 1996]. Their structures can be conceived as derivatives of the $C_{11}N_5$ skeleton of oroidin through; (i) isomerization of double bond and oxidation or reduction, (ii) dimerization, and (iii) cyclization [Fattorusso and Scafati, 2000]. Important biological activities of these classes of molecules are antifouling activity [Tsukamoto *et al.*, 1996], α-adrenoceptor blocking [Kobayashi *et al.*, 1986], antifungal, antitumor and immunosuppressive activity [Kinnel *et al.*, 1998]. Some reported compounds which were isolated from the genus *Agelas* are shown below.



Oroidin

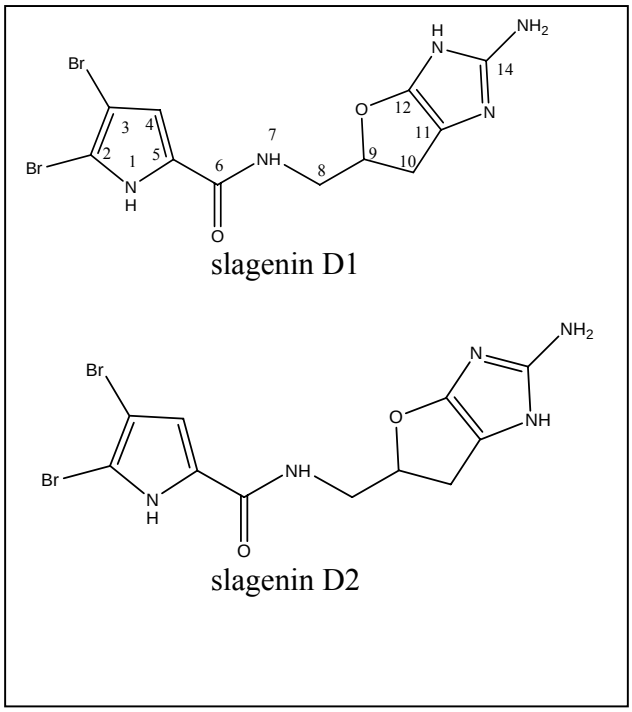


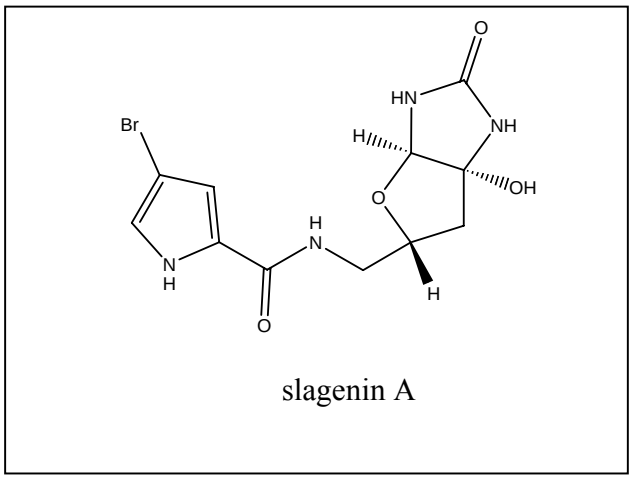
Dibromophakellin



Mauritiamine

3.5.1. Slagenin D1 and D2 (18, new compounds)





Slagenin D1 and D2 are new metabolites which were isolated as white oily residues with UV absorbances at λ_{max} 221 and 284 nm. These compounds were isolated as a mixture at a ratio of 2:1. HRESIMS (**Figure 52**) showed the typical bromine substitutent pattern at *m/z* 404.942, 406.942 and 408.941 $[M+H]^+$ that calculated for 404.9436 $[M+H]^+$ as molecular

weight and was compatible with the molecular formula $C_{11}H_{13}Br_2N_5O_2$. The ^{1}H-NMR spectra data were comparable to those of slagenin A. Slagenin D has 18 mass units less than the known derivative slagenin A. An identical brominated pyrrole ring was found in both compounds as shown by the integrals which were twice as high as those of the aliphatic region. Due to tautomerism occurring in the imidazole ring, the presence of the two tautomers (**D_1** and **D_2**) could be observed in its ^{1}H-NMR spectrum in a 2:1 ratio. ^{1}H-NMR (**Figure 53** and **Table 12**) exhibited two broad singlets at δH 6.97 and at δH 6.95 which belong to the respective protons in the pyrrole ring of both tautomers. Five sp^3 proton resonances at the upfield region were assigned for a $CH_2CH(X)CH_2$ spin system of both tautomers which was proved through its COSY spectra.

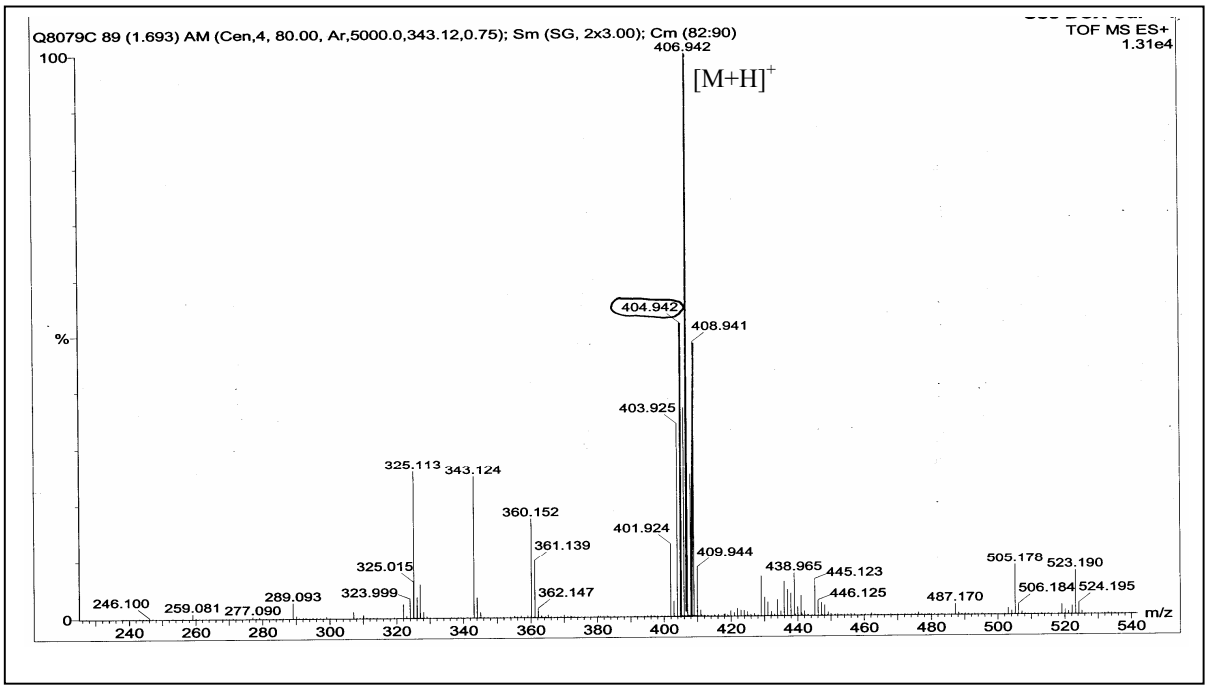


Figure 52 HRESIMS data of **slagenin D_1** and **D_2**

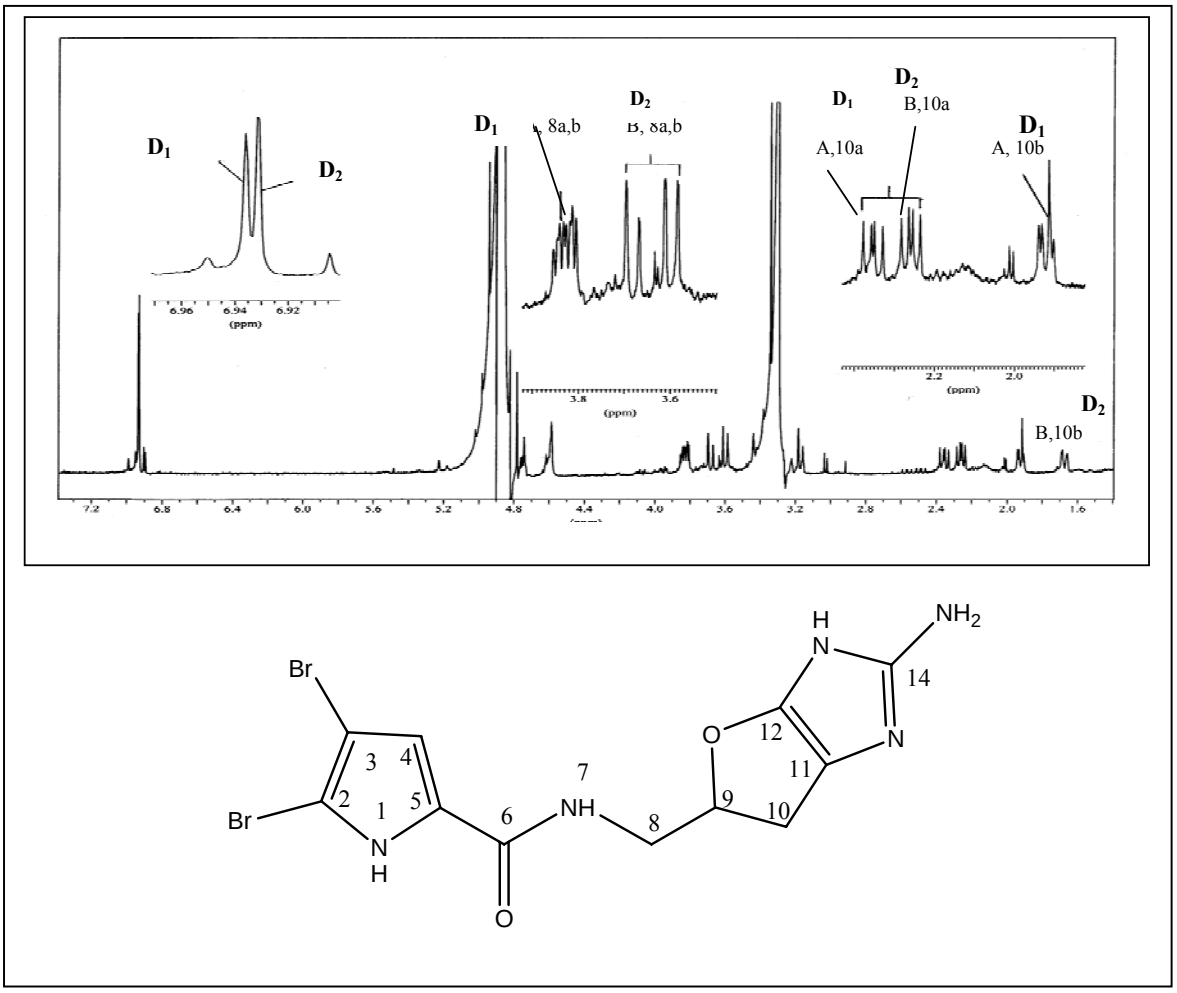


Figure 53 ^{1}H-NMR of **slagenin D1** and **D2** (MeOH-d_4)

The COSY spectra (**Figure 54**) was used to analyze the coupling protons in the compound. It supported the presence of two sets of a $CH_2CH(X)CH_2$ spin system. The first tautomer consisted of sp^3 protons resonating at δH 3.90 (H-8a and H-8b), δH 4.70 (H-9), and at δH 2.35 and δH 1.95 ppm (H-10a and H-10b). The second tautomer revealed sp^3 protons at δH 3.83 (H-8a and H-8b), δH 4.85 (H-9), and δH 2.41 and 1.82 ppm (H-10a and H-10b), respectively. Due to the very small amount isolated, no HMBC spectrum could be determined, proton assignments were established only by COSY and analysis of the chemical shifts in comparison with the literature data. The proton at δH 4.70 and 4.85 were assigned for the position H-9 for tautomers D1 and D2, respectively. This assignment was comparable with slagenin A [Tsuda, Uemoto and Kobayashi, 1999]. In slagenin A, the proton at δH 4.00 was assigned to the methine H-9 while the resonance at δH 2.06 (dd, 3.6, 11.6) was assigned to the methylene H_2-10. These assignments were compatible to those of salagenin D1/D2 (**18**), which gave proton resonances at δH 2.10 and 2.41 (dd, 3.7, 10.4) for H_2-10, respectively. Slagenin D has no oxygenated proton at C-11 as found in slagenin A, which formed a double bond with C-12. This also explained the disappearance of an extra methine singlet at δH 4.90 as observed in slagenin A. The first tautomer was determined to exhibit the structure of D1 because it showed a slight downfield shift for H-10b (δH 1.95) when compared to slagenin D2 due to the shielding effect of an unpaired electron on the nitogen atom adjacent to it. On the one hand, the second tautomer was assigned to be slagenin D2 because it showed an upfield shift at H-10b (δH 1.85) due to the deshielding effect of the NH group adjacent to the methylene at C-11. Compound **18** was identified as slagenin D, a new derivative of slagenin A which was previously isolated from the Okinawan marine sponge *Agelas nakamurai* [Tsuda *et al.*,1999; Assmann *et al.*, 2001].

Structure elucidation was performed by COSY and comparison of proton chemical shifts with those in the literature.

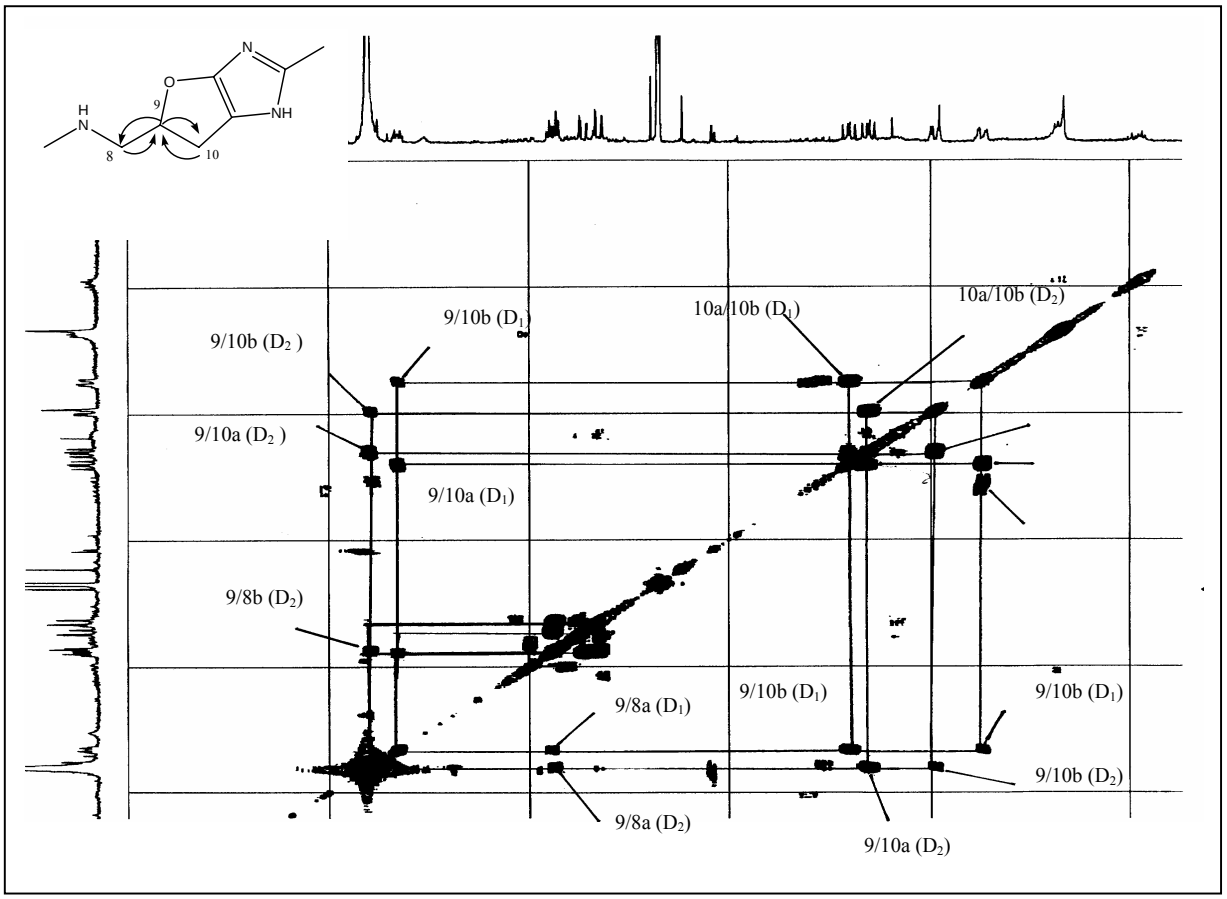


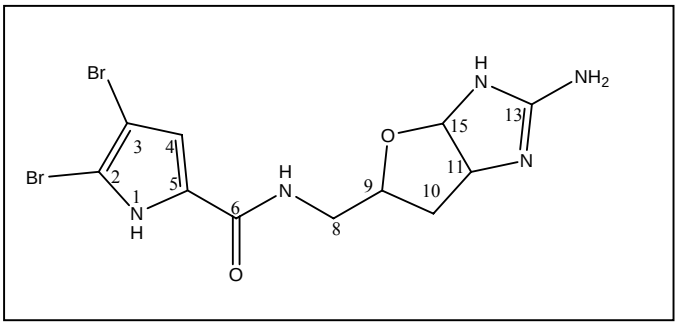
Figure 54 COSY spectra of compounds **18** (slagenin D_1 and D_2)

Table 12 NMR data of compound **18** comparison with slagenin A

Position	δ H (multiplicities, J in Hz) (MeOH-d_4)		COSY		Slagenin A* (DMSO-d_6)	
	18 D_1	**18 D_2**	**18 D_1**	**18 D_2**	δ H	δC
1, NH	-	-	-	-	11.81 (brs)	-
2	-	-	-	-	6.97 (brs)	126.7, d
3	-	-	-	-	-	94.9, s
4	6.97 (s)	6.95 (s)	-	-	6.87 (brs)	111.7, s
5	-	-	-	-	-	-
6	-	-	-	-	-	-
7	-	-	9	9	-	-
8	3.90 (m)	3.83 (m)	8a,b and 10a,b	8a,b and 10a,b	a, 3.38, m b, 3.34, m	41.5, t
9	4.70 (m)	4.85 (m)	9	9	4.00, m	76.1, d
10	2.10 (dd, 3.7, 10.4) b, 1.95 (dd, 3.7, 10.4)	a, 2.41 (dd, 3.7, 10.4) b, 1.82 (dd, 3.7, 10.4)	-	-	2.06 (dd, 3.6, 11.6)	43.0, t
11	-	-	-	-	OH	93.3, s
12	-	-	-	-	4.9 (brs)	91.9, d
13	-	-	-	-	-	159.7, s
14	-	-	-	-	-	-
15	-	-	-	-	-	-

* Tsuda, Uemoto and Kobayashi (1999)

3.5.2. Slagenin E (19, new compound)



Compound **19** was obtained as a white oil (see **Scheme 6**) with UV absorbance at λ_{max} 237 and 286 nm. This compound gave a very small yield and was quite unstable. Therefore, no HRMS and 2D NMR data could be obtained. Structure elucidation was performed by means of ^{1}H-NMR and mass fragmentation analysis. The ESIMS spectrum (**Figure 55**) showed the molecular ion peak cluster at *m/z* 400, 402, and 404 in the negative mode, which indicated that it has two bromine substituents in the molecule. Compound **19** has two mass units more than compound **18.** The MS/MS fragmentation showed the loss of 80 mass units at *m/z* 331.1 which suggested the presence of furan imidazole ring.

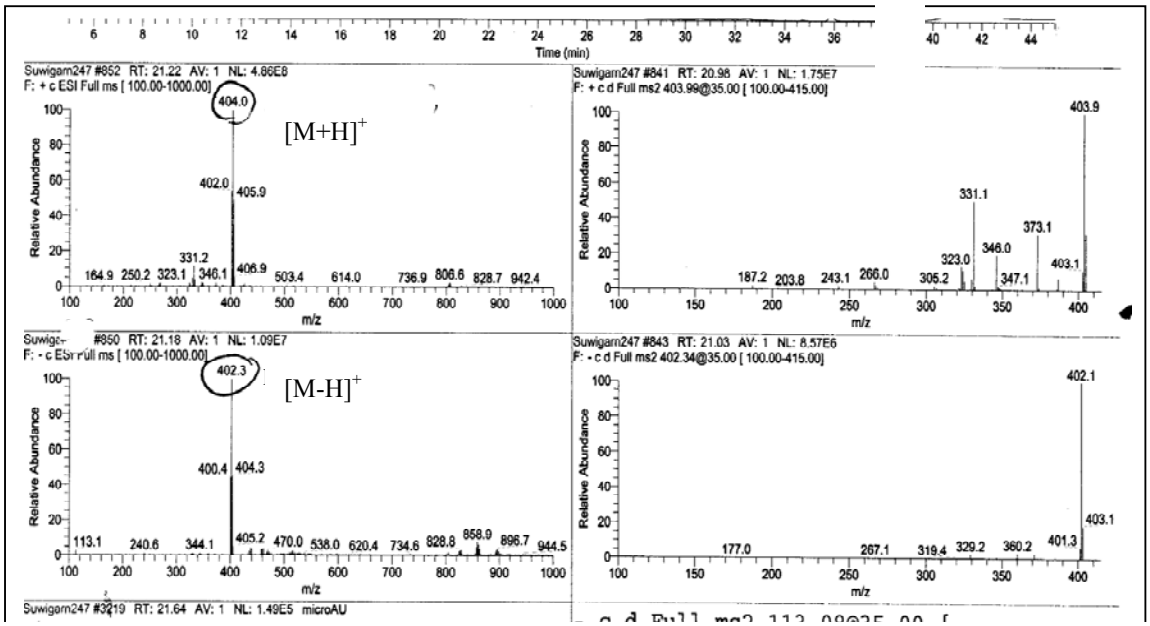


Figure 55 ESIMS spectra of **slagenin E** .

The ^{1}H-NMR spectra of this compound (**Figure 53**), showed a typical proton resonance at δH 6.90 as found in a pyrrole ring and it showed also a $CH_2CH(O)CH_2CH$ spin system. This was slightly different from the presence of $CH_2CH(O)CH_2$ spin system as found in **slagenin D**. Protons at C-10 were under the solvent peak at δH 2.20 ppm. Exchangeable protons were clearly shown in the ^{1}H-NMR spectrum such as δH 12.5 (1-NH), δH 8.30 (7-NH) and δH 7.90 (13-NH_2), repectively. Compound **19** showed an additional sp^3 proton at δH 2.20 (H-11) which did not appear in slagenin A and D. Its ^{1}H-NMR spectra also showed an extra oxygenated proton at δH 4.90 (H-12) which was comparable to that of **slagenin A** at δH 4.94. These data confirmed the assignment for a tetrahydrofuran ring instead of a dihydrofuran ring as found in compound **18**.

Based on the comparison with the proton chemical shifts of slagenin A [Tsuda *et al.*,1999] (**Table 13)** and with the former compound, the structure of compound **19** was concluded to be **Slagenin E**.

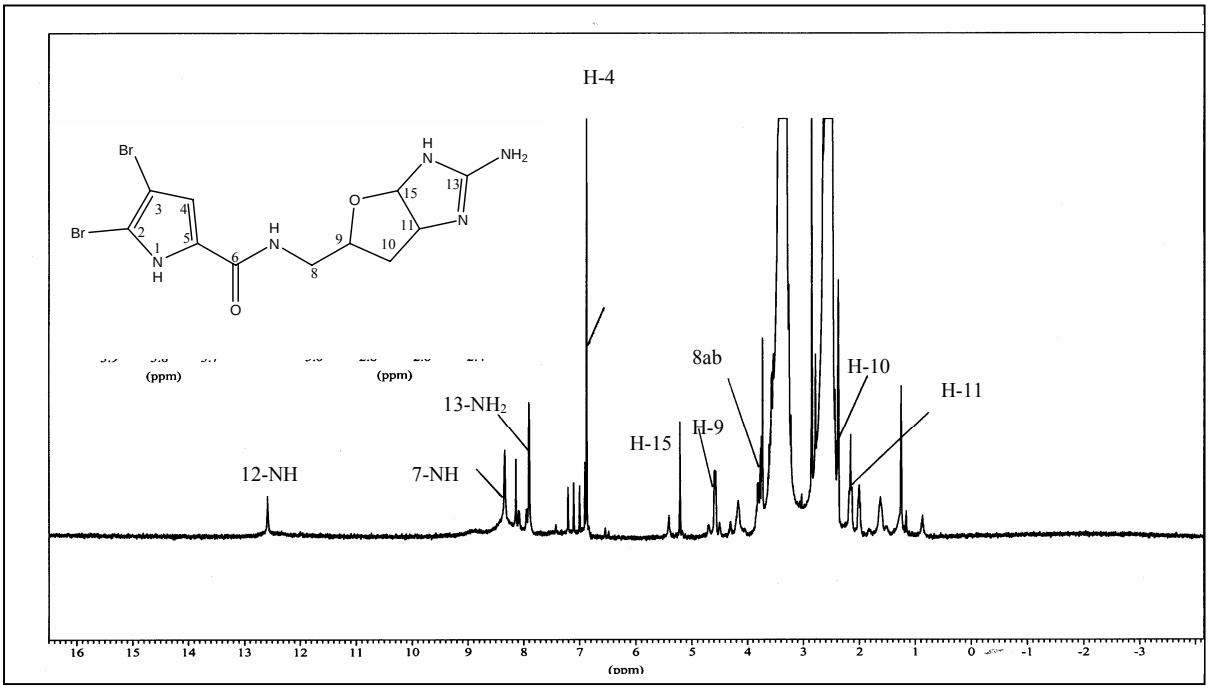


Figure 56 ^{1}H-NMR spectra of compound **19** (DMSO-d_6)

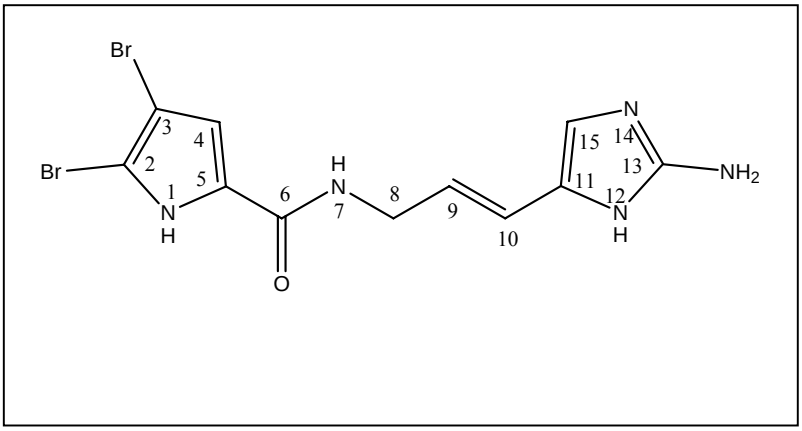
Table 13 The comparison of NMR data of slagenin E (**19)** with slagenin A

Position	slagenin A* (DMSO-d_6)		slagenin E **(19)** (MeOH-d_4) δ H (multiplicities, J in Hz)
	δ H	δC	
1, NH	11.81 (brs)	-	12.5 (brs)
2	6.97 (brs)	126.7, d	-
3	-	94.9, s	-
4	6.87 (brs)	111.7, s	6.90 (s)
5	-	-	-
6	-	-	-
7,NH	8.21, brs	-	8.30 (brs)
8	a, 3.38, m b, 3.34, m	41.5, t	3.70 (m)
9	4.00, m	76.1, d	4.25 (m)
10	2.06 (dd, 3.6, 11.6)	43.0, t0	a and b, 2.20 **
11	OH	93.3, s	2.20 (m)
12	-	-	-
13	-	159.7, s	-
14	-	-	-
15	4.94 (brs)	91.9, d	4.90 (brs)

* Tsuda, Uemoto and Kobayashi (1999)

** Under solvent peak

3.5.3. Oroidin (20, known compound)



Compound **20** was isolated as a slight yellow oil containing two bromine substituents in the molecule as shown by the quasimolecular ion clusters at *m/z* 386, 388 and 390 $[M+H]^+$ (**Figure 57**) in its ESIMS spectra. It showed UV absorbances at λ_{max} 276 and 215 nm. Its ^{1}H-NMR (**Figure 58** and summarized in **Table 14**) spectrum showed four sp^2 signals in the molecule, which contained the typical singlet protons in a pyrrole ring and an imidazole ring at δH 7.06 and δH 6.81, respectively. Moreover, the allylic spin system at δH 3.39 (H-8, brt, 5.0, 11.3), δH 6.12 (H-9, ddd, 10.0, 15.7, 5.0) and 6.21 (H-10,brd, 16.3) was also defined. Typical $^2J_{NH}$ triplet with coupling constant of 5.5 Hz at δH 8.53 to the methylene protons at δH 3.39 ppm was confirmed by HMBC correlations.

