Studies on Some Important Secondary Metabolites from Marine Organisms

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For the degree of

Doctor of Philosophy

(In Chemistry)

By

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CERTIFICATE

Certified that the work incorporated in the thesis entitled "**Studies on Some Important Secondary Metabolites from Marine Organisms**" submitted by **Mr. Rajesh Ramnath Parvatkar**, was carried out by the candidate under our supervision and the same has not been submitted elsewhere for the award of a degree.

All the corrections suggested by external examiners are incorporated in the thesis

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June 2011

Declaration

As required under the University ordinance 0.19.8 (vi), I hereby declared that the work embodied in the thesis entitled "**Studies on Some Important Secondary Metabolites from Marine Organisms**" is the result of investigation carried out by me under the guidance of Dr. Chandrakant G. Naik (NIO) and Prof. Shrivallabh P. Kamat (Goa University) and it has not previously formed basis for any other titles. In keeping with the general practice of reporting scientific observations, due acknowledgement has been made wherever the work described is based on the findings of other investigators. Due acknowledgements have been made wherever facilities and suggestions have been availed of.

Rajesh R. Parvatkar

(Research Student)

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Rajesh

Dedicated To My Late Father

CONTENTS

GENERAL REMARKS

1) The compound numbers, figure numbers, scheme numbers and reference numbers given in each chapter refer to that particular chapter only.

2) All melting points and boiling points were recorded using electronic Melting point machine and are uncorrected.

3) Commercial reagents were used without further purification.

4) All solvents were distilled prior to use and then dried using standard procedures.

5) Petroleum ether refers to the hydrocarbon fraction collected in the boiling range 60 – 80° C.

6) All reagents were prepared using literature methods.

7) For normal and flash silica gel chromatography silica gel (60 – 120 mesh size) and (230-400 mesh size) were used.

8) SephadexLH-20 (Pharmacia) was used gel permeation column chromatography.

9) Thin layer chromatography (TLC) was carried out on Precoated kiesegel 60 F_{254} (Merck)

10) The UV and IR spectra were recorded on Shimadzu UV-2401 PC and Shimadzu FTIR-8201 PC spectrometers.

11) UPLC was performed on Waters ACQUITY machine.

12) NMR (^{1}H, ^{13}C, DEPT, HSQC and HMBC) data were obtained on Bruker Avance 300 and Bruker Avance 500 spectrometer with TMS as internal standard.

13) EI-MS and HRESITOFMS were recorded on Shimadzu 2010 and QSTARXL MS/MS, Applied Biosystems, Switzerland.

14) Optical rotations were measured on optical polarimeter ADP220 (Bellingham + Stanley Ltd)

General Abbreviations and Symbols

anhyd	Anhydrous
aq	Aqueous
atm	Atmospheric
cat	Catalytic
°C	Degree Celcius
Conc	Concentrated
dil	Dilute
DW	Distilled water
E	Eentegegen (opposite)
et al	Et alia (and others)
Fig	Figure
g	Gram
h	Hour
lit	Literature
m	Meta
mg	Milligram
min	Minute
ml	Milliliter
mmol	Millimole
MNP(s)	Marine natural product(s)
Mp / mp	Melting point
MW	Molecular weight
NP(s)	Natural product(s)
o	Ortho
p	Para
PDA	Potato dextrose agar
PDB	Potato dextrose broth
psi	Pounds per square inch
R	Rectus
RT / r.t.	Room temperature
S	Sinister
Sat	Saturated

sec	Second
Temp	Temperature
TLC	Thin layer chromatography
SW	Sea-water
Z	Zussamen (together)

Spectroscopic abbreviations and Symbols

br s	Broad singlet
$CDCl_3$	Deuterated chloroform
CID	Collision induced dissociation
cm^{-1}	unit of wavenumber
COSY	Correlation spectroscopy
d	Doublet
δ	Delta (Chemical shift in ppm)
DEPT	Distortionless Enhancement by Polarization Transfer
DMSO-d6	Deuterated dimethyl sulfoxide
HMQC	Heteronuclear multiple quantum coherence
HMBC	Heteronuclear multiple bond correlation
Hz	Hertz
IR	Infrared
J	Coupling constant
m	Multiplet
MHz	MegaHertz
MS	Mass spectrometry
NMR	Nuclear magnetic resonance
NOESY	Nuclear overhauser effect spectroscopy
υ_{max}	Frequency maximum
ppm	Parts per million
q	Quartet
s	Singlet
t	Triplet
UV	Ultra violet
XRD	X-Ray diffractometry

LIST OF PUBLICATIONS

1. **Parvatkar, R.R.,** DeSouza, C., Tripathi, A., Naik, C.G.; Aspernolide A and B, butenolides from a marine derived fungus *Aspergillus terreus*. *Phytochemistry*: 70(1); 2009; 128-132 **(IF 3.15)**

2. **Parvatkar, R. R.,** Mendon, M., Almeida A., Devi, P., Naik. C. G., Anti-inflammatory agents from mangrove-derived fungus *Aspergillus flavipes* (ready for submission)

3. Verma, A. K., Raghukumar C., **Parvatkar R.** and Naik, C. G. (2012) A Rapid Two-Step Bioremediation of the Anthraquinone dye, Reactive Blue 4 by a Marine-derived Fungus. *Water, Air, and Soil Pollution* **(IF 1.765)** (article in press, DOI 10.1007/s11270-012-1127-3).

4. Thakur, N. L., Salvi, J., Naik, D., **Parvatkar, R. R.,** Meena, R. M., Naik, C. G., Beta-lactamase mediated antibiotic resistance in marine sponge-associated bacteria: A selected metabolomic study of ampicillin (Submitted to "*Journal of Chemical Ecology*" **IF 2.486**)

CONFERENCE/SYMPOSIUM PUBLICATIONS

1. **Rajesh R Parvatkar**, Chandrakant G. Naik. Comparison of Closely Related Fungi Aspergillus flavipes and Aspergillus terreus Using Electrospray Ionization Tandem Mass Spectrometry (ESI-MS/MS). *7th Junior National Organic Symposium Trust (JNOST) Conference for Research Scholars* at IISER Mohali (December 15-18, **2011**). (Poster Presentation)

2. Celina D'Souza**; Rajesh R. Parvatkar**; Prabhadevi; Naik, C.G. Mangrove associated fungi and bioactive metabolites from *A. flavpies*. National conference on *Marine Biology to Marine Biotechnology"* 18-20 January **2008**, Ruparel College, Mumbai. (Oral Presentation)

3. **Parvatkar R. R.**, D'Souza C., Prabha D. and Naik C. G. Butenolides and cyclopentane diols from marine derived fungus *Aspergillus flavipes*:

Structure elucidation using advanced spectroscopic techniques. *RSC West India Section Ph.D. Students Symposium* **2007**, Goa (Oral Presentation).

4. Celina Rodrigues, Tonima Kamat, **Rajesh Parvatkar**, Ashootosh Tripathi, Naik, C.G. Antibacterial Potential of Marine Associated Fungi. National Seminar on *Prospecting fungal diversity in Tropical India, it's conservation and applications in biotechnology,* April **2006**, Agharkar Research Institute. Pune, India

Introduction

Introduction

I. Natural products

Natural products (NPs) in simplest term are the chemical compounds, produced by living organisms. The cells of living organisms can be considered as micro chemical reactors producing large number of chemical compounds through metabolic reactions. These compounds can be categorized into primary and secondary metabolites. Primary metabolites such as proteins, carbohydrates, etc. are required as nutrients and source of energy for the organisms. The secondary metabolites, which may be categorized into different chemical classes depending on their biosynthetic origin such as terpenes, polyketides, alkaloids, etc. do not play a direct role in different functions within the organism, but have prominent role to play in an ecosystem.

NPs unlike synthetic chemicals have low toxicity, complete biodegradability, availability from renewable sources and some cases low cost. It is because of these reasons that health care products and environmentally acceptable agricultural compounds are mainly natural products or are derived by modification of natural product leads. Moreover, nature provides compounds having unique and diverse chemical structures with potential biological properties, which are beyond the reach of human imagination. For the past many centuries man has been using these NPs or the extracts of the organisms (especially plants) containing these NPs for human benefit. Today agriculture along with health care industry has been the prime area of application of NPs, but it was mainly the treatment of diseases with the aid of NPs or more specifically extract containing these NPs was the main use of NPs in the ancient time.

II. Evolution of natural product science

Historically, the use of NPs as medicinal agents presumably predates the earliest recorded history as the earliest humans used various, but specific plants (terrestrial) to treat an illness. In India, Rishis or Vaidyas (doctors), who treated the diseases using

herbs, passed the knowledge orally to their disciples. It was only between 1,400 and 1,000 BC that ayurveda got a written reference material. Ebers papyrus, written in about 1550 B.C., includes many of the plants used in Egyptian medicine. In the late 1700s, studies like William Withering's *An Account of the Foxglove, and its Medicinal Uses* (1785) began to appear.

The effectiveness of natural products in mitigating illnesses has inspired pharmaceutical scientists to search for new directions in drug discovery. In 1928, the accidental, but the most important discovery of antibiotic penicillin from *Penicillium notatum* by A. Fleming revolutionized medicinal sciences. This discovery diverted the attention of the researchers from plants, towards microorganisms as new source of drugs, and many new antibiotics resulted from such studies in mid 1900s. The transition from fortuitous discovery to systematic screening through validation in experimental models has taken place since the 1930s. High-throughput screening (HTS) and high-throughput chemistry technologies developed in the last two or three decades have significantly improved the speed, scale and quality of this process. Many important natural products, like anticancer agent, taxol from Pacific yew tree, *Taxus brevifolia* in 1967 (Goodman, 2001), a cholesterol lowering agent, lovastatin from *A. terreus* in 1970 were discovered (Alberts et al 1990) from terrestrial sources.

With ineffectiveness of some drugs in severe diseases, development of bacterial resistance to the antibiotics in use, emergence of deadly infections and diseases (Swine flu, SARS, Cancer, AIDS, etc) and limitations of the terrestrial resources, a need was felt for search of new resources and potent drugs. The oceans which were untouched until 1960 were recognized to be an alternative and undoubtedly the final frontier to be explored for the much needed source of NPs that could lead to the potent drugs.

III. Marine Biosphere (Oceans): The final frontier of bioactive natural products.

Over the past three decades or so, marine organisms have been recognized as an untapped resource for novel bioactive compounds. The oceans cover more than 70% of the earth's surface, and contain more than 300,000 described species of plants and

animals. Yet until relatively recently, chemists were confirmed landlubbers and until about 1960, no one even thought of the ocean. The ocean environment is massively complex, consisting of extreme variations in pressure, salinity, temperature, and biological habitats. The marine environment ranges from nutrient-rich regions to nutritionally sparse locations, where only a few organisms can survive. The surface and internal spaces of the sessile marine organisms provide a unique micro-habitat, and several symbiotic microorganisms are reported to occupy sometimes up to 40% of their tissue volume. These microorganisms are proven to be highly specific in their relationship with filter-feeding organisms like sponges, soft corals, ascidians and marine plants. The unique metabolic and physiological functions resulting due to various stresses in marine environment offer a potential for the production of novel enzymes and bioactive metabolites having novel chemistry by these organisms for potential exploitation.

The fact that the marine organisms have overcome, and adapted to all sorts of environments, and these creatures are constantly under tremendous selection pressure, including nutrition, space competition, predation, surface fouling and reproduction, is the opportunity for the researchers, to search for the potential drugs from the sea.

IV. Marine natural product chemistry

i) Opportunities and challenges

Natural product chemistry represents enormous opportunity for the drug development, and therefore drug discovery has been the prime aim for the scientist to have diverted their attention towards marine natural products (Chin et al., 2006). Before a drug comes into market, its discovery goes through various phases of scientific studies which require several years. The scientists from various disciplines such as biology, chemistry, pharmacy, medicine and interdisciplinary sciences like biotechnology, biophysics, biochemistry and medicinal chemistry are engaged in the process. The process of drug discovery goes through mainly following phases; drug target identification, target validation, lead compounds identification, and its

optimization and lastly preclinical and clinical evaluation. It is the lead identification and optimization stages, where chemists with mainly organic chemistry knwoledge, are involved in the job. The process involves extraction of the compounds from an organism, their purification using various chemical and chromatographic techniques, followed by structural elucidation using chemical methods and spectroscopic analysis like IR, NMR, MS. etc. Furthemore efficient synthesis and structral modification of the NPs are needed to facilalitate the further processes.

As the process of drug discovery being a multidciplinary, technical deficiencies of one discipline has been the limitations to the development of the other, which has hampered the discovery of drugs from the ocean. Moreover, men fear the ocean as it is considered very hostile and inhospitable place to deal with. Intial obstacles of collection of organisms living beneath and on sediments below the ocean were overcome by development of SCUBA diving. The difficulties of retrieving a sustained, reliable harvest of marine organisms; leading to insufficient quantities of extracts and NPs was the main concern for natural product chemists. As the availability and reproducibility of sample was limited, natural product chemists did not have the luxury of working with large amount of the extracts, for the purification of NPs from the complex mixture, to allow the completion of chemical structure analysis with the available underdeveloped technologies.

Organic chemistry itself is evolved from the study of NPs (terrestrial origin). Earlier large quantities of plant material were used to prepare the extract, and then further purification using solvent partition and chromatographic techniques. The structural elucidation would follow various degradative chemical reactions for determining elemental composition, molecular weight, carbon framework, functional groups and stereochemistry. For example elemental composition would be determined by estimation of CO_2, H_2O and N_2 produced by pyrolysis of the sample and then the molecular formula hence, molecular weight would be determined. To test the presence of a functional group, specific chemical reactions would be carried out. For example, carbonyl functionality would be tested by reaction with 2,4-dinitrophenylhydrazine or hydroxyl amine. A carbon-carbon double bond would be tested by $KMnO_4$ or Br_2-Acetic acid. Sterochemistry would be determined by

degradation or conversion of the compound to a chiral compound, whose chirality was known. These analysis would take decades to characterize the structure after its purification. The synthesis would follow this cumbersome process for the confirmation of the structure. Neverthless, these processes has led to the discovery of so many important reactions in organic chemistry. Glucose, the most important chemical as source of energy for living being and cholesterol almost found in all the organisms were assigned the structure after anylyses that took decades together.

It would have been virtually impossible for the chemist to characterize the NPs from the seas, if the same methodologies had to be followed over last three decades. Fortunately, continuous parallel development in the purification methologies, such as flash chromatography, GC, HPLC, and very recently UPLC, SEP box and the spectroscopic methods such as UV-VIS, IR, MS and NMR, chemists were able to characterize the chemical structures with fewer evidences from classical chemical methods. Especially the discovery of the NMR in 1950 is conisdered to be the most wonderful discovery for the natural product chemistry. The advent and commercial availability of instrument with broadband excitation and fourier transform methods has been the boost for sensitivity and speed in the analyses. Today, a spectrum can be recorded on sample quantities down to submilligram level. These advances in the instrumentaion techniques coupled with discoveries of two dimentional NMR correaltion methods such as COSY, HMBC, NOESY etc, have enabled chemist to assign complete structure even wthout the use of XRD. A relatively small number of marine plants, animals and microbes have already yielded more than 22,000 novel chemicals (Blunt et al, 2011), but majority of marine chemical biodiversity still remains to be fully realized.

ii) Current status

The technological advances over the past half century have seen the identification of several NPs from marine sources as well the minor constituents of terrestrial sources, which remained uninvestigated. As per the MarineLit database more than 22000 new compounds have been reported from the marine sources. These compounds have been

derived from 3,700 species from 2,255 genera belonging to 40 phyla (Blunt et al, 2007). The number of metabolites reported annually is increasing every year. In the 1960s decade, which marked the beginning of the marine natural product chemistry the number of new MNPs discovered were just over hundred (**fig 1**). The next decade (1970s) saw sharp rise in discovery of new MNPs and over 350 compounds were reported in this decade. A significant increase in the discovery of new MNPs was also observed in the subsequent decade. The marine natural product chemistry blossomed in 1990s and the last two decades have seen the discovery of nearly 14000 new MNPs accounting for 60% of total new MNPs discovered. More than 1000 new MNPs have been discovered in each 2008 and 2009 calendar years.

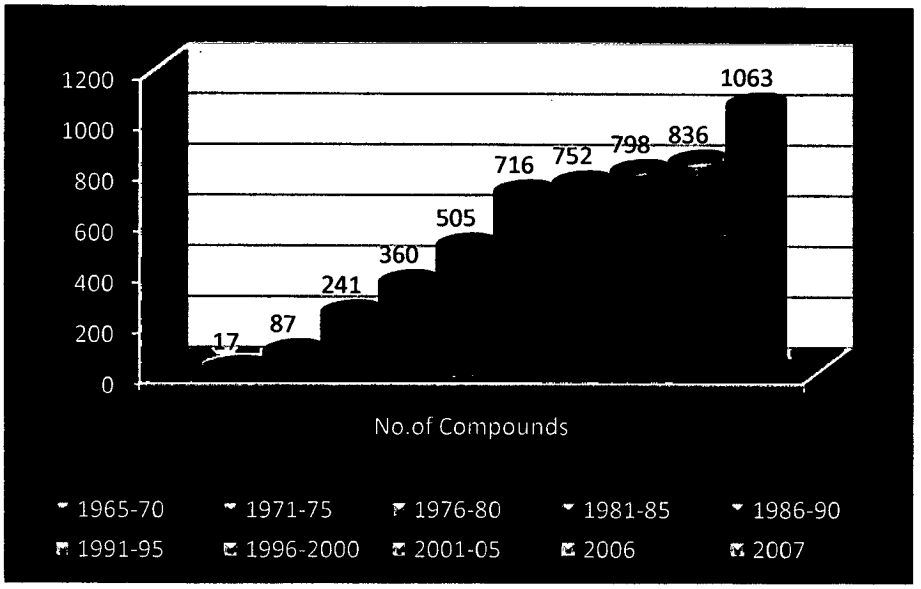

Fig 1: Annual output of marine natural products, 1965–2007. The average output/year for the period 1965 to 2005, in five-year periods, has been plotted with that for 2006 and 2007

The output by geographic region of collection of sources examined shows that seas around Japan, western Pacific region, Caribbean Sea and China Sea have been the major contributors to new MNPs for 1965-2007 as shown in the **fig 2.** Not far behind them are Mediterranean Sea, Indian Ocean and seas around Australia. However, the comparison of this data with average annual distribution for 2001–2007 generates a snapshot of the differential changes in output by region that has taken place over the years. In the recent years China Sea has emerged as highly significant source of new

MNPs. The discovery of new compounds from China Sea from 2001-2007 has risen to around 230 per year from 50 compounds per year for 1965-2007, which is far ahead from other geographical regions. Nearly 75 new compounds on an average have been discovered from the seas around India and ranks 4th behind China Sea, western pacific (100 compounds per year) and Caribbean Sea (80 compound per year) for the period 2001-2007 (Blunt et al., 2009).

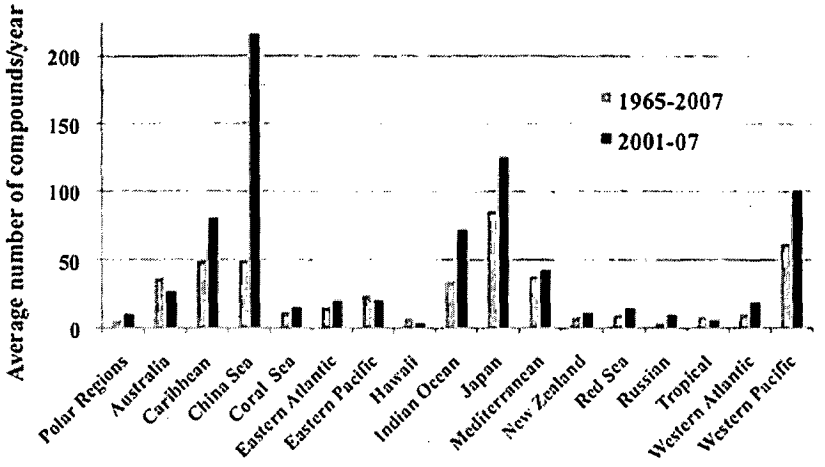

Fig. 2 Trends in geographic distribution of collection sources. For clarity the Polar Regions include Antarctic and Arctic; North Sea and Baltic Sea records are included with the Eastern Atlantic; Caspian Sea and Black Sea records have been added to the Russian region.

Of the total new MNPs discovered 5% of them belong to bacteria (prokaryotes), while 95% are eukaryota in origin (Blunt et al., 2008). It is noteworthy that just three Phyla, Porifera, Cnidaria and Chromophyta, constitute 60% of the species examined, and that if a further five Phyla are added (Mollusca, Rhodophyta, Chordata, Cyanobacteria, Echinodermata), nearly 94% of all of the species recorded are accounted for (**fig 3**). The remaining 6% are scattered across another 14 Phyla. As in the recent years the marine natural product science has been bioactivity driven rather than chemistry, the sessile benthic organisms, mainly invertebrates such as sponges, ascidians, bryozoans etc attracted maximum attention.

Marine Natural Products Chemistry is essentially a child of the 1970's that developed rapidly during the 1980's and matured in the two last decades. Several compound

have entered into the clinical trials and few of them have entered the market as well (Newman and Cragg, 2004, Jimenez et al., 2009; Faulkner, 2000).

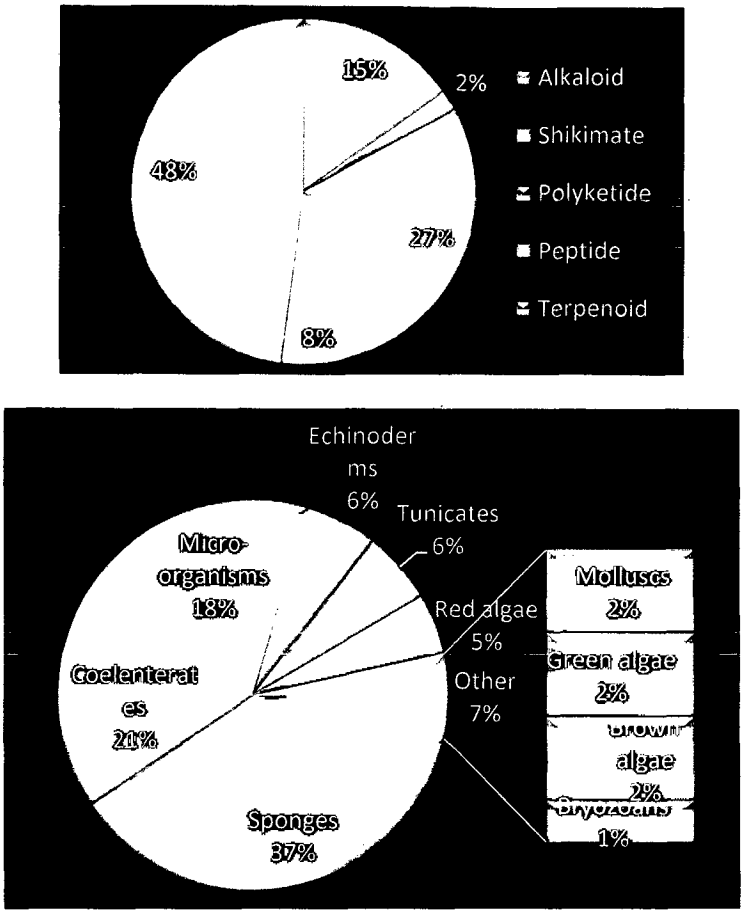

Fig 3: New marine natural products reported in 2002 divided according to phyla (1) and biosynthetic origin (2)

iii) Future of MNP science

It was a big question whether marine natural products will play a major role in drug discovery in the future, when marine natural product research was in the beginning or even perhaps before the two decades (Grabbley et al., 2000). The steady arithmetic rate of discovery of new natural products is increasing since 1965, but recent few years have not seen any increase in the discovery of natural products. It might be that marine natural product discovery research have attained its peak, as last three years have seen nearly or over 1000 metabolites being discovered per year. It appears that trend will continue over next several years at least due to shift towards unexplored

microorganisms. There is any doubt left that discovery of natural products from macro-organisms is going to slow down as many of them have been explored but their associated fauna and flora represents huge biodiversity for exploration. Microbial diversity constitutes an infinite pool of novel chemistry, making up a valuable source for innovative biotechnology. So far we have only scratched the surface of it. The most recent estimates suggest that by now we only know approx. 5% of the total species of fungi and may be as little as 0.1 % of the bacteria. Among the ones already described, only a small fraction has been examined for metabolite profile (Lange, 1996; Debbab et al., 2010). If the previous era saw change from terrestrial natural products to marine natural products, the coming era will turn out to be a change from marine macroorganisms to marine micro-organisms natural products.

V. Marine Natural product chemistry at National institute of Oceanography (NIO), Goa

A group of scientist at Bio-organic Chemistry Laboratory, NIO is engaged on a major project "Drugs from the sea" and several other related projects funded by various Indian government organization and a project "Marex" funded by European commission involving European institutes besides NIO. The group also has collaborations with CNR Institute of Biomolecular Chemistry, Italy; Piramal Life Sciences Limited, Mumbai and these programs uses different strategies for discovery and development of MNPs.

The MNP research at NIO has resulted in isolation of several novel and known bioactive molecules from marine resources obtained from seas around India. The novel compounds discovered by the research group at NIO include antitumor alkaloid jorumycin from *Joruna funebris* (Fontana et al., 2000), caulerpenyne from alga *Caulerpa* sp. (Fontana et al., 1999), kahalalide R and S from green alga *Bryopsis* sp (Tilvi et al., 2007), γ-pyrone propionates from molluscs *Placobranchus ocellatus* (Manzo et al., 2005), peroxycarnoic acid C and D (Fontana et al., 1999), bromotyrosine alkaloids from sponge *Psammaplysilla purpurea* (Tilvi et al., 2004),

ceramide from sponge *Haliclona* sp. (Tilvi et al., 2005), armatols A-F and glycerolipids from red alga *Chondria armata* (Al-Fadhli et al., 2006; Ciavatta et al., 2001); pregnenes from octocoral *Carijoa sp* (Ciavatta et al., 2004) and several other known compounds from these macroorganisms.

The microbial bioactive products those have been discovered include mainly known compounds cyclosporine and terrein from fungus *A. terreus*; kojic acid and cyclopiazinic acid from fungus *A. sulphereus*; auroglocinnsa and anthraquinone derivatives from fungus *Eurotium* sp.; 4,4'-oxybis[3-phenylpropionic acid] from bacterium *Bacillus licheniformis.* A novel antibiotic, cyclic peptide PM181104 from a bacterium *Kocuria* sp. associated with a marine sponge having potent antibacterial activity has been patented (Patent application number: 20090076094).

A strong research interest in the field of organic chemistry made me to join the bioorganic chemistry group to pursue research career in the field of marine natural product chemistry. During the course of my research work, I was involved in isolation, structure elucidation and bioactive properties of the metabolites from marine-derived fungi of genus *Aspergillus*; marine invertebrates, a sponge and a soft coral; and synthesis of amathamide alkaloids, the metabolites of a bryozoan. The investigation carried out is the subject of present thesis. The thesis is divided into four chapters.

Chapter 1 contains a review of literature on the metabolites of marine fungi with special reference to metabolites isolated in year 2010

'The content of **Chapter 2** forms chemical investigation of three marine derived fungi. It has been sub-divided into five sections

In sections 2.1, 2.2 and 2.3 isolation, structure elucidation and bioactivity of metabolites of the fungi *A. flavipes*, *A. terreus*, and *A. aculeatus* respectively has been described.

Section 2.4 deals with comparison of metabolic profile of the two fungi *A. flavipes* and *A. terreus* using electrospray ionization tandem mass spectrometry (ESI-MS/MS).

Section 2.5 describes the effect of seawater concentration on production of an important metabolite, butyrolactone I from *A. terreus.*

Chapter 3 presents the investigation carried on chemical constituents of two marine invertebrates and it is divide into two sections.

Section 3.1 describes the isolation and characterization of metabolites from antibacterial extract of rarely investigated a marine sponge *Oceanapia* sp.

Section 3.2 describes the metabolites isolated from a soft coral *Lobophytum crassum*

In **Chapter 4**, the studies carried out toward the synthesis of amathamides and their biological activity is reported.

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Chapter 1

MARINE-DERIVED FUNGI: A SOURCE OF BIOLOGICALLY POTENT AND NOVEL CHEMICALS-A REVIEW

I. Introduction

i) General

Terrestrial microbes are well recognized as a source of novel metabolites, many of which possess valuable biological properties. Indeed, many of the pharmaceutical, agrochemical and crop protection industry are based on the exploitation of microbial natural products. Over the last five decade the search for new drugs has expanded, to those produced by marine macro-organisms and over a last two decade or so it has extended towards microbes obtained from marine sources (Bugni and Ireland, 2010). Fungi isolated from substrates such as marine plants (algae, sea grasses), invertebrates (ascidians, sponges, tunicates, etc), vertebrates (fish), sands, sediments and seawater itself, have been found to yield novel bioactive natural products. This review covers the literature on novel bioactive metabolites of marine-derived fungi published in the first half of 2010. The review also presents the trend so far observed in isolation of novel molecules from different types of species of fungi, source of fungi, their biosynthetic classification and the biological activities. It also gives brief overview of progress of the marine fungal chemistry over the years.

ii) Marine habitats and associated fungi as bioactive resource

The world's oceans represent a resource of huge dimension for natural product chemists; however, the chemistry of marine fungi until last two decades was relatively neglected area of marine natural products chemistry. Published reviews shows the importance of these organisms as potential sources of pharmaceutical leads (Ebel, 2006; Ebel, 2010; Rateb and Ebel, 2011). More specifically, fungi from the marine environment are fast growing as source of potential bioactive resource as suggested by the diversity of secondary metabolites isolated so far.

Though sourced from marine substrates, the marine character of these microbes may not be absolute. Therefore most fungi are referred as "marine-derived" since the fungi obtained from marine ecosystem can be obligate and facultative fungal species. Kohlmeyer (1979) defined obligate marine fungi as those "that grow and sporulate exclusively in a marine or estuarine (brackish water) habitat; facultative

marine are fungi from freshwater or terrestrial areas also able to grow in the natural marine environment." Apparently, the majority of fungi inhabiting the world have not yet been described, which implicates fungi to represent an enormous source for natural products with diverse chemical structures and biological activities. Of special interest are creative fungal strains. Creativity in this sense is defined as the ability to produce compounds of interest for human activities (Dreyfuss & Chapela, 1994). Even if the natural function of secondary metabolites often is unknown, it is assumed that they play an important role in chemical defence and communication (Gloer 1995). This suggests that the biosynthesis of secondary metabolites does not occur randomly but it is correlated with ecological factors. Many of them have been suggested to act as pheromones, antifeedants or repellents, and as regulators in the development of organism (Sterner, 1995). Whether a marine derived microbe is obligate or facultative, or its status remains unknown, these organisms are quite capable of contributing to bio-prospecting and the discovery of new generation drugs.

iii) *Bioactive metabolite symbioses: fungi as an alternative for the supply of natural products.*

Marine invertebrates have been and continue to be a prolific source of novel and structurally diverse natural products. Often these compounds display potent and selective bioactivities that trigger biomedical interest. Unfortunately, the supply of the bioactive natural product is usually insufficient to meet the demands of pre-clinical and clinical development. A large-scale collection of the source marine invertebrate can be difficult due to scarcity of the organism, and can also have negative environmental consequences. In addition, natural supplies can fluctuate, either seasonally or due to environmental changes. Ideally, an efficient chemical synthesis of the desired natural product could be achieved; however the structural complexity of many natural products such as bryostatin 1 and swinholide A requires inefficient multistep syntheses that cannot meet the demands of pre-clinical and clinical development. Identification of a simpler, more easily synthesized structure which retains the biological activity is another option, but

the best scenario would be to have a supply of the natural product that can be generated inexpensively and reproducibly in the lab under controlled conditions.

In many cases, marine chemical ecological studies have revealed that the molecule is biosynthesized by associated microbial symbionts. Symbiotic fungi that can be cultivated in the laboratory and still produce the bioactive metabolite could be subjected to fermentation technology to produce larger amounts of the compound. Such a process eliminates large scale collection of the source marine macro organisms.

iv) Biology and ecology of marine fungi

Based on the distinction between obligate and facultative marine fungi, it can clearly be stated that among the former there are taxa which are exclusively found in the marine environment, while by definition, this is not the case for the latter. A recent monographic treatment of filamentous marine fungi listed at total of 530 species in 321 genera, among them 424 species within Ascomycota (in 251 genera), 94 species of anamorphic fungi (in 61 genera) and only 12 species within Basidiomycota (in 9 genera) (Jones et al., 2008). Marine fungal strains have been obtained from virtually every possible marine habitat, including inorganic matter (soil, sediments, sandy habitats, artificial substrates, and the water column), marine microbial communities, marine plants (algae, sea grasses, driftwood and other higher plants, especially mangrove plants), marine invertebrates (most notably sponges, but also corals, ascidians, holothurians, bivalves, crustaceans) and vertebrates (mainly fishes). However, it is worth mentioning that the fraction of culturable isolates is very low, i.e. in the range of 4% or less, with regard to the overall estimated biodiversity, similar to the situation with bacteria. As described above, marine plants, especially from mangrove habitats, harbour an enormous diversity of fungal associates. Several individual marine habitats are tremendous sources of fungal diversity and they have been so far underestimated. There is a pronounced difference regarding the biological diversity accessible by cultivation-based methods, and the diversity evident from molecular biological studies which rely on gene sequences present in environmental samples. In terms of global fungal diversity, a commonly used estimate is that while 74,000 fungal species

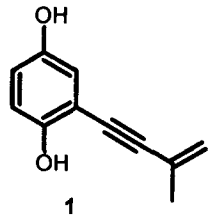
have been described, the overall expected diversity amounts to 1.5 million species.

v) Historical perspective

Historically, fungi have influenced many aspects of human life and development. It was the antibiotic production by fungi that have provided the stimulus for an entire era of drug discovery research. The earliest report indicating the antibacterial potential of fungi occurred in 1876 when Tyndall described the antagonistic effect of a *Penicillium* sp. on bacteria (Florey et al., 1949). In 1896, Gosio reported that he had isolated small amounts of a crystalline substance that had antibacterial properties. The compound was later confirmed to be mycophenolic acid. Although, there were numerous reports describing antibiotic properties of fungi in the late 1800s and early 1900s, the landmark discovery came in 1929 when Sir Alexander Fleming described the effects of *Penicillium notatum* and its metabolite, penicillin on bacteria. Unfortunately, the importance of his work was not fully realized until the early 1940s when a group at Oxford began investigating penicillin for use as an antibiotic in humans. After the potential of penicillin was elaborated, Giuseppe Brotzu 1945, began investigating seawater samples near a sewage outlet in Sardinia for antibiotic producing microbes (Abraham and Loder, 1972). Brotzu hypothesized that self-purification of water might be due in part to bacterial antagonism. Later, he obtained a fungus from a seawater sample that exhibited antibacterial activity and noted that it was similar to *Cephalosporium acremonium* (now named *Acremonium chrysogenum*). This observation led to the discovery of cephalosporin C.

In the intervening years, the fungi were not in the focus of drug discovery work, but interest in fungi resurged when cyclosporin A was isolated from *Tolypocladium inflatum* in 1976 and was approved for clinical use as an immunosuppressant in 1983. However, the rediscovery of high numbers of previously described metabolites has to some extent precluded the study of traditional terrestrial sources of fungi and led researchers to explore unique habitats, such as the marine environment, for fungi with potentially new biosynthetic diversity. Over the past many years fungi have proven to be a

valuable resource for the discovery of novel natural products. Although cephalosporin C was isolated from a marine derived fungus, studies reporting chemistry from marine derived fungi were rare until the 1990s. A few studies were performed between 1970 and 1990 followed by a nearly exponential growth through the 1990s. Although, siccayne (1) was most likely the first antibiotic isolated from an obligate marine fungus, the compound had been previously isolated from a terrestrial species (Kupka et al., 1981).



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vi) Current status of marine mycochemistry

Overall, research on marine-derived fungi has led to the discovery of more than 1000 new natural products including many that have novel carbon skeletons, indicating that marine-derived fungi have the potential to be a rich source of pharmaceutical leads.

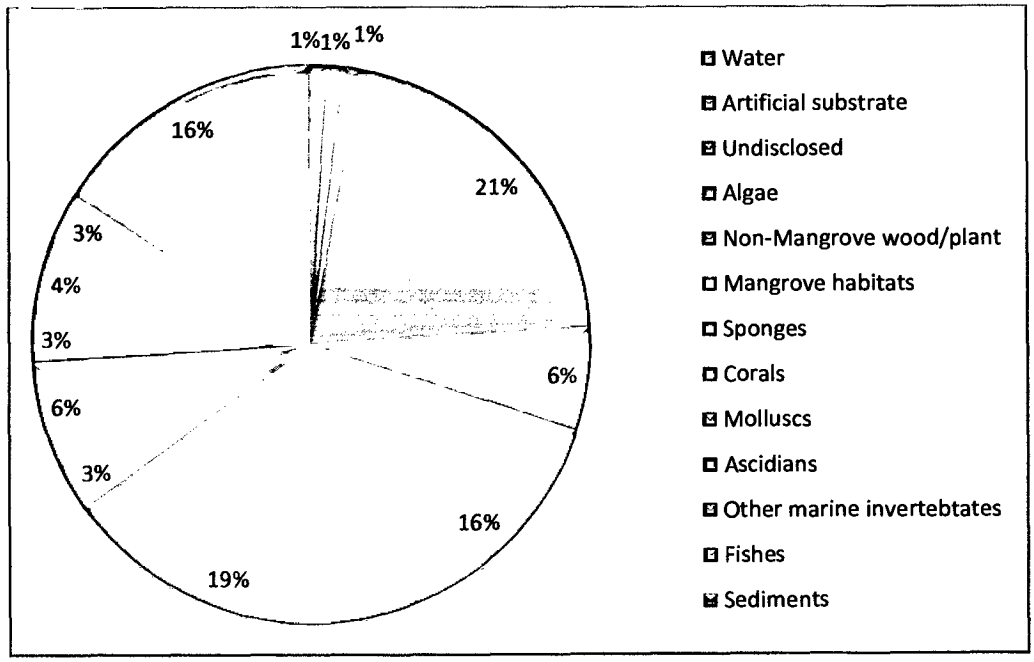


Fig 1.1: Distribution of fungal metabolites according to source of fungi

Fig. 1.1 gives an overview of new chemical structures from marine-derived fungi reported in the literature until mid-2010 classified according the source of the fungi. Based on these considerations, it can be estimated that roughly two-thirds of all new compounds reported from marine fungi are derived from isolates from living matter, while the remaining compounds are due to fungi from non-living sources, most notably sediments. Within the individual groups, algae are the predominant sources for fungal diversity, closely followed by sponges and mangrove habitats. An interesting newly emerging source is the deep sea, and only a handful of reports exist describing new secondary metabolites from fungi derived from this habitat, all of them actually obtained from deep sea sediments.

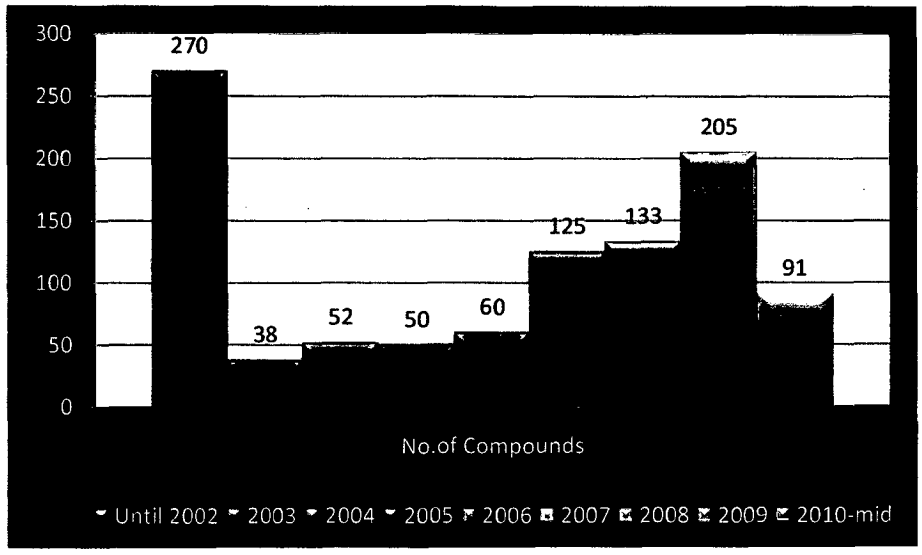


Fig 1.2: No. of fungal metabolites isolated over each year

From the number of new compounds published each year (**Fig.1.2**), it is evident that natural products chemistry of marine-derived fungi is rapidly developing, and still has not reached its climax. While, from the beginning until 2002, 272 new structures had been reported (Bugni and Ireland, 2004); in 2009 alone more than two-thirds of this figure was reached and it appears that similar figure will also be achieved in 2010, which is evident from the number of metabolites isolated in first half the year (95 new metabolites). The total number of new natural products from marine-derived fungi currently exceeds 1000.

Fig 1.3 gives an overview of new chemical structures from marine-derived fungi reported in the literature until mid-2010, based on their putative biogenetic origin. As evident from **Fig 1.3**, polyketides play a dominant role, and if prenylated polyketides and nitrogen-containing polyketides (grouped as alkaloids in this overview) are taken into account, their total share will exceed 50% of all new natural products from marine-derived fungi, which is similar to the situation of terrestrial fungi.

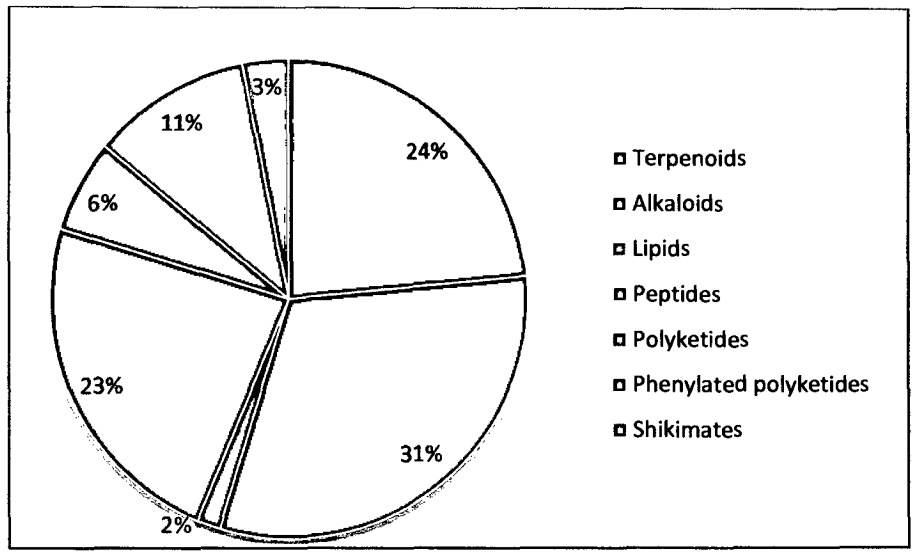


Fig 1.3: Fungal metabolites distribution according to their biosynthetic classification

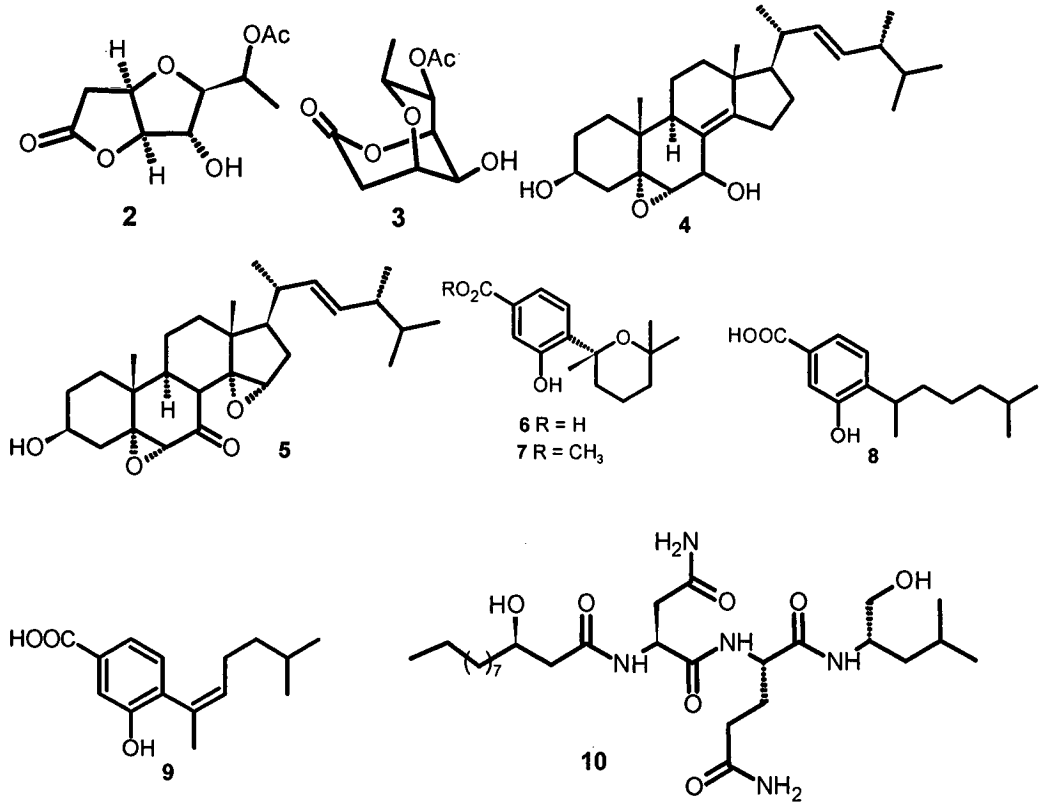
II. Metabolites from marine derived fungi reported in first half of 2010

i) Metabolites from Aspergillus sp.

Protulactones A (**2**) and B (**3**) were reported from an *Aspergillus* sp., isolated from an inter-tidal sediment sample collected in Korea (Sohn and Oh, 2010). Both the unusual furo[3,2-b] furan ring system present in **2** and the dioxabicyclo[3.3.1]nonane ring system as found in **3** appear to be unprecedented in fungi, at least in the genus *Aspergillus*, but are present in plant-derived metabolites such as goniofufurone from *Goniothalamus giganteus* (Fang et al., 1990), or a tetraketide from *Euscaphis japonica* (Takeda et al., 2000).

A. awamori isolated from soil around the mangrove plant *Acrostichum speciosum* in Hainan, China, yielded the two new oxidized sterols, (22*E*)-7α-methoxy - 5α,6α-epoxyergosta-8(14),22-dien-3β-ol (**4**) and (22*E*)-3β-hydroxy-5α,6α,8α,14α-

diepoxyergosta-22-en-7-one (**5**), both of which displayed weak cytotoxic activity towards the lung cancer cell line A549 (Gao et al., 2010). Conformational analysis on the basis of the observed NOEs in the ROESY spectrum indicated that the cyclohexene oxide system in ring B of **5** adopted an *endo*-boat rather than a half-chair conformation.

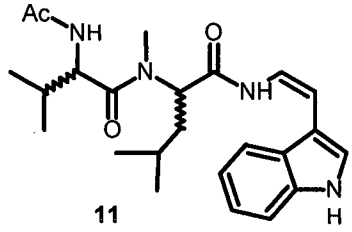


Four new phenolic bisabolane-type sesquiterpenoids, (+)-sydowic acid (**6**), (+)-methyl sydowate (**7**), 7-deoxy-7,14-didehydrosydonic acid (**8**) and 7-deoxy-7,8-didehydrosydonic acid (**9**), together with the known (+)-sydonic acid were isolated from an *Aspergillus* sp., obtained from the Chinese gorgonian *Dichotella gemmacea* (Wei et al., 2010a). The enantiomer of **6** [(-)-sydowic acid], as well as the corresponding racemate and sydonic acid, had previously been obtained from terrestrial strains of *Aspergillus sydowi* (Hamasaki et al., 1978; 1975a, 1975b). Compounds **6**, **7** and sydonic acid exhibited weak antibacterial activity against *Staphylococcus aureus*, but were inactive against methicillin resistant *S. aureus*.

A new lipopeptide, fellutamide C (**10**), was isolated from a strain of *A. versicolor,* which was obtained from the Korean sponge *Petrosia* sp (Lee et al., 2010). Compound **10** displayed mid-range cytotoxicity against several cancer cell lines,

and is a derivative of the previously described fellutamides A and B, metabolites of a marine fish-derived *Penicillium fellutanum*, (Shigemori et al 1991), which however were not detected in the extract of *A. versicolor.*

Japanese brown alga *Sargassum* sp. derived fungus *Aspergillus* sp. produced tereptin derivatives, JBIR-81 (**11**) and JBIR-82 (**12**), along with terpeptin (Izumikawa et al., 2010). The latter is a mammalian cell cycle inhibitor initially described from *A.terreus* (Kagamizono et al, 1997). All three terpeptins displayed free radical scavenging activities as expressed by their ability to protect N18- RE-105 cells against L-glutamate toxicity, and were considerably more potent than the positive control, a-tocopherol.



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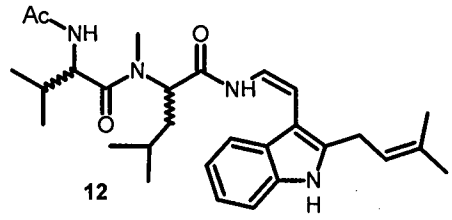

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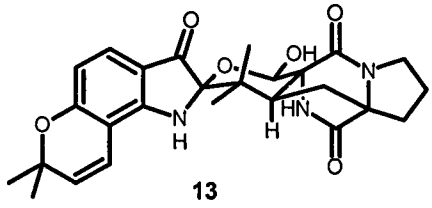
Notoamides O–Q (**13-15**) and notoamide R (**16**) were reported from Aspergillus sp. (Tsukamoto et al., 2010), but the latter compound appears to be identical to 21-hydroxystephacidin A, a metabolite of Marine fungi *A. ostianus,* which also produced notoamide F (Kito et al., 2009). Compound **13** possesses a hemiacetal/hemiaminal ether function, which is unprecedented in the notoamide class of compounds.



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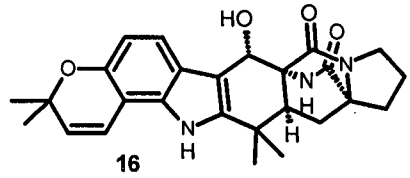

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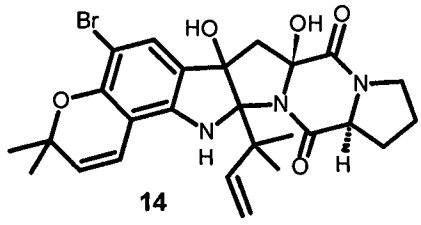

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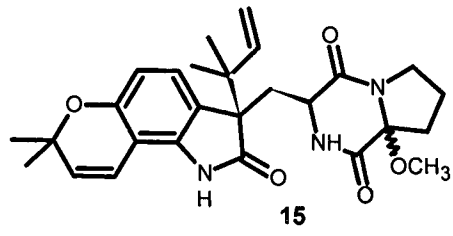

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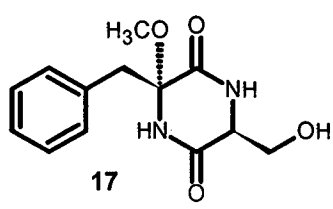
Three new dioxopiperazine metabolites **17-19** were isolated from a strain of *A. fumigatus* which was obtained from marine sediments collected in China.

Compounds **17-19** were devoid of cytotoxic activity (Zhao et al., 2010). The fungus *A. sydowi*, isolated from marine sediments collected in China, was identified as the producer of two additional pseurotin derivatives, azaspirofurans A (**20**) and B (**21**), characterized by the presence of an additional furan ring in the side chain (Ren et al., 2010). Compound **20** exhibited moderate cytotoxicity against the A549 cell line.



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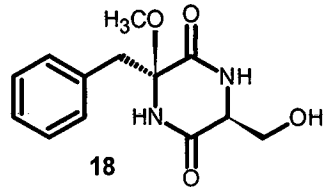

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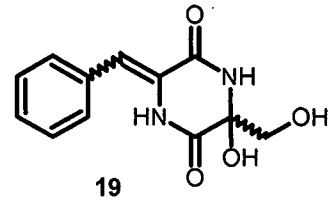

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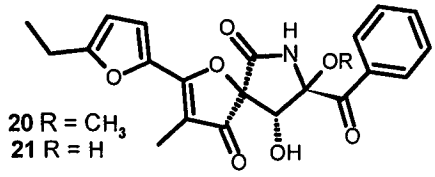
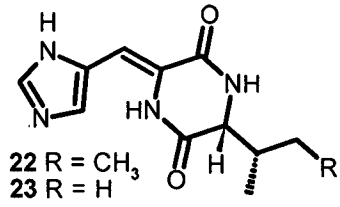
The fungus *Aspergillus*, isolated from an unidentified Japanese calcareous sponge, produced JBIR-74 (**22**) and JBIR- 75 (**23**), which were devoid of cytotoxic or antimicrobial activity (Takagi et al., 2010).



20 R = CH_3
21 R = H



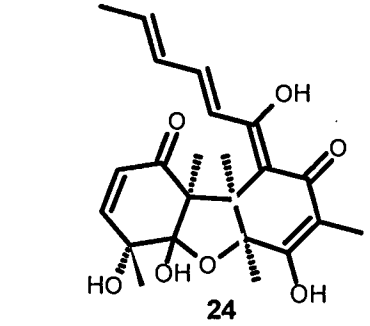
22 R = CH_3
23 R = H

ii) *Metabolites from Penicillium sp.*

An isolate of *P. citrinum* obtained from an unidentified Japanese demosponge, yielded JBIR-59 (**24**), along with a series of known sorbicillinoids (Ueda et al., 2010). Based on structural considerations, **24** is assumed to be formed by reaction of sorbicillin and the known trihydroxyquinol USF 406A, a metabolite of the soilborne fungus *Mortierella* sp.

A *Penicillium* sp., isolated from the red alga *Laurencia* sp. collected in the Bahamas Islands, yielded penilumamide (**26**), an unusual alkaloid combining a lumazine system with L-methionine sulfoxide and an anthranilic acid ester (Meyer et al., 2010). The 1,3-dimethyllumazine-6-carboxamide moiety within a natural product had previously only been reported from them freshwater leech *Limnatis nilotica* (Voerman et al., 2005). Compound **25** was inactive when tested for

cytotoxic and antimicrobial properties, and also did not affect cellular Ca^{2+} signalling in neuroendocrine cells. The fungus *Penicillium* sp., isolated from the tree *Clerodendrum inerme* growing in the inter-tidal zone of the South China Sea, yielded three new esters of desoxypatulinic acid **26-28** (Wu et al., 2010). Desoxypatulinic acid, which was also detected in the culture broth, is a common mycotoxin initially reported from *Penicillium patulum* (Scott et al., 1972).



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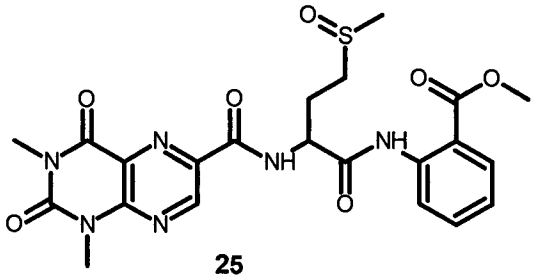

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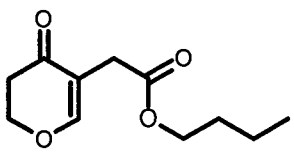

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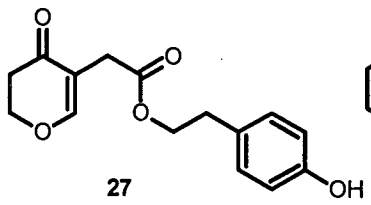

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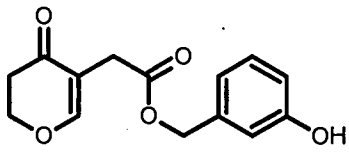

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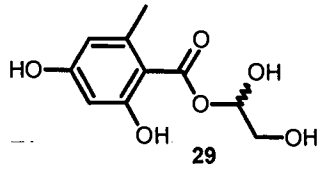
The endophytic fungus *P. commune*, which was isolated from the Chinese semi-mangrove plant *Hibiscus tiliaceus*, afforded a new ester of orsellinic acid with glycerol (**29**), besides a suite of mostly structurally simple known metabolites (Yan et al., 2010).

Another Chinese mangrove (*Excoecaria agallocha*) derived fungus *P. expansum* was identified as the producer of expansols A (**30**) and B (**31**), bisabolane sesquiterpenoids substituted with a diphenyl ether unit, and two new phenolic bisabolane sesquiterpenoids, (S)- (+)-11-dehydrosydonic acid (**32**) and (7S,11S)-(+)-12-acetoxysydonic acid (**33**) (Lu et al., 2010). Compound **30** exhibited moderate cytotoxicity against the HL-60 cell line, while **31** inhibited the proliferation of A549 and HL-60 cells, while **32** and **33** were inactive.



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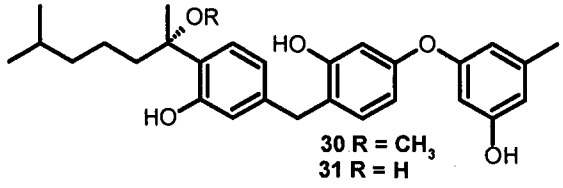
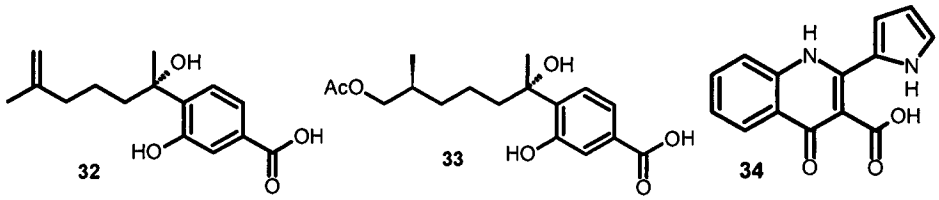
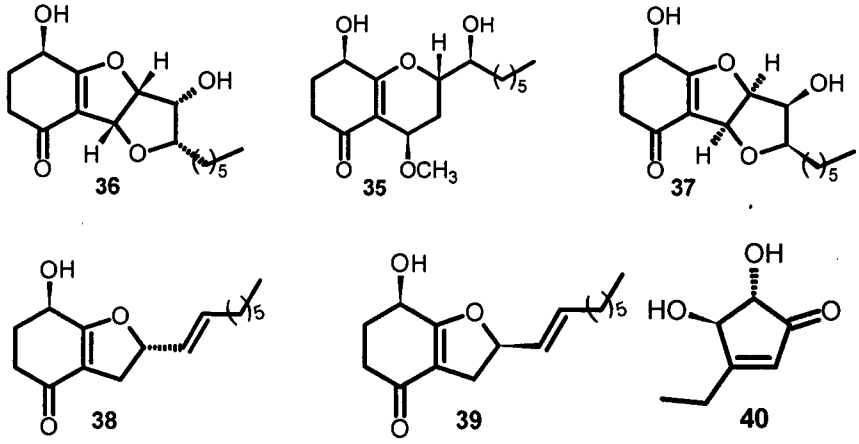




Yet another *Penicillium* sp., isolated from the Chinese mangrove plant *Acanthus ilicifolius* was the producer of isomeric pyrrolyl 4-quinolinone alkaloid penicinoline (**34**) (Shao et al., 2010a). Upon methylation of this compound, an N-methylated lactam was unexpectedly formed. Compound **34** exhibited potent cytotoxicity towards the 95-D and HepG2 cell lines, and also displayed strong insecticidal activity against *Aphis gossypii*.

iii) *Metabolites from Trichoderma sp.*

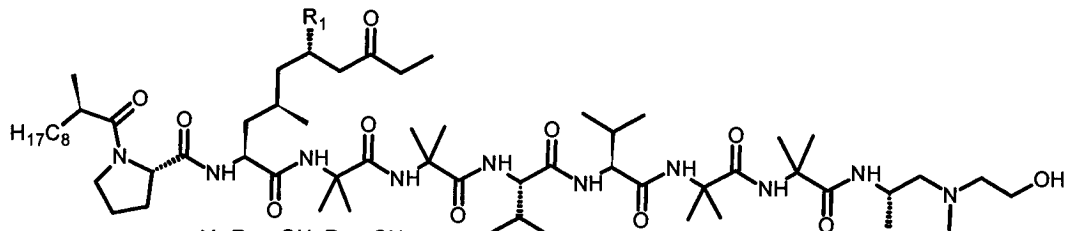
The fungus *T. koningii*, isolated from marine mud of the South China Sea, produced 7-O-methylkoninginin D (**35**) and trichodermaketones A–D (**36-39**), together with four koninginin derivatives (Song et al., 2010). Koninginin D (**39**) had previously been reported form a terrestrial strain of *T. Koningii* (Dunlop et al., 1989). Compounds **35-39** did not display antimicrobial activity against methicillin-resistant *Staphylococcus aureus* or *Candida albicans*, but **36** showed synergistic antifungal activity against *C. Albicans* with ketoconazole, but at rather high concentrations.



The fungus *Trichoderma* sp. was isolated from marine sediment in the South China Sea and found to produce a new cyclopentenone, trichoderone (**40**), besides a known steroid (You et al., 2010). Compound **40** displayed selective cytotoxicity

towards six cancer cell lines and were moderately active, probably by inducing apoptosis.

The fungal strain *Trichoderma* sp., isolated from an unidentified marine sponge of undisclosed geographic origin, produced three new aminolipopeptides, trichoderins A (**41**), A1 (**42**) and B (**43**) (Pruksakorn et al., 2010). Compounds **41-43** are characterized by the presence of a 2-amino-6-hydroxy-4-methyl-8-oxodecanoic acid (AHMOD) moiety, which they share with previously described aminolipopeptides such as the leucinostatins or trichopolyns, metabolites of *P. lilacinum* (Arai et al., 1973) and *T. polysporum*, (Fuji et al, 1978) respectively. Compounds **41-43** showed potent anti-mycobacterial activity against *Mycobacterium smegmatis*, *M. bovis* and *M. tuberculosis*, with MIC values in the range 0.02–2.0 mg/ml, and were effective against both actively growing and dormant states



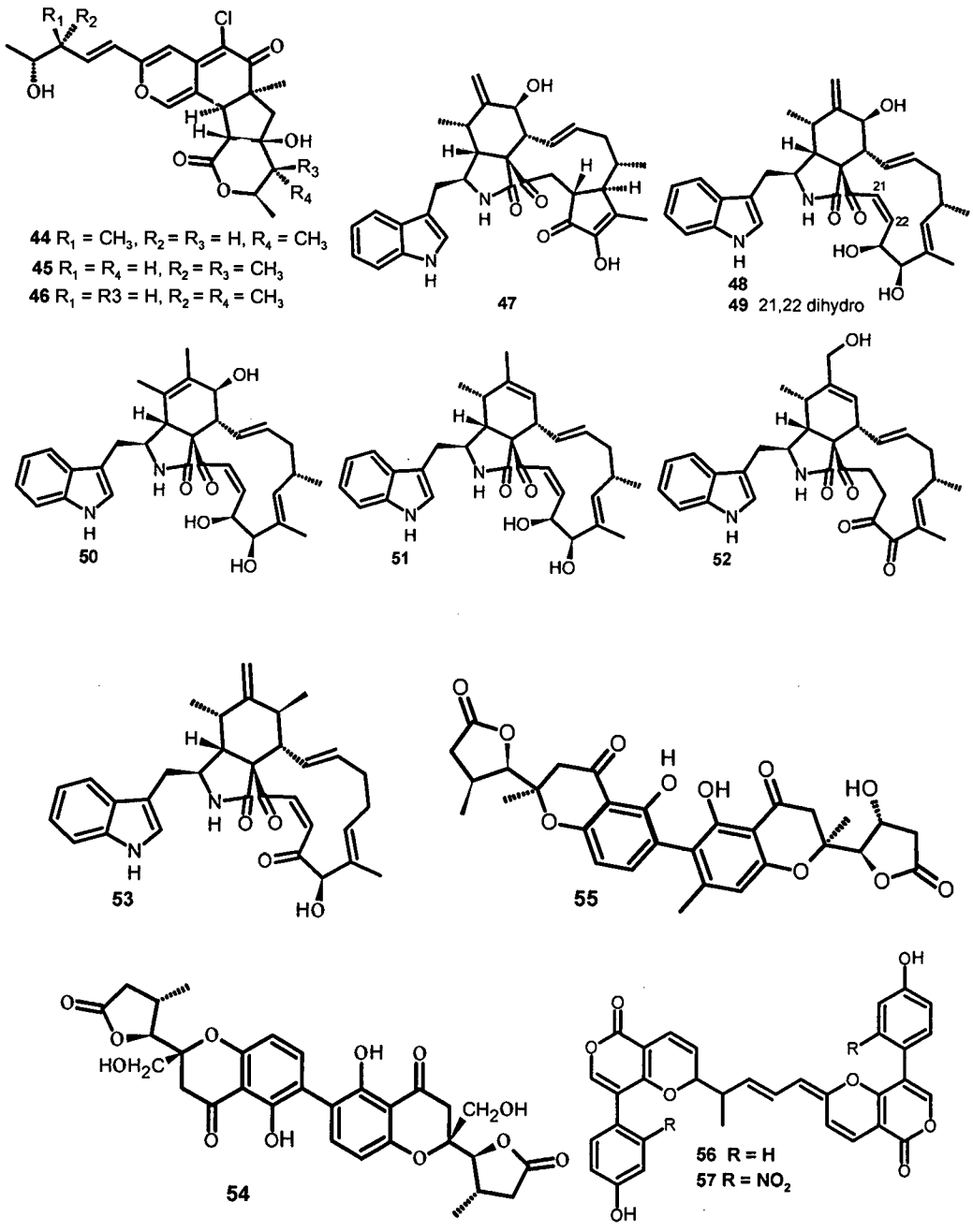
41 R_1 = OH, R_2 = CH_3
42 R_1 = H, R_2 = CH_3; C6=C7
43 R_1 =OH, R_2 = H

iv) *Metabolites from Chaetomium globosum*

11-epi-chaetomugilin A (**44**) and 40-epichaetomugilin A (**45**), were discovered from fungus *Chaetomium globosum*, originally isolated from the Japanese fish *Mugil cephalus* (Muroga et al., 2010). Both the compounds are steroisomers of chaetomugilins A (**46**) previousely isolated from the same culture (Yamada et al., 2008). Compound **44** exhibited moderate activity against P388 and HL-60 cell lines, but marginal activity against L1210 and KB cell lines, whereas **45** was only marginally active against all cell lines.

Another endophytic fungus *Chaetomium globosum* derived from the Chinese green alga *Ulva pertusa* produced seven new cytochalasan derivatives of the chaetoglobosin class, cytoglobosins A–G (**47-53**), together with the structurally related known compounds, isochaetoglobosin D and chaetoglobosin F.

Compounds **49** and **50** exhibited moderate cytotoxic activity against the A-549 cell line.

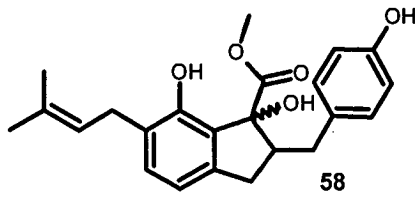


v) Metabolites from Phomopsis sp

The endophytic fungus *Phomopsis* sp., isolated from the Chinese mangrove tree *Excoecaria agallocha*, produced phomopsis-H76 A (**54**), a dimeric chromanone closely related to monodictyochrome B (**55**), as well as phomopsis-H76 B (**56**),

and C (**57**), structurally unique dimeric pyrano[4,3-b]pyran-5(2H)-ones (Yang et al., 2010).

Phomoindene A (**58**), a rare example of an indene derivative from nature, was described from the fungus *Phomopsis* sp., obtained from a sediment sample in a Chinese mangrove habitat, but was inactive when tested for cytotoxic properties (Chen et al., 2010).



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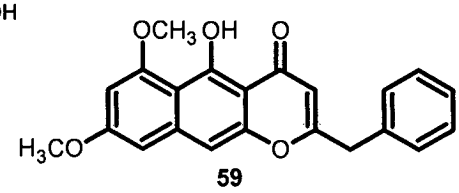
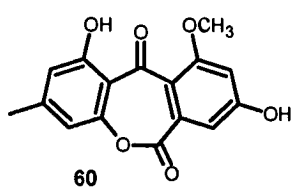


59

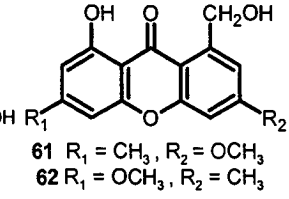
A new naphtho-γ-pyrone, 2-benzyl-5-hydroxy-6,8-dimethoxy- 4H-benzo[g]chromen-4-one (**59**), was reported from the endophytic fungus *Phomopsis* sp., isolated from the Chinese mangrove tree *Excoecaria agallocha* (Huang et al., 2010a).

vi) *Metabolites from Phoma sp.*

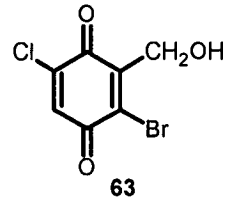
A new lactone, 1,8-dihydroxy-10-methoxy-3-methyldibenzo[b,e]oxepine-6,11-dione (**60**), and two new xanthones, 1-hydroxy-8-(hydroxymethyl)-6-methoxy-3-methyl-9H-xanthen-9-one (**61**) and 1-hydroxy-8-(hydroxymethyl)-3-methoxy-6-methyl-9H-xanthen-9-one (**62**) were reported from the endophytic fungus *Phoma* sp., isolated from the roots of the Chinese mangrove plant *Avicennia marina* (Pan et al., 2010). Compounds **60-62** were inactive when tested for cytotoxic properties.



60



61 $R_1 = CH_3, R_2 = OCH_3$
62 $R_1 = OCH_3, R_2 = CH_3$



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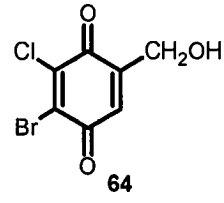
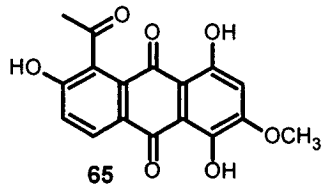


64

Bromochlorogentisylquinones A (**63**) and B (**64**) are metabolites of the fungus *Phoma herbarum*, isolated from the Korean red alga *Gloiopeitis tenax*, which were formed upon addition of calcium bromide to the culture medium, together with the known chlorogentisyl alcohol and gentisyl alcohol (Nenkep et al., 2010).

All compounds displayed significant radical scavenging properties in the DPPH assay.

vii) *Metabolites from Fusarium sp.*

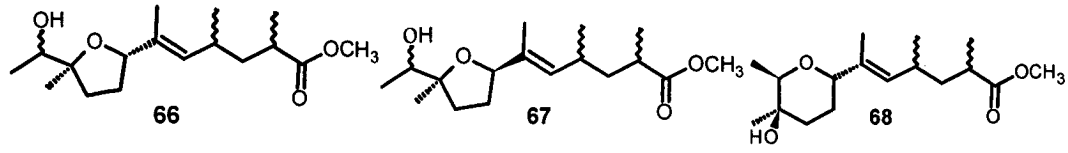


65

A new anthraquinone, 5-acetyl-2-methoxy-1,4,6-trihydroxy-anthraquinone (**65**), besides a series of known naphthoquinones from the endophytic fungus *Fusarium* sp. from an undisclosed Chinese mangrove plant is the only report on this genus in first half of 2010 (Shao et a., 2010b).

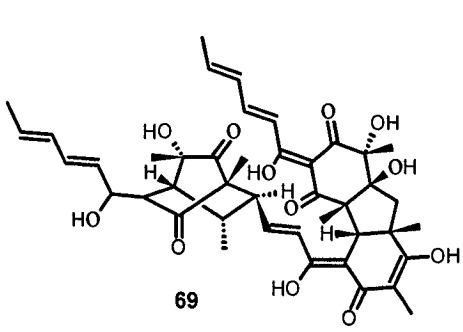
viii) *Metabolites from other species*

The fungus *Rhizopus* sp. was isolated from the Chinese bryozoan *Bugula* sp., and was found to produce aspericins A–C (**66-68**), along with the related asperic acid (Wang et al., 2010). Asperic acid, so far the only compound reported from nature with the same carbon skeleton as present in **66-68**, had previously been obtained from a saltwater culture of sponge-derived *Aspergillus niger* (Varoglu and Crews, 2000). When tested for cytotoxic properties against four different cell lines, only **68** displayed moderate activity.



A deep-sea isolate of *Phialocephala* sp., collected from sediments at a depth of 5059 m yielded three new metabolites, trisorbicillinones B–D (**69-71**), all of which exhibited only very weak cytotoxicity (Li et al., 2010a). The former member of the series trisorbicillinone A was obtained from the same strain in previous studies, which was moderately cytotoxic against P388 and HL60 cells (Li et al., 2007).

In a screening effort directed at discovering tyrosine kinase inhibitors using humanumbilical vein endothelial cell lysate, out of a total of 200 fungal strains tested, only the fungus *Hypocrea vinosa* isolated from beach sand collected in Japan displayed activity. Chemical analysis revealed the presence of two new bisnaphtho-g-pyrones, hypochromins A (**72**) and B (**73**), besides the closely related known SC2051, the diketocongener of **72**, which so far has only been reported in the patent literature (Miki et al., 2010). All three compounds inhibited tyrosine kinase activity in HUVEC lysate, and in addition also inhibited HUVEC proliferation, migration, and tubule formation, thus displaying antiangiogenic potential.



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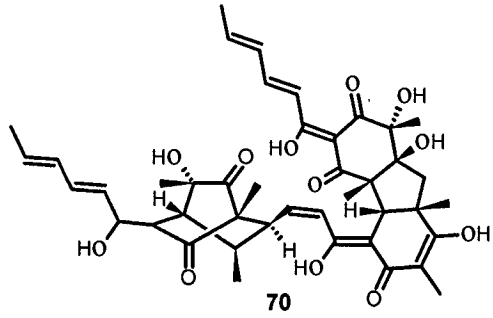

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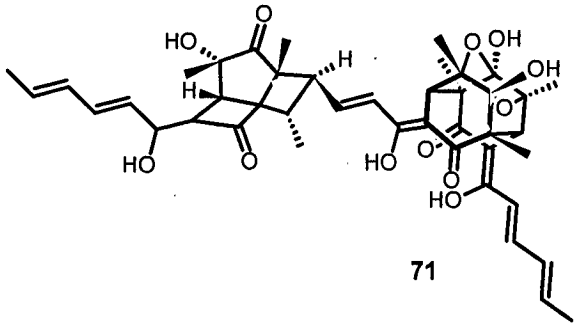

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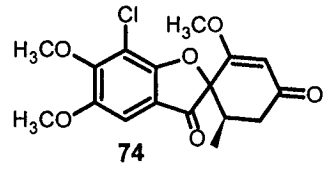

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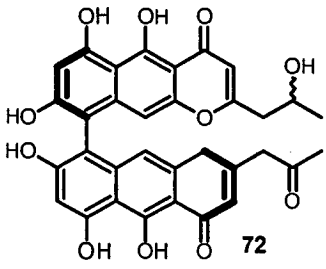

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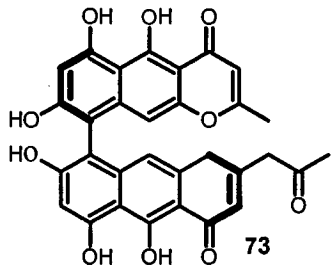

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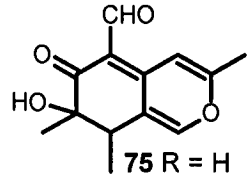


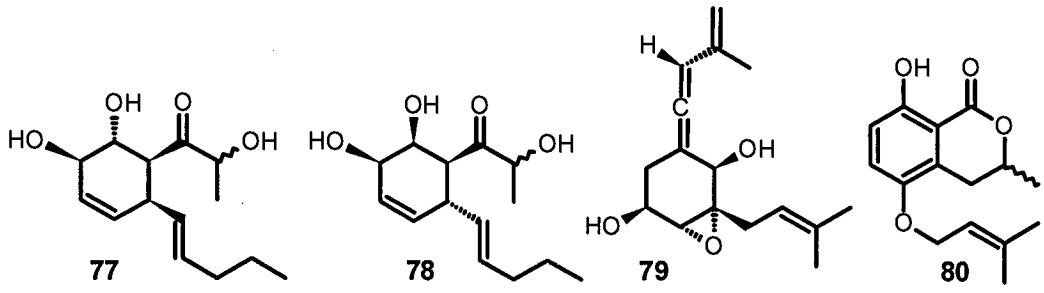
75 R = H
76 R = CH_3

The fungus *Sporothrix* sp., isolated from an undisclosed mangrove at the South China Sea coast, yielded the new griseofulvin derivative **74** (Wen et al., 2010).

