Isolation and Structure Elucidation of Bioactive Secondary Metabolites from Marine Sponges

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

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Erklärung

Hiermit erkläre ich ehrenwörtlich, dass ich die vorliegende Dissertation mit dem Titel ``Isolierung and Struktur-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. Außerdem erkläre ich, dass ich bisher noch keine weiteren akademischen Grade erworben oder zu erwerben versucht habe.

Düsseldorf, den 18. 04. 2005

Sabrin R. M. Ibrahim

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Sabrin R. M. Ibrahim
Düsseldorf, Germany

To my Family

and

the memory of my Mother

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Zusammenfassung

Aus marinen Organismen wie Schwämmen, Pilzen und Bakterien isolierte Naturstoffe zeigen eine einzigartige stukturelle Vielfalt, die zu neuen, für medizinische Zwecke verwendbaren Verbindungen führen kann. Diese Arbeit umfasst die Isolierung, Strukturaufklärung und das Screening auf Bioaktivität der aktiven Inhaltsstoffe einiger in Indonesien gesammelter Schwämme unter Anwendung verschiedener chromatographischer Methoden und zahlreicher Mittel der Spektralanalytik. In dieser Arbeit wurden vier Schwämme untersucht, die daraus isolierten und strukturaufgeklärten Verbindungen sind in der folgenden Tabelle aufgeführt (Tabelle 5.1).

5.1. Der indonesische Schwamm *Callyspongia aerizusa*

Der Schwamm *Callyspongia aerizusa* wurde erschöpfend mit Methanol beziehungsweise Aceton extrahiert. Die vereinigten Extrakte wurden zur Trockne aufkonzentriert und mit Hexan, Ethylacetat und *n*-Butanol gegen Wasser ausgeschüttelt. Die Ethylacetat-Fraktion wurde einer Kieselgel-Säulenchromatographie gefolgt von semipräparativer HPLC und Umkehrphasen (RP-18)-Kieselgel-Säulenchromatographie unterzogen, um die bekannten Reinsubstanzen Callyaerine B, E, F, D sowie die neue Verbindung Callyaerin S zu isolieren. Die Callyaerine B, E und S zeigten starke zytotoxische Aktivität. Callyaerin E zeigte auch antivirale Aktivität.

5.2. Der indonesische Schwamm *Diacarnus megaspinorhabdosa*

Der Schwamm *Diacarnus megaspinorhabdosa* wurde erschöpfend mit Methanol beziehungsweise Aceton extrahiert. Die vereinigten Extrakte wurden zur Trockne aufkonzentriert und mit Hexan, Ethylacetat und *n*-Butanol gegen Wasser ausgeschüttelt. Die Ethylacetat-Fraktion wurde einer Vakuum-Flüssigkeitschromatographie gefolgt von semipräparativer HPLC unterzogen, um die beiden bekannten Verbindungen Indol-3-carbaldehyd und p-Hydroxy-benzylidenaceton sowie die neue Verbindung Diacarmin zu isolieren. Die *n*-Butanol-Fraktion wurde einer Vakuum-Flüssigkeitschromatographie gefolgt von semipräparativer HPLC unterzogen, wobei

vier Reinsubstanzen isoliert werden konnten, Thymin-2´-Desoxyribosid, 2´-Desoxyadenosin, 2´-Desoxyinosin und 2´-Desoxyguanosin. Die Hexan-Fraktion wurde einer Vakuum-Flüssigkeitschromatographie unter Verwendung eines Hexan-Ethylacetat-Gradienten unterzogen. Die erhaltenen Fraktionen wurden einer Flash-Chromatographie, Kieselgel-Säulenchromatographie, Sephadex (LH-20) –Säulenchromatographie beziehungsweise Umkehrphasen(RP-18)-Kieselgel-Säulenchromatographie unterzogen, wobei neun neue Norterpen-Cycloperoxid-Diacarperoxid (A-I) und desweiteren vier bekannte Verbindungen isoliert wurden. Alle isolierte Verbindungen zeigten interessante zytotoxische Aktivität gegen verschiedene Krebszell-Linien.

5. 3. Der indonesische Schwamm *Acanthostronglylophora ingens*

Der Schwamm *Acanthostronglylophora ingens* wurde erschöpfend mit Methanol beziehungsweise Aceton extrahiert. Die vereinigten Extrakte wurden zur Trockne aufkonzentriert. Der Vollextrakt wurde einer VLC unter Verwendung eines Dichlormethan:Methanol Gradientensystems unterzogen. Die eluierten Fraktionen wurden einer Sephadex- beziehungsweise Kieselgel-Säulenchromatographie und semipräparativer HPLC unterzogen, um zwei bekannte Substanzen und eine neue Verbindung zu erhalten. Annomontin zeigte starke zytotoxische Aktivität.

5. 4. Der indonesische Schwamm *Theonella swinhoei*

Der Schwamm *Theonella swinhoei* wurde erschöpfend mit Methanol beziehungsweise Aceton extrahiert. Die vereinigten Extrakte wurden zur Trockne aufkonzentriert und mit Hexan, Ethylacetat und *n*-Butanol gegen Wasser ausgeschüttelt. Die Ethylacatat-Fraktion wurde einer Kieselgel-Säulenchromatographie gefolgt von semipräparativer HPLC unterzogen, um drei bekannte Verbindungen zu isolieren. Die Hexan-Fraktion wurde einer Kieselgel–Säulenchromatographie unterzogen, um eine bekannte Verbindung zu erhalten, Cholest-4-ene-3-on.

Introduction

1.1. The significance of the study

Natural products play a highly significant role in drug discovery, which has been reviewed recently [Newman, *et al*., 2004]. It was reported that at least 119 compounds derived from 90 plant species can be considered as important drugs [Farnsworth, *et al*., 1985]. Drugs of natural origin have been classified as original products, products derived semi-synthetically from natural products, or synthetic products based on natural product models [Cragg, *et al*., 1997].

The Ocean, which is called the ´mother of origin of life´, is also the source of structurally unique natural products. Several of these compounds show pharmacological activities and are helpful for the invention and discovery of bioactive compounds [Jha, *et al*., 2004]. They cover more than 70 % of the world surface (see Fig. 1.1) [Ellis, 2001]. It was found that about 80 % of all known animal species are living in or on this water environment [Marderosian, 1969, Proksch, 1991].

Research into pharmacological properties of marine natural products has led to the discovery of many potently active agents considered worthy of clinical evaluation. The marine environment is an exceptional reservoir of bioactive natural products, many of which exhibit structural/chemical features not found in terrestrial natural products [Carte´, *et al*., 1996]. Almost every class of marine organism exhibits a variety of molecules with unique structural features. But beyond the chemical diversity, the sea also provides amazing biological diversity. Among 34 fundamental phyla of life, 17 occur on land whereas 32 occur in the sea. From the fundamental point of view of biodiversity, the ocean is far more diverse and really would have been the better place to start to develop a natural pharmacy. To date, researchers have isolated approximately 7000 marine natural products, 25 percent of which are from algae, 33 percent from sponges, 18 percent from coelenterates (sea whips, sea fans and soft corals), and 24 percent from representatives of other invertebrate phyla such as ascidians (also called tunicates), opisthobranch molluscs (nudibranchs, sea hares etc), echinoderms (starfish, sea cucumbers etc) and bryozoans (moss animals) [Faulkner, 1995]. A simplistic analysis of these data reveals that as the research for “Drugs from the Sea” progresses at the rate of a 10 percent increase in new compounds per year, researchers are concentrating their efforts on slow-moving or sessile invertebrate phyla

that have soft bodies, and lack spines or a shell, *i.e.* animals that require a chemical defence mechanism [Faulkner, 1995]. In recent years many marine natural products which are promising candidates for new drugs have been discovered.

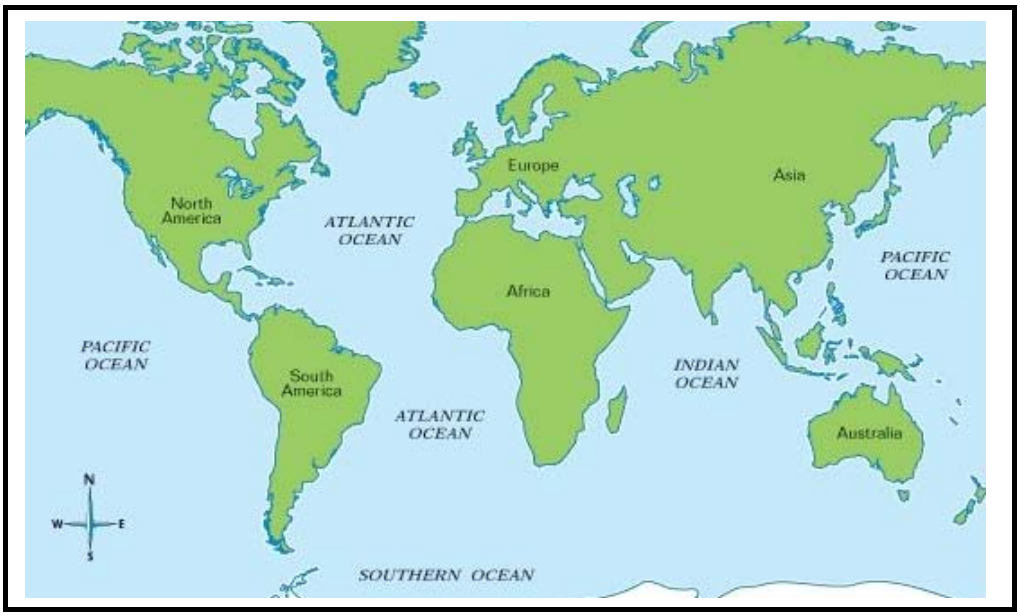


Fig. 1.1: World map

1.2. The requirements and difficulties of the marine natural products research

Sample collection

Sample collection is the first step in these studies and may be much more difficult in the marine environment than if working with terrestrial organisms. This is not only due to the difficulties inherent to collection in the marine environment but also due to problems associated with the taxonomy and the lack of sufficient biological material. The presence of symbionts (fungi, bacteria, microalgae) living on or inside the macrorganisms being studied introduces real uncertainty about the exact metabolic origin of the active compounds isolated. This fact is further complicated by the difficulties encountered in the search for adequate condition for growth and cultivation of marine organisms, be these invertebrates (i.e. sponges, bryozoans) or microbe [Fenical, 1993].

Number of samples screened

The probability of finding useful active metabolites is obviously dependent on the number of samples screened. Bulk collection is necessary and amounts of ≥ 1 kg of lyophilised material may be needed just to get the minimum amount of pure compound to check its activity and determine its structure.

Selection of the active sample

Selection of the active sample is based on fast, economic and representative primary tests. To do this, selective extractions, separations and purification procedures are followed. If the pure compound shows really interesting activity, further pharmacological assays (*in vitro*, *in vivo*, tolerated dose, and so on) and chemical work (structure modification, preparation of analog, structure-activity relationship, total synthesis, cultivation, etc.) should be carried out in order to enter the development step [Riguera, 1997]. A general view of the search for useful bioactive compounds is presented in Fig. 1.2.

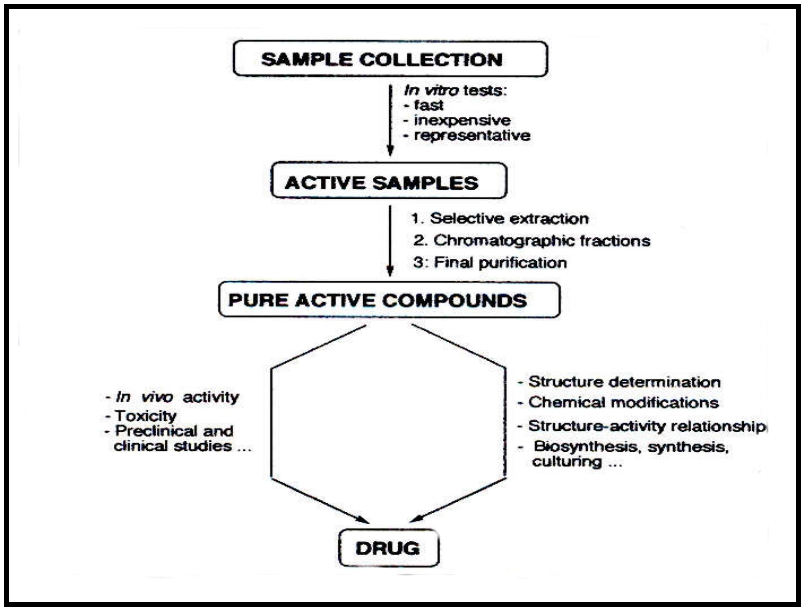


Fig. 1.2: A general view of the search for useful bioactive compounds

1.3. The biological importance of marine natural products

1.3.1. Anticoagulant and antiplatelet marine natural products

Fucoidan [Thorlacius, *et al*., 2000] is a sulfated polysaccharide. It inhibited thrombus formation in arterioles and venules *in vivo* with no effect on P- and L-selectin function suggesting that the anticoagulant effect of fucoidan was mainly responsible for its powerful antithrombotic property *in vivo*.

Sulfated D-galactan [Farias, *et al*., 2000] isolated from the red algae *Botryocladia occidentalis*. It had potent anticoagulant activity, similar to that of unfractionated heparin, owing to the enhanced inhibition of thrombin and factor Xa by antithrombin or heparin cofactor II.

Eryloside F is a penasterol isolated from the marine sponge *Erylus formosus* [Stead, *et al*., 2000]. It inhibited thrombin-induced human platelet aggregation *in vitro* in a concentration-dependent manner.

1.3.2. Antifungal marine natural products

The antifugal properties of the novel marine natural product **lyngbyabellin B**, a depsipeptide isolated from the marine cyanobacterium, *Lyngbya majuscula* [Milligan, *et al*., 2000] showed activity against *Candida albicans* in a disk diffusion assay.

Naamine D a novel imidazole alkaloid, isolated from the Red Sea sponge *Leucetta* cf. *chagosensis* [Dunbar, *et al*., 2000] had a MIC of 6.25 μg/mL against *Cryptococcus neoformans*. Interestingly, naamine D at 1 μM competitively inhibited murine macrophage-inducible nitric oxide synthase by 50 %.

1.3.3. Antituberculosis marine natural products

In a systematic study involving more than 30 selected marine compounds belonging to several structural classes and derived from both marine algae and invertebrates, antimycobacterial activities against either *Mycobacterium tuberculosis* or *Mycobacterium avium* were observed in approximately one third of the screened compounds. Of interest was the observation that molecules with an isonitrile group were the most active [Konig, *et al*., 2000].

Axisonitrile-3 was isolated from the sponge *Acanthella kletra*. It was shown to be active against *M. tuberculosis*, with a concomitant low toxicity [Konig, *et al*., 2000].

Elisapterosin B is a novel diterpene isolated from the gorgonian *Pesudopetrogorgia elisabethae* [Rodriguez, *et al*., 2000]. It demonstrated inhibitory activity against *M. tuberculosis* at a concentration of 12.5 μg/mL.

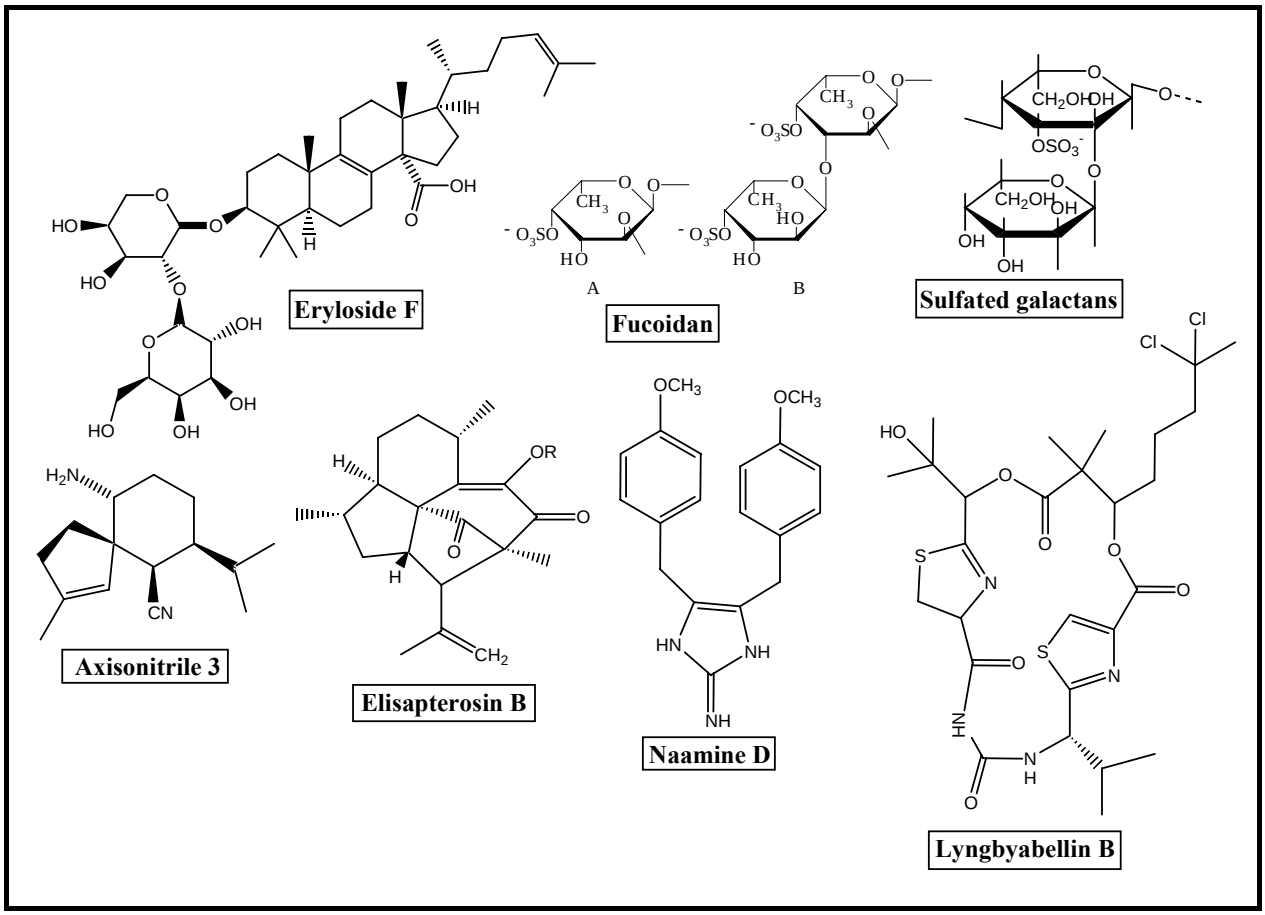


Fig. 1.3: Marine compounds with anticoagulant, antifungal and antituberculosis activities

1.3.4. Antiviral and antitumour marine natural products

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. Both antiviral and anticancer drugs are cell-growth inhibitory substances, which act more or less selectively hopefully without damage of the cells of host organisms. Nature has been a relevant resource for the discovery of anticancer entities. Today, more than 60 % of the anticancer drugs commercially available are of natural origin [Craigg, *et al.*, 1997]. The relevance of the sea as a tool to discover novel anticancer compounds was validated by the discovery, development and marketing approval of 1-beta-D-arabinofuranosylcytosine (ARA-C) [Bergmann, *et al.*, 1985]. ARA-C is a basic component in the curative setting of acute myeloid leukaemia [Wolf, *et al.*, 1985]. The available results clearly anticipated the potential of the marine ecosystem in cancer therapy. During the last decade about 2500 new metabolites with antiproliferative activity have been reported; about 68 of which are new marine derived anticancer

chemical entities, most of them with undetermined modes of action [Mayer, *et al*., 2003].

Ecteinascidin-743 (ET-743) (Trabectedin, Yondelis^{TM}) is a tetrahydroisoquinoline alkaloid derived from the colonial tunicate *Ecteinascidia turbinata* [Rinehart, *et al*., 1999], a tunicate that lives in clusters in the Caribbean and Mediterranean seas. The compound demonstrated very potent activity against a broad spectrum of tumour types in animal models [Rinehart, 2000]. Early trial results have shown promising activity of ET-743 in the treatment of advanced soft tissue sarcoma (STS), osteosarcoma and metastatic breast cancers. It was found that ET-743 prevents the formation of P-glycoprotein, a protein associated with multidrug-resistant tumours [Zewail-Foote, *et al*., 1999].

Kahalalide F is a depsipeptide discovered in *Elysia rufescens*, a marine mollusc found in Hawaii. It caused a disruption of lysosomal membranes and consequently the formation of large vacuoles. This mechanism is unique among anticancer agents and may cause increasing acidification of the intra-cellular space, a stimulatory event that initiates a pathway for apoptosis [Hamann, *et al*., 1993]. In addition, kahalaide F leads to an inhibition of *erb* 2 transmembrane tyrosine kinase activity and inhibits TGF–a gene expression.

Discodermolide is a polyhydroxylated lactone, isolated from the deep-sea sponge *Discodermia* sp. It is an immunosuppresive and cytotoxic agent [Gunasekera, *et al*., 1990]. The study of its mechanism has revealed that discodermolide was able to stabilise microtubules. In 1998, **Novartis Pharma AG** licensed this compound for development as a candidate agent for treatment of cancers.

Bryostatin 1 is a macrocyclic lactone isolated from the marine bryozoan *Bugula neritina* (Bugulidae). It has many properties including activation of T-cells, immunomodulation and stimulation of haematopoietic progenitor cells. Bryostatin 1 was found to bind to protein kinase C with high affinity, which may be the mechanistic basis for both observed anticancer and immunostimulating activities [de Vries, *et al*., 1995].

Didemnin B is a depsipeptide isolated from the Caribbean tunicate *Tridemnum solidum*. It was found to display antineoplastic, antiviral and

subsequently immunosuppresive activities [Rinehart, *et al*., 1988]. Mechanistically, didemnin B acts at the GTP binding protein elongation factor.

Ziconotide (**Prialt**) is a 25 amino acid peptide from the venom of the predatory snail *Conus magnus*. It acts by binding to and inhibiting presynaptic calcium channels, thereby preventing neurotransmitter release [Oliveira, *et al*., 1985]. It is licensed by Elan Pharmaceuticals as **Prialt**. Prialt blocks nerve impulses in a key region of the spinal cord, where pain fibres from the body connect with the nerve cells that send pain to the brain. This is why Prialt is 50 times more potent than morphine.

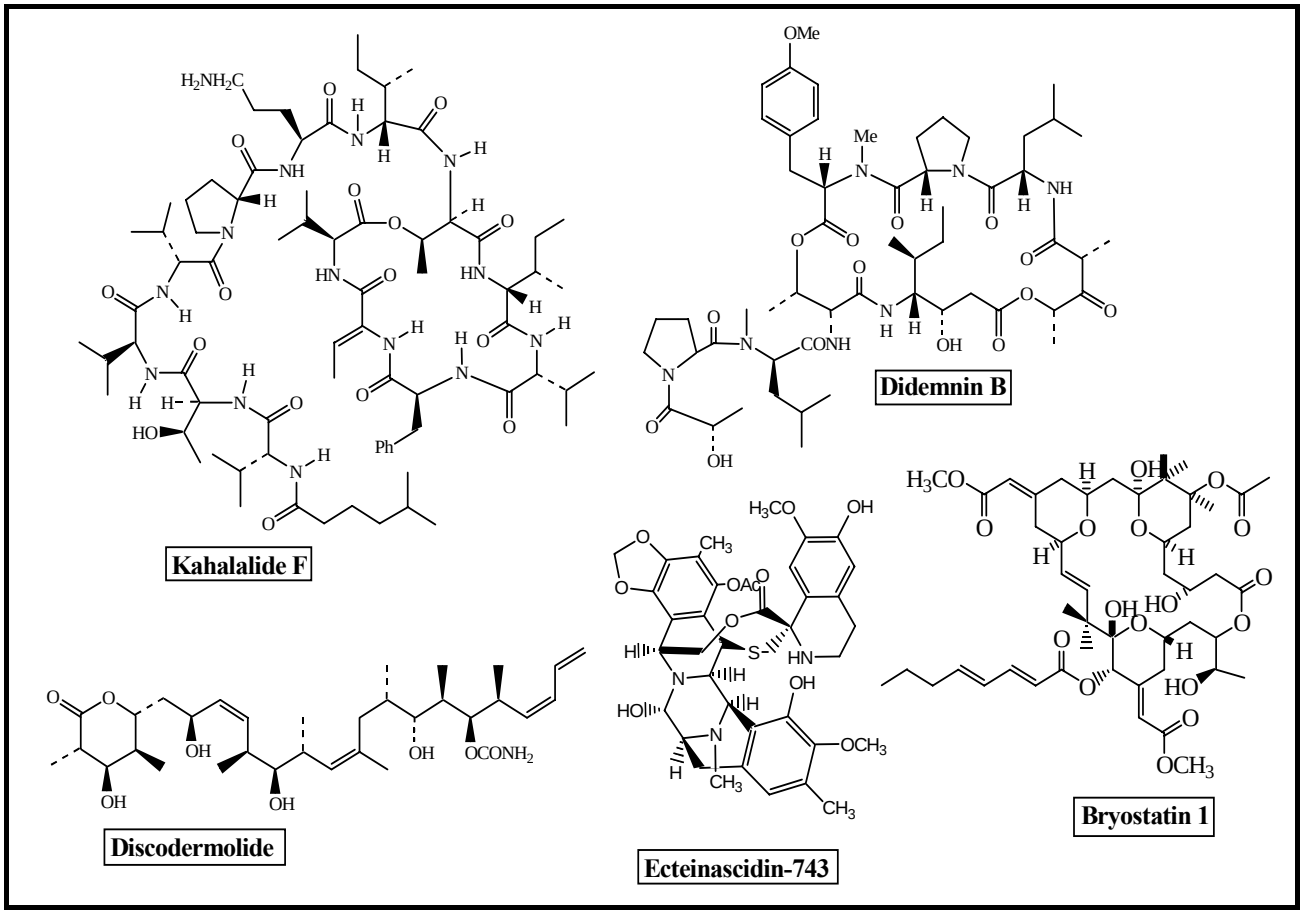


Fig. 1.4: Marine natural products with antiviral and antitumour activities

1.3.5. Anti-inflammatory activity of marine natural products

Cavernolide is a C21 terpene lactone isolated from the sponge *Asciospongia cavernos -a*. It causes potent inhibition of tumour necrosis factor-a, nitric oxide, and prostaglandin E_2 *in vitro* as a result of both human synovial phospholipase A_2 inhibition and inhibition of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) gene expression in intact cells [Posadas, *et al*., 2000].

Contignasterol is a highly oxygenated sterol isolated from the sponge *Petrosia contignata*. It appears to be as potent as nedocromil in inhibiting allergen-induced bronchoconstriction *in vivo*. The study demonstrated that contignasterol dose-dependently inhibited plasma exudation *in vivo* in response to ovalbumin, indicating that this is a potential anti-inflammatory compound [Coulson, *et al*., 2000].

Cyclolinteinone is a sesterterpene isolated from the sponge *Cacospongia linteiformis*, It causes inhibition of nuclear transcription factor-jB binding activity, a decrease of both iNOS and COX-2 expression, and the concomitant weak *in vitro* inhibition of both prostaglandin E_2 (IC_{50} = 50 µM), and nitric oxide (IC_{50} = 50 µM) [D'Acquisto, *et al*., 2000].

Manoalide is a sesquiterpenoid isolated from the sponge *Luffariella variabilis*. It is a potent analgesic and anti-inflammatory agent. Manoalide is by far the best characterised PLA_2 inhibitor from natural sources. At low concentrations manoalide inhibited calcium channels with no effect on phosphoinositide metabolism [Potts, *et al*., 1992].

Pseudopterosins are tricyclic diterpene glycosides isolated from the Caribbean sea whip (gorgonian) *Pseudopterogorgia elisabethae* (Gorgoniidae). They are potent anti-inflammatory and analgesic agents and appear to inhibit eicosanoid biosynthesis by inhibition of both PLA_2 and 5-lipoxygenase [Potts, *et al*., 1992].

1.3.6. 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, Williams, *et al*., 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].

1.3.7. 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

[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 mosquitos belonging to the genus *Anopheles*. Since it is almost impossible to eliminate the vector of transmission, there will always be a need of new antiplasmodium drugs [Wright, *et al.*, 1996]. The marine organisms are considered as a source for some antimalarial drugs.

Ascosalipyrrolidinone is a novel polyketide from the marine fungus *Ascochyta salicoriniae* [Osterhage, *et al.*, 2000]. It has antiplasmodial activity against *Plasmodium falciparum* strain K1 (a strain resistant to chloroquine) and NF 54 (a strain susceptible to standard antimalarials).

Homofascaplysin A and **fascaplysin** are sesterterpenes isolated from the marine sponge *Hyrtios* cf. *erecta* [Kirsch, *et al.*, 2000]. They demonstrated potent activity against both chloroquine susceptible (NF-54) and chloroquine-resistant (K-1) *Plasmodium falciparuim* strains.

Finally, **manzamine A** is a *ß*-carboline alkaloid, which was shown to inhibit the growth of the rodent malaria parasite *Plasmodium berghei*, not only *in vitro* but also *in vivo*. It caused reduction of parasitemia by either oral or intraperitoneal route of administration, suggesting that it is a potentially promising antimalarial agent [Ang, *et al.*, 2000].

Fig. 1.5: Geographic distribution of malaria

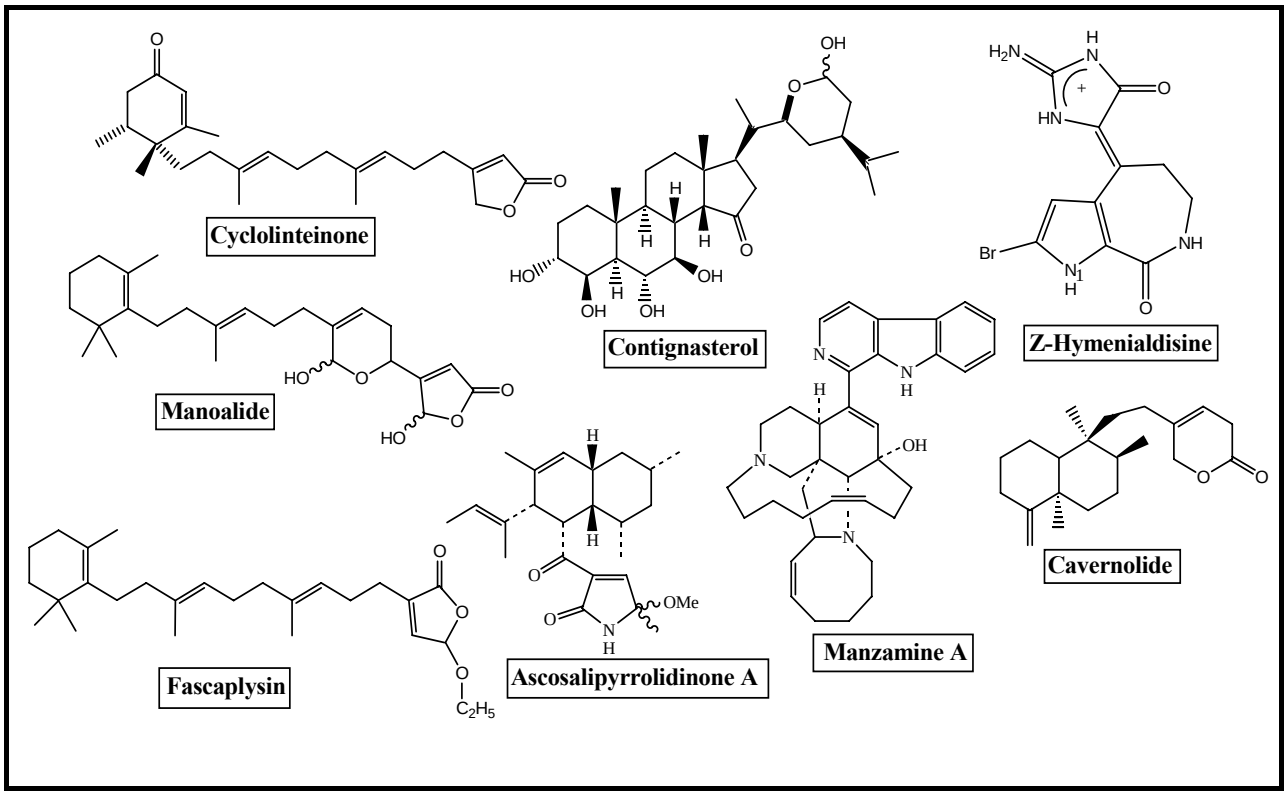


Fig. 1.6: Anti-inflammmatory, antimalarial and protein kinase inhibiting marine natural products

1.3.8. Anthelmintic activity of marine natural products

The parasitic nematodes pose a great health threat to both livestock and human beings. The growing resistance of nematodes to anthelmintics drugs necessitates the discovery of new and potent bioactive compounds against nematodes.

Dihydroxytetrahydrofuran isolated from *Notheia anomala* shows selective nematocidal activity [Capon, *et al*., 1998].

Amphilactams are isolated from a sponge *Amphimedon* sp. and proved to be effective against free-living stages of the parasitic nematode [Ovenden, *et al*., 1999].

Geodin A magnesium salt is a macrocyclic polyketide lactam tetramic acid was isolated from the sponge *Geodia* sp.. It shows a potent nematocidal activity [Capon, *et al*., 1999].

1.3.9. Immunosuppressive activity of marine natural products

Theonellapeptolides Ia, Id, and IId [Roy, *et al*., 2000] are cyclic peptides isolated from the marine sponge *Theonella swinhoei*. They have some common structural features with the cyclosporins, currently used clinically as immunosuppressants. The

tridecapeptide lactones were strongly immunosuppressive, possibly as a result of their cytotoxic effect.

Eicosapentaenoic and docosahexanoic acids [Mori, *et al*., 2000] are omega-3 fatty acids. Docosahexanenoic acid, but not eicosapentaenoic acid, enhanced the vasodilator mechanisms and constrictor responses in forearm circulation, thus contributing to selective blood-pressure-lowering effects. The authors proposed that these observations could be relevant to the food industry with respect to the possible inclusion of omega-3 fatty acids into food stuffs and animal feeds, and hence the human food chain.

4α-Methyl-5α-cholest-8-en-3β-ol and **4, 5-dibromopyrrole-2-carboxylic acid** were highly active in suppression of murine splencytes in the two-way mixed lymphocyte reaction (MLR). Both compounds were isolated from the marine sponge *Agelas flabelliformis* and could be useful in organ transplantations [Gunasekera, *et al*., 1989].

1.3.10. Nervous System and marine natural products

Reports on both central and autonomic nervous system pharmacology of marine natural products increased slightly over 1998 and 1999 [Mayer, *et al*., 2000].

Conantokin-G and **conantokin-T(G)** are small peptide toxins derived from the venom of marine snails of the genus *Conus*. They potentiated contralateral rotation induced by L-3,4-dihydroxyphenylalanine (L-DOPA) in 6-hydroxydopamine-treated rats [Adams, *et al*., 2000]. The 2 conantokins potentiated the behavioral effects of L-DOPA, probably by alterations in the function of striated efferent neurons.

Halitoxins are marine 1, 3 alkyl-pyridinium salts isolated from the marine sponge *Callyspongia ridleyi*, and reported by [Scott, *et al*., 2000]. Most notable is that because halitoxins form ion-permeable pores in biological and artificial membranes, allowing flux of monovalent (K^+, Na^+) and divalent (Ca^{2+}) cations, they could be useful in a number of applications, including the development of novel cytotoxic molecules and possibly as agents for intracellular drug delivery.

Stolonidiol is a marine diterpenoid isolated from the soft coral *Clavularia* sp. It demonstrated potent choline-acetyltransferase-inducible activity in neuronal cultures *in vitro* suggesting that stolonidiol or some of its derivatives may serve as leads in the

discovery of more useful agents with potential benefit to the cholinergic nervous system [Yabe, *et al*., 2000].

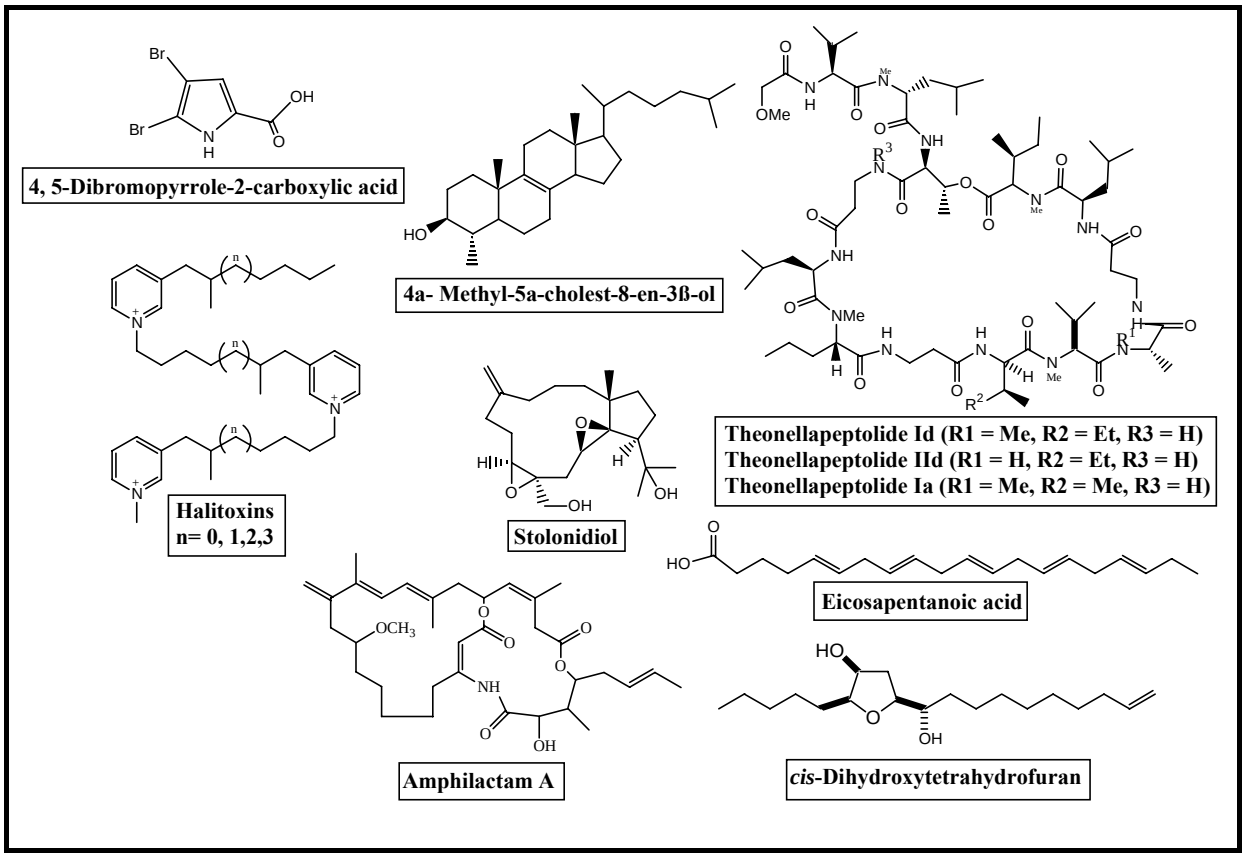


Fig. 1.7: Marine natural products with anthelmintic, immunosuppressant and central nervous system activities

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
Tridemnum 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 (IZP-94,005)

[d] semisynthetic pseudopterosin derivative

1.5. Anatomy of sponges

Sponges are aquatic animals that occur in all oceans and also in fresh water and have a wide distribution from tropical to temperate to arctic regions. They grow at all depths and in brackish and fresh water also. They may occur as thin encrusting coatings on rocks and wood, or as long thin branching fingers attached to the bottom, or in the typical rounded form. The outer surface of a sponge is covered with inhalant pores where the ambient water is drawn into sponge. These pores represent why sponges have been called the ´´Porifera´´ (pore-bearing) [Pearse, *et al*., 1987]. They lead internally to a system of canals and eventually out to one or more larger holes. These canals exist to move water through the sponge's body. Lining these canals are special collar cells. The collar cells force water through the sponge which brings oxygen and nutrients while removing carbon dioxide and waste.

A sponge has differentiated cells and functionally distinct layers. The amoebocytes secrete spicules which stack up together to form the sponges skeleton. The choanocytes are composed of a flagellum and a collar (collar cells). The amoebocytes and choanocytes are the work force of the sponge. They create the body of the sponge. The sponges reproduce either sexually or asexually. When they reproduce sexually, they usually cross-fertilize. Eggs and sperm unite to make a free-swimming larva that settles on a surface. Asexually, the sponge produces small, internal buds called gemules. These gemules each produce a new sponge. Sponges can also reconstitute themselves if their cells are separated into a suspension [Pechenik, 2000].

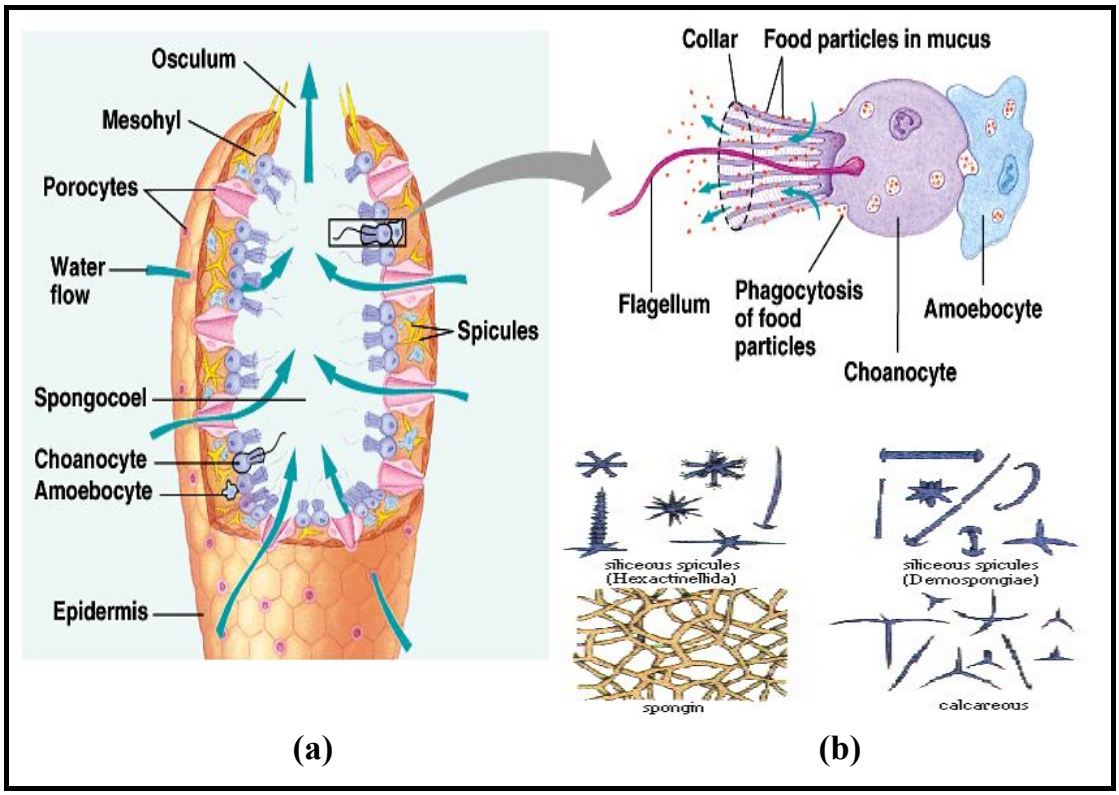


Fig. 1.8: Anatomy of sponges (a), and types of spicules (b)

1.6. Aim of the present study

Even today, after more than 100 years of research in the pharmaceutical industry, 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. Sponges are among the well researched marine organisms primarily because of their size and abundance. This study is focused on the isolation of biologically active secondary metabolites from different sponge extracts. The sponges used in this study were collected from Indonesia. The isolated compounds were evaluated for their cytotoxic and antiviral activities. Cytotoxicity was studied *in vitro* using mouse lymphoma (L5178Y), human cervix carcinoma (HeLa), and rat brain tumour (PC12) cell lines and with the brine shrimp (*Artemia salina*) lethality test. Antiviral experiments were conducted for the determination of the β-galactosidase activitiy caused by infecting a P4CCR5 cell line with HIV-1-wild type as vector. Different chromatographic techniques were used for the isolation of biologically active compounds like TLC, CC, VLC or semi-preparative HPLC.

2. Material and Methods

2.1. Biological materials

Samples include sponges collected in Indonesia. The sponges were collected by scuba diving and voucher specimens were identified by Dr. Rob W. M. van Soest of the Zoological 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 a series of biological assays for cytotoxicity and antibiotic activity.

2.1.1. *Callyspongia aerizusa*

Taxomony

Phylum: Porifera

Class : Demospongiae

Order : Haplosclerida

Family : Callyspongiidae

Genus : *Callyspongia*

Species: *Callyspongia aerizusa*

The sponges were collected by scuba diving at Ambon in Indonesia in 1996. The sponge consists of a dense mass of black-colored branches, which may reach an overall length of more than 1 m. The branches are 0.5-1 cm in diameter and have large numbers of evenly distributed small oscules with raised rims. The surface is smooth, and the ectosome is obscured by black-pigmented grains. The ectosomal skeleton is made from the usual double meshed reticulation of primary and secondary fibres (10-25 μm of diameter), making larger meshes of up to 400 μm subdivided into smaller meshes of 80-150 μm. All fibres are cored by a single spicule. The interior skeleton is irregularly rectangular. Primary fibres are 30-50 μm in diameter and have a core of 2-5 spicules. Secondary fibres are 12-20 μm and are singly cored. Spicules are composed of straight equidiametrical strongyles of 55-65 x 0.5-1.5 μm [Hooper, *et al*., 2002]. A voucher specimen has been deposited in the Zoological Museum, University of Amsterdam, under the registration no. ZMAPOR. 17717 (see Fig. 2.1).

2.1.2. *Diacarnus megaspinorhabdosa*

Taxomony

Phylum: Porifera

Class : Demospongiae

Order : Hadromerida

Family : Latrunculiidae

Genus : *Diacarnus*

Species: *Diacarnus megaspinorhabdosa*

The sponge was massive-amorphous, subspherical to tubular in shape, with one large atrium, and oscules on the walls of the atrium. The surface was tuberculate, rubbery, and sometimes with regularly distributed holes, 1-3 mm in diameter. The sponges were of a tough consistency, reddish pink and yellow colour internally [Hooper, *et al*., 2002]. The sponge was collected by diving at a depth of 8 m from Pulau Baranglompo Island south in Indonesia in July 1997. The sample was directly frozen after collection and stored at - 20° C. A voucher specimen has been deposited in the Zoological Museum, University of Amsterdam, under the registration no. ZMA POR. 17057 (see Fig. 2.2).

2.1.3. *Acanthostrongylophora ingens*

Taxomony

Phylum: Porifera

Class : Demospongiae

Order : Petrosida

Family : Petrosiidae

Genus: *Acanthostrongylophora*

Species: *Acanthostrongylophora ingens*

The sponges form irregular thick columns with a coarse surface. The oscules were several mm in diameter, on low hummoks or flush. Consistency is firm, incompressible, and crumbly. The colour is brown. The skeleton consists of reticulation of thick spicule tracts averaging 75 µm in diameter, forming rather squarishly rounded meshes of 200-280 µm in diameter [Hooper, *et al*., 2002]. Specimens of Acanthostronglyphora *ingens* were collected by scuba diving at depth of 4-5 m from Ujung Pandang Island in Indonesia in 1996. Freshly collected sponges

were frozen immediately after collection and then freeze-dried. A voucher specimen has been deposited in the Zoological Museum, University of Amsterdam, under the registration no. ZMAPOR. 17795 (see Fig. 2.3).

2.1.4. *Theonella swinhoei*

Taxomony

Phylum: Porifera

Class : Demospongiae

Order : Lithistida

Family : Theonellidae

Genus : *Theonella*

Species: *Theonella swinhoei*

The sponge was collected by diving at a depth of 11 m from Pulau Baranglompo Island in Indonesia in July 1997. A voucher specimen has been deposited in the Zoological Museum, University of Amsterdam, under the registration no. ZMAPOR.17078. The sponge's surface was uneven, pitted, ridged, and grooved, but smooth in between. Its consistency was hard. The skeleton of the surface was made up of a tangential layer of phyllotriaenes with cladomes up to 400-450 μm and individual cladi of 200-240 x 12-20 μm which formed a characteristic honeycombed-surface network with rounded spaces of 150-240 μm diameter. Scattered over the surface were numerous curved or flexuous 15-18 x 3 μm microrhabds with spined or rugose surface. The skeleton of the interior was almost completely "lithistid"; that is, there were comparatively few bundles of strongyles and all desmas appeared to be zygosed from the surface downward. The strongyles measured 250- 400 x 3-6 μm, often with wide axial canals or frequently broken. Desmas were polyrhabdose, with cladome up to 540 μm. Cladi were 35-40 μm in diameter, tuberculated, but smooth between tubercles. In the interior, microrhabds were frequent [Hooper, *et al*., 2002] (see Fig. 2.4).

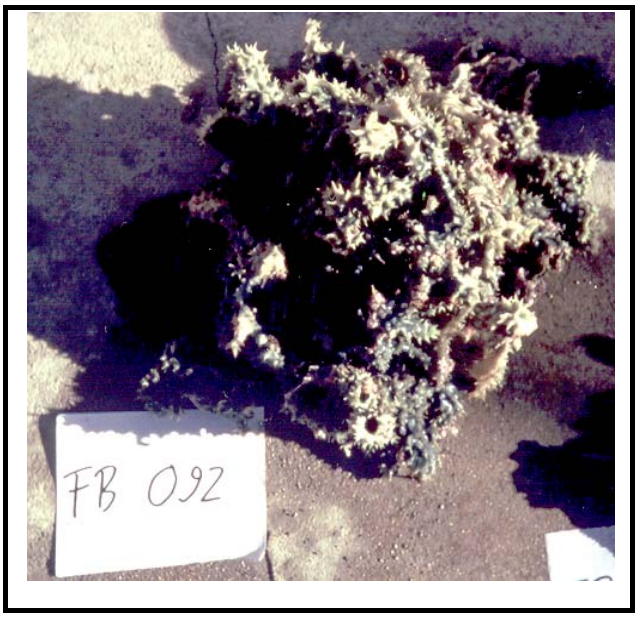


Fig. 2.1: ***Callyspongia aerizusa***

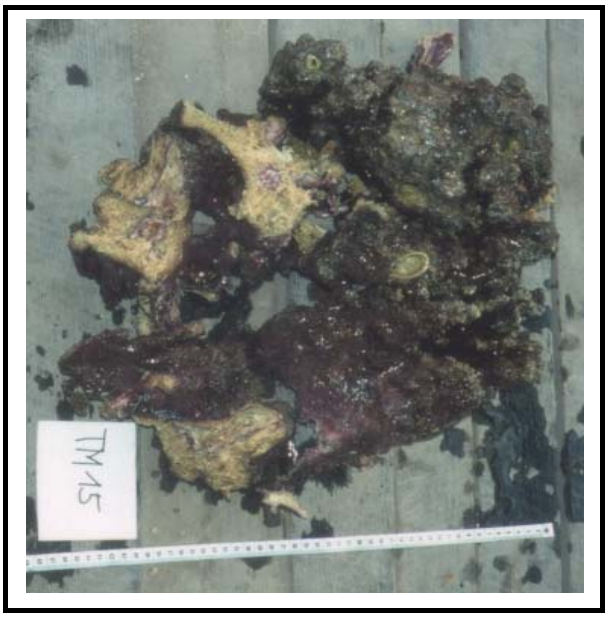


Fig. 2.2: ***Diacarnus megaspinorhabdosa***

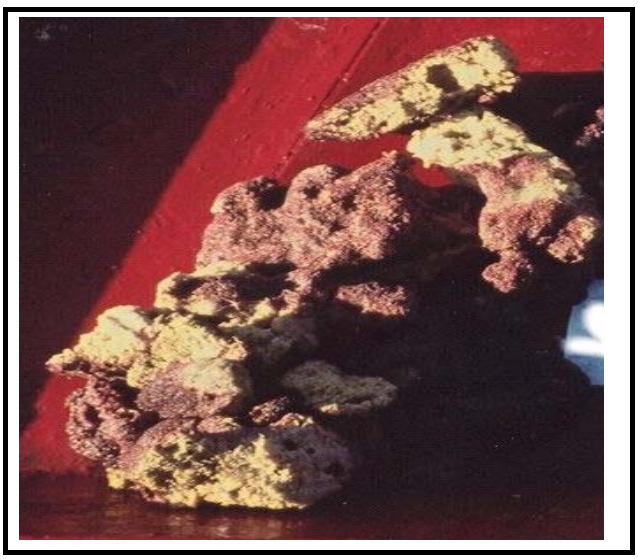


Fig. 2.3: ***Acanthostrongylophora ingens***

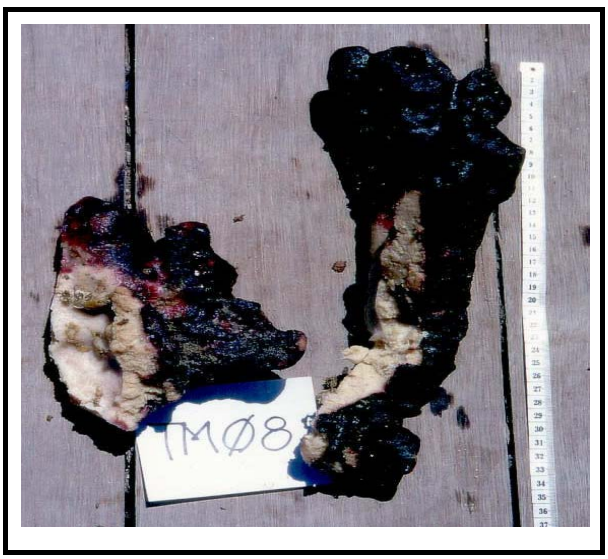


Fig. 2.4: ***Theonella swinhoei***

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
Hydrochloric acid	Merck
Potassium hydroxide	Merck
Pyridine	Merck
Concentrated sulphuric acid	Merck
Trifloroacetic acid (TFA)	Merck
Concentrated ammonia solution	Fluka
Acetic anhydried	Merck
Ortho-phosphoric acid 85% (p.a.)	Merck
Ninehydrin	Sigma
N-(5-flouro-2,4-dinitrophenyl)-L-Leucinamide	Tokyo Kasi
Amino acids standards	Sigma
Sodium hydrogen carbonate	Sigma

2.2.2. Solvents

2.2.2.1. General solvents:

Acetone

Acetonitrile

Dichloromethane

Ethanol

Ethyl acetate

Hexane

Methanol

Petroleum ether

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.

2.2.2.2. Solvents for HPLC:

Methanol was LiChroSolv HPLC (Merck), nano-pure water (distilled and heavy metals free water) was obtained by passing distilled water through nano- and ion-exchange filter cells (Barnstead, France).

2.2.2.3. Solvents for optical rotation:

Chloroform spectral grade	Sigma

Methanol spectral grade	Sigma
Water spectral grade	Fluka

2.2.2.4. Solvents for NMR:

Deuterated methanol, chloroform, dimethylsulfoxide, benzene and pyridine (Uvasol, Merck) were used for NMR measurements.

2.2.3. Chromatography

Pre-coated TLC plates (Aluminium, Silica Gel 60 F_{254}, layer thickness 0.2mm)	Merck
Silica Gel 60, 0.04-0.063 mm mesh size	Merck
Pre-coated TLC plates (glass), RP-18, F_{254} S, layer thickness 0.25 mm	Merck
RP-18, 0.04-0.063 mm mesh size	Merck
Amberlit XAD. Diaion PH 20, Dowex MCI Gel	Merck
Sephadex LH 20, 0.25-0.1 mm mesh size	Merck

2.3. Equipments used

Balances	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

Drying Ovens	Heraeus T 5050
Sonicator	Bandelin Sonorex RK 102
UV Lamp	Camag (254 and 366)
Vacuum desiccators	Solvent speed vac SPD 111V
	Savant Refrigerator Vapour Trap
	RVT400, Pump Savant VLP80

HPLC equipment

I. Semipreparative HPLC:

Pump:	Gynkotek M40
HPLC Program:	Gynkosoft (V. 5.4)
Detector:	Gynkotek Photodiode Array Detector UVD 340
Autosampler:	Gynkotek Autosampler GINA 50
Printer:	NEC P60

II. Dionex

HPLC program:	Chromeleon (V. 6.3)
Pump:	Dionex P580A LPG
Detector:	Dionex Photodiode Array Detector UVD 340S
Autosampler:	ASI-100T
Column thermostat:	STH 585

III. LC-MS:

MS spectrometer	Finnigan LCQ-DECA
HPLC system (pump, Detector and autosampler)	Agilent 1100 series

Column Knauer (125 mm L, 2 mm ID), pre-packed with Eurosphere-100 C18 (5 μm) and with integrated pre-column.

2.4. Chromatographic methods

2.4.1. Thin layer chromatography (TLC)

Chromatography refers to any separation method in which the components are distributed between stationary phase and mobile phase. The separation occurs because sample components have different affinities for the stationary and mobile phases and therefore move at different rates along the TLC plates and the column. TLC was

performed on pre-coated TLC plates with silica gel 60 F_{254} (layer thickness 0.2 mm, E. Merck**,** Darmstadt, Germany**)** with either CH_2Cl_2:MeOH (95:5, 90:10 and 80:20) for semi-polar compounds or Hexane:CH_2Cl_2:Aetone (90:10:10, 90:20:20 and 90:40:40) as eluent for non-polar compounds. TLC on reversed phase RP-18 F_{254} (layer thickness 0.25 mm, Merck, Darmstadt, Germany) was used for polar substances and using the different solvent systems of MeOH:H_2O (90:10, 80:20, 70:30 and 60:40). The band separation on the TLC describing the separation of compounds were detected under UV absorbance at 254 and 366 nm, followed by spraying the TLC plates with anisaldehyde reagent and subsequent heating at 110° C. TLC was always conducted for each fraction prior to further chemical work, to get an overview of the identity of each fraction and the qualitative purity of the fraction and the isolated compound.

Anisaldehyde/H_2SO_4 Spray Reagent (Per 100 mL)

Methanol:	85 mL
Glacial Acetic Acid:	10 mL
Conc. H_2SO_4:	5 mL (added slowly)
Anisaldehyde:	0.5 mL

The reagent was stored in an amber-coloured bottle and kept refrigerated until use. TLC was used to monitor the identity of each of the fractions and the qualitative purity of the isolated compounds. It was also utilised to optimise the solvent system that would be applied for column chromatography.

2.4.2. Vacuum liquid chromatography

Vacuum liquid chromatography (VLC) is a useful method for initial isolation procedure for large amounts of sample. The apparatus consists of a 500 cm sintered glass Büchner filter funnel with an inner diameter of 12 cm. Fractions were collected in Erlenmeyer flasks. Silica gel 60 was packed to a hard cake at a height of 5-10 cm under applied vacuum. The sample used was covered onto a small amount of silica gel using volatile solvents. The resulting sample mixture was then packed onto the top of the column. Step gradient elution with a non-polar solvent (Hexane) and increasing the amounts of a polar solvents (EtOAc, MeOH) gave successive fractions. The flow was produced by vacuum and the column was allowed to run dry after each fraction collected.

2.4.3. Column chromatography:

Fractions derived from VLC were subjected to repeated separation through column chromatography using appropriate stationary and mobile phase solvent systems previously determined by TLC. The following separation systems were used:

a) Normal phase chromatography uses a polar stationary phase, typically silica gel in conjunction with a non-polar mobile phase (*n*-Hexane, CH_2Cl_2, etc). Thus hydrophobic compounds elute more quickly than do hydrophilic compounds.

b) Reversed phase (RP) chromatography uses a non polar stationary phase and a polar mobile phase (water, methanol). The stationary phase consists of silica packed with n-alkyl chains covalently bound. For instance, C-8 signifies an octanyl chain and C-18 an octadecyl ligand in the matrix. The more hydrophobic the matrix on each ligand, the greater the tendency of the column to retain hydrophobic moieties. Thus hydrophilic compounds elute more quickly than do hydrophobic compounds.

c) Size exclusion chromatography involves separations based on molecular size of compounds being analysed. The stationary phase consists of porous beads. The larger compounds will be excluded from the interior of the bead and thus will elute first. The smaller compounds will be allowed to enter the beads and elute according to their ability to exit from the small sized pores they were internalised through.

2.4.4. Semi-preparative high pressure liquid chromatography (HPLC):

This process is used for isolation and purification of compounds. Semi-preparative HPLC equipment was used for the isolation of pure compounds from fractions previously separated using column chromatographic separation. The most appropriate solvent system should be found before running semi- preparative HPLC separation. The mobile phase combination was MeOH or acetonitrile and nanopure water with or without 0.01 % TFA, pumped in gradient or isocratic manner depending on the compounds retention time. Each injection consisted of 3 mg of the fraction dissolved in 1 mL of the solvent system. The solvent system was pumped through the column at a rate of 5 mL/min. The eluted peaks were detected by the online UV detector and collected separately in Erlenmeyer flasks. The separation column (125 × 4 mm, i.d.) was pre-filled with Eurospher C-18 (Knauer, Berlin, Germany).

2.4.5. Analytical HPLC (Dionex):

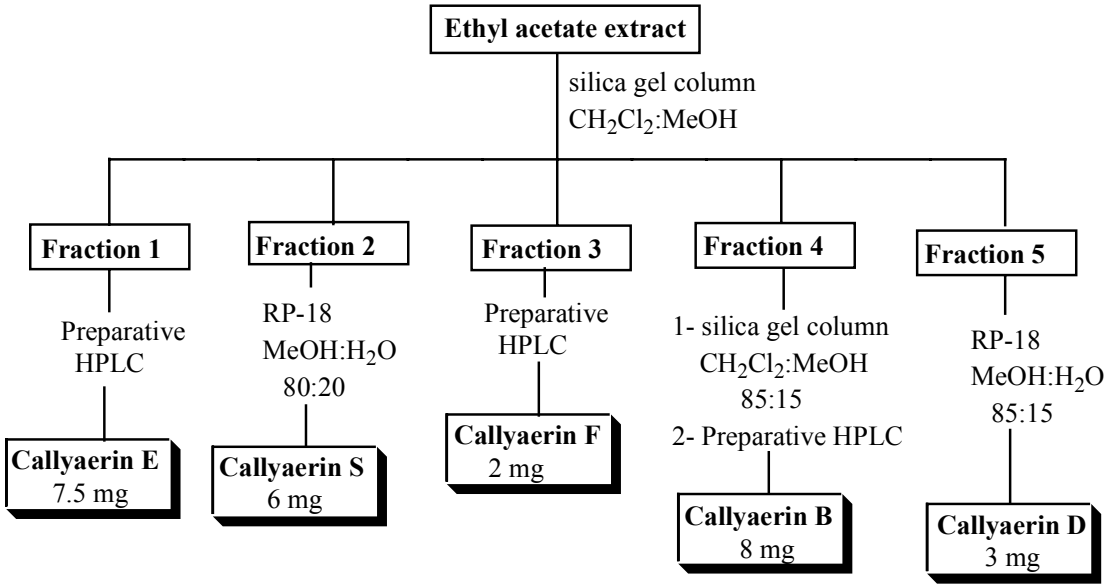
Analytical HPLC was used to identify the distribution of peaks either from extracts or fractions, as well as to evaluate the purity of isolated compounds. The solvent gradient used started with 10:90 [MeOH:nanopure H_2O (adjusted to pH 2 with phosphoric acid) to 100 % MeOH in 35 minutes. The autosampler took 20 µL sample. All peaks were detected by UV-VIS photodiode array detector. In some cases, special programs were used. HPLC instrument consists of the pump, the detector, the injector, the separation column and the reservoir of mobile phase.

2.4.6. Flash chromatography

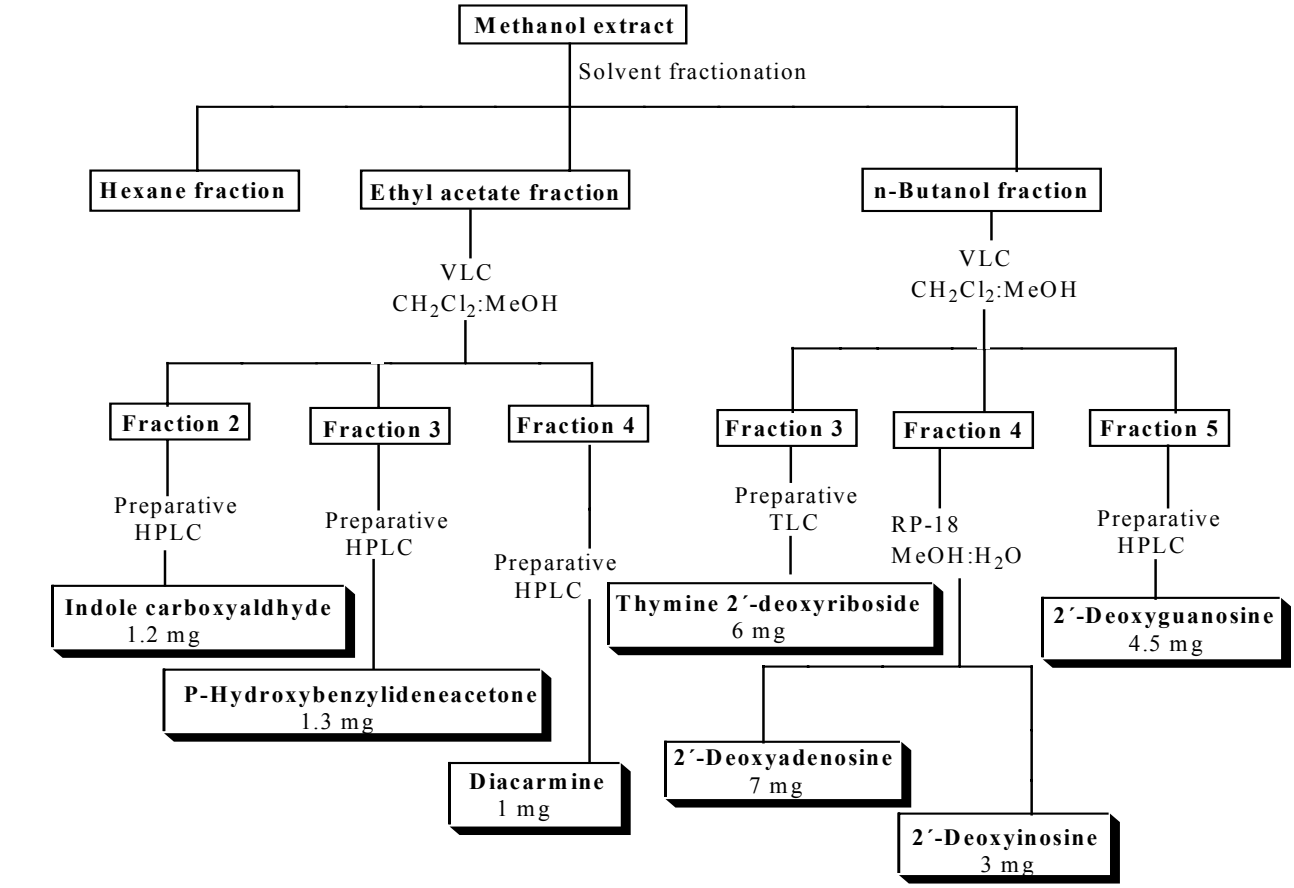
Flash chromatography was used for rapid isolation of compounds. A silica gel 60 GF_{254} pre-packed column was used and the sample was dissolved in a small volume of the initial solvent used. The resulting mixture was then packed onto the top of the column using special syringe. The flow rate was maintained by an air pump. The eluent was collected by a fraction collector and the separation was followed up by TLC.

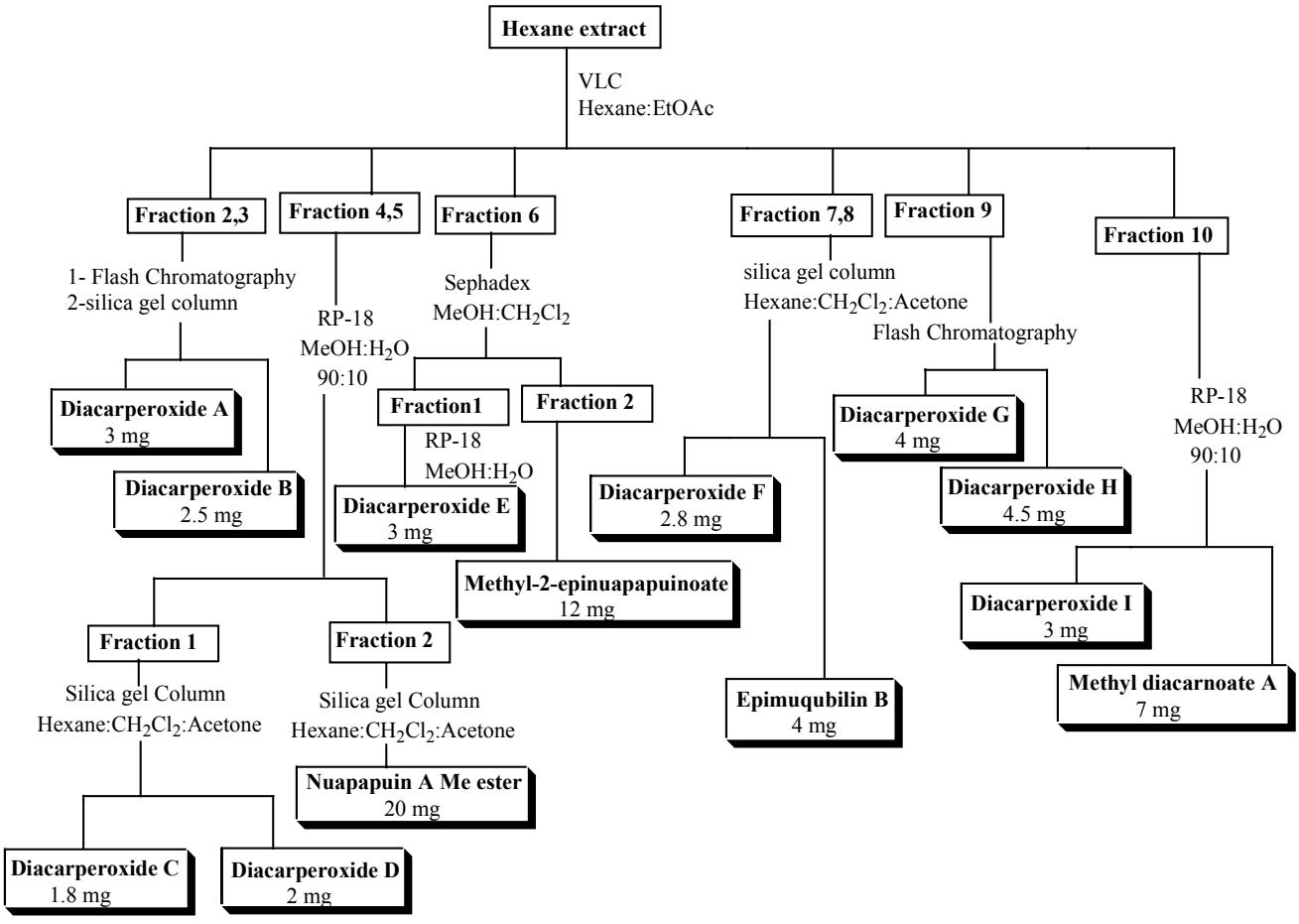
2.5. Procedure for the isolation of the secondary metabolites

2.5.1. Isolation of the secondary metabolites from *Callyspongia aerizusa*

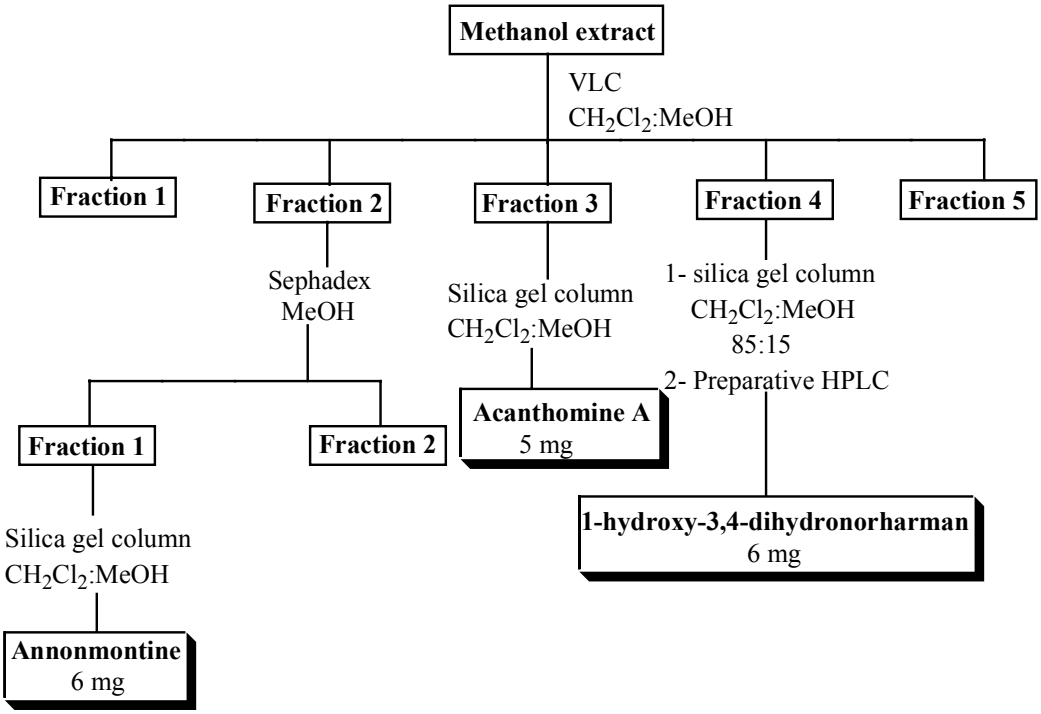


2.5.2. Isolation of metabolites from *Diacarnus megaspinorhabdosa*

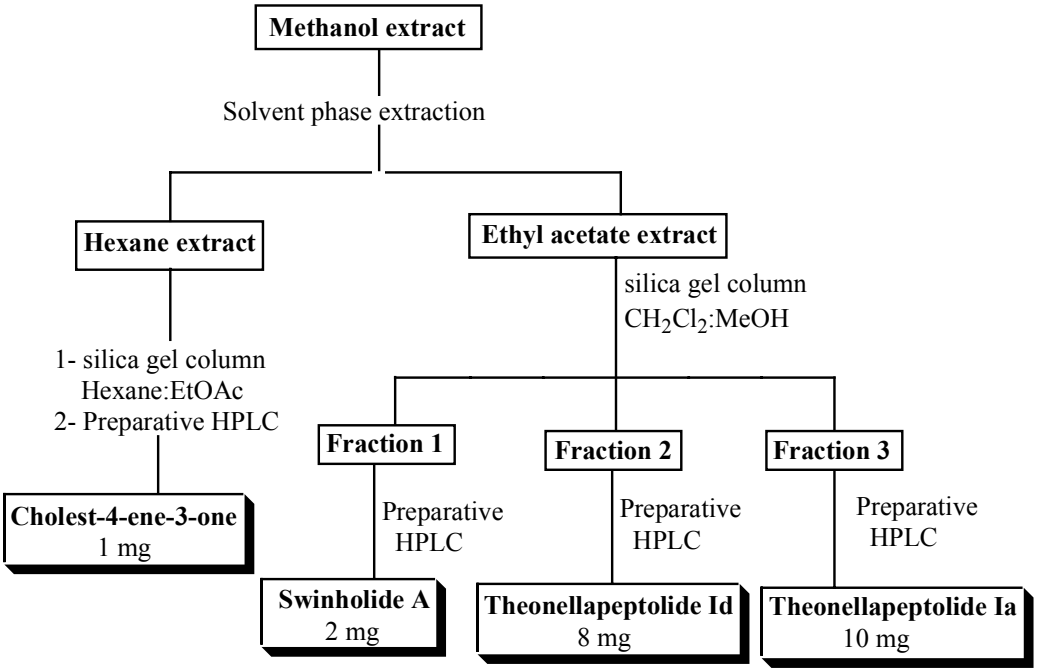




2.5.3. Isolation of metabolites from *Acanthostrongylophora ingens*



2.5.4. Isolation of metabolites from *Theonella swinhoei*



2.6. Structure elucidation of the isolated secondary metabolites:

2.6.1. Mass spectrometry (MS)

Mass spectrometers use the difference in mass-to-charge ratio (*m*/*z*) of ionised molecules to separate them from each other. Mass spectrometry is therefore useful for quantification of atoms or molecules and also for determination of chemical and structural information of molecules. Mass spectrometer consists of an ion source, ion detector and mass-selective analyser. The output of mass spectrometer shows a plot of relative intensity *vs* the mass-to-charge ratio (*m*/*z*).

Low resolution mass spectra were measured by EI and FAB-MS on a Finnigan MAT 8430 mass spectrometer. Measurements were done by Dr. Peter Tommes, Institute of Anorganische and Strukturchemie, Heinrich-Heine University, Düsseldorf.

EIMS (Electron impact mass spectroscopy)

Analysis involves vaporizing a compound in an evacuated chamber and then bombarding it with electrons having 25.80 eV (2.4-7.6 MJ/mol) of energy. The high energy electron stream not only ionises an organic molecule (requiring about 7-10 eV) but also causes extensive fragmentation (the strongest single bonds in organic molecules have strengths of about 4 eV). The advantage is that fragmentation is extensive, giving rise to a pattern of fragment ions which can help to characterise the compound. The disadvantage is the frequent absence of a molecular ion.

FABMS (Fast atom bombardment mass spectrometry)

It was the first widely accepted method that employs energy sudden ionisation. FAB is useful for compounds, especially polar molecules, unresponsive to either EI or CI mass spectrometry. It enables both non-volatile and high molecular weight compounds to be analysed. In this technique, a sample is dissolved or dispersed in a polar and relatively non-volatile liquid matrix, introduced into the source on a copper probe tip. Next, this matrix is bombarded with a beam of atoms of about 8 Kev. It uses a beam of neutral gas (Ar or Xe atoms) and both positive and negative ion FAB spectra can be obtained.

HRMS (High resolution mass spectrometry)

High resolution is achieved by passing the ion beam through an electrostatic analyser before it enters the magnetic sector. In such a double focusing 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 ions can be determined. HREIMS was measured at HKI für Naturstofforschung, Jena.

ESIMS (Electron spray ionisation mass spectrometry)

A sample solution was sprayed at atmospheric pressure into the source chamber to form droplets. The droplets carry charge when they exit the capillary, and as the solvent evaporates, the droplets disappear leaving highly charged analyte molecules. ESI is particularly useful for large biological molecules those are difficult to vaporise or ionise.

LC/MS (High pressure liquid chromatography (HPLC))

High pressure liquid chromatography is a powerful method for the separation of complex mixtures, especially when many of the components may have similar polarities. If a mass spectrum of each component can be recorded as it elutes from the LC column, quick characterisation of the components is greatly facilitated. Usually, ESI-MS (electron spray ionisation mass spectrometry) is interfaced with LC to make an effective on-line LC/MS. HPLC/ESI-MS was carried out using a Finnigan LC QDECA mass spectrometer connected to a UV detector. The samples were dissolved in water/MeOH mixtures and injected to HPLC/ESI-MS set-up. HPLC was run on a Nucleosil C-18 reversed-phase column. Measurements were done at the Institute of Pharmaceutical Biology, Heinrich-Heine University, Düsseldorf. For standard MS/MS measurements, a gradient of 10:90 (acetonitrile:nanopure H_2O (0.1 % HCOOH) to 100 % acetonitrile in 35 minutes was used.

MALDI TOF-MS (Matrix Assisted Laser Desorption Ionisation-Time of Flight mass spectrometry)

Matrix Assisted Laser Desorption Ionisation deals well with thermolabile, non-volatile organic compounds especially those of high molecular weight and is used successfully for the analysis of proteins, peptides, glycoproteins, oligosaccharides, and oligonucleotides. MALDI is based on the bombardment of sample molecules with a laser light to bring about sample ionisation. The sample is pre-mixed with a highly absorbing matrix compound. The matrix transforms the laser energy into excitation energy for the sample, which leads to sputtering of analyte and matrix ions from the surface of the mixture. The laser is fired, the energy arriving at the sample/matrix surface optimised, and data accumulated until a *m*/*z* spectrum of reasonable intensity

has been amassed. The time-of-flight analyser separates ions according to their mass (*m*)-to-charge (*z*) (*m*/*z*) ratios by measuring the time it takes for ions to travel through a field free region known as the flight.

2.6.2. Nuclear magnetic resonance spectroscopy (NMR):

Nuclear magnetic resonance is a phenomenon which occurs when the nuclei of certain atoms are immersed in a static magnetic field and exposed to a second oscillating magnetic field. Some nuclei experience this phenomenon, and others do not, dependent upon whether they possess a property called spin. It is used to study physical, chemical, and biological properties of matter. As a consequence, NMR spectroscopy finds applications in several areas of science. NMR spectroscopy is routinely used by chemists to study chemical structure using simple one-dimensional techniques. Two-dimensional techniques are used to determine the structure of more complicated molecules.

NMR measurements were done at the Institut für Anorganische Chemie und Makromolekulare Chemie of Heinrich-Heine University, Düsseldorf. ^{1}H and ^{13}C spectra were recorded at 300º 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 different solvents (i.e. DMSO-d_6, $CDCl_3$ and CD_3OD), the choice of which was dependent on the solubility of the samples. Residual solvent signals were used as internal standards (reference signal). The observed chemical shift (δ) values were given in ppm and the coupling constants (*J*) in Hz.

2.6.3. Optical activity

Optically active compounds contain at least one chiral centre. Optical activity is a microscopic property of a collection of these molecules that arises from the way they interact with light. Optical rotation was determined on a Perkin-Elmer-241 MC polarimeter. The substance was stored in a 0.5 mL cuvette with 0.1 dm length. The angle of rotation was measured at the wavelength of 546 and 579 nm of a mercury vapour lamp at room temperature (25º C). The specific optical rotation was calculated using the expression:

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

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

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

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

Where α = the measured angle of rotation in degrees,

I = the length in dm of the polarimeter tube,

c = the concentration of the substance expressed in g/100 mL.

2.7. Amino acid analysis of callyaerin peptides

N-(5-flouro-2,4-dinitrophenyl)-L-leucinamide contains a reactive flourine atom which can be used for the reaction with a mixture of L- and D-amino acids. The resulting diastereomers which are obtained in a quantitative yield can be separated and estimated by HPLC. L-diastereomers were eluted from the reverse-phase column before D-diastereomers, because of the stronger intramolecular hydrogen bonding (more hydrophobic) in the latter diastereomers [Marfey, 1984].

Hydrolysis of peptide

A solution (0.5-1 mg) of peptide was hydrolysed by treatment with 2 mL 6 N HCl (p.a.) and left at 112° C for 24 hr in a sealed ampoule. The solution was concentrated using N_2 gas.

Derivatization of the amino acids

Aqueous solutions of (50 mM) of amino acids were used. To 2.5 µmol amino acid (50 µL of 50 mM amino acid dissolved in H_2O) in Eppi, 3.6 µmol of F-DNP-L (N-(5-flouro-2,4-dinitrophenyl)-L-leucinamide) (100 µL of 1 % in acetone) was added, followed by 20 µmol of $NaHCO_3$ (= 20 µL of 1 M solution), the contents were mixed and heated over a hot plate at 40° C for 1 hr with frequent mixing. After cooling at

room temperature, 20 μmol of 2 M HCl was added to each reaction mixture. Complete the volume to 1000 μL with methanol. The samples were measured in LC-MS, the molecular weights and retention times were compared with standard amino acids.

2.8. Mosher reaction

Degradation of the cyclic peroxide

To a solution of nuapapuin A methyl ester (**6**) [10 mg in 1 mL dry ether] was added in excess $LiAlH_4$ (4 mg) and the resulting mixture stirred under reflux for 2 hr. The reaction was quenched by the addition of 10 % HCl (0.4 mL) and extracted with EtOAc. The reaction product was concentrated under vacuum, then purified by silica gel column using Hexane:EtOAc (9:1) to yield the triol derivative (**6a**), then ^{1}H NMR was measured in C_5D_5N.

Reaction of (6a) with (*R*)-MTPA acid

Compound **6a** (2 mg) was transferred into a NMR tube and was dried under vacuum. Deuterated pyridine (0.5 mL) and (*R*)-(-)-α-(trifluoromethyl) phenylacetyl chloride (6 μL) were added into the NMR tube immediately under a N_2 gas stream and then the NMR tube was shaken carefully to mix the sample and MTPA chloride evenly. The reaction NMR tube was permitted to stand at room temperature and monitored by ^{1}H NMR. The reaction was found to be complete after 72 hr. ^{1}H-^{1}H COSY was performed to confirm the assignment of the signals (Fig. 3.39 and Table 3.9).

Reaction of (6a) with (S)-MTPA acid

Another portion of compound **6a** (2 mg) was transferred into NMR tube. The reaction was performed in the same manner as described before to yield the (*S*)-MTPA ester (**6c**).

2.9. Biological Test Methods:

2.9.1. Brine-shrimp assay

This technique is an *in vivo* lethality test involving the whole body of a tiny crustacean, brine shrimp (*Artemia salina* Leach). It has been previously utilised in various bioassay systems including the analysis of pesticide residues, mycotoxins, stream pollutants, anaesthetics, dinoflagellate toxins, morphine-like compounds, toxicity of oil dispersants, cocarcinogenicity of phorbol esters and toxicants in marine

environments [Meyer, *et al*., 1982]. This test takes into account the basic premise that pharmacology is simply toxicology at a lower dose, and that toxic substances might indeed elicit, at a lower non-toxic dose, interesting pharmacological effects. The procedure determines LC_{50} values in μg/mL of active compounds and extracts in the brine medium.

Sample preparation. The test samples were dissolved in a suitable organic solvent and the appropriate amount was transferred to a 10 mL sample vial. Bioassay was done on 0.5 mg crude extract and various concentration of the pure compounds. The samples were then dried under nitrogen and reconstituted with 20 μL DMSO. Control vials containing the same amount of DMSO were also prepared.

Hatching of 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 a commercial salt mixture (33 g, Sera Sea-Salt, Aquaristik GmbH, Bonn, Germany) and distilled water (1000 mL). After 48 hr, 20 nauplii were collected by pipette (counted macroscopically in the stem of the pipette against a lighted background) and transferred into each test sample vial. Artificial sea water was then added to make 5 mL. The vials were maintained under illumination. Survivors were counted, with the aid of a magnifying glass after 24 hours and the mortality at each dose and control were determined. The LC_{50} values were determined using the probit analysis method. The LD_{50} was derived from the best fit line obtained by linear regression analysis.

2.9.2. Cytotoxicity test

Cytotoxicity tests were carried out by Prof. Dr. W. E. G. Müller (Mainz University, Germany), cancer cells used were L5178Y, Hela, and PC12.

The cytotoxicity against L5178Y mouse lymphoma cells, Hela cervix carcinoma cells, and PC12 brain tumour cells of the rats was determined using the microculture tetrazolium (MTT) assay, and compared to that of untreated controls [Carmichael, *et al.,* 1987]. From the test samples, stock solutions in ethanol 96 % (v/v) were prepared. Exponentially growing cells were harvested, counted and diluted appropriately. Of the cell suspension, 50 μL containing 3750 cells were pipetted into 96-well microtiter plates. Subsequently, 50 μL of a solution of the test samples containing the appropriate concentration was added to each well. The concentration range was 3 and 10 μg/mL. The small amount of ethanol present in the wells did not affect the experiments. The

test plates were incubated at 37° C with 5 % CO_2 for 72 hr. A solution of 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) was prepared at 5 mg/mL in phosphate buffered saline (PBS; 1.5 mM KH_2PO_4, 6.5 mM Na_2HPO_4, 137 mM NaCl, 2.7 mM KCl; pH 7.4) and from this solution, 20 µL was pipetted into each well. The yellow MTT penetrates the healthy living cells and in the presence of mitochondrial dehydrogenases, MTT is transformed to its blue formazan complex. After an incubation period of 3 hr and 45 min at 37° C in a humidified incubator with 5 % CO_2, the medium was centrifuged (15 min, 20° C, 210 x g) with 200 µL DMSO, the cells were lysed to liberate the formed formazan product. After thorough mixing, the absorbance was measured at 520 nm using a scanning microtiter-well spectrophotometer. The colour intensity is correlated with the number of healthy living cells. Cell survival was calculated using the formula:

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

All experiments were carried out in triplicates and repeated three times.

2.9.3. Test on the HIV-1-wild type and P4CCR5 cell lines

The isolated callyaerins were tested for antiviral activity. This experiment was conducted for the determination of the β-galactosidase activity caused by infecting a P4CCR5 cell line with HIV-1-wild type as vector, as well as cell proliferation (BrdU-test for determination of the cytotoxicity).

For the determination of the galactosidase activity the medium was separated from the cells after an incubation period of 2 days. The cells were washed one time with PBS solution. For fixation a 0.5 % glutaraldehyde solution was then equally distributed on the cells and left to stand for 10 min at room temperature. Then the cells were washed three times with PBS solution (1 x short; 10 min; 1 x short). The X-Gal solution was added to the cells. The tested concentrations were 1, 3, 10 and 15 µg/ml.

Consequently, the infected cells get blue colour and can be counted. The results were then compared to controls, which were not preincubated with the test substances. The activity could then be calculated as percentage of the control being considered as 100 % activity.

In this case cytotoxicity was determined by a cell proliferation-ELISA-test (BrdU) through chemoluminescence. This vital colouration is composed of a double colouration with flourescindiacetate and ethidiumbromide. Thereby the membrane permeability can be determined.

The cell line (P4CCR5) occurs in a density of 6 x 10^3 in white 96-well-plates (cellstar). On the following day, the medium was replaced using different concentrations of the compounds to be tested (1, 3, 10, and 15 µg/ml). Then it was incubated for 2 days at 37° C and 5 % CO_2, prior to labelling the cells with BrdU (5-bromo-2´-deoxuridine) for 19-22 hrs at 37° C. Following this FixDenate (Roche Molecular Biochemicals) was added and all were incubated for additional 30 min at room temperature.

Then Anti-BrdU POD-solution was added for labelling and incubated again for 30 min at room temperature. Finally, the plate was washed three times with the washing solution before pipetting the substrate and incubating for 3 min. Then the chemoluminescence was measured using a luminometer.

