Isolation and structure elucidation of bioactive secondary metabolites from sponges collected at Ujungpandang and in the Bali Sea, Indonesia

(Isolierung und Strukturaufklärung bioaktiver Sekundärstoffe aus Schwämmen aus Ujungpandang und der Balisee, Indonesien)

Inaugural – Dissertation

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Erlangung des Doktorgrades der
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Vorgelegt von
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To my Family who gave me spirit to fight

Erklärung

Hiermit erkläre ich ehrenwörtlich, daß ich die vorliegende Dissertation **„Isolierung und Strukturaufklärung bioaktiver Sekundärstoffe aus Schwämmen aus dem Ujungpandang und der Balisee, Indonesien"** selbständig angefertigt und keine anderen als die angegebenen Quellen und Hilfsmittel angefertigt habe.

Diese Dissertation wurde weder in gleicher noch in ähnlicher Form in einem anderen Prüfungsverfahren vorgelegt. Außerdem erkläre ich, daß ich bisher noch keine weiteren akademischen Grade erworben oder zu erwerben versucht habe.

Düsseldorf, den 6 Juni 2006

Yosi Bayu Murti

Acknowledgements

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ZUSAMMENFASSUNG

Naturstoffe aus marinen Schwämmen besitzen eine einzigartige strukturelle Vielfalt, welche zur Findung neuer Leitstrukturen im Arzneimittelbereich und bei der Pflanzenschädligsbekämpfung von Interesse sind. Auf der Suche nach bioaktiven Inhaltsstoffen mariner Schwämme, konnten innerhalb dieser Arbeit sechzehn Verbindungen isoliert werden, darunter auch zwei neue Metabolite. Die Proben wurden in Ujungpandang und Balisee, Indonesien, gesammelt und extrahiert. Die erhaltenen Extrakte wurden mit chromatographischen Verfahren aufgetrennt, um die Reinsubstanzen zu erhalten. Deren Struktur wurde durch verschiedene analytische Verfahren, unter anderem Massenspektroskopie und ein- und zweidimensionale NMR – spektroskopische Experimente identifiziert.

1. *Agelas nakamurai*
Aus *A. nakamurai* wurden 5 sekundäre Stoffwechselprodukte isoliert, darunter vier bromierte Verbindungen und ein Sesquiterpen. Bei einer der bromierten Substanzen handelt es sich um die neue Verbindung 4-(4-Brom-1H-Pyrrol-2-carboxamid)-Buttersäure. In diesem Zusammenhang sind für die Gattung *Agelas* bromierte Pyrrolalkaloide bereits beschrieben, denen eine wichtige Rolle bei der Abwehr des Schwammes gegen Frassfeinde, wie zum Beispiel Fische, zukommt.

2. *Theonella swinhoei*

Vier Hochmolekulare Verbindungen wurden aus *T. swinhoei* erhalten, drei Theonellapeptolidderivate und Swinholid A. Theonellapeptolide sind einzyklische Depsipeptide, die einen ungewöhnlich methylierten Aminosäurerest besitzen.

3. *Acanthostrongylophora ingens*
Hier wurden drei Alkaloide vom Manzamintyp und eine neue Verbindung, 1-Dehydroircinol A, gefunden. Manzaminderivate sind vielversprechende Substanzen in der Malaria- und HIV-Therapie.

4. *Petrosia(Petrosia) hoeksemai*
Aus diesem Schwamm wurden zwei Alkaloide vom Manzamintyp isoliert: Manzamin A und Xestomanzamin A.

5. *Axynissa aculeata*
Ebenso konnten zwei Verbindungen vom Manzamintyp aus A. aculeata aufgereinigt werden: Indole-3-carboxaldehyd und die antibakteriell wirksame Verbindung Curcuphenol.

1. INTRODUCTION

Natural products have been selected over millions of years by evolution as effectors of biological processes. Throughout the past few decades, hundreds of these have been identified as useful therapeutic drugs, or the starting point for medicinal chemistry programs, for a wide range of human, animal and plant diseases. Additional novel chemical structures are yet to be discovered that will be incorporated into future medicines. The investigation of secondary metabolites produced by plant and animals has also resulted in the discovery of numerous organic chemicals which have found applications as insecticides, biomedical tools, or fragrances.

The search for new pharmacologically active agents obtained by screening natural sources has led to the discovery of many clinically useful drugs that play a major role in the treatment of human diseases. Approximately 60% of the antitumor and anti-infective agents that are commercially available or in late stages of clinical trials today are of natural product origin. Natural products are still major sources of innovative therapeutic agents for infectious diseases (both bacterial and fungal), cancer, lipid disorders and immunomodulation (Clardy and Walsh, 2004; Cragg *et al.*, 1997).

The importance of natural products as drugs for life science applications can easily be drawn from actual sales figures of marketed products and their impact as biomechanical tools, as well as inspiring starting point for chemical/biological optimization to become a useful product. About 30% of drugs on the worldwide

market are natural products or are derived from natural products. A similar ratio accounts for clinical candidates currently under development (Grabley and Sattler, 2002). However, regarding the identification of new innovative bioactive compounds from nature, the value assessment of certain biological material towards molecular diversity and thus improvement of probability of success, is still challenging.

1.1. What is natural product?

The term "natural product" is a term used commonly in reference to chemical substances found in nature that have distinctive pharmacological effects. This term is also usually reserved for secondary metabolites produced by living organisms. Such a substance is considered as a natural product even it can be prepared by total synthesis. Secondary metabolites are a very broad group of metabolites, with no distinct boundaries, and grouped under no single unifying definition. Concepts of secondary metabolism include products of overflow metabolism as a result of nutrient limitation, or shunt metabolites produced during idiophase, defence mechanisms, regulator molecules, and so on (Cannel, 1998).

1.2. Why isolate natural product?

About half of all the drugs currently in use are derived from natural products. These include many of the anti-infective and anti-tumour drugs developed during the past twenty years. Terrestrial plants are still the principal source but marine organisms are a most rapidly expanding sector. More recent research

has developed a cancer therapy made of algae and a painkiller derived from toxins of a cone snail venom. Anti-viral drugs Ara-A and AZT and anti-cancer agent Ara-C were developed from a Caribbean coral reef sponge, and Dolastatin 10 (Figure 1.1), extracted from an Indian Ocean sea hare, is undergoing clinical trials for the treatment of breast cancer, tumours, and leukaemia.

Dolastatin 10

ARA-A

Bryostatin 1

Ecteninascidin 743
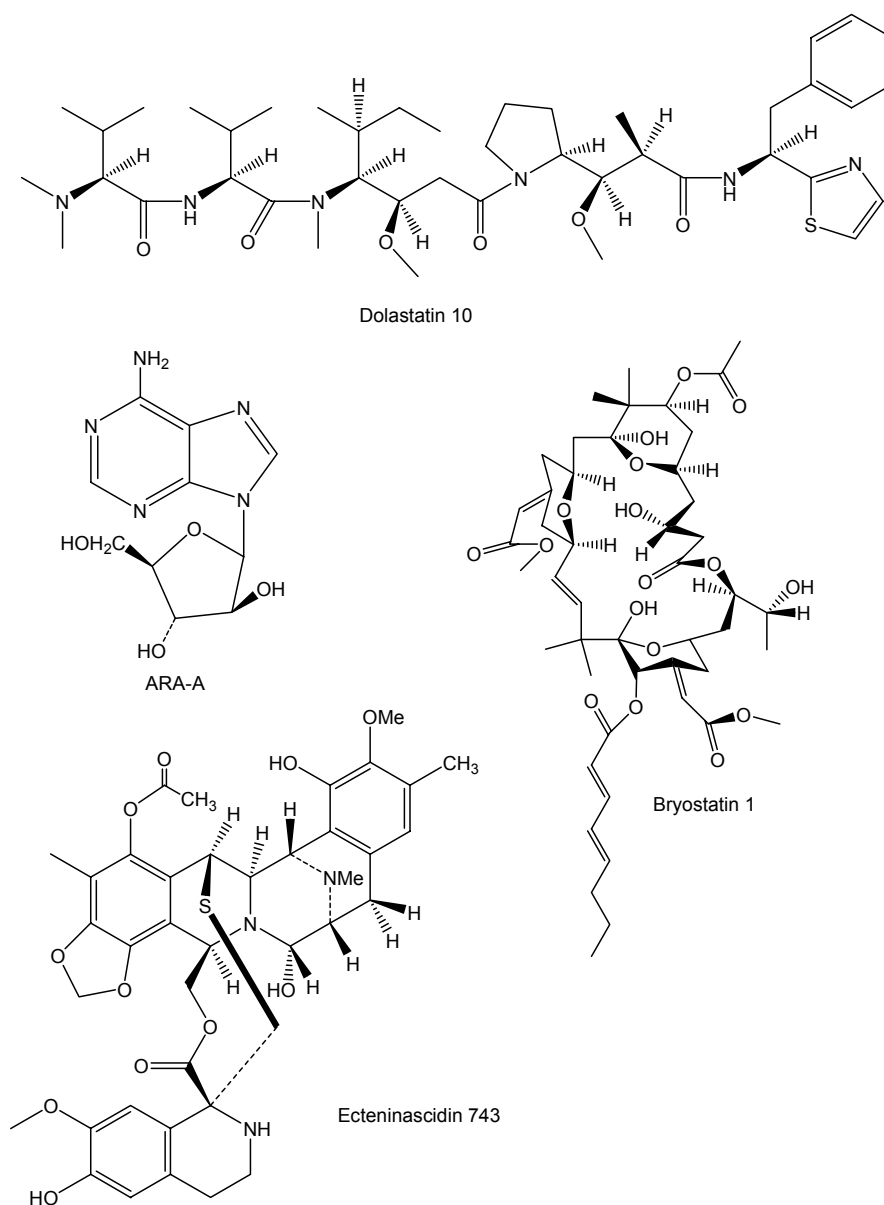
Figure 1.1. Selected compounds found from marine organisms.

At first, organisms tended to be collected in huge quantities, more or less at random, in the vain hope that some useful compound might be extracted later. This involved the unpleasant homogenisation (blending) of tonnes of material to extract minute quantities of chemicals for testing. In one case, 1600 kg of a sea hare were used to isolate just 10 mg of a compound to treat melanoma, though in explorative experiments as much as 1 kg many be need for bioassays. Genetic engineering has made prospecting for new drugs much more environmentally friendly, it being routine to collect as little as 1 kg of living material. DNA is then extracted from this and cloned into host bacterial cells which produce large quantities of the chemical in the laboratory (Blunden, 2001).

1.3. Marine natural products

The lack of obvious physical defence mechanisms made sessile, soft-bodied marine invertebrates to generate a chemical arsenal to survive from predation. Therefore, they have evolved the ability to synthesize toxic compounds or to obtain them from marine microorganisms. These compounds help them to deter predators, keep competitors at bay or paralyze their prey. If it is assumed that secondary metabolites evolved from primary metabolites in a random manner, any newly produced secondary metabolite that offered an evolutionary advantage to the producing organism would contribute to the survival of the new strain. The specific evolutionary pressures that led to chemically rich organisms need not be defined but the longer the period of evolution, the more time the surviving organism has had to perfect its chemical arsenal. Sessile marine

invertebrates have a very long evolutionary history and have had ample opportunity to produce a vast diversity of uniquely sophisticated chemical entities and perfect their chemical defense (Cooper, 2004; Faulkner, 2000b).

There are compounds with potent biological activities among these chemical defenses which have been developed as a form of biochemical warfare to survive in a highly competitive environment. Today, toxic principles dominate the spectrum of biological activities isolated from marine sources. Natural products released into the water are rapidly diluted and, therefore, need to be highly potent to have any effect. Of course, chemical defense mechanisms cannot be directly correlated to the biomedical activity, but in fact it is empirically visible how well both correlate. Thus sessile, soft-bodied marine invertebrates that lack obvious physical defences are therefore prime candidates to possess bioactive metabolites. For this reason, and because of the immense biological diversity of life form in the sea as a whole, it is increasingly recognized that a huge number of natural products and novel chemical entities exist in the oceans, with biological activities that may be useful in the quest for finding drugs with greater efficacy and specificity for the treatment of human diseases. During the relatively few years that marine organisms have been mined for useful compounds, a large number of bioactive chemicals have been discovered, many of which have useful biotechnological applications either as research tools to understand other living systems or as potential drugs. Based on this experience, some of the most promising organisms for the discovery of

future drugs are believed to be marine invertebrates and microorganisms (Haefner, 2003; Proksch *et al.*, 2002; Wallace, 1997).

Marine chemicals often possess quite novel structures which in turn lead to pronounced biological activity and novel pharmacology. The study of such chemicals therefore is a very promising endeavour. There are three parallel tracks in marine natural products chemistry: marine toxins, marine biomedicinals, and marine chemical ecology. Integration of these three fields of study gives marine natural products chemistry its unique character and strength. The search among marine chemicals for medically useful agents involves two steps, discovering the type of biological activity and studying the pharmacological mechanism of the activity (Lei and Zhou, 2002). Research into the pharmacological properties of marine natural products has led to the discovery of many potently active agents considered worthy of clinical application. 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, 1996).

In recent years, a significant number of novel metabolites with potent pharmacological properties have been discovered from marine organisms. Although there are only a few marine-derived products currently on the market, several robust new compounds derived from marine natural products are now in the clinical pipeline, with more clinical development. While the marine world offers an extremely rich resource for novel compounds, they are often present

Table 1.1. Status of Marine-Derived Natural Products in Clinical and Preclinical Trials (Newman and Cragg, 2004).

Name	Source	Status (disease)	Comment
Bryostatin 1	*Bugula neritina*	Phase II (cancer)	now in combination therapy trials;
TZT-1027	synthetic dolastatin	Phase II (cancer)	also known as auristatin PE and soblidotin
Cematodin	synthetic derivative of dolastatin 15	Phase I/II (cancer)	some positive effects on melanoma pts in Phase II; dichotomy on fate
ILX 651, synthatodin	synthetic derivative of dolastatin 15	Phase I/II (cancer)	in Phase II for melanoma, breast, NSCLC
Ecteinascidin 743	*Ecteinascidia turbinata*	Phase II/III (cancer)	produced by partial synthesis from microbial metabolite
Aplidine	*Aplidium albicans*	Phase II (cancer)	dehydrodidemnin B, made by total synthesis
E7389	*Lissodendoryx* sp	Phase I (cancer)	Eisai's synthetic halichondrin B deriv.
Discodermolide	*Discodermia dissoluta*	Phase I (cancer)	licensed to Novartis by Harbor Branch Oceanographic Institution
Kahalalide F	*Eylsia rufescens /Bryopsis* sp.	Phase II (cancer)	licensed to PharmaMar by Univ. Hawaii; revision of structure
ES-285	*Spisula polynyma*	Phase I (cancer)	Rho-GTP inhibitor
HTI-286	*Cymbastella* sp	Phase II (cancer)	synthetic derivative made by Univ. British Columbia; licensed to Wyeth
KRN-7000	*Agelas mauritianus*	Phase I (cancer)	an agelasphin derivative
Squalamine	*Squalus acanthias*	Phase II (cancer)	antiangiogenic activity as well
NVP-LAQ824	*Synthetic*	Phase I (cancer)	derived from psammaplin, trichostatin, and trapoxin structures
Laulimalide	*Cacospongia mycofijiensis*	preclinical (cancer)	synthesized by a variety of investigators
Curacin A	*Lyngbya majuscula*	preclinical (cancer)	synthesized, more soluble combi-chem derivatives being evaluated
Diazonamide	*Diazona angulata*	preclinical (cancer)	synthesized and new structure elucidated
Eleutherobin	*Eleutherobia* sp.	preclinical (cancer)	synthesized and derivatives made by combichem
Sarcodictyin	*Sarcodictyon roseum*	Preclinical (cancer) (derivatives)	combi-chem synthesis performed around structure
Peloruside A	*Mycale hentscheli*	preclinical (cancer)	
Salicylihalimides A	*Haliclona* sp.	preclinical (cancer)	first marine Vo-ATPase inhibitor; synthesized
Thiocoraline	*Micromonospora marina*	preclinical (cancer)	DNA polymerase R inhibitor

Name	Source	Status (disease)	Comment
Ascididemnin		preclinical (cancer)	reductive DNA-cleaving agents
Variolins	*Kirkpatrickia variolosa*	preclinical (cancer)	Cdk inhibitors
Dictyodendrins	*Dictyodendrilla verongiformis*	preclinical (cancer)	telomerase inhibitors
Manoalide	*Luffariaella variabilis*	Phase II (anti-psoriatic)	discontinued due to formulation problems
IPL-576,092 and derivates	*Petrosia contignata*	Phase II (antiasthmatic)	derivative of contignasterol; licensed to Aventis
Ziconotide	*Conus magus*	Phase III (neuropathic pain)	licensed by Elan to Warner Lambert
CGX-1160	*Conus geographus*	Phase I (pain)	contulakin G

as highly complex chemical structures. It also represents a great challenge that requires inputs from various scientific areas to bring the marine chemical diversity up to its therapeutic potential (Grabley and Thiericke, 1999; Kijjoa and Sawangwong, 2004).

Extraordinary chemical diversity is the hallmark of marine natural products structures. Marine natural products, such as the ion-channel modulators gamma-conotoxins, ciguatoxins and tetrodotoxin, have found extensive use in neuropharmacology and their properties continue to be the subject of new discovery. Novel classes of marine alkaloids have been found to intervene in key receptors of cell-cycle, enzymes or other targets and lead to insights into new therapeutics. Investigational drugs such as bryostatin-1, from the common fouling bryozoan, *Bugula neritina* and ecteinascidin-743 (ET-743), obtained from *Ecteinascidia turbinata* an inhabitant of Caribbean mangroves (Rinehart *et al.*, 1990) have been developed both by the National Cancer Institute and

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private pharmaceutical companies PharmaMar through to late-phase human clinical trials as effective anti-cancer treatments. ET-743 emerges as the first active agent developed in sarcomas in the last 25 years, with very promising results as single agent in pretreated ovarian cancer as well (Amador *et al.*, 2003; Jimeno *et al.*, 2004; Moore *et al.*, 1997).

Oceans are unique resources that provide a diverse array of natural products, primarily from invertebrates such as sponges, tunicates, bryozoans and molluscs as well as from marine bacteria and cyanobacteria. Since infectious diseases evolve and develop resistance to existing pharmaceuticals, the marine environment provides novel leads against fungal, parasitic, bacterial and viral diseases. Several marine natural products, including dolastatin 10, ecteinascidin-743, kahalalide F and aplidine, have successfully advanced to late stages of clinical trials, and a growing number of candidates have been selected as promising leads for extended preclinical assessment. Although many marine-product clinical trials have been conducted for cancer chemotherapy, factors such as drug resistance, emerging infectious diseases and the threat of bioterrorism, have contributed to the interest for assessing natural ocean products in treating infectious and parasitic diseases (Amador *et al.*, 2003; Donia and Hamann, 2003).

Initial attempts at determining the chemistries of marine organisms were simply applications of established phytochemical separation techniques. Thus, easily accessible organisms (generally sponges and encrusting organisms such as ascidians) were collected by hand or using simple snorkel, and then their

chemical components were extracted and identified. The developments of SCUBA diving technique clearly improved the capability to access deeper ocean floor and further the finding of more fascinating marine natural products. Any biological activity was found as an afterthought in these initial experiments. Since then, thousands of new compounds have been determined by marine natural products chemists. Research targeting organisms such as sponges, soft corals, algae, ascidians, bryozoans, and molluscs has demonstrated that marine organisms produce unique secondary metabolites unlike those found in terrestrial organisms (Davidson, 1995; Newman and Cragg, 2004). To date, researchers have isolated approximately 7000 marine natural products (MarinLit, 2004), 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 (Figure 1.2). A simplistic analysis of these data reveals that as the search 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 of spines or a shell, i.e. animals that require a chemical defense mechanism (Faulkner, 1995; Lei and Zhou, 2002).

Marine Natural Products

Distribution by Source
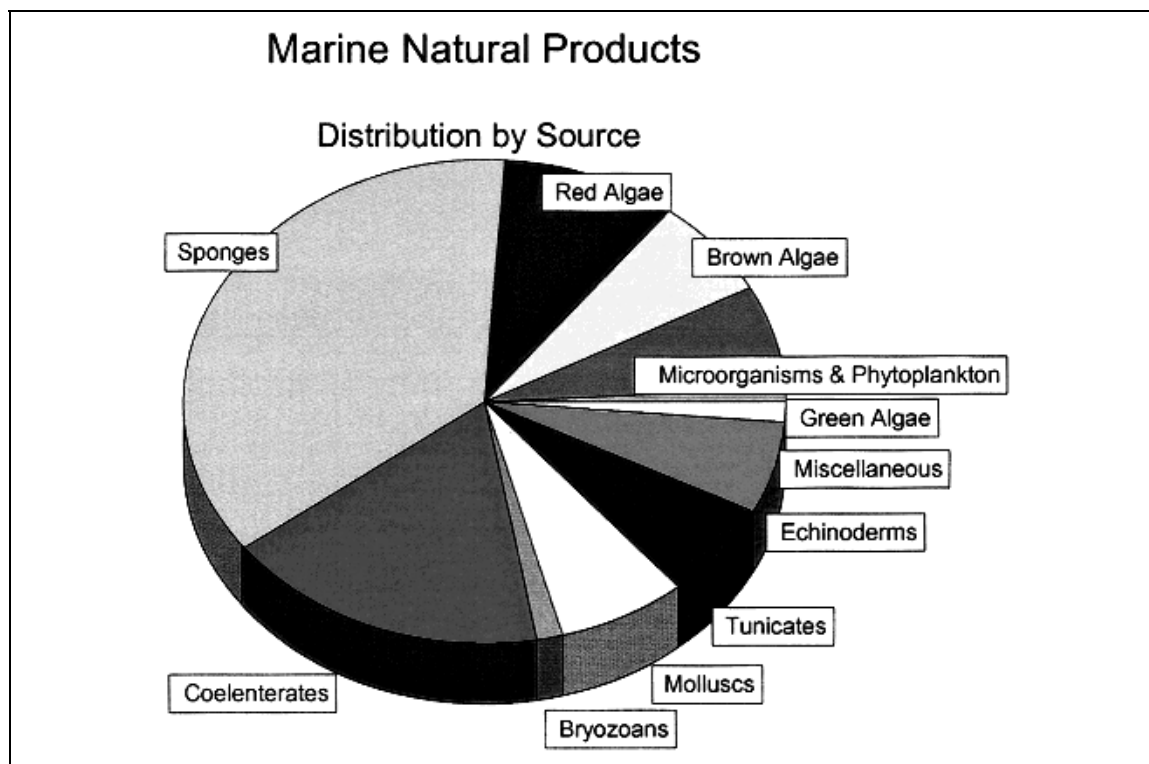
Figure 1.2. Marine natural product distribution by source (Lei and Zhou, 2002).

Apart from human medicines, the research on marine natural products in the last three decades has also brought to the discoveries of many chemically and biologically interesting molecules. Some of them, e.g., kainic acid, okadaic acid, tetrodotoxin, manoalide, palytoxin etc., have become indispensable tools in biochemical research and played significant roles in the recent advancement of life sciences (Higa *et al.*, 2001).

1.4. Marine invertebrates

Many marine invertebrates are immobile, attached to the ocean floor, and use highly evolved chemical compounds to attract food, block the growth of intruding neighbours or repel predators. Biologists believe that these survival demands triggered the evolution of a particularly abundant mixture of bioactive

compounds. Marine sponges, for example, are one of the richest sources of interesting chemicals from the marine environment. To survive on incredibly crowded and complex coral reef ecosystems, they must compete for food and attachment; thousands of species of sponges have now been identified and each species produces a different set of secondary metabolites to help them occupy their particular niche. These can have novel and enormously complex structures. Secondary metabolites are not involved in the primary pathways of life such as food digestion or energy metabolism, but provide sophisticated chemical defences or communication (Wallace, 1997). Marine organisms have evolved biochemical and physiological mechanisms that include the production of bioactive compounds for such purposes as reproduction, communication, and protection against predation, infection and competition (Halvorson, 1998). Because of the physical and chemical conditions in the marine environment, almost every class of marine organism exhibits a variety of molecules with unique structural features.

Diversity at higher taxonomic levels (phyla and classes) is much greater in the sea than on land or in freshwater. Of the 82 or so eukaryote phyla currently recognized around 60 have marine representatives compared with around 40 found in freshwater and 40 on land. Amongst animals the preponderance is even higher, with 36 out of 38 phyla having marine representatives. Some 23 eukaryote phyla, of which 18 are animal phyla, are confined to marine environments. Most of these are relatively obscure and comprise few species. The major exceptions are the Echinodermata (including starfish, sea urchins

and sea stars, sea cucumbers), of which some 6000 species are known, and the Foraminifera, with around 4000 known, extant species. A number of other important phyla including the Cnidaria (including corals and anemones), sponges (Porifera) and brown and red algae (Phaeophyta and Rhodophyta, respectively) are very largely marine organisms, each with only a small number of non-marine species. The reason for this predominance of marine higher taxa is believed to be because most of the fundamental patterns of organization and body plan, i.e. the different basic kinds of organism that are distinguished as phyla, originated in the sea and remain there, but only a subset of them has spread to the land and into freshwaters. It is noteworthy that only a third or so of marine phyla are found in the pelagic realm, the remainder being confined to sea bottom (benthic) areas. The habitat where eukaryotic organisms are believed to have evolved (Medlin, 1998).

1.5. Sponges (Porifera)

Sponges are among the simplest of multicellular animals and often described as the most primitive of all. They differ from other groups of invertebrates in that they maintain an almost protozoan independence for their constituent cells, which form no true tissue layers or organs, although they are composed of a variety of different cells, these cells display significant independence. They are highly organized colonies of unicellular nomads composed of loosely integrated cells covered by a skin and, with few exceptions, supported internally by a skeleton of silica, calcite, or sponging. The life of a sponge centers on pumping a high volume of water through its tissues, filtering out food and oxygen. The sponge body is organized around a system of pores, ostia, canals, and

chambers, which conduct water current from the inhalant sponge surface to the exhalant apertures, the osculum. Sponges come in many colours, shapes, and sizes, from millimeter-thin encrustations to branching ropes to giant barrel sponges more than six feet in height. Sponges are sessile marine animals which are commonly found in seas where there are rocks, coral or other suitable substrata. There are more than 5000 species and they are found in almost every sea from mid-tide levels to the deepest parts of the oceans (Carte, 1996; Russell, 1984; Whitehead, 1999).
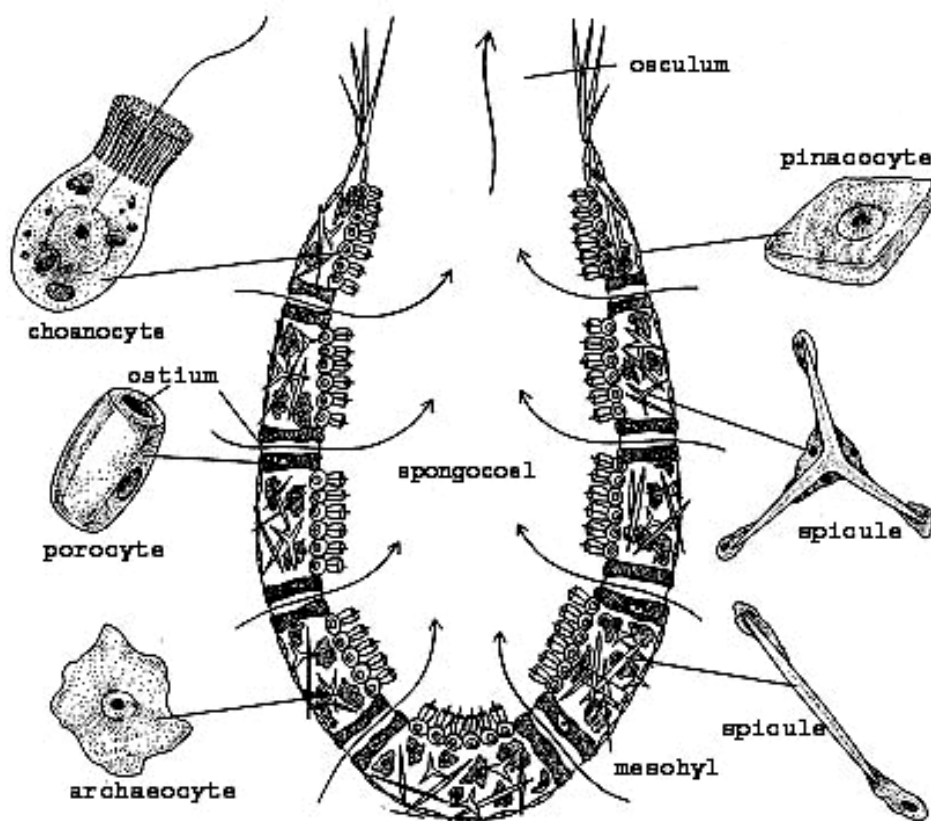
Figure 1.3. Diagrammatic representation of a simple (asconoid) sponge, illustrating its various cellular and structural components. In asconoids, the incurrent canal is simply a tube passing through a modified pinacocyte, called a porocyte. Note that 6 cells are involved in producing a triradiate spicule (Pechenik, 2000).
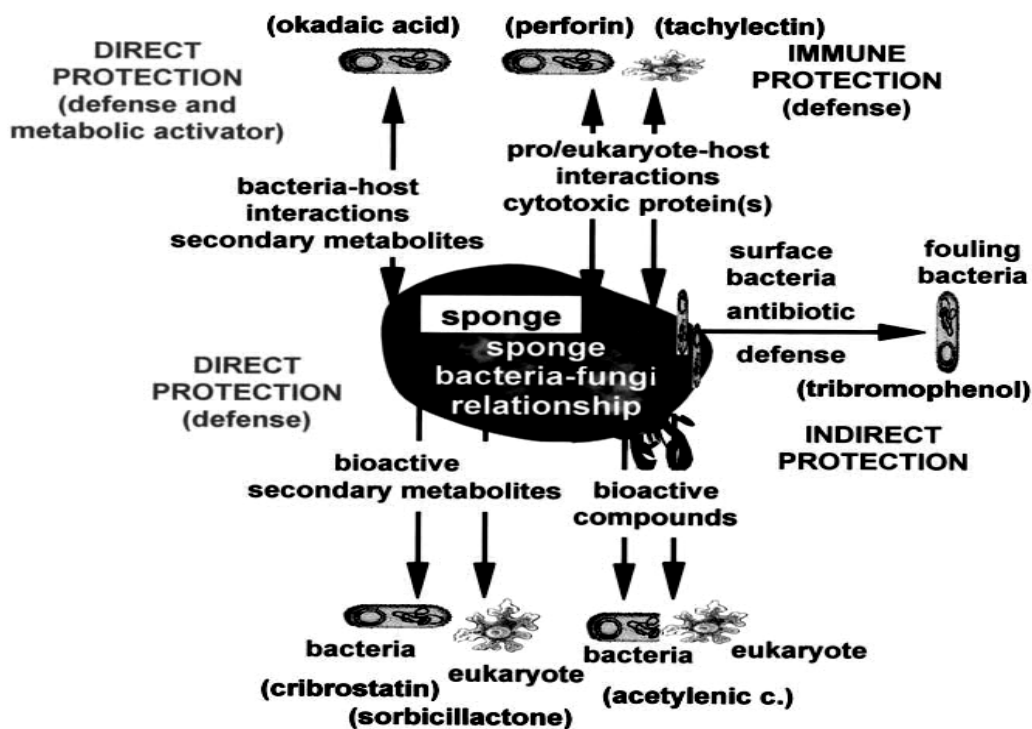
Figure 1.4. Protection strategies of sponges, together with their symbiotic microorganisms, bacteria and fungi against attackers. The red arrows indicate bacterial origin, and the blue ones indicate that the compounds are produced by sponges. Examples of secondary metabolites and proteins involved in these strategies of protection are provided (Müller *et al.*, 2004).

Sponges act as hosts to a variety of symbiotic/parasitic organisms including blue-green algae and bacteria. The question therefore arises as to which organisms are the true producers of the structurally diverse compounds isolated by extraction of whole marine sponges. It is quite widely accepted that many such metabolites are produced by symbiotic microorganisms, a conclusion supported by the finding that the natural products from some classes of sponge are very similar to those known from terrestrial microorganisms (Whitehead, 1999).

According to Koenig and Wright, biosynthesis of secondary metabolites may be influenced by: (a) external factors such as prevailing environment condition e.g., presence of predators, infection with parasites, water temperature, depth, and nutrient levels; (b) internal factors e.g., stage of development/reproduction and chemical races; (c) for marine macroorganisms the occurrence and type of symbionts within or on the tissue of host animal may be of major importance, as may be the storage or further modification of secondary metabolites obtained from food sources. Due to these changing parameters the chemical content of marine species often varies depending on where and when an organism is collected (Koenig and Wright, 1996).

1.6. Sponges and Drug Discovery

Approximately 10,000 sponges have been described in the world and most of them live in marine waters. A range of bioactive metabolites has been found in about 11 sponge genera. The discovery of spongouridine, a potent tumor-inhibiting arabinosyl nucleoside in Caribbean sponge *Cryptotethia crypta*, focused attention on sponges as a source of biomedically important metabolites. The compound manoalide from a Pacific sponge has spawned more than 300 chemical analogs, with a significant number of these going on to clinical trials as anti-inflammatory agents. Halichondrin-B, a polyether macrolide from Japanese sponge *Theonella* spp., has generated much interest as a potential anticancer agent (Fusetani *et al*., 1992).

Metabolites by Phylum

Number of Compounds
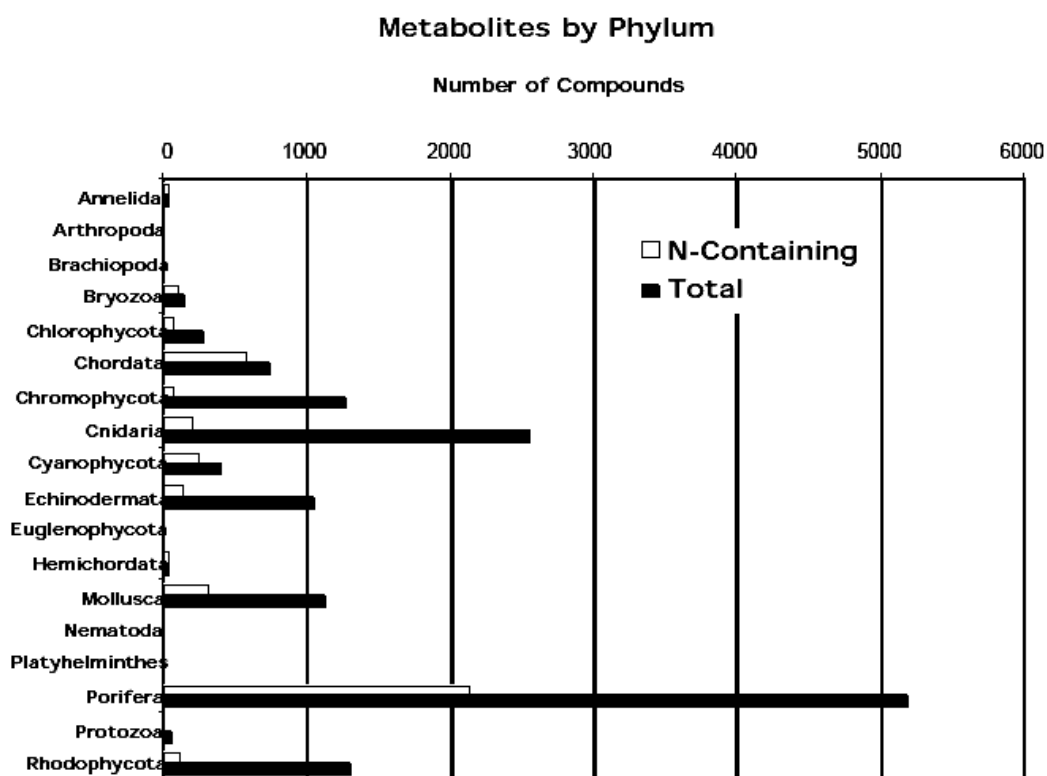
Figure 1.5. Distribution of metabolites among marine phyla (Urban *et al.*, 2000).

The theopederins are structurally related to mycalamide-A from marine sponge, *Mycale* spp. collected in New Zealand (Perry *et al.*, 1988) and onnamide-A from marine sponge, *Theonella* spp. collected in Okinawa (Sakemi *et al.*, 1988), which show *in vitro* cytotoxity and *in vivo* antitumour activity in many leukemia and solid tumour model systems (Burres and Clement, 1989). Isoquinolinequinone metabolite cribostatin from the Indian Ocean sponge *Cribrochalina* spp. shows selective activity against all nine human melanoma cells in National Critical Technologies (NCT) panel. Spongistatin, a macrocytic lactone from the Indian Ocean collection of *Spongia* spp., is the most potent substance known against a subset of highly chemoresistant tumour types in the

NCI tumour panel (Pettit *et al.*, 1993). Two new pyrones (herbarin) along with a new phthalide, herbaric acid, were isolated from two cultured strains of the fungus *Cladosporium herbarum* isolated from the sponges *Aplysina aerophoba* and *Callyspongia aerizusa* collected in the French Mediterranean and in Indonesian waters, respectively. Herbarins displayed activity in the brine shrimp assay (Jadulco *et al.*, 2002). Lembehynes B and C, isolated from an Indonesian species of *Haliclona* were found to possess neuritogenic activity against neuroblastoma cells (Aoki *et al.*, 2002).
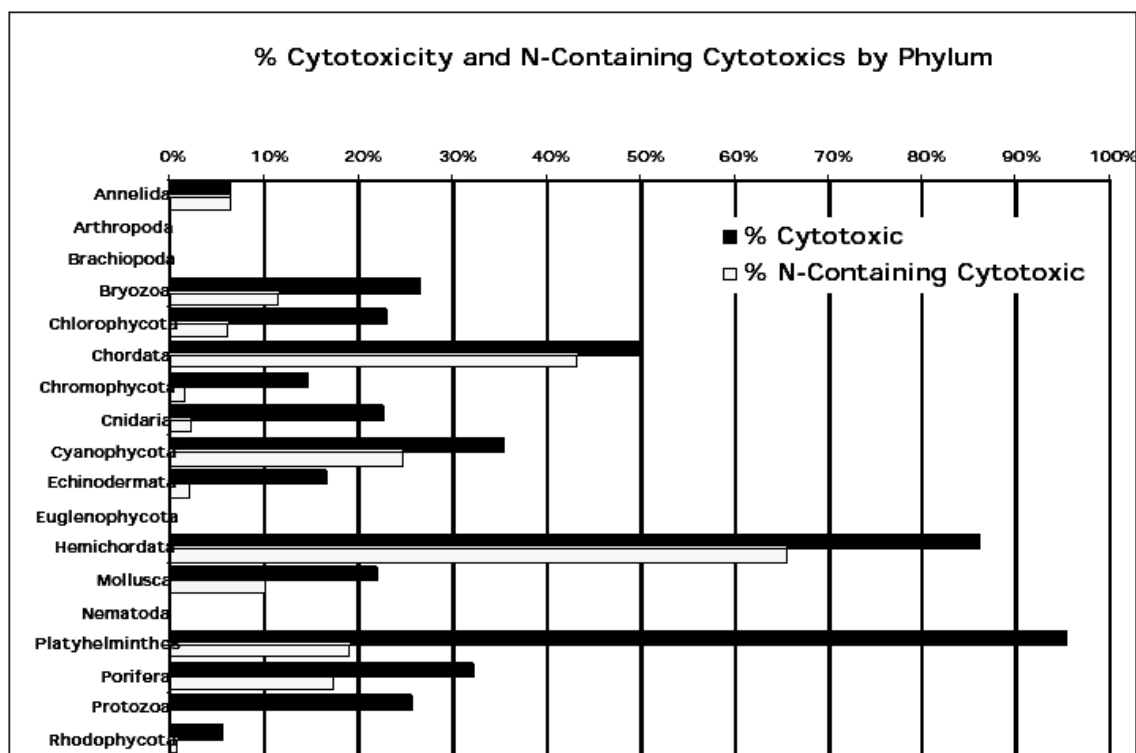
Figure 1.6. Relative metabolite cytotoxicity among marine phyla (Urban *et al.*, 2000).
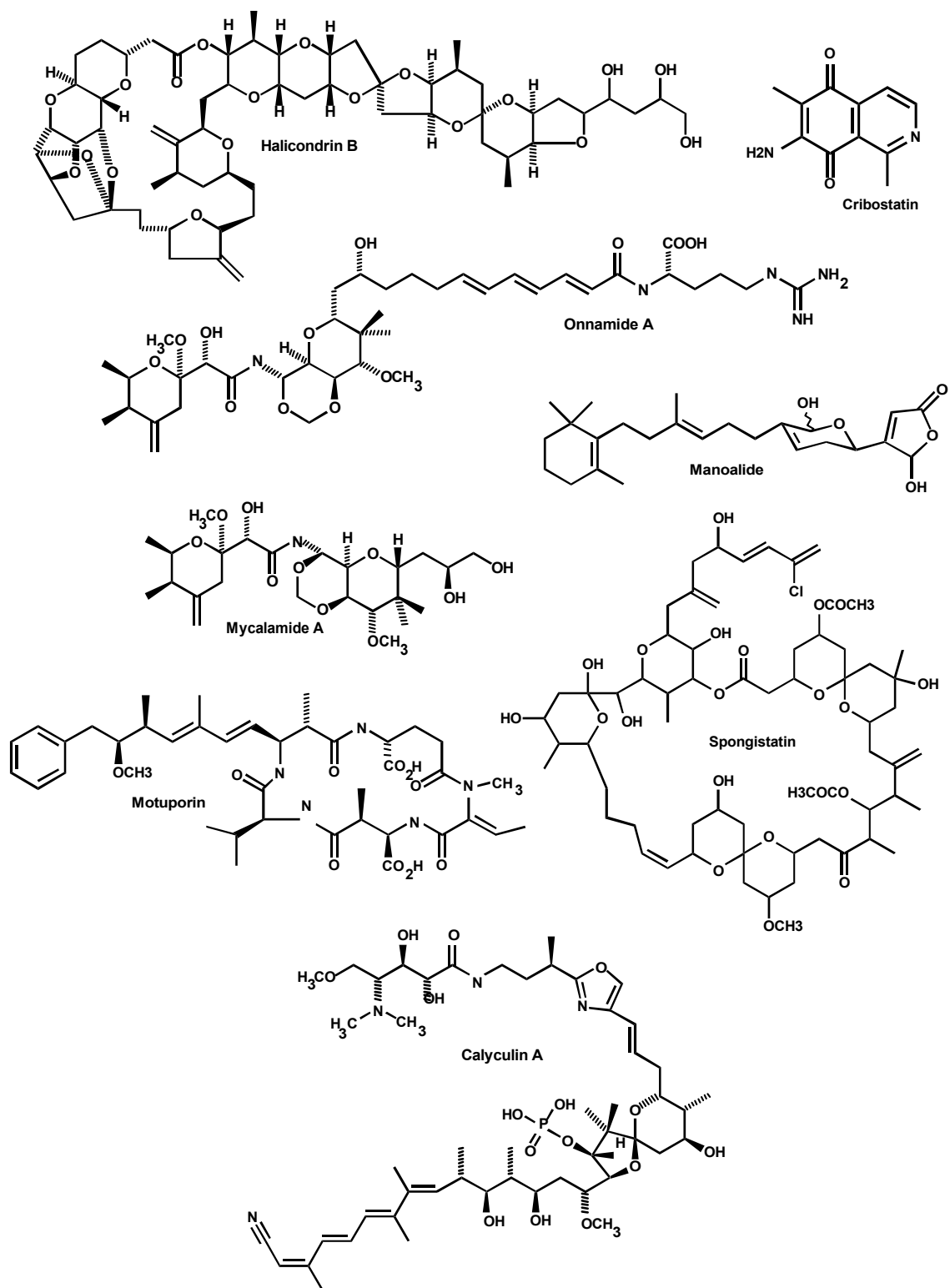
Figure 1.7. Selected compounds found in marine sponges.

Potent phosphate inhibitors have been isolated from sponges like, okadaic acid from *Halichondria okadai*, motuporin from *Theonella swinhoei* and calyculin-A from *Discodermia calyx*. Inhibitors of phospholipase such as manoalide and scalaradial have proved to be useful tools to study the role of this enzyme in the release of arachidonic acid, which is a key molecule, involved in the biochemical processes leading to inflammation (Kato *et al.*, 1986; Potts and Faulkner, 1992).

A number of receptor antagonists with potential as biochemical tools or structural leads to the development of therapeutics have been isolated from sponges. Examples include xestobergsterol (isolated from *Xestospongia berguista*), which inhibits immunoglobulin E mediated histamine release from mast cells and is 5000 times more potent than the antiallergic drug disodium cromoglycate (Shoji *et al.*, 1992). Leucettamine A isolated from *Leucetta microraphis*, is a potent and selective antagonist for the receptor for leukotrine, a non-peptide metabolite of arachidonic acid produced mainly in inflammatory cells (Chan *et al.*, 1993). Batzelladine A & B, novel polycyclic guanidine alkaloids from the Carribean sponge *Batzella* spp., exhibit potent inhibition to the binding of HIV glycoprotein, on CD4 receptors of T cells (Carte, 1996). *Plakortis nigra*, collected from a depth of 115 m in Palau, was found to contain epiplakinic acid G and H, and the -lactones along with several carbolines (vide infra) (Sandler *et al.*, 2002). All compounds have been found to inhibit the growth of HCT-116 cells. The moderately cytotoxic thioester irciniamine has been isolated from an *Ircinia* spp. collected in Japan (Kuramoto *et al.*, 2002).

The previously reported motuporins A–C along with the new congeners, motuporins D–F have been found to inhibit the invasion of breast carcinoma cells into new tissues. These compounds have been isolated from *Xestospongia exigua* collected in Papua New Guinea along with an unresolved mixture of three isomers of motuporins (Williams *et al.*, 1998). *Hyrtios erecta* collected from the Egyptian Red Sea has been found to contain salmahyrtisol A and B and sesterstatins, all of which have shown significant cytotoxicity in human cancer cell-lines (Yousaf *et al.*, 2002). A peroxy steroid, from an Okinawan species of the genus *Axinyssa*, has been found to inhibit the growth of several human cancer cell-lines (Iwashima *et al.*, 2002).

Marine invertebrates have been recognized as an important source of bioactive compounds having medicinal potential. Although no major therapeutic drugs have yet been developed from the sea, several compounds have so far entered clinical trials as anticancer drugs. A limitation that plagues the development of marine invertebrate metabolites as clinical agents, however, is supply. Many of the compounds are present in small quantities and have complicated structures that preclude economical industrial syntheses (Davidson, 1995). The development of biotechnological production of marine natural product such as aquaculture (bryostatin, esteinacidin), through symbiotic microorganism production (swinholide, manzamine) and also development of synthetical production (Ara-A) might become the solution of those plagues. Thus prospects of marine natural products as future medicine are still promising.

1.7. Aims and scope of study

Based on the knowledge on terrestrial biodiversity, it is believed that the greatest biodiversity supplied by organisms are from tropical and sub-tropical water regions. Sponges are primarily among the well-researched marine organisms because of their size and abundance. About one third of discovered marine substances have been isolated from sponges and their symbiotic microorganisms. On the other side, Indonesian waters cover about a quarter of world tropical waters. Both facts drive the interest of isolating bioactive metabolites from Indonesian marine sponges.

Hence, this study is focused on the isolation of metabolites from Indonesian marine sponges which exhibit antibacterial, antifungal, and cytototoxic activites. This study contains three main processes: first is the isolation and purification of natural products, second is the bioassay and third is the structure elucidation. For the isolation of the metabolites, a chemical and biological approach will be used. Extracts are screened for antibacterial, antifungal, insecticidal, cytotoxic, and general toxicity activities. As complement to the bioassay-guided approach, TLC, UV, HPLC and LC-MS are used to guide the isolation and purification of the compounds responsible for the activity of the extract or compound that are most interesting. Thereby, purified compounds which are not responsible for the initial bioactivity screening may have a chance to be evaluated for other more sophisticated pharmacological assays.

2. MATERIALS 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.

2.1.1. *Agelas nakamurai*

The sponge (Figure 2.1.B.) was collected from a depth of 12 m near Menjangan Island (North of Bali Island) in Indonesia on September 1997. It forms an irregular thick-walled flabellate mass with an irregular surface. The skeleton is a fine-meshed reticulation of sponge fibers of which the primary ones are cored with one to four spicules in cross section, and the connecting fivers are uncored. All fibers are lightly echinated. The spicules are the usual verticillated acanthostyles 180-285 x 12-20 µm with 17-26 whorls of spines. A voucher specimen has been deposited in the Zoological Museum Amsterdam, reg. no. ZMA POR 16735.