The endophytic fungus *Talaromyces* sp., isolated from the Chinese mangrove plant *Kandelia candel*, yielded two new isochromenones 7-epiaustdiol (**75**) and 8-O-methylepiaustdiol (**76**), besides a diverse series of known polyketides Liu et al., 2010).

Nigrosporanenes A (**77**) and B (**78**) were isolated from fungus *Nigrospora* sp, which was obtained from the sea fan *Annella* sp. collected Thailand (Rukachaisirikul et al. 2010). Compound **77** displayed moderate cytotoxicity towards MCF-7 and Vero cells, while **77** and **78** also showed weak radical scavenging activity.

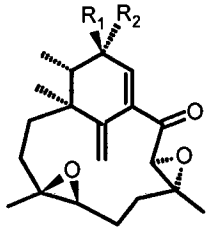
The endophytic fungus *Phaeosphaeria spartinae*, isolated from a German red alga of the genus *Ceramium*, was the source of spartinoxide (**79**), which is the enantiomer of the known compound A82775C (Elsebai, et al., 2010). The latter was previously reported from an unknown fungus of the order *Sphaeropsidales* (Sanson et al., 1991). Compound **79** showed potent inhibition of human leukocyte elastase (HLE), but was inactive against bovine trypsin acetyl cholinesterase from electrophorus electricus, and porcine cholesterol esterase.



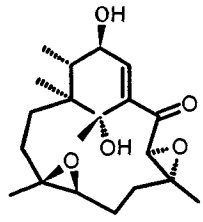
8-Hydroxy-3-methyl-5-(3-methylbut-2-enyloxy)isochroman-1-one (**80**) was detected in the culture broth of the fungus *Cephalosporium* sp., isolated from an undisclosed Chinese mangrove plant (Wei et al., 2010b).

An unidentified fungus that was isolated from the surface of the Japanese brown alga *Ishige okamurae*, and based on DNA sequence analysis was grouped into the order *Dothideales* was identified as the producer of phomactin I (**81**), 13-epi-phomactin I (**82**) and phomactin J (**83**) (Ishino et al., 2010). Phomactins are an intriguing class of fungal diterpenes which have raised considerable attention due

to their activity as platelet-activating factor (PAF) antagonists (Goldring and Pattenden, 2006), and were initially discovered in cultures of the fungus *Phoma* sp. obtained from the shell of the crab *Chionoecetes opilio* (Sugano et al., 1994), and it is therefore remarkable that the unidentified fungus in this study was taxonomically not closely related to the genus *Phoma*.



81 R_1 = OH, R_2 = H
82 R_1 = H, R_2 =O H



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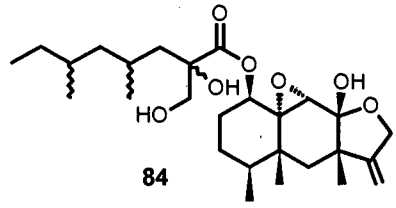


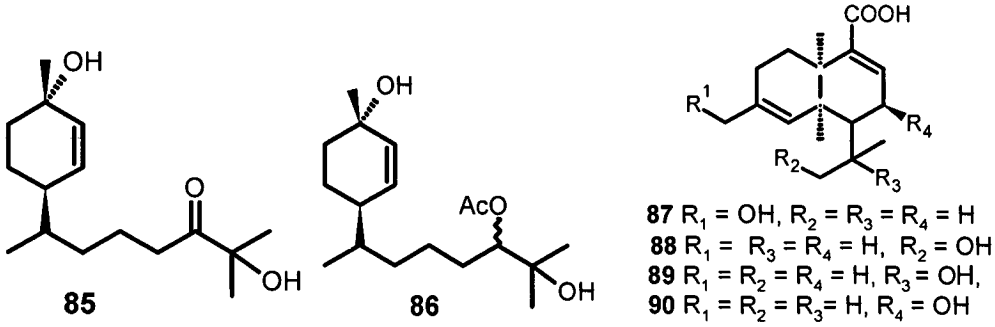
84

A marine-derived ascomycete related to the genus *Cryptosphaeria*, isolated from an unidentified ascidian in the Bahamas, produced cryptosphaerolide (**84**), an eremophilane type sesquiterpenoid bearing both an exo-methylene and an ester function (Oh et al., 2010). **84** is related to berkleasmin A, a metabolite of the terrestrial saprobic fungus, *Berkleasmium nigroapicale* (Isaka et al., 2009). In the Mcl-1/Bak fluorescence resonance energy transfer (FRET) assay, **84** displayed inhibitory activity towards the Mcl-1 protein, a cancer drug target involved in apoptosis. In addition, **84** also showed significant cytotoxicity against the HCT-116 human colon carcinoma cell line, with IC50 values in the lower mM range.

Verticinols A (**85**) and B (**86**) are two new hydroxylated bisabolane-type sesquiterpenes which were reported from the fungus *Verticillium tenerum*, isolated from an unidentified marine alga (Almeida et al., 2010a). Although **85** and **86** were tested for a variety of effects, i.e. antibacterial, antifungal, antialgal, antiplasmodial, antiviral, and cytotoxic activity as well as protein kinase inhibition or fat-accumulation inhibitory activity against 3T3-L1 murine adipocytes, they did not display significant activity in any of these test systems.

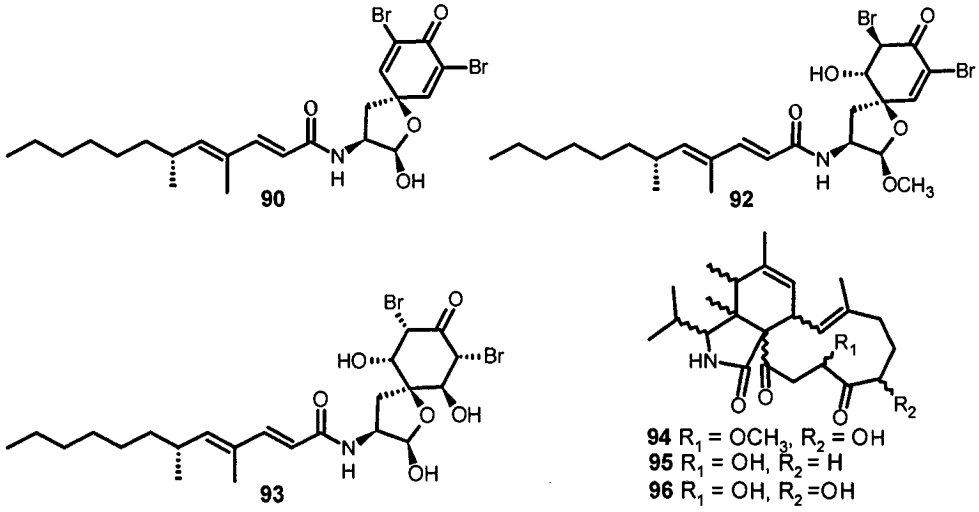
Four new hydroxylated sclerosporin derivatives 15-hydroxysclerosporin (**87**), 12-hydroxysclerosporin (**88**), 11-hydroxysclerosporin (**89**) and 8-hydroxysclerosporin (**90**), besides the known (+)-sclerosporin were produced by the fungus *Cadophora malorum*, isolated from the green alga *Enteromorpha* sp. Upon long-term fermentation in a medium supplemented with artificial sea salt

(Almeida et al., 2010a). Sclerosporin was initially characterised as a sporogenic metabolite of a terrestrial isolate of *Sclerotinia fruticula*, and is a rare example of a fungal-derived cadinane-type sesquiterpenes (Katayama et al., 1979). Compounds **87-90** were subjected to a variety of assays, but were found devoid of significant biological activity, apart from **90**, which showed a weak fat-accumulation inhibitory activity against 3T3-L1 murine adipocytes.



87 R_1 = OH, R_2 = R_3 = R_4 = H
88 R_1 = R_3 = R_4 = H, R_2 = OH
89 R_1 = R_2 = R_4 = H, R_3 = OH,
90 R_1 = R_2 = R_3 = H, R_4 = OH

Fungus *Gymnascella dankaliensis*, isolated from *Halichondria japonica* collected off Osaka in Japan was known to produce gymnastatins F–H and the fatty acid- or polyketide-derived gymnamide (Amagata et al., 2006). The same fungus yielded the brominated analogues gymnastatins I–K (**91-93**), when cultured in a bromine-containing medium. All of them exhibited potent cytotoxicity towards the murine P388 lymphocytic leukemia cell line. Moreover, **91** and **92** displayed pronounced activity against a panel of 39 human cancer cell lines (Amagata et al., 2010).

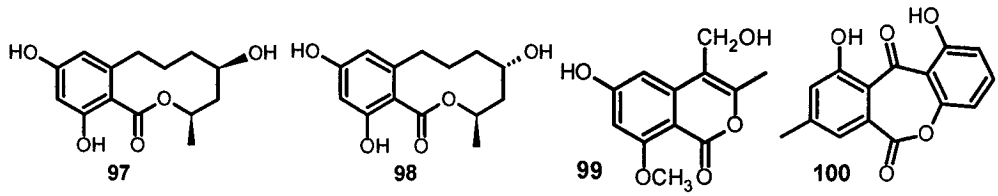


94 R_1 = OCH_3, R_2 = OH
95 R_1 = OH, R_2 = H
96 R_1 = OH, R_2 =OH

An investigation of fungus *Spicaria elegans* yielded aspochalasin congeners, aspochalasins R–T (**94-96**), all of which proved inactive in terms of cytotoxic effects (Lin et al., 2010).

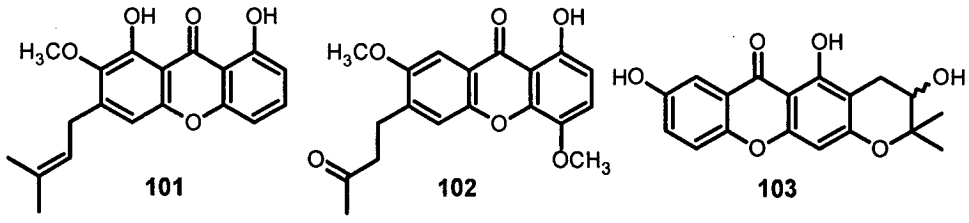
Metabolites from Unidentified fungi

An unidentified endophytic fungus, isolated from the Chinese mangrove plant *Sonneratia apetala*, produced two new 10- membered lactones, (3*R*,5*R*)-sonnerlactone (**97**) and its (3*R*,5*S*) diastereoisomer (**98**) (Li et al., 2010b). Both **97** and **98** displayed weak inhibitory effects towards the multi-drug resistant cancer cell line KV/MDR.



An unidentified endophytic fungal strain isolated from the Chinese mangrove *Bruguiera gymnorrhiza* produced two new aromatic lactones, 1,10-dihydroxy-8-methyldibenzo[b,e]oxepine-6,11-dione (**99**) and 3-(hydroxymethyl)-6,8-dimethoxy-2Hchromen- 2-one (**100**), along with a known coumarin derivative and the 8-demethyl congener of **100**, which so far had only been obtained synthetically, but not as a natural product (Huang et al., 2010b).

1,7-Dihydroxy-2-methoxy-3-(3-methylbut-2-enyl)-9Hxanthen-9-one (**101**) and 1-hydroxy-4,7-dimethoxy-6-(3-oxobutyl)-9H-xanthen-9-one (**102**) were reported from an unidentified endophytic fungus, isolated from the Chinese mangrove tree *Avicennia marina* (Huang et al., 2010c). Compounds **101** and **102** displayed mild cytotoxicity towards the KB and KBV200 cell lines.



3,5,8-Trihydroxy-2,2-dimethyl-3,4-dihydropyrano[3,2-b]xanthen-6(2H)-one (**103**) was reported from an unidentified endophytic fungus, obtained from a Chinese mangrove tree of the genus *Avicennia* (Huang et al., 2010d).

x) Concluding remarks

It may be concluded that the ubiquitous fungi *Aspergillus* and *Penicillium* continues to be the prolific producers of new metabolites (**Fig 1.4**)

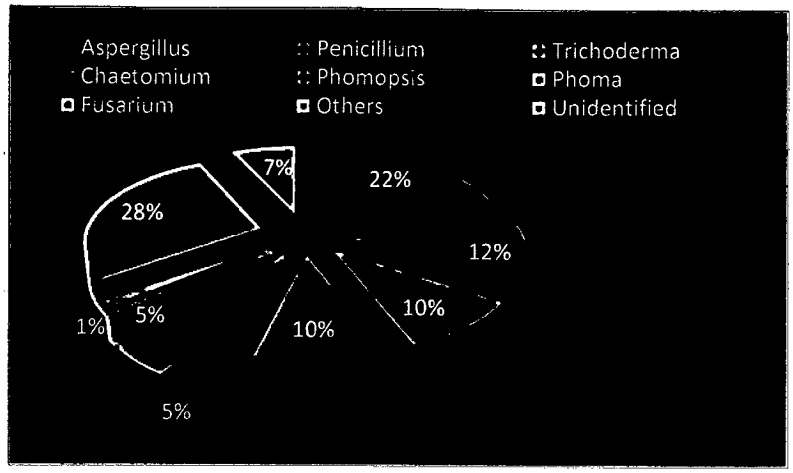


Fig 1.4 Distribution of metabolites reported in first half of 2010 according to fungal genus.

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Chapter 2

SECONDARY METABOLITES FROM THREE DIFFERENT MARINE DERIVED *ASPERGILLUS* SPECIES

I. Introduction

i) General:

Given the recent evidences, marine-derived fungi are proving to be excellent source of chemical entities with novel structures and biological potential (Ebel, 2008; Rateb and Ebel, 2011). A review of literature on fungal metabolites, (chapter 1) shows that even the marine derived *Aspergillus* spp. are one the richest producer of secondary metabolites. *Aspergillus* is one of the oldest named genera of fungi, received its name from Micheli in 1729. *Aspergilli* grow abundantly as saprophytes on decaying vegetation, but they can also live on substrates as diverse as dung, human tissues, and antique parchment. *Aspergillus* is ubiquitous genera of fungus commonly isolated from soil, plant debris, and indoor air environment and also from several marine habitats. It has ability to disperse globally in air currents and to grow almost anywhere when appropriate food, water are available and can even tolerate high salt concentration. As a result, isolates (facultative marine) similar to terrestrial ones have been obtained from marine habitats. Marine-derived strains may produce unusual secondary metabolites as an environmental adaptation. The genus *Aspergillus* includes over 250 species (Geiser, 2008) and around 20 species have so far been reported as causative agents of opportunistic infections in man.

ii) Role of Genus Aspergillus in Human Economy:

Aspergilli are industrially important microorganisms due to their production of organic acids, statins and extracellular enzymes. Citric acid is a true 'bulk chemical' with an estimated production of more than 1.6 billion kg each year (Dodds and Gross, 2007) and gluconic acid, which is used for therapy for iron and calcium deficiency are obtained from *Aspergillus niger*. *Aspergillus terreus* is used for itaconic acid production, a synthetic polymer (Calam et al., 1939). *Aspergillus. oryzae* is fermented for kojic acid production which is used for skin whitening and as a precursor for synthesis of flavour enhancers (Ruijter et al., 2002). The first statin approved for

human use, was lovastatin, a secondary metabolite of *A. terreus* was developed by a group of scientists at Merck Inc. in the USA (Alberts et al., 1980). Lovastatin was sold under the brand named Mevacor™ and became a blockbuster drug for Merck. Other compounds with pharmacological activities include cholecystokinin and neurokinin antagonists, ion channel ligands, antifungal drugs and a host of other compounds. A useful comprehensive review of fungal drug discovery is provided by An (2005) in *Handbook of Industrial Mycology.*

Food fermentations are ancient technologies that harness microorganisms and their enzymes to improve the human diet. Hydrolytic enzymes from *A. orzyae* and *A. sojae* suit them for growth on starch and other carbohydrate-rich substrates, and are effective in production of rice wine. These enzymes are also used in legume fermentation to obtain miso and soy sauce. Although this genus produces many industrially useful enzymes, chemicals, and biomedicals, they are also known as producer of deadly carcinogenic toxins such as aflatoxin from *A. flavus* (Reddy et al., 2005). There is probably no other genus of fungi so useful to humans that is also so harmful to humans.

iii) Scope of our study

Fungi for many decades have played a pivotal role as a source of interesting new natural products, mainly due to their highly developed and diverse secondary metabolism. Thus, terrestrial fungi have been intensively studied over the three quarters of 20th century. The lack novelty in chemistry of the terrestrial fungi diverted the attention of mycochemists towards unexplored fungi from marine habitats as source of new chemical entities with potent bioactivity. The work described in this chapter is an effort to discover novel structures with potent biological activity from three marine-derived fungi, belonging to genus *Aspergillus.*

The chapter is divided into five sections. The first three sections, section 2.1, section 2.2 and section 2.3 deals with the purification, structure elucidation and biological activities of metabolites from *A.flavipes, A. terreus* and *A. aculateus* respectively. Section 2.4 deals with the detection of minor constituents and chemotaxonomic

comparison of the two fungi, *A. flavipes* and *A. terreus* using electrospray ionization mass spectrometry. The last section *i.e* section 2.5 describes the effect of sea water on the production of butyrolactone I **(FM-1)** in *A. terreus*.

Section 2.1: Bioactive secondary metabolites from the mangrove-derived fungus *Aspergillus flavipes*

This section is further sub-divided into two subsections the subsection I gives the review of literature on *A. flavipes* and its metabolites and the subsection II describes the purification of fungal strains and structure elucidation of bioactive metabolites from *A. flavipes* isolated from pneumatophores of mangrove *Acanthus illicifolius.*

I. Review of Literature on metabolites from *Aspergillus flavipes*

Aspergillus flavipes produces colonies, which are white or silvery white, reverse the medium yellow to orange brown or reddish brown. The conidia are colourless, smooth globose 2-3 μm in diameter (**Fig 2.1**).

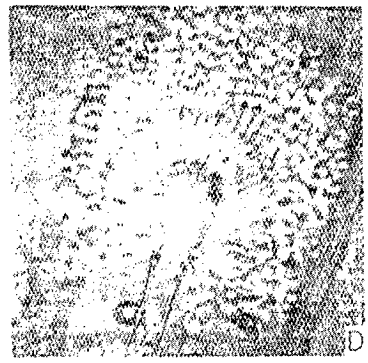


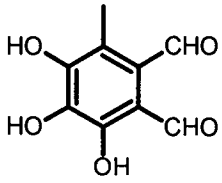
Fig 2.1: Photomicrograph of typical head of *A. flavipes*

A review of literature indicates that over 15 novel and several known compounds have been identified from this species, most of them being soil-derived fungal strains and there being one a solitary reference on the metabolites from marine derived strain. The first metabolite with complete structure elucidation to be reported from this species was flavipin **(1)** having powerful antifungal property in 1956 (Raistrick and Rudman). It was identified as 1,2-diformyl-4,5,6-trihydroxy-3-methylbenzene, by using classical organic chemical methods (without the use of any available spectroscopic evidences). This was followed by the isolation of yet another new

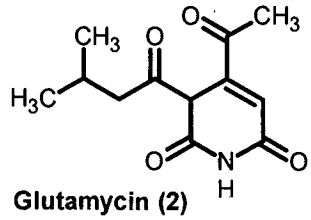
compound glutamycin **(2)**, from *A. flavipes by* Casonovi et al., in 1968. Glutamycin **(2)** was highly potent against both gram +ve and –ve bacteria. In 1972, a pyridine base antibacterial, named flavipucine **(3)** was isolated from the same species by Findlay and Radices (1972) and identified as 3'-isovaleryl-6-methyl pyridine-3-spiro-2(1H),4(3H)-dione .

The first cytotoxic compound to be reported from *A. flavipes* was TMC-169 **(4)** (Kohno et al., 1999). Through modern spectroscopic analysis including 2D-NMR techniques such as HMBC and ROESY, **4** was identified as a new analogue of the aspochalasin group, represented by aspochalasins A-G (Fang et al., 1997) and phomactin C (Alvi et al., 1997). Aspochalasins consist of 11,13, or 14-membered carbocyclic (or oxygen-containing) rings connecting the C-8 and C-9 positions of a perhydroisoindol-1-one moiety bearing 2-methylpropyl group substituent at C-3. Later on, three new moderately cytotoxic aspochalasins I **(5)**, J **(6)** and K **(7)** were isolated from *A. flavipes* obtained from rhizosphere of *Ericameria laricifolia* (Guang et al., 2004). Yet another aspochalasin L **(8)** having activity against HIV-1 integrase was isolated from the extract of a soil derived fungal culture of *A flavipes* (Rochfort et al., 2005).

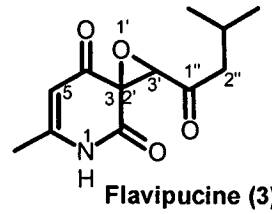
Barrow and Sun (1994) reports the isolation of spiroquinazoline **(9)**, a novel P inhibitor with a new carbon skeleton. Structurally **9** is related to fumiquinazoline C **(10)**, a metabolite of a marine fungus *A. fumigatus* (Numata et al., 1992),but differs from **10** in having a methyl and oxygen less in its structure. Additionally, a new but inactive metabolite, benzodiazepinedione **(11)** along with seven known diketopieperazine derivatives and known compounds acyl aszonalenin and N-benzoyl- L-phenylalaninol, were also isolated from this fungus. As mentioned earlier there is a single report on metabolites from marine derived strain of *A. flavipes*, namely flavicerebrosides A (**12**) and B (**13**), beside two known glycosphingolipids cerebrosides D and C (Jiang et al., 2004). The latest report on the metabolites from *A. flavipes* is the isolation of two highly hydroxylated 1,3 dihydrobenzofurans FR198248 (**14**) and FR202306 **(15)** from soil derived culture (Kwon et al., 2010).



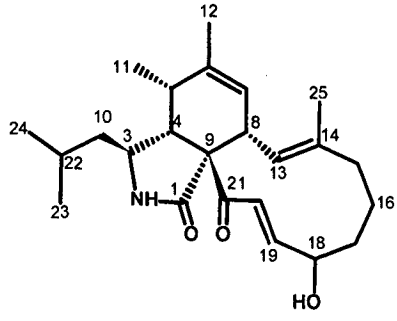
Flavipin (1)



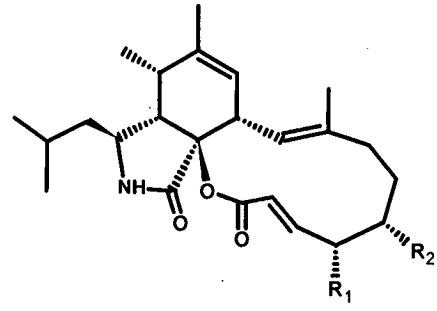
Glutamycin (2)



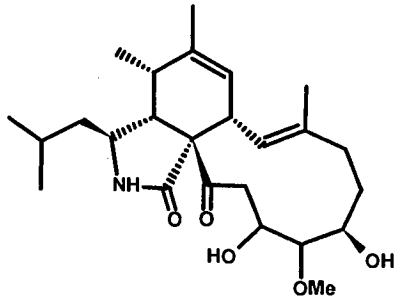
Flavipucine (3)



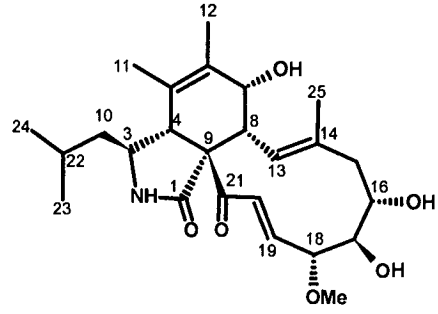
TMC-169 (4)



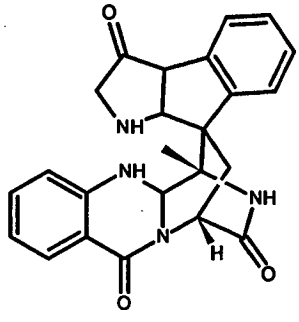
Aspochalasin I, $R_1 = R_2 = OH$ (5)
Aspochalasin J, $R_1 = OH$ $R_2 = H$ (6)



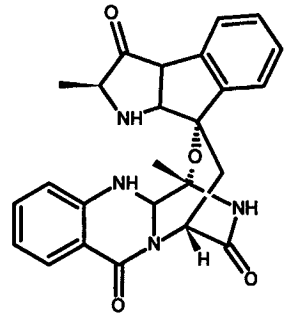
Aspochalasin K (7)



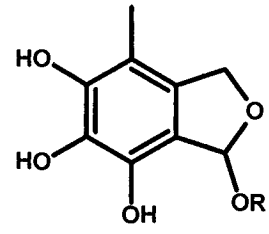
Aspochalasin L (8)



Spiroquinazoline (9)

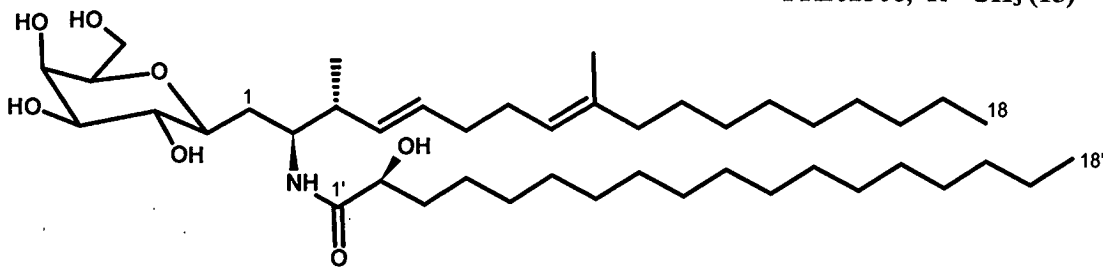


Fumiquinazoline C (10)

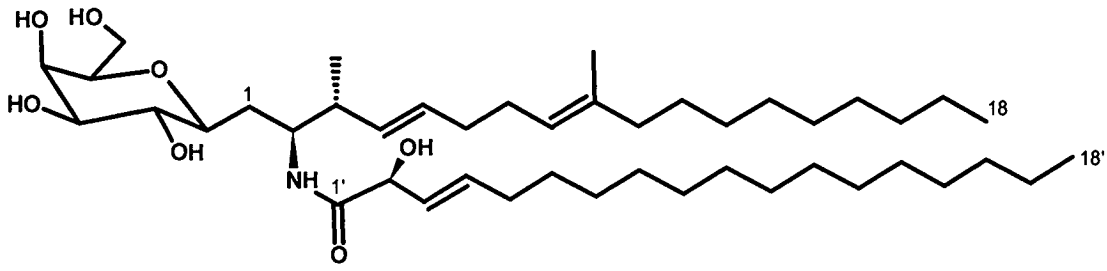


FR198248, R = H (14)

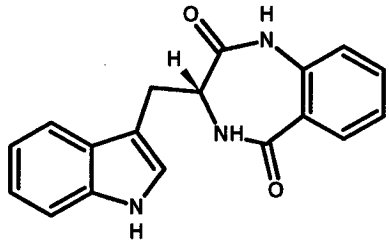
FR202306, R =CH_3 (15)



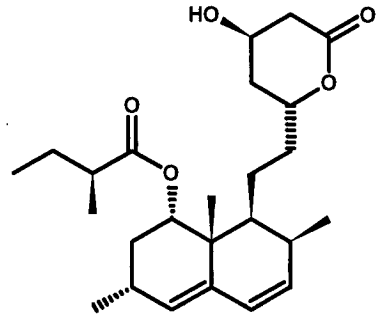
Flavicerebrosides A (12)



Flavicerebrosides B (13)



Benzodiazepinedione (11)



Lovastatin (16)

A. flavipes has resemblance to another well-known terrestrial fungus *A. terreus.* Morphologically, the young cultures of the two species are indistinguishable (Raistrick and Rudman, 1956). Genetically *A. terreus* is discriminated from *A. flavipes* only by presence pksM (6-methylsalicylic acid synthase) gene (Pazoutova et al., 1999). These similarities are also reflected in their metabolite chemistry. Lovastain **(16)** (Valera et al., 2005; Alberts et al., 1980) and flavipin **(1)** are common metabolites to both species.

A. flavipes is also known to produce enzymes such as α-galactosidase, L-Methioninase (Ashraf et al., 2009) and endo-1,4-beta-D-xylanase.

II. Results and discussion:

i) Isolation and cultivation mangrove associated fungi

Mangroves are highly specialized ecosystem, which are characterized by salt resistant plants growing in the inter-tidal areas along sheltered seacoasts and estuaries in the tropical and subtropical regions. More than 80 species of mangroves have been recorded all over the world, out of which, 61 species are found in India. 19 of these species find their home in the saline waters of Goa

Pneumatophores of mangrove plant, *A. illicifolius* used for the isolation of associated fungi of the present study were collected from the bank of Cumbarjua canal, Banastari, Goa. After cleaning with sterile water pneumatophores were placed onto potato dextrose agar prepared in seawater-distilled water (75:25). Seven different fungi were purified by repetitive sub culturing. Morphological features visible with naked eyes of these fungi along with their identification and biological activities are tabulated below **(Table 2.1)**.

Table 2.1: Fungal epiphytes isolated from pneumatophores of *Acanthus illiccifolius*

Culture No.	Description	Species	Biological activity
1.	Light green	*Aspergillus ornatus*	Anticancer
2.	Dark green	*Aspergillus cervinus*	Anticancer
3.	Brown		
4	Dark green		
5	Green with red base		
6	Greenish brown		
7.	Off white pigmented yellow	*Aspergillus. flavipes*	Anti-inflammatory

All the fungi were grown on 50 ml scale and extracted with ethyl acetate, which was assessed for bioactivity in high throughput screening (HTS). Culture no.1 and 2 were found to be anticancer positive, whereas culture no. 7 exhibited anti-inflammatory activity (IL-6 and TNF-α inhibitor). Examination of morphological features of condiophores of these fungi led to the identification of culture No. 1, 2 and 7 as *Aspergillus ornatus*, *Aspergillus cervinus* and *Aspergillus flavipes* respectively (**Fig 2.2a**). *A. ornatus* culture stored in 10% glycerol at -20°C for several days could not be re-grown or revived and *A. cervinus* did not produce sizeable quantity of the extract, therefore these two fungi could not be taken up for chemical studies. *A. flavipes* was grown on a potato dextrose broth (**Fig 2.2 b**) prepared in 1:1 seawater-distilled water under static condition for 21 days.

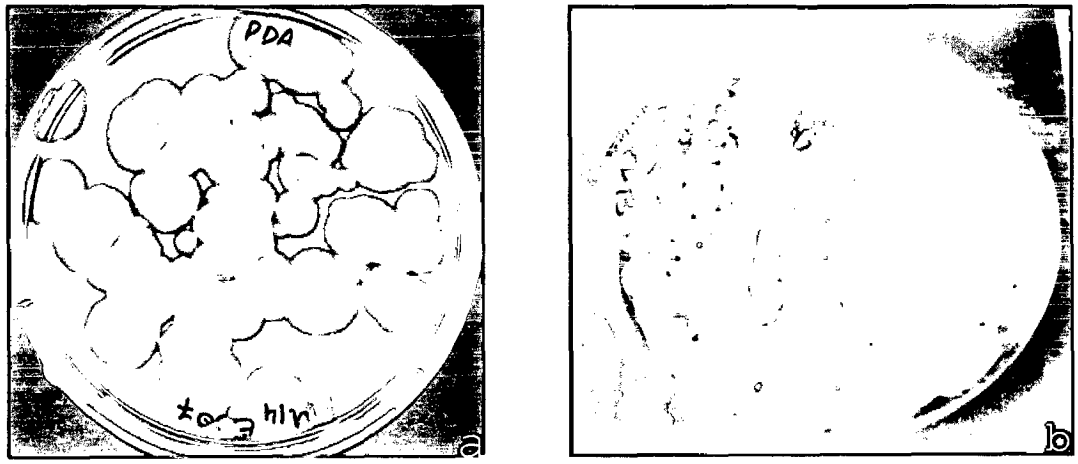
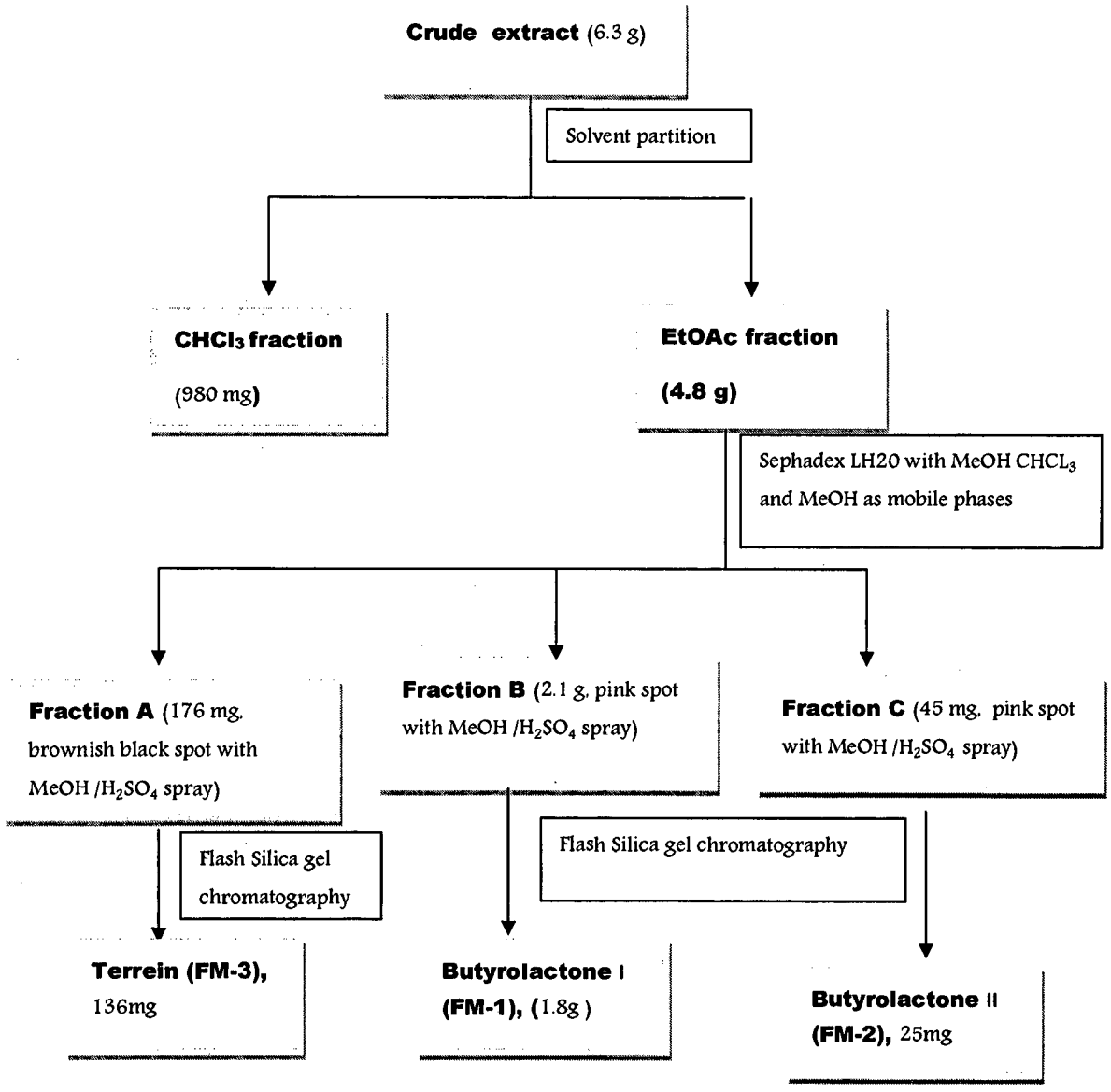


Fig 2.2: *A. flavipes* cultured on a) PDA, b) PDB

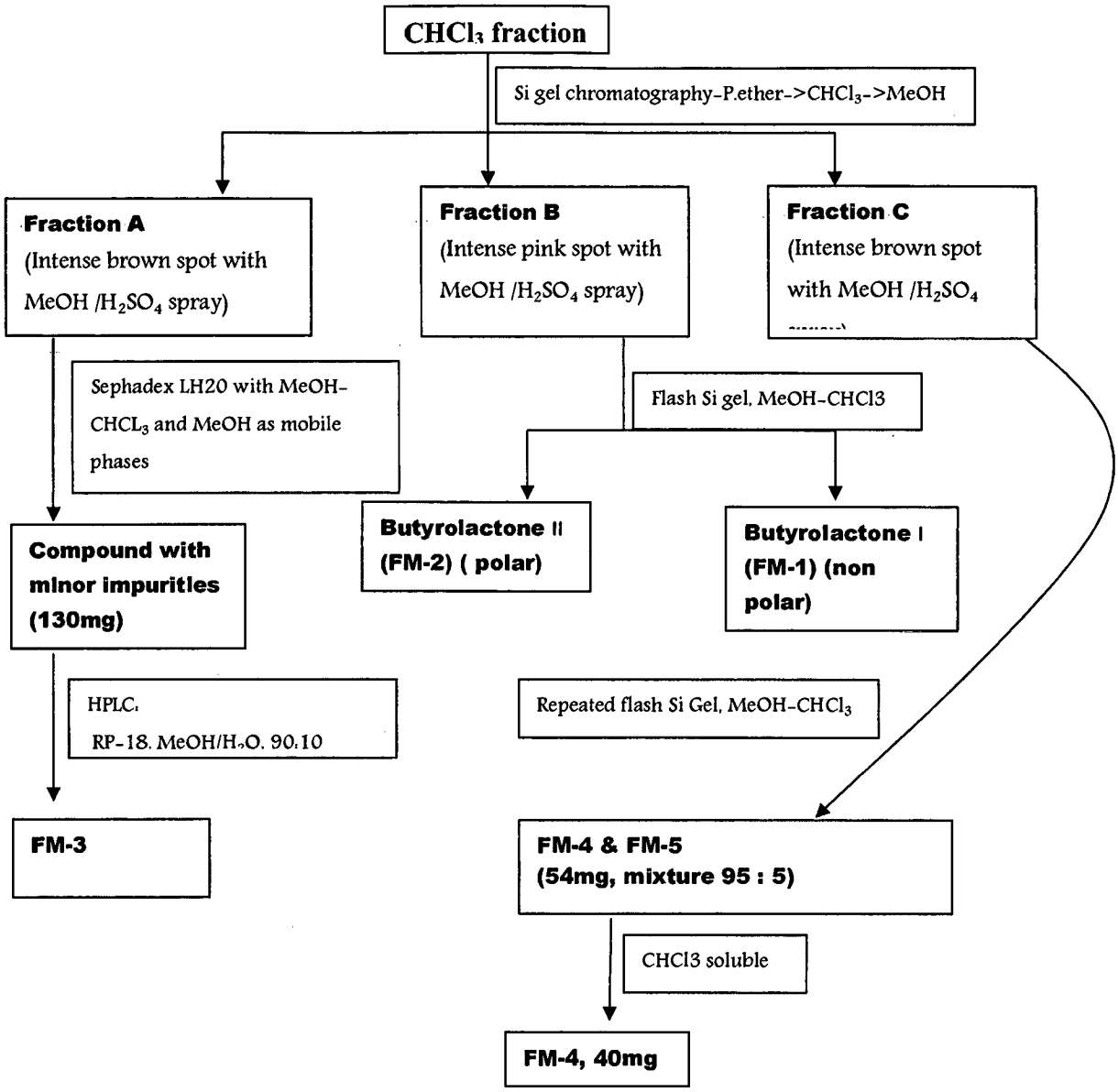
ii) Purification of metabolites

after filtration, 21 days old culture broth yellow in colour was extracted with ethyl acetate and the mycelium was extracted with methanol. Both the extracts on TLC analysis showed similarities in metabolite profiles. Hence, these extracts were combined, and then fractionated between $CHCl_3$ and EtOAc. Each organic extract was purified either with repeated Sephadex LH 20 gel filtration (MeOH/$CHCl_3$, 1:1, v/v and MeOH) or on silica gel column ($CHCl_3$/Petroleum ether, 0-100% to

MeOH/$CHCl_3$, 0-30%) as depicted in flow diagram **(Scheme 2.1 and 2.2)**. These chromatographic fractionation led to the isolation of five pure compounds designated as, butyrolactone I **(FM-1)**, and butyrolactone II **(FM-2)** (+) terrein **(FM-3)**, *meso*-4β-(1'-propenyl)cyclopentane-1β,2β-diol **(FM-4)** and (+) 4-(1'-propenyl) – *trans*-cyclopentane-1,2-diol **(FM-5)**



Scheme 2.1: Purification of metabolites from EtOAc Extract

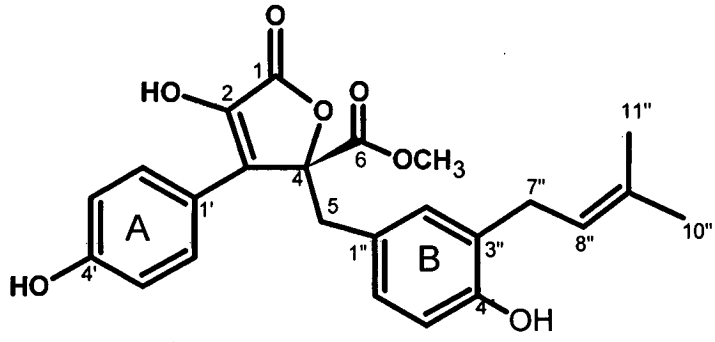


Scheme 2.2 : Purification of metabolites from $CHCl_3$ fraction

iii) Structure elucidation of metabolites

Butyrolactone I (**FM-1**) was obtained as colourless crystalline solid, which would eventually turn into a gummy material on storage for long time. **FM-1** showed

negative Lassigne's test for nitrogen, sulfur and halides (Cl, Br and I) indicating that molecule was mainly made up of C, H and O.



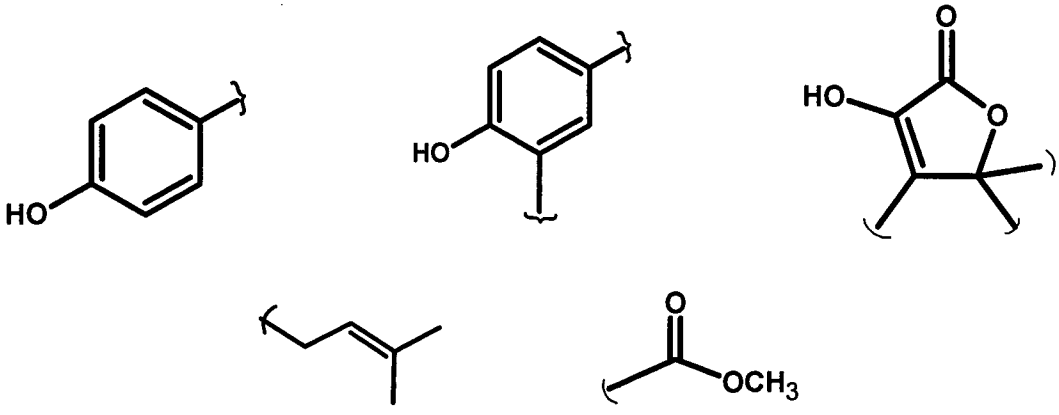
(+) Butyrolactone I (**FM-1**)

Molecular formula of the **FM-1** was determined to be $C_{24}H_{24}O_7$ from its HRESITOFMS. It showed a pseudomolecular ion peak $[M+H]^+$ at 425.1516 (calcld. 425.1522 for $C_{24}H_{25}O_7$). IR spectrum (**Fig 2.3a**) showed presence of ester/lactone carbonyl 1739 cm^{-1}, strong OH_{str} at 3384 cm^{-1} and presence of absorption at 1610 cm^{-1} was suggestive of aromaticity in the molecule. ^1H NMR (**Fig 2.3b**) recorded in CD_3OD accounted for 21 hydrogens, therefore **FM-1** contained three hydroxyls. Analysis of ^1H, ^{13}C and DEPT NMR data (**Table 2.2**) revealed the nature of all 24 carbons as three methyls of which one was methoxyl and two vinylic; two benzylic methylenes; eight methines of which three belonged to trisubtituted aromatic ring A and two equivalent pairs belonged to p-substituted aromatic ring B; and eleven quartenaries of which two were ester carbonyls, eight were olefenic/aromatic and only one was sp3 hybridized (C-5, δ_c 84.6), which was also the only chiral centre in the molecule.

In ^1H NMR spectrum of **FM-1** exihibited aromatic A_2B_2 system of ortho coupled doublets (J = 8.7 Hz) attributed to two pairs of symmetric protons δ_H 7.54 and 6.82 ring A and another was ABM system of ring B δ_H 6.50, dd, J = 8.7, 1.8 Hz; C-6"; δ_H 6.46, d, J = 8.7 Hz; C-5" and δ_H 6.36, d, J = 1.8 Hz; C-2" . In ^{13}C NMR and DEPT (**Fig 2.3c**) two phenolic carbons were observed at δ_C 157.1, C-4" (ring A) and 152.8, C-4' (ring B) and three alkylated aromatic carbons were evident from the signals at δ_C

120.9, C-1' (ring A), 122.8, C-1" and 127.0, C-3" (both for ring B). Therefore, one of the aromatic systems was p-substituted phenol; the other was unsymmetrical trisubstituted phenol.

NMR signals δ_H 3.02, d, J = 7.2 Hz 2H; δ_C 26.46 C-7" coupled to a vinyllic proton δ_H 5.0, t, J = 7.2 Hz; δ_C 122.8, C-8," and two vinyllic methyls δ_H 1.52, s; δ_C 15.5 C-11" and δ_H 1.61 s; δ_C 23.7, C-10" both attached to quarternary olefinic carbon δ_C 130.7, C-9" suggested presence of a prenyl group. The chemical shift of the methylene δ_H 3.02, indicated the prenyl to be attached to the aromatic ring. NMR signals δ_H 3.73, s; δ_C 51.6, C-5OMe and δ_C 169.4 s, C-5 indicated presence of carbomethoxy group. Two mutually coupling doublets of geminal protons δ_H 3.35, d, J = 13.0 Hz; δ_H 3.41, d, J = 13.0 Hz; δ_C 37.4, C-6 is indicative of another benzyllic methylene probably attached to the chiral centre.



Substructures of FM-1 identified from spectroscopic data

Presence of two aromatic rings, two ester carbonyls and two C-C double bonds accounted for 12 degrees of unstauration out of 13, therefore presence of an alicyclic ring in the **FM-1** was evident. The above NMR data accounted for 20 carbons out of 24 carbons present in the molecule which meant three quarternary sp2 carbon signals δ_C 166.1, C-1; 137.4, C-2; 126.2, C-3 and only quaternary sp carbon signals 84.6, had to be accounted for. IR absorption of 1739 cm^{-1} was strong evidence that the **FM-1** had be α, β unsaturated γ-lactone ring comprising of four quarternary carbons (13^{th} degree of unsaturation). Saturated γ-lactones are known absorb at 1770 cm-1,

while α, β unsaturated γ-lactones absorb around 1740 cm^{-1}. Out of the three -OHs two are accounted for phenolic; hence last one had to be enolic, as there was no evidence of alcoholic hydroxyl in the molecule.

The comparison of the above data revealing the presence of above five substructures with a literature report (Rao et al., 2000) suggested that the molecule under investigation is no different from a butenolide named butyrolactone I (Kiriyama et al., 1977), which was isolated for the first time in 1977 from a terrestrial isolate of *A. terreus*.

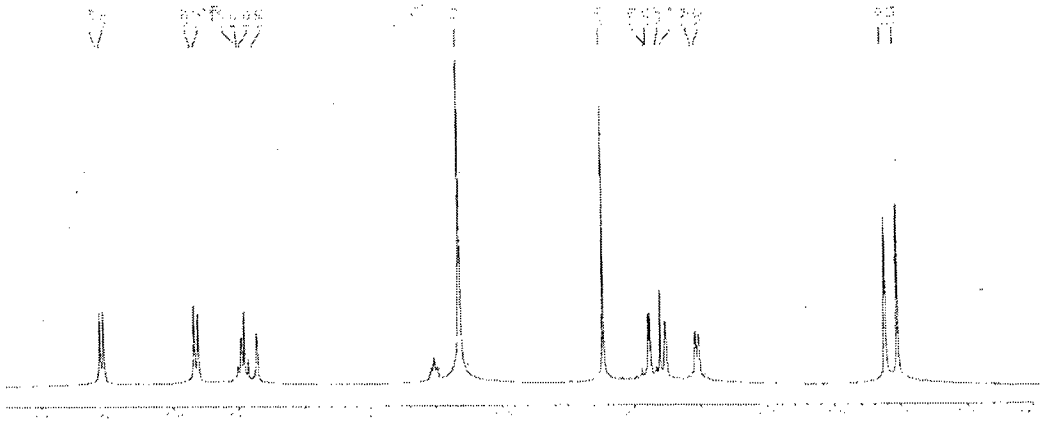


Fig 2.3b: ^{1}HNMR of FM-1

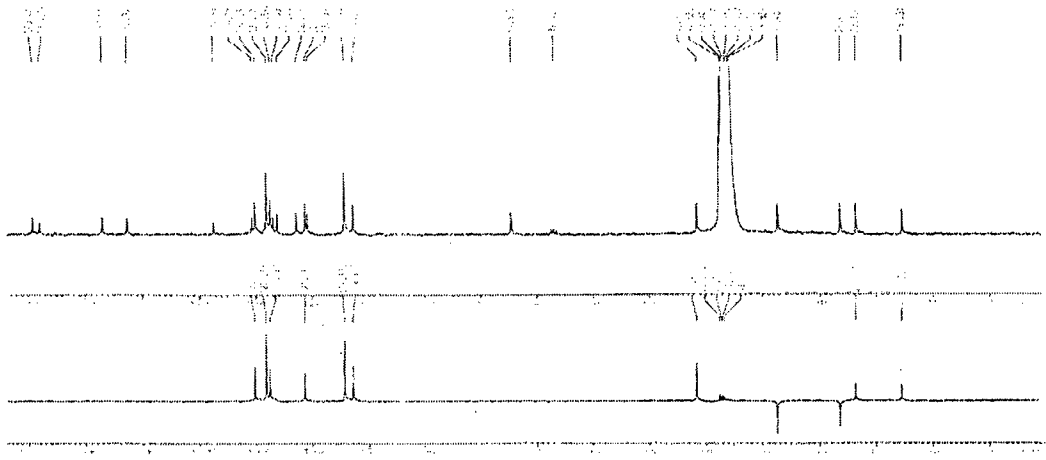


Fig 2.3c: ^{13}C NMR and DEPT of FM-1

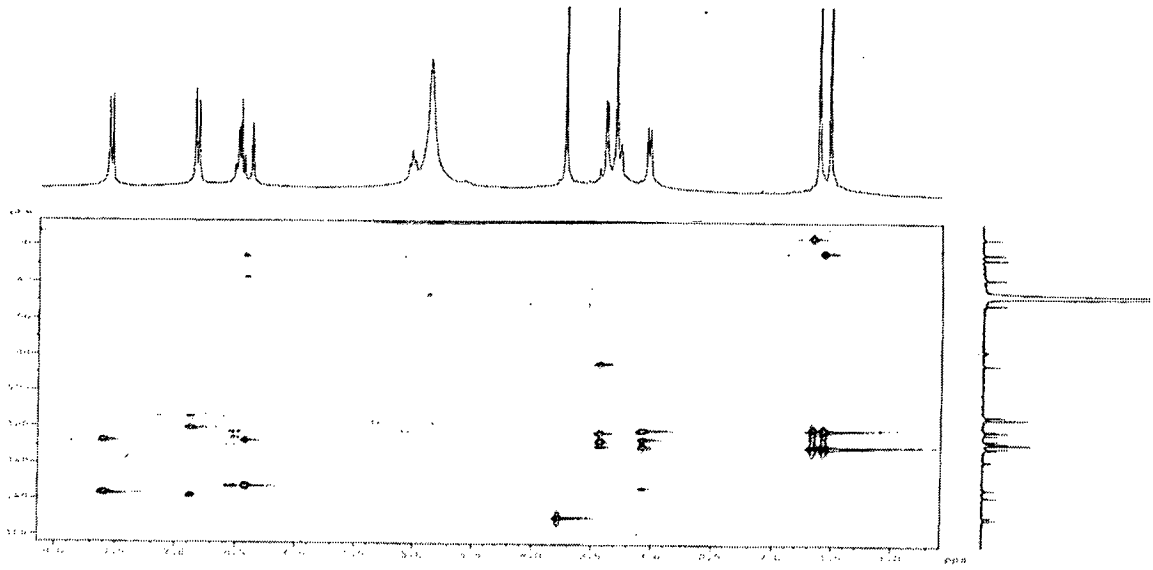
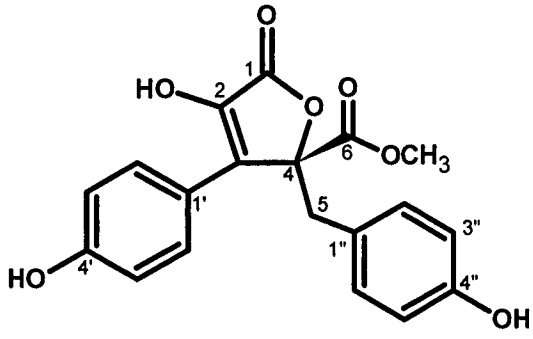


Fig 2.3d: HMBC of FM-1

FM-2 was obtained as gummy solid$[\alpha]^{28.8}_{D}$ +85 (c = 1, EtOH). It gave spot on TLC more polar, but having colour and appearance like **FM-1**, indicating it to have similar structure. IR spectrum showed presence of ester/lactone carbonyl 1739 cm^{-1}, phenolic/enolic OHs at 3360 cm^{-1} and presence of absorption at 1608 cm^{-1} was suggestive of aromaticity in the molecule. The molecular formula $C_{19}H_{16}O_7$ was revealed by ESI-MS, which showed $[M+H]^+$ at m/z 357, and was 68 units lesser compared to **FM-1**, suggesting absence of prenyl chain.



(+) Butyrolactone II (**FM-2**)

The absence of prenyl chain was also evident from the absence of signals for vynilic methyls, allylic methylene, and olefinic methine in its NMR and complemented by the presence of signals for two pairs of *p*-disubstituted benzene rings in ^{1}H NMR (**Fig 2.4a**) and 15 signal accounting for 19 carbons in ^{13}C NMR (**Fig 2.4b**). Therefore, the **FM-2** was identified as deprenyl derivative of **FM-1** by comparison f its NMR data (**Table 2.2**) with **FM-1**. **FM-2** is also a known terrestrial natural product.

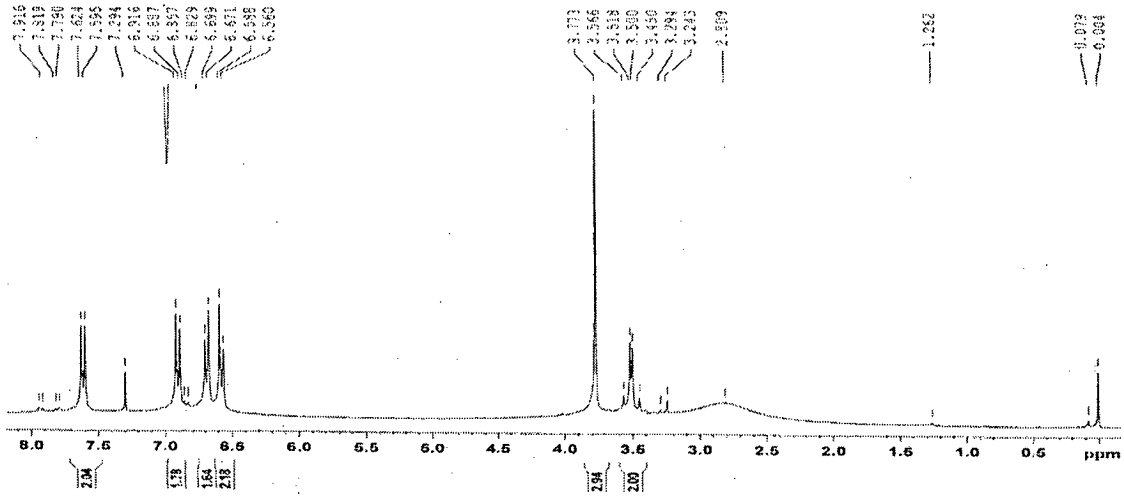


Fig 2.4a: ^{1}HNMR of FM-2

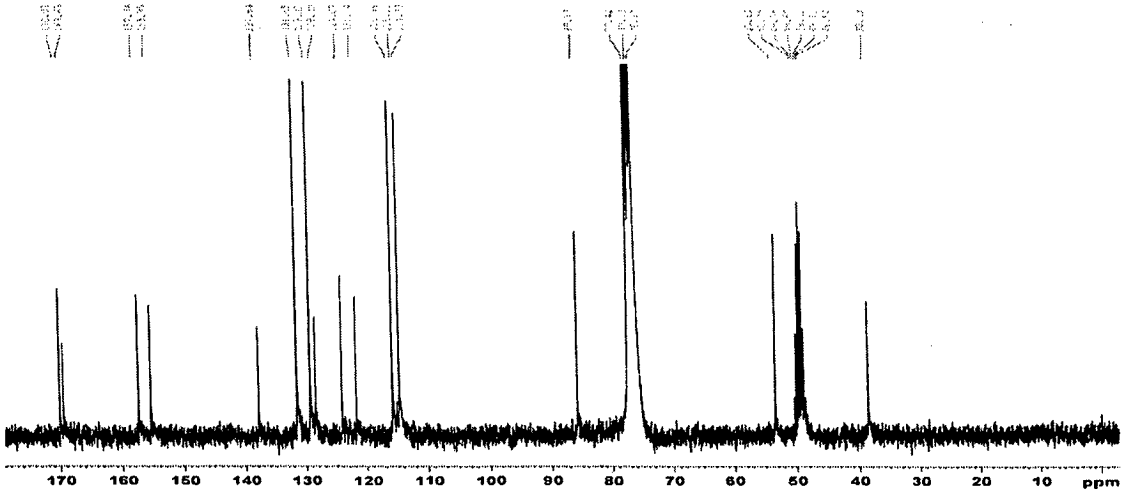
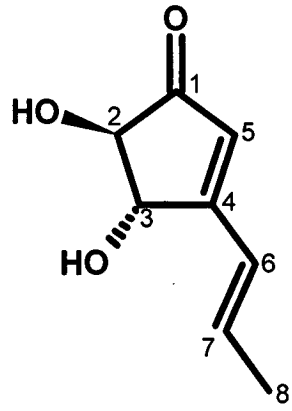


Fig 2.4b: ^{13}CNMR of FM-2

Table 2.2: NMR data of FM-1 and FM-2

Position	Butyrolactone I (FM-1) (CD_3OD)		Butyrolactone II (FM-2) ($CDCl_3$)	
	δ_H, mult., J(Hz)	δ_C, mult.	δ_H, mult., J(Hz)	δ_C, mult.
1		166.1 s		169.4, s
2		137.4 s		137.8, s
3		126.2 s		128.3, s
4		84.6 s		85.7, s
5	3.35, d, 13.0 3.41, d, 13.0	37.4 t	3.47, d, 15.0 3.54, d, 14.4	38.4, t
6		169.4 s		170.0, s
1'		120.9 s		124.0, s
2' (6')	7.53, d, 8.7	128.1 d	7.61, d, 8.7	131.4, d
3'(5')	6.82, d, 8.7	114.3 d	6.90, d, 8.7	115.8, d
4'		157.1 s		157.3, s
1"		122.8 s		121.7, s
2"	6.36, d, 1.8	130.1 d	6.68, d, 8.4	129.3, d
3"		127.0 s	6.57, d, 8.4	114.7, d
4"		152.8 s		155.9, s
5"	6.44, d, 8.7	112.8 d	H-3" eq.	C-3" eq.
6"	6.48, d, 8.7, 1.8	127.5 d	H-2" eq.	C-2" eq.
7"	3.02, d, 7.2	26.4 t		
8"	5.0, t, 7.2	121.3 d		
9"		130.7 s		
10"	1.61, s	23.7 q		
11"	1.52, s	15.5 q		
6-OMe	3.72, s	51.6 q	3.77, s	53.4, q

FM-3 (terrein) was obtained as colourless crystalline needles. It gave large intense brownish black spot with H_2SO_4-MeOH spray on TLC. Its molecular formula $C_8H_{10}O_3$ with four sites of unsaturation was deduced from HRESITOFMS, which showed pseudomolecular ion at $[M+H]^+$ at m/z 155.1312. IR spectra gave very sharp bands at 3396 and 3209 cm^{-1} due to presence of hydroxyl groups, C-H str. at 3075, 2900 and 2858 cm^{-1}, CH bending at 1444 cm^{-1}, conjugated five membered ketonic C=O str. at 1693 cm^{-1}, C=C str. 1633 cm^{-1} and C-O str. 1083 cm^{-1}.



(+) Terrein (**FM-3**)

Its ^{13}C NMR (**Fig 2.5b**) signal at δ_c 205.6 s suggested presence of a cyclic ketone functionality. Proton signals (**Fig 2.5a**) at δ_H 6.00 s, 1H, 6.44, d, J = 15.8 Hz, 1H coupled to another sp2 methine δ_H 6.92 dq, J = 15.8 Hz, 6.9 Hz, which in turn showed coupling to a methyl at δ_H 1.96, d, J= 6.9 Hz and the carbon signals δ_c 125.9 d, 170.8, s 126.4 d, 141.8 were indicative of two c-c double bonds in conjugation with carbonyl. The mutually coupling oxygenated methines at δ_H 4.07 , δ_c 82.4 C-2 δ_H 4.67, δ_c 78.1 with small coupling constant of 2.4 Hz was indicative trans stereochemistry of two hydroxyls. These data (**Table 2.3**) were found identical to that of a known metabolite terrein reported in the literature (Dunn et al., 1975).

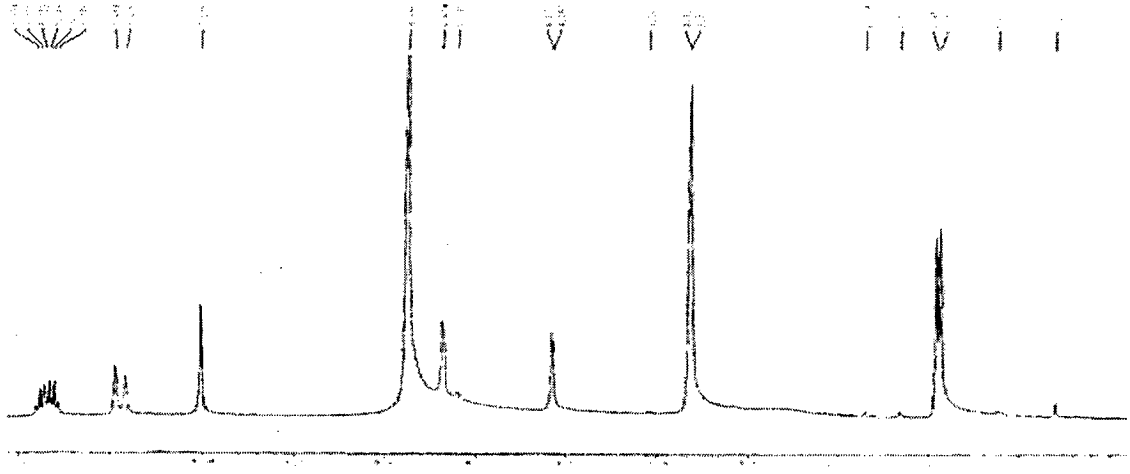


Fig 2.5a: ^{1}HNMR of FM-3

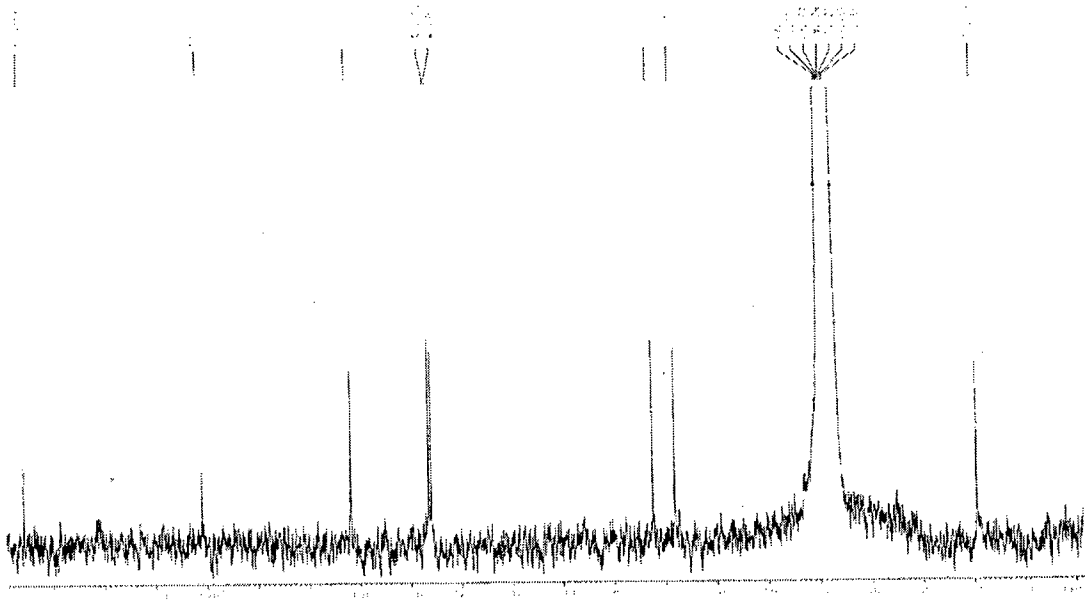
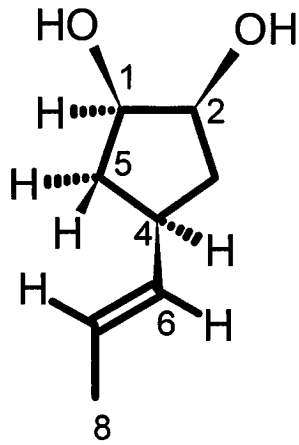


Fig 2.5b ^{1}HNMR of FM-3

FM-4 was obtained as optically inactive colourless liquid. It gave a large, intense brown spot with methanolic H_2SO_4 acid spray on TLC. It showed M^+ at m/z 142 in EIMS. From its MS and NMR data molecular formula $C_8H_{14}O_2$ was assigned to it. It showed absorption band in its IR spectrum at 3360 cm^{-1} indicative of hydroxyl groups. The ^{13}C NMR (**Fig 2.6b**) showed six carbons signals suggesting that compound had element of symmetry and revealed the constitution of all the carbons in the molecule. It included signals for 1-propenyl moiety, oxymethine carbons, methylene carbons and a methine carbon. The molecular formula requires 2 degrees of unsaturation. An alkene functionality of propenyl system accounts for one degree of unsaturation, which means other five carbons are involved in constructing a symmetric cyclopentane system. In the proton NMR (**Fig 2.6a**), the methyl of 1-propenyl resonated at δ_H 1.63, d J = 5Hz. and the olefenic methines showed merged multiplet at δ_H 5.39, 2H. One of the olefenic proton was coupled to a methine δ_H 2.35 ddd. (J = 6, 8, 9.5 Hz), (actually appeared as sextet due to merging of peaks) which in turn showed coupling of 9.5 to a pair of protons signal at δ_H 1.46 (ddd, J=5,9.5, 13.5 Hz) and smaller coupling of 8 Hz to a pair of protons at δ_H 2.09, (ddd, J = 5 , 8, 13.5

Hz). Each of the pairs of hydrogen showed correlation to the same carbon signal δ_c 38.5..indicating the pairs of hydrogen were sterochemically different (α and β hydrogens of C-3 and C-5). Each of these sets of protons showed coupling to a signal at δ_H 3.98 (2H, br t, J = 5 Hz) attributed to oxymethine protons. From these data, planar structure of **FM-4** was deduced. The *E* stereochemistry of double bond was rather easily assigned from the chemical shift of the vynilic methyl (δ_c 17.6) as was difficult to assign by using coupling constants of the merged olefenic hydrogens. The syn relationship of two hydroxyls was established from the observation that the compound was optically inactive and so the two hydroxyls had to be syn, so as to maintain the C-2 symmetry in the molecule for the compound to be meso. The 1-propenyl can either be syn or anti with respect to two hydroxyls. The same molecule with all three substituents with syn stereochemistry has been reported as natural product, wherein the stereochemistry of the propenyl unit has been assigned from NOESY data. Our IR and NMR spectroscopic data was in agreement with the reported (Ghisalberti et al., 1990), but in order to confirm their findings, we obtained the NOESY spectrum (**Fig 2.6c**) on our compound. The NOESY data (**Fig 2.6d**) established the propenyl group oriented syn with the two hydroxyls.



FM-4

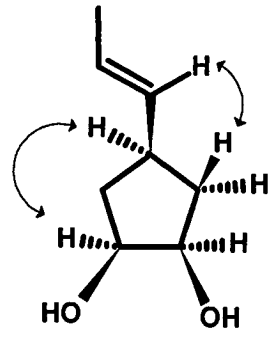


Fig 2.6d : NOESY correlation of FM-4

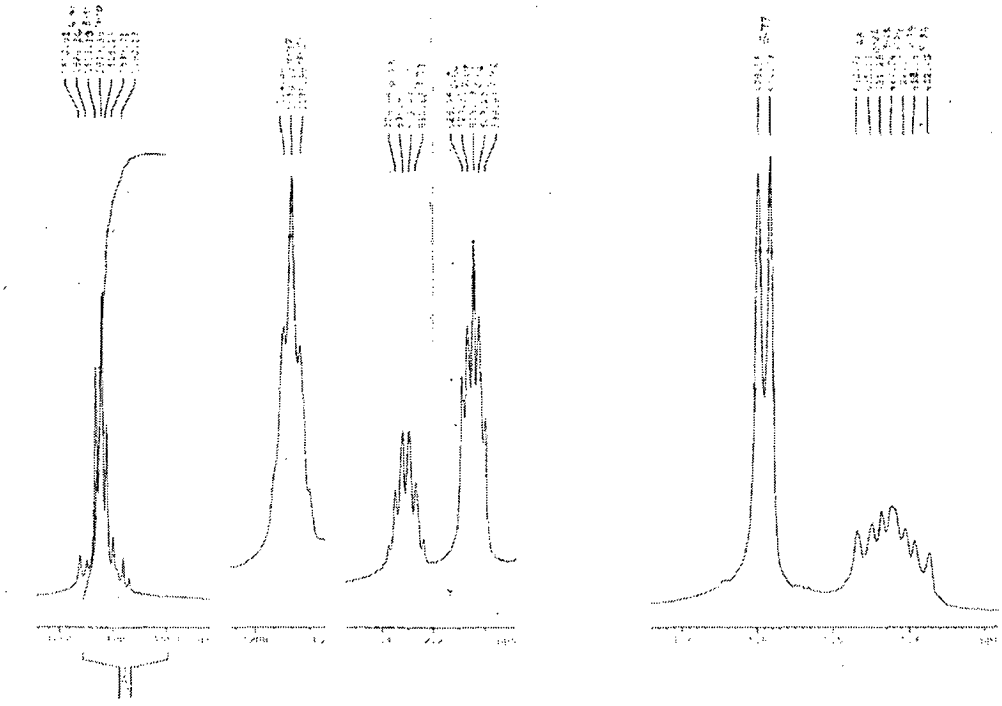


Fig 2.6a: ^{1}HNMR (expansion) of FM-4

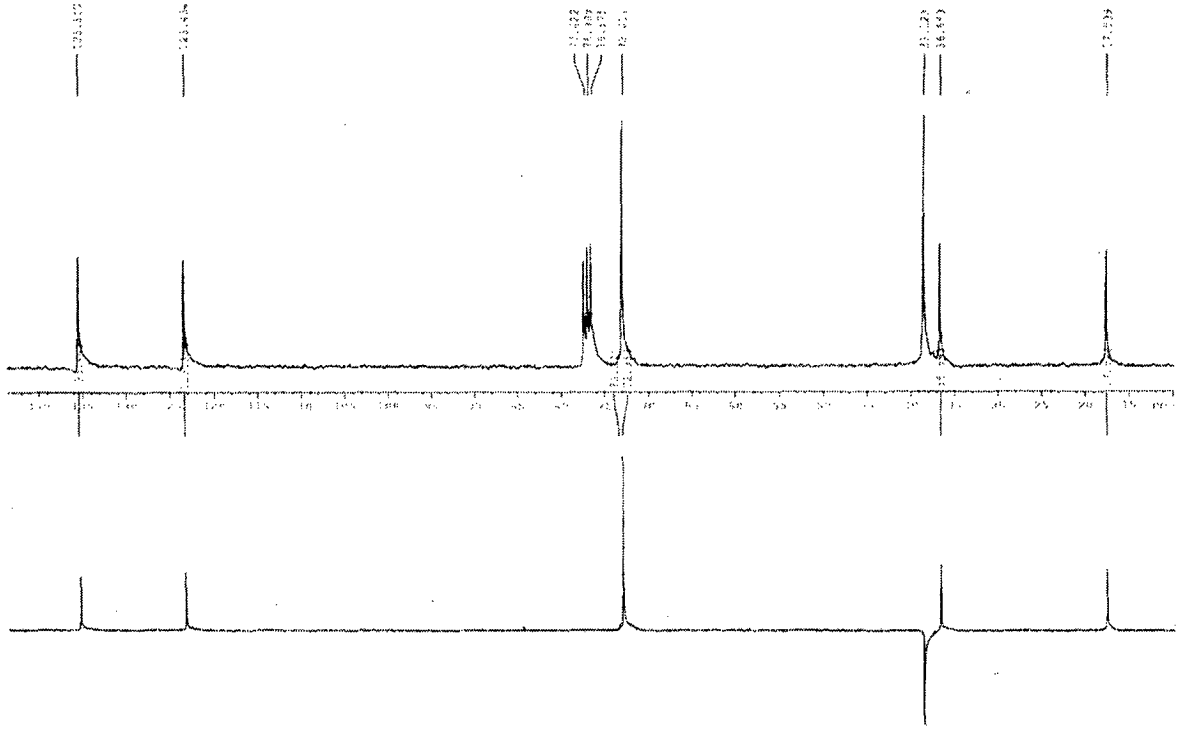
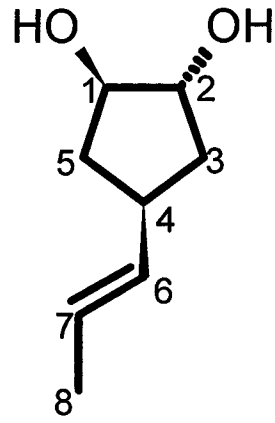


Fig 2.6b ^{13}C NMR and DEPT of FM-4

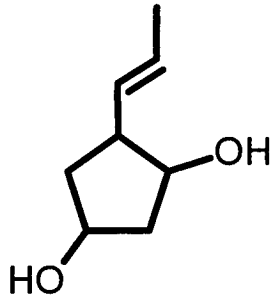
FM-5 was obtained as optically active viscous liquid. It gave large intense bluish black spot with H_2SO_4-MeOH spray similar to compound **FM-3** and **FM-4** and also identical in retardation factor with **FM-3**. Unlike **FM-3, FM-5** was soluble in $CHCl_3$ and showed no fluorescence on TLC under UV light (254nm). Absence of fluorescence could be the indication that the **FM-5** lacked the conjugated carbonyl functionality in the molecule. As anticipated, IR spectrum of **FM-5** did not show any carbonyl absorption. It showed absorption bands at 3360 cm^{-1} for hydroxyls like **FM-4**. EIMS gave M^+ at m/z 142 indicating molecular formula $C_8H_{14}O_2$ same as that of **FM-4**. The ^{13}C nmr (**Fig 2.7b**) showed eight carbons signals, which included signals for *E*-1-propenyl moiety, two oxymethine carbons, two methylenes and a methine. These ^{13}C nmr data when compared with **FM-4** suggested that **FM-5** had same constitution, but lacks C-2 symmetry due to which it shows eight carbons signals and also exhibited optical activity.