3. Results

3.1. Isolated compounds from the sponge *Callyspongia aerizusa*

Sponges belonging to the order Haplosclereida have been proven to be a rich source of polyacetylenic compounds with different chain lengths. Many of the reported polyacetylenic compounds showed significant biological activity [Youssef, *et al*., 2003]. Depsipeptides have been isolated from several species of the genus *Callyspongia*. New nematocidal depsipeptides, phoriospongin A and B were isolated from the Australian marine sponge *Callyspongia bilamellata* [Capon, *et al*., 2002]. Furthermore, callynormine A, is a cyclic peptide, which has been isolated from the southern Kenyan sponge *Callyspongia abnormis* [Berer, *et al*., 2004]. Similarly, callyaerins are also cyclic peptides that have been isolated from the Indonesian sponge *Callyspongia aerizusa* [Min, *et al*., 2001]. The ethyl acetate fraction of the sponge *Callyspongia aerizusa* collected from Indonesia in 1996 lead to the isolation of five callyaerin derivatives (**1-5**). The structures were elucidated on the basis of spectroscopic data (^{1}H NMR, ^{13}C NMR, COSY, TOCSY, ROESY and HMBC) in combination with mass spectrometery.

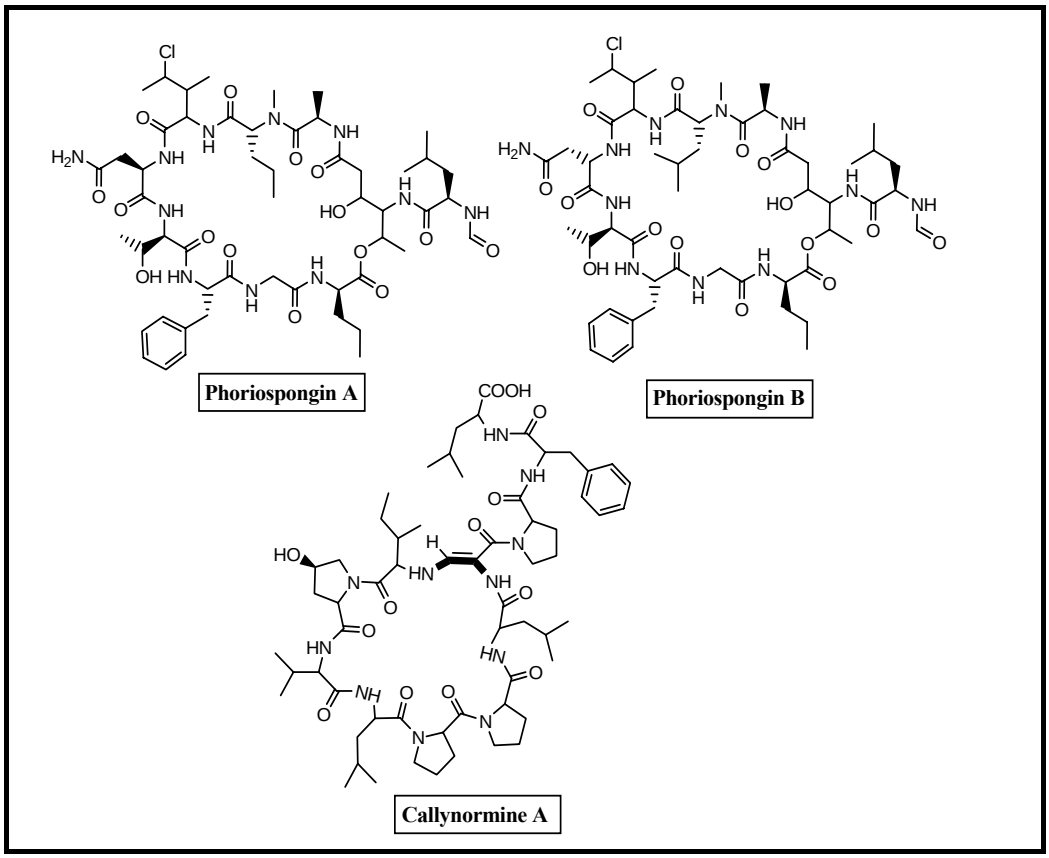
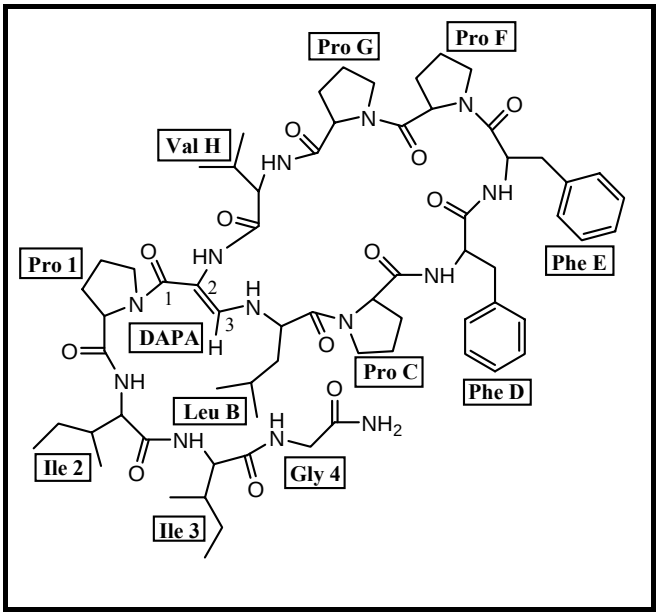


Fig. 3.1: Some reported peptides isolated previously from the genus *Callyspongia*

3.1.1. Callyaerin E (1, known compound)



Callyaerin E (**1**) was obtained as a white amorphous powder, with an $[\alpha]_D$ of - 68° (*c* 0.25, CH_3OH). It has UV absorbances at λ_{max} 206 and 282 nm. (+) ESIMS showed molecular ion peaks at *m*/*z* 1262 $[M+H]^+$ and 1284 $[M+Na]^+$, suggesting a molecular weight of 1261 g/mol. The molecular weight was also confirmed by the MALDI-TOF spectrum with intense ion peaks at *m*/*z* 1284 $[M+Na]^+$, 1300 $[M+K]^+$ and 865 $[M-(Pro+Ile+Ile+Gly+NH_2)]^+$. HRMS gave the molecular formula of $C_{66}H_{99}N_{13}O_{12}$. The ^1H NMR spectrum analysis revealed the peptidic nature of compound **1**, particularly noteworthy were the numerous deshielded amide N*H* resonances, cluster of α-amino acid methines, and a rich selection of aromatic protons, aliphatic primary and secondary methyls indicative of lipophilic amino acid residues. Careful inspection of TOCSY and COSY spectra showed the existence of eleven spin systems for eleven amino acid residues. One leucine was assigned at δ 5.27 (dd, *J* = 13.2, 10.4 Hz, B-N*H*), 4.32 (d, *J* = 10.4 Hz, H-Bα), 1.77, 1.23 (m, H_2-Bβ), 1.60 (H-Bγ), 0.96 (d, *J* = 6.6 Hz, H_3-Bδ), 0.94 (d, *J* = 6.6 Hz, H_3-Bδ′)]. Four proline spin systems were observed at [δ 4.01(m, H-Cα), 2.16, 1.67 (H_2-Cβ), 2.06, 1.88 (m, H_2-Cγ) and 3.93, 3.59 (H_2-Cδ)], [δ 4.36 (dd, *J* = 7.0, 8.2 Hz, H-1α), 2.22, 1.57 (H_2-1β), 1.78, 1.68 (m, H_2-1γ) and 3.57, 3.21 (H_2-1δ)], [δ 4.41(m, H-Fα), 2.15, 1.82 (H_2-Fβ), 1.93, 1.89 (m, H_2-Fγ) and 3.49, 3.42 (H_2-Fδ)], and [δ 4.16 (dd, *J* = 9.8, 7.9 Hz, H-Gα), 2.22, 1.41 (H_2-Gβ), 1.74, 1.59 (m, H_2-Gγ) and 3.34, 2.48 (H_2-Gδ)] attributed to Pro C, Pro 1, Pro F and Pro G,

respectively. Furthermore, two phenylalanine spin systems were assigned at δ 7.76 (d, *J* = 6.9 Hz, D-N*H*), 4.39 (m, H-Dα), 3.00 (m, H_2-Dβ), 7.17-7.30 (phenyl ring protons) (Phe D) and δ 7.31 (d, *J* = 6.9 Hz, E-N*H*), 4.63 (m, H-Eα), 3.00, 2.71 (H_2-Eβ), 7.17-7.30 (phenyl ring protons) (Phe E). The TOCSY spectrum also showed a spin system characteristic for valine amino acid at δ 7.03 (d, *J* = 10.0 Hz, H-N*H*), 4.56 (dd, *J* = 10.0, 3.8 Hz, H-Hα), 2.33 (m, H-Hβ), 0.97 (d, *J* = 6.8 Hz, H_3-Hγ) and 1.06 (d, *J* = 6.9 Hz, H_3-Hγ′). In addition, two spin systems for two isoleucine amino acid residues were observed at [δ 7.62 (d, *J* = 7.9 Hz, 2-N*H*), 3.98 (m, H-2α), 1.95 (m, H-2β), 1.45, 1.21 (H_2-2γ) and 0.85 (d, *J* = 6.9 Hz, H_3-2δ), 0.88 (d, *J* = 6.9 Hz, H_3-2δ′)] and [δ 7.37 (d, *J* = 8.2 Hz, 3-N*H*), 4.07 (t, *J* = 7.6 Hz, H-3α), 1.82 (m, H-3β), 1.39, 1.27 (H_2-3γ) and 0.80 (d, *J* = 6.7 Hz, H_3-3δ), 0.87 (d, *J* = 6.9 Hz, H_3-3δ′)]. A spin system for a glycine amino acid was observed at [δ 7.88 (t, *J* = 6.3 Hz, 4-N*H*) and 3.72, 3.57 (H_2-4α)]. An uncommon spin system at [δ 8.36 (s, DAPA-N*H*) and 7.28 (d, *J* = 14.2 Hz, H-3)] was assigned to 2,3-diamino-2-propenoic acid (DAPA). The assignment of the spin systems was supported by the correlations appearing in the ^{1}H-^{1}H COSY spectrum. The sequence of amino acids, the position of the DAPA, the terminal NH_2 and the cyclic structure of callyaerin **E** were established by interpretation of HMBC and ROESY experiments. ROESY correlations from NH_2 terminal to Gly 4-N*H* and Gly 4-αCH_2 established the attachment of NH_2 to Gly 4. Gly 4-N*H* showed correlations to Ile 3-N*H*, Ile 3-αC*H*, Ile 3-βC*H*, and Ile 3-δCH_3 established the connectivity between Gly 4 and Ile 3 [substructure **A**], which was confirmed by the HMBC correlations of Gly 4-N*H* to Ile 3-CO and Ile 3-αC. ROESY correlations of Ile 2-N*H* to Pro 1-αC*H* and Pro 1-βCH_2 indicated the amide linkage of Ile 2 and Pro 1 [substructure **B**], which was proven by the HMBC correlations of Pro 1-δCH_2 to Ile 2-CO, Ile 2-βC and Ile 2-γC. The amide bond between substructures **A** and **B** was established by the ROESY correlations of Ile 2-N*H* to Ile 3-N*H* and Ile 3-αC*H*. This substructure was further confirmed by ESI and MALDI-TOF MS spectra, which gave a fragment ion at *m*/*z* 865 for M-(NH_2+Gly 4+Ile 3+Ile 2+Pro 1). The DAPA-N*H* showed a ROESY correlation to Leu B-αC*H*, and also a COSY correlation of H-3 to Leu B-N*H* assigned the amide linkage of DAPA to Leu B, which was justified by the

HMBC correlation of H-3 to Leu B-αC and Leu B-N*H* to C-2 to give substructure **C**. The DAPA-N*H* correlations to Pro 1-αC*H* and Pro 1-δCH_2 assured the connectivity between substructures **C** and **B**. Pro C and Phe D were linked to give the dipeptide substructure **D**, which was proven by the ROESY correlations of Phe D-N*H* to Pro C-αC*H*, Pro C-βCH_2 and Pro C-γCH_2, as well as, HMBC correlation of Phe D-N*H* to Pro C-αC. Substructures **C** and **D** were connected through the observed ROESY correlations of Leu B-αC*H* to Pro C-αC*H*, Pro C-βCH_2. From its ROESY spectrum, correlations of Phe E-αC*H* and Phe E-βCH_2 to Pro F-δCH_2 indicated the connectivity of Phe E and Pro F [substructure **E**]. The amide bond between substructures **E** and **D** was again confirmed by the ROESY correlation of Phe E-N*H* to Phe D-N*H*, and also the HMBC correlation of Phe E-N*H* to Phe D-CO. Pro G-αC*H* showed correlation to Val H-αC*H* which established the amide linkage of Pro G and Val H, and was confirmed by the HMBC correlation of Val H-N*H* to Pro G-αC [substructure **F**]. The linkage between substructures **E** and **F** was established through the ROESY correlation of Pro G-αC*H* to Pro F-αC*H*. ROESY correlation of the DAPA-N*H* to Val H-N*H* and Val H-αC*H*, and also its HMBC correlation to Val H-CO established the cyclisation via the amide bond formed between DAPA and Val H. The absolute stereochemistries of the individual amino acid constituents of Callyaerin E (**1**) were determined following acid hydrolysis of the parent peptide and consequent derivatization with Marfey´s reagent. Stereochemical assignments were made using LC-MS technique to compare the amino acids of callyaerin **E** with appropriate standards (Fig. 3.10). The LC-MS analysis of the resulting 1-flouro-2,4-dinitrophenyl-5-L-leucinamide derivatives established the presence of two L-phe, one L-Val, three L-Pro, one D-Pro, one L-Leu, two L-Ile and Gly. Comparison of the NMR data and molecular weight of compound **1** with those of the previously reported callyaerin **E** isolated from *Callyspongia aerizusa* in combination with a careful inspection of the COSY, HMBC, TOCSY and ROESY spectra allowed the complete assignment of all signals. Compound **1** was identical with the known compound **callyaerin E** [Min, *et al*., 2001].

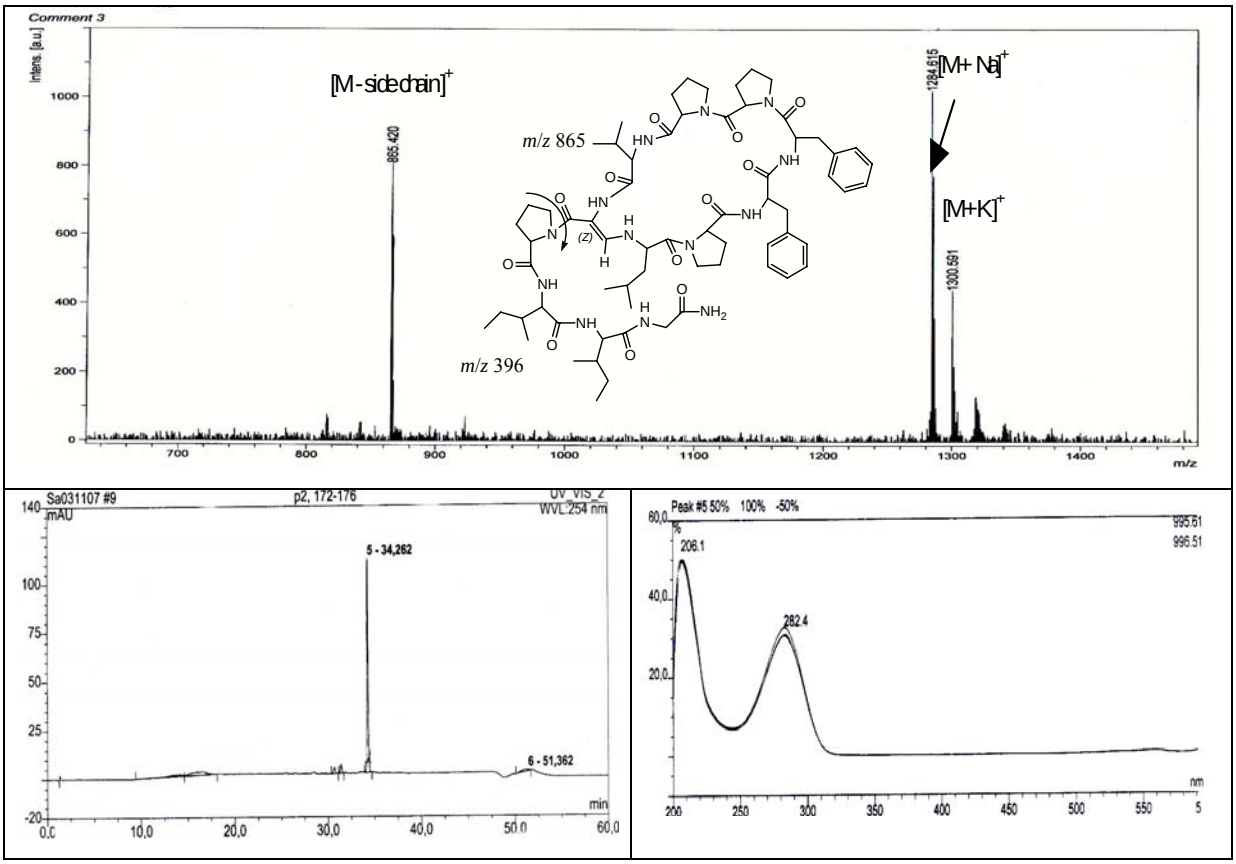


Fig. 3.2: MALDI-TOF MS and UV spectra of compound 1

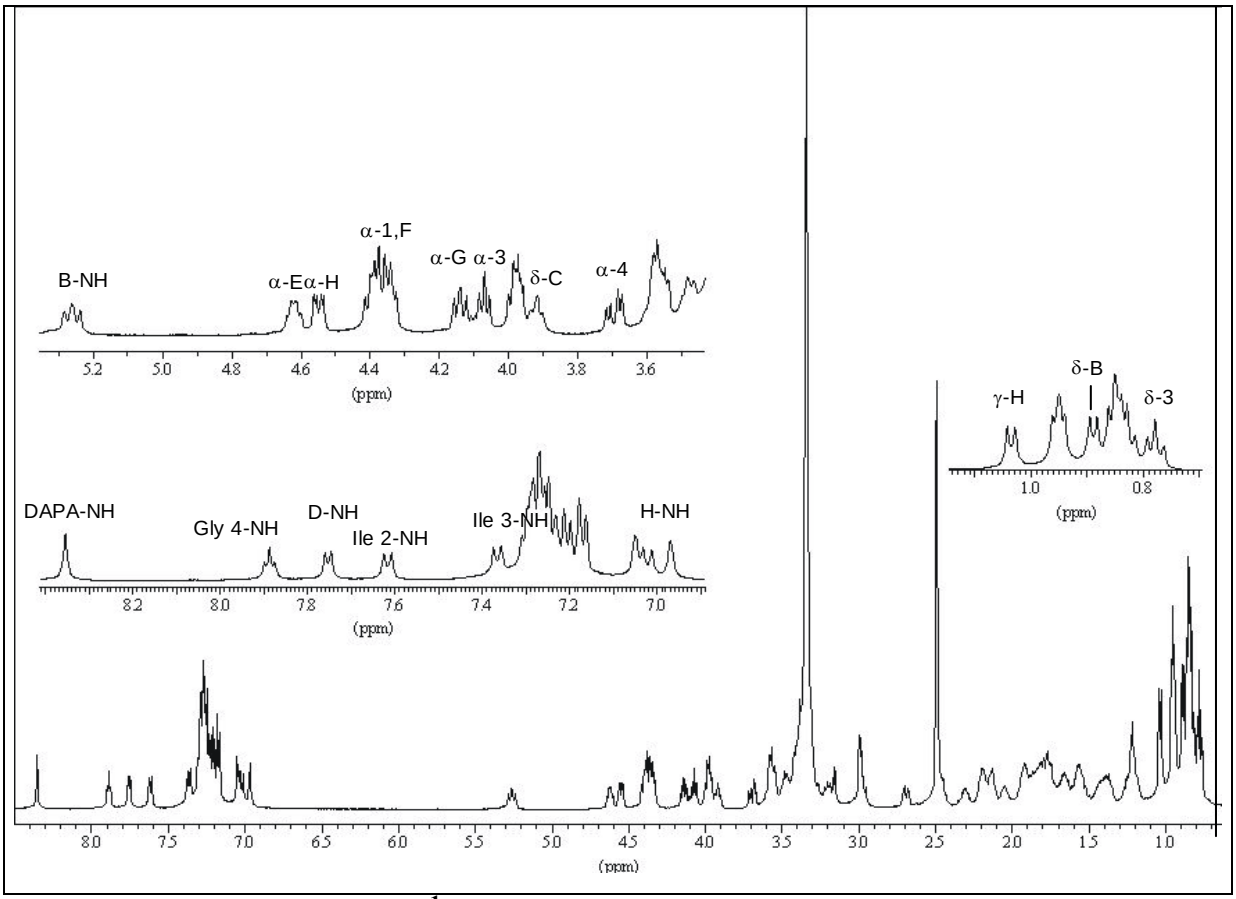


Fig. 3.3: ^{1}H NMR spectrum of compound 1

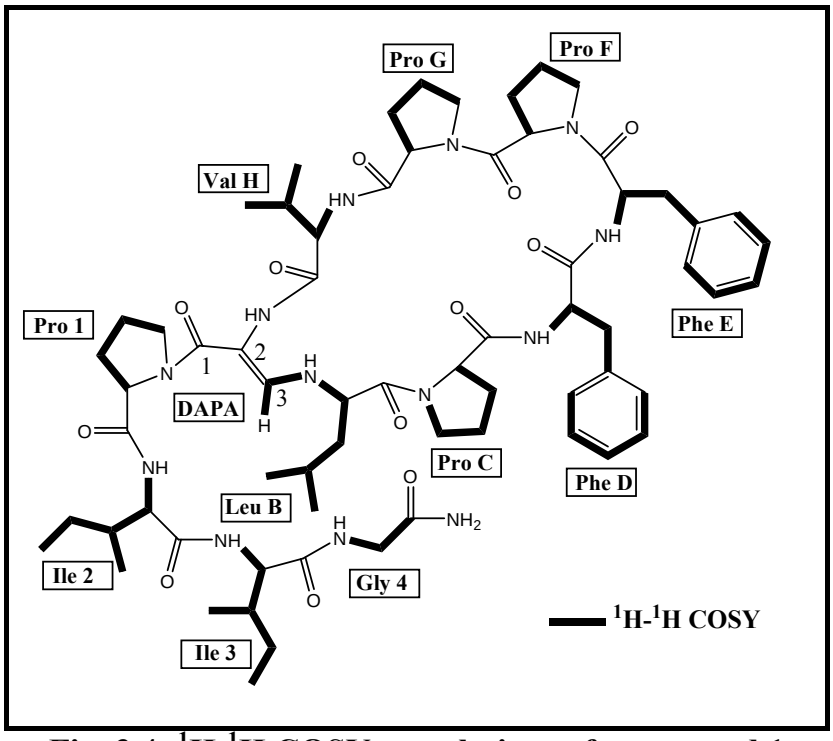


Fig. 3.4: ^{1}H-^{1}H COSY correlations of compound 1

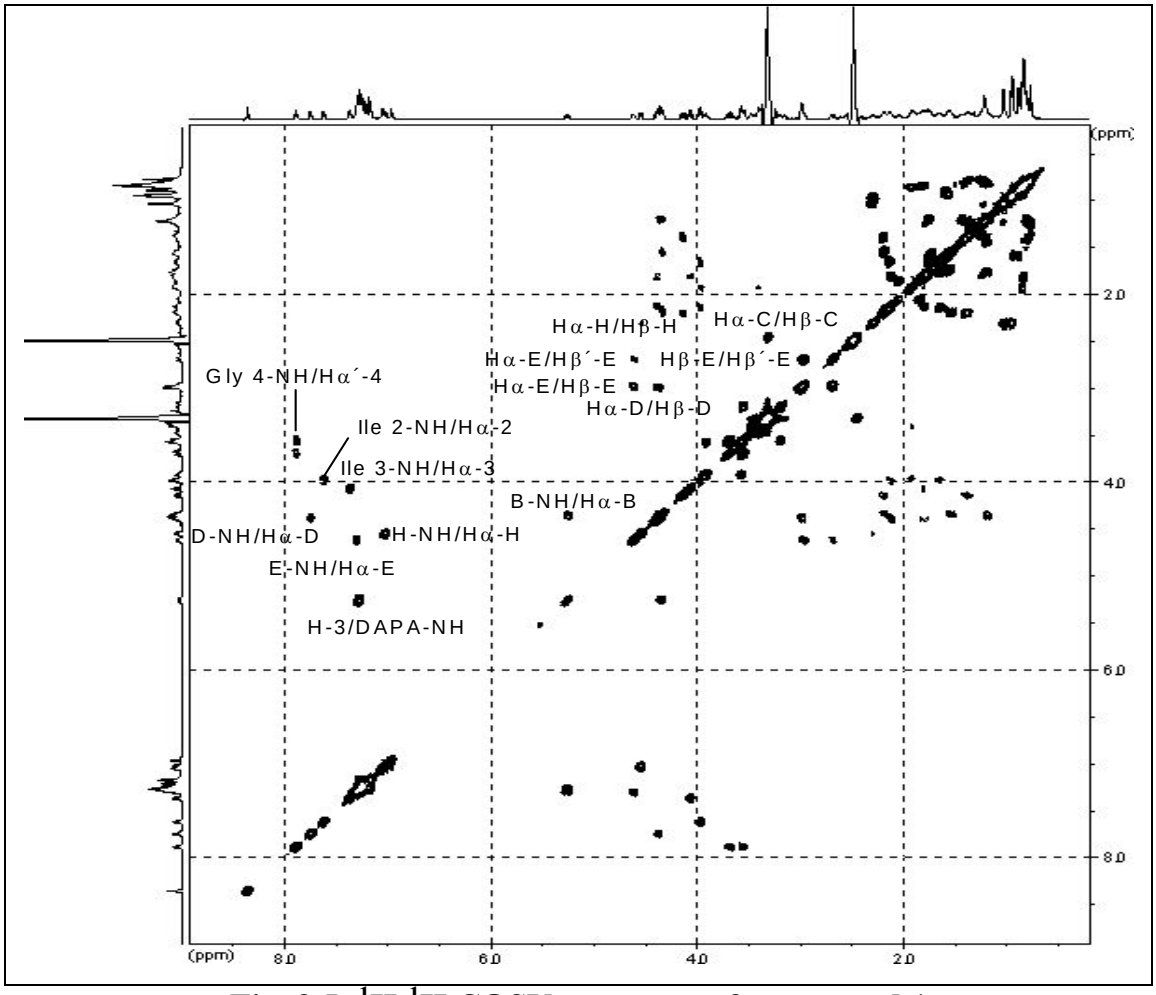


Fig. 3.5: ^{1}H-^{1}H COSY spectrum of compound 1

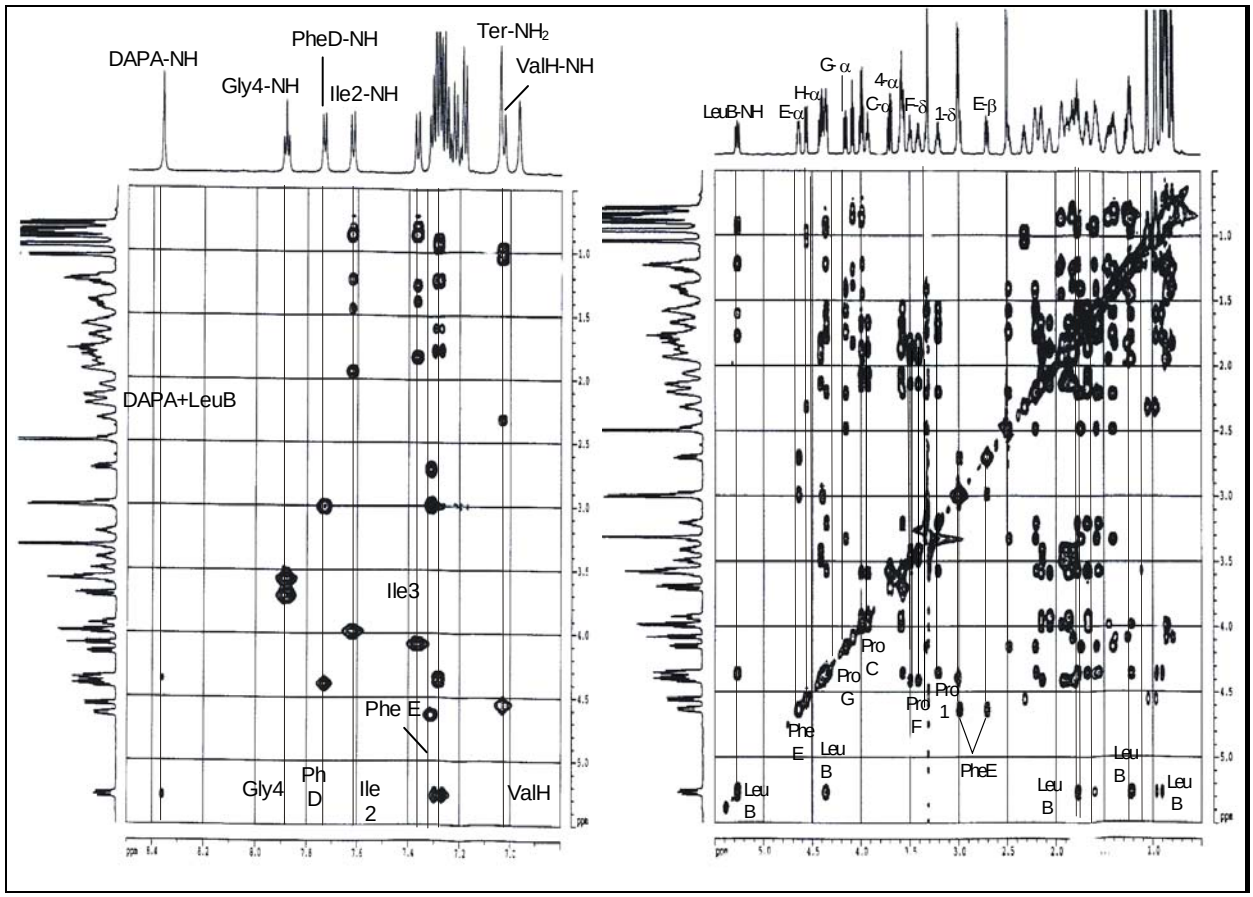


Fig. 3.6: TOCSY spectrum of compound 1

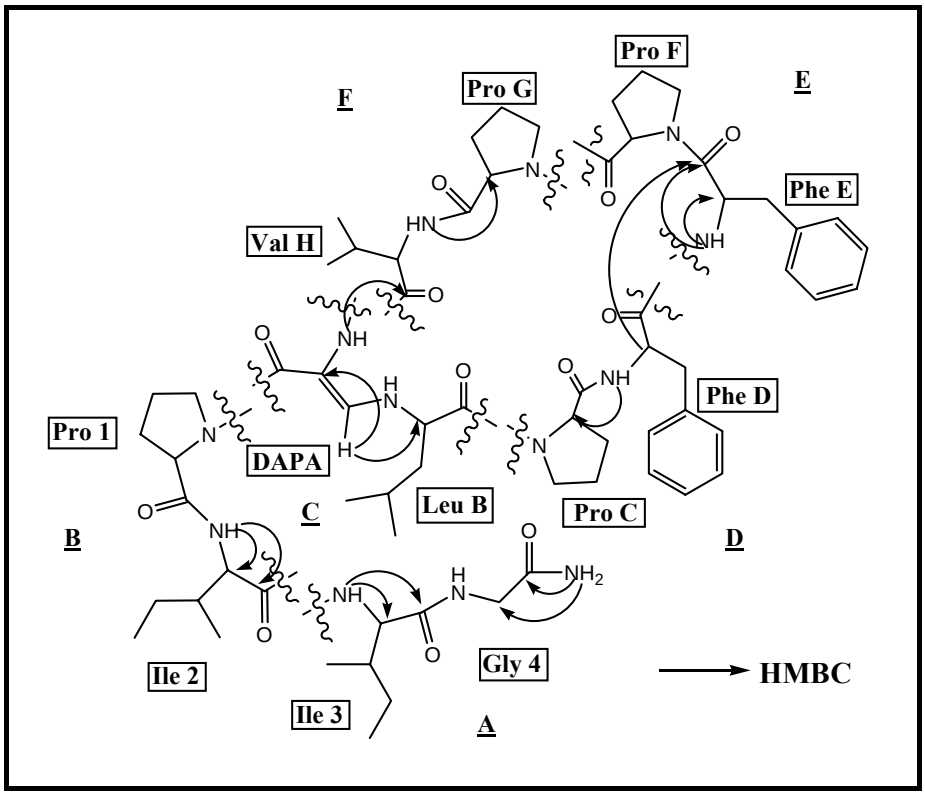


Fig. 3.7: Partial substructures A-F and HMBC correlations of compound 1

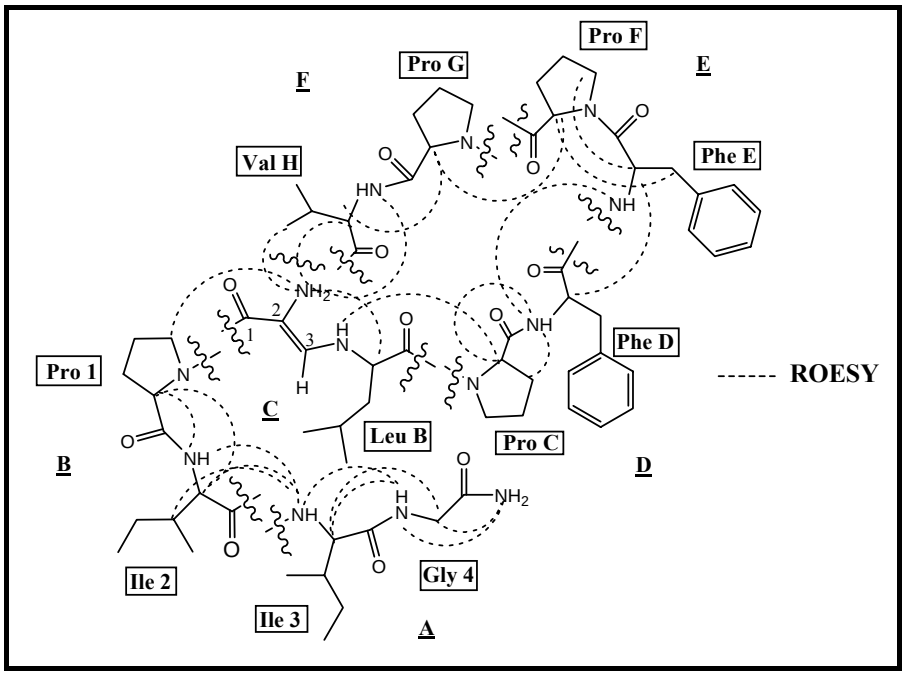


Fig. 3.8: Partial substructures A-F and ROESY correlations

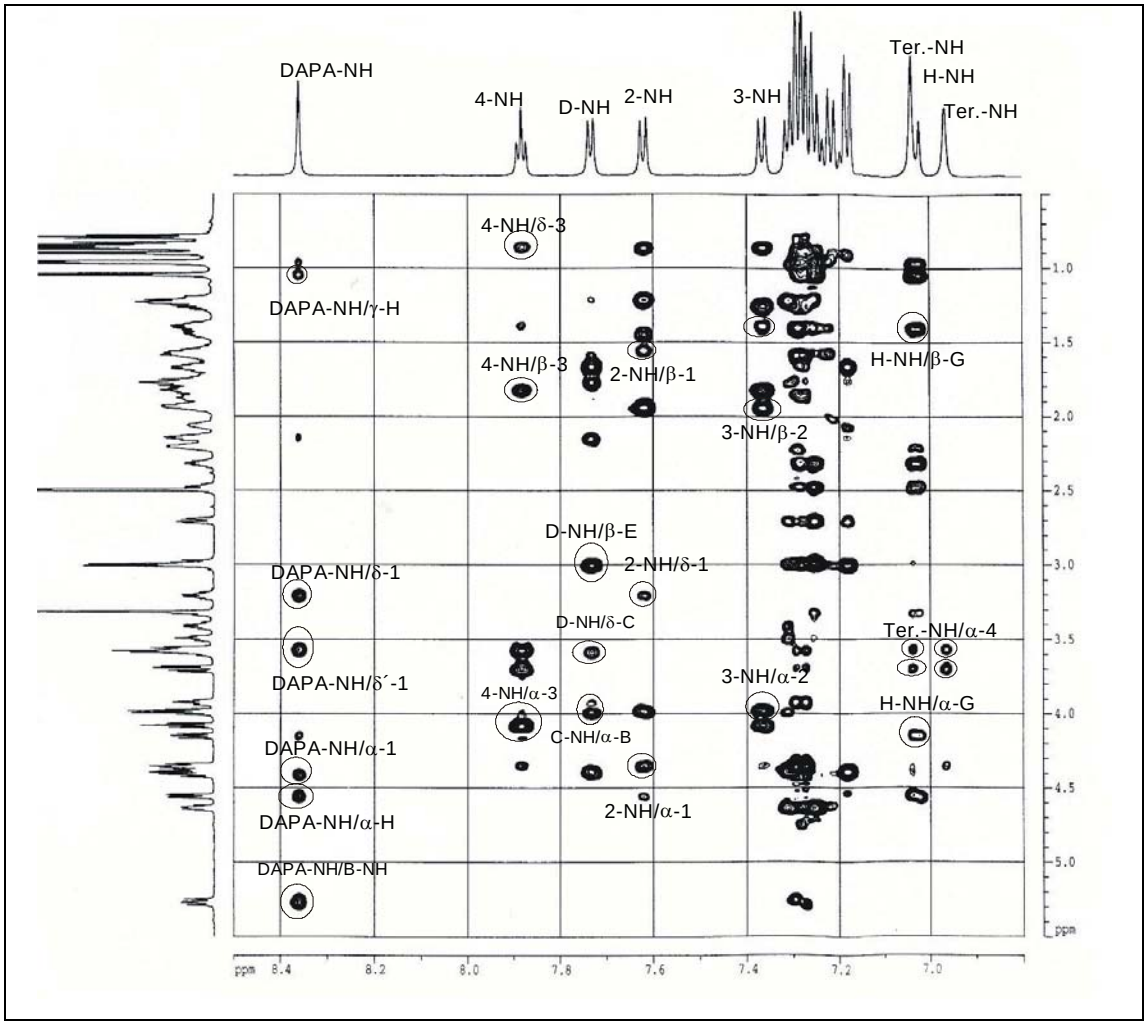


Fig. 3.9: Part of the ROESY spectrum of compound 1

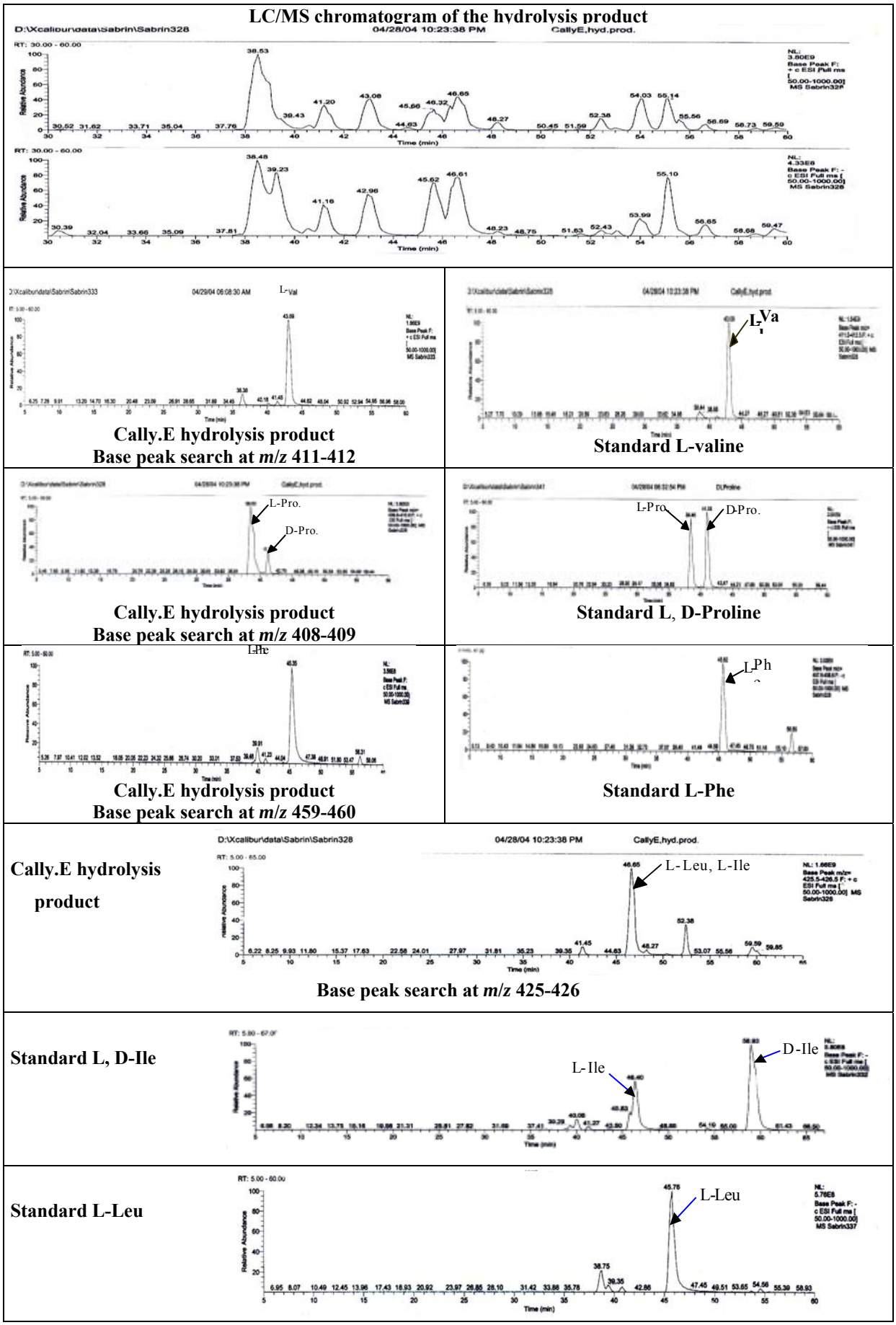
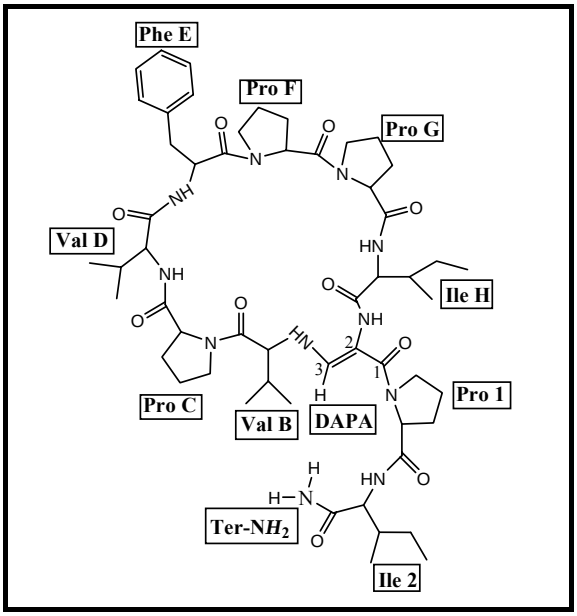


Fig. 3.10: Amino acids analysis results of compound 1

Table 3.1: NMR data of compound 1 (DMSO-d_6, 500 MHz)

A. A.	Position	δ_H (m, *J* in Hz)	ROESY	COSY	HMBC
DAPA	N*H*	8.36 (s)	H-N*H*, H-γ, B-α, H-α, 1-δ		DAPA-C=O, H-C=O
	H-3	7.28 (d, *J* = 14.2 Hz)		B-N*H*	DAPA -C=O,C-2
NH_2 *terminal*		7.04 (brs)	4-N*H*, 4-α		4-C=O, 4-α
		6.97 (brs)			
Leu B	α	4.32 (d, *J* = 10.4 Hz)	C-α, C-β, 1-β, H-γ	B-N*H*	
	β	1.77 (m), 1.23 (m)			
	γ	1.60 (m)			
	δ, δ`	0.96 (d, *J* = 6.0 Hz), 0.94 (d, *J* = 6.6 Hz)			
	N*H*	5.27 (dd, *J* = 13.2, 10.4 Hz)		C-α	DAPA-α
Pro C	α	4.01 (m)	B-γ, B-δ	C-β	C-C=O, C-γ
	β	1.67 (m), 2.16 (m)	B-α		
	γ	1.88 (m), 2.06 (m)			
	δ	3.59 (m), 3.93 (t, *J* = 8.8 Hz)			
Phe D	α	4.39 (m)	E-α	D-β	
	β	3.00 (m)		D-α	D-α, D-C=O, 1´´, 2´´
	N*H*	7.76 (d, *J* = 6.9 Hz)	E-N*H*, E-α, C-α, C-β, C-γ	D-α	D-α, D-β, D-C=O
	others	7.17-7.30			
Phe E	α	4.63 (m)	F-δ, D-β	E-β	E-β, E-C=O, D-C=O
	β	3.00 (m), 2.71 (dd, *J* = 13.2, 4.1 Hz)	F-α, H-γ		E-α, E-C=O, 1´ , 2´, 6´
	N*H*	7.31 (d, *J* = 6.9 Hz)	D-N*H*	E-α	E-β, E-C=O, D-C=O
	others	7.17-7.30			
Pro F	α	4.41 (m)		F-β	
	β	2.15 (m), 1.82 (m)			
	γ	1.89 (m), 1.93 (m)			
	δ	3.42 (m), 3.49 (t, *J* = 8.8 Hz)	D-α		
Pro G	α	4.16 (dd, *J* = 7.9, 9.8 Hz)	H-α, F-α	G-β	G-C=O
	β	2.22 (m), 1.41 (m)			
	γ	1.59 (m), 1.74 (m)			
	δ	3.34 (m), 2.48 (m)			
Val H	α	4.56 (dd, *J* = 10.0, 3.8 Hz)	C-α, C-β, 1-β, H-γ	H-β	H-γ, H-β, H-C=O
	β	2.33 (m)			
	γ	0.97 (d, *J* = 6.8), 1.06 (d, *J* = 6.9 Hz)			
	N*H*	7.03 (d, *J* = 10.0 Hz)		H-α	H-α, H-β, H-C=O
Pro 1	α	4.36 (dd, J = 8.2, 7.0 Hz)	2-α, B-α, 2-δ, H-γ, 2-β	1-β	
	β	2.22 (m), 1.57 (m)	H-α, 2-γ		
	γ	1.68 (m), 1.78 (m)			
	δ	3.21 (m), 3.57 (m)	B-α, B-β		
Ile 2	α	3.98 (m)	1-α, 1-β, 3-β, 1-δ	2-β	2-β, 2-C=O, 2-γ, 2-δ
	β	1.95 (m)			
	γ	1.45 (m), 1.21 (m)			
	δ, δ`	0.85 (t, *J* = 6.9 Hz), 0.88 (d, *J* = 6.9 Hz)			
	N*H*	7.62 (d, *J* = 7.9 Hz)	3-N*H*, 1-α, 1-β, 3-δ, 1-δ	2-α	2-α, 2-β, 2-C=O
Ile 3	α	4.07 (t, *J* = 7.6 Hz)	2-α, 2-β, 2-γ	3-β	3-β, 3-δ, 3-γ, 3-C=O
	β	1.82 (m)			
	γ	1.39 (m), 1.27(m)			
	δ, δ`	0.80 (t, *J* = 6.7 Hz), 0.87 (d, *J* = 6.9 Hz)			
	N*H*	7.37 (d, *J* = 8.2 Hz)	2-N*H*, 4-N*H*, 2-α, 2-δ, 2-γ	3-α	3-α, 3-C=O
Gly 4	α	3.72 (dd, *J* = 16.7, 6.3 Hz) 3.57 (d, *J* = 16.7 Hz)	3-δ	4-N*H*	4-C=O
	N*H*	7.88 (t, *J* = 6.3 Hz)	Ter.-NH_2, 3-N*H*, 3-α, 3-β	4-α	4-C=O, 4-α

3.1.2. Callyaerin S (2, new compound)



Callyaerin S (**2**) was obtained as a white amorphous powder, with an $[\alpha]_D$ of - 93° (*c* 0.3, CH_3OH). It has UV absorbances at λ_{max} 208 and 284 nm. The (+) ESIMS showed pseudomolecular ion peaks at *m*/*z* 1044 $[M+H]^+$, 1066 $[M+Na]^+$ and 817 [M-side chain]$^+$, which was consistent with the molecular formula $C_{54}H_{81}N_{11}O_{10}$ as determined by HRMS. The structure required 20 double-bond equivalents (DBE). The MALDI-TOF MS revealed intense peaks at *m*/*z* 1066 $[M+Na]^+$, 1082 $[M+K]^+$ and 817 [M-side chain]$^+$. Compound **2** had a 219 mass unit difference from **1**. When compound **2** was compared with **1**, it had the same ring size but differed in the length of the side chain. The mass difference suggested that compound **2** chain had two amino acids less than **1** in the side chain. Its peptidic nature was determined after inspection of its ^1H and ^{13}C NMR spectra. ^{13}C NMR and DEPT spectra revealed 10 amide carbons at [δ 173.2 (2-CO), 172.5 (C-CO), 172.4 (B-CO), 172.2 (H-CO), 171.9 (1-CO), 171.6 (G-CO), 171.3 (F-CO), 170.7 (D-CO), 168.9 (E-CO) and 167.2 (DAPA-CO)], 6 aromatic carbons at δ 137.9 (C-1′), 128.1 (C-3′, 5′), 128.9 (C-2′, 6′) and 126.6 (C-4′) for a monosubstituted benzene ring, olefinic double bond resonance at 140.2 (C-3), 98.8 (C-2), 13 methine carbons, 15 methylene carbons and 8 methyl carbons. The HMQC spectrum assigned the ^1H resonances associated with those of the carbons. The ^1H NMR spectrum of compound **2** exhibited 6 deshielded amide N*H* groups between δ 8.47 and δ 5.27 and terminal NH_2 signals at δ 6.98 and 6.88. Interpretation of COSY and TOCSY spectra

led to the assignment of two valines at δ 7.61 (d, *J* = 5.0 Hz, D-N*H*), 3.85 (t, *J* = 5.0 Hz, H-Dα), 2.10 (m, H-Dβ), 0.93 (d, *J* = 6.9 Hz, H_3-Dγ), 0.84 (d, *J* = 6.9 Hz, H_3-Dγ′) and 5.27 (dd, *J* = 13.8, 10.0 Hz, B-N*H*), 4.42 (d, *J* = 10.0 Hz, H-Bα), 2.35 (m, H-Bβ), 0.86 (d, *J* = 6.9 Hz, H_3-Bγ), 0.67 (d, *J* = 6.9 Hz, H_3-Bγ′), two isoleucines at δ 7.54 (d, *J* = 6.6 Hz, H-N*H*), 4.50 (dd, *J* = 6.7, 3.8 Hz, H-Hα), 1.85 (m, H-Hβ), 1.77 (H_2-Hγ) and 0.81 (H_3-H δ, δ′) and 7.49 (d, *J* = 6.3 Hz, 2-N*H*), 4.01 (dd, *J* = 8.8, 6.4 Hz, H-2α), 1.92 (m, H-2β), 1.49, 1.21 (H_2-2γ) and 0.86 (H_3-2δ, δ′), one phenylalanine [δ 8.86 (brs, E-N*H*), 4.55 (m, H-Eα), 3.03 (dd, *J* = 13.6, 8.5 Hz, H-Eβ), 2.76 (dd, *J* = 13.6, 8.5 Hz, H-Eβ′), 7.18-7.21, 7.25-7.28 (H-2′-H-6′)], four prolines and again the unusual acid 2,3-diamino-2-propenoic acid [DAPA] residue. The sequence of **2** and the position of DAPA, and terminal NH_2 were deduced by detailed interpretation of ROESY and HMBC spectra. The terminal NH_2 showed ROESY correlations to Ile 2-N*H* and Ile 2-αC*H*, which assigned the attachment of NH_2 to Ile 2. This was confirmed by its HMBC correlation to Ile 2-CO. Amino acid ROESY correlation of Ile 2-N*H* to Pro 1-αC*H*, in addition to its HMBC correlation to Pro 1-CO assured the attachment of Ile 2 and Pro 1 to give substructure **A**. Substructure **A** was also confirmed by ESI and MALDI-TOF MS, which showed a fragment ion at *m*/*z* 817 for M-(NH_2+Ile 2+Pro 1). DAPA-N*H* showed ROESY correlations to Val B-N*H* and H-3 to Val B-N*H*, Val B-αC*H*, Val B-γCH_3 which indicated its connectivity with Val B [substructure **B**]. HMBC correlations of Val B-αC*H* to C-3 and Val B-N*H* to C-2 further assured the attachment of DAPA and Val B. The connectivity of substructures **A** and **B** was confirmed by ROESY correlations of Pro 1-αC*H*, Pro 1-δCH_2 to DAPA-N*H*, and was further confirmed by the HMBC correlation of Pro 1-αC*H* to DAPA-CO. Pro C and Val D were linked to give the dipeptide substructure **C**. This was justified by the ROESY correlations of Pro C-αC*H* and Pro C-βCH_2 to Val D-N*H*, while the HMBC correlation of Val D-αC*H* to Pro C-CO proved this substructure. The amide bond between substructures **C** and **B** was demonstrated by the ROESY correlations of Pro C-δCH_2 to Val B-αC*H* and Val B-βC*H* and HMBC correlation of Pro C-αC*H* to Val B-CO. ROESY correlations of Pro F-δCH_2 to Phe E-αC*H*, Phe E-N*H* and Pro F-αC*H* to Phe E-αC*H* indicated the connectivity between Phe E and Pro F which

resulted in substructure **D**. The linkage between substructures **C** and **D** was accomplished by the ROESY of Val D-αC*H* to Phe E-N*H* and Val D-N*H* to Phe E-N*H*. ROESY correlations of Pro G-αC*H* and Pro G-βCH_2 to Ile H-N*H*, and of Pro G-δCH_2 to Ile H-αC*H* established the amide linkage between Pro G and Ile H for substructure **E**, which was further confirmed by HMBC correlation of Ile H-αC*H* to Pro G-CO. The subunits **E** and **D** were linked together which was distinguished by the characteristic HMBC correlation of Pro G-αC*H* with Pro F-CO. ROESY correlations of DAPA-N*H* to Ile H-N*H*, Ile H-αC*H*, and also its HMBC correlation with Ile H-CO established the cyclisation via amide bond formation between DAPA and Ile H. Comparison of the NMR data and molecular weight of compound **2** with those of the previously reported callyaerins isolated from *Callyspongia aerizusa* [Min, *et al*., 2001] in combination with a careful inspection of the COSY, HMBC, TOCSY and ROESY spectra allowed the complete assignment of all signals. The name **callyaerin S** was proposed for this new natural product. The absolute stereochemistries of the individual amino acid constituents of callyaerin S (**2**) were determined following acid hydrolysis of the parent peptide and consequent treatment with Marfey´s reagent [Marfey, 1984]. This proved the presence of one L-Phe, two L-Val, three L-Pro, one D-Pro, and two L-Ile.

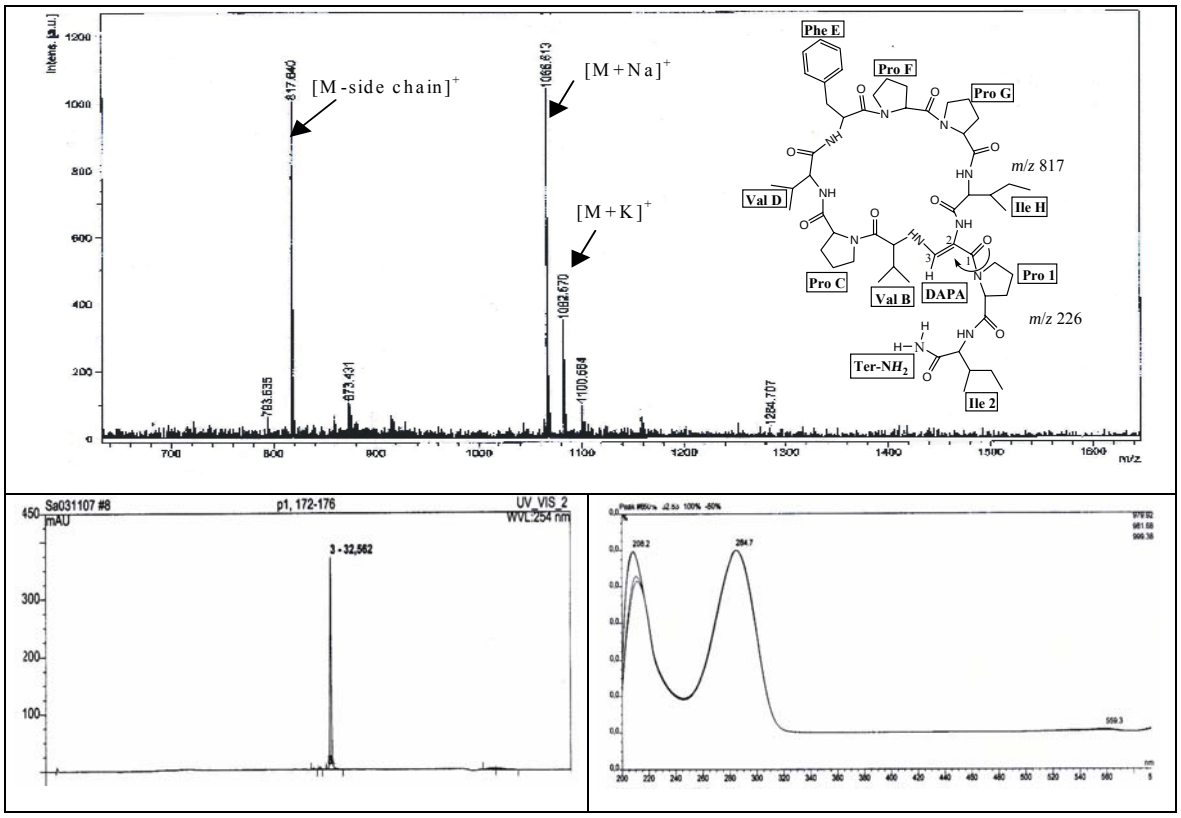


Fig. 3.11: MALDI-TOF MS and UV spectra of compound 2

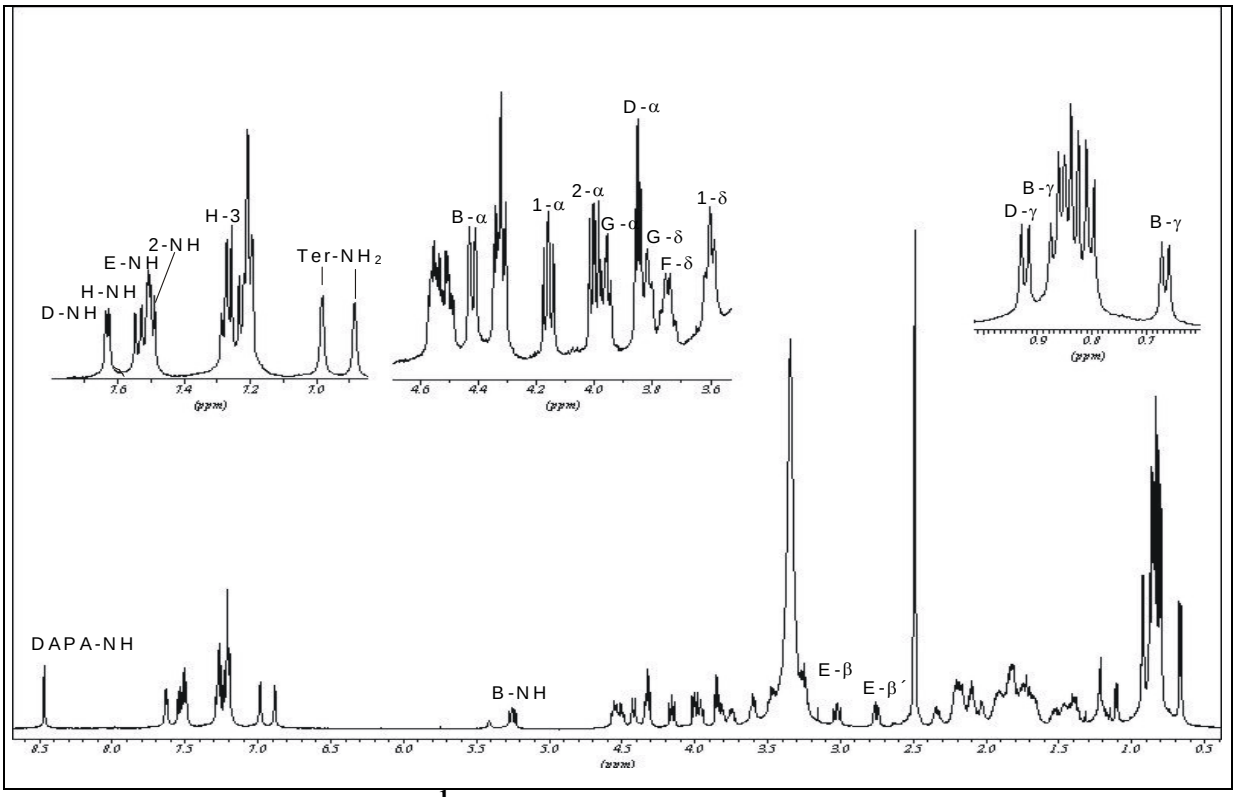


Fig. 3.12: ^{1}H NMR spectrum of compound 2

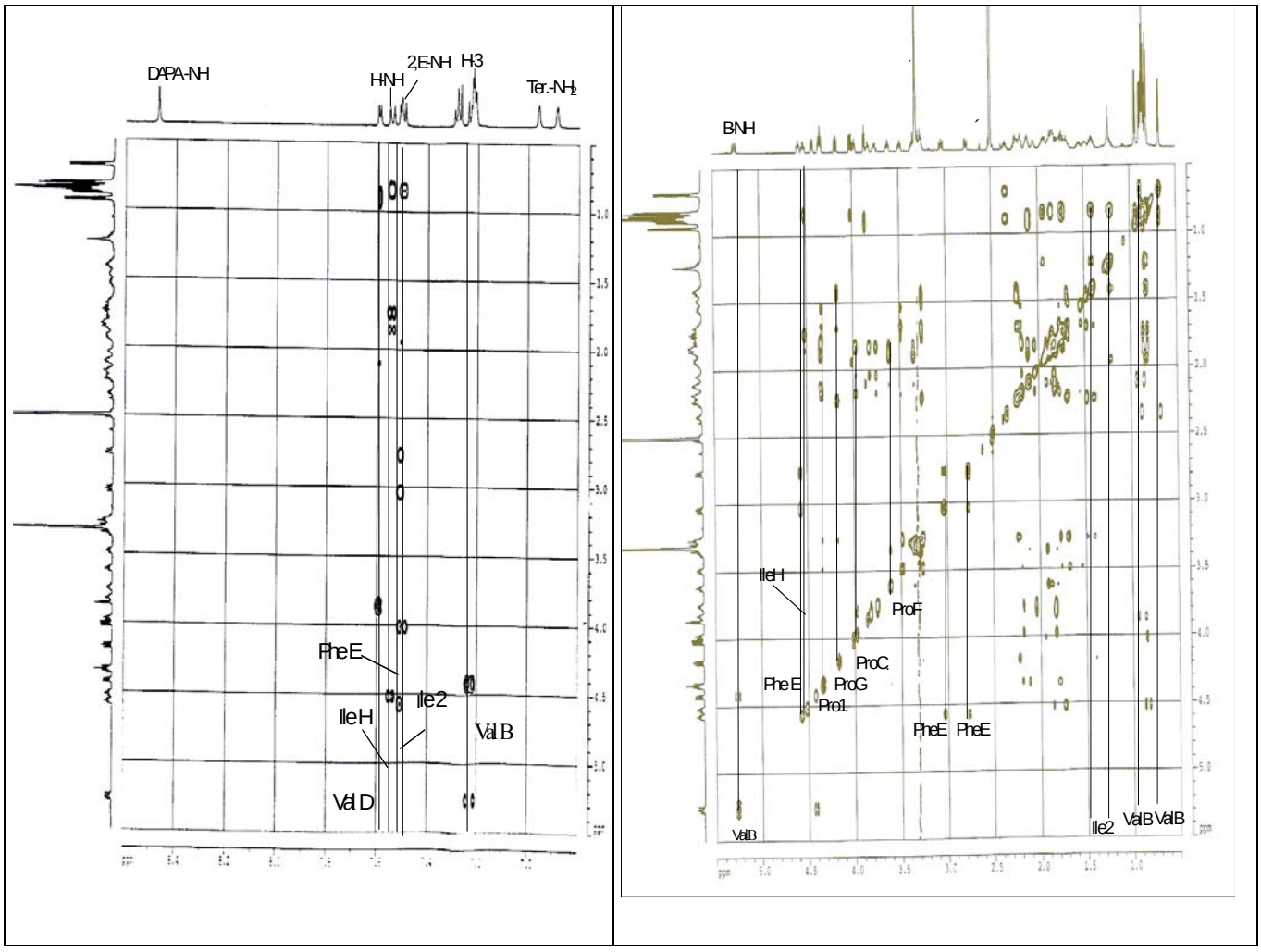


Fig. 3.13: TOCSY spectrum of compound 2

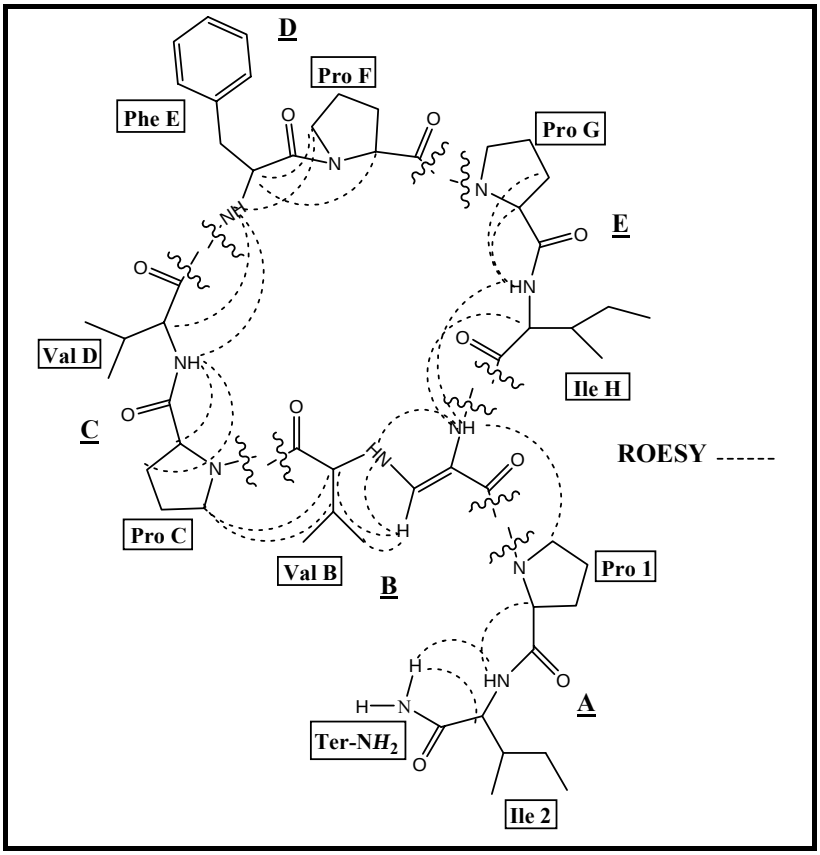


Fig. 3.14: Substructures A-E and ROESY correlations of compound 2

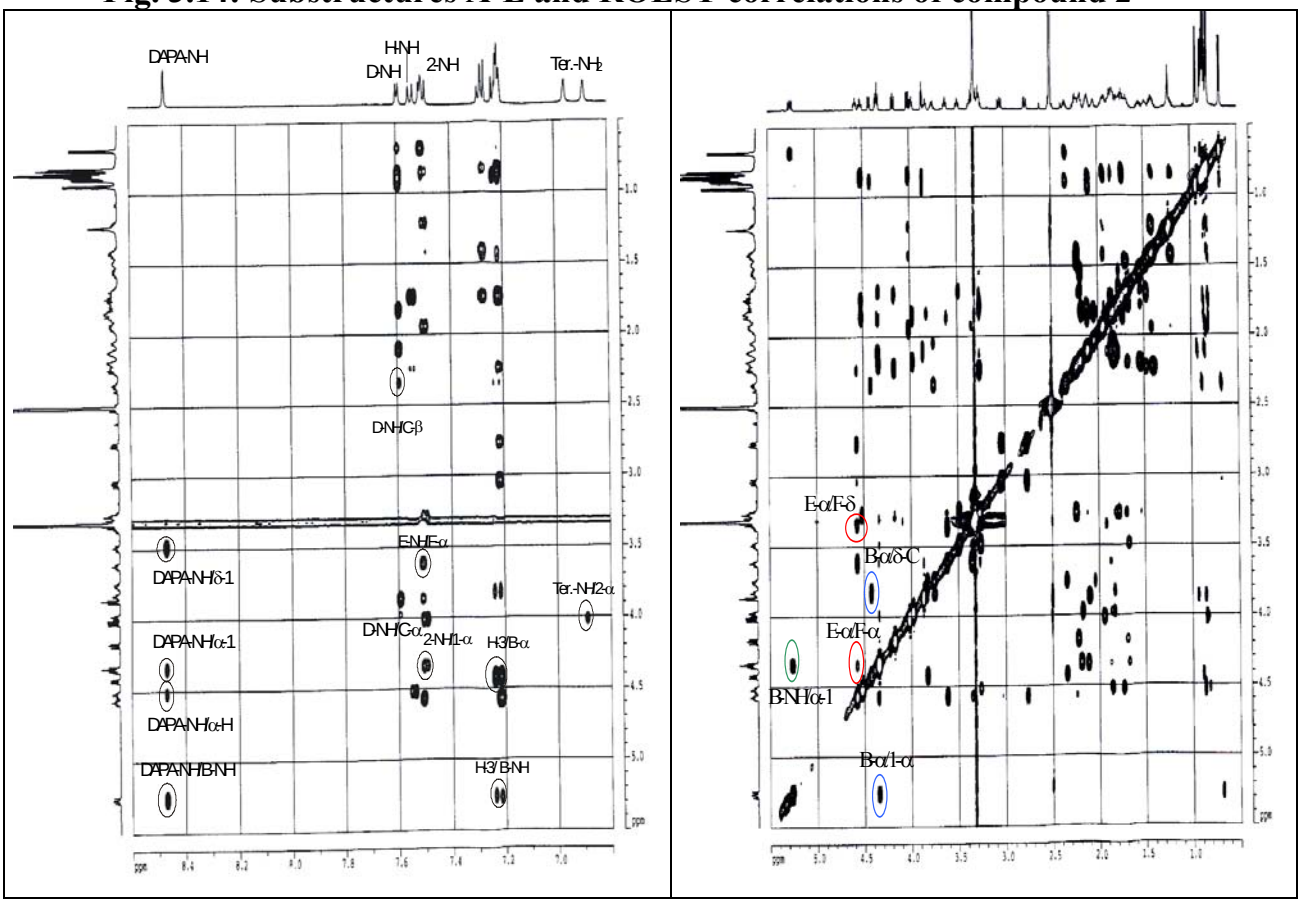


Fig. 3.15: ROESY spectrum of compound 2

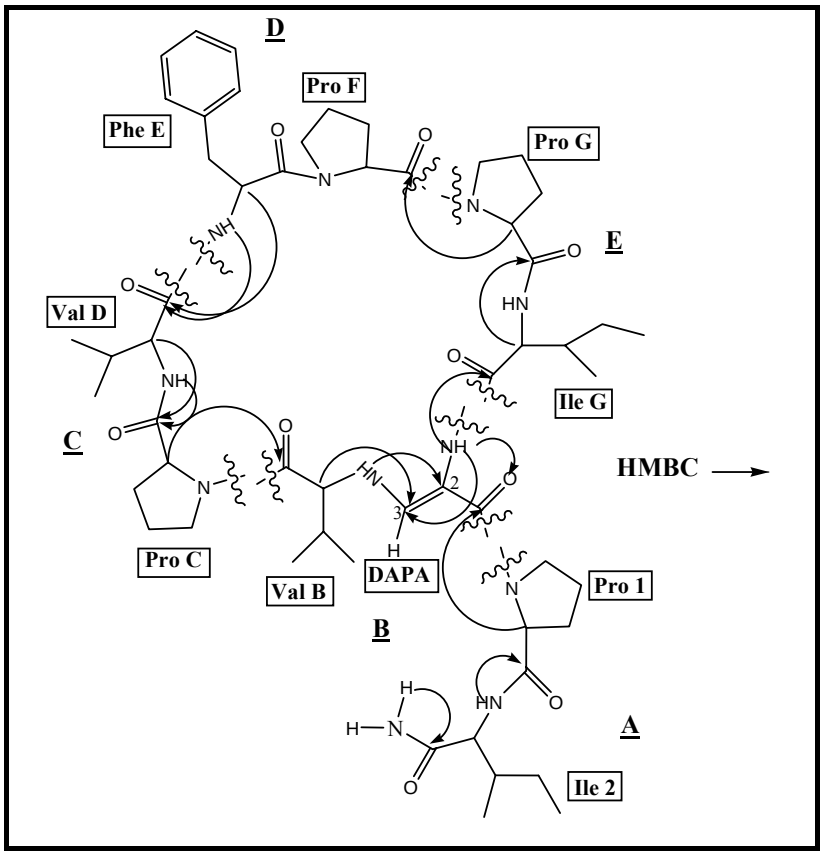


Fig. 3.16: Substructures A-E and HMBC correlations of compound 2

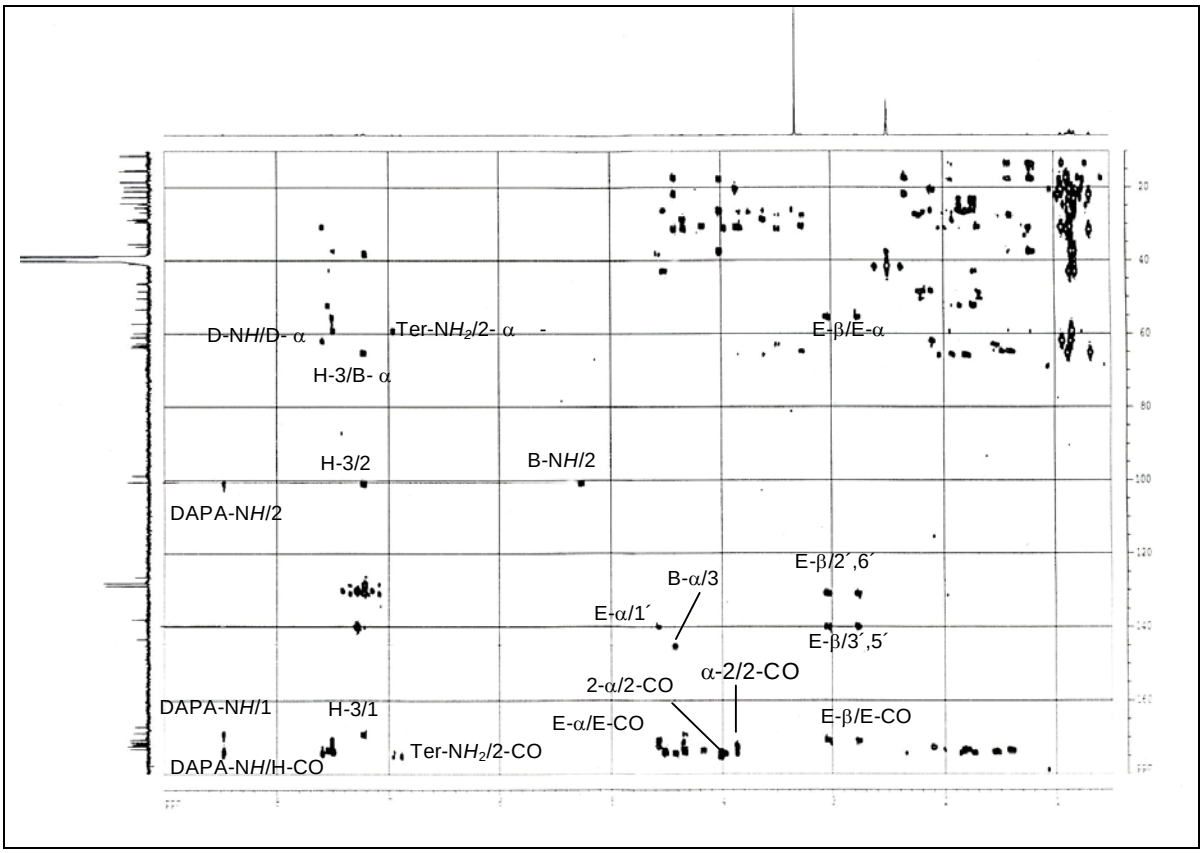


Fig. 3.17: HMBC spectrum of compound 2

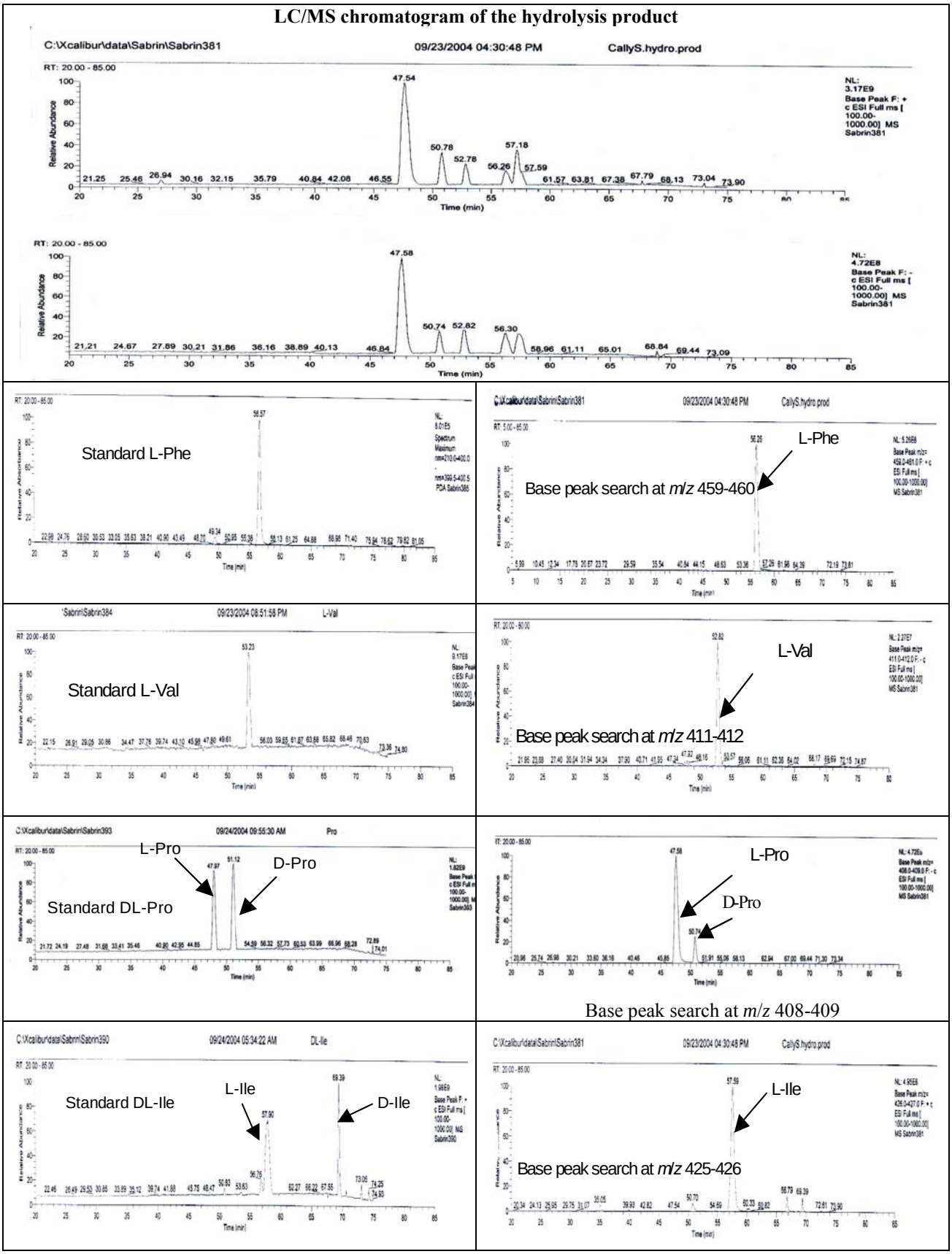
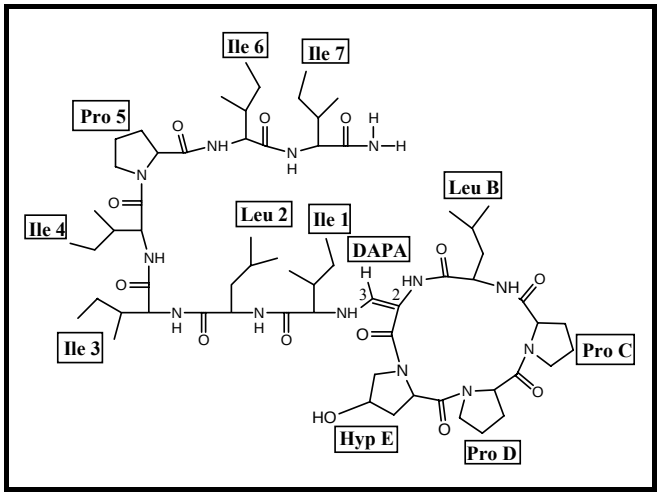


Fig. 3.18: Amino acids analysis results of compound 2

Table 3.2: NMR data of compound 2 (DMSO-d_6, 500 MHz)

A.A.	Position	δ_H (m, *J* in Hz)	ROESY	HMBC
DAPA	N*H*	8.47 (brs)	H-N*H*, B-N*H*, H-α, 1-α	DAPA-α, H-C=O
	2			
	3	7.23 (s)	B-α, B-γ, B-N*H*	DAPA-C=O, 3
	C=O			
NH_2 terminal		6.88 (brs)	2-N*H*, 2-α	2-C=O
		6.98 (brs)		
Val B	C=O			
	α	4.42 (d, *J* = 10.0 Hz)	C-δ, DAPA-N*H*	B-C=O, 3
	β	2.35 (m)	C-δ	B-C=O
	γ	0.67(d, *J* = 6.9 Hz), 0.86 (d, *J* = 6.9 Hz)		
	N*H*	5.27 (dd, *J* = 13.8, 10.0 Hz)	1-α	DAPA-α
Pro C	C=O			
	α	3.96 (m)	D-N*H*	B-C=O, C-C=O
	β	2.03 (m), 1.82 (m)	D-N*H*	
	γ	2.18 (m), 2.11 (m)		
	δ	3.82 (m), 3.75 (m)	B-α, B-β	
Val D	C=O			
	α	3.85 (t, *J* = 5.0 Hz)	E-N*H*	D-C=O, C-C=O
	β	2.10 (m)		D-C=O
	γ	0.93 (d, *J* = 6.9 Hz), 0.84 (d, *J* = 6.9 Hz)		
	N*H*	7.61 (d, *J* = 5.05 Hz)	C-β, C-α , E-N*H*	D-C=O, C-C=O
Phe E	C=O			
	α	4.55 (m)	F-α, F-δ, 2´	4-C=O, 2-C=O, 1´
	β	2.76 (dd, *J* = 13.6, 8.5 Hz), 3.03 (dd, *J* = 13.6, 8.5 Hz)		2-C=O, 1´, 2´
	others	7.18-7.21,7.25-7.28		
	N*H*	8.86 (brs)	D-α, F-δ, D-N*H*	E-C=O, D-C=O
Pro F	C=O			
	α	3.61 (t, *J* = 8.2 Hz)	E-α	F-C=O
	β	2.10 (m)		F-C=O
	γ	1.80 (m)		
	δ	3.37 (m)	E-α, E-N*H*	
Pro G	C=O			
	α	4.16 (t, *J* = 7.6 Hz)	H-N*H*	G-C=O, F-C=O
	β	2.21 (m), 1.69 (m)	H-N*H*	G-C=O
	γ	1.45 (m)		
	δ	3.27 (m)	H-α	
Ile H	C=O			
	α	4.50 (dd, *J* = 6.7, 3.8 Hz)		H-C=O, G-C=O
	β	1.85 (m)	C-α	H-C=O
	γ	1.77 (m)		
	δ, δ`	0.81		
	N*H*	7.54 (d, *J* = 6.6 Hz)	H-3, DAPA-N*H*, G-α	
Pro 1	C=O			
	α	4.33 (m)	B-N*H*, 2-N*H*, H-3	1-C=O, DAPA-C=O
	β	2.16 (m), 1.72 (m)		1-C=O
	γ	1.81 (m), 1.55 (m)		
	δ	3.48 (t, *J* = 8.0 Hz), 3.24 (m)	DAPA-N*H*	
Ile 2	C=O			
	α	4.01 (dd, *J* = 8.8, 6.4 Hz)	Ter.-NH_2	2-C=O
	β	1.92 (m)		
	γ	1.21(m), 1.40 (m)		
	δ, δ`	0.86		
	N*H*	7.49 (d, *J* = 6.3 Hz)	1-α, Ter.-NH_2	1-C=O

3.1.3. Callyaerin B (3, known compound)



Callyaerin B (**3**) was obtained as a yellowish-white amorphous powder. The $[\alpha]_D$ value was - 89° (*c* 0.2, CH_3OH). It has UV absorbances at λ_{max} 210 and 284 nm. The (+) ESIMS showed pseudomolecular ion peaks at *m*/*z* 1280.6 $[M+H]^+$ and 1303 $[M+Na]^+$. The molecular formula $C_{65}H_{109}N_{13}O_{13}$ was confirmed by HRESI-TOF MS. Compound **3** had a 20 mass unit difference from compound **1**. It had the same amino acid number as **1**. In opposite to **1**, compound **3** had four amino acids in the ring, while the chain consisted of seven amino acids. The structure of compound **3** was derived on the basis of spectroscopic analysis. Assignments of the ^{1}H NMR and ^{13}C NMR spectra are depicted in Table (3.3). The ^{1}H NMR spectrum showed resonances of seven dieshelded amide N*H* protons at δ 9.00 (brs, 4-N*H*), 8.86 (brs, 3-N*H*), 7.58 (d, *J* = 10.4 Hz, 6-N*H*), 7.37 (d, *J* = 6.6 Hz, 2-N*H*), 7.23 (d, *J* = 9.5 Hz, 7-N*H*), 6.63 (d, *J* = 10.0 Hz, B-N*H*) and 5.70 (dd, *J* = 13.6, 10.8 Hz, 1-N*H*) which revealed the peptidic nature of compound **3**. In addition, clusters of α-amino acid methines, aliphatic primary and secondary methyls indicated the presence of lipophilic amino acid residues. Inspection of ^{1}H NMR and TOCSY spectra showed eleven spin systems for eleven amino acid residues (see Table 3.4). The ^{1}H NMR and TOCSY spectra showed signals at δ 8.28 (DAPA-N*H*) and 7.04 (H-3) attributed to 2,3 diamino-2-propeneoic acid (DAPA). Two singlet signals at δ 7.17 (brs) and 6.97 (brs) corresponded to the terminal NH_2. The assignment of the spin systems was aided through the correlations observed in its ^{1}H-^{1}H COSY spectrum. Full inspection of the ^{13}C NMR and DEPT spectra revealed the presence of 65 carbons consisting of 12 amide carbonyl signals, one olefinic

double bond, 16 methine, 18 methylene, and 14 methyl carbons. The direct correlation of all protons to their carbons was determined by HMQC experiment. The sequence of amino acids, the position of DAPA and the cyclic structure of callyaerin **B** were established by careful interpretation of its ROESY spectrum. Ile 7-N*H* showed correlations to Ile 6-N*H*, Ile 6-αC*H* and Ile 6-N*H* to Ile 7-αC*H* and established the connectivity between Ile 7 and Ile 6 for substructure **A**. The dipeptide substructure of Pro 5 and Ile 4 [substructure **B**] was proven through the correlation of Ile 4-N*H* to Pro 5-αC*H*. The connectivity of substructures **A** and **B** was established by the characteristic correlation of Ile 6-N*H* to Pro 5-αC*H* and Pro 5-δCH_2. ROESY correlations of Ile 3-βC*H*, Ile 3-γCH_2 to Leu 2-αC*H* and of Leu 2-N*H* to Ile 3-αC*H* assured the attachment of Leu 2 and Ile 3 for substructure **C**. Ile 1 and DAPA were linked to give the dipeptide substructure **D**. This was justified by the ROESY correlations from Ile 1-αC*H* to DAPA-N*H*. An observed cross peak between Leu 2α-C*H* and Ile 1-αC*H* established the amide linkage of substructures **C** and **D**. The connectivity of Leu B and Pro C was confirmed by ROESY correlations of Leu B-N*H* with Pro C-αC*H* and Pro C-δCH_2 [Substructure **E**]. The linkage of substructures **E** and **D** was confirmed by the ROESY correlations of Leu B-N*H* to DAPA-N*H* and DAPA-N*H* to Leu B-αC*H*. Furthermore, ROESY correlations of Pro D-αC*H*, Pro D-δCH_2 to Hyp E-O*H* established the connectivity between Pro D and Hyp E [substructure **F**]. Treatment of the acid hydrolysate of compound **3** with Marfey´s reagent [Marfey, 1984] and LC-MS analysis of the resulting 1-flouro-2,4-dinitrophenyl-5-L-leucinamide derivatives revealed the presence of one (*cis*) L-Hyp, three L-Pro, two L-Leu and five L-Ile. Comparison of the NMR data and molecular weight of compound **3** with those of the previously reported **callyaerin B** isolated from *Callyspongia aerizusa* indicated that both compounds were identical [Min, *et al*., 2001].