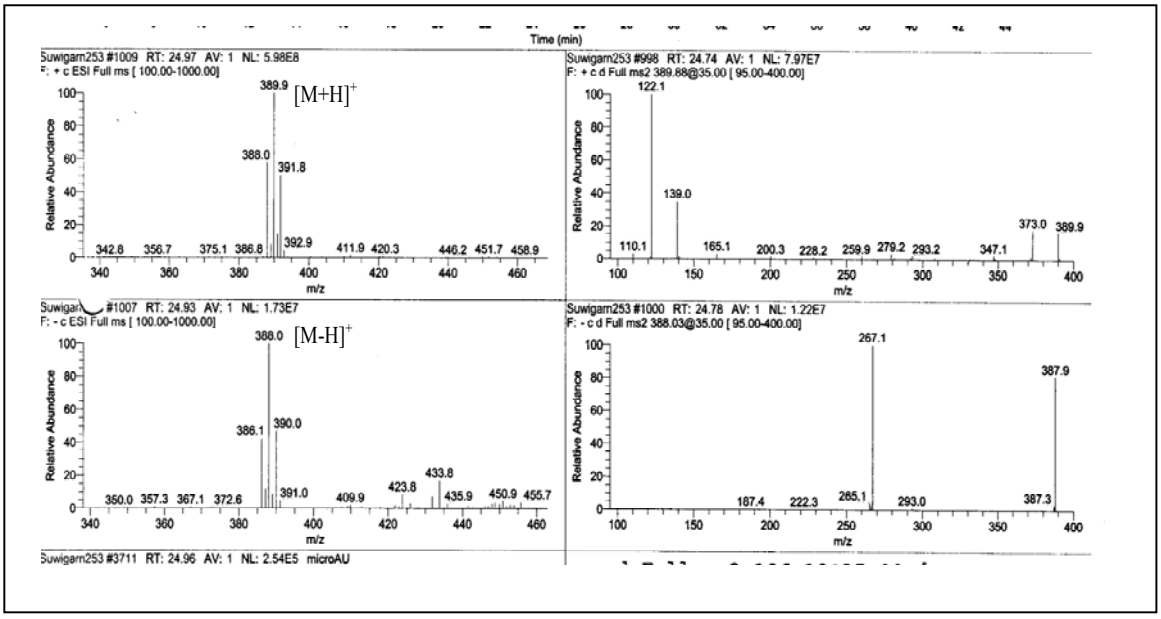


Figure 57 ESIMS spectra of compound **20**

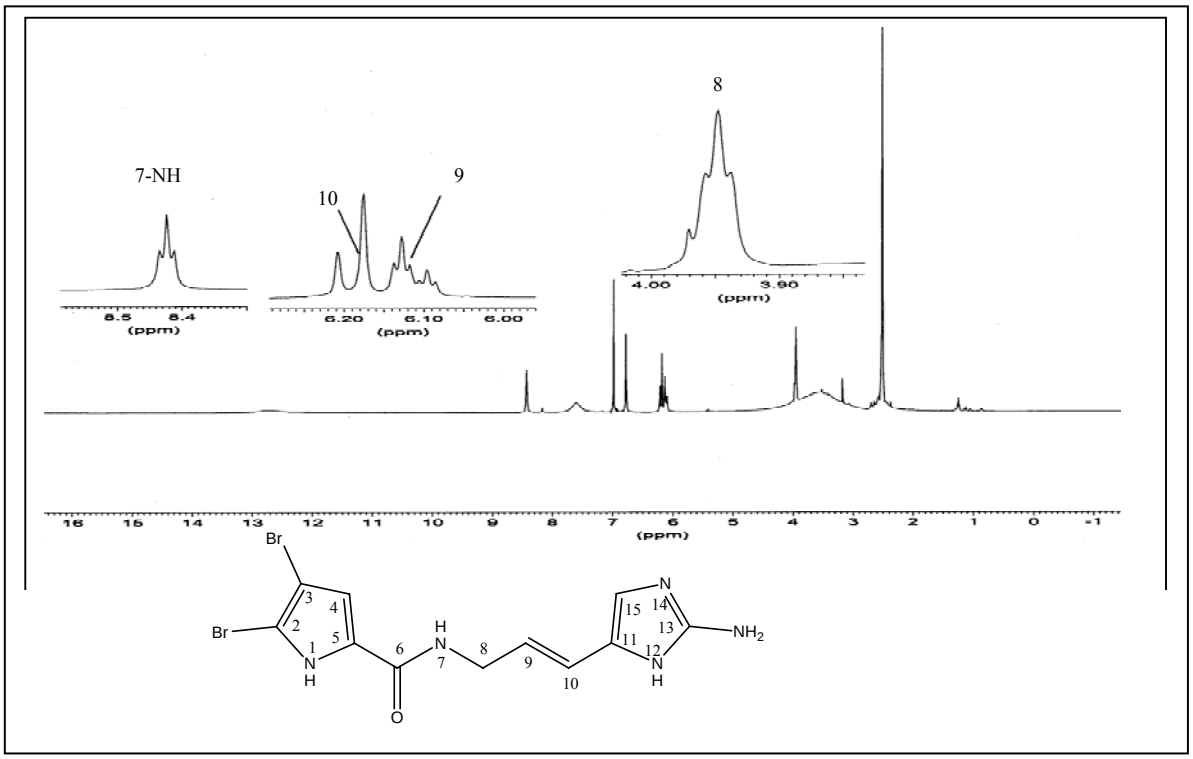


Figure 58 ^{1}H-NMR spectrum of compound **20** (DMSO-d_6).

Its HMBC spectrum (**Figure 59)** was used to establish the connectivities of the pyrrole ring, imidazole moiety, and allylic system in order to complete the structure. The pyrrole ring was connected with the side chain through the correlation of the amine carbonyl carbon at C-6 with the proton at δH 7.06 (H-4) which also correlated with C-3 (δC 104.4) and C-5 (δC 128.1). The C-H long range correlation of the methylene proton at δH 3.93 (H-8) with C-6 (δC 158.6) also strongly supported this assignment and its attachment to the amide moiety. An imidazole ring was directly attached to the allylic system at C-11 (δC 124.1) by using the correlation of the singlet at δH 6.81 ppm (H-15) to sp^2 carbon at δC 117.1 ppm (C-10). Based on the above data, the chemical structure of compound **20** was identified as oroidin, which has been previously isolated from the marine sponge, *Agelas oroides* collected from the Naples Bay, Italy [Forenza, Minale, Ricco and Fattorusso, 1971].

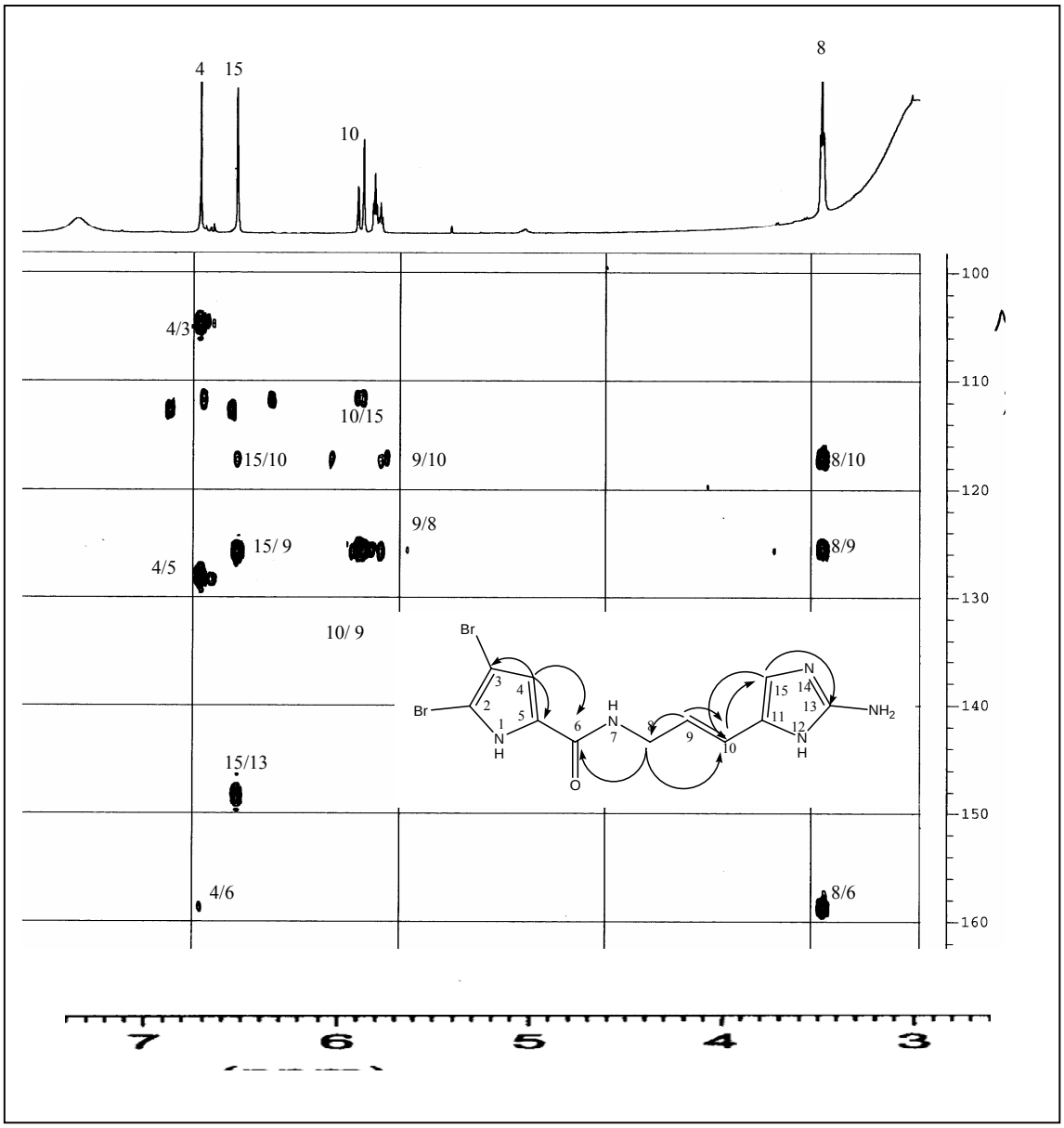
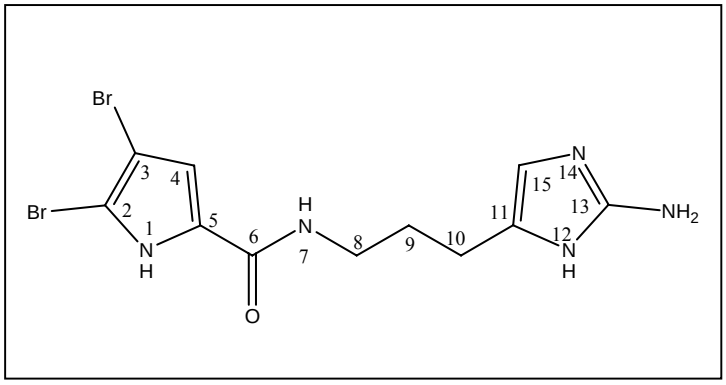


Figure 59 Important C-H long range correlations of compound **20**

Table 14 NMR data of compound **20** (DMSO-d_6)

position	δ ^{1}H (multiplicities, J in HZ	δ ^{13}C, multiplicities	HMBC
1,NH	10.43(brt, 5.6, 5.5)	-	-
2	-	-	-
3	-	104.4, s	-
4	7.06 (s)	112.5, s	3, 5, 6
5	-	128.1, s	-
6	-	158.6, s	-
7,NH	8.53 (brt, 5.0, 10.1)	-	6
8	3.93 (brt, 5.0,11.3)	39.8, t	6, 9, 10
9	6.12 (ddd, 10.0, 15.7, 5.0)	125.6, d	8, 10
10	6.21 (brd, 16.3)	117.1, d	8, 9, 11,15
11	-	128.1, s	-
12,NH	12.70 (brs)	-	-
13	-	148.3, s	-
14,N	-	-	-
15	6.81 (s)	111.5.,s	9, 10 , 13
NH_2	7.60 (brs)	-	-

3.5.4. Dihydrooroidin (21, new compound)



Compound **21** was obtained as a colorless oil with UV absorbance at λ_{max} 210 nm. The (+) ESIMS spectrum (**Figure 60**) showed a pseudomolecular ion pattern at *m/z* 388, 390 and 392, which indicated the presence of two bromines in the molecule and was two mass units higher than the molecular weight of compound **20**. Its proton NMR spectrum (**Figure 61**) was comparable to that of compound **20** but it has a NH-$CH_2CH_2CH_2$ instead of a NH-CH_2CH=CH spin system. This difference in the spin system was revealed by comparison of the chemical shifts at δH 6.12 (H-8), 6.21 (H-9) for oroidin (compound **20**), and at δH 3.55 (H-8), 2.22 (H-10) for compound **21**, respectively. The olefinic peak at δH 6.12 and 6.21 in oroidin disappeared in compound **21**. Compound **21** has also the 2,3-dibromo-1*H*pyrrole-2-caboxylic acid amide [Forenza, Minale and Riccio 1971] as substructure which is the same as found in compound **20** as indicated by the pyrrole singlet for H-4 (δH 7.06). The imidazole proton at H-15 (δH 6.81) was also observed.

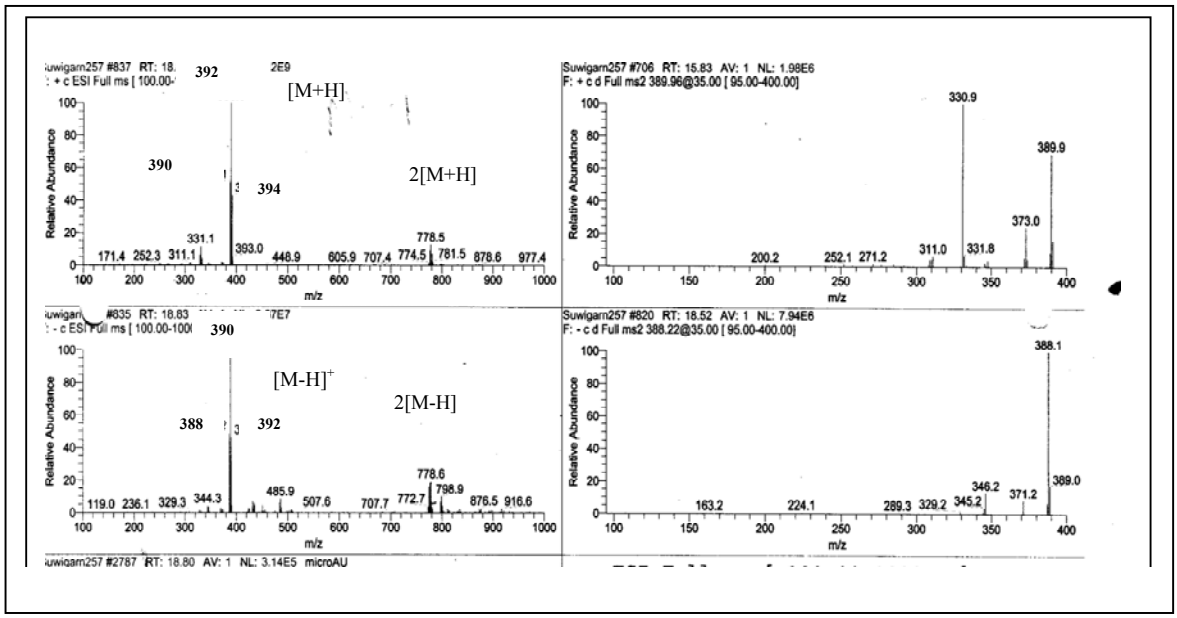


Figure 60 ESIMS spectra of dehydrooroidin (compound **21**)

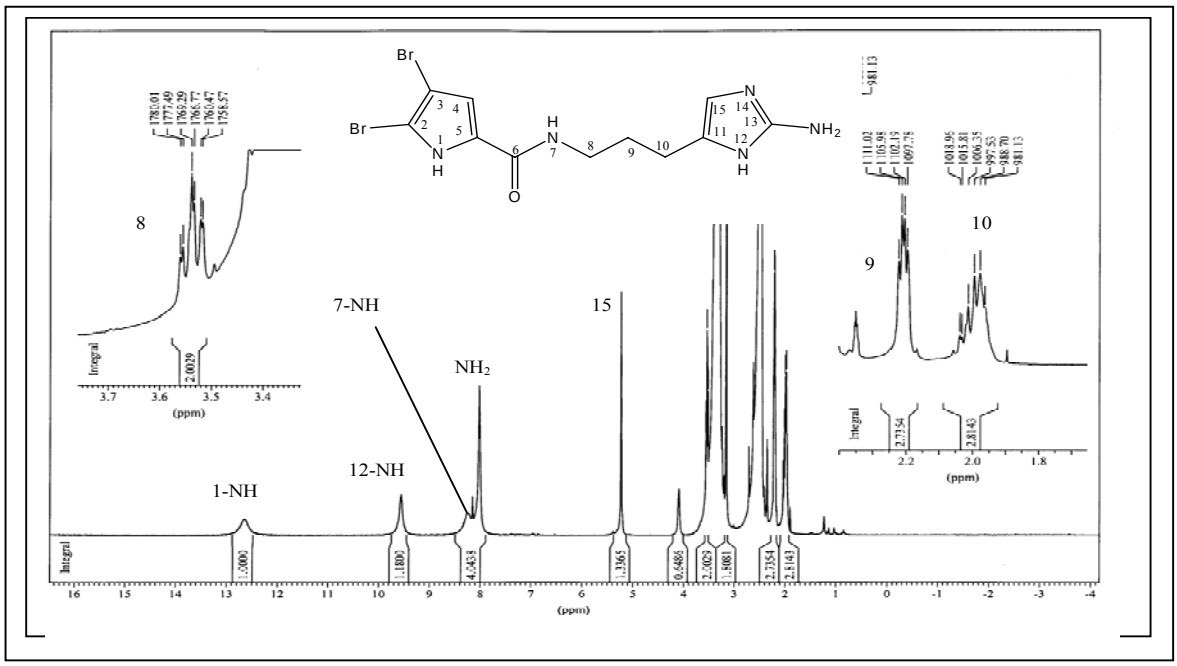
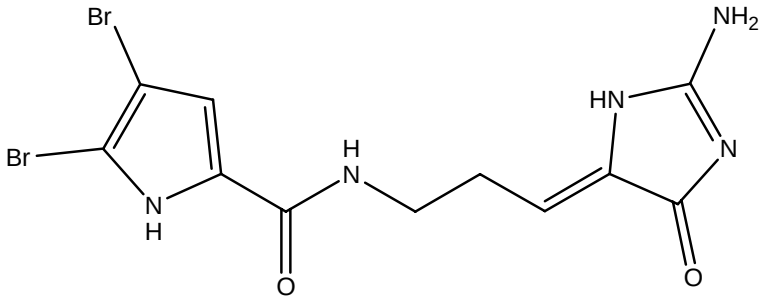


Figure 61 ^{1}H-NMR spectra of dehydrooroidin (compound **21**, DMSO-d_6)

The $NH\text{-}CH_2CH_2CH_2$ spin system was established through its HMBC data (**Figure 63**) and proton multiplicity pattern. The proton at δH 3.55 ppm (H-8) was directly attached to a hetero atom which exhibited the NH coupling constant of 2.5 Hz with the methylene proton at C-8. The HMBC spectrum showed the correlation of the methylene proton at δH 2.22 (H_2-9) with C-11 (δC 83.5) while the imidazole methine singlet at δH 6.81 (H-15) correlated with C-10 (δC 39.9). This information suggested that the imidazole ring was directly connected to the chain $NHCH_2CH_2CH_2$ at C-11 which was similar to the previous compound, oroidin. Compound **21** was transformed from oroidin (compound **20**) which could be explained by the electron transfer between the nucleophilic C-9 and electrophilic C-10 of oroidin that afforded the intermediate skeleton **21.I** which could be transformed to compound **21** as shown in **Figure 62.** The intermediate **20.I** is closely related to dispacamide, a compound isolated from a Carribean *Agelas* sponge [Cafieri, Fattorusso, Mangoni and Scafati, 1996). Compound **21** was identified as dehydrooroidin which is a new derivative of oroidin. The compound was quite unstable , therefore no HRMS could be determined.



dispacamide

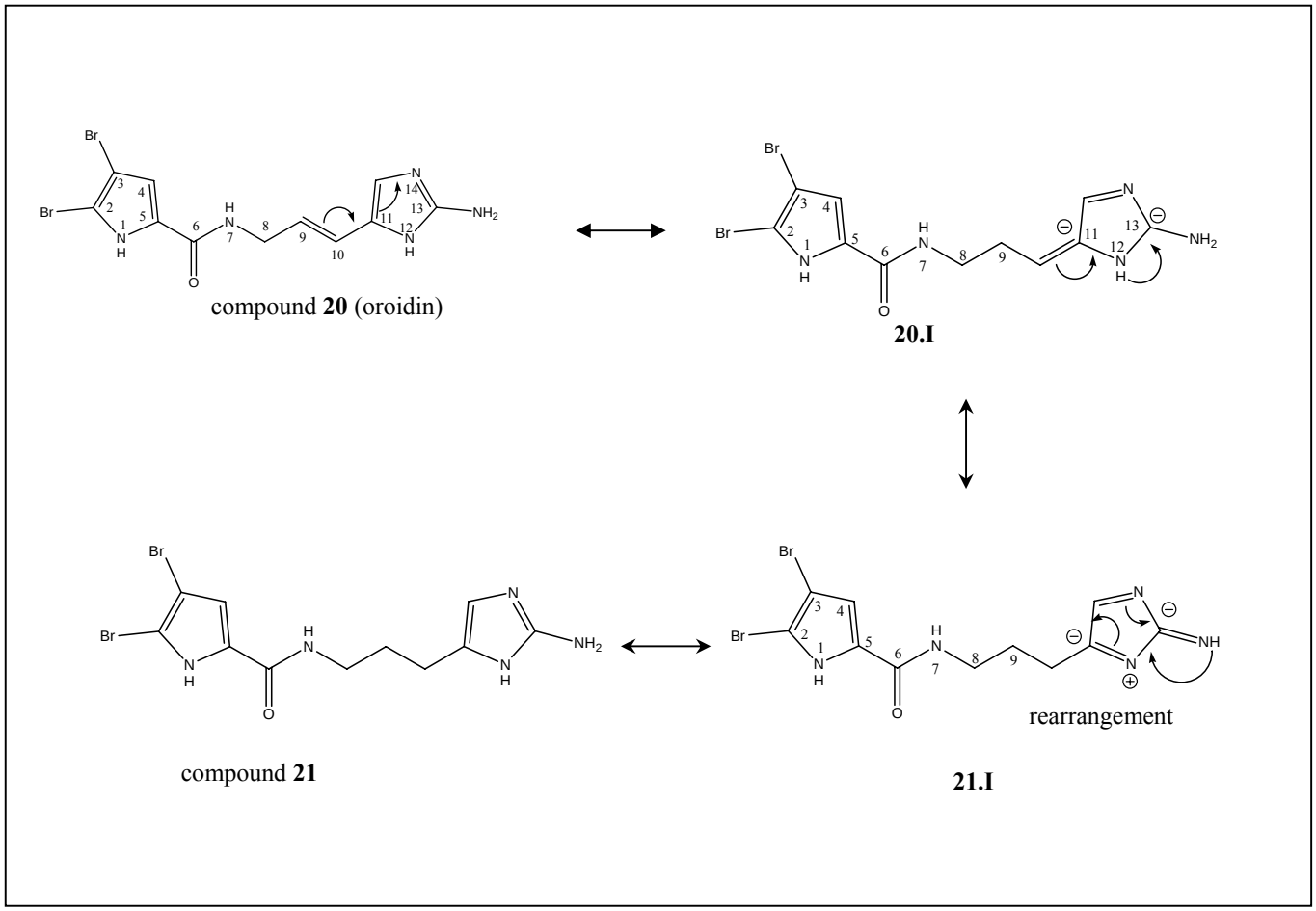


Figure 62 The proposed transformation mechanism from oroidin to dihydrooriodin (compound **21**)

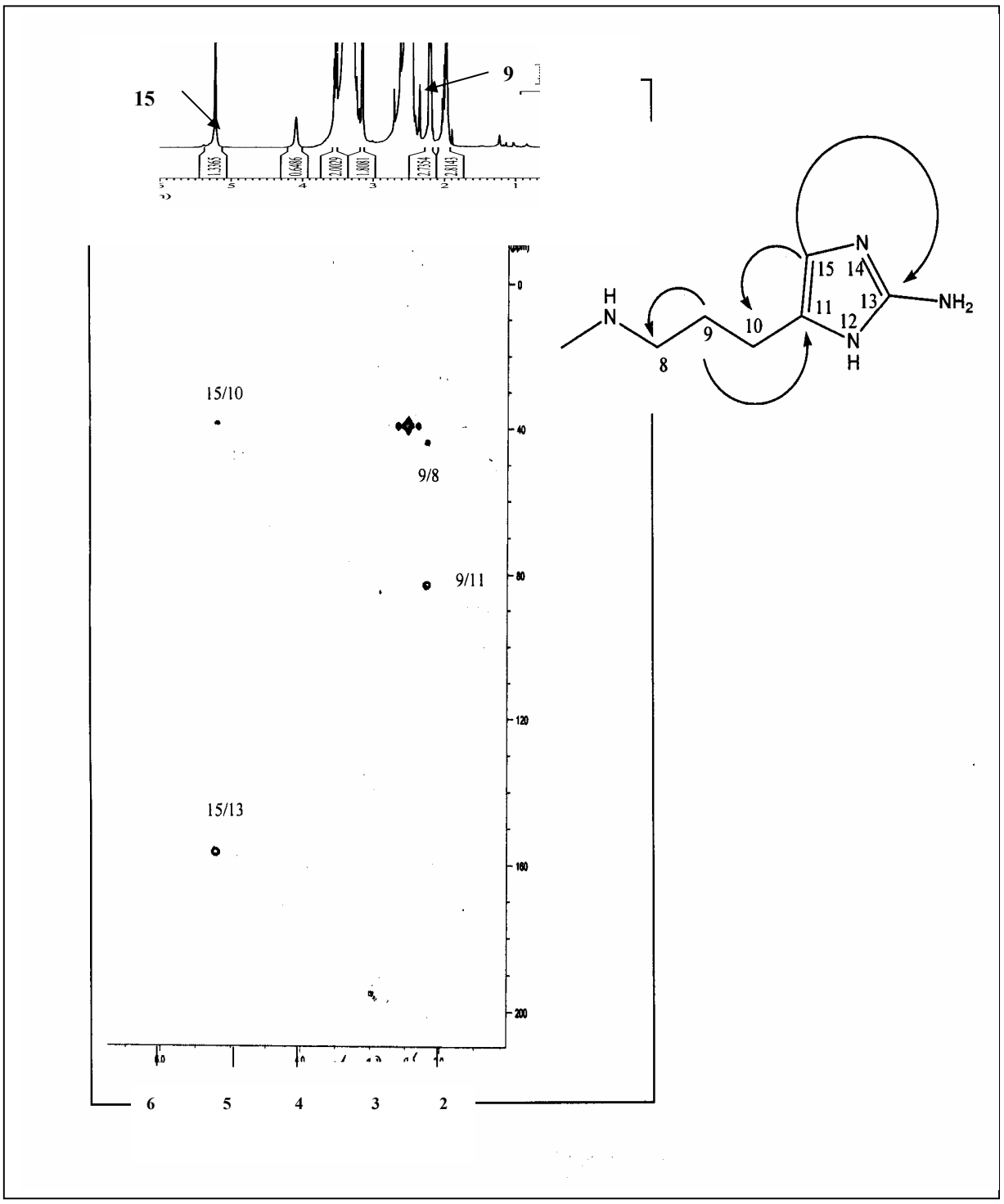
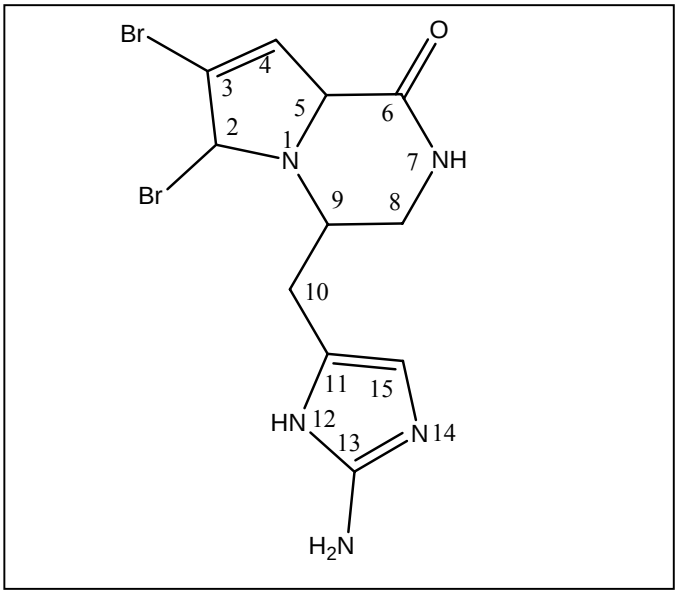


Figure 63 HMBC spectra of compound **21**

Table 15 NMR data of compound **21** (DMSO-d_6)

position	Compound **21**		HMBC	δ H (multiplicities, J in Hz) of compound **20**
	δH (multiplicities, J in Hz)	δ C		
1, NH	12.70 (brs)	-	-	10.43(brt, 5.6, 5.5)
2	-	-	-	-
3	-			-
4	8.10 (brs)	-	-	7.06 (s)
5	-	-	-	-
6	-	-	-	-
7,NH	8.21, brs	-	-	8.53 (brt, 5.0, 10.1)
8	3.55 (brt, 2.5, 13.2)	44.4, t	-	3.93 (brt, 5.0,11.3)
9	2.22 (td, 6.3, 13.2)	-	8, 11	6.12 (tt, 10.0, 15.7, 5.0)
10	2.00 (m)	39.9, d	-	6.21 (brd, 16.3)
11	4.10 (m)	83.5,d	-	-
12,NH	9.60 (brs)	-	-	12.70 (brs)
13	-	-	-	-
14	-	-	-	-
15	5.20 (brd)	-	10, 13	6.81 (s)
NH_2	7.90, brs	-	-	7.60 (brs)

3.5.5. Cyclooroidin (22, known compound)



Compound **22** was isolated as a white oil with UV absorbance at λ_{max} 222 and 286 nm. It has a pseudomolecular ion peak pattern at *m/z* 387.942, 389.939 and 391.938 with a ratio of 1:2:1 as determined by HRESIMS (**Figure 64)**, and indicated that it has two bromine substituents in the molecule. It was compatible with the molecular formula as $C_{11}H_{13}Br_2O$. Its proton NMR spectrum (**Figure 65** and summarized in **Table 16**) revealed again the typical proton resonances for a pyrrole and an imidazole ring at δH 7.00 and δH 6.54, respectively.

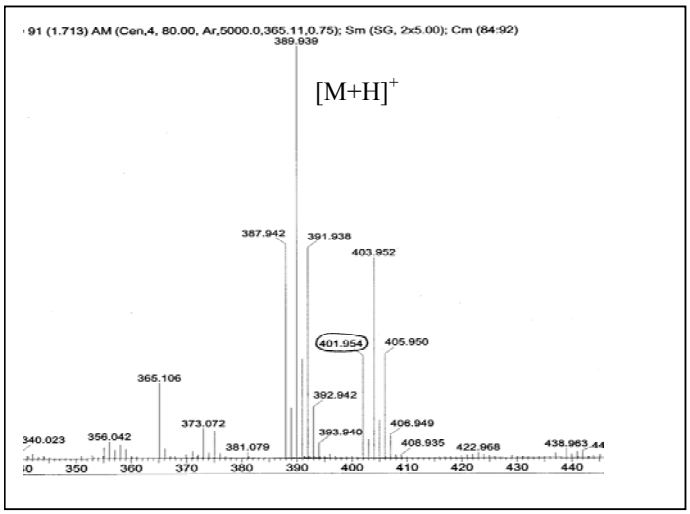


Figure 64 HRESIMS spectra of compound **22**

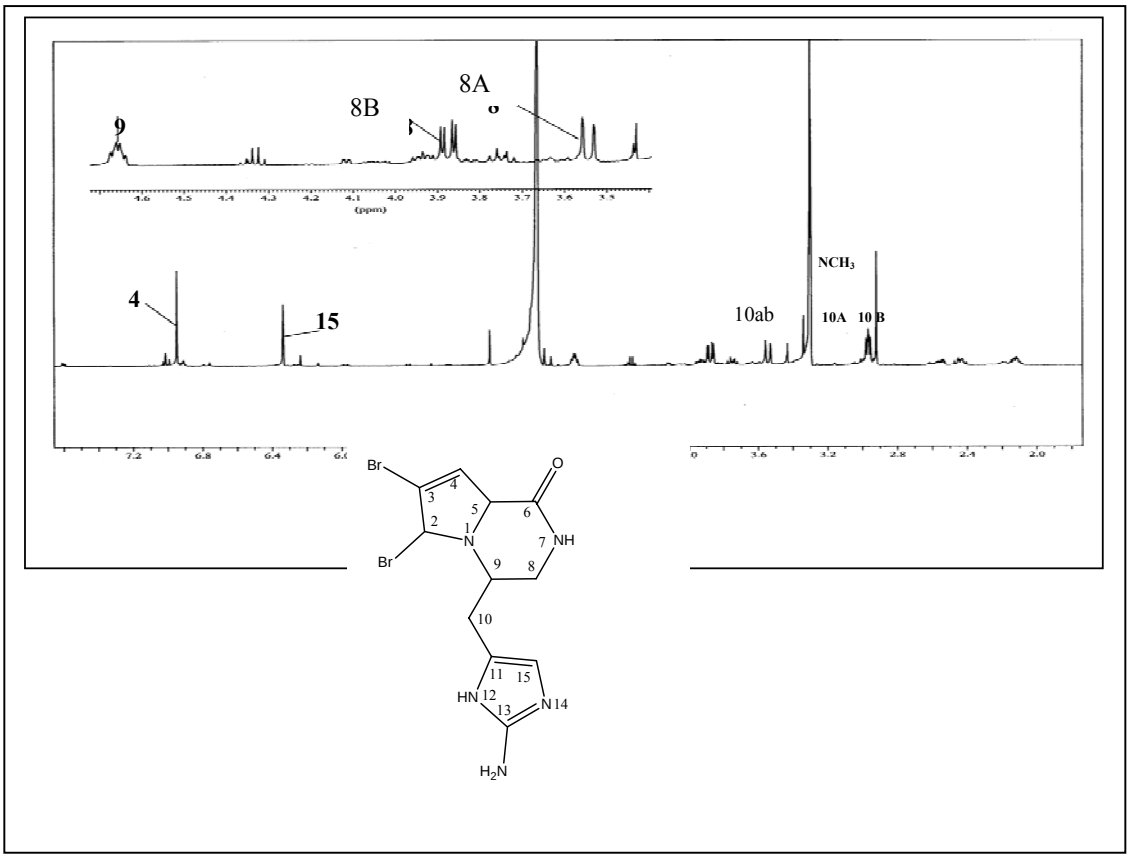


Figure 65 ^{1}H-NMR spectra of compound **22** (MeOH-d_4)

Furthermore, this compound contained the $NHCH_2CH_2(N)CH_2$ spin system, which was established from its COSY spectrum. The COSY spectrum (**Figure 66**) was used to confirm the spin system involving the resonances at δH 3.59 and 3.54 (H-8a and H-8b), δH 4.52 (H-9) and δH 3.05 (H-10), respectively.