FM-5

The certain similarities between **FM-4** and **FM-5** were also seen in their ^1H NMR (**Fig 2.7a**). The ^1H NMR gave signals for 1-propenyl moiety (δ_H 1.62, d, 4.5 Hz, 3H δ_C 17.74 and δ_H 5.38, m, 2H δ_C 123.6 and 135.3). Additionally, there were five sets of proton multiplets. Their multiplicities and coupling constant could not be properly derived, as the splitting had complex pattern due to coupling with multiple protons.

One of the olefinic hydrogen showed coupling to apparent sextet at δ_H 2.64, 1H, which showed coupling to two three sets of proton signals at δ_H 2.15 m 1H; δ_H 1.75, m, 2H and δ_H 1.29,m 1H. Out of the three sets of proton signals, two sets of proton signals δ_H 2.15, m, 1H and δ_H 1.29, m, 1H were due to the stereochemically non-equivalent hydrogens of a methylene (C-3). β-hydrogen of C-3 is shielded due to bulky groups (β-hydroxyl and a β-propenyl) and appears upfield, whearas α-hydrogen of C-3 which is less shielded due to α-hydrogen on adjacent carbons appears downfield. The third set of signal were due to two methylene protons of C-5, whose both α and β-hydrogens experiences nearly similar electronic environment due to one bulky group on adjacent carbon (either C-1-OH or C-4-propenyl) on their face. The hydroxymethines were observed at δ_H 3.95, m and coupled with both the methylenes. Although, these data (**Table 2.3**) reveals the compound to have trans orientation of the two adjacent hydroxyls in **FM-5,** the fact that the asymmetry or chirality in the molecule is caused due to the geometry of the two hydroxyls (with 1,2,4 constitution of cyclopentane ring) and not due to the some other constitution (shown in **17-19**) is confirmed from the ^{1}H-^{1}H-COSY (**Fig 2.7c**).



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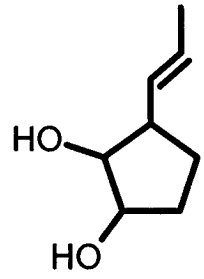

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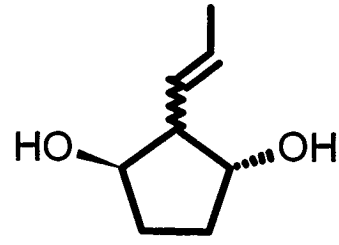

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Each sets of the three proton signal resulting due to the two methylene C-3 and C-5 showed correlation to a methine C-4 and also to oxymethines C-1 and C-2, while no correlations were observed between the two methylene protons and also between any of the oxymethines and C-3 methine (**Fig 2.7d**).

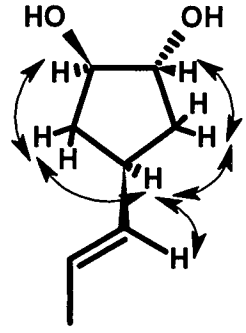


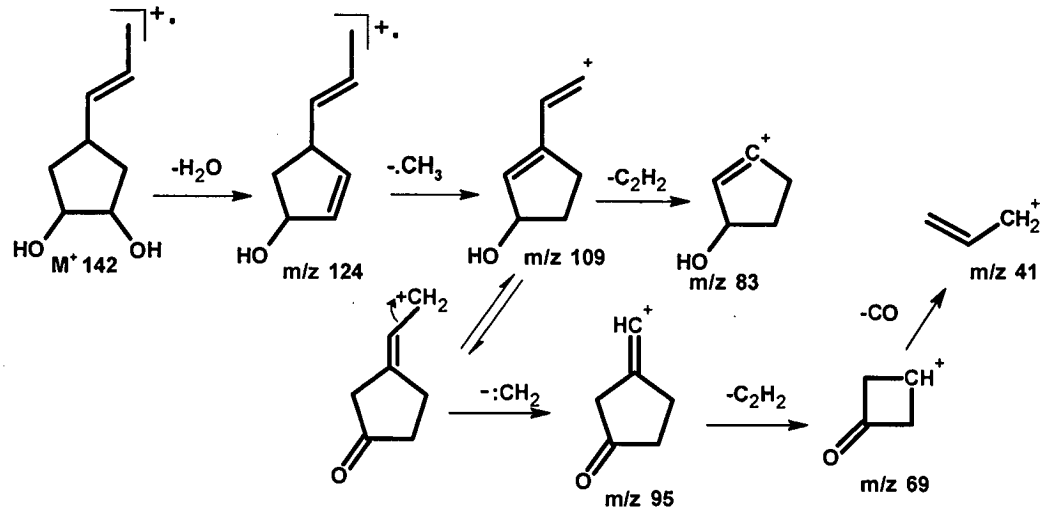
Fig 2.7d: H-H COSY of FM-5

Table 2.3 NMR data of FM-3, FM-4 and FM-5 at 300 MHz

Carbon	(FM-3) (CD_3OD)		(FM-4) ($CDCl_3$)		(FM-5) ($CDCl_3$)	
	δ_H, mult., J(Hz)	δ_C, mult.	δ_H, mult., J(Hz)	δ_C, mult.	δ_H, mult., J(Hz)	δ_C, mult.
1		208.6, s	3.93, br t, 5	73.0 d	3.91-3.98, m	79.4 d
2	4.59, d, 2.3	82.4, d	H-1 eq	C-1eq	3.91-3.98, m	78.5, d
3	4.06, d, 2.4	78.1, d	2.05, ddd, 13.5, 8,0, 5.0 (H-3α) & 1.43, ddd,13.5, 9.5, 5.0 (H-3β)	38.5 t	2.15, ddd, H-α 1.29, ddd, H-β	39.1, t
4		170.8, s	2.30, ddd, 9.5, 8.0, 6.0	36.6 d	2.65, m	37.1, d
5	6.00, s	126.4, d	H-3 eq.	C-3 eq.	1.76, m	38.3, t
6	6.26 d, 15.9	125.9, d	5.29-5.43, m	135.5 d	5.37-5.40, m	135.0, d
7	6.84, dq, 15.9, 6.9	141.8, d	5.29-5.43, m	123.4 d	5.37-5.40, m	123.1, d
8	1.94, d, 6.9	19.4, q	1.59, d, 4.8	17.5 q	1.63, d, 4.8	17.7,t

The EI-MS spectrum of two epimers, **FM-4** and **FM-5** displayed the same fragmentation pattern. The molecular ion of both compounds initially eliminate a

molecule of water to yield fragment ion m/z 124, and the smaller fragment ions m/z 109, 95, 83, 69, 56 and 41 are then generated from this ion. The formation of this mass fragments are explained in the **scheme 2.3** below.



Scheme 2.3: Mass fragmentation of FM-4/FM-5

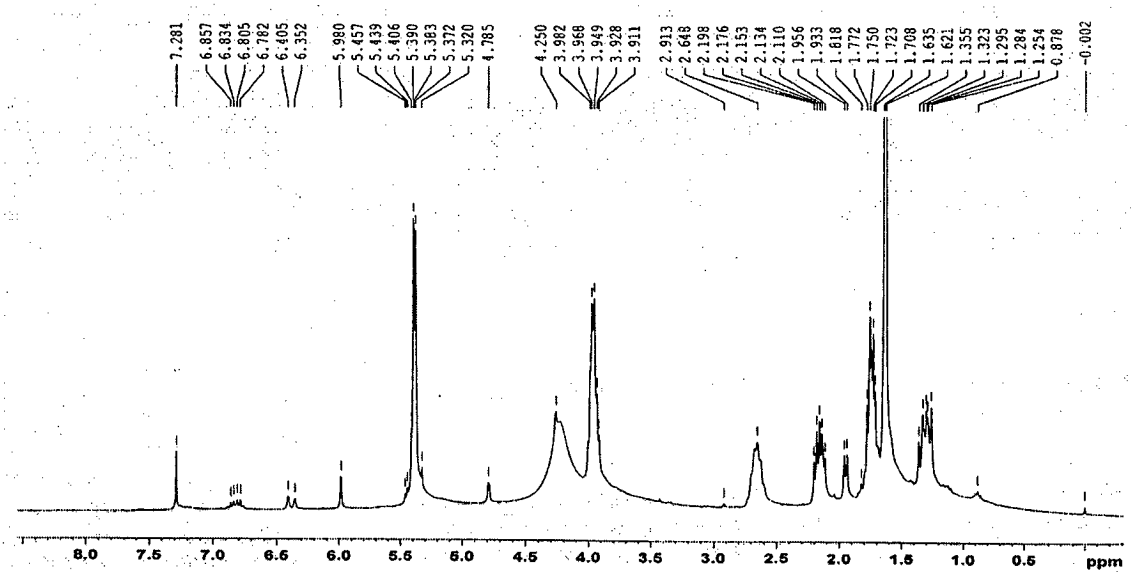


Fig 2.7a : ^{1}HNMR of FM-5

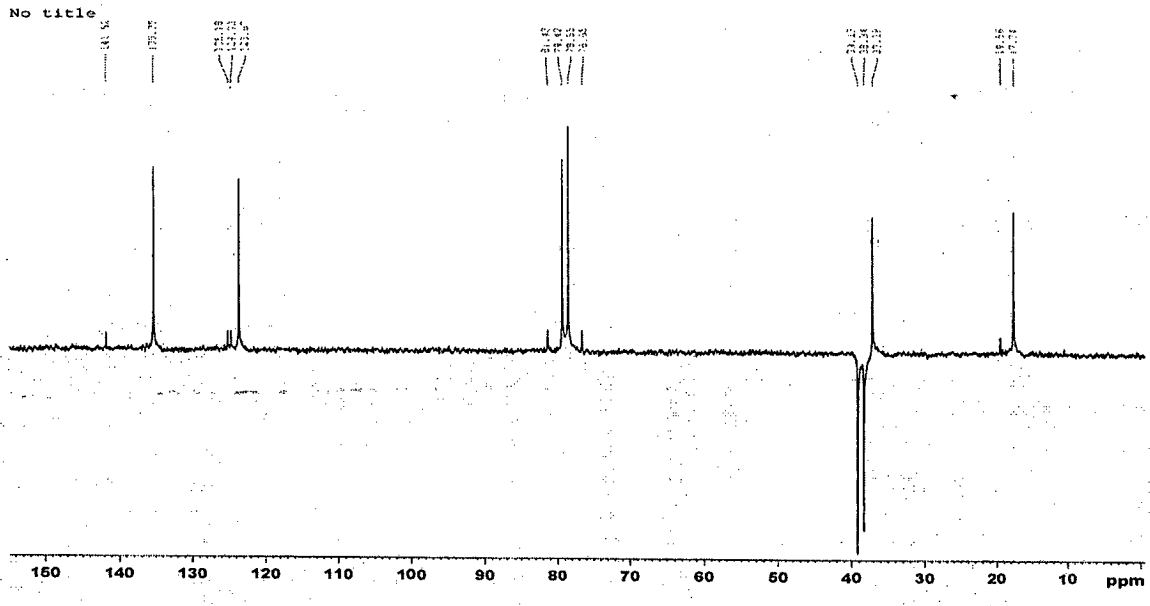


Fig 2.7b: DEPT showing signals for all the carbons of FM-5

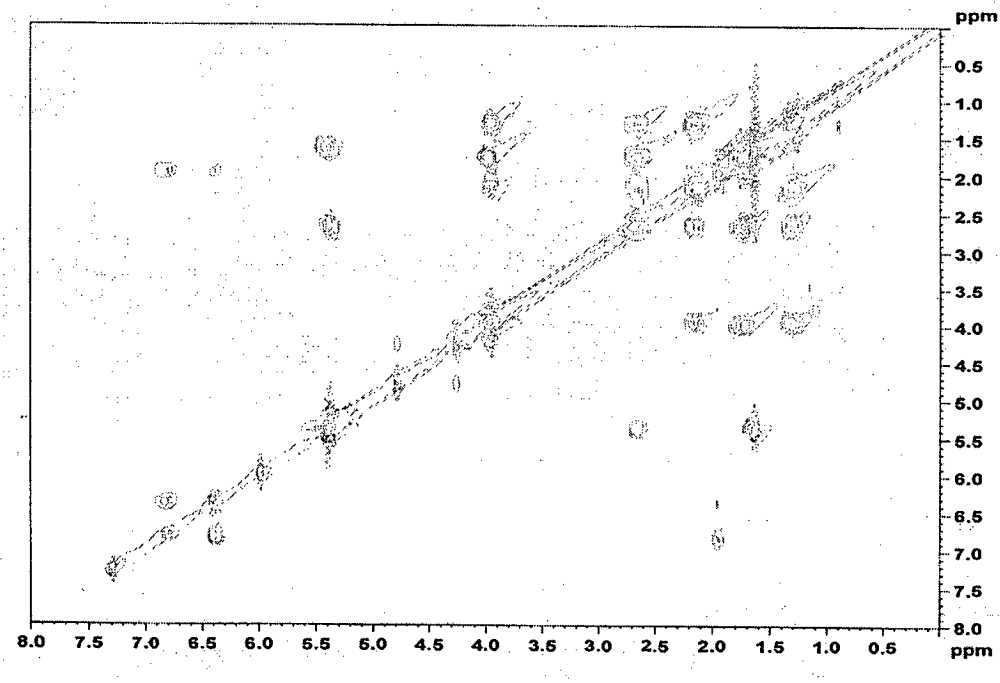


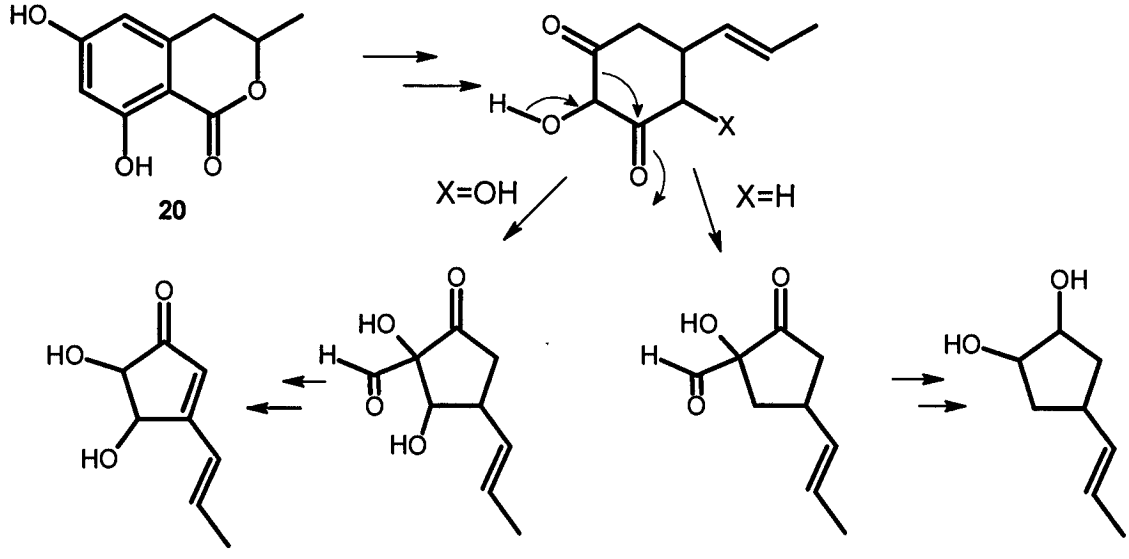
Fig 2.7c: ^{1}H-^{1}H COSY of FM-5

All the known compounds were first reported as metabolites of terrestrial isolates of *A. terreus*. **FM-3** is known from various terrestrial as well as marine-derived fungi, whereas **FM-4** *A. terreus* is the only soure of **FM-4** (Ghisalberti et al., 1990). **FM-1**

is also known to be a metabolite of *A. fischeri* TISTR 3272 (Phattanawasin et al., 2007). The known metabolites are isolated for the first time from *A. flavipes*, a fungus, which is closely related to *A. terreus*.

iv) Biogenesis of FM-1 to FM-5

The biosynthesis of butenolides (**FM-1** and **FM-2**) in *A. terreus* has been well known and the same has been discussed in **section 2.4, subsection iii** of this chapter. Among cyclopentanediols, **FM-3** is known to biosynthesize from a resorcyllic acid lactone (**20**) (polyketide), whereas as proposed by Ghilsaberti et al. (1990) for **FM-4**, its epimer **FM-5** must also be derived from the same common precursor of **FM-3** as depicted in **Scheme 2.4**



Scheme 2.4 : Biogenesis of cyclopentanediols FM-3, FM-4 and FM-5

v) Biological properties of the metabolites: Interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) activity

The crude extract was able to inhibit IL-6 and TNF-α production from LPS stimulated THP-1 cells (IC_{50} = 2.69 ± 0.5 μg/ml and 6.26 ± 0.4 μg/ml suggesting discovery of discovery of molecules that would be potent anti-inflammatory. Interleukins are a family of cytokines that govern leukocyte function in response to any challenge to the immune system. Over-expression of interleukins has been implicated in a variety of inflammatory diseases. One such interleukin-6 (IL-6) is an ubiquitous and multi-functional cytokine involved in the early steps of T-cell activation, B-cell differentiation, in the regulation of the acute phase response (which is characterized by the release of a set of serum proteins in response to injury) and in hematopoiesis (the maturation of blood cells from bone marrow). Consistently elevated levels of IL-6 are found in the synovial fluid associated with rheumatoid arthritis (Hossiau and Deuxchaisnes, 1990) and in multiple myeloma tumors (Malik, 1992). Their modulation, particularly via chemical entities, is currently of therapeutic interest.

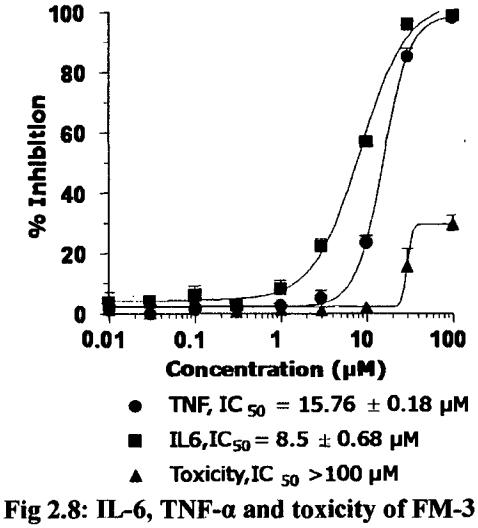


Fig 2.8: IL-6, TNF-α and toxicity of FM-3

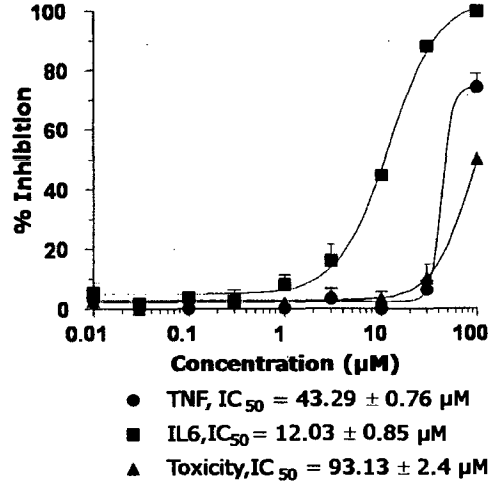


Fig 2.9: IL-6, TNF-α and toxicity of FM-1

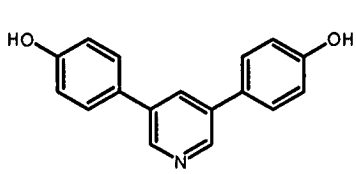
All the metabolites (**FM-1** to **FM-5**) were tested for inhibition of IL-6 and TNF-α in LPS stimulated THP-1 cells according to method described by Hwang and co-workers (Hwang et al., 1993). **FM-3** was found to be the most active. It showed IC_{50} value of 8.5 ± 0.68 μM and 15.76 ± 0.18 μM for the inhibition of IL-6 and TNF-α respectively without affecting the host cells (**Fig 2.8**). The second best was compound **2**, which showed IC_{50} of 12.03 ± 0.85 μM for the inhibition of IL-6, but inhibited TNF-α at comparatively higher concentration IC_{50} 43.29 ± 0.76 μM (**Fig 2.9**). Other compounds did not show any significant activity below 30 μM.

vi) Structure-activity relationship

The two inactive cyclopentanediols (**FM-4** and **FM-5**) differs from each other only by stereochemistry of two hydroxyls, whereas terrein (**FM-3**) (also a cyclopentanediol) has additional functionalities (a keto and endo double bond). These additional functionalities could be responsible for **FM-3** being highly active compared to **FM-4** and **FM-5**.

Among butenolides, the fact that **FM-1** is active and its precursor, **FM-2** is inactive could be attributed to the presence of prenyl chain in **FM-1,** because a similar trend has been also observed for these compounds towards the inhibition of cyclin dependant kinases CDK1 and CDK2. The importance of the prenyl group for maintaining CDK activities have been demonstrated by computer aided molecular modeling using automated docking methods and molecular dynamics simulations studies (Brana et al., 2004).

Till date synthetic derivatives of diarylpyridines (**21**), m-terphenyls (**22**), (Tagat et al., 1995a) and 4-aryl substituted cyclopentane amd imadozole fuzed tryclic pyridinones (**23**) (Tagat et al., 1995b) are the only compounds reported as inhibitors of IL-6. To best our knowledge, except the compound of present study no natural product possessing IL-6 activity has been reported so far.



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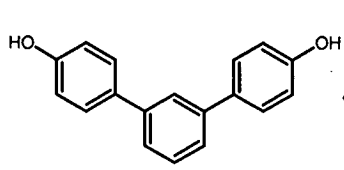

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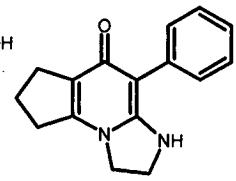

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III. Experimental

i) General experimental procedure

Mixture of methanol and chloroform (10:90, v/v) was used as mobile phase for TLC analysis. Cylopentanediols and butenolides were visualized as blue-black and intense rose colour spots respectively on spraying with methanolic sulphuric acid (95:5, v/v) followed by heating at 120°C.

ii) Fungal isolation and identification

Pneumatophores of *Acanthus illicifolius* were collected from the bank of Mandovi River, Banastarim, Goa, India. Fungi were isolated using standard isolation technique as epiphytes on potato dextrose agar (PDA) from the pneumatophores of mangrove plant *Acanthus illicifolius*. PDA was prepared in 75% natural SW supplemented with penicillin benzyl sodium salt (0.02 g/l) to avoid any bacterial growth. After 6-7 days cultures subtending in medium were sub cultured repeatedly to obtain seven pure fungal cultures.

iii) Fungal mass cultivation

Fungi were grown in Erlenmeyer flasks containing 50 ml. PDB prepared in SW/DW-75:25 and incubated for 21 days under static condition and used for biological activity testing. Similarly 10 L culture of *A. flavipes* was made for isolation of metabolites.

iv) Extraction of metabolites

On 21st day mycelium was separated from the broth by filtration through cotton wool. Broth (50 ml) was extracted with ethyl acetate (30 ml x 3). This extract was used for biological activity screening.

v) Biological activities

Anticancer and anti-inflammatory results were obtained from other laboratories in India.

vi) Identification of fungi

Three active fungi were identified using molecular techniques. Additionally *A. flavipes* was identified from morphological characteristics of its conidiophores (Dr. Sanjay Singh, Agharkar Research Institute, Pune). A voucher specimen of *A. flavipes* with MTCC 5220 is deposited at IMTEC, Chandigarh

vii) Purification of metabolites from A. flavipes

Mycelium was filtered and extracted with methanol (300ml x 3). Culture filtrate (10 L) was concentrated on a rotary evaporator to a smaller volume (2.5 L) under reduced pressure and extracted with ethyl acetate (500ml x 4). Methanol and ethyl acetate extracts were concentrated under vacuum and mixed due to their similar TLC profile. The combined organic extract (6.3 g) was suspended in 10% aqueous MeOH (200ml) and partitioned between $CHCl_3$ (150ml x 4) and EtOAc (150ml x 4). The organic extracts were concentrated under vacuum to yield dry $CHCl_3$ extract (980mg) and EtOAc extract (4.8g). EtOAc extract was fractionated on Sephadex LH20 [gel permeation chromatography (GPC)] using MeOH-$CHCl_3$ (1:1) as mobile phase and common fractions were pooled to give three fractions A B and C. Fraction A, B and C were eluted with MeOH-$CHCl_3$ (3:97, v/v) (2:98, v/v) and (3:97, v/v) respectively on flash Si gel to yield pure compounds **FM-3** (136mg) **FM-1** (1.8g) and **FM-2** (28mg) respectively.

$CHCl_3$ extract was fractionated on Si gel by eluting with increasing concentration of petroleum ether in $CHCl_3$ (0 – 100%) to yield several fractions which were pooled

into fractions A B and C on the basis of their TLC profile. Fraction A was eluted with MeOH –$CHCl_3$ (1:1) on Sephadex LH20 (GPC) to obtain a **FM-4** (130mg) with closely resolved impurities. This compound (12mg) was purified by semipreparative HPLC (discovery® C18, 5μ, 1 x 25 cm, detection at λ 215nm) by eluting with MeOH-H_2O (1:9) to give pure compound (6.4 mg) . Fraction B was flash Si gel chromatographed with increasing conc of MeOH in $CHCl_3$ (0-10, v/v) to serially give **FM-1** (43mg) and **FM-2** (19mg). Fraction C on repeated Si gel chromatography with MeOH-$CHCl_3$ (0:100 – 10:90, v/v) gave mixture (~5:95, 54mg) of **FM-3** and **FM-5. FM-5** was purified by repeatedly dissolving and filtering through Whatman 1 filter paper.

viii) Butyrolactone I (FM-1)

White powder; $[\alpha]^{28.8}_D$ + 84.32 (c 0.617, MeOH); and $[\alpha]^{28.8}_D$ + 87.56 (c 0.617, $CHCl_3$); HRESITOFMS: $[M+H]^+$ *m/z* 425.1516 (calcd. 425.1522 for $C_{24}H_{25}O_7$). UV (MeOH) λmax 306, 230, 210 nm .; IR (KBr) ν_{max} 3271, 2927, 1741, 1703, 1609, 1516, 1436, 1361, 1222, 1180, 840 cm^{-1}; ^1H NMR and ^{13}C NMR (300 MHz, CD_3OD) see table 2.2

ix) Butyrolactone II (FM-2)

White sticky solid, $[\alpha]_D^{25}$ = +85 (c = 1, EtOH), ESITOFMS $[M+H]^+$ m/z 357, UV (MeOH) λmax 303, 285 nm: IR (NaCl) ν_{max} 3290, 1740, 1730, 1660 cm^{-1}; ^1H NMR and ^{13}C NMR (300 MHz, $CDCl_3$) see table 2.2

x) Terrein (FM-3)

White crystalline solid; mp 121-122; $[\alpha]^{28.8}_D$ + 144 (C = 0.8 in MeOH); UV λmax 273 345 nm; IR (KBr) ν_{max} 3320, 3160, 1690, 1635, 1570 cm-^{1}; EIMS, m/e 154 (M), 139 (M-Me), 121 (M-Me-H_2O); ^1H NMR and ^{13}C NMR (300 MHz, CD_3OD) see table 2.3

*xi) 4β-(1'-Propenyl)cyclopentane-1β,2β-diol (**FM-4**)*

Colourless viscous liquid; optically inactive; IR (KBr) ν_{max} 3400, 2968, 2931, 2856, 1660, 1436 cm^{-1}; EI-MS, m/e 142 (M), for fragments see scheme 1.4; ^{1}H NMR and ^{13}C NMR (300 MHz, $CDCl_3$) see table 2.3

xii) 4-(1'-Propenyl) cyclopentane-1β,2α-diol (FM-5)

Colourless viscous liquid; $[\alpha]^{25}_{D}$ = +17.44 (c = .0.043, $CHCl_3$) ; IR (KBr) ν_{max} 3400, 2968, 2931, 2856, 1660, 1436 cm^{-1}; EI-MS, m/e 142 (M), for fragments see scheme 1.4; ^{1}H NMR and ^{13}C NMR (300 MHz, $CDCl_3$) see table 2.3

***xiii)** Screening of compounds to identify inhibitors of TNF-α and IL-6 production: Screening in LPS stimulated THP-1 cells (Primary Screening)(as carried outby technicians at piramal life science, Mumbai)*

IL-6 and TNF- α production by lipopolysaccharide (LPS) in THP-1 cells was measured according to the method described by Hwang et al., (1993). Briefly, THP-1 cells were cultured in RPMI 1640 culture medium (Gibco BRL, Pasley, UK) containing 100 U/ml penicillin and 100mg/ml streptomycin, (100X solution, Sigma Chemical Co. St Louis, MO) containing 10% fetal bovine serum (FBS, JRH). The cells were differentiated with phorbol myristate acetate (PMA, Sigma) and treated with the test compounds or vehicle. The plate was incubated for 30 min at 37°C. Finally, LPS (Escherchia coli 0127:B8, Sigma Chemical Co., St. Louis, MO) was added at a final concentration of 1 μg/ml. Plates were incubated at 37°C for 24h, 5% CO_2. Supernatants were harvested, and assayed for TNF-α and IL-6 by ELISA as described by the manufacturer (BD Biosciences). The cells were parallely evaluated for cytotoxicity using CCK-8. Percent inhibition of cytokine release compared to the control was calculated. The 50% inhibitory concentration (IC_{50}) values were calculated by a nonlinear regression method.

Section 2.2: Bioactive secondary metabolites from the soft coral associated fungus *Aspergillus terreus*

This section is further subdivided into Part A and Part B. Part A gives the review of literature on *A. terreus* and its metabolites. Part B describes the purification of fungal strains and structure elucidation of bioactive metabolites from *A. terreus* isolated from an octocoral, *Sinularia kavarattiensis*

I. Bioactive metabolites from *Aspergillus terreus*- a review

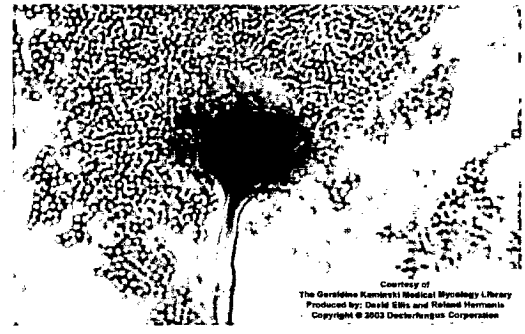
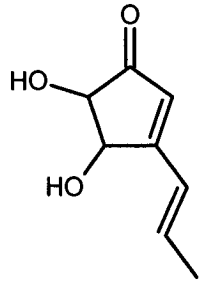


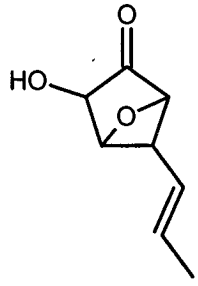
Fig 2.10: Microscopic view *A. terreus* conidia

A. terreus is a cosmopolitan fungus, which is primarily isolated from compost, plant material and from soil. It is more common in tropical or sub-tropical areas. It is an occasional causative agent of among immune-compromised patients. A few cases of cerebral infection have been reported. *A. terreus* is isolated occasionally from outer ear canal colonizations. The name *A. terreus* is used by Thom and Church (1918; 1926) to cover a series of strains, which have a number of well-marked morphological features in common, but which show a certain amount of variation in colour and type of growth. The fungus produces colonies which are velvety to floccose, buff, cinnamon to sand brown, velvety; reverse a medium yellow to deep dirty brown; heads columnar upto 500µm diameter; vesicles dome shaped, bearing closely packed metulae (**Fig 2.10**).

The earliest report on the chemical constituents of *A. terreus* appeared in 1934, when Raistrick and Smith, (1935) isolated a compound and named it as terreic acid. Subsequently, this substance was named terrein, as highly purified sample gave *pH* of 6.8 in 1% aq. solution, an indication of compound not having acidic proton. After studying the several reactions of terrein, the authors arrived at two possible structures for terrein (**FM-3** and **24**) and accepted compound to have most likely the structure **24**, on the basis of following two facts. Terrein gave only a mono-p-bromobenzoate and secondly it did not react with maleic anhydride to give Diels alder adduct. Eventually, **FM-1** was later proved to be the correct structure of terrein on the basis of spectroscopic evidences.



FM-3



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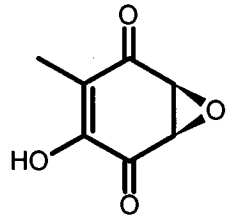

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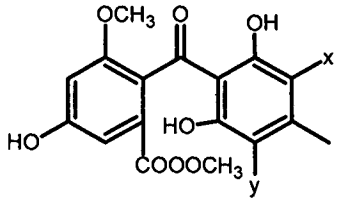
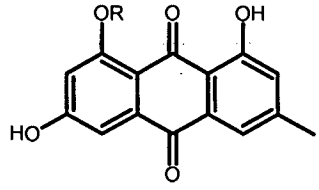
In 1949 (Abraham and Florey) an acidic metabolite having good antibiotic activity isolated from *A. terreus* was named terreic acid **(25)** and structure was assigned to be 5,6-epoxy-3-hydroxytoluquinone (Sheehan, 1958). It was later identified as potent inhibitor of Bruton's tyrosine kinase (Kawakami, 1999) and very recently as covalent inhibitor of the bacterial cell wall (Han, H. 2010).

A. terreus (Calam et al., 1939) was found to be a highly productive source of itaconic acid **(26)**, a metabolite isolated first from *Aspergillus itaconicus* (Dwiarti, 2002). It is very useful industrial chemical and produced in bulk quantity from fermentation of *A. terreus.* (Petuchow et al., 1980). Geodin and erdin, the first chlorine containing fungal metabolites were isolated in 1936 from a strain of *A. terreus* Thom (Raistrick and Smith, 1936). After the initial efforts by Clutterback et al., (1937), the correct relative structures were assigned by Barton and Scott (1958). Some other chlorine containing metabolites include dihydrogeodin (**27**) sulochrin (**28**) and monochloro derivative of

sulochrin (**29**) isolated as antimicrobial metabolites of *A. terreus* (Inamori et al., 1983). Biosynthetically related previously known metabolites, questin (**30**) and emodin (**31**) were also isolated from this fungus.

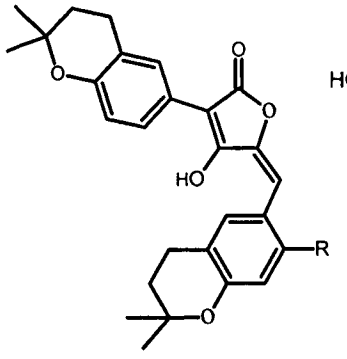


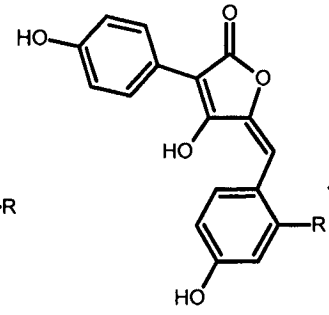


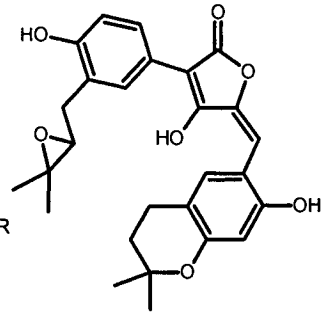
Dihydrogeodin (27) x = Cl, y = Cl
Sulochrin (28) x = H, y = H
29 x = Cl, y = H

emodin (30) R = H
questin (31) R = CH_3

Aspulvinones were the first phenylpropanoid (C6-C3) derived metabolites obtained from strain of *A. terreus* IAM 2054 (Nobutoshi et al., 1973). This type of compounds were previously known synthetically (Claisen and Ewan, 1895) and also as natural products in higher fungi (Edwards and Gill, 1973). Biosynthesis of aspulvinones involves dimerization of hydroxylphenyl pyruvate derived from tyrosine/phenyl alanine. Additional feature of these aspulvinones isolated from *A. terreus* was that some have one or two C5 units on aromatic rings (Nobutoshi et al., 1975). So far seven pulvinone derivatives, aspulvinones A-G (**32-38**) have been reported.



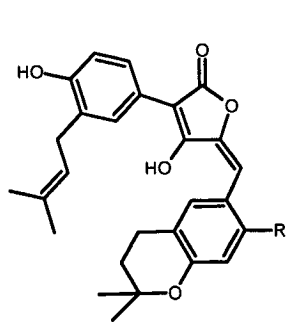




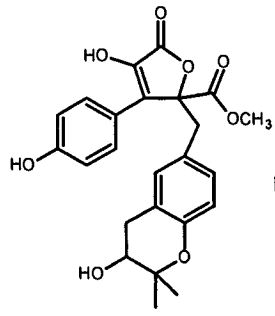
Aspulvinone A (32) R = H
Aspulvinone C (34) R= OH

Aspulvinone E (36) R = H
Aspulvinone G (38) R= OH

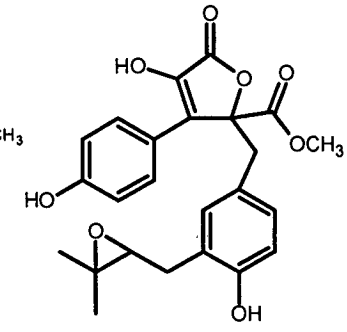
Aspulvinone F (37)



Aspulvinone B (33) R = H
Aspulvinone D (35) R= OH

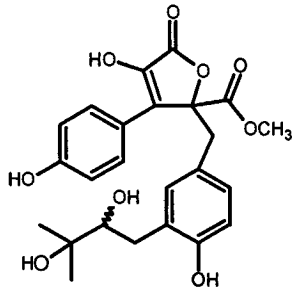


Butyrolactone V (FM-11)

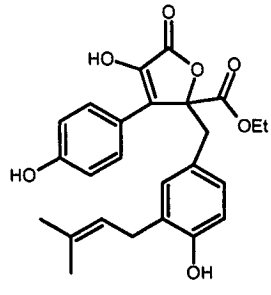


Butyrolactone III (FM-9)

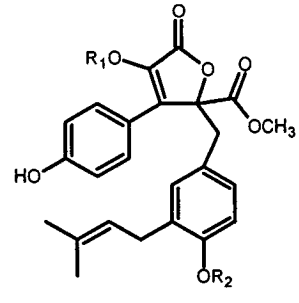
Since the isolation of aspulvinones, many phenyl propanoid derived metabolites, some of them also having C5 unit on aromatic ring, but differing in constitution of central furanone ring have been isolated from *A. terreus*. The first such compound to be isolated was butyrolactone I (**FM-1**) from strain of *A. terreus* IFO 8835 by Kiriyama et al., (1977). This compound proved to be a rare potent metabolite that selectively inhibits CDK2 and CDK1 kinases, both of which play important roles in cell progression from G1 to S phase and from G2 phase to M phase, respectively, in mammalian cell. Later, the derivatives of **FM-1**, butyrolactones II-VII (**FM-2** and **FM-9 to FM-13**) (Rao et al., 2000; Haritakun et al., 2010) and two butyrolactone I sulfates (**FM-14** and **FM-15**) (Niu et al., 2008) were isolated from different strains of *A. terreus*. Incidentally, **FM-12** is identical to aspernolide D (Nuclear et al., 2010) reported in separate article during same time as a metabolite of *A. terreus,* along with a biosynthetically unusual six membered anhydride, asperterone (**39**). Some of these metabolites exhibit various activities those include anticancer, antiplasmodial and antioxidant.



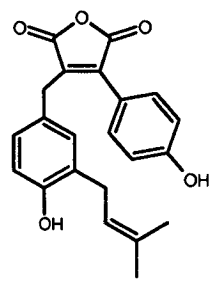
FM-12 butyrolactone VI



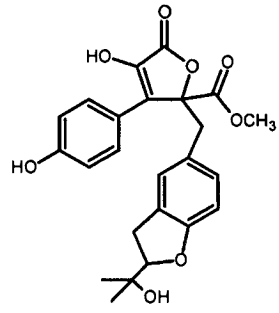
FM-13 Butyrolactone VII



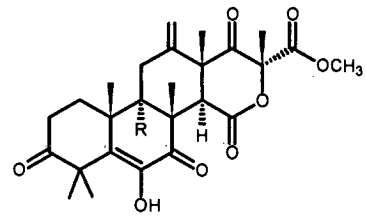
FM-14 R_1= SO_3H, R_2 = H
FM-15 R_1 = H, R_2= SO_3H



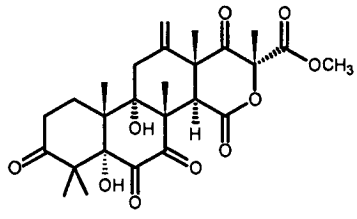
Asperterone (39)



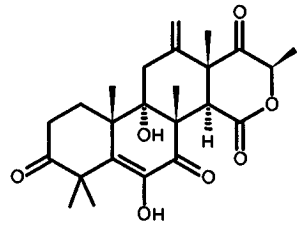
Butyrolactone IV (FM-10)



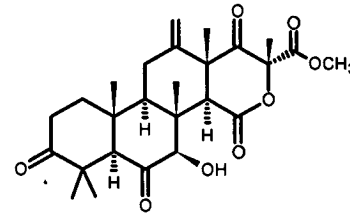
Terrentonin (40) R= OH
Terretonin A (41) R= H



Terrentonin B (42)



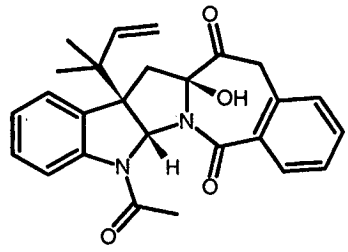
Terrentonin C (46)



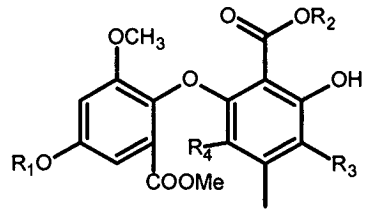
Terrentonin D (47)

Some strains of *A. terreus* produces metabolites those are biosynthetically distinct. **FM-11** was previously isolated from an unidentified strain of *Aspergillus* sp. This strain produced metabolites those belong to five structure types namely, butenolides (**FM-1**, **FM-2** along with **FM-15**); seterterpenoids terrentonin (**40**) (Springer et al., 1979), terretonin A (**41**) and B (**42**) (Li et al., 2005), questin (**31**) (Mahmoodian and Stickings, 1964), dihydrogeodin (**27**) (Harold and George, 1936), asterric acid (**43**) (Hiroshi, et al., 1992) and four diphenyl ethers of which two were new (**44-45**) (Hargreaves et al., 2002). It appears that unidentified strain used to isolate these compounds must be *A. terreus*, because **FM-1** and **FM-2**, terrentonins (**40-42**) and compounds (**27**, **31** and **43**) have been previously isolated from *A. terreus.* Terretonin (**40**) (Springer et al., 1979) and terretonins A-D (**41-42** and **46-47**) (Li, et al., 2005), the only sesterpenoid class of metabolites were first obtained from *A. terreus.* The fungus that produced **41-42** and **46-47** also produced biosynthetically distinct metabolite, a novel alkaloid asterrelenin (**48**) (Li et al., 2005) None of these terrentonins were cytotoxic, but similar terretonins E and F metabolites of marine

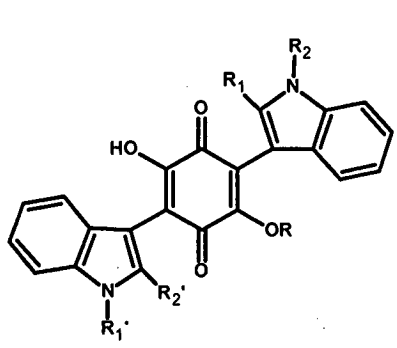
sponge-derived fungus *Aspergillus insuetus* have been shown to be inhibitors of the mammalian mitochondrial respiratory chain (Pilar et al., 2009).

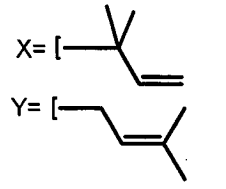


Asterlennin (48)



Asterric acid (43) R_1= R_2=R_3=R_4=H
44 R_1=H R_2= n-butyl R_3= R_4= Cl
45 R_1= R_2= Me R_3= R_4= Cl





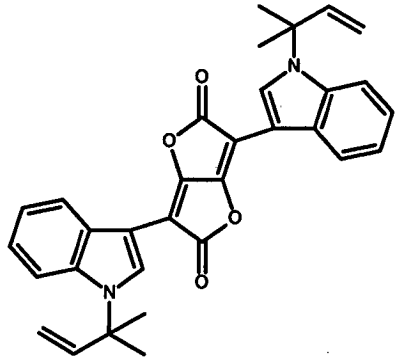
	R_1	R_2	R_1'	R_2'	R
49	X	H	X	H	H
50	X	H	X	H	Ac
51	X	H	H	Y	H
52	H	Y	H	Y	H

Asterrriquinone (49) and its derivatives (50-52)

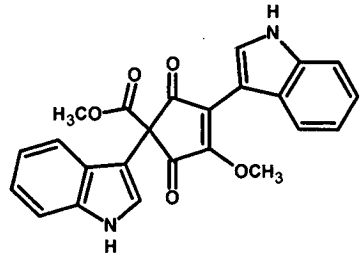
An investigation by Yamamoto et al., (1976) on *A. terreus* IFO 6123 led to the isolation of asterriquinone (**49**), a dimer biosynthized by pyruvate derived from tryptophan. As in aspulvinones (**32-38**) and aspernolides (butyrolactone I derivatives) **49** also have C5 unit on aromatic rings. It showed good antitumor properties. Further investigation of the same strain by kaji (2003) led to the isolation of three new derivatives of **49**, a monoacetate (**50**) and other two (**51-52**) differing by attachment of C5 unit on aromatic systems, beside a new congener having a different framework formed by oxidation and internal cycliztion of quinone functionality of **49**. The new congener was named asterridinone (**53**).

Asterridione (**54**) (not asterridinone, **55**) was cytotoxic metabolic product of *A. terreus* isolated from sonaran desert (Wijeratne et al., 2003). Two new derivatives of

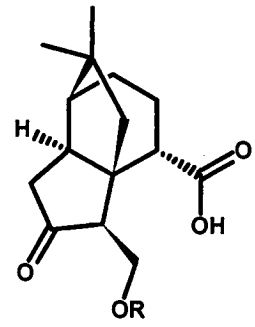
terricyclic acid (**56-57**) were also isolated from this source along with seven known molecules. **55** is supposed to be biosynthesized from asterriquinone D, which involves contraction of six membered ring to five membered ring.



54 Asterridinone
57 R = H

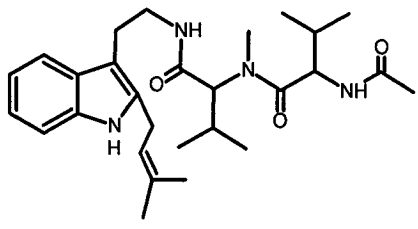


55 asterridione

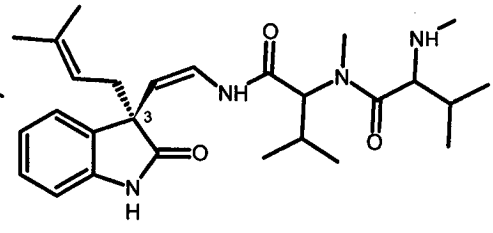


56 R = CH_3

A new prenylated indolic enamide, terpeptin (**58**) was metabolic product of *Aspergillus terreus* 95F-1 (Kagamizono et al., 1997). It showed strong inhibition of cell cycle progression of mouse tsFT210 cells in the C2/M. A Chinese mangrove-derived unidentified strain of *Aspergillus* sp. also produced **58** along with two new derivatives, terpeptins A (**59**) and B (**60**) (Lin et al., 2008).



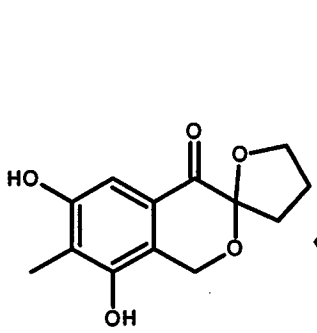
58



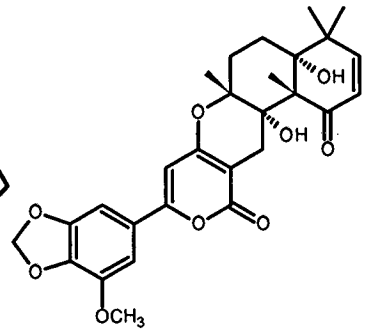
59
60 3- epimer

Terreineol (**61**), a novel polyketide, containing spiro furan isochromone moiety biosyntheisized from six acetyl/malonyl units added by CoA was metabolic product of A. terreus (maceda et al., 2004). A metabolite, named aspergiketal was also obtained from *A. terreus* associate with *Opuntia ficusindica* Mill (Wu et al., 2008). Interestingly, this compound has been assigned the structure identical to terreineol

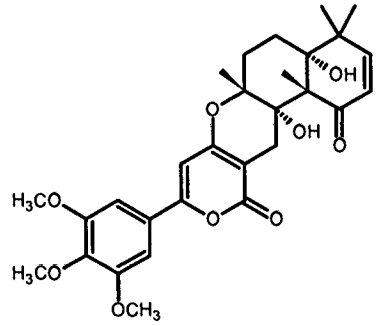
(**61**), but claimed it to be new metabolite. The spectroscopic data presented for terreineol and aspergiketal are identical.



Terreineol (61)



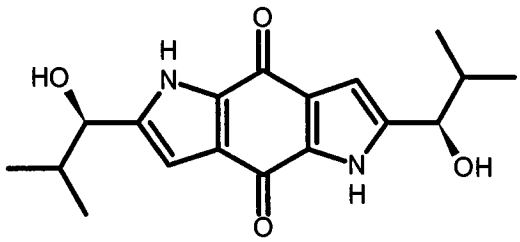
Territrem A (62)



Territrem B (63)

Territrem A (**62**) and B (**63**) as the name indicates were isolated as tremorgenic metabolites from *A. terreus*. The tremorgenic effects of territrems arise from their being a potent inhibitor of acetylcholinesterase (AChE) (Arvanov et al., 1993). Analogous to territrems are arisugacins obtained from *penicllium* sp. (Omura et al., 1995) are also highly specific and potent AChE inhibitors as well, but less active than **63**.

A. terreus have yielded large number of various types of secondary metabolites from terrestrial isolates. Some of these metabolites have been also obtained from marine sources, but there is only one report that exists on novel metabolite from marine derived *A. terreus*. A novel chiral dipyrrolobenzoquinone derivative, terreusinone (**64**), has been the only compound to be obtained from a marine-derived fungus (Lee et al., 2003). The fungus was isolated from a marine red alga *Halymenia acuminata* collected in the Bijin Island. It exhibited a UV-A absorbing activity with ED50 value of 70 μg/mL



Terreusinone (64)

II. Results and discussion

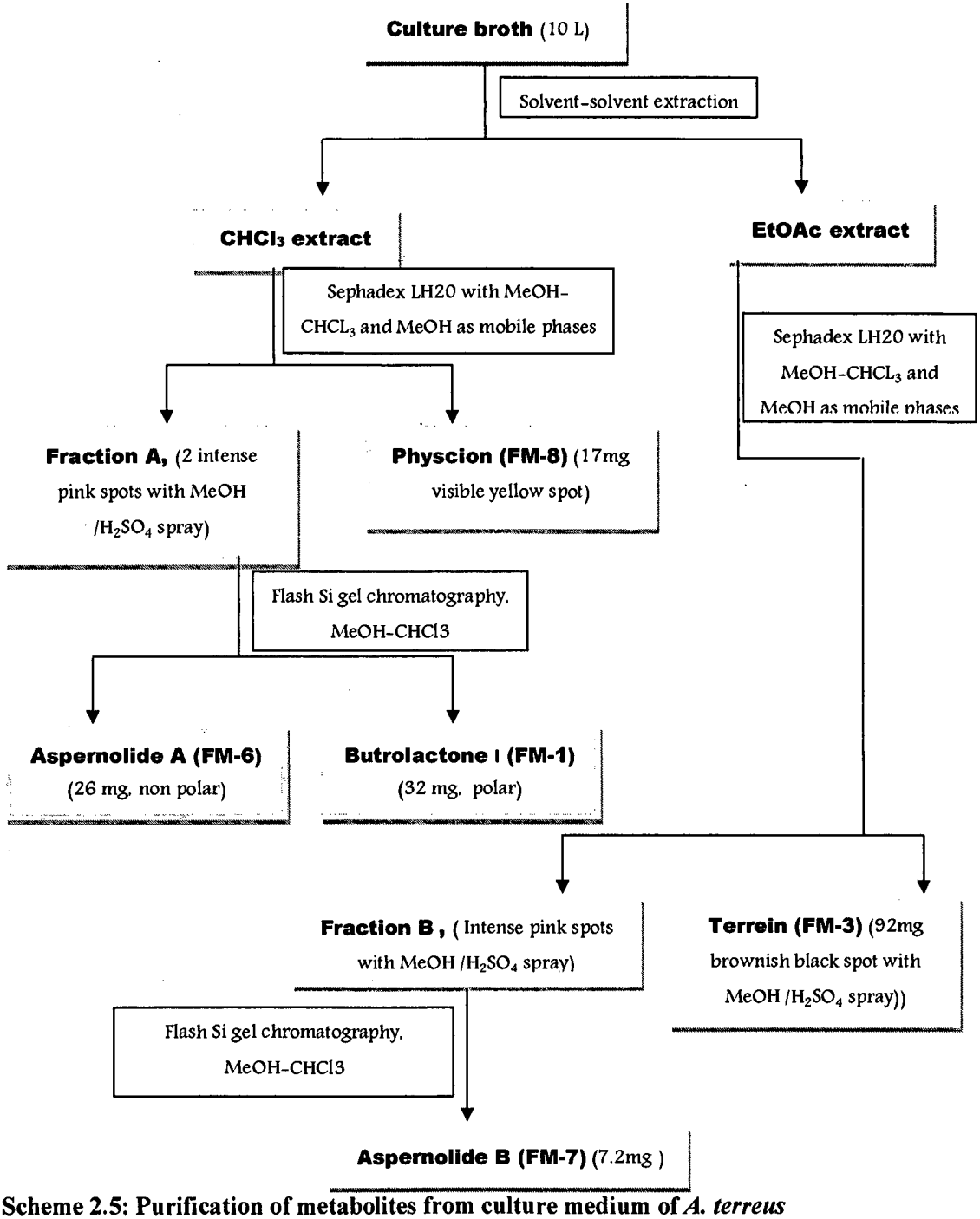
i) Biological materials

Indian coastline is potential source of marine organisms such as sponges soft corals, tunicates, algae etc and exhibits various biological activities. Each of these marine organisms harbours vast biodiversity of microorganisms. Numerous bioactive compound of marine origin are in fact microbial metabolites originating from dietary commensalic or endosymbiotic microorganisms.

One among these, a soft coral for our study was collected from Mandapam, Tamil Nadu and used for the isolation of associated fungi. Only one epiphyte grew on both PDA and Czapek Dox Agar, while on treatment of octocoral with hypochlorite no endophyte could be obtained. From the morphological features the epiphytic fungi was identified as *Aspergillus terreus.* Its 5.8SR gene sequences had 99% similarity to *Aspergillus terreus* isolates NRRL 4609, NRRL 260 NRRL 255 and NRRL 2399. The fungus was mass cultured on potato dextrose broth prepared in seawater and the ethyl acetate extract of cultured broth showed good anticancer activity.

Fig 2.11: *Aspergillus terreus* cultured on PDA

ii) Purification of metabolites



Scheme 2.5: Purification of metabolites from culture medium of *A. terreus*

After filtration the culture broth, was fractionated between chloroform and ethyl acetate. A new secondary metabolite aspernolide A (**FM-6**), along with a known

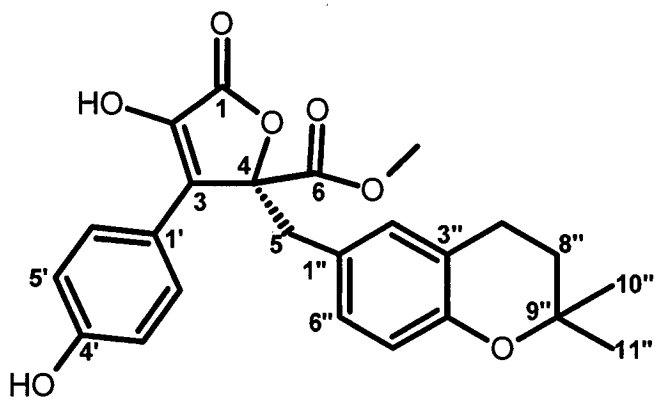
metabolite physcion (**FM-8**) were purified from the chloroform extract by successive fractionation with silica gel and Sephadex LH20 gel filtration chromatography. Repeated fractionation of the ethyl acetate extract by silica gel and Sephadex LH20 gel filtration chromatography yielded a new compound aspernolide B (**FM-7**) and two known compounds butyrolactone I (**FM-1**) and terrein (**FM-3**). The systematic scheme of isolation these metabolites is depicted in **Scheme 2.5.**

iii) Structure elucidation of metabolites

FM-1 was obtained as colourless crystalline solid, which would eventually turn into a gummy material on storage for long time, and **FM-3** was isolated as crystalline needles. On TLC **FM-1** and **FM-3** gave a spots identical in all respect to butyrolactone I (**FM-1**) and terrein (**FM-3**) respectively, isolated from *A. flavipes* (section 2.1). The spectroscopic data (UV-Vis, IR, NMR and MS) **of FM-1** and **FM-3** isolated from this fungus were well in agreement with the **FM-1** and **FM-3** respectively. The detail description of the structure and it spectroscopic data is discussed in preceding section of this chapter.

FM-6 was obtained as white sticky solid. The molecular formula $C_{24}H_{24}O_7$ of **FM-6** was determined by HRESITOFMS(**Fig 2.12a**), which showed pseudomolecular ion peaks $[M+Na]^+$ at m/z 447.1433 (calcd. 447.1420 for $C_{24}H_{24}O_7Na$) and $[2M+Na]^+$ at m/z 871.2959 (calcd. 871.2942 for $C_{48}H_{48}O_{14}Na$) and was isomeric with FM-**1**. IR spectrum (**Fig 2.12b**) showed presence of ester/lactone carbonyls at 1731 cm^{-1} and 1738 cm^{-1}, phenolic OHs were evident at 3330cm^{-1} and presence of absorption at 1660 cm^{-1} was suggestive of aromaticity in the molecule. ^1H NMR signals (**Fig 2.12c**) of the A_2B_2 system at δ_H 7.56 (d, 2H, J = 8.7Hz) and 6.86 (d, 2H, J = 8.7Hz) revealed the presence of para substituted benzene moiety. Two aromatic signals 6.53 (s, 1H) and 6.47 (s, 2H) (two doublets merged into a singlet) were indicative of the presence of additional unsymmetrical trisubstituted benzene ring in the molecule. Its ^{13}C NMR (**Fig 2.12d**) showed the presence of 10 aromatic signals for two aromatic rings, two ester carbonyls δ_c 169.3 (s), and 169.6 (s), olefenic carbon signals δ_c 137.2 (s) and 128.8 (s), three sp^3 CH_2s δ_c 22.1 t, 32.5 t and 38.6 t, a carbomethoxy δ_c 53.4 q and

two oxygenated quartenaries δ_c 86.1 (s) and 74.2 (s). The molecular formula $C_{24}H_{24}O_7$ requires 13 degrees of unsaturation. Presence of two aromatic rings accounts for eight while two carbonyls and two olefenic carbons accounts for another three, which makes total of eleven degrees of unsaturation. Therefore **FM-6** must possess two aliphatic rings in addition to two aromatic rings.



Aspernolide A (FM-6)

A detail comparison of NMR data (**Table 2.4**) of **FM-6** with that of **FM-1**, confirmed a common hydroxoyphenyl pyruvate dimer type of network in the molecule. The significant difference observed in the NMR spectra of **FM-6** as compared to that of **FM-1** was the absence of a olefenic proton signals δ_H 5.0 (t, 1H), two olefenic carbon signals δ_c 121.0 (d) and 130.7 (s) and the presence of three methylenes and two oxygenated sp3 quarternaries compared to two methylenes and one oxygenated sp3 quartenary in **FM-1** in both ^{13}C NMR spectra. These data was indicative of the presence of a dihydropyran ring fused to a trisubstituted benzene ring in place of the open prenyl chain present in **FM-1**.

HMBC (**Fig 2.12e**) was well in agreement to the given structure (**Fig 2.12f**). Key HMBC correlations from H-2" to C-3" and C-7", from H-7" to C-2", C-3" and C-4" and from H-8" to C-9" and C-10"(11") established dihydropyran ring fused through C3"-C4" bond of benzene ring. HMBC correlations from H-2" and H-6" to C-6 and from H-6 to C-1", C-6", C-2", C-4, C-5 and C-3 was evident of the benzodihydropyranmethylene moiety linked to lactone ring at C-4. Furthermore,

HMBC correlation from H-2'(6') to C-3 established p-substituted phenolic moiety at C-3. Out of two carbonyls, C-5 and C-1, δ_c 169.6 was assigned to C-5 on the basis of its HMBC correlation to the protons H-6 and H_3-5OMe. **FM-6** is known previously as reaction product of **FM-1**, but was known as natural product.

Table 2.4 NMR spectroscopic data of aspernolide A (FM-6) (500 MHz, $CDCl_3$)

Position	δ_C, mult.	δ_H, mult., *J*(Hz)	HMBC
1	169.3 s		
2	137.2 s		
3	128.8 s		
4	86.1 s		
5	38.6 t	3.39, d, (15.0) 3.59, d, (15.0)	C4, C1", C6", C3, C2", C5
6	169.6 s		
1'	122.2 s		
2'(6')	129.5 d	7.56, d, (8.7)	C4', C3
3'(5')	115.9 d	6.86, d, (8.7)	C1', C4'
4'	156.4 s		
1"	123.5 s		
2"	131.4 d	6.53, s	C7", C6, C6", C4"
3"	120.3 s		
4"	152.9 s		
5"	116.6 d	6.47, s	C1", C3"
6"	129.0 d	6.47, s	C6, C2", C4"
7"	22.1 t	2.53m	C8", C9", C2", C3", C4"
8"	32.5 t	1.66, t, (6.5)	C3", C7", C9", C10"(11")
9"	74.2 s		
10"(11")	26.6 q	1.21, s	C7", C8", C9"
5-OMe	53.4 q	3.72, s	C5

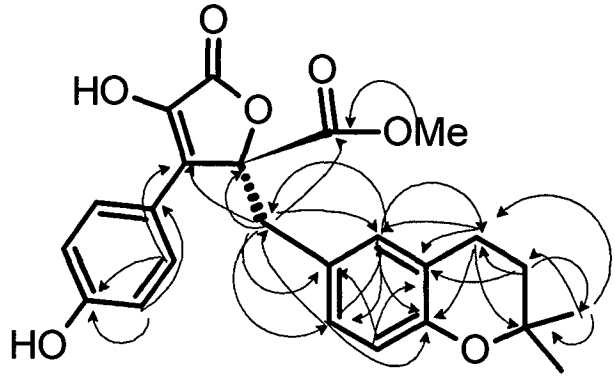


Fig 2.12f: HMBC correlation of FM-6

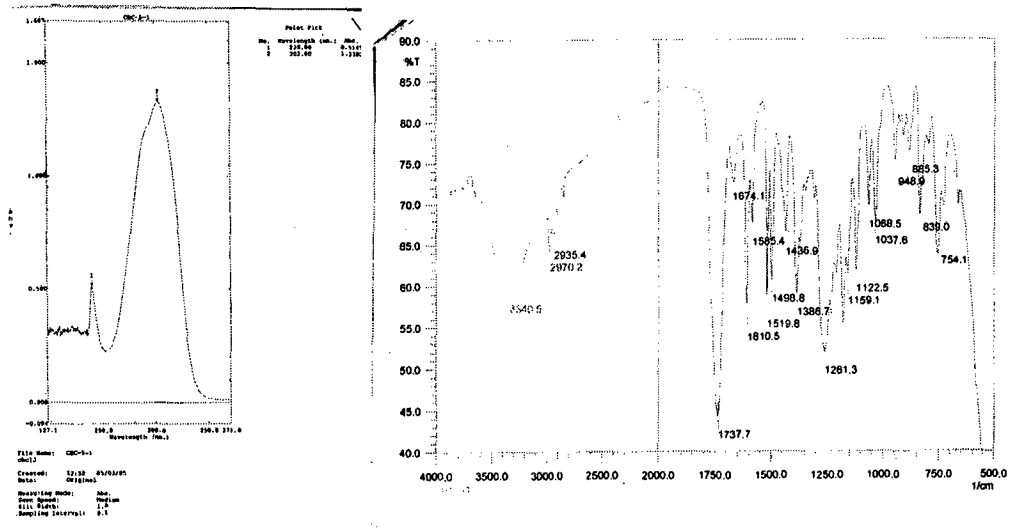


Fig 2.12g: UV-Vis spectrum of Aspernolide A **Fig 2.12b: IR spectrum of Aspernolide A**

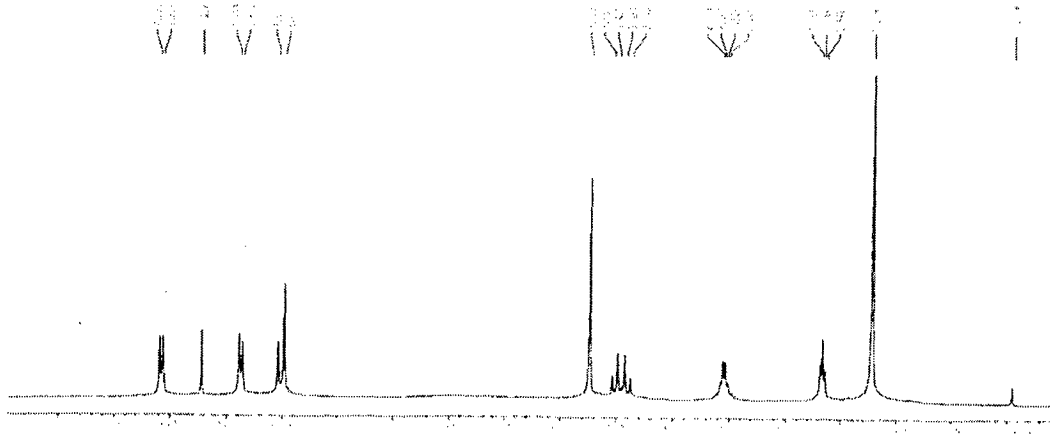


Fig 2.12c: ^{1}H NMR of Aspernolide A

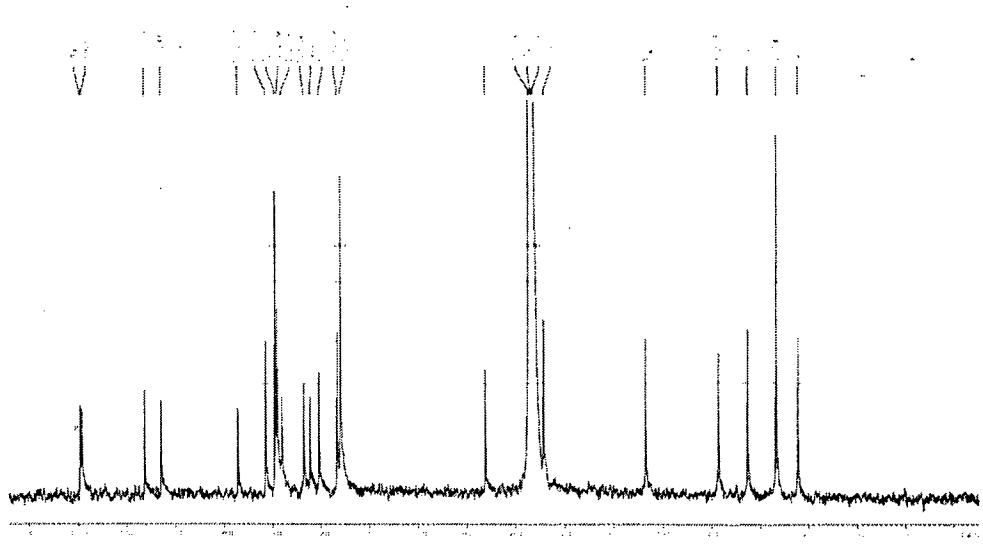


Fig 2.12d: ^{13}C NMR of Aspernolide A

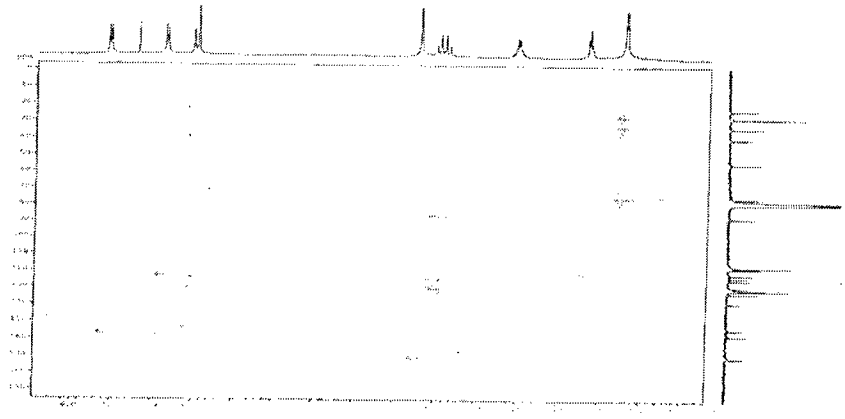


Fig 2.12e HMBC of Aspernolide A

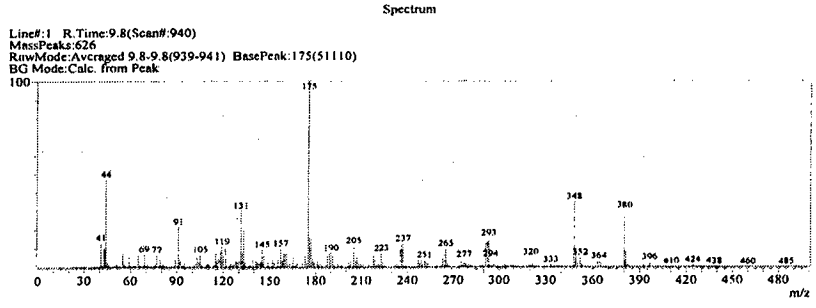
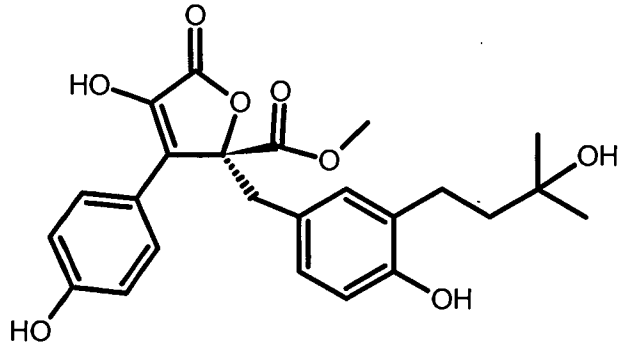


Fig 2.12f: EI-MS of Aspernolide A

Aspernolide B (**FM-7**) (Rf, 0.51), more polar than **FM-6** (Rf, 0.81) was obtained as light brown syrup $[\alpha]_D$ + 48.27 (c 0.29, MeOH). The IR spectra showed presence of OHs at 3330 cm^{-1}, ester/lactone carbonyls overlapping peaks at 1732 and 1747 cm^{-1} and 1610 and 1519 cm^{-1} for aromatic rings very similar to **FM-6**. It gave ^1H (**Fig 2.13a**) and ^{13}C (**Fig 2.13b** anf **Fig 2.13c**) NMR patterns very similar to **FM-6.** Although, spectral pattern matched with **FM-6** chemical shift variations were observed in the ^1H and ^{13}C NMR spectra. Significant variations in the chemical shifts were observed for ring carrying iso-pentyl chain wherein C-1", C-3", C-7", C-8" C-10"and C-11" were considerably deshielded to resonate at δ_c 128.1($\Delta\delta$ 4.6ppm), 124.0($\Delta\delta$ 3.7ppm), 24.2($\Delta\delta$ 2.1ppm), 43.2($\Delta\delta$ 10.7ppm), 28.4($\Delta\delta$ 1.8ppm) and 28.5($\Delta\delta$ 1.9ppm) while C-5" and C-9" were considerably shielded to resonate at δ_c 114.6 ($\Delta\delta$ 2.0ppm) and 70.8($\Delta\delta$ 3.4ppm) as compared to **FM-6**, suggesting a change on the aromatic ring carrying iso-pentyl chain (**Table 2.5**).



Aspernolide B (FM-7)

FM-7 was well distinguished from ESI-MS spectrum (**2.13d**), which showed pseudomolecular ions $[M+H]^+$ at m/z 443.1699 (calcd. 443.1706 for $C_{24}H_{27}O_8$) and $[M+Na]^+$ at m/z 465.1516 (calcd. 465.1525 for $C_{24}H_{26}O_8Na$) suggesting molecular weight of 442 for the compound, which was 18 units more than **FM-6**. Based on the above observations it was evident that **FM-7** had open chain hydroxylated prenyl chain ortho to phenolic –OH(C-4"). **FM-7** is a new natural product.

