# *Secondary Metabolites from Selected Marine Invertebrates and their Associated Microorganisms*

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**Doctor of Philosophy in Chemistry**
*to*
**Goa University**

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*by*

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T-566

*To*

*My parents*

# *Declaration*

*As required under the University Ordinance 0.19.8 (vi), I state that the present thesis entitled* ***"Secondary metabolites from selected marine invertebrates and their associated microorganisms"*** *is my original contribution and the same has not been submitted on any previous occasion. To the best of my knowledge the present study is the first comprehensive work of its kind from the area mentioned. The literature related to the problem investigated has been cited. Due acknowledgements have been provided wherever facilities and suggestions have been availed of.*

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# *Certificate*

*This is to certify that the thesis entitled* ***"Secondary metabolites from selected marine invertebrates and their associated microorganisms"****, submitted by* ***Ms. Divya Shridhar M.P.*** *for the award of the degree of* ***Doctor of Philosophy in Chemistry*** *is based on her original studies carried out by her under my supervision. The thesis or any part thereof has not been previously submitted for any other degree or diploma in any University or Institution.*


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*Divya Shridhar M.P.*

## General Remarks

The compound numbers, figures, references and table numbers given in each section refers to that particular section only.

Solvents were distilled prior to use.

Petroleum ether refers to that fraction of boiling range 60-80°C.

Thin Layer chromatography was performed on Merck aluminimum backed silica gel Kieselgel 60 $F_{254}$.

UV-Vis and IR spectra were recorded on Shimadzu 2401-PC and Shimadzu FTIR-8201PC spectrometer.

NMR were recorded on Bruker Avance 300 and 500 MHz Instrument.

HRESITOFMS were recorded on QSTARXL MS/MA Applied Biosystems Instrument.

## Abbreviations

| | |
|---|---|
| α | alpha |
| ACHN | Human kidney adenocarcinoma cell line |
| AcOH | Acetic acid |
| APL | Acute promyelocytic leukemia |
| ARA-C | Arabinofuranosyl Cytidine/cytarabine |
| ATCC | American type culture collection |
| β | beta |
| ben | bending |
| BOC | t-butyloxycarbamate |
| $Br_2$ | Bromine |
| br s | broad singlet |
| BuOH | Butanol |
| Calu-1 | Human pancreatic carcinoma, epithelial-like cell line |
| CDK | Cyclin-dependent kinase |
| $CHCl_3$ | Chloroform |
| CID | Collision Induced Dissociation |
| conc. | concentrated |
| COSY/$^1$H-$^1$H COSY | Correlation Spectroscopy/Proton Correlation Spectroscopy |
| d | doublet |
| δ | delta |
| $\delta_C$ | (delta) chemical shift or downfield shift in carbon $^{13}$ NMR |
| DCC | Dicyclohexylcarbodiimide |
| DCM | Dichloromethane |
| dd | double doublet |
| ddd | double of double doublet |
| DEPT | Distorsionless Enhancement by Polarisation Transfer |
| $\delta_H$ | (delta) chemical shift or downfield shift in proton NMR |
| DMAP | Di methyl amino pyridine |
| DNA | Deoxyribonucleic acid |
| DP | declustering potential |
| DPPH | 2,2-Diphenyl -1-picrylhydrazyl |
| *E* | *entgegen*German for opposite |
| EDTA-ATPase | Ethylene diamine tetra acetic acid-Adenosine triphosphate enzyme |
| EFG | Epidermal growth factor |
| ELISA | Enzyme-linked immunosorbent assay |
| eq | equivalent |
| ESI-MS | Electro Spray Ionization-Mass Spectrometry |
| ET743 | Ecteinascidin-743 |
| EtOAc/EA | Ethyl acetate |

| | |
|---|---|
| $Et_2O$ | diethyl ether |
| FABMS | Fast Atom Bombardment Mass Spectrometry |
| FAB MS/MS | Fast Atom Bombardment Tandem Mass Spectrometry |
| FDA | Food and Drug Administration |
| FTIR | Fourier Transform Infra Red Spectroscopy |
| γ | gamma |
| g | gram |
| GI-Phase | Gap 1 phase, is the first of four phases of the cell cycle that takes place in eukaryotic cell division |
| h | hour |
| H460 | human large-cell large carcinoma cell line |
| HIV | Human Immunodeficiency Virus |
| HMBC | Heteronuclear multiple Bond correlation spectroscopy |
| HMQC | Heteronuclear Multiple-Quantum Correlation spectroscopy |
| HPLC | High Performance Liquid Chromatography |
| hr | hour |
| HR(TOF)ESI-MS | High Resolution Time of Flight Electrospray Mass Spectrometry |
| HSQC | Heteronuclear single quantum coherence spectroscopy |
| Hz | Hertz |
| IC50 | Inhibitory concentration at 50% |
| J | coupling constant |
| Kg | Kilogram |
| KRN7000 | alpha-galactosylceramide |
| LAF389 | Cyclohexanecarboxylic acid 7-oxo-6(S)-[(2R,3R,4S,5R,6E)-3,4,5-trihydroxy-2-methoxy-8,8-dimethyl-6-nonenamido]perhydroazepin-3(R)-yl ester |
| LcK | lymphocyte-specific protein tyrosine kinase |
| LTB4 | Leukotriene B4 |
| m | multiplet |
| M | Molar |
| MDR1 gene | human multidrug resistance gene |
| MeI | Methyl Iodide |
| mg | milligram |
| μg/mL | microgram per milliLitre |
| Min | Minutes |
| MIC | Minimum Inhibitory Concentration |
| MIC50 | Minimum Inhibitory concentration at 50% |
| MIC90 | Minimum Inhibitory concentration at 90% |
| μL | microLitre |
| μM | micromolar |
| mM | milli molar |

| | |
|---|---|
| mmol | millimoles |
| MRSA | Methicillin resistant *Staphylococcus aureus* |
| MSSA | Methicillin sensitive *Staphylococcus aureus* |
| MS/MS | Tandem mass spectroscopy |
| m/z | mass to charge ratio |
| NCCLS | National Committee for Clinical Laboratory Standards/Clinical and Laboratory Standards Institute |
| nM | nanomolar |
| NMR | Nuclear Magnetic Resonance |
| ω | omega |
| Panc-1 | Human pancreatic carcinoma, epithelial-like cell line |
| PE | Petroleum Ether |
| PLA | Polylactic acid |
| ppm | parta per million |
| Py | Pyridine |
| q | quartet |
| R | Latin *rectus* for right |
| RPMI | Rosewell Park Memorial Institute medium |
| s | singlet |
| S | Latin *sinister* for left. |
| SCUBA | Self-contained underwater breathing apparatus |
| Sf9-cells | a clonal isolate of Spodopterafrugiperda Sf21 cells |
| sp. | species singular |
| spp. | species plural |
| SRB | Sulphorodhamine B assay |
| SRC | sarcoma |
| str | stretching |
| t | triplet |
| TCA | Trichloroacetic acid |
| TC-NER | Transcription-coupled Nucleotide excision repair |
| TFA | Trifluoro acetic acid |
| TkB | tyrosine kinase-binding |
| TLC | Thin Layer Chromatography |
| TMS | Tetra methyl silane |
| TOF | Time of Flight |
| TOF MS/MS | Time of Flight Tandem Mass Spectrometry |
| TPA | Tissue plasminogen activator |
| TsCl | p-Toluene sulphonyl chloride |
| UV | Ultra Violet |
| VRE | Vancomycin resistant Enterococci |
| VSE | Vancomycinsensitivet Enterococci |

| | |
|---|---|
| v-SRC | viral sarcoma |
| WHO | World Health Organisation |
| w/v | weight per volume |
| *Z* | *zusammen*German for together |
| Zn-Se ATR | Zinc-Selenium Attenuated Total Reflectance |

**Papers Published:**

Antibacterial Activity of 2-(2', 4'-Dibromophenoxy)-4,6-dibromophenol from *Dysidea granulosa*
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# *Introduction*

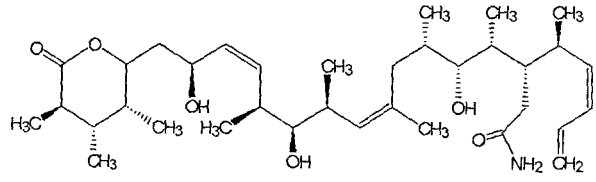
## Introduction

In recent years demand for new drugs to treat diseases is steadily increasing. Nature has provided a diverse array of bioactive compounds that are utilized as drugs against a variety of diseases. Until recently, most of the pharmaceuticals compounds obtained from nature have been from terrestrial sources. There is very less evidence of marine organisms being used in traditional medicine. Therefore the sea has received much attention as a potential source among the natural source of drugs.[1] Thus the oceans represent the next frontier as a source for drugs.

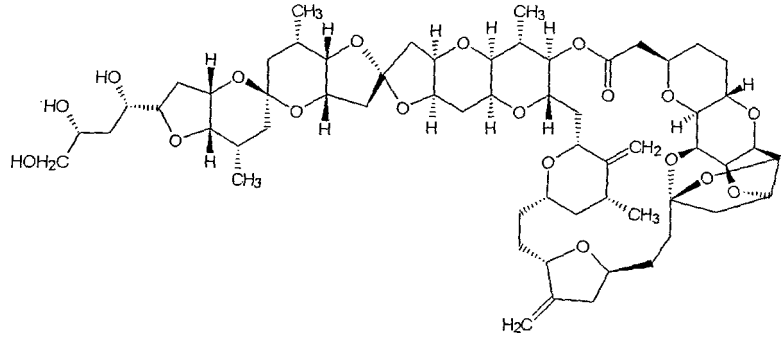
With the ocean comprising of 70% of the earth's surface; marine species comprise approximately half of the global diversity ranging from 3 million to 500 million different species.[2] These include the bacteria, fungi, sponges, soft corals, nudibranchs, tunicates and the algae. The rationale of searching for drugs from marine environment stems from the fact that marine plants and animals have adapted to all sorts of marine environments and these creatures are constantly under tremendous selection pressure including space competition, predation, surface fouling and reproduction. Over hundred million years of selection, has led to the evolution and production of various secondary metabolites to offset negative effects of selection force. The outcome is that there exist vast arrays of natural compounds that could benefit human beings if these compounds could be identified and examined for their effects. The vast diversity of marine fauna and flora offer human beings the last frontier to explore the existence of potential drugs for use in disease treatment. The compounds that are identified so far represent only the tip of the iceberg.[3]

It is interesting to note that majority of marine natural products currently in clinical trials or under pre-clinical evaluation is produced by invertebrates such as sponges, tunicates, molluscs and bryozoans. Anticancer agents like (+)-Discodermolide (1) isolated from *Discodermia dissoluta* (sponge),[4] Halichondrin B (2) from *Halichondria okadai* (Sponge),[5] Aplidine (3) from *Aplidium albicans* (tunicate),[6] Kahalaide F (4) from *Elysia rufescens* (Mollusk)[7] are currently in clinical trials. Manoalide (5) isolated from *Luffariella variabilis* (sponge)[8] and Ziconotide (6) from *Conus magnus* (cone snail).[9,10] are also under clinical trials against inflammation and pain respectively. The wealth of bioactive metabolites

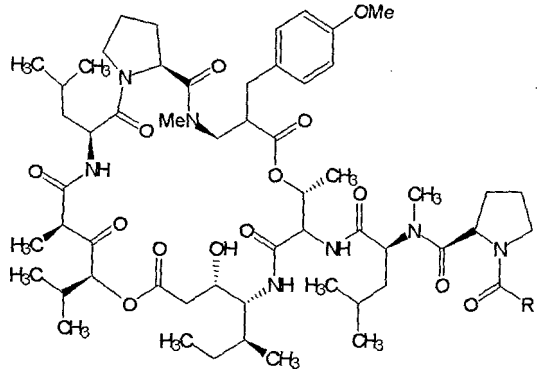
isolated from soft-bodied, sessile or slow moving marine invertebrates that usually lack morphological defense structures such as spines or a protective shell is no coincidence but reflects the ecological importance of these constituents for the respective invertebrates. It has been repeatedly shown that chemical defense through accumulation of toxic or distasteful natural product is an effective strategy to fight off potential predators.[11]



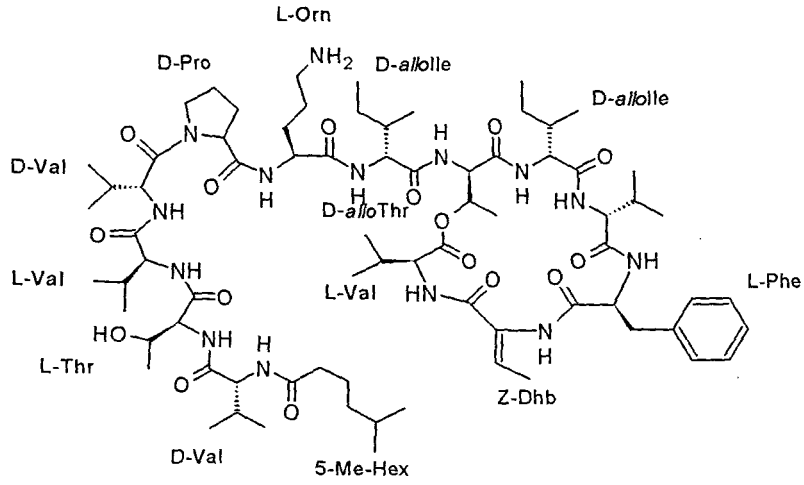
**(+)-Discodermolide (1)**



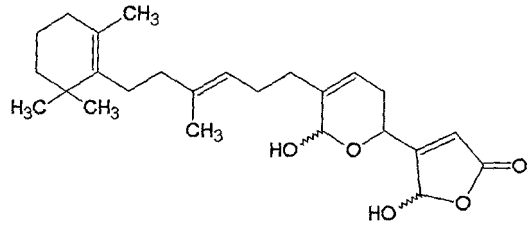
**Halichondrin B (2)**



**Dihydrodidemnin B R = $COCH_3$ (3) (Aplidine)**




**Kahalalide F (4)**



**Manoalide (5)**

C-K-G-K-G-A-K-C-S-R-L-M-Y-D-C-C-T-G-S-C-R-S-G-K-C-$NH_2$

**Ziconotide (6)**

## MARINE MICROORGANISMS

The biological and chemical investigations of marine microorganisms have also been identified as an area that will play a major role in natural product chemistry during the next century. The past few years have already seen a rapidly developing interest in the chemistry of marine organisms. A large number of structurally unique and bio chemically diverse natural products have been discovered and this has been reflected by an increasing number of literature reports related to microbial metabolites.[12] The importance of symbiosis in the marine environment has been acknowledged by many researchers during the past

decade, who have speculated that numerous marine products are produced by a symbiotic microorganism and not the invertebrate host.[13,14]

## MARINE NATURAL PRODUCTS LEADS FOR DRUG DEVELOPMENT

Approximately 30 % of the drugs in the market worldwide are natural products or their derivatives. Natural products show a diversity of chemical structures that are not accessible even by the most sophisticated synthetic concepts. Moreover, natural products have often opened up completely new therapeutic approaches. They have contributed to identifying and understanding novel biochemical pathways and proved to make not only valuable drugs available but also essential tools in biochemistry and molecular cell biology.[15]

Until recently most of the pharmaceutical compounds obtained from nature have been from terrestrial sources. 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. The marine environment is an exceptional reservoir of bioactive natural products, many of which exhibit structural/chemical features not found in terrestrial natural products.[16] Thus prospects of marine natural products as future drugs are very promising with novel structures, novel pharmacology and pronounced biological activity.

## OBJECTIVES

- Isolation and structure elucidation of new structural classes of natural products produced by marine organisms like sponges, soft corals, ascidians including their associated marine microorganisms (fungi, bacteria, algae and actinomycetes).
- Biological screening of crude extract and pure compounds.
- Synthesis of some selected marine natural products.

## SIGNIFICANCE OF WORK

- Resource for structurally unique and biochemically diverse bioactive natural products.
- New biological resource for medicines and therapeutics.
- Source of new structural class of compounds.
- Additionally marine organisms also provide agrochemicals, cosmetic products, nutrition products and much more.

## SCOPE OF STUDY

Marine invertebrates are primarily among the well-researched marine organisms because of their size and abundance. About one third of MNPs have been reported from marine invertebrates. It has been proven that many of the molecules isolated from the invertebrates have been produced by an associated microorganism rather than the host organism. Hence this study is focused on the isolation of metabolites from marine invertebrates and their associated microorganisms. This study contains three main processes: first is isolation and purification of natural products, second is biological activity and third structure elucidation.

For the isolation of the metabolites, a chemical and biological approach is used. Extracts are screened for antibacterial, antifungal, cytotoxicity and general toxicity activities. As a complement to the bioassay-guided approach, TLC, UV, column chromatography, 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. Purified compounds, which are not responsible for the initial bioactivity screening, may have a chance to be evaluated for other more sophisticated pharmacological assays and may also, find interest in academics for their unique skeletal structures.

As a student of Goa University and a researcher at National Institute of Oceanography (NIO), Goa I had an opportunity to work with the marine Natural Product Chemists at NIO under the Ministry of Earth Sciences (MoES) funded project "Development of Potential Drugs from the Seas around India." During the

course of the research I was involved in the isolation, structure elucidation, screening for anti-infective, anticancer and other biologically active metabolites from marine organisms. The investigation carried out on some selected marine organisms is the subject of the present thesis.

The work presented in the thesis is divided into four chapters.

**Chapter 1:** This chapter deals with the review of literature on bioactive metabolites isolated from marine invertebrates based on specific biological action.

**Chapter 2:** Deals with the identification of secondary metabolites from marine sponges. It has been subdivided into two sections

**Section 1:** Investigation of chemical constituents and bioactivity of marine sponge *Dysidea granulosa*

**Section II:** Deals with the chemical investigation of marine sponge *Psammaplysilla purpurea.*

**Chapter 3:** Deals with the chemical investigation of soft corals. It has been subdivided into two sections

**Section 1:** Investigation of chemical constituents and bioactivity of *Sinularia kavarattiensis.*

**Section II:** Deals with the chemical investigation of *Lobophytum crassum*

**Chapter 4:** Miscellaneous Synthetic studies: Synthesis of bryozoan metabolites Alternamides

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# *Chapter I*

# *Marine Invertebrates and Bioactive metabolites under clinical trials: A literature Review*

**Marine Invertebrates and Bioactive Metabolites under clinical trials: A literature Review**

In recent years, marine natural product bioprospecting has yielded a considerable number of drug candidates. Most of these molecules are still in preclinical or early clinical development but some are already on the market, such as Cytarabine, and some are predicted to be approved soon, such as ET743 (Yondelis™). Research into the ecology of marine natural products has shown that many of these compounds function as chemical weapons and have evolved into highly potent inhibitors of physiological processes in the prey, predators or competitors of the marine organisms that use them. Such molecules isolated from marine invertebrates are likely to become a rich source of novel chemical entities for the discovery of more effective drugs.

More than 70% of our planet's surface iscovered by oceans and life on Earth has itsorigin in the sea. In certain marine ecosystems,such as coral reefs or the deep sea floor, experts estimate that the biological diversity is higher than in tropical rain forests. Many marine invertebrates are soft bodied and have a sedentarylife style necessitating chemical means of defense.Therefore, they have evolved the abilityto synthesize toxic compounds or to obtainthem from marine microorganisms. Thesecompounds help them deter predators, keepcompetitors at bay or paralyze their prey. Marine invertebrates have thus been recognized as an important source of bioactive compounds with therapeutical potential.

Natural products released into the water are rapidly diluted and, therefore, need to be highly potent to have any effect. For this reason, and because of the immense biological diversity in the sea as a whole, it is increasingly recognized that a huge number of natural products and novel chemical entities exist inthe oceans, with biological activities that maybe useful in the quest for finding drugs withgreater efficacy and specificity for the treatment of many human diseases.[1-2] Interestingly many marine natural products produced by invertebrates are currently in clinical trials.

The marine natural products reviewed here include only those molecules which are under development based on specific biological action.

**Molecules targeting ion channels**

Cone snail venoms are highly potent and actas selective peptide antagonists or agonistsof ligand or voltage-gated ion channels and G-protein-coupled receptors (GPCR).[3,4] Nicotinic acetylcholine receptors (nAChR) are competitively inhibited by α- and αA-conotoxins, whereas ψ-conotoxins show non competitive inhibition of this family of receptors. The σ-conotoxins antagonize the related 5-HT3serotonin receptor and the conopressins areagonists of vasopressin receptors, a family ofGPCR; ω-conotoxins are calcium-channel blockers, κ-conotoxins are potassium-channel blockers and μ-, as well as μO-conotoxins, aresodium channel blockers, to name but a few of the conotoxin families. The high potencyof these toxins, which are potentially lethal tohumans, may, in the future, be turned to ouradvantage by pharmacologists who have beeninvestigating their potential use as adjuncts in anaesthesia, analgesics or as drugs for thetreatment of conditions such as epilepsy, cardiovascular disease and psychiatric disorders.[4] The strong commercial interest in thesemolecules is reflected by more than 100patents and patent applications with the word'conotoxin(s)' in their title. The available conotoxin libraries are thought to containmore than 50,000 distinct molecules. Themain players in the field of conotoxin commercialization are Cognetix, Neurex, AMRAD and Xenome. Ziconotide, previously referred to as CI1009 or SNX111, is the synthetic form of ω-conotoxin MVIIA, a neuron-specific, N-typecalcium-channel blocker and the first conotoxin drug to receive an approval letter from the FDA.[5]Ziconotide was developed by Neurex as an intrathecal treatment for chronic pain. Unlike morphine it does not induce thedevelopment of tolerance, constipation or respiratorysuppression. Another ω-conotoxin, AM336, is in clinical development at AMRAD for the treatment of severe morphine-resistant pain. This molecule was found to be 100-foldmore selective than Ziconotide for N-type over P/Q-type calcium channels with reduced adverse neurological effects,which suggests that it may be preferable to Ziconotide.GTS21, 3-(2,4-dimethoxybenzylidene)-anabaseine, is aselective α7 nAChR partial agonist in clinical development at Taiho to

treat Alzheimer's disease and schizophrenia.GTS21 was isolated from the nemertine worm *Amphiporus lactifloreus*. The compound has been shown to improvelearning performance and memory retention in passiveavoidance models in nucleus basalis magnocellularis(NbM)-lesioned rats as well as in active avoidance modelsin aged rats. It also reduced neocortical cell loss in NbM lesioned rats and cell death induced by β-amyloid or glutamatein cultures of neuronal cells.

**Compounds targeting enzymes**

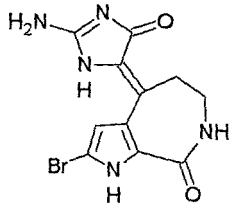
*Protein serine/threonine kinase inhibitors*

Bryozoans are sessile animals with a life style very similarto that of corals but, owing to their unique body plan, theyconstitute a phylum of their own. One particular bryozoan, *Bugula neritina*, has been the source of a family ofprotein kinase C (PKC) inhibitors called bryostatins currentlyin clinical trials for cancer. Bryostatin-1 has beengranted Orphan Drug status by the FDA and has beendesignated an Orphan Medicinal Product in Europe for oesophageal cancer in combination with paclitaxel.[6-7]Bryostatins are complex polyketides based on the bryopyran ring system. They have been studied for theircellular activity and therapeutic potential since 1968. Thefirst isolation and structure determination of a bryostatinwas performed by George Pettit in the 1980s. Bryostatins inhibit protein serine/threonine kinases of the PKC familyand have antineoplastic activity. Strictly speaking, bryostatins are not inhibitors but activators of PKC. However, bryostatin-induced activation results in the down regulationof this protein kinase by proteolysis more efficientlyand rapidly than that induced by 12-*O*-tetradecanoylphorbol13-acetate (TPA). Because of the resemblance of these macrolides to certain bacterial secondary metabolites, ithas long been suspected that bryostatins are actually synthesized by symbiotic bacteria. It appears that the symbiont '*Candidatus Endobugula sertula*', a so far uncultivated γ-proteobacterium, is the true source of bryostatin-1.[8] Fermentation of this bacterium would solve the currentsupply problem that exists for bryostatin-1, which, untilnot so long ago, had to be extracted from *B. neritina* harvested from the sea. For example, it took 2 years for diversemployed by the National Cancer Institute to collect 17tons of the organism off the southern California coastwhere it is most abundant. However,

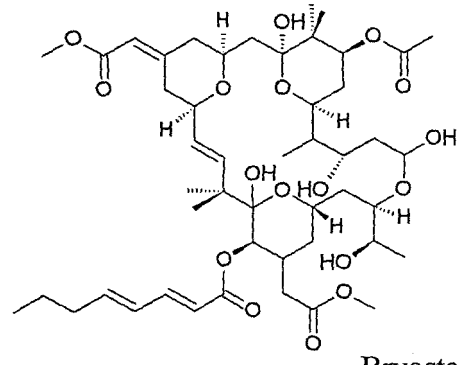
CalBio Marine has recently been able to establish aquaculture for the bryozoan. Total synthesis of bryostatins has also been makingprogress. Bryostatin-2, 3 and 7 have been produced synthetically but synthesis of the commercially most interestingbryostatin-1 has not been achieved, yet.[9-11] Owing to the therapeutic potential of the molecule, the generation of bryostatin-1 analogues is an area of intensive research. Scientists at Stanford University have synthesizeda series of such analogues, some of which were two to three orders of magnitude more potent against tumour cell lines. Neristatin-1, a biologically active yet structurally simplerbiosynthetic precursor of bryostatin-1, was obtained at Arizona State University Cancer Research Institute.

The screening of compounds isolated from marine invertebrates for cyclin-dependent kinase (CDK) inhibition led to the discovery of hymenialdisine as a potent($IC_{50}$=10–40 nM) inhibitor of the protein serine/threoninekinases glycogen synthase kinase 3β, CDK1, CDK2, CDK5,casein kinase 1 and mitogen-activated protein kinase kinase-1.[12,13]Hymenialdisine is a sponge alkaloid named after *Hymeni acidonaldis*. The compound inhibited the*in vitro* phosphorylation of human microtubule-associated protein tau, which is implicated in the pathogenesis of Alzheimer's disease, and in Sf9 cells expressing the protein.[13] Additionally, the molecule reduced the production ofinterleukin-8 and prostaglandin E2 in human cells, indicating that it may have anti-inflammatory activity.[14-15]Scytonemin is a protein serine/threonine kinase inhibitorreferred to as a marine natural product [16], but the moleculewas in fact isolated for structural analysis and pharmacological characterization from the cyanobacterium *Stigonema sp.* collected from Waldo Lake, Oregon. The compoundis a yellow-green ultraviolet sunscreen pigment present in the extracellular sheaths of different genera of aquatic and terrestrial blue-green algae. It has a unique symmetrical dimeric ring structure (**Fig.1**). The monomeric subunit is closely related to nostodione A (**Fig.1**), a mitoticspindle poison from the terrestrial blue-green alga *Nostoc commune*. Scytonemin was found to inhibit human polo like kinase ($IC_{50}$=2 μM), which has an important role in the regulation of mitotic spindle formation as well as other kinases involved in cell cycle control, including checkpoint kinase 1 and

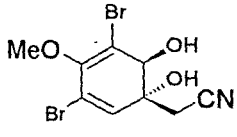
CDK1, with similar potency. Furthermore, the compound inhibits proliferation of human fibroblasts andendothelial cells in culture and reduces TPA-induced ear oedema in mice when administered topically.In this respect, it is interesting to note that nostodione A and the scytonemin monomer show a striking overall structural similiarity to PS1145, an inhibitor of the anti-inflammatory drug target IκB kinase **(Fig.1)**. It has been suggested that scytonemin may provide a novel pharmacophore for the development of protein kinase inhibitors as antiproliferative and anti-inflammatory drugs.[17]



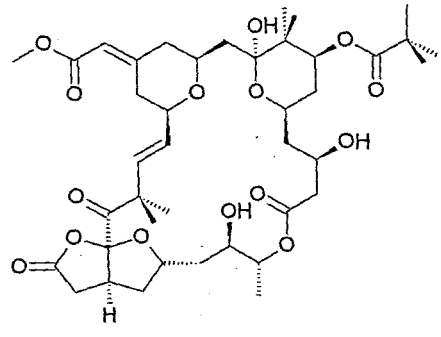

Hymenialdisine



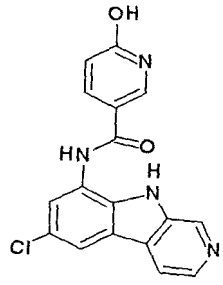

Bryostatin-1

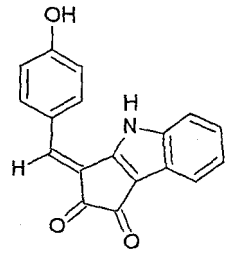



(+)-Aeroplysinin

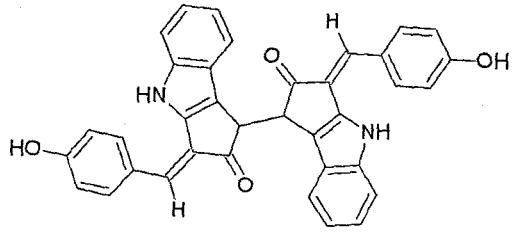



Neristatin-1




PS1145 Nostodione A




Scytonemin

**Fig1: Marine natural product Protein Kinase Inhibitors and similarity of Nostodione A to PS1145**

*Protein tyrosine kinase inhibitors*

Marine organisms have also been the source of several proteintyrosine kinase inhibitors. Aeroplysinin-1, a brominatedtyrosine metabolite from the sponge *Verongia aerophoba* ,has been found to inhibit purified epidermal growth factor (EGF) receptor protein tyrosine kinase activity, to block EGF-stimulated proliferation of cancer cell lines, to inducetheir apoptosis at high nanomolar concentrations and to suppress angiogenesis *in vivo*.[18–20] The polyketide halenaquinone, isolated from the Okinawan sponge *Xestospongia exigua,* has also been shown to inhibit the protein tyrosinekinase activity of the EGF receptor as well as that of theviral oncogene product v-Src.[21] The concentrations requiredare in the low micromolar range. The sulfated steroid derivative halistanol trisulfate, extracted from sponges of the genus *Topsentia,* has also been reported to be a v-Srcinhibitor at these concentrations.[22] The brown alga *Stypopodium zonale* has yielded stypoquinonic acid and the fungus

*Asochyta salicornia* has yielded ascosalipyrrolidinone A, which are two novel inhibitors of the Src-family protein tyrosine kinase Lck.[23-24]

*Phospholipase A2 inhibitors*

In humans, secreted type IIA phospholipase A2 (PLA2) isinvolved in the pathogenesis of a variety of inflammatory diseases via the production of arachidonic acid, the precursor of prostaglandins and leukotrienes. Secreted PLA2 is,therefore, seen as a promising target for the developmentof anti-inflammatory drugs and a considerable research effort has been focused on this family of enzymes.[25-26] The first natural marine human synovial PLA2 inhibitor, the sesterterpene Manoalide ($IC_{50}$=3.9 μM) **(Fig.2)**, was isolated from the Palauan sponge *Luffariella variabilis* and was found to have analgesic and anti-inflammatory activity.[27] This compound was taken into phase I clinical trialsby Allergan but its development was later discontinued.

Other marine sponge terpenoid PLA2 inhibitors are the sesterterpenes variabilin from *Ircinia variabilis*, cacospongionolide B from *Fasciospongia cavernosa* and petrosaspongiolide M from *Petrospongia nigra,* as well as the sesquiterpenes bolinaquinone from *Dysidea sp.* **(Fig.2)**. Variabilin inhibits human synovial PLA2 ($IC_{50}$=6.9 μM) but not 5-lipoxygenase or cyclooxygenases 1 and 2 *in vitro.*[28] These sesquiterpenes were found to inhibit human neutrophildegranulation, superoxide generation, leukotrieneB4 (LTB4) production (variabilin), TPA-induced ear oedema (variabilin, bolinaquinone, topically) as well as carrageenan induced paw oedema (variabilin, petrosaspongiolide Mandbolinaquinone, p.o.) in mice.[29–32] The long-known anti-inflammatory sponge PLA2 inhibitors are the scalaranes, sesterterpenes, named after scalaradial isolated from *Cacospongia mollior,* and the pseudopterosins.[33] diterpene-pentoseglycosides, which also have analgesic properties and are used as additives in certain skin-care products. One compound in this family, the semi synthetic methopterosin (OAS1000) **(Fig.2)**, has entered clinical development for the promotion of woundhealing. OAS1000 has also shown anti-inflammatory activity in animal models, which is thought to be due to its inhibition of LTB4 synthesis in neutrophils.

**Microtubule-interfering agents**

The development of resistance in tumours to the microtubule-stabilizing taxanes has prompted the search for novel microtubule-interfering agents in the hope of finding a way to circumvent this problem. Marine organisms have yielded the largest number of such compounds.[34]:

• discodermolide, a polyketide, purified from the sponge *Discodermia dissoluta*;

• eleutherobin, a diterpenoid, from the soft coral *Eleutherobia* sp;

• sarcodictyins, diterpenoids, from the Mediterranean coral *Sarcodictyon roseum*;

• dolastatins, short peptides containing unique amino acids, from the sea hare *Dolabella auricularia*[35], a nudibranch snail and, later, from marine cyanobacteria.[36];

• halichondrin B (**Fig.3**), a polyether macrolide from the sponge *Halichondria okadai.*[37];

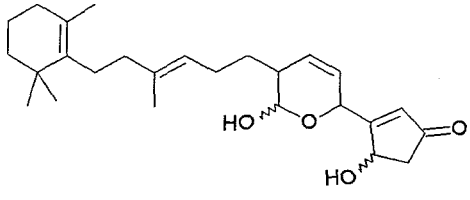
• laulimalide, as well as its rearrangement product isolaulimalide, which are 18-membered macrocycliclactones, from the sponge *Cacospongia mycofijiensis.*[38];

• peloruside A, related to bryostatin 1, from the sponge *Mycale* sp. [39];

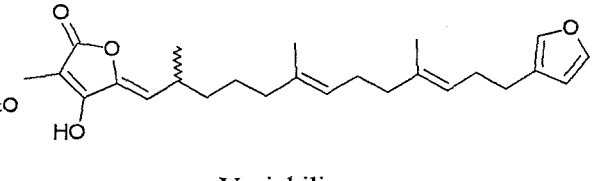
• hemiasterlins, tripeptides from the sponges *Auletta* sp. and *Siphonochalina* sp. [40];

• vitilevuamide (**Fig.3**), a bicyclic marine peptide, from the ascidians *Didemnum cuculiferum* and *Polysyncraton lithostrotum.*[41]

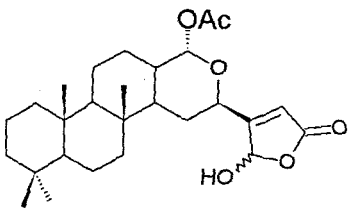
All of these compounds have been reported to have general cytotoxic activity and to kill cancer cells *in vitro* butonly dolastatin 10, the dolastatin 15 analogues ILX651 andcemadotin, discodermolide and the hemiasterlin analogue HTI286 have so far reached clinical development. Halichondrin B analogues are in preclinical development at Eisai. The laulimalides and discodermolide are of special interest to anticancer drug discovery researchers becausethey have been shown to remain active in cells over expressing multidrug-resistant P-glycoprotein.[38,42]



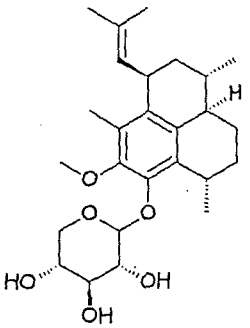

Manoalide



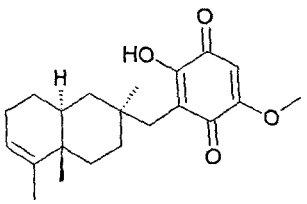

Variabilin



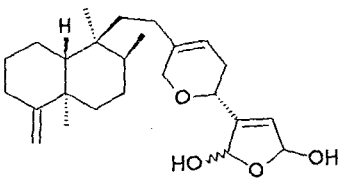

Petrosaspongiolide M



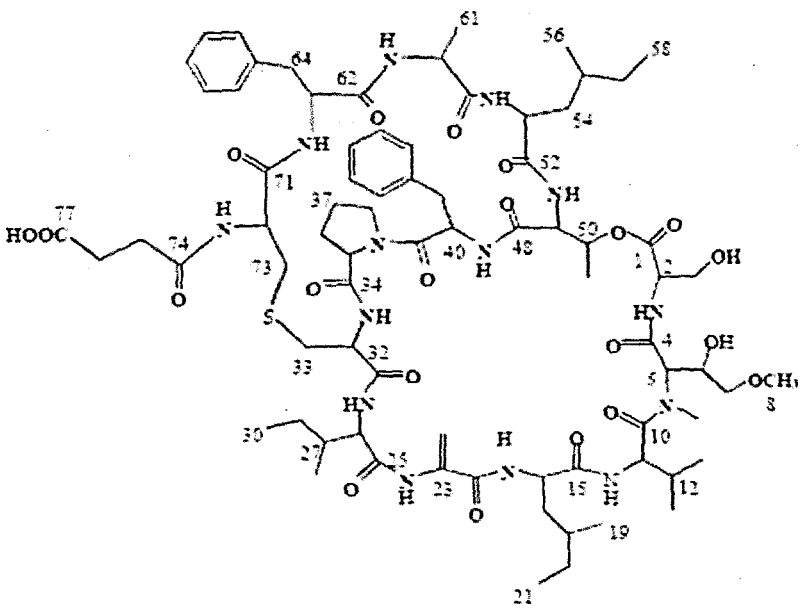

Methopterosin




Bolinaquinone




(+)-Cacospongionolide B

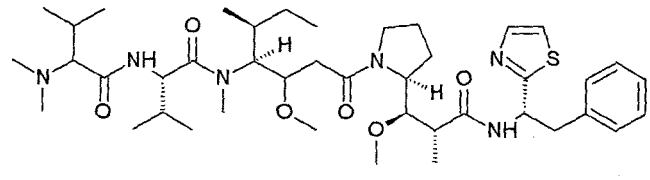



Vitilevuamide

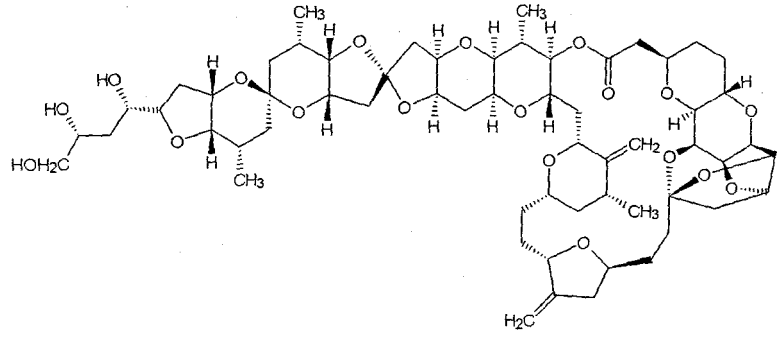
**Fig. 2: Marine Natural Product $PLA_2$ Inhibitors**

**DNA-interactive agents**

Several established anticancer drugs are DNA-interactiveagents. These compounds, including the platinum drugs cisplatin and carboplatin, are characterized by strong cytotoxicactivity, but they also show a total lack of specificityfor cancer versus normal cells. The question of whether DNA can be a suitable target for the development of more specific, second-generation anticancer drugs has, therefore, been raised.[43] Support for the idea that DNA isstill a target worth aiming at has come from clinical trials with a marine natural product, ET743 (Yondelis™) (**Fig.4**). ET743 is one of a family of tunicate-derived antitumour compounds originally identified in 1988 by Kenneth Rinehart's team at the University of Illinois, Urbana-Champaign[44] and licenced to PharmaMar in 1994. These ecteinascidins, named after *Ecteinascidia turbinata*, acolonial ascidian from which they were isolated, are not the only class of marine DNA-interactive compounds active in cytotoxicity screens. Others include dercitin, anacridine alkaloid isolated from a sponge of the genus *Dercitus*[45], and the topsentins, a class of sponge-derived bisindole alkaloids[46],but ET743 is so far the only compound that has gone into clinical development.



Dolastatin-10



HalichondrinB

**Fig. 3: Microtubule interfering agents from Marine sources**

ET743 is an isoquinoline compound consisting of three tetrahydroisoquinoline rings. The first total synthesis of the molecule was achieved in 1996 by David Gin in Elias Corey's team at Harvard University. [47] Owing to its extreme potency (picomolar to low nanomolar $IC_{50}$ values for the inhibition of growth in sensitive human tumour cell lines), extraction of the drug is a viable strategy for obtaining adequate amounts for clinical trials even though more than half a ton of the sea squirt needs to be harvested to obtain 1 g of the compound. Nevertheless, a hemisynthetic process starting from Safracin B, an antibiotic obtained by fermentation of the bacterium *Pseudomonas fluorescens*, has been developed at PharmaMar and successfully used to synthesize ET743 in sufficient quantities for further commercial development.[48] Corey and his student EduardoMartinez designed a structurally simpler, easier to synthesizeand more stable analogue of ET743 named phthalascidin(PT650) **(Fig.4)**. PT650 and ET743 show undiminishedpotency in cell lines resistant to the topoisomerase inhibitors camptothecin and etoposide.[49] Structural modelling has suggested that two of the tetrahydroisoquinoline rings of ET743 bind to the minor grove of DNA resulting in DNA adduct formation with aguanine residue.[50] The binding of ET743 to the minor groove results in bending of the DNA towards the major groove, induction of a conformational change that appearsto be unique to ET743. The protruding third ring is predictedto interact with so far unknown DNA-binding proteins that are believed to be responsible for the pharmacodynamic properties of the compound. ET743 was shown to cause a prolonged cell cycle blockadein G2/M and to inhibit induced gene transcriptionof several genes including HSP70 and p21 while having little effect on basal gene expression.[51-52] Moreover, ET743 can enhance the activities of other chemotherapeutic agents by inhibiting multi drug resistant MDR1 gene expressionupon co-administration.[53] Cells inthe G1 phase of the cell cycle, and of these softtissuesarcoma cells in particular, are extremely sensitive to ET743-induced cell killing. Othercancer cells that have shown sensitivity towards ET743 are cell lines and xenografts derived from tumours of the breast, kidney, lung, ovariesand prostate as well as from glioblastomas and melanomas.[54] The cytotoxic effects of ET743 are entirely dependent on the presence of functional transcription-coupled nucleotide excision repair (TC-NER) in the

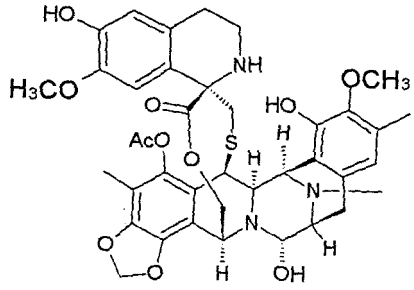
treated cells but independent of wild-type p53 tumour suppressor protein function.[55] Interference with this DNA repair pathway is, therefore,thought to be at the heart of the unique mode of action ofET743. Loss of TC-NER renders cancer cells exquisitely sensitive to cisplatin.[56] This effect may open up new avenuesfor combination chemotherapy because it should be feasibleto stop the outgrowth of ET743-resistant tumour cell populations by simultaneous treatment with cisplatin. This approachis currently being tested by PharmaMar in a phase Iclinical trial. ET743 has also been found to act synergisticallywith doxorubicin or paclitaxel in soft-tissue sarcomacell lines, suggesting that a combination of the drug with anthracyclins or taxanes may also result in increased therapeutic benefit.[57]ET743 has completed clinical trials for soft-tissue sarcoma and is currently under regulatory review in Europe for the treatment of this type of cancer for which it was granted Orphan Medicinal Product designation by The European Agency for the Evaluation of Medicinal Products in 2001. In the same year, Zeltia, the parent company of PharmaMar, announced a licensing agreement with OrthoBiotech, a Johnson and Johnson subsidiary, to develop and market ET743. A comprehensive development programme to study the potential use of ET743 for the treatment of other forms of cancer was agreed. So far, promising results have been released from phase II clinical trials for ovarian and breast cancer. The efficacy of ET743 in endometrial and non-small cell lung cancer is also being investigated in phase II trials. To date, more than 1,600 patients have been treated with ET743.[58]

**Other marine natural product drug candidatesinclinical development**

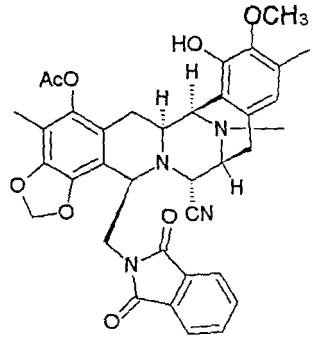
APL (dehydrodidemnin B, Aplidin™) a cyclic depsipeptide derived from *Aplidium albicans*, is another tunicate marine natural product discovered by Rinehart and coworkers[43] and licensed to PharmaMar. The compound triggers rapid and persistent activation of the apoptotic processas a consequence of the induction of oxidative stress and the sustained activation of the protein kinases Jun Nterminal kinase, p38 stress-activated protein kinase, EGFreceptor and Src.[59-60] At present, more than 200 patients have been treated with APL in phase I clinical trials for cancer. Kahalalide F is a third marine natural product anticancer drug candidate in clinical development at

PharmaMar.[61] This cyclic depsipeptide was isolated from the sea slug *Elysia rufescens* but is most probably derived from *Bryopsis sp.*, its green algal diet. The compound has shown antitumour activity, probably by interfering with lysosome function in prostate, colorectal and lung cancer cell lines as well as in animal models of lung and breast cancer. It is currently in phase I trials for prostate cancer and other solid tumours. Kirin Brewery is developing KRN7000, a novel α-galactosylceramide derived from agelasphin-9b, which, in turn, was isolated from the sponge *Agelas mauritianus*, for the potential treatment of cancer and other diseases.[62] KRN7000 has been shown to have immune stimulatory and antimetastatic activity possibly by enhancing antigen presenting cell function. Phase I clinical trials for solid tumours showed no drug-related toxicity, no signs ofaccumulation or saturation and an increase in interferon-γ, interleukin-4, interleukin-12 and granulocyte macrophage colony-stimulating factor levels, as well as natural killercell activity at least in some of the patients. Squalamine lactate, a novel antiangiogenic aminosteroid from the dogfish shark *Squalusa canthias* is currently in phase II clinical trials for ovarian and non-small cell lung cancer at Genaera and was granted Orphan Drug status for the treatment of ovarian cancer by the FDA. Reported objective response rates were around 30% for oneor more cycles of treatment in combination with standard chemotherapy. The compound is thought to act by sequestration of calmodulin[63] resulting in the inhibition of a sodium/proton antiport regulating intracellular pH and, consequently, in reduced cell proliferation in endothelial cells. IPL512602, a synthetic analogue of the steroid contigna sterol isolated from the sponge *Petrosia contignata*[64], is in phase II clinical trials as a leukocyte-suppressing anti-inflammatory drug for the treatment of asthma.

LAF389 is an easier to synthesize and more water-soluble synthetic analogue of bengamide B, a heterocyclic aminoacid derivative from the sponge *Jaspis digonoxea.*[65] LAF389 induces cell cycle arrest by inhibition of methionine amino peptidase.




Ecteinascidin 743




Phthalascidin

**Fig 4: ET743 and its synthetic analogue PT650**

**A promising future for marine natural products in drug discovery**

It has recently been argued that biodiversity prospecting is not necessary because structural diversity is of less importance than functional diversity as potent natural inhibitors for any given biological target can be found virtually infront of one's doorstep, their number greatly exceeding that of all potential drug targets encoded by the human genome.[66] However, in drug discovery it is frequently observed that a certain series of compounds that yielded the most potent inhibitors *in vitro* turned out not to be the ideal drug candidate *in vivo*. Moreover, it is likely that for every compound that does make it to the market, a better drug with distinct chemistry, improved bio availability and less unwanted side effects can be found either in chemical libraries or among the much larger number and diversity of natural products. This has been pointed out in a reply by natural product researchers Gordon Cragg and David Newman of the National Cancer Institute [67], to which the authors of the article responded by saying, among other things, that 'the world's oceans are likely to harbour the largest biodiversity but can it not be argued that land, the natural environment in which mankind evolved, is more likely to contain compounds with relevant mechanisms of action?'.[68] This argument does not take into consideration thefact that the molecular physiology of eukaryotic cells evolved in our early marine ancestors and that it is highly conserved molecular mechanisms that are targeted by many novel drug candidates. However, it is not always necessaryto travel to far away,

exotic places to find a specific natural product, considering, for example, the case of scytonemin. Moreover, pharmaceutical companies do not have to bioprospect for natural products, marine or terrestrial, to benefit from the diversity of small, pharmacologically interesting molecules that exist in nature. They may just use structural scaffolds of published compounds as templates for the identification of similar molecules in their own chemical libraries, which they can use as starting points for chemical modification[69] as was done for the development of acyclovir, for example. With three drugs from the deep, the cephalosporins, cytarabine (Ara-C) and vidarabine (Ara-A), already well-established on the market [70], the granting of Orphan Drug status to bryostatin-1, squalamine and ET743 in the past 2 years, and more than a dozen further marine compoundsor derivatives currently in clinical trials (**Table 1**), the answer to the question of whether marine natural products will have a promising future in drug development looks likely to be good. Given that marine natural product bioprospecting has only begun relatively recently but has already yielded several thousand novel molecules, that deep sea biodiversity research is still in its infancy, that affordable technology for the exploration of the abyss is currently being developed, and that the total number ofspecies and, therefore, most likely biochemical diversity in the oceans is higher than on land, there is good reason to believe that approval of the above-mentioned drugs will only be the beginning of the arrival in our pharmacies of a larger number of marine natural products, or compounds derived from them, for the improved treatment of human illnesses.

**Table 1: Marine Natural Products and derivatives in clinical development**

| Compound name | Source | Chemical class | Company | Disease area | Status |
|---|---|---|---|---|---|
| *Compounds targeting ion channels* | | | | | |
| Ziconotide | Cone snail | Peptide | Neurex | Chronic pain | Phase III |
| AM336 | Cone snail | Peptide | AMRAD | Chronic pain | Phase I/II |
| GTS21 | Nemertine Worm | Anabaseine-derivative | Taiho | Alzheimer's disease Shizophrenia | Phase I/II |
| Compounds targeting enzymes | | | | | |
| *Methionine/Aminopeptidase inhibitors* | | | | | |
| LAF 389 | Sponge | Amino acid derivative | Novartis | Cancer | Phase I |
| *Protein Kinase Inhibitors* | | | | | |
| Bryostatin-1 | Bryozoan | Polyketide | GPC Biotech | Cancer | Phase II |
| *$PLA_2$ Inhibitors* | | | | | |
| OAS 1000 | Soft coral | Diterpenepentoseglycosidase | Osteo arthritis Sciences | Wound healing Inflamation | Phase I/II |
| *Microtubule interfering agents* | | | | | |
| Dolastatin-10 | Sea slug | peptide | NCL/Knoll | Cancer | Phase II |
| ILX651 | Sea slug | peptide | Ilex Oncology | Cancer | Phase I |

| | | | | | |
|---|---|---|---|---|---|
| Cemadotin | Sea slug | peptide | Knoll | Cancer | Phase II |
| Discodermolide | Sponge | Polyketide | Novartis | Cancer | Phase I |
| HTI286 | Sponge | tripeptide | Wyeth | Cancer | Phase I |
| *DNA interactive agents* | | | | | |
| Yondelis TM | Sea Squirt | isoquinolone | PharmaMar/ Jhonson&Jhonson | Cancer | Phase II/III |
| *Oxidatve stress inducers* | | | | | |
| Aplidin TM | Sea Squirt | Cyclic depsipeptide | Pharma Mar | Cancer | Phase II |
| *Lysosomotropic compounds* | | | | | |
| Kahalalide F | Sea Slug/algae | Cyclic depsipeptide | Pharma Mar | Cancer | Phase I |
| *Immunostimulatory agents* | | | | | |
| KRN 7000 | Sponge | α-galactosylceramide | Kirin | Cancer | Phase I |
| *Calcium binding protein antagonists* | | | | | |
| Squalamine lactate | shark | Aminosteroid | Genaera | Cancer | Phase II |
| Compound with unknown mechanism of action | sponge | steroid | Inflazyme/ Aventis | Inflammation,Asthma | Phase II |

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# *Chapter II*

## *Chemical Investigation of marine sponges Dysidea granulosa & Psammaplysilla purpurea....*

## Introduction

Sponges are the oldest metazoan group, belonging to Phylum Porifera, with about 5000 species known across the world and having an outstanding importance as a living fossil.[1] Sponges are found in virtually all-aquatic habitats, although they are most common and diverse in the marine environment. These sessile organisms synthesize toxic substances, probably to discourage predators. Certain other marine animals take advantage of this characteristic of sponges by placing adult sponge on their bodies, where the sponges attach and grow. The chemicals also probably play a role in competition among sponges and other organisms, as they are released by sponges to insure themselves space in the marine ecosystem. Some of these chemicals have been found to have beneficial pharmaceutical effects for humans, including compounds with respiratory, cardiovascular, gastrointestinal, anti-inflammatory, antitumor, and antibiotic activities. Sponges also provide a home for a number of small marine plants, which live in and around their pore systems. Symbiotic relationships with bacteria and algae have also been reported, in which the sponges provide its symbionts support and protection and the symbionts provides the sponge with food. Some sponges (boring sponges) excavate the surface of corals and mollusks, sometimes causing significant degradation of reefs and death of mollusk. The corals and mollusks are not eaten; rather the sponge is probably seeking protection for itself by sinking into the hard structures it erodes. Even this process has some beneficial effects, in that it is an important part of the process by which calcium is recycled.

Chemicals i.e. secondary metabolites produced by the sponges not only play various roles in the metabolism of the producer but also in their strategies in the given environment. The diversity of secondary metabolites produced in sponges has been highlighted in several reviews.[1,2] They range from derivatives of amino acids and nucleosides to macrolides, porphyrins, terpenoids to aliphatic cyclic peroxides and sterols.

This chapter has been divided into two sections;

**Section I:**deals with thechemical investigation and identification of antimicrobial and anti-cancer properties of sponge *Dysidea granulosa.* This section has been further subdivided into:

**Part-1:** gives a brief and concise review of literature on the secondary metabolites from the sponges belonging to genus *Dysidea.*

**Part- II:** deals with bioactive constituent isolated and identified from the chloroform fraction of the crude methanolic extract of sponge *Dysidea granulosa,* collected off the coast of Lakshadweep Islands.

**Section II:** deals with the chemical investigation of marine sponge*Psammaplysillapurpurea*. It has been divided into two parts:

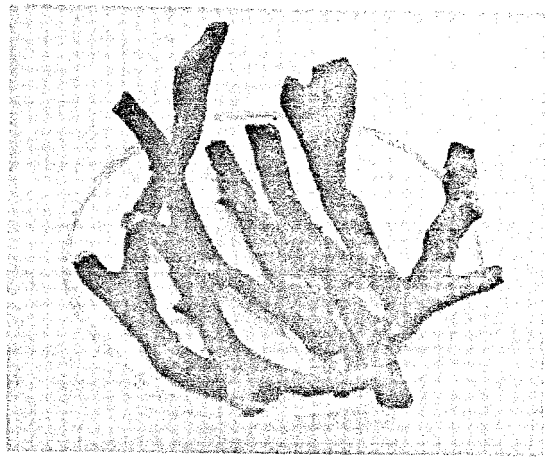
**Part-1:** gives a review of literature on the different chemical constituents of the genus *Psammaplysilla*

**Part- II:** chemical constituents of *Psammaplysilla purpurea* and their anticancer properties

# *Section I*

## *Dysidea granulosa....*

## Section 1.1: Genus *Dysidea*: A review of literature



*Dysidea granulosa* is a pinkish green sponge belonging to the Phylum:Porifera, Class :Demospongiae, Order:Dictyoceratida, Family:*Dysideae*, Genus:*Dysidea* and Species:*granulosa* (Bergquist). It has a thick encrusting, massive, or a branching growth form, often with a marked conulose. The skeleton consists of a regular, usually rectangular, arrangement of concentrically laminated primary and secondary fibers. Textures are usually soft and compressible. Presently thirteen different species are known from the genus *Dysidea* viz *herbaceae, etheria, granulosa, avara, cinerea, arenaria, amblia, frondosa, robusta, pallescens, villosa, spetosa & fragilis.*

Sponges belonging to this genus are rich source of metabolites with promising biological activities. A number of molecules belonging to different class of compounds such as sesquiterpenes and spirolactones, tricyclic furans based on furodysin skeleton, amino acid derived chlorinated metabolites, bromodiphenyl ethers, modified sterols and terpenesand polychlorinated alkaloids are known in the literature.This section deals mainly with thebioactivity and chemical investigation of the sponge *Dysidea granulosa*. But, before going into the details of this investigation a review of literature, particularly on the compounds derived mainly from the sponge belonging only to genus *Dysidea* is presented in the first part of this section.

