Isolation and Structure Elucidation of Bioactive Secondary
Metabolites from Marine Sponges

(Isolierung und strukturelle Identifizierung von biologisch
aktiven Naturstoffen aus marinen Schwämmen)

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Vorgelegt von

Wafaa Hassan

aus Sharkia, Ägypten

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Erklärung

Hiermit erkläre ich ehrenwörtlich, daß ich die vorliegende Dissertation (Isolierung und strukturelle Identifizierung von biologisch aktiven Naturstoffen aus marinen Schwämmen) selbständig angefertigt und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt habe. Ich habe diese Dissertation in gleicher oder ähnlicher Form in keinem anderen Prüfungsverfahren vorgelegt.

Düsseldorf,   06.11.2003

Wafaa Hassan

Acknowledgements

To my Family

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Zusammenfassung

Im Rahmen der Suche nach bioaktiven Verbindungen aus marinen Schwämmen wurden in dieser Arbeit insgesamt 35 Sekundärstoffe isoliert, wobei es sich bei dreizehn um neue Verbindungen handelt. Die Schwämme wurden in Indonesien und bei der Insel Elba gesammelt.

Für die Entdeckung neuer bioaktiver Substanzen wurde eine Kombination von biologischen Methoden angewendet: Fischfütterungversuche, Untersuchungen mit gram-positiven und gram-negativen Bakterien.

1. *Leucetta chagosensis*

Nach Extraktion des Schwammes *Leucetta chagosensis* konnten sechs verschiedene Imidazol-Alkaloide isoliert und identifiziert werden. Drei der aufgefundenen Extraktkomponenten, Naamine A (bereits zuvor bekannt), das Naamine F (neue Substanz) und Naamine G (ebenfalls neu) gehören der Gruppe der Naamine an. Bei den übrigen isolierten neuen Komponenten Kealiinine A, B und C handelt es sich um mit dem Kealiiquinon verwandte Stoffe.

Naamine G erwies sich als cytotoxisch aktiv gegen Maus-Lymphom-Zellen (L5178Y) und das menschliche Cervix-Karziniom (HeLa). Naamine F zeigte darüber hinaus eine starke fungizide Aktivität gegen *Cladosporium herbarum*. Eine Konzentration der Substanz von 100 µg/ Disk verursachte in diesem Assay einen Hemmhof von 20 mm Durchmesser.

2. *Axinyssa aplysinoides*

Aus dem Schwamm *Axinyssa aplysinoides* wurden vier bekannte Substanzen isoliert. Hierbei handelt es sich um ein Sesquiterpen-Isothiocyanat (Epipolasin A), zwei Bisabolen-Sesquiterpene (6*R*,7*S*)-7-Amino-7,8-dihydro-α-bisabolene Harnstoff, (6*R*,7*S*)-7-Amino-7,8-dihydro-α-bisabolene hydrochlorid und das Phenol [*E*-(Hydroxystyryl) trimethyl ammonium chlorid]. Letztere Substanz zeigte bei einer Konzentration von 100 µg/ Disk eine starke fungizide Aktivität gegen *C. herbarum*.

(6*R*,7*S*)-7-Amino-7,8-dihydro-α-bisabolene hydrochlorid erwies sich als stark cytotoxisch aktiv gegen über Maus-Lymphom-Zellen, L5178Y, (HK_{50}= 2.2 µg/ml, dem menschliche Cervix-Karziniom, HeLa Zellen, (HK_{50} = 0.3 µg/ml), und PC12 Zellen (HK_{50} = 2.14 µg/ml).

3. *Hamigera hamigera*

Aus dem Extrakt von *Hamigera hamigera* wurden acht Substanzen gewonnnen. Vier davon, Hamigeroxalamensäure, Hamigeramine, Hamiguanasinol und Hamigeramide erwiesen sich als neu. Vier weitere, Indole-3-carboxaldehyde und drei Steroide waren bereits bekannt. Hamigunansinol zeigte eine schwache antibakterielle Aktivität gegen *B. subtilis*.

4. *Axinella damicornis*

Die Extraktion von *Axinella damicornis* erbrachte eine Reihe von Bromopyrrol-Alkaloiden, von denen vier, 3,4-Dibromopyrrol-2-carbamid, 3,4-Dibromopyrrol-2-Carbonsaüre, 3-Bromoaldisin und 2,3-Dibromoaldisin, neue Naturstoffe sind. Darüber hinaus wurden dreizehn bekannte Substanzen (2-Bromoaldisin, 4,5-Dibromopyrrol-2-carbamid, Dibromocantharelline, Oroidin, 4,5-Dibromopyrrol-2-Carbonsaüre, Manzacidin A, Z-Hymenialdisin, Longamide, Hymenidin, Stevensin, Hymenin, 4-Bromopyrrol-2-carbamid, und 4,5-Dibromopyrrol-2-methylcarboxylat) aus diesem Extrakt isoliert.

Mit Hilfe eines Fischfütterungsversuches konnte gezeigt werden, daß die beiden Extraktkomponenten 4,5-Dibromopyrrol-2-Carbonsäure und Oroidin verantwortlich für die fraßhemmende Wirkung des Rohextraktes von *A. damicornis* sind. Damit spielen diese beiden Substanzen eine wesentliche Rolle beim Fraßschutz des Schwammes gegen räuberische Fische.

1. Introduction

1.1. The significance of the study

A large number of today's drugs have been developed from natural sources. In the last decade the research in the field of marine natural products has increased. Due to the oceans, which cover almost 75% of the earth surface (Fig. 1) (George and George, 1979), the marine world represents an attractive source for the search of new natural products which could be used as candidates for new drugs (McConnell *et al*, 1994).

Prior to 1995, a total of 6,500 marine natural products have been isolated and by Database 2003 this figure had risen to approximately more than 14,800 (Marinlit, 2003). When compared with the 150,000 (DNP, 2000) natural products obtained from terrestrial plants, this is a relatively small number of compounds, but an increase of 100% over a period of few years clearly represents an explosion of interest in natural products obtained from the marine environment. The discovery of prostaglandins, which are mediators involved in inflammatory diseases, fever, and pain, from the coral reef *Plexaura homomalla* directed the search for drugs discovery from the sea (Weinheimer and Spraggins*,* 1969), and from this time more than 10,000 marine natural products have been isolated from marine organisms. The major research effort has centred on sessile or slow moving organisms or invertebrates such as sponges, coelenterates, ascidians, molluscs and bryozoans. Many of the members of these phyla are brightly coloured and the absence of the spines or protective shell means that they require an effective self-defence. It is therefore not surprising that many of them produce unusual biologically active natural products, as a means of defence (Whitehead, 1999).

The sponges are the source of the greatest diversity of marine natural products. About one-third of all marine natural products have been isolated from sponges, which make them currently the most popular source of novel compounds (Whitehead, 1999). The marine sponges are considered not only as a very important source of new natural products but also a source for bioactive compounds. These compounds are interesting

candidates for new drugs, primarily in the area of cancer, anti-inflammatory and analgesic (Proksch *et al*, 2002).
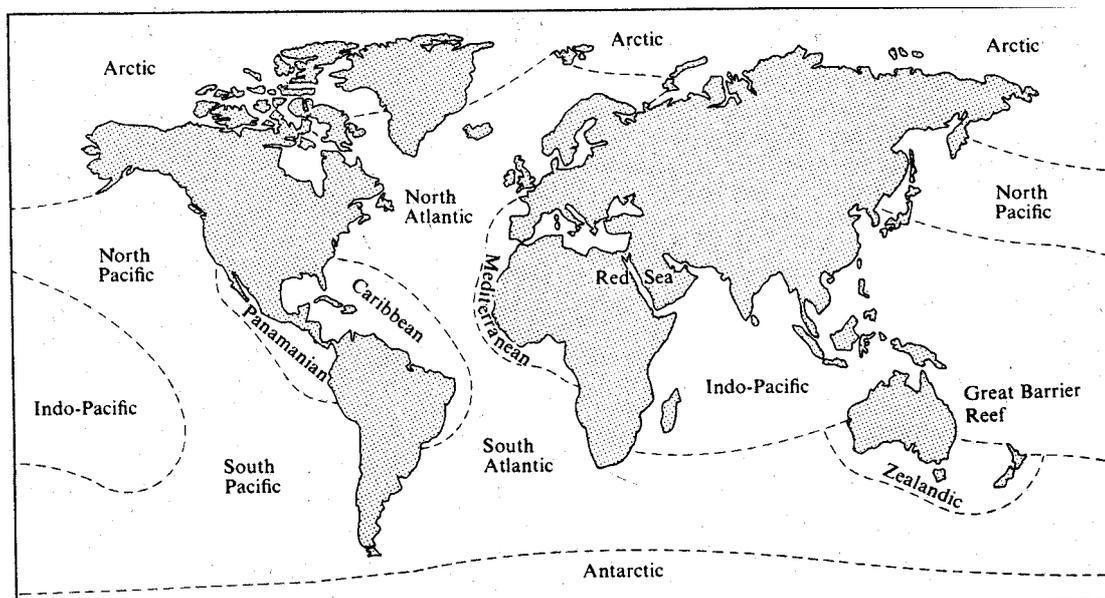
Fig. 1.1 :   World map showing the main marine zoo-geographic regions

1.1.1. The biological importance of marine natural products

1.1.1.1. Antiviral and antitumour marine natural products

The sea is considered as the most likely source of antiviral and anticancer drugs. Both antiviral and anticancer drugs are cell-growth inhibitory substances, which act selectively without damage of the cells of host organisms. Cancer is the second leading cause of death in the first world, but, it is estimated that 60% of all illness in the developed countries is a consequence of viral infections. The leading work of Bergmann and Feeny in 1951, which reports the presence of the unusual arabinosyl nucleoside,   spongothymidine, spongosine and sponguridine from the sponge *Cryptoptethya crypta* (Bergmann and Feeney, 1951, Bergmann and Burke 1955, and Bergmann *et al*, 1957), have provided the lead compounds for the development of the therapeutically used **Ara-C** for treatment of leukaemia, in addition to the virustatic agent **Ara-A** (Vidarabin), which was introduced to the market and used therapeutically against *Herpes encephalitis* since the late of 1970 (Miller *et al*, 1968 and Whitley *et al*, 1977).

Variabilin (sesterterpene tetronic acid) was in 1973 isolated by Faulkner in a high yield from the sponge *Ircinia variabilis* (Faulkner, 1973), and from other sponges of genus *Ircinia* (Rothberg and Shubiak, 1975, Kazlauskas *et al*, 1976, and Gonzalez *et al*, 1983). The initial reports of its antibiotic properties have been extended to include cytotoxicity and variabilin is also an effective antiviral agent.

Eudistomins (Fig .1.3) were in 1981 isolated from colonial tunicate *Eudistoma olivaceum* (Rinehart *et al*, 1981). There are four distinct structural categories of eudistomins. The oxathiazepine containing eudistomins possess a pronounced antiviral activity toward the DNA virus HSV-1.

Didemnins, A, B and C (Fig. 3) are cyclic depsipeptides and have been isolated from a tunicate of the *Trididemnum* genus (family *Didemnidae*). The didemnins inhibit the growth of both RNA and DNA viruses and are highly cytotoxic to L1210 leukaemic cells and P-388 leukaemia (Martin *et al*, 1986). Didemnin A inhibits *Coxackie* A21 virus and *Herpes simplex* virus. Didemnin B shows cytotoxicity to L1210, P-388 leukaemic cells and B-16 melanoma (Rinehart *et al*, 1981).

Bryostatins are a group of macrocyclic polyether lactones isolated from the bryozoan *Bugula neritina*. This group of compounds are extremely potent against P-388 mouse leukaemia and human ovarian sarcoma in vivo (Pettit *et al,* 1982 and 1993).

1.1.1.2. Protein kinase inhibition activity of marine natural products

Kinases are ATP-dependent enzymes that add phosphate groups to proteins. Protein phosphorylation is the key regulatory mechanism that is utilised to regulate the activity of enzymes and transcription factors (Meijer *et al*, 2000). Therefore protein-kinases play an essential role in virtually all cellular processes and are involved in most diseases. Hymenialdisine is a marine constituent which has been isolated from many marine sponges (Pettit *et al*, 1990 and Williams and Faulkner, 1996). It is a potent inhibitor of cycline-dependent kinase, glycogen synthase kinase-3β and casein kinase 1. Hymenialdisine could also be used in the treatment of neuro-

degenerative disorders which cause many diseases such as Alzheimer′s disease (Meijer *et al*, 2000).

1.1.1.3. Antimalarial marine natural products

In the last two decades, malaria has been considered as a great threat to humans. It is estimated that 1.5 billion persons are living in regions where malaria is endemic (Fig. 1.2, Rang, *et al*, 1999) and that 1.5 million persons die from this disease annually. Malaria is caused by the most dangerous parasite *Plasmodium falciparum* which is transmitted through the bite of the mosquito *Anopheles*. Since it is almost impossible to eliminate the vector of transmission, there will always be a need of new anti-plasmodium drugs (Wright and König, 1996).

The marine organisms are considered as a source for some antimalarial drugs. Axisonitrile-3, a bicyclic-spirosesquiterpene, has been isolated from the sponge *Acanthella klethra* and is a potent *in vitro* anti-plasmodium agent (Angerhofer and Pezzuto, 1992). The isocycloamphilectanes are an important class of compounds with pronounced in vitro anti-plasmodium activity, that rivals the results obtained with some clinically used anti-plasmodium drugs (chloroquine and quinine), an example of this class is the diisocyanadociane from *Adocia* sponges (König and Wright, 1996).
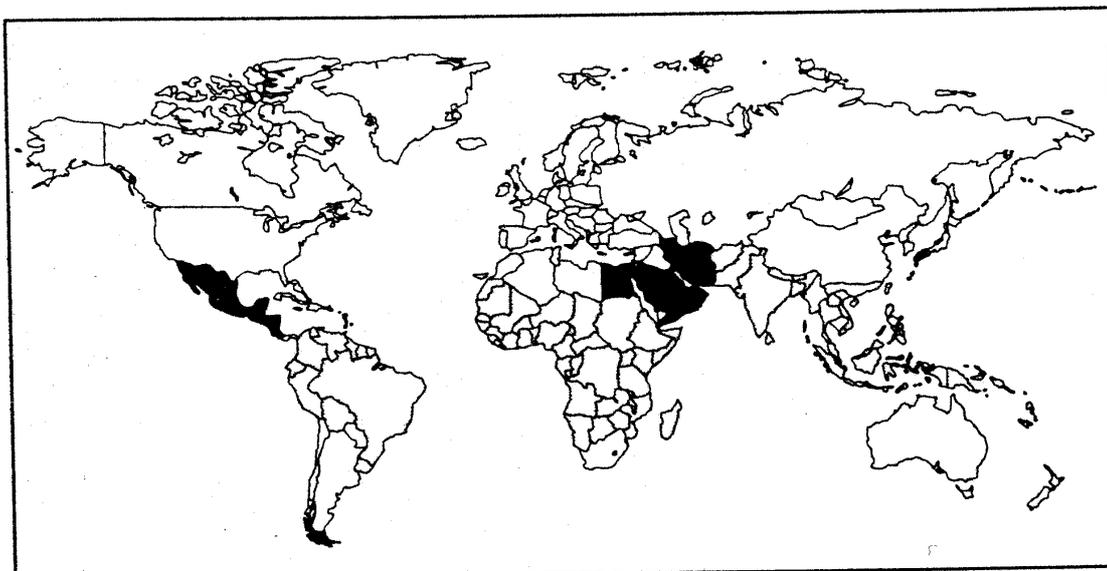
Fig. 1.2: Geographic distribution of malaria (Rang *et al*, 1999)

Spongouridine　　**Spongothymidine**　　**Spongosine**

Name	R	R′	R′′	R′′′
C	H	OH	Br	H
E	Br	OH	H	H
F	H	OH	Br	C$_2$H$_3$O
K	H	H	Br	H
L	H	Br	H	H

Eudistomins

Didemnin-B

Variabilin

Bryostatin-1

Fig. 1.3 : Antiviral and cytotoxic marine natural products
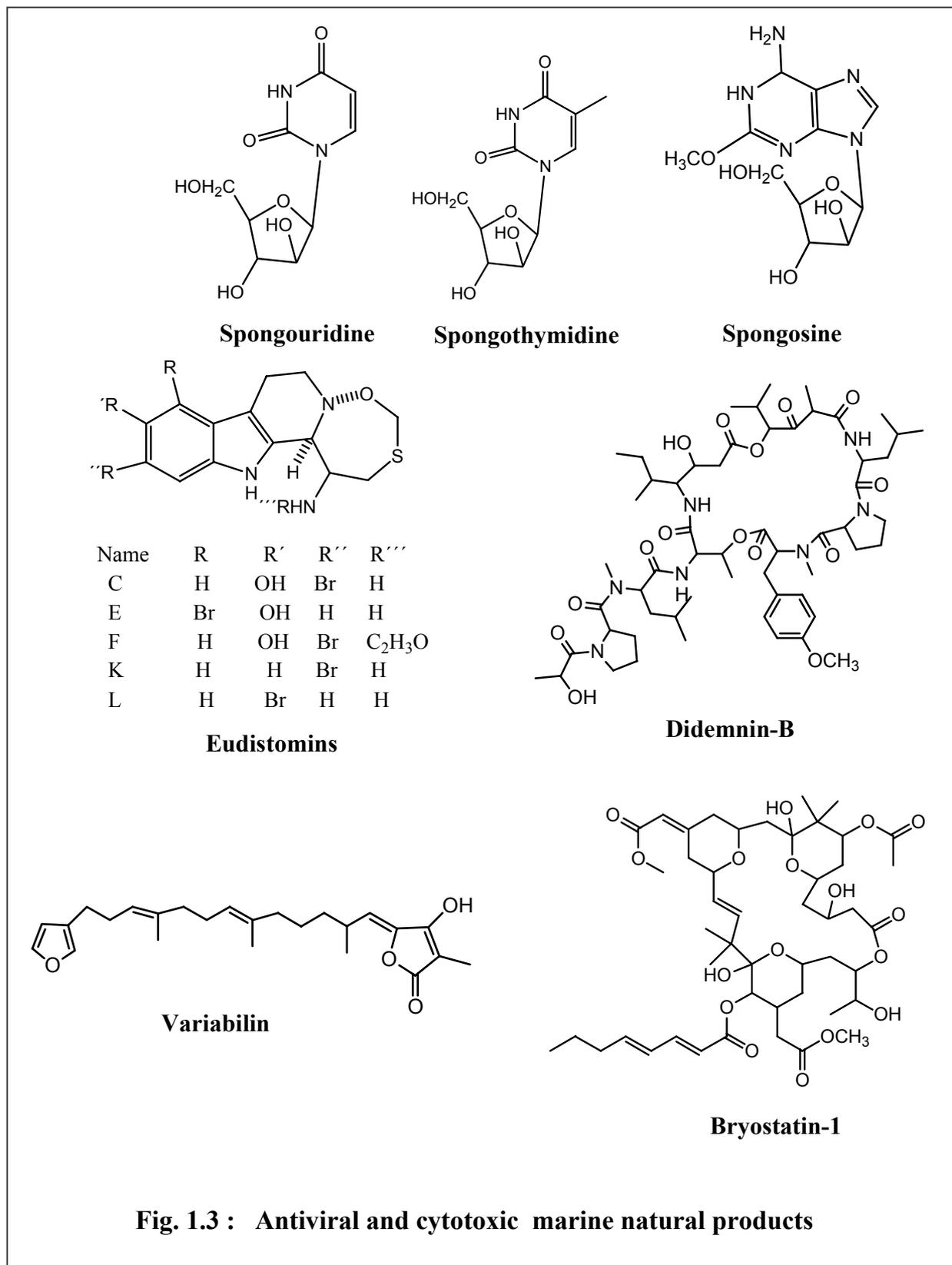
1.1.1.4. Anthelmintic activity of marine natural products

Chemotherapy is important in the treatment of parasitic infections. The search for new anthelmintic agents is necessary for medicinal consideration. Some marine metabolites exhibit anthelmintic activity, such as the indole alkaloids chondriamide A, C, indole-3-acrylamide, and the trimethylderivative of chondriamide A (Fig.1.5). These were isolated from the red alga *Chondria atropurprrea* and exhibit anthelmintic activity against *Nippostrongylus brasiliensis* (Davyt *et al*, 1998).

1.1.1.5. Reversing multidrug resistance (MDR) activity

Multidrug resistance (MDR) in tumour cells has been recognised as a major obstacle to successful cancer chemotherapy. Overexpression of certain membrane glycoproteins has been observed in MDR tumour cell lines. The substance which inhibits the action of those membrane glycoprotein would have high possibility for solving the MDR problems in cancer chemotherapy. Agosterol A (Fig. 1.5) completely reversed the resistance to colchicine in KB-C2 cells and also the resistance to vincristine in KB-CV60 cells. Agosterol A is a polyhdroxylated sterol acetate and was isolated from a marine sponge of genus *Spongia*. It reverses MDR caused by over expression of multidrug resistance associated protein (MRP). It may be a pharmaceutical candidate for reversing MDR (Aoki *et al*, 1998).

1.1.1.6. Immunosuppressive activity

There are also other marine natural products which show in vitro immuno-suppressive activity such as 4α-methyl-5α-cholest-8-en-3β-ol and 4,5-dibromopyrrole-2-carboxylic acid. Both compounds were highly active in suppression of murine splencytes in the two-way mixed lymphocyte reaction (MLR) with little or no demonstrable cytotoxicity. Both compounds were isolated from the marine sponge *Agelas flabelliformis* and could be useful in organ transplantations (Gunasekera *et al*, 1989).

1.2. The importance of marine natural products in agriculture

Marine natural products are not only a useful source for the development of new drugs but also represent a source for new insecticides, 40 active compounds have

been reported (El Sayed *et al,* 1997). The most important one is jaspamide from *Jaspis* sponges (Zabriske *et al,* 1986) and halichondramide from the sponge genus *Halichondria* (El Sayed *et al,* 1997). Both are active against tobacco bud worm *Heliothis viriscens*. Laurepinnacin isolated from the red algae *Laurecia pinnata* (Fukusawa and Masamume, 1981) and calyculins E and F from the sponge *Discodermia* sp. show activity against the mosquito larvae *C. pipiens pallens* (Okada *et al,* 1991).

Laurapinnacin

Jaspamide

Halichondriamide

Fig. 1.4: Some marine natural products with potential uses in agriculture
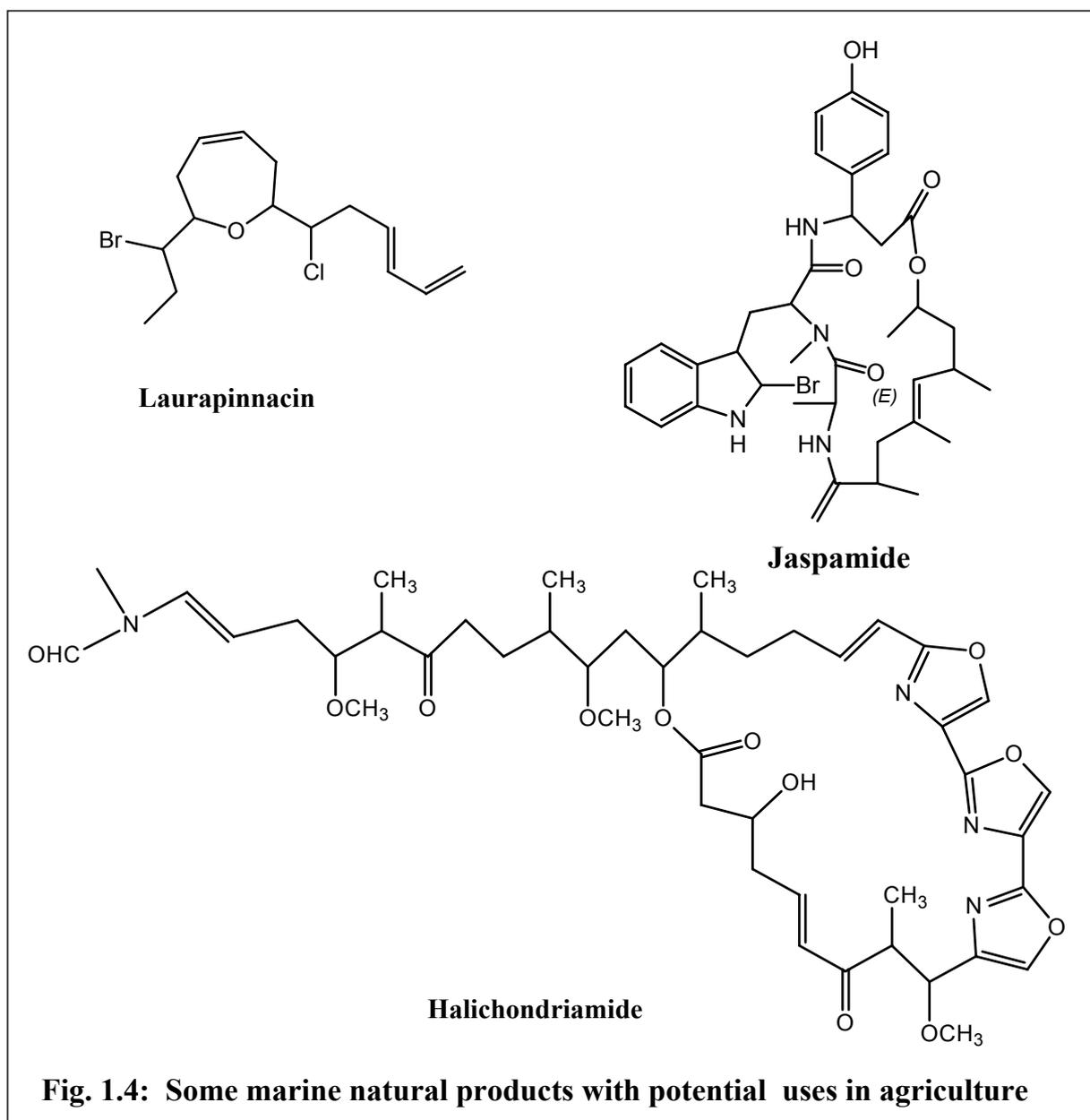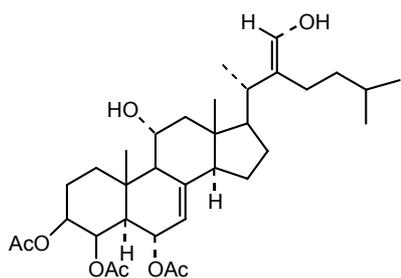
Agosterol A
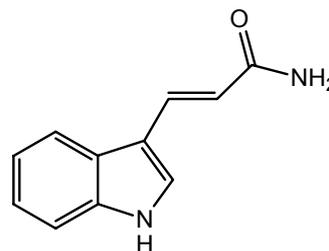
Indole-3-acrylamide

Z-Hymenialdisine
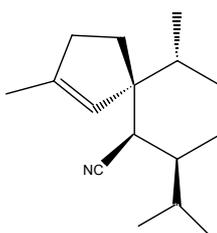
Axisonitrile-3
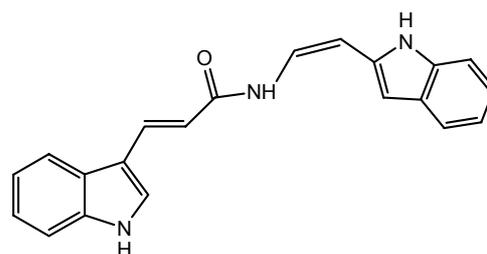
Chondriamide C
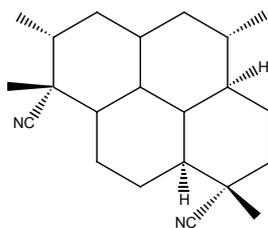
Diiosocyanoadociane
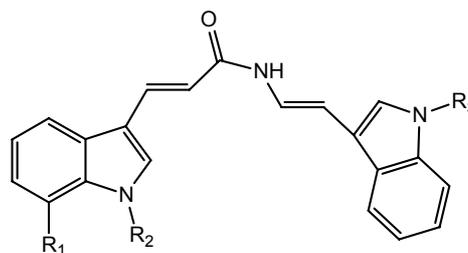
$R_1 = R_2 = R_3 = H$ **Chondriamide A**

$R_1 = OCH_3$ $R_2 = R_3 = CH_3$

Trimethylderivative of Chondriamide A
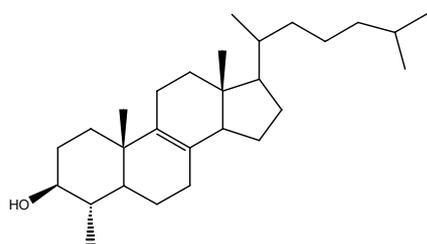
4α- Methyl-5α-cholest-8-
en-3β-ol
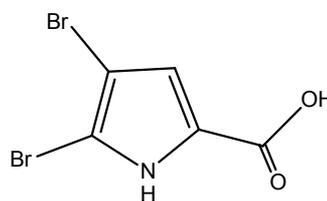
4, 5-Dibromopyrrole-2-carboxylic acid

Fig. 1.5. : Some biologically active marine natural products

1.3. The importance of marine metabolites for the source organisms

Some marine metabolites play a very important role for the source organisms such as:

1.3.1. Chemical defence against fouling and spatial competition

It was demonstrated that the sponge *Aplysina fistularis* exudes aerothionin and homo-aerothionin into the surrounding sea water at sufficient concentrations to protect it following mechanical injury. Both compounds are able to prevent the settlement of fouling organisms on *A. fistularis*. Aerophobin-2 and isofistularin-3 are present in *A. aerophoba,* these are after injury converted to dienone and aeroplysinin-1 respectively, which protect the sponge from invasion of pathogenic microrganisms (Proksch and Ebel, 1998 and Ebel *et al,* 1997).

Marine sponges also contain a wealth of antibiotically active natural products (Krebs, 1986 and Faulkner, 1993) that have been suggested to be involved in the suppression of epibiotic bacteria. For example, eudistomins G and H were found to be responsible for the epibiont-free surface of *E. olivaceum* (Davis, 1991). Antifouling sesquiterpenes from genus *Axinyssa* protect the sponge from settlement of barnacles (Hirota *et al*, 1998).

1.3.2. Chemical defence against fish

Latrunculin-A and B protect the sponge *Latrunculina magnifica* from fishes (e.g. *Gambusia affinis*) (Neeman *et al*, 1975). The cyclic monoterpene from marine alga *Achtodes crockeri* acts as a feeding deterrent and protects the sponges from the attack of predators (Paul *et al*, 1980). The unpalatiblity of the ascidian *Trididemnum solidum* was shown to be due to alkaloids of didemnin B and nor-didemnin which inhibited the reef fishes from feeding (Lindquist *et al*, 1992).

1.4. The current status of marine natural products research

The growing interest in marine natural products has led to the discovery of an increasing number of potently active metabolites considered worthy for clinical applications. A recent review provided an updated list of marine natural products which are currently under clinical trials (Table 1) (Proksch *et al*, 2002).

Table 1. Selected natural products from marine sources which are currently undergoing clinical trials.

Source	Compounds	Disease area	Phase of clinical trial
Conus magnus (cone snail)	Ziconotide	Pain	III
Ecteinascidia turbinata (tunicate)	Ecteinascidin	Cancer	II/III
Dolabella auricularia (sea hare)	Dolastatin 10	Cancer	II
Dolabella auricularia (sea hare)	LU103793 [a]	Cancer	II
Bugula neritina (bryozoan)	Bryostatin 1	Cancer	II
Trididemnum solidum (tunicate)	Didemnin B	Cancer	II
Squalus scanthias (shark)	Squalamine lactate	Cancer	II
Aplidium albicans (tunicate)	Aplidine	Cancer	I/II
Agelas mauritianus (sponge)	KRN7000[b]	Cancer	I
Petrosia contignata (sponge)	IPL 576,092[c]	Inflammation / Asthma	I
Pseudopterogorgia elisabethae (soft coral)	Methopterosin[d]	Inflammation/ Wound	I
Luffariella variabillis (sponge)	Manoalides	Inflammation/ Psoriasis	I
Amphiporius lactifloreus (marine worm)	GTS-21[c]	Alzheimer/ Schizophrinia	I

Reported from Proksch *et al*, 2002

[a] synthetic analogue of dolastatin 15

[b] Agelasphin analogue (α- galactosylceramide derivative)

[c] synthetic analogue of contignasterol 8IZP-94,005)

[d] semisynthetic pseudopterosin derivative

1.5. The aim of the present study

Even today, after more than 100 years of research in the pharmaceutical industries, there is still a great need for innovative drugs. Only one third of all diseases can be treated efficiently (Müller *et al,* 2000). This means that there is need for new drugs to enable therapeutic innovations. In this work, the isolation and structure elucidation of the metabolites of some sponges collected from Elba and Indonesia are presented. The extracts and isolated compounds were subjected to a series of bioassays such as antimicrobial, feeding deterrence, insecticidal, and cytotoxic activities. The anti-microbial activity was studied using agar diffusion assay. Cytotoxicity was studied using mouse lymphoma, (L5178Y), human cervix carcinoma, (HeLa), and rat brain tumour, (PC12) cell lines. For the strongly active compounds the dose response relationships were also performed to determine the IC_{50}. The fish feeding assay was performed for the crude extracts and for the pure compounds which were isolated in sufficient amount, using *Blennius sphinx* which is *a* fish from Mediterranean Sea.

2. Materials and methods

2.1. Animal materials

The vast majority of living sponges, more than 95%, are members of the class *Demospongiae*. Marine sponges range in distribution from shallow water to great depths. Coloration is frequently brilliant because of the pigment granules located in the amebocytes (Barnes, 1987).

Sponges, which constitute the phylum *Porifera* were long believed to be plants and were only recognised as animals in 1825, (scheme 2.1). Adult sponges are attached and motionless. Even when touched they do not draw away. About 5,000 poriferan species are currently recognised. They occur must abundantly in shallow, coatal waters, attached to the bottom or to submerged objects. But they are also found in deep water, and some 200 species are adapted to fresh water (Goerge and Goerge, 1979).

Kingdom Animalia ———→ Subkingdom Parazoa
                     └———→ Subkingdom Metazoa

Subkingdom Metazoa ———→ Phylum Porifera

Phylum Porifera   (Sponges) approx. 10000 species

Phylum	Subphylum	Class	Subclass	Order
	Gelatinosa	Calcarea		Homocoela
				Heterocola
				Pharetronida
		Demospongia	Homosclerinomorpha	
			tetractinomorpha	Astrophorida
				Spirophorida
				Desmophorida
				Other lithistids
				Hadromerida
				Axinellida
Porifera			Ceractinomorpha	Halichondrida
				Poecilosclerida
				Haplsclerida
				Dictyoceratida
				Dendroceratida
		sclerospongiae		Ceratoporillida
				Tabulospongida
	Nuda	Hexactinellida		Amphidiscophora
				Hexasterophra

Scheme 2.1 : Taxonomy of animal kingdom (Goerge and Goerge, 1979)
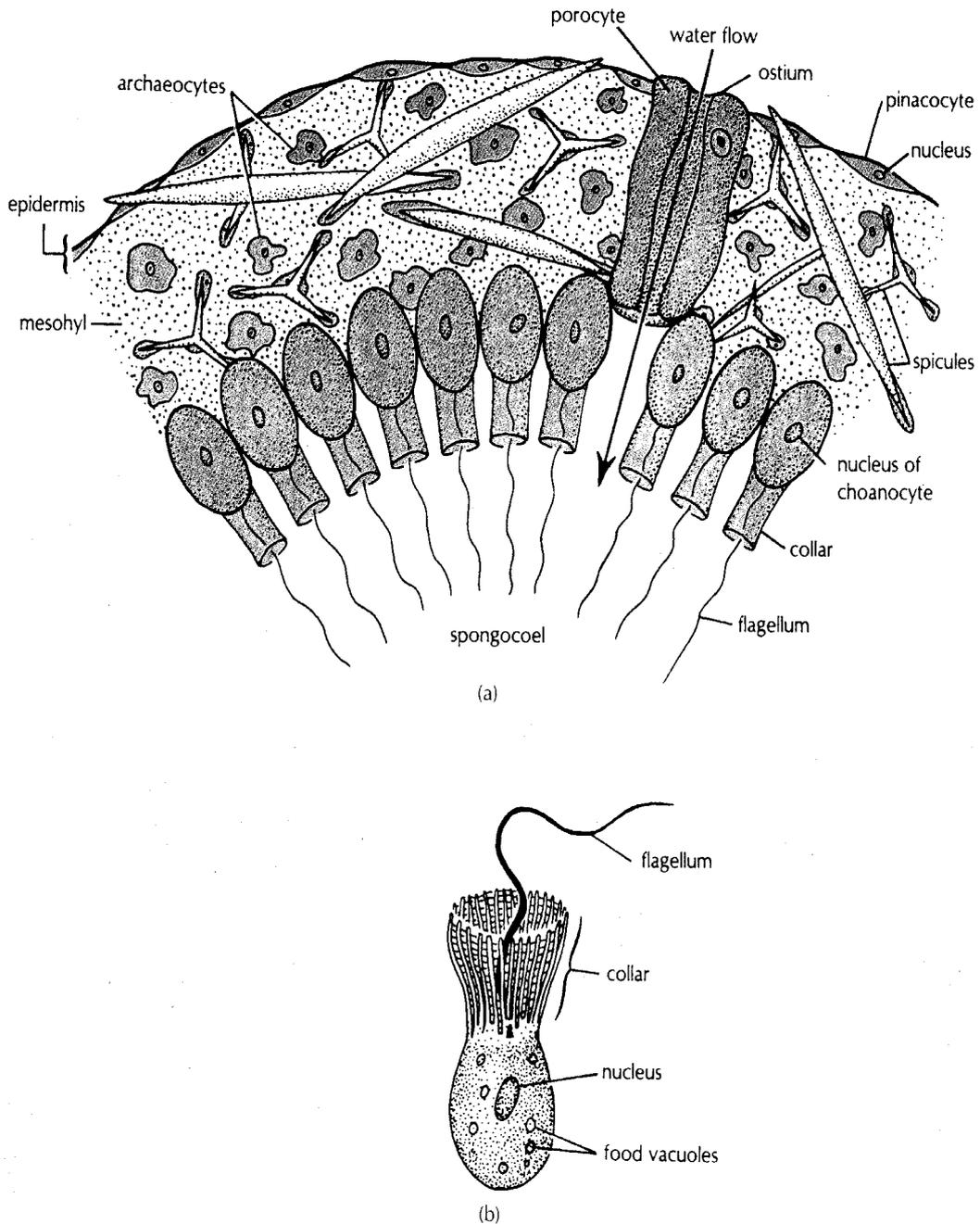
Fig. 2.1: (a) Diagramatic illustration of the body wall of a sponge

(b) Detail of a choanocyte (Pechenik, 2000)

The samples which included two sponges (*Axinella damicornis* and *Hamigera hamigera*), were collected around Elba (Mediterranean Sea) in May 2000 and April 2001 at a depth of 25-30 ft. In addition, two samples (*Axinyssa aplysinoides* and *Leucetta chagosensis*), were collected in Indonesia. All samples were identified by Dr. Rob W.M. van Soest of Zoologisch Museum, Amsterdam. The samples were frozen at 20°C until work-up.

The freeze dried samples were extracted with organic solvents and the resulting extracts were subjected to series of biological assays such as antibacterial and fish-feeding assays.

2.1.1. *Leucetta chagosensis*

The sponge *Leucetta chagosensis* Dendy (Fig. 2.2) (order *Leucettida*, family *Leucettidae*) is a lemon-yellow, soft sponge. It was collected in Kapoposang island (Indonesia, 07.08.1997) at a depth of 41 ft. A voucher specimen has been deposited in the Zoological Museum Amsterdam under the registration number ZMA POR.

2.1.2. *Axinyssa aplysinoides*

The sponge *Axinyssa aplysinoides* (Fig. 2.3) (order *Halchondriida*, family *Anchinoidae*) it is soft sponge with a yellow outer surface and a brown violet in the middle. It was collected in Taka Bako (Indonesia) at depths of 20 to 30 ft. (31.07. 1997) in Indonesia. A voucher specimen has been deposited in the Zoological Museum Amsterdam under the registration number ZMA POR. 17054.

2.1.3. *Axinella damicornis*

The sponge *Axinella damicornis* Esper (order *Axinellida*, family *Axinellidae*) (Fig. 2.4) is present as a small branching erect sponge with a short stalk. The branches are compressed and webbed together. The colour is distinctively bright to deep yellow sometimes verging toward deep orange at the margins. Small oscles are borne on the apices. The surface has a characteristic meanly appearance, as though dusted with small yellow particles. The sample was collected at Elba (Mediterranean Sea) in May 2000 and April 2001 at depth of 25-30 ft. A voucher specimen has been deposited in the Zoological Museum Amsterdam under the registration number ZMA POR 14393.

2.1.4. *Hamigera hamigera*

The sponge *Hamigera hamigera* Schmidt (Fig. 2.5) (order *Poecilosclerida*, family *Anchinoidae*) occurs at a depth of 15 to 35 ft as meaty crust. It has a bright red colour with extract of the same colour. The sample was collected at depth of 15 to

21 ft. at Elba (Mediterranean Sea). A voucher specimen has been deposited in the Zoological Museum Amsterdam under the registration number ZMA POR 14397.

Fig. 2. 2: *Leucetta chagosensis*

Fig. 2.3: *Axinyssa aplysinoides*

Fig. 2.4: *Axinella damicornis*

Fig. 2.5: *Hamigera hamigera*

2.2 Chemicals used

2.2.1. General laboratory chemicals

Agar-Agar	Merck
Anisaldehyde (4-methoxybenzaldehyde)	Merck
(-)-2- butanol	Merck
Dimethylsulfoxide	Merck
Formaldehyde	Merck
L-(+) - Ascorbic acid	Merck
Nipagin A	Sigma
Hexamethyldisilazane	Merck
Hydrochloric acid	Merck
Potassium hydroxide	Merck
Pyridine	Merck
Concentrated sulphuric acid	Merck
Trifloroacetic acid (TFA)	Merck

2.2.2. Solvents
Acetone

Acetonitrile

Dichloromethane

Ethanol

Ethyl acetate

Hexane

Methanol

The solvents were purchased from the institute of chemistry university of Duesseldorf. They were distilled before using and special grade were used for spectroscopic measurements.

2.3. Equipments used

Balance	: Mettler 200
	: Mettler AT 250
	: Mettler PE 1600 Sartorious RC210P
Centrifuge	: Kendro D-37520 osterde
Fraction collector	: ISCO Cygnet

Freeze dryer	: LYOVAC GT2;
	: Pump TRIVAC D10E
Hot plate	: Camag
Syringe	: Hamilton 1701 RSN
Mill	: Molinex 354
Magnetic stirrer	: Variomag Multipoint HP
Mixer	: Braun
PH-Electrode	: Inolab
	: Behrotest PH 10-Set
Rotary evaporator	: Büchi Rotavap RE 111; Buchi Rotavap R -200
Drying Ovens	: Heraeus T5050
Sonicator	: Bandelin sonorex RK 102
UV Lamp	: Camag (254 and 366)
Vacuum Exicator	: Solvent speed vac SPD 111V

2.3.1. HPLC equipment

I   Gynkotek

    Pump: : Gynkotek, M40

    HPLC program: : Gynkosoft (v.5.4)

    Detector: : Gynkotek, Photoiode Array Detector UVD 340

    Autosampler: : Gynkotek Autosampler GINA 50

    Printer: : NEC P60

II   Dionex

    HPLC program   : Chromeleon Ver 6.3

    Pump   : Dionex P580A LPG

    Detector   : Dionex, Photoiode Array Detector UVD 340S

    Autosampler   : ASI-100T

    Column Thermostat: : NEC P60

2.4. Chromatographic methods

2.4.1. Thin layer chromatography

TLC was performed on pre-coated TLC plates with Si gel 60 F_{254} (Layer thickness 0.2 mm, E. Merk. Darmstadt, Germany) with Hexane : EtOAc (90 : 10, 80 : 20 and 70 : 30) were used as eluent for semi-polar compounds; CH_2Cl_2 : MeOH : NH_4OH (70: 30: 3% and 75: 25: 3%) for the brominated alkaloids. TLC on reversed

phase (RP)-C18 F_{254} (layer thickness 0.25 mm, E. Merk. Darmstadt, Germany) was also used for polar alkaloids using the solvent system MeOH : H_2O : TFA, 50 : 50 : 1%, 40 : 60 : 1%, and 60 : 40 : 1%). The compounds were detected from the UV absorbance at 254 and 366 nm or by spraying with Anisaldehyde reagent followed by heating at 110°C.

Anisaldehyde /H_2SO_4 spray reagent (DAB10)

Anisaldehyde   :5 parts

Glacial Acetic Acid   : 100 Parts

Methanol   : 85 parts

Conc. H_2SO4 : 5 parts (added slowly)

The solution was stored in amber-coloured bottles and kept refrigerated until use. TLC was used for the fractions and for the pure compounds to identify the fractions and determine the purity of the isolated compounds.

2.4.2. Column chromatography

The crude extracts were subjected to series of chromatographic columns using different stationary phases and solvent systems. Further purification of the fractions were performed by semi-preparative HPLC.

Type of columns used (according to the stationary phase)

Stationary phase   : Sephadex LH-20 (15-100 cm)

Solvent system   : Methanol

Stationary phase   : RP-18 (different size)

Solvent system   : Methanol : Nanopure water

Stationary phase   : Silica gel (different size)

Solvent system   : DCM:MeOH:NH_4OH , DCM: MeOH and Hexane: EtOAc

Stationary phase   : Amberlite XAD, Diainon, HP-20

Solvent system   : Methanol : Nanopure water

2.4.3. Semi-preparative HPLC

The semi-preparative HPLC was used for the isolation of pure compounds from fractions eluted by column chromatography. Each injection was in concentration of 3

mg of the dried fraction dissolved in 1 ml of solvent system. The injection volume up to 1 ml was injected into the column and the flow rate 5 ml/min. The eluted peaks were detected by UV.

The solvent system used for isolation of the brominated alkaloids and imidazole alkaloids

1)

Time	MeOH	H_2O (0.1% TFA)
0	0	100
35	100	0
40	100	0

2)

Time	MeOH	H_2O (0.1% TFA)
0	0	100
5	10	90
40	100	0

The solvent system used for isolation of sterols

Time	MeOH	H_2O
0	50	50
20	100	0
25	100	0

The solvent system used for isolation of nucleoside derivative

Time	MeOH	H_2O
0	0	100
10	0	100
40	100	0

2.4.4. Analytical HPLC

Analytical HPLC was used to identify the content of the fractions and to check the degree of purity of the isolated compounds. The gradients used were different:

1)   start with 100% H_2O (pH, 2 with o-phosphoric acid) to 100% methanol in 35 minutes.

2)   start with 90% H_2O (pH, 2 with o-phosphoric acid) to 100% methanol in 35 minutes, the detection of the peaks was by UV-VIS diode detector.

2.4.5. Vacuum liquid chromatography

Vacuum liquid chromatography (VLC) is a useful method as an initial isolation procedure for the large amount of sample. The apparatus consists of a 500 ml sintered

glass büchner filter funnel, with an inner diameter of 12 cm. Fractions are collected in Erlenmeyer flasks, (see Fig. 2.5). Silica gel 60 was packed to a hard cake at a height of 5 cm under applied vacuum. The sample used was incorporated in a small amount of silica gel using volatile solvent. The resulting sample mixture was then packed onto the top of the column. Step gradient elution with non-polar solvent (Hexane) then increasing the amount of the polar solvent (EtOAc, MeOH) is added to each successive fraction. The flow is produced by vacuum and the column is allowed to run dry after each fraction collected.

Fig. 2. 6: Vacuum liquid chromatography apparatus
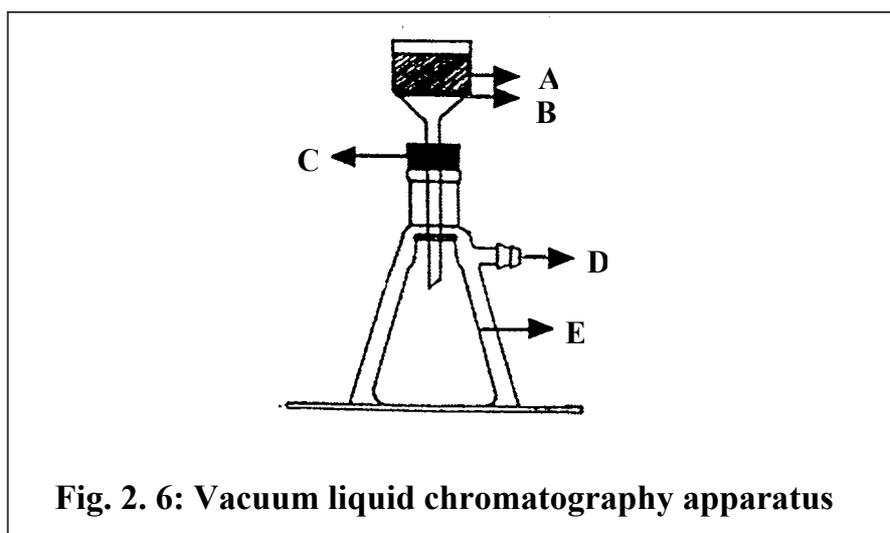
A- Sintered glass adsorbent, Si 60

B- Sintered glass Büchner filter funnel

C- Rubber gasket

D- To vacuum pump

E- Flask collector

2.4.6. Flash chromatography

Flash chromatography was used for isolation of certain compounds which showed no absorbance under UV (sesquiterpenes). A silica gel G pre-packed column was used and the sample was dissolved in a small volume of the solvent used. The resulting mixture was then packed onto the top of the column using special syringe. The flow rate is activated by an air pump and 5 ml fractions were collected and monitored by TLC.

2.5. Schemes of isolation

2.5.1. Isolation of secondary metabolites from *Leucetta chagosensis*

```
                        ┌─────────────────────┐
                        │ Leucetta chagosensis│
                        │    Total extract    │
                        └─────────────────────┘
                                  │
                        ┌─────────────────┐
                        │    Solvent      │
                        │  partitioning   │
                        └─────────────────┘
                                  │
      ┌──────────────┬────────────┴────────────┬──────────────┐
      │              │                          │              │
┌───────────┐  ┌──────────────┐         ┌──────────────┐ ┌─────────────┐
│ EtOAc phase│  │ Hexane phase │        │ Butanol phase│ │ Water phase │
└───────────┘  └──────────────┘         └──────────────┘ └─────────────┘
      │                                         │
┌──────────────────┐              ┌──────────────────────────┐
│ Sephadex column  │              │        RP column         │
│     (MeOH)       │              │   MeOH : H₂O : TFA       │
└──────────────────┘              │    50  :  50  : 0.1%     │
      │                           └──────────────────────────┘
┌──────────┐                                    │
│  Fr. 10  │                        ┌───────────┴──────────┐
└──────────┘                        │                      │
      │                      ┌──────────────┐      ┌──────────┐
┌──────────────────┐         │  Compound 2  │      │  Fr. 10  │
│ Semi-preparative │         └──────────────┘      └──────────┘
│      HPLC        │                                     │
└──────────────────┘                          ┌──────────────────┐
      │                                        │ Semi-preparative │
┌─────┴──────┐                                 │      HPLC        │
│            │                                 └──────────────────┘
```

2.5. Schemes of isolation

2.5.1. Isolation of secondary metabolites from *Leucetta chagosensis*

Leucetta chagosensis Total extract → Solvent partitioning → EtOAc phase, Hexane phase, Butanol phase, Water phase

EtOAc phase → Sephadex column (MeOH) → Fr. 10 → Semi-preparative HPLC → Compound 4, Compound 5, Compound 6

Butanol phase → RP column MeOH : H_2O : TFA 50 : 50 : 0.1% → Compound 2, Fr. 10 → Semi-preparative HPLC → Compound 1, Compound 3

2.5.2. Isolation of secondary metabolites from *Axinyssa aplysinoides*

Axinyssa aplysinoides
Total Extract

Solvent partitioning

Hexane phase | Ethyl acetate phase | Butanol phase

Silica gel column

1-Hex:EtOAC
95:5 ➤ 100%
2- EtOAC:MeOH
9:1 ➤ 100%

Sephadex column (MeOH)

Sephadex column (MeOH)

Fr. 4

Fr.1

Flash Chromatography
1-DCM
2-DCM : MeOH
9 : 1
8 : 2

F.r. 1   **Fr. 2**   **Fr.3**

Silica gel column
Hex: EtOAc
95 : 5

Silica gel column
(Hex.)

Semi-preparative HPLC

Compound 9 | **Compound 8** | **Compound 7** | **Compound 10**

2.5.3.1. Isolation of secondary metabolites from *Axinella damicornis*

```
                    ┌─────────────────────┐
                    │  Axinella damicornis │
                    │   (First collection) │
                    │    Total extract     │
                    └──────────┬──────────┘
                               │
                    ┌──────────┴──────────┐
                    │      Solvent        │
                    │    partitioning     │
                    └──────────┬──────────┘
                               │
                ┌──────────────┴──────────────┐
        ┌───────────────┐          ┌───────────────┐
        │  Water phase  │          │  EtOAc phase  │
        └───────────────┘          └───────┬───────┘
                                           │
                                ┌──────────┴──────────┐
                                │  Sephadex column    │
                                │      (MeOH)         │
                                └──────────┬──────────┘
```

Fraction 1   Fraction 2   Fraction 3   Fraction 4   Fraction 5

Silica gel column
DCM : MeOH : NH$_4$OH
70 : 30 : 3%

RP column
MeOH : H$_2$O : TFA
80 : 20 : 0.1%

Compound 23

Compound 19   **Compound 16**

Fraction 1   Fraction 2

Semi-preparative
HPLC

Compound 13

Compound 22   **Compound 26**   **Compound 17**   **Compound 15**   **Compound 27**

2.5.3.2. Isolation of secondary metabolites from *Axinella damicornis*

```
                          Axinella damicornis
                           (second collection)
                             Total extract
                                   │
                                   ▼
                              Solvent
                             partitioning
                                   │
        ┌──────────────┬───────────┴────────┬──────────────┐
        ▼              ▼                    ▼              ▼
   Water phase    EtOAc phase          Hexane phase    Butanol phase
                      │                                     │
                      ▼                                     ▼
                  VLC column                         Sephadex column
                      │                                  (MeOH)
                      │                                     │
            ┌─────────┴──────┐                      ┌───────┴───────┐
            ▼                ▼                       ▼               ▼
        Fraction 5       Fraction 6             Fraction 1      Fraction 2
            │                │                       │               │
            ▼                ▼                       ▼               ▼
      Semi-preparative  Semi-preparative      Semi-preparative   RP Column
          HPLC              HPLC                  HPLC        MeOH : H₂O : TFA
            │                │                       │          80  :  20  : 0.1%
            │                │                       │               │
      ┌─────┴─────┐          ├──────────┐            │               │
      ▼           ▼          ▼          ▼            ▼        ┌───────┴───────┐
 Compound 24  Compound 25  Compound 11  Compounds  Compounds ▼               ▼
                                        12, 14      17, 27  Compounds    Compound 20
                                                            16,, 17, 18
```

Semi-preparative HPLC

RP Column
MeOH : H_2O : TFA
80 : 20 : 0.1%

2.5.4. Isolation of secondary metabolites from *Hamigera hamigera*

```
                        ┌─────────────────────┐
                        │ Hamigera hamigera   │
                        │ Total extract       │
                        └─────────────────────┘
                                  │
                        ┌─────────────────┐
                        │ Solvent         │
                        │ partitioning    │
                        └─────────────────┘
```

Aqueous Phase	Butanol Phase	Ethyl acetate phase

Sephadex column (MeOH)

Sephadex column (MeOH)

HP -20- Column (H$_2$O to MeOH)

Fr.5   Fr 2

Fr. 2   Fr.3   Fr.4

Fr 2

RP Column MeOH : H$_2$O 1 : 1

RP Column MeOH : H$_2$O 1 : 1

Silica gel Hexane: EtOAc 9 : 1

Semi-preparative HPLC

Fr.4

Compounds 28, 35

Fr.3

Semi-preparative HPLC

Semi-preparative HPLC

Compound 28

Compound 30   **Compound 34**

Sephadex column (MeOH)

Compound 29   **Compound 32**   **Compound 31**   **Compound 33**

2.6. Structure elucidation of the isolated compounds

2.6.1. Mass spectrometry (MS)

Low resolution MS. low resolution mass spectra were measured by ESI, EI, and FAB-MS on a Finnigan MAT 8430 mass spectrometer.

EIMS (Electron impact mass spectroscopy) analysis involves vaporising a compound in an evacuated chamber and then bombarding it with electrons having 25-80 ev of energy. The high energy electron stream not only ionises an organic molecule (requiring about 7-10 eV) but also causes extensive fragmentation. The advantage is that fragmentation gives rise to a pattern of fragment ions which can help to characterise the compounds. The disadvantage is the frequent absence of a molecular ion.

ESIMS (electro spray ionisation) is a method for ejecting ionised molecules from a solution by creating a fine spray of highly charged droplets in the presence of a strong electric field. This type of ionisation is highly conductive to the formation of multiply charged molecules.

High resolution MS High resolution mass is achieved by passing the ion beam through an electrostatic analyser before it enters the magnetic sector. In a double focussing mass spectrometer, ion masses can be measured with an accuracy of about 1 ppm. With measurement of this accuracy the atomic composition of the molecular ion can be determined.

LC/MS High pressure liquid chromatography (HPLC) is a powerful method for the separation of complex mixtures. If a mass spectrum of each component can be determined as it elutes from LC column a quick characterisation of the components can be done. ESIMS (electron spray ionisation mass spectrometry) is interfaced with LC to make an effective online LC/MS. HPLC/ESIMS was carried out using a Finnigan QDECA-7000 mass spectrometry connected to a UV detector. The sample is

dissolved in water or methanol or mixture of both and injected to HPLC/ESI-MS set up. HPLC was run on a Eurospher C-18 reversed phase column.

2.6.2. Nuclear magnetic resonance spectroscopy (NMR)

^{1}H and ^{13}C-NMR spectra were recorded at 300° K on Bruker DPX 300, ARX 400, 500 or AVANCE DMX 600 NMR spectrometers. All 1D and 2D spectra were obtained using the standard Bruker software. The samples were dissolved in deuterated solvents (DMSO-d_6, CDCl$_3$, CD$_3$OD), the choice of the solvent depends mostly on the solubility of the compound. Solvent signals at 3.3 ppm. and 49.0 ppm. (CD$_3$OD) and 7.26 ppm and 77.0 ppm (CDCl$_3$) and 2.49 ppm and 39.5 ppm (DMSO-d_6) were considered as internal reference signal for calibration. The observed chemical shift values (δ) were given in ppm. and the coupling constant (J) in Hz.

2.6.3. The optical activity

Optical rotation was determined on a Perkin-Elmer-241 MC Polarimeter by measuring the angle of rotation at a wavelength of 546 and 579 nm. A mercury vapour lamp was used and the sample was run at room temperature (25°C) in a 0.5 ml cuvette with 0.1 dm length. The specific optical rotation was calculated using the equation:

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

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

$[\alpha]_{579}$ and $[\alpha]_{546}$ = the optical rotation at the wavelength 579 and 546 nm respectively calculated using the formula:

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

Where α = the measured 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 of the solution.

2.7. Bioassays

2.7.1. Fish-feeding assay

In the experiment with *Blennius sphinx* fishes, food pieces treated with sponge extracts were given to the fishes at the same time as untreated food pieces. If the fishes show a preference to the untreated food pieces this means the extract has fish feeding deterrence activity.

In the natural habitat some sponges show self defence against predators with the aid of their secondary metabolites, due to this the extracts have been tested in their natural concentration as present in the sponge. The amount of extract in 10 ml sponge was determined, by the following equation:

$$\text{Density} = \frac{\text{Wet weight}}{\text{Volume}}$$

The preparation of food pieces

The total extract was dissolved in certain volume of suitable solvent and a volume equal to 10 ml sponge was calculated. 1.053 g food granules were grinned to a fine powder in a mortar and put in round bottom flask, then the volume of the extract solution (10 ml sponge) was added, mixed well and evaporated to dryness. At the end the food/ extract mixture was detached from the wall of the flask by spatula. .... **(Flask A)**.

0.189 g agar was taken in Erlenmeyer flask, then 9.5 ml distilled water was added to it. The flask was closed with aluminium foil, boiled on a water bath for four minutes and then cooled to 55ºC. .... **(Flask B)**
 The content of flask **B** was added to flask **A** and mixed well to give the treated food ....**(Experiment, E)**.
The untreated food was prepared according to the previous steps, but instead of the extract containing solvent pure solvent was used ....**(Blank, B)**.

Both types of food (experiment and blank) were poured into a rectangular frame, this frame containing two openings 2.5 x 25 cm. Under the frame there was a network and plastic foil. One of the openings was filled with E and the other was filled

with B, the extract/food mixture was distributed to be of 2 mm thickness. After cooling the agar the frame was removed and the food remained at the network. Six pieces were cut from B and E, each 10 squares wide. Each of the blank and the experiment diet was attached to metallic body to sink in the aquarium.

The experiment

Six food bands were put in the aquarium at the same time. The food pieces were observed until one third of the food in each band had been consumed. The empty squares were counted and the percentage of eaten food from both E and B were calculated.

$$\text{Percentage of eaten food from } E = \frac{e \times 100}{t}$$

$$\text{Percentage of eaten food from } B = \frac{b \times 100}{t}$$

e = number of empty squares from **E**

b = number of empty squares from **B**

t = e + b

The fishes used in this experiment (*Blennius sphinx*) were collected from the Mediterranean Sea near Elba Island, Italy.

Quantification of the pure compounds for the fish feeding assay

Before carrying out fish feeding assays for the pure compounds (oroidin and 4,5-dibromopyrrole-2-carboxylic acid), the amount of pure compounds in 10 ml sponge had been determined by injecting known concentrations of the substances in HPLC (Table 2.1). A standard curve was obtained, showing the given area under the peak at different known injected concentrations (Fig. 2. 7 and 2. 8). It was found that the amount of oroidin and 4,5-dibromopyrrol-2-carboxylic acid in 10 ml of *Axinella damicornis* sponge were 60 and 48 mg, respectively.

Table 2.1: Quantification of oroidin and 4,5-dibromopyrrole-2-carboxylic acid in *Axinella damicornis* **extract**

Oroidin		4, 5-Dibromopyrrole-2-carboxylic acid	
(µg)	Area under the peak	(µg)	Area under the peak
3.75	76.8	1.25	30.21
5	100.8	2.5	59.37
1.25	26.68	3.75	87.36
10	198.5	6.25	144.7
6.25	125.8	5	116.6
7.5	150.5	7.5	172.1

Oroidin

Fig. 2. 7: Quantifcation of oroidin in *Axinella damicornis*

4, 5-dibromopyrrole-2-carboxylic acid
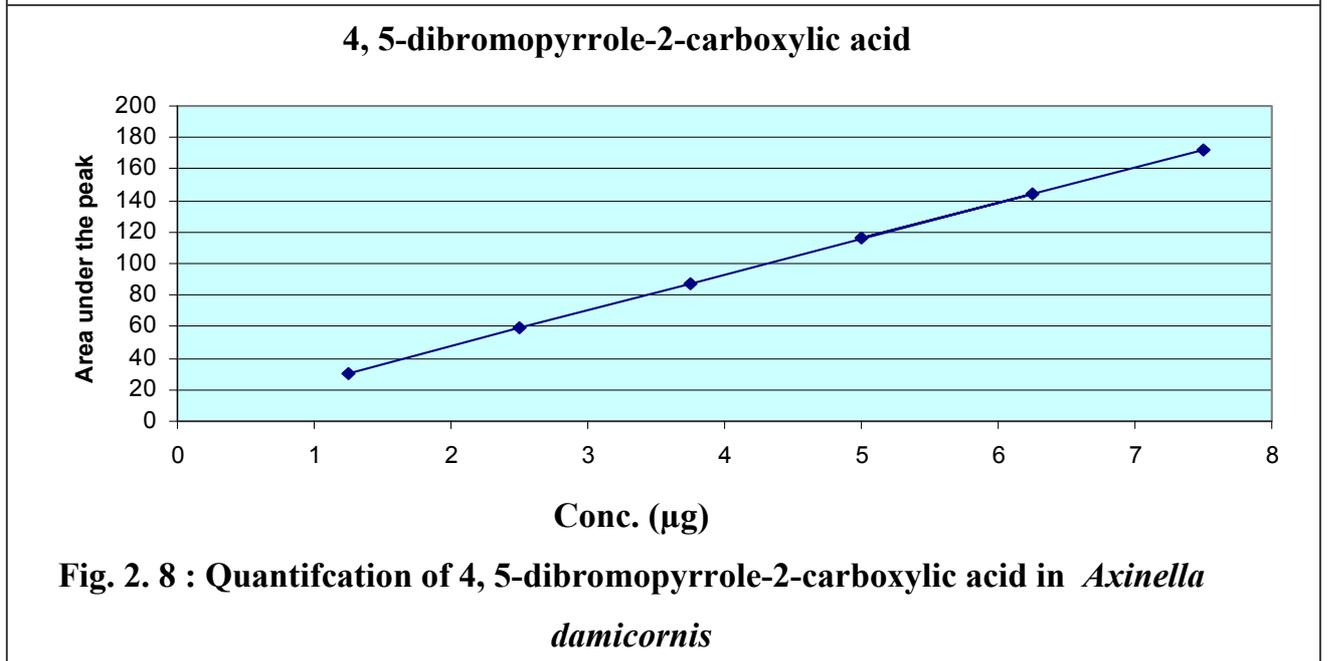
Fig. 2. 8 : Quantifcation of 4, 5-dibromopyrrole-2-carboxylic acid in *Axinella damicornis*

2.7.2. Brine shrimp assay

This technique is an *in vivo* lethality test with the brine shrimp (*Artemia salina* Leach). The test determines the LC_{50} in µg/ml of active compounds and extracts (Meyer *et al*, 1982).

Sample preparation

The test samples were dissolved in organic solvents and the required amount was transferred to 10 ml vials. For the crude extract 0.5 mg was used and for the pure compounds different concentrations (50-500 µg). The samples were taken to dryness and redistributed in 20 µl DMSO. Control vials were prepared using only DMSO.

Hatching the eggs

Brine shrimp eggs (Dohse, Aquaristik GmbH, Bonn, Germany) were hatched in a small tank filled with artificial sea water which was prepared with sea salts (Sera Sea-Salt, Aquaristik GmbH, Bonn, Germany) and distilled water. After two days, 20 nauplii were taken by pipette and transferred to the sample vial and artificial sea water was added to the volume of 5 ml and the vials were maintained under illumination. After one day the surviving brine shrimps were counted using a magnifying glass and the percent dead at each concentration was determined.

2.7.3. Antimicrobial activity
Micro-organisms

The crude extracts and the pure compounds were tested for activity against the following standard strains: gram positive *Bacillus subtilis*; gram negative bacteria *Escherichia coli*; the yeast *Sacharomyces cerevisiae* and two fungal strains *Cladosporium herbarum* and *C. cucumerinum*.

Culture preparation

The agar diffusion assay was performed according to the Dauer Kirby- Test (DIN 58940, Bauer *et al*, 1966). Prior to testing, a few colonies (3 to 10) of the organism to be tested, were subcultured in 4 ml of tryptose-soy broth (Sigma, FRG) and incubated for 2 to 5 h to produce a bacterial suspension of moderate cloudiness.

The suspension was diluted with sterile saline solution to a density visually equivalent to that of a $BaSO_4$ standards. The standards were prepared by adding 0.5 ml of 1 % $BaCL_2$ to 99.5 ml of 1% H_2SO_4 (0.36 N). The prepared bacterial broth is inoculated onto Müller-Hinton-Agar plates (Difco, USA) and dispersed by means of sterile beads.

Agar diffusion assay

For screening, aliquots of the test solution were applied to sterile filter- paper discs (5 mm diameter, Oxid 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 discs containing solvent blanks. The plates were incubated at 37°C for 24 hr. and anti-microbial activity was recorded as the clear zones of inhibition surrounding the discs. The diameter was measured in mm.

2.7.4. Cytotoxicity test

Cytotoxicity tests were carried out by Prof. Dr. Müller (Univ. Mainz). The assay used mouse lymphoma (L5178Y), rat brain (PC12) and human cervix cancer cells (HELA). L5178Y mouse lymphoma cells were grown in Eagle's minimal essential medium supplement with 10% horse serum in roller tube culture (Kreuter *et al*, 1992 and Müller *et al,* 1985). All cells were mycoplasma-free and cultures were propagated under standardised conditions (Drexler *et al*, 1995). Stock solutions of the freeze-dried test compounds were dissolved in EGMME (ethylene glycol monomethyl ether) or DMSO, diluted with culture medium and stored at $–20^oC$.

For all experiments, exponentially growing cells were used, with viability more than 90% as determined by trypan blue staining. For analysis, the cells were harvested, washed and re-suspended in fresh medium at a final concentration of 2-4 $x10^5$ cells/mL according to the cell line used. Total cell number and viability were determined in a cell counting chamber after staining the cells with trypan blue. The cells were seeded out in volumes of 90 μL into 96-well flat-bottom culture plates (Nunc, Wiesbaden, Germany). The test compounds stock solutions were diluted with suitable amounts of culture medium to concentration of 10 μL, were added to each well and incubated for 48 h at 37 ^oC in 5% CO_2 humidified incubator.

The cytotoxicity was evaluated by the [^{3}H] thymidine assay (Steube *et al* 1992). Radioactive incorporation was carried out for the last 3 h of the 48 h incubation. One µCi of [methyl-^{3}H]-thymidine (Amersham-Buchler, Braunschweig, Germany; specific activity 0.25 mCi/µmol) was added in a 20 µL volume to each well. Cells were harvested on glass fibre filters with a multiple automatic sample harvester, and radioactivity was determined in a liquid scintillation counter (1209 Rackbeta, LKB, Freiburg, Germany). Media with 0.1% EGMME and DMSO were incubated in the experiment as control.

2.7.5. Protein kinase screening assays

The protein kinase screening assays were done by Dr. Monsier Lozach at CNRS Station Biologique (France). Some of the isolated secondary metabolites were subjected to protein kinase assays. Protein kinases catalyze the phosphorylation of serine, thereonine and tyrosine residues of protein using adenosin triphosphate (ATP) as phosphate donor. Protein phosphorylation is considered as one of the main post translational mechanisms used by cells to finely tune their metabolic and regulatory pathways. Cycline dependent kinases (CDKs) are involved in controlling the cell cycle, apoptosis, neuronal functions and neurodegeneration. Glycogen synthase kinase-3 (GSK-3) is involved in multiple physiological processes including cell cycle regulation, in which it controls the levels of cyclin D1 and β-catenin, insulin action on glycogen synthesis and phosphorylation of tau. CDK/GSK-3 inhibitors are very important in treatment of cancers, neurodegenerative disorders such as Alzheimer´s disease (Metty *et al*, 2003).

3. Results

3.1. Imidazole alkaloids from *Leucetta chagosensis*

Almost all of the natural products reported to date from marine sponges are based on studies of the class Demospongia (Marinlit, 2003 and Faulkner, 2001). From the Calcarea group, the genus *Leucetta*, has received the widest attention. Early investigation of the sponges genus *Leucetta* has led to the isolation of interesting imidazole alkaloids such as naamines, isonaamines, naamidines, isonaamidines and calcaridines (Carmely and Kashman, 1987 and 1989, Kong and Faulkner*,* 1993, Fu *et al* 1997, Plubrukarn *et al*, 1997, Dunbar *et al,* 2000, Edrada *et al*, 2003, and Crews *et al*, 2003). The alkaloids of this group are similar in that each possesses a central imidazole ring to which one or two benzyl groups are attached at the C-4, C-5 or N-3 positions. In addition, kealiiquinone, preclathridines and leucettamines have been isolated (Akee *et al*, 1990 and Edrada *et al,* 2003). From the same concern imidazole alkaloids exhibit very interesting biological activities such as antimicrobial (Carmely *et al*, 1997, Kong and Faulkner*,* 1993, Ciminiello *et al,* 1989, and Mancini *et al,* 1995) anti-cryptococcal, inhibition of nitric oxide synthase and cytotoxic activity (Plubrukarn *et al,* 1997, Akee *et al,* 1990 and Gross *et al*, 2002). It was revealed that naamidine A exhibited anti-tumour activity by regulation of ERK1 and ERK2 resulting in stimulation of p21 level and consequently arresting cells in growth phase 1, G1, which is a new mechanism for anticancer agents (Tasdemir *et al,* 2002).