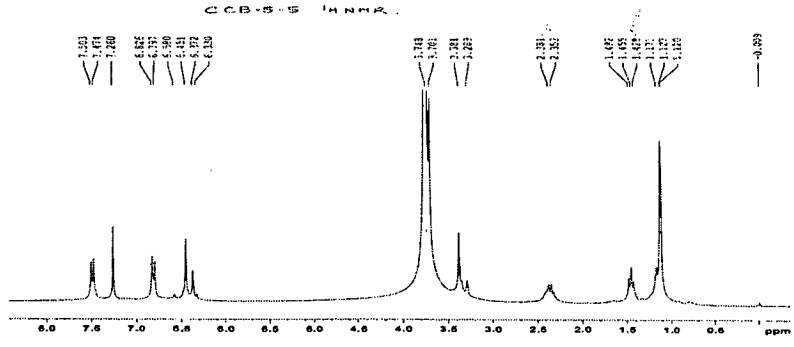


Fig 2.13a: ^{1}H NMR of Aspernolide B

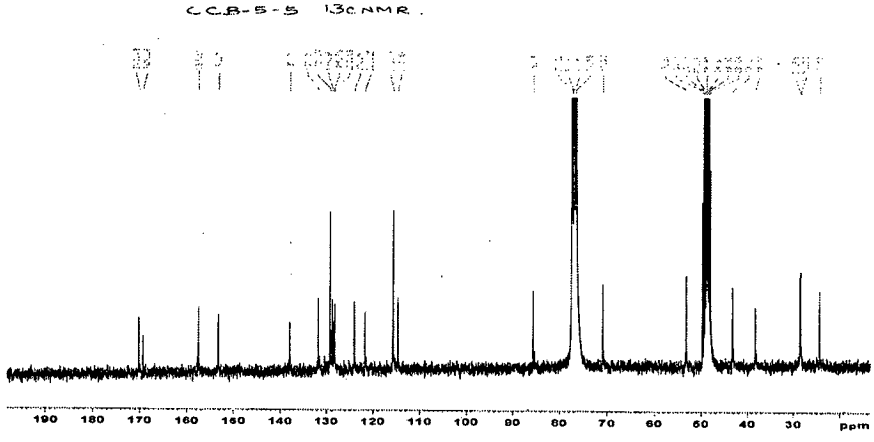


Fig 2.13b: ^{13}C NMR of Aspernolide B

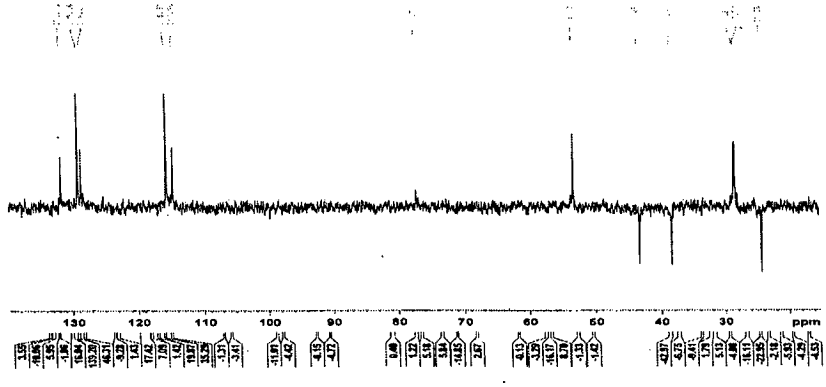
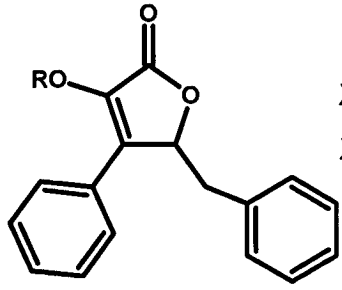


Fig 2.13c: ^{13}C NMR of Aspernolide B

iv) Biogenesis and stereochemistry of Aspernolide A (FM-6) and B (FM-7)

To determine and confirm the stereochemistry of **FM-6** and **FM-7**, **FM-1** was converted to **FM-6** and **FM-7**. Based on the reported feeding experiments for establishing the biosynthesis of xenofuranones A (**65**) and B (**66**) together with compound **1** (Brachmann et al., 2006; Nitta et al., 1983) and isolation of **FM-6** and **FM-7** from *A. terreus,* it is apparent that the structures of **FM-6** and **FM-7** are extension of the biosynthesis of **FM-1**, which is derived from p-hydroxy phenylpyruvate. The enzyme catalyzed cyclization or addition of water across the double bond of the prenyl chain of **FM-1** results in the formation of **FM-6** and **FM-7** respectively (**Scheme 2.6**). The last step in the biogenetic scheme was mimicked using mild acid catalysis to confirm the absolute structure of **FM-6**.

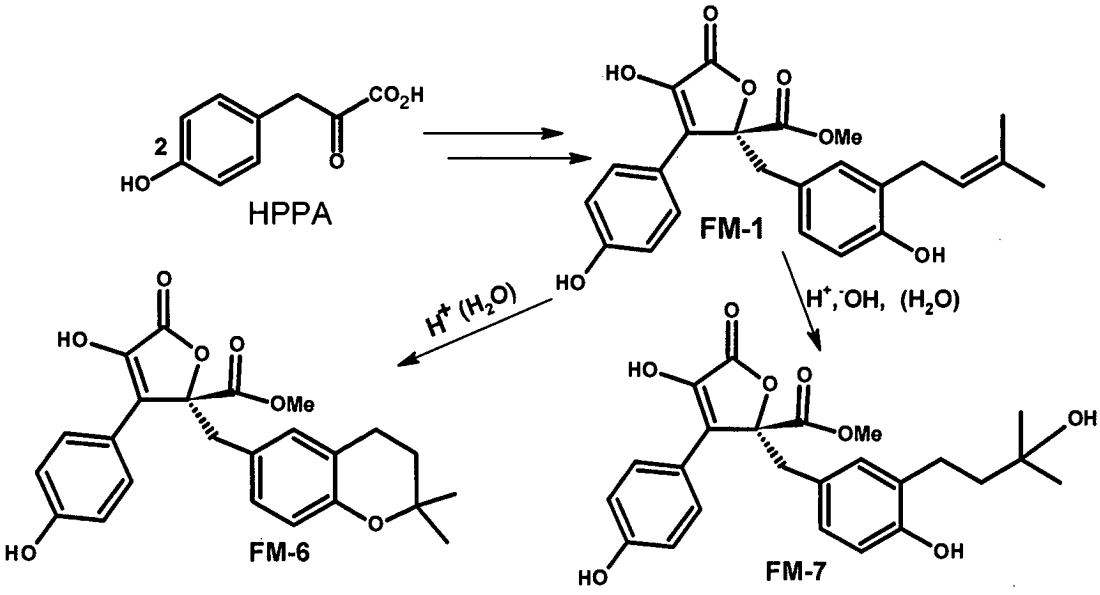


Xenofuranone A (**65**), R = H

Xenofuranone B (**66**), R = Me

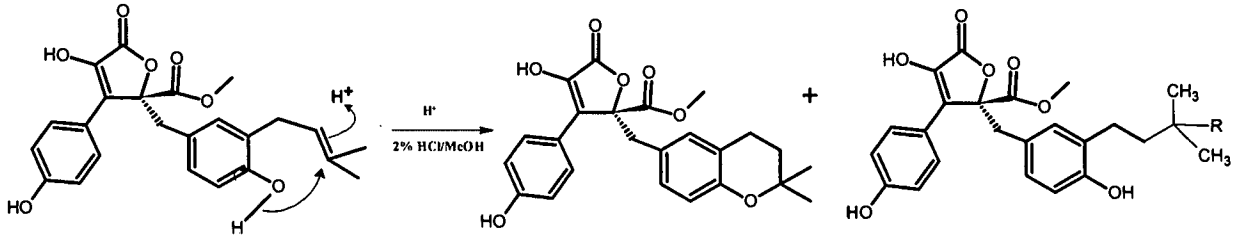
On heating with 1% aqueous sulphuric acid, **FM-1** was converted to **FM-6** as well as small amount of **FM-7** (**Scheme 2.7**), but only 50% conversion was observed. When the same reaction was carried out using 2% conc. HCl in methanol, complete conversion of **FM-1** was observed as indicated by TLC (90:10, $CHCl_3$/MeOH) giving three products. On chromatographic separation, 75% yield of **FM-6** was obtained along with minor amounts of **FM-7** and a new product Aspernolide C **(67).** Compound **67** displayed an extra methoxy signal δ_H 3.15, s, δ_C 49.0 q in NMR spectra (**Fig 2.14a** and **2.14b**) compared with **FM-7** (**Table 2.5**). In the rest of the NMR spectra it was seen that C-7", C-8", C-10" and C-11" were shielded and observed at δ_c 23.2($\Delta\delta$ 1.0), 40.0($\Delta\delta$ 3.2), 24.0 ($\Delta\delta$ 4.4), and 24.4($\Delta\delta$ 4.1), while only methoxylated quartenary carbon C-9" was deshielded to 75.4($\Delta\delta$ 4.6) compared to **FM-7**. Pseudomolecular ion peaks $[M+H]^+$ at m/z 457.1851 and $[M+Na]^+$ at m/z 479.1669 observed in ESI-MS (**Fig. 2.14c**) indicated molecular weight of 456 for the

compound **67** On the basis of these observations, the structure was assigned as shown in **67. FM-7** and **67** are formed by Markovnikoff's addition of water and methanol respectively, across the double bond of the prenyl chain.



Scheme 2.6: Plausible biogenetic pathway for Aspernolide A (FM-6) and B (FM-7)

During the structure elucidation of **FM-1, FM-7** has been reported as the product of its reaction with ethanolic HCl (Kiriyama et al., 1977). In the present investigation, the same reaction in methanolic HCl yielded besides **FM-6**, two additional products **FM-7** and **57.** These butenolides **FM-1**, **FM-6** and **FM-7** have one chiral centre C-4. Co-metabolite **FM-1** used in the above reaction was determined to be 4*R* configured by comparison of specific rotation data $[\alpha]_D$ + 84.32 with the previously reported result (Kiriyama et al., 1977). The absolute configurations of **FM-6** and **FM-7,** therefore could actually be deduced as 4*R* based on the biogenetic grounds and similarity of the specific rotations $[\alpha]_D$ +88.73 for **FM-6** and +48.27 for **FM-7** with **FM-1.** Further confirmation of the absolute stereochemistry of **FM-6** and **FM-7** results from the fact that, the natural **FM-6** and **FM-7** and those obtained as products of acid catalyzed reaction had identical spectral data and specific rotations. Therefore, we conclude that the compounds **FM-6** and **FM-7** also have 4*R*-configuration.



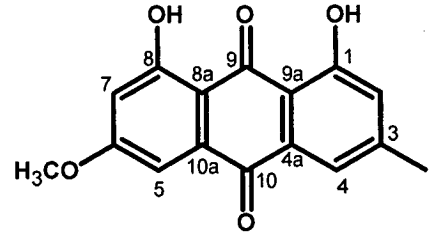
FM-1 FM-6 FM-7 R = OH
57 R = OMe

Scheme 2.7: Conversion of butyrolactone I to Aspernolides A (FM-6), B (FM-7) and C (57)

Table 2.5: ^{1}H and ^{13}C NMR spectroscopic data of aspernolides B (FM-7) and C (57) (300 MHz, $CDCl_3$+ 2drops CD_3OD)

	FM-7		57	
Position	δ_C, mult	δ_H, mult., J(Hz)	δ_C, mult	δ_H, mult., J(Hz)
1	169.2 s		169.3 s	
2	137.9 s		137.9 s	
3	128.3 s		128.2 s	
4	85.6 s		85.7 s	
5	170.1 s		170.1 s	
6	38.3 t	3.46, s	38.4 t	3.43, s
1'	121.6 s		121.8 s	
2'(6')	129.1 d	7.55, d, (8.7)	129.2 d	7.54, d, (8.7)
3'(5')	115.6 d	6.87, d, (8.7)	115.0 d	6.85, d, (8.7)
4'	157.5 s		157.5 s	
1"	128.1 s		128.1 s	
2"	131.7 d	6.41, d (1.8)	131.8 d	6.40, d (1.8)
3"	124.0 s		124.0 s	
4"	153.2 s		153.4 s	
5"	114.6 d	6.52, d, (8.7)	114.4 d	6.49, d, (8.1)
6"	128.6 d	6.55, dd, (8.7, 1.8)	128.9 d	6.54, dd, (8.1, 1.8)
7"	24.2 t	2.37, m	23.2 t	2.35, m
8"	43.2 t	1.53, t, (7.8)	40.0 t	1.54, t, (7.8)
9"	70.8 s		75.4 s	
10"	28.4 q	1.20, s	24.0 q	1.12, s
11"	28.5 q	1.19, s	24.4 q	1.12, s
9"-OMe	---		49.0 q	3.15, s
5-OMe	53.5 q	3.76, s	53.3 q	3.74, s

Physcion (**FM-8**) was obtained as a bright yellow solid. Pseudomolecular ion $[M+H]^+$ at m/z 285 indicated molecular formula to be $C_{16}H_{12}O_5$. The IR absorption band of 1629 cm^{-1} and bright yellow colour of the compound was indication that compound had quinone functionality. Its ^{13}C NMR along with DEPT 135 (**Fig 2.15b**) indicated presence of 16 carbon atoms distributed as 2 methyls, 4 methines and 10 quaternary carbons. The carbon signals at δ 181.8 and 190.6 were indicative of presence of quinone carbonyl groups. Presence of 14 sp2 carbons including two carbonyls indicated it to be a anthraquinone derivative The ^1H (**Fig 2.15a**)and ^{13}C spectra indicated presence of a methoxy (δ_H 3.88s, δ_C 55.9q), an aromatic methyl (δ_H 2.39 s, δ_C 22.04), four distinct aromatic methines (δ_H 7.02 brs, δ_C 124.3 d; 7.56 brs, 121.1d; 7.31 d, 2Hz, 108.1 d and 6.63 d, 2Hz,106.7) and two chelated phenolic hydroxyl groups (δ_H 12.06 s and 12.27 s). Comparison of these NMR data with those reported in the literature (Bachmann et al., 1979; Parmeswaran et al., 2004) revealed the compound to be known anthraquinone derivative Physcion (**FM-8**).



Physcion (**FM-8**)

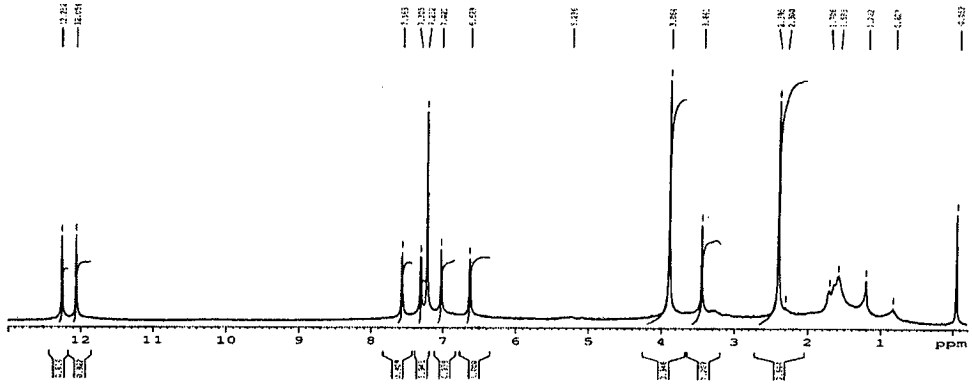


Fig. 2.15a ^1H NMR spectra of Physcion

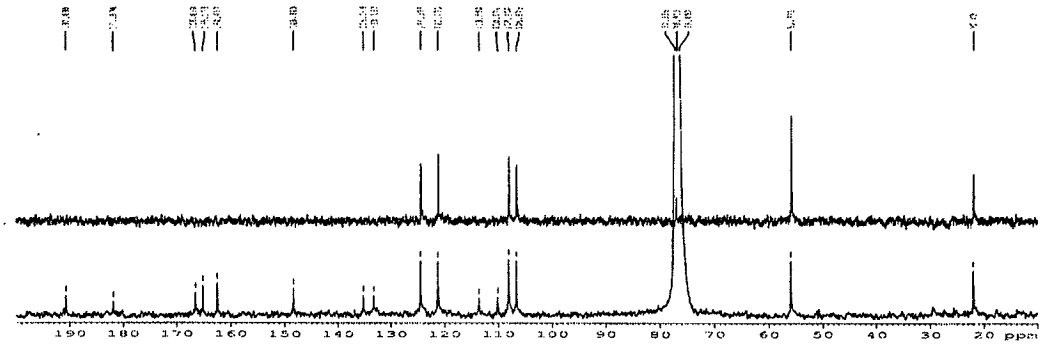


Fig 2.15b ^{13}C NMR spectra of Physcion

v) Biological activity

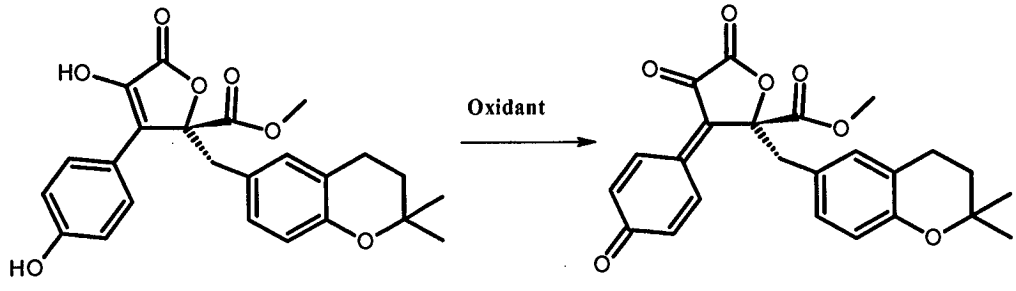
Terrein (**FM-3**) and Physcion (**FM-8**) are well known metabolites and known mainly for their antimicrobial and antioxidant activities respectively. **FM-7** on standing in pure form or in the solution would change to **FM-6** indicating it to be unstable. Therefore, only **FM-6** along with **FM-1** was evaluated for anticancer, and antinflammatory activities.

Butyrolactone I (**FM-1**) is a selective inhibitor of cyclin dependant kinases cdk1/cyclin B and cdk2/cyclin A (Fischer et al., 2000) and it is as potent as roscovitine, a drug currently undergoing phase IIb clinical evaluation (www.cyclacel.com). CDKs are involved in numerous diseases, among which are cancer, Alzheimer's disease, Parkinson's disease, stroke, diabetes, polycystic kidney disease, glomerulonephritis, inflammation, and AIDS (Knockaert, et al 2002.; Shapiro 2006). FM-1 has been demonstrated to exhibit antiproliferative activity against colon and pancreatic carcinoma, human lung cancer (Nishio et al., 1996), and prostatic cancer cell lines (Suzuki et al., 1999).

In anticancer activity testing done by us, FM-6 displayed inhibition against H460 (large cell lung cancer), ACHN (renal cell adenocarcinoma), Calu (epidermoid carcinoma), Panc1 (epithelioid carcinoma) and HCT116 (colorectal carcinoma) cell lines with IC_{50} 88, 103, 147, 130, 121μM respectively. This activity was marginally better than observed for **FM-1**. It has been shown that elimination of hydroxyl groups

or changing them with other polar groups such as nitro, chloro, amino alkyl in **FM-1** does enhances the antiploliferative activity, but elimination of prenyl chain make the compound (**FM-2**) completely inactive (Brana et al., 2004). As the antiproliferative activity was maintained in the **FM-6** having prenyl chain, in the form of dimethyl pyran ring further provides the experimental proof for the conclusion derived by Brana et al (2004) that alkenyl chain (prenyl) is an essential component in maintaining antiproliferatve activity.

There is a recent report on **FM-6** and its soybean lipoxygenase inhibitory activities. **FM-6** has been found to be exhibit this activity with IC_{50} 55.9 μM. The inhibitory activity was much higher than that for 138.2 μM, **FM-1**, 343.8 μM for **FM-2** and 147.5 μM for a standard compound kojic acid suggesting dimethyl pyran ring has role to play (Sugiyama et al 2010). The mechanism of lipooxygenase has been elucidated. **FM-6** is oxidized to a quinone methide (**68**) by loss of two hydrogens. It is difficult here to rationalize from the observed transformed product (**68**) scheme 2.7 as to why **FM-6** has higher activity than **FM-1** and **FM-2m,** as other two also would form similar products. From the observed activity trend it is apparent that **68** is more stable than the corresponding quinone methide of **FM-1** which in turn more stable than quinone methide of **FM-2**.



FM-6 **68**

Scheme 2.8: Lipooxygenase oxidatation of FM-6

III. Experimentals

i) General experimental procedures

Mixture of methanol and chloroform (10:90, v/v) was used as mobile phase for TLC analysis. Compounds were visualized as intense rose colour spots on spraying with methanolic sulphuric acid (95:5, v/v) followed by heating at 120°C.

ii) Fungal isolation, identification and cultivation

Soft coral *Sinularia kavarattiensis* was collected by scuba diving at a depth of 8-10m from the coast of Mandapam, Tamil Nadu, India in May 2004. After washing the soft coral with sterile sea water, fungus *Aspergillus terreus* was isolated as an epiphyte using Czapek agar containing (g/l) $NaNO_3$ (2.0), $MgSO_4.7H_2O$ (5.0) $FeSO_4.7H_2O$ (0.1), KH_2PO_4 (1.0), KCl (0.5), agar (3.0) sucrose (30.0) at pH 5.5 prepared in seawater supplemented with penicillin benzyl sodium salt (0.02) to avoid any bacterial growth. After 6-7 days sand brown, velvety, reverse the medium deep dirty brown colonies were observed. The strain was identified as *Aspergillus terreus* from the morphological features of conidiophores (by Dr. Sanjay K. Singh, mycologist, Agharkar Research Institute, Pune). Straun has A voucher specimen of the fungus is deposited at National Institute of Oceanography, Dona Paula, Goa, India. Stock cultures of the fungus, maintained at -20^0C preserved with 20% glycerol was used to inoculate 500 ml of seed medium in Erlenmeyer flask (4 L) containing 24 g/l of potato dextrose broth in seawater. It was then cultured at 27±2°C on a rotary shaker at 200 rpm. The flask was incubated for 72 hr and used as a first stage inoculum. The same medium (1 L) was made in 10 Erlenmayer flasks (4 L) and inoculated with 5% of first stage inoculum. The flasks were incubated for 21 days at 27±2^{0}C on a rotary shaker at 200 rpm for 10 hr/day.

iii) Extraction and isolation of metabolites

21 days old fermentation broth (10 L) was separated from fungal mat and concentrated to a volume of 1 L under reduced pressure. The broth was extracted first with chloroform (200 ml X 4) followed by ethyl acetate (200 ml X 4). Chloroform

and ethyl acetate layers were separately concentrated under reduced pressure to yield chloroform extract (470 mg) and ethyl acetate extract (430 mg). The ethyl acetate extract was chromatographed over Sephadex LH-20 using MeOH-$CHCl_3$ (1:1) to yield pure crystalline compound, **FM-3** (92 mg) (Dunn et al., 1975) and a fraction containing an intense rose colour spot. The fraction containing intense rose colour spot was flash chromatographed over silica gel using gradient elution of MeOH-$CHCl_3$ (5:95 - 20:80) to yield **FM-7** (7.2 mg; Rf, 0.51). The chloroform extract was repeatedly chromatographed over Sephadex LH-20 using MeOH-$CHCl_3$ (1:1) and 100% MeOH, which yielded a pure yellow coloured compound, **FM-8** (17 mg) Other fractions giving two prominent rose colour spots on TLC was further purified over silica gel column using gradient elution of MeOH-$CHCl_3$ (0:100- 20:80) to afford **FM-1** (32.0 mg; Rf, 0.64) and another nearly pure compound which was fine purified over Sephadex LH-20 using MeOH/$CHCl_3$/Pet. ether (40 :40 :20) to yield **FM-6** (26.3 mg; Rf, 0.81).

iv) Aspernolide A (FM-6)

White sticky solid; $[\alpha]^{28}_D$ +88.73 (c 0.58, $CHCl_3$); UV (MeOH) λ_{max} nm : 303, 240; IR (NaCl) ν_{max} cm^{-1} 3340, 3024, 2970, 2935, 1737, 1732, 1610, 1519, 1498, 1436, 1386, 1261, 1182, 1122, 1068, 1037, 948, 839, 754; For NMR data see Table 1; HRESITOFMS $[M+Na]^+$ *m/z* 447.1433 (calcd. 447.1420 for $C_{24}H_{24}O_7Na$), $[2M+Na]^+$ *m/z* 871.2959 (calcd. 871.2942 for $C_{48}H_{48}O_{14}Na$); EIMS *m/z*(%): M^+ 424(2.7), 380(37.8), 348(35.1), 320(6.8), 293(13.5), 265(8.1), 237(10.8), 218(5.4), 205(6.8) 189(6.8), 175(100), 157(8.1), 145(10.8), 131(16.2), 119(13.5), 107(5.4), 91(24.3), 77(3.5), 69(12.1), 44(37.8).

v) Aspernolide B (FM-7)

Light brown syrup; $[\alpha]^{28}_D$ +48.27 (c 0.29, MeOH); UV (MeOH) λ_{max} nm : 303, 240; IR (NaCl) ν_{max} cm^{-1} 3380, 3024, 2975, 2933, 1745, 1610, 1519, 1442, 1386, 1182, 1070, 1037, 838, 762; For ^1H and ^{13}C NMR spectroscopic data see Table 2; HRESITOFMS: $[M+H]^+$ *m/z* 443.1699 (calcd. 443.1706 for $C_{24}H_{27}O_8$), $[M+Na]^+$ *m/z* 465.1516 (calcd. 465.1525 for $C_{24}H_{26}O_8Na$); EIMS *m/z*(%): $[M-CO2]^+$ 398(13),

380(70), 348(100), 333(18), 320(15), 293(40), 205(26), 249(10), 237(23), 218(17), 205(25), 188(16), 175(77), 145(13), 131(41), 119(23), 107(15), 91(35), 77(17), 69(20), 59(33), 43(23), 41(11).

*vi) physcion (**FM-8**)*

Yellow crystalline neddles (EtOAc) mp 202-204 "C; UV (MeOH) λ_{max} 224, 255, 265 , 287, 435 nm ; IR (KBr) ν_{max} 2940-3040, 2840, 1680, 1629, 1614, 1570, 1486, 1390, 1368, 1306, 1262, 1105, 1100, 1034,992 cm-I; ^{1}H NMR ($CDC1_3$,) δ_H 2.39 (s, C-3-CH_3), 3.90 (s, C-6-OCH_3), 6.63 (d, J = 2.0 Hz, H-7), 7.02 (brs H-2) 7.31 (d, J = 2.0 Hz, H-5), 7.55 (brs, H-4), 12.06 (s, C-1-OH), 12.26 (s, C-8-OH); EI-MS, M^+ 284, 255 (M - CO - H), 241 (M - CO - CHJ,- CH20), 185 (M - 3CO - CH3).

*v) Conversion of butyrolactone I (**FM-1**) to aspernolides A (**FM-6**), B (**FM-7**) and C (**67**)*

FM-1 (76.9 mg) was dissolved in MeOH (10 ml) containing conc. HCl (0.2 ml). Mixture was stirred at r.t. approximately for 2 hr. or till complete conversion of **FM-1** as indicated from TLC. Solvent was removed under vacuum and resulting residue was separated on flash Si-gel column using gradient elution of MeOH/$CHCl_3$ (0:100 – 20:80, v/v) to yield in the order of increasing polarity **FM-6** (58.6 mg, 75 %), **57** (8.8 mg, 10.4%) and **FM-7** (6.9 mg, 8.3%).

vi) Aspernolide C (57)

Light brown syrup; ^{1}H and ^{13}C NMR spectroscopic data see Table 2; HRESITOFMS: $[M+H]^+$ *m/z* 457.1851 (calcd. 457.1862 for $C_{25}H_{29}O_8$) and $[M+Na]^+$ *m/z* 479.1669 (calcd. 479.1682 for $C_{25}H_{28}O_8Na$).

Section 2.3: Antifungal secalonic acid D from the marine sponge associated fungus *Aspergillus aculeatus*

I. Introduction

Marine animals, especially sponges, have been demonstrated to represent a rich source of fungal diversity (Proksch et al. 2008; Proksch et al. 2010). Of the total new metabolites discovered from marine derived fungi, 19% have been obtained from sponge associated fungi, which is second only to metabolites from fungi associated marine algae (Rafteb and Ebel, 2011). Sponges are filter feeders; therefore the origin of fungal endophytes and the true nature of their interaction with their hosts remain largely unclear. The fact that many fungi so far isolated from sponges are well known from terrestrial habitats where they ubiquitously occur might suggest that fungal spores which are washed into the sea are sequestered by sponges through filter feeding and are retained in the inhalant canals from which they can be isolated and subsequently cultivated. Furthermore, there has been no evidence reported so far indicating that fungi actively grow inside sponges suggesting that their presence in these invertebrates is in fact limited to spores. On the other hand, compounds obtained from marine isolates of ubiquitously occurring fungal genera such as *Aspergillus* or *Penicillium* differ often significantly from those of terrestrial strains (Edrada et al., 2002; Lin et al., 2003) indicating that at least on a biochemical level many sponge derived fungi are distinguished from their terrestrial counterparts.

In an investigation on an endophytic fungus *Asprgillus aculateus* from a marine sponge led to the identification of secalonic acid D to be a potent antifungal against *Candida albicans.* The same along with short review on metabolites of *A. aculateus* and secalonic acids and its biosynthesis is presented in this section.

II. Metabolites from *Aspergillus aculeatus*-literature review

Aspergillus aculeatus is member of *Aspergillus* section *Nigri* (formerly *A. niger* group). These fungi develop black colonies and therefore also called black *Aspergilli.* The morphological differences between the species are very subtle and many of them are sometime incorrectly identified as *Aspergillus niger.* Although, cladification of *Aspergillus* section nigri using the β-tubulin and calmodulin genes have been shown that three clades could be distinguished, it can be preliminary distinguished into two varieties by the uniseriate (single palisade of cells which give rise to conidia) or biseriate (two palisades of cells) structures of the conidiophores (**Fig 2.16**) the colour of the colony and the conidial heads, and the shape and ornamentation of the conidia (Abarca et al 2004). *A. aculateus* which is very similar in morphology as well as on molecular level with *Aspergillus japonicus* are uniseriate species. Recently new species *Aspergillus aculeatinus* and *Aspergillus uvarum* two uniseriate black *Aspergillus* species have been described. (Samson et al 2007).

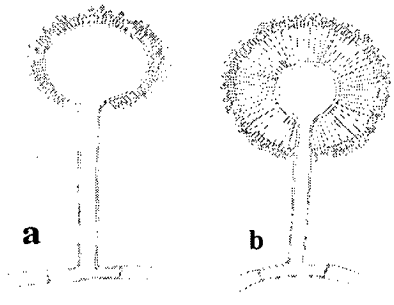
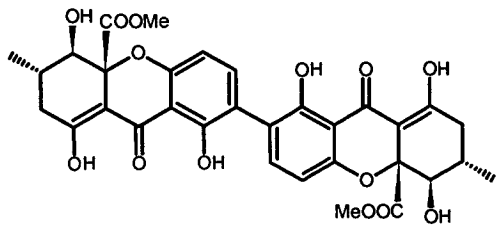
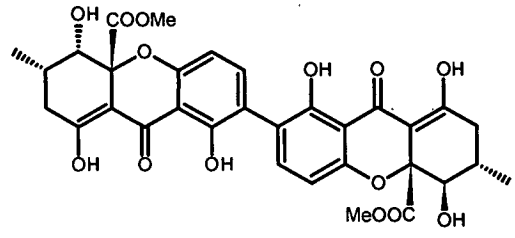


Fig 2.16: Microscopic view of Black aspergilli condiophores a uniseriate species; b biseriate species

Only four reports have appeared so far on new metabolites of *A. aculeatus*, of which two are on marine derived strains. The first two metabolites to be unveiled from this fungus were secalonic acids D (SAD) (**FM-16**) and F (SAF) (**69**) (Andersen et al 1977). SAD was previously known, while SAF was new member of its group. SAD proved to be stronger antimicrobial than SAF against *Bacillus magaterium.*

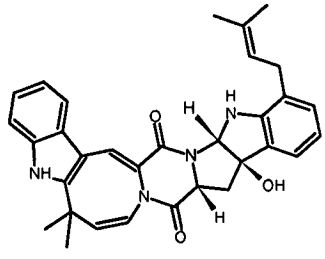


SAD (FM-17)



SAF (69)

A soil derived isolate of *A. aculeatus* when cultured on okara (the water-insoluble residue of whole soybean) produced two new metabolites okaramines H (**70**) and I (**71**) along with known, okaramines A (**72**) and B (Hayashi et al., 1998). The known metabolite **72** showed much better insecticidal activity than its new congeners **70** and **71**. Okaramines A (**72**) B, D, E, F, and G were known from a strain of *Penicillium simplicissimum* ATCC 90288.



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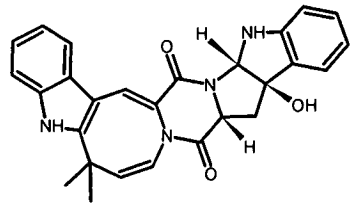

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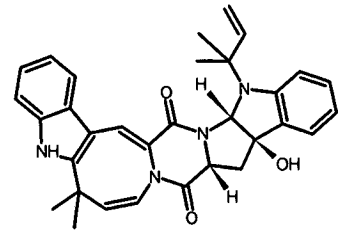

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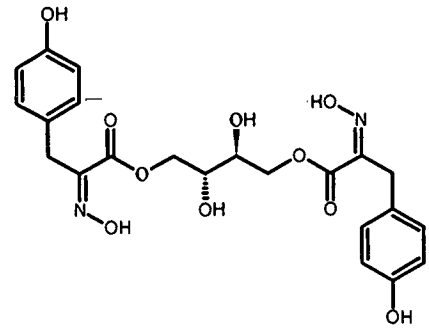
The Chemical exploration of the fungus *A. aculeatus* CRI323-04 derived from marine sponge *Xestospongia testudinaria* led to the isolation of one new and one known tyrosine-derived metabolites aspergillusol A (**73**) and methyl ester of 4-hydroxyphenylpyruvic acid oxime (Ingavat et al 2009). Aspergillusol A (**73**), has structure similar to that of a brominated tyrosine-derived metabolite, psammaplin A (**74**), a metabolite found in different types marine sponges. This is only instant wherein metabolites isolated from fungus derived from hydroxyphenylpyruvic acid have an oxime moiety in it resembling **74**. An investigation by same group researcher on metabolites from another marine sponge, *Stylissa flabelliformis* derived fungal strain of *A. aculeatus* resulted in isolation of three new metabolites, pre-aurantiamine (**75**), (-)-9-hydroxyhexylitaconic acid (**76**) and (-)-9-hydroxyhexylitaconicacid-4-

methyl ester (**77**), together with two known compounds, paraherquamide E and secalonic acid D (**FM-16**) (Antia et al., 2011).



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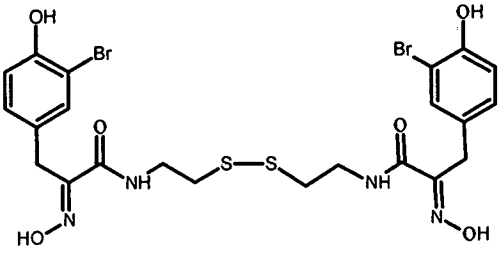

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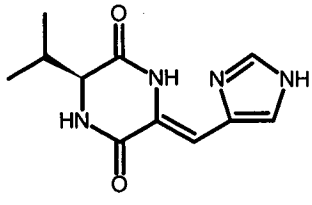

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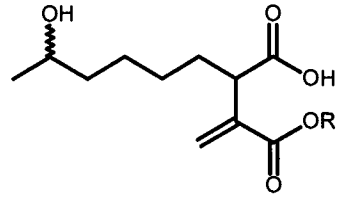


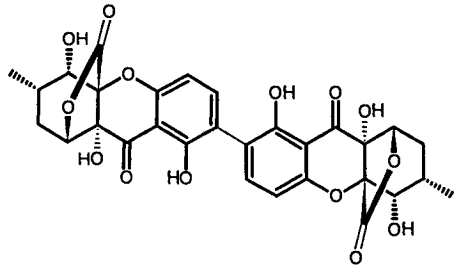
76 R=H

77 R = CH_3

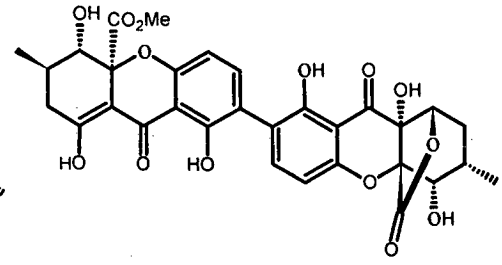
III. Secalonic acids and their biosynthesis

The secalonic acids along with ergoflavins [e.g. ergoflavin (**78**)] and ergochrysins [e.g. ergochrysin A (**79**)] are members of the ergochrome family. They represent important group of biologically highly active mycotoxins, produced by fungi and also from other microorganisms and plants (Franck, 1980). So far at least twenty-two members of the ergochrome family are known as natural products. They are dimers of six different monoxanthones [hemisecalonic acids A–F (HAS A-F)], and ergochrome diversity is attributable to different homo- and heterodimers of these six monomeric units (Franck, 1980). The first of these type fungal metabolites, named ergoflavins, were isolated in pure form from the fungus *Claviceps purpurea* (ergot) in 1958 (Eglinton et al.). Secalonic acids have 2,2'-linkage, but secalonic acids A-D were incorrectly assigned to have 4,4'-linkage, eventually rectified by Hopper et al., (1971) to have 2,2'-linkage from transformational studies and their NMR data. The 4,2'-linkage and 4,4'-linkage in ergochrome dimers have also been reported. Isoergochrysin (**80**), and another class of dimers, the eumitrins are coupled through the 4,2'

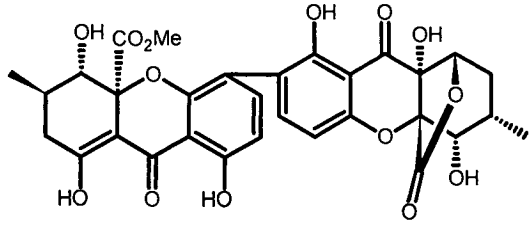
positions (Yang et al., 1973), while phomoxanthon A (**81**), a dimer isolated from *Phomopsis* sp. is coupled through 4,4'-position (Isaka et al., 2001).



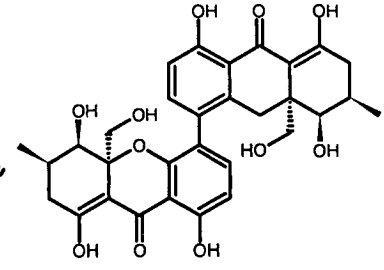
Ergoflavin (78)



Ergochrysin A (79)



Isoergochrysin (80)

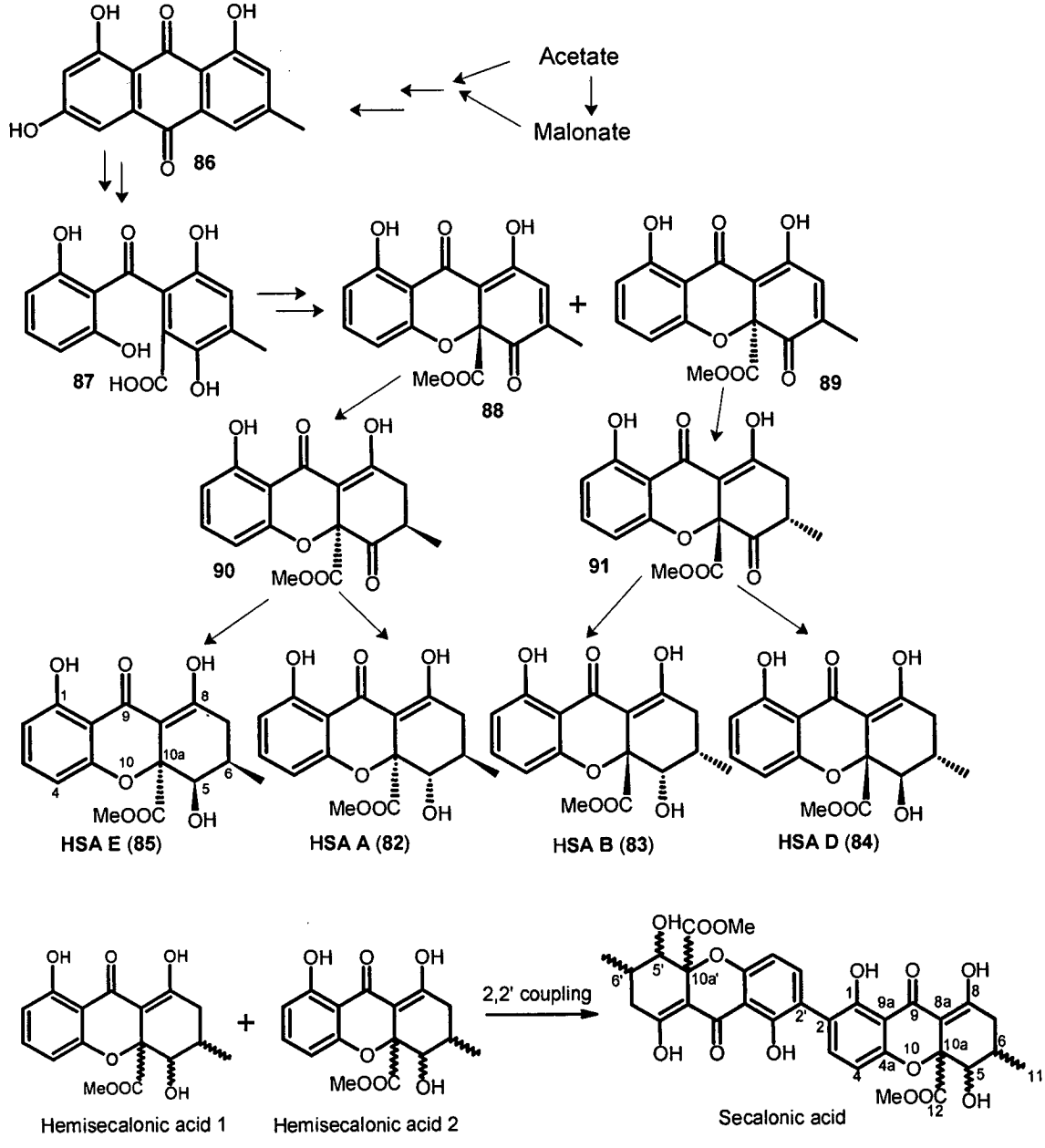


Phomoxanthone (81)

Secalonic acid have six chiral centres (three in each monomer) at 5, 6 and 10a in one half and 5', 6' and 10a' in another half of the molecule. Theoretically, large number of steroisomers is possible for the secalonic acids, but the number is restricted to only ten isomers considering the specificity of the biosynthetic pathway, which can give only four stereoisomeric monomers of tetrahydroxanthones [hemisecalonic acids A (**82**), B (**83**), D (**84**) and E (**85**)]

The biosynthesis of the secalonic acid from emodin has been postulated (**Scheme 2.9**) (Kurobane and Vining, 1979). It is assumed that the emodin (**86**), which is a co metabolite in the culture producing secalonic acids is a intermediate precursor to these secalonic acids. Emodin (**86**) is first reduced at C-3 and oxidized at C-5, which is followed by Bayer-Villager type oxidation through C5-C10a bond giving rise to benzophenone carboxylic acid (**87**), which is methyl esterified, cyclized and reduced at C6-C7 double bond and C-5 carbonyl to HSAs. It is the final three steps involving a cyclization step and two reduction steps that govern the stereochemistry of **82-84** and thus stereochemistry of secalonic acids. The cyclization step followed by

reduction steps in principle should give rise to eight tetrahydroxanthones (HSAs), but the that fact no ergochromes with *cis* stereochemistry of substituents at C-6 and C-10a have been so far reported, restricts to formation of HSAs to only four. This implies that the reduction of C6-C7 double bond is catalysed by specific enzyme and configuration of C-10a directs the stereochemistry of reduction.



Scheme 2.9: Biosynthesis of secalonic acids

The different combinations (2,2'-linkage) of these four HSAs thus can give rise to only ten secalonic acids A-J. The self coupling of HSAs generates SAA (secalonic acid A), SAB, SAC and SAD, while cross coupling should give six hybrid secalonic acids of which four SAB (AB, coupling of hemisecalonic acid A and B), SAF (BD), SAG (AE), SAH (DE) should be chiral due to coupling of disteromeric monomers, while two SAI (AD) and SAJ (BE) will be meso due to coupling of enantiomeric monomers.

Although four HSAs (**82-85**) can be formed during cyclization and reduction of benzophenone carboxylic ester intermediate, any organism that produces secalonic acid appears to make only two HSAs. The pair of HSAs produced either differ by position at C-5 or at the trans substituted C-6 and C10a. The pair of HSAs composing secalonic acids in fungus *Penicillium terrestris* differs only at C-5 which produces SAA, SAE and SAG. Same is also true in fungus *A. aculeatus* which produces SAB, SAD and SAF. These observations suggest that the cyclization of benzophenone methyl ester is very specific in these fungi giving only one isomer, while the reduction of double bond is specific in generating trans stereochemistry at C-6 and C10a the reduction of C-5 carbonyl is not specific leading to two HSAs (HSAs A and E in *P. terrestris,* whereas A and E in *A. aculeatus*) having opposite configuration at C-5.

On the other hand, fungus *Claviceps purpurea* produces SAA, SAB and SAC, whose pair of monomers had a same configuration C-5, but differed by having opposite configurations at trans substituted C-6 and C-10. This observation indicates that in this organism cycliztion is not specific and produces both the enantiomers, but both reduction steps are specific giving rise to only two HSAs A (**82**) and B (**83**). A similar case can be formation of HSAs D (**84**) and E (**85**), which should lead to the formation of SAD, SAE and SAH, but likewise no reports have appeared yet.

The dimerization of HSAs is suggested to be the last step in the biosynthesis. The absence of any dimeric product in which two halves of the molecules are at different oxidation levels argues in favour of this.

In conclusion, the stereochemistry in the biosynthesis of secalonic acids is governed by three steps (a cyclization and two reductions) and the reduction of C6-C7 double is always specific. Of the other two steps at least one or both might be specific. Therefore, it appears any organism that will produce HSA A or B will not produce its enantiomer D or E respectively. The consequence is that it further reduces the number of secalonic acids that can be obtained naturally to eight as the meso secalonic acids SAI (AD) and SAJ (BE) cannot be formed.

IV. Results and Discussion

i) Biological materials

Marine sponge derived fungi represent a huge resource for discovery novel chemicals. South east coast of India harbours rich diversity of marine sponges. A marine sponge *Cinachyra cavernosa* was collected by scuba diving at a depth of 8 to 10 m from Mandapam, Tamil Nadu, India. Out of several fungi isolated from this sponge only one was identified initially as *Aspergillus* species. Initially it was identified to be *Aspergillus niger* (section nigri, also called black Aspergilli) *from* its morphological features. As our interest was to study metabolites from *Aspergillus* fungi, we took this fungus for chemical investigation, which yielded secalonic acids. The literature survey revealed *A. niger* is not known for production of secalonic acid but other species *A. aculeatus and A. aculeatinus* belonging to same section nigri are known to produce secalonic acids. Therefore, we got the phylogentic analysis done on this strain using molecular techniques, and as to our expectation the fungus was identified as *A. aculeatus.*

ii) Purification of metabolites

The organic constituents were adsorbed on HP 20 diaon resin from the culture medium and the resin was washed with MeOH and MeOH washings were dried to obtain yellow powder. This yellow powder on filtration through Sephadex LH20 (GPC) was collected in 45 fractions. Fractions 17-19 (**FM-16**) showed long yellow

bands in visible light on TLC (**Fig 2.17**), but at the centre of this band it had a bright spot visible in UV at 254 nm.

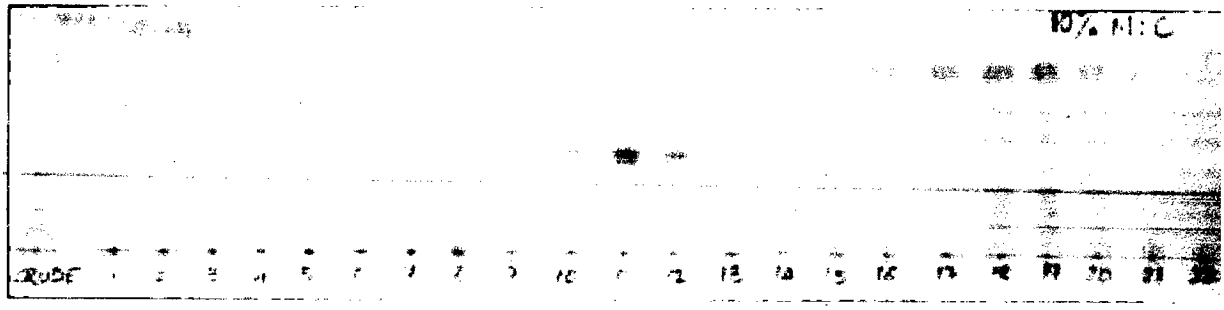


Fig 2.17: TLC of chromatographic fractions of extract of *A. aculeatus*

Fractions 17-20 were pooled together and upon evaporation of solvent gave bright yellow solid (**FM-16**).

iii) Structure elucidation of FM-16

FM-16 was obtained as bright yellow solid$[\alpha]^{28}_{D}$ = 0 in $CHCl_3$ or in MeOH. The molecular formula of the **FM-16** was determined to be $C_{32}H_{30}O_{14}$ from its ESIMS (**Fig 2.18a**), which displayed a pseudomeolecular ions at $[M+H]^+$ at m/z 639 and $[M+Na]^+$ at m/z 661. Its IR spectra (**Fig 2.18b**) showed absorption band at 3552 cm^{-1} for hydroxyl. The ^1H NMR spectra (**Fig 2.18c**) of **FM-16** showed every set of signal in duplicates. It showed signals for two methyl doublets δ_H 1.5 (J = 6 Hz) and 1.7 (J = 6 Hz); a set of signals in the region δ_H 2.25-2.77 HZ corresponding to pairs of methine, methylenes and hydroxyl; a signal at δ_H 3.73 (s) corresponding a pair of methoxyls; pair of hydroxymethines, a doublets at δ_H 4.02 (J = 10.4) and broad singlet at δ_H 4.12; two pairs of aromatic ortho hydrogen doublets (J = 8.4 Hz) at δ_H 6.56 and 7.42 and δ_H 6.62 and 7.44; and two chelated hydrogens at δ_H 11.74 and 1.88Hz and another pair of chelated hydrogens at δ_H 13.77 and 13.96. As in ^1H NMR in ^{13}C NMR (**Fig 2.18d**) also signals were displayed in pairs. These NMR data accounts for all the 34 hydrogens in the compound and the signals obtained in pairs is suggestive of dimeric molecule like secalonic acid having one half of the molecule stereochemically different from the other half. If the two halves of the molecule are stereochemically different than the compound has to be optically active, which

contradicts with our earlier observation of optically inactivity of the compound, which suggest molecule to be achiral.

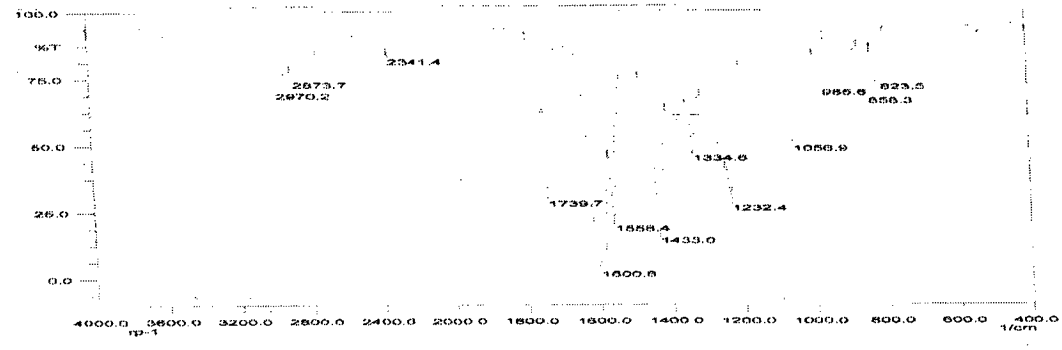


Fig 2.18b: ^{1}H NMR spectra of FM-16

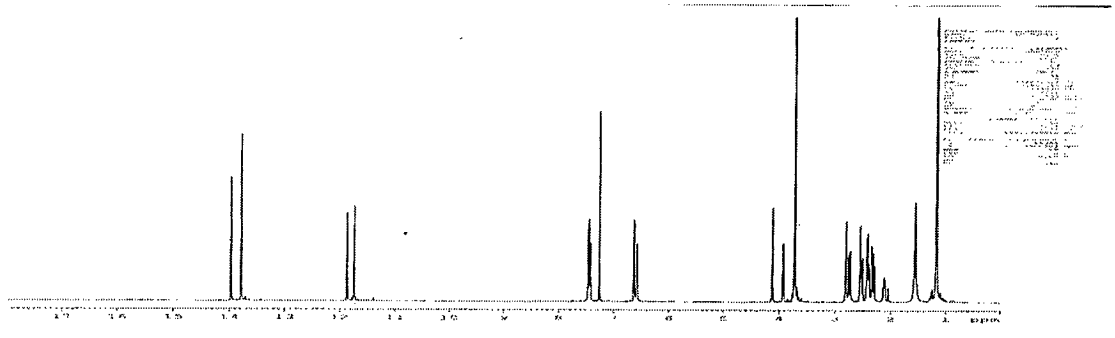


Fig 2.18c: ^{1}H NMR spectra of FM-16

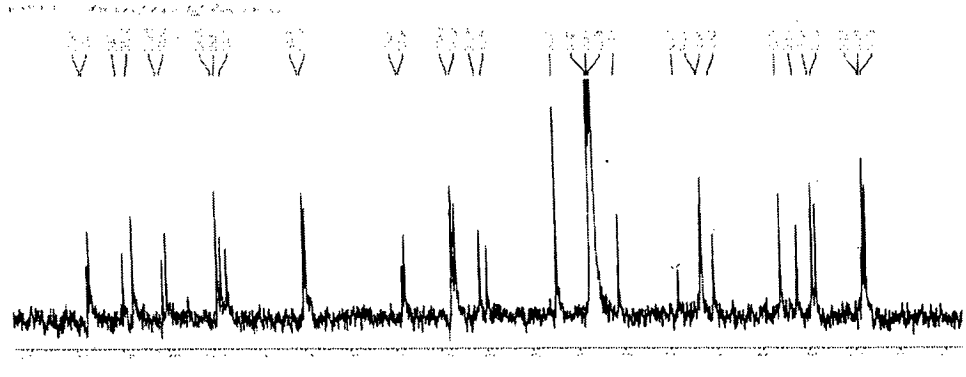


Fig 2.18d: ^{1}H NMR spectra of FM-16

As discussed in **Part B** that in nature only eight types of secalonic acids can be formed by combinations of four monomeric HSAs, which are stereochemical variants

of each other. As per the spectroscopic data the **FM-16** has to be one or mixture of two secalonic acids from the possible ten.

The HSA A (**72**) is enantiomer of HSA D (**74**), while HSA B (**73**) is enantiomer of HSA E (**75**), hence SAA, SAB, SAC and SAF are indistinguishable from their enatiomers SAD, SAE, SAH and SAG respectively from their NMR data. While, the homo dimers SAA, SAB, SAD and SAE gives NMR signals for only one half of the molecule, the heterodimers SAC (AB), SAF (BD) and SAG (AE) gives separate signals for both the halves. The remaining three heterodimers, which are not reported yet, the two *meso* SAI (AD) and SAJ (BE) are expected to give signals for only one half, and the third one SAH (DE),which is chiral should give different signals for the monomeric units.

From the optical inactivity and NMR data for the compound (**FM-16**) under investigation following three cases can be considered.

1. **FM-16** can be mixture of two meso isomers SAI (AD) or SAJ (BE)

2. **FM-16** is a heterodimer SAC (AB) or SAH (DE)

3. **FM-16** might be mixture diasteromers SAA with SAB or SAD with SAE

Case 1. No *meso* secalonic acids have been yet reported as natural product, which suggests that any culture that produces the hemisecalonic acid A do not produces its entantiomer hemisecalonic acid D and culture that produces hemisecalonic acid B do not produces it enantiomer, hemisecalonic acid E. Therefore, it has been observed so far that SAA or SAB has never been isolated from the culture producing their enantiomer SAD or SAE respectively and vice versa. This goes to suggest that it is very unlikely even one of the *meso* secalonic acids will be obtained as natural product. Therefore the possibility of the **FM-16** as mixture of two meso secalonic acid is ruled out.

Case 2. SAC or SAH being heterodimers will give different NMR signals for both the half of the molecule. About optical rotation, SAC, has low optical rotation and SAH

should also have low rotation, the dimers made of momeric units of opposite rotation should have a low optical rotation and, as we had low amount of the **FM-16** (~12 mg) the optical rotation recorded at very low concentration might not have been sensitive to the instrument we used.

Case 3. Another possibility is that the **FM-16** is mixture of two diastereomers such as SAA with SAB or SAD with SAE. These pairs diastereomers nearly have or expected to have same optical rotations and opposite sign as result their mixture in certain proportion would show very low or no optical rotation. Their NMR spectra would also show two sets of signals. As discussed in the biosynthesis of secalonic acids any organism to produce triplet of diasteromeric secalonic acids SAA, SAB and SAC or SAD, SAE and SAH the cyclization of benzophenone benzoic acid ester (**87**) should not be specific. Whereas the production of SAA, SAB and their hybrid dimer SAC has been observed in *C. purpurea* the production of triplet of secalonic acids SAD, SAE and SAH has not been observed in organisms so far. In contrary, previous study on *A. aculeatus* indicates that cyclization of benzophenone benzoic acid ester is specific and therefore cannot produce the triplet of secalonic acids SAD, SAE and SAH but known to produce triplet of SAD SAB and SAF indication the reduction of carbonyl step is no-specific.

So, to put an end to this ambiguity we crystallized the **FM-16** from a mixture of MeOH-$CHCl_3$ to obtain crystals for X-ray diffraction studies. On single X-ray diffraction studies on the sample it was revealed that the compound analyzed was SAD (**FM-17**). **Fig 2.19** shows the thermal elliposoid representation of **FM-17**.The unit cell parameters of a = 17.647Å, b = 7.165Å, c = 13.127Å, $\alpha = \gamma = 90°$, and β = 93.720 were in agreement with the reported for SAD (Barnes et al., 1999). So, it was assumed that during crystallization fractional crystallization between the two isomers had taken place leading to isolation of crystals of SAD.

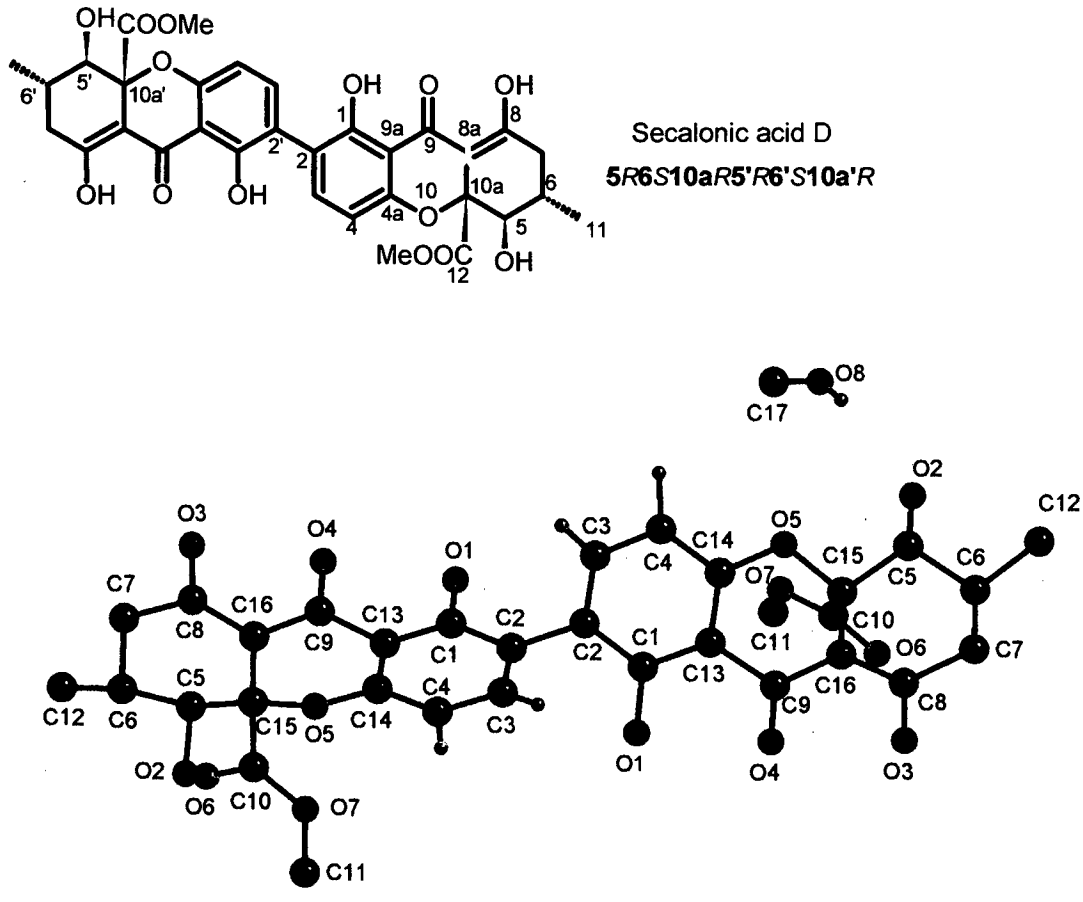


Fig 2.19: Thermal ellipsoid reprentation of SAD

The little crystalline sample of **FM-17** which was available with us, we recorded NMR on it, and to our expectations one set of signals were observed with larger intensity matching for SAD in its NMR spectra (**Fig 2.20a** and **2.20b**). The other set of signals were observed in very low intensity, indicating the crystallized sample still had the other isomer in minor amount in it, which we expect it to be SAE on the basis of optical inactivity of the mixture. Surprisingly the optical activity data taken on **FM-17** was still nearly zero. Therefore it cannot be conclusively stated, whether other isomer is SAE or SAB. The NMR data of SAD (**FM-17**) obtained on crystallized sample and NMR data of other stereoisomer obtained on diasteromeric mixture with SAD (**FM-16**) are tabulated below (**Table 2.6**)

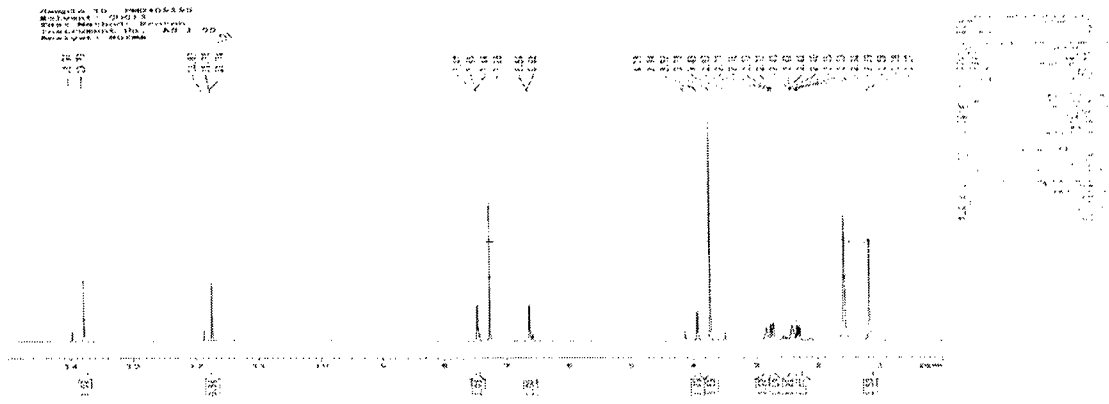


Fig 2.20a: ^{1}H NMR spectra of FM-17

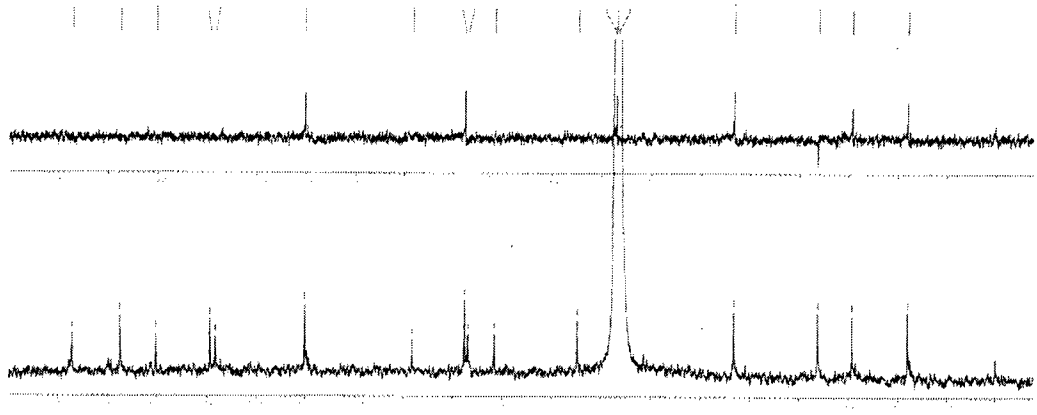


Fig 2.20b: ^{13}C NMR spectra of FM-17

Table 2.6: NMR data of FM-17 in $CDCl_3$

Position	δ_H, mult., J(Hz)	δ_C, mult.	Position	δ_H, mult., J(Hz)	δ_C, mult.
1		158.3 s	11	1.17, d, 6	18.0 q
2		118.2 s	12		170.3
3	7.45, d, 8.4	140.2 d	13	3.73, s	53.2 q
4	6.20, d, 8.4	107.5 d	4a		159.4
5	3.91,d, 10.8	77.0 d	8a		101.5
6	2.24, m	28.4 d	9a		106.8
7	2.74, dd , 18.4, 5.4 2.31 dd, 18.4, 10.5	32.6 t	10a		84.7
8		177.5 s	1-OH	13.79	
9		187.1 s	8-OH	11.75, s	

iv) Antifungal activity

The strong antifungal activity of the extract against a human pathogen *Candida albicans* had prompted us to investigate its active principles. The purified metabolite **FM-17** also showed strong antifungal activity and therefore its minimum inhibition concentration on disc diffusion assay was determined. **FM-17** showed inhibition zone of 6 mm at 25 µg/disc and at conc. from 25 - 3µg/disc it consistently showed inhibition zone between 4-5 mm (**Fig 2.21**) while it showed inhibition zone of 2-3 mm at concentration as low as 0.5 µg/disk. A standard compound, flucanozole, a drug presently in the market showed inhibition zone of 2 mm at 25 µg/disc and at any concentration below this it was inactive (**Table 2.6**).

Table 2.6: Antifungal activity of SAD and Flucanozole (std) against *Candida albicans*

Concentration(µg/disc)	Flucanozole (Std) Inhibition zone in mm	Secolanic Acid D Inhibition zone in mm
25	2	6
20	-	5
15	-	4-5
10	-	4-5
5	-	4-5
4	-	4-5
3	-	4
2	-	2-3
1	-	2-3
0.5	-	2-3

Fig 2.21: Antifungal (*C. albicans*) assay plate at concentrations 4, 5, 10 and 15 µg/disc

V. Experimentals

i) Fungal isolation, identification and cultivation

Marine sponge *Cinachyra cavernosa* was collected by scuba diving at a depth of 8 to 10 m from Mandapam, Tamil Nadu. The fungus *Aspergillus aculateus* was isolated as an endophyte using standard isolation techniques on PDA. It was identified as *A. niger* from its morphological characteristics (Dr. Sanjay Singh, ARI, Pune) and *A. aculateus* by molecular techniques. The pure isolate was inoculated from plates into flasks containing PDB (24g/l) in sea water (2 L x 3). The flasks were incubated at room temperature for 20 days on a rotary shaker at 200 rpm.

ii) Extraction and isolation of metabolites

HP-20 diaon resin (250 g) column was prepared in distilled water. 20 days old fermentation broth (6 L) was filtered through cotton wool to remove mycelium and was passed through HP-20 diaon. Column was washed first with water (2 L) till the effluent was colourless and then with methanol (1 L). Methanol fraction was collected and evaporated under vacuum to yield yellow powder (0.785g). This bright yellow powder was loaded onto Sephadex LH 20 column and eluted with MeOH-$CHCl_3$ (1:1) and collected in 25 fractions. Fractions 16-22 were collected and evaporated to bright yellow solid (**FM-16**) (17 mg). This solid was recrystallized from $CHCl_3$-MeOH to give thin yellow needles of secalonic acid D (**FM-17**).

iii) Secalonic acid D (FM-17)

Yellow crystalline needles; $[\alpha]^{28}_D$ 0 (c 0.140, MeOH or $CHCl_3$); HRESITOFMS: $[M+H]^+$ *m/z* 639.1753; IR (KBr) ν_{max} 3552. 2968, 2931, 2873, 1739, 1604 1589 1433, 1232, 1058; ^1H NMR and ^{13}C NMR (500 MHz, CD_3OD) see **Table 2.6**

iv) Antifungal activity

Antifungal activity was using standard disk diffusion assay in which first an inoculum of pathogen was spread over sterile nutrient broth agar plates. In the center of the

plate a paper disk impregnated with the compound/extract was placed. The plates were incubated for 2-3 days at rt and inhibition zone was measured.

Section 2.4 Comparison and Identification of metabolites from the fungi *Aspergillus flavipes* and *Aspergillus terreus* using tandem mass spectrometry

I. Introduction

The chemical investigation of the marine-derived fungi *Aspergillus flavipes* (sections 2.1) and *Aspergillus terreus* (sections 2.2) led to the isolation of butyrolactone I (**FM-1**) and its derivatives, some of these were common to both the fungi. Additionally, terrein (**FM-3**), was a common metabolite of both the fungi, while a cyclopentandiol (**FM-4**) though reported earlier from *A. terreus* (Ghisalberti et al., 1990) was isolated only from *A. flavipes*. *A. terreus* is known to biosynthesize butenolides (hydroxyphenyl pyruvate dimers) through aryl pyruvic acids derived from amino acids. The same precursor with a different coupling pathway leads to other type of dimers called aspulvinones (Nobutoshi et al., 1973) whereas the biosynthetic pathway of aspulvinone, when followed for a pyruvate derived via tryptophan leads to asterriquinones (Yamamoto et al., 1976). Several **FM-1** derivatives have been reported from *A. terreus* which include sulfated derivatives and derivatives carrying a change on C5 unit.

Morphologically, the young cultures of both the fungi appears identical, but by molecular techniques it has been shown that *A. flavipes* unlike *A. terreus* do not contain pksM (6-methylsalicylic acid synthase) gene (Pazoutova et al., 1999). Although, there exist reports of isolation of metabolites like flavipin (Raistrick and Rudman, 1956) and lovastatin (Alberts et al., 1980) from both these fungi, which supports the closeness of these fungi, no **FM-1** derivatives or cyclopentanediols (**FM-4** and **FM-5**) have been reported from *A. flavipes* prior to our study (section 2.1). A section of the present thesis uses tandem mass spectrometry (MS/MS) approach and molecular biology tools to study differences and similarities in two cultures. The following studies have been incorporated in this section.

1. Identification of new and known metabolites in *A. terreus* and *A. flavipes* using ESI-MS/MS and

2. Study of taxonomic relation between the two fungi using metabolite profile and molecular biology data.

Before going into the application of ESI-MS/MS, we thought it is desirable to give brief introduction to electrospray ionization tandem mass spectrometry (ESI-MS/MS) and also present a brief discussion on biogenesis of aryl pyruvate dimers. The section is initiated with a brief introduction to electrospray ionization tandem mass spectrometry (ESI-MS/MS) and discussion on biogenesis of aryl pyruvate dimers in *A. terreus*

II. Introduction to electrospray ionization tandem mass spectrometry (ESI-MS/MS)

The interest in studying novel structures and functions of the molecules in the biological systems has created an impetus for the development of efficient, effective and information-rich methods and techniques of analysis. In this regard, mass spectrometry (MS) is gaining lot of attention for studying minor and unstable metabolites or their interactions in the system due to development of soft ionization methods such as electrospray ionization (ESI), atmospheric pressure chemical ionization (APCI) and matrix assisted laser desorption ionization (MALDI).

MS is an important analytical tool in biological and biochemical research. The speed, accuracy and sensitivity is unmatched by conventional analytical techniques. For over ten decades, mass spectrometry has become a virtually ubiquitous research tool. Scientific breakthroughs made possible by MS have included the discovery of isotopes, the exact determination of atomic weights, characterization of new elements, quantitative gas analysis, stable isotope labeling, fast identification of trace pollutants and drugs and the characterization of molecular structure.

Soft ionization techniques like ESI are capable of producing ions of low energy, and hence have the power to generate ions from biological macromolecules of molecular masses in excess of 100,000, and when ESI is coupled with highly sensitive mass analyzers such as time of flight (TOF) in many cases these masses can be measured with accuracies better than 0.01%.

In early days of mass spectrometry two separate ionizations techniques had to be used for detection of molecular ions and fragment ions. Electron impact ionization (EI), the earliest technique of ionization produces molecular ion with high energy thereby quick disintegration of the molecular ion into fragment ions and hence in many cases molecular ions could not be detected. The chemical ionization (CI) method was required to serve the purpose as it is softer than EI. These techniques ionizes molecules in vapour state and hence are not suitable for non volatile and polar substances. In ESI-tandem mass spectrometry instruments, generally two mass analyzers are used, wherein the soft ionization techniques such as ESI or MALDI first produces molecular ions and then collision induced dissociation (CID) of the selected molecular ion leads to the fragmentation of the molecular ion. This method is suitable for wide range of substance from non polar lipids to high molecular weight polar compounds such as proteins and peptides.

In ESI, exact molecular ion (M^+) of the compound is not produced, but multiple pseudomolecular ions of such as protonated $[M+H]^+$; alkali metal adduct ions such as $[M+Na]^+$ and $[M+K]^+$ and polymeric ions such as $[2M+H]^+$, $[2M+Na]^+$ and $[3M+H]^+$, etc in positive ionization mode and $[M-H]^-$, $[M+X-H]^-$ in negative ionization mode where X is small molecules such as H_2O, CH_3OH, HCOOH, etc are produced. The multiple pseudomolecular ions thus give additional confirmation of molecular weight of the compound being investigated especially when analyzing mixture. Applications of ESI-MS/MS include the sequencing and analysis of peptides and proteins; studies of non-covalent complexes and immunological molecules; DNA sequencing; and the analysis of intact viruses (Watts, et al., 1997; Keki, et al 2003).

In our studies, we have used QSTAR XL-MS/MS Quadrupole-Time of Flight (QTOF) system in combination with electrospray ionization technique (ESI), which delivers sensitivity, selectivity and reproducibility while performing excellent performance for the characterization of proteins and peptides, analysis of drug metabolite information from small quantities of metabolites etc. The technique is well suited to mixture analysis because the characteristic product ion spectra can be obtained for each component in a mixture without interference from the other components, assuming that the product ions have unique m/z ratio. The sample introduction system manages the introduction of a sample into the QSTAR XL system and the conversion of the sample into ions, which can be analysed in the quadrupole. In a precursor ion scan, QSTAR XL detects precursor ions which generate a specific product ion. The instrument uses Q_1 (Mass Filter Quadrupole) in mass resolving mode to scan over the mass range of interest, while the time of flight (TOF) section records product ion spectra for each precursor ion. The Q_1 mass spectrum shows all precursor ions, which produce ion of interest. In a product ion scan, Q_1 selects a precursor ion which fragments in Q_2 (LINAC Collision Cell Quadrupole), generating product ions by a process termed 'collision-induced dissociation' (CID) which are detected in the time of flight (TOF) section. Product ions provide information on the molecular structure of the original (precursor) ions. This technique provides structural information by establishing relationships between precursor ions and their fragmentation products.