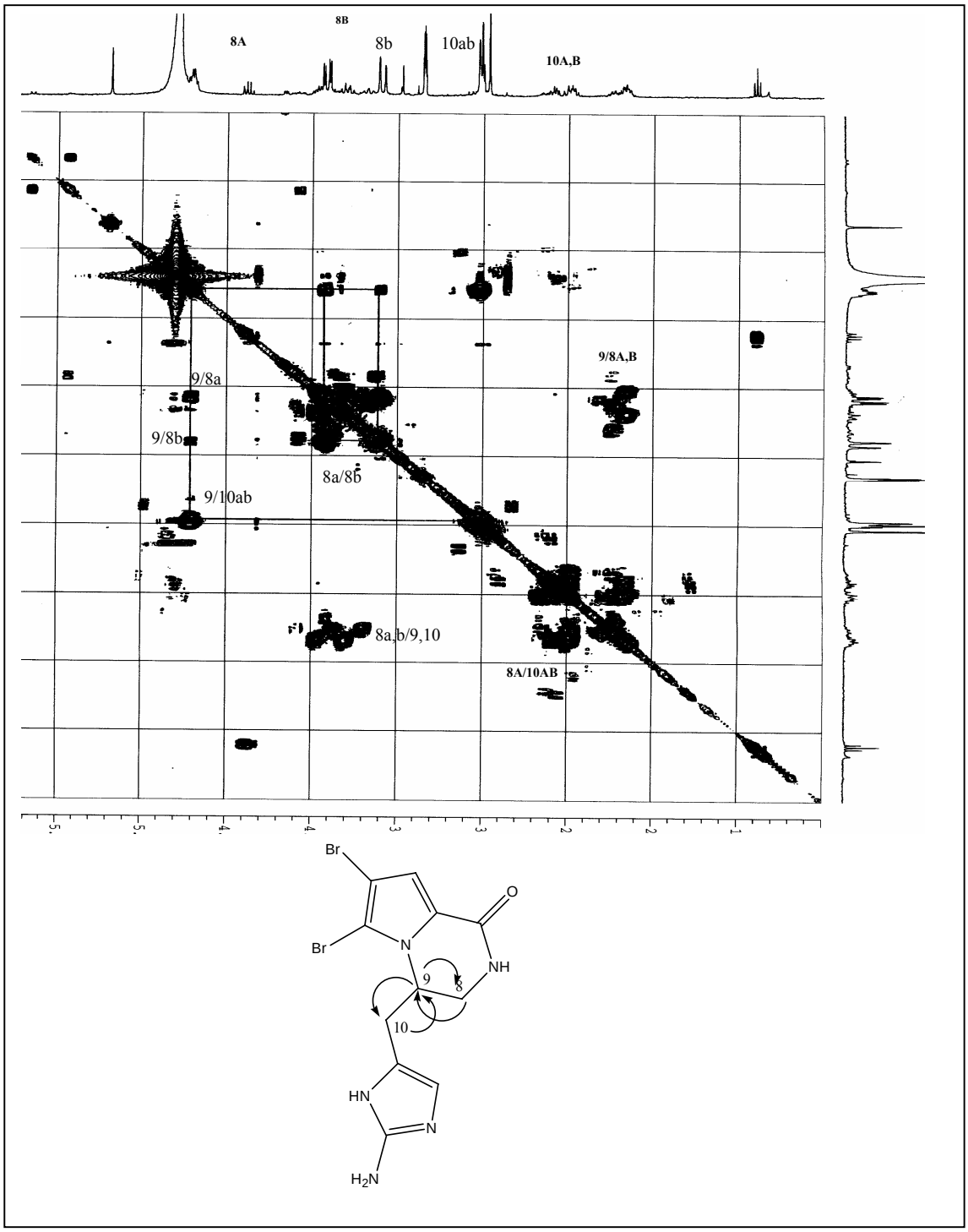
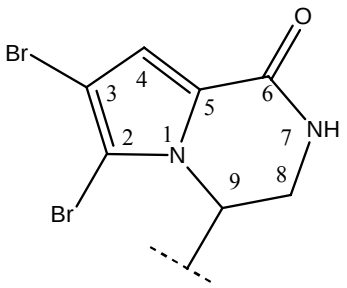


Figure 66 COSY spectrum of compound **22**

The 2D HMBC spectrum (**Figure 67**) was used to interconnect three partial substructures which also include the pyrrole and the imidazole moiety. This also includes an unambiguous assignment of all carbon resonances. The key cross peaks were between H-9 (δH 4.52) and C-2 (δC 126.1) and C-5 (δC 125.6), then H-8 (δH 3.59 and 3.54) with C-6 (δ161.0) to prove the pyrroloketopiperazine nucleus. The cross-peaks between H-9(δH 4.52) and C-11 (δC123.4) and H-10 (δH 3.05) and C-15 (δC 112.6) indicated that this nucleus must be connected to the imidazole ring through the methylene carbon at C-11.



Pyrroloketopiperazine nucleus

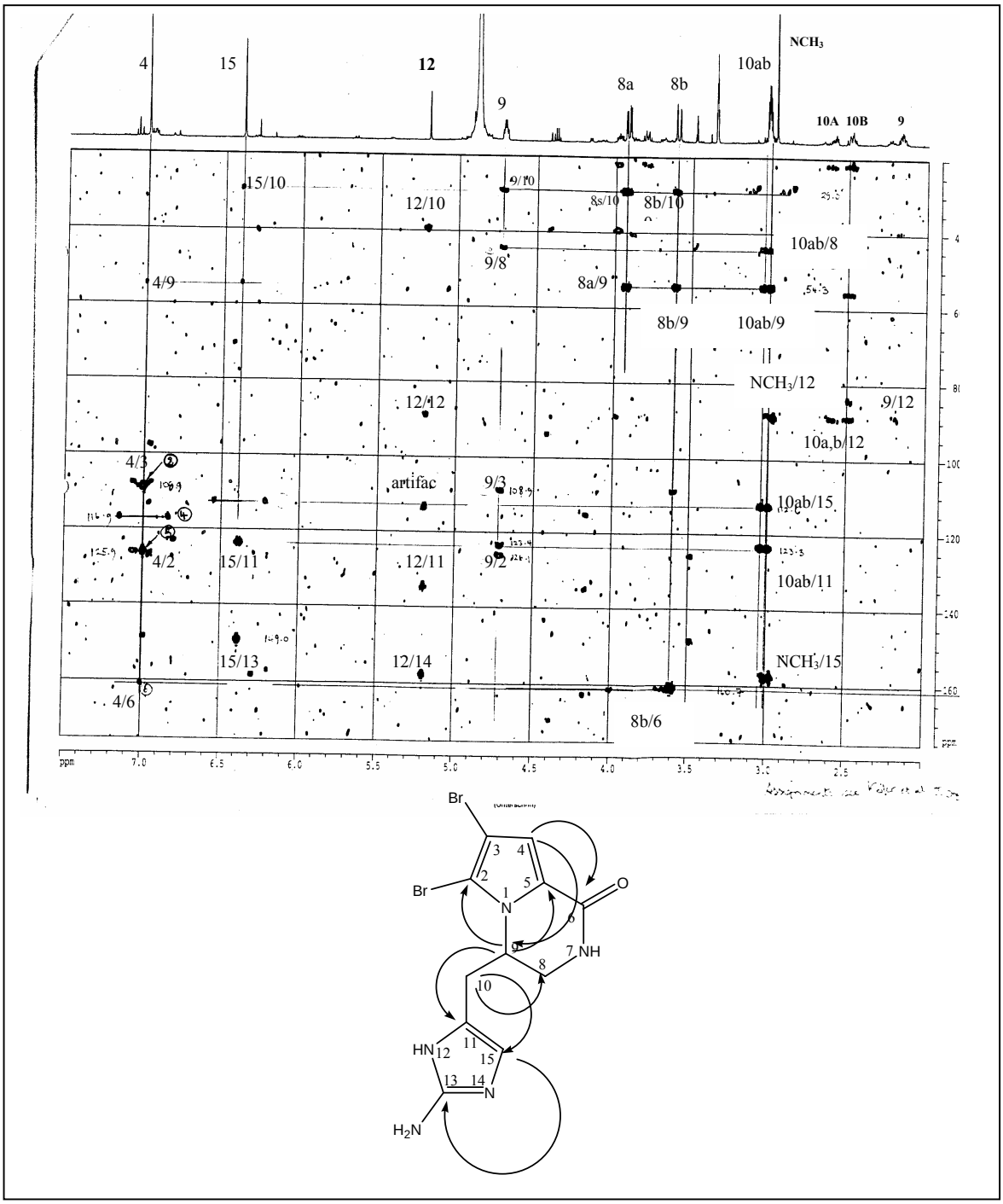


Figure 67 Important C-H long range correlations of compound **22**

Based on the previous discussion, the basic structure of compound **22** was therefore completely defined. It corresponds to the cyclization between N1 and C-9 of compound **20** which was also encountered in agelastatin as shown below.

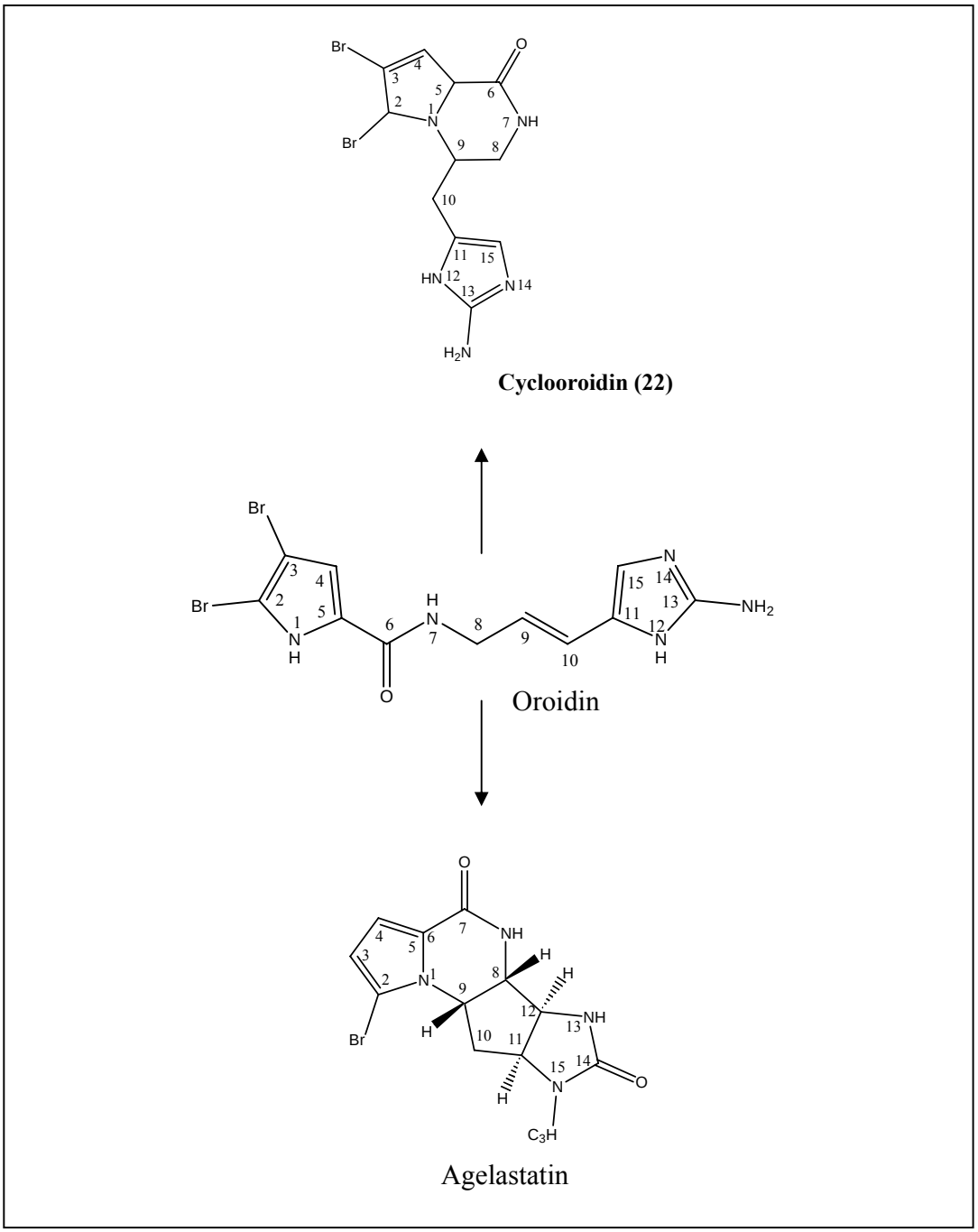


Figure 68 Cyclization of oroidin to cyclooroidin and agelastatin

The intramolecular cyclization mechanism of compound **22** can be explained by the electron delocalization of the linear structure of oroidin (compound **20)**. Cyclization is formed between N-1 and C-9 which commenced at the lone pair electron on NH-1 and was delocalized to C-5 resulting in the positive charge on N-1 which showed an electrophilic property. Therefore, the lone pair electron of nucleophilic C-9 migrated to N-1 to form a ring closure as illustrated below. Compound **22** was identified as cyclooroidin which was previously isolated from the Mediteranean marine spone, *Agelas oroides* [Fattorusso and Scafati, 2000].

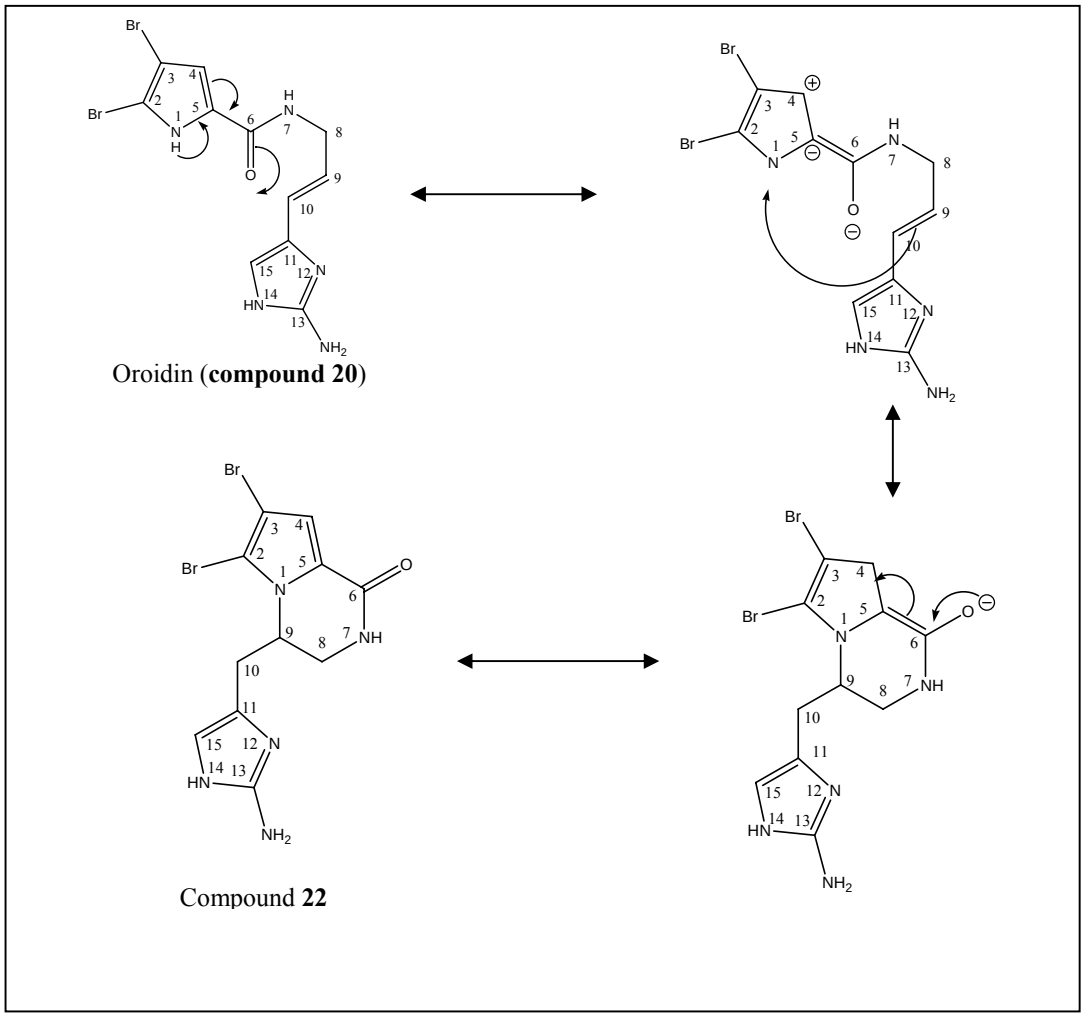
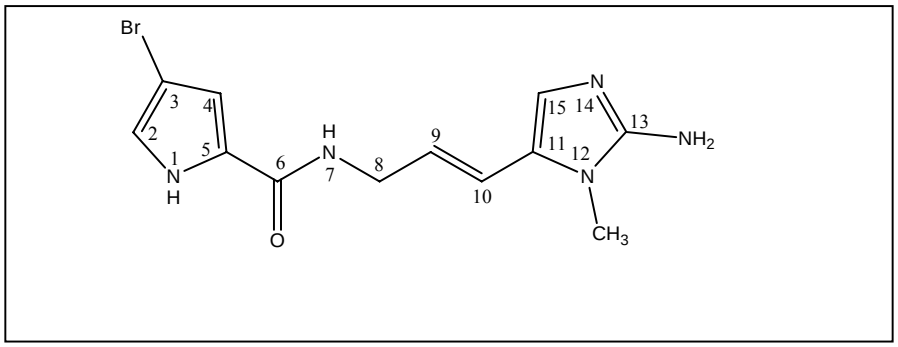


Figure 69 Intramolecular cyclization mechanism proposal of compound **22**

Table 16 NMR data of compound **22** (MeOH-d_4)

Position	δH (multiplicities, J in Hz)	δC	COSY	HMBC
1,N	-	-	-	-
2	-	126.1, s	-	-
3	-	108.9, s	-	-
4	7.00 (s)	116.9, d	-	3, 5, 6, 9
5	-	125.6, s	-	-
6	-	161.0, s	-	-
7,NH	-	-	-	-
8	a, 3.59 (dd, 4.4, 13.6) b, 3.54 (dd, 1.2, 13.6)	44.5, t	8b, 9a and b 8a, 9a and b	9, 10
9	4.52 (m)	54.3, d	8 a and b, 9a and b	2, 3, 5, 8, 10
10	3.05 (dd, 4.7, 6.2)	29.01, t	8a and b, 9	8, 9, 11
11	-	123.4, s	-	-
12,NH	-	-	-	-
13	-	159.0, s	-	-
14,N	-	-	-	-
15	6.54 (s)	112.6, d	-	9, 10, 11,13

3.5.6. Keramidine (23, known compound)



Compound **23** was obtained as a slightly yellow oil with UV absorbance at λ_{max} 271 nm. It showed a pseudomolecular ion peak pattern at *m/z* 324 and 326 in the positive ESIMS (**Figure 70**) which indicated that it contained one bromine in the molecule. The ^{1}H-NMR spectrum (**Figure 71)** showed again the typical proton resonance at δH 7.06 for a broad singlet in a pyrrole ring and an additional doublet proton at δH 6.91. These two protons were deduced to be *meta* oriented from their typical coupling constant of $^{2}J_{HH}$ = 1.8 Hz. A singlet proton of an imidazole ring (H-15) and allylic spin system at δH 4.10 (H-8), δH 5.91 (H-9) and 6.25 ppm (H-10) were also observed in compound **23**.

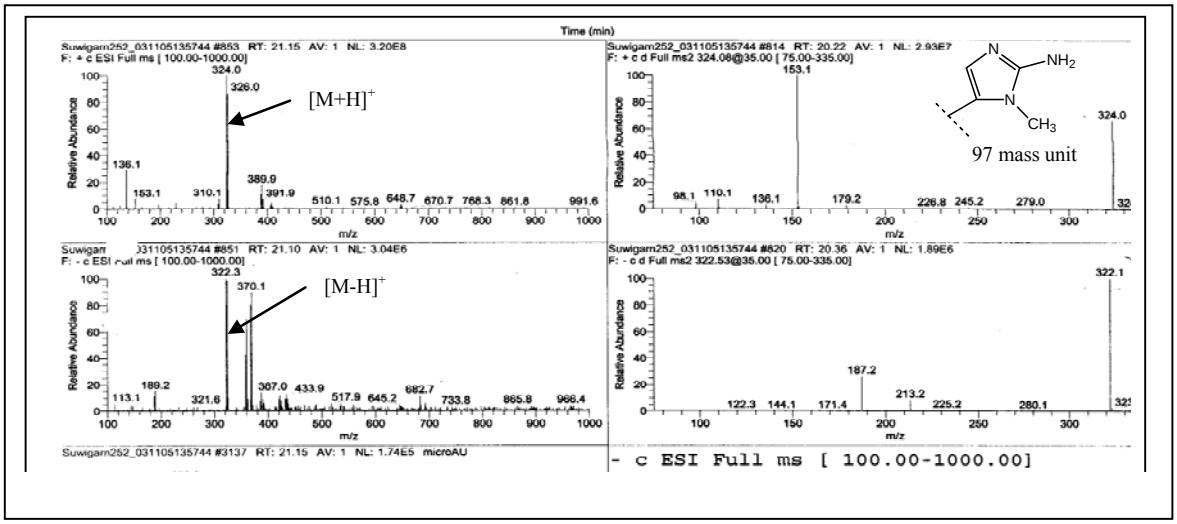


Figure 70 ESIMS spectra of compound **23**

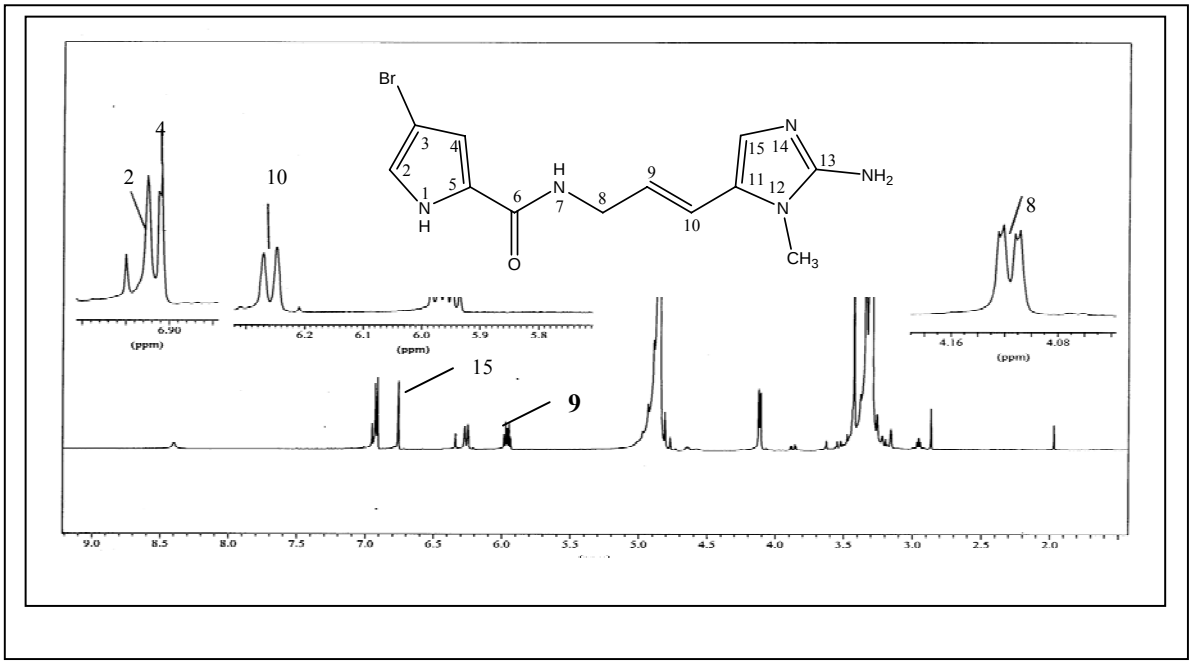
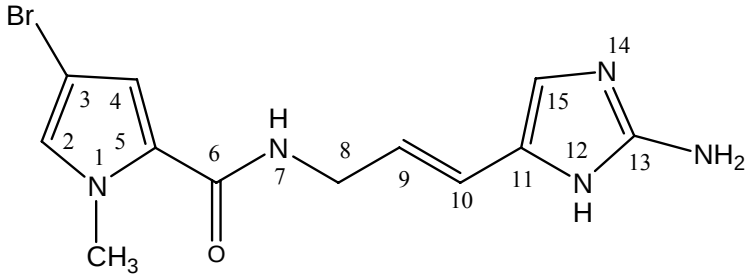


Figure 71 ^{1}H-NMR spectra of compound **23 (**MeOH-d_4)

The COSY spectrum data (**Figure 72)** confirmed the presence of the allylic spin system. Furthermore, its COSY spectrum revealed a long range correlation from H-15 to H-10 which showed that the imidazole ring was directly attached to the allylic system at C-11 as found in compound **20**. The fragmentation of 97 mass units at *m/z* 226 by MS/MS further suggested the presence of a methyl imidazole ring moiety. This confirmed to the occurrence of the N-CH_3 group in the imidazole ring rather than in the pyrrole ring. This differs from sventrin where the methyl substituent is at N-1. The N-CH_3 for sventrin occurred at δH 3.89 while that of keramidine appeared at δH 3.38. Moreover, the isolated compound **23** gave almost an identical chemical shift as that of keramidine. Therefore, compound **23** was identified as keramidine which was previously isolated from the Okinawan sponge *Agelas* sp [Nakamura, *et al*., 1984].



Sventrin

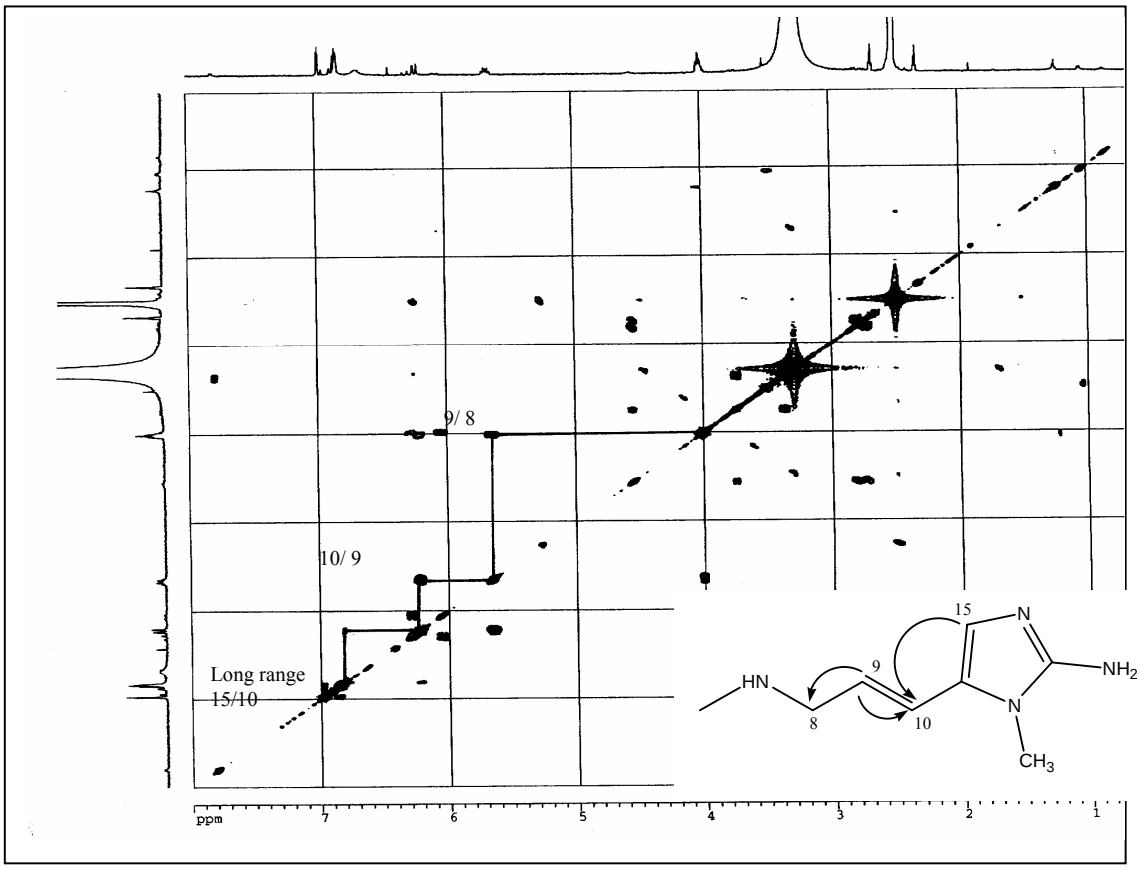


Figure 72 COSY spectrum of compound **23** (keramidine)

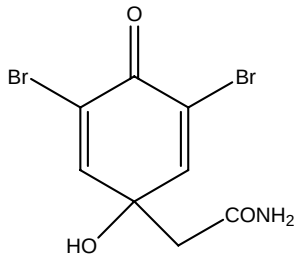
Table 17 NMR data of compound **23** comparison with reported data*

position	Sventrin (MeOH – d_4)		Compound **23** (MeOH –d_4)		Keramidine (DMSO-d_6)
	δ H (multiplicities, J in Hz	δ ^{13}C	δ H (multiplicities, J in Hz	COSY	
1, NCH_3	3.89 (s)	35.4, q	-	-	-
2	6.94 (d, 1.5)	110.8, s	6.91 (brd, 1.8)	-	6.92 (dd, 2.9, 1.5)
3	-	97.0, s	-	-	-
4	7.06 (s)	116.9, s	6.90 (brd, 1.8)	-	6.80 (dd, 2.9, 1.5)
5	-	127.6, s	-	-	-
6	-	159, s	-	-	-
7, NH	-	-	-	-	-
8	3.93 (t, 5.5, 5.7)	39.9, t	4.10 (dd, 1.9, 6.3)	9	4.10 (t, 5.6)
9	6.09 (t, 5.5, 16.1)	126.9, d	5.91 (dt, 11.3, 6.3)	10, 8	5.81(dt, 11.0, 5.6)
10	6.23 (d, 16.1)	116.4, d	6.25 (d, 11.3)	9	6.20 (d, 11.0)
11	-	124.9, s	-	-	-
12, NH	-	-	-	-	-
13	-	147.9, s	-	-	-
14, N	-	-	-	-	-
15	6.90 (s)	110.8, d	6.79 (`s)	10 (long range)	7.02(s)
NCH_3	-	-	3.40 (s)	-	3.38 (s)

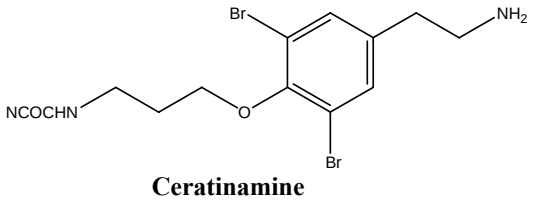
*Assman *et al.*, 2001, exchangeable protons determined in DMSO-d_6

3.6. Secondary metabolites from the sponge *Pseudoceratina purpurea*

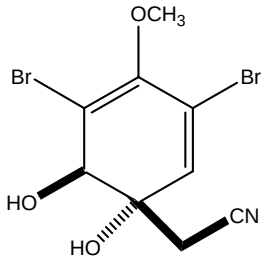
Marine sponges of the genus *Pseudoceratina* belong to the order Verongida. They were reported to produce unique secondary metabolites containing the bromotyrosine structure [Cimino *et al.,* 1994]. They all show also a peculiar biochemistry characterized by elaboration of a diverse group of brominated derivatives biogenetically related to tyrosine [Ciminiello *et al*., 2000]. Some examples of compounds of isolated from the genus *Pseudoceratina* are shown below.