Taxonomy data

Phylum : Porifera

Class : Demospongiae

Order : Agelasida

Family : Agelasidae

Genus : Agelas

Species : *Agelas nakamurai*
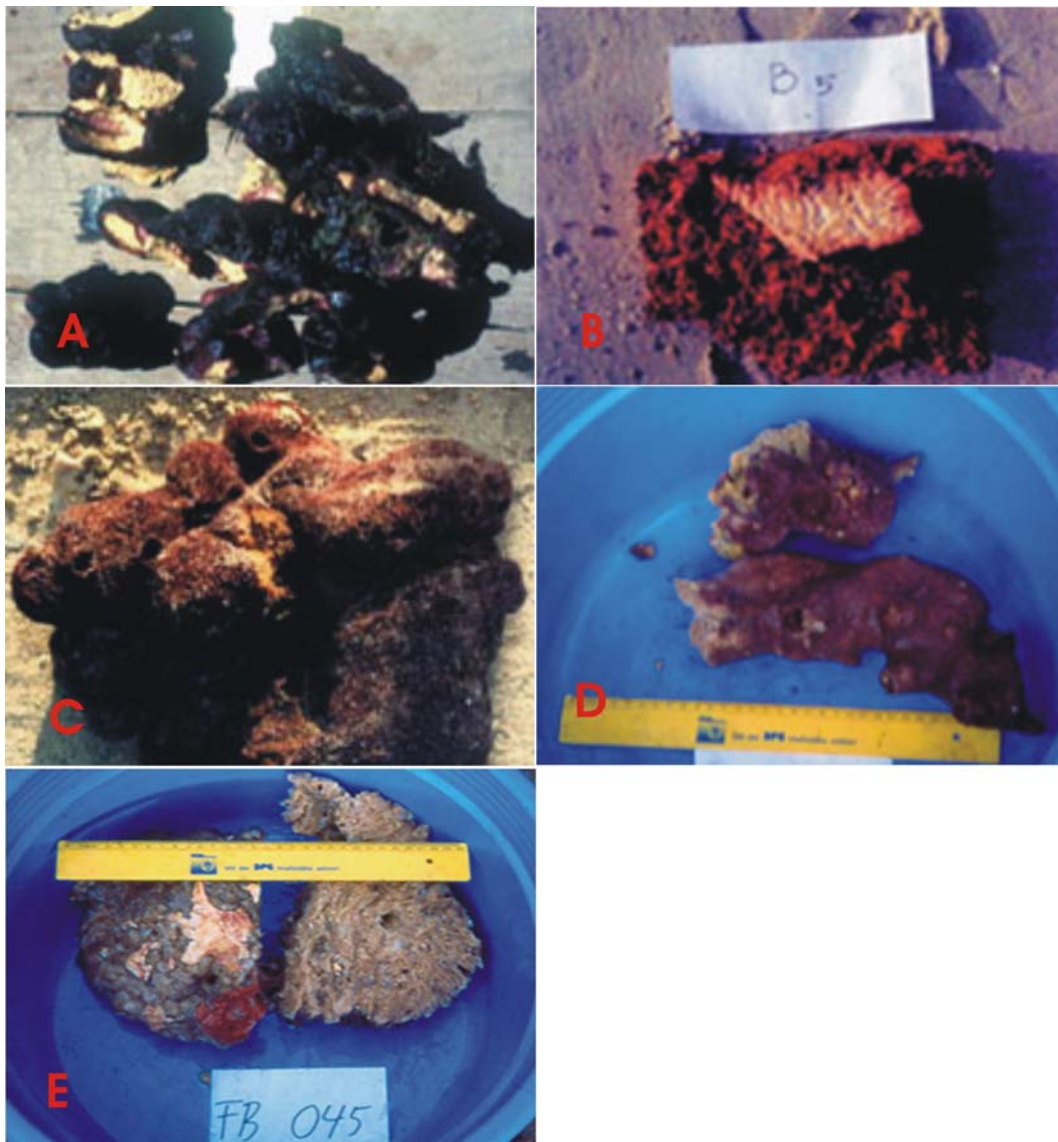
Figure 2.1. Sponge samples picture: A. *Theonella swinhoei*, B. *Agelas nakamurai*, C. *Acanthostongylophora ingens*, D. *Petrosia(Petrosia) hoeksemai*, E. *Axinyssa aculeata*.

2.1.2. *Theonella swinhoei*

The sponge (Figure 2.1.A.) was collected from Menjangan Island (North of Bali Island) in Indonesia on September 1997. Massive, short, shallow cup-shaped sponge with a broad base by which it is attached to the rocky substrate, supplied with a deep and narrow axial cavity. The sponge measures 3.5 cm

high and about 4 cm wide; walls are about 6 mm thick. A single large osculum, about 1 x 0.7 cm wide, is located in the middle of the cup-shaped depression leading to a rapidly narrowing axial cavity. Outer side of the sponge is supplied with irregular thick ridges. Ectosomal spicules are strongly differentiated phyllotriaenes with a very short rhabd; they may have relatively long and very slender (narrow) clads, or conversely, relatively massive flattened clads in various portions of the sponge. Those with narrow clads occur mostly in the inhalant areas on the outer sponge surface, while those with massive clads occur mostly on the inner surface of the cup. The clads may bear sparse tubercles, especially in the central portion of the cladome. Phyllotriaenes are 460-560 µm in diameter (Pisera and Levi, 2002). A voucher specimen has been deposited in the Zoological Museum Amsterdam, reg. no. ZMA POR

Taxonomy data

Phylum   : Porifera

Class   : Demospongiae

Order   : Lithistida

Family   : Theonellidae

Genus   : Theonella

Species   : *Theonella swinhoei*

2.1.3. *Acanthostrongylophora ingens*

The sponge (Figure 2.1.C.) was collected in 1996 in Ujung Pandang, Indonesia. It was identified by Dr. Van Soest of the Zoological Museum Amsterdam. A

voucher specimen has been deposited in the Zoological Museum Amsterdam, reg. no. ZMA POR 18287.

Taxonomy data

Phylum   : Porifera

Class   : Demospongiae

Order   : Petrosida

Family   : Petrosiidae

Genus   : Acanthostrongylophora

Species   : *Acanthostrongylophora ingens*

2.1.4. *Petrosia (Petrosia) hoeksemai*

The sponge (Figure 2.1.D.) was collected from Menjangan Island (North of Bali Island) in Indonesia on September 1997. It was identified by Dr. Van Soest of the Zoological Museum Amsterdam. A voucher specimen has been deposited in the Zoological Museum Amsterdam, reg. no. ZMA POR 18288.

Taxonomy data

Phylum   : Porifera

Class   : Demospongiae

Order   : Petrosida

Family   : Petrosiidae

Genus   : Petrosia

Species   : *Petrosia (Petrosia) hoeksemai*

2.1.5. *Axinyssa aculeata*

The sponge (Figure 2.1.E.) was collected in 1996 in Ujung Pandang, Indonesia. It was identified by Dr. Van Soest of the Zoological Museum Amsterdam. A voucher specimen has been deposited in the Zoological Museum Amsterdam, reg. no. ZMA POR 18289.

Taxonomy data

Phylum   : Porifera

Class   : Demospongiae

Order   : Halichondrida

Family   : Halichondriidae

Genus   : Axinyssa

Species   : *Axinyssa aculeata*

2.2. Chemicals used

2.2.1. General laboratory chemicals

Anisaldehyde (4-methoxybenzaldehyde)	Merck
L-(+)-Ascorbic acid	Merck
Bismuth nitrate	Sigma
Glacial acetic acid	Merck
Hydrochloric acid	Merck
Potassium iodide	Sigma
Sodium hydroxide	Merck

Concentrated sulfuric acid   Merck

Trifluroacetic acid (TFA)   Merck

2.2.2. Solvents

Solvents were purchased from the Chemical Department, University of Duesseldorf as technical degree. They were distilled prior to use for extractions, separation and chromatography applications. Used solvents were:

Acetone

Acetonitrile

Butanol

Chloroform

Dichloromethane

Diethyl ether

Dimethylformamide

Dimethylsulfoxide

Ethanol

Ethyl acetate

Hexane

Isopropanol

Methanol

Due to NMR measurement, deuterated solvents such D_2O, MeOD, $CDCL_3$ and DMSO-d_6 were used.

2.2.3. Chromatography:

Chemicals:	Company
1. Pre-coated TLC plates (AluO), Silica gel 60 F254, layer thickness 0.2 mm	Merck
2. Pre-coated TLC plates (Glass), RP-18, F254 S, layer thickness 0.25 mm	Merck
3. Silica gel 60, 40-63 µm mesh size	Merck
4. RP-18, 40-63 µm mesh size	
5. Sephadex LH 20, 25-100 µm mesh size	Merck
6. HPLC solvents (Methanol Chromanorm for HPLC)	VWR
6. Phosphoric acid (85% p.a.)	Merck
7. Trifluroacetic acid	Merck
8. Nanopure water	Institute of Botany
9. Pre-packed LOBAR® with columns packing materials	Merck

Silica gel 60 and RP C-18, size A & B respectively:

Size A: 10 mm i.d. x 240 mm

Size B: 25 mm i.d. x 310 mm

2.3. Equipments used

Balances	: Mettler AT 200, Mettler AT 250, Mettler PE 1600, and Sartorius RC210P
Centrifuge	: Biofuge
Fraction collector	: Retriever II

Freeze Dryer	: a)	Christ Alpha 2-4 with Equipment Control; LDC-IM and Vacuum Pump DVO 004B
	b)	LYOVAC GT2 Pump TRIVAC D10E
Hot plates	:	Camag
Syringe	:	Hamilton 25, 100, 250 and 500 µL
Mill	:	Moulinex 354
Magnetic Stirrer	:	Variomag Multipoint HP
PH-meter	:	Inolab
Vacuum Pump	:	Vacuubrand Diaphragm Vacuum Pump
Rotary Evaporator	:	Buchi Rotavap RE111; Buchi Rotavap R-200
Drying Ovens	:	Heraeus T 5050
Ultrasonic Bath	:	Bandelin Sonorex RK 102
UV Lamp	:	Camag (254 and 366 m,)
UV/Vis Spectrophotometer	:	Perkin-Elmer Lambda 2
Shaker	:	Janke & Kunkel, VF 1
Vacuum Filtration	:	Supelco
Vacuum Exsiccator	:	Savant SpeedVac SPD111V, Savant Refrigerator Vapor Trap RVT400, and Pump Savant VLP80

HPLC equipment:

I.   Analytical HPLC (Dionex)

Pump	:	Dionex P580A LPG
HPLC Program	:	Chromeleon Ver. 6.3
Detector	:	Dionex, Photodiode Array Detector UVD 340S

Column thermostat : STH 585

Column : Knauer, 5.0 mmID. Packing material 5 µm

Eurospher-100 C-18

Auto sampler : ASI-100T

II. Semi-preparative HPLC (LaChrom Merck Hitachi)

Pump : LaChrom L-7100

Detector : UV detector LaChrom L-7400

Printer : Kipp & Zonen Flatbed Recorder type BD 11E

Column : Knauer, 8.0 mmID. Packing material 10 µm

Eurospher-100 C-18

2.4. Extraction and partition

Natural product sample often contains a mixture of many components in a complex matrix. The components must be separated from each other so that each individual component can be identified by other analytical methods. A mixture can be separated using the differences in physical or chemical properties of the individual components. Initially, natural products are extracted from the complex matrix by organic solvent such as methanol, acetone, ethyl acetate or hexane. Extraction solvent is dependent on the type of compounds to be isolated. Methanol is a common solvent of choice for the isolation unknown samples.

When two immiscible solutions are mixed, they will form two layers of liquid. Based on "like-dislike" principle, compounds will migrate from solvent that it dislikes (less soluble) to the layer of solvent that it likes (more soluble). They will be separated by means of the solubility character of each of the compound. This is the idea of partitioning an aqueous solution or suspension of two components A and B is mixed with an immiscible organic liquid, and the mixture is shaken vigorously and then allowed to stand. If one of the components is more soluble in the organic layer as in the aqueous layer, then this component will be extracted into the organic layer. Assuming another component is more soluble in the aqueous layer then this component goes to the aqueous layer, resulting in two different layers. Partition was commonly initiated between hexane-methanol 90% and water (resulting in the hexane fraction), then continued with ethyl acetate and water (resulting in the ethyl acetate fraction), and ended by butanol and water (resulting to the butanol fraction and water fraction).

2.5. Chromatography method

Chromatography refers to any separation method in which the components to be separated are distributed between two phases: a *stationary phase* bed and a *mobile phase* which percolates through the stationary bed. The stationary phase is either a porous solid used alone or coated with a stationary liquid phase. The mobile phase is called the eluent and the process by which the eluent causes a compound to move along the column is called elution. Sample components have different affinities for the stationary and mobile phases and therefore move at different rates along the column. Repeated sorption-

desorption acts that take place during the movement of the sample over the stationary bed determine the rates difference. The smaller the affinity a molecule has for the stationary phase, the shorter is the movement of the molecule through a column. These differential rates of migration as the mixture moves over adsorptive materials provide separation.

The separation properties of the components in a mixture are constant under constant conditions, and therefore once determined they can be used to identify and quantify each of the components. Based on this advantage, chromatographical methods can be used in isolation and analysis of natural products.

2.5.1.   Thin layer chromatography

Thin layer chromatography (TLC) is a simple and rapid method to detect mixtures of compounds in samples or to check the purity level of compounds through separation. The mobile phase is a solvent and the stationary phase is a solid adsorbent immobilised on a flat support like a glass, plastic or aluminium plate. The sample, either liquid or dissolved in a volatile solvent, is deposited as a spot on the stationary phase. The bottom edge of the plate is placed in a solvent reservoir, and the solvent moves up the plate by capillary action. When the solvent front reaches the other edge of the stationary phase, the plate is removed from the solvent reservoir. The separated spots are visualized with ultraviolet light detector or spray dye reagents (e.g. Dragendorf or anisaldehyde-sulfuric acid). The different components in the mixture move up

the plate at different rates due to differences in their partition and adsorption properties.

TLC's were performed on pre-coated TLC plates with silica gel 60 F_{254} (layer thickness 0.2 mm) with CH_2Cl_2: MeOH (90:10 or 95:5) for less polar compound, CH_2Cl_2:2-C_3H_7OH (75:25) or EtOAc:n-Hexane (70:30) for semi-polar compounds. TLC on reversed phase (RP)-C18 F_{254} (layer thickness 0.25 mm) was also used for polar substances, utilizing the solvent system MeOH:H_2O (85:15, 80:20, 70:30 or 60:40).

The bands separating on the TLC plate indicate the separation of compounds which were detected under UV absorbancy at 254 and 366 nm, and/or by spraying the TLC plates with anisaldehyde reagent with subsequent heating at 110^oC. Anisaldehyde/H_2SO_4 Spray Reagent (DAB 10) was prepared as follows: anisaldehyde (5 parts), glacial acetic acid (100 parts), methanol (85 parts), were mixed, to which 5 parts of concentrated H_2SO_4 were added slowly. The reagent was stored in an amber-colored bottle and kept refrigerated until use. Dragendorf reagent was also often used for showing alkaloids in the samples. Dragendorf reagent contains mixture of bismuth nitrate (0.85 g), distilled water (40 ml), acetic acid concentrate (10 ml), and potassium iodide 40% (8g KI in 20 ml water) (20 ml).

TLC's were always conducted for each fraction prior to further chemical work, to monitor the identity of each and the qualitative purity of the fractions or the

isolated compounds. Band separations in TLC ware also very helpful in optimizing the solvent system that would be later applied for column chromatography.

2.5.2. Vacuum liquid chromatography

Vacuum liquid chromatography (VLC) technique is essentially a preparative thin layer chromatographic (PTLC) separation run as a column, the flow of which is activated by vacuum. It differs from flash chromatography, in that VLC involves step gradient elution, and the column is allowed to run dry after each fraction is collected. The apparatus is simple and universally available, and the separations are efficient in terms of time, amount of adsorbent, and volume of solvent (Coll and Bowden, 1986).

The elution scheme is started with lipophilic solvent e.g. hexane and the polarity is gradually increase with increasing portion of ethyl acetate until 100% ethyl acetate is reached, then was continued with step gradient mixture of ethyl acetate-methanol until 100% methanol is reached. It provides an ideal pre-treatment of large amount of samples prior to fine chromatography separation like Lobar column or HPLC separations, and enables the fractionation of up to 30 g of extract in several hours.

2.5.3. Column chromatography

Fractions derived from VLC were subjected to repeated separation through series of column chromatography using appropriate stationary and mobile

phase solvent system previously determined by TLC. Purification of fractions was later performed on semi-preparative HPLC. Based on type of stationary phase, liquid chromatography can be distinguished into four general classes: normal phase, reversed phase, ion exchange, and size exclusion chromatography.

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

Reversed phase (RP) chromatography uses a non-polar stationary phase and a polar mobile phase (water, methanol, acetonitrile, and tetrahydrofuran). RP operates on the basis of hydrophilicity and lipophilicity. The stationary phase consists of silica packed with n-alkyl chains covalently bound. For instance, C-8 signifies a n-decyl chain and C-18 an octadecyl ligand in the matrix. The more hydrophobic the matrix on each ligand, the greater is the tendency of the column to retain hydrophobic moieties. Thus hydrophilic compounds elute more quickly than do hydrophobic compounds. RP is the most common form of liquid chromatography, primarily due to the wide range on analytes that can dissolve in the mobile phase.

Ion exchange chromatography involves ionic interactions. The mobile phase supports ionization to ensure solubility of ionic solutes. The stationary phase must be partially ionic to promote retention. Consequently, the interactions with

the stationary phase are strong, and this is usually reflected in longer retention time and broad peaks.

Gel permeation chromatography or size-exclusion chromatography involves separations based on molecular size of compounds being analyzed. The stationary phase consists of porous beads. The bigger 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 pores. Sephadex LH-20 is the preferred gel in this mode since it swells adequately in organic solvents (e.g. methanol). The useful fractionation range is approx 100-4000. Thus, compounds of molecular weight of approx 4000 are usually not retained by Sephadex LH-20 (Salituro and Dufresne, 1998).

In this study, Sephadex LH-20 columns with 100% methanol or mixture of methanol and dichloromethane (1:1) as eluent were commonly applied to initiate chromatography separation. Further separation was conducted using silica gel columns with several eluent combinations (dichloromethane and methanol, dichloromethane and isopropanol, n-hexane and ethyl acetate). RP-18 columns with methanol and nanopure water as eluent were frequently used, especially for isolating relatively polar compounds.

2.5.4. Low pressure liquid chromatography

Further purifications of fractions from were later performed on prepacked LOBAR® columns. In order to increase efficiency of the separation, low

pressure pump (40-60 mbar) was applied to run the mobile phase through the column. The following separation systems were used:

a) Stationary Phase: RP-18, 40-63 µm (LOBAR, size A and B)

   Solvent system: step gradient mixture of methanol-nanopure water

b) Stationary Phase: Silica gel 60, 40-63 µm (LOBAR, size A and B)

   Solvent system: step gradient mixture of dichloromethane-methanol or

   dichloromethane-isopropanol-methanol.

2.5.5. High performance liquid chromatography

High performance liquid chromatography (HPLC) was developed in the mid 1970's and quickly improved with the development of column packing materials and the additional convenience of on-line detectors. In the late 1970's, new methods including reverse phase liquid chromatography allowed for improved separation of very similar compounds.

A modern HPLC instrument consists of the reservoir of mobile phases, the pump, solvent mixer, auto sampler, auto injector, the separation column, and the detector. The main difference between HPLC and other modes of column chromatography is that the size of stationary phase particles is comparatively low (3-10 µm), and these particles are tightly packed to give a very uniform column bed structure. Thus HPLC utilizes high pressure pumps to run the solvent through the bed which increase the efficiency of the separation (Stead, 1998).

2.5.5.1. Analytical HPLC

The use of analytical HPLC was meant to identify the distribution of peaks either from crude extracts or fractions, as well as to evaluate the purity of isolated compounds.   Compounds are separated by injecting a plug of sample mixture onto the column.   The different components in the mixture pass through the column at different rates due to differences in their partitioning behavior between the mobile liquid phase and the stationary phase.

As standard analytical program, column was initially equilibrated isocratically with 10:90 [methanol: acidic-nanopure water (adjusted to pH 2 with phosphoric acid)] in 5 minutes then the solvent was gradually changed to 100% methanol in 30 minutes which was continued to 10 minutes with 100% methanol. Injection volume was 20 μl. Compounds that have UV absorption were detected by UV-VIS diode array detector.   Analytical HPLC was used to identify interesting peaks from extracts and fractions as well as to evaluate the purity of isolated compounds.

2.5.5.2. Semi-preparative HPLC

Natural products are commonly available only as minor components or mixtures of closely related structures in the extract; in these circumstances the sensitivity and resolving power of HPLC has an advantage to ease their separation.

The semi-preparative HPLC was utilized for the purification or isolation of pure compounds from fractions previously obtained from other methods of column

chromatography. The solvent system, either gradient or isocratic depends primarily on the retention time of the compound. Every injection has a maximum amount of 2 mg of the fraction dissolved in the solvent system in a volume of 1 ml. The solvent system was pumped through the column at a flow rate of 5 ml/min. The eluted peaks, which were clearly detected by the online UV detector and recorded on chromatointegrator, were separately collected in Erlenmeyer flasks.

2.6. Procedure for the isolation of the secondary metabolites

2.6.1. Isolation of metabolites from *Agelas nakamurai*
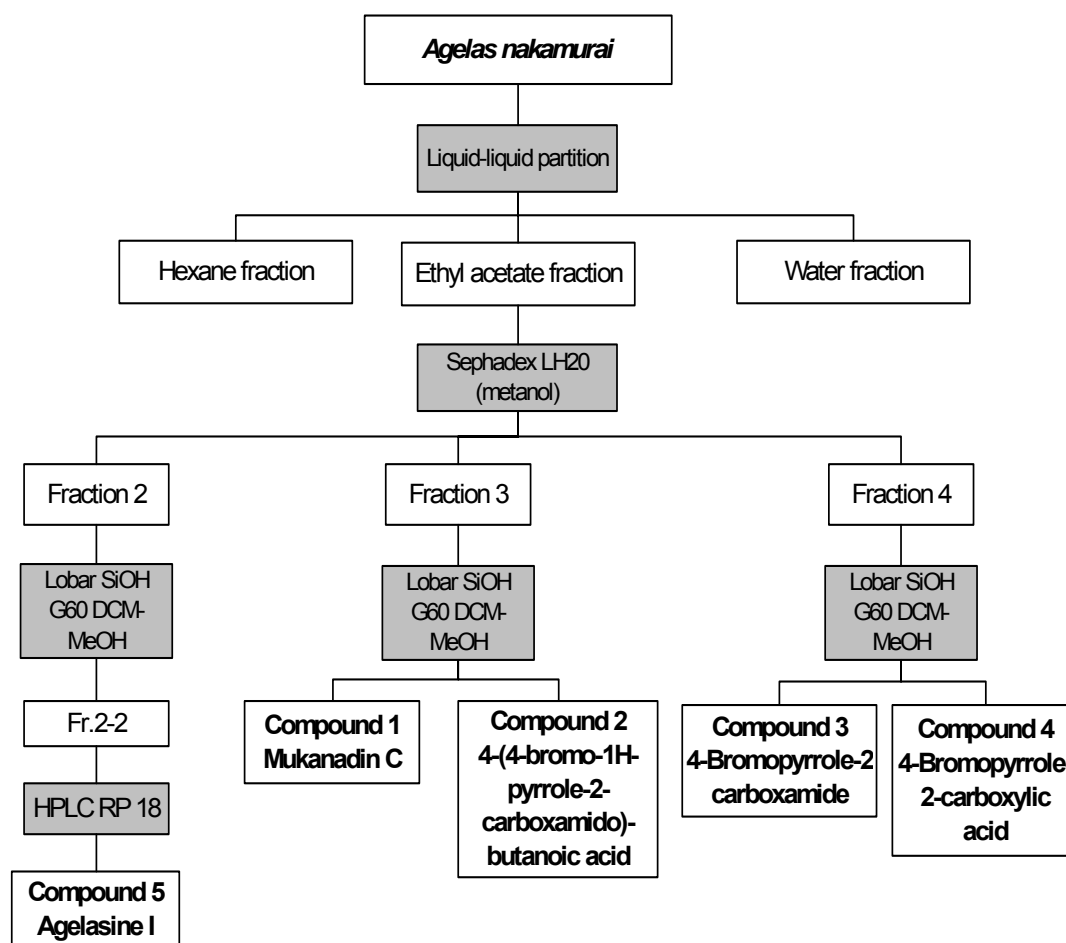
Figure 2.2. Isolation scheme of compounds from *Agelas nakamurai.*

2.6.2. Isolation of metabolites from *Theonella swinhoei*
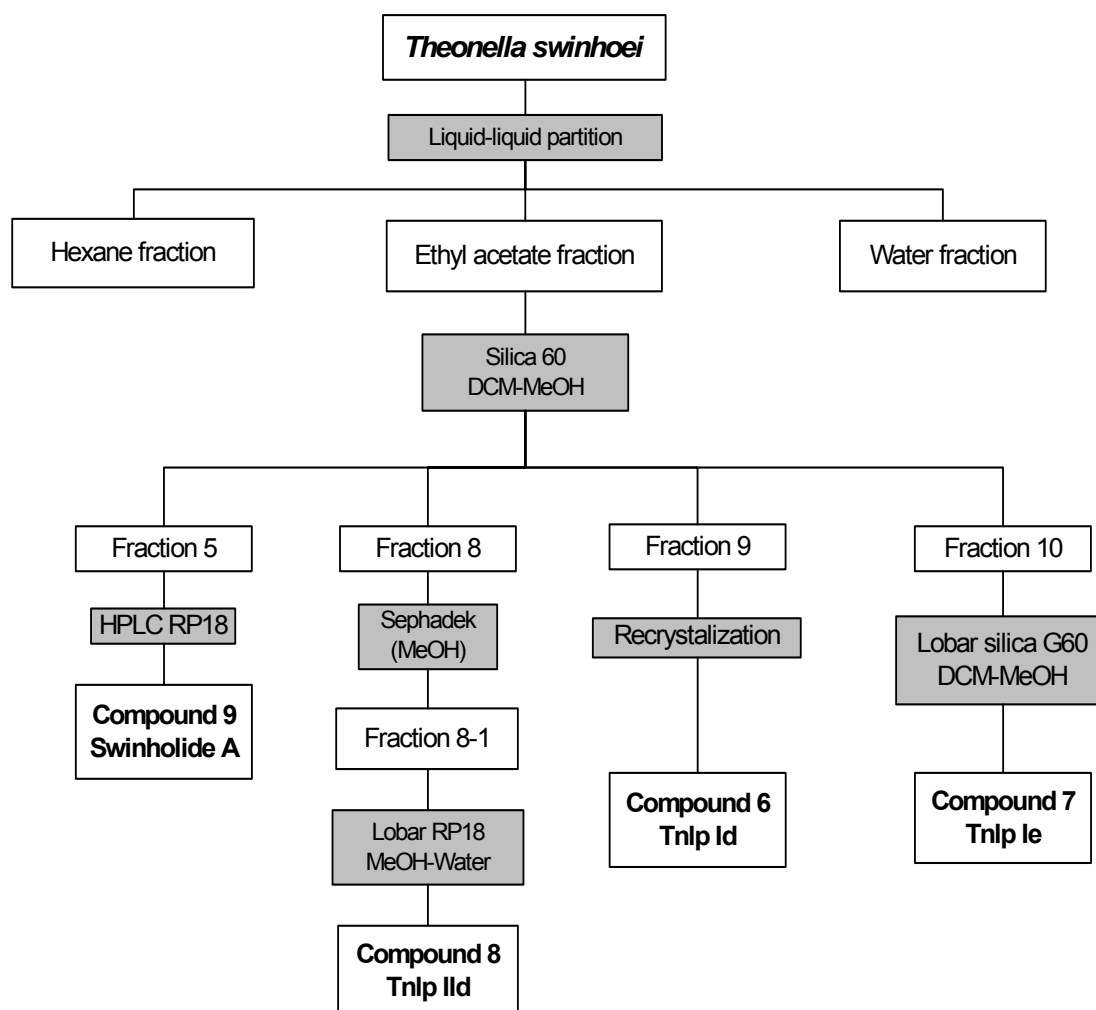
Figure 2.3. Isolation scheme of compounds from *Theonella swinhoei*.

2.6.3. Isolation of metabolites from *Acanthostongylophora ingens*
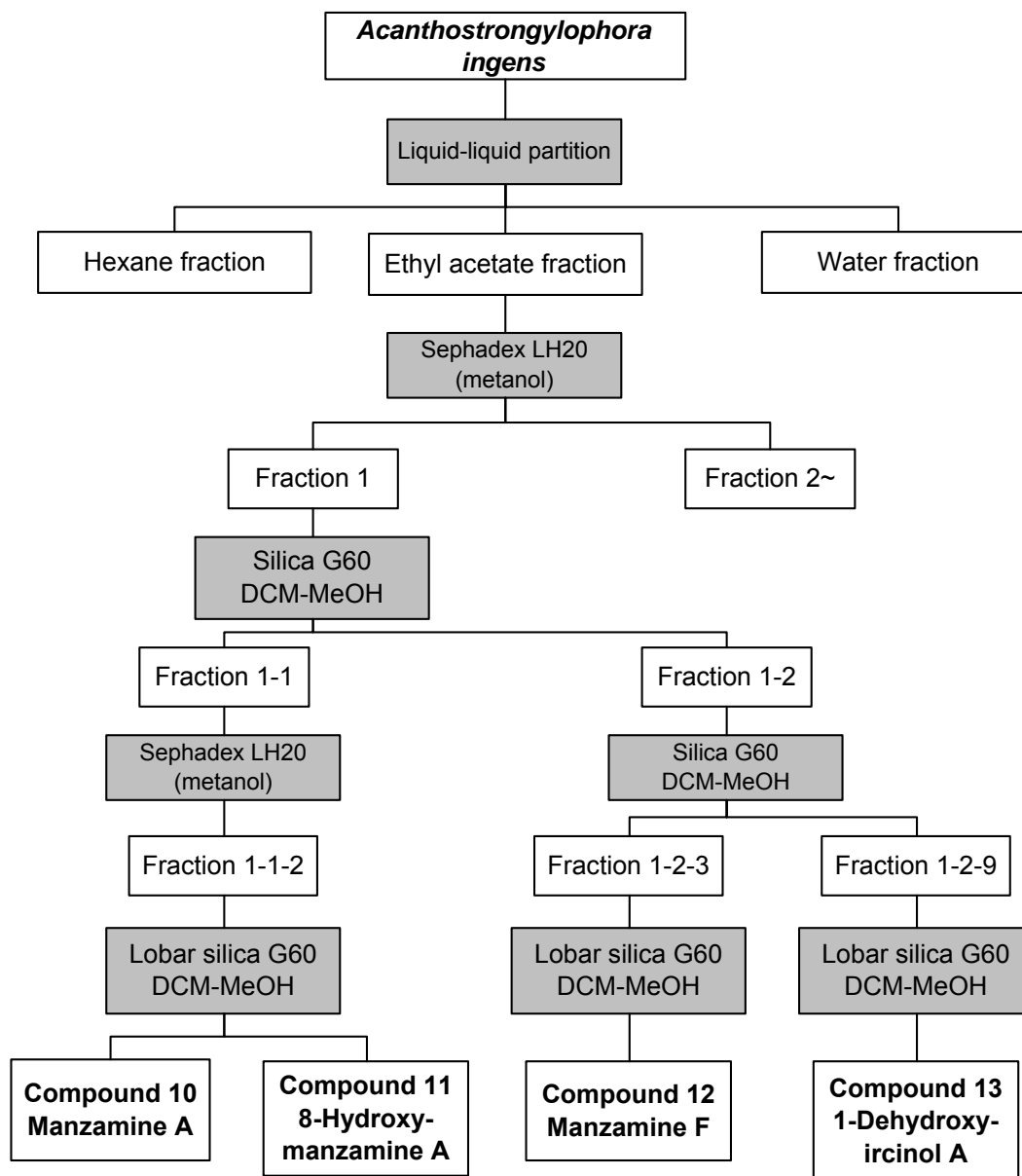
Figure 2.4. Isolation scheme of compounds from *Acanthostrongylophora ingens*.

2.6.4. Isolation of metabolites from *Petrosia hoeksemai*
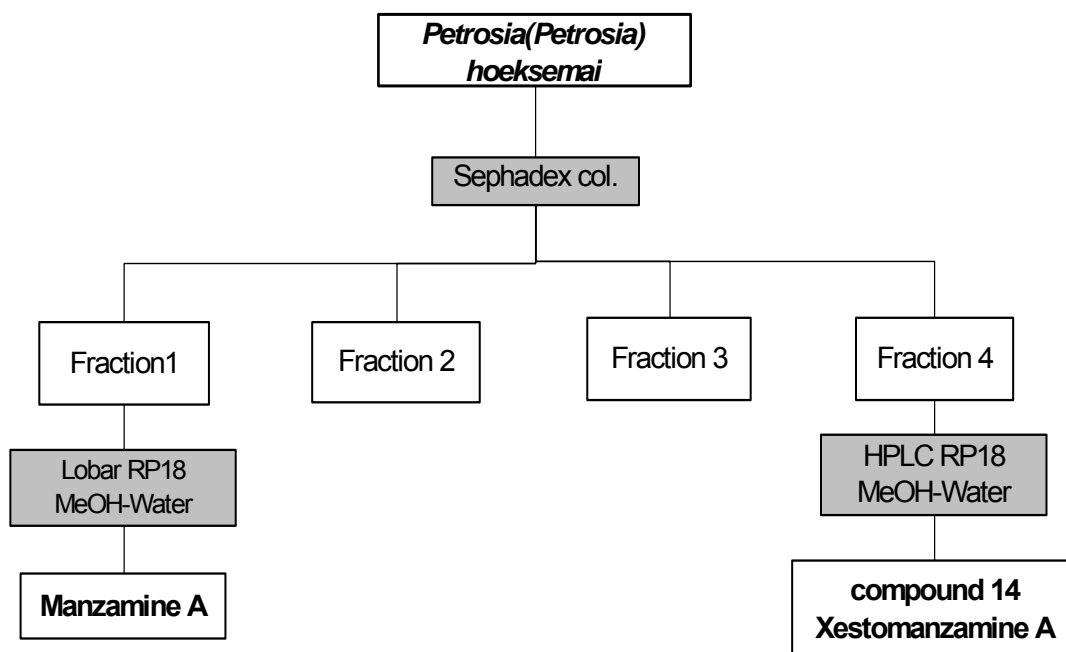
Figure 2.5. Isolation scheme of compounds from *Petrosia(Petrosia) hoeksemai.*

2.6.5. Isolation of metabolites from *Axynissa aculeata*
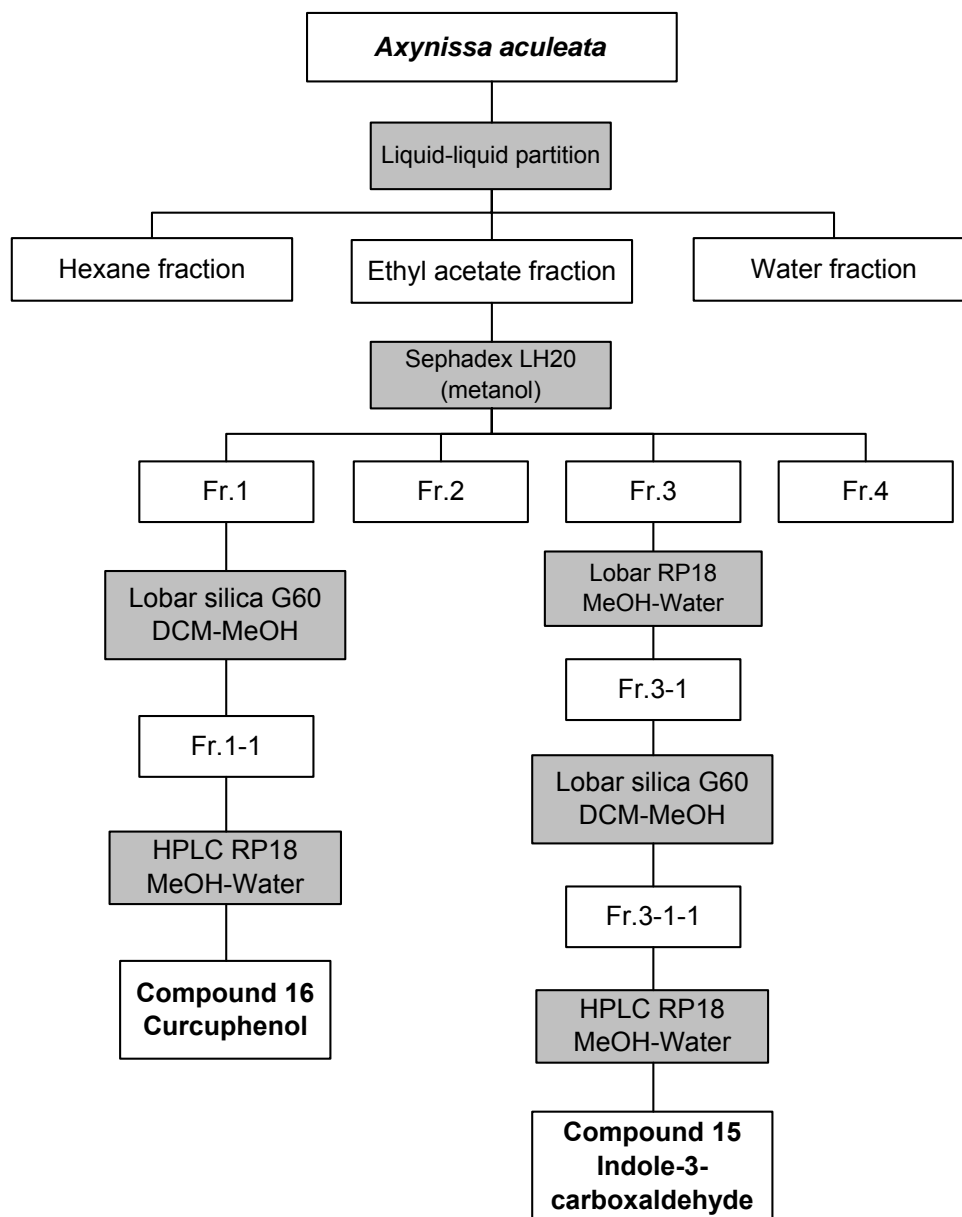
Figure 2.6. Isolation scheme of compounds from *Axynissa aculeata.*

2.7. Structure elucidation of the isolated secondary metabolites

Structure elucidation and identification is an integral part of natural products chemistry. Spectroscopic methods, such as mass spectrometry (MS), nuclear magnetic resonance (NMR), and IR and UV/ VIS spectroscopy have, thus, become indispensable tools in chemistry. In the field of marine natural products, levels of 1 mg of compound per 3 kg of organism combined with complicated structure were not uncommon. Thus high-field NMR and mass spectrometry that involved Tandem-MS techniques were used. Development of computerized techniques has permitted utilization of Fourier transform methods for acquisition of spectra and greatly increased the capabilities of these methods.

2.7.1. Mass spectrometry (MS)

Low resolution mass spectra were measured by EI-, CI- and FAB-MS on a Finnigan MAT 8430 mass spectrometer. Measurements were done by Dr. Peter Tommes of HHU Düsseldorf. ESI- and APCI-MS was measured on a Finnigan LCQ DECA-7000 mass spectrometer in Institute for Pharmaceutical Biology and Biotechnology, HHU Düsseldorf.

Electron Impact Mass Spectroscopy (EI-MS) 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 ionize an organic molecule (requiring about 7-10 eV) but also cause 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 characterize the compound. The disadvantage is the frequent absence of a molecular ion.

Fast Atom Bombardment Mass Spectroscopy (FAB-MS). The analyte is dissolved in a non volatile liquid matrix such as glycerol, thioglycerol, m-nitro benzyl alcohol, or diethanolamine and a small amount (about 1 microliter) is placed on a target. The target is bombarded with a fast atom beam (for example, 6 keV xenon atoms) that desorbs molecular-like ions and fragments from the analyte. Cluster ions from the liquid matrix are also desorbed and produce a chemical background that varies with the matrix used. This type of ionization presents the molecular ion as base peak.

Electron Spray Ionization Mass Spectrometry (ESI-MS). The sample solution is sprayed across a high potential difference (a few kilovolts) from a needle into an orifice in the interface. Heat and gas flows are used to desolvate the ions existing in the sample solution. Electrospray ionization can produce multiply charged ions with the number of charges tending to increase as the molecular weight increases. This kind of ionization method, give possibility to connect this spectrometer onto HPLC instrument as detector (see LC-MS).

Atmospheric Pressure Chemical Ionization Mass Spectrometry (APCI-MS) is having similar interface to that used for ESI. In APCI, a corona discharge is used to ionize the analyte in the atmospheric pressure region. The gas-phase

ionization in APCI is more effective than ESI for analyzing less-polar species. ESI and APCI are complementary methods.

Liquid Chromatography – Mass Spectrometry (LC-MS) is combination of the power of HPLC as a separation method and mass spectrometry as a detector. If a mass spectrum of each component can be recorded as it elutes from the HPLC column, quick characterization of the components is greatly facilitated. Usually, ESI or APCI is interfaced with LC to make an effective on-line LC/MS. HPLC/ESI-MS or APCI-MS was carried out using a Finnigan Deca mass spectrometry connected to a UV detector. The samples were dissolved in water/MeOH mixtures and injected to HPLC set-up. HPLC was run on a Eurospher C-18 reversed-phase column. Measurements were done at HHU Düsseldorf.

2.7.2.   Nuclear magnetic resonance (NMR) spectroscopy

The measurement of 1D and 2D NMR spectra was carried out by Dr. W. Peters at the NMR service, Heinrich-Heine University, Düsseldorf, and Dr. Victor Wray at the Institute for Biotechnology (Gesellschaft für Biotechnologische Forschung/GBF), Braunschweig, Germany.   Proton (^{1}H) and carbon (^{13}C) NMR spectra were recorded at 300°K on Bruker DPX 300, ARX 500 or AVANCE DMX 600 NMR spectrometers.

Several deuterated solvents (DMSO-d6, CDCl$_3$, CD$_2$Cl$_2$, CD$_3$OD, or D$_2$O) were used to dissolve samples for NMR measurement. The selection of which was

primarily dependent on the solubility of the sample and the consideration of obtaining hydroxyl and amine group. Spectra of pure compounds were processed using Bruker 1D WIN-NMR or 2D WIN-NMR software.

They were calibrated using solvent signals of carbon. There is a list of calibration value for deuterated solvents against trimethylsilane (TMS): carbon (^{13}C) CDCl$_3$ 77.00 ppm, MeOD 49.00 ppm, CD$_3$COCD$_3$ 30.50 ppm, DMSO-d6 39.70 ppm) and proton (^{1}H) CDCl$_3$ 7.26 ppm, D$_2$O 4.79, MeOD 3.35 ppm, DMSO-d6 2.50 ppm, acetone-d6 2.05 ppm). The observed chemical shift (δ) values were given in ppm and the coupling constants (J) in Hz.

Structural assignment was based on spectra resulting from one or more of the following NMR spectra measurement: ^{1}H, ^{13}C, DEPT, ^{1}H $\rightarrow$ ^{1}H COSY, ^{1}H $\rightarrow$ ^{13}C direct correlation (HMQC), ^{1}H $\rightarrow$ ^{13}C long range correlation (HMBC), TOCSY and NOESY. HMQC experiment yield correlations between directly bonded carbon and hydrogen atoms, while HMBC experiment assign ^{1}H and ^{13}C signals on the basis of ^2J(C,H) and ^{2+n}J(C,H) values.

Multiplicity for ^{13}C was deduced from DEPT experiments; singlet (s) = C, doublet (d) = CH, triplet (t) = CH$_2$, quartet (q) = CH$_3$. A procedure which enhances the intensities of ^{13}C signals and also provides information on the number of attached protons. Quaternary carbons are not observed. The 135° pulse results in positive signals for CH and CH$_3$ groups and negative signals for CH$_2$ groups. The 90° pulse results in positive signals for CH groups and null

signals for CH_2 and CH_3 groups. The 45° pulse results in positive signals for CH, CH_2 and CH_3 groups.

Coupled protons on the molecule were deduced from COSY experiment. The diagonal and the projection on each axis are the one-dimensional spectrum. The off-diagonal peaks indicate the presence of coupling between pairs of protons. As enhancement of COSY experiment, a TOCSY experiment could be conducted to show the proton member of any spin system. The TOCSY pulse sequence is similar to that of the normal COSY experiment, except that here the second 90° pulse is replaced by a spin-lock stage. The off-diagonal of TOCSY spectrum peak shows correlation between protons that belong to the same coupled spin system. This experiment is very useful in the field of peptides, since here the correlation peaks enable one to identify the protons belonging to the individual amino acids (Friebolin, 1998).

To have a knowledge of all atoms that are close in space, NOESY experiment was conducted. In simple word, NOESY is a 2D version of NOE experiment, a procedure of irradiation at specific frequencies before signal acquisition enhances the intensities of nearby nuclei. Nearby nuclei induce relaxation, which leads to signal enhancement, through dipole-dipole interactions. The effect usually diminishes as function of $1/\chi^6$. The diagonal and the projection on each axis are the one-dimensional spectrum. The off-diagonal peaks indicate the presence of Overhauser enhancements between pairs of protons.

2.8. Bioassay

The methods for the detection of biological activity of extracts, fractions, and pure compounds can best be divided into two groups for screening purposes: general screening bioassays and specialized screening bioassays. The search for specific pharmacologic activities often overlooks other useful activities which are not detected or are ignored, in the screening process. Furthermore specific test methods are often cumbersome and expensive. Hence, for bioassay-guided isolation of natural products single, inexpensive 'bench-top' bioassays for the rapid screening of extracts and fractions have to be employed. Since most active principles are toxic at elevated doses, a possible approach to develop an effective general bioassay might be simply to screen for substances that are toxic to zoological systems. Once such substances have been isolated, a battery of specific and more sophisticated bioassays could then be employed.

2.8.1. Brine-shrimp assay

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

procedure determines LC_{50} values in µg/ml of active compounds and extracts in the brine medium (Carballo *et al.*, 2002; Meyer *et al.*, 1982).

2.8.1.1. Preparation of samples

The test samples were dissolved in an organic solvent and the appropriate amount is transferred to a 10-ml sample vial. For crude extracts, 0.5 µg of the compound were used and various concentrations of the pure compound. The samples were then dried under nitrogen and the dried samples were reconstituted with 20 µL DMSO. Control vials containing DMSO were also prepared.

2.8.1.2. Hatching the eggs

Brine shrimp eggs (Dohse, Aquristik GmbH, Bonn, Germany) were hatched in a small tank filled with artificial sea water which was prepared with a commercial salt mixture (Sera Sea-Salt, Aquaristik GmbH, Bonn, Germany) and distilled water. A plastic divider with several 2 mm holes was clamped in the dish to make two unequal compartments. The eggs were sprinkled into the larger compartment which was darkened, while the smaller compartment was illuminated. After 48 hours, the nauplii will move to lighted side and ready to be used for assay.

2.8.1.3. Bioassay

Ten nauplii were collected using Pasteur pipette and transferred into each test sample vial. Artificial sea water was then added to make 5 mL. A drop of dry

yeast suspension (3 mg in 5 ml artificial sea water) was added as food to each vial. The vials were maintained under illumination. Survivors were counted, with the aid of a magnifying glass after 24 hours and the percent deaths at each dose and control were determined. The LC_{50}'s were determined using the probit analysis method.

2.8.2.   Insecticidal bioassay

The polyphagous pest insect *Spodoptera littoralis* Boisd. (Noctuidae, Lepidoptera), commonly known as Egyptian cotton worm, has proved to be a good test model for an insecticidal bioassay on a broad spectrum of chemical classes of natural products. The larvae of *S. littoralis* feed on a wide variety of plant species and are one of the most robust and hazardous pest insects in the Mediterranean as well as in Africa. *S. littoralis* has a short life cycle of 4 weeks and is easy to maintain in the laboratory by utilizing an artificial diet consisting mainly of leguminous beans in agar.

2.8.2.1. Culture conditions

Larvae of *S. littoralis* were from a laboratory colony reared on artificial diet under controlled conditions (Srivastava and Proksch, 1991). Optimum growth was achieved under standardized conditions at a temperature of 28°C and a controlled day and night phase of 16 and 8 h, respectively.

Larvae at their pre-pupal stage were regularly separated (to prevent from being cannibalized) and set on Vermiculit, a kind of silicate mineral, until they reach

the pupal stage. The pupae were then stored to a dark humid chamber at 28°C until they develop to their final adult stage. The adults were then transferred to a 10-liter plastic pail, lined with filter paper on which the female can lay their eggs. The adults feed on saccharose solution at this stage; they were maintained at a controlled day and night phase of 16 and 8 h, respectively, and at a temperature of 28°C. The laid eggs were collected every two days and again transferred to the dark chamber until the neonate larvae hatch.

2.8.2.2. Artificial diet

The artificial diet consisting mainly of leguminous beans in agar were first left to stand overnight in 460 mL water and then homogenized in a mixer. To the bean suspension, the following substances were added:

3.0	g	L(+)-Ascorbic acid
3.0	g	Nipagin
30.0	g	Baker's Yeast
0.180	g	Gentamycin Sulfate
1.0	mL	Formaldehyde Solution

To this bean homogenate, warm agar suspension (10 g agar cooked in 315 mL water and cooled down to 50°C) was added and thoroughly mixed. The agar bean mixture was then poured into a 3-liter GERDA box and cooled to room temperature to solidify. The solidified agar-bean diet was then stored in the refrigerator until use.

2.8.2.3. Chronic feeding assay

The neonate larvae of *S. littoralis* are forced to feed on a diet treated with a known concentration of a test sample, and then the larvae are monitored after 6 days. The diet consists of a freeze-dried form of the above-described bean homogenate mixture, this time consisting only of nipagin, baker's yeast and formaldehyde solution. The test sample, dissolved in 2 mL carrier solvent, was incorporated to 0.735 g of the freeze-dried homogenate, and the mixture is allowed to stand overnight to allow evaporation of the carrier solvent. For biological screening studies, concentrations of 5.0 and 1.0 mg were used for crude extracts and pure compounds, respectively. To the treated homogenate, 2.2 mL of warm agar suspension was added and then left to solidify at room temperature. Twenty neonate larvae of S. littoralis were used per assay whose growths are then monitored after 6 days of incubation at $28^{o}C$ in a dark humid chamber. Activity is measured as the survival rate or growth rate of the surviving larvae when compared to controls.

$$\% \text{ survival rate } = \frac{\text{number of surviving larvae (sample)}}{\text{number of surviving larvae (control)}} \times 100\%$$

$$\% \text{ growth rate } = \frac{\text{Average larval weight (sample)}}{\text{Average larval weight (control)}} \times 100\%$$

A subsequent experiment on the active compound was analyzed for activity at a range of doses. Potency was determined as the effective concentration (EC_{50}) of the test substance added to that diet necessary to cause a 50% reduction in weight. Lethal concentrations that cause 50% mortality (LC_{50}) were also

determined. The ED_{50} and LC_{50} were calculated by probit analysis from the dose response curve.

2.8.3.   Antimicrobial activity

Crude extracts and pure compounds were tested for antibacterial activity against the following standard strains: gram positive bacteria *Bacillus subtilis* and *Staphylococcus aureus*, gram negative bacteria *Escherichia coli*. Three fungal test strains were applied for antifungal activity: *Saccharomyces cerevisiae*, *Candida albicans*, and *Cladosporium herbarium*. The agar diffusion assay was performed according to the Kirby-Bauer Test (Bauer *et al.*, 1966).

2.8.3.1. Culture preparation

Prior to testing, a few colonies (3 to 10) of the organism to be tested were subcultured in 4 ml of tryptose-soy broth (Sigma, FRG) and incubated for 2 to 5 h to produce a bacterial suspension of moderate cloudiness. The suspension was diluted with sterile saline solution to a density visually equivalent to that of a $BaSO_4$ standards, prepared by adding 0.5 ml of 1% $BaCL_2$ to 99.5 ml of 1% H_2SO_4 (0.36 N). The prepared bacterial broth is inoculated onto Müller-Hinton-Agar plates (Difco, USA) and dispersed by means of sterile beads.

2.8.3.2. Agar diffusion assay

For screening, aliquots of the test solution were applied to sterile filter-paper discs (5 mm diameter, Oxoid Ltd.) to give a final disc loading concentration of 500 µg for crude extracts and various concentrations (50, 25, 10, 5 and 1µg/disk) for pure compounds. The impregnated discs were placed on agar

plates previously seeded with the selected test organisms, along with discs containing solvent blanks. The plates were incubated at 37°C for 24 h then antimicrobial activity was recorded as a clear zone of inhibition surrounding the disc at which the diameter was measured in mm.