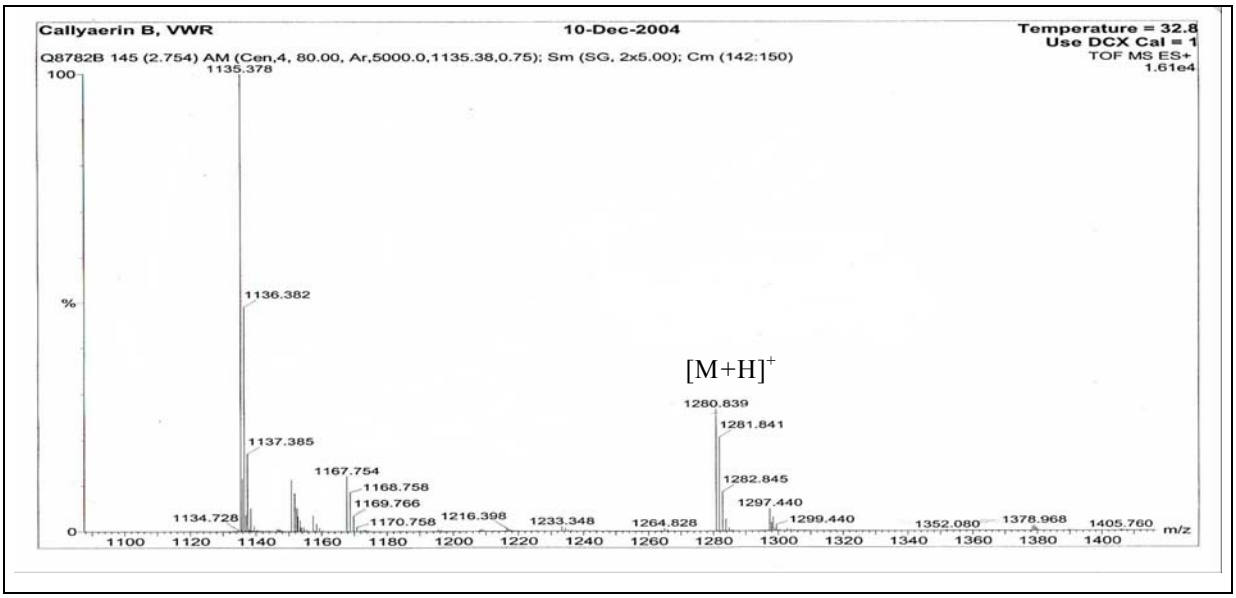


Fig. 3.19: HRESI-TOF MS spectrum of compound 3

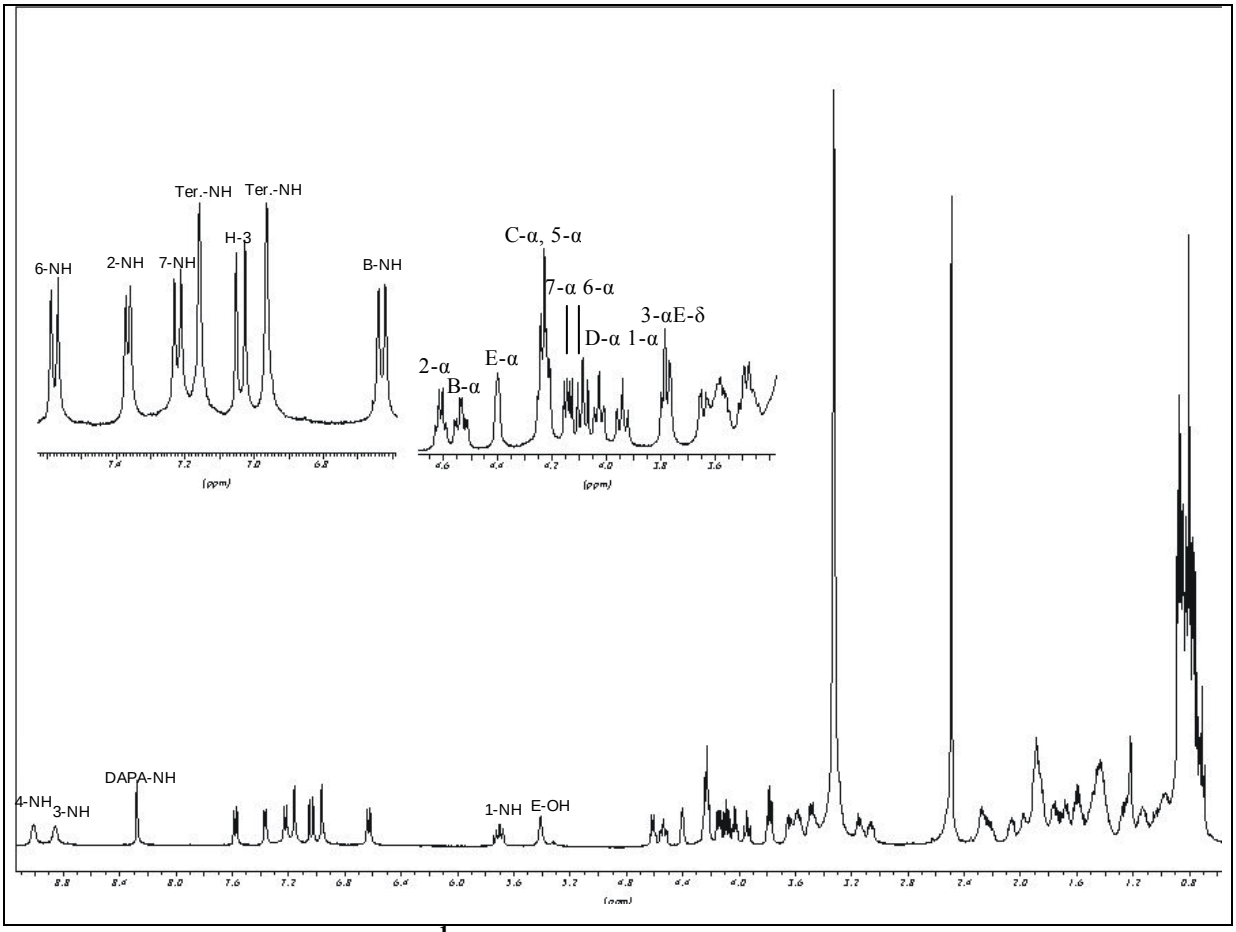
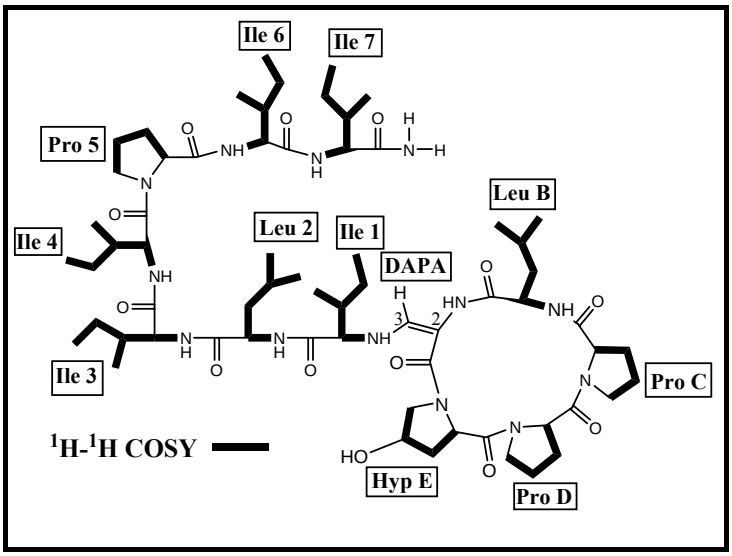


Fig. 3.20: ^{1}H NMR spectrum of compound 3



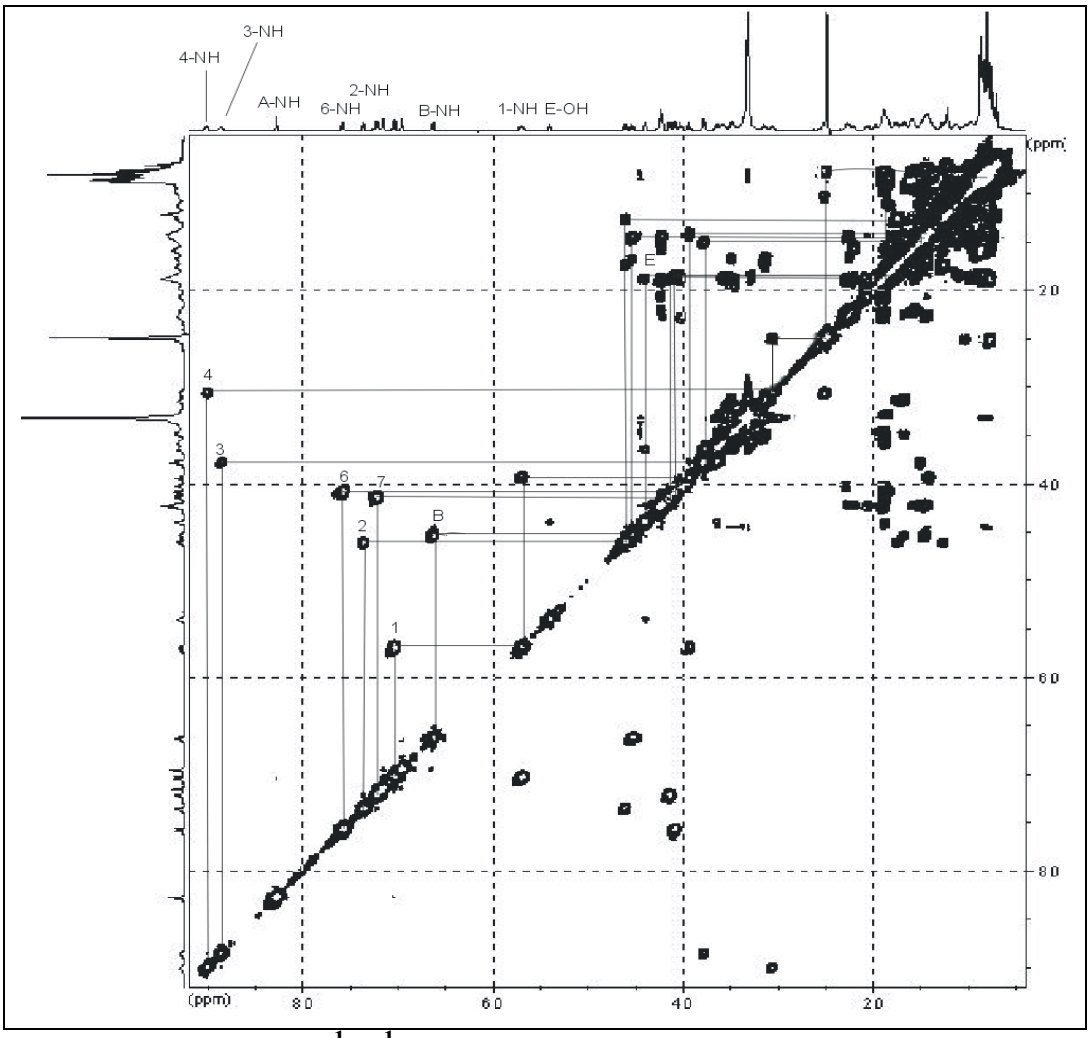


Fig. 3.21: ^{1}H-^{1}H COSY spectrum of compound 3

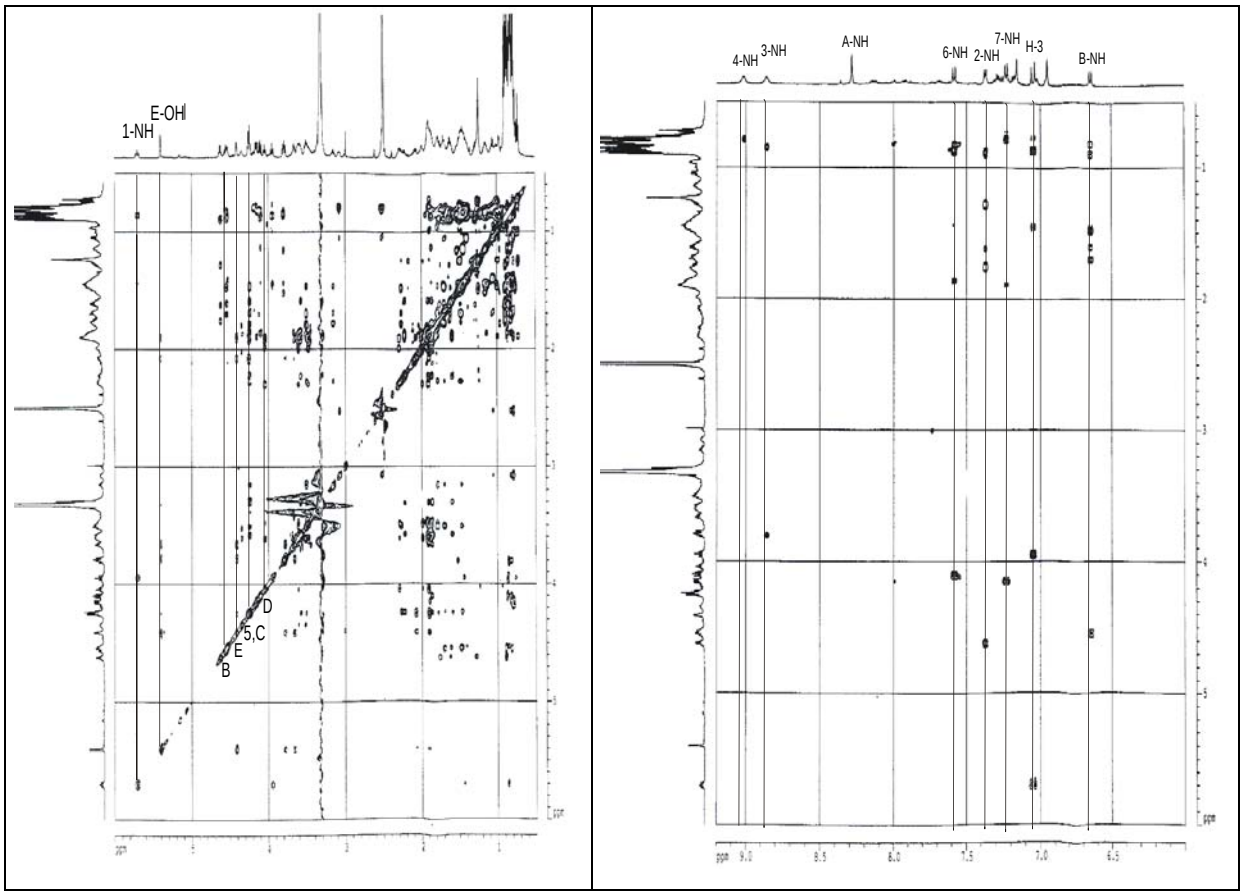


Fig. 3.22: Total TOCSY spectrum compound 3

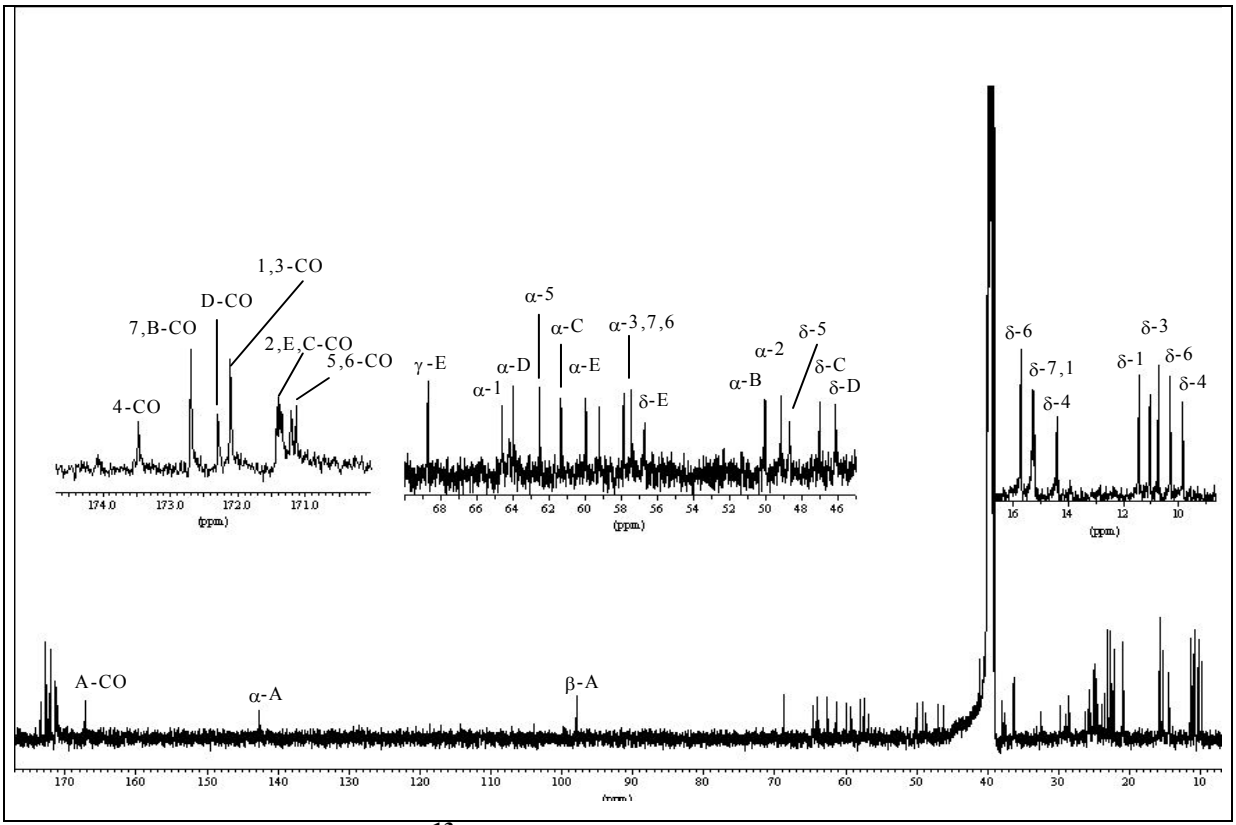


Fig. 3.23: ^{13}C NMR spectrum of compound 3

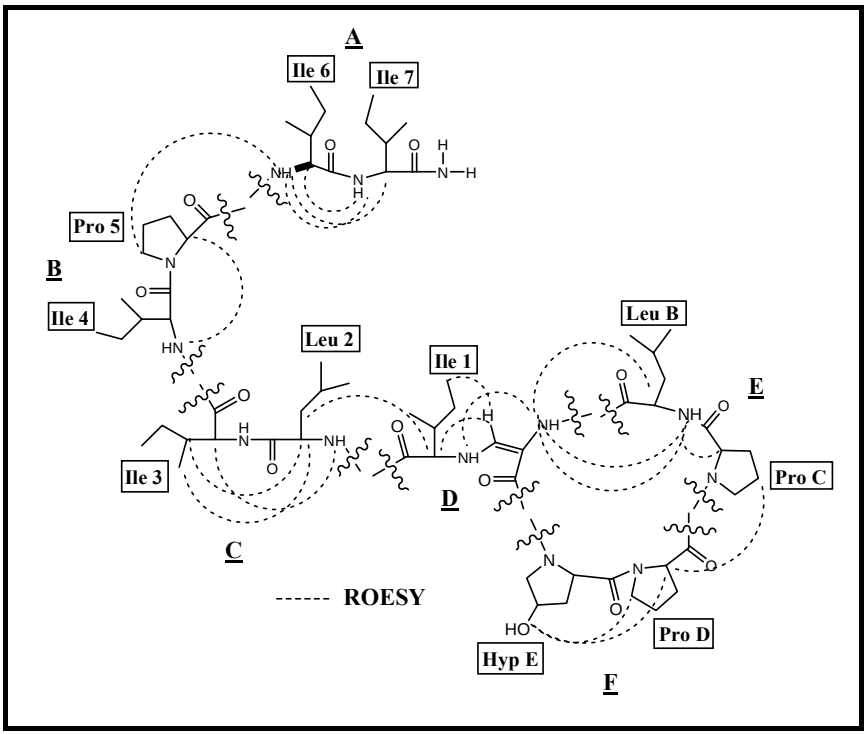


Fig. 3.24: Partial substructures A-F with ROESY correlations

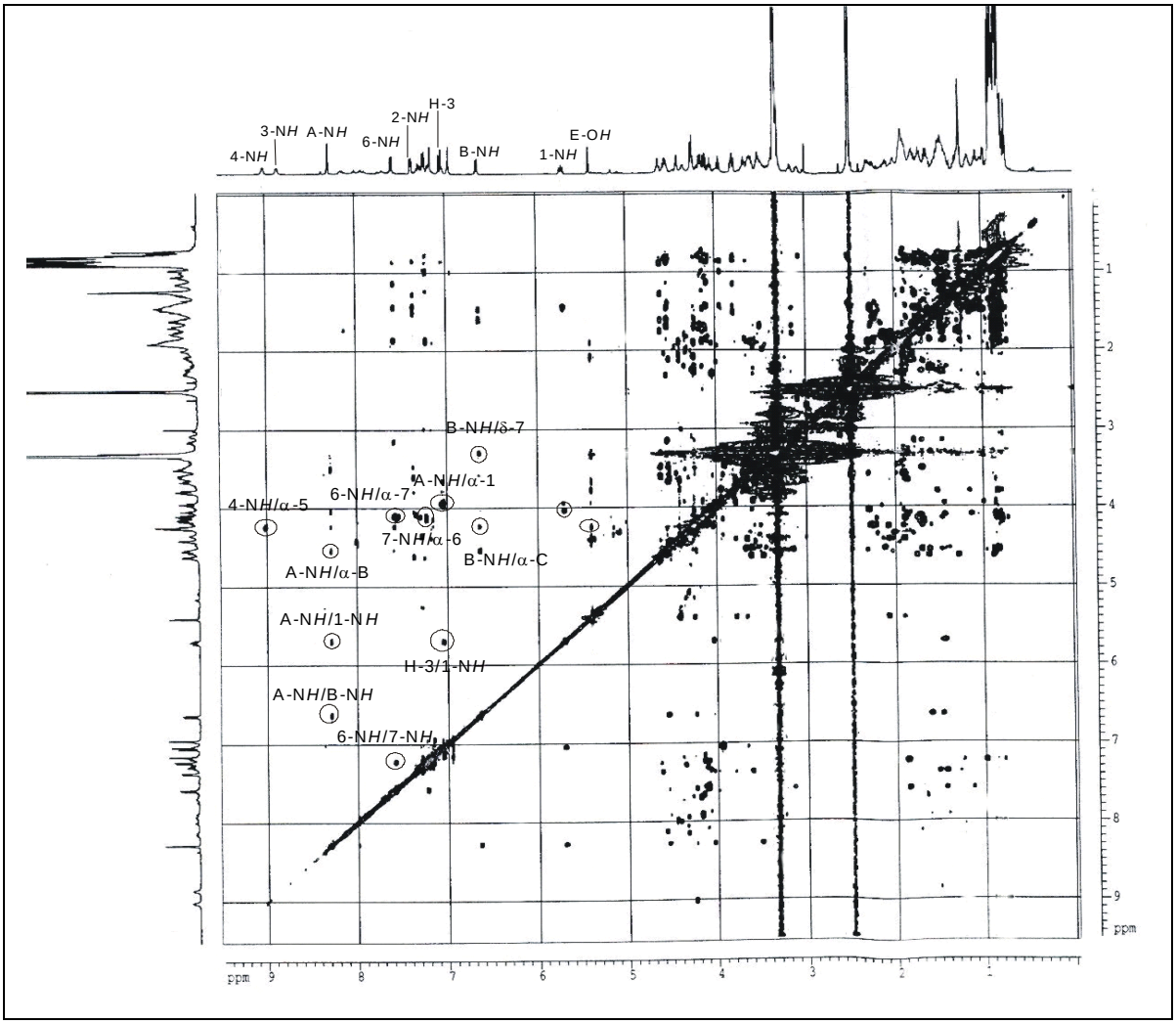


Fig. 3.25: ROESY spectrum of compound 3

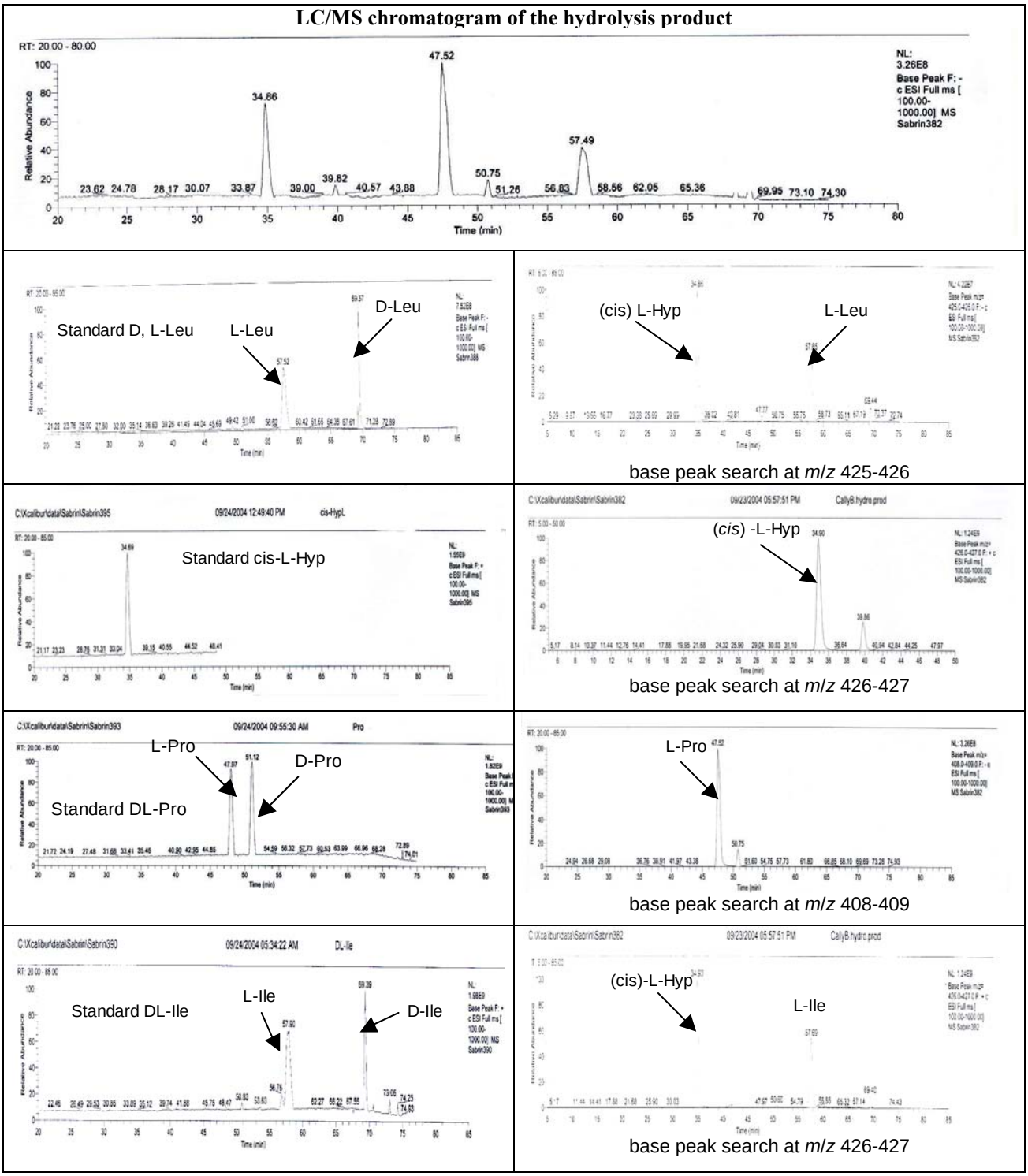


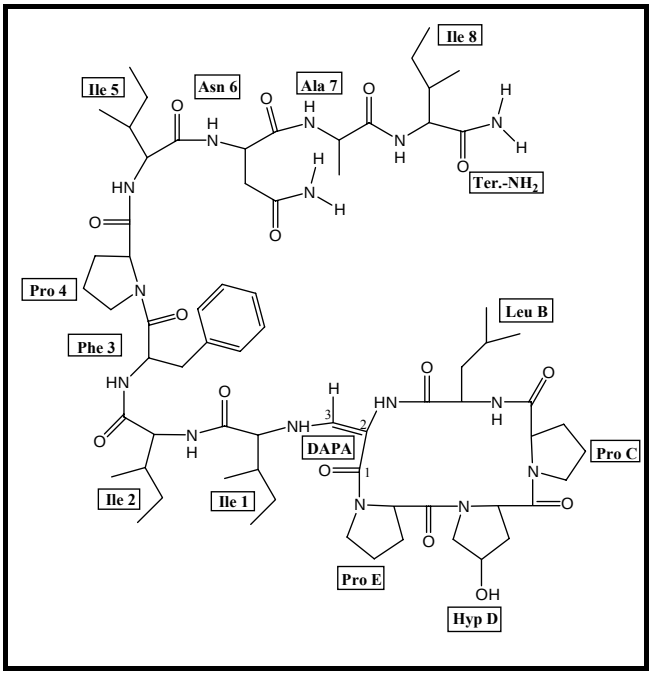
Fig. 3.26: Results of the amino acid analysis of compound 3

Table 3.3: NMR data of compound 3 (DMSO-d_6, 500, 125 MHz)

A.A.	Position	δ_H (m, *J* in Hz)	COSY	δ_C (m)	ROESY
DAPA	N*H*	8.28 (brs)			1-N*H*, B-N*H*, B-α
	2			97.9 s	
	H-3	7.04 (d, *J* = 13.6 Hz)	1-N*H*	142.6 d	1-α, 1-δ
	C=O			167.2 s	
NH_2 *terminal*		7.17 (brs)			
		6.97 (brs)			
Ile 1	C=O			172.1 s	
	α	3.94 (t, *J* = 9.8 Hz)	1-N*H*	64.6 d	DAPA-N*H*
	β	1.45 (m)	1-γ, 1-α	37.9 d	
	γ	1.40 (m), 1.00 (m)	1-β	29.1 t	
	δ, δ`	0.75 (d, *J* = 6.9 Hz), 0.80 (d, *J* = 6.9 Hz)		11.4 q, 15.3 q	
	N*H*	5.70 (dd, *J* = 13.6, 10.8 Hz)	1-α, H-3		
Leu 2	C=O			171.4 s	
	α	4.62 (dd, *J* = 6.9, 6.6 Hz)	2-β, 2-N*H*	49.1 d	1-α
	β	1.76 (m), 1.60 (m)	2-γ, 2-α	41.1 t	1-α
	γ	1.30 (m)	2-β, 2-δ	24.7 d	
	δ, δ`	0.90 (d, *J* = 6.8 Hz), 0.89 (d, *J* = 6.8 Hz)	2-γ		
	N*H*	7.37 (d, *J* = 6.6 Hz)	2-α		3-α
Ile 3	C=O			172.1 s	
	α	3.81 (t, *J* = 8.8Hz)	3-N*H*, 3-β	57.8 d	
	β	1.55 (m)	3-γ, 3-α	36.2 d	2-α
	γ	1.45 (m), 1.15 (m)	3-β, 3-δ	25.5 t	2-α
	δ, δ`	0.80 (d, *J* = 6.7 Hz), 0.81 (*J* = 6.7 Hz)		10.7 q	
	N*H*	8.86 (brs)			
Ile 4	C=O			173.4 s	
	α	3.07 (m)	4-β, 4-N*H*	64.2 d	6-N*H*
	β	2.55 (m)	4-γ, 4-α	32.4 d	
	γ	1.40 (m), 1.10 (m)	4-β, 4-δ	24.5 t	
	δ, δ`	0.80 (d, *J* = 6.9 Hz), 0.81 (d, *J* = 6.9 Hz)		9.8 q, 14.4 q	
	N*H*	9.00 (brs)	4-α		5-α
Pro 5	C=O			171.1 s	
	α	4.25 (m)		62.5 d	4-N*H*
	β	2.55 (m), 1.80 (m)		26.2 t	
	γ	1.70 (m), 1.45 (m)		29.7 t	
	δ	3.52 (m), 3.15 (m)		48.6 t	6-N*H*
Ile 6	C=O			172.1 s	
	α	4.10 (t, *J* = 8.8 Hz)	6-β, 6-N*H*	57.8 d	
	β	1.82 (m)	6-γ, 6-α	36.4 d	
	γ	1.45 (m), 1.15 (m)	6-β, 6-δ	25.0 t	
	δ, δ`	0.80 (d, *J* = 6.8 Hz), 0.82 (d, *J* = 6.8 Hz)		9.8 q, 14.4 q	
	N*H*	7.58 (d, *J* = 10.4 Hz)	6-α		7-N , 5-δ, 7-α
Ile 7	C=O			172.7 s	
	α	4.15 (dd, *J* = 9.5, 5.0 Hz)	7-β, 7-N*H*	57.4 d	
	β	1.90 (m)	7-γ, 7-α	36.2 d	
	γ	1.25 (m), 1.00 (m)	7-β, 7-δ	23.5 t	
	δ, δ`	0.87 (d, *J* = 6.8 Hz), 0.85 (d, *J* = 6.8 Hz)		15.2 q	
	N*H*	7.23 (d, *J* = 9.5 Hz)	7-α		6-N*H*, 6-α
Leu B	C=O			172.7 s	
	α	4.55 (ddd, *J* = 9.5, 5.0, 3.8 Hz)	B-β, B-N*H*	50.0 d	
	β	1.70 (m), 1.60 (m)	B-γ, B-α	40.5 t	
	γ	1.47(m)	B-β, B-δ	24.6 d	
	δ, δ`	0.88 (d, *J* = 6.9 Hz), 0.82 (d, *J* = 6.9 Hz)		20.8 q, 22.1 q	

	N*H*	6.63 (d, *J* = 10.0 Hz)	B-α		A-N*H*, C-α, C-δ
Pro C	C=O			171.4 s	
	α	4.25 (m)	C-β	61.3 d	B-N*H*
	β	2.20 (m), 1.82 (m)	C-γ, C-α	25.7 t	
	γ	1.90 (m), 1.50 (m)	B-β, B-δ	29.7 t	
	δ	3.55 (m), 3.35 (m)		47.0 t	B-N*H*
Pro D	C=O			172.3 s	
	α	4.03 (t, *J* = 8.2 Hz)		63.9 d	C-β, E-O*H*
	β	2.30 (m), 1.90 (m)		24.9 t	
	γ	2.00 (m), 1.90 (m)		29.1 t	
	δ	3.60 (m), 3.48 (m)		46.1 t	E-O*H*
Hyp E	C=O			171.4 s	
	α	4.24 (m)		59.2 d	
	β	2.10 (m),1.85 (m)		37.5 t	
	γ	4.40 (brs)		68.7 d	
	δ	3.67 (m), 3.80 (m)		56.7 t	
	O*H*	5.40 (d, *J* = 2.4 Hz)			D-α, D-δ

3.1.4. Callyaerin D (4, known compound)



Callyaerin D (**4**) was isolated as a white amorphous powder. The $[\alpha]_D$ value was - 49° (*c* 0.2, CH_3OH). It has UV absorbances at λ_{max} 205 and 285 nm. The ESIMS spectrum showed pseudomolecular ion peaks at *m*/*z* 1387 $[M+H]^+$, 1409.7 $[M+Na]^+$ and 1385 $[M-H]^+$, suggesting a molecular weight of 1386 g/mol and a molecular formula of $C_{69}H_{107}N_{15}O_{15}$ which was established by HRMS. In addition, a fragment ion at *m*/*z* 861 was observed which suggested the nature of the side chain for M-(Ile+Ala+Asn+Ile+Pro+N*H*$_2$). Compound **4** had a 103 mass unit difference from callyaerin **B** which suggested the presence of an additional amino acid in the side

chain. The ^{1}H NMR and COSY spectra again suggested that compound **4** was a peptide, due to the presence of a series of N*H* signals between δ 5.30 - 7.95 coupled to α-protons in the region from δ 3.95 - 4.64. The TOCSY and COSY experiments indicated that there were 12 amino acids. The presence of DAPA was indicated by signals at δ 8.42 (1H, s, DAPA-N*H*) and 7.38 (1H, d, *J* = 8.8 Hz, H-3). Two downfield singlet signals at δ 7.03 and 7.11 were assigned to the terminal NH_2 group. The 12 amino acid subunits could be linked to form the substructures (**A**-**F**). This was accomplished using the correlations observed from the ROESY spectrum. The terminal NH_2 position was defined from the diagnostic ROESY correlation with Ile 8-αC*H*. The dipeptide substructure **A** [Ile 8 and Ala 7] was justified on the basis of the correlation of Ile 8-N*H* with Ala 7-βCH_3. The Asn 6 and Ile 5 amino acids were linked to give the dipeptide substructure **B**, which was confirmed by the cross peak observed between Asn 6-N*H* to Ile 5-αC*H*. The ROESY correlation from Ala 7-N*H* to Asn 6-αC*H* justified the connection of substructure **A** to substructure **B**. The correlations observed from the α-proton of Pro 4 to the α-proton of Phe 3 and from the δ-proton of Pro 4 to N*H* proton of Phe 3 indicated that Pro 4 and Phe 3 were amide linked to give substructure **C**. The connectivity of substructure **B** to **C** was proven by the correlation of Ile 5-N*H* to Pro 4-αC*H*. Furthermore, Ile 2 and Ile 1 were bound to each other, as shown by correlation of Ile 2-N*H* to the β- and γ-protons of Ile 1 [substructure **D**]. The Phe 3-N*H* correlation with Ile 2-αC*H* assured the linkage of substructure **C** to **D**. The position of the DAPA and the assignment of substructure **E** were established by the correlations observed from DAPA-N*H* to Leu B-αC*H*, Ile 1-N*H*, and Pro E-αC*H*. Pro C and Hyp D amino acids were linked to give substructure **F**. Assignment of the structure of compound **4** was confirmed by comparison of the NMR spectra data to those of **callyaerin D**, which was previously isolated from *Callyspongia aerizusa* [Min, *et al*., 2001]. The absolute stereochemistries of the individual amino acid constituents of callyaerin D (**4**) were determined following acid hydrolysis of the parent peptide and treatment with Marfey´s reagent [Marfey, 1984]. The LC-MS analysis of the resulting 1-flouro-2,4-dinitrophenyl-5-L-leucinamide derivatives established the presence of one (*cis*) L-Hyp, two L-Pro, one D-Pro, one L-Leu, four L-Ile, one L-Phe and Asn.

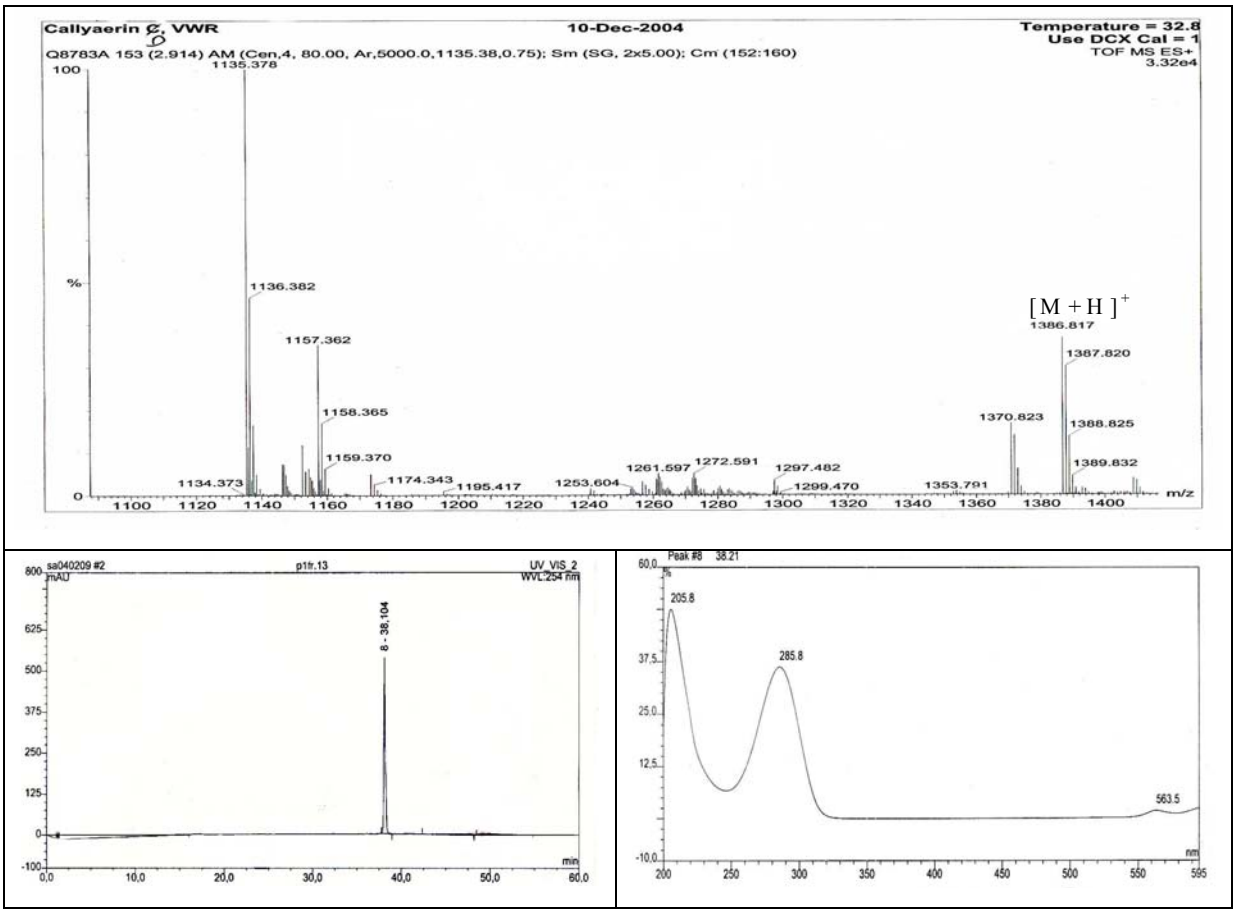


Fig. 3.27: HRESI-TOF MS and UV spectra of compound 4

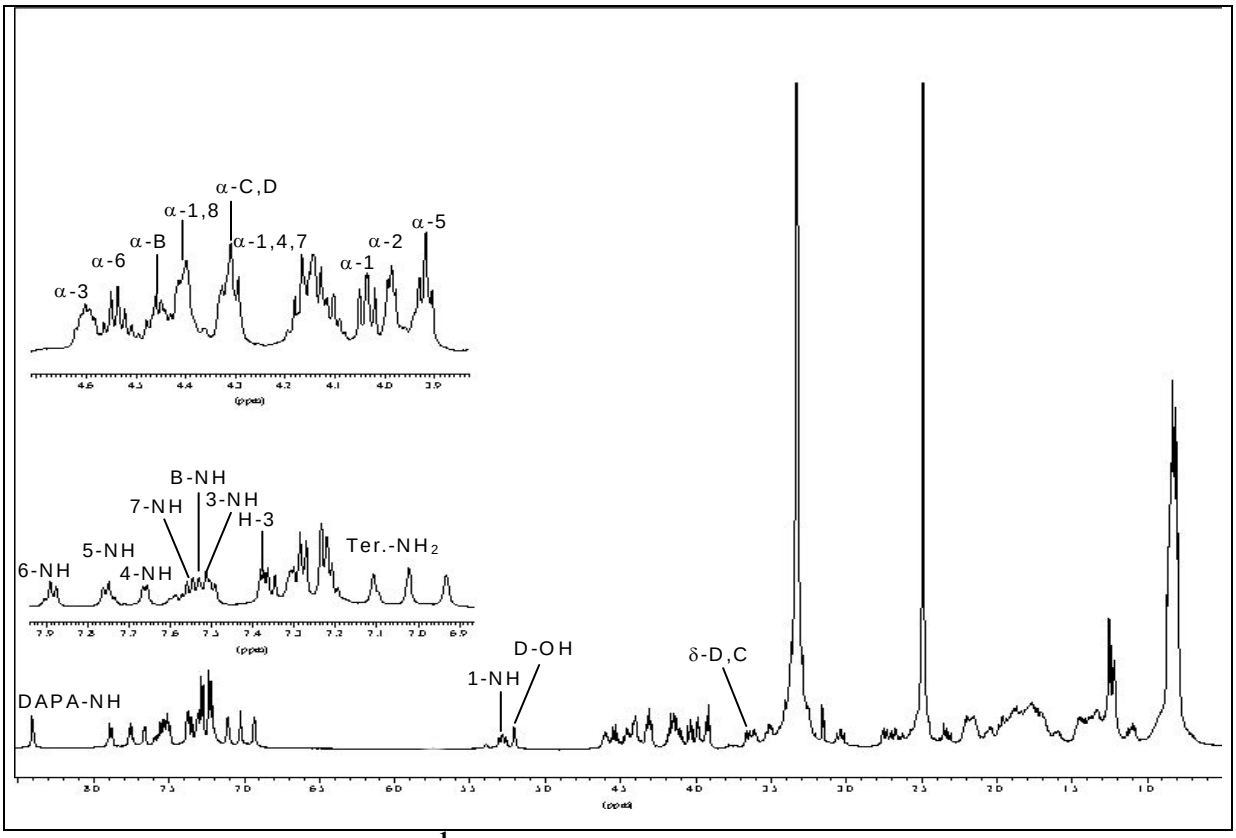
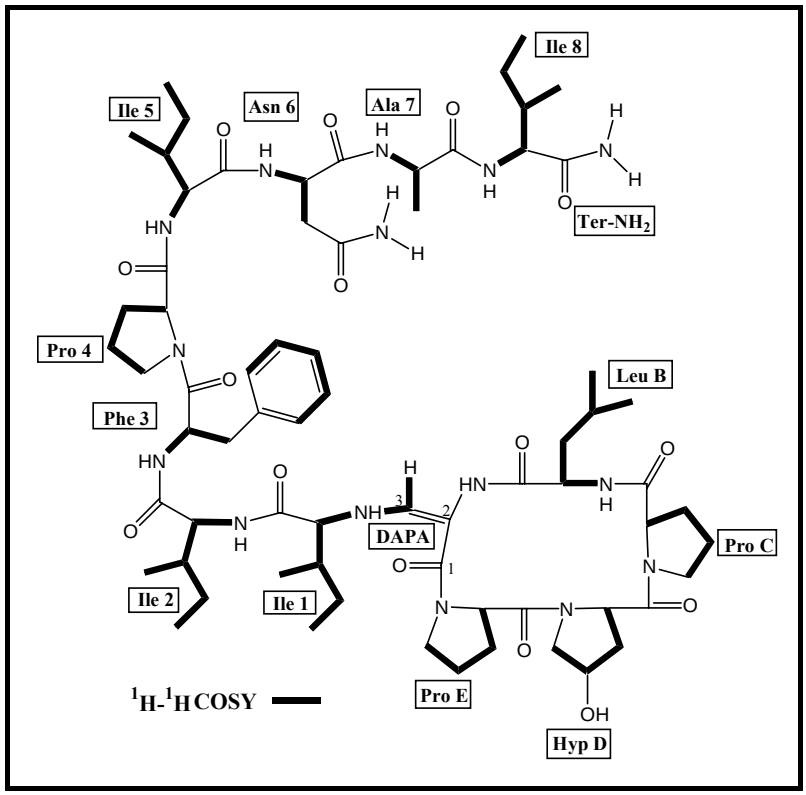


Fig. 3.28: ^{1}H NMR spectrum of compound 4



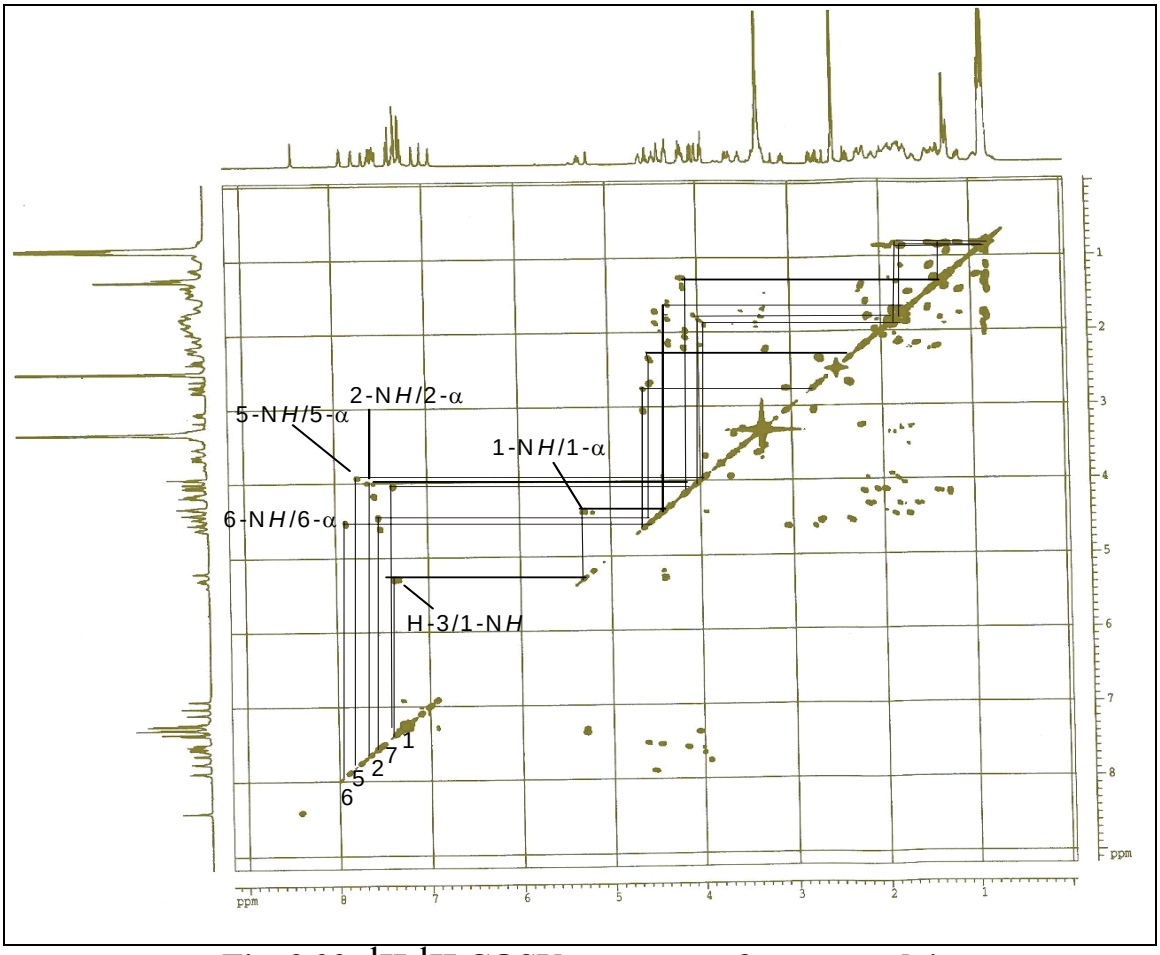


Fig. 3.29: ^{1}H-^{1}H COSY spectrum of compound 4

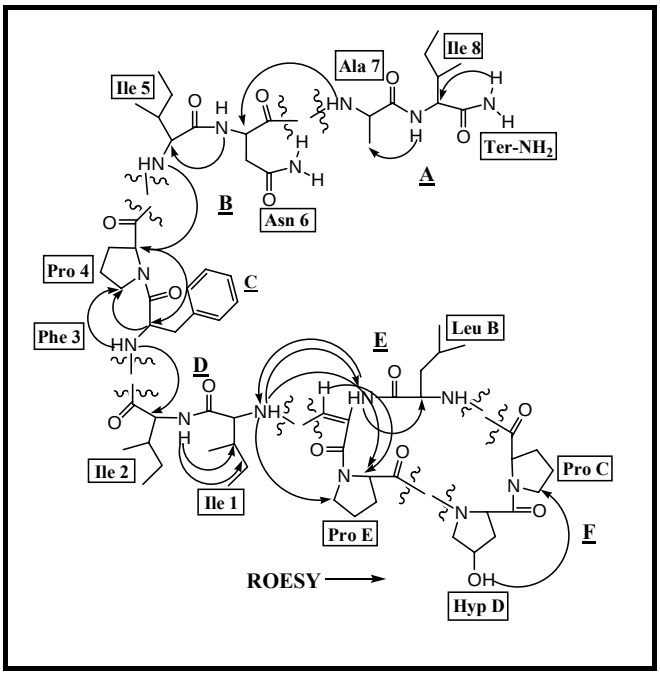


Fig. 3.30: Partial substructures A-F and ROESY correlations.

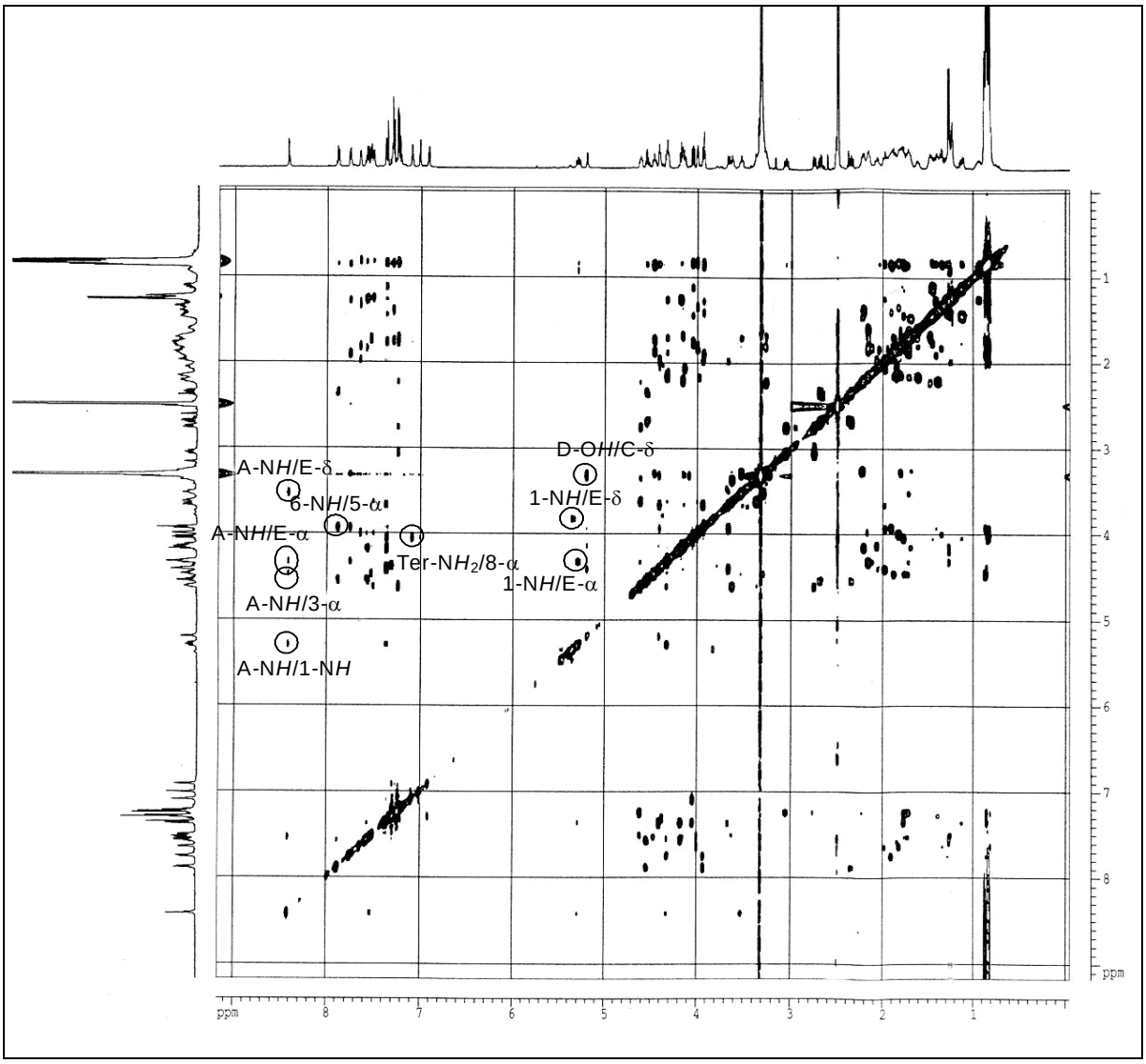
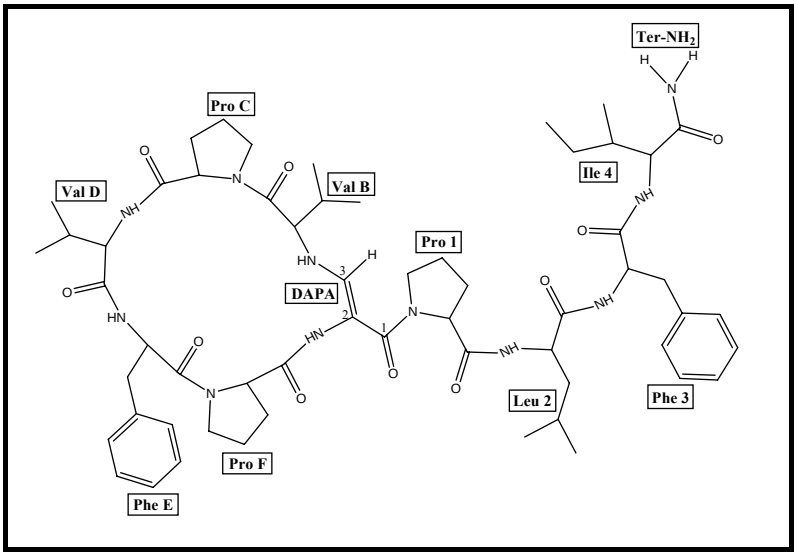


Fig. 3.31: ROESY spectrum of compound 4

Table 3.4: NMR data of compound 4 (DMSO-d_6, 500 MHz)

A. A.	Position	δ_H (m, *J* in Hz)	COSY	ROESY
DAPA	N*H*	8.42 (brs)		B-α, E-α, 1-N*H*
	H-3	7.38 (d, *J* = 8.8 Hz)	1-N*H*	E-α
NH_2 *terminal*		7.11 (brs)		8-α
		7.03 (brs)		
Ile 1	α	4.41 (d, *J* = 10.7 Hz)	1-N*H*, 1-β	
	β	1.98 (m)	1-α	2-N*H*
	γ	0.94 (m), 1.20 (m)	1-β, 1-δ	2-N*H*
	δ, δ`	0.83(m), 0.84 (m)	1-γ	
	N*H*	5.30 (t, *J* = 10.7 Hz)	1-α	E-α, E-δ, DAPA-N*H*
Ile 2	α	4.00 (t, *J* = 7.6 Hz)	2-N*H*	3-N*H*
	β	1.81 (m)	2-α	
	γ	1.25 (m), 1.30 (m)	2-β	
	δ, δ`	0.80 (m), 0.80 (m)	2-γ	
	N*H*	7.66 (d, *J* = 7.6 Hz)	2-α	1-β, 1-γ
Phe 3	α	4.61 (m)	3-N*H*	4-α, 4-δ
	β	3.05 (t, *J* = 16.0 Hz), 2.77 (t, *J* = 16.0 Hz)	3-α	
	N*H*	7.50 (d, *J* = 6.0 Hz)	3-α	2-α, 4-δ
	others	7.25 (d, *J* = 8.8 Hz), 7.23 (d, *J* = 6.9 Hz), 7.21 (d, *J* = 7.6 Hz)		
Pro 4	α	4.32 (t, *J* = 8.6 Hz)	4-β	3-α, 5-N*H*
	β	1.70 (m), 2.16 (m)	4-γ	
	γ	1.80 (m), 1.90 (m)	4-δ	
	δ	3.61 (m), 3.38 (m)	4-γ	3-N*H*, 3-α
Ile 5	α	3.93 (t, *J* = 6.3 Hz)	5-N*H*, 5-β	6-N*H*
	β	1.90 (m)	5-α	
	γ	1.40 (m), 1.28 (m)	5-δ	
	δ, δ`	0.80 (m), 0.85 (m)		
	N*H*	7.76 (d, *J* = 6.3 Hz)	5-α	4-α
Asn 6	α	4.55 (t, *J* = 6.9 Hz)	6-N*H*	7-N*H*
	β	2.69 (dd, *J* = 15.1, 6.9 Hz), 2.35 (dt, *J* = 15.1, 6.9 Hz)	6-α	
	NH_2	6.93 (s), 7.31 (s)		
	N*H*	7.88 (d, *J* = 7.6 Hz)	6-α	5-α
Ala 7	α	4.16 (t, *J* = 6.9 Hz)	7-N*H*, 7-β	8-N*H*
	β	1.25 (d, *J* = 6.9 Hz)	7-α	
	N*H*	7.57 (d, *J* = 6.9 Hz)	7-α	6-α
Ile 8	α	4.05 (d, *J* = 6.7 Hz)	8-N*H*, 8-β	Ter.-NH_2
	β	1.77 (m)	8-α	
	γ	1.12 (m), 1.45 (m)	8-β	
	δ, δ`	0.85 (m), 0.87 (m)	8-γ	
	N*H*	7.34 (d, *J* = 6.7 Hz)	8-α	7-β
Leu B	α	4.45 (d, *J* = 9.5 Hz)	B-N*H*	DAPA-N*H*
	β	1.85 (m), 1.71 (m)	B-α	
	γ	1.70 (m)	B-β	
	δ, δ`	0.80 (m), 0.82 (m)	B-γ	
	N*H*	7.54 (d, *J* = 9.5 Hz)	B-α	
Pro C	α	4.30 (t, *J* = 8.6 Hz)	C-β	
	β	1.75 (m), 2.17 (m)	C-α, C-γ	
	γ	1.60 (m), 1.70 (m)	C-β, C-δ	
	δ	3.30 (m), 3.53 (t, *J* = 8.8 Hz)	C-γ	D-O*H*
Hyp D	α	4.40 (m)	D-β	
	β	2.00 (m), 2.21 (m)	D-α, D-γ	
	γ	1.91(m)	D-β, D-δ	
	δ	3.65 (m), 3.93 (t, *J* = 6.3 Hz)	D-γ	
Pro E	α	4.17 (m)	E-β	H-3
	β	1.40 (m), 1.70 (m)	E-α, E-γ	
	γ	1.42 (m), 1.66 (m)	E-β, E-δ	
	δ	2.23 (m), 3.27 (m)	E-γ	1-N*H*

3.1.5. Callyaerin F (5, known compound)



Callyaerin F (**5**) was isolated as a white amorphous powder, with an $[\alpha]_D$ of - 32° (*c* 0.15, CH_3OH). It has UV absorbances at λ_{max} 204 and 279 nm. It has a molecular weight of 1094 g/mol and a molecular formula $C_{58}H_{83}N_{11}O_{10}$ as confirmed by HRMS. This was also supported by signals found in the ESIMS spectrum at *m*/*z* 1095 $[M+H]^+$, 1117 $[M+Na]^+$ (positive), 1093 $[M-H]^+$ and 1140 $[M+HCOOH]^+$. The ^1H NMR spectrum (Table 3.5, Fig. 3.33) indicated the peptidic nature of compound **5**. It showed six deshielded amide N*H* protons at δ 7.66 (d, *J* = 7.8 Hz, 2-N*H*), 7.57 (d, *J* = 6.3 Hz, 4-N*H*), 7.54 (d, *J* = 8.2 Hz, D-N*H*), 7.50 (d, *J* = 6.7 Hz, E-N*H*), 6.18 (d, *J* = 8.2 Hz, 3-N*H*), 5.14 (dd, *J* = 10.0, 4.0 Hz, B-N*H*), and a cluster of α amino acid methines at δ 3.80-4.68, signals for methylenes, aliphatic primary and secondary methyls which indicated the presence of lipophilic amino acid residues. ^1H-^1H COSY spectrum showed 9 spin systems for 9 amino acid residues (see Table 3.5) consisting of two valines, three prolines, two phenylalanines, one leucine, and one isoleucine. Compound **5** was identified by comparing NMR and MS data with those of callyaerin **F**, previously isolated from marine sponge *Callyspongia aerizusa* [Min, *et al*., 2001] stereochemistries of the individual amino acid constituents were determined by acid hydrolysis of the parent peptide and reaction with Marfey´s reagent [Marfey, 1984]. The resulting 1-flouro-2,4-dinitrophenyl-5-L-leucinamide derivatives established the presence of two L-Val, three L-Pro, one L-Leu, one L-Ile, and two L-Phe.

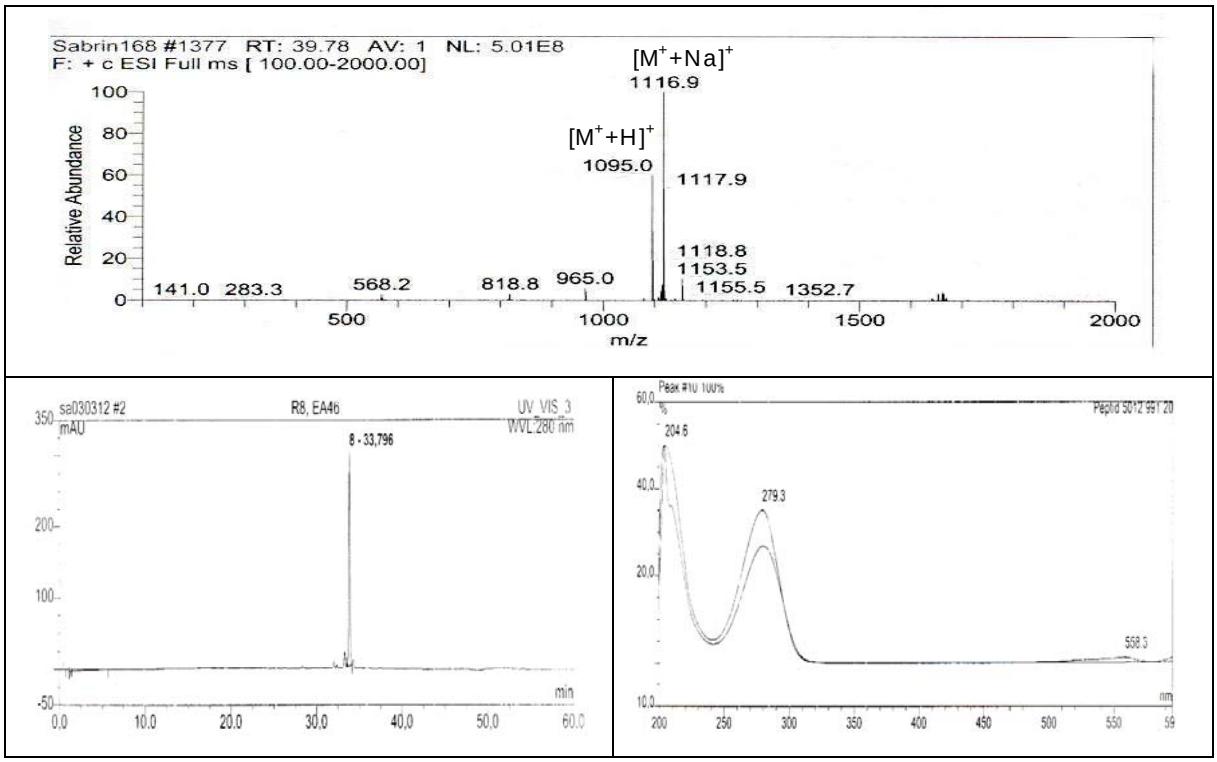


Fig. 3.32: ESIMS and UV spectra of compound 5

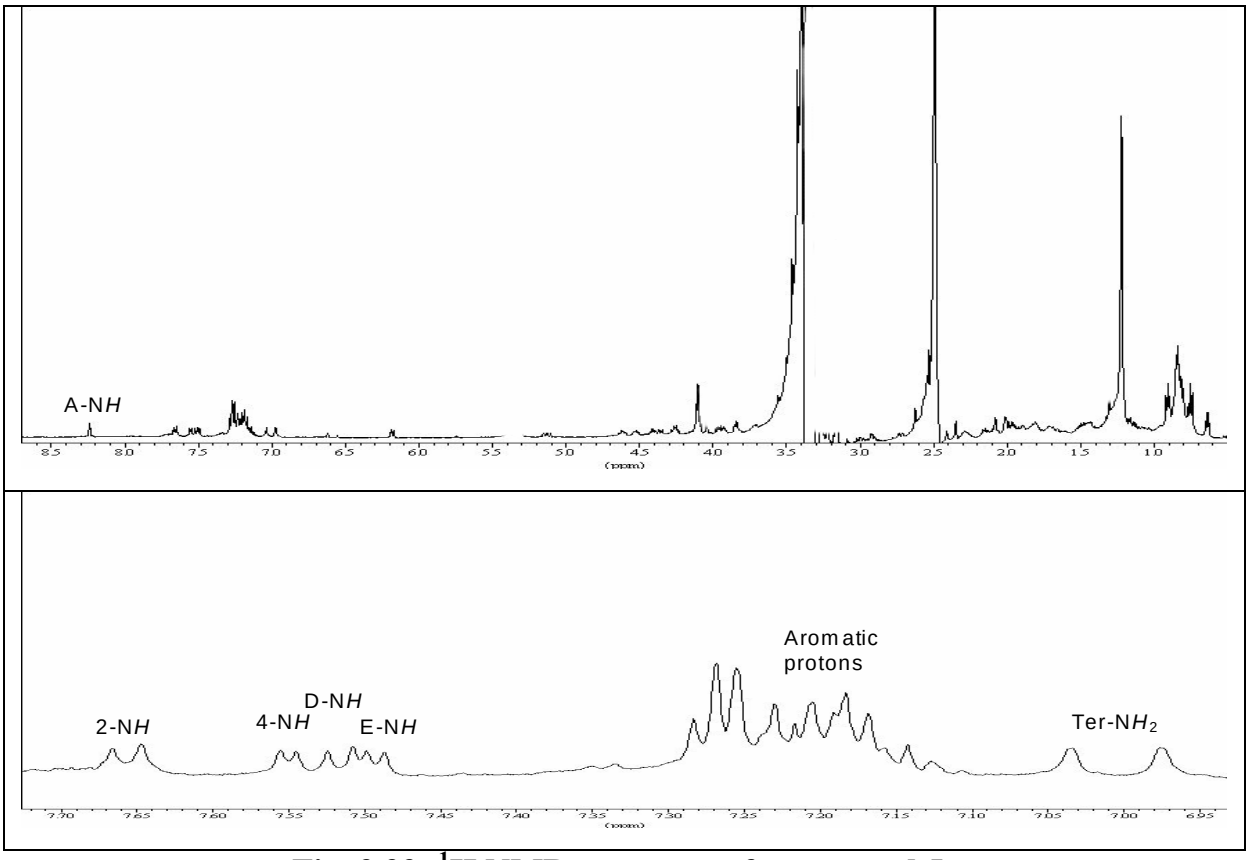
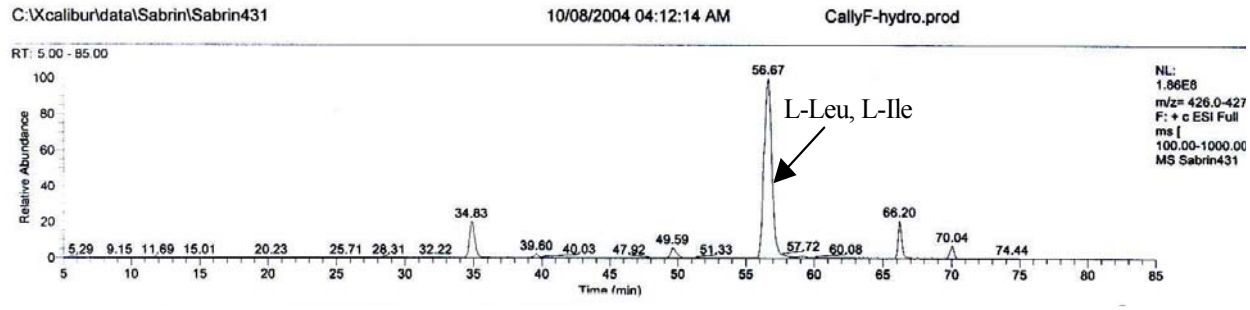
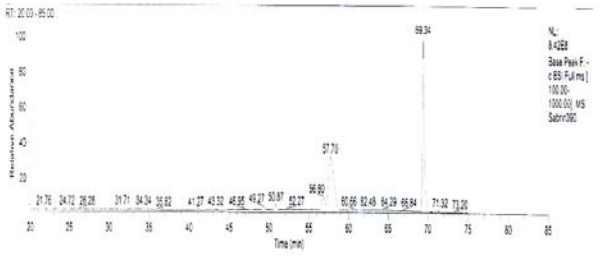


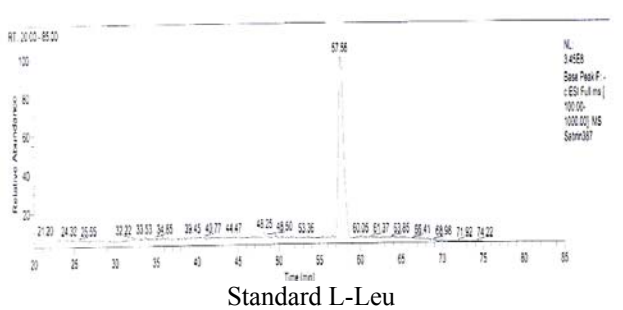
Fig. 3.33: ^{1}H NMR spectrum of compound 5



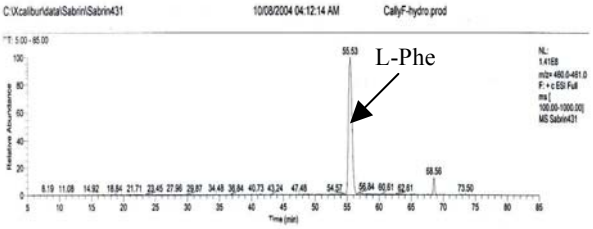
LC/MS chromatogrm of cally. F hydrolysis product
Base peak search at *m/z* 426-427



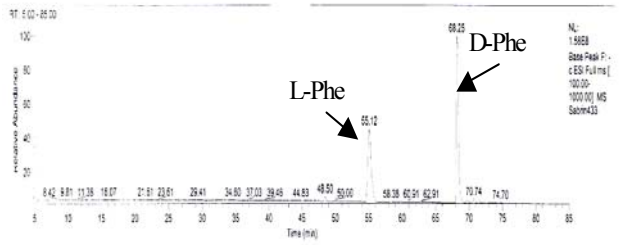
Standard D, L-Ile



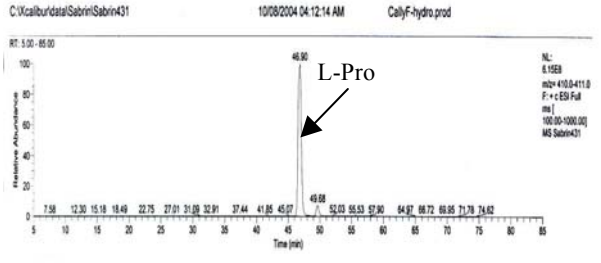
Standard L-Leu



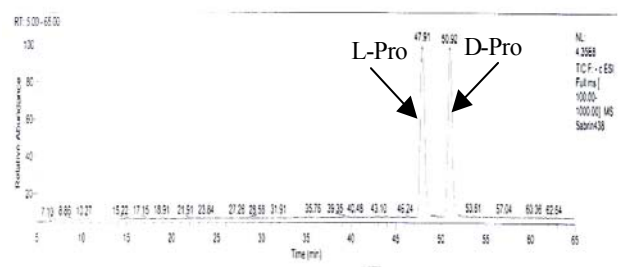
Cally. F hydrolysis product
Base peak search at *m/z* 459-460



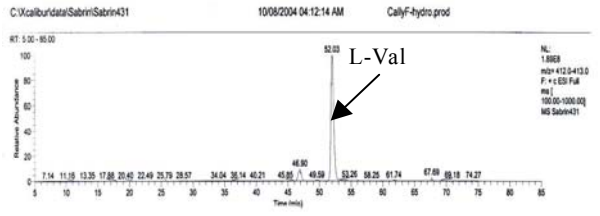
Standard D, L-Phe



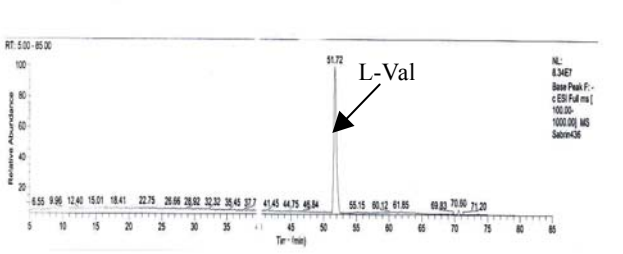
Cally. F hydrolysis product
Base peak search at *m/z* 410-411



Standard D, L-Pro



Cally. F hydrolysis product
Base peak search at *m/z* 412-413



Standard L-Val

Fig. 3.34: Amino acid analysis results of compound 5

Table 3.5: ^{1}H NMR and COSY data of compound 5 (DMSO-d_6, 500 MHz)

A. A.	Position	δ_H (m, J in Hz)	COSY
DAPA	N*H*	8.25 (s)	
	H-3	7.25	B-N*H*
NH_2 terminal		7.04 (brs)	
		6.95 (brs)	
Val B	α	4.38 (brd, *J* = 10.0 Hz)	B-N*H*
	β	2.07 (m)	B-α
	γ	0.87 (d, *J* = 6.9 Hz), 0.66 (d, *J* = 6.9 Hz)	B-β
	N*H*	5.14 (dd, *J* = 10.0, 4.0 Hz)	B-α, H-3
Pro C	α	3.95 (m)	C-β
	β	2.17 (m), 1.81 (m)	C-α, C-γ
	γ	2.03 (m), 1.84 (m)	C-β
	δ	3.86 (m), 3.74 (m)	C-γ
Val D	α	3.86 (d, *J* = 8.2 Hz)	D-N*H*
	β	2.11 (m)	D-α
	γ	0.93 (d, *J* = 6.9 Hz), 0.86 (d, *J* = 6.9 Hz)	
	N*H*	7.54 (d, *J* = 8.2 Hz)	2-α
Phe E	α	4.54 (dd, *J* = 6.9, 6.7)	E-N*H*
	β	3.02 (dd, *J* = 15.1, 6.7 Hz), 2.71 (dd, *J* = 15.1, 6.9 Hz)	E-α
	N*H*	7.50 (d, *J* = 6.7 Hz)	E-α
	others	7.14-7.28	
Pro F	α	4.28 (m)	F-β
	β	2.09 (m), 1.80 (m)	
	γ	1.91 (m), 1.83 (m)	
	δ	3.58 (m), 3.35 (m)	F-γ
Pro 1	α	4.26 (m)	1-β
	β	2.31 (m), 1.42 (m)	
	γ	1.73 (m), 1.45 (m)	1-δ
	δ	3.26 (m), 2.19 (m)	1-γ
Leu 2	α	4.64 (dt, *J* = 10.0, 3.8 Hz)	2-N*H*
	β	1.93 (m), 1.67 (m)	2-α
	γ	1.72 (m)	
	δ, δ`	0.94 (d, *J* = 6.9 Hz), 0.84 (d, *J* = 6.9 Hz)	2-γ
	N*H*	7.66 (d, *J* = 7.8 Hz)	2-α
Phe 3	α	4.42 (dt, *J* = 7.6, 4.4 Hz)	3-N*H*
	β	2.93 (m)	3-α
	N*H*	6.18 (d, *J* = 8.2 Hz)	3-α
	others	7.14-7.28	
Ile 4	α	3.98 (dd, *J* = 8.2, 6.3 Hz)	4-N*H*
	β	1.75 (m)	4-α
	γ	1.29 (m), 1.10 (m)	
	δ, δ`	0.77 (d, *J* = 6.9 Hz), 0.77 (d, *J* = 6.6 Hz)	
	N*H*	7.57 (d, *J* = 6.3 Hz)	4-α

Bioactivity

The extracts and some of the isolated compounds of the sponge *Callyspongia aerizusa* were screened for activity against brine shrimp. It was found that the total and ethyl acetate extracts exhibited strong activities with 100 % mortality rates at concentrations of 20 µg/mL and 50 µg/mL. Compounds **1** and **2** showed moderate activities, while compound **3** was less active. On other hand, the isolated callyaerins were tested for the cytotoxicity against mouse lymphoma cells (L5178Y), human cervix carcinoma (HeLa), and rat brain tumour (PC12) cell lines. It was observed that callyaerin B and S exhibited strong activities against L5178Y cell line, whereas callyaerin E exhibited strong activity against L5178Y and Hela cell lines, while callyaerin F was inactive on any of the cell lines tested. All the results are summarised in (Table 3.6, 3.7). In the antiviral test (anti-HIV), only callyaerin E showed moderate activity, while the other callyaerins B, D, F, and S have no activity.

Table 3.6: Results of the brine shrimp test of *Callyspongia aerizusa*

Samples	Mortality (%)	
	20 µg/mL	50 µg/mL
Total extract	100 %	100 %
Ethyl acetate extract	95 %	100 %
Hexane extract	30 %	50 %
Callyaerin B (3)	15 %	35 %
Callyaerin E (1)	45 %	70 %
Callyaerin S (2)	30 %	55 %

Table 3.7: **Cytotoxicity test of the isolated callyaerins**

Compound	% Growth inhibition								
	L5178Y			Hela cell			PC12 cell		
	3 µg/mL	10 µg/mL	ED50 (µg/mL)	3 µg/mL	10 µg/mL	ED50 (µg/mL)	3 µg/mL	10 µg/mL	ED50 (µg/mL)
Callyaerin B	21	100	5.3	4	2	> 10	2	3	> 10
Callyaerin E	100	100	0.49	48	100	4.4	47	88	4.9
Callyaerin F	0	7	> 10	0	0	> 10	0	0	> 10
Callyaerin S	100	100	0.51	n.t.	n.t.		n.t.	n.t.	

* n.t. not tested

3.2. Isolated compounds from the sponge *Diacarnus megaspinorhabdosa*

Terpenoid peroxides have been commonly isolated from the sponge family Latrunculiidae. Terpene peroxides have also been found in plants. In 1979, two different research groups independently reported the first examples of sponge-derived terpene peroxides. These consisted of muqubilin from Red Sea *Prianos* species [Kashman, *et al*., 1979] and sigmosceptrellin A methyl ester from the Papua New Guinea *Sigmosceptrella laevis* [Sperry, *et al*., 1998]. The isolated marine-derived terpenoid peroxides were attributed to the sponge families Latrunculiidae (order Poecilosclerida and/or Hadromerida) and Mycalidae (order Poecilosclerida), respectively. In the search for biologically active metabolites from marine invertebrates, seven new cytotoxic norterpene peroxides (**8, 10-16**) and norsesterpene (**18**) were isolated from the Indonesian marine sponge *Diacarnus megaspinorhabdosa* in addition to the known compounds nuapapuin A methyl ester (**6**) [Manes, *et al*., 1984], methyl-2-epinuapapuanoate (**7**), methyl diacarnoate A (**9**) [D´ Ambrosio, *et al*., 1997], and epimuqubilin B (**17**) [Sperry, *et al*., 1998].

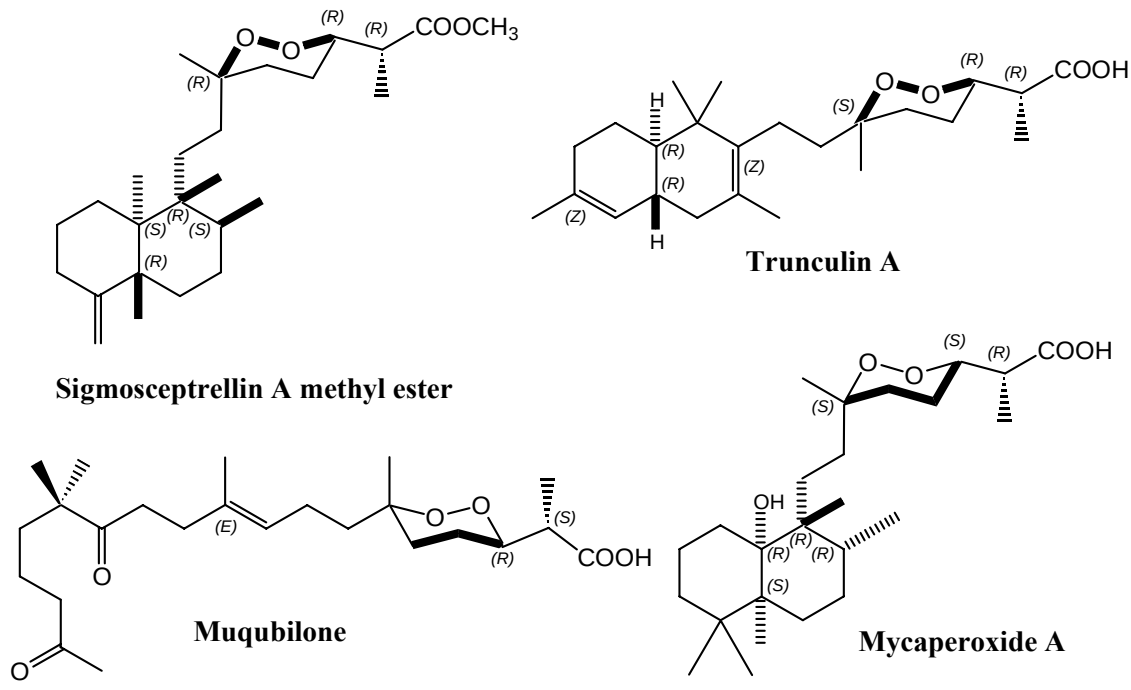
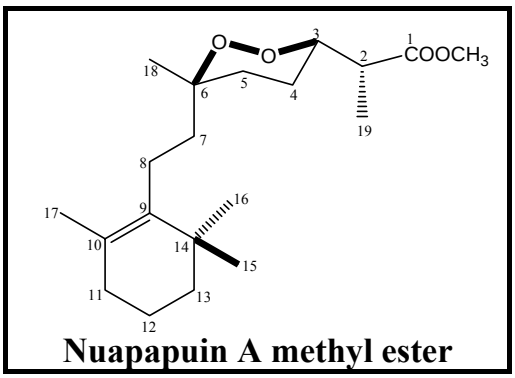


Fig. 3.35: Some norterpene peroxides isolated from marine sponges

3.2.1. Nuapapuin A methyl ester (6, known compound)



Nuapapuin A methyl ester

Compound **6** was isolated as a colorless oil. The $[\alpha]_D$ value was + 52.7° (*c* 0.175, $CHCl_3$). The EIMS spectrum showed a pseudomolecular ion peak at *m*/*z* 338 $[M]^+$. The significant fragment ion at *m*/*z* 137 suggested the presence of a cyclohexene moiety. The ^1H and ^{13}C NMR spectral data were identical to those of nuapapuin A methyl ester [Manes, *et al*., 1984]. The ^1H NMR spectrum revealed the presence of four methyl singlet signals: two of them were geminal methyls at δ 1.00 (H_3-15/16), one at δ 1.14 (H_3-18) which was bound to a quaternary carbon and a vinyl methyl singlet at δ 1.60 (H_3-17). Furthermore, a doublet methyl signal at δ 1.12 (H_3-19) was also observed. Two coupled methylene signals were present at δ 1.55 (H_2-7) and 1.92, 2.07 (H_2-8). A methoxy signal at δ 3.68 indicated the methyl ester nature of compound **6**. The oxymethine signal at δ 4.25 (1H, dt, *J* = 6.9, 2.8 Hz, H-3) indicated the presence of the cyclic peroxide moiety [Sperry, *et al*., 1998]. These findings were supported by the ^{13}C NMR and DEPT spectral data, which revealed the presence of 20 carbons. The ^{13}C NMR signals at δ 136.6 (C-9), 127.0 (C-10), 32.7 (C-11), 19.5 (C-12), 39.8 (C-13), 34.9 (C-14), 28.6 (C-15), 28.4 (C-16), and 19.7 (C-17) indicated the presence of a 2, 6, 6-trimethyl cyclohexene moiety [Yunker, *et al*., 1978, Tsuda, *et al*., 1992, Albizati, *et al*., 1987]. This also coincided with the fragment ion peak at *m*/*z* 137, which is characteristic of the polyalkylated-cyclohexene moiety encountered in the manoalide-related sesterpenes [Butler, *et al*., 1992]. The signals at δ 81.1 (C-3), 32.5 (C-4), 34.7 (C-5), and 80.0 (C-6) indicated the presence of the cyclic peroxide moiety [Capon, *et al*., 1998, Ovenden, *et al*., 1998]. The ^{13}C NMR and DEPT data confirmed the presence of the propionic acid methyl ester moiety in **6** from the characteristic signals at δ 174.2 (C-1), 42.6 (C-2), 12.5 (C-19) and 51.8 (OCH_3). The

^{1}H-^{1}H COSY and HMQC data allowed the unambiguous assignment of four spin systems. The first spin system of the methylenes at δ 1.90 (H_2-11), 1.55 (H_2-12) and 1.40 (H_2-13) corresponded to the cyclohexene moiety. The second spin system consisted of two isolated methylene signals at δ 1.55 (H_2-7) and 2.07, 1.92 (H_2-8) for the ethylene moiety (C*H*$_2$-C*H*$_2$). The methylene signal at δ 1.76 (H_2-5) showed correlation with the methylene protons signal at δ 1.56 (H_2-4), and this further correlated with the oxymethine signal H-3 to complete the peroxide ring. The last spin system consisted of a methine proton at δ 2.55 (H-2) correlated with the doublet methyl signal at δ 1.12 (H_3-19), which was characteristic of a propionic acid methyl ester moiety [Manes, *et al*., 1984]. The connectivities of the different spin systems were established by HMBC. The HMBC spectrum of **6** showed correlations of the geminal dimethyl group (H_3-15/16) with C-9, C-13, and C-14 and of the vinyl methyl (H_3-17) with C-9, C-10, and C-11. These resonances were characteristic of the cyclohexene function. The location of the ethylene group adjacent to the cyclohexene moiety was confirmed through the HMBC correlations of the methylene protons (H_2-8) with C-9, C-10, and C-14 of the latter ring system. The cyclic peroxide structure was also validated by the correlations observed in the HMBC (Table 3.9). The connectivity of the cyclic peroxide with that of ethylene moiety at C-7 was established through the HMBC correlations of H_2-7 with C-5 and of H_2-8 with C-6. The attachment of the propionic acid methyl ester with the cyclic peroxide was proved by the correlations of H_3-19 with C-3 and of H-3 with C-1. Comparison of the NMR data and $[\alpha]_D$ of compound **6** with those reported for nuapapuin A methyl ester [+ 53.7° (*c* 0.13, $CHCl_3$)] isolated from *Prianos* species [Manes, *et al*., 1984] indicated that both compounds were identical. The stereochemistry was assigned to be 2*R*, 3*R*, 6*R*, which agreed to that in the literature as based on Capon and MacLeod empirical rules. To prove these empirical rules, a Mosher analysis of **6** was done after hydrolysis of the peroxide ring to yield the triol derivative (**6a**), which was then converted to the (*R*)-MTPA ester (**6b**) and the (*S*)-MTPA ester (**6c**) [Capon, *et al*., 1998, Ovenden, *et al*., 1999]. Diagnostic ^{1}H NMR chemical shift differences between these MTPA diastereomers [Δ δ*S* - δ*R*] confirmed the 2*R*, 3*R*, 6*R* absolute stereochemistry (see Table 3.8).