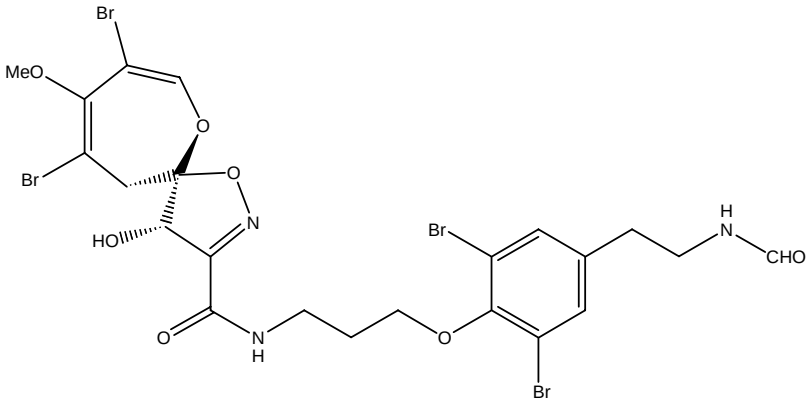
Dienone



Ceratinamine

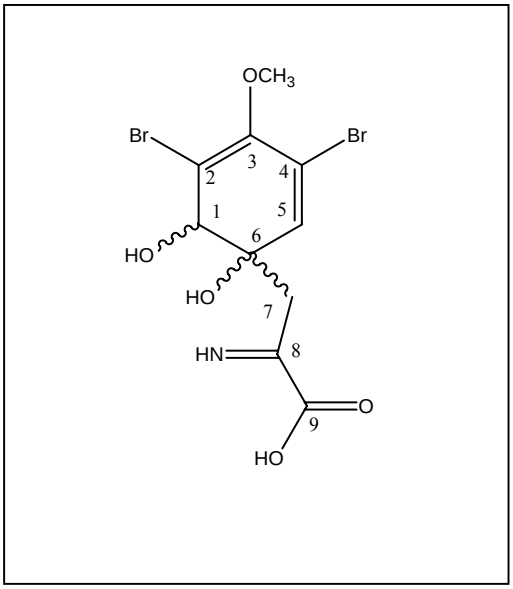


Aeroplysinin-1



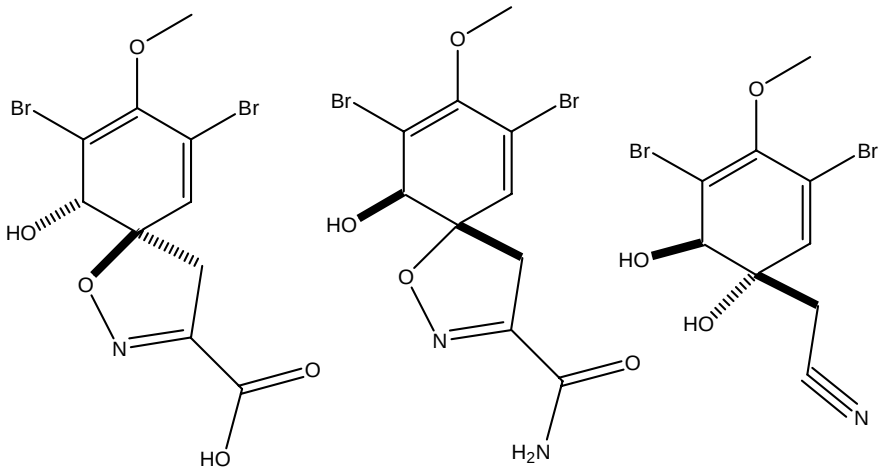
Ceratinamide A

3.6.1. 7-(2,4-dibromo-1,6-dihydroxy-3- methoxy-cyclohexa-2,4-dienyl)-8-imino-propionic acid (24 new compound)



Compound **24** was a diastereomeric mixture which was isolated as a brown amorphous residue with UV absorbance at λ_{max} 238 and 286 nm. The negative ESIMS (**Figure 73**) displayed a molecular ion triplet clusters at *m/z* 382, 384, and 386, which indicated the presence of two bromine atoms and suggested a molecular weight of 385. Inspection of their ^{1}H-NMR data (**Figure 74** and summarized in **Table 18**) revealed two singlet signals at δH 6.40 and δH 6.50 which indicated that the mixture contained two isomers at a ratio of 2:1. The methoxy protons of both isomers were observed at δH 3.62 and 3.52, respectively. The methylene protons of the first isomer resonated as pairs of doublets at δH 2.88 and 3.12 while the methylene group of the second compound was observed as a broad singlet at δH 2.80. Compound **24** was structurally related to bromo-spiranic acid (**24C**) which was previously isolated from a

Caribbean *Pseudoceratina* sponge. However, **24** has two mass units more than that of the known compound **24C**.



Bromo-spiranic acid (**24C**) | Purealidine R | Aeroplysinin-1

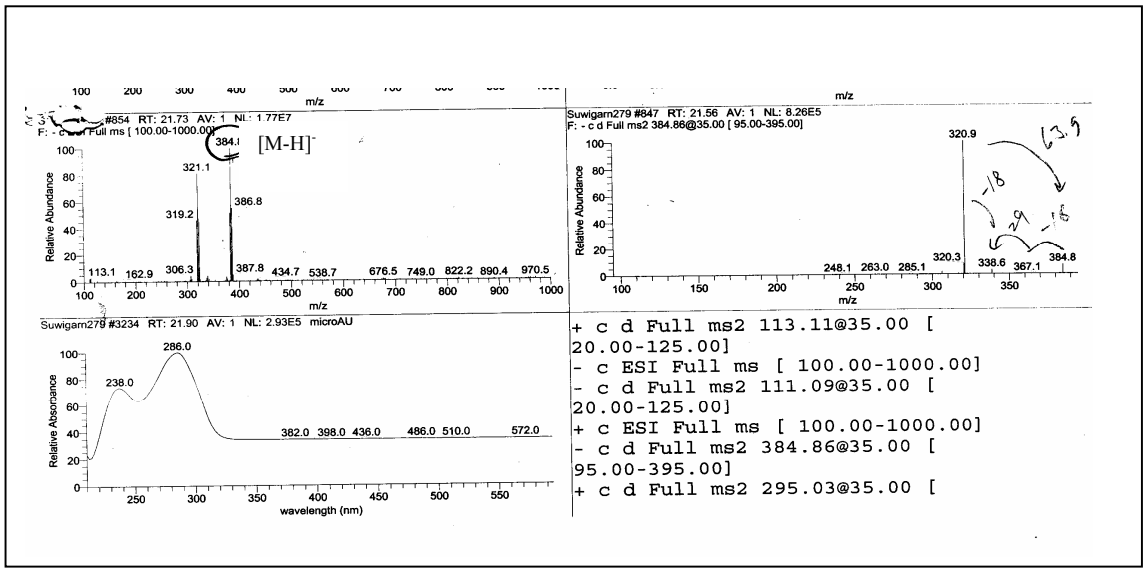


Figure 73 ESIMS spectra of compound **24**

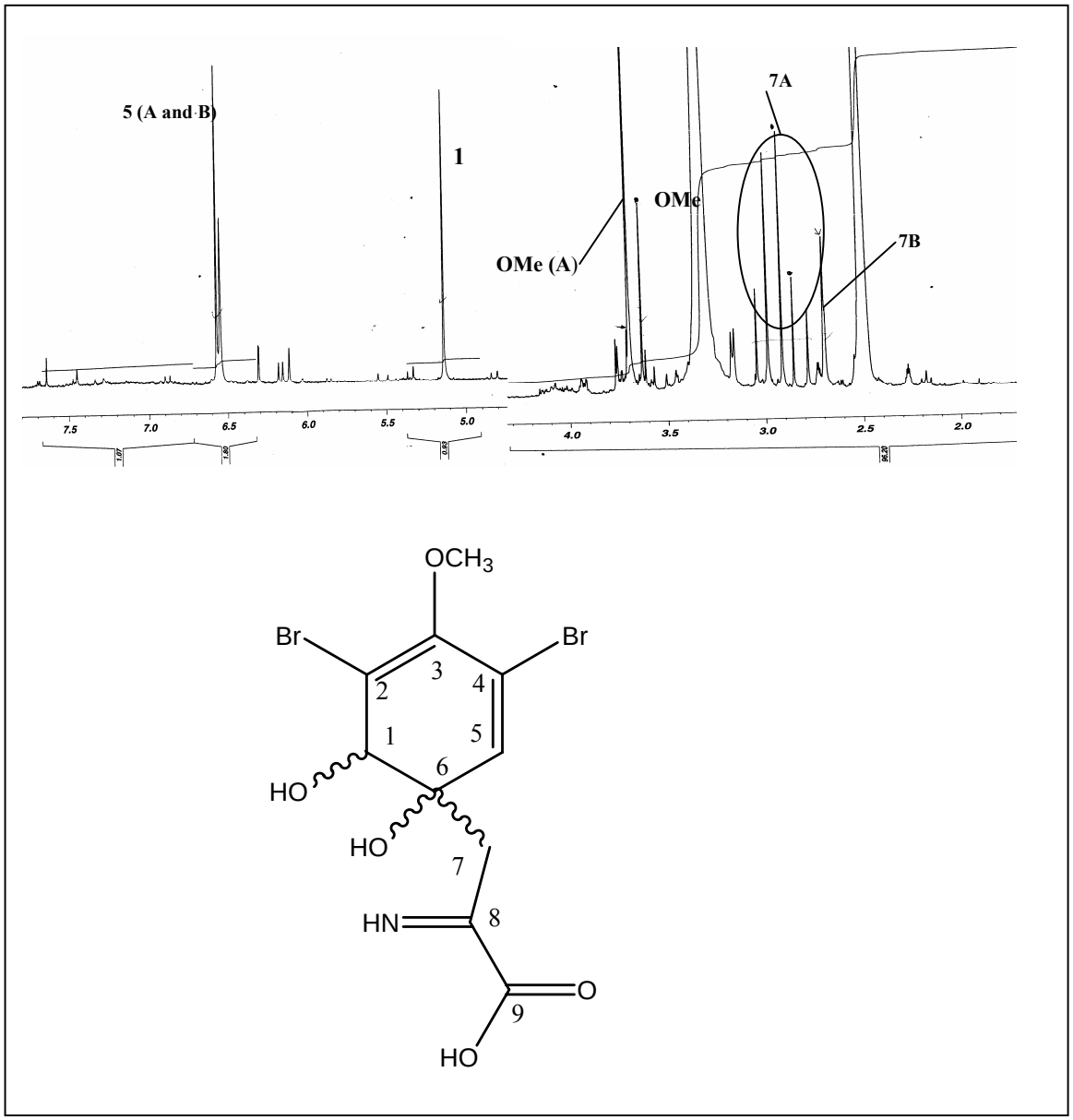


Figure 74 ^{1}H-NMR spectra of compound **24** (MeOH-d_4)

Although the NMR data of structurally related known congeners were acquired in methanol while that of **24** was recorded in DMSO (due to its insolubility in methanol), the ^{1}H and ^{13}C NMR data of **24,** are clearly different to those of bromo-spiranic acid (**24C**) and its amide derivative purealidin R. However, the HMBC spectra of **24** proved that its basic structure is identical to those of the known congeners**.** The methoxy proton was attributed at C-3 as it showed a long range correlation to δC 146.4 as observed from its HMBC spectra (**Figure 75**). Cross peaks between δH 6.40 (C*H*-5) and hydroxyl-bearing methine carbon at δC 86.8 (C-1) were observed while its hydroxyl methyl proton at δH 5.12 (H-1) gave cross peaks with δC 146 (C-3) and 134.7 (C-5) which indicated the presence of the dibromo-cyclohexadiene substructure. The connection of this substructure to the side chain can be proven by the relevant long range correlation from H-5 to C-7 (δC 40.7) and from H-7 to the carboxylic carbon at C-9 (δC 172.3).

The difference of two mass units between **24** and the known congener **24C** could only suggest that the dihydroisoxazole ring is possibly open in which case the structure of the **24** would be comparable to that of aeroplysinin-1. To prove this, aeroplysinin-1 was measured in methanol, to be able to determine the changes in chemical shifts in comparison to those of bromo-spiranic acid and purealidin R. The ^{13}C NMR data of the cyclohexadiene ring of aeroplysinin-1 were comparable to those of the latter known compounds with the exception of the carbon resonance for position 6 which was detected at δC 74.4 instead of *ca.* 90 ppm as in bromo-spiranic acid and purealidin R (**Table 18**). This further confirms that the dihydroisoxazole ring is open in **24.** Hence, the ring was concluded to be opened by hydrogenation to give the corresponding OH and NH substituents.

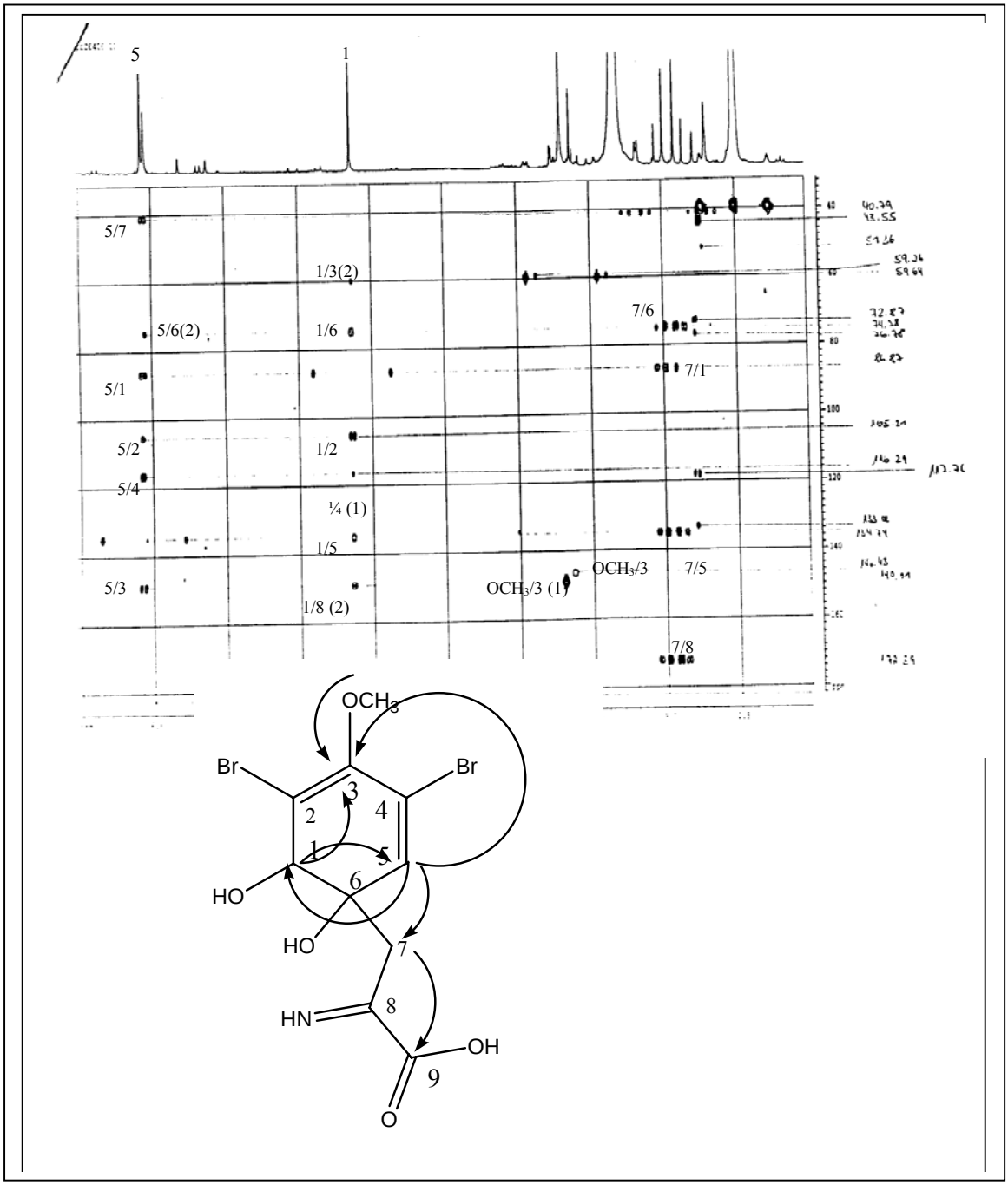


Figure 75 HMBC spectrum of compound **24**

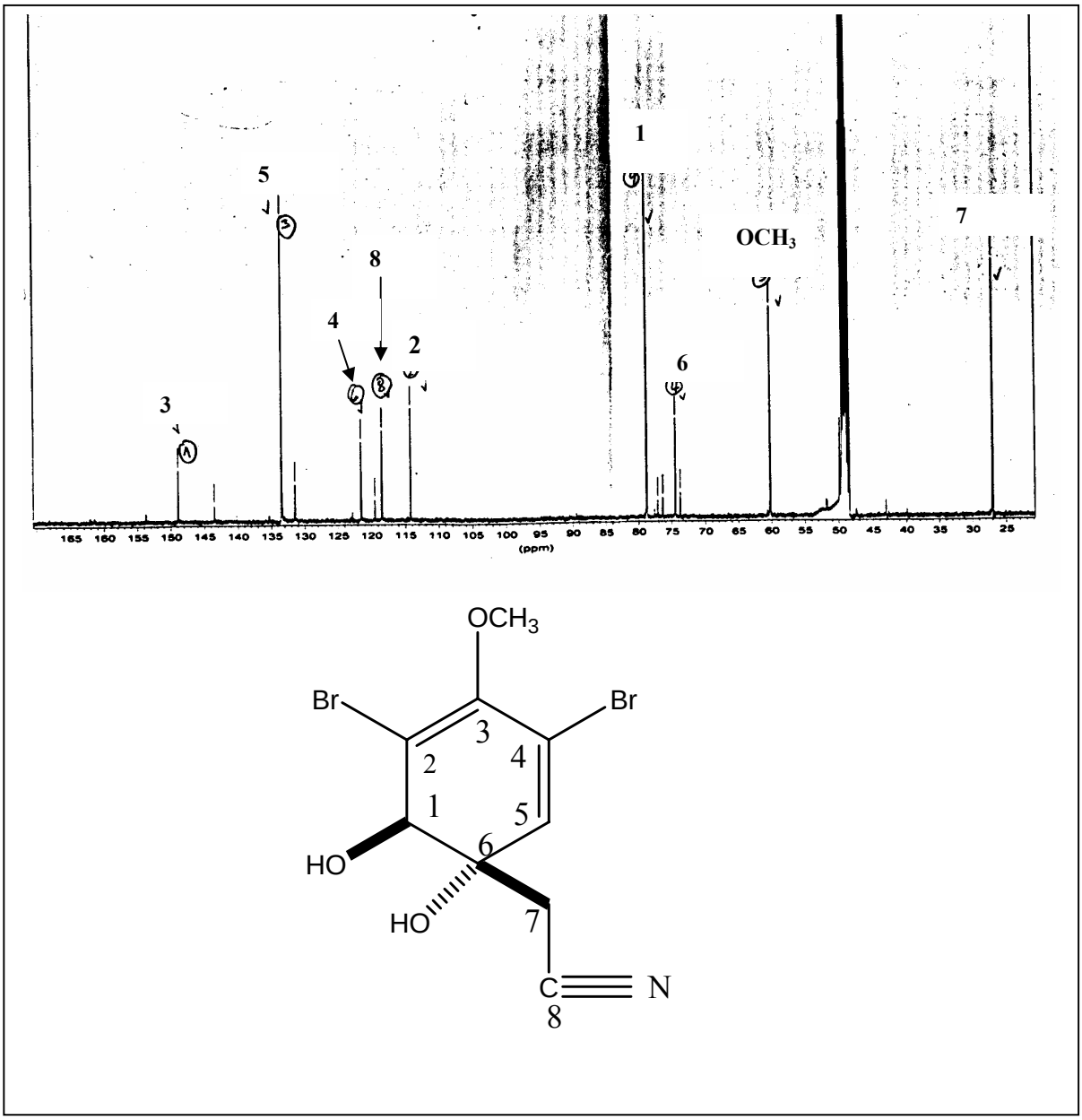
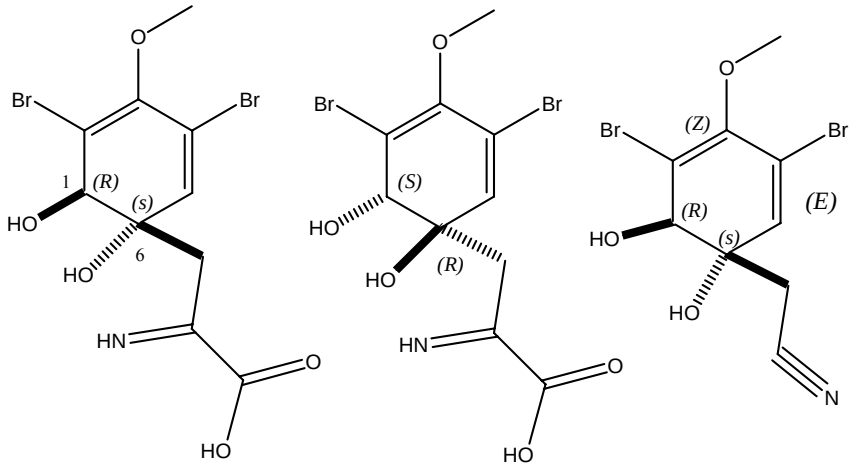


Figure 76 ^{13}C-NMR spectra of aeroplysinin-1 (MeOH-d_4)

Table 18 The comparison NMR data of the isolated compounds from the sponge *Pseudoceratina purpurea* with the reported data.

Position	**24C** (MeOD)[1]		Aeroplysinin-1 (MeOD)		Purealidin R[4]		**24 A** (DMSO-d_6)			**24 B** (DMSO-d_6)		
	δH	δC	δH[2]	δC[3]	δH (DMSO-d_6)	δC (MeOD)	δH	δC[5]	HMBC	δH	δC[5]	HMBC
1	4.10 (brs)	75.8, d	5.87 (s)	79.2, d	3.92	75.5, d	5.12 (s)	86.8, d	2,4,5,6,8	5.12 (s)	76.8, d	2,3,5,7
2	-	114.9, s	-	114.2, s	-	114.2, s	-	105.1, s	-	-	105.1, s	-
3	-	149.1, s	-	148.9, s	-	149.3, s	-	149.0, s	-	-	146.4, s	-
4	-	123.5, s	-	121.5, s	-	122.7, s	-	116.2, s	-	-	117.8, s	-
5	6.40 (brs)	133.8, d	7.53 (brs)	133.4, d	6.58	132.3, d	6.50 (s)	133.4, d	1,2,3,6,7	6.40 (s)	134.7, d	1,3,6,7
6	-	91.9, s	-	74.4 (s)	-	92.6, s	-	74.2, s		-	74.2, s	-
7	Ha, 3.80 (d, 18.0) Hb, 3.06 (d, 18.0)	42.0, t	3.70 (s)	26.9, s	Ha, 3.18 (d, 18.0) Hb, 3.60 (d, 18.0)	40.0, t	2.88 (d, 16.1) 3.12 (d, 16.1)	40.8, d	1, 5, 6, 9	2.80 (br s)	43.5, d	1 5, 6, 9
8	-	157.9, s	-	119.4, s	-	155.2, s	-	159.1, s	-	-	159.1, s	-
9	-	172.0, s	-	-	-	163.6, s	-	172.3, s	-	-	172.3, s	-
OMe	3.98 (s)	60.0, q	3.88, s	60.2, q	3.65	60.4, q	3.62 (s)	60.4, q	3	3.52 (s)	59.2, q	-

[1] 2,4-dibromo-3-hydroxy-10-oxa-spiro[6,7] deca-3,5,8-triene-11-carboxylic acid, Aiello et al, 1995.
[2] Fattorusso, Minale, and Sodono, 1971.
[3] NMR data determined from available aeroplysinin-1.
[4] Kobayashi *et al*., 1995
[5] NMR data determined from HMBC spectrum.

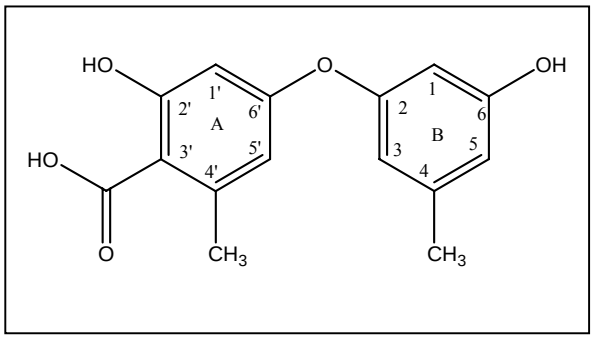


The structurally related known compounds have been reported to have either the 1*S*, 6*R* configuration as in bromo-spiranic acid or to follow the 1*R*, 6*S* stereochemisty as found in purealidin R and aeroplysin-1. The NMR data revealed chemical shift differences of 1-3 ppm between bromo-spiranic acid and purealidin R. Likewise, in the case of compound **24**, which has been isolated as a 2:1 diastereomeric mixture, a similar phenomenon was observed. The differences in the NMR resonances of both isomers, (**24A** and **24B**) as acquired in DMSO, are even more discernible for position 1 (δ_C 76.8 and 86.8, respectively), appeared as by the 10 ppm shift. In addition, changes in stereochemistry at position 6 could be revealed by the splitting pattern difference of C*H*$_2$-7. The methylene group for **24A** appeared as a pair of doublets with a large coupling constant of 16.1 Hz while that of **24B** appeared as a broad singlet. The NMR data for the cyclohexadiene ring of the major compound **24A** are comparable to those of purealidin R as exemplified by the chemical shifts of *C*H$_2$-7 (δ 40.0 ppm) and C*H*-5 (δ 6.50 ppm) while the known congener gave the respective resonances at δ_C 40.8 and δ_H 6.58. This may suggest that **24A** follows the 1*R*, 6*S* configuration. On the other hand, the second compound, **24B**, is more comparable to that of bromo-spiranic acid as revealed by resonances of *C*H$_2$-7 (δ 43.5 ppm) and

C*H*-5 (δ 6.40 ppm). For the known derivative, these were observed at at δ_C 42.0 and δ_H 6.40, respectively. This proposes that **24B** follows the 1*S*, 6*R* configuration. The stereochemistry in both isomers could only be relatively assigned. Compound **24** was thus concluded to be 3-(-3,5-dibromo-1,6-dihydroxy-4-methoxycyclohexa-2,4-dienyl)-2-iminopropanoic acid. No HRMS or further NMR experiments could be done due to the instability of the compounds.

3.7. Secondary metabolites from the mangrove fungus *Eurotium clevalieri*

3.7.1. 2-hydroxy-6-(6-hydroxy-4-methy-phenoxy)-4-methyl-benzoic acid (25, known compound)



Compound **25** was isolated as a dark purple solid residue which showed a pseudomolecular ion peak at *m/z* 275 $[M+H]^+$ as determined by ESIMS (**Figure 77**). It showed UV absorbance at λ_{max} 219 and 262nm. Its ^{1}H-NMR spectra (**Figure 78**) revealed five proton resonances in the aromatic region, and it contained also two methyl protons at δH 2.20 and 2.75, respectively. ^{13}C-NMR spectral data (**Figure 79)** showed the presence of two methyl functions

at δC 21.2 and 22.0, and four oxygenated carbon atoms at δC 161.2, 157.0, 157.2 and 157.3, together with a carboxylic acid moiety at δC 175.0. Protons in the aromatic region can be divided to two sets, first the *meta* orientation of the broad doublet protons of ring A at δH 6.16 (H-1) and 6.28 (H-5), and second the broad *meta* triplet protons of ring B resonanting at δH 6.22 (H-1'), 6.35 (H-3') and 6.40 (H-5'), respectively.