3. RESULTS

3.1. Secondary metabolites of the sponge *Agelas nakamurai*

Numerous interesting compounds have been reported from the sponge *Agelas nakamurai*. Majority of these compounds found in this sponge are purinoditerpenes and bromopyrrole derivatives, exhibiting interesting bioactivities such as antimicrobial, inhibition of Na and K-ATPase, as well as antiserotonergic activity (Iwagawa *et al*., 1998; Wu *et al*., 1986). Mono and dibromopyrrole derivative alkaloids are characteristic metabolites of sponges of the genus Agelas. These compounds often show interesting biological properties such as antibiotic and antifouling activities. Selected examples of compounds found from sponges of the genus *Agelas* are presented below. Five secondary metabolites were isolated from the sponge *Agelas nakamurai* collected from Menjangan Island, Bali-Indonesia.
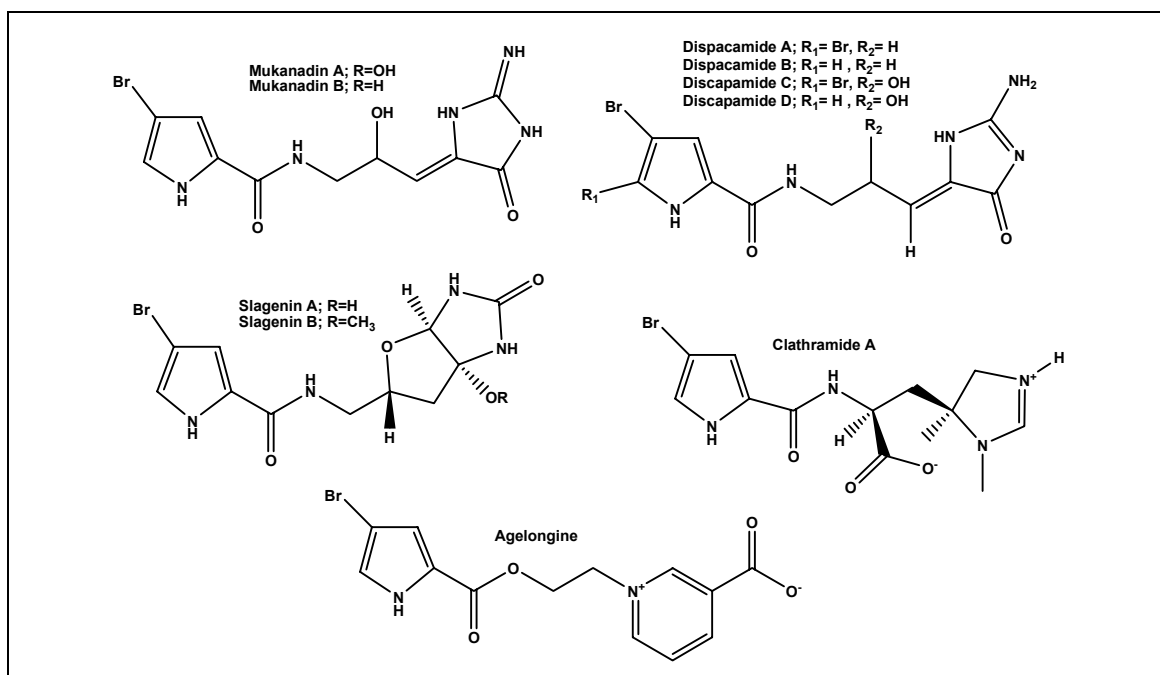
Figure 3.1. Selected compounds occurring in the sponge *Agelas nakamurai*.

3.1.1. Compound 1: Mukanadin C
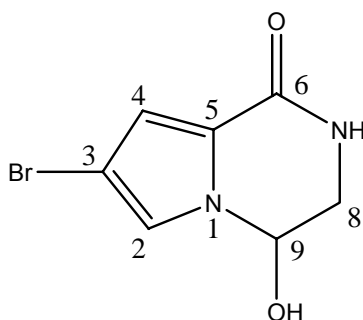
Compound **1** was isolated as a white powder with UV (MeOH) absorbance at

λmax 232 and 278 nm. The positive ESI-MS of compound **1** showed a cluster of

molecular ions [M]+ peaks at m/z 230:232 with ratio 1:1 indicating the presence

of a monobromine subtituent. This molecular weight is compatible with the

molecular formula $C_7H_7BrN_2O_2$. The 1H NMR spectrum of **1** (see Figure 3.3)

revealed the presence of two doublet protons at 7.08 and 6.79 (H-2 and H-4)

which suggested the occurrence of an asymmetric pyrrole ring. A coupling

constant of 1.7 Hz suggested a meta coupling between these two protons and

confirmed the positition of the bromine attached at carbon number 3. In the

upperfield region, one proton triplet signal appeared at 5.61 ppm with a coupling

constant of 3.5 Hz. The chemical shift of this proton suggested a proton under

the influence of two electronegative units and was assigned as H-9 that coupled

with methylene protons at C-8, which gave a double doublet at 3.71 and 3.46

ppm with coupling constants of 3.5 and 13.4 Hz. The chemical shift of these two

protons, suggested the position of the methylene attached to the amide moiety.

ESI-MS/MS showed four major fragments at m/z 230/232(30%), 202/204(27%),

172/174(100%) and 143/145(23%). Major fragmentation included the alkyl

amine cleavage which yielded an ion peak at m/z 172/174 with 100%

abundance. Second pattern goes through amide cleavage which yielded an ion

peak 202/204 with an abundance of 27%. The structure of compound **1** was

established as mukanadin C from ESI-MS fragmentation analysis (see Figure

3.4) and by comparison of its 1H NMR data with the literature (Uemoto et al.,

1999).

Figure 3.2. Proton NMR data of compound **1**.

Position	δ **1H (ppm), multiplicity, J (Hz)**
2	6.79 (d, 1H, J=1.7Hz)
4	7.08 (d, 1H, J=1.7Hz)
8	3.71 (dd, 1H, J=3.5Hz, J=13.4Hz) 3.49 (dd, 1H, J=3.5Hz, J=13.4Hz)
9	5.61 (t, 1H, J=3.5Hz)
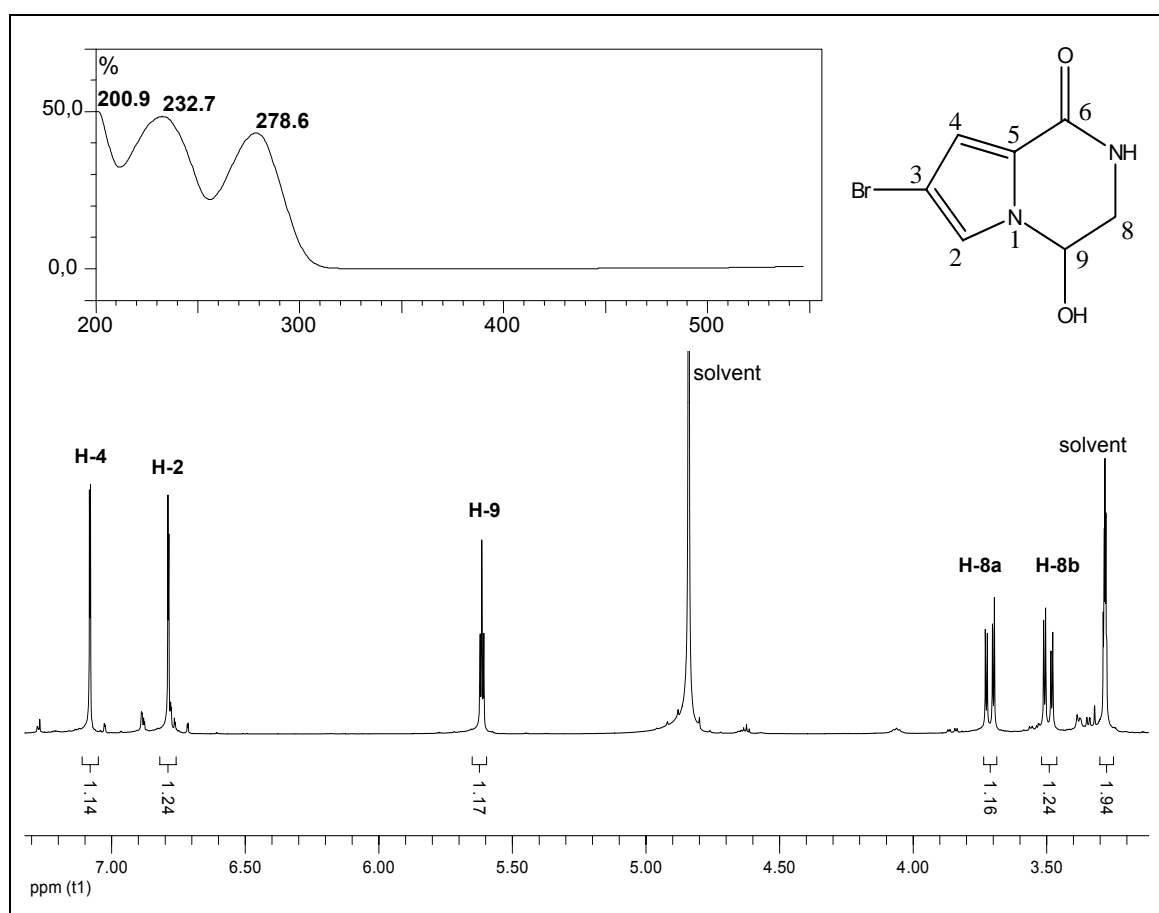
Figure 3.3. Ultraviolet and proton NMR spectrum of compound **1**.
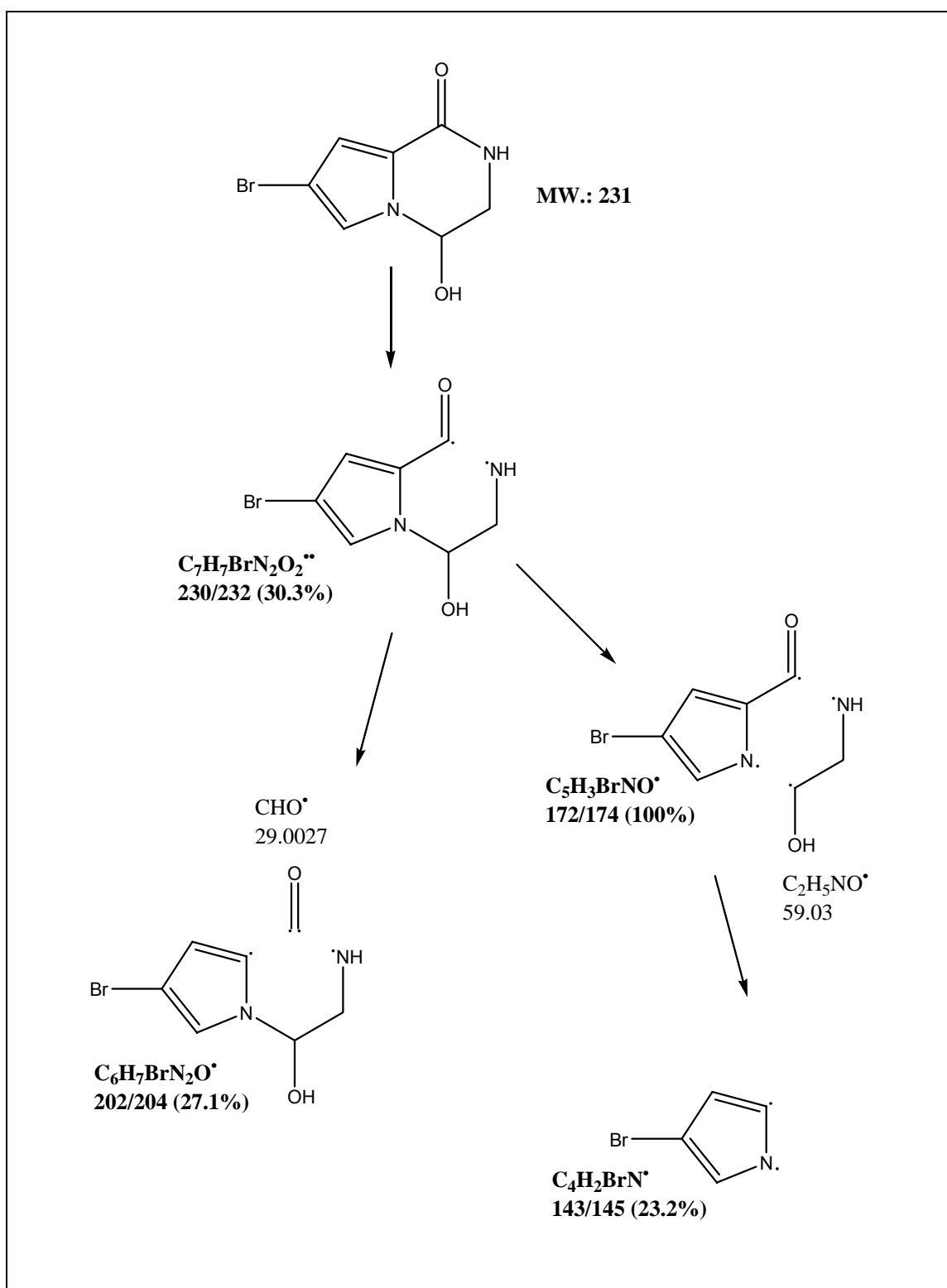
Figure 3.4. Mass spectrometric fragmentation of compound **1**.

3.1.2. Compound 2: 4-(4-bromo-1H-pyrrole-2-carboxamido)-butanoic acid (New)
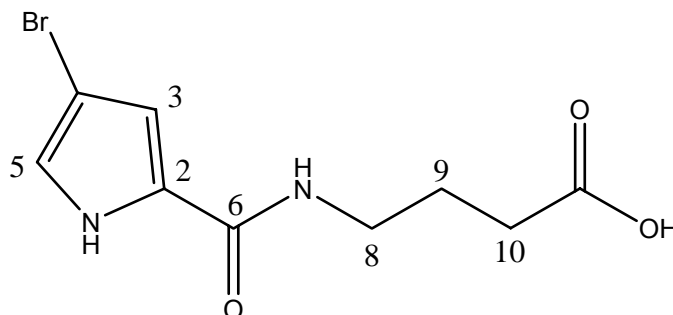
Compound **2** was isolated as a white powder with UV absorbance at λmax 269.

The positive ESIMS of compound **2** showed a cluster of molecular ions at *m/z*

274:276 [M+H]+ with a ratio of 1:1 indicating the presence of a monobromine

substituent. The molecular weight is compatible with the molecular formula

$C_9H_{11}BrN_2O_3$. The ^{1}H NMR spectrum of **2** (see Figure 3.5) revealed the

presence of two doublet protons at 6.89 and 6.75 (H-3 and H5) which

suggested the presence of an asymmetric pyrrole ring. A coupling constant of

1.5 Hz suggested a *meta* coupling between these two protons and confirmed

the positition of the bromine attached at C-4. There were three signals in the

upper field region; a triplet (2H) at 3.34 ppm with coupling constant of 6.9 Hz

which suggested a methylene moiety adjacent to another methylene and amide

function, a triplet (2H) at 2.34 ppm with coupling constant of 7.4 Hz suggested

that the methylene moiety is next to another methylene and carbonyl unit, and a

quintet (2H) at 1.86 ppm with a coupling constant of 7.12 Hz suggested a

methylene coupled to two methylene neighbours. These three peaks were

assigned as H-8, H-10 and H-9, respectively. HMBC analysis (see Figure 3.6)

showed correlations of H-3 to C-2(J^2) and C-5(J^3) and correlations of H-5 to C-

$2(J^3)$, C-3(J^3) and C-4(J^2) proved the disubstituted-4-monobromopyrrole substructure. Correlations of H-9 to the carbon at 177.1 ppm assigned as the carbonyl unit at C-11 and two J^2 correlations to C-8 and C-10. These data together with the correlation of H-8 to C-10 (J^3) proved a butanoic acid substructure. In addition, a cross peak from H-8 to the carbon at 162.3 ppm proved the connectivity of the butanoic acid side chain to the amide carbon (C-6). ESI-MS fragmentation showed three major peaks (see Figure 3.7) at m/z 274/276 (9.7%), 172/174 (100%), and 144/146 (29%). Major fragmentation revealed an amide bond cleavage which yielded peaks at m/z 172/174 for a 4-bromopyrrole-2-carboxylic acid ion. The structure of compound **2** was established as 4-(4-bromo-1H-pyrrole-2-carboxamido)butanoic acid from analysis of its ^{1}H NMR and HMBC spectrum together with its ESI-MS fragmentation data (see Figure 3.7).

Table 3.1. NMR data of compound **2** in MeOD.

Nr.	^{13}C (ppm)	δ ^{1}H (ppm), multiplicity, *J* (Hz)	HMBC (H → C)
2	127.1 s	--	
3	112.8 d	6.75 (1H, d, j 1.5)	C2; C5
4	97.2 s	--	
5	122.6 d	6.89 (1H, d, j 1.5)	C2; C3; C4
6	162.3 s	--	
8	39.2 t	3.34 (2H, t, j 6.9)	C6; C8(direct); C9; C10
9	25.6 t	1.86 (2H, q, j 7.12)	C8; C9(direct); C10; C11
10	32.3 t	2.34 (2H, t, j 7.4)	C8; C9; C11
11	177.1 s	--	
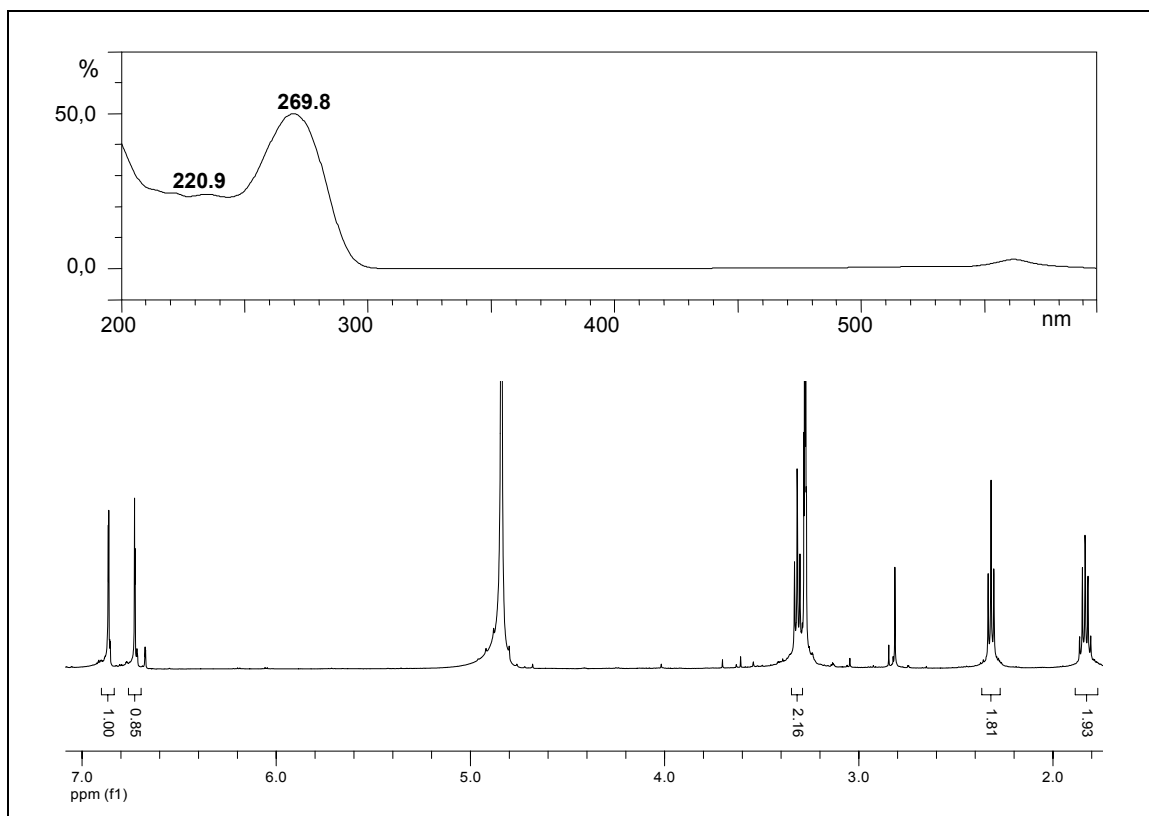
Figure 3.5. UV and proton NMR of compound **2**.
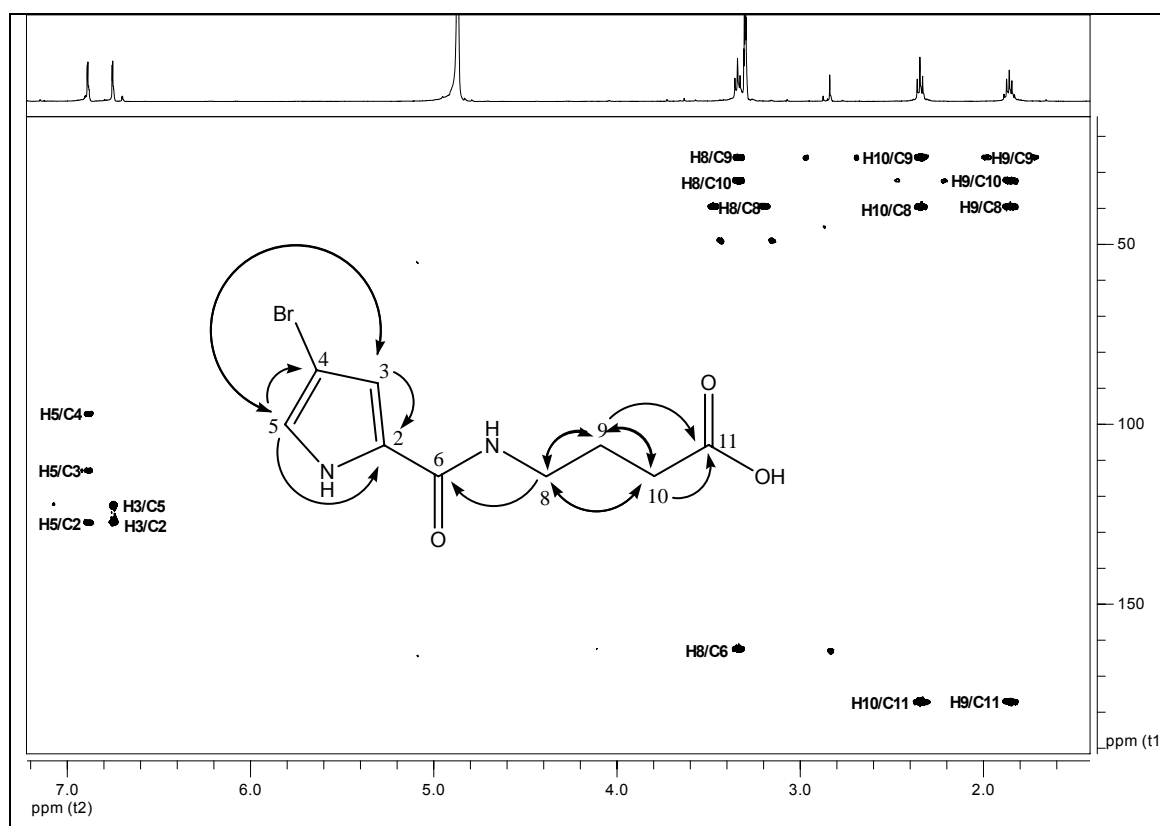
Figure 3.6. HMBC correlation of compound **2**.
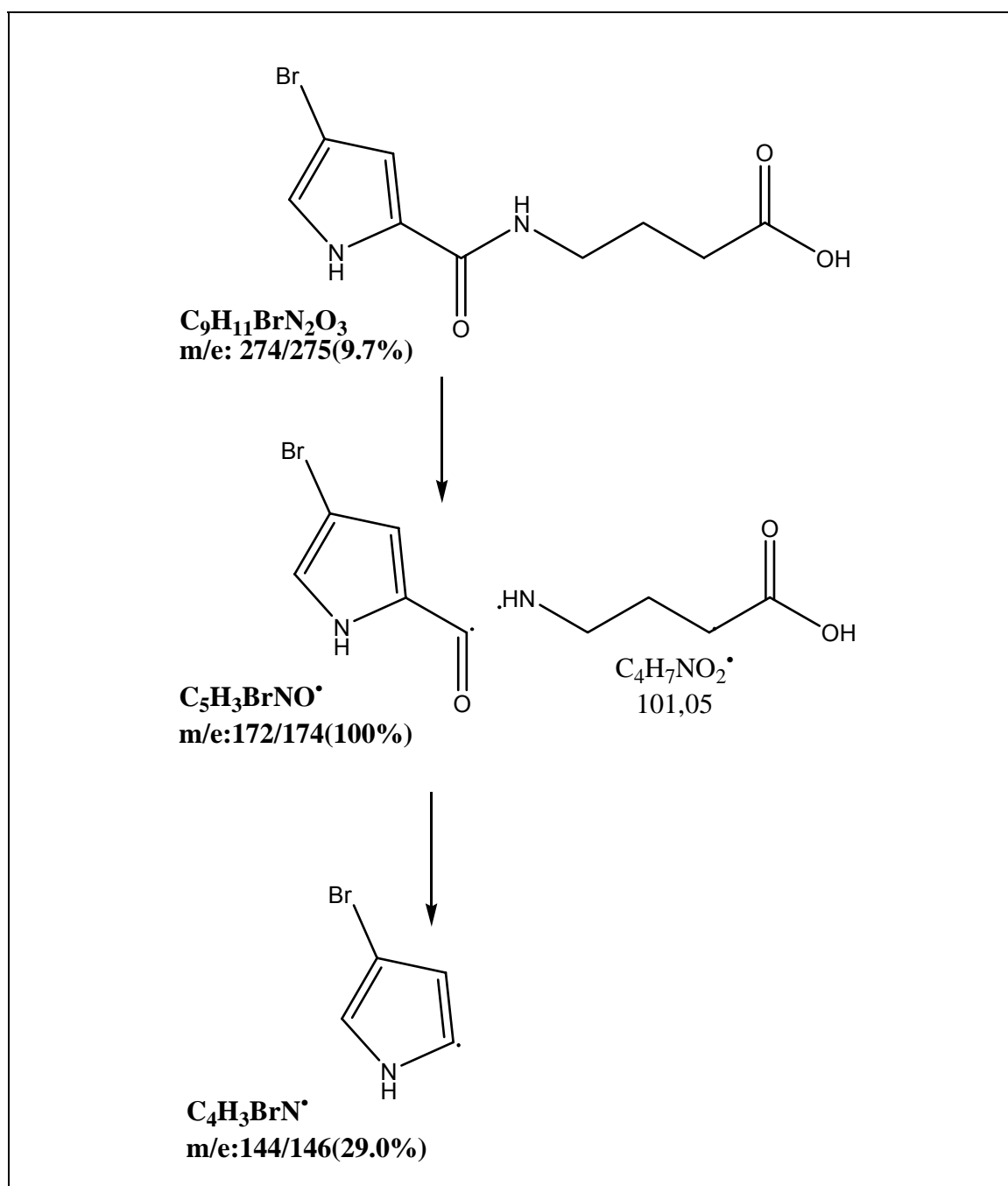
Figure 3.7. Mass spectrometric fragmentation of compound **2**.

3.1.3. Compound 3: 4-Bromopyrrole-2-carboxamide
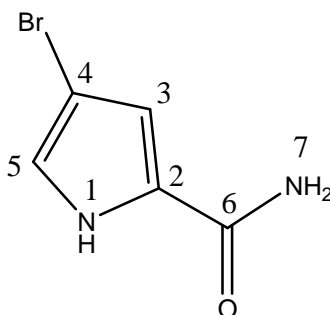
Compound **3** was isolated as a white powder with UV absorbance at λ_{max} 270 nm (MeOH). The positive ESIMS of compound **3** showed a cluster of molecular ion peaks at *m/z* 188:190 [M+H]$^+$ with ratio of 1:1 indicating the presence of one bromine substituent. This molecular weight is compatible with the molecular formula $C_5H_5BrN_2O$. The ^{1}H NMR spectrum of compound **3** (see Figure 3.9) revealed the presence of one singlet proton at 11.79 assigned as 1-NH and two double-doublet protons at 6.97 and 6.85 (H-3 and H5) which suggested the presence of an asymmetric pyrrole ring. A coupling constant of 1.5 Hz suggested a *meta* coupling between these two protons. Two protons at 7.58 and 7.10 ppm was assigned as amide protons ($CONH_2$). The structure of compound **3** was established as 4-bromopyrrole-2-carboxamide after comparison of its ^{1}H NMR data with the literature (Mancini *et al.*, 1997).

Table 3.2. The ^{1}H NMR data of compound **3** in DMSO.

Nr.	δ ^{1}H (ppm), multiplicity, *J* (Hz)
1	11.79 (s, 1H)
3	6.97 (dd, 1H, J=1.5Hz, J=2.8Hz)
5	6.85 (dd, 1H, J=1.5Hz, J=2.5Hz)
7	7.58 (b, 1H) 7.10 (b, 1H)
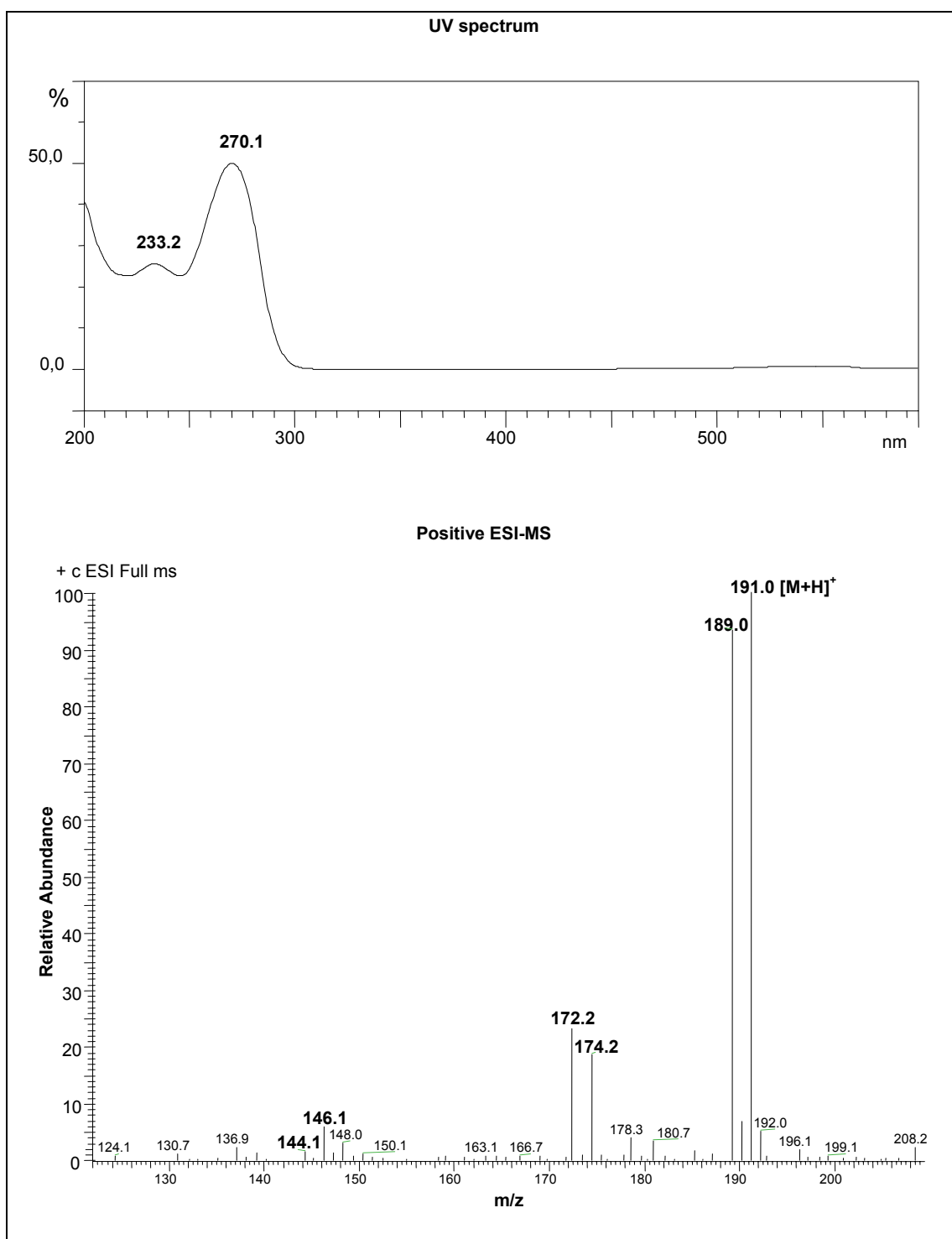
Figure 3.8. UV and ESI-MS spectrum of compound **3**.
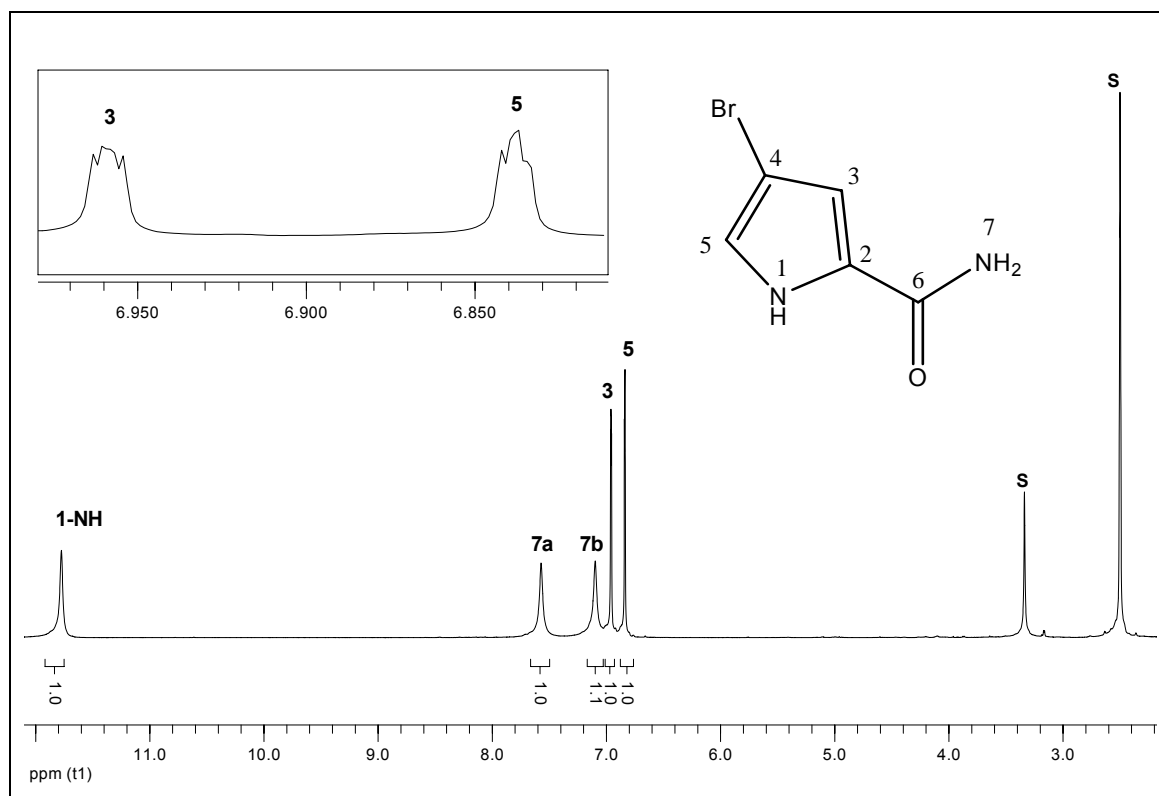
Figure 3.9. Proton NMR spectra of compound **3**.
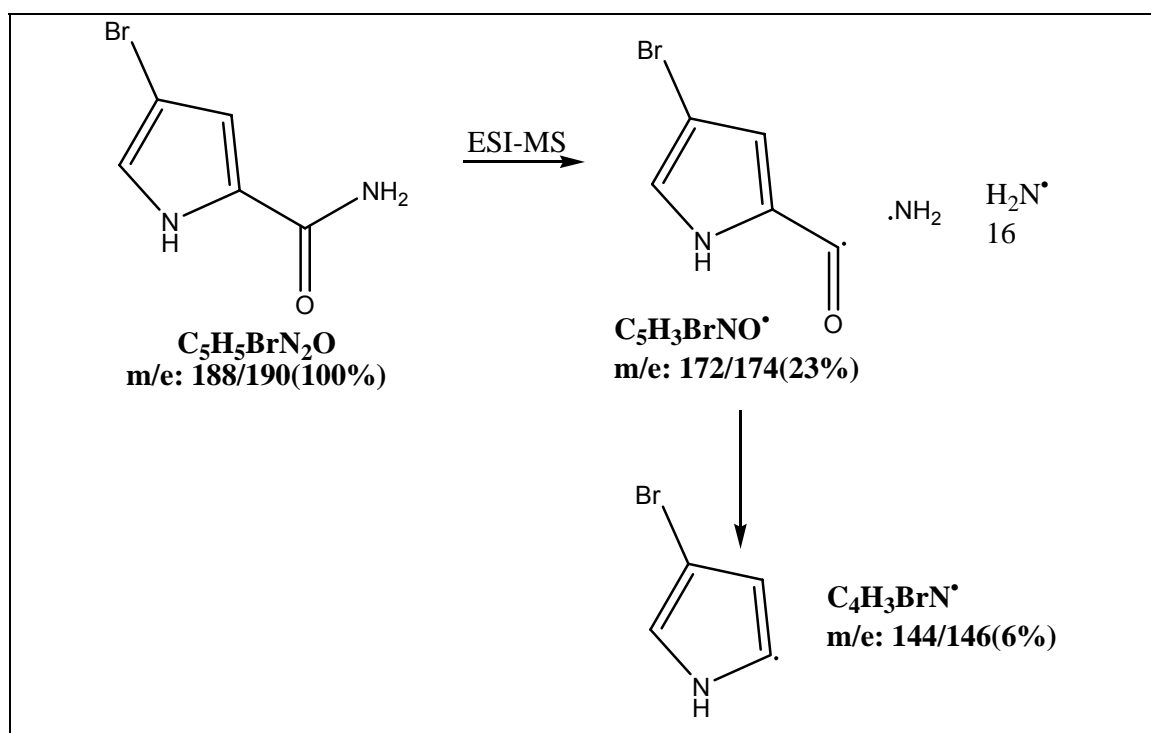
Figure 3.10. Mass spectrometric fragmentation of compound **3**.

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3.1.4.   Compound 4: 4-Bromopyrrole-2-carboxylic acid
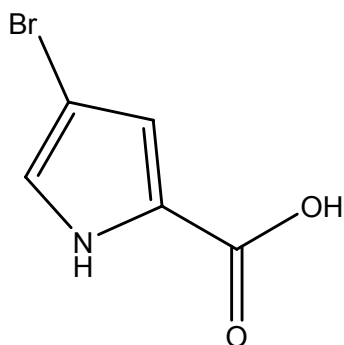
Compound **4** was isolated as a white powder with UV absorbance at λ_{max} 270 nm (MeOH). The negative ESI-MS of compound **4** showed a molecular ion cluster at *m/z* 188:190 [M-H] with a ratio of 1:1 indicating again the presence of a bromine substituent. This molecular weight is compatible with the molecular formula $C_5H_4BrNO_2$. The ^{1}H NMR spectrum of **4** (see Figure 3.12) revealed similar appearance to that of compound **3**, except for the presence of one broad singlet at 12.56 ppm which was assigned as OH and the absence of two amide protons. One singlet appeared at 12.10 ppm and assigned as 1-NH proton and two double-doublet protons at 7.10 and 6.74 ppm which were assigned to H-3 and H-5 that again suggested the presence of an asymmetric pyrrole ring. A coupling constant 1.7 Hz suggested a *meta* coupling between these two protons and the chemical shift indicating the position of bromine attached to C-4. The structure of compound **4** was established as 4-bromopyrrole-2-carboxylic acid from ESI-MS fragmentation analysis and ^{1}H NMR data with comparison to the data of compound **3** as reference.

Table 3.3. Proton NMR data of compound **4** in DMSO.

Nr.	H (ppm), multiplicity (Hz) in DMSO (500 MHz)
1-NH	12.10 (s, 1H)
3	7.10 (dd, 1H, J=1.7Hz, J=2.8Hz)
5	6.74 (dd, 1H, J=1.7Hz, J=2.5Hz)
6-OH	12.56 (bs, 1H)
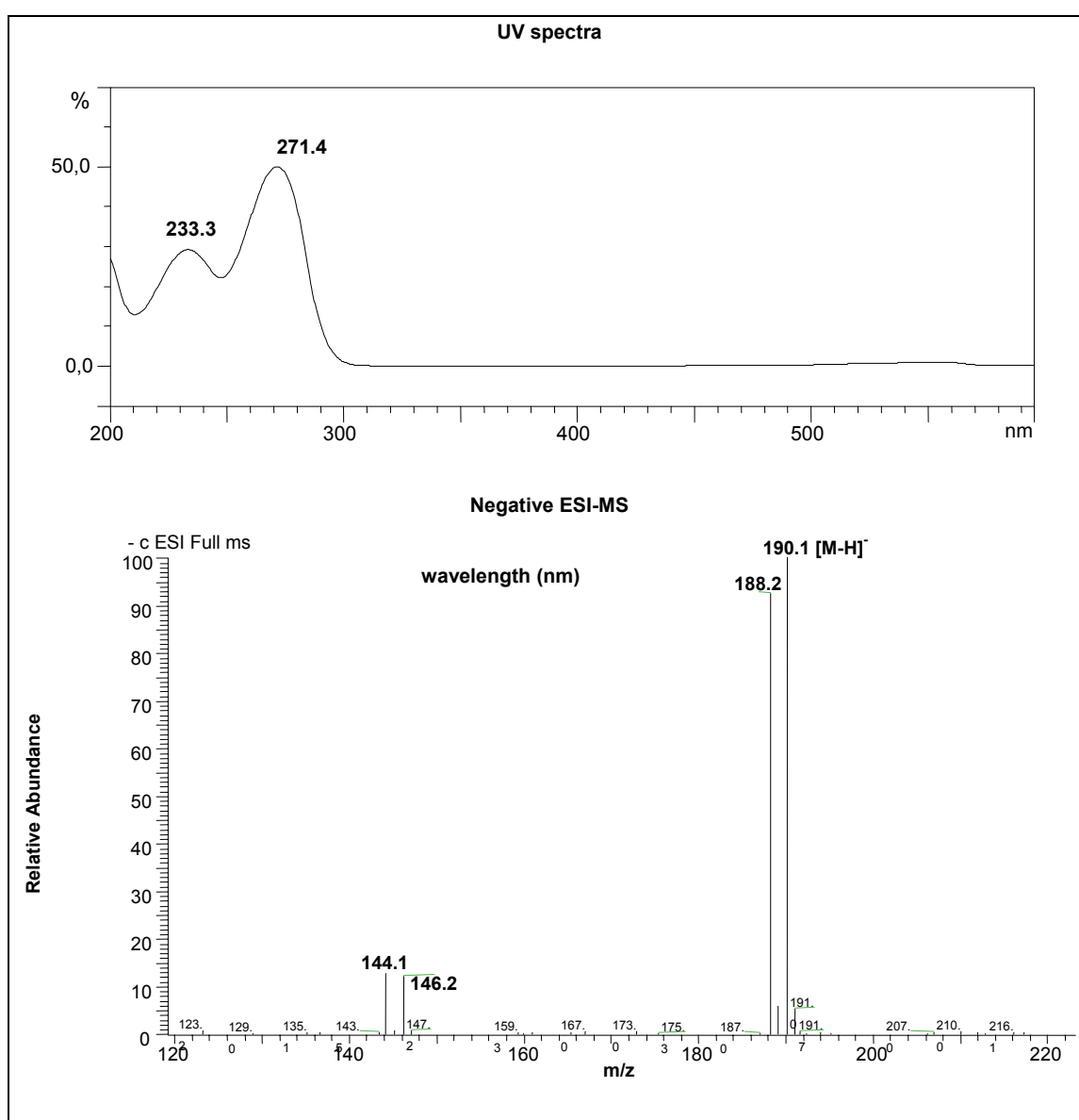
Figure 3.11. UV and ESI-MS spectra of compound **4**.
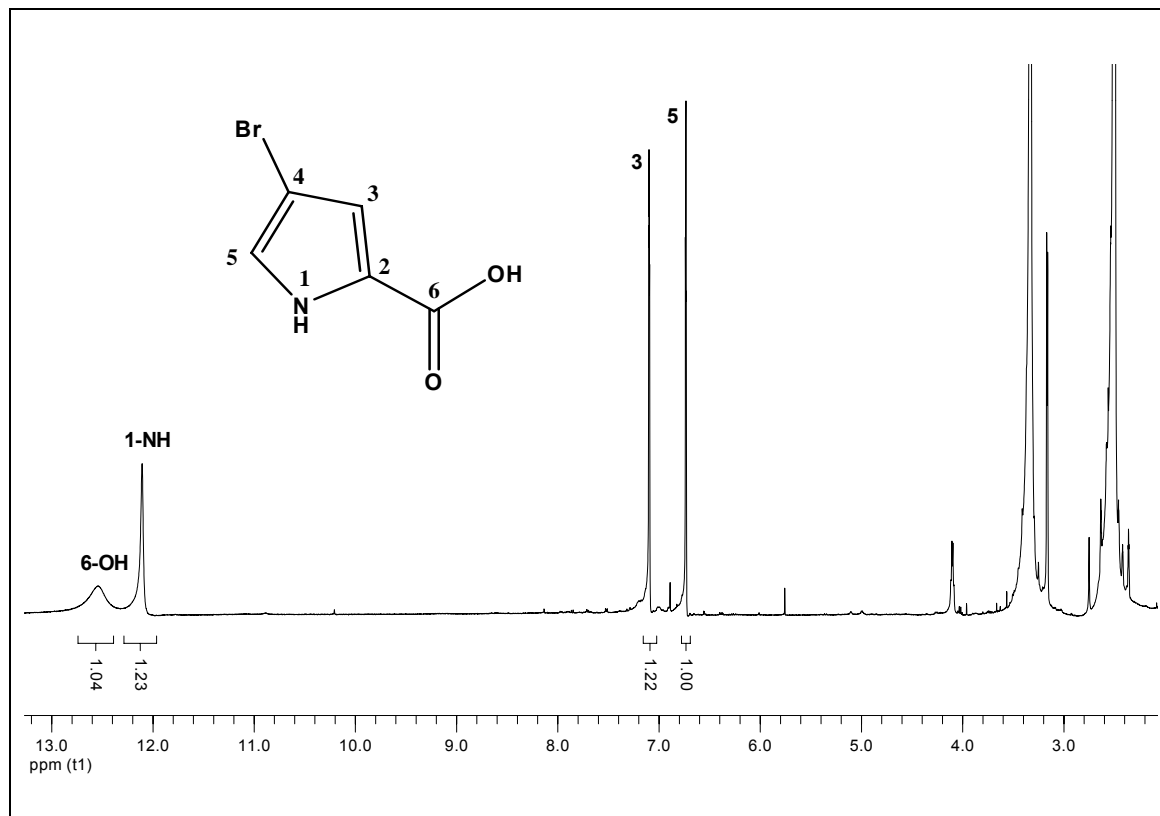
Figure 3.12. Proton NMR spectrum of compound **4**.
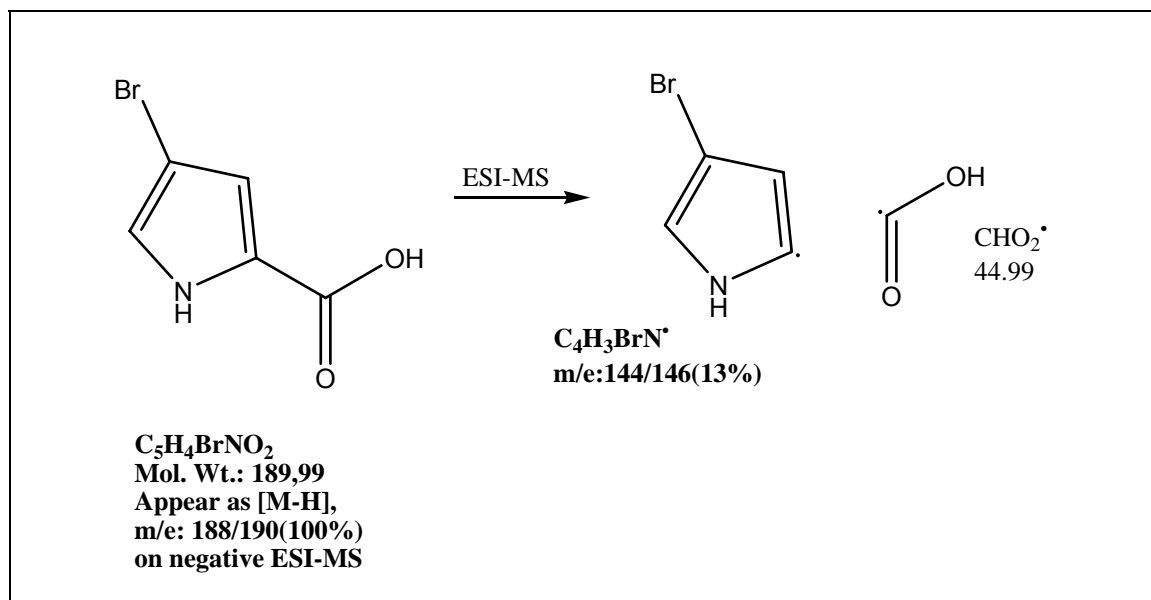
Figure 3.13. Mass spectrometric fragmentation scheme of compound **4**.