Recently, ESI-MS/MS technique have been successfully employed in identification of new cyclic depsipeptides, kahalalides R and S from the ethyl acetate fraction of marine mollusk *Elysia gradifolia* without any prior purification (Tilvi et al., 2007). The authors initially characterized the known anticancer agent cyclic depsipeptides, kahalalides F based on the mass fragmentation pattern. The two new homologous molecules, kahalalides R and S were successfully characterized by comparing the mass fragmentation pattern of new compounds with the known Kahalalide F. Kahalalide F was first isolated from *Elysia rufescens* collected from Black point, O'ah by Hamann et al. in May 1993. It showed anticancer activity *in vitro* and *in vivo*

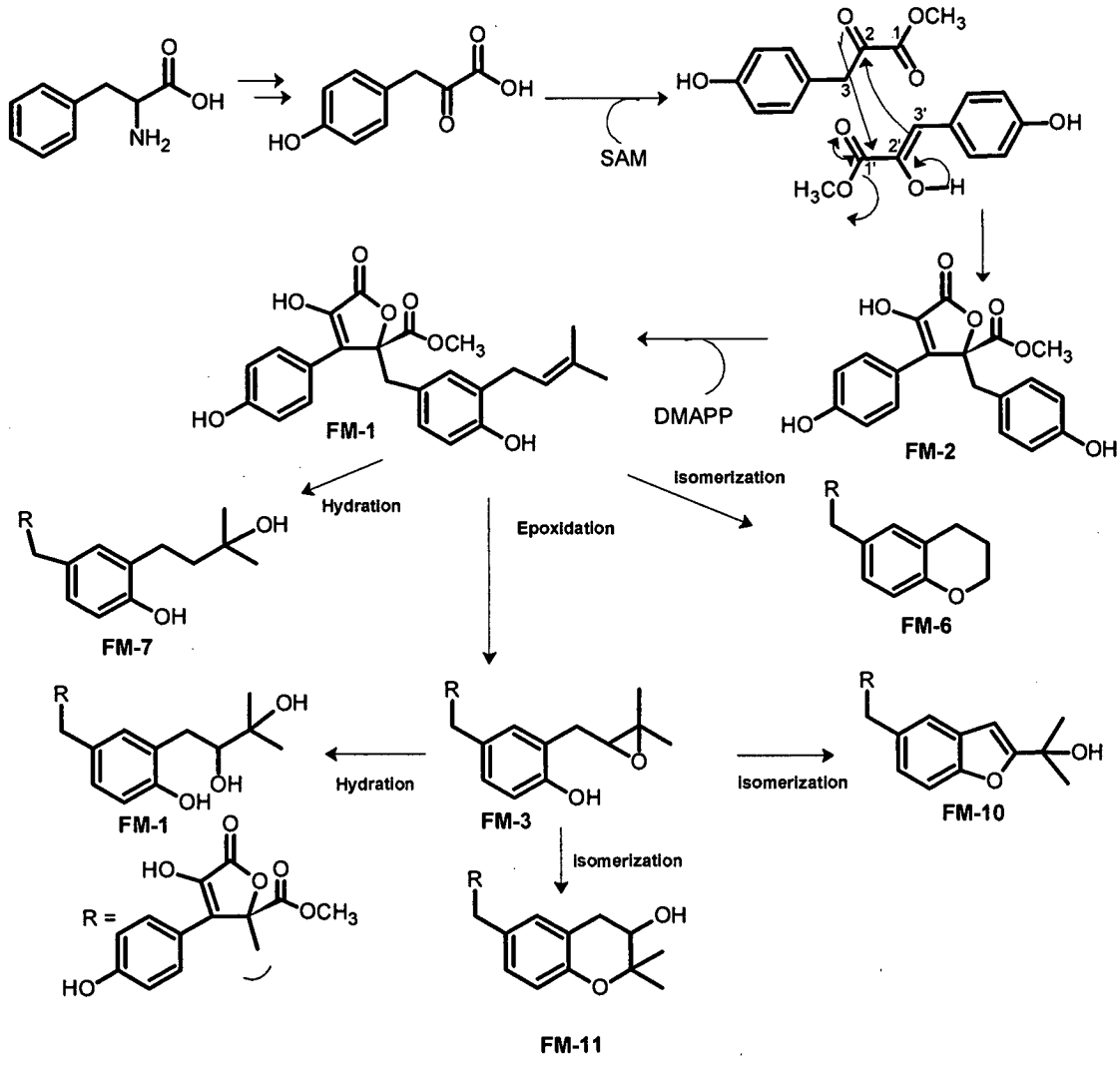
against breast cancer, colon cancer, non-small cell lung cancer (NSCLC), ovarian cancer and, in particular, against androgen-independent prostate cancer (Hamann et al.1993). PharmaMar has announced that kahalalide F has begun Phase II clinical trials for non small cell lung carcinoma (NSCLC) and melanoma (http://www.cancersolution.tk/2011/05/kahalalide-f-phase-ii-trials-for-lung.html).

We therefore, have made an attempt to characterize metabolites; especially butenolides derived from phenyl pyruvates from the crude extracts of fungi *A. flavipes* and *A. terreus* using ESI-MS/MS.

III. Biosynthesis of aryl pyruvate dimers in *Aspergillus terreus*

Three distinct types of phenyl pyruvate dimers are produced by *A. terreus* from aryl amino acid. They are butyrolactones/aspernolides, aspulvinones and asterriquinones. Compounds of these classes are derived by homodimerization of pyruvate derived via an amino acid. In the biosynthesis of **FM-1** (Nitta et al, 1983), after the formation of p-hydroxyphenyl pyruvic acid from phenyl alanine the methyl esterification of the carboxyl group take place (**Scheme 2.10**).The methyl group in the ester is incorporated from *S*-adenosyl methionine (SAM). The esterification takes place before coupling, and has been proved by the observation that the parent carboxylic acid do not undergo condensation to give butenolide carboxylic acids. The intact condensation of two pyruvate ester molecules between 2 and 3' followed by lactonization gives butyrolactone II (**FM-2**). **FM-2** is enzymatically prenylated in presence of dimethylallyl pyrophosphate (DMAPP) to give **FM-1**. This biosynthetic pathway has been very well proved using tracer technique (Nitta et al, 1983; Brachmann et al., 2006). It is possible that other **FM-1** derivatives such as butyrolactone III-VII (**FM-9 to FM-13**) or aspernolides A (**FM-6**) and B (**FM-7**), which are obtained from long standing cultures (culturing time more than 15 days) might have been chemically derived from the **FM-1** and may not be enzymatically formed. It has also been observed that every time a derivative of **FM-1** has been isolated from the fungal culture **FM-1** has been always obtained and that too in major

amount, which suggest that other compounds might be chemically converted products of **FM-1** depending upon the pH and the aeration of the culture media.



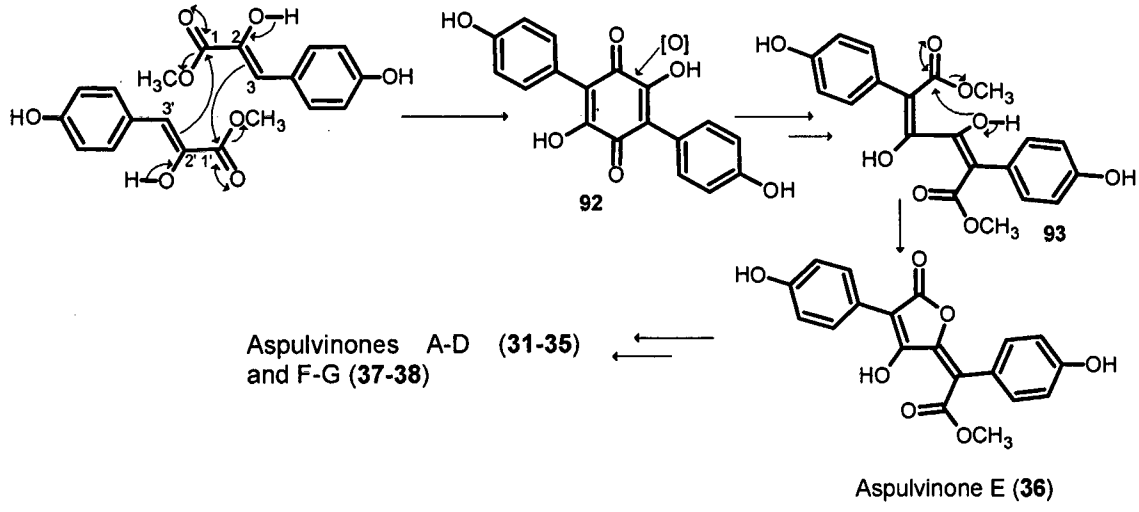
Scheme 2.10: Biosynthesis of butyrolactone I (FM-1) and its derivatives

Aspulvinones (**32-38**), another class of butenolides are also biosynthesized from the same precursor (phenyl pyruvate) involving a different pathway (**Scheme 2.11**). It involves condensation of phenyl pyruvate at two points between 1 and 3' and 3 and 1', to first give terphenyl benzoquinone (.). This terphenyl benzoquinone (**92**) is cleaved into a diketodicarboxylate (**79**) by Bayer-Villiger type oxidation followed by hydrolysis. Subsequent lactonisation and decarboxylation leads to butenolide aspulvinone E (**36**).

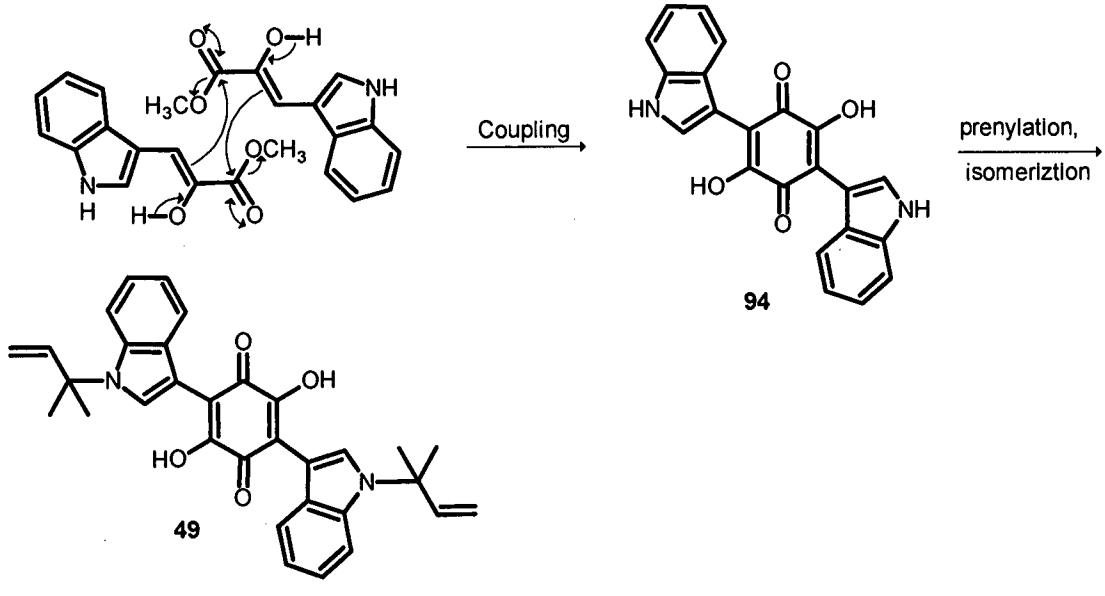
Aspulvinone E (**36**) is then hydroxylated, and prenylated at various positions on aromatic ring which gives series of aspulvinones (**32-35** and **37-38**). Interestingly, no derivatives of intermediate benzoquinone (**92**) derived from the phenyl pyruvate (derived via phenyl alanine) have been obtained as natural products so far from this fungus, but natural products called asterriquiones (**49-52**) derived from tryptophan via same pathway have been obtained from this fungus. More interestingly, butenolides, neither of aspulvinone type nor of butyrolactone I type derived via tryptophan have been obtained as natural products from these fungi. These observations leads to conclusion that the enzymes responsible for coupling of pyruvates and oxidation of benzoquinone derived from pyruvates are substrate specific beside its site specificity. The fungi produce two different enzymes responsible for coupling of pyruvates. Enzyme responsible for condensation between 2 and 3' acts only on *p*-hydroxylphenyl pyruvates leading to **FM-1** type of butenolides, whereas the other responsible for coupling in two points between 1 and 3' and 3 and 1' acts on pyruvates derived via phenyl alanine giving aspulvinones as well as tryptophan giving asterriquinones. A substrate specific enzyme oxidizes (Bayer-Villiger type oxidation) benzoquinone derived from phenyl alanine, but not benzoquinone derived from tryptophan to dienol dicarboxylates (**93**), which on series of transformations give aspulvinones. The activity of the oxidase is so strong in these fungal strains that the intermediate quinones (**92**) have never been isolated.

Interestingly, similar benzoquinones, but derived via tryptophan called asterriquinones (**49-52**) have been isolated as natural products from these fungal strain (**Scheme 2.12**) and the fact that no oxidation products of the intermediated benzoquinone (**94**) similar to aspulvinones are known as natural products is an indication that the oxidase is highly substrate specific. Nevertheless, a natural product called asterridinone (**54**) (Yamamoto et al., 1976) and asterridione (**55**) (Wijeratne et al 2003), which were metabolic products of *A. terreus* is proposed to be formed by oxidation of quinone moiety in asterriquinones. In this case another substrate specific enzyme must be responsible for this transformation as up to now no metabolites

similar to **54-55** derived from hydroxyphenyl pyruvates have been reported from *A. terreus*.



Scheme 2.11: Biosynthesis of aspulvinones



Scheme 2.12: Biogenesis of asterriquinone

IV. Results and discussion

i) Positive ESI-MS/MS analysis

Isolation and identification of the two fungi *A. flavipes* and *A. terreus* has been discussed in sections 2.1 and 2.2. These two fungi were cultured using identical conditions and the ESI-TOF-MS of EtOAc extracts of their culture media were recorded.

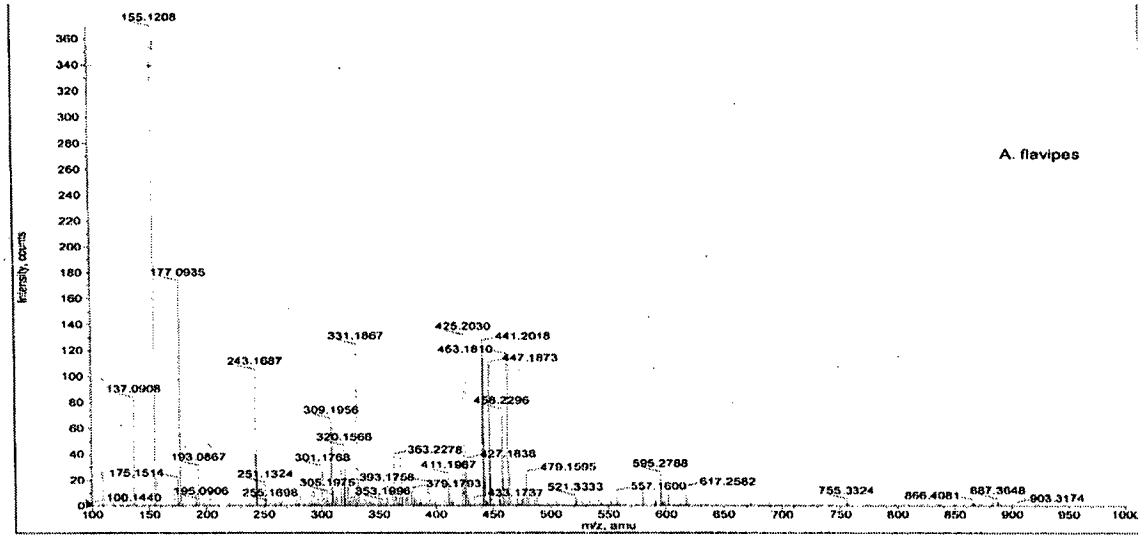


Fig 2.22: +ve ESI-TOF-MS of EtOAc extract of *A. flavipes*

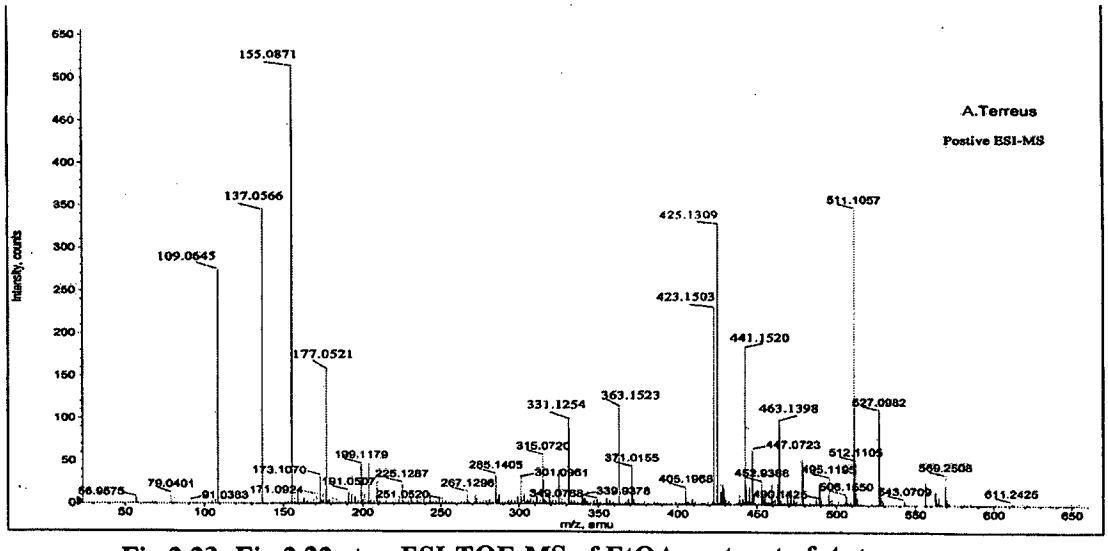


Fig 2.23: Fig 2.22: +ve ESI-TOF-MS of EtOAc extract of *A. terreuse*

The positive ESI-MS Spectra (**Fig 2.22** and **2.23**) of the two fungi *A. flavipes* and *A. terreus* showed some identical peaks but several peaks were uncommon indicating the two fungi have different metabolism. The molecular masses obtained from the MS spectra were compared with molecular weights of known metabolites of *A. terreus* of the type class butyrolactone I, aspulvinones and asterriquinones those listed in the **Table 2.7**. No pseudomolecular ions of any of the aspulvinones or asterriquinone were observed, but pseudomolecular ions of certain butenolides corresponding to molecular formulae of butyrolactone I/aspernolide A and butyrolactones III-V were observed in both the fungi.

The important peaks which were common in both the spectra were $[M+H]^+$ at m/z 425 and $[M+H]^+$ at m/z 441 signifying the presence of butyrolactone I or Aspernolide A and probably butyrolactone III respectively, and also $[M+H]^+$ at m/z 155 was indicative of due to presence of terrein in both the fungal cultures. The $[M+H]^+$ at m/z 357 corresponding to the butyrolactone II (**FM-2**) was observed in *A. flavipes* but not in *A. terreus*.

Table 2.7 Metabolites of *A. terreus*, their molecular formulae and molecular weights

Metabolite	Molecular formula	Molecular weight	Metabolite	Molecular formula	Molecular weight
Butyrolactone I/Aspernolide A	$C_{24}H_{24}O_7$	424	Butyrolactone I monosulphate	$C_{24}H_{23}O_{10}S$	504
Butyrolactone II	$C_{19}H_{16}O_7$	356	Aspulvinones A/B	$C_{27}H_{28}O_5$	432
Butyrolactones III/IV/V	$C_{24}H_{24}O_8$	440	Aspulvinones C/D	$C_{27}H_{28}O_6$	448
Aspernolide D	$C_{24}H_{26}O_9$	458	Aspulvinone E	$C_{17}H_{12}O_5$	296
Butyrolactone VII	$C_{25}H_{26}O_7$	438	Aspulvinone F	$C_{27}H_{28}O_7$	464
Aspernolide B	$C_{24}H_{26}O_8$	442	Aspulvinone G	$C_{17}H_{12}O_6$	312
			Asterriquinone	$C_{32}H_{30}N_2O_4$	506

Besides, simple pseudomolecular ions such protonated, sodiated, potassiated and homodimeric pseudomolecular ions, the protonated or sodiated or potassiated adducts

of terrein (**FM-3**) with butyrolactone I (**FM-1**) and III (**FM-9**) were also observed. For e. g. terrein gave pseudomolecular ions $[M+H]^+$ at m/z 155, $[M+Na]^+$ at m/z 177, $[M+K]^+$ at m/z 193, $[2M+H]^+$ at m/z 309, $[2M+Na]^+$ at m/z 331 and $[2M+K]^+$ at m/z 347 and **FM-1** gave pseudomolecular ions at $[M+H]^+$ at m/z 425, $[M+Na]^+$ at m/z 447, $[M+K]^+$ at m/z 463, $[2M+H]^+$ at m/z 849, $[2M+Na]^+$ at m/z 871 and $[2M+K]^+$ at m/z 887, while a protonated adduct ion of **FM-3** (MW 154) and **FM-1** (MW 424) was observed at m/z 579.

To detect the presence of any new molecules (or known molecules, which were missed during purification) related to **FM-1**, some of the pseudomolecular ions between the mass range 400-500 amu were selected for fragmentation study. It is obvious that the prenyl chain in **FM-1** is most vulnerable to biogenetic alteration in the molecule, which is evident from the type of **FM-1** derivatives isolated from *A. terreus*. Initially, the mass fragmentation pattern of **FM-1** was studied using tandem mass spectrometry (MS/MS), in which the $[M+H]^+$ ion at m/z 425 obtained from pure butyrolactone I was fragmented by collision induced dissociation (CID) by applying electrical potential between 20-40 ev. Similarly, the ions suspected to be originating from related molecules were fragmented by CID and their fragmentation was compared with **FM-1** fragments to deduce their structure.

MS/MS spectra of butyrolactone I (FM-1)

The CID (or MS/MS) of **FM-1** $[M+H]^+$ at m/z 425 gave several fragment ions (**Fig 2.24**) which were m/z 407 (ΔM = 18), 393 (ΔM = 32), 389 (ΔM = 36), 369 (ΔM = 56), 363 (ΔM = 62), 347 (ΔM = 78), 331 (ΔM = 94), 307 (ΔM = 118), 231 (ΔM = 194), 217 (ΔM = 208), 203 (ΔM = 222), 175 (ΔM = 250), 157 (ΔM = 268), 121 (ΔM = 304), 69 (ΔM = 356). Out of these peaks four peaks at m/z 175, 331, 393 and 369 were assigned to the specific fragments and of diagnostic value considering the former three of them retains the ring B carrying prenyl chain. The mass difference of these ions with the corresponding fragment ion resulting from another pseudomolecular ion under investigation would give the information regarding the

change to be made in the **FM-1** structure to arrive at a structure of unknown molecule. The fragment at m/z 393 is formed by elimination of CH_3OH molecule from protonted molecular ion. An m/z 331 fragment ion is supposed to be formed by elimination of phenol molecule and a fragment ion m/z is obtained due to formation of stable benzyllic cation of ortho-prenylhydroxy benzyl by benzyllic cleavage. A benzyllic cleavage on prenyl group by elimination of C_4H_8 leads to the formation of fragment ion at m/z 369.

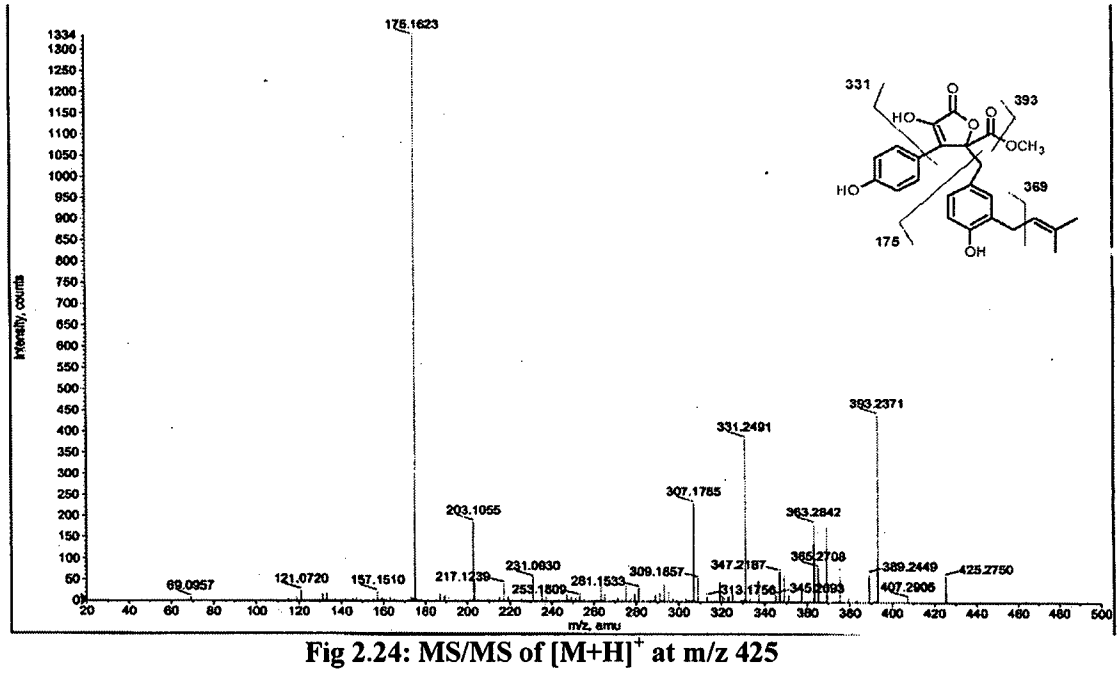


Fig 2.24: MS/MS of $[M+H]^+$ at m/z 425

*MS/MS of $[M+H]^+$ ion at m/z 411: Identification of **FM-17***

One of the protonated ion that was analyzed by MS/MS (**Fig 2.25**) was m/z 411 assuming the existence of molecule (**FM-17**) with MW= 410 amu, which was further confirmed from the observation of the sodiated molecular ion $[M+Na]^+$ at m/z 433. These ion peaks were present only in spectra of *A. flavipes*. The molecular weight of 410 amu is less from **FM-1** by 14 amu suggesting a one methyl group is replaced by hydrogen atom in the molecule. One of the changes that can be done in **FM-1** is to replace one methyl group with hydrogen from the prenyl chain. In doing so the

fragment ions those are expected in MS/MS are m/z 161, 317, 379. None of these peaks were observed in MS/MS spectra **(Fig 2.25)**; instead the peaks at m/z 175 and 393 along with the m/z 369 were retained. This suggested that prenyl chain in **FM-17** is present without any change. The only other possibility is that molecule is the carboxylic acid of **FM-1**. In this case the methyl group of **FM-1** is replaced with hydrogen as that would display the fragment ions m/z 175, 393 and 369. Therefore, most likely structure of the molecule yielding pseudomeolcular ion at m/z 411 is butyrolactone I carboxylic acid as shown in **FM-17**

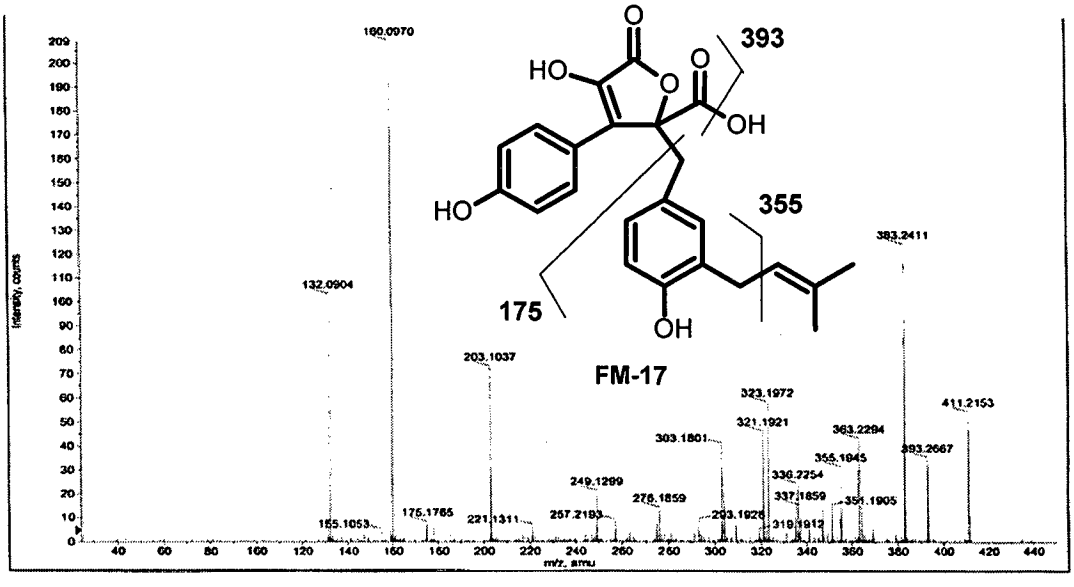


Fig 2.25: MS/MS of $[M+H]^+$ at m/z 411

*MS/MS of $[M+H]^+$ ion at m/z 441: Identification of **FM-9***

A pseudomolecular ion $[M+H]^+$ at m/z 441 was present in both the extracts indicating presence of molecule with molecular weight 440 amu, which is 16 units more compared to **FM-1**. Corresponding pseudomolecular ions such as $[M+Na]^+$ at m/z 463, $[M+K]^+$ at m/z 479, $[2M+H]^+$ at m/z 849, $[2M+Na]^+$ at m/z 881 and $[2M+K]^+$ at m/z 903 and protonated adduct ion with terrein at m/z 595 were also evident from

ESI-MS spectrum (**Fig 2.22**). Three **FM-1** derivatives, butyrolactone III (**FM-9**), IV (**FM-10**) and V (**FM-11**) of molecular weight 440 amu are known from *A. terreus*. The $[M+H]^+$ at m/z 441 in MS/MS (**Fig 2.26**) spectra displayed fragment peaks at m/z 191, 347, which were also 16 units more than corresponding fragments of **FM-1**. The presence of fragment at m/z 369 clearly suggests that the additional oxygen is present on terminal C-4 unit of prenyl chain and must been derived from **FM-9** as other two should give corresponding fragment ion at m/z 368 (**Fig 2.27**). The absence of m/z 368 may not be enough evidence to suggest that the **FM-10** and **FM-11** are not present in the extract. It is also possible that the **FM-10** and **FM-11** are first converted into **FM-9** in ionization chamber then yielding fragment ion at m/z 369. Therefore, the ion under investigation might have been produced due to any of the three known natural products (**FM-9, FM-10** and **FM-11**) or their mixture. As explained in proceeding biosynthetic section **FM-9** is precursor to **FM-10** and **FM-11,** and therefore, we decided to synthesize **FM-9** by simplest route and then **FM-10** and **FM-11** from it.

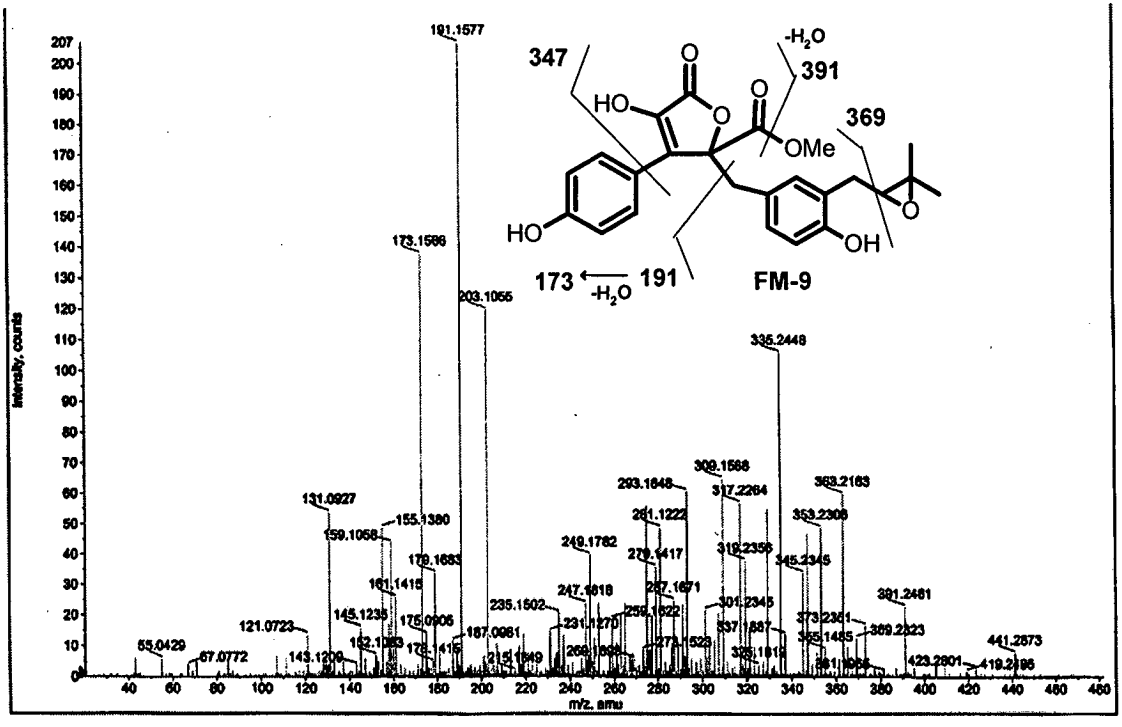
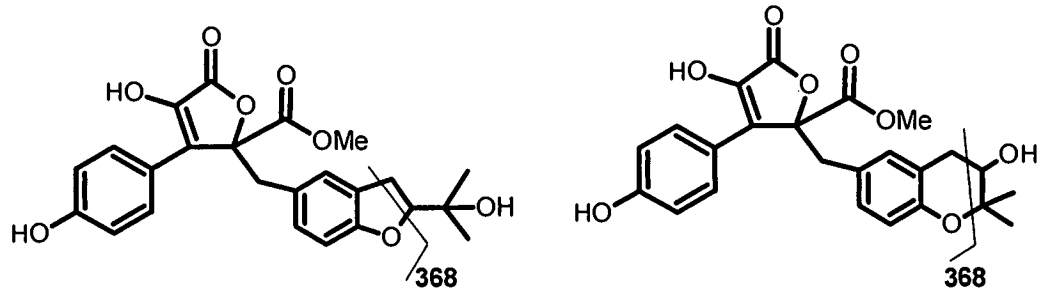


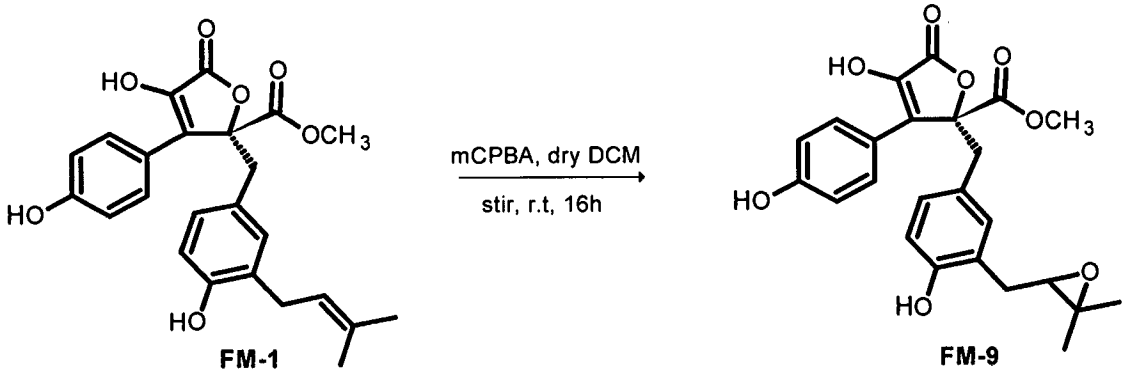
Fig 2.26: MS/MS of $[M+H]^+$ at m/z 441



FM-10 **FM-11**

Fig 2.27: Structures of FM-10 and FM-11 showing expected mass fragment of 368 amu

Thus, butyrolactone III (**FM-9**) was synthesized by treating **FM-1** with mCPBA in anhydrous DCM in 78% yield (**Scheme 2.13**). The compound was characterized using ^{1}H and ^{13}C NMR (**Figs 2.27a** and **2.27b**). Due to epoxidation of the prenyl double bond of **FM-1** the signal due vinyllic proton at δ_H disappeared and a new signal for oxymenthine appeared at δ_H 3.73, m. The proton signal for H-2' (6') was observed as quartet at δ_H 7.58 and one of the benzyllic proton H-7" showed two double doublets suggesting presence of C-8" epimer in the compound. This is further supported from the observation that several carbons signals appeared in duplicates in ^{13}C NMR (**Fig 2.27b**). The complete NMR data is listed in **table 2.8**. As expected, ESI-TOF-MS (**Fig 2.27c**) displayed a pseudomolecular ion at $[M+H]^+$ at m/z 441, $[M+Na]^+$ at m/z 463 and $[M+K]^+$ at m/z 479 and its MS/MS of m/z 441 spectra (**Fig 2.27d**) was identical to MS/MS obtained on the $[M+H]^+$ at m/z 441 of the extract. Thus, it is confirmed that at least **FM-9** is present in the EtOAc extract of *A. flavipes* and *A. terreus*.



Scheme 2.13: Preparation of FM-9 from FM-1

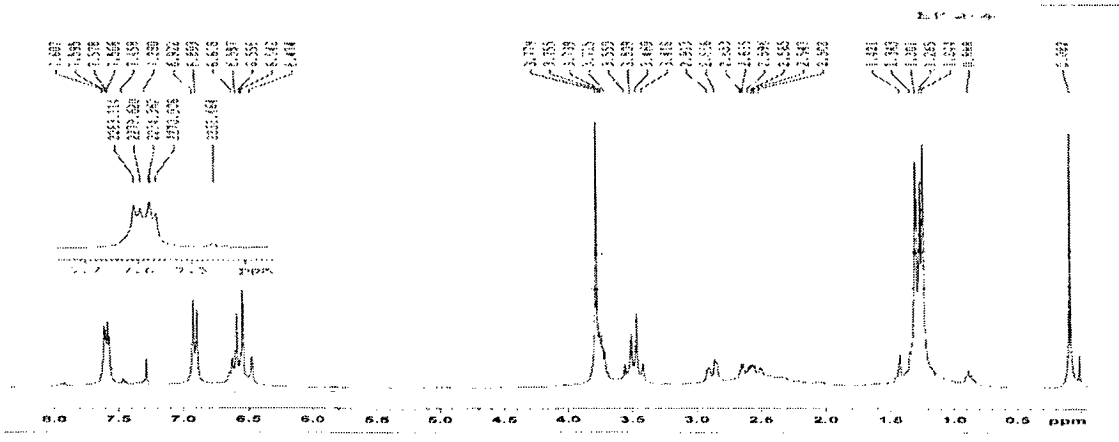


Fig 2.27a: ^{1}H NMR of FM-9 (synthetic)

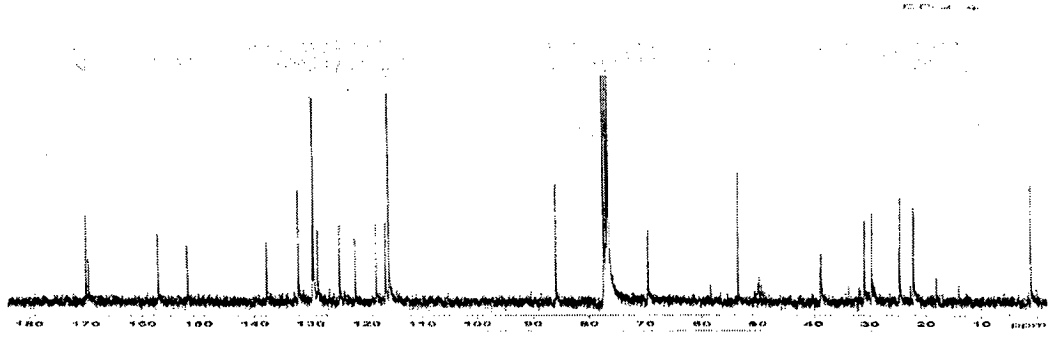


Fig 2.27b: ^{13}C NMR of FM-9 (synthetic)

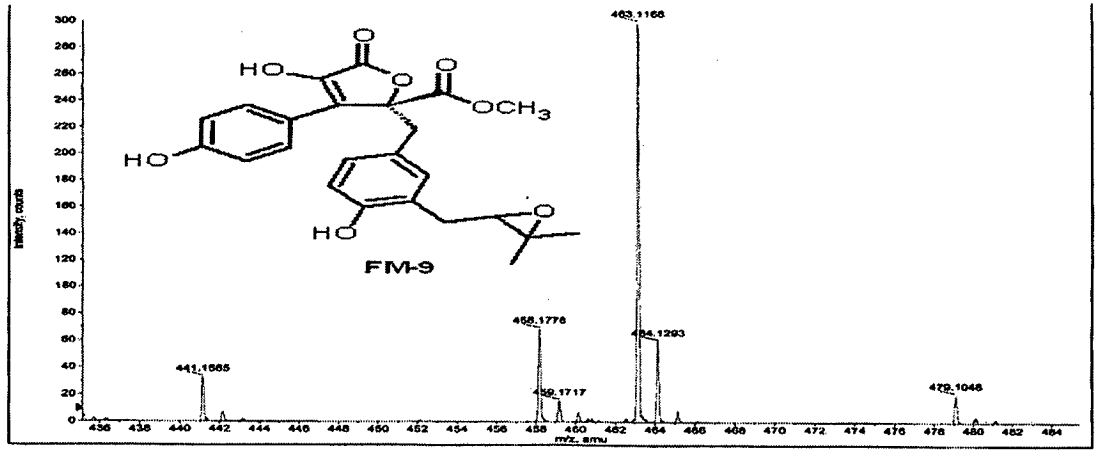


Fig 2.27c: ^{1}H ESI-TOF-MS of FM-9 (synthetic)

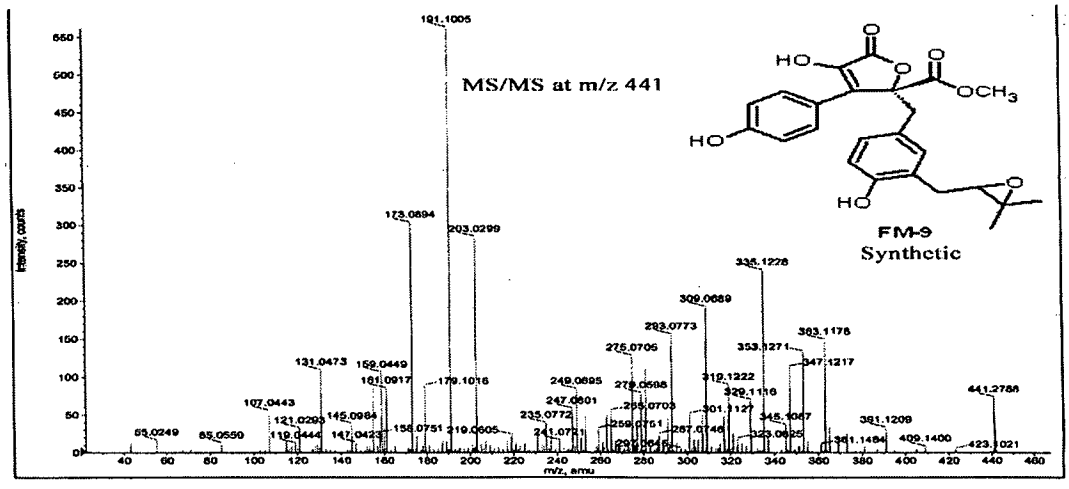


Fig 2.27d: MS/MS of FM-9 (synthetic)

Next, we treated the **FM-9** with a Lewis acid, $BF_3.Et_2O$ in anticipation of obtaining **FM-10** or **FM-11**, but instead an isomerized dehydrated product (**81**) of **FM-10** was obtained (**Scheme 2.13**). Compound **81** has been reported as transformation product of **FM-9** obtained by treatment with pyridine/HCl during structure elucidation of **FM-9** (Nitta et al., 1983). Our product gave pseudomolecular ions $[M+H]^+$ at m/z at 423, $[M+Na]^+$ at m/z 445 and $[M+K]^+$ at m/z 461. Its ^1H NMR showed singlet at δ_H 6.91 for the methine of furan ring. The quartarnay carbon C-8" was observed at δ_C 165.1 in ^{13}C NMR. The complete 1D NMR data given in **table 2.8** accounts well for the given structure (**81**).

The MS/MS spectra (**Fig 2.28a**) of m/z 423 displayed fragment ions at m/z 391, 329 and 173, all of them as expected were two units smaller than corresponding fragment ions of **FM-1**.

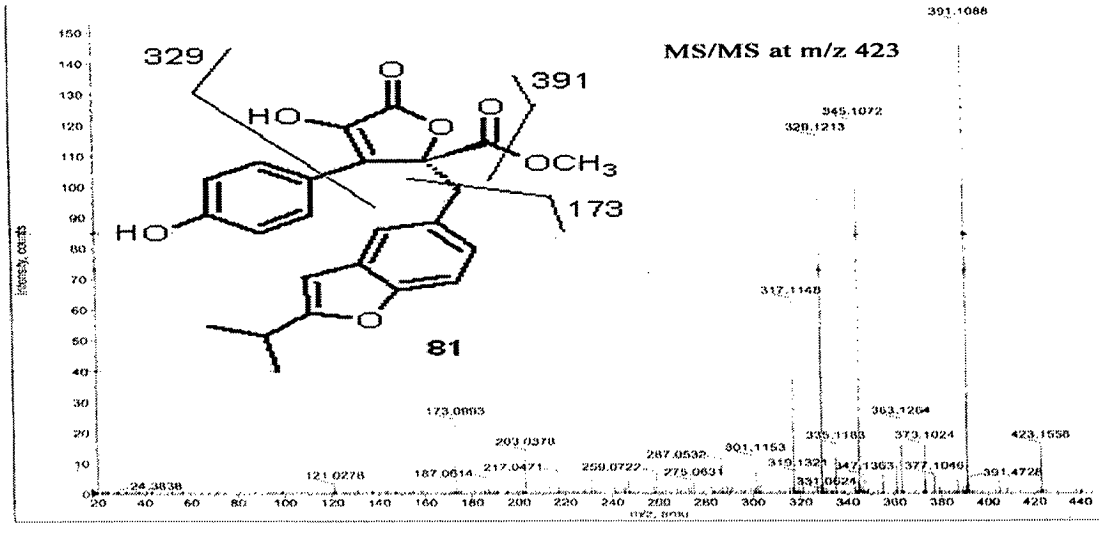
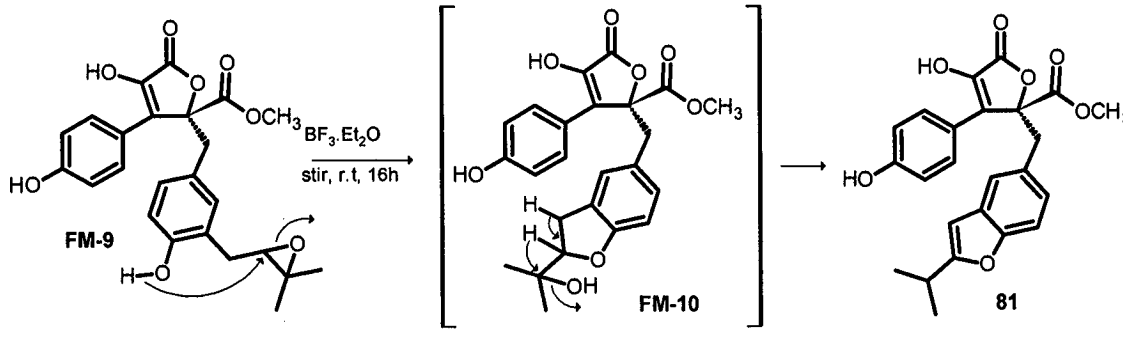


Fig 2.28a: MS/MS of 81



Scheme 2.13: Transformation of FM-9 into 81

Table 2.8: NMR data of FM-9 and 81 ($CDCl_3$, 300 Mhz)

	FM-9		81	
Position	δ_C, mult	δ_H, mult., J(Hz)	δ_C, mult	δ_H, mult., J(Hz)
1	169.4 s		168.9 s	
2	137.7 s		137.0 s	
3	128.5 s		128.7 s	
4	85.8 s		86.0 s	
5	38.6 t		169.7 s	
6	169.8s	3.44, d, (15.3) 3.53, d, (15.3)	39.2 t	3.68, d, (12.0)
1'	121.9 s		122.4 s	
2'(6')	129.6 d	7.59 d, (8.7)	129.6 d	7.64, d, (8.1)
3'(5')	115.9 d	6.91, d, (8.7)	116.0 d	6.97, d, (8.7)
4'	157.1 s		156.4 s	
1"	128.5 s		129.6 s	
2"	132.1 d	6.47, s	122.2 d	6.92, s
3"	124.7 s		127.5 s	
4"	151.9 s		153.9 s	
5"	116.6 d	6.59, d, (9.3)	110.0 d	6.66, d, (8.1)
6"	129.6 d	6.55, d, (9.3)	125.4 d	7.15, d, (8.4)
7"	30.9 t	2.52, dd (13.0, 5.4) 2.88, dd (13.0, 3.3)	99.7 d	6.19, s
8"	69.5 d	3.75, m	165.1 s	
9"	76.7 s		28.1 s	3.00, m
10"	22.2 q	1.24, s	14.1 q	1.30, s, (6.6)
11"	24.6 q	1.30, s	14.1 q	1.30, s, (6.6)
6-OMe	53.5 q	3.78, s	53.5 q	3.79 , s

ii) Negative ESI-MS/MS analysis

A negative ESI-TOF-MS spectra (**Figs 2.29** and **2.30**) of ethyl acetate extracts of the both fungi showed some common peaks while several peaks were not common. Likewise, pseudomolecular ions for **FM-1** and **FM-9** $[M-H]^-$ at m/z at 423 and 439 respectively were present in both. Additionally, two pseudomolecular ions $[M-H]^-$ at m/z 503 and 519 were observed only in the extract of *A. flavipes,* which differed from **FM-1** and **FM-9** respectively by 80 units, an indication of the presence of molecules with molecular weight of 504 and 520 amu. The corresponding ions were not observed in positive ion mode. The mono sulfates (**FM-13** and **FM-14**) of **FM-1** has molecular weight of 504 and has been shown to give pseudomolecular ions $[M-H]^-$ in

negative mode (Niu et al., 2008). The presence of sulfate in the molecule was evidenced from the presence of signals at m/z 423 and 439 of pseudomolecular ions $[M-H]^-$ at m/z 503 and 519 respectively formed by elimination of SO_3. The lower region of the MS/MS of the $[M-H]^-$ at m/z 503 (**Fig 2.32**) and 519(**Fig 2.354**) had same ions as those observed for $[M-H]^-$ at m/z 423 (**Fig 2.31**) and 439 (**Fig 2.33**). It was not possible to predict the position of $-SO_3H$ group, but it could be present on any of the three existing hydroxyl groups in the molecule. It is also possible that pseudomolecular ions might be resulting out of the mixture of two or three isomeric sulfates of **FM-1** and **FM-9.** the possible structures of sulfated compounds are shown in **FM-13, FM-14** and **FM-18** to **FM-21**

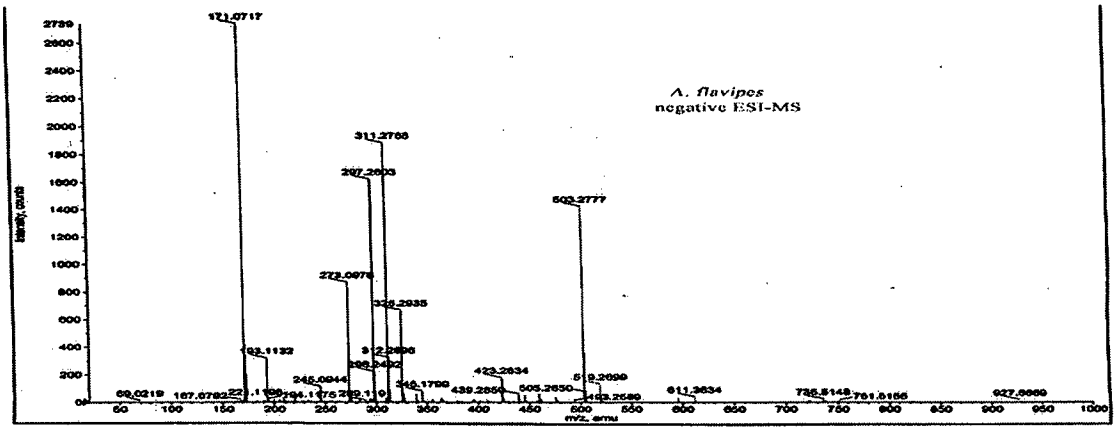


Fig 2.29: -ve ESI-TOF-MS of EtOAc extract of *A. flavipes*

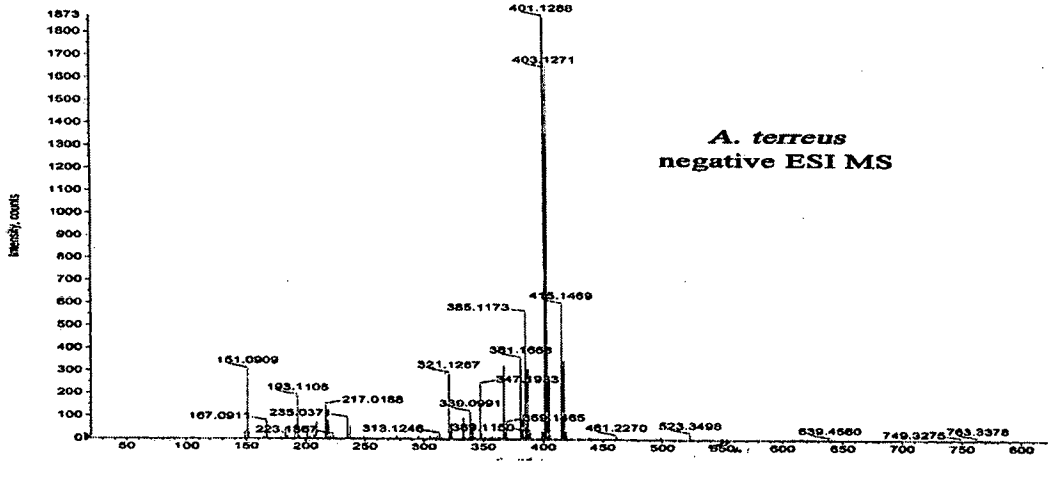


Fig 2.30: +ve ESI-TOF-MS of EtOAc extract of *A. terreus*

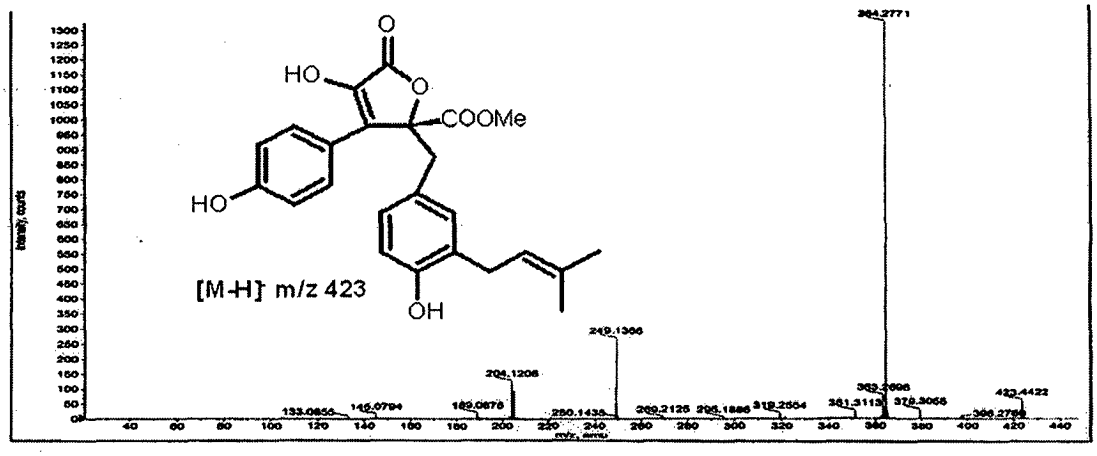


Fig 2.31: MS/MS of [M+H]. at m/z 423

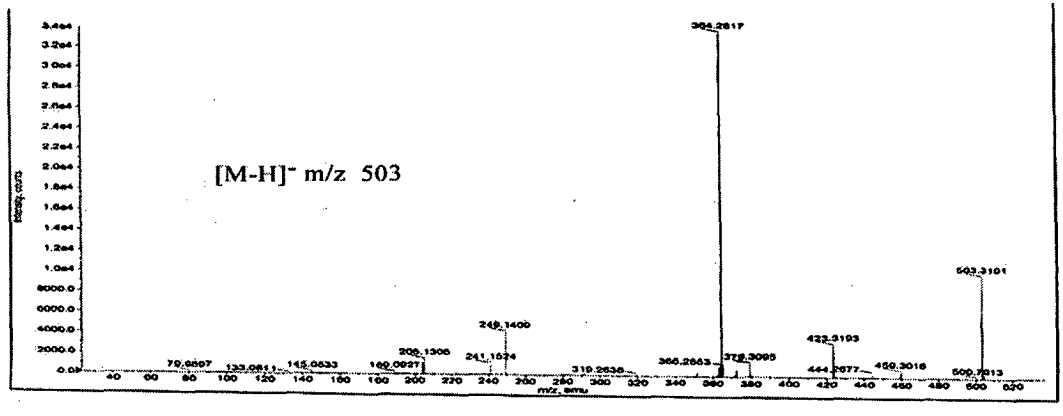


Fig 2.32: MS/MS of [M+H]. at m/z 503

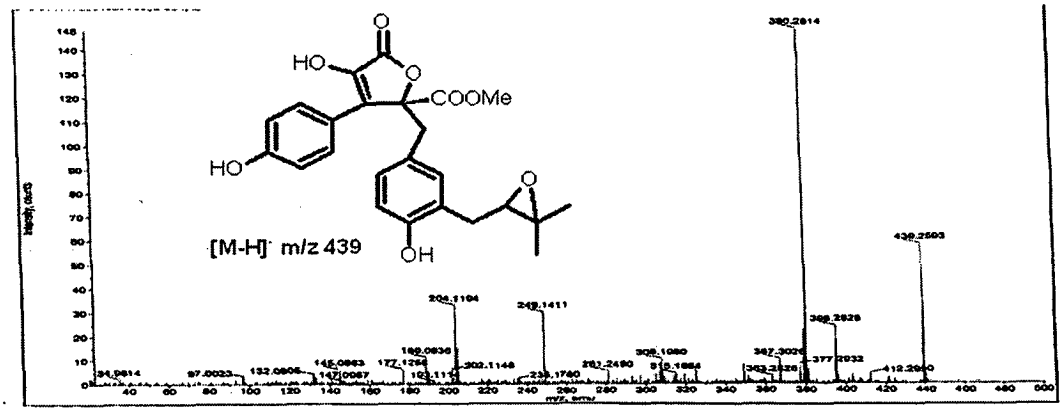


Fig 2.33: MS/MS of [M+H]. at m/z 439

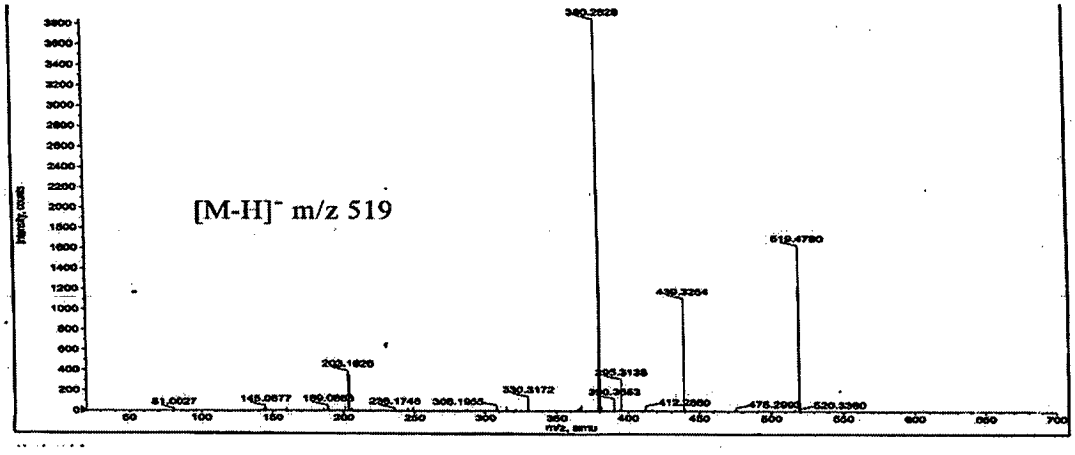
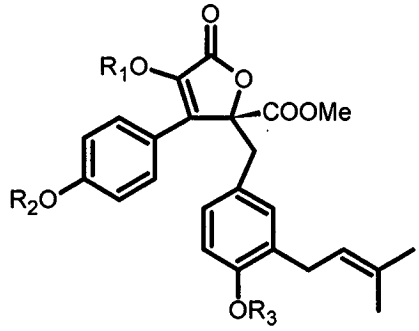
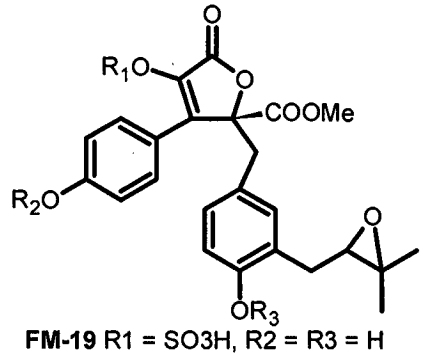


Fig 2.34: MS/MS of [M+H]. at m/z 519



FM-13 R1 = SO3H, R2 = R3 = H
FM-18 R1 = R3 =H, R2 = SO3H
FM-14 R1 = R2 = H, R3 = SO3H



FM-19 R1 = SO3H, R2 = R3 = H
FM-20 R1 = R3 =H, R2 = SO3H
FM-21 R1 = R2 = H, R3 = SO3H

iii) Phylogenetic analysis

The sequence of the ITS region gene has been widely used as a molecular marker to estimate relationships among fungi (phylogeny), but more recently it has also become important as a means to identify an unknown fungi to the genus or species level. It was recently reported that slight sequence differences in the ITS region gene could be used for species identification. Herein, to have comparison between the two fungi *A. flavipes* and *A. terreus* the two strains were identified using ITS region gene sequences.

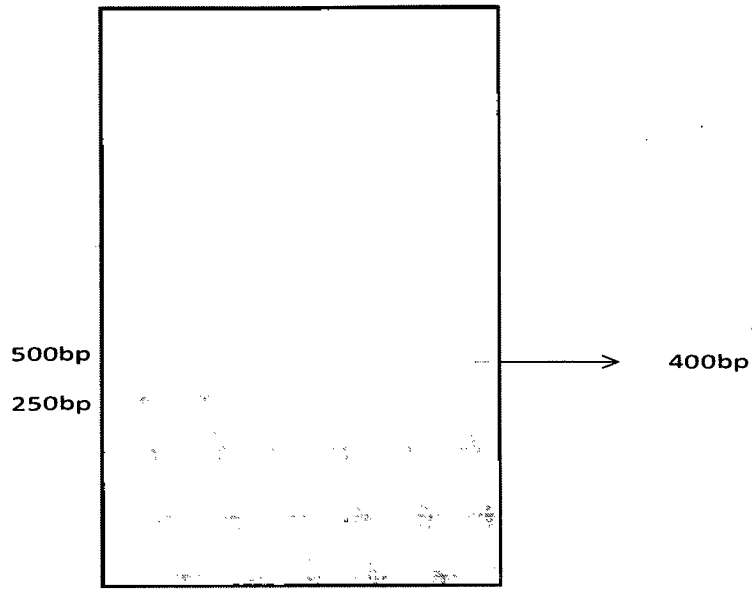


Fig 2.35: ITS gene amplification for fungi at approx. 400bp : Lane 1: 1Kb DNA ladder, Lane 2: Amplified product of *Aspergilus flavipes* and Lane 3: Amplified product of *Aspergilus terrcus.*

DNA of both the fungi was isolated, amplified on PCR (polymerase chain reactor) and the PCR product was further purified. The purified PCR product was sequenced using 5.8sr (5' TCGATGAAGAACGCAGCG 3') as sequencing primer. After amplification a PCR product of size 400 bp was obtained (**Fig 2.35**) and the sequences of PCR products bin FASTA format were analyzed by using Basic Local Alignment Search Tool (BLAST).

The 5.8SR gene sequence in FASTA format obtained for the two fungi were

Aspergillus flavipes Nucleotides sequenced: 329

AGGAAATGGTATTGCAGATTCAGTGATCATCGAGTCTTTGAACGCACATT
GCGCCCCCTGGTATTCCGGGGGGCATGCCTGTCCGAGCGTCATTGCTGCC
CTCAGCCCGGCTTGTGTGTTGGGCCCTCGTCCCCCGGCTCCCGGGGGACG
GGCCCGA
AAGGCAGCGGCGGCACCGCGTCCGGTCCTCGAGCGTATGGGGCTTCGTCT
TCCGCTCCGTAGGCCCGGCCGGCGCCCGCCGACGCATTTATTTGCAACTT

GTTTTTTTCCAGGTTGACCTCGGATCAGGTAGGGATACCCGCTGAACTTAA
GCATATCAATAAGCGGAGGAA

Aspergillus terreus Nucleotides sequenced: 332

GGGAAAAAGTGAATTGCAGATTCAGTGAATCATCGAGTCTTTGAACGCAC
ATTGCGCCCCCTGGTATTCCGGGGGGCATGCCTGTCCGAGCGTCATTGCT
GCCCTCAGCCCGGCTTGTGTGTTGGGCCCTCGTCCCCCGGCTCCCGGGGG
ACGGGCCCGAAAGGCAGCGGCGGCACCGCGTCCGGTCCTCGAGCGTATG
GGGCTTCGTCTTCCGCTCCGTAGGCCCGGCCGGCGCCCGCCGACGCATTT
ATTTGCAACTTGTTTTTTTCCAGGTTGACCTCGGATCAGGTAGGGATACCC
GCTGAACTTAAGCATATCAATAAGCGGAGGAA

Table 2.9:

Sr. No.	Gene Accession No.	Phylogenetic neighbours	% Similarity	
			A. terreus	*A. flavipes*
1	*EF669603*	*Aspergillus terreus* isolate NRRL 4609 internal transcribed spacer 1, 5.8S ribosomal RNA gene	99	98
2	*EF669587*	*Aspergillus terreus* isolate NRRL 260 internal transcribed spacer 1, 5.8S ribosomal RNA gene	99	98
3	*EF669586*	*Aspergillus terreus* isolate NRRL 255 internal transcribed spacer 1, 5.8S ribosomal RNA gene	99	98

The comparison of these fungi using basic local alignment search tool (BLAST) showed that our fungus *A. terreus* had 99% homology with several *A. terreus* isolates, three of which are listed below (**Table 2.9**) Interestingly, the fungus *A. flavipes* had 98% similarity to the same isolates indicating it is marginally different from our *A. terreus*. Phylogenetic analysis by Varga et al., (2005) have led to the

understanding that *A. flavipes* isolates are as closely related to *A. terreus* and have put the argument that these two fungi should be grouped in a same section separately as flavipedes and terri as presently done.

iv) Concluding remarks

By using positive and negative ESI-MS/MS we were able to detect presence of new compounds in *A. flavipes* **FM-17** to **FM-21** while known compounds such **FM-1** **FM-3** and **FM-9** in both fungi, *A. flavipes* and *A. terreus*. **FM-1** or its derivatives have not been previously reported to be metabolites of *A. flavipes*. The phylogentic anlalysis revealed that two fungi differed only marginally. Therefore, our study using metabolic profiling reveals that the two fungi, *A. flavipes* and *A. terreus* are very closely related. Phylogenetic analysis have led to the understanding that *A. flavipes* isolates are as closely related to *A. terreus*. The results obtained by us on these fungi showing high similarities are further proof of the earlier findings by Varga et al., (2005) that two fungi are phylogenetic neighbors.

V. Experimentals

i) General experimental procedures

The experiments carried for the phylogentic analysis were performed by trained molecular biologists and the procedures are not reported here. Cultivation of fungi and extraction of metabolites is given in sections 2.1 and 2.2

ii) Analysis on mass spectrometer

Solutions (10 ppm approx.) of ethyl acetate extracts of the fungi *A. terreus* and *A. flavipes* were prepared. These solutions were injected in an ESI-QTOF MS/MS at 5ev to record ESI-TOF MS. Certain individual molecular ions were selected and

fragmented using collision induced dissociation (CID) by applying suitable potential between 20-40ev to obtain MS/MS spectra.

iii) Preparation of butyrolactone III (FM-9)

A mixture of **FM-1** (75 mg, 0.177 moles), mCPBA (55%) (39.6 mg, 0.230 moles) in dry DCM (10 ml) and stirred under N_2 atmosphere for 16 hrs. TLC (5% MeOH-$CHCl_3$, v/v) indicated partial conversion of the **FM-1** to slightly polar compound upon which reaction was terminated by addition of liquor ammonia and the resultant mixture was extracted with EtOAc (20 ml x 3). Upon evaporation a sticky solid was obtaine which was purified over flash Si gel by gradient elution of MeOH in $CHCl_3$ (0-5% v/v) to give **FM-9** as colourless solid (68 mg, 78%).

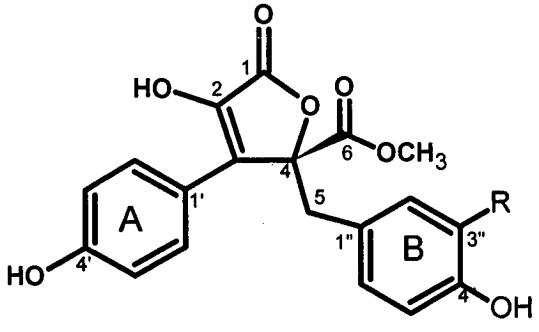
iv) Conversion of butyrolactone III (FM-9) to 81

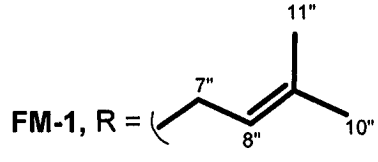
$BF_3.Et_2O$ (10 ml) was added to **FM-9** (30 mg) in RB flask and stirred under anhydrous condition for 16 hrs. TLC showed complete conversion of **FM-9** into a non polar compound. The reaction mixture was neutralized with aq NH_3 and extracted in EtOAc (20 ml x 3). Upon evaporation of the solvent a thick liquid compound was obtained which was purified on flash Si gel by gradient elution of MeOH in $CHCl_3$ (0-5% v/v) to give **81** as sticky solid (10 mg).

Section 2.5 Effect of seawater concentration on the production of butyrolactone I in *Aspergillus terreus*

I. Introduction

Butyrolactone I (**FM-1**) has been one of the most characteristic metabolite of the fungus *Aspergillus terreus*. Its unique structural properties and highly selective and potent cyclin dependent kinase (CDK) inhibition activity, makes it one of the most studied metabolite from this fungus. It has one chiral centre having 4*R* configuration and prenyl chain on ring B, and both the features are very important in maintaining high antitumor activity. **FM-1** selectively inhibit CDK2 and CDK1 kinases, both of which play important roles in cell progression from G1 to S phase and from G2 phase to M phase, respectively, in mammalian cells. Butyrolactone I has been shown to present antitumor effects on several lung cancer cell lines with IC_{50} values in the order of 0.12 μM (Nishio et al., 1996) and prostatic cancer cell lines with IC_{50} values in the order of 70 μM (Suzuki et al., 1999).





FM-1, R =

FM-2, R = H

Although three decades have passed the isolation of **FM-1**, the synthesis of this molecule has not been reported. Butyrolactone II (**FM-2**), the immediate precursor of **FM-1**, has been synthesized, but it only in racemic form. Not only efficient chiral catalysis will be needed to produce optically pure **FM-2**, but selective prenylation of ring B in **FM-2** will also be problem to tackle as both the aromatic rings (rings A and B) are equally susceptible to prenylation. It is remarkable to know as of now no

synthetic route is available to access this molecule for biochemical studies; the alternative followed for its access is large scale fermentation. A procedure developed by Schimmel et al., (1999) yields 700 mg/l of **FM-1** from fermentation *A. terreus* ATCC 20542 using optimized basic medium of glucose peptone yeast extract.

Here in this section we wish to show that **FM-1** production in *A. terreus* can be elevated by altering the seawater concentration in the medium.

II. Results and discussion

As discussed in section 2.2 the fungus *A. terreus* produced 3.2 mg/l of **FM-1** in PDB in seawater medium. Surprisingly, the re-culturing of this strain under same condition did not yield any **FM-1** and only a co-metabolite terrein (**FM-3**) was detected in major quantity. On the contrary, *A. flavipes* had produced over 200 mg/l of **FM-1** in PDB made in 75% SW medium (section 2.1). A careful observation of the culturing medium of the two fungi, goes to suggest that **FM-1** production in these fungi is function of seawater (SW) concentration in the culture medium. Natural SW composition changes with seasons and the change in SW composition of SW used for re-culturing might have been responsible for non production of **FM-1** in *A. terreus* in repeat cultures. To test the hypothesis that **FM-1** production is function of SW concentration, we carried experiments of growing the *A. terreus* in medium having different SW concentrations.

i) Cultivation of fungus

The fungus *A. terreus* was cultured in PDB dextrose media containing different SW concentrations form 0-100% (**Table 2.10**). One more set of experiment was carried using medium additionally containing 4-hydroxyphenypyruvic acid (HPPA), which is a precursor to **FM-1.** After the incubation of the fungus under static condition for 21 days broth was extracted in EtOAc, and the extract was quantitatively analyzed for **FM-1** on reverse phase ultra performance liquid chromatography (UPLC).

Table 2.10: Culture media composition

Experiment	DW (%)	SW (%)	Media components
Set I	100	0	PDB
Set II	50	50	PDB
Set III	0	100	PDB
Set IV	100	0	PDB + (..)

ii) Analysis of the extracts on UPLC

First a method to analyze butyrolactone I (**FM-1**) from the extract was developed by varying the gradient of AcCN and H_2O on RP-18 column. The column used has AQUITY UPLC® BEH C18, 1.7µm, 2.1 x 100mm. The parameters used in the method is tabulated in **table 2.11**

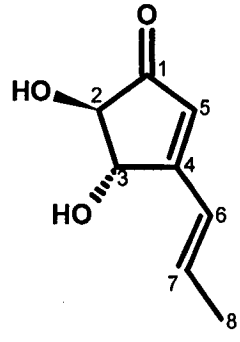
Table 2.11: UPLC mobile phase parameters

Time (min.)	Flow (ml/min)	H_2O (%)	AcCN (%)
0	0.25	100	0.0
2.5	0.25	100	0.0
3.5	0.25	60	40
4.5	0.25	20	80
6.5	0.25	0	100
10	0.25	0	100

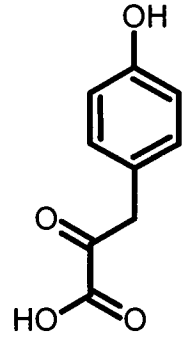
The wavelength of 254 nm was used on UV detector. First the standard methanolic solution of **FM-1** was analyzed, which gave a retention time (RT) of 5.57 min and area under the peak was recorded (**Fig 2.36**). Similarly, the EtOAc extracts of four experiments (**table 2.10**) were analyzed and the area under the peak at 5.57 was recorded (**Figs 2.37a -2.37d**). The amount of **FM-1** in the extracts was calculated by

correlating their area with area recorded for pure **FM-1**. The calculation of amount of **FM-1** present per milligram in the extracts is tabulated in **table 2.12** and the amount of **FM-1** present in the total EtOAc extract is given **table 2.12.**

As observed in one of the previous experiments hardly any amount (0.0067 mg) of **FM-1** was detected on UPLC in *A. terreus* culture in 100% SW. The change from 100% SW to 100% DW (0% SW) resulted in marginal decrease of EtOAc extract from 73 mg in 100% SW to 69 mg in 100% DW, but the amount of butyrolactone I in the extract was 19.87 mg which very high considering negligible amount in 100% SW. This indicates that the production of other components such as terrein (**FM-3**) (peak at RT 2.6 min) was getting reduced in 100% DW cultures. A change from 0% SW towards 50% SW saw increase in total extract (103 mg from 63 mg in 0% SW) as well increase in production of **FM-1** to 27.93 mg in 100ml culture. A peak at 2.60 min appears due to **FM-3**, the production of which also is affected by change in SW concentration of culture media. An experiment carried out with a 50 mg HPPA in 100 ml DW (Set IV) based medium as expected saw rise in production of **FM-1** from 19.87 mg in set I to 21.95 mg.