A chemical study by Perez-Garcia (2005) of two unidentified species of the genus *Dysidea* collected in the Gulf of California led to the isolation of the new merosesquiterpenes 20-*O*-acetyl-21-hydroxy-*ent*-isozonarol(**1**), 20-*O*-acetylneoavarol (**2**), *ent*-yahazunol(**3**), and dysienone(**4**). Compounds 1 and 4 showed cytotoxic activity against three human tumor cell lines.[3]

Sesquiterpenoids, arenarol(**5**) and arenarone(**6**)possessing the cis -4,9-friedo drim-4(11)-ene skeleton bearing hydroquinol and hydroquinone moieties have been reported from the Pacific sponge *Dysidea arenaria.*[4]These compounds have attracted much interest due to their biological properties which include antitumor, antimicrobial, anti- HIV, antioxidants and others.[5]

Recently Utkina et al. (2010) identified four sesquiterpenoidarenarone derivatives, 18-aminoarenarone (**7**), 19-aminoarenarone (**8**), 18-methylaminoarenarone (**9**), and 19-methylaminoarenarone (**10**), and the new dimericpopolohuanone F (**11**) a derivative of 19-aminoarenarone (**8**) and arenarol(**5**), from the Australian marine sponge *Dysidea* sp. together with the known compounds arenarol(**5**) and popolohuanone A (**12**). PopolohuanonesA(**12**) and F (**11**) and arenarol(**5**) are antioxidants exhibiting DPPH radical scavenging activity with $IC_{50}$ values of 35, 35, and 19 μM, respectively.[6] Thepopolohuanones A (**12**) and B (**13**) were first reported from a Pacific *Dysidea* sp. The popolohuanones are inhibitors of topoisomerase-II and exhibit highly selective cytotoxicity against human non-small cell lung cancer cells.[7]

The *Dysideae*, including several species of the genus *Dysidea,* usually yield bi- or tricyclic sesquiterpenes ($C_{15}$ compounds) with various carbon skeletons and frequently possessing a furan functionality; typical examples are the furanoidsesquiterpenes, herbadysidolide(**14**) and herbasolide,(**15**)[8] and spirodysin(**16**)[9], constituents of *Dysidea herbacea.*

Dysetherin(**17**), an epoxy lactone has been also reported from the sponge of the *Dysidea* sp.[10] Sesquiterpenoid(**18**) was identified as the didehydro analogue of herbadysidolide(**14**).[11] A lactone furodysinin-O-methyl lactone (**19**) and its 6-hydroxy derivative (**20**) has also been reported from *Dysidea* sp.[11-13]

Furodysin(**21**) and furodysinin(**22**) have been isolated with two related thioacetates; thiofurodysin acetate (**23**) and thiofurodysinin acetate (**24**) from *Dysidea* sp. from Sydney, Australia.[14] Four samples of *Dysideaherbacea* contained a variety of new sesquiterpenes; five furodysinin derivatives (**25-29**), a furodysin derivative (**30**), a linear sesquiterpenes furan (**31**) and euryfuran(**32**).[15-17] The Pallescensins, a group of sesquiterpene furans are also known from *D. pallescens*(**33-38**).[18] *Dysidea etheria* from Bermuda contained a furodysinin lactone (**39**).[19]

Nakafuran-8 (**40**) and nakafuran-9 (**41**) two furanosesquiterpenes reported from Hawaian *D. fragilis* inhibit fish feeding.[20] An aldehyde (**42**) possibly derived from furanosesquiterpenes precursor was isolated from *D. fragilis*.[21] *D. fragilis* from Brittany contained penlanfuran(**43**) which is a new structural variation of the furano sesquiterpenes.[22]

Chemical investigation of a *Dysidea* sponge, collected at Siquijor, Philippines, has afforded two sesquiterpenoid metabolites, furodysin lactone (**44**) and pyrodysinoic acid (**45**). The known 3,6,11-trihydroxy-9,11-seco-ergostane-7,24(28)-dien-9-one (**46**)was also isolated from the sponge sample.[23]

Three nitrogen containing terpenes related to pyrodysinoic acid (**45**), Isopyrodysinoic acid (**47**), 13-hydroxy isopyrodysinoic acid (**48**) and pyrodysinoic acid B (**49**) have been identified from the Brazilian sponge *Dysidea robusta*. Pyrodysinoic acid B (**49**) is the first furodysin or furodysinin sesquiterpene derivative with a *trans* junction between the two six-membered rings of the 1,2,3,4,4a,7,8,8a-octahydro-1,1,6-trimethylnaphthalene moiety.[24]

A Bermudian sponge *Dysidea etheria* yielded sesquiterpenes, 5-acetoxy and 5-hydroxynakafuran 8(**50-51**).[25] Sesquiterpenes, lingshuiolides A (**52**) and B (**53**), lingshuiperoxide(**54**), isodysetherin(**55**), and spirolingshuiolide(**56**) were isolated from the Hainan marine sponge *Dysidea septosa*. The structures of new compounds were determined by detailed analysis of 1D and 2D NMR spectra and by comparison with related model compounds. The absolute configuration of lingshuiolide B (**53**) was established by using the modified Mosher's method. Spirolingshuiolide(**56**) represents the first example of a sesquiterpene with a rearranged drimane skeleton.[26]

Modified sterol, herbasterol, an ichthyotoxic 9, 11-Secosterol(**57**) has been reported from the sponge *Dysidea herbacea*.[27]The marine sponge *Dysidea amblia* contained two major metabolites, Ambliol-A (**58**) and Ambliol-B (**59**).The diterpenes are the first to be isolated from a *Dysidea* species.[28]

A new meroterpenoid avinosol(**60**), have been isolated from the marine sponge *Dysidea* sp. collected in Papua New Guinea. Avinosol(**60**), which is apparently the first example of a naturally occurring meroterpenoid-nucleoside conjugate, showed anti-invasion activity in a cell-based assay.[29]

Two polybrominated diphenyl ethers, 3,5-dibromo-2-(2′,4′-dibromophenoxy)phenol (**61**) and 4,6-dibromo-2-(2′,4′- dibromophenoxy)phenol (**62**) and (**63**) were isolated from an extract prepared from *Dysidea* sp. Tie2 kinase, an enzyme that supports angiogenesis and is essential for tumor growth and survival, was selected as a target in a search for naturally occurring inhibitors of potential utility for antitumor therapy and (**61-63**) were obtained after bioassay-guided fractionation.[30]

A sample of *D. herbacea* from Figi contained 2-(3,5-dibromo-2-methoxy-phenoxy)-3,5-dibromo anisole (**64**).[31]A polybrominated oxydiphenol derivative (**65**) was identified from the sponge *Dysidea herbacea*.[32] Brominated metabolites (**66-73**) were also reported from a tropical marine sponge of the genus *Dysidea*.[33-34] A pentabrominated phenolic diphenyl ether (**74**) that has not previously been reported

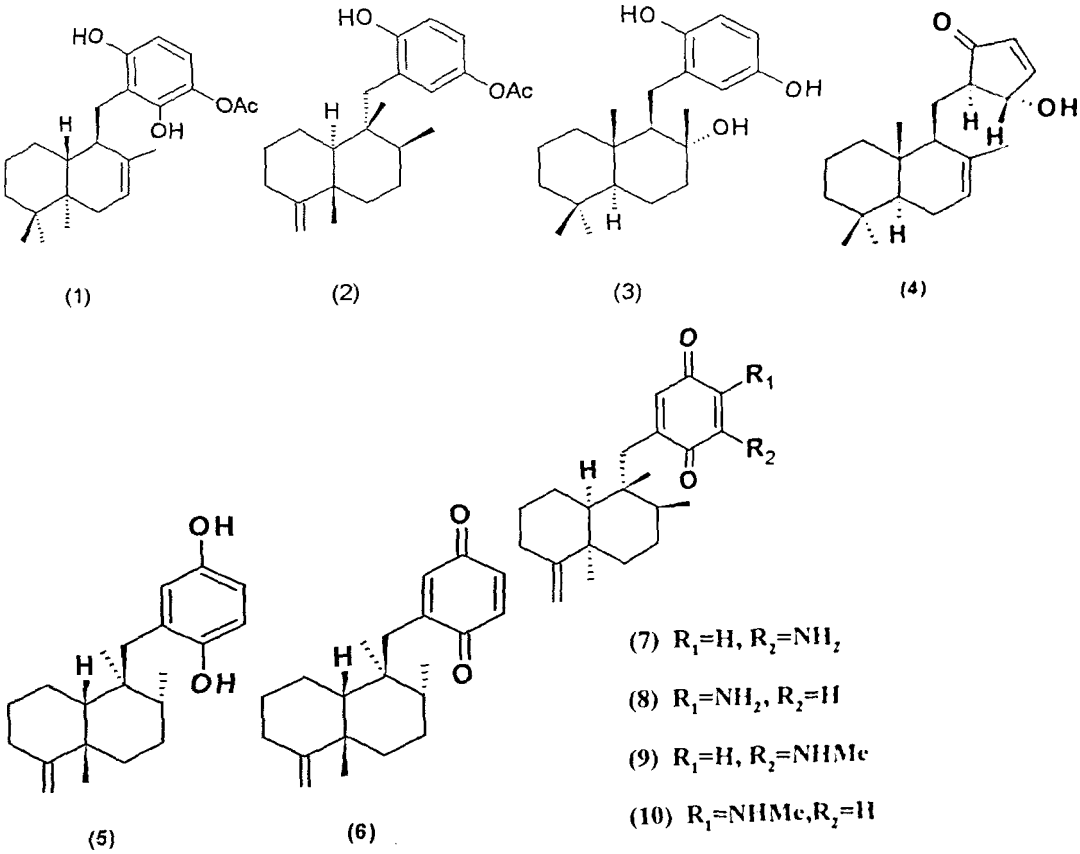
from marine sources has been isolated from *Dysidea herbacea* collected at Pelorus Island, Great Barrier Reef, Australia.[35]

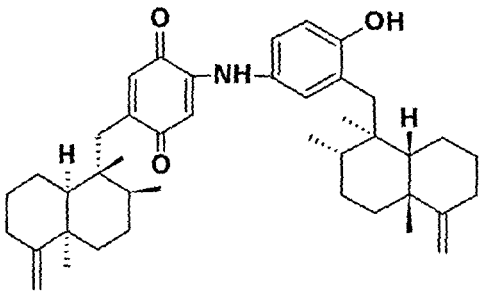
Chemical investigation of a marine sponge, *Dysidea* sp., collected at Bararin Island, Philippines, led to the novel metabolites(**75-80**) proline-derived analogues of dysidenin (**81**). The compounds (**75-80**) were termed Dysideaprolines A–F, respectively. Also isolated were barbaleucamides A(**82**) and B(**83**), structural analogues of barbamide(**84**) and (**10**), metabolites originally isolated from the cyanobacterium *Lyngbya majuscula*. It is most probable that the compounds presented here are actually derived from a symbiotic cyanobacterium found in close association with the *Dysidea* sp. Structure elucidation of the isolated metabolites involved high-field 2D NMR spectroscopy including $^{1}H-^{1}H$ COSY, HSQC, and HMBC.[36]

A chlorinated metabolite, herbacic acid (**85**) has been isolated from sponge *D.herbacea*.[34,37] Red Sea *Dysidea herbacea* afforded a hexachloro metabolite, dysidamide (**86**) whose structure has been established from spectroscopic and chemical evidence, and confirmed by X-ray crystal structure determination. Crystals of (**86**) are orthorhombic $P2_12_12_1$, a 20.509(13), b 18.411(11), c 11.356(9)Å, Z 8. The structure was refined by least-squares methods to a residual of 0.049 for 2277 'observed' reflections. Two different conformations of the molecule were present in the crystal.[38]

A number of amino acid derived chlorinated metabolites have also been reported from the genus *Dysidea*. Diketopiperazines(**87-88**) derived from trichloroleucine were isolated from the sponge *Dysidea herbacea*.[39] Chlorinated peptide sintokamide A(**89**) has been isolated from the marine sponge *Dysidea* sp. collected in Indonesia. Its structure was elucidated by a combination of spectroscopic and single-crystal X-ray diffraction analyses. Sintokamide A (**89**) is an inhibitor of N-terminus transactivation of the androgen receptor in prostate cancer cells.[40]

The polychlorinated alkaloids and brominated diphenyl ethers produced by the sponge have been shown to be associated with the filamentous cyanobacterium *Oscillatoria spongeliae* and are therefore likely synthesised by the cyanobacterial symbiont; in contrast, the terpene metabolites present in the sponge are localised in the sponge cells of *Dysidea*. The origin of metabolites of sponges has been a subject of discussion, as to whether they are products of bacterial, algal or sponge metabolism. Currently, it is believed that the brominated metabolites of the tropical sponge are the products of symbiotic metabolism while terpene metabolites result from metabolism of the sponges themselves.[41-42]





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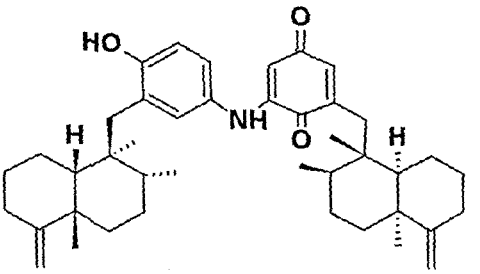

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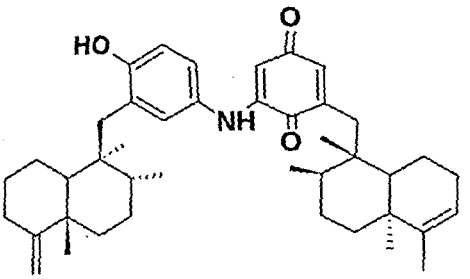

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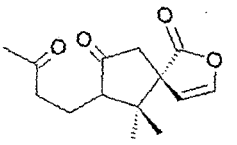

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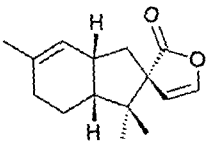

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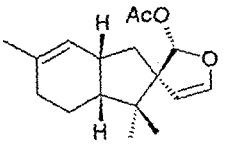

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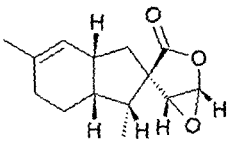

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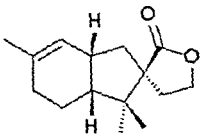

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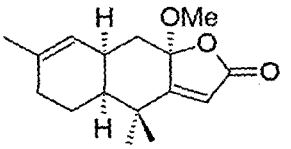

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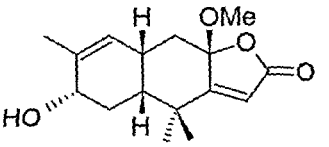

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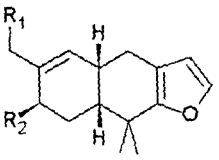
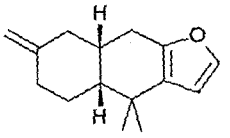


(21) $R_1$=$R_2$=H

(23) $R_1$=SAc, $R_2$=H

(30) $R_1$=H, $R_2$=OH



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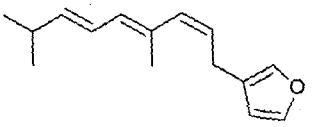

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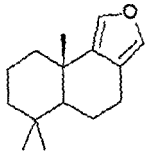

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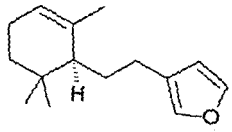

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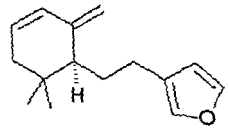

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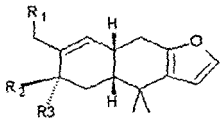


(22) $R_1=R_2=R_3=H$

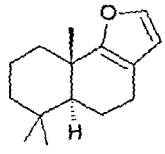
(24) $R_1=SAc$, $R_2=R_3=H$

(25) $R_1=R_3=H$, $R_2=OH$

(26) $R_1=R_3=H$, $R_2=OAc$

(27) $R_1=R_2=H$, $R_3=OH$

(28) $R_1=OH$, $R_2=R_3=H$



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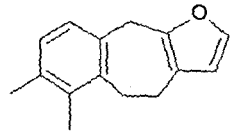

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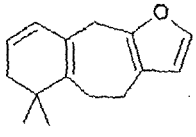

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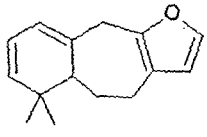

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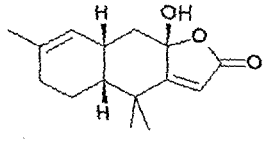

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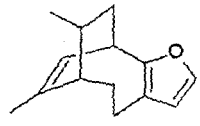

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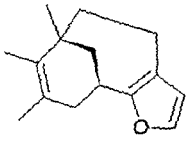

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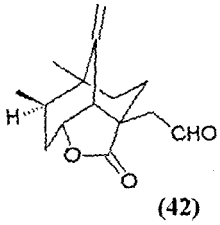

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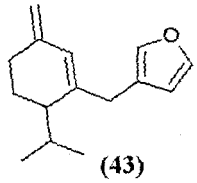

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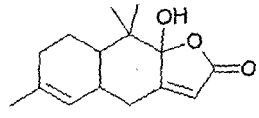

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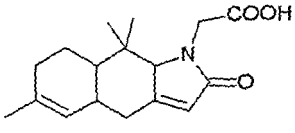

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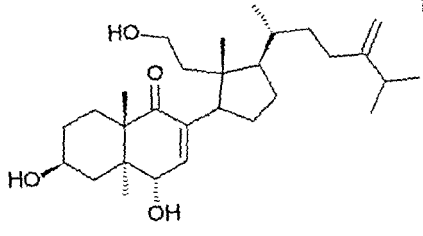

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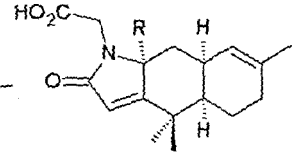
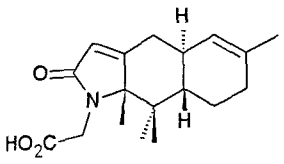


(47) R=H

(48) R=OH



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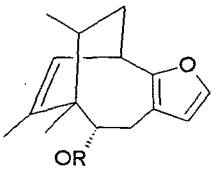
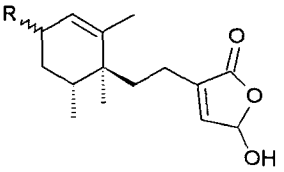


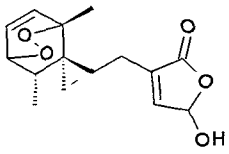
(50) R= Ac

(51) R= H



(52) R= alpha OH

(53) R= beta OH



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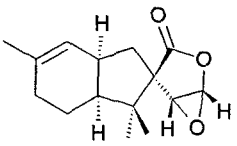

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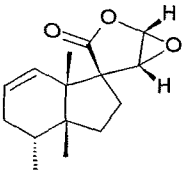

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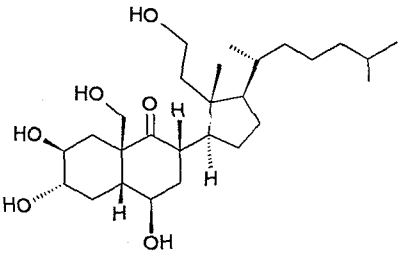

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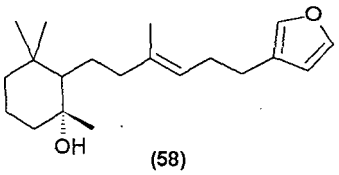

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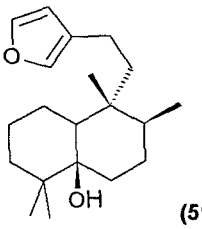

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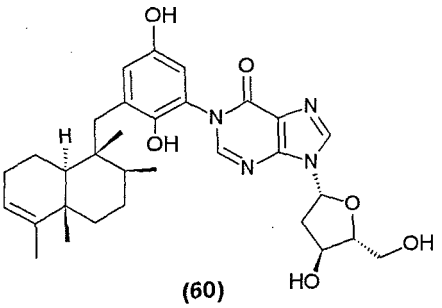

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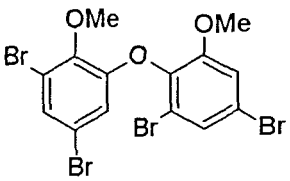

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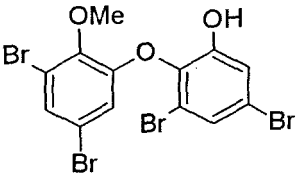

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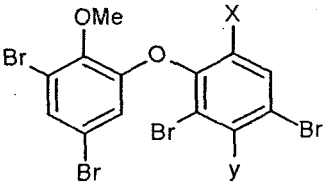
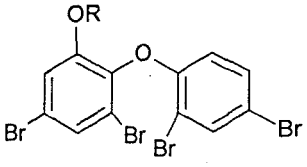


(66) X = Y = H

(67) X=OMe; Y= H

(68) X=OMe; Y=Br



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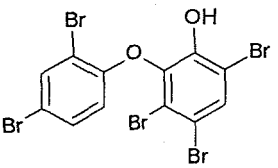

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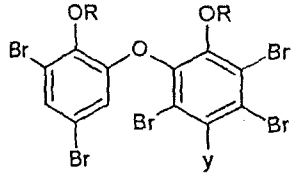
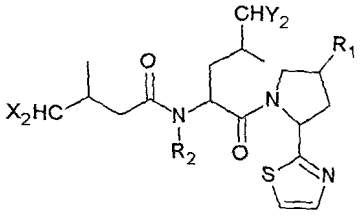


(69) Y= H; R= H

(70) Y=Br;R= H

(71) Y=H; R=Me

(72) Y=Br;R=Me



(75) $R_1$ = H, $R_2$ = $CH_3$, X = Cl, Y = Cl

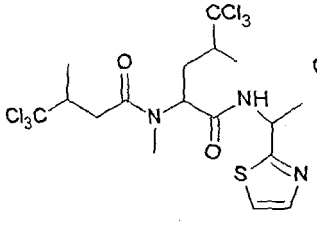
(76) $R_1$ = $CH_3$, $R_2$ = $CH_3$, X = Cl, Y = Cl

(77) $R_1$ = H, $R_2$ = H, X= Cl, Y = Cl

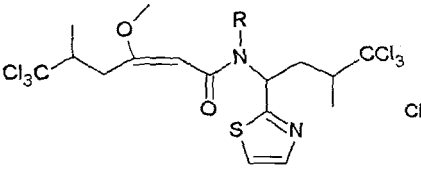
(78) $R_1$ = H, $R_2$ = $CH_3$, X = H, Y = Cl

(79) $R_1$ = H, $R_2$ = $CH_3$, X = Cl, Y = H

(80) $R_1$ = H, $R_2$ = $CH_3$, X = Cl, $Y_2$= ClH

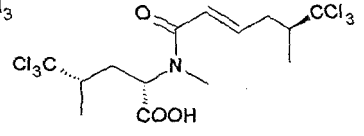


(81)



(82) R = H

(83) R = $CH_3$



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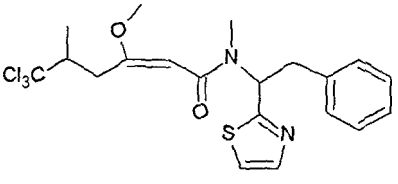

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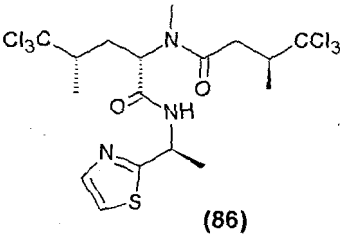

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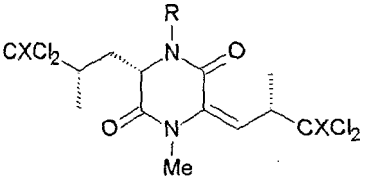
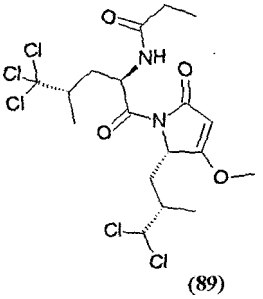


(87) X=Cl ; R=Me

(88) X= H; R=Me



(89)

In the section, which follows chemical investigation and bioactivity of the sponge ***Dysidea granulosa*** has been discussed.

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## Section 1.2: Antimicrobial and anticancer metabolite from the marine sponge, *Dysidea granulosa*

The striking rise in the prevalence of bacterial antibiotic resistance currently poses a serious threat to public health worldwide. Of particular concern are infections caused by methicillin-resistant *Staphylococcus aureus* (MRSA), penicillin-resistant *Streptococcus pneumoniae*, vancomycin-resistant *Enterococcus*[1] and *Mycobacterium tuberculosis.*[2] Many of these organisms have developedresistance to several classes of established antibiotics.[1,2] The most significant problem in clinical practice is the increase in incidence of MRSA infections. At present, the only effective treatment for multiple resistant MRSA infections is vancomycin. However, there are number of reports of emerging vancomycin resistance in some MRSA isolates [3]. Another group of clinically relevant multiple drug resistant bacteria that has emerged recently are *Enterococci*, some of which also exhibit vancomycin resistance. The appearance of vancomycin resistant *Enterococci* (VRE) infections has caused a dilemma upon physicians –linezolid and streptogramin combinations are the new drugs of choice for treating MRSA infections, but linezolid resistance have been reported in VRE and MRSA isolates.[4] Resistance to these oxazolidinone[5] streptogramin combinations [6,7] and various glycopeptides[5] requires expanded development of agents with alternative targets or modes of action. *Staphylococcus aureus* is the most clinically important of the Gram positive pathogens because of its exceptional virulence, stress tolerance, and capacity to accumulate antimicrobial resistances. MRSA is known as amajor nosocomial pathogen which has also developed resistance to many other antibiotics. Moreover, MRSA and other *S. Aureus* strains have been reported to acquire resistance to the last-resort antibiotic, vancomycin.[8,9] These facts suggest that *S. aureus* (MRSA) could fully acquire resistance to vancomycin in the near future.

The emergence and spread of resistant nosocomial and community-acquired pathogens is becoming a great menace to global public health. Therefore, a need is perceived to find new classes of antimicrobials. 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 [10,11].

Given the diverse array of bioactive secondary metabolite chemical structures with a wide variety of biological activities isolated from marine sponges, natural product chemists have long been fascinated by these sessile marine invertebrates [11]. One of the most extensively studied species of sponge is the tropical marine sponge *Dysidea*sp. (family *Dysideidae*, Order *Dendroceratida*), a pinkish green sponge that occurs in a number of distinct chemotypes as a result of an association with symbiotic microorganisms. The chemistry reported from the genus *Dysidea* has already been discussed in the review presented in the first section of this chapter.

**1.2a.Screening of *Dysidea granulosa* for biological activity:**

In our continuous search for biologically active metabolites from marine organisms under the programme 'Development of Potential Drugs from the India Ocean' the sponge *Dysidea granulosa* (Bergquist) collected off the coast of Lakshadweep Islands, India, was screened for its bioactivity particularly antimicrobial and anticancer properties in search of new promising molecules. For the purpose, the frozen sample was lyophilized and extracted with methanol. The extract was then screened for antimicrobial and anticancer activities and found to be active.This property of this extract led us to investigate further leading to the isolation of the active principle. It was found that methanolic extract of the sponge *Dysidea granulosa* (Bergquist) was antibacterial, antifungal and also exhibited anticancer activity against 5 cancer cell lines.Bioassay guided fractionation determined that the activity was located in petroleum ether and chloroform fractions. As both, petroleum ether as well as chloroform fractions were active fresh sample of *Dysidea granulosa*

was extracted successively with ethyl acetate and methanol. Ethyl acetate fraction was used for further investigation i.e. for the isolation and identification of active principle, such that wedo not miss potent molecules (antimicrobial and anticancer constituent), present in both the active fractions.

In this section we have reported the isolation of 2-(2',4'-dibromophenoxy)-4,6-dibromophenol from a new source *D. granulosa*, its potent *in-vitro* antibacterial activity, and as a possible lead molecule for drug development especially against strains of MRSA, methicillin sensitive *S. aureus*(MSSA), VRE and vancomycin sensitive *Enterococci* (VSE).

In initial screening the crude methanolic extracts exhibited antibacterial activity against; *E.coli, Salmonella typhi, Shigella flexineri, Klebsiella pneumoniae, Pseudomonas aeruginosa, Staphylococcus aureus,* Methicillin-resistant *Streptococcus (MRS)*, and was antifungal against *E. faecium, A. fumigatus* and *Candida albicans.* Anticancer activity was observed against 5 cancer cell lines: 96% against Pancreatic (Panc-I), 81% against cervical (SiHa), 99% against human lung large cell carcinoma (H460), 92% against renal adenocarcinoma (ACHN) and 93% against human lung carcinoma (Calu-1). After repeat screening and confirmation of the observed antimicrobial activity against pathogenic strains in the ethyl acetate extract, the active fraction was chromatographed over Sephadex LH20 using 1:1 methanol-chloroform as the eluent. Chromatographed fractions showing similarprofile by thin-layer chromatography were combined. The partially purified sample was further purified through stepwise gradient elution over silica gel (60–120 mesh, 2% ethyl acetate: petroleum ether) to afford the single major compound identified as polybrominated secondary metabolite2-(2',4'-dibromophenoxy)- 4, 6-dibromophenol **(Fig.1.1)** on the basis of detailed spectral analysis.

### 1.2b. Identification of 2-(2',4'-dibromophenoxy)-4,6-dibromophenol-Spectral Data

2-(2',4'-dibromophenoxy)-4,6-dibromophenol was obtained from the sponge *Dysidea granulosa* as a colourless optically inactive crystalline solid, mp 179°C. In methanol

it showed UV $\lambda_{max}$ at 340 nm. In its Infra-Red (KBr) (**Fig.4**) major peaks were observed at 3,492.8 (phenolic O-H str), 3,084.0 (aromatic C-H str), 1,591.5 (aromatic C=C str), 1,463.9(C-H bend), 1,147.6, 1039.6 (C-O str), 918.1, 869.8, and 719.4 cm-1 (C-Br str). Its NMR data including $^{1}$H-NMR (300 MHz, CDCl3), $^{13}$C-NMR, COSY, HMQC and HMBC of the compound is given in **Table 1.1** and **Fig.1.2** and **Figs.1.5-1.10**.

**Table 1.1:**$^{1}$H, $^{13}$CNMR, COSY and HMBC of 2-(2',4'-dibromophenoxy)-4,6-dibromophenol

| **Carbon No.** | **$^{13}$CNMR $\delta_C$, ppm** | **$^{1}$HNMR $\delta_H$, ppm** | **COSY correlations** | **HMBC correlations** |
|---|---|---|---|---|
| 1 | 138.60 | - | - | - |
| 2 | 150.30 | - | - | - |
| 3 | 119.70 | 7.20 (d, 2.3Hz) | - | 1, 2, 5 |
| 4 | 117.30 | - | - | - |
| 5 | 127.80 | 7.34 (d, 2.3Hz) | - | 4, 6 |
| 6 | 119.80 | - | - | - |
| 1' | 152.00 | - | - | - |
| 2' | 112.60 | - | - | - |
| 3' | 136.20 | 7.77 (d, 2.3Hz) | - | 1', 5' |
| 4' | 116.00 | - | - | - |
| 5' | 131.50 | 7.29(dd, 8.8Hz) | H6' | 3', 1' |
| 6' | 115.90 | 6.43 (d, 8.8Hz) | H5' | 1', 2' |

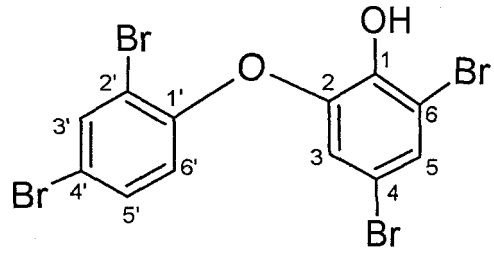



**Fig.1.1: Structure of 2-(2',4'-dibromophenoxy)-4,6-dibromophenol**

Its ESI-MS spectrum **(Fig.1.11)** showed a deprotonated $[M-H]^-$ ion peak at m/z 500.7023 (calculated: 500.6982); the corresponding $[2M-2H]^-$ peak was observed at m/z 1001.7274 (calculated: 1001.3964). The mass spectral pattern with the respective spectral peaks at $[M-H]^-$ at 496.7042, 498.7054, 500.7023, 502.7010 and 504.6974 (observed) and 496.7023, 498.7003, 500.6982, 502.6962 and 504.6942 (calculated) in the ratio 17.30:68.0:100.0:65.70:16.50 were indicative of the presence of four bromine atoms. The presence of bromine atoms were further confirmed by the twin peaks at 79 and 81 in its MS/MS spectrum **(Fig.1.12)**

The spectral data ($^1$H-NMR, $^{13}$C-NMR) obtained was compared with data reported and found to be in good agreement with the literature values for 2-(2',4'-dibromophenoxy)-4,6-dibromophenol. [12,13] The two dimensional NMR data is being reported here for the first time.

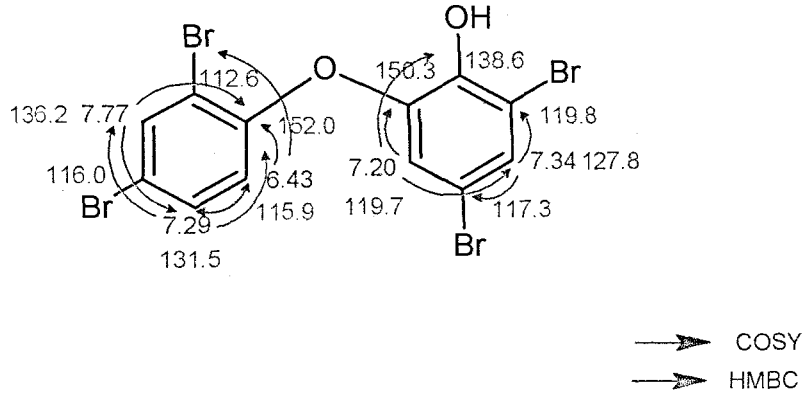



**Fig.1.2: COSY and HMBC correlations of 2-(2',4'-dibromophenoxy)-4,6-dibromophenol**

## Section 1.3: Biological Activity

Since the active chloroform fraction contained 2-(2',4'-Dibromophenoxy)-4,6-dibromophenol as the major constituent it was quite possible that the activity observed was due to this constituent of the fraction. This led us to check the activity of this halogenated metabolite.

### 1.3.a. Antimicrobial activity

2-(2',4'-Dibromophenoxy)-4,6- dibromophenol was observed to be a potent inhibitor of the growth of clinically relevant Gram positive bacteria, especially MRSA and VRE. Preliminary testing by the agar diffusion method revealed that the compound is strongly antibacterial, but has no inhibitory effect against fungal strains. To evaluate its *in-vitro* antibacterial potency and characterize its activity spectrum, the compound was tested against 57 bacterial test strains. The efficacy is measured in terms of minimum inhibitory concentration (MIC) and expressed in μg/mL.

Minimum Inhibitory Concentration (MIC) values were determined in Mueller Hinton Broth by the broth macrodilution method according to NCCLS guidelines, as per document no. M7-A5.[14] The medium with a twofold serial dilution of compound in Mueller Hinton broth was inoculated with $10^5$ colony forming units/mL of test culture and incubated at 37 °C for 24 hrs. The MIC of the bromoether was determined against 57 test organisms including different clinical and standard strains of *S. aureus*, both MRSA and MSSA, clinical and inhouse strains of *Enterococci*, both VRE and VSE and some gram negative strains. American type culture collection (ATCC) strains were also screened. Linezolid (Glenmark Pharmaceuticals Ltd, India) was used as control agent for antibacterial activity.

The spectrum of bacterial test cultures included 20 clinically derived and 10 standard strains of MRSA, two standard strains of MSSA; 11 clinical strains of VRE, four standard strains of VRE, five standard strains of VSE; one each standard strains of *E. coli, K. pneumoniae, S. typhi, S. flexneri*and *P. aeruginosa,* including three methicillin resistant strains of *S. aureus*3066, E710, ATCC 33591 and two

vancomycin resistant strains of *E. faecium* 02 D3 IP1, R-2 and one vancomycin resistant strain of *E. faecalis* ATCC 51299, which were subjected to antibacterial activity tests.

The compound showed potent, broad-spectrum *in-vitro* Gram-positive activity, as evident from **Table 1.2**. The compound exhibited no cross-resistance to other key antibiotics, which was evident from its potent activity against key antibiotic-resistant bacteria including MRSA, erythromycin-resistant *S. aureus*, and VRE (**Table 1.3**). *Escherichia coli* and *Pseudomonas* sp. were the least susceptible, with MIC values above 15 µg/mL(**Table 1.2**). However, it was active against other Gram negative bacteria such as *Klebsiella pneumoniae*, *Shigellaflexneri*and *Salmonella typhi*(**Table 1.2**).

The bromoether was tested by the agar diffusion assay against pathogenic fungal strains; *C. albicans*, *A. fumigatus*, *C. tropicalis*, *C. glabrata*and was found to be inactive against fungi even up to 100 µg/mL(**Table 1.4**). Agar well diffusion assay was used with a well size of 6mm diameter and volume of the sample loaded was 50 µL. Amphoterecin B was used as control for antifungal activity
The compound is active in *A. fumigatus* at 50-100 µg/mL and is poorly active in *C.albicans*being activeonly above 500 µg/mL and did not exhibit significant activity in *C. tropicalis* and *C. glabrata*. Thus, the compound specifically shows antibacterial activity. Our work has first time revealed the wide spectrum of activity of this compound.

**Table 1.2:** Antibacterial activity of 2-(2',4'-dibromophenoxy)-4,6-dibromophenol

| Test Organisms | MIC Range (µg/ml) | $MIC_{50}$ (µg/ml) | $MIC_{90}$ (µg/ml) |
|---|---|---|---|
| ***S. aureus**** **MRSA (20)** | 0.131-1.25 | 0.313 | 0.625 |
| ***S.aureus***** **MRSA (10)** | 0.131-10 | 0.313 | 0.625 |

| | | | |
|---|---|---|---|
| ***S.aureus*** ** MSSA (2) | 0.117-0.313 | - | - |
| ***Enterococci**** **VRE (11)** | 0.625-2.5 | 0.625 | 1.25 |
| ***E. faecalis***** **VSE (4)** | 1.25-2.5 | - | - |
| ***E. faecium***** **VSE (1)** | 1.25 | - | - |
| ***E. faecium***** **VRE (3)** | 1.25 | - | - |
| ***E. faecalis***** **VRE (1)** | 1.25 | - | - |
| ***E. coli***** **(1)** | >15 | - | - |
| ***K. pneumoniae***** **(1)** | 2 | - | - |
| ***Salmonella typhi***** **(1)** | 0.5 | - | - |
| ***Shigellaflexneri*** **Type IV**** **(1)** | 2 | - | - |
| ***P.aeruginosa***** **M-35 (1)** | >15 | - | - |

*: Clinical derived strains,

**: Standard strains procured from culture banks.

-: Not applicable,

# the numbers in brackets indicates the number of strains tested.

**Table 1.3:** Antibacterial activity of 2-(2',4'-dibromophenoxy)-4,6-dibromophenol against some resistant strains

| **Test organisms** | **MIC (µg/ml)** | **Standard Linezolid (µg/ml)** |
|---|---|---|
| *S .aureus* (3066), MRSA,Ery$^R$ | 0.313 | 0.313 |
| *S. aureus (E710)*, MRSA, *Ery$^R$* | 0.625 | 2.5 |
| *S. aureus ATCC 33591*, MRSA | 0.313 | 2.5 |
| *E. faecium (02 D3 IP1), Vanco$^R$, Teicho$^R$* | 1.25 | 2.5 |
| *E. faecium (R-2), Vanco$^R$ (VanA)* | 1.25 | 2.5 |
| *E. faecalis ATCC 51299*, VRE, *Gen$^R$, Strept$^R$(VanB)* | 2.5 | 2.5 |

**Table 1.4:** Antifungal activity of 2-(2',4'-dibromophenoxy)-4,6-dibromophenol.

| Fraction number | Conc. (µg/ml) | *A.fumigatus* | *C.albicans* | *C. tropicalis* | *C. glabrata* |
|---|---|---|---|---|---|
| 1 | 1 | - | - | - | - |
| 2 | 5 | - | - | - | - |
| 3 | 20 | 8.2h | - | - | - |
| 4 | 50 | 9.3 | - | - | - |
| 5 | 100 | 10.5 | - | - | - |
| 6 | 500 | 12.6 | 8 | - | - |
| 7 | 1000 | 13 | 8 | 9 | 8 |
| Amphotericin B | 100µg/disc | 14 | 11.4 | 11 | 13 |

h-hazy zone

Its activity with a variety of resistant strains confirmed that 2-(2',4'-dibromophenoxy)-4,6-dibromophenol did not show cross resistance with widely marketed antibiotics such as erythromycin, β-lactams, vancomycin, teichoplanin, gentamicin and streptomycin. Thus it reflects that the type of bacterial strains used for antibacterial efficacy testing has no resistance to this compound. Therefore it could be a potential molecule to develop further to address the resistance issue.

This is the first report of 2-(2',4'-dibromophenoxy)-4, 6-dibromophenol from the sponge *Dysidea granulose* and the first attempt to exhaustively test the *in-vitro* activity of this molecule. Results obtained indicate that the compound shows good lead properties for antibacterial drug development and represent a promising agent for the treatment of *S. aureus* as well as enterococcal infections caused by drug-resistant strains.

### 1.3.b. Antimycobacterial activity of 2-(2',4'-dibromophenoxy)-4,6-dibromophenol

Mycobacteria have gained a very special place in the bacterial world due to their clinical importance. Alone, *Mycobacterium tuberculosis* is responsible for about two

million deaths annually worldwide making tuberculosis one of the most influential diseases in the history of mankind. Almost half of the Non-tuberculous *Mycobacteria* species identified are associated with opportunistic infections in animals and humans. Although having been studied for centuries, it is still responsible for more human deaths than any other single infectious disease, and was declared a global public health emergency by the World Health Organization (WHO) in 1993. Since 2-(2',4'-dibromophenoxy)-4,6-dibromophenol,exihibited good antibacterial activity against multidrug resistant bacteria, the compound was tested for antimycobacterial activity.

**1. Preliminary assays:** The assays were performed on *Mycobacterium fortuitum* and *Mycobacterium abscessus*, 2 non-tuberculous mycobacteria usually more susceptible to drugs than *Mycobacterium tuberculosis.*

A concentration of 256 micrograms per mL significantly inhibited growth. MIC *for M. Abscessus* and *M. fortuitum* should be close to 300 micrograms per mL as per International Standards

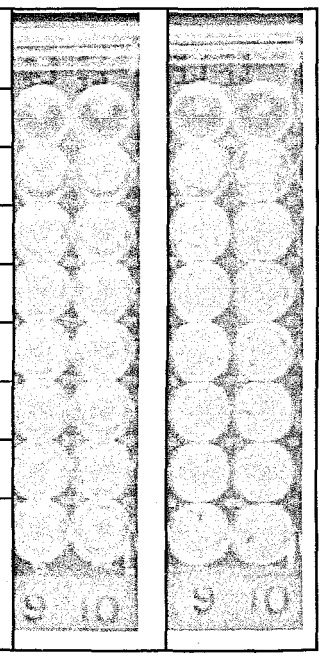
| 2-(2',4'-dibromophenoxy)-4,6-dibromophenol **(µg/mL)** |  |
|---|---|
| **256** | |
| **128** | |
| **64** | |
| **32** | |
| **16** | |
| **8** | |
| **4** | |
| **0 Pos. Control.** | |

**Fig.1.3: Antimicobacterial activity of 2-(2',4'-dibromophenoxy)-4,6-dibromophenol**

### 1.3.c. Anticancer activity:

**1.In vitro evaluation of cytotoxic activity: methodology**

A protocol of 48 hr continuous drug exposure was used and a sulforhodamine B (SRB) protein assay was used to estimate cell viability or growth. The cell lines were grown in RPMI 1640 medium containing 10% foetal bovine serum and 2 mM L-glutamine, and were inoculated into 96-well microtiter plates in 90 μL at plating densities depending on the doubling time of individual cell lines. The microtiter plates were incubated at $37^0$C, 5% $CO_2$, 95% air and 100% relative humidity for 24 hr prior to addition of experimental drugs. Aliquots of 10 μL of the drug dilutions were added to the appropriate microtiterwells already containing 90 μL of cells, resulting in the required final drug concentrations. Each compound was evaluated for four concentrations (0.1, 1, 10 and 100 μL) and each was done in triplicate wells. Plates were incubated further for 48 hr, and assay was terminated by the addition of 50 μL of cold trichloro acetic acid (TCA) (final concentration, 10% TCA) and plates were again incubated for 60 min at 4°C. The plates were washed five times with tap water and air-dried. Sulforhodamine B (SRB) solution (50 μL) at 0.4% (w/v) in 1% acetic acid was added to each of the wells, and plates were incubated for 20 min at room temperature. The residual dye was removed by washing five times with 1% acetic acid. The plates were air-dried. Bound stain was subsequently eluted with 10 mMtrizma base, and the absorbance was read on an ELISA plate reader at a wavelength of 540 nm with 690 nm reference wavelengths. Percent growth was calculated on a plate-by-plate basis for test wells relative to control wells. The above determinations were repeated three times.

The percentage cytotoxicity / antiproliferative activity of the extracts and the isolated compound 2-(2',4'-dibromophenoxy)-4,6-dibromophenol was evaluated in vitro against 21 tumor cell linesusing the standard Sulphorodhamine blue (SRB) assay . They showed strong Cytotoxic activity against; Human cervical cancer (ME180), Human bladder cancer (T24), Human breast cancer(ZR-75-1), Human prostate cancer (DU145), Human oral cancer DWD, Human colorectal

cancer(SW620), Human colon cancer (HCT15), Human prostate cancer(PC3), Human lung cancer (HOP62) and Human renal cell cancer(786-O). The $IC_{50}$ values are given in **Table 1.5**

**Table 1.5:Anticancer activity of *Dysidea granulosa* extracts and the active molecule**

| Cell line used for study | Name of the extract | | | |
|---|---|---|---|---|
| | Crude methanol extract | Hexane fraction | Chloroform fraction | 2-(2',4'-dibromophenoxy)-4,6-dibromophenol ($IC_{50}$ µM) |
| SiHa | 24 | - | - | $>10^{-4}$ |
| ME180 | 26 | - | - | $1.92x10^{-7}$ |
| T24 | 14 | <10 | <10 | $2.84x10^{-6}$ |
| MCF7 | <10 | <10 | <10 | $>10^{-4}$ |
| Zr-75-1 | 29 | - | - | $2.0x10^{-6}$ |
| DU145 | <10 | 21 | 21 | $3.76x10^{-5}$ |
| DWD | 20 | <10 | >80 | $3.31x10^{-5}$ |
| SW620 | 27 | - | - | $2.4x10^{-6}$ |
| Colo205 | 10 | 20 | 11 | $>10^{-4}$ |
| HCT15 | <10 | 20 | 11 | $2.2x10^{-6}$ |
| HT29 | <10 | - | - | - |
| MIA-PA-CA-2 | <10 | 15 | 15 | $>10^{-4}$ |
| A2780 | <10 | 11 | <10 | - |
| A549 | <10 | - | - | $>10^{-4}$ |
| U373MG | 11 | - | - | $>10^{-4}$ |
| PC3 | 13 | - | - | $2.0x10^{-6}$ |
| Hop62 | 29 | - | - | $2.3x10^{-6}$ |
| HL60 | <10 | - | - | $>10^{-4}$ |
| A498 | >80 | - | - | $>10^{-4}$ |

| 786-O | 27 | - | - | $2.03x10^{-6}$ |
|---|---|---|---|---|
| Total cell lines active | **19** | **8** | **7** | **10** |

Most halogenated phenols are found to be strongly antimicrobial. As a group, the halogenated bis(hydroxyphenyl) methanes and polybrominated diphenyl ethers exhibit a wide range of activities in bioassays, ranging from antibacterial activity (against *S. Aureus* and *T. mentagrophytes*), to cytotoxicity (Ehrlich ascite tumor cells).[15,16] Polybrominated biphenyl ethers from *Dysidea herbacea* are found to be active against eubacteria, cyanobacterium*Synechococcus*sp. [17]

The polychlorinated alkaloids and brominated diphenyl ethers produced by the sponge have been shown to be associated with the filamentous cyanobacterium *Oscillatoria spongeliae*, and are therefore likely synthesized by the cyanobacterialsymbionts.[17-20]

**1.4. Experimental Section**

**a. General Experimental Procedures**

Column chromatography was carried out on silica gel 60-120 mesh(Sisco, India). Gel filtration on Sephadex LH20 (Pharmacia Biotech, Sweden). Fractions were monitored on TLC kieselgel 60 F254aluminum backed sheets and visualized under UV (254 nm) and iodine vapours. UV-VIS spectrum was recorded in methanol using a Shimadzu UV-2401PC spectrophotometer. IR spectrum was recorded on a Shimadzu FTIR-8201 PC spectrophotometer. NMR spectra ($^{1}H$, $^{13}C$, COSY, HMQC and HMBC) were recorded on a BrukerAvance 300MHz instrument. Chemical shifts, relative to TMS as internal standard, are given in δ values. J values are given in Hz. Mass spectra were obtained on a QSTAR XL MS/MS system.

**b.Collection of Animal Material and Chromatography**

The sponge was collected off the coast of Lakshadweep Islands by scuba diving at a depth of about 8-10 meters and identified by Dr. P.A. Thomas of the Vizhingam

Research Center of the Central Marine Fisheries Research Institute, Kerala, India. A voucher specimen is deposited at the National Institute of Oceanography, Dona Paula, Goa, India. The frozen sample of *D. granulosa* was lyophilized to obtain 200 g of dried sample, which was extracted successively with ethyl acetate and methanol (200 mL × 3 times each). These extracts were concentrated under reduced pressure and temperature to obtain the crude extracts. The ethyl acetate extract exhibited antimicrobial activity against pathogenic strains and was chromatographed over Sephadex LH20 (1:1 methanol-chloroform). Fractions that were similar in composition as shown by thin-layer chromatography were combined. The partially purified sample was further purified through step gradient elution over silica gel (60–120 mesh) (2% ethylacetate:petroleum ether) to afford the single major polybrominated secondary metabolite2-(2',4'-dibromophenoxy)- 4, 6-dibromophenol(198 mg).

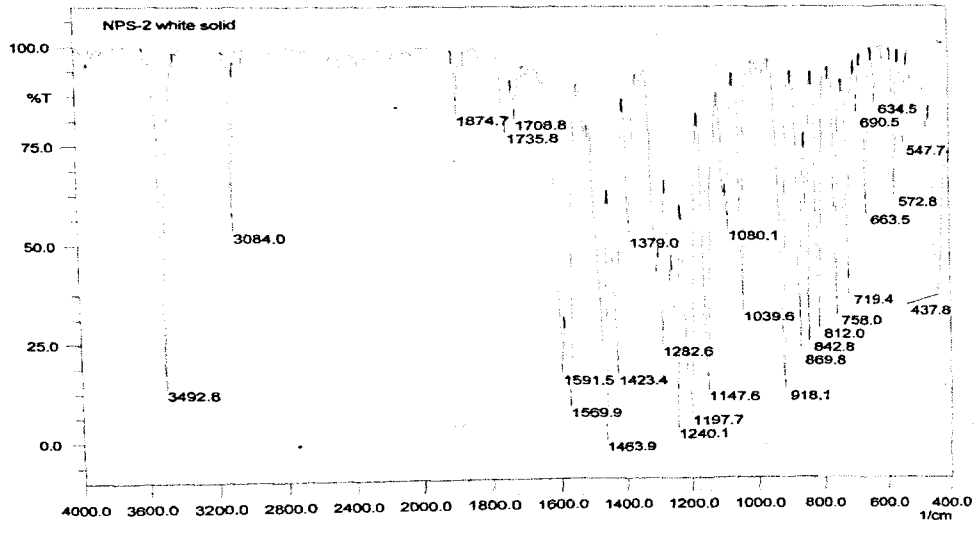



**Fig.1.4:** IR spectrum of 2-(2',4'-dibromophenoxy)-4,6-dibromophenol

**Fig.1.5:** $^{1}$H NMR spectrum of 2-(2',4'-dibromophenoxy)-4,6-dibromophenol

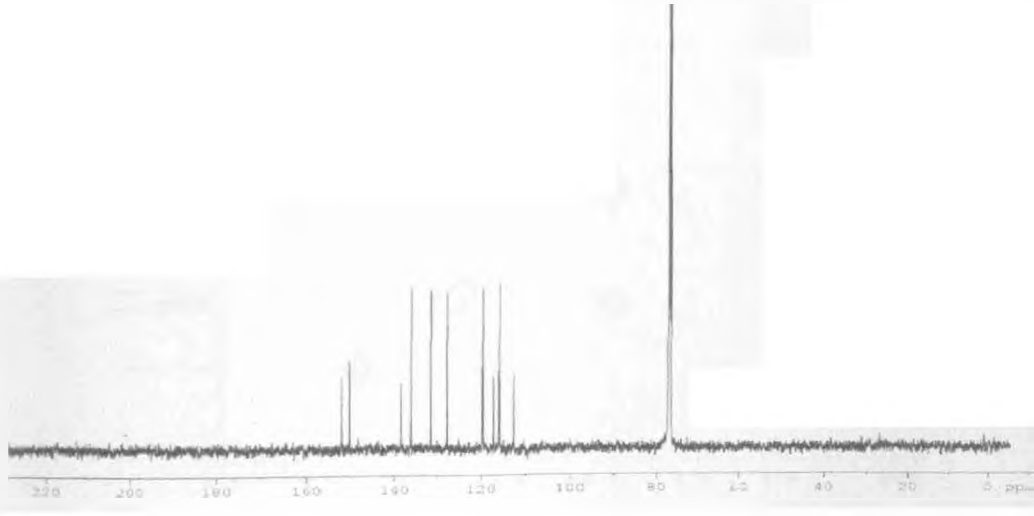



**Fig.1.6:** $^{13}$C NMR spectrum of 2-(2',4'-dibromophenoxy)-4,6-dibromophenol

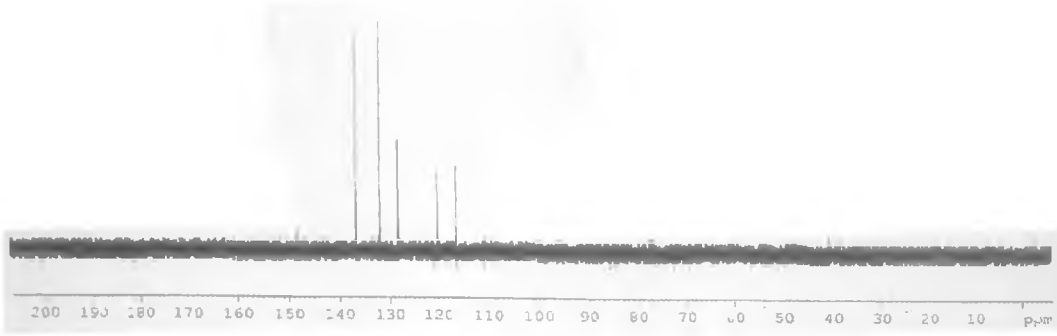



**Fig.1.7:** DEPT spectrum of 2-(2',4'-dibromophenoxy)-4,6-dibromophenol

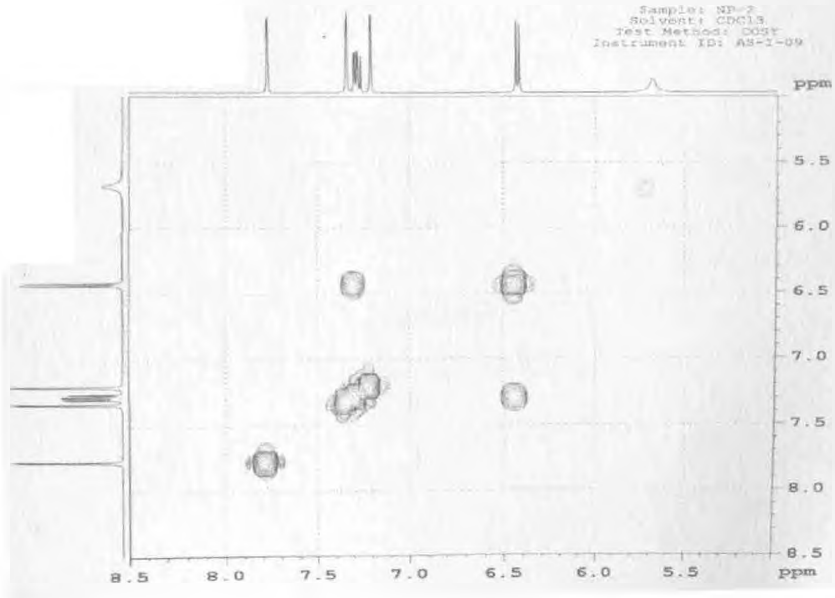



**Fig.1.8:** $^{1}H$-$^{1}H$ COSY spectrum of 2-(2',4'-dibromophenoxy)-4,6-dibromophenol

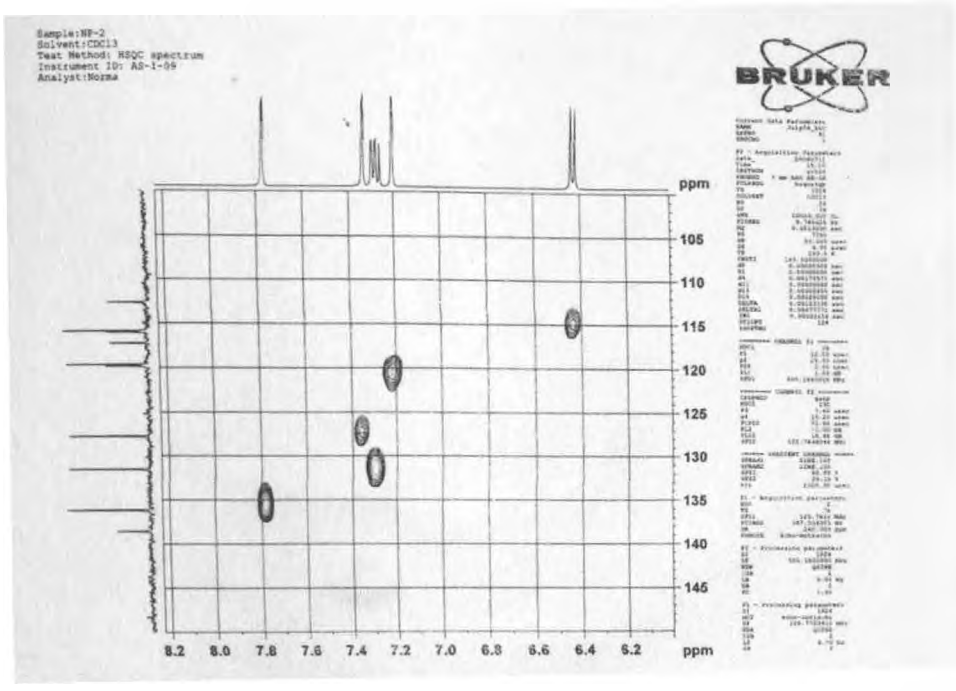



**Fig.1.9:** HSQC spectrum of 2-(2',4'-dibromophenoxy)-4,6-dibromophenol

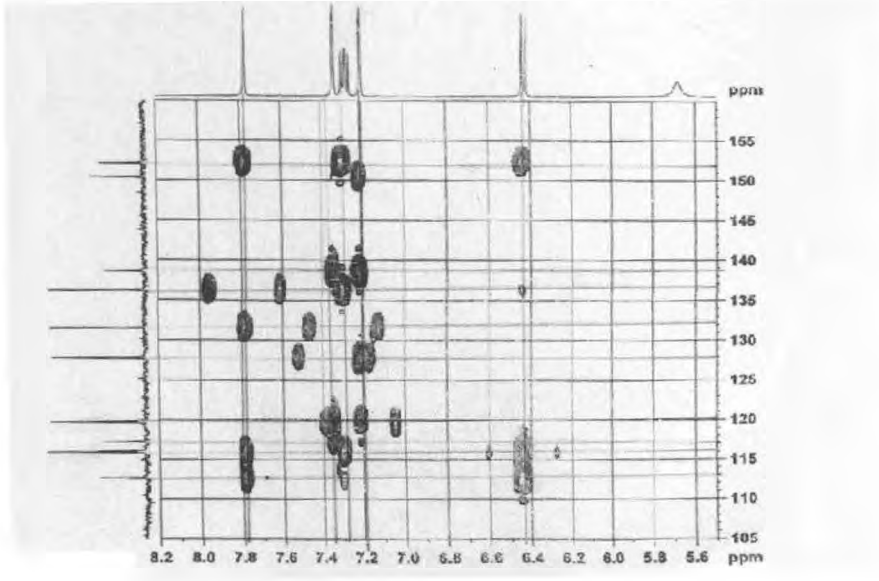



**Fig.1.10:** HMBC spectrum of 2-(2',4'-dibromophenoxy)-4,6-dibromophenol

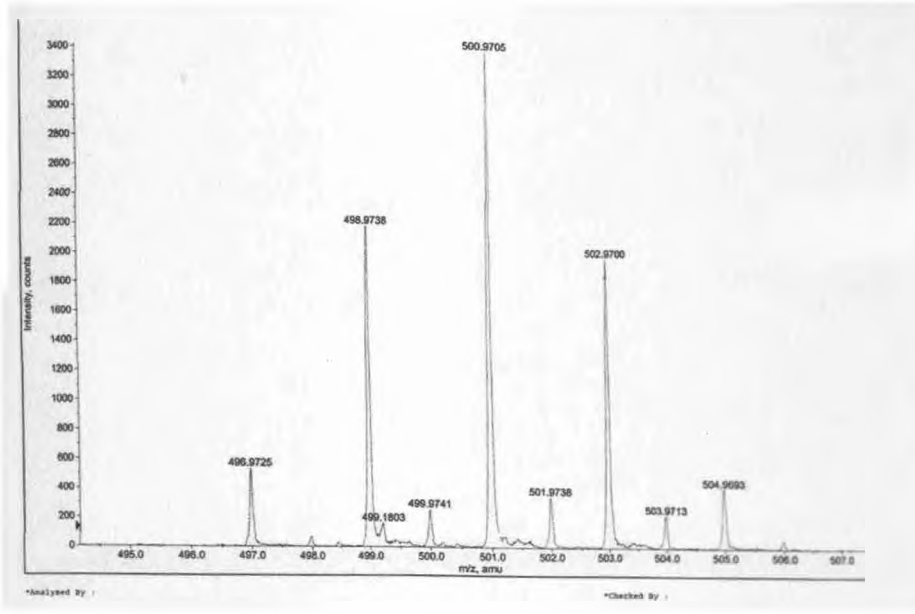



**Fig.1.11:** ESI-MS spectrum of 2-(2',4'-dibromophenoxy)-4,6-dibromophenol showing the ratio of peaks 1:4:6:4:1 indicating presence of 4 bromine atoms.

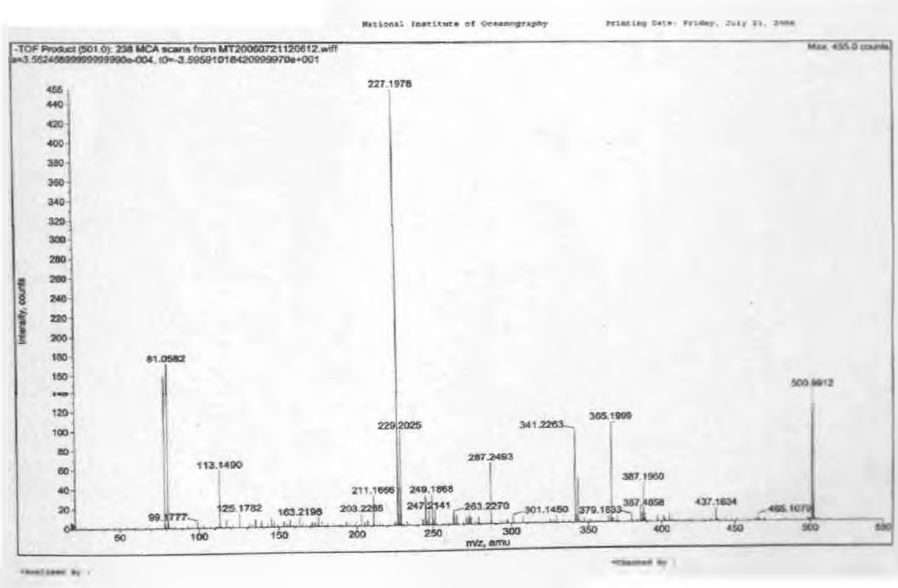



**Fig.1.12:** MS/MS spectrum of 2-(2',4'-dibromophenoxy)-4,6-dibromophenol

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# *Section II*

## *Psammaplysilla purpurea...*

**Section 2.1: *Psammaplysilla purpurea:* A review**

*Psammaplysilla purpurea* is a dark purple/black sponge, belonging to Phylum: Porifera, Class: Demospongiae, Order: Verongida, Family: Aplysinellidae, Genus: *Psammaplysilla* and Species: *purpurea.* The matrix of the sponge is extremely dense and heavily reinforced by collagen and the texture of the sponge may hence vary from rubbery to often extremely hard and incompressible. The surface is tuberculate and the purple pigmentation is uniform throughout the sponge.