The present work investigated a member of class Calcarea, the sponge *Leucetta chagosensis*. The methanolic extract of the freeze-dried sponge tissue was subjected to solvent partitioning resulting in phases of hexane, ethyl acetate, butanol and water. The butanol phase was subjected to reversed phase silica gel column, then to semi-preparative HPLC to give three compounds **1**, **2**, and **3** of the naamine class of alkaloids. The ethyl acetate fraction was subjected to a Sephadex column, then to semi-preparative HPLC to give three compounds **4**, **5**, and **6** of a new class of compounds related to kealiiquinone.
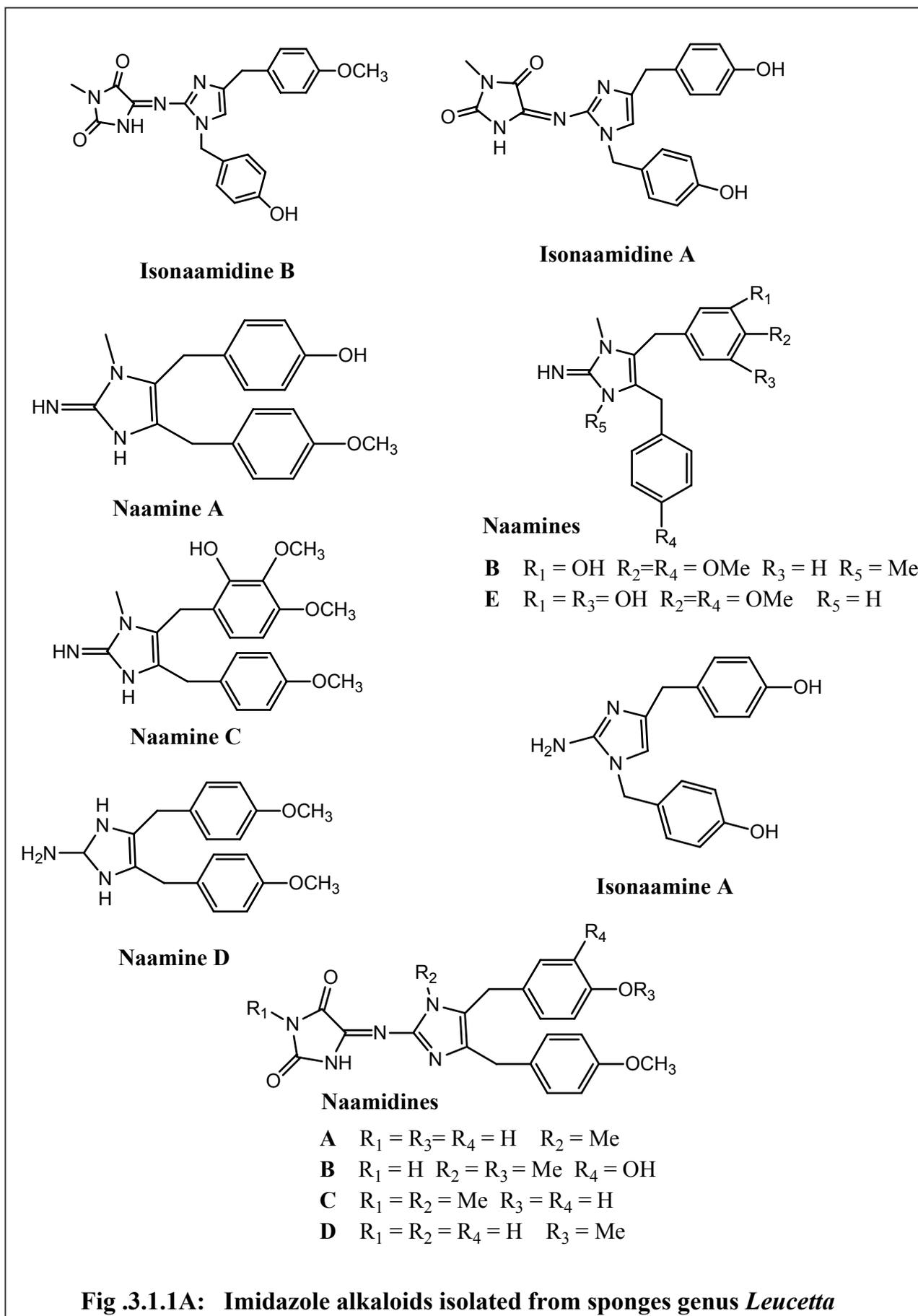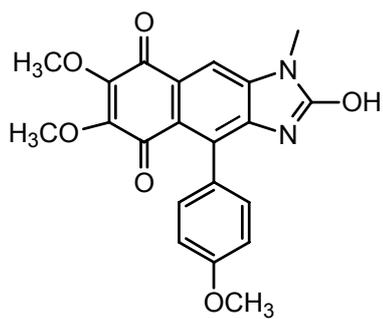
Isonaamidine B

Isonaamidine A

Naamine A

Naamines

B R_1 = OH $R_2=R_4$ = OMe R_3 = H R_5 = Me

E R_1 = R_3= OH $R_2=R_4$ = OMe R_5 = H

Naamine C

Isonaamine A

Naamine D

Naamidines

A R_1 = R_3= R_4 = H R_2 = Me

B R_1 = H R_2 = R_3 = Me R_4 = OH

C R_1 = R_2 = Me R_3 = R_4 = H

D R_1 = R_2 = R_4 = H R_3 = Me
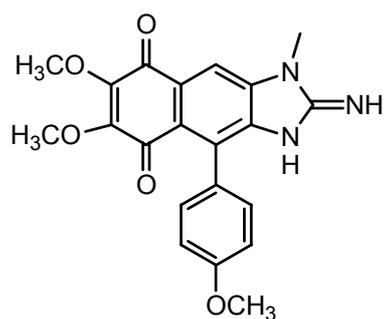
Fig .3.1.1A: Imidazole alkaloids isolated from sponges genus *Leucetta*
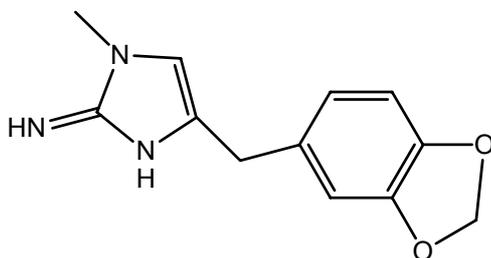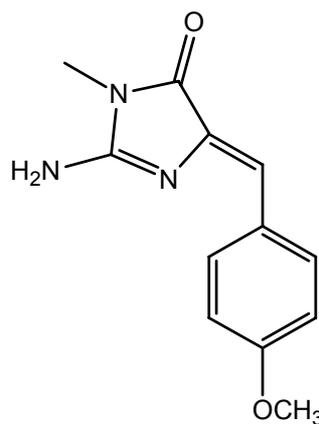
Fig .3.1.1B: Imidazole alkaloids isolated from sponges genus *Leucetta*

3.1.1. Structure elucidation of the isolated compounds

3.1.1.1. Structure elucidation of naamine A (1, known compound)

Naamine A
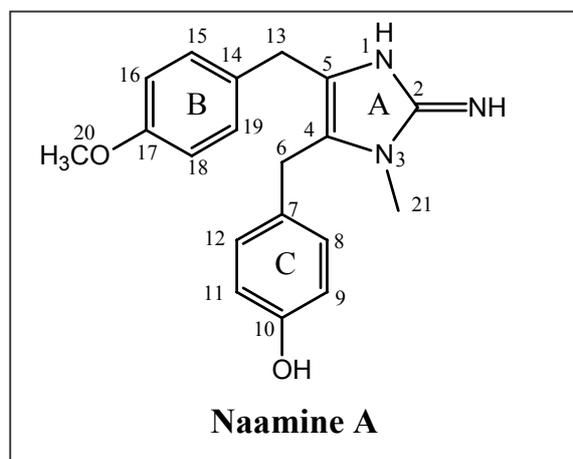
Compound **1** was obtained together with compound **2** as a dark brown amorphous powder. It has UV absorbances at λ_{max} 226 and 279 nm. The ESIMS (Fig. 3.1.3) showed a pseudo-molecular ion peak at *m/z* 324 [M+H]$^+$ which was compatible with the molecular formula $C_{19}H_{21}N_3O_2$. This was confirmed by HRESIMS (324.1712 [M+H]$^+$). The ^{1}H-NMR spectrum of compound **1** (Fig. 3.1.5, Table 3.1.1) showed four signals in the aromatic region for eight protons at δ_H 6.98 (2H, d, 8.8 Hz), δ 6.66 (2H, d, 8.8 Hz), δ 7.13 (2H, d, 8.8 Hz), δ 6.83 (2H, d, 8.8 Hz), this revealed the presence of two sets of AA′BB′ spin systems (Dunbar *et al*, 2000). The COSY spectrum of compound **1** (Fig. 3.1.6) showed two separate spin systems which confirmed the presence of two sets of 1,4-disubstituted benzene rings. The ^{1}H-NMR data showed also two singlet signals at δ_H 3.71 (3H) and δ_H 3.13 (3H) which were assigned to a methoxy and an N-methyl groups, respectively (Gross *et al*, 2002). Two singlet signals at δ_H 3.88 and δ_H 3.73 each for two protons were assigned to C*H₂*-6 and C*H₂*-13, respectively were observed. The low-field chemical shifts of C*H₂*-6 and C*H₂*-13 meant that they were benzylic methylenes. Two exchangeable protons for N*H₂* group at δ_H 7.35 were also observed. The HMBC correlations of compound **1** (Fig. 3.1.2) showed the C-H long range correlations of C*H₂*-6 with C-8, C-12, C-5 and correlations of H-8, and H-12 with C-10 which established the presence of a *para-*

hydroxybenzyl moiety. The ESIMS/MS gave a fragment ion peak at m/z 230 [M-C_6H_5O]$^+$ due to the loss of a hydroxy benzene moiety which confirmed the presence of a *para*-hydroxybenzyl moiety. The second *para*-methoxybenzyl moiety was established from the HMBC correlations of CH_2-13 with C-15, C-19, and H-20 with C-17. The chemical shifts of C-2, C-4 and C-5 at δc 146.2 (s), 122.0 (s) 122.3 (s) revealed the presence of a 2-amino-4,5-dibenzyl-imidazole (Fu *et al*, 1997). The HMBC correlations of H-21 (NCH_3) with C-2 and C-4 confirmed its attachment at N-3. From the above mentioned data and through the comparison with the literature (Kong and Faulkner, 1993 and Carmely *et al*, 1987) it was concluded that compound **1** was identical with the reported compound **naamine A**.

Table 3.1.1: NMR data of compound 1 (DMSO-d_6, 500 MHz)

NO.	δH	HMBC	δC^a	COSY	^{13}C-NMR of naamine A (Kong, 1993)
1	-	-	-	-	-
2	N*H* 7.35 (br s)	-	146.2	-	146.4 s
3	-	-	-	-	-
4	-	-	122.3	-	122.4 s
5	-	-	122.0	-	121.9 s
6	3.88 (s)	5, 8, 12	-	-	26.9 t
7	-	-	-	-	127.3 s
8	6.98 (d, 8.8 Hz)	10	128.9	9	129.1 d
9	6.66 (d, 8.8 Hz)	7	-	8	115.0 d
10	-	-	156.1	-	156.3 s
11	6.66 (d, 8.8 Hz)	7	115.0	12	115.0 d
12	6.98 (d, 8.8 Hz)	12	128.9	11	129.1 d
13	3.73 (s)	15, 19	-	-	28.2 t
14	-	-	-	-	130.3 s
15	7.13 (d, 8.8 Hz)	13	129.3	16	129.6 d
16	6.83 (d, 8.8 Hz)	-	-	15	114.1 d
17	-	-	157.9	-	158.2 s
18	6.83 (d, 8.8 Hz)	-	-	19	114.1 d
19	7.13 (d, 8.8 Hz)	13	129.3	18	129.6 d
20	3.71 (s)	17	-	-	55.3 q
21	3.13 (s)	2, 3	-	-	29.7 q

^{a} carbon assignments were determined from the HMBC spectrum which did not allow a complete assignment

Fig. 3.1.2: Important HMBC correlations of compound 1

Fig. 3.1.3: ESIMS spectrum of compound 1
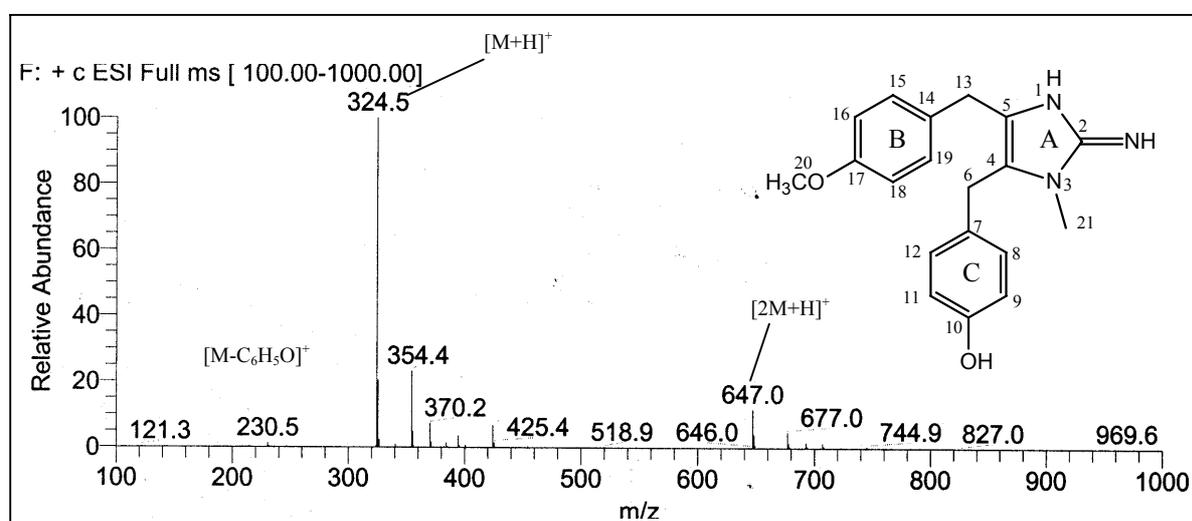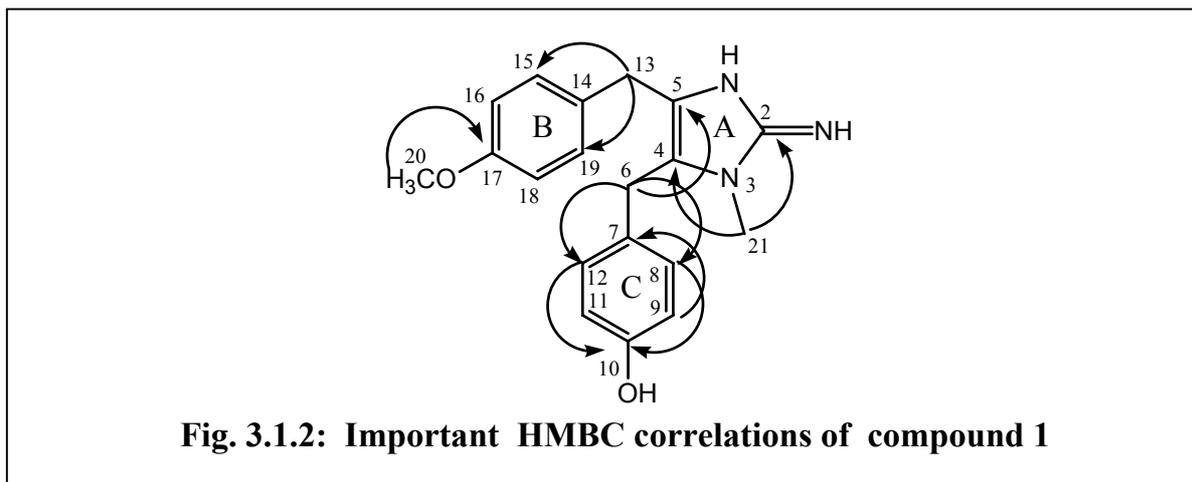
3.1.1.2. Structure elucidation of naamine F (2, new compound)

Naamine F
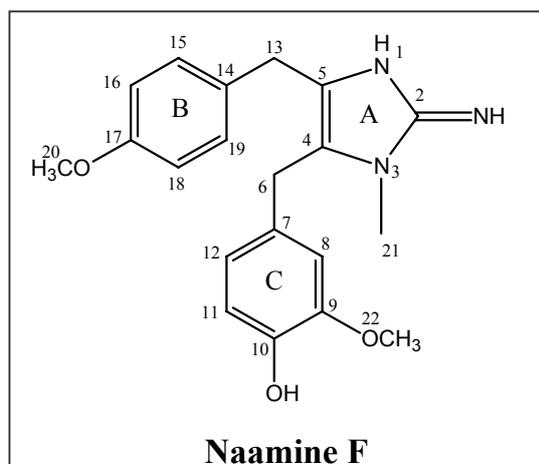
      Compound 2 was isolated together with compound **1** as a yellowish brown amorphous substance. The ESIMS spectrum of compound **2** (Fig. 3.1.4) showed a pseudo-molecular ion peak at *m/z* 354 [M+H]$^+$ which was compatible with the molecular formula $C_{20}H_{23}N_3O_3$ as established by HRESIMS (*m/z*, 354.1817, [M+H]$^+$). Inspection of the ^{1}H-NMR spectrum of compound **2** (Fig. 3.1.5, Table 3.1.2.) showed that it was closely related to that of naamine A (**1**). It showed two signals at δ_H 7.10 (2H, d, 8.2 Hz), and 6.80 (2H, d, 8.2 Hz) which were assigned to an AA′BB′ spin system of ring B and a signal at δ_H 3.12 for the N-methyl function. Two methylene signals at δ_H 3.85 and δ_H 3.80 were observed which were assigned to C*H$_2$*-6 and C*H$_2$*-13, respectively. The low-field chemical shifts of these suggested that they belonged to benzylic groups. An exchangeable proton for a N*H$_2$* group at δ_H 7.35 was also observed. The ^{1}H-NMR data (Table 3.1.2) showed an additional methyl singlet signal at δ_H 3.72 which was assigned to a methoxy group. The presence of a *para*-methoxybenzyl moiety as in naamine A was established from the HMBC correlations of the methylene signal at δ_H 3.80 (C*H$_2$*-13) with the carbon signal at δ_C 129.0 for C-15 and C-19 while the methoxy singlet at δ_H 3.72 (OC*H$_3$*-20) correlated with C-17 at δ_C 157.8. Irradiation of the methoxyl singlet (OC*H$_3$*-20) at δ_H 3.72 gave a NOE response for the AA′BB′ methine protons (H-16/18) at δ_H 6.80 while the methylene proton at δ_H 3.80 enhanced the AA′BB′ methine protons (H-15, H-19) at δ_H 7.10 which confirmed the substitution pattern in ring B of compound **2**. The ESIMS/MS spectrum gave a fragment ion peak at *m/z* 246 [M-C$_7$H$_7$O]$^+$ due to the loss of a methoxyphenyl

moiety which further confirmed the presence of this substructure. The presence of a 2-amino-4,5-dibenzylimidazole was evident from the chemical shifts of C-2, C-4 and C-5 at δ_C 145.9, 122.7 and 122.4, respectively (Fu *et al*, 1997). The HMBC correlations of NCH_3-21 with C-2 and C-5 proved its attachment at N-3 which was further established through the NOE of the NCH_3 singlet at δ_H 3.12 on the proton (H-8) at δ_H 6.60. However, due to the broadness of the signal at δ_H 6.50 which was assigned for H-12, NOE effect of NCH_3-21 on this proton was not discernible. The major difference between compounds **1** and **2** was that the second AA´BB´ spin system found in naamine A (**1**) for ring C was replaced by an ABC spin system which was comparable with naamine B (Gross *et al*, 2002). The signals at δ_H 6.60 (1H, d, 2.0 Hz), 6.50 (1H, dd, 8.2 and 2.0 Hz), and 6.67 (1H, d, 8.2 Hz) signified the presence of 1,3,4-trisubstituted benzene. An additional methoxyl singlet was also observed at δ_H 3.63 which satisfied the 30 mass units difference in the molecular weight when compared with naamine A. Irradiation of the methoxyl singlet at 3.63, gave a NOE effect at δ_H 6.60 which identified the position of the methoxyl group, OCH_3-22, at C-9. Compound **2** is at present the six member of the group known as naamines and it was thus named **naamine F**.

Naamine F (compound 2)

Chemical name: 4-[2-Amino-5-(4-methoxy-benzyl)-3-methyl-3H-imidazol-4-ylmethyl]-3-methoxy-phenol
Character: yellowish brown amorphous substance
Amount: 1.5 mg   Sample code: TM84 EtOAc fr.10 wash of prep. P2
Molecular Weight: 353 g/mol
Biological source : marine sponge *Leucetta chagosensis*
Molecular formula: $C_{20}H_{23}N_3O_3$

New natural product
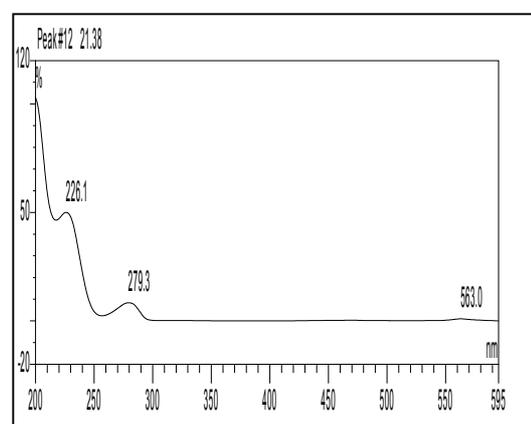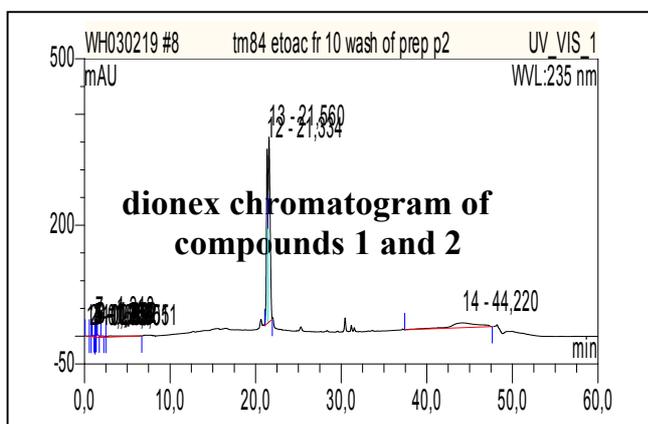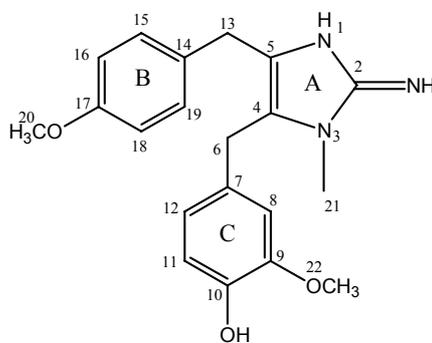
ESIMS: *m/z*, 354 [M+H]$^+$, 707 [2 M+H]$^+$

ESIMSMS: *m/z*, 354 [M+H]$^+$, 246, 231, 215, 190 and 122
HRESIMS: Experimental: 354.1817[M+H]$^+$, calculated: 354.1817 [M+H]$^+$
          for $C_{20}H_{23}N_3O_3$

Table 3.1.2: NMR data of compound 2 (DMSO-d_6, 500 MHz)

No.	δH	δC[a]	HMBC	COSY
1		-	-	-
2	N*H*, 7.35 (br s)	145.9	-	-
3	-	-	-	-
4	-	122.7	-	-
5	-	122.4	-	-
6	3.85 (s)	-	4, 8, 12	-
7	-	-	-	-
8	6.60 (d, 1.89 Hz)	110.3	-	12
9	-	147.3	-	-
10	-	-	-	-
11	6.67 (d, 8.2 Hz)	-	-	12
12	6.50 (dd, 8.2, 1.89 Hz)	127.8	10	8, 11
13	3.80 (s)	-	15, 19	-
14	-	-	-	-
15	7.10 (d, 8.2 Hz)	129.0	-	16
16	6.80 (d, 8.2 Hz)	116.0	16	15
17	-	157.8	-	-
18	6.80 (d, 8.2 Hz)	116.0	-	19
19	7.10 (d, 8.2 Hz)	129.0	18	18
20	3.72 (s)	-	17	-
21	3.12 (s)	-	2, 5	-
22	3.63 (s)	-	9	

[a] carbon assignments were determined from the HMBC spectrum which did not allow a complete assignment.
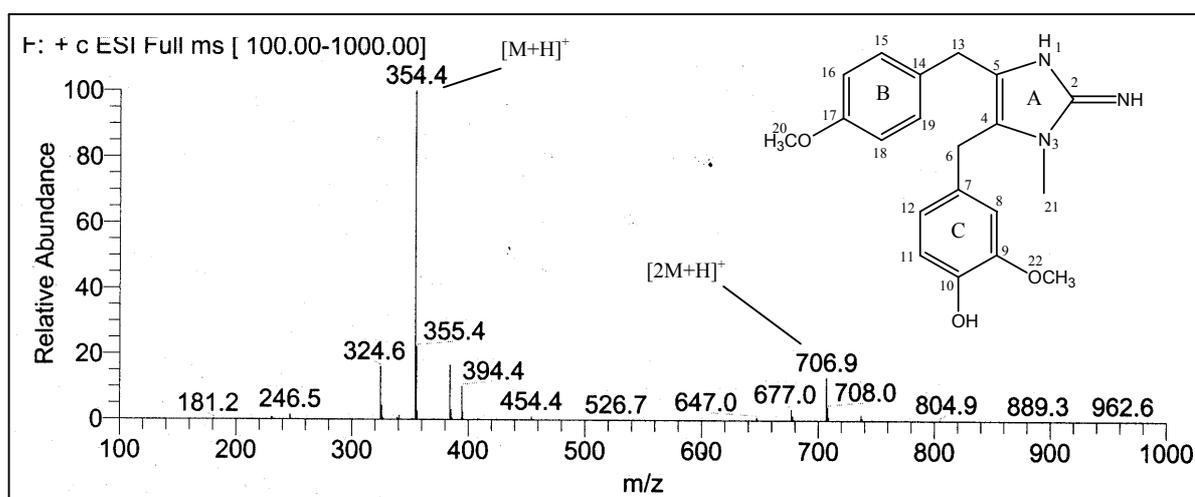
Fig. 3.1.4: ESIMS spectrum of compound 2
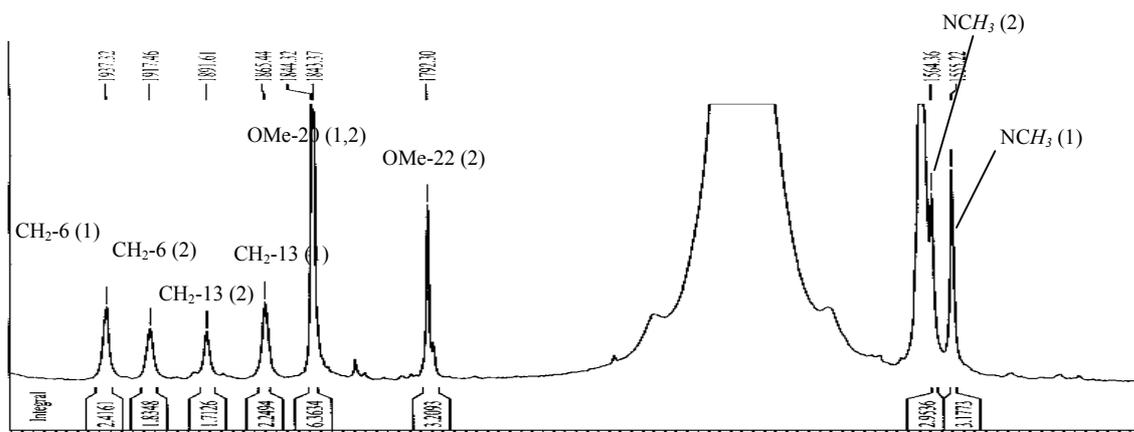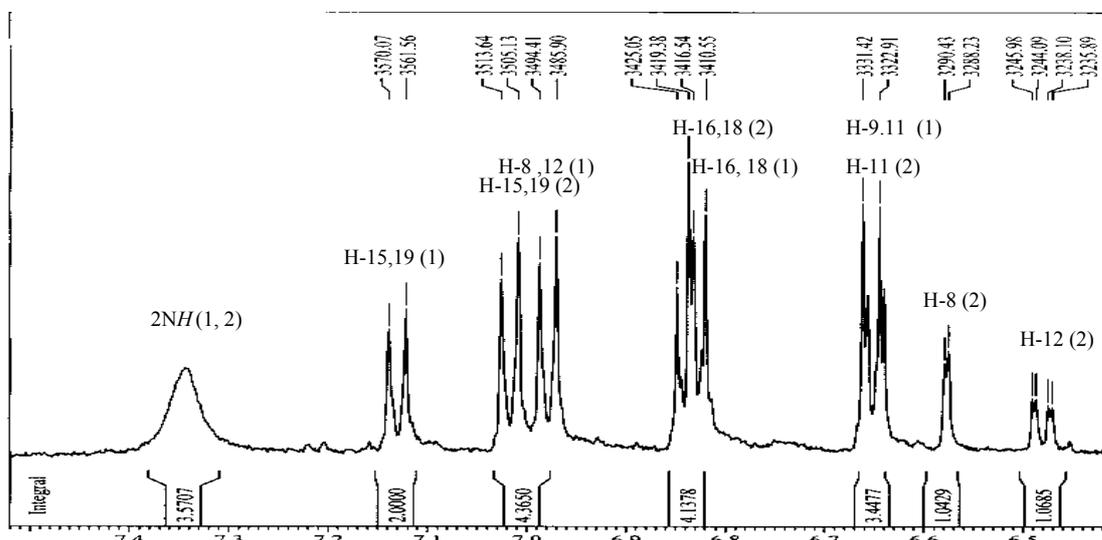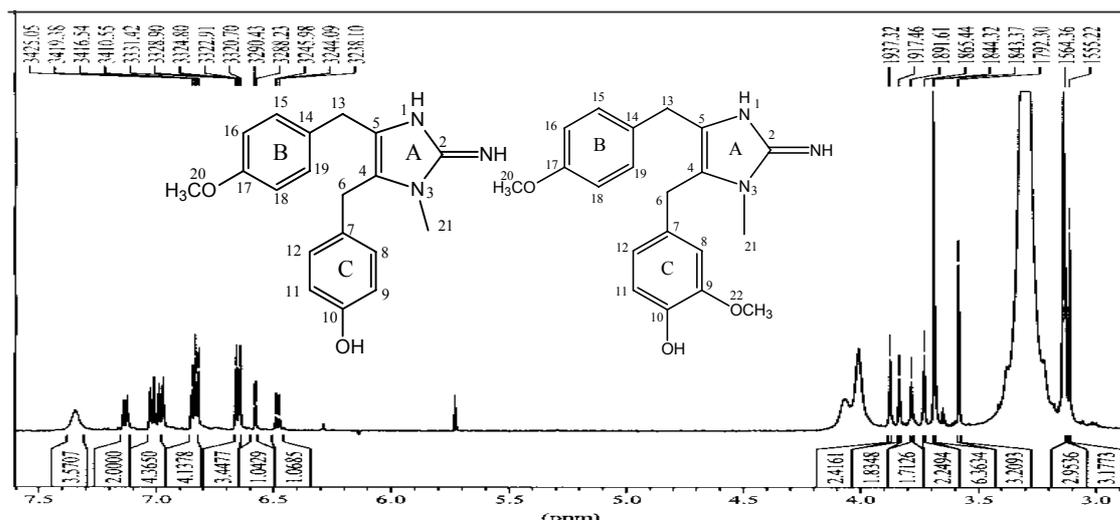
Number of signal (number of compound)

Fig. 3.1.5: ¹H-NMR spectrum of compounds 1 and 2
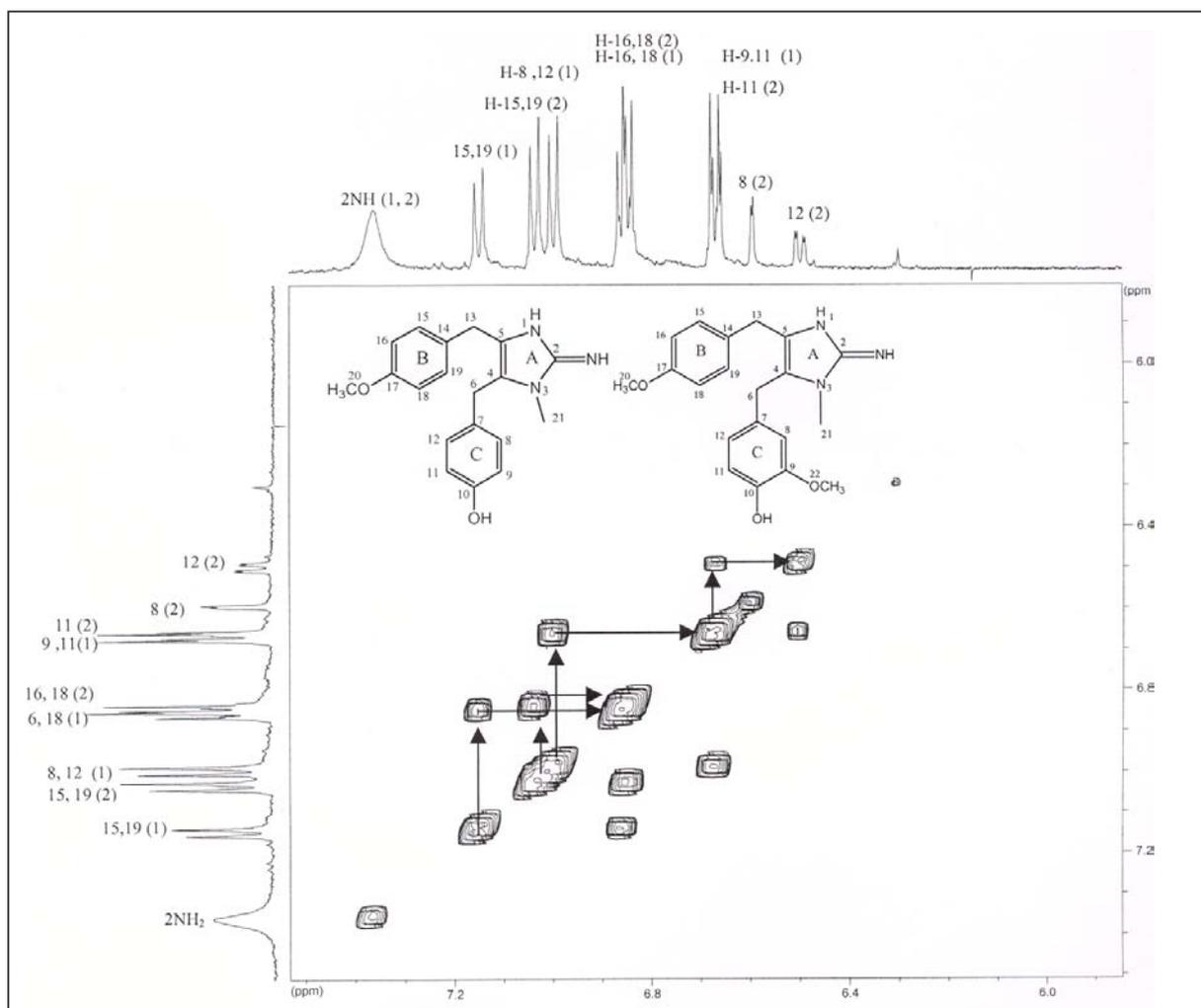
Fig. 3.1.6: COSY spectrum of compounds 1 and 2
Number of signal (number of compound)
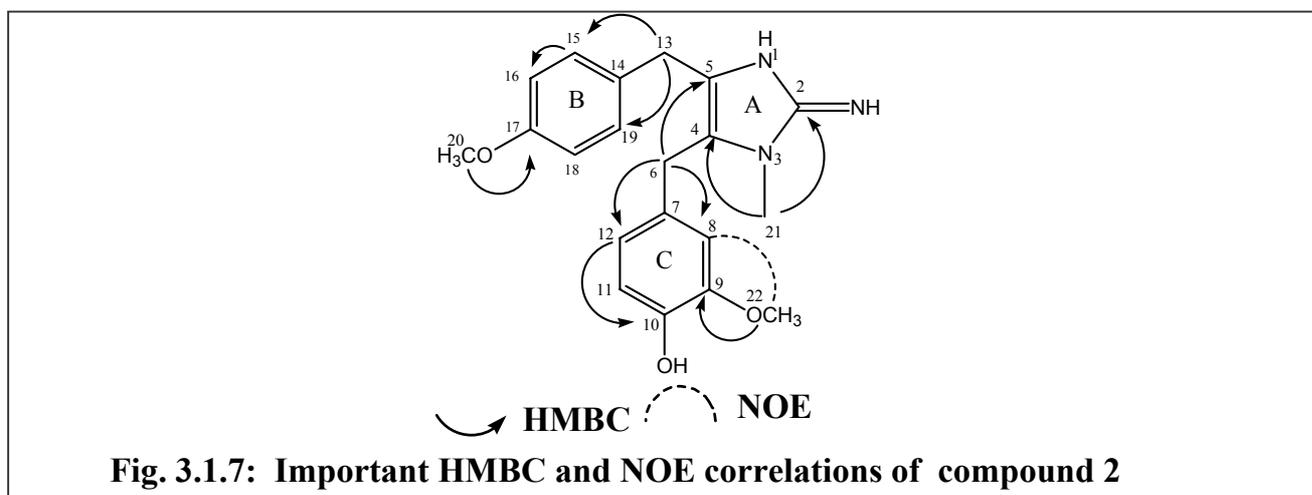
Fig. 3.1.7: Important HMBC and NOE correlations of compound 2

3.1.1.3. Structure elucidation of naamine G (3, new compound)

Naamine G
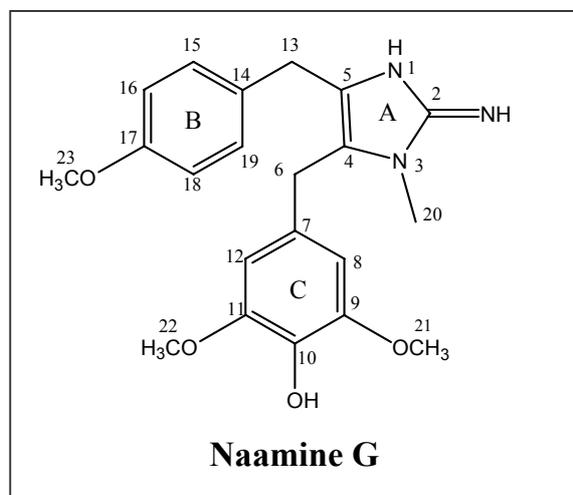
Compound 3 was isolated as a yellowish brown oil, the molecular formula was determined as $C_{21}H_{25}N_3O_4$ from the EIMS spectrum which showed the molecular ion peak at m/z 383 $[M]^+$. This was confirmed by HREIMS (383.18451). The EIMS (Fig. 3.1.8) showed fragmentation peaks at *m/z* 262 $[M-C_8H_9O]^+$ and *m/z* 216 $[M-C_9H_{11}O_3]^+$ due to the subsequent loss of *p*-methoxy-benzyl function (ring B + C*H$_2$*-13) and *p*-hydroxy-3,5-dimethoxy-benzyl residue [ring C + C*H$_2$*-6]. The ^{13}C-NMR and DEPT of compound **3** (Fig. 3.1.10, 11 and Table 3.1.3) showed twenty one carbons which consisted of six doublets, nine singlets, four quartets and two triplets. The ^{1}H-NMR spectrum (Fig. 3.1.9, Table 3.1.3) was very simple and showed signals for twenty five protons at which comprised the three exchngeable protons at δ_H 12.47, δ_H 8.23, and δ_H 7.55; a pair of AA´ BB´ doublets at δ_H 7.17 and 6.85; an aromatic singlet for two protons at δ_H 6.31; two methylene singlets at δ_H 3.86 and δ_H 3.81; methoxy singlets at 3.70 (3H) and δ_H 3.60 (6H); and a N-methyl singlet at δ_H 3.16. Comparison of the NMR data of compound **3** with those of naamines A (**1**) and F (**2**) showed that the compounds were closely related. Inspection of their ^{1}H and ^{13}C-NMR spectra showed that the AA´ BB´ spin system for ring **B** and 3-*N*-methyl imidazole ring were present in all congeners and this was confirmed through a series of NOE experiments. Compound **3** however, contained a symmetrically 1,3,4,5-substituted benzyl moiety which is comparable to that of naamine E (Gross *et al*, 2002). The symmetry of ring **C** was evidenced from the ^{1}H-NMR singlet signals observed at δ_H 3.60 which integrated

for two OCH_3 functions and two aromatic methine protons at δ_H 6.31 which gave both a HMQC and HMBC cross peak with the methine carbon at δ_C 105.5. The ^{1}H-NMR data for ring **C** were comparable with the ^{13}C-NMR spectral data which consisted of a quaternary signal at δ_C 148.1 for C-9 and C-11, a methine signal at δ_C 105.5 for C-8 and C-12, and a methyl signals at δ_C 55.8 for OCH_3-21 and OCH_3-22. Through the HMBC spectrum, the attachment of the methoxyl groups was established to be at C-9 and C-11. This was shown by the HMBC correlations of the methine singlet at δ_H 6.31 (H-8 and H-12) with the methylene carbon at δ_C 27.9 (C-6) and also with quaternary carbons at δ_C 127.0 (C-7), δ_C 134.2 (C-10), and δ_C 148.1 (C-9, C-11). The carbon resonance at δ_C 148.1 further gave a correlation with the methoxyl singlets at δ_H 3.60 assigned to OCH_3-21 and OCH_3-22. The NOE experiment finally confirmed the substitution pattern of ring **C**. Irradiation of the methyl singlet at δ_C 3.60 for OCH_3-21 and OCH_3-22 gave an enhancement of the methine singlet at δ_H 6.30 for H-8 and H-12. Compound **3**, named **naamine G**, has a similar disposition of the oxygen substituents as naamine E (Gross *et al*, 2002) but differed in the proportions of hydroxyl and methoxyl groups.

Naamine G (compound 3)

Chemical name: 4-[2-Amino-5 -(4-methoxy-benzyl)-3-methyl-3 H-imidazol-4-ylmethyl]-3,5-dimethoxy-phenol

Character: yellowish brown oil   **Amount**: 7.2 mg

Sample code: TM084 But. 9-10   **Molecular Weight**:   383 g / mol

Biological source: marine sponge *Leucetta chagosensis* (TM 084)

Molecular formula: $C_{21}H_{25}N_3O_4$

New natural product
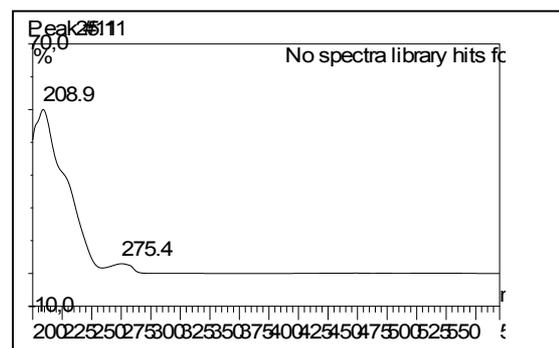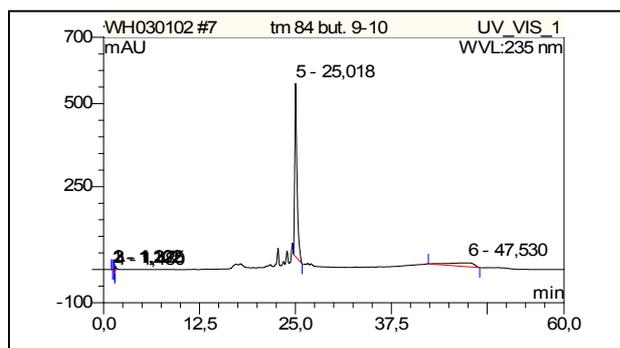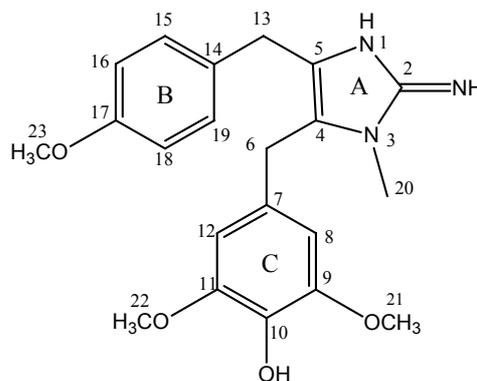
ESIMS: *m/z* 384 [M+H]$^+$ (Positive)

EI/MS (*m/z*, rel. int.) : 383 [M]$^+$ (100), 353 (20), 331 (2), 262 (7), 230 (12), 216 (36), 186 (6), 167 (7), 143 (11), 121 (14), 84 (17), 69 (69), and 44 (94).

HREIMS: *m/z* = 383   [M]$^+$ Experimental = 383.1848
                                Calculated   = 383.1845
                                 for $C_{21}H_{25}N_3O_4$

Table 3.1.3: ^{1}H, ^{13}C-NMR and HMBC correlations of compound 3 (DMSO -d_6, 500 MHz)

No.	δH	δC	HMQC	HMBC
1	-	-	-	-
2 N*H*	7.55 (s)	146.2 s	-	-
3	-	-	NC*H$_3$*	-
4	-	121.7 s	-	-
5	-	122.1 s	-	-
6	3.86 (s)	27.9 t	6	4, 8, 12
7	-	127.0 s	-	-
8	6.31 (s)	105.5 d	8	7, 9, 10, 12
9	-	148.1 s	-	-
10 O*H*	8.23 (br s)	134.2 s	-	-
11	-	148.1 s	-	-
12	6.31 (s)	105.5 d	12	7, 8, 9, 10
13	3.81 (s)	27.6 t	13	5, 15, 19
14	-	130.3 s	-	-
15	7.17 (d, 8.5 Hz)	129.3 d	15	17, 19
16	6.85 (d, 8.5 Hz)	113.9 d	16	15, 17, 18
17	-	157.9 s	-	-
18	6.85 (d, 8.5 Hz)	113.9 d	18	15, 16, 17
19	7.17 (d, 8.5 Hz)	129.3 d	19	15, 17
20	3.16 (s)	29.4 q	20	2, 5
21	3.60 (s)	55.8 q	21	9
22	3.60 (s)	55.8 q	22	11
23	3.70 (s)	55.1 q	23	17
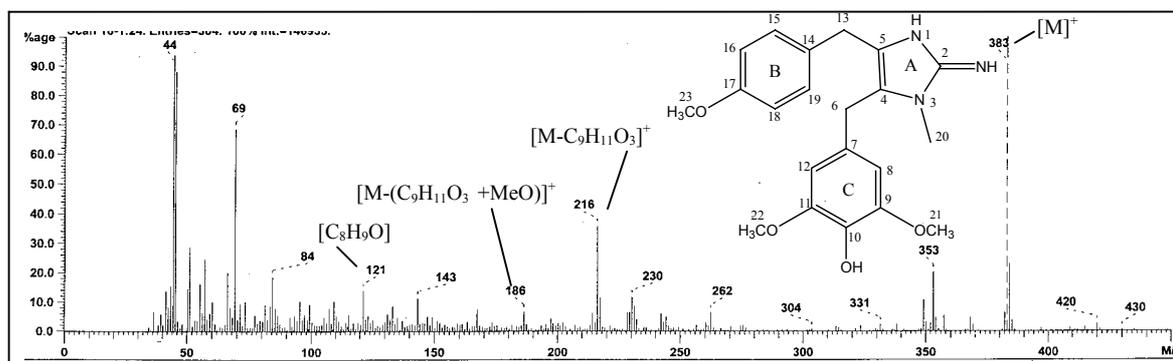
Fig. 3.1.8 : EIMS spectrum of compound 3
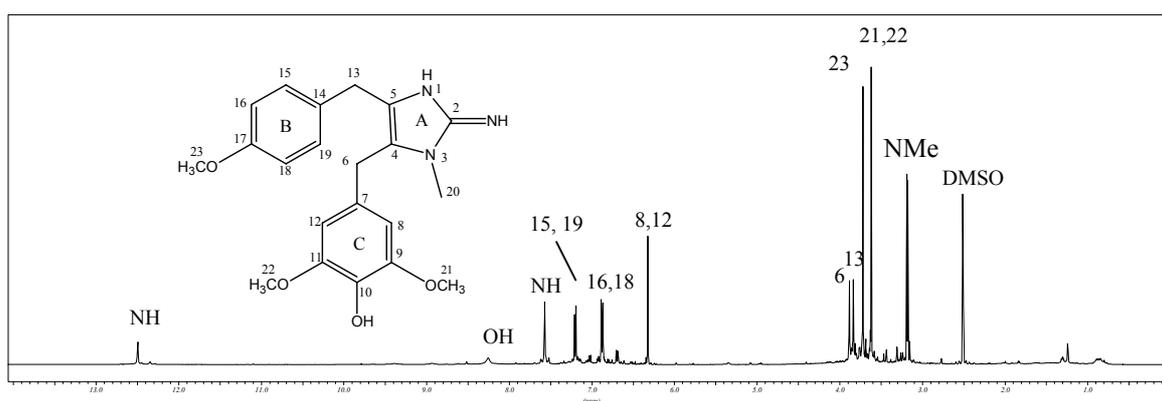
Fig. 3.1.9: ¹H-NMR spectrum of compound 3
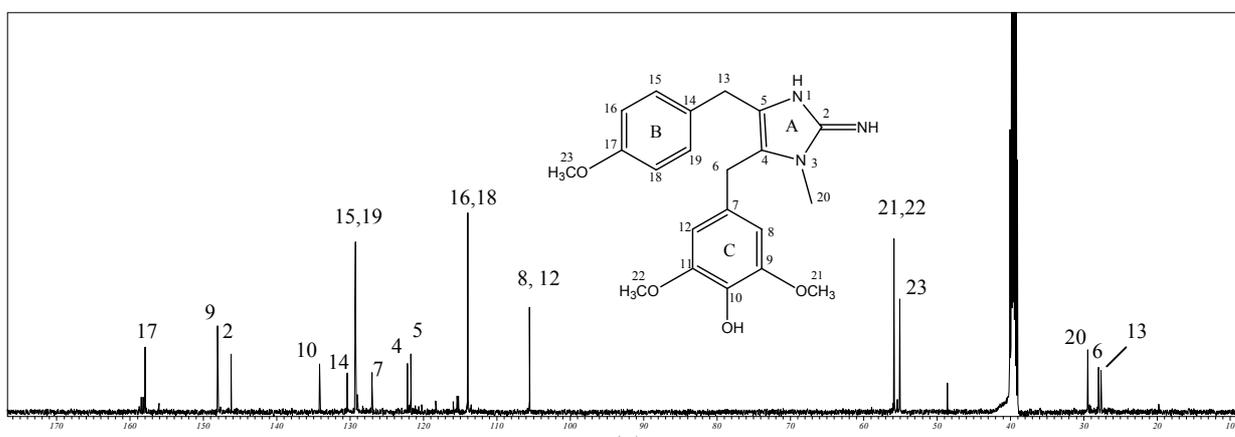
Fig. 3.1. 10: ¹³C-NMR spectrum of compound 3
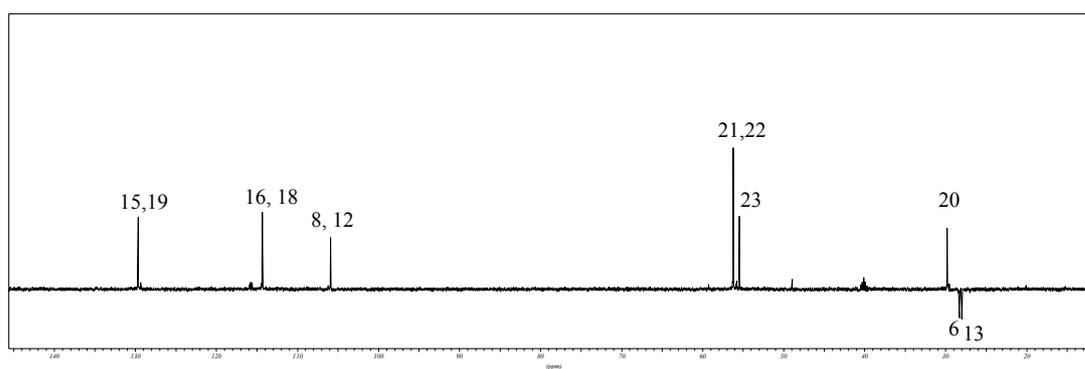
Fig. 3.1.11 : DEPT spectrum of compound 3
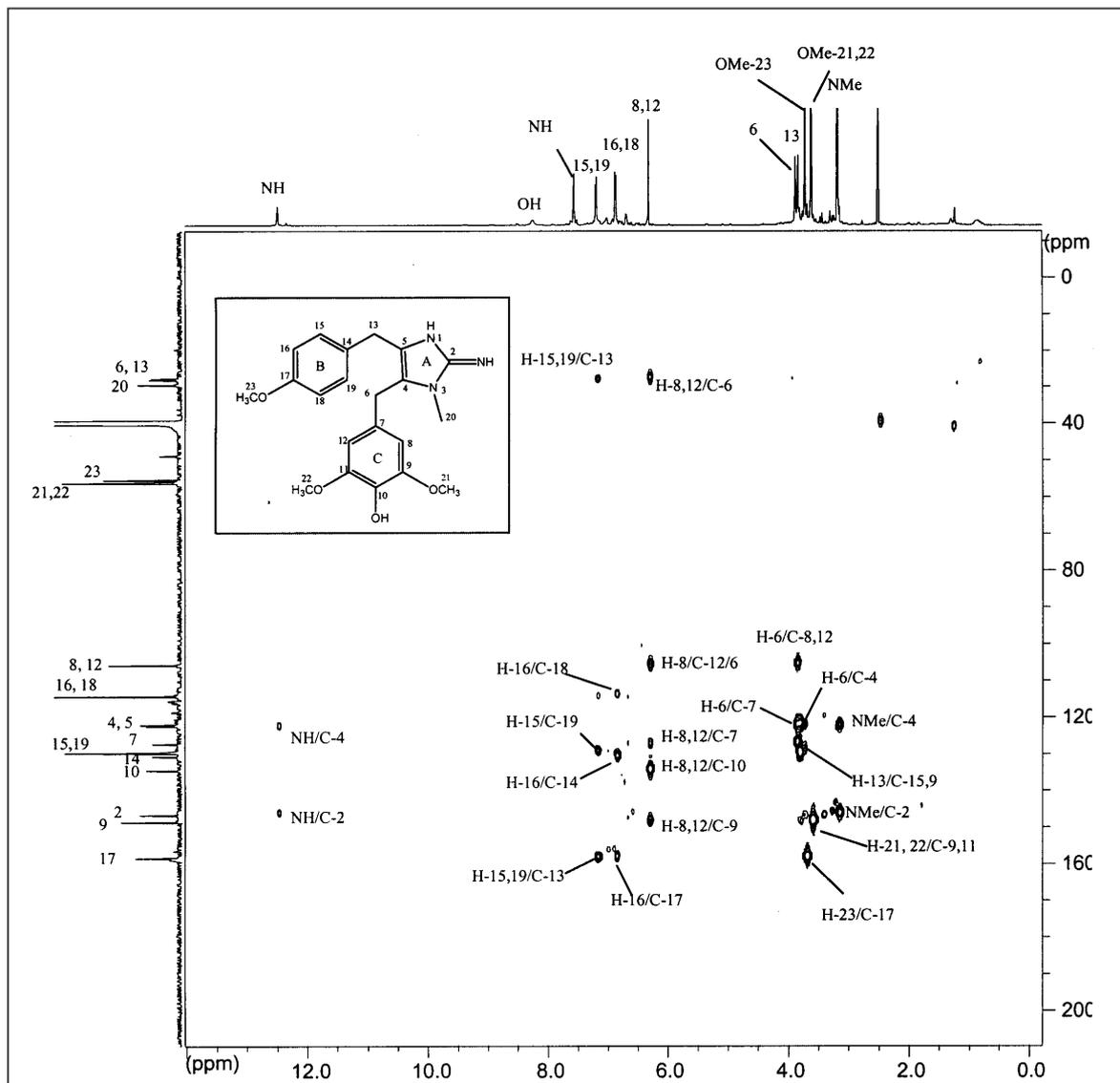
Fig. 3.1.12: HMBC spectrum of compound 3
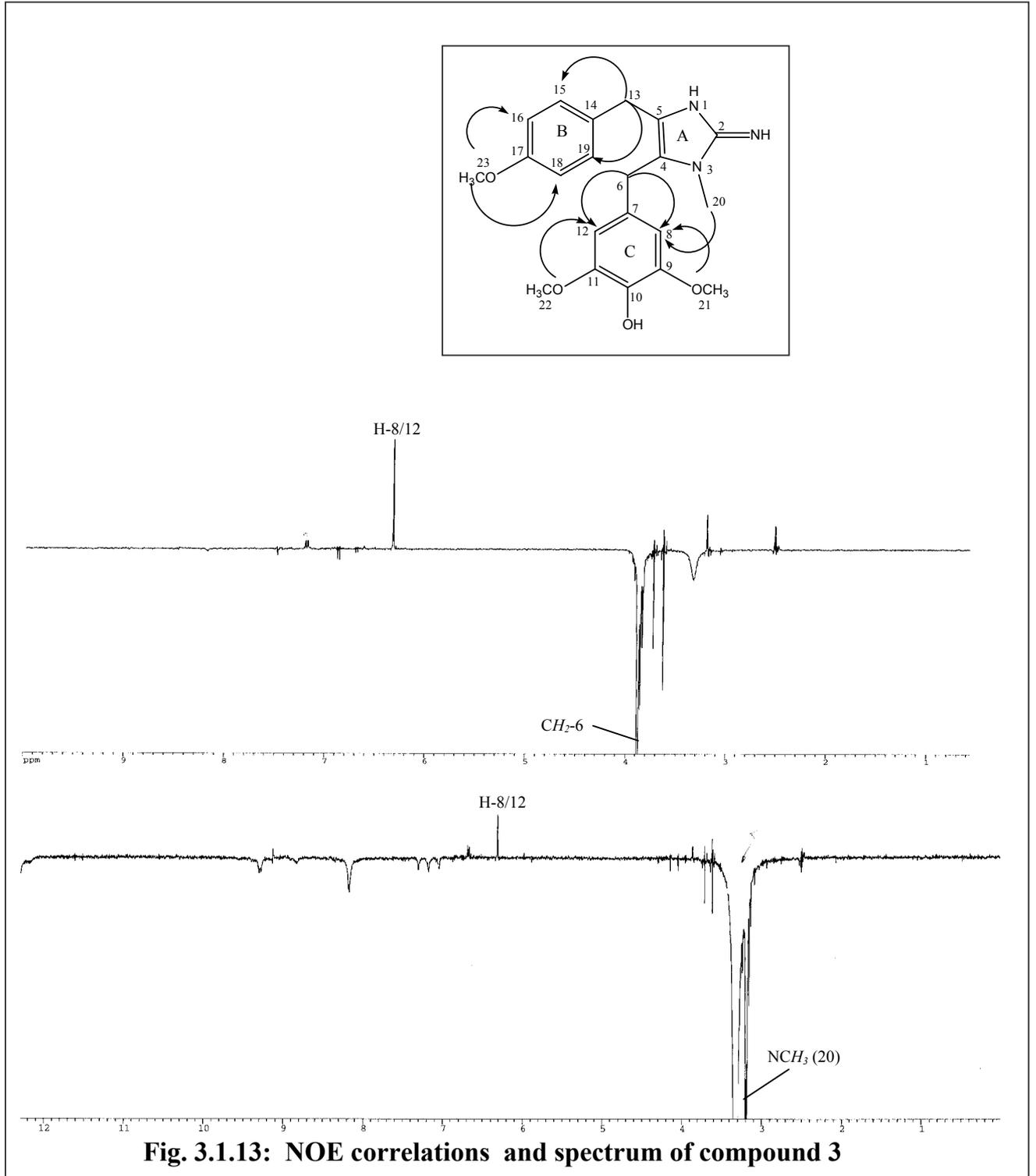
Fig. 3.1.13: NOE correlations and spectrum of compound 3

3.1.1.4. Structure elucidation of kealiinine A (4, new compound)

Kealiinine A
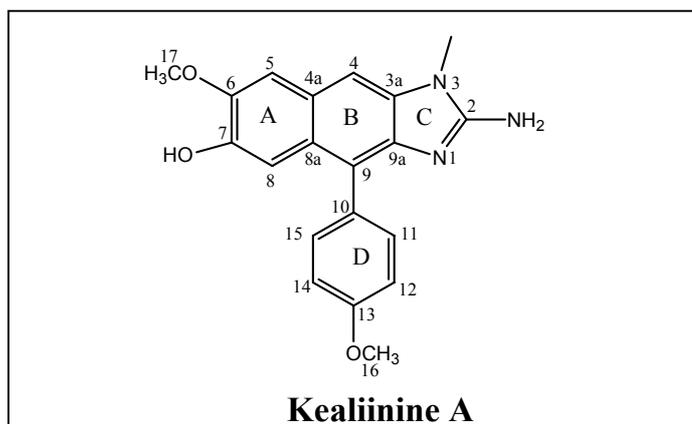
Compound 4 was isolated as a yellowish brown powder. The ESIMS (Fig. 3.1.15) showed a pseudo-molecular ion peak at *m/z* 350 [M+H]$^+$ corresponding to the molecular formula $C_{20}H_{19}N_3O_3$ as determined by HRESIMS (m/z 350.1507 [M+H]$^+$). The ^{1}H- and ^{13}C-NMR data of 4 (Table 3.1.4) were comparable to those of the naamines and those of 2-aminokealiiquinone (Fu *et al*, 1997). Inspection of the ^{1}H- and ^{13}C-NMR spectral data of compound 4 showed that the AA'BB' spin system for ring D and the 1-*N*-methyl imidazole ring were also present. Although shifted to low field, the ^{1}H-NMR signals at δ_H 7.35 (2H, d, 8.5 Hz) and δ_H 7.18 (2H, d, 8.5 Hz) also indicated the presence of an AA'BB' spin system. The presence of the 1-*N*-methyl imidazole ring was confirmed from the ^{13}C-NMR quaternary signals at δ_C 152.0, δ_C 125.0, δ_C 129.5, and the N*CH$_3$* resonance at δ_C 28.1. The attachment of the methyl function at N-3 was established from the HMBC correlations of the N*CH$_3$* singlet signal at δ_H 3.67 with the quaternary carbon signals at δ_C 152.0 (C-2) and δ_C 129.5 (C-3a) in addition to its ROESY correlation with that of the methine singlet at δ_H 7.71 for H-4. The ^{13}C-NMR spectrum of 4 showed the presence of twenty carbons which consisted of ten quaternary carbons, seven methine carbons, and three methyl carbons as determined from its DEPT spectrum. The ^{13}C-NMR data of compound 4 revealed a structural similarity with 2-aminokealiiquinone except for the replacement of the two carbonyl groups at δ_C 181.8 and δ_C 182.3 ppm by two methine carbons at δ_C 106.5 (C-5) and δ_C 107.0 (C-8) which was compatible with the methine singlets at δ_H 7.40 (H-5) and δ_H 6.95 (H-8) in its ^{1}H-NMR spectrum as shown by their direct correlations in the

HMQC spectrum. The hydrogenation occurring at C-8 caused an upfield shift for C-9 to 118.5 ppm compared to 129.5 ppm in 2-aminokealiiquinone. The loss of the keto functions was confirmed from the HMBC spectrum (Fig. 3.1.18) which showed correlations of δ_H 7.40 (H-5) with C-4, C-4a, C-6, and C-7 (δ_C 104.4, 125.0, 148.1, and 146.5, respectively) and δ_H 6.95 (H-8) with C-6, C-7, C-8a, and C-9 (δ_C 148.1, 146.5, 124.8, and 118.5, respectively). This was further established from the ROESY correlations of δ_H 7.40 (H-5) with the singlet at δ_H 7.71 (H-4) and of δ_H 6.95 (H-8) with the AA'BB' doublet at δ_H 7.35 (H-11/15) in ring D. Apart from the additional aromatic methine singlets, the ^{1}H-NMR data of **4** was comparable to that of 2-aminokealiiquinone. The absence of a third methoxyl singlet suggested that the OC*H*$_3$ attached at C-10 in 2-aminokealiiquinone had been replaced by a hydroxyl function that afforded a broad singlet signal at δ_H 9.49 in kealiinine A (**4**). In kealiinine A, the methyl signals at δ_H 3.67, 3.88, and 3.90 corresponded to the presence of a NC*H*$_3$, and two OC*H*$_3$ functions, respectively. The attachment of the methoxyl groups were determined from the HMBC spectrum through the correlations of δ_H 3.90 (OC*H*$_3$-17) with δ_C 148.1 for C-6 of ring A and of δ_H 3.88 (OC*H*$_3$-16) with δ_C 159.0 for C-13 of ring D. This was further confirmed by the ROESY correlations of the methoxyl singlets at δ_H 3.90 and δ_H 3.88 with the methine singlet δ_H 7.40 for H-5 and with the AA'BB' doublet protons at δ_H 7.18 for H-12/14, respectively. The HMBC correlations of δ_H 7.35 (H-11/15) with δ_C 118.5 (C-9) and δ_C 159.0 (C-13) confirmed the attachment of ring D at C-9. Irradiation of δ_H 7.35 (H-11/15) showed an enhancement of δ_H 6.95 (H-8) confirming ring D is attached at C-9. Compound **4** is the first member of a new class of compounds related to kealiiquinone and was thus named **kealiinine A**.

Fig. 3.1.14: HMBC and ROESY correlations of compound 4
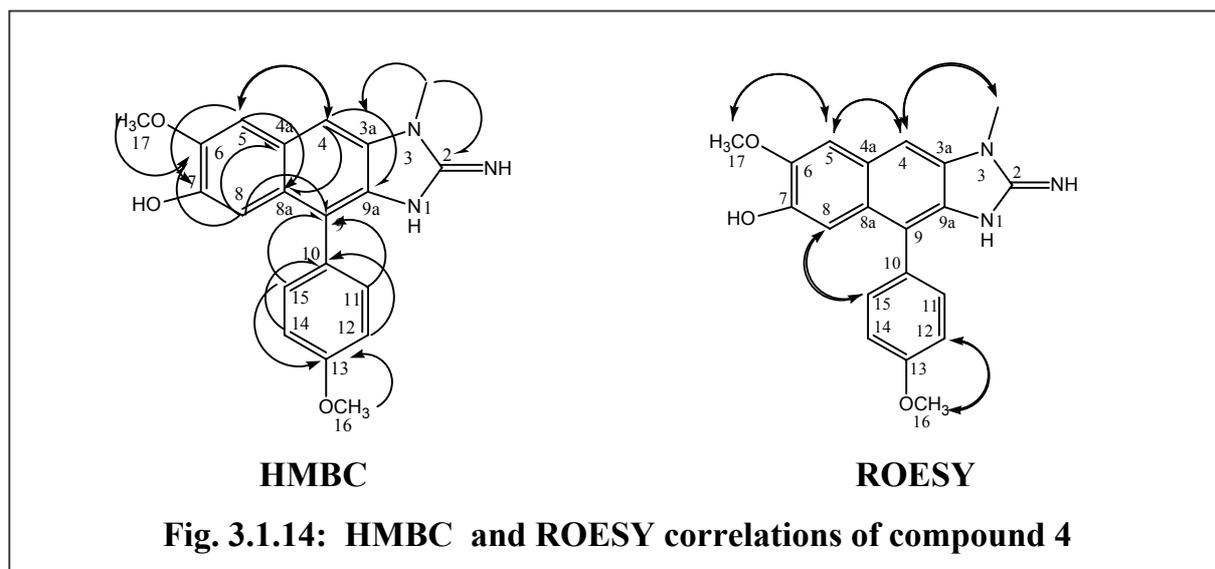
Table 3.1.4: ^{1}H and ^{13}C-NMR data and HMBC correlations of compound 4 (DMSO-d$_6$, 500 and 400 MHz)

No.	δH	δC	HMBC
2	N*H* 8.30 (s)	152.0 s	-
3	NC*H$_3$* 3.67 (s)	28.1 q	3a , 2
3a	-	129.5 s	-
4	7.71 (s)	104.4 d	5, 8a , 9a
4a	-	125.0 s	-
5	7.40 (s)	106.5 d	4 , 4a , 6, 7
6	-	148.1 s	-
7	O*H*, 9.49 (s)	146.5 s	-
8	6.95 (s)	107.0 d	6 , 7, 8a, 9
8a	-	124.8 s	-
9	-	118.5 s	-
9a	-	125.0 s	-
10	-	127.0 s	-
11	7.35 (d, 8.5 Hz)	131.5 d	9, 13, 15
12	7.18 (d, 8.5 Hz)	114.2 d	10, 13, 14
13	-	159.0 s	-
14	7.18 (d, 8.5 Hz)	114.2 s	10, 12, 13
15	7.35 (d, 8.5 Hz)	131.5 s	9, 11, 13
16	3.88 (s)	55.5 q	13
17	3.90 (s)	55.8 q	6

Kealiinine A (compound 4)

Chemical name: 2-Amino-6-methoxy-9-(13-methoxy-phenyl)-3-methyl-3H-naphtho[2,3-d] imidazol-7-ol

Character: yellowish brown powder   **Amount:** 2.1 mg

Sample code: TM84 EtOAc fr.10 P5   **Molecular weight:** 349 g/mol

Biological source: marine sponge *Leucetta chagosensis*

Molecular formula: $C_{20}H_{19}N_3O_3$

New natural product
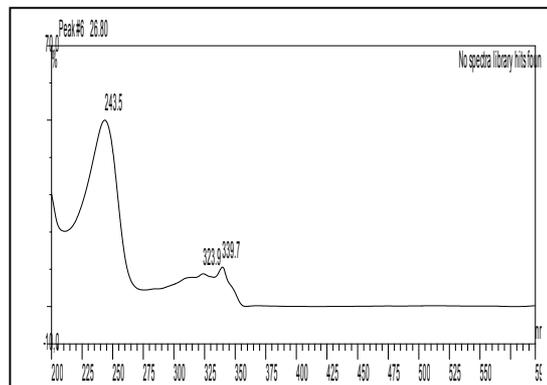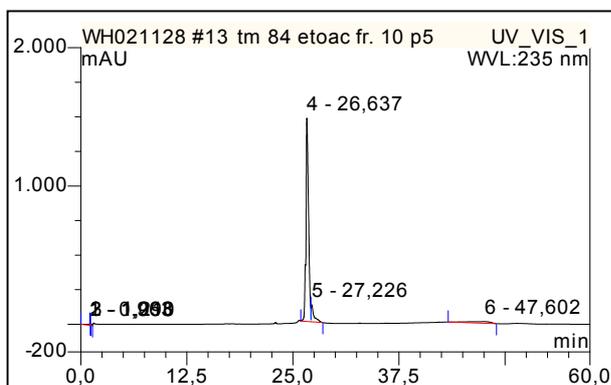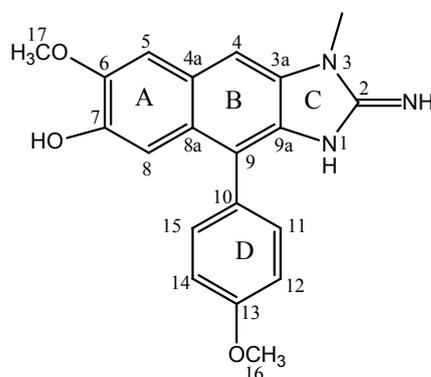
ESIMS: 350 $[M+H]^+$, 699 $[2M+H]^+$ Positive

EIMS: *m/z* (rel. Int.) 349 (6), 334 (2), 253 (3), 231 (3), 216 (5), 151 (19), 121 (5), 84 (15), 69 (59) and 45 (100).