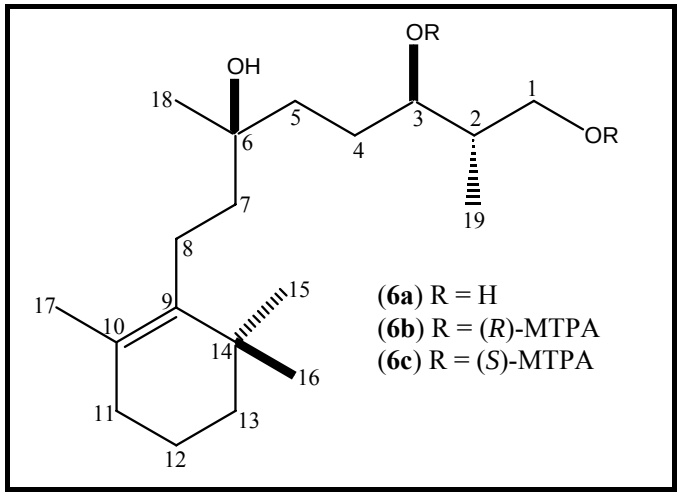


Fig. 3.36: Mosher analysis of compound 6

Table 3.8: ^{1}H NMR results of Mosher analysis in C_5D_5N

Proton no.	Triol	(*S*)-MTPA ester	(*R*)-MTPA ester	Δ δ*S* - δ*R*
1	4.08 4.03	4.607 4.394	4.483 4.329	0.124 0.065
2	2.39	2.456	2.426	0.030
3	3.97	5.465	5.464	0.001
18	1.46	1.323	1.402	- 0.079
19	1.16	1.007	0.938	0.069

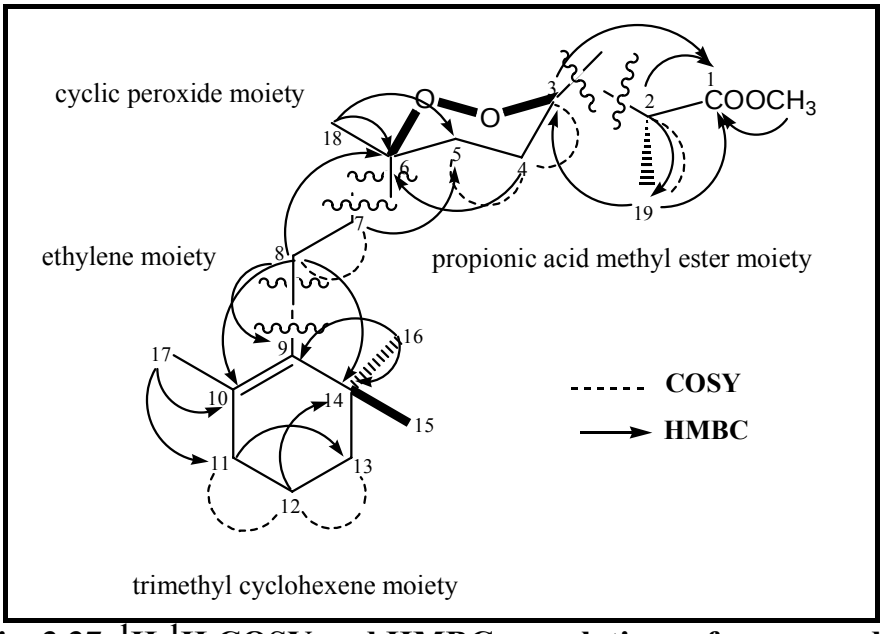


Fig. 3.37: ^{1}H-^{1}H COSY and HMBC correlations of compound 6

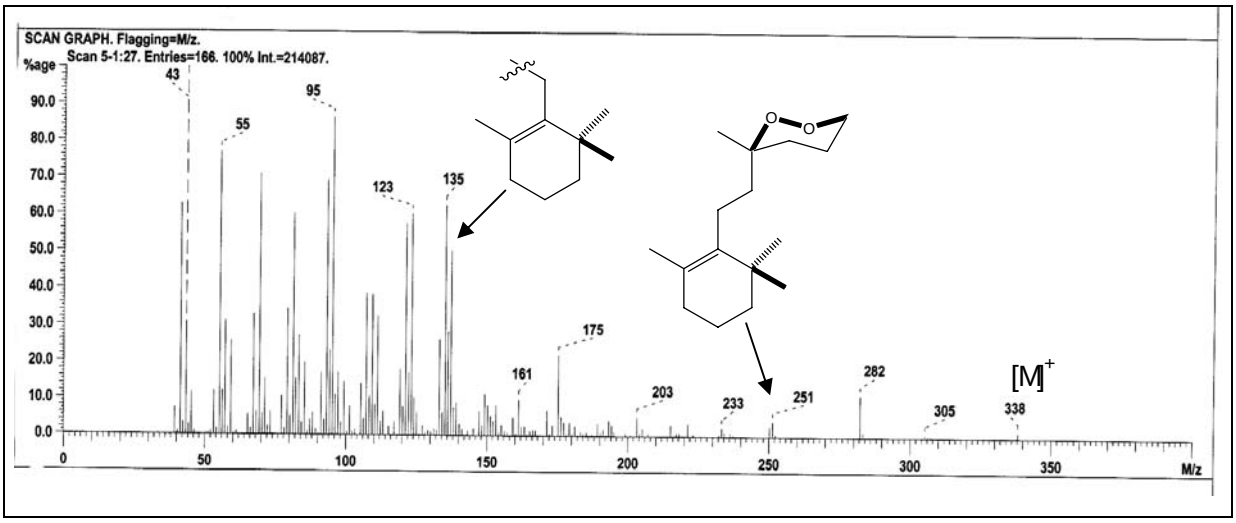


Fig. 3.38: EIMS spectrum of compound 6

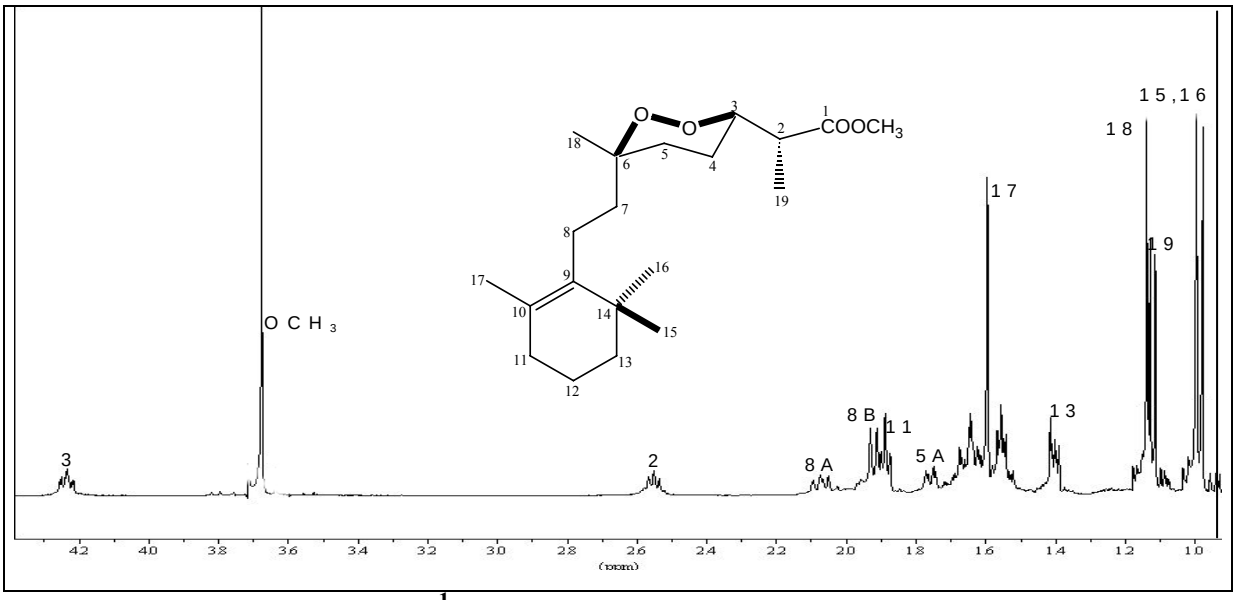


Fig. 3.39: ^{1}H NMR spectrum of compound 6

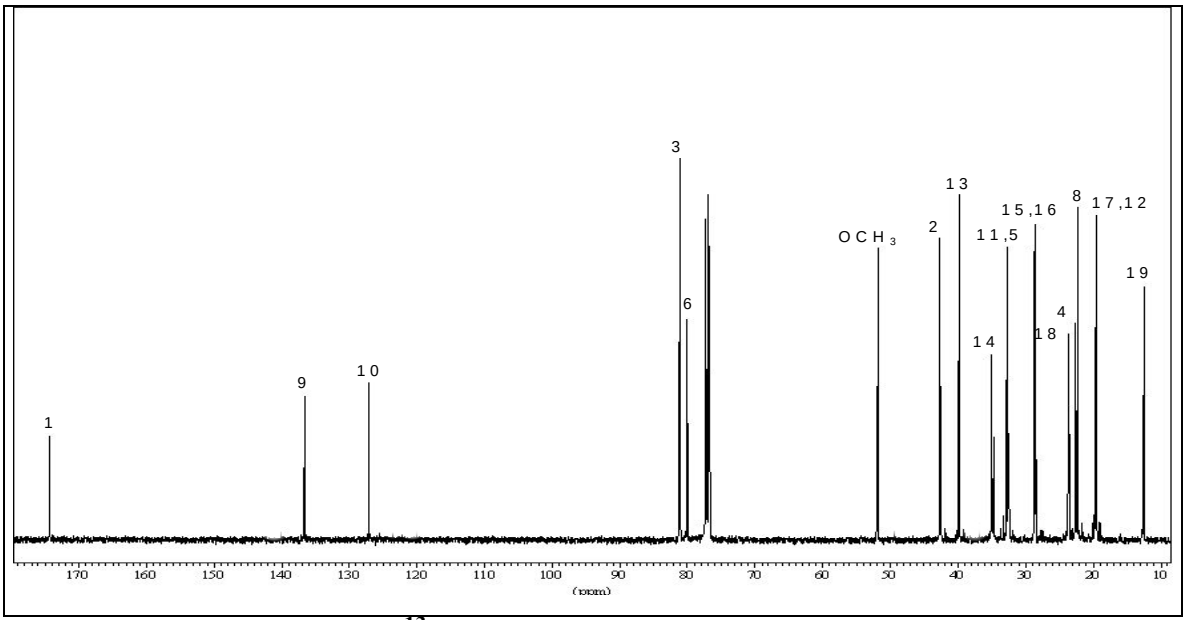


Fig. 3.40: ^{13}C NMR spectrum of compound 6

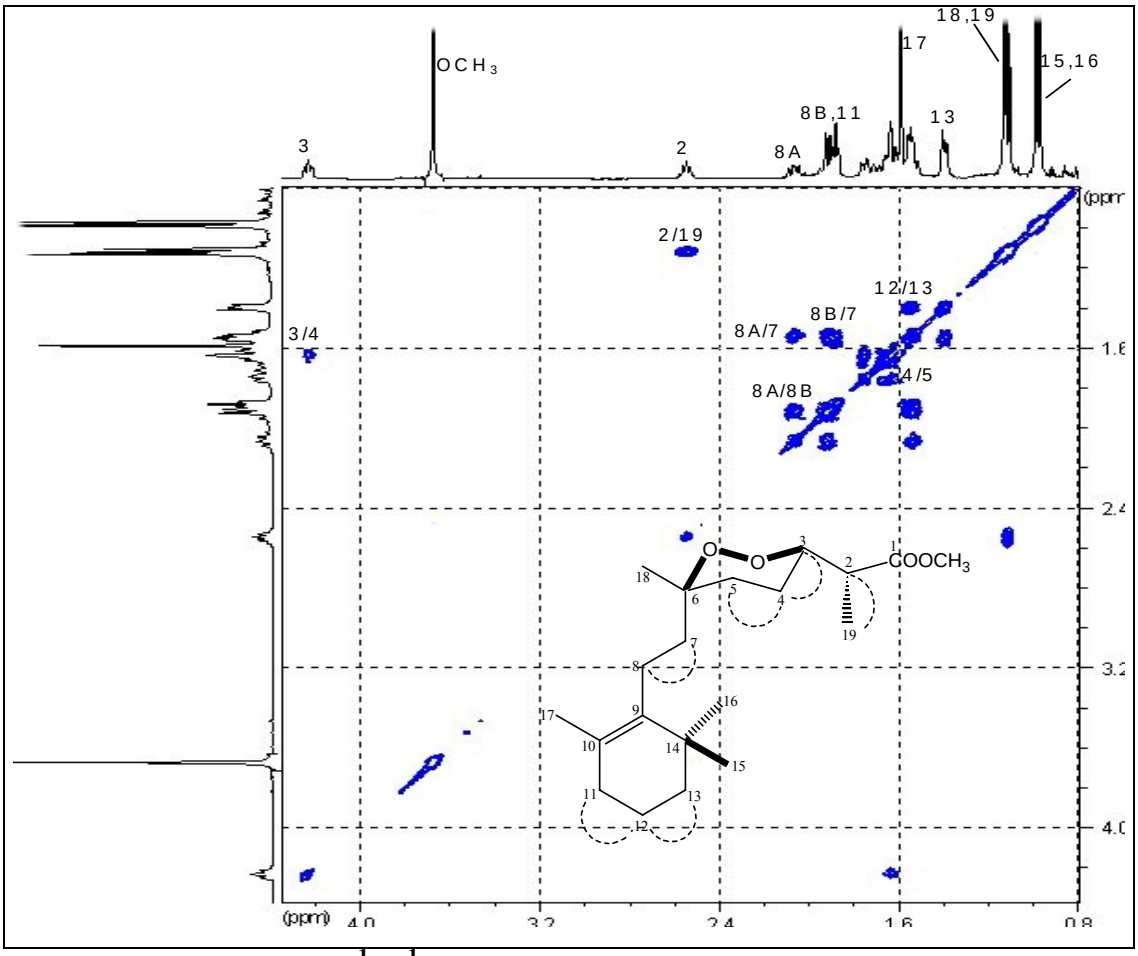


Fig. 3.41: ^{1}H-^{1}H COSY spectrum of compound 6

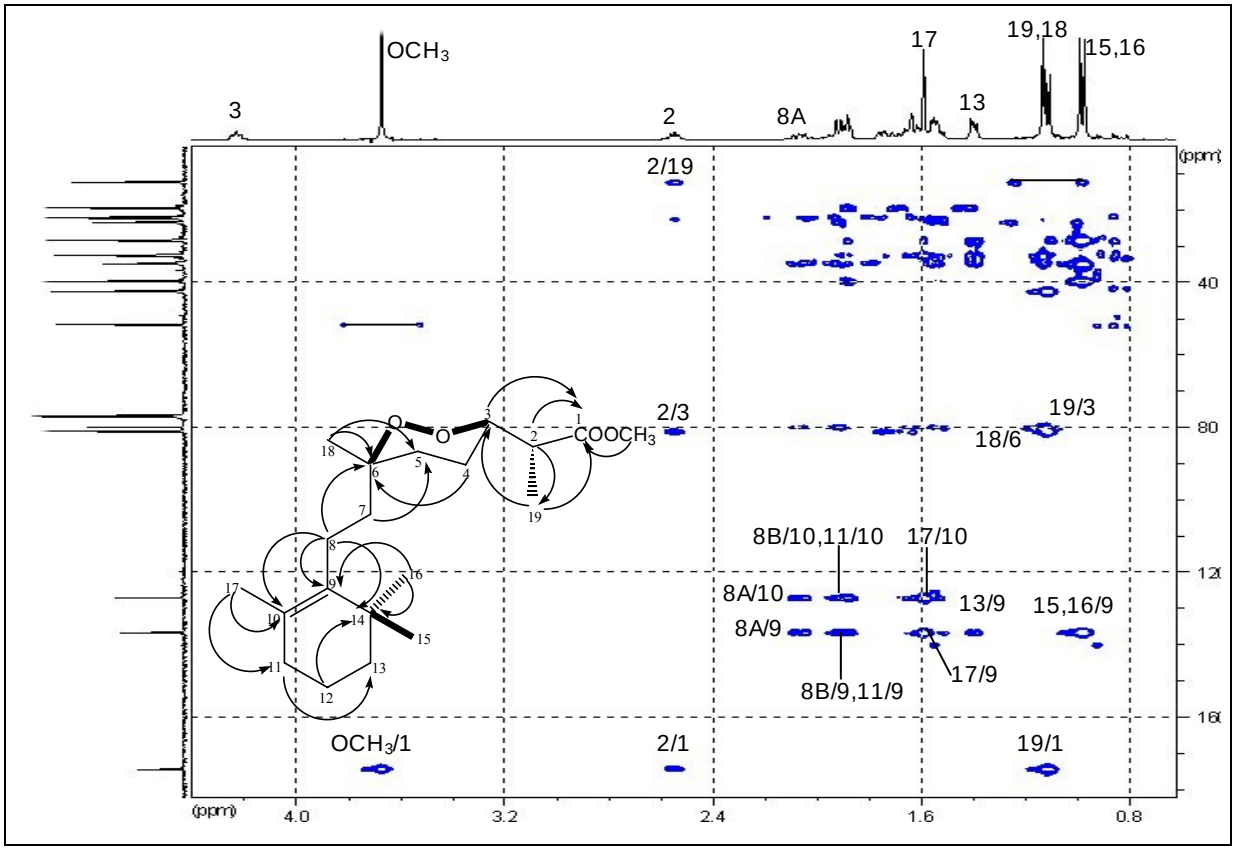
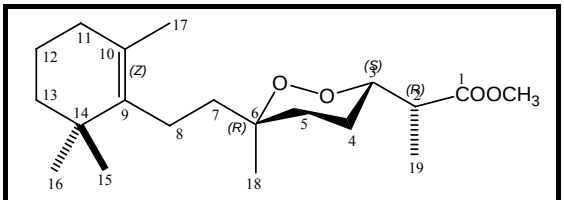


Fig. 3.42: HMBC spectrum of compound 6

Table 3.9: NMR data of compound 6 ($CDCl_3$, 500, 125 MHz)

Position	δ_H (m, *J* in Hz)	δ_C (m)	δ_C (m) of nuapapuin A methyl ester [Manes, *et al*., 1984 in $CDCl_3$]	COSY	HMBC
1		174.2 s	174.1 s		
2	2.55 (p, *J* = 7.2 Hz)	42.6 d	42.7 d	19	1, 3, 19
3	4.25 (dt, *J* = 6.9, 2.8 Hz)	81.1 d	81.1 d	4	1, 2
4	1.56 (m)	32.5 t	32.8 t	5	3, 6
5	1.76 (m)	34.7 t	34.9 t	4	3
6		80.0 s	79.9 s		
7	1.55 (m)	22.2 t	22.2 t	8	5
8	2.07 (dd, *J* = 14.3, 8.2 Hz) 1.92 (d, *J* = 14.3 Hz)	22.6 t	22.6 t	7	6, 7, 9, 10, 14
9		136.6 s	136.8 s		
10		127.0 s	127.0 s		
11	1.90 (t, *J* = 6.3 Hz)	32.7 t	32.8 t	12	9, 10, 13, 14, 17
12	1.55 (m)	19.5 t	19.5 t	13	14, 11
13	1.40 (m)	39.8 t	40.0 t	12	9, 11, 15, 16
14		34.9 s	34.9 s		
15	1.0 (s)	28.6 q	28.6 q		13, 14, 16, 9
16	1.0 (s)	28.4 q	28.6 q		13, 14, 16, 9
17	1.60 (s)	19.7 q	19.6 q		8, 9, 10, 11
18	1.14 (s)	23.6 q	23.6 q		5, 6, 4
19	1.12 (d, *J* = 8.2 Hz)	12.5 q	12.5 q	2	2, 3, 1
OCH_3	3.68 (s)	51.8 q	51.8 q		1

3.2.2. Methyl-2-epinuapapuinoate (7, known compound)



Methyl-2-epinuapapuinoate

Compound **7** was isolated as a colorless oil. The $[\alpha]_D$ value was - 30.3° (*c* 0.20, $CHCl_3$). The EIMS spectrum exhibited a pseudomolecular ion peak at *m*/*z* 338 $[M]^+$. The diagnostic fragment ion at *m*/*z* 251 $[M-87]^+$ could be due to the loss of a propionic acid methyl ester. The fragment ion at *m*/*z* 137 representing the trimethyl cyclohexyl moiety was also present. The ^1H and ^{13}C NMR spectral data of compound **7** were very similar to those of **6** and indicated it had the identical carbon skeleton. The NMR data of **7** were identical to those of methyl-2-epinuapapuinoate [D´Ambrosio, *et al*., 1997]. When **7** was compared to **6**, differences in the ^1H and ^{13}C NMR chemical shifts were observed for C-2 and C-6 methyl groups, which suggested the change in the

stereochemistry at these positions. C-2 methyl (H_3-19) was shifted downfield to δ 1.25, which indicated a *threo* configuration at C-2 and C-3 (*R*, *S* or *S*, *R*). While C-6 methyl (H_3-18) was shifted upfield to δ 20.5 which signified an axially-oriented methyl substituent. These findings indicated that compound **7** was a diastereoisomer of **6**. The stereochemistry was assumed to be identical with that reported in the literature. On the basis of a comparable optical rotation value $[\alpha]_D$ - 30.3° (*c* 0.25, $CHCl_3$) with that in the literature - 33° (*c* 0.40, $CHCl_3$) and also the similar chemical shifts for the methyl functions at C-2 and C-6, the stereochemistry of **7** was concluded to be 2*R*, 3*S*, 6*R* as in **methyl-2-epinuapapuinoate** [Capon, *et al*., 1985].

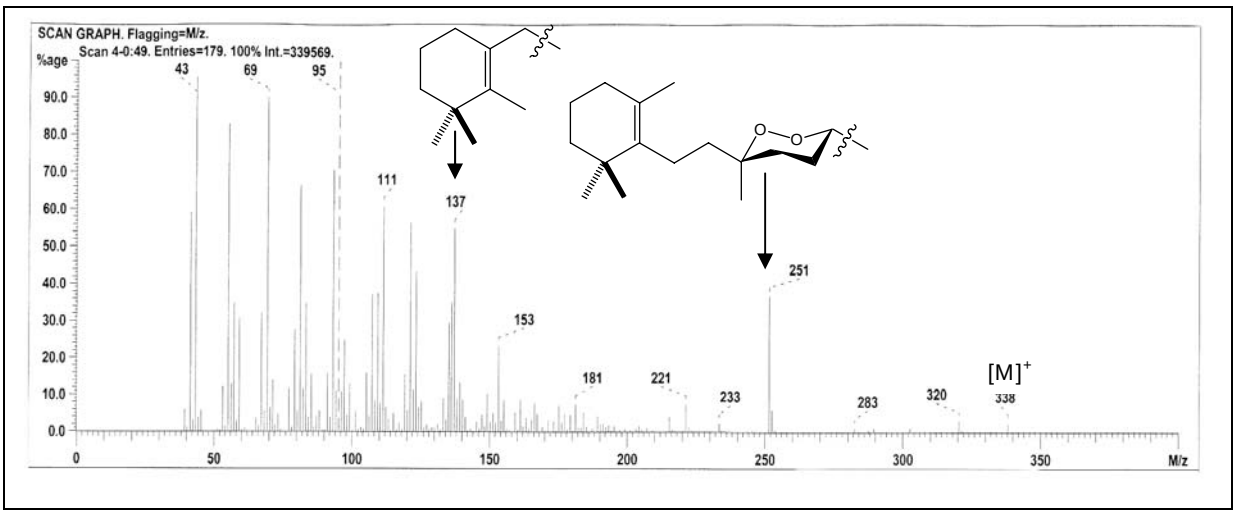


Fig. 3.43: EIMS spectrum of compound 7

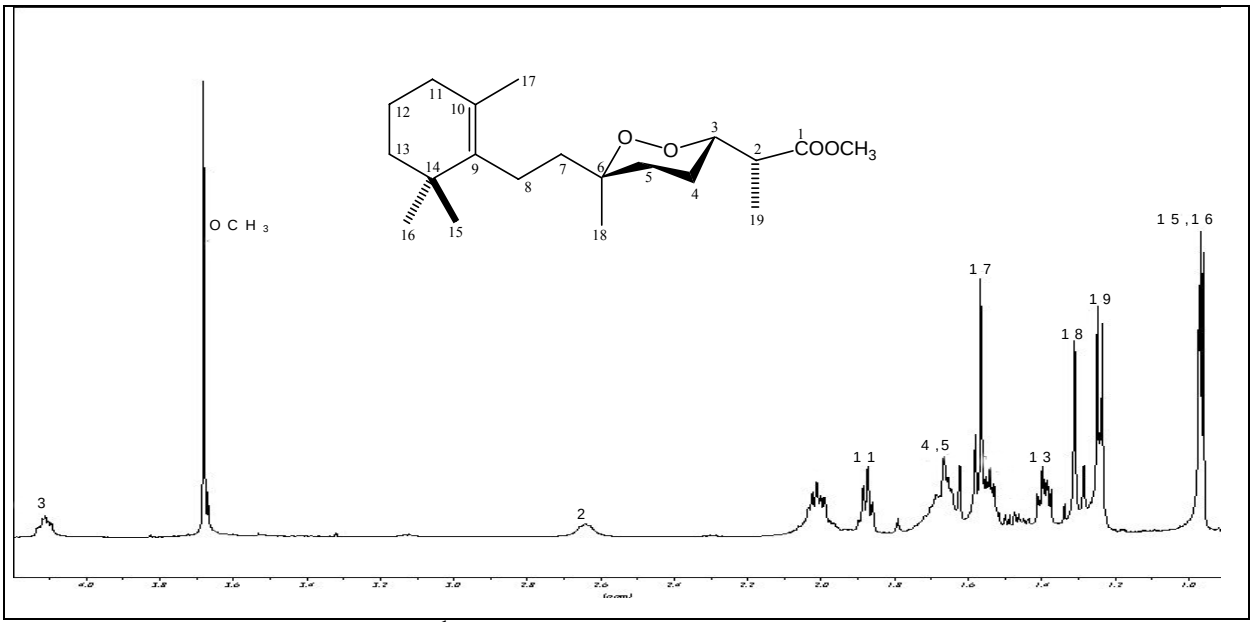


Fig. 3.44: ^{1}H NMR spectrum of compound 7

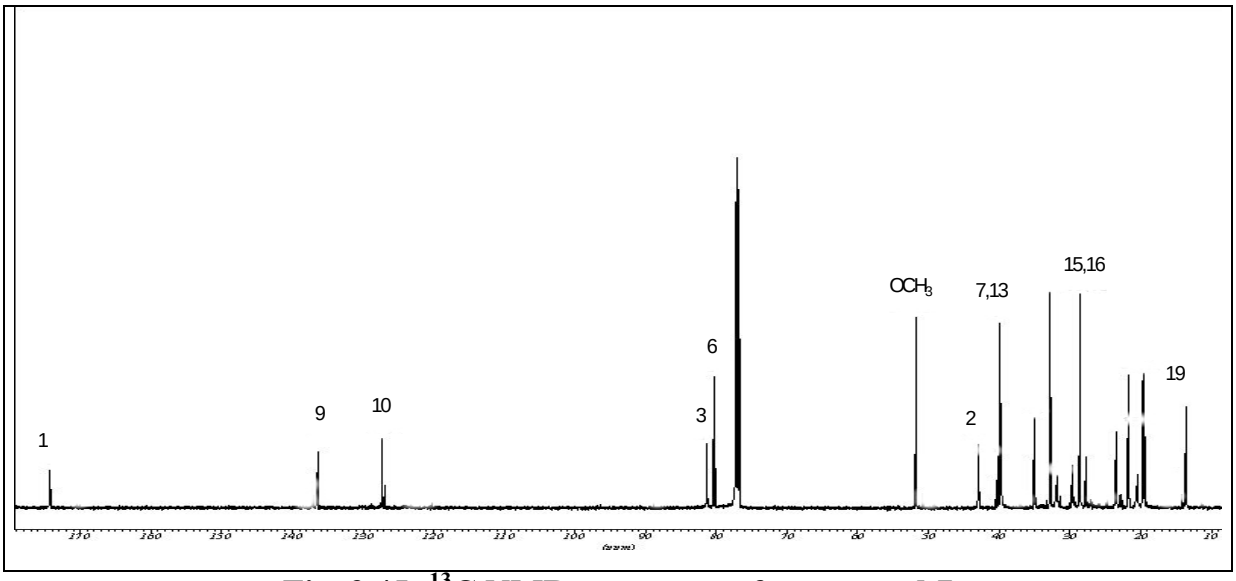


Fig. 3.45: ^{13}C NMR spectrum of compound 7

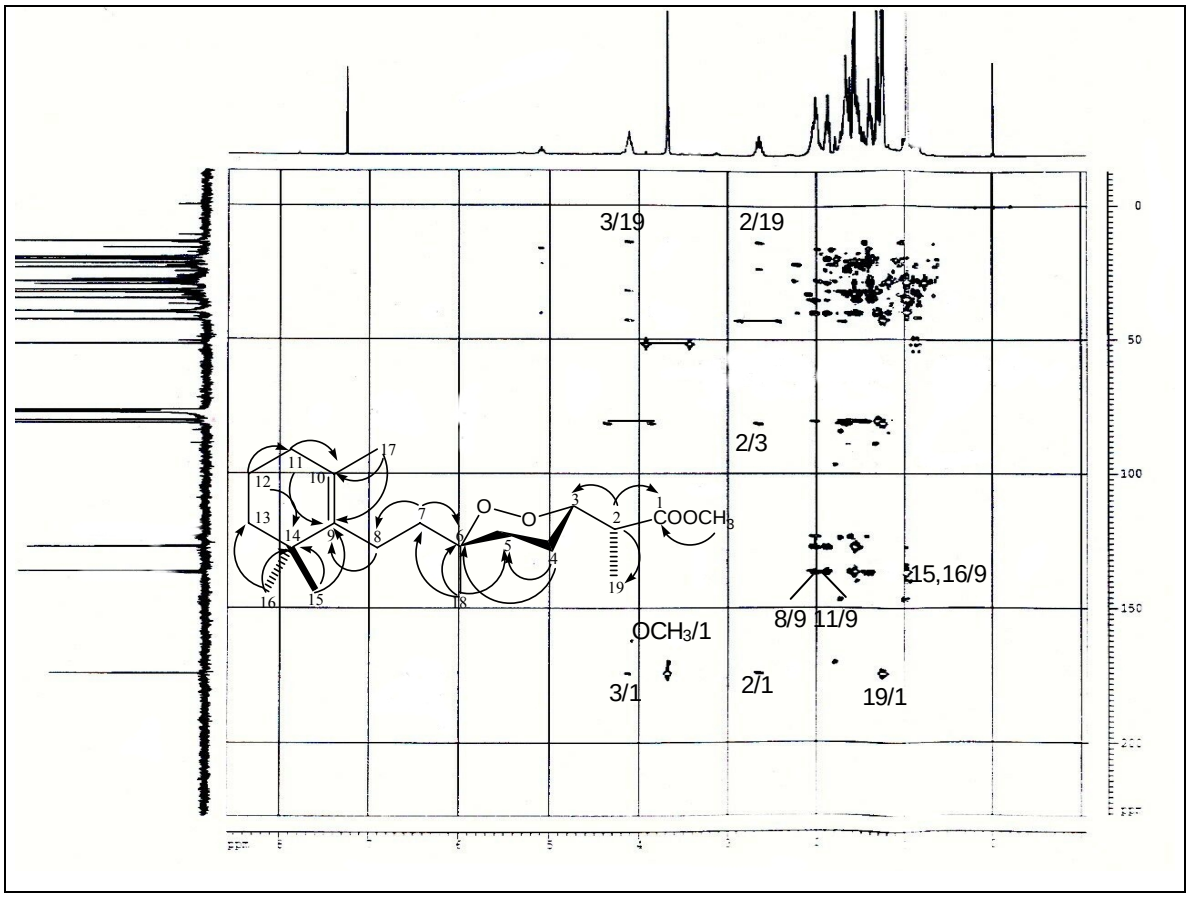
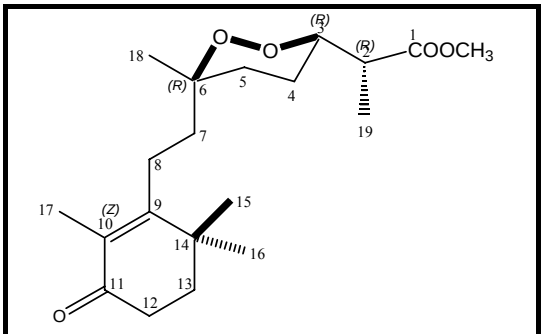


Fig. 3.46: HMBC spectrum of compound 7

Table 3.10: NMR data of compound 7 ($CDCl_3$, 500, 125 MHz)

Position	δ_H (m, *J* in Hz)	δ_C (m)	δ_C (m) of methyl-2-epinuapapuanoate [D´ Ambrosio, *et al*., 1997 in $CDCl_3$]	COSY	HMBC
1		174.3 s	174.3 s		
2	2.65 (t, *J* = 7.3 Hz)	43.0 d	43.0 d	19	1, 3, 19
3	4.12 (dt, *J* = 8.2, 4.4 Hz)	81.3 d	81.3 d	4	1
4	1.67 (m)	23.5 t	21.7 t	5	5, 6
5	1.65 (m)	31.8 t	31.8 t	4	3, 4, 7
6		80.4 s	80.4 s		
7	1.46 (m), 1.55 (m)	39.7 t	39.0 t	8	5, 6, 8
8	2.02 (m)	21.8 t	21.8 t	7	6, 7, 9, 10, 14
9		136.4 s	136.4 s		
10		127.3 s	127.3 s		
11	1.88 (t, *J* = 6.3 Hz)	32.8 t	32.8 t	12	9, 10, 13
12	1.54 (m)	19.5 t	19.5 t	13	11, 13, 14
13	1.40 (m)	39.9 t	39.0 t	12	9, 11, 12, 15, 16
14		35.1 s	35.0 s		
15	0.95 (s)	28.6 q	28.6 q		9, 13, 14, 16
16	0.96 (s)	28.6 q	28.6 q		9, 13, 14, 15
17	1.56 (s)	19.7 q	19.7 q		9, 10
18	1.31 (s)	20.5 q	20.5 q		5, 6 , 7
19	1.25 (d, *J* = 6.9 Hz)	13.6 q	13.6 q	2	1, 2, 3
OCH_3	3.67 (s)	51.8 q	51.8 q		1

3.2.3. Diacarperoxide A (8, new compound)



Diacarperoxide A

Diacarperoxide A (**8**) was isolated as a colorless oil, with an $[\alpha]_D$ of + 53.4° (*c* 0.25, $CHCl_3$). The EIMS of **8** showed a pseudomolecular ion peak at *m/z* 352 $[M]^+$. Through HREIMS, **8** had a molecular formula $C_{20}H_{32}O_5$, which required five degrees of unsaturation instead of 4 as in **6**. The significant fragment ions were different from those of **6** and the base peak at *m*/*z* 137 was also not present, which attested the loss of the cyclohexene moiety. Compound **8** had a 14 mass unit difference from compound **6**. The ^1H and ^{13}C NMR data (Table 3.11) of **8** and **6** were similar for the C_1-C_8 region.

The ^{13}C NMR spectrum revealed the presence of 20 carbons as in **6** with the appearance of new signals at δ 164.7 (s, C-9), 130.0 (s, C-10), 199.0 (s, C-11), 34.2 (t, C-12), 37.4 (C-13), 36.5 (s, C-14), 26.8 (q, C-15/16), and 11.4 (q, C-17). The signals at δ 164.7 and 130.0 indicated the presence of an olefinic double bond. The ^{1}H-^{1}H COSY spectrum showed an additional spin system which consisted of two methylene triplet signals at δ 2.45 (H_2-12) and 1.82 (H_2-13) coupled to each other. In the HMBC spectrum, the additional aliphatic carbonyl signal at δ 199.0 (C-11) correlated with the olefinic methyl singlet at δ 1.78 (H_3-17) and the methylene groups H_2-12 and H_2-13. The downfield shift of the C-9 to δ 164.7 was explained by the presence of an α,β-unsaturated unit in which the carbonyl function could only be situated at C-11 [Elkhayat, *et al*., 2004]. This was confirmed by the HMBC correlations of H_3-17 with C-9, C-10, C-11, and C-12. These resonances were characteristic of a trimethyl cyclohexenone moiety [Jimenez, *et al*., 1991, Montagnac, *et al*., 1994, D´ Ambrosio, *et al*., 1997]. The connectivity of the cyclohexenone function with that of the C_1-C_8 moiety was established through the HMBC correlations of H_2-8 with C-9 and C-10 of the cyclohexenone. This unambiguously led to the elucidation of structure **8**, which was named **diacarperoxide A**. Comparison of the proton and carbon NMR chemical shifts with those of methyl diacarnoate A afforded further confirmation of this structure [D´ Ambrosio, *et al*., 1997]. The stereochemistry was assigned to be 2*R*, 3*R*, 6*R* as in **6** based on the established empirical rules by Capon´s and MacLeod [Capon, *et al*., 1985].

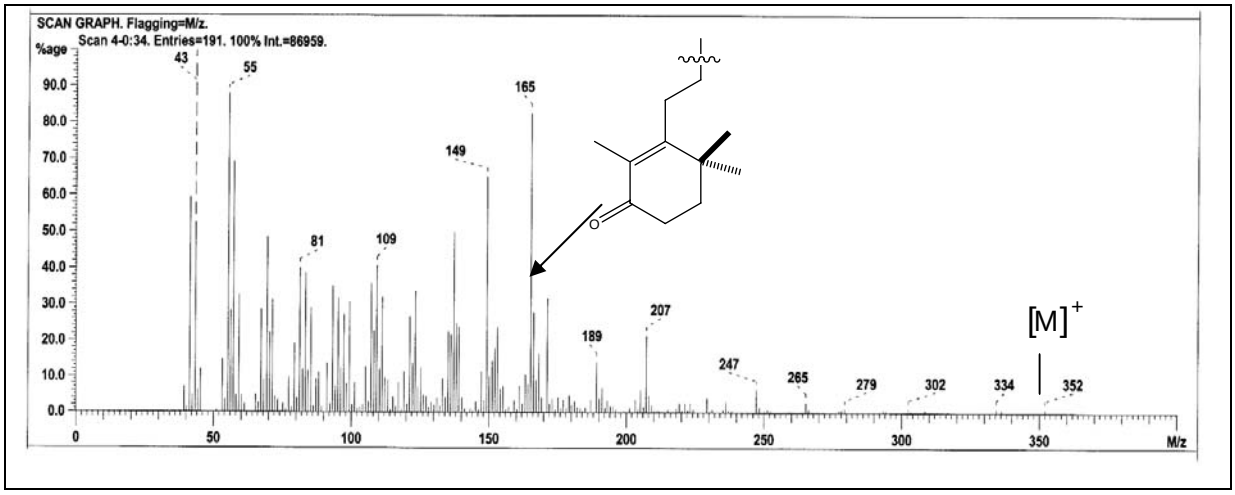


Fig. 3.47: EIMS spectrum of compound 8

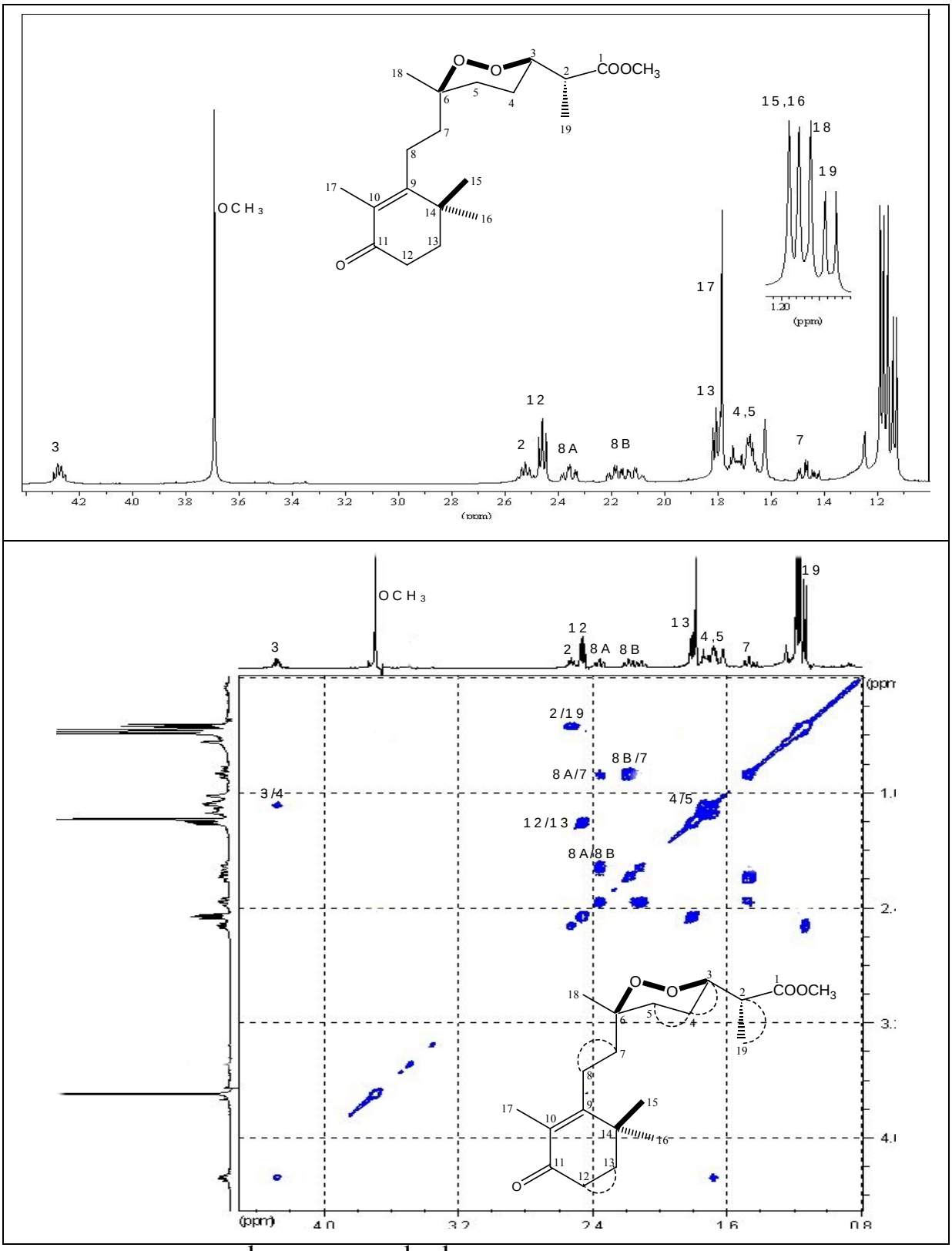


Fig. 3.48: ^{1}H NMR and ^{1}H-^{1}H COSY spectra of compound 8

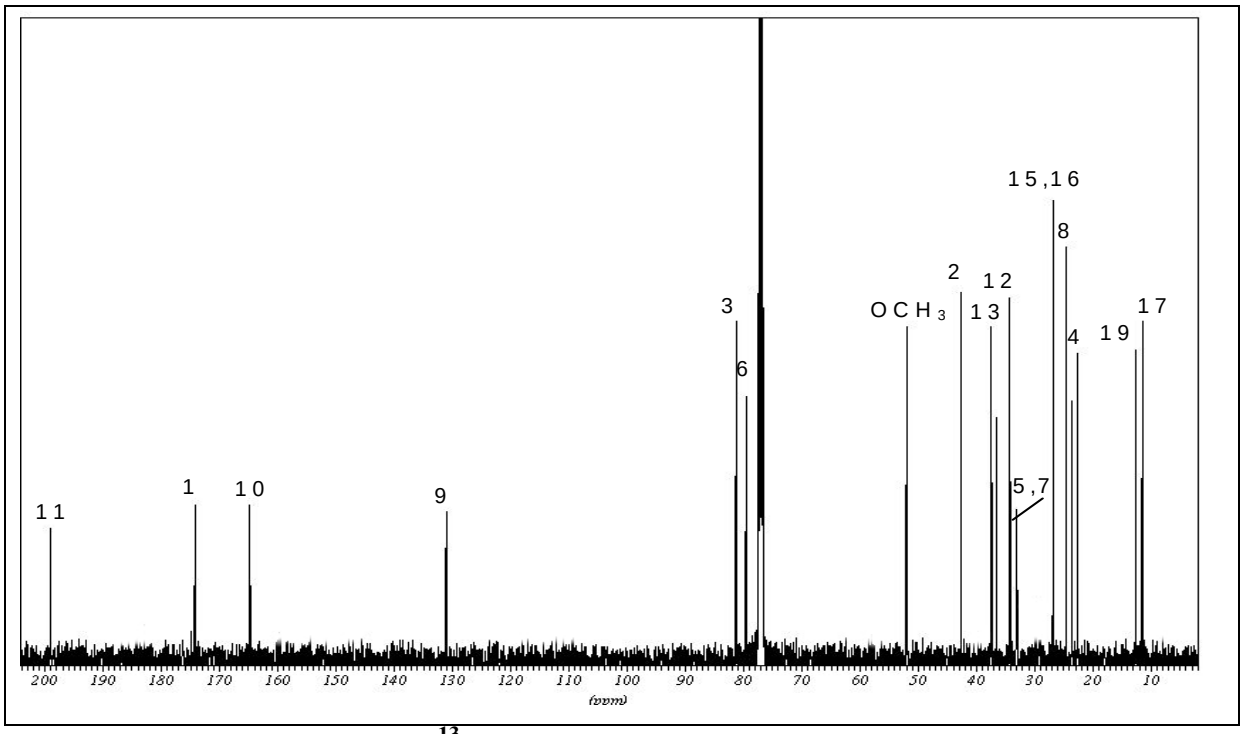


Fig. 3.49: ^{13}C NMR spectrum of compound 8

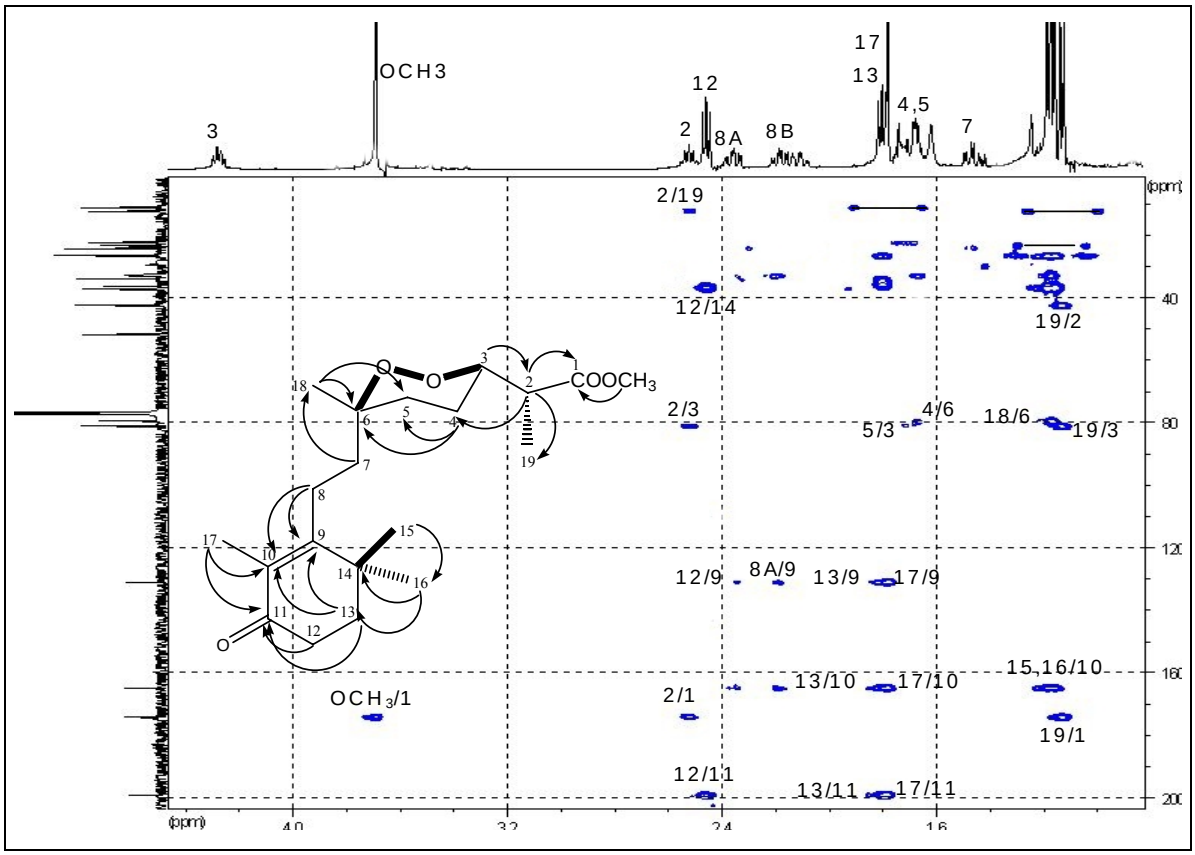
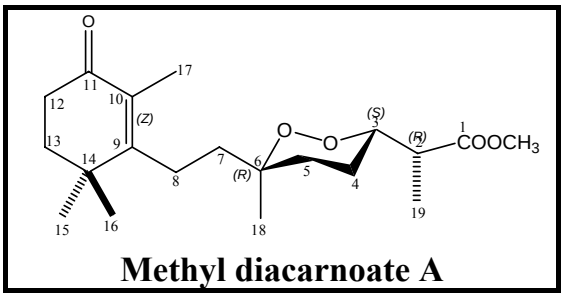


Fig. 3.50: HMBC spectrum of compound 8

Table 3.11: NMR data of compound 8 ($CDCl_3$, 500, 125 MHz)

Position	δ_H (m, *J* in Hz)	δ_C (m)	COSY	HMBC
1		174.1 s		
2	2.54 (q, *J* = 6.6 Hz)	42.7 d	19	1, 4, 19
3	4.28 (ddd, *J* = 7.6, 6.3, 1.6 Hz)	81.1 d	4	2
4	1.68 (m)	22.6 t	5	5, 6, 7
5	1.73 (m)	31.8 t	4	3, 4
6		79.5 s		
7	1.46 (ddd, *J* = 12.6, 8.2, 3.1 Hz)	32.9 t	8A, 8B	18
8	2.36 (dt, *J* = 17.0, 3.8 Hz) 2.15 (ddd, *J* = 17.0, 8.2, 3.8 Hz)	24.5 t	7	5, 7, 9, 10
9		164.7 s		
10		130.0 s		
11		199.0 s		
12	2.45 (t, *J* = 6.9 Hz)	34.2 t	13	14, 9, 11
13	1.82 (t, *J* = 6.9 Hz)	37.4 t	12	9, 10, 11, 15, 16
14		36.5 s		
15	1.19 (s)	26.8 q		10, 14, 16
16	1.16 (s)	26.8 q		10, 14, 15
17	1.78 (s)	11.4 q		9, 10, 11, 12
18	1.18 (s)	23.5 q		5, 7
19	1.13 (d, *J* = 6.9 Hz)	12.5 q	2	1, 2, 3
OCH_3	3.70 (s)	51.9 q		1

3.2.4. Methyl diacarnoate A (9, known compound)



Methyl diacarnoate A

Compound **9** was obtained as a colorless oil, with an $[\alpha]_D$ of - 52° (*c* 0.1, $CHCl_3$). The EIMS spectrum showed a pseudomolecular ion peak at *m*/*z* 352 that was similar to **8**. The ^{1}H NMR spectrum of compound **9** revealed the presence of four methyl singlets, one methyl doublet, two methines, six methylenes, and one methoxy signal. These findings were supported by its ^{13}C NMR and DEPT spectra, which revealed the presence of 20 carbons. The ^{13}C NMR resonances at δ 164.1 (C-9), 131.0 (C-10), 198.9 (C-11), 34.2 (C-12), 37.3 (C-13), 36.4 (C-14), 26.9 (C-15, 16) and 11.4 (C-17) indicated the presence of a trimethyl cyclohexenone moiety [Jimenez, *et al*., 1991, Montagnac, *et al*., 1994, D´ Ambrosio, *et al*., 1997]. The ^{1}H and ^{13}C NMR spectra

(Fig. 3.52 and 3.53) were identical to those of methyl diacarnoate A [D´ Ambrosio, *et al*., 1997]. When the NMR data of compound **9** when compared to those of **8**, differences in the chemical shifts were observed for C-2 methyl (H_3-19) and C-6 methyl (H_3-18). The C-2 methyl was deshielded to δ 1.26, while the C-6 methyl was shielded to δ 20.6. This indicated that compound **9** was a diastereoisomer of **8**. The stereochemistry of **9** was concluded to be 2*R*, 3*S*, 6*R* [Ambrosio, *et al*., 1997, Capon, *et al*., 1985].

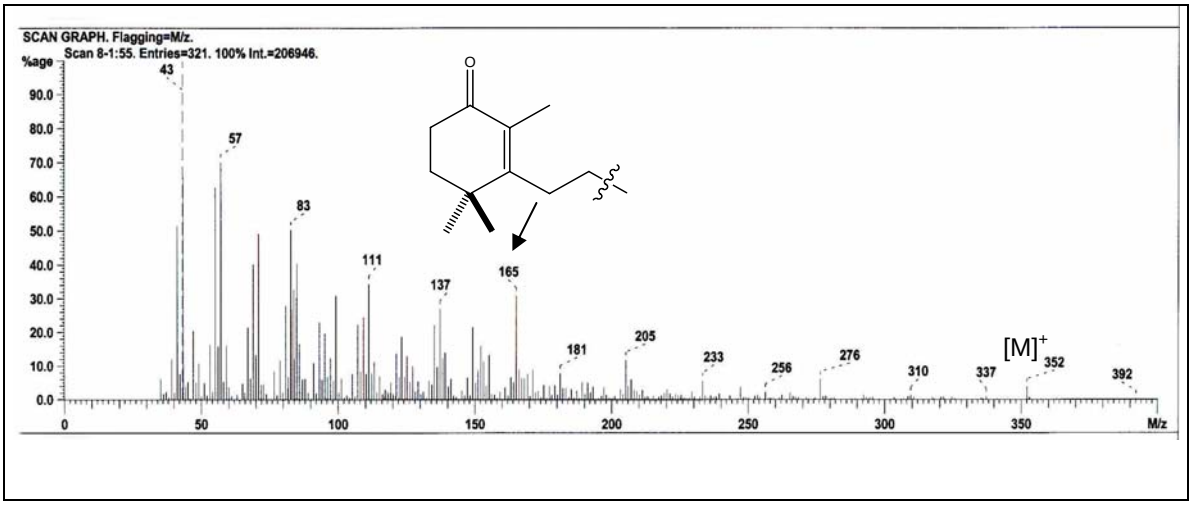


Fig. 3.51: EIMS spectrum of compound 9

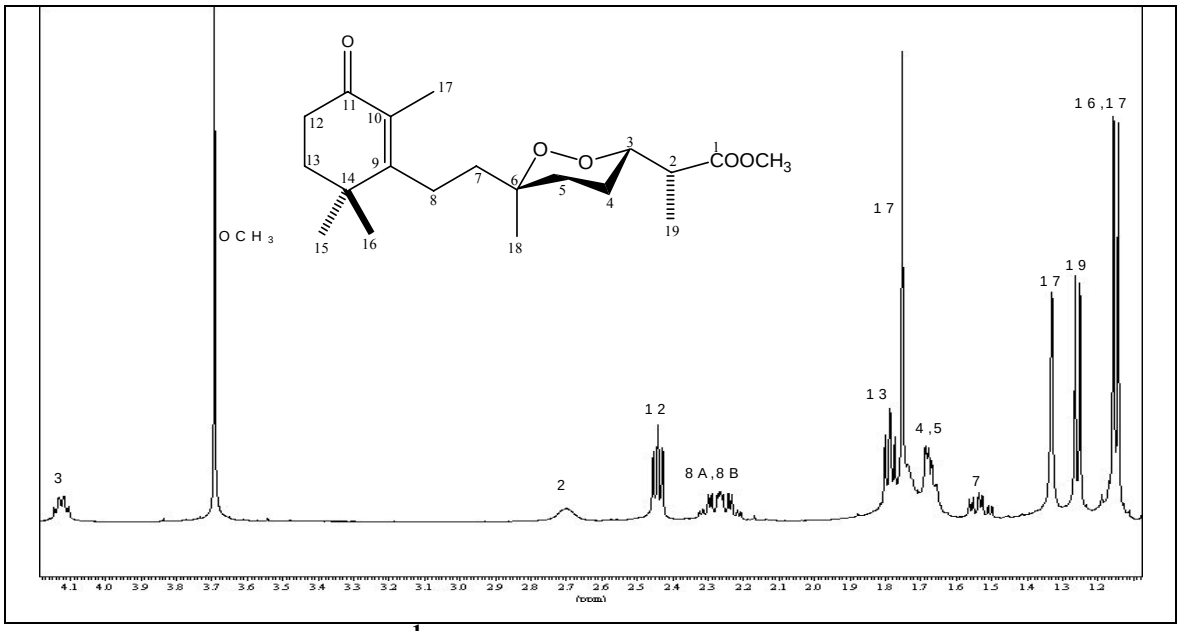


Fig. 3.52: ^{1}H NMR spectrum of compound 9

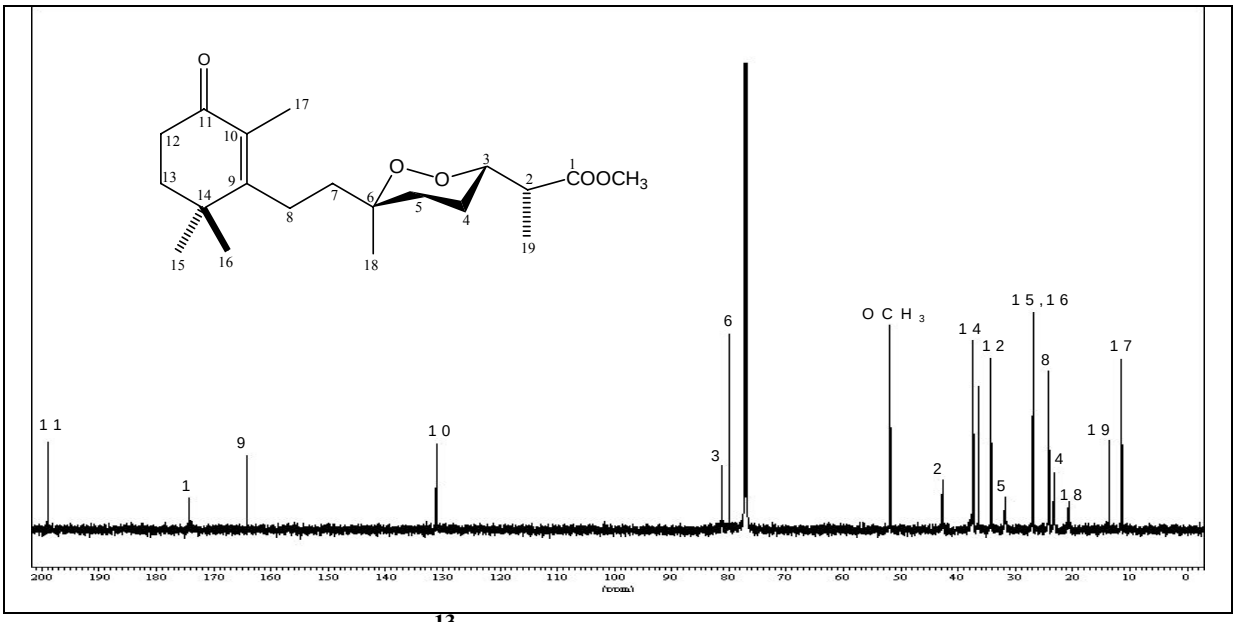
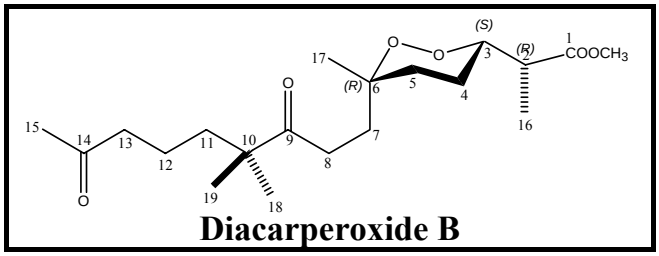


Fig. 3.53: ^{13}C NMR spectrum of compound 9

Table 3.12: NMR data of compound 9 ($CDCl_3$, 500, 125 MHz)

Position	δ_H (m, *J* in Hz)	δ_C (m)	δ_C (m) of methyl diacarnoate A [D´ Ambrosio, *et al*., 1997 in $CDCl_3$]
1		174.2 s	174.1 s
2	2.71 (brs)	42.4 d	42.4 d
3	4.13 (brq, *J* = 6.3 Hz)	81.2 d	81.2 d
4	1.70 (m)	23.5 t	23.3 t
5	1.67 (m)	31.7 t	31.8 t
6		79.8 s	79.8 s
7	1.53 (dt, *J* = 14.0, 5.3 Hz)	37.7 t	37.7 t
8	2.30 (dt, *J* = 12.6, 5.0 Hz) 2.24 (dt, *J* = 12.6, 5.0 Hz)	24.0 t	24.1 t
9		164.1 s	164.0 s
10		131.0 s	131.1 s
11		198.9 s	198.6 s
12	2.46 (t, *J* = 6.9 Hz)	34.2 t	34.2 t
13	1.79 (t, *J* = 6.9 Hz)	37.3 t	37.4 t
14		36.4 s	36.5 s
15	1.15 (s)	26.9 q	26.9 q
16	1.16 (s)	26.9 q	26.9 q
17	1.75 (s)	11.4 q	11.4 q
18	1.33 (s)	20.6 q	20.7 q
19	1.26 (d, *J* = 6.9 Hz)	13.6 q	13.6 q
OC*H*$_3$	3.70 (s)	51.8 q	51.7 q

3.2.5. Diacarperoxide B (10, new compound)



Diacarperoxide B

Diacarperoxide B (**10**) was obtained as a colorless oil, with an $[\alpha]_D$ of - 35° (*c* 0.11 CH_3Cl). The EIMS showed the pseudomolecular ion peak at *m/z* 370 $[M]^+$. The fragment ion peak at m/z 137 was not detected, which suggested the loss of the cyclohexene moiety. Compound **10** had a 32 mass unit difference from **6**. The ^1H and ^{13}C NMR data of **10** and **6** were similar for the C_1-C_6 region. The signals associated with the cyclohexene or cyclohexenone moiety as in **6** and **8** were not evident. Instead new signals for two aliphatic carbonyls at δ 215.0 (s, C-9) and 208.5 (s, C-14) were observed. The presence of two aliphatic carbonyl signals together with a signal for a methyl ketone residue (δ 2.12 and δ 29.9) suggested the presence of an open chain dicarbonyl function [Montagnac, *et al*., 1994], which could be derived from oxidative opening of the cyclohexene ring at C-9-C-10 olefinic bond [El Sayed, *et al*., 2001]. This was compatible with the 32 mass unit difference in the molecular weight of compound **10** and **6**. The ^1H-^1H COSY and HMQC spectral data allowed the assignment of two spin systems which consisted of two and three methylene groups. One methylene of each group was adjacent to a ketone substituent, as indicated by their chemical shifts of δ 2.59, 2.50 (H_2-8) and 2.40 (H_2-13). The first spin system consisted of two coupled methylene groups H_2-7 (δ 1.84) and H_2-8. The second spin system consisted of the methylenes at δ 1.47 (H_2-11), 1.42 (H_2-12) and 2.40 (H_2-13). These spin systems were confirmed by the correlations observed in the HMBC spectrum. HMBC spectrum showed correlations of H_2-11 with C-12, H_2-12 with C-11 and C-13 and of H_2-13 with C-12 and C-14. The geminal dimethyls at δ 1.12 (H_3-18/19) correlated with C-10 and C-11, which suggested the attachment of H_3-18/19 at C-10. The methyl ketone at δ 2.12 (H_3-15) implied that this was terminus, as it showed a cross peak with the carbonyl function at C-14. The ^1H and ^{13}C NMR data of **10** were comparable to those found in aikupikoxide C isolated from *Diacarnus erythraenus*

[Youssef, *et al*., 2001]. When compound **10** was compared to aikupikoxide C, difference in the ^{1}H NMR chemical shift of C-2 methyl group was also evident, which suggested a change in the stereochemistry at this position. C-2 methyl was shifted downfield to δ 1.24, which indicated *threo* configration at C-2-C-3. In addition, the observed $[\alpha]_D$ value of compound **10** was - 35° (*c* 0.11, $CHCl_3$), while that of aikupikoxide C was + 88° (*c* 0.2, CH_2Cl_2). These findings indicated compound **10** was a diastereoisomer of aikupikoxide C. Thus, the structure of **10** was unambiguously determined, and the name of **diacarperoxide B** was proposed for this new natural product. The stereochemistry of **10** was concluded to be 2*R*, 3*S*, 6*R* [Capon, *et al*., 1985].

Table 3.13: NMR data of compound 10 ($CDCl_3$, 500, 125 MHz)

Position	δ_H (m, *J* in Hz)	δ_C (m)	COSY	HMBC
1		174.0 s		
2	2.68 (m)	42.7 d	16	
3	4.11(q, *J* = 6.9 Hz)	81.2 d	2, 4, 16	
4	1.73 (m)	23.2 t	5	4
5	1.67 (m)	32.0 t	4	
6		79.4 s		
7	1.84 (m),1.68 (m)	33.0 t	8A, 8B	
8	2.59 (ddd, *J* = 15.7, 10.7, 5.8 Hz) 2.50 (ddd, *J* = 15.8, 10.1, 5.0 Hz)	30.4 t	7A, 7B	
9		215.0 s		
10		47.5 s		
11	1.47 (m)	39.1 t	12	10, 12
12	1.42 (m)	18.9 t	11, 13	11, 13
13	2.40 (t, *J* = 6.9 Hz)	43.8 t	12	12, 14
14		208.5 s		
15	2.12 (s)	29.9 q		14
16	1.25 (d, *J* = 6.0 Hz)	13.5 q	2	1, 2, 3
17	1.26 (s)	29.6 q		6, 8
18	1.12 (s)	24.4 q		10, 11, 19
19	1.12 (s)	24.4 q		10, 11, 18
OC*H*$_3$	3.71 (s)	51.8 q		1

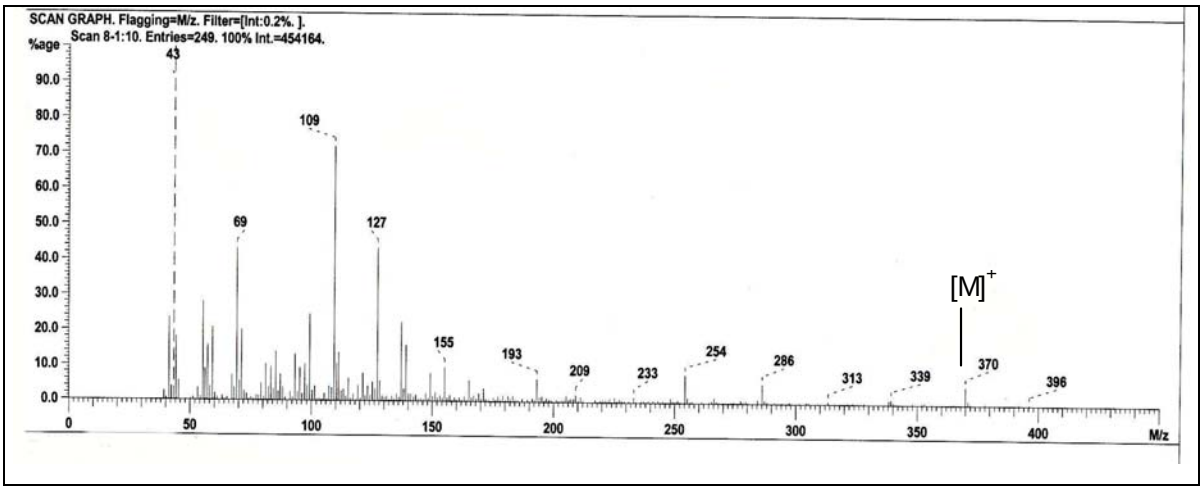


Fig. 3.54: EIMS spectrum of compound 10

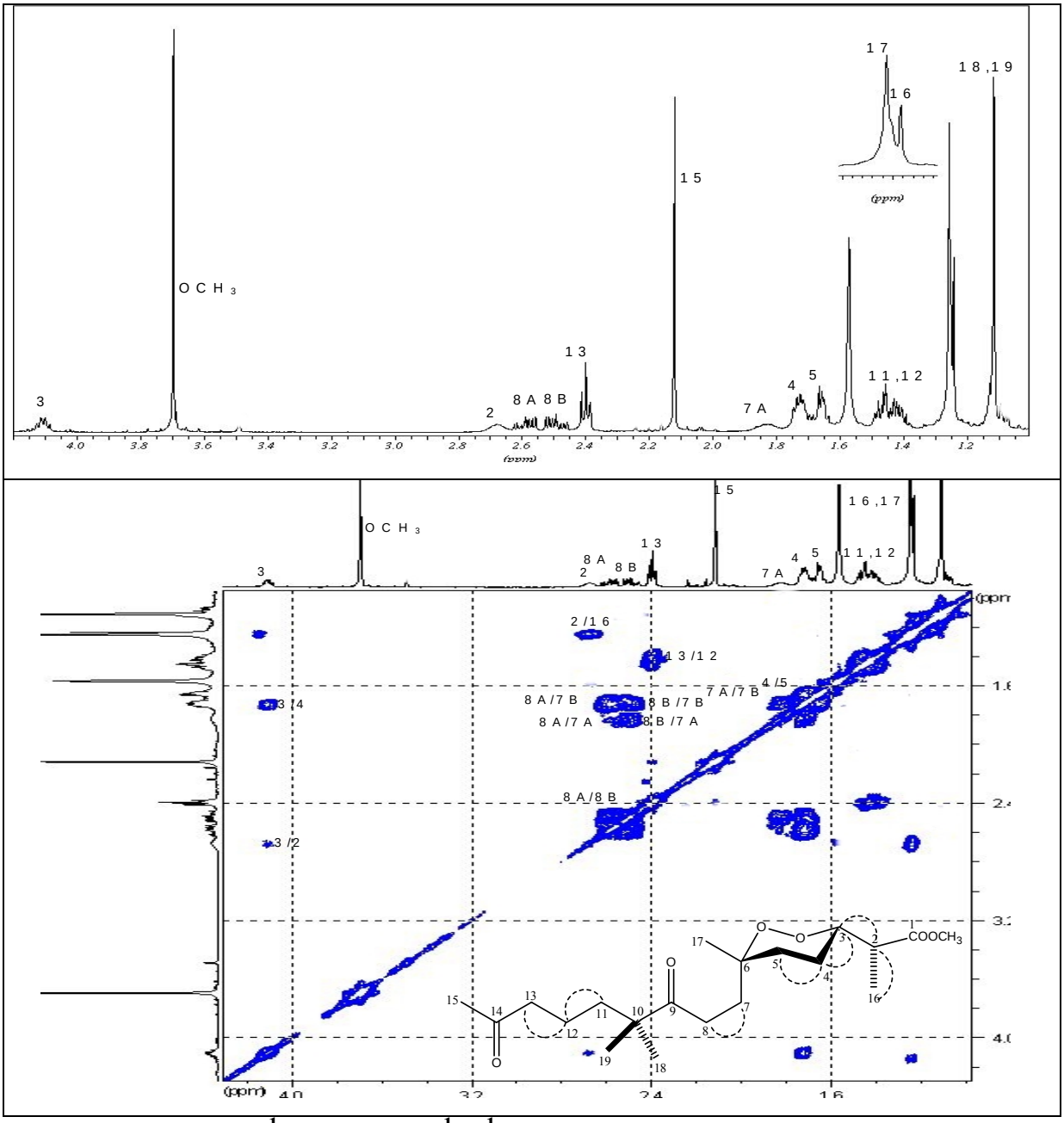


Fig. 3.55: ^{1}H NMR and ^{1}H-^{1}H COSY spectra of compound 10

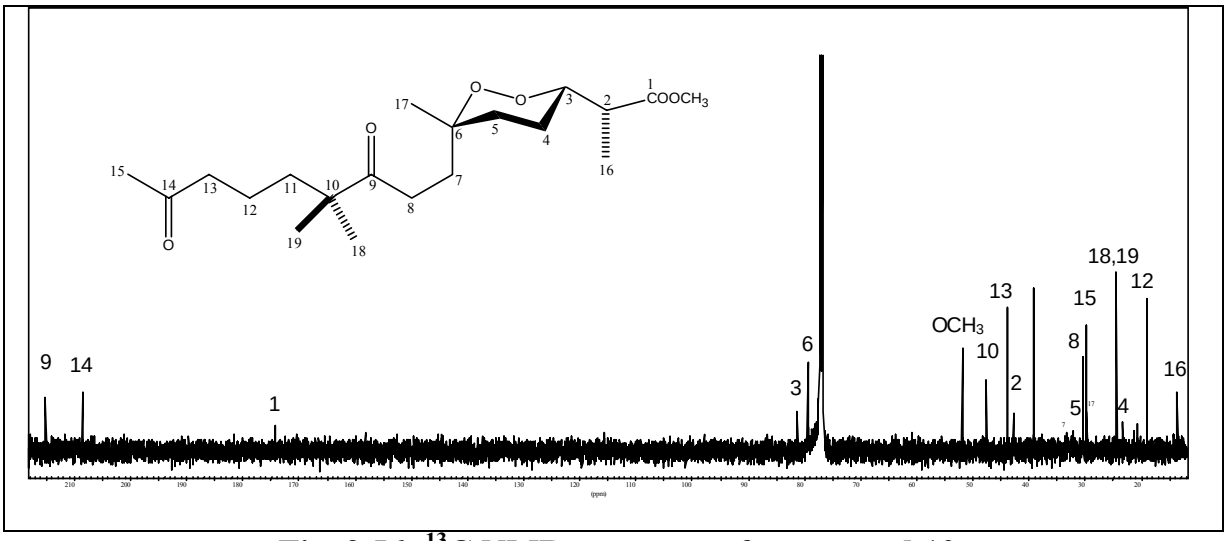


Fig. 3.56: ^{13}C NMR spectrum of compound 10

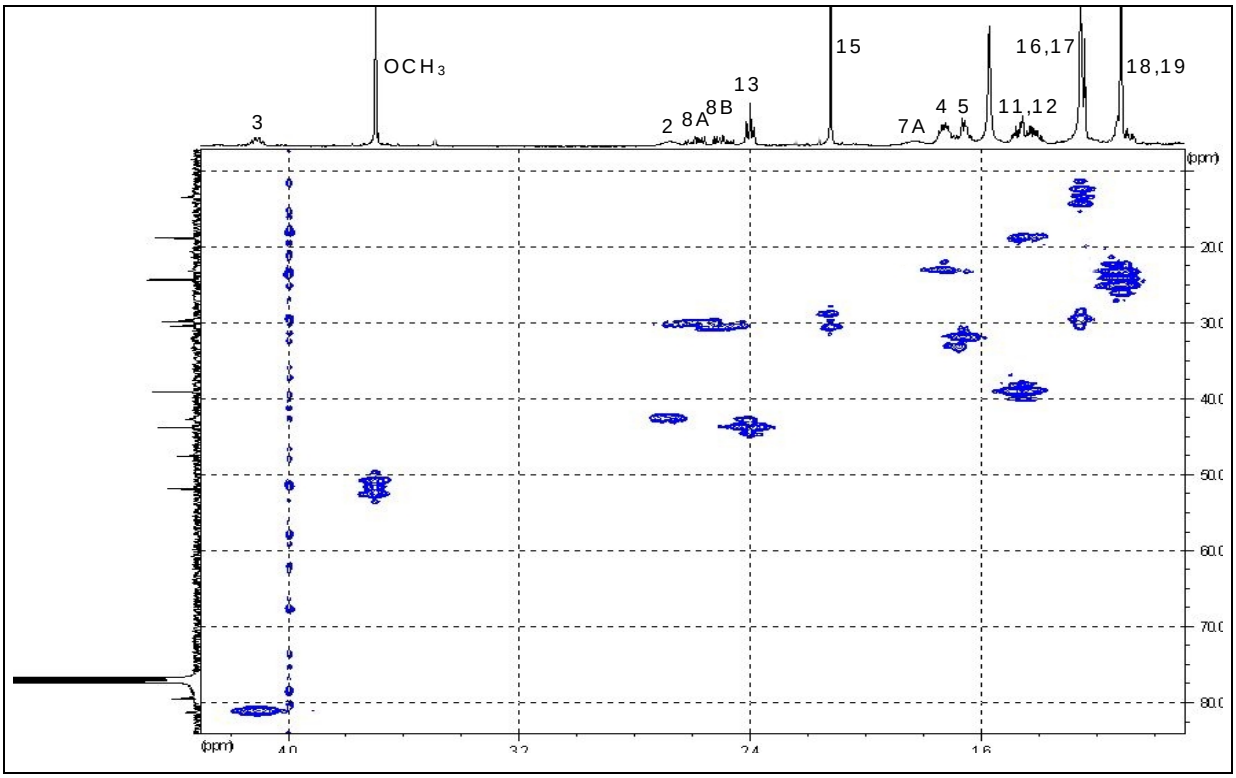
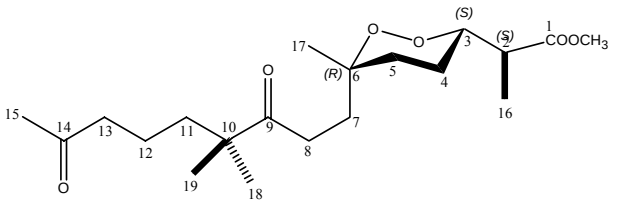


Fig. 3.57: HMQC spectrum of compound 10

3.2.6. Diacarperoxide C (11, new compound)



Diacarperoxide C

Diacarperoxide C **(11)** was obtained as a colorless oil. Th $[\alpha]_D$ value was - 55° (*c* 0.06 $CHCl_3$). The EIMS showed a pseudomolecular ion peak at *m/z* 370 $[M]^+$. HREIMS established the molecular formula $C_{20}H_{34}O_6$ for **11**, which required 4 degrees of unsaturation. The fragment ion peak at *m*/*z* 137 was not present, which indicated the absence of the cyclohexene moiety. Inspection of the ^1H and ^{13}C NMR spectra (Fig. 3.59, Table 3.14) indicated that it had the same carbon skeleton as **10**. Compound **11** was compared to dicarperoxide B (**10**) and aikupikoxide C [Youssef, *et al*., 2001]. When **11** was compared to compound **10**, a difference in the chemical shift of the C-2 methyl suggested that there was a change in stereochemistry at this position. C-2 methyl was shifted upfield to δ 1.12, which indicated an *erythro* configuration at C-2-C-3. Dicarperoxide C (**11**) was a diastereoisomer of **10**. The stereochemistry of **11** was therefore 2*S*, 3*S*, 6*R*.

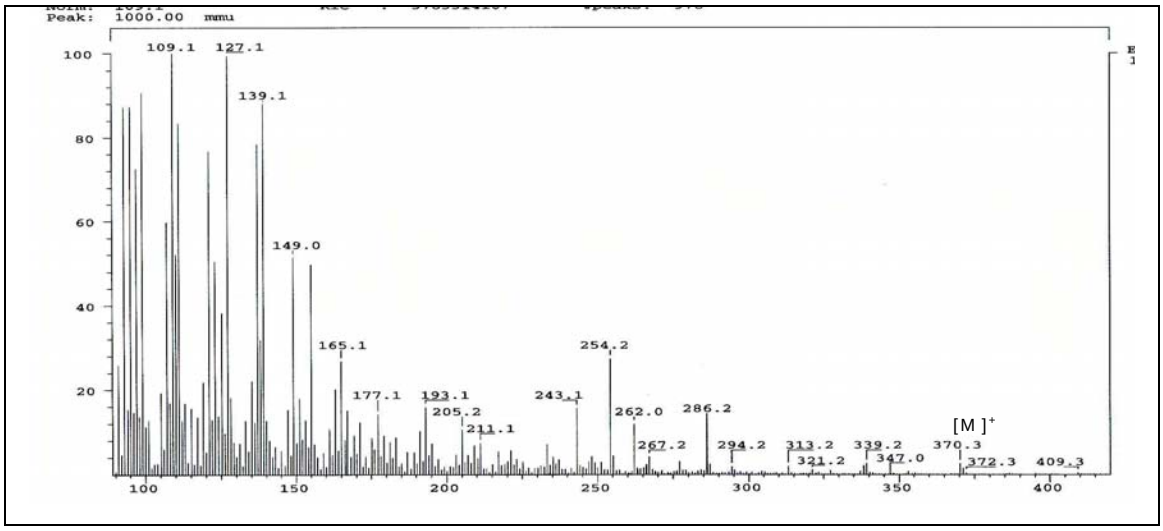


Fig. 3.58: HREIMS spectrum of compound 11

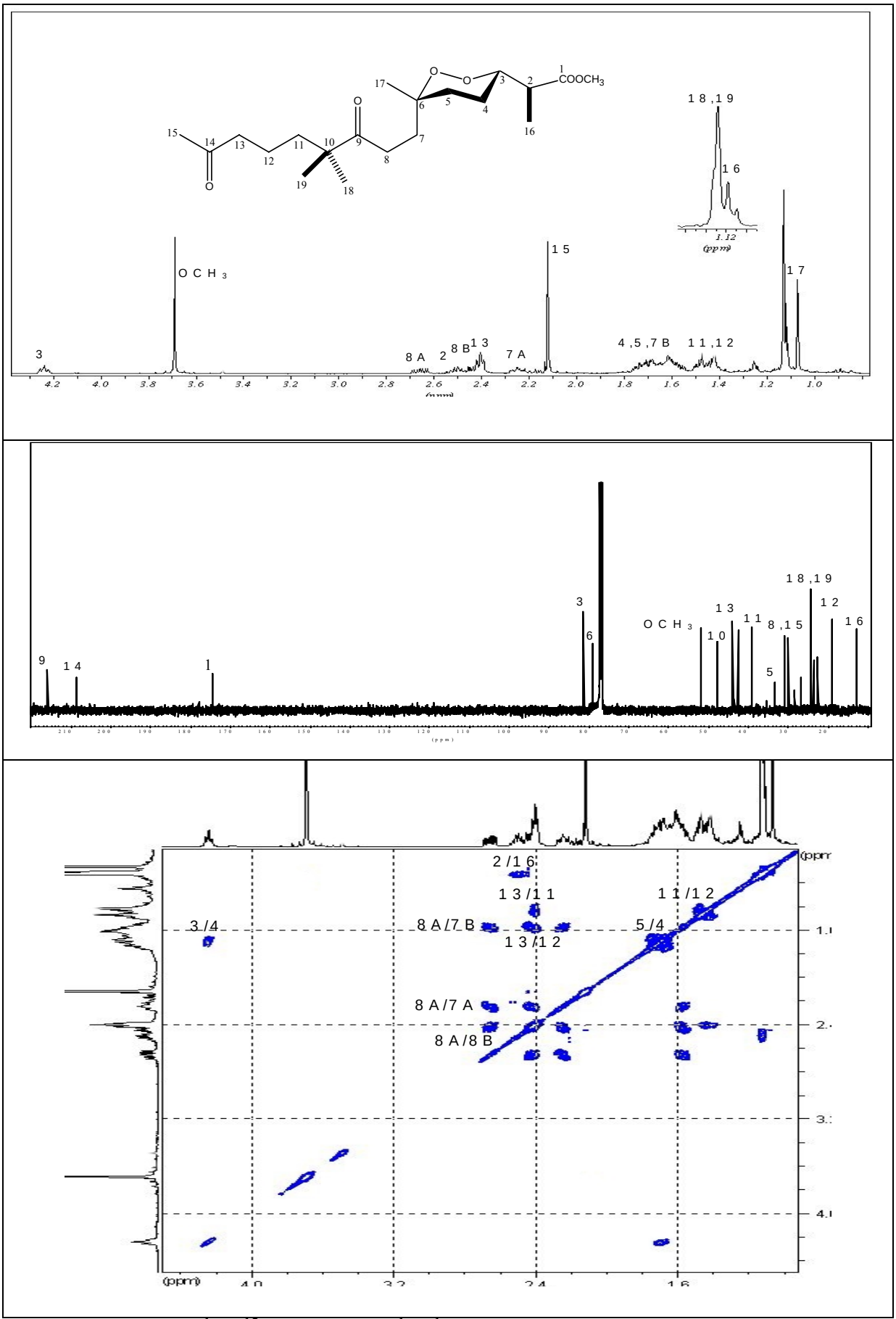
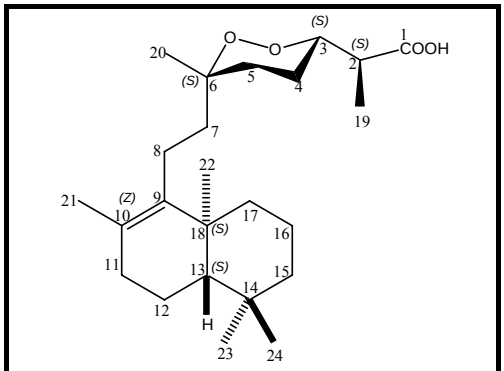


Fig. 3.59: ^{1}H, ^{13}C NMR and ^{1}H-^{1}H COSY spectra of compound 11

Table 3.14: NMR data of compound 11 ($CDCl_3$, 500, 125 MHz)

Position	δ_H (m, *J* in Hz)	δ_C (m)	COSY	HMBC
1		174.3 s		
2	2.49 (quin, *J* = 7.2 Hz)	42.6 d	16	
3	4.24 (dt, *J* = 8.8, 2.2 Hz)	81.3 d	4	
4	1.65 (m)	22.5 t	3, 5	
5	1.73 (m)	33.4 t	4	
6		79.2 s		
7	2.24 (ddd, *J* = 14.5, 11.0, 5.0 Hz) 1.57 (m)	26.9 t	8A, 8B	
8	2.65 (ddd, *J* = 17.3, 11.6, 6.6 Hz) 2.45 (m)	30.9 t	7A, 7B	
9		215.7 s		
10		47.6 s		
11	1.48 (m)	39.2 t	12	12
12	1.43 (m)	19.0 t	11, 13	
13	2.41 (dt, *J* = 7.2, 1.9 Hz)	43.9 t	12	12
14		208.6 s		
15	2.12 (s)	29.9 q		
16	1.11 (d, *J* = 6.9 Hz)	12.8 q	2	1, 2
17	1.07 (s)	23.8 q		6
18	1.12 (s)	24.3 q		10
19	1.12 (s)	24.3 q		10
OCH_3	3.68 (s)	51.9 q		1

3.2.7. Diacarperoxide D (12, new compound)



Diacarperoxide D

Diacarperoxide D (**12**) was isolated as a colorless oil. The $[\alpha]_D$ value was - 11.3° (*c* 0.1, $CHCl_3$). The EIMS spectrum showed a pseudomolecular ion peak at *m/z* 392 $[M]^+$. The significant fragment ions were different from those of **6**. The ion peak at *m*/*z* 137 was also not detected, which also suggested the absence of the cyclohexene group. The significant fragment ion at *m*/*z* 205 could be rationalized as arising through the facile bisallylic cleavage, which indicated the presence of a bicyclic unit, tetramethyl octahydronaphthalene [Butler, *et al*., 1991, Butler, *et al*., 1992], which was further

confirmed by the base peak at *m/z* 191 $[M\text{-}C_{14}H_{23}]^+$. ^1H and ^{13}C NMR data of **12** and **6** were comparable for the C_1-C_8 region. ^{13}C NMR and DEPT spectra revealed the presence of 24 carbons consisting of six methyl carbons, nine methylenes, three methines and six quaternary carbons. ^1H and ^{13}C NMR resonances of the bicyclic moiety were in excellent agreement with those found in luffarins [Butler, *et al*., 1992] and mycaperoxides [Capon, *et al*., 1998]. From its ^1H-^1H COSY spectrum, two new spin systems replacing the cyclohexene moiety in **6** could be unambiguously assigned. The first spin system consisted of two methylenes at δ 2.02 (H_2-11) and 1.91 (H_2-12) and one methine at δ 1.11 (H-13). The second spin system consisted of the methylenes at δ 1.60, 1.40 (H_2-15), 1.98 (H_2-16) and 1.95 (H_2-17). These spin systems were confirmed from the correlations observed on the HMBC spectrum. H_2-11 showed correlations with the quaternary carbons at δ 140.1 (C-9) and 125.7 (C-10) and the methine carbon at δ 51.9 (C-13). The methine proton H-13 correlated with C-9, C-18, and C-22. Moreover, the correlations of H_2-15 with C-13 and of H_2-16 with the quaternary carbon at δ 39.1 (C-18) were observed. The connectivity of the ethylene function to the bicyclic unit was proven by the correlations of H_2-8 with C-9 and C-10 and H_2-7 with C-9. The presence of the geminal dimethyl functionality was evident from the correlations of the methyl resonances at δ 0.83 (H_3-23) and 0.88 (H_3-24) with their corresponding carbons in addition to similar set of correlations with the neighbouring carbons. The position of the geminal dimethyl group (H_3-23/24) was assigned at C-14 from the correlations with C-13, C-14 and C-15. By comparison with the literature data together with the data obtained from ^1H-^1H COSY, HMQC, and HMBC spectra, the structure of **12** was unambiguously elucidated and named **diacarperoxide D**. The stereochemistry of **12** was assigned to be 2*S*, 3*S*, 6*S* by applying the established empirical rules by Capon and MacLeod [Capon, *et al*., 1985].