The genus *Psammaplysilla* exists in 3 known species: *purpurea, purea and arabica.* Marine sponges of the genus *Psammaplysilla* are known to synthesize brominated tyrosine derivatives, many of which possess potent antimicrobial and cytotoxic activities. Purealins, Lipopurealins, Purealidins, Psammaplysins, Purpuramines, Aplysamines and Macrocyclic peptides Bastadins, have been previously reported form the sponge Psammaplysilla sp. This section of the thesis deals with a survey of literature on the different secondary metabolites isolated and identified from the genus *Psammaplysilla.*

Earlier studies on the sponge *P. purpurea* afforded, a dibromotyrosine metabolite, 2-hydroxy-3,5-dibromo-4-methoxyphenyl acetamide(**1**)[1] characterised through interpretation of spectral data. In 1978, Ayanoglu *et al.*,[2] isolated four sterols,

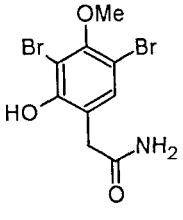
aplysterol(**2**), 3β-hydroxy-26,27-bis-norcholest-5-en-24-one (**3**), 3β-hydroxypregna-5-en-20-one (**4**) and 3β-hydroxy-5α-pregnan-20-one (**5**) along with the usual sterols in minor amounts. Three new bromotyrosine-cysteine derivatives, psammaplins B, C & D (**6-8**) and known 3-bromo-4-hydroxybenzenealdehyde (**9**) and 3-bromo-4-hydroxyphenyl acetonitrile (**10**) were isolated from the sponge *P. purpurea*. Psammaplin D (**8**) showed antimicrobial and mild tyrosine kinase inhibitory activity.[3]

Psammaplin-A (**9a**), containing a disulfide bridge along with 3-bromo-4-hydroxyphenylacetonitrile (**10**) has been isolated from the cytotoxic extract of an unidentified species of *Psammaplysilla*.[4] Kim *et al.* (1999) reported that psammaplin A (**9a**), possesses antibacterial activity against methicillin-resistant gram-positive *Staphylococcus aureus* that was almost comparable to ciprofloxacin, a quinolone antibiotic currently used in the US. Furthermore, extensive mechanisms of action studies completed by these investigators determined that psammaplin A did not bind to penicillin-binding proteins, and inhibited DNA synthesis of *S. aureus* SG 511, not in a dose-dependent manner ($IC_{50}$=2.83 μg/mL). It inhibited the super coiling activity of DNA gyrase, similar to the quinilones, although less efficiently than ciprofloxacin.[5] 14-Debromo araplysillin(**11**) and 14-debromo prearaplysillin(**12**) together with araplysillin(**13**) have been reported from *Druinella* (=*Psammaplysilla*) *purpurea* by James *et al.*[6] Copp *et al.*[7] described cytotoxic psammaplysins A-C (**14-16**) from the same source. All these compounds possess moderate *in vitro* cytotoxicity towards the human colon tumor cell HCT116.

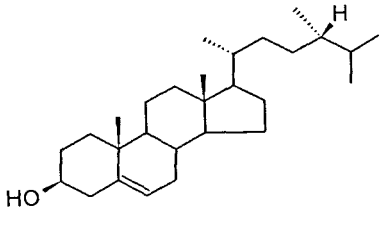
A macrocyclic peptides bastadin 2 (**17**) and the previously reported bastadins 5, 7 & 12 (**18-20**) were identified from Pohnpei *P. purpurea* by Carney *et al.*[8] Compound (**20**) is mildly cytotoxic against several cell lines and inhibits the enzymes topoisomerase II and dehydrofolate reductase.

Nine bromotyrosine metabolites purpuramines A-I **(21-29)** were isolated from the marine sponge *P. purpurea*.[9] All the compounds exhibited antibacterial activity against *S. aureus*. Jurek *et al.*[10] reported three cytotoxic bromotyrosine metabolites, aplysamines 3-5 **(30-32)** from the same source. In 1994, Pakrashi *et al.*[11] reported two new bromotyrosine metabolites **(33-34)** from the sponge *P. purpurea* collected from Bay of Bengal and the structures were assigned on the basis of spectroscopic analysis of their acetylated products **(33a & 34a)**.

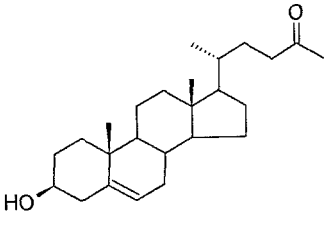
Venkateswarlu *et al.*[12] identified three compounds, 3,5-dibromo-4-hydroxyphenyl acetonitrile **(35)**, 3-bromo-4methoxyphenyl acetonitrile **(10)** and 3,5-dibromo-4-methoxybenzoic acid **(36)** from the *P. purpurea* collected from Tuticorin coast. Subsequently, same group reported[13-14] three bromotyrosine derivative **(37a, 37b, 38)** besides the known molakaimine**(39)**, 2,6-dibromo-4-hydroxyacetamido-1,1-dimethoxy-2,5-cyclohexadiene **(39a)**, aerothionin **(39b)**, bastadin-6 **(40)**, bastadin-16 **(41)**, Purealidin P, purealidin G and aplysamine-2 **(42)** from the same source collected off the Mandapam coast, India.



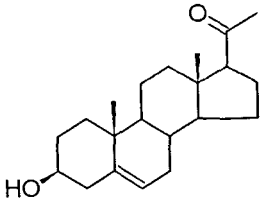

**(1)**



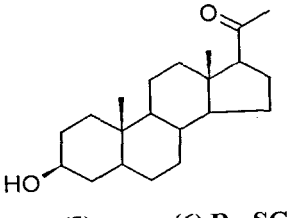

**(2)**




**(3)**

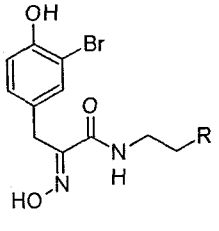



**(4)**



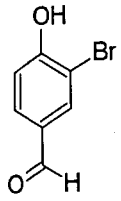

**(5)**

**(6) R= SC**

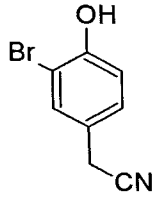



**(7) R= $SO_2NH_2$**

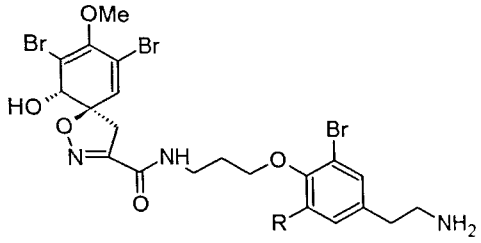
**(8) R=$S_2CH_2NHCOOMe$**



(9)

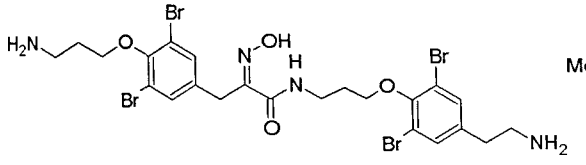


(10)



(11) R= H

(12) R=Br



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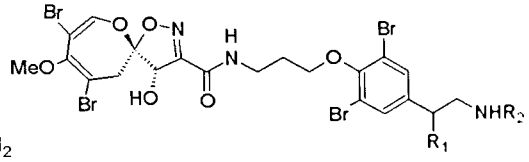
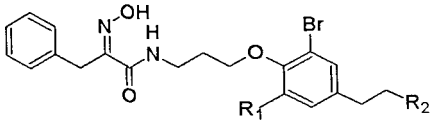


(14) $R_1$=OH, $R_2$=Me

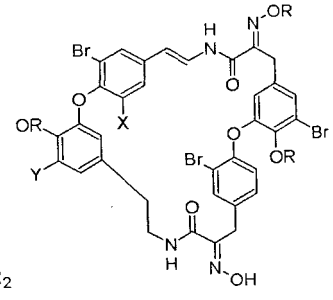
(15) $R_1$=R2=H

(16) $R_1$=OH, $R_2$=H



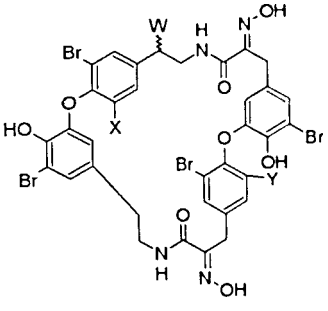
(24) R=H

(25) R=Me



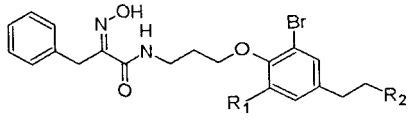
(17) X=Br, Y=Br, R=H

(18) X=H, Y=Br, R=H



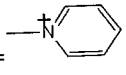
(19) W=H, X=H, Y= Br

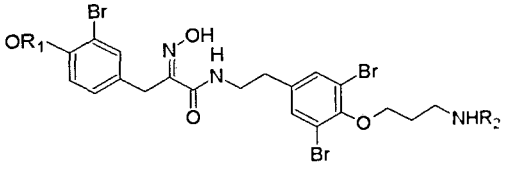
(20) W=OH, X=Br, Y=H



(21) $R_1$= Br, $R_2$=$NH_2$;

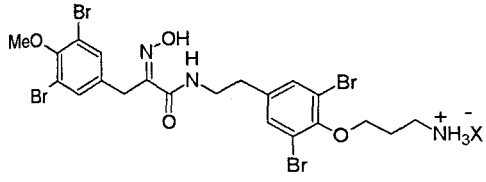
(22) $R_1$=H, R2=$NH_2$

(23) $R_1$=Br, $R_2$= 



(26) $R_1$=H, $R_2$=H; (27) $R_1$=H, $R_2$=Me

(28) $R_1$=Me, $R_2$=H; (29) $R_1$=Me, $R_2$=Me



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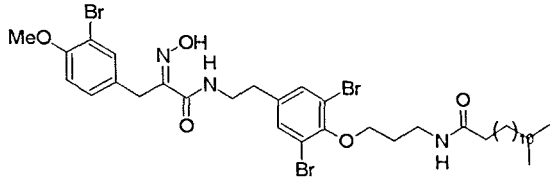

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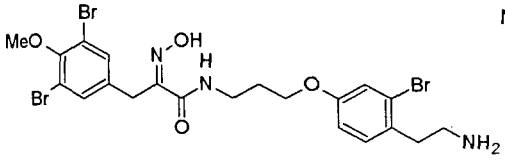

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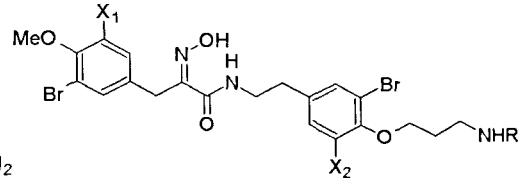
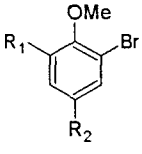
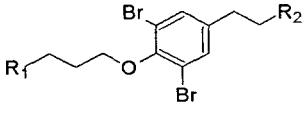
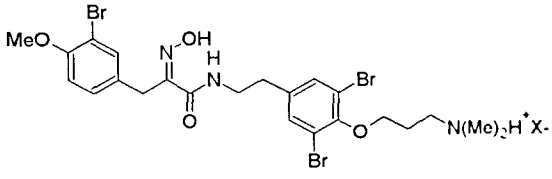


(33a) R=Ac, $X_1$=$X_2$=H

(34a) R=Ac, $X_1$=Br, $X_2$=H







(35) $R_1$=Br, $R_2$=$CH_2CN$

(36) $R_1$=Br, $R_2$=COOH

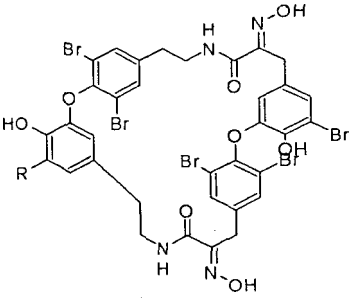
(37a) $R_1$=$N(CH_3)_2H^+X^-$ ;$R_2$=$NH_2$

(37b) $R_1$=$N(CH_3)_2H^+X^-$;$R_2$=NHAc

(38) $R_1$= $N(CH_3)_2$; $R_2$=NHAc

(39) $R_1$=$R_2$=$NH_2$

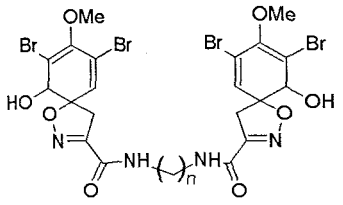
(42)



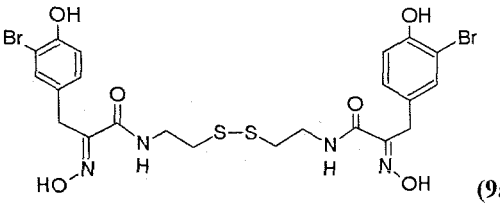
(40) R=Br

(41) R=H

(39a)




**(39b) n=4**



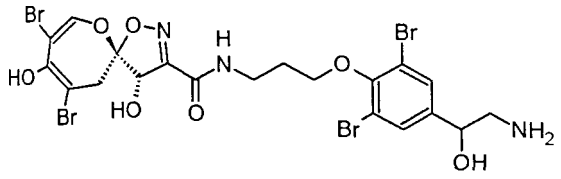

**(9a)**

Rotem*et al.*,[15] isolated bromotyrosine metabolite **(43)** along with the known 14-debromo prearaplysillin**(12)** from *P. purea.* Same sponge yielded two antimicrobial constituents psammaplysin A and B **(14-15)** characterized by spectroscopic analysis, which was further confirmed by single crystal X-ray diffraction studies on its acetamide acetate.[16] The FABMS of the known metabolites aerophobin-1 **(44)** and aerophobin-2 **(45)** has been discussed in detail by Cimino *et al.*[17] Nakamura *et al.* [18] isolated purealin **(46)** from the Okinawan, *P. purea.* Purealin **(46)** moderates enzymic reactions of ATPases. From the same source lipopurealins A-C **(47-49)** inhibitors of Na, K-ATPase were obtained.[19] Takido *et al.* [20] & Nakamura *et al.* [21-22] independently established the structure of purealin **(50)** and its methoxy derivative **(51)**, which activates myosin EDTA-ATPase at 0.3-30μM and inhibited myosin Ca-ATPase and $Na^+$, $K^+$-ATPase.

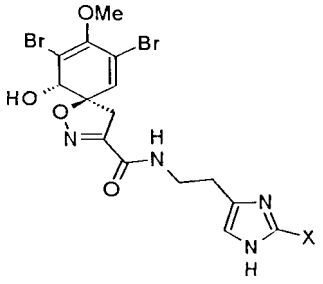
Okinawan *P. purea* yielded cytotoxic bromotyrosine alkaloid purealidin-A **(52)**[23] with promising antileukemic activity. Later, Kobayashi[24] reported purealidins B **(53)** and C**(54)**. Of these **(53)** was antibacterial and **(54)** exhibited antifungal & antineoplastic activities. Tsuda *et al.*[25-26] reported purealidin D **(55)** and purealidins E-G **(56-58)** from the same source.

Two new guanidine alkaloids, aplysillamides **(59-60)**[27] having antimicrobial activity were isolated from *P. purea*. Okinawan *P. purea* yielded lipopurealidins D-E **(61-62)** and purealidin H **(63)**.[28] The structures were elucidated on the basis of 2D NMR and FAB MS/MS data. Further, Kobayashi *et al.*[29-30] reported nine bromotyrosine alkaloids, purealidins J-R **(64-71, 71a)**. Purealidins N, P and Q **(68, 70 & 71)** were cytotoxic to tumor cell lines, while purealidins J, K, P and Q **(64, 65, 70 & 71)**

showed moderate inhibitory activity against epidermal growth factor (EGF) inhibitor kinase.

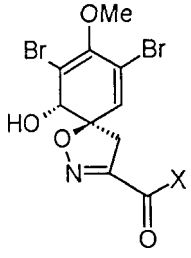


(43)



(44) X=H

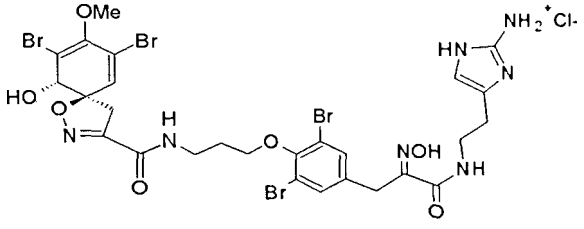
(45) X=$NH_2$



(47) X=$CO(CH_2)_{11}CH_2CH_3$

(48) X=$CO(CH_2)_{11}CH(CH_3)_2$

(49) X=$CO(CH_2)_{13}CH_2CH_3$



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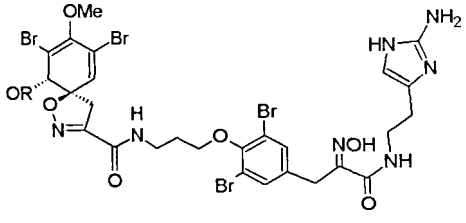
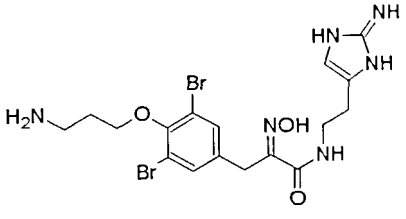


(50) R=Me

(51) R=H



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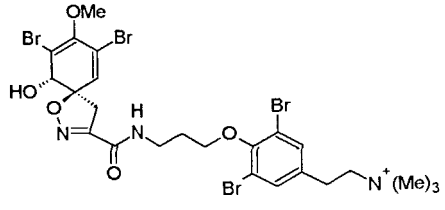

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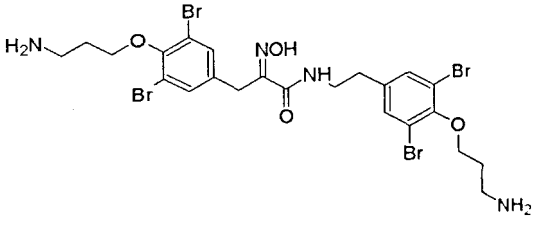

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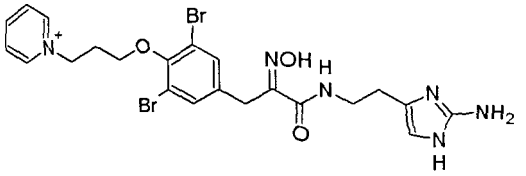

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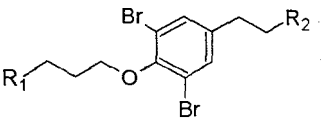
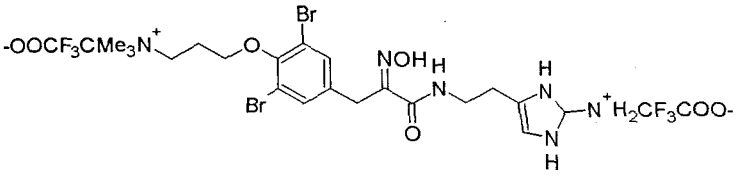


(56) $R_1$=$N^+H_2MeCl^-$;$R_2$=$NHMe_2Cl^-$

(57) $R_1$= $N^+HMe_2Cl^-$;$R_2$= $NH_2MeCl^-$



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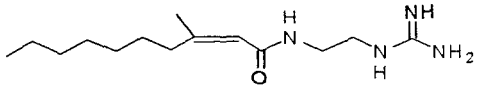

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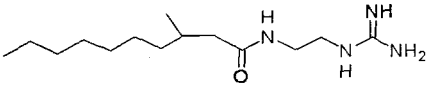

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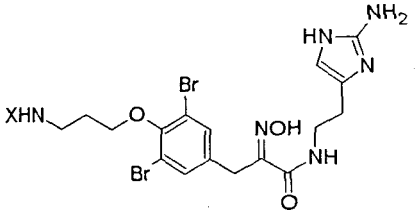
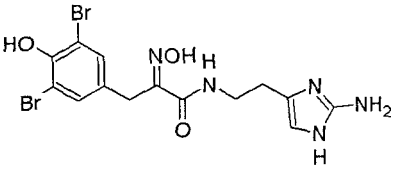


(61) X=CO $(CH_2)_7$-CH=CH-$(CH_2)_4$-$CH(CH_3)_2$

(62) X=CO $(CH_2)_{17}$ $CH_3$



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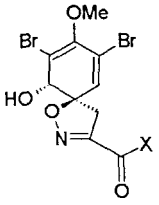
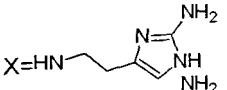
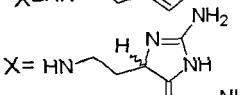


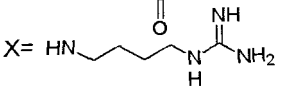
(64) X=HN

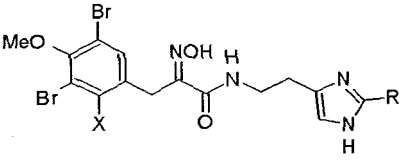


(65) X= HN

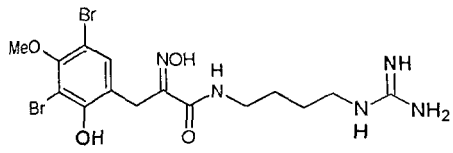


(66) X= HN





(67) R=$NH_2$, X=OH; (68) R=H, X=OH



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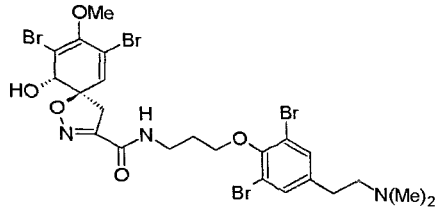

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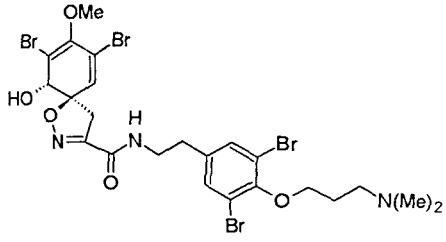

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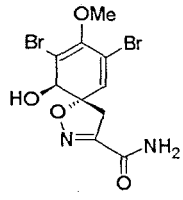
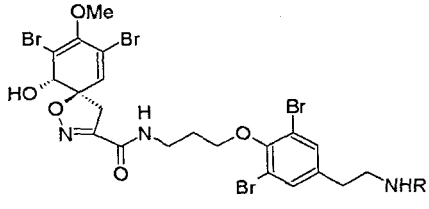


(71a)

Longeon *et al.* [31] isolated antimicrobial agents araplysillin-I & II (**72** & **73**), from the sponge *P. arabica* that inhibited of $Na^+/K^+$-ATPase.

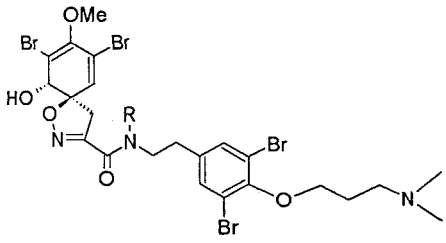


**(72) R=H**

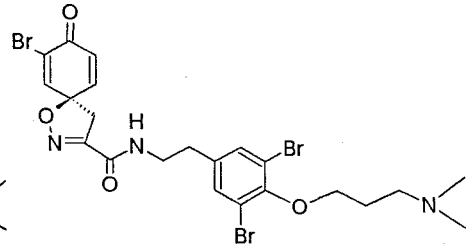
**(73) R=$COCH_2(CH_2)_nCH(CH_3)_2$**

## Section 2.2: Chemical Investigation of the marine sponge *Psammaplysilla purpurea*

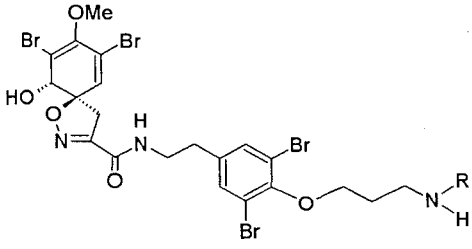
Earlier studies from our laboratory by Tilvi et al., 2004 [32] on the isolation, structure elucidation and *in vitro* bioactivity of bromotyrosine metabolites from the sponge *Psammaplysilla purpurea* have resulted in identification of Purpurealidin A-D and F-H along with Purealidin Q, Purpurealidin E, 16-debromoaplysamine-4 and purpuramine I, many of which possess antimicrobial properties.



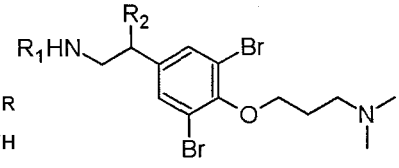
**Purealidin Q ; R=H**
**Purpurealidin A ; R=Me**



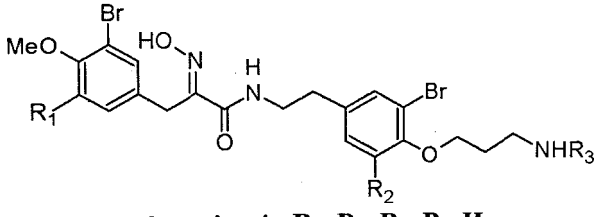
**Purpurealidin B**



**Purpurealidin C ; R=-CO(CH$_2$)$_{11}$CH(CH$_3$)$_2$**
**Purpurealidin D ; R=-CO(CH$_2$)$_{12}$CH$_2$CH$_2$CH$_3$**



**Purpurealidin E ; R$_1$=R$_2$=H**
**Purpurealidin F ; R$_1$=H, R$_2$=OH**
**Purpurealidin G; R$_1$=-COCH$_2$CH$_3$, R$_2$=OH**



**16-Debromoaplysamine-4 ; R$_1$=Br, R$_2$=R$_3$=H**
**PurpurealidinH ; R$_1$=Br, R$_2$=H, R$_3$=Me**
**PurpuramineI ; R$_1$=H, R$_2$=Br, R$_3$=Me**

Continuing with the investigation of the same sponge we collected fresh specimen from Muttom, Tamil Nadu, India.

The methanolic crude extract of the sponge was found to inhibit growth of human cancer cell lines and therefore subjected to chemical fingerprinting by ESI-MS studies. The mass fingerprint studies (**Fig.2.8**) revealed additional signals for non-brominated compounds which were different from the ones observed and reported earlier. Significantly, there is no such report of non-brominated molecules from *P. purpurea.* This led to the isolation of 3 compounds from the sample on fractionation and further chromatography. These alkaloids incidentally belonged to the Lamellarin class isolated originally from prosobranch mollusc *Lamellaria* sp.[33] and the marine ascidian, *Didemnum chartaceum.*[34] Subsequently they have also been isolated from an Australian sponge *Dendrilla cactos.*[35] Interestingly, the Lamellarin class of alkaloids are being reported for the first time from the sponge *P. purpurea.*

**2.1a.Collection of Biological Material**

The marine sponge *Psammaplysilla purpurea* was collected off the coast of Muttom, Tamil Nadu, India. The organism was identified by Dr. P. A. Thomas of the Vizhingam Research Center of the Central Marine Fisheries Research Institute, Kerala, India. A voucher specimen is deposited at the National Institute of Oceanography, Goa, India.

**2.1b.Extraction and Isolation**

Freshly collected animals were frozen at -20 °C and stored until use. The frozen samples were lyophilized and extracted with different solvents according to their increasing polarity. The chloroform and methanol fractions were found to be active for anticancer activity.

Subsequently for isolation and identification of the active principle fresh sample (1.2Kg) was collected, lyophilized and defatted first with hexane (500x3ml) followed by methanol (500x3ml) extraction of the material. This methanol fraction (2.47g) was chromatographed over Sephadex LH-20 using methanol as eluent. The partially purified lamellarins (0.457g) distinguished by TLC as lemon yellow spots on spraying with 5% sulphuric acid in methanol and purple fluorescence in methanol were further purified over silica gel column and HPLC to obtain pure compounds,

Lamellarin 1-3 in decreasing order of polarity. The most polar Lamellarin 1 (7mg) was soluble in methanol, and the others Lamellarin 2 (12mg) and 3 (3mg) were soluble in chloroform.

### 2.1c. Characterization of lamellarins 1-3

**Lamellarin 1:** The most fluorescent of all the lamellarins was obtained as a white amorphous crystalline solid, melting point 124°C.The molecular formula $C_{25}H_{11}NO_8$ which requires 18 degrees of unsaturation was determined by HR(TOF)ESI-MS in combination with $^1H$,$^{13}C$ and 2D NMR studies. The IR band at 3392.6cm$^{-1}$ and 1616 cm$^{-1}$ indicated the presence of phenolic and aromatic functionalities. Signal at 1676cm$^{-1}$was suggestive of a highly conjugated carbonyl group. The $^1H$ NMR data of Lamellarin 1 revealed two methylenes mutually coupled indicated by the presence of two triplets at δ 4.64 and δ2.99. The upfield $^1$HNMR signals indicated that they are attached to an electronegative atom and an aromatic ring respectively. Its $^{13}C$ NMR spectrum had 25 carbon signals which on the basis of DEPT 135 experiment were identified as two methylenes, seven methines, and sixteen quaternary carbons inclusive of a carbonyl carbon. The integration of the aromatic region showed 7 aromatic protons. The signals at 6.75 (s,1H), 6.71 (s,1H), 6.70 (s,1H), 6.63 (s,1H), 6.85 (d, 1H, J=1.8), 6.99 (d,1H, J=8.1), 6.75 (dd, 1H,$J_1$=8.1, $J_2$=1.8) in the $^1$HNMR spectrum indicated tetra and 1,3,4 tri substituted ring systems. The signals at δ 147.37, 147.23, 143.35, 146.75, 144.89, 146.57, 147.23 and 157.65 along with its molecular formula $C_{25}H_{11}NO_8$ and IR spectrum revealed 7 phenolic groups and a conjugated ester moiety in the molecule. The compound was acetylated and a hexaacetyl derivative was obtained which suggested that the compound had 6 phenolic –OH groups. The other phenolic –OH was assumed to form a lactone with the ester carbonyl at δ157.65.

The positions of the phenolic groups were determined by the analysis of their COSY, HMQC and HMBC data (**Table 2.1** and **Fig.2.2**) and on comparison with literature values. Lamellarin 1 was found to be 10, 11-dihydro analogue of Lamellarin H previously isolated from the ascidian *Didemnum sp.*[34]

**Table 2.1. NMR assignments for Lamellarin1 (DihydroLamellarin H)**

| Carbon No. | $^{13}$CNMR $\delta_C$, ppm | $^{1}$H NMR $\delta_H$, ppm | COSY | HMBC |
|---|---|---|---|---|
| 1 | 157.65 | - | - | - |
| 2 | 113.77 | - | - | - |
| 3 | 130.18 | - | - | - |
| 4 | 111.40 | - | - | - |
| 5 | 147.37 | - | - | - |
| 6 | 104.16 | 6.75 (s,1H) | | C4,C8 |
| 7 | 147.23 | - | - | - |
| 8 | 143.35 | - | - | - |
| 9 | 109.10 | 6.71 (s,1H) | - | C3, C8 |
| 10 | 43.62 | 4.64 (t, 2H) | - | C12 |
| 11 | 29.64 | 2.99 (t, 2H) | - | C12 |
| 12 | 127.88 | - | - | - |
| 13 | 115.79 | 6.7 (s,1H) | - | C12, C15 |
| 14 | 146.75 | - | - | - |
| 15 | 144.84 | - | - | - |
| 16 | 114.66 | 6.63 (s,1H) | - | C12,C18,C14 |
| 17 | 116.60 | - | - | - |
| 18 | 138.18 | - | - | - |
| 19 | 120.52 | - | - | - |
| 20 | 127.91 | - | - | - |
| 21 | 118.88 | 6.85 (d, 1H, J=1.8) | H25 | - |
| 22 | 146.57 | - | - | - |
| 23 | 147.23 | - | - | - |
| 24 | 117.56 | 6.99 (d,1H, J=8.1) | H25 | C20,C22 |
| 25 | 123.51 | 6.75 (dd, 1H, $J_1$=8.1, $J_2$=1.8) | H21,H24 | C19,C23 |

ESI-MS of the Lamellarin 1 gave quasimolecularion $[M+H]^+$ peak at m/z 460.0264 (calculated: 460.4117); and the corresponding $[M+Na]^+$ peak was observed at m/z 482.0065 (calculated: 482.3936).

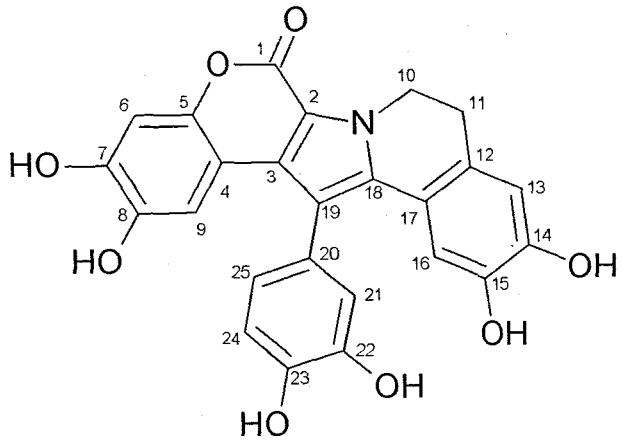



**Fig.2.1: Structure of Lamellarin1 (DihydroLamellarin H)**

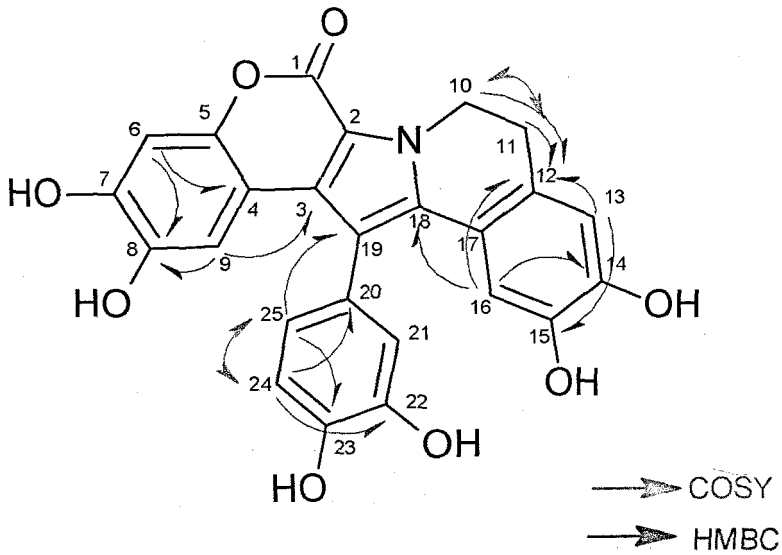



**Fig.2.2: COSY &HMBC correlations of Lamellarin1 (DihydroLamellarin H)**

**Lamellarin 2** had structural characteristics similar to lamellarin 1. IR of compound 2 showed the presence of hydroxyl group due to the presence of a broad band at $3400cm^{-1}$. Absorption at $1683cm^{-1}$ and corresponding peak at δ155.9 in the $^{13}C$ NMR indicated the presence of carbonyl functionality. $^{1}H$ NMR showed the presence of 5 singlets from δ 2.8- 4.0 for methoxy groups, 2 methylenes at δ 3.07 and δ 4.64

indicating that compound 2 belonged to a 10,11-dihydrolamellarin group. Signals for aromatic protons (6 protons) were evident between δ 6.50 to δ 7.05. Acetylation of the compound yielded a diacetyl product indicating the presence of 2 free hydroxyl groups. $^{13}C$ and DEPT experiments revealed the presence of 30 carbons which include 2 methylenes, 5 methoxy, 6 methines and remaining 17 carbons as quaternary carbons. ESI-MS of the compound gave a quasimolecular ion $[M+H]^+$ at m/z 546.0694 (calculated: 546.5440).

The above data along with extensive 2D NMR data (**Table 2.2**) & (**Fig.2.5**), single crystal X-ray diffraction studies (**Fig.2.4**) and also comparing with the previously reported analogues confirmed the structure of Lamellarin 2 as shown below (**Fig.2.3**). Lamellarin 2 on comparison with the reported literature on lamellarins, was identified as Lamellarin C previously isolated by Anderson et al in 1985 from the prosobranch mollusk *Lamellaria* sp.[33]

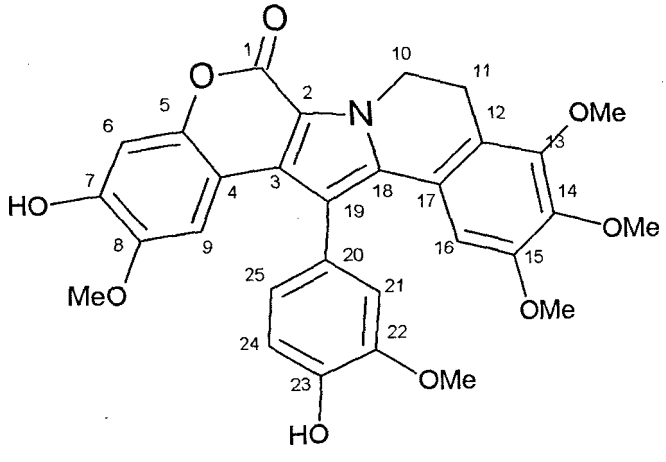



**Fig.2.3: Structure of Lamellarin 2**

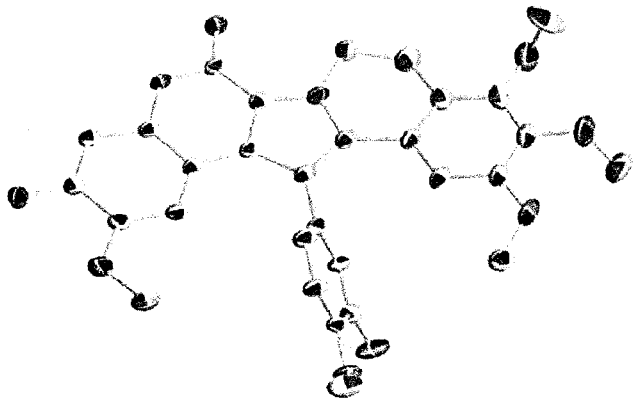


**Fig.2.4: X-ray crystal structure of Lamellarin 2**

**Table 2.2: NMR assignments for Lamellarin 2**

| Carbon No. | $^{13}$CNMR $\delta_C$, ppm | $^{1}$H NMR $\delta_H$, ppm | COSY | HMBC |
|---|---|---|---|---|
| 1 | 155.90 | - | - | - |
| 2 | 115.32 | - | - | - |
| 3 | 128.35 | - | - | - |
| 4 | 109.82 | - | - | - |
| 5 | 145.76 | - | - | - |
| 6 | 103.32 | 6.84 (s,1H) | - | C4,C7 |
| 7 | 146.08 | - | - | - |
| 8 | 143.82 | - | - | - |
| 9 | 104.43 | 6.64 (s,1H) | | C3,C7 |
| 10 | 41.98 | 4.64(t, 2H) | H11 | C12 |
| 11 | 21.78 | 3.08(t,2H) | H10 | C12 |
| 12 | 120.02 | - | - | - |
| 13 | 150.42 | - | - | - |
| 14 | 142.07 | - | - | - |

| | | | | |
|---|---|---|---|---|
| **15** | 151.65 | - | - | - |
| **16** | 105.41 | 6.58 (s,1H) | - | C12,C18,C14,C15 |
| **17** | 113.52 | - | - | - |
| **18** | 135.46 | - | - | - |
| **19** | 122.98 | - | - | - |
| **20** | 128.35 | - | - | - |
| **21** | 117.49 | 7.06 (d, 1H, J=1.8) | - | C25,C22 |
| **22** | 146.83 | - | - | - |
| **23** | 146.58 | - | - | - |
| **24** | 111.67 | 6.97 (d,1H, J=8.1) | H25 | C20,C22 |
| **25** | 122.65 | 6.95 (dd, 1H, $J_1$=8.1, $J_2$=1.8) | H24 | C21,C23 |
| **26** | 55.05 | 3.47(s,3H) | - | C21 |
| **27** | 56.16 | 3.93(s,3H) | - | C25 |
| **28** | 55.37 | 3.34(s,3H) | - | C27 |
| **29** | 60.92 | 3.85(s,3H) | - | C20 |
| **30** | 60.82 | 3.85(s,3H) | - | C26 |

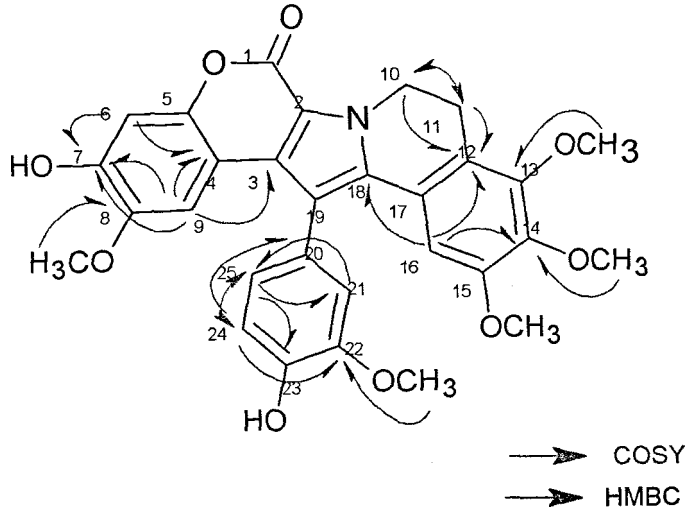



**Fig.2.5: COSY& HMBC correlations of Lamellarin 2**

**Lamellarin 3** was obtained as a pale yellow solid. Its spectral data was similar to the above two compounds (1& 2) differing only in the absence of double bond at $C_{10-11}$. As expected the Infra red spectroscopic studies (**Fig.2.26**) of the compound showed a very strong absorption in the region 3300cm$^{-1}$ indicating it to be a polyhydroxylated molecule. A band at 1681cm$^{-1}$ with corresponding $^{13}$C NMR (**Fig.2.28**) value at δ155.40 revealed it to be a carbonyl group. $^{1}$H NMR (**Fig.2.27**) signals in the region δ 6.70 to δ 9.30 corresponded to 8 aromatic protons. $^{13}$C experiment indicted the presence of 30 carbons, multiplicities of which indicated the presence of 5 methyls, 2 methylenes, 7 methines and 15 quaternary carbons. The signals for 4 singlets in the aromatic region of its $^{1}$H NMR were attributed to two 1,2,4,6 tetra substituted ring systems as in compound 1. The two meta coupling doublets at δ 7.11 (J=1.5Hz) and δ7.21 (J=1.5Hz) were assigned to two protons on the central phenyl ring meta in position to each other to form a 1,3,4,5 tetra substituted ring. The absence of 2 methylene protons in the region δ2-4 and instead, two additional low field signals at δ 9.21 (J=7.5Hz) and δ 7.39 (J=7.5Hz) present in its $^{1}$H NMR spectrum indicated unsaturation at $C_{10-11}$. Five methoxy groups were evident as singlets at δ3.47, 3.53, 3.93, 4.01 & 4.02 in the proton NMR.

ESI-MS (**Fig.2.32**) of the compound gave quasimolecular ions $[M+H]^+$ at m/z 544.0352 (calculated: 544.5281) and $[2M+H]^+$at m/z 1087.1276. Acetylation of the compound yielded a diacetyl product at m/z 628.2767 indicating the presence of 2 free hydroxyl groups (**Fig.2.33**).

The relative positions of the atoms and groups (methoxy and hydroxyl) were deduced on the basis of the two dimentional NMR data, $^{1}$H-$^{1}$H COSY (**Fig.2.29**), HSQC (**Fig.2.30**) & HMBC (**Fig.2.31**).

The structure of Lamellarin **3** (**Fig.2.6**) was arrived at on the basis of the spectral data (**Table 2.3**) and on comparison with the spectral data reported in the literature for Lamellarins.

Table 2.3. NMR assignments for Lamellarin 3

| Carbon No. | $^{13}$CNMR $\delta_C$, ppm | $^{1}$H NMR $\delta_H$, ppm | COSY | HMBC |
|---|---|---|---|---|
| 1 | 155.40 | - | - | - |
| 2 | 110.00 | - | - | - |
| 3 | 129.00 | - | - | - |
| 4 | 110.00 | - | - | - |
| 5 | 146.55 | - | - | - |
| 6 | 101.79 | 7.02(s,1H) | - | C4 |
| 7 | 146.20 | - | - | - |
| 8 | 143.30 | - | - | - |
| 9 | 104.83 | 6.73 (s,1H) | | C3,C7 |
| 10 | 123.47 | 9.21 (d, 1H, J=7.5) | H11 | C11,C12,C18 |
| 11 | 106.93 | 7.38 (d,1H,J=7.5) | H10 | C17 |
| 12 | 119.46 | - | - | - |
| 13 | 122.94 | 7.12(s,1H) | - | C16 |
| 14 | 142.20 | - | - | - |
| 15 | 153.40 | - | - | - |
| 16 | 103.58 | 7.01(s,1H) | | C12,C18,C14,C15 |
| 17 | 116.00 | - | - | - |
| 18 | 133.40 | - | - | - |
| 19 | 121.20 | - | - | - |
| 20 | 128.00 | - | - | - |
| 21 | 117.82 | 7.21(d,1H,J=1.5Hz) | - | C22 |
| 22 | 147.06 | - | - | - |
| 23 | 148.6 | - | - | - |
| 24 | 146.32 | - | - | - |
| 25 | 111.38 | 7.11(d,1H,J=1.5Hz) | - | C21,C23 |
| 26 | 55.55 | 3.53 (s,3H)2 | - | C8 |
| 27 | 61.68 | 4.03(s,3H)5 | - | C23 |

| | | | | |
|---|---|---|---|---|
| **28** | 61.14 | 3.98 (s,3H)3 | - | C22 |
| **29** | 55.14 | 3.47 (s,3H)1 | | C15 |
| **30** | 56.39 | 4.01 (s,3H)4 | - | C14 |

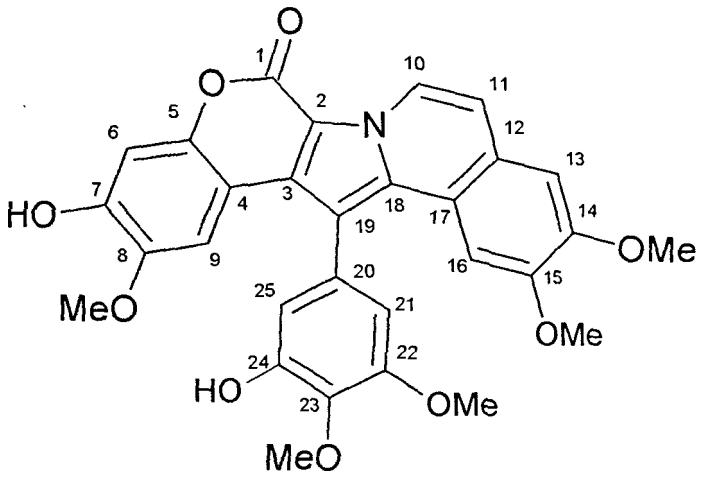



**Fig.2.6: Structure of Lamellarin 3**

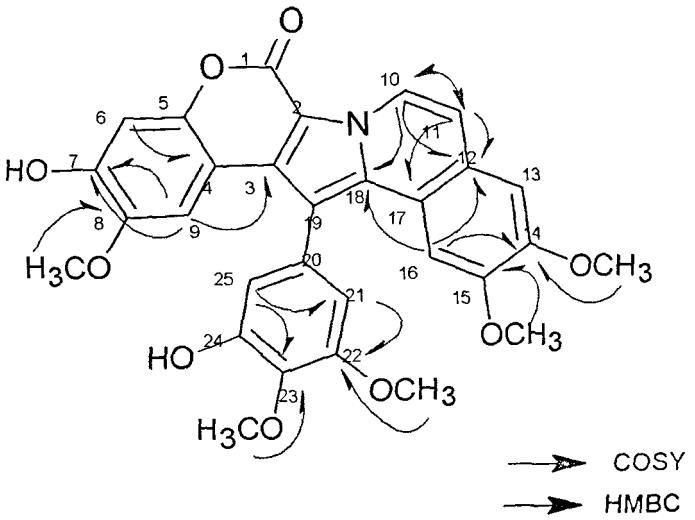



**Fig.2.7: COSY & HMBC correlations of Lamellarin 3**

The structure of Lamellarin 3 was assumed to be similar to Lamellarin γ (**Fig.2.6a**), reported by Krishnaiah et al. in 2004 [36] but since the $^{1}H$ NMR data indicated 4 singlets and two doublets with meta coupling, 4 protons needed to be placed para to each other on two rings and the remaining 2 protons needed to be placed meta on a 1,3,4,5 tetra substituted middle ring (**Fig.2.6**).

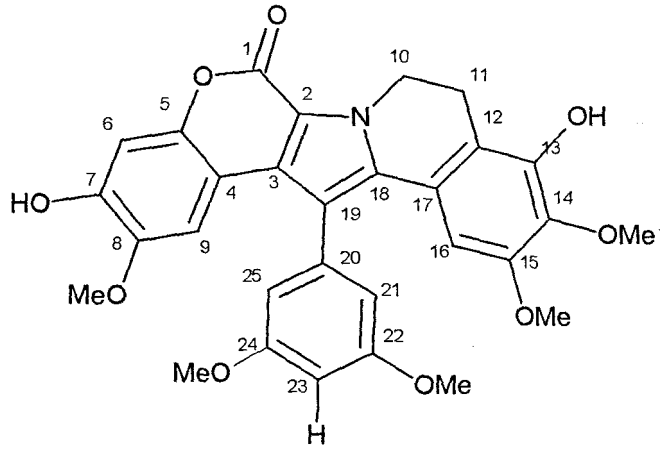
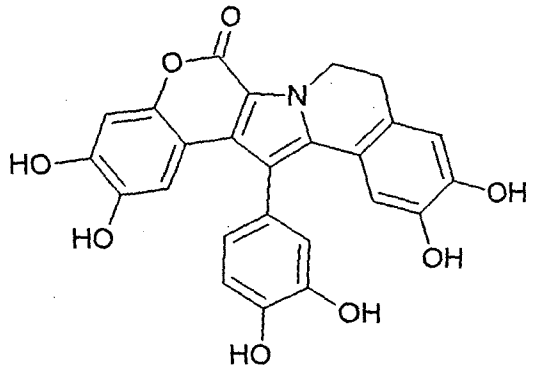



**Fig.2.6a: Structure of Lamellarin γ**

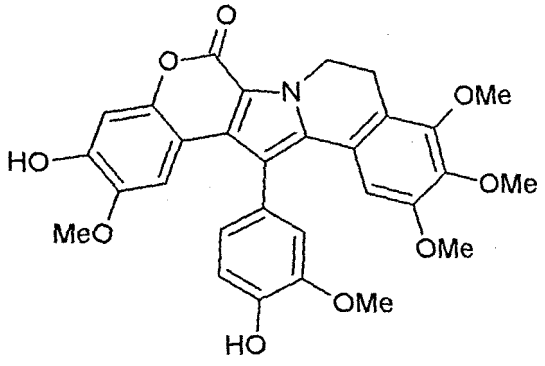
Investigation of the sponge *Psammaplysilla purpurea* has previously resulted in the isolation of alkaloids such as psammaplysins, purpuramines, puramines and bastadin alkaloids. Ours is a first report of the occurrence of cytotoxic lamellarin class of alkaloids in the marine sponge *Psammaplysilla purpurea* and of natural occurrence of Lamellarin 1 a dihydro analogue of Lamellarin H. This has been previously synthesized by Baunbaek et al., (2008) taking into consideration the immense cytotoxic activity of the lamellarins as a group[37]. Lamellarin 3 is a new Lamellarin being reported here for the first time from any natural source.

Pentacyclic lamellarins are observed to show diverse biological activities, many of which are of pharmacological importance. The potent biological activity and multifunctional properties of these particular marine alkaloids allow them to be considered as potential leads for drug development.

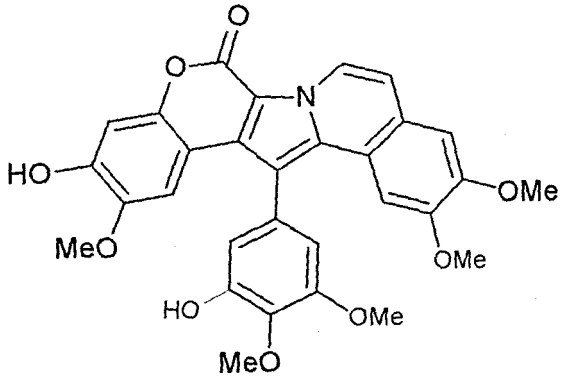
**Systematic names of the isolated lamellarins**




Lamellarin (1) 8,9-dihydro-2,3,11,12-tetrahydroxy-14(3,4-dihydroxyphenyl) 6H[1] benzopyrano - [4',3';4,5]pyrrolo [2,1-α] isoquinoline-6-one




Lamellarin (2) 8,9-dihydro-3hydroxy-2,10,11,12-tetramethoxy-14(4-hydroxy,3-methoxyphenyl)6H [1] benzopyrano-[4',3';4,5] pyrrolo [2,1-α]isoquinoline-6-one




Lamellarin(3)3-hydroxy-2,11,12-trimethoxy-14(5-hydroxy,3,4-dimethoxyphenyl) 6H[1] benzopyrano -[4',3';4,5] pyrrolo [2,1-α]isoquinoline-6-one

### 2.1d.General experimental procedures

Column chromatography was performed by using Silica gel (60-120 mesh, Qualigens) and using Sephadex (LH20 17-0090-01 Pharmacia Biotech). Fractions were monitored on TLC using alumina-backed sheets (silica gel 60 $F_{254}$, 0.25mm thick) with visualization under UV (254nm) and 5% sulphuric acid in methanol spray reagent.

UV spectra were recorded in MeOH, using Shimadzu UV-Vis 2401PC Spectrophotometer and IR spectra were recorded on a Shimadzu FT-IR spectrophotometer. Optical rotations were recorded in MeOH using Optical Polarimeter ADP220 (Bellingham Stanley Ltd.).

Mass spectra were recorded on a PE Sciex-QSTAR and QSTAR-TOF MS/MS Applied Biosystems.

NMR ($^{1}H$, $^{13}C$, COSY, HMQC and HMBC) experiments were obtained on a Bruker (Avance 300) Spectrometer with TMS as the internal standard.

## Section 2.3: Anticancer activity:

### 2.3a. Cytotoxicity Assay

The cancer cell lines were grown in RPMI 1640 with 10% fetal bovine serum in 5% $CO_2$ atmosphere. Cytotoxicity/ antiproliferative activity of crude extract and purified compounds were evaluated in vitro against 21 cell lines using the standard Sulphorodhamine Blue (SRB) Colorimetric assay.

They crude extract showed activity against the following cell lines;

Human cervical cancer (SiHa), Human cervical cancer (ME180), Human bladder cancer (T24), Human breast cancer (MCF7), Human breast cancer (ZR-75-1), Human prostate cancer (DU-145), Human oral cancer (DWD), Human colorectal cancer (SW620), Human colon cancer (COLO205), Human colon cancer (HCT15), Human pancreatic cancer (MIAPACA-2), Human ovarian cancer (A2780), Human lung cancer (A549), Human prostate cancer (PC3), Human lung cancer (HOP62), Human promyelocytic leukemia (HL60), Human renal cell cancer (786-O), Human

glioblastoma (brain tumour) cell lines (U373MG), Human colon cancer (HT29), Human renal carcinoma (A498) and Human breast cancer (T47D).

**2.3b. In vitro evaluation of cytotoxic activity**

A protocol of 48 hr continuous drug exposure was used and a sulforhodamine B (SRB) protein assay was used to estimate cell viability or growth. The cell lines were grown in RPMI 1640 medium containing 10% fetal bovine serum and 2 mM L-glutamine, and were inoculated into 96-well microtiter plates in 90 μL at plating densities depending on the doubling time of individual cell lines. The microtiter plates were incubated at $37^0$C, 5% $CO_2$, 95% air and 100% relative humidity for 24 hr prior to addition of experimental drugs. Aliquots of 10 μL of the drug dilutions were added to the appropriate microtiter wells already containing 90 μL of cells, resulting in the required final drug concentrations. Each compound was evaluated for four concentrations (0.1, 1, 10 and 100 μL) and each was done in triplicate wells. Plates were incubated further for 48 hr, and assay was terminated by the addition of 50 μL of cold trichloro acetic acid (TCA) (final concentration, 10% TCA) and plates were again incubated for 60 min at 4°C. The plates were washed five times with tap water and air-dried. Sulforhodamine B (SRB) solution (50 μL) at 0.4% (w/v) in 1% acetic acid was added to each of the wells, and plates were incubated for 20 min at room temperature. The residual dye was removed by washing five times with 1% acetic acid. The plates were air-dried. Bound stain was subsequently eluted with 10 m Mtrizma base, and the absorbance was read on an ELISA plate reader at a wavelength of 540 nm with 690 nm reference wavelengths. Percent growth was calculated on a plate-by-plate basis for test wells relative to control wells. The above determinations were repeated three times.

Extracts, fractions and pure compounds were tested for anticancer activity against 21 human cancer cell lines (**Table 2.4**) and found to be very potent anticancer agents with $GI_{50}$ values in the range of <0.1μM.