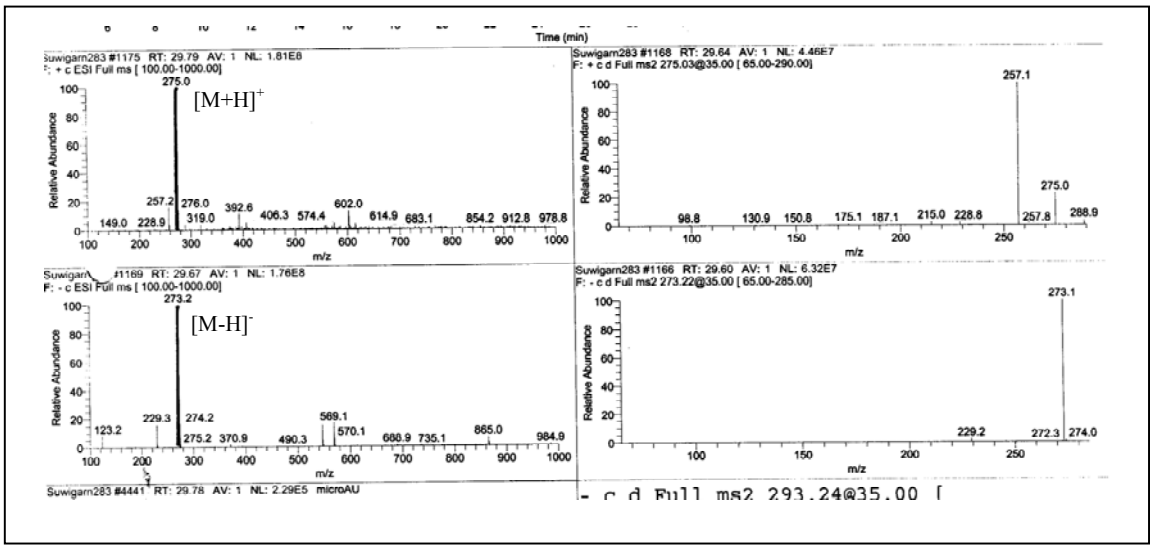
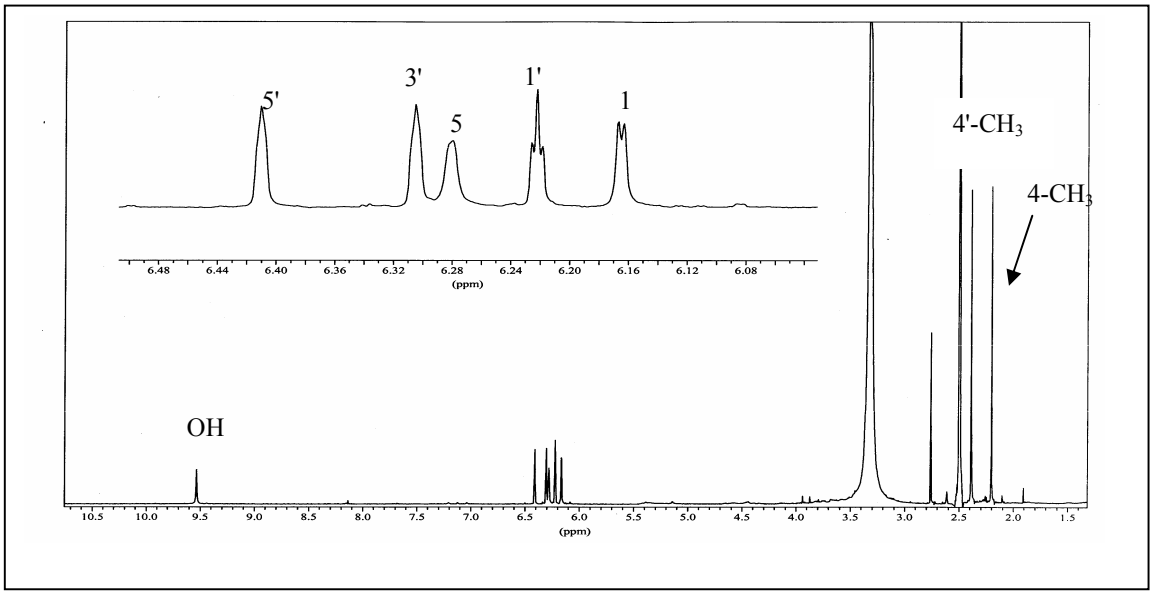


Figure 77 ESIMS spectra of compound **25**



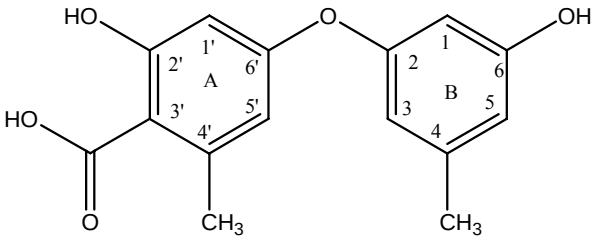


Figure 78 ^{1}H-NMR spectra of compound **25** (DMSO-d_6)

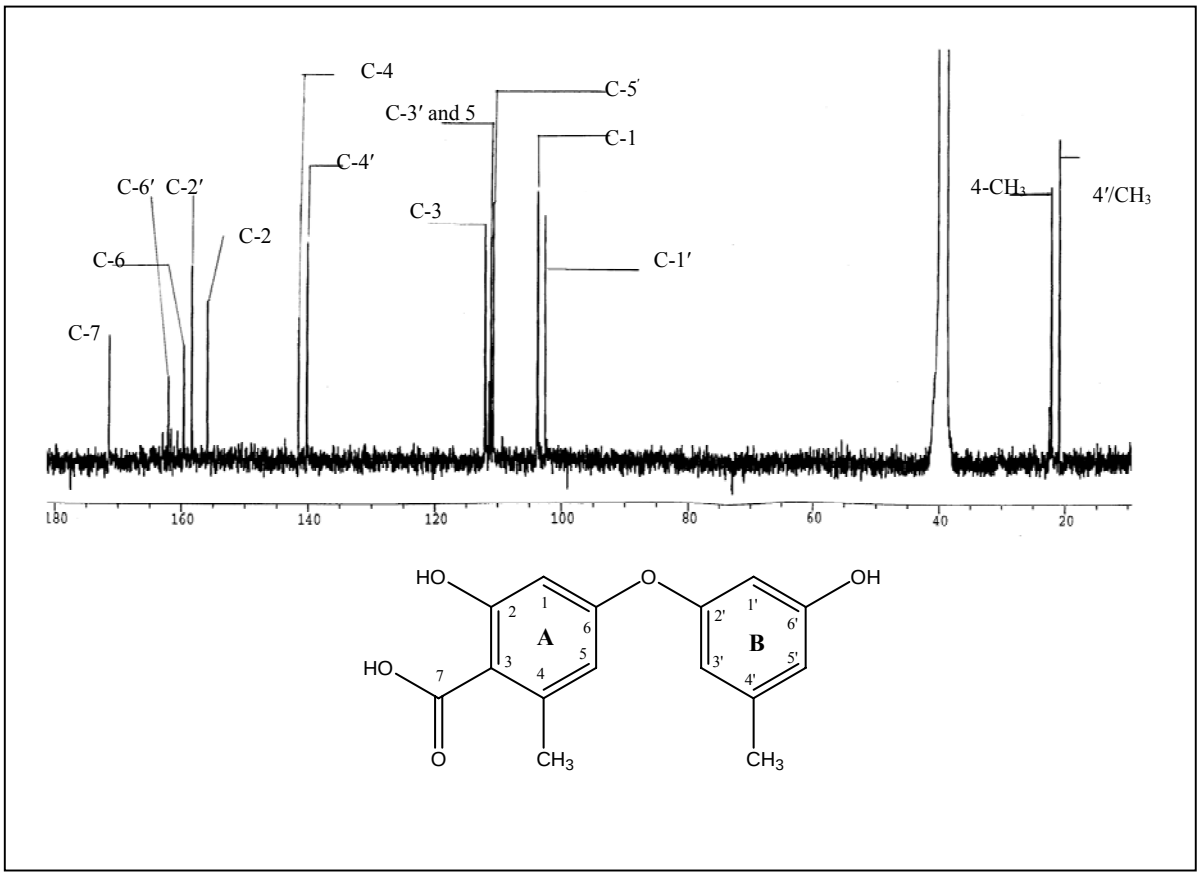


Figure 79 ^{13}C-NMR spectra of compound **25** (DMSO-d_6)

HMBC spectral data (**Figure 80** and summarized in **Table 19**) were used to establish these substitutions. The correlations from δH 6.16 (H-1) to δC 112.9 (C-5) and δH 6.28 (H-5) to δC 103.7 (C-1) and 116.2 (C-3) supported the assignment of the *meta* oriented protons for ring A. Cross peaks of δH 6.22 (H-1') with δC 114.7 (C-5'), and δH 6.35 (H-3') with δC 105.2 (C-1') and 114.7 (C-5'), and δH 6.40 (H-5') with δC 105.2 (C-1') and 113.2 (C-3') were also unambiguous and proved the assignments for ring B. HMBC correlation data were also used to establish the position of methyl groups. C-H long range correlations of the methyl singlet of ring A at δH 2.75 with the quaternary carbon at δC 142.0 (C-4) and the methine carbon at δC 112.9 (C-5) confirmed its position at C-4. Similar to ring A, the HMBC cross peaks of the methyl singlet at δH 2.20 with δC 141.5 (C-4') and 114.7 (C-5') proved also the assignment of the methyl function at C-4' for ring B. The oxygenated carbon atoms were established through the HMBC cross peaks of δH 6.16 (H-1) with δC 157.0 and 157.2 which were assigned to the ether linkage and the hydroxyl bearing carbon, respectively. The important cross peaks of the proton at δH 6.28 (H-5) with δC 157.0, 116.2 (H-3) and 103.7 (H-1), but not with 157.2 were the evidences used to establish the ether linkage at C-6 (δC 157.0) and the hydroxyl function at C-2 (δC 157.2) for ring A. The carboxylic acid function at δC 175.0 (C-7) asignated to C-3 can be explained by the shielding effect at C-3 (δC 116.2) which was caused by the mesomeric property of C-7. Moreover, an HMBC cross peak between δ 6.28 (H-5) and C-7 was also observed.

The *meta* oriented protons, methoxy function as well as the ether linkage in ring B were also established from its HMBC spectra. Cross peaks between δH 6.22 (H-1') and δC 114.7 (C-5'), and a correlation of δH 6.35 (H-3') with δC 105.2 (C-1'), 114.7 (C-5'), and δH 6.40 (H-5') with δC 105.2 (C-1') and 113.2 (C-3') confirmed the proton and carbon assignments. C-H long range correlations of the hydroxyl proton at δH 9.50 with δC 105.2 (C- 1'), 114.7 (C-5')

and 161.2 designated its position on C-6' (δC 161.2). The methyl proton at δH 2.20 showed correlations with δC 141.5 and 114.7 (C-5') supporting its position at C-4' (δC 141.5). The proton resonance at δH 6.35 (C-3') correlated with δC 157.3 but not with δC 161.2. On the other hand, the proton at δH 6.16 (H-1') showed only one cross peak with δC 161.2 (C-6'). These correlations were used to assign the ether linkage of ring B at C-2' (δC 157.3). Compound **25** was therefore identified as 2-hydroxy-6-(6-hydroxy-4-methyl-phenoxy)-4-methyl-benzoic acid.

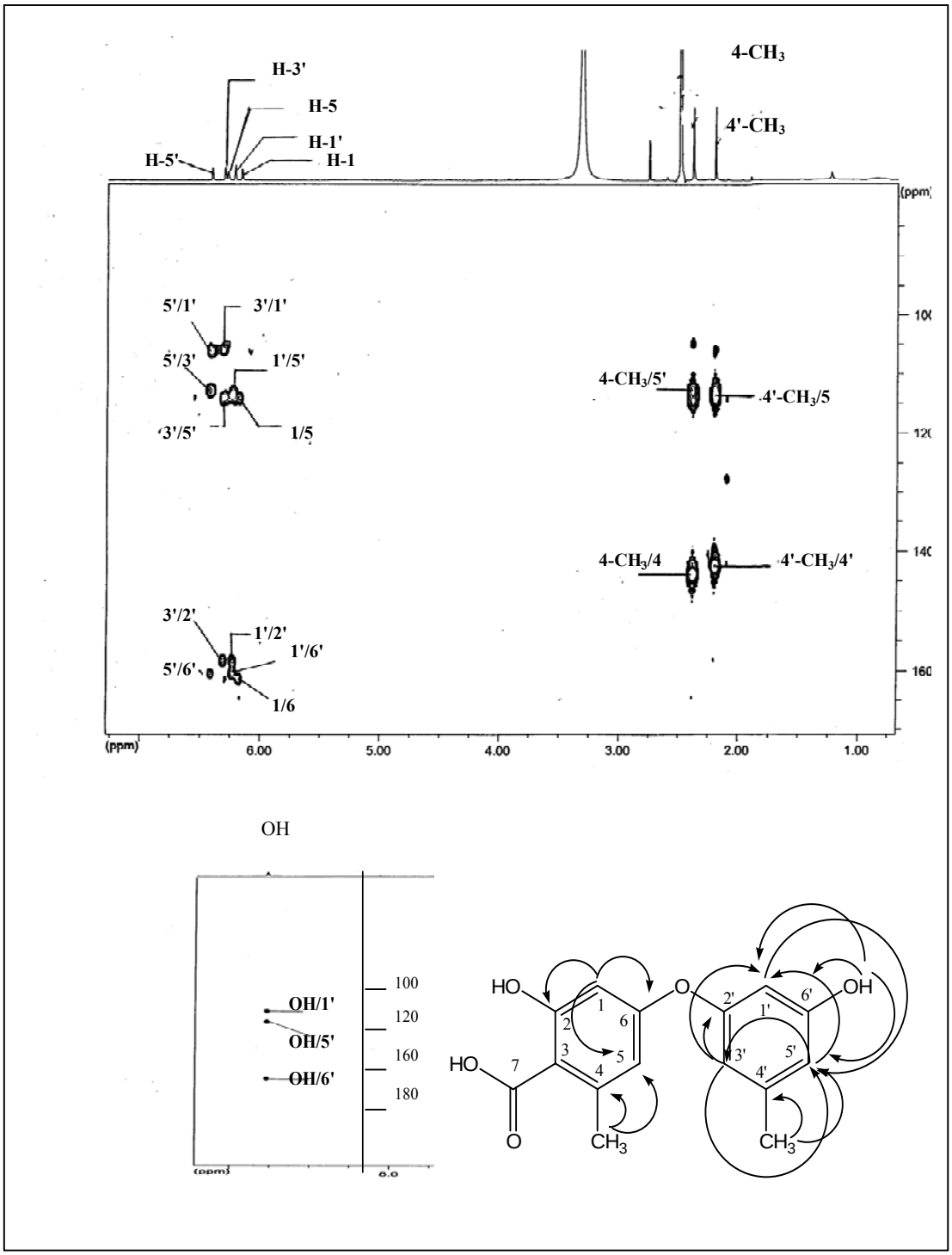
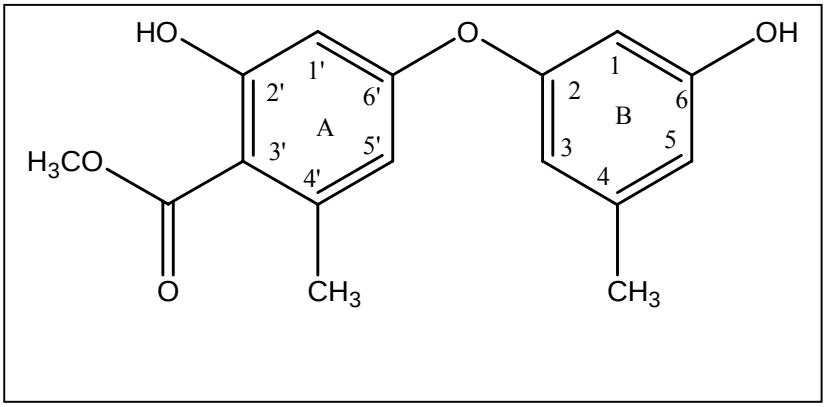


Figure 80 Important C-H long range correlation of compound **25**

3.7.2. 2-hydroxy-6-(6-hydroxy-4-methyl-phenoxy)-4-methyl-bensoic acid methyl ester (26, known compound)



Compound **26** was isolated as a dark solid residue with a pseudomolecular ion peak at *m/z* 289 $[M+H]^+$ as determined by ESIMS spectra data (**Figure 81)**. It showed UV absorbance at λ_{max} 221 and 263 nm. It has a 14 mass units difference composed to **26** which implied that it contained an additional methyl group**.** Its ^{1}H-NMR spectral data (**Figure 82)** revealed the presence of an additional methoxy proton at δH 3.75. The two methyl protons appeared as one broad singlet at δH 2.40 as determined by their integration.

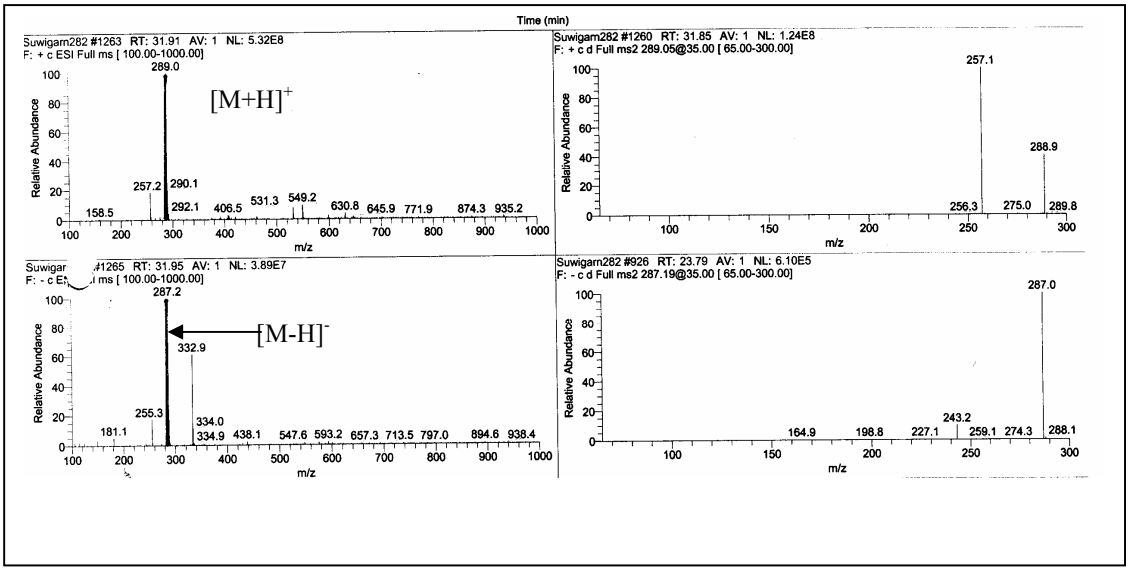


Figure 81 ESIMS spectra of compound **26**

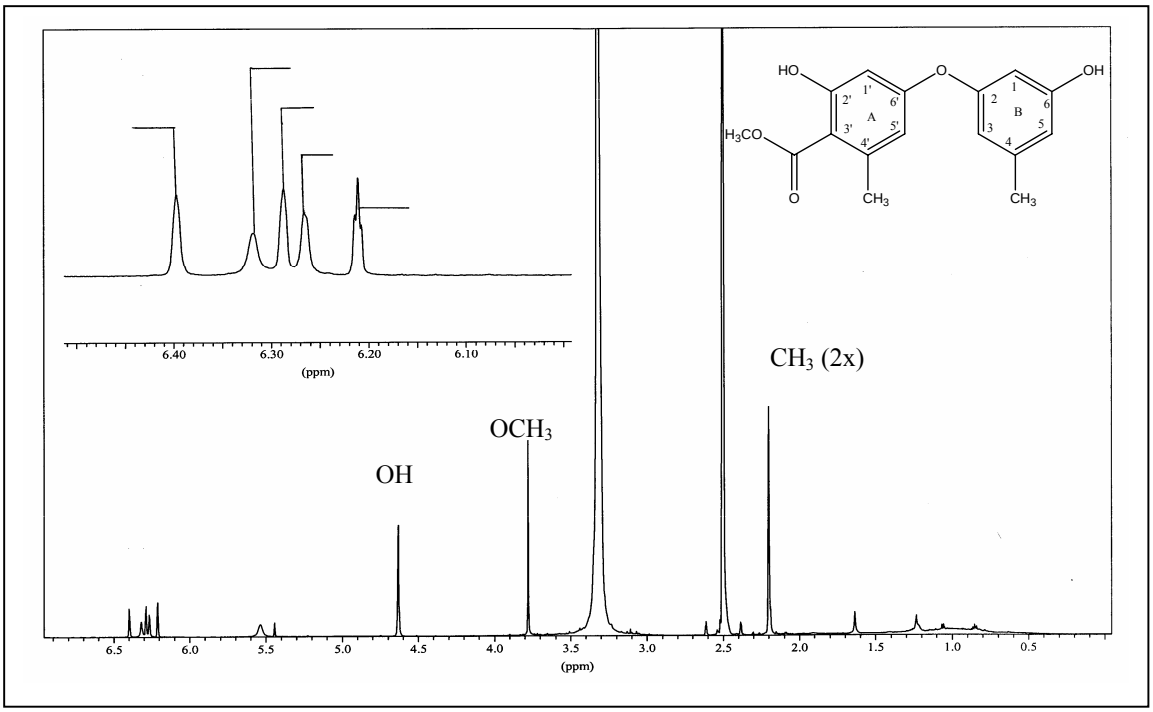
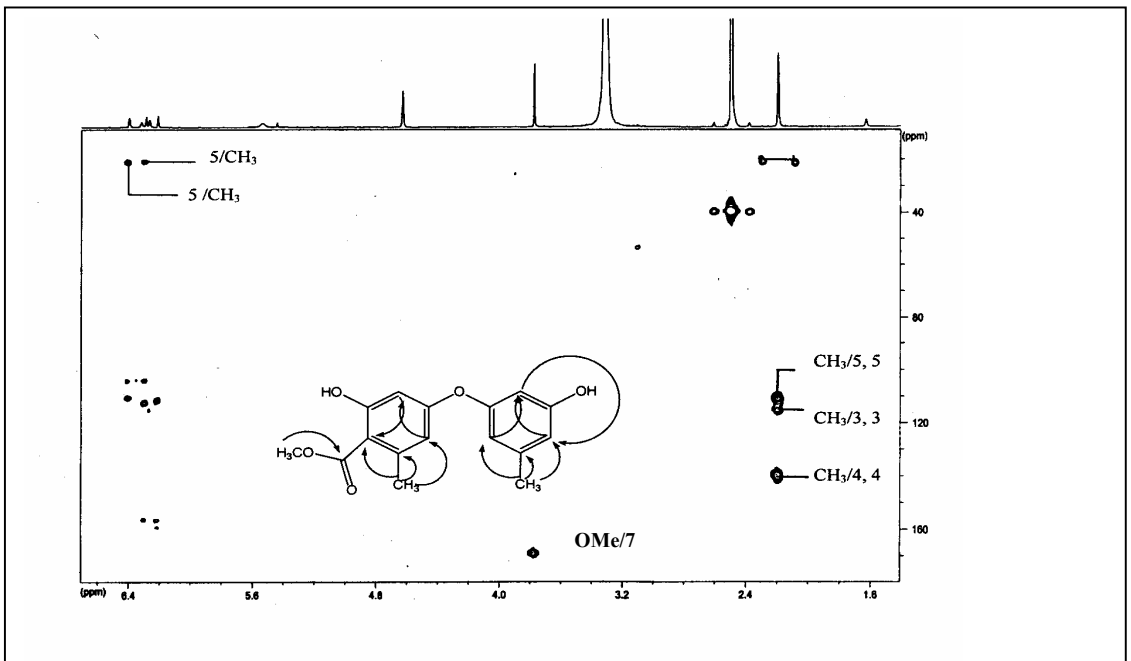


Figure 82 ^{1}H-NMR spectra of compound **26** (DMSO-d_6)

HMBC spectral data (**Figure 83)** revealed the strong intensity of a cross peak of the methoxy proton at δH 3.75 with the carbonyl carbon at δC 169.0, which confirmed its position at C-7'. For the other assignments, they were almost identical as found in compound **25**. Thus, compound **26** was identified as 2-hydroxy-6-(6-hydroxy-4-methyl-phenoxy)-4-methyl-bensoic acid methyl ester.

Both compounds have been previously isolated from the fungus *Aspergillus fumigatus* [Takahashi *et al*., 1986]. This is the first report in which a complete unambiguous assignments of both proton and carbon data have been presented.



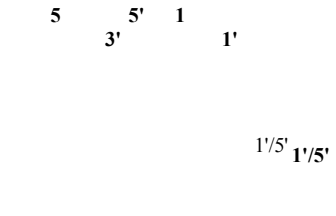


Figure 83 Important C-H long range correlation of compound **26**

Table 19 NMR data of compound **25** and **26** (DMSO-d_4)

Position	Compound **25**			Compound **26**		
	δ H, (multiplicities, J in Hz)	δC, multiplicities	HMBC	δ H, multiplicities, J in Hz	δC, multiplicities	HMBC
1	6.16 (brd,2.10)	103.7,d	2,5,6	6.29 (brs)	102.5, d	2,3

2-OH	-	157.2, s	-	-	159.2,	-
3	-	116.2, s	-	6.32,brs	114.2, d	-
4	-	142.0, s	-	-	139.5, s	-
5	6.28 (brs)	112.9, s	1,3,6	6.41 (brs)	111.5, d	4-CH_3
6	-	161.2, s	-	-	159.0, s	-
7	-	172.5, s	-	-	162.5, s	-
1'	6.22 (brt, 2.10)	105.2, d	5',6'	6.21 (brt,2.10, 2.10)	102.3, d	2',5',6'
2'	-	157.3, s	-	-	159.2, s	-
3'	6.35 (brs)	113.2, d	1',2',5'	6.32 (brs)	114.2, d	-
4'	-	141.5, s	-	-	139.5, s	-
5'	6.40 (brs)	114.7, s	1',3'	6.31 (brs)	111.5, d	2',5', 4'-CH_3
6 '-OH	9.50 (s)	157.0, s	1', 5', 6'	-	159.2, s	-
Me-4	2.75 (s)	22.0, q	4,5	2.40	22.0, q	-
Me-4'	2.20 (s)	21.2, q	3', 4', 6'	2.40 (s)	22.0, q	3, 4, 6
7	-	175.0 (s)	-	-	169.0	7

3.3. Secondary metabolites from the marine sponge *Dysidea granulosa*

Sponges from the genus *Dysidea* have been reported as a source of various bioactive secondary metabolites which include chlorinated metabolites derived from amino acids [Kazlauskas, Lidgard and Wells, 1997], sesquiterpenes [Carmerson *et al.*, 2000], spirol/lactones [Kaslauskas, Murphy and Wells, 1978], tricyclic furans, some based on the furodysidinin

skeleton [Ksebati and Schmitz, 1988], polychlorinated alkaloids, and bromodiphenyl ether congeners [Carte and Faulkner, 1981, Handayani *et al*., 1997]. Bromodiphenyl ethers have been reported to be produced by the filamentous cyanobacterium, *Oscillatoria spongeliae*, associated with the sponge genus *Dysidea* [Berthod, Borowitzka, and Mackay, 1982]. It has been hypothesized that this class of compounds should play an important ecological role as potential defense substances against predators and bacterial invasion [Faulkner, Unson and Bewley, 1983, 1994; Holland and Faulkner, 1994]. Bromodiphenyl ether compounds have also been reported to exhibit strong antibiotic activity toward *B. subtilis* (Handayani *et al.,*1997). Some reported compounds which were isolated from the genus *Dysidea* are shown below.

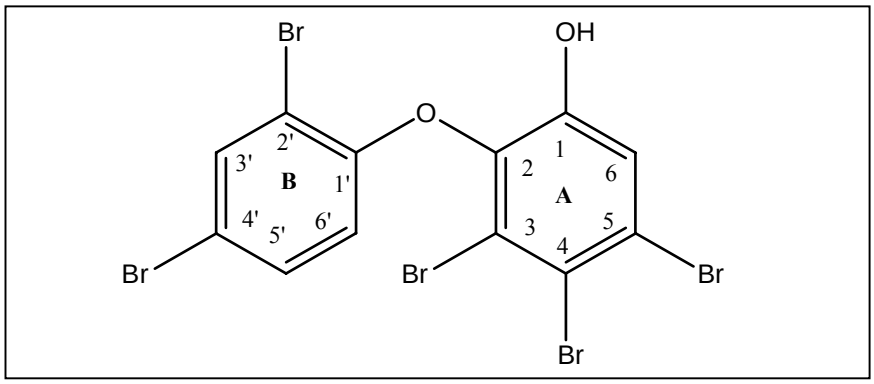
Four compounds were isolated from a marine sponge, *Dysidea granulosa* collected from the Andaman Sea. Structure elucidations were performed by ^{1}H-NMR, 2D-NMR experiment together with analysis of EIMS spectroscopic data. All of the isolated compounds exhibited antimicrobial activity toward *B. subtilis* as shown in **Table 9.**

3.3.1. Structure elucidation of compounds 12 and 13

Compounds **12** and **13** were isolated as white powders. All compounds showed the molecular ion cluster peaks at *m/z* 575, 577, 579, 581, 583, 585 with intensities of 1:4:6:6:4:1 as shown by their EIMS spectrum (**Figure 33**). This implied the occurrence of five bromine substituents in the molecules. They showed UV absorbance at λ_{max} 210, 214 and 215, respectively.

3.3.1.1. 3,4,5-tribromo-2-(2,4-dibromo-phenoxy)-phenol

(12, known compound)



^{1}H-NMR of compound **12** (**Figure 34)** showed five resonances which consisted of four signals in the aromatic region and one OH functional group at δH 10.90. Three proton signals at δH 7.31, 7.85 and 6.51 indicated an ABC spin system as revealed by their *ortho*, *ortho-meta*, and *meta* coupling, respectively. This is typical of a 1,2,4-trisubstituted benzene ring (ring B). The singlet proton at δH 7.41 was assigned to a penta-substituted benzene ring (ring A).