HRESIMS: Experimental: 350.1507$[M+H]^+$, calculated: 350.1504 $[M+H]^+$ for $C_{20}H_{19}N_3O_3$
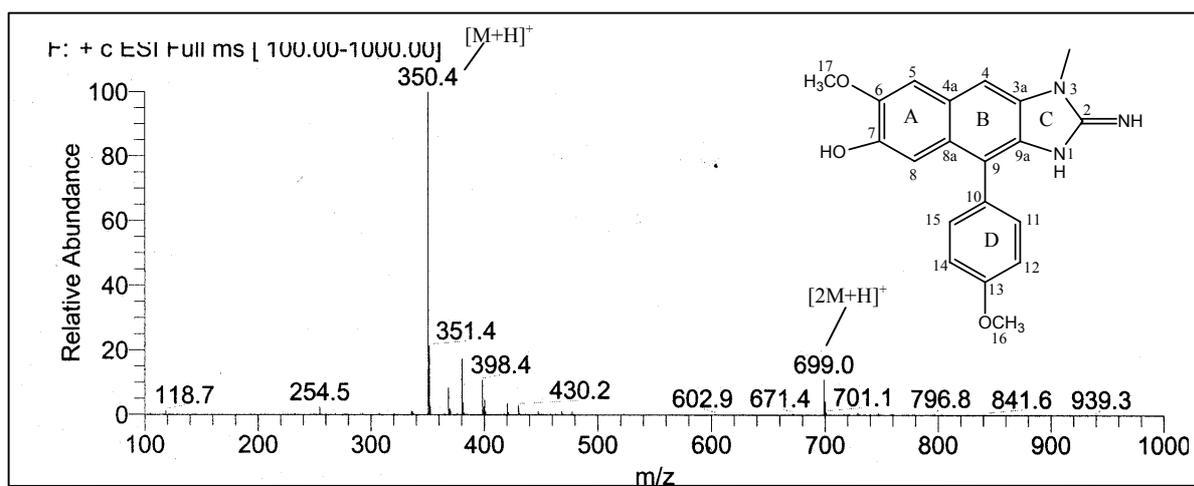
Fig. 3.1.15: ESIMS spectrum of compound 4
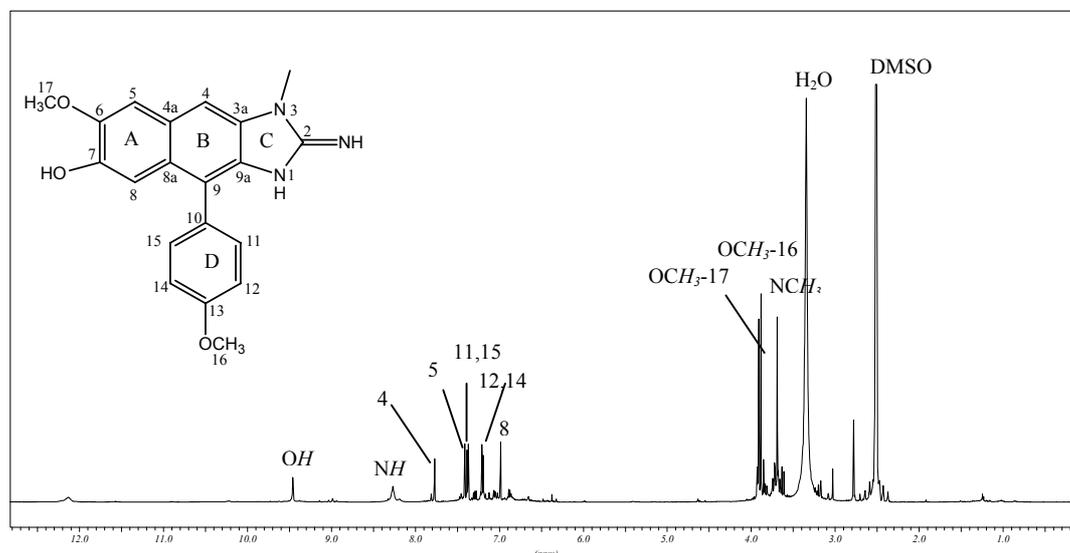
Fig. 3.1.16: ¹H-NMR spectrum of compound 4

Fig. 3.1.17: ROESY spectrum of compound 4

Fig. 3.1.18: HMBC spectrum of compound 4
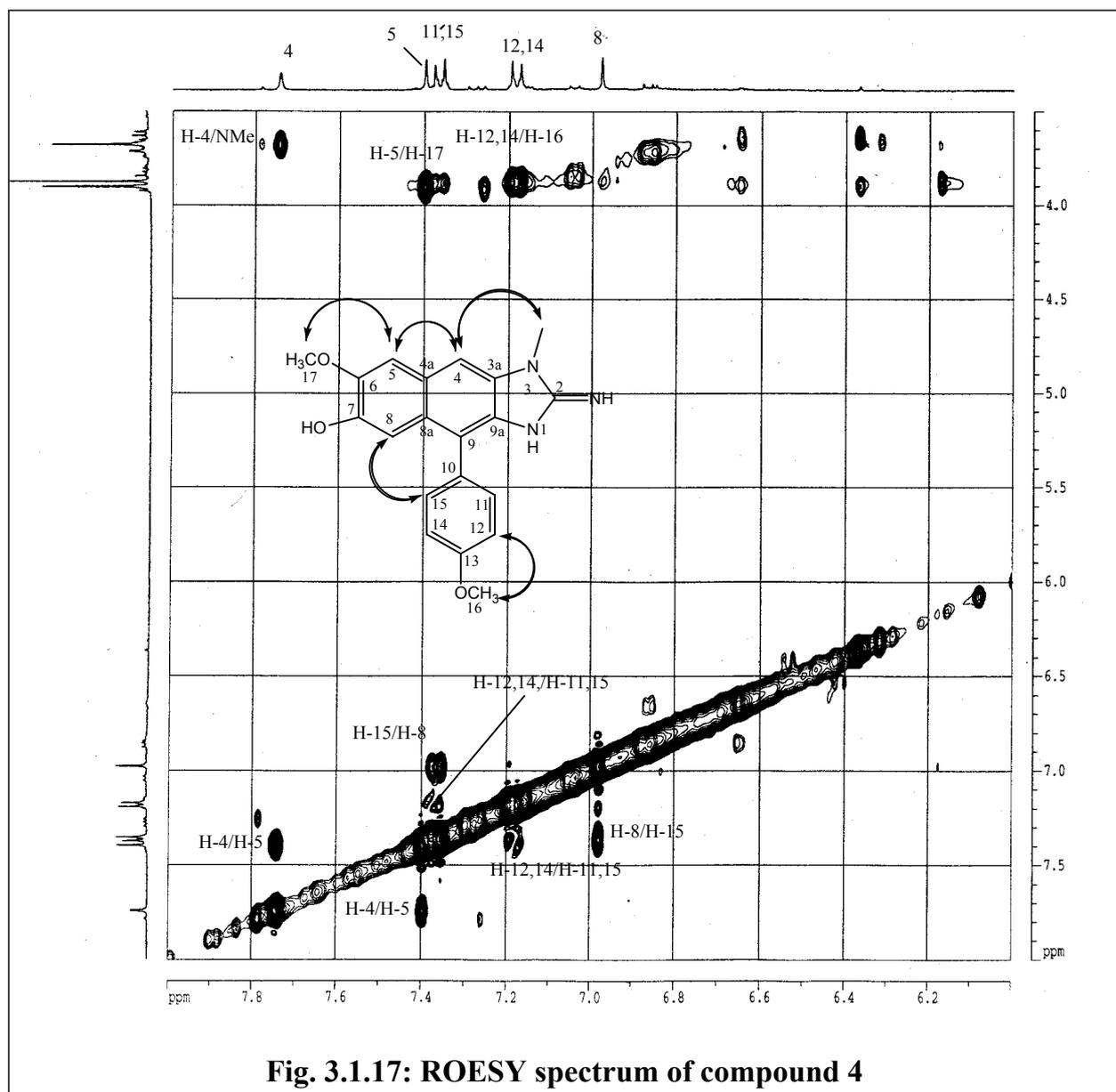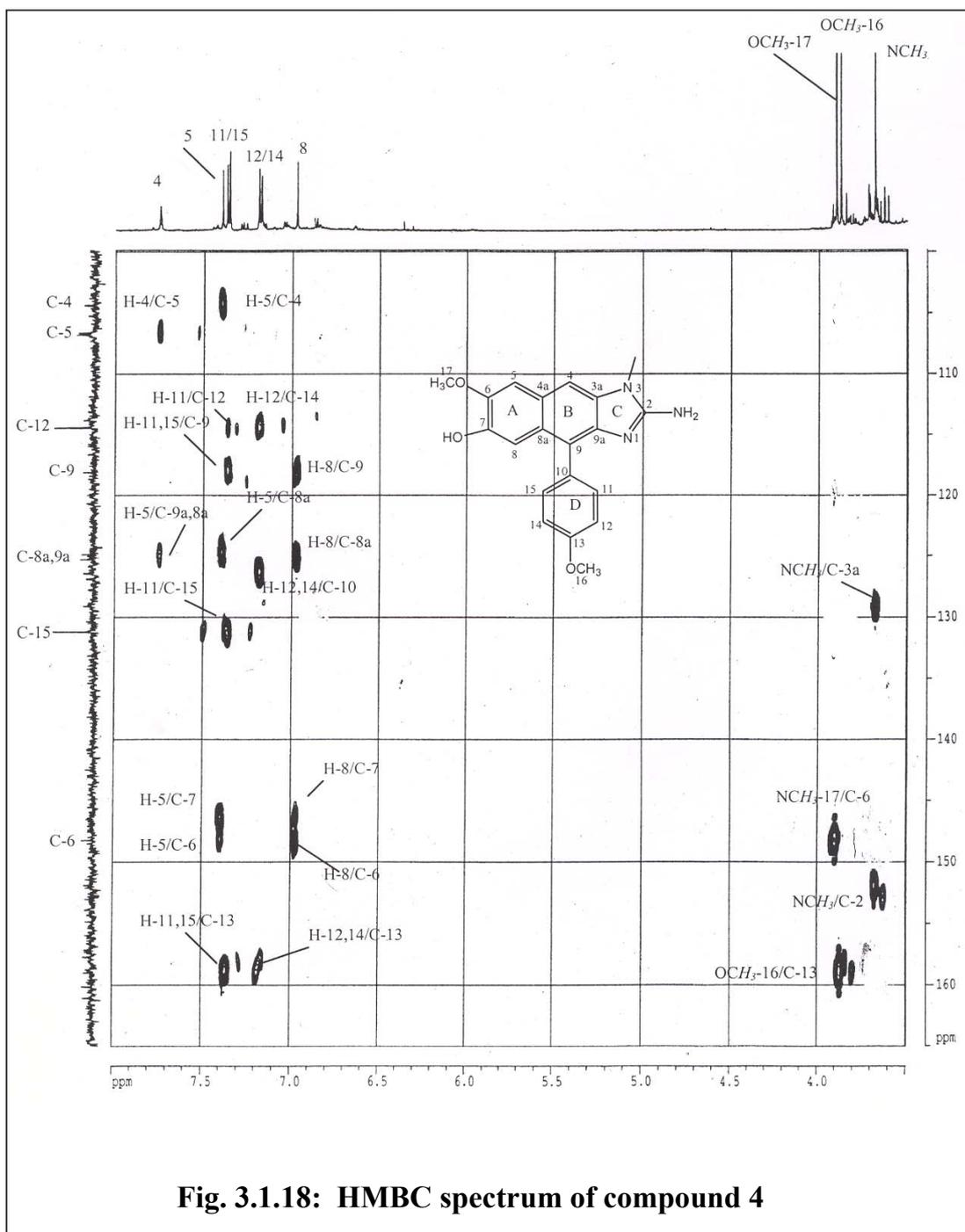
3.1.1.5. Structure elucidation of kealiinine B (5, new compound)

Kealiinine B
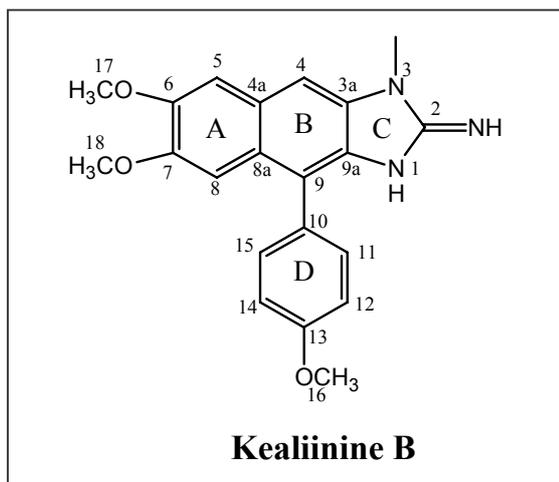
     Compound 5 was isolated as a dark brown amorphous solid. The ESIMS spectrum (Fig. 3.1.20) showed a pseudo-molecular ion peak at *m/z* 364 [M+H]$^+$ corresponding to the molecular formula $C_{21}H_{21}N_3O_3$. Its UV spectrum, ^{1}H-NMR, COSY, and NOE data (Table 3.1.5) showed that it is closely related to kealiinine A (**4**). Due to the very small yield, the structure could only be established from a series of 1D ^{1}H NOE performed on all the protons observed in the ^{1}H-NMR spectrum. This spectrum showed three aromatic methine singlet signals at δ_H 7.90, δ_H 7.47, and δ_H 7.10 for H-4, H-5, and H-8, respectively and four methyl singlets at δ_H 3.79, δ_H 3.96, δ_H 4.02, and δ_H 3.77 which were assigned to NCH_3, OCH_3-16, OCH_3-17 and OCH_3-18, respectively. Detailed comparison of the ^{1}H-NMR spectrum (Table 3.1.5) of compound **5** with that of kealiinine A, revealed that the two compounds were quite similar, except for the presence of an additional methoxyl group. This suggested the possible methylation of the hydroxyl function at C-7 which was also compatible to the 14 mass units difference in molecular weight between kealiinine A (**4**) and the latter compound. This was confirmed as irradiation of the singlet at δ_H 3.77 gave an enhancement of the methine singlet at δ_H 7.10 (H-8) which confirmed its attachment to C-7. The similarity between compound **5** and kealiinine A was also revealed by the almost identical results of the NOE experiments performed on both compounds. Irradiation of the methyl protons at δ_H 3.79 (NCH_3), δ_H 3.96 (OCH_3-16), and δ_H 4.02 (OCH_3-17) enhanced the methine singlets for H-4 (δ_H 7.90), H-12/14 (δ_H 7.24) and H-5 (δ_H 7.47) while irradiation of these methine singlets gave the same NOE effects on

the complimentary methyl resonances. The NOE effect of H-11/15 on H-8 also confirmed that ring D is bound at C-9. From these data, it was concluded that compound **5** is the 10-methoxylated congener of kealiinine A and was named **kealiinine B**.

Fig. 3.1.19: NOE correlations of compound 5
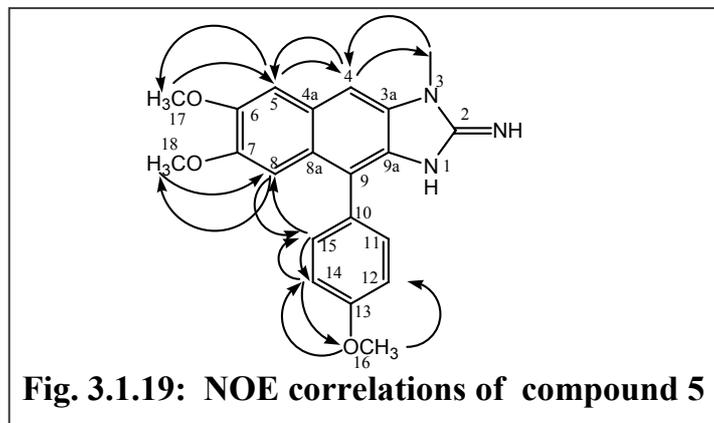
Kealiinine B (compound 5)

Chemical name: 6,7-Dimethoxy-9-(13-methoxy-phenyl)-3-methyl-3H-naphtho[2,3-d]imidazol-2-ylamine
Character: dark brown amorphous solid   **Amount :** 2.2 mg
Sample code: TM84 EtOAc fr.10 P6   **Molecular Weight:** 363 g/mol
Biological source: marine sponge *Leucetta chagosensis*
Molecular formula : $C_{21}H_{21}N_3O_3$

New natural product
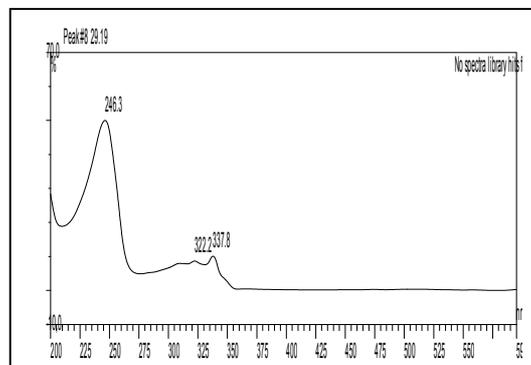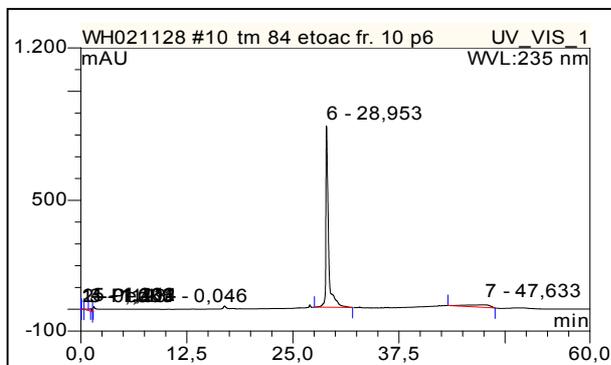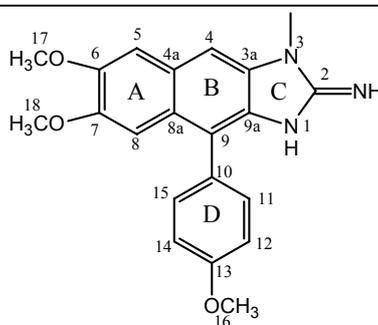
ESIMS: *m/z* 364 [M+H]$^+$, 727 [2M+H]$^+$ Positive
HRESIMS: Experimental, 364.1650 [M+H]$^+$,   calculated, 364.1661 [M+H]$^+$,
          for $C_{21}H_{21}N_3O_3$

Table 3.1.5: NMR data of compound 5 (MeOD, 400, 500 MHz)

Proton No.	δH	COSY	NOE	
			Proton irradiated	**Proton enhanced**
1	-	-	-	-
2	-	-	-	-
3	NCH_3 3.79 (s)	-	NCH_3	4
3a	-	-	-	
4	7.90 (s)	5	4	NCH_3, 5
4a	-	-	-	
5	7.47 (s)	4	5	5, 17
6	-	-	-	-
7	-	-	-	-
8	7.10 (s)	5	8	11, 15, 18
8a	-	-	-	-
9	-	-	-	-
9a	-	-	-	-
10	-	-	-	-
11	7.47 (d, 8.5 Hz)	12	-	-
12	7.24 (d, 8.5 Hz)	11	-	-
13	-	-	-	-
14	7.24 (d, 8.5 Hz)	15	14	16, 15
15	7.47 (d, 8.5 Hz)	14	15	14, 8
16	3.96 (s)	-	16	12, 14
17	4.02 (s)	-	17	5
18	3.77 (s)	-	18	8
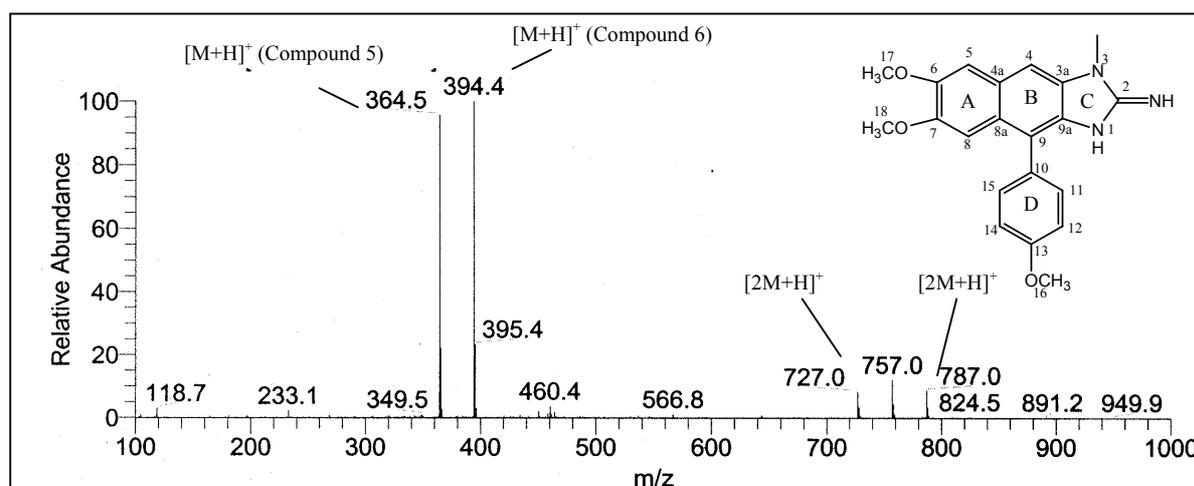
Fig. 3.1.20: ESIMS spectrum of a mixture of compounds 5 and 6

Fig. 3.1.21: ^{1}**H-NMR spectrum of compounds 5 and 6**
Number of signal (number of compound)
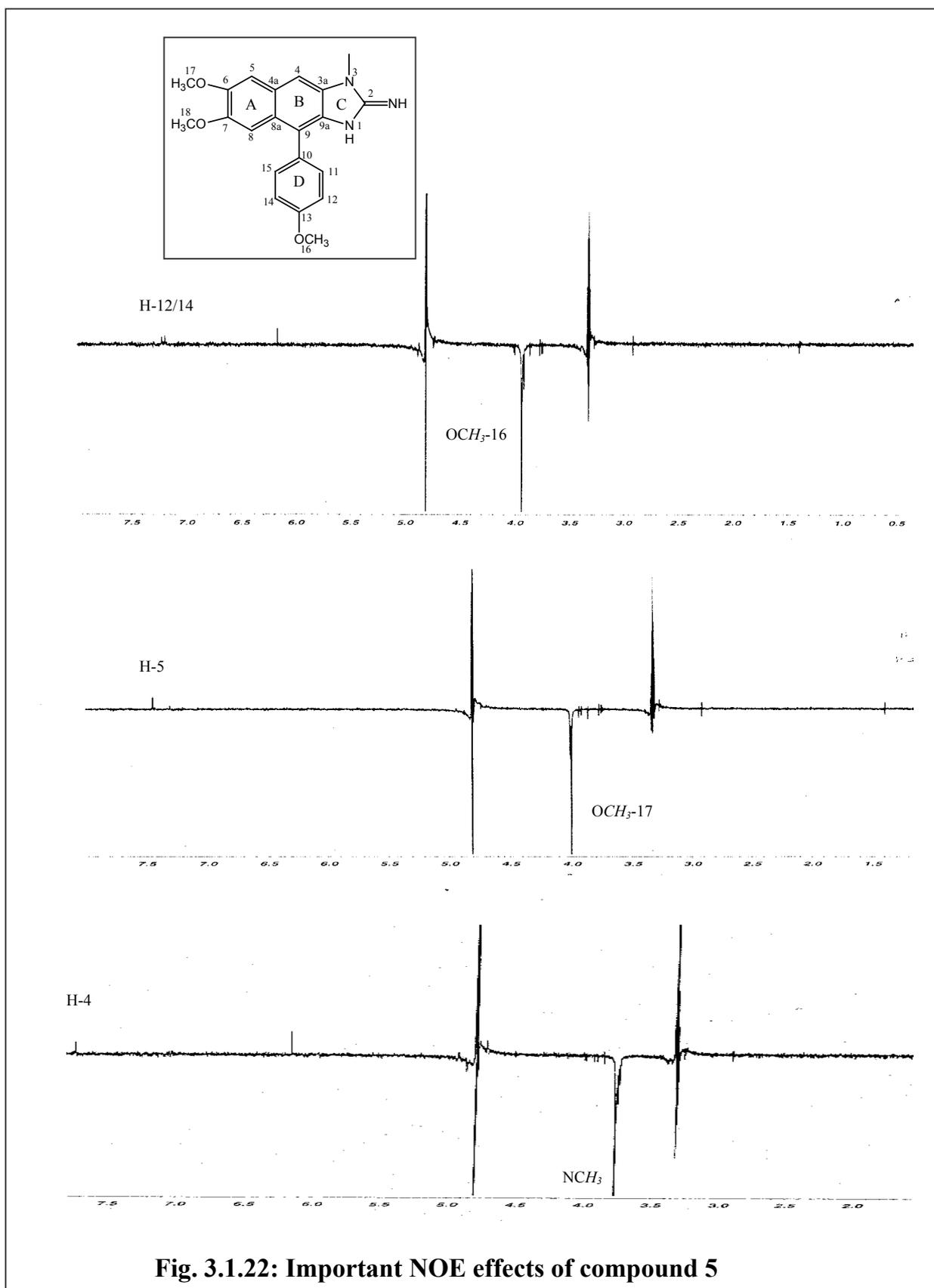
Fig. 3.1.22: Important NOE effects of compound 5

3.1.1.6. Structure elucidation of kealiinine C (6, new compound)

Kealiinine C
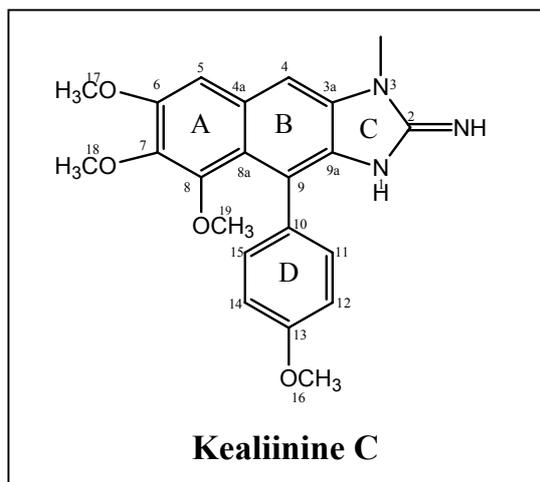
Compound **6** was isolated together with compound **5** as a dark brown amorphous solid. The ESIMS spectrum (Fig. 3.1.20) showed the molecular ion peak at *m/z* 394 [M+H]$^+$ which was 30 amu higher than that of compound **5** and was compatible with the molecular formula $C_{22}H_{25}N_3O_4$ established by HRESIMS (*m/z*, experimental 394.1765 [M+H]$^+$ and calculated 394.1767 [M+H]$^+$). Its UV spectrum and ^{1}H-NMR data (Table 3.1.6) showed that it is closely related to kealiinine A (**4**) and B (**5**) except for the disappearance of the methine singlet at *ca.* 7.10 ppm previously assigned to H-8 in the latter compounds and also the emergence of an additional NC*H*$_3$ or OC*H*$_3$ methyl signal. The 30 atomic mass unit difference could be accounted for the presence of an additional methoxyl group at C-8. A NOE experiment showed an enhancement effect of δ$_H$ 3.78 (NC*H*$_3$-1) on H-4 (δ$_H$ 7.81) and *vice versa* which confirmed the presence of a methyl substituent at N-3. Irradiation of the methine singlet at δ$_H$ 7.81 enhanced the singlet at δ$_H$ 7.33 assigned to H-5, which when irradiated, also enhanced the methyl signal at δ$_H$ 4.03 assigned to OC*H*$_3$-17, confirming the methoxyl substituent at C-6. A NOE effect on the AA'BB' doublet protons at δ$_H$ 7.12 for H-12/14 was observed when the methoxyl singlet at δ$_H$ 3.94 was irradiated and confirmed the methoxyl bound to C-13. This also established that ring D in compound **6** is identical to that in kealiinine A and B. Although no NOE effect was observed when the methoxyl signals at δ$_H$ 3.89 and δ$_H$ 3.30 were irradiated, the above data were only compatible with further methoxyl groups at C-7 and C-8.

Fig. 3.1.23: NOE correlations of compound 6
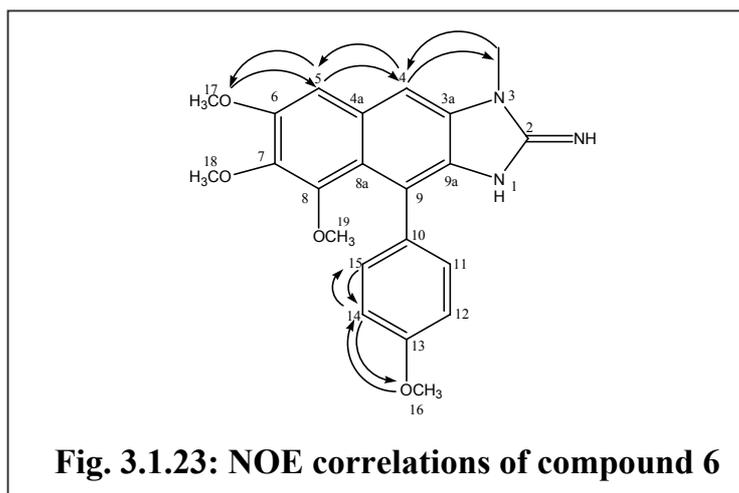
Table 3.1.6.: NMR data of compound 6 (MeOD, 500, 400 MHz)

Proton No.	δH	COSY	NOE Proton irradiated	NOE Proton enhanced
1		-	-	-
2	-	-	-	-
3	NC*H₃* 3.79 (s)	-	NC*H₃*	4
3a	-	-	-	
4	7.81 (s)	5	4	NC*H₃*, 5
4a	-	-	-	
5	7.33 (s)	4	5	4, 17
6	-	-	-	-
7	-	-	-	-
8	-	-	-	-
8a	-	-	-	-
9	-	-	-	-
9a	-	-	-	-
10	-	-	-	-
11	7.35 (d, 8.5 Hz)	12	-	-
12	7.12 (d, 8.5 Hz)	11	-	-
13	-	-	-	-
14	7.12 (d, 8.5 Hz)	15	14	15, 16
15	7.35 (d, 8.5 Hz)	14	15	14
16	3.94 (s)	-	16	14
17	4.03 (s)	-	17	5
18	3.89 (s)	-	18	-
19	3.30 (s)	-	-	-

3.1.2. Biological activities

3.1.2.1. Cytotoxic activity

For the cytotoxicity against mouse lymphoma cells (L5178Y), human cervix carcinoma (HeLa), and rat brain tumour (PC12) cell lines, it was observed that

naamine G exhibited mild activity against L5178Y and HeLa cell lines. The other compounds were inactive.

Table 3.1.7: cytotoxic activity of imidazole alkaloids

Name of the compound	% of Growth inhibition					
	L5178Y		HeLa		PC12	
	10µg/ml	3µg/ml	10µg/ml	3µg/ml	10µg/ml	3µg/ml
Naamine G	46 %	0	29	0	0	0
Kealiinine A	0	0	0	0	0	0

3.1.2.2. Anti-microbial activity

For anti-microbial activity against *E. coli, B. subtilis,* and *S. cerevisiae,* it was observed that naamine G was completely inactive. For the fungicidal activity, naamine G exhibited strong anti-fungal activity against *C. herbarum* at a concentration of 100 µg/ disc, causing zone of inhibition 20 mm in diameter.

Table. 3.1.8: Results of anti-microbial assay of extracts and pure compounds of *Leucetta chagosensis*

Sample	*B. subtilis*	*E. coli*	*S. cerevisiae*	*C. herbarum*		*C. cucumerinum*	
	20 µl	20 µl	20 µl	10 µl	20 µl	10 µl	20 µl
Hexane extract	n.a	n.a	n.a	n.t	n.a	n.t	n.a
EtOAc extract	n.a	n.a	n.a	n.t	n.a	n.t	n.a
Butanol extract	n.a	n.a	n.a	n.t	n.a	n.t	n.a
Aqueous extract	n.a	n.a	n.a	n.t	n.a	n.t	n.a
Naamine G	n.a	n.a	n.a	n.t	n.a	n.t	20 mm[*]
Kealiinine A	n.a	n.a	n.a	n.t	n.a	n.t	n.a

20µl contains 100µg   n.a.   no activity   n.t.   not tested   [*] diameter of inhibition zone

3.2.3. Brine shrimp assay

From the brine shrimp assay, compound **4** (kealiinine A) exhibited mild activity with a mortality rate of 50% at concentration of 100 µg/ml.

Table .3.1.9: Results of brine shrimp assay of extracts and pure compounds of
Leucetta chagosensis.

Samples	Brine shrimp mortality rate	
	10µg/ml	100µg/ml
EtOAc extract	15%	40%
Hexane extract	0%	25%
Aqueous extract	0%	0%
Butanol extract	10%	25%
Naamine G	0%	10%
Kealiinine A	10%	50%

Fig. 3.1.24: Results of brine shrimp mortality test of *Leucetta chagosensis* extracts and pure compounds
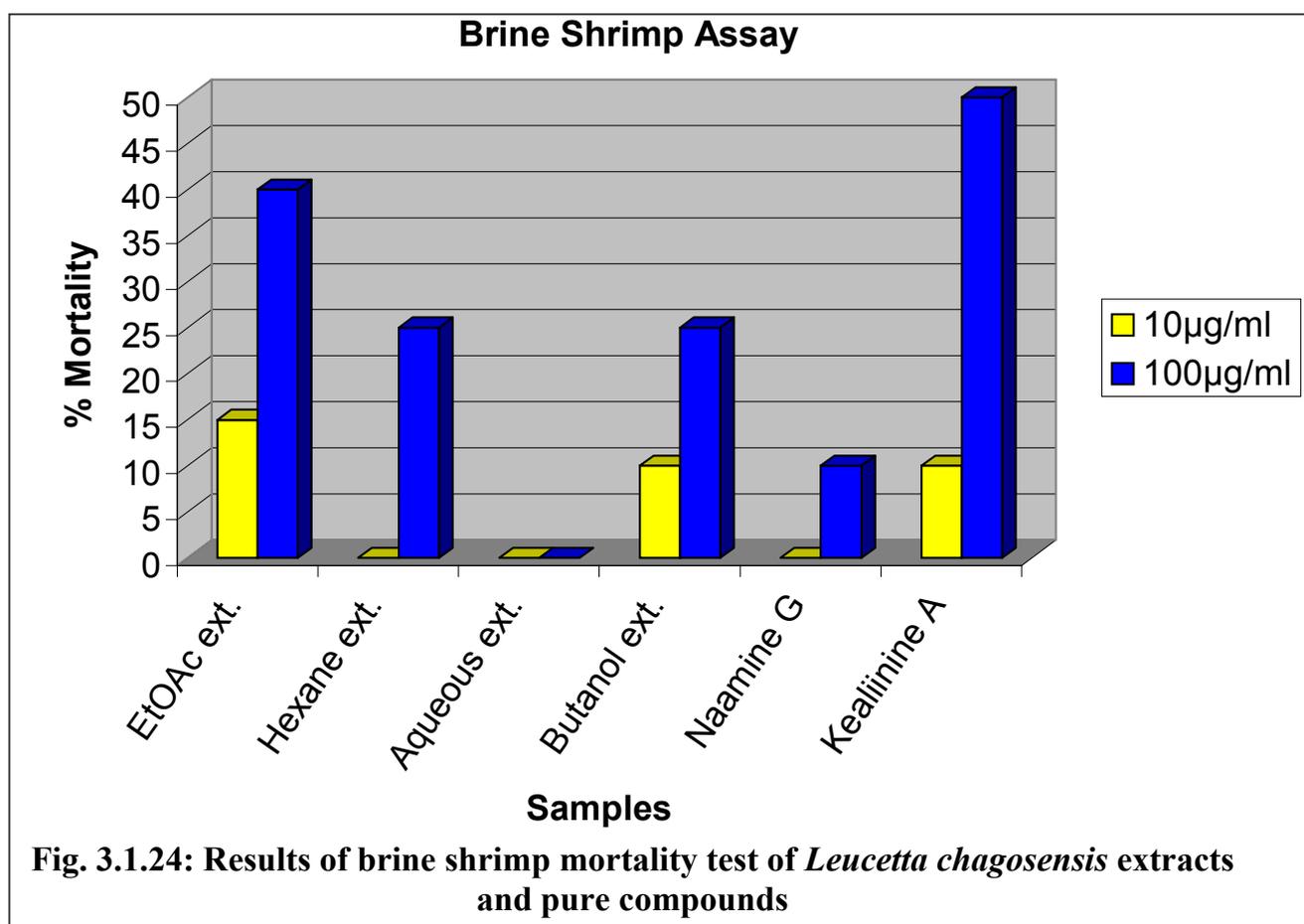
3.2. Secondary metabolites isolated from the sponge *Axinyssa aplysinoides*

Marine sponges of the order *Halichondrida* have provided an array of sesquiterpenes containing isonitriles, iosthiocyanates, and formamides (Marcus *et al*, 1989). Some of these metabolites exhibited very interesting biological activities such as, antimicrobial (Compagnone and Faulkner, 1995), anti-tumour (Patil *et al*, 1997), and anti-feeding activities against fish (Thompson *el al*, 1982). The present work investigated a member of this order, the sponge *Axinyssa aplysinoides.* It has been reported that the total extract of the sponge *Axinyssa aplysinoides* exhibited anti-feeding activities against the pufferfish *Canthigaster solandri* (Marcus *et al*, 1989).

The alcoholic extract of the sponge (*Axinyssa aplysinoides,* Dendy, 1922, *Halichondrida*) was subjected to liquid-liquid extraction to give ethyl acetate, butanol, hexane and water fractions which were screened using the brine-shrimp assay (Fig. 3.2.1.). The ethyl acetate fraction exhibited strong activity with mortality rate of 100% against brine shrimp (50 µg and 500 µg extract), hexane fraction exhibited activity with mortality rate of 10% (50 µg extract) and 55% (500 µg extract), the butanol fraction showed a mild activity with mortality rate of 25% (500 µg extract). The most important fraction eluted from the ethyl acetate extract, fraction 4, showed also strong activity exhibiting mortality rate of 70% (50 µg extract) and 100% (500 µg extract) in the brine shrimp assay.

The chemical investigation of the active extracts led to the isolation of four compounds including, one sesquiterpene isothiocyanate, two bisabolene sesquiterpenes and one phenolic compound.
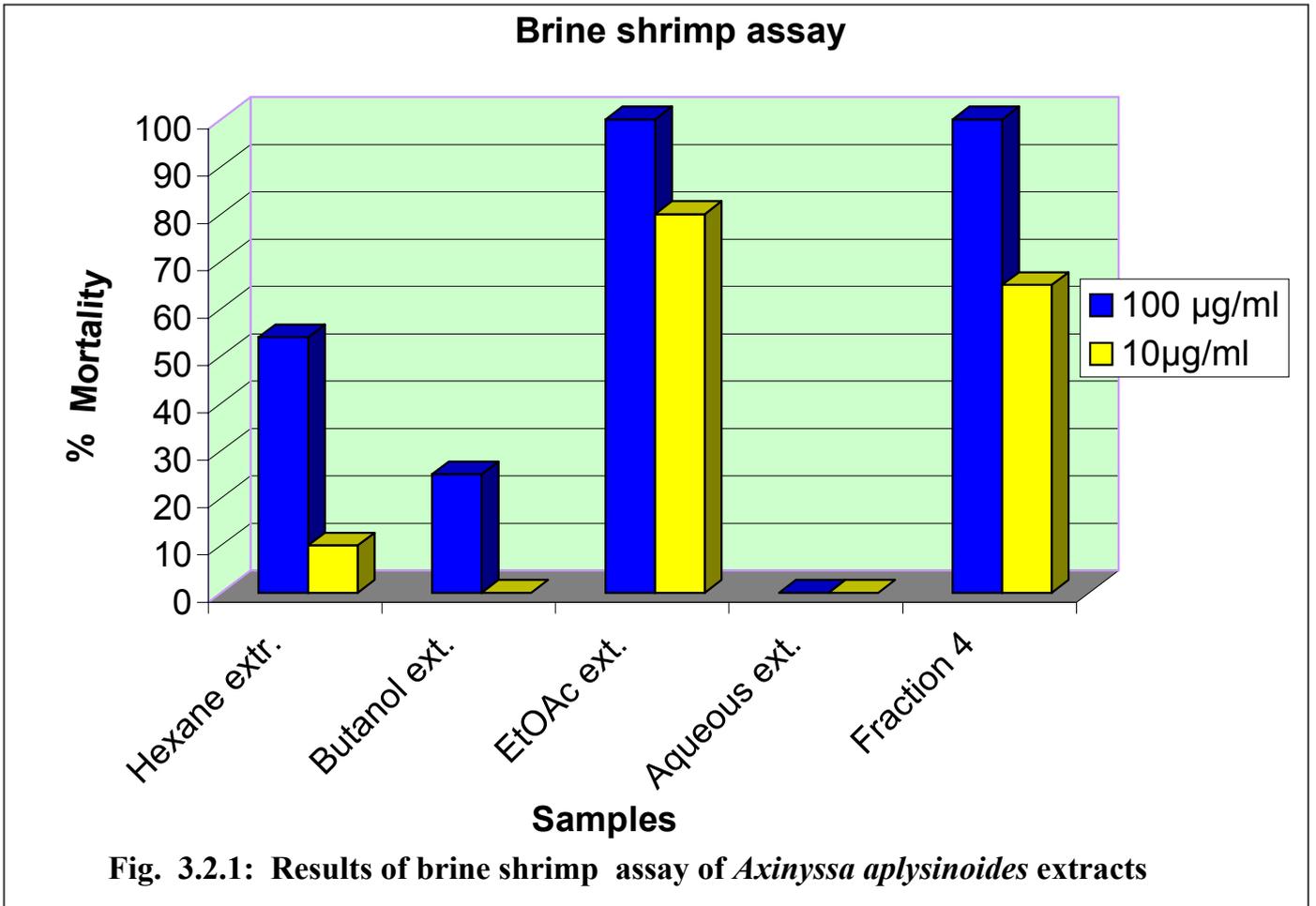
Fig. 3.2.1: Results of brine shrimp assay of *Axinyssa aplysinoides* extracts
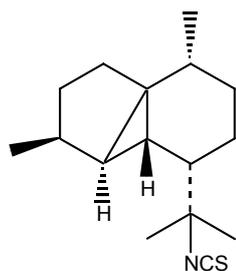
(1S, 2R, 5S ,6S, 7R, 8S)-13-
Isothiocyanatocubebane
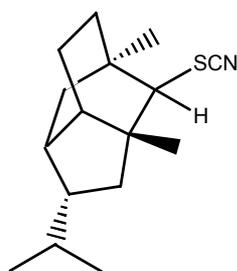
(1S, 2R,3R, 5S ,6S, 7S)-2-
Thiocyanatopupukeanane
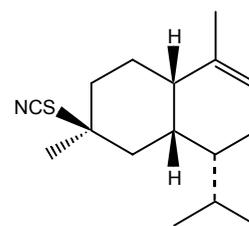
(1S, 4S ,6S, 7R) -4-Thiocyano-9-
cadenine
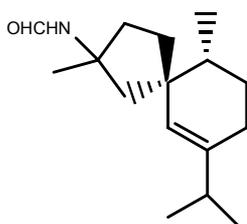
(2R, 5R , 10R)-2-
Formamido-6-axine
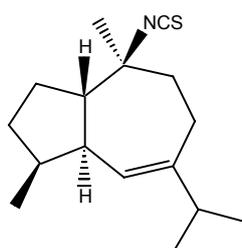
(1S, 4S, 5R ,10S)-10-
Isothiocyanatoguaia-6-ene
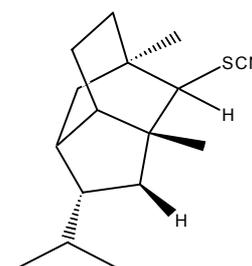
(2R, 5R , 10R)-2-
Isothiocyanato-6-axine

13-(Methylamino) cubebane

(3S, 5R, 6R, 9R)-3-Formamido-(10) cadinene
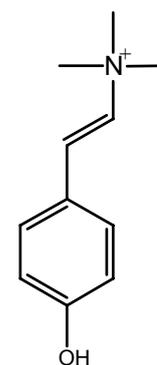
[E-(-4- Hydroxystyryl) trimethyl ammonium chloride]
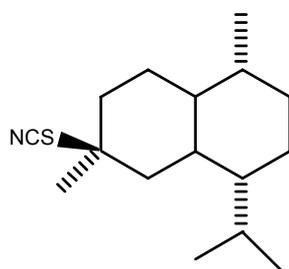
(1S, 4R, 6S, 7R)-3-Thiocyanato-(9) cadinene   (1R, 2R, 3R, 5R, 6S, 7S)-2-Thiocyanatopupukeanane
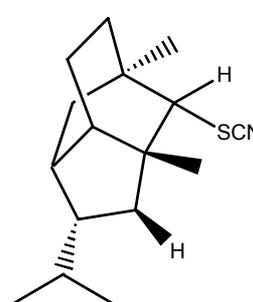
Fig. 3.2.2: Some sesquiterpenes isolated from the sponge *Axinyssa aplysinoides*

3.2.1. Structure elucidation of the isolated compounds

3.2.1. 1. Structure elucidation of (6R,7S)-7-amino-7, 8-dihydro-α-bisabolene hydrochloride (7, known compound)

(6R,7S)-7-Amino -7, 8-dihydro-α-bisabolene hydrochloride

      Compound 7 was isolated as a yellow oily substance. The FABMS (Fig. 3.2.4) showed the molecular ion peak at *m/z* 222 [M+H]$^{+}$ which was compatible with the molecular formula $C_{15}H_{27}N$. The ^{13}C-NMR spectrum and DEPT (Fig.3.2.6, 7) showed signals for fifteen carbons (sesquiterpene nucleus), which consisted of four quartets, three doublets, five triplets and three singlets. The ^{13}C-NMR spectrum showed the presence of two double bonds which was signified by the two singlet signals at δ_C 133.6, 132.1 and two doublet signals at δ_C 123.0 and 119.6. This was confirmed from the ^{1}H-NMR signals at δ_H 5.35 (1H, br s) and 5.05 (1H, t, 6.0 Hz) which indicated the presence of double bonds at H-10 and H-2 which coupled with H-9 and H-1, respectively as observed from the COSY spectrum. The COSY spectrum showed also homoallylic coupling between H-10 and Me-12, Me-15 and between H-2 and Me-13. The ^{13}C-NMR signals at δ_C 23.1 (q), 21.6 (q), 25.5 (q) and 17.7 (q) suggested the presence of four methyl groups as also shown in its DEPT specrtum. This was confirmed from the ^{1}H-NMR signals at δ_H 1.60 (3H, s), 1.58 (3H, s), 1.65 (3H, s), and 1.38 (3H, s) which were assigned to Me-12, Me-15, Me-13, and Me-14, respectively. The downfield shift of the three methyl groups 12, 13 and 15 indicated that these methyls were vinylic. This was established from the HMBC correlations

(Fig. 3.2.4) of CH_3-12 and CH_3-15 with C-10, C-11, C-15; which indicated that Me-12 and Me-15 were geminal to each other and the presence of $(CH_3)_2$-C=CH- moiety. The correlations of CH_3-13 with C-2, C-4 which revealed the presence of a trisubstituted olefin with one methyl substituent. The downfield shift of C-7 at δ_C 59.6 suggested the presence of a heteroatom-bearing carbon. This was established from the ^{1}H-NMR signal at δ_H 8.35 (2H, br s) for NH_2 group. The HMBC spectrum showed [3]J correlations of Me-14 with C-6, and C-8 and [2]J correlation with C-7 which confirmed that the Me-14 was bound to C-7. The two functions $(CH_3)_2$-C=CH- and trisubstituted olefin with one methyl in addition to the tertiary methyl (Me-14) were characteristic for the bisabolene nucleus. The NMR data of compound **7** matched those of **(6R,7S)-7-amino-7,8-dihydro-α-bisabolene hydrochloride** from the literature (Gulavita and Faulkner, 1986 and Sullivan and Faulkner, 1986).

Stereochemistry

The stereochemistry of compound **7** was assumed to be identical with that of the reported (6R,7S)-7-amino-7,8-dihydro-α-bisabolene hydrochloride due to the similarity in $[\alpha]_D$. The $[\alpha]_D$ of compound **7** is + 84 (*c*, 0.1, CHCl$_3$) which is similar to that of the known compound + 64 (*c*, 4.2, CHCl$_3$) which was isolated from the marine sponge *Halichondria sp.* (Gulavita *et al*, 1986, and Sullivan and Faulkner, 1986). It is the first time that (6R,7S)-7-amino-7,8-dihydro-α-bisabolene hydrochloride has been isolated from sponge *Axinyssa aplysinoides.*

Fig.3.2.4: Important HMBC correlations of compound 7
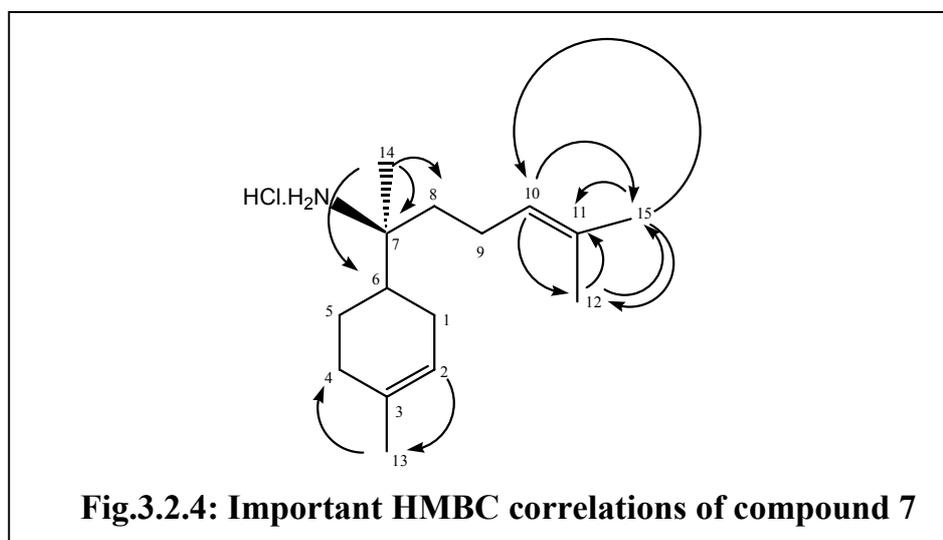
Table 3.2.1: NMR data of compound 7 (CDCl₃, 500 MHz)

NO.	δC	δH	COSY	HMBC
1	26.2 t	2.00 (m)	2	3
2	119.6 d	5.35 (br s)	1, 13	13
3	132.1 s	-	-	
4	30.6 t	1.30-2.20 (m)	*	-
5	21.9 t	1.30-2.20 (m)	*	-
6	40.4 d	2.13 (m)	1	-
7	59.6 s	-	-	-
8	36.0 t	1.30-2.20 (m)	*	-
9	23.6 t	2.20 (m)	10	11
10	123.0 d	5.05 (t, 6.0 Hz)	9, 12, 15	15, 12
11	133.6 s	-	-	-
12	23.1 q	1.60 (s)	10	10, 11, 15
13	21.6 q	1.65 (s)	2	2, 4
14	25.5 q	1.38 (s)	-	6, 7, 8
15	17.7 q	1.58 (s)	10	10, 11, 12
16	-	N*H₂*, 8.35 (br s)	-	-

* signals were overlapping

Fig. 3.2.4 : FABMS spectrum of compound 7
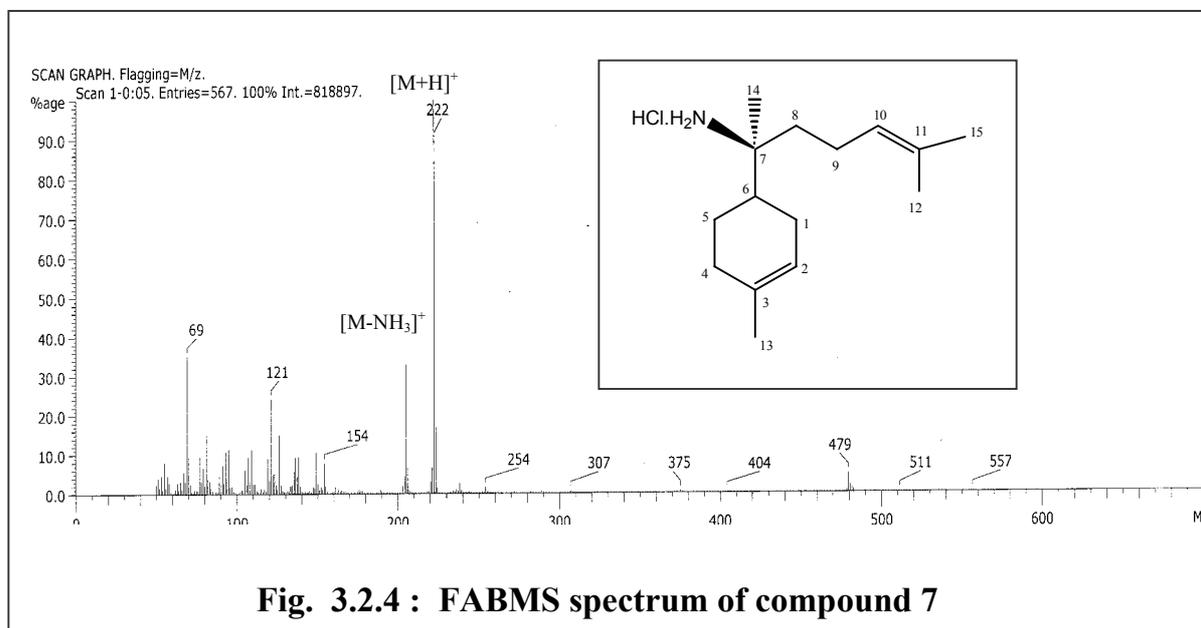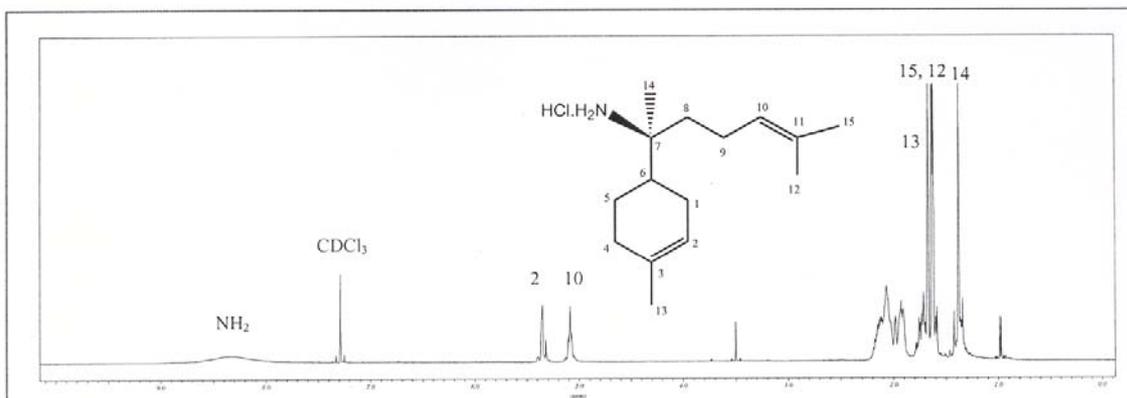
Fig. 3.2.5: ¹H-NMR spectrum of compound 7
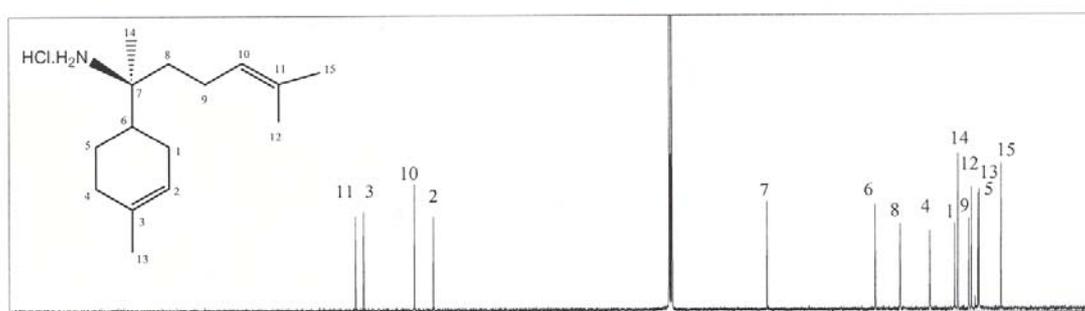
Fig. 3.2.6: ¹³C-NMR spectrum of compound 7
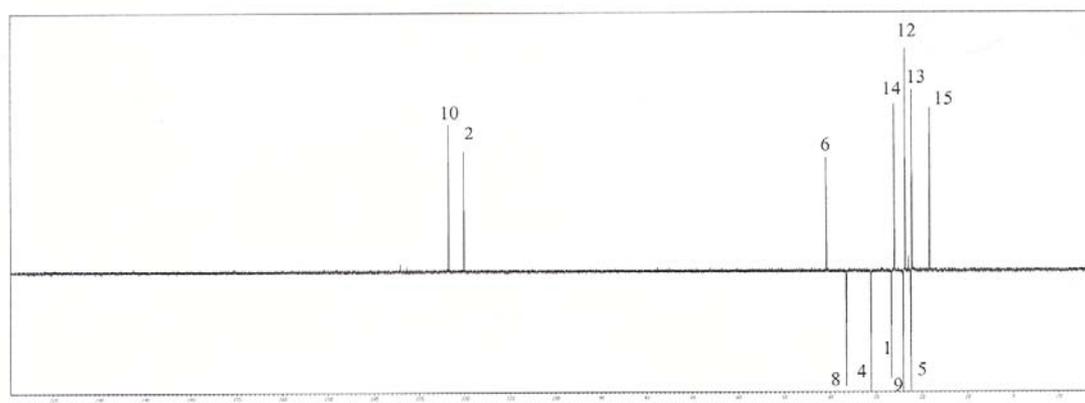
Fig. 3.2.7: DEPT spectrum of compound 7

3.2.1.2. Structure elucidation of (6*R*,7*S*)-7-amino-7, 8-dihydro-α-bisabolene urea
(8, known compound)

(6*R*,7*S*)-7-amino-7, 8-dihydro-α-bisabolene urea
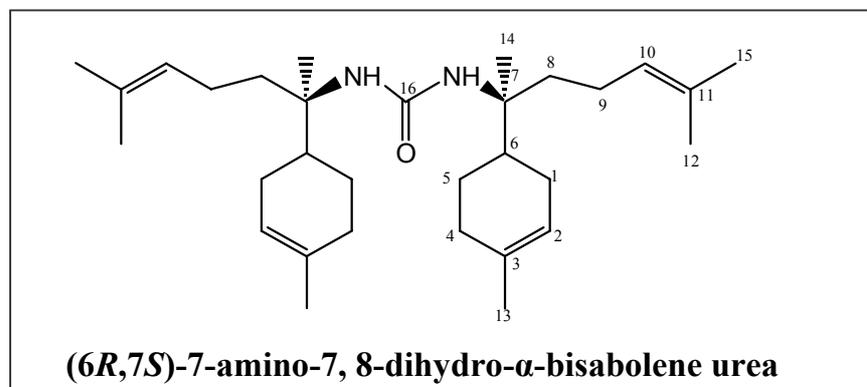
Compound 8 was isolated as a white crystalline powder. The FABMS spectrum (Fig. 3.2.8) showed a molecular ion peak at *m/z* 469 [M+H]$^+$ suggesting the molecular formula $C_{31}H_{52}N_2$. But ^{1}H and ^{13}C-NMR spectra showed only signals for twenty six protons and sixteen carbons, respectively. This suggested that compound **8** consists of a symmetrical dimer and that the NMR spectra (Fig. 3.2.9,10) showed only signals corresponding to the monomer which is closely related to compound **7**. Detailed comparison of the ^{1}H-NMR data (Table 3.2.2) showed the upfield chemical shift of the signal at δ_H 1.05 (3H, s) for Me-14 and C-7 at δ_C 57.3 in comparison to that of compound **7**. In addition, the signal at δ_C 8.35 (2H, br s) for compound **7** was replaced by a singlet signal at δ_H 3.75 (1H) in compound **8** which revealed that the N*H*$_2$.HCl moiety in compound **7** was replaced by an amide group in compound **8**. This was confirmed from the ^{13}C-NMR group which showed an additional signal at δ_C 157.8 for the amide function. Further evidence for the presence of the amide unit was revealed by the HMBC spectrum (Fig. 3.2.12) which showed a 4-bond correlation of Me-14 with C-16 at δ 157.8. The NMR data agreed with those of (6*R*,7*S*)-7-amino-7,8-dihydro-α-bisabolene urea isolated from marine sponge *Halichondria* sp (Gulavita *et al,* 1986 and Sullivan and Faulkner, 1986).

Stereochemistry

The observed [α]$_D$ value of compound **8** + 43.3 (*c*. 0.1 in CHCl$_3$) was almost identical with that of the known   (6*R*,7*S*)-7-amino-7,8-dihydro-α-bisabolene urea (+

44) (*c*. 1.1 in CHCl₃,) suggesting that the two compounds are identical (Gulavita *et al,*1986 and Sullivan and Faulkner, 1986). It is also the first time that (6*R*,7*S*)-7-amino-7,8-dihydro-α-bisabolene urea has been isolated from marine sponge *Axinyssa aplysinoides*.

Table 3.2.2: ¹H, ¹³C-NMR, COSY data and HMBC of compound 8 (CDCl₃, 500 MHz)

NO.	δC	δH	HMBC	COSY
1	26.4 t	A 1.80 (m) B 1.95 (m)	7, 6, 2, 3	1B, 6
2	120.7 d	5.35 (br s)	1	1, 13
3	131.2 s	-	-	-
4	31.3 t	1.80 (m)	5	5
5	22.5 t	A 1.90 (m) B 1.20 (m)	-	5B, 4, 6 5A , 6, 4
6	41.0 d	2.20 (m)	-	5A, 1A
7	57.3 s	-	8	
8	36.6 t	A 2.00 (m) B 1.55 (m)	9, 10	8B / 9
9	24.4 t	A 1.90 (m) B 2.00 (m)	10, 11	8, 10
10	124.8 d	5.15 (t, 6 Hz)	9	-
11	133.8 s	-	-	-
12	23.2 q	1.67 (s)	10, 11, 15	10
13	21.7 q	1.63 (s)	2, 3, 4	2
14	25.7 q	1.05 (s)	6 / 7 / 8 / 16	-
15	17.5 q	1.59 (s)	10, 11, 12	10
16	157.8 s	N*H* 3.75 (s)	-	-
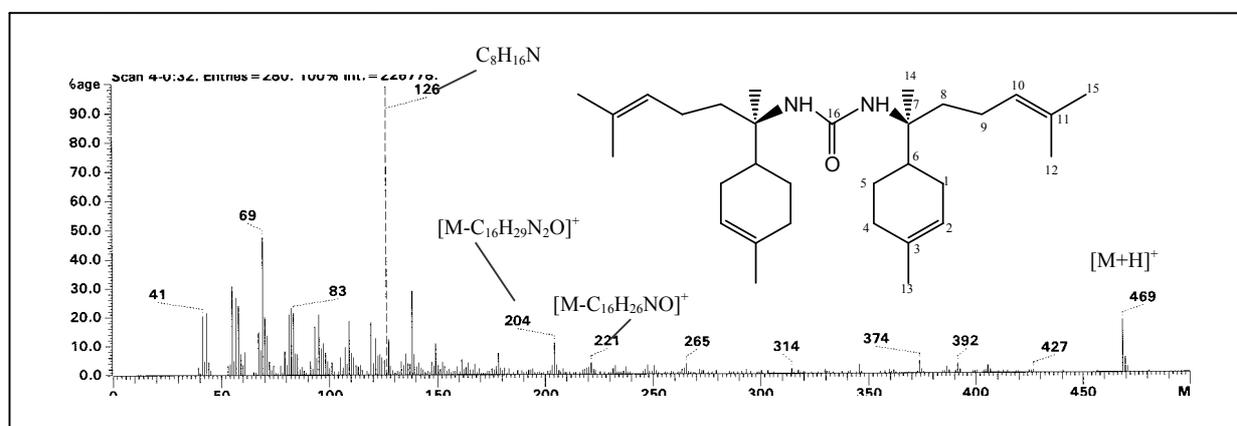
Fig. 3.2.8: FABMS spectrum of compound 8

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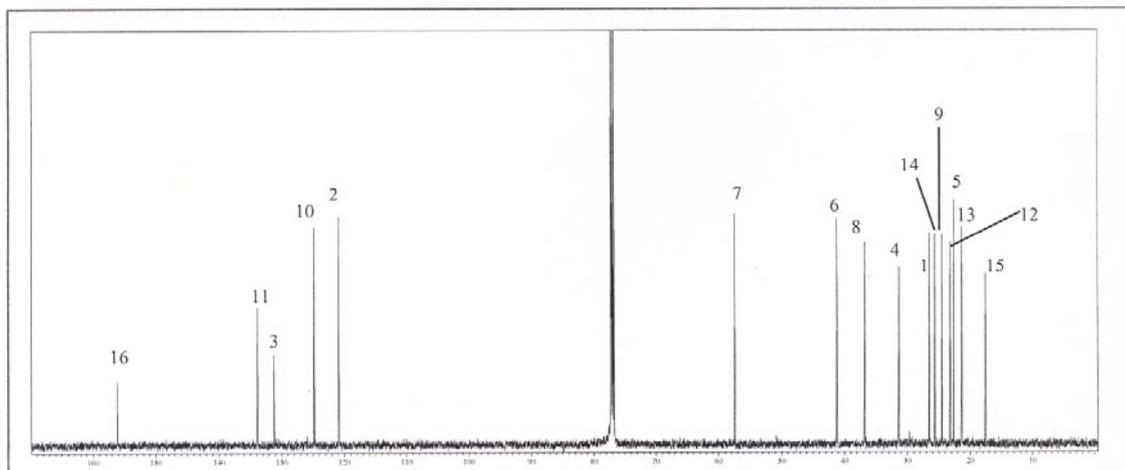
Fig. 3.2.9: ^{13}C-NMR spectrum of compound 8
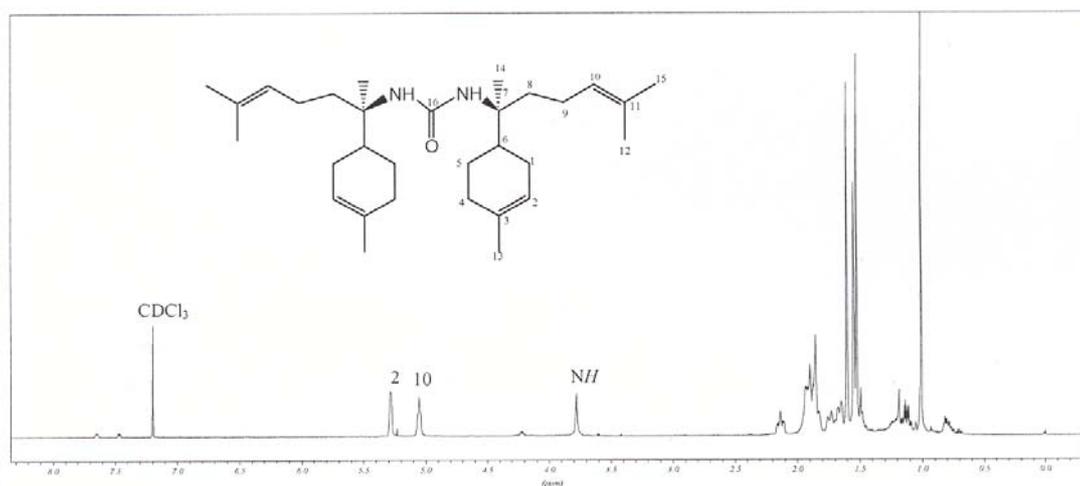
Fig. 3.2.10: ^{1}H-NMR spectrum of compound 8
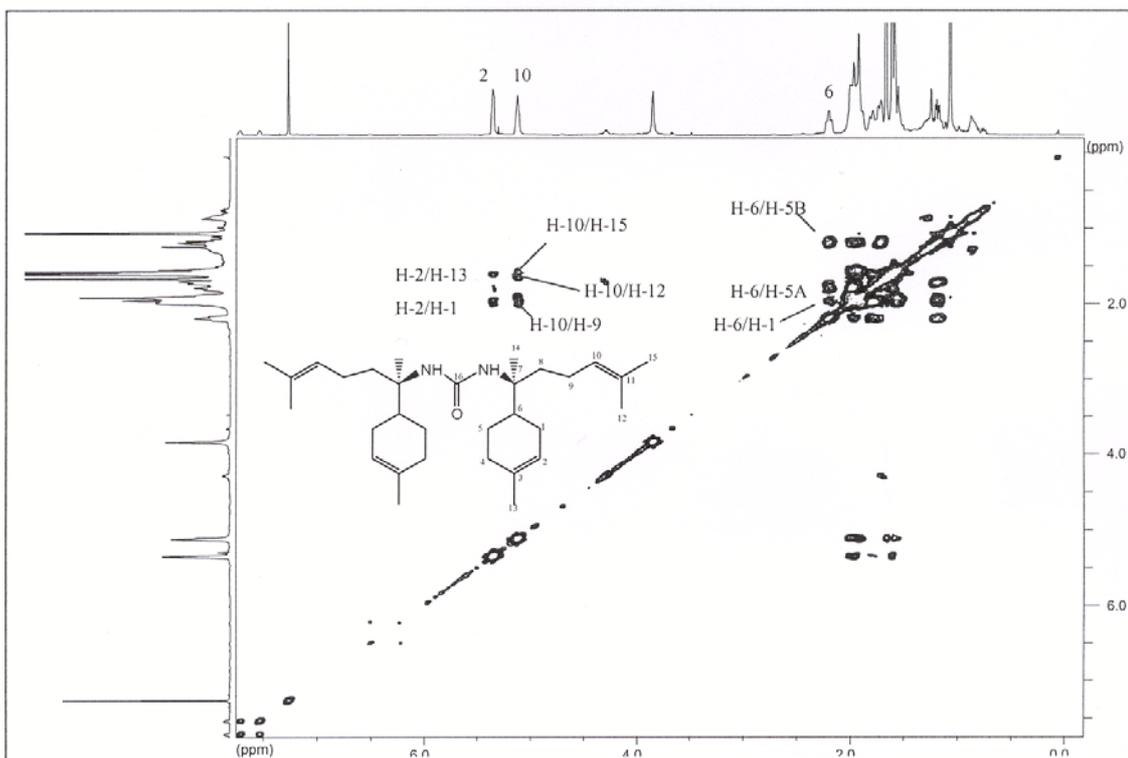
Fig. 3.2.11: COSY spectrum of compound 8
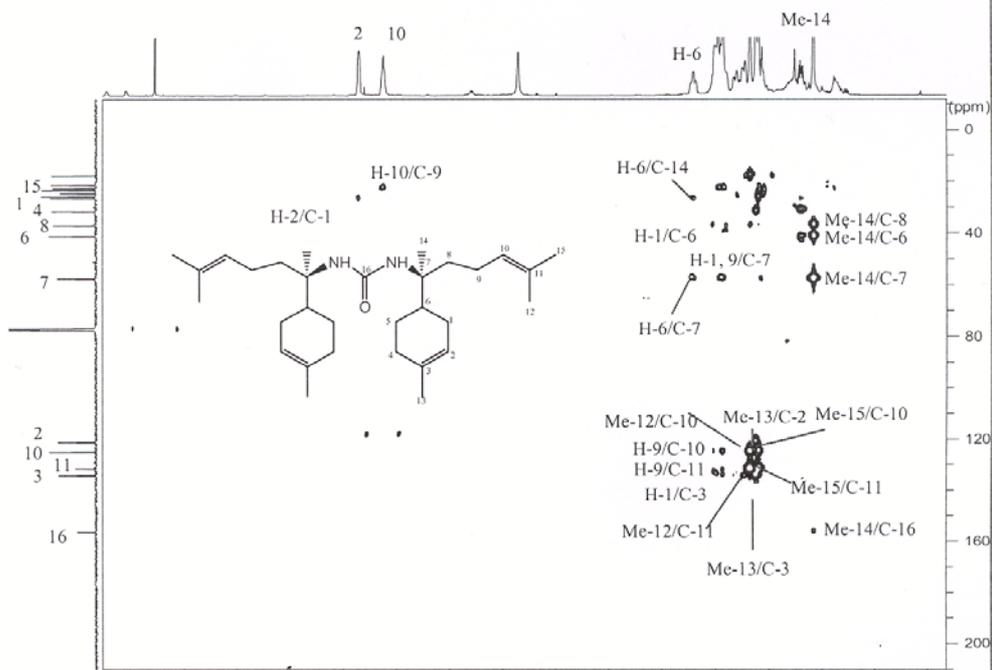
Fig. 3.2.12: HMBC spectrum of compound 8

3.2. 1.3. Structure elucidation of epipolasin A (9, known compound)

Compound 9 was isolated as a colourless oil $[\alpha]_D$, + 7.9 *c*. 0.1, CHCl$_3$. The EIMS spectrum (Fig. 3.2.13)   showed the molecular ion peak at m/z 263 which corresponded to the molecular formula C$_{16}$H$_{25}$NS. In addition, the important fragment at *m/z* 205 indicated the loss of NCS or SCN group which revealed the presence of a sesquiterpene isothiocyanate or thiocyanate. The presence of an isothiocyanate was confirmed from the ^{13}C-NMR signal at δ 128.2 which is characteristic for an isothiocyanate group (He *et al*, 1989). The signal at δ 64.7 (s) indicated that NCS group was attached to a quaternary carbon as confirmed by its DEPT spectrum. Furthermore the ^{1}H-NMR spectrum (Fig. 3.2.14 and 15) showed a signal at δ_H 1.42 (3H, s) for a methyl group being geminal to the NCS function. This was confirmed from the HMBC correlation of Me-15 with NCS. Three signals at δ_H 1.01, 1.12 and 0.89 were assigned to the methyl groups 12, 13, and 14, respectively. The presence of the cyclopropane ring was assigned from the signals at δ_H 0.56 (1H, dd, *J* = 9.0, and 6.5 Hz) and δ_H 0.64 (1H, dt, *J* = 9.0 and 7.5 Hz). The COSY spectrum (Fig. 3.2.18) showed that the proton at δ_H 0.56 coupled vicinally to H-5 and H-7, and the signal at δ_H 0.64 coupled to the methylene signals at δ_H 1.77 (8α) and 1.58 (8β).The ^{13}C-NMR and DEPT spectra (Fig. 3.2.16,17) showed four quartet signals at δ_C 15.2, 19.0, 21.9, and 29.3 for the four methyl groups 12, 13, 14, and 15, respectively. The position of attachment of the methyl groups 12 and 13 at carbon 11 was confirmed from the HMBC correlations of Me-12 with C-13, C-6, C-7 and Me-13 with C- 12, C-6 and C-7. The HMBC cross peaks between Me-14 and C-1, C-5, C-9 and C-10 established the position of attachment   at C-10. The structure of compound **9** was established as **epipolasin A** from ^{1}H-NMR, ^{13}C-NMR, HMQC, COSY and HMBC spectra and through the comparison with the literature (Capon and MacLeod, 1988, and Thompson *et al*, 1982).