3.1.5. Compound 5: Agelasine I
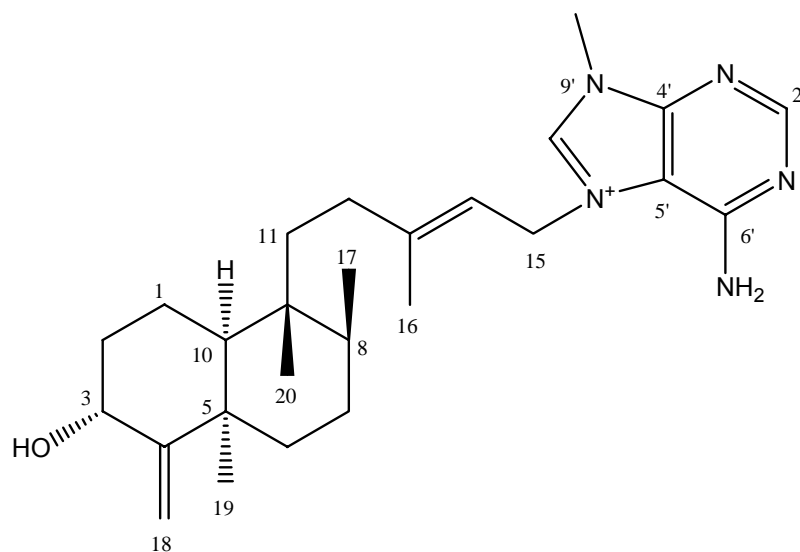
Compound **5** was isolated as a white powder with UV absorbance at λ_{max} 228 and 270 nm (MeOH). The positive ESI-MS of compound **5** showed the pseudo molecular ion at *m/z* 440 [M+H]$^+$. The molecular weight of 439 Da was compatible with the molecular formula $C_{26}H_{40}N_5O$. Proton NMR of compound 5 showed two aromatic protons at δ 8.15 (1H, s, H-2') and 7.98 (1H, s, H-8') and three olefinic protons   at δ 5.31 (1H, m, H-14), 4.79 (1H, brs, H-18a), 4.42 (1H, brs, H-18b). From the carbon and DEPT NMR data, two doublet carbons at δ 164.3 (C-2') and 157.1 (C-8') and four singlet carbon at δ 163.9 (C-4), 160.9 (C-6'), 159.2 (C4'), 148.4 (C-13) were found in the aromatic region. Carbon 2', 4', 6', and 8' belong to methyladenine substructrure, while carbon 4 and 13 were olefinic carbons that was shifted downfield. C-4 was extremelly shifted downfield due to the electron withdrawing group attached to the neighbourhood carbon. The next olefinic carbon appeared at δ 106.8 (t, C-18). Carbon triplet at this region was characteristic for an exomethylene group. HMBC spectra showed correlation of H-18 to C-5, H-20 to C-5, 10 and 11, H-17 to C-20, H11

to C-8, H12 to C11 and 13, H16 to C11, 13 and 14, H16 to C13 and 14, and H15 to C13, which built the diterpenoid substructure. Correlation of proton 9'NMe to C-4' proved the attachment position of a methyl to the adenine substructure. Finally correlation of H-15 to C-5' proved the connection of diterpernoid side chain to the 9-methyl adenine.
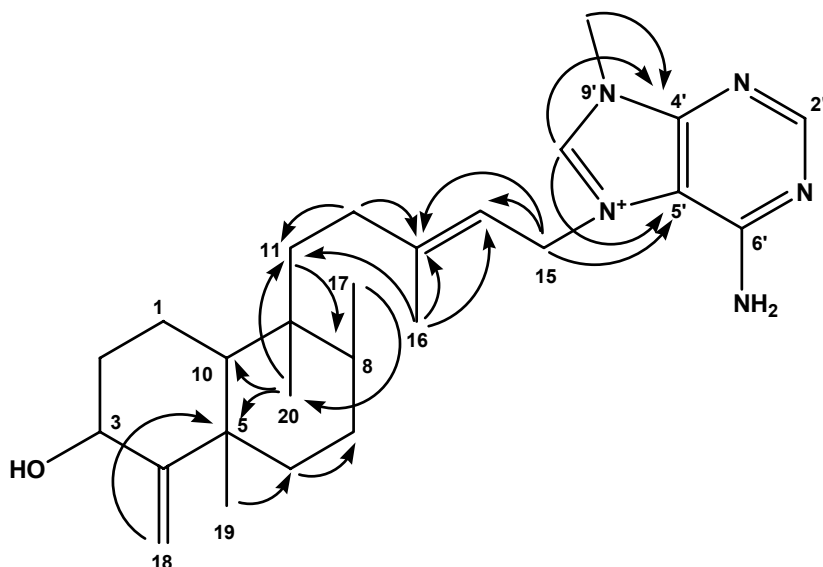
Compound **5** was established as agelasine I from its molecular weight and NMR data inspection and comparison to the data of previously reported compound (Fu *et al.*, 1998). Proof of exomethylene presence, distinguished this compound from agelasine H.

Table 3.4. NMR data assignable peaks of compound **4** in DMSO.

position	Carbon	Proton	HMBC
1	19.3 (t)		
2	24.6 (t)		
3	77.5 (d)		
4	163.9 (s)	---	
5	46.5 (s)	---	

position	Carbon	Proton	HMBC
6	42.1 (t)		
7	22.41 (t)		
8	55.1 (d)		
9	36.8 (s)	---	
10	55.7 (d)		
11	37.8 (t)	0.85 (1H, m), 0.77 (1H, m)	C-8, C-9, C-12, and C-20
12	33.2 (t)	0.96	C-11, C-13, and C-15
13	148.4 (s)	---	
14	118.1 (d)	5.31 (1H, m)	
15	42.8 (t)	4.74 (1H, m), 4.13 (1H, m)	C-5', C-13, and C-14
16	16.3 (q)	1.59 (3H, s)	C-11, C-13, and C-14
17	14.45 (q)	0.87 (3H, b)	
18	106.8 (t)	4.79 (1H, brs), 4.42 (1H, brs)	C-8
19	33.5 (q)	1.24 (3H, s)	
20	21.7 (q)	0.64 (3H, s)	C5, C10, C11
2'	164.3 (d)	7.98 (1H, s)	
4'	159.2 (s)	---	
5'	99.7 (s)	---	
6'	160.9 (s)	---	
8'	157.1 (d)	8.15 (1H, s)	C-4' and C-5'
9'-N-Me	28.1 (q)	2.93	C-4'
6'NH2	--	7.98	
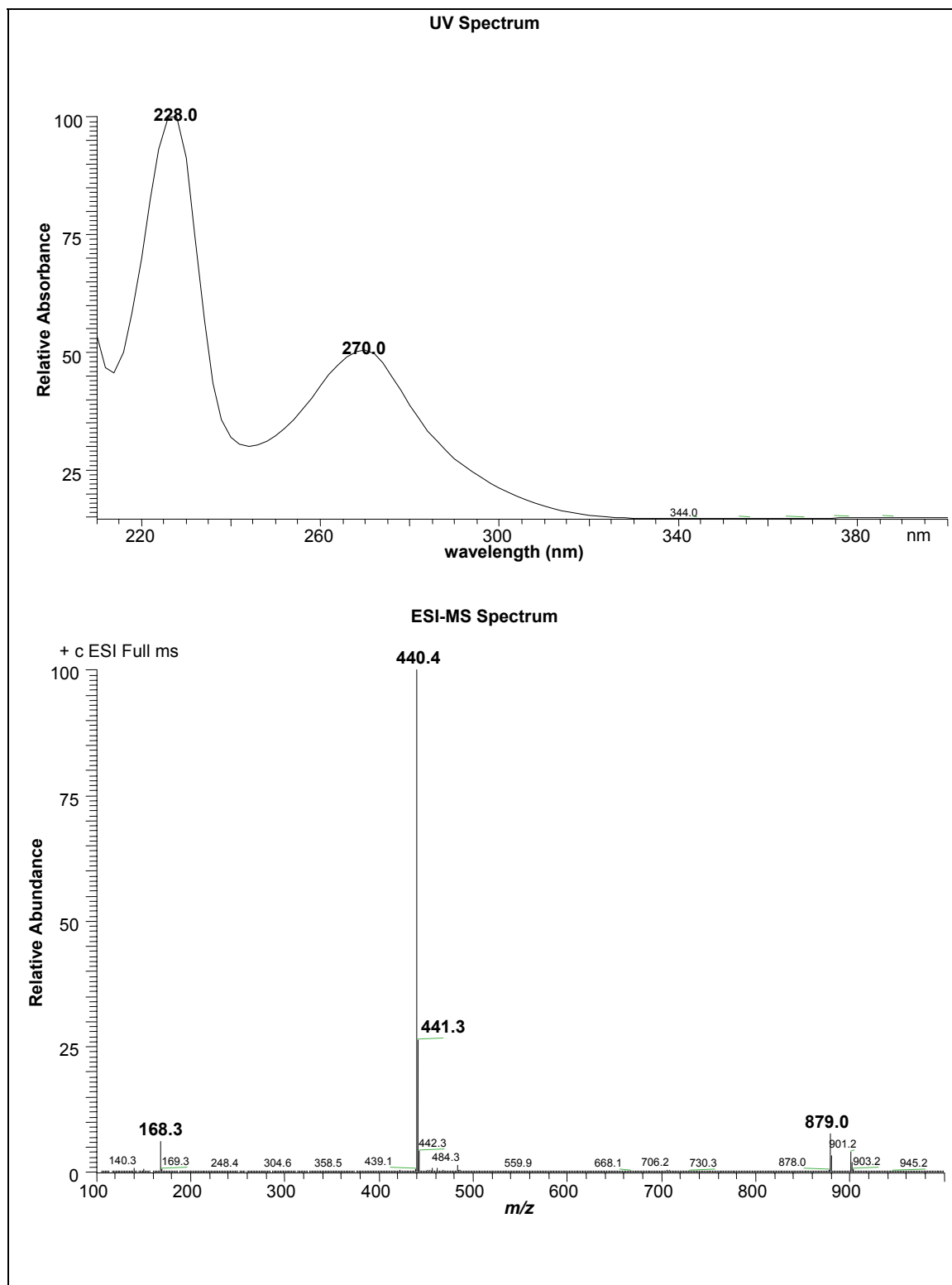
Figure 3.14. UV and ESI-MS spectrum of compound **5**.
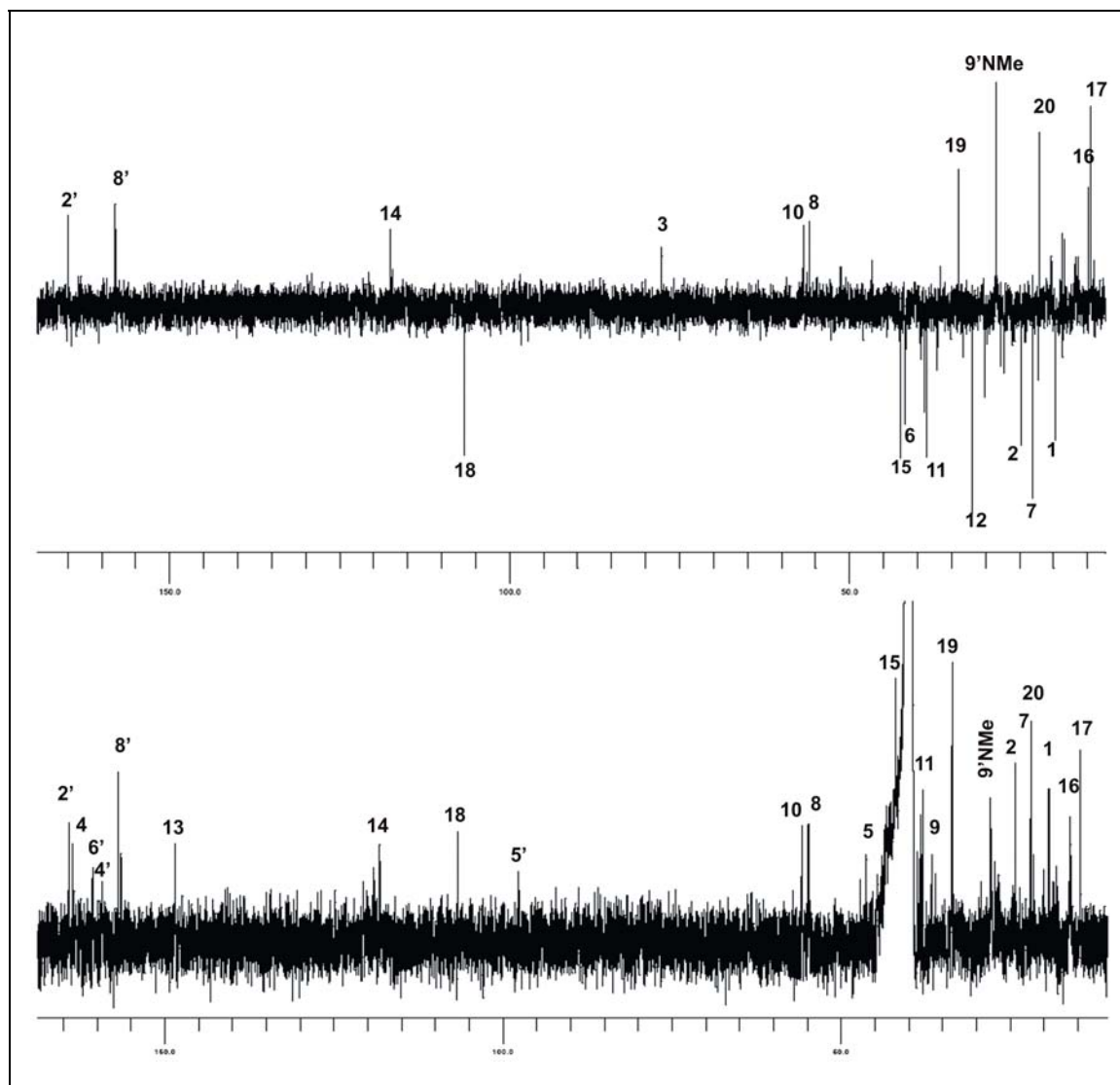
Figure 3.15. Carbon and DEPT spectra of compound **5**.
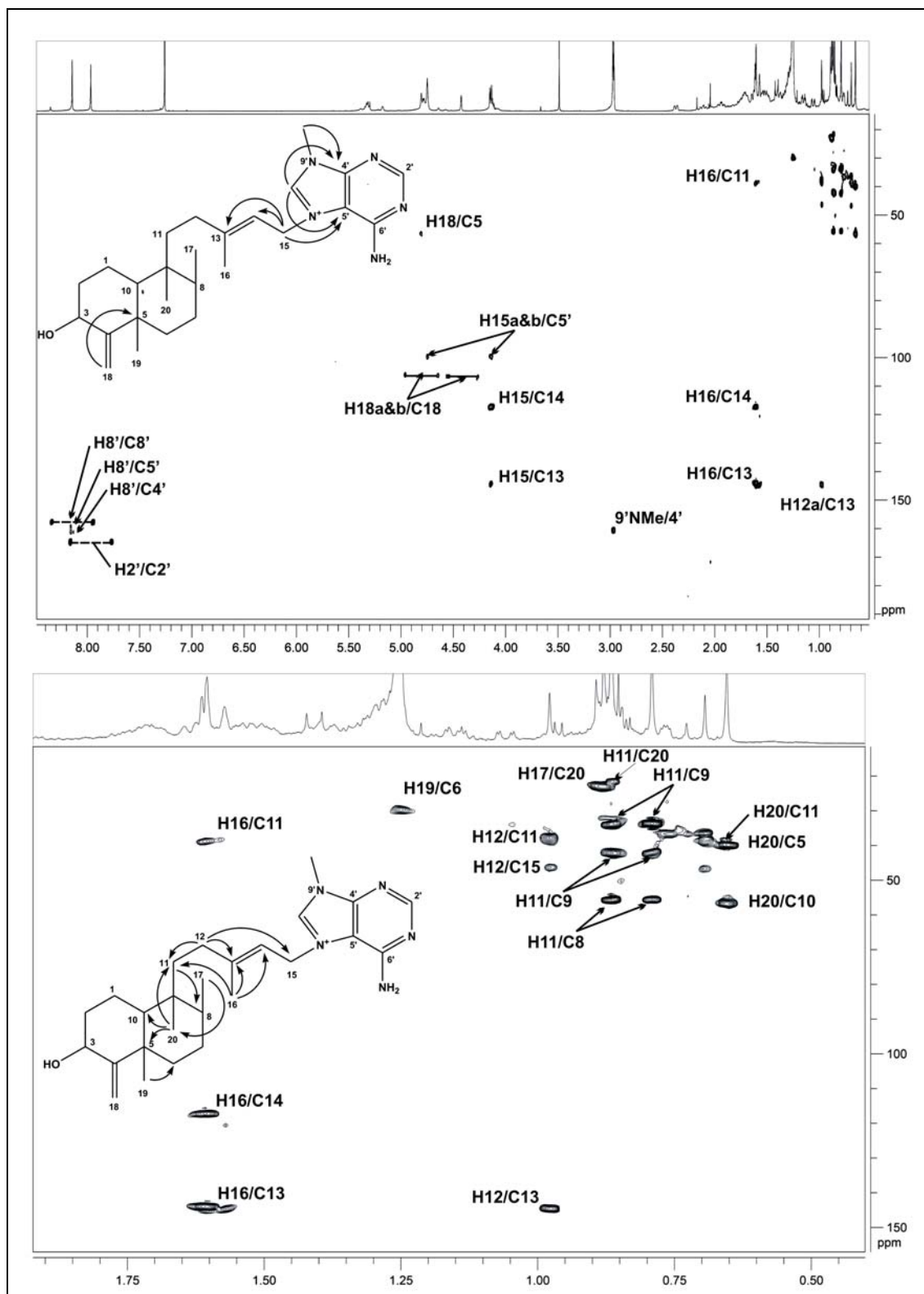
Figure 3.16. HMBC correlation of compound **5**.

3.2. Secondary metabolites of the sponge *Theonella swinhoei*

Numerous interesting compounds have been reported from the sponge *Theonella swinhoei*. Examples include peptides ranging from cyclic depsipeptides to large bicyclic peptides. Microsclerodermins C–E are antifungal cyclic peptides that were isolated from specimens of *Theonella* sp. and *Microscleroderma* sp. both collected from the Philippines. A Japanese species of *Theonella* contained cyclotheonamides E2 and E3, which are cyclic pentapeptides possessing potent inhibitory activity against thrombin, trypsin, and plasmin. Theopalauamide is a bicyclic glycopeptide that was isolated from specimens of *T. swinhoei* collected from Palau and Mozambique and from filamentous bacterial symbionts found within the sponge. An Okinawan species of *Theonella* yielded two new cytotoxic cyclic peptides, keramamides K and L, that both contain unusual tryptophan residues. An Indonesian specimen of *T. swinhoei* contained the antifungal cyclodepsipeptide cyclolithistide A (Faulkner, 2000a). Five secondary metabolites were isolated from the sponge *Theonella swinhoei* collected from Menjangan Island, Bali-Indonesia. They are theonellapeptolide Id, Ie, IId and swinholide A.
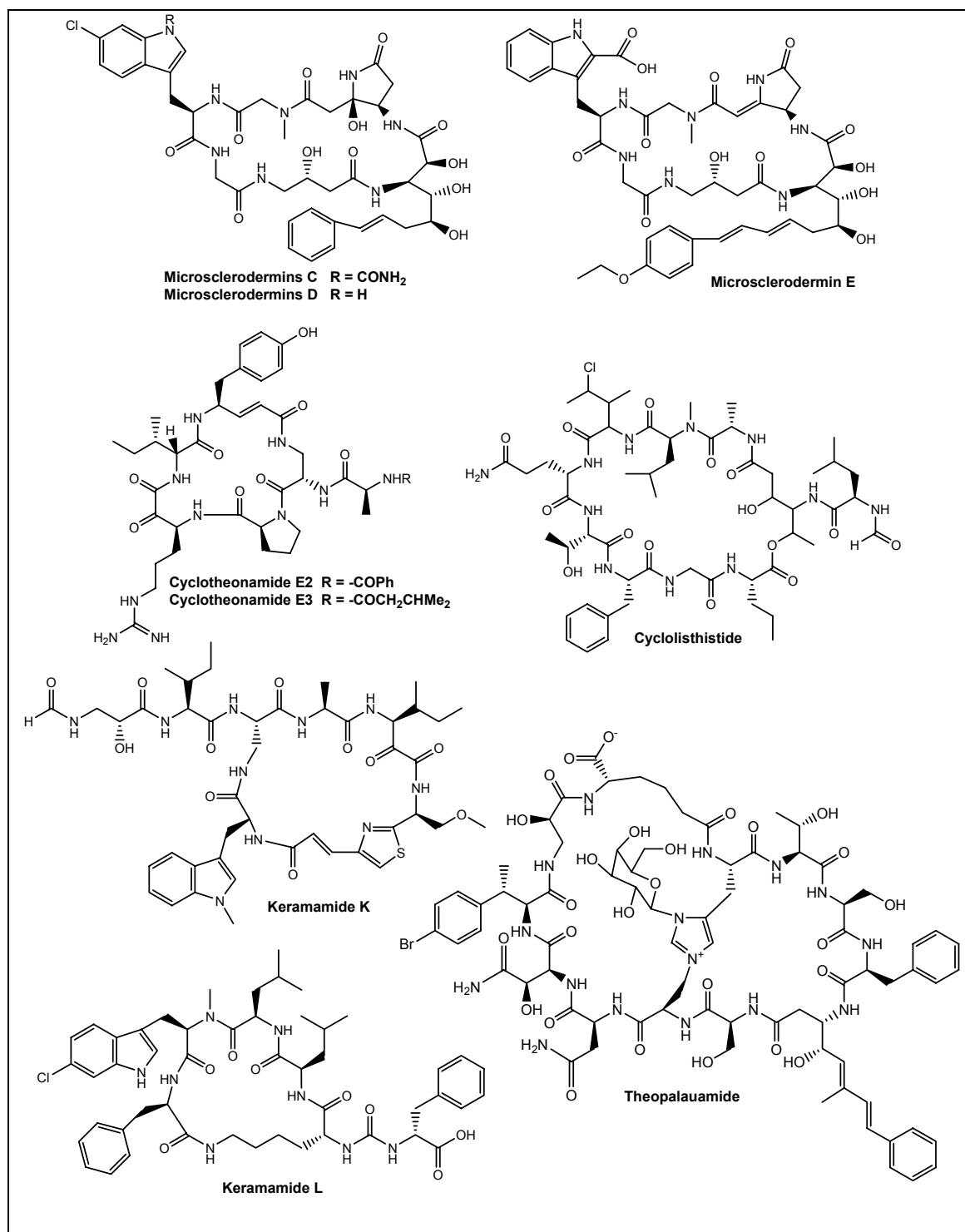
Figure 3.17. Several examples of compounds isolated from *Theonella swinhoei.*

3.2.1. Compound 6: Theonellapeptolide Id

Theonellapeptolide 1d
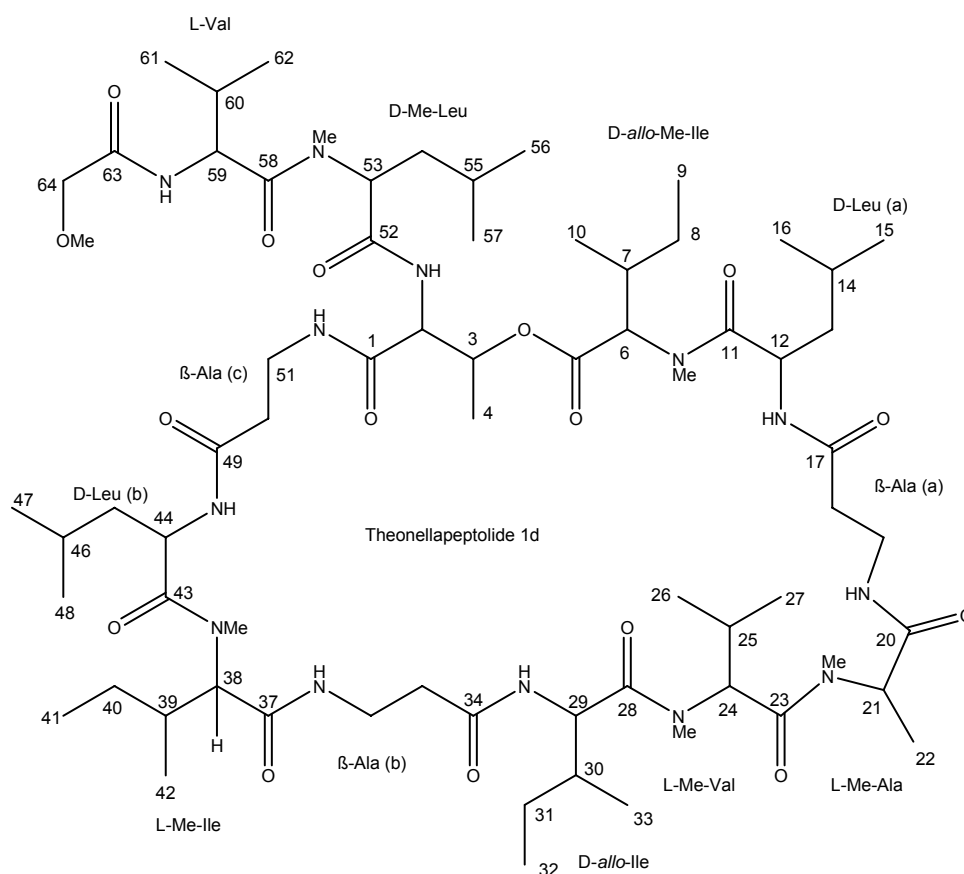
Compound **6** (TNLPId) was isolated through a series of normal phase liquid chromatographic separation steps. It readily formed colourless crystals on slow evaporation of aqueous methanol. Crystal formation was improved using combination of dimethylformamide and water. UV spectrum of compound **6** exhibited a maximum absorption at λ 208 nm. Compound **6** was negative to the ninhydrin test. Thus, it was presumed that, compound **6** is a cyclic peptide lactone in which the N-terminal is protected with a methoxyacetyl group and the C-terminal is connected through a lactone linkage to the ß-hydroxyl group of threonine (Thr). Since this compound has amine groups in the molecule, it could be detected with the Dragendorf reagent. This spray reagent is commonly used to detect alkaloids and other quaternary nitrogenous compounds. The FAB-MS

spectrum of compound **6** presented molecular ion peak at *m/z* 1405 [M+H]$^+$ and 1427 [M+Na]$^+$. The molecular weight 1404 is compatible to molecular formula $C_{70}H_{125}N_{13}O_{16}$.

The ^{1}H NMR spectrum of compound **6** showed resonances for eight amide protons at δ 8.32 (1H, brs, 2-NH), 8.22 (1H, brs, 29-NH), 8.09 (1H, d, *J* = 9.1 Hz, 12-NH), 8.03 (1H, brs, 44-NH), 7.20 (1H, d, *J* = 9.1 Hz, 59-NH), 6.89 (1H, dd, *J* = 7.8, 3.0 Hz, 36-NH), 6.66 (1H, d, *J* = 6.2 Hz, 19-NH), 6.56 (1H, brt, *J* = 5.6 Hz, 51-NH) and five N-methyl groups at δ 3.33 (24- NCH$_3$), 3.25 (6-NCH$_3$), 3.20 (53-NCH$_3$), 3.19 (38-NCH$_3$), 2.77 (21-NCH$_3$) which indicated the peptide nature of this compound. One methoxy-acetyl at δ 3.94 (d, *J* = 15.0 Hz, H-64), 3.87 (d, *J* = 15.0 Hz, H-64´) and 3.40 (3H, s, 64-OCH$_3$) was also observed. The ^{13}C NMR spectrum showed resonances due to 12 amide carbons at δ 176.1 (C-28), 174.9 (C-43), 174.3 (C-11), 173.7 (C-58), 172.9 (C-52), 171.3 (C-49), 171.2 (C-34), 170.4 (C-5), 170.3 (C-17), 169.8 (C-37), 168.9 (C-20), 170.3 (C-23), and 168.3 (C-1) and one lactone carbon at δ 169.2 (C-63) which indicated the presence of 13 amino acids. One methoxyacetyl group was indicated by resonances of C-63, methylene C-64 at δ 71.8 and one methoxy group 64-OCH$_3$ at δ 59.1 ppm.

The COSY and TOCSY spectrum showed thirteen spin systems for thirteen amino acids: eight ordinary amino acids and five N-methylated amino acid residues. One threonine (thr) spin system was indicated by the signals at δ 8.31 (2-NH), 4.38 (H-2), 5.16 (H-3) and 1.09 (CH$_3$-4). A lactone linkage of the

C-terminal from *allo* methylisoleucine to the ß-hydroxyl group of threonine (thr) was confirmed from the HMBC correlation between protons CH_3-4 (thr) to carbonyl C-5 of *allo* methylisoleucine. An *allo* methylisoleucine (*allo*-Me-Ile) amino acid residue was indicated by the TOCSY signal at δ 5.15 (H-6), 2.43 (H-7), 1.80 (H-8), 0.96 (H_3-9) and 0.75 (H_3-10). HMBC correlation proved the connection from 6-NMe to α-carbon C-6 of this amino acid sequence and carbonyl carbon of leucine amino acid (C-11). The first leucine (leu) amino acid was evident from the COSY spectrum, which indicated the connectivity of protons at 12-NH (δ 8.08), H-12 (δ 5.02), CH_2-13 (δ 1.60 and 1.26), H-14 (δ 1.71), CH_3-15 (δ 0.97) and CH_3-16 (δ 0.96). The HMBC spectrum of compound **6** showed the correlation of proton 12-NH to carbonyl carbon of ß-alanine (C-17). The first ß-alanine (ß-ala) residue was assembled through the COSY correlations, which established the connectivity of proton 19-NH (δ 6.68), CH_2-19 (δ 3.87 and 3.11), and CH_2-18 (δ 2.37 and 2.10). Its HMBC spectrum inferred the correlation of 19-NH to the carbonyl carbon of methylalanine (C-20). A methyl alanine spin system was indicated by the signals at δ 4.92 (H-21) and 1.38 (CH_3-22). HMBC spectrum proved the presence of N-Methyl with correlation of 21-NMe to C-21 and connectivity to the methyl valine with correlation of 21-NMe to carbonyl C-23. A methylvaline (Me-Val) amino acid residue was indicated by the TOCSY signal at δ 4.95 (H-24), 2.37 (H-25), 0.91 (H_3-26), and 0.87 (H_3-27). HMBC spectrum confirmed the presence of N-methyl with correlation of 24-NMe to C-24 and its linkage to *allo* isoleucine with correlation of 24-NMe to carbonyl C-28. A spin system for *allo* isoleucine was identified from the COSY which consisted of 29-NH (δ 8.21), H-29 (δ 5.42), H-

30 (δ 1.76), CH_2-31 (δ 1.44 and 1.20), CH_3-32 (δ 0.98), and CH_3-33 (δ 0.74).

NOESY spectrum confirmed the connection of the second ß-alanine, indicated

by the correlation of proton 29-NH to proton H-35. The second β-alanine

residue commenced at 36-NH (δ 6.89) to CH_2-36 (δ 4.18 and 3.09) and CH_2-35

(δ 2.40 and 2.24). NOESY spectrum proved the connectivity from the second

ß-alanine to methyl isoleucine as indicated by a cross peak between proton 36-

NH to 38-NMe. A methylisoleucine (Me-Ile) amino acid residue was indicated by

the TOCSY signal at δ 4.98 (H-38), 2.10 (H-39), 1.29 (H-40), 0.97 (H-40'), 0.85

(CH_3-41) and 0.95 (CH_3-42). HMBC correlation proved the connection from 38-

NMe to α carbon (C-38) of this amino acid sequence and carbonyl carbon of

second leucine amino acid (C-43). The connectivity of second leucine residue

protons was detected from the COSY spectrum as protons: 44-NH (δ 8.02), H-

44 (δ 5.0), CH_2-45 (δ 1.60 and 1.24), H-46 (δ 1.78), CH_3-47 (δ 0.90) and CH_3-

48 (δ 0.97). The third ß-alanine started at 51-NH (δ 6.56) to CH_2-51 (δ 3.70 and

3.28) and CH_2-50 (δ 2.27 and 2.11) were assembled together through the

COSY correlations. A methylleucine residue was indicated by the TOCSY

signal at δ 5.16 (H-53), 1.92 (H-54), 1.39 (H-54'), 1.36 (H-55), 0.94 (H_3-26), and

0.79 (H_3-57). HMBC correlation proved the connectivity from 53-NMe to α

carbon (C-53) of this amino acid sequence and NOESY spectrum proved the

connection from methylleucine to valine with cross peak between proton 53-

NMe to α proton of valine (H-59). From the COSY spectrum a spin system for

valine was also determined which was comprised of 59-NH (δ 7.20), H-59 (δ

4.98), H-60 (δ 2.05) CH_3-61 (δ 0.99) and CH_3-62 (δ 0.88). HMBC spectrum

confirmed the connection of valine to methoxyacetyl group. Compound **6** was established as theonellapeptolide Id through comparison of the ^{1}H-^{13}C NMR data and molecular weight of compound **6** with literature (Roy *et al.*, 2000) in combination with careful analysis of the 2D-NMR data: COSY, TOCSY, ROESY, NOESY and HMBC spectra.

Theonellapeptolide Id is a cyclic tridecapeptide lactone, which consists of thirteen amino acids; five of which are methylated. The eight non methylated amino acids are three ß-alanine (ß-Ala), two leucines (Leu), threonine (Thr), *allo*-isoleucine (*allo*-Ile), valine (Val), and five N-methylated amino acids are *allo*-isoleucine (*allo*-Me-Ile), alanin (Me-Ala), valine (Me-Val), leucine (Me-Leu). Cyclization through the ester bond of carboxyl residue from D-*allo* methylisoleucine and hydroxyl residue from threonine was indicated by HMBC correlations.

Table 3.5. NMR data of compound **6**.

Amino acid monomer	number	^{13}C-NMR	^{1}H-NMR
L-Thr	1	168.3 s	---
	2	57.4 d	4.38 dd
	3	69.1 d	5.16 m
	4	17.3 q	1.12 d
	2-NH	---	8.33 brd
D-allo-Me-Ile	5	170.4 s	---
	6	68.8 d	5.15 br
	7	33.9 d	2.43 m
	8	28.4 t	1.80 m
	9	11.6 q	0.96 t
	10	14.5 q	0.75 d
	6-NMe	39.0 q	3.25 s

Amino acid monomer	number	¹³C-NMR	¹H-NMR
D-Leu (a)	11	174.3 s	---
	12	47.9 d	5.03 ddd
	13	40.3 t	1.60 brt 1.28 m
	14	24.8 d	1.71 m
	15	23.6 q	0.96 d
	16	20.9 q	0.98 d
	12-NH	---	8.09 d
ß-Ala (a)	17	170.3 s	---
	18	36.7 t	2.37 m 2.10 m
	19	37.0 t	3.87 m 3.11 m
	19 NH	---	6.68 dd
L-Me-Ala	20	168.9 s	---
	21	55.7 d	4.92 q
	22	14.6 q	1.38 d
	21-NMe	28.7 q	2.77 s
L-Me-Val	23	170.3 s	---
	24	57.9 d	4.95 d
	25	28.2 d	2.37 m
	26	19.8 q	0.91 d
	27	19.4 q	0.87 d
	24-NMe	31.4 q	3.33 s
D-allo-Ile	28	176.1 s	---
	29	52.6 d	5.42 dd
	30	37.0 d	1.76 m
	31	26.6 t	1.44 m 1.20 m
	32	12.0 q	0.98 t
	33	13.9 q	0.74 d
	29-NH	---	8.21 brs
ß-Ala (b)	34	171.2 s	---
	35	35.2 t	2.40 m 2.24 m
	36	35.1 t	4.18 m 3.09 m
	36-NH	---	6.89 dd

Amino acid monomer	number	^{13}C-NMR	^{1}H-NMR
L-Me-Ile	37	169.8 s	---
	38	60.5 d	4.98 d
	39	32.2 d	2.10 m
	40	24.8 t	1.29 m 0.97 m
	41	10.2 q	0.85 t
	42	15.6 q	0.95 d
	38-NMe	31.1 q	3.19 s
D-Leu (b)	43	174.9 s	----
	44	48.2 d	5.09 dd
	45	40.2 t	1.61 m 1.24 brt
	46	24.9 d	1.78 m
	47	23.2 q	0.92 d
	48	21.2 q	0.97 d
	44-NH	---	8.03 d
ß-Ala (c)	49	171.3 s	---
	50	36.9 t	2.27 m 2.11 m
	51	36.5 t	3.70 m 3.28 m
	51-NH	---	6.56 brt
D-Me-Leu	52	172.9 s	---
	53	55.4 d	5.16 m
	54	37.8 t	1.92 brt 1.39 m
	55	25.1 d	1.36 m
	56	23.3 q	0.94 d
	57	20.6 q	0.79 d
	53-NMe	31.6 q	3.20 s
L-Val	58	173.7 s	---
	59	53.6 d	4.98 dd
	61	19.6 q	0.99 d
	62	16.9 q	0.88 d
	59-NH	---	7.20 d
MeOAc	63	169.2 s	---
	64	71.8 t	3.94 d 3.87 d
	64-OMe	59.1 q	3.40 s
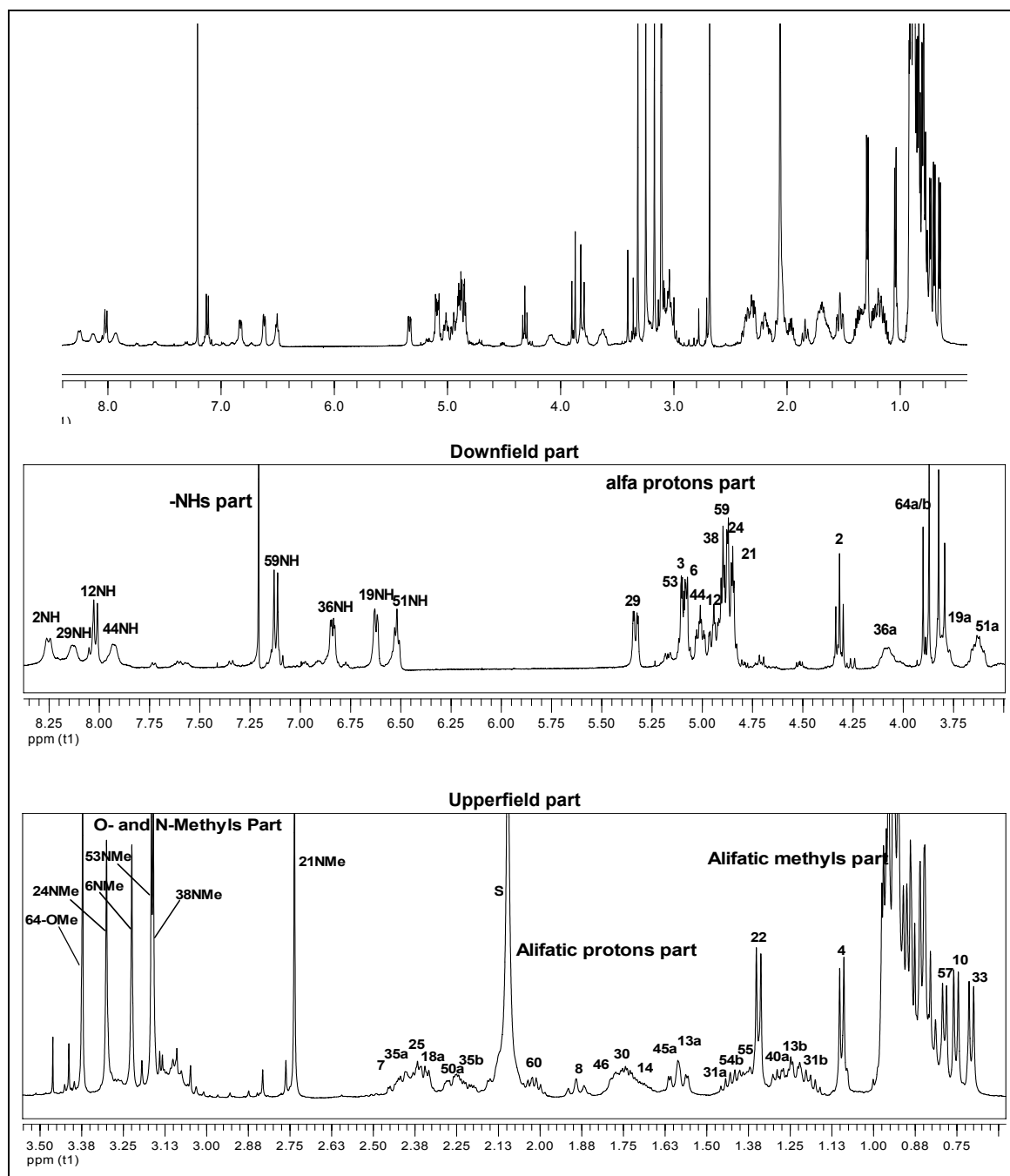
Figure 3.18. Proton spectrum of compound **6**.
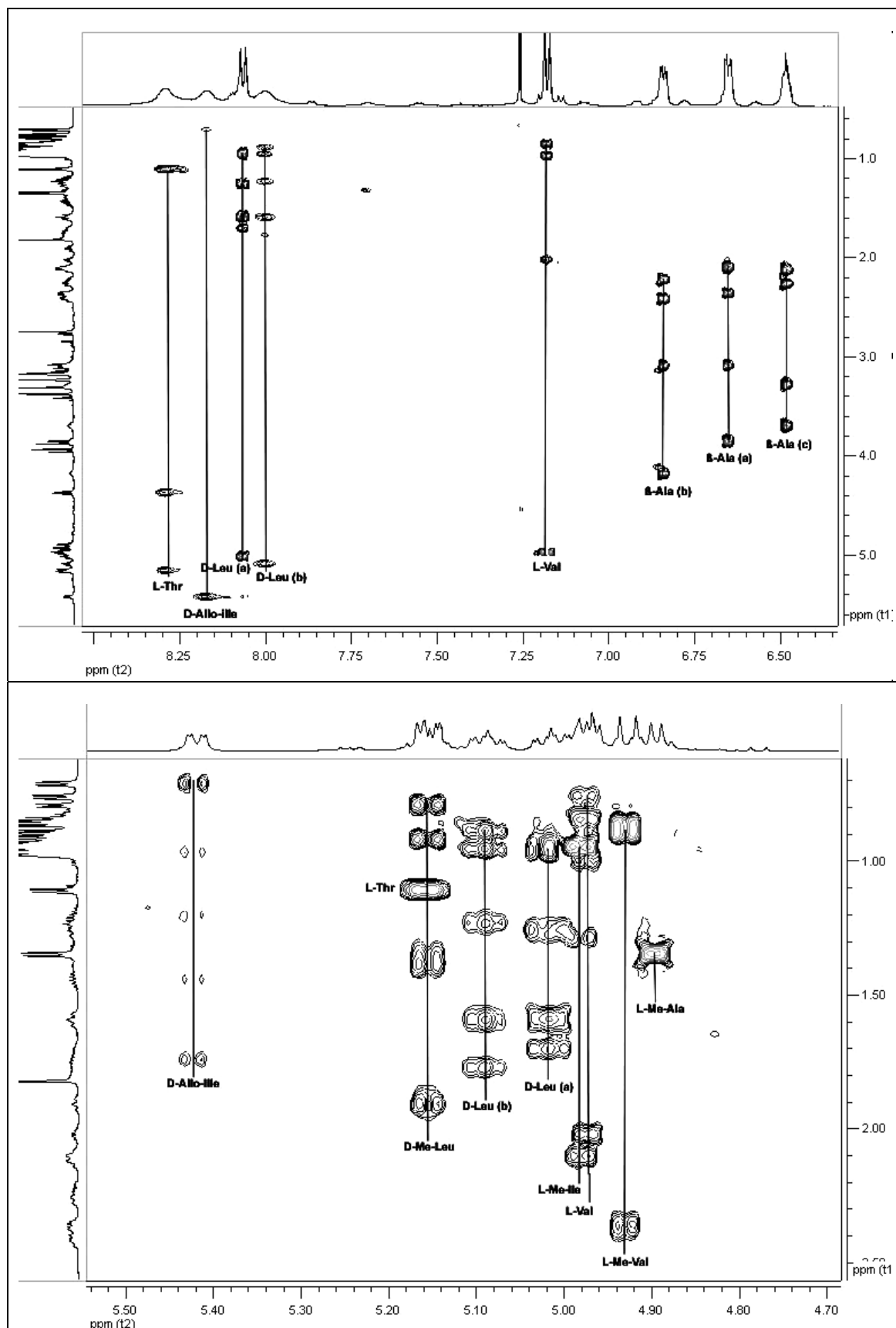
Figure 3.19. TOCSY of compound **6**.
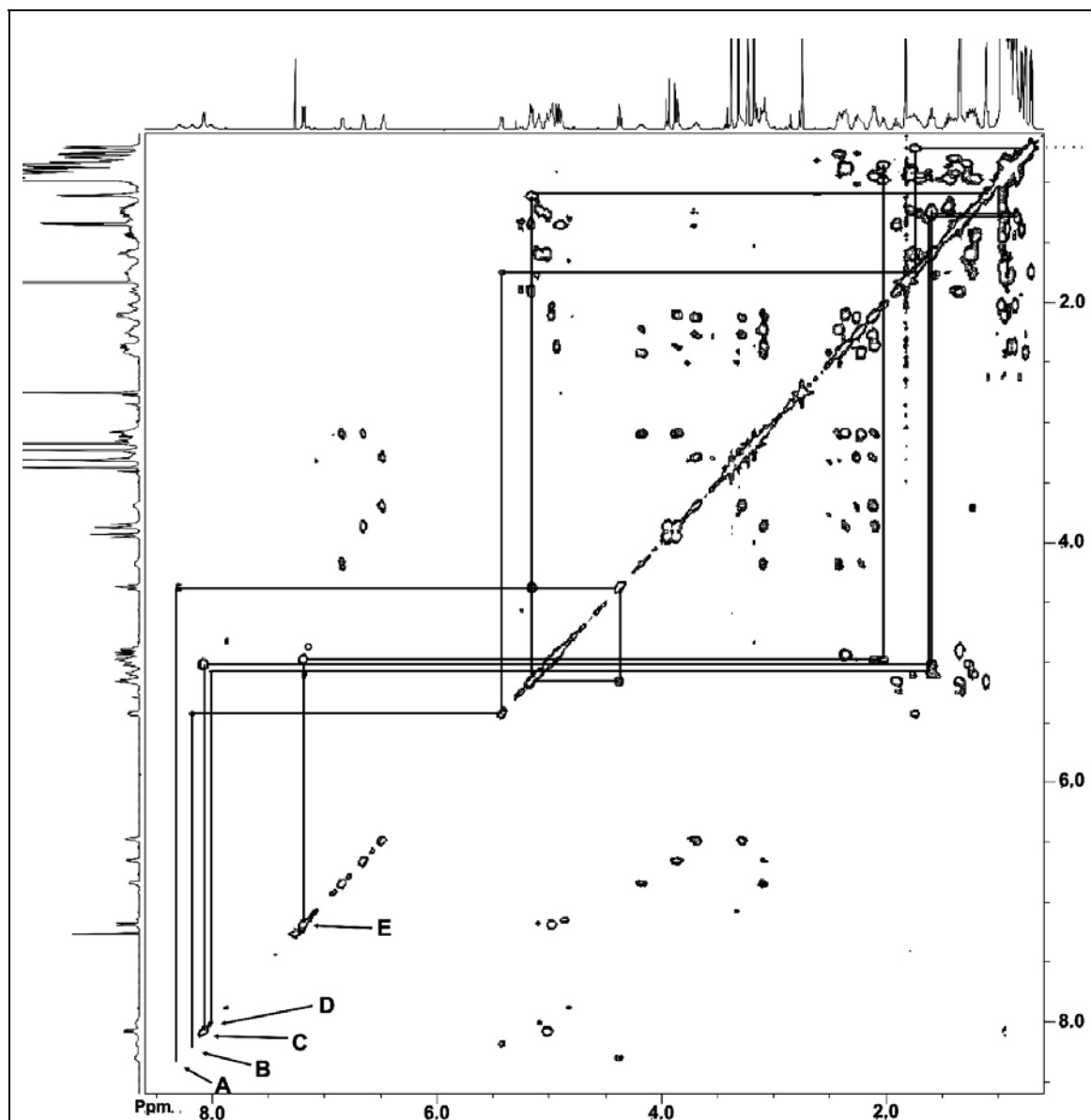
Figure 3.20. COSY correlation five amino acids: A. L-threonine; B. D-allo isoleucine; C. D-leucine(a); D. D-leucine(b); and L-valine.
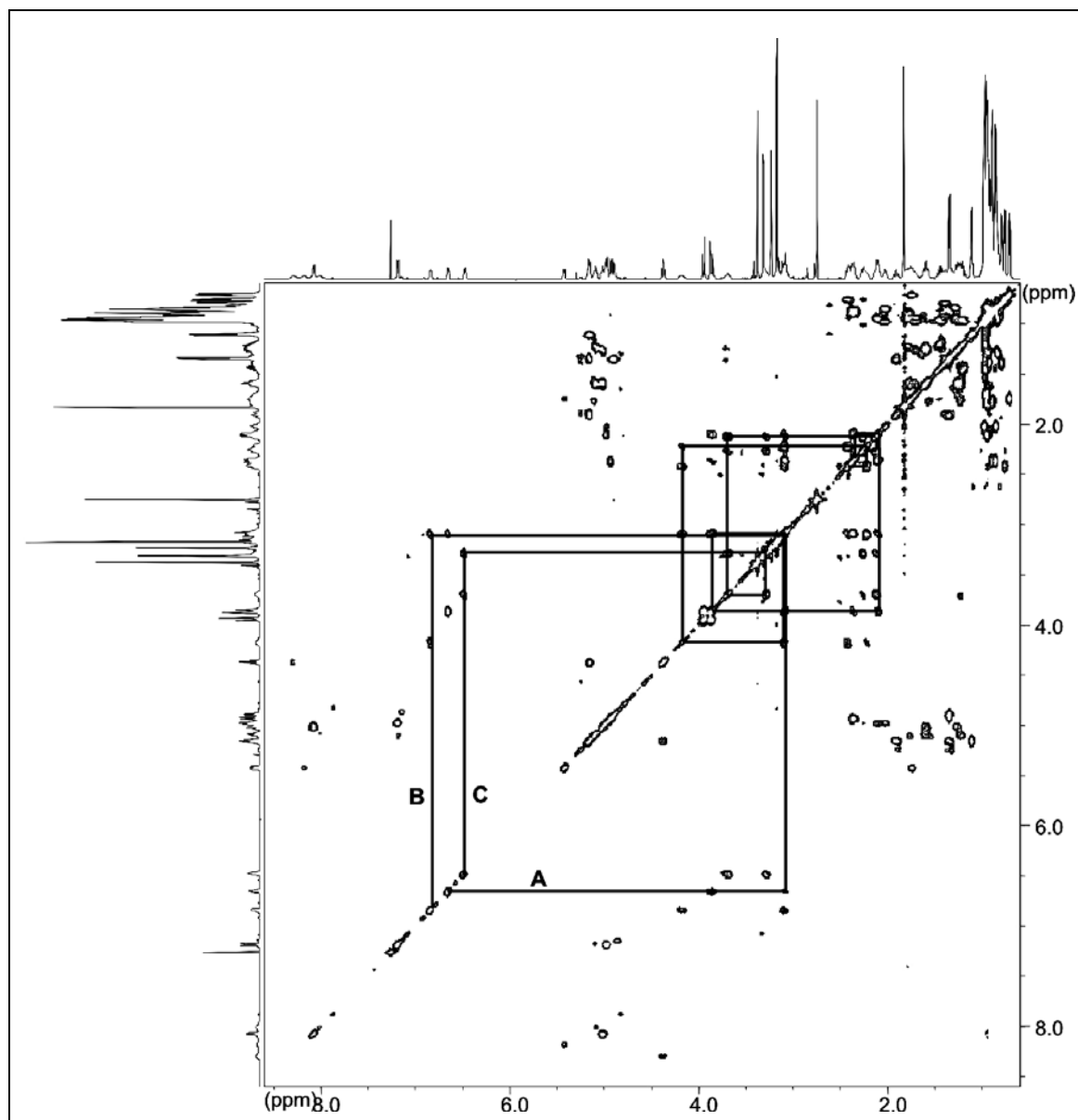
Figure 3.21. COSY correlation of three ß-alanine amino acids.
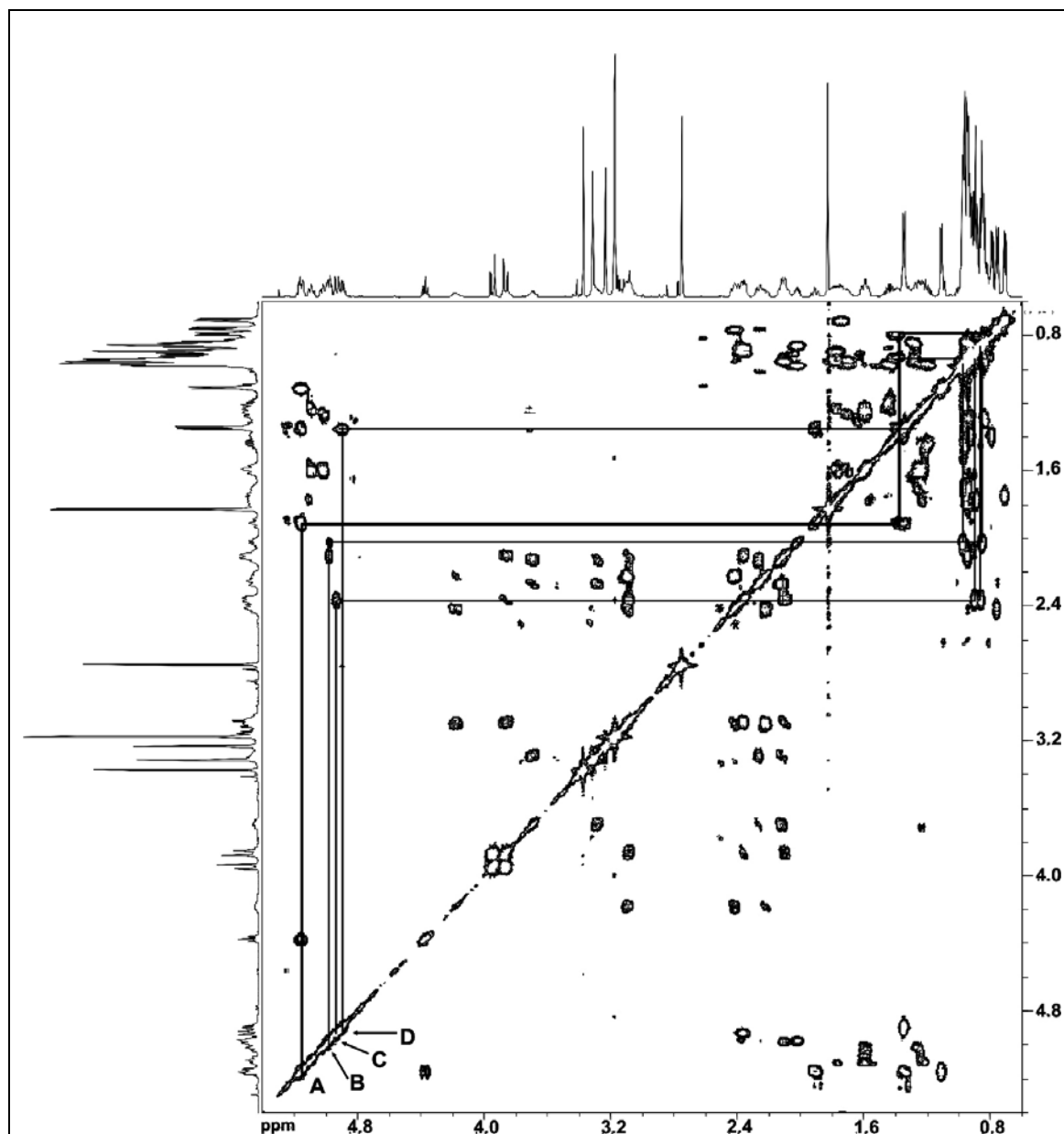
Figure 3.22. COSY correlation of four N-methylated amino acid: A. D-methyl leucine; B. D-methyl alanine; C. D-methyl valine; and D. L-methyl isoleucine.
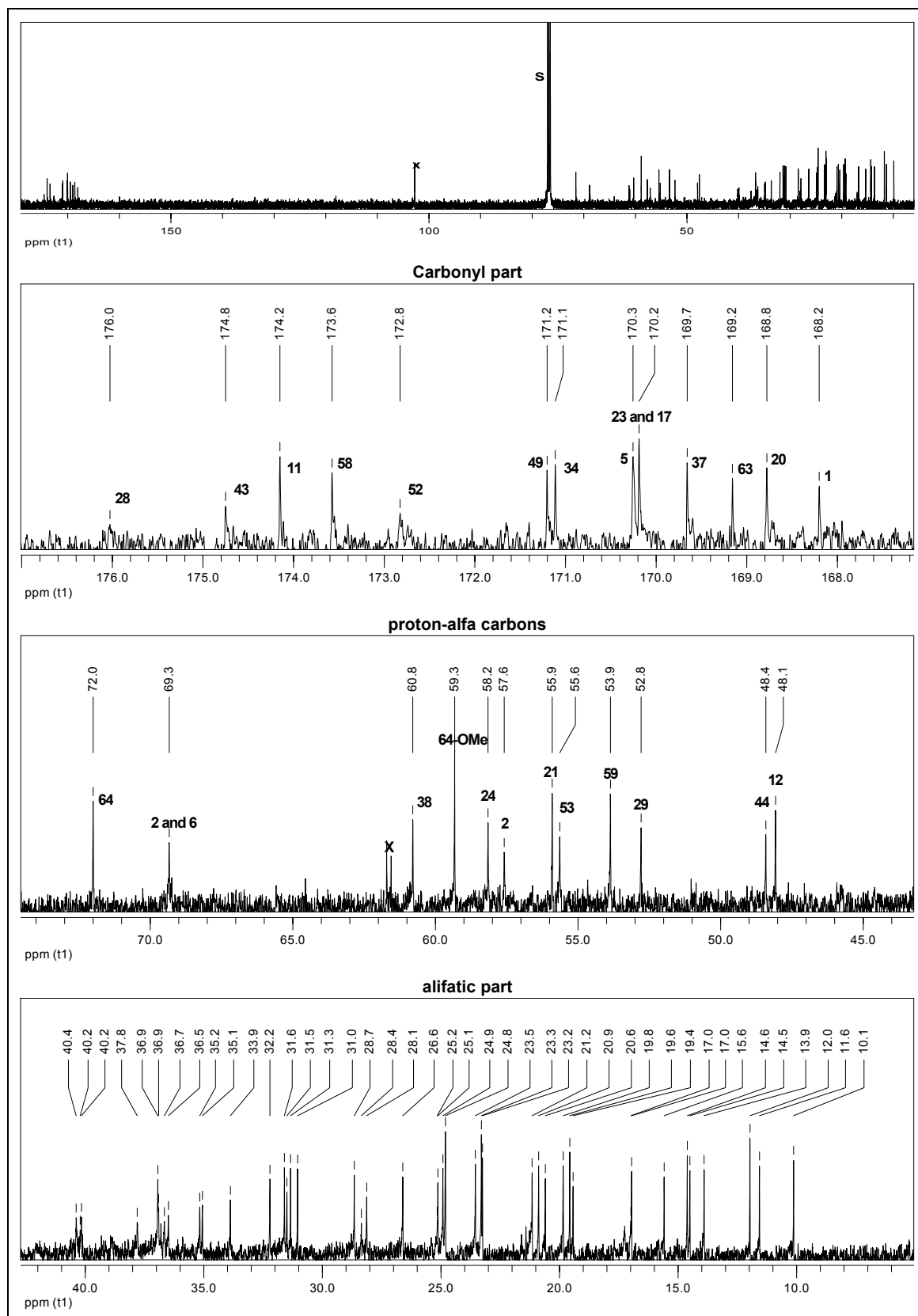
Figure 3.23. Carbon spectrum of compound **6**.
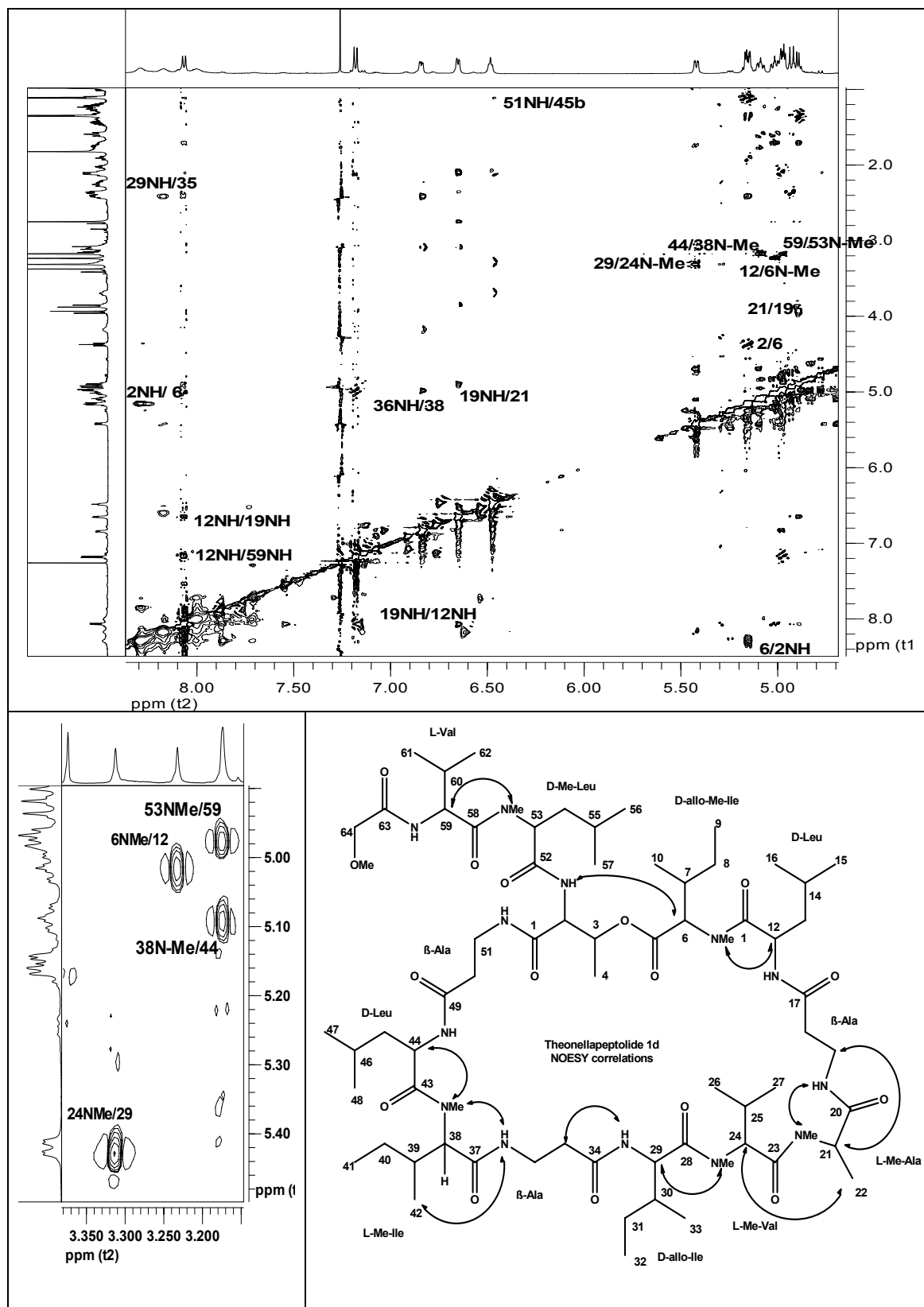
Figure 3.24. NOESY correlation of compound **6**.
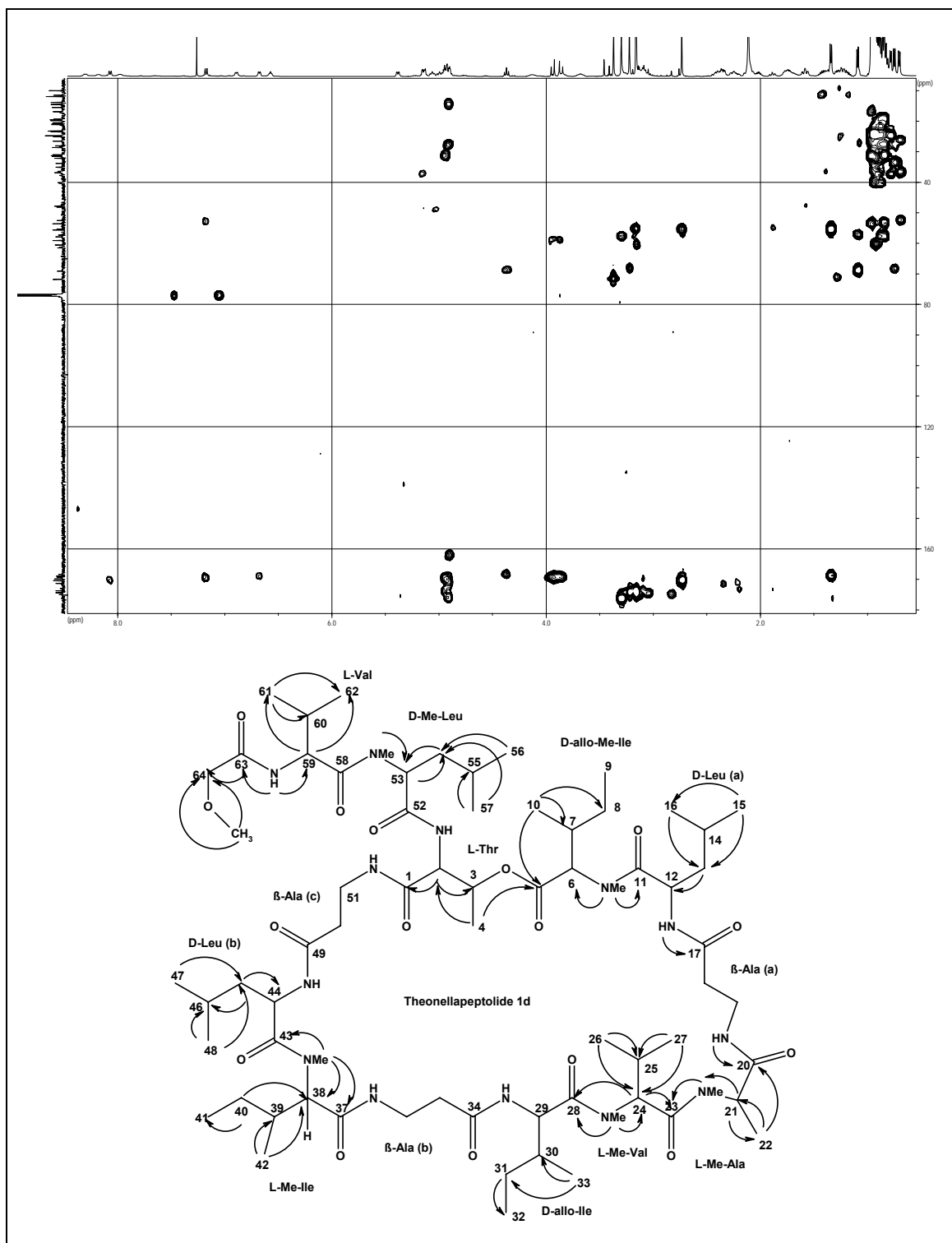
Figure 3.25. HMBC correlation of compound **6**.