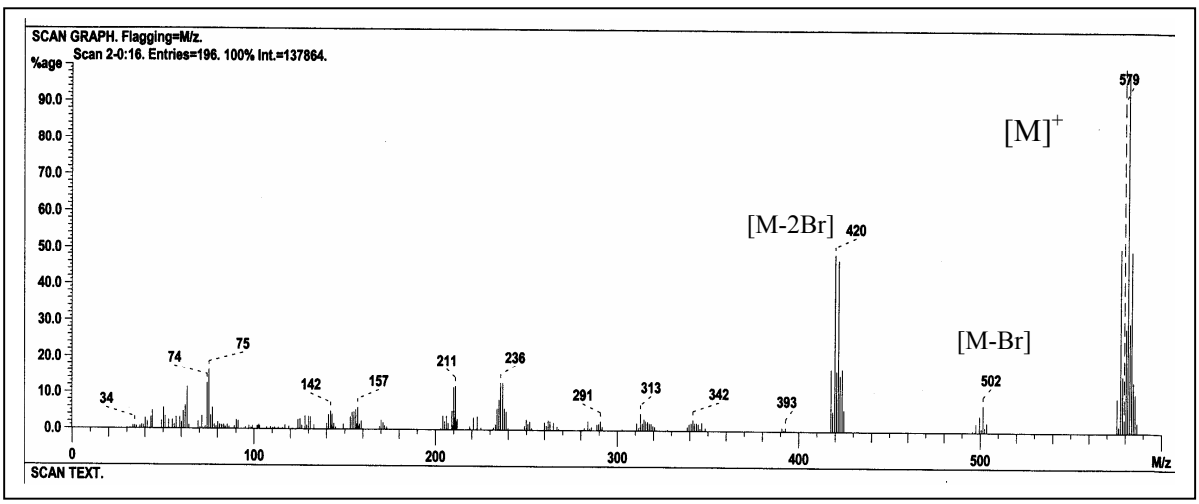


Figure 33 EIMS of compound **12** and **13**

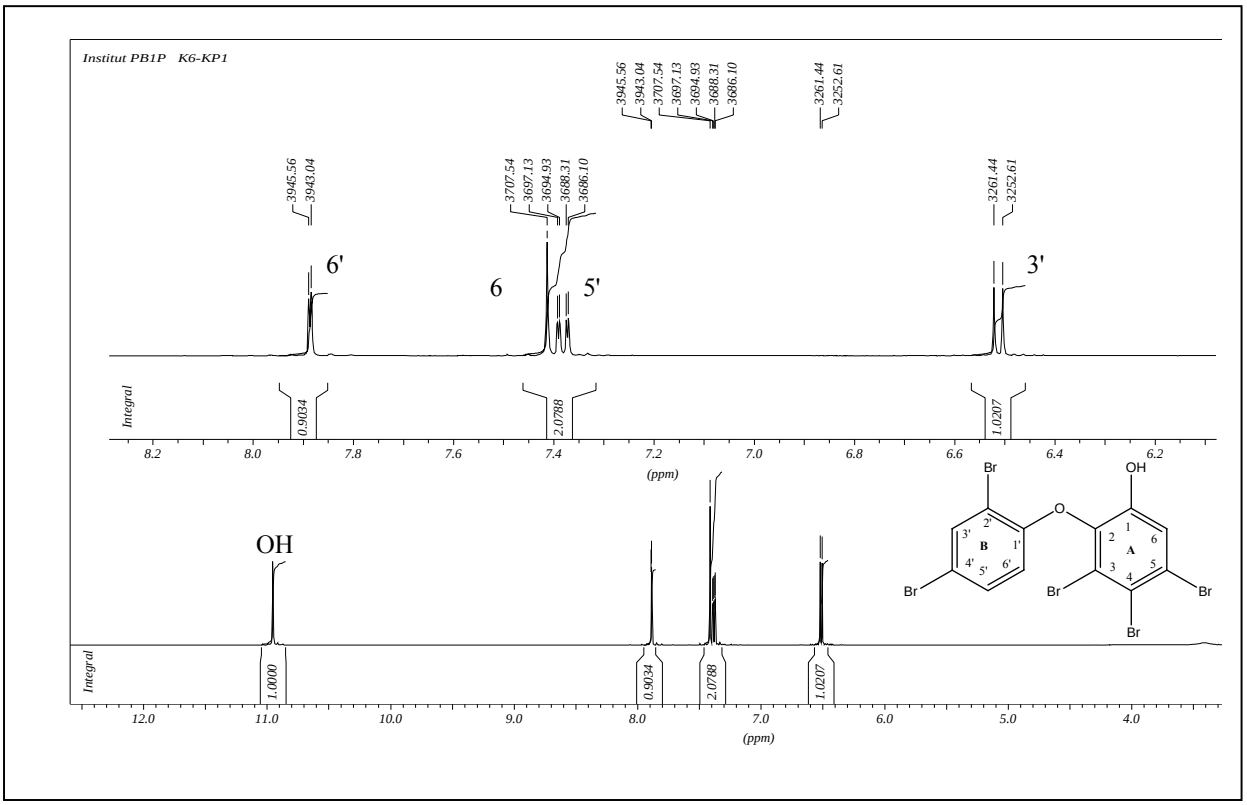


Figure 34 ^{1}H-NMR of compound **12** (DMSO-d_6)

HMBC spectral data (**Figure 35)** was used to confirm the position of the hydroxyl at C-1, by inspection of the correlation of the oxygenated proton at δH 10.90 to C-1 (δC 151.4), C-2 (δC 139.7) and the methine carbon (δC 121.9) at position 6. Furthermore, the singlet proton at δH 7.41 (H-6) correlated with C-2 (δC 139.7) and C-4 (δC 116.2), which confirmed its assignment at C-6. The upfield shift at δC 139.7 (C-2) was assigned to a carbon carrying an

oxygen atom which is also the ether linkage to the B ring. This upfield shift of C-2 was due to the shielding effect of the OH substituent at C-1, as well as to its inductive effect on C-2. The assignment was also proven by HMBC cross peaks of the hydroxyl proton and H-6 (δH 7.41) with C-2. The lone pair electron located on the oxygen atom is delocalized through the sigma bond resulting in high electron density at C-2. The π electron at C-1 is rearranged to the carbonyl function causing the withdrawal of electrons from the OH group which showed a higher electronegativity than C-1. This mechanism is shown below. On the one hand, the more downfield shift at δC 152.6 was assigned to C-1' of ring B due to the deshielding effect of the oxygen atom at the ether linkage. It was also confirmed by C-H long range correlations from H-5' (δH 7.31) and H-3' to C-1'. Thus, the ether linkage was concluded to be between C-1' and C-2. Compound **12** was determined as 3,4,5-tribromo-2-(2,4-dibromo-phenoxy)phenol which was previously isolated from a Micronesian marine sponge, *Dysidea* sp. [Fu *et al*., 1995].

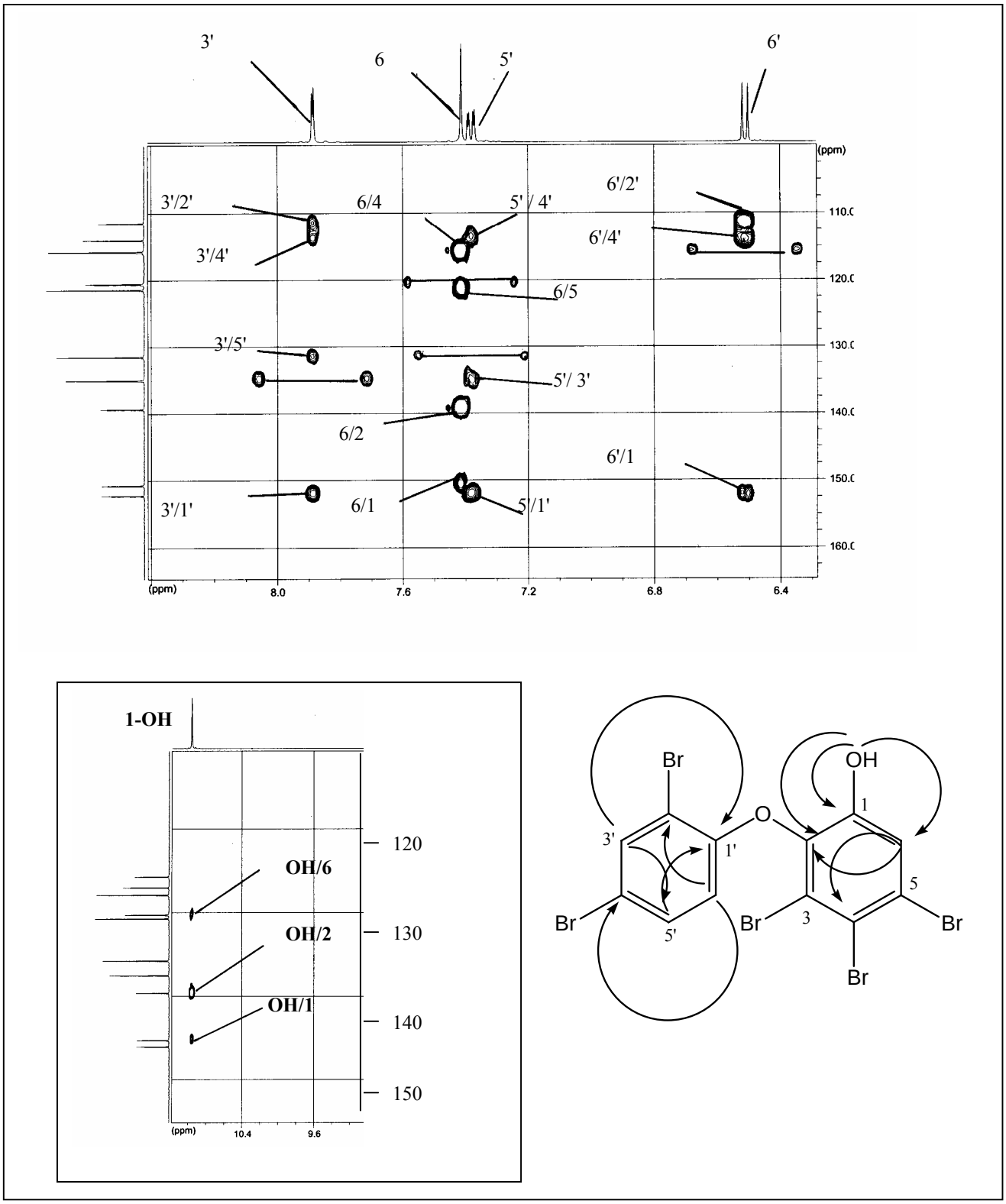
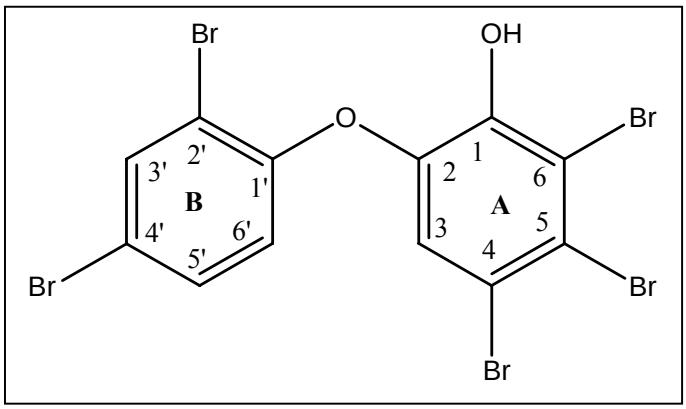


Figure 35 HMBC spectra of compound **12**

3.3.1.2. 4,5,6-tribromo-2-(2,4-dibromo-phenoxy)-phenol

(13, known compound)



Compound **13** is related to compound **12.** They have the same ^{1}H-NMR data for ring B which was indicated by the occurrence of a similar spin system. The ^{1}H-NMR of compound **13** (**Figure 36**) also revealed the presence of a 1,2,4-trisubstituent benzene ring as found in compound **12**. The only difference is the more upfield shift of the singlet proton at δH 7.21 (H-3) which belong to ring A. From its HMBC spectra (**Figure 37)**, this methine proton singlet showed correlations to C-1 (δC 152.0) and C-5 (δC 122.6) which indicated its position at C-3. The oxygenated carbon at δC 152.0 (C-2) of compound **13** is more downfield than observed for compound **12** because of the delocalization of the π electron system of the phenol ring resulting in a deshielded carbon at position 2. This argument and the HMBC cross peak between H-3 (δH 7.21) and C-2 (δC 152.0) confirmed the ether linkage at C-2 and C-1'. Based on above discussion and in comparison to literature data, compound **13** was identified as 4,5,6-tribromo-2-(2,4-dibromo-phenoxy)phenol which was previously isolated from the sponge *D. herbacea* obtained from Western Caroline Island [Sharma and Vig, 1972].

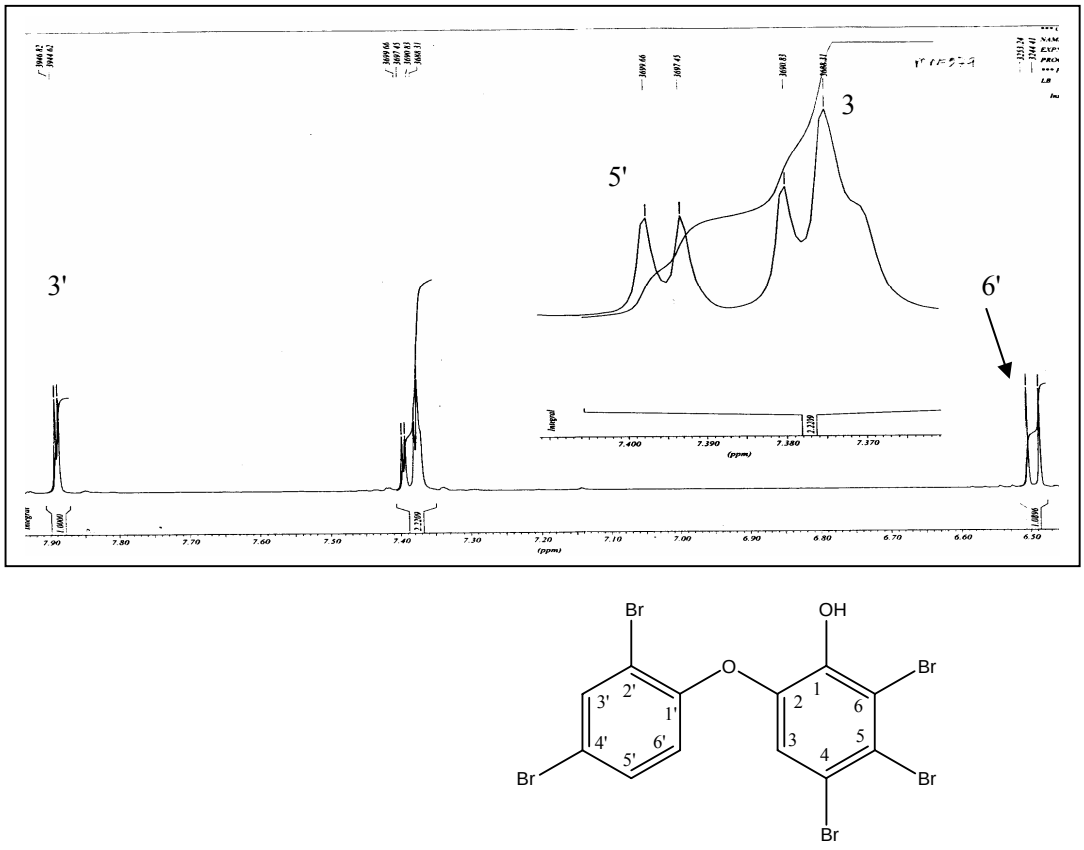


Figure 36 ^{1}H-NMR spectra of compound **13** (DMSO-d_6)

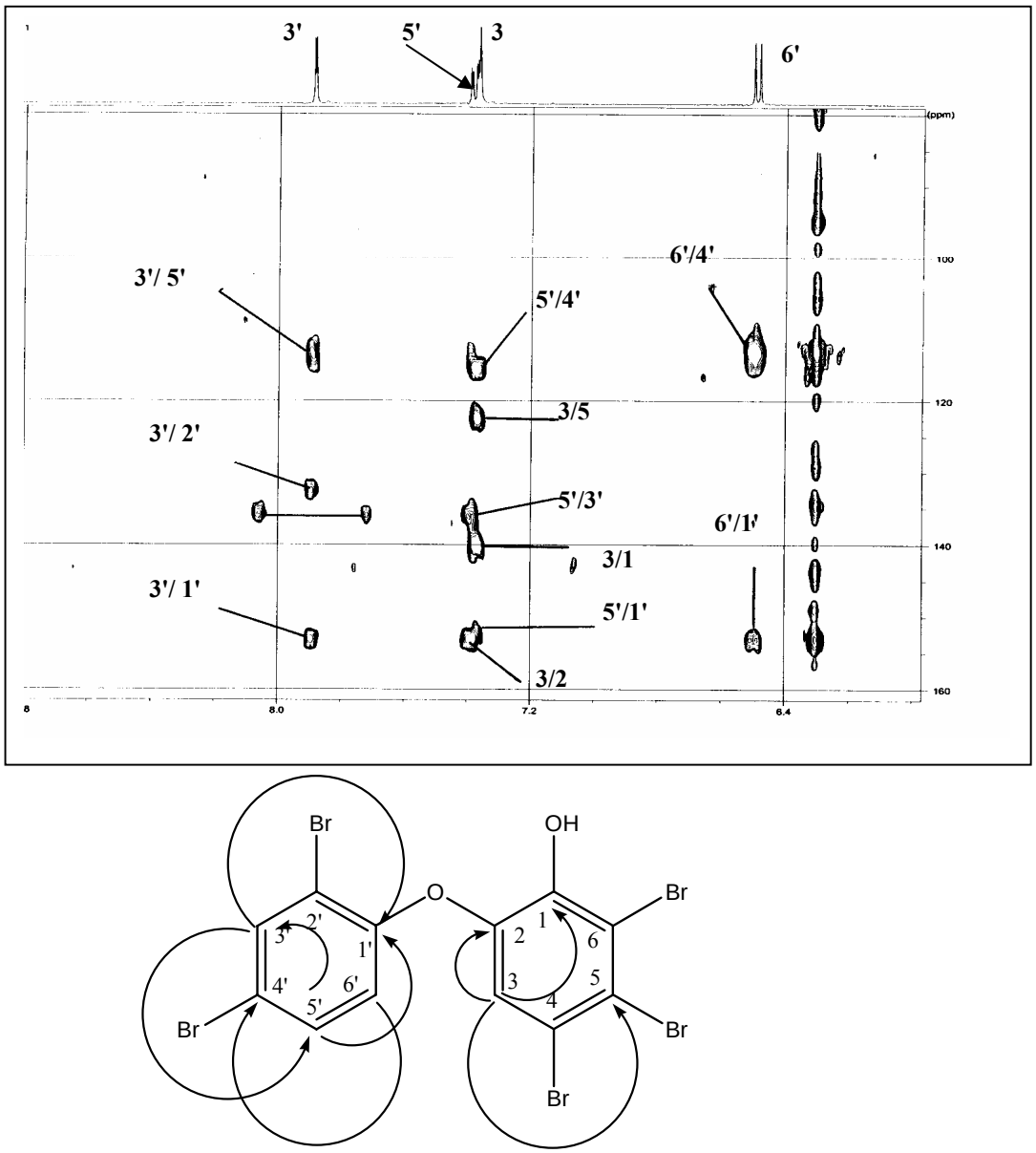
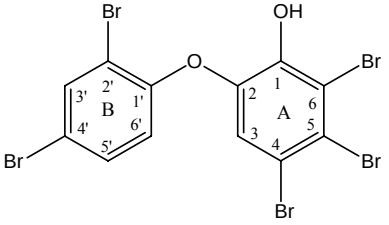
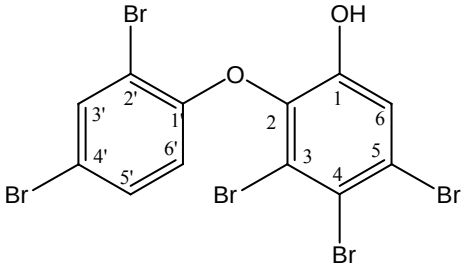


Figure 37 Important C-H long range correlations of compound **13**

In order to facilitate structure elucidation of brominated diphenylether derivatives it has been observed that the brominated substituent at C-3 affects the chemical shift of the C-6' proton significantly. The chemical shift of C-6' proton is approximately 6.60 – 6.80 ppm when the phenol ring B does not have a bromine substitutent at C-3. This shift of the C-6' proton decreases to 6.20 – 6.40 ppm if there is a bromine substituent at C-3 in ring B. The ^{13}C chemical shift can be predicted in the same way as the ^{1}H chemical shift. The chemical shift of C-6' is 121.0 – 122.0 ppm, but if C-3 carries a bromine, the ^{13}C shift of C-6' decreases to 116.0 ppm as shown below [Fu and Schmitz, 1996].



C-3 = H
δH-6' = 6.60 – 6.80 ppm
δC-6' = 121.0 – 122.0 ppm



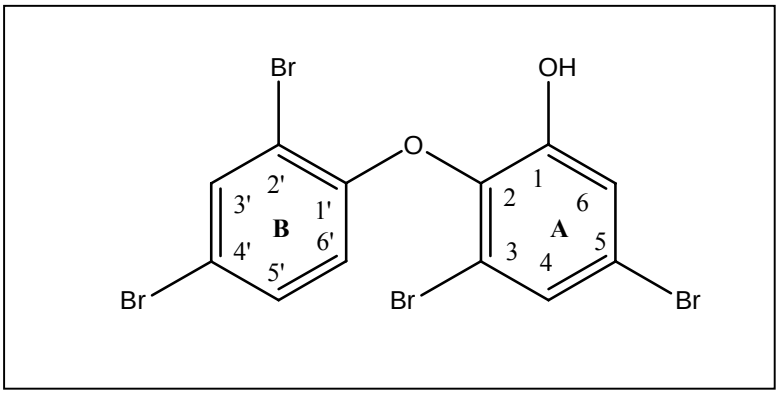
C-3 = Br
δH-6' = 6.20 – 6.40 ppm
δC-6' = 116.0 ppm

Table 7 NMR data of compounds **12** and **13** (DMSO-d_6)

Positision	Compound **12**			Compound **13**		
	δH, multiplicities, J in Hz	δC, multiplicities	HMBC	δH, multiplicities, J in Hz	δC, multiplicities	HMBC
1	-	151.4, s	-	-	-	-
2	-	139.7, s	-	-	152.0, s	-
3	-	121.9, s	-	7.21(s)	116.6, d	1, 2, 5
4	-	116.2, s	-	-	122.6, s	-
5	-	122.2, s	-	-	122.6, s	-
6	7.41(s)	121.9,d	1,2,4	-	-	-
1′	-	152.6, s	-	-	153.1, s	-
2′	-	111.9, s	-	-	132.6, s	-
3′	7.85 (d, 2.5)	135.9, d	1′, 2′, 4′ , 5′	7.85 (d 2.52)	135.9, d	1′, 2′, 3′
4′	-	114.5, s	-	-	115.5, s	-
5′	7.31 (dd, 8.8, 2.0)	131.9, s	1′, 4′	7.48 (dd, 2.5, 8.8)	133.8, d	1′, 3′ ,4′
6′	7.85 (d, 8.8)	114.5, s	1′, 2′, 4′	6.51 (d, 8.8)	121.6, d	1′, 2′, 4′
OH	10.96	-	-	-	-	-

3.3.2. Structure elucidation of compounds 14 and 15

3.3.2.1. 3,5-dibromo-2-(2,4-dibromo-phenoxy)-phenol (14, known compound)



Compound **14** was obtained as a white powder with quasi molecular ion clusters at *m/z* 498, 500, 504, 506 and 508 determined by EIMS spectra as shown in **Figure 38**, which indicated that it has four bromine substituents in the molecule. It showed UV absorbance at λ_{max} 215 nm. Its ^{1}H-NMR (**Figure 39**) also exhibited the presence of a 1,2,4-trisubstituent benzene ring (ring B) as found in compounds **12** and **13.** Two doublet protons at δH 7.95 and 7.13 ppm with a coupling constant of 2.2 Hz revealed the typical *meta-meta* orientation of these protons. They were assigned as protons belonging to C-4 and C-6. This was confirmed by their C-H long range correlation through its HMBC spectra (**Figure 40)**. The methine proton at δH 7.13 (H-6) correlated with C-2 (δC 139.4) and C-4 (δC 126.0) while the methine proton at δH 7.95 (H-4) showed cross peaks with C-2. Therefore, compound **14** was identified as 3,5-dibromo-2-(2,4-dibromo-phenoxy)phenol which was previously isolated from Indo-Pacific *Dysidea* sp. [Fu and Schmitz, 1996]

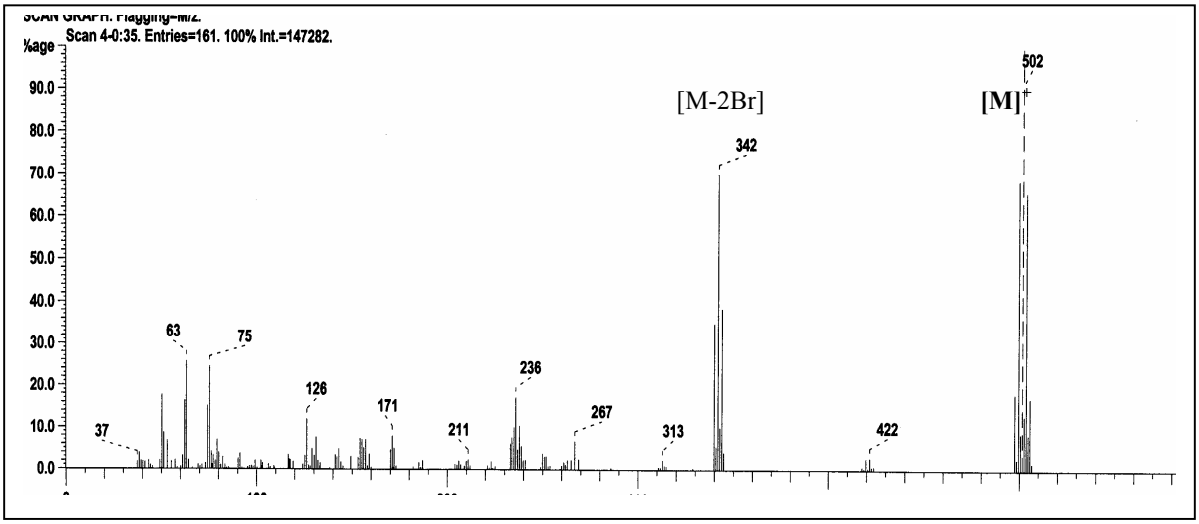


Figure 38 EIMS of compound **14**

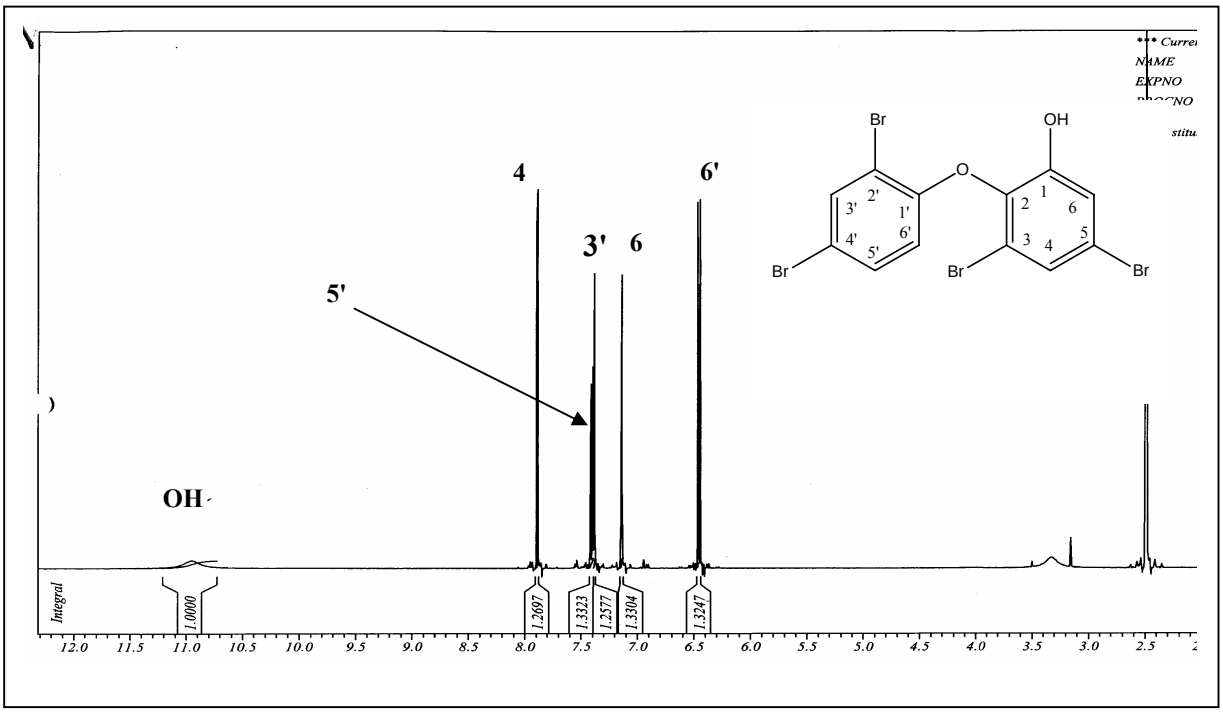


Figure 39 ^{1}H-NMR of compound **14** (DMSO-d_6)

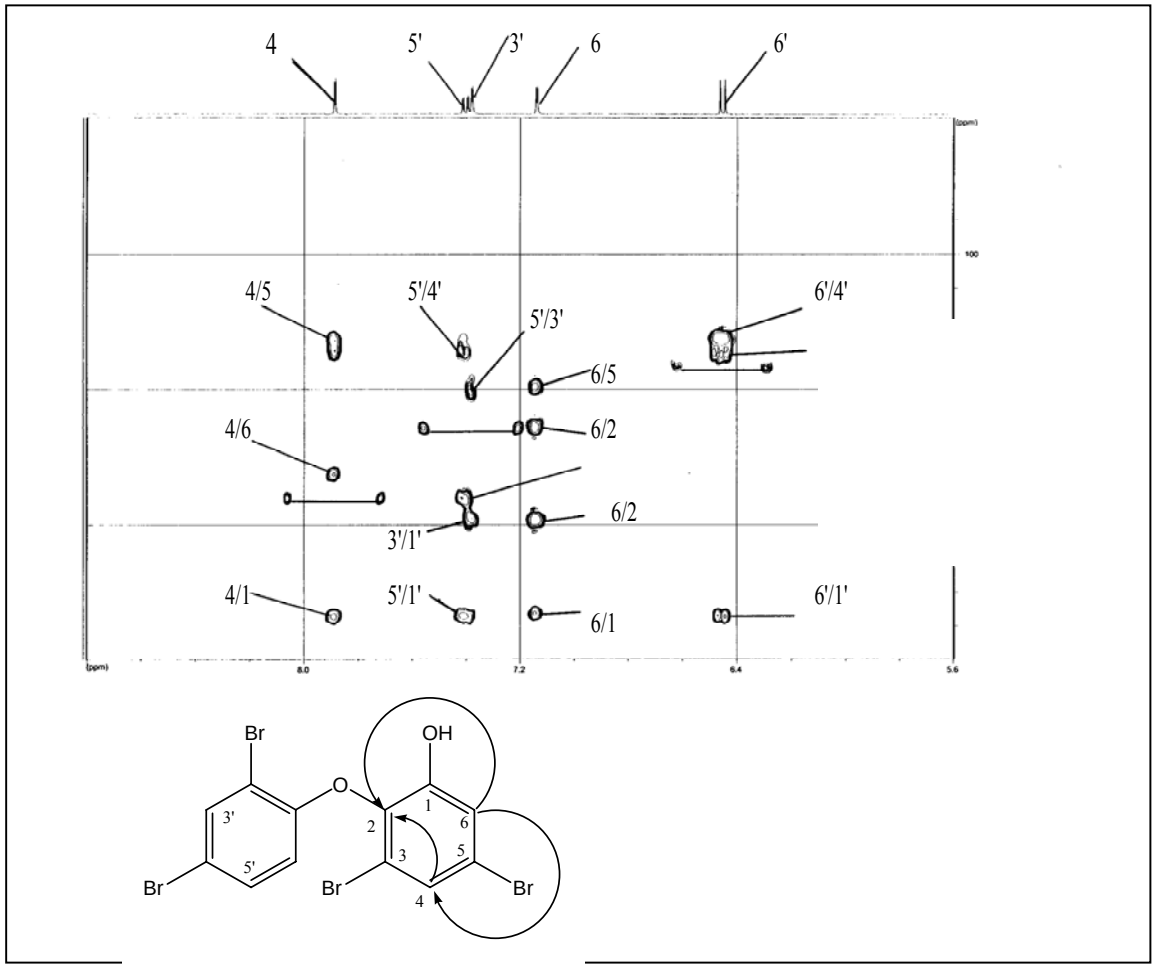
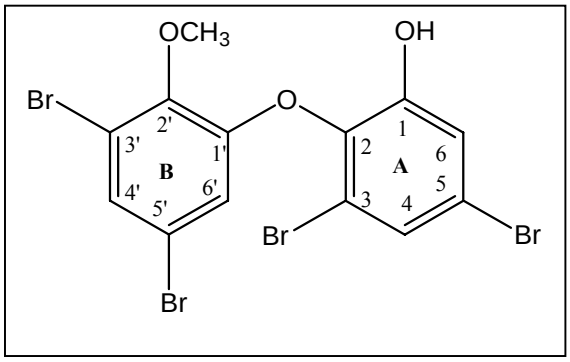


Figure 40 Important C-H long range correlations of compound **14**

3.3.2.2. 3,5-dibromo-2-(3,5-dibromo-2-methoxy-phenoxy)-phenol (15, known compound)



Compound **15** was also isolated as a white powder with UV absorbance at λ_{max} 201 nm and exhibited four bromine substituents in the molecule as determined by the molecular ion peaks at *m/*z 528, 530, 532, 534, 536 in its EIMS spectrum (**Figure 41**). ^{1}H-NMR spectra of this compound (**Figure 42**) revealed that it contained four resonance signals in the aromatic region, which were divided into two sets of AB spin systems. Analysis of their multiplicities indicated the presence of two *meta* oriented pairs of proton with the coupling constant of 2.2 Hz, respectively. The methoxy proton at δH 3.61, which was connected on C-2**'** (δC 146.0) was determined by the HMBC cross peak between the O*CH*$_3$ proton with C-2'. Its HMBC spectrum (**Figure 43** and summarized in **Table 8**) was used to identify the positions of the *meta* protons. The correlation of H-4 (δH 7.85) with the quaternary carbon C-2 (δC 138.9) and the methine carbon C-6 (δC 121.6) confirmed the presence of the AB system in phenol ring B. In addition, H-4' (δH 7.58) correlated with C-2' (δC 146.0) and C-6' (δC 116.8) and strongly supported the assignment of the second AB spin system for the dibromophenoxy ring A. Therefore, compound **15** was classified as 3,5-tibromo-2-(3,5-dibromo-2-methoxy-phenoxy)phenol which was previously isolated from the Indo-Pacific marine sponge *D. herbacea* [Levy *et al.,* 1972].