Stereochemistry

The stereochemistry of compound **9** is similar to that of epipolasin A, where the observed $[\alpha]_D$ value of compound **9** is + 7.9 (*c*. 0.1 in CHCl$_3$), which was identical with that of the reported epipolasin A + 7.6 (*c*. 1.0 in CHCl$_3$) isolated from the marine

sponge *Epipolasis kushimotoensis*. This suggested that the two compounds are identical (Tada and Yasudo, 1985).
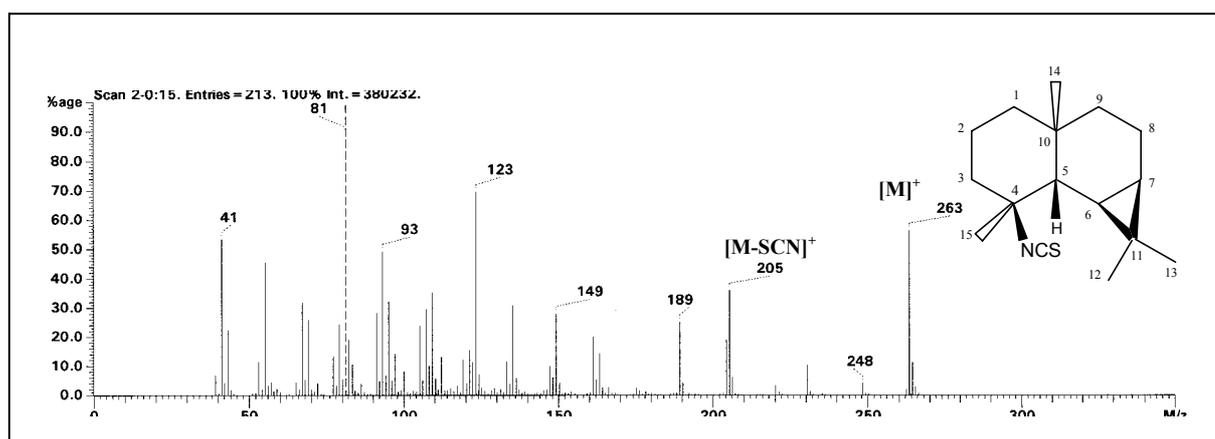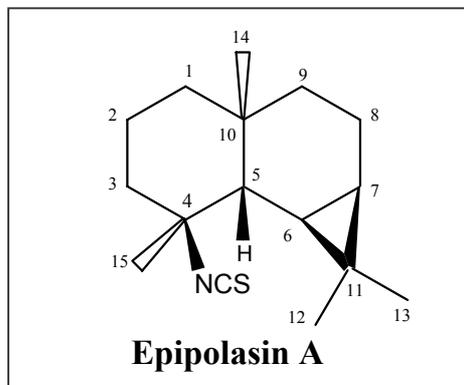
Epipolasin A

Fig. 3.2.13: EIMS spectrum of compound 9

Table 3.2.3 : ¹H, ¹³C-NMR data, HMBC and COSY correlations of epipolasin A in CDCl₃ at 500, 400 MHz)

No.	δH	δC	HMBC	COSY
1α β	1.33 (m) 1.02 (m)	39.0 t	3, 9, 5	1 β, 2, 3 α* 1 α, 2 α/β
2α β	1.58 (m) 1.58 (m)	19.0 t	1, 3, 4, 10	1, 3
3α β	1.96 (m) 1.75 (m)	41.3 t	1, 5, 4	3 β, 2 α/β, 1 α 3α, 2 α/β
4	-	64.7 s	-	
5	1.23 (d, $J_{5\text{-}6}$ = 6.5 Hz)	49.1 d	14, 7, 15, 10, 3, 4	6
6	0.56 (dd, $J_{6\text{-}5}$ = 6.5 Hz) $J_{6\text{-}7}$ = 9 Hz	18.9 d	12, 11, 13, 4	5, 7
7	0.64 (dt, $J_{7\text{-}6}$ = 9.0 Hz, $J_{7\text{-}8}$ = 7.5 Hz)	20.6 d	11, 13, 9, 5	6, 8α
8α β	1.77 (m) 1.58 (m)	15.5 t	6 , 10, 9	7, 9 α/β, 8 β 7, 8α, 9 β
9α β	1.13 (m) 0.81 (m)	41.5 t	1, 5, 4, 7	9 β 8 α/β, 9α
10	-	33.2 s	-	-
11	-	18.1 s	-	-
12	1.01 (s)	15.2 q	13, 6, 7	-
13	1.12 (s)	29.3 q	12 , 6 , 7	-
14	0.89 (s)	19.0 q	10, 9, 5, 1	-
15	1.42 (s)	21.9 q	3, 5, 4, NCS	-
NCS	-	128.2 s	-	-

* 4-bond coupling
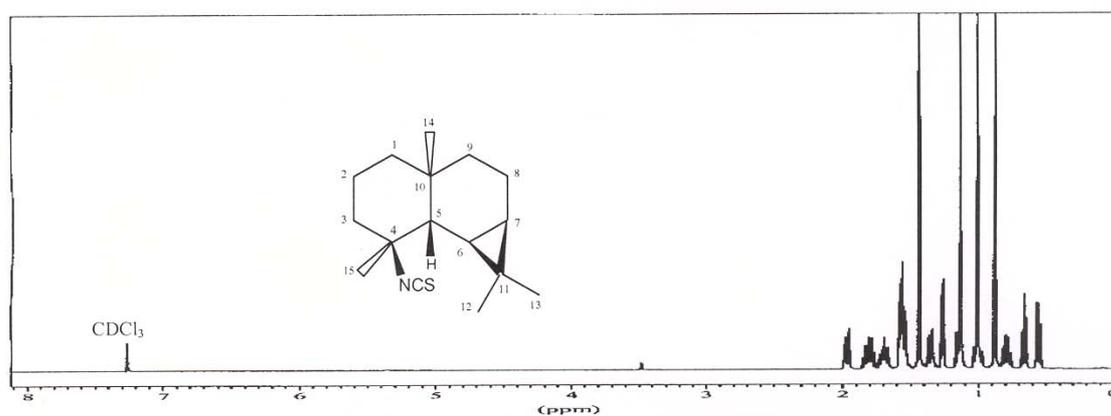
Fig. 3.2.14: ¹H-NMR spectrum of compound 9
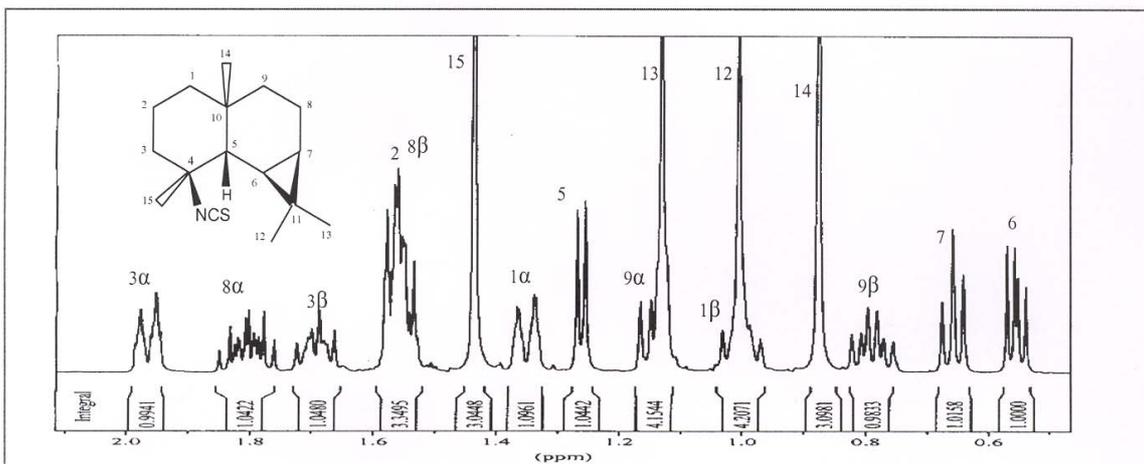
Fig. 3.2.15: Expansion of the ¹H-NMR of compound 9
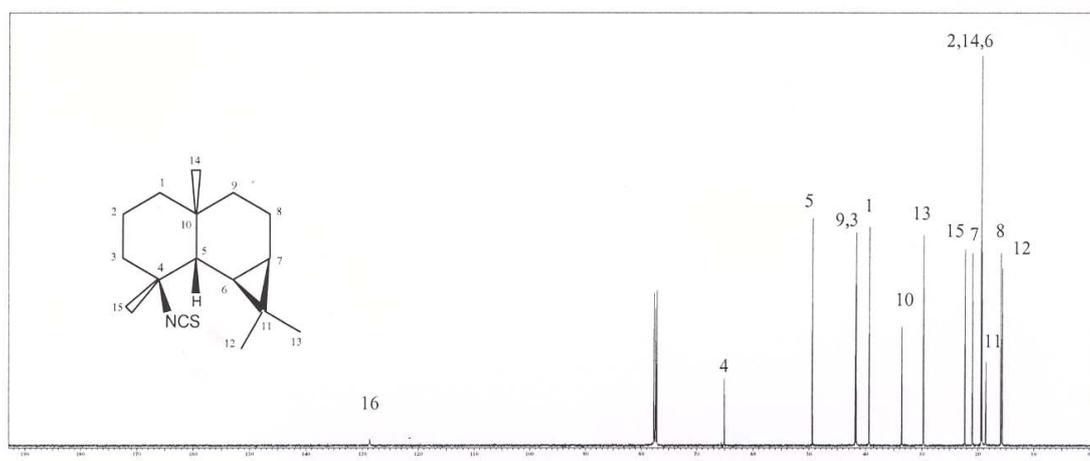
Fig. 3.2.16: ¹³C-NMR spectrum of compound 9
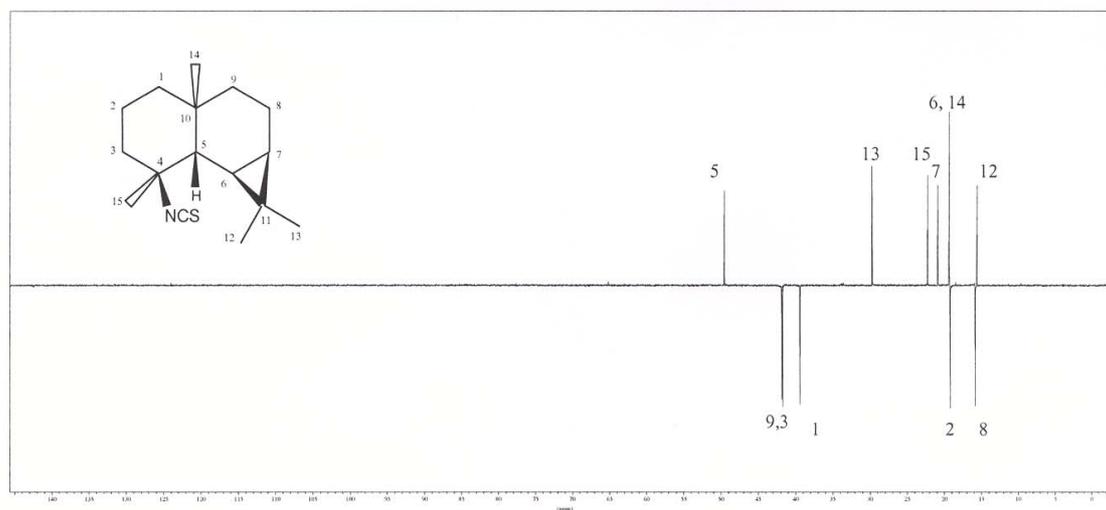
Fig. 3.2.17: DEPT spectrum of compound 9

Fig. 3.2.18: COSY spectrum of compound 9
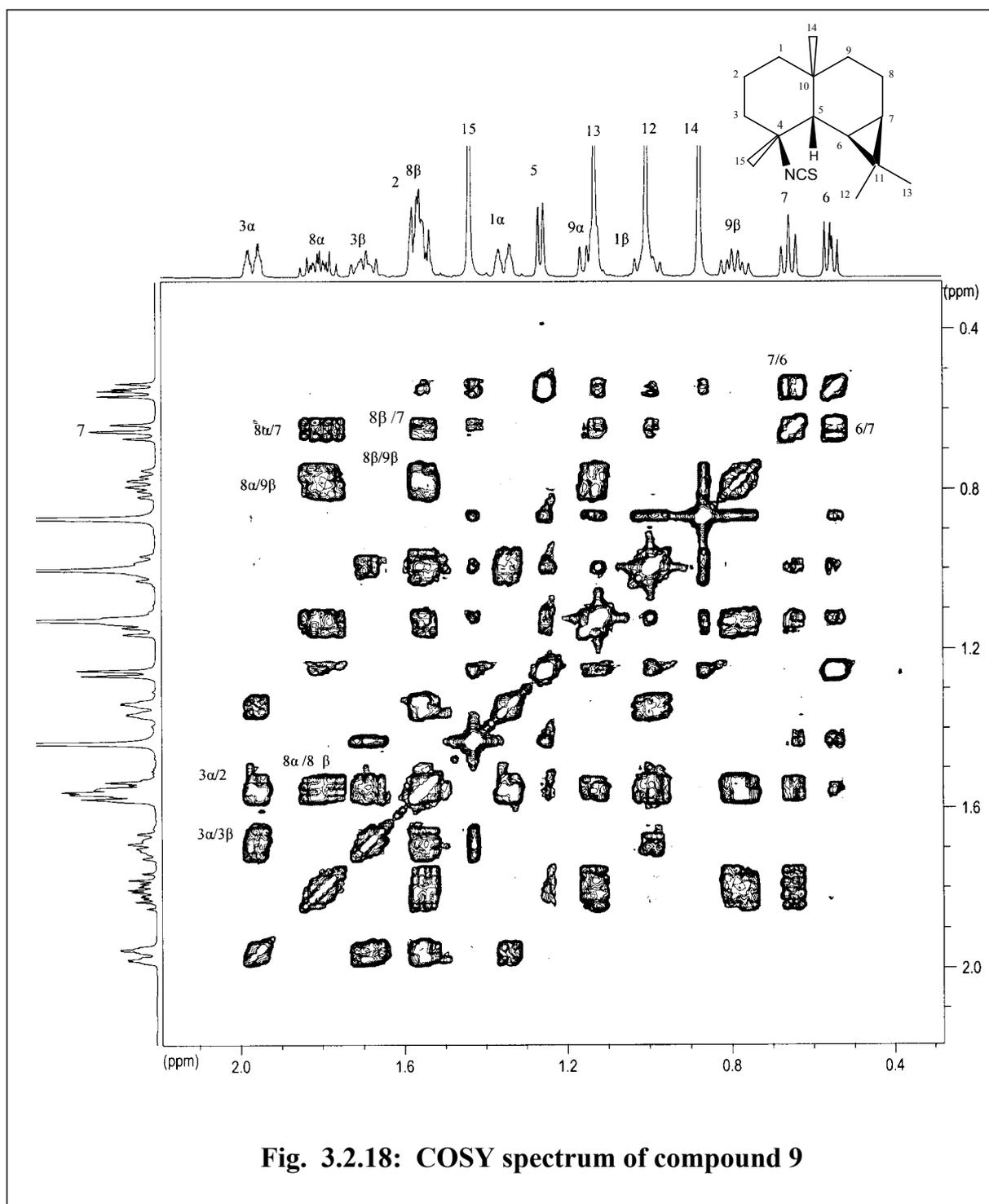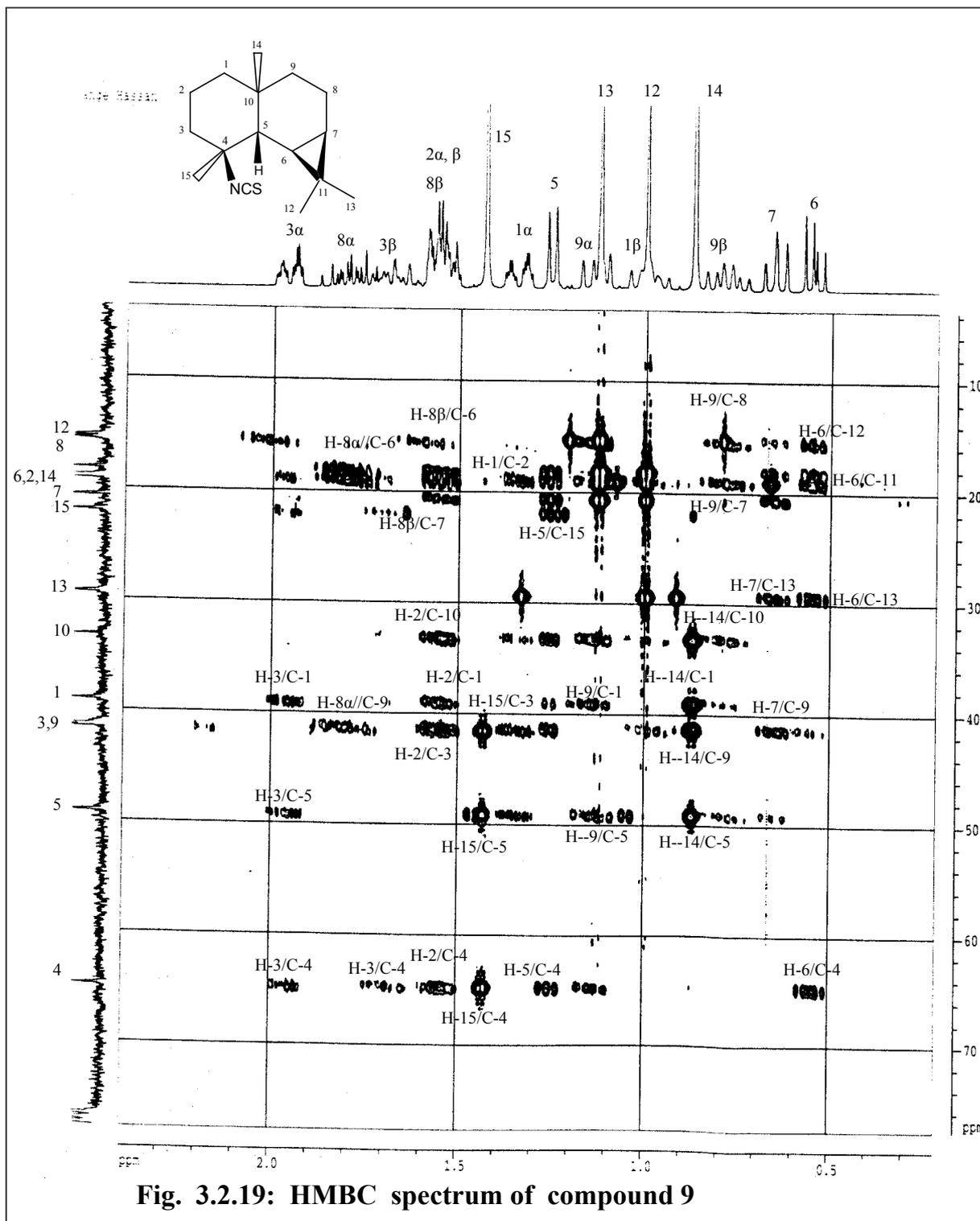
Fig. 3.2.19: HMBC spectrum of compound 9

3.2.1.4. Structure elucidation of [*E*-(-4-Hydroxystyryl) trimethyl ammonium chloride] (10, known compound)

Compound 10 was isolated as a yellowish white amorphous powder. It gave a molecular ion peak at *m/z* 178 in the FABMS (Fig. 3.2.20) which was compatible with the molecular formula $C_{11}H_{16}NOCl$. The ^{13}C-NMR data (Table 3.2.4) showed seven signals for eleven carbons, one signal for three NMe groups, two quaternary for C-1 and C-4, six doublets which includes two AA′BB′ doublet pair for C-2, C-3, C-5, C-6 and two olefinic methines for C-7 and C-8. The ^1H-NMR spectrum (Fig. 3.2.21, Table 3.2.4) showed two signals at δ_H 7.40 (2H, d, *J* = 8.8 Hz), and δ_H 6.81 (2H, d, *J*= 8.8 Hz) indicating an AA′BB′ spin system for a *para*-disubstituted benzene. The downfield shift of C-4 at δ_C 158.9 indicated that compound **10** was a phenolic compound. Furthermore the ^1H-NMR signals at δ_H 7.13 (1H, d, *J*=14.2), and δ_H 6.90 (1H, d, *J*= 14.2) suggested a *trans*-disubstituted olefin conjugated with the benzene ring. This was established from the HMBC correlations of H-8 with C-1, C-7, and H-2/6 with C-1, C-4, C-7. The ^1H-NMR signal at δ_H 3.41 (9H, s) was assigned to three N-methyl groups having the same chemical shift. The HMBC correlations of the three methyl groups, Me-9, Me-10 and Me-11 with C-8 confirmed the presence of a trimethylammonium moiety vicinal to an olefinic double bond. The assignment of ^{13}C and ^1H-NMR signals was established by HMBC and HMQC and all of these data agreed with those of the literature (Compagnone and Faulkner, 1995).

***E*-(-4-Hydroxystyryl) trimethyl ammonium chloride**
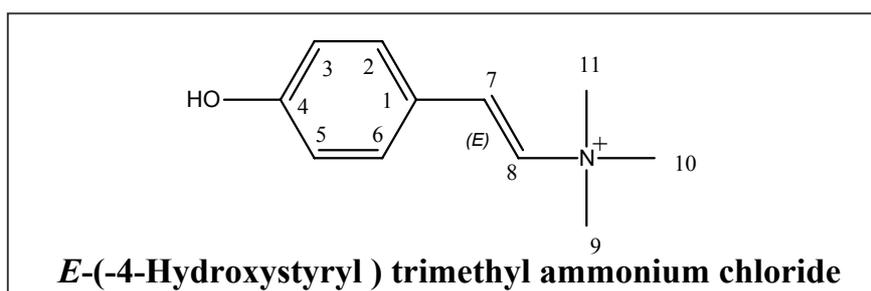
Table 3.2.4: NMR data of compound 10 (500 MHz)

No.	δH (MeOD)	δC (DMSO)	HMBC (MeOD)
1	-	122.4 s	
2, 6	7.40 (d, 2H, 8.8 Hz)	128.9 d	2, 4, 6, 7
3, 5	6.81 (d, 2H, 8.8 Hz)	115.8 d	1, 3, 4, 5
4	-	158.9 s	-
7	6.90 (d, 1H, 14.2 Hz)	125.7 d	8
8	7.13 (d, 1H, 14.2 Hz)	133.4 d	1, NCH$_3$
9, 10, 11	3.40 (s, 9H)	54.5 s	8, 10
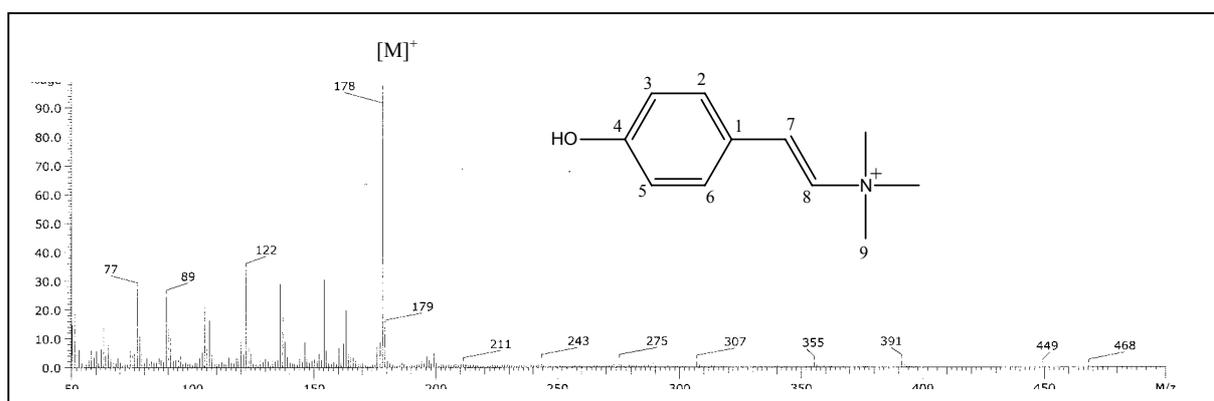
Fig. 3.2.20: FABMS spectrum of compound 10
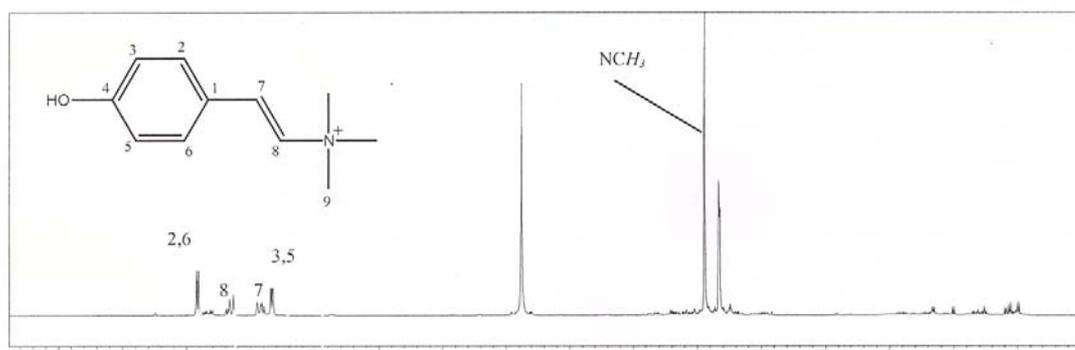
Fig. 3.2.21: ^{1}H-NMR spectrum of compound 10
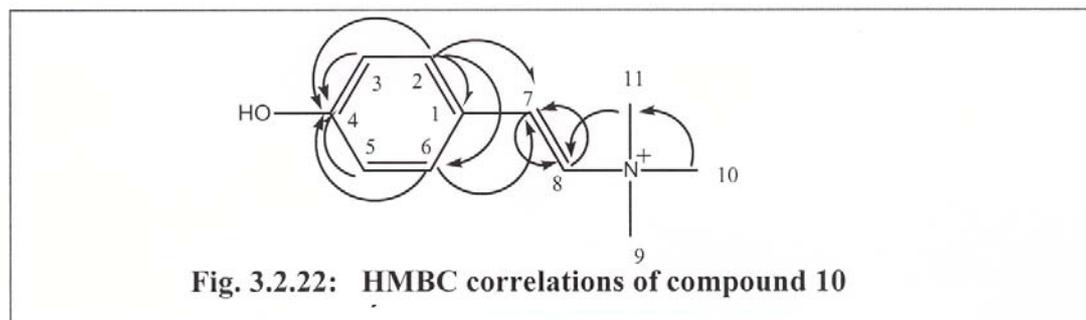
Fig. 3.2.22: HMBC correlations of compound 10

3.2.2. Biological activity of the secondary metabolites from *Axinyssa aplysinoides*

3.2.2.1. Cytotoxic activity

The cytotoxicity of the compounds isolated from *Axinyssa aplysinoides* was studied *in vitro* against human cervix cancer cells (HeLa), rat brain tumour (PC12) and mouse lymphoma (L5178Y) cell lines using the microculture tetrazolium (MTT) technique.

  From Tables 3.2.5 and 3.2.6 it can be seen that compound **7** (bisabolene HCl derivative) showed strong cytotoxic activity against L5178Y (ED_{50} = 0.6 µg/ml), HeLa cell   (ED_{50} = 2.5 µg/ml), and PC12 cell (ED_{50} = 2.5µg/ml).   The other bisabolene urea derivative (compound **8**) exhibited mild activity against L5178Y and no activity against the other cell lines.

Table 3.2.5: Cytotoxic activity of the compounds isolated from *Axinyssa aplysinoides*

Compound	% Growth inhibition (10µg/ml)	
	L5178Y	**HeLa**
7	100	100
8	36	0
9	0	0
10	0	0

Table 3.2.6: Dose response relationship of cytotoxic activity of compound 7

Comound 7 (Conc.)	% Growth inhibition		
	L5178y	**HeLa**	**PC12**
0.1 µg/ml	0	0	0
0.3µg/ml	25 %	0	0
1.0µg/ml	83%	0	11 5
3.0µg/ml	100%	68 %	70 %
10µg/ml	**100 %**	**100 %**	**85 %**
ED_{50}	0.6 µg/ml	2.5 µg/ml	2.5 µg/ml

3.2.2.2. Brine shrimp assay

The sponge *Axinyssa aplysinoides* was studied because its extract exhibited strong activity in the brine-shrimp assay (Fig. 3.2.1). Compound **7** (bisabolene HCl derivative) showed considerably higher activity than the other metabolites isolated from the sponge *Axinyssa aplysinoides* (LC_{50} of 41.07 µg). Compounds **8** and **9** exhibited only mild activity in this assay. This means that compound **7** is responsible for the activity of the extract.

Table3.2.7: **The result of brine-shrimp assay with secondary metabolites of** *Axinyssa aplysinoides*

Compound	Brine shrimp mortality rate	
	50µg	500µg
7	60%	100%
8	25%	30%
9	0%	40%
10	0%	20%
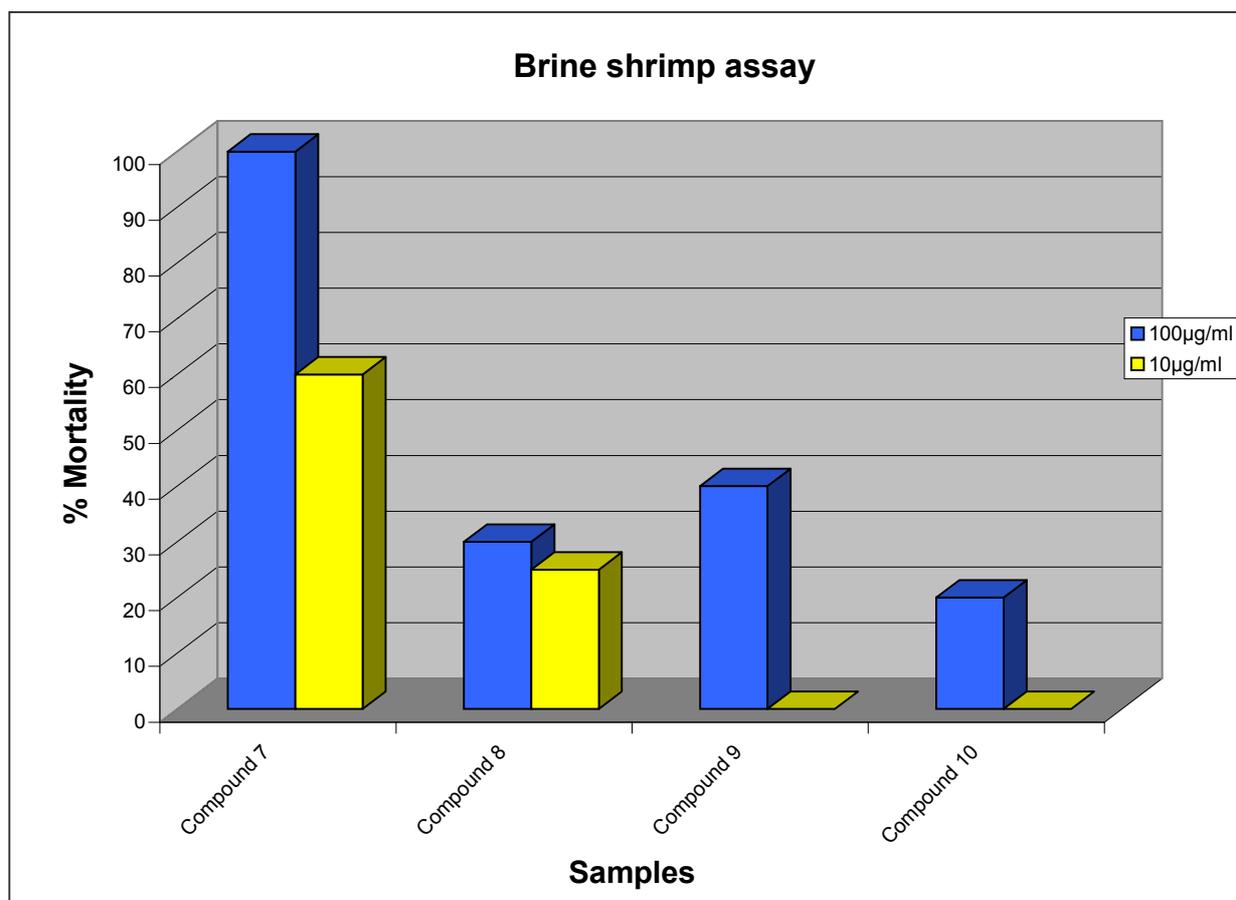
Fig. 3.2.22: The result of brine-shrimp assay with secondary metabolites of *Axinyssa aplysinoides*

3.2.2.3. Antimicrobial activity

The isolated compounds were inactive against *B. subtilus*, *E. coli* and *S. cervisia.* Compound **10** showed strong activity against *Cladosporium herbarum* at a concentration of 100µg exhibiting a zone of inhibition of 14 mm, but was inactive against *C. cucumerinum*.

3.3. Secondary metabolites from *Axinella damicornis*

Marine sponges are considered as a rich source of halogenated compounds with predominance of bromine containing metabolites. It is believed that this is due to the greater ease with which bromide ions are oxidised to give reactive bromonium species which react as electrophiles with unsaturated species (Whitehead, 1999). The sponges belonging to genera *Agelas* and *Axinella* have been found to be rich sources of bromopyrrole alkaloids. These alkaloids are represented by 5-bromopyrrole-2-carbamide (Iwagawa *et al,* 1998), sceptrins (Eder *et al*, 1999, and Vassas *et al,* 1996), oroidin and its cyclized metabolites (Kitagawa *et al,* 1983, and Garcia *et al*, 1973) dispacamides (Whitehead, 1999) and clathramides (Cafieri *et al*, 1996). Many of these metabolites exhibit promising biological activities due to anti-mycobacterial (Shen *et al,* 1998), anti-microbial (Cafieri *et al*, 1996 and Eder *et al*, 1999, Iwagawa *et al*, 1998, and Keifer *et al*, 1991), cytotoxic activity (Inaba *et al*, 1998) and antihistaminic activity (Whitehead, 1999). Sceptrin and ageleferine are two alkaloids isolated from *Agelas novaecaledoniae* which were reported as somatostatin and vasoactive intestinal peptide inhibitors (Vassas *et al,* 1996).

$R_1 = R_2 = H$ clathrodin
$R_1 = R_2 = Br$ oroidin
$R_1 = H$ $R_2 = Br$ hymenidin

$R_1 = R_2 = Br$ $R_3 = NH_2$ 4,5-dibromopyrrole-2-carbamide
$R_1 = R_2 = Br$ $R_3 = OH$ 4,5-dibromopyrrole-2-carboxylic acid
$R_1 = Br$ $R_2 = H$ $R_3 = NH_2$ 4-bromopyrrole-2-carbamide
$R_1 = Br$ $R_2 = H$ $R_3 = OH$ 4-bromopyrrole-2-carboxylic acid
$R_1 = H$ $R_2 = Br$ $R_3 = NH_2$ 5-bromopyrrole-2-carbamide
$R_1 = Br$ $R_2 = H$ $R_3 = OH$ 4-bromopyrrole-2-carbxylic acid

Fig. 3.3.1 : Some brominated compounds isolated from sponges

Spongiacidin B

Z-Hymenialdisine
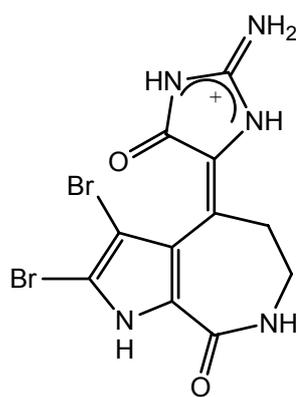
Spongiacidin A
(*E*-3-Bromohymenialdisine)
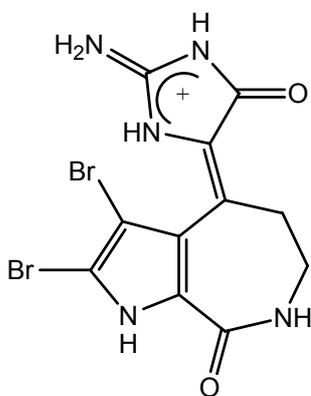
Z-3-Bromohymenialdisine
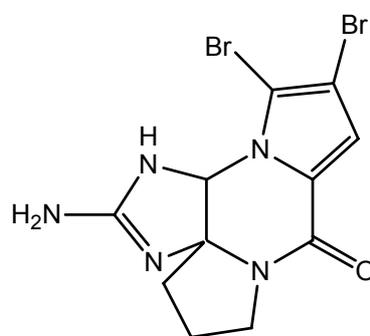
Dibromophakellin
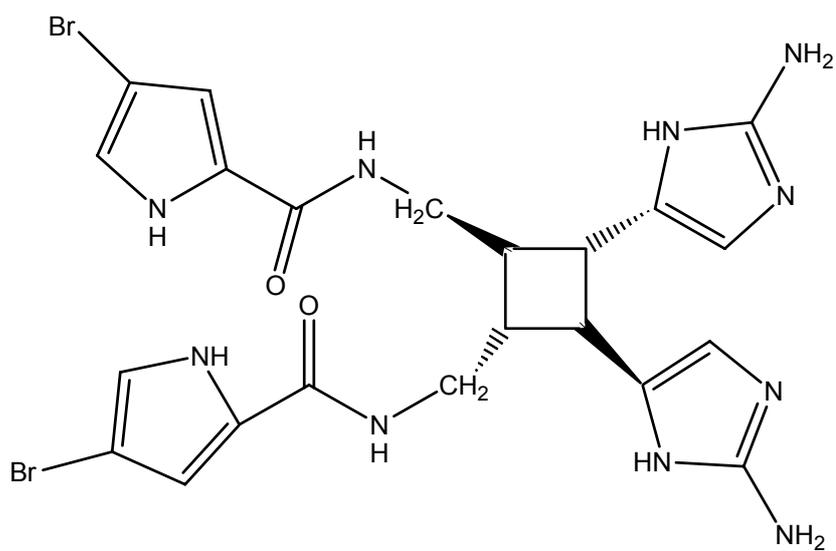
Sceptrin

Fig. 3.3.2 : Some brominated compounds isolated from sponges

In our search for biologically active metabolites, freeze dried sponges collected from Elba were extracted with organic solvents and the resulting extracts were subjected to a biological screening assay for feeding deterrence activity against *Blennius sphinx*. Among the sponges which exhibited strong activity was *Axinella damicornis* (Fig. 3.3.3).

Table 3.3.1: The result of fish-feeding assay (*B. sphinx*) for the total extract of some sponges from Elba

Total Extract of	% of eaten food			
	Experiment		Blank	
	1	2	1	2
Axinella damicornis	**0.8**	**0.8**	**99.2**	**99.2**
Agelas oroides	**3.1**	**3.6**	**96.9**	**96.4**
Hamigera hamigera	16.7	18.3	83.3	81.7
Ircinia fasciculata	15.9	17.3	84.1	82.7
Acanthella acuta	23.0	17.7	77.0	82.3
Aplysina cavernicola	**7.0**	**0.8**	**93.0**	**99.2**
Spongia officinalis	7.7	18.2	92.8	81.8
Chondrosia reniformis	37.8	40.4	62.2	59.6
Petrosia ficifomis	40.4	42.1	59.6	57.9
Ircinia spinosula	40.0	38.9	60.0	61.1

The total extract of the sponge *Axinella damicornis* was subjected to solvent-solvent partitioning to yield ethyl acetate, hexane, butanol, and water phase. The ethyl acetate and butanol extracts exhibited strong activity in the fish-feeding assay (Fig. 3.3.4). The chemical investigation of the active ethyl acetate and butanol extracts yielded seventeen brominated compounds. Compounds **11** (3,4-dibromopyrrole-2-carbamide), and **15** (2,3-dibromoaldisin) were new natural products, while compounds **12** (3,4-dibromopyrrole-2-carboxylic acid) and **14** (3-bromoaldisin) were new as natural products but had been reported before as synthetic products.
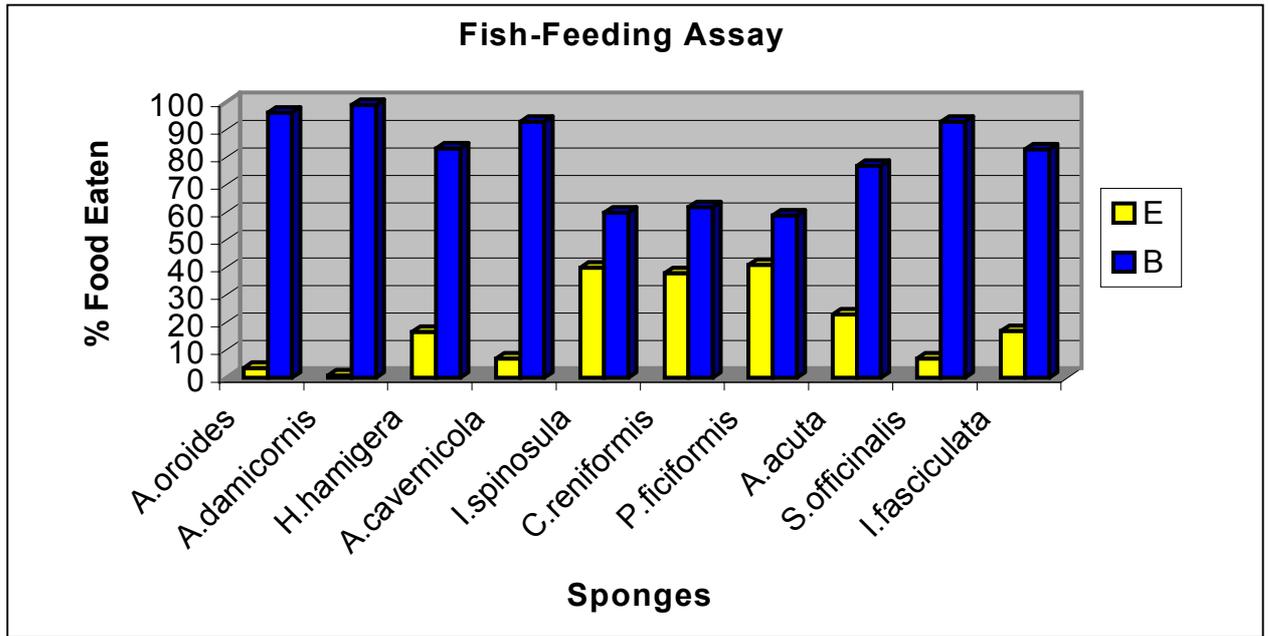
E = experiment, B = blank

Fig. 3.3.3: Results of fish-feeding assay of 10 sponges collected from Elba, Italy
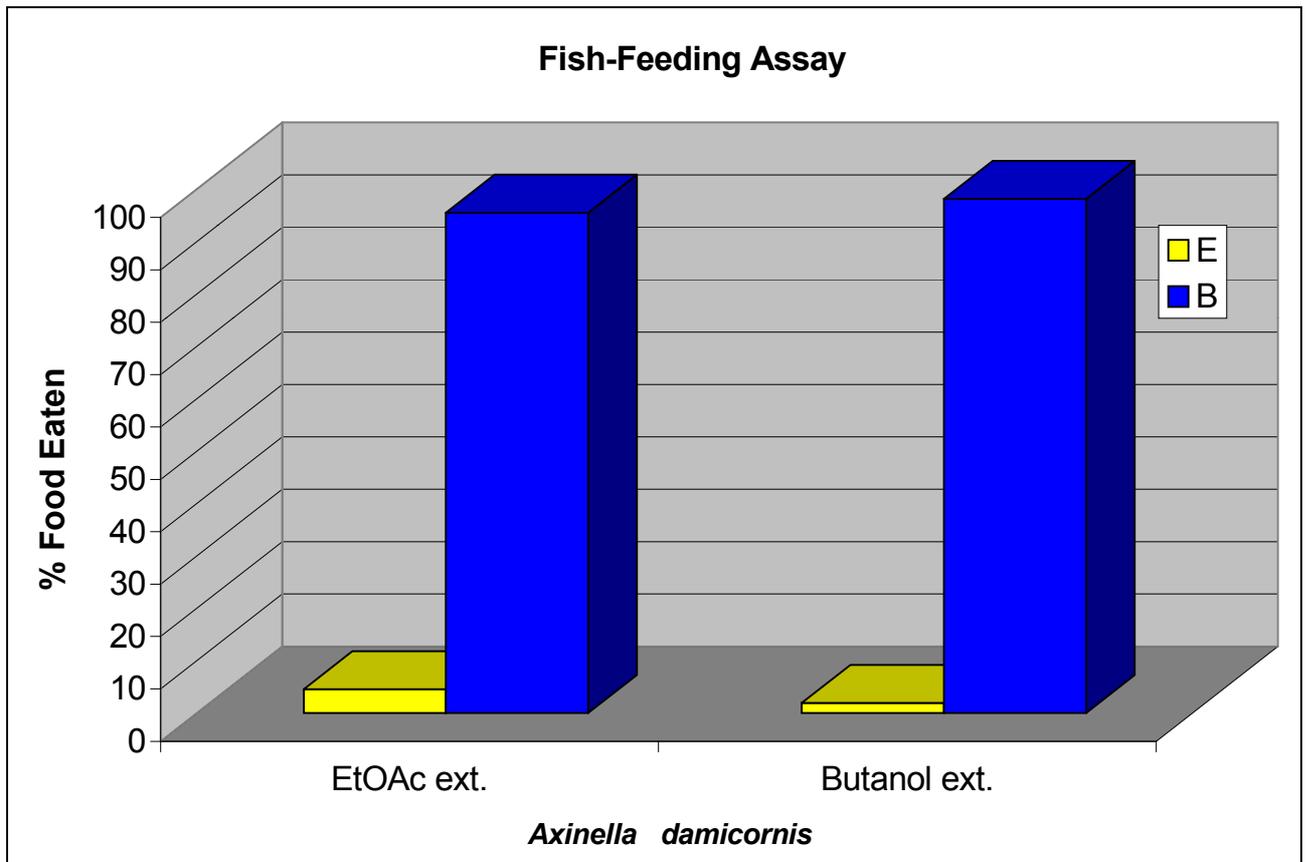
E = experiment, B = blank

Fig. 3.3.4: Results of fish-feeding assay of ethyl acetate and butanol extracts of
Axinella damicornis

3.3.1. Structure elucidation of the isolated compounds

3.3.1.1. Structure elucidation of 3,4-dibromopyrrole-2-carbamide (11, new compound)

Compound 11 was isolated as a yellowish white amorphous powder. It has UV absorbances at λ_{max} 267 and 200 nm. The EIMS spectrum (Fig. 3.3.5A) gave a cluster of peaks at *m/z* 266, 268 and 270 in the ratio of 1:2:1, which revealed that compound **11** is a dibrominated compound. The fragment cluster at *m/z* 249/251/253 $[M-NH_3]^+$ indicated the presence of a terminal amine group. By HREIMS (Fig. 3.3.5B), the molecular formula was determined as $C_5H_4N_2OBr_2$. The UV absorption of compound **11** at 276 nm suggested the presence of a pyrrole ring in conjugation with a carbonyl group (Eder, 1998). The ^{13}C-NMR spectrum (Fig. 3.3.9, Table 3.3.2) and DEPT showed resonances at δ_C 125.0 (s), 104.6 (s), 97.7 (s) and 113.1 (d) corresponding to a trisubstituted pyrrole ring, which was confirmed from the signals at δ_H 6.90 (1H, d, *J* = 2.52 Hz) and 12.63 (1H, br.s, N*H*-1) in the ^1H-NMR spectrum (Fig. 3.3.7). Furthermore a carboxamide signal at δ_C 160.2 (s) and two ^1H-NMR signals for N*H*$_2$ group at δ_H 7.17 and 7.58 (each, 1H, br s) were observed for the terminal amide function. The coupling constant value of the doublet signal at δ 6.90 (2.5 Hz) indicated an *ortho* coupling with the pyrrole N*H*-1 proton (Pretsch *et al*, 2000). Accordingly the positions of the substituents were assigned to be at C-2, C-3, and C-4. By comparison of the chemical shifts with those of literature data, (Iwagawa *et al*, 1998, Pretsch *et al*, 2000 and Stierle and Faulkner, 1980), it was deduced that the position of the CON*H*$_2$ group was at C-2 and the two bromines were at C-3 and C-4. This assignment was confirmed through the HMBC spectrum (Fig. 3.3.11), where the doublet proton, H-5, showed correlations with two quaternary carbons C-2 and C-3. In addition, the absence of the correlation between H-5 and the CON*H*$_2$ was observed which means that the amide function is not sitting at C-4. According to the results mentioned before, compound **11** was concluded to be the new natural product **3,4-dibromopyrrole-2-carbamide**.

3,4-Dibromopyrrole-2-carbamide (Compound, 11)

Chemical name: 3,4-Dibromo-1H-pyrrole-2-carboxylic acid amide
Character: yellowish white amorphous powder
Sample code: Ax.dam.vlc fr.6 p5   **Amount:** 3.5 mg
Molecular Weight: 266/268/270   **Molecular formula** $C_5H_4Br_2N_2O$
Biological source: Marine sponge *Axinella damicornis*

New Natural Product

HPLC chromatogram

UV absorbance
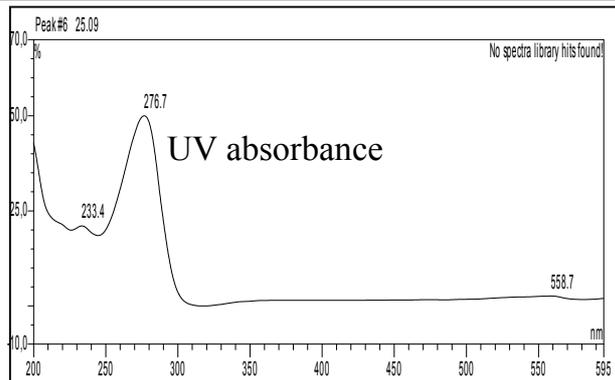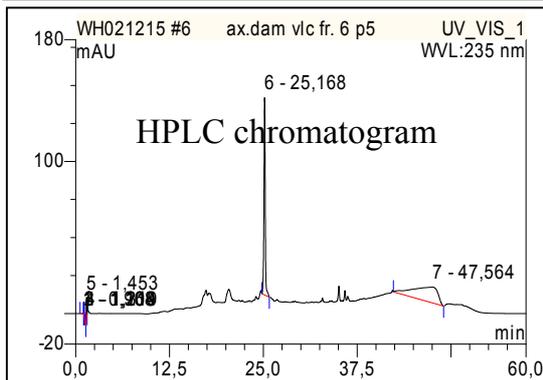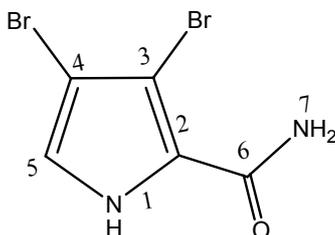
ESI-MS: 265/267/269 $[M-H]^+$, 533/535/537 $[2M-H]^+$ (Negative)

EI-MS: *m/z* (rel. int.) 266/268/270 (33:60:25) $[M]^+$, 249/251/253 (38:69:31) 242 (6), 186 (35), 133 (18), 118 (7), 97 (79, 77 (10), 60 (66) and 44 (100)

HREIMS: for 266/268/270 $[M]^+$ Experimental = 265.87148
                              Calculated = 265.86904
                                    for $C_5H_4Br_2N_2O$
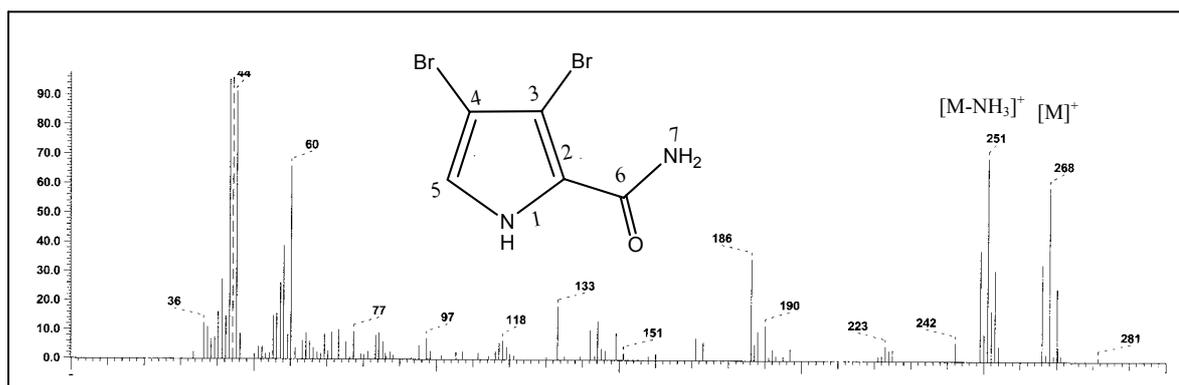
Fig. 3.3.5A: EIMS spectrum of compound 11
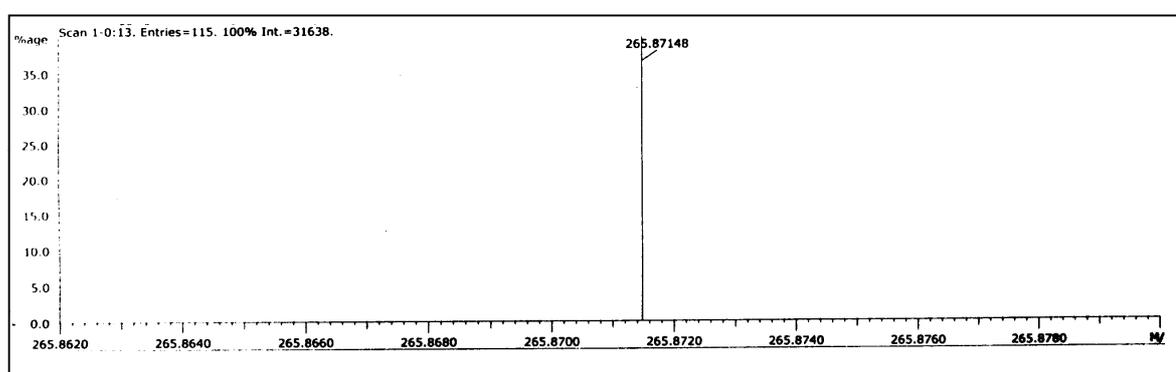
Fig. 3.3.5B: HREIMS spectrum of compound 11
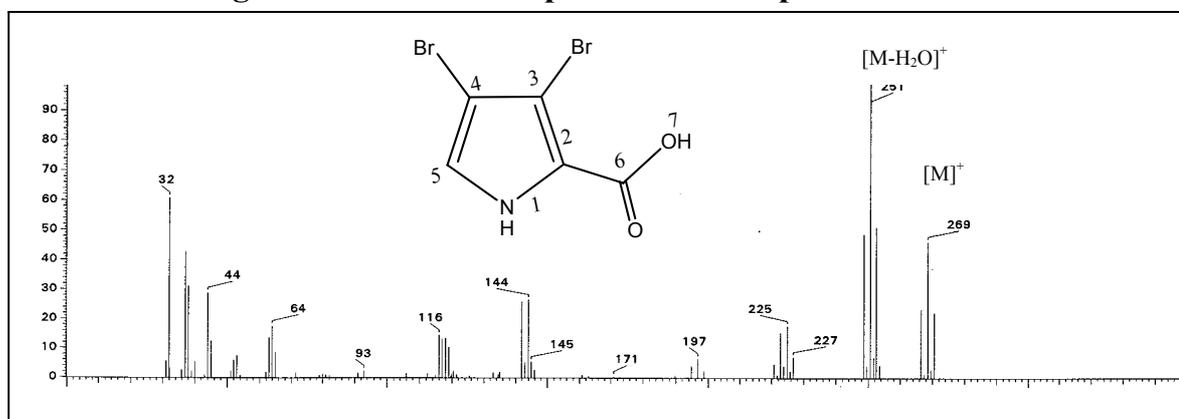
Fig. 3.3.6A: EIMS spectrum of compound 12
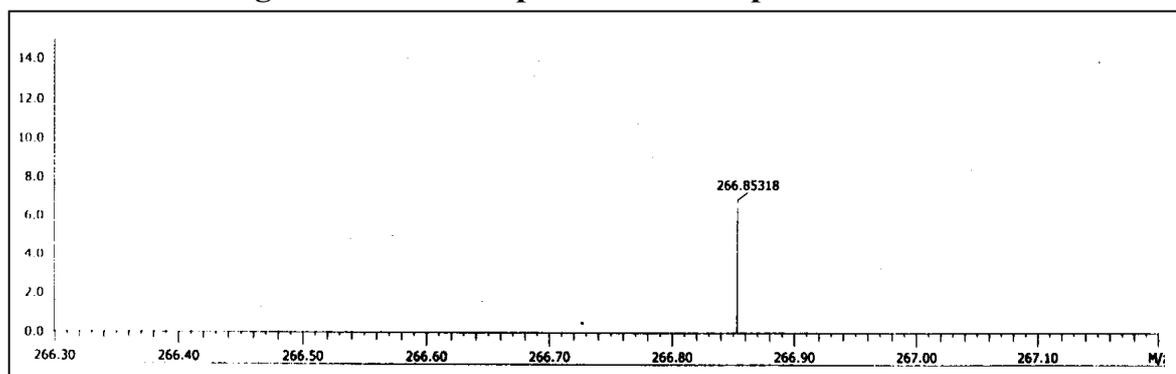
Fig. 3.3.6B: HREIMS spectrum of compound 12
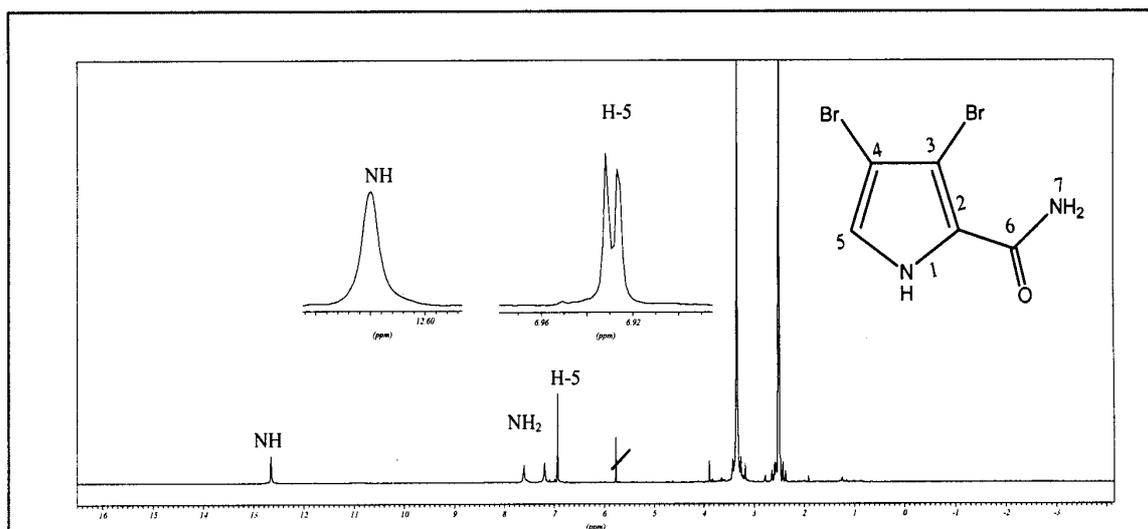
Fig. 3.3.7: ¹H-NMR spectrum of compound 11

Fig. 3.3.8: ¹H-NMR spectrum of compound 12
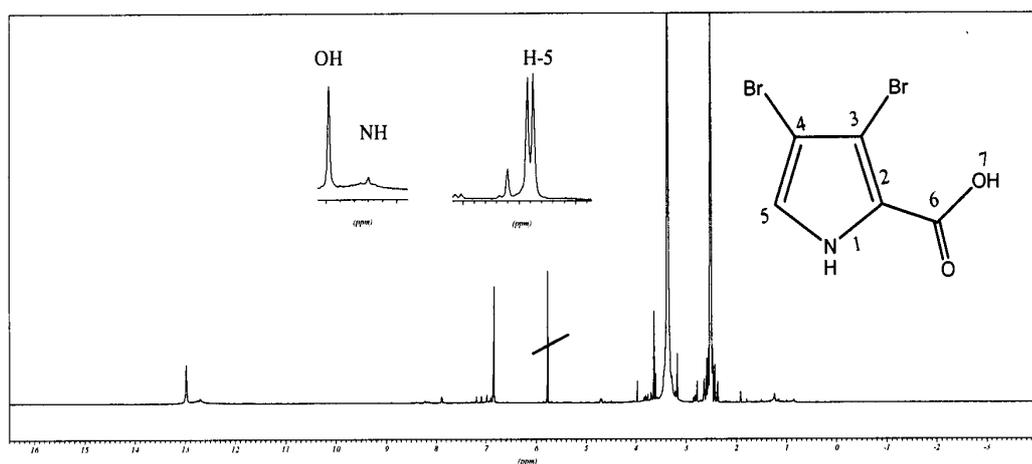
Fig. 3.3.11: HMBC spectrum of compound 11
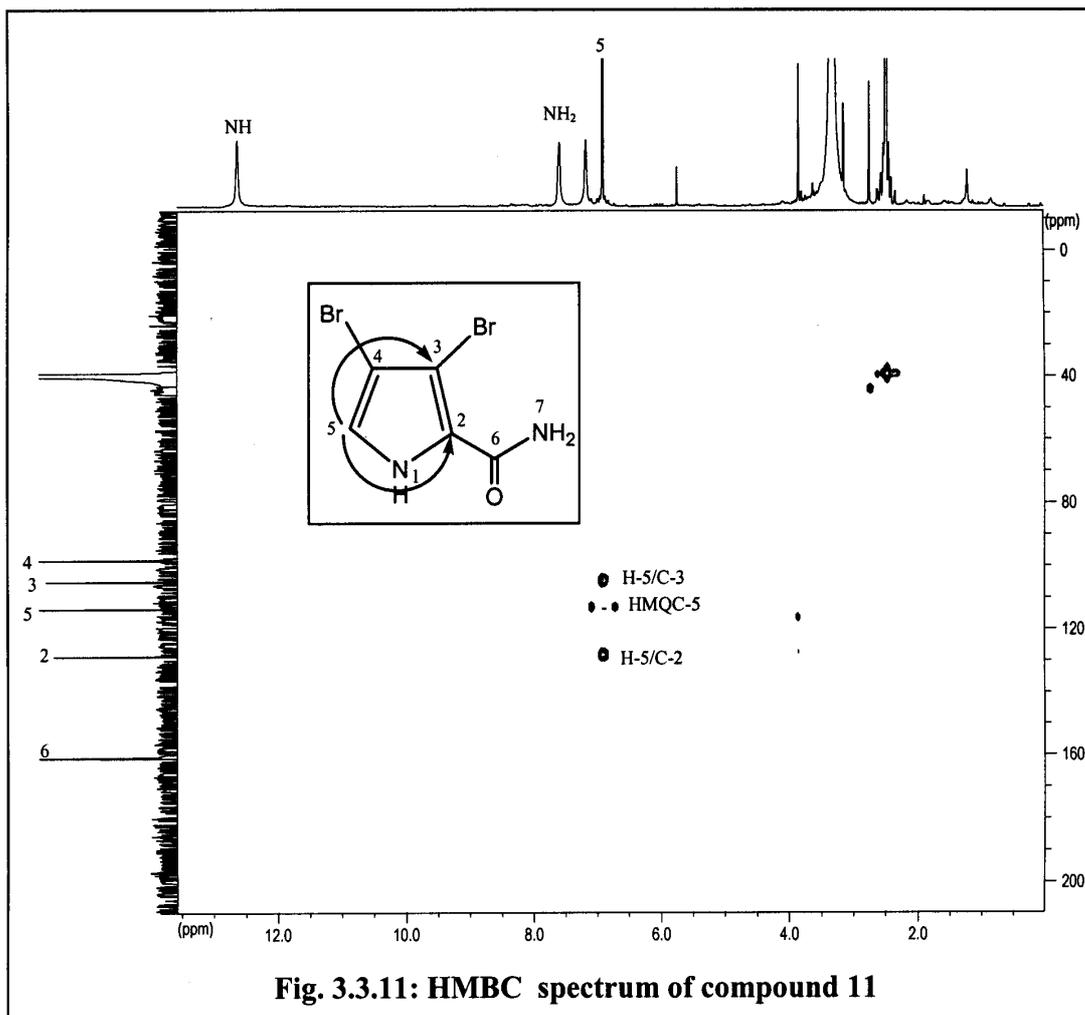
3.3.1.2. Structure elucidation of 3,4-dibromopyrrole-2-carboxylic acid (12, new as a natural product)

Compound 12 showed similar chromatographic behaviour as compound **12**, it has UV absorbances at λ_{max} 277 and 234 nm. Compound **12** was isolated as a yellowish amorphous powder. The EIMS spectrum (Fig. 3.3.6A) showed a cluster of peaks similar to that of compound **11** but at a higher mass unit, at *m/z* 267, 269 and 271 in the same ratio of 1:2:1. This revealed that compound **12** is also a dibrominated compound. By HREIMS (Fig. 3.3.6B), the molecular formula was determined as $C_5H_3NO_2Br_2$. The ^{1}H-NMR (Table 3.3.2, Fig. 3.3.8), showed a similar spectrum to that of compound **11**, with a doublet signal at δ_H 6.80 (1H, d, *J*= 2.8 Hz). The difference in the ^{1}H-NMR spectrum was the presence of an additional signal at δ_H 13.00 (1H, br s) which was assigned to an acidic O*H* and disappearance of the two signals for the

NH_2 group at δ_H 7.17 and 7.58. The presence of a terminal carboxyl group was confirmed from the cluster fragment in the EIMS spectrum at *m/z* 249/251/253 [M-H$_2$O]$^+$. The ^{13}C-NMR spectrum (Table 3.3.2, Fig. 3.3.10) and DEPT were also similar to those of compound **11** except for the slight downfield shifts of C-2 (Δ 3.1 ppm), C-3 (Δ 2.0 ppm), C-4 (Δ 0.9 ppm) and C-5 (Δ 3.6 ppm) which resulted from the replacement of NH_2 group by an OH function. The positions of the carboxylic group and the two bromine atoms were elucidated from the HMBC data (Fig. 3.3.12, Table 3.3.2). According to the results mentioned above, compound **12** was concluded to be **3,4-dibromopyrrole-2-carboxylic acid** and this is the first isolation of **12** as a natural product. Compound **12** was previously reported as a synthetic product obtained in the synthesis of lukianol A and camellarino dimethyl ether (Fürstner *et al*, 1995).

Fig. 3.3.12: HMBC correlations of 3,4-dibromopyrrole-2-carboxylic acid
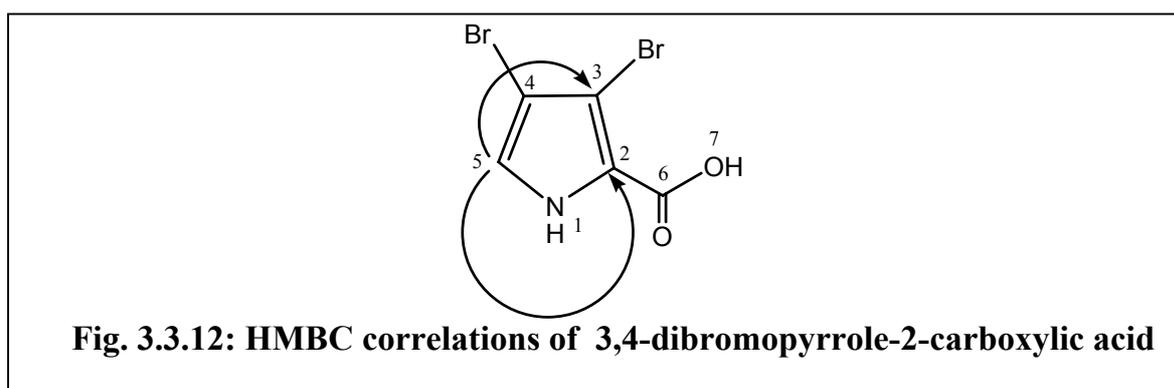
Table 3.3.2: ^{1}H and ^{13}C-NMR data of compounds 11 and 12 (DMSO-d_6, 500 MHz)

No.	Compound 11		Compound 12	
	δH	δC	δH	δC
1	12.63 (br s)	-	12.70 (br s)	-
2	-	125.0 s	-	128.1 s
3	-	104.6 s	-	106.6 s
4	-	97.7 s	-	98.6 s
5	6.90 (d, *J*=2.5 Hz)	113.1 d	6.80 (d, *J*=2.8 Hz)	116.7 d
6	-	160.2 s	-	160.4 s
7	7.17 (br s)	-	13.00 (br s)	-
	7.58 (br s)			

3,4-Dibromo-1H-pyrrole-2-carboxylic acid (Compound 12)

Chemical name: 3,4-Dibromo-1H-pyrrole-2-carboxylic acid
Character : yellowish white amorphous powder **Amount**: 3.4 mg
Sample code : Ax.dam.vlc fr.6 p7
Molecular Weight: 267/269/271
Biological source: Marine sponge *Axinella damicornis*
Molecular formula: $C_5H_3Br_2NO_2$

New natural product
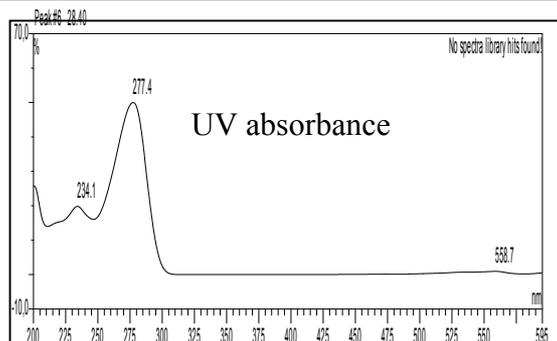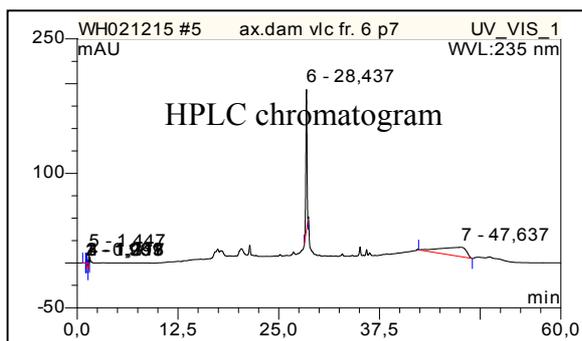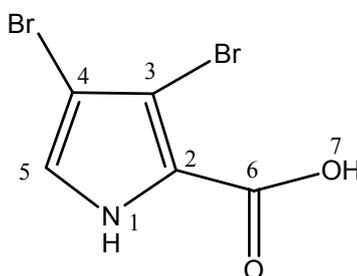
ESI-MS: 266/268/270 [M-H]$^+$, 535/537/539 [2M-H]$^+$ (Negative)

EI-MS: *m/z* (rel. int.) 267/269/271 (22:43:22) [M]$^+$, 249/251/253 (50:100:50), 221/223/225 (5:10:5), 195/197/199 (2:4:2), 142/144 (26:25), 116/118 (9:9) 64 (9) and 37 (22).

HREIMS: for 267/269/271 [M]$^+$ Experimental = 266.85318
Calculated = 266.85306
for $C_5H_3Br_2NO_2$

3.3.1.3. Structure elucidation of 2-bromoaldisin (13, known compound)

2-Bromoaldisin was isolated as a yellowish white powder, it has UV absorbances at λ_{max} 310 and 228 nm. The EIMS spectrum (Fig. 3.3.13) gave isotopic clusters at *m/z* 242 and 244 in ratio of 1:1 [M]$^+$, indicating a monobrominated compound with the molecular formula $C_8H_7N_2O_2Br$. In addition, other peaks at *m/z* 214/216 [M-C=O]$^+$, 185/187 [M-C*H*$_2$-N*H*-C=O]$^+$, *m/z* 171/173 [M-C*H*$_2$-C*H*$_2$-N*H*-C=O]$^+$ were observed. The ^{13}C-NMR (Table 3.3.3, Fig. 3.3.15) and DEPT showed resonances at δ_C 105.1 (s), 111.2 (d), 124.5 (s) and 129.4 (s) which suggested the presence of a trisubstituted pyrrole ring. This was established from the ^{1}H-NMR signals at δ_H 6.54 (1H, s, H-3), and 13.00 (1H br s, N*H*-1). The resonances at δ_C 36.1 (t), 43.4 (t) δ 161.2 (s) and 193.5 (s) suggested the presence of a CO-N*H*-C*H*$_2$-C*H*$_2$-CO function. The ^{1}H-NMR spectrum (Fig. 3.3.14) confirmed this assumption. It showed resonances at δ_H 2.80 (2H, m), 3.40 (2H, m) and 8.40 (1H, br. t, *J* = 5.05 Hz) which were assigned to C*H*$_2$-5, C*H*$_2$-6 and N*H*-7 respectively. The COSY spectrum of compound **13** showed a spin system consisting of a N*H*-C*H*$_2$-C*H*$_2$ residue. The above mentioned data indicated the presence of an aldisin nucleus. The HMBC spectrum (Fig. 3.3.16) confirmed this, showing correlations of H-3 with C-2, C-8a, C-4; H-6 with C-8, C-5 and H-5 with C-4, C-6. According to these data and through comparison with the literature (Nanteuil *et al*, 1985 and Schmitz *et al*, 1985), compound **13** was concluded to be **2-bromoaldisin**.

Table 3.3.3: NMR Data of 2-Bromoaldisin (DMSO-d_6, 500 MHz)

No.	δH	δC	COSY	HMBC
1	13.00 (br s)	-		-
2		105.1 s		-
3	6.54 (s)	111.2 d		2, 4, 8a
3a	-	124.5 s		-
4	-	193.5 s		-
5	2.80 (m)	43.4 t	6	4, 6
6	3.40 (m)	36.1 t	5, 7	8
7	N*H*, 8.40 (t, *J*= 5.1 Hz)	-	6	-
8	-	161.2 s		-
8a	-	129.4 s		-
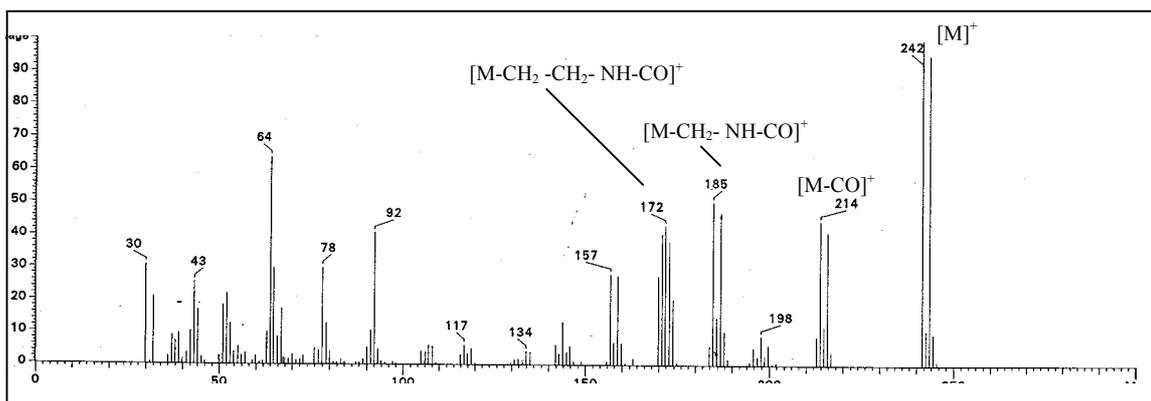
Fig. 3.3.13: EIMS spectrum of compound 13

Fig. 3.3.14: ^{1}H-NMR spectrum of compound 13

. Fig. 3.3.15: ^{13}C-NMR spectrum of compound 13
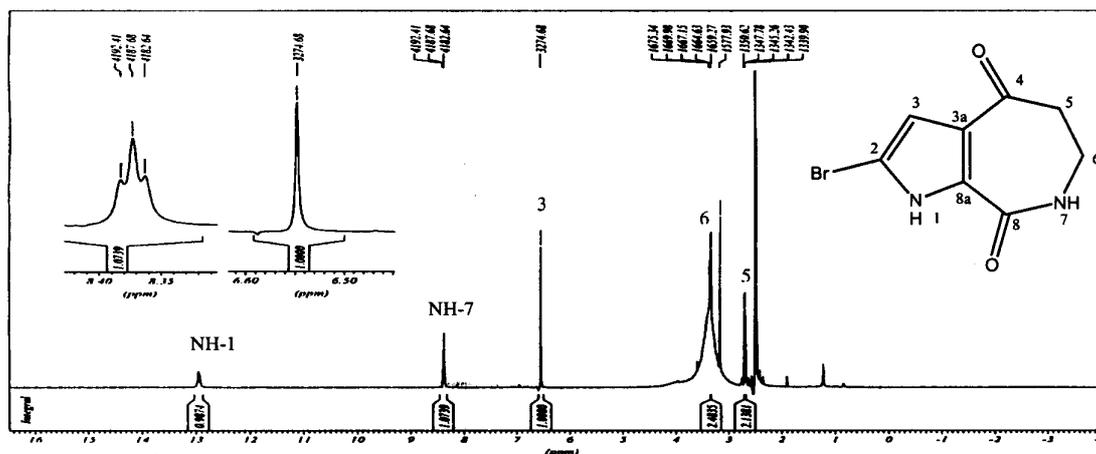
Fig. 3.3.16: Important HMBC correlations of compound 13

3.3.1.4. Structure elucidation of 3-bromoaldisin (14, new natural product)
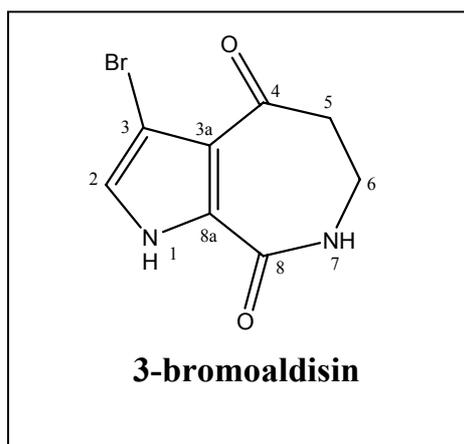
3-bromoaldisin

3-Bromoaldisin was isolated as a yellow amorphous solid. The EIMS spectrum (Fig. 3.3.17A) showed isotopic clusters at *m/z* 242 and 244 in the ratio of 1:1, revealing a monobrominated compound. The molecular formula was established as $C_8H_7N_2O_2Br$ by HREIMS (Fig. 3.3.17B). The ^{1}H-NMR spectrum of **14** (Fig. 3.3.18) showed the general characteristics of 2-bromoaldisin (**13**) which was also isolated in this study. There were two exchangeable protons at δ_H 13.00 and 8.40 for N*H*-1 and N*H*-7, respectively, two methylenes at δ_H 2.80 (m) and 3.20 (m) for C*H*$_2$-5 and C*H*$_2$-6 respectively, with the exception that, the olefinic signal at δ_H 6.54 appeared as doublet with a coupling constant of 2.84 Hz. In comparison to compounds **11** and **12**, the coupling value reflected an ortho coupling with the N*H*-1 of the pyrrole ring, which suggested a 3-bromoaldisin structure. The ^{13}C-NMR (Fig. 3.3.19, Table 3.3.4), showed the resonances associated with a bromoaldisin structure, as it showed five quaternary carbons at δ_C 193.5, 161.3, 124.6, 129.4 and 105.2, two methylenes at δ_C 36.3 and 43.3 and one methine at δ_C 111.2. The HMBC spectrum (Fig. 3.3.20), showed the correlations of H-2 with C-3 and C-8a, and absence of the 2-bromaldisin diagnostic correlation with the carbonyl at δ_C 193.5 C-4 thus confirming that compound **14** was 3-bromoaldisin. A literature search on 3- bromoaldisin revealed that it was first isolated as an inseparable mixture with 2-bromoaldisin (1:1) during the synthesis of hymenialdisine (Annoura and Tatsouka, 1995). This is the first isolation of **3-bromoaldisin** as a natural product.