**Table 2.4.Anticancer analysis of crude extract, fractions and pure compounds**

| | Name of the extract (conc. in µM) | | | | | | |
|---|---|---|---|---|---|---|---|
| Cell line used for study | Crude methanol extract | Crude extract (Repeat) | Chloroform | Methanol | 01 | 02 | 03 |
| SiHa | <10 | <10 | <10 | 29 | $>10^{-4}$ | $2.6x10^{-6}$ | $2.0x10^{-6}$ |
| ME180 | <10 | <10 | <10 | <10 | $1.9x10^{-7}$ | $1.58x10^{-7}$ | $1.06x10^{-7}$ |
| T24 | <10 | <10 | <10 | <10 | $2.75x10^{-6}$ | $1.85x10^{-7}$ | $<10^{-7}$ |
| MCF7 | <10 | <10 | <10 | <10 | $>10^{-4}$ | $>10^{-4}$ | $3.0x10^{-5}$ |
| Zr-75-1 | <10 | <10 | <10 | 12 | $>10^{-4}$ | $2.4x10^{-6}$ | $<10^{-7}$ |
| DU145 | <10 | <10 | <10 | <10 | $>10^{-4}$ | $1.89*10^{-7}$ | $<10^{-7}$ |
| DWD | <10 | <10 | <10 | <10 | $2.29x10^{-6}$ | $2.05x10^{-6}$ | $<10^{-7}$ |
| SW620 | 15 | <10 | <10 | 18 | $>10^{-4}$ | $2.6x10^{-6}$ | $1.9x10^{-6}$ |
| Colo205 | <10 | <10 | <10 | 42 | $>10^{-4}$ | $>10^{-4}$ | $>10^{-4}$ |
| HCT15 | 12 | <10 | <10 | 22 | $>10^{-4}$ | $2.2x10^{-6}$ | $1.1x10^{-7}$ |
| HT29 | 34 | | | | - | | |
| MIA-PA-CA-2 | <10 | <10 | <10 | 13 | $>10^{-4}$ | $2.32x10^{-6}$ | $1.52x10^{-7}$ |
| A2780 | <10 | <10 | <10 | <10 | | | |
| A549 | 14 | | | | $2.5x10^{-6}$ | $>10^{-4}$ | $>10^{-4}$ |
| U373MG | 28 | | | | $3.0x10^{-5}$ | $3.0x10^{-5}$ | $1.0x10^{-7}$ |
| PC3 | <10 | | | | $>10^{-4}$ | $2.2x10^{-6}$ | $1.8x10^{-7}$ |
| Hop62 | <10 | | | | $>10^{-4}$ | $2.5x10^{-6}$ | $>10^{-4}$ |
| HL60 | 20 | | | | $>10^{-4}$ | $>10^{-4}$ | $>10^{-4}$ |
| A498 | >80 | | | | $2.8x10^{-6}$ | $>10^{-4}$ | $2.5x10^{-6}$ |
| 786-O | >10 | | | | $1.7x10^{-7}$ | $1.6x10^{-7}$ | $1.05x10^{-7}$ |
| T47D | >80 | | | | | | |
| Total cell lines active | | | | | 7 | 13 | 10 |

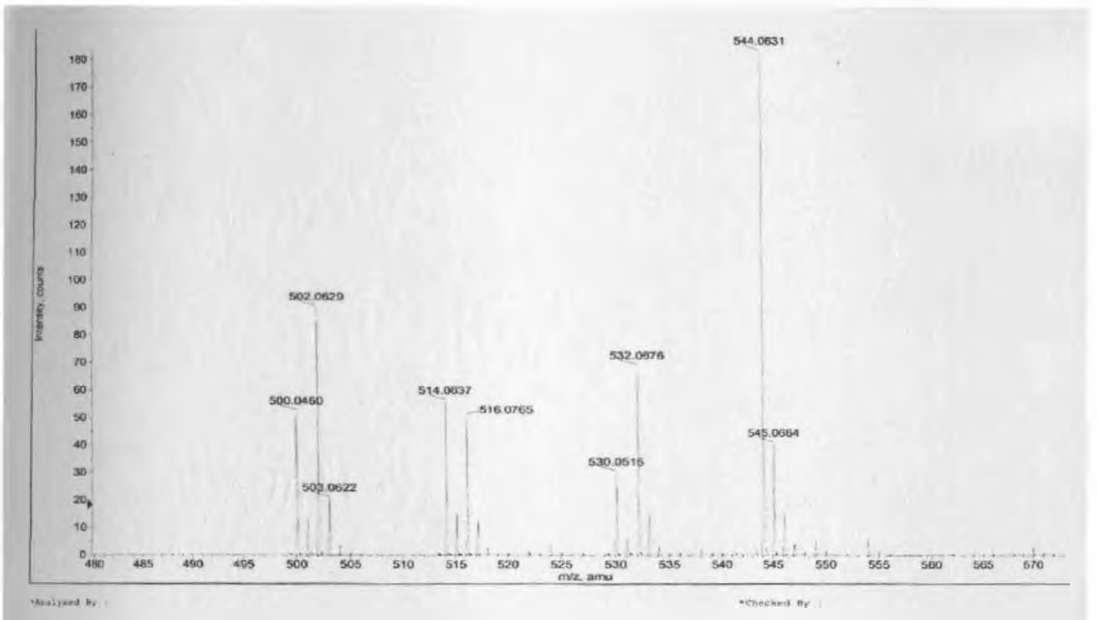



**Fig.2.8: ESI-MS spectrum of the crude methanol extract of *Psammaplysilla purpurea***

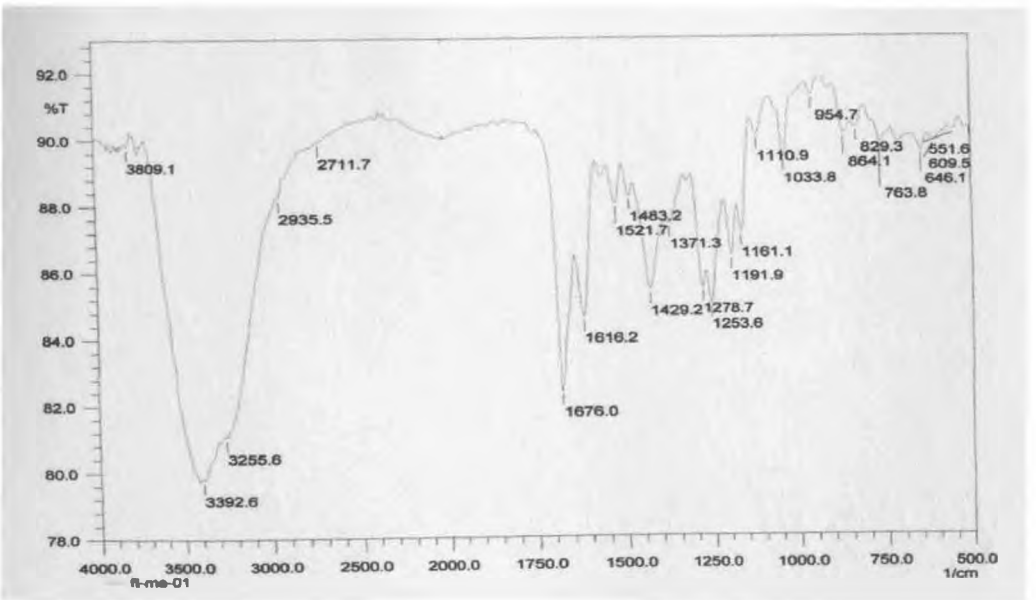



**Fig.2.9: IR spectrum of Lamellarin 1**

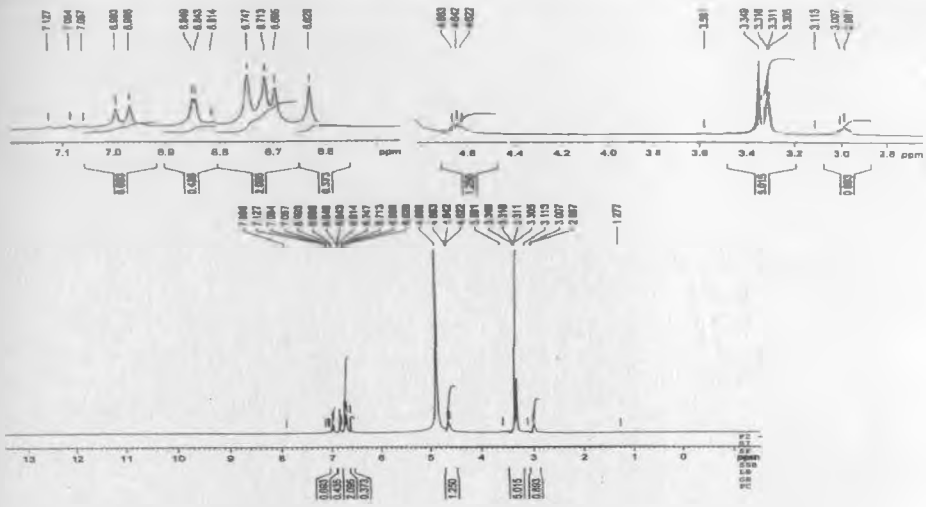


**Fig.2.10: $^{1}$H NMR spectrum of Lamellarin 1**

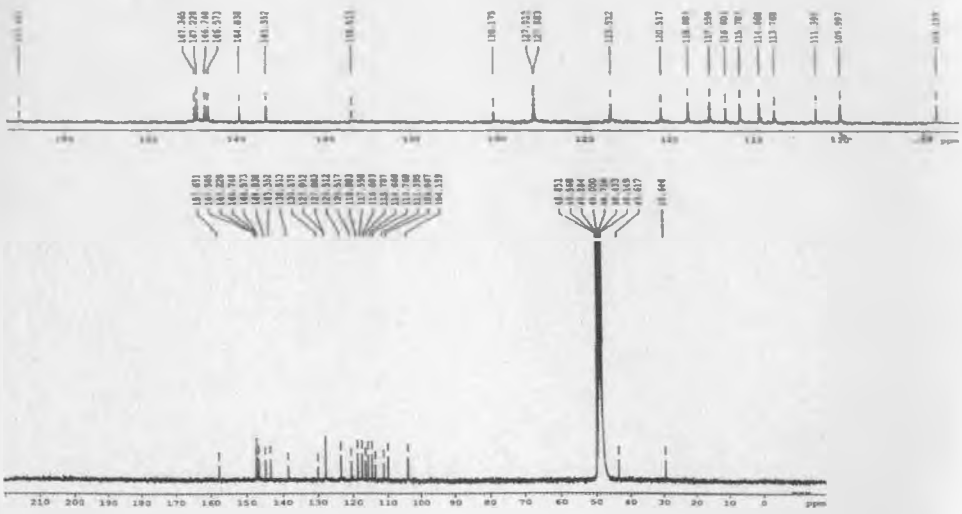


**Fig.2.11: $^{13}$C NMR spectrum of Lamellarin 1**

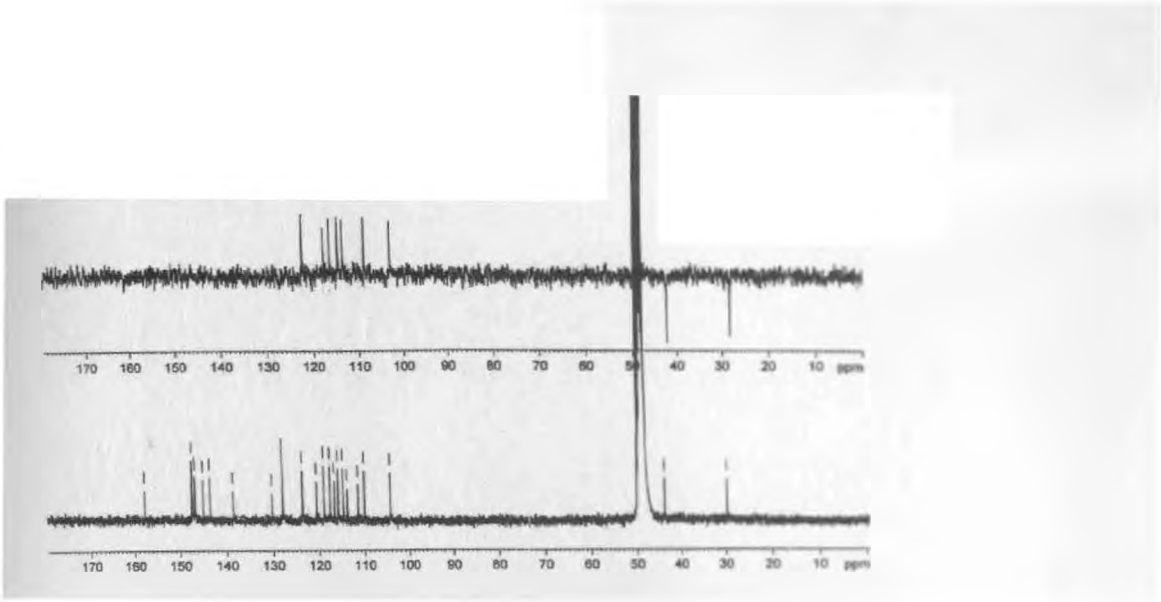



**Fig.2.12: DEPT spectrum of Lamellarin 1**

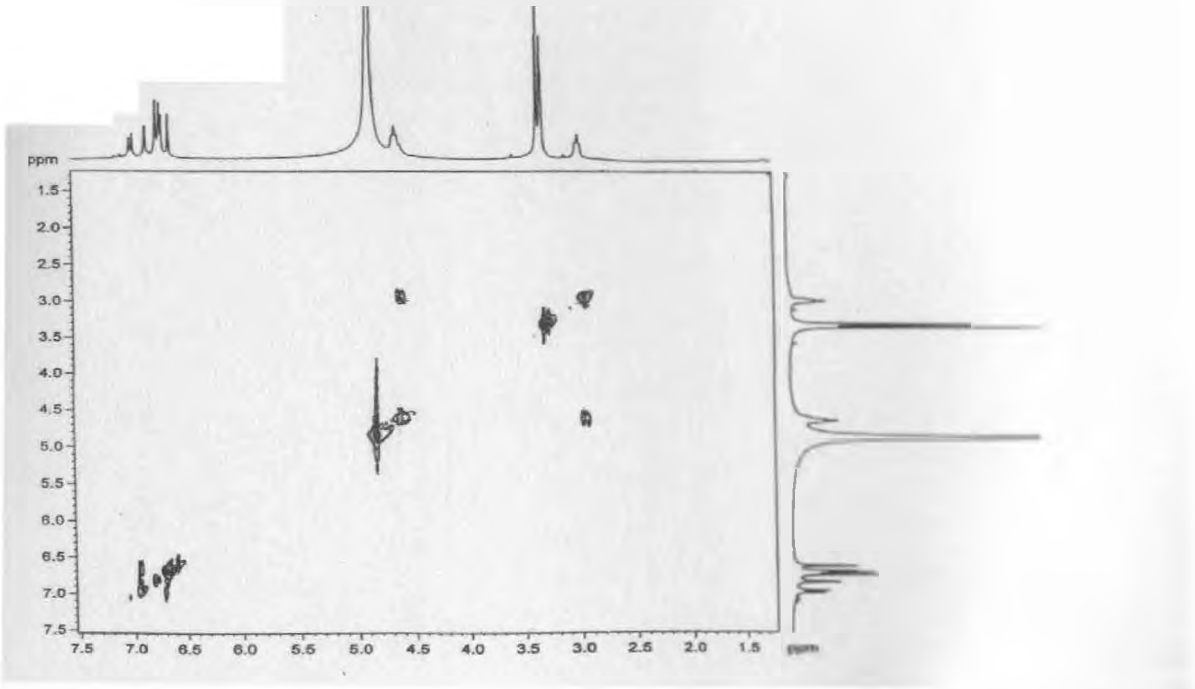



**Fig.2.13: $^{1}H$-$^{1}H$ COSY spectrum of Lamellarin 1**

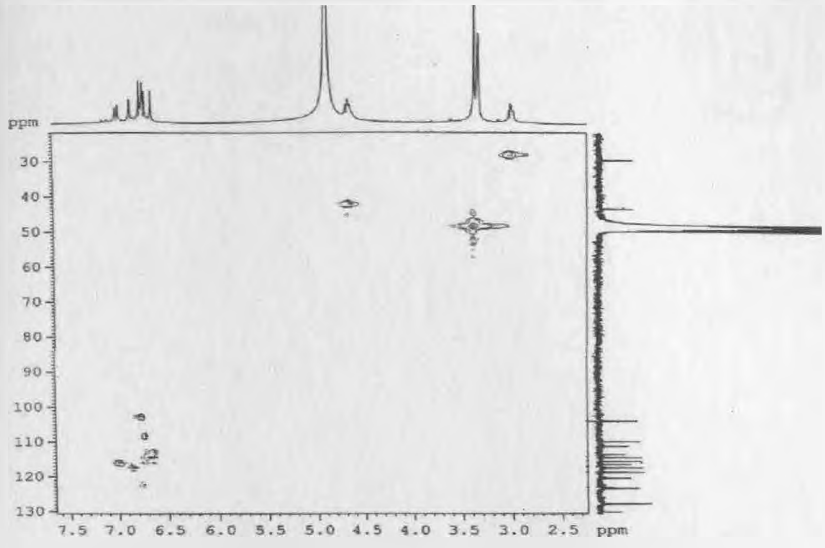



**Fig.2.14: HSQC spectrum of Lamellarin 1**

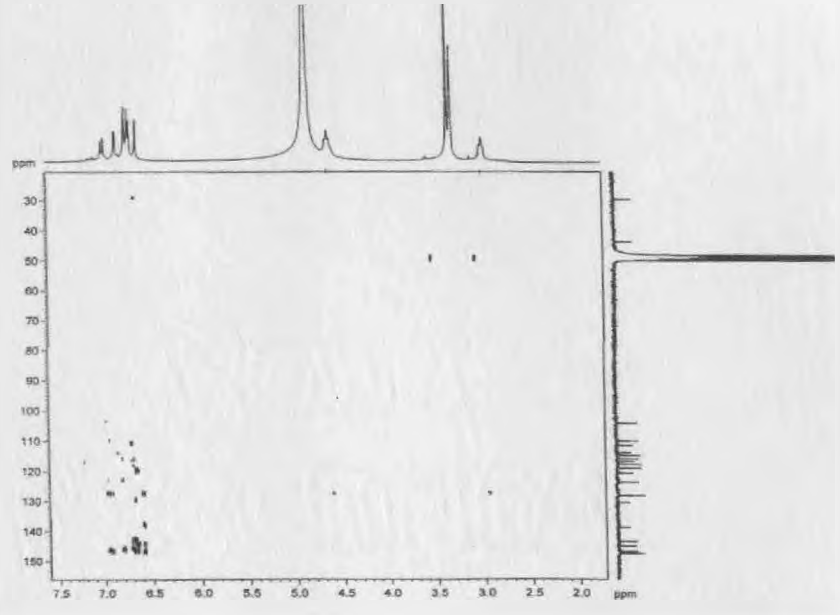



**Fig.2.15: HMBC spectrum of Lamellarin 1**

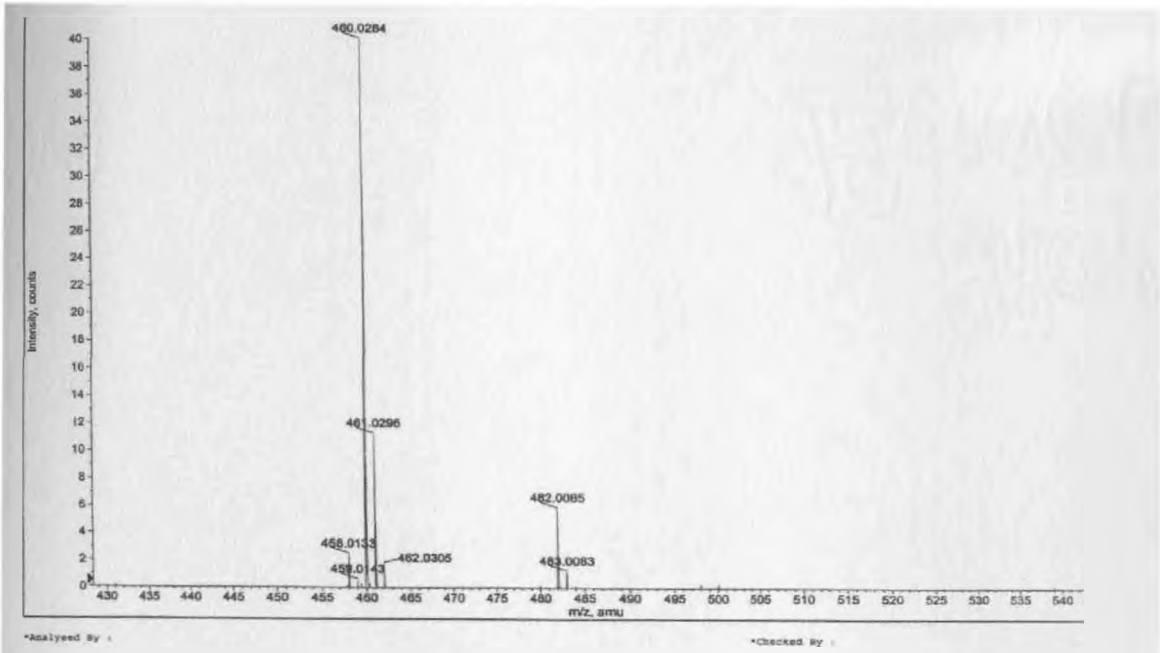



**Fig.2.16: ESI-MS spectrum of Lamellarin 1**

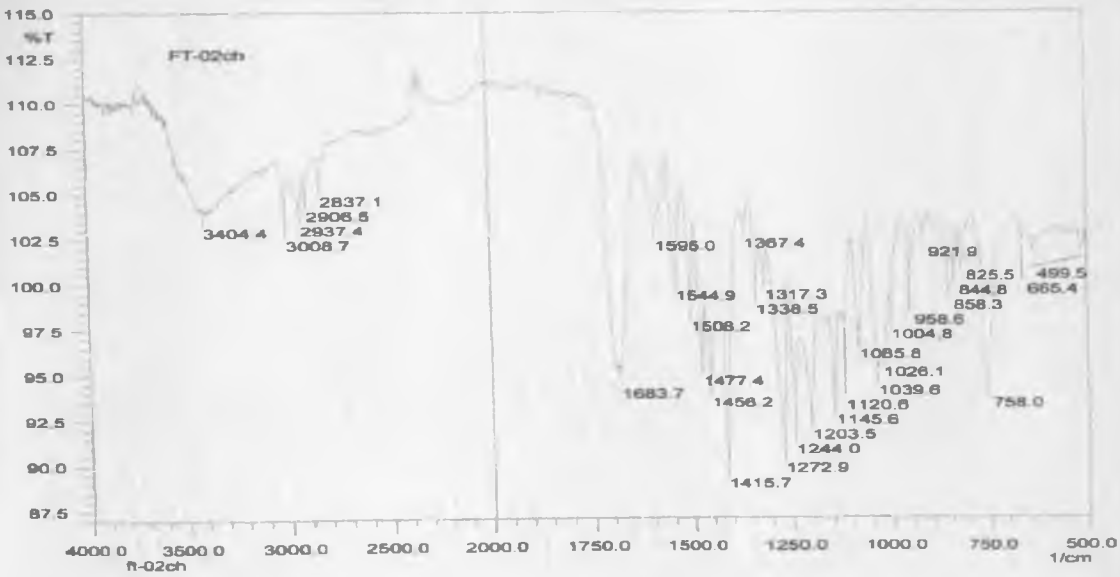



**Fig.2.17: IR spectrum of Lamellarin 2**

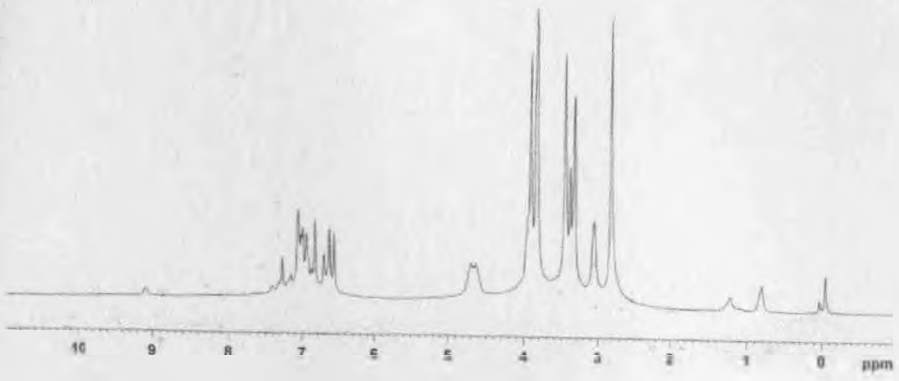



**Fig.2.18: $^{1}H$ NMR spectrum of Lamellarin 2**

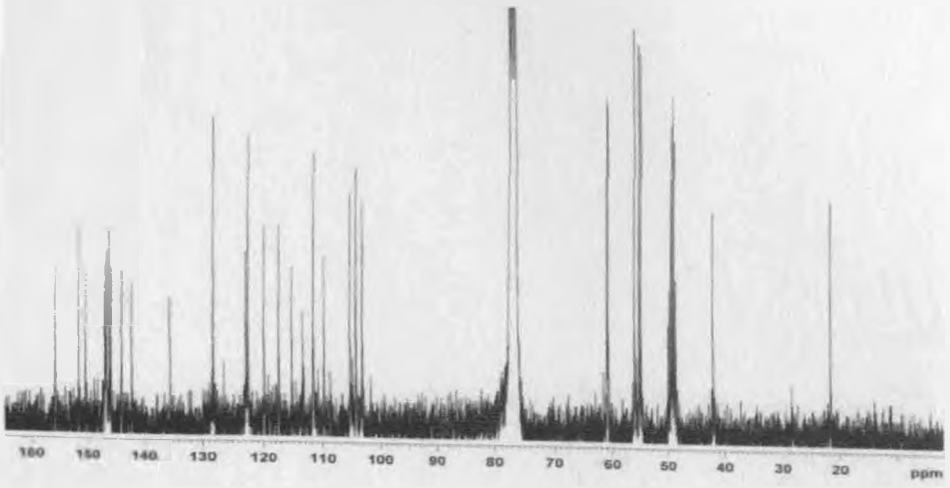



**Fig.2.19: $^{13}C$ NMR spectrum of Lamellarin 2**

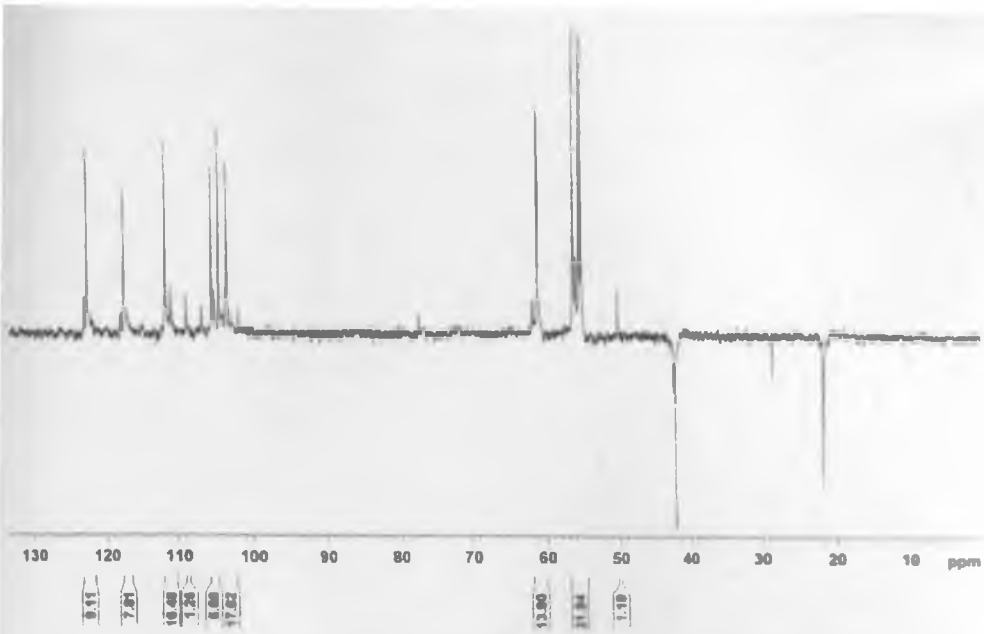



**Fig.2.20: DEPT spectrum of Lamellarin 2**

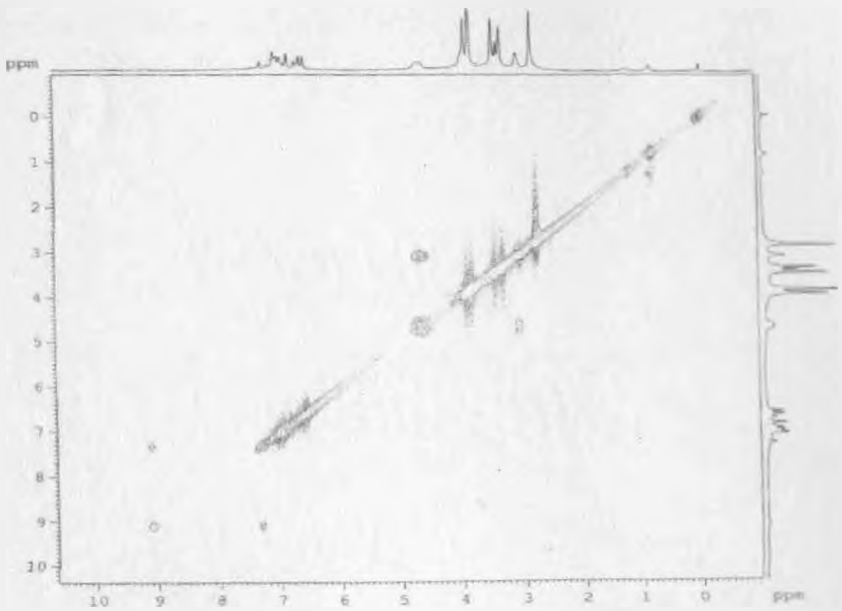



**Fig.2.21: $^{1}H$-$^{1}H$ COSY spectrum of Lamellarin 2**

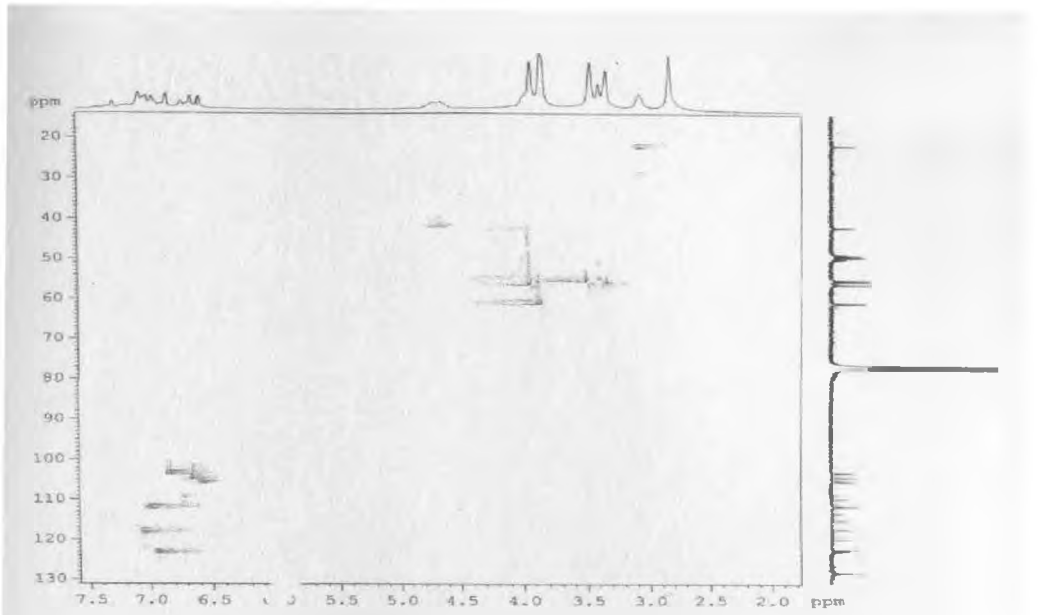



**Fig.2.22: HSQC spectrum of Lamellarin 2**

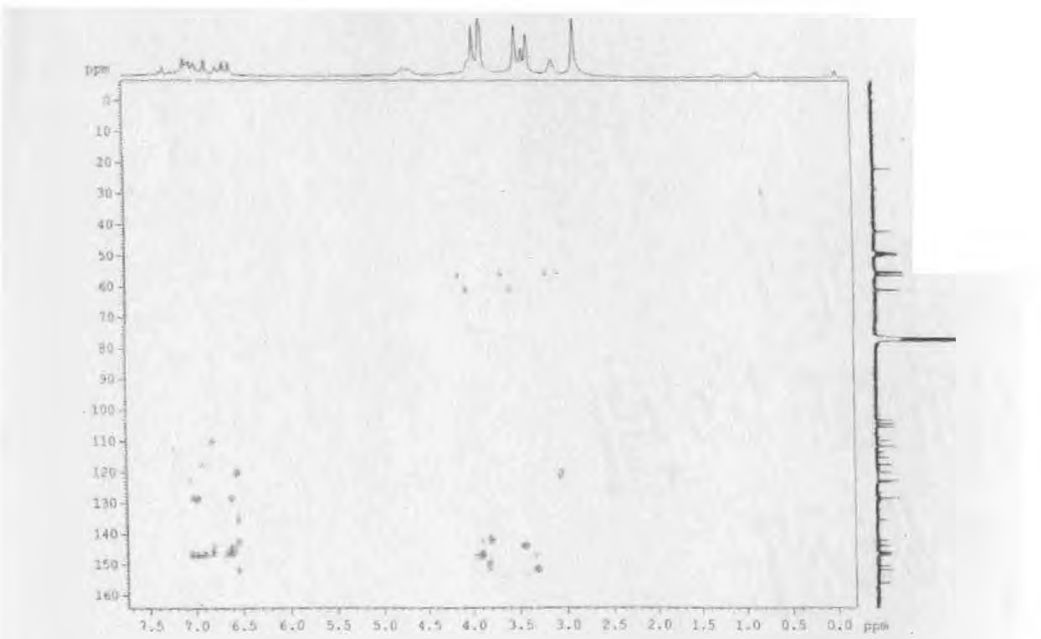



**Fig.2.23: HMBC spectrum (a) of Lamellarin 2**

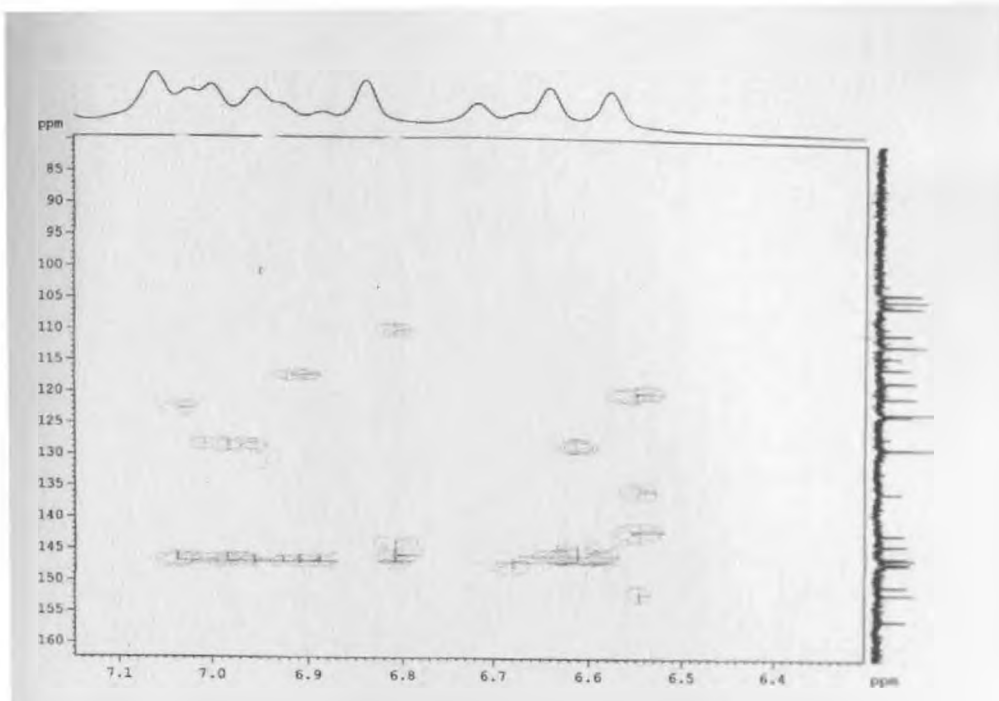



**Fig.2.24: HMBC spectrum (b) of Lamellarin 2**

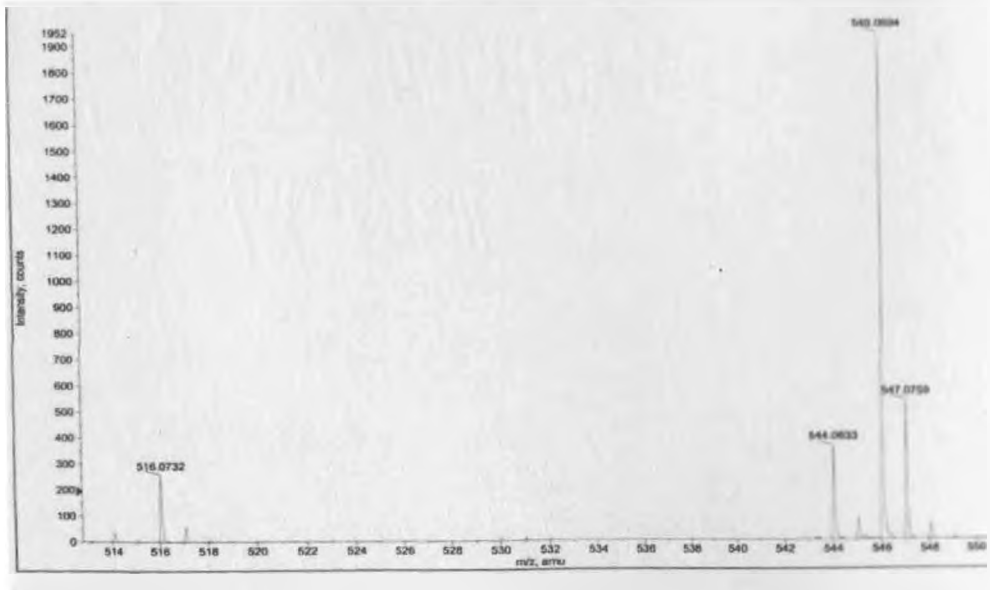



**Fig.2.25: ESI-MS spectrum of Lamellarin 2**

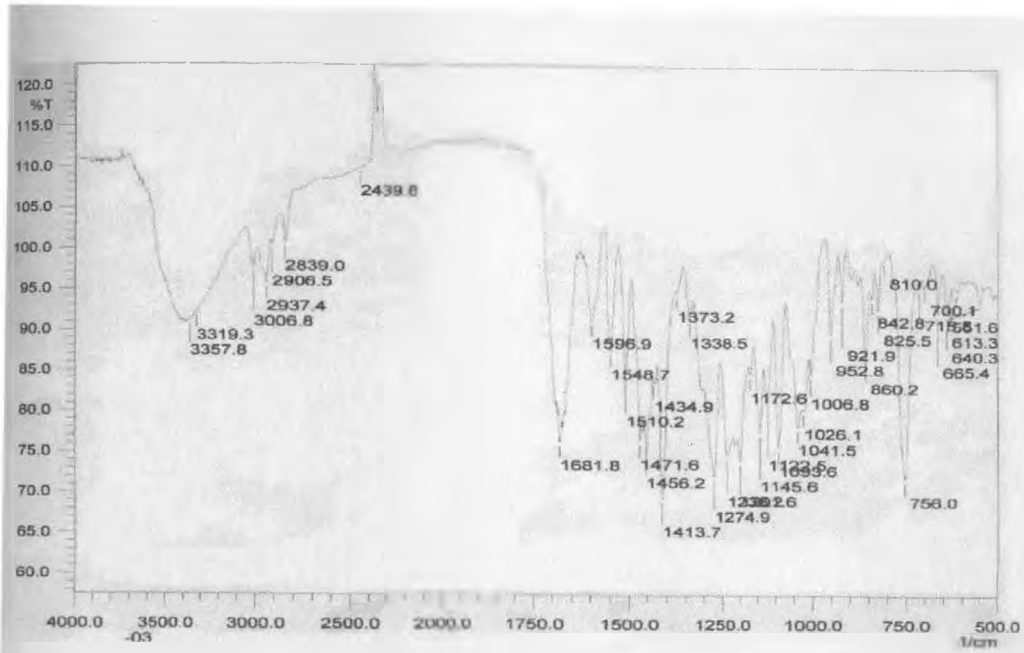



**Fig.2.26: IR spectrum of Lamellarin 3**

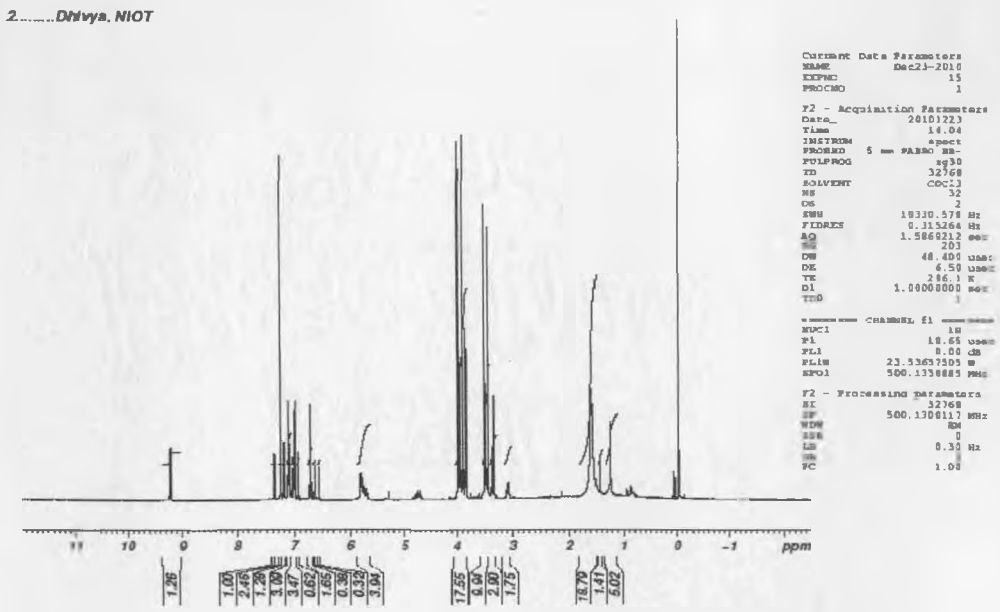



**Fig.2.27:1H NMR spectrum of Lamellarin 3**

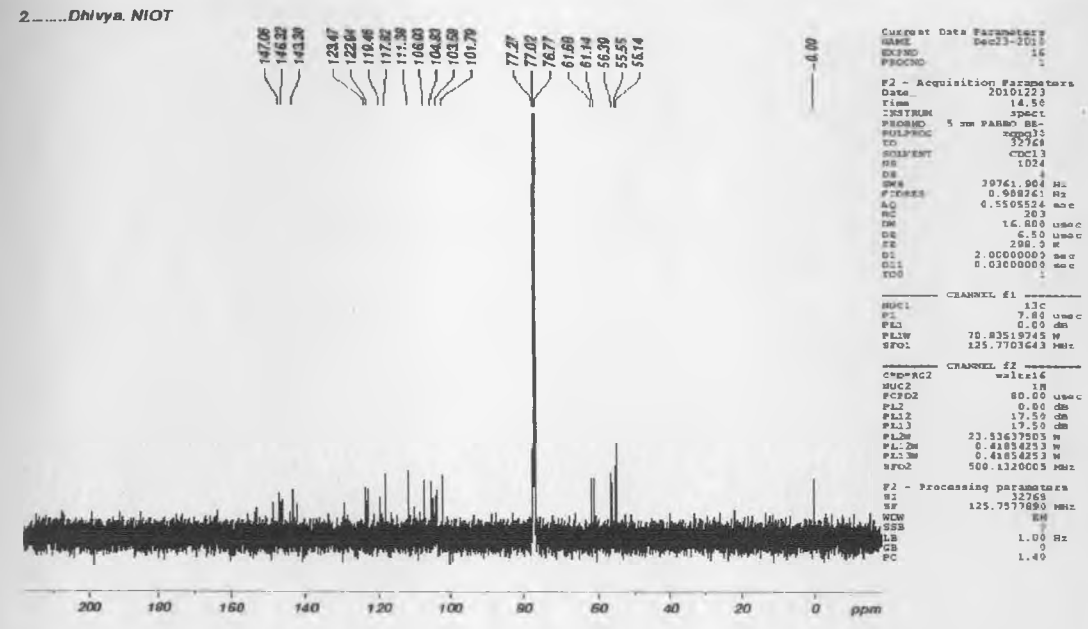



**Fig.2.28: $^{13}$C NMR spectrum of Lamellarin 3**

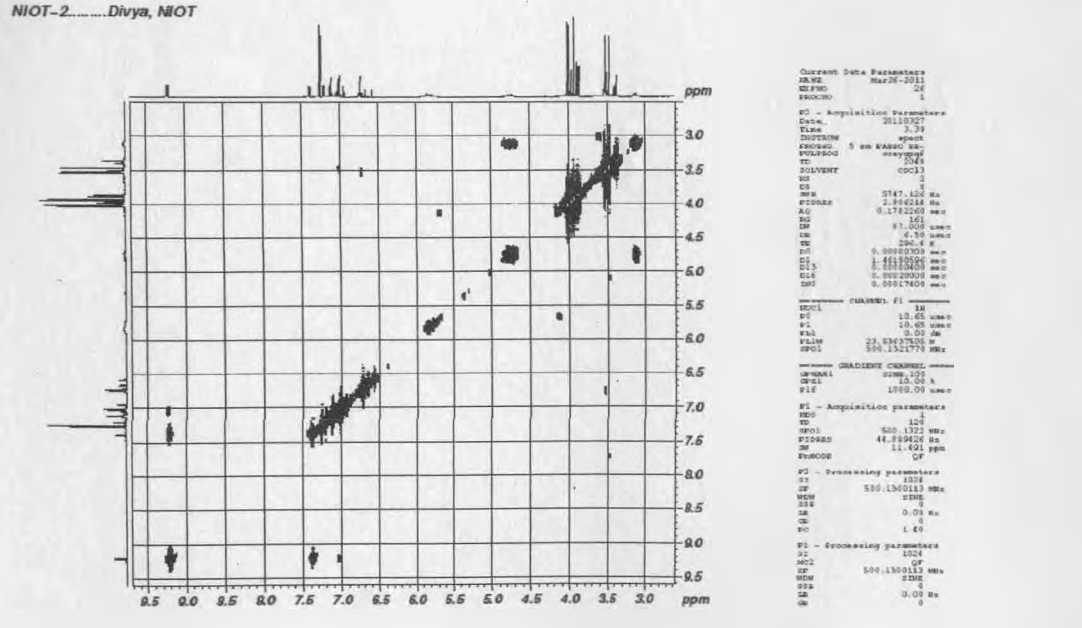



**Fig.2.29: $^{1}$H-$^{1}$H COSY spectrum of Lamellarin 3**

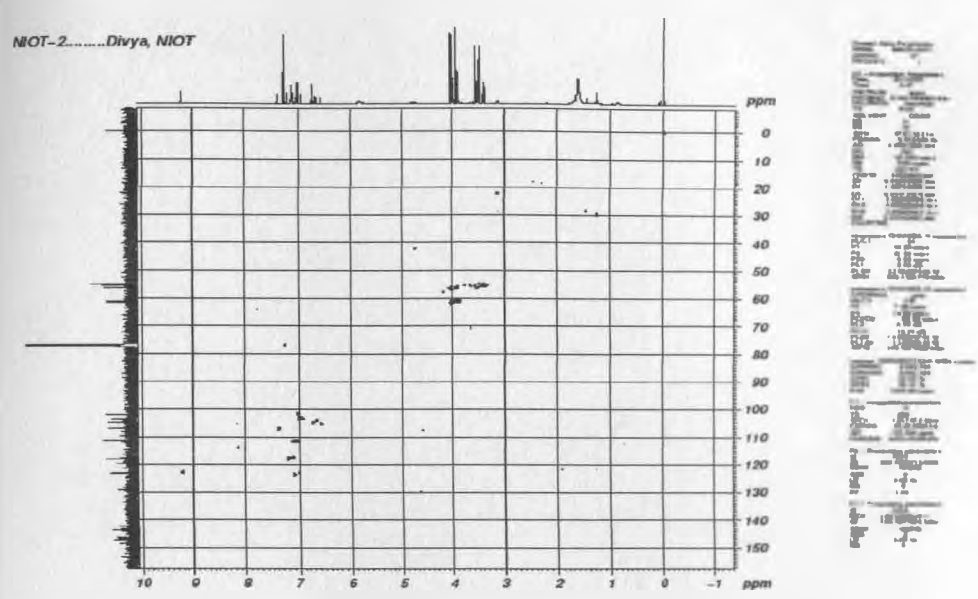



**Fig.2.30: HSQC spectrum of Lamellarin 3**

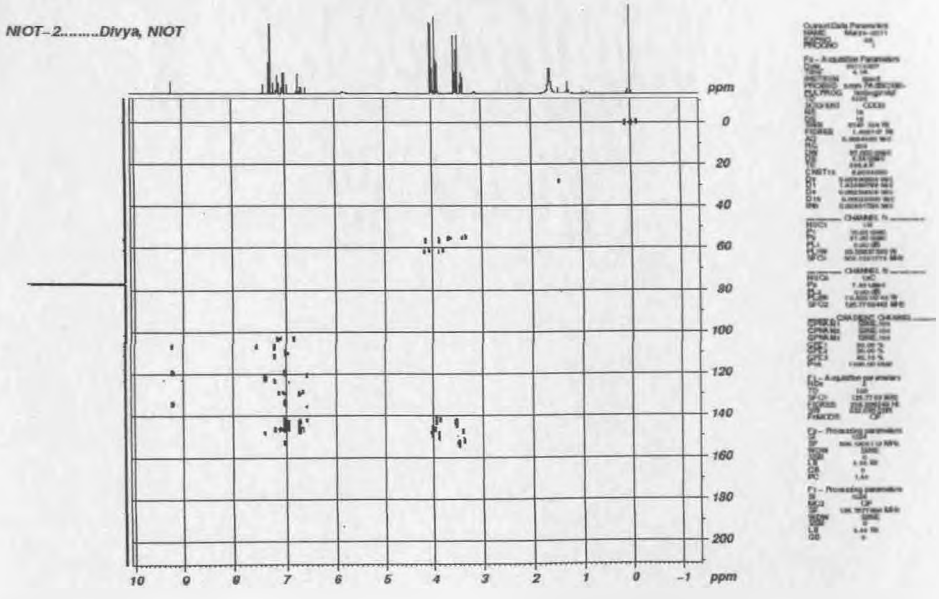



**Fig.2.31: HMBC spectrum of Lamellarin 3**

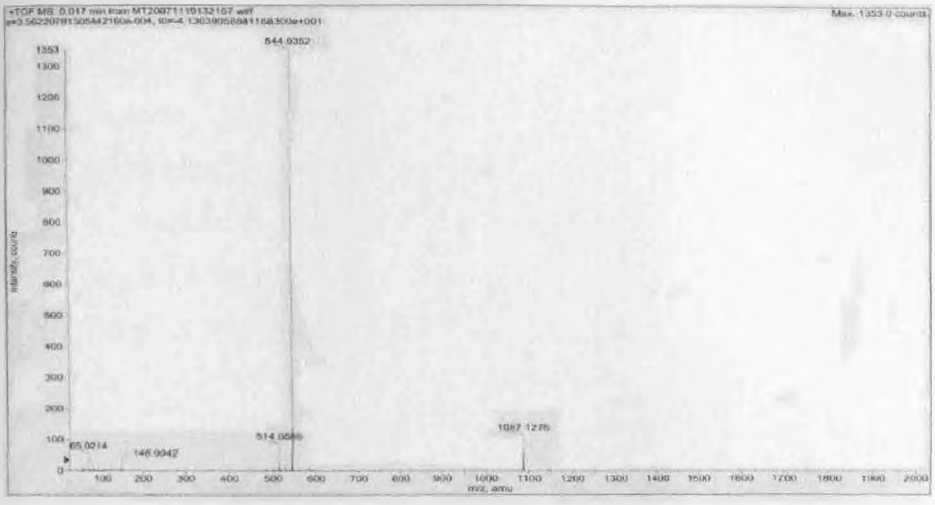



**Fig.2.32: ESI-MS spectrum of Lamellarin 3**

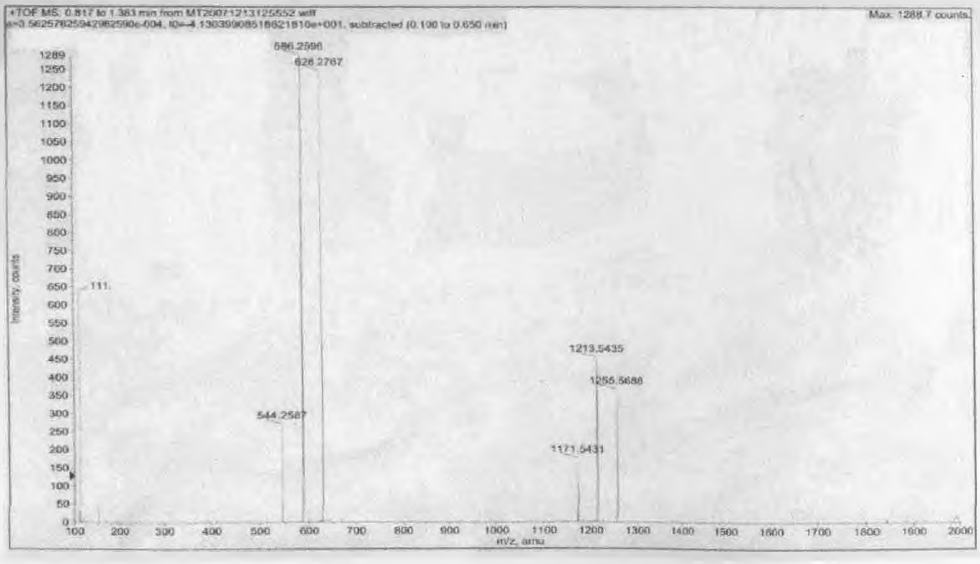



**Fig.2.33: ESI-MS spectrum of acetylation product of Lamellarin 3**

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# *Chapter III*

# *Chemical Investigation of Soft Corals Sinularia kavarattiensis & Lobophytum crassum....*

## Introduction

Soft corals are a rich source of bioactive molecules such as terpenes, steroids and steroidal glycosides.[1] It was reported that 50% of the soft coral extracts exhibited ichthyotoxic characteristics.[2] Many of the secondary metabolites of cembranoid type diterpenes from the soft corals may be involved in ecological interactions,[3] whereas other metabolites have biological activities such as antifungal, antineoplastic, ichthyotoxic,[4] cytotoxic, HIV-inhibitory[5] and anti-inflammatory activity.[6] Soft corals are widely distributed but have marked preference for tropical waters of a depth between 5 and 30 m than temperate reefs. The genera of *Sinularia*, *Lobophytum* and *Sarcophyton* are the most prolific soft corals. The range of biological activity that has been reported from these are reportedly wide. They are found to function as insect trial pheromones, termite allomones, neurotoxins, cytotoxins, anti-inflammatory and antimitotic agents.[7]

The prespective that a significant number of bioactive metabolites have been isolated from the genus of *Sinularia* and *Lobophytum*, two soft corals *Sinularia kavarattiensis* and *Lobophytum crassum* have been studied and their terpene metabolites have been presented in this chapter.

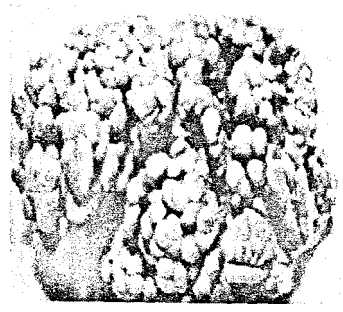
**Section 1** deals with the anti-inflammatory furanoid metabolites isolated from the soft coral *Sinularia kavarattiensis* and their ESI-MS/MS study.

**Section 2** is based on the secondary metabolites from *Lobophytum crassum*

# *Section I*

## *Sinularia kavarattiensis.....*

## Section1.1: *Sinularia kavarattiensis*: A Review



The genus *Sinularia* is characterized by a leathery soft tree like branching with heavy stalks containing finger like projections. It is sometimes called as dead man's fingers. The different species in *Sinularia* may vary in colour from brown to tan, yellow, green, pink or grey. The grey green soft coral *Sinularia kavarattiensis* discussed in this section belongs to the Phylum: Cnidaria, Class: Anthozoa, Subclass: Octocorallia, Order: Alcyonacea, Family: Alcyoniidae, Genus: *Sinularia* & Species: *kavarattiensis* (Alderslade & Shirwaikar 1991).

Soft coral of the genus *Sinularia* has been found to be a rich source of several biologically active compounds withdiverse structures including diterpenoids, sesquiterpenoids, peroxyhemiketal terpenoids, polyhydroxylated sterols etc. Many of these compounds are known to possess strong antibacterial, anti-inflammatory, antioxidant, antitumour and cytotoxic properties.

Chemical investigation on the dichloromethane-soluble fraction from the extract of *Sinularia gibberosa* Tixier- Durivault led to the isolation of three new polyoxygenated sterols, gibberoketosterols B (**1**) and C (**2**) and gibberoepoxysterol (**3**), along with two known steroids, gibberoketosterol (**4**) and 24-methylenecholest-5-en-3*â*-ol (**5**). These cholestane-type sterols possessing a 22,24(28)-conjugated diene (**1** and **2**) in the side chain or a 5R,6R-epoxide in the B-ring (**3**) were isolated for the first time from marine sources. Compound (**4**) showed significant inhibition against the up-regulation of the pro-inflammatory iNOS and COX-2 proteins of LPS-stimulated RAW264.7 macrophage cells, while **1** was found to be inactive. The

cytotoxicity of compounds **(1-4)** against the Hep G2, MCF- 7, MDA-MB-231, and A-549 cell lines was studied and steroid **(4)** exhibited moderate cytotoxicity against the growth of all cancer cell lines (IC50 13.0, 14.1, 14.4, and 14.5 μg/mL, respectively). The 5,6-epoxide-possessing sterol **(3)** also showed moderate cytotoxicity but only against MDA-MB-231 and A-549 cells (IC50 15.9 and 15.5 μg/mL, respectively).[8]

Chemical examination of a new soft coral species of *Sinularia genus* of the Indian Ocean resulted in the isolation of 4 new and rare aromadendrane type of diterpenoids **(6-9)**.The crude extract of the organism has showed moderate larvicidal activitiy.[9]

Softcoral*Sinulariakavarattiensis*collected from Mandapam coast afforded a rare sesquiterpenespathulenol**(10)** [10(14)-aromadendren-4-ol].[10]

Two sesquiterpenoids, sinularianin A **(11)** and sinularianin B **(12)** were isolated from the soft coral Sinularia sp., collected off the northeastern Taiwan coast. Sinularianin A possesses an unprecedented bicyclic skeleton, namely sinulariolane. Sinularianin B has a valerenane skeleton, with a spirobutenolide moiety. Previously, metabolites with valerenane skeleton had been isolated mostly from the plant *Valeriana officinalis.*[11]

Continuing their studies of Australian soft corals, Bowden *et al.* have isolated twelve structurally related furanosesquiterpenes, **(13-24)**, from *Sinularia capillosa.*[5] Compounds **(13,14 and 16)** were extremely acid sensitive and decomposed on storage. The product **(14)** was also isolated from *Sinularia firma.*[12]

Arepalli et al in 2009 later reported the furanosesquiterpene **(21)** from *Sinularia kavarattiensis* which was found to induce apoptosis via the mitochondrial-mediated caspase-dependent pathway in THP-1, leukemia cell line.[13]

Park et al reported the isolation and structure determination of two furanosesquiterpenes, **25** and **26**, from the soft coral *Sinularia lochmodes.*[14] Another

*Simularia* sp. collected from Aulong Island, Palau, contained the furanosesquiterpene (25).[15]

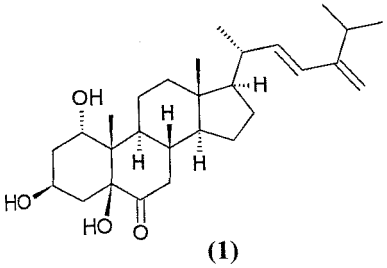
Experiments on incubation of small colonies of *Simularia capillosa*, collected from Magnetic Island (Australia), with labeled mevalonolactone afforded the furanoquinol(**27**), containing the label in the terpenoid portion of the sesquiterpene hydroquinone; this confirmed how mevalonate is incorporated into terpenoids *in vivo* in this soft coral.[16]

In 1994, the furoic acid (**28**), which had been isolated from an Indian specimen of *Simularia* sp., was shown to inactivate bee venom PLA 2 *in vitro*.[17]

Intrigued by the idea that such a simple compound might be a useful anti-inflammatory agent, Williams et al synthesized the anti-inflammatory furanosesquiterpene(**22**), a metabolite of *Simularia* sp.,[12] which employs a strategy based on the Claisen rearrangement.[18] The furoic acid (**22**) inhibited bee venom PLA 2 (46% at 0.8 μg/mL)

Soft coral *Simularia gibberosa*, collected from northern Taiwan, was investigated and resulted in the isolation of five new xeniaphyllane-type diterpenoids with a rare cyclic peroxyhemiketal (3,6-dihydro-1,2-dioxin-3-ol) moiety, sinugibberosides A–E (**28-33**). Sinugibberosides A–E were the first example of marine terpenoids possessing the rare 3,6-dihydro-1,2-dioxin-3-ol group.[19]

A rearranged norcembrane, designated sinularectin (**34**), was isolated from the Kenyan soft coral *Simularia erecta*. Sinularectin is a chlorinated highly oxygenated norcembrane with an unprecedented functionalisation of the cembrane isopropyl group.[20]



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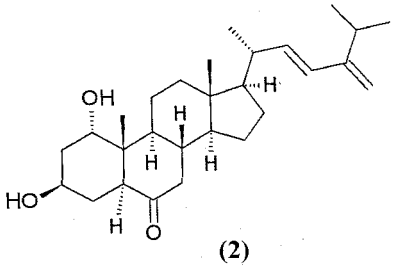

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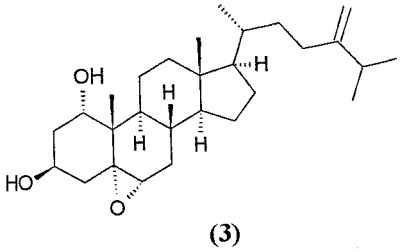
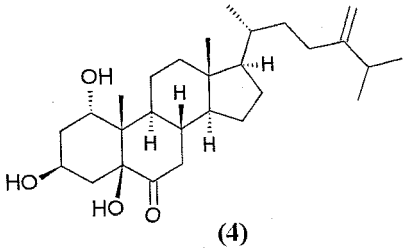


(3)



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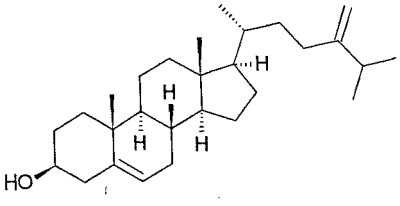

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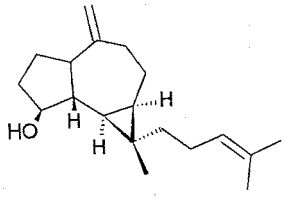

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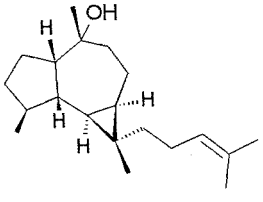

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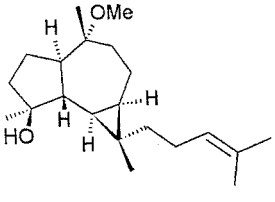

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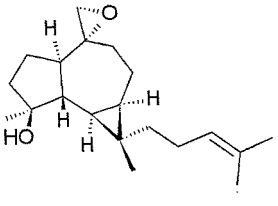

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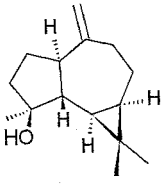

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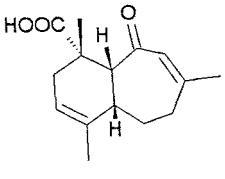

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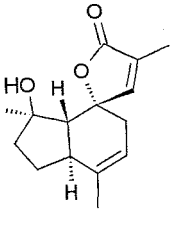

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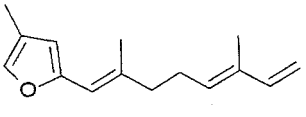

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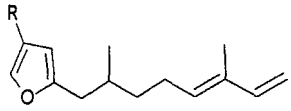
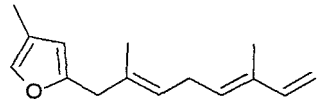


(14) R = Me

(15) R= COOMe

(16) R= COOH



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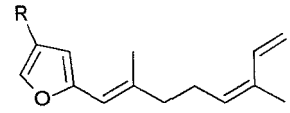
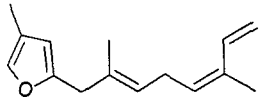


(18) R=Me

(19) R=COOMe



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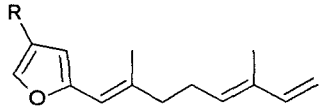
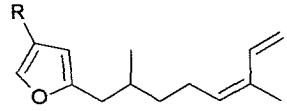


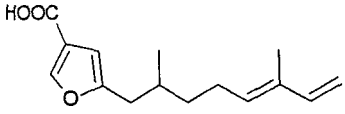
(21) R=COOMe

(22) R=COOH



(23) R=COOMe

(24) R=COOH



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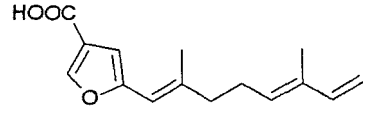

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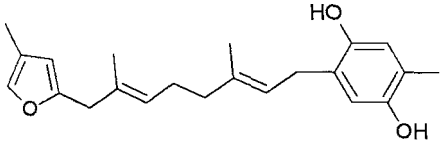

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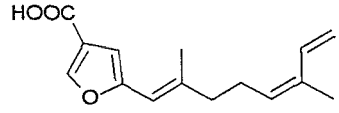

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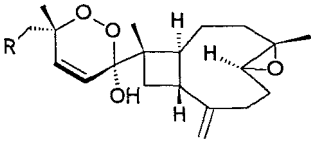
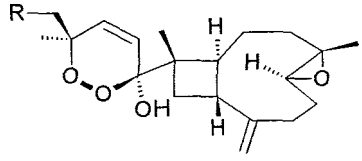


(29) R= OAc

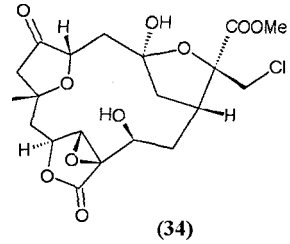
(31) R= OH

(32) R= H



(30) R= OAc

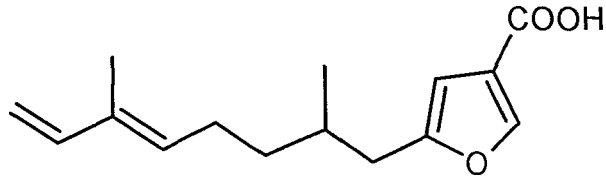
(33) R= H



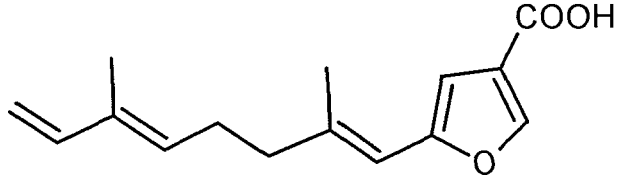
(34)

## Section 1.2 Chemical investigation of the Soft Coral *Sinularia kavarattiensis*

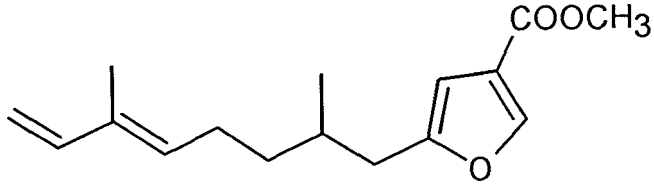
During the course of investigation of bioactive chemical constituents from marine invertebrates, we have identified sesquiterpenoids from the soft coral *Sinularia* sp., collected off Sethukarai, Mandapam the south coast of India at a depth of 10m.