Terrein (FM-3)



HPPA

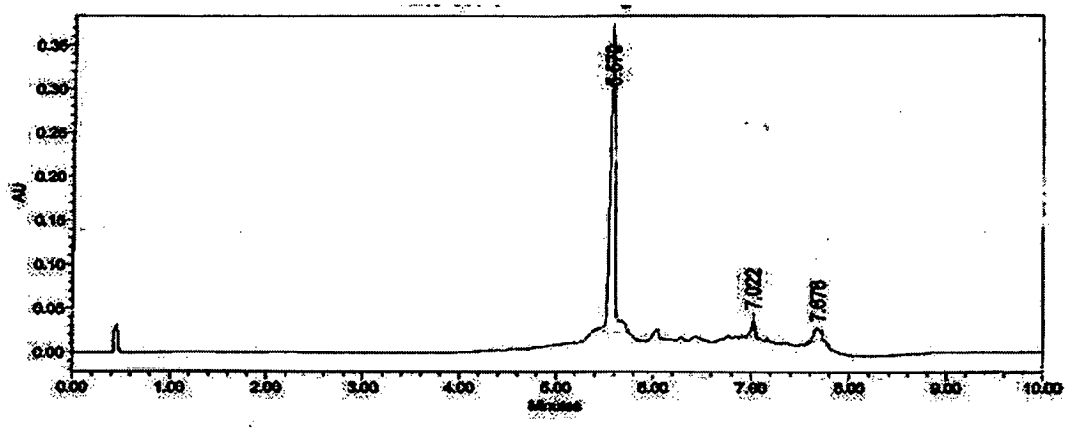


Fig 2.36: UPLC Chromatogram of Pure FM-1

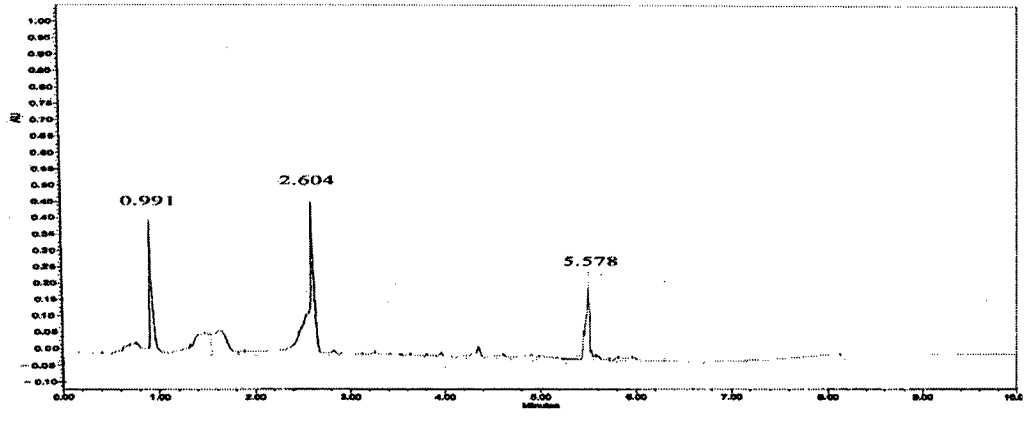


Fig 2.37a: UPLC Chromatogram of EtOAc of Set I

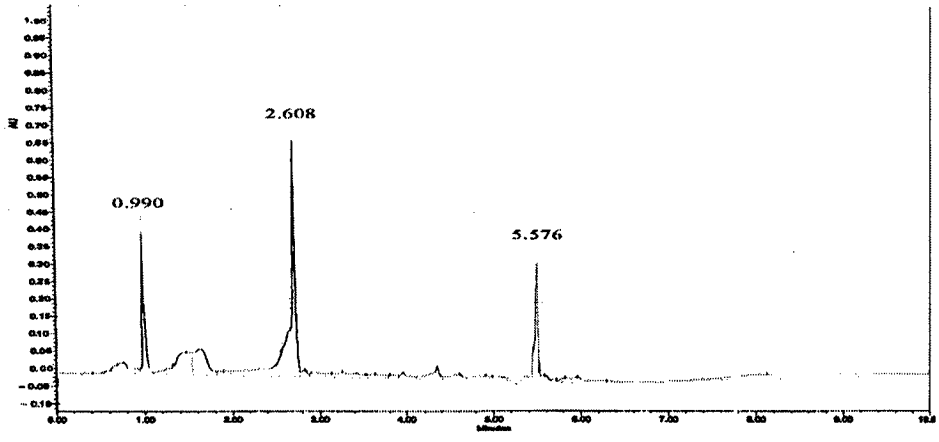


Fig 2.37b: UPLC Chromatogram of EtOAc of Set II

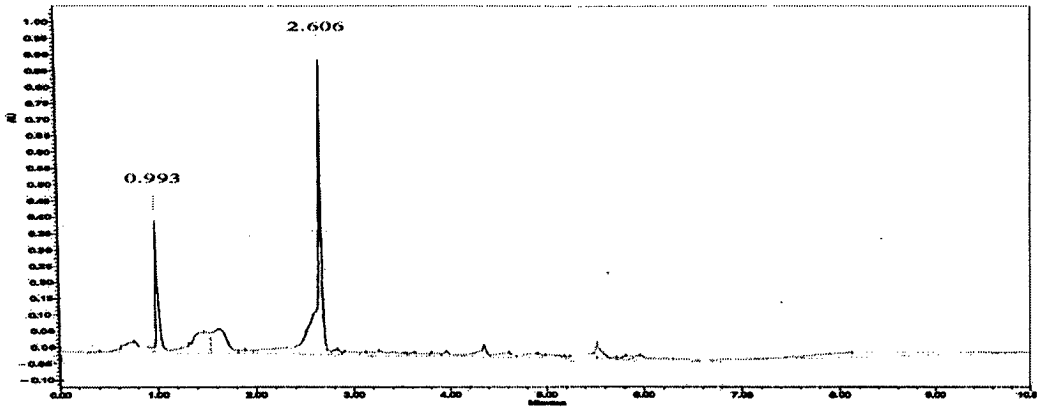


Fig 2.37c: UPLC Chromatogram of EtOAc of Set III

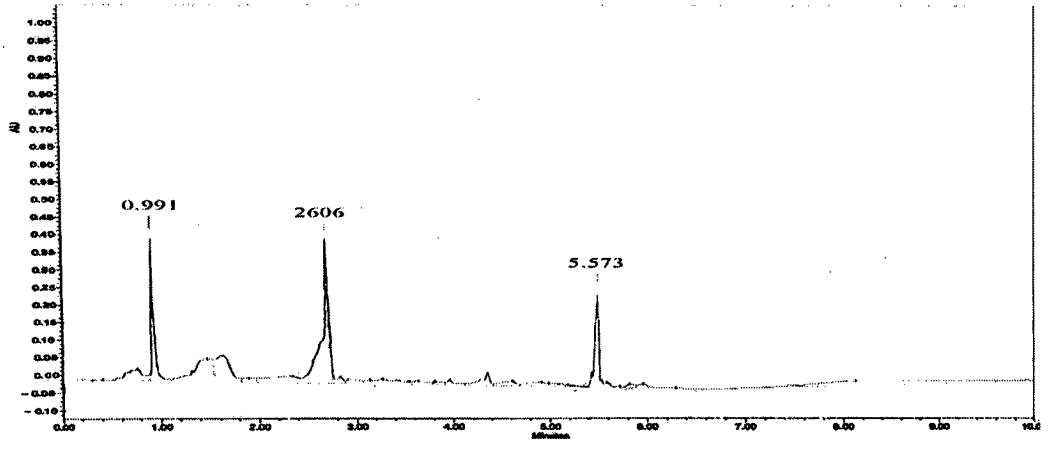


Fig 2.37d: UPLC Chromatogram of EtOAc of Set IV

Table 2.12 : Amount of FM-1 in per milligram of extract

Sample	Conc of Sample (mg/ml)	Peak area (units) (RT 5.57 min)	Area % w.r.t. FM-1	Conc of FM-1 sample solution (mg/ml)	Amount of FM-1 per mg of EA extract (X) (mg)
FM-1	1.3	19482003	100.00	1.30	1.000
Set I	1.7	7361024	37.78	0.49	0.288
Set II	1.4	5622312	28.86	0.38	0.271
Set III	1.5	114564	0.59	0.008	0.006
Set IV	1.4	5167613	26.53	0.34	0.257

Table 2.12: Total amount of FM-1 produced in each set

Experiment	Total weight of EtOAc Extract (Y mg)	Total FM-1 In 100ml culture (X*Y mg)
Set I	69.0	19.87
Set II	103.0	27.93
Set III	73.0	0.0067
Set IV	81.0	21.95

Our above results clearly indicate that the salinity of water has role to play in production of **FM-1** and other metabolites in *A. terreus* and the optimum production of **FM-1** occurs at intermediate concentration of SW. It is therefore suggested that an experiment designed by Schimmel et al., (1999) which produces 70 mg/l of FM-1 in DW glucose-peptone-yeast based medium would produce larger amount of FM-1, if carried with certain concentration of SW in the medium.

III. Experimentals

i) *Cultivation of fungus*

Three media (100 ml each) containing PDB (24 g/l) having SW concentration 0%, 50% and 100% were prepared. Another PDB medium (2.4g in 100ml) containing 4-hydroxyphenypyruvic acid (50mg) in DW (0% SW) was also prepared. All the media were inoculated with fungus *A. terreus* culture (0.5ml) grown on PDB. Flasks were incubated for 21 days under static condition at 27±2°C.

ii) Preparation of extracts

Each culture medium free of mycelium was extracted in ethyl acetate (40ml x 3). Organic extracts were washed with water, dried on anhyd. Na_2SO_4, concentrated under vacuum and weighed.

iii) Analysis of Extracts on UPLC

Accurately weighed amount between 1-2mg of EtOAc extracts were dissolved in MeOH and made up to the mark in std. flasks (10ml). These solutions were analyzed on UPLC as per the method parameters given in table No.

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Chapter 3

CHEMICAL INVESTIGATION OF MARINE INVERTEBRATES *OCEANAPIA* SP. AND *LOBOPHYTUM CRASSUM*

I. Introduction

i) General

In terrestrial environment, plants are the richest sources of natural products. However, in oceans this leading position is taken primarily by invertebrates such as sponges, tunicates, bryozoans, soft corals and molluscs. One has to realize that these marine invertebrates with very few exceptions are sessile and require a "foot-hold" on a nonmoving, fixed substrate (rock or coral) that permits them to feed by filtration of the seawater flowing in and around them. Sessile or sluggish organisms must defend themselves from predation or encroachment of competitors since their lack of mobility precludes evasion. Many organisms have been found to defend themselves by producing toxins or other bioactive small molecules (*i.e.*, M.W. < 1,000). The molecular architectures of marine metabolites are distinct from those of their terrestrial relatives in that the physicochemical requirements of adaptation to an aqueous world, the biosynthetic pathways used, and even the elements employed in crafting their arsenal of defensive molecules are quite different. As a consequence of their structural diversity and uniqueness, marine natural products (MNPs) are providing a prominent share of the recent clinical and preclinical lead compounds for the treatment of various diseases, most prominently cancer. Of late, the marine microbes have been the preferred sources novel chemicals due to their vast biodiversity. Although, majority of invertebrates have been studied and the focus is on their microbes, the reinvestigation of marine invertebrates continues to yields novel molecules due to advances in analytical techniques. As per the review of literature on MNPs by Blunt et al., (2004); of the total MNPs so far discovered, the maximum share of 37% comes from sponges, which is followed by 21%, contributed by another class of invertebrates, coelenterates (**Fig 3.1**).

The studies on MNPs have provided molecules, which are undergoing clinical evaluation. Out of 13 MNPs (or analogues derived from them) those are currently undergoing in clinical trials as new drug candidates, 12 are derived from invertebrates (Proksch *et. al.* 2003).

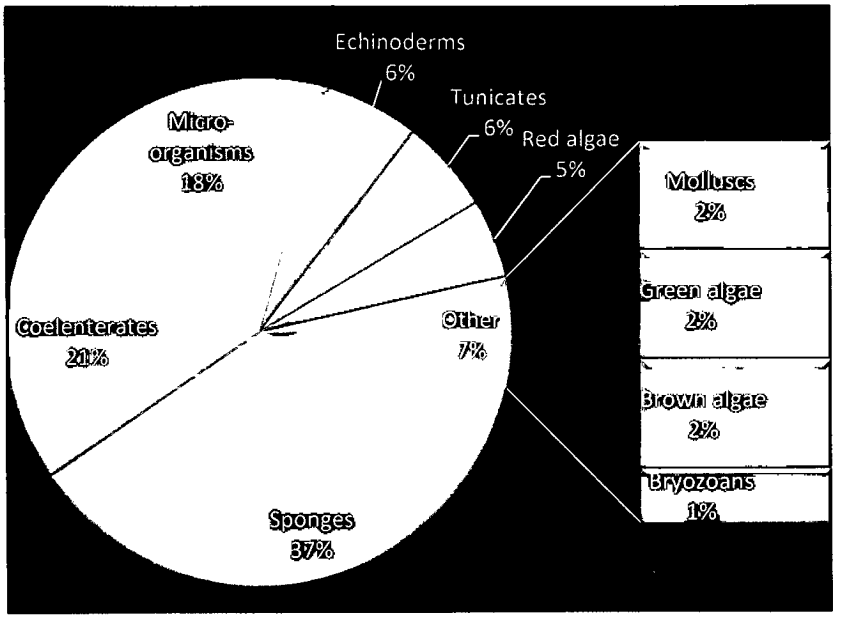
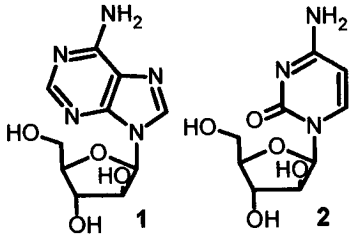


Fig 3.1: Distribution of marine natural products by phylum, 2002

ii) Natural products from marine invertebrates as source of lead compound

A large number of novel metabolites with potent pharmacological properties have been discovered from marine invertebrates. Historically, the first two compounds to make it to market from a marine source are adenine arabinoside Ara-A (**1**) (Vidarabine®, Vidarabin®, Thilo®) and cytosine arabinoside Ara-C (**2**) (Cytarabine, Alexan®, Udicil®). Ara-A is an anti-viral compound and Ara-C has anti-leukemic properties.Both the compounds were isolated from the same sponge (*Cryptotethya crypta*) (Bergmann and Feeney, 1951; 1955) and Ara-C is commercialized by Upjohn (now Pharmacia) and (Ara-A), by Burroughs Wellcome (now Glaxo Smith-Kline). These compounds are still prescribed today.

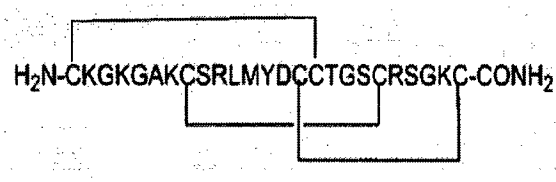


Unfortunately since then marine natural products have not found their way to store shelves with much success. This is because compounds isolated from marine sources have failed to progress to the research stage in the past due to numerous

reasons. But, the continuous advancement of technology has brought significant number of compounds from marine sources especially invertebrates into preclinical and clinical trials since the early 1980s. The result of this led to the development of two drugs ziconotide (prialt^{TM}) (**3**) and ecteinascidin 743 (ET 743) (yondelis^{TM}) (**4**).

Ziconotide (**3**) is 25-residue peptide with three interlocking cystinyl bridgeswas isolated by Olivera's group from a snail *Conus magus* and was known as MVIIA toxin (Ferber et al., 2003). It demonstrated a potent activity against voltage-gated Ca2+ channels and significant effects as an analgesic and was licensed to Neurex Inc. and entered into the market as neuropathic pain reliever

ET-743 (**4**) was isolated from the ascidian *Ecteinascidia turbinate* as antitumor compound (Rinehart et al.,1990; Wright et al., 1990)



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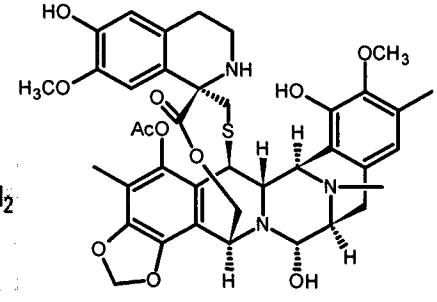


4

Other compounds those in clinical and preclinical trials include bryostatin 1 from bryozoan *Bugula neritina* (Pettit, 1996*)* Dolastatin Derivative, TZT-1027, ILX651 (Synthadotin) (Auristatin PE or Soblidotin**)** originally isolated in very low yield from the Indian Ocean mollusk *Dolabella auricularia* (Pettit et al., 1989), dehydrodidemnin B (aplidine) from *Aplidium albicans* (tunicate) (Sakai et al., 1996) and several other, while few MNPs such as didemin B, dolastain 10 have been withdrawn having failed to deliver at advance stages of clinical trials (Newman and Cragg, 2004).

This chapter deals with isolation and characterization of bioactive metabolites from a marine sponge *Oceanapia sp.* and a marine soft coral *Lobophytum crassum.* The investigation carried out on each organism is discussed two separate sections.

Section 3.1 Secondary Metabolites from marine sponge *Oceanapia sp.*

I. Introduction to marine sponges

Marine sponges (Porifera) are the oldest metazoan group, having an outstanding importance as a living fossil. (Muller, 1998). There are approximately 8000 described species of sponges and perhaps twice as many un-described species. (Hooper, 2002). They are among the simplest of multi-cellular animals often described as the most primitive of all. Sponges come in various shapes, and sizes, from millimeter thin encrustations to branching ropes to giant barrel sponge more than six feet in height. They are sessile marine animals commonly found in seas where there are rocks, corals or other suitable substrata. Sponges inhabit every type of marine environment, from polar seas to temperate and tropical waters and also thrive and prosper at all depths. Sponges have the capacity of filtering out several tons of water to get nutrition. As a consequence of this, marine sponges are exposed to vast number pathogenic and non-pathogenic microorganisms. In order to cope up with these microorganisms sponges has developed strong immune system during the course of evolution and they have possessed efficient chemical defense mechanism against the predators.

Marine sponges have attracted significant attention from various scientific disciplines. These fields include bio-technology, chemical and drug industries etc. From the organic chemists point of view marine sponges are of great value considering discovery of diverse and novel molecules. They are the largest contributors of MNPs (**Fig 3.1**) (Blunt et al., 2004).

A marine sponge of genus *Oceanapia* which is relatively less known for its chemical constituents has been investigated by us. The results of these investigations along with the literature on metabolites from the sponge *Oceanapia* is presented in this section.

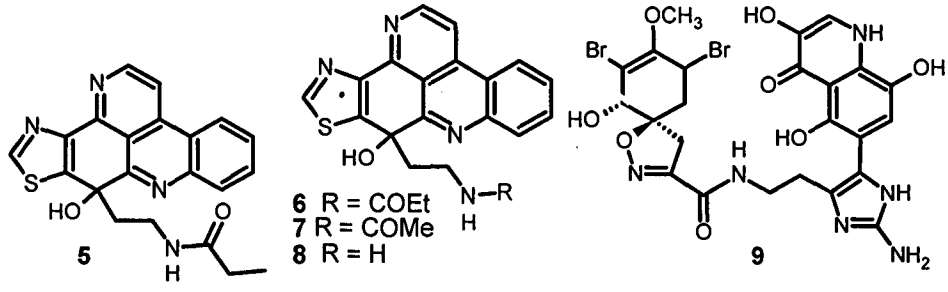
II. Review of literature on metabolite of marine sponge genus *Oceanapia*

Species of *Oceanapia* are common, with more than 50 nominal species recorded for the Indo-west Pacific region alone, relatively abundant in both soft and hard

substrates, and widely distributed (Hooper et. al., 1993). However, only few compounds have been isolated from this sponge. The majority of the compounds isolated from this sponge are alkaloids, but several non-alkaloids constituents have also been isolated. In our following discussion metabolites from *Oceanapia* sp. are divided into alkaloids and non-alkaloids.

i) Alkaloids

Sagitol (**5**) is a pyridoacridine alkaloid was perhaps the first compound to be isolated from the sponge *Oceanapia* (species *sagittaria*). It has disrupted aromatic system but its CD spectrum suggests that it is not entirely an artifact. (Salomon and Faulkner, 1996).

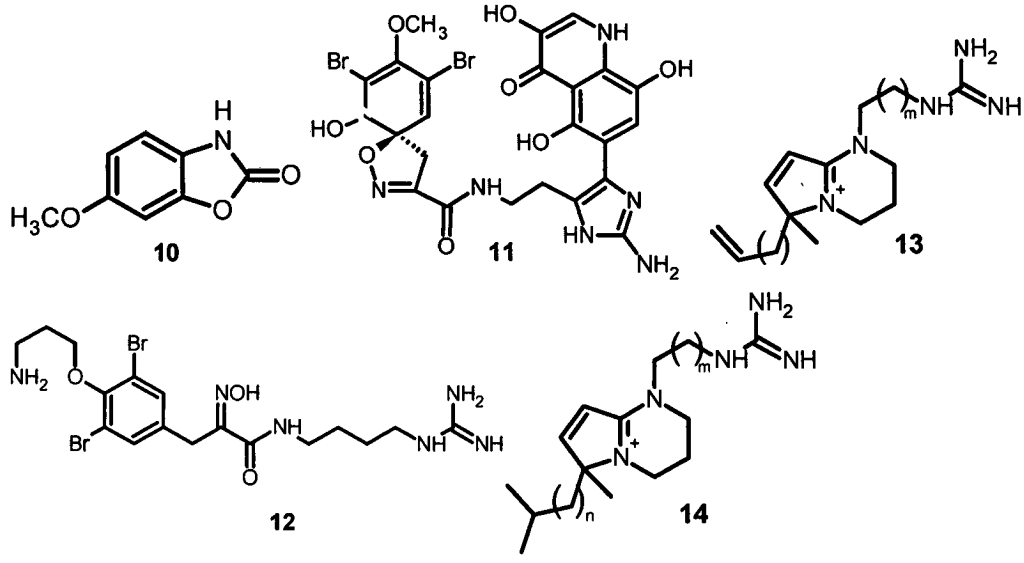


Similar to **5** were insecticidal and cytotoxic metabolites kuanoniamines C (**6**) and D (**7**), an additional pyridoacridine alkaloid, *N*-deacetylkuanoniamine C (**8**) obtained *Oceanapia* sp. from Truk, *Micronesia* (Eder et al., 1998). Metabolites **6** and **7** along with kuanoniamines A and B were previously isolated from a tunicate and its prosobranch mollusk predator *Chelynotus semperi* (Carroll and Scheuer, 1990).

Oceanapia phillipenesis, collected from the coast Southern Australia led to the isolation of new α,ω-bis-aminohydroxylipid glycoside, oceanapiside (9), which showed good antifungal activity (Nicholas et al., 1999).

A chemical investigation on Indian marine sponge resulted in isolatation 6-methoxy-2(3*H*)-benzoxazolinone, coixol (**10**). It was toxic to brine shrimp (Venkateswarlu et al., 1999).

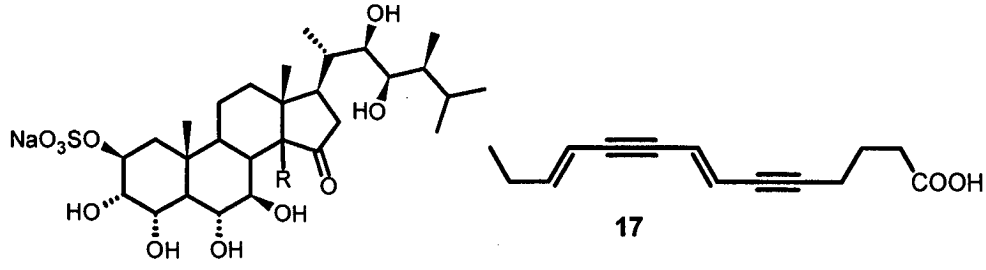
The novel bromotyrosine-derived alkaloids **11** and **12** were isolated from an Australian *Oceanapia* species and were found to be inhibitors of S-conjugate amidase (Nicholas et al., 2001).



Oceanapia fistulosa (New Caledonia) was found to be producer of several phloeodictine alkaloids of the type shown in **13** and **14**, of which twenty were new (Mancini et al., 2004).

ii) Non-alkaloids

Only few non-alkaloids are known from sponge *Oceanapia* sp. The C-14 epimeric pair of polyhydroxy sterols tamasterone sulfates **15** and **16** were the new metabolic products of a new species of the genus *Oceanapia* (Fu et al., 1999).

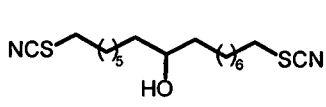


15 R = α-H

16 R = β-H

In an investigation on the antimicrobial constituent of a Japanese *Oceanapia* sp., bis-acetylene (**17**) was identified active principle (Matsunaga et al 2000).

Three dithiocyanates, thiocyanatins A (**18**), B (**19**), and C (**20**), were isolated from an *Oceanapia* species collected from South West Australia. These compounds had nematocidal activity and their structures were confirmed by synthesis (Capon et al., 2001). Similar, but branched dithiocyanates, a pair of isomeric thiocyanatins D1 (**21**) and D2 (**22**), and related thiocyanate-thiocarbamates, thiocyanatins E1 (**23**) and E2 (**24**), were obtained as inseparable pairs from a South Australian *Oceanapia* species. These were also potent nematocides. (Alam et al., 2001)



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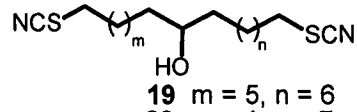
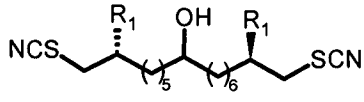
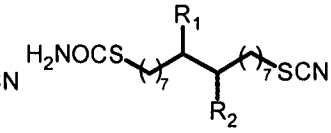


19 m = 5, n = 6
20 m = 4, n = 7



21 R_1 = Me, R_2 = H
22 R_1 = H, R_2 = Me



23 R_1 = OH, R_2 = H
24 R_1 = H, R_2 = OH

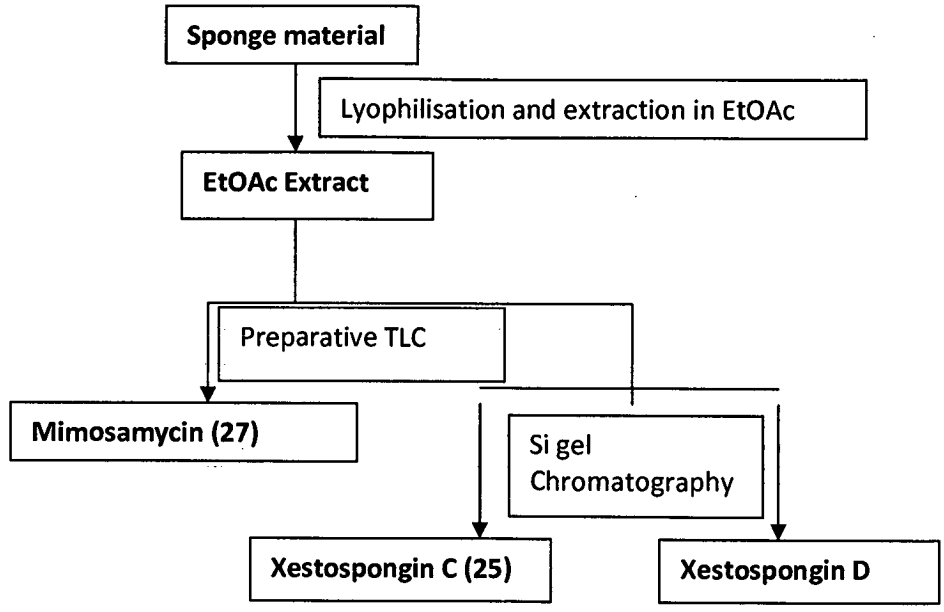
III. Results and Discussion

i) Biological materials

The marine sponge *Oceanapia* sp. was collected from bio-diverse coast of Rameshwaram coast, Tamil Nadu, by SCUBA diving at a depth of 8-10 m. Its EtOAc extract showed strong antimicrobial activity. Its strong antimicrobial activity and only few reports on the chemistry of this sponge prompted us for the investigation of its chemical constituents.

ii) Extraction and purification

Lyophilized sponge was extracted in EtOAc and solvent evaporated to give crude EtOAc extract. Two compounds **25** and **26** were purified by column chromatography by eluting with gradients of MeOH-$CHCl_3$, which is depicted in **Scheme 3.1**. A bright yellow spot seen in visible on TLC (10% MeOH-$CHCl_3$, v/v) was purified from EtOAc extract using preparative TLC to yield bright yellow solid (**27**).



Scheme 3.1: Purification of metabolites from *Oceanapia* sp.

iii) *Structure elucidation of metabolites*

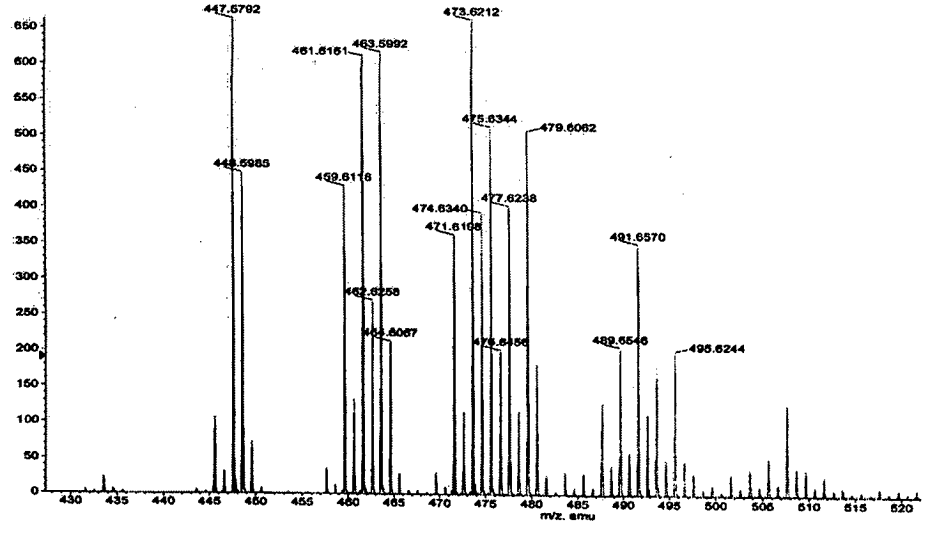
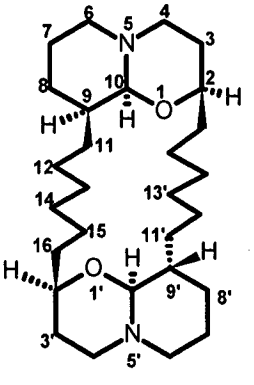


Fig 3.2: ESI-TOF-MS of EtOAc extract of *Oceanapia* sponge

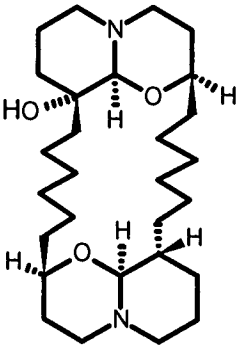
The initial examination of EtOAc extract using ESI-MS (**Fig 3.2**) gave very interesting mass peaks in the region 440-500 amu. In this spectrum there were pairs of peaks, which had difference of 16 or 14 units. The difference between pairs of peaks at m/z 447 and 463, m/z 463 and 479, and m/z 479 and 495 was 16 units indicating the compound with lower mass unit in the pair lacked hydroxyl group. Similarly peaks at m/z 447 and 461, m/z 463 and 477, m/z 479 and 493 had

a difference of 14 units indicating the compound with lower molecular weight lacked a methyl group. Subsequent to chromatographic purification of EtOAc extract, only two compounds **25** and **26** were purified, which were analyzed using IR, NMR and MS. The attempts to isolate any more compounds were unsuccessful.

Compound **25** was obtained as sticky solid and showed very low but negative optical rotation. It displayed the pseudomolecular ion $[M+H]^+$ at m/z 447.3464 and $[M+Na]^+$ at m/z 469.3723 suggesting molecular weight of the compound to be 446 accountable to molecular formula $C_{28}H_{50}N_2O_2$. Its IR spectrum (**Fig 3.3a**) showed strong absorptions at 2850 and 2929 cm^{-1} for methyls and methylenes but no OH and NH_2 groups were observed. The ^1H NMR spectrum (**Fig 3.3b**) of **25** showed broad multiplets in the region δ_H 1-2. It showed complex set of multiplets in the region δ_H 2-4 suggesting CH_2 and CHs for ring protons. A signal at δ_H 4.33 suggested CH probably attached to oxygen and nitrogen. Its ^{13}C NMR (**Fig 3.3c**) showed 28 signals of which 24 carbon signals were observed upfield below δ_C 55, whereas two of the remaining four signals were observed as oxymethines at δ_C 75.4, and 75.5 and α-amino-oxymethines at δ_C 87.8 and 95.8. A comparison of the above IR, NMR, MS and optical activity data with the literature reports (Nakagawa and Endo 1984; Baldwin et al., 1998) revealed that molecule under investigation is identical to previously known bis 1-oxaquinolizidine alkaloid, xestospongin C (**25**).



Xestospongin C (25)



Xestospongin D (26)

Compound **26** was isolated as dextrorotatory solid, more polar than **25**. It showed pseudomolecular ion $[M+H]^+$ at m/z at 463, which was 16 units more than

molecular weight of xestospongin C (**25**). More polar nature and molecular weight more by 16 units compared to **25** was suggestive of a hydroxyl group in the **26**, which was confirmed from the strong absorption band at 3340 cm^{-1} IR spectrum (**Fig 3.4a**). In comparison to **25** an additional signal as expected due to hydroxylated carbon in the ^{13}C NMR spectrum was observed at δ_C 70.1 (C-9) (**Fig 3.4b**). These data is identical to a known sponge metabolite xestospongin D (**26**), suggesting the molecule under investigation is Xestospongin (D).

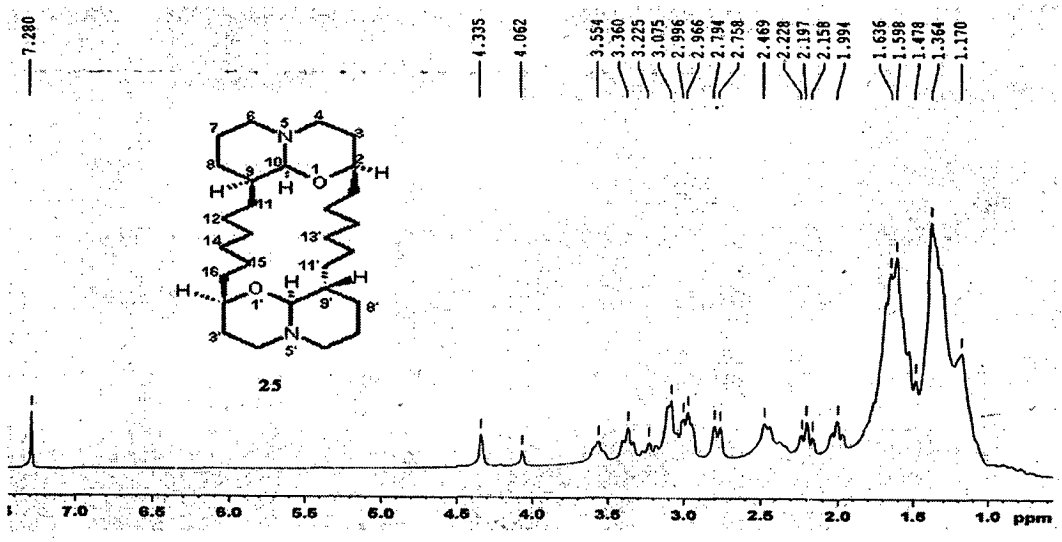


Fig 3.3b: ^1H NMR spectrum of xestospongin C (25)

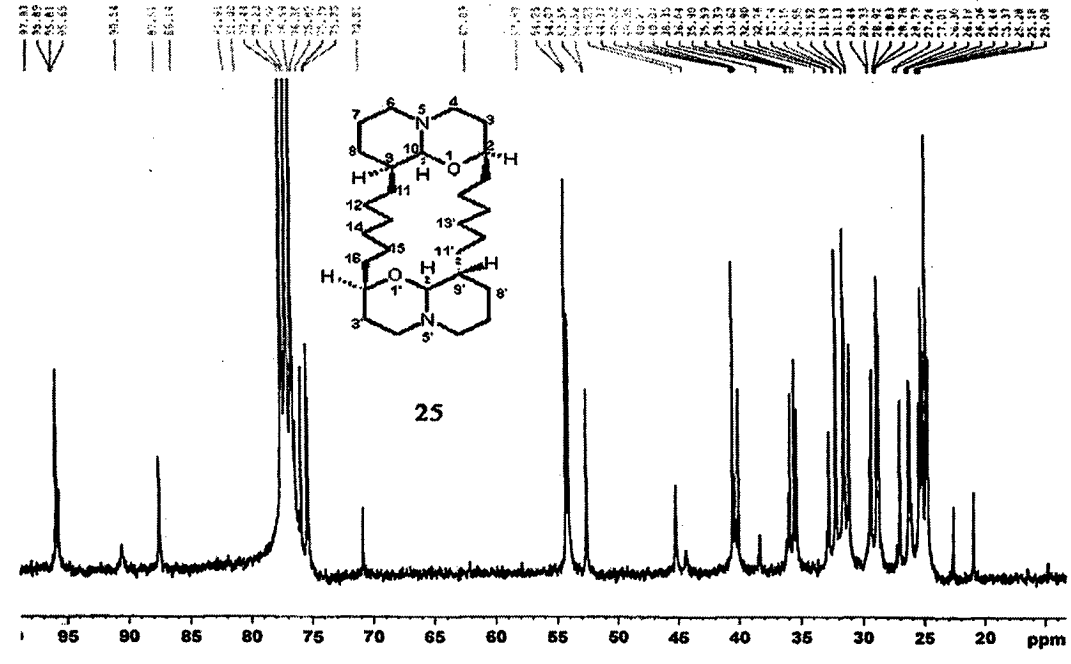


Fig 3.3c: ^{13}C NMR spectrum of xestospongin C (25)

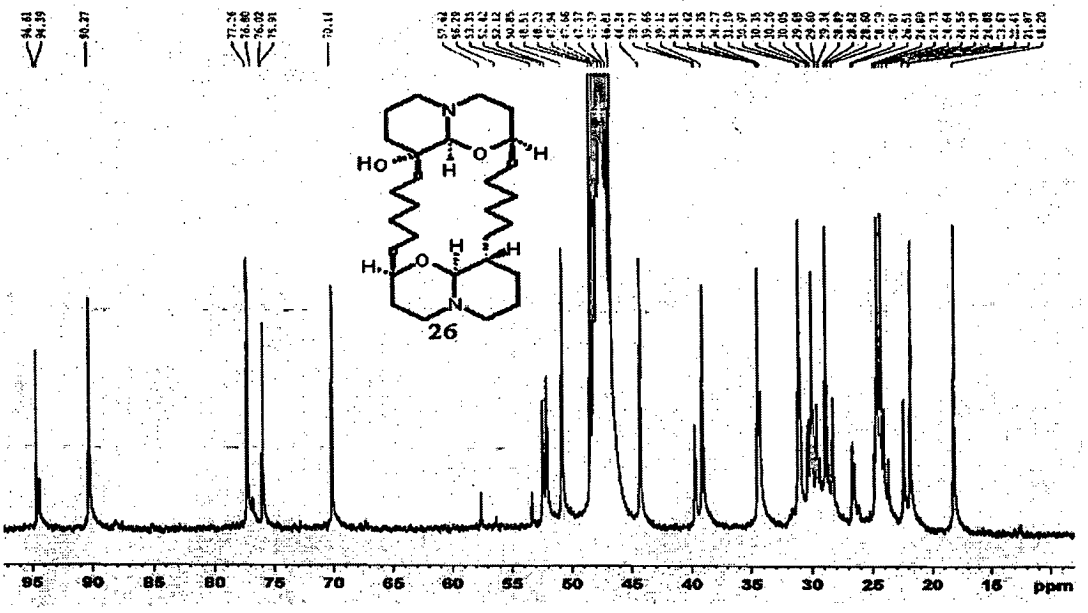
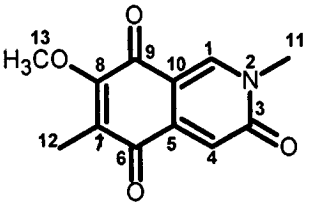


Fig 3.4b: ^{13}C NMR spectrum of xestospongin D (26)

Both xestospongin C (**25**) and D (**26**) along with xestospongin A and B were first isolated from sponge x*estosponqia exigua* (Nakagawa and Endo, 1984). They are bis-1-oxaquinolizidine alkaloids and other members of the family includes araguspongins A-J (Kobayashi et al., 1989), 3β,3'β-dimethylxestospongin C (Reddy and Faulkner, 1997), (+)-7*S*-Hydroxyxestospongin (Moon et al., 2002). The stereochemistry of the xestospongin/araguspongine alkaloids is complex, and it is clear that several stereostructural issues were unresolved until Baldwin's biomimetic syntheses (Baldwin et al., 1998). The biomimetic synthesis allowed unambiguous assignments and revision of absolute configurations of **25** as 2*R*,2'*R*,5*S*,5'*R*,9*S*,9'*R*,10*S*,10'*S*, which was actually assigned exactly opposite in its first report (Nakagawa and Endo 1984), while **26** was determined to be 2*R*,2'*R*,5*S*,5'*R*,9*S*,9'*R*,10*S*,10'*S* from XRD studies (Moon et al., 2002).



Mimosamycin (27)

Compound **27** gave bright yellow fluorescent spot on TLC plate indicating the presence of chromophoric group such as quinone. It was bright yellow and

optically inactive solid. Its IR spectrum showed peaks at 1686, 1646, 1636 and 1616 cm^{-1} indicating presence of quinone carbonyls, amides and C=C bonds. There were no absorption bands for NH or OH stretching. The EIMS of **27** revealed to molecular weight to be 233 indicating presence of odd number nitrogen atoms in the molecular formula. ^1H (**Fig 3.5a**) and ^{13}C NMR (**Fig 3.5b**) data coupled with its molecular weight, the molecular formula of the compound was deduced to be $C_{12}H_{11}NO_4$. Its ^1H NMR gave signals for three methyls, of which signal at δ_H 2.05 was due to a vinyllic methyl and signals at δ_H 3.65 and 4.40 were due NCH_3 and OCH_3 respectively. The additional two signals were due to highly deshielded vinyllic protons at δ_H 7.08 and 8.25. The ^{13}C NMR suggested the presence of two carbonyls at δ_C 183.4 and 177.2, amide carbonyl at δ_C 162.7, two vinyllic methines at δ_C 142.0 and 116.6, and 4 quartenary sp2 carbons at δ_C 159.4, 138.3, 133.1 and 111.2 of which one at δ_C 159.4 is oxygenated. These data accounts for six δ_C 159.4 degrees of unsaturation, while its molecular formula requires eight. Considering the molecule to have one quinone type of ring and three carbons are accounted by methyls the remaining three carbons and a nitrogen atom should be involved six membered fused rings. Using the above data, the molecular structure of the compound was interpreted to be of mimosamycin (**27**), a known natural product (Parameswaran et al., 1998).

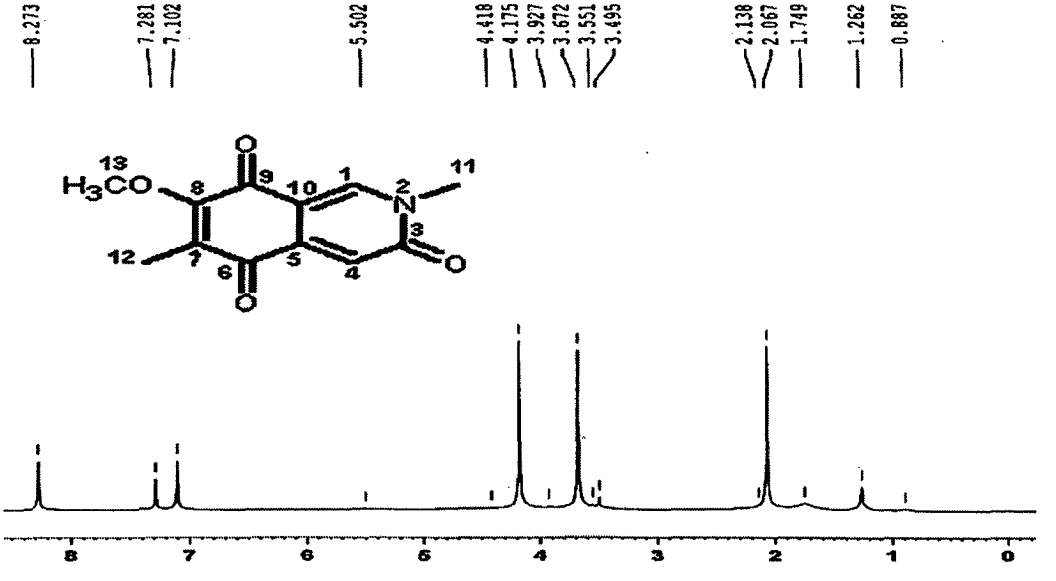


Fig 3.5a: ^1H NMR spectrum of Mimosamycin (27)

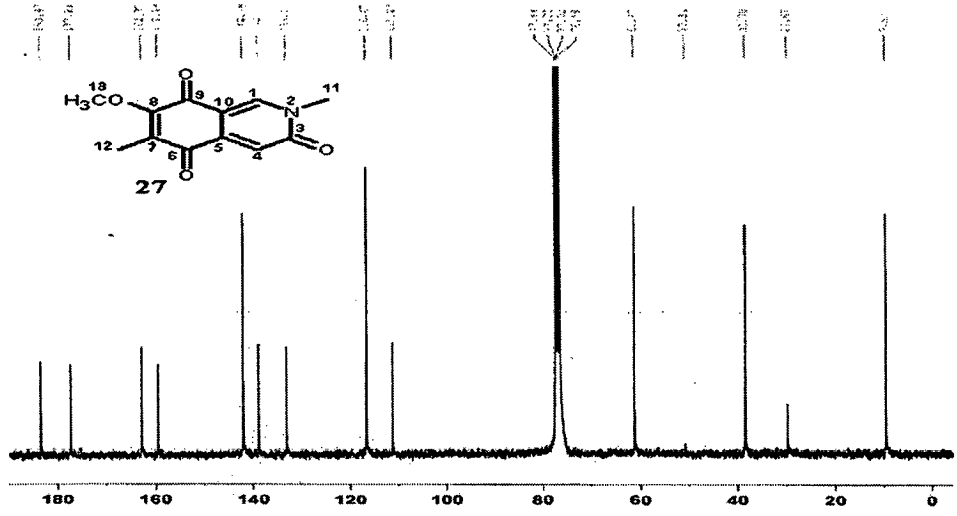


Fig 3.5b: ^{13}C NMR spectrum of Mimosamycin (25)

iv) Biological activity

EtOAc extract of the sponge *Oceanapia* showed very potent antibacterial activity against several pathogenic strains those were tested. Mimosamycin (**27**) is known to be active against mainly mycobacteria, including the streptomycin-resistant strains of *Mycobacterium tuberculosis* and against gram positive bacteria (Arai et al., 1976; Fukami et al., 1978). As expected **27** showed very good activity against *Staphylococcus aureus*, whereas xestospongins **25-26** were moderately active against *S. aureus* and *Escherichia coli*. Xestospongins are better known for their vasodilative activity.

IV. Experimental

i) Biological material

The sponge *Oceanapia sp.* was collected from Rameshwaram coast, Tamil Nadu, by SCUBA diving at a depth of 8-10 m in March 2007 and brought to the laboratory under frozen condition. It was identified by P. A. Thomas.

ii) Extraction and purification of metabolites

Sponge sample (500 g) was dried in a freeze drier and extracted with EtOAc (500 ml x 4) under sonication. EtOAc extract was evaporated under vacuum. A bright yellow spot (10% MeOH-$CHCl_3$ TLC mobile phase) was purified from EtOAc

extract (250 mg) on preparative TLC to yield mimosamycin (2.7 mg). EtOAc (500mg) extract was loaded on flash Si-gel column and eluted with increasing concentration MeOH (0-100%, v/v) in $CHCl_3$. Elution with 4% MeOH-$CHCl_3$ gave xestospongin C (4.8 mg). Further elution with 7% MeOH-$CHCl_3$ gave xestospongin D (3.9 mg)

a) Xestospongin C (25): colorless crystals, $C_{28}H_{50}N_2O_2$, mp ($CHCl_3$-MeOH); $[\alpha]^{28}_D$ -2.7° (c 0.29, $CHCl_3$); IR (KBr): ν_{max} 2990, 1636, 1581, 1549 cm^{-1}. (^1H NMR, $CDCl_3$, 300 MHz, δ_H) 4.38 (s, H-10), 3.57 (br t, J = 12.6, H-2), 3.36 (br t, J = 10.8 Hz, H-2;), 3.11 (br d, , J = 7.7 H-10'), 3.09 (br d, J = 10.5, H-4, H-6'α), 2.95 (br d, J = 10.7, H-4, 2xH4'), 2.77 (br t, H-6α, J = 16.8), 2.50 (br d, H-4), 2.17 (br t, H-6'β), 1.93 (br t, H-6β), 1.17-1.70 (m, 38H); (^{13}C NMR, $CDCl_3$, 75 MHz, δ_C) 95.7, 87.3, 75.7, 75.3, 54.2, 54.0, 52.7, 45.2, 40.5, 40.2, 35.9, 32.8, 32.5, 32.2, 31.5, 31.1, 29.3, 28.8, 28.7, 27.0, 26.4, 26.2, 25.7, 25.2, 25.0, 24.9, 24.7.

b) Xestospongin D (26): Colorless crystals, $C_{28}H_{50}N_2O_3$, mp 158-159°C ($CHCl_3$-MeOH); $[\alpha]^{28}_D$ +17.5° (c 0.33, $CHCl_3$); (^1H NMR, $CDCl_3$, 300 MHz, δ_H) 4.59 (s, H-10), 3.94 (br t, J = 10.3, H-2'), 3.32 (br d, J = 6.0, H-6'α), 3.07 (br d, J = 11.1, 2xH-4', H-10), 2.79 (br d, J = 11.1, H-6α), 2.25 (br d, J = 10.8, H4), 2.17 (br t, J = 8.7, H-6'β), 1.98 (br t, J = 8.7, H-6β), 1.17-1.69 (m, 38H). (^{13}C NMR, CD_3OD, 75 MHz, δ_C) 95.8, 90.2, 77.2, 75.9, 70.1, 54.0, 53.9, 52.4, 45.0, 40.2, 39.4, 34.5, 34.2, 32.3, 31.8, 30.3, 30.0, 29.6, 28.7, 26.6, 25.6, 25.2, 25.0, 24.5, 22.2, 18.2.

c) Mimosamycin (27): Yellow crystalline solid, $C_{12}H_{11}NO_4$, mp 228-230 °C; IR (KBr): ν_{max} 2990, 1636, 1581, 1549 cm^{-1}. (^1H NMR, $CDCl_3$, 300MHz, δ_H): 2.06 (3H, s), 3.67 (3H, s,), 4.17 (3H, s), 7.09 (1H, s), 8.27 (1H, s); (^{13}C NMR, CDCl3, δ_C): 9.6 (q), 38.4 (q), 61.3 (q), 111.3 (s), 116.7 (d), 133.2 (s), 138.9 (s), 142.1 (d), 159.5 (s), 162.8 (s), 177.3 (s), 183.5 (s). MS *m/z* (%): 233 (M^+), 218, 205, 190, 162, 134.

iii) Biological activity

Test cultures were grown on nutrient broth with paper disk impregnated with the extract or pure compounds. Antibacterial assay plates were incubated at 37° C, for 24 hr. Antifungal assay plates were incubated at room temperature for 24- 48 hr. The plates were observed for zones of inhibition.

Section 3.2: Secondary metabolites from soft coral *Lobophytum crassum*

I. Introduction to soft corals and their metabolites

Soft corals or the Alcyonacea, are an order of corals which do not produce calcium carbonate skeletons and so are neither reef-building corals nor do they lay new foundations for future corals. Unlike stony corals, most soft corals thrive in nutrient-rich waters with less light intensity. Soft corals often contain novel secondary metabolites, which serve antifoulant, predator deterrence, competitor exclusion, and reproductive functions (reviewed by Sammarco & Coll, 1992). They are the second largest contributors of the secondary metabolites after marine sponges.

Here in this section the chemical investigation carried out on the soft coral *Lobophytum crassum* is discussed.

II. Results and discussion

i) Biological material, extraction and purification of metabolites

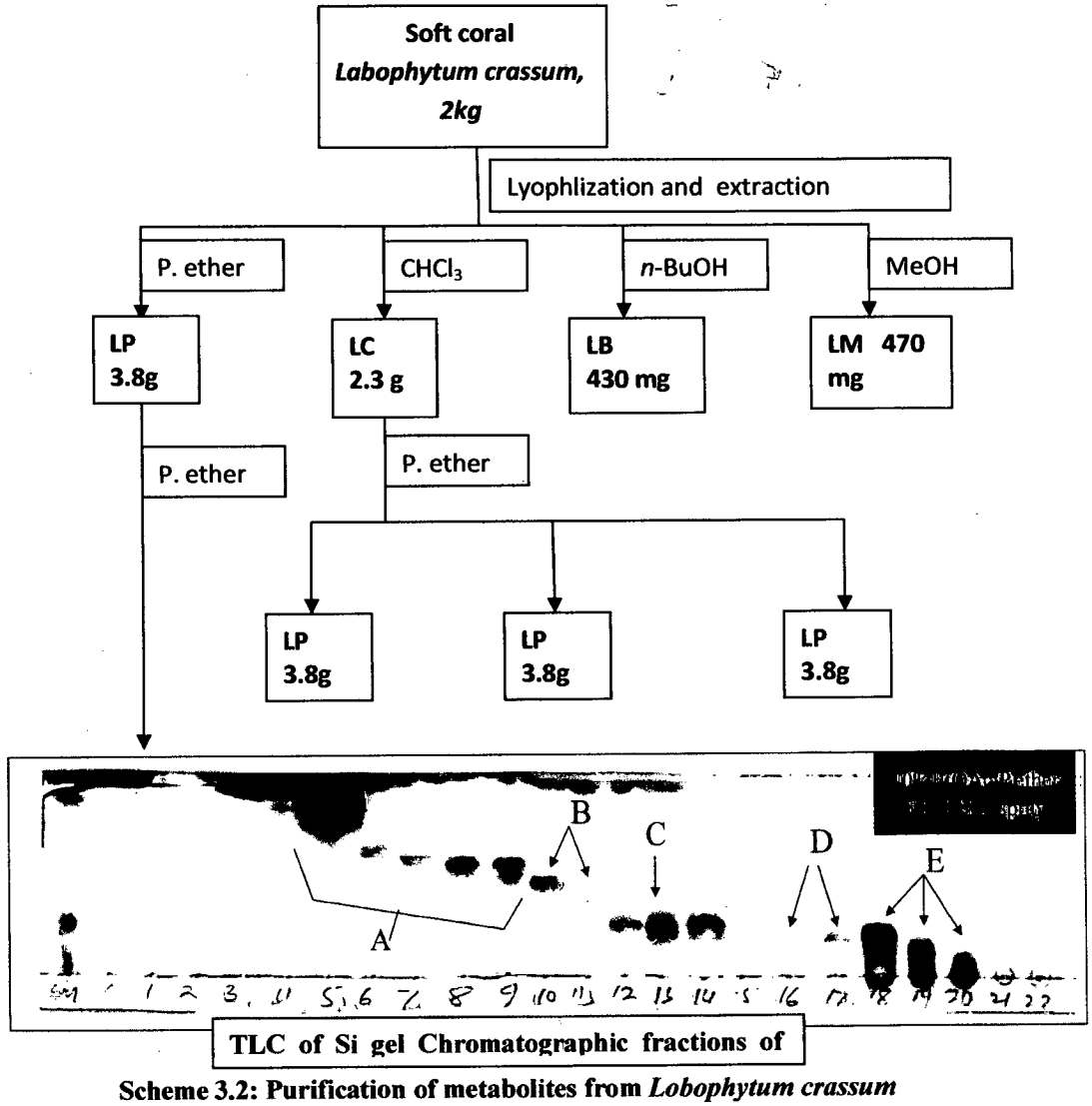
The coastal waters of south east coast of India is a habitat for variety of soft corals. The soft coral, *Lobophytum crassum* was collected from coast of Rameshwaram, Tamil Nadu at a depth of 8-10 m in march 2007. According to scientific classification it belongs to kigdom Anamalia, phylum Cnidiaria, class Anthazoa, order Alcyonacea, family Alcyoniidae, genus *Lobophytum*, species *crassum.*

Lyophilized organism was extracted sequentially with pet ether, $CHCl_3$, EtOAc, n-BuOH and MeOH and the extracts were labeled as **LP**, **LC**, **LB** and **LM** (**Scheme 3.2**). More than 90% of the organic substances were extracted in pet ether and $CHCl_3$.

LP had very pleasant smell probably due to presence of terpenoids in it. It was chromatographed on Si gel by eluting with gradient of EtOAc in pet ether (0-50%, v/v), collected in 22 fractions and analyzed with TLC (10% EtOAc-P.ether, 5% H_2SO_4 sprayed and heated at 120°C) (**Scheme 3.2**). The substances in fractions 5-

9 (fraction A) had peculiar smell of fatty acids and were obtained in the form of wax. Its IR spectrum showed characteristic peaks of fatty esters in the region 2800-3000, 1740 and 1463 cm^{-1}. They were not further analyzed.

The contents of fractions 9-10 (fraction B) had very sweet and pleasant odour and were expected to be terpenoids. On repeated column chromatography on flash Si gel with gradient elution of EtOAc in pet ether (0-10% v/v) gave a pure liquid compound (**28**).



Scheme 3.2: Purification of metabolites from *Lobophytum crassum*

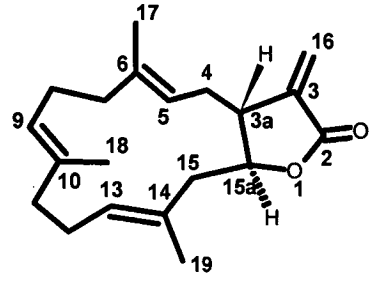
Fraction 13 (fraction C) had solids with green pigments in it. TLC showed green spots on spraying with sulfuric acid and heating at 120° C. A crystalline solid

compound (**29**) was purified by repeated column chromatography on flash Si gel by elution with EtOAc in pet ether (0-20% v/v).

Fraction 16-17 (fraction D) as seen from the TLC had a pure compound (**30**) and Fraction 18-20 (fraction E) was found to be mixture of known sterols when analyzed on GC-MS.

LC was chromatographed on Si gel with gradient elution first with EtOAc in pet ether (0-30% v/v) $CHCl_3$ in MeOH (0-40%, v/v). The initial non polar fraction mainly contained the same spots corresponding to spots for the sterols present in LP. Fractions eluted with gradients of $CHCl_3$-MeOH mainly gave 3 polar spots on TLC (10% MeOH- $CHCl_3$). All the three compounds (**31-33**) were purified on repeated flash Si gel chromatography with gradient elution of MeOH-$CHCl_3$.

ii) Structure elucidation of purified compounds



Cembrenoid 1 (28)

Compound **28** was obtained as sweet smelling liquid. Its IR spectra showed strong absorption bands for C-H str 2854, 2925 and 2960 cm^{-1} and for lactone carbonyl str at 1768 cm^{-1}. No other functional groups were detected from IR spectrum. Its molecular $C_{20}H_{28}O_2$ was deduced from the combination of 1D NMR and EI mass spectrum which showed molecular ion (M^+) at m/z 300. The molecular formula indicated it to be diterpene. Its ^1H NMR (**Fig 3.6a**) showed downfield doublets (J = 2.6) at δ_H at 5.70 (H-3) and 6.23 (H-16) indicating an exomethylene in conjugation with lactone carbonyl. A set of multipltes corresponding to three protons were observed between δ_H 4.86-5.07 revealing the presence of three vinyllic hydrogens (H-5, H-9 and H-13). A signal due to lactonic methine (H-15a) appeared as triplet at δ_H 4.15 (J = 9.6Hz). Another methine (H-3a) appeared as multiplet at δ_H 2.66. Set of multiplets appeared in the region between δ_H 2.02-

2.29 for 12 hydrogen atoms (6 methylenes *viz* C-4, C-7 C-8, C-11, C-12, and C-15). Three vinyllic methyl singlets were observed at δ_H 1.57, 1.65 and 1.71. These signals accounted for all the 28 hydrogen atoms in the molecule. The data was equally supported by its ^{13}C (**Fig 3.6b**) and DEPT NMR (**Fig 3.6c**). They revealed presence of a lactone carbonyl δ_C 170.4, three sp olefinic methines, three olefinic quarternaries, a exo-methylene δ_C 120.5, a oxymethine at δ_C 81.8 a methine at δ_C 44.9, six methylenes between δ_C 24. 4 and 38.7, and three methyls at δ_C 15.9, 16.5 and 17.4. The molecular formula of the compound requires 7 degrees of unsturation. A lactone and 4 ene functionalities accounted for 6 degrees of unsaturation, hence compound most have another alicyclic ring, which suggested a cembrenolide type of ring aystem as shown in the structure **28**. The comparison of our NMR data with reported data for cemrenolide 1 isolated from *Lobophytum crassospiculatum* (Ahond et al., 1979) indicated our compound to be identical to cembrenolide 1. Cembernolide 1 (**28**) has been later isolated as an antimalarial constituent of *Lobophytum crassum* collected from Pange reef in Zanzibar (Said, 2005).

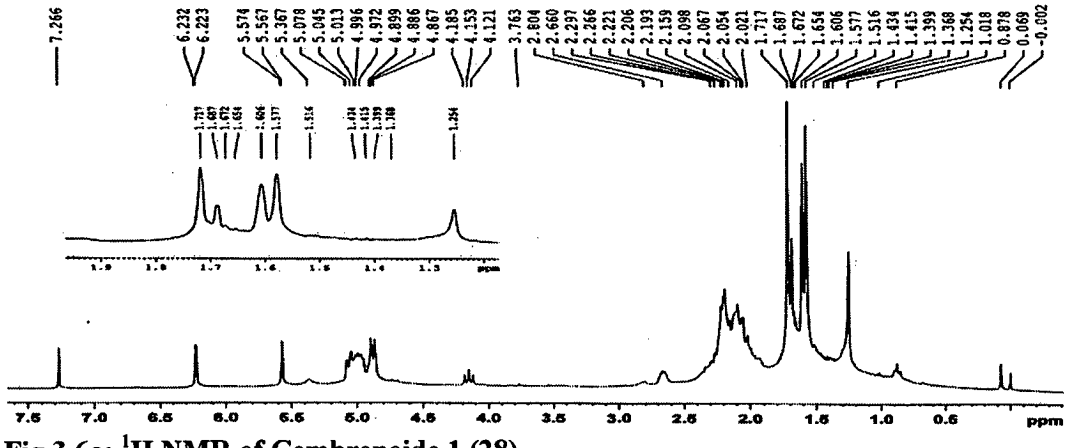


Fig 3.6a: ^{1}H NMR of Cembrenoide 1 (28)

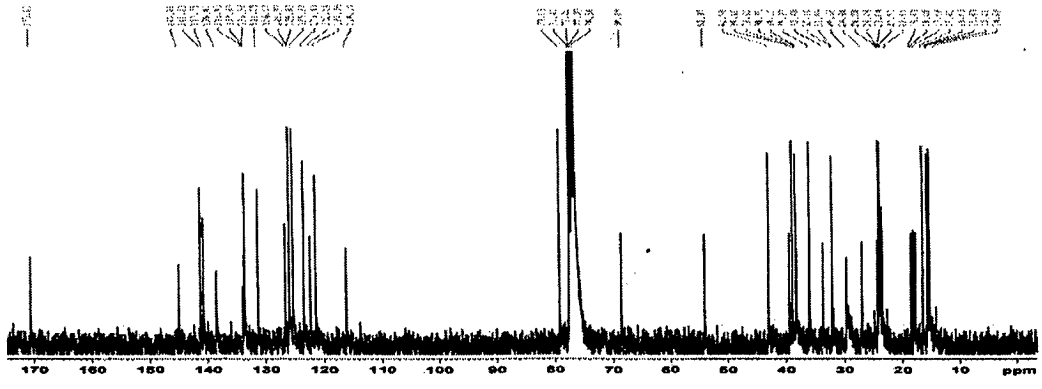


Fig 3.6b: ^{13}C NMR of Cembrenolide 1 (28)

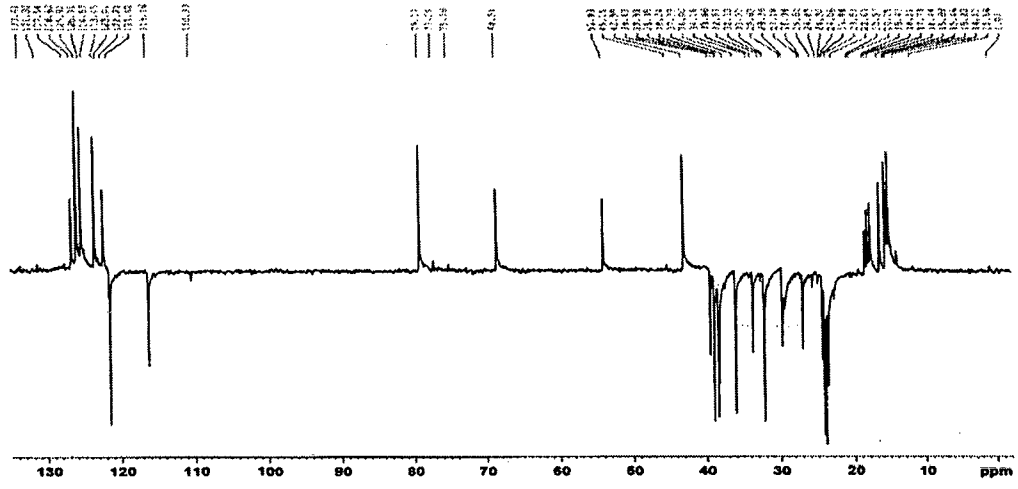
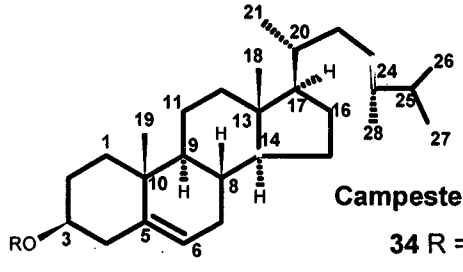


Fig 3.6c: DEPT NMR of Cembrenolide 1 (28)



Campesterol (29) R = H

34 R = Ac

Compound **29** was isolated as crystalline solid and showed intense bands at 2946, and 2854 cm^{-1} and less intense bands at 1464 and 1470 cm^{-1} for C-H stretching and bending vibrations respectively. It showed band at 3320 cm^{-1} revealing presence of hydroxyl in the molecule. A weak intensity band at 1640 cm^{-1} due to C=C stretching was evidence of alkene functionality in the molecule. The ^1H NMR (**Fig 3.7a**) spectra showed characteristics typical of sterols. A vinyllic proton signal was observed as broad singlet at δ_H 5.35. A hydroxymethine signal was observed as multiplet at δ_H 3.55. Six methyls were evident from the signals at δ_H 1.01 (s, C-19), 1.00 (d, C-27), 0.91, (d, C-26), 0.85 (d, C-28), 0.77 (d, C-21) and 0.70 (s, C-18). Several multiplets were observed between 1.0-2.5 due to methylens and methines. These data suggested compound G to be 24-methyl Cholesterol (campesterol). The compound was acetylated using anhydrous pyridine Ac_2O mixture. The acetylated compound (**34**) was a monoacetate which was evident from the signals at δ_H 2.03 (s, 3H) and δ_C 21.4 and 170.5 (s), which confirms that natural product is campesterol (Pouchert and Behnke 1993).

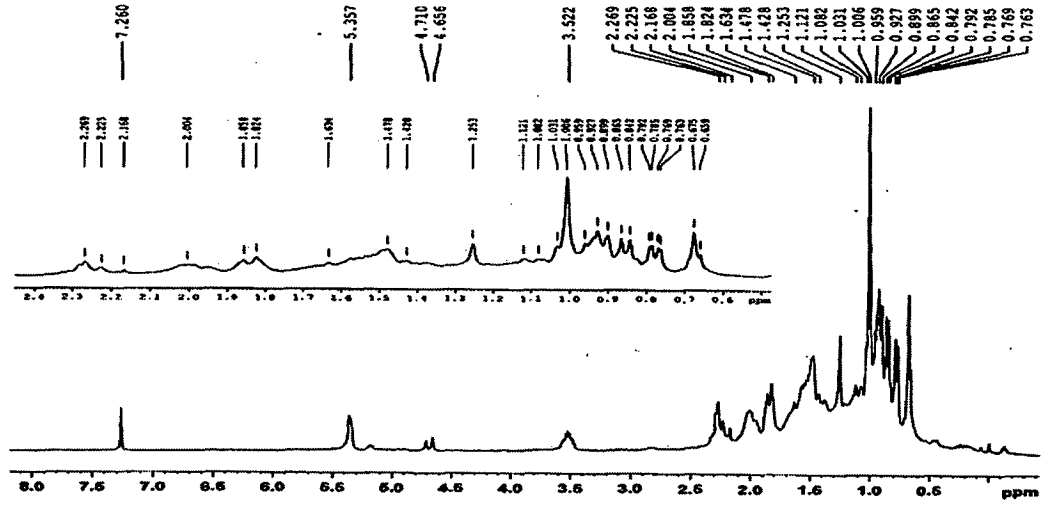


Fig 3.7c: ^{1}H NMR of campesterol (29)

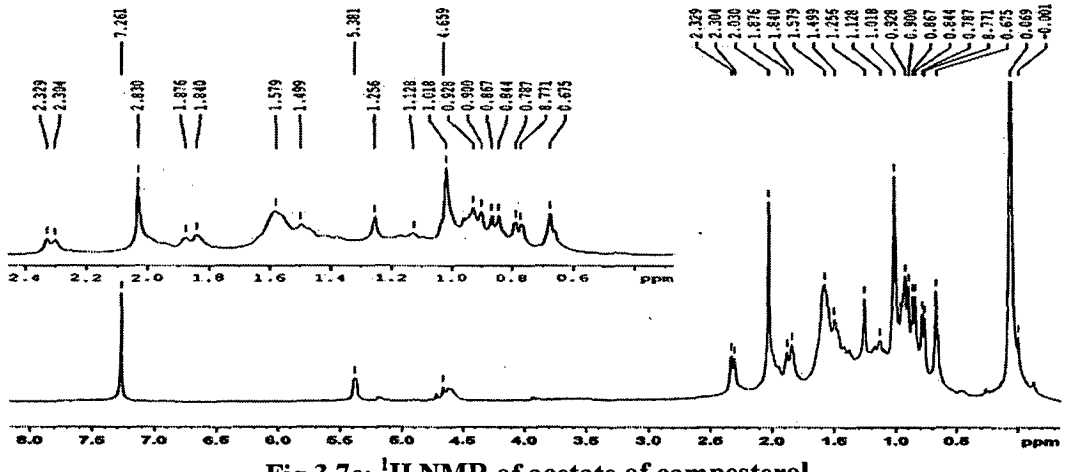


Fig 3.7c: ^{1}H NMR of acetate of campesterol

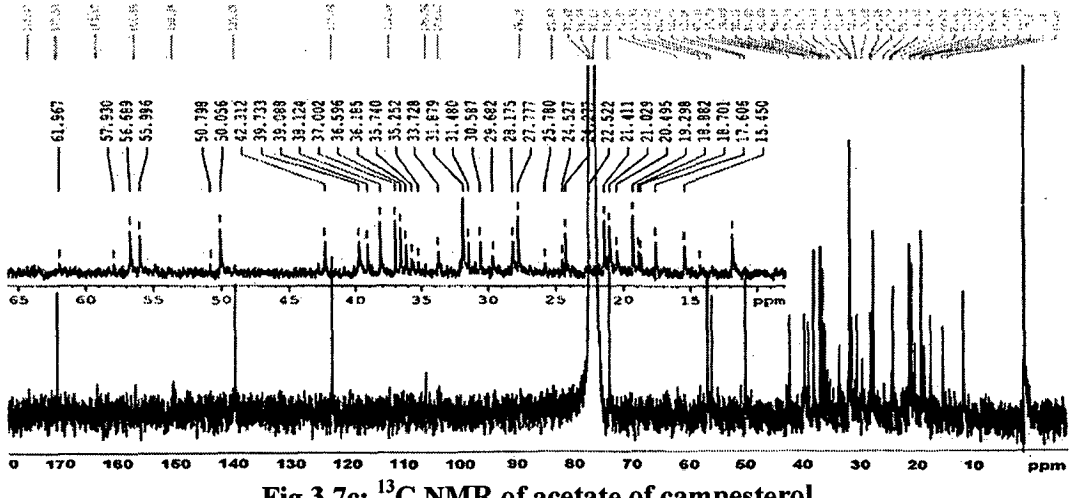
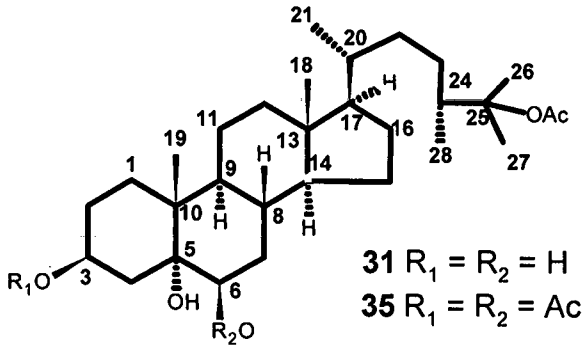


Fig 3.7c: ^{13}C NMR of acetate of campesterol

Compound **31** was obtained as crystalline solid which was identified as cholesterol by comparison of its TLC (10% EtOAc, v/v; sprayed with methanolic H_2SO_4) with authentic sample.