Table 3.3.4: ^{1}H- and ^{13}C-NMR Data and HMBC of 3-Bromoaldisin (DMSO-d_6, 500 MHz)

No.	δH	δC	COSY	HMBC
1	13.00 (br s)	-	-	-
2	6.54 (d, 2.8 Hz)	111.2 d	-	8a
3	-	105.2 s	-	-
3a	-	124.6 s	-	-
4	-	193.5 s	-	-
5	2.80 (m)	43.3 t	6	4, 6
6	3.20 (m)	36.3 t	5, 7	4, 5, 8
7	N*H*, 8.40 (t, 5.1 Hz)	-	6	-
8	-	161.3 s	-	-
8a	-	129.4 s	-	-

3-Bromoaldisin (Compound 14)

Chemical name: 3-Bromo-5,6-dihydro-1H,7H-pyrrolo[2,3-c]azepine-4,8-dione
Character: Yellow amorphous solid
Amount: 2.4 mg   **Sample code:** Ax. dam. Übrig P1
Molecular Weight : 242/244   **Molecular formula**: $C_8H_7BrN_2O_2$
Biological source: marine sponge *Axinella damicornis*

New natural product
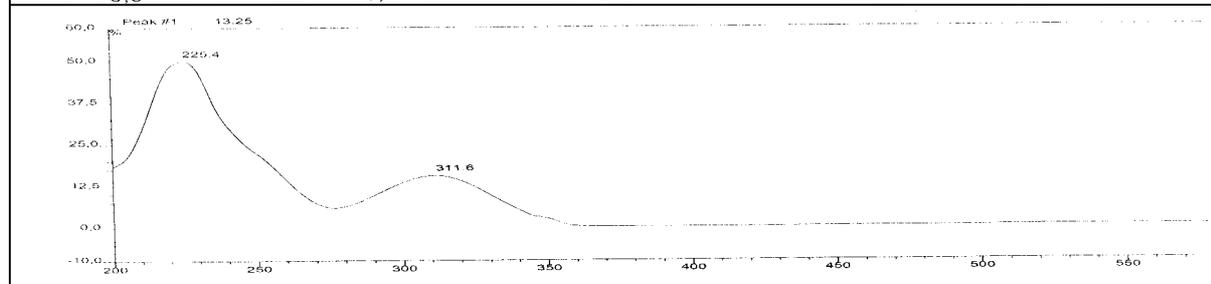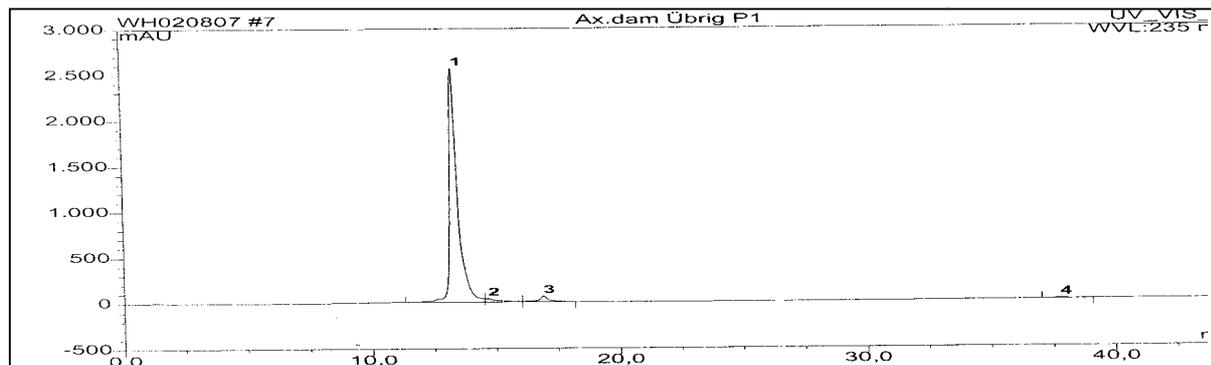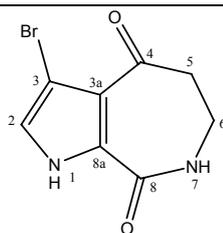
EISMS: 243/245 $[M+H]^+$ (Positive)

EIMS: *m/z* (rel. int.) 242/244 (24: 22) $[M]^+$
HREIMS: Experimental   241.97012
       Calculated   241.96909
         for $C_8H_7BrN_2O_2$
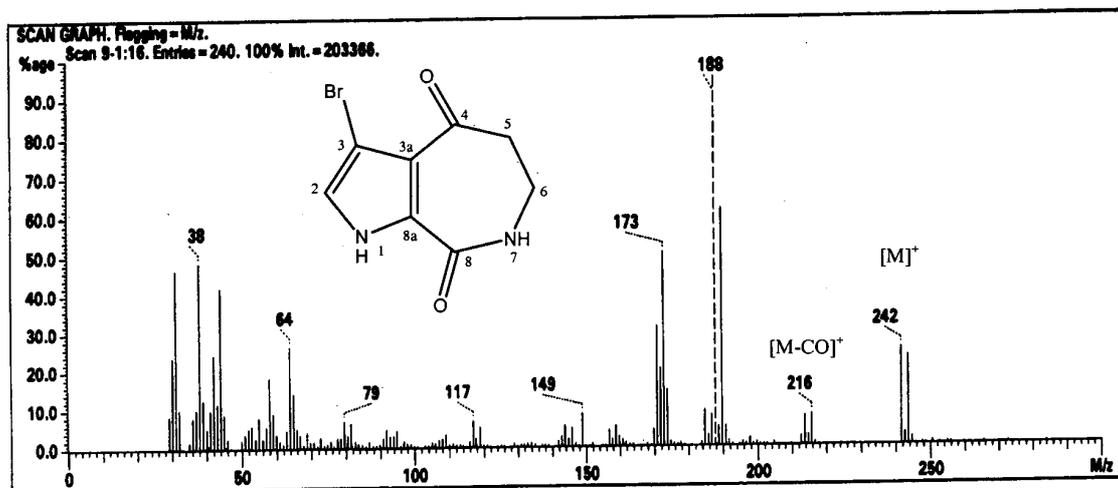
Fig. 3.3.17A: EIMS spectrum of compound 14
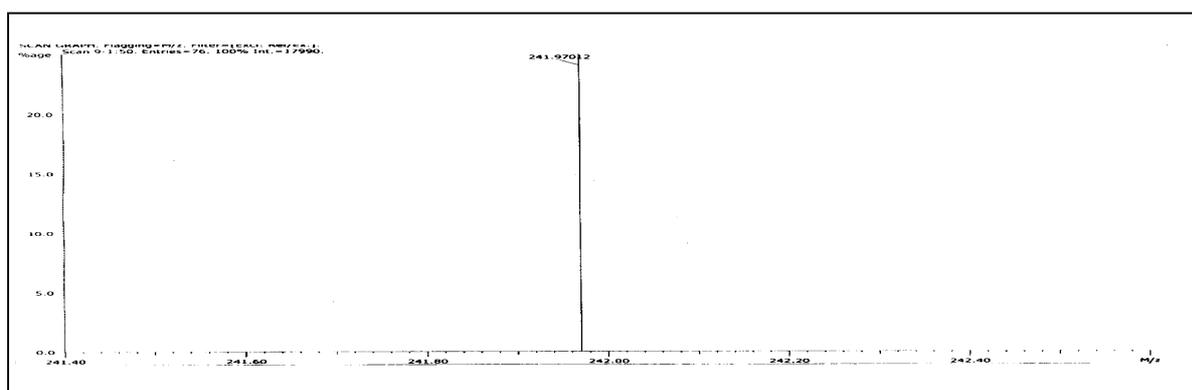
Fig. 3.3.17B: HREIMS spectrum of compound 14
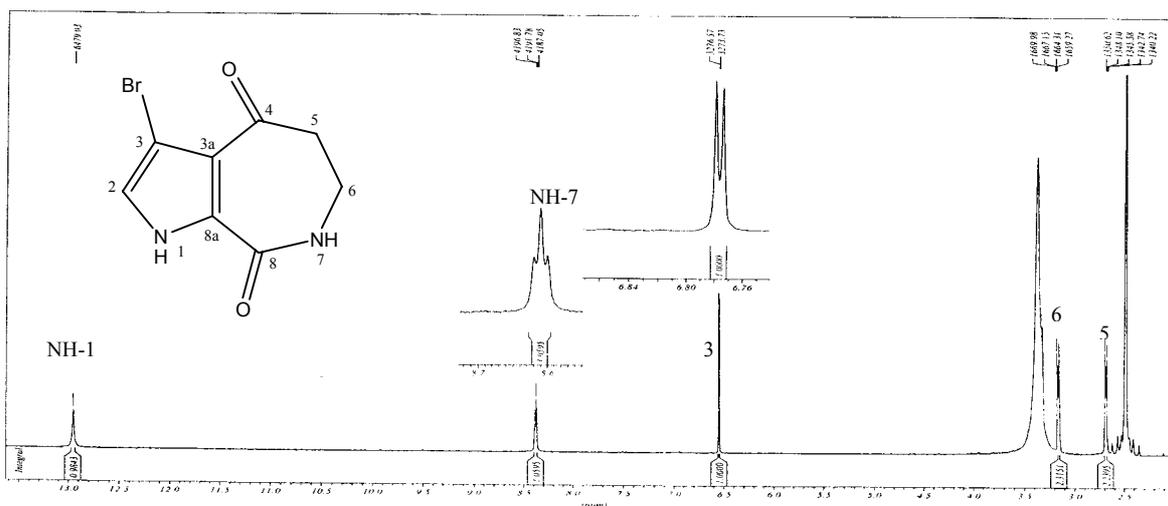
Fig. 3.3.18: ^{1}H-NMR spectrum of compound 14
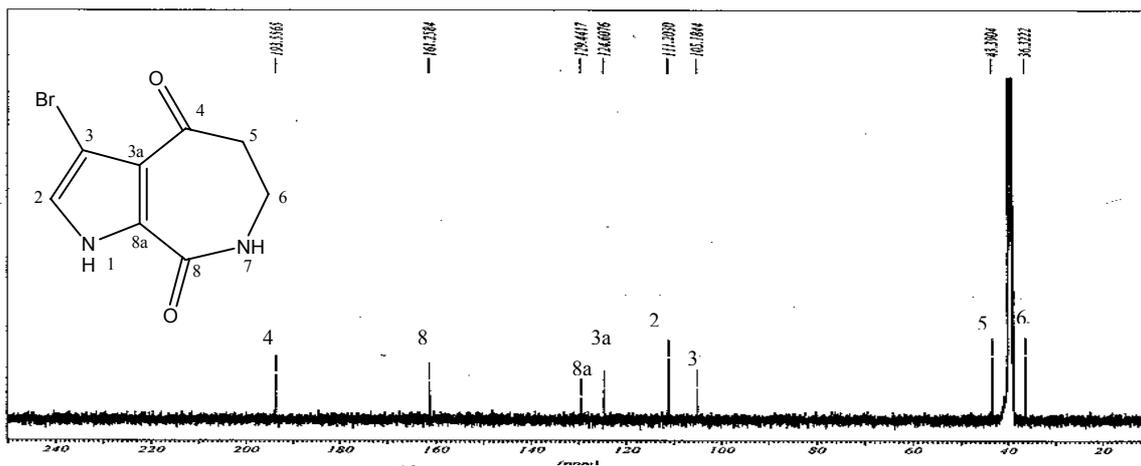
Fig. 3.3.19: ^{13}C-NMR spectrum of compound 14
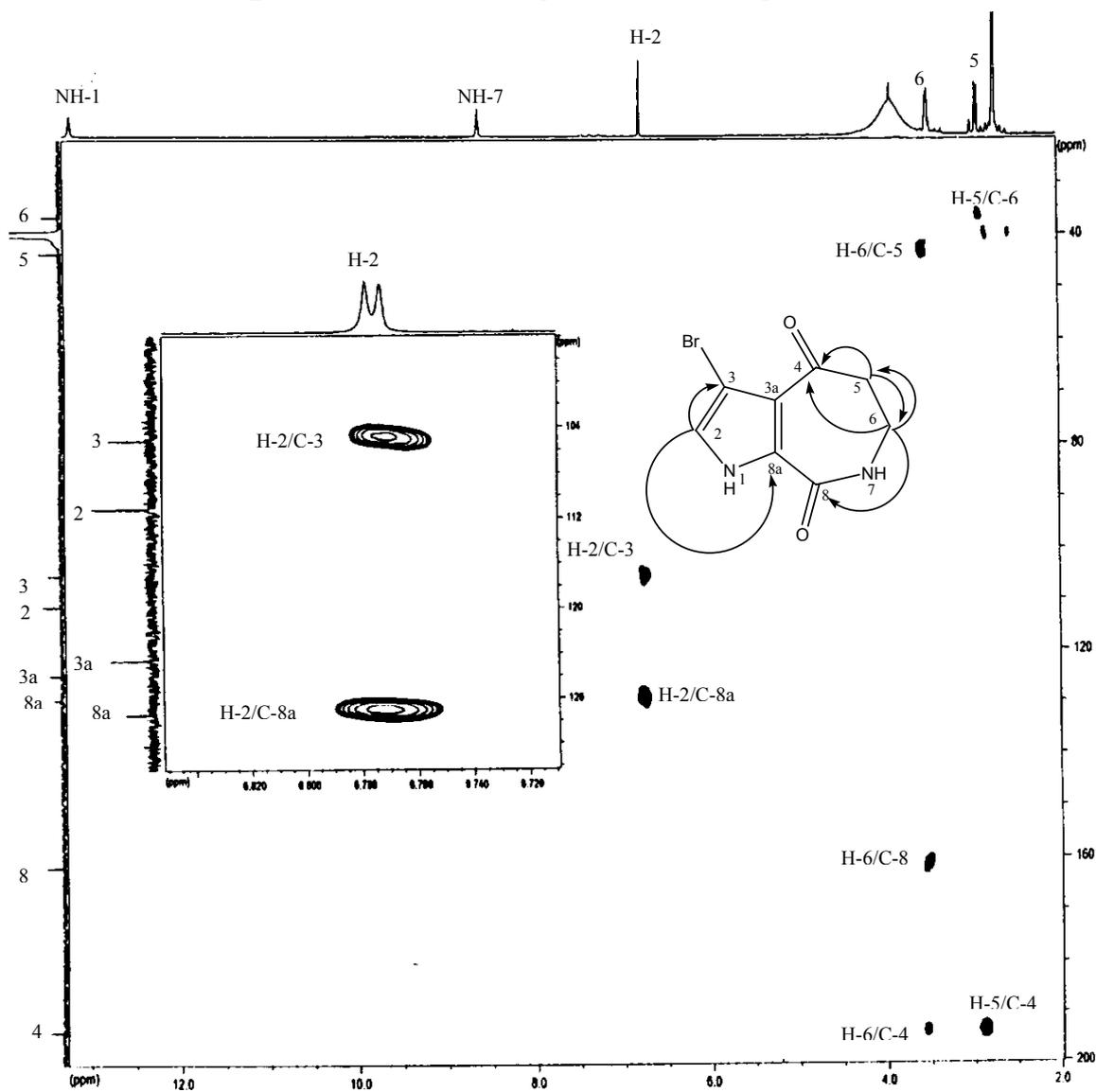
Fig. 3.3.20: HMBC spectrum of compound 14

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3.3.1.5. Structure elucidation of 2,3-dibromoaldisin (15, new compound)

2,3-dibromoaldisin
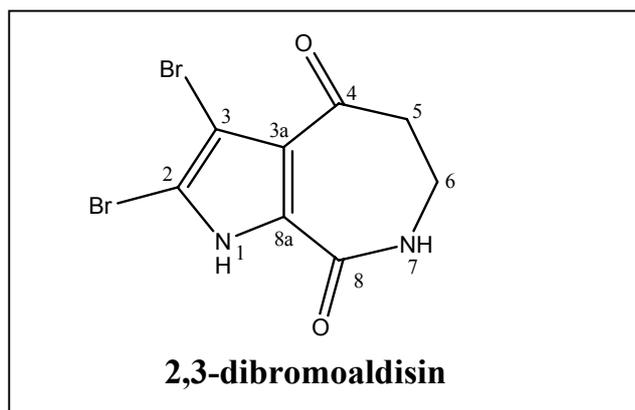
Compound 15 was isolated as a yellow amorphous solid. It has UV absorbances at λ_{max} 228 and 313 nm. The EIMS spectrum (Fig. 3.3.21) of this compound showed the molecular ion peak at *m/z* 320, 322, and 324 in the ratio of 1:2:1, indicating that compound **15** is a dibrominated compound. In addition the mass spectral fragmentation pattern with intense ion peaks at *m/z* 292/294/296 [M-CO]$^{+}$, 277/279/281 [M-NH-CO]$^{+}$, 263/265/267 [M-CH$_2$-NH-CO]$^{+}$, 249/251/253 [M-NH-CO-CH$_2$-CH$_2$]$^{+}$ suggested an aldisin nucleus (Schmitz, *et al,* 1985). The ^{13}C-NMR data (Table 3.3.5) showed the resonances for an aliphatic carbonyl at δ_C 193.5 (s), an amide carbon at δ_C 161.5 (s), four quaternary carbons at δ 129.5 (s), 124.0 (s), 106.0 (s), and 98.0 (s), in addition two methylene carbons at δ_C 43.5 (t) and 36.5 (t) which again suggested the presence of an aldisin nucleus. The ^{1}H-NMR spectrum recorded in *d$_6$*-DMSO (Table 3.3.5, Fig. 3.3.22) of **15** confirmed this. It showed two exchangeable protons at δ_H 13.50 (1H, br s) and 8.53 (1H, br t, *J* = 5.05) for N*H*-1 and N*H*-7, respectively, which were identical to those of 2-bromoaldisin, except for the absence of the signal at δ_H 6.54 (1H, s) in **15**. A mass difference of 80 amu between compound **15** and **14** in addition to the disappearance of the doublet carbon at δ 111.2 in ^{13}C-NMR of compound **15** revealed that **15** is a dibrominated derivative of compound **13**. The COSY spectrum (Fig. 3.3.23) of compound **15** showed a spin system consisting of two methylenes and one exchangeable proton, where it showed correlation of C*H$_2$*-5 with C*H$_2$*-6 which in turn coupled with N*H*-7. To confirm the structure a partial synthesis of **15** from 2-bromoaldisin (**13**) was performed in order to compare their ^{1}H and ^{13}C-NMR spectrum. The HMBC spectrum of the partially synthesised **15** (Fig.

3.3.24) showed the correlations of H-5 with C-4, C-6 and H-6 with C-4, C-5, C-8. The NMR data of the partially synthesised **15** and the isolated product were also identical. From the above data it was concluded that compound **15** was the new natural product **2,3-dibromoaldisin**.

Table 3.3.5: NMR data of compound 15 (DMSO-d_6, 500 MHz)

NO.	δH	δC	COSY	HMBC
1	N*H* 13.50 (br s)	-	-	3a
2	-	106.0 s	-	-
3	-	98.0 s	-	-
3a	-	124.0 s	-	-
4	-	193.5 s	-	-
5	2.75 (m)	43.5 t	6	4, 6
6	3.33 (m)	36.5 t	5	4, 5, 8
7	N*H*, 8.53 (t, *J*= 5.0 Hz)	-	6	8a
8	-	161.5 s	-	-
8a	-	129.5 s	-	-

2,3-Dibromoaldisin (Compound 15)

Systematic name: 2,3-Dibromo-5,6-dihydro-1H,7H-pyrrolo[2,3-c]azepine-4,8-dione

Character: yellow amorphous solid

Amount: 2.1 mg   **Sample code**: *Ax. dam.* p2

Molecular Weight : 320/322/324   **Molecular formula**: $C_8H_6Br_2N_2O_2$

Biological source: marine sponge *Axinella damicornis*

New natural product

HPLC chromatogram

UV absorbance
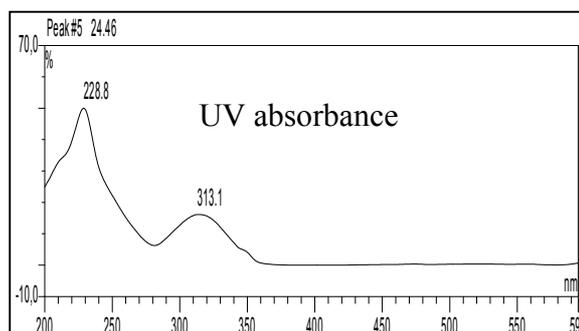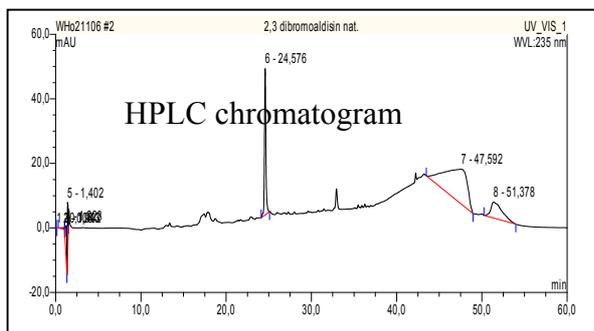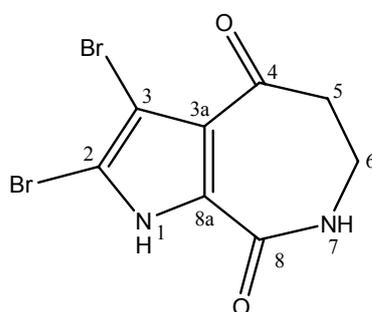
ESIMS: *m/z*,   319/321/323 [M-H]$^+$ (Negative)

          321/323/325 [M+H]$^+$ (Positive)

EIMS: (*m/z*, rel. int.) 320/322/324 (38:66:24) [M]$^+$, 292/294/296 (37:55:25), 263/265/267 (11.22:9), 214/216 (21:11), 170/172 (16:20), 142/144 (13:12), 80 (54), 68 (51), 50 (53) and 44 (100)
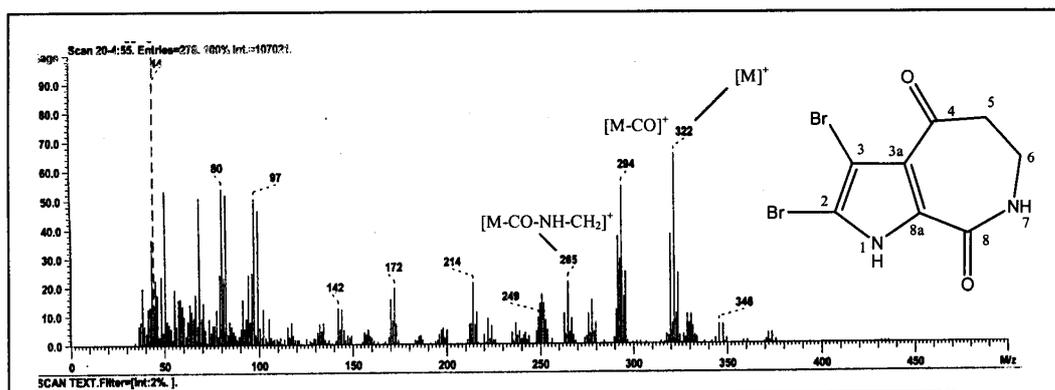
Fig. 3.3.21 : EIMS spectrum of compound 15
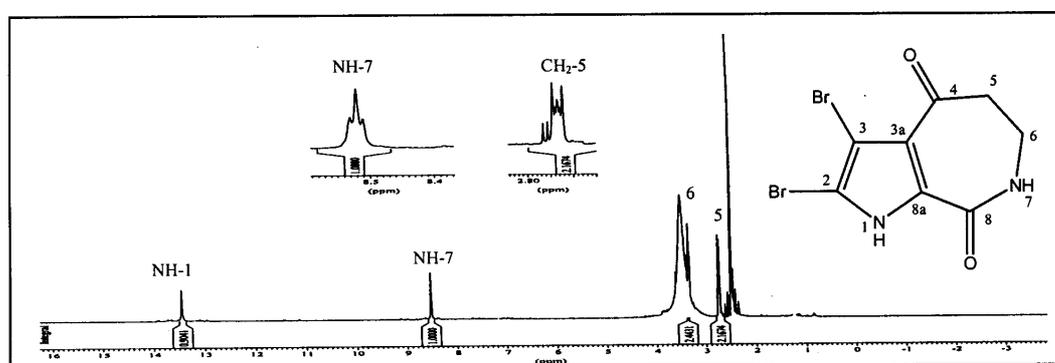
Fig. 3.3.22 : ^{1}H-NMR spectrum of compound 15
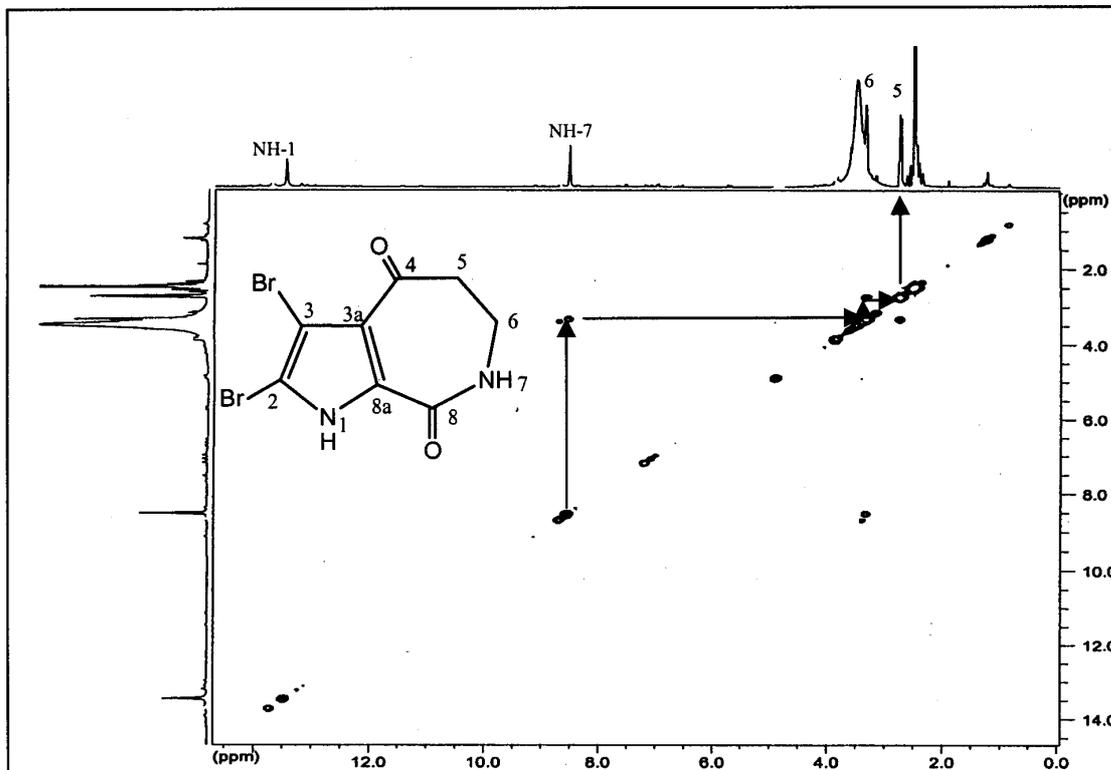
Fig. 3.3.23 : COSY spectrum of compound 15
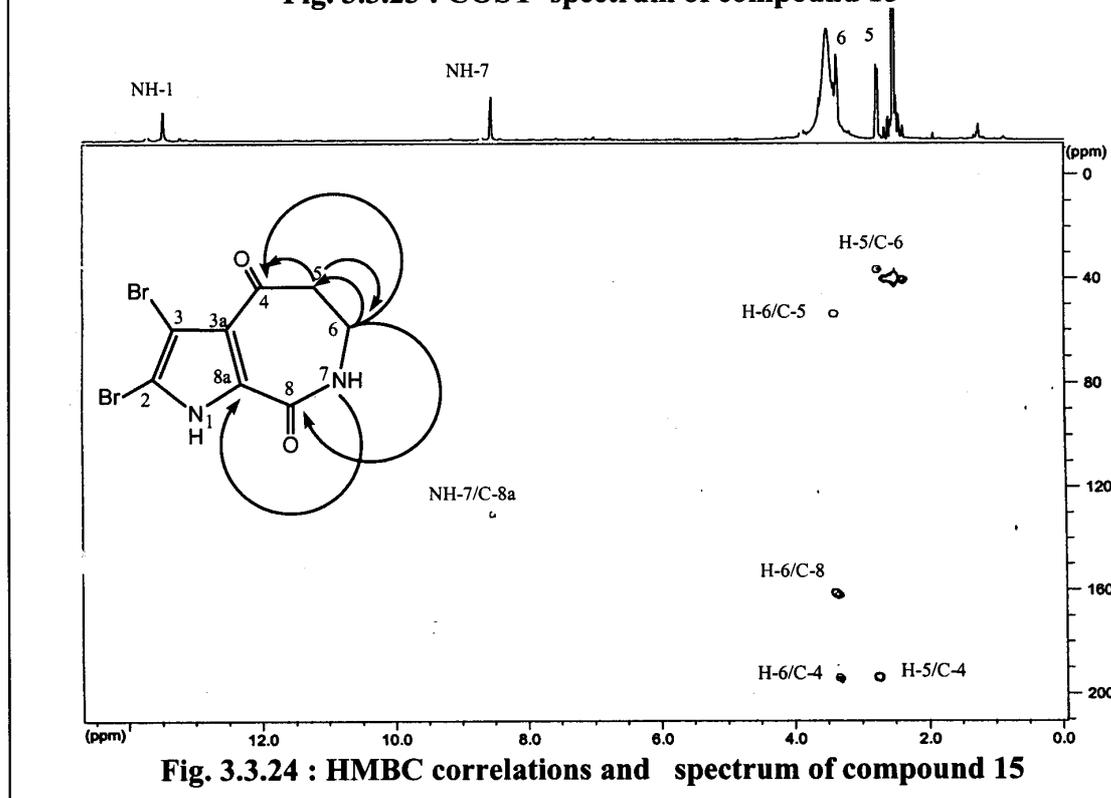
Fig. 3.3.24 : HMBC correlations and spectrum of compound 15

3.3.1.6. Structure elucidation of oroidin (16, known compound)

Oroidin has previously been isolated for the first time from the sponge genus *Agelas* (Forenza *et al*, 1971), then from the marine sponge *Agelas oroides* (König *et al*, 1998). It was reported to have moderate anti-microbial activities against a broad spectrum of bacteria (Cafieri *et al*, 1995).

Compound 16 was obtained as a yellowish white powder, it has UV absorbances at λ_{max} 201 and 277 nm. The FABMS (Fig. 3.4.25 A) showed the molecular ion peak at *m/z* 388, 390, and 392 $[M+H]^+$ in a ratio of 1:2:1, typical for a dibrominated compound. The ^{13}C-NMR and DEPT spectra (Fig. 3.3.27 A and B) showed signals at δ_C 104.6 (s), 97.9 (s), 112.9 (d) and 128.0 (s) suggesting the presence of a trisubstituted pyrrole ring. This was confirmed from the ^{1}H-NMR spectrum (Fig.3.3.26A, Table.3.3.6) which showed signals at δ_H 12.9 (1H, s) and 6.92 (1H, s) corresponding to N*H*-1 and H-4. The resonances at δ_C 124.8 (s), 147.5 (s), 111.0 (d) were assigned to an amino imidazole ring. This was confirmed also from the ^{1}H-NMR signals at δ_H 7.50 (2H, s), 11.90 (1H, s), 12.70 (1H, brs) and 6.95 (1H, d, 2.5 Hz) which were assigned to N*H₂*-13, N*H*-12, N*H*-14 and H-15 respectively. The signals at δ_C 126.8 (d), 116.2 (d) for the olefinic carbons H-9 and H-10 were also observed. The remaining ^{1}H-NMR and ^{13}C-NMR signals at δ_H 8.50 (1H t, 5.6 Hz), 3.99 (2H, t, 5.6 Hz), 6.10 (1H, dd, 5.6, 15.8 Hz) and 6.20 (1H, d, 15.8 Hz) and at δ_C 39.8 (t), 126.8 (d) and 116.2 (d) indicated the presence of a NH-CH₂-CH=CH- function. The coupling constant values of H-9 and H-10 indicated a *trans*-olefinic function. By careful inspection of ^{1}H-NMR and ^{13}C-NMR data and comparison with the literature (König *et al*, 1998), compound **16** was concluded to be **oroidin**.

R₁ = R₂ = Br   Oroidin

R₁ = H   R₂ = Br Hymenidin
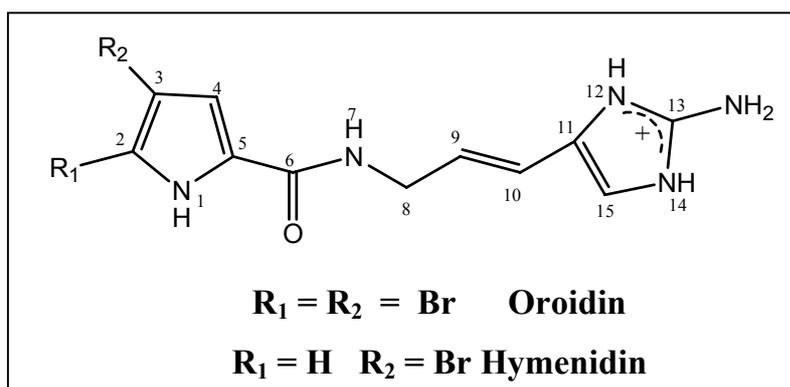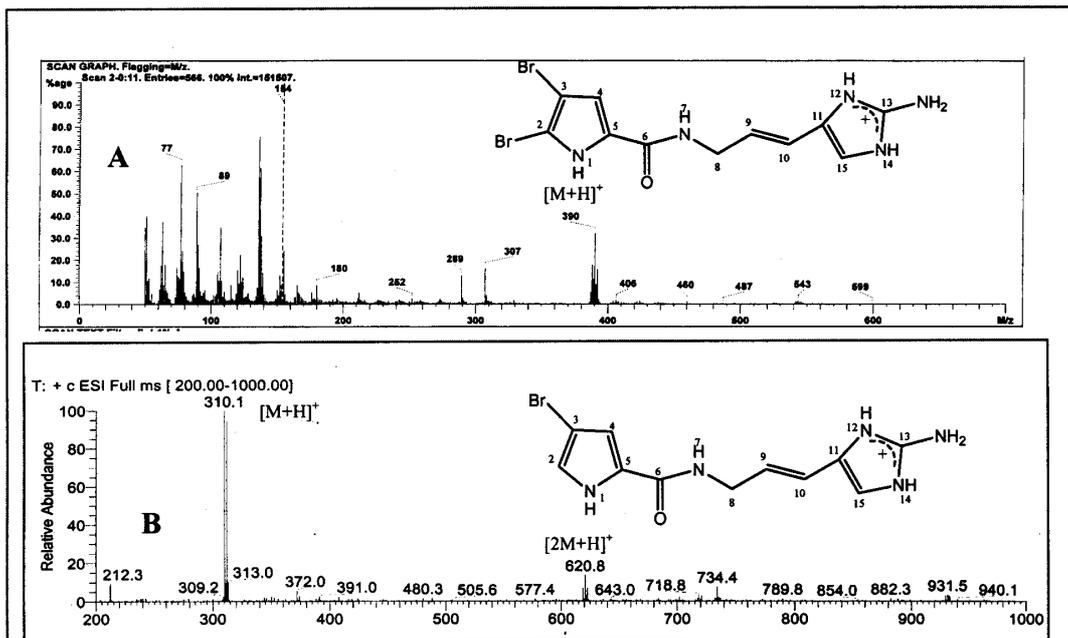
Fig. 3.3.25: A FABMS spectrum of compound 16

B ESIMS spectrum of compound 17
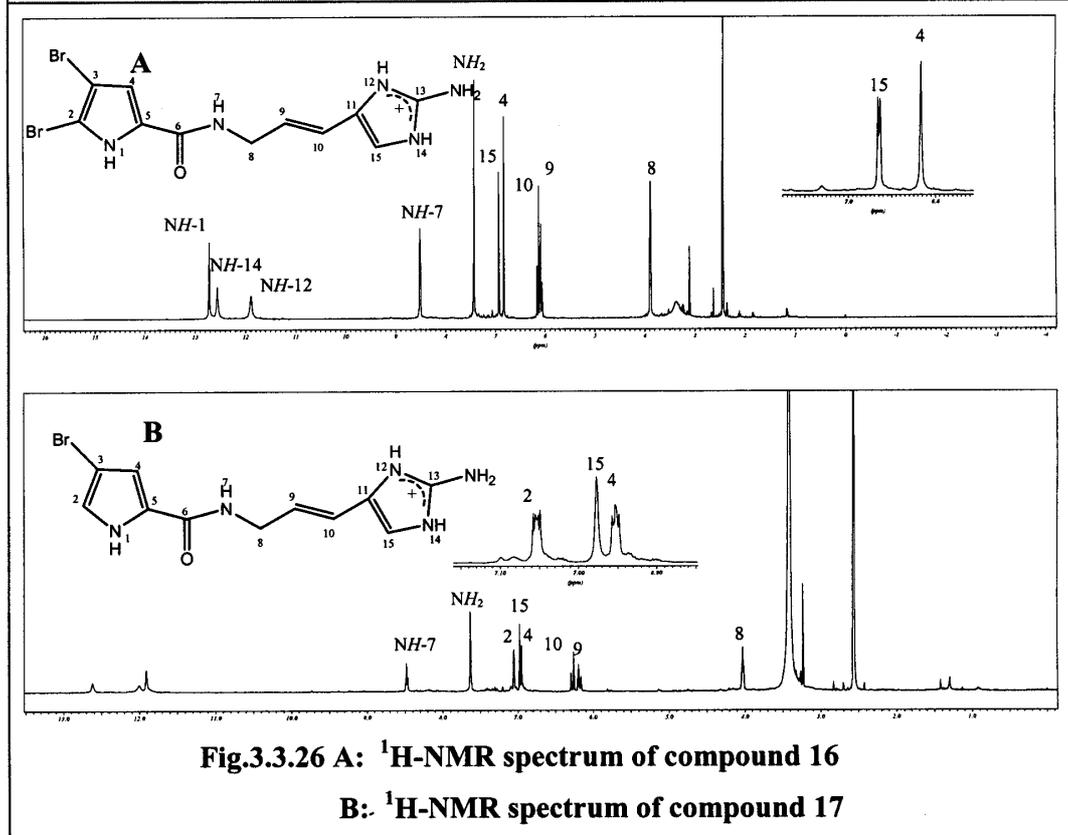
Fig.3.3.26 A: ¹H-NMR spectrum of compound 16

B:- ¹H-NMR spectrum of compound 17

Fig. 3.3.27 A: ^{13}**C-NMR spectrum of oroidin**

B: DEPT spectrum of oroidin
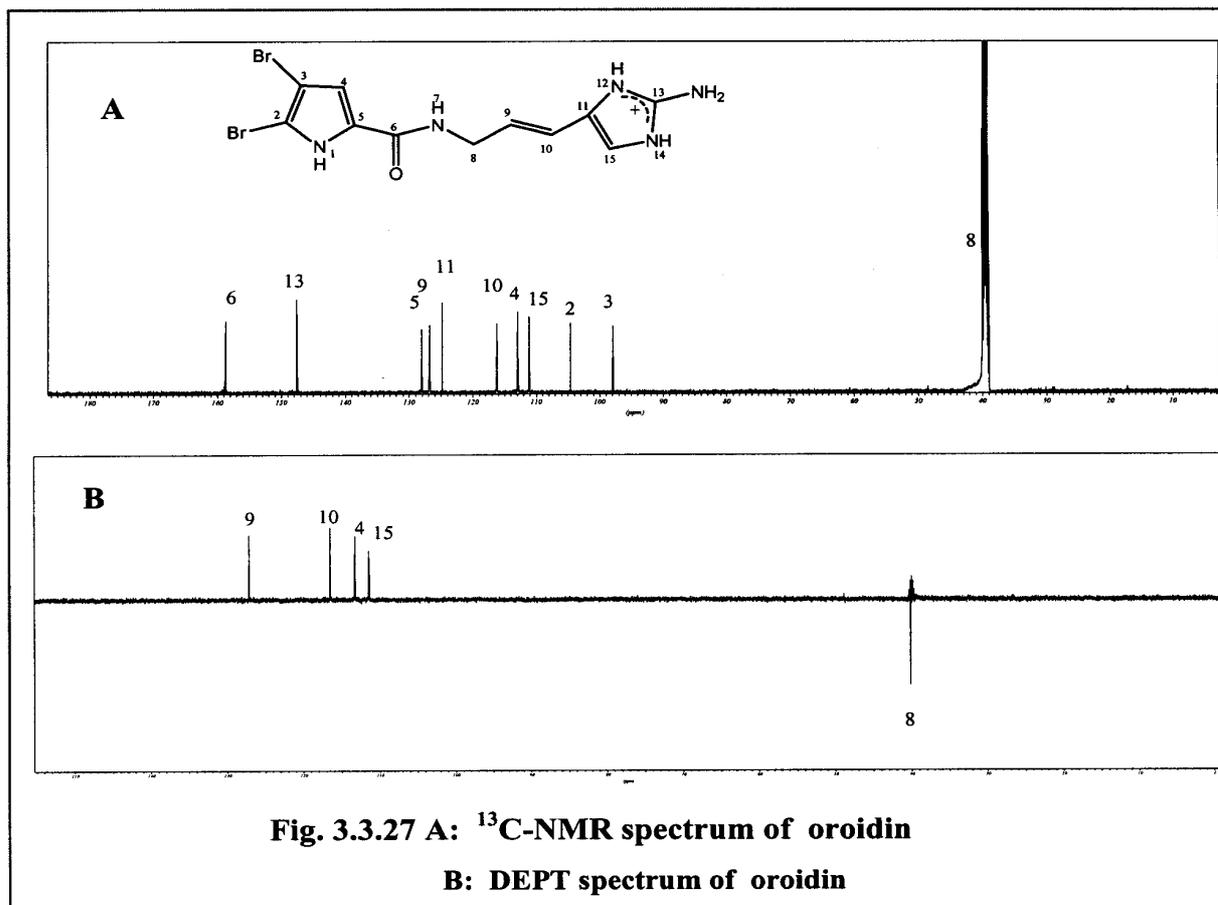
Table 3.3.6: ^{1}**H and** ^{13}**C-NMR data of compounds 16 and 17 (DMSO-d_6, 500 MHz)**

No	Compound 16		Compound 17
	δH	δC	δH
1	12.90 (s)	-	12.60 (s)
2	-	104.6 s	7.10 (dd, 2.5, 1.9 Hz)
3	-	97.9 s	-
4	6.92 (s)	112.9 d	6.97 (t, 1.9 Hz)
5	-	128.0 s	-
6	-	158.7 s	-
7	8.50 (t, 5.6 Hz)	-	8.50 (t, 5.7 Hz)
8	3.99 (t, 5.6 Hz)	39.8 t	4.00 (dd, 5.0, 5.7 Hz)
9	6.10 (dd, 5.6, 15.8 Hz)	126.8 d	6.19 (dd, 16.0, 5.0 Hz)
10	6.20 (d, 15.8 Hz)	116.2 d	6.22 (d, 16.0 Hz)
11	-	124.8 s	-
12	N*H*, 11.90 (br s)	-	N*H*, 12.00
13	N*H$_2$*, 7.50 (s)	147.5 s	N*H$_2$*, 7.50 (br s)
14	N*H*, 12.70 (br s)	-	N*H*, 11.90 (s)
15	6.95 (d, 2.5 Hz)	111.0 d	6.99 (s)

3.3.1.7. Structure elucidation of hymenidin (17, known compound)

Compound 17 was isolated as a pale yellow amorphous substance, it has UV absorbances at λ_{max} 213 and 272 nm; the molecular formula was established as $C_{11}H_{12}N_5OBr$ from the ESIMS (Fig.3.3.25B) which showed the molecular ion peak at *m/z* 310 and 312 $[M+H]^+$ in a ratio of 1:1 typical for a monobrominated compound. The eighty mass units difference between compound **17** and compound **16** suggested that compound **17** is a debrominated derivative of oroidin. This was confirmed from the ^{1}H-NMR spectrum (Fig. 3.3.26B) of compound **17**, which was very similar to that of oroidin except for the presence of an extra signal at δ_H 7.10 (1H, dd, *J* =2.5 and 1.89 Hz), and the difference in the multiplicity of H-4 in compound **17** as double doublet with coupling constant 2.5 and 1.89 Hz in contrast to the singlet found in oroidin. It was clear from the coupling constant of both H-2 and H-4 that the two protons coupled together and with N*H*-1 of the pyrrole ring. ^{1}H-NMR data revealed the presence of a disubstituted pyrrole ring in compound **17** instead of trisubstituted ring as in compound **16**. The structure of compound **17** was established as **hymenidin** from ESIMS, ^{1}H-NMR data and through the comparison with the literature (Supriyono *et al*, 1995 and Kobayashi *et al*, 1986).

3.3.1.8. Structure elucidation of dibromocantharelline (18, known compound)

Dibromocantharelline
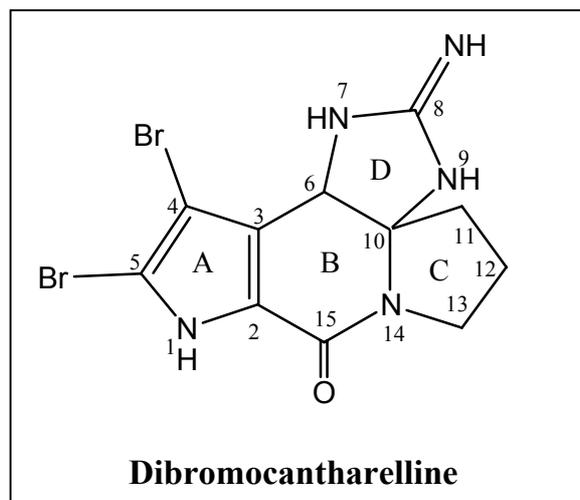
Compound 18 was isolated as white needles, it has UV absorbances at λ_{max} 287 and 208 nm and $[\alpha]_D$ - 91 (*c*, 0.1 in MeOH). The ESIMS spectrum (Fig. 3.3.28) showed the molecular ion peak at *m/z* 388/390/392 [M+H]$^+$ in a ratio of 1:2:1 indicating a dibrominated compound. The NMR data of this compound were identical with those of dibromocantharelline. The ^{1}H-NMR spectrum (Fig. 3.3.29) of compound **18** showed five exchangeable protons at δ_H 13.20 (1H, br s), 8.80 (1H, br s), 7.90 (2H, br s) and 9.80 (1H, br s) which were assigned to N*H*-1, N*H*-9, N*H*$_2$-8 and N*H*-7, respectively. The three signals at δ_H 2.10, 2.00 (2 H, m), 2.27 (2H, m) and 3.45 (1H, q, 11.03 Hz), 3.56 (1H, dt, 11.03, 1.9 Hz) (AB system) were assigned to C*H*$_2$-12, C*H*$_2$-11 and C*H*$_2$-13, respectively. This was established from the ^{13}C-NMR spectrum (Fig. 3.3.30, Table 3.3.7) and DEPT, which showed three methylene signals at δ_C 19.1 (t), 40.0 (t) and 44.1 (t) for C*H*$_2$-12, C*H*$_2$-11 and C*H*$_2$-13, respectively. The downfield shift of C-13 in comparison to the other methylenic carbons C-11 and C-12 was indicative for a carbon-bearing heteroatom. The COSY spectrum of compound **18** established the presence of a -C*H*$_2$-C*H*$_2$-C*H*$_2$- moiety as it showed correlations of C*H*$_2$-11 with C*H*$_2$-12 which in turn coupled with C*H*$_2$-13. The important signal in the ^{1}H-NMR spectrum of compound **18** was at δ_H 5.27 (1H, s) for H-6 which connected the four ring systems. The ^{13}C-NMR spectrum (Fig. 3.3.30) with DEPT showed also resonances at δ_C 96.2 (s), 108.4 (s), 122.4 (s) and 122.7 (s) assigned for C-4, C-5, C-3 and C-2 of a tetrasubstituted pyrrole ring, ring A. In the ^{13}C-NMR spectrum an

additional carbonyl signal at δ 156.8 assigned for a amide group, C-15, was observed. The ^{13}C-NMR showed also resonances at δ_C 53.9 (d), 154.6 (s) and 83.9 (s) for C-6, C-8 and C-10 of ring D. The downfield shift of the three carbons easily revealed that these carbons were attached to heteroatoms. The HMBC spectrum (Fig. 3.3.31) showed the long range correlations which confirmed the attachment of the four ring systems (A, B, C and D) together, where it showed the correlations of H-6 with C-11, C-10, C-4, C-5, C-2 and C-8. The correlations of H-11 with C-6, C-10, C-12; H-12 with C-10, C-11, C-13 and H-13 with C-10, C-11, C-12 were also observed. From the HMBC spectrum the correlations between N*H*-9 with C-6, C-8, C-10, and the correlations of N*H*-7 with C-6, C-8, C-10 were indicative of the tautomeric form for ring D. The structure of this compound as **dibromocantharelline** was established from the NMR data and through the comparison with these of the literature data (Nanteuil *et al*, 1985). However, the reported chemical shifts of C-4 and C-2 in ^{13}C-NMR were found to be interchanged, and through the comparison with the other brominated compounds isolated in this study and with the previously reported compounds (König *et al*, 1998 and Eder, 1998) it was established that the chemical shifts of the two carbons should be as given in Table 3.3.7. This compound may be a new compound due to the negative ($[\alpha]_D$ -91, *c*. 0.1, CHCl$_3$) compared to the known compound dibromocantharelline ($[\alpha]_D$ +95, *c*. 1.0, CHCl$_3$).
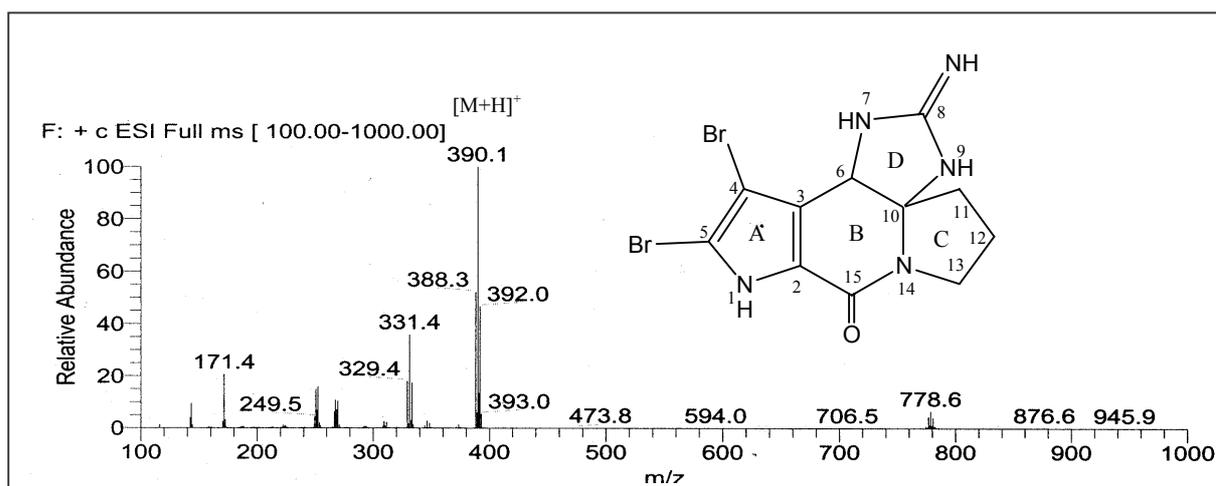
Fig. 3.3.28: ESIMS spectrum of compound 18

Table 3.3.7: NMR Data of dibromocantharelline (DMSO-d_6, 500 MHz)

No.	δC	δH	COSY	HMBC
1	-	N*H*, 13.20 (br s)	-	-
2	122.7 s	-	-	-
3	122.4 s	-	-	-
4	96.2 s	-	-	-
5	108.4 s	-	-	-
6	53.9 d	5.27 (s)	-	2, 4, 5*, 10, 11
7	-	N*H*, 9.80 (s)	-	6, 8, 10
8	154.6 s	N*H*, 7.90 (br s)	-	-
9	-	N*H*, 8.80 (br s)	-	6, 8, 10
10	83.9 s	-	-	-
11	40.0 t	2.27 (m)	12	6, 10, 12
12	19.1 t	A 2.10 (m) B 2.00 (m)	11, 13	10, 11, 13
13	44.1 t	A 3.56 (dt, 11.0, 1.9) B 3.45 (q, 11.0)	12, 13 B 12, 13 A	10, 11, 12
15	156.8 s	-	-	-

* 4-bond coupling
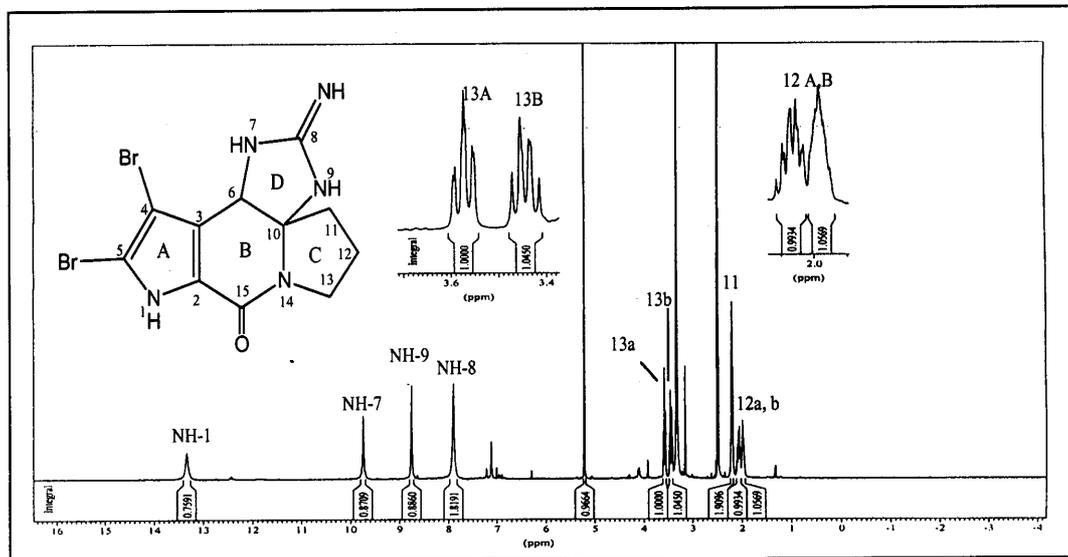
Fig. 3.3.29: ^{1}H-NMR spectrum of compound 18
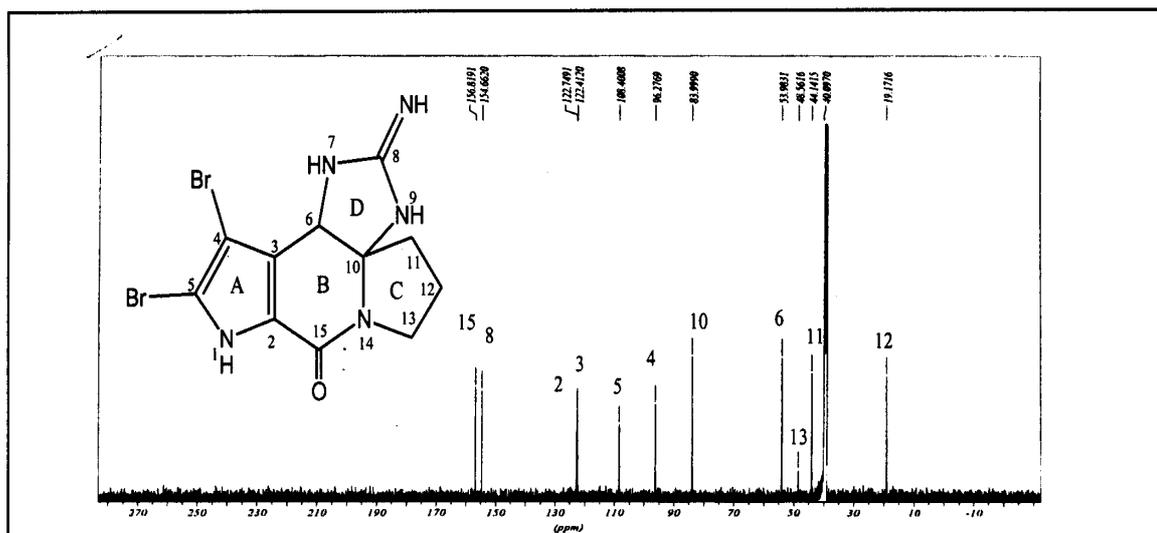
Fig. 3.3.30: ^{13}C-NMR spectrum of compound 18
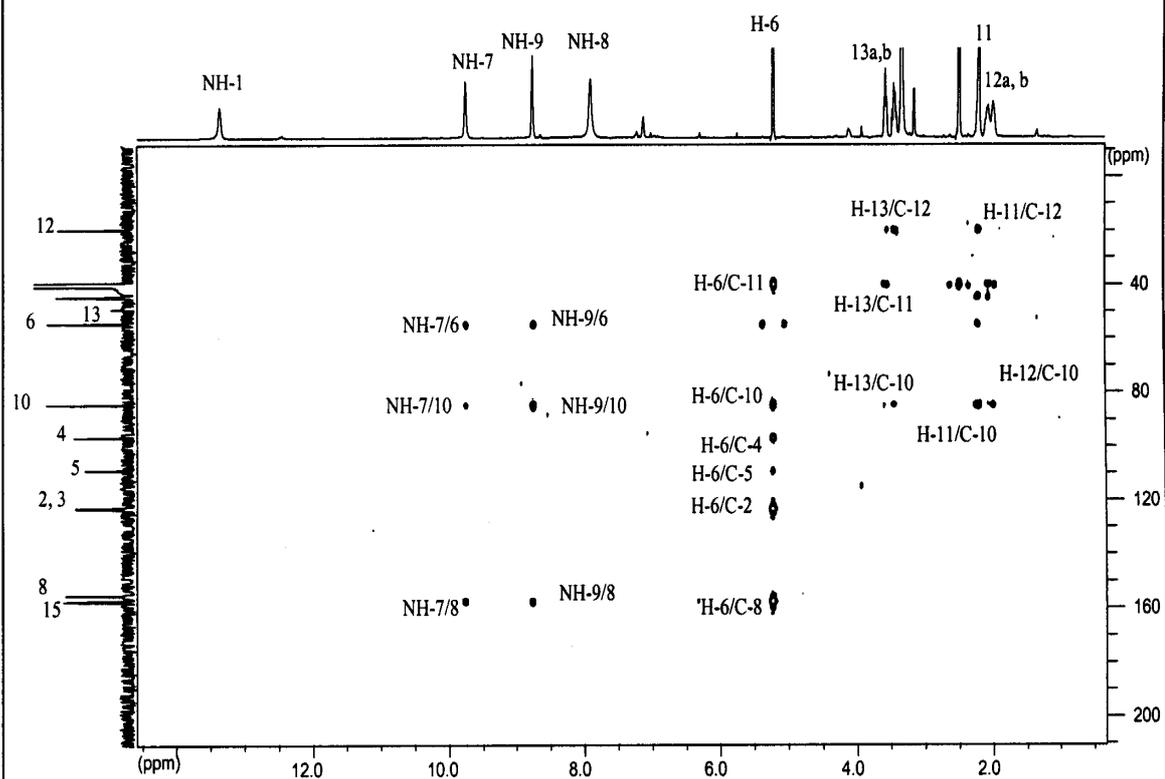
Fig. 3.3.31. HMBC spectrum of compound 18

3.3.1.9. Structure elucidation of *Z*-hymenialdisine (19, known compound)

Z-Hymenialdisine has been previously isolated from the sponges *Axinella verrucosa*, *Acanthella aurantiaca* and *Hymeniacidon aldis* (Cimino *et al*, 1982 and Kitagawa *et al*, 1983) and also from *Stylissa cateri* (Eder, 1998). It has been reported to have a moderate cytotoxic activity against KB cell lines (human oropharengeal epidermoid carcinoma) (Cimino *et al*, 1982) and to inhibit protein kinases, PKC-ε, CDK2/cyclin E, CDK4/cyclin D_1, EGFR, and PDGFR-β activities (Eder, 1998).

Z-Hymenialdisine
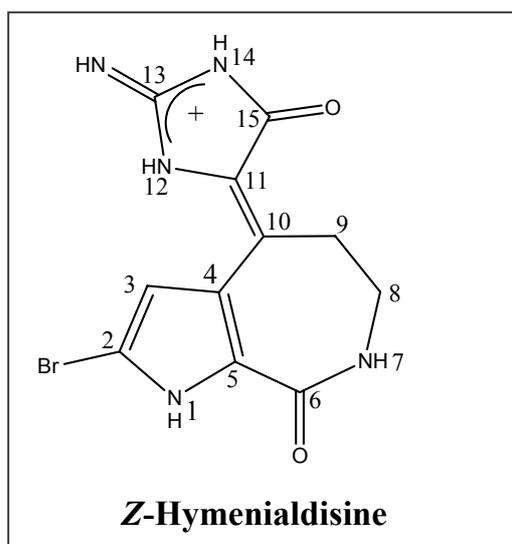
Compound 19 was isolated as a yellow crystalline powder, UV (MeOH) absorbances were at λ_{max} 212, 262 and 354 nm. The FABMS (Fig.3.3.32) showed the molecular ion peak at *m/z* 324/ 326 in a ratio of 1:1 [M+H]$^+$ corresponding to a monobrominated compound with the molecular formula $C_{11}H_{11}N_5O_2Br$. The ^{1}H-NMR spectrum (Table 3.3.8 Fig. 3.3.33) showed five exchangeable protons at δ_H 12.80 (1H, br s), 8.20 (1H, br s), 8.40 (2H, br s) and 9.00 (1H, br s), which were assigned to N*H*-1, N*H*-7, N*H*-14 and N*H*-12, respectively, as well as a signal at δ_H 3.10 (4H, s) for the methylene groups C*H*$_2$-8 and C*H*$_2$-9. The four protons of the methylenic groups, C*H*$_2$-8 and CH$_2$-9 appeared at the same chemical shift due to the anisotropic effect of carbonyl group at position 15 on C*H*$_2$-9. The ^{13}C-NMR spectrum of **19** (Fig. 3.3.34) confirmed the presence of two methylene carbons, as it showed resonances at δ_C 39.1 and 31.9 for C-8 and C-9, respectively. The downfield shift of C-8 in ^{13}C-NMR was due to its position adjacent to N*H*-7. While the other ^{1}H-NMR

and ^{13}C-NMR signals at δ_H 6.40 (1H, s) and 12.80 (1H, br s) and at δ_C 104.8 (s), 111.3 (d), 121.7 (s), and 128.2 (s) established the presence of the trisubstituted pyrrole ring. The signals at δ_C 164.0 (s) 154.7 (s) and 128.2 (s) were assigned to C-15, C-13 and C-11 of the amino-imidazolinone ring. Careful inspection of ^{1}H and ^{13}C-NMR data in comparison with compound **13** indicated the presence of a 2-bromoaldisin moiety. The assignments of the carbon and proton signals were based on the comparison with those of literature data (Eder, 1998 and Kitagawa *et al*, 1983).

3.3.1.10. Structure elucidation of hymenin (20, known compound)

Hymenin
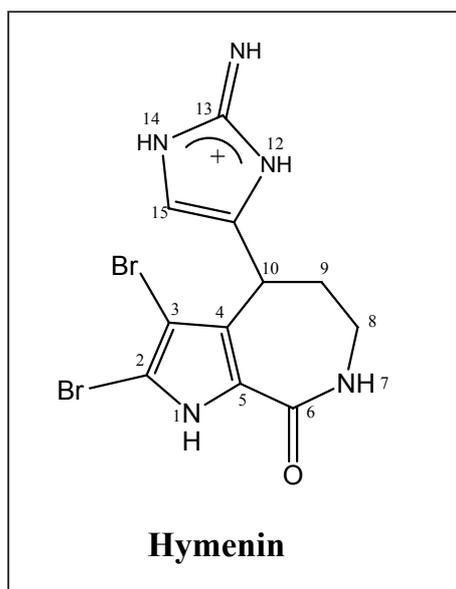
Compound 20 was isolated as a brown amorphous substance, UV (MeOH) absorbances at λ_{max} 220 and 278 nm were observed. The ESIMS spectrum (Fig.3.3.35) of compound **20** showed a cluster of peaks at *m/z* 388, 390, 392 [M+H]$^+$ corresponding to a dibrominated compound with the molecular formula $C_{11}H_{13}N_5OBr_2$. The ^{1}H and ^{13}C NMR data of compound **20** were closely related to those of compound **19**. The ^{13}C NMR spectrum (Fig. 3.3.36) showed eleven carbons divided as seven singlets, two triplets, and two doublets. The ^{1}H NMR signals at δ_H 11.83 (1H, br s), 12.26 (1H, br s), 5.62 (2H, br s) and 6.00 (1H, s) with the ^{13}C NMR resonances at δ_C 125.9 (s), 148.6 (s), and 110.5 (s) indicated the presence of an

amino-imidazole ring instead of an amino-imidazolinone ring as in compound **19**. The other ^{1}H NMR at δ_H 12.4 (1H, br s), with the ^{13}C NMR resonances at δ_C 123.0 (s), 106.7 (s), 100.8 (s) and 128.8 (s) were assigned to a tetrasubstituted pyrrole ring instead of a trisubstituted ring as in **19**. While the remaining ^{1}H NMR and ^{13}C NMR signals at δ_H 3.20 (1H, m), 3.02 (1H, m), 2.25 (1H, m), 1.95 (1H, m), and 3.95 (1H, t, 4.3 Hz) and at δ_C 36.6 (t), 31.8 (t) and 34.9 (d) were assigned to CH_2-8 and CH_2-9 both of which represented an AB system and CH-10, respectively. Detailed comparison of NMR data of compound **20** with those of **hymenin** in the literature (Kobayashi *et al,* 1986) revealed that the two compounds were identical.

3.3.1.11. Structure elucidation of stevensin (21, known compound)

Stevensin
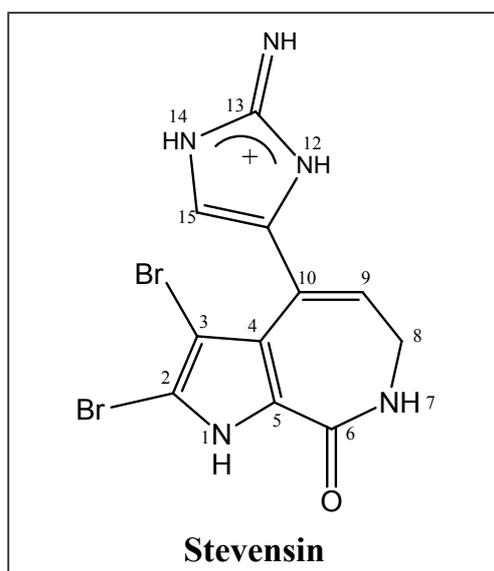
Compound 21 was isolated as a dark orange amorphous substance, it has UV absorbances at λ_{max} 236, 263, and 288 nm. The ESIMS spectrum (Fig. 3.3.37) showed a cluster of peaks at *m/z* 384/386/388 [M-H]$^+$ indicative for a dibrominated compound. The ^{13}C-NMR of this compound is closely related to that of compound **20**, and showed eleven carbons distributed as eight singlets, two doublets and one triplet, except for the absence of the two signals at δ_C 31.8 (t) and 34.9 (d) of compound **20** and the appearance of two olefinic carbons at δ_C 128.5 (d) and 128.8 (s) in compound **21**. This indicated the presence of an extra double bond in compound **21** which was compatible with the molecular weight. The ^{1}H-NMR data of compound **21** (Table 3.3.8) confirmed the presence of an extra double bond. It showed signals for

two olefinic protons at δ_H 6.50 (s) and 6.21 (t, $J = 6.9$ Hz) instead of one at δ_H 6.0 (s) as in compound **20**. The assignment of carbon and proton NMR signals was based on comparison with the literature (Eder, 1998).