3.2.2. Compound 7: Theonellapeptolide Ie

Theonellapeptolide 1e
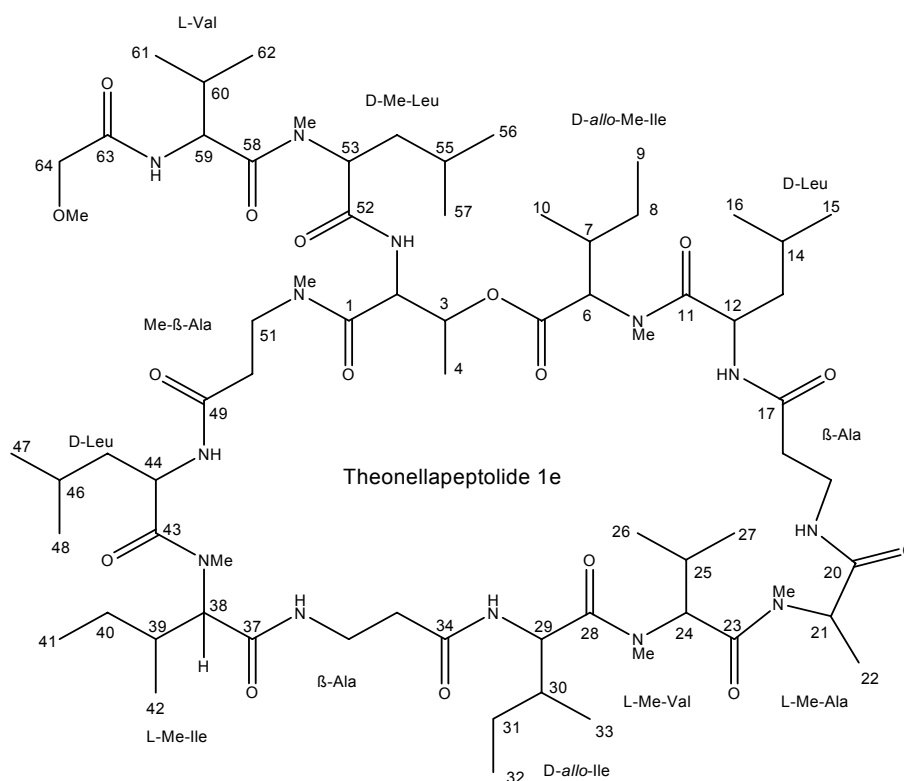
Compound **7** was isolated as a colorless crystalline with UV absorbance at λ_{max} 208 nm due to the lack of chromophore. It did not show any fluorescence or did not quench on UV detection by TLC analysis. It was negative to the ninhydrin test, but positive to Dragendorf reagent. Thus, it was again presumed that, compound **7** is a cyclic peptide lactone in which the N-terminal is protected with a methoxy acetyl group and the C-terminal is connected through a lactone linkage to the ß-hydroxyl group of threonine (Thr). Since this compound has amine groups in the molecule, it could be observed by Dragendorf reagent. This spray reagent commonly used to detect alkaloids and other quaternary nitrogenous compounds. The FAB-MS spectra showed molecular ion peaks at *m/z* 1418 [M]$^+$. The molecular weight 1418 Da is compatible to molecular formula $C_{70}H_{125}N_{13}O_{16}$. Compound **7** also revealed several similar properties to

theonellapeptolides. It has 14 mass units difference from theonellapeptolide Id, which suggested an additional methyl subtituent in this structure relative to theonellapeptolide Id.

The ^{1}H NMR spectrum of compound **7** showed resonances for seven amide protons at δ 9.06 (1H, brs, 2-NH), 8.20 (1H, brs, 29-NH), 8.08 (1H, d, *J* = 9.3 Hz, 12-NH), 7.85 (1H, d, *J* = 7.5 Hz, 44-NH), 7.14 (1H, dd, *J* = 3.1 and 7.4 Hz, 59-NH), 6.69 (1H, d, *J* = 9.2 Hz, 36-NH), 6.70 (1H, d, *J* = 6.2 Hz, 19-NH) and six N-methyl groups at δ 3.30 (24-NCH$_3$), 3.26 (6-NCH$_3$), 3.22 (53-NCH$_3$), 3.15 (38-NCH$_3$), 2.74 (51-NCH$_3$), 2.72 (21-NCH$_3$) which indicated the peptide nature of this compound. The missing amide proton signal in the downfield region and the additional N-methyl resonance at δ 2.74 ppm suggested one ß-Ala was N-methylated in comparison to theonellapeptolide Id. One methoxy-acetyl at δ 3.92 (d, *J* = 15.0 Hz, H-64), 3.86 (d, *J* = 15.0 Hz, H-64´) and 3.41 (3H, s, 64-OCH3) was also observed

The COSY and TOCSY spectrum showed thirteen spin systems for thirteen amino acids: seven ordinary amino acids and six N-methylated amino acid residues. One Thr spin system was indicated by the signals at δ 8.31 (2-NH), 4.68 (H-2), 5.25 (H-3) and 1.01 (CH$_3$-4). ROESY spectra showed correlation from H-3 to H-7 and 53-NCH$_3$, which indicated connections from Thr to *allo*-Ile and Me-Leu. It also showed correlation from H-2 to 51-NCH$_3$, that indicated the connectivity from Thr to Me-ß-Ala. These correlations proved the cyclic nature of this compound. An *allo*-Me-Ile amino acid residue was indicated by the

TOCSY signal at δ 5.03 (H-6), 2.42 (H-7), 1.26 and 1.20 (H-8), 0.96 (CH$_3$-9) and 0.75 (CH$_3$-10). ROESY spectrum showed correlation from proton 6-NCH$_3$ to H-4, H-12 and H-18, confirmed the connectivity of Me-Ile to Thr, Leu 1, showed an NOE effect on the N-methyl proton of Me-*allo*-Ile to ß-Ala 1. The first Leu amino acid was evident from COSY spectrum, which was indicated by connectivity of protons at 12-NH (δ 8.08), H-12 (δ 5.02), CH$_2$-13 (δ 1.60 and 1.26), H-14 (δ 1.70), CH$_3$-15 (δ 0.94) and CH$_3$-16 (δ 0.98). ROESY spectrum showed the correlation of proton 19-NH to α proton H-21 and 21-NCH$_3$, which established the connectivity of ß-Ala to Me-Ala. A Me-Ala spin system was indicated by the TOCSY signals at δ 4.88 (H-21) and 1.34 (CH$_3$-22). ROESY spectrum showed the correlation of proton H-21 to H-19 and 21-NCH$_3$ to H-24, confirmed the connectivity to ß-Ala and Me-Val. A Me-Val amino acid residue was indicated by the TOCSY signal at δ 4.91 (H-24), 2.36 (H-25), 0.96 (CH$_3$-26), and 0.88 (CH$_3$-27). ROESY spectrum confirmed the correlation of proton H-21 to 21-NCH$_3$ from Me-Ala and proton 24-NCH$_3$ to H-29 from next amino acid sequence *allo*-Ile.

Compound **7** was established as theonellapeptolide Ie through comparison of the ^{1}H NMR data and molecular weight of compound **7** with compound **6** and literature (Roy *et al.*, 2000) in combination with careful analysis of the 2D-NMR: COSY, TOCSY, and ROESY spectra.

Theonellapeptolide Ie is a cyclic tridecapeptide lactone, which was differing from previous compound by an additional N-methyl at ß-Ala. This difference

was revealed as missing peak in the amine protons regions and additional peak at 274 ppm assigned as N-Me, which showed ROESY correlation to H-50 of Me-ß-Ala and H-2 from Thr. The rest of NMR properties of this molecule showed similar pattern to compound **6**.

Table 3.6. NMR data of compound **7** (CD$_3$Cl, 500 MHz).

Amino acid monomer	number	¹H-NMR	ROESY
L-Thr	2 3 4 2-NH	4.68 dd 5.25 m 1.01 d 9.06 brd	1.01, 2.74(51NMe) 1.01, 2.42(7), 3.18(53NMe)
D-allo-Me-Ile	6 7 8 9 10 6-NMe	5.13 2.39 1.80 0.95 0.75 3.26 s	0.75, 0.95 0.75, 0.96, 1.01(4), 1.26, 2.34(18a), 5.02(12)
D-Leu (a)	12 13 14 15 16 12-NH	5.02 ddd 1.60 brt 1.26 m 1.70 m 0.94 d 0.98 d 8.08 d	0.98, 1.26, 1.60, 1.70, 3.26(6NMe)
ß-Ala (a)	18 19 19 NH	2.34 m 2.11 m 3.89 m 3.07 m 6.70 dd	2.11,3.07 2.34, 3.07 2.32, 3.85, 2.11 2.74(21NMe), 4.88(21)
L-Me-Ala	21 22 21-NMe	4.88 q 1.34 d 2.74 s	1.34, 3.07(19b) 1.34, 4.88, 4.91(24)
L-Me-Val	24 25 26 27 24-NMe	4.91 d 2.36 m 0.96 d 0.88 d 3.30 s	2.74(21NMe) 2.11, 3.30, 4.91 0.88, 0.96, 1.75(30), 2.36, 4.91, 5.40(29)
D-allo-Ile (continue to next page)	29 30 31	5.40 dd 1.75 m 1.46 m 1.23 m	1.46, 1.75, 3.30(24NMe)

Amino acid monomer	number	¹H-NMR	ROESY
D-allo-Ile	32 33 29-NH	0.98 t 0.71 d 8.20 brs	
ß-Ala (b)	35 36 36-NH	2.40 m 2.22 m 4.15 m 3.14 m 6.69 dd	4.95,
L-Me-Ile	38 39 40 41 42 38-NMe	4.95 d 2.11 m 1.29 m 0.94 m 0.82 t 0.90 d 3.15 s	0.82, 0.94, 1.29, 3.14(36b) 0.82, 0.90, 0.94, 1.20, 1.29, 2.11, 5.10(44)
D-Leu (b)	44 45 46 47 48 44-NH	5.10 dd 1.59 m 1.20 brt 1.71 m 0.89 d 0.92 d 7.85 d	0.89,0.92, 1.20, 1.71, 3.15(38NMe)
Me-ß-Ala	50 51 51-NMe	2.28 m 2.21 m 4.49 m 2.72 m 2.74 s	3.41(64OMe) 1.36, 2.28, 4.65(2)
D-Me-Leu	53 54 55 56 57 53-NMe	5.12 m 1.92 brt 1.40 m 1.36 m 0.93 d 0.79 d 3.22 s	 0.93, 1.40, 1.92, 2.02, 4.95(59)
L-Val	59 60 61 62 59-NH	4.93 dd 2.02 dsept 0.97 d 0.89 d 7.14 d	0.92, 0.98,1.36, 2.02, 3.22(53NMe) 0.89, 0.99, 2.02, 3.40(64OMe), 3.89&3.92(64), 4.95
MeOAc	64 64-OMe	3.92 d 3.86 d 3.41 s	3.41 1.71(46), 2.21&2.28(50a&b), 3.86&3.92(64)
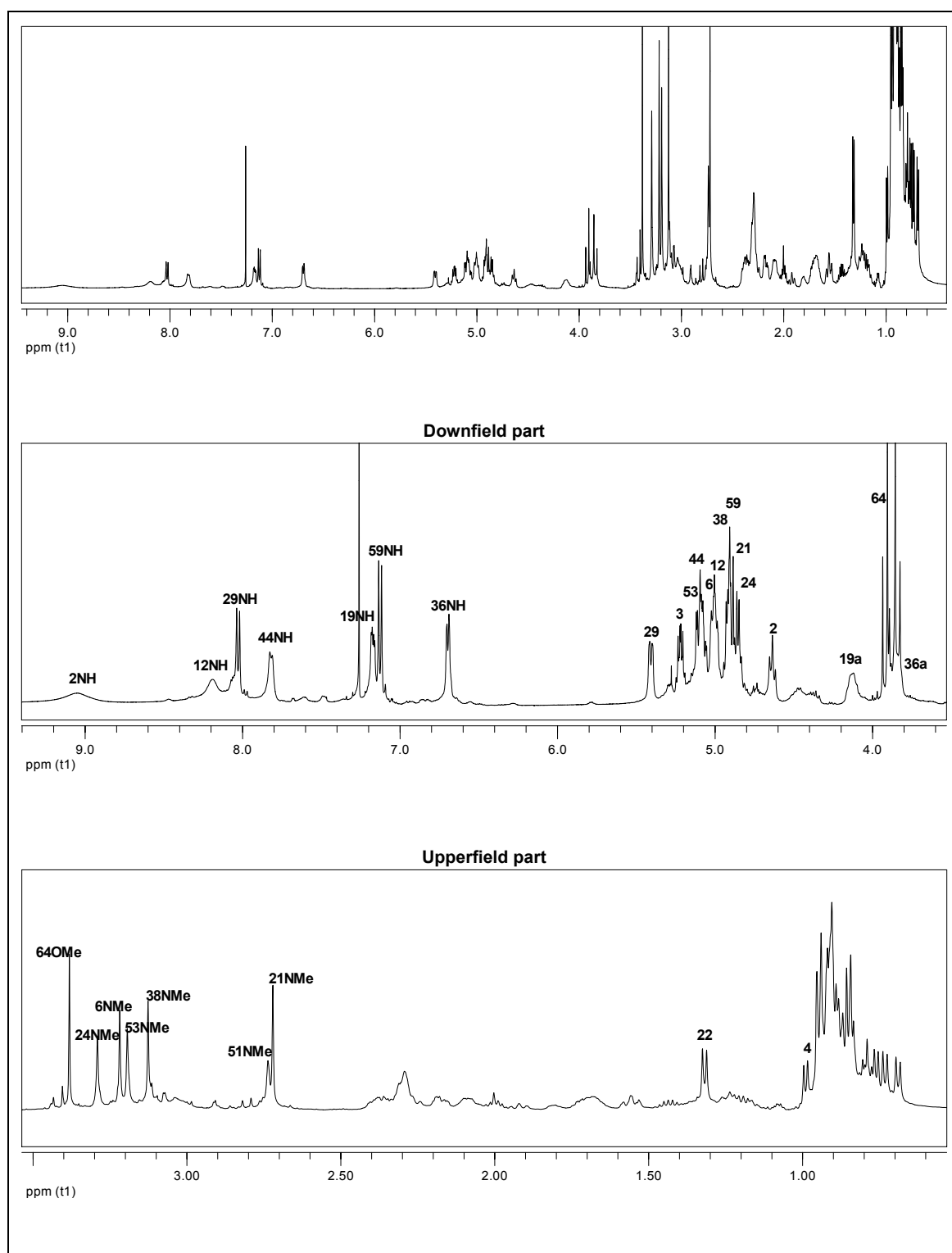
Figure 3.26. Proton spectrum of compound **7**.
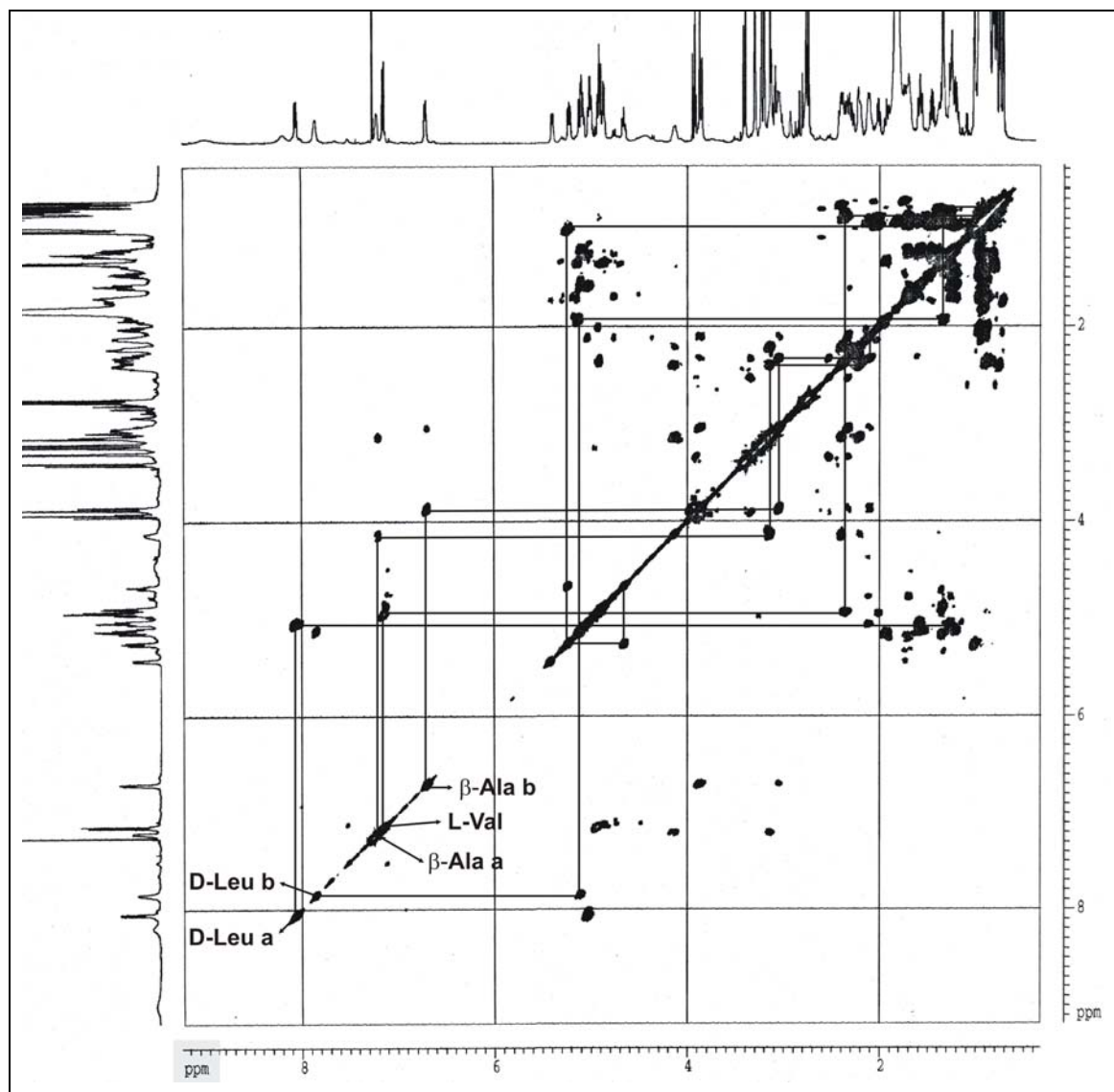
Figure 3.27. COSY spectrum of compound **7**: ordinary amino acids part.
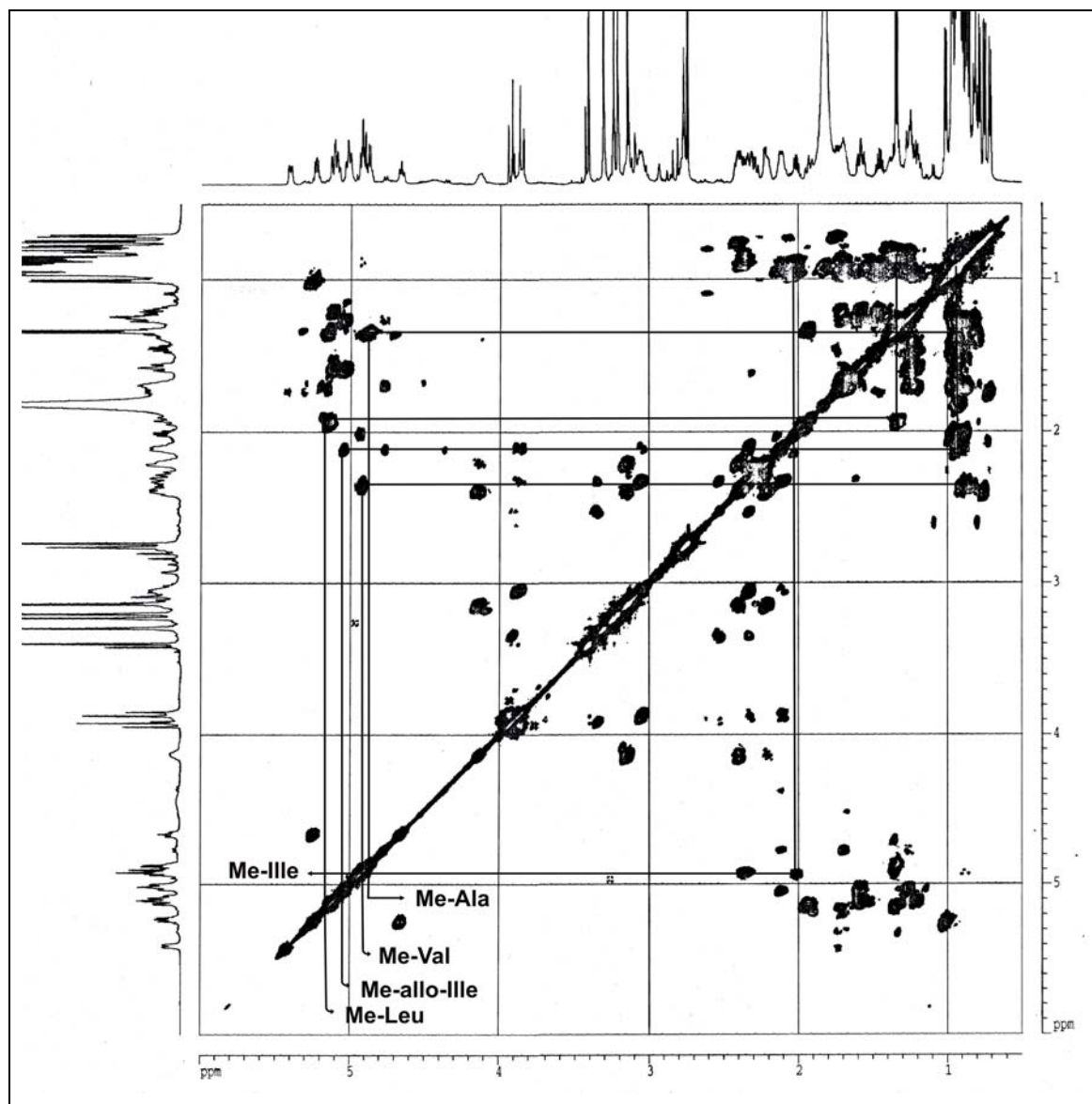
Figure 3.28. COSY spectrum of compound **7**: methyl amino acids part.
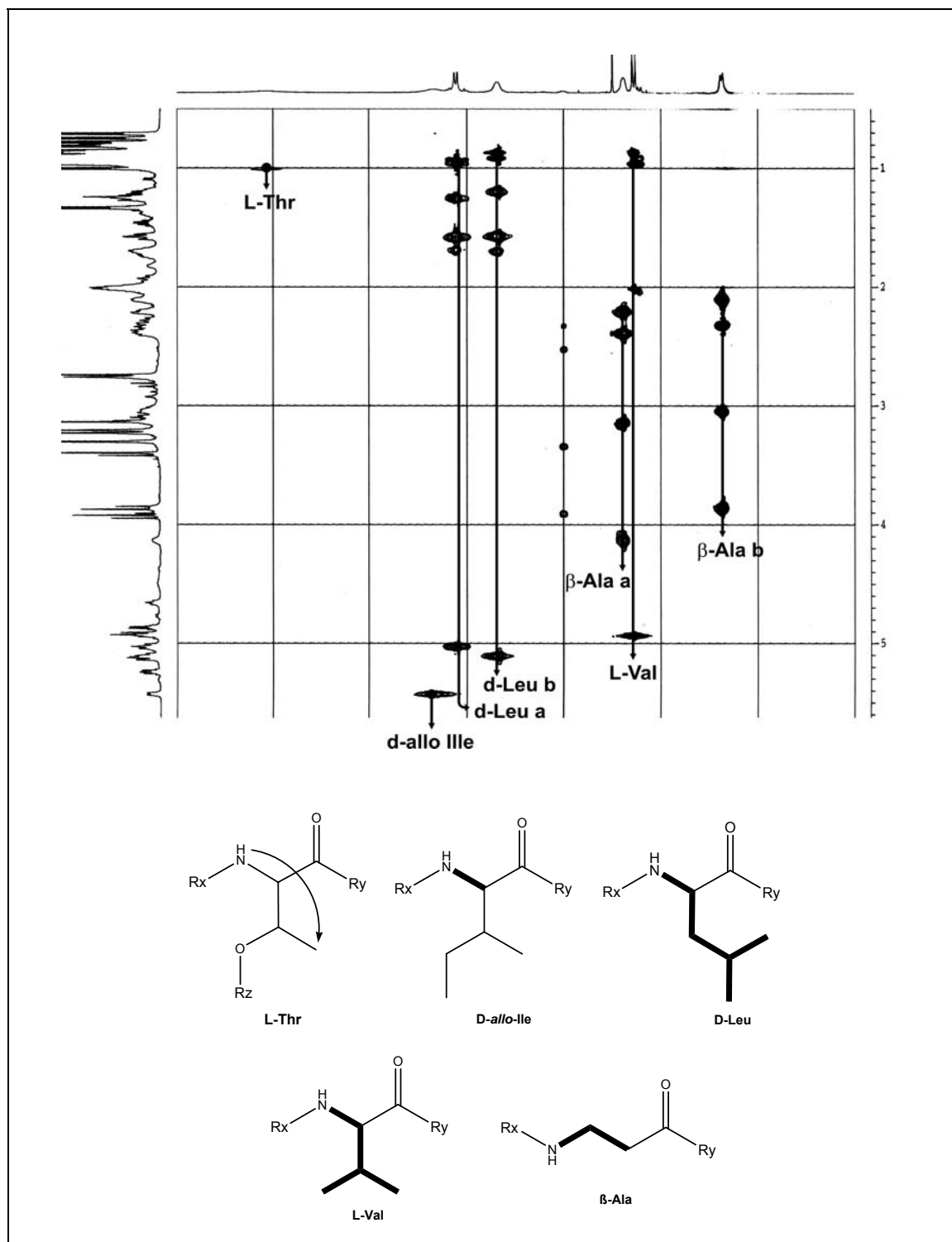
Figure 3.29. TOCSY spectrum of amin region from compound **7**.
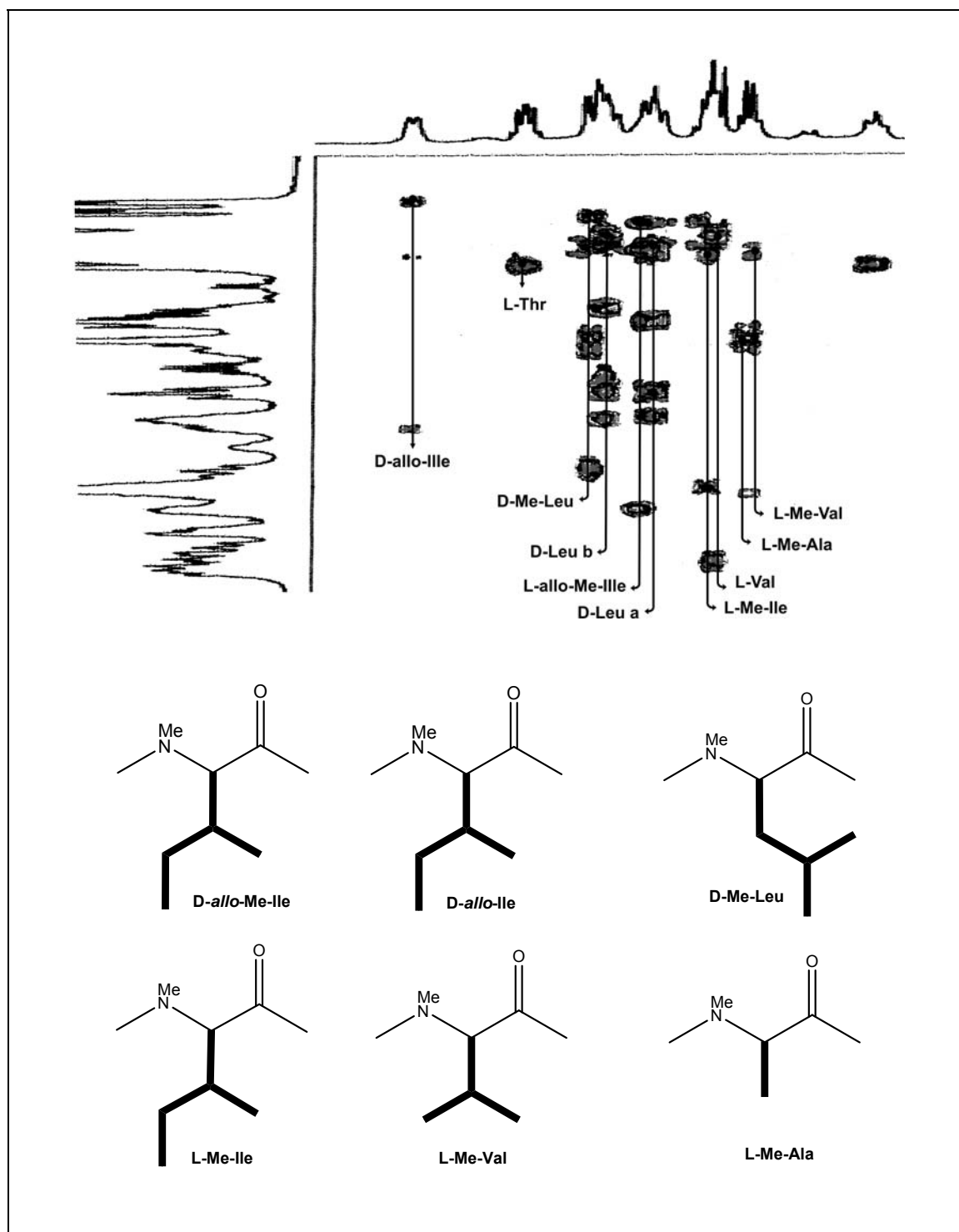
Figure 3.30. TOCSY spectrum in the α proton region from compound **7**.
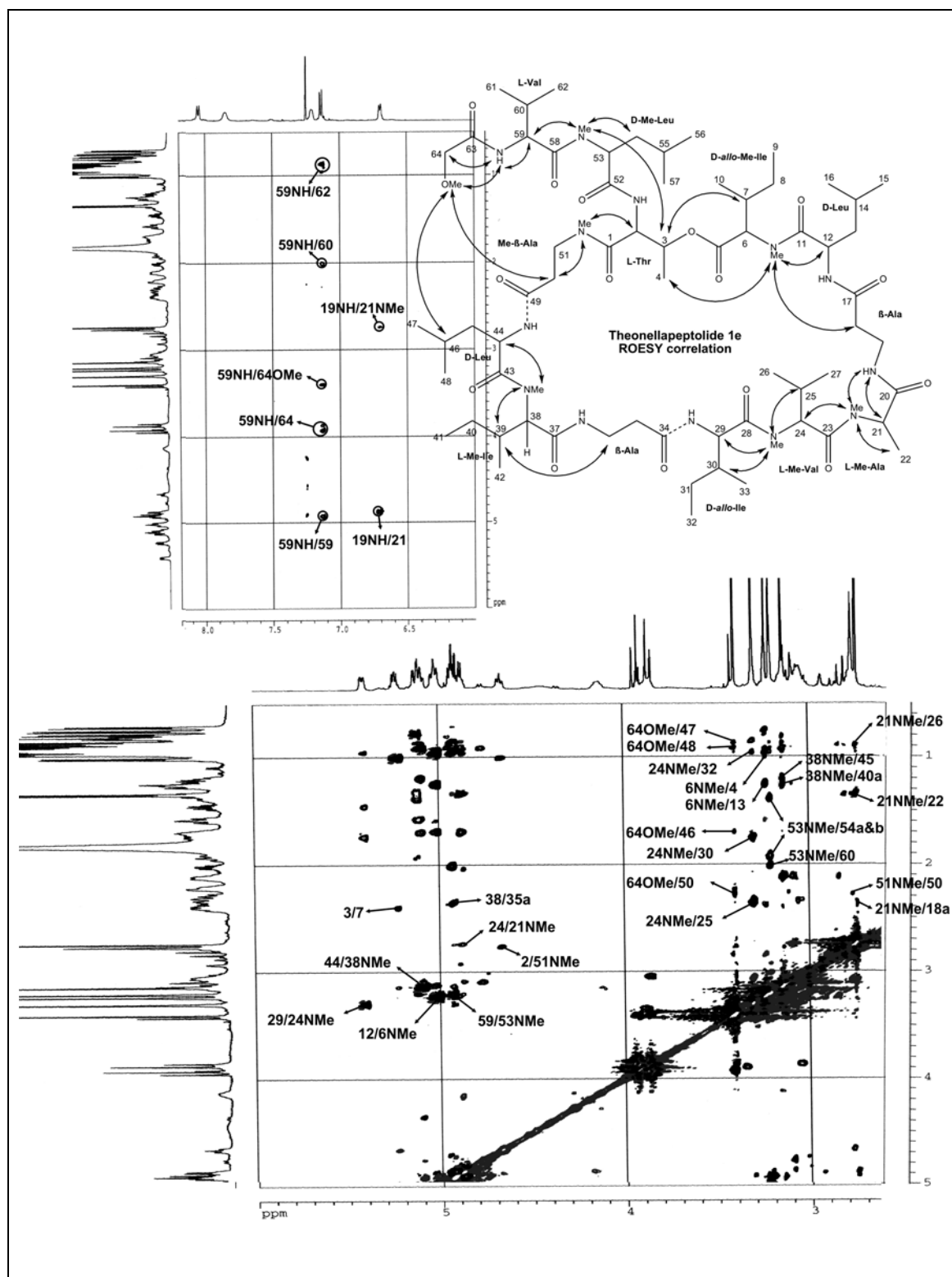
Figure 3.31. ROESY spectrum of compound 7.