Compound **31** was obtained as crystalline solid $[\alpha]^{25}_D$ -21.1° (c 1.3, MeOH). Its ESI-TOF-MS gave pseudomolecular ion $[M+Na]^+$ at m/z 515.4124 $[M+K]^+$ 531.3912 revealing the molecular formula of compound to be 492. From the molecular weight and NMR data the molecular formula of **31** was determined to be $C_{30}H_{52}O_5$. ^1H ^{13}C and DEPT NMR (CD_3OD) (**Fig 3.8a-c**) spectrum of **31** corroborated the presence of four oxygenated carbon atoms, of which two were secondary hydroxyls (δ_H 3.44 brs, δ_C 67.4, C-3 and δ_H 3.99, δ_C 75.5, C-6) one tertiary hydroxyl (δ_C 75.8) and one tertiary acetoxy (δ_H 2.14, δ_C 22.4, 170.7). The acetoxy was considered to be tertiary on the basis that two oxymethine signals (δ_H 3.99 and 3.44) were below δ_H 4. The ^1H shifts of the C-26 and C-27 methyl groups (δ_H 1.35) in **31** pointed to a substituent at C-25. Therefore, either hydroxyl or acetoxy must be present at this carbon. The presence of 3 singlets [δ_H 0.66 (3H), 1.06 (3H), and 1.35 (6H)] for 4 methyls, and two doublets [0.85 (J = 6.7) and 0.87 (J = 6.33)] suggested a ergostane skeletone similar to campesterol (**29**). Therefore, one of the secondary hydroxyl is established at C-3. There were no signals for alkene functionality in the molecule indicating absence of any double bond such as Δ^5 in **29**. The two remaining oxygenated carbons were assumed to be C-5 and C-6. The second secondary hydroxyl must be present at C-6, but the third hydroxyl which tertiary must be present on C-5, if acetoxy is present in C-25 or vice versa. In HMBC (**Fig 3.8d**) acetate methyl and the C-28 methyl protons showed correlations to a common carbon signal (C-25, 86.0) establishing acetoxy group at C-25, hence tertiary hydroxyl has to be present at C-5. Therefore the

structure of polyhydroxy sterol **31** is 24*S*-24-methyl cholestane- 3β,5α,6β,25-tetraol-25-*O*- acetate. It is a known compound, which was isolated first from soft coral species e.g., *Sarcophyton elegans* (Raju et al., 1992) and then from a gorgonian *Junceella juncea* (Rubinstein et al., 1976) and very recently from sponge *Callyspongia* sp (Rao et al., 2010). Its optical activity and spectral characteristics are in good agreement with the literature records also confirming the stereochemistry as shown in its structure **31**. The spectral properties of the triacetate (**35**) obtained by Ac_2O/Py treatment of **31** was also in accord for 24*S*-24-methylcholestane-3β,5α,6β,25-tetraol-3,6,25-*O*-triacetate [^{1}H NMR (**Fig 3.10**)] (Radhika et al., 2004). Recently, compound **31** has been shown to exhibit good antimalarial activity. It showed good activity against chloroquine-resistant strain of *P. falciparum,* but was less active against chloroquine-sensitive strain (Rao et al., 2010)

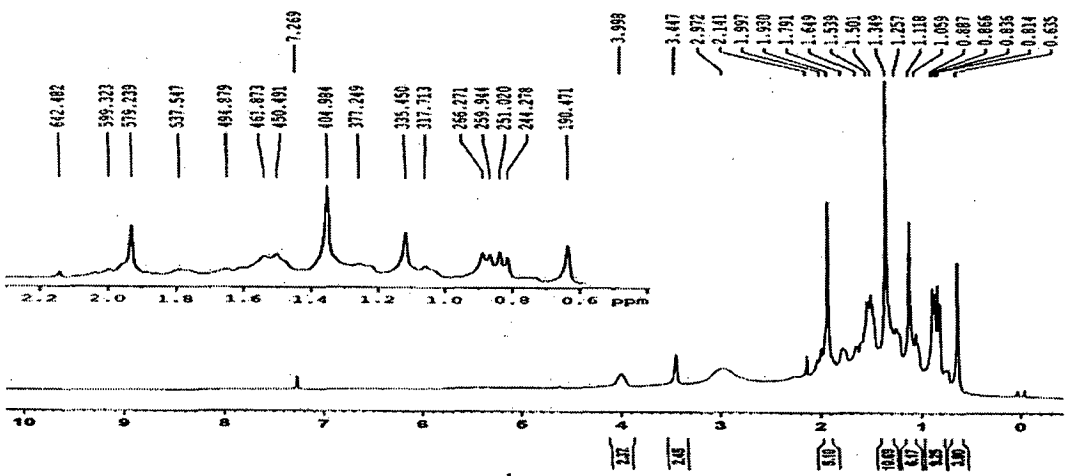


Fig 3.9a: ^{1}H NMR of 32

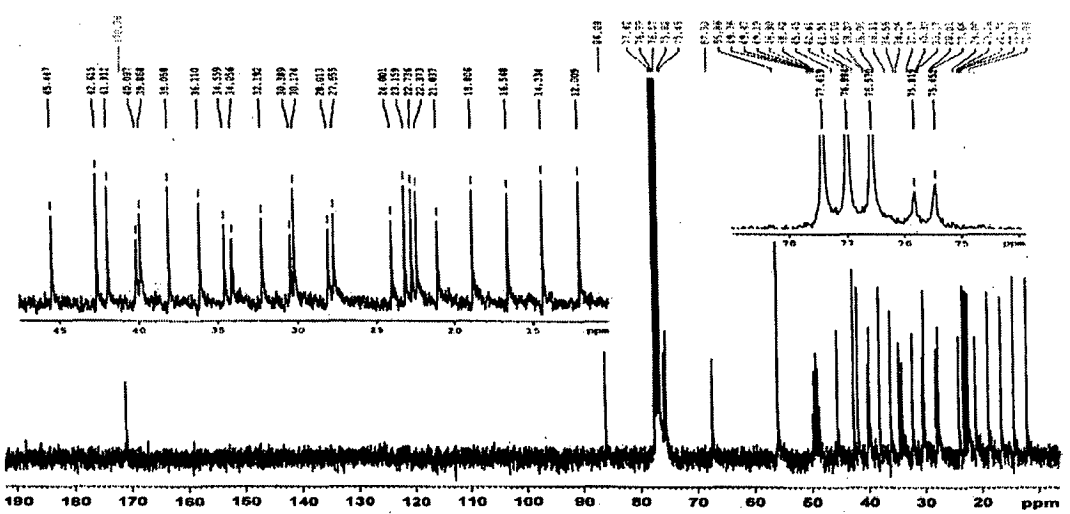


Fig 3.9b: ^{13}C NMR of 32

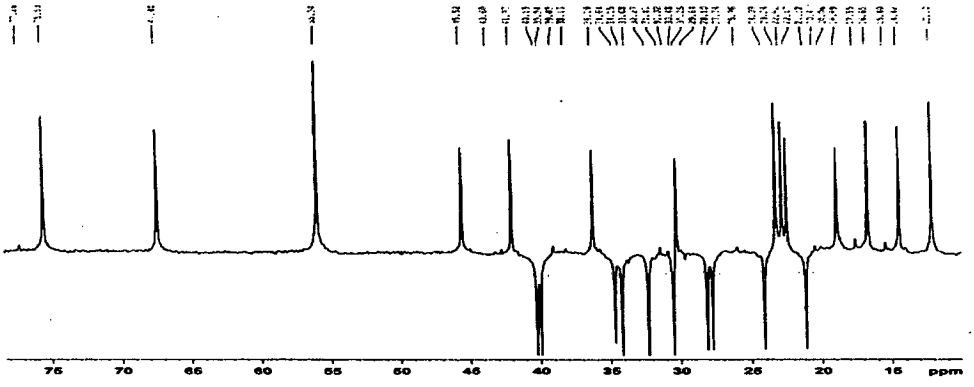


Fig 3.9c: DEPT NMR of 32

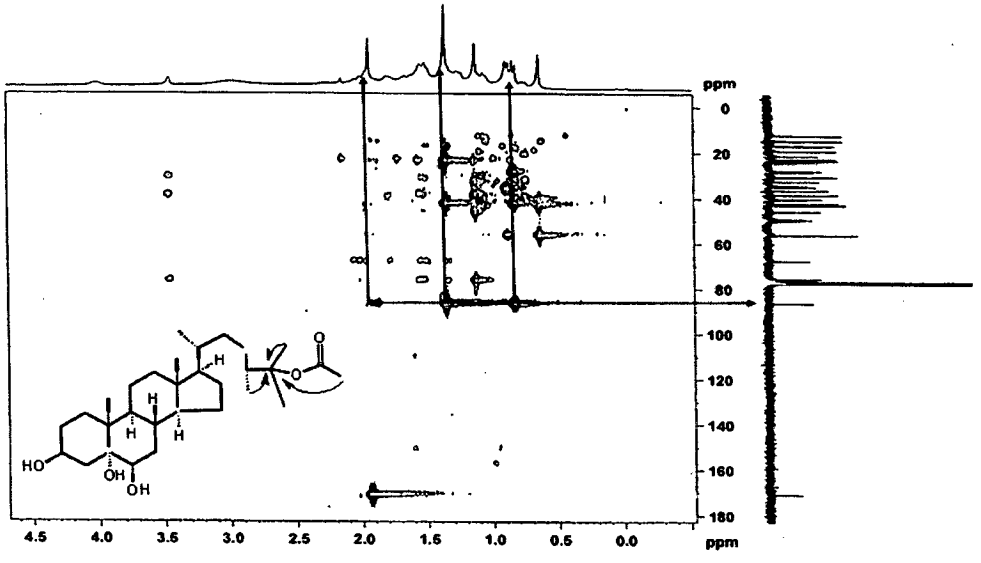


Fig 3.9d: HMBC NMR of 32

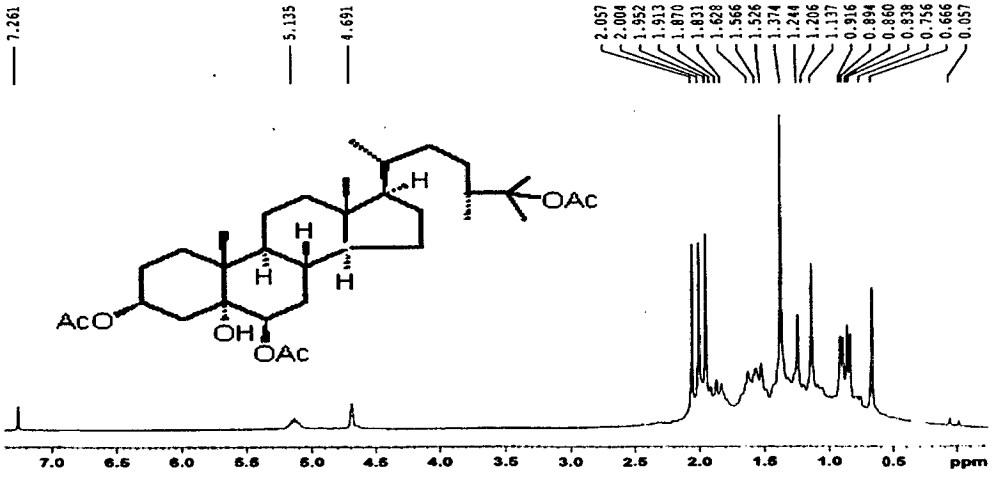


Fig 3.10: ^{1}H NMR of 35

The other two compounds **32** and **33** also appear to be polyhydroxy sterols from their NMR spectra but the exact positions of hydroxyls have not been yet determined. **Fig 3.11** is ^{13}C NMR spectra of **32**.

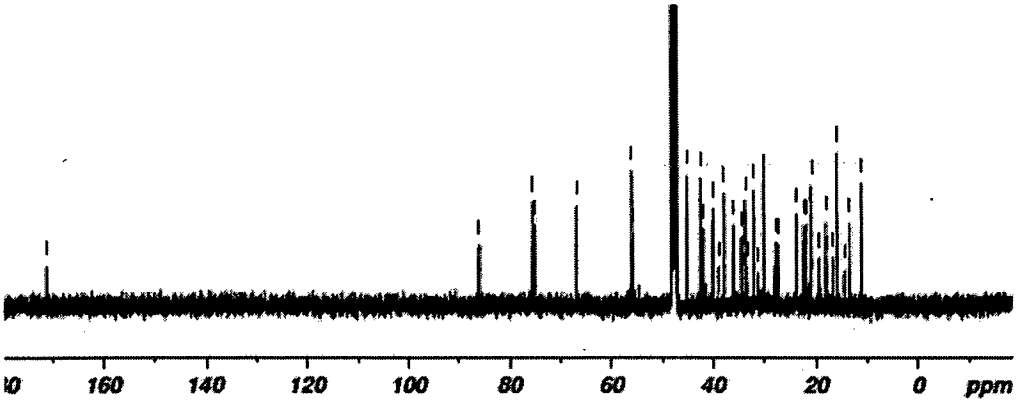


Fig 3.11: ^{13}C NMR of 32

III. Experimental

i) Biological material

The soft coral *Lobophytum crassum* was collected from Rameshwaram coast, Tamil Nadu, by SCUBA diving at a depth of 8-10 m in March 2007. It was brought to lab under frozen condition. The organism was identified by P. A. Thomas.

ii) Extraction of metabolites from the soft coral

The organism (2 Kg approx.) was lyophilized and cut into small pieces. It was sequentially extracted by sonicating with petroleum ether (300 ml x 5), $CHCl_3$ (300 ml x 4), n-BuOH (300 ml x 3) and MeOH (200 ml x 3). Organic extracts were evaporated under vacuum to yield concentrated extracts petroleum ether (3.8 g), $CHCl_3$ (2.3 g), n-BuOH (430 mg), and MeOH (470 mg).

iii) Purification of metabolites from Petroleum ether extract

Petroleum ether extract was chromatographed on Si gel column by eluting with increasing volumes of EtOAc in petroleum ether (0-50% v/v) and collected in 22 fractions. Each of these fractions were analyzed with TLC and IR and similar fractions were pooled together to make 5 fractions. TLC was developed in mixture of EtOAc-pet ether (10%, v/v), sprayed with H_2SO_4-MeOH (5%, v/v) and heated

to 120°C to develop spots. On the basis of TLC, fractions 5-9, 10-11, 12, 16-17 and 18-20 were pooled, solvent evaporated. These fractions were labeled as A, B, C, D and E. Fraction B (65 mg) was purified on flash Si gel by eluting with EtOAc- petroleum ether (1-10%, v/v) to yield pure compound **28** (13 mg). Fraction C (85 mg) was flash chromatographed on Si gel using EtOAc- petroleum ether (0-12%, v/v) to give pure campesterol (**29**) (60 mg).

Cembrenoid 1 (**28**)- Colourless liquid, IR (KBr) ν_{max} 2960, 2925, 2854, 1768, 1661, 1437, 1281, 1120, 947, 942, 833 cm^{-1}. ^1H NMR (300 MHz, $CDCl_3$) δ_H 6.23 (d, J = 2.7, 1H, H-16), 5.70 (d, J = 2.1, 1H, H-16), 4.86-5.07 (m, H-5, H-9-H-13), 4.15 (t, J = 9.6 Hz, 1H, H-15a), 2.02-2.29 (H-4, H-7, H-8, H-11, H-12, H-15), 1.75, (s, 3H), 1.65 (s, 3H), 1.57 (s, 3H). ^{13}C NMR (75 MHz, $CDCl_3$) 170.4 (C-2), 139.6 (C-3) 137.1 (C-6), 133.4 (C-14), 129.5 (C-10), 128.1 (C-5), 124.5 (C-9), 122.0 (C-16), 120.5 (C-13), 81.8 (C-15'), 44.9 (C-15), 38.7 (C-11), 38.1 (C-12), 33.7 (C-7), 24.48 (C-8), 24.41 (C-4), 17.4 (C-17), 16.5 (C-19) 15.9 (C-19).

Campesterol- White solid IR (KBr) ν_{max} 3320, 2946, 2854, 1470, 1464 cm^{-1}. ^1H NMR (300 MHz, $CDCl_3$) 5.35 (m, 1H, H-6), 3.35 (m, 1H, H-3), 1.01 (s, 3H, H-19), 1.00 (d, 3H H-27), 0.91 (d, 3H, H-26), 0.85 (d, 3H, H-28), 0.77, (d, 3H, H-21), 0.70 (s, 3H, H-18). 1.20- 2.35 (sets of merged multiplets for rest of the methines and methylenes).

iv) Purification of $CHCl_3$ extract

$CHCl_3$ extract (200 mg) was fractionated on Si gel column by eluting with MeOH-$CHCl_3$ (0-40%, v/v). Fraction 3 was purified on flash Si gel column by eluting with MeOH-$CHCl_3$ (3-7%, v/v) to yield pure compound **31** (51 mg). Fraction 4 was purified on flash Si gel column by eluting with MeOH-$CHCl_3$ (5-10%, v/v) to yield **32** (7 mg). Fraction 5 was purified on flash Si gel column by eluting with MeOH-$CHCl_3$ (5-15%, v/v) to yield pure sterol **33** (11 mg).

Polyhydroxy sterol (**31**): Colourless needles (MeOH-$CHCl_3$) m.p >200°C $[\alpha]^{25}_D$- 19.9° (c 2.1, MeOH); FTIR (KBr): ν_{max} 3512, 2992, 1732, 1418, 1262, 935 cm^{-1}; ^1H NMR (300 MHz, CD_3OD) δ_H 3.44 (m, 1H, 3β-H), 3.99 (br s, 1H, 6β -H), 0.66 (s, 3H, 18-H), 1.06 (s, 3H, 19-H), 0.85 (d, *J* = 5.4Hz, 3H, 21-H), 1.35 (s, 6H, 26,27-H), 0.87 (d, *J* = 6.33 Hz, 3H, 28 H), 1.98 (s, 3H, $OCOCH_3$), several

multiplets in the region 0.5-2.0. ^{13}C NMR (75 MHz, $CDCl_3$): 30.3 (C-1), 22.3 (C-2), 67.0 (C-3), 38.0 (C-4), 75.4 (C-5), 75.8 (C-6), 34.5 (C-7), 34.0 (C-8), 41.9 (C-9), 36.1 (C-10), 21.0 (C-11), 40.0 (C-12), 45.4 (C-13), 55.8 (C-14), 24.0 (C-15), 28.0 (C-16), 42.6 (C-17) 12.0 (C-18), 16.5 (C-19), 36.1 (C-20), 18.8 (C-21), 32.1 (C-22), 23.1 (C-23), 42.6 (C-24), 86.0 (C-25), 22.7 (C-26), 27.6 (C-27), 14.3 (C-28), 22.3 ($OCO\underline{C}H_3$), 170.7 ($O\underline{C}OCH_3$) ESIMS $[M+Na]^+$ m/z 515.4214, $[M+K]^+$ m/z 531.3912.

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Chapter 4

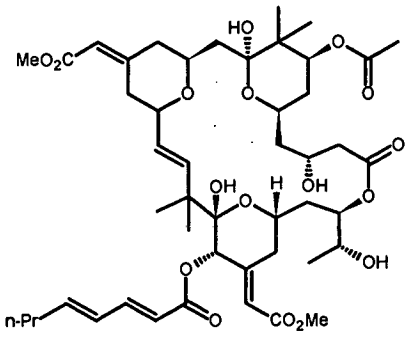
STUDIES TOWARDS THE SYNTHESIS OF AMATHAMIDES AND THEIR ANTIMICROBIAL ACTIVITY

I. Introduction

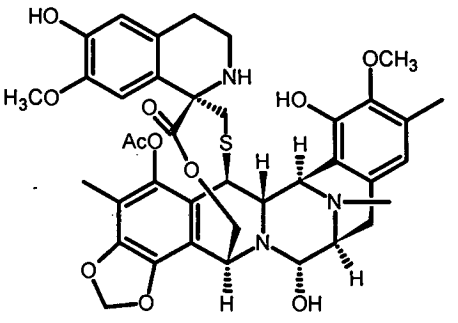
i) General

During past 30 to 40 years, numerous novel compounds have been isolated from marine organisms and many of these have been reported to exhibit various biological activities, some of which are of interest from the point of view of potential drug development. Despite, over 22000 compounds isolated from marine organisms and the biological activities attributed to many of them, those that have either been marketed or are under development are very few (Blunt et al., 2011). There are probably several reasons why only few compounds originating from marine plants and animals have been developed as drugs. Much of the work undertaken in the 1960-1970s and probably the early 1980s was driven by an interest in the chemistry of new compounds rather than their biological activities. Another issue was to tackle with the supply of MNPs, which has been major limiting factor for further pharmaceutical development. Often, a metabolite occurs in trace amounts in an organism, and a steady source of supply from wild harvest cannot provide enough of the target compound for preclinical studies. In general, the natural abundance of the source organisms do not support production based on wild harvest. Unless, there is a feasible alternative to harvesting, promising chemicals will remain undeveloped.

The total synthesis or perhaps semi-synthesis in some cases has been the one of the best solutions to avail these substances in sufficient amount for further development (Cuevas et al., 2000). Moreover, the considerable interest in the synthesis of marine natural products is developed from the fact that many marine natural products have unique structures not found in terrestrial metabolites and so their syntheses represent a challenge for the organic chemists. The synthesis of some important marine natural products those are under clinical evaluation for e.g. bryostatins 1 (**1**) (Evans et al., 1999), or in the market as potential drug for e.g. ecteinascidin 743 (**2**) (Endo et al., 2002) have been achieved. Herein, we intend to synthesize amthamides, the secondary metabolites of a bryozoan, *Amathia wilsoni* and assess their antimicrobial activity. The synthetic studies towards amathamides along with literature review on amathamides and related compounds from bryozoan genus *Amathia* and the ecology and biological activity of amathamides is presented in this chapter.



Bryostatin 1 (1)

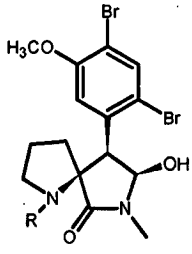


Ecteinascidin 743 (2)

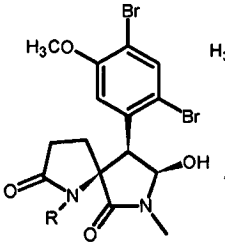
ii) Amathamides and related metabolites from bryozoan genus Amathia

Bryozoans, also known as seamats or sea mosses, are found in both freshwater and marine environments. It is interesting to note that as reviewed by Sharp et al., (2007), the metabolites so far reported from bryozoans are exclusively from marine habitats. A bryozoan of genus *Amathia* is a member of phylum Stenolaemata, order Cyclostomata, family Vesiculariidae.

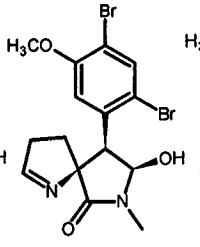
A species, *Amathia wilsoni* Kirkpatrick, a reasonably common foliose bryozoan from Tasmanian coastal waters, has been shown to contain a series of brominated alkaloids, amathamides A-F (**3-8**) (Blackman and Matthews, 1985; Blackman, and Green, 1987) and another group of biosynthetically related alkaloids, amathaspiramides A-F (**9-14**) (Morris and Prinsep, 1999). The isolation of 2-(2,4-dibromo-5-methoxyphenyl)ethanamine (**15**) from this species suggest that amathamides and amathaspiramides are biosynthesized from phenylalanine by a series of aromatic substitution reactions and a decarboxylation giving the amines of the type **15**, which then reacts with a proline derivative (Blackman and Fu, 1989).



9 R = CH_3
11 R = H



10 R = CH_3
12 R = H



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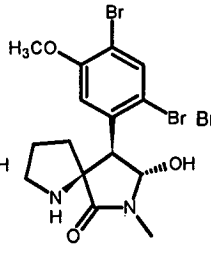

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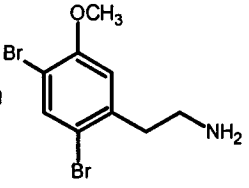

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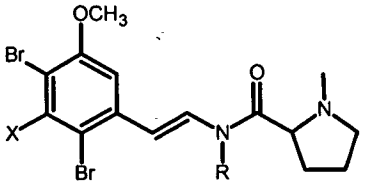
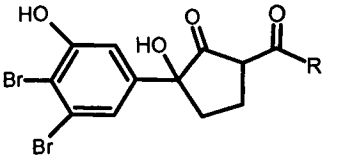
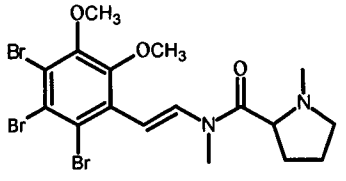


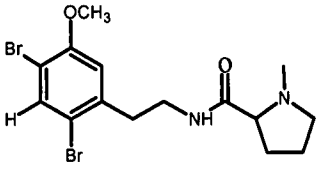
3 R = H, X = H
5 R = H, X = Br
7 R= H, X = H
4 R= H, X = H *cis* geometry
8 R= H, X= Br *cis* geometry



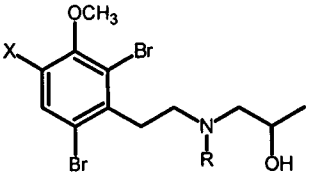
17 R = $(CH_2)_{12}CH_3$
18 R= $(CH_2)_7(CH)_2(CH_2)_5CH_3$
19 R=$(CH_2)_{14}CH_3$
20 R=$(CH_2)_7(CH)_2(CH_2)_7CH_3$
21 R=$(CH_2)_{16}CH_3$
22 R=$(CH_2)_6(CH)_2(CH_2)_{10}CH_3$



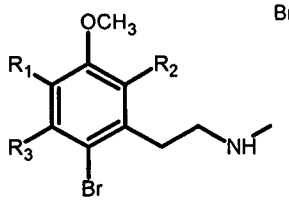
Amathamide G (**16**)



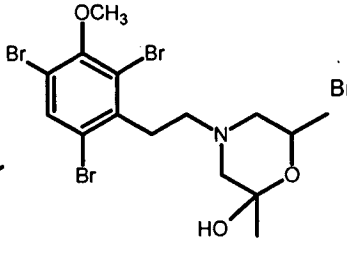
Amathamide E (**6**)



23 X = Br, R = CH_3
24 X = H R = CH_3
25 X = Br R = H



28 $R_1 = R_2 = Br$ $R_3 = H$
29 $R_1 = R_3 = H$ $R_2 = Br$
30 $R_1 = R_3 = Br$ $R_2 = OCH_3$



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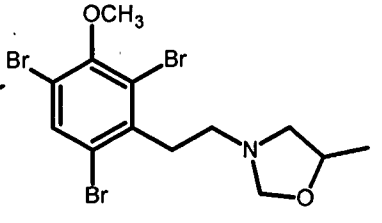


26

Amathamide G (**16**), a new member of amathamide series was isolated from another species *Amathia convoluta,* (Narkowicz et al., 2002) which also produces related metabolites that includes convolutamides A–F (**17-22**) (Zhang et al., 2002), convolutamines A–H (**23-30**) (Narkowicz, et al., 2002; Zhang et al., 1994b; Kamano et al., 1999), convolutamydines A–E (Kamano et al., 1999; Zhang et al., 1995; Kamano et al, 1995), convolutindole A, and volutamides A–E (Montanari et al., 1996). Interestingly, the species *Amathia convoluta* has been also shown to produce a chemically different class of compounds called bryostatins (Petit et al., 1985), although their production by *A. convoluta* cannot be confirmed due to the presence of another bryozoan *Bugula neritina* growing epiphytically on the sampled colonies.

The bryostatins are a group of compounds usually associated with *Bugula neritina* of which, bryostatin 1 (**1**) is at advance stage of clinical evaluation as anticancer drug. Another species in the same genus, *Amathia alternata*, is known to produce antibacterial brominated dipeptides alternamides A–D (Lee et al., 1997)

iii) *Ecology and biological activity of amathamides*

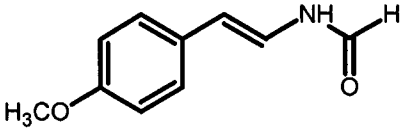
A. wilsoni has been the subject of extensive ecological studies. The amathamide content of *A. wilsoni* varies between sampling sites, but individual sites remain constant over the time, exhibiting no seasonal variation or individual colony variation (Blackman, and Green, 1987). In addition, the distribution of the amathamides within individual colonies decreases from the colony tip (9%) to the base, with the basal areas being totally devoid of metabolites (Walls et al., 1991). Although, the function of such a gradient is unknown, Walls et al., (1991) have suggested that new growth would be more susceptible to predation and larval settlement, therefore, enhanced chemical defence in these areas could reduce grazing and fouling, although high metabolite content in one area does not necessarily indicate that metabolites are produced in these areas. However, Walls et al., (1995) suggest that the surface-associated bacteria may be responsible for the production of the amathamides. High bromine levels on the bryozoan surface, but not in the cells, indicate that the amathamides are present on the surface but are not found in the cells. The surface bromine distribution correlates with the presence of surface bacteria, suggesting that production is associated with these bacteria. Although, in previous studies it had been reported that there were differing amounts of amathamides from *A. wilsoni* collected in different Tasmanian sites, none had recorded an absence of amathamides. However, a study of *A. wilsoni* from New Zealand reported the presence of amathaspiramides A–E **99-13**) (biogenetically modified amathamides) but no amathamides (Morris and Prinsep, 1999). The authors of this study suggest that the different chemical profiles reflect genetic variability or differing environmental conditions between the populations studied which contradicts the opinion of Narcowicz et al., (2002) who suggested that the difference in chemical profiles of the closely related species, *A. convoluta*, could be the result of bacterial symbionts

producing the products associated with the bryozoan. There is currently no conclusive evidence as to which of these hypotheses is most likely to be a true representation of the situation.

None of the amathamides have been reported for any biological activities, but extract containing amathamide G (**16**) has been shown to be nematocidal (Narcowicz et al., 2002). The predicted ecological functions of these amathamides as discussed above and biosynthetically related amathaspiramides found in the New Zealand *A. wilsoni*, of which A and E found to exhibit moderate cytotoxicity to cancer cell lines and also mild antimicrobial activity implies that amathamides could be potential antimicrobial agents.

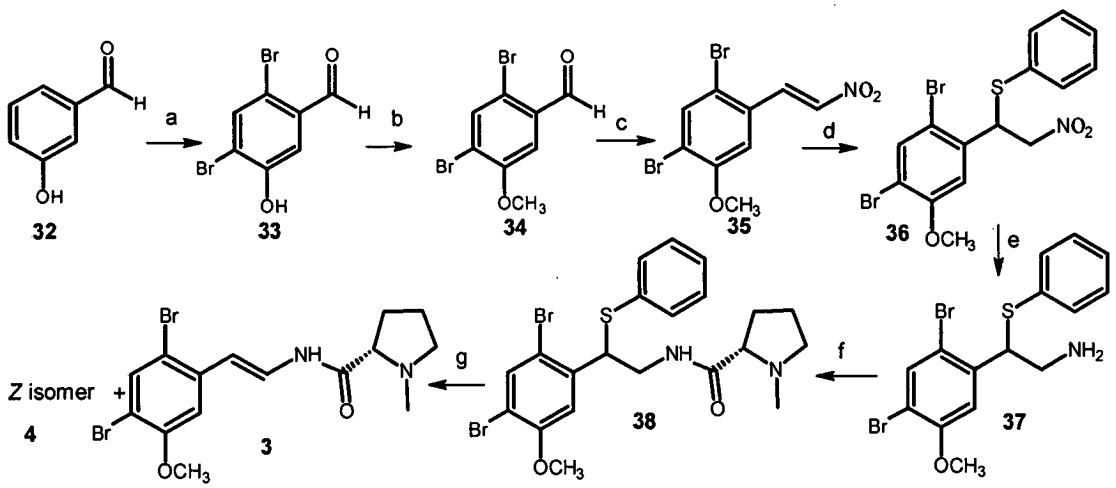
iv) Previous synthetic studies on amathamides

Amathamides are enamides of a styrylamine and proline derivative. Only one synthetic route is known towards the synthesis of amthamides. This method uses the methodology used for the synthesis of tuberine (**31**), an enamide of styrylamine and formic acid. Tuberine (**31**) was synthesized from a nitrostyrene prepared from 3-methoxybenzaldehyde (Somanathan et al., 1996). The ene functionality of nitrostyrene was protected using thiophenol and nitro group was reduced to amine with Zn/HCl. The resulting amine was coupled with acetic formic anhydride and thiophenol was removed by oxidation with $NaIO_4$ and elimination with a base to get **31**.



Tuberin (**31**)

Riding on the similar strategy Osuna et al., (2002) prepared amathamides A (**3**) and B (**4**) by coupling *N*-methylproline (**39**) with a suitable amine derivative as shown in **Scheme 4.1**.



***Reagents and conditions*:** (**a**) $CHCl_3$, Br_2, 92%; (**b**) CH_3I, DMF, K_2CO_3, 98%; (**c**) CH_3NO_2, AcOH, $AcONH_4$, 50%; (**d**) PhSH, Base, CH_2Cl_2, 95%; (**e**) SmI_2, THF, 65%; (**f**) DCC, DMAP, HOBT, **39**, 33%; (**g**) i. $NaIO_4$, MeOH; ii. K_2CO_3, Toluene, reflux, **3** 23%, **4** 1.15%.

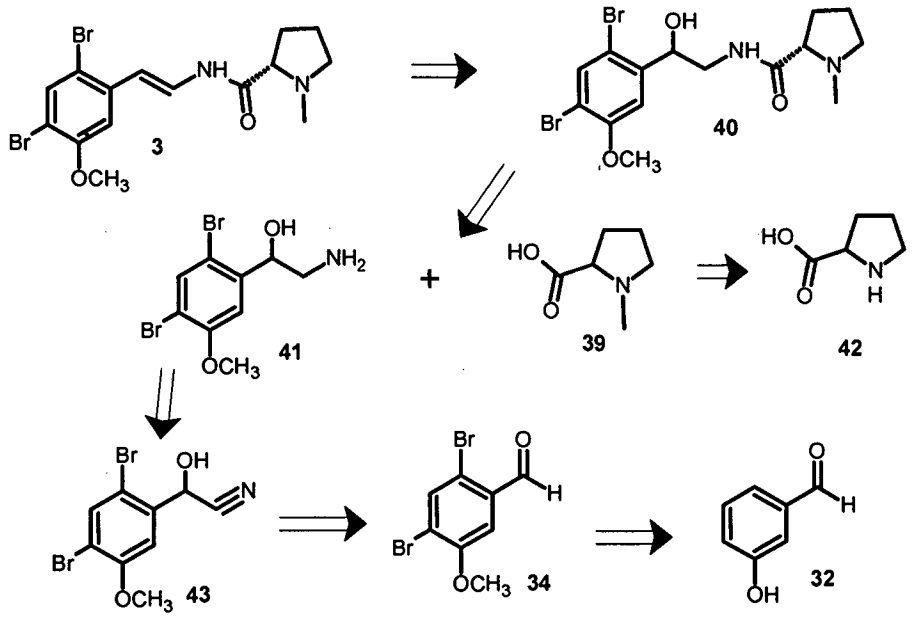
Scheme 4.1: Synthesis of amathamides A (3) and B (4)

II. Results and Discussion

i) Synthetic studies

Biological activity of amathamide alkaloids has not been well studied, but related metabolites have shown to possess nematocidal, antifungal, and antibacterial activity against few strains, which is discussed in our earlier sections. Their ecological roles suggest that these metabolites could be potentially antimicrobial. This prompted us in finding a new pathway for the synthesis of amathamides and their analogous for discovery of new antimicrobial agents.

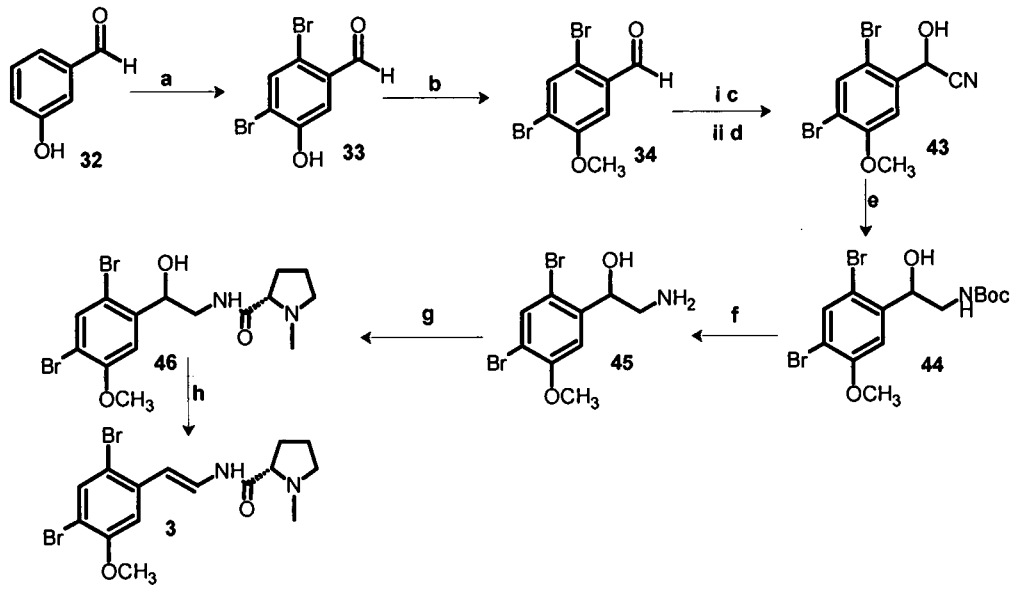
On retrosynthetic analysis, we considered the key precursor is to prepare amathamide alcohol, which can be eventually dehydrated to obtain amathamide A (**Scheme 4.2**). The key precursor thus can be obtained by coupling of 2-(2,4-dibromo-5-methoxyphenyl)-1-aminoethan-2-ol (**41**) (a β-amino alcohol) and *N*-methyl proline (**39**). The β-amino alcohol (**41**) can be prepared by reduction of a cyanohydrin (**43**) obtained from corresponding benzaldehyde (**34**).



Scheme 4.2: Retrosynthetic analysis of amathamide A (3)

Accordingly, the synthetic scheme was designed as shown **scheme 4.3**. Thus, 2,4-dibromo-5-methoxybenzaldehyde (**34**) was prepared in two steps from commercially available 3-hydroxybenzaldehyde (**32**). 3-hydroxybenzaldehyde (**32**) was dibrominated with two moles of bromine in acetic acid to give solid compound (**33**), in 91% yield which was methylated using methyl iodide and anhyd. K_2CO_3 in DMF to give **34**. Compound (**34**) was obtained as solid in 98% yield and has been characterized using spectroscopic methods.

Once we had in our hand, the required benzaldehyde derivative (**34**), the next task was to prepare cyanohydrin (**43**) from it. The simplest and primitive way of preparing cyanohydrin is to react an aldehyde with HCN. HCN is generated *in situ* from cyanide salt such as NaCN, KCN, etc by treatment with a mineral acid. It is not safe to use HCN or its salts owing to its toxic effects that are fatal. Moreover, the yields of these reactions are poor and also direct synthesis from aldehyde/ketone and HCN may be difficult owing to inherent thermodynamic instability of the aldehyde/ketone derivatives. Several alternatives to prepare cyanohydrins, which avoid using directly HCN or its salts, have been developed. They mainly involve process of trialkylcyanosilylation and then hydrolysis leading to cyanohydrin.



Reagents and conditions: (**a**) Br_2, $CHCl_3$, 3days, rt, stir, 93% (**b**) CH_3I, DMF, K_2CO_3, 4h, rt stir, 98% (**c**) Me_3SiCN, cat. LiCl/THF, rt, stir, 1h (**d**) 1N HCl/MeOH-aq, 1h. rt 96% (**e**) $NaBH_4$, cat $NiCl_2.H_2O$, Boc_2O, MeOH 0° C, 30 min and rt, 2 h., 78% (**f**) 6N, HCl, reflux 3h. 76% (**g**) **39**, oxyma, DIPC, DMF, rt, 30 min, 92% (**h**) CuCl-DCC, DMF, 24 h, 50° C.

Scheme 4.3: Synthesis of Amthamide A (3)

A process developed by Cabirol et al., (2008) uses NaCN and TMSCN to give cyanotrimethylsilylhydrins of ketones in 60-99% yield in 30 min at rt. Kim et al., (2004) has shown the preparation of cyanotrimethylsilylhydrins using *N*-methylmorpholine-*N*-oxide as catalyst in 91-99% yield in 5-20 h at rt, whereas Lewis acid catalysis using ZnI_2 in CH_2Cl_2 yields 90% cyanotrimethylsilylhydrins of benzophenone (Talley and Gassman, 1990). The above three procedures have not been used earlier for the preparation of cyanotrimethylsilylhydrins of aldehydes.

Even though, ZnI_2 was reported to be the catalyst for cyanosilyaltion for ketones (Talley and Gassman 1990), we used the same to prepare cyanohydrins of aromatic benzaldehydes, assuming that aldehydes being more reactive than ketones should also easily react, but to our disappointment we recovered our starting material **34** after the reaction. Therefore, we thought of using a method specifically used to prepare cyanotrimethylsilylhydrins of aldehydes.

There are several procedures for preparations of cyanotrimethylsilylhydrins of aldehydes and some are preferably used to obtain the product in enantiopure form. Those used for chiral synthesis include dinuclear chiral (Salen) titanium complex

catalysis (Belokon et al., 2000), chiral oxazaborolidinium ion catalysis (Ryu and Corey, 2004), $(DHQ)_2PHAL$ catalysis (Denmark and Chung, 2006) and vanadium(V) salen complex catalysis (Khan et al., 2006).

There are at least four methods in the literature used to prepare cyanotrimethylsilylhydrins in racemic form. They are

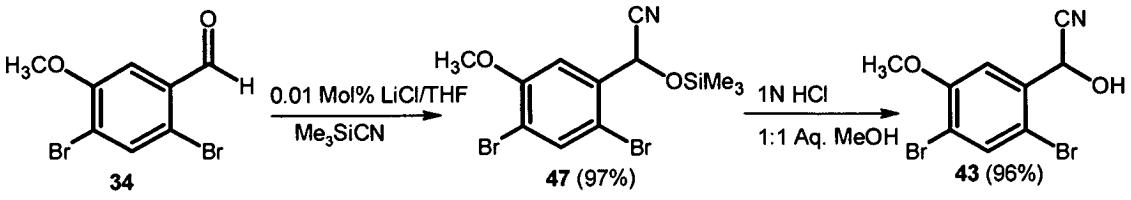
1. A method developed by Suzuki et al., (2006) gives over 93% of yield in 30-180 min catalyzed by nitrogen-heterocyclic carbenes (NHCs) in THF. If chiral NHCs are used then optically pure compounds can also be obtained.

2. A Lewis base catalysis using triethylamine (TEA) or *N,N*-(dimethylamino)pyridine (DMAP) in acetonitrile have also been used to obtain cyanotrimethylsilylhydrins. Yields of >90% have been achieved on aromatic aldehydes using DMAP (Denmark and Chung, 2006)

3. Another method makes use of NbF_5 catalysis in solvent free condition (Kim and Rajgopal, 2007). Yields of 80-99% can be obtained in 10-30 min at rt depending upon the type of aldehyde.

4. Yet another method to obtain cyanotrimethylsilylhydrins is by using LiCl catalysis in solvent free condition at room temperature (Kurono et al., 2005). The reaction is completed in 10-30 min at rt with quantitative yields.

NHCs and NbF_5 were not readily available to us, so we did not opt for methods 1 and 3. Thus, the reaction was carried out using DMAP as per the reported method 2, but again no conversion of 2,4-dibrom-5-methoxybenzaldehyde was observed. Therefore, we opted for 4^{th} method *i.e.* LiCl catalysis. The reaction in presence of 0.01 mol % of LiCl in solvent free condition showed complete conversion of starting material in 15 min, monitored by TLC. After stirring the reaction mixture for another half an hour it was concentrated under vacuum to remove any traces of TMSCN and THF left in the reaction mixture to obtain 2-(2,4-dibromo-5-methoxyphenyl)-2-trimethylsilyloxyacetonitrile (**47**) in 97% yield. This product without characterization was used for further reaction, but a similar compound 2-(2-bromo-5-methoxyphenyl)-2-trimethylsilyloxyacetonitrile (**50**) has been isolated and characterized with NMR data.



Scheme 4.4: Preparation of a cyanohydrins through cyanosilylation of an aldehyde

Compound **47** was then hydrolysed to give corresponding cyanohydrin, 2-(2,4-dibromo-5-methoxyphenyl)-2-hydroxyacetonitrile (**43**) in 1N HCl in aqueous MeOH (**Scheme 4.4**). The product was extracted in $CHCl_3$ to give buff colour solid in 96% yield. In its IR spectrum it showed a very weak absorption for cyano at 2250 cm^{-1}, and strong absorption for hydroxyl at 3350 cm^{-1}. No Peak in the aldehydic region was observed. Usually, cyano compounds show very strong absorption in IR for cyanide stretching, but it has been observed that certain cyanohydrins either show very weak or no absorption in IR for cyano group.

In NMR spectra (**Fig 4.1a** and **b**) it showed two aromatic methines at δ_H 7.76 (s, 1H) δ_C 136.7 (d, C-5) and δ_H 7.23 (s, 1H) and δ_C 111.2 (d, C-8). The cyanide group was evident form a singlet at δ_C 117.6 (C-1) and benzyllic hydroxymethine from the signal at δ_H 5.76 (br s, 1H), 3.67 (br s, OH) and δ_C 62.6, (d, C-2).

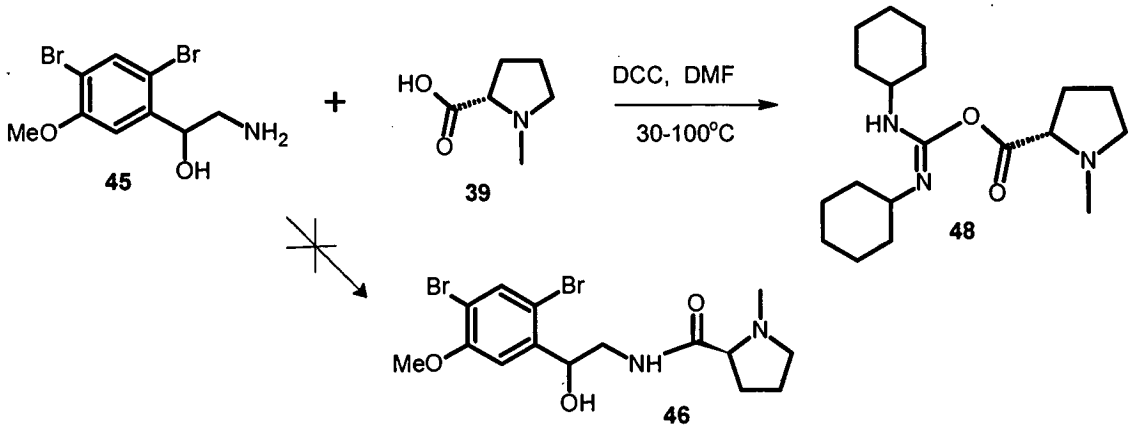
The next step in the scheme was to reduce cyanohydrin to β-amino alcohol without hydrogenolysis of benzylic hydroxyl. In reduction of nitriles, the yields are low due to formation of some unwanted dimeric products resulting from intermediates. Through literature survey we found that $NiCl_2$ catalysis of sodium borohydride reduction is one of the best ways of selectively reducing nitriles to *N*-protected amines (Caddick et al., 2003). Only catalytic amount of $NiCl_2$ is necessary to carry out the reaction and the active species is $Ni(BH_4)_2$. Accordingly, the reaction was carried out on **43** with Boc_2O as protecting agent. At the end of the reaction the solvent (MeOH) was evaporated under vacuum, the product was extracted in EtOAc and washed with sat. bicarbonate solution. After evaporation of EtOAc, a viscous liquid compound (**44**) was obtained in 76% yield, which was characterized using NMR (**Fig 4.2a** and **b**). Proton NMR showed signals at δ_H 5.03, br t for benzylic hydroxyl methine (C-3) coupled to a vinylic methylene at δ_H 3.45, ddd (α-HC-2, J = 15.0, 6.1, 1.8 Hz) and

3.36, m (β-HC-2). The three equivalent methyls of Boc group were observed at δ_H 1.45, s.

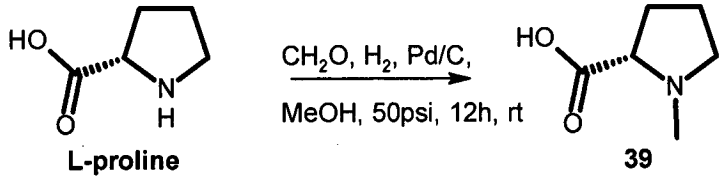
The next task was to deprotect the amine (**43**) by removing Boc group, which was done by refluxing the protected amine in 6N HCl for 3 h. On completion of reaction, fine drops of *tert*-butanol were seen floating on reaction mixture, which were extracted in ethyl acetate. Aqueous portion was basified with dil. NaOH and the free amine was extracted in $CHCl_3$. After evaporation of the solvent the amine, 2-(2,4-dibromo-5-methoxyphenyl)-1-aminoethan-2-ol (**45**) was obtained as brown solid in 63% yield. In NMR (**Fig 4.3a** and **b**) spectra it showed pattern of signals similar to **43** except that the signals for Boc group disappeared.

The next task was to prepare amathamide A alcohol (**46**), the immediate precursor to amathamide A, which requires formation of amide bond between the amine (**45**) and L-*N*-methyl proline (**39**).

Formation of amide bond by coupling an amine with a carboxylic acid is one of the most important processes in organic chemistry and the same is required in syntheses of several biologically important peptides. A lot of attention has been given to the development of efficient coupling reagents that give high yields in less reaction time with prevention of racemization. For the formation amide bond, a carboxyl group needs to be activated to react with an amine nucleophile. The simplest way in which carboxylic acid is converted into an amide is by converting carboxylic acid into a more reactive acyl chloride and reacting it with an amine. But this methodology has limitations due to several reasons such as high reactivity and non-selectivity of acyl chlorides towards nucleophiles. Therefore, one of the most preferred strategies is carbodiimide approach of activation of carboxyl in amide formation. The carbodiimide alone either gives poor yields or no yields, as also observed in our coupling reaction of **45** with (S)-*N*-methyl-L-proline **(39) (Scheme 4.5).** *N*-methyl L-proline (**39**)was prepared by reacting L-proline with aq. formaldehyde and reducing it *in situ* with palladized charcoal in MeOH in quantitative yield as shown in **scheme 4.6** (Han et al., 2005).



Scheme 4.5: Coupling of an amine with *N*-methyl proline using DCC

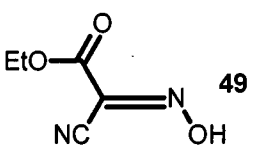


Scheme 4.6: Synthesis of *N*-methyl-L-proline

The reaction of an amine (**45**) with proline derivative (**39**) using dicyclohexyl carbodiimide (DCC) saw no attack of **45** on the activated adduct (**48**) (**Scheme 4.5**). After the reaction, the adduct **48** was isolated and characterized by NMR spectroscopy. In ESI-MS, it showed pseudomolecular ions $[M+H]^+$ at m/z 336 $[M+Na]^+$ at m/z at 358 $[M+K]^+$ at m/z 374. Its ^{13}C NMR showed a signal at δ_C 173 for ester carbonyl and showed a signal at δ_C 153 for the sp2 carbon attached to the two nitrogen atoms and an oxygen atom. The signals for the other thirteen carbon atoms were observed in the region δ_C 20-70 accounting for two non equivalent cyclohexane rings and *N*-methyl proline ring.

Poor yield or no reaction are not the only problem with carbodiimide approach, but it also bring racemization, hence an additive, which can enhance yield and suppress racemization is also used in the coupling. Of these compounds, the most intensively used display a benzotriazole core: 1-hydroxybenzotriazole (HOBt), probably the most common reagent found in a peptide synthesis laboratory, whereas later the use of the

more powerful 1-hydroxy-7-azabenzotriazole (HOAt) (Carpino, 1993) and, more recently, 6-chloro-1-hydroxybenzotriazole (6-Cl-HOBt) has been reported (Azev et al., 1976). Other coupling strategies, such as the combination of base and stand-alone coupling reagents, such as immonium (HATU, HBTU/TBTU, and HCTU/TCTU) (El-Faham and Albericio 2008), or phosphonium salts (PyAOP, PyBOP, and PyClock) have also been enhanced by the use of these additives. However, a report of a potentially explosive character of HOBt and its related additives have recently led to the finding of an alternative reagents, such as oxyma (**49**) (Funosas et al., 2009) and uronium salts (the adducts of oxyma/isonitrosoMeldrum's acid and morpholonium-based immonium moiety) such as COMU and HTMU as a superior and safer coupling reagent for amide formation (El-Faham et al., 2010).



49

The reported synthesis of **3** **(Scheme 4.1)** makes use of DCC/HOBt/DMAP for coupling of their amine with **39**, which gave them only 33% yield of coupling product (**38**) after 18 h. Considering the low yield of coupling using HOBt and also due to its explosive nature we opted for **49** as a coupling reagent. In contrast to the reported reaction, the coupling between **45** with **39** using DIPC/oxyma fetched us the amide (amathamide A alcohol) (**46**) in 92% yield in just 1 h. The amide from the reaction mixture was isolated by washing the acidified reaction mixture with EtOAc. The acidic aqueous part was then basified with dil NaOH and extracted in EtOAc. Upon evaporation of EtOAc, **46** was obtained as colourless crystalline needles. The compound has been characterized using spectroscopic methods.

Table 4.1 : NMR data of amathamide alcohols 46and 60

Position	46		60	
	δ_C, mult	δ_H, mult	δ_C, mult	δ_H, mult., J(Hz)
2	56.46 t (56.42)	3.05 m, 2.17 m	54.6 t	
3	24.3 t (24.2)	1.75 m, 1.57 m	20.0 t	
4	31.1 t (31.0)	2.17 m, 1.76 m	28.8 t (28.9)	
5	68.5 d	2.95 m	67.1	
6	178.0 s (177.9)		174.0 s (173.9)	
8	41.6 t (41.7)	3.64 m, 3.57 m	42.7 t (42.4)	3.43, s
9	73.9 d (77.9)	5. 07 br s	69.3 d (69.2)	
10	141.5 s (141.4)		140.7 s (140.6)	7.54, d, (8.7)
11	111.0 s		110.2 s (110.1)	6.85, d, (8.7)
12	136.1d (135.9)	7.72, s	131.4 d	
13	111.4 s		112.0 d (111.9)	
14	155.5 s (155.3)		157.9 s (157.8)	6.40, d (1.8)
15	111.3 d	7.23 s	113.1 d (112.9)	
16	45.9 q, (45.8)	2.38 s	39.7 q	
17	56.6 q (56.5)	3.89 s	53.0 q	6.49, d, (8.1)

* The values given parenthesis are observed due C-9 epimer

The ESI-TOF-MS (**Fig 4.4d**) of **46** showed the presence of the required product containing two bromine atoms by giving triplets of pseudomolecular ions $[M+H]^+$ at m/z at 435/437/437 and $[M+Na]^+$ 457/459/461, corresponding to average molecular weight of 436 as required for **3**. In the MS/MS spectrum (**Fig 4.4e**) the base peak occurred at *m/z* 84, which is characteristic of *N*-methylpyrrolidines, also observed for natural amathamides (Blackman and Green, 1985). Its IR spectrum (**Fig 4.4a**) showed conjugated amide carbonyl absorption at 1666 cm^{-1}, a hydroxyl and amide N-H absorption centred at 3340 cm^{-1} and various C-H str peaks in the region 2790-3050 cm^{-1}. In ^1H NMR (**Fig 4.4b**) it showed peaks in the region for two aromatic protons as singlets at δ_H 7.72 and 7.23. A benzylic hydroxymethine at δ_H 5.07 (br s); methoxy at δ_H 3.89, s; and two multiplets of diastereotopic protons of methylene at δ_H 3.64 and 3.57 for the amine part of the compound. The signals for proline part were observed as δ_H 3.04 (m, 1H), 2.92, (m, 1H), 2.35 (s, 3H), 2.44 (m 2H), 1.76 (m, 2H) and 1.57 (m, 1H). The ^{13}C NMR (**Fig 4.4c**) spectrum showed either peaks in duplicate or larger peaks (merging of two peaks) indicating it to be a mixture of epimers at C-9. The amino alcohol (**45**) used in the reaction was racemic, while the *L*-proline

derivative (**39**) used was chiral, therefore the reaction between them produced a mixture of epimers (at C-9) of **46**. The formation of mixture of epimers is not concern to us as the epimeric chiral centre (C-9) would be destroyed in the dehydration step leading to single stereoisomer. The NMR data of **46** and **60** are tabulated in **table no. 4.1**

The final step in our synthesis was to dehydrate **46** to give **3**. In the literature, methods of eliminations of alcohols using carbamates (Atkinson et al., 1981), acetates (DePuy and King, 1960) and xanthates (O'Connor and Nace, 1953) are reported. These methods give good yield, but require high temperatures and long reaction times. A pseudourea-mediated dehydration of tertiary and benzylic alcohols using CuCl-DCC reported by Majetich et al., (1998) requires comparatively lower temperatures. We chose this method for dehydration of our compound **46**. Accordingly, the reaction was carried out, and although, disappearance of the **46** was observed as monitored by TLC, to our disappointment mixture of products, inseparable from by-product dicyclohexyl urea (DCU) was obtained. The formation of product is inferred from the triplet (due to two bromine atoms) of the pseudomolecular ions observed at $[M+H]^+$ m/z at 417/419/421 in the ratio 1:2:1 in its ESI-MS(**Fig 4.5a**). The ^1H NMR(**Fig 4.5b**) spectrum showed multiple signals due to the mixture of compounds dominated by the signal in the region δ_H 1-2 due cyclohexyls of DCC.

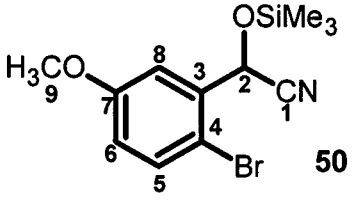
The effort to dehydrate the 46 by replacing DCC with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) is in progress (Sai et al., 2003). The by-product 1-ethyl-3-(3-dimethylaminopropyl) urea formed from EDC is unlike DCU is fairly soluble in water, thus can be easily separated from the main product.

Once we had achieved the synthesis of key intermediate amathamide A alcohol (**46**), the next task was to synthesize its analogues for screening against human pathogens for antimicrobial activity. In a time period, we were able to prepare three more analogous amathamide alcohols, **55**, **60** and **65** from three different stating materials, 3-methoxybenzaldehyde (**51**), 2-bromo-4-methoxybenzaldehyde (**56**) and 3-bromo-4-methoxybenzaldehyde (**61**) using methodology followed for amathamide A alcohol

(**46**). The yields of each step in the synthesis of the different analogues were comparable to the corresponding synthetic step of **3**.

3-Methoxybenzaldehyde (**51**) was prepared by methylation using CH_3I/K_2CO_3 in acetone in 98% yield. 2-bromo-5-methoxybenzaldehyde (**56**) was made available by bromination of **51** using Br_2 and NaOAc in AcOH, and in same way 3-bromo-4-methoxybenzaldehyde (**61**) was prepared from commercially available *p*-anisaldehyde. Benzaldehydes **51**, **56** and **61** were used to synthesize the amathamide alcohols **55**, **60** and **65** respectively. The structures of products of each step and their yields are tabulated in **table 4.2**

In the synthesis of amathamide alcohol (**60**) the intermediate silyloxy cyanohydrin compound (**50**) prepared from 2-bromo-5-methoxybenzaldydehyde (**50**) was the only compound in its series which was characterized using NMR data while all other derivatives were used for further reaction without purification. In IR spectrum, **50** showed no signal for any aldehydic carbonyl which confirms the conversion of aldehyde, but also no signal for a cyanide group was observed. It has been observed that in all our cyanohydrins cyanide stretching is either weak or not observed. The NMR (**Fig 4.9**) spectrum of **50** showed the benzylic methine at δ_H 5.79, s, δ_C 63.1, d cyano carbon signal at δ_C 11.8.9, s a highly deshielded methyls were observed at δ_H 0.27, δ_C –0.34.



50

Table 4.2: Products of each synthetic step of amathamide alcohol and their yields

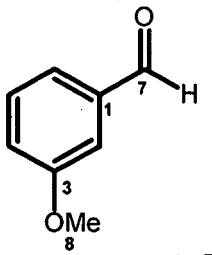
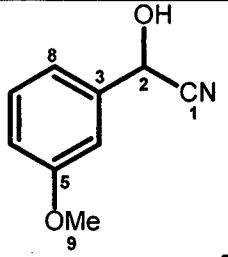
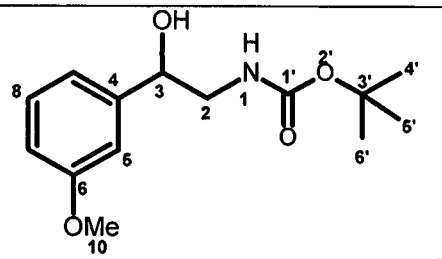
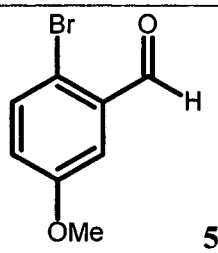
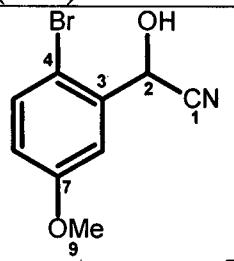
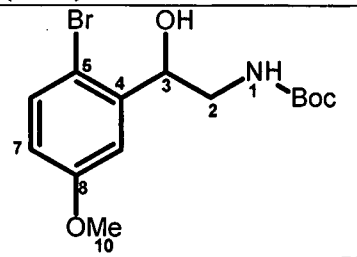
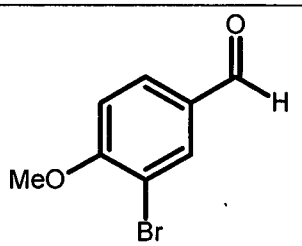
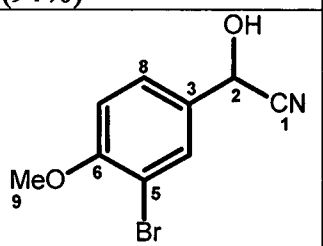
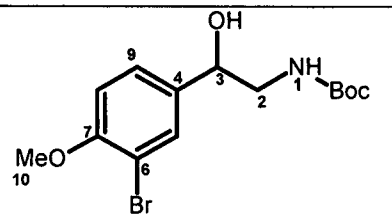
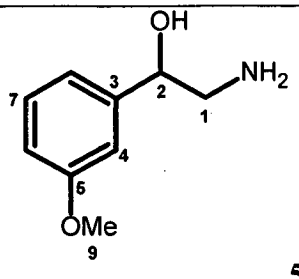
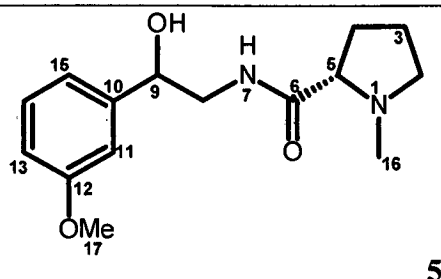
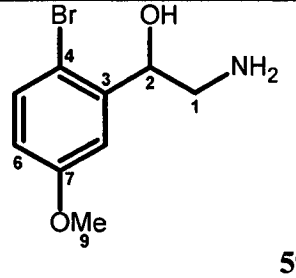
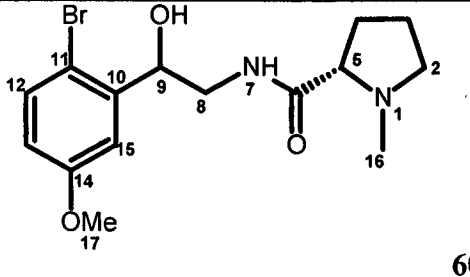
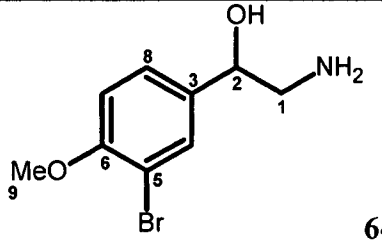
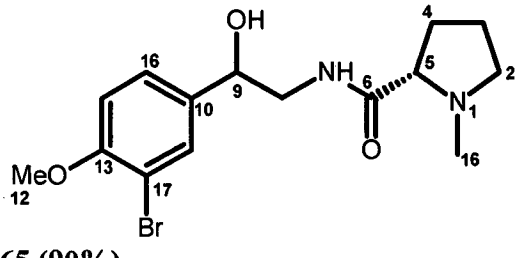
Sr.No.	Aldehyde	Cyanohydrin	*N*-Boc amino alcohol
1	 **51**	 **52** (93%)	 **53** (71%)
2	 **56**	 **57** (94%)	 **58 (69%)**
3	 **61**	 **62 (95%)**	 **63 (72%)**

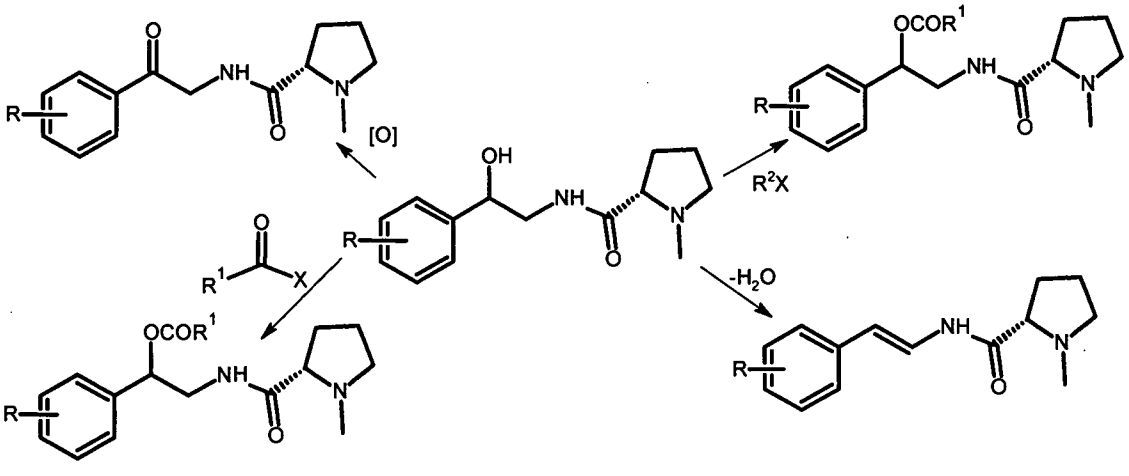
Table 4.2 continues..

Sr. No	Amino alcohol	Amathamide alcohol
1.	 **54 (62%)**	 **55 (85%)**
2.	 **59 (82%)**	 **60 (89%)**
3.	 **64 (60%)**	 **65 (90%)**

Our method towards the synthesis of amathamides is second only to Osuna et al., (2002). A comparison drawn between our synthetic method (**scheme 4.3**) and the reported method (**scheme 4.1**) indicates that our methodology of preparing amathamides through cyanohydrins instead of nitrostyrenes requires a step less, as our route do not require a step to protect the styryl double bond prior to reduction. Moreover, the yield of key step of our method that is preparation of cyanohydrins in were between 92-98%, whereas the yield of corresponding step of preparation of nitro styrene in literature method are 44-50%. An important step in the both the synthetic method is coupling of an amine and proline derivative. The DCC/HOBt

coupling methodology has given Osuna et al., (2002) the yieds of 30-33%, while the use of oxyma/DIPC in similar coupling by us has yielded of 88-95% of amides.

Additionally, our synthetic scheme of amathamides through amathamide alcohols represents diversity oriented approach towards amathamides and their analogues as depicted in the scheme below (**scheme 4.7**). The amathamide alcohols in addition to precursor to amathamides also can be treated with various carboxylic acids/alkyl halides to obtain esters/ethers or can be oxidized to ketones, which themselves can give several more derivatives.



Scheme 4.7: Diversity oriented synthesis of amathamide alcohols

ii) Biological activity

Our main aim was to discover potent antimicrobial agents through synthesis of various analogues of amathamides. It has been discussed in one of the above sections that amathamides could be potential antibacterial agent. The four syntheisized amathamide alcohols were tested against 14 clinical pathogens. The pathogens include 9 bacterials strains of which two were multidrug resistant strains and five fungal strains. The name of the strains and disease caused by them is listed **table 4.3**. Unfortunately, none of our synthesized amathamide alcohols (**46**, **55**, **60**, and **65**) showed activity at concentration of 100 μg/disc in a disc diffusion assay.

Table 4.3: details of pathogens used ion antimicrobial activity

Name of the pathogens	Disease caused	Code
Bacterial Strains		
Escherichia coli	Neonative meningitis	B1
Pseudomonas aeruginosa	Urinary tract infection	B2
Staphylococcus aureus	Skin infection	B3
Salmonella typhi	Typhoid	B4
Shigella flexineri	Gastrointestineal infection	B5
Klebsiella sp	Urinary tract infection	B6
Vibrio cholera	Cholera	B7
Multi Drug Resistant Strains		
Acinetobacter sp.	Urinary tract infection	D1
Methicillin resistant staphylococcus aureus (MSRA)	Skin infection	D2
Fungal Strains		
Aspergillus fumigates	Skin infection	F1
Rhodotorulla sp	Skin infection	F2
Candida albicans	Candiasis	F3
Aspergillus niger	Skin infection	F4
Cryptococcus neoformans	Skin infection	F5

III. Experimental

i) Bromination of aromatic aldehydes

a) 2-Bromo-5-methoxybenzaldehyde (56)

To a solution of 3-methoxybenzaldehyde (2g, 14.7 mmol), and NaOAc (2.4g, 17.6 mmol) in AcOH (20 ml) was added Br_2 (0.91ml, 17.6 mmol) in AcOH (5 ml) at 0-5°C over 30 min. The reaction mixture was stirred at room temperature for a day. Reaction was quenched by addition of saturated $NaHSO_3$ to decompose excess Br_2. Further reaction mixture was diluted with chilled DW (60 ml) and extracted in $CHCl_3$ (30 ml x 3). $CHCl_3$ layer was washed with DW, dried over anhyd Na_2SO_4 and solvent evaporated to get crude solid mixture. Crude mixture was purified over Si gel using EtOAc-petroleum ether (2%-10% v/v) as mobile phase. Elution with 2% EtOAc in PE gave **56** (1.8g, 58%) and 4% EtOAc in PE gave 4-bromo-3-methoxybenzaldehyde (0.82g, 26%).

2-Bromo-5-methoxybenzaldehyde (**56**) crystalline solid; IR (KBr) 3074, 3006, 2979, 2943, 2875, 2844, 2746, 1681, 1639, 1596, 1571, 1463, 1244, 1197, 1058, 933, 821, 867, 821, 755 cm^{-1}.

b) 2,4-Dibromo-5-hydroxybenzaldehyde (33)

To a solution of 3-hydroxybenzaldehyde (40.9 mmol, 5.00g) in $CHCl_3$ (60ml) was added solution of bromine (81.7mmol, 4.2ml) in $CHCl_3$ (20 ml) over 30 min at 0-5°C. The resulting solution was stirred at rt for 3 days. The excess bromine was removed by addition of a chilled saturated solution of sodium sulphite (20 ml). Chilled DW (50 ml) was added to the reaction mixture and the organic phase was separated. The aqueous portion was washed with $CHCl_3$ (30 ml x 2) and the combined organic phase was washed with water and dried over anhyd. Na_2SO_4. Removal of solvent gave a solid (**33**) which was recrystallized from hexane/$CHCl_3$ to give (10.3 g, 91%) light brown solid; mp 134–5°C; IR (KBr) 3379, 3072, 2896, 1687, 1531, 1440, 1292, 1176, 1002, 968, 866, 792 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ_H 10.23 (s, 1H, H-7), 7.80 (s, 1H, H-3), 6.33 (s, 1H, H-6) ppm; MS (EI) *m/z* (%) 278 (M^+, 15), 63 (100).

c) 3-Bromo-4-methoxybenzaldehyde (61)

To a solution of 4-methoxybenzaldehyde (1.0 g, 7.35 mmol), and NaOAc (1.2g 8.8 mmol) in AcOH (15 ml) was added Br_2 (0.46 ml, 8.8 mmol) in AcOH (5 ml) at 0-5°C over 30 min. The reaction mixture was stirred at room temperature for a day. Reaction was quenched by addition of saturated $NaHSO_3$ to decompose excess Br_2. reaction mixture was diluted with chilled DW (100 ml) and extracted in $CHCl_3$ (30 ml x 3). $CHCl_3$ layer was washed with DW, dried over anhyd. Na_2SO_4 and solvent evaporated to get orange solid, which was recrystallized from MeOH-water to give light brown needles (1.40g 89%). ^1H NMR (300 MHz, $CDCl_3$) δ_H 9.81 (s, 1H, H-7), 8.09 (d, J = 1.9, 1H, H-2), 7.83 (d, J = 8.4, 1.9, 1H, H-6), 7.02 (d, J = 8.4, 1H, H-5), 3.99 (s, 1H, H-9) ppm;

ii) General procedure for methylation of phenols

To a solution of phenol (1 mmol) and K_2CO_3 (0.165g, 1.2 mmol) in dried DMF (5 ml) or dried acetone (5 ml) was added methyl iodide (1.1 mmol, 0.068 ml) and the mixture was stirred for 4-6 h at rt. The reaction mixture was quenched with water (50ml) and the ppt/emulsion was either filtered or extracted in $CHCl_3$ (30 ml x 3). After extraction, solvent was evaporated to obtain methoxy derivative in pure form.

a) 3-Methoxybenzaldehyde (51)

Reactants and reagents used were 3-hydroxybenzaldehyde (6.0g, 49.18 mmol), K_2CO_3 (8.15g, 59.01 mmol), acetone (150 ml). **51** was obtained as yellowish liquid, yield (6.28, 94%).; IR (KBr) 3066, 3004, 2960, 2837, 2731, 1701, 1683, 1593, 1558, 1490, 1458, 1263, 1047, 792, 738 cm^{-1}. ^1H NMR (300 MHz, $CDCl_3$) δ_H 7.16 (m, , 1H), 6.90 (br s, 7.8 Hz, 1H) 6.81 (dd, J =7.8Hz, 2.1 Hz) 3.67, (s, 3H).

c) 2,4-Dibromo-5-methoxybenzaldehyde (34)

Reactants and reagents used were **33** (1.0 g, 3.40 mmol), K_2CO_3 (0.96 g), DMF (40 ml). **34** was obtained as crystalline needles, light brown yield (0.79, 96%), mp 110-111°C,; IR (KBr) 3010, 2993, 1679, 1575cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) 10.25 (s, 1H), 7.85 (s, 1H), 7.40 (s, 1H), 3.95 (s, 3H).

iii) General procedure for cyanotrimethylsilylation of aldehydes

LiCl (130.3 mg, 3.07 mmol) and THF (10 ml) were placed in a round bottom flask, sonicated for 10 min and this mixture was used as a catalyst stock solution. An aldehyde (100.0 mmol) and $(CH_3)_3SiCN$ (115 mmol) were placed in a 50 ml two necked flask, and the mixture was stirred at 20° C. The catalyst solution (33 µl, 10.1 µmol) was added to the mixture, and the reaction proceeded exothermically, which was kept in control by keeping reaction mixture under water bath. Reaction mixture stirred for 1-2 h and traces of THF and excess TMSCN was removed under vacuum to obtain trimethylsilyl cyanohydrin.