Table.3.3.8: ^{1}H, ^{13}C-NMR data of compounds 19, 20 and 20 (DMSO - d_6, 500 MHz)

No.	*Z*-Hymenialdisine (19)		Hymenin (20)		Stevensin (21)	
	δH	**δC**	**δH**	**δC**	**δH**	**δC**
1	12.80 (br s)	-	12.40 (br s)	-	-	-
2	-	104.8 s	-	106.7 s	-	107.3 s
3	6.40 (s)	111.3 d	-	100.8 s	-	97.9 s
4	-	121.7 s	-	123.0 s	-	120.7 s
5	-	128.2 s	-	128.5 s	-	125.0 s
6	-	162.2 s	-	161.3 s	-	162.0 s
7	8.20 (br s)	-	7.80 (t, 5.1 Hz)	-	8.10 (t, 5.1 Hz)	-
8	3.10 (s)	39.1 t	A 3.02 (m) B 3.20 (m)	36.6 t	3.30 (m)	37.4 t
9	3.10 (s)	31.9 t	A 1.95 (m) B 2.25 (m)	31.8 t	6.21 (t, 6.9 Hz)	128.5 d
10	-	121.7 s	3.95 (t, 4.3 Hz)	34.9 d	-	128.8 s
11	-	128.1 s	-	125.9 s	-	130.0 s
12	N*H* 9.00 (br s)	-	11.83 (br s)	-	-	-
13	-	154.7 s	5.62 (br s)	148.6 s	7.00 (s)	149.0 s
14	N*H* 8.40 (br s)	-	12.26 (br s)	-	-	-
15	-	164.0 s	6.00 (s)	110.5 d	6.50 (s)	111.0 d
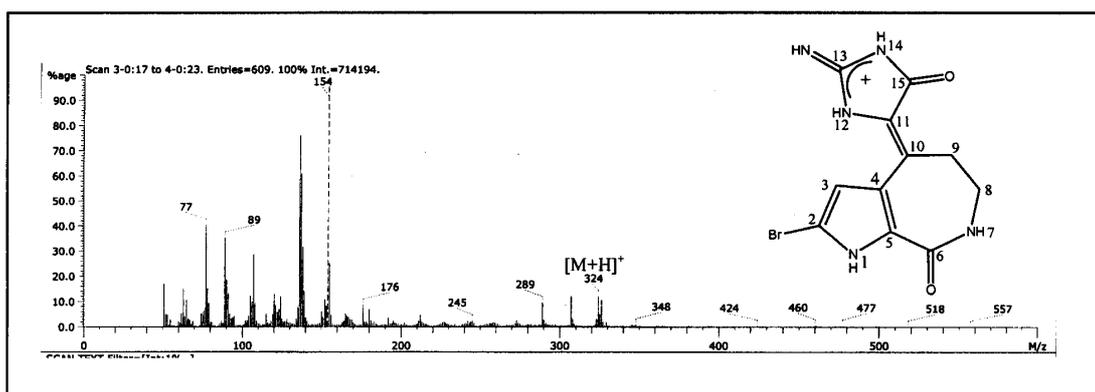
Fig.3.3.32: FABMS spectrum of compound 19
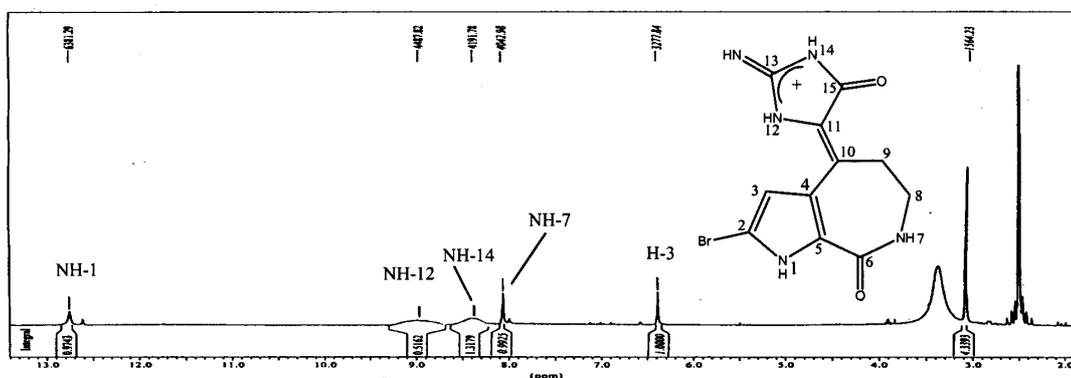
Fig. 3.333: ¹H-NMR spectrum of compound 19
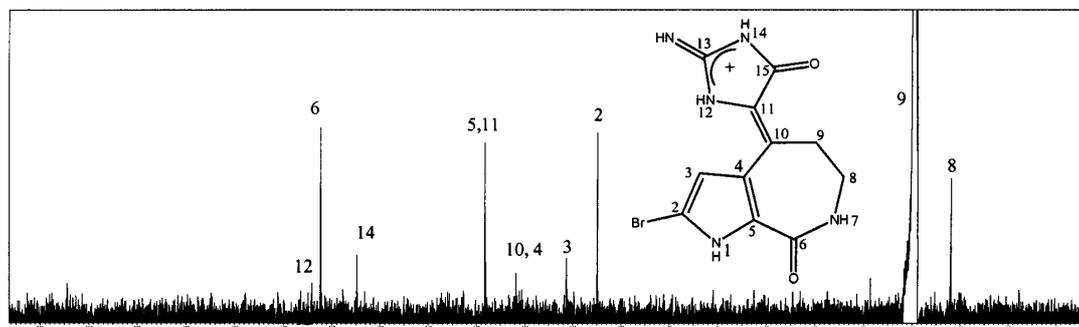
Fig. 3.3.34: ¹³C-NMR spectrum of compound 19

F: + c ESI Full ms [100.00-1000.00]   [M+H]⁺
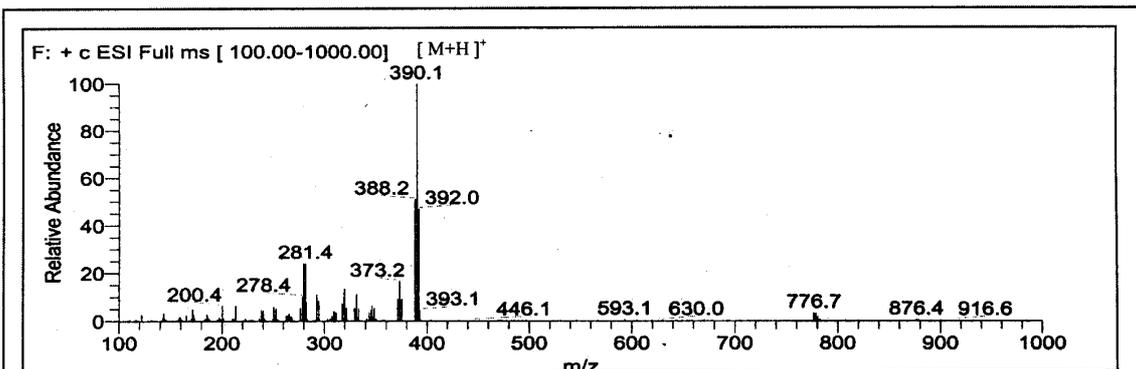
Fig.3.3. 35: ESIMS spectrum of compound 20
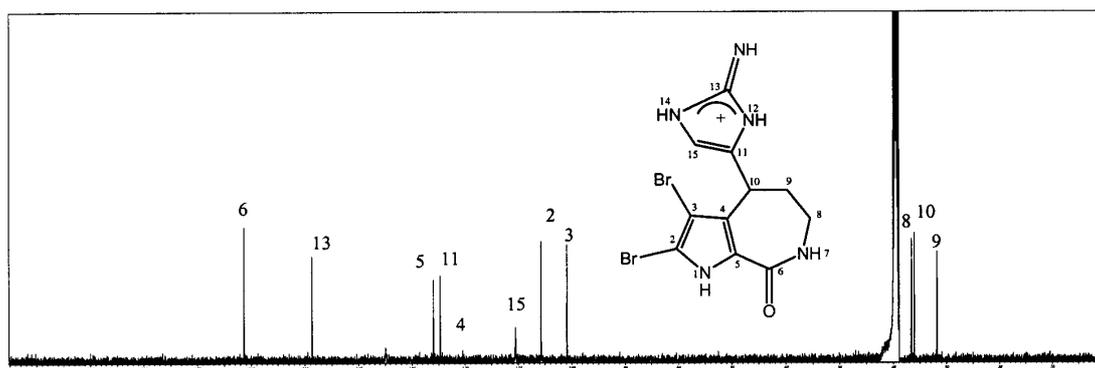
Fig.3.3.36: ^{13}C-NMR spectrum of compound 20

[M-H]⁺

F: - c ESI Full ms [100.00-1000.00]
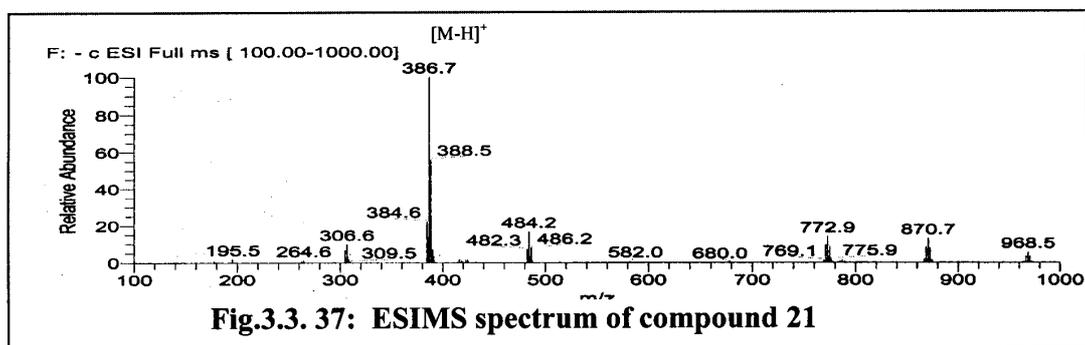
Fig.3.3. 37: ESIMS spectrum of compound 21
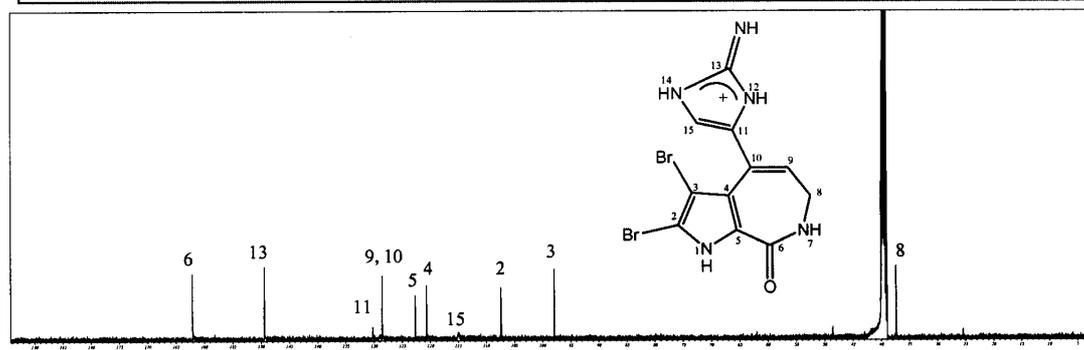
Fig.3.3. 38: ^{13}C-NMR spectrum of compound 21

3.3.1.12. Structure elucidation of 4, 5 dibromopyrrole-2-carbamide (22, known compound)

4,5-Dibromopyrrole-2-carbamide has been previously isolated from the sponge *Agelas oroides* (Forenz *et al*, 1971) and subsequently from *Agelas mauritiana* (Tsukamoto *et al*, 1996). It has been reported to have antifouling activity against the ascidian *Ciona savignyi* at a concentration of 2.5 µg/ml (Tsukamoto *et al*, 1996) and cytotoxic activity against NSCLC-N6 human non-small-cell-lung carcinoma (IC$_{50}$ 9.4 µg/ml) (Mancini *et al*, 1997).

4,5-Dibromopyrrole-2-carbamide
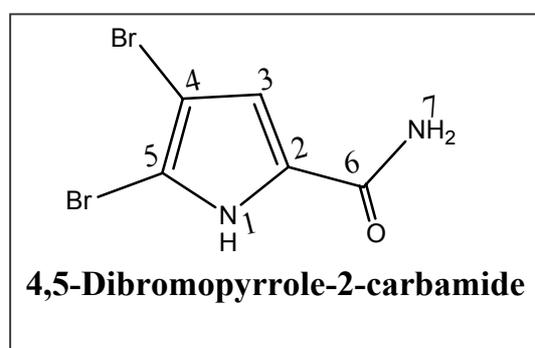
Compound 22 was isolated as a yellowish white amorphous solid. It has UV absorbances at λ_{max} 277 and 233 nm. The ESIMS spectrum (Fig. 3.3.40) showed a cluster of peaks at *m/z* 265, 267 and 269 in the ratio of 1:2:1 [M-H]$^{+}$. This revealed that **22** was a dibrominated compound with the molecular formula $C_5H_4N_2OBr_2$. The ^{13}C-NMR data (Table 3.3.9) showed resonances for a trisubstituted pyrrole derivative at δ_C 128.0 (s), 103.4 (s), 98.0 (s) and 113.4 (d), which were confirmed from the ^{1}H-NMR spectrum (Fig. 3.3.41), that showed proton signals at δ_H 6.93 (1H, s), and 12.60 (1H, br.s, N*H*), in addition to a signal typical for a amide function at δ_C 160.0 (s). This was confirmed from the ^{1}H-NMR signals at δ_H 7.15 and 7.60 (each, 1H, br s) of N*H$_2$* group. The multiplicity of the signal at δ_H 6.90 as singlet, indicated the positions of the substituents which were assigned to be at C-2, C-4, and C-5. According to these results, and through comparison with the literature data (Eder, 1998), compound **22** was concluded to be **4,5-dibromopyrrole-2-carbamide**.

3.3.1.13. Structure elucidation of 4,5-dibromopyrrole-2-carboxylic acid (23, known compound)

4,5-Dibromopyrrole-2-carboxylic acid
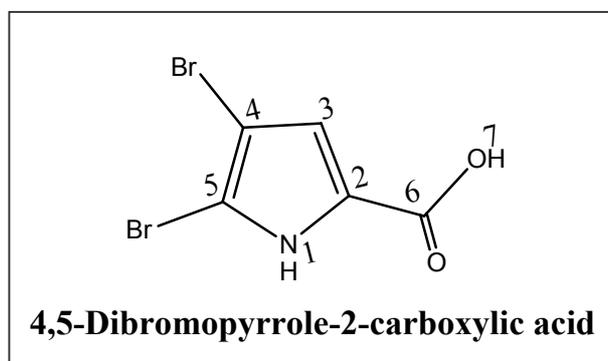
Compound 23 showed spectral characteristics similar to those of compound **22**. It has   UV absorbances at λ_{max} 277 and 234 nm. It was isolated as a yellowish amorphous solid. The ESIMS spectrum (Fig. 3.3.43) showed a cluster of peaks at *m/z* 266, 268 and 270 in a ratio of 1:2:1 [M-H][+]. This revealed that **23** was a dibrominated compound with the molecular formula $C_5H_3NO_2Br_2$. The ^{1}H-NMR spectrum (Table 3.3.9, Fig. 3.3.45), was similar to that of compound **22** except for an additional signal at δ 13.00 (1H, br s) which was assigned to an acidic O*H* and the absence of two signals for the amide moiety. The multiplicity of the signal at δ_H 6.86 (s), in addition to the HMBC correlations (Fig. 3.3.46) of H-3 with C-2, C-5 and C-6 indicated the positions of the substituents which were assigned to be at C-2, C-4 and C-5. The ^{13}C-NMR spectrum (Fig. 3.3.44, Table 3.3.9), was also similar to that of compound **22** except for the slight differences of the chemical shifts of C-2, C-3, C-4 and C-5. The differences in the chemical shift of these carbons were due to the replacement of N*H*$_2$ in compound **22** by O*H* in compound **23** (König *et al*, 1998). According to the results mentioned before and   through the comparison with the literature data (König *et al*, 1998), compound **23** was concluded to be **4,5-dibromopyrrole-2-carboxylic acid**.

3.3.1.14 Structure elucidation of 4-bromopyrrole-2-carbamide (24, known compound)

4-Bromopyrrole-2-carbamide has been previously isolated from the sponge *Acanthella carteri* (Mancini *et al*, 1997). It has been reported to exhibit antimicrobial activity against gram-positive bacteria and fungi (Iwagawa *et al,* 1998), and cytotoxic activity against NSCLC-N6 human non-small-cell-lung carcinoma ($IC_{50} = 4.8$ µg/ml).

4-Bromopyrrole-2-carbamide
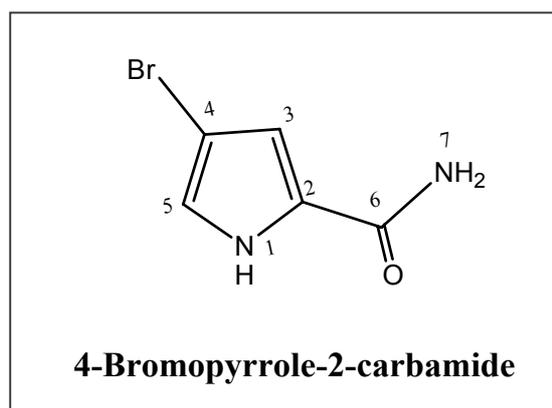
Compound 24 was isolated as a yellowish brown amorphous powder, it has UV absorbances at λ_{max} 233 and 270 nm. The EIMS spectrum (Fig. 3.3.48) showed two peaks at *m/z* 188/190 in a ratio of 1:1 which indicated the compound to be a monobrominated. The position of the bromine atom was confirmed from the coupling constants of H-3 (d, *J*= 2.9 and 1.7 Hz). and H-5 (d, *J*= 2.9 and 1.7 Hz) (Mancini *et al*, 1997, and Pretsch 2000). The structure of compound **24** was established as **4-bromopyrrole-2-carbamide** from EIMS spectrum, ^{1}H-NMR spectrum (Fig. 3.3.49) and the COSY data (Table 3.3.9) and by the comparison with the literature (Mancini *et al*, 1997).

3.3.1.15. Structure elucidation of 4,5-dibromopyrrole-2-methylcarboxylate (25, known compound)

4,5-Dibromopyrrole-2- methylcarboxylate
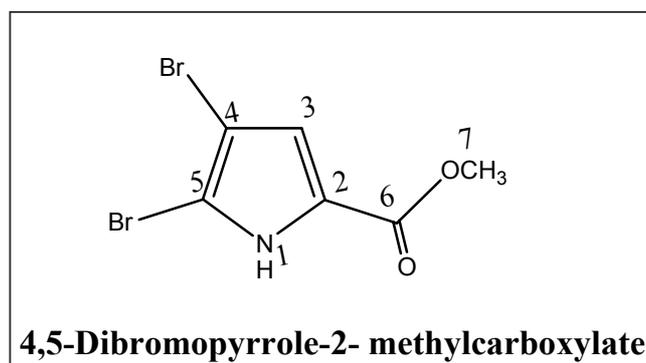
Compound 25 was isolated from the sponge *Axinella damicornis* and its structure was established from ESIMS (Fig.3.3.51) and ^{1}H-NMR data (Table 3.3.9) and through comparison with the literature data (Foreza *et al*, 1971 and König *et al*, 1998).

Table 3.3.9: NMR data of compounds 22-25 (DMSO-d_6, 500 MHz)

No	22		23		24		25
	δ H	δ C	δ H	δ C	δ H	COSY	δ H
1	12.60 (br s)	-	12.86 (br s)	-	11.80 (br s)	-	-
2	-	128.0 s	-	125.3 s	-	-	-
3	6.93 (s)	113.4 d	6.86 (s)	116.7 d	6.97 (dd, 1.7, 2.9 Hz)	1, 5	6.90 (s)
4	-	98.0 s	-	99.0 s	-	-	-
5	-	103.4 s	-	107.3 s	6.85 (dd, 1.7, 2.9 Hz)	1, 3	-
6	-	160.0 s	-	160.5 s	-	-	-
7	N*H₂*, 7.15 (s) 7.60 (s)	-	OH, 13.00 (br s)	-	N*H₂*, 7.10 (s) 7.50 (s)	-	3.80 (3 H, s)
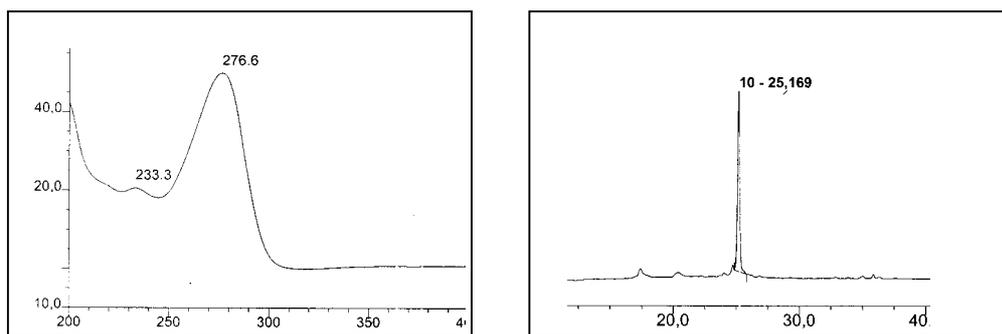
Fig. 3.3.39: UV and HPLC chromatogram of compound 22
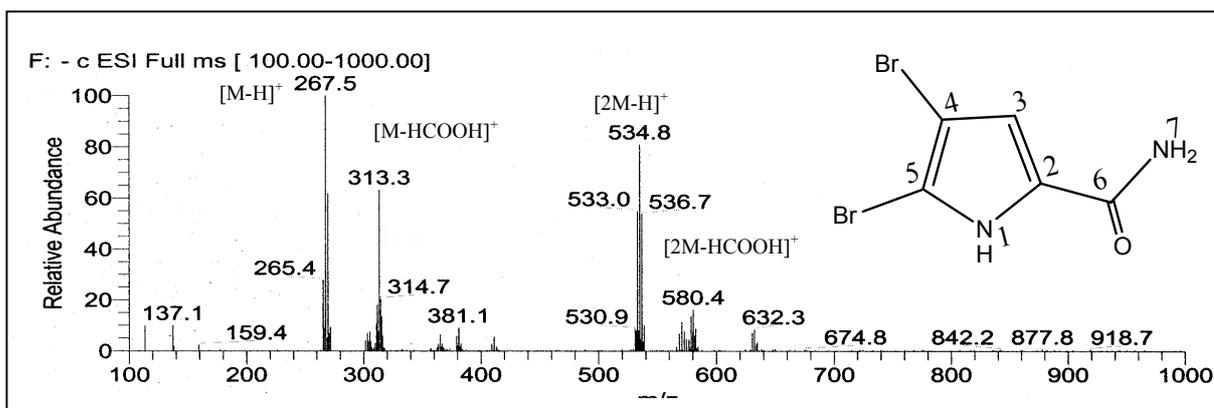
Fig. 3.3.40: ESIMS spectrum of compound 22
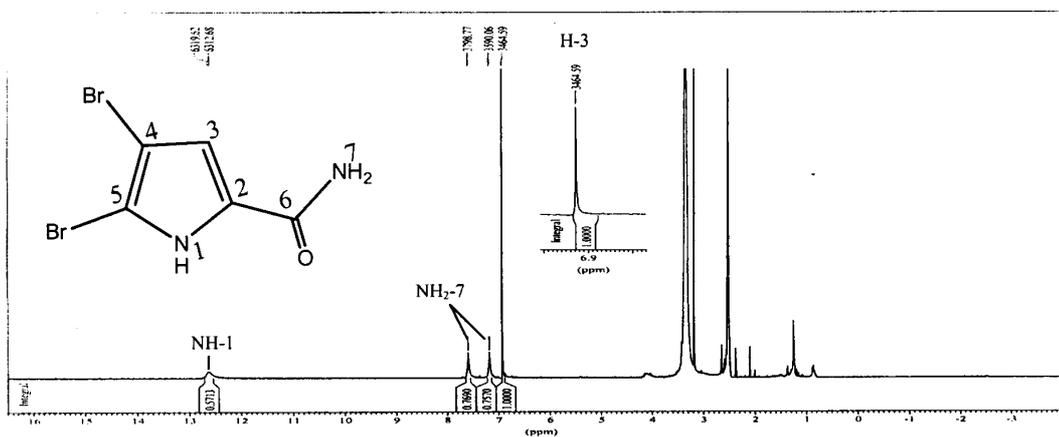
Fig. 3.3.41: ^{1}H-NMR spectrum of compound 22
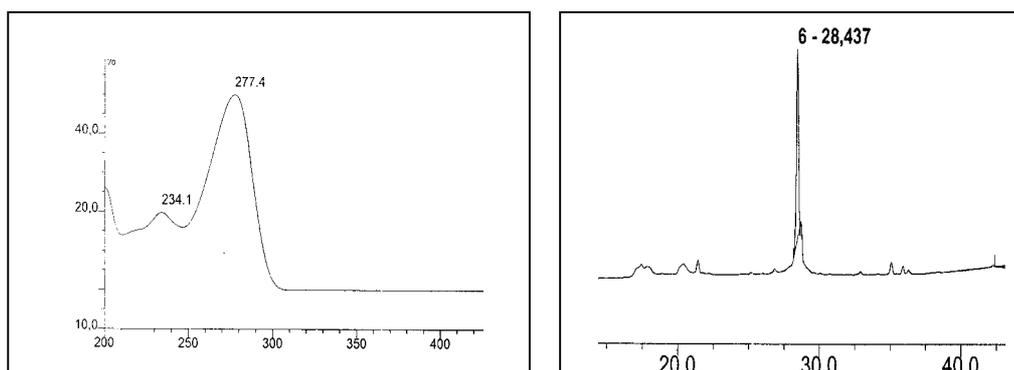
Fig. 3.3.42: UV and HPLC chromatogram of compound 23
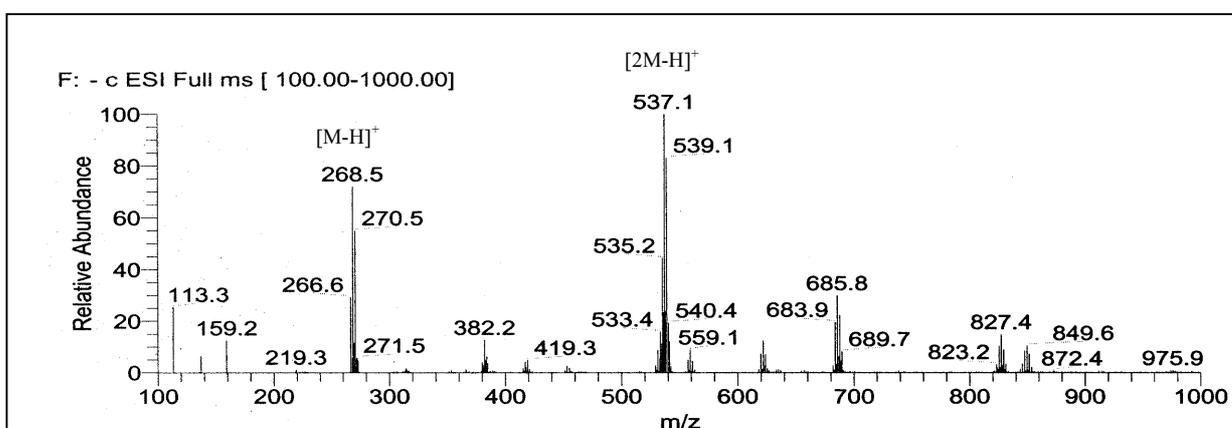
Fig.3.3.43: ESIMS spectrum of compound 23
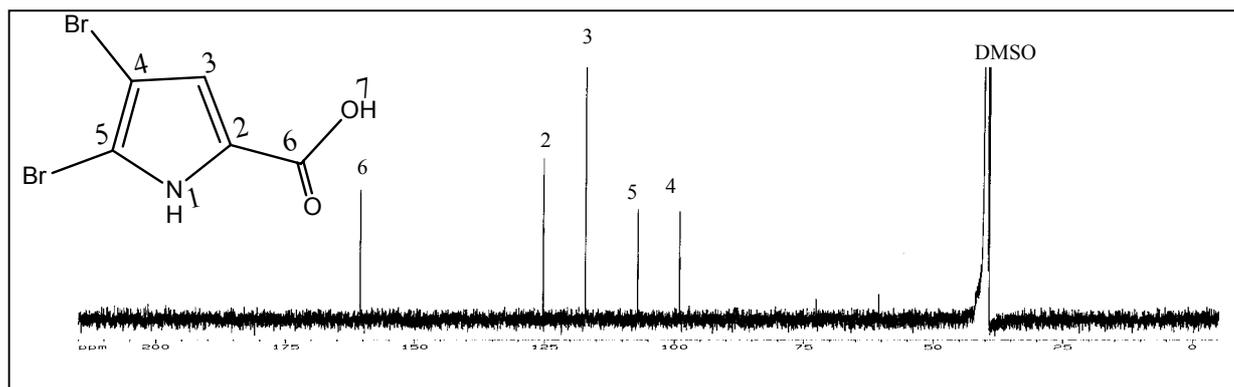
Fig..3.3.44. ^{13}C-NMR spectrum of compound 23
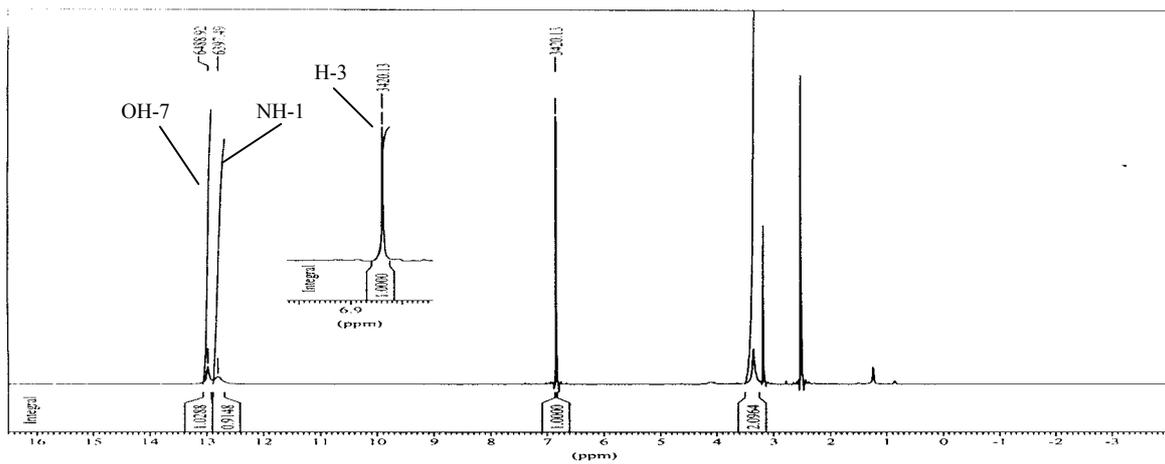
Fig. 3.3.45: ^{1}H-NMR spectrum of compound 23
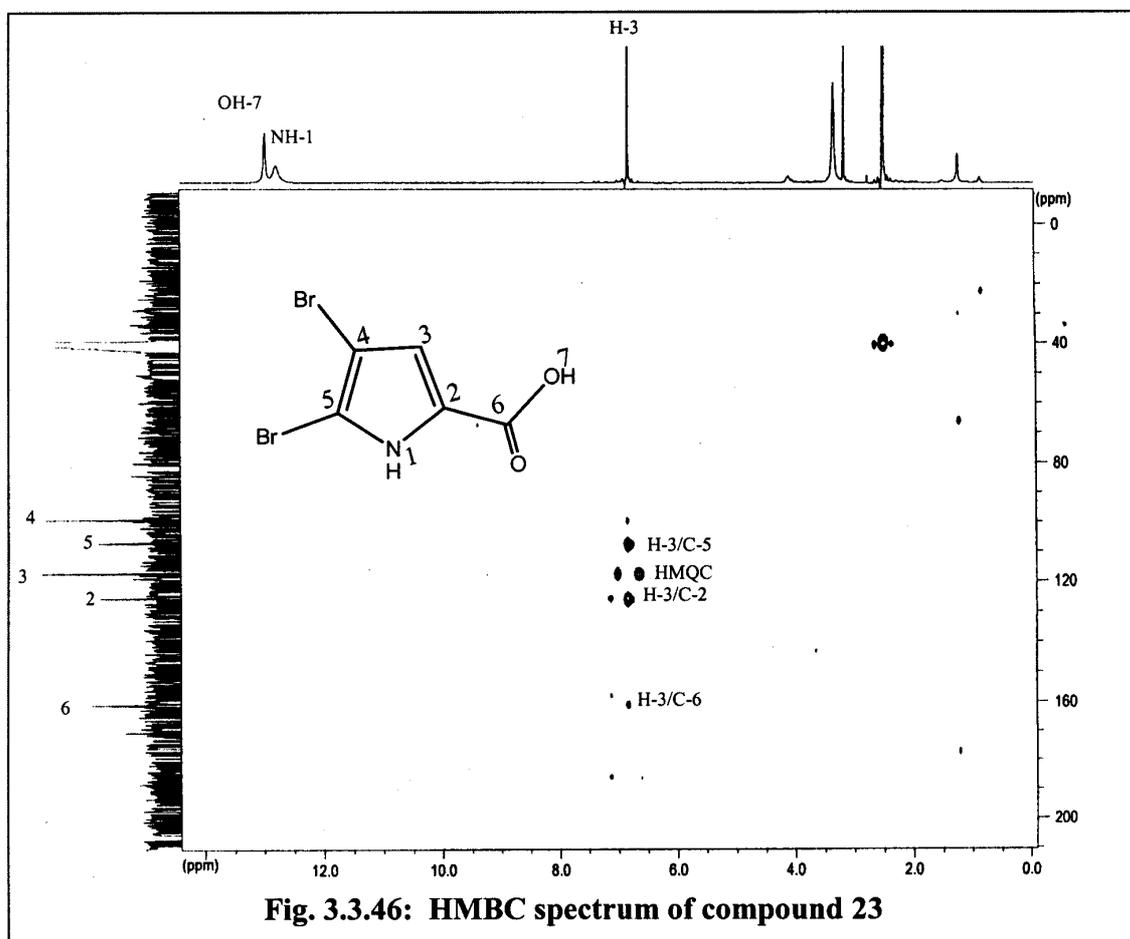
Fig. 3.3.46: HMBC spectrum of compound 23
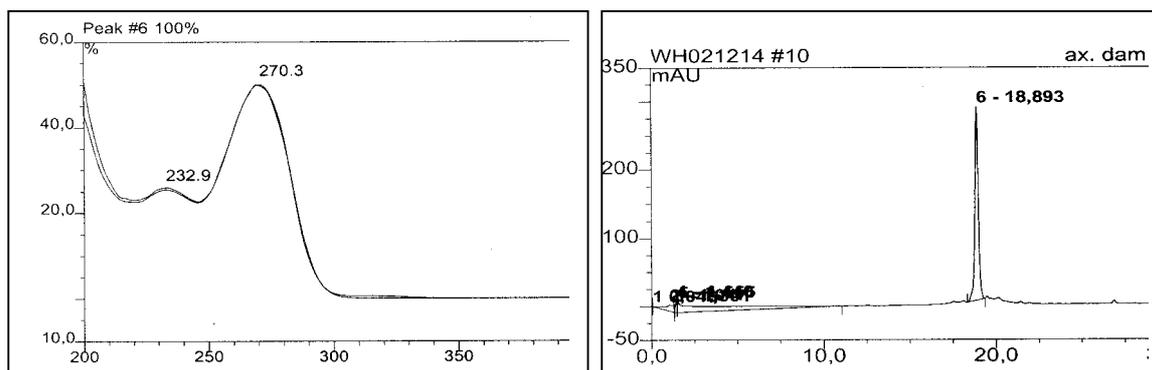
Fig.3.3.47: UV and HPLC chromatogram of compound 24
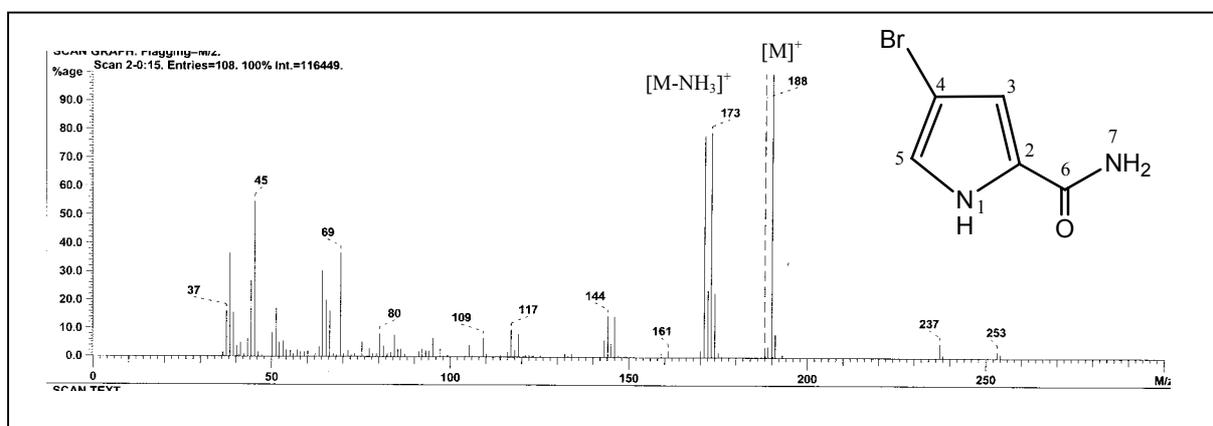
Fig. 3.3.48: EIMS spectrum of compound 24
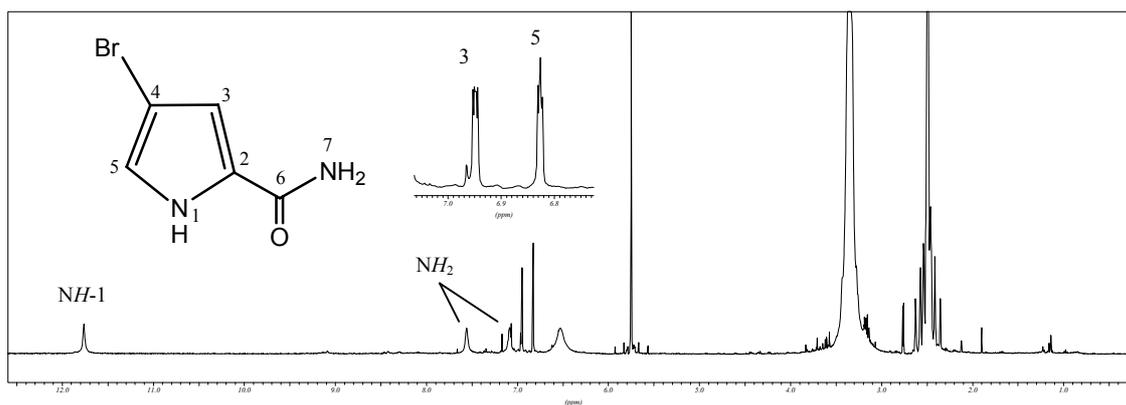
Fig. 3.3.49: ^{1}H-NMR spectrum of compound 24
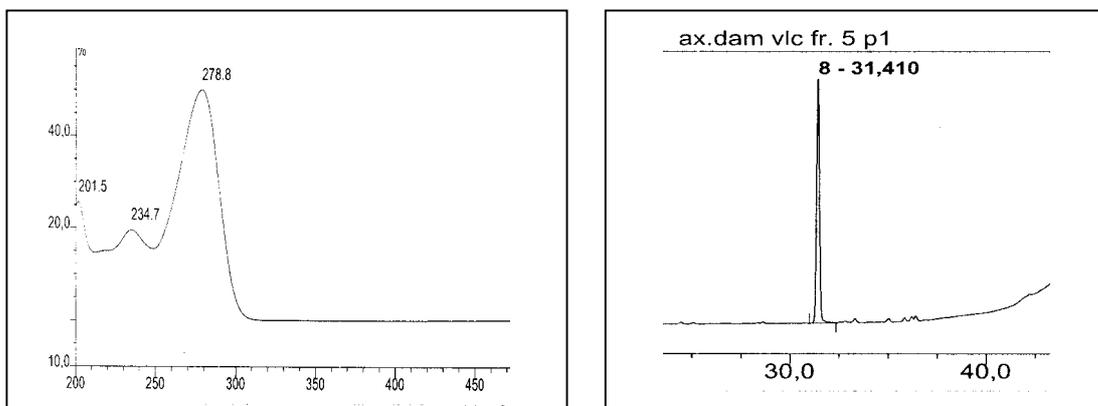
Fig. 3.3.50: UV and HPLC chromatogram of compound 25
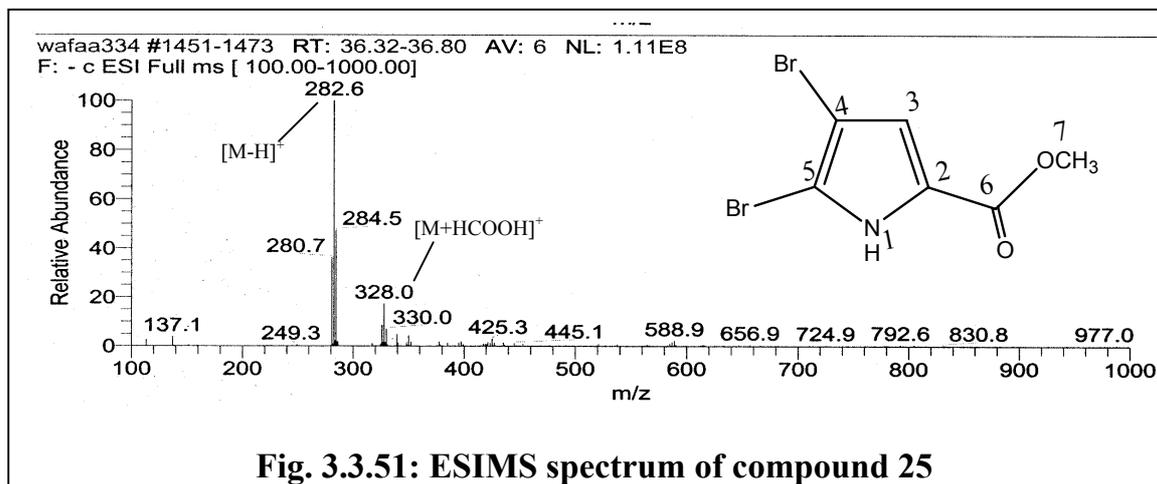
Fig. 3.3.51: ESIMS spectrum of compound 25

3.3.1.16. Structure elucidation of longamide and manzacidin A (26 and 27, known compounds)

Longamide (26)   **Manzacidin A (27)**
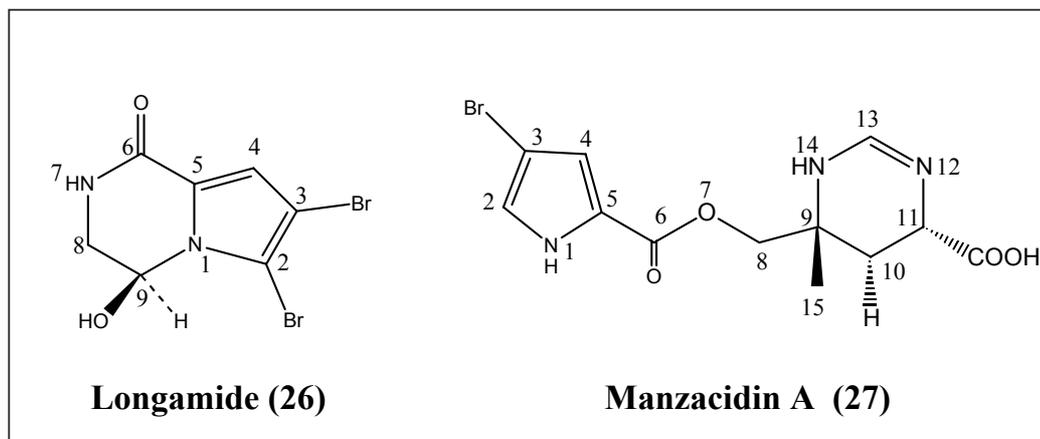
Compound 26 was isolated as a pale yellowish white amorphous powder. The EIMS spectrum (Fig.3.3.52) showed the molecular ion peak at *m/z* 308, 310 and 312 in a ratio of 1:2:1 which was compatible with the molecular formula $C_7H_6N_2O_2Br_2$. The ^{1}H-NMR data (Table 3.3.10) matched those of **longamide** which was previously isolated from *Agelas longissima* (Cafieri *et al*, 1995).

Manzacidin A (compound 27) was previously isolated from sponge genus *Hymeniacidon* (Kobayashi *et al*, 1991). From *Axinella damicornis*, compound **27** was isolated as a pale yellow amorphous substance, the ESIMS spectrum showed an isotopic cluster at *m/z* 242/244 [M-1][+]: 344/ 346 [M+1][+] with equal intensities, which indicated a monobrominated compound with the molecular formula $C_{12}H_{15}N_3O_4Br$. The structure of this compound was established from ESIMS, ^{1}H-NMR (Table 3.3.10) and through comparison with the literature data (Kobayashi *et al*, 1991).

Table 3.3. 10: ^{1}H-NMR data of longamide and manzacidin A

No.	δH	
	Longamide (MeOD)	**Manzacidin A (DMSO)**
1	-	12.40 (br s)
2	-	7.25 (s)
4	6.92 s	7.00 (s)
8a	3.81 (dd, J= 13.9, 2.8 Hz)	4.10 (d, 11.0 Hz)
8b	3.58 (dd, J= 13.9, 1.5 Hz)	4.28 (d, 11.0 Hz)
9	5.75 (br s)	-
10 eq.	-	2.20 (dd, 13.6, 5.1 Hz)
10 ax.	-	2.00 (dd, 13.6, 10.9 Hz)
11	-	4.20 (dd, 5.1, 10.9 Hz)
13	-	8.10 (br s)
14	-	10.05 (br s)
15	-	1.30 (3H, s)
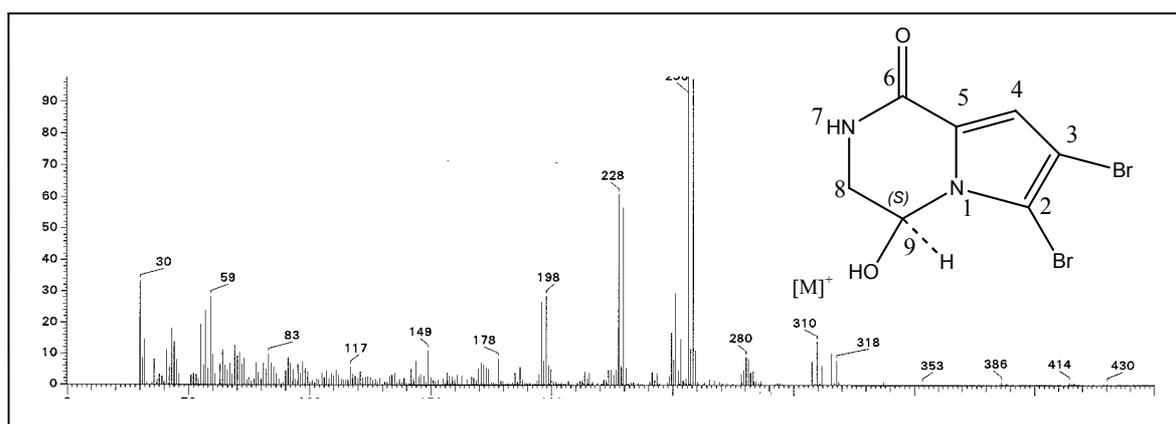
Fig. 3.3.52: EIMS spectrum of compound 26

3.3.2. The results of biological assays

3.3.2.1. Cytotoxic activity

Cytotoxicity of the compounds isolated from *Axinella damicornis* was studied *in vitro* against human cervix uteri tumour (HeLa), rat brain tumour (PC12) and mouse lymphoma (L5178Y) cell lines using the microculture tetrazolium (MTT) technique.

Table 3.3.11   shows the results of the cytotoxicity test based on growth inhibition of the cells. It was found that *Z*-hymenialdisine was the most active compound towards human cervix uteri tumour and mouse lymphoma cell lines. Dibromocantharelline was also active against mouse lymphoma cell line only, (ED_{50}, 8.3 µg/ml), while 2-bromoaldisin showed only mild activity towards the same cell line (ED_{50}, 13.8 µg/ml) and 2,3-dibromoaldisin exhibited activity towards L5178Y cell line.

Table 3.3.11:   Cytotoxic activities of some brominated compounds (10 µg/ml)

Name of Compound	% Growth inhibition against two cell lines (10 µg/ml)	
	L5178Y	**HeLa**
Oroidin	0	0
Stevensin	0	0
4, 5 DBPCA	0	0
4,5 DBPC	0	0
Hymenin	0	0
Hymenidin	0	0
Dibromocantharelline	60%	0
2-Bromoaldisin	36 %	0
Manzacidin A	0	0
***Z*-Hymenialdisin**	**100 %**	**100%**
Longamide	0	0
3,4-DBPCA	0	0
3,4-DBPC	0	0
3-bromoaldisin	0	0
2,3-dibromoaldisin	70%	-

Table 3.3.12: Dose response relationship of the cytotoxic activity of dibromocantherilline

Conc.	% Growth inhibition		
	L5178Y	**HeLa**	**PC12**
0.1 µg/ml	0	0	0
0.3µg/ml	0	0	0
1.0µg/ml	0	0	0
3.0µg/ml	11 %	0	0
10µg/ml	35 %	0	0
ED$_{50}$	0.6 µg/ml	>10 µg/ml	>10 µg/ml

3.3.2.2: Anti-microbial assay

Agar diffusion assay

The anti-microbial activity of extracts and pure isolated metabolites of *Axinella damicornis* was assessed using agar diffusion assay.

For all the tested extracts and pure metabolites of *Axinella damicornis*, the ethyl acetate extract exhibited weak anti-microbial activity against gram-positive and gram-negative bacteria and strong antifungal activity against *Cladosporium herbarum* and moderate activity against *Cladosporium cucumerinum*. Manzacidin A exhibited strong activity against *Cladosporium herbarum* and weak activity against *Cladosporium cucumerinum* while it showed moderate activity against gram-positive and gram-negative bacteria.

Table 3.3.13: Anti-microbial activities of pure compounds isolated from *Axinella damicornis*

Name of the compound	*B. subtilis*	*E. coli*	*S. cerevisiae*	*C. herbarum*		*C. cucumerinum*	
	100 µg	**100 µg**	**100 µg**	**50 µg**	**100 µg**	**50 µg**	**100 µg**
Hymenin	6 mm[*]	n. a.	n. a.	n. t	n. a.	n. t	n. a.
Manzacidin A	13 mm[*]	9 mm[*]	10 mm[*]	n. t	30 mm[*]	n. t	8 mm[*]
3-Bromoaldisin	n.a.	n.a.	n.a.	n. t	n.a.	n. t	n.a.
2-Bromoaldisin	n.a.	n.a.	n.a.	n. t.	n.a.	n. t	n.a.
2,3-Dibromoaldisin	n.a.	n.a.	n.a.	n. t	n.a.	n. t	n.a.
Dibromocantharelline	n.a.	n.a.	n.a.	n. t	16 mm[*]	n. t	n.a.
3, 4 DBPCA	n.a.	n.a.	n.a.	n. t	n.a.	n. t	n.a.
3, 4 DBPC	n.a.	n.a.	n.a.	n. t	n.a.	n. t	n.a.

[*] diameter of inhibition zone

3.3.2.3: Brine shrimp assay

Brine shrimp mortality rate of extract and isolated compounds

The pure compounds were screened for bioactivity in the brine-shrimp assay and the results were based on the percentage of mortality. Nearly all the tested compounds showed no or only mild activity in this assay.

Table3.3.14: The result of brine-shrimp assay of *Axinella damicornis* extracts and pure compounds

Samples	Brine shrimp mortality rate	
	50µg	**500µg**
Ax. dam. **EtOAc**	0%	40%
Ax. dam. **Hexane**	0%	0%
Ax. dam **H₂O**	0%	0%
Ax. dam. **Butanol**	n. t	n. t
Oroidin	0%	30%
Stevensin	0%	15%
Hymenin	0%	0%
Hymenidin	0%	0%
4,5 DBPCA	0%	50%
4,5-DBPC	0%	5%
Z-**Hymenialdisine**	0%	0%
Dibromocantharelline	0%	0%

Ax. dam. = Axinella damicornis

3.3.2.4: Fish-Feeding Assay with *Blennius sphinx*

Table 3.3.15: Results of fish-feeding assay for some pure compounds

Name of compound	% of food eaten	
	Experiment (E)	Blank (B)
Oroidin (60 mg) [*]	3.5	96.5
Oroidin (48 mg)	3.3	96.7
Oroidin (25 mg)	6.7	93.3
Oroidin (10 mg)	13.6	86.4
4,5-dibromopyrrole-2-carboxylic acid (4,5-DBPCA, 48 mg) [*]	16.5	83.5

[*] natural concentration as present in *Axinella damicornis*

Fig. 3.3.53: Results of fish-feeding assay of oroidin and 4,5-dibromopyrrole-2-carboxylic acid (4,5-DBPCA)
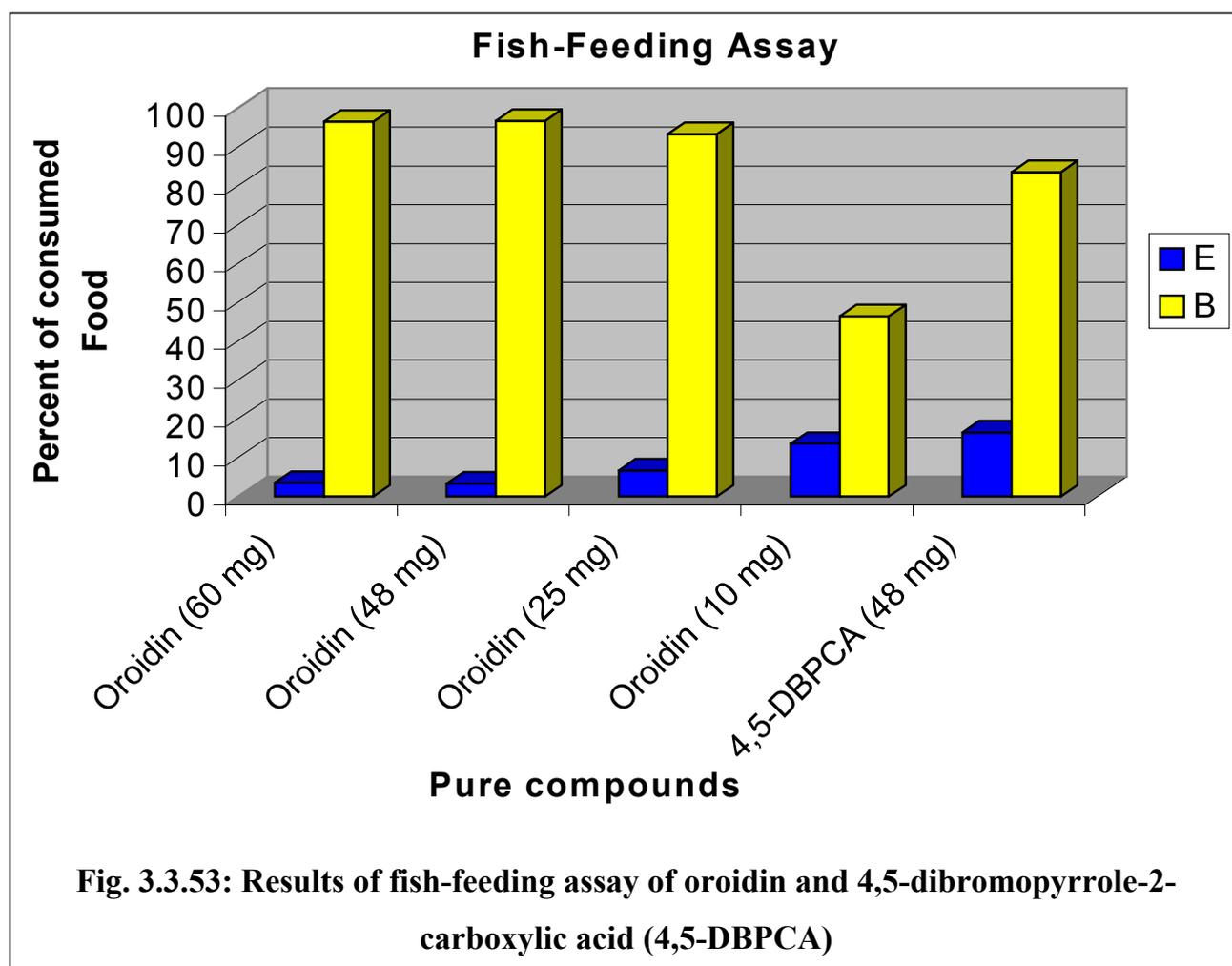
E = experiment,   B = blank

3.3.2.5. Protein kinase inhibition test

Several brominated alkaloids were screened in protein kinase inhibition assays towards cyclin-dependent kinase-1, cyclin-dependent kinase-5 and glycogen synthase kinase-3. The IC_{50} in μM for each compound tested is given in Table 3.3.16.

Table. 3.3.16: Results of protein kinase inhibition test

Name of compound	IC_{50} (μM)		
	CDK-1	**CDK-5**	**GSK-3**
Oroidin	0.26	2.2	2.4
Manzacidin A	n.t.	> 10	> 10
Hymenin	0.50	0.34	0.12
Stevensin	5.3	6.3	7.4
Longamide	n.t.	> 10	> 10
4,5-Dibromopyrrole-2-carboxylic acid	n. t.	> 10	> 10
2-Bromoaldisin	n.t.	> 10	> 10
3-Bromoaldisin	n.t.	> 10	> 10
Dibromocantherelline	n.t.	> 10	> 10
***Z*-Hymenialdisine**	0.45	0.14	0.11
3,4-Dibromopyrrole-2-carbamide	n. t.	> 10	> 10
Hymenidin	> 10	> 10	> 10

n. t. = not tested

3.4. Metabolites isolated from the sponge *Hamigera hamigera*

In the search for bio-active substances from marine sponges, the family *Anchinoidae* was found as a rich source of bioactive metabolites which include peptide alkaloids, sulfur-containing compounds (Casapullo, 1993, 1994 A and B) and chlorine-containing phenolic compounds (Rudi *et al*, 1994). Many of these metabolites exhibited very interesting anti-fungal and cytotoxic activities (Philip *et al*, 1995, 1996 and Molinski, 1996).

The marine sponges were also found to be the most diverse source of sterols, particularly in term of unique side chain and unusual functional groups (Faulkner, 1988, Gunasekera *et al*, 1989, 1983, Yamada *et al*, 1980 and Aoki *et al*, 1998). From the genus *Hamigera* (family *Anchinoidae*), bioactive brominated and debrominated compounds have previously been isolated from *Hamigera tarangensis* (Wellington *et al*, 2000 and Cambie *et al,* 1995) Bisformamidodiphenylbutadiene was isolated from the sponge-associated fungus of *Hamigera avellanea* (Breinholt *et al*, 1996). The sponge *Hamigera hamigera* was analysed in this study because its extracts showed biological activity in the fish-feeding deterrence assay using *Blennius sphinx* (Fig 3.3.2 and 3.4.1). This investigation led to the isolation of four new compounds including, **28** and **29** which are phenolic compounds, **30** as a nucleosidic compound, and **36** as an indole alkaloid. In addition, four known compounds **31**, **32**, **33**, and **34** were also isolated.

Table 3.4.1: Results of fish-feeding assay (*Blennius sphinx)* of *Hamigera hamigera* extract

Extract	% of food eaten	
	Experiment	**Blank**
Total ext.*	18.3	81.7
EtOAc ext.*	31.4	68.6
Aqueous ext.*	16.0	84.0
Butanol ext.*	46.2	53.8
Hexane ext.*	38.6	61.4

* 10 ml sponge (see materials and methods)

Fig. 3.4.1. Result of fish-feeding assay (*Blennius sphinx*) of *Hamigera hamigera* extracts
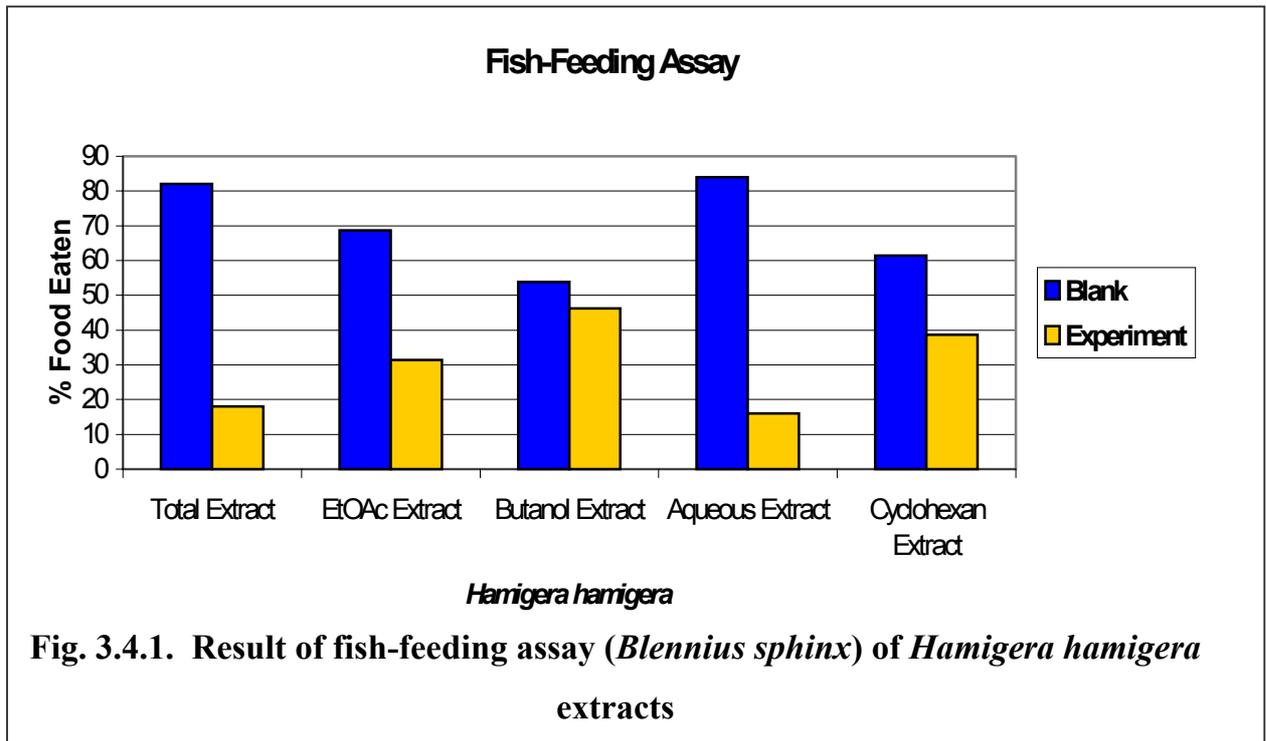
3.4.1. Structure elucidation of the isolated compounds

3.4.1. 1. Structure elucidation of hamigeroxalamic acid (28, new compound)

Hamigeroxalamic acid
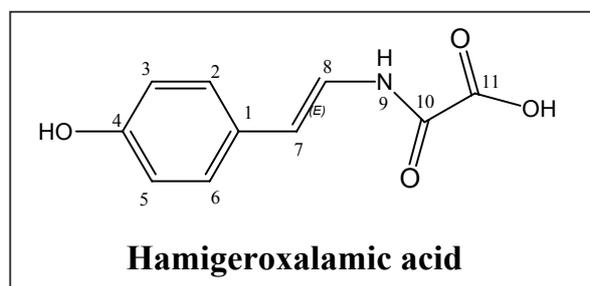
Compound 28 was isolated as a pale yellowish white amorphous powder. The ESIMS (Fig. 3.4.2) showed a pseudo-molecular ion peak at *m/z* 206 [M-H]$^+$, 252.1 [(M+HCOOH)-H]$^+$, and 413.2 [2M-H]$^+$. The ^{1}H-NMR spectrum (Table 3.4.1, Fig. 3.4.3) revealed an AA′BB′ system for 2H doublets at δ_H 7.09 and 6.67 which indicated a *para*-disubstituted benzene ring. The broad proton signal at 9.32 (O*H*) suggested that compound **28** was a phenolic compound as it gave a ^2J correlation with ^{13}C-NMR resonance at δ_C 158.3 and ^3J correlations to C-3 and C-5 at δ 116.5 for the AA`BB′ system. The presence of a 1,2-di-substituted double bond adjacent to the N*H* group or *trans-enamide* structure were observed as implied by the coupling constant and proton resonances at δ_H 6.35 (H-7, d, *J* = 14.5 Hz) and δ 7.10 (H-8, dd, *J* = 14.5 and 10.7 Hz) which coupled further with the doublet broad proton (NH) at δ 10.07 (d, *J* =10.7 Hz) (Palermo *et al*, 1996 and Bokesh *et al*, 2000).

The ^{1}H and ^{13}C-NMR spectra suggested a close similarity to an alkaloid isolated previously from the Australian marine sponge *Spongosorites* sp. (Urban *et al*, 1994). From the ^{13}C-NMR signals (Table. 3.2.4) of compound **28** an additional resonance at δ_C 163.0 was observed indicating the presence of two carbonyl groups. The carbonyl resonances at δ_C 163.0 and δ_C 156.7 were compatible with the terminal oxalamic (NHCOCOOH) functionality as present for example in psammaplin F with chemical shifts at δ 161.9 and δ 159.2 (Piňa *et al*, 2003). The ESI-MS/MS performed on the negative pseudo-molecular ion at *m/z* 206 [M-H]$^+$ gave a daughter ion at *m/z* 134.5 indicating the loss of an oxo-acetic acid [CO-COOH]. This confirmed that **28** was *N*-[2-(4-hydroxy-phenyl)-vinyl]-oxalamic acid which was assigned the trivial name **hamigeroxalamic acid**.

Hamigeroxalamic acid (compound 28)

Chemical name: N-(2-(4-Hydroxy-phenyl -vinyl)-oxalamic acid
Character: yellowish white amorphous powder
Sample code: HH5   **Amount**: 2.5 mg
Molecular weight 207   g/mol   **Molecular formula**: $C_{10}H_9O_4N$
Biological source: Marine sponge *Hamigera hamigera*

New natural product
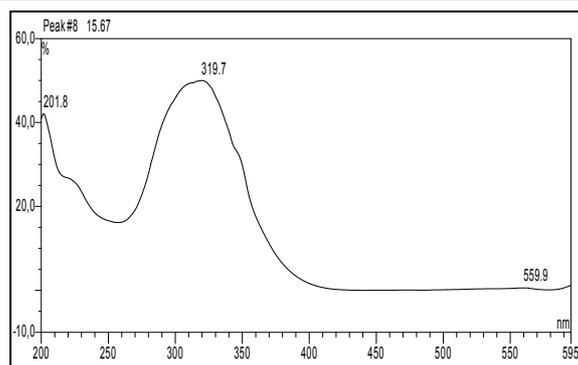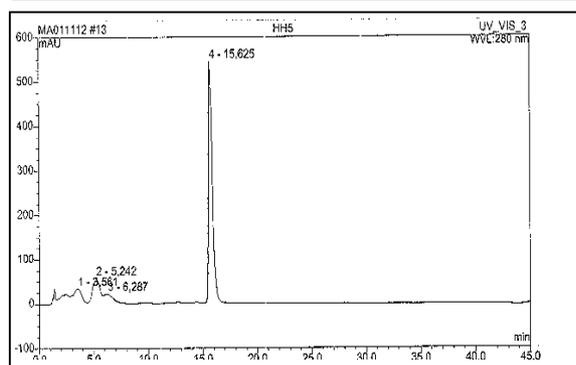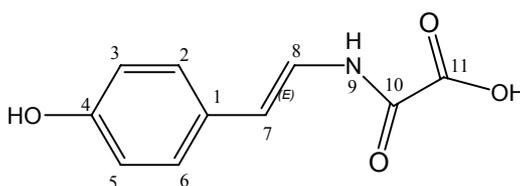
ESIMS: *m/z*, 206 [M-H]$^+$, 413   [2M-H]$^+$, 134.4 [M-C$_2$O$_3$H]$^+$ (negative)

Fig. 3.4.2: ESIMS spectrum of compound 28
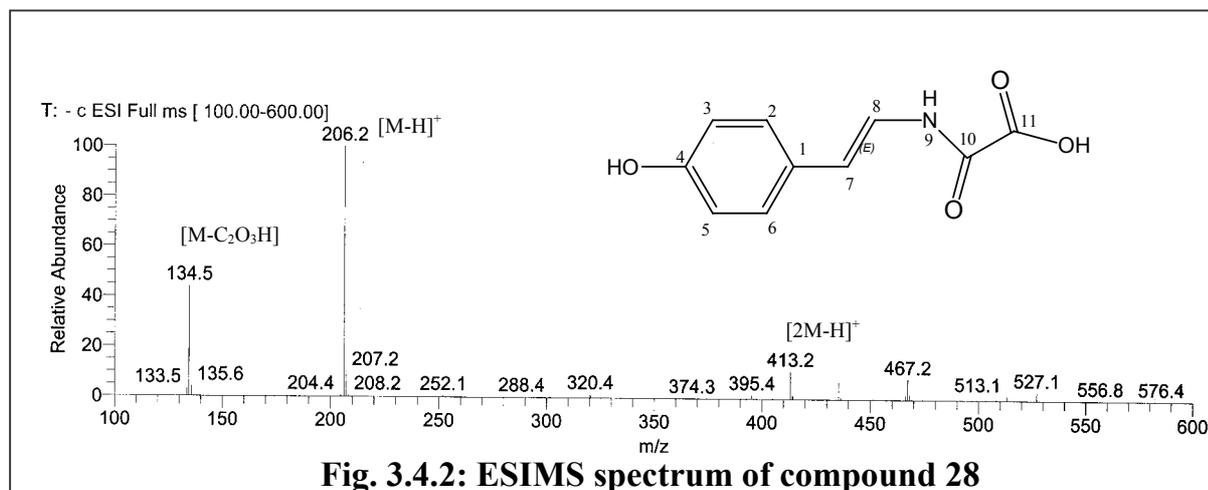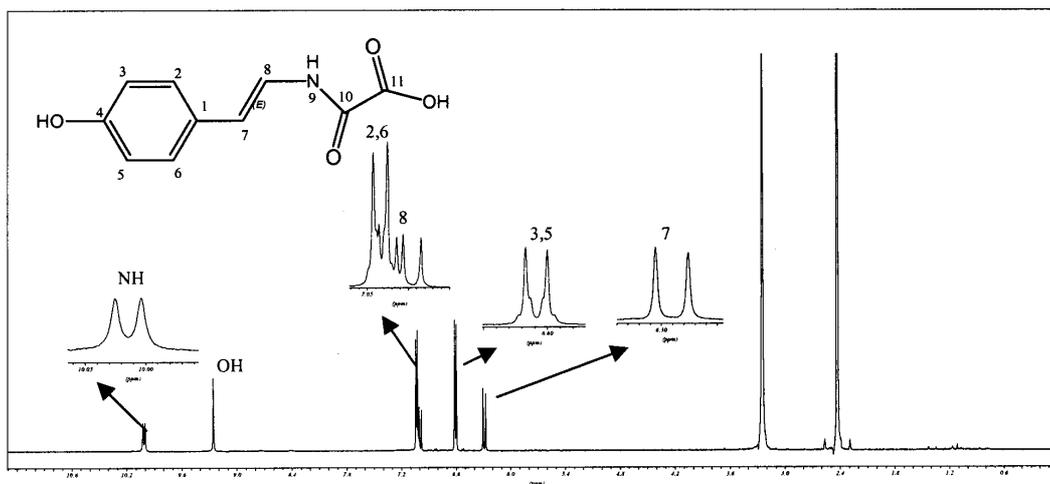
Fig. 3.4.3: ¹H-NMR spectrum of compound 28
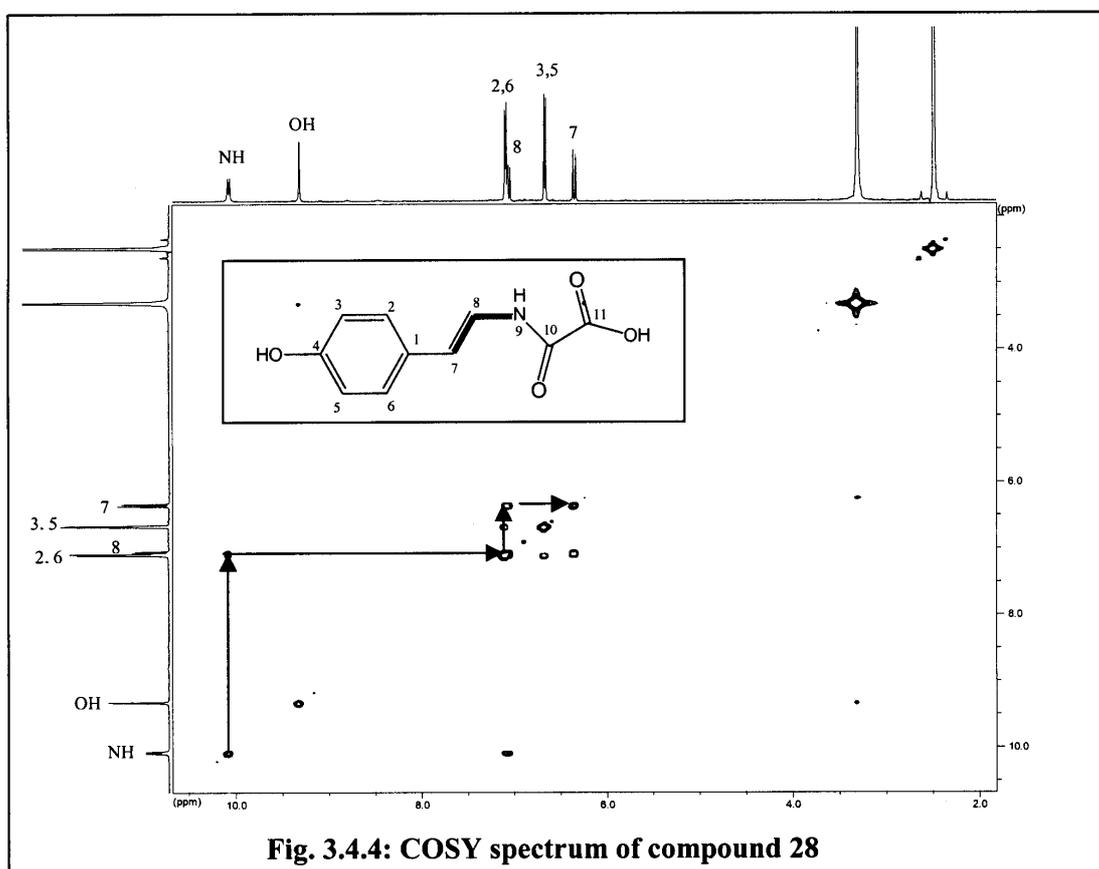
Fig. 3.4.4: COSY spectrum of compound 28
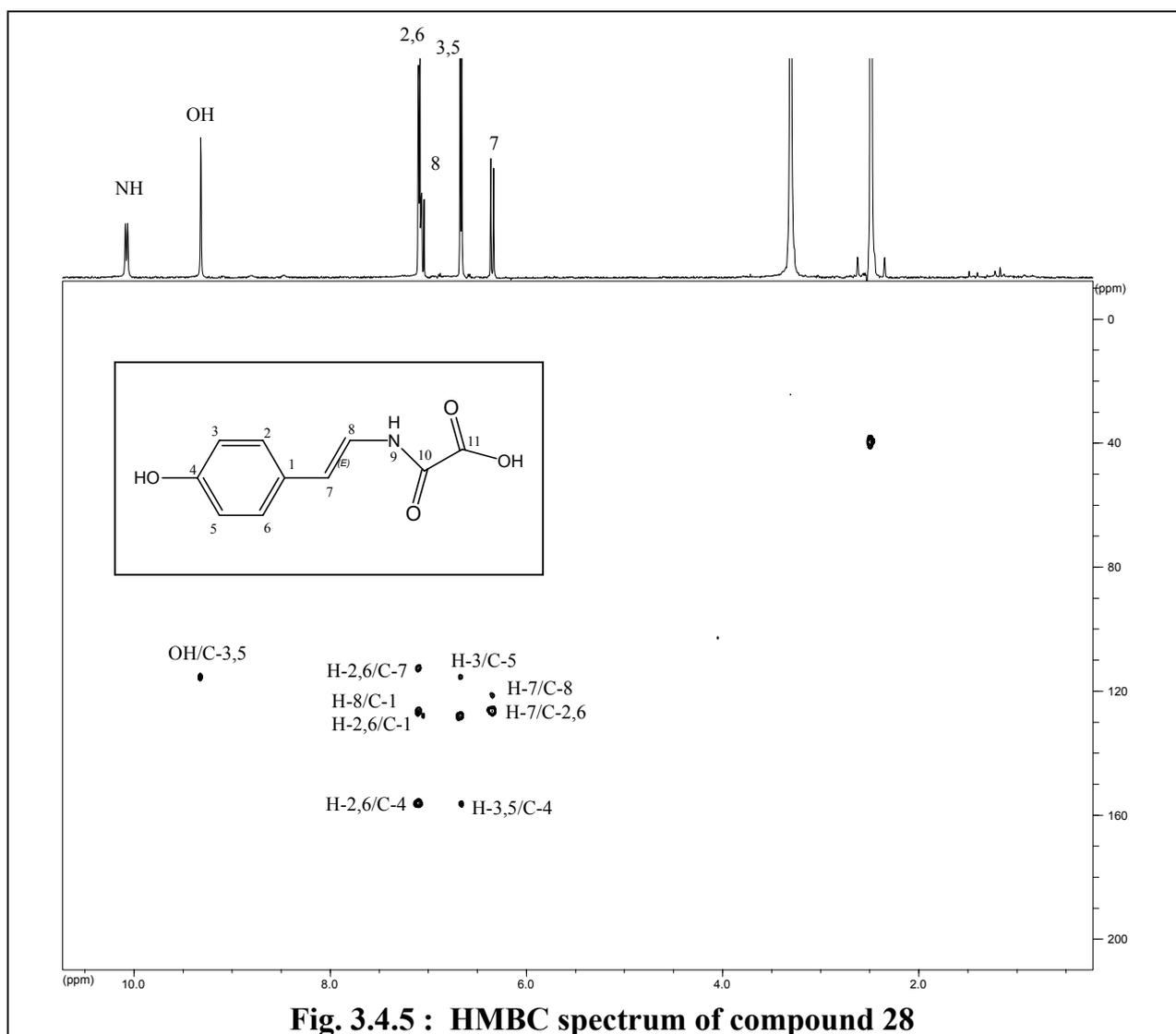
Fig. 3.4.5 : HMBC spectrum of compound 28

3.4.1.2. Structure elucidation of hamigeramine (29, new compound)
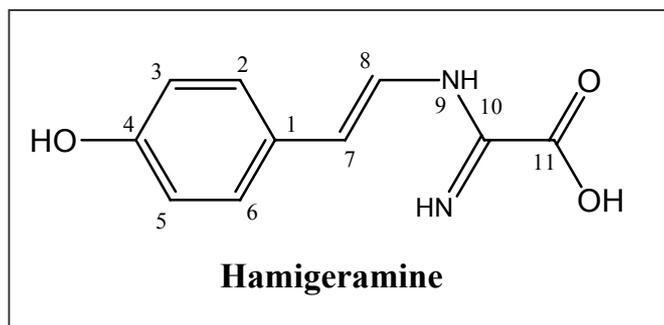
Hamigeramine

Hamigeramine 29 was isolated as a yellowish white powder. The ESIMS spectrum of hamigeramine (Fig. 3.4.6) showed a peak at *m/z* 207 [M+H]⁺ in accordance with the molecular formula $C_{10}H_{10}N_2O_3$ as determined by HRESIMS. The ¹H and ¹³C-NMR data (Table 3.4.2) were largely comparable to those of hamigeraoxalamic acid, which also revealed the presence of a 4-hydroxy styryl unit, a similar substructure as in **28**. Its ¹H NMR spectrum (Fig. 3.4.7) also showed both the AA´BB´ spin system and *trans* enamide pattern of resonance. However, the resonance for the N*H* at δ 10.97 and the corresponding olefinic proton at δ_H 7.29 appeared very broad. The expected doublet for a *trans* enamide was not resolved. However, the COSY spectrum (Fig. 3.4.10) confirmed the presence of the *trans* enamide as a correlation was observed between δ_H 10.97 and δ_H 7.29. The ¹³C NMR spectrum of hamigeramine (Fig. 3.4.8) was very similar to that of **28** except for a significant change in chemical shifts for the carbonyl functionalities (δ 156.1 and δ 155.4) which resonated more upfield as compared to the latter.