**(1)** (5'*E*)-2-(2',6'-dimethylocta-(5',7'-dienyl)-4-furoic acid




**(2)** (1'E, 5'E)- 2-(2',6'-dimethylocta-(1',5',7'-trienyl)-4-furoic acid




**(3)** (5'*E*)-2-(2',6'-dimethylocta-(5',7'-dienyl)-4-furoic acid methyl ester

**Fig1: Structures of furanosesquiterpenes from the soft coral *Sinularia kavarattiensis***

## 1. Experimental Section

### a. General Experimental Procedures

Column chromatography was carried out on silica gel 60-120 mesh, Sisco, India. Gel filtration on Sephadex LH20, Pharmacia Biotech, Sweden. Fractions were monitored on TLC kieselgel 60 F254aluminum backed sheets and visualized under UV (254 nm) and iodine vapors. UV-VIS spectrum was recorded in chloroform using a Shimadzu UV-2401PC spectrophotometer. IR spectrum was recorded on a Shimadzu FTIR-8201 PC spectrophotometer. NMR spectra ($^{1}H$, $^{13}C$, COSY, HMQC and HMBC) were recorded on a BrukerAvance 300MHz instrument. Chemical shifts, relative to TMS as internal standard, are given in δ values. J values are given in Hz. Mass spectra were obtained on a QSTAR XL MS/MS system.

### b. Collection of Animal Material and Chromatography

*Simularia kavarattiensis* was collected from Sethukarai, Mandapam coast of Tamil Nadu, India, from a depth of 12 meters by SCUBA diving. The sample was identified by Dr. P.A. Thomas of the Vizhingam Research Center of the Central Marine Fisheries Research Institute, Kerala, India. A voucher specimen is deposited at the National Institute of Oceanography, Dona Paula, Goa, India.

1.2 kg of the wet sample was washed and frozen and transported to the laboratory. The frozen specimen was lyophilized and extracted successively with hexane, ethyl acetate and methanol. The sweet smelling ethyl acetate fraction was sub fractionated by passing through a Sephadex LH20 column (1:1 Methanol:Chlororoform) to obtain 8 fractions. The fraction (3) was further purified successively over silica gel columns gradient hexane: ethylacetate to obtain the pure compounds 1, 2 and 3.The structures of the purified compounds were deduced based on NMR ($^{1}H$,$^{13}C$,COSY,HSQC &HMBC), MS and Infra Red Spectroscopy.

**Table 1:$^{1}$H, $^{13}$CNMR, COSY and HMBC of compound(1) (5'*E*)-2-(2',6'-dimethylocta-(5',7'-dienyl)-4-furoic acid**

| Carbon No. | $^{13}$CNMR $\delta_C$, ppm | $^{1}$HNMR $\delta_H$, ppm | $^{1}$H -$^{1}$H COSY Correlations | $^{1}$H -$^{13}$C HMBC Correlations |
|---|---|---|---|---|
| **2** | 157.19 | - | - | - |
| **3** | 141.51 | 6.4(1H,s) | - | 3,5 |
| **4** | 119.13 | - | - | - |
| **5** | 147.68 | 7.9(1H,s) | - | 2,3 |
| **1'** | 35.12 | 2.6 (2H,m) | H2' | 2'Me,2 |
| **2'** | 32.08 | 1.8(1H,m) | H 1',H2',H3' | - |
| **3'** | 36.09 | 1.3-1.4(2H,m) | H 2',H4' | 2'Me,1',5' |
| **4'** | 25.07 | 2.2(2H,m) | H3',H5' | 6',3' |
| **5'** | 132.80 | 5.4(1H,t,7.2Hz) | H4' | - |
| **6'** | 134.10 | - | - | - |
| **7'** | 105.98 | 6.37(1H,dd,17.3 & 10.7 Hz) | H8' | 6'Me,5' |
| **8'** | 110.57 | 4.95(1H,d,10.6Hz) 5.10(1H,d,17.3Hz) | H7' | 6' |
| **6'-Me** | 11.67 | 1.77,s | - | - |
| **2'-Me** | 19.39 | 0.9(3H,d) | H2' | 1',3' |
| **4'-COOH** | 168.96 | - | - | - |

**Table 2:$^{1}$H, $^{13}$CNMR of compounds (2) (1'E, 5'E)- 2-(2',6'-dimethylocta-(1',5',7'-trienyl)-4-furoic acid, (3) (5'*E*)-2-(2',6'-dimethylocta-(5',7'-dienyl)-4-furoic acid methyl ester**

| Carbon No. | $^{13}$CNMR $\delta_C$, ppm(2) | $^{1}$HNMR $\delta_H$, ppm(2) | $^{13}$CNMR $\delta_C$, ppm(3) | $^{1}$HNMR $\delta_H$, ppm(3) |
|---|---|---|---|---|
| 2 | 155.02 | - | 157.19 | - |
| 3 | 141.33 | 6.53(1H,s) | 141.48 | 6.37(1H,s) |
| 4 | 119.77 | - | 119.05 | - |
| 5 | 146.83 | 8.00(1H,s) | 147.59 | 7.98(1H,s) |
| 1' | 113.51 | 6.09(1H,s) | 35.11 | 2.5(2H,m) |
| 2' | 140.99 | - | 32.08 | 2.1(1H,m) |
| 3' | 40.28 | 2.3(2H,m) | 36.08 | 1.27(2H,m) |
| 4' | 26.20 | 2.2(2H,m) | 25.68 | 2.2(2H,m) |
| 5' | 131.66 | 5.4(1H,t ,7.2Hz) | 132.77 | 5.4(1H,t,7.2Hz) |
| 6' | 134.59 | - | 134.09 | - |
| 7' | 106.67 | 6.37(1H,dd,16.6 &11.2Hz) | 105.98 | 6.37(1H,dd,16.6 &11.2Hz) |
| 8' | 110.85 | 4.95(1H,d,11.2Hz ) 5.11(1H,d, 16.6Hz) | 110.26 | 4.95(1H,d,11.2Hz 5.11(1H,d,16.6Hz) |
| 6'-Me | 11.69 | 1.77(3H,s) | 11.67 | 1.75(3H,s) |
| 2'-Me | 18.72 | 2.00(3H,s) | 19.37 | 0.94(3H,d) |
| 4'-COOH | 168.07 | - | 168.22 | - |
| 4'-COOMe | - | - | 50.69 | 3.50(3H,s) |

## 2. Identification of the isolated compounds:

Compound (1) was obtained as a pale yellow liquid with a pleasant smell. The UV-Visible spectrum of the compound in chloroform showed λmax at 247 nm. The infra red spectrum was typical of acids with a characteristic broad band from 2500cm$^{-1}$ to 3200cm$^{-1}$. Acid carbonyl was observed at 1693.4cm$^{-1}$. The olefinic and aliphatic C-H signals were observed at 2800- 3050cm$^{-1}$. C-O signals were observed at 1215.1 and 1143.7cm$^{-1}$.

The $^{1}$H NMR spectrum of the compound exhibits 12 different signals indicating 12 different types of protons present in the molecule. $^{13}$C NMR is indicative of 15 different types of carbons with the DEPT 135spectrum distinguishing 2-methyls, 4-methylenes, 4-methines and the rest quaternary carbons in the spectrum. The quarternary carbon at δ 168.96 was indicative of an acidic carbonyl, which is supported by the signal at 1693.4cm$^{-1}$in the infrared spectrum. Similarly the methylene at δ 110.9(2H) could be attributed to an exomethylene group.
This was further supported by the $^{1}$H NMR spectrum, which indicated presence of 7 vinyl protons;3 singlets(1H) each, one multiplet(1H) and the remaining 3 protons as a mutually coupled AMX pattern at δ 4.96,5.12 & 6.38(1H each)5.0,5.4 &6.4
Based on its$^{1}$H, $^{13}$CNMR, COSY and HMBC spectra of compound **(1)** it was identified as (5'*E*)-2-(2',6'-dimethylocta-(5',7'-dienyl)-4-furoic acid which was further supported by a pseudomolecular ion $[M+H]^{+}$ at *m/z* 249 in its ESI-MS spectrum. Compound1 was earlier reported by Mizobuchiet al[15] in 1994 from a *Sinularia sp.* collected from Aulong Island, Palau. This is the first report of (5'*E*)-2-(2', 6'-dimethylocta-(5', 7'-dienyl)-4-furoic acid from *Sinularia kavarattiensis.*

The $^{13}$C and DEPT 135 spectra of compound 1 (yield=0.235g,0.025 based on wet weight) m/z=249$[M+H]^{+}$, indicated the presence of 15 carbons, distributed as 5 quarternary, 5 methines(CH), 3 methylenes($CH_2$) and 2 methyls($CH_3$). Among these the signal at δ167.7 was attributed to a carboxylic acid, which was also confirmed by the strong peak at m/z 202[M-CO2]+ in the ESI-MS spectrum. Similarly the doublet at δ 110.9(2H) could be attributed to an exomethylene group. This was further

supported by the 1H NMR spectrum, which indicated presence of 7 vinyl protons;3 singlets(1H) each, one multiplet(1H) and the remaining 3 protons as a mutually coupled AMX pattern at δ 4.96,5.12 & 6.38(1H each).Detailed 2D NMR studies (1H-1H COSY,HMQC & HMBC) confirmed the structure as (1'E,5'E)-2-(2',6'-dimethylocta-1',5',7'-trienly)-4-furoic acid 1

This is the first report of the occurrence of (1'E,5'E)-2-(2',6'-dimethylocta-1',5',7'-trienly)-4-furoic acid 1 from *Sinularia kavarattiensis*. Earlier compound 1 was isolated from the Australian Soft Coral *Sinularia capillosa*.[12]

Compound (2) was isolated as a yellow liquid. The NMR spectrum of the compound shows an additional double bond at 1' position in the side chain to the furan ring as evident by the additional singlet in $^{1}$H NMR at δ 6.09 and the corresponding $^{13}$C signals at δ 113.51 and δ 140.99 and the disappearance of the signals in proton NMR at δ 2.6, ddd, δ 1.8, m and$^{13}$C signal at δ 32.08 when compared to compound (1) (5'*E*)-2-(2', 6'-dimethylocta-(5', 7'-dienyl)-4-furoic acid. This was further supported by the 1H NMR spectrum, which indicated presence of 7 vinyl protons;3 singlets(1H)each, one multiplet(1H) and the remaining 3 protons as a mutually coupled AMX pattern at δ 4.95,5.11& 6.37(1H each).
The structure of the compound was identified as (1'E,5'E)-2-(2',6'-dimethylocta-1',5',7'-trienly)-4-furoic acidwhich was supported by a pseudomolecular ion $[M+H]^{+}$ at *m/z* 247 in its ESI-MS spectrum.

The compound was previously reported by Bowden et al in 1983 from the Australian soft coral *Sinularia capillosa*.[12]

It was also reported by Limna mol et al., 2009[21] from *Sinularia kavarattiensis* collected from gulf of Mannar, Tamil Nadu, India and was reported to show antifouling activity against the barnacle *Balanus amphitrite*.

Compound (3) was obtained as a sweet smelling liquid and was found to be the methyl ester of compound (1) as evident by the additional methoxy signal at δ3.5, s in $^{1}$H NMR and the corresponding carbon signal at δ 50.69, and the rest of the signals

being remained unchanged. The structure was further supported by a pseudo molecular ion $[M+H]^+$ at *m/z* 263 in its ESI-MS spectrum

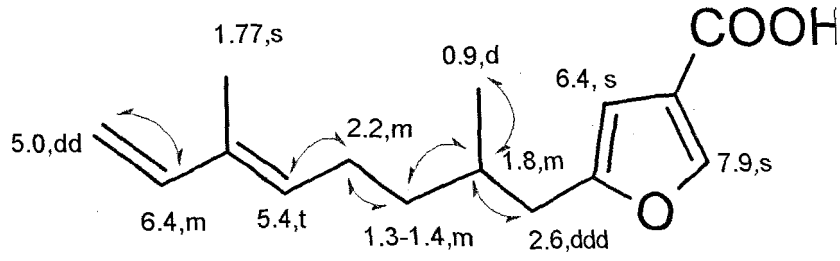



**Fig 2: COSY correlations of furanosesquiterpene1**

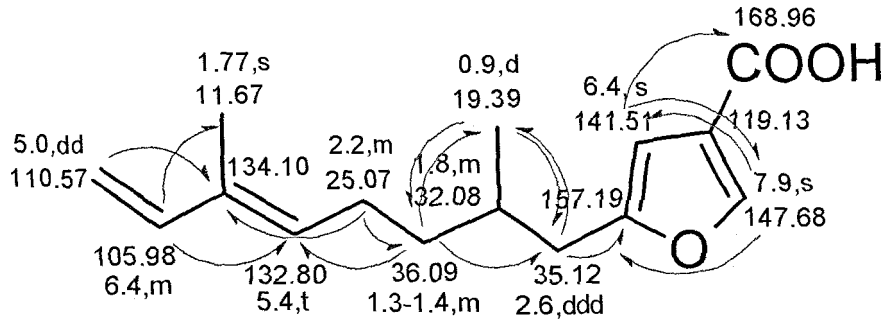



**Fig 3: HMBC correlations of furanosesquiterpene1**

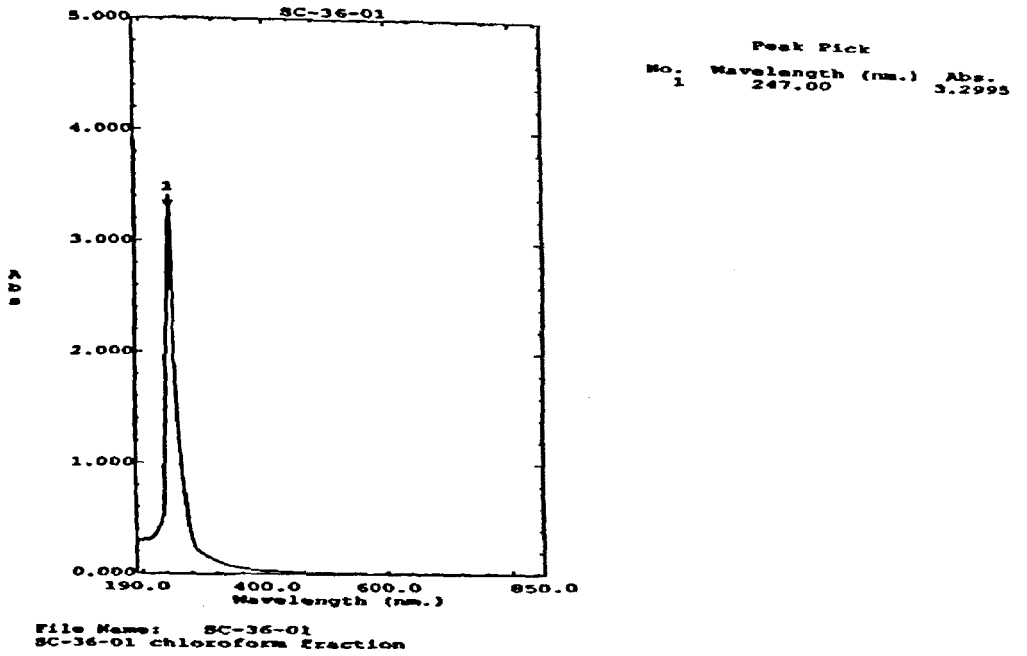



**Fig 4: UV-Visible spectrum of compound 1**

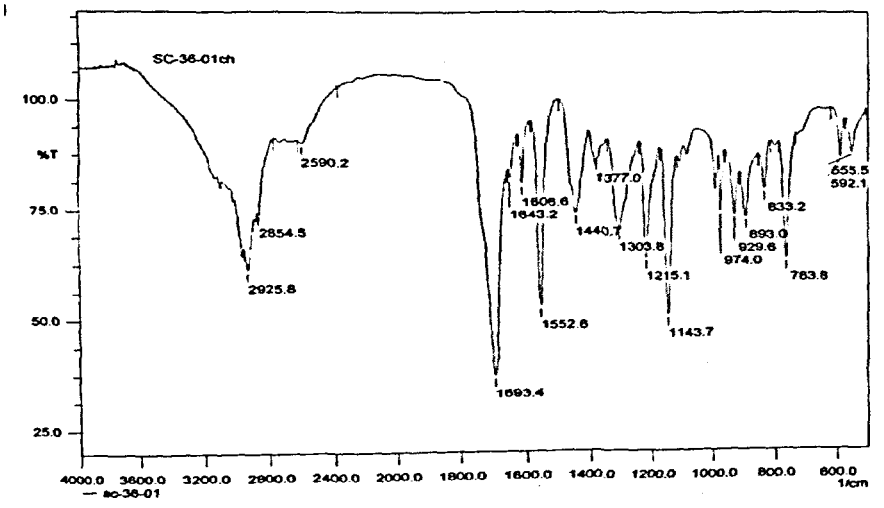



**Fig 4: IR spectrum of compound 1**

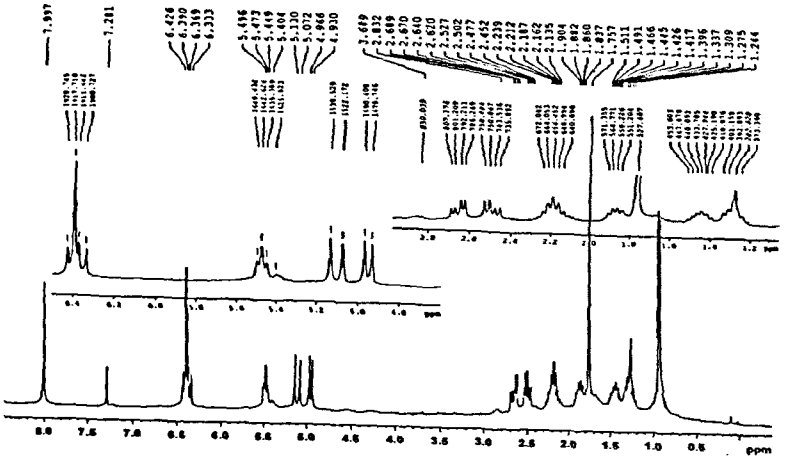


**Fig 5: $^{1}$H NMR spectrum of compound 1**

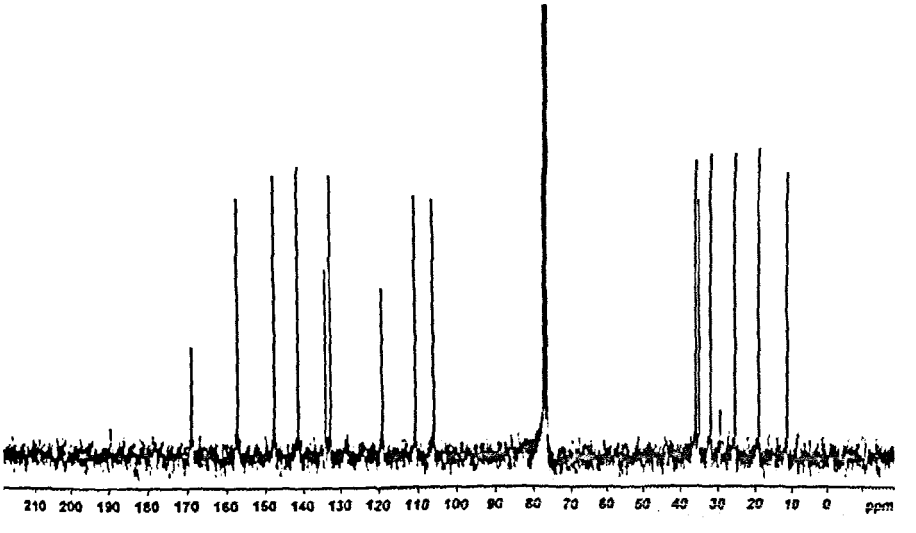


**Fig 6: $^{13}$C NMR spectrum of compound 1**

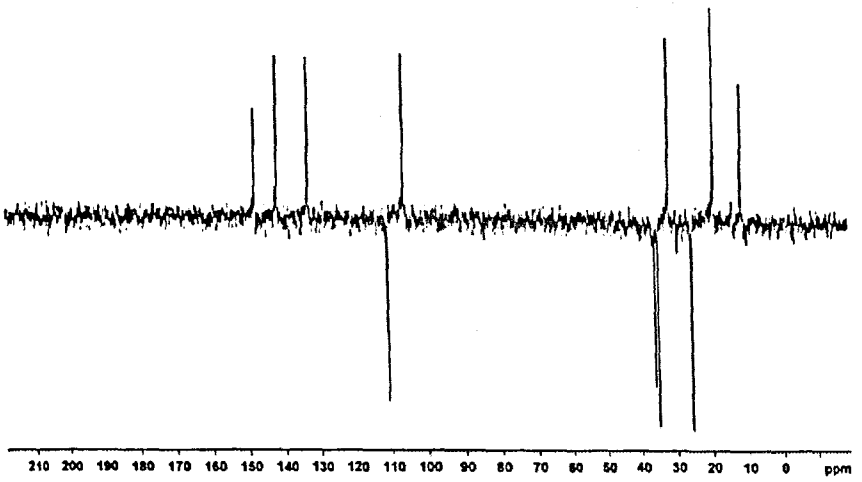



**Fig 7: DEPT spectrum of compound 1**

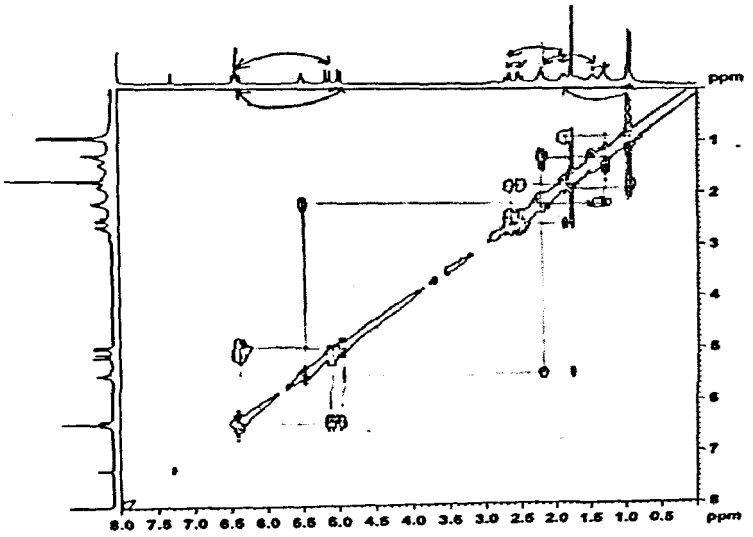



**Fig 8: $^{1}H$-$^{1}H$ COSY spectrum of compound 1**

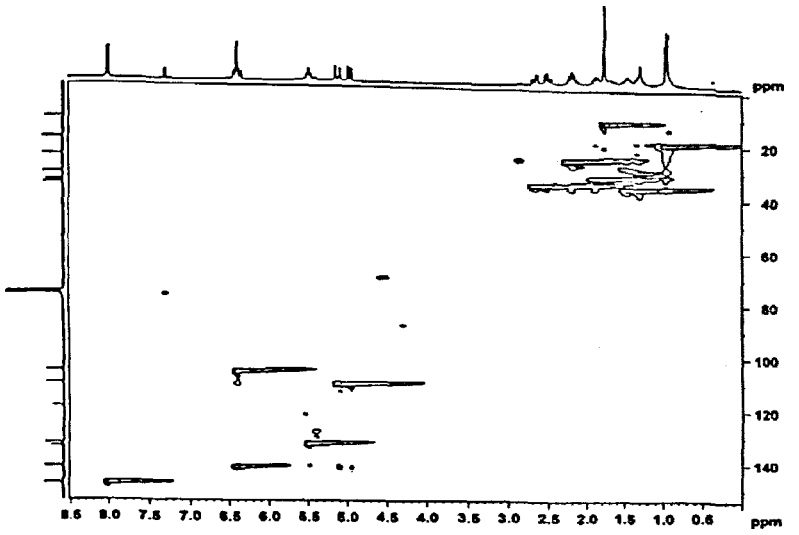



**Fig 9: HSQC spectrum of compound 1**

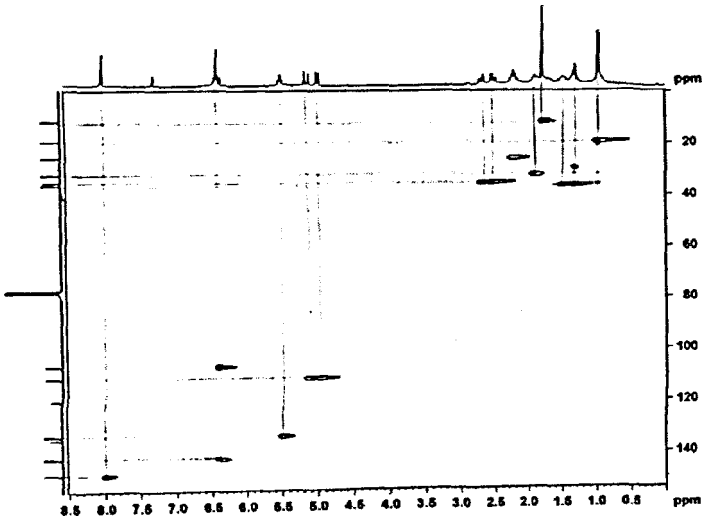



**Fig 10: HSQC spectrum of compound 1**

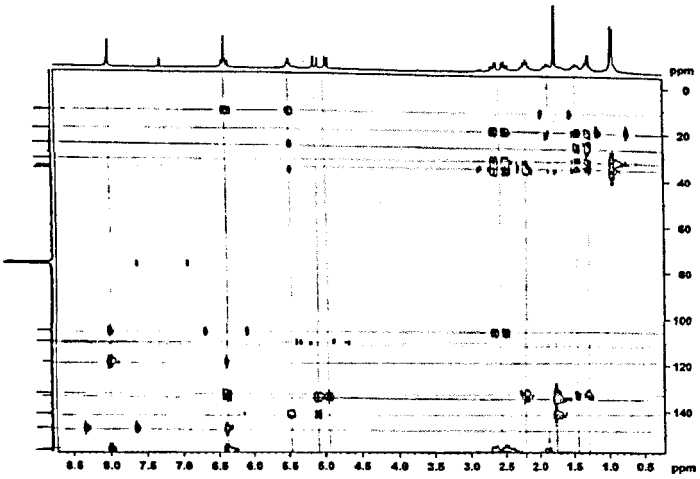



**Fig 11: HMBC spectrum of compound 1**

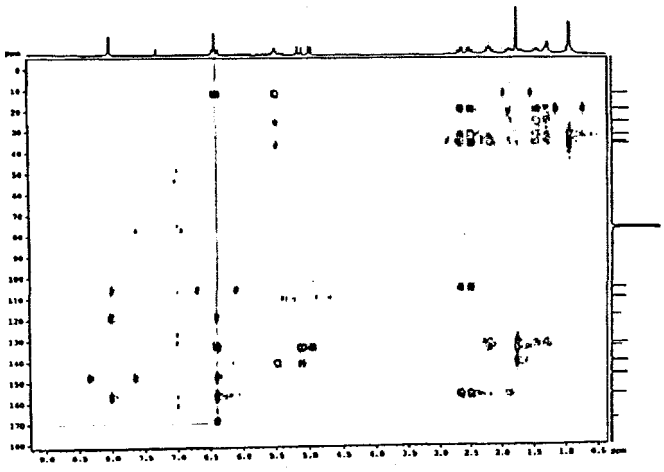


**Fig 12: HMBC spectrum of compound 1**

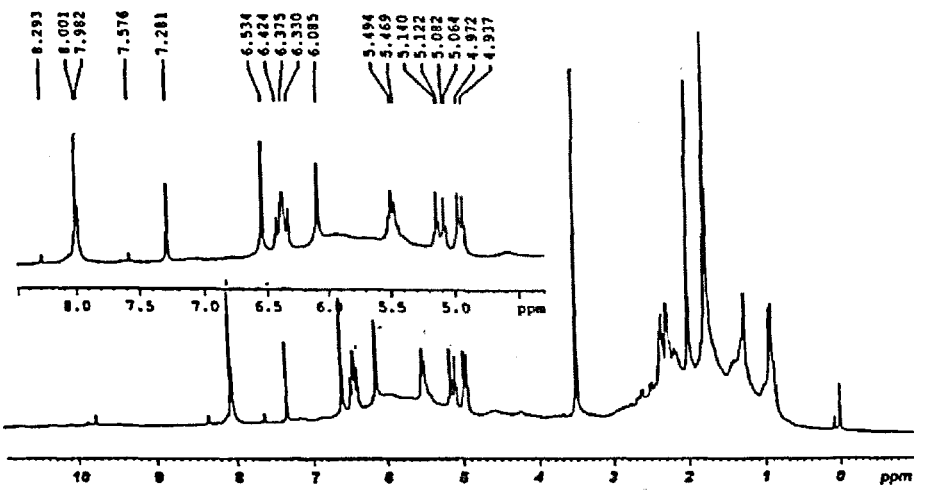



**Fig 13: $^{1}$H NMR spectrum of compound 2**

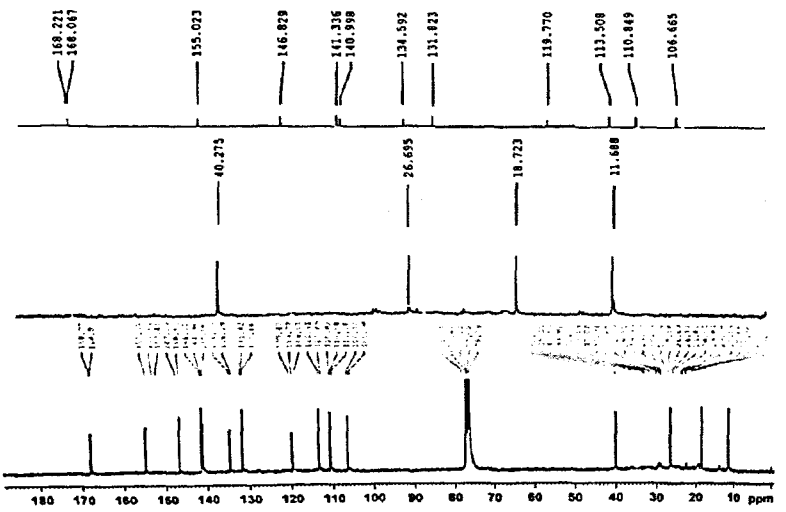



**Fig 14: $^{13}$C NMR spectrum of compound 2**

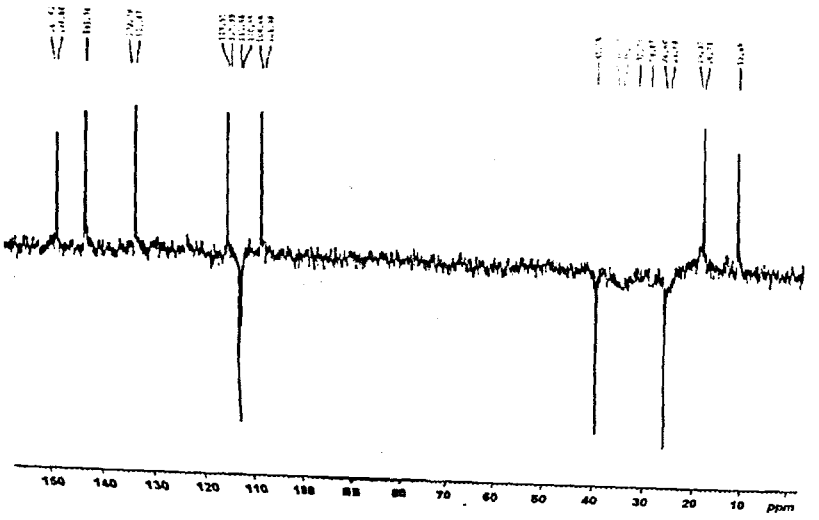



**Fig 15: DEPT spectrum of compound 2**

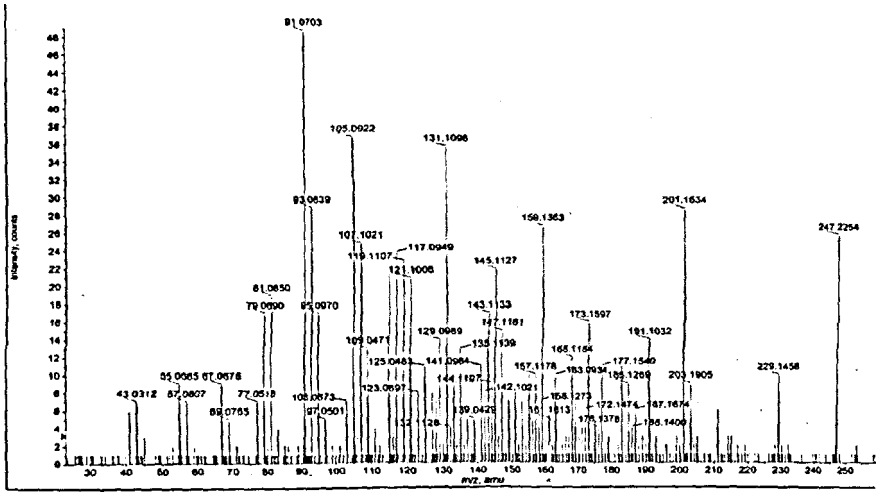



**Fig 16: ESI-MS spectrum of compound 1**

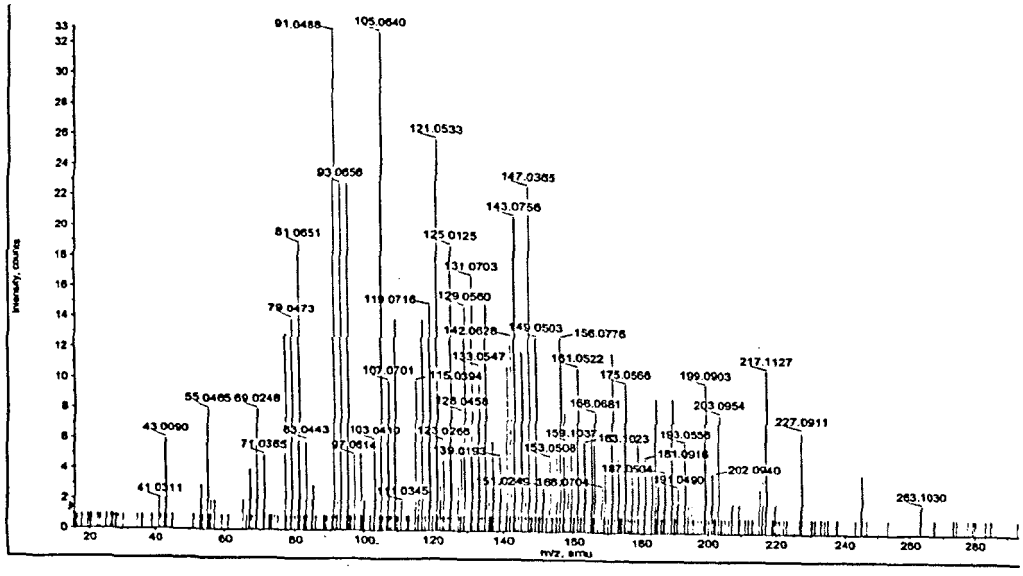



**Fig 17: ESI-MS spectrum of compound 2**

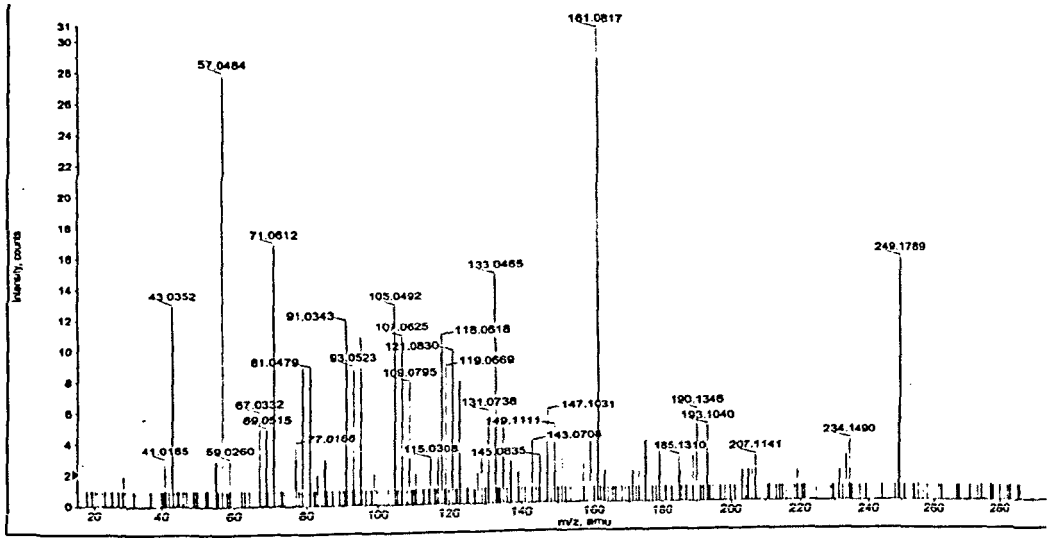



**Fig 18: ESI-MS spectrum of compound 3**

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**Section 1.2: ESI- MS study of furano sesquiterpenes from Indian soft coral *Sinularia kavarattiensis*.**

In modern drug discovery and development programmes, it has been possible to identify unknown compounds in complex biological mixtures, with the highest possible accuracy because of modern analytical techniques. Conventional analytical methods approach for characterization of molecular species is time consuming, involving multistep procedures and often lacks the sensitivity required for analysis of low concentration of molecules present in biological samples.

Electrospray mass spectrometry is a relatively new technique for soft ionization of biological molecules. In electrospray mass spectrometry, the molecules (present in a mixture) dissolved in a suitable solvent are charged and droplets are formed at atmospheric pressure and room temperature by electrostatic nebulization. The solvent is evaporated by a curtain gas until only the charged solute remains, which is subsequently drawn into the low vacuum of a mass analyzer. This technique gives accurate molecular mass and molecular formula under ESI-MS conditions of all the constituents present in the mixture and measures the mass of its fragment ions generated in the collision cell when the intact molecule is subjected to MS/MS analysis. Because the fragmentation of similar, ionized, intact molecule and structures follows similar pathways, the mass of the protonated molecule and its fragmentation pattern readily lead to the unambiguous identification of molecules belonging to a specific class of compound. Although used extensively in protein chemistry, this technique has now been extended for the structure determination of any class of compounds [1-6]

Determination of molecular structure by mass spectrometry is analogous to solving a jigsaw puzzle. A molecule is ionized with sufficient internal energy to break it into several fragments which are then individually identified and reassembled according to valence rules to determine the structure of the original ion. Thus fragmentation of

molecular ions is central to the characterization by tandem mass spectrometry (ESI-MS/MS analysis).

ESI-MS/MS studies have been reported in literature to elucidate structures of unstable molecules or molecules present in the biological mixture, in trace amounts.
Electrospray ionization mass spectrometry was used by Pulfer et al. (2003) to study the somewhat unstable ozonolysis product of cholesterol which was found to display unique ionization and fragmentation properties when subjected to Collision Induced Dissociation (CID).[7]
Tilvi and Naik (2007) deduced the structures of new kahalalides R & S analogues of the anticancer metabolite Kahalalide F from the mollusk *Elysia* and its algal diet *Bryopsis plumosa* from their methanolic extract.[8]
Tilvi et al (2005) also reported the identification of eight molecular species of ceramides from the marine sponge *Haliclona cribricutis* using Electrospray Ionization Tandem Mass Spectrometry (ESI-MS/MS).[9]

In this section of chapter 3 we have applied electrospray ionization tandem mass spectrometry, for identification of furano sesquiterpenes from Indian soft coral *Sinularia kavarattiensis* collected from Sethukarai, Mandapam, the south coast of Tamil Nadu.

Though 3 furanosesquiterpene molecules were isolated from the terpene rich fraction, as detailed in section 3.1.2, we found that they were quite unstable leading to several artifacts on standing or degrading during the purification process. Therefore it was felt necessary to use the above soft ionization technique which was found to be quite suitable for detection and determination of structure of these unstable terpenes in the mixture. This led to the detection of 10 additional sesquiterpenes based on their fragmentation pattern.

The furano sesquiterpenes contains two parts: the furan ring and the olefin portion. Olefins/alkenes are found to undergo allylic bond cleavage by hemolytic or

heterolytic means leaving the charge upon either fragment. Alkenes with γ hydrogen also undergo a rearrangement involving a six membered transition state called McLafferty rearrangement common for carbonyl compounds. Alkenes usually form fragments corresponding to $C_nH_{2n+1}^+$, $C_nH_{2n}^+$, and $C_nH_{2n-1}^+$ (the latter two fragment ion series are more abundant). It is very difficult to locate the position of the double bond in an alkene because of the easy migration of the double bond by hydride and hydrogen atom shifts and rearrangements. For this reason, the mass spectra of alkene isomers are nearly identical and almost impossible to distinguish. Herein the sesquiterpenes were distinguished based on the molecular ion peaks and the elimination of small molecules such as $H_2O$, HCOOH, and the methyl groups from the parent ion and their biogenesis.

The different peaks in the ESI-MS spectra of the partially purified terpene rich faction were subjected to Collision Induced Dissociation (CID) to obtain fragments which are characteristic for each class of compounds.

### 1. ESI-MS/MS analysis of furano sesquiterpenes rich fraction:

The ESI-MS profile **(Fig.3.1)** of the terpene rich fraction gave major peaks at $[M+H]^+$ =217.18, 219.14, 221.12; 229.09, 231.10, 233.12, 235.14, 237.13; 245.08, 247.10, 249.11; 263.09 & 265.11 corresponding to elemental composition having molecular formula $C_{15}H_{20}O$, $C_{15}H_{22}O$, $C_{15}H_{24}O$, $C_{15}H_{16}O$, $C_{15}H_{16}O_2$, $C_{15}H_{18}O_2$, $C_{15}H_{22}O_2$, $C_{15}H_{24}O_2$, $C_{15}H_{16}O_3$, $C_{15}H_{18}O_3$, $C_{15}H_{20}O_3$, $C_{16}H_{22}O_3$, and $C_{16}H_{24}O_3$ respectively.

Furano sesquiterpenes [compounds **1(3a)**, **2(3b)** & **3(3c)**] with pseudo molecular ions at m/z at 249.11, 247.10 and 263.09, identified on the basis of NMR and Mass spectral data and reported in section 3.1.2 of this chapter were subjected to ESI-MS/MS i.e. tandem mass spectroscopy and their fragmentation pattern was utilized as markers for the detection of the other similar terpenes in the ESI-MS spectrum.

ESI-MS/MS spectra **(Fig.3.2, 3.3, 3.4)** and the fragmentation pathway observed for $[M+H]^+$ = 249, 247 & 263 are given in **Fig.3a**, **3b** and **3c** respectively.

Based on the interpretation of the tandem mass spectral data **(Fig.3.5-3.14)** these molecular ions have been grouped into five clusters viz.

**Cluster 1:** The protonated molecular ion peaks observed at m/z 217.18 **(3d)**, 219.14 **(3e)**, 221.12 **(3f)**; were assigned for furanosesquiterpenes with the carboxylic acid group as in 247 **(3b)** and 249 **(3a)** unoxidised i.e a methyl at the 4 position of the furan ring.

**Cluster 2:** The protonated molecular ion peaks observed at m/z 231.10 **(3g)**, 233.12 **(3h)**, 235.14 **(3i)**, 237.13 **(3j)**; were assigned for furanosesquiterpenes with the carboxylic acid group replaced by a $CH_2$ OH (alcohol) group at the 4 position of the furan ring.

**Cluster 3:** The protonated molecular ion peak observed at m/z 229.09 **(3k)**; was assigned for furanosesquiterpene with a CHO (aldehyde) group at the 4 position of the furan ring.

**Cluster 4:** The m/z peak at 245.08 **(3l)**, were assigned for a carboxylic acid group at the 3 position on the furan ring, as in 247.10 **(3b)** and 249.11**(3a)**.
**Cluster 5:** The m/z peak at 265.11 **(3m)** was assigned for methyl ester at the 3 position on the furan ring as in m/z 263 **(3c)**.

The most abundant fragment ion following collision induced dissociation was observed at *m/z* 81, resulting from an allylic cleavage in most of the molecules. Other abundant peaks were observed for each set of compounds either due to the loss of the methyl, $CH_2OH$, COOH or the COOMe from positon 4 on the furanoid ring.
The positions of the double bonds were difficult to assign due to the hydride shifts therefore it was interpreted that the conjugated double bonds are more stable and placed accordingly. The structures assigned to the furanosequiterpenoids identified on the basis of fragmentation pattern observed are shown in **Fig.3n.**

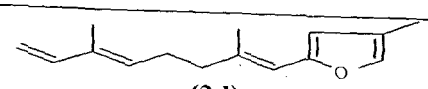
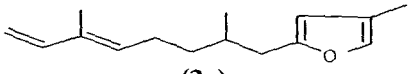
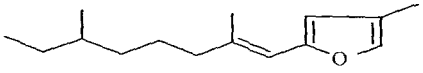
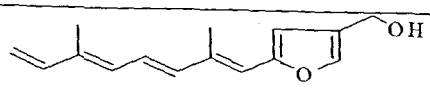
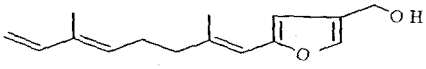
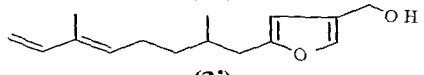
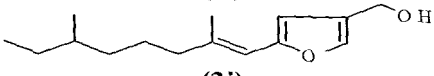
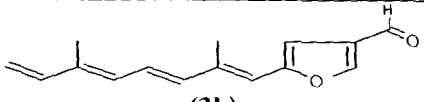
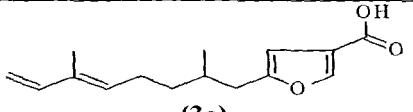
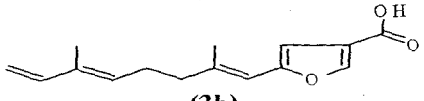
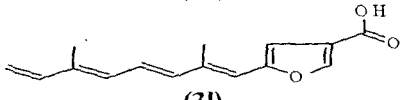
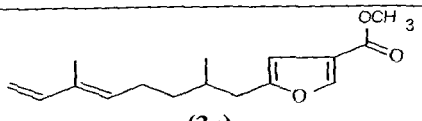
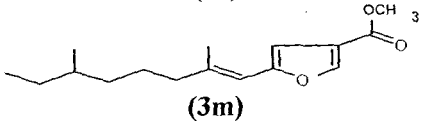
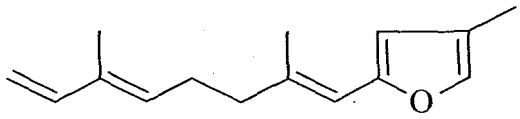
| | |
|---|---|
| Cluster 1 |  (3d)  (3e)  (3f) |
| Cluster 2 |  (3g)  (3h)  (3i)  (3j) |
| Cluster 3 |  (3k) |
| Cluster 4 |  (3a)  (3b)  (3l) |
| Cluster 5 |  (3c)  (3m) |

**Fig.3n:** Furanosequiterpenoids identified on the basis of fragmentation pattern in ESI-MS/MS

Cluster **1** with the molecular ions $[M+H]^+$ at m/z 217.2 **(3d),** 219.1420 **(3e)** and 221.1250 **(3f)** with corresponding elemental composition $C_{15}H_{20}O$, $C_{15}H_{22}O$ and $C_{15}H_{24}O$ appears to be 4 methyl furan compounds of which compound **6** was earlier reported from the Australian soft coral by Bowden *et al.*[10] The compound with elemental composition $C_{15}H_{24}O$ $[M+H]^+$ 221.2, appears to be a new compound proposed having structure **3f**.



**(3d)**

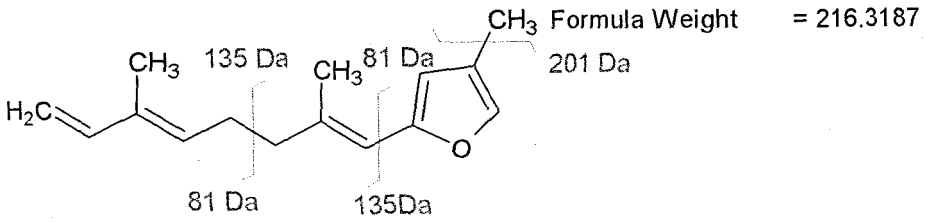
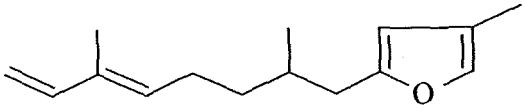



**Fig.3d : Fragmentation of $[M+H]^+$ =217**



**(3e)**

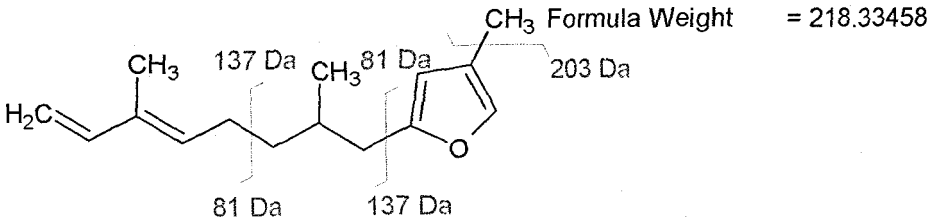
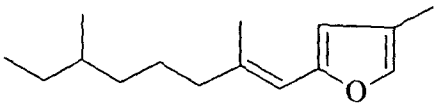



**Fig.3e : Fragmentation of $[M+H]^+$ =219**



**(3f)**

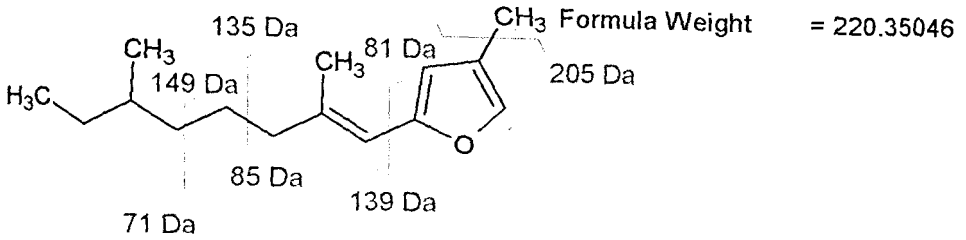
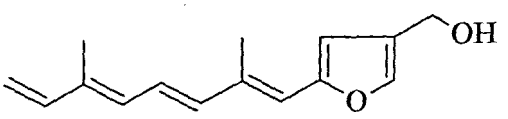



**Fig.3f: Fragmentation of $[M+H]^+$ =221**

Cluster **2**, having molecular ions $[M+H]^+$ ; m/z 231.1070 (3g), m/z 233.1223(3h), m/z 235.1415(3i) and 237.1390(3j), with their respective elemental composition $C_{15}H_{18}O_2$, $C_{15}H_{20}O_2$, $C_{15}H_{22}O_2$ and $C_{15}H_{24}O_2$ were analysed. All the pseudomolecular ions showed the loss of 18 mass units indicating the presence of hydroxyl groups in all the compounds in the cluster group. There is yet another similarity with the lower fragment regions of these compounds specially in the range of 40-150 amu, which are the characteristic of acyclic mono and sesquiterpenoids with different degree of unsaturation . Based on these observations four sesquifurano alcohols were assigned structures **3g, 3h, 3i,** and **3j**. Their respective fragmentation patterns are given in **Fig.3g, 3h, 3i** and **3j.**



**(3g)**

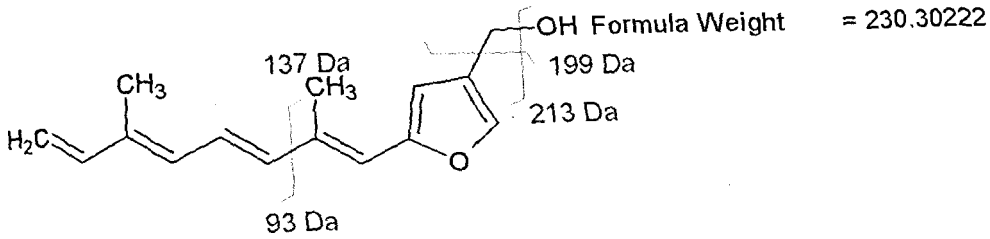
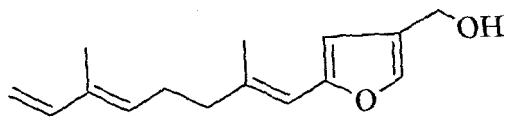



**Fig.3g: Fragmentation of $[M+H]^+$ =231**



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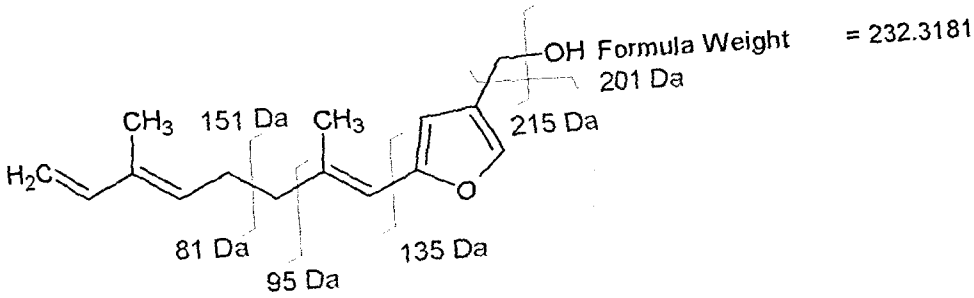
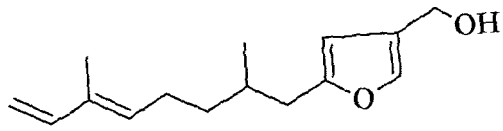



**Fig.3h: Fragmentation of $[M+H]^+$ =233**



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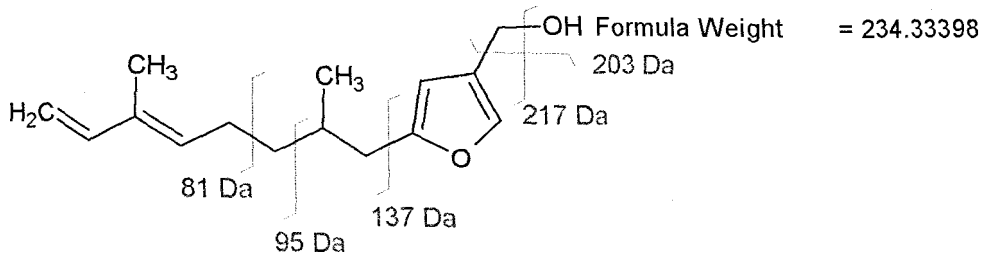
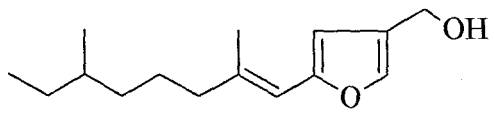



**Fig.3i: Fragmentation of [M+H] $^{+}$ =235**




**(3j)**

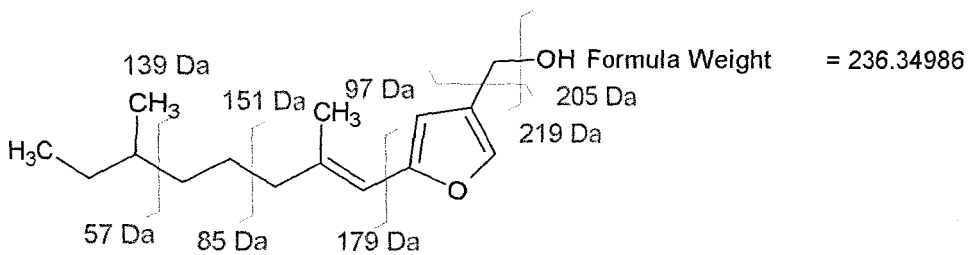
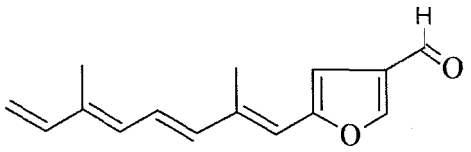



**Fig.3j: Fragmentation of [M+H] $^{+}$ =237**

Cluster **3**: The pseudomolecular ion $[M+H]^{+}$ m/z 229.0952 **(3k)**, $C_{15}H_{18}O_2$ did not show any loss of 18 amu showed the absence of hydroxyl group in the molecule instead a loss of 15 amu of prenyl methyl having m/z 214.1352 was observed followed by a fragment of 29 amu (CHO) of the furan moiety at around m/z 199. Fragment ion m/z 185.1255 is observed due to loss of (CHO) from the molecular ion indicating end aldehyde. EI-MS study of furanoid molecules on synthetic compounds showed the rupture of furan ring at C1 and C4 of the furan moiety to give correspond fragment ions[11], based on these observations and proposed fragmentation pattern **Fig.3k** structure **3k** was assigned to the proposed molecular ion.



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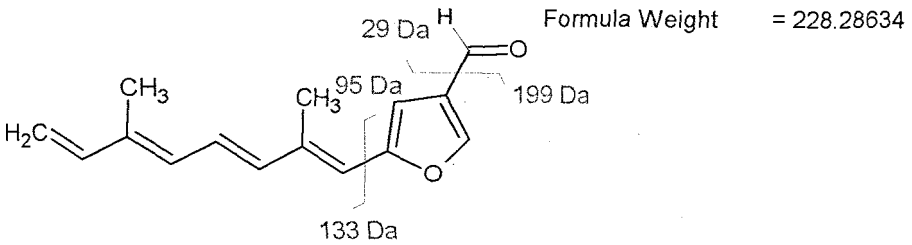


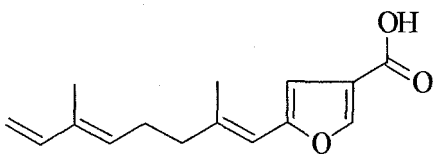

**Fig.3k: Fragmentation of $[M+H]^+$ =229**

Cluster **4** showed $[M+H]^+$ at m/z 245.0867 **(3l)**, m/z 247.1049 **(3b)** and m/z 249.1144 **(3a)**.

Section 3.1.2 of this chapter described the isolation and complete characterization of compounds **3b** and **3a**, **3a** is the dihydro product of **3b**.

Compound **3b**, product ion study of molecular ion $(M+H)^+$ = m/z 247.1049 at collision energy 30 eV gave characteristic fragment ion at m/z 229.1458 , a loss of $H_2O$( 18 amu) , fragment m/z 201.1634, loss of $CO_2$ (44 amu) and allylic cleavage at m/z 81.0850.