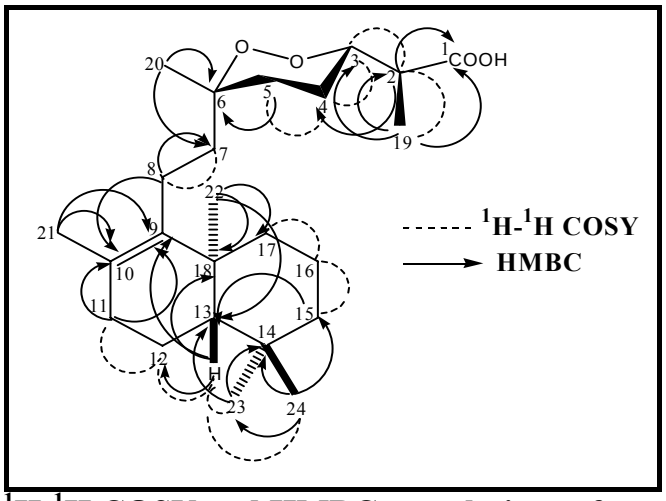


Fig. 3.60: ^{1}H-^{1}H COSY and HMBC correlations of compound 12

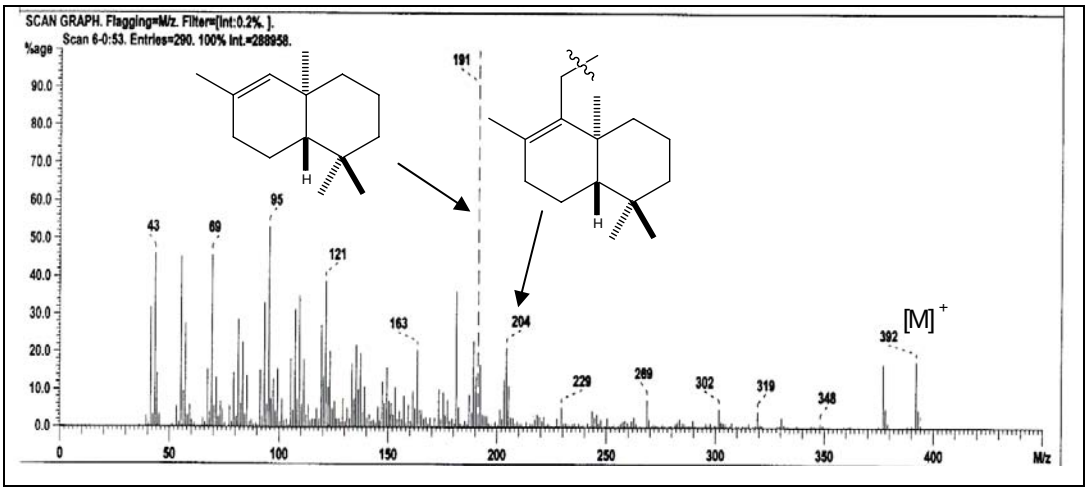


Fig. 3.61: EIMS spectrum of compound 12

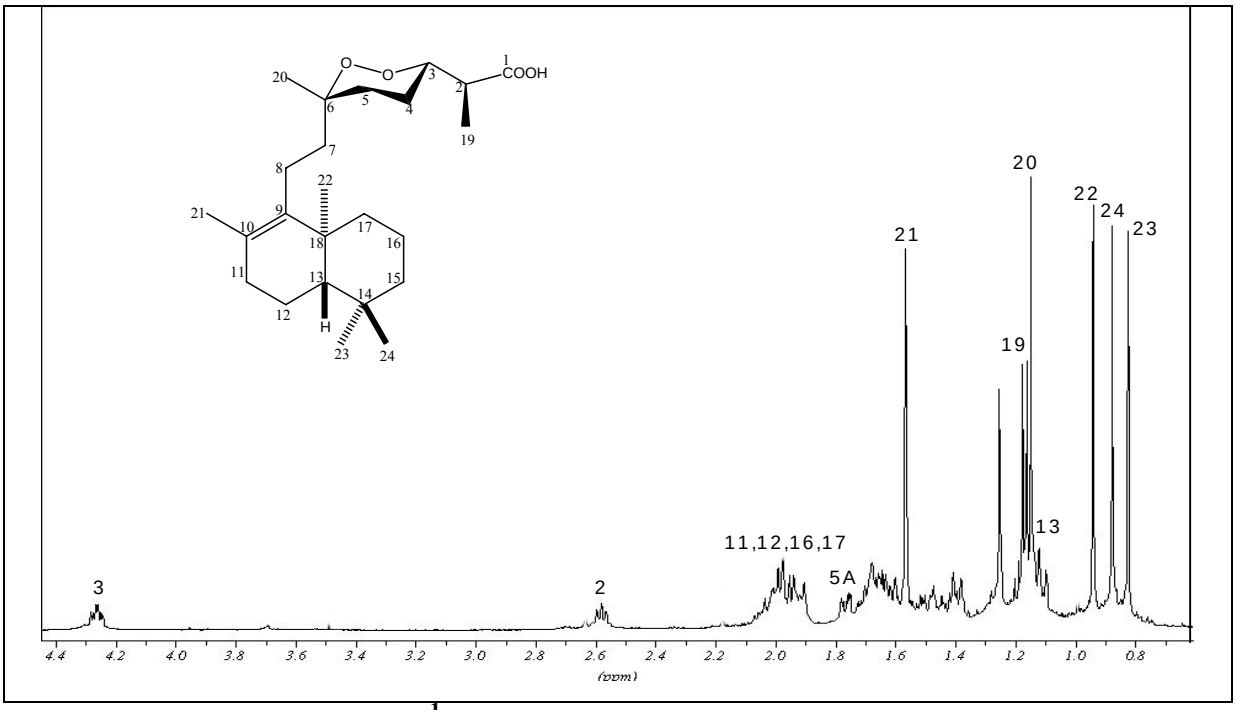


Fig. 3.62: ^{1}H NMR spectrum of compound 12

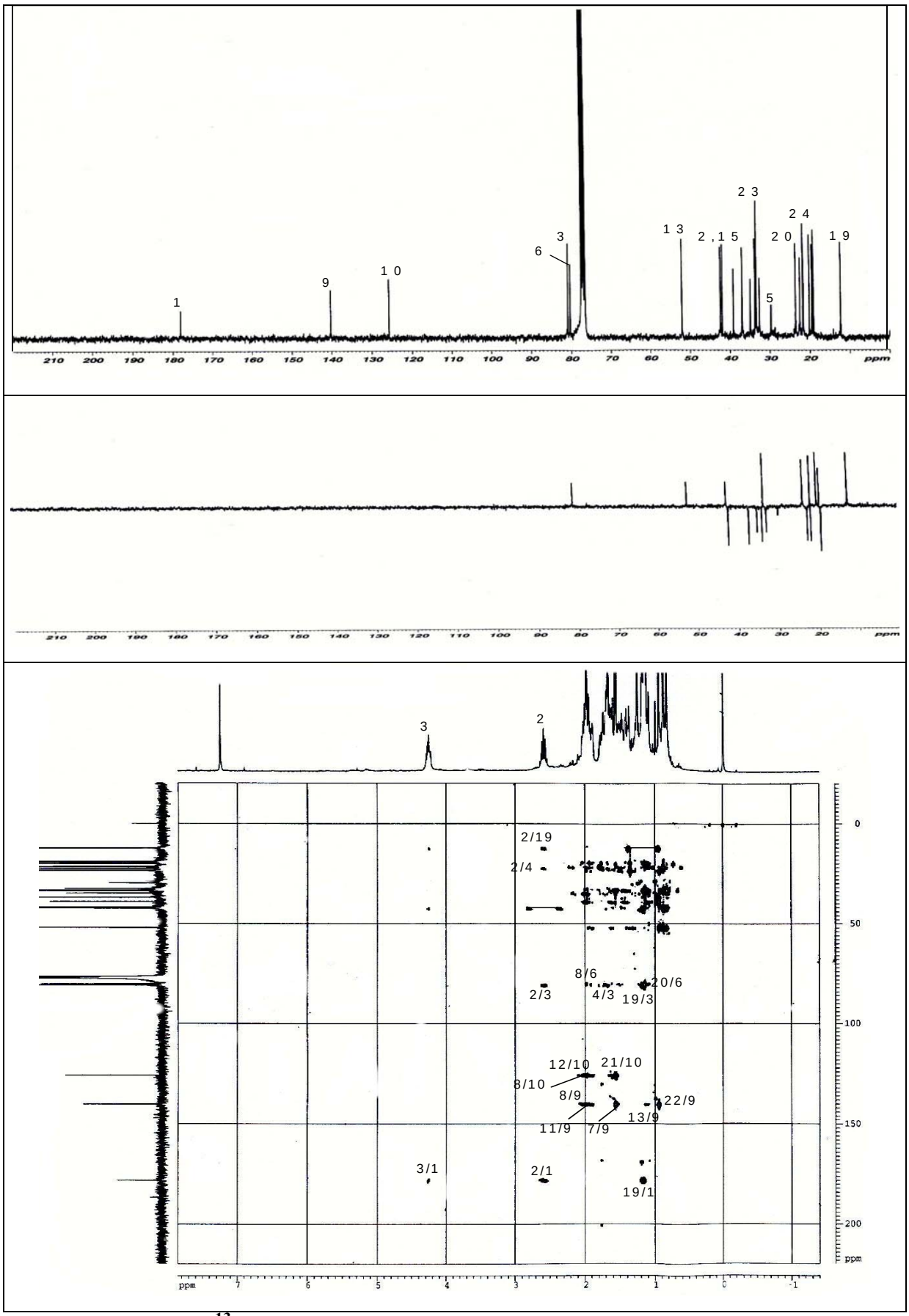


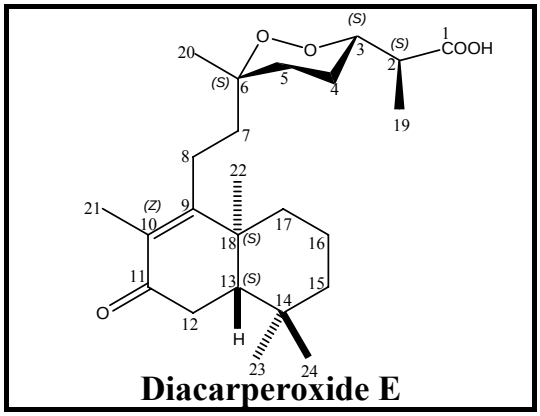
Fig. 3.63: ^{13}C NMR, DEPT and HMBC spectra of compound 12

Table 3.15: NMR data of compound 12 ($CDCl_3$, 500, 125 MHz)

Position	δ_H (m, *J* in Hz)	δ_C (m)	COSY	HMBC
1		178.0 s		
2	2.59 (p, *J* = 6.9 Hz)	42.3 d	3, 19	1, 3, 4, 19
3	4.27 (dt, *J* = 8.8, 3.1 Hz)	80.8 d	4	1, 2, 19
4	1.67 (m)	22.4 t	5	2, 3
5	1.52 (m)	32.5 t	4	6
6		80.2 s		
7	1.48 (m), 1.39 (m)	33.6 t	8	9
8	1.99 (m)*	19.0 t	7	7, 9, 10
9		140.1 s		
10		125.7 s		
11	2.02 (m)*	33.6 t	12	9, 10, 13
12	1.91 (m)*	19.1 t	13	10, 14, 18
13	1.11 (m)	51.9 d	12, 22, 24	9, 12, 18, 22
14		33.8 s		
15	1.60 (m), 1.40 (m)	41.8 t	16	13
16	1.98 (m)*	21.6 t	15, 17	18
17	1.95 (m)*	36.8 t	16	
18		39.1 s		
19	1.17 (d, *J* = 6.9 Hz)	12.3 q	2	1, 2, 3
20	1.15 (s)	23.5 q		5, 6, 7
21	1.56 (s)	19.4 q		9, 10, 11
22	0.94 (s)	20.0 q		9, 13, 17, 18
23	0.83 (s)	32.5 q		13, 14, 15, 24
24	0.88 (s)	21.7 q		13, 14, 15

* overlapped

3.2.8. Diacarperoxide E (13, new compound)



Diacarperoxide E

Diacarperoxide E (**13**) was isolated as a colorless oil, with an $[\alpha]_D$ of - 12.6° (*c* 0.3, $CHCl_3$). EIMS showed a pseudomolecular ion peak at *m*/*z* 406 $[M]^+$. The fragment ion at *m*/*z* 191 was not present, which attested to the loss of a tetramethyl octahydronaphthalene function. Compound **13** had a 14 mass unit difference from compound **12**. However the ^1H and ^{13}C NMR data of **13** were comparable to those of diacarperoxide D (**12**). An additional aliphatic carbonyl signal at δ 200.7 (C-11) was observed as the major difference to compound **12**. The ^1H-^1H COSY and HMQC allowed the unambiguous assignment of two spin systems which consisted of one methylene, one methine and three methylenes units. The first spin system consisted of a methylene at δ 2.35, 2.51 for H_2-12 and one methine at δ 1.70 for H-13. The second spin system consisted of methylenes at δ 1.49, 1.25 (H_2-15), 1.58, 1.66 (H_2-16) and 2.07, 1.39 (H_2-17). The HMBC spectrum showed correlations of the olefinic methyl at δ 1.76 (H_3-21) with quaternary carbons at δ 168.8 (C-9), and 130.6 (C-10) and carbonyl carbon at δ 200.7 (C-11). The deshielding effect on C-9 was explained by the presence of α,β-unsaturated carbonyl unit in which the carbonyl function was located to be at C-11. This was confirmed by the HMBC correlations of H_2-12 with the carbonyl at δ 200.7 (C-11) and quaternary carbon at δ 41.5 (C-18) and of H_3-22 with C-11. These resonances were again characteristic of a tetramethyl hexahydronaphthalenone [D´ Ambrosio, *et al.*, 1998]. This unambiguously led to the elucidation of structure **13**, which was named **diacarperoxide E**. The stereochemistry of **13** was assigned to be the same as **12** (2*S*, 3*S*, 6*S*).

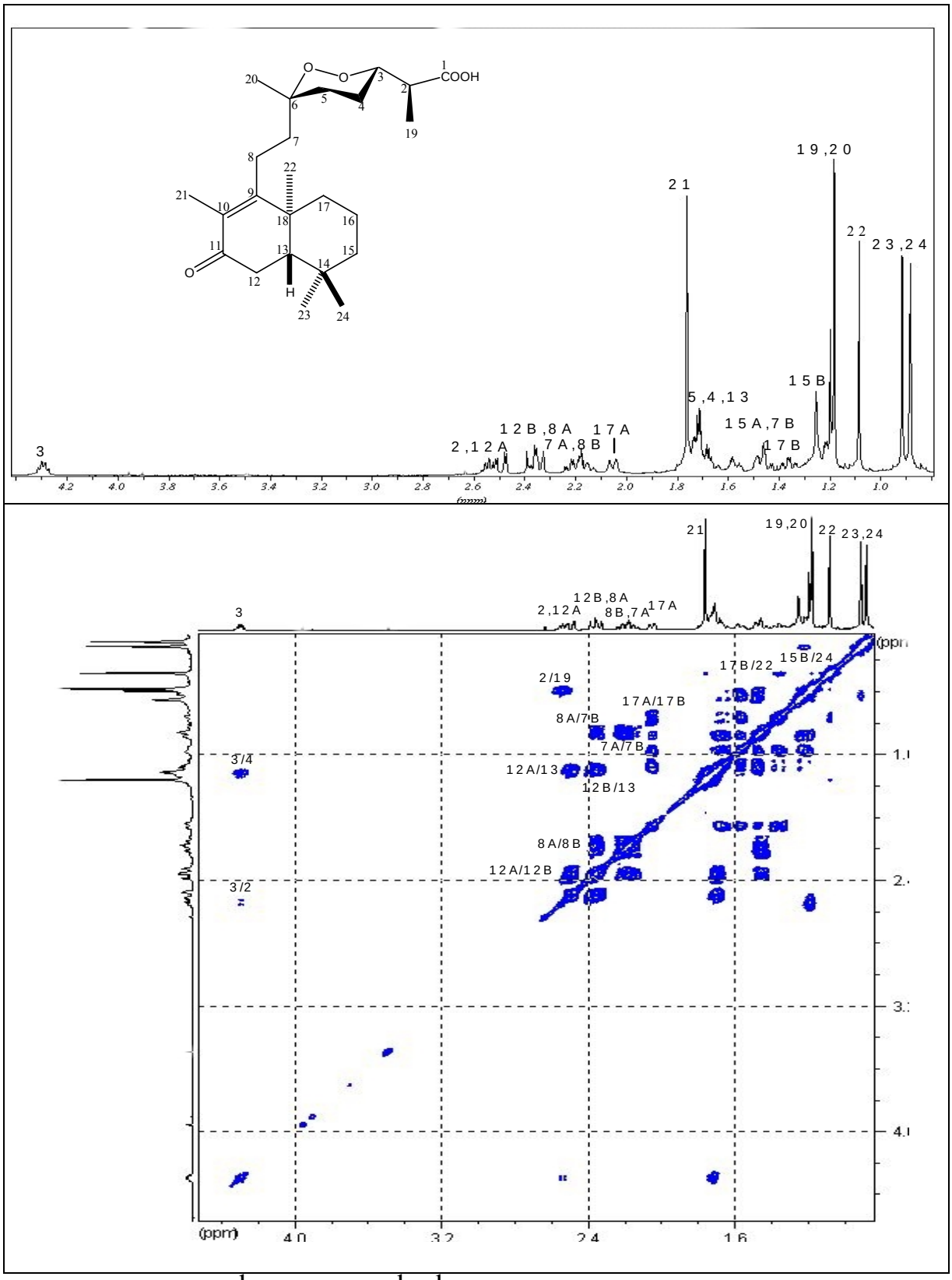


Fig. 3.64: ^{1}H NMR and ^{1}H-^{1}H COSY spectra of compound 13

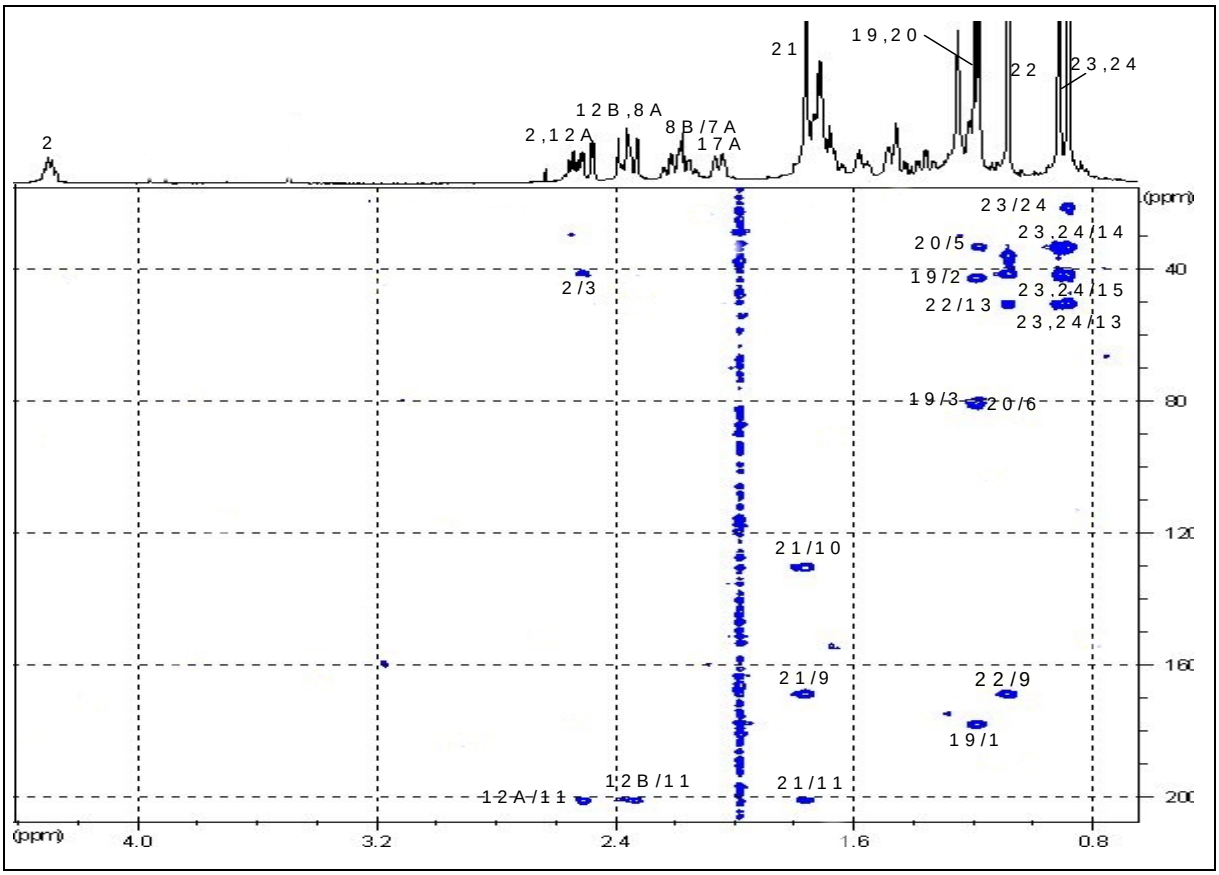
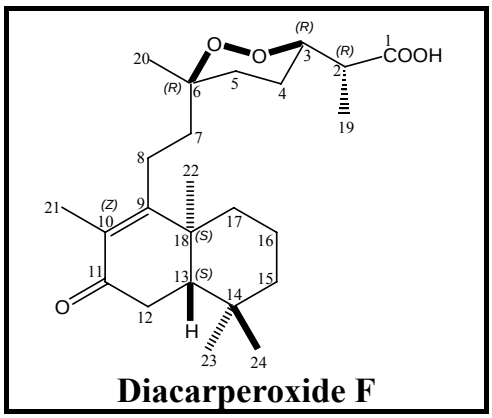


Fig. 3.65: HMBC spectrum of compound 13

Table 3.16: NMR data of compound 13 ($CDCl_3$, 500, 125 MHz)

Position	δ_H (m, *J* in Hz)	δ_C (m)	COSY	HMBC
1		178.1 s		
2	2.54 (q, *J* = 7.6 Hz)	42.9 d	19	3
3	4.03 (q, *J* = 6.6 Hz)	81.4 d	2, 4	
4	1.71 (m)	23.1 t	3	
5	1.73 (m)	33.4 t	4	
6		80.4 s		
7	2.21 (dd, *J* = 4.4, 3.2 Hz), 1.45 (m)	33.5 t	8	
8	2.37 (dd, *J* = 13.2, 4.1 Hz), 2.16 (dd, *J* = 13.2, 3.5 Hz)	23.6 t	7	
9		168.8 s		
10		130.6 s		
11		200.7 s		
12	2.35 (dd, *J* = 17.3, 3.1 Hz), 2.51 (dd, *J* = 17.3, 3.5 Hz)	35.3 t	13	11,18
13	1.70 (m)	50.7 d	12	
14		33.7 s		
15	1.49 (m), 1.25 (m)	41.9 t	16	
16	1.66 (m), 1.58 (m)	19.1 t	15, 17	
17	2.07 (dd, *J* = 14.8, 2.5 Hz), 1.39 (dd, *J* = 14.8, 3.5 Hz)	36.1 t	16	
18		41.5 s		
19	1.19 (d, *J* = 7.5 Hz)	12.8 q	2	1, 2, 3
20	1.80 (s)	23.8 q		5, 6
21	1.76 (s)	11.6 q		9, 10, 11
22	1.08 (s)	18.5 q		9, 13, 17, 18
23	0.87 (s)	32.9 q		13, 14, 15, 24
24	0.92 (s)	21.8 q		13, 14, 15

3.2.9. Diacarperoxide F (14, new compound)



Diacarperoxide F

Diacarperoxide F (**14**) was obtained as a colorless oil. The $[\alpha]_D$ value was + 40° (*c* 0.2, $CHCl_3$). The EIMS spectrum showed a pseudomolecular ion peak at *m*/*z* 406 $[M]^+$. The fragment ion at *m*/*z* 205 suggested the presence of a tetramethyl hexahydronaphthalenone moiety as in **13**. The ^1H and ^{13}C NMR spectra were very similar to those of **13**. Inspection of ^1H-^1H COSY and HMBC spectral data (Table 3.17) indicated compound **14** had the same carbon skeleton as **13**. When **14** was compared with **13**, differences in the ^1H and ^{13}C NMR chemical shifts were observable for the oxymethine as well as the carbonyl carbon (C-1), which suggested a change in the stereochemistry at C-2 and C-3. The oxymethine proton was deshielded to δ 4.31 (brs, H-3), while the carbonyl carbon (C-1) was shielded to δ 175.1. Moreover the methyl group (H_3-19) was overlapped with H_3-20. In addition compound **14** had $[\alpha]_D$ + 40°, while diacarperoxide E (**13**) had $[\alpha]_D$ - 12.6°. This indicated that diacarperoxide F was a stereoisomer of **13**. The stereochemistry was determined by applying Capon´s empirical rules [Capon, *et al*., 1985] to be 2*R*, 3*R*, 6*R*.

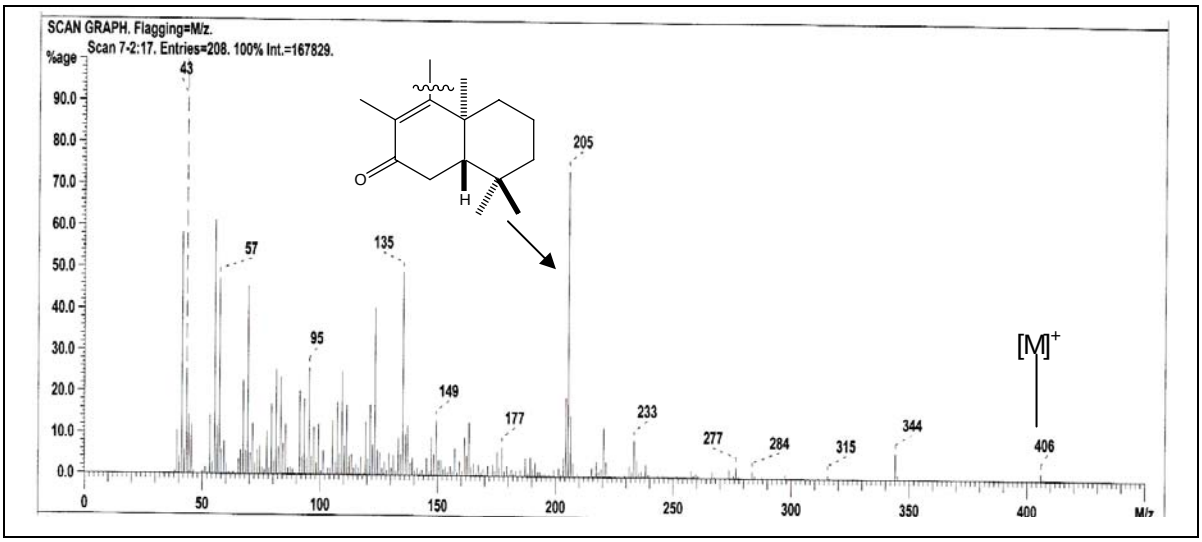


Fig. 3.66: EIMS spectrum of compound 14

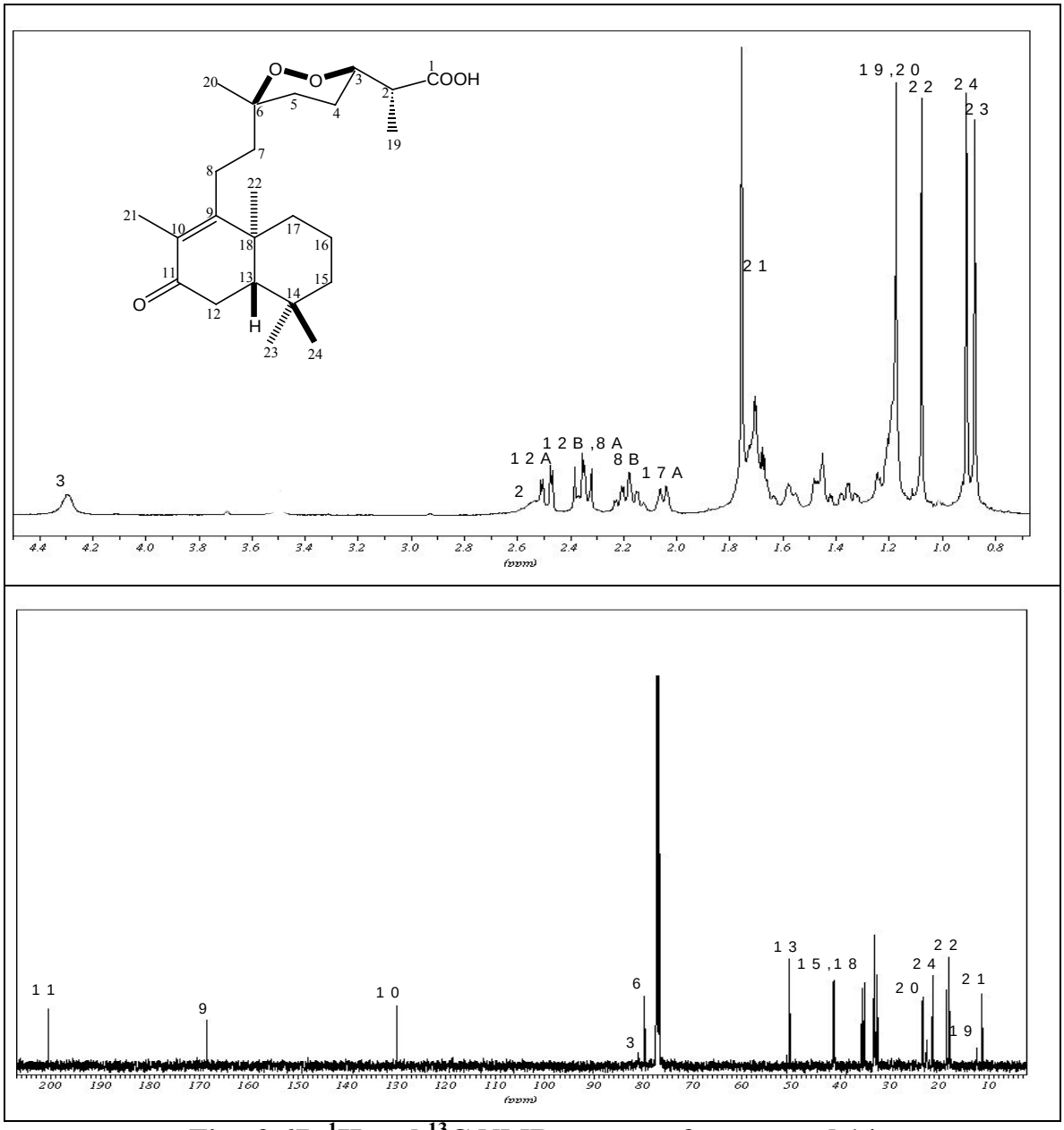


Fig. 3.67: ^{1}H and ^{13}C NMR spectra of compound 14

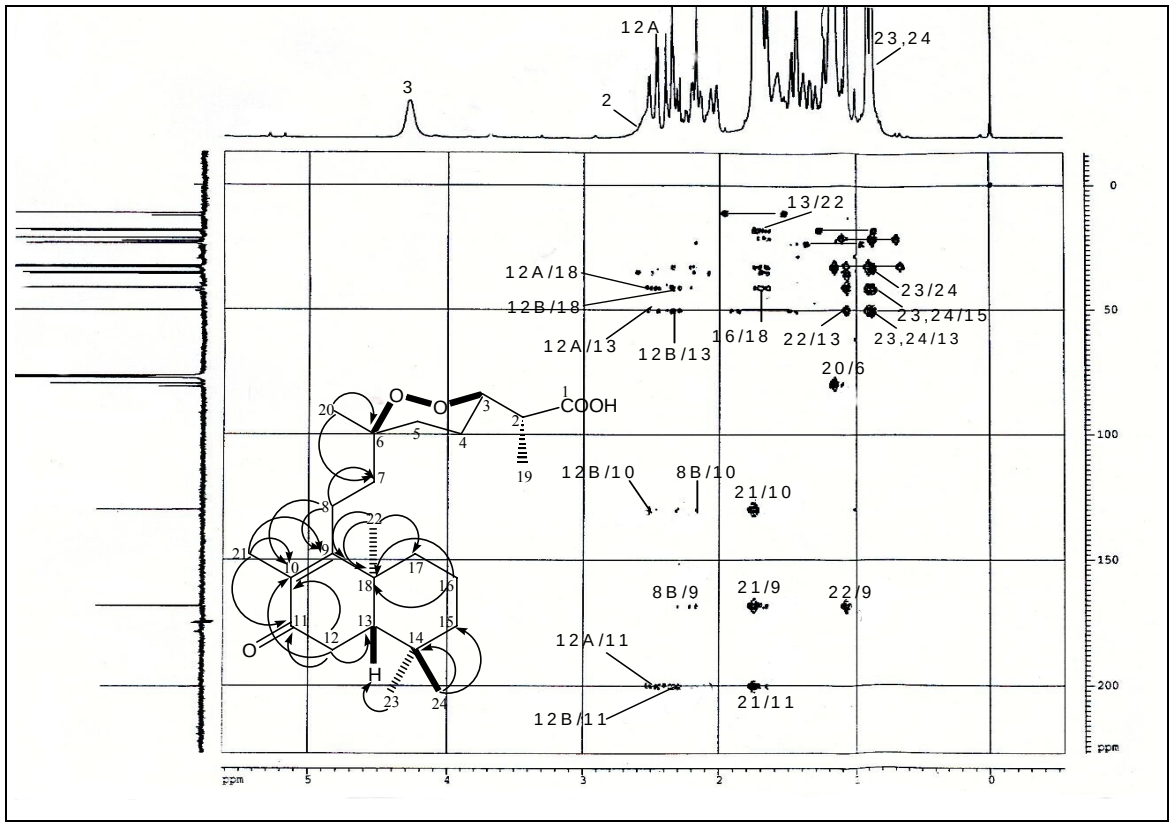


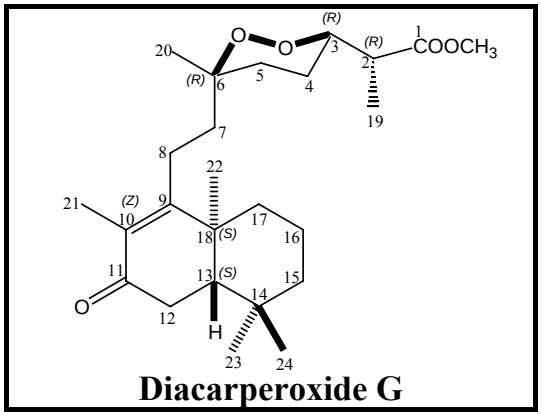
Fig. 3.68: HMBC spectrum of compound 14

Table 3.17: NMR data of compound 14 ($CDCl_3$, 500, 125 MHz)

Position	δ_H (m, *J* in Hz)	δ_C (m)	COSY	HMBC
1		175.1 s		
2	2.55 (brs)	42.7 d	19	
3	4.31 (brs)	80.9 d	4	
4	1.71 (m)	22.5 t	5	
5	1.72 (m)	32.8 t	4	
6		79.6 s		
7	2.21 (m), 1.45 (m)	33.1 t	8	
8	2.37 (dd, *J* = 14.5, 3.5 Hz), 2.15 (dd, *J* = 14.5, 3.5 Hz)	23.2 t	7	6, 7, 9, 10
9		168.4 s		
10		130.0 s		
11		200.3 s		
12	2.49 (dd, *J* = 17.3, 3.4 Hz), 2.33 (d, *J* = 17.3 Hz)	35.2 t	13	11,13, 18
13	1.67 (m)	50.3 t	12	12, 22, 24
14		33.5 s		
15	1.47 (m), 1.25 (m)	41.4 t	16	
16	1.64 (m), 1.57 (m)	18.5 t	17	18
17	2.05 (d, *J* = 13.2 Hz), 1.35 (dd, *J* = 13.2, 3.1 Hz)	35.7 t	16	
18		41.1 s		
19	1.18 *	12.5 q	2	
20	1.18 (s)	23.5 q		6, 7
21	1.75 (s)	11.2 q		9, 10, 11
22	1.08 (s)	17.9 q		9, 13, 18, 17
23	0.88 (s)	32.5 q		13, 14, 15, 24
24	0.91 (s)	21.3 q		13, 14, 15, 23

* overlapped

3.2.10. Diacarperoxide G (15, new compound)



Diacarperoxide G (**15**) was isolated as a colorless oil, with an $[\alpha]_D$ of + 12.6° (*c* 0.10, $CHCl_3$). The EIMS showed a pseudomolecular ion peak at *m/z* 420 $[M]^+$. A diagnostic fragment ion was observed at *m/z* 205 representing the tetramethyl hexahydronaphthalenone moiety was also present. Compound **15** had the molecular formula $C_{25}H_{40}O_5$ as established by HREIMS, which required 6 degrees of unsaturation. Compound **15** had a 14 mass unit difference from compound **14**. The ^1H and ^{13}C NMR spectra of **15** (Table 3.18) were comparable to those of **14** and indicated that it had an identical carbon skeleton. The only difference between the two compounds was the appearance of an additional methoxy group signal at δ 3.70/51.9. Thus, compound **15** was the methyl ester of **14**, which was established by the HMBC correlation of the methoxy group with the acetyl carbonyl carbon at δ 174.1 (C-1). Compound **15** had the same stereochemistry as diacarperoxide F (**14**) (2*R*, 3*R*, 6*R*).

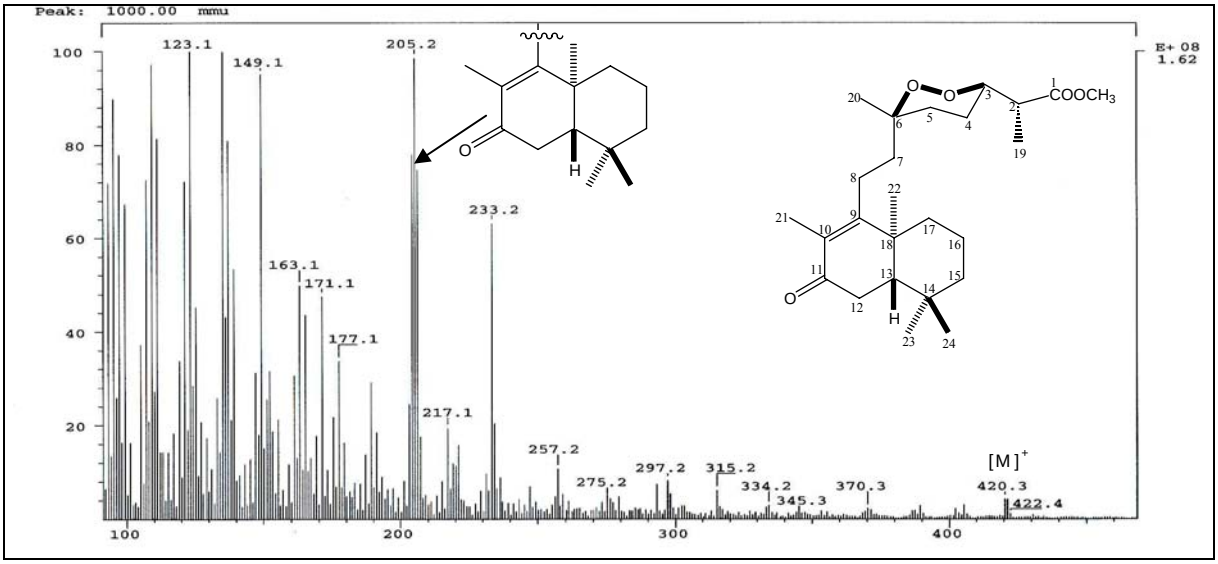


Fig. 3.69: HREIMS spectrum of compound 15

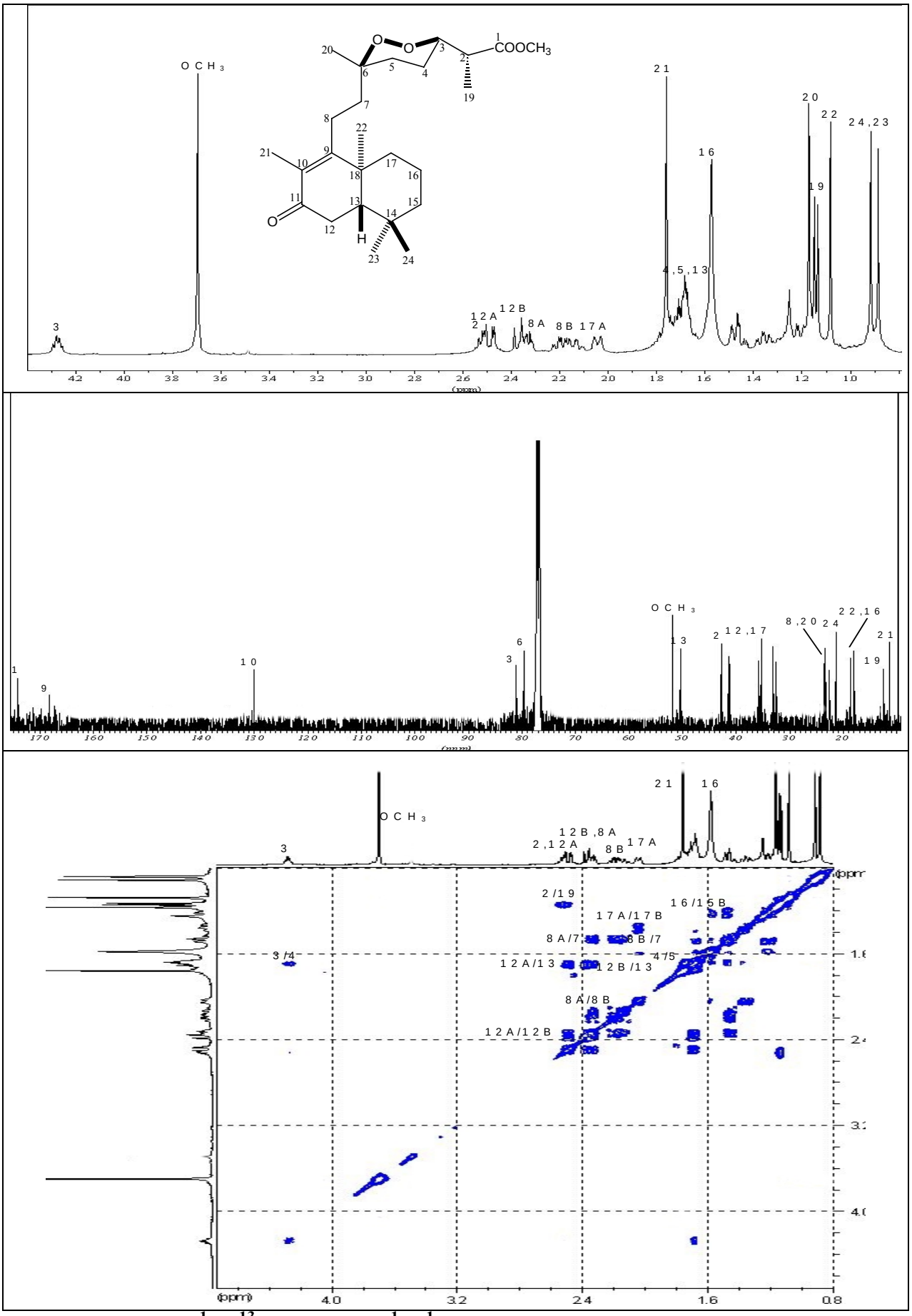


Fig. 3.70: ^{1}H, ^{13}C NMR and ^{1}H-^{1}H COSY spectra of compound 15

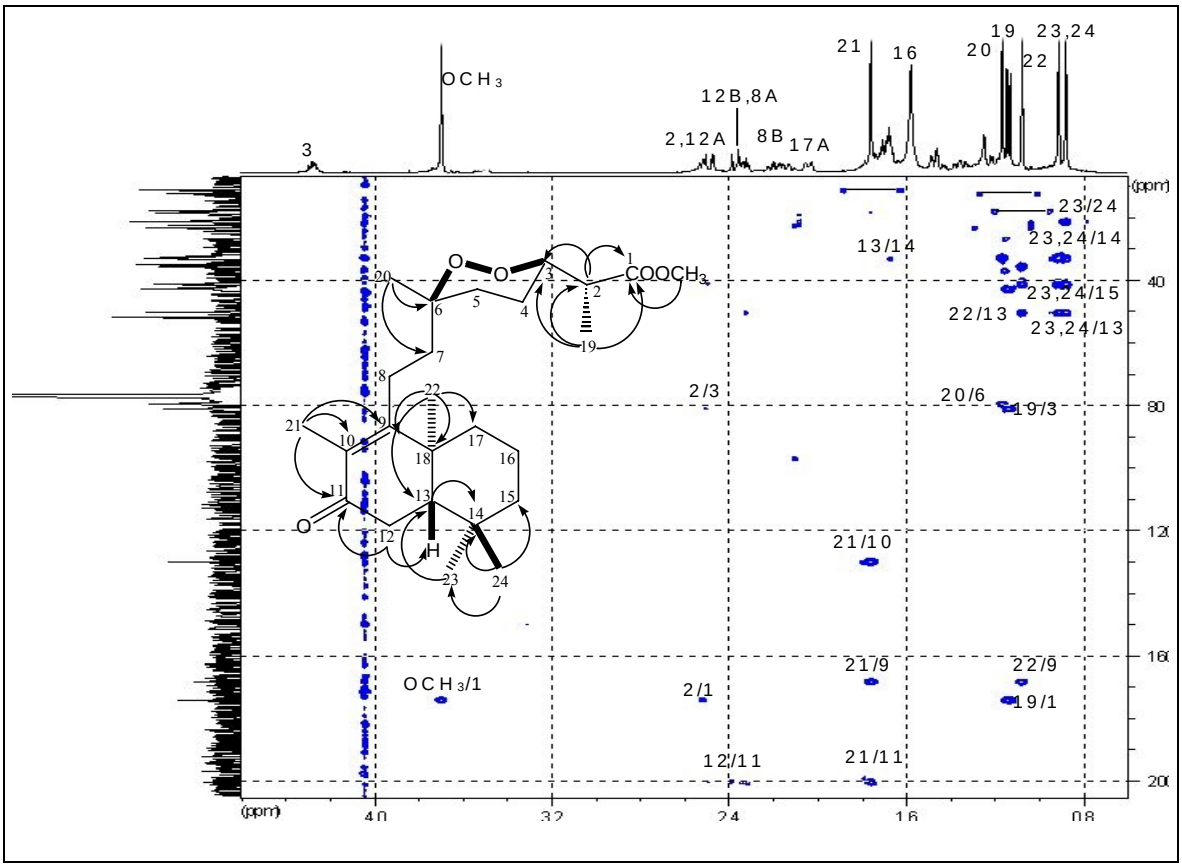
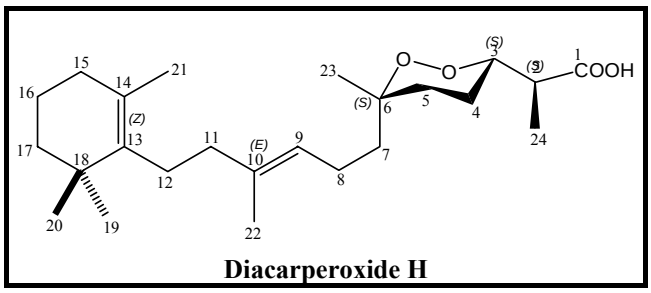


Fig. 3.71: HMBC spectrum of compound 15

Table 3.18: NMR data of compound 15 ($CDCl_3$, 500, 125 MHz)

Position	δ_H (m, *J* in Hz)	δ_C (m)	COSY	HMBC
1		174.1 s		
2	2.52 (q, *J* = 7.6 Hz)	42.7 d	19	3, 1
3	4.26 (q, *J* = 7.6 Hz)	81.0 d	4	
4	1.72 (m)	22.6 t	5	
5	1.67 (m)	33.0 t	4	
6		79.6 s		
7	1.48 (m)	32.8 t	8A, 8B	
8	2.33 (ddd, *J* = 17.2, 12.0, 3.8 Hz), 2.15 (ddd, *J* = 17.0, 13.2, 3.8 Hz)	23.2 t	7	
9		168.3 s		
10		129.9 s		
11		200.2 s		
12	2.50 (dd, *J* = 17.0, 3.8 Hz), 2.37 (dd, *J* = 17.0, 12.8 Hz)	35.2 t	13	11, 13
13	1.66 (m)	50.3 d	12	14
14		33.1 s		
15	1.19 (m), 1.46 (m)	41.1 t	16	
16	1.58 (m)	18.5 t	17	
17	2.05 (m),1.35 (dd, *J* = 12.6, 3.0 Hz)	35.7 t	16	
18		41.1 s		
19	1.14 (d, *J* = 6.9 Hz)	12.5 q	2	1, 2, 3
20	1.17 (s)	23.5 q		6, 7
21	1.77 (s)	11.2 q		9, 10, 11
22	1.08 (s)	17.9 q		9, 13, 17, 18
23	0.98 (s)	32.5 q		13, 14, 15, 24
24	0.92 (s)	21.3 q		13, 15, 23
OCH_3	3.70 (s)	51.9 q		1

3.2.11. Diacarperoxide H (16, new compound)



Diacarperoxide H

Diacarperoxide H (**16**) was isolated as a colorless oil. The $[\alpha]_D$ value was - 20.5° (*c* 0.54, $CHCl_3$). The ESIMS showed a pseudomolecular ion peak at *m*/*z* 392 $[M]^+$. The ^1H and ^{13}C NMR data of **16** (Table 3.19) and compound **13** were similar for the C_1-C_6 region. The signals associated with the cyclic peroxide and cyclohexene moieties were also evident. The ^1H NMR spectrum showed the presence of additional signals: olefinic methine at δ 5.15 (H-9), olefinic methyl at δ 1.65 (H_3-22) and four methylenes at δ 1.57 (H_2-7), 1.67 (H_2-8), 2.01 (H_2-11) and 2.04 (H_2-12). The ^{13}C NMR signals at δ 137.5 (C-13), 127.2 (C-14), 31.1 (C-15), 40.2 (C-16), 19.9 (C-17), 35.4 (C-18), 28.2 (Me-19), 28.8 (Me-20) and 19.4 (Me-21) indicated the presence of the 2,6,6-trimethyl cycohexene moiety [Kobayashi, *et al*., 1993]. The occurrence of one set of methyl monosubstituted olefinic carbons was in agreement with the ^{13}C NMR signals at δ 123.8 (C-9), 136.8 (C-10) and 19.4 (C-22) [D´ Ambrosio, *et al*., 1997, Sperry, *et al*., 1998]. From its ^1H-^1HCOSY spectrum typical allylic coupling of the methine at δ 5.15 with the methyl at δ 1.65 (H_3-22) was evident. The COSY spectrum showed correlation of the methine proton H-9 with the methylene protons at 1.67 (H_2-8), which further correlated with the methylene at δ 1.57 (H_2-7). The latter part consisted of two coupled methylenes H_2-11 and H_2-12, which was confirmed by the correlations observed from its COSY spectrum. The HMBC spectrum showed correlations of the olefinic methine H-9 with C-8, C-11, and C-22, which established the position of the double bond. This was again confirmed by the correlations of H_3-22 with C-9, C-10 and C-11. The connectivity of the cyclohexene moiety with that of the C_1-C_{12} region was established by the HMBC correlations of H_2-12 with C-13, C-14 and C-18 of the cyclohexene moiety. The connectivity of the cyclic peroxide group was established by the HMBC correlations of H_3-23 with C-5, C-6 and C-7. The NMR data of **16** were very similar to those of epimuqubilin A isolated from *Diacarnus* cf. *spinopoculum*

[Sperry, *et al*., 1998]. Compound **16** ($[\alpha]_D$ - 20.5°) and epimuqubilin A ($[\alpha]_D$ + 61.7°) were identified as enantiomers, because they displayed identical NMR data but gave opposite optical rotations.

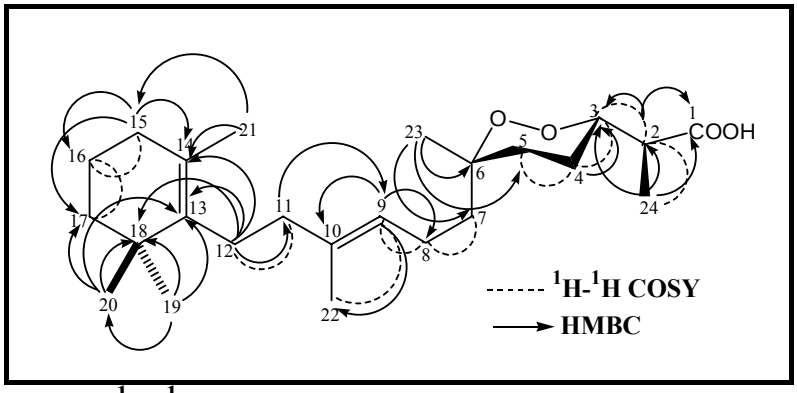


Fig. 3.72: ^{1}H-^{1}H COSY and HMBC correlations of compound 16

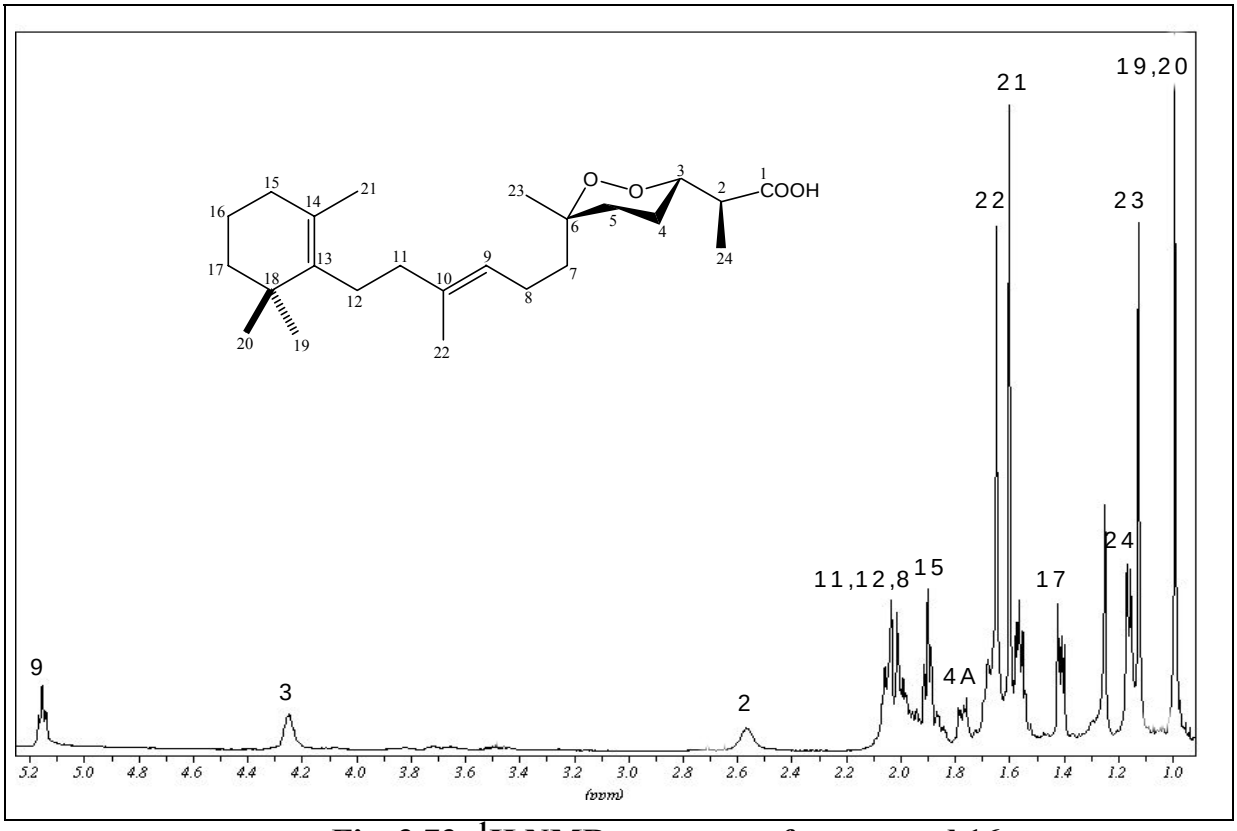


Fig. 3.73: ^{1}H NMR spectrum of compound 16

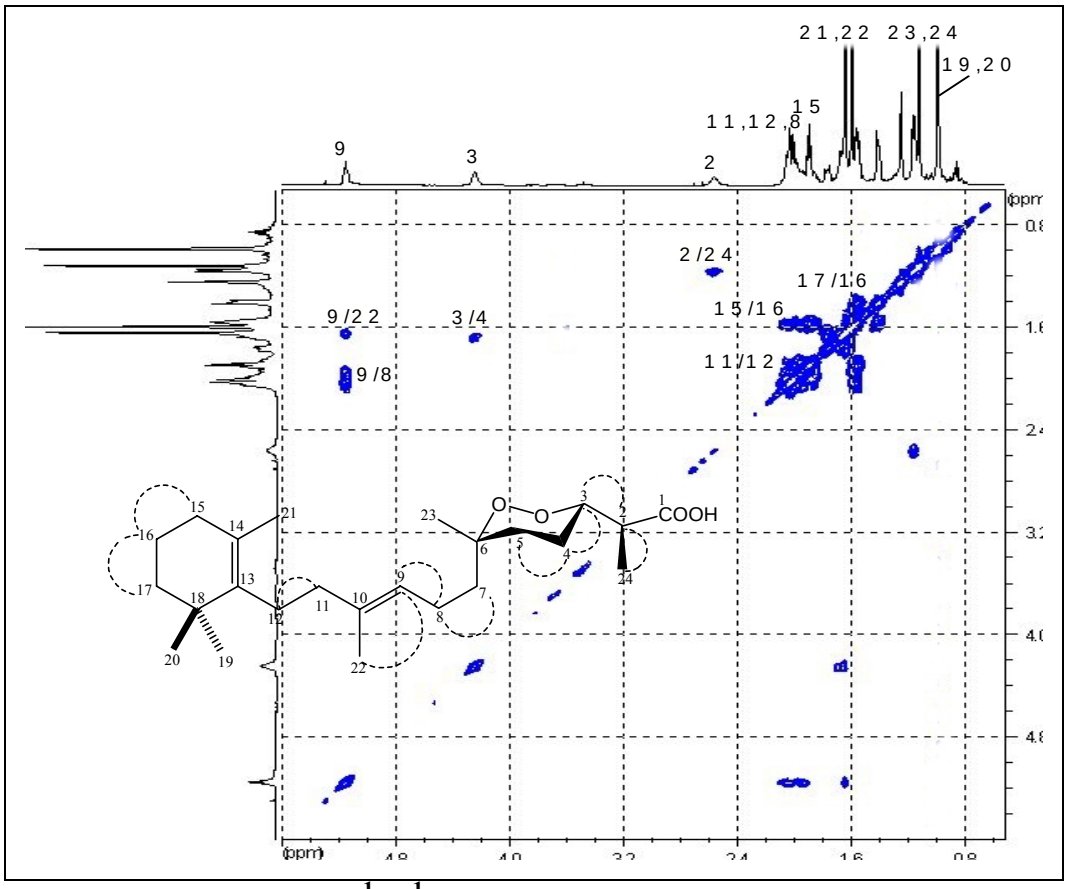


Fig. 3.74: ^{1}H-^{1}H COSY spectrum of compound 16

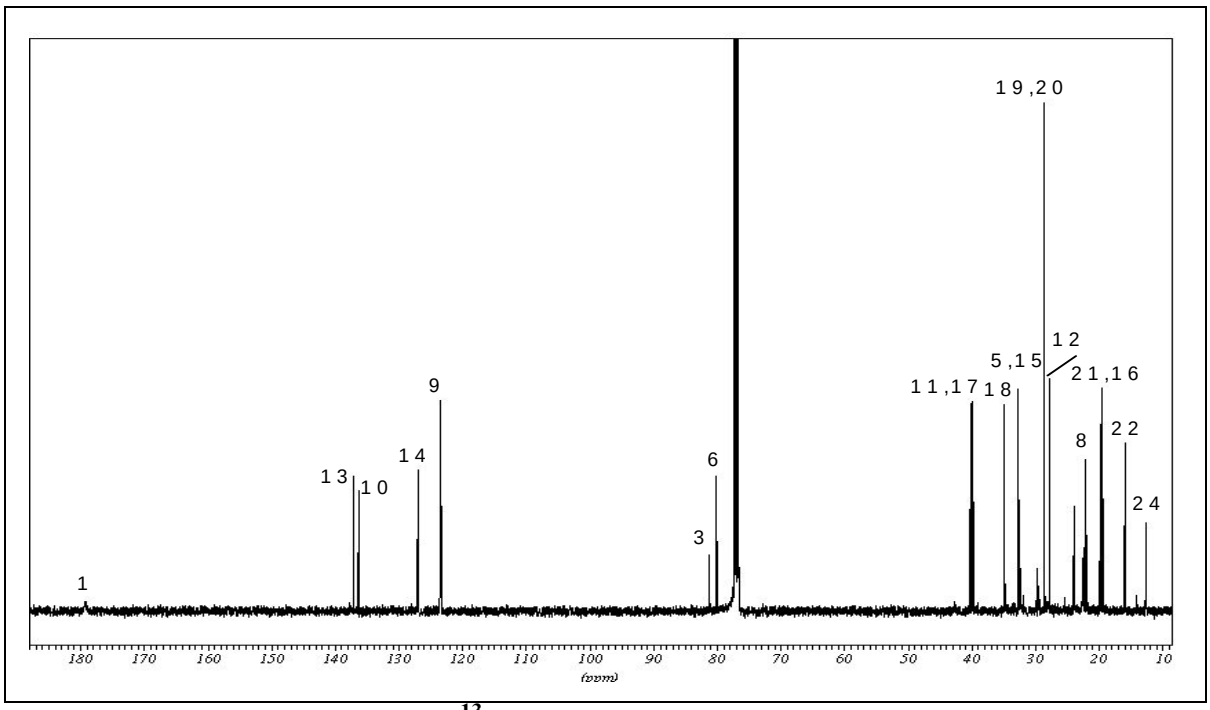


Fig. 3.75: ^{13}C NMR spectrum of compound 16

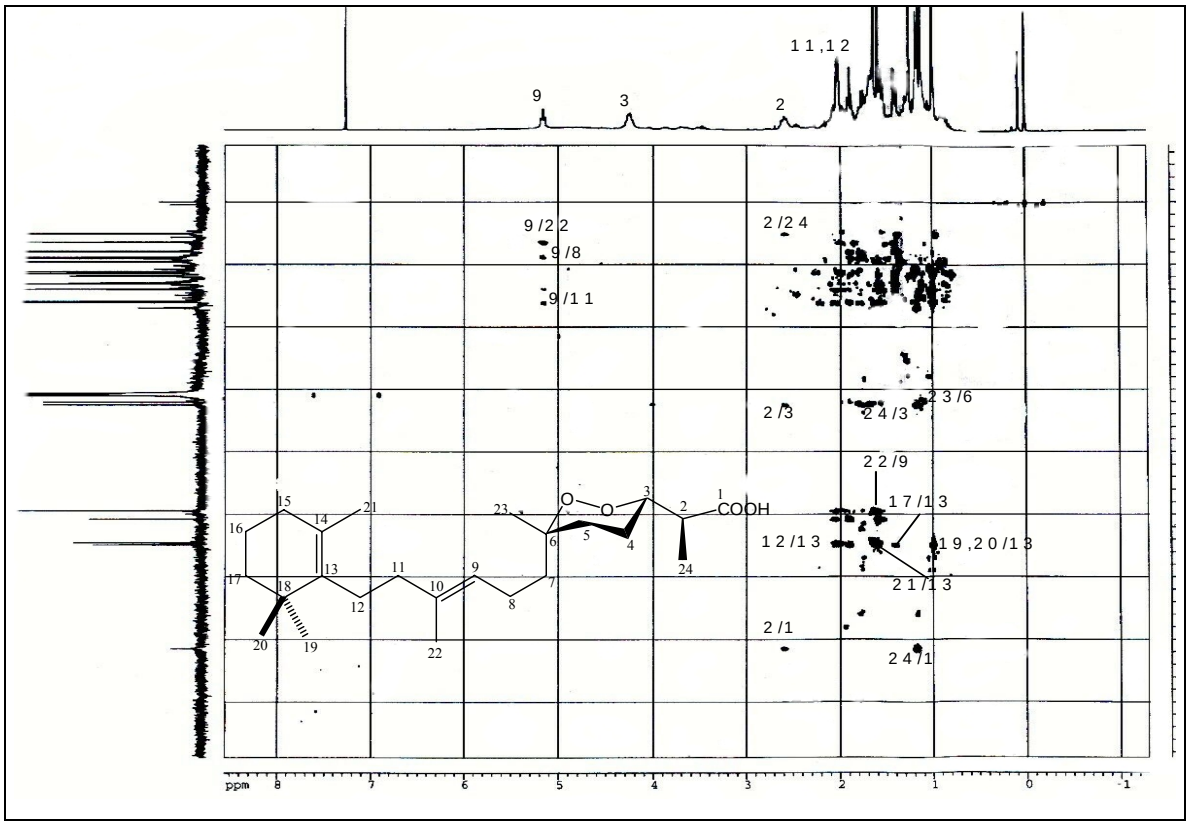
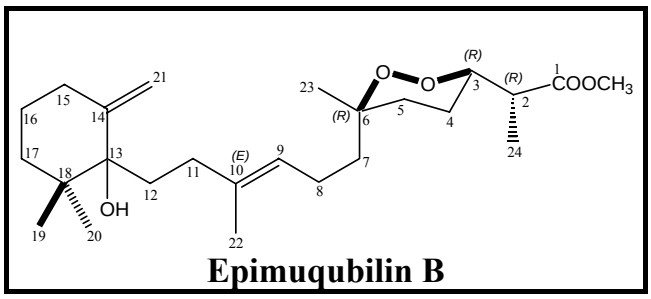


Fig. 3.76: HMBC spectrum of compound 16

Table 3.19: NMR data of compound 16 ($CDCl_3$, 500, 125 MHz)

Position	δ_H (m, *J* in Hz)	δ_C (m)	COSY	HMBC
1		179.5 s		
2	2.57 (brs)	43.2 d	3, 24	1, 3, 24
3	4.25 (brs)	81.1 d	2, 4	2
4	1.77 (m), 1.67 (m)	32.1 t	5	3
5	1.56 (m)	32.8 t	4	
6		80.5 s		
7	1.57 (m)	35.2 t		
8	1.67 (m)	22.6 t		
9	5.15 (t, *J* = 6.6 Hz)	123.8 d	8, 22	22, 8, 11
10		136.8 s		
11	2.01 (m)	40.5 t	12	9, 10
12	2.04 (m)	27.6 t	11	11, 13, 14, 18
13		137.5 s		
14		127.2 s		
15	1.91 (t, *J* = 6.3 Hz)	33.1 t	16, 17	13, 14, 16, 17
16	1.41 (m)	40.2 t	15, 17	13, 18
17	1.58 (m)	19.9 t	16	
18		35.4 s		
19	1.00 (s)	28.2 q		13, 18, 20
20	1.00 (s)	28.8 q		13, 17, 18
21	1.60 (s)	19.4 q		13, 14, 15
22	1.65 (s)	15.8 q		9, 10, 11
23	1.12 (s)	23.6 q		5, 6, 7
24	1.17 (d, *J* = 6.9 Hz)	12.5 q	2	1, 2, 3

3.2.12. Epimuqubilin B (17, known compound)



Epimuqubilin B

Compound **17** was obtained as a colorless oil, with an $[\alpha]_D$ of + 3.09° (*c* 0.135, $CHCl_3$). The EIMS spectrum showed a pseudomolecular ion peak at *m*/*z* 422 $[M]^+$. The significant ion peak at *m*/*z* 137 was not detected which suggested the absence of a cyclohexene moiety. The ^1H and ^{13}C NMR data of compound **17** and dicarperoxide H (**16**) were similar for the C_1-C_{12} region. The signals associated with the cyclohexene moiety were not evident. The major differences from the signals in **16** were discernible in the segment of the methyl-substituted olefins. The signals for one such olefin were absent, and instead, additional signals were observed at δ 4.89 (1H, d, *J* = 1.2 Hz, H-21A), 4.83 (1H, d, *J* = 1.2 Hz, H-21B), three methylenes and two methyls at δ 0.97 (H_3-19), 0.89 (H_3-20). A singlet methoxy signal at δ 3.70 indicated the methyl ester nature of **17**. The ^1H-^1H COSY showed correlation of H-21A with H-21B. An additional spin system consisted of three methylenes at δ 2.31, 1.99 (H_2-15), 1.49, 1.36 (H_2-16) and 1.58 (H_2-17) was observed. The ^{13}C NMR and DEPT data confirmed the presence of the exomethylene functionality from the characteristic methylene signal at δ 108.2 (t, CH_2-21) and its respective quaternary carbon at δ 150.7 (C-14). The HMQC correlations of the two signals at δ 4.89 and 4.83 to the carbon at δ 108.2 also corroborated this assignment. The occurrence of an oxygenated quaternary carbon was deduced from the carbon resonance at δ 80.0 (C-13). From the HMBC spectrum, it was shown that the oxygenated quaternary carbon was allylic to the exomethylene unit, as the olefin protons at δ 4.89 and 4.83 correlated with the quaternary carbon at δ 80.0. The substructure C_{13}-C_{18} was established by the HMBC correlations of the methylenes at δ 2.31, 1.99 (H_2-15) and 1.58 (H_2-17) with C-13. The location of the geminal dimethyls at C-18 was confirmed by the correlations of H_3-19 with C-13 and C-18. The NMR data and $[\alpha]_D$ value of compound **17** were similar to those of

epimuqubilin B [+14.8 (*c* 0.27, $CHCl_3$)] isolated from *Diacarnus* cf. *spinopoculum* [Sperry, *et al*., 1998]. The stereochemistry of **17** was assigned to be 2*R*, 3*R*, 6*R*, while the configuration at C-13 was undefined [Sperry, *et al*., 1998]. Due to very small amount isolated, it was not possible to further characterise the stereochemistry at C-13.

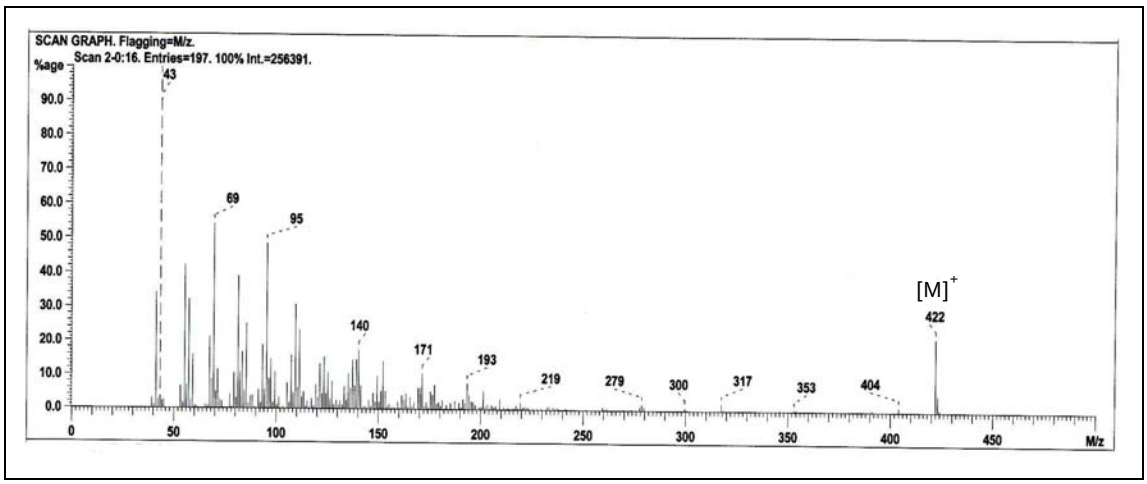


Fig. 3.77: EIMS spectrum of compound 17

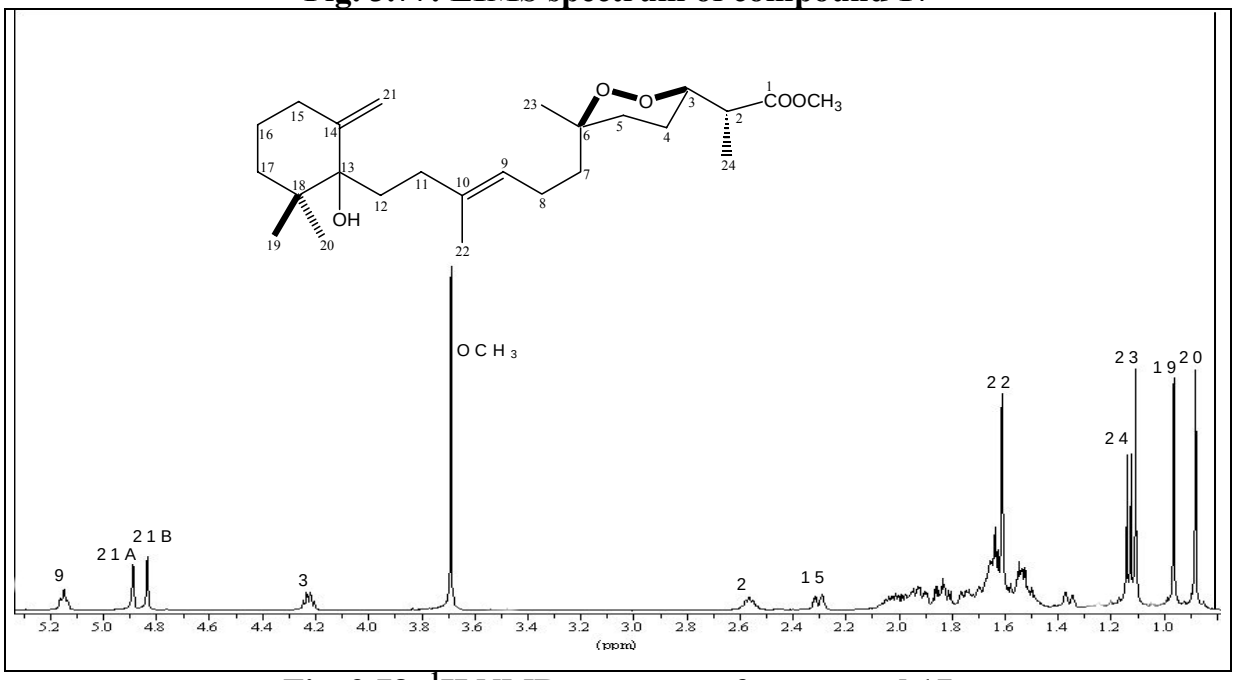


Fig. 3.78: ^{1}H NMR spectrum of compound 17

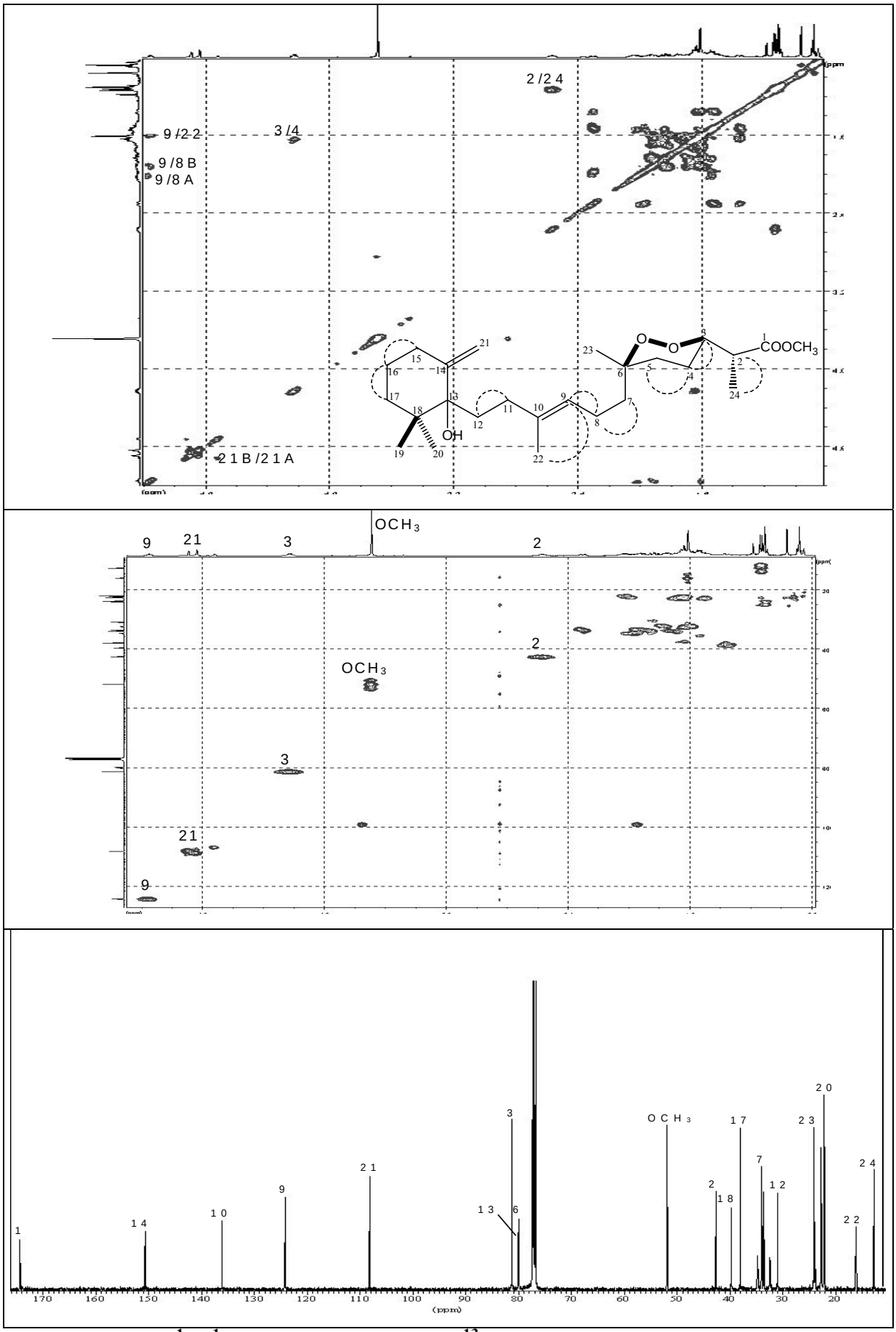


Fig. 3.79: ^{1}H-^{1}H COSY, HMQC and ^{13}C NMR spectra of compound 17

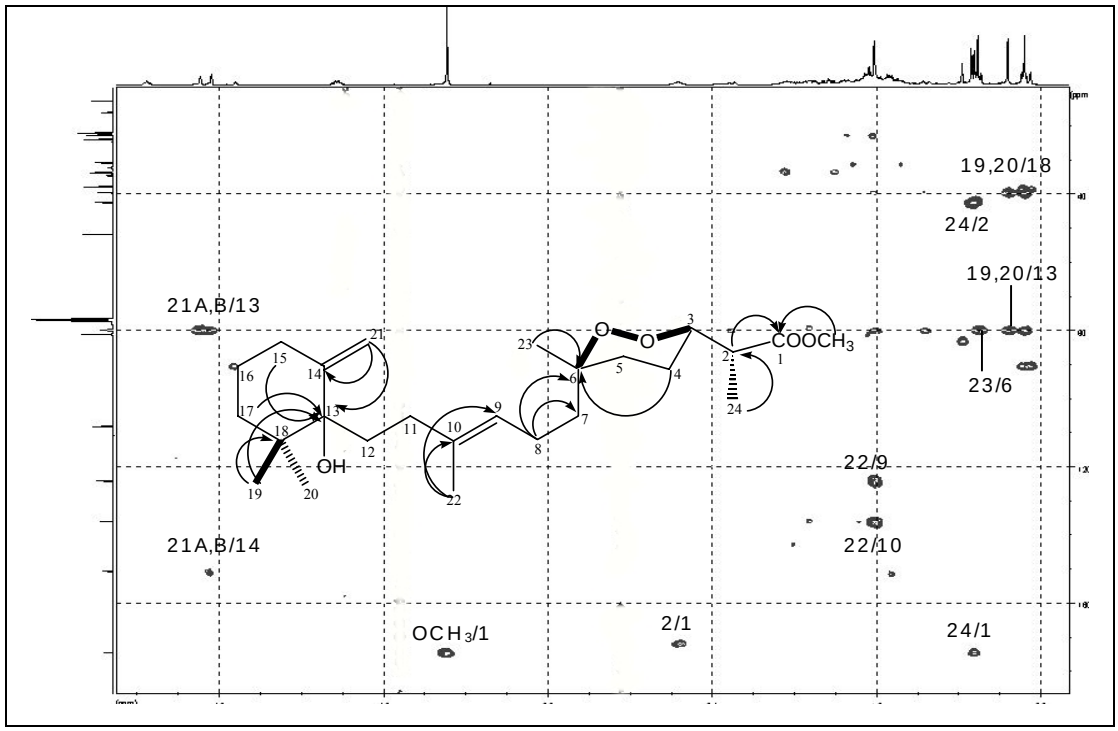
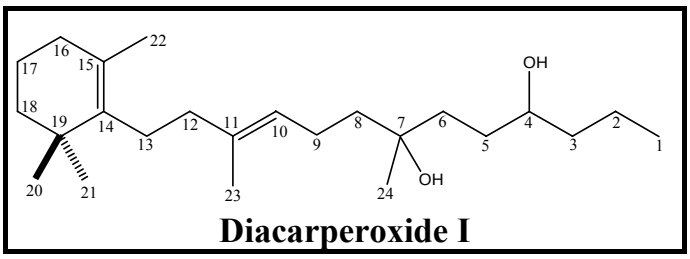


Fig. 3.80: HMBC spectrum of compound 17

Table 3.20: NMR data of compound 17 ($CDCl_3$, 500, 125 MHz)

Position	δ_H (m, *J* in Hz)	δ_C (m)	δ_C (m) of epimuqubilin B [Sperry, *et al.*, 1998 in C_6D_6]	COSY	HMBC
1		174.4 s	174.0 s		
2	2.57 (p, *J* = 6.9 Hz)	42.6 d	43.0 d	24	1
3	4.23 (q, *J* = 8.2 Hz)	81.3 d	81.6 d	4	
4	1.62 (m)	22.6 t	23.0 t	5	6
5	1.76 (m), 1.65 (m)	32.4 t	33.2 t	4	
6		79.9 s	79.4 s		
7	1.53 (m)	34.0 t	35.2 t	8	
8	2.03 (m), 1.98 (m)	22.1 t	22.6 t	7	6, 7
9	5.16 (t, *J* = 6.9 Hz)	124.2 d	124.9 d	8, 22	
10		136.0 s	136.3 s		
11	1.94 (m), 1.71 (m)	33.6 t	34.3 t	12	
12	1.85 (m), 1.62 (m)	30.9 t	31.7 t	11	
13		80.0 s	79.9 s		
14		150.7 s	151.1 s		
15	2.31 (m), 1.99 (m)	33.6 t	34.3 t	16	13
16	1.49 (m), 1.36 (m)	22.7 t	23.3 t	15, 17	
17	1.58 (m)	37.9 t	38.2 t	16	13
18		39.7 s	40.0 s		
19	0.97 (s)	24.0 q	24.4 q		13, 18
20	0.89 (s)	22.1 q	22.4 q		13, 18
21A 21B	4.89 (d, *J* = 1.2 Hz) 4.83 (d, *J* = 1.2 Hz)	108.2 t	108.7 t	21B 21A	13, 14
22	1.62 (s)	16.1 q	16.4 q		9, 10
23	1.12 (s)	24.1 q	24.2 q		6
24	1.14 (d, *J* = 7.2 Hz)	12.8 q	12.9 q	2	2
OCH_3	3.70 (s)	51.9 q	51.9 q		1

3.2.13. Diacarperoxide I (18, new compound)



Diacarperoxide I

Diacarperoxide I (**18**) was isolated as a colorless oil, with an $[\alpha]_D$ of - 10.3° (*c* 0.125, $CHCl_3$). The EIMS spectrum displayed the pseudomolecular ion peak at *m/z* 332 $[M-2H_2O]^+$ and suggested the presence of two hydroxyl functions in the molecule. The base peak at *m*/z 137 suggested the presence of cyclohexyl moiety as in **16**. The ^1H and ^{13}C NMR data (Table 3.21) were comparable to those of **16**, which showed similar resonances for the C_9-C_{19} region. However, the signals associated with the cyclic peroxide and propionic acid methyl ester functions, which were encountered in the terpene cyclic peroxides were not evident [Capon, 1991, Faulkner, *et al*., 1991]. In its ^1H NMR spectrum, there were new signals at δ 3.54 (1H, m, H-4), triplet methyl signal at δ 0.95 (3H, t, *J* = 8.0 Hz, H_3-1) and 5 methylene signals. The ^{13}C NMR spectrum showed 24 carbons as in **16**, with the appearance of new signals at δ 10.0 (q, C-1), 31.0 (t, C-2), 30.3 (t, C-3), 73.7 (d, C-4), 37.8 (t, C-5), 41.7 (t, C-6), 72.7 (s, C-7), 29.0 (t, C-8), and 26.9 (q, C-24). The ^1H-^1H COSY spectrum showed a new 2-methyl-octane-2, 5-diol spin system replacing the cyclic peroxide and propionic acid methyl ester units in **16**. The triplet methyl signal at δ 0.95 (H_3-1) showed correlation with the signal at δ 1.48 (H_2-2), which further correlated with the methylene signal at δ 1.49 (H_2-3), and gave a cross peak with the oxymethine at δ 3.54 (H-4), which also correlated with the signal at δ 1.52 (H_2-5) and the methylene signal at δ 1.54 (H_2-6). The methylene signal at δ 1.53 (H_2-8) showed correlations with the signal at δ 2.07 (H_2-9) and with the olefinic methine proton at δ 5.15 (H-10). The 2-methyl-octane-2, 5-diol was also validated by the correlations observed in the HMBC spectrum. The triplet methyl signal showed correlations with C-3 and the four-bond coupling to C-4. H_2-3 correlated with the oxygen bound C-4 and the methylene C-5. The methyl singlet at δ 1.20 (H_3-24) correlated with C-6, C-7, and C-8. These resonances were comparable to those found in the triol derivatives of muqubilin [Kashman, *et al*., 1979,

Manes, *et al*., 1984]. The connectivity of 2-methyl-octane-2, 5-diol with that of C_9-C_{19} was established through the HMBC correlations of H_2-8 with C-24 and C-9. By comparison of the literature data with the data obtained from the ^{1}H-^{1}H COSY, HMQC and HMBC, the structure of **18** was unambiguously elucidated and named **diacarperoxide I**. It was not possible to ascertain the absolute configuration of C-4 and C-7 due to the very small amount of compound isolated.