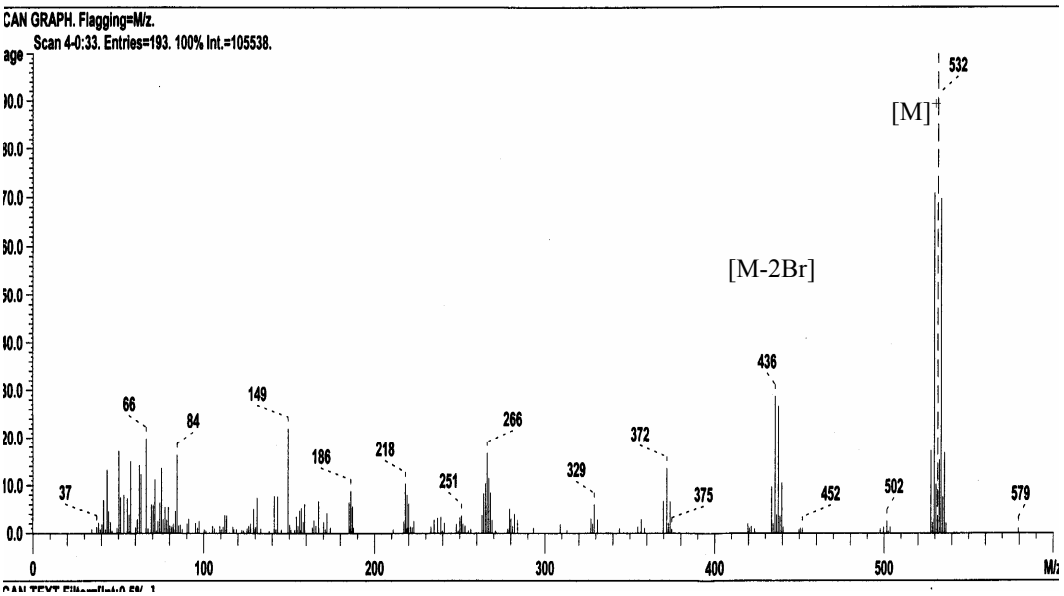


Figure 41 ESIMS spectra of compound **15**

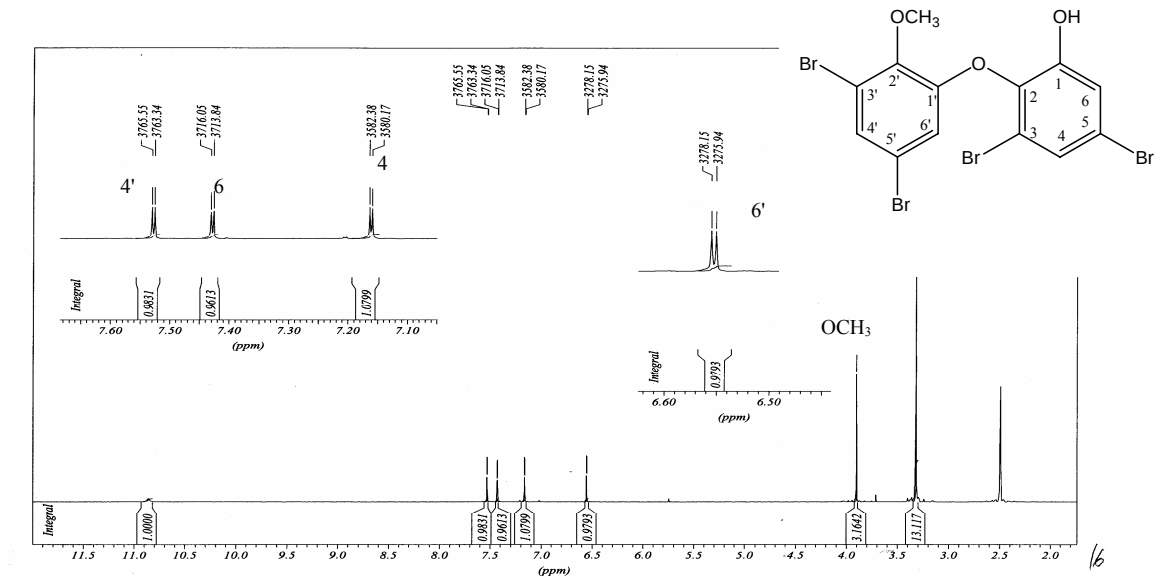


Figure 42 ^{1}H-NMR of compound **15** (DMSO-d_6)

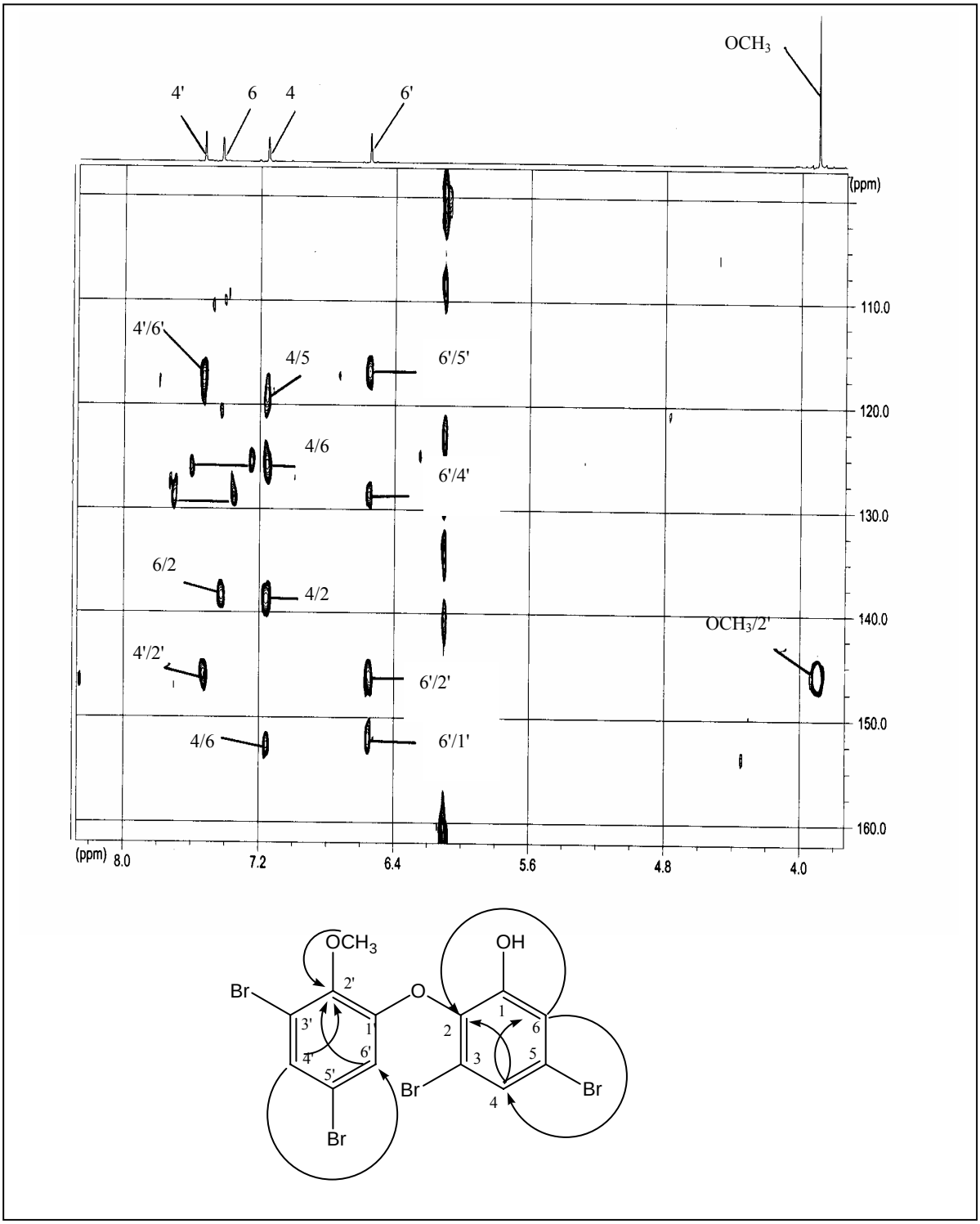


Figure 43 Important C-H long range correlations of compound **15**

Table 8 NMR data compounds **15** and **16** (DMSO-d_6)

Positi-on	Compound **15**			Compound **16**		
	δH, multiplicities, J in Hz	δC, multiplicities	HMBC	δH, multiplicities	δC, multiplicities, J in Hz	HMBC
1	-	152.5, s	-	-	152.7, s	-
2	-	139.4, s	-	-	138.9, s	-
3	-	121.0, s	-	-	119.5, s	-
4	7.95 (d, 2.2)	126.0, d	2, 3	7.85 (d 2.2)	126.1, d	1,5,6
5	-	119.0, s	-	-	119.5, s	
6	7.13 (d, 2.2)	153.0, s	1,5,6	7.12 (d, 2.2)	121.6, d	1, 2, 4, 5
1′	-	113.0, s	-	-	152.0, s	-
2′	-	136.0, d	-	-	146.0, s	-
3′	7.4 (d, 2.2)	115.0, s	1′, 5′, 4′	-	118.0, s	-
4′	-	115.0, s	-	7.58 (d, 2.2)	118.0, s	2′, 6′
5′	7.8 (dd, 2.5, 8.3)	132.5, d	1′, 3′, 4′	-	128.5, s	-
6′	6.43 (d, 8.8)	117.0, s	1′, 2′, 4′	6.56 (d, 2.2)	116.8, s	1′, 4′, 5′
OH	11.0	-	-	11.0	116.8, d	-
OCH_3	-	-	-	3.60,	61.4, q	2′

Table 9 Inhibition zone (mm) of anti-bacterial assay of isolated compounds from *Dysidea granulosa*

Compound No	*B.subtilis* at concentration of 20μg/ml* (inhibition zone)
12	9
13	8
14	10
15	10

*
The soluble sample was applied about 10 μg/disc.

4. Discussion

4.1. Metabolites isolated from the sponge *Dragmacidon* sp.

Secondary metabolites produced by the order Axinellidae have been dominated by compounds derived from the mevalonate and the deoxyxylulose phosphate pathway. However, the genus *Dragmacidon* also contains a lot of natural products derived from the amino acid pathway [Harper *et al*., 2001]. The first secondary metabolites reported from *Dragmacidon* sponges was the bisindole alkaloid, dragmacidin, [Komoto *et al*., 1998] which was biosynthetically derived from a tryptamine-containing an unoxidized piperazine ring positioned between differently substituted indole residues [Jiang *et al*., 1994]. The second report of secondary metabolites was an antiviral compound, dragmacidin F, which was isolated from *Halicortex* sp [Cutignano *et al*., 2000].

All the isolated compounds in this study were tryptophan-derived. Tryptophan is an aromatic amino acid containing an indole ring system which originated from the shikimate pathway via anthranilic acid. It gave rise to three simple indole alkaloids (**1-3**) which were isolated in this study. Compound **3** was classified to be biogenetically related to 4-hydroxy-5-(indole-3-yl)-5-oxo-pentan-2-one, which was previously isolated from the Bermudian sponge *Hyrtios erecta* [Kobayashi *et al.,* 1990]. It has shown cytoxicity against human epidermoid carcinoma KB cell *in vitro* [Kobayashi *et al*., 1990]. The two new β-carboline alkaloid which were named dragmacidonamine A and B were isolated.

4.1.1. β-carboline alkaloids from marine invertebrates

The first isolated β-carboline and its derivative were norharman and harman. Both of them were isolated from a bioluminescent marine dinoflagellate, *Noctiluca miliaris* [Inoue *et al*., 1980]. Dragmacidonamine A and B are related to hyrtiomanzamines which were previously isolated from the sponge *Hyrtios erecta* [Kondracki and Guyot, 1990] and xestomanzamine A and B from *Xestospongia* sp. [Kobayashi *et al.*, 1995]. These compounds (**Figure 84**) consisted of two parts where the β-carboline is linked at position one to a betaine unit. Naturally occurring β-carbolines are formed by the condensation of a tryptophan derivative with a second amino acid producing a compound bearing an amino acid side chain pendant to the tricyclic β-carboline nucleus [Wagoner, *et al*., 1999]. The indole unit of these metabolites is derived from tryptophan via tryptamine [Still and Mcnulty, 2000]. It has also been suggested that the dragmacidonamines could be derived from the same biosynthetic pathway as the metabolites mentioned above and that the betaine part originated from histamine. This group of compounds exhibits a great range of biological activities particular immunosuppressive and cytotoxic activity against several cancer cell lines [Kondracki and Guyot, 1996].

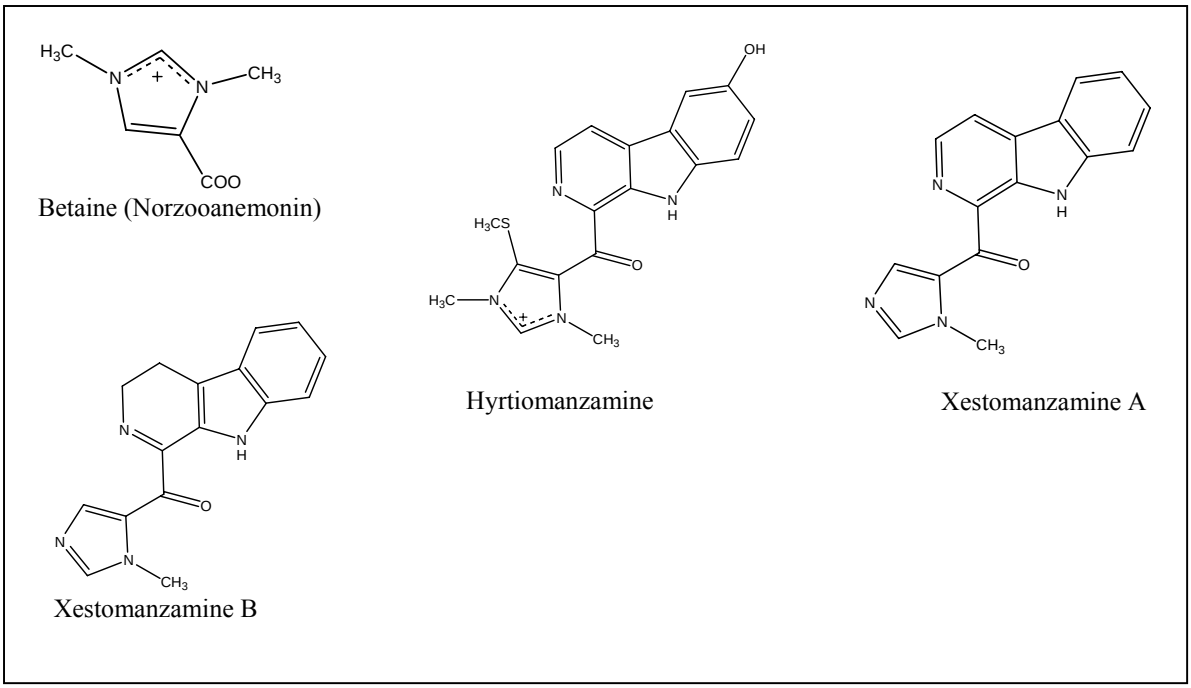


Figure 84 The betaine unit and some manzamine compounds are related to dragmacidonamines

4.1.2. Biosynthesis of the β-carboline alkaloids

Alkaloids based on a β-carboline system are formed from a new six membered heterocyclic ring using the ethylamine side chain of tryptophan. This is a process analogous to generating tetrahydroisoquinoline alkaloids. The position two of the indole ring system is nucleophilic due to the adjacent nitrogen and can participate in a Mannich/Pictet-Spengler type of reaction, which attacks a Schiff base generated from tryptamine and an aldehyde or keto acid as shown below.

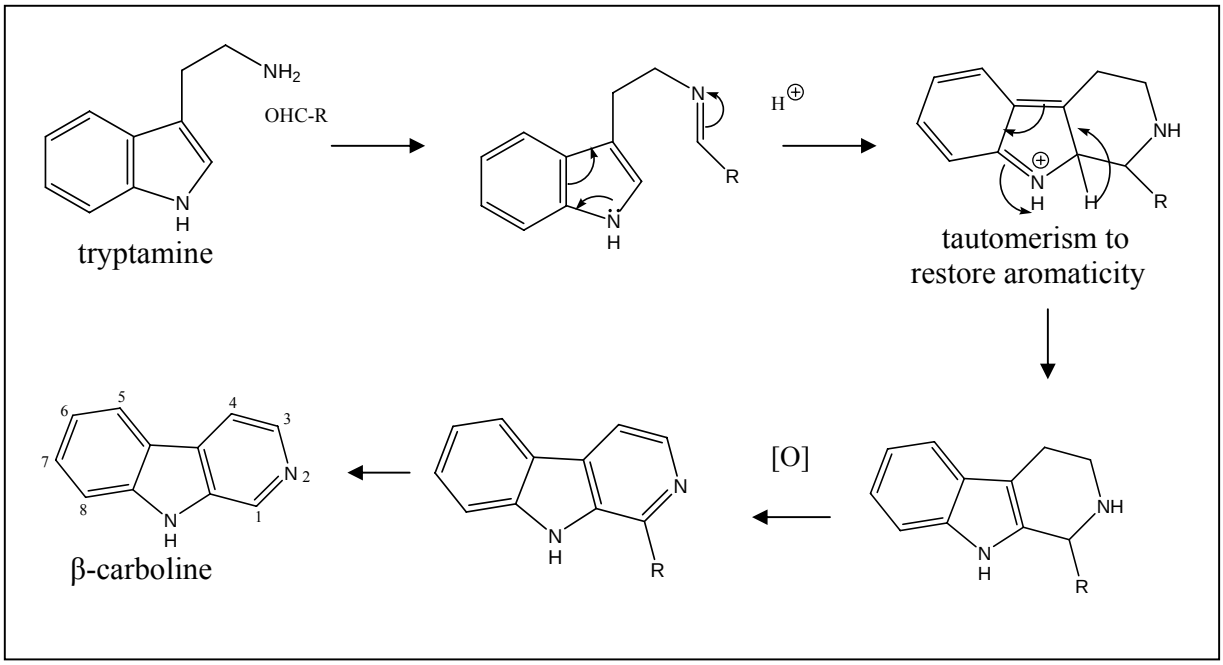
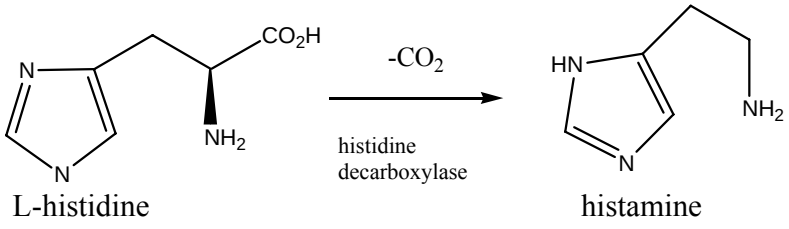


Figure 85 The biosynthetic pathway of simple β-carboline alkaloids (Dewick, 2003)

4.1.3. Alkaloids derived from histidine

The amino acid L-histidine contains an imidazole ring, and is a likely precursor of alkaloids containing this ring system. There are relatively few examples and definite evidences linking them, but it is suggested that L-histidine is converted to histamine by histamine decarboxylase as shown below [Dewick, 2003].



4.2. Brominated secondary metabolites from marine sponges

The most abundant naturally synthesized organohalogens are bromine-containing metabolites. Due to the high bromine concentration in the sea water, these metabolites are preferentially produced by marine invertebrates. Marine invertebrates apparently make use of a facile oxidation of bromide into bromine (or hypobromite), a process called biobromination and the result is an astounding array of organobromine metabolites. Protoporphyrin IX bromoperoxidase is the catalyzing enzyme of the biobromination process and it is found in algae, bacteria, and marine fungi [Van Pée, 1996; Gribble, 1999]. The biobromination process can be explained by a sequence of events leading from natural bromine to organobromine compounds (**Figure 86)**. Enzyme-bound bromine complexes have been isolated from nearly 100 different species of marine algae and phytoplankton, acorn worms, and marine annelids. Chloroperoxidase and other peroxidase also have the ability to oxidize bromide. Examples are enzymes involved in the bromophenol production in acorn worm and in the biomimetic syntheses in *Laurencia* sp. [Gribble, 1999].

Many marine invertebrates, particularly sponges, produce an astonishing array of organobromine metabolites. It should be noticed that the bromo-metabolites isolated in the recent work may actually be biosynthesized by bacteria or micro-algae associated with the sponge.

This study allocates three groups of brominated metabolites isolated from different marine sponges. They consisted of three groups: (i) bromopyrrole alkaloids isolated from *Stylissa flabelliformis* and *Agelas nemoecinata*, (ii) bromophenolic compounds from *Dysidea granulosa*, and (iii) bromotyrosine alkaloids isolated from *Pseudoceratina purpurea*.

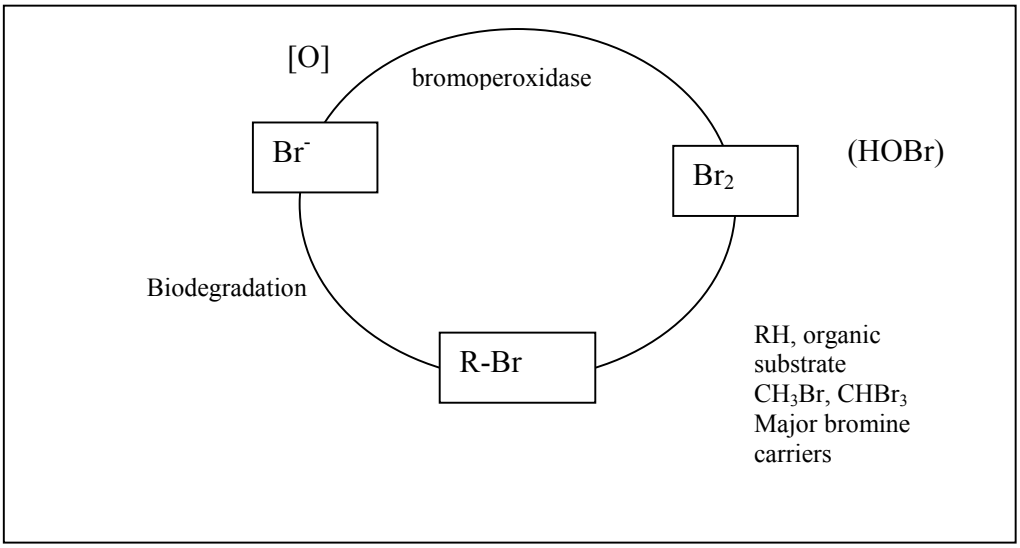


Figure 86 The biobromination process in the marine environment (modified from Gribble, 1999)

4.2.1. Bromo-pyrrole metabolites isolated from the sponge

Stylissa flabelliformis

A number of structurally unique C_{11}-N_5 (fused bicyclic pyrrole azepine) marine metabolites containing brominated or nonbrominated guanidine – based alkaloids with pyrrole moieties have been isolated from the order Axinellidae. In this study, four compounds with the C_{11}-N_5 skeleton such as stevensine (**6**), spongiacidin A (**7**), *E* and *Z* – bromohymine aldisine (**8** and **9,** respectively) have been isolated. Bromoaldisin (**10**) and dibromoaldisin (**11**) were also isolated from the Andaman Sea sponge, *Stylissa flabeliformes*. Marine natural products with the fused bicyclic pyrrole [2,3-*c*]azepin-8-one ring system were reported to either bear a 2-aminoimidazole (AI) or glycocyamidine appendage [Xu, *et al*., 1997; Cimino *et al*., 1982]. Actually, aldisine has been known to be a degradation product of hymenialdisin through an

oxidation process [Schmitz *et al.*, 1985]. Therefore, it was assumed that bromoaldisin should also be derived from hymenialdisin. The conversion between *E* and *Z* debromohymenialdisin was proposed by Eder *et al* (1999). The smooth conversion of (*E*)-debromohymenialdisin into its respective *Z* isomer by *E/Z* isomerization at the C-C double bound at position C_{10} and C_{11} was explained by a push-pull character of the two substituents at this double bond. This is best illustrated by a zwitterionic mesomeric structure as illustrated in **Figure 87**.

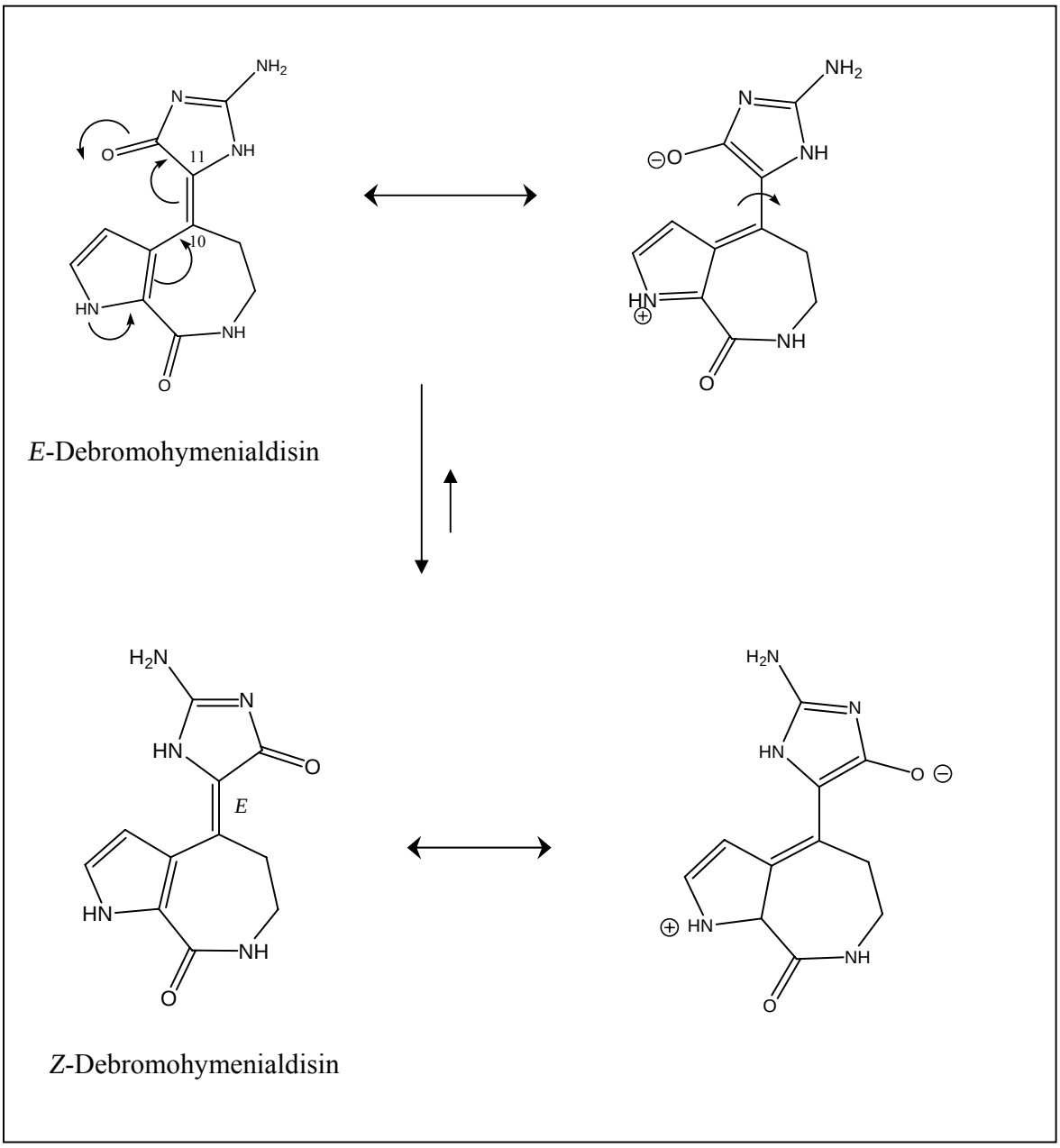


Figure 87 Proposed mechanism of interconversion of *E*-debromohymenialdisin into the *Z* isomer (Eder, *et al*., 1999)

4.2.2. Bromopyrrole alkaloids from the sponge *Agelas nemoecinata*

Marine sponges belonging to the genus *Agelas* produce bromo-pyrrole alkaloids, which are chemically characteristic for this group of sponges. Pyrrole-imidazole alkaloids are interesting because they show various biological activities. Bromo-pyrrole alkaloids were described to be antimicrobial and also to block α-adreno and serotonergic receptors [Nakamura *et al.,* 1984; Cafieri *et al.,* 1996]. They also showed antifouling activity toward barnacle larvae [Fusetani, 2004; Keifer *et al*, 1991; Tsukamoto *et al*., 1996 and Shen *et al*., 1998]. Furthermore, they play an ecological role as feeding deterrents [Assmann *et al.,* 2001]. One example of this group of compounds is oroidin (**compound 20**), which was the first bromo-pyrrole alkaloid reported from the sponge *Agelas* in 1971 [Forenza *et al*.,1971; Faulkner *et al*., 1981]. Common structural features for this group of secondary metabolites included a brominated or nonbrominated pyrrole carboxamide unit connected to a functionalized or unfunctionalized three-membered carbon bridge [Fresnesda, *et al*., 2001]. Examples are tetrahydrofuro[2,3-*d*] imidazolinin-2-one congeners like keramidine, dispacamides [Nakamura and Kobayashi, 1984; Cafieri, *et al*., 1996], slagenins, and their derivatives [Tsuda *et al.,* 1999].