The detail fragmentation pattern is represented in **Fig.3b**



**(3b)**

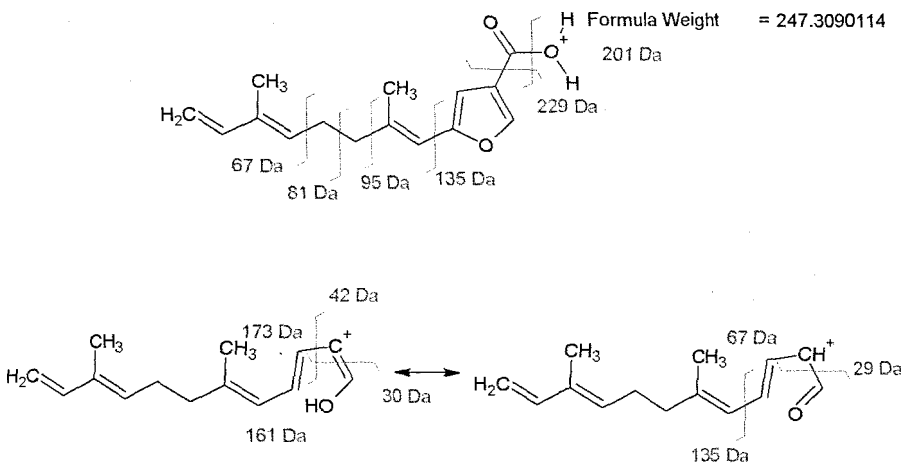
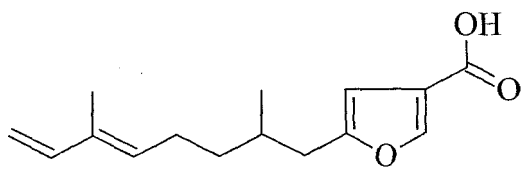



**Fig.3b: Fragmentation of $[M+H]^+$ =247**

Comparing the lower order fragment ions of compound **3b,** $C_{15}H_{18}O_3$, m/z 247.1207 and compound **3a**, $C_{15}H_{20}O_3$, 249.1789, the maximum intensity ions have shifted up by 2 amu for compound **3a** indicating the hydrogenated product of **3b**. **Fig.3a** shows proposed fragmentation of m/z 249.1789



**(3a)**

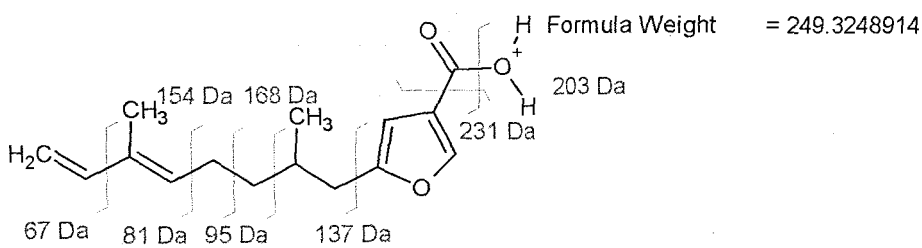
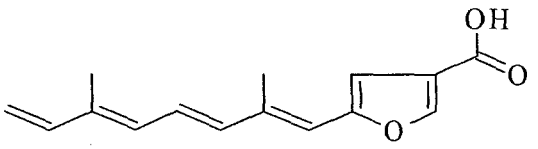



**Fig.3a: Fragmentation of $[M+H]^+$ =249**

Similarly, the product ion study of $[M+H]^+$ 245.0867, $C_{15}H_{16}O_3$, appears to be dehydro product of **3b** having structure **3l**. **Fig.3l** represents the proposed fragmentation pattern of **3l**.




**(3l)**

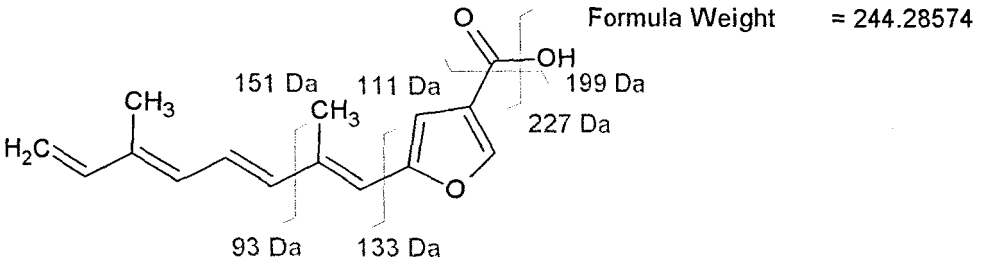
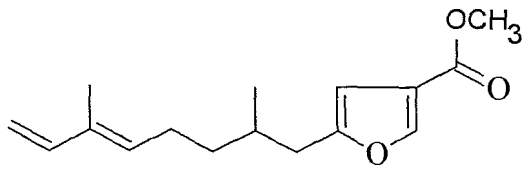



**Fig.3l: Fragmentation of $[M+H]^+$ =245**

Cluster **5**, comprised of molecular ions $[M+H]^+$ m/z 263.0985 **(3c)** and m/z 265.1144 **(3m)**, having corresponding molecular composition $C_{16}H_{22}O_3$ & $C_{16}H_{24}O_3$.

Section **3.1.2** of this chapter describes the detailed NMR and IR study giving full characterization of the compound, assigning structure **3c**, which was previously isolated from an Australian *Sinularia sp.*[10] We describe here the product ion study of the compound **3c**. **Fig.3c** shows the probable fragmentation pattern of **3c**. The presence of fragment ion m/z 247, 203, 81 observed indicate the presence of furnoic acid methyl ester with the rupture of methyl group followed by carboxylate ion and allylic cleavage respectively.




**(3c)**

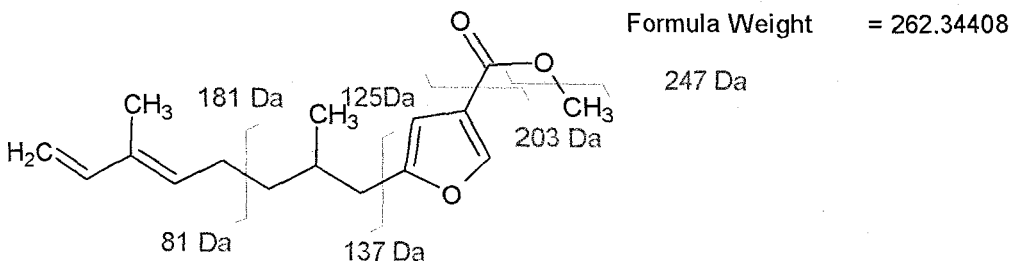
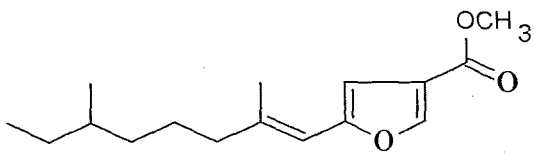



**Fig.3c: Fragmentation of $[M+H]^+$ =263**

The molecular ion $[M+H]^+$, m/z 265.1144, $C_{16}H_{24}O_3$, showed similar pattern as compound **3c** with little variation in the fragment ions in the lower region of product ion spectrum, the proposed fragmentation pattern is shown in **Fig. 3m** and fits well with compound **3m** reported earlier.[10]




**(3m)**

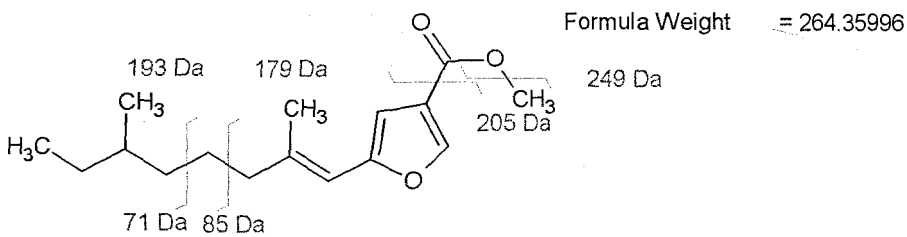



**Fig.3m : Fragmentation of $[M+H]^+$ =265**

## 2. Probable biogenesis of furanosesquiterpenoids

As a result of pioneering studies by Lynen et al., 1958, Chaykin et al. and Cornforth in 1959, the essential steps in the biosynthetic transformation of mevalonic acid to geranyl pyrophosphate and then into squalene via farnesyl pyrophosphate are now well understood.

Based on biogenetic rules the following biogenetic pathway has been proposed for the furanosesquiterpenoids from *Sinularia kavarattiensis.*

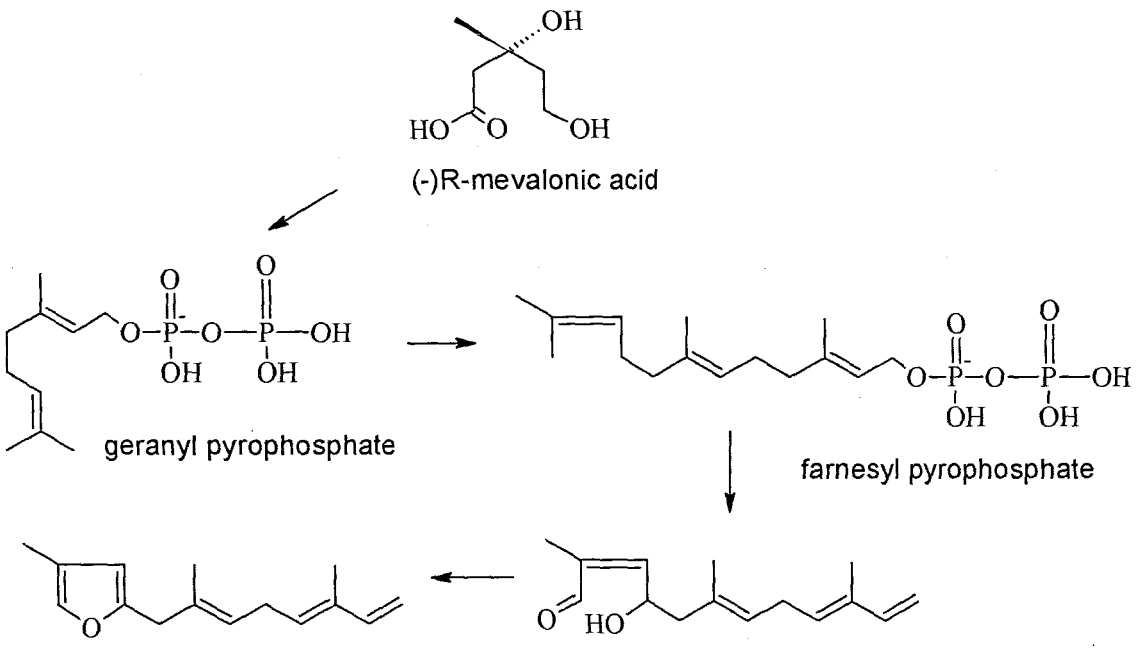



**Fig 4 : Proposed biogenetic pathway of furanoterpenoids**

Biogenetically furanoterpenoids isolated from *sinularia sp.*, seems to have been derived from farnesyl pyrophosphate with the head end involved in the formation of furan moiety. Though, 4-methyl furansesquiterpene is the basic precursor for these compounds, only methyl and carboxylate compounds have been reported. This investigation reveals the presence of hydroxy analogues, a 4-methyl furan **(3f)** and an aldelyde **(3k)** from Indian soft coral *Sinularia kavarattiensis.*

## 3. General Experimental procedures:

### a. Sample:

The sweet smelling terpene rich fraction from which the sesquiterpenes **1(3a)**, **2(3b)** & **3(3c)** (Section 3.1.2) obtained was used for ESI-MS/MS studies.

### b. Mass Spectrometry (ESI-QTOF MS/MS)

Mass spectra were recorded, in the positive mode, on a QSTARXL MS/MS Applied Biosystems, instrument (Canada) equipped with analyst software application.
The sample dissolved in MeOH:$CHCl_3$ (95.5: 0.5) containing traces of 0.1% Trifluoro acetic acid (TFA) was directly infused at a constant flow rate of 10 μL/ml into the ion spray source using integrated syringe pump. The instrument was operated in positive ionization mode with the following settings: nebulizer gas ($N_2$) 28 (arbitrary units); curtain gas ($N_2$) 18 (arbitrary units); ion spray voltage 5700V; declustering potential (DP) 120V; focussing potential (FP) 365V; declustering potential (DP2) 14 V and collision gas (CAD) 3 (arbitrary units). Full scan data acquisition was performed, scanning from m/z 100 to m/z 1500 in the profile mode and using a cycle time of 1s. The MS/MS products were produced by collision induced dissociation (CID) of the selected precursor ions at collision energy between 25-40V and mass analyzed using TOF analyser of the instrument. The declustering potential and the collision energy were optimised for MS/MS experiments so as to cause fragmentation of the selected molecular ion species as evident by the appearance of fragment ions and decrease in the intensity of the molecular ion.

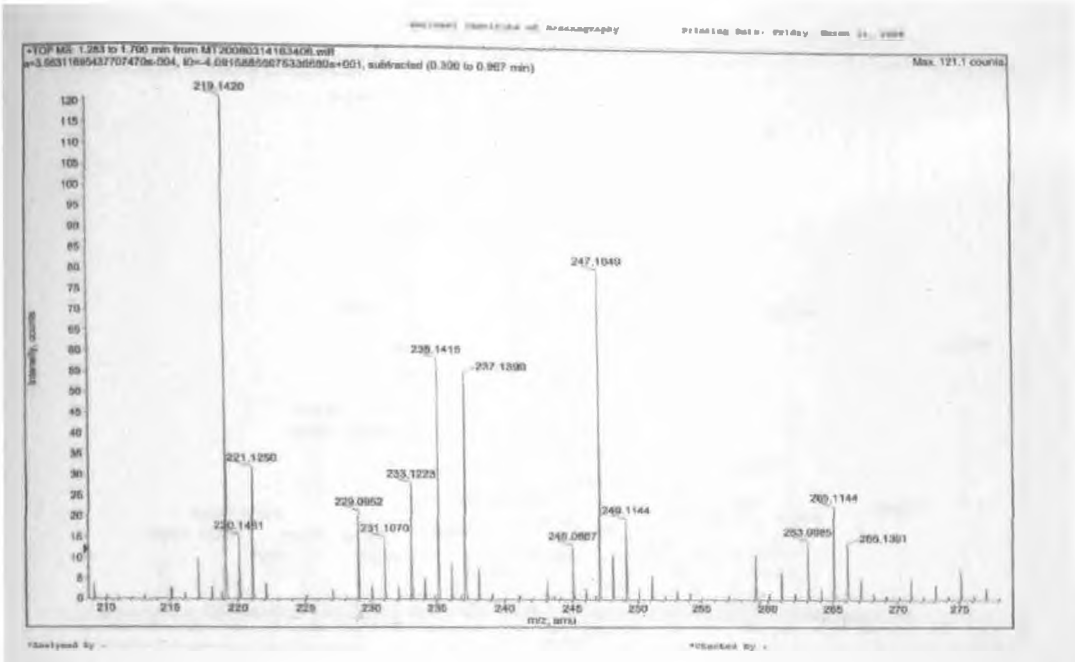



**Fig.3.1: ESI-MS spectrum of terpene rich fraction of *Sinularia kavarattiensis***

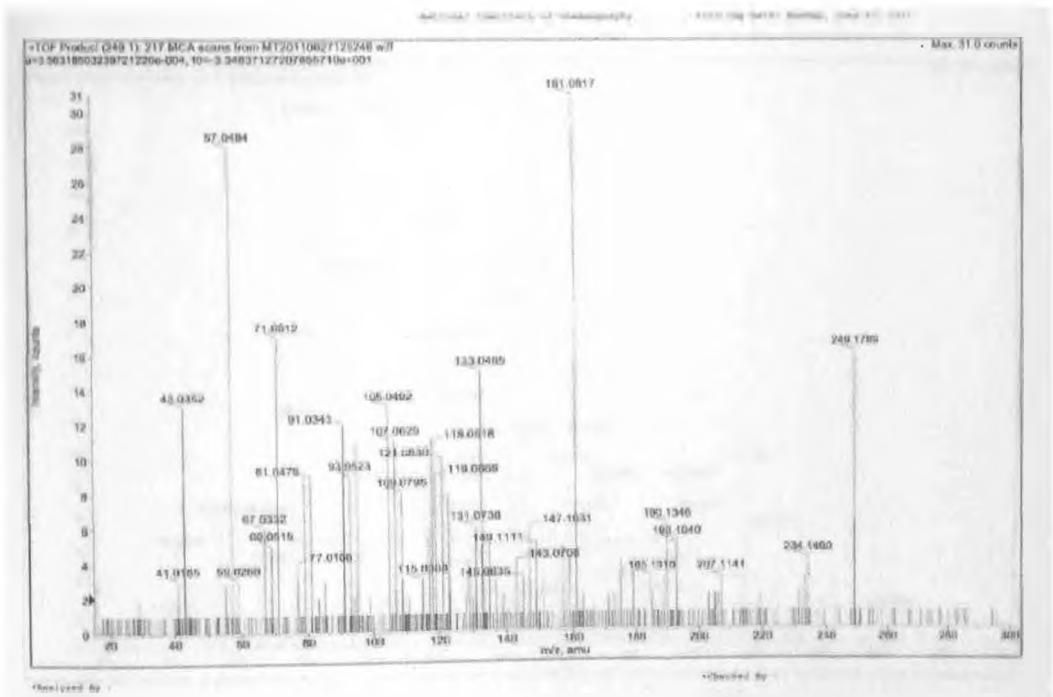



**Fig.3.2: CID spectrum at m/z 249.1 $[M+H]^+$**

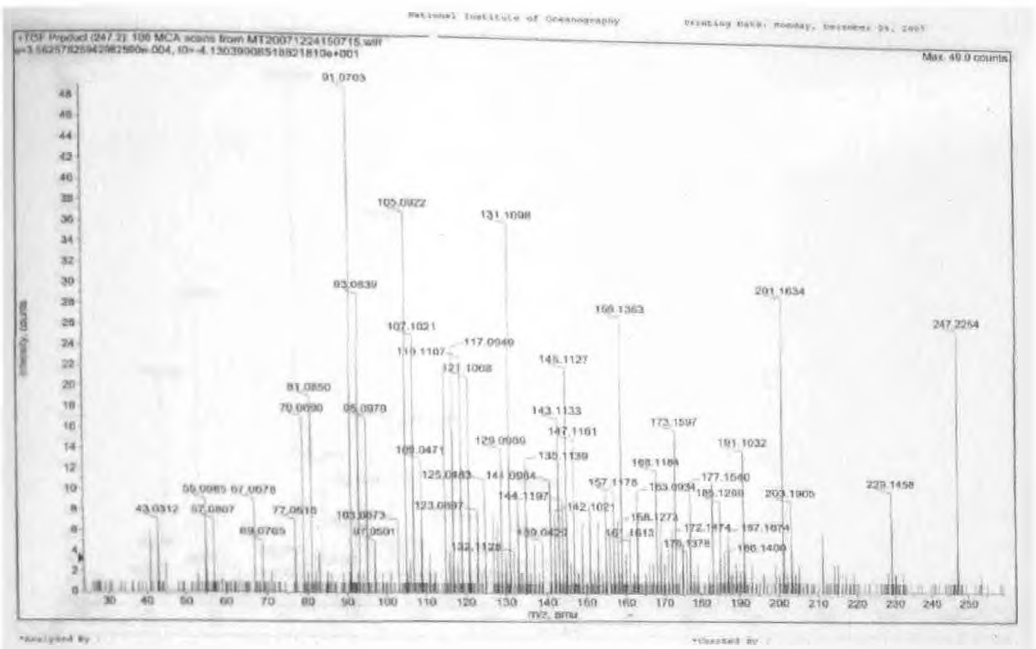


**Fig.3.3: CID spectrum at m/z 247.2 $[M+H]^+$**

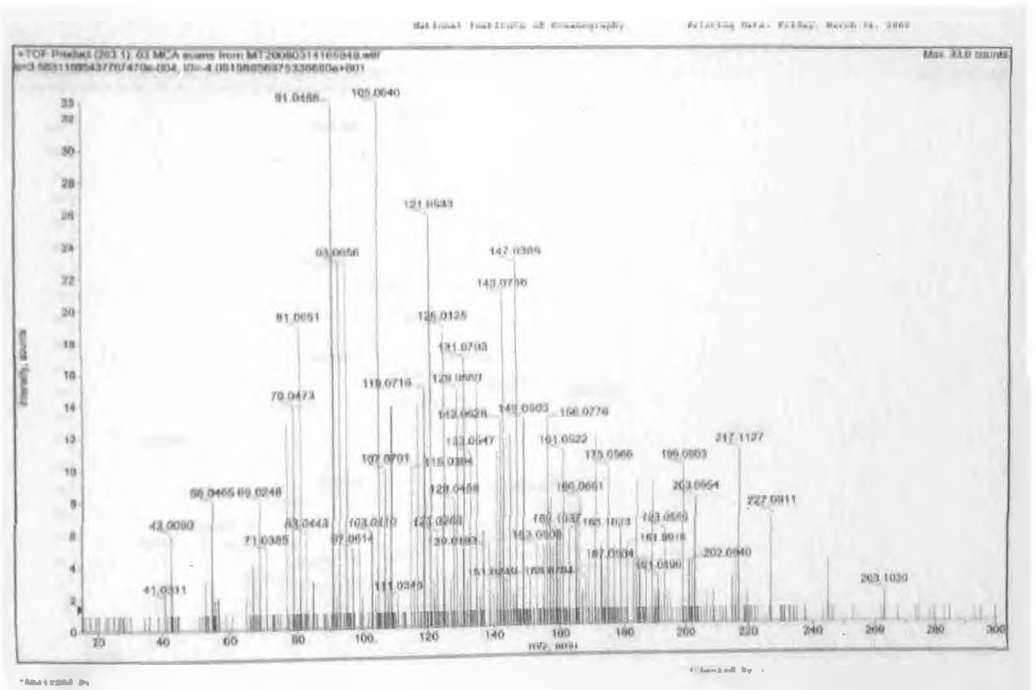


**Fig.3.4: CID spectrum at m/z 263.1 $[M+H]^+$**

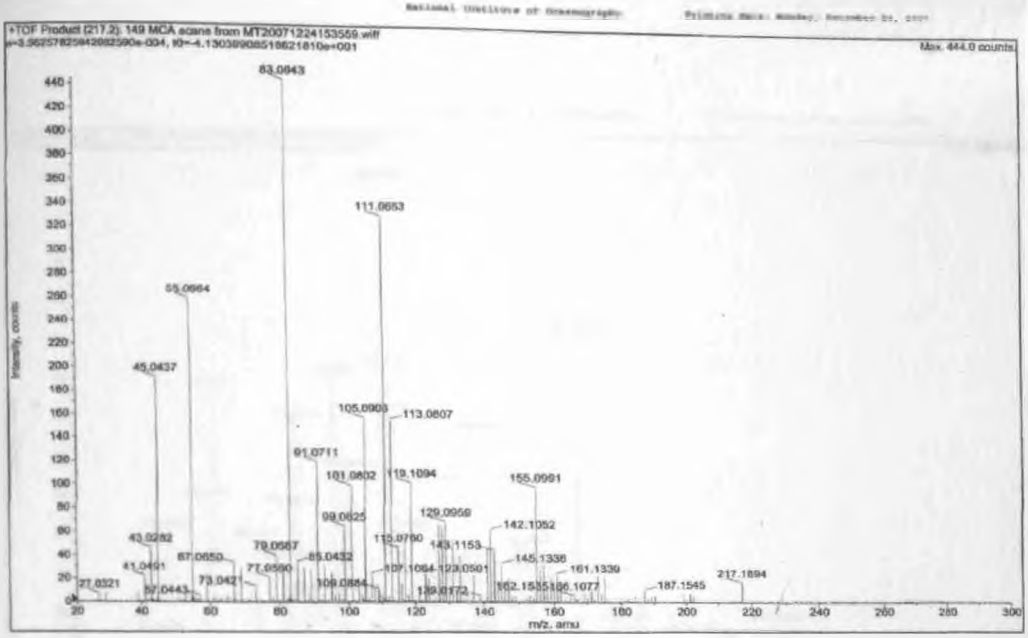



**Fig.3.5: CID spectrum at m/z 217.2 $[M+H]^+$**

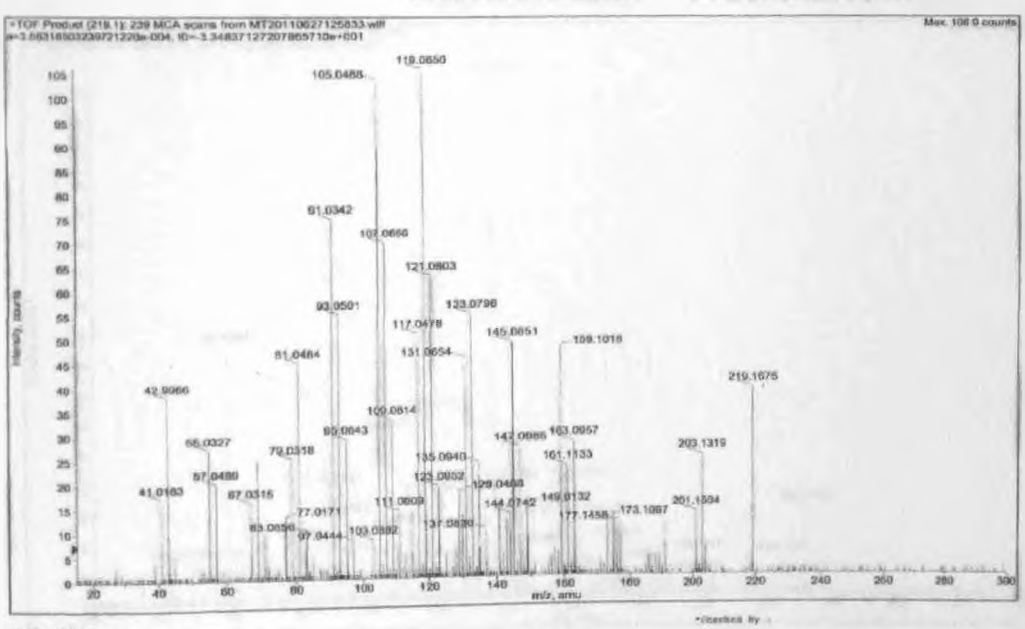



**Fig.3.6: CID spectrum at m/z 219.1 $[M+H]^+$**

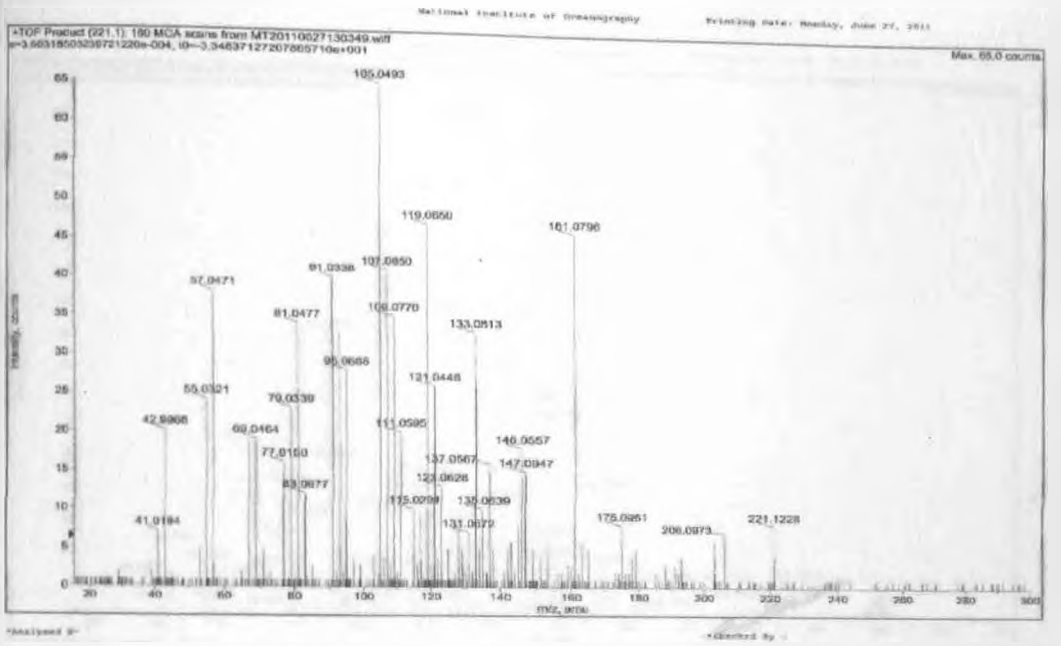



**Fig.3.7: CID spectrum at m/z 221.1 $[M+H]^+$**

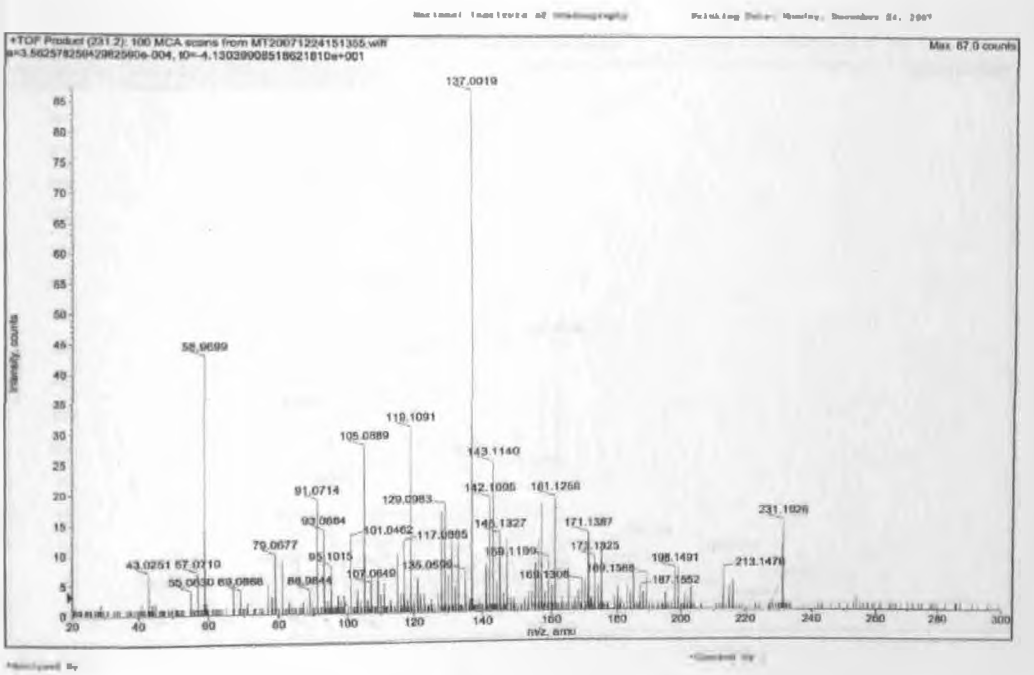



**Fig.3.8: CID spectrum at m/z 231.2 $[M+H]^+$**

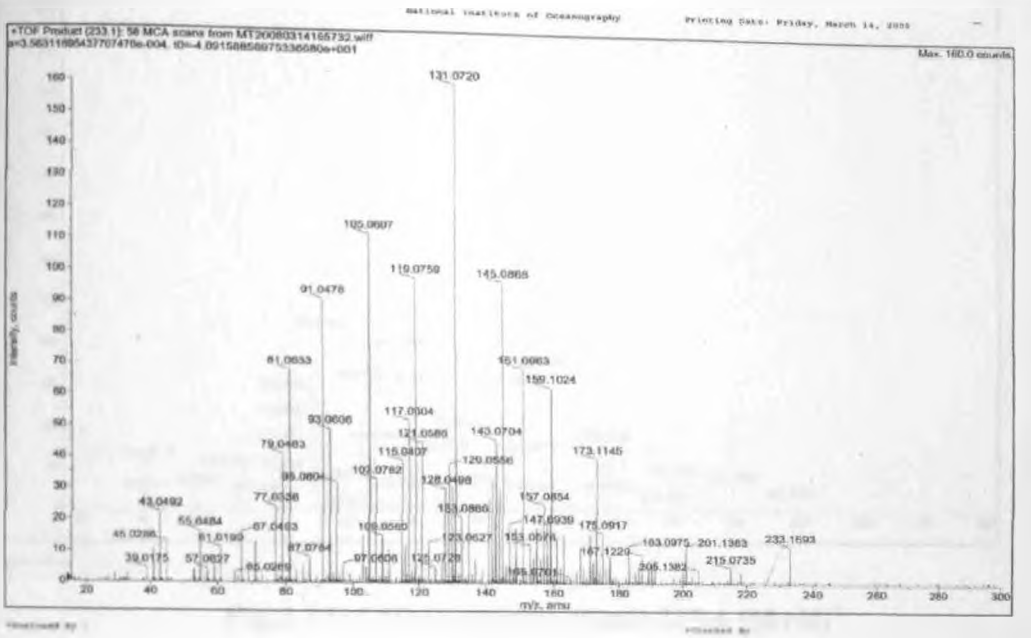



**Fig.3.9: CID spectrum at m/z 233.1 $[M+H]^+$**

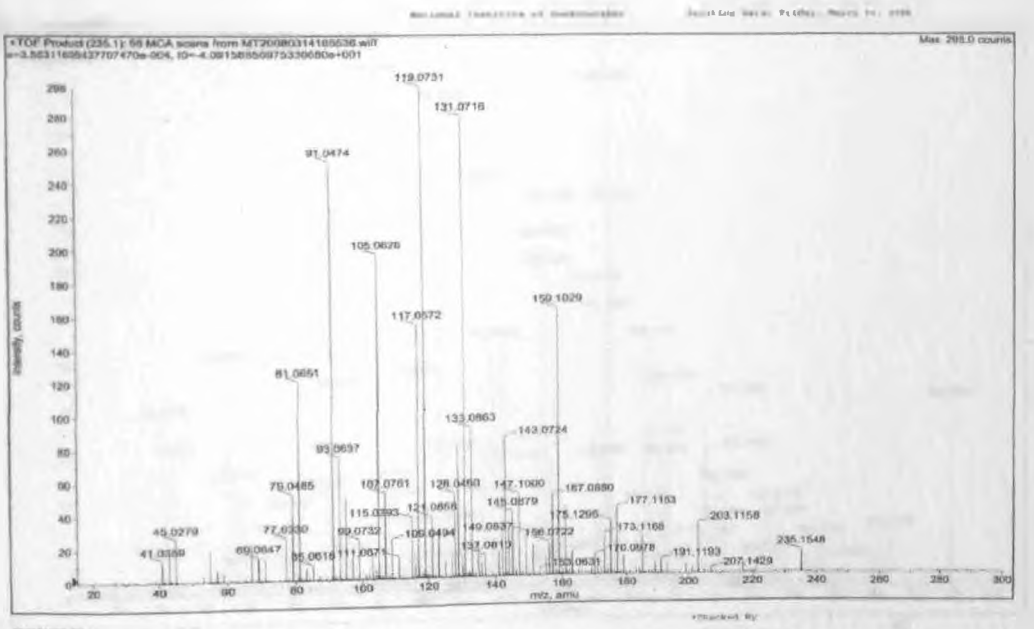



**Fig.3.10: CID spectrum at m/z 235.1 $[M+H]^+$**

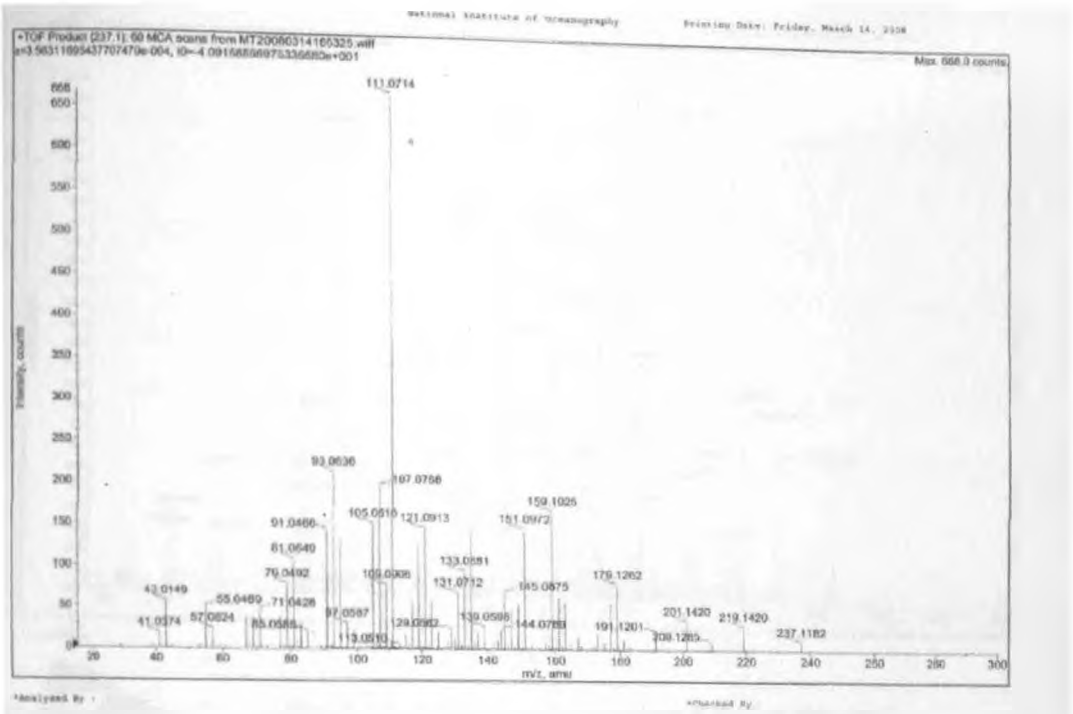



**Fig.3.11: CID spectrum at m/z 237.1 $[M+H]^+$**

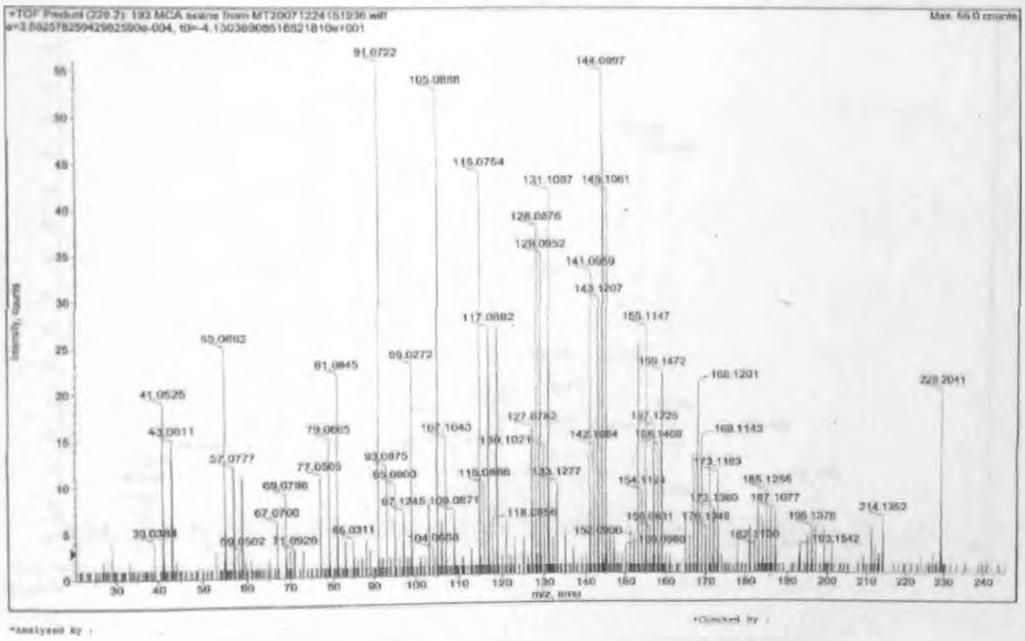



**Fig.3.12: CID spectrum at m/z 229.2 $[M+H]^+$**

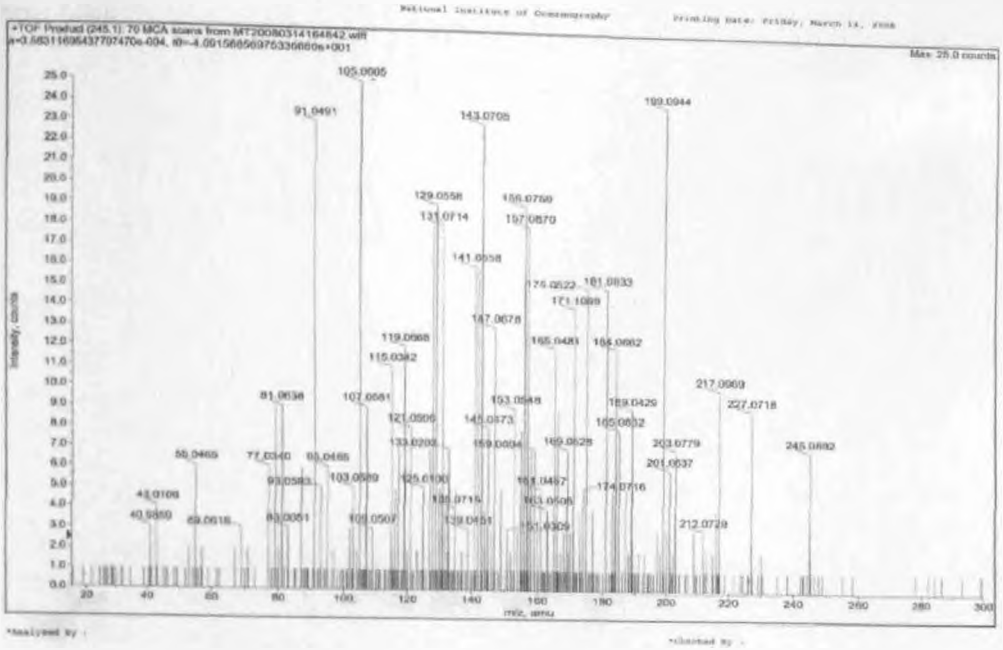


**Fig.3.13: CID spectrum at m/z 245.1 $[M+H]^+$**

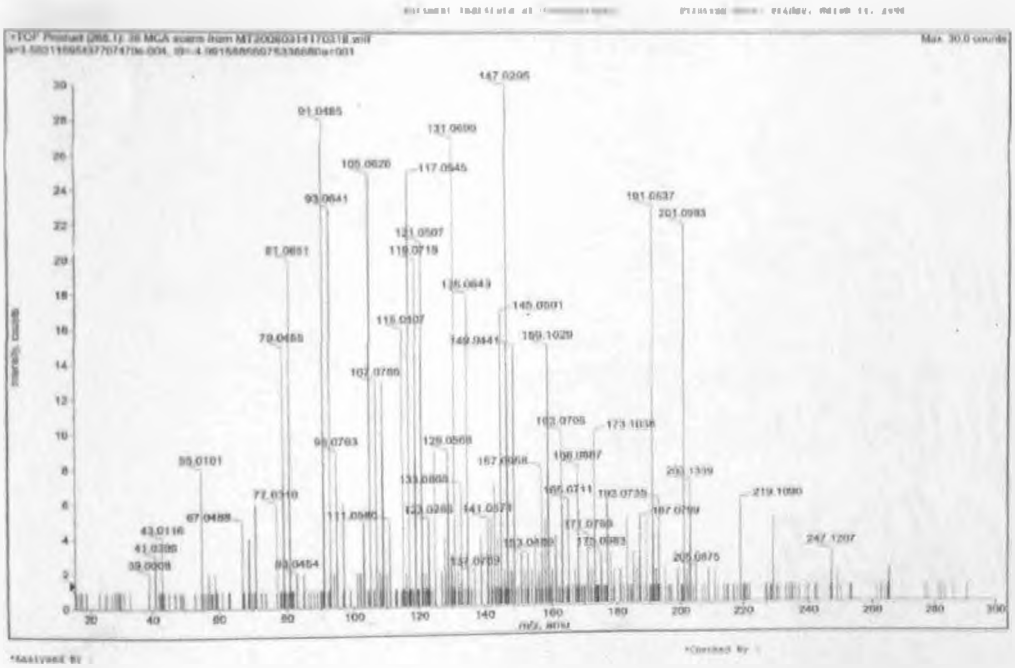


**Fig.3.14: CID spectrum at m/z 265.1 $[M+H]^+$**

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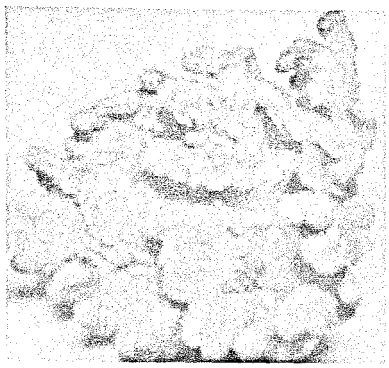
14. Cornforth J W. *J. Lipid Res.* 1959, 1, 3-28.

# *Section II*

## *Lobophytum crassum...*

## Section 2.1: *Lobophytum crassum:* A review



The soft coral *Lobophytum* has a thick and heavy "skin" with lobed projections that form low encrusting colonies. It resembles cabbage in appearance, and hence commonly called as the cabbage coral. The polyps are equally distributed on the coral. The texture is grainy, and the colors range from dull gray, pink, cream, brownish, green, yellow and many colors.

The marine soft coral investigated in this section belongs to the Phylum: Cnidaria, Class: Anthozoa, Order: Alcyonacea, Family: Alcyoniidae, Genus: *Lobophytum* and Species: *crassum* (von Marenzeller, 1886 ).

Investigations on *Lobophytum crassum* has resulted in the identification of various bioactive secondary metabolites which include cytotoxic and anti-inflammatory cembranoids, antimalarial cembraniods, a new diterpene Lobocrassol, cytotoxic Crassumolides, polyhydroxylated sterols and ethers.[1-2]

As a group working on the National Project "Development of Potential Drugs from the Ocean"in our search for bioactive substances from marine organisms, *L. crassum* was selected based on its rich chemical diversity and biological activity.

## 1. Collection of animal material and chromatography

The soft coral was collected off the shore of Rameshwaram, Tamil Nadu, during September 2009. The freeze-dried sample of *Lobophytum* (520 g) was extracted with $CH_2Cl_2$–MeOH 1:1 (500 mL × 4). The total extract was evaporated under reduced pressure and partitioned between EtOAc and $H_2O$. The organic fraction was evaporated to dryness to provide 1.8 g of extract which was subjected to gel filtration chromatography on a Sephadex LH 20 1:1 $CH_2Cl_2$ - MeOH column. The terpene rich fraction was further fractionated on activated silica gel column with gradient mixtures of hexane, dichloromethane and ethyl acetate as the mobile phase. Fractionation on silica column resulted in the purification of two cembrenoid diterpenes (1 & 2) and two sterols (4 & 5).

**Compound 1** was obtained as a pale yellow oily liquid, soluble in chloroform. The IR absorption peaks at 3353, 1583 and 1256 cm-1 indicated the presence of hydroxyl, olefin, and epoxide functionalities in the molecule. The $^{13}C$ NMR spectrum exhibited 20 signals divided by DEPT into four quaternary carbons, five methines, six methylenes, and five methyl groups. The olefinic region consisted of six signals, 3 methines and 3 quarternarycarbons. The $^{1}H$ NMR spectrum showed two olefinic proton doublets resonating at δ ppm 5.84 (d, 1H, J=11.7Hz) and 5.93 (d, 1H J=11.7Hz) indicating them to be conjugated doublets. A singlet at δ 5.21 integrated for 1H was indicative of an isolated olefenic proton. The isopropyl group was observed in the form of two methyl signals at δ 22.20 and δ 22.27 as coupled doublets. Three methyl signals were observed as singlets δ 18.05, δ 17.40& δ 14.93. A methine signal at δ 60.37 and a quarternary carbon at δ 61.06 were indicative of anepoxy functionality.

The above spectral data confirmed the structure of compound **1** as 11,12-epoxy-1,3,7-cembratriene.

**Table 3.1. NMR assignments for compound (1) 11, 12-epoxy-1, 3, 7-cembratriene&(2) 1, 3, 7, 11-cembratetraene-14-ol**

| Carbon No. | $^{13}$CNMR (1) $\delta_C$, ppm | $^{1}$H NMR (1)$\delta_H$, ppm | $^{13}$CNMR (2) $\delta_C$, ppm | $^{1}$H NMR (2) $\delta_H$, ppm |
|---|---|---|---|---|
| 1 | 146.98 | - | 146.91 | - |
| 2 | 127.07 | 5.84(1H,d,J=11.7Hz) | 125.37 | 5.99(1H,d,11.4Hz) |
| 3 | 120.82 | 5.93(1H,d,J=11.7Hz) | 124.50 | 6.14(1H,d,11.4Hz) |
| 4 | 135.86 | - | 136.00 | - |
| 5 | 24.24 | 2.20 (2H,m) | 24.49 | 2.30(2H,m) |
| 6 | 24.44 | 2.30 (2H,m) | 38.76 | 2.40(2H,m) |
| 7 | 118.38 | 5.22(1H,t, J=7.2Hz)) | 120.52 | 4.99(1H,m) |
| 8 | 133.33 | - | 131.39 | - |
| 9 | 25.25 | 2.17 (2H,m) | 25.51 | 2.20 (2H,m) |
| 10 | 38.35 | 2.01 (2H,m) | 39.63 | 2.10 (2H,m) |
| 11 | 60.37 | 2.88(1H,t, J=7.2Hz)) | 121.17 | 4.99(1H,m) |
| 12 | 61.06 | - | 134.61 | - |
| 13 | 36.83 | 1.92 (2H,m) | 44.55 | 2.60(2H,m) |
| 14 | 36.78 | 2.15 (2H,m) | 69.95 | 3.48(1H,m) |
| 15 | 17.40 | 1.40 (1H,m) | 18.18 | 1.60(1H,m) |
| 16 | 22.21 | 1.02(3H,d) | 15.52 | 1.05(3H,d,6.6Hz) |
| 17 | 22.27 | 1.03(3H,d) | 16.33 | 1.11(3H,d,6.6Hz) |
| 18 | 14.93 | 1.26 (3H,s) | 24.36 | 1.47(3H,s) |
| 19 | 18.05 | 1.59(3H,s) | 25.37 | 1.60(3H,s) |
| 20 | 34.05 | 1.72(3H,s) | 27.08 | 1.74 (3H,s) |

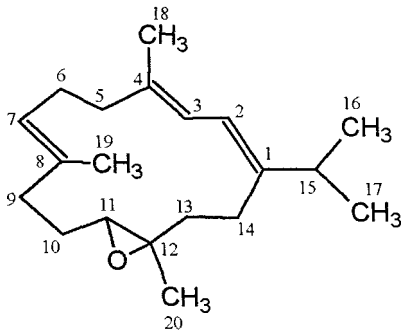



**Fig.3.1:Structure of compound (1) 11, 12-epoxy-1, 3, 7-cembratriene**

**Compound 2** was also obtained as a colourless liquid. The $^{13}C$ and DEPT spectra of the compound exhibited 20 signals; five methyls, five methylenes, six methines and and 4 quarternary carbons. Eight signals were observed in the olefinic region indicating four double bonds unlike compound 1 which exhibited 6 signals indicated 3 double bonds. Compound 2 was similar to compound 1 since both contained the isopropyl group and the conjugated double bond. A signal at 60.92 was indicative of a methine attached to a hydroxyl group. All these physical and spectral data suggested that **1& 2** have a cembranoid-type carbon skeleton. Further confirmation to their structure, was based on their 2D NMR and mass spectral studies and identified as Sarcophytol A reported in literature by Kobayashi et al., 1979, from the soft coral *Sarcophyton glaucum.*[18] Compound2 was obtained as a colorless oil: $[\alpha]_{20}D+122.0°$ (c 0.38, $CHCl_3$) (lit. +141.0°); $^1H$ and $^{13}C$ NMR data were in agreement with previously reported values.

Sarcophytol A has been reported to show antitumor activity[19] as well as potent inhibitory activities against several kinds of tumor promoters.[20]

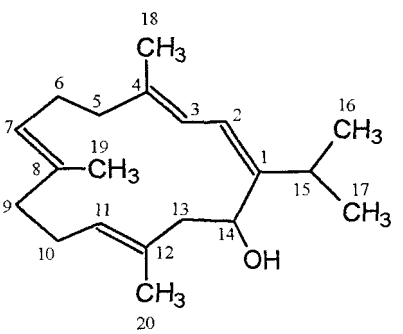



**Fig.3.2: Structure of compound (2) 1, 3, 7, 11-cembratetraene-14-ol**

**Compound 3:**The molecular formula of the compound (4) was determined as $C_{30}H_{50}O_6$ by the high resolution positive ion ESI-MSspectrum (**Fig.30a**) of the compound, which gave a pseudomolecular ion $[M+H]^+$ at 507.3587(calculated 507.3607), [M+Na] at 529.3407 and [M+K] at 545.3093. An m/z peak was observed at 447.3487 for $[M-CH_3COOH]$ and m/z 429.3363 for $[M-(CH_3COOH+H_2O)]$.

1H NMR spectrum (**Fig.9**) was typical of a sterol. The only difference was that instead of five methyls, the compound has six methyl groups.

The proton spectrum of compound 4 showed four methyl singlets (0.66,0.76,1.39 and 1.97) of which the integration of the signal at δ1.39 was suggestive of its being for two methyl groups.Two methyl doublets were observed at $\delta_H$ 0.94 and $\delta_H$0.88., two oxymethines broad doublets were observed at $\delta_H$ 4.19 (d,J=3.9Hz) and at $\delta_H$ 3.98 (dd,J=4.5,1.5Hz). Two additional doublets were observed in the same region at $\delta_H$3.71 (d,J=6.6Hz) and $\delta_H$2.97 (d,J=6.3Hz) probably due to coupling of –OH protons with the oxymethine protons as observed in the $^1$H-$^1$H COSY experiment and also due to no linkage to any carbon as observed in the HSQC experiment. These data clearly indicated polyhydroxy sterol structure. The $^{13}$C NMR spectrum (**Table 3.3, Fig.13**) confirmed the presence of two oxymethines, six quaternary carbons of which one was a six membered ring ketone ($\delta_c$ 210.64), an acetyl carbonyl ($\delta_c$ 170.43), two were assigned to carbons carrying the C18 and C19 methyls, and the remaining two down field signals ($\delta_c$ 85.64 & δc 85.87) were assigned respectively to two carbons with an acetyl group and carbon linked to the remaining hydroxyl group observed at $\delta_H$ 4.54 in its proton spectrum.

The above spectral data along with its correlation data (**Table 3.3, Fig.3.5**) confirmed the structure of the compound as given below (**Fig.3.4**).

**Table 3.3.** NMR assignments for **compound (3)(24-methyl-25(29(30)-methylester)-3one-5,6,7-triol)**

| Carbon No. | $^{13}$CNMR $\delta_C$, ppm | $^1$H NMR $\delta_H$, ppm | COSY | HMBC |
|---|---|---|---|---|
| 1 | 23.97 | 1.65(dd,2H, (J=3.5Hz,J=3.5Hz) | H2 | - |

| | | | | |
|---|---|---|---|---|
| **2** | 26.82 | 1.76(dd,2H, J=3.5Hz, J=3.5Hz) | H1 | - |
| **3** | 210.64 | - | - | - |
| **4** | 27.88 | 1.87(m,2H) | - | C2,C5,C6 |
| **5** | 85.87 | - | - | - |
| **6** | 70.21 | 3.99(br s,1H) | H7 | - |
| **7** | 69.84 | 4.19(br s,1H) | H6 | - |
| **8** | 37.89 | 1.78(m,1H) | H9 | C11 |
| **9** | 42.92 | 1.40(m,1H) | H8 | - |
| **10** | 46.50 | - | - | - |
| **11** | 21.65 | 1.52(m, 2H) | H12 | - |
| **12** | 39.41 | 2.06(dt,2H,J=12.5Hz, J=3.0Hz) | H11 | - |
| **13** | 43.19 | - | - | - |
| **14** | 55.79 | 1.12(m,1H) | - | - |
| **15** | 24.18 | 1.58(m,2H) | H16 | - |
| **16** | 41.48 | 2.40(m,2H) | H15,H17 | C13,C14 |
| **17** | 56.85 | 1.18(m,1H) | H16 | - |
| **18** | 11.97 | 0.66 (s,3H) | - | C12,C14,C16 |
| **19** | 16.88 | 0.76(s,3H) | - | C1,C5,C9 |
| **20** | 36.08 | 1.38(m,1H) | H21,H22 | - |
| **21** | 18.88 | 0.93(d,3H,J=6.5Hz) | H20 | C17,C22 |
| **22** | 34.56 | 1.55(m,2H) | H20,H23 | - |
| **23** | 27.72 | 1.31(m,2H) | H24 | - |
| **24** | 41.88 | 1.95(m,1H) | H23,H28 | - |
| **25** | 85.64 | - | - | - |
| **26** | 22.54 | 1.40(s, 3H) | - | C24, C25 |
| **27** | 23.35 | 1.40(s, 3H) | - | C24, C25 |
| **28** | 14.49 | 0.88(d,3H,6.5Hz) | H24 | C23,C25 |
| **29** | 170.43 | - | - | - |
| **30** | 22.89 | 1.98(s,3H) | - | C29 |

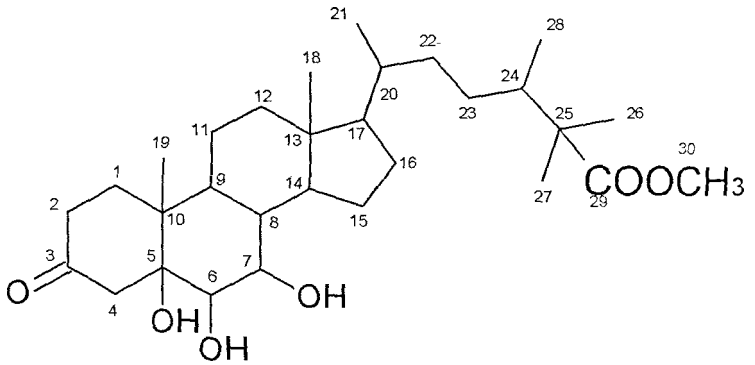



**Fig.3.4: Structure of compound (3) 24-methyl-25(29(30)-methylester)-3one-5,6,7-triol**

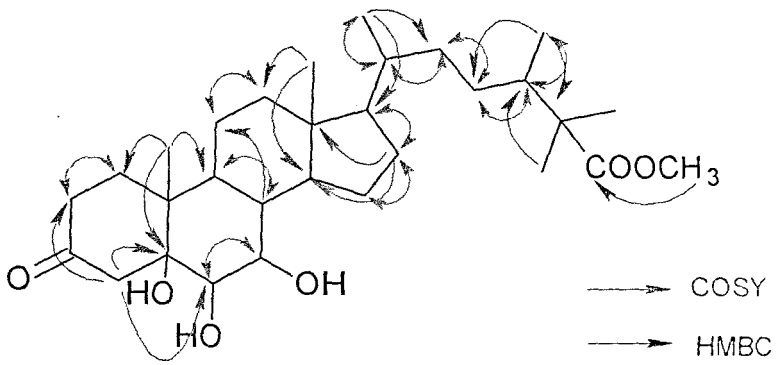



**Fig.3.5: COSY& HMBC correlations of compound 3**

**Compound 4** was obtained as white crystals.Its spectral data (**Table 3.4, Fig.31-38**) on comparison with literature data revealed it to be the common sterol Cholest-5,24(28)diene-3β-ol(24-methylene cholesterol).

**Table 3.4.NMR assignments forcompound (4) Cholest-5,24(28)diene-3β-ol**

| Carbon No. | $^{13}$CNMR $\delta_C$, ppm | $^{1}$H NMR $\delta_H$, ppm |
|---|---|---|
| 1 | 39.09 | 1.80 (m,2H) |
| 2 | 31.68 | 0.90 (m,2H) |
| 3 | 71.82 | 3.55 (m,1H) |
| 4 | 39.80 | 2.01 (s,2H) |
| 5 | 140.78 | - |
| 6 | 121.71 | 5.37 (m,1H) |
| 7 | 21.09 | 1.45 (m,2H) |
| 8 | 31.92 | 1.38 (m,1H) |
| 9 | 50.16 | 0.90 (m,1H |
| 10 | 36.52 | - |
| 11 | 22.00 | 1.05 (m,2H) |
| 12 | 28.21 | 1.58 (m,2H) |
| 13 | 42.32 | - |
| 14 | 56.77 | 1.20 (m,1H) |
| 15 | 24.29 | 1.57(m,2H) |
| 16 | 37.27 | 1.83 (m,2H) |
| 17 | 56.02 | 1.38 (m,1H) |
| 18 | 11.86 | 0.67 (s,3H) |
| 19 | 15.45 | 0.75 (s,3H) |
| 20 | 35.76 | 1.41 (m,1H) |
| 21 | 17.61 | 0.77 (d, 1H, J=6.5Hz) |
| 22 | 36.19 | 1.38 (m,2H) |
| 23 | 31.48 | 1.27 (m, 2H) |
| 24 | 158.00 | - |
| 25 | 33.82 | 1.39 (m, 1H) |
| 26 | 18.72 | 1.03 (d,3H,J=6.5Hz) |
| 27 | 19.39 | 1.03 (d,3H, J=6.5Hz) |
| 28 | 115.00 | 4.68(d,2H, J=1Hz)<br>4.74 (d,2H, J=1Hz) |

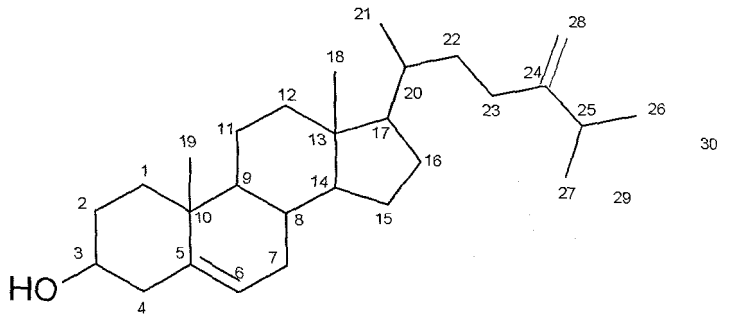



**Fig.3.6: Structure of compound (5) Cholest-5,24(28)diene-3β-ol**

**Experimental Section**

Column chromatographies were carried out using Silica gel (60-120 mesh, Qualigens), gel filtrations were carried out using Sephadex LH20 (Pharmacia Biotech).Fractions were monitored on TLC using alumina backed sheets (silica gel 60, F254, 0.25mm thick) with visualization under UV (254nm) and 5% sulphuric acid in methanol spray reagent.