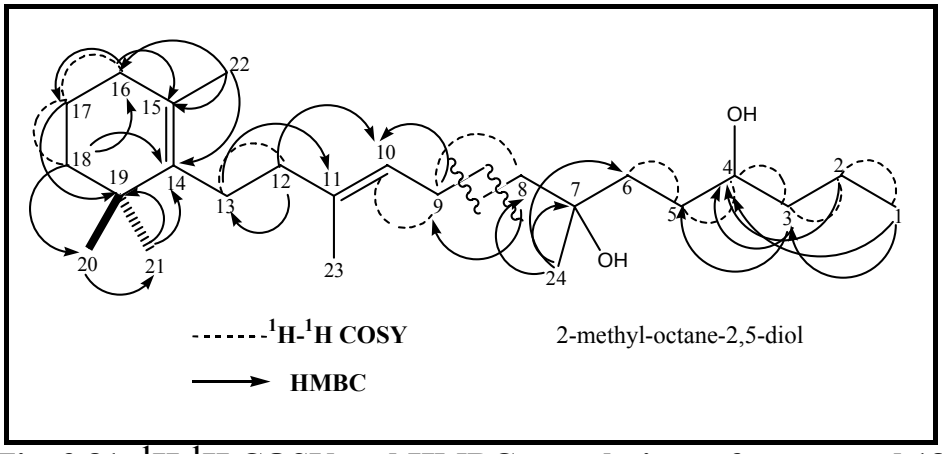


Fig. 3.81: ^{1}H-^{1}H COSY and HMBC correlations of compound 18

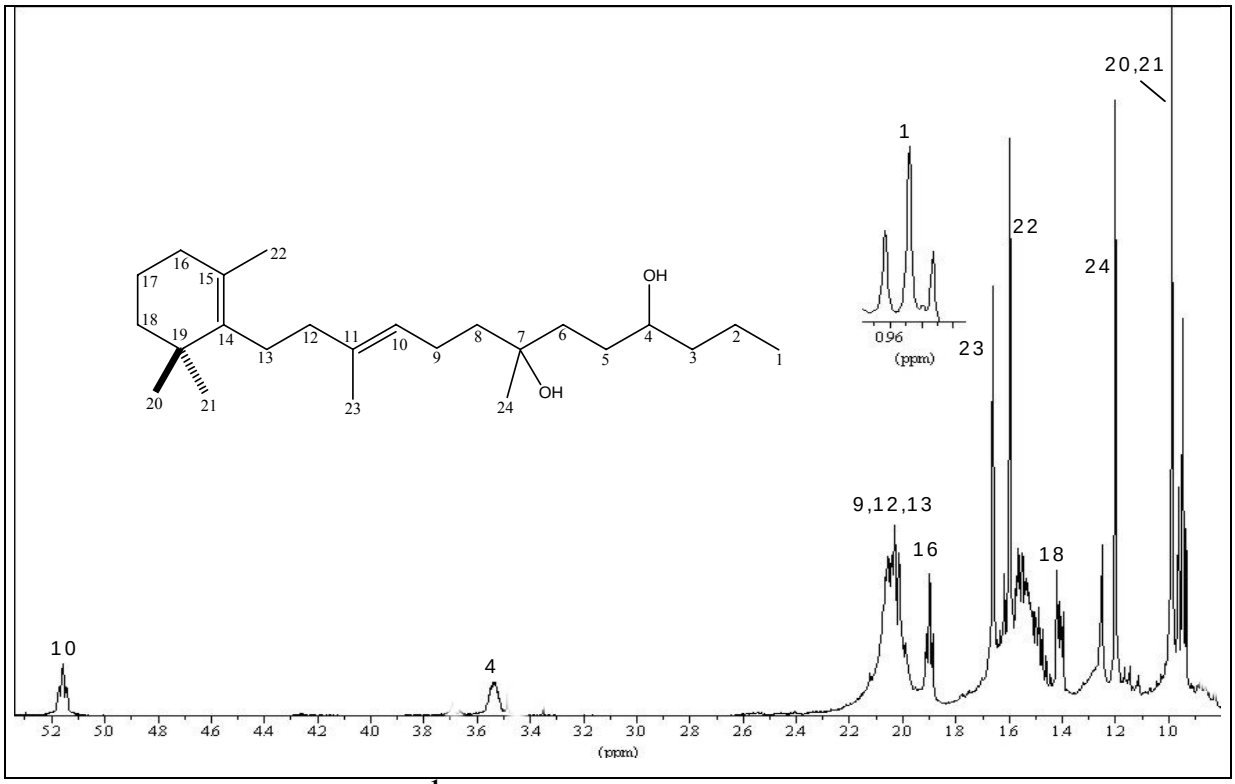


Fig. 3.82: ^{1}H NMR spectrum of compound 18

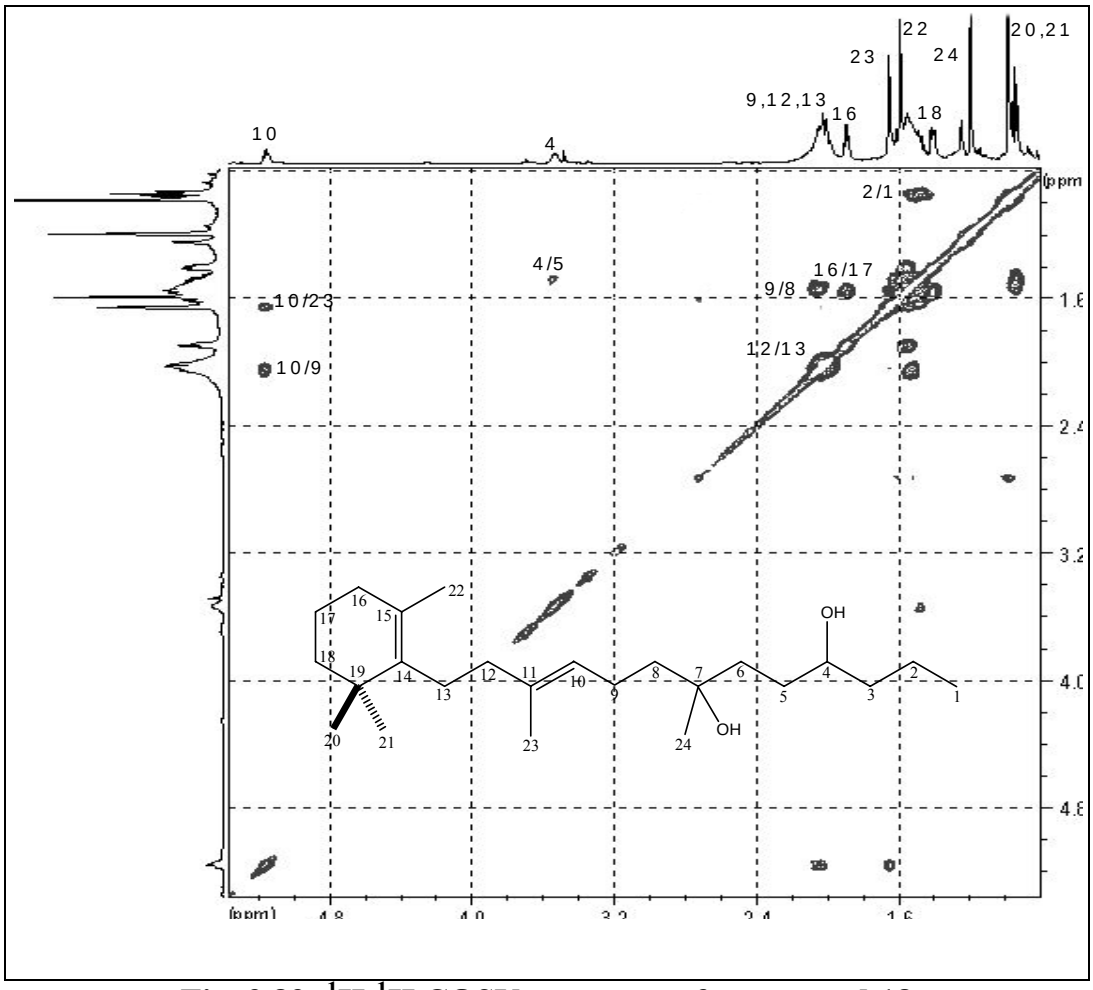


Fig. 3.83: ^{1}H-^{1}H COSY spectrum of compound 18

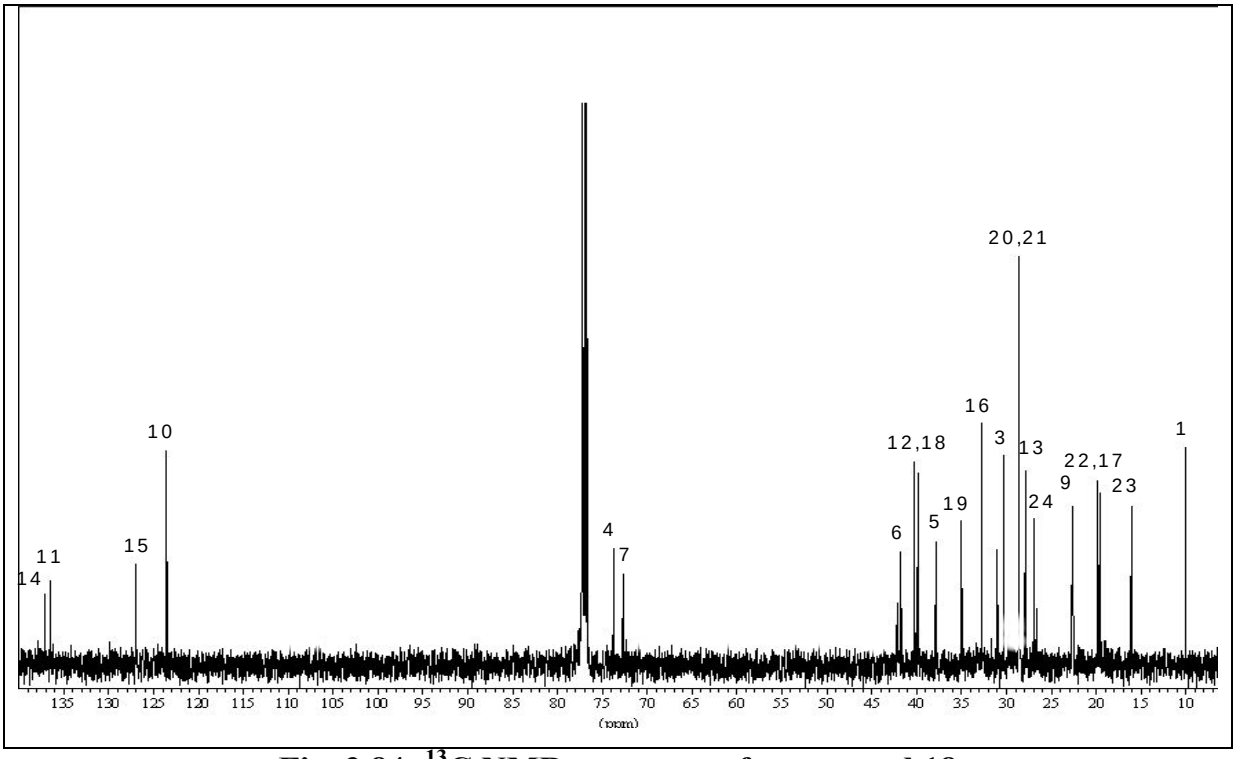


Fig. 3.84: ^{13}C NMR spectrum of compound 18

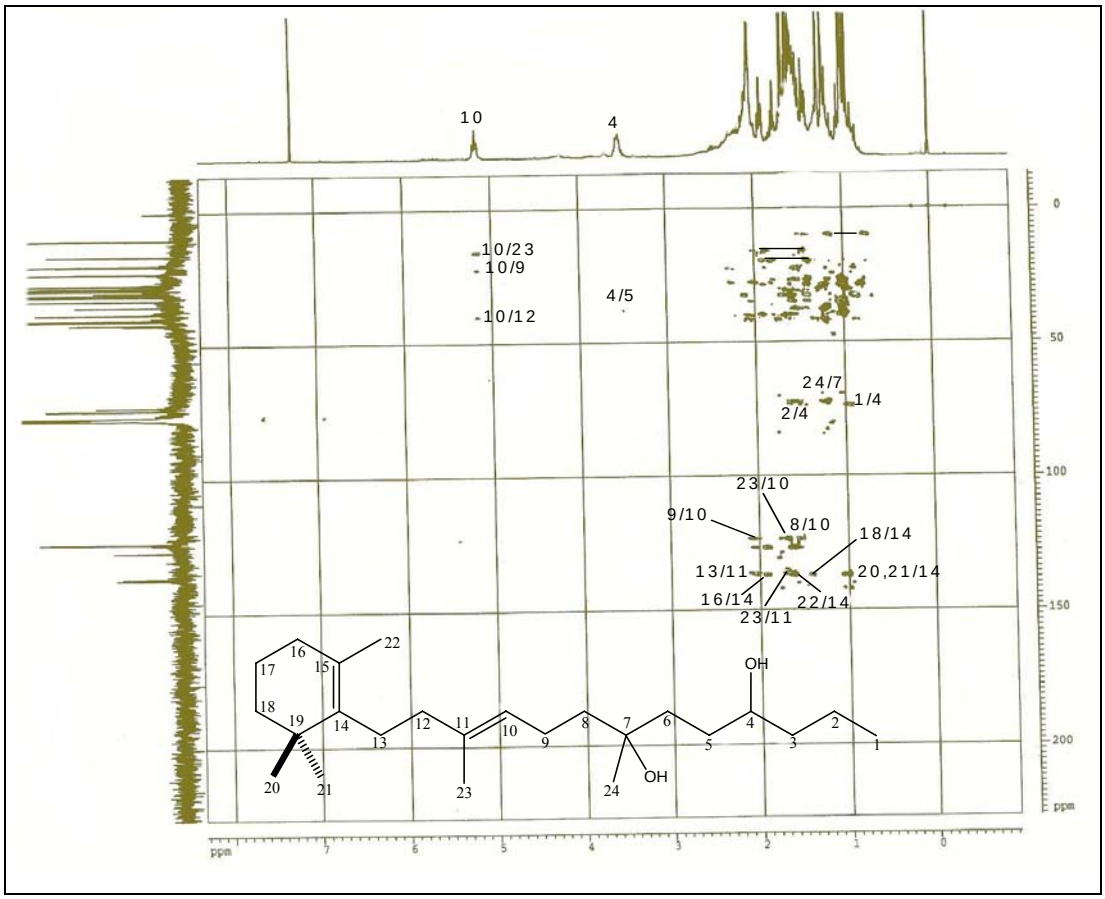


Fig. 3.85: HMBC spectrum of compound 18

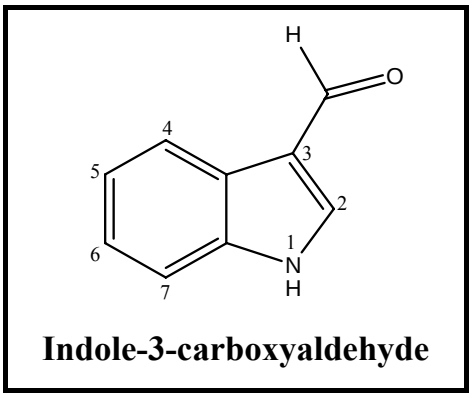
Table 3.21: NMR data of compound 18 ($CDCl_3$, 500, 125 MHz)

Position	δ_H (m, *J* in Hz)	δ_C (m)	COSY	HMBC
1	0.95 (t, *J* = 8.0 Hz)	10.0 q	2	3, 4
2	1.48 (m)	31.0 t	3	4, 5
3	1.49 (m)	30.3 t	4	4, 5
4	3.54 (m)	73.7 d	5	
5	1.52 (m)	37.8 t	6	
6	1.54 (m)	41.7 t	5	
7		72.7 s		
8	1.53 (m)	29.0 t	9	9, 24
9	2.07 (m)	22.6 t	8	10, 12
10	5.15 (t, *J* = 6.6 Hz)	123.5 d	9, 23	9, 12, 23
11		136.5 s		
12	2.02 (m)	40.2 t	13	10, 13
13	2.0 (m)	27.8 t	12	11, 12
14		137.0 s		
15		126.9 s		
16	1.90 (t, *J* = 6.3 Hz)	32.7 t	17	14, 15, 17, 18
17	1.57 (m)	19.5 t	18	18, 19
18	1.41 (m)	39.8 t	17	14, 16, 17, 19, 20, 21
19		35.0 s		
20	0.99 (s)	28.6 q		14, 18, 19, 21
21	0.99 (s)	28.6 q		14, 18, 19
22	1.60 (s)	19.8 q		14, 15, 16
23	1.66 (s)	16.0 q		10, 11
24	1.20 (s)	26.9 q		5, 6, 7, 8

Table 3.22: Stereochemistry and $[\alpha]_D$ values of the isolated norterpene peroxides

Compound (no.)	$[\alpha]_D$	stereochemistry
Nuapapuin A methyl ester (6)	+ 52.7° (*c* 0.175, $CHCl_3$)	2*R*, 3*R*, 6*R*
Methyl-2-epinuapapuinoate (7)	- 30.3° (*c* 0.20, $CHCl_3$)	2*R*, 3*S*, 6*R*
Diacarperoxide A (8)	+ 53.4° (*c* 0.25, $CHCl_3$)	2*R*, 3*R*, 6*R*
Methyl diacarnoate A (9)	- 52.0° (*c* 0.10, $CHCl_3$)	2*R*, 3*S*, 6*R*
Diacarperoxide B (10)	- 35.0° (*c* 0.11, $CHCl_3$)	2*R*, 3*S*, 6*R*
Diacarperoxide C (11)	- 55.0° (*c* 0.06, $CHCl_3$)	2*S*, 3*S*, 6*R*
Diacarperoxide D (12)	- 11.3° (*c* 0.10, $CHCl_3$)	2*S*, 3*S*, 6*S*
Diacarperoxide E (13)	- 12.6° (*c* 0.30, $CHCl_3$)	2*S*, 3*S*, 6*S*
Diacarperoxide F (14)	+ 40.0° (*c* 0.20, $CHCl_3$)	2*R*, 3*R*, 6*R*
Diacarperoxide G (15)	+ 12.6° (*c* 0.10, $CHCl_3$)	2*R*, 3*R*, 6*R*
Diacarperoxide H (16)	- 20.5° (*c* 0.54, $CHCl_3$)	2*S*, 3*S*, 6*S*
Epimuqubilin B (17)	+ 3.09° (*c* 0.135, $CHCl_3$)	2*R*, 3*R*, 6*R*

3.2.14. Indole-3-carboxyaldehyde (19, known compound)



Indole-3-carboxyaldehyde

Compound **19** was isolated as a yellowish white amorphous powder. It had UV absorbances at λ_{max} 208, 243, and 297 nm. The (+) ESIMS spectrum showed the pseudomolecular ion peak at *m*/*z* 146 $[M+H]^+$. The ^1H NMR spectrum showed a doublet at δ 8.05 (1H, d, *J* = 7.9 Hz, H-4), two double triplets in the downfield region at δ 7.16 (1H, dt, *J* = 7.2, 1.3 Hz, H-6) and 7.21 (1H, dt, *J* = 7.2, 1.3 Hz, H-5), and one doublet at δ 7.48 (1H, d, *J* = 7.6 Hz, H-7), indicative of an ABCD spin system. This revealed the presence of an *ortho*-disubstituted benzene ring. The other singlet signal at δ 8.31 (1H, s) taken together with the above mentioned signals suggested the presence of C3-substituted indole moiety. The signal at δ 9.88 (1H, s), which was not exchangeable suggested the presence of an aldehydic group. The singlet at δ 8.22 (1H, s) was assigned for N*H*. From the above data and through comparison with the literature (Aldrich, 1992, Hiort, 2002) it was confirmed that compound **19** was **indole-3-carboxyaldehyde**.

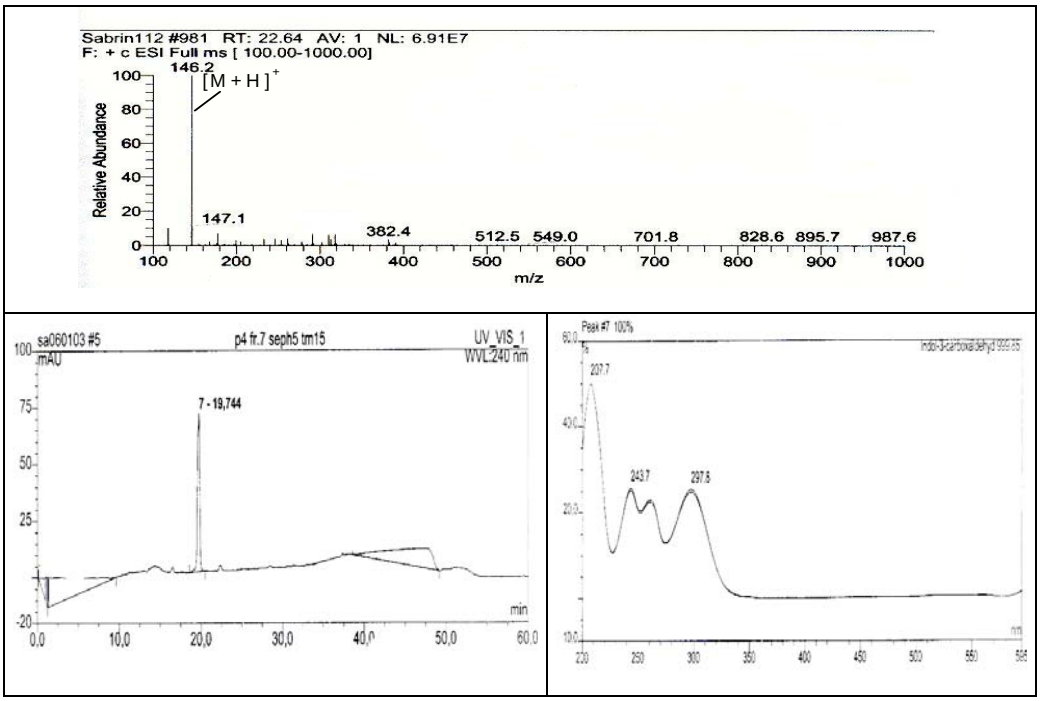


Fig. 3.86: (+) **ESIMS and UV spectra of compound 19**

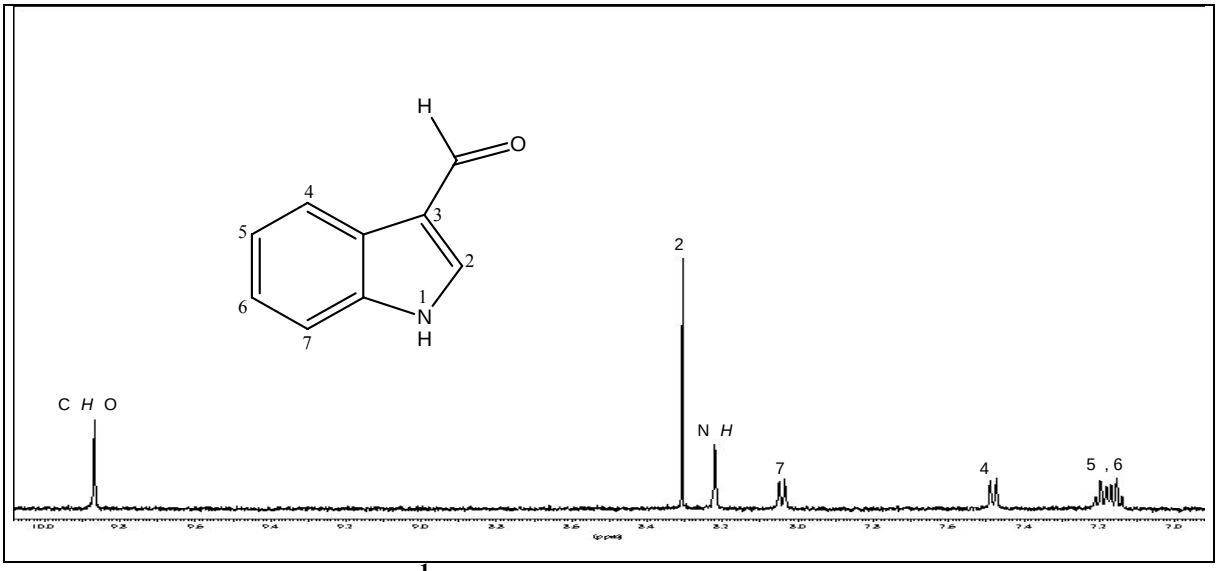
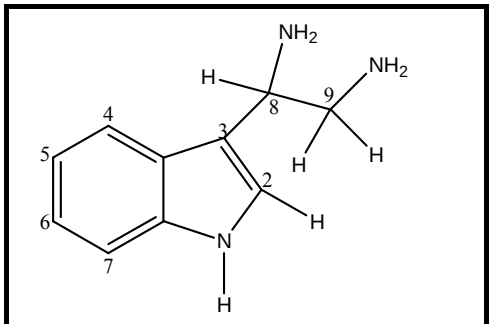


Fig. 3.87: ^{1}H NMR spectrum of compound 19

Table 3.23: ^{1}H NMR of compound 19 (DMSO-d_6, 500 MHz)

Proton	δ_H (m, *J* in Hz)
1	
2	8.31 (1H, s)
3	
3a	
4	8.05 (1H, d, *J* = 7.88 Hz)
5	7.21 (1H, dt, *J* = 7.2, 1.3 Hz)
6	7.16 (1H, dt, *J* = 7.2, 1.3 Hz)
7	7.48 (1H, d, *J* = 7.6 Hz)
7a	
C*H*O	9.88 (1H, s)
N*H*	8.22 (1H, s)

3.2.15. Diacarmine (20, new compound)



Diacarmine

Diacarmine (**20**) was isolated as a white amorphous powder. The $[\alpha]_D$ value was + 37.2° (*c* 0.08, H_2O). It had UV absorbances at λ_{max} 208, 241 and 299 nm which are similar to those of **19**. The ESIMS spectrum showed the pseudomolecular ion peak at *m*/*z* 176 $[M+H]^+$. The fragment ion peak at *m*/*z* 116 $[M\text{-}C_2H_7N_2]^+$ suggested the presence of the indole moiety as in **19**. The ^1H NMR spectrum of compound **20** (Fig. 3.90) was comparable to this of compound **19** (Fig. 3.87). The ^1H NMR signals at δ 8.17 (1H, dd, *J* = 6.5, 1.2 Hz, H-4), 7.47 (1H, dd, *J* = 6.6, 1.9 Hz, H-7), 7.23 (1H, dt, *J* = 6.9, 1.2 Hz, H-6), 7.20 (1H, dt, *J* = 6.9, 1.2 Hz, H-5), and the singlet signal at δ 8.34 (1H, s, H-2) indicated the presence of a C3-substituted indole derivative, which coincided with the fragment peak at *m*/*z* 116 [Riemer, *et al*., 1997, Lysek, *et al*., 2003]. The signal at δ 12.00 (1H, s, N*H*) represented the N*H* group of the indole moiety. The new signals of one methine at δ 4.87 (1H, t, *J* = 5.7 Hz, H-8) and one methylene at δ 4.56 (2H, d, *J* = 6.3 Hz, H_2-9) were observed. The substructure C*H*(NH_2)-CH_2(NH_2) was not found in any of the available databases. Through the MS fragmentation, which showed significant fragment ions at *m*/*z* 146 $[M\text{-}CH_4N]^+$ and 130 $[M\text{-}CH_6N_2]^+$ the compound was concluded as **20** (Fig. 3.89). From its COSY spectrum the new spin system replacing the aldehyde group in **19** could be unambiguously assigned. The NOE spectrum showed the effect of irradiation at two different positions. Irradiation of H-2 showed enhancement on H_2-9 and irradiation of H-8 showed enhancement of H-2 and H_2-9. The stereochemistry of compound **20** was assumed to be identical to that of *R*-tryptophan due to the similarity in the observed $[\alpha]_D$ [+ 33°, H_2O] for tryptophan and [+ 37.2° (*c* 0.08, H_2O)] for compound **20**. According to the results mentioned

above, compound **20** was concluded to be the new natural product 1-(1*H*-Indol-3-yl)-ethane-1,2-diamine and was named **diacarmine**.

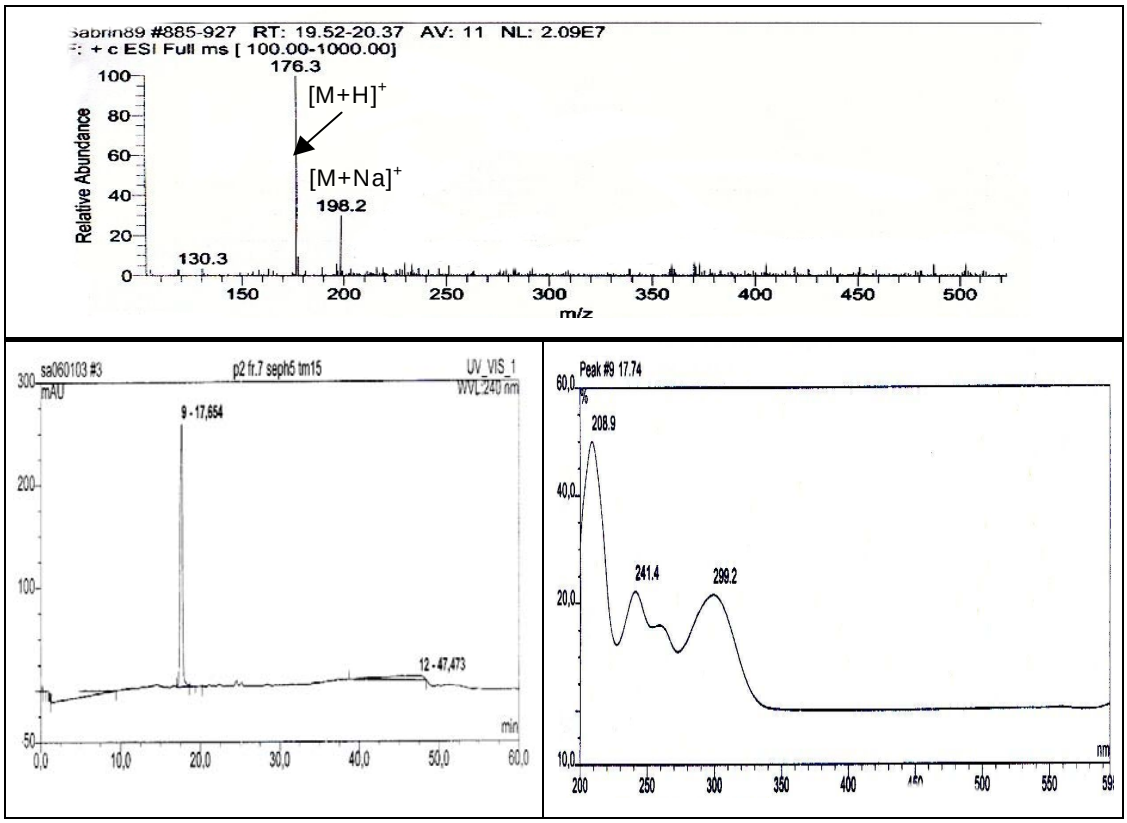


Fig. 3.88: ESIMS and UV spectra of compound 20

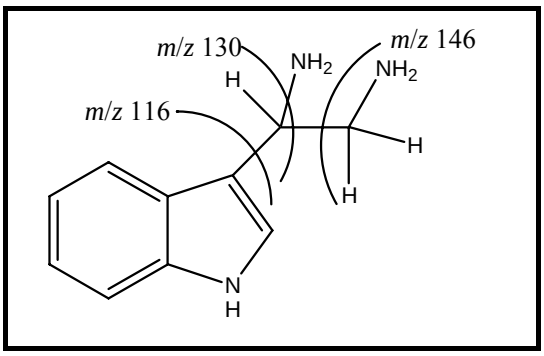


Fig. 3.89: Fragmentation pattern of diacarmine

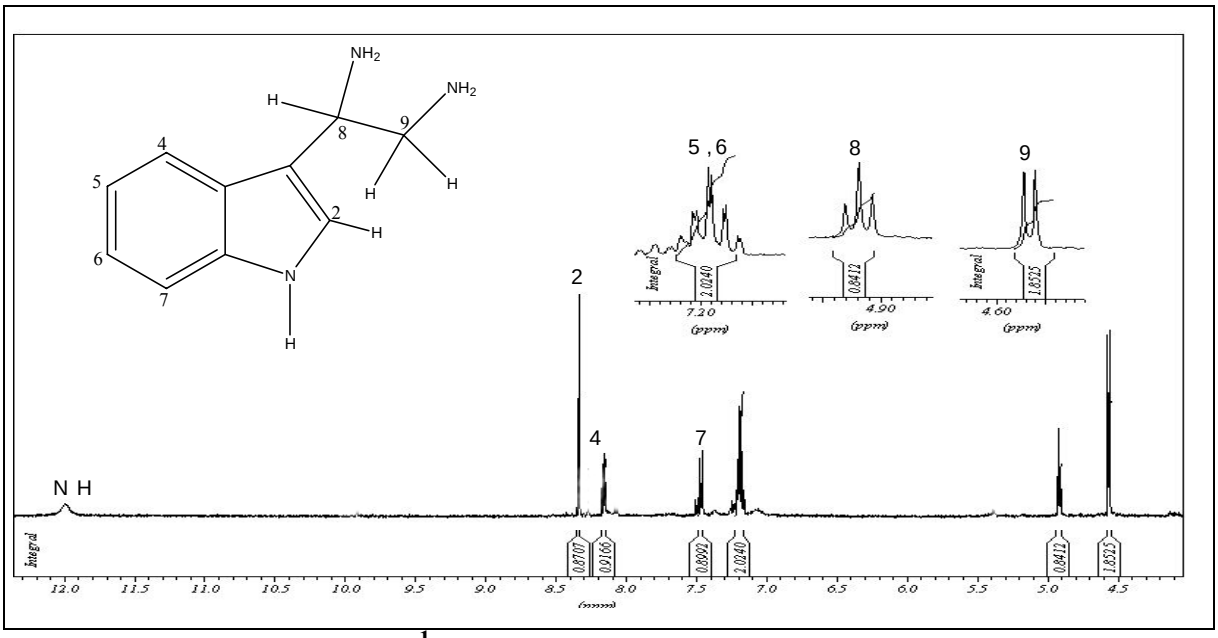


Fig. 3.90: ^{1}H NMR spectrum of compound 20

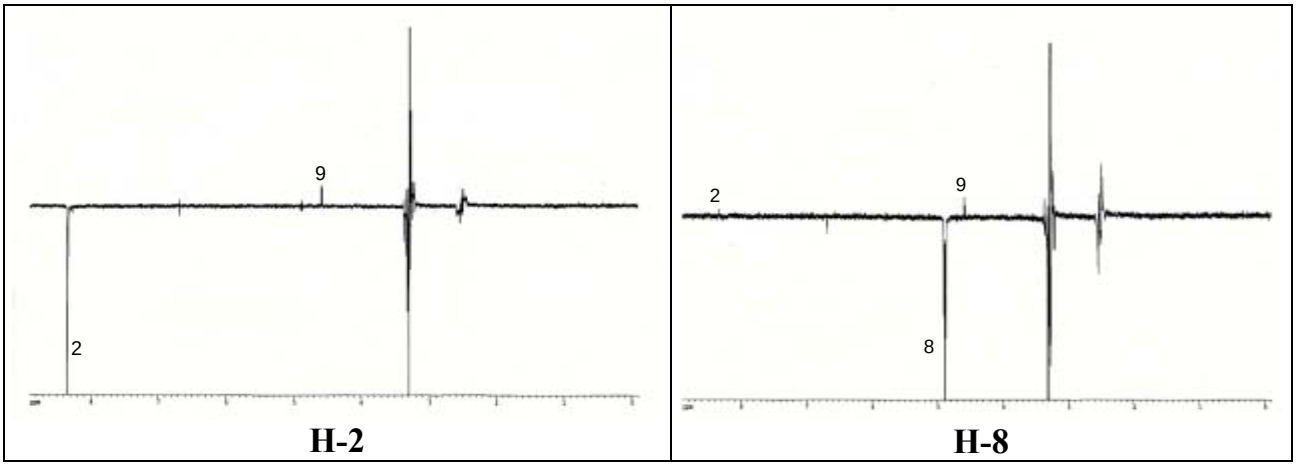
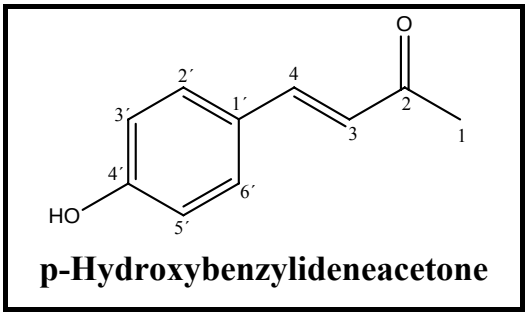


Fig. 3.91: NOE spectrum of compound 20

Table 3.24: NMR data of compound 20 (DMSO-d_6, 500 MHz)

Position	δ_H (m, *J* in Hz)	COSY	NOE	
			Proton irradiated	Proton enhanced
1	12.00 (1H, brs, N*H*)			
2	8.34 (1H, s)		2	9
4	8.17 (1H, dd, *J* = 6.5, 1.2 Hz)	5		
5	7.20 (1H, dt, *J* = 6.9, 1.2 Hz)	4		
6	7.23 (1H, dt, *J* = 6.9, 1.2 Hz)	7		
7	7.47 (1H, dd, *J* = 6.6, 1.9 Hz)	6		
8	4.87 (1H, t, *J* = 5.7 Hz)	9	8	2, 9
9	4.56 (2H, d, *J* = 6.3 Hz)	8		

3.2.16. p-Hydroxybenzylideneacetone (21, known compound)



p-Hydroxybenzylideneacetone

Compound **21** was isolated as a yellow solid. It has UV absorbances at λ_{max} 234 and 322 nm. The ESIMS spectrum showed pseudomolecular ion peaks at *m*/*z* 163 $[M+H]^+$ (positive) and 161 $[M-H]^+$ (negative). The ^1H NMR spectrum showed the presence of a *para*-substituted phenyl ring containing two pairs of *ortho*-coupled proton at δ 7.53 (2H, d, *J* = 8.2 Hz, H-2′, H-6′) and 6.78 (2H, d, *J* = 8.2 Hz, H-3′, H-5′). This AA′BB′ spin system of the phenyl ring was confirmed by the COSY spectrum, through the correlation between H-2′/H-6′ and H-3′/H-5′. A singlet signal at δ 2.27 (3H, s, H_3-1) was assigned to a methyl ketone. An AB system of two *trans*-coupled vinylic protons was detected at δ 7.52 (1H, d, *J* = 16.4 Hz, H-3) and 6.58 (1H, d, *J* = 16.4 Hz, H-4). This spin system was assured by the COSY correlation of H-3 to H-4, in addition to the large coupling constant 16.4 Hz. Comparison of the NMR data of compound **21** with those of **p-hydroxybenzylideneacetone** isolated from the bird′s nest fungus *Nidula niveo-tomentosa* [Ayer, *et al.*, 1980] revealed that the two compounds were identical.

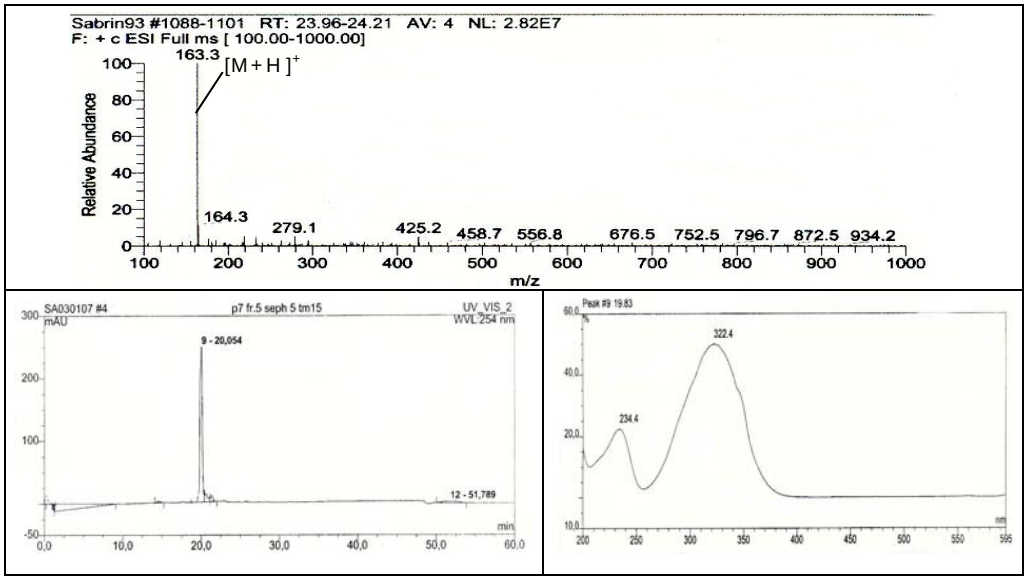


Fig. 3.92: (+) ESIMS and UV spectra of compound 21

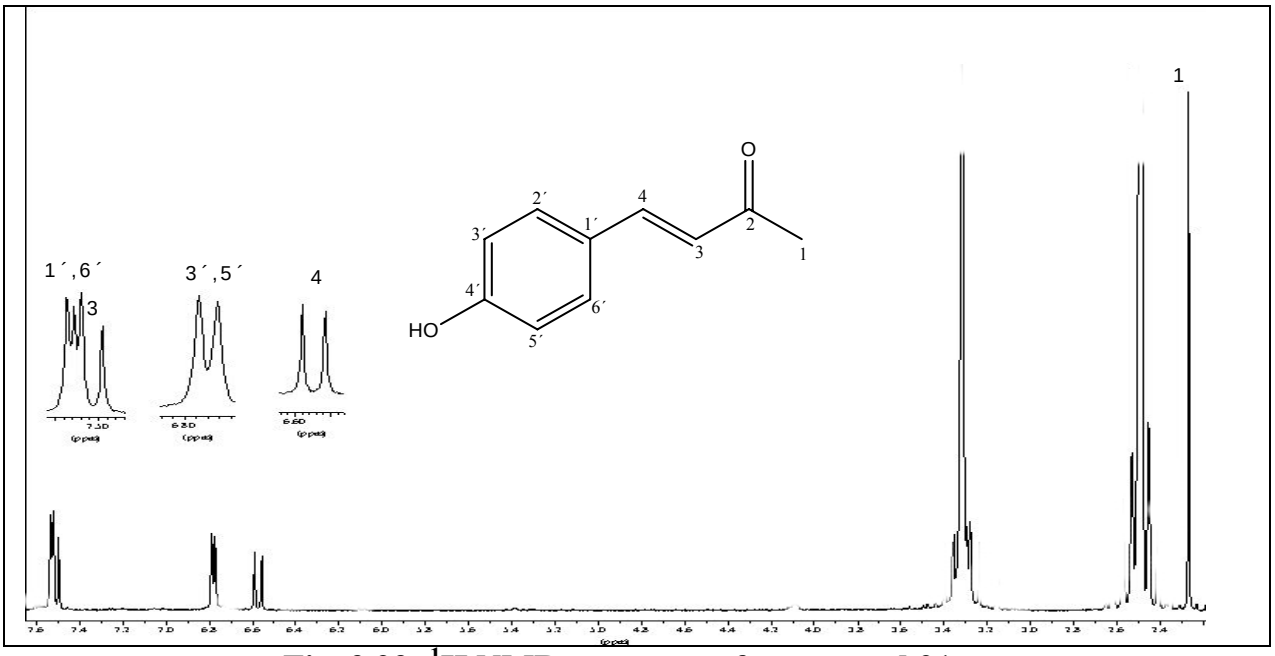
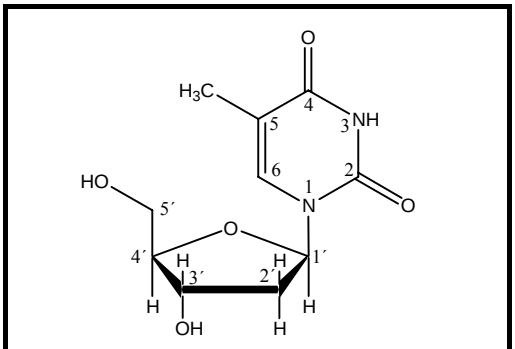


Fig. 3.93: ^{1}H NMR spectrum of compound 21

Table 3.25: NMR data of compound 21 (DMSO-d_6, 500 MHz)

Proton	δ_H (m, *J* in Hz)
1	2.27 (3H, s)
3	7. 52 (d, *J* = 16.4 Hz, 1H)
4	6.58 (d, *J* = 16.4 Hz, 1H)
2´, 6´	7.53 (d, *J* = 8.2 Hz, 2H)
3´, 5´	6.78 (d, *J* = 8.2 Hz, 2H)

3.2.17. Thymine 2´-desoxyriboside (22, known compound)



Thymine 2´-desoxyriboside

Compound **22** was isolated as a white amorphous powder. It has UV absorbances at λ_{max} 208 and 267 nm. In the (+) ESIMS, this compound exhibited the molecular ion peak at *m*/*z* 243 $[M+H]^+$. The fragment ion at *m*/*z* 127 [M+H-(2´-deoxyribose)] indicated the presence of 5-methyl-1*H*-pyrimidine-2, 4-dione. The ^{1}H NMR spectrum

showed only one proton in the aromatic region at δ 7.68 (1H, s) representing the free proton in the pyrimidine ring. The triplet signal at δ 6.15 (1H, t, *J* = 6.3 Hz) was assigned to the anomeric proton of sugar. The existence of two pairs of diastereomeric protons at position 2`and 5` indicated the presence of deoxyribose. Other protons of the deoxyribose are between δ 3.50-4.23, along with H-2` at δ 2.04. According to these data and through comparison with the literature [Aldrich, 1992, Pretsch, *et al*., 2000], compound **22** was concluded to be **thymine 2´-desoxyriboside**.

Table 3.26: ^{1}H NMR data of compound 22 (DMSO-d_6, 500 MHz)

Proton	δ_H (m, *J* in Hz)
1	
2	
3	
4	
5	
6	7.68 (1H, s)
7	1.74 (3H, s)
1´	6.15 (1H, t, *J* = 6.3 Hz)
2´	2.04 (2H, m)
3´	4.23 (1H, dt, *J* = 6.6, 3.5 Hz)
4´	3.75 (1H, dd, *J* = 6.6, 3.5 Hz)
5´	3.55 (1H, ddd, *J* = 14.0, 4.0 Hz)

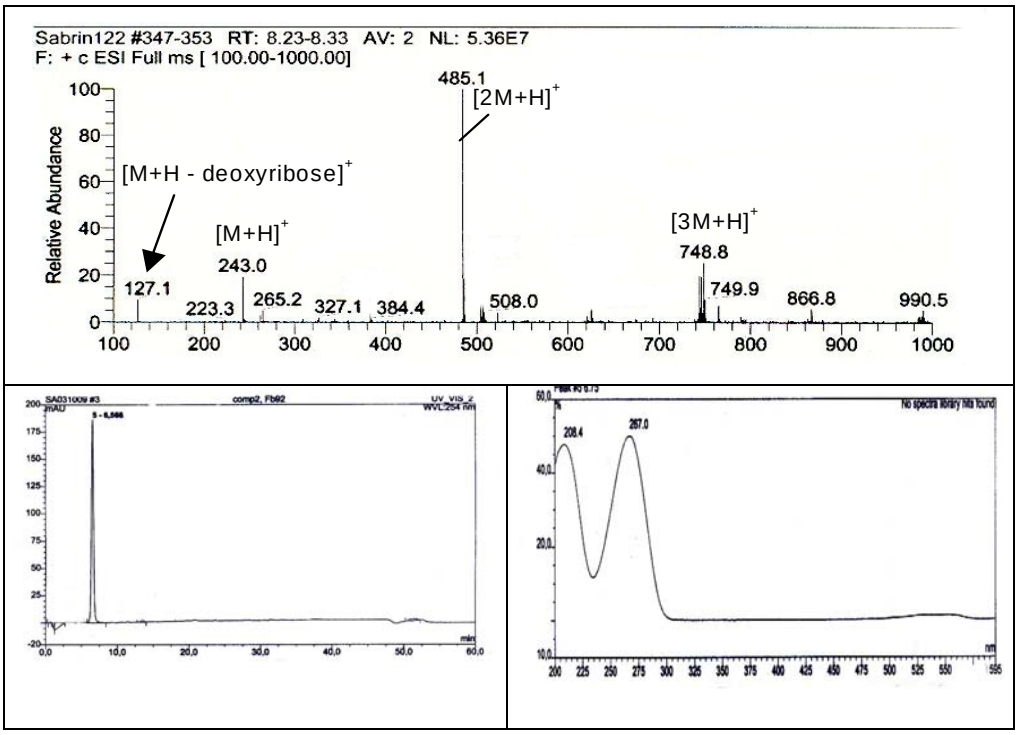


Fig. 3.94: (+) ESIMS and UV spectra of compound 22

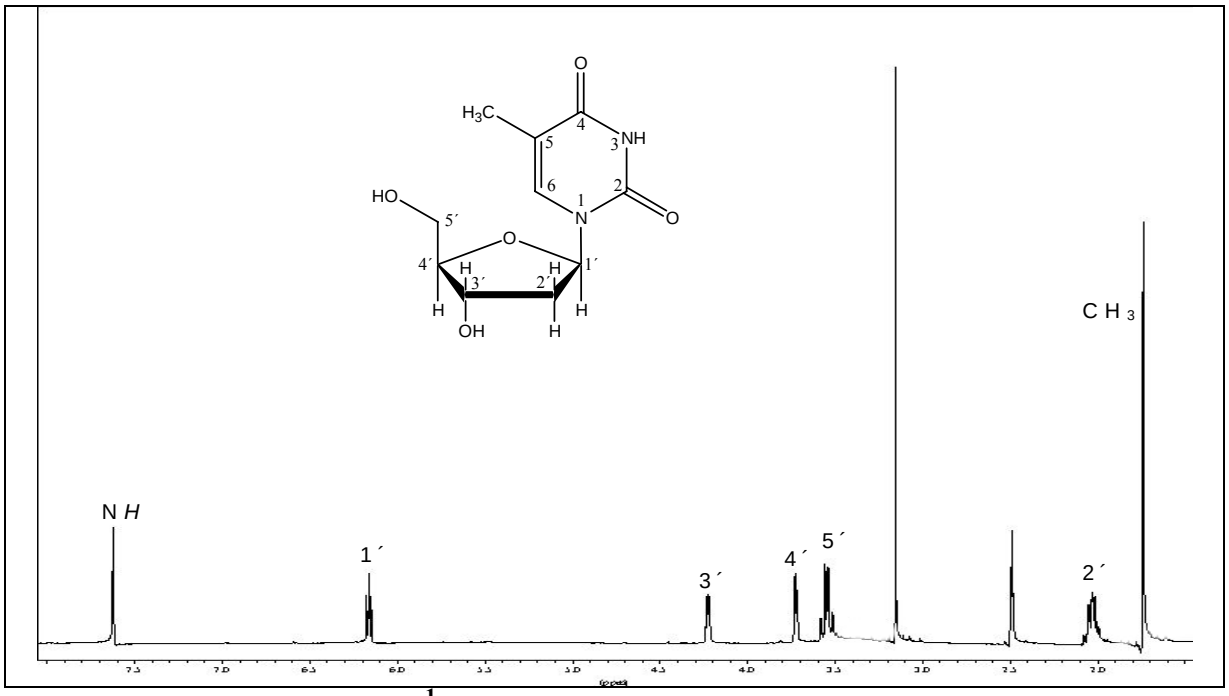
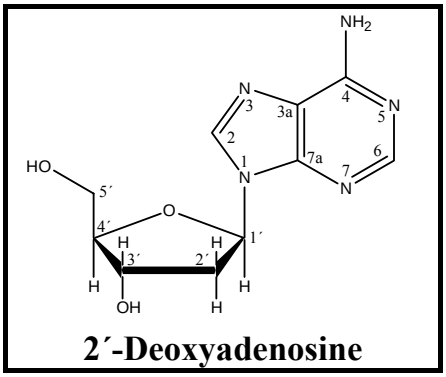


Fig. 3.95: ^{1}H NMR spectrum of compound 22

3.2.18. 2´-Deoxyadenosine (23, known compound)



Compound **23** was isolated as a white amorphous powder. It has UV absorbances at λ_{max} 216 and 256 nm. The (+) ESIMS spectrum showed the pseudomolecular ion peak at *m/z* 252 $[M+H]^+$. The fragment ion at *m/z* 136 $[M + H\text{-}(2´\text{-deoxyribose})]^+$ suggested the presence of a 9*H*-purin-6-ylamine moiety. The existence of a sugar moiety in this compound was evident by a number of protons resonating between δ 2.25 and 6.32. The triplet proton signal at δ 6.32 represented the anomeric proton (H-1´). Two singlet protons encountered in the downfield region of the ^{1}H NMR spectrum were assigned to positions 2 and 6 of the purine moiety. Two splitted AB methylene proton signals for positions 2´ and 5´ were observed at δ 2.74 (H-2´A), 2.25 (H-2´B) and δ 3.65 (H-5´A), 3.52 (H-5´B) because of the effect of the chiral centers at H-1´, H-3´ and H-4´. A

singlet integrated for two protons at δ 7.31 was assigned to NH_2 group. The ^{1}H NMR data of compound **23** matched those of **2′-deoxy adenosine** [Evidente, *et al*., 1989, Pretsch, *et al*., 2000].

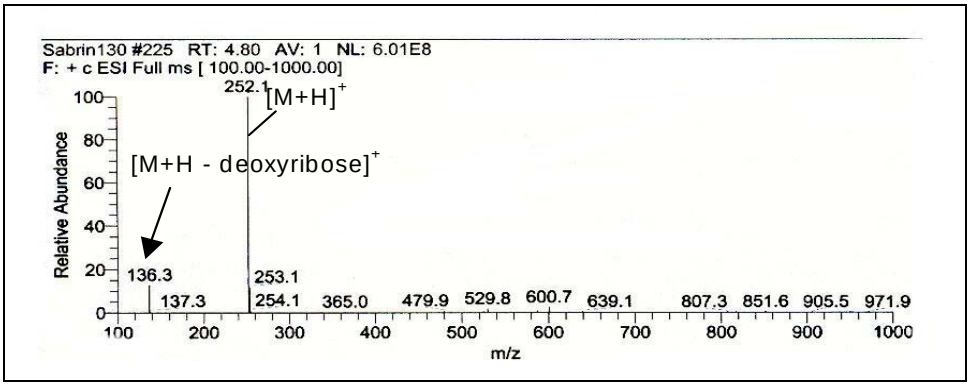


Fig. 3.96: (+) ESIMS spectrum of compound 23

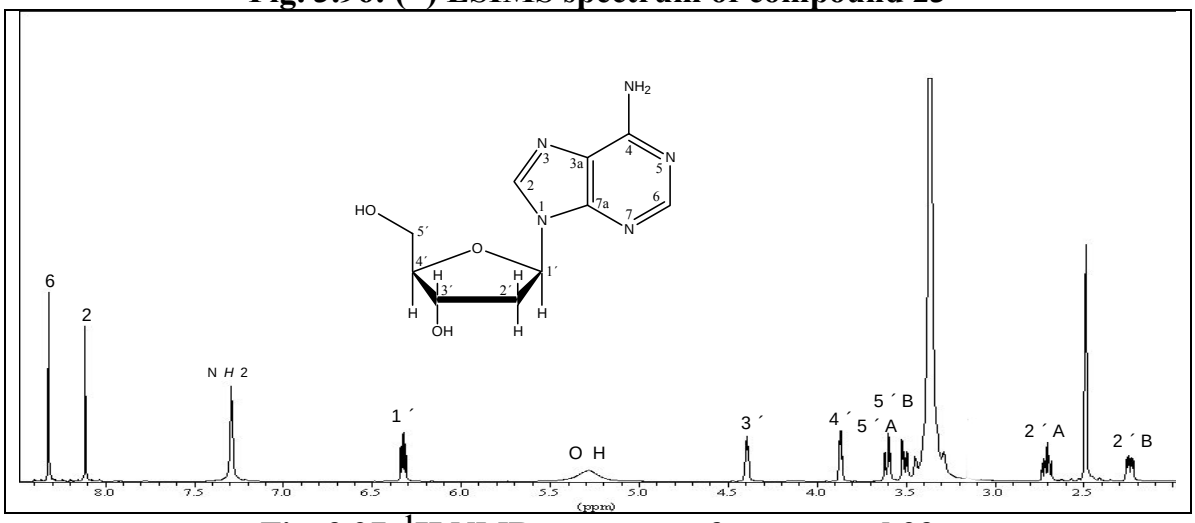
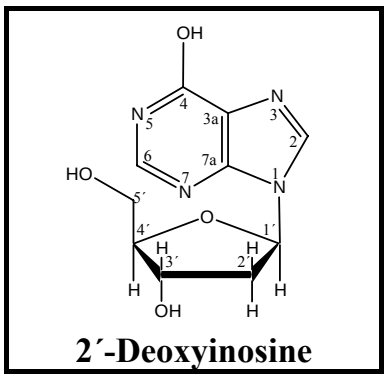


Fig. 3.97: ^{1}H NMR spectrum of compound 23

Table 3.27: NMR data of compound 23 (DMSO-d_6, 500 MHz)

Proton	δ_H (m, *J* in Hz)
1	
2	8.12 (1H, s)
3	
4	
5	
6	8.31 (1H, s)
1′	6.32 (1H, dd, *J* = 7.6, 6.3 Hz)
2′A 2′B	2.74 (1H, ddd, *J* = 13.3, 7.6, 5.6 Hz) 2.25 (1H, ddd, *J* = 13.3, 7.6, 5.7 Hz)
3′	4.42 (1H, t, *J* = 3.6 Hz)
4′	3.85 (1H, dd, *J* = 6.9, 4.4 Hz)
5′A 5′B	3.65 (1H, dd, *J* = 12.0, 3.8 Hz) 3.52 (1H, dd, *J* = 12.0, 4.4 Hz)
NH_2	7.31 (2H, s)

3.2.19. 2´-Deoxyinosine (24, known compound)



Compound **24** was isolated as a white amorphous powder. It has UV absorbances at λ_{max} 205 and 248 nm. The (+) ESIMS spectrum exhibited the molecular ion peak at *m*/*z* 253 $[M+H]^+$. Compound **24** had a 1 mass unit difference from 2´-deoxyadenosine (**23**). The fragment ion at *m*/*z* 137 $[M + H – (2´\text{-deoxyribose})]^+$ suggested the presence of a 9*H*-purin-6-ol moiety in **24** instead of 9*H*-purin-6-ylamine moiety in **23**. The ^{1}H NMR data (Table 3.29) were comparable to those of **23**. The examination of the signal systems of the sugar moiety indicated the presence of deoxyribose linked at position 1 of the purine ring. Two downfield singlets at δ 8.05 and 8.27 were observed representing protons 2 and 6 of the purine ring, respectively. The triplet signal at δ 6.32 belonged to the anomeric proton of the sugar. Six other signals from 2.28 to 4.38 ppm were assigned to H-2´A, H-2´B, H-3´, H-4´, H-5´A and H-5´B. Comparison of the ^{1}H NMR data with those reported for **2´-deoxyinosine** [Atta-Ur-Rahman, *et al*., 1999, Pretsch, *et al*., 2000] proved both compounds to be identical.

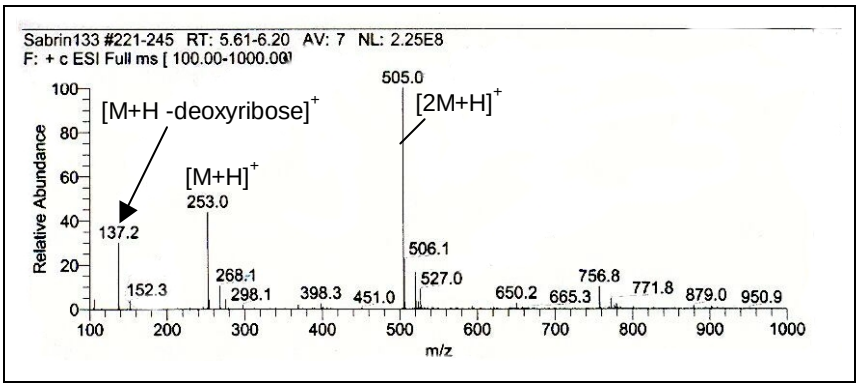


Fig. 3.98: (+) ESIMS spectrum of compound 24

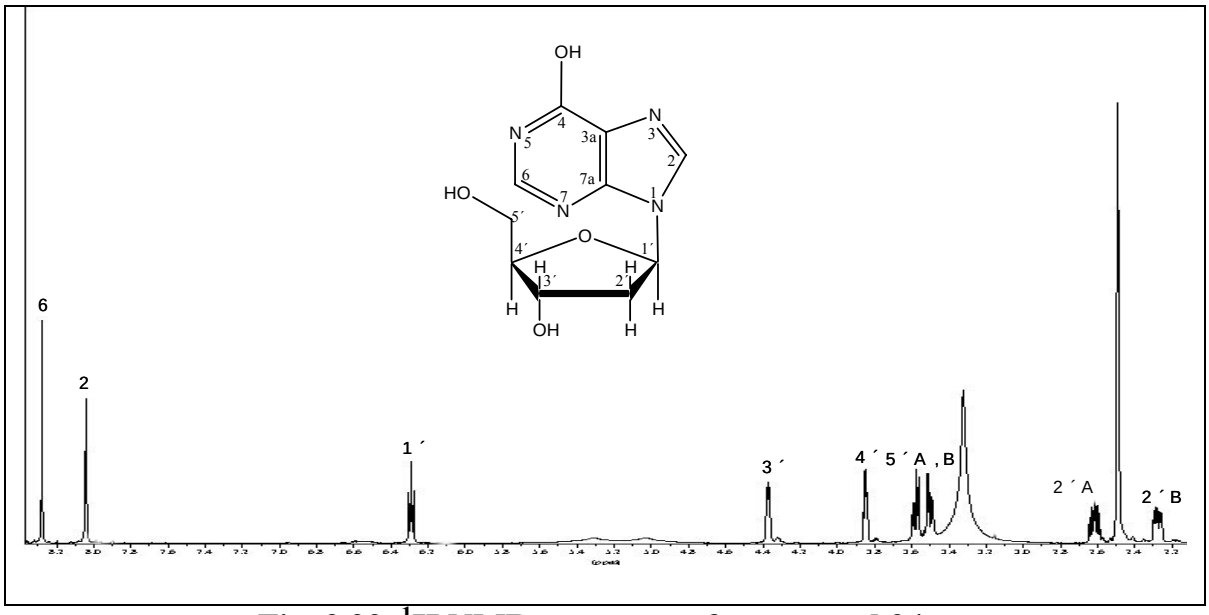
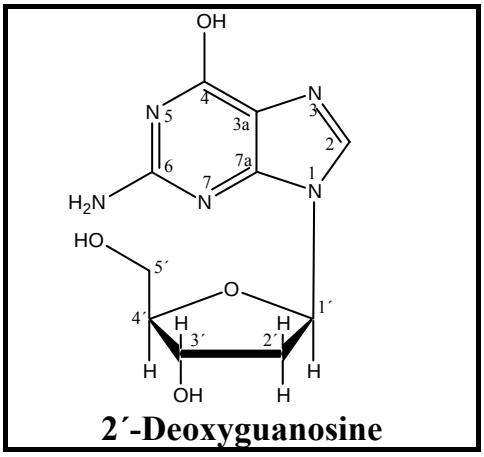


Fig. 3.99: ^{1}H NMR spectrum of compound 24

Table 3.28: NMR data of compound 24 (DMSO-d_6, 500 MHz)

Proton	δ_H (m, *J* in Hz)
1	
2	8.05 (1H, s)
3	
3a	
4	
5	
6	8.27 (1H, s)
1´	6.32 (1H, t, *J* = 6.9 Hz)
2´A	2.62 (1H, dd, *J* = 13.2, 6.3 Hz)
2´B	2.28 (1H, ddd, *J* = 13.2, 6.3 Hz)
3´	4.38 (1H, p, *J* = 7.5 Hz)
4´	3.85 (1H, q, *J* = 7.5 Hz)
5´A	3.85 (1H, dd, *J* = 11.3, 4.4 Hz)
5´B	3.51 (1H, dd, *J* = 11.3, 4.4 Hz)

3.2.20. 2´-Deoxyguanosine (25, known compound)



Compound **25** was isolated as a white amorphous powder. It has UV absorbances at λ_{max} 202 and 254 nm. In the (+) ESIMS, this compound exhibited the molecular ion peak at *m/z* 268 $[M+H]^+$ suggesting a molecular weight of 267 g/mol. The fragment ion at *m*/*z* 152 [M + H- (2´-deoxyribose)]$^+$ suggested the presence of a 2-amino-9*H*-purin-6-ol moiety. The ^{1}H NMR spectrum exhibited a downfield singlet at δ 7.91 representing the heterocyclic proton of the purine ring at position 2. The singlet at δ 6.85 equivalent to two protons was assigned to the NH_2 group at position 6. The existence of a deoxyribose moiety was evident by a number of protons resonating between δ 2.18 and 6.11. The triplet proton signal at δ 6.11 represented the anomeric proton (H-1´). Compound **25** was identical with the known compound **2´-deoxyguanosine** [Aldrich 1992, Pretsch, *et al*., 2000].

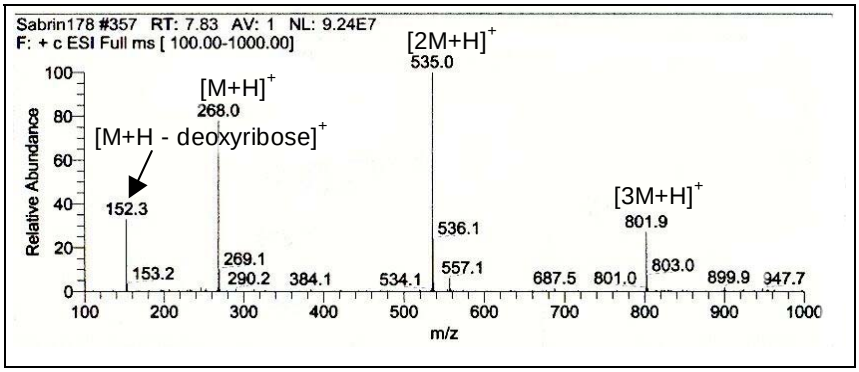


Fig. 3.100: (+) ESIMS spectrum of compound 25

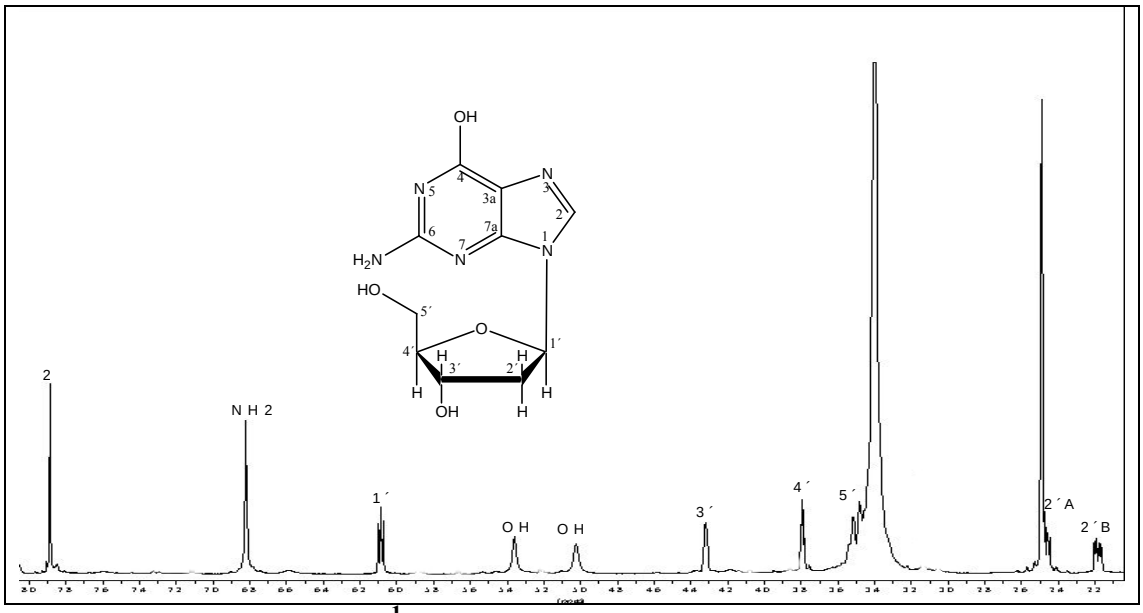


Fig. 3.101: ^{1}H NMR spectrum of compound 25

Table 3.29: NMR data of compound 25 (DMSO-d_6, 500 MHz)

Proton	δ_H (m, *J* in Hz)
1	
2	7.91 (1H, s)
3	
3a	
4	
5	
6	
1´	6.11 (1H, dd, *J* = 8.0, 6.3 Hz)
2´A 2´B	2.18 (1H, ddd, *J* = 8.8, 6.0, 3.0 Hz) 2.46 (m)
3´	4.35 (1H, brs)
4´	3.78 (1H, m)
5´	3.52 (1H, m)
NH_2	6.85 (2H, s)

Bioactivity

The terpene cyclic peroxides isolated from the genus *Diacarnus* were known to exhibit significant cytotoxicity. In our search for new biologically active natural products, all the isolated norterpene peroxides were evaluated for their cytotoxic activity. In the brine shrimp assay, nuapapuin A methyl ester (**6**) is the most active one and diacarperoxide I (**18**) is less active probably due to the lack of the cyclic peroxide moiety, see Table 3.30. It also was interesting to note that all of the compounds showed strong or moderate activity against different cancer cell lines like Hela human cervix carcinoma, L5178Y mouse lymphoma or PC12 brain tumour cells of rats. The tests were determined using the microculture tetrazolium (MTT) assay. The results showed inhibition of cell growth at concentration of 3 and 10 μg/mL and the data were compared to those of untreated controls [Carmichael, *et al*., 1987]. The results are listed in Tables 3.31, 3.32. Nucleosides were also tested for their cytotoxic activities, it was found that thymidine 2′-deoxyriboside (**22**) showed strong activity against L5178Y, while 2′-deoxy adenosine (**23**) and 2′-deoxyinosine (**24**) were less active Table 3.33.

Table 3.30: Brine shrimp bioassay results of peroxides (μg/mL)

Compound	Mortality %	
	20 μg/mL	50 μg/mL
Nuapapuin A methyl ester (6)	100	100
Methyl-2-epinuapapuinoate (7)	25	65
Diacarperoxide A (8)	35	55
Diacarperoxide B (10)	45	55
Diacarperoxide C (11)	35	55
Diacarperoxide G (15)	50	60
Diacarperoxide H (16)	25	50
Epimuqubilin B (17)	45	60

Table 3.31: **Cytotoxicity results of the isolated cyclic peroxides**

Compound	% Growth inhibition					
	L5178Y		Hela cell		PC12 cell	
	3 µg/mL	10 µg/mL	3 µg/mL	10 µg/mL	3 µg/mL	10 µg/mL
Nuapapuin A methyl ester (6)	100	100	21	95	33	76
Methyl-2-epinuapapuinoate (7)	n.d.	64.7	0	0	0	0
Diacarperoxide A (8)	n.d.	26.1	0	0	0	0
Methyl diacarnoate A (9)	96	98	0	0	0	0
Diacarperoxide B (10)	n.d.	51.9	0	0	0	0
Diacarperoxide C (11)	n.d.	36.6	0	0	0	0
Diacarperoxide D (12)	100	100	100	100	24	60
Diacarperoxide E (13)	99	100	n.d.	n.d.	n.d.	n.d.
Diacarperoxide F (14)	93	97	95	95	82	86
Diacarperoxide G (15)	70	100	0	0	18.1	29.6
Diacarperoxide H (16)	100	100	20.2	67.7	0	0
Epimuqubilin B (17)	100	100	70	73	36.4	65.8
Diacarperoxide I (18)	45	92	6	13	0	0

*n.d. not detected

Table 3.32: ED50 (µg/mL) of the cyclic peroxides

Compound	ED50 (µg/mL)		
	L5178Y	Hela cell	PC12 cell
Nuapapuin A methyl ester (6)	0.76	5.5	6.2
Methyl diacarnoate A (9)	< 3		
Diacarperoxide D (12)	< 0.1	0.17	8.1
Diacarperoxide E (13)	< 3		
Diacarperoxide F (14)	0.06	0.6	0.8
Diacarperoxide G (15)	2	> 10	> 10
Diacarperoxide H (16)	0.28	7.2	> 10
Epimuqubilin B (17)	< 0.1	1	6
Diacarperoxide I (18)	4.8	> 10	> 10

Table 3.33: **Cytotoxicity results of the isolated nucleosides against L5178Y**

Compound	% Growth inhibition		ED50 (µg/mL)
	3 µg/mL	10 µg/mL	
Thymidine 2´-deoxyriboside (22)	83	97	< 3
2´-Deoxyadenosine (23)	27	41	> 10
2´-Deoxyinosine (24)	8	31	> 10

3.3. Isolated compounds from the sponge *Acanthostrongylophora ingens*

Marine sponges are sources of interesting groups of nitrogenous metabolites with unique chemical features and pronounced chemical activities. Sponges belonging to the order Haplosclerida are a rich source of alkaloids such as ingenamines A-F [Kong, *et al*., 1995, Kong, *et al*., 1994], manzamines [Ichiba, *et al*., 1988, Kobayashi, *et al*., 1995] and madangamines [Kong, *et al*., 1998]. These compounds exhibit a diverse range of bioactivities including cytotoxicity, insecticidal, and antibacterial activity, as well as activity against malaria [Roa, *et al*., 2003]. As part of our search for biologically active metabolites from marine sponges, one new alkaloid acanthomine A (**27**) was isolated form the Indonesian sponge *Acanthostrongylophora ingens* in addition to the known compounds annomontine (**26**) [Leboeuf *et al*., 1982] and 1-hydroxy-3,4-dihydronorharman (**28**) [Roa, *et al*., 2003].

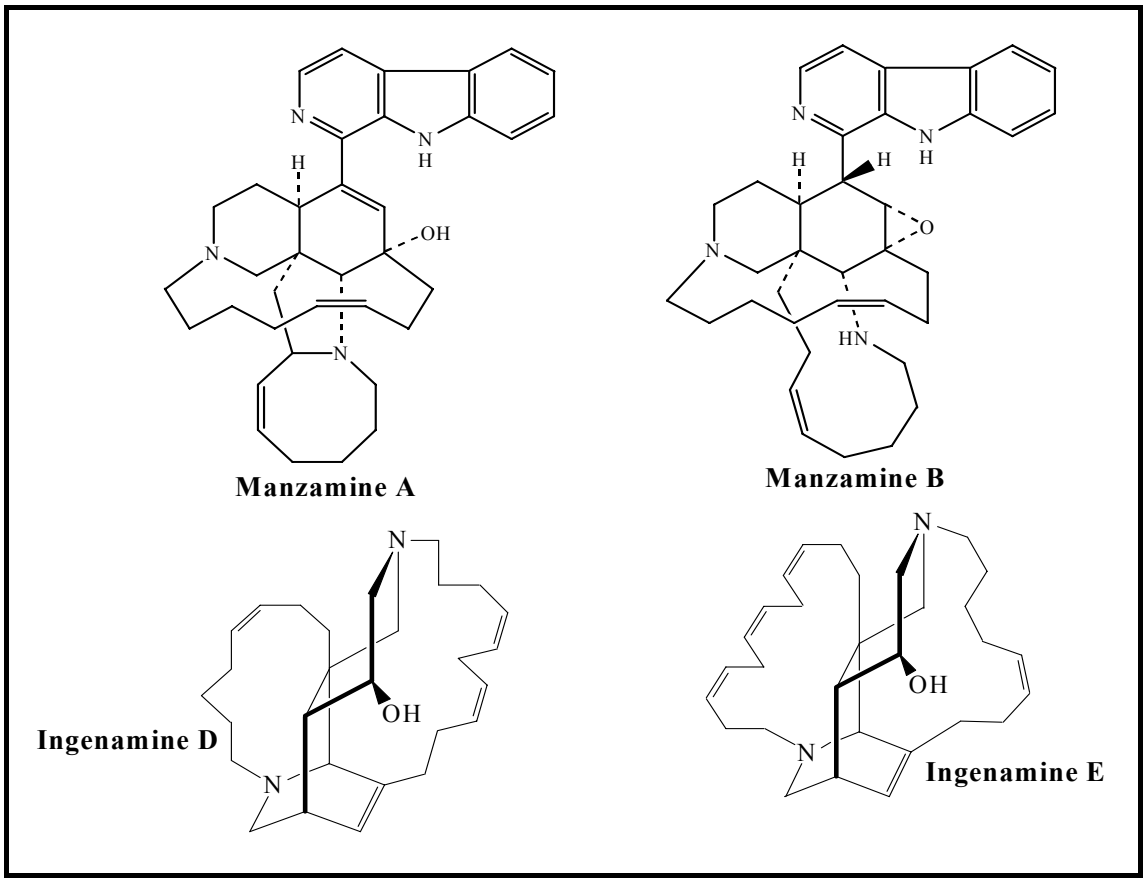
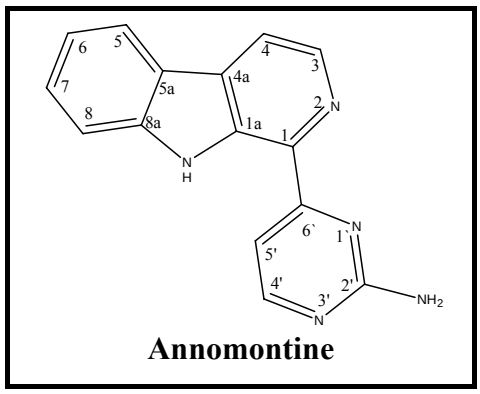


Fig. 3.102: Some alkaloids previously isolated from the order Haplosclerida

3.3.1. Annomontine (26, known compound)



Annomontine

Compound **26** was isolated as a yellow crystalline solid. It has UV absorbances at λ_{max} 229, 289 and 311 nm. Compound **26** had a molecular weight of 261 g/mol, as derived from the ESIMS measurement that showed the ion peak at *m*/*z* 262 $[M+H]^+$. The ^1H NMR spectrum disclosed that the molecule consisted of a disubstituted phenyl ring, containing four *ortho*-coupled protons at δ 8.31 (d, *J* = 7.9 Hz, H-5), 7.77 (d, *J* = 8.2 Hz, H-8), 7.64 (dt, *J* = 8.2, 0.9 Hz, H-7) and 7.29 (dt, *J* = 7.9, 0.9 Hz, H-6). This ABCD system was confirmed by the COSY spectrum. HMBC correlations from H-5 to C-7, H-6 to C-7 and C-8, H-7 to C-5 and H-8 to C-6 corroborated this spin system. The second spin system consisted of two downfield doublet proton signals at δ 8.51 (d, *J* = 5.0 Hz, H-3) and 8.29 (d, *J* = 5.0 Hz, H-4) with a small coupling constant, indicating that these two protons occur in a nitrogen-containing ring system as in pyridine. The downfield singlet at δ 11.54 was assigned to an indolic N*H* group. The characteristic HMBC correlations of N*H* to C-8a, C-5a, C-1a and C-4a, H-3 to C-4a, H-4 to C-1a and C-4a allowed the connection of the two previously mentioned spin systems to give the *β*-carboline moiety. Comparison of the ^1H and ^{13}C NMR data with literature data [Coune, *et al*., 1980, Leboeuf, *et al*., 1982, Corbally, *et al*., 2000] indicated the presence of a norharmane structure as found in compound **26**. Proton doublets at δ 8.44 and 7.68 with coupling constants of 5.3 Hz were positioned *ortho* to each other, as also indicated by their COSY correlations. This was also confirmed by their correlations in the HMBC spectrum. H-4′ showed also cross peaks to C-5′ and C-6′ and H-5′ to C-4′ which led to the elucidation of a 2-aminopyrimidine as the second substructure. The NMR data were comparable with the published data for 2-aminopyrimidine [Leboeuf, *et al*., 1982]. ^{13}C NMR together with the HMQC spectra

showed the presence of seven heteroatom-bound carbons observed at δ 164.4 (C-6′), 163.4 (C-2′), 159.0 (C-4′), 140.8 (C-8a), 137.9 (C-3), 136.2 (C-1) and 134.3 (C-1a). In addition, as determined from its DEPT spectrum five doublet carbons at δ 128.8 (C-7), 121.9 (C-5), 119.8 (C-6), 116.9 (C-4), 112.3 (C-8) and 105.6 (C-5′) and two singlet carbons at δ 130.2 (C-4a) and 120.2 (C-5a) were encountered. The attachment of the 2-aminopyrimidine moiety at C-1 was established by the HMBC correlations of H-5′ and H-4′ to C-1. Compound **26** was identified as a *β*-carboline alkaloid substituted at C-1 by a 2-aminopyrimidine unit which is known as **annomontine**. Annomontine was first isolated from the bark of *Annona montana* (family *Annonaceae*) [Leboeuf, *et al*., 1982]. This is the first report that a natural product, which originally occurred in a terrestrial plant, is also isolated from a marine invertebrate.

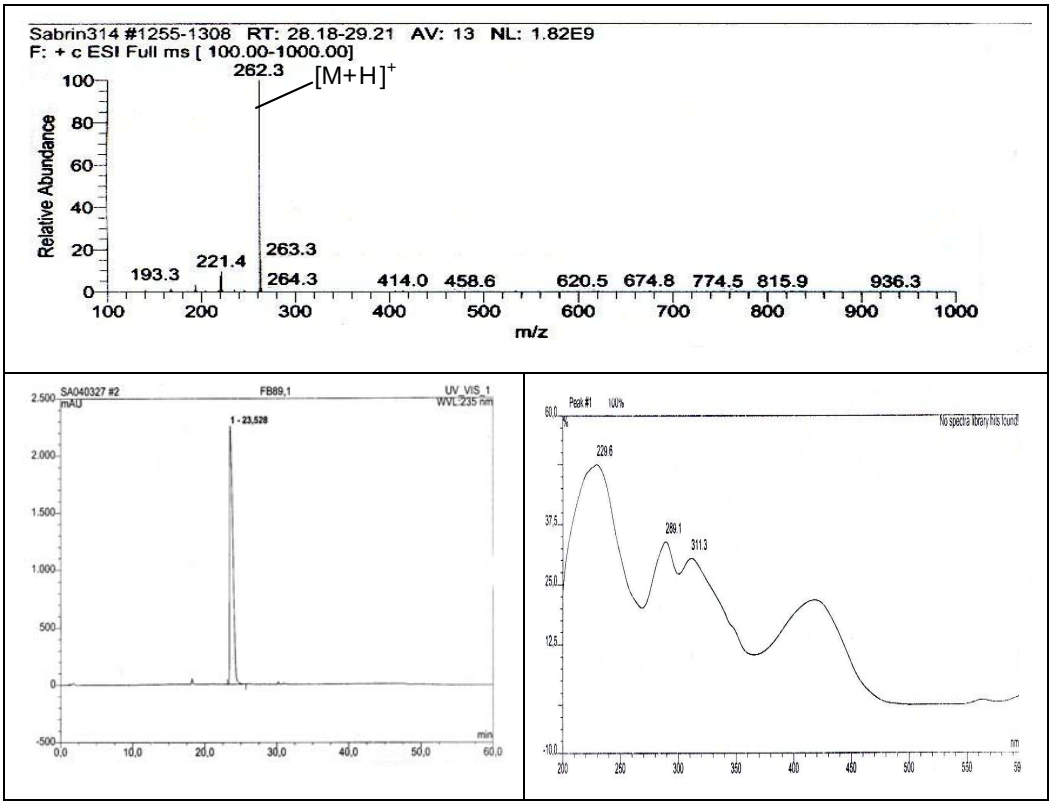


Fig. 3.103: (+) **ESIMS and UV spectra of compound 26**

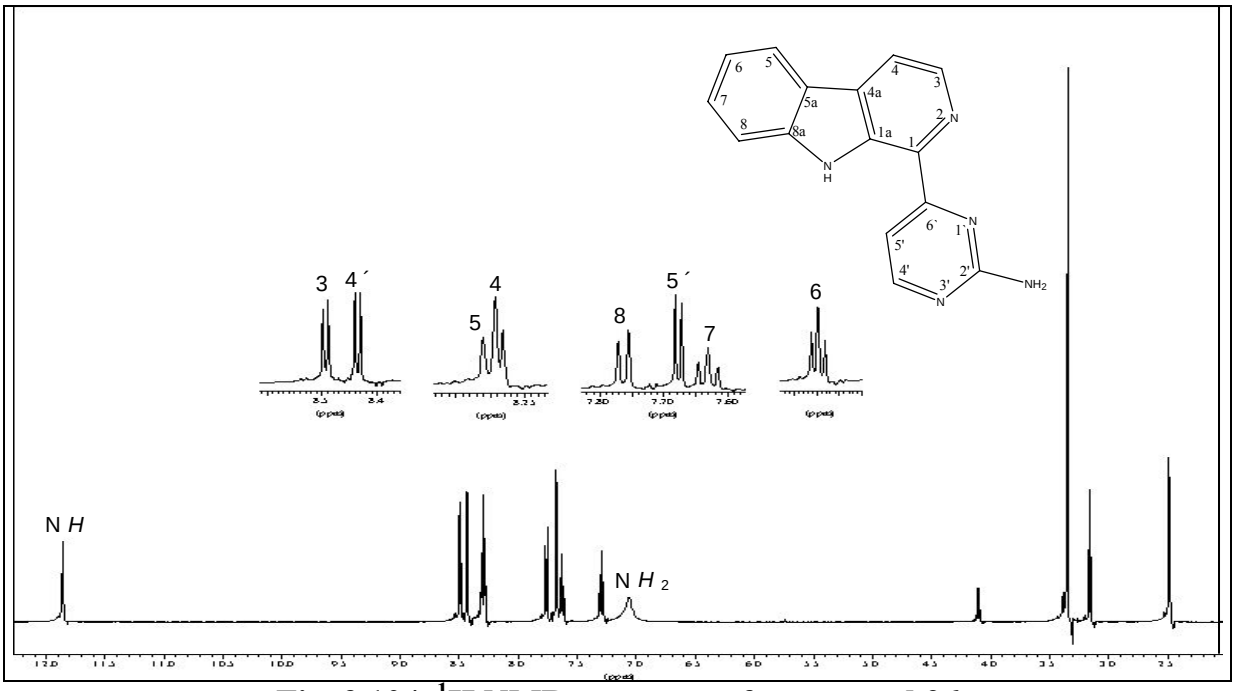


Fig. 3.104: ^{1}H NMR spectrum of compound 26

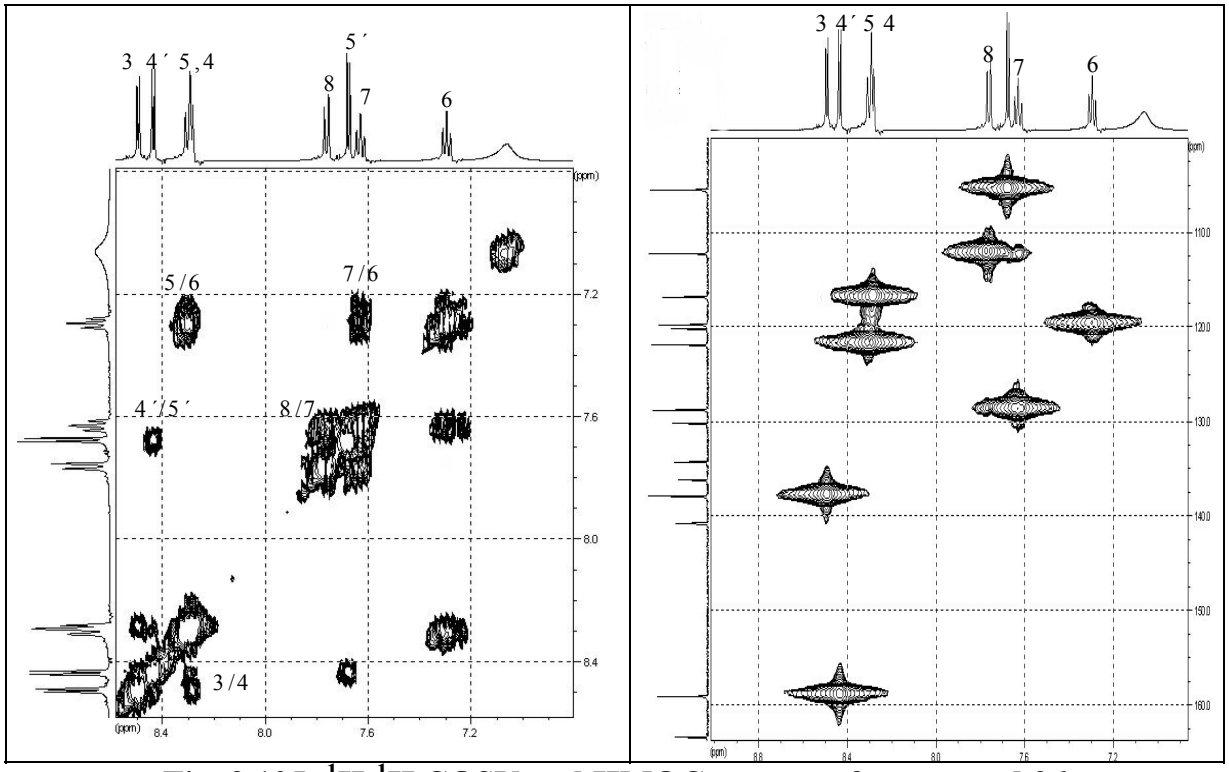


Fig. 3.105: ^{1}H-^{1}H COSY and HMQC spectra of compound 26

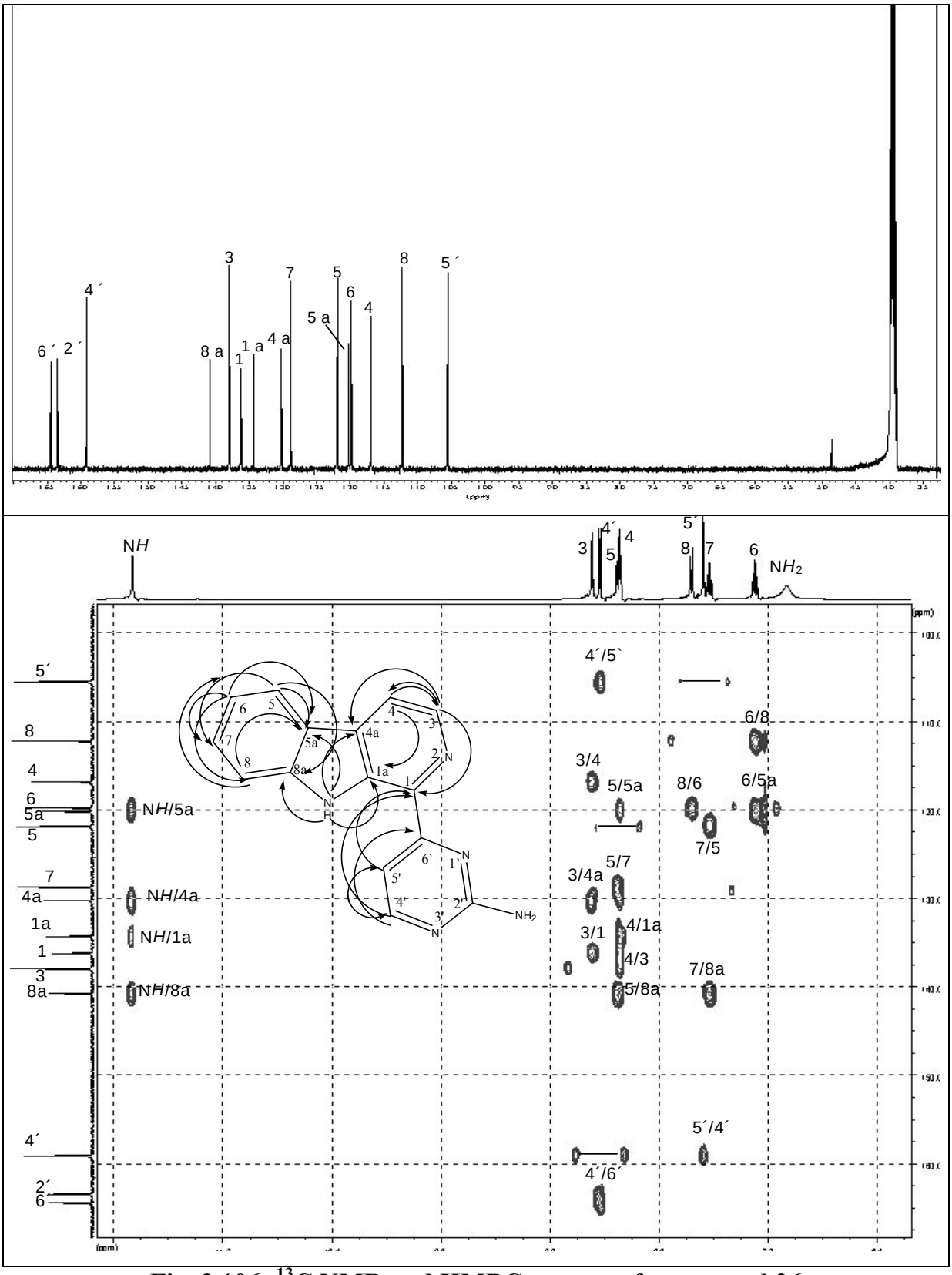
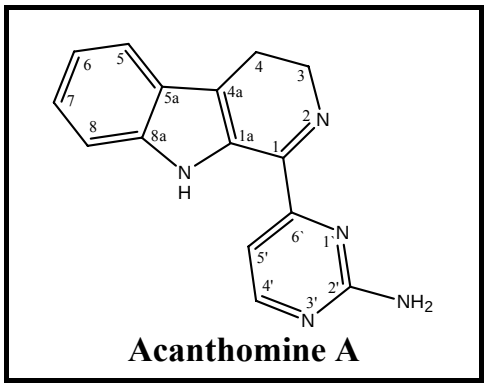


Fig. 3.106: ^{13}C NMR and HMBC spectra of compound 26

Table 3.34: NMR data of compounds 26 (DMSO-d_6, 500, 125 MHz)

Position	δ_H (m, *J* in Hz)	δ_C (m)	COSY	HMBC
N*H*	11.54 (s)			1a, 4a, 5a, 8a
1		136.2 s		
2				
3	8.51 (d, *J* = 5.0 Hz)	137.9 d	4	4, 4a, 1
4	8.29 (d, *J* = 5.0 Hz)	116.9 d	3	3, 1a
4a		130.2 s		
5a		120.2 s		
5	8.31 (d, *J* = 7.9 Hz)	121.9 d	6	5a, 7, 8a
6	7.29 (dt, *J* = 7.9, 0.9 Hz)	119.8 d	5, 7	5a, 7, 8
7	7.64 (dt, *J* = 8.2, 0.9 Hz)	128.8 d	6, 8	5, 8a
8	7.77 (d, *J* = 8.2 Hz)	112.3 d	7	6
8a		140.8 s		
1a		134.3 s		
1`				
2`		163.4 s		
3`				
4`	8.44 (d, *J* = 5.3 Hz)	159.0 d	5`	5`, 6`, 1
5`	7.68 (d, *J* = 5.3 Hz)	105.6 d	4`	4`, 1
6`		164.4 s		

3.3.2. Acanthomine A (27, new compound)



Acanthomine A

Compound **27** was obtained as a red crystalline solid. It has UV absorbances at λ_{max} 225 and 391 nm. The (+) ESIMS showed a pseudomolecular ion peak at *m*/*z* 264. Compound **27** had 2 mass unit more than **26**. The structure of compound **27** was derived on the basis of spectroscopic analysis. Assignments of ^{1}H and ^{13}C NMR spectra are depicted in Table 3.35. Comparison of the NMR data of compound **27** with those of compound **26** revealed that compound **27** is closely related to annomontine. Its ^{1}H NMR spectrum showed 2 methylene protons at δ 2.85 (H_2-4) and 4.05 (H_2-3), two *ortho*-coupled aromatic protons at δ 8.38 (H-4′) and 7.24 (H-5′), along with an ABCD system of four protons at δ 7.61 (H-5), 7.55 (H-8), 7.27 (H-7) and 7.07 (H-6).