From the ¹H NMR spectrum of **29**, it can be observed that the resonances for the *trans* olefinic protons were shifted more downfield when compared to those of compound **28**. In addition there was the appearance of additional broad singlets resonating at δ 9.29 and δ 9.10 which could indicate the presence of a terminal N*H₂* group as in oxalamide which would also satisfy the proposed molecular formula $C_{10}H_{10}N_2O_3$. However, significant discrepancies were observed for the carbonyl resonances in the ¹³C-NMR spectrum (Fig. 3.4.8) of **29** when compared to those of psammaplin E (Piňa *et al*, 2003), 3-bromotyramine amide (Pettit *et al*, 1996), and

igzamide (Dumdei and Andersen, 1993), all of which possess an oxalamide terminal functional group. The carbonyl shift of the oxalamide unit for these known compounds was reported to resonate at ca. 161 and 165 ppm. For hamigeramine, the quaternary carbons resonating at 155.7 and 155.1 ppm rather suggested an amino imino moiety as in the guanidino unit of tubastrine (Sakai and Higa, 1987, Sperry and Crews, 1998) and the alkaloid isolated from the the sponge S*pongosorites* sp. (Urban *et al*, 1994). The carbonyl resonances of hamigeramine were closely related to those of acetylbenzeneamidinocarboxylic acid which had been isolated from the fungus *Gibberella saubinetii* (Munekata *et al,* 1982).

The presence of an amino imino acetic acid moiety [NH(C=NH)COOH] as found in acetylbenzeneamidinocarboxylic acid in **29** was confirmed by the cross peak between the imino proton at δ_H 9.29 and the carboxylic keton group at δ_C 155.1 as observed in the HMBC spectrum (Fig. 3.4.9) of **29**. The ESI-MS/MS fragmentation of the negative pseudo-molecular ion at *m/z* 205 [M-H]$^+$ gave a daughter ion at *m/z* 161.1 instead of at *m/z* 134.1 as in **28**. This indicated the loss of [COOH] unit and the absence of the amide linkage found in **28**. It was then confirmed that compound **29** was **2-[4-hydroxy-phenyl)-vinylamino]-imino-acetic acid**.

Table 3.4.2: ^{1}H, ^{13}C-NMR data and HMBC correlation of compounds 28 and 29 (DMSO-d_6, 500 MHz)

No	28			29		
	δH	**δC**	**HMBC**	**δH**	**δC**	**HMBC**
1	-	128.2 s	-	-	125.9 s	-
2	7.09 (d, 8.5 Hz)	127.9 d	1, 4, 7	7.25 (d, 8.4 Hz)	127.5 d	1, 6, 7
3	6.67 (d, 8.5 Hz)	116.5 d	1, 4, 5	6.75 (d, 8.4 Hz)	115.6 d	1, 4, 5
4	O*H*, 9.32 (s)	158.3 s	3, 4, 5	9.60 (br s)	157.3 s	3, 4, 5
5	6.67 (d, 8.5 Hz)	116.5 d	1, 3, 4	6.75 (d, 8.4 Hz)	115.6 d	1, 3, 4
6	7.10 (d, 8.5 Hz)	127.9 d	1, 4, 7	7.25 (d, 8.4 Hz)	127.5 d	1, 2, 7
7	6.35 (d, 14.5 Hz)	114.0 d	2, 6, 8	6.81 (d, 13.9 Hz)	121.3 d	2, 6, 8
8	7.07 (dd, 14.5, 10.7 Hz)	122.1 d	1, 7	7.29 (br d, 13.9 Hz)	119.5 d	7, 9, 10
9	N*H*, 10.07 (d, 10.7 Hz)	-	11	N*H*, 10.97 (br s)		-
10	-	163.0 s	-	N*H*, 9.29 (br s)	155.7 s	11
11	-	156.7 s	-	O*H*, 9.10 (br s)	155.1 s	-

Hamigeramine (compound 29)

Chemical name: 2-[4-hydroxy-phenyl)-vinylamino]-imino-acetic acid

Character: yellowish white amorphous powder

Sample code: H2   **Amount:** 7.5 mg

Molecular Weight 206 g/mol   **Molecular formula**: $C_{10}H_{10}N_2O_3$

Biological source: marine sponge *Hamigera hamigera*

New natural product
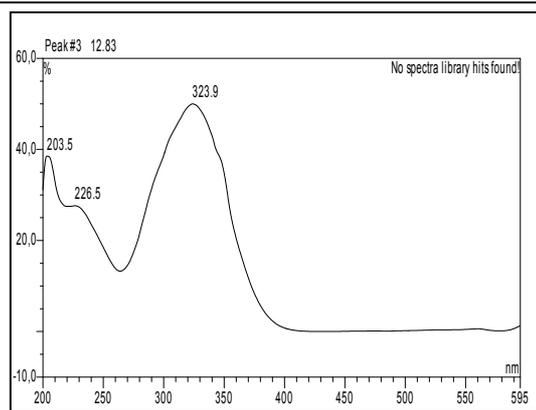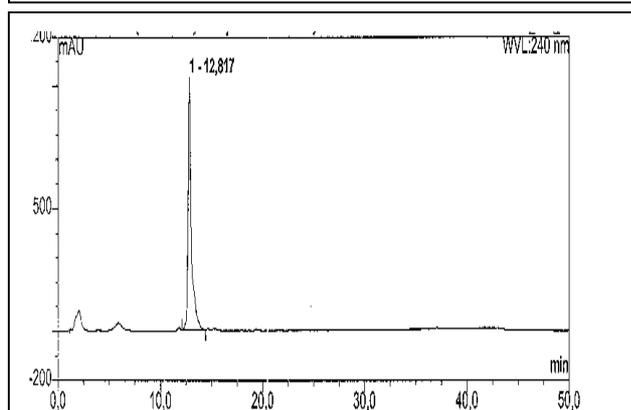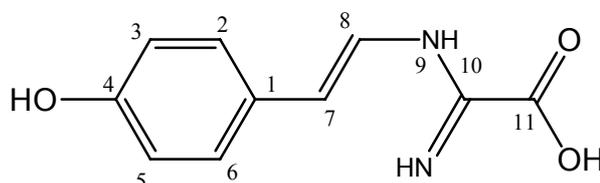
ESIMS: *m/z*, 207 [M+H]$^+$, 229 [M+Na]$^+$, 413 [2M+H]$^+$, 435 [2M+Na]$^+$ (positive)

ESIMSMS: (*m/z*, rel. int.) 190 (35),162 (28), 134 (25) and 119 (10)

HRESIMS: *m/z* = 206   Experimental = 207.0953 for [$C_{10}H_{10}N_2O_3$]

Experimental = 205.0742 for [$C_{10}H_{10}N_2O_3$]

Fig. 3.4.6: ESIMS spectrum of compound 29

Fig. 3.4.7: ¹H-NMR spectrum of compound 29

Igzamide

Psammaplin F

Acetylbenzeneamidinocarboxylic acid

Tubastrine

155
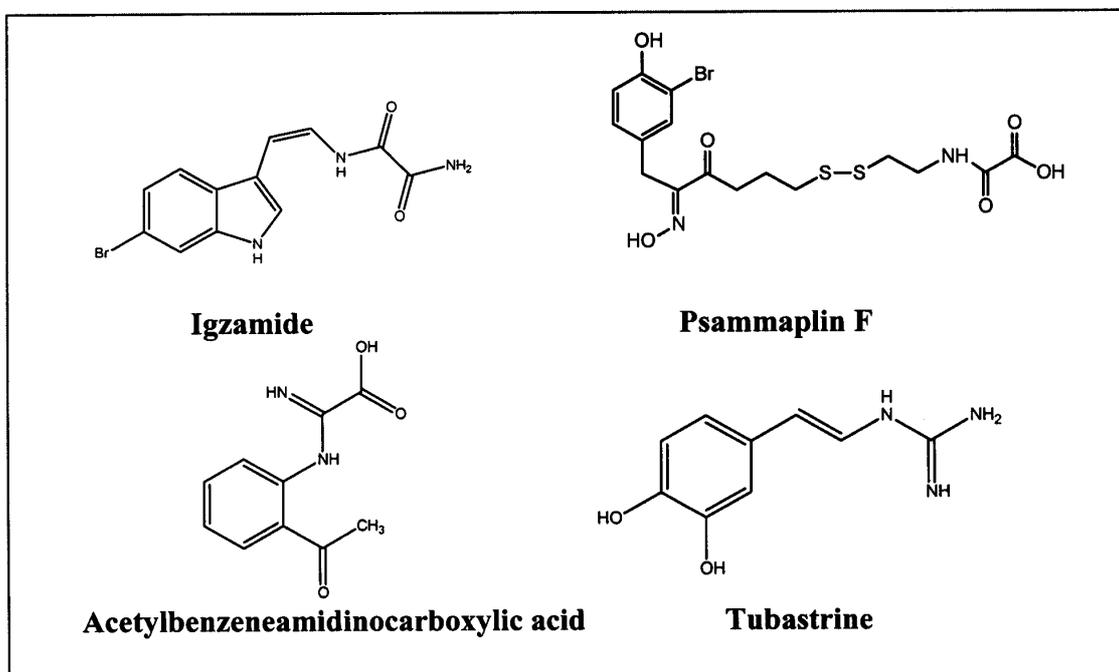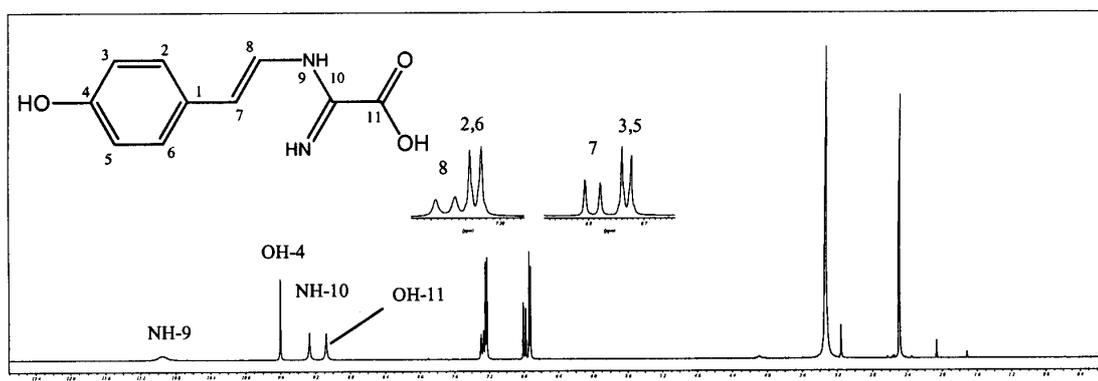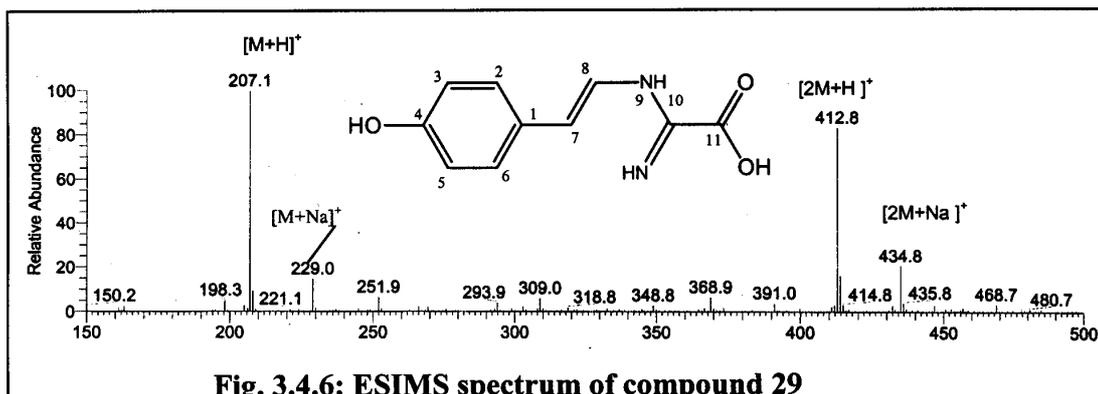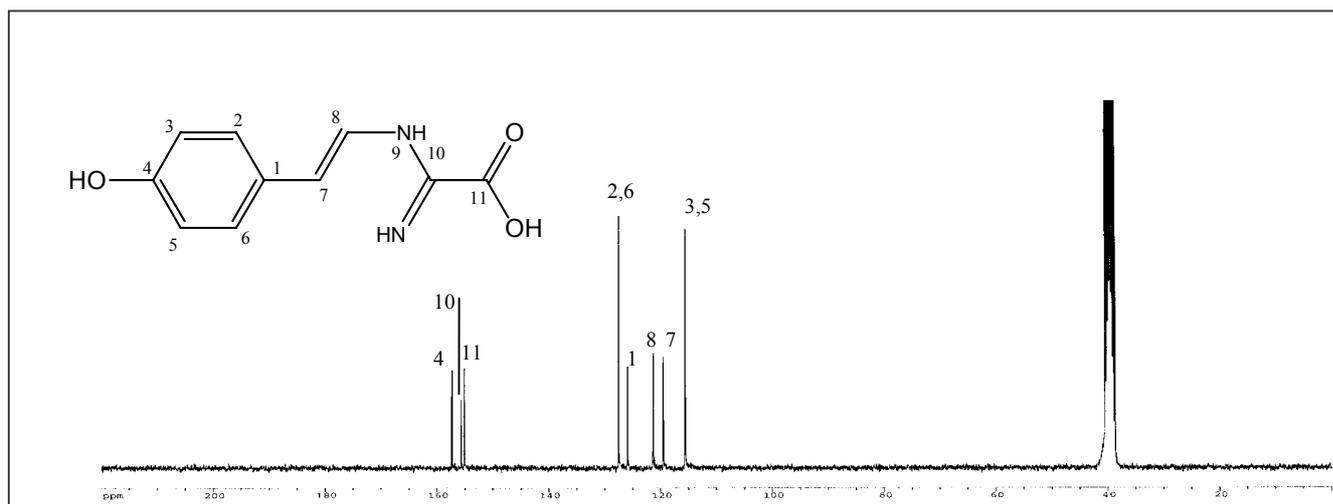
Fig. 3.4.8: ^{13}C-NMR spectrum of compound 29

Fig. 3.4.9 : HMBC spectrum of compound 29
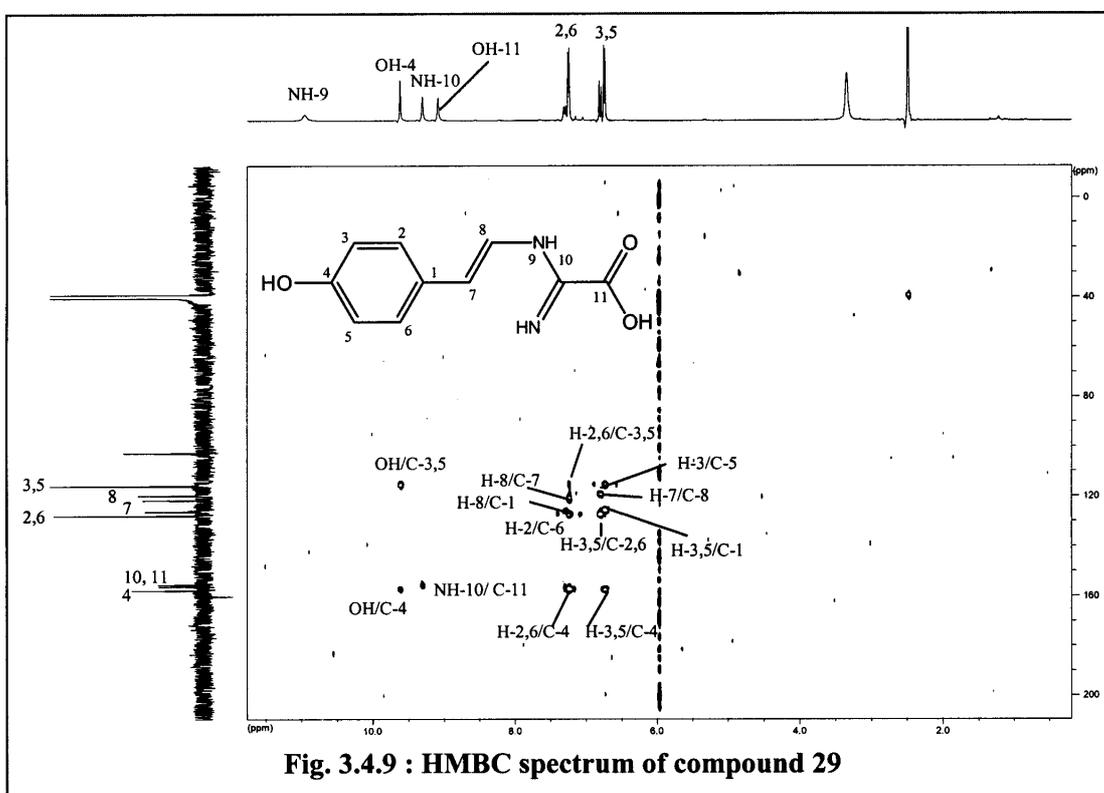
Fig. 3.4.10: COSY spectrum of compound 29
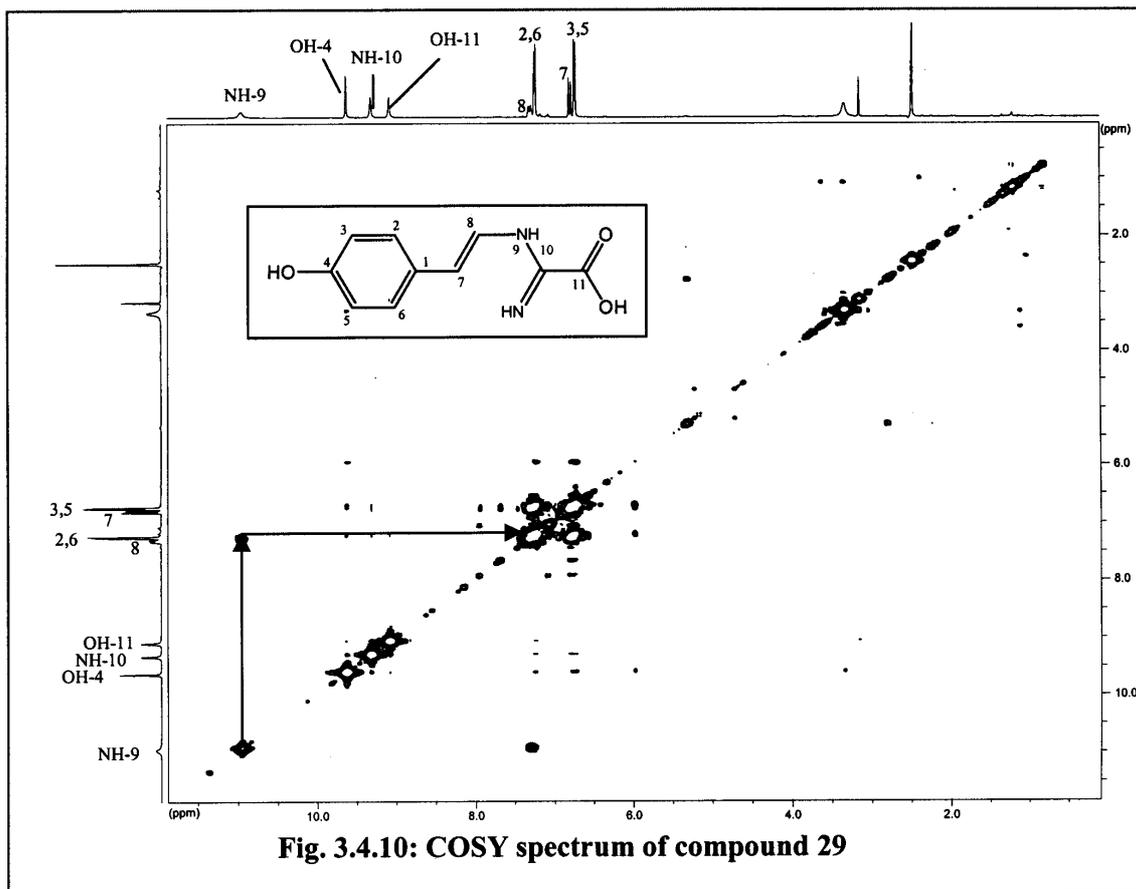
3.4.1. 3. Structure elucidation of hamiguanasinol (30, new compound)
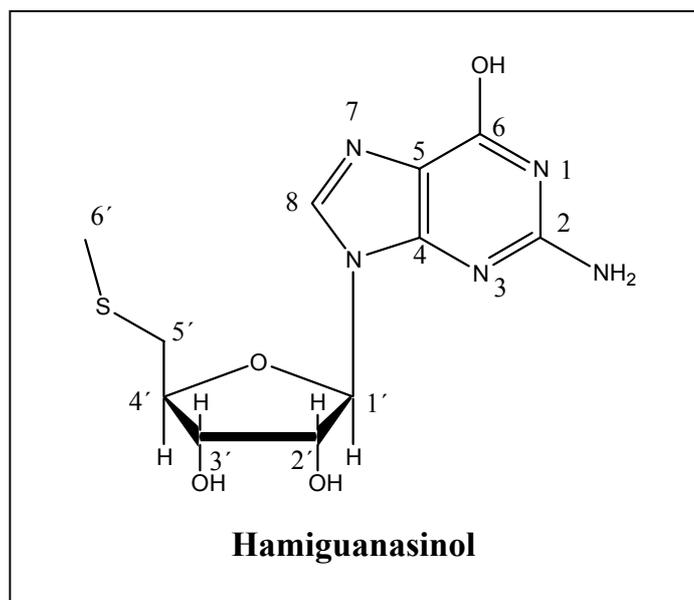
Hamiguanasinol

Hamiguanasinol 30 was isolated as a pale yellowish white amorphous solid. The ESI-MS showed the molecular ion peak at *m/z* 314 [M+H]$^+$, corresponding to the molecular formula $C_{11}H_{15}N_5O_4S$. The ^{1}H-NMR spectrum (Fig. 3.4.12) and ^{13}C NMR data of hamiguanasinol (**30**) were similar to those of 6-hydroxy guanosine (Pretsch *et al*, 1990). The ^{1}H-NMR signals at δ 5.70, 4.50, 3.95, 4.05, 2.80, and 2.70, together with the ^{13}C-NMR resonances at δ 87.0, 84.0, 74.0, 71.5 and 35.0 indicated the presence of a β–ribosyl moiety. The remaining ^{1}H- and ^{13}C NMR resonances at $δ_H$ 10.57 (1H, s), 7.90 (1H, s) and 6.40 (2H, br s) and $δ_C$ at 135.0 (d), 118.0 (d), 157.5 (s) and 153.5 (s), respectively were assigned to the 2-amino purine nucleus. The occurrence of the β–ribosyl moiety was confirmed by the HMBC spectrum which showed correlations between exchangeable hydroxyl protons that resonated at $δ_H$ 5.40 and 5.20 with C-1′, C-3′ and C-2′, C-4′, respectively. The only difference from the known nucleoside A was the presence of a methyl singlet at $δ_H$ 2.05 which gave a CH direct correlation with the quaternary carbon at $δ_C$ 16.0 and attested the presence of a SCH$_3$ group. This was confirmed from the HMBC correlations of H-5′ with C-6,C-3′,C-4′, and from the ESI-MS/MS experiment which showed daughter ions at *m/z* 163 for methylthioribosyl moiety, while the subsequent fragment at *m/z* 97 indicated the presence of SCH$_3$ moiety. For the guanine nucleus, the presence of a hydroxyl group at C-6 instead of a keto group was confirmed from the resonance at $δ_H$ 10.57

and δ_C at 157.2 instead of a ^{13}C resonance at ca. 170 ppm for the keto amide function. The hydroxyl substituent at C-6 was also evident by the coupling of δ_H 7.5 (H-8) with δ_C 157.2 (C-6). The attachment of the ribosyl moiety with the purine unit at N-9 was confirmed by the HMBC correlations of the anomeric proton (H-1´) with C-4 and C-8. From the above mentioned results compound **30** was concluded to be [**2-(2-amino-6-hydroxy-purin-9-yl)-5´-methylsulfanylmethyl-tetrahydro-furan-2´,3´-diol**].

Stereochemistry

The observed $[\alpha]_D$ value of compound **31** is + 15.8 (*c*, 0.1 in MeOH). The stereochemistry of the ribose sugar was determined from the coupling constant of the anomeric proton (1 H, d, 5.7 Hz) which indicated the presence of β a ribosyl moiety (Tabudravu and Jaspars, 2001).

Table 3.4.3: ^{1}H, ^{13}C-NMR data and HMBC correlation of compound (30) (DMSO-d$_6$, 500 MHz)

No.	δH	δC	COSY	HMBC
1	-	-	-	-
2 (N*H$_2$*)	6.40 (s, 2H)	153.5 s	-	-
3	-	-	-	-
4	-	153.0 s	-	-
5	-	118.0 s	-	-
6	10.57 (br s)	157.5 s	-	-
7	-	-	-	-
8	7.90 (s)	135.0 d	-	1´, 4, 5,
9	-	-	-	-
1´	5.70 (d, 5.7 Hz)	87.0 d	2´ (H)	4, 3´, 8
2´ (H)	4.50 (q, 5.7 Hz)	74.0 d	1´, 3´	1´, 4´
2´ (O*H*)	5.40 (d, 6.3 Hz)	-	-	1´, 3´
3´(H)	3.95 (m)	71.5 d	2´, 4´	1´
3´(O*H*)	5.20 (d, 5.1 Hz)	-	-	2´, 4´
4´ (H)	4.05 (t, 6.9 Hz)	84.0 d	3´, 5´ A, B	3´
5´ A B	2.80 (dd, 13.9, 6.9 Hz) 2.70 (dd, 13.9, 6.9 Hz)	35.0 t	5´ B, 4´	3´, 4´, 6
6´	2.05 (s, 3H)	16.0 q	5´A, 4´	5´

Hamiguanasinol (compound 30)

Chemical name: [2-(2-amino-6 hydroxy-purin- 9-yl)-5′-methylsulfanyl

methyl-tetrahydro-furan-2′, 3′-diol]

Character: yellowish white amorphous powder

Sample code: 281-300 P4   **Amount:** 2.3 mg
Molecular weight: 313 g/mol   **Molecular formula**: $C_{11}H_{15}N_5O_4S$
Biological source: Marine sponge *Hamigera hamigera*
$[\alpha]_D$: + 15.8 (*c*, 0.1 in MeOH)

New natural product
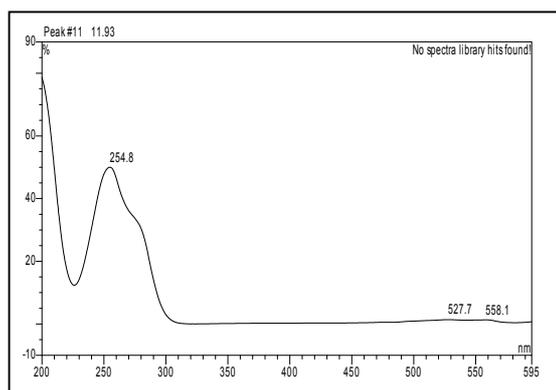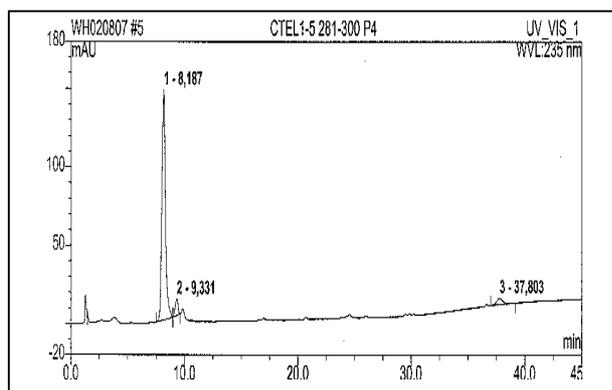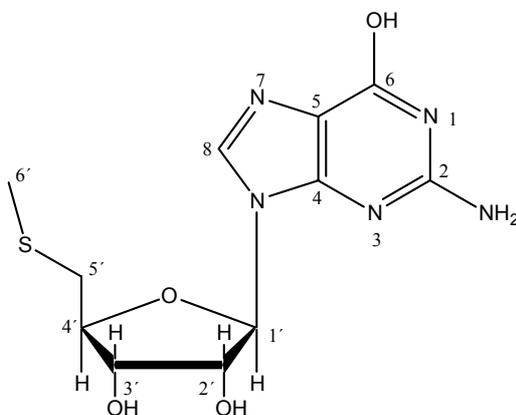
ESIMS: *m/z*, 314 [M+H]$^+$, 627 [2M+H]$^+$, 940 [3M+H]$^+$ (positive)

Fig. 3.4.11: ESIMS spectrum of compound 30

Fig. 3.4.12 : ¹H-NMR spectrum of compound 30

Fig. 3.4. 13 : Important HMBC correlations of compound 30

161

Fig. 3.4.14: COSY spectrum of compound 30
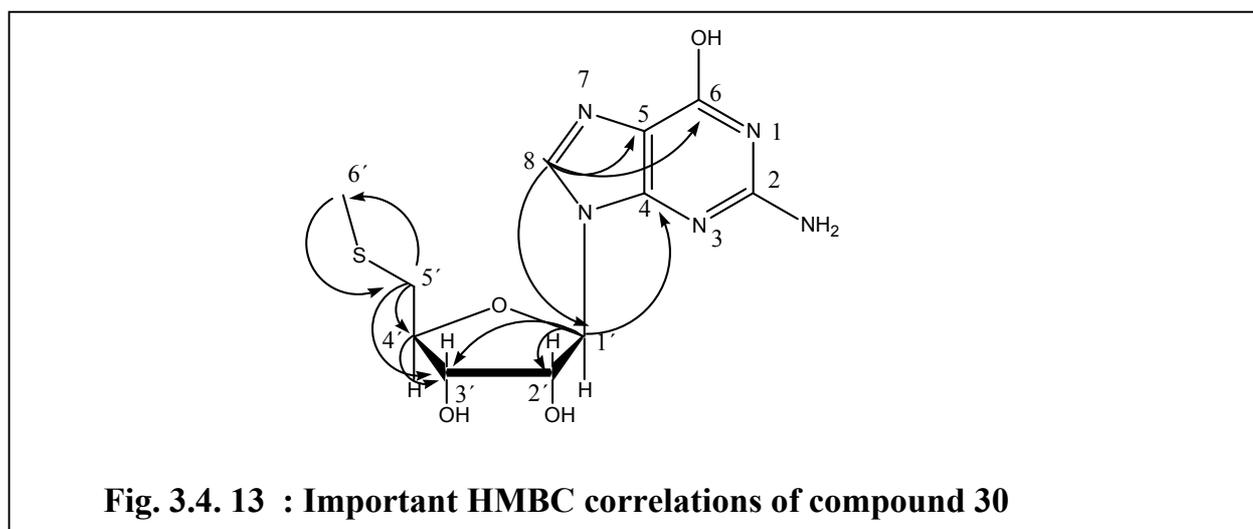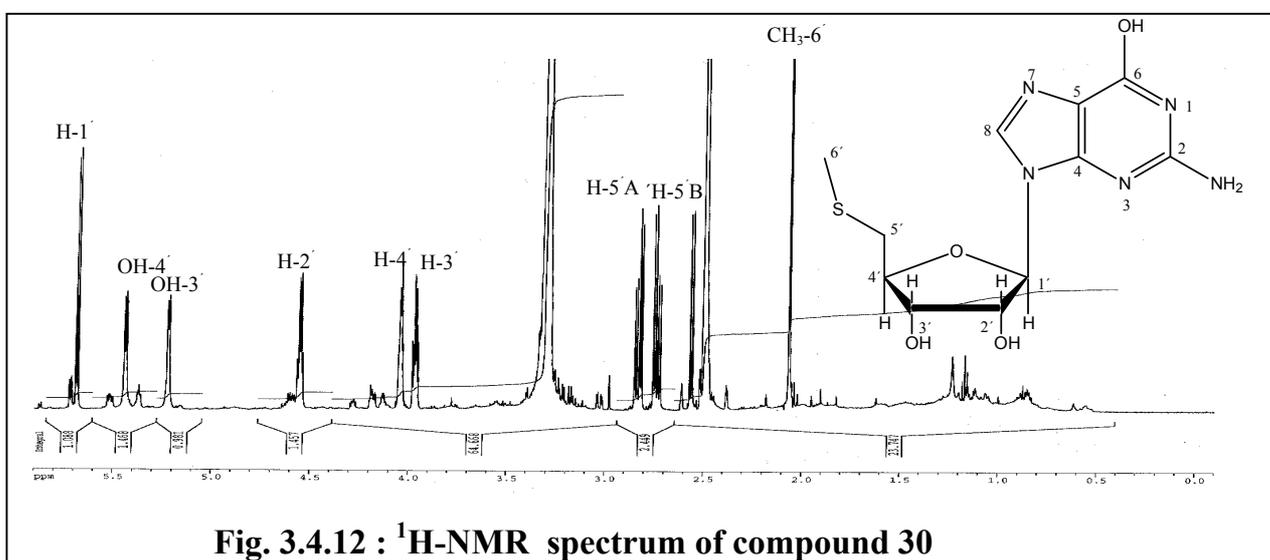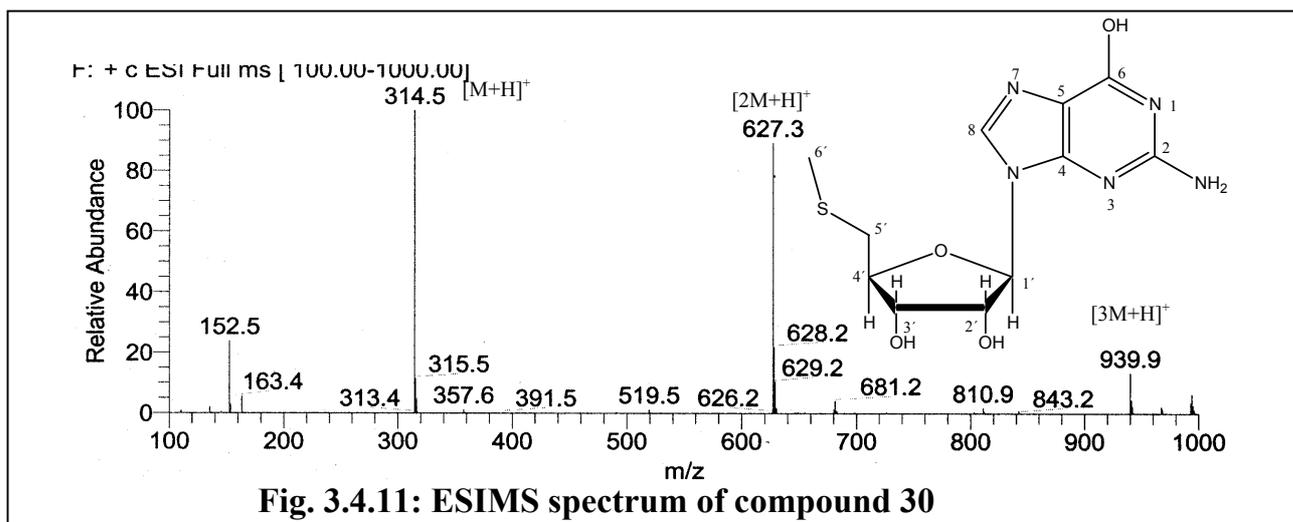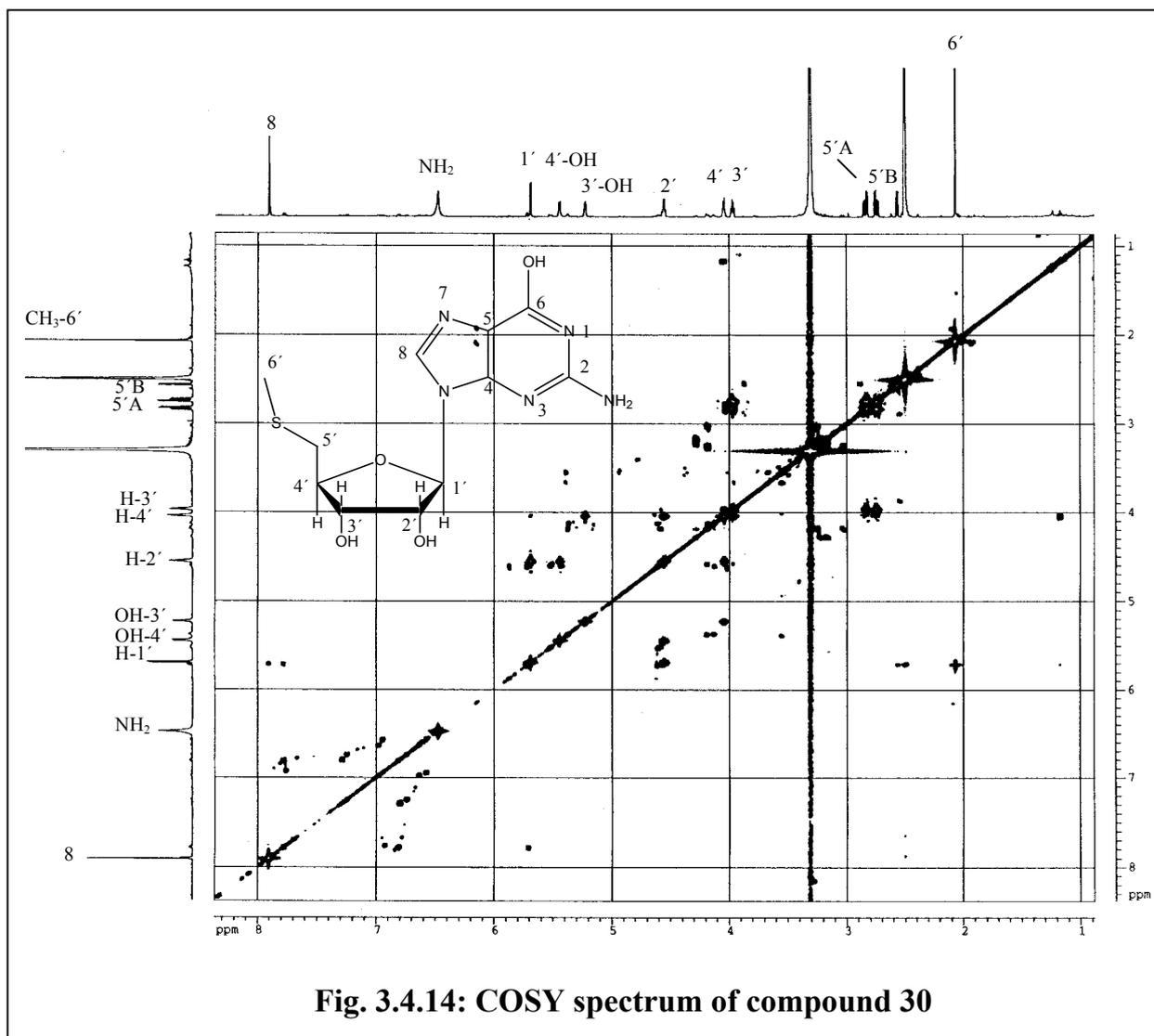
3.4.1.4. Structure elucidation of 22-dehydrocampesterol (31, known compound)

Compound 31 was isolated as a white crystalline powder. The UV spectrum (MeOH) (Fig. 3.4.15) showed absorbances bands at λ_{max} 207 and very weak shoulders at 271.6, 281.5 and 291.9 nm. The EIMS showed signals at m/z [M]$^+$ (C$_{28}$H$_{46}$O); 398 (69), 386 (26), 369 (6), 355 (4.5), 337 (6.2), 314 (41.2), 300 (30.2), 273 (14.5), 271 (34.3), 255 (32.0), 213 (14.6), 199 (7.2), 159 (24.4), 125 (22.79), 107 (36.0), 69 (100) and 43 (59.5).

22-Dehydrocampesterol
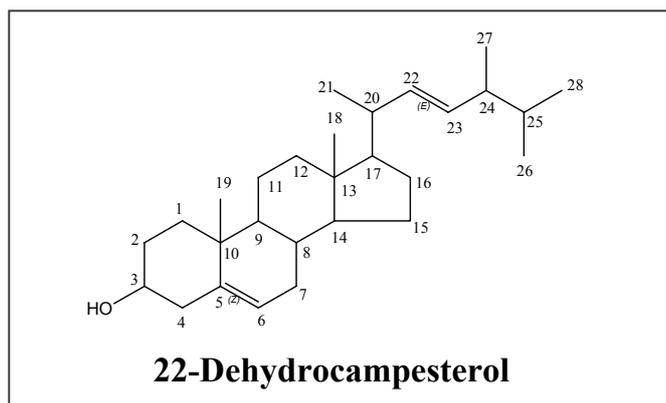
Compound 31 showed the molecular ion peak at m/z 398 in the EIMS (Fig. 3.4.18) which was compatible with the molecular composition of $C_{28}H_{46}O$. Fragments at m/z 355 $[M-C_3H_7]^+$, and *m/z* 337 $[M-C_3H_7-H_2O]^+$ due to loss of a isopropyl group and one molecule of water, as well as the two ion fragments at *m/z* 271 $[M–C_9H_{19}]^+$ and *m/z* 273 $[M–C_9H_{17}]^+$, due to loss of side chain with or without transfer of two protons and the fragment at *m/z* 255 $[M-C_9H_{17}-H_2O]^+$ suggested the presence of mono-hydroxylated steroid compound with a mono-unsaturated side chain. The presence of a stong peak in the EIMS at *m/z* 125 confirmed the presence of the C_9H_{17} side chain. The ^{1}H-NMR (Fig. 3.4.19, Table 3.4.4) showed resonances for six methyl groups at δ 0.67 (s), 0.99 (s), 1.02 (d), 0.79 (d), 0.89 (d) and 0.82 (d) which were assigned to the methyl groups 18, 19, 21, 26, 27, and 28, respectively. The resonances at $δ_H$ 3.50 (1H, m) and 5.33 (1H, t, *J* = 5.2 Hz) assigned for H-3 and H-6, respectively, were indicative for $Δ^{5-6}$ mono-hydroxylated steroidal nucleus (Itoh *et al*, 1983), the other resonance at $δ_H$ 5.18 (2H, m) indicated that it has a $Δ^{22-23}$ mono-unsaturated side chain (Itoh *et al*, 1983). The ^{13}C-NMR spectrum of compound **31** (Fig. 3.4.20), showed six methyl signals at $δ_C$ 12.1, 20.4, 21.0, 19.4, 18.1, and 19.7 for the methyl groups 18, 19, 21, 26, 27, and 28 respectively, in addition to resonances for ten methines, nine methylenes and three quaternary carbons. The structure of the two parts, the steroidal nucleus and the side chain, of compound **31** were established from the CH long range spectrum (Fig. 3.4.21), which showed correlations between H-28 and C-24, C-25, C-26; H-26 and C-24, C-25, C-28; H-27 and C-23, C-24, as well as, between H-22 and C-23; H-23 and C-22; plus the correlation between H-21 and C-22. All of these data revealed the presence of a $Δ^{22}$ mono-unsaturated side chain. The other CH long range

correlations between H-18 and C-12, C-13, C-14, C-17; H-19 and C-1, C-5, C-9, C-10; H-6 and C-4, C-5, C-7, as well as the correlations between H-3 and C-4; H-1 and C-3 were indicative for Δ^{5-6} mono-hydroxylated steroid nucleus. All the ^{1}H and ^{13}C-NMR resonances were assigned by careful inspection of the COSY, HMQC, and HMBC spectra and by the comparison with the previously reported steroid 22-dehydrocampesterol isolated from *Phaeodactylum tricornutum*. Regarding the stereochemistry of compound **31**, the configuration at C-24 can not be determined because the reported difference in the chemical shift of C-24 in ^{13}C-NMR between *S* and *R* epimers is very small (Wright *et al*, 1978, Shirane *et al*, 1996, Anjaneyulu *et al*, 1995).
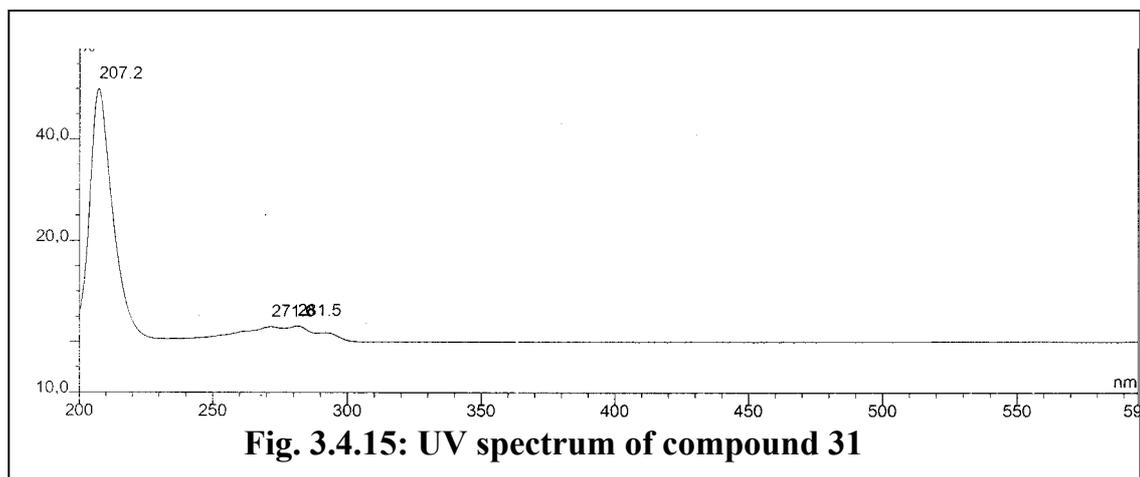
Fig. 3.4.15: UV spectrum of compound 31

3.4.1.5. Structure elucidation of 27-nor-22-dehydrocampesterol (32, known compound)

Compound 32 was isolated as a white amorphous powder; the UV absorbance was identical to that of compound **31**. The EIMS spectrum of **32** (Fig. 3.4.23) showed the molecular ion peak at *m/z* [M]$^+$ 384 (90 %) corresponding to the molecular formula $C_{27}H_{44}O$; in addition to the other fragments at *m/z* 369 ($C_{26}H_{41}O$, 28%), 351 ($C_{26}H_{39}$, 13 %), 300 ($C_{21}H_{32}O$, 45%), 273 ($C_{19}H_{29}O$, 22.2), 271 ($C_{19}H_{27}O$, 75%), 255 ($C_{19}H_{27}$, 33%), 246 ($C_{17}H_{26}O$, 3%), and 213 ($C_{16}H_{21}$, 15%). The ^{1}H-NMR spectrum (Fig. 3.4.24) and the ^{13}C-NMR data (Table 3.4.4) of compound **32** showed that it was closely related to compound **31**. Detailed comparison of the ^{1}H, ^{13}C-NMR and COSY

data of the two compounds showed that the major difference between the two compounds is the disappearance of the doublet signal at 0.89 ppm of methyl-27 of compound **31**. This was confirmed from the absence of the peak at δ 18.1 in the ^{13}C-NMR data (Table 3.4.4). The ^{13}C-NMR data showed resonances for 27 carbons, five methyls, nine methylene, ten methine and three quaternary carbons and this is compatible with the molecular weight of compound **32**. The presence of ^{1}H-NMR signals at δ_H 0.80 (6 H, d, 6.7 Hz), as well as the presence of a strong ion fragment at m/z 43 (45 %) in EIMS spectrum confirmed the terminal isopropyl group and gave further evidence for the   loss of methyl 27. The presence of the C_8H_{15} mono-unsaturated side chain was established from the EIMS fragments at *m/z* 271 [M-C_8H_{17}]$^+$ and *m/z* 273 [M-C_8H_{15}]$^+$, due to the loss of the side chain (C_8H_{15}) with or without transfer of two protons. Another evidence for the presence of C_8H_{15} side chain was the presence of fragment at *m/z* 111 (38%) in the EIMS spectrum. The ^{1}H and ^{13}C-NMR data of compound **32** (Table 3.4.4) agreed with those of **27-nor 22-dehydrocampesterol** (Wright *et al*, 1978, Shirane *et al*, 1996, Anjaneyulu *et al*, 1995).

27-Nor 22-dehydrocampesterol
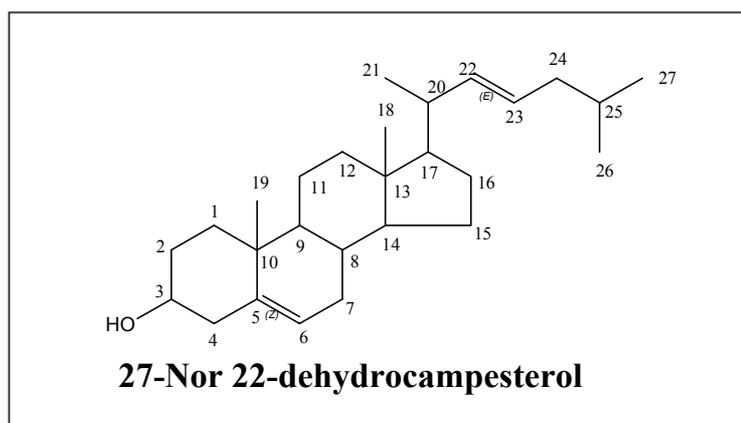
3.4.1.6. Structure elucidation of 24-methylcholesta-5,22-dien-3β-ol (33, known compound)

Compound **33** was isolated as a white amorphous powder. The EIMS (Fig. 3.4.25) showed the molecular ion peak at *m/z* [M]$^+$ 384 (85) corresponding to the molecular formula $C_{27}H_{44}O$; in addition to other fragments at *m/z* 369 ($C_{26}H_{41}O$, 24%), 351 ($C_{26}H_{39}$, 8%), 271 ($C_{19}H_{27}O$, 35%), 255 ($C_{19}H_{27}$, 48%), 246 ($C_{17}H_{26}O$, 3%) 213 ($C_{16}H_{21}$, 10%), and 111 (C_8H_{15}, 43%). The ^{1}H, ^{13}C-NMR and COSY spectra of compound **33** revealed that it was closely related to compound **31**. The ^{1}H-NMR

spectrum of compound **33** (Fig. 3.4.26) showed the absence of the methyl signal at 0.79 ppm and the presence of a triplet signal at 0.83 ppm (3H, t, 7.5 Hz). The ^{13}C-NMR and DEPT spectra showed resonances for 27 carbons, five methyls, nine methylenes, ten methines and three quaternary, and this was compatible with the molecular formula $C_{27}H_{44}O$. Detailed comparison of the ^{13}C-NMR data with those of compound **31** established that the two compounds were completely identical in the nucleus. The appearance of a triplet methyl signal in the ^{1}H-NMR at 0.83 ppm ($J =$ 7.5 Hz) which coupled in the COSY spectrum with CH_2-25 at 1.2 ppm indicated the presence of a terminal CH_2-CH_3 group. Another evidence for the presence of a terminal CH$_2$-CH$_3$ was the upfield shift of C-26 in ^{13}C-NMR at 11.9 ppm in **33** instead of 19.9 in **31**. These signals revealed the presence of a terminal CH$_2$-CH$_3$ moiety in the side chain of compound **33** instead of an isopropyl in compound **31**. The structure of this compound was established as **24-methylcholesta-5, 22-dien-3β-ol** by inspection of ^{1}H, ^{13}C-NMR and COSY data and by comparison with the literature (Kobayashi and Mitsuhashi, 1974, and Dauria *et al*, 1992).

24-Methylcholesta-5, 22-dien-3β-ol
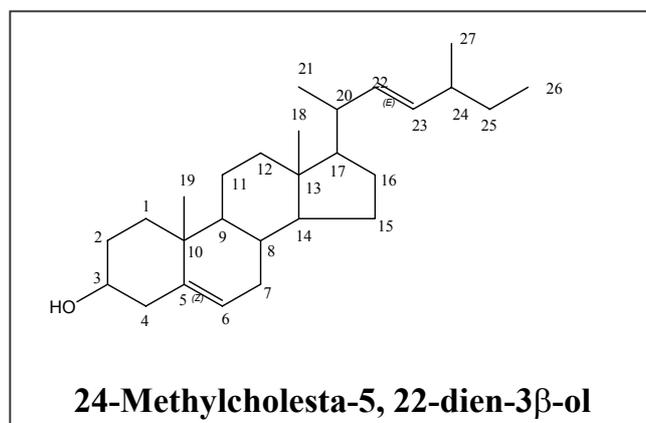
Table 3.4.4: ^{1}H, ^{13}C-NMR data for compounds 32-34 in (CDCl$_3$, 500, 400 MHz)

No.	32		33		34	
	δH	δC	δH	δC	δH	δC
1	1.85, 1.10 (m)	37.3 t	1.00-2.20 (m)	37.3 t	1.50, 1.10 (m)	37.3 t
2	1.83, 1.48 (m)	31.7 t	1.00-2.20 (m)	31.8 t	1.85, 1.50 (m)	31.7 t
3	3.50 (m)	71.9 d	3.50 (m)	71.79 d	3.50 (m)	71.8 d
4	2.30, 2.22 (m)	42.3 t	1.00-2.20 (m)	42.3 t	2.25 (m)	42.4 t
5	-	141.0 s	-	140.7 s	-	140.8 s
6	5.33 (t, 5.2)	121.7 d	5.40 (m)	121.7 d	5.28 (m)	121.7 d
7	2.20, 1.90 (m)	31.9 t	1.00-2.20 (m)	31.6 t	2.30, 2.00 (m)	32.0 t
8	1.25 (m)	29.8 d	1.00-2.20 (m)	31.6 d	1.52 (m)	31.9 d
9	0.92 (m)	50.2 d	1.00-2.20 (m)	50.1 d	1.00-2.00 (m)	50.2 d
10	-	36.6 s	-	36.1 s	-	36.6 s
11	1.45 (m)	21.15 t	1.00-2.20 (m)	21.0 t	1.00-2.00 (m)	21.1 t
12	2.00, 1.50 (m)	39.7 t	1.00-2.20 (m)	38.5 t	1-2.00 (m)	40.1 t
13	-	43.10 s	-	41.9 s	-	42.3 s
14	1.10 (m)	56.4 d	1.00-2.20 (m)	56.8 d	1.00-2.00 (m)	56.0 d
15	1.05, 1.53 (m)	24.4 t	1.00-2.20 (m)	24.7 t	1.00-2.00 (m)	24.3 t
16	-	28.8 t	1.00-2.20 (m)	28.6 t	1.00-2.00 (m)	28.7 t
17	1.12 (m)	56.2 d	1.00-2.20 (m)	55.9 d	1.00-2.00 (m)	56.0 d
18	0.67 (s)	12.1 q	0.68 (s)	12.0 q	0.69 (s)	12.1 q
19	0.99 (s)	20.4 q	1.05 (s)	19.4 q	1.10 (s)	19.4 q
20	2.00 (m)	40.1 d	1.00-2.20 (m)	39.7 s	1.00-2.00 (m)	40.4 d
21	1.02 (d, 6.9 Hz)	21.0 q	1.10 (d, 6.9)	20.8 q	1.10 (d, 6.9)	21.0 q
22	5.18 (m)	136.1 d	5.20 (m)	139.7 d	5.10 (m)	135.5 d
23	5.18 (m)	131.9 d	5.20 (m)	125.2 d	5.10 (m)	133.5 d
24	2.25 (m)	43.1 d	1.00-2.20 (m)	41.9 t	2.00 (m)	38.5 d
25	1.48 (m)	33.3 d	1.00-2.20 (m)	28.6 d	1.20 (m)	32.0 t
26	0.79 (d, 6.6 Hz)	19.4 q	0.80 (d, 6.7 Hz)	22.3 q	0.83 (t, 7.5 Hz)	11.9 q
27	0.89 (d, 6.9 Hz)	18.1 q	0.80 (d, 6.7 Hz)	22.60 q	0.93 (d, 6.9 Hz)	20.9 q
28	0.82 (d, 6.6 Hz)	19.7 q	-	-	-	-
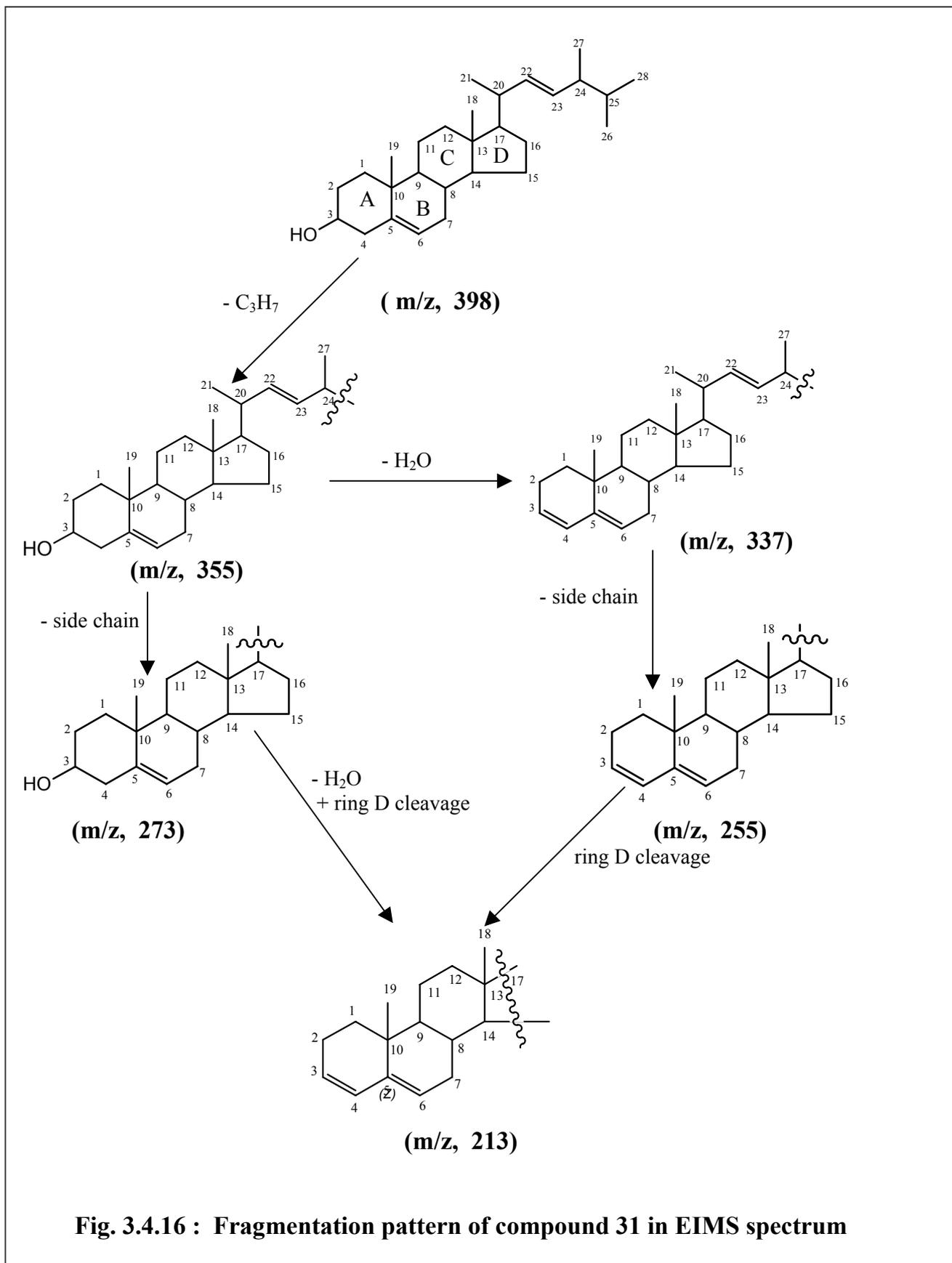
Fig. 3.4.16 : Fragmentation pattern of compound 31 in EIMS spectrum
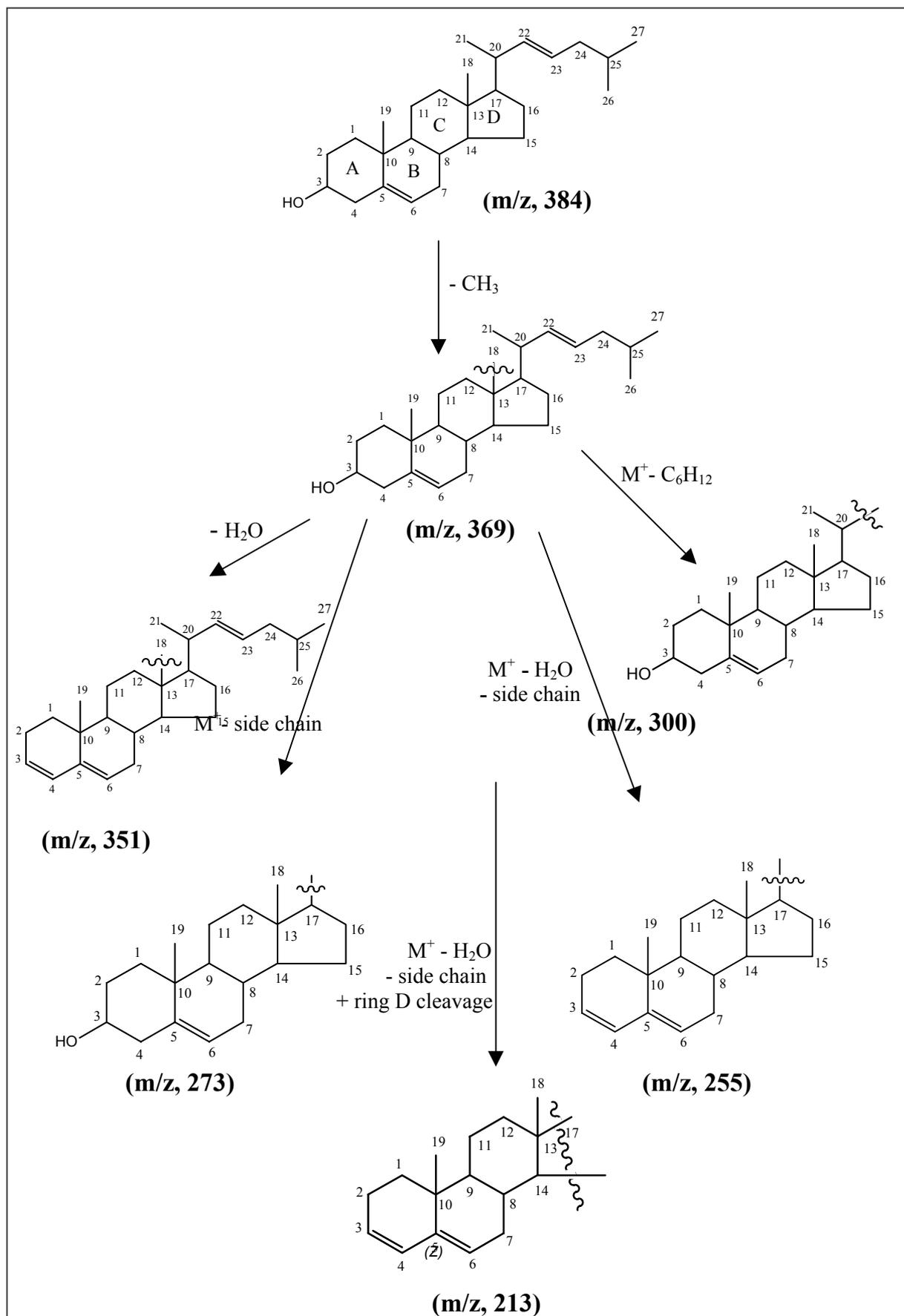
Fig. 3.4.17 : Fragmentation pattern of compound 32 in EIMS spectrum
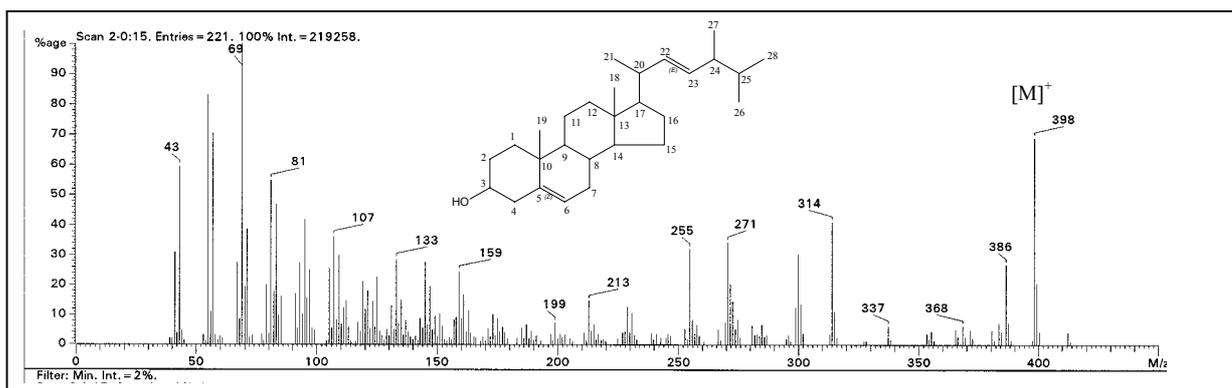
Fig. 3.4.18: EIMS spectrum of compound 31
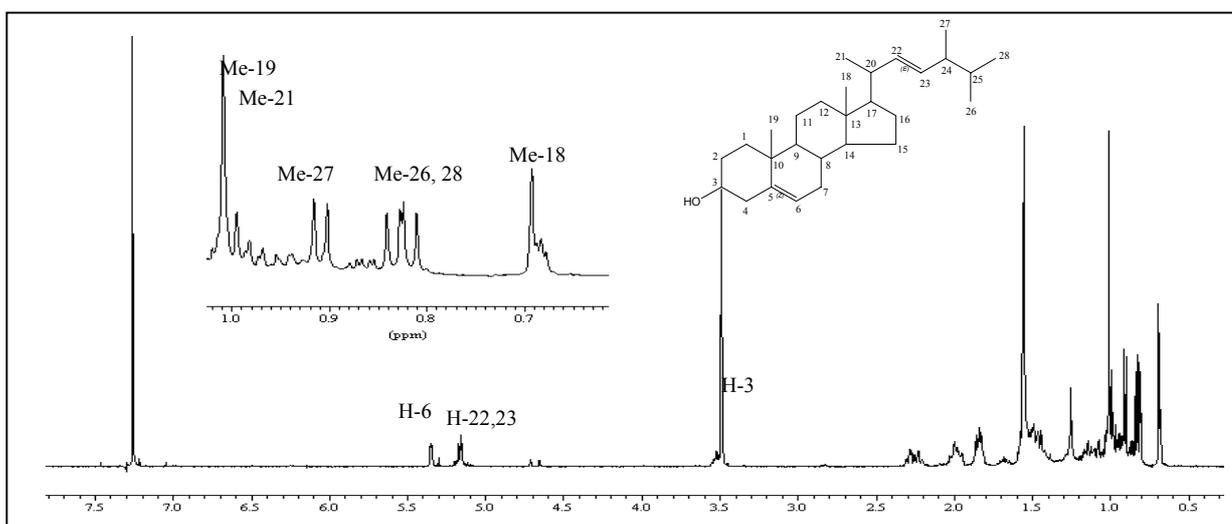
Fig. 3.4.19: ^{1}H-NMR spectrum of compound 31
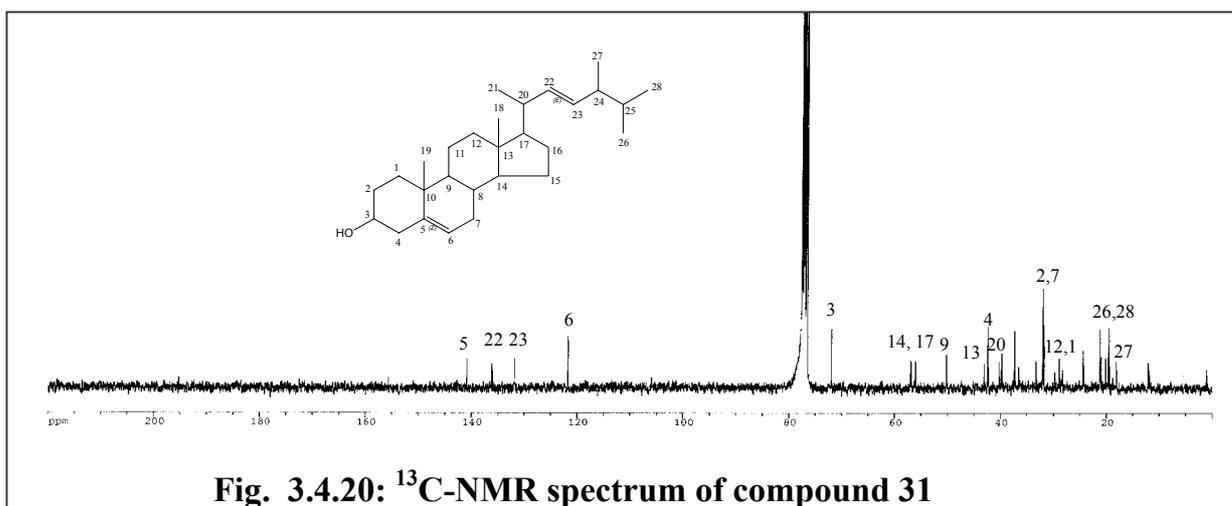
Fig. 3.4.20: ^{13}C-NMR spectrum of compound 31