3.2.3. Compound 8: Theonellapeptolide IId
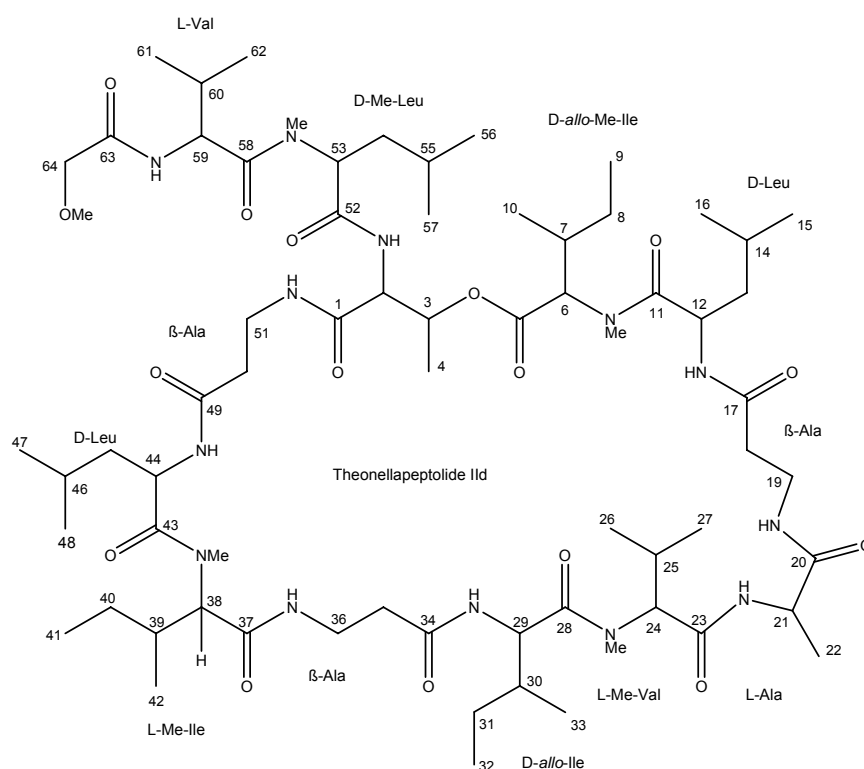
Compound **8** was isolated as a colorless crystalline with UV absorbance at λ_{max} 208 nm due to the lack of chromophore. It did not show any fluorescence or did not quench on UV detection by TLC analysis. It was negative to the ninhydrin test, but positive to Dragendorf reagent. Thus, it was again presumed that, compound **8** is a cyclic peptide lactone in which the N-terminal is protected with a methoxy acetyl group and the C-terminal is connected through a lactone linkage to the ß-hydroxyl group of threonine (Thr). Since this compound has amine groups in the molecule, it could be observed by Dragendorf reagent. This spray reagent is commonly used to detect alkaloids and other quaternary nitrogenous compounds. The FAB-MS spectra showed molecular ion peaks at *m/z* 1390 [M]$^{+}$. The molecular weight 1390 Da is compatible to the molecular formula $C_{68}H_{121}N_{13}O_{16}$. Compound **8** also revealed several similar properties to other theonellapeptolides. It has a 14 mass units difference from

theonellapeptolide Id, which suggested a missing methyl subtituent in this structure relative to theonellapeptolide Id.

Proton spectra did not show NH peak due to the mixture of CDCl₃ and MeOD used as NMR solvent. In comparison to the proton spectra of compound **6**, there was a missing N-methyl signal at δ 2.74 ppm, which suggesting that the amine residue of alanine was not methylated. The COSY and TOCSY spectrum showed thirteen spin systems for thirteen amino acids, which had similar pattern to compound **6**. NOESY data analysis was helpful in building three substructures of compound **8**. The first substructure was the connection of *allo*-Me-Ile, Leu, and then ß-ala. The second substructure connected Me-Val, *allo*-Ile, ß-Ala, Me-Ile, Leu. The last substructure was ß-Ala, Thr, Me-Leu, Val, and a CH₃OCH₂CO- tail.
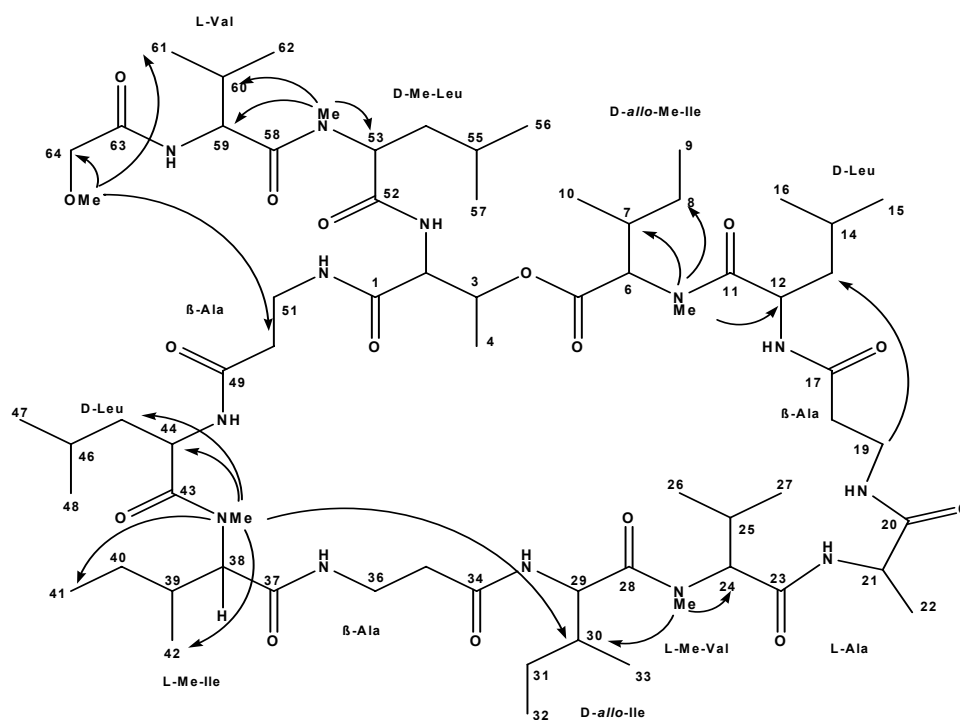
Figure 3.32. NOESY correlation of compound **8**.

Compound **8** was proposed as theonellapeptolide IId through comparison of its ^{1}H NMR data and molecular weight of compound **8** with compound **6** and literature data (Roy *et al.*, 2000). Theonellapeptolide IId is a cyclic tridecapeptide lactone, which is differing from compound **6** with a missing N-methyl unit at Ala. This difference is revealed as missing peak of the N-methyl at δ 2.74. The rest of NMR properties of this molecule showed similar pattern to compound **6**.

Table 3.7. NMR data of compound **8**.

Amino acid monomer	number	Data	
		^{1}H-NMR	NOESY
L-Thr	2	4.38 dd	
	3	5.12 m	
	4	1.12 d	
D-allo-Me-Ile	6	4.93 m	
	7	2.09 brt	
	8	1.38 m 1.29 m	
	9	1.02 d	
	10	0.81 d	
	6-NMe	3.13 s	H-7, H-8, H-9, H-12
D-Leu (a)	12	4.98 dd	
	13	1.49 m 1.34 brt	
	14	1.61 m	
	15	0.91 d	
	16	0.91 d	
ß-Ala (a)	18	2.48 m 2.34 m	
	19	3.46 m 3.29 m	
L-Ala	21	4.24 q	
	22	1.32 d	
L-Me-Val	24	4.68 d	
	25	2.05 m	
	26	0.90 d	
	27	0.80 d	
	24-NMe	3.03 s	H-30, H-35, H-38

Amino acid monomer	number	Data	
		¹H-NMR	NOESY
D-allo-Ile	29	3.87 dd	
	30	2.20 m	
	31	1.55 m 1.07 m	
	32	0.79 t	
	33	0.92 d	
ß-Ala (b)	35	2.18 m 2.32 m	
	36	3.40 m 3.29 m	
L-Me-Ile	38	4.63 d	
	39	1.77 m	
	40	1.42 m 1.23 m	
	41	0.80 t	
	42	0.90 d	
	38-NMe	3.12 s	H-30, H-41, H-42, H-44, H-45
D-Leu (b)	44	4.86 ddd	
	45	1.50 brt 1.38 m	
	46	1.61 m	
	47	0.91 d	
	48	0.91 d	
ß-Ala (c)	50	2.42 m 2.32 m	
	51	3.68 m 3.16 m	
D-Me-Leu	53	5.11 dd	
	54	1.76 m 1.58 m	
	55	1.41 m	
	56	0.93 t	
	57	0.85 d	
	53-NMe	3.08 s	H-53, H-59, H60
L-Val	59	4.75 dd	
	60	2.02 dsept	
	61	0.92 d	
	62	0.78 d	
MeOAc	64	3.90 s	
	64-OMe	3.40 s	H-50a&b, H-61, H-64
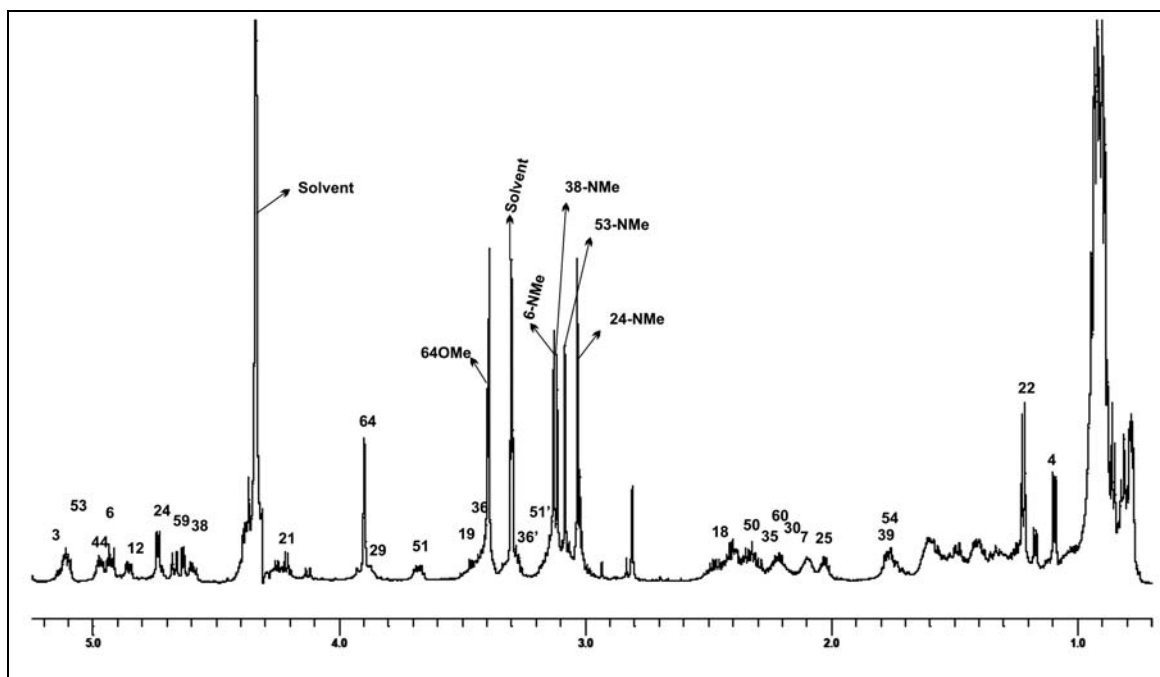
Figure 3.33. Proton NMR of compound **8.**
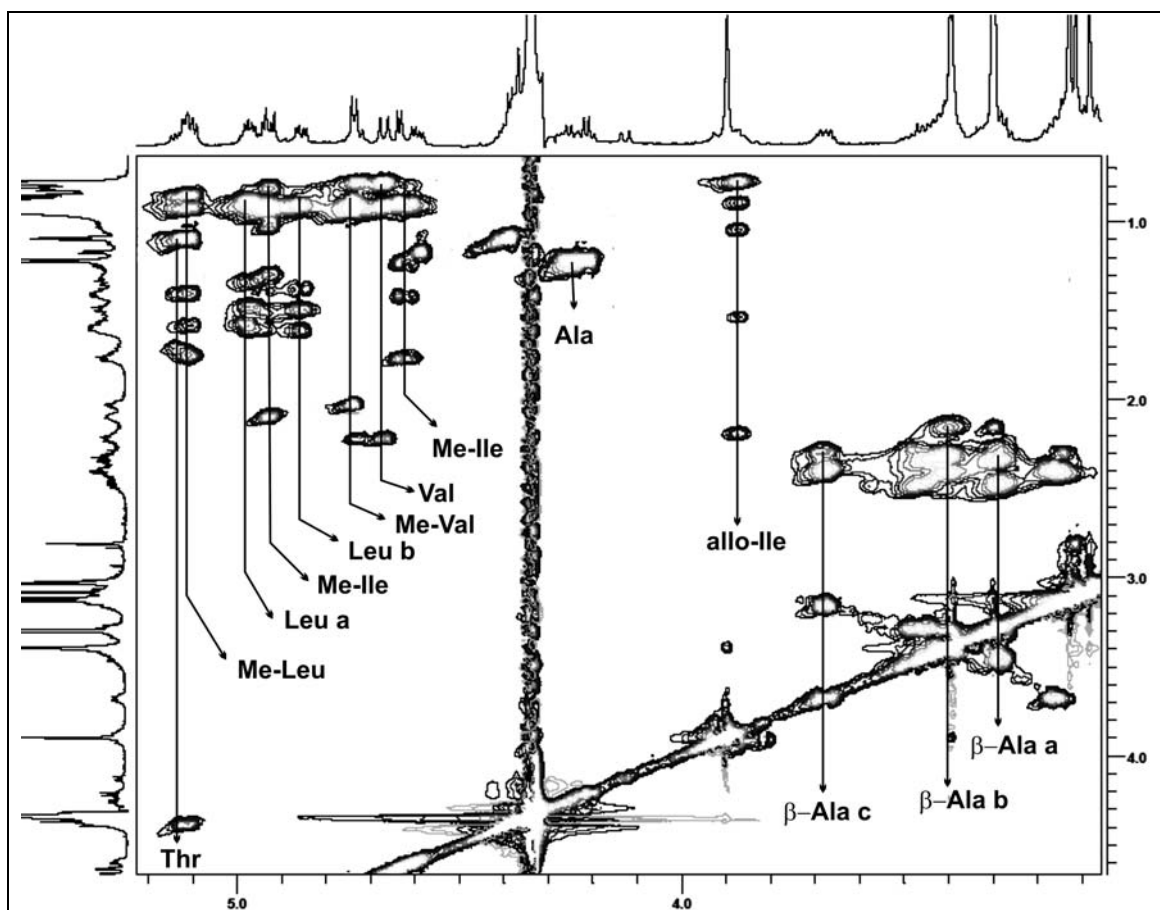
Figure 3.34. TOCSY spectra of compound **8**.

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3.2.4. Compound 9: Swinholide A
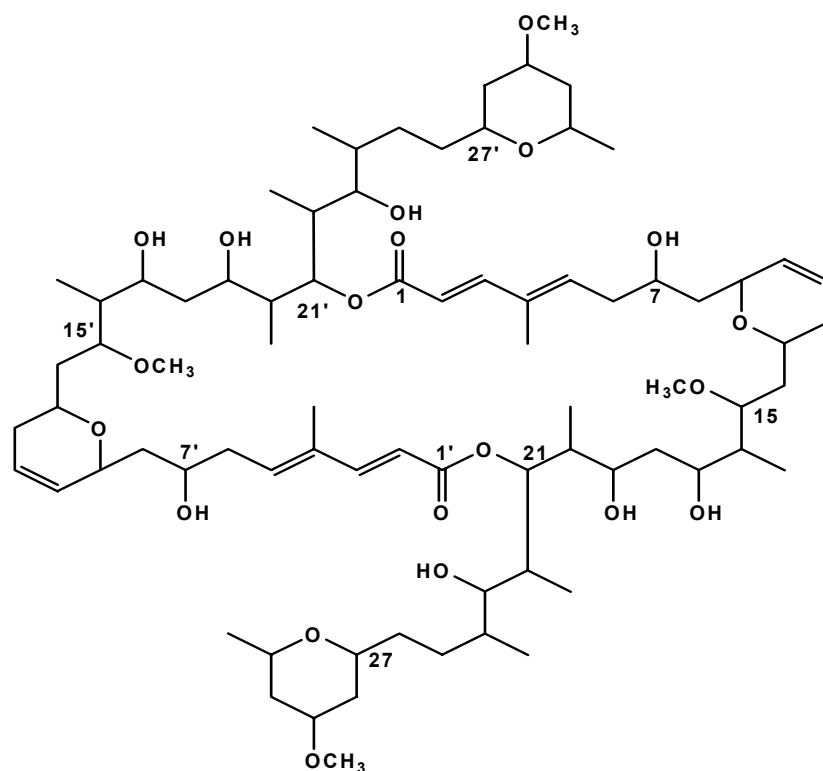
Compound **9** was isolated as a white amorphous powder. It has a UV absorbance at λ_{max} 272 nm and appeared as a quenched spot under UV_{254nm} on TLC analysis. This compound has a molecular weight of *m/z* 1390, as derived from the FABMS spectrum that showed a quasimolecular ion peak at *m/z* 1391 [M+H]$^{+}$. The ^{1}H NMR spectrum of the compound was considerably simplified showing only half of the expected signals in agreement with the molecular weight, which suggested a dimeric nature of compound **9** which was comprised of two identical subunits (Figure 3.36). Five olefinic protons at δ 7.58 (1H, d, *J*= 15.4 Hz, H-3), 6.08 (1H, dd, *J*= 9.5, 1.9 Hz, H-5), 5.78 (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. The ^{1}H NMR spectrum also presented one olefinic methyl singlet at δ 1.81 (3H, s, CH$_3$-4) and five methyl doublets at δ 1.20 (3H, d, *J*= 6.3 Hz, 31-
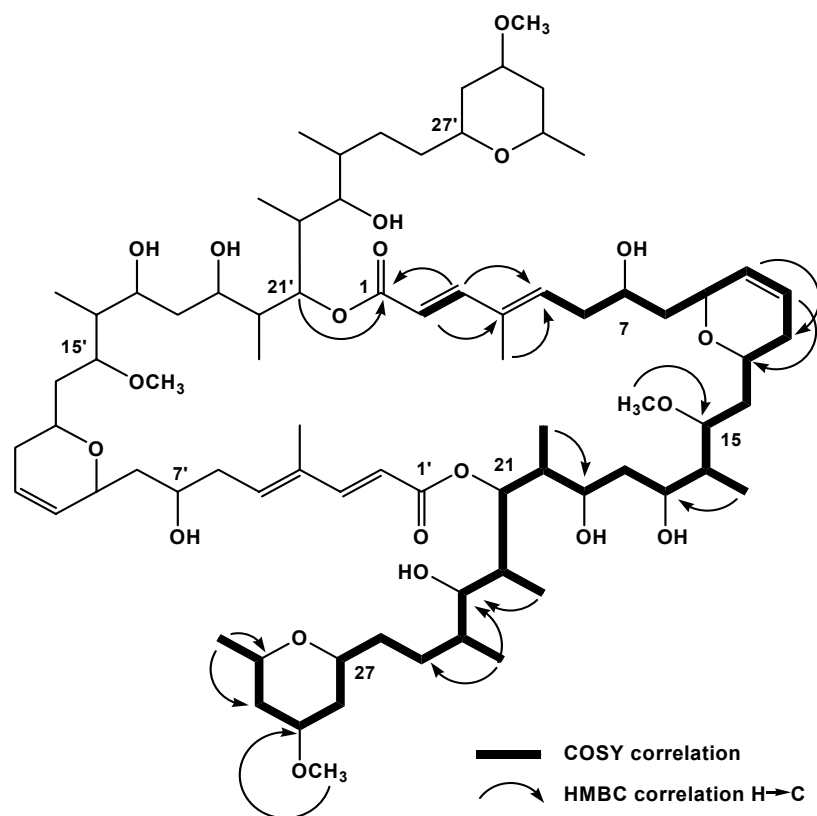
Figure 3.35. Diagram of COSY and HMBC correlation of compound **9**.

CH$_3$), 0.99 (3H, d, J= 7.2 Hz, 24-CH$_3$), 0.97 (3H, d, J= 8.5 Hz, 20-CH$_3$), 0.83

(3H, d, J= 6.9 Hz, 22-CH$_3$), and 0.80 (3H, d, J= 6.9 Hz, 16-CH$_3$). Two methoxy

singlets were observed at δ 3.35 (3H, s, 15-OCH$_3$) and 3.34 (3H, s, 29-OCH$_3$).

The COSY spectrum allowed assignment of all protons and revealed obviously

protons connection for two spin systems (Figure 3.37), first spin system started

from H-2 to H-3 and second one from H-5 to 31-Me. HMBC showed correlation

of H-3 to C-4 (j^2) and C-5 (j^3) confirmed the connection of both spin systems.

HMBC spectra (Figure 3.39) confirmed the dimeric connection with a cross

peak assigned as correlation of proton H-21 to carbon C-1' or proton H-21' to

carbon C-1. Compound **9** was established as swinholide A through comparison

of its ^{1}H NMR data with those of swinholde A previously isolated from the

Okinawan marine sponge *T. swinhoei* (Kobayashi *et al.*, 1989) and deep inspection on COSY, HMQC and HMBC spectrum.

Table 3.8. NMR data of compound **9** compared with literature (Kobayashi *et al.*, 1989).

No.	Literature		Data			
	¹³C-NMR	¹H-NMR	¹³C-NMR	¹H-NMR	COSY	HMBC (H→C)
1	169.6 s	---	169.9 s	---	---	---
2	113.3 d	5.79 d	113.6 d	5.79 d	H3	C1,C4
3	152.5 d	7.58 d	153.5 d	7.57 d	H2	C1,C5
4	133.9 s	---	134.1 s	---	---	---
4-Me	12.0 q	1.83 s	11.9 q	1.81 s		C3,C4,C5
5	141.2 d	6.08 dd	141.4 d	6.08 dd	H4, H6, 4-Me	
6	37.4 t	2.18 brd 2.46 ddd	37.9 t	2.18 brd 2.46 ddd	H5, H7	
7	66.6 d	4.14 brdd	66.7 d	4.14 brdd	H8,H6	
8	40.4 t	1.58 m 1.63 m	39.7 t	1.6 m	H7, H9	
9	66.7 d	4.51 brd	66.8 d	4.51 brd	H8, H10	
10	129.7 d	5.69 brdd	129.2 d	5.69 dd	H9, H11	
11	123.1 d	5.78 brd	124.0 d	5.75 brd	H12ab, H10	
12	30.2 t	1.82 m 2.27 brd	29.8 t	1.82 m 2.27 brd	H11, H13	
13	65.1 d	3.86 m	65.0 d	3.88 m	H12ab,H14	
14	34.6 t	1.46 ddd 2.14 ddd	34.5 t	1.45 ddd 2.14 ddd		
15	75.6 d	4.01 m	75.5 d	4.00 m	H14ab,H16	
15-OMe	56.9 q	3.35 s	58.1 d	3.35 s	---	C15
16	41.4 d	1.68 m	41.2 d	1.68	16-Me	
16-Me	9.0 q	0.81 d	10.2 q	0.81 d	H16	C16, C17

No.	Literature		Data			
	¹³C-NMR	¹H-NMR	¹³C-NMR	¹H-NMR	COSY	HMBC (H→C)
17	73.5 d	3.83 dd	73.7 d	3.83 dd	H18, H17	
18	38.1 t	1.62 m 1.69 m	38.0 t	1.63 m 1.68 m	H17, H18	C19
19	70.9 d	3.98 m	71.1 d	3.99 m		
20	40.7 d	1.75 dq	40.5 d	1.75 q	20-Me, H21	
20-Me	8.9 q	0.97 d	10.3 q	0.97 d	H20	C20,C19
21	74.1 d	5.36 d	75.5 d	5.36 d	H20	C1' (dimeric)
22	37.2 d	1.95 m	37.2 d	1.95 m	H23,22-Me	
22-Me	8.8 q	0.84 d	8.5 q	0.83 d	H22	C22, C21
23	75.8 d	3.12 d	75.5 d	3.12 d	H22	
24	32.9 d	1.65 m	32.6 d	1.65	24-Me, H25	
24-Me	17.4 q	0.99 d	17.2 q	0.99 d	H24	C23, C24
25	23.7 t	1.27 m 1.38 m	24.1 t	1.24 m 1.37 m	H24, H26	C26
26	29.0 t	1.30 m 1.90 m	29.3 t	1.25 m 1.90 m	H25, H27	
27	70.9 d	4.02 m	71.1 d	4.01 m	H26, H28	
28	34.6 t	1.60 m 1.82 m	34.5 t	1.64 m 1.83 m	H29, H27	
29	72.9 d	3.53 dddd	72.9 d	3.53 dddd	H28,H30	
29-OMe	54.8 q	3.33 s	54.6 q	3.34 d	---	C29
30	38.3 t	1.18 ddd 1.96 m	38.3 t	1.18 ddd 1.95 m	H29, H31	
31	64.3 d	3.69 ddq	64.1 d	3.70 ddq	31-Me	
31-Me	21.4 q	1.20 d	---	1.20 d	H31	C30,C31

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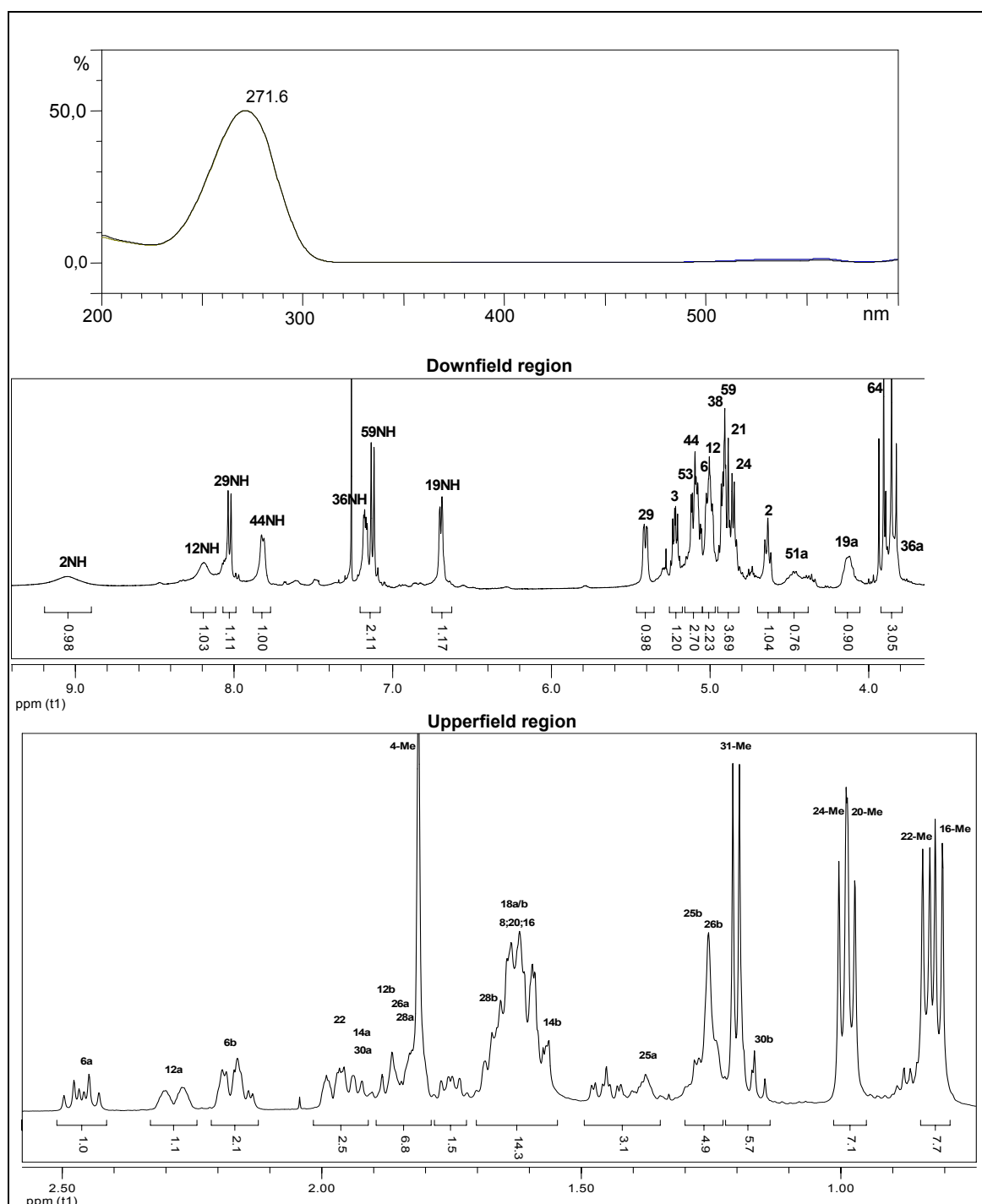
Figure 3.36. UV and proton spectrum of compound **9**.

Figure 3.37. COSY spectrum of compound **9**.
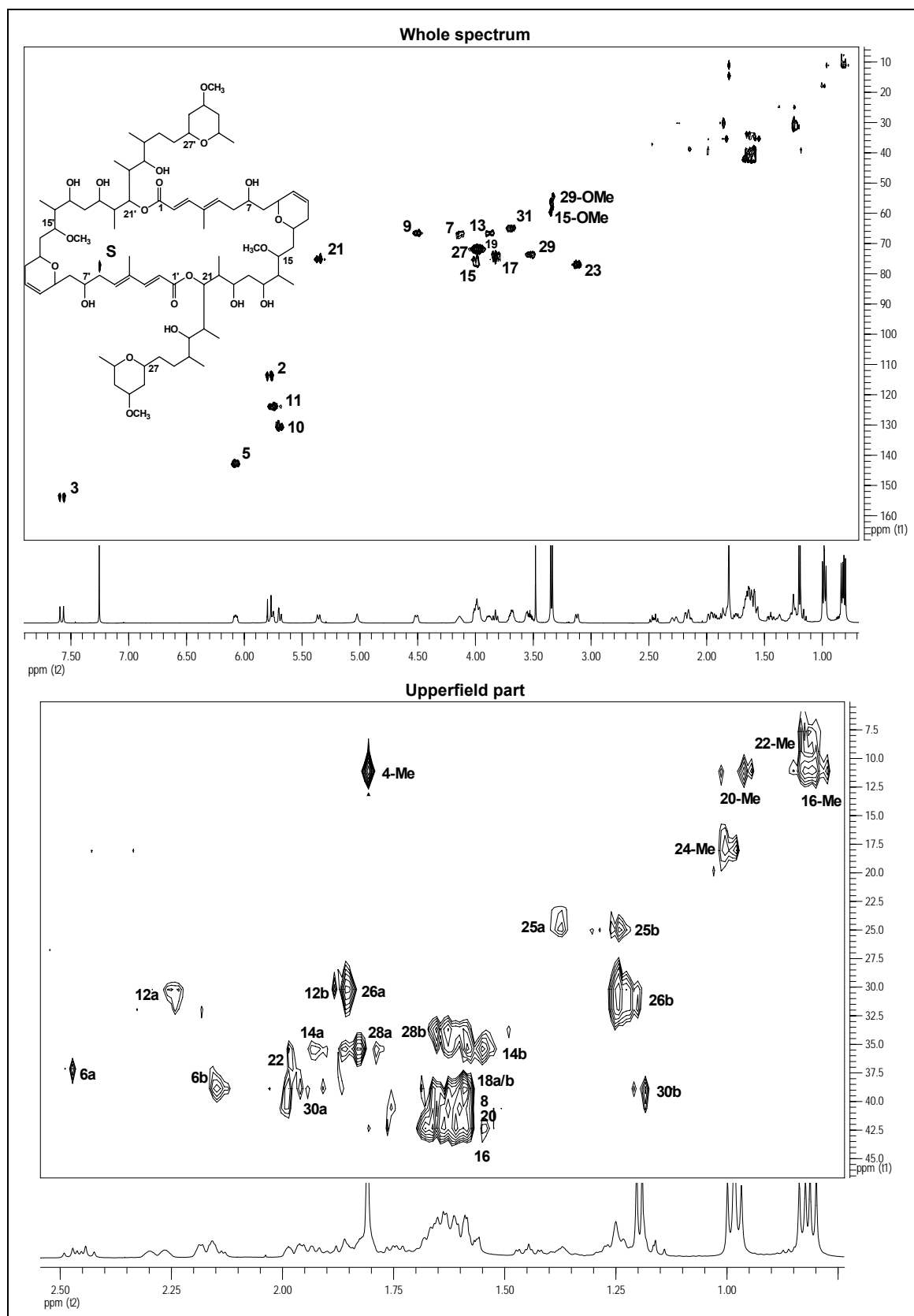
Figure 3.38. HMQC spectrum of compound **9**.
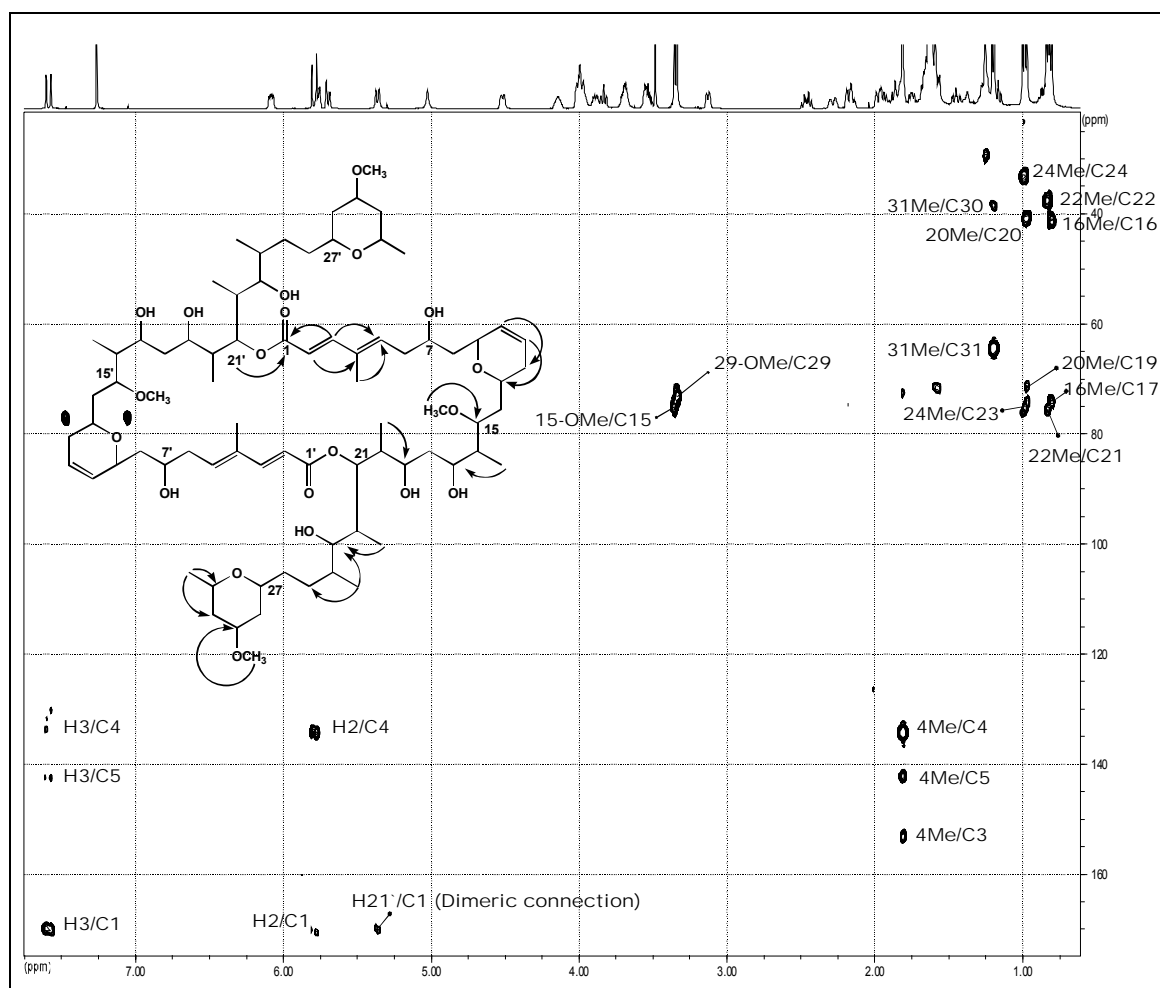
Figure 3.39. HMBC spectrum of compound **9**.

3.3. Secondary metabolites of sponge *Acanthostrongylophora ingens*

This species was previously reported taxonomically as *Xestospongia* sp. Like other members of the family Petrosiidae (eg. *Haliclona* sp. and *Petrosia* sp.), this sponge was also reported to contain manzamine alkaloids. Four secondary metabolites were isolated from sponge *Acanthostrongylophora ingens* collected from Ujung Pandang, Indonesia. They are manzamine A, 8-hydroxymanzamine A, manzamine F, and 1-deoxyircinol A. The following description elaborates more detailed on these compounds.
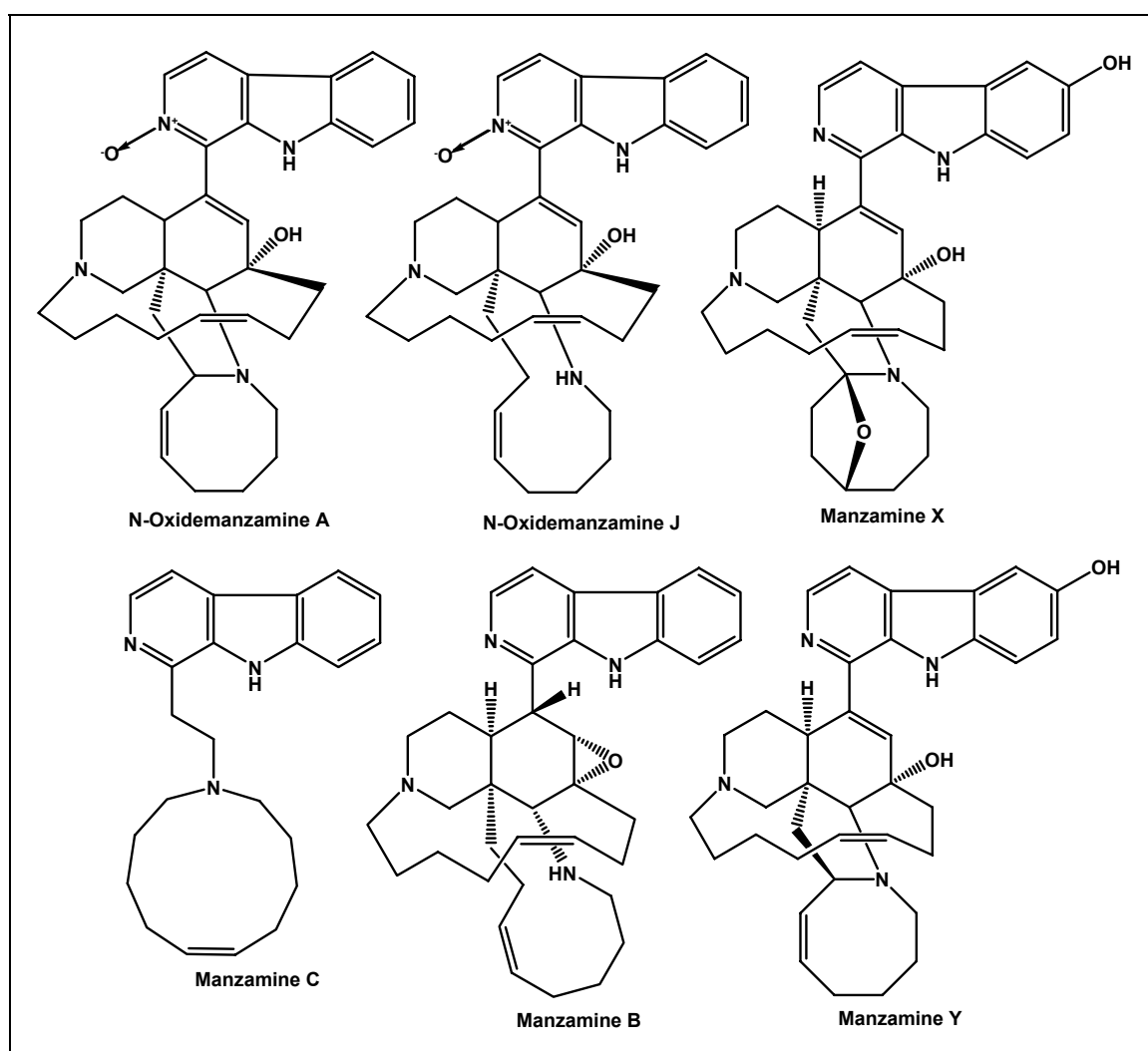
Figure 3.40. Several manzamine derivatives found from sponges of the family Petrosidae.

3.3.1. Compound 10: Manzamine A
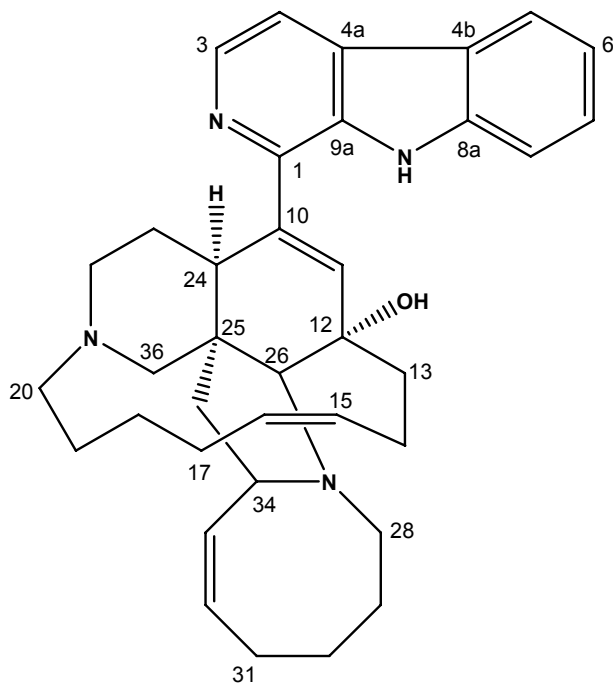
Compound **10** showed a yellow spot upon spraying with Dragendorf reagent on TLC. UV spectra of this compound showed λ_{max} 240, 282 and 358 nm. Its present positive ion mass [M+H]$^+$ at m/z 549 in ESI-MS, indicated the molecular weight of 548 Da. This molecular weight is compatible with $C_{36}H_{44}N_4O$. Proton NMR showed six aromatic protons at δ 8.34 (1H, d, *J*= 5.2, H-3), 8.07 (1H, d, *J*= 7.8, H-5), 7.85 (1H, d, *J*= 5.2, H-4), 7.83 (1H, d, *J*= 8.2, H-8), 7.51 (1H, t, *J*= 7.9, H-7), and 7.22 (1H, t, *J* = 7.8, H-6), which characteristically belong to a ß-carboline moiety. Coupling between H-3 and H-4 revealed the value of 5.2 Hz which is a characteristic *ortho*-coupling constant in a pyridine ring system. Multiplicity pattern of H-3 to H-4, H-5 to H-6, H-6 to H-7 and H-7 to H-8 presented an ABCD spin system which was characteristic of an *ortho*-substituted benzene ring (see Figure 3.41). The olefinic region presented 5 protons which were evident at δ 6.54 (1H, s, H-11), 5.55 (1H, m, H-15), 5.52

(1H, m, H-16), 6.29 (1H, m, H-32), and (1H, m, H-33). By inspection of its

COSY spectrum, this confirmed the presence of five spin systems.
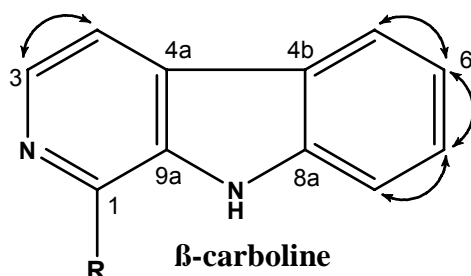
Figure 3.41. COSY correlation of ß-carboline moiety in compound **10**.

Compound **10** was established as manzamine A through comparison of its

molecular weight and ^{1}H NMR data with those previously isolated from the

marine sponge *Xestospongia* sp. (Kobayashi *et al.*, 1995).

Table 3.9. Proton NMR data of compound **10** compared with literature (Kobayashi *et al.*, 1995).

Nr.	^{1}H-NMR δ ^{1}H (ppm), multiplicity, *J* (Hz)	
	Manzamine A (Lit.)	**Compound 10**
3	8.34 (d, *J* =5.1-5.3 Hz)	8.34 (1H, d, *J* = 5.2 Hz)
4	7.85 (d, *J* =5.1-5.3 Hz)	7.85 (1H, d, *J* = 5.2 Hz)
5	8.08 (d, *J* =7.9 Hz)	8.07 (1H, d, *J* = 7.8 Hz)
6	7.23 (t, *J* =7.9 Hz)	7.22 (1H, t, *J* = 7.8 Hz)
7	7.52 (t, *J* =7.9 Hz)	7.51 (1H, t, *J* = 7.9 Hz)
8	7.83 (d, *J* =7.9 Hz)	7.83 (1H, d, *J* = 8.2 Hz)
11	6.52 (s)	6.54 (1H, s)
13	1.75 (m) 2.15 (m)	1.69 (1H, m) 2.00 (1H, m)