An exchangeable proton for a N*H* group at δ 11.40 was also observed. The presence of an unsubstituted indole moiety was evident by the COSY correlations between H-5, H-6, H-7 and H-8, which was further confirmed by the HMBC correlations from H-5 to C-5a, C-8a, H-6 to C-8, C-5a, H-7 to C-8a and H-8 to C-6. The HMBC correlations of H_2-3 to C-4a, as well as, H_2-4 to C-4a, C-1a suggested that a dihydropyridine system was attached to the indole ring at C-4a and C-1a, respectively. A further COSY correlation of H-4′ to H-5′, in addition the HMBC correlations of H-4′ to C-2′, C-5′ and C-6′ proved valuable for assigning the presence of the 2-aminopyrimidine system. The ^{1}H and ^{13}C NMR data for the 2-aminopyrimdine part in compound **27** were in excellent agreement with the published data [Leboeuf, *et al*., 1982] and also to those of compound **26**. The ^{13}C NMR together with the HMQC spectra showed the presence two methylene carbons at δ 48.5 (C-3), 18.5 (C-4), six methine carbons at δ 159.2 (C-4′), 124.1 (C-7), 119.6 (C-5), 119.4 (C-6), 112.6 (C-8) and 105.4 (C-5′) and seven quaternary carbons at δ 163.3 (C-6′), 162.7 (C-2′), 154.7 (C-1), 136.5 (C-8a), 127.0 (C-1a), 124.2 (C-5a) and 116.5 (C-4a). Compound 27 was considered as a new natural product and named **acanthomine A**. It is 3, 4-dihydroannomentine.

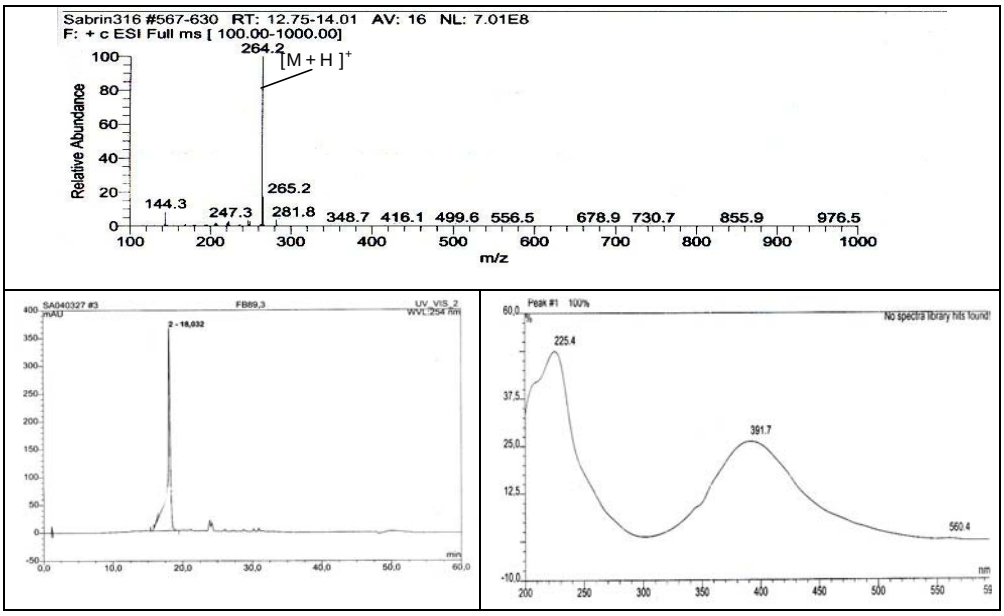


Fig. 3.107: (+) **ESIMS and UV spectra of compound 27**

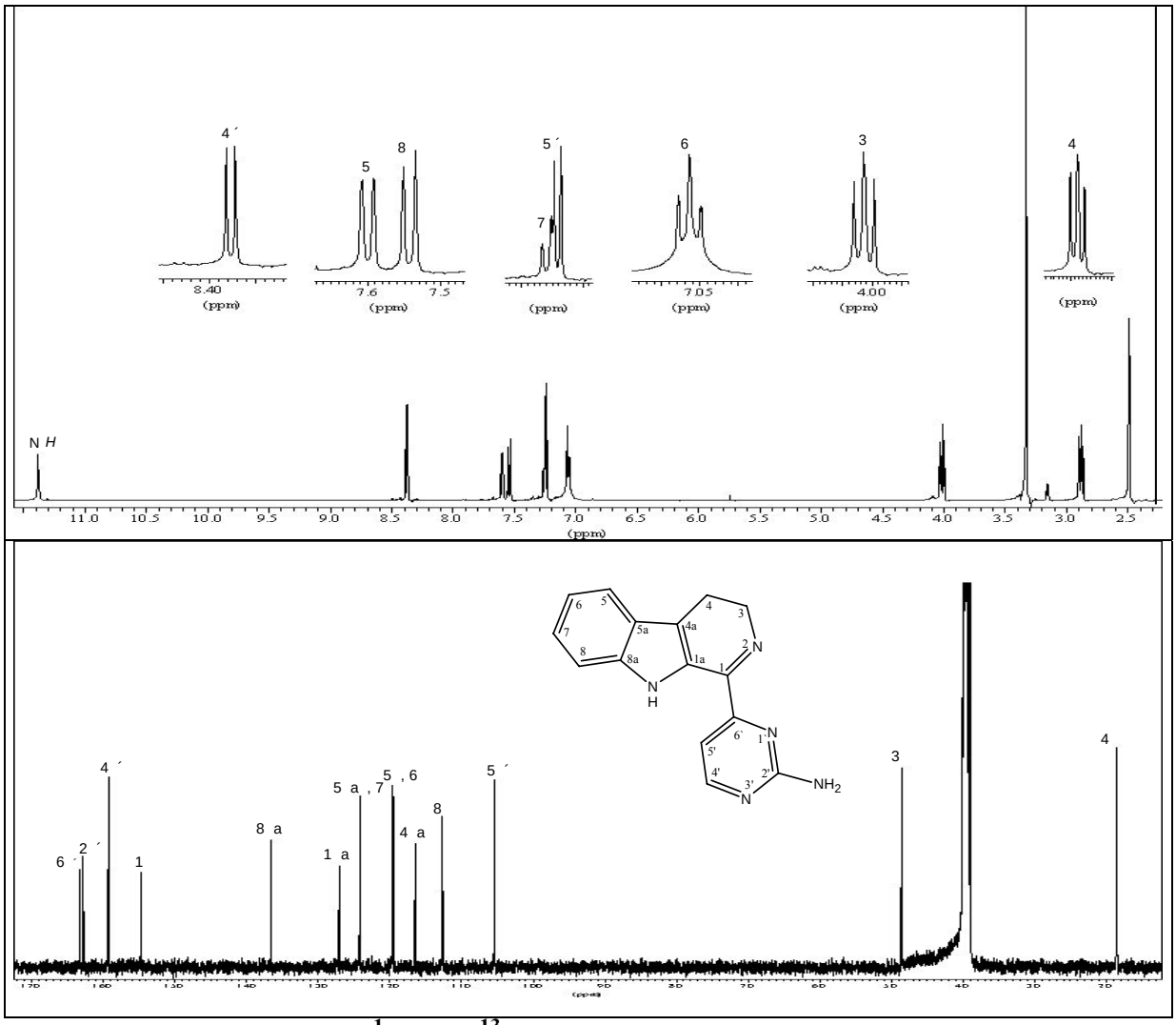


Fig. 3.108: ^{1}H and ^{13}C NMR spectra of compound 27

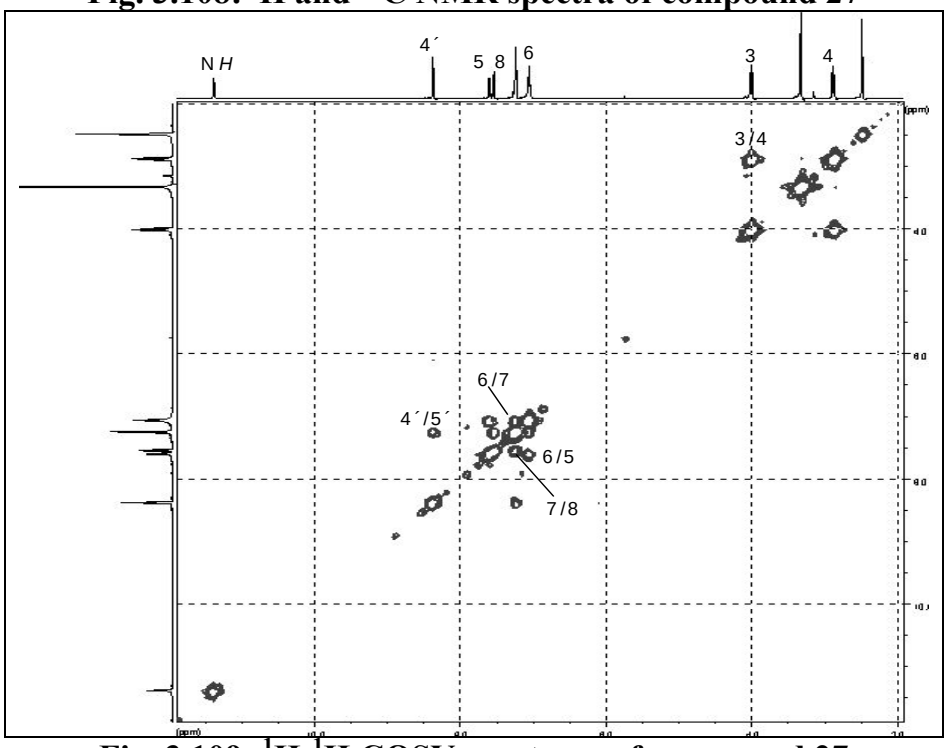


Fig. 3.109: ^{1}H-^{1}H COSY spectrum of compound 27

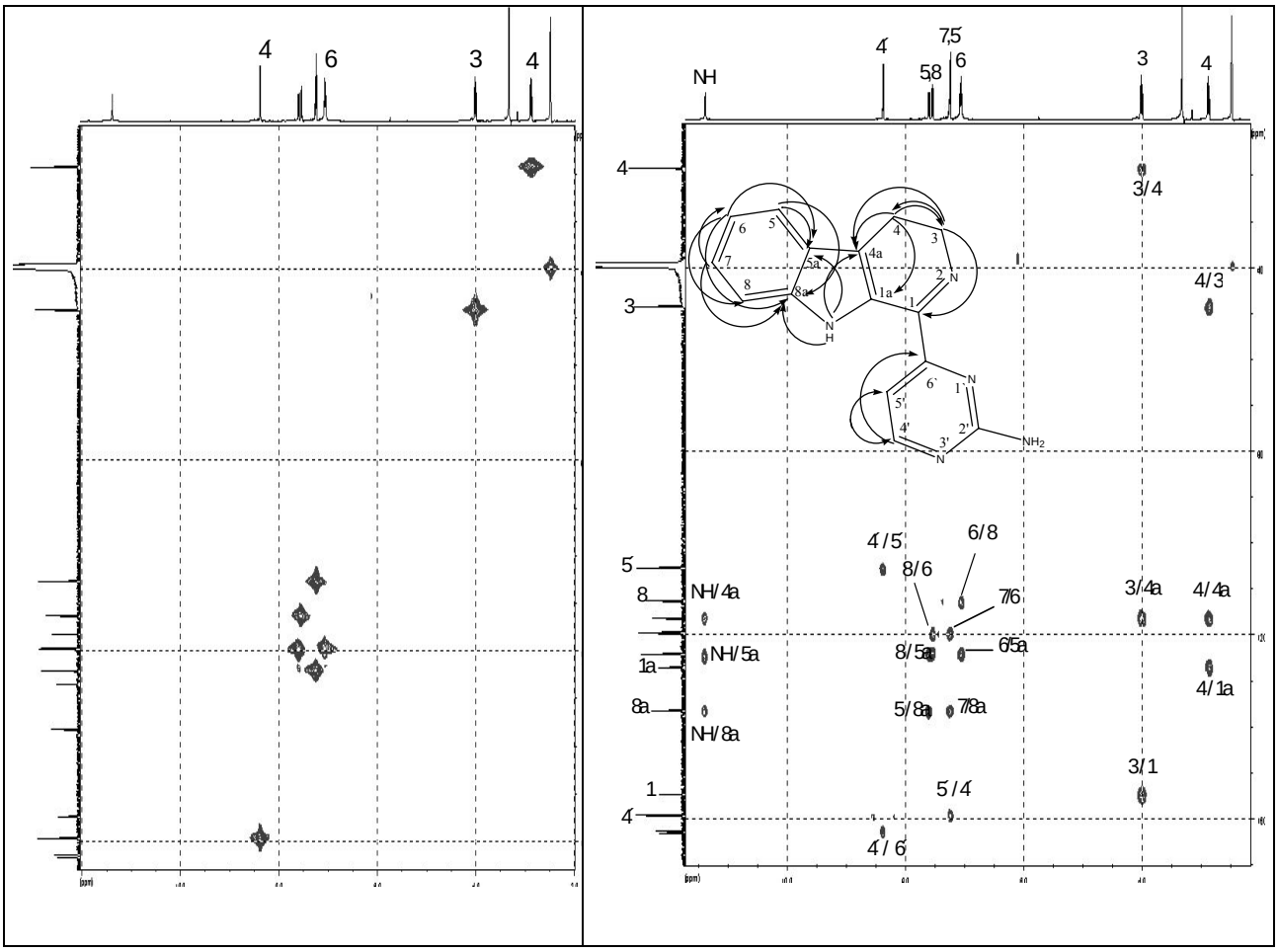
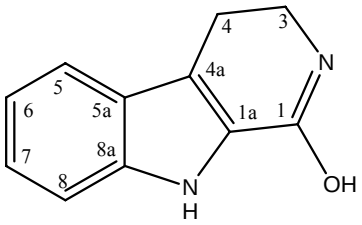


Fig. 3.110: HMQC and HMBC spectra of compound 27

Table 3.35: NMR data of compound 27 (DMSO-d_6, 500, 125 MHz)

Position	δ_H (m, *J* in Hz)	δ_C (m)	COSY	HMBC
N*H*	11.40 (s)			4a, 5a, 8a
1		154.7 s		
2				
3	4.05 (t, *J* = 8.2 Hz)	48.5 t	4	1, 4, 4a
4	2.85 (t, *J* = 8.2 Hz)	18.5 t	3	1a, 3, 4a
4a		116.5 s		
5a		124.2 s		
5	7.61 (d, *J* = 7.9 Hz)	119.6 d	6	5a, 8a
6	7.07 (t, *J* = 7.3 Hz)	119.4 d	5, 7	5a, 8
7	7.27 (d, *J* = 8.2 Hz)	124.1 d	6	6, 8a
8	7.55 (d, *J* = 8.2 Hz)	112.6 d	7	5a, 6
8a		136.5 s		
1a		127.0 s		
1`				
2`		162.7 s		
3`				
4`	8.38 (d, *J* = 5.0 Hz)	159.2 d	5`	5`, 2`, 6`
5`	7.24 (d, *J* = 5.0 Hz)	105.4 d	4`	4`
6`		163.3 s		

3.3.3. 1-Hydroxy-3, 4-dihydronorharman (28, known compound)



1-Hydroxy-3,4-dihydronorharman

Compound **28** was obtained as a white amorphous powder. It has UV absorbances at λ_{max} 206, 226, and 300 nm. The (+) ESIMS spectrum exhibited an ion peak at *m*/*z* 187 $[M+H]^+$, implying a molecular weight of 186 g/mol. The ^{1}H NMR spectrum showed four protons in the aromatic region at δ 7.57 (d, *J* = 8.2 Hz, H-5), 7.37 (d, *J* = 8.2 Hz, H-8), 7.20 (dt, *J* = 8.2, 0.9 Hz, H-7) and 7.04 (dt, *J* = 8.2, 0.9 Hz, H-6). Along with the COSY correlations of these protons, they suggested the presence of an ABCD spin system. Protons at δ 3.51 (t, *J* = 6.9 Hz, H_2-3) and 2.91 (t, *J* = 6.9 Hz, H_2-4) were positioned close to each other as indicated by their COSY correlation. Furthermore, a downfield singlet at δ 11.51 represented an indolic N*H*, while a broad singlet at δ 7.55 indicated an O*H* group. The ^{13}C NMR spectrum showed the presence of three nitrogen-bound carbons at δ 137.0 (C-8a), 127.2 (C-1a) and 41.1 (C-3). In addition, the presence of four methine carbons at δ 123.9 (C-7), 120.1 (C-5), 119.3 (C-6) and 112.4 (C-8), two quaternary carbons at δ 124.9 (C-5a), 118.1 (C-4a) and two methylene carbons at δ 41.1 (C-3), 20.3 (C-4) was confirmed by its DEPT spectrum. One oxygen-bound carbon was determined by a signal at δ 161.2 (C-1). Direct correlations of protons to their respective carbons were revealed in the HMQC spectrum. The HMBC spectrum clearly pinpointed correlations of H-5 to C-7, C-8a and C-4a, N*H* to C-1a, C-4a, C-5a and C-8a which established the position of the N*H* group. The two methylene groups were connected to the indole moiety at position C-4a. This connection was confirmed by the HMBC spectrum, which showed three bond couplings between H_2-3 to C-4a, H_2-4 to C-5a and C-1a, as well as the two bond coupling between H_2-4 and C-4a. The position of the hydroxyl group at C-1 was established by the cross peak of the O*H* group with C-1a and also the downfield carbon chemical shift of C-1. Comparison of the NMR data of compound **28** with

those **27** revealed the absence of the 2-aminopyrimidine ring, which was replaced by a hydroxyl function. Compound **28** was identified as **1-hydroxy-3,4-dihydronorharman**. It was identified that **28** was a tautomer of 1,2,3,4-tetrahydronorharman-1-one [Roa, *et al*., 2003].

Table 3.36: NMR data of compound 28 (DMSO-d_6, 500, 125 MHz)

Position	δ_H (m, *J* in Hz)	δ_C (m)	COSY	HMBC
1		161.2 s		
1a		127.2 s		
2				
3	3.51(t, *J* = 6.9 Hz)	41.1 t	4	1, 4, 4a
4	2.91 (t, *J* = 6.9 Hz)	20.3 t	3	1a, 3, 4a, 5a
4a		118.1 s		
5a		124.9 s		
5	7.57 (d, *J* = 8.2 Hz)	120.1 d	6	4a, 7, 8a
6	7.04 (dt, *J* = 8.2, 0.9 Hz)	119.3 d	5, 7, 8	5a, 8
7	7.20 (dt, *J* = 8.2, 0.9 Hz)	123.9 d	5, 6, 8	5, 8, 8a
8	7.37 (d, *J* = 8.2 Hz)	112.4 d	7	5a, 6
8a		137.0 s		
N*H*	11.51(s)			1a, 4a, 5a, 8a
O*H*	7.55 (brs)			1a

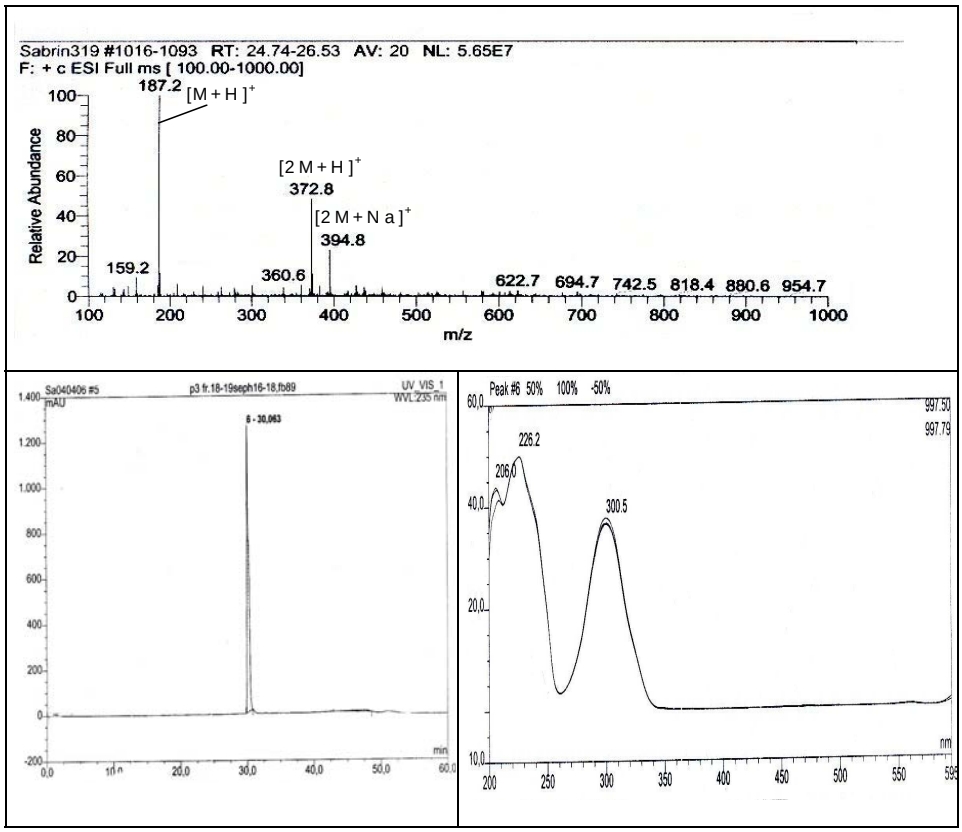


Fig. 3.111: (+) **ESIMS and UV spectra of compound 28**

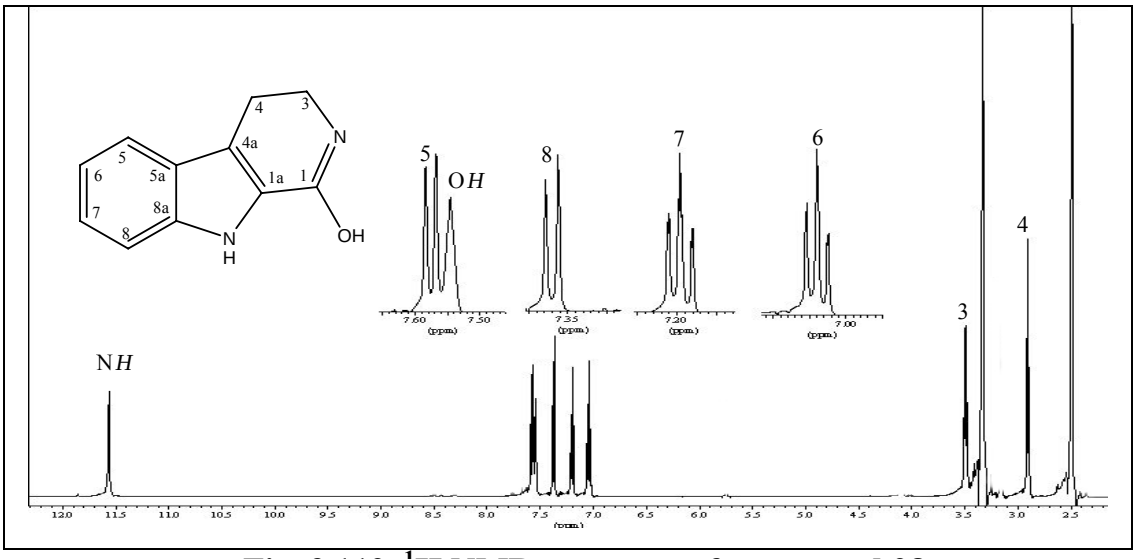


Fig. 3.112: ^{1}H NMR spectrum of compound 28

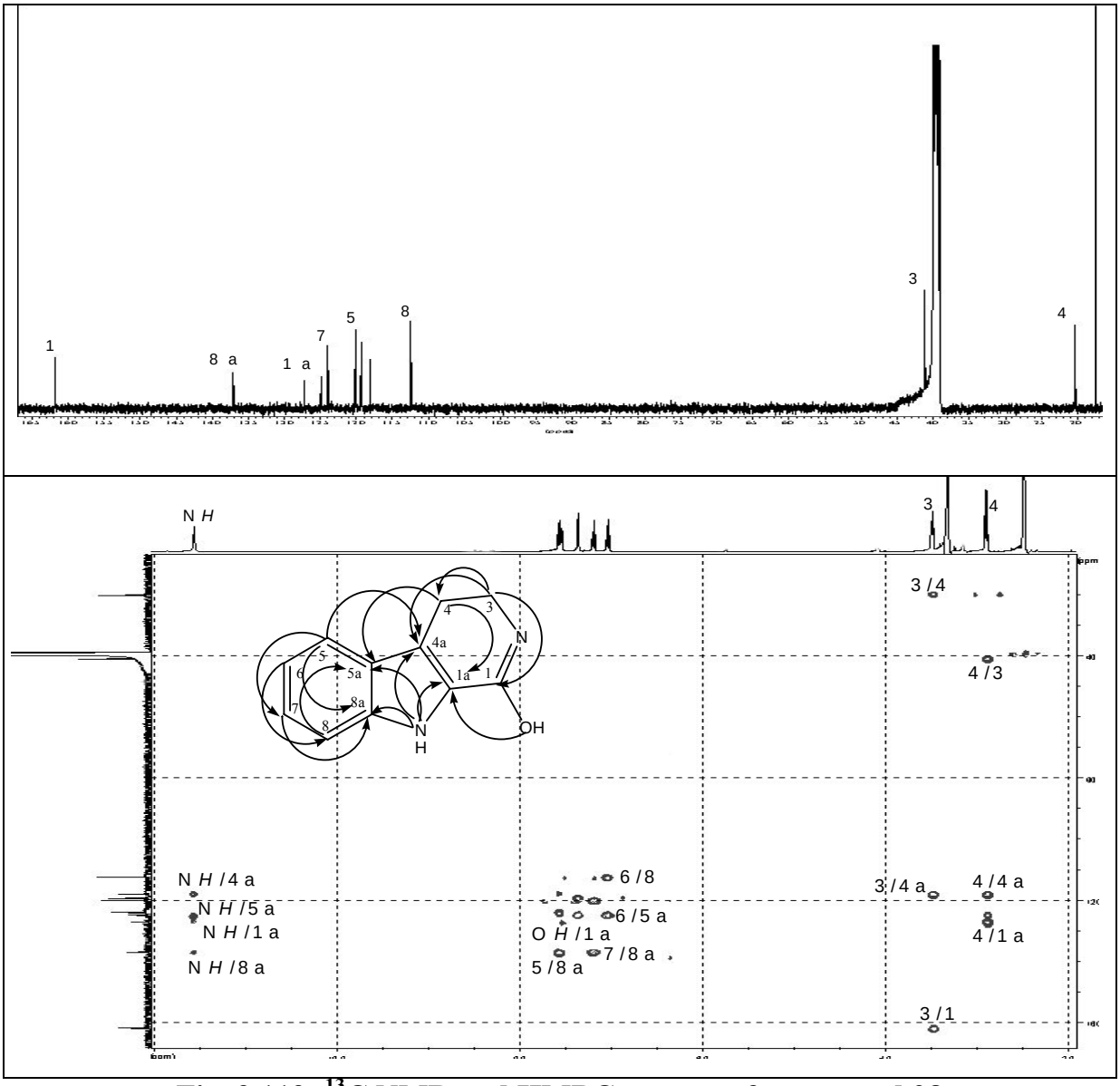


Fig. 3.113: ^{13}C NMR and HMBC spectra of compound 28

Bioactivity

In our search for new cytotoxic drugs all of the isolated compounds from *Acanthostrongylophora ingens* were tested against L5178Y cancer cell line. Annomontine (**26**) showed strong activity at concentration of 3 µg/mL while the other compounds were only moderately active. In the brine shrimp bioassay, the isolated alkaloids showed activities with different percentages. The results are summarised in Table 3.37.

Table 3.37: **Cytotoxicity of the isolated carbolines against L5178Y**

Compound	% Growth inhibition L5178Y		ED50 µg/mL
	3 µg/mL	10 µg/mL	
Annonomontine (26)	100	100	0.49
Acanthomine A (27)	44	71	4.7
1-Hydroxy-3,4-dihydronorharman (28)	34	42	> 10

3.4. Isolated compounds from the sponge *Theonella swinhoei*

Sponges of the order *Lithistida* are one of the most prolific sources of structurally diverse marine natural products. The genus *Theonella* belonging to the order *Lithistida* has been shown to be a rich source of secondary metabolites with pronounced biological activities [MarinLit., 2002]. Examples of these secondary metabolites are po -lytheonamides [Hamada, *et al*., 1994], cyclotheonamides [Fusetani, *et al*., 1990], theonellapeptolides [Kobayashi, *et al*., 1994], theonellamides [Matsunaga, *et al*., 1989, Matsunaga, *et al*., 1995], theonegramides [Bewley, *et al*., 1994], keramamides [Kobayashi, *et al*., 1995], mozamides [Schmidt, *et al*., 1997], mutoporins [de Silva, *et al*., 1992] microsclerodermins [Schmidt, *et al*., 1998], cupolamides [Bonnington, *et al*., 1997], swinholides [Spector, *et al*., 1999, Tong, *et al*., 2001, Segal, *et al*., 2001] bistheonellides [Watabe, *et al*., 1996, Saito, *et al*., 1996, Saito, *et al*., 1998], and swinhoeiamide [Edrada, *et at*., 2002]. Many *Theonella* derived peptides demonstrate potent cytotoxicity [Hamada, *et al*., 1994, Bonnington, *et al*., 1997], thrombin inhibition [Fusetani, *et al*., 1990], phosphotase inhibition [de Silva, *et al*., 1992], protease inhibition, antifungal [Matsunaga, *et al*., 1989, Matsunaga, *et al*., 1995] and human immunodeficiency virus inhibition (HIV) [Ford, *et al*., 1999] activities. Theonellapeptolides are tridecapeptide lactones which have been isolated from Okinawa marine sponges of genus *Theonella* [Kitagawa, *et al*., 1986]. Chemical investigation of the Indonesian sponge *Theonella swinhoei* resulted in the isolation of theonellapeptolides (Ia and Id) (compound **29**, **30**), swinholide A (compound **31**) and cholest-4-ene-3-one (compound **32**).

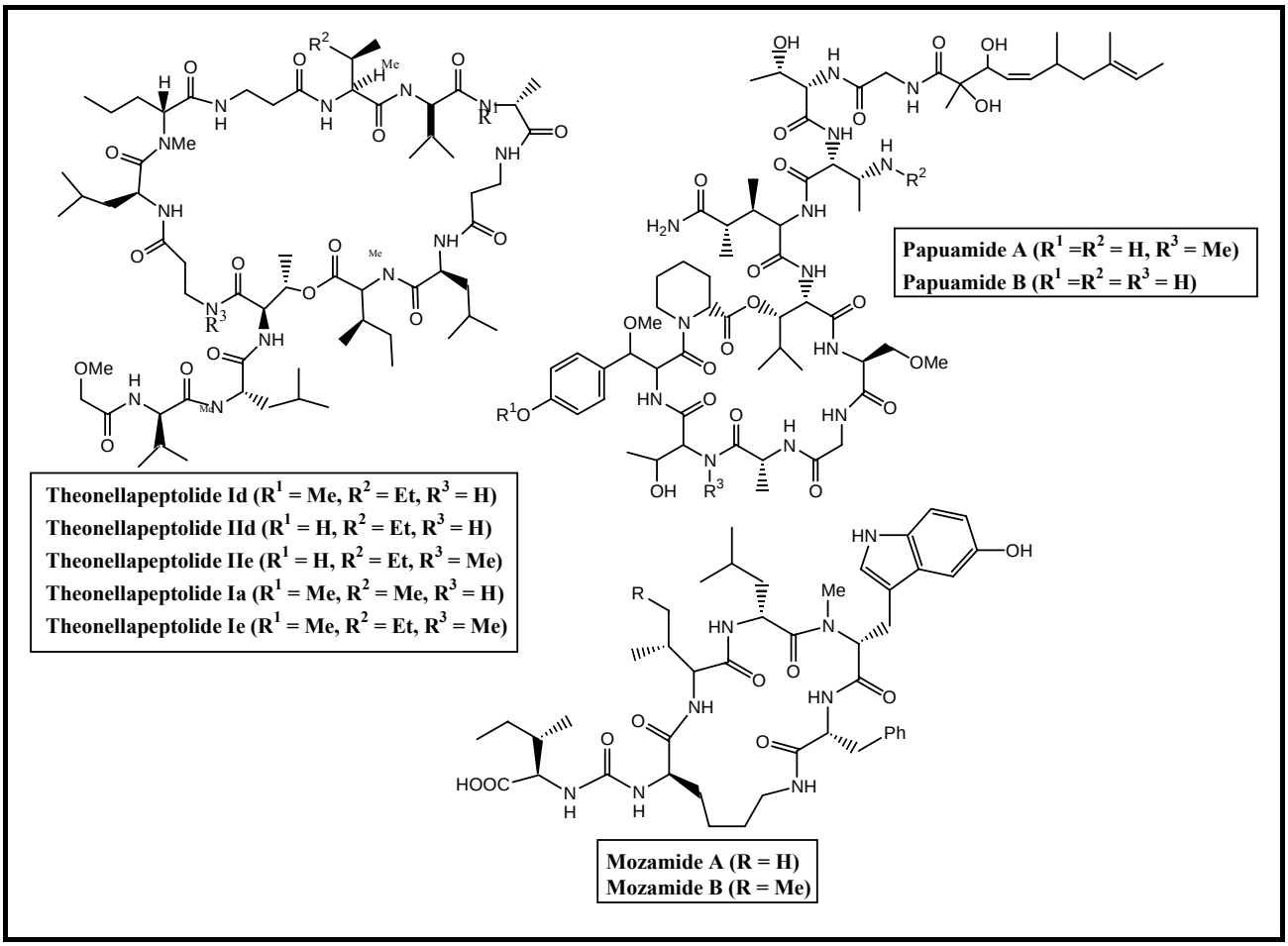
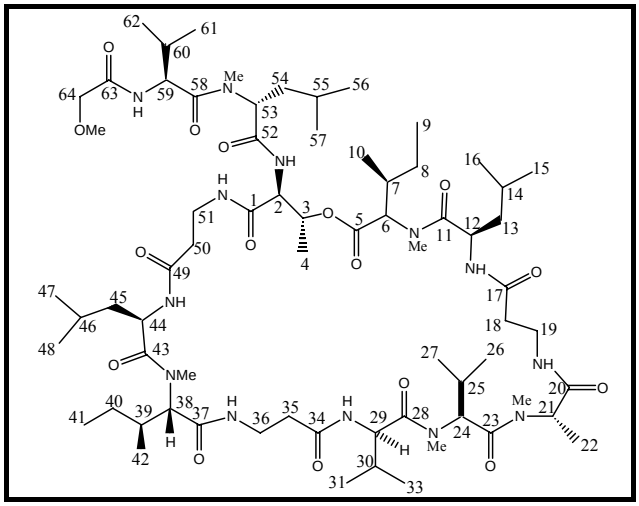


Fig. 3.114: Examples of some secondary metabolites isolated from *Theonella swinhoei*

3.4.1. Theonellapeptolide Ia (29, known compound)



Compound **29** was isolated as a white crystalline solid. It has a UV absorbance at λ_{max} 209 nm. Compound **29** has a molecular weight of 1390 g/mol, as derived from the APCIMS and FABMS measurements which showed molecular ion peaks at *m/z* 1391 $[M+H]^+$ and 1413 $[M+Na]^+$. The ^1H NMR spectrum of compound **29** disclosed that the

molecule had a peptidic nature, containing eight N*H* protons at δ 8.32 (1H, brs, 2-N*H*), 8.09 (1H, d, *J* = 9.1 Hz, 12-N*H*), 6.66 (1H, brs, 19-N*H*), 7.18 (1H, d, *J* = 8.5 Hz, 29 N*H*), 6.88 (1H, t, *J* = 3.7 Hz, 36-N*H*), 8.04 (1H, brs, 44-N*H*), 6.53 (1H, brs, 51-N*H*), 7.19 (1H, d, *J* = 8.5 Hz, 59-N*H*) and five *N*-methyl groups at δ 3.24 (3H, s, 6-NCH_3), 2.74 (3H, s, 21-NCH_3), 3.30 (3H, s, 24-NCH_3), 3.15 (3H, s, 38-NCH_3), 3.17 (3H, s, 53-NCH_3). One methoxyacetyl at δ 3.77 (3H, s, 64-OCH_3), 3.90 (1H, d, *J* = 15.1 Hz, H-64) and 3.87 (1H, d, *J* = 15.1 Hz, H-64′) was also determined. Analysis of the ^{13}C NMR spectrum pointed out twelve amide carbons at δ 168.2 (C-1), 174.2 (C-11), 170.2 (C-17), 168.8 (C-20), 170.2 (C-23), 176.3 (C-28), 171.1 (C-34), 169.7 (C-37), 175.0 (C-43), 171.2 (C-49), 173.0 (C-52), 173.7 (C-58), and one lactone carbon at δ 170.4 (C-5) proving the existence of thirteen amino acids. One methoxyacetyl group was present as observed by the resonances at δ 169.1 (CH_3O-CH_2-CO) and 72.0 (t, CH_3O-CH_2-CO). The DEPT spectrum revealed ninteen methine carbons, eleven methylene carbons, eighteen methyl carbons, five *N*-methyl carbons and one methoxy carbon at δ 59.1. These findings led to the presumption that compound **29** was an oligopeptide containing five *N*-methyl amino acids and one methoxyacetyl moiety. Furthermore, compound **29** was negative in the ninhydrin test from which it can be presumed that it could be a peptide lactone (peptolide). In its structure the *C*-terminal amino acid participates in the lactone ring and the *N*-terminal amino acid is blocked with a methoxyacetyl group. Inspection of the COSY and TOCSY spectra revealed the existence of thirteen spin systems for thirteen amino acid residues. The sequence of amino acids was established by detailed interpretation of the HMBC and inter-amino acid ROESY correlations. In its ROESY spectrum, 29-N*H* showed correlations to 24-NCH_3, H-35 and H-36, which proved the amide linkages between Me-Val, Val and β-Ala to give substructure **A**. The HMBC correlations of 24-NCH_3 to C-28 and H-29 to C-34 confirmed this substructure. 19-N*H* showed ROESY correlations to 21-NCH_3 and H-21 which indicated the connectivity of β-Ala to Me-Ala, which was further confirmed by the HMBC correlations of 19-N*H* to C-20 and H-19 to C-21 for substructure **B**. The characteristic HMBC correlation of 21-NCH_3 to C-23 assured the connection between substructures **A** and **B**. The dipeptide substructure **C** of Leu and *allo*-Me-Ile, was confirmed by the ROESY correlation of 6-NCH_3 to H-12.

Substructure **C** was further confirmed through the HMBC correlation 6-NC*H*$_3$ to C-11. Likewise the HMBC correlation of 12-N*H* to C-17 confirmed the connection of substructure **B** to **C**. The ROESY spectrum showed correlations of 51-N*H* to H-2 and H-3 and proved the connectivity of threonine to β-alanine which resulted in substructure **D**, which was further confirmed by HMBC correlations of 51-N*H* and H-51 to C-1. The connectivity of substructure **C** with **D** was confirmed through the HMBC correlations of H_3-4 and H-3 to C-5. This also established cyclization via the ester bond formation between *C*-terminal *allo*-Me-isoleucine and the hydroxy group of threonine. In addition, ROESY correlation of 38-NC*H*$_3$ to H-44 confirmed the connectivity of Me-isoleucine to leucine for substructure **E**. Further correlations of 36-N*H* to H-38 and H-38 to H-36 assumed that β-alanine was amide linked to Me-isoleucine. The connectivity between substructures **A** and **E** was proven by the HMBC correlations of 38-NC*H*$_3$ to C-43, H-36´ to C-38 and 36-N*H* to C-37. The HMBC correlation observed for H-2 to C-52 confirmed the position of Me-leucine. The key ROESY correlation of 53-N*CH*$_3$ to H-59 confirmed the connectivity of Me-leucine to valine for substructure **F**. This was again confirmed by the HMBC correlation of 53-NC*H*$_3$ to C-58. The position of the methoxyacetyl group was confirmed by the HMBC correlations of 59-N*H*, H-59, H_2-64 and 64-OC*H*$_3$ to C-63, H_2-64 to 64-OC*H*$_3$. Comparison of the NMR data and careful assignment of COSY, TOCSY, ROESY and HMBC spectra and comparison with literature data led to the identification of compound **29** as **theonellapeptolide Ia**. This compound was also previously isolated from the Indonesian sponge *Theonella swinhoei* [Roy, *et al*., 2000].

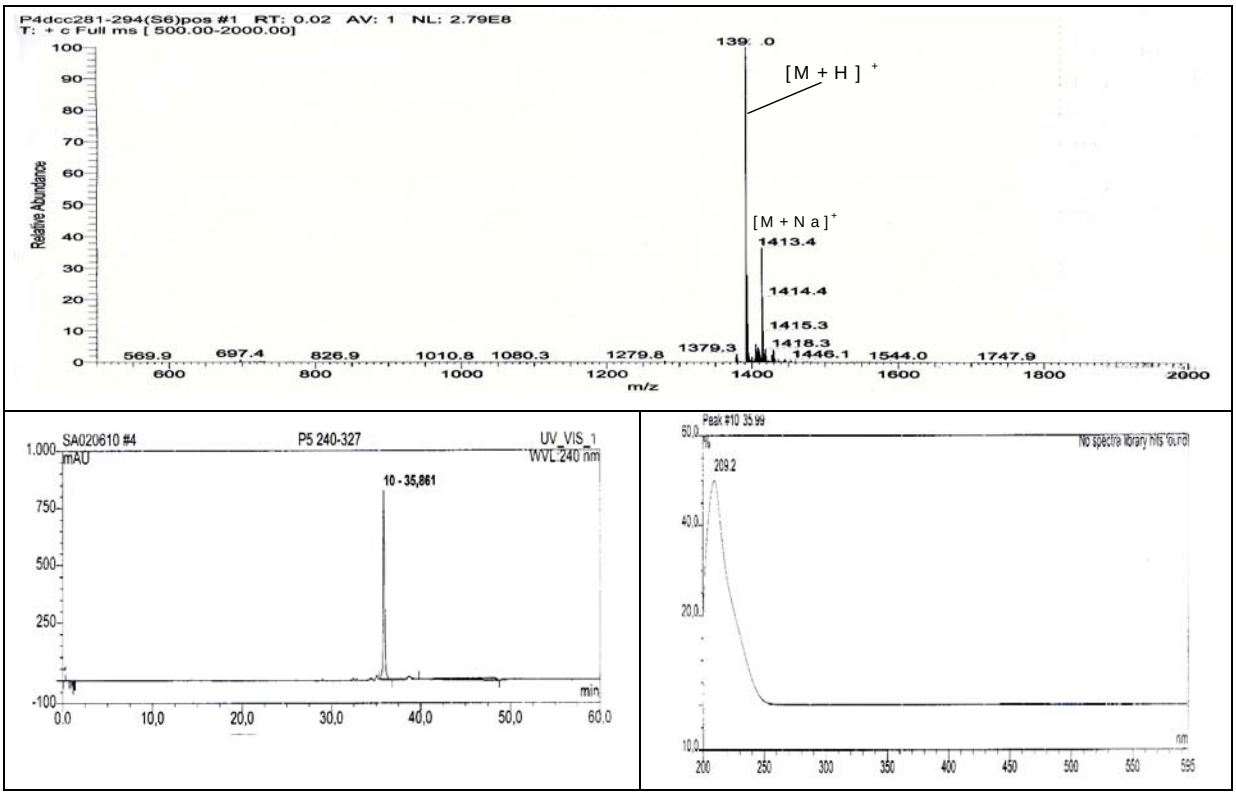


Fig. 3.115: APCIMS spectrum of compound 29

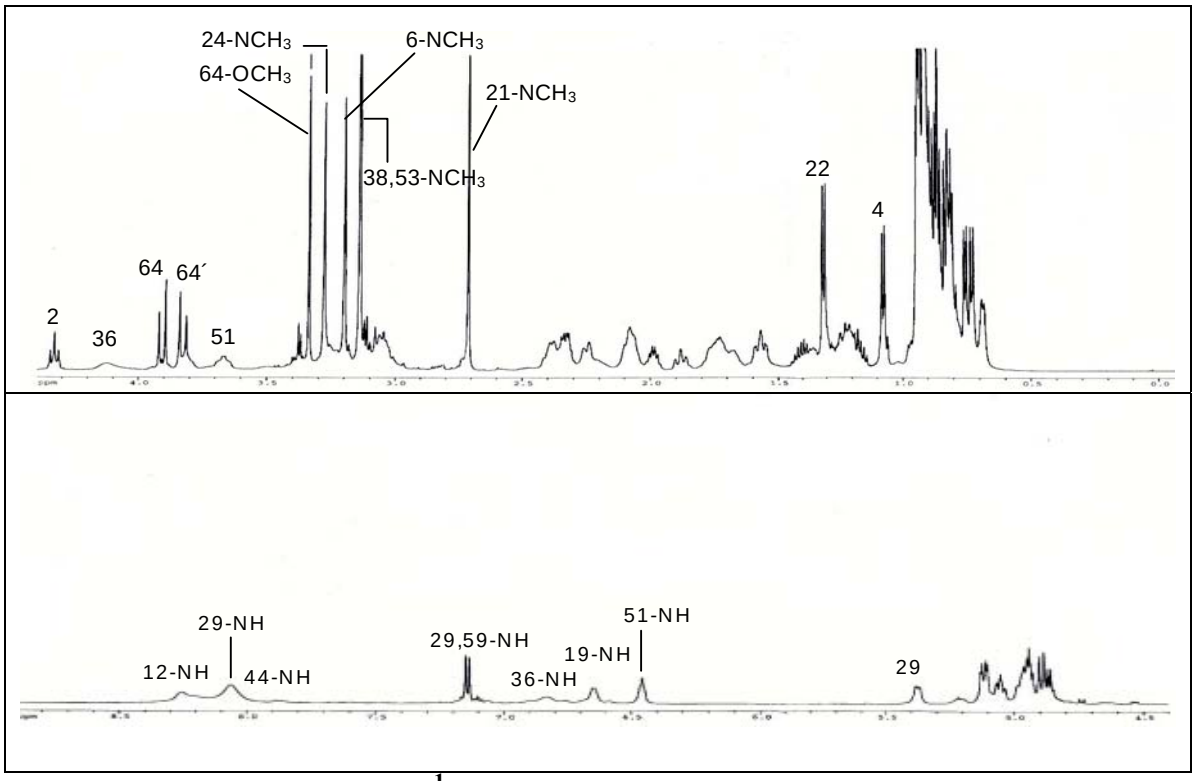


Fig. 3.116: ^{1}H NMR spectra of compound 29

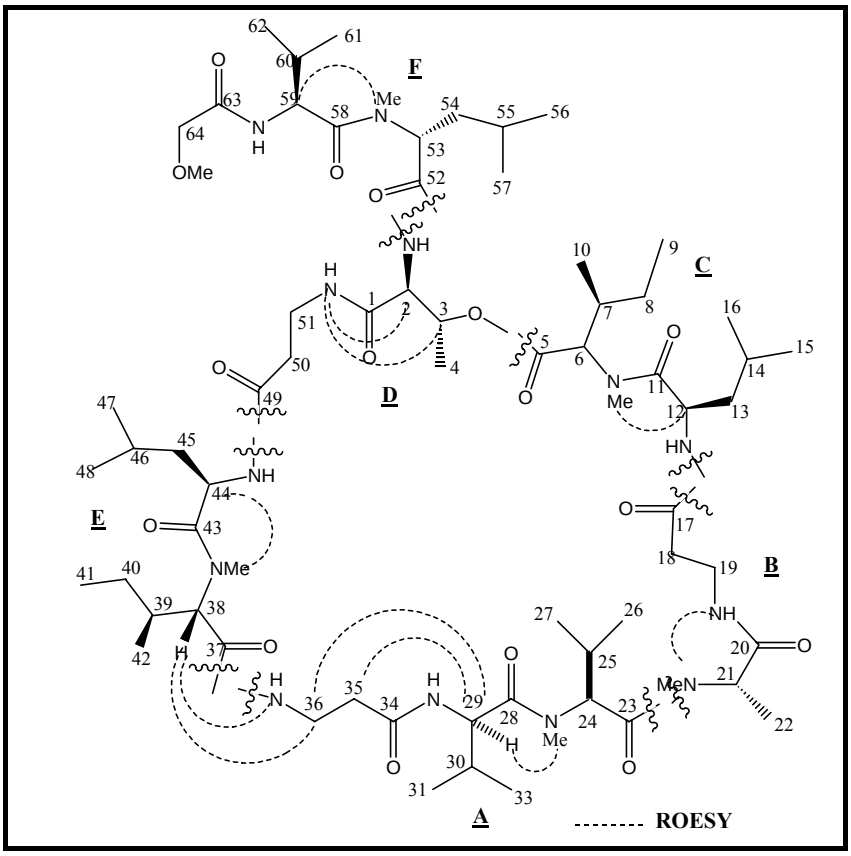


Fig. 3.117: Partial substructures A-F and ROESY correlations

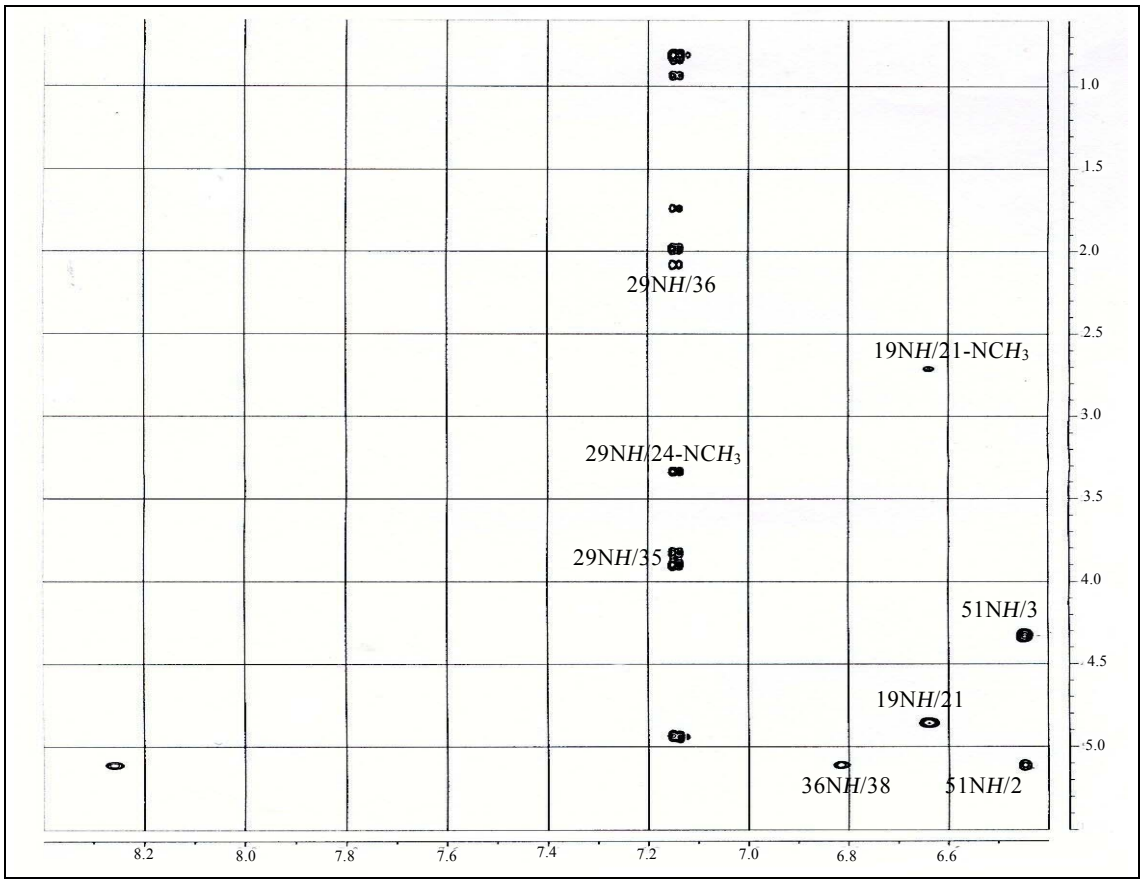


Fig. 3.118: Part of ROESY spectrum showing some important correlations

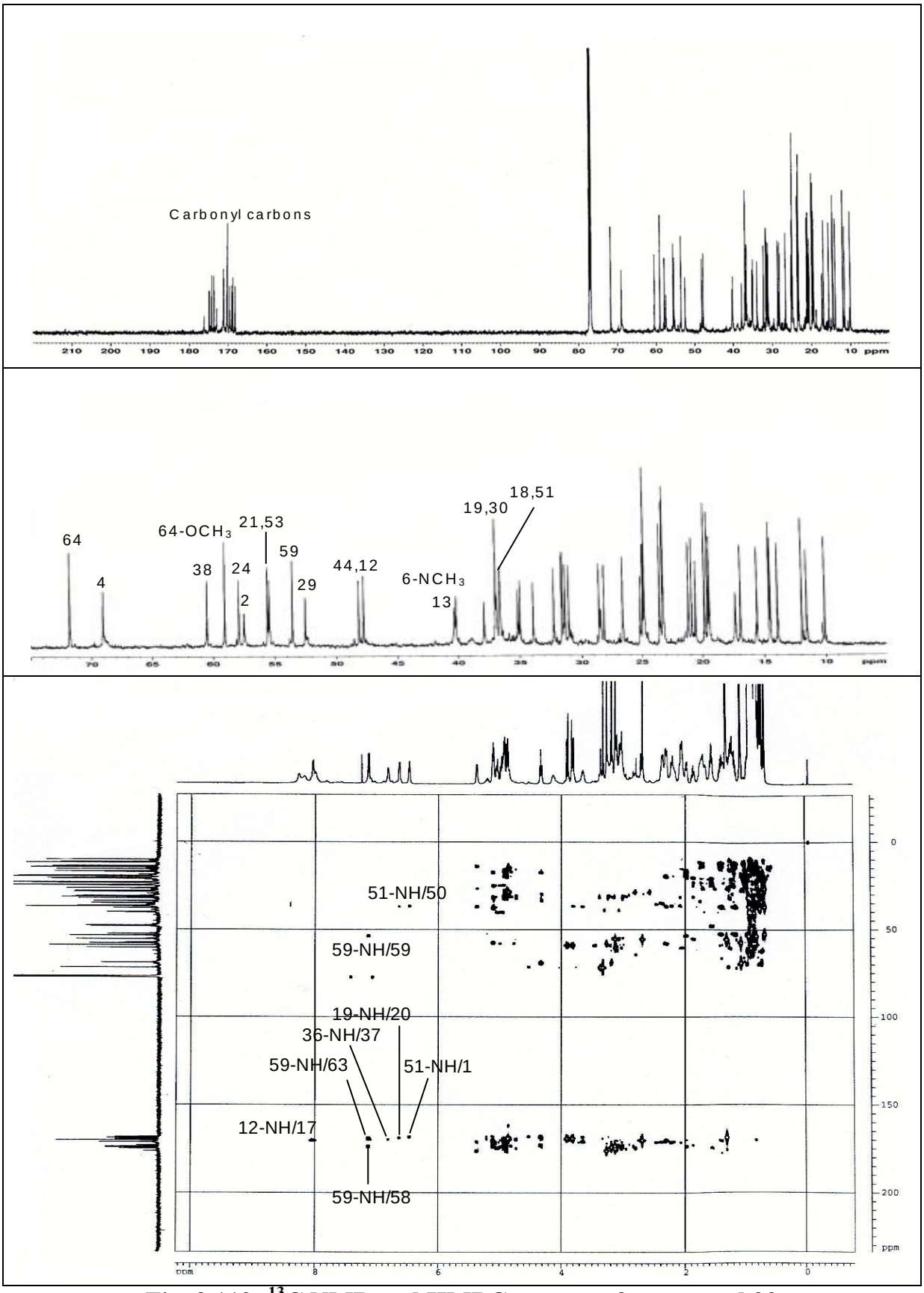


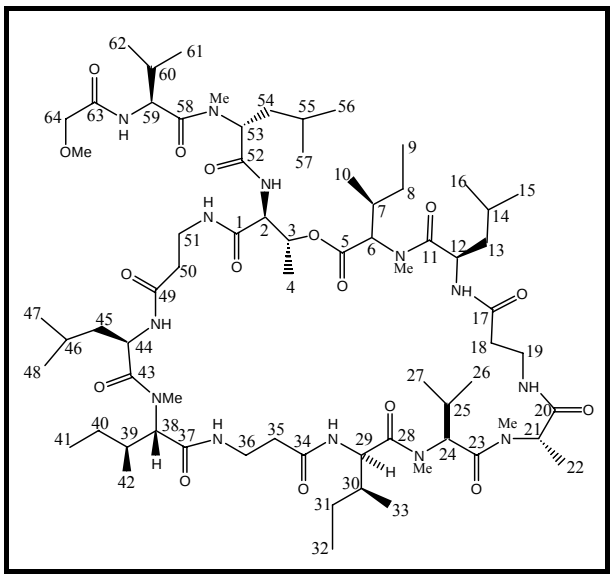
Fig. 3.119: ^{13}C NMR and HMBC spectra of compound 29

Table 3.38: NMR data of compound 29 ($CDCl_3$, 500, 125 MHz)

A.A.	Position	δ_H (m, *J* in Hz)	δ_C (m)	HMBC	ROESY
L-Thr	1		168.2 s		
	2	4.38 (m)	57.5 d	1, 3, 4, 52	51-N*H*
	3	517 m	69.1 d	1, 2, 4, 5, 52	6
	4	1.12 (d, *J* = 6.9 Hz)	17.3 q	2, 3	
	2-N*H*	8.32 (brs)			
	O*H*				
D-allo-Me-Ile	5		170.4 s		
	6	5.20 (m)	69.0 d		3
	7	2.43 (m)	33.9 d		
	8	1.80 (m)	28.4 t		
	8′	1.35 (m)			
	9	0.96 (d, *J* = 6.9 Hz)	11.6 q		
	10	0.77 (d, *J* = 6.9 Hz)	14.5 q		
	6-NC*H*$_3$	3.24 (s)	38.9 q	11	12
D-Leu	11		174.2 s		
	12	4.96 (m)	48.0 d		
	13	1.60 (m)	40.3 t	11	
	13′	1.26 (m)			
	14	1.71 (m)	24.8 d		
	15	0.95 (d, *J* = 6.6 Hz)	23.5 q	13	
	16	0.89 (d, *J* = 6.9 Hz)	20.8 q	13	
	12-N*H*	8.09 (d, *J* = 9.1 Hz)			
β-Ala	17		170.2 s		
	18	2.37 (m)	36.6 t		
	18′	2.10 (m)			
	19	3.88 (m)	36.9 t		
	19′	3.12 (m)		21	
	19-N*H*	6.66 (brs)		20	21, 21-NC*H*$_3$
L-Me-Ala	20		168.8 s		
	21	4.92 (m)	55.6 d	20	19-N*H*, 21-NC*H*$_3$
	22	1.36 (d, *J* = 6.6 Hz)	14.6 q	20, 21	
	21-NC*H*$_3$	2.74 (s)	28.6 q	21, 23	19-N*H*
L-Me-Val	23		170.2 s		
	24	4.93 (m)	57.9 d	23, 28	24-NC*H*$_3$
	25	2.37 (m)	28.1 d	23	
	26	0.91 (d, *J* = 6.9 Hz)	19.8 q	24	
	27	0.87 (d, *J* = 6.9 Hz)	19.4 q	23, 24	
	24-NC*H*$_3$	3.30 (s)	31.4 q	24, 28	
D-Val	28		176.3 s		
	29	4.92 (m)	52.5 d	30, 33, 34, 28	
	30	2.12 (m)	36.9 d		
	31	0.85 (d, *J* = 6.6 Hz)	12.0 q		
	33	0.88 (d, *J* = 6.6 Hz)	13.9 q	29	
	29-N*H*	7.18 (d, *J* = 8.5 Hz)			24-NC*H*$_3$, 35, 36
β-Ala	34		171.1 s		
	35	2.39 (m)	35.2 t	34	
	35′	2.22 (m)			
	36	4.12 (brs)	35.0 t		
	36′	3.07 (m)		38	38
	36-N*H*	6.88 (brt, *J* = 3.7 Hz)		37	38
L-Me-Ile	37		169.7 s		
	38	5.12 (m)	60.5 d		36-N*H*, 36, 45
	39	1.88 (m)	32.3 d		
	40	1.35 (m)	24.9 t		
	40′				

	41	0.78 (d, *J* = 6.8 Hz)	10.1 q		
	42	0.88 (d, *J* = 6.8 Hz)	15.6 q	38	
	38-NCH_3	3.15 (s)	31.0 q	43, 38	44
D-Leu	43		175.0 s		
	44	5.08 (m)	48.2 d		38-NCH_3
	45	1.58 (m)	40.2 t		
	45´	1.24 (m)			
	46	1.72 (m)	24.9 d		
	47	0.85 (d, *J* = 6.9 Hz)	23.2 q		
	48	0.92 (d, *J* = 6.9 Hz)	21.2 q		
	44-N*H*	8.04 (brs)			
β-Ala	49		171.2 s		
	50	2.27 (m)	36.9 t	49	
	50´	2.13 (m)			
	51	3.70 (m)	36.6 t	1, 49	
	51´	3.28 (m)			
	51-N*H*	6.53 (brs)		50, 1	2, 3
D-Me-Leu	52		173.0 s		
	53	5.37 (m)	55.4 d		
	54	1.70 (m)	37.9 t		
	54´	1.41 (m)			
	55	1.22 (m)	25.1 d		
	56	0.95 (d, *J* = 6.5 Hz)	23.3 q		
	57	0.78 (d, *J* = 6.5 Hz)	20.6 q		
	53-NCH_3	3.17 (s)	31.6 q	58, 53	59
L-Val	58		173.7 s		
	59	4.98 (m)	53.6 d	58	53-NCH_3
	60	2.03 (m)	31.6 d	58, 59	
	61	0.99 (d, *J* = 6.7 Hz)	19.6 q	59	
	62	0.86 (d, *J* = 6.7 Hz)	16.9 q		
	59-N*H*	7.19 (d, *J* = 8.5 Hz)		59, 58, 63	
MeOAc	63		169.1 s		
	64	3.90 (d, *J* = 15.1 Hz)	72.0 t	63, 64-OCH_3	
	64´	3.87 (d, J = 15.1 Hz)		63, 64-OCH_3	
	64-OCH_3	3.77 (s)	59.1 q	64	

3.4.2. Theonellapeptolide Id (30, known compound)



Compound **30** was isolated as a white crystalline solid. It has a UV absorbance at λ_{max} 209 nm similar to that of theonellapeptolide Ia. The APCI and FABMS spectra showed molecular ion peaks at *m/z* 1405 $[M+H]^+$ and 1427 $[M+Na]^+$. Compound **30** had a 14 mass unit difference from theonellapeptolide Ia (**29**), which indicated that compound **30** contained one methylene group more than compound **29**. The ^{1}H NMR spectrum of compound **30** showed resonances for eight amide protons at δ 8.31 (1H, brs, 2-N*H*), 8.20 (1H, brs, 29-N*H*), 8.08 (1H, d, *J* = 9.1 Hz, 12-N*H*), 8.02 (1H, brs, 44-N*H*), 7.18 (1H, d, *J* = 9.1 Hz, 59-N*H*), 6.85 (1H, dd, *J* = 7.9, 2.8 Hz, 36-N*H*), 6.66 (1H, d, *J* = 6.0 Hz, 19-N*H*), 6.50 (1H, brt, *J* = 4.4 Hz, 51-N*H*) and five *N*-methyl groups at δ 3.30 (24-NCH_3), 3.23 (6-NCH_3), 3.16 (53-NCH_3), 3.15 (38-NCH_3), 2.73 (21-NCH_3) which indicated the peptidic nature of this compound. One methoxy-acetyl at δ 3.93 (d, *J* = 15.0 Hz, H-64), 3.85 (d, *J* = 15.0 Hz, H-64′) and 3.36 (3H, s, 64-OCH_3) was also observed. The ^{13}C NMR spectrum showed resonances due to 12 amide carbons and one lactone carbon at δ 176.3 (C-28), 174.9 (C-43), 174.2 (C-11), 173.7 (C-58), 173.0 (C-52), 171.2 (C-49), 171.1 (C-34), 170.4 (C-5), 170.2 (C-17, 23), 169.7 (C-37), 168.8 (C-20) and 168.2 (C-1) which confirmed the presence of 13 amino acids. One methoxyacetyl group was indicated by resonances at δ 168.8 (CH_3O-CH_2-<u>CO</u>) and 71.8 (CH_3O-<u>CH_2</u>-CO). From the DEPT spectrum, the presence of one methoxy group at δ 59.0 (<u>CH_3</u>O-CH_2-CO), 19 methines, 12 methylenes and 17 methyl groups was shown. The COSY spectrum showed thirteen spin systems for thirteen amino acids:

eight ordinary amino acids and five *N*-methylated amino acid residues. One threonine spin system was indicated by the signals at δ 8.31 (2-N*H*), 4.38 (H-2), 5.16 (H-3) and 1.09 (H_3-4). This was confirmed by the HMBC correlations of H-2 to C-1 (δ 168.2) and C-3 (δ 69.0), H_3-4 to C-2 (δ 57.5) and C-3, 2-N*H* to C-1. Two leucine amino acid residues were evident from the COSY spectrum, which indicated the connectivity of the protons 12-N*H* (δ 8.08), H-12 (δ 5.02), H_2-13 (δ 1.60 and 1.26), H-14 (δ 1.71), H_3-15 (δ 0.97) and H_3-16 (δ 0.96). As well as, the connectivity of protons 44-N*H* (δ 8.02), H-44 (δ 5.0), H_2-45 (δ 1.60 and 1.24), H-46 (δ 1.78), H_3-47 (δ 0.90) and H_3-48 (δ 0.97) was detected. Three β-alanine residues were assembled through the COSY correlations, which established the connectivity of the protons 19-N*H* (δ 6.66), H_2-19 (δ 3.88 and 3.10) and H_2-18 (δ 2.37 and 2.10). Another β-alanine residue started at 36-N*H* (δ 6.85) to H_2-36 (δ 4.18 and 3.10) and H_2-35 (δ 2.40 and 2.20), while the third one commenced at 51-N*H* (δ 6.50) to H_2-51 (δ 3.71 and 3.28) and H_2-50 (δ 2.27 and 2.12). From its COSY spectrum a spin system for valine was also determined which was comprised of 59-N*H* (δ 7.18), H-59 (δ 4.98), H-60 (δ 2.02) H_3-61 (δ 0.99) and H_3-62 (δ 0.86). Furthermore a spin system for *allo*-isoleucine was identified which consisted of 29-N*H* (δ 8.20), H-29 (δ 5.4), H-30 (δ 1.75), H_2-31 (δ 1.44 and 1.20), H_3-32 (δ 0.98), and H_3-33 (δ 0.72). From the HMBC and COSY spectra, the *N*-methyl amino acid residues were identified as *allo*-Me-Ile, Me-Ala, Me-Ile, Me-Leu and Me-Val. Comparison of the NMR data and molecular weight of compound **30** with those of previously reported theonellapeptolide Id in combination with careful inspection of the ^{13}C NMR, COSY and HMBC spectra allowed the complete assignment of all signals. Compound **30** was identical with the known compound **theonellapeptolide Id** [Roy, *et al*., 2000].

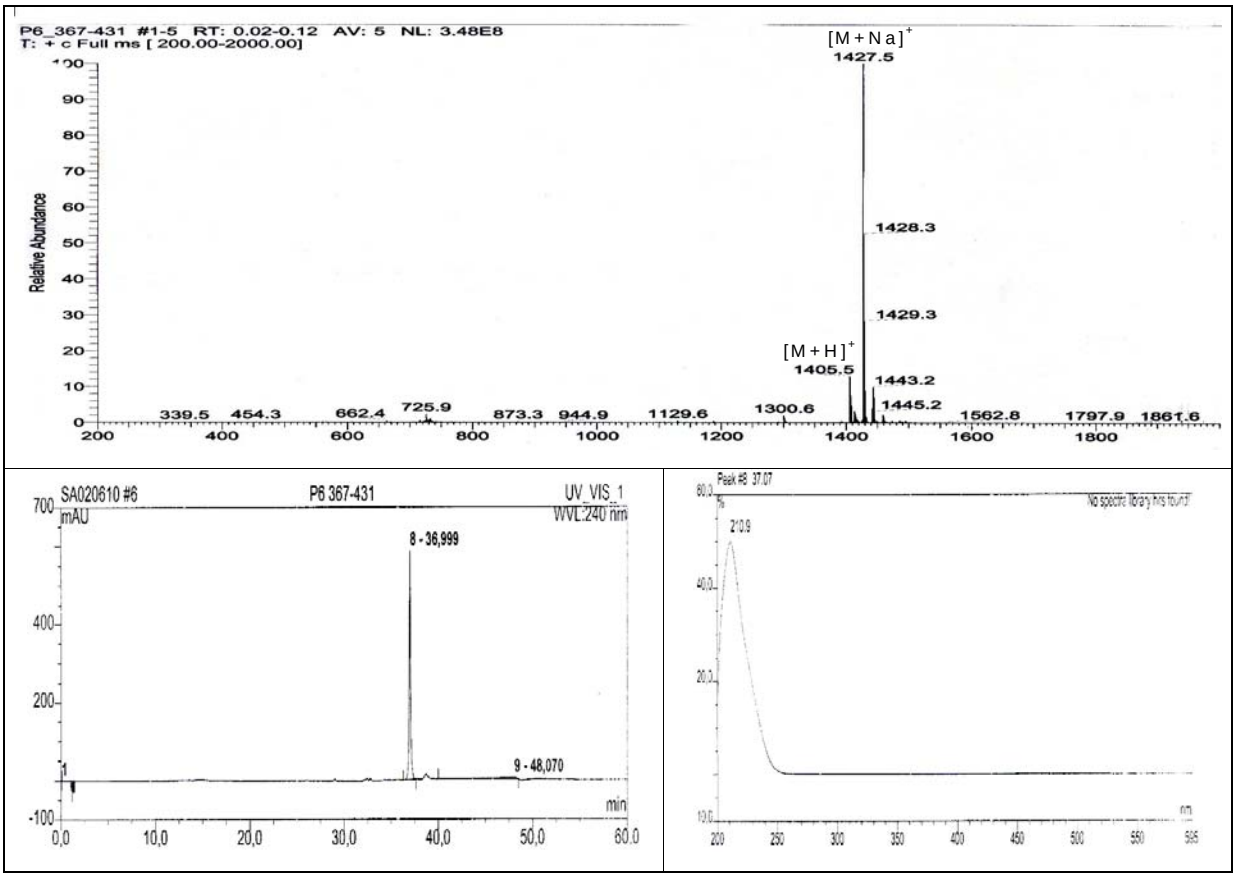


Fig. 3.120: APCIMS and UV spectra of compound 30

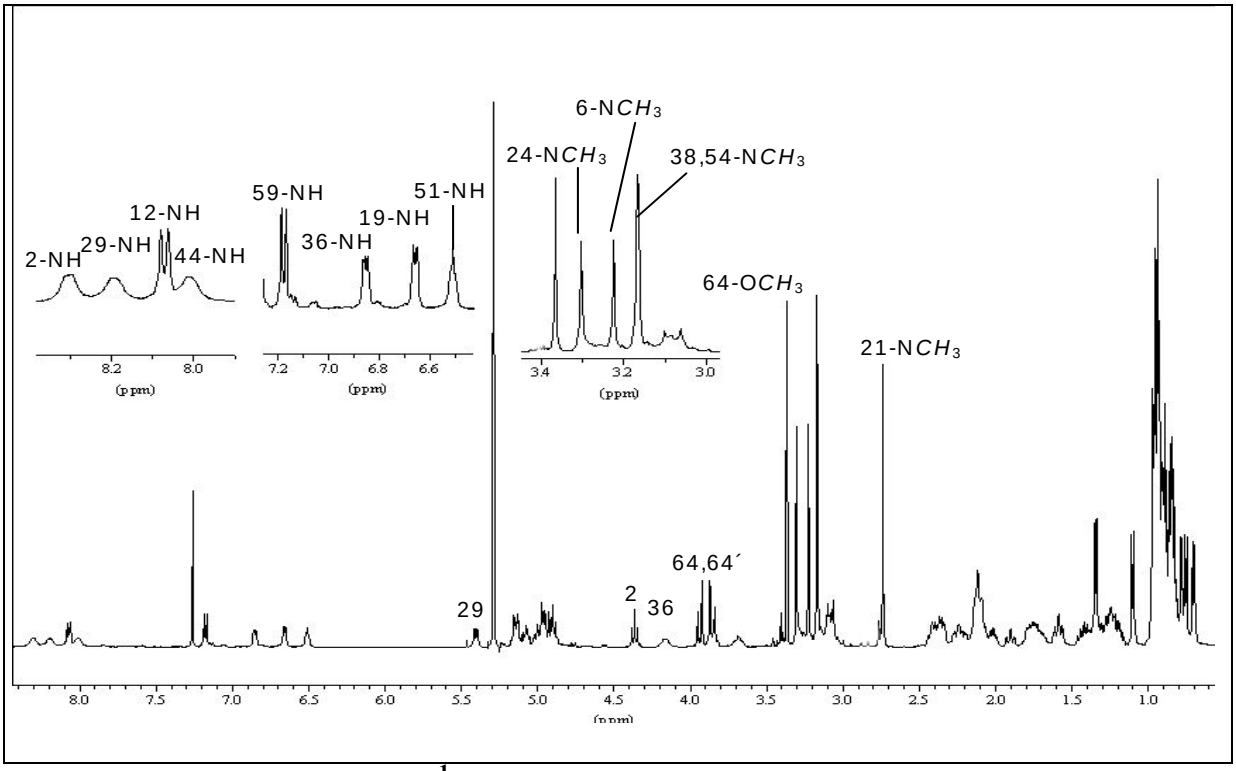


Fig. 3.121: ^{1}H NMR spectrum of compound 30

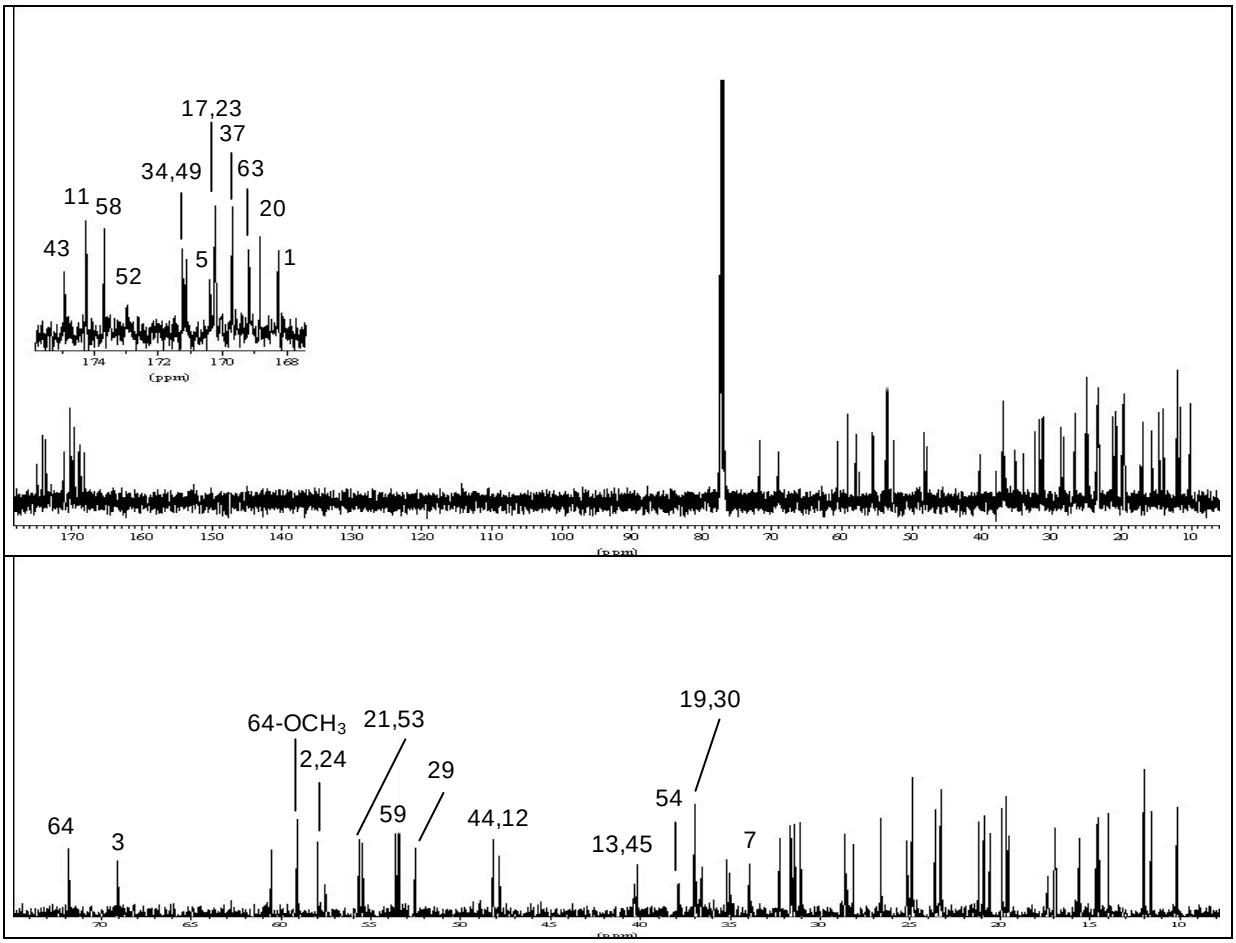
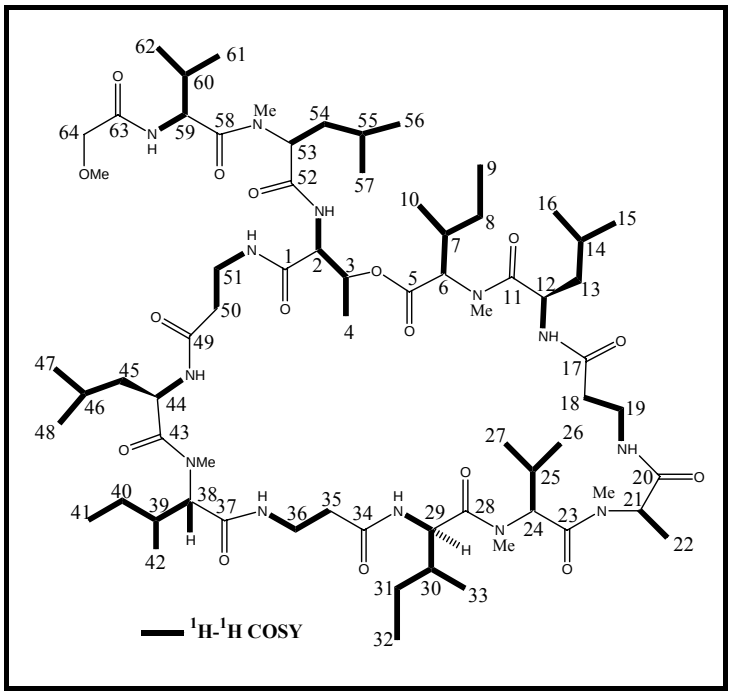


Fig. 3.122: ^{13}C NMR spectrum of compound 30



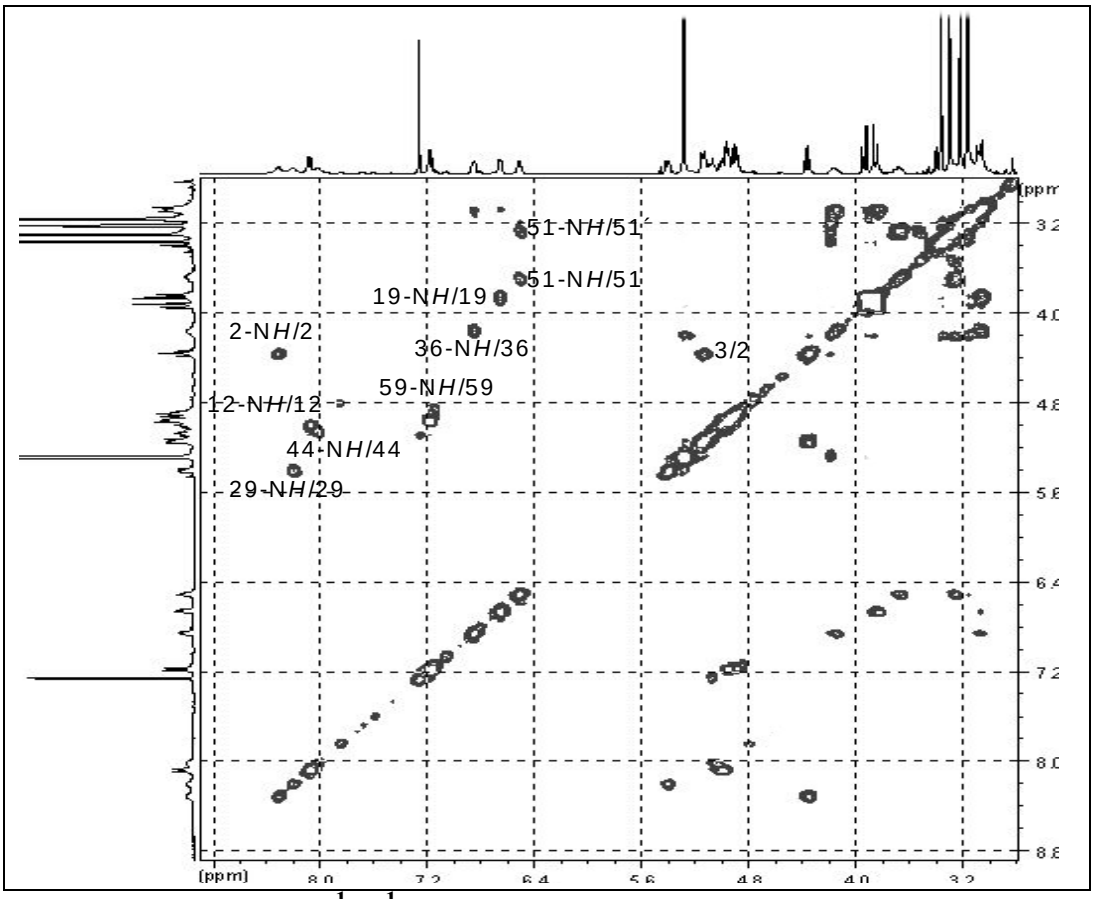


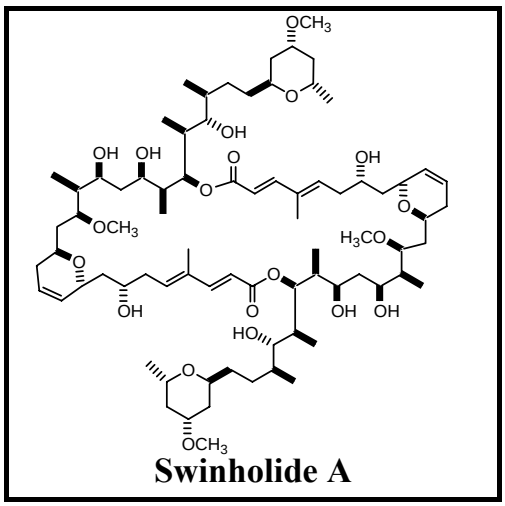
Fig. 3.123: ^{1}H-^{1}H COSY spectrum of compound 30

Table 3.39: NMR data of compound 30 ($CDCl_3$, 500, 125 MHz)

A. A.	Position	δ_H (m, *J* in Hz)	δ_C (m)	COSY	HMBC
L-Thr	1		168.2 s		
	2	4.38 (m)	57.5 d	3, 2-N*H*	1, 3
	3	5.16 (m)	69.0 d	2, 4	
	4	1.09 (d, *J* = 6.9 Hz)	17.3 q	3	2, 3
	2-N*H*	8.31 (brs)		2	1
	O*H*				
D-allo-Me-Ile	5		170.4 s		
	6	5.15 (m)	69.0 d	7	
	7	2.43 (m)	33.9 d	6	
	8	1.78 (m)	28.4 t	6, 9	7
	8´	1.35 (m)			
	9	0.96 (t, *J* = 6.9 Hz)	11.6 q		8
	10	0.78 (d, *J* = 6.9 Hz)	14.5 q		8
	6-NCH_3	3.23 (s)	40.2 q		11
D-Leu	11		174.2 s		
	12	5.02 (m)	48.0 d	12-N*H*	
	13	1.60 (m)	40.3 t	13´	
	13´	1.26 (m)		13, 14	
	14	1.71 (m)	24.8 d	16, 13´	
	15	0.97 (d, *J* = 6.7 Hz)	23.5 q		14
	16	0.96 (d, *J* = 6.7 Hz)	20.8 q		
	12-N*H*	8.08 (d, *J* = 9.1 Hz)		12	
β-Ala	17		170.2 s		
	18	2.37 (m)	36.6 t	18´	
	18´	2.10 (m)		18	
	19	3.88 (m)	36.9 t	19-N*H*, 19´	
	19´	3.10 (m)		18´, 19-N*H*, 19´	20
	19-N*H*	6.66 (d, *J* = 6.0 Hz)		19, 19´	
L-Me-Ala	20		168.8 s		
	21	4.92 (m)	55.6 d	22	22
	22	1.36 (d, *J* = 6.6 Hz)	14.6 q	20, 21	20, 21
	21-NCH_3	2.73 (s)	28.6 q	21, 23	21, 23
L-Me-Val	23		170.2 s		
	24	4.93 (m)	57.9 d		25
	25	2.37 (m)	28.1 d	27	
	26	0.91 (d, *J* = 6.9 Hz)	19.8 q		25
	27	0.87 (d, *J* = 6.9 Hz)	19.4 q	25	25, 26
	24-NCH_3	3.30 (s)	31.4 q	24, 28	24, 28
D-allo-Ile	28		176.3 s		
	29	5.40 (dd, *J* = 9.7, 2.8 Hz)	52.5 d	29-N*H*, 30	
	30	1.75 (m)	37.0 d	31, 32, 33	
	31	1.44 (m)	26.6 t	30, 31´, 32	
	31´	1.20 (m)		31, 30	
	32	0.98 (d, *J* = 6.8 Hz)	12.0 q		
	33	0.72 (d, *J* = 6.8 Hz)	13.9 q	30	29, 30, 31
	29-N*H*	8.20 (brs)		29	
β-Ala	34		171.1 s		
	35	2.40 (m)	35.2 t	35´, 36´	
	35´	2.20 (m)		35, 36´	
	36	4.18 (brs)	35.0 t	36´, 38, 36-N*H*	
	36´	3.10 (m)		36, 35, 35´, 36-N*H*	35
	36-N*H*	6.85 (dd, *J* = 7.9, 2.8 Hz)		36, 36´	
L-Me-Ile	37		169.7 s		
	38	4.98 (m)	60.5 d		39

	39	2.10 (m)	32.2 d	42	
	40	1.29 (m)	24.8 t	40´, 41	
	41	0.97 (t, *J* = 6.9 Hz)	10.1 q		
	42	0.85 (d, *J* = 6.9 Hz)	15.6 q	39	38, 39
	38-N-C*H*$_3$	3.15 (s)	31.0 q		38
D-Leu	43		174.9 s		
	44	5.0 (m)	48.2 d		
	45	1.60 (m)	40.2 t	45´	
	45´	1.24 (m)		45, 44, 46	
	46	1.78 (m)	24.9 d	45, 45´, 47, 48	
	47	0.90 (d, *J* = 6.6 Hz)	23.2 q		45
	48	0.97 (d, *J* = 6.6 Hz)	21.2 q		
	44-N*H*	8.02 (brs)		44	
β-Ala	49		171.2 s		
	50	2.27 (m)	36.9 t	50´, 51, 51´	
	50´	2.12 (m)		50, 51	52
	51	3.71 (m)	36.6 t	51´	
	51´	3.28 (m)		50, 50´, 51	
	51-N*H*	6.50 (brt, *J* = 4.4 Hz)		51, 51´	
D-Me-Leu	52		173.0 s		
	53	5.10 (m)	55.4 d		54
	54	1.92 (m)	37.9 t	53, 54´, 55	
	54´	1.40 (m)		54	
	55	1.36 (m)	25.1 d	54, 57	
	56	0.93 (d, *J* = 6.9 Hz)	23.3 q		
	57	0.80 (d, *J* = 6.9 Hz)	20.6 q	55, 54	55, 54
	53-NC*H*$_3$	3.16	31.6 q	58, 53	53, 58
L-Val	58		173.7 s		
	59	4.98 (m)	53.6 d	29-N*H*	
	60	2.02 (m)	31.6 d	61, 62	
	61	0.99 (d, *J* = 6.5 Hz)	19.6 q		59, 60
	62	0.86 (d, *J* = 6.5 Hz)	16.9 q		59, 60, 61
	59-N*H*	7.18 (d, *J* = 9.1 Hz)		59	63
MeOAc	63		168.8 s		
	64	3.93 (d, *J* = 15.0 Hz)	71.8 t	63, 64-OC*H*$_3$	63
	64´	3.85 (d, *J* = 15.0 Hz)		63, 64-OC*H*$_3$	63, 64-OC*H*$_3$
	64-OC*H*$_3$	3.36 (s)	59.0 q	64	64

3.4.3. Swinholide A (31, known compound)



Swinholide A

Compound **31** was isolated as a white amorphous powder. It has a UV absorbance at λ_{max} 254 nm. This compound has a molecular weight of 1390 g/mol, as derived from the FABMS spectrum that showed molecular ion peaks at *m*/*z* 1391 $[M+H]^+$ and 1413 $[M+Na]^+$. The ^{1}H NMR spectrum of the compound was considerably simplified showing only half of the expected signals, which suggested a dimeric nature of compound **31** which comprises two identical subunits (Fig. 3.125). Five olefinic protons at δ 7.56 (1H, d, *J* = 15.4 Hz, H-3), 6.08 (1H, dd, *J* = 9.5, 1.9 Hz, H-5), 5.81 (1H, d, *J* = 15.7 Hz, H-11), 5.77 (1H, d, *J* = 15.4 Hz, H-2), 5.70 (1H, dd, *J* = 15.7, 1.9 Hz, H-10) were observed. Two spin systems were pointed out clearly in the COSY spectrum. The ^{1}H NMR spectrum also showed five methyl doublets [δ 1.16 (3H, d, *J* = 6.3 Hz, H_3-31), 0.99 (3H, d, *J* = 7.2 Hz, H_3-24), 0.97 (3H, d, *J* = 8.5 Hz, H_3-20), 0.84 (3H, d, *J* = 6.9 Hz, H_3-22) 0.80 (3H, d, *J* = 6.9 Hz, H_3-16)] and one olefinic methyl singlet at δ 1.75 (3H, s, H_3-4). Two methoxy singlets were observed at δ 3.25 (3H, s, 15-OCH_3) and 3.23 (3H, s, 29-OCH_3). Comparison of ^{1}H NMR data of compound **31** with those of swinholde A previously isolated from the Okinawan marine sponge *Theonella swinhoei* [Kobayashi, *et al*., 1990] proved that both compounds were identical in terms of their chemical shifts.