Fig. 3.4.21: Important HMBC (A) and COSY (B) correlations of compound 31

Fig. 3.4.22: HMBC spectrum of compound 31
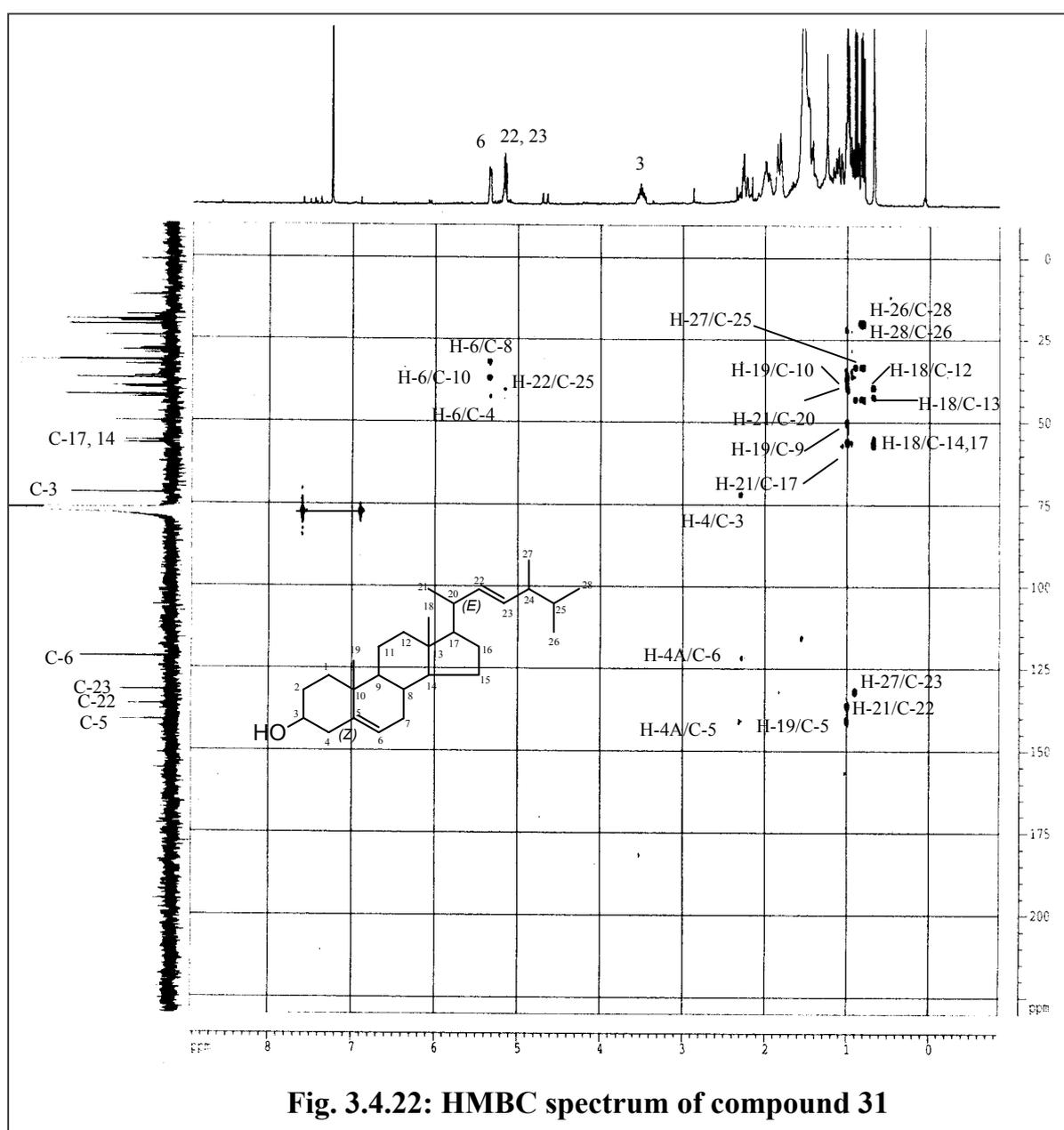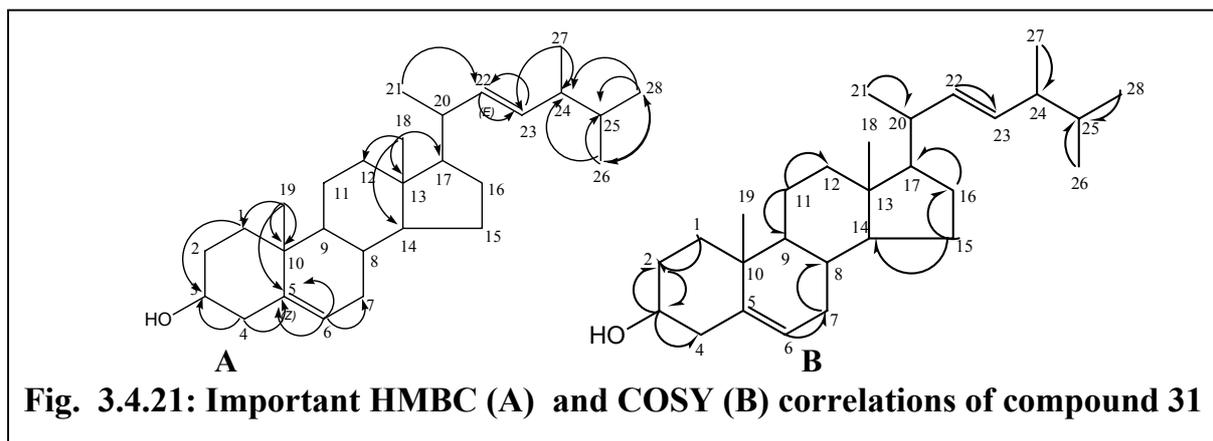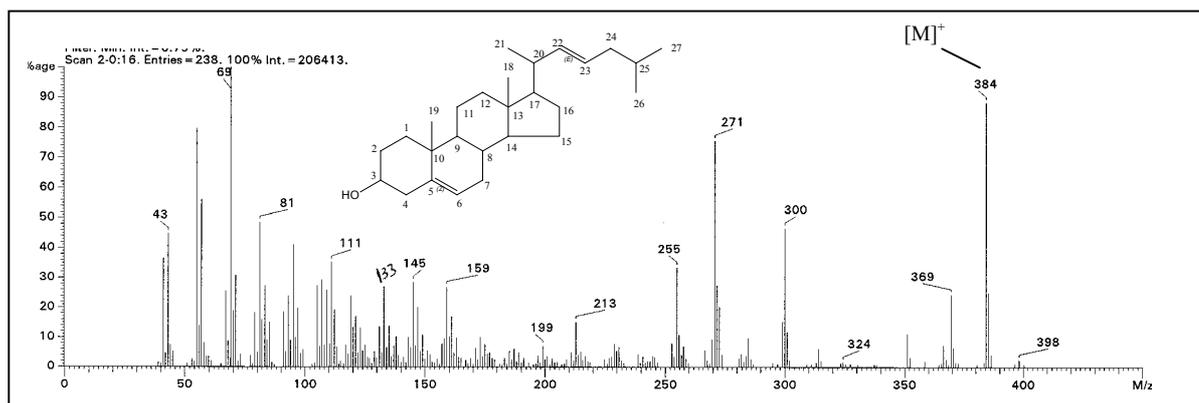
Fig. 3.4.23 : EIMS spectrum of compound 32
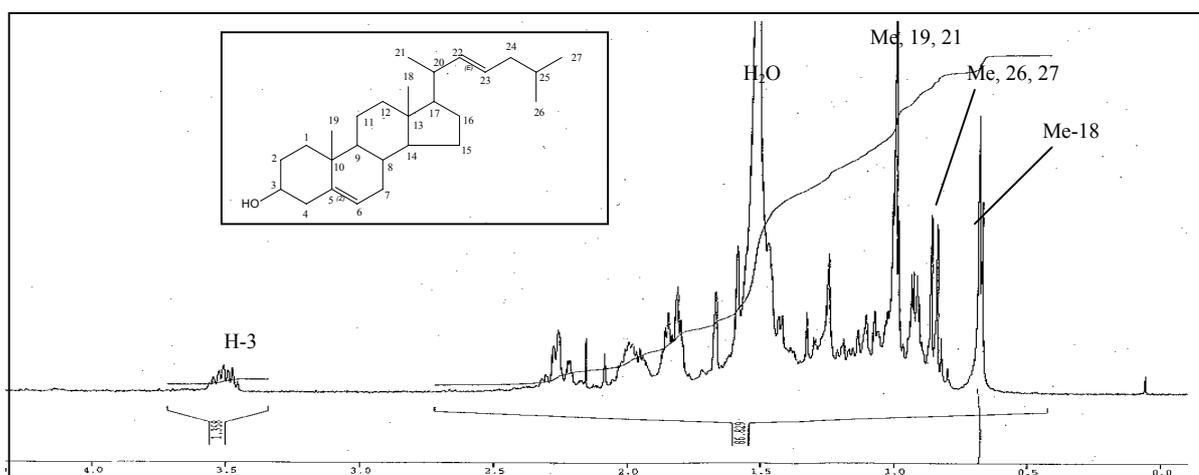
Fig. 3.4.24 : ¹H -NMR spectrum of compound 32
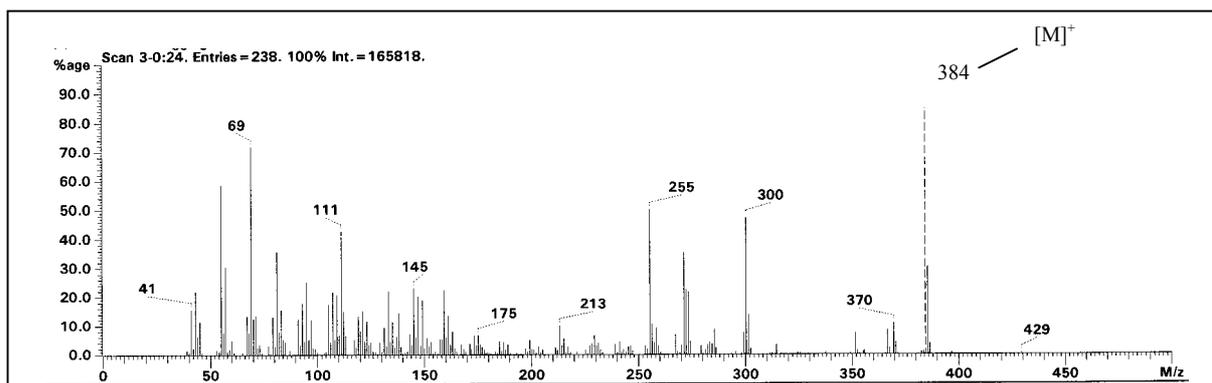
Fig. 3.4.25: EIMS spectrum of compound 33
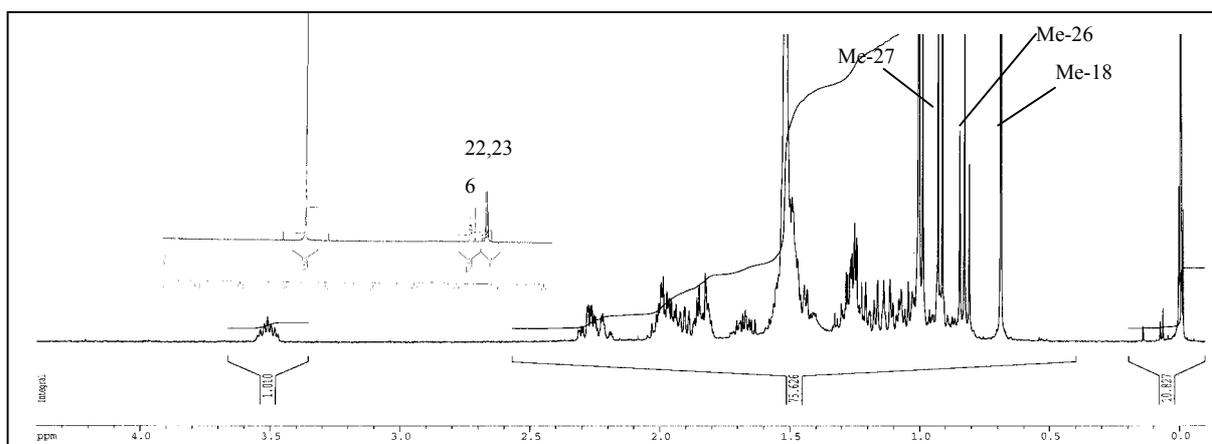
Fig. 3.4.26: ^{1}H-NMR spectrum of compound 33
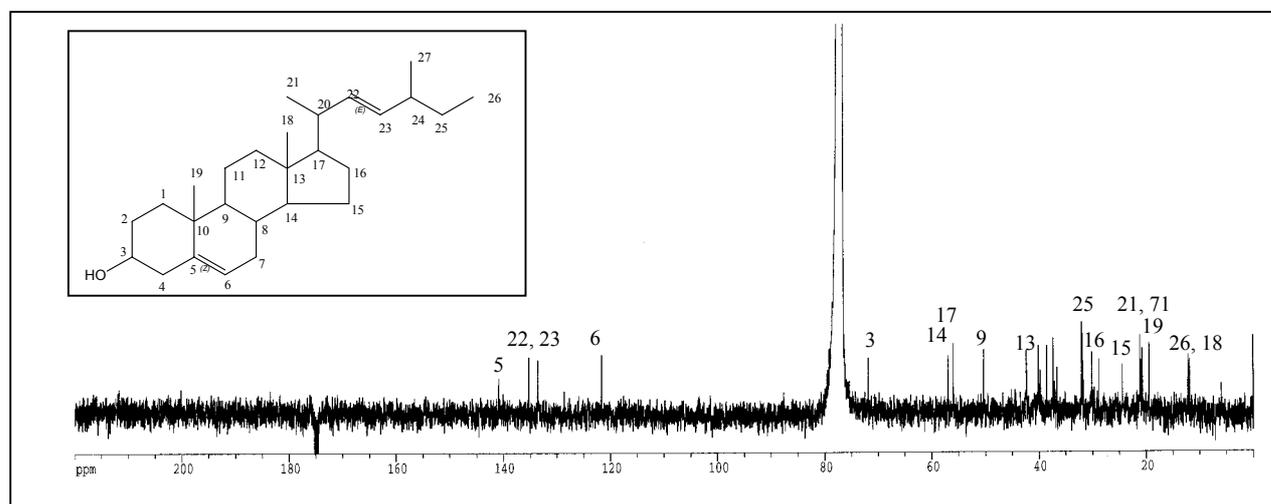
Fig. 3.4.27: ^{13}C-NMR spectrum of compound 33

3.4.1.7. Structure elucidation of indole-3-carboxyaldehyde (34, known compound)

Indole-3-carboxyaldehyde
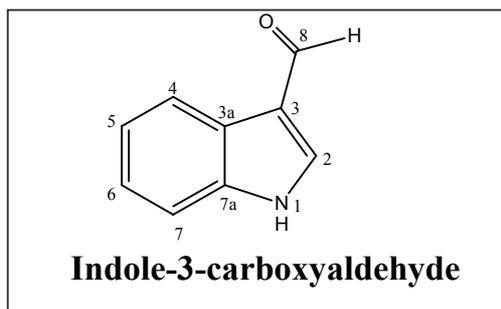
Compound 34 was isolated as a yellowish white amorphous powder. It has UV absorbances at λ_{max} 206, 243 and 298 nm. The ESIMS spectrum showed the pseudo-molecular ion peak at *m/z* 146 [M+H]$^+$. The ^{1}H-NMR spectrum (Fig. 3.4.28) data (Table 3.3.5) showed signals at δ_H 8.07 (1H, d, *J* = 7.4 Hz), 7.10 (1H, t, *J* = 7.4 Hz), 7.30 (1H, t, *J* = 7.4 Hz), and 7.60 (1H, d, *J* = 7.4 Hz), indicative for an ABCD aromatic spin system. This revealed the presence of an *ortho*-disubstituted benzene ring. The other signal at δ_H 8.30 (1H, s) taken together with the above mentioned signals suggested the presence of 3-substituted indole. The signal at δ_H 9.95 (1H, s) which was not exchangeable suggested the presence of an aldehydic group. This suggestion was confirmed from HMQC data (Table 3.4.5) which showed the direct correlation of H-8 with C-8 at δ_C 175.0 and from the HMBC spectrum (Fig. 3.4.29) which showed the correlations of H-2 with C-3, C-3a, C-7a, in addition to the correlations of H-5 with C-3 and C-7; H-4 with C-6 and C-7, and the correlations of H-8 with C-3 and C-3a. From the above data and through the comparison with the literature (Aldrich, 1992, Hiort, 2002) it was confirmed that compound **34** was **indole-3-carboxaldehyde**.

Table 3.4.5: NMR data of compound 35 (DMSO-d_6, 500 MHz)

No.	δH	δCª	HMQC	HMBC
1	12.10 (s)	-	-	-
2	8.30 (s)	139.5 d	2	3, 3a, 7a
3	-	119.1 s	-	-
3a	-	126.5 s	-	-
4	8.07 (d, 7.4 Hz)	120.0 d	4	6, 7a
5	7.10 (dt, 7.4, 2.4 Hz)	121.0 d	5	3a, 7
6	7.30 (dt, 7.4, 2.4 Hz)	121.5 d	6	4, 7a
7	7.60 (d, 7.4 Hz)	114.1 d	7	3a, 5
7a	-	138.5 s	-	-
8	9.95 (s)	175.0 d	8	3, 3a

ª carbon assignments were determined indirectly from the HMBC spectrum
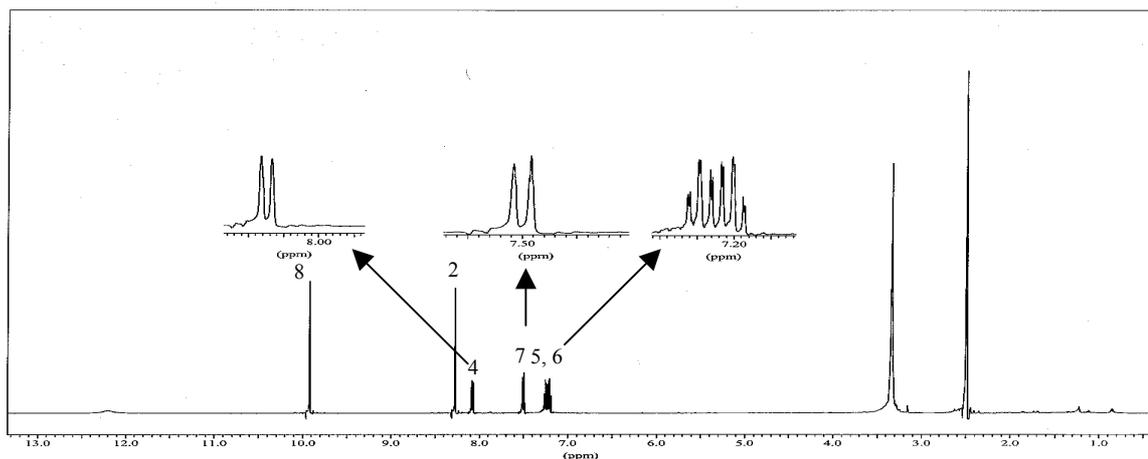
Fig.3.4.28: ¹ H-NMR spectrum of compound 34
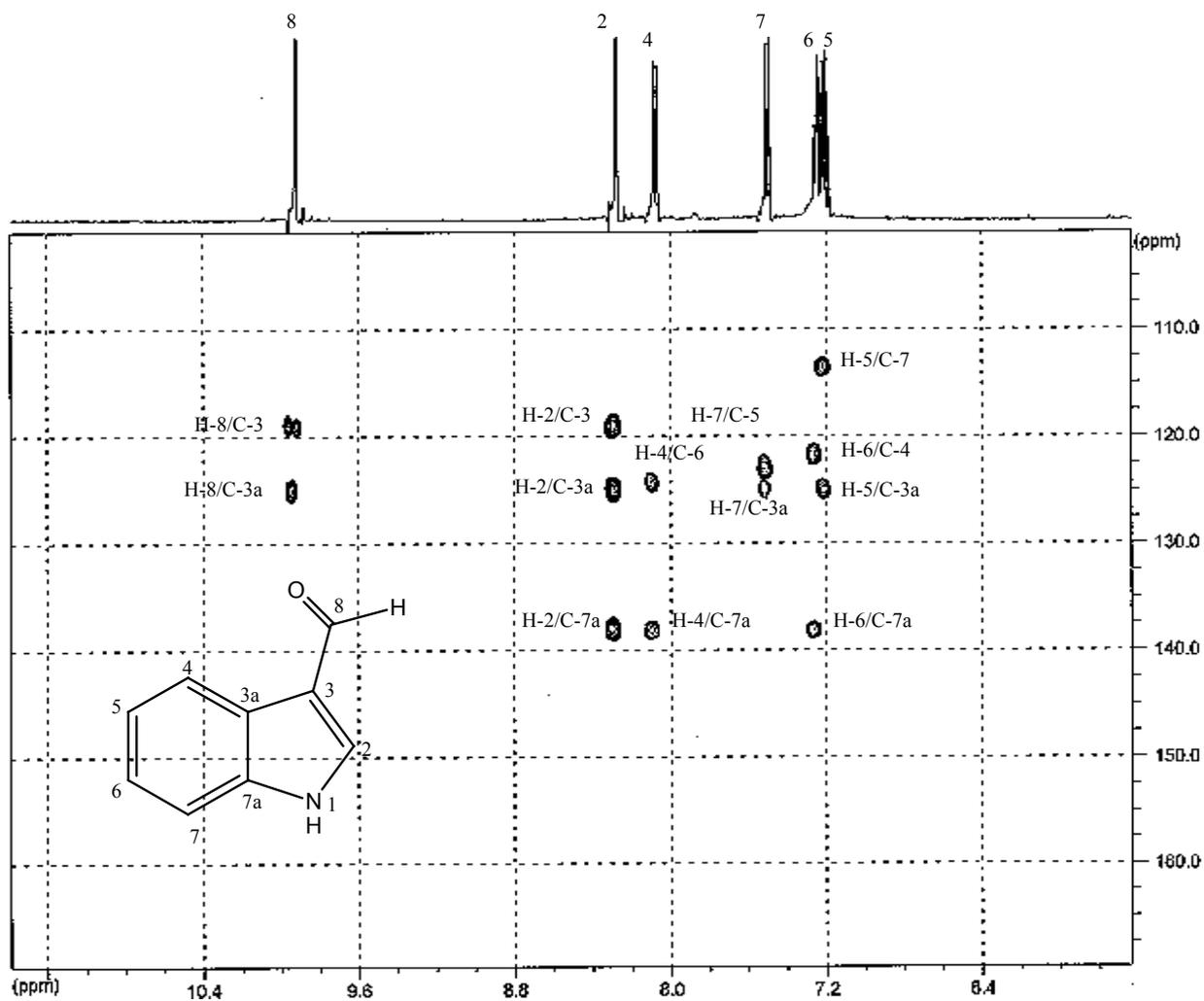
Fig. 3.4.29: HMBC spectrum of compound 34

3.4.1.8. Structure elucidation of hamigeramide (35, new compound)

Hamigeramide **35** was isolated as a yellowish white powder from the biologically active (fish feeding experiment) ethyl acetate extract. The ESIMS spectrum showed the pseudo-molecular ion peak at *m/z* 230 [M+H]$^+$ in accordance with the molecular formula $C_{12}H_{11}N_3O_2$. The ^{1}H-NMR spectrum (Fig. 3.4.31) was very similar to that of 3-indoleacrylamide and its acid congener (Davyt *et al*, 1998) except for the difference in resonances observed for the 1,2-*trans*-disubistituted olefinic protons. The carbon resonances for **35** were obtained through the HMBC spectrum and significant differences with the known compounds were also observed for the olefinic carbons. The proton resonances for the olefinic bonds of indoleacrylamide and its acid congener were observed ca. 6.80 and 8.00 ppm while their carbon shifts were detected at ca. 115 and 138 ppm. The same chemical shifts were also observed for the bis indole alkaloids, chondriamides, isolated from the red alga *Chondria atropurpurea* (Palermo *et al*, 1992, Davyt *et al*, 1998). The functionality common to both the chondriamides and indolacrylamide is the presence of a carbonyl moiety adjacent to the *trans* olefinic bond. The proton resonances for the olefinic bond of **35** were observed at 7.18 and 7.10 ppm while their carbon shifts were detected at 118.2 and 117.3 ppm which suggested that **35** had a unique terminal functionality. Since the ^{1}H-NMR and ^{13}C-NMR chemical shifts of the olefinic protons were relatively comparable to those of hamigramine (**29**), this could suggest the presence of an imino amino moiety adjacent to it. Due to the very small amount of hamigeramide isolated, it was not possible to obtain a ^{13}C-NMR spectrum to prove the presence of imino group. The HMBC spectra also did not show any discernible correlation between any olefinic protons to the expected imino carbon chemical shift at ca. 155 ppm. From the HMBC correlations (Fig. 3.4.32) the presence of a carbonyl amide was determined by the correlation between the N*H* resonance at 9.00 ppm and carbon resonance at 173.0 ppm. Through MS/MS experiments by ion trap ESIMS, the presence of the imino amino moiety and its connectivity were corroborated. ESI (+) fragmentation of the [M+H]$^+$ ion at *m/z* 230.21 gave a major daughter ion peak at m/z 186.1 [M-COOH +H]$^+$ which indicated the loss of the terminal acid unit. This was confirmed by the ESI (-) fragmentation of the [M-H]$^+$ ion at *m/z* 228.62

which gave a major daughter ion peak at *m/z* 184.1 [M-COOH-H]$^+$. The daughter ion peak at *m/z* 169.3 with a relative abundance of 50% was also observed upon ESI (+) fragmentation of [M+H]$^+$ ion at *m/z* 230.21 which indicated the subsequent loss of an NH_3 unit. A daughter ion peak at *m/z* 142.1 suggested the sequential loss of [C=NH]$^+$. The daughter ion peak at *m/z* 169.3 also indicated the absence of an enamide unit as in **28** and **29** which further suggested that the imino unit was adjacent to the 1,2- *trans* disubistituted double bond. This accounted for the relatively sharp doublet resonance of both the olefinic protons in the ^{1}H-NMR spectrum of **35** when compared to those of **29**. The fragmentation pattern suggested the presence of an imino carbamic acid terminal moiety and thus hamigeramide was elucidated as **1-imino-3-(1H-indol-3-yl)-allyl) carbamic acid**.

Table 3.4.6: NMR data of compound 35 (DMSO-d_6, 500 MHz)

No.	δH	δC^a	HMBC
1	11.20 (br s)	-	-
2	7.50 (s)	127.3 d	3, 3a, 7a
3	-	111.2 s	-
3a	-	124.5 s	-
4	7.90 (d, *J*= 7.8 Hz)	120.1 d	6, 3a, 7a
5	7.08 (t, *J*= 7.8 Hz)	119.0 d	7, 3a
6	7.15 (t, *J*= 7.8 Hz)	121.5 d	7a
7	7.40 (d, *J*= 7.8 Hz)	112.3 d	5, 3a
7a	-	138.4 s	-
8	7.18 (d, *J*= 14.0 Hz)	118.2 d	2, 3, 9
9	7.10 (d, *J*= 14.0 Hz)	117.3 d	2, 8
10	8.95 (br s)	-	-
11	9.00 (br s)	-	12
12	-	173.0 s	-

^{a} carbon assignments were determined indirectly from the HMBC spectrum

Hamigeramide (compound 35)

Chemical name: 1-imino-3-(1H-indol-3-yl)-allyl) carbamic acid.

Character: yellowish white amorphous powder

Sample code: H3   **Amount:** 3.2 mg
Molecular Weight 229 g/mol   **Molecular formula**: $C_{12}H_{11}N_3O_2$
Biological source: Marine sponge *Hamigera hamigera*

New natural product
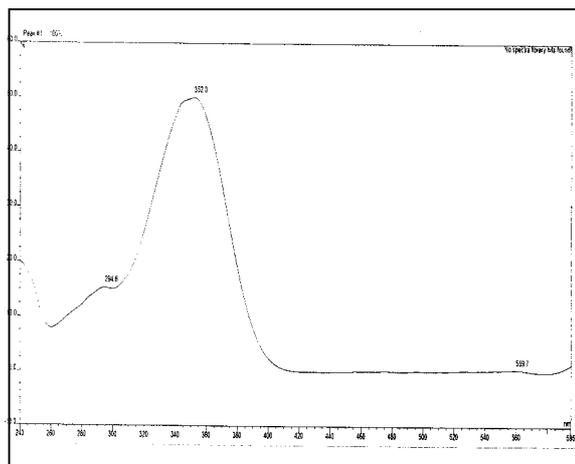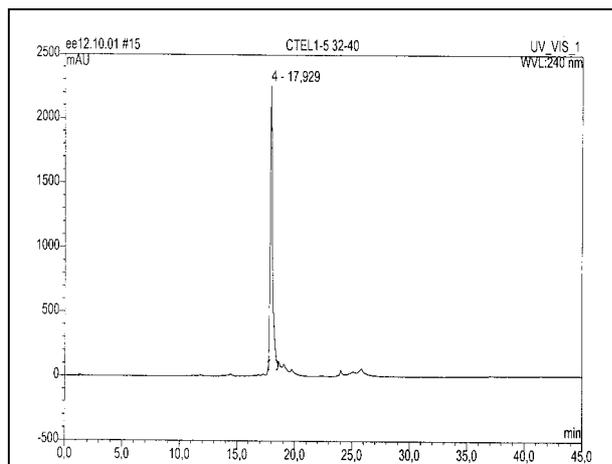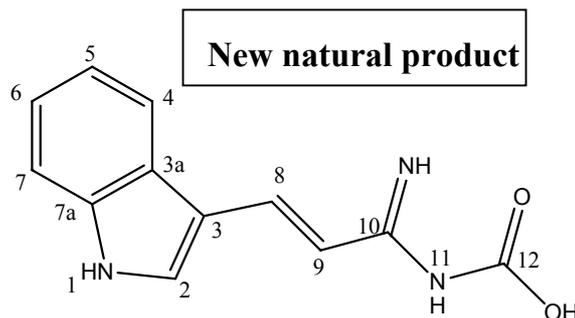
ESIMS: *m/z*, 230 [M+H]$^+$, 459 [2M+H]$^+$ (positive)
       228 [M-H]$^+$ 457 [2M-H]$^+$ (negative)
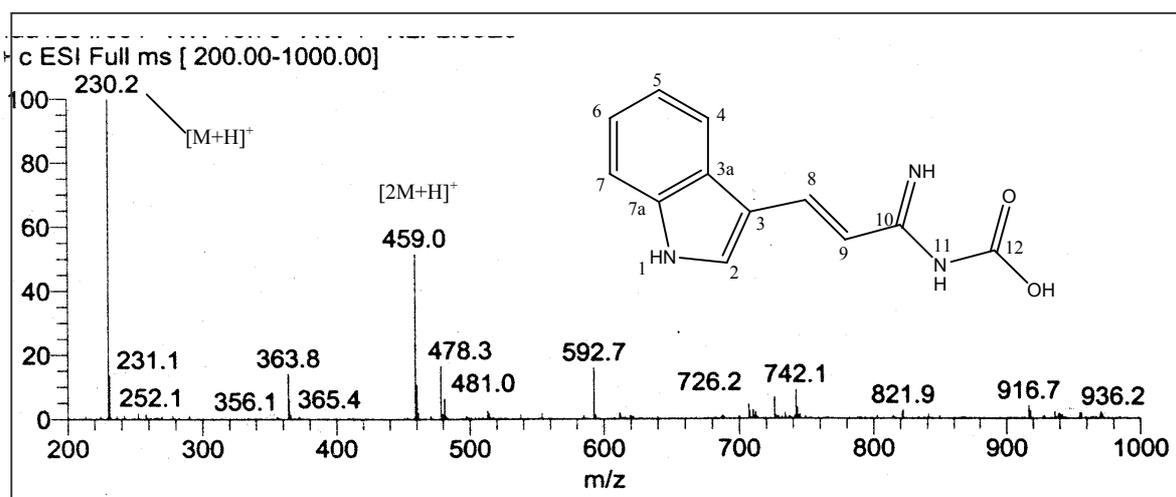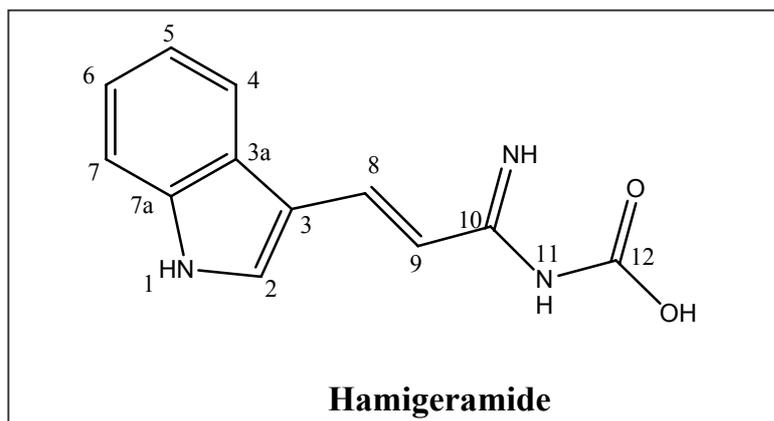
Hamigeramide

Fig. 3.4.30 A : ESIMS spectrum of compound 35

m/z 213 (rel. abund. 20%)

m/z 184, 186 (rel. abund. 100 %)
m/z 169 (rel. abund. 50 %)

m/z 142 (rel. abund. 10%)
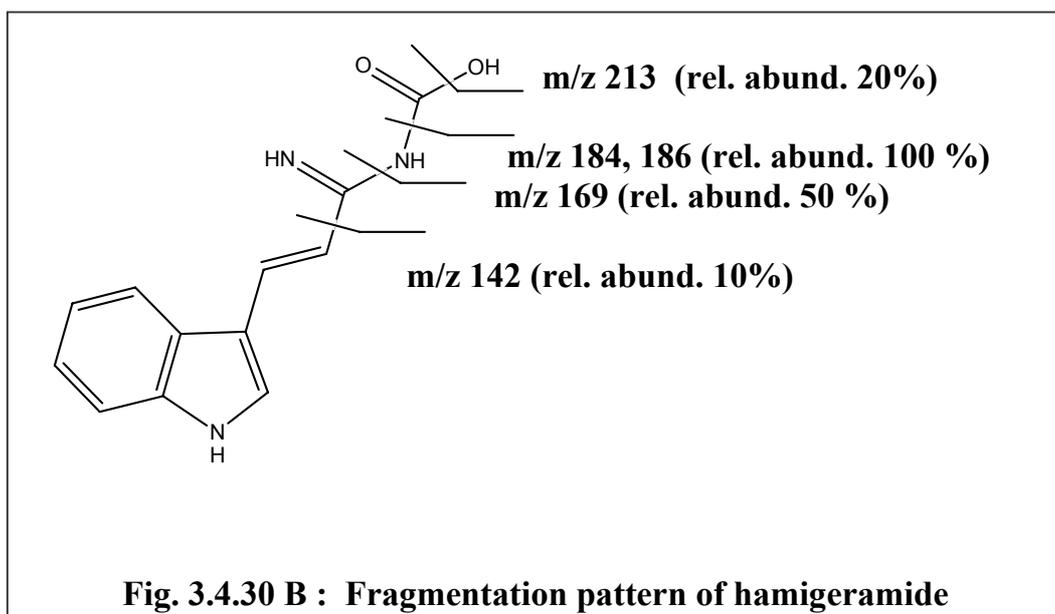
Fig. 3.4.30 B : Fragmentation pattern of hamigeramide

Fig. 3.4.31: ¹H-NMR spectrum of compound 35

Fig. 3.4.32: Important HMBC correlations of compound 35
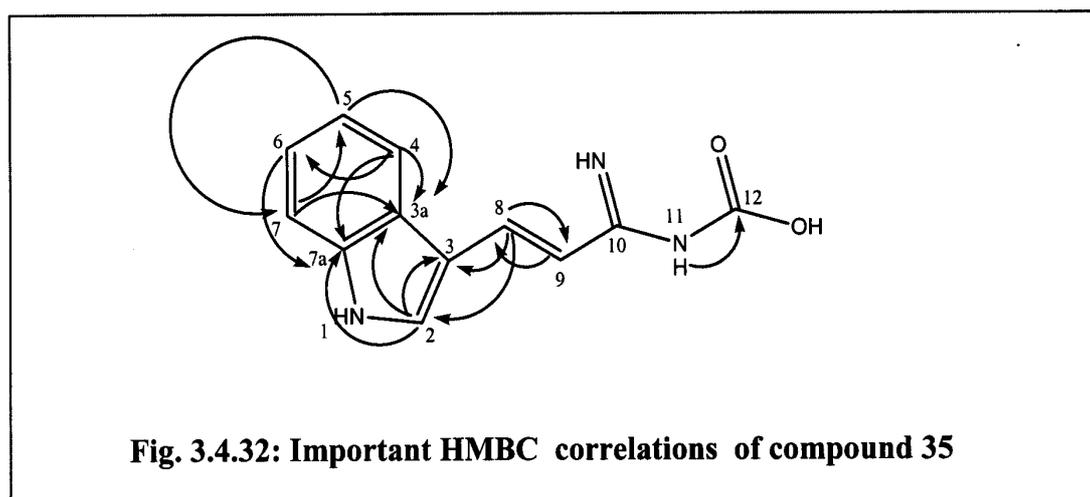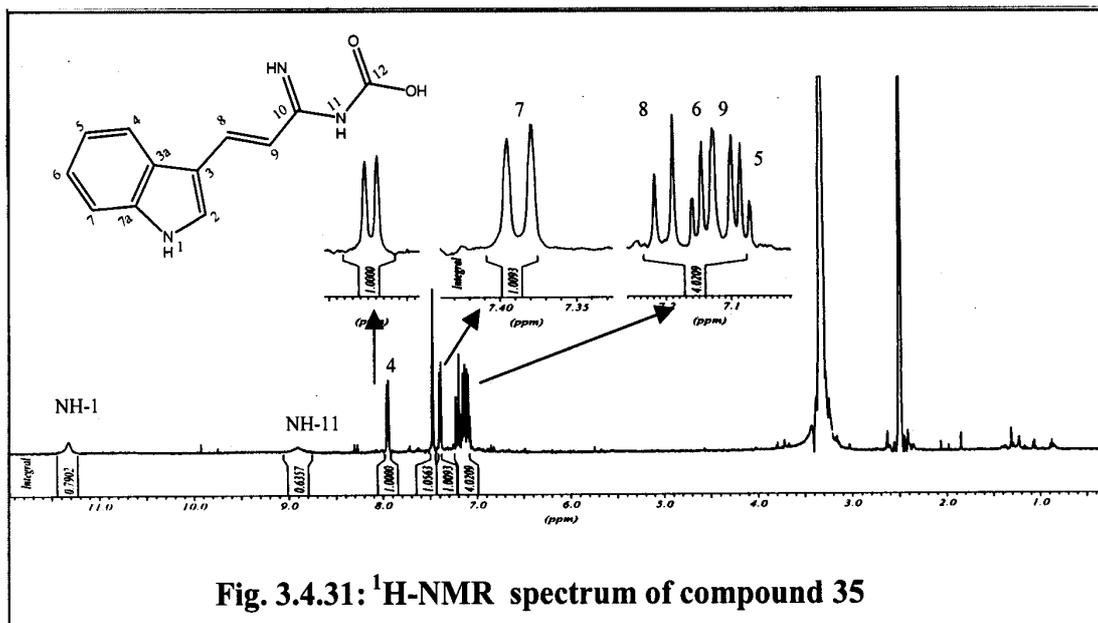
3.4.2. Biological activities

3.4.2.1. Cytotoxic activity

The cytotoxicity of some compounds isolated from *Hamigera hamigera* was studied in vitro against human cervix uteri tumour (HeLa), rat brain tumour (PC12) and mouse lymphoma (L5178Y) cell lines.

Table 3.4.7 shows the results of the cytotoxicity test depending on the percentage of rate of cell growth inhibition. It was found that compound **34** exhibited weak cytotoxic activity against mouse lymphoma cell line only (L5178Y).

Table 3.4.7: Results of cytotoxicity test of the metabolites of *Hamigera hamigera*

Compound No.	% of Growth inhibition of different types of cells (10 µg/ml)		
	L5178Y	**HeLa**	**PC12**
28	0	0	n. t
29	0	0	n. t
34	47%	0	0

n. t. = not tested

3.4.2.2. Brine shrimp assay

The pure compounds and the extracts of *Hamigera hamigera* were screened for bioactivity in the brine-shrimp assay. The tested compounds showed no activity or only very weak activity in this assay.

Table 3. 4. 8: The result of brine-shrimp assay of extracts and secondary metabolites of *Hamigera hamigera*

Samples	Brine shrimp mortality rate	
	10 µg/ml	**100 µg/ml**
Butanol extract	0%	0%
MeOH extract	20%	50%
Hexane extract	0%	0%
Compound 28	0%	0%
Compound 29	0%	15%
Compound 30	0%	15%
Compound 31	0%	0%

3.4.2.3. Antimicrobial assay

Agar diffusion assay

For the agar diffusion assay method, the test samples are considered active when the zone of inhibition is greater than 7 mm. The anti-microbial activity was categorised according to the zone of inhibition (diameter in mm) as follow.

8 to 10 mm	weakly active	(+)
11 to 15	Moderately active	(++)
> 15 mm	very active	(+++)

For the pure compounds isolated from the *Hamigera hamigera*, it was found that only the new compound **31** had weak activity against *B. subtilis*.

Table 3.4.9: Anti-microbial activities of some pure compounds isolated from *Hamigera hamigera*

No. of compound	*B. subtilis*	*E. coli*	*S. cerevisiae*	*C. herbarum*		*C. cucumerinum*	
	100μg	100μg	100μg	50μg	100μg	50μg	100μg
28	n. a.	n. a.	n. a.	n.t	-	n.t	n. a.
29	n. a.	n. a.	n. a.	n.t	-	n.t	n. a.
30	8 mm*	n. a.	n. a.	n.t	-	n.t	n. a.
31	n. a.	n. a.	n. a.	n.t	-	n.t	n. a.
32	n. a.	n. a.	n. a.	n.t	-	n.t	n. a.
33	n. a.	n. a.	n. a.	n.t	-	n.t	n. a.

20 μl contains 100 μg   *diameter of inhibition zone   n.t. not tested   n.a. no activity

The actvities of the isolated compounds were not quantified in the fish feeding assay due to the very small yields isolated.

4. Discussion

The phylum *Porifera* represents a rich source for the discovery of biologically active compounds. From this phylum four sponges, *Leucetta chagosensis*, *Axinyssa aplysinoides*, *Axinella damicornis* and *Hamigera hamigera*, were studied and diverse classes of compounds have been isolated, including alkaloids, terpenes, sterols, brominated compounds, phenolic compounds and nucleosides. Some of these metabolites exhibited interresting biological activities.

4.1. Imidazole alkaloids isolated from *Leucetta chagosensis*

Many marine imidazole alkaloids have been recently isolated, and most of them exhibit biological activities such as antimicrobial and antitumour activities (Kawasaki *et al*, 1997).

The chemical investigation of the butanol extract of the sponge *Leucetta chagosensis* yielded two new compounds named naamine F and G (compounds **2** and **3**) in addition to the known compound naamine A (compound **1**), while the ethyl acetate extract gave the three new compounds kealiinine A, B and C (compounds **4, 5** and **6**). From the results of bioassays, it was found that the new compound naamine G exhibited cytotoxic activity against mouse lymphoma (L5178Y) and human cervix carcinoma (HeLa) cell lines. In the brine shrimp assay it was found that kealiinine A (compound **4**) was more active than naamine G. This deduced that this compound is responsible for the activity of the ethyl acetate extract in the brine shrimp lethality test. The imidazole alkaloid (naamine G) was found to be strongly active against the fungal strain *C. herbarum* with a zone of inhibition of 20 mm at 100 µg/disc loading concentration.

Naamine G   Kealiinine A

Naamidine A

Pyronaamidine

Naamine D

2E, 9E-pyronaamidine 9-(N-methylimine)

Fig. 4.1: Some biologically active imidazole alkaloids previously isolated from sponges
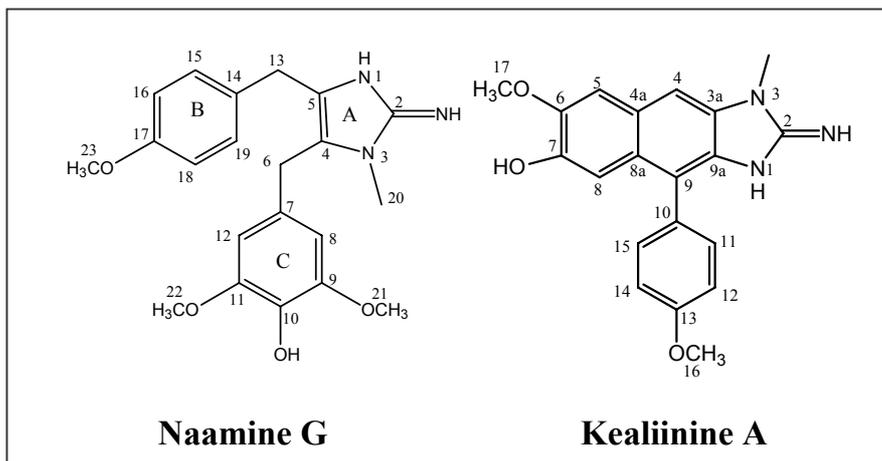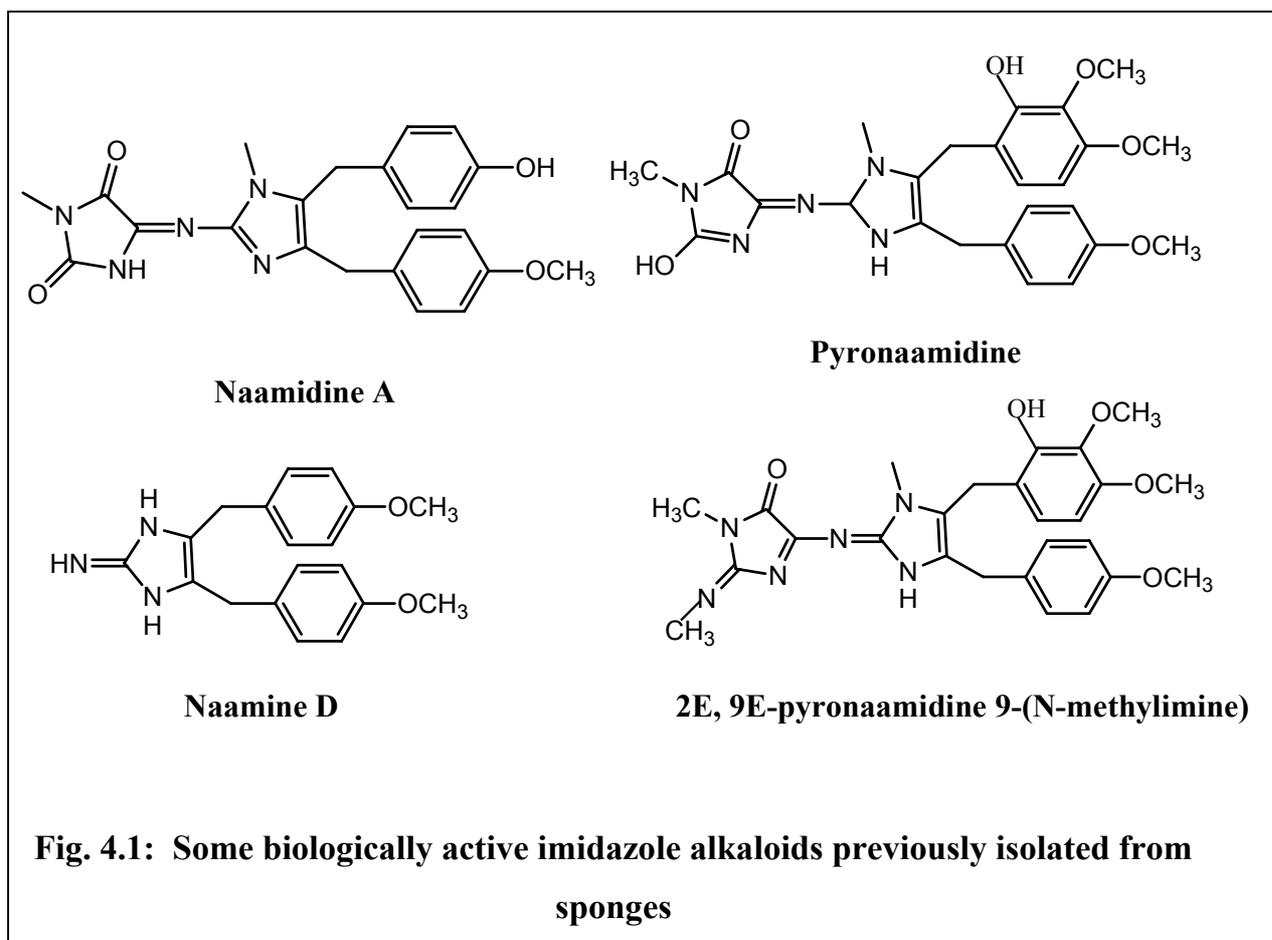
4.2. Biologically active metabolites from the sponge *Axinyssa aplysinoides*

The hexane extract of the sponge *Axinyssa aplysinoides* exhibited strong antifungal activity against *C. herbarum* (zone of inhibition 30 mm), and weak activity against *C. cucumerinum* (zone of inhibition 10 mm), while the butanol extract exhibited weak activity against *C. herbarum* (zone of inhibition 10 mm) but no activity against *C. cucumerinum*. The ethyl acetate extract showed also strong activity in the brine shrimp mortality test with 100% rate of mortality at a concentration of 100μg/ml.

Chemical investigation of the active extracts yielded the antifungal metabolite [*E*-(-4-hydroxystyryl) trimethyl ammonium chloride], (compound **10**), which showed antifungal activity against *C. herbarum* (zone of inhibition 14 mm at concentration 100 μg/ disc) (see Table 3.2.8), in addition to (6*R*,7*S*)-7-amino-7,8-dihydro-α-bisabolene hydrochloride (compound **7**), which exhibited strong cytotoxic activity against mouse lymphoma (L5178Y), human cervix carcinoma (HeLa) and tumour of rat brain (PC12) cell lines. In addition to (6*R*,7*S*)-7-amino-7,8-dihydro-α-bisabolene urea (compound **8**) which exhibited cytotoxic activity against L5178Y (see Table 3.2.5 and 3.2.6). From the result of bioassays it was found that the monomeric compound (compound **7**) was more active than the dimeric one (compound **8**), where the first compound (**7**) was active as antifungal and the second (**8**) was inactive, also compound **7** was more active than compound **8** in the brine shrimp lethality test and in the cytotoxicity assay. It seems that the free NH_2 group of compound **7** plays an important role in the bioactivity of the compound, as in case of sulphadiazine, (sulphonamide antibiotics), which is a prodrug that must be activated in the body since the free amino group is necessary for antibacterial activity (Rang *et al*, 1999). Or the nature of compound **7**, as a salt, increases its polarity and also the dispersion of the compound in an aqueous biological system, thus, the monomer was found to be more active than the dimer.

Compound 7

Compound 8

Compound 10

Sulphadiazine

(Prodrug)

Sulphanilamide
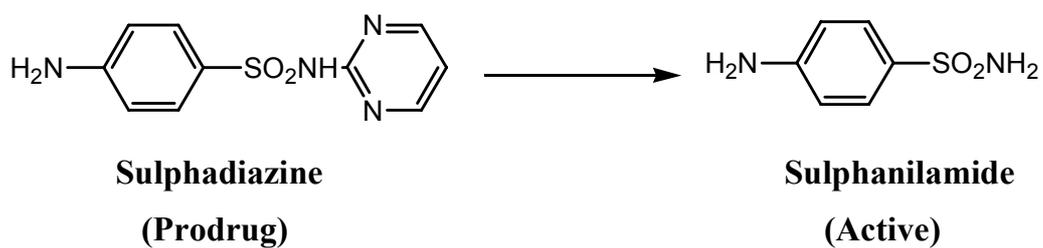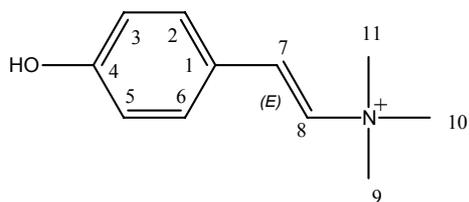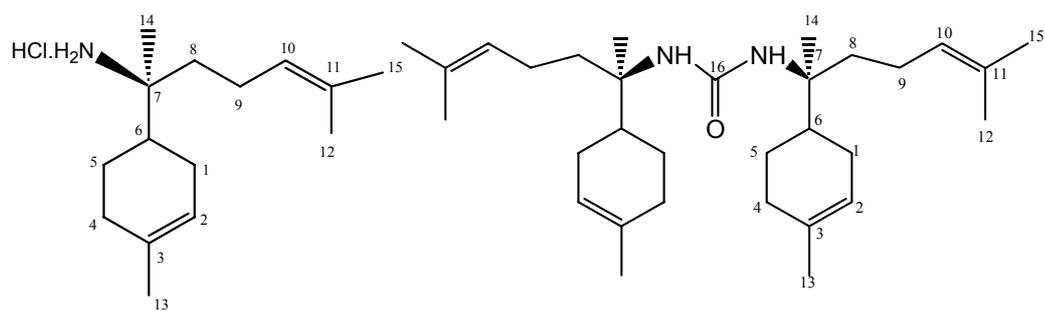
(Active)

4.3. Biologically active metabolites from the sponge *Axinella damicornis*

4.3.1. Bromopyrrole alkaloids

Biological activity of bromopyrrole alkaloids

The last year has seen significant interest in halogen containing heteroaromatic natural products which are a particularly common class of sponge-derived secondary metabolites. In particular brominated pyrroles have been isolated on several occasions as major constituents of marine sponges. The cyclic bromopyrrole alkaloid longamide B isolated from the marine sponge *Acanthella carteri* showed activity against gram-positive bacteria. Hanishin, another bromopyrrole alkaloid exhibited activity *in vitro* against NSCLC-N6 human non-small-cell-lung carcinoma (Mancini *et al*, 1997). Agelastatins are even more complicated bromopyrrole alkaloids which possess a unique fused tetracyclic skeleton that derived from an oroidin like precursor. Agelastatin A was isolated from the sponge *Cymbastela* sp, it was highly toxic to brine shrimp, and was also reported to be insecticidal with a biological activity comparable to a commercial preparation of the biopesticide *Bacillus thuringiensis* (Hong *et al*, 1998).

Bromopyrrole alkaloids as protein kinase inhibitors

Cyclin-dependent kinase (CDK) and glycogen synthase kinase (GSK)

The significance of appropriate CDK regulation is apparent in the role of cyclins in cancer cells. CDKs have been identified to be over-expressed in mammary carcinoma, oesophageal carcinoma, lymphoid adenoma, and other human cancers. CDK inhibitors are being important in case of undesired cell proliferation (Kamb *et al*, 1994). Cyclin-dependent kinase-1 is involved in the control of cell cycle, while cyclin-dependent kinase-5 and glycogen synthase kinase are involved in neuronal functions. It has been reported that hymenialdisine is a very potent inhibitor of CDK1, CDK2, CDK5, GSK-3β and CK1. It inhibits the phosphorylation of specific neural proteins by GSK-3β and CDK5 which cause Alzaheimer´s disease (Meijer *et al*, 2000). Dibromocantherelline displayed a significant inhibitory effect towards GSK-3β (IC$_{50}$ = 3µg). Hymenidin was a selective inhibitor for CDK5 (Meijer *et al*, 2000). Spongiacidin A-D have been isolated from marine sponge *Hymeniacidon* sp.

Spongiacidin A and B, which both possess (7*E*) geometry, displayed inhibitory activity against C-erbB-2 kinase and cyclin-dependent kinase 4. Spongiacidin C and D, which possess (7*Z*) geometry and have a hydantioin ring in place of the aminoimidazolinone ring, showed no inhibition of either kinase (Inaba *et al*, 1998). Tauroacidin A and B, both alkaloids were reported to show inhibitory activity against EGF receptor kinase and C-erbB-2 kinase (Kobayashi *et al*, 1998).

Longamide B

Hanishin

Spongiacidin B

Spongiacidin A

Agelastatin A

Fig. 4.2. Some biologically active bromopyrrole alkaloids
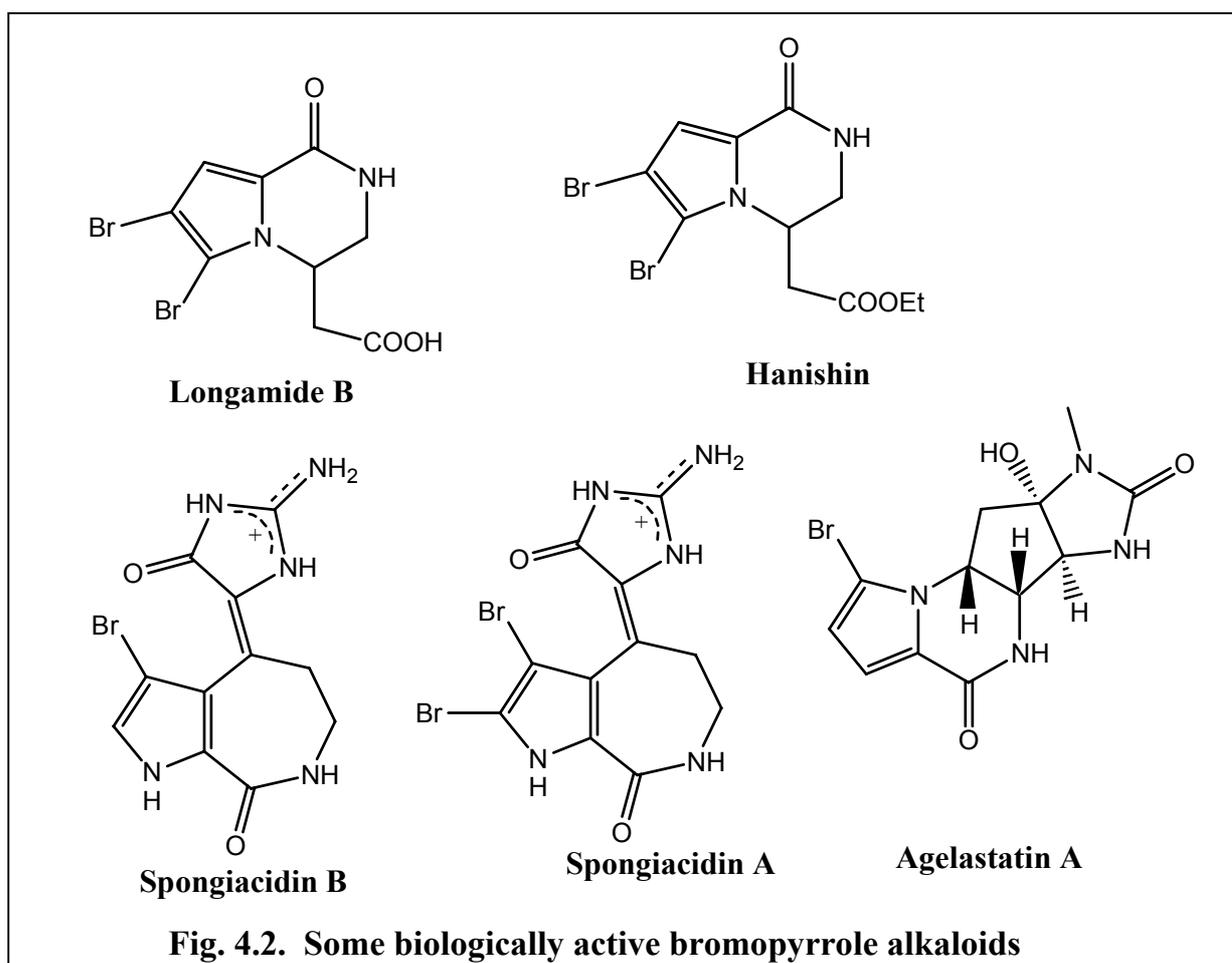
Structure activity relationship of bromopyrrole alkaloids in inhibition of fish feeding

The relationship between the structures of bromopyrrole alkaloids from marine sponges of the genus *Agelas* and their capacity to deter feeding by the polyphagous Caribbean fish *Thalassoma bifasciatum* for seven natural products, dispacamide A, keramadine, oroidin, midpacamide, 4,5-dibromopyrrole-2-carboxylic acid, 4,5-

dibromopyrrole-2-carbamide and longamide A, were assayed. It was found that the pyrrole moiety was required for feeding inhibition activity, while addition of an imidazole group enhanced this activity. Imidazole alkaloids lacking the pyrrole moiety were not deterrent (Lindel *et al*, 2000).

3.3.2. Bromopyrrole alkaloids from *Axinella damicornis*

The ethyl acetate and the butanol extracts of the sponge *Axinella damicornis* exhibited strong activity in the fish feeding assay using *Blennius sphinx*. The ethyl acetate extract exhibited also strong antimicrobial activity. By chemical investigation, many brominated metabolites were isolated from the sponge *Axinella damicornis*. Oroidin, (this compound is composed of two rings, an imidazole ring and pyrrole ring), is present in the sponge *Axinella damicornis* at high concentrations, so it is likely to be metabolically important for the sponge to make and store, and should have adaptive purpose.

Reversed-phase HPLC proved to be an excellent analytical tool, as it provided a rapid and effective method for the separation, identification and quantification of the brominated secondary metabolites in the crude extract of *Axinella damicornis*. It was found that the concentration of oroidin and 4,5-dibromopyrrole-2-carboxylic acid in ten ml volume of sponge *Axinella damicornis* were 60 mg and 48 mg respectively. The two compounds were tested in the fish feeding assay using *Blennius sphinx* at physiological concentration (the concentration of the compounds in the sponge), (see Table 3.3.15, Fig. 3.3.53). It was found that oroidin is still active at a concentration of 10 mg/10 ml food i.e. one sixth of the natural concentration. From the results of the fish-feeding assay it was deduced that the two compounds oroidin and 4,5-dibromopyrrole-2-carboxylic acid were the primary chemical defences of *Axinella damicornis* sponge.

In addition to oroidin and 4,5-dibromopyrrole-2-carboxylic acid, several bromo-pyrrole alkaloids exemplified by *Z*-hymenialdisine, manzacidin A, 2-bromoaldisin, longamide, 4,5-dibromopyrrole-2-carbamide, 4-bromopyrrole-2-carbamide and 4,5-dibromopyrrole-2- methylcarboxylate have been isolated.

The chemical investigation of the methanol extract of *Axinella damicornis* has led to isolation of four new compounds including, 3,4-dibromopyrrole-2-carboxylic

acid, 3,4- dibromopyrrole-2-carbamide, 3-bromoaldisin and 2,3-dibromoaldisin. The last compound was isolated in a very small amount which was insufficient to complete the structure elucidation. Partial synthesis of this compound was done by bromination of the naturally occurring compound 2-bromoaldisin (compound **13**) through electrophilic addition reaction (Heinz, 1996). 2-Bromoaldisin was dissolved in dichloromethane, followed by drop-wise addition of bromine water until persistent straw-yellow colour was obtained, and it was left overnight in a dark place at room temperature. The resulting precipitate was washed with dichloromethane and freeze dried.

2-Dromoaldisin
(Compound 13)

2,3-Dibromoaldisin
(Compound 15)
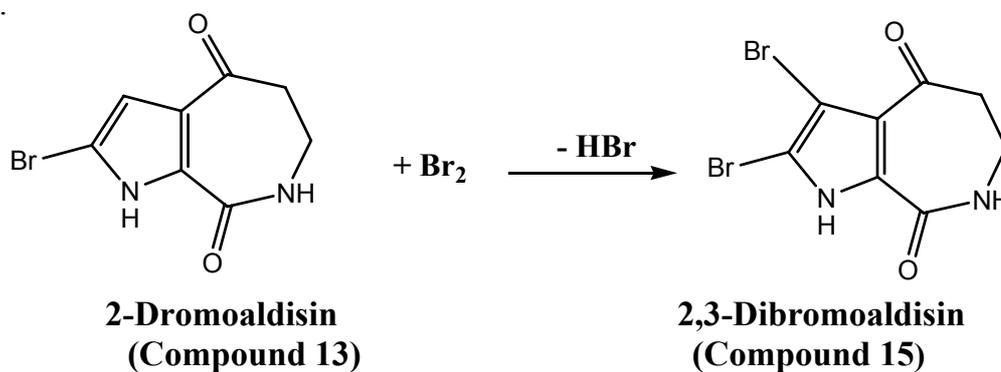
Fig. 4. 3. Bromination of 2-bromoaldisin

Regarding the biological activities of the brominated compounds, it was found that *Z*-hymenialdisine exhibited strong cytotoxic activity towards mouse lymphoma (L5178Y), human cervix carcinoma (HeLa) cell lines. Dibromocantharelline and 2-bromoaldisin showed activity only against the first type of cells (Table 3.3.11). In addition to the cytotoxic activity some brominated metabolites exhibited antimicrobial activity such as dibromocantharelline which showed antifungal activity against *C. herbarum* (zone of inhibition 16 mm). Manzacidin A exhibited strong antimbacterial and antifungal activities (see Table 3.3.13). It was deduced that manzacidin A was responsible for the antimicrobial activity of the ethyl acetate extract of the sponge *Axinella damicornis*.

In the protein kinase inhibition assay several brominated compounds were tested. *Z*-Hymenialdisine was found to be specifically active on cyclin-dependent kinase CDK1, CDK5 and GSK3 with IC$_{50}$ 0.45, 0.14 and 0.11 μM, respectively), stevensin, hymenin and oroidin were observed to display inhibitory effect towards the

three enzymes with IC$_{50}$ 5.3, 6.3 and 7.3 µM respectively for stevensin, IC$_{50}$ 0.50, 0.34 and 0.12µM respectively for hymenin, and IC$_{50}$ 0.26, 2.2 and, 2.4 µM respectively for oroidin. From the results of protein kinase inhibition assay it was observed that, dibromocantharellene exhibited no activity towards CDK5 and GSK3. As reported in the literature (Meijer *et al*, 2000), dibromocantharelline exhibited inhibitory effect towards GSK-3β (IC$_{50}$ 3 µM) (Meijer *et al*, 2000). The negative results in case of (–) dibromocantherelline isolated from *Axinella damicornis,* suggests that the (+) configuration of the chiral centre at C-6 in dibromocantharelline is necessary for the kinase inhibitory activity.

4.4. Different sets of metabolites isolated from the sponge *Hamigera hamigera*

Sterols from *Hamigera hamigera*

The total extract of the sponge *Hamigera hamigera* yielded three steroidal compounds (compounds **31**, **32** and **33**). The mass spectra of the three steroids showed that a diagnostically important cleavage was associated with the presence of a double bond in the side chain. Thus an intense peak at m/z 271 was found in the three compounds. This indicated that the steroids had the Δ^5-3-β-ol moiety together with a double bond in the side chain. The presence of the Δ^{22} double bond is indicated by the allylic cleavage peak at m/z 273 and in addition by a peak at m/z 300 (Wyllie *et al*, 1968). All the sterols which were isolated from the sponge *Hamigera hamigera* were screened for antimicrobial and brine shrimp lethality assays, but all of them were inactive.

Indole, phenolic and nucleoside derivatives isolated from the sponge *Hamigera hamigera*

Chemical investigation of the total extract of the sponge *Hamigera hamigera* yielded in addition to the three steroidal compounds (compounds **31**, **32**, and **33**), five other compounds, two of them were new phenolic compounds (compounds **28** and **29**), one was a new nucleoside (compound **31**) and two were indole alkaloids (compounds **34** and **35**). Compound **30** exhibited mild antibacterial activity against *B. subtilis* (zone of inhibition 8 mm) and compound **34** showed cytotoxic activity against the mouse lymphoma (L5178Y) cell line.
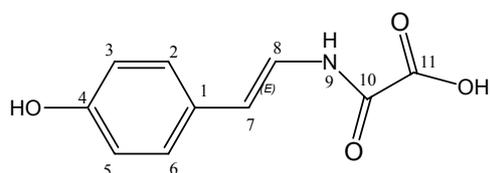
Hamigeroxalamic acid
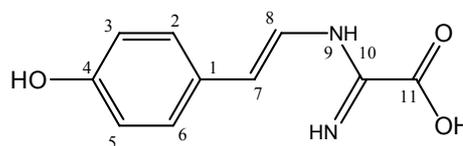
(Compound 28)

Hamigeramine

(Compound 29)
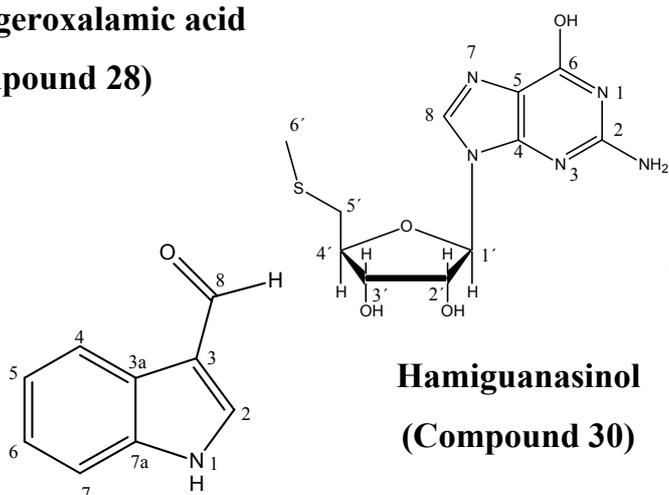
Hamiguanasinol

(Compound 30)
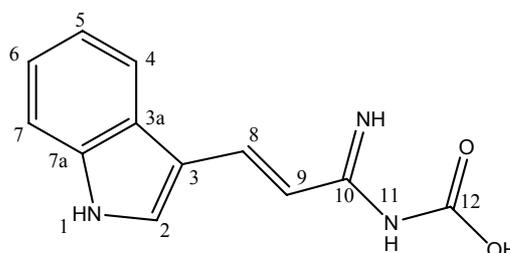
Indole-3-carboxaldehyde

(Compound 34)

Hamigeramide

(Compound 35)

Three compounds, hamigeroxalamic acid (**28**), hamigeramine (**29**) and hamigeramide (**35**) were isolated from the ethyl acetate extract, and the nucleoside hamiguanasinol (**30**) was isolated from the aqueous extract of the sponge. The two extracts, ethyl acetate and aqueous, were active in the fish feeding assay. Nucleoside compounds are known for their antiviral activity. They were first isolated from a

Caribbean sponge in the 1950, and later served as lead structures for the development of nowadays commercially important anti-viral drugs such as Ara A known in the market as aciclovir (Balzarini *et al*, 2001). Hamiguanasinol (**30**) exhibited mild activity against the gram positive bacteria *Bacillus subtilis*. Indole-3-carboxaldehyde (**34**) showed cytotoxic activity against mouse lymphoma (L5178Y) cell line. Hamigeroxalamic acid (**28**) is among the few examples of the natural products which carry the oxalamic functionality. Another important natural product with the same oxalamic moiety is psammaplin F which was found to selectively inhibit histone deacetylase (HDAC), which is an epigenetic modifier in the silencing of tumour suppression genes. It is also important to note that among the ten known psammaplin derivatives, it was only psammaplin F, the oxalamic congener that was found to inhibit HDAC (Piňa *et al*, 2003). Hamigeramine (**29**) is related to O-acetylbenzeneamidinocarboxylic acid, a metabolite of the fungus *Gibberella saubinetii* Hamigeramine (**29**) and O-acetylbenzeneamidinocarboxylic acid are the only two natural product that possess the imino amino acetic acid functionality. Hamigeramine (**29**) is related to O-acetylbenzeneamidinocarboxylic acid.

O-acetylbenzeneamidinocarboxylic acid was reported to have selective cytotoxicity against SV40-transformed cells and was also found to inhibit Ehrlich ascites tumour growth (Munekata *et al*, 1982). Compound **35** is basically an indole alkaloid. Related compounds are the bisindole alkaloids chondramides which were reported to exhibit anthelmentic and antiviral activity against HSV II.
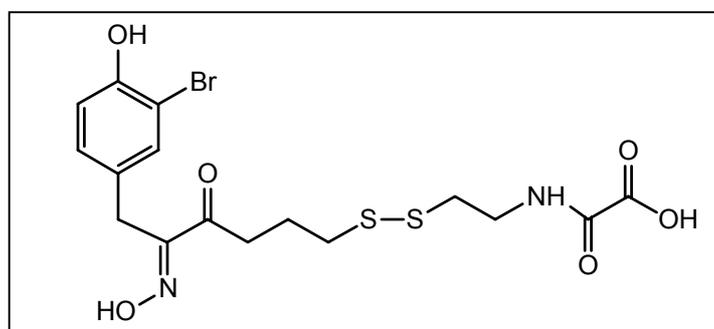
Psammaplin F (Piňa, 2003)

5. Summary

In the search for bioactive compounds from marine sponges, this study led to the isolation of thirty-six compounds, thirteen of which are new metabolites. The sponges were collected from Indonesia and at Elba island. For the discovery of biologically active metabolites, a combination of biological screening assays, fish feeding assay and screening for antimicrobial activities was performed.

1- *Leucetta chagosensis*

The extract of the sponge *Leucetta chagosensis* afforded six imidazole alkaloids, three of them are of the naamine class of compounds including, naamine A (known compound), naamine F (new compound) and   naamine G (new compound), in addition to another three new compounds which are related to kealiiquinone such as, kealiinine A, B and C. Naamine G exhibited cytotoxic activity against mouse lymphoma cells (L5178Y) and human cervix carcinoma cells (HeLa). It was found that naamine G exhibited strong antifungal activity against *C. herbarum* at a concentration of 100 µg causing zone of inhibition 20 mm in diameter.

2- *Axinyssa aplysinoide*s

The sponge *Axinyssa aplysinoide*s yielded four known compounds including, one   sesquiterpene isothiocyanate (epipolasin A), two bisabolene sesquiterpenes [(6*R*,7*S*)-7-amino-7,8-dihydro-α-bisabolene hydrochloride and (6*R*,7*S*)-7-amino-7,8-dihydro-α-bisabolene urea)] and one phenolic compound [E-(-4-Hydroxystyryl) trimethyl ammonium chloride]. The last compound showed strong antifungal activity against *Cladosporum herbarum* at a concentration 100µg. (6*R*,7*S*)-7-Amino-7,8-dihydro-α-bisabolene hydrochloride showed strong cytotoxic activity against L 5178Y cells (IC$_{50}$ = 0.3 µg/ml), HeLa cells (IC$_{50}$ = 2,2 µg/ml), and PC$_{12}$ cells (IC$_{50}$ = 2.14 µg/ml).

3- *Hamigera hamigera*

Eight compounds have been isolated from the marine sponge *Hamigera hamigera*. Four new compounds included hamigeroxalamic acid, hamigeramide, hamigeramine and hamigunansinol. Additionally the known compounds indole-3-carboxaldehyde and three steroidal compounds were also obtained. Hamigunansinol exhibited mild antibacterial activity against *B. subtilis*.

4- *Axinella damicornis*

The sponge *Axinella damicornis* yielded several bromopyrrole alkaloids, four were new compounds including, 3,4-dibromo-pyrrole-2-carbamide, 3,4-dibromo-pyrrole-2-carbxylic acid, 2,3-dibromoaldisin, and 3-bromoaldisin. In addition, thirteen known compounds, oroidin, 2-bromoaldisin, Z- hymenialdisin, manzacidin A, 4,5-dibromo-pyrrole-2-carbamide, 4,5-dibromo-pyrrole-2-carbxylic acid, 4,5-dibromo-pyrrole-2-methylcarboxylate, 4-bromo-pyrrole-2-amide, longamide, stevensin, hymenidin, hymenin and dibromocantharelline were also isolated.

It was found from the fish-feeding assay that the two compounds, oroidin and 4,5-dibromo-pyrrole-2-carbxylic acid, are responsible for the fish feeding deterrence activity of the total extract of the sponge and play an important role in the chemical defence of the sponge against fishes predators.

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Curriculum Vitae

Personal Data:

Name: Wafaa Hassan Badr Hassan

Birth date: November, 27, 1965

Marital status: Married, two children

Position: Assistant Lecturer of Pharmacognosy and Medicinal Plants

        Dept. of Pharmacognosy, Faculty of Pharmacy,   Zagazig

        University, Zagazig, Egypt

Address: Safor, Dyarb-Nigm, Sharkia, Egypt

Telephone: 002/055-3830762 (Home)

Fax: 002/055-345452

Post Address: Department of Pharmacognosy, Faculty of Pharmacy,

        Zagazig University, Zagazig, Egypt

Education Career:

1)- B.Sc. Pharmacy and Pharmaceutical Science 05/1989, Faculty of Pharmacy,
Zagazig University, Zagazig, Egypt.

2)- M. Sc. of Pharmacognosy, Jan, 1996, Zagazig University, Zagazig, Egypt.

Professional Career:

a)- Demonstrator of Pharmacognosy and Medicinal Plants 1990-1996 Faculty of
Pharmacy, Zagazig University, Zagazig, Egypt.

b)- Assistant Lecturer of Pharmacognosy and Medicinal Plants 1996-till now, Faculty
of Pharmacy, Zagazig University, Zagazig, Egypt.

Membership held:

1)- Egyptian Pharmacist Syndicate

2)- Egyptian Society of Pharmaceutical Sciences

List of publications

Wafaa Hassan, Ru Angelie Edrada, Rainer   Ebel, Victor Wray , Albrecht Berg, Rob
   van Soest, Sumali Wiryowidagdo, and Peter Proksch
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