Nr.	^{1}H-NMR δ ^{1}H (ppm), multiplicity, J (Hz)	
	Manzamine A (Lit.)	**Compound 10**
14	2.1-2.2 (m)	2.17 (2H, m)
15	5.57 (m)	5.55 (1H, m)
16	5.57 (m)	5.52 (1H, m)
17	1.60 (m) 2.50 (m)	1.52 (1H, m) 2.42 (1H, m)
18	1.20 (m) 1.45 (m)	1.28 (1H, m) 1.53 (1H, m)
19	1.45 (m) 1.81 (m)	1.38 (1H, m) 1.79 (1H, m)
20	2.38 (m) 2.58 (m)	2.34 (1H, m) 2.56 (1H, m)
22	1.88 (m) 2.93 (m)	1.81 (1H, m) 2.89 (1H, m)
23	1.78 (m) 2.95 (m)	1.73 (1H, m) 2.91 (1H, m)
24	2.55 (m)	2.53 (1H, m)
26	3.72 (s)	3.72 (1H, s)
28	3.27 (m) 4.03 (m)	3.27 (1H, m) 4.03 (1H, m)
29	2.00 (m)	1.96 (2H, m)
30	1.45 (m)	1.40 (2H, m)
31	2.30 (m)	2.30 (2H, m)
32	6.29 (m)	6.29 (1H, dt, J=7.0, 11.8 Hz)
33	5.39 (t)	5.39 (1H, dd, J=8.2, 11.4 Hz)
34	4.94 (m)	4.94 (1H, dd, J=7.5, 13.2 Hz)
35	1.85 (m) 2.40 (m)	1.82 (1H, m) 2.40 (1H, m)
36	2.32 (m) 2.88 (m)	2.32 (1H, m) 2.87 (1H, m)
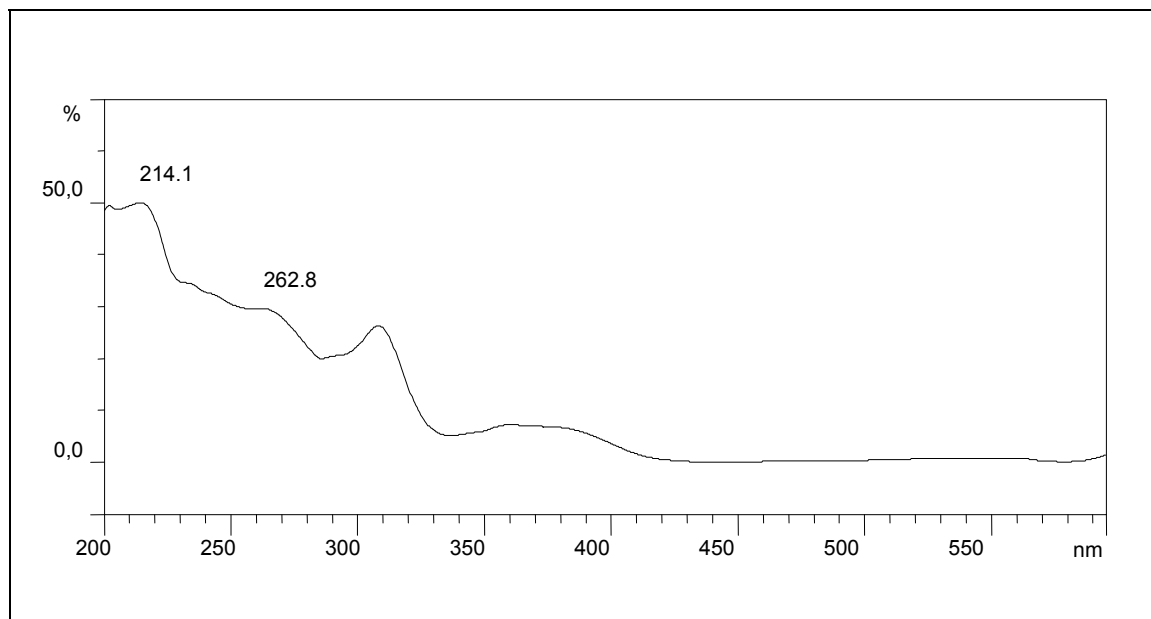
Figure 3.42. Ultraviolet spectra of compound **10**.
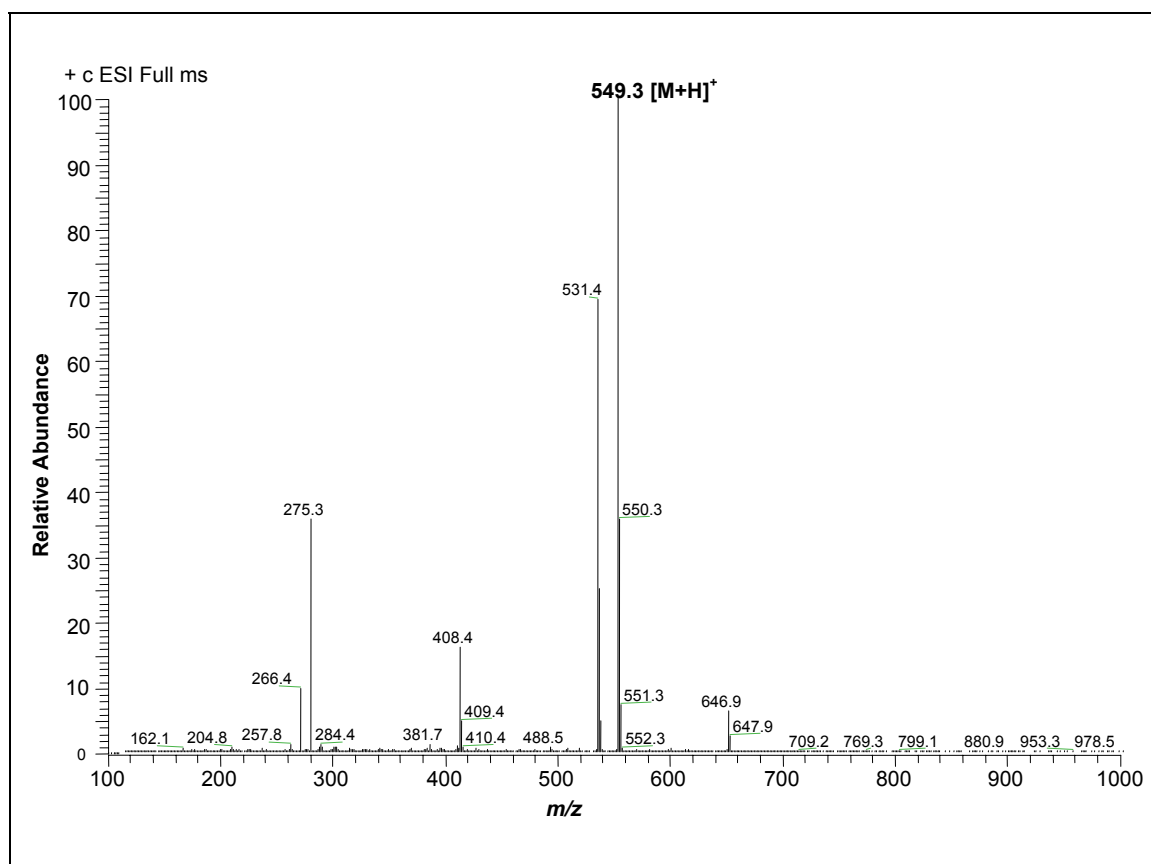
Figure 3.43. ESI-MS of compound **10**.
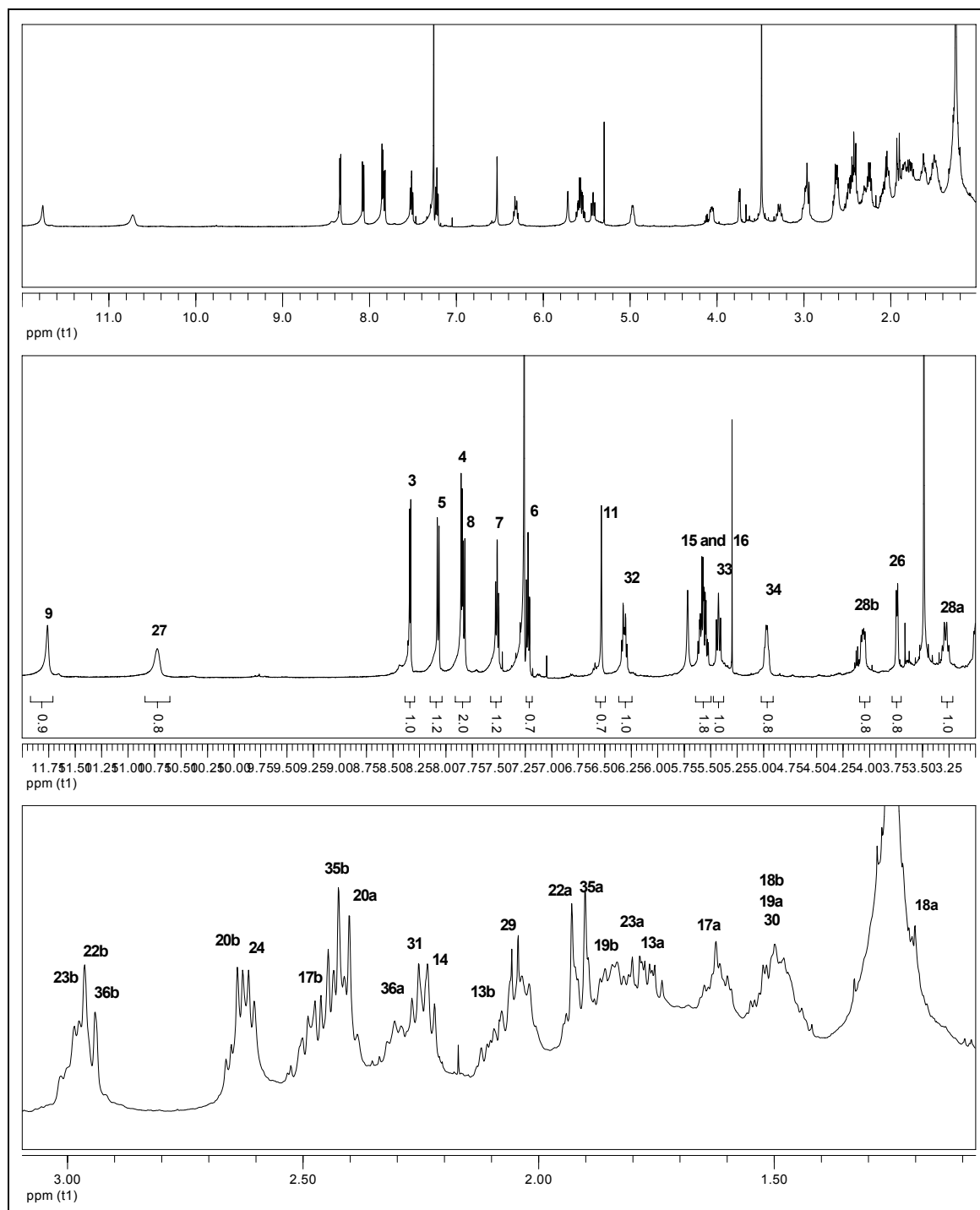
Figure 3.44. Proton assignment of compound **10**.
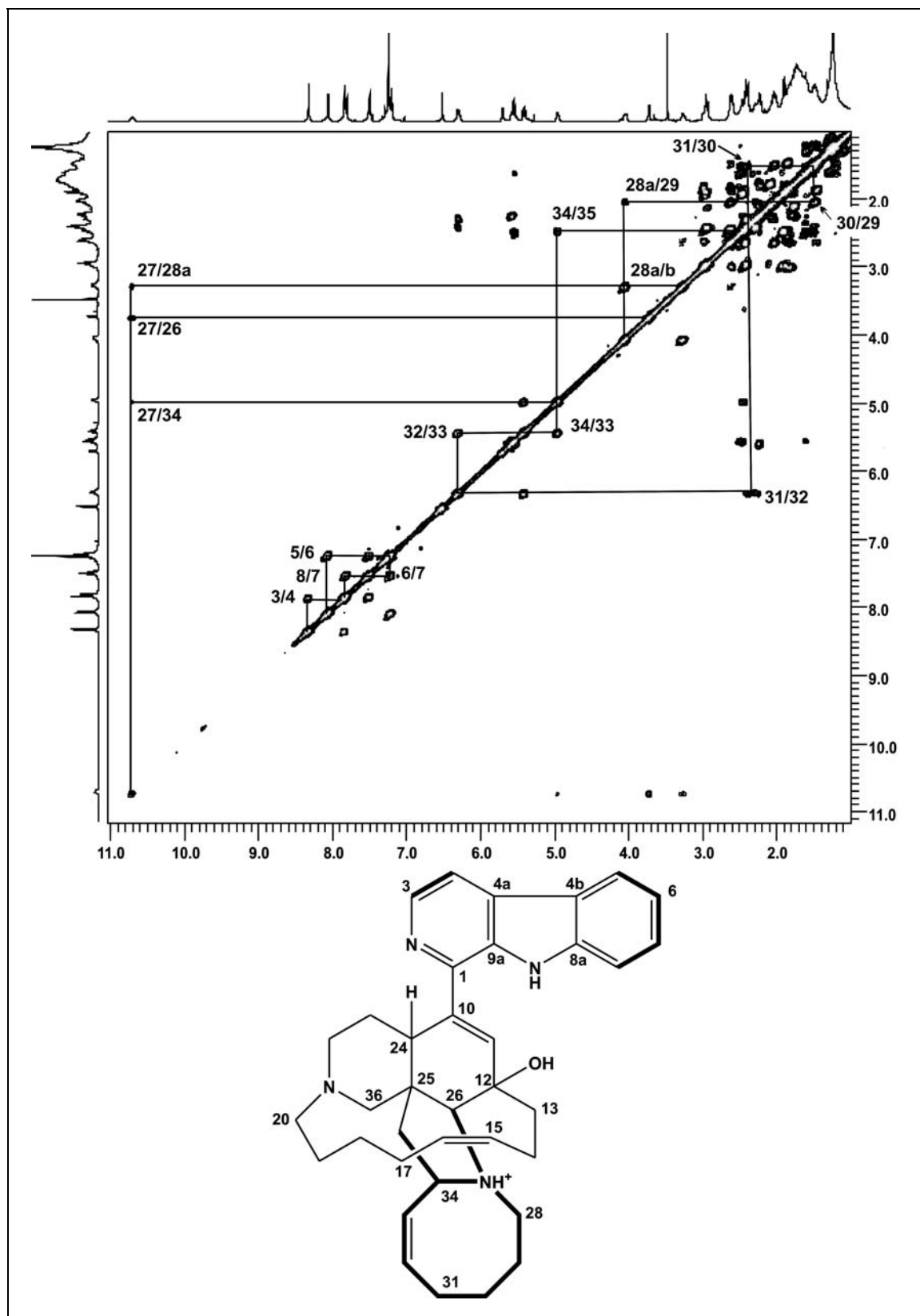
Figure 3.45. COSY spectrum of compound **10** showed proton connection from H3 to H-4, H-5 to H-6 and from H-26 to H-35.
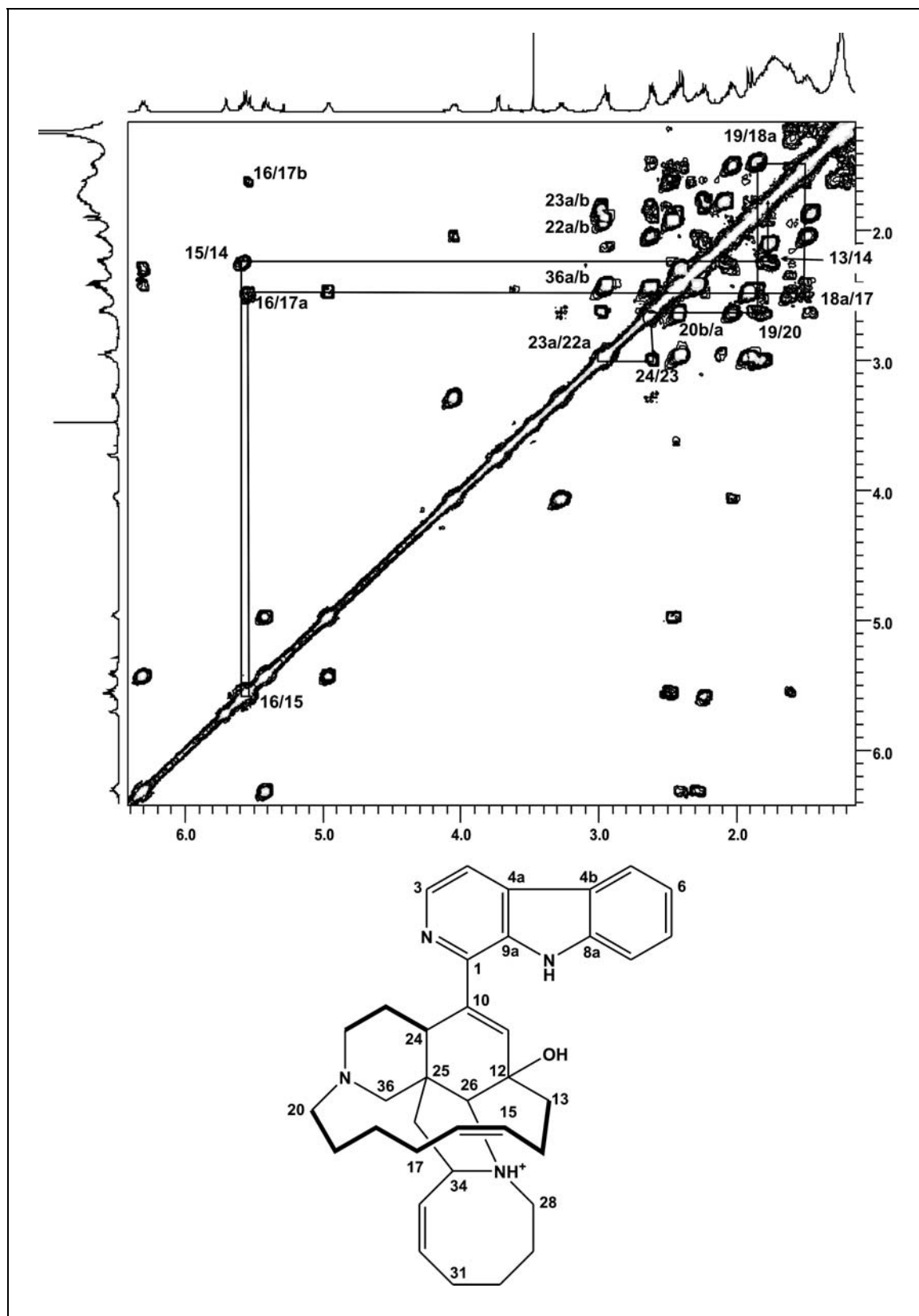
Figure 3.46. COSY correlation of compound **10** showed protons connection from H-13 to H-20 and H-22 to H-23.

3.3.2. Compound 11: 8-Hydroxymanzamine A
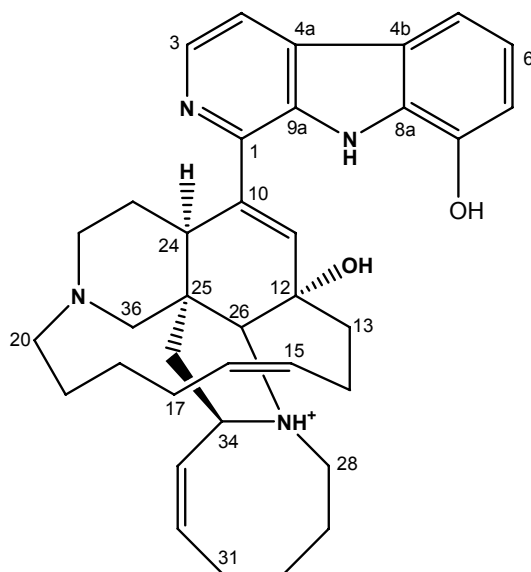
Compound **11** was isolated as a yellowish powder with UV absorption at λ_{max} 246, 286 and 358 nm. In TLC analysis, it gave a blue fluorescence under UV_{366nm} and was positive to Dragendorf reagent. This compound has a molecular weight of 564 g/mol, as derived from the ESI-MS spectrum that showed quasimolecular ion peak at m/z 565 [M+H]$^+$. This mass revealed a 16 mass difference from compound **10** (see page 119), that suggested an additional oxygen atom to the molecule relative to compound **10**. The ^{1}H NMR showed five aromatic protons at δ 8.79 (d, 1H, $J=$ 6.16 Hz, H-3), 8.27 (d, 1H, $J=$ 6.16 Hz, H-4), 7.72 (d, 1H, $J=$ 7.8 Hz, H-5), 7.34 (t, 1H, $J=$ 7.8 Hz, H-6), and 7.29 (d, 1H, $J=$ 7.9 Hz, H-7). There was one missing signal in this aromatic region particularly at the benzene part of ß-carboline moiety in comparison to compound **10**. There were an AB and an ABCD spin system present in compound **10** but only an AB and an ABC system were present in compound **11**. COSY data show the *ortho* correlation of H-3 to H-4, and also *ortho* correlations from H-5 to H-6 and H-6 to H-7. There was a remarkable shielding

effect of H-7 to upperfield when compared to the same proton position on compound **10** due to OH substitution at C-8. The rest of COSY data showed three spin systems with similar pattern compared to that of compound **10**. These facts suggested the substitution at position 8 with a hydroxyl group, which was elucidated as 8-hydroxymanzamine A. Compound **11** was established as 8-hydroxymanzamine A through comparison of its ^{1}H NMR data with those previously isolated from the marine sponge *Pachipellina* sp. (Ichiba *et al.*, 1994).

Table 3.10. Proton NMR data of compound **11**.

Nr.	[1]H (CDCl$_3$, 500 MHz) δ (ppm), multiplicity, *J* (Hz)	Nr.	[1]H (CDCl$_3$, 500 MHz) δ (ppm), multiplicity, *J* (Hz)
3	8.79 (d, 1H, *J*=6.16)	22	3.38 (t, 1H, *J*=10.4) 3.09 (m, 1H)
4	8.27 (d, 1H, *J*=6.16)	23	2.72 (m, 1H) 1.99 (m, 1H)
5	7.72 (d, 1H, *J*=7.8)	24	3.12 (m, 1H)
6	7.34 (t, 1H, *J*=7.8)	26	3.57 (s, 2H)
7	7.29 (d, 1H, *J*=7.9)	27	10.35 (s, 1H)
11	6.46 (s, 1H)	28	4.21 (t, 1H, *J*=9.7) 3.21 (q, 1H, *J*=9.4)
13	3.01 (m, 1H) 1.98 (m, 1H)	29	2.22 (m, 1H) 2.10 (m, 1H)
14	2.64 (m, 1H) 2.37 (m, 1H)	30	2.03 (m, 1H) 1.53 (m, 1H)
15	5.38 (t, 1H, *J*=10.2)	31	2.42 (m, 2H)
16	5.56 (t, 1H, *J*=8.7)	32	6.52 (dt, 1H, *J*=7.8, 9.9)
17	2.79 (m, 1H) 2.10 (m, 1H)	33	5.57 (t, 1H, *J*=9.4)
18	1.73 (m, 1H) 1.56 (m, 1H)	34	4.99 (brs, 1H)
19	2.02 (m, 1H) 1.79 (m, 1H)	35	3.03 (m, 1H) 2.49 (m, 1H)
20	2.43 (m, 1H) 2.24 (m, 1H)	36	3.68 (m, 1H) 3.00 (m, 1H)
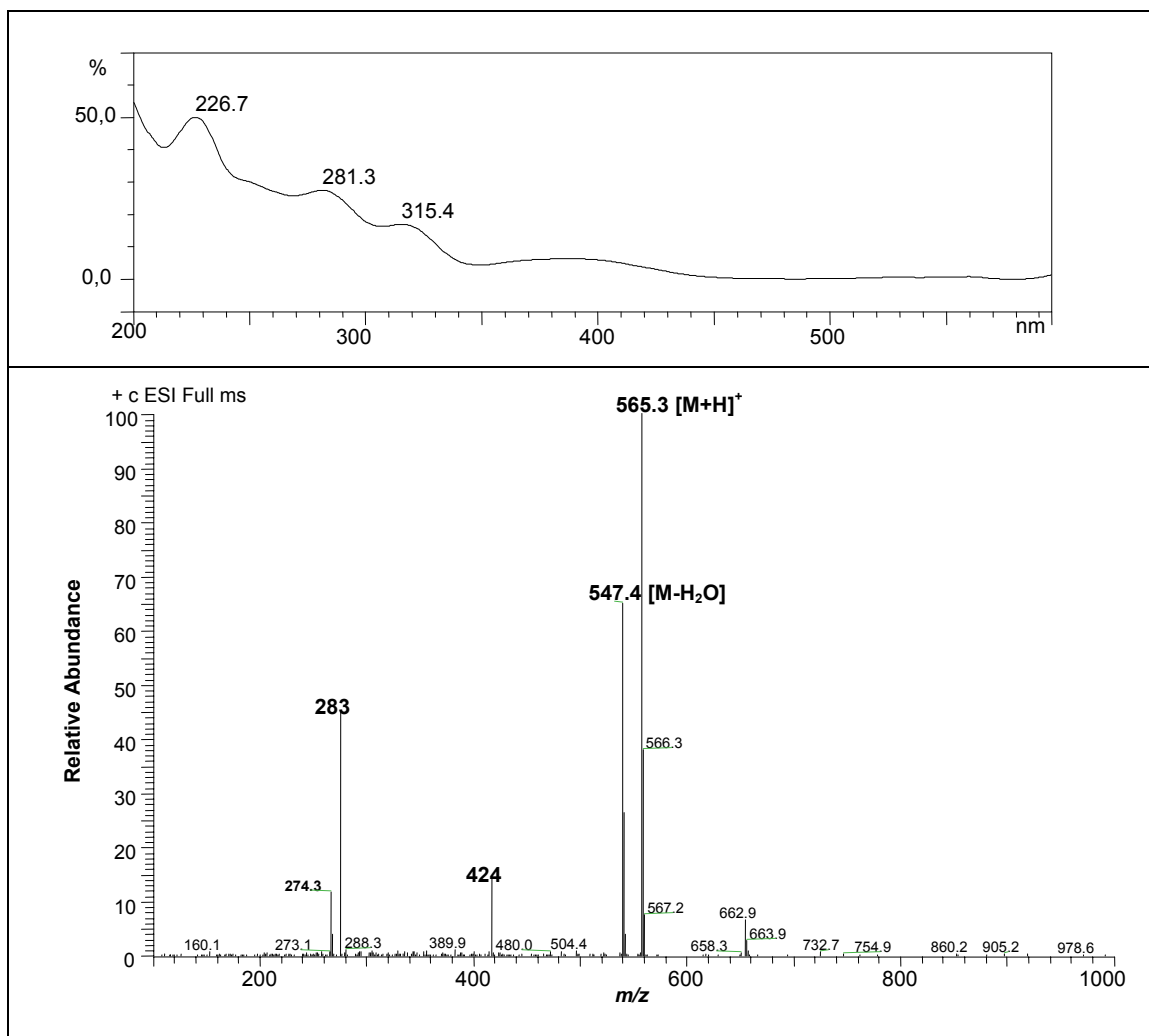
Figure 3.47. UV and ESI-MS spectra of compound **11**.
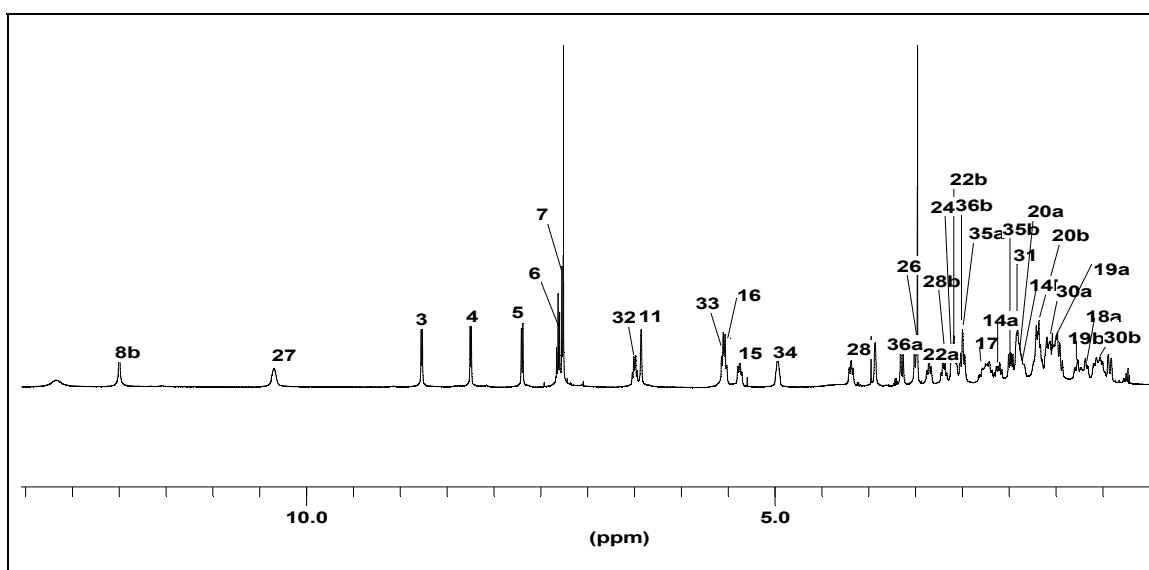
Figure 3.48. Proton NMR spectrum of compound **11**.

3.3.3. Compound 12: Manzamine F
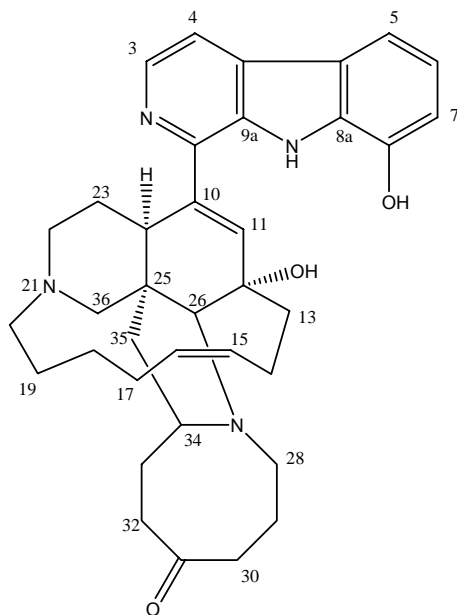
In ESI-MS compound **12** presented a quasimolecular ion peak at 581 [M+H]$^+$ in

positive mode and 579 [M-H]$^-$ in negative mode. It suggested a molecular

weight of 580 that is compatible with $C_{36}H_{44}N_4O_3$. This compound has UV λ_{max}

at 226, 281, and 316 nm. The downfield region showed five aromatic protons

which belong to the 8-OH-ß-carboline moiety at δ 8.48 (d, 1H, *J*= 5.7 Hz, H-3),

8.41 (d, 1H, *J*= 5.7 Hz, H-4), 7.83 (d, 1H, *J*= 7.9 Hz, H-5), 7.27 (t, 1H, *J*= 7.9 Hz,

H-6), and 7.14 (d, 1H, *J*= 7.6 Hz, H-7) that was comparable to compound **11**. In

the olefinic region, there are only one singlet at δ 6.66 (s, 1H, H-11) and two

overlapping signals for H-15 and H-16 at δ 5.69 (m, 2H). There were two

missing signals at δ 6.52 and 5.57 ppm when compared to compound **11**.

COSY showed six spin systems H-3 to H-4, H-5 to H-7, H-13 to H-20, H-22 to

H-24, H-28 to H-30, and H-32 to H-35. It showed an additional spin system

relative to compound **11** since the spin system from H-28 to H-34, present in

compound **11** was split into two spin systems by the carboxyl substitution at C-

31. These facts indicated that compound **12** is manzamine F. Manzamine F and 8-hydroxymanzamine A has 16 mass difference due to the missing double bond at position C-32 and additional carbonyl moiety at C-31. Compound **12** was established as manzamine F through comparison of their NMR data to those in literature (Ichiba *et al.*, 1994).

Table 3.11. The ^{1}H NMR data of compound **12**.

H no.	^{1}H (MeOD): δ (ppm), multiplicity, *J* (Hz)	H no.	^{1}H (MeOD): δ (ppm), multiplicity, *J* (Hz)
3	8.48 d, 1H, 5.7 Hz	20	3.32 m, 3.35 m
4	8.41 d, 1H, 5.7 Hz	22	3.66 m, 3.98 m
5	7.83 d, 1H, 7.9 Hz	23	2.42 m, 2.71 m
6	7.27 t, 1H, 7.9 Hz	24	3.32 m
7	7.14 d, 1H, 7.6 Hz	26	3.34 s
11	6.66 s, 1H	28	3.86 m, 4.51 m
13	2.32 m, 2.41 m	29	1.89 m, 2.31 m
14	2.32 m, 2.60 m	30	2.85 m, 3.17 m
15	5.72 m, 1H	32	2.39 m, 2.51 m
16	5.69 m, 1H	33	2.17 m, 2.41 m
17	2.13 m, 2.49 m	34	4.47 m
18	1.49 m, 1.91 m	35	2.23 m, 2.92 m
19	1.86 m, 1.94 m	36	2.51 m, 2.82 m
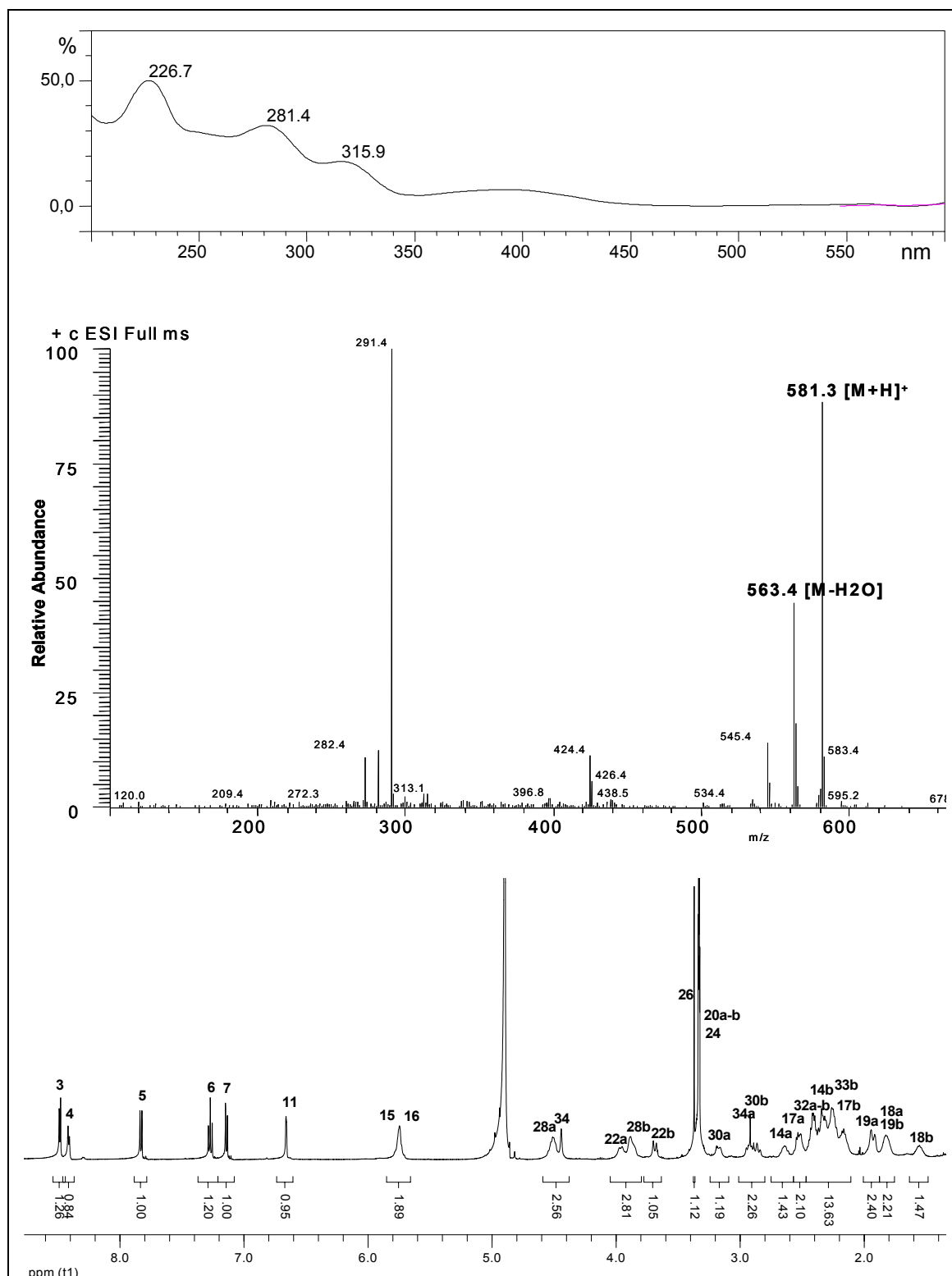
Figure 3.49. UV spectrum, ESI-MS and ^{1}H NMR of compound **12**.
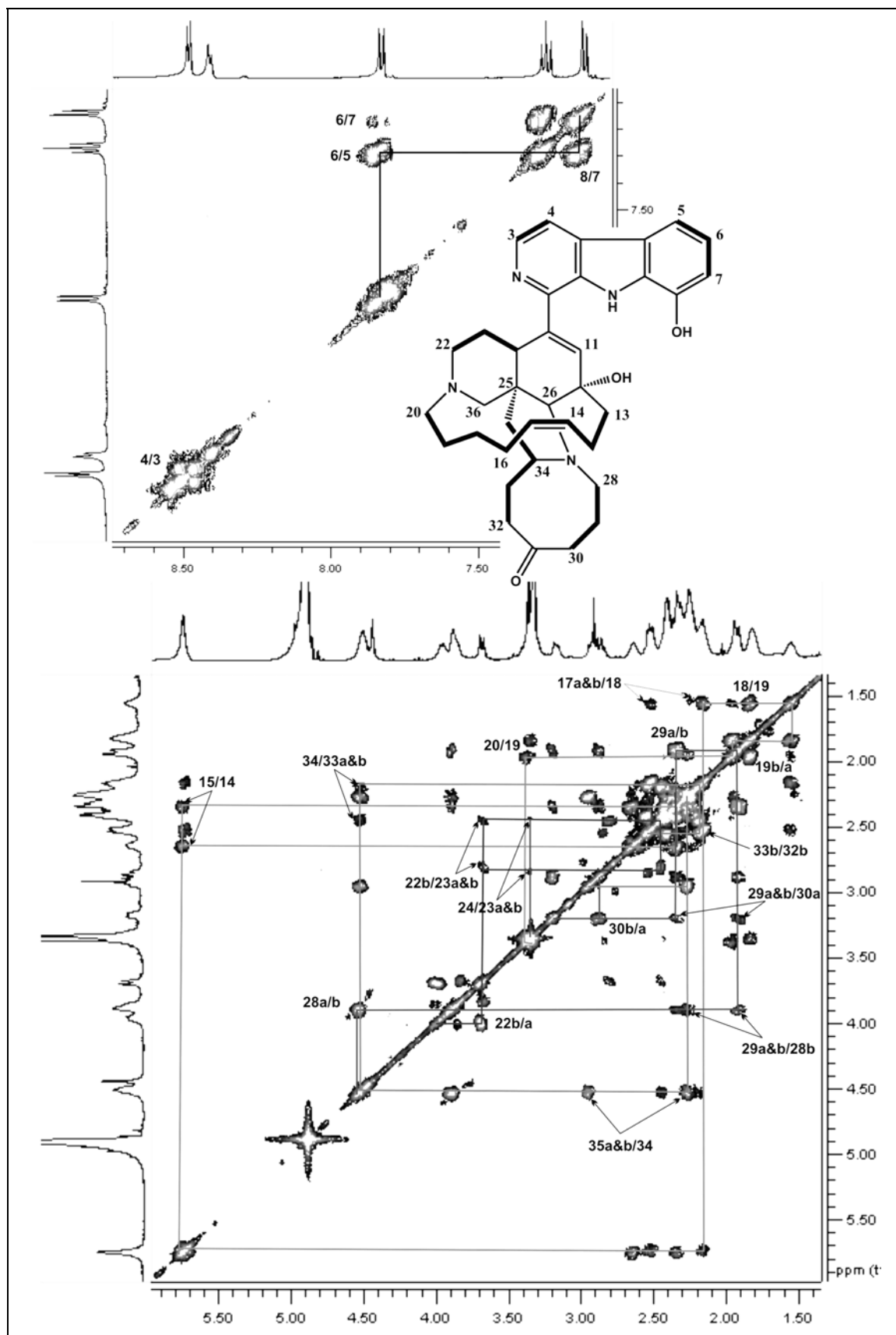
Figure 3.50. COSY spectrum of compound **12**.

3.3.4. Compound 13: 1-Dehydroxyircinol A (New)
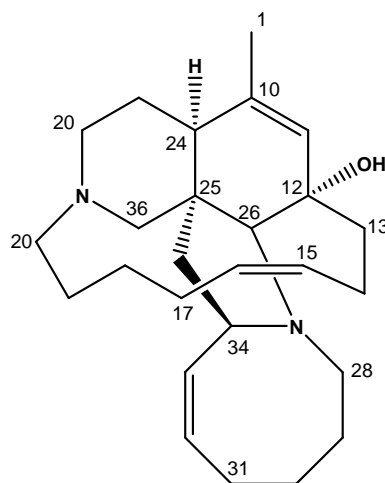
Compound **13** showed a yellow spot after spraying with Dragendorf reagent on TLC. UV spectra of this compound showed λ_{max} 240, 282 and 358 nm. Its positive molecular ion at *m/z* 397 [M+H]$^+$ in ESI-MS, suggested the molecular weight of 396 Da. This molecular weight is compatible with $C_{26}H_{40}N_2O$. Proton NMR did not show any characteristic signal for a ß-carboline structure but the rest of the protons in the olefinic and aliphatic region exhibited a similar pattern evident to the lower part of manzamine A. The olefinic region presented 5 protons at δ 6.54 (1H, s, H-11), 5.55 (1H, m, H-15), 5.52 (1H, m, H-16), 6.29 (1H, m, H-32), and (1H, m, H-33). COSY spectrum again showed three spin systems with similar pattern to that of manzamine A. Previously isolated compounds that show similar substructure were ircinol A and ircinal A (Kondo *et al.*, 1992; Tsuda *et al.*, 1994). Compound **13** differed from Ircinal A with 14 mass units (mass 410) while a 16 mass units difference was observed to ircinol A (mass 412). It suggested that compound **13** differed by one oxygen atom relative to ircinol A. ^{1}H NMR data of compound **13** were compared to ircinal A as reference. In comparison to ircinal A, there was a missing signal at δ 9.45

(1H, s, H-1), which was substituted by an additional signal at δ 1.29 (3H, s, H-1). Replacement of aldehyde group by a methyl on C-10 drew the chemical shift of H-11 and H-24 upperfield. Compound **13** was established as 1-deoxyircinol A through its molecular weight, ^{1}H NMR data and inspection of COSY spectrum, with comparison to ircinal A data as a reference compound. Compound **13** is a new derivate of ircinal A.

Table 3.12. NMR data of compound **13** compared to the ircinal A as a reference compound (Kondo *et al.*, 1992).

Nr.	Ircinal A ^{1}H-NMR (lit.)	Compound 13 δ [1]H (ppm), multiplicity
1	9.45 (1H, s)	1.29 (3H, s)
11	6.75 (1H, s)	5.95 (1H, s)
13	1.61 (1H, m), 1.75 (1H, m)	1.51 (1H, m), 1.64 (1H, m)
14	2.11 (1H, m), 2.24 (1H, m)	2.03 (1H, m), 2.17 (1H, m)
15	5.56 (1H, m)	5.32 (1H, m)
16	5.50 (1H, m)	5.43 (1H, m)
17	1.63 (1H, m), 2.43 (1H, m)	1.59 (1H, m), 2.28 (1H, m)
18	1.25 (1H, m), 1.41 (1H, m)	1.04 (1H, m), 1.20 (1H, m)
19	1.38 (1H, m), 1.71 (1H, m)	1.24 (1H, m), 1.59 (1H, m)
20	2.39 (1H, m), 2.57 (1H, m)	2.79 (1H, m), 3.18 (1H, m)
22	1.88 (1H, m), 2.93 (1H, m)	2.32 (1H, m), 2.95 (1H, m)
23	1.86 (1H, m), 2.77 (1H, m)	1.49 (1H, m), 1.59 (1H, m)
24	2.58 (1H, m)	1.93 (1H, m)
26	3.44 (1H, s)	3.09 (1H, s)
28	3.04 (1H, m), 3.38 (1H, m)	2.56 (1H, m), 3.33 (1H, m)
29	1.73 (1H, m), 1.93 (1H, m)	1.58 (1H, m), 1.73 (1H, m)
30	1.35 (1H, m), 1.89 (1H, m)	1.49 (2H, m)
31	2.14 (1H, m), 2.29 (1H, m)	2.09 (2H, m)
32	6.03 (1H, m)	5.66 (1H, m)
33	5.26 (1H, t)	5.63 (1H, m)

Nr.	Ircinal A ¹H-NMR (lit.)	Compound 13 δ ¹H (ppm), multiplicity
34	4.36 (1H, m)	4.66 (1H, m)
35	1.67 (1H, m), 1.86 (1H, m)	1.55 (2H, m)
36	2.29 (m), 2.81 (m)	2.19 (1H, m), 2.69 (1H, m)
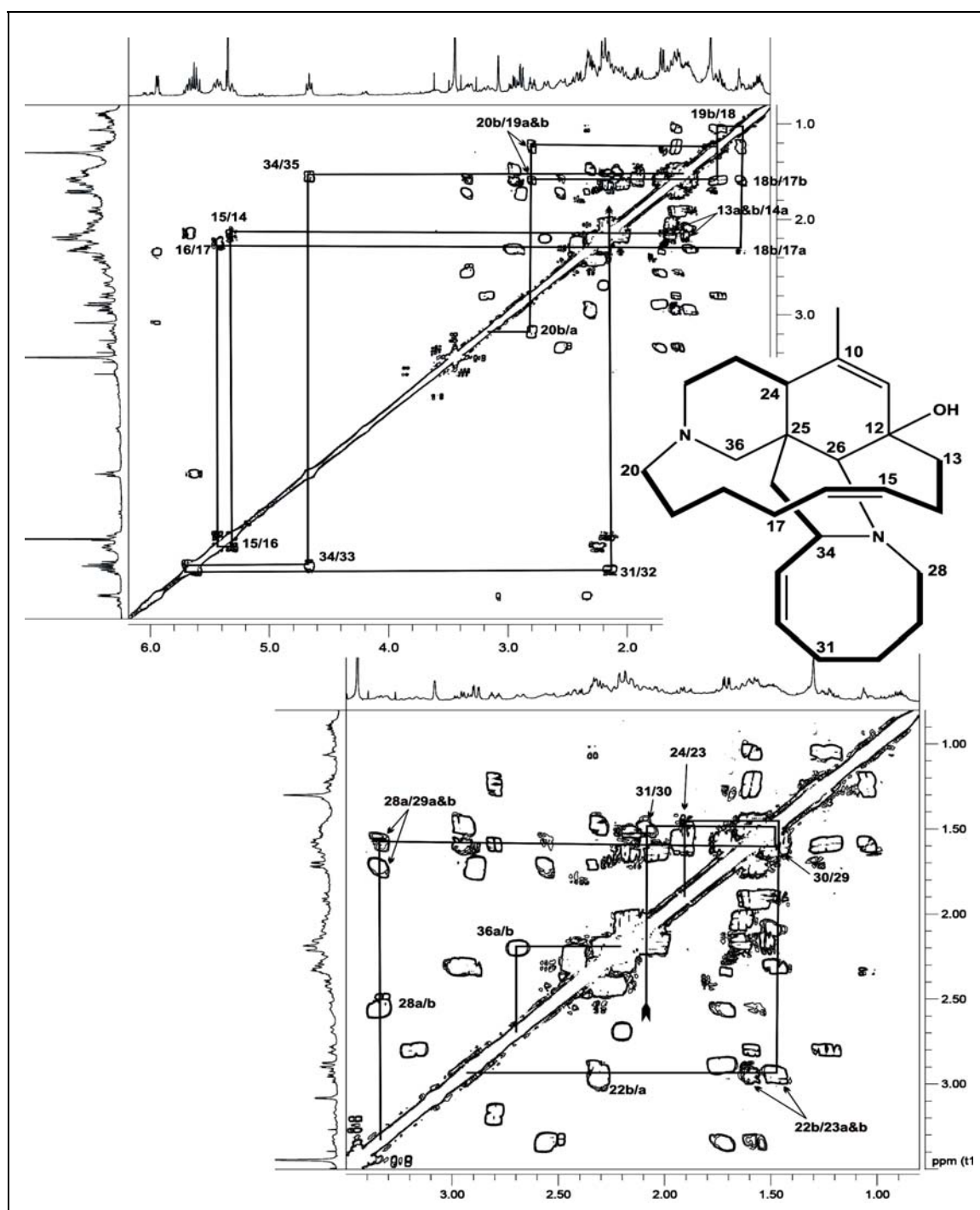
Figure 3.51. COSY schema of compound **13**.

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3.4. Secondary metabolites of sponge *Petrosia(Petrosia) hoeksemai*

Like other member of family Petrosiidae (eg. *Xestospongia* sp. and *Petrosia* sp.), the sponge *Petrosia(Petrosia) hoeksemai* was also reported to contain manzamine alkaloids. Two secondary metabolites were isolated from sponge *Petrosia(Petrosia) hoeksemai* collected from Menjangan Island, Bali-Indonesia. They were manzamine A and xestomanzamine A. The first isolated compound was assigned as manzamine A through direct comparison of its ^{1}H NMR data to those of compound **10** (see page 119).

3.4.1. Compound 14: Xestomanzamine A
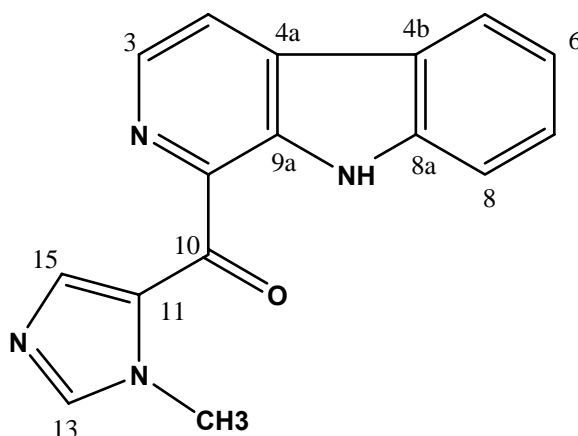
Compound **14** was isolated as yellowish powder that gave a positive reaction to Dragendorf reagent. ESI-MS of compound **14** presented a quasimolecular ion peak at *m/z* 277 [M+H]$^+$. It suggested the molecular weight 276 that was compatible with $C_{16}H_{12}N_4O$. This compound has λ_{max} at 220, 300, and 404 nm. The UV absorption maximum at 404 nm of compound **14** sugested that an additional chromophore is conjugated to the ß-carboline moiety as judged from the λ_{max} at 348 nm of manzamine A. The down field region of H^1-NMR showed

seven aromatic protons which belong to the ß-carboline moiety at δ 12.03 (s, 1H, 9-NH), 8.57 (d, 1H, *J*= 4.8 Hz, H-3), 8.47 (d, 1H, *J*= 4.8 Hz, H-4), 8.33 (d, 1H, *J*= 7.9 Hz, H-5), 7.83 (d, 1H, *J*= 7.9 Hz, H-8), 7.61 (dd, 1H, *J*= 7.4 and 8.2 Hz, H-7) and 7.32 (dd, 1H, *J*= 7.2 and 7.8 Hz, H-6) that showed two spin systems in the COSY spectrum. The upperfield region showed a NCH$_3$ signal at δ 4.09 (s, 3H). Compound **14** was established as xestomanzamine A through comparison of molecular weight, UV absorption spectrum and NMR data to those of the previously isolated compound (Kobayashi *et al.*, 1995).
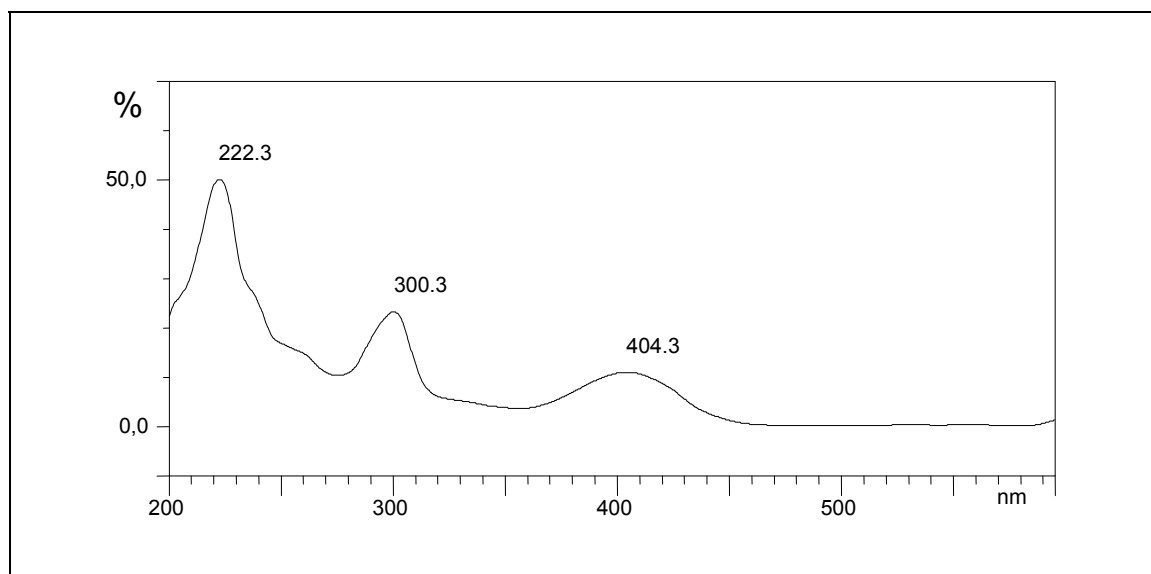
Figure 3.52. UV spectrum of compound **14**.
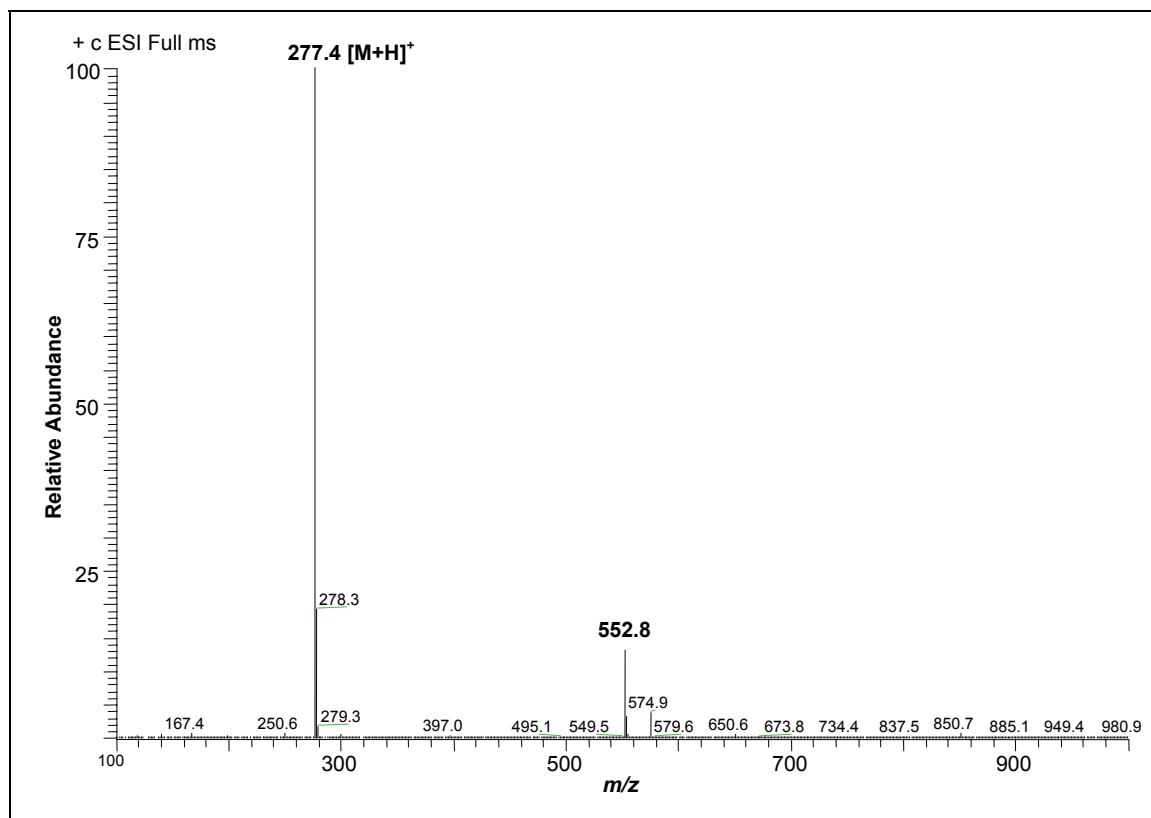
Figure 3.53. ESI-MS spectrum of compound **14**.
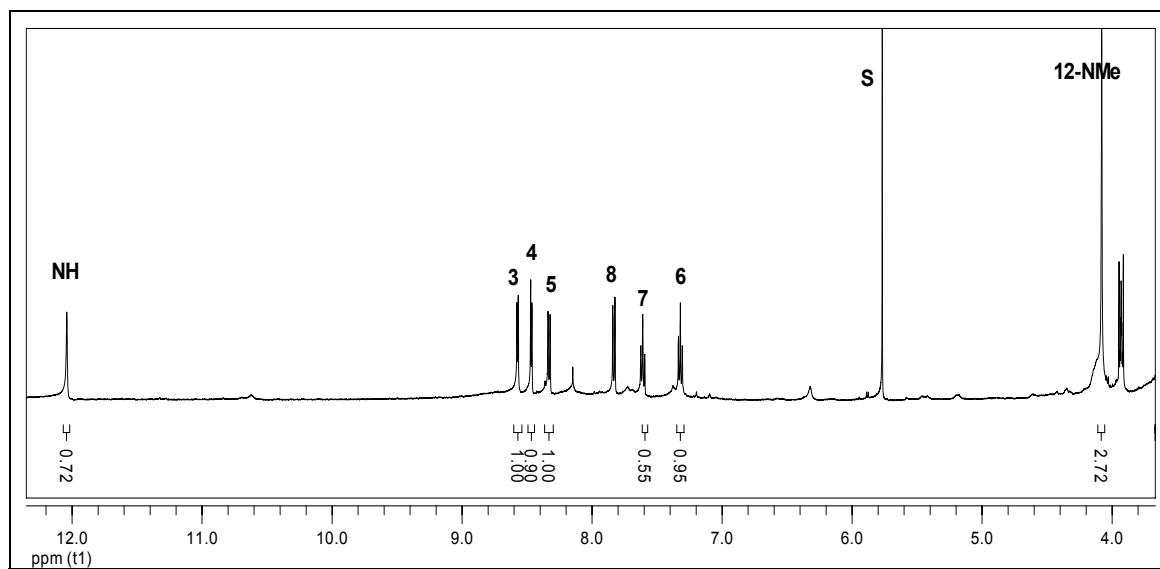
Figure 3.54. Proton NMR spectrum of compound **14**.