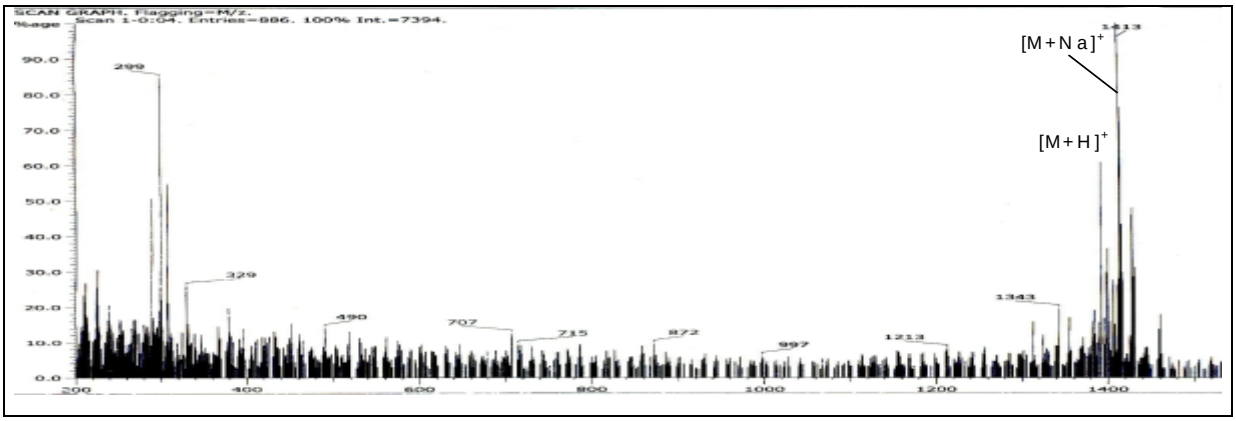


Fig. 3.124: FABMS spectrum of compound 31

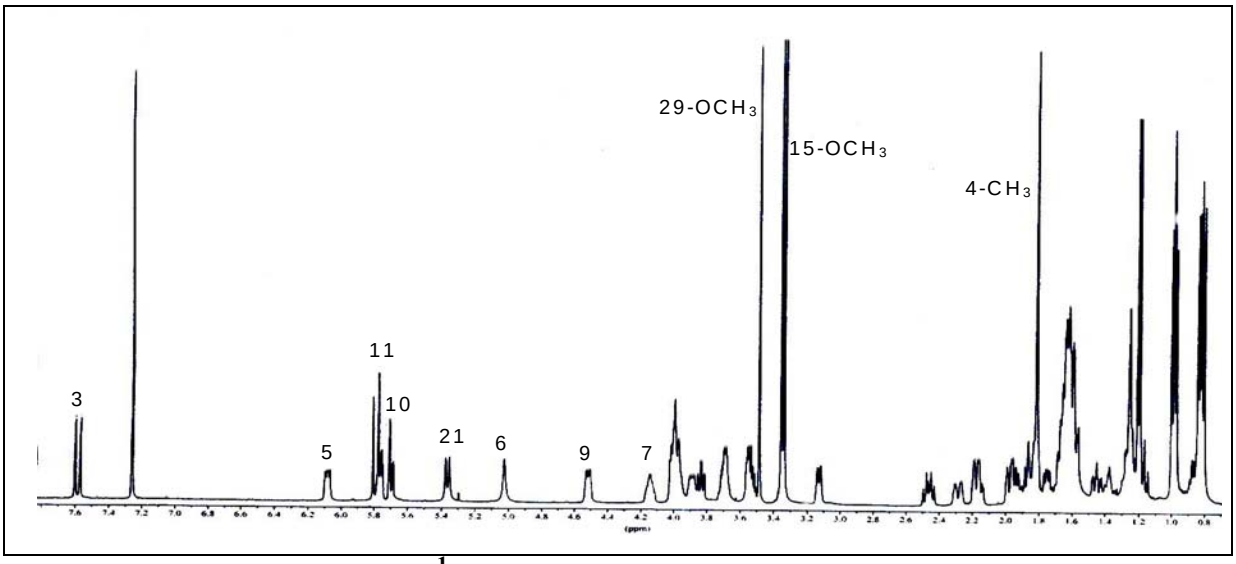


Fig. 3.125: ^{1}H NMR spectrum of compound 31

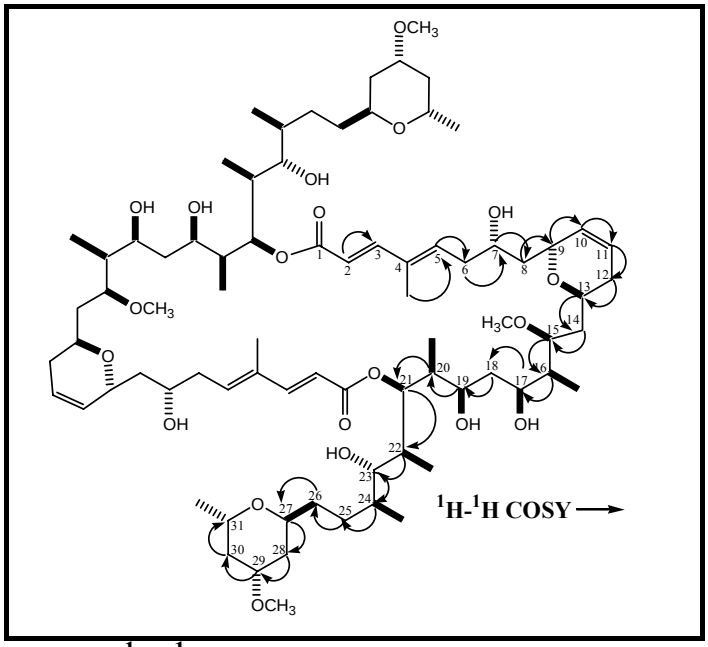
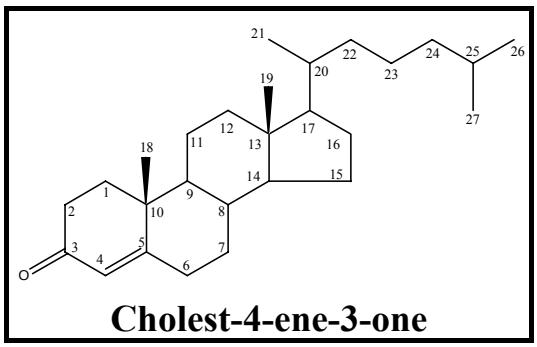


Fig. 3.126: ^{1}H-^{1}H COSY correlations of compound 31

Table 3.40: NMR data of compound 31 ($CDCl_3$, 500 MHz)

Position	δ_H (m, *J* in Hz)	COSY
2	5.77 (d, *J* = 15.4 Hz)	3
3	7.56 (d, J = 15.4 Hz)	2
4-CH_3	1.75 (s)	5
5	6.08 (dd, *J* = 9.5, 1.9 Hz)	6a, 6b, 4-CH_3
6	2.16 (dd, *J* = 15.5, 5.4 Hz), 2.46 (m)	
7	4.14 (brs)	8, 6a
8	1.58 (m), 1.63 (m)	7, 9
9	4.51 (brd, *J* = 8.2 Hz)	8
10	5.70 (d, *J* = 15.7, 1.9 Hz)	9
11	5.81 (d, *J* = 15.7 Hz)	12a, 12b
12	1.82 (m), 2.27 (d, *J* = 5.7 Hz)	11, 13
13	3.86 (m)	12a, 12b, 14a, 14b
14	1.46 (m), 2.14 (m)	15
15	4.01 (m)	14a, 14b, 16
15-OCH_3	3.25 (s)	
16	1.68 (m)	16- CH_3
16-CH_3	0.80 (d, *J* = 6.9 Hz)	16
17	3.83 (m)	18
18	1.62 (m), 1.69 (m)	17
19	3.98 (m)	
20	1.75 (m)	21, 20-CH_3
20-CH_3	0.97 (d, *J* = 8.5 Hz)	20
21	5.3 (d, *J* = 10.4 Hz)	20
22	1.95 (m)	23, 22-CH_3
22-CH_3	0.84 (d, *J* = 6.9 Hz)	22
23	3.1 (d, *J* = 8.4 Hz)	22
24	1.65 (m)	25a, 25b, 24-CH_3
24-CH_3	0.99 (d, *J* = 7.2 Hz)	24
25	1.27 (m), 1.38 (m)	26
26	1.30 (m), 1.90 (m)	25, 27
27	3.95 (m)	26a, 26b
28	1.60 (d, *J* = 7.2 Hz), 1.82 (m)	29
29	3.46 (m)	28a, 28b, 30a, 30b
29-OCH_3	3.23 (s)	
30	1.8 (m)	30b
	1.95 (m)	30a
31	3.65 (m)	30, 31-CH_3
31-CH_3	1.16 (d, *J* = 6.3 Hz)	31

3.4.4. Cholest-4-ene-3-one (32, known compound)



Compound **32** was isolated as a yellowish white amorphous powder. It has UV absorbances at λ_{max} 201 and 240 nm. The EIMS spectrum showed intense peaks at *m*/*z* 369, 342, 327, 299 and 271, corresponding to the following hypothetical fragmentation $[M\text{-}CH_3]^+$, $[M\text{-}C_3H_6]^+$, $[M\text{-}C_4H_9]^+$, $[M\text{-}C_6H_{13}]^+$ and [M-side chain]$^+$, respectively [Sheikh, *et al*., 1974]. The EIMS gave molecular weight of 384 g/mol. The downfield singlet signal at δ 5.73 (H-4) in the ^{1}H NMR spectrum revealed the existence of a double bond in the steroid skeleton. Other characteristic signals were four methylene groups protons at δ 2.23-2.46 (H_2-2,6), 2.02 (H_2-7) and 1.86 (H_2-1). In the upfield region of ^{1}H NMR spectrum three doublet methyl groups at δ 0.91 (d, J = 6.3 Hz, H_3-21), 0.87 (d, J = 6.9 Hz, H_3-26) and 0.85 (d, J = 6.9 Hz, H_3-27) and two methyl singlets at δ 1.17 (H_3-19) and 0.70 (H_3-18) were observed. Comparison of the ^{1}H NMR spectrum of compound **32** with that of cholest-4-ene-3-one from Aldrich (1992) confirmed that both compounds were identical. Also the MS fragmentation was identical to the one reported for **cholest-4-ene-3-one** [Sheikh, *et al*., 1974].

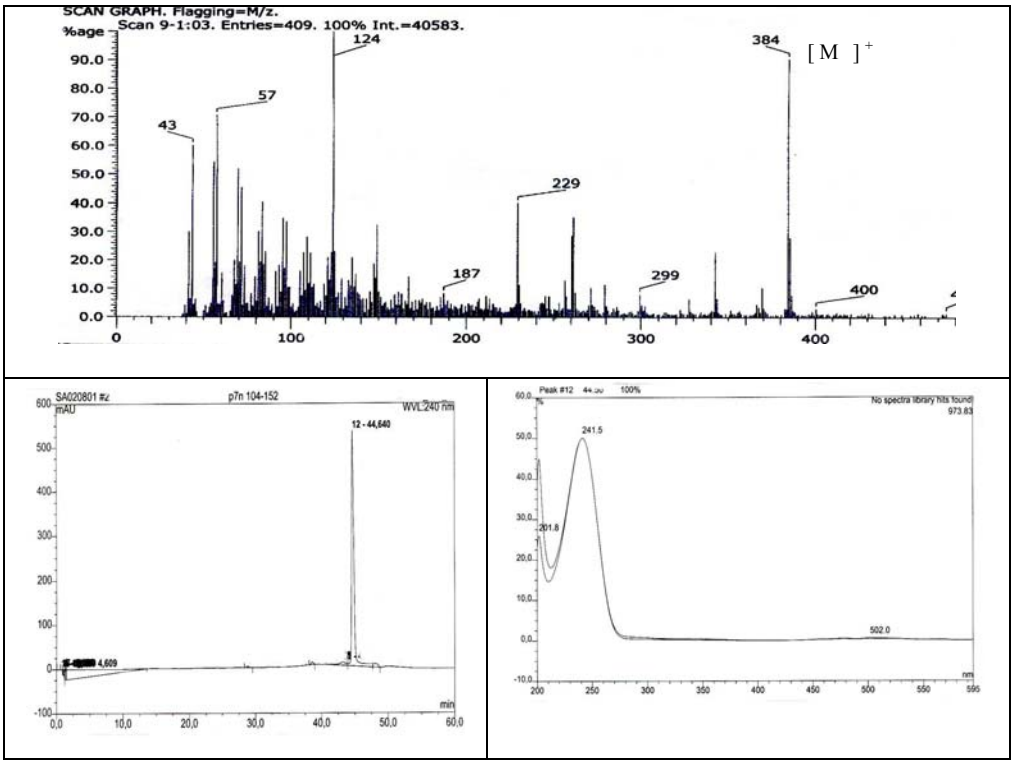


Fig. 3.127: EIMS and UV spectra of compound 32

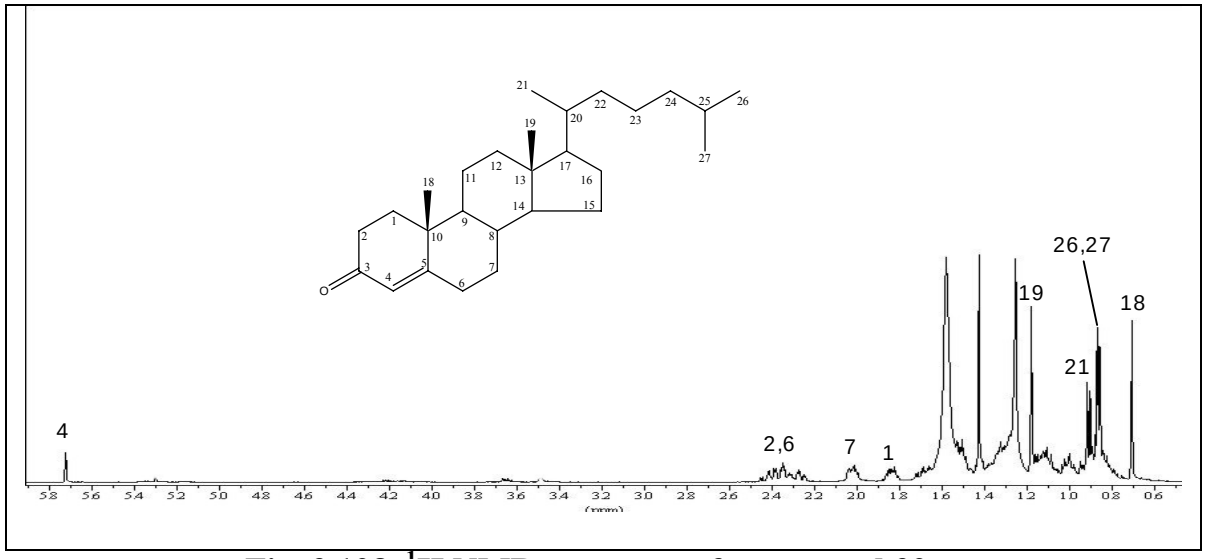


Fig. 3.128: ^{1}H NMR spectrum of compound 32

Table 3.41: NMR data of compound 32 ($CDCl_3$, 500 MHz)

proton	δ_H (m, *J* in Hz)
1	1.86 (m)
2	2.43, 2.32
3	
4	5.73 (s)
18	0.70 (s)
19	1.17 (s)
21	0.91 (d, *J* = 6.3 Hz)
26	0.87 (d, *J* = 6.9 Hz)
27	0.85 (d, *J* = 6.9 Hz)

4. Discussion

4.1. Metabolites isolated from the sponge *Callyspongia aerizusa*

Cytotoxic cyclic peptides (callyaerins)

Marine sponges continue to attract attention as a rich source of chemically diverse pharmacologically active secondary metabolites. A number of cyclic peptides has been isolated from the organic extract of the marine sponge belonging to the genus *Callyspongia* [Min, *et al*., 2001, Berer, *et al*., 2004]. The ethyl acetate extract of the investigated sponge *Callyspongia aerizusa* from Indonesia, exhibited strong activity in the brine shrimp assay. Chemical investigation of the ethyl acetate extract yielded the new cyclic peptide callyaerin S (**2**), together with the known peptides callyaerin E (**1**), B (**3**), D (**4**), and F (**5**).

The structures of callyaerins comprise a cyclic peptide part and a linear peptide chain joined together by a structurally unusual 2,3-diamino-2-propenoic acid (DAPA) functional group [Svete, *et al*., 1997]. This functionality is formed by the condensation of the aldehyde of α-formyl glycine (Fgly) and the N-terminal amino acid amino group [Berer, *et al*., 2004] to give the conjugated acrylamide functionality via a carbinol amine through isomerization of the Schiff base (Fig. 4.1) [Berer, *et al*., 2004].

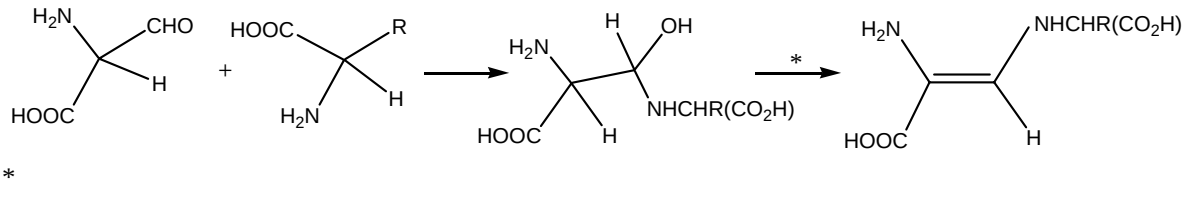


Fig. 4.1: Suggested mechanism for the formation of the acrylamide functionality [Berer, *et al*., 2004]

The occurrence of α-formyl glycine has already been reported for both eukaryotic and prokaryotic sulfatases within their catalytic sites [Dierks, *et al*., 1998, Chruzcz, *et al*., 2003]. It was shown that Fgly is generated by oxidation of cysteine or serine and furthermore Fgly hydrate is covalently sulfated [Dierks, *et al*., 1998] or could be covalently phosphorylated [Chruzcz, *et al*., 2003] during catalysis.

Biosynthesis of peptides

Non ribosomal cyclic peptides and depsipeptides are a large group of compounds known from terrestrial microorganisms. The first report of these peptides was their occurrence in *Fusarium* and *Streptomyces*, as well as in marine organisms [Schemjakin, 1960, Faulkner, 2002].

The majority of cyclic peptides are synthesised non-ribosomally on large multifunctional enzymes called nonribosomal peptide synthetases (NRPSs). They are assembled from diverse groups of precursors including pseudo, non proteinogenic, hydroxy, *N*-methylated, and D-amino acids. NRPS derived peptides also undergo further modifications that lead to additional structural diversity [Marahiel, *et al*., 1997, Schwarzer, *et al*., 2003]. A typical NRPS module consists of three steps as shown in Fig. 4.2:

Step 1. Adenylation (**A**) domain: responsible for amino acid activation.

Step 2. Thiolation (**T**) domain or peptidyl carrier protein (PCP), for thioesterification of the activated amino acids by the attachment to the cofactor, phosphopantetheine.

Step 3. Condensation (**C**) domain which catalyzes the peptide bond formation to elongate the growing peptide chain.

The modification of the amino acyl and/or peptidyl substrates during this process is shown in Fig. 4.3, such as epimerization (**E**) domain for the conversion of an L- to D- configuration of an amino acid [Marahiel, *et al*., 1997, Schwarzer, *et al*., 2003]; methylation (**MT**) domain is responsible for the *N*-methylation of the amide nitrogen [Marahiel, *et al*., 1997, Schwarzer, *et al*., 2003]; a cyclization (**Cy**) domain for the formation of heterocyclic rings [Konz, *et al*., 1997]; a reduction (**R**) for reductive release of an aldehyde product [Ehmann, *et al*., 1999]; and oxidation (**Ox**) domain for the conversion of a thiazoline to thiazole or for α-hydroxylation of the incorporated amino acid (**Ox´**) [Silakowski, *et al*., 1999, Schwarzer, *et al*., 2003].

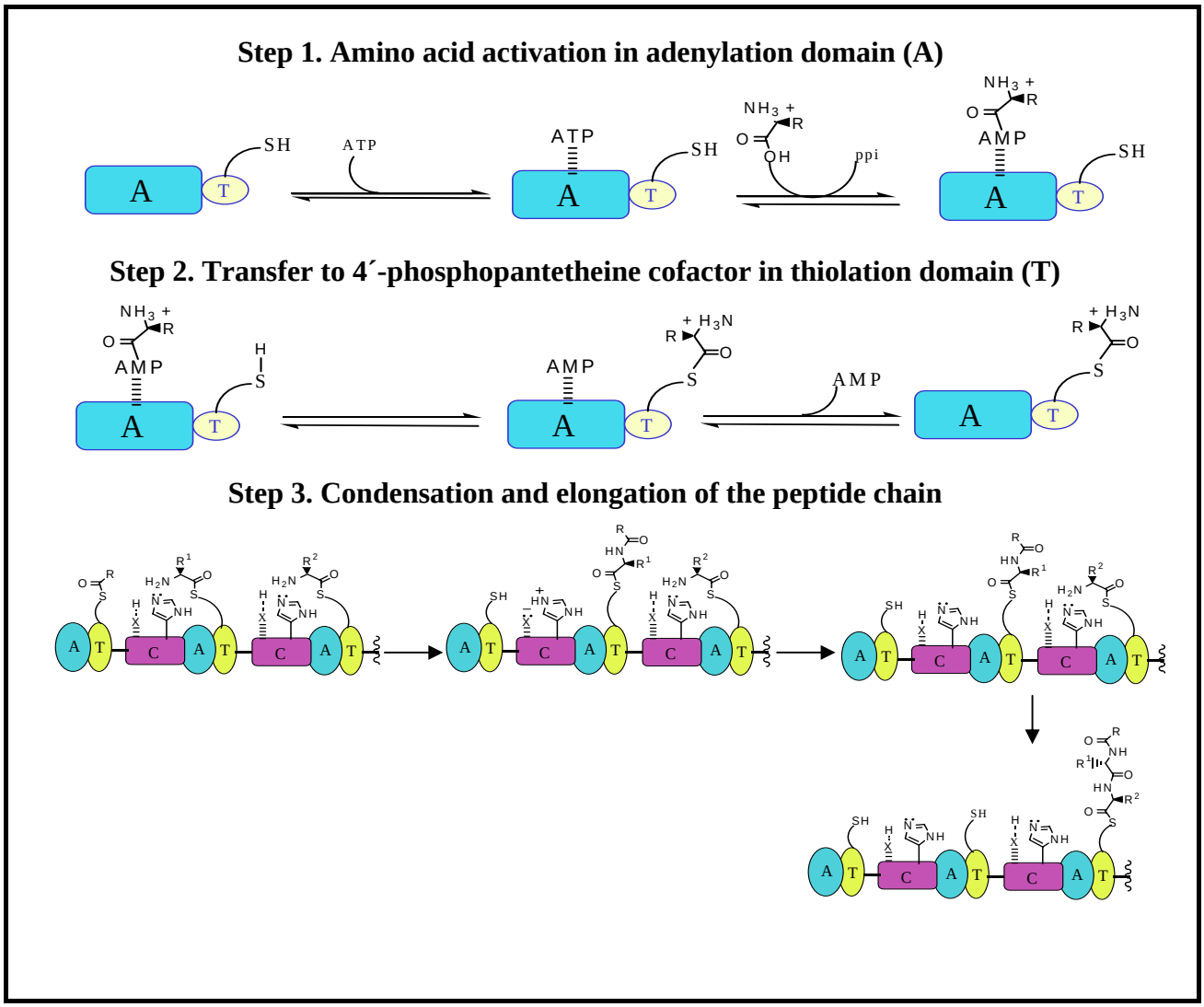


Fig. 4.2: Modular peptide synthesis [Marahiel, *et al.*, 1997]

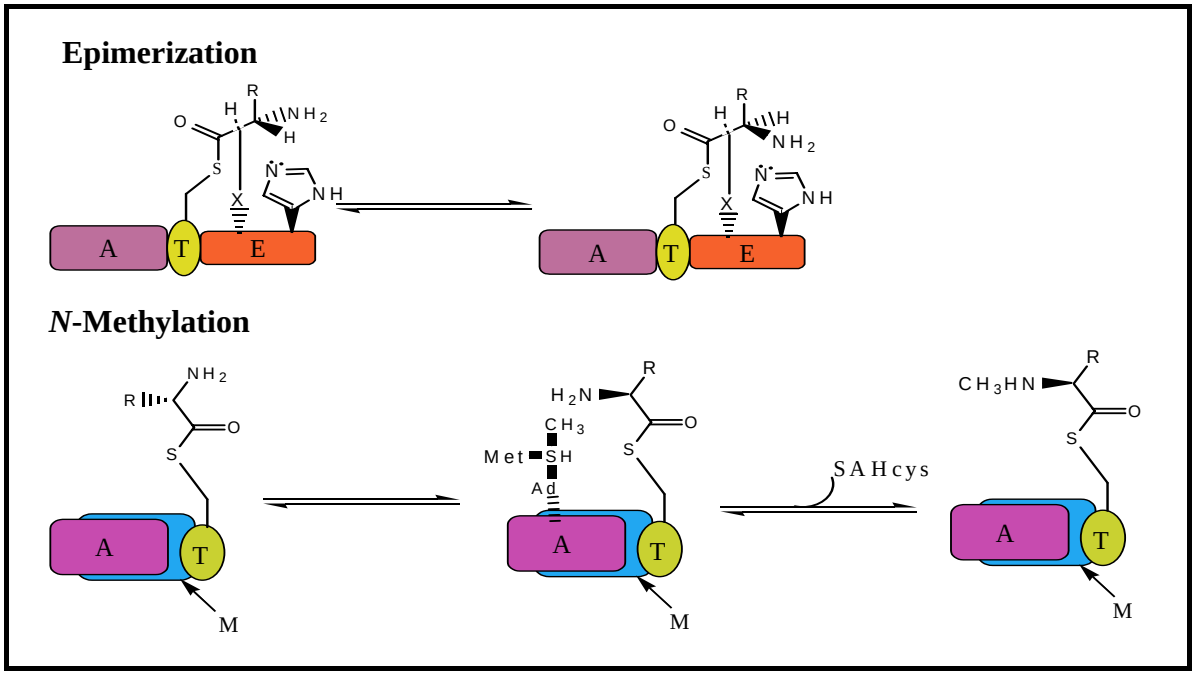


Fig. 4.3: Modification in peptide synthesis [Marahiel, *et al.*, 1997]

Peptides are of extraordinary pharmacological importance. The activity of peptides reaches from antibiotics over immunosuppressive, cytostatic to cytotoxic activities. These compounds often have molecular weights in the range of 300 – 2000 mass units. For example, the ACV-tripeptide is a precursor of the antibiotic penicillin and cephalosporine [Byford, *et al*., 1997]. Cyclosporin A is an immunosuppressive agent, and is used in the aftercare of organ transplantations [Du, *et al*., 2001]. Bleomycin A_2 exhibits cytostatic activity [Du, *et al*., 2000]. Ziconotide has successfully undergone phase III clinical trials for two therapeutic applications, to alleviate pain associated with malignant diseases (cancer and AIDS) and as an analgesic for non malignant neuropathic pain [Olivera, 2000, Proksch, *et al*., 2002]. Natural peptides of varying complexity are also abundant. For example, tripeptide glutathione (stimulates tissue growth), bradykinin (hypotensive vasodilator acts on smooth muscle), oxytocin (uterus contracting hormone also stimulates lactation) and insulin (pancreatic hormone, used in treatment of diabetes) are widely distributed natural peptides.

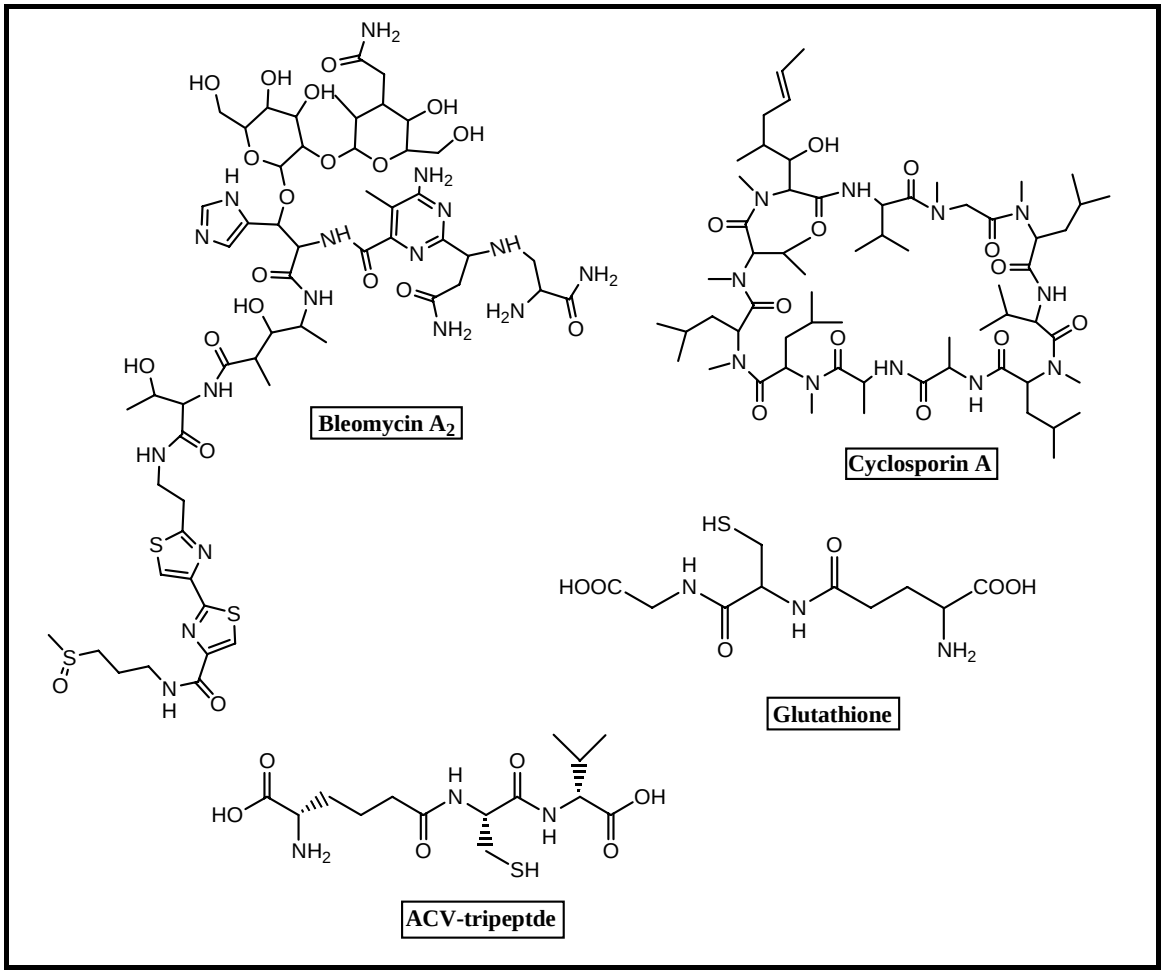


Fig. 4.4: Examples of some bioactive peptides

Relationship between structures and cytotoxic activities of callyaerins

In the brine shrimp assay of the isolated compounds from *C. aerizusa*, callyaerin E was active, callyaerin S showed moderate activity, while callyaerin B was inactive. Like in the brine shrimp assay, callyaerin E was also found to have strong activity against mouse lymphoma cell L5178Y, HELA cervix carcinoma, and brain tumour PC12 cells. On the other hand, callyaerins S and B showed only strong activity towards the mouse lymphoma cell L5178Y and no activity against either HELA cervix carcinoma or brain tumour PC12 cells. Callyaerin F exhibited no activity on any of the cell lines tested (see Tables 3.6 and 3.7). These results appear to prove that the difference between the size of the cyclic peptide and the peptide chain plays a role in the activity. Callyaerins E and S were found to be potent. They possess seven amino acid residues in the cyclic peptide, four and two amino acids in the chain, respectively. Callyaerin B which was active has four amino acids in the ring and seven in the chain. While, callyaerin F was inactive and has five amino acids in the ring and four in the chain. The bigger cyclic peptide part as in callyaerins E and S may allow the compounds to fit its receptors, respectively. Furthermore, it is suggested that the presence of phenylalanine in the side chain may decrease the activity of the peptides as in callyaerin F, while its presence in the ring may increase the activity as found in callyaerins E and S. In the antiviral (HIV) test, only callyaerin E showed moderate activity, while the other callyaerins B, D, F and S have no activity.

4.2. Different metabolites isolated from the sponge *Diacarnus megaspinorhabdosa*

4.2.1. Cytotoxic norterpene peroxides

Investigation of the Indonesian sponge *Diacarnus megaspinorhabdosa* (family *Latrunculiidae*) led to the isolation of interesting terpene peroxide derivatives. Sponges of the genus *Diacarnus* are already known to produce terpene peroxides and other related metabolites, which had also been reported to have a wide variety of biological activities [El-Sayed, *et al*., 2001, Youssef, *et al*., 2003]. A literature survey showed that no study on the sponge species *D. megaspinorhabdosa* has been published [MarinLit. 2004]. Terpene peroxides had mainly been isolated from the hexane fractions. Chemical investigation of the hexane fraction yielded a series of terpene peroxides with cytotoxic activity. The compounds isolated include norditerpene cylic

peroxides nuapapuin A methyl ester (**6**), methyl-2-epinuapapuinoate (**7**), diacarperoxides A-C (**8, 10 and 11**), and methyl diacarnoate A (**9**), norsesterpene cyclic peroxides including monocyclic terpenes, epimuqubilin B (**17**) and diacarperoxide I (**18**), and bicyclic terpenes, diacarperoxide D (**12**), E (**13**), F (**14**), and G (**15**), in addition to the norsesterpene diacarperoxide H (**16**).

Terpene peroxides are a fascinating class of compounds isolated from both plants and marine organisms [Sperry, *et al*., 1998]. Additional challenges have also been addressed in defining the chirality of their multiple stereocenters [Sperry, *et al*., 1998, Youssef, *et al*., 2003]. There are several families of norterpene cyclic peroxides and they could be structurally classified according to their carbon skeleton frame work: acylic, monocyclic, and bicyclic [Capon, *et al*., 1997]. Norterpene peroxide acids and their methyl esters have been frequently isolated from marine sponges of different genera, which include *Prianos* [Kashman, *et al*., 1979, Manes, *et al*., 1984], *Mycale* [Capon, *et al*., 1987], *Sigmosceptrella* [Albericci, *et al*., 1979], *Latrunculia* [Ovenden, *et al*., 1998], and *Diacarnus* [D'Ambrosio, *et al*., 1998].

Proposed biosynthesis of terpene peroxides

The norditerpene dienes (**1**) and (**2**) and the norsesterpene dienes (**3**) and (**4**) (Fig. 4.5) represent plausible biosynthetic precursors to norterpene cyclic peroxides. Concerted oxygen addition to the (*E, E*)-diene followed by reduction of the resulting unsaturated peroxide intermediate would yield a saturated cyclic peroxide [Capon, *et al*., 1997] as shown in Fig. 4.6. The relative stereochemistry of cyclic peroxide moieties formed from (**1**)-(**4**) in this way would be the same as that found in the norsesterpene cyclic peroxide sigmosceptrellin C (**5**). Similar precursors differing in stereochemistry at C2, and with different double-bond geometry, could yield the other norterpene cyclic peroxides [Capon, *et al*., 1985, Capon, *et al*., 1997].

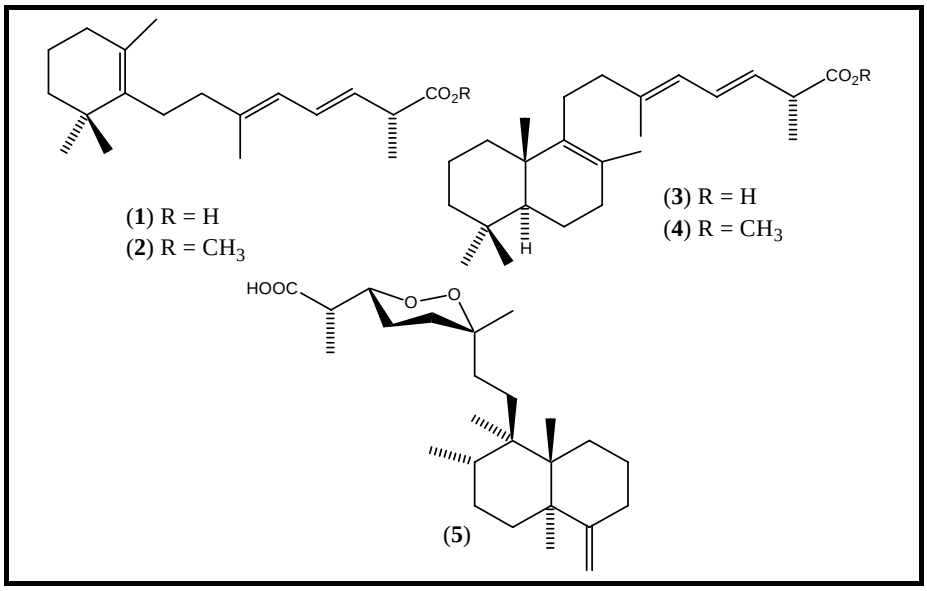


Fig. 4.5: Biosynthetic precursors of norterpene cyclic peroxides [Capon, *et al.*, 1997]

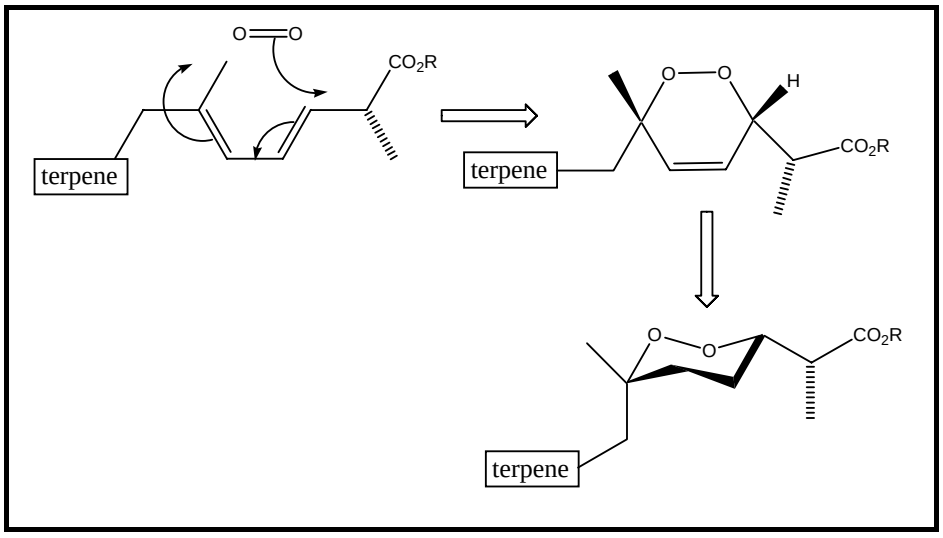


Fig. 4.6: Postulated biosynthesis of norterpene cyclic peroxides by oxygen addition to norterpene diene precursors [Capon, *et al.*, 1997]

Interest has usually been focused on this class of compounds because they frequently possess a variety of biologically active properties such as antimicrobial [Capon, *et al.*, 1985, Faulkner, *et al.*, 1991], ichthyotoxicity [Albericci, *et al.*, 1979, Albericci, *et al.*, 1982], sea urchin egg cell division inhibitor [Manes, *et al.*, 1984], cytotoxicity [Sperry, *et al.*, 1998], antiviral [Tanaka, *et al.*, 1993], antitoxoplasmosis [El-Sayed, *et al.*, 2001] and antimalarial activity [D´Ambrosio, *et al.*, 1998].

Mechanism of the antimalarial activity of cyclic peroxides

Malaria is endemic in Mexico, most of South America and Africa, the Middle East, Indochina, and Indonesia. Each day, 0.8 - 1.4 million people become sick, and 4800 – 7200 children die from malaria [D´ Ambrosio, *et al*., 1998]. Prospects about malaria are of a reemerging disease that may threaten even traditionally non-endemic areas and areas that had been freed of, or never touched by this plague [Mons, *et al*., 1998]. The multiform nature of this illness, with variability and acquired resistance of the responsible parasite, cause much concern and demand for new drugs. Cyclic peroxides proved more effective against chloroquine-resistant strains than against chloroquine-sensitive strains of the parasite. This suggests that the antimalarial mode of action of these epidioxy-terpenes on parasite growth may be different from that of the quinolinamines. Earlier studies have evidenced the susceptibility of plasmodium-infected erythrocytes to oxidant-mediated damages [Wozencraft, 1986, Vennerstrom, *et al*., 1988]. Artemisinin (qinghaosu) and its derivatives are characterized by a peroxide bridge embedded in a 1,2,4-trioxane (six-membered ring containing three oxygen atoms) pharmacophore, which is deemed essential for their antimalarial activity, since the corresponding acyclic compounds without the peroxidic bridge were found to be biologically inactive [Gu, *et al*., 1980, Fattorusso, *et al*., 2002]. Indeed, artemisinin induces lipid peroxidation *in vitro*. Several authors have suggested that the free radicals arising from its endoperoxide moiety are responsible for the death of malaria parasites inside the red blood cells [Beekman, *et al*., 1997]. Cyclic peroxides having the peroxide group in a very different arrangement than in artemisinin, exert their effects similarly to the latter in creating an oxidant stress on a similar model [D´ Ambrosio, *et al*., 1998].

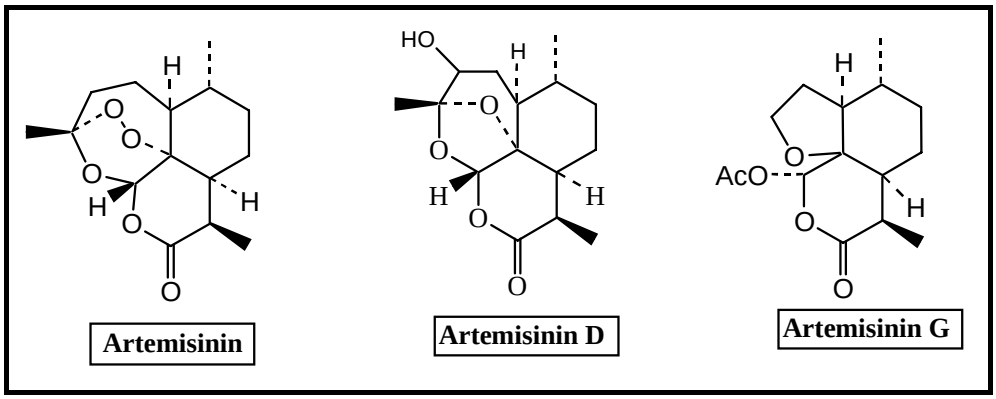


Fig. 4.7: Structures of artemisinin and its derivatives

Structure activity relationship of norterpene cyclic peroxides

According to the results of the cytotoxicity test of the isolated terpene peroxides from the sponge *D. megaspinorhabdosa* (see Tables 3.31 and 3.32), it is suggested that the presence of the ester moiety which was found in all of the congeners has no effect on the activity of the compounds. Replacement of the allylic methylene in diacarperoxide D (**12**) by a carbonyl group to give diacarperoxides E (**13**), F (**14**) and G (**15**) does not improve their cytotoxicity [D´Ambrosio, *et al.*, 1997]. Moreover, similar cytotoxicity for diacarperoxides E (**13**) and F (**14**) showed the unimportance of the configuration at C-2 [D´Ambrosio, *et al.*, 1997]. It was also suggested that the oxidative opening of the cyclohexene ring as in diacarperoxide B (**10**) and C (**11**) decreased the activity. Insertion of a prenyl unit in the central portion of the molecule made norsesterpenes cyclic peroxides more active than their norditerpene cyclic congeners [Benet, *et al.*, 1996, D´Ambrosio, *et al.*, 1997]. The longer side chain allowed the norsesterpenes to cross the cell membranes (lipophilicity effect) and made them to fit receptors (size effect) [D´Ambrosio, *et al.*, 1997]. It was also found that the role played by the peroxide group is difficult to judge since this group can not be removed without changing the nature of the molecule and because the literature terms of comparison of this functionality are scarce, except for antimalarial agents [Haynes, *et al.*, 1997].

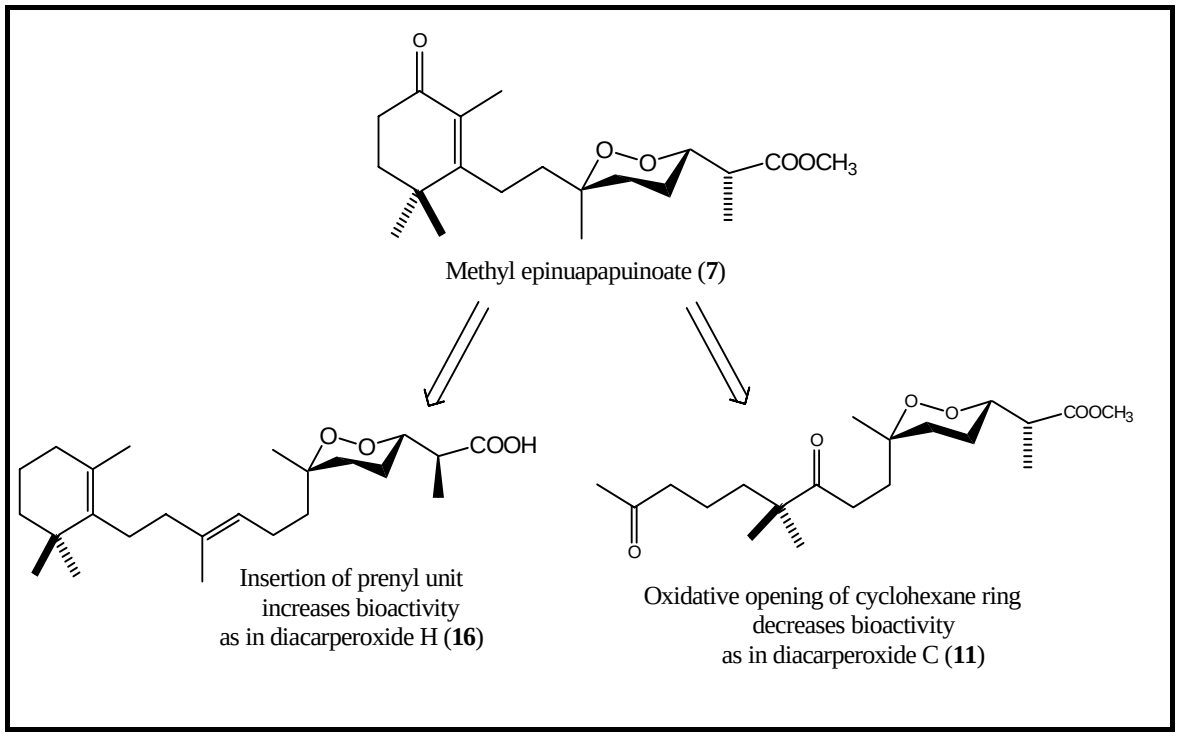


Fig. 4.8: Bioactivity of the cyclic peroxides

4.2.2. Indole, phenolic and nucleoside derivatives.

Chemical investigation of the ethyl acetate and *n*-butanol fractions of *Diacarnus megaspinorhabdosa* led to the isolation of seven compounds, two of them are indole alkaloids (diacarmine, indole-3-carboxyaldehyde), one is a phenolic compound (p-hydroxybenzylideneacetone), and four are nucleosides (compounds **22**, **23**, **24** and **25**).

Nucleoside antibiotics have been found to belong to diverse groups of secondary metabolites of microbial origin. A total of 163 nucleosides antibiotics has been classified on the basis of their structures. The biological activity of nucleoside antibiotics is also wideranging, comprising antibacterial, antifungal, immunosuppressive, antitumor, antiviral, herbicidal, insecticidal, and immunostimulating properties. Not only nucleic acid synthesis but also protein synthesis, glycan synthesis, and glycoprotein synthesis are targets of nucleoside antibiotics. Nucleoside antibiotics are thus potential candidates for the regulation of all aspects of cell growth and differentiation [Isono, 1998]. In the early 1950s, Bergmann discovered the nucleosides spongouridine and spongothymidine (Arabinose A and B). These compounds had antiviral activity. These discoveries aided in the development of antitumor agents and a generation of nucleoside antivirals. Zidovudine (AZT) was the first antiretroviral agent indicated for the treatment of HIV [Corey, *et al*., 1996]. Didanosine (ddI, Videx) and Zalcitabine (ddc, Hivid) are also used to treat HIV infection [Gulick, *et al*., 1997]. They inhibit viral reverse transcriptase [Pereira, *et al*., 2003]. Acyclovir inhibits the viral RNA polymerase and is active against herpes viruses (HSV) [Morfin, *et al*., 2003].

Cytotoxicity test of the isolated nucleoside compounds from the sponge *D. megaspinorhabdosa* against L5178Y mouse lymphoma cells showed that thymine 2´-deoxyriboside (**22**) has strong activity, while 2´-deoxyadenosine (**23**) and 2´-deoxyinosine (**24**) were inactive.

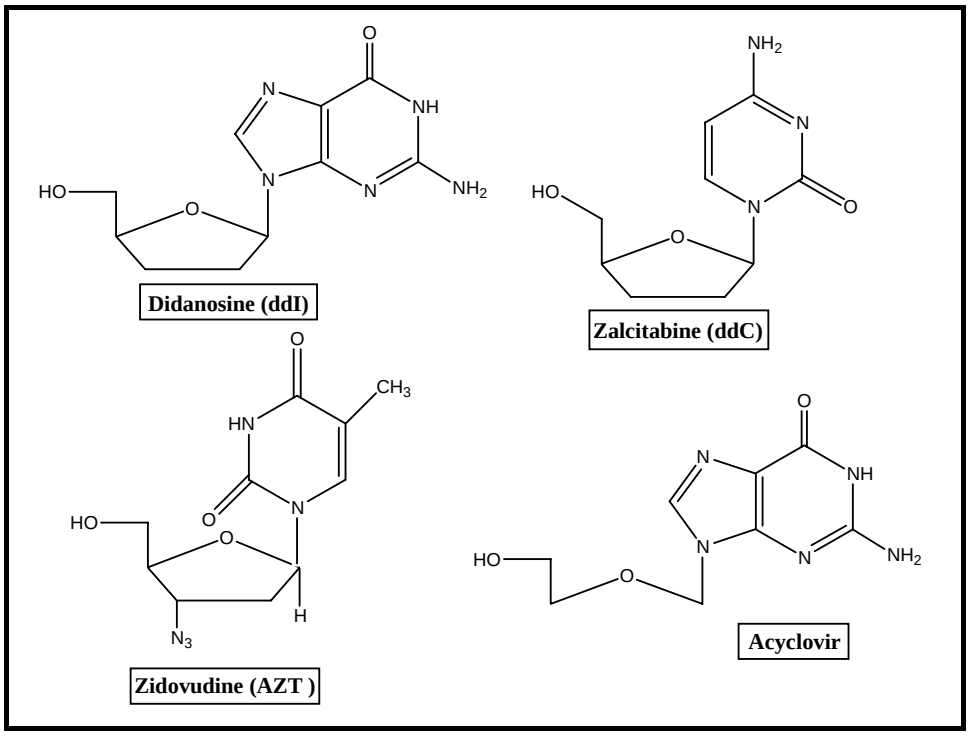


Fig. 4.9: Structures of nucleoside analogs

Mechanism of action of nucleoside analogs

As with endogenous nucleosides, the nucleoside analogs must be triphosphorylated into a nucleotide. Initially, the nucleoside analog biotransformation requires enzymes such as thymidine kinase, deoxycytidine kinase, or inosine phosphotransferase to form the monophosphate metabolite. Once in the triphosphorylated form, the dideoxynucleotide can compete with naturally occurring nucleotides for DNA incorporation. The dideoxynucleotide will for example be accepted by reverse transcriptase and become incorporated into the elongating viral complement DNA (cDNA). The absence of the 3'-hydroxyl group on the ribose moiety of these nucleosides will cause DNA elongation to be terminated [Graham, *et al.*, 1991, Collier *et al.*, 1996, Englund, *et al.*, 1997] as shown in figure 4.10.

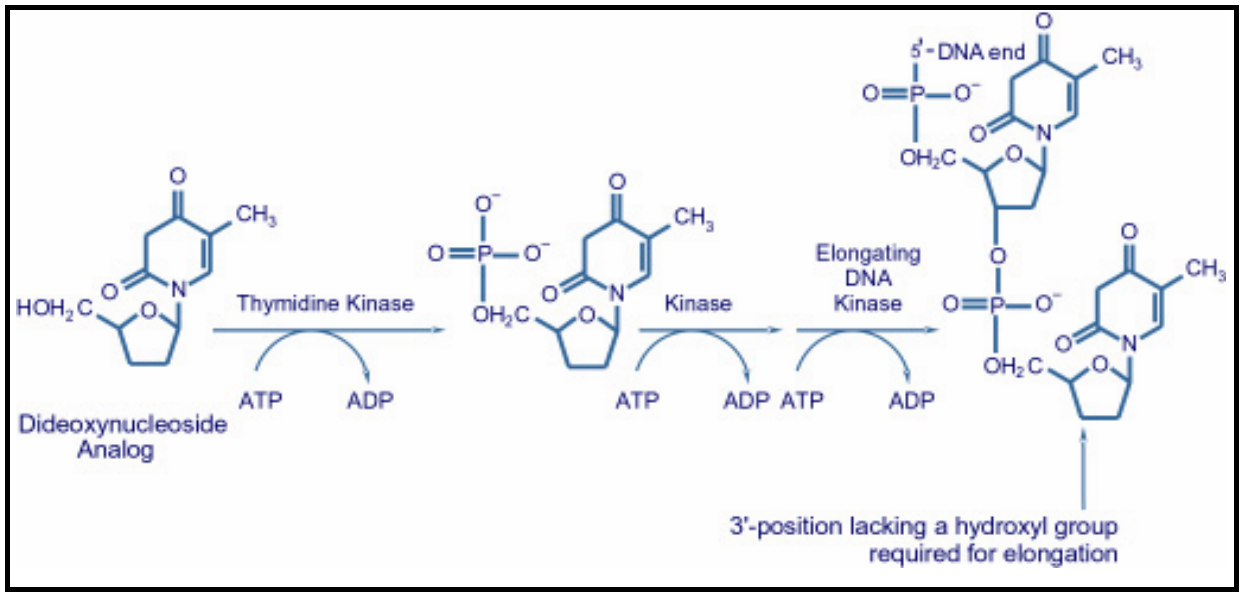


Fig. 4.10: Mechanism of action of nucleoside analogs

4.3. Metabolites isolated from the sponge *Acanthostrongylophora ingens*

4.3.1. β-carboline alkaloids.

This study describes the isolation and structure elucidation of pyrimidinyl carboline alkaloids. Compounds of this class were isolated previously from the bark of *Annona montana* [Leboeuf, *et al*., 1982] (family *Annonaceae*). They are the only examples of alkaloids in which a harmane moiety is linked to a 2-aminopyrimidinyl moiety. It is interesting to see compounds of this structural type isolated from marine sponges. Chemical investigation of the ethyl acetate extract of the sponge *Acanthostrongylophora ingens* afforded one new pyrimidinyl-β-carboline alkaloid, acanthomine A (**27**), together with the known annomontine (**26**) and 1-hydroxy-3,4-dihydronorharman (**28**). Acanthomine A is the 3,4-dihydro congener of annomontine.

Biosynthesis of β-carbolines

Indole alkaloids comprise a group of compounds of widespread natural occurrence, displaying considerable structural diversity. Many of the indole alkaloids contain an intact β-carboline skeleton. β-carbolines are defined as compounds comprising a tricyclic pyrido (3,4-b) indole ring system with alkyl substituents. The most commonly used trivial nomenclature is based on the root-harm, derived from an early isolation from *Peganum harmala* [Allen, *et al*., 1980].

Tryptophan is the major precursor for the indole moiety of the indole alkaloids containing a β-carboline skeleton [Gröger, 1969, Cordell, 1974]. Incorporation of

pyruvate -[^{14}C] and acetate -[^{14}C] into the pyridyl ring of simple β-carbolines has been demonstrated. It was presumed to take a place via Schiff base intermediates [Bernfeld, 1967, Dalton, 1979, Trossell, 1997]. Another biosynthetic pathway includes incorporation of a tryptophan moiety. A pyridoxyl phosphate-catalyzed decarboxylation yields tryptamine, which condenses with acetyl coenzyme A to give the *N*-acetyl tryptamine and subsequent cyclo-dehydration of the 3,4-dihydro-β-carbolines [Slaytor, *et al*., 1968, Dalton, 1979] as shown in figures 4.11 and 4.12.

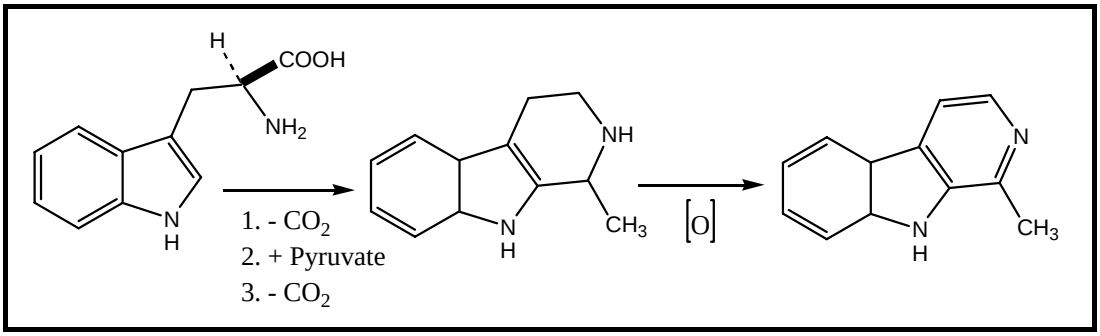


Fig. 4.11: Biosynthetic pathway of harmane from tryptophan [Trossell, 1997]

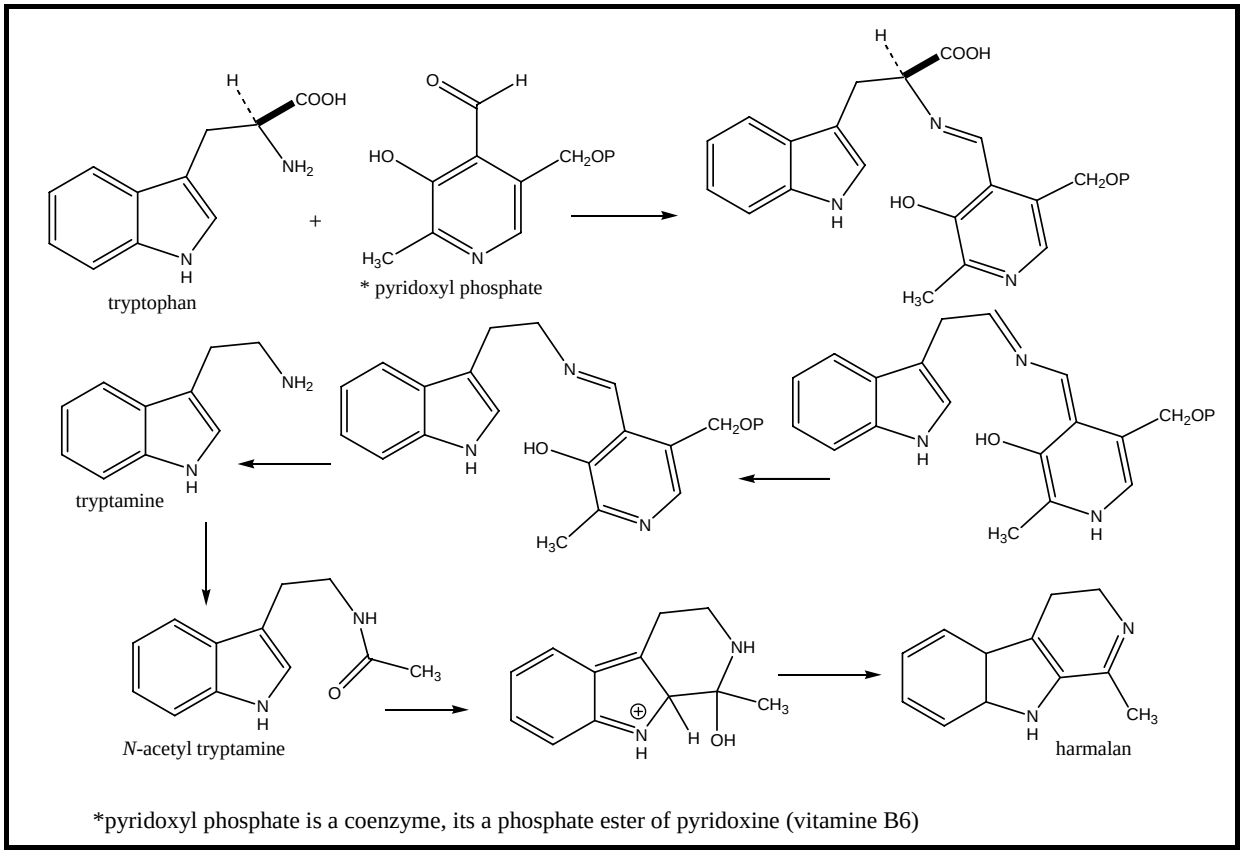


Fig. 4.12: Biosynthetic pathway of harmalan from tryptophan [Dalton, 1979]

Natural occurrence of β-carbolines

In addition to their natural occurrence in plants and marine organisms these alkaloids have been found to be present in tabacco smoke [Schumacher, *et al*.,1977], marijuana smoke, charred insects, and well-cooked food stuffs [Proliac, *et al*., 1976] which reflect their formation from pyrolysis [Poindexter, *et al*., 1962]. The presence of simple 1,2,3,4-tetrahydro-β-carbolines has been recorded in mammalian tissues [Mandel, *et al*., 1974, Honecker, *et al*., 1978] and their formation by the condensation of tryptamine with aldehyde formed as by-products *in-vitro* metabolism of animal tissue preparations has also been demonstrated [Lauwers, *et al*., 1975].

Role of β-carbolines in monoamine oxidase inhibation

β-carbolines were reported to inhibit ion transport and to blockade monoamine uptake [Sepulveda, *et al*., 1978, Drucker, *et al*., 1990]. Monoamine oxidase (MAO) is a critical enzyme in the oxidative deamination of biogenic amines throughout the body. β-Carbolines (BC), by themselves, may have some psychoactivity and thus may contribute to the overall psychotropic activity [Airaksinen, *et al*., 1981]. As MAO inhibitors, β-carbolines can increase brain levels of serotonin, and the primarily sedative effects of high doses of β-carbolines are thought to result from their blockade of serotonin deamination [Ho, *et al*., 1973, Bonnet, *et al*., 2004]. The natural β-carbolines closely resemble the neurotoxin 1-methyl-4-phenyl-1, 2, 3, 6- tetrahydro pyridine (MPTP) in structure [Collins, *et al*., 1986]. The *N*-methylated β-carbolinium ions (BC (+)) are potent inhibitors of mitochondrial respiration and are nigrostriatal neurotoxins [Collins, *et al*., 1992, Bonnet, *et al*., 2004]. There are several proteins to which BC bind with high affinity such as the enzyme carboxylesterase [Greube, *et al*., 2003], cytochrome P (450) 2E1, enzyme monoamine oxidase B and also a small G-protein of the Rho subfamily [Greube, *et al*., 1993]. It was found in a recent study that 2,9-dimethyl-BC (+) was the most potent inhibitor of TPI (triosephosphate isomerase) [Esnouf, *et al*., 1982]. Thus, TPI inhibition could contribute to neurodegeneration [McKenna, *et al*., 1984, Crosby, *et al*., 2003].

The simple β-carbolines have been found to act principally on the central nervous system and muscle [Allen, *et al*., 1980]. It was reported that the β-carboline alkaloids exhibited cytotoxic, antibacterial, and antiviral activities [Jha, *et al*., 2004]. In addition,

these compounds are widely studied for their activity in antiradialization and antitrypanosome as well as their mutagenic and co-mutagenic properties. Also they are potent and selective inhibitors of cyclin-dependent kinases [Li, *et al*., 1995, Pan, *et al*., 1997, Duan, *et al*., 1998].

The cytotoxicity test of the *β*-carboline alkaloids isolated from the ethyl acetate fraction of the Indonesian sponge *Acanthostrongylophora ingens* (Table 3.38) against mouse lymphoma cells showed that annomontine (**26**) has strong activity, while acanthomine A (**27**) and 1-hydroxy-3,4-dihydronorharman (**28**) have moderate activities. According to these results, it is suggested that the presence of the 2-aminopyrimidine moiety may increase the activity. Furthermore, the conversion of the harmane substructure in annomontine (**26**) to harmalan may decrease the activity of the compounds (see Fig. 4.13).

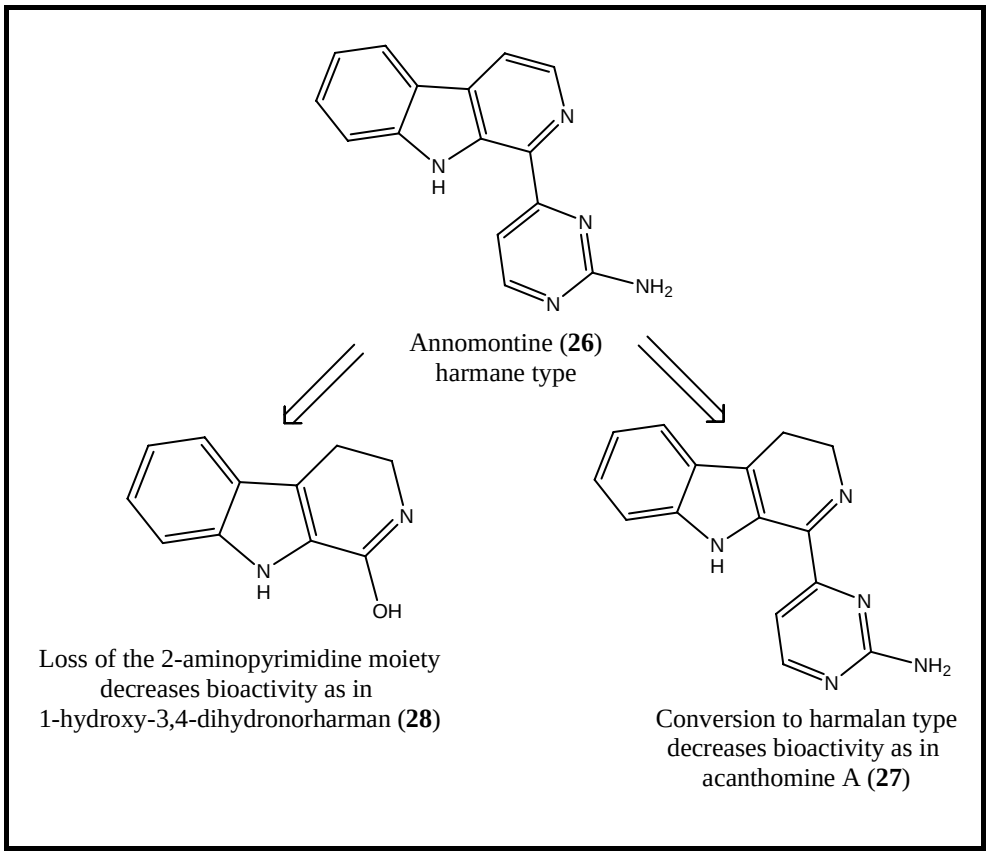


Fig. 4.13: Bioactivity of the *β*-carboline alkaloids

4.4. Metabolites isolated from the Indonesian sponge *Theonella swinhoei*

Numerous interesting compounds have been reported from the sponge *Theonella swinhoei* belonging to the order Lithistida. Theonellapeptolides (Ia and Id) (**29**, **30**) and swinholide A (**31**) (dimeric macrolide) were isolated from the EtOAc fraction of the Indonesian sponge *Theonella swinhoei*, in addition to cholest-4-ene-3-one (**32**) from the hexane fraction.

Theonellapeptolides are tridecapeptide lactones and have been reported from the Okinawan marine sponge *Theonella swinhoei* [Roy, *et al*., 2000]. These peptides are characterized by the presence of high proportions of D-amino acids, *N*-methyl amino acids, and β-amino acids. The biological activity of these compounds includes cytotoxicity [Roy, *et al*., 2000], ion-transport activity for Na^+ and K^+ ions [Kobayashi, *et al*., 1991], and Na^+, and K^+-ATPase inhibitory activity [Nakamura, *et al*., 1986]. Other theonellapeptolides have also been reported from a sponge *Lamellomorpha strongylata*, belonging to a different order [Li, *et al*., 1998].

Swinholide A is a dimeric macrolide, which contains a 44-membered dilactone moiety. It was shown to exhibit potent cytotoxic activity [Spector, *et al*., 1999, Tong, *et al*., 2001, Segal, *et al*., 2001].

5. Summary

Natural products isolated from marine organisms such as sponges, fungi and bacteria show a unique structural diversity which may lead to new metabolites which can be used for medicinal purposes. This study involved the isolation, structural elucidation and biological screening of the active constituents of some marine sponges collected from Indonesia using different chromatographic techniques and various tools of spectral analysis.

Four sponges have been used in this study and the compounds which have been isolated and structurally elucidated from them are summarized in the next table (Table 5.1)

5.1. The sponge *Callyspongia aerizusa*

The sponge *Callyspongia aerizusa* was successively extracted with MeOH and acetone, respectively. The combined extracts were concentrated to dryness and partitioned between hexane, EtOAc, *n*-BuOH and H_2O, respectively. EtOAc fraction was subjected to silica gel column chromatography followed by semi-preparative HPLC and RP-18 silica column chromatography to isolate the known compounds callyaerin B, E, F and D and the new compound callyaerin S. Callyaerin B, E and S showed strong cytotoxic activity. Callyaerin E had moderate antiviral activity, while the other callyaerins showed no activity.

5.2. The sponge *Diacarnus megaspinorhabdosa*

The sponge *Diacarnus megaspinorhabdosa* was successively extracted with MeOH and acetone, respectively. The combined extracts were concentrated to dryness and partitioned between hexane, EtOAc, *n*-BuOH and H_2O. The EtOAc fraction was subjected to vacuum liquid chromatography followed by semi-preparative HPLC to isolate the known compounds indole-3-carboxyaldehyde and p-hydroxybenzylideneacetone and the new compound diacarmine. The BuOH fraction was subjected to vacuum liquid chromatography followed by semi-preparative HPLC, by which four known compounds were isolated, including thymine 2´-deoxyriboside, 2´-deoxyadenosine, 2´-deoxyinosine and 2´-deoxyguanosine. The hexane fraction was subjected to VLC using a hexane-EtOAc gradient. The resulting fractions were

subjected to normal phase flash chromatography, normal phase silica gel column, sephadex LH-20 and reversed phase RP-18 column chromatography to afford nine new norterpene cyclic peroxides, diacarperoxides (A-I), in addition to four known compounds. All the isolated compounds showed interesting cytotoxic activities against different cancer lines.

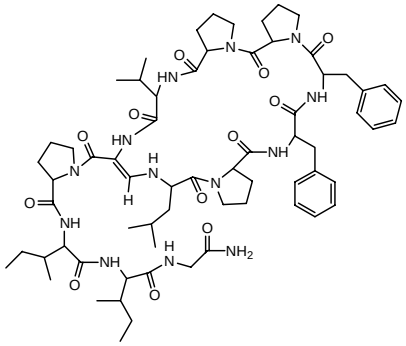
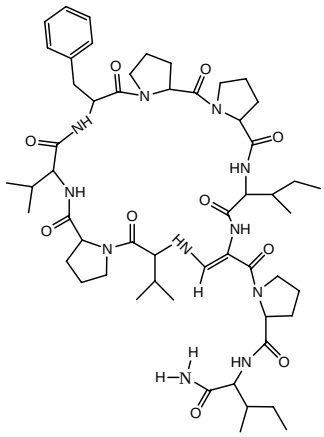
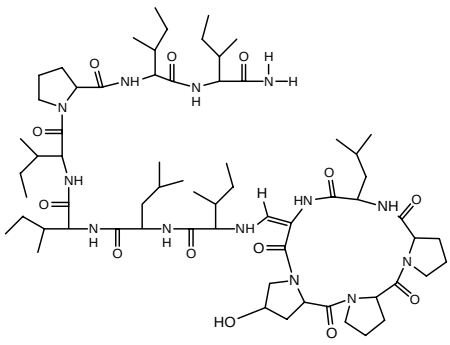
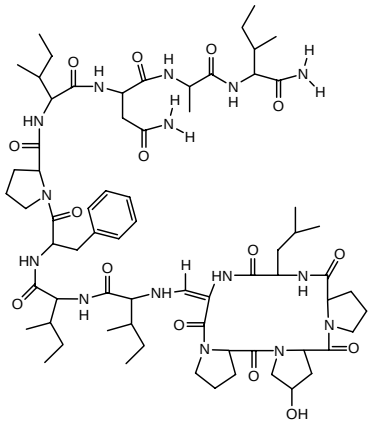
5.3. The sponge *Acanthostrongylophora ingens*

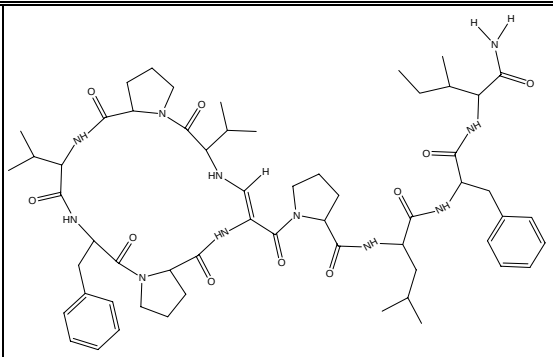
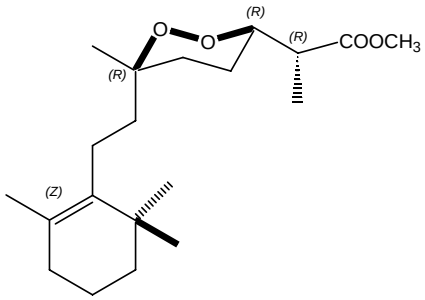
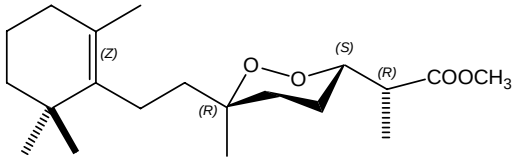
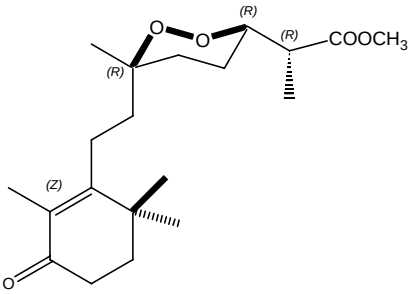
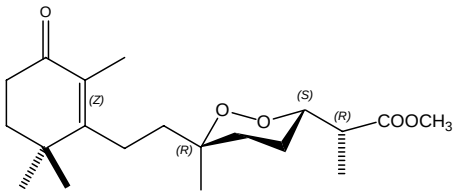
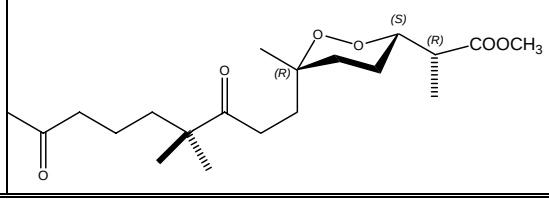
The sponge *Acanthostrongylophora ingens* was successively extracted with MeOH and acetone, respectively. The combined extracts were concentrated to dryness. The total extract was subjected to VLC using a CH_2Cl_2:MeOH gradient system. The eluted fractions were subjected to Sephadex LH20, silica gel column and semi-preparative HPLC to afford two known and one new pyrimidinyl β-carboline alkaloids. Annomontine showed strong cytotoxic activity.

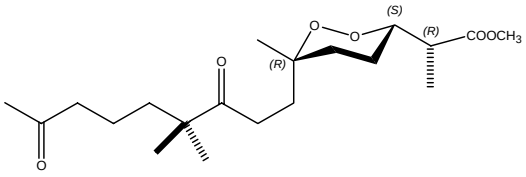
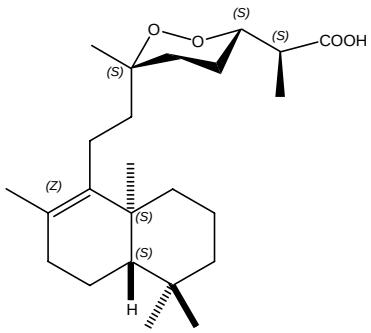
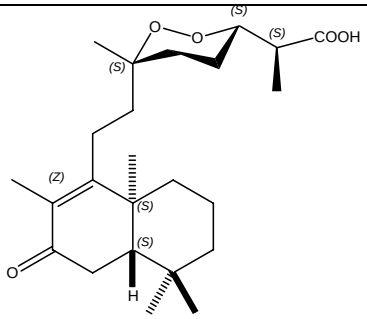
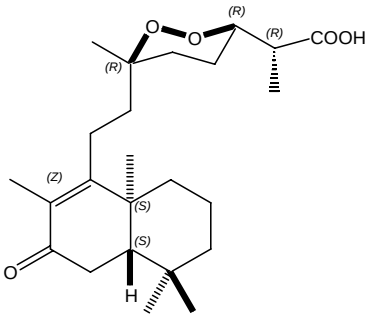
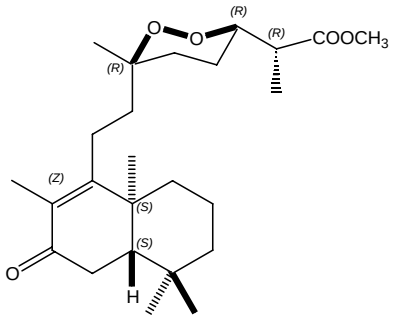
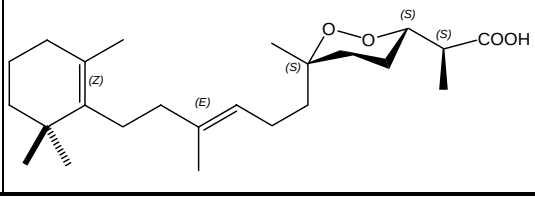
5.3. The sponge *Theonella swinhoei*

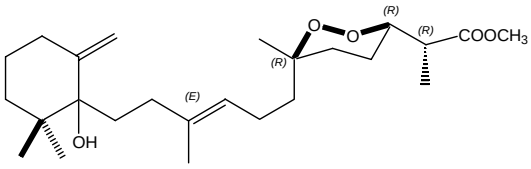
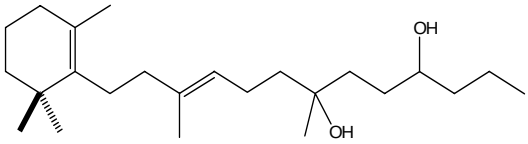
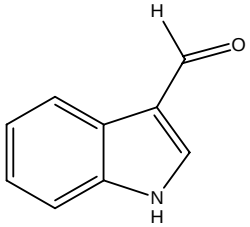
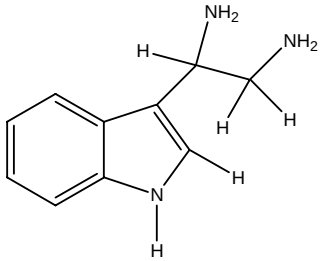
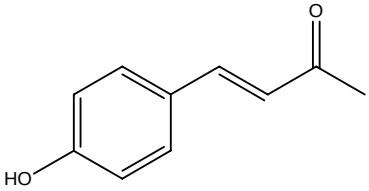
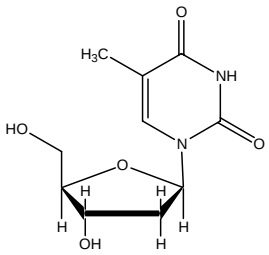
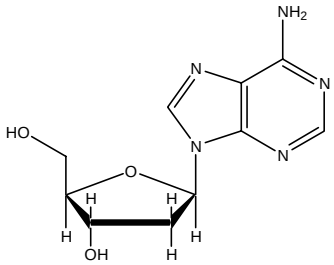
The sponge *Theonella swinhoei* was successively extracted with MeOH and acetone, respectively. The combined extracts were concentrated to dryness and partitioned between hexane, EtOAc, *n*-BuOH and H_2O, respectively. The EtOAc fraction was subjected to silica gel column chromatography followed by semi-preparative HPLC to obtain three known compounds including theonellapeptolides (Ia and Id) and swinholide A. The hexane fraction was subjected to normal phase column chromatography to afford the known compound cholest-4-ene-3-one.

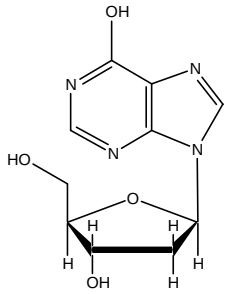
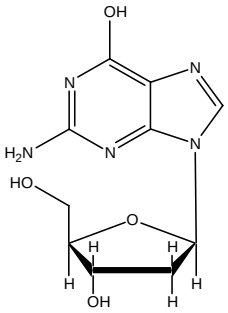
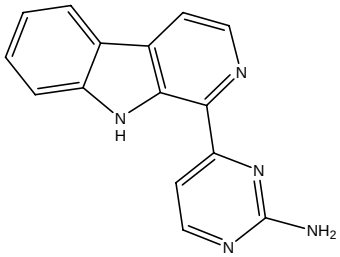
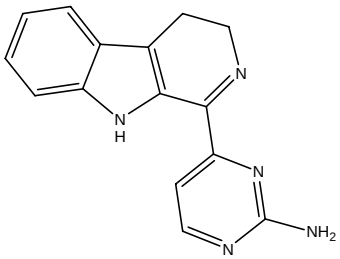
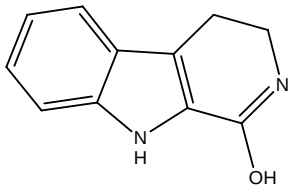
Table 5.1: Summary of the isolated compounds

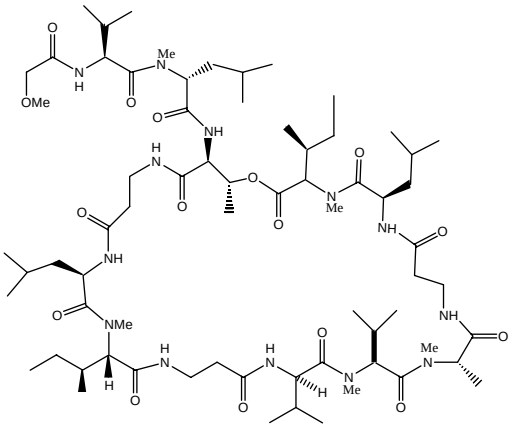
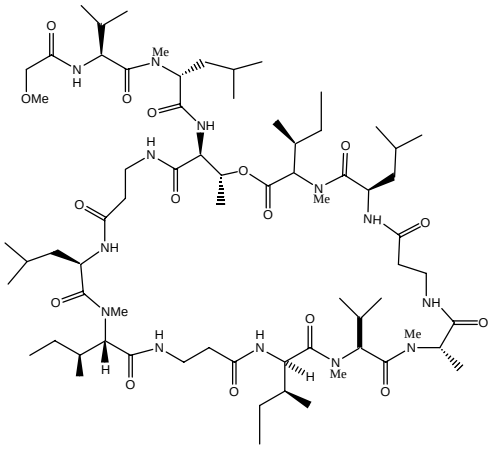
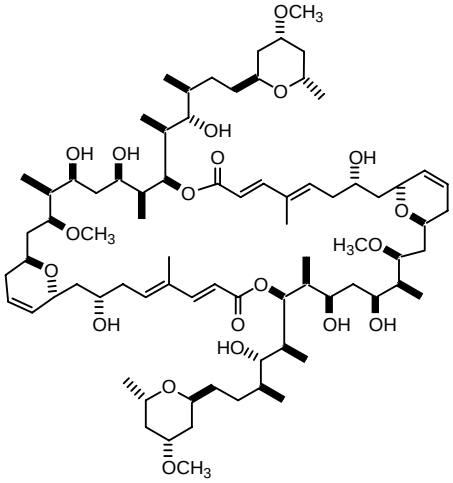
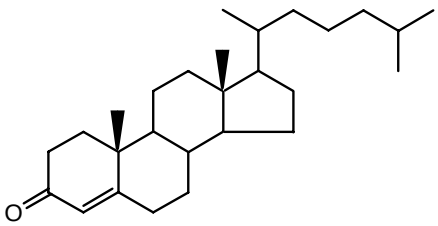
Compounds	Structure	Name of the sponge	Comment
Callyaerin E (**1**)		*Callyspongia aerizusa*	known
Callyaerin S (**2**)		*Callyspongia aerizusa*	new
Callyaerin B (**3**)		*Callyspongia aerizusa*	known
Callyaerin D (**4**)		*Callyspongia aerizusa*	known

Callyaerin F (**5**)		*Callyspongia aerizusa*	known
Nuapapuin A methyl ester (**6**)		*Diacarnus megaspinorhabdosa*	known
Methyl epinuapapuinoate (**7**)		*Diacarnus megaspinorhabdosa*	known
Diacarperoxide A (**8**)		*Diacarnus megaspinorhabdosa*	new
Methyl diacarnoate A (**9**)		*Diacarnus megaspinorhabdosa*	known
Diacarperoxide B (**10**)		*Diacarnus megaspinorhabdosa*	new

Diacarperoxide C (**11**)		*Diacarnus megaspinorhabdosa*	new
Diacarperoxide D (**12**)		*Diacarnus megaspinorhabdosa*	new
Diacarperoxide E (**13**)		*Diacarnus megaspinorhabdosa*	new
Diacarperoxide F (**14**)		*Diacarnus megaspinorhabdosa*	new
Diacarperoxide G (**15**)		*Diacarnus megaspinorhabdosa*	new
Diacarperoxide H (**16**)		*Diacarnus megaspinorhabdosa*	new

Epimuqubilin B (**17**)		*Diacarnus megaspinorhabdosa*	known
Diacarperoxide I (**18**)		*Diacarnus megaspinorhabdosa*	new
Indole-3-carboxyaldehyde (**19**)		*Diacarnus megaspinorhabdosa*	known
Diacarmine (**20**)		*Diacarnus megaspinorhabdosa*	new
P-hydroxybenzy lideneacetone (**21**)		*Diacarnus megaspinorhabdosa*	known
Thymine 2´-desoxyriboside (**22**)		*Diacarnus megaspinorhabdosa*	known
2´-Deoxyadenosine (**23**)		*Diacarnus megaspinorhabdosa*	known

2´-Deoxyinosine (**24**)		*Diacarnus megaspinorhabdosa*	known
2´-Deoxyguanosine (**25**)		*Diacarnus megaspinorhabdosa*	known
Annomontine (**26**)		*Acanthostrongylophora ingens*	known
Acanthomine A (**27**)		*Acanthostrongylophora ingens*	new
1-Hydroxy-3,4-dihydronorharman (**28**)		*Acanthostrongylophora ingens*	known

Theonellapeptolide Ia (**29**)		*Theonella swinhoei*	known
Theonellapeptolide Id (**30**)		*Theonella swinhoei*	known
Swinholide A (**31**)		*Theonella swinhoei*	known
Cholest-4-ene-3-one (**32**)		*Theonella swinhoei*	known

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