UV spectra were recorded in MeOH, using Shimadzu UV-Vis 2401PC Spectrophotometer and IR spectra were recorded on a Shimadzu FT-IR spectrophotometer. Optical rotations were recorded in MeOH using Optical Polarimeter ADP220(Bellingham Stanley Ltd.).

Mass spectra were recorded on a PE Sciex-QSTAR and QSTAR-TOF MS/MS Applied Biosystems.

NMR ($^{1}H$, $^{13}C$, COSY, HSQC and HMBC) experiments were obtained on a Bruker(Avance 300) Spectrometer with TMS as the internal standard.

**Animal Material**

Specimens of the soft coral *Lobophytum crassum* were collected off the coast of Mandapam, southernTamil Nadu, India. A voucher specimen was deposited in the National Institute of Oceanography, Goa.

**Extraction and Separation**

The frozen soft coral (2.0 kg, fresh wt.) was freeze-dried. The freeze-driedmaterial was minced and extracted exhaustively with EtOAc (5 × 2 L). The organic extract wasevaporated to yield a residue (6.5 g), which was fractionated by open column chromatography onsilica gel using *n*-hexane–EtOAc and EtOAc–acetone mixtures of increasing polarity to yield15 fractions. Fraction 6, eluting with *n*-hexane-EtOAc (15:1), was further separated by silica gelcolumn chromatography with gradient elution (*n*-hexane-EtOAc, 15:1 to 10:1) to yield fivesubfractions (6A–6E). Subfraction 6C was subjected to normal phase HPLC (*n*-hexane-EtOAc, 15:1)to obtain compound **4** (3.0 mg). Fraction 8, eluting with *n*-hexane-EtOAc (5:1), was further separatedby silica gel column chromatography with gradient elution (*n*-hexane-EtOAc, 5:1 to 2:1) to give sixsubfractions (8A–8F). Subfraction 8B was separated by normal phase HPLC using *n*-hexane-EtOAc(5:1) to afford **5** (2.5 mg). In the same manner, compound **3** (2.0 mg) was obtained from subfraction8 D normal phase HPLC (*n*-hexane-EtOAc, 3:1). Fraction 9, eluting with *n*-hexane-EtOAc (3:1), wasfurther separated by silica gel column chromatography with gradient elution (*n*-hexane-EtOAc, 3:1 to1:1) to yield five subfractions (9A–9E). Subfraction 9C was subjected tonormal phase HPLC (*n*-hexane-EtOAc, 2:1) to obtain compounds **1**(4.2 mg) and **2** (3.0 mg).

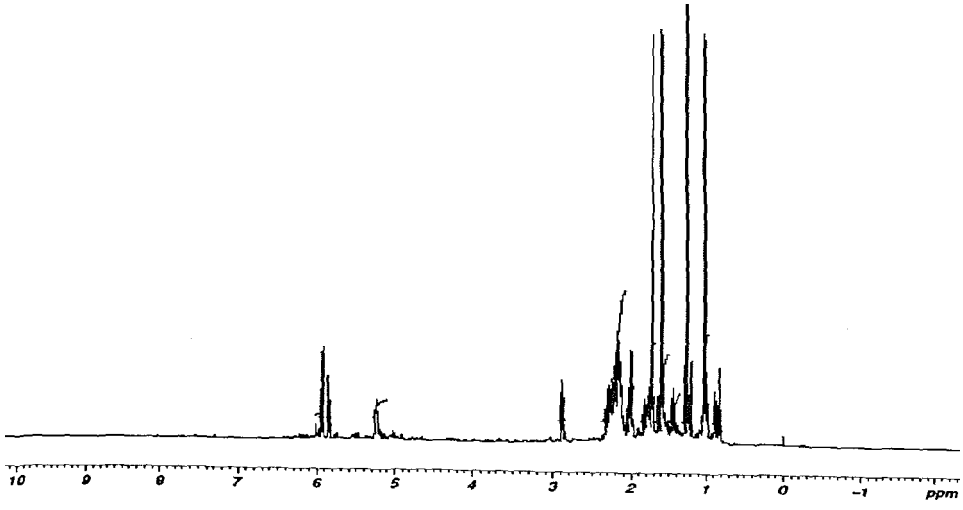



**Fig.1: $^{1}$H NMR spectrum of compound 1**

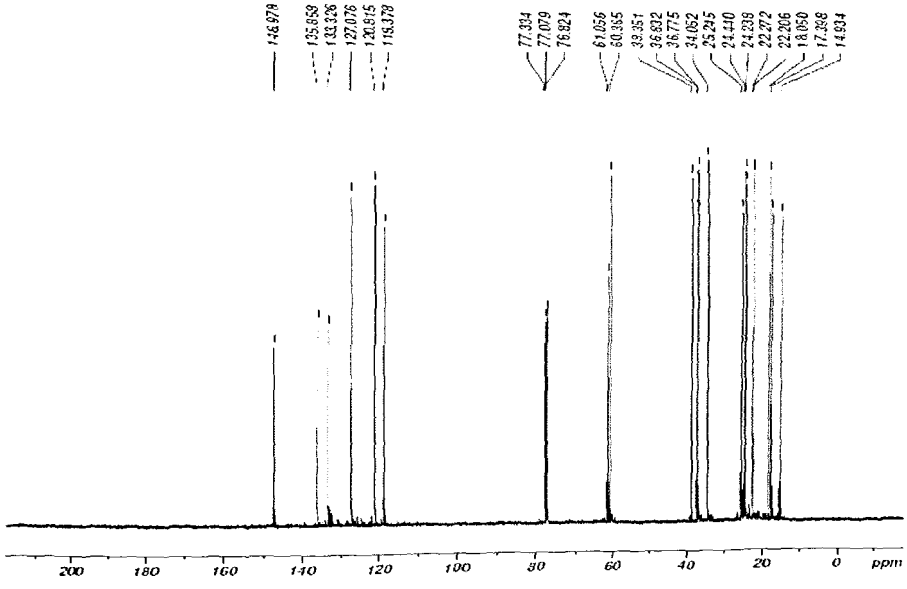



**Fig.2: $^{13}$C NMR spectrum of compound 1**

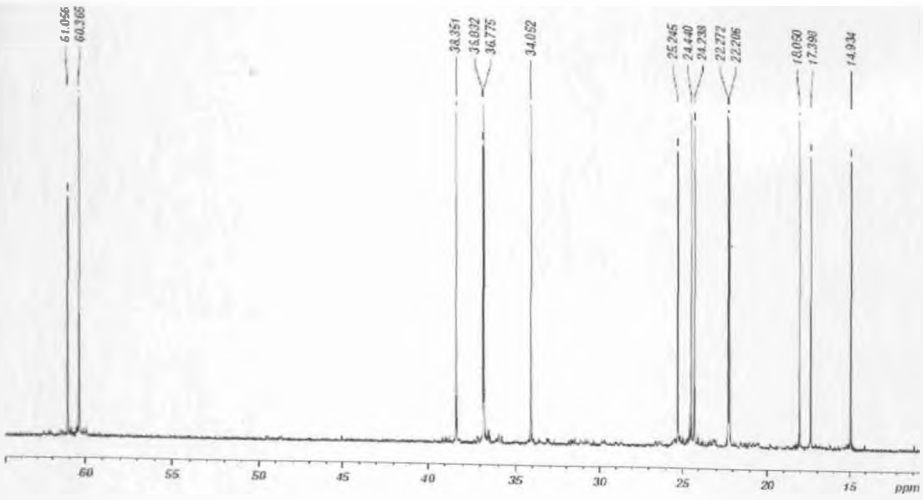



**Fig.3: $^{13}C$ NMR spectrum (expanded from 10-70ppm) of compound 1**

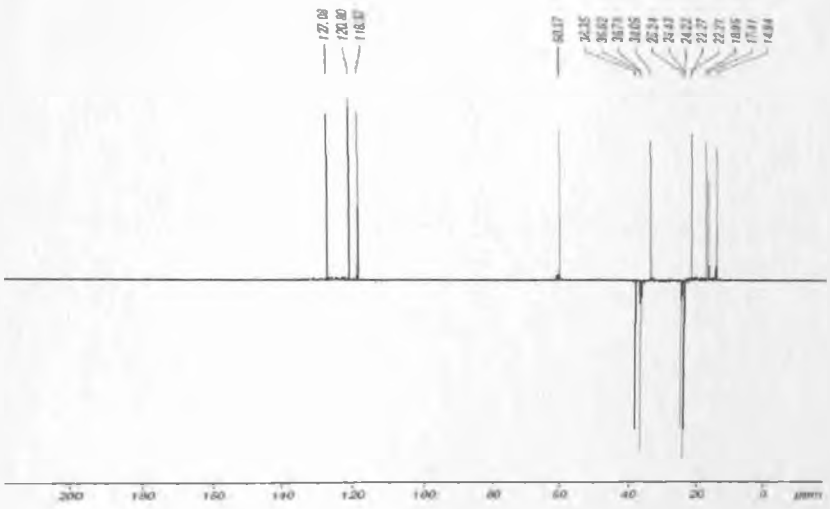



**Fig.4: DEPT spectrum of compound 1**

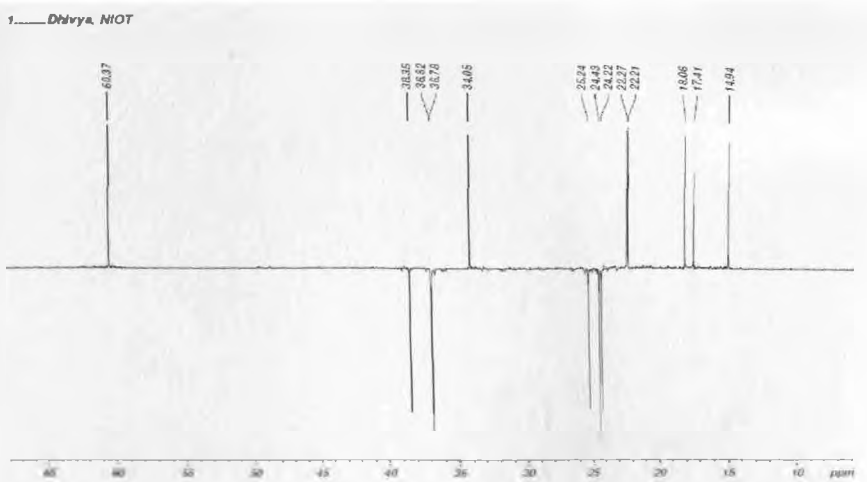



**Fig.5: DEPT spectrum of compound 1**

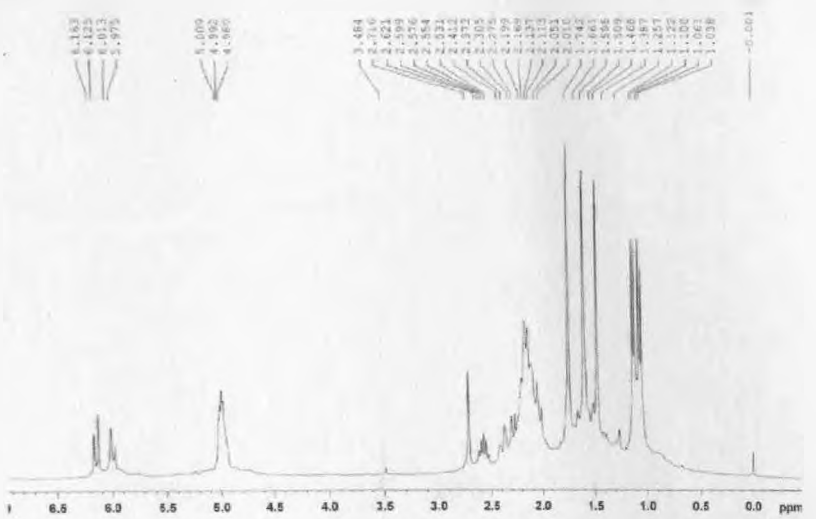



**Fig.6: $^{1}$H NMR spectrum of compound 2**

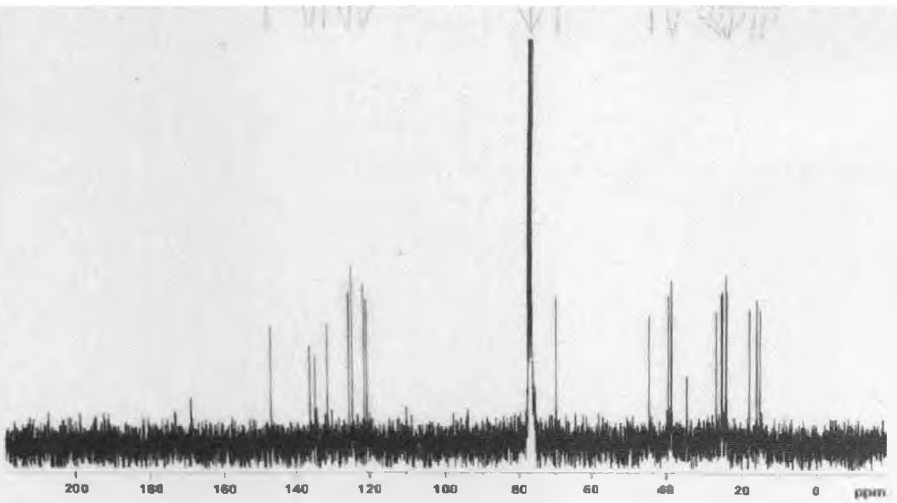



**Fig.7: $^{13}C$ NMR spectrum of compound2**

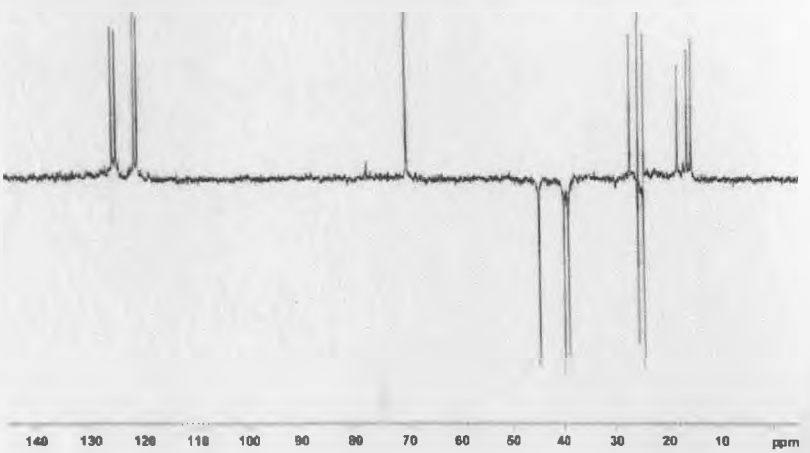



**Fig.8: DEPT spectrum of compound 2**

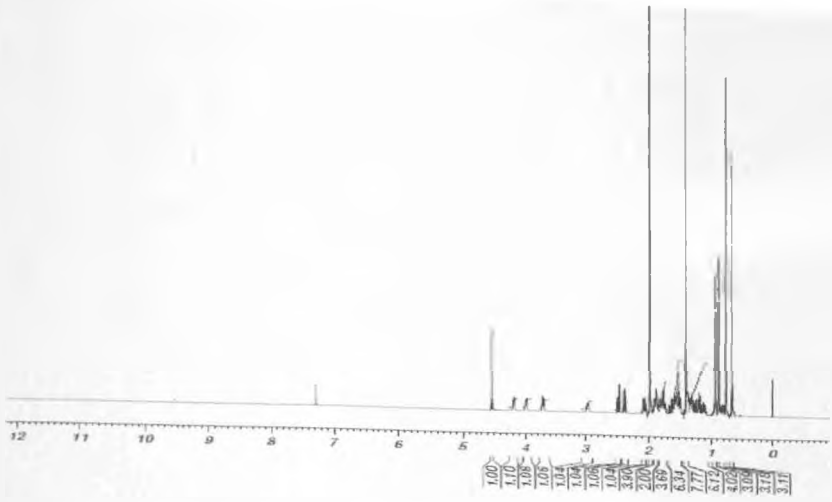


**Fig.9: $^{1}$H NMR spectrum of compound 3**

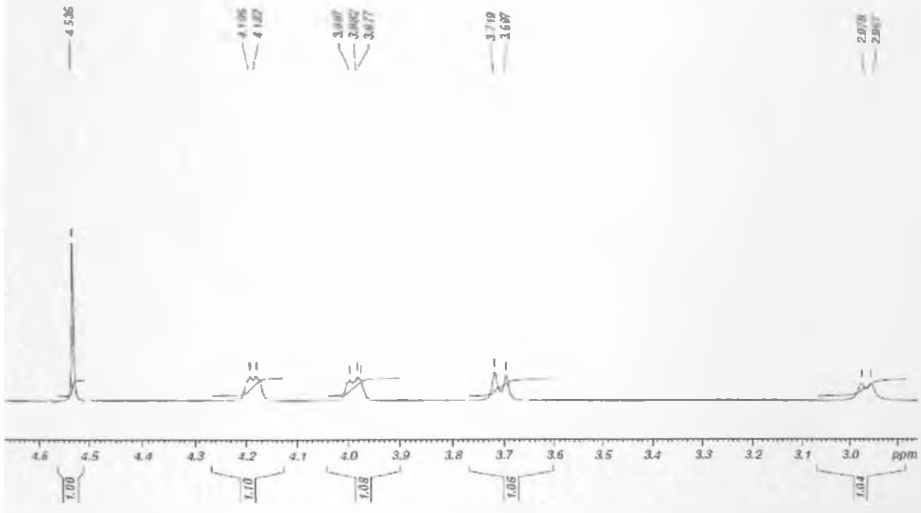


**Fig.10: $^{1}$H NMR spectrum of compound 3**

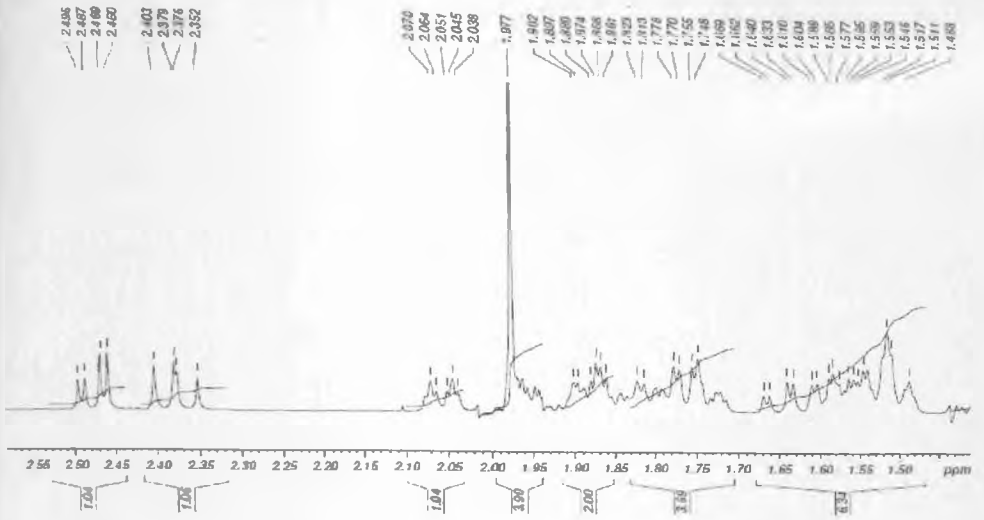


**Fig.11: $^{1}$H NMR spectrum of compound 3**

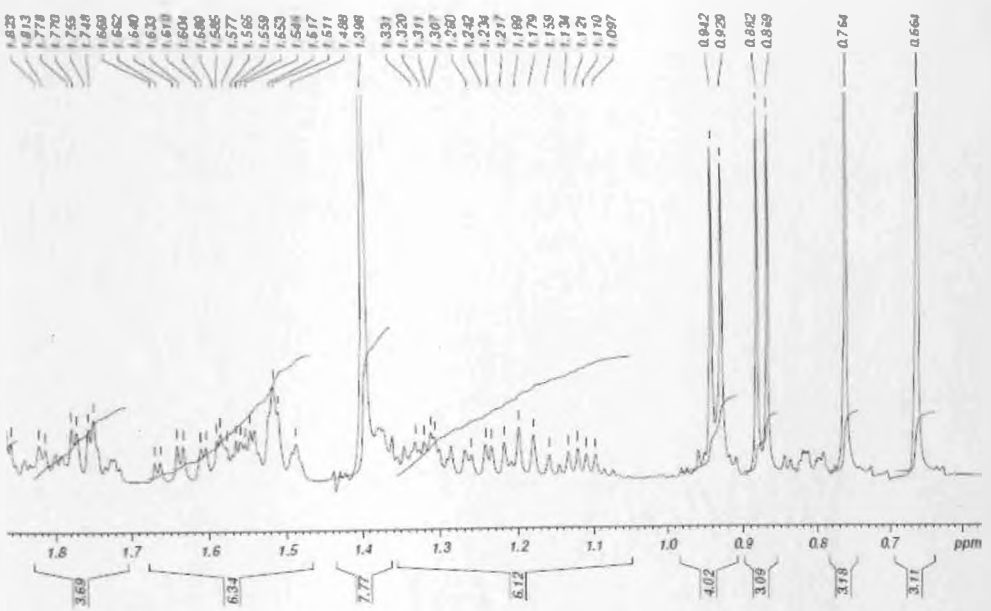


**Fig.12: $^{1}$H NMR spectrum of compound 3**

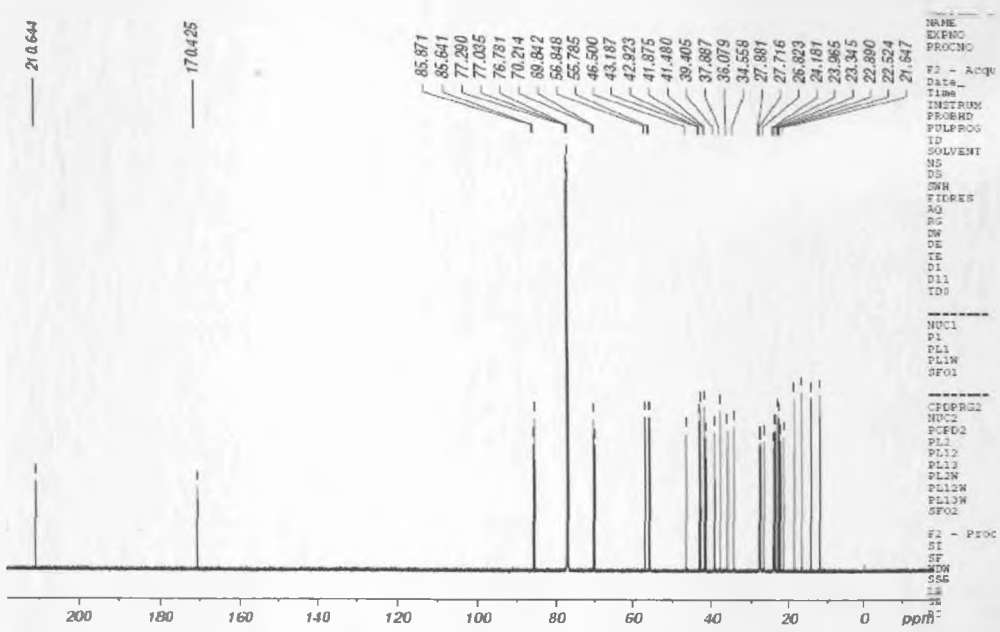



**Fig.13: $^{13}$C NMR spectrum of compound 3**

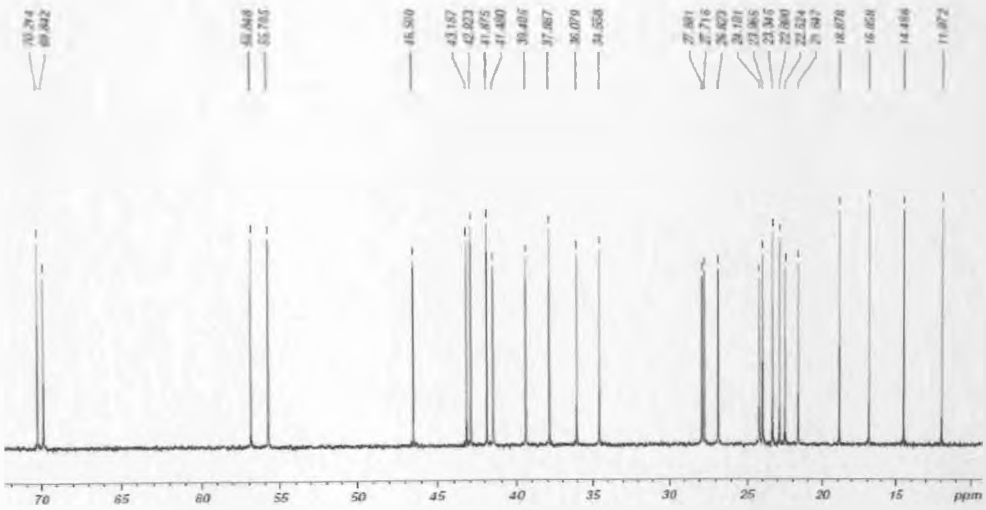



**Fig.14: $^{13}$C NMR spectrum of compound 3**

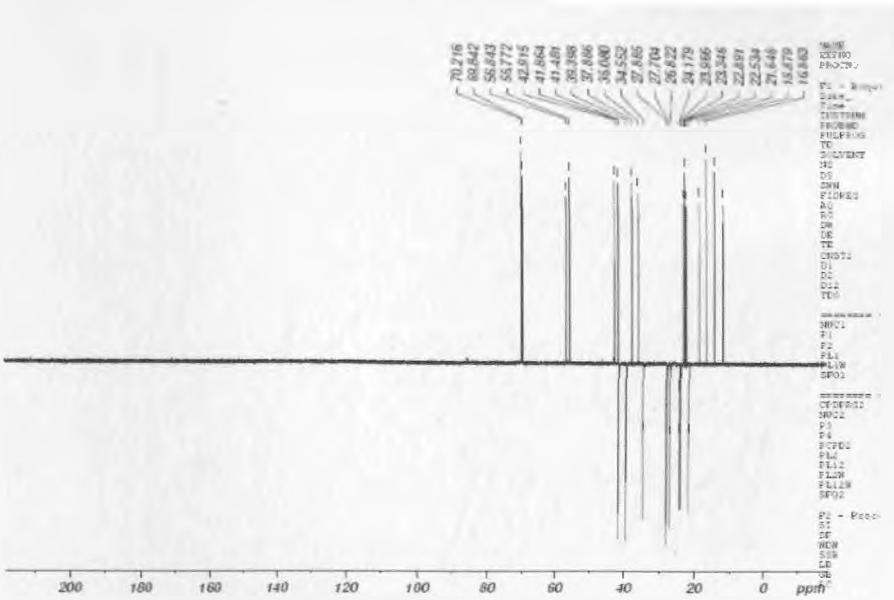



**Fig.15: DEPT spectrum of compound 3**

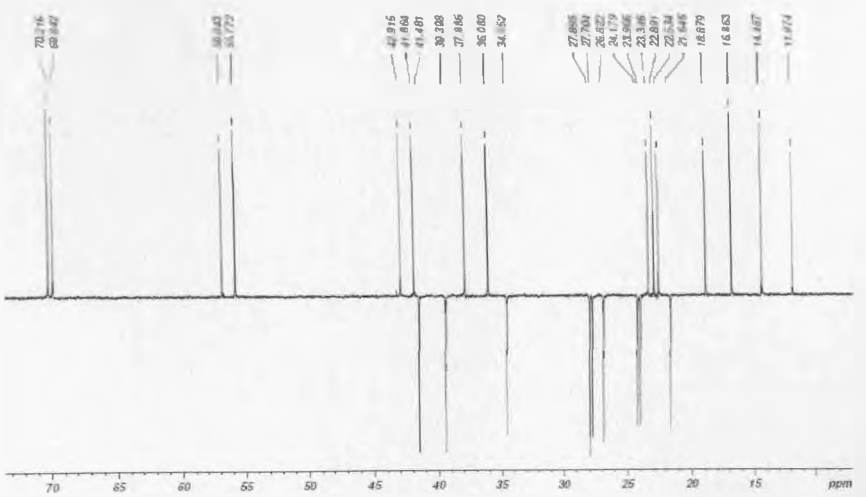



**Fig.16: DEPT spectrum of compound 3**

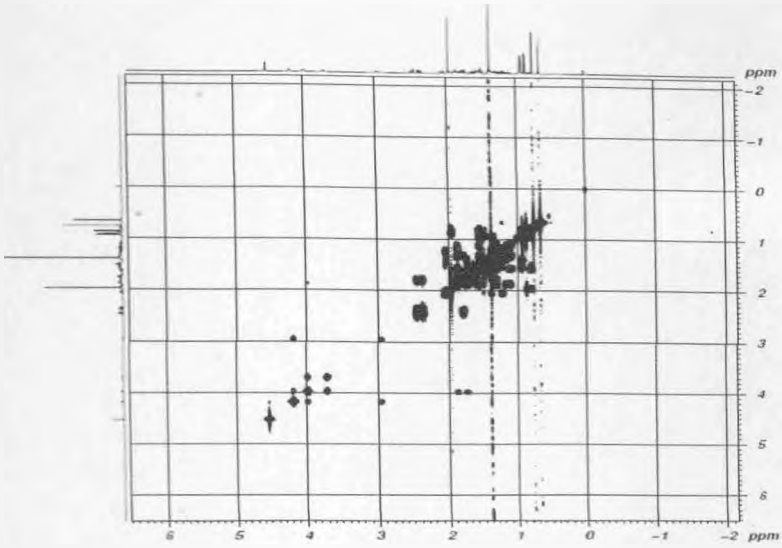



**Fig.17: $^{1}H$-$^{1}H$ COSY spectrum of compound 3**

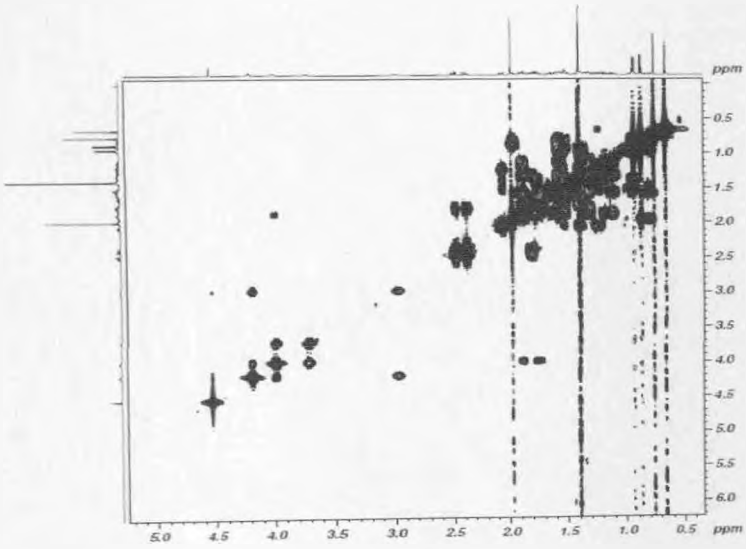



**Fig.18: $^{1}H$-$^{1}H$ COSY spectrum of compound 3**

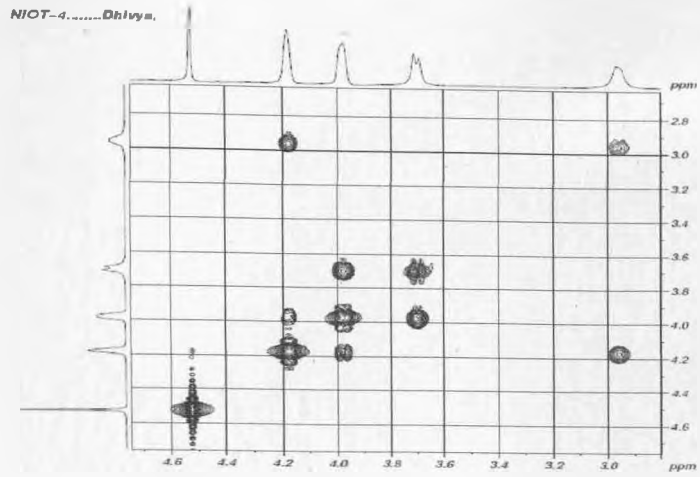



**Fig.19: $^{1}H$-$^{1}H$ COSY spectrum of compound 3**

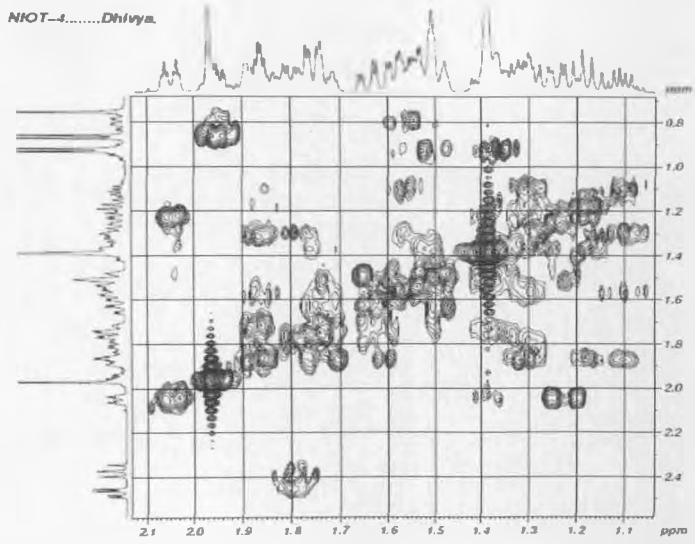



**Fig.20: $^{1}H$-$^{1}H$ COSY spectrum of compound 3**

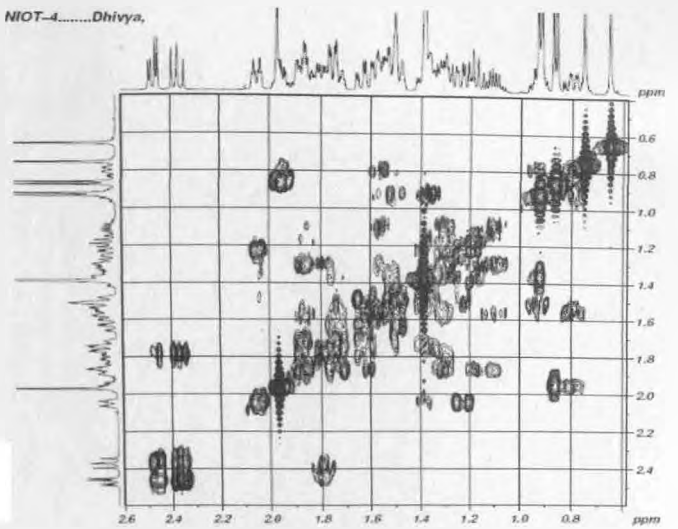



**Fig.21: $^{1}H$-$^{1}H$ COSY spectrum of compound 3**

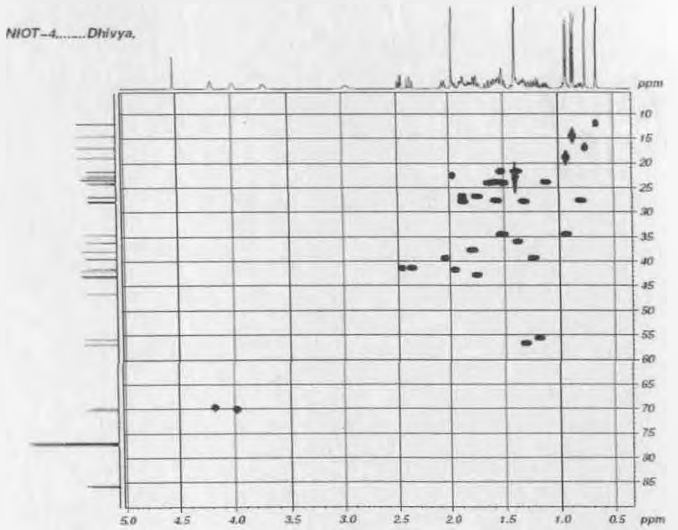



**Fig.22: HSQC spectrum of compound 3**

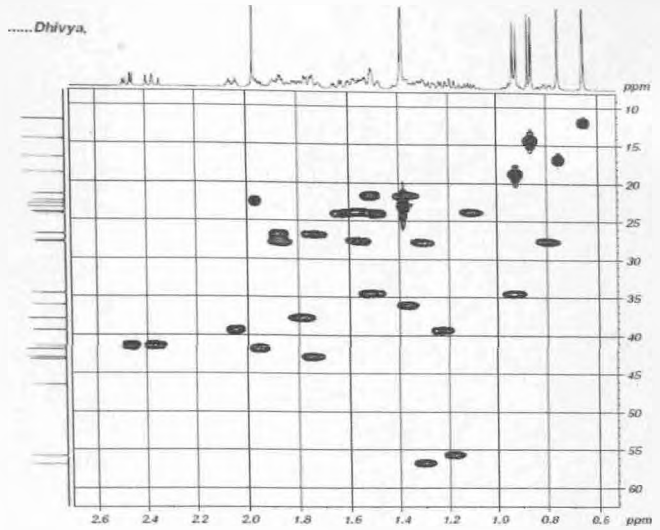



**Fig.23: HSQC spectrum of compound 3**

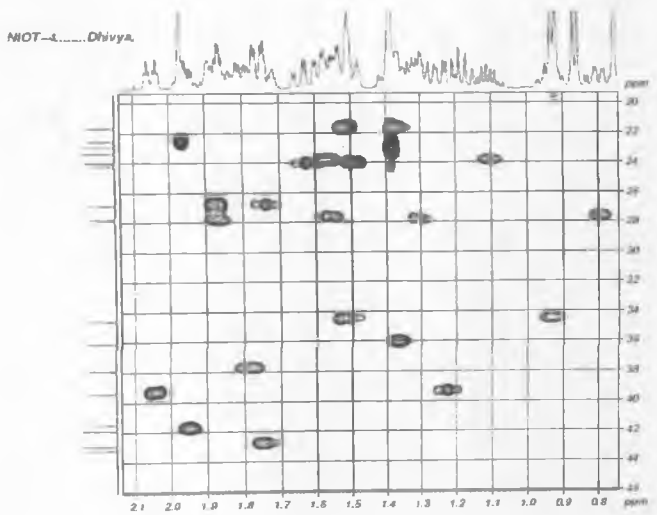



**Fig.24: HSQC spectrum of compound 3**

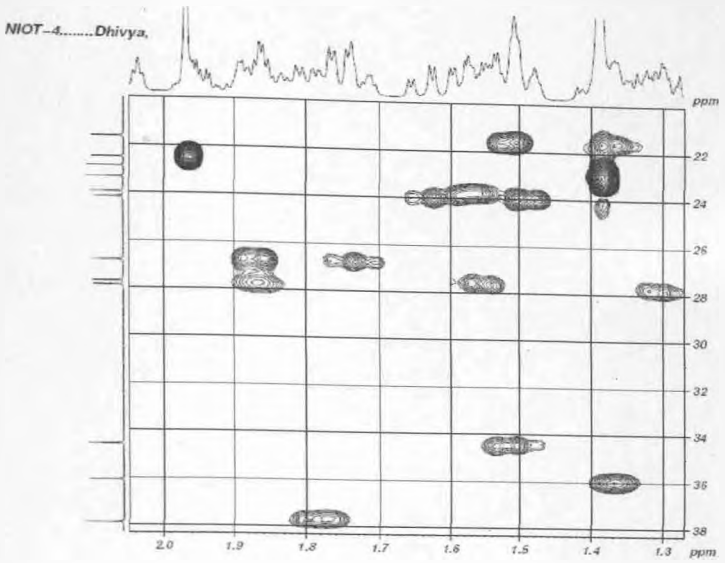



**Fig.25: HSQC spectrum of compound 3**

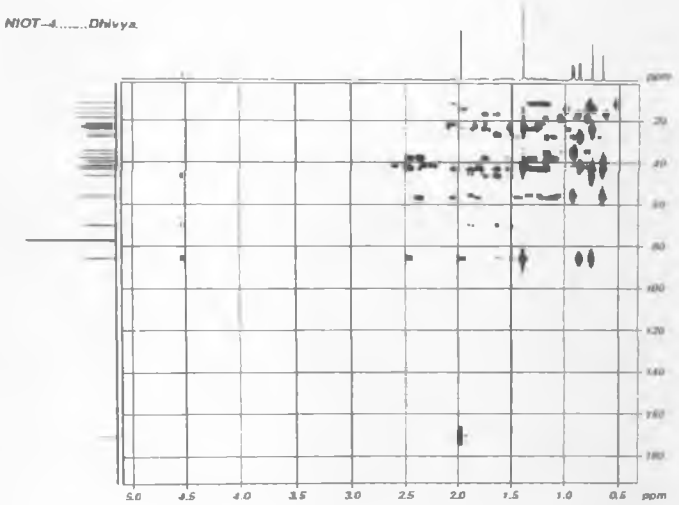



**Fig.26: HMBC spectrum of compound 3**

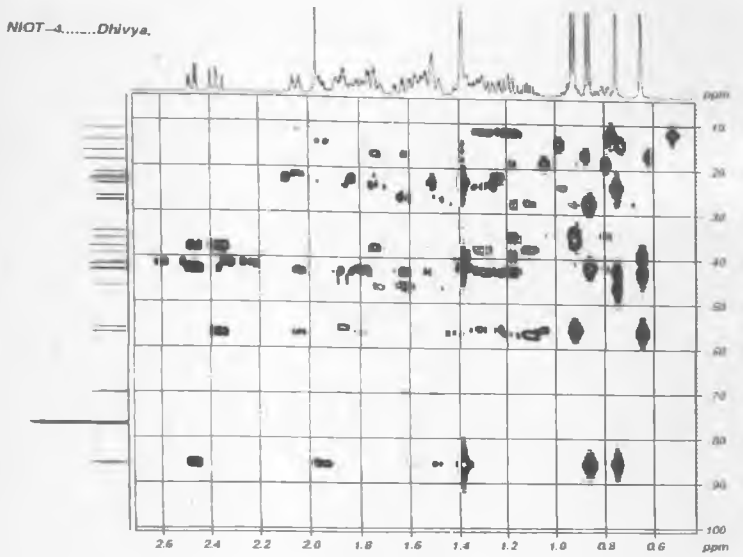



**Fig.27: HMBC spectrum of compound 3**

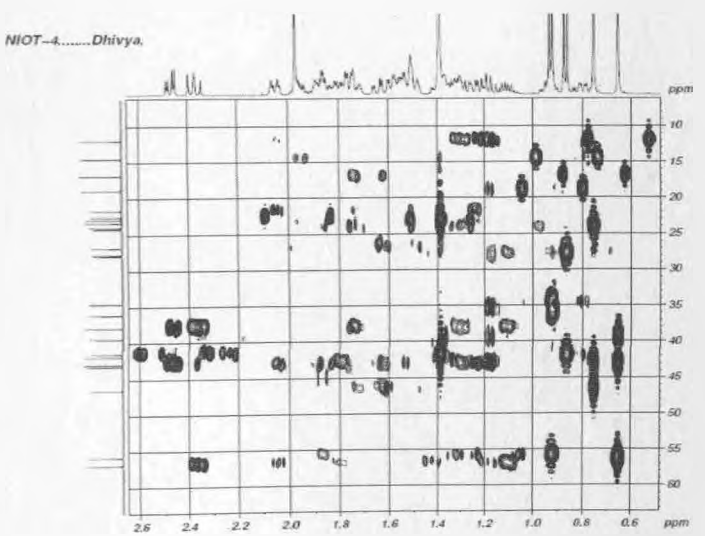



**Fig.28: HMBC spectrum of compound 3**

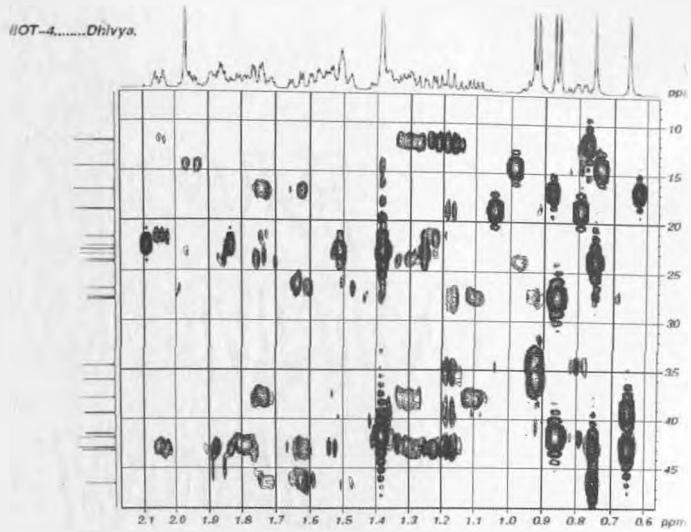



**Fig.29: HMBC spectrum of compound 3**

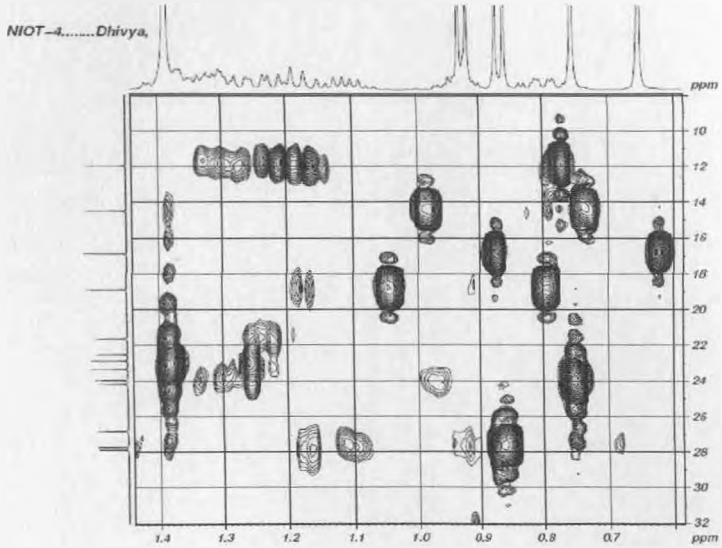



**Fig.30: HMBC spectrum of compound 3**

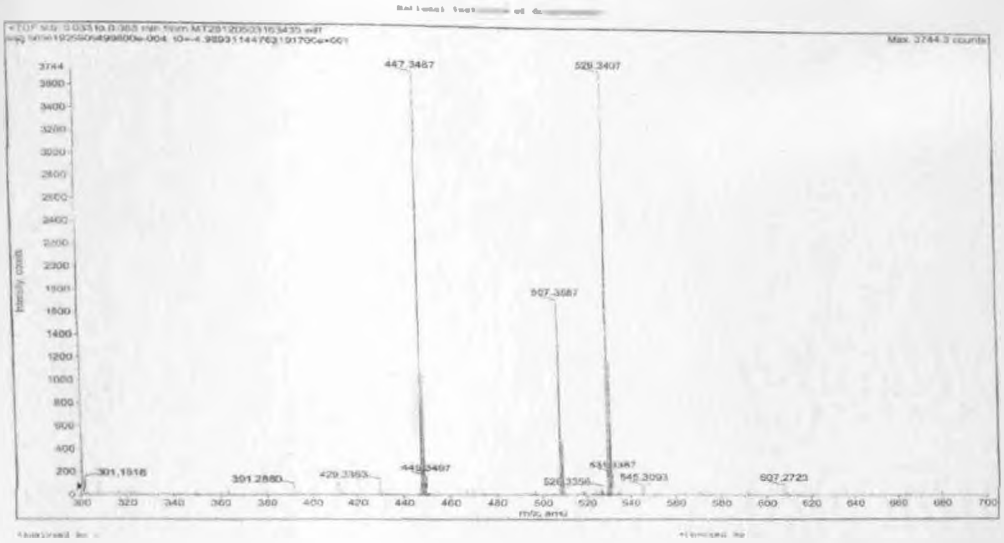



**Fig.30a: ESI-MS spectrum of compound 3**

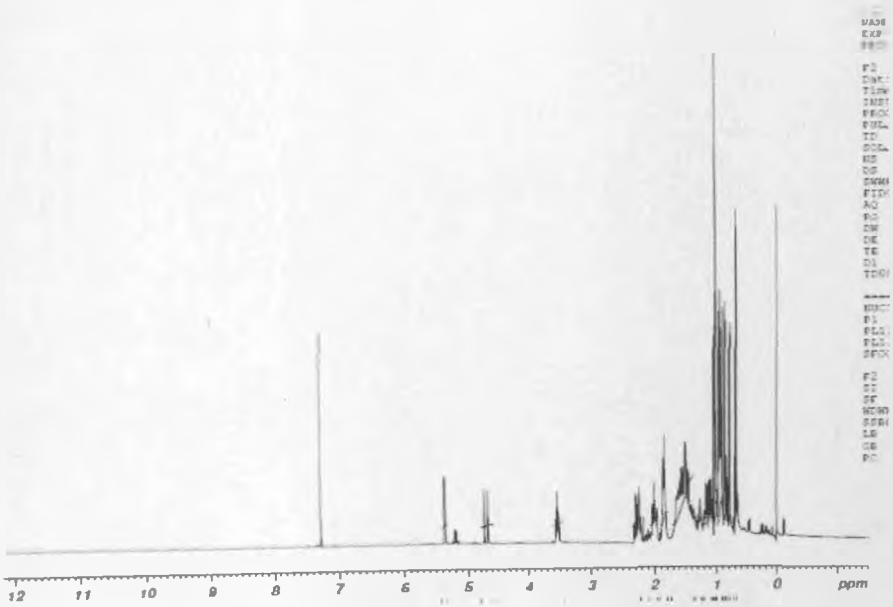



**Fig.31: $^{1}$H NMR spectrum of compound 4**

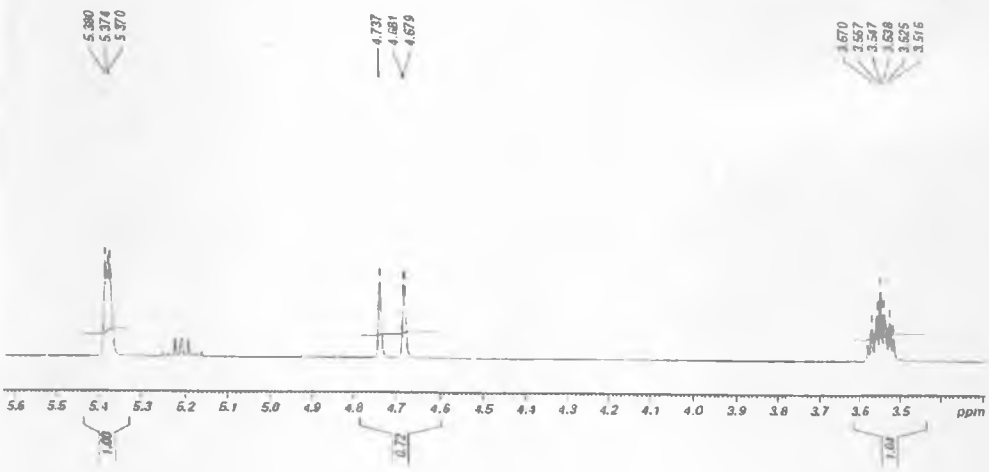



**Fig.32: $^{1}$H NMR spectrum of compound 4**

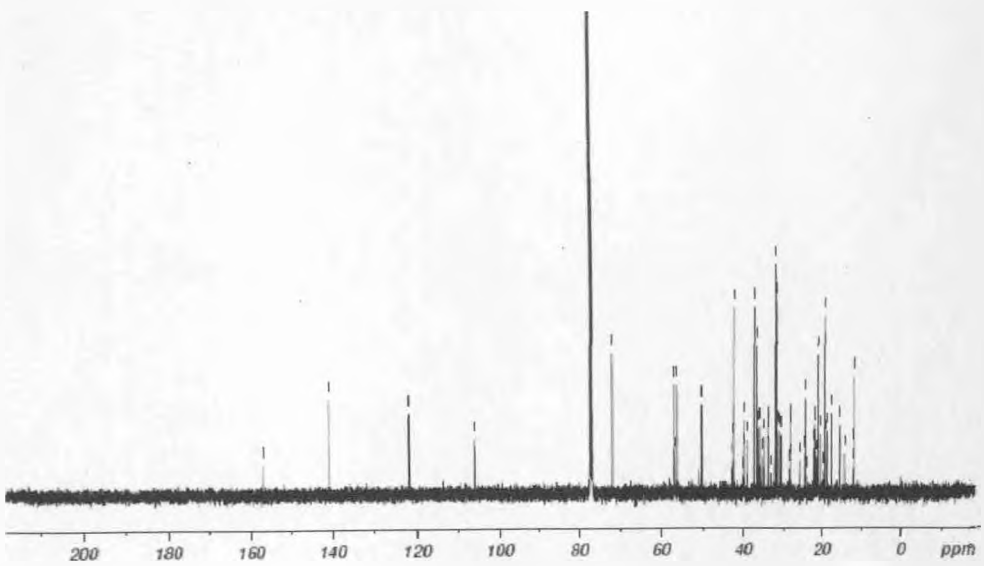



**Fig.33: $^{13}$C NMR spectrum of compound 4**

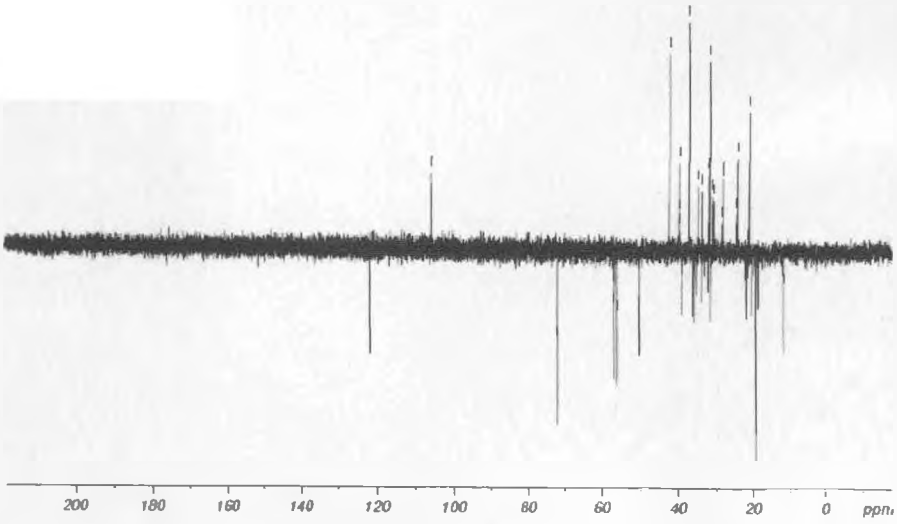



**Fig.34: DEPT spectrum of compound 4**

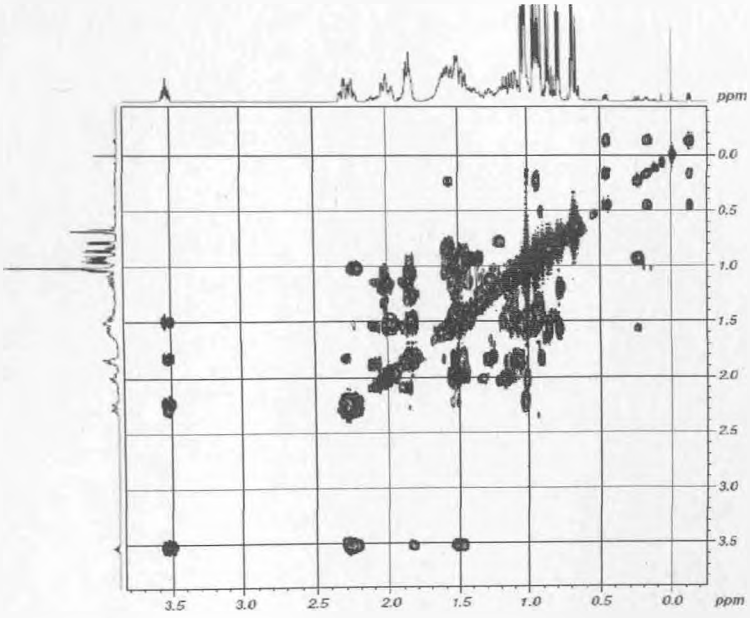



**Fig.35: $^{1}H$-$^{1}H$ COSY spectrum of compound 4**

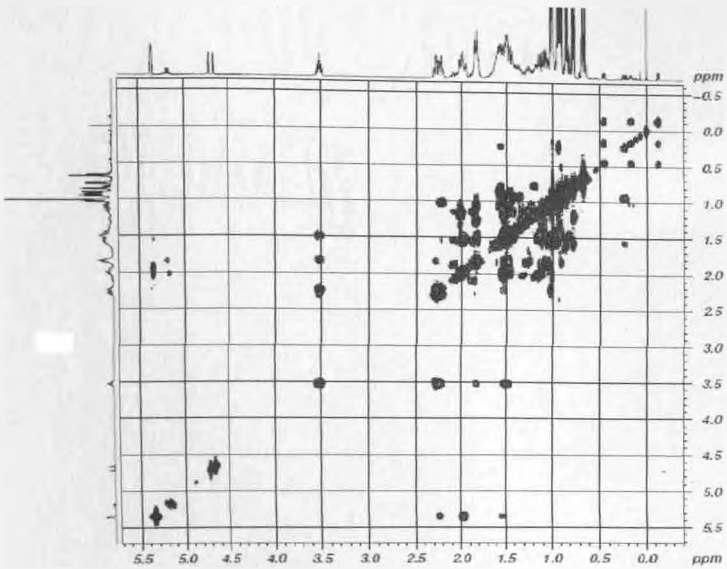



**Fig.36: $^{1}H$-$^{1}H$ COSY spectrum of compound 4**

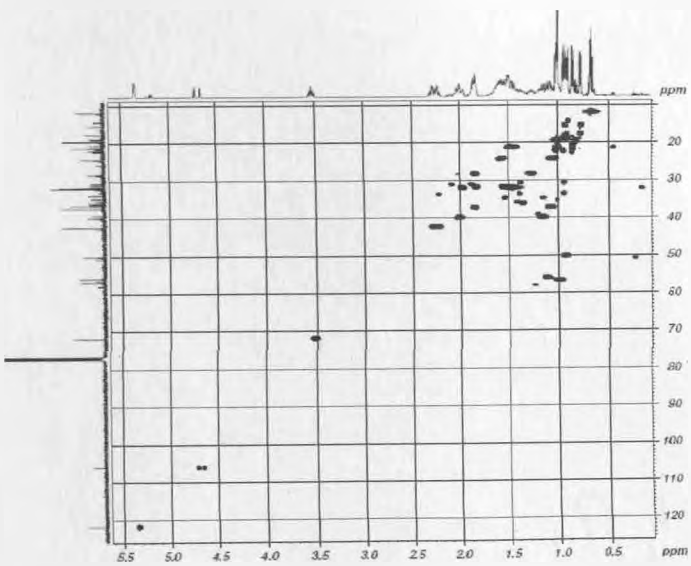



**Fig.37: HSQC spectrum of compound 4**

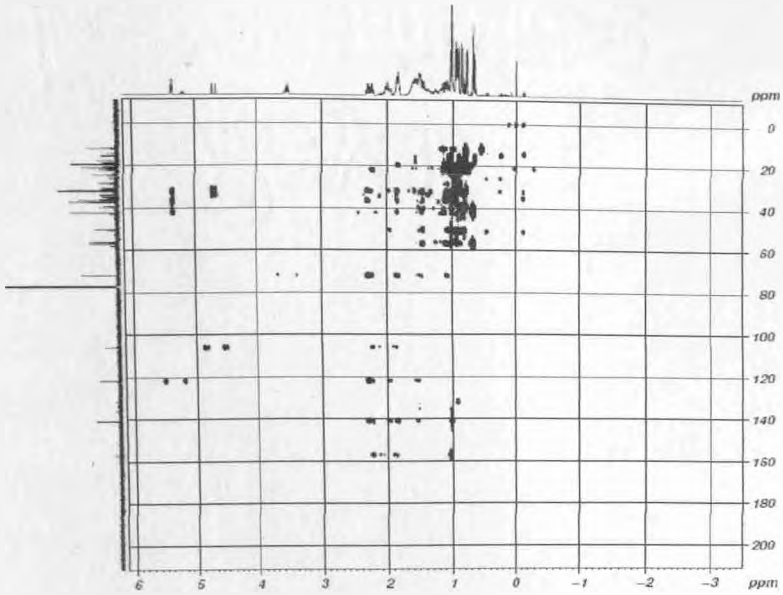



**Fig.38: HMBC spectrum of compound 4**

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# *Chapter IV*

## *Miscellaneous synthetic studies*

## *Synthesis of Alternamides*

## Chapter IV: Miscellaneous synthetic studies

### Introduction:

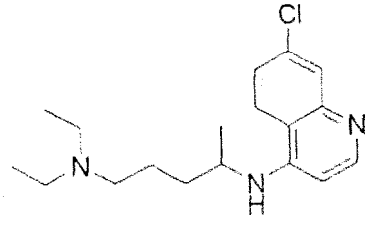
Natural products research has played a critical role in the development of modern medicine. Natural products, their derivatives, and their analogues are among the most important sources for new drug candidates and tools for chemical biology and medicinal chemistry research. Despite the rise of synthesis, and in particular combinatorial chemistry, in drug design, natural products continue to play an important role to this day. Analyses show that of all the new small molecule chemical entities approved as drugs in this time period, 39% were either natural products, directly derived from natural products or synthetic compounds whose design was based on a natural product pharmacophore.[1] In the areas of anti-infectives and anti-cancer drug discovery, natural product research has been particularly productive. Amongst anticancer drugs (approved between the 1940's and today), 57%, and of anti-infectives 63% (approved between 1983 and 1994) were natural products, natural product-derived or synthetic analogues of natural product pharmacophores.[2-4]

However, there are a number of clear disadvantages associated with natural products as a source of drug leads. These relate mainly to identification of the active natural product, and issues of supply. The use of large libraries of extracts from various natural sources against high-throughput assay systems often brings to light potential drug leads. However, once an extract has been selected, identification of the active component in what may be a very complex mixture of compounds can be difficult. The complexity of natural products can make elucidation of the structure of the active component an arduous task. Once the active component has been identified, reasonably significant quantities of this natural product are required for initial biological evaluation, and much more will be required for *in vivo* testing and clinical supply if the compound is successful. Most natural products are produced on a very small scale by their host organisms and isolation of the compound of interest directly from the natural source is often (although not always) uneconomical on large scales. An approach used to circumvent these supply issues has been the synthesis of these molecules.