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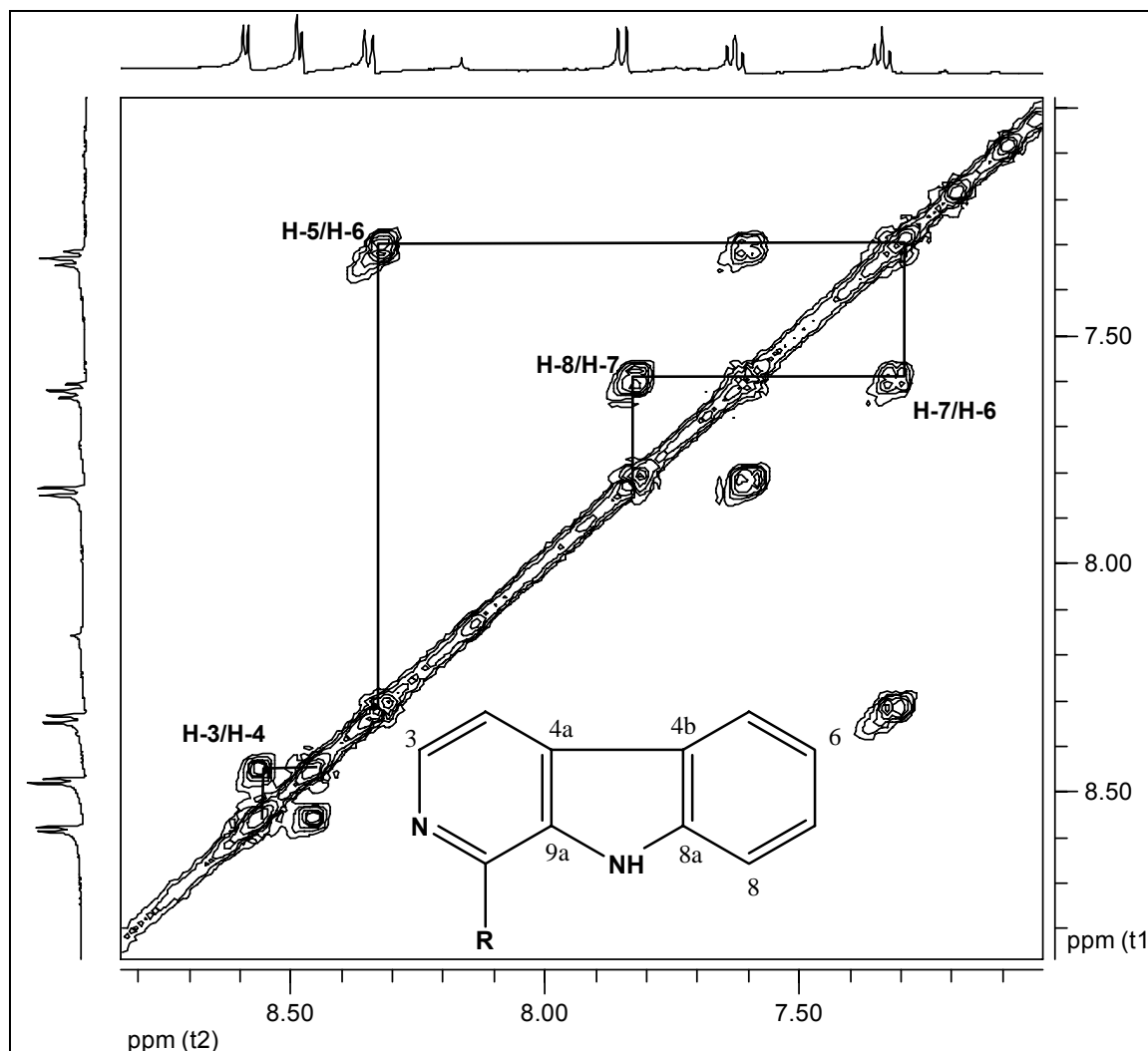
Figure 3.55. COSY spectrum of compound **14**.

3.5. Secondary metabolites of sponge *Axynissa aculeata*

In this study an indole-3-carboxaldehyde and curcuphenol were isolated from sponge the *Axynissa aculeata*. Previously isocyanate and thiocyanate terpenoids were reported from this sponge. It is the first time that an indole compound was found in this sponge.

9-isocyanopupukeanae　　**epi-9-isocyanopupukeanae**　　**9-isothiocyanatopupukeanae**
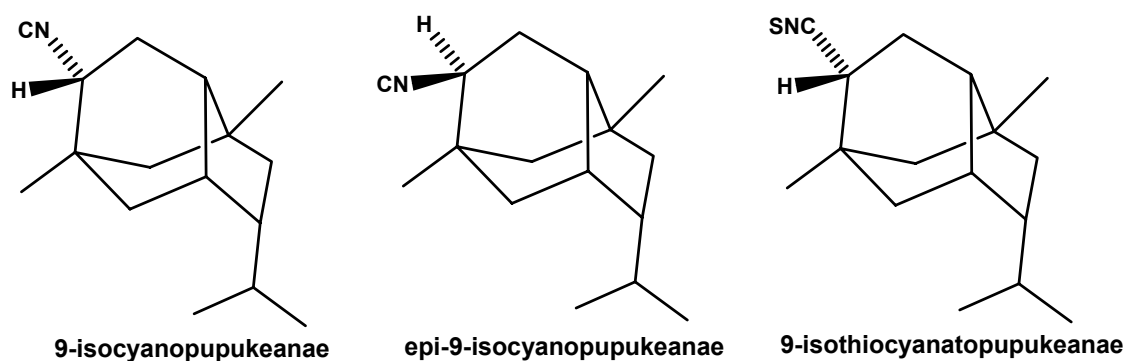
Figure 3.56. Selected compounds occurring in the *Axynissa aculeata*.

3.5.1. Compound 15: Indole-3-carboxaldehyde
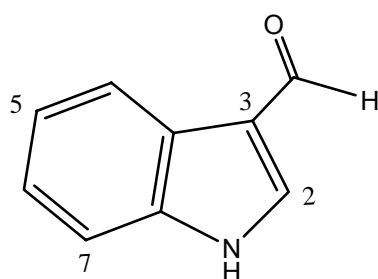
Compound **15** was found as a white amorphous powder with UV absorbance at λ_{max} at 265 nm. The positive ESI-MS spectrum showed the molecular ion at 146 *m/z* [M+H]$^+$ and an ion fragment at 118 *m/z*. These data suggested a nitrogenous compound with molecular weight at 145 Da. The fragmentation

pattern revealed the presence of a CO function and the ion fragment at 118 suggested an indole backbone.

The ^{1}H NMR spectrum showed a characheristic resonance for an aldehyde proton at δ 9.86 (1H,s, CHO) and a cluster of aromatic peaks at δ 8.13 (1H, d, J=7.7, H-4), 7.45 (1H, d, J=8.1, H-7), 7.25 (1H, dt, J= 7.1, 1.2 Hz, H-6), 7.21 (1H, dt, J= 7.5, 1.2 Hz, H-5) which formed an ABCD spin system. In addition one aromatic singlet proton at δ 8.07 (1H, s) was assigned as H-2 for the pyrrole ring. Proton NMR spectra did not show any peak in the aliphatic region. Compound **15** was established through comparison of NMR data to previously isolated indol-3-carboxaldehyde was reported in the literature (Cardellina *et al.*, 1986).

Table 3.13. The ^{1}H NMR data of compound **15**.

Nr.	δ ^{1}H (ppm), multiplicity, *J* (Hz)
CHO	9.86 (1H,s)
2	8.07 (1H, s, H-2)
4	8.13 (1H, d, *J*=7.7, H-4)
5	7.21 (1H, dt, *J*= 7.5, 1.2, H-5)
6	7.25 (1H, dt, *J*= 7.1, 1.2, H-6)
7	7.45 (1H, d, *J*=8.1, H-7)
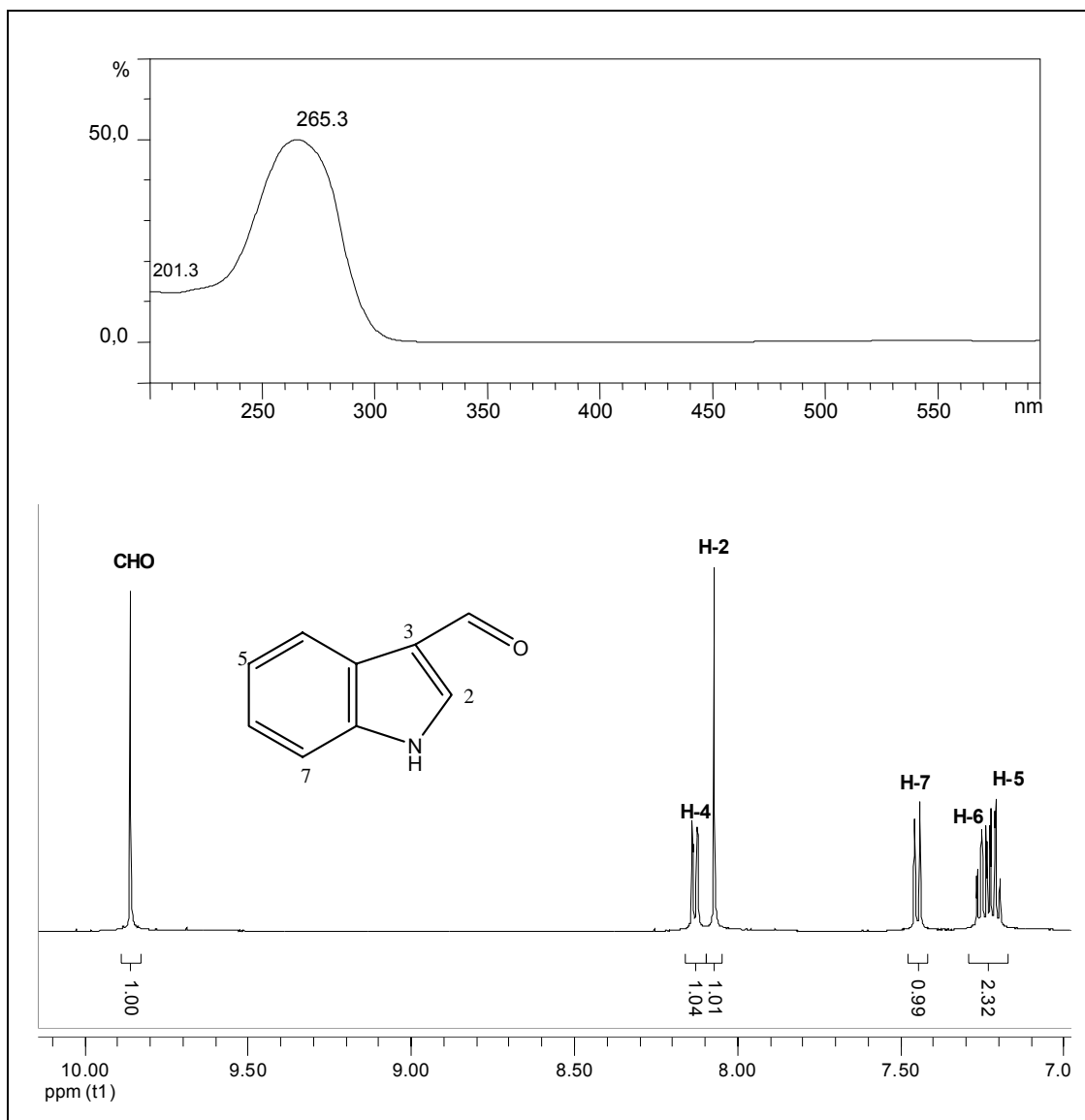
Figure 3.57. UV and Proton NMR spectra of compound **15**.

3.5.2. Compound 16: Curcuphenol
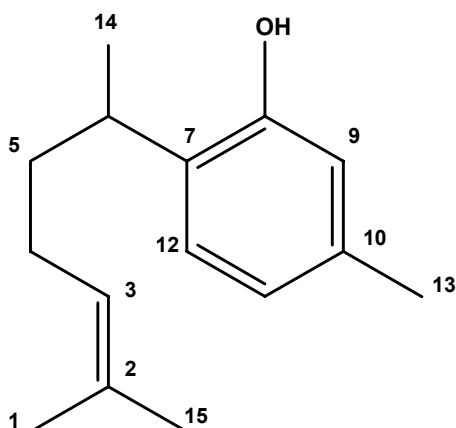
Compound **16** was found as colorless oily substance with UV absorbances at λ_{max} at 220 and 276 nm which were characteristic for a phenol functionality. The positive ESI-MS spectra showed the molecular ion at 219 *m/z* [M+H]$^+$ and suggested that compound **16** had a molecular weight of 218. The ^{1}H NMR spectra showed characheristic of 1,2,4-subtituted benzene signals at δ 7.03 (1H, d, *J*= 7.4, H-12), 6.72 (1H, d, *J*= 7.4, H-11), and 6.59 (1H, s, H-9). H-9 and 11 were shifted upfield due to the presence of an electron donating group at *ortho* position to H-9 and *para* to H-11. Two geminal methyls at δ 1.19 (3H, s) and 1.17 (3H, s), exchangeable assigned as CH$_3$-1 and CH$_3$-15, while the vinyl proton at δ 5.30 (1H, br) was assigned as H-3. An aromatic methyl group appeared at δ 2.26 (3H, s, H-13) and a benzyl substituted secondary methyl group was detected at δ 1.25 (3H, d, *J*= 10 Hz), which coupled to a methine group at δ 3.07 (1H, m, H-6). Compound **16** was established as curcuphenol through comparison of its ^{1}H NMR data with those previously reported in the literature (Mcenroe and Fenical, 1978).

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Table 3.14. NMR data of compound **16**.

Nr.	δ ^{1}H (ppm), multiplicity, *J* (Hz)
1 and 15	1.19 (3H, s) and 1.17 (3H, s)
3	5.30 (1H, br)
4	1.64 (2H, m)
5	1.40 – 1.50 (2H, m)
6	3.07 (1H, m)
9	6.59 (1H, s)
11	6.72 (1H, d, *J*= 7.4)
12	7.03 (1H, d, *J*= 7.4)
13	δ 2.26 (3H, s)
14	1.25 (3H, d, *J*= 6.9 Hz)
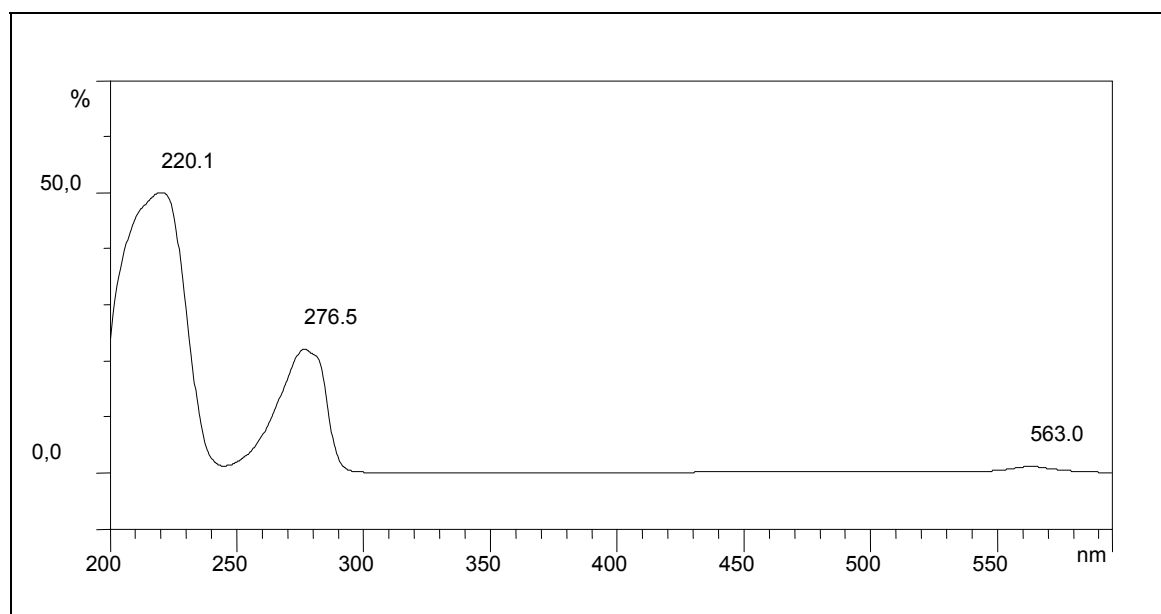
Figure 3.58. UV spectrum of compound **16**.
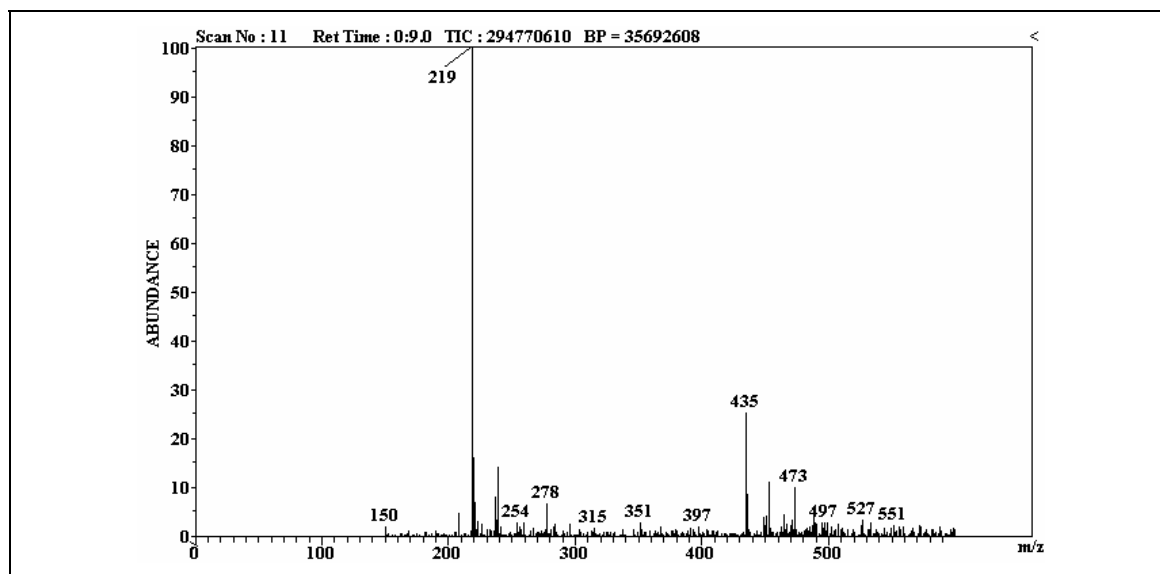
Figure 3.59. ESI-MS spectra of compound **16**.
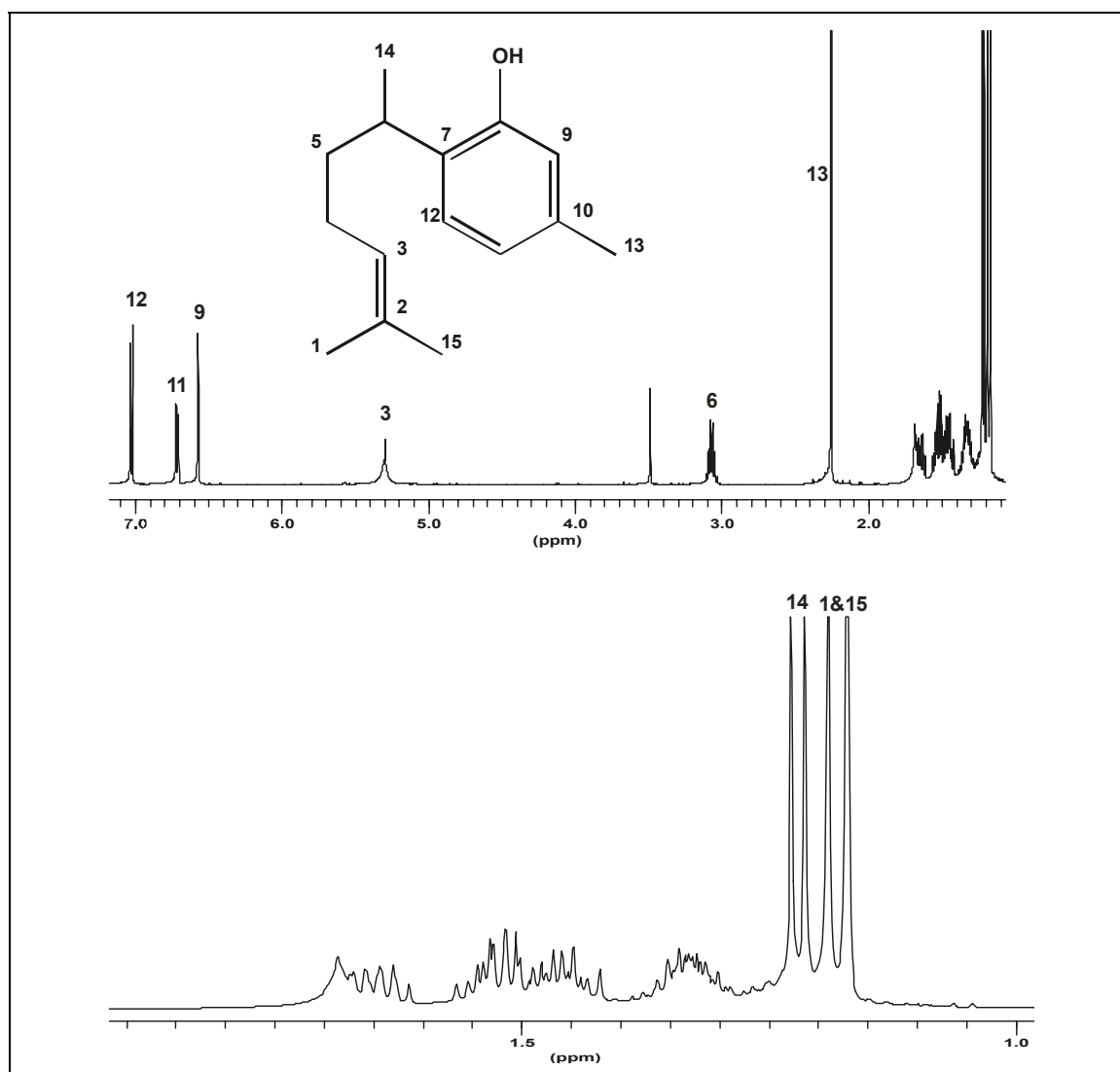
Figure 3.60. Proton NMR spectra of compound **16.**

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4. DISCUSSION

The Indonesian archipelago attracts high attention due to its abundance of marine life biodiversity. It is believed that there are over 4,000 sponges living in this archipelago.

4.1. Secondary metabolites from sponge *Agelas nakamurai*

Bromopyrrole derivatives are often found in marine sponges of the genera *Axinella*, *Agelas*, *Hymeniacidon*, *Stylotella*, and *Pseudoceratina*, and show a variety of biological activities including tyrosine kinase inhibition, and antifouling activity (Tsukamoto *et al.*, 2001).

Several bromopyrrole alkaloids found in marine sponges have been shown to exhibit pharmacologically useful activities which include c-erbB-2 kinase and cyclin-dependent kinase 4 inhibition, R-adrenoceptor blockers, serotonergic receptor antagonists, and antihistamine and actomyosin ATP-ase activators, etc. (Jiang *et al.*, 2002). Bromopyrrole alkaloids are well-known metabolites of the genus *Agelas* and are proven to play an important role in the chemical defense of the sponge against predation from fishes. This group of metabolites is also known to posses antibacterial and antifungal activity. A bromo substituent at C-4 and an amide, carboxyl or a cyano group are required for antimicrobial activity (Jadulco, 2002). Another group of compounds found from *A. nakamurai* included agelasine I. Agelasine I is a member of a unique group of diterpenoids possessing a 9-methyladeninium subtituent. It was reported to have weak antifungal activity but was inactive as antibacterial (Fu *et al.*, 1998).

4.2. Secondary metabolites from the sponges *Acanthostrongylophora ingens* and *Petrosia hoeksemai*

Manzamines are a series of unique ß-carboline alkaloids with unique polycyclic systems. Related alkaloids have been previously isolated from eight genera of marine sponges: *Haliclona*, *Pellina*, *Xestospongia*, *Pachypellina*, *Petrosia*, *Cribochalina*, *Ircinia*, and *Amphimedon* (Peng *et al.*, 2003; Watanabe *et al.*, 1998; Yousaf *et al.*, 2004). Manzamine A was isolated from several species of *Xestospongia*, and attracted researcher interest due to its antimalarial activity. Recent progress was presented by Hill and Hamann, who succeded to isolate and cultivate a manzamine A producing-unknown *Micromonospora* bacteria from the Indonesian sponge *Xestospongia* (Proksch *et al.*, 2006).

Manzamines possess an intricate nitrogen-containing ring system at C-1 of the ß-carboline ring. They are substituted at C-1 by complex polycyclic isoquinoline subtituents, or in the case of manzamine C, by an azacycloundecenyl moiety linked to ß-carboline by a two-carbon chain. Therefore subtituents at C-1 of manzamines are classified into the following two types; one is represented by manzamines A and B possessing penta- and tetracyclic isoqunoline chromophores, respectively, while another group has an azacycloundecene ring such as manzamine C. These unusual ring systems have attracted great interest as one of the most challenging targets for total synthesis or for unprecedented biosynthetic pathways to be resolved (Tsuda and Kobayashi, 1997). Four isolated manzamine derivatives showed characteristic UV spectra

from the chromophoric ß-carboline backbone, which also exhibited high intensity of blue fluorescene under radiation of UV 366 nm.
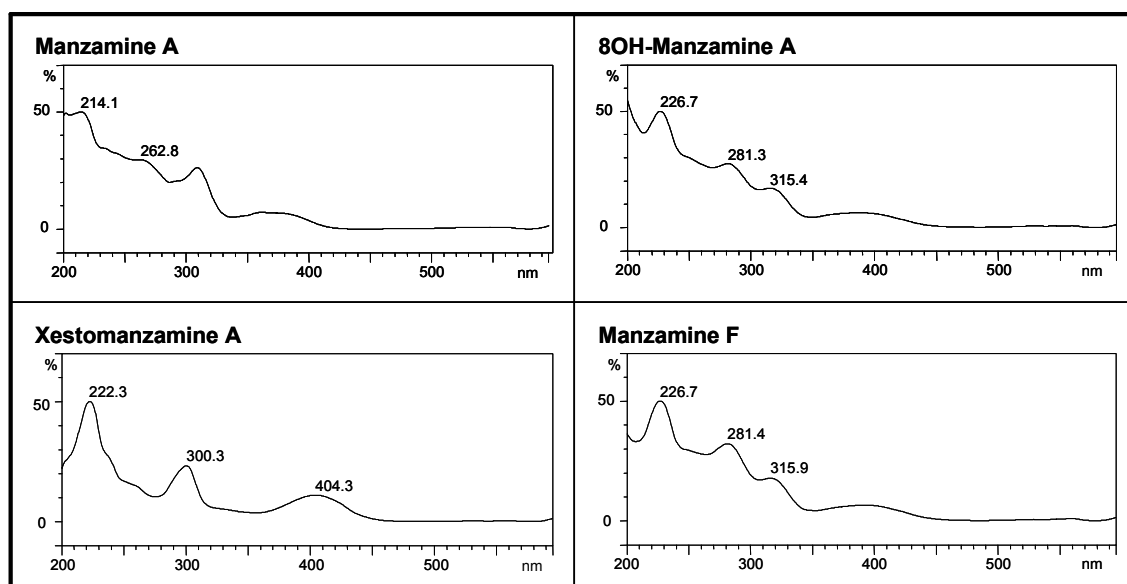
Figure 4.1. UV spectra comparison of isolated manzamine derivates.

4.2.1. Biosynthesis of Manzamines

It was proposed by Baldwin *et al.* (1992) that each structure could be reduced into four building blocks: ammonia, a C10 unit (a symmetrical dialdehyde), a C3 unit (an acrolein equivalent), and tryptophan, as shown in Figure 4.2 for manzamine B. Biosynthesis starts with reaction of the first three building blocks resulting in an tricycle iminium ion (7). Complex lower part of manzamine structure is biosynthesized via an intramolecular Diels-Alder reaction of the tricycle (7) wherein the diene component is a dihydropyridine and the dienophile is a conjugated iminium ion. Subsequently, disproportionation followed by hydrolysis of the intermediate iminium ion gives a tetracyclic aldehyde (ircinal B) which is finally transformed to the manzarnines via reaction with tryptophan. The key step in the proposal was the intramolecular endo Diels-Alder

cycloaddition of the bisdihydropyridine. So far it is not known whether a Diels-Alderase exists in the nature (Baldwin *et al.*, 1998).
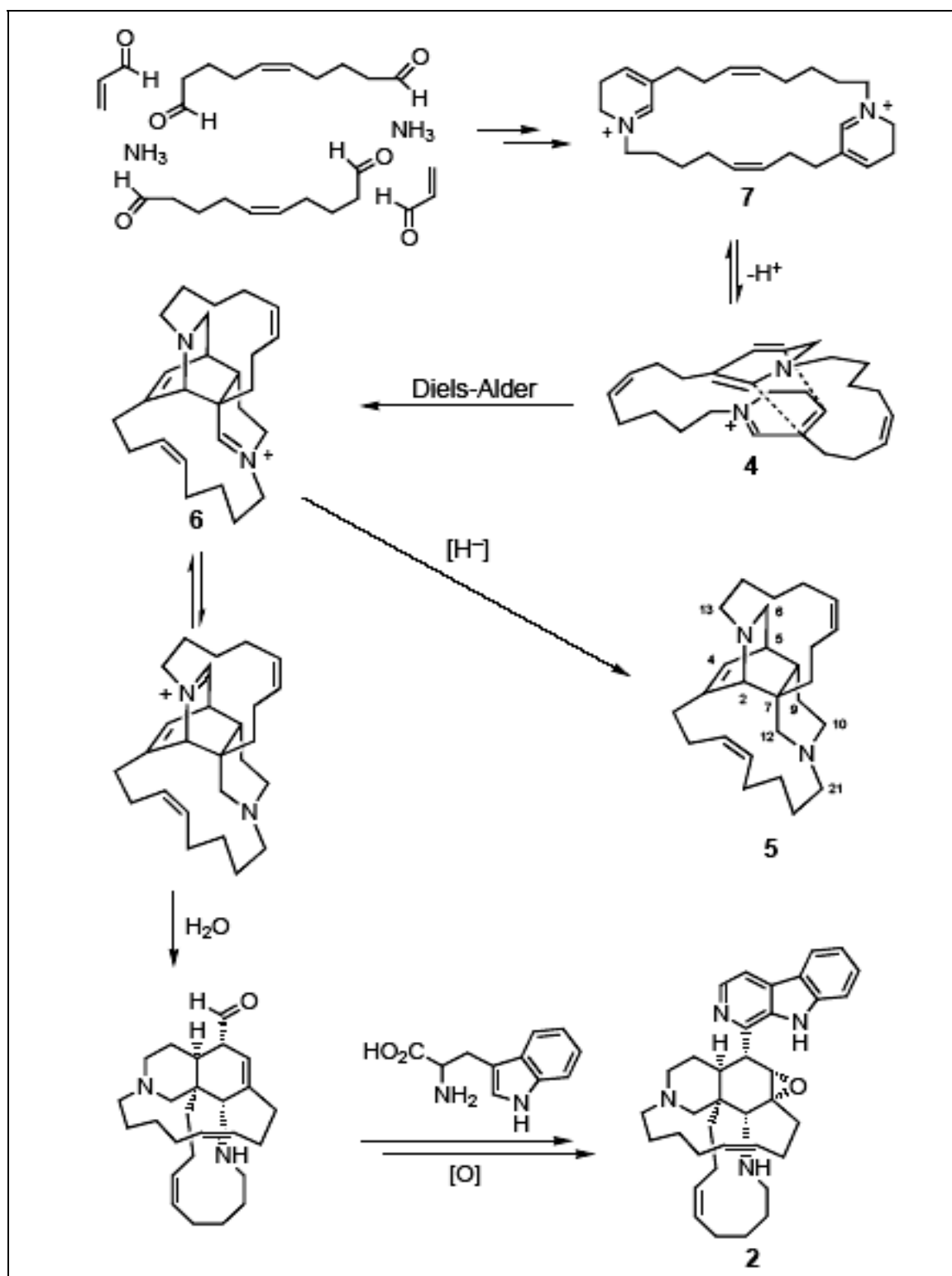
Figure 4.2. A hypothesis for the biosynthesis of manzamine B (Baldwin *et al.*, 1998; Baldwin and Whitehead, 1992).

4.2.2. Bioactivity of Manzamines

Manzamines were reported to posses antimicrobial, antiviral, and antimalarial activities. In vitro analysis of manzamine A and 8-hydroxymanzamine A against *Toxoplasma gondii* indicated significant activity. Manzamine A showed a 70% inhibition of the parasite at 0.1 μM concentration without toxicity to the host cells, while 8-hydroxymanzamine A showed 35% and manzamine F 37% inhibition without toxicity to the host cells at the same concentration (El Sayed *et al.*, 2001). It was also reported that 8-OH-manzamine A in a single intraperitoneal dose of 100 μmoles/kg effectively reduced parasitemia with an increase in the average survival days of *Plasmodium berghei* infected mice (9-12 days), as compared with: untreated controls mice (2-3 days), mice treated with artemisinin (2 days), and chloroquine (6 days). The increase in survival days in mice treated with manzamine appears to be attributed in part to an observed immunostimulatory effect (El Sayed *et al.*, 2001).

Table 4.1. Bioactivities comparison of Manzamine A and 8-Hydroxy-manzamine A (El Sayed *et al.*, 2001; Ichiba *et al.*, 1994; Sakai and Higa, 1986).

Assay	Manzamine A	8-OH-manzamine A
KB (IC_{50} μg/mL)	0.05	0.30
LoVo (IC_{50} μg/mL)	0.15	0.26
P388 (IC_{50} μg/mL)	0.07	Inactive
HSV-II (MIC_{50} μg/mL)	0.05	0.1
T. gondii (% inhibition of 10μM)	70%	35%

Bioactivity comparison of Manzamine A and 8-Hydroxymanzamine A
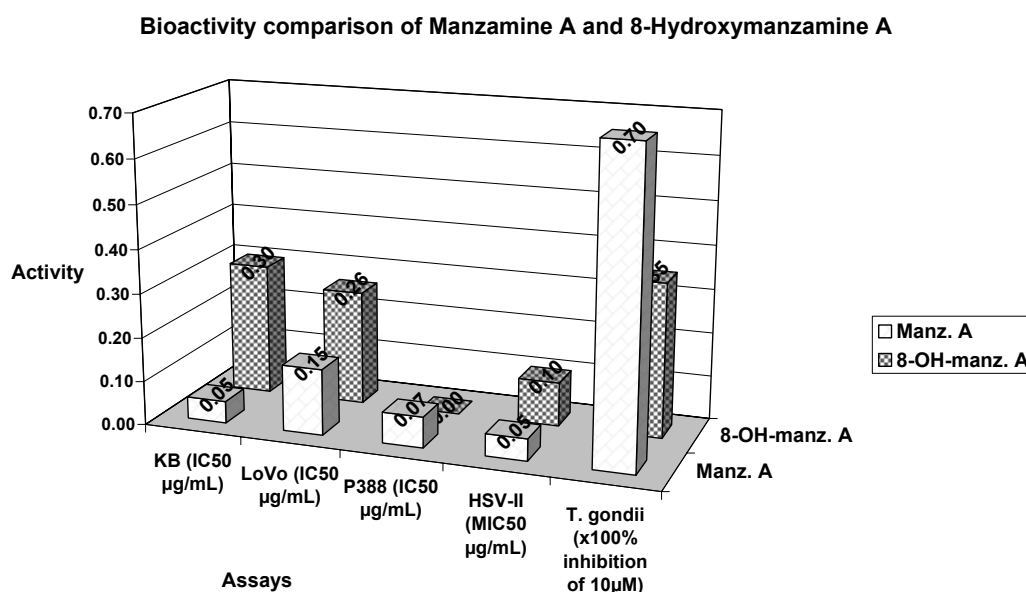
Figure 4.3. Bioactivities comparison histogram of Manzamine A and 8-Hydroxy-manzamine A (El Sayed *et al.*, 2001; Ichiba *et al.*, 1994; Sakai and Higa, 1986).

4.3. Secondary metabolites from sponge *Theonella swinhoei*

Numerous interesting compounds have been reported from the sponge *T. swinhoei* belonging to the Order Lithistida. Examples include swinholide A, a potent cytotoxic macrolide, cyclotheonamide A, a cyclic pentapeptide possessing potent inhibitory activity against thrombin, trypsin and plasmin, and other acyclic and cyclic polypeptides ranging from cyclic depsipeptide to large bicyclic peptides. Theonellapeptolides (Ia-e and IId), tridecapeptide lactones, have been reported from an Okinawan specimen of *T. swinhoei*. These peptides are characterized by the presence of high proportions of d-amino acids, N-methyl amino acids, and ß-amino acids. Biological activity of these compounds include cytotoxicity, ion-transport inhibitory activity for Na^+ and K^+ ions, and Na^+, K^+-ATPase inhibitory activity. Other theonellapeptolides (III series) exhibiting cytotoxicity have also been reported from a New Zealand

deep water sponge, *Lamellomorpha strongylata*, belonging to a different Order (Roy *et al.*, 2000).

Theonellapeptolide-Id (TNLP) is a cyclic tridecapeptide lactone, which has been isolated from the Okinawa marine sponge *Theonella swinhoei* with associated macrolides and peptolides. Their chemical structures contain unusual amino acids, ß-alanine, and allo-isoleucine, and most of the amide bonds are methylated. In TNLP-Id, the terminal N atom is capped by the methoxyacetyl group, and the C-terminus atom bonds through an ester linkage to the hydroxyl group of the threonine residue creating a cyclic structure (Doi *et al.*, 1999).

The TNLP family have high hydrophobicity due to the methylations on the amide bonds and the aliphatic side-chains, and the peptides are soluble in relatively lipophilic organic solvents such dichloromethane or chloroform. TNLPs have strong cytotoxicities, which are advantageous for survival from predation by other organisms. Furthermore, the notable functions, Na^+ and Ca^{2+} transport/binding activities and prevention of fertilization, have also been reported (Doi *et al.*, 2000; Kobayashi *et al.*, 1994).

Similar structural features are found in the cyclosporins, cyclic undecapeptides, some of which are widely used as immunosuppressants after organ transplant surgery. It was reported that theonellapeptolide IId showed stronger immunosuppressive activity than theonellapeptolide Ia and Id. It is interesting to note that small variation in the structures of these compounds significantly affect

their biological activity. Theonellapeptolides Ia and Ie have been reported to be cytotoxic (IC50 1.3±2.4 mg/mL) against L1210 tumor cells. The observed immunosuppressive activity of theonellapeptolides may largely originate from their cytotoxic effect (Roy *et al.*, 2000).

4.4. Secondary metabolites from sponge *Axynissa aculeata*

In this study an indole-3-carboxaldehyde and curcuphenol were isolated from sponge *Axynissa aculeata*. This sponge was reported to contain isocyanate and thiocyanate terpenoids. It is the first time that an indole compound was found from this sponge.

The sesquiterpenoid curcuphenol was reported previously as a metabolite of the gorgonian coral *Pseudopterogorgia rigida*, the terrestrial plant *Lasianthaea*
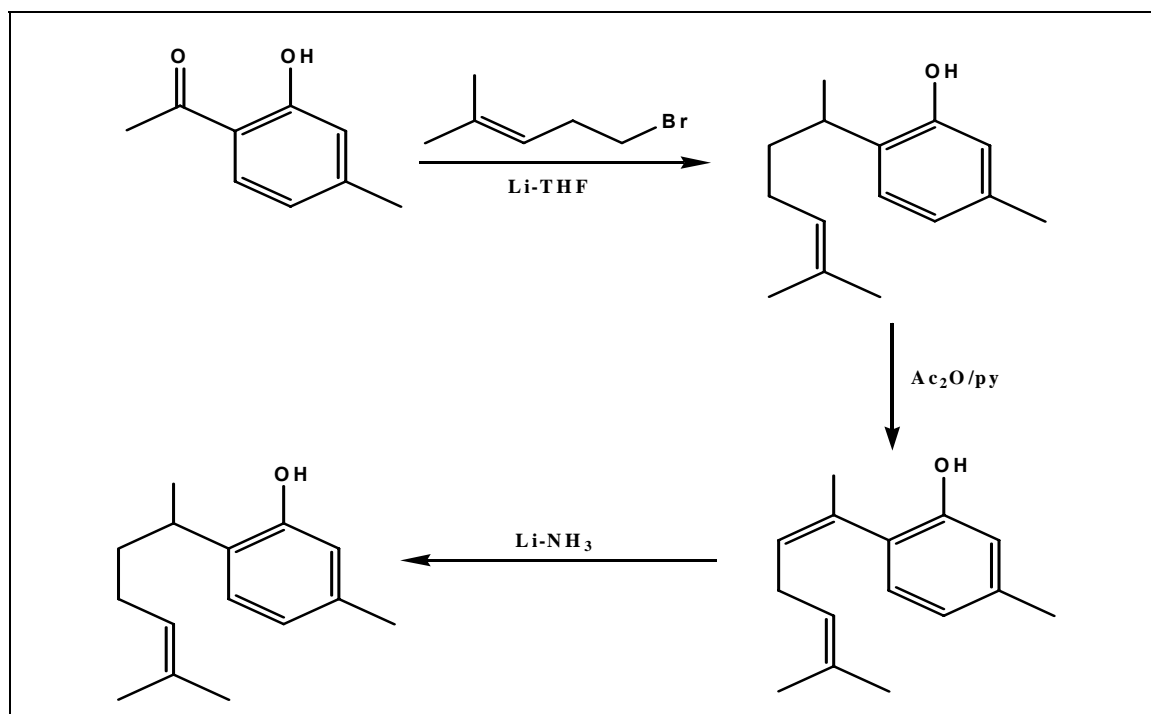
Figure 4.4. Schema of synthetic reaction of curcuphenol (Mcenroe and Fenical, 1978).

podocephala, and the marine sponge *Didiscus flavus*. It was reported as having antibacterial, antifungal, and cytotoxic activity. It inhibited gram positive bacteria *Staphylococcus aureus* with and the yeast *Candida albicans*. This compound was also reported to be successfully synthesized at high yield. The starting materials were 2-hydroxy-4-methyl acetophenone and 5-bromo-2-methyl-2-pentene, which were condensed in Li/NH$_3$/THF solution, and gave the benzyl alcohol. The tertiary alcohol dehydrated on base-catalyzed acetylation (Ac$_2$O/Py), yielding styrene acetate. The conjugated olefin and the ester functionality could then be reduced with lithium in ammonia to give 97% yield of curcuphenol (Mcenroe and Fenical, 1978; Wright *et al*., 1987).

5. SUMMARY

Natural products from marine sponges exhibit unique structural diversity which is of interest for the identification of new lead structures for drugs and agrochemicals. In the search for bioactive compounds from marine sponges, this study resulted in the isolation of sixteen compounds, two of which are new metabolites. The sponges were collected from Ujungpandang and Bali Sea Indonesia. They were extracted and isolated through a series of chromatographic techniques. A combination of analytical chromatography, mass spectrometry and NMR analysis was necessary to identify the isolated compounds.

1. *Agelas nakamurai*

The sponge *A. nakamurai* yielded five secondary metabolites, including four brominated compounds and one sesquiterpenoid, one of them is the new compound 4-(4-bromo-1H-pyrrole-2-carboxamido)-butanoic acid. Bromopyrrole alkaloids are well-known metabolites of the genus *Agelas* and are proven to play an important role in the chemical defense of the sponge against predation from fishes.

2. *Theonella swinhoei*

Four high molecular weight compounds were isolated from the sponge *T. swinhoei*, three theonellapeptolide derivates and one macrolide swinholide A. Theonellapeptolides are unique cyclic depsipeptides which contain uncommon amine-methylated amino acid residues.

3. *Acanthostrongylophora ingens*

This sponge afforded three manzamine type alkaloids and one new compound 1-dehydroircinol A. Manzamine derived alkaloids are known to be promising candidate for antimalarial and anti-HIV drug.

4. *Petrosia(Petrosia) hoeksemai*

The sponge yielded two manzamine type alkaloids: manzamine A and xestomanzamine A.

5. *Axynissa aculeata*

There were two compounds found in this sponge: indole-3-carboxaldehyde and the antibacterial sesquiterpenoid curcuphenol.

Table 5.1. Isolated Natural Products from Indonesian marine sponges

Sponge name	Compound	MW	Yield*	Known or new	Known activity
A. nakamurai	(**1**) Mukanadine C	230	11.3 mg (0.35%)	Known	Antibacterial
A. nakamurai	(**2**) 4-(4-Bromo-1H-pyrrole-2-carboxamido)-butanoic acid	274	7.2 mg (0.22%)	New	---
A. nakamurai	(**3**) 4-Bromopyrrole-2-carboxamide	189	58.9 mg (1.80%)	Known	Antibacterial
A. nakamurai	(**4**) 4-Bromopyrrole-2-carboxylic acid	188	2.1 mg (0.06%)	Known	Antibacterial
A. nakamurai	(**5**) Agelasine I	439	64.1 mg (1.96%)	Known	Antifungal
T. swinhoei	(**6**) Theonellapeptolide Id	1404	117 mg (4.25%)	Known	Cytotoxic
T. swinhoei	(**7**) Theonellapeptolide Ie	1418	59.6 mg (2.17%)	Known	Cytotoxic
T. swinhoei	(**8**) Theonellapeptolide IId	1390	26.2 mg (0.95%)	Known	Cytotoxic
T. swinhoei	(**9**) Swinholide A	1390	3.6 mg (0.13%)	Known	Cytotoxic
A. ingens	(**10**) Manzamine A	548	14.7 mg (0.63%)	Known	Anti malarial, antibacterial, antiparasitic

Sponge name	Compound	MW	Yield*	Known or new	Known activity
A. ingens	(**11**) 8-OH-manzamine A	564	20.3 mg (0.87%)	Known	Anti malarial, antibacterial, antiparasitic
A. ingens	(**12**) Manzamine F	580	2.0 mg (0.09%)	Known	Anti malarial, antibacterial, antiparasitic
A. ingens	(**13**) 1-Dehydroxyircinol A	396	1.6 mg (0.07%)	New	---
P. hoeksemai	(**14**) Xestomanzamine A	276	4.4 mg (0.26%)	Known	cytotoxic
A. aculeata	(**15**) Indole-3-carboxaldehyde	265	2.4 mg (0.007%)	Known	---
A. aculeata	(**16**) Curcuphenol	218	10.0 mg (0.029%)	Known	antifungal

* Yield calculated as percentage of isolated compound to crude extract.

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List of Abbreviations

$[\alpha]_D$: specific rotation at the sodium D-line

APCI : atmospheric pressure chemical ionization

BuOH : butanol

br : broad signal

CD : circular dichroism

CI : chemical ionization

COSY : correlation spectroscopy

δ : chemical shift

d : doublet

dd : double doublet

ddd : double of double doublet

DEPT : distortionless enhancement by polarization transfer

DMSO : dimethylsulfoxide

DNA : deoxyribonucleic acid

ED : effective dose

EI : electron impact

ESI : electron spray ionization

EtOAc : ethyl acetate

eV : electron volt

FAB : fast atom bombardement

Fig : Figure

g : gram

h : hour

HMBC : heteronuclear multiple bond connectivity

HMQC : heteronuclear multiple quantum coherence

HPLC : high performance liquid chromatography

Hz : hertz

IR : infrared spectroscopy

λ_{max} : maximum absorbance (UV)

LC : lethal concentration

lit.	: literature
m	: multiplet
MeOD	: deuterated methanol
MeOH	: methanol
mg	: milligram
mL	: milliliter
μg	: microgram
μL	: microliter
MS	: mass spectrometry
m/z	: mass per charge
n.a	: no activity
ng	: nanogram
nm	: nanometer
NMR	: nuclear magnetic resonance
NOE	: nuclear Overhauser effect
NOESY	: nuclear Overhauser and exchange spectroscopy
ppm	: parts per million
q	: quartet
RNA	: ribonucleic acid
ROESY	: rotating frame Overhauser effect spectroscopy
RP-18	: reversed phase C-18
s	: singlet
t	: triplet
TFA	: trifluoroacetic acid
THF	: tetrahidrofuran
TLC	: thin layer chromatography
TOCSY	: total correlation spectroscopy
UV	: ultraviolet

Curriculum vitae

Biodata:

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1996 – 1999	Apothecary speciality education, graduated as Apothecary (Apt.)
1999 – 2001	Master Degree (S2) on Gadjah Mada University Yogyakarta, graduated as Magister Sains (M.Si. =

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