All of the other isolated secondary metabolites in this study are pyrrole alkaloids derived from ornithine where the pyrrolidine ring system is formed initially as a Δ^1-pyrrolinium cation [Samuelson, 2005]. The biogenetic synthesis of this group of compounds involve 3-amino-1(2-aminoimidazolyl)-prop-1-ene and pyrrole-5-carboxylic acid as building blocks. The biosynthesis is catalyzed by enzymatic oxidoreduction, hydrolysis, hydration, and alkylation (Mourabit and Potier, 2001). The variation of this group of secondary metabolites depend upon the substitutions in the pyrrole ring and the oxidation, reduction, or hydrolysis state of the 2-amino-4(5)-vinylimidazole unit. The pyrrole-5-caboxaminde moiety can be mono-, or

dibrominated at positions C-2 or C-3 but the bromination at position C-4 at the imidazole ring has not been reported [Hoffmann and Lindel, 2003; Jin, 2005]. Furthermore, the pyrrol-5-carboxylic acid and its 2- or 3-brominated derivatives are an important building block for this structural group as proposed by Mourabit and Potier in 2001. The four possibilities of 5-carboxylic acid building blocks are shown in **Figure 88**. Therefore, the pyrrole-5- carboxamide portion of all isolated compounds in this study could be derived from these respective 5-carboxylic acid building blocks.

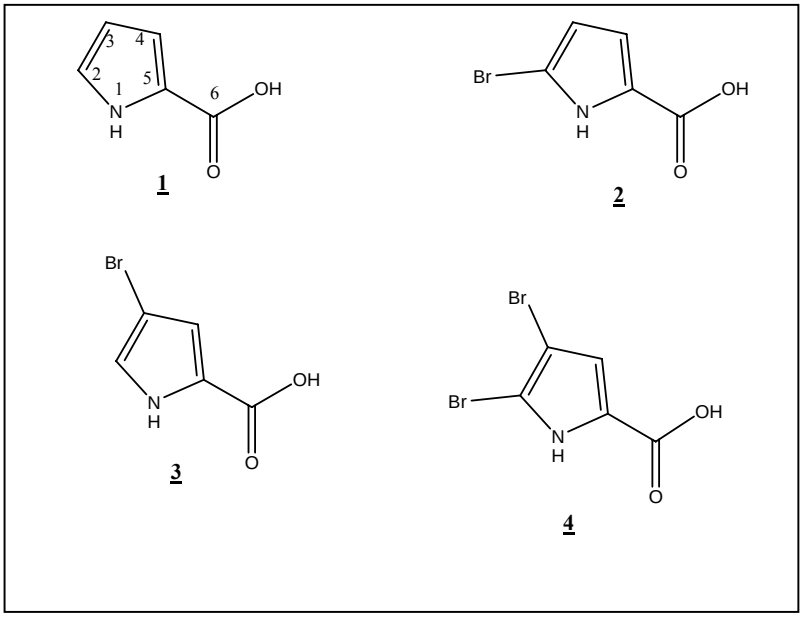
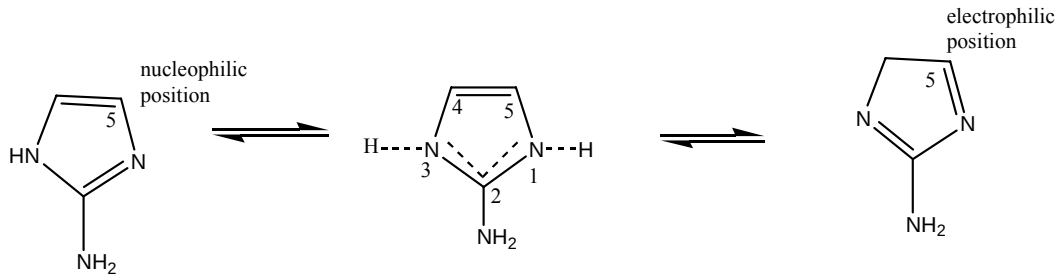
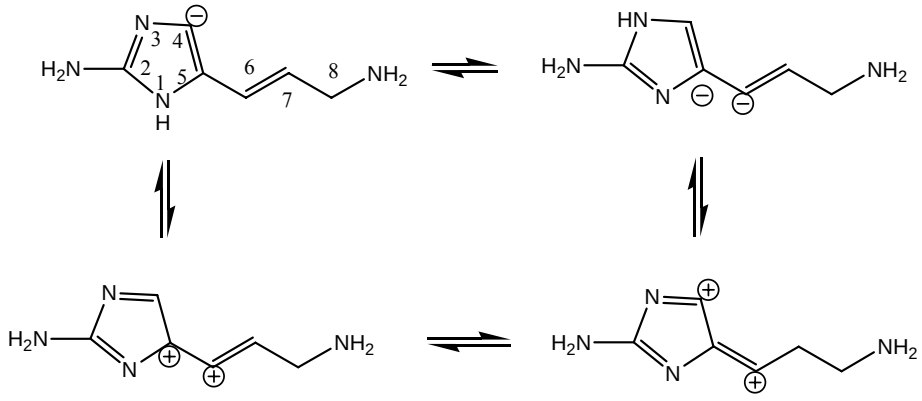


Figure 88 Four pyrrole building blocks proposed by Mourabit and Potier (2001)

The ambivalent reactivity of 2-aminoimidazole is responsible for the molecular diversity observed in this group of alkaloids. The electrophilic or nucleophilic characteristic of position 4 is dependent on the tautomerism occurring in the ring as shown below.



The tautomeric forms and the behavior in the imidazole ring system are probably controlled by the catalytic ability of the host enzyme to exchange protons within the structure [Jin, 2005]. The protonation-deprotonation property of the 2-aminoimidazole ring is undoubtedly crucial for this protonation transfer and this is also exhibited by the vinyl nature of the side chain as illustrated below (Mourabi and Potier, 2001).



4.2.3. Bromophenol metabolites isolated from the sponge *Dysidea granulosa*

Four polybrominated diphenyl ethers were isolated from the Andaman Sea sponge, *Dysidea granulosa*. Bromophenol metabolites are found in several marine sponges, but polybrominated diphenyl ethers are characteristic of the family Dysideidae. There are two chemotypes of the *Dysidea* sponges. One chemotype contains both polychlorinated amino acid derived metabolites and sesquiterpenes while the second chemotype contains only diphenyl ethers. The polychlorinated and brominated diphenyl ether metabolites are produced by filamentous cyanobacterium symbionts associated with the sponge, *Oscillatoria spongeliae*, while terpenes are considered to be true sponge metabolites [Sun *et al*., 1985; Faulkner, *et al*., 1994; Handayani, *et al*., 1997; Cameron *et al*., 2000]. The distribution of terpenes or brominated diphenyl ether metabolites in the genus *Dysidea* is dependent on the algal or bacterial symbionts associated with this sponge which are wide spread and geographically different. For example, the green sponge samples of *D. herbacea* collected from the Pacific Ocean, Palau Island, Indo-Pacific, Western Australia, and Caroline Island contain brominated diphenyl ether as the major metabolites [Capon *et al*., 1981; Fu and Schmitz, 1996; Norton and Wells, 1980; Sharma and Vig, 1972]. On the other hand, some of these sponges devoid of the green coloration which were collected from Papua New Guinea, Great Barrier reef, and Lizard Island yielded terpenes as the major metabolites [Carmeron *et al*., 2000; Clark and Crew, 1995; Norton *et al*., 1981; Horton, *et al*.,1990]. In case of this study, only polybrominated diphenyl ether compounds were found from the green sponge, *D. granulosa* collected from the Andaman Sea. This indicated that those isolated metabolites should be produced by microalgae associated with this sponge. Natural occurrence of this group of compounds is proposed to be a result of a phenolic oxidative coupling mechanism. Bioactivity properties of this group of compounds were described to include inhibition of inosine monophosphate dehydrogenase, guanosine monophosphatase

synthetase, or 15-lipoxygenase [Xu and Schmitz, 1996]. They were also antibacterial toward positive and negative bacteria [Sharma and Vig, 1972] and antifungal against the phytopathogenic fungus *Cladosporium cucumerinum* [Handayani *et al*., 1997].

In this study, compounds **14** and **15** exhibited stronger antimicrobial activity against *B.subtilis* at the concentration of 5µg/ml than compounds **12** and **13.** Antimicrobial activity suggests that these compounds, particularly **14** and **15**, may serve a role in the chemical defense of the sponge against bacterial invasion. Compound **14** has also been reported as feeding deterrent agent to a generalist fish at a low natural concentration [Paul, 1992]. The chemical structures of these compounds differed by the substitution pattern of the bromine function in ring B. Compounds **14** and **15** were bromine-substituted at C-3 and C-5 while compounds **12** and **13** were tri-brominated at either C-3, 4, and 5 or C-4, 5, and 6, respectively. Therefore, it should be noted that the differences in the bromine substitution in ring B can contribute to the biological activity of this group of compounds.

4.2.3.1. Phenolic oxidative coupling mechanisms

Many natural products are produced by the coupling of two or more phenolic ring systems in a process readily rationalized by means of free radical reactions. The reaction can be brought about by oxidase enzymes known to be radical generators, which includ peroxidases and laccases. Other enzymes catalyzing phenolic oxidative coupling have been characterized as cytochrome P-450 dependent proteins, which require NADPH and O_2 as cofactors, but no oxygen is incorporated into the substrate. Oxidation of a phenol results in a free radical and the unpaired electron can then be delocalized via resonance forms in which the free electron is dispersed to position *ortho* and *para* to the original oxygen function. Coupling of two of these mesomeric structures gives a range of dimeric systems as exemplified in **Figure 89**. The final

products are then derivatized by enolization, which then restores the aromaticity in the ring. Thus, carbon-carbon bonds or ether linkages involving positions *ortho* or *para* to the original phenols may be formed. The reactive dienone system formed as intermediate may in some case be attacked by other nucleophilic groups, extending the range of structures ultimately derived from the basic reaction sequence [Dewick, 2003].

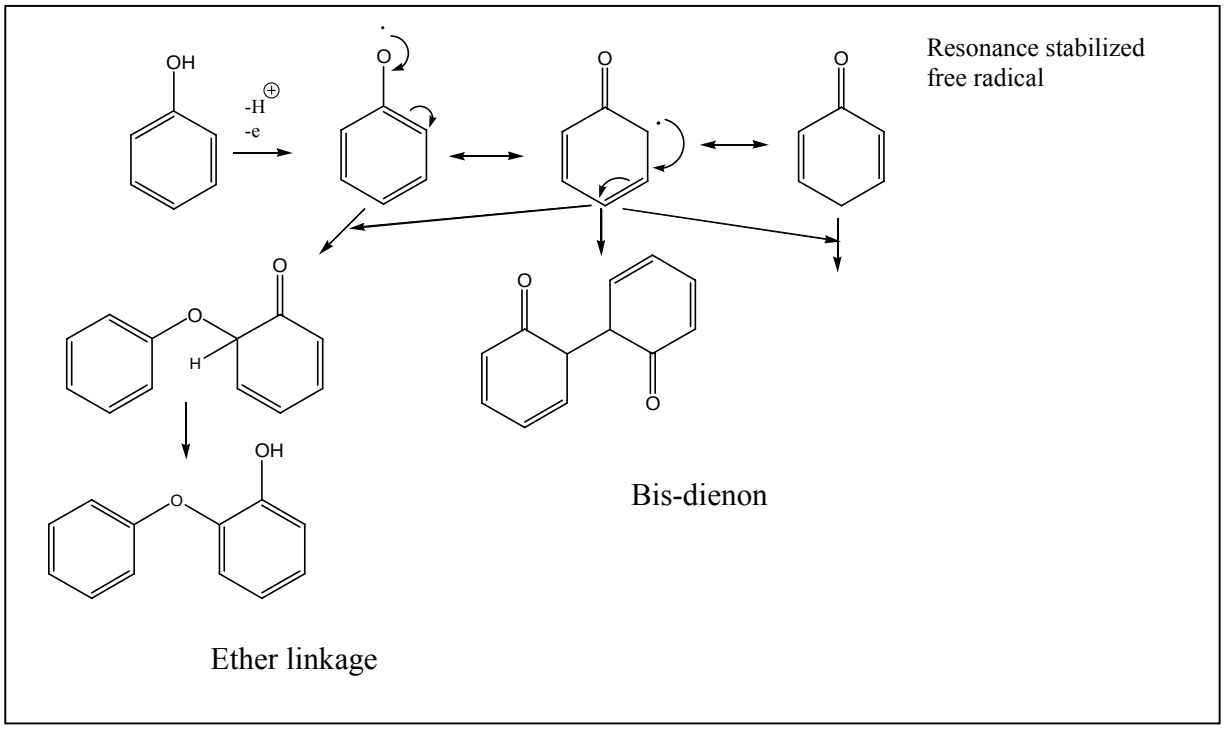


Figure 89 Phenolic oxidative coupling mechanisms (modified from Dewick, 2003)

4.2.4. Brominated tyrosine alkaloids from the sponge

Pseudoceratina purpurea

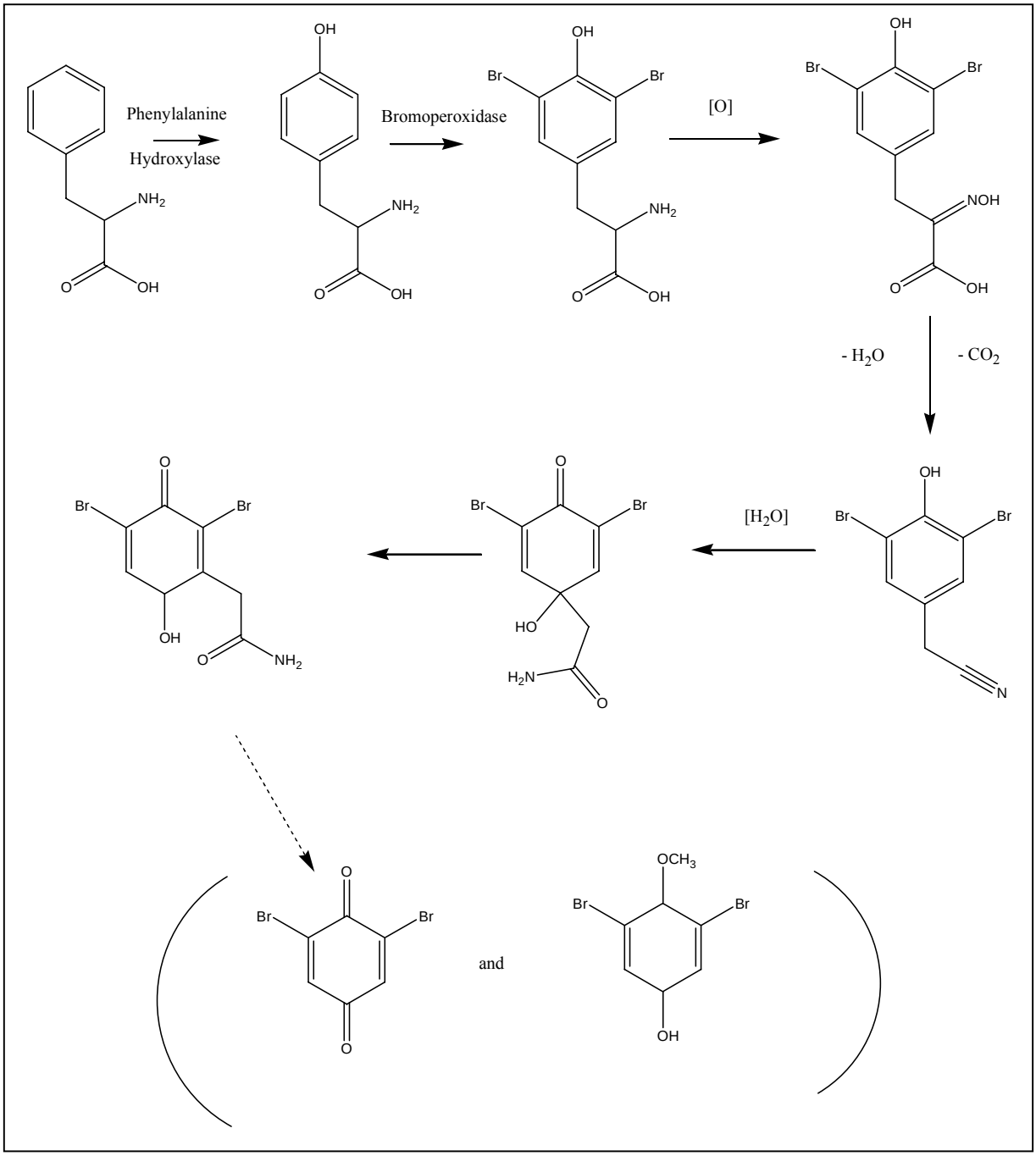
The sponges of the genus *Pseudoceratina* belong to the order Verongida (family Aplysinellidae). Secondary metabolites of Verongida are characterized by typical brominated compounds which are biogenetically related to tyrosine. All the species belonging to this order, so far examined, have been shown to contain remarkable quantities of such metabolites. The structures of these metabolites, in most cases, comprise a unique dibromo-cyclohexadienyl-dihydroisoxazole moiety [Ciminiello *et al.,* 1996]. Isolated metabolites from this order have proven to be valuable chemotaxonomic markers and show various biologically activities such as antihistaminic, cytotoxic, and antimicrobial activities [Sakai *et al.*, 2002].

Because Verongid sponges have a high assemblage of symbiotic bacteria, it is possible that these symbiotic bacteria should synthesize the metabolites found in this sponge. But the presence of brominated metabolites located within the sponge tissue should be circumstantial evidence that no brominated materials are associated with bacterial cells [Faulkner, 1994]

4.2.4.1 Biosynthesis of dibromotyrosine derivatives

The isolated major metabolite from *Pseudoceratina purpurea* (compound **24**) is a derivative of aeroplysinin-1. It is related to *O*-methyltyrosine where both phenylalanine and tyrosine are shown to be biosynthetic precursors. This implies the ready conversion of phenylalanine to tyrosine in the sponge [Rinehart and Carney, 1995; Rinehart and Tymiak, 1981]. The biosynthetic pathway of this group of metabolites was proposed in 1981 by Rinehart and Tymiak (**Figure 90)**. However, typical secondary metabolites produced by the order Verongida are the simple small molecules such as areoplysinin-1 and dienone which are related

to the isolated compound. They are biotransformation products originating from higher molecular weight precursors as for instance isofistularin and aerophobin-2 [Teeyapant, 1994].



Scheme 90 Biosynthetic approach of dibromotyrosine compounds from Verongid sponges (Rinehart and Tymiak, 1981)

4.3. Napthyridine derive alkaloids from the sponge *Aaptos suberitoides*

Two compounds containing the *1H*-benzo[*de*]1,6- naphthyridine ring system, **aaptamine** and **demethylaaptamine**, were isolated from *A. suberitoides*. The sponge genus *Aaptos* was previously reported as a source of aaptamine and several other derivatives [Nakamura, Kobayashi and Ohizumi, 1987; Kazman and Rudi, 1993]. This group of compounds exhibit strong α-adrenoceptor blocking activity, cytotoxicity against many cell lines, antimicrobial, and antioxidation activities [Nakamura, Kobayashi and Ohizumi, 1982; Shen, Lin, Sheu and Duh, 1999; Takamatsu, *et al*., 2003]. Recently, aaptamine from the sponge *Aaptos aaptos* was launched in the chemical market as a potent anti-tumor agent by A.G. Scientific, Inc. [A.G. Scientific, 2004]. Aaptamine (**17**) showed both a potent cytoxicity and a potent anti-microbial activity against *Bacillus subtilis* (see **3.4.4**). Based on the chemical resemblance to quinoline, quinazoline, and naphthyridine, it could be assumed that this group of marine metabolites should be derived from anthranilic acid.

4.3.1 Biosynthesis of alkaloids derived from anthranilic acid

Anthranilic acid (2-aminobenzoic acid) is another shikimate-derived compound. CoA ester anthraniloyl-CoA is the precursor unit for the malonated chain extension. Aromatization of the acetate-derived portion then leads to quinoline or arcridine alkaloids, depending on the number of acetate units incorporated. There are many examples of which anthralinic acid itself functions as an alkaloid precursor as found in quinazoline and quinoline showing in **Figure 91** [Dewick, 2003].

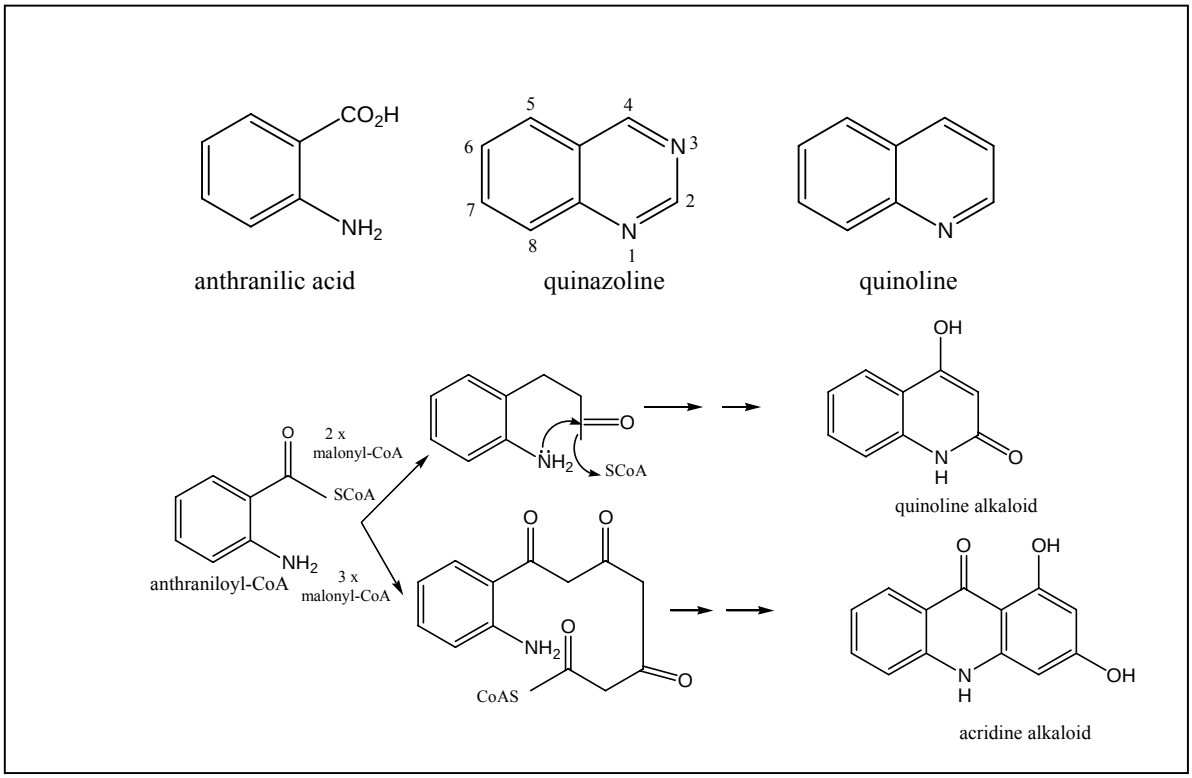


Figure 91 The pathway of alkaloids derived from anthranilic acid. (Dewick, 2003)

4.3.2. Structural activity relationship (SAR) of aaptamine and their derivatives

The activity of this group of compounds is dependent on the substitutions at positions C-9 and N-4. Methoxy substituents at C-9 and N-4 caused a decrease in cytotoxicity as found in 4-*N*-acethyldihydroaaptamine. On the other hand, a hydroxyl or a carbonyl substituent at C-9 increases the cytotoxicity as exemplified by demethyl(oxy)aaptamine [Shen *at al*., 1999]. In case of this study, aaptamine (**16**) exhibited higher cytotoxicity and antibacterial activity against *B. subtilis* than demethylaaptamine (**17**). Therefore, it should be noted that the methoxy substituent at C-9 may increase the biological activity.

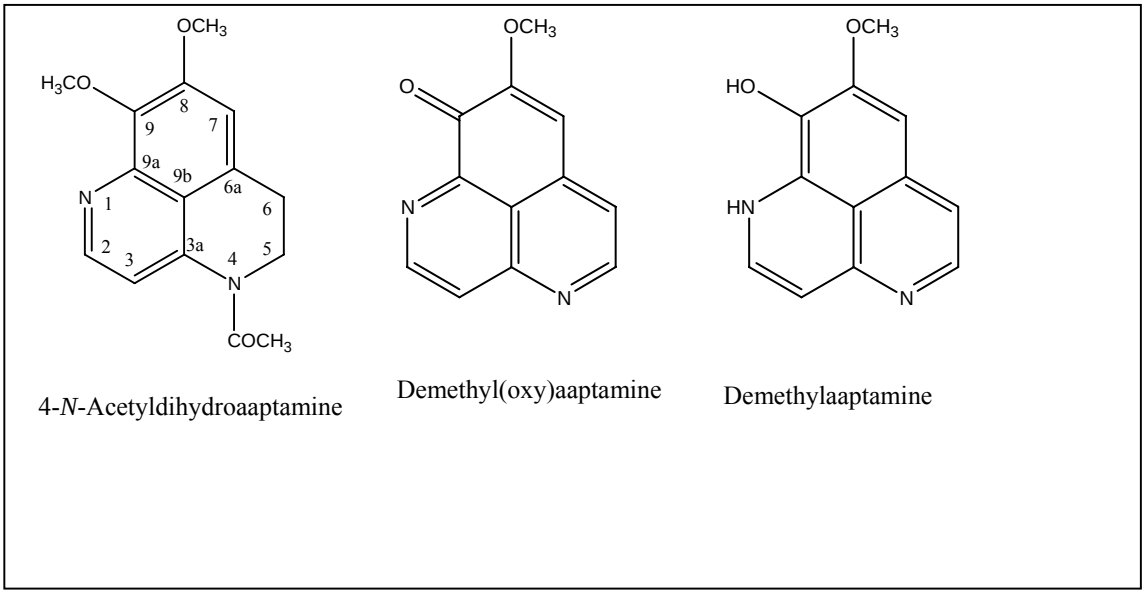


Figure 92 Some aaptamine derivatives.

4.4. Diphenyl ether metabolites isolated from a mangrove fungus, *Eurotium clevariere*

This study is the first report for the chemical investigation of the genus *Eurotium*. Two diphenyl ethers were isolated from the mangrove fungus *Eurotium clevariere*. This group of metabolites is one of the major metabolites found in the genus *Aspergillus* [Hamasaki *et al*.,1980; Furukawa, *et al*., 1972]. They show biological activity against gram-positive bacteria [Koenig, *et al*., 1980]. Naturally occurrence of diphenyl ether, perhaps, originates from a phenolic oxidative coupling mechanism as explained in section **4.2.3.**

5. Summary

Secondary metabolites produced by marine invertebrates are novel and have diverse chemical structures. They are important as new potential drug candidates and/or used as prototype structures for apply to different pharmacological and clinical purposes (see **Table 2** and **3**). This study involved the isolation, structure elucidation, and biological screening of pharmacologically active metabolites. The isolation of these metabolites was accomplished using various chromatographic techniques. Structures were elucidated through MS and NMR spectroscopy. Biological screening was done on the basis of antimicrobial activity and cytotoxicity.

5.1. Metabolites isolated from the Andaman sea sponges

5.1.1. *Dragmacidon* sp.

The sponge *Dragmacidon* sp. was extracted with aqueous methanol. The extract was partitioned with ethyl acetate which was then subjected to Sephadex LH-20 column chromatography and using methanol as eluent. Interesting fractions were analyzed through Dionex HPLC coupled to a DAD detector and by LCMS. Purification was done through RP-18 reversed phased column chromatography using different ratios of methanol and water as eluents. All isolated metabolites have been purified by means of preparative HPLC on a RP-18 column with 0.1% trifuoroacetic acid (TFA) in nanopure water and methanol. Indole alkaloids were isolated from this sponge which included of three known indole carboxylic acid derivatives (**3-5**) and two new β-carboline alkaloids (**1** and **2**) which exhibited moderate cytotoxicity.

5.1.2. *Stylissa flabelliformis*

The clued extract of this sponge specimen was fractionated by vacuum liquid chromatography on silica gel using a step gradient elution starting from cyclohexane, dichloromethane, and methanol. The fractions which showed interesting LCMS and HPLC chromatograms were subjected to RP-18 column chromatography with mixture of methanol and H_2O as eluents. The HPLC and LCMS chromatograms guided the purification of five known bromopyrrole alkaloids (**6** – **10**) and one new dibromoaldisine derivative (**11**).

5.1.3. *Dysidea granulosa*

The *D. granulosa* sponge sample was macerated in aqueous methanol, then extracted with ethyl acetate. The resulting extract was fractionated using hexane and dichloromethane through step gradient elution on silica gel. Four brominated diphenylethers were isolated from this sponge. 3,4,5,-tribromo-2-(2,4-dibromo-phenoxy)phenol **(12)** was purified by normal phase chromatography using hexane and dichloromethane as eluents. Compounds **13** -**15** were purified by semi-preparative HPLC. Each of these compounds showed antimicrobial activity against *Bacillus subtilis* but 1,5,6-tribromo-2-(2,4-dibromo-phenoxy)phenol **(14)** and 3,5-dibromo-2-(4-bromo-2-methoxy)phenol **(15)** showed highest activity at a concentration of 100 μg exhibiting 9 mm and 10 mm zones of inhibition, respectively.

5.2. Metabolites isolated from marine sponges collecting from Indonesia

5.2.1. *Aaptos suberitoides*

The freeze-dried sponge sample was extracted with different organic solvents; hexane, dichloromethane, and methanol, respectively. The dichloromethane extract was subjected to RP-18 reversed phase column chromatography with gradient elution starting with MeOH : H_2O 7:3 and increasing to 100 % MeOH. Purification of the compounds was monitored by HPLC. Compound **16** (aaptamine) and **17** (8-methoxy-1*H-1*-aza-phenalen-9-ol) were further purified by preparative HPLC using MeOH and H_2O with 0.1% TFA (47:53, v/v) as the eluent. Both compounds showed antimicrobial activity against *B. subtilis* and cytotoxicity towards L5178Y mouse lymphoma cells, Hela and PC-12 cell lines.

5.2.2. *Agelas nemoecinata*

The sponge sample was macerated in MeOH and extracted with hexane. The MeOH extract was subjected to RP-18 reversed phase column chromatography using 45 % MeOH in H_2O as eluent. Monitoring was done by HPLC-DAD. The isolated compounds were further purified by RP-18 semi-preparative HPLC eluted isocratically with 30 % MeOH and 70 % H_2O with 0.1 % TFA. Six compounds were isolated. All isolated compounds are new except for compounds **20** (oroidin), **22** (cyclooroidin) and **23** (keramidine). Unfortunately, these compound proved to be unstable.

5.2.3. *Pseudoceratina purpurea*

The sponge sample was extracted with MeOH and subjected to a Sephadex LH-20 column which was eluted with MeOH : CH_2Cl_2 (10 : 0.5, v/v). The major fraction was further purified by Si-gel normal phase chromatography and eluted with MeOH : CH_2Cl_2 (10 :1, v/v) with NH_4OH (0.1%), affording a new 3-(3,5-dibromo-1,6-dihydroxy-4-methoxy-cyclohexa-2,4-dienyl)-2-imino-propionic acid **(24).**

5.3. Metabolite isolated from mangrove fungus *Eurotium clevalieri*

The fungus was isolated from the mangrove soil and enriched in a marine broth medium which was extracted with EtOAc. The EtOAc extract was subjected to HP-20 and eluted with 20 % MeOH in H_2O. Compound **26** (2-hydroxy-4-(3-hydroxy-5-methyl-phenoxy)-6-methyl-benzoic) acid was isolated through Sephadex LH-20 chromatography and eluted with MeOH. Compound **25** (2-hydroxy-4-(3-hydroxy-5-methyl-phenoxy)-6-methyl-benzoic acid methyl ether was finally isolated by semi-preparative HPLC eluted with 25 % MeOH. Both compounds **25** and **26** were reported to be active against gram positive bacteria [Yamamoto *et al.*, 1972].

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