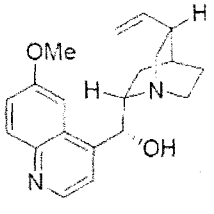
The intelligent merger of these two approaches is likely to be the most useful approach for future drug discovery programs.

The massive improvement in analytical techniques continues to simplify the elucidation of natural product drug leads. In those cases where supply issues are critical to the success of the emerging pharmaceutical, total synthesis or the synthesis of more accessible natural product analogues will continue to facilitate the viability of natural products as lead compounds in drug discovery.

The synthetic targets chosen were often driven by interest in the biological activity of a natural product whose supply was questionable. An example is the total synthesis of a quinine precursor by Woodward and Doering in 1944.[5] The supply issue of this strategic drug led to intense interest in a possible synthetic source. When the successful synthesis was announced there was much fanfare amongst the public, although realistically the synthesis could not have provided a genuine alternative source, and the careful management of natural resources and discovery of synthetic analogues such as chloroquine (1), made the synthesis of quinine (2), a more academic pursuit.

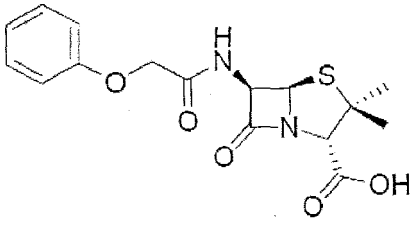



**(1)**



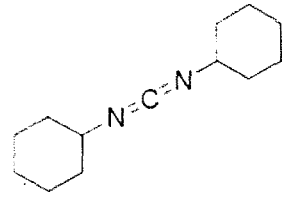

**(2)**

An early example of the advances in methodology which resulted directly from the synthesis of a natural product is the development of mild dehydrating agents by Sheehan, used in the synthesis of penicillin V (3).[6-7]




**(3)**

Although fermentation methods were able to generate significant amounts of penicillin, the synthesis of penicillin was also actively pursued. The synthesis of the penicillins had been hampered by an inability to form the highly labile β-lactam moiety which was so critical to the activity of these natural products. All the dehydrating agents for amide bond formation of the day involved harshly acidic conditions (eg thionyl chloride and phosphorous trichloride), and Sheehan appreciated that a more mild system would be required for the sensitive penicillin skeleton. His development of dicyclohexylcarbodiimide (4) as a mild and efficient dehydrating agent allowed the synthesis of this challenging target to be completed. In addition, the development of a mild dehydrating agent for amide bond formation had a huge effect on the field of peptide synthesis and aided important new developments such as solid-phase peptide synthesis.



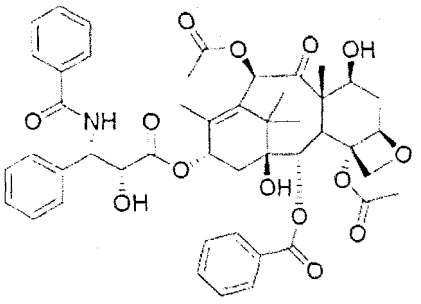

**(4)**

There are three main ways in which synthesis has aided natural product research; firstly in the validation of proposed structures for new metabolites, secondly by allowing improved supply for metabolites produced in insufficient quantities, and thirdly by generating analogues of the natural product pharmacophore which are, in some way, better than the original natural product.

The validation of proposed structures and the confirmation of the assigned structure are regularly only recognized on complete synthesis of the molecule.[8-9] The misassignments are generally not the result of overlooked work on the part of the researcher, but the result of an unexpected ambiguity in the spectroscopic data.[10] This demonstrates the continued value total synthesis has in validating natural product structures.

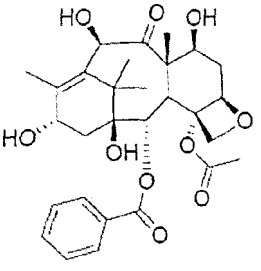
One of the chief complaints about natural products in drug discovery is the issue of supply. The sustainable production of sufficient drug to supply demand in an

economical fashion is a critical factor in the viability of any pharmaceutical candidate. However, total synthesis also plays an important role in alleviating the problem of supply. Galantamine used for the treatment of Alzheimer's disease and mycophenolate sodium as immunosuppressant, are two natural product drugs which have recently reached the market and both are produced by total synthesis.[11] Semi-synthesis is another approach for alleviating problems of supply, with paclitaxel or taxol (5) being a good example.



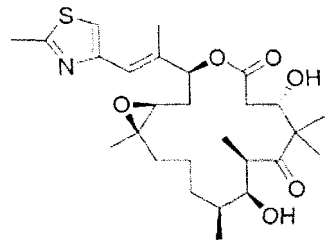
**(5)**

This clinically important anti-cancer drug is a natural product originally isolated from the bark of the Pacific yew tree (*Taxus brevifolia*) and its potential was recognised during research in the 1960s and 70s.[12] However, only 1 g of the metabolite could be extracted from 24 kg of dried bark. Since a 125 year old tree was only capable of producing around 2 kg of bark and this species was relatively rare, the issue of supply was particularly acute in the case of this natural product and slowed its development as a drug. The total synthesis of taxol was completed by Holton and Nicolaou independently.[13-15] However, it was taxol's semi-synthesis from a natural congener, 10-deacetyl baccatin III (6) more sustainably obtained from the needles of the *Taxus baccata*, which allowed for its large-scale manufacture and commercial supply.[16]



**(6)**

Finally, synthetic studies can be used to improve the natural product pharmacophore in some way. This may be by directly improving an aspect of a natural product's biological activity, for example by reducing toxicity, increasing potency or selectivity or improving bioavailability. Alternatively, synthesis may lead to an improvement in the economy of production. A combination of these factors was achieved during the synthetic studies of epothilone B (7) by Danishefsky and co-workers, leading to several very promising lead compounds, which are currently at various stages of clinical testing.[17-18]




(7)

Investment in natural product programs by pharmaceutical companies was seriously reduced during the last twenty years as emerging technologies, in particular combinatorial chemistry, became popular. Few would claim that natural products no longer have a role to play in drug discovery. Scientists have expressed surprise that such a time-honoured and successful approach had been abandoned so readily for far less validated drug discovery platforms.[17] In more recent years, however, natural products have experienced a resurgence of interest from the pharmaceutical industry, and there is an increasing realization that a combination of natural product libraries, more focused combinatorial chemistry, and rational design will provide the best opportunities for drug discovery.[19-20] Synthesis is likely to continue to feature heavily in drug development programs. The increased interest in and success of "green" chemistry is likely to improve the sustainability of using synthesis for commercial pharmaceutical supply.[21]

There is a need for the development of efficient synthesis methods which give access to natural product derived and inspired compound collections. To meet this challenge, the requirements of multistep stereoselective syntheses, and the logic and methodology of total synthesis need to be translated and adapted to the methods and

formats for the synthesis of natural product compound collections. Recent developments in the synthesis of natural product inspired compounds have highlighted the fact that this goal can be successfully attained. The progress made has paved the way for the integration of natural product inspired compound collections into medicinal chemistry and chemical biology research.[22]

The work presented in this chapter is to utilize synthesis as a tool in the study of natural products. As discussed, it can be noted that the ultimate aim would be the synthesis of the isolated bioactive natural product. Keeping in view the significance of synthesis, an attempt was made towards the synthesis of Alternamides, antibacterial natural products isolated from the marine invertebrate, bryozoan *Amathia alternata*.[23]

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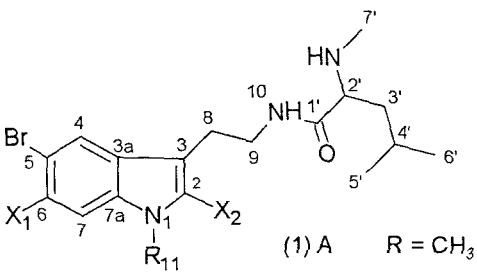
## Chapter 3: Section I: Synthesis of Alternamides

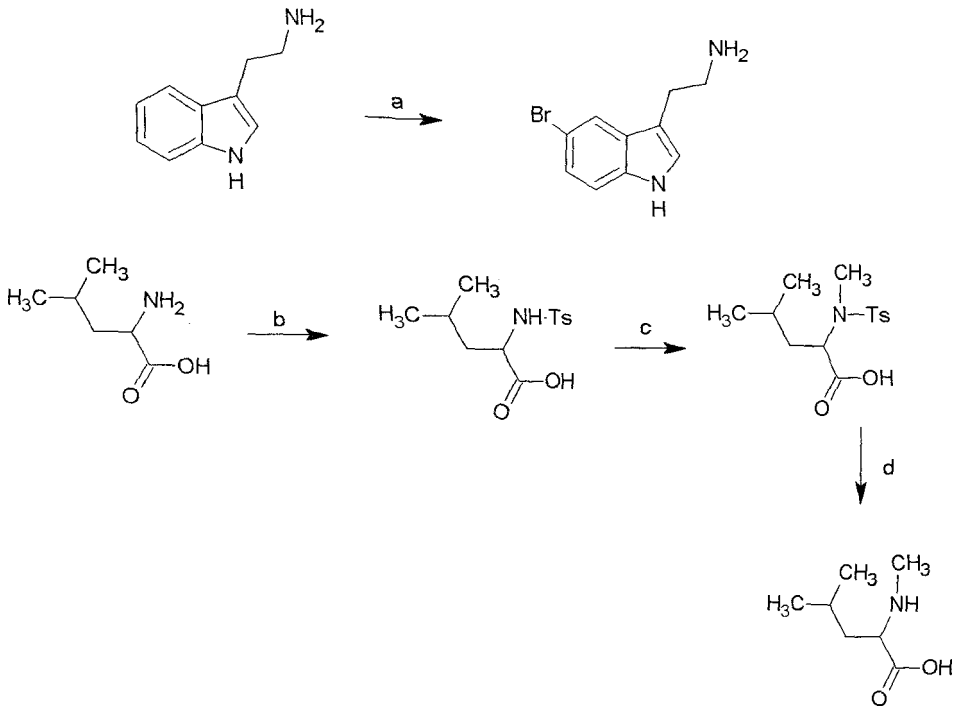
Alternamides isolated by N.K.Lee et. Al., 1997 from the bryzoan *Amathia alternata* are found to have antibacterial activity against several Gram-positive bacteria. including *Staphylococcus aureus*, *B. subtilis* and *Enterococcus faecium*.[23] This activity could potentially prevent bacterial surface fouling or prevent infection by some bacteria.

In this section, an attempt was made towards the synthesis of these molecules.




| | | | |
|---|---|---|---|
| (1) A | $R = CH_3$ | $X_1 = X_2 = Br$ | |
| (2) B | $R = H$ | $X_1 = X_2 = Br$ | |
| (3) C | $R = H$ | $X_1 = H$ | $X_2 = Br$ |
| (4) D | $R = H$ | $X_1 = Br$ | $X_2 = H$ |

Alternamides are monopeptides basically made up of tryptamine; a decarboxylated natural amino acid tryptophan and N-methyl derivative of the amino acid leucine. The synthetic strategy was to synthesize these two compounds separately and couple them by dehydration to form the amide linkage. The synthetic scheme is as given below **(Fig.1)**




a.Br2 / AcOH b.TsCl,Py c.MeI, CHCl3 d.Conc.H2SO4

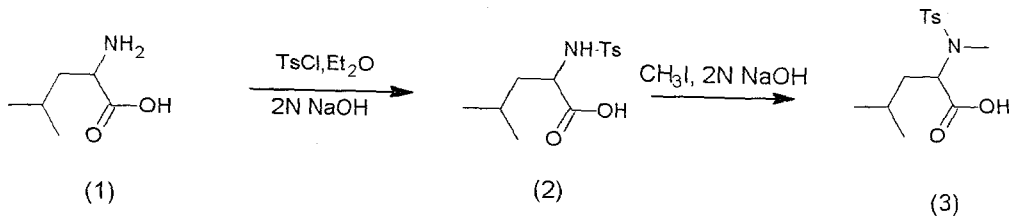
**Fig 1: Synthetic scheme towards Alternamides**

1. The first synthetic scheme proposed was to prepare the N-methyl Leucine by protection of one of the N-H with a p-Toluene sulphonyl group. After protection, methylation of the free N-H with methyl iodide and subsequent deprotection of the p-Ts group with a suitable deprotecting agent.

### 1.1. Preparation of N-methyl leucine from the amino acid leucine.

p- Toluene sulphonyl leucine was obtained in 62% yield from L-Leucine in 2N NaOH when treated with p-toluene sulphonyl chloride in diethyl ether.

From p-tosyl leucine in 2N sodium hydroxide reacted with equimolar amounts of methyl iodide at 60-70° C was obtained N-Methyl, N-tosyl derivative in 82% yield. For detosylation of N-methyl, N-tosyl leucine, conc. Sulphuric acid was used.




**Experimental:**

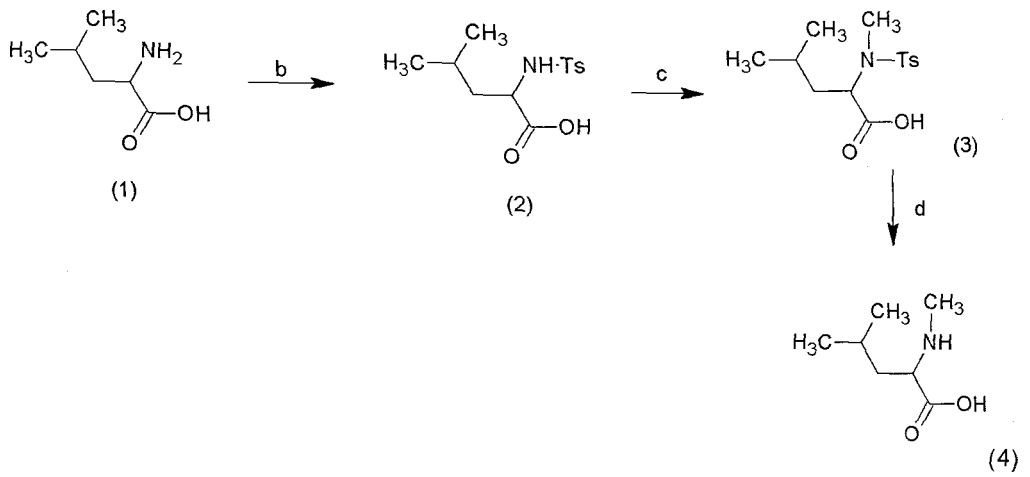
**1.1a. Preparation of N-methyl leucine from leucine.**

1. L-Leucine(1) (2g, 40mmol) was dissolved in 2N NaOH (30ml) and stirred with equimolar quantities of p-toluene sulphonyl chloride in diethyl ether (30ml) and treated in 1hr intervals with small portions of the same sodium hydroxide solution. After 4 hours the reaction mixture was neutralised with 5N hydrochloric acid and extracted with diethyl ether. p- Toluene sulphonyl leucine (2) was obtained in 62% yield.

**1.1b.** N-tosyl leucine (2) (1.7g, 31mmol) was dissolved in 2N sodium hydroxide (20ml). An equimolar amount of methyl iodide was added dropwise to the reaction mixture which was kept at 60-70° C. The reaction mixture was kept for 20 minutes more at the same temperature, cooled and precipitated with 5N HCl. The precipitate was filtered. The filtrate was washed with water and dried over sodium sulfate and later concentrated under vacuum to obtain the product N-methyl,N-tosyl leucine (3) in 82% yield.

**1.1c. Detosylation of N-methyl, N-tosyl leucine.**

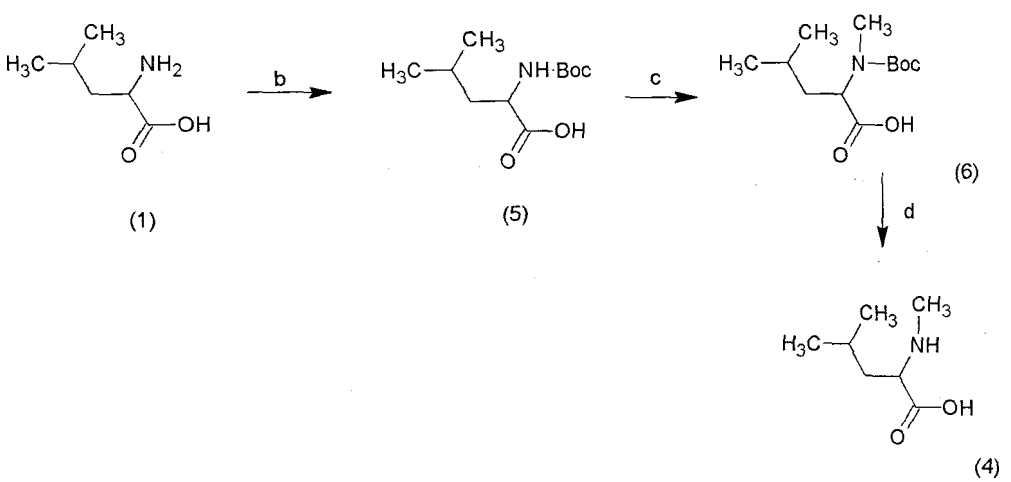
N-methyl, N-tosyl leucine (1.5g, 26 mmol) was dissolved in concentrated sulphuric acid (15ml) and left for 4 hrs at room temperature. The reaction mixture was later neutralized with NaOH and extracted with ethyl acetate to obtain the detosylated product.




**b.**TsCl,$Et_2O$,2N NaOH **c.**MeI, 2N NaOH, 60-70° C **d.**Conc.$H_2SO_4$

**1.2.** In the synthesis of n-mono methylated leucine , the first two intermediates; the n-tosylated leucine and the n-methyl, n-tosyl leucine were successfully obtained. Detosylation of the n-methyl, n-tosyl leucine using conc. $H_2SO_4$ yielded only 5% of the product n-mono methyl leucine.

In order to improve the yield of N-methyl leucine the scheme was modified by protecting leucine with t-butyloxy carbamate (boc) reagent, which is stable under basic conditions and could be easily removed with TFA.




**b.**BOC anhydride,$Na_2CO_3$,dioxane **c.**MeI, 2N NaOH, 60-70° C **d.**TFA

### 1.2a. Preparation of N-Boc leucine.

The amine (1.04g, 7.9 mmol) is dissolved in water and sodium bicarbonate (2 eq) is added with stirring. The resulting solution is cooled to 5°c and BOC anhydride (2.756ml, 1.5 eq) is added slowly as a solution in *para*-dioxane (also cooled). The mixture is stirred at 0° for 1 h and allowed to warm to room temperature overnight. Water is then added and the aqueous layer is extracted 2 times with ethylacetate. The organic layer is back extracted twice with saturated sodium bicarbonate solution. The combined aqueous layers are acidified to a ph of 1 with 10% HCL, and extracted 3 times with ethyl acetate. The organic layer was dried over sodium sulfate and concentrated *in vacuo*. The resulting residue was then purified by flash chromatography.

### 1.2b. Preparation of N-methyl, N-boc leucine.

N-boc leucine (1.8, 7.7mmol) was dissolved in 6N sodium hydroxide solution, stirred with equimolar amounts of methyl iodide at 60-70° c until completely dissolved. The reaction mixture was kept for 30 minutes more at the same temperature, cooled, precipated with 5N HCL to obtain the n-methyl derivative in 80% yield.

### 1.2c. Removal of boc (t-butyloxy carbamate)

N-methyl, N-boc leucine (1.4g, 5.7mmol) is dissolved in 1:1 trifluoroacetic acid: water (30ml) and stirred at room temperature till the formation of the product N-methyl leucine as indicated by TLC (usually <2 h). The solution is concentrated *in vacuo* and purified by flash chromatography.

### 1.2d. Removal of Boc (t-butyloxy carbamate)

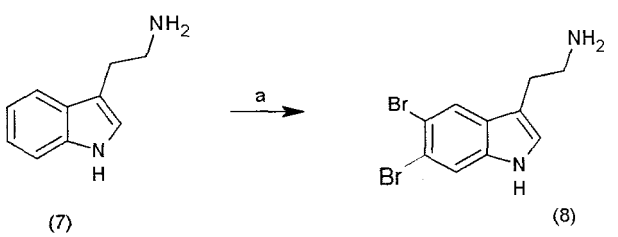
In view of the difficulty encountered in isolating the N-methyl leucine from the aqueous fraction due to its solubility in water, the method was modified as follows. Instead of a solution of 1:1 trifluoroacetic acid: water, the N-methyl, N-BOC leucine was dissolved in dichloro methane and trifluoroacetic acid was added dropwise,

keeping the reaction mixture at 0°C. The reaction mixture was left stirring for 3 hrs until completion. The insoluble product obtained was filtered and recrystallised in alcohol. Yield=40%

**2. Bromination of tryptamine.**

Tryptamine (2g, 12.48mmol) was dissolved in acetic acid 20ml, cooled in ice and equimolar amounts of bromine in acetic acid added dropwise and stirred for 30 minutes. The reaction mixture was later poured over ice and later extracted with ethyl acetate. The organic layer was washed thoroughly with water to remove traces of acetic acid and bromine. The organic layer was dried to obtain the buff coloured solid of the mono, di or the tri substituted bromo derivatives of tryptamine. These were separated by flash chromatography to obtain 5-bromotryptamine, 6-bromotryptamine and 5,6-di bromotryptamine.

Bromination using bromine in acetic acid in various molar ratio yielded mono di and tri substituted bromo derivatives of tryptamine.

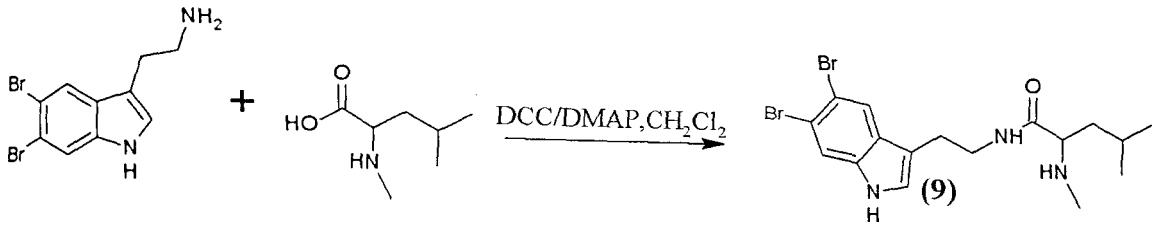



a.$Br_2$ / AcOH

**3. Preparation of Amide:**

5,6-dibromo tryptamine (0.662g, 2.08mmol) was added to N-methyl leucine (0.703g,1.86mmol), DCC(0.430g, 2.1mmol) and catalytic amount of DMAP (11mg) in DCM (40ml). The reaction mixture was stirred for 2 hrs at room temperature. At the end of 2hrs it was filtered through celite and the filtrate was poured into a separatory funnel containing saturated sodium bi carbonate solution (15ml), the aqueous layer was extracted with DCM (3x15ml).The combined organic layers were washed with brine and dried over sodium sulphate and concentrated under vacuum to

yield light yellow oil. The compound (9) was purified by flash chromatography (1:2 Ethyl acetate : hexane). Yield=11%




**Discussion:** In this section an attempt was made towards the synthesis of bryozoan metabolites Alternamides from the species *Amathia alternata.* These compounds were selected because on their antibacterial activity.

Alternamides are monopeptides basically made up of tryptamine; a decarboxylated natural amino acid tryptophan and N-methyl derivative of the amino acid leucine. The synthetic strategy was to synthesize these two compounds separately and couple them by dehydration to form the amide linkage.

N-methyl leucine was prepared by first protecting one of the N-H with a suitable protecting agent, t-butoxy carbamate , then methylating with Methy iodide, to obtain N-methyl N-boc leucine. The deprotection of the Boc group was done with TFA. The bromo tryptamine was prepared by bromination utilizing Bromine in acetic acid as the brominating agent.

The final step of coupling needs to be improved with other coupling agents as coupling of the N-methyl leucine with bromo tryptamine gave low yields of the product.

Compound 5: white crystalline solid; IR (Zn-Se ATR) $\nu_{max}$ 3444.37, 3334.92, 2984.00, 2855.02, 1712.70, 1674.21, 1244.00, 1170.79, 1047.35 $cm^{-1}$; $^{1}H$ NMR ($CDCl_3$) ($\delta_H$) 0.970 (6H, d), 1.465 (9H, s), 1.56(1H, m), 1.706(1H, m), 1.767 (2H,m); $^{13}C$ NMR($CDCl_3$) ($\delta_C$) 178.0, 156.0, 80.2, 52.0, 41.4, 28.3, 24.7, 22.939, 21.7

Compound 6: white crystalline solid; IR (Zn-Se ATR) $\nu_{max}$ 3331.67, 2964.59, 1716.58, 1383.38, 1247.94, 1166.66, 1043.49 $cm^{-1}$; $^{1}H$ NMR ($CDCl_3$) ($\delta_H$) 0.970 (6H, d), 1.465 (9H, s), 1.56(1H, m), 1.706(1H, m), 1.767 (2H,m),2.4 (3H, s); $^{13}C$ NMR($CDCl_3$) ($\delta_C$) 178.0, 155.7, 80.2, 53.1, 51.9, 41.3, 28.3, 24.7, 22.8, 21.7

Compound 4: white crystalline solid; $^{1}H$ NMR ($CD_3OD$) ($\delta_H$) 0.970 (6H, d), 1.465 (9H, s), 1.56(1H, m), 1.706(1H, m), 1.767 (2H, m) 3.2(3H,s); $^{13}C$ NMR($CDCl_3$) ($\delta_C$)178.00, 53.222, 40.382, 24.399, 21.910, 20.548

Compound 8: pale red yellow solid: $^{1}H$ NMR ($CDCl_3$) ($\delta_H$) 7.08(1H,s), 7.45(1H, s), 7.66(1H.s), 2.92(2H,m), 2.91(2H,m); $^{13}C$ NMR($CDCl_3$) ($\delta_C$)110.912(C2), 111.916(C3), 119.227(C4), 117.907(C5), 122.219(C6), 120.994(C7), 136.871(7A), 28(C8), 41.656(C9)

Compound 9: pale brown solid; $^{1}H$ NMR ($CD_3OD$) ($\delta_H$) 7.08(1H,s), 7.85(1H, s), 7.66(1H.s), 2.92(2H,m), 3.58(2H,m), 3.63(1H,m) 1.25(1H,m), 1.56(1H,m), 1.56(1H,m), 0.91(3H,d), 0.11(3H,d), 2.31(3H,s) $^{13}C$ NMR($CDCl_3$) ($\delta_C$) 124.2(C2), 112.6(C3), 128.4(C3a), 123.0(C4), 117.0(C5), 114(C6), 115.9(C7), 135.9(C7a), 25.0(C8), 39.3 (C9), 171.4(C1'), 62.5(C2'), 41.4(C3'), 25.0 (C4'), 22.3(C5'), 22.7(C6'), 33.9 (C7')

**References:**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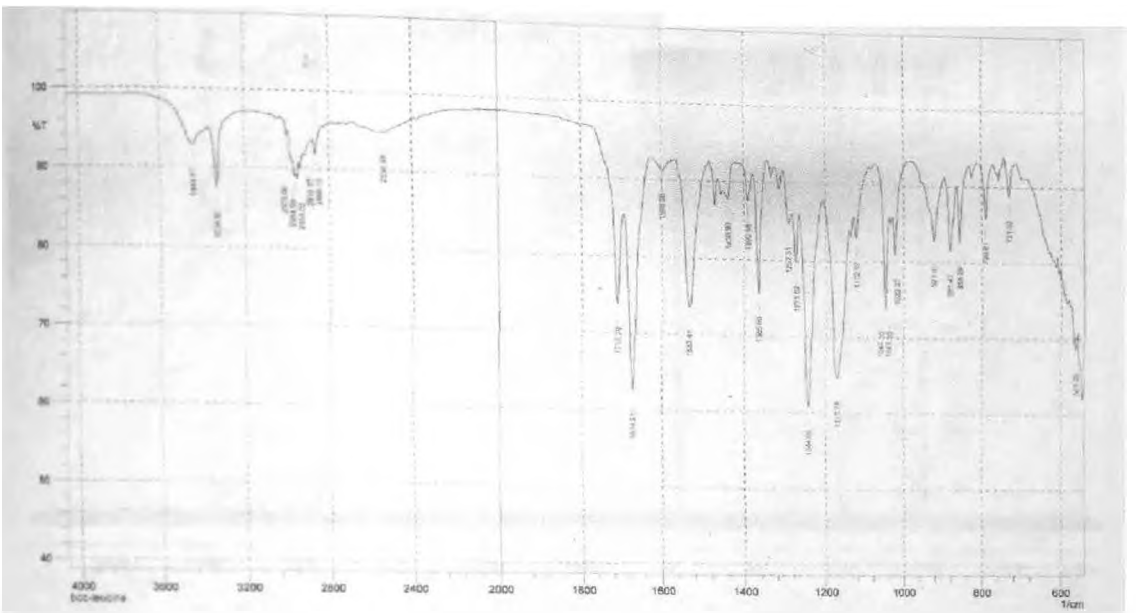



Fig1.1: IR spectrum of (5)

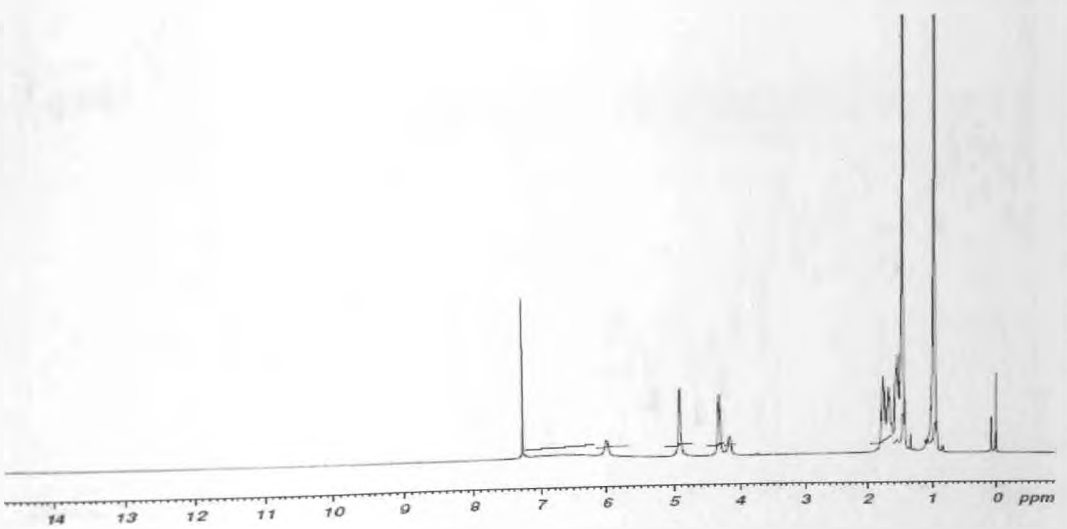



Fig1.2: $^{1}$HNMR spectrum of (5)

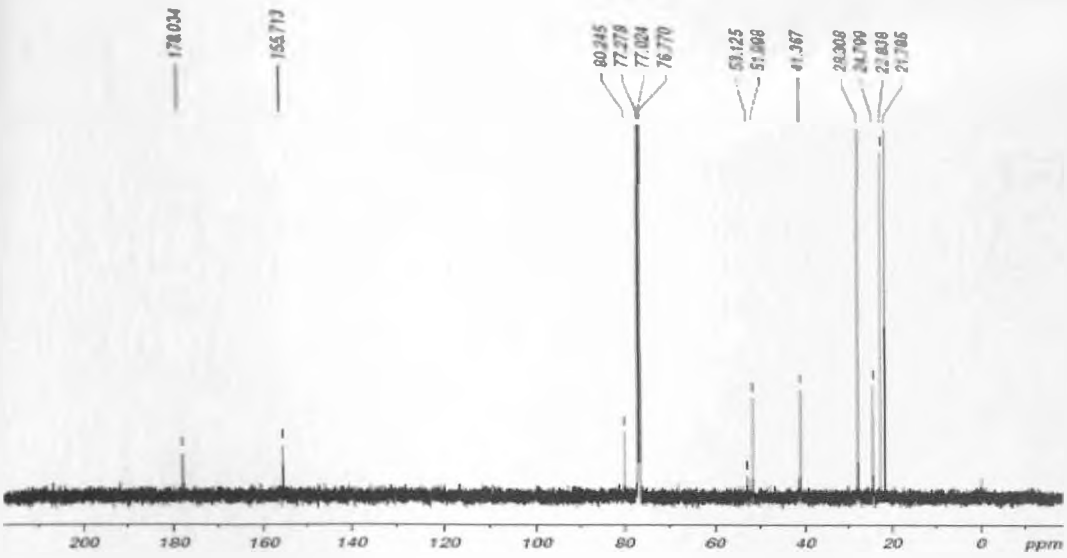



Fig1.3: $^{13}$CNMR spectrum of (5)

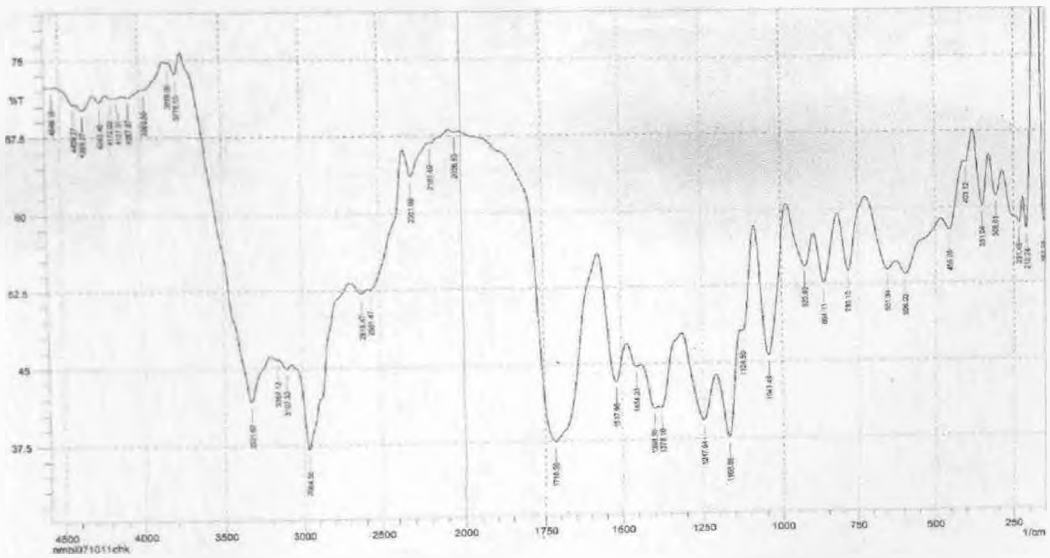



Fig1.4: IR spectrum of (6)

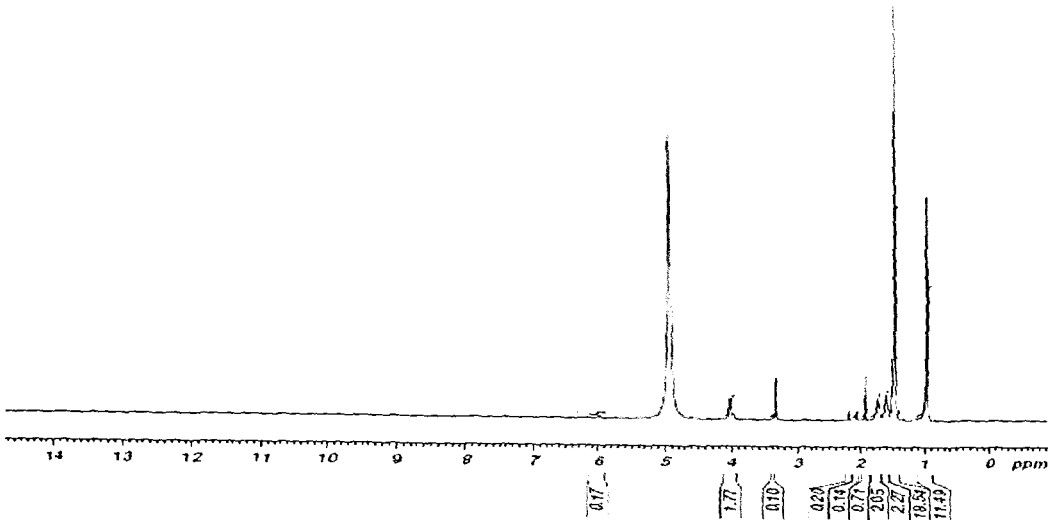



Fig1.5: $^{1}$HNMR spectrum of (6)

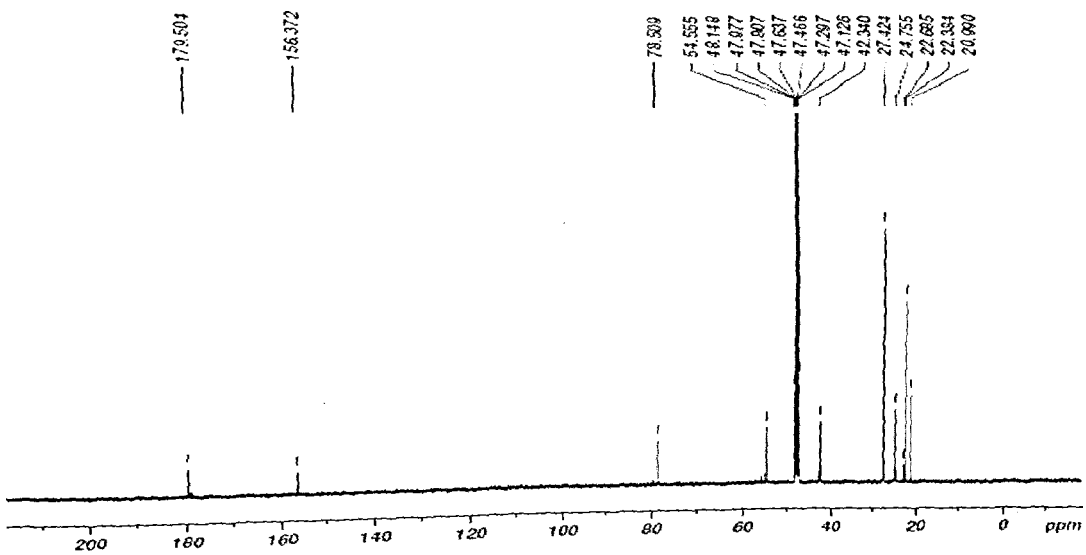



Fig1.6: $^{13}$CNMR spectrum of (6)

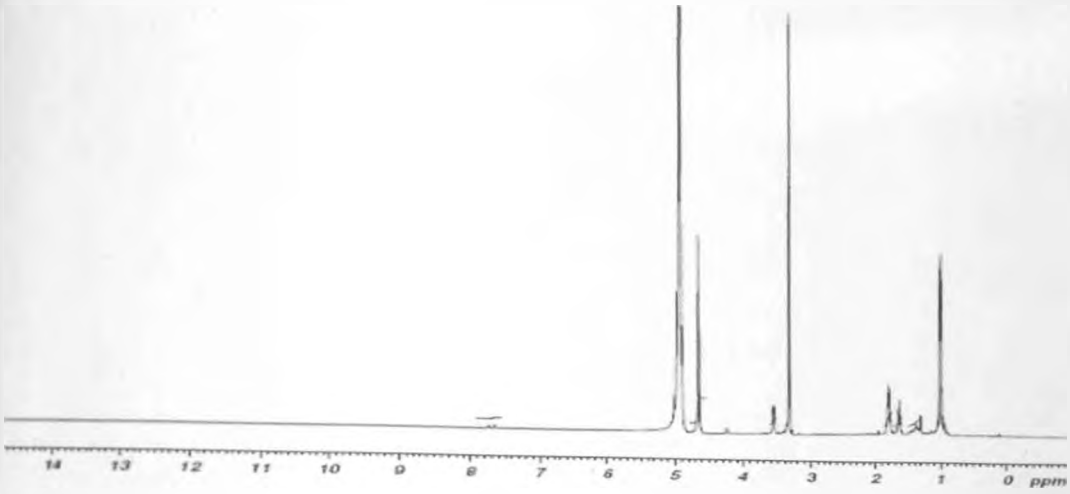



Fig1.7: $^{1}$HNMR spectrum of (4)

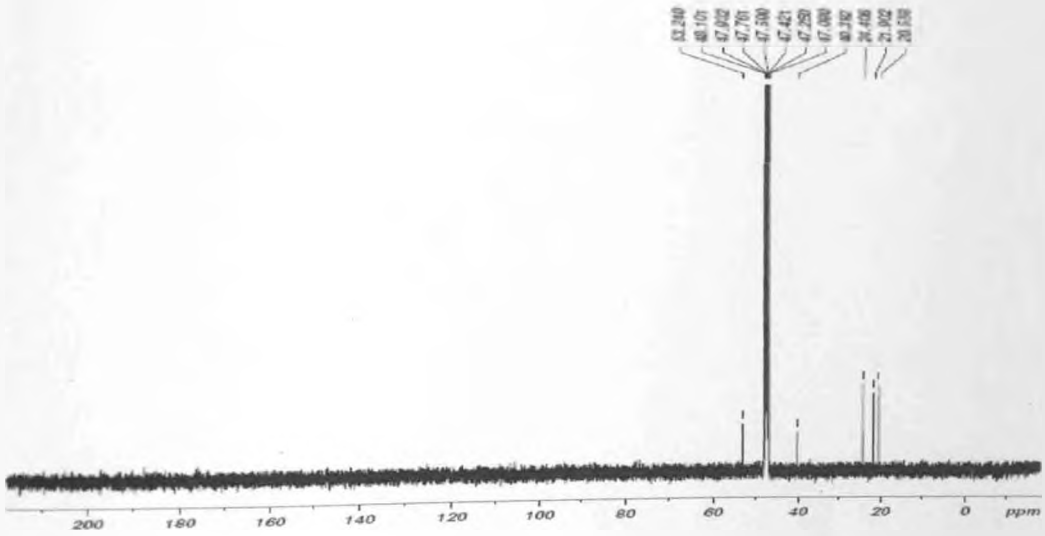



Fig1.8: $^{13}$CNMR spectrum of (4)

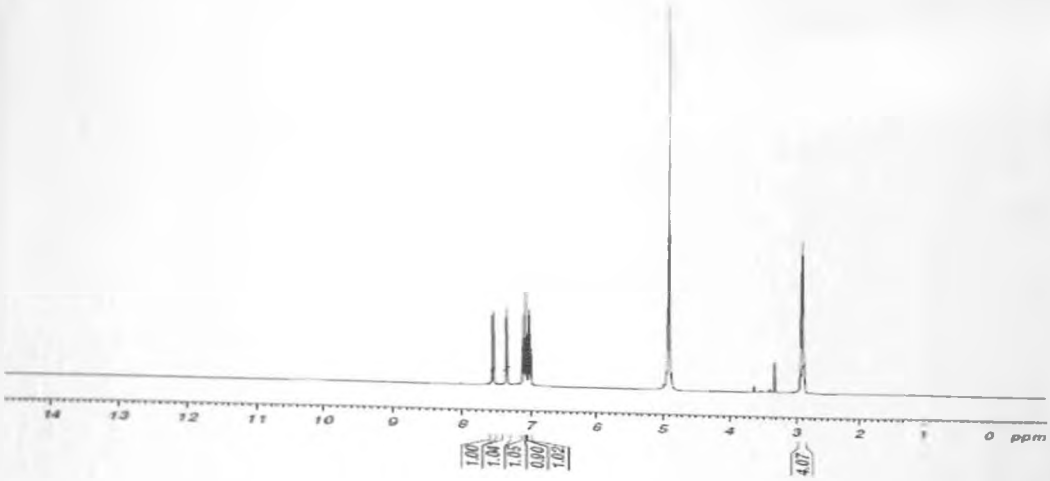



Fig19: $^{1}$HNMR spectrum of bromotryptamine (8)

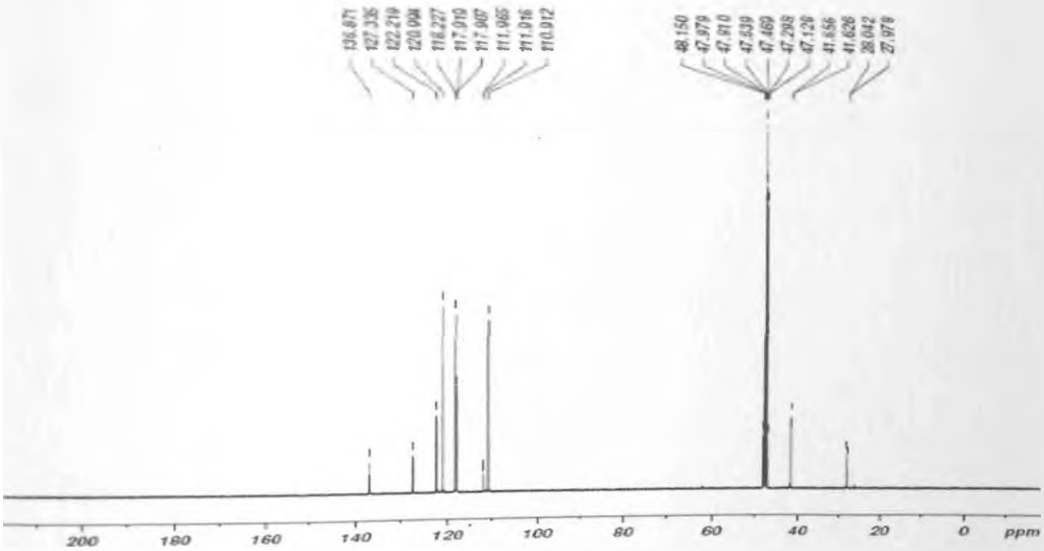



Fig1.10: $^{13}$CNMR spectrum of bromotryptamine (8)

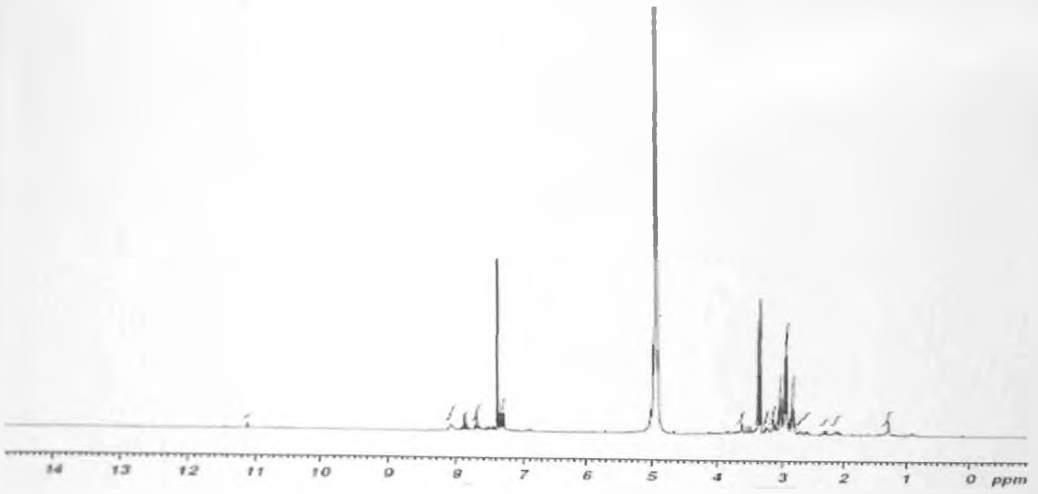



Fig1.11: $^{1}$HNMR spectrum of product (9)

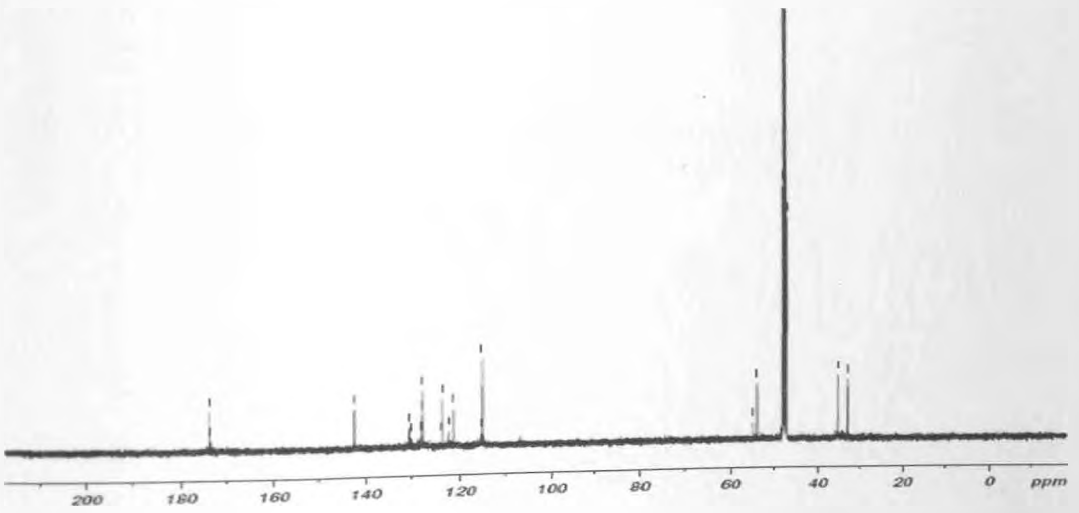



Fig1.12: $^{13}$CNMR spectrum of product (9)

# *Summary*

# *Secondary Metabolites from Selected Marine Invertebrates and their Associated Microorganisms*

## *Summary*

The work presented in this thesis is based on the isolation of metabolites from marine invertebrates and their associated microorganisms. This study focuses on three main aspects: isolation and purification of natural products, biological activity testing and structure elucidation of compounds from marine invertebrates. It is speculated that some of the compounds isolated from the invertebrates are basically not a product of their metabolism, but are from a microorganism symbiotically associated with it.
Additionally an attempt was also made here towards the synthesis of some reported bioactive molecules.
For the isolation of the metabolites, a chemical and biological approach was used. Extracts were screened for antibacterial, antifungal and anticancer activities. As complement to the bioassay-guided approach, TLC, UV-Visible & Infra Red Spectroscopy, column chromatography, HPLC and LC-MS/MS were used to guide the isolation and purification of the compounds responsible for the activity of the extract or compounds that were most interesting. Purified compounds, which were not responsible for the initial bioactivity screening, may have a chance to be evaluated for other more sophisticated pharmacological assays and may also find interest in academics for their unique skeletal structures.

The work presented in the thesis is divided into four chapters.

**Chapter 1:** This chapter deals with the review of literature on bioactive metabolites isolated from marine invertebrates presently in clinical trials, based on their specific biological action. Molecules targeting ion channels, compounds targeting enzymes (*Protein serine/ threonine kinase inhibitors, Protein tyrosine kinase inhibitors, Phospholipase A2 inhibitors),* microtubule-interfering agents, DNA-interactive agents and other marine natural product drug candidates in clinical development have been reviewed.

**Chapter 2:** Describes the identification of secondary metabolite from marine sponges.

It has been subdivided into two sections

**Section 1:** Investigation of chemical constituents and bioactivity of marine sponge *Dysidea granulosa*. A major metabolite 2-(2',4'-dibromophenoxy)-4, 6-dibromophenol **(Fig.1)** was isolated. It was tested against 57 strains of multidrug resistant bacteria and was found to be better than the standard drug Linezolid. This is the first report of 2-(2',4'-dibromophenoxy)-4, 6-dibromophenol from the sponge *Dysidea granulosa* and the first attempt to exhaustively test the *in-vitro* activity of this molecule. Results obtained indicate that the compound shows good lead properties for antibacterial drug development and represent a promising agent for the treatment of *S. aureus* as well as enterococcal infections caused by drug-resistant strains.

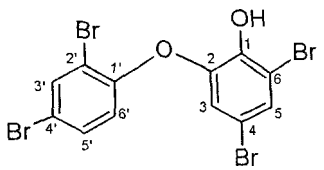


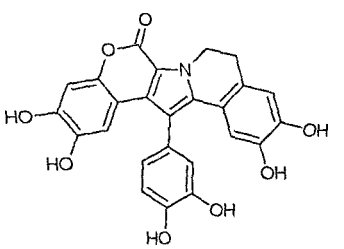

**Fig.1: 2-(2',4'-dibromophenoxy)-4,6-dibromophenol from *Dysidea granulosa***

The compound was also tested against *Mycobacteria*. Mycobacteria have gained a very special place in the bacterial world due to their clinical importance. Alone, *Mycobacterium tuberculosis* is responsible for about two million deaths annually worldwide making tuberculosis one of the most influential diseases in the history of mankind. Almost half of the Nontuberculous *Mycobacteria* species identified are associated with opportunistic infections in animals and humans. The assays were performed on *Mycobacterium fortuitum* and *Mycobacterium abscessus*, 2 non-tuberculous mycobacteria usually more susceptible to drugs than *Mycobacterium tuberculosis*. A concentration of 256 micrograms per mL significantly inhibited growth. MIC *for M. abscessus* and *M. fortuitum* should be close to 300 micrograms per mL as per International Standards.

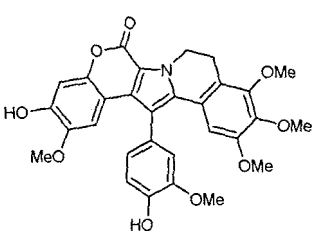
**Section II:** Deals with the chemical investigation of marine sponge *Psammaplysilla purpurea*.

Previous studies of *Psammaplysilla purpurea* has resulted in the isolation of brominated metabolites. The methanolic extract of the sample exhibited significant anti cancer activity and chemical fingerprinting of the same by ESI-MS revealed additional signals for non-brominated compounds which were different from the ones observed and reported earlier. This led to the isolation of 3 compounds **(Fig.2)** from the sample on careful bioassay guided fractionation and purification. The compounds incidentally belonged to the Lamellarin group. Interestingly, the Lamellarin class of alkaloids has been isolated for the first time from the sponge *P.purpurea.*

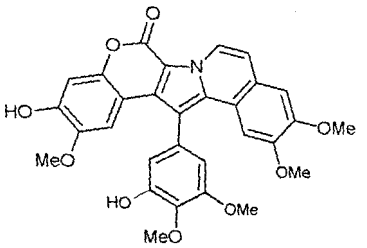
The lamellarins have been previously reported from the prosobranchmollusc *Lamellaria* sp., and the marine ascidian of the *Didemnum sp.* Subsequently they had also been isolated from an Australian sponge *Dendrilla cactos.* Spectroscopic structure elucidation of the compounds determined the Lamellarin 1 to be a dihydro analogue of Lamellarin H & Lamellarin 3 is a new Lamellarin isolated first time from any natural source. The Lamellarins are significant because of their immense biological activity.




Lamellarin(1)8,9-dihydro-2,3,11,12-tetrahydroxy-14(3,4-dihydroxyphenyl) 6H[1] benzopyrano -[4',3';4,5]pyrrolo [2,1-α]isoquinoline-6-one




Lamellarin(2)8,9-dihydro-3hydroxy-2,10,11,12-tetramethoxy-14(4-hydroxy,3-methoxy phenyl) 6H[1]benzopyrano-[4',3';4,5]pyrrolo [2,1-α]isoquinoline-6-one

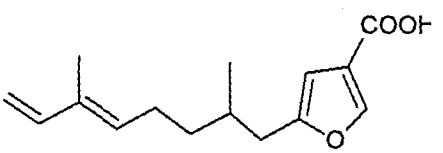



Lamellarin(3)3-hydroxy-2,11,12-trimethoxy-14(5-hydroxy,3,4-dimethoxyphenyl) 6H[1]benzopyrano-[4',3';4,5]pyrrolo [2,1-α]isoquinoline-6-one

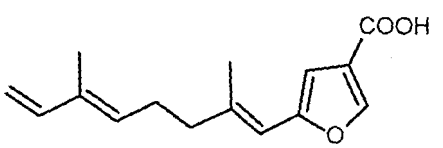
**Fig.2: Lamellarins from *Psammaplysilla purpurea***

**Chapter 3:** Deals with the chemical investigation of soft corals. It has been subdivided into two sections

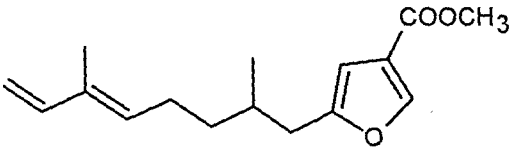
**Section 1:** Investigation of chemical constituents and bioactivity of soft coral *Sinularia kavarattiensis*. Three furanosesquiterpenes **(Fig.3)** have been isolated, two compounds are isolated for the first time from this species, however all the compounds have been previously reported from other species of *Sinularia* and are known to have significant anti-inflammatory activity. Further attempt was made to identify minor furanosesquiterpenes by the method of tandem mass spectroscopy. Ten furanosesquiterpenes, some previously reported and new have been identified here.




(1) (5'*E*)-2-(2',6'-dimethylocta-(5',7'-dienyl)-4-furoic acid



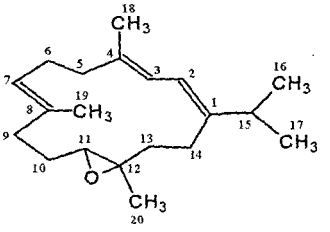

(2) (1'E, 5'E)- 2-(2',6'-dimethylocta-(1',5',7'-trienyl)-4-furoic acid



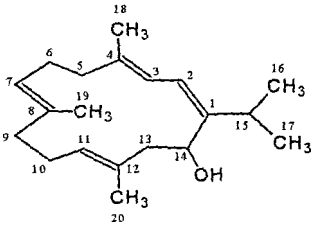

(3) (5'*E*)-2-(2',6'-dimethylocta-(5',7'-dienyl)-4-furoic acid methyl ester

**Fig.3: Furanosesquiterpenes from the soft coral *Sinularia kavarattiensis***

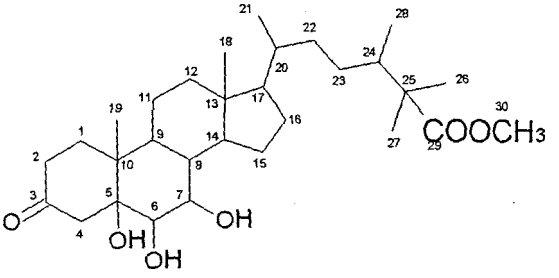
**Section II:** Deals with the chemical investigation of *Lobophytum crassum*. In our search for bioactive substances from marine organisms, *L. crassum* was selected based on its rich chemical diversity and biological activity. Two cembrenoid diterprenes and two sterols **(Fig.4)** were isolated and their structures determined by NMR spectroscopy and by comparison of their previously reported data.



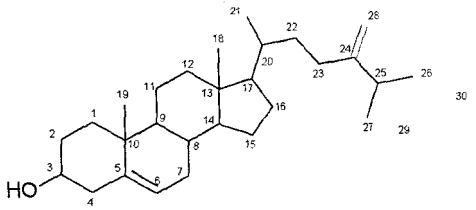

(1) 11, 12-epoxy-1, 3, 7-cembratriene




(2) 1, 3, 7, 11-cembratetraene-14-ol



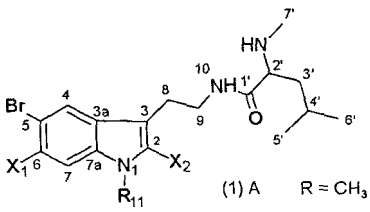

(3)(24-methyl-25(29(30)-methylester)-3one-5,6,7-triol)



(4) Cholest-5,24(28)diene-3β-ol

**Fig.4. Compounds from soft coral *Lobophytum crassum***

**Chapter 4:** Miscellaneous Synthetic studies: In this chapter an attempt was made towards the synthesis of bryozoan metabolites Alternamides **(Fig.5)** from the species *Amathia alternata.* These compounds were selected because of their activity against several human pathogenic bacteria. Alternamides are monopeptides basically made up of tryptamine; a decarboxylated natural amino acid tryptophan and N-methyl derivative of the amino acid leucine. N-methyl leucine was prepared by first protecting one of the N-H with a suitable protecting agent, t-butoxy carbamate and methylating with Methy iodide. The bromo tryptamine was prepared by bromination utilizing Bromine in acetic acid as the brominating agent. Bromotryptamine and N-methyl leucine coupling was achieved with DCC/DMAP reagent.



| | | |
|---|---|---|
| (1) A | R = $CH_3$ | $X_1 = X_2 = Br$ |
| (2) B | R = H | $X_1 = X_2 = Br$ |
| (3) C | R = H | $X_1 = H$ $X_2 = Br$ |
| (4) D | R = H | $X_1 = Br$ $X_2 = H$ |

**Fig.5: Alternamides from *Amathia alternata***