Caution: TMSCN must be used in a well-ventilated hood due to its high toxicity.

a) 2-(3-methoxyphenyl)-2-trimethylsilyloxyacetonitrile

Reagents and reactants used were **51** (500 mg, 3.67 mmol), TMSCN (539.28 µl, 4.04 mmol) and catalyst solution (1.19 µl, 0.37 µmol). This product was obtained as thick liquid (846.69 mg 98%) and used for further reaction without characterization.

b) 2-(2-bromo-5-methoxyphenyl)-2-trimethylsilyloxyacetonitrile (50)

Reagents and reactants used were **56** (500 mg, 2.32 mmol), TMSCN (341µl, 2.55 mmol) and catalyst solution (0.755µl, 0.23µmol); **50** was obtained as brownish thick liquid (715.63 mg, 98%); IR (KBr) 3004, 2960, 2902, 2839, 1595, 1575, 1473, 1257, 1163, 1099, 956, 867, 756 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$, δ_H ppm) 7.46 (d, J = 8.7Hz, 1H), 7.26, (br s, 1H), 6.83 (d, J = 8.7Hz, 1H), 3.84 (s, 3H). 0.20 (s, 9H), ^{13}C NMR (75 MHz, $CDCl_3$, δ_C ppm) 159.4, 136.2, 133.5, 118.1, 116.8,113.8, 111.6, 63.1, 55.5, -0.34.

c) 2-(2,4-dibromo-5-methoxyphenyl)-2-trimethylsilyloxyacetonitrile (47)

Reagents and reactants used were **34** (1.0 g, 3.4 mmol), TMSCN(0.499 ml, 3.74 mmol) and catalyst solution (1.11 µl 0.34 µmol). This product was obtained as thick liquid (1.30 g, 69 mg 97%) and used for further reaction without characterization.

d) 2-(3-bromo-4-methoxyphenyl)-2-trimethylsilyloxyacetonitrile

Reagents and reactants used were **61** (500 mg, 2.32 mmol), TMSCN (341µl, 2.55 mmol) and catalyst solution (0.755µl, 0.23µmol). This product was obtained as thick liquid (716.03 mg, 97%) and used for further reaction without characterization.

iv) *General procedure to prepare cyanohydrins by hydrolysis of trimethylsilyl cyanohydrins*

To a trimethylsilyl cyanohydrin (1 mmol) was added solution of hydrochloric acid (0.5 mmol) in aq-MeOH (1:1 v/v, 5 ml) and stirred at rt for 1-2 h. Reaction mixture was concentrated under vacuum to remove MeOH. DW (5 ml) was added to the reaction mixture and extracted with $CHCl_3$ (3 ml x 3) The organic layer was washed with DW, dried over anhyd Na_2SO_4 and evaporated under vacuum to get cyanohydrin

a) 2-(3-methoxyphenyl)-2-hydroxyacetonitrile (52)

Reagents and reactants used were *2-(3-methoxyphenyl)-2-trimethylsilyloxyacetonitrile* (864 mg, 3.67 mmol), 0.2 N HCl in aq-MeOH (20 ml, 1:1 v/v); product was obtained as reddish-brown liquid, (557.36 mg, 93%), ^{1}H NMR (300 MHz, $CDCl_3$, δ ppm) 7.16 (dd, J = 8.1, 7.9 Hz, 1H), 6.94, (brd, J = 7.9, 1H), 6.90 (br s, 1H), 6.81 (dd, J = 8.1, 2.1Hz, 1H). 5.33 (s, 1H), 4.38 (brs, OH), 3.67 (s, 3H),

b) 2-(2-bromo-5-methoxyphenyl)-2-hydroxyacetonitrile (57)

Reagents and reactants used were **50** (730 mg, 2.32 mmol), 0.2 N HCl in aq-MeOH (13 ml, 1:1 v/v) low melting colourless solid (528.25 mg, 94%), IR (KBr) 3400, 3014, 2939, 2906, 2839, 2250, 1595, 1475, 1288, 1163, 1053, 956, 860, 813 , ^{1}H NMR (300 MHz, $CDCl_3$, δ ppm) 7.39 (d, J = 9 Hz, 1H, H-5), 7.16, (br s, 1H, H-8), 6.75 (dd, J = 9.0, 3.0Hz, 1H, H-6), 5.70 (d, J = 6.0 Hz, 1H, H-2), 3.74 (s, 1H); ^{13}C NMR (75 MHz, $CDCl_3$, δ ppm), 159.4 (s, C-7), 135.2 (s, C-3), 133.8 (d, C-5), 118.0 (s, C-1), 117.1 (d, C-6 or C-8) 113 (d, C-6 or C-8), 112 (s, C-4), 62.8 (d, C-2), 55.8 (q, C-9).

c) 2-(2,4-dibromo-5-methoxyphenyl)-2-hydroxyacetonitrile (43)

Reagents and reactants used were **47** (1.34g, 3.40 mmol), HCl in aq-MeOH (18.5 ml, 1:1, v/v), product was obtained as low solid (1.04 g, 95%) IR (KBr) 3400, 3016, 2942, 2829, 2250, 1597, 1475, 1167, 1053, 956, 864, 813; ^{1}H NMR (300 MHz, $CDCl_3$, δ ppm) 7.86 (s, 1H, H-5), 7.24, (s, 1H, H-8), 5.70 (br s, 1H, H-2), 3.93 (s, 1H); ^{13}C NMR (75 MHz, $CDCl_3$, δ ppm), 156.1 (s, C-7), 136.7 (s, C-5), 134.4 (d, C-3), 1187.6 (s, C-1), 114.0 (s, C-4 or C-6) 112.2 (s, C-4 or C-6), 111.2 (d, C-8), 62.6 (d, C-2), 56.6 (q, C-9).

d) 2-(3-bromo-4-methoxyphenyl) -2-hydroxyacetonitrile (62)

Reagents and reactants used were 2-(3-bromo-4-methoxyphenyl)-2-trimethylsilyloxyacetonitrile (730 mg, 2.32 mmol), 0.2N HCl in aq-MeOH (13 ml). Product was obtained as low melting colourless solid (534.48 mg, 95%); ^{1}H NMR (300 MHz, $CDCl_3$, δ ppm) 7.51 (d, *J* = 2.1 Hz, 1H, C-4), 7.24, (dd, J = 8.7, 2.1 Hz 1H, C-8), 6.64 (d, J = 9.0Hz, 1H, C-9), 5.31 (s, 1H, H-2). 4.35 (br s, OH), 3.75 (s, 3H, H-9); ^{13}C NMR (75 MHz, $CDCl_3$, δ ppm), 156.7 (s, C-9), 131.6 (d, C-8 or C-4), 128.6

(s, C-3), 127.2 (d, C-8 or C-4), 118.9 (s, C-1) 112.1 (d, C-7) 112.0 (s, C-5), 62.2 (d, C-2), 56.8 (q, C-9).

v) General procedure for reduction of cyanohydrins to N-Boc-2-(3-aryl)-1-aminoethan-2-ol

To a stirred solution of a cyanohydrin (2 mmol) in dry methanol (15 ml), cooled to 0°C, were added Boc_2O (873 mg, 4.0 mmol) and $NiCl_2 \cdot 6H_2O$ (48 mg, 0.2 mmol). $NaBH_4$ (530 mg, 14.0 mmol) was then added in small portions over 30 min. The reaction was exothermic and effervescent. The resulting reaction mixture containing a finely divided black precipitate was allowed to warm to the room temperature and left to stir for a further 2-3 h, before solvent evaporation under vacuum. The residue was dissolved in EtOAc (50 ml) and washed with saturated aq.$NaHCO_3$ (2 x 50 ml). The organic layer was dried over anhyd Na_2SO_4 and the solvent was removed in vacuum to obtain to yield....

a) N-Boc-2-(3-methoxyphenyl)-1-aminoethan-2-ol (53)

Reagents and reactants used were **52** (1.72 g, 10.55 mmol), $NiCl_2.6H_2O$ (259 mg, 1.09 mmol), $NaBH_4$ (2.79 g, 73.75 mmol) and Boc_2O (4.843 ml, 21.1 mmol) and MeOH (80 ml); **53** was obtained as viscous liquid (2.00 g, 71%); ESI MS $[M+H]^+$ at m/z 168.1494, $[M+Na]^+$ at m/z 190.1824. ^1H NMR (300 MHz, $CDCl_3$, δ ppm) 7.08 (t, J = 8.1Hz, 1H, H-7) 6.76 (m, 2H), 6.65 (br d, J = 7.2 Hz), 5.19 (br s, 1H, H-2), 4.70, (br d, OH), 3.62, (s, H-9), 3.26 (m, 1H, βH-1), 3.037 (m, 1H αH-1), 1.28 (s, 9H, H-4',H-5',H-6'); ^{13}C NMR (75 MHz, $CDCl_3$, δ ppm) 159.6 (s, C-2'), 156.5 (s, C-5), 143.7 (s, C-2), 129.3 (d), 118.2 (d), 112.9 (d), 111.2 (d), 79.6 (s, C-4'), 73.3 (d, C-2), 55.0 (q, C-9), 48.1 (t, C-1), 28.1 (q, C-4', C-5', C-6')

b) *N-Boc-2-(2-bromo-5-methoxyphenyl)-1-aminoethan-2-ol (58)*

Reagents and reactants used were **57** (500 mg, 2.07 mmol), $NiCl_2.6H_2O$ (48.97 mg, 0.21 mmol), $NaBH_4$ (547.1 mg, 14.45 mmol) and Boc_2O (0.95 ml, 4.13 mmol) and MeOH (15.5 ml). **58** was obtained as viscous liquid viscous liquid (493.69 mg, 69%); ESIMS $[M+H]^+$ at m/z 346/348, $[M+Na]^+$ at m/z 368/370 $[M+K]^+$ at m/z 384/386,

$[2M+H]^+$ 713/715/717; MS/MS at m/z 346 328, 272,254, 228,193,175,149,134,106; ^1H NMR (300 MHz, $CDCl_3$, δ ppm) 7.27 (d, J = 8.7, Hz, 1H, H-5), 7.08 (d, J = 3 Hz 1H, H-8), 6.60 (dd, J = 8.7, 3.0Hz, 1H, H-6), 5.25 (br s, OH), 4.95 (br d, 1H, H-2), 3.69 (s, 3H, H-9), 3.40 (m, 1H, βH-1), 3.22 (m, 1H, αH-1), 1.34 (s, 9H, H-5', H-6',H-7'); ^{13}C NMR (75 MHz, $CDCl_3$, δ ppm) 158.7 (s, C-7), 157.3 (s, C-2'), 141.6 (s, C-3), 132.8 (d, C-5), 114.9 (d, C-6 or C-8), 112.9 (d, C-6 or C-8), 111.7 (s, C-4) 79.71 (s, C-4') 72.9 (d, C-2) 55.1 (q, C-9) 46.2 (t, C-1) 28.1 (q, C-5', C-6' C-7')

c) *N-Boc-2-(2,4-Dibromo-5-methoxyphenyl)- 1-aminoethan-2-ol* ***(44)***

Reagents and reactants used were **43** (800 mg, 2.49 mmol), $NiCl_2.6H_2O$ (59 mg, 0.249 mmol), $NaBH_4$ (660 mg, 17.84 mmol) and Boc_2O (1.14 ml, 4.98 mmol) and MeOH (18.7 ml). **44** was obtained as viscous liquid (804.98 mg, 76%). ^1H NMR (300 MHz, $CDCl_3$, δ ppm) 7.61 (s, 1H, H-6), 7.15 (s, 1H, H-9), 5.04 (m, 1H, H-3), 3.86 (s, 3H, H-10), 3.45 (ddd, J = 15.0, 6.1, 1.8 Hz, 1H, βH-2), 3.31 (m, 1H, αH-2), 1.40 (s, 9H, H-5', H-6',H-7') ^{13}C NMR (75 MHz, $CDCl_3$, δ ppm) 155.8 (s, C-8), 155.5 (s, C-1'), 141.1 (s, C-4), 133.0 (d, C-6), 111.8 (s, C-5 or C-7), 111.5 (s, C-5 or C-7), 111.5 (d, C-9), 80.3 (s, C-3'), 73.4 (d, C-3), 56.3 (q, C-10), 39.9 (t, C-2), 28.2 (q, C-5', C-6' C-7').

d) N-Boc-2-(3-bromo-4-methoxyphenyl)-1-aminoethan-2-ol ***(63)***

Reagents and reactants used were **62** (500 mg, 2.07 mmol), $NiCl_2.6H_2O$ (48.97 mg, 0.21 mmol), $NaBH_4$ (547.1 mg, 14.45 mmol) and Boc_2O (0.95 ml, 4.13 mmol) and MeOH (15.5 ml) **63** was obtained as viscous liquid (515.15 mg, 72%). ESIMS $[M+H]^+$ at m/z 346.2061/348.2863, $[M+Na]^+$ at m/z 368.843/370.2843 $[M+K]^+$ at m/z 384.2766/386.7776, $[2M+H]^+$ 713.6253/715.6278/717.6295; ^1H NMR (300 MHz, $CDCl_3$, δ ppm) 7.51 (br s, 1H, H-5), 7.21 (d, 1H, *J* = 8.4 Hz, H-9), 6.83 (d, *J* = 8.4 Hz, 1H, H-8), 5.05 (t, 1H, H-2), 4.55 (d, 1H, OH), 3,85 (s, 3H, H-10), 3.36 (m, 1H, βH-2), 3.17 (m, 1H, αH-2), 1.41 (s, 9H, H-5', H-6',H-7').

vi) Procedure for deprotection of N-Boc-2-(3-aryl)-1-aminoethan-2-ol (54, 45, and 64)

To an *N*-Boc amine (10 mmol) was added 6N HCl (100 ml) and refluxed for 3 h. Reaction mixture was cooled and washed with EtOAc (3 x 30 ml). The aqueous portion was basified with liq NH_3, extracted with EtOAc (3 x 30 ml) and dried over anhyd Na_2SO_4. Upon evaporation of solvent an amine was obtained.

a) 2-(3-methoxyphenyl)-1-aminoethan-2-ol (54)

Reagents and reactants used were **53** (1.0 g, 3.74 mmol), 6 N HCl (37.5 ml); **54** was obtained as solid (387.35, 62%); IR (KBr) 3404, 2929, 2839, 1558, 1413, 1261, 1159, 1045, 786, 700 cm-1; ESIMS $[M+H]^+$ at m/z 168.1494, $[M+Na]^+$ at m/z 190.1824; ^1H NMR (300 MHz, $CDCl_3$, δ ppm) 7.11 (t, J = 8.1Hz, 1H, H-7), 6.82 (m, 2H), 6.69 (d, J = 7.5 Hz), 4.82 (br s, H-2), 3.65 (s, 3H, H-9), 3.00 (m, 1H, βH-1), 2.91 (m, 1H αH-1); ^{13}C NMR (75 MHz, $CDCl_3$, δ ppm), 159.6 (s, C-5), 142.8 (s, C-3), 129.5 (d), 118.0 (d), 13.1 9d), 111.5 (d), 70.3 (d, C-2), 55.0 (q, C-9), 46.6 (t, C-6).

b) 2-(2-bromo-5-methoxyphenyl)-1-aminoethan-2-ol (59)

To the **58** (500 mg, 1.45 mmol) in RB flask was added 1:1 TFA/CH_2Cl_2 (10 ml) and stirred for 2 h and the solvent and the TFA was removed under vacuum. The resultant residue was dissolved and $CHCl_3$ (50 ml) and was washed with water (20 ml x 3). The organic layer was dried over anhyd. Na_2SO_4 and solvent evaporated to solid compound (291.50 mg, 82%), ^1H NMR (300 MHz, $CDCl_3$, δ ppm) 7.38 (d, J = 8.7 Hz, 1H, H-5), 7.14 (d, J = 2.7 Hz, 1H, H-8), 6.69 (dd, J = 8.4, 2.7 Hz, 1H, H-6), 4.92 (dd, J = 7.5, 3.9 Hz, 1H, H-2), 3.80 (s, 3H, H-9), 3.10 (d, J = 12.6 Hz, 1H, βH-1), 2.69 (dd, J = 12.6, 7.5 Hz, αH-1).

c) 2-(2,4-Dibromo-5-methoxyphenyl)- 1-aminoethan-2-ol (45)

Reagents and reactants used were **44** (1.1g, 2.59 mmol), 6 N HCl (26.0 ml). **45** was obtained as solid (529.94, 63%);NMR data recorded on HCl salt. ^1H NMR (300 MHz, D_2O, δ ppm) 7.75 (s, 1H, H-5), 7.18 (s, 1H, H-8), 5.18 (dd, 1H, H-2), 3.84 (s, 3H. H-9), 3.29 (dd, 1H, βH-1), 3.06 (dd, 1H, αH-1). ^{13}C NMR (75 MHz, D_2O, δ ppm)

158.9 (s, C-7), 139.4 (s, C-3), 136.2 (d, C-5), 111.8 (s, C-6 or C-4), 111.4 (s, C-6 or C-4), 111.1 (d, C-8), 68.7 (d, C-2), 56.5 (q, C-9), 43.7 (t, C-1).

d) 2-(3-bromo-4-methoxyphenyl)-1-aminoethan-2-ol(64)

Reagents and reactants used were **63** (700 mg, 2.02 mmol), 6 N HCl (21.0 ml) **64** was obtained as solid (298.61 mg, 60%). IR (KBr), 3354, 2929, 2841, 1598, 1496, 1460, 1259, 1053, 1016, 900, 815 cm^{-1}. ^{13}C NMR (75 MHz, $CDCl_3$, δ ppm) 155.2 (s, C-7), 135.8 (s, C-3), 130.9 (d, C-4 or C-8), 126.4 (d, C-4 or C-8), 111.7 (d, C-7), 96.4 (s, C-5), 77.8 (d, C-2), 54.5 (q, C-9), 49.1 (t, C-1)

***vii)** General procedure for preparation of amathamide alcohol by coupling 2-Aryl-1-aminoethan-2-ol with N-methyl-L-proline (39)*

A solution of oxyma (21.9 mg, 0.154 mmol), diisopropyl carbodiimide (DIC) (39.0 mg, 47.94 µl, 0.31 mmol), **39** (19.87 mg, 0.154 mmol) in dry DMF (5 ml) was dtirred at rt. for 2 min (preactivation). To this was added solution of *2*-aryl-1-aminoethan-2-ol (0.154 mmol) in DMF (1 ml), stirred at rt. for 30-60 min and monitored with TLC. On completion of reaction, the reaction mixture was added to cold 1N HCl (30 ml) and extracted with EtOAc (10 ml x 3). The aqueous portion was basified with liq. NH_3 and extracted with EtOAc (15 ml x 3). This EtOAc portion was washed with water and dried over anhyd. Na_2SO_4. Upon evaporation of the solvent a sticky solid was obtained which was purified on flash Si gel column by eluting with gradients of MeOH-$CHCl_3$ to give crystalline solid.

a) 2(S)-N-[2-(5-methoxyphenyl)-2-hydroxyethenyl]-1-methyl-2-pyrrolinecarboxamide (55)

Reagents and reactants used were oxyma (30.25 mg, 0.209 mmol), diisopropyl carbodiimide (DIC) (52.9 mg, 64.90 µl, 0.419 mmol), **39** (27.07 mg, 0.209 mmol), **54** (35.0 mg, 0.209 mmol) and DMF (6.8 ml); **55** was obtained as colourless crystalline needles (49.52 mg, 85%);

b) 2(S)-N-[2-(2-Bromo-5-methoxyphenyl)-2-hydroxyethenyl]-1-methyl-2-pyrrolinecarboxamide (60)

Reagents and reactants used were oxyma (87.14 mg, 0.613 mmol), diisopropyl carbodiimide (DIC) (151.4 mg, 185.7 µl, 1.20 mmol), **39** (78.57 mg, 0.610 mmol), **59** (150.0 mg, 0.610 mmol) and DMF (20.0 ml); **55** was obtained as colourless crystalline needles(193.73mg, 89 %);

c) 2(S)-N-[2-(2,4-Dibromo-5-methoxyphenyl)-2-hydroxyethenyl]-1-methyl-2-pyrrolinecarboxamide[amathamide A alcohol (46)]

Reagents and reactants used were oxyma (75.64 mg, 0.532 mmol), diisopropyl carbodiimide (DIC) (165.9 µl, 1.07 mmol), **39** (68.75 mg, 0.532 mmol), **59** (173.0 mg, 0.534 mmol) and DMF (17.5 ml); **46** was obtained as colourless crystalline needles (93.35 mg, 92%);

d) 2(S)-N-[2-(3-Bromo-4-methoxyphenyl)-2-hydroxyethenyl]-1-methyl-2-pyrrolinecarboxamide (65)

Reagents and reactants used were oxyma (61.0 mg, 0.429 mmol), diisopropyl carbodiimide (DIC) (106.4 mg, 130.7 µl, 0.844 mmol), **39** (55.0 mg, 0.426 mmol), **59** (105 mg, 0.420 mmol) and DMF (14.0 ml); **65** was obtained as colourless crystalline needles(79.67 mg, 90%);

IV. Spectra

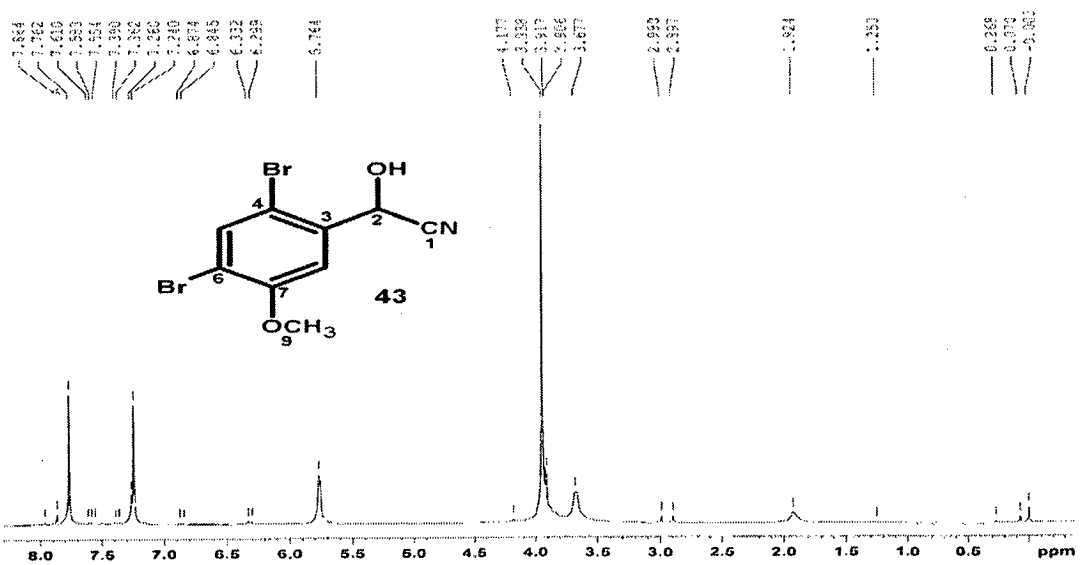


Fig 4.1a ^{1}H NMR of 43

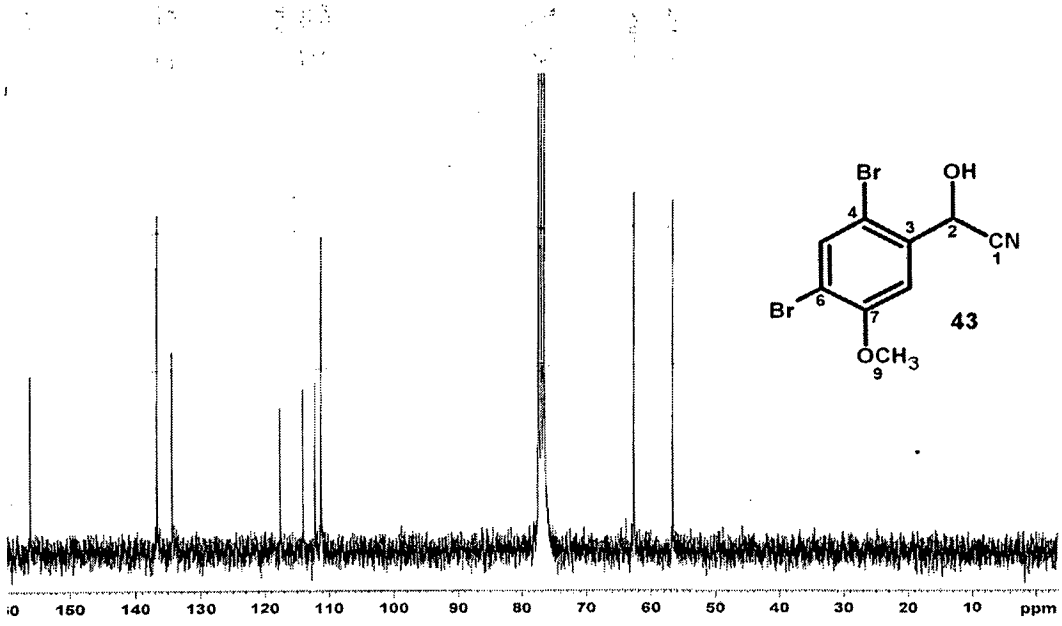


Fig 4.1b: ^{13}C NMR of 43

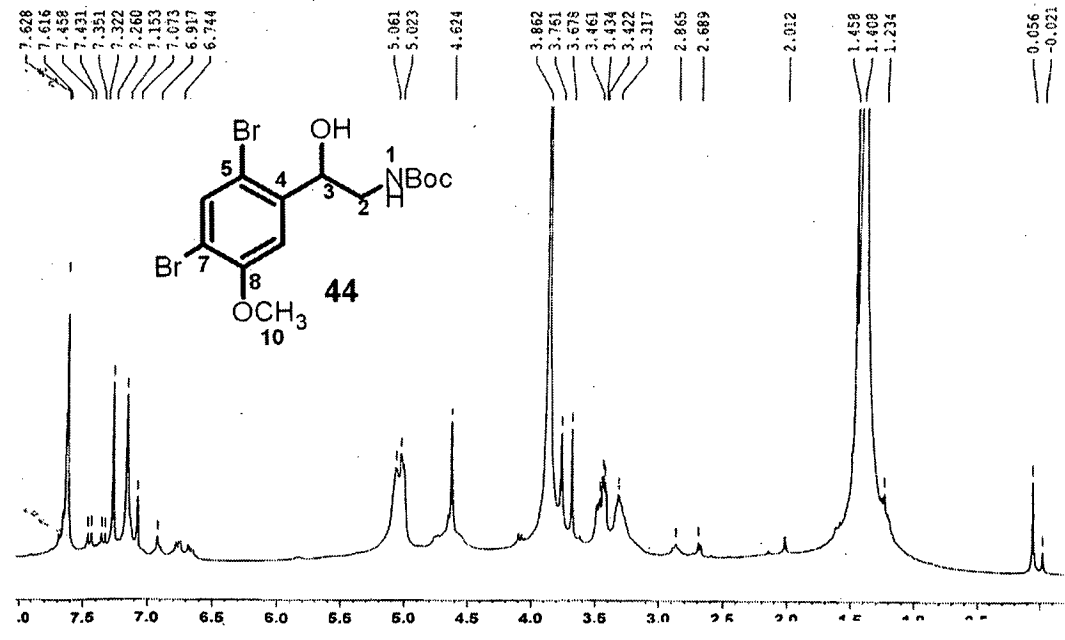


Fig4.2a: ^{1}H NMR of 44

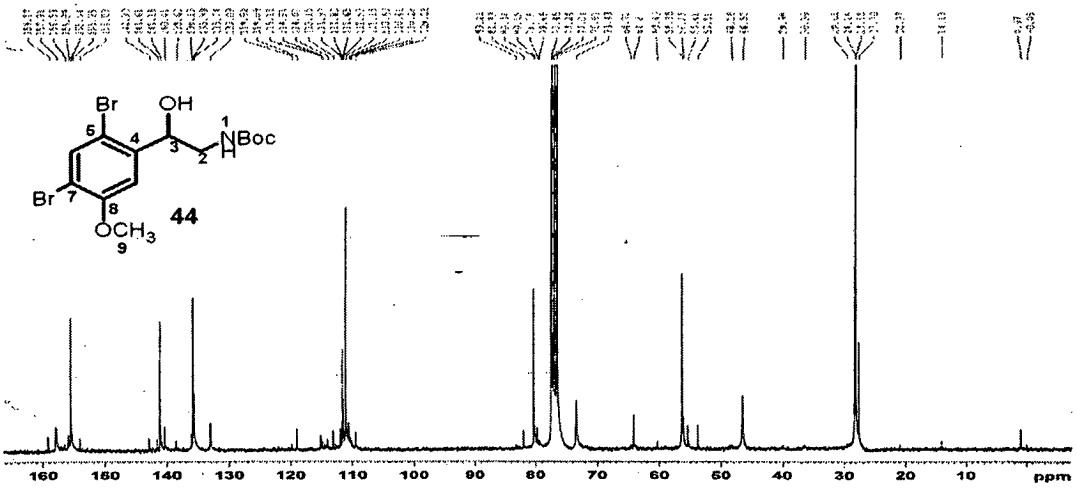


Fig 4.2b ^{13}C NMR of 44

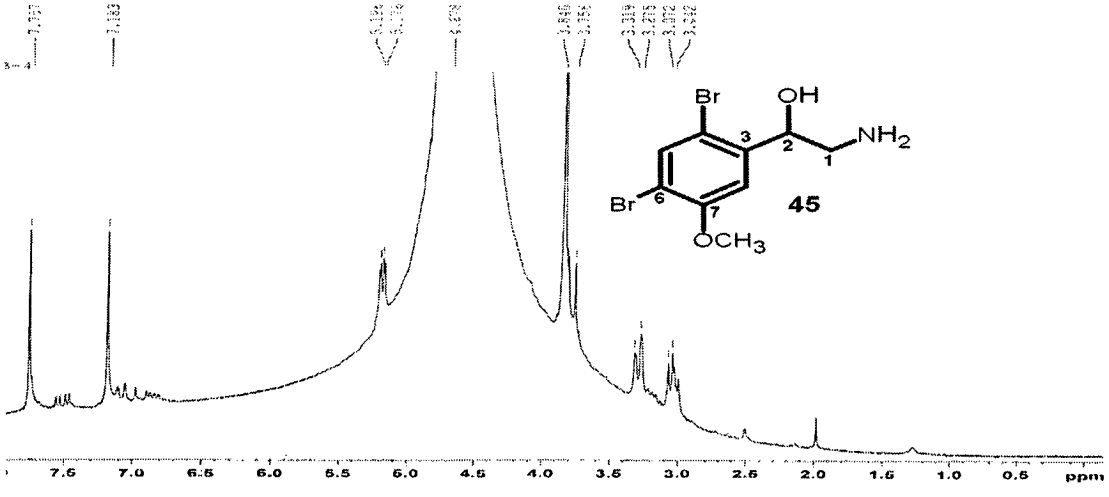


Fig 4.3a ^{1}H NMR of 45

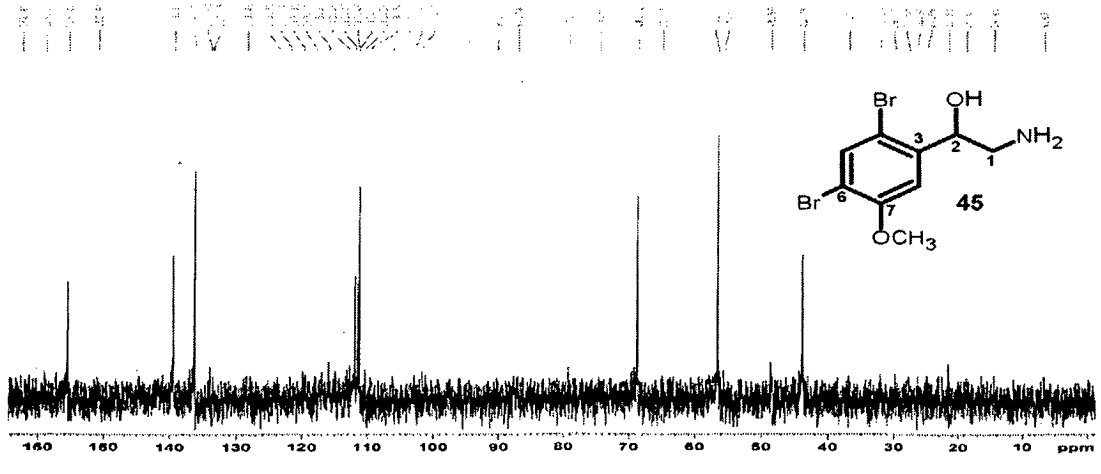


Fig 4.3 b ^{13}C NMR of 45

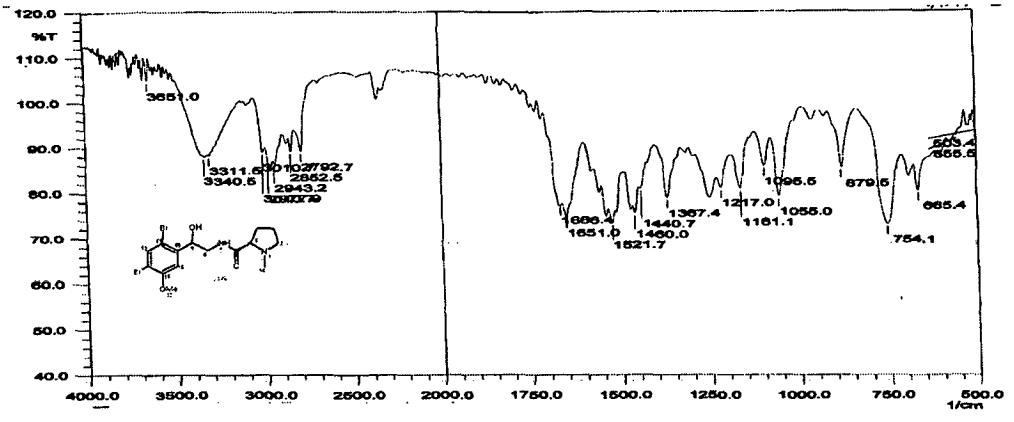


Fig: 4.4a IR spectra of 46

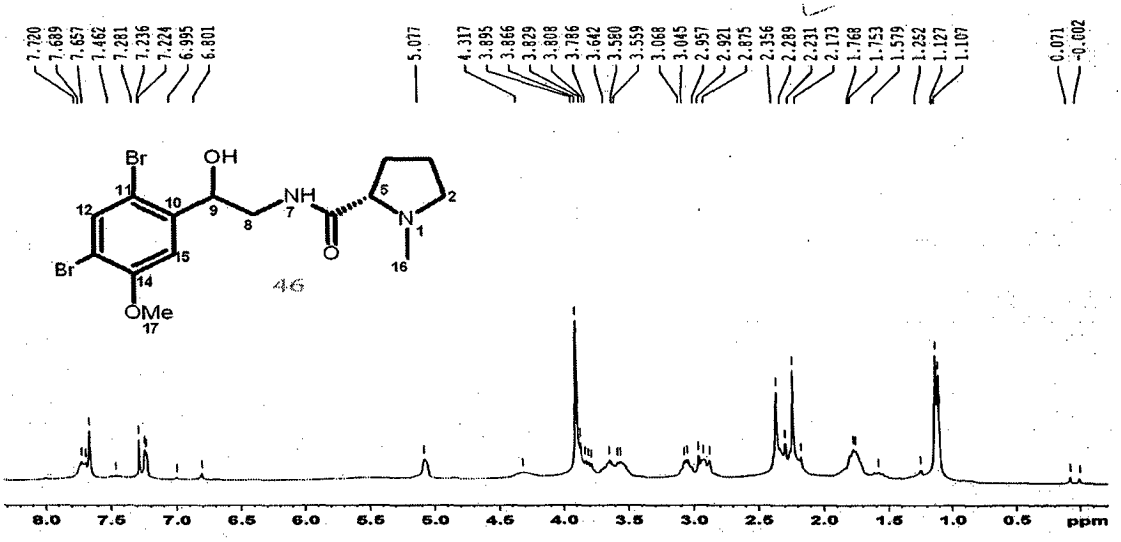


Fig 4.4b ^{1}H NMR of 46

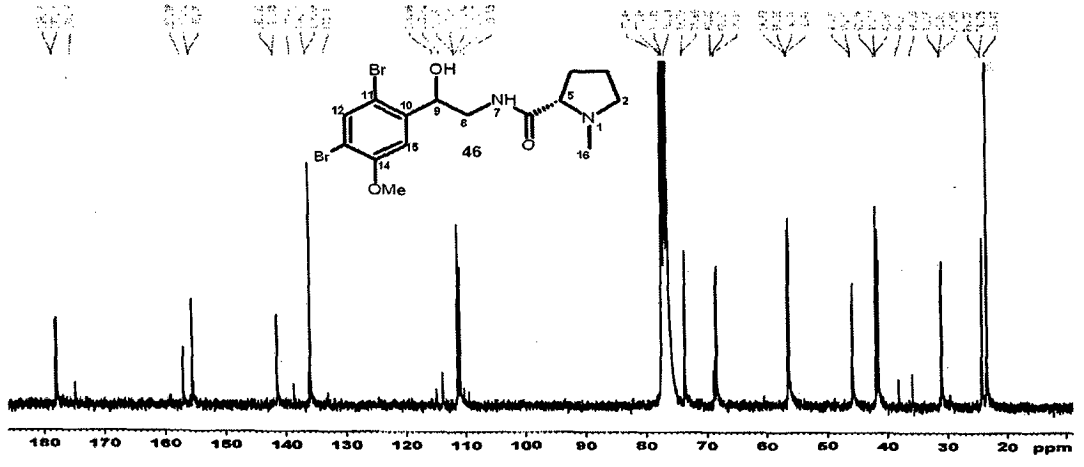


Fig 4.4c: ^{13}C NMR of 46

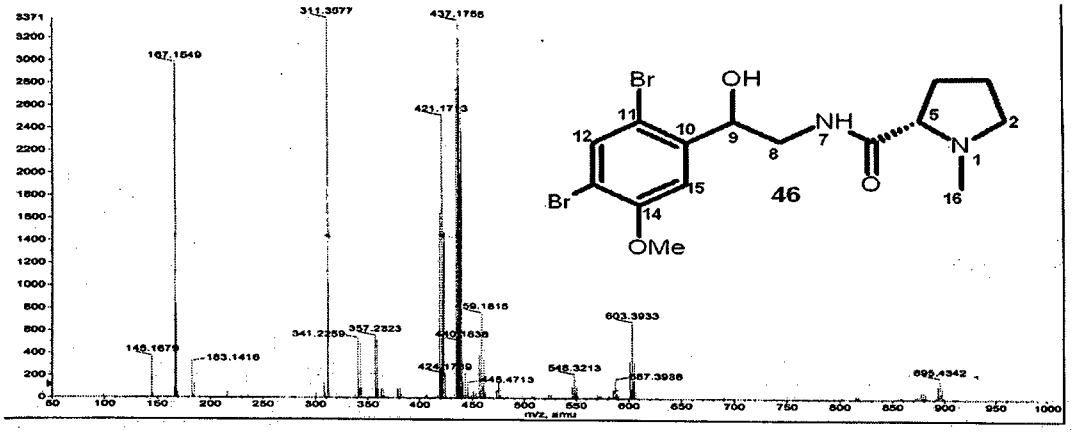


Fig 4.4d: ESITOFMS of 46

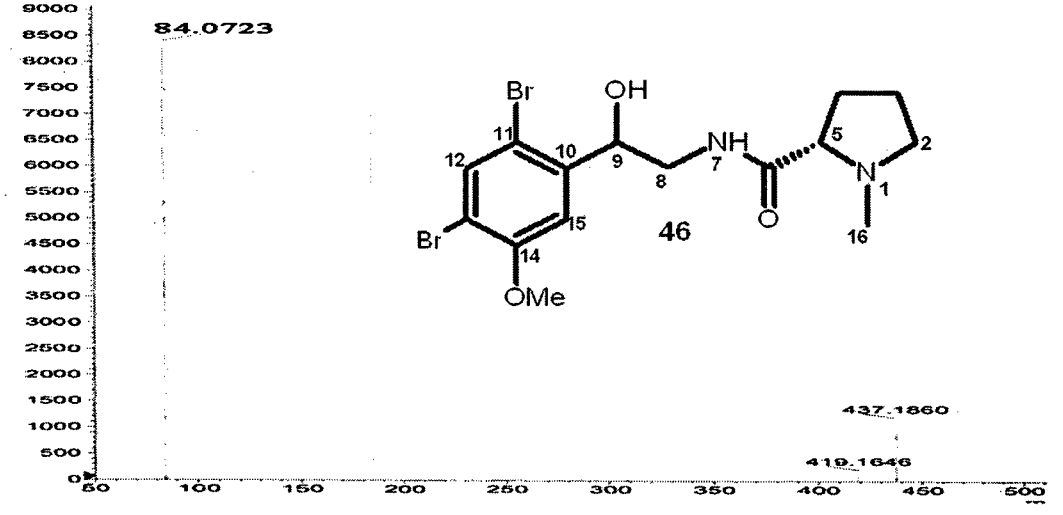


Fig 4.4e: MS/MS at m/z 437 of 46

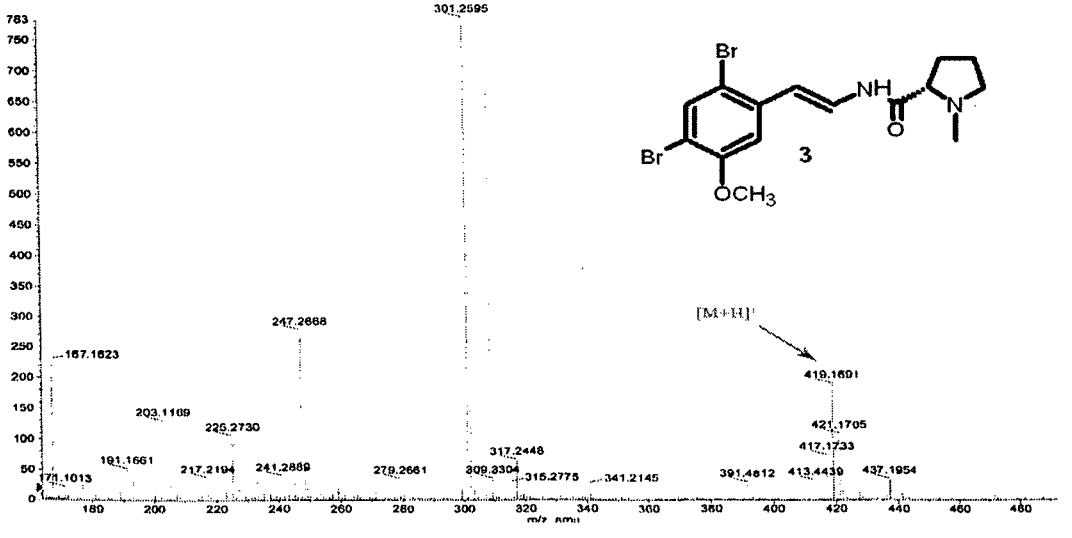


Fig 4.5a: ESITOFMS of amamthamide A (3)

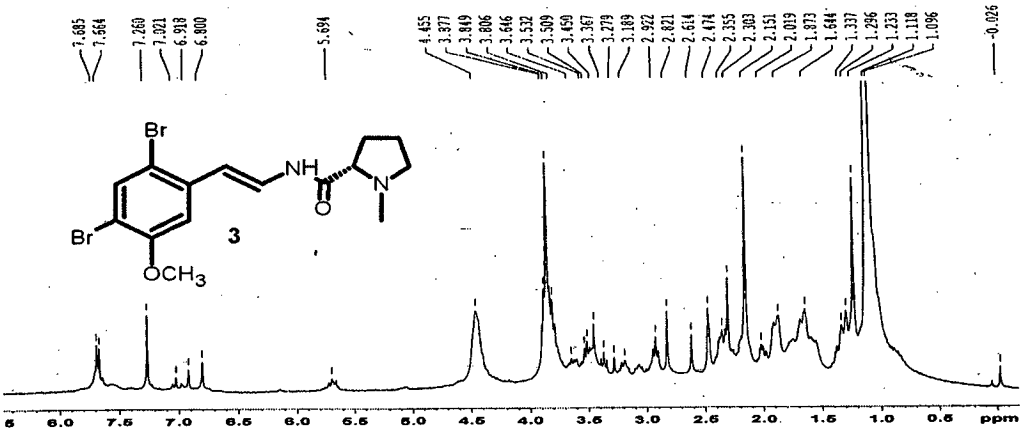


Fig 4.5b ^{1}H NMR of mixture containing amathamide A (3)

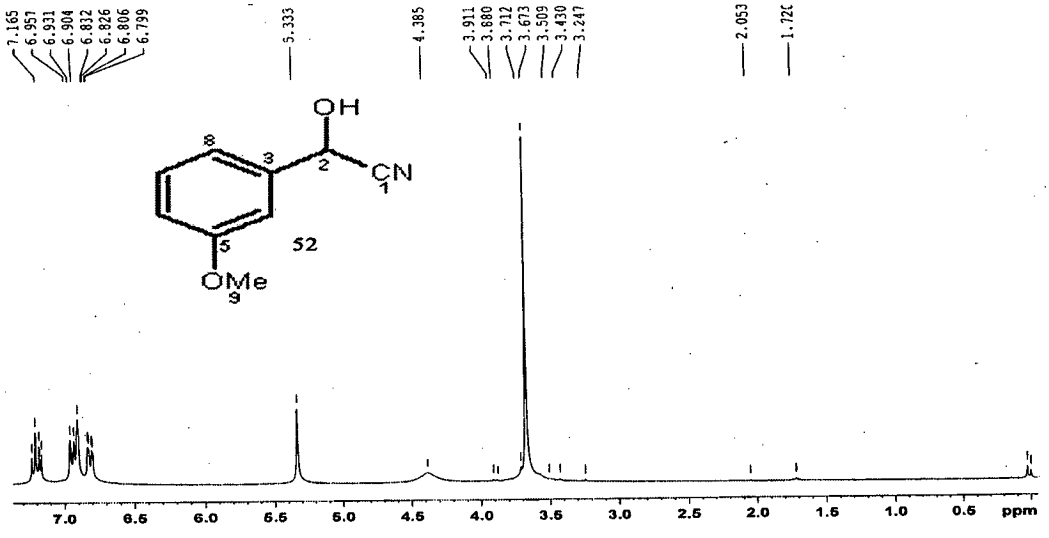


Fig 4.6: ^{1}H NMR of 52

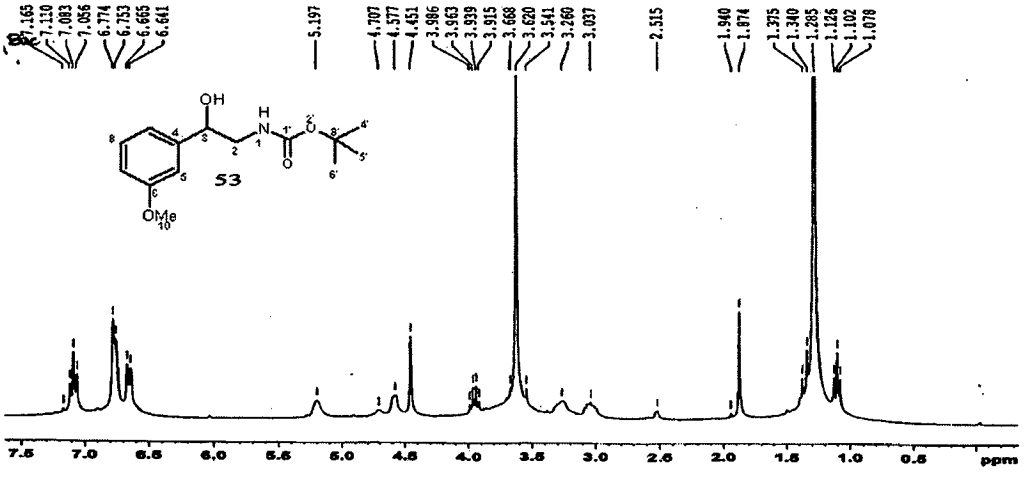


Fig 4.7: ^{1}H NMR of 53

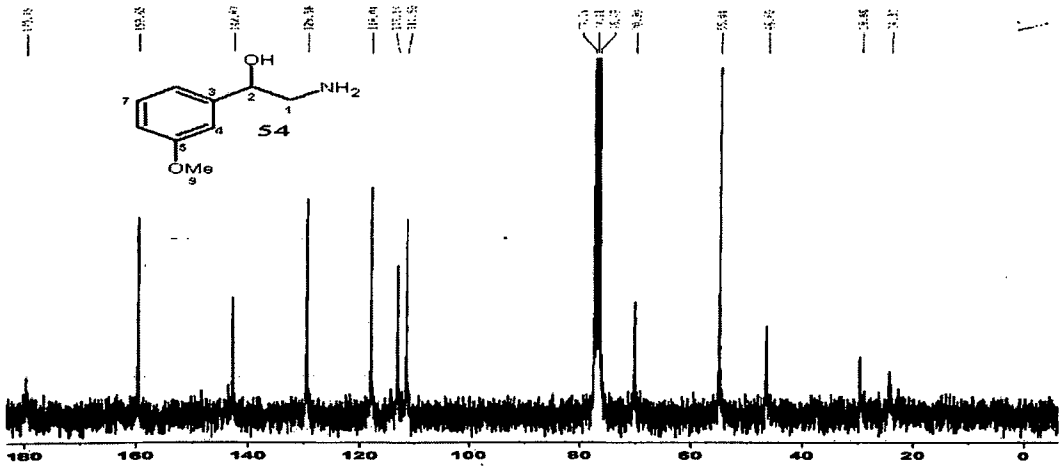


Fig 4.8: ^{13}C NMR of 54

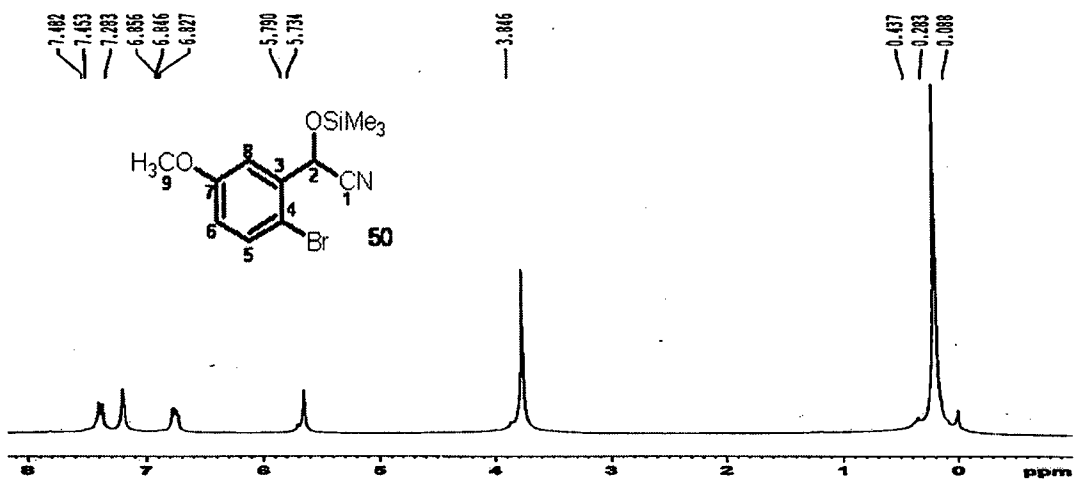


Fig 4.9: ^{1}H NMR of 50

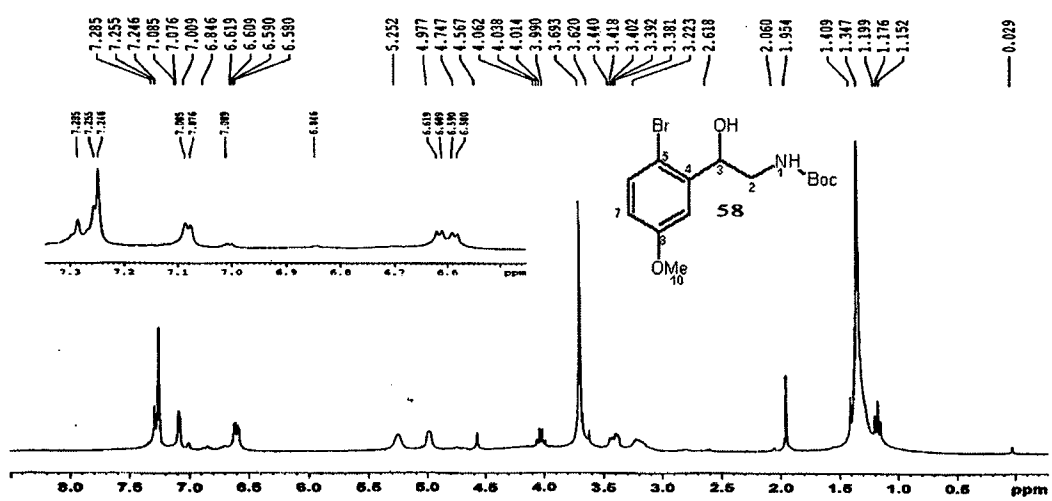


Fig 4.10: ^{1}H NMR of 58

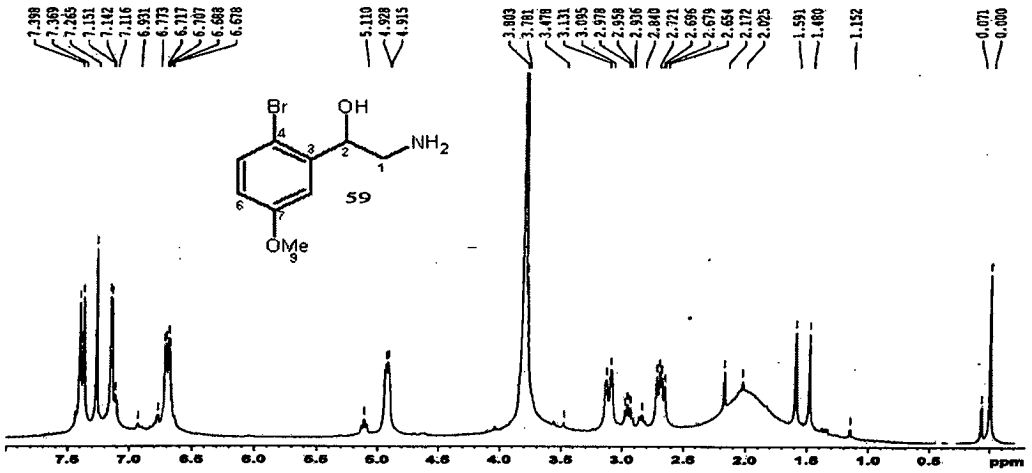


Fig 4.11: ^{1}H NMR of 59

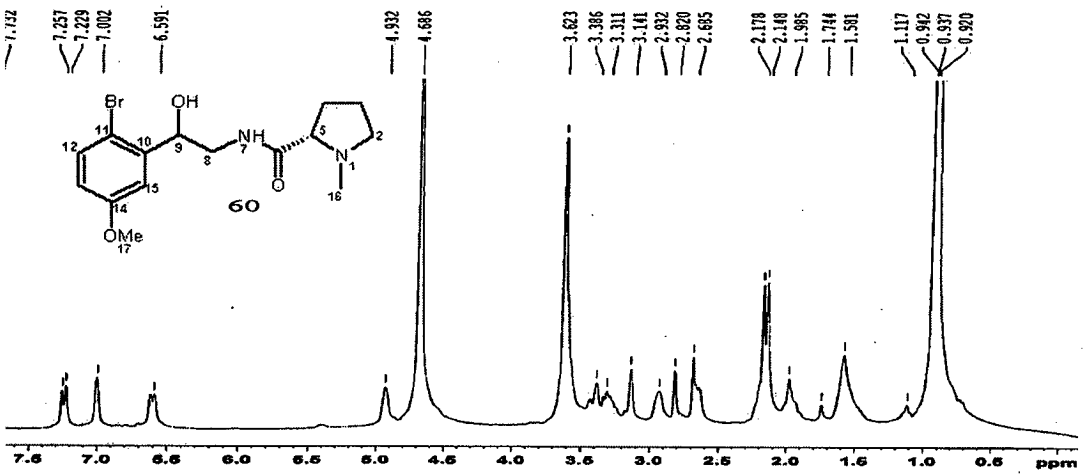


Fig 4.12: ^{1}H NMR of 60

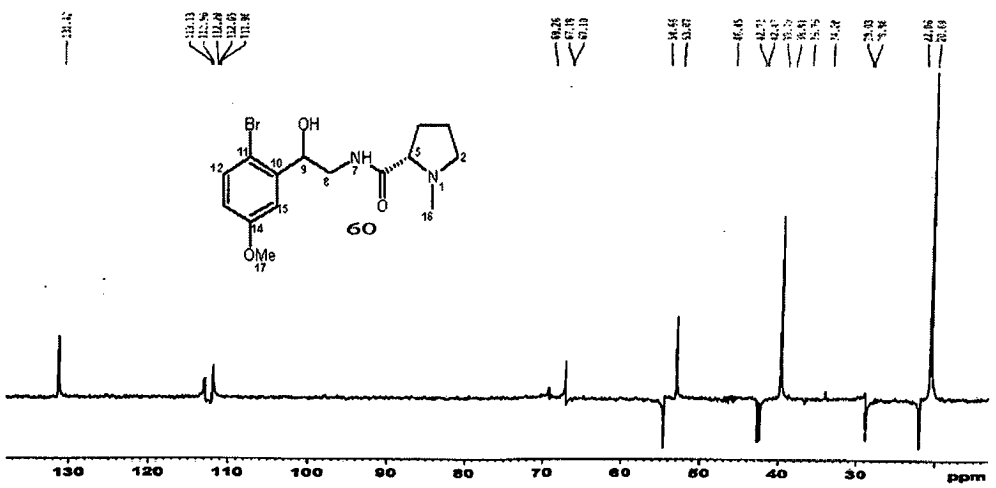


Fig 4.13: DEPT NMR of 60

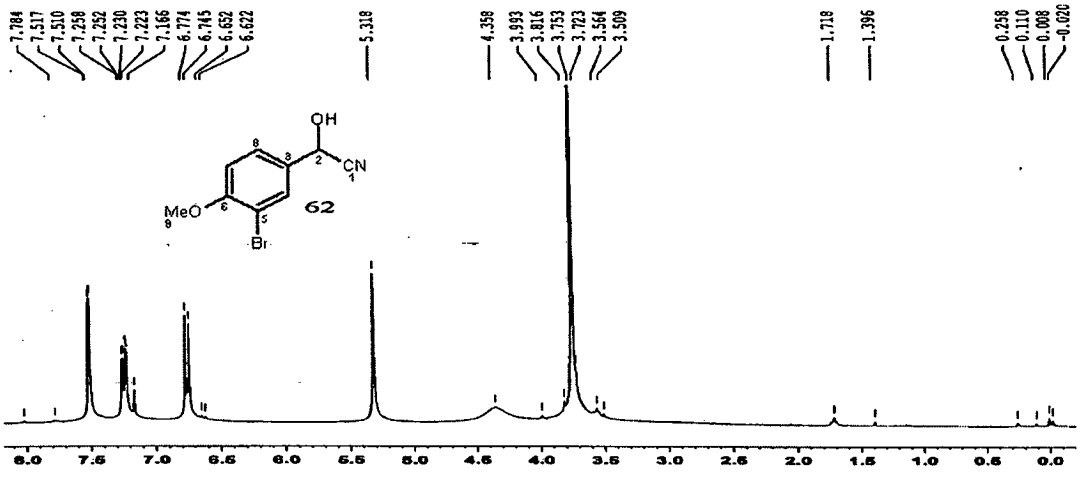


Fig 4.14: ^{1}H NMR of 62

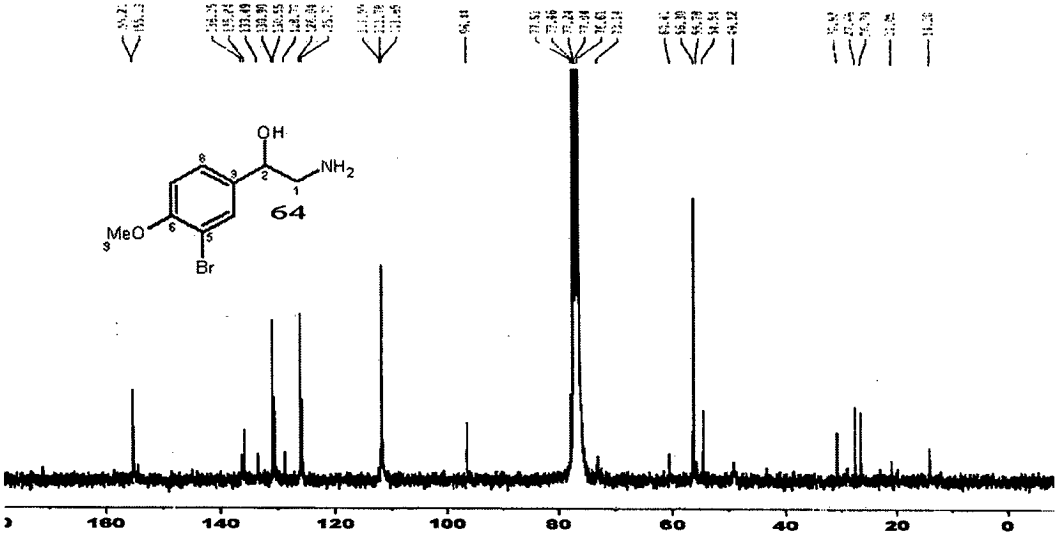


Fig 4.15: ^{13}C NMR of 64

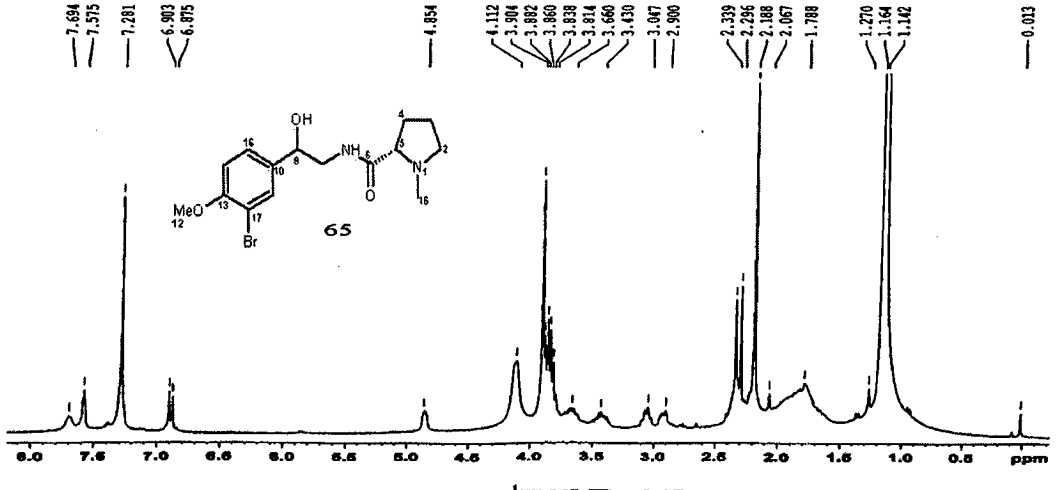


Fig 4.16: ^{1}H NMR of 65

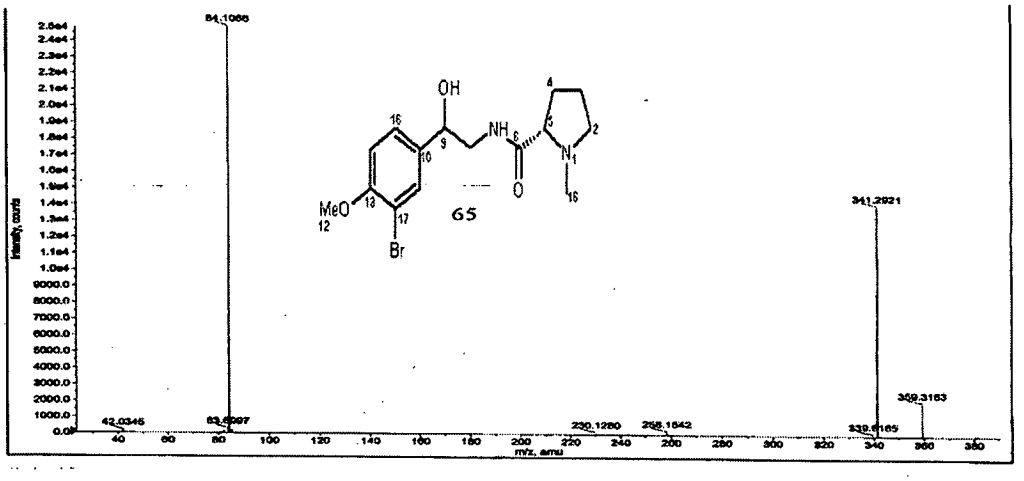


Fig 4.17: MS/MS of 65 at m/z 359

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SUMMARY

SUMMARY

The thesis entitled "Studies on some Important Secondary Metabolites from Marine Organisms" is divided into four chapters, and describes the efforts directed towards the discovery of bioactive metabolites from marine-derived *Aspergillus* species of fungi, marine invertebrates such as sponge *Oceanapia* and soft coral *Lobophytum crassum,* and diversity oriented synthetic route towards the synthesis of bryozoan metabolites, amathamides.

Chapter 1: A review of literature published on the secondary metabolites of marine-derived fungi during the first half of 2010 has been presented with current status on marine mycochemistry.

Chapter 2 section 2.1: Seven epiphytic fungi were isolated from pnematophores of mangrove *Acanthus illicifolius.* High throughput screening revealed *Aspergillus cervinus and Aspergillus ornatus* to have anticancer activity and *Aspergillus flavipes* to have anti-inflammatory activity. The chemical investigation of the fungus *A. flavipes* led to the isolation one new compound, (+) 4-(1'-propenyl) – *trans*-cyclopentane-1,2-diol **(FM-5)** and four known compounds , butyrolactone I **(FM-1),** butyrolactone II **(FM-2),** (+) terrein **(FM-3),** and *meso*-4β-(1'-propenyl)cyclopentane-1β,2β-diol **(FM-4). FM-1, FM-2** and **FM-4** have been isolated for the first time from marine derived source and also for the first time from *A. flavipes.* The structures of the metabolites were mainly elucidated using spectroscopic techniques those included 1D, 2D NMR and MS. **FM-1** and **FM-3** exhibited strong anti-inflammatory activity. A review of the metabolites of *A. flavipes,* which includes 13 structures, has been also presented in the beginning of this section.

Section 2.2: Two new aromatic butenolides, aspernolides A (**FM-6**) and B (**FM-7**) along with the known metabolites, butyrolactone I (**FM-1**), terrein (**FM-3**) and physcion (**FM-8**) were isolated from the fermentation broth of a soft coral derived fungus *Aspergillus terreus.* The structures of these metabolites were assigned on the basis of detailed spectroscopic analysis. The absolute stereochemistry of **FM-6** and **FM-7** was established by their preparation from the known butenolide **FM-1.** When tested, **FM-6** exhibited mild cytotoxicity against cancer cell lines. These type of butenolides are previously known only from terrestrial *A. terreus* isolates.

Some of the important metabolites from *A. terreus* with reference to their structure and bioactivity have also been reviewed in this section.

Section 2.3: A marine sponge derived fungus *Aspergillus aculeatus* produced diastereomers of secalonic acids, one of these was characterized to be secalonic acid D (**FM-17**) from its NMR and XRD data. **FM-17** showed antifungal activity against *Candida albicans*, superior to flucanozole, a drug currently in use. The section is initiated with a review of literature on *A. aculeatus* and its metabolites, and brief introduction to secalonic acids and their biosynthesis.

Section 2.4: The section depicts the closeness of two marine-derived fungi *A. flavipes* and *A. terreus* by comparison of their metabolites and phylogentic analysis. Using electrospray ionization-tandem mass spectrometry (ESI-MS/MS) the metabolites were characterize from the crude extracts (without purification) of fungi *A. flavipes* and *A. terreus*. Both the fungi displayed similar metabolic profile. Butyrolactone I (**FM-1**), butyrolactone III (**FM-9**) and terrein (**FM-3**) were the common metabolites to both the fungi whereas, derivatives of butyrolactone I, such as butyrolactone sulfates, butyrolactone carboxylic acid have been detected only in the extract of *A. flavipes*. The phylogenetic analyses revealed that *A. terreus* and *A. flavipes* have 99% and 98% similarity to the several *A. terreus* isolates. The section also presents the discussion on biosynthesis of butyrolactones, aspulvinones and asterriquinones.

Section 2.5: Culture of *A. terreus* grown in medium containing different seawater concentration was analysed for butyrolactone I production which revealed that butyrolactone I production in *A. terreus* is optimum at 50% conc of sea water, whereas negligible amount or no butyrolactone I is produced at 100% sea water concentration. This study proves that butyrolactone I production in *A. terreus* is function of sea water concentration of culture medium.

Chapter 3 Section 3.1: From the antibacterial extract of sponge *Oceanapia* sp. three known compound mimosamycin and xestospongins C and D were purified, while several known xestospongin were detected to be present from the ESI-MS spectrum. The three isolated metabolites are known sponge metabolites but not from *Oceanapia* sp. Sponge *Oceanapia* is relatively less known for its metabolites and majority of the metabolites known from it are alkaloids, which are presented in a short review.

Section 3.2: A diterpene, cembrenoid 1, campesterol, cholesterol, and three polyhydroxy sterols have been isolated from a soft coral *Lobophytum crassum* and their structures were mainly elucidated by comparison of their spectreoscopic data (IR and NMR) with the literature records.

Chapter 4: Amathamide alkaloids are the enamides of stryl amines and *N*-methyl prolines. They are the metabolites of-bryzoan *Amathia wilsoni* and are not well studied for biological activity. We have synthesized amathamide alkaloid A and four amathamide alcohols using a new diversity oriented approach, which involves preparation of β-hydroy-β-arylethamine by reduction of cyanohydrin and then coupling of β-hydroy-β-arylethamine with *N*-methyl proline using oxyma as coupling agent to yield amthamide alkaloids. One such amathamide alkaloid was dehydrated using DCC-$CuCl_2$ to give amathamide A. This synthesis is second synthetic route for amathamides and superior to the previous known method.

PUBLICATIONS

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Aspernolides A and B, butenolides from a marine-derived fungus *Aspergillus terreus*

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ABSTRACT

Two aromatic butenolides, aspernolides A and B along with the known metabolites, butyrolactone I, terrein and physcion were isolated from the fermentation broth of a soft coral derived fungus *Aspergillus terreus*. The structures of these metabolites were assigned on the basis of detailed spectroscopic analysis. The absolute stereochemistry of aspernolides A (**1**) and B (**2**) was established by their preparation from the known butyrolactone I. Biogenetically aspernolides A and B must be derived from butyrolactone I, a well known specific inhibitor of cyclin dependent kinase (cdk) from *A. terreus*. When tested, aspernolide A exhibited mild cytotoxicity against cancer cell lines.

1. Introduction

Marine-derived microbes, fungi in particular have long been recognized as potential source of structurally novel and biologically potent metabolites (Faulkner, 2000; Bugni and Ireland, 2003; Saleem et al., 2007). Fungi belonging to *Aspergillus* genera are one of the major contributors to the secondary metabolites of fungal origin. *Aspergillus terreus* is a ubiquitous fungus in our environment and although *Aspergillus* sp. are normally considered terrestrial species, the genus is tolerant to high salt concentrations. In recent years, we sought to draw marine microbial diversity into the arena of drug discovery. The present investigation is an outcome of such a study on the fungus *A. terreus* associated with a soft coral *Sinularia kavarattiensis*.

Terrestrial isolates of *A. terreus* are well known for the production of butenolides. Cytotoxic butyrolactones I–IV, biogenetically derived from tyrosine (Rao et al., 2000; Kiriyama et al., 1977; Nitta et al., 1983) and non prenylated-decarboxylated butenolides, xenofuranones A (**4**) and B (**5**) (Morishima et al., 1994), biogenetically produced from phenyl alanine are known to be derived from *A. terreus*. Xenofuranones A (**4**) and B (**5**) are also known as metabolites of bacterium *Xenorhabdus szentitmaii* (Brachmann et al., 2006). There is a recent report on the identification of **3** and its sulfated derivatives (**6** and **7**) (Niu et al., 2008) from a strain *A. terreus* (HKI0499).

This report focuses on the isolation and structure elucidation of new butenolides, aspernolides A (**1**) and B (**2**) and other known metabolites from culture medium of fungus *A. terreus*. Although, **1** has been described in the literature (Kiriyama et al., 1977) as a reaction product in the structure elucidation of **3**, neither its NMR data is reported nor it is known to be a natural product. Moreover this is the first report describing the isolation of **3** and other related butenolides from marine-derived fungus.

2. Results and discussion

Fungus, *A. terreus* was isolated as an epiphyte from a soft coral *Sinularia kavarattiensis* collected from the coast of Mandapam, Tamil Nadu, India. This fungus was grown on potato dextrose broth prepared in seawater. New secondary metabolites, aspernolides A (**1**) and B (**2**) were identified from the chloroform and ethyl acetate extracts of the culture broth respectively. These butenolides along with their plausible biogenetic precursor **3** and the known metabolites physcion and terrein were purified using repeated silica gel and Sephadex LH-20 gel filtration chromatography.

Aspernolide A (**1**) was obtained as white sticky solid. The molecular formula $C_{24}H_{24}O_7$ of **1** was determined by HRESITOFMS which showed pseudomolecular ion peaks $[M+Na]^+$ at 447.1433 (calcd. 447.1420 for $C_{24}H_{24}O_7Na$) and $[2M+Na]^+$ at 871.2959 (calcd. 871.2942 for $C_{48}H_{48}O_{14}Na$). The IR spectrum showed the presence of ester/lactone carbonyl at 1731 and 1738 cm^{-1}, phenolic OHs were evident at 3330 cm^{-1} and the presence of an absorption at 1660 cm^{-1} was suggestive of aromaticity in the molecule.

The ^1H NMR signals of the A_2B_2 system at δ_H 7.56, *d*, 2H, $J = 8.7$ Hz and 6.86, *d*, 2H, $J = 8.7$ Hz revealed the presence of para di-substituted benzene moiety. Two aromatic signals 6.53, *s*, 1H and 6.47, *s*, 2H (two doublets merged into a singlet) were indicative of the presence of additional unsymmetrical trisubstituted benzene ring in the molecule. Its ^{13}C NMR showed the presence

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of 10 aromatic signals for two aromatic rings, two ester carbonyls δ_c 169.3 s, and 169.6 *s*, olefenic carbon signals δ_c 137.2 *s* and 128.8 *s*, three sp^3 CH_2 *s* δ_c 22.1 *t*, 32.5 *t* and 38.6 *t*, a carbomethoxy δ_c 53.4 *q* and two oxygenated quaternaries δ_c 86.1 s and 74.2 s. The molecular formula $C_{24}H_{24}O_7$ requires 13 degrees of unsaturation. The presence of two aromatic rings accounts for eight while two carbonyls and two olefenic carbons account for another three, which makes a total of eleven degrees of unsaturation. Therefore **1** must posses two aliphatic rings in addition to two aromatic rings.

A detailed comparison of the NMR data of **1** with that of butyrolactone I (**3**) (Rao et al., 2000), confirmed a common hydroxyphenylpyruvate dimer type of network in the molecule. The significant difference observed in the NMR spectra of **1** as compared to that of **3** was the absence of an olefinic proton signal δ_H 5.0, *t*, 1H, two olefinic carbon signals δ_c 121.0 *d* and 130.7 s and the presence of three methylenes and two oxygenated sp^3 quaternaries compared to two methylenes and one oxygenated sp^3 quaternary in **3** in both ^{13}C and DEPT NMR spectra. This data was indicative of the presence of a dihydropyran ring fused to a trisubstituted benzene ring in place of the open prenyl chain present in **3**. HMBC was in good agreement with the structure **1** (Fig. 1). Key HMBC correlations from H-2″ to C-3″ and C-7″, from H-7″ to C-2″, C-3″ and C-4″ and from H-8″ to C-9″ and C-10″ (11″) established a dihydropyran ring fused through the C3″–C4″ bond of a benzene ring. HMBC correlations from H-2″ and H-6″ to C-6 and from H-6 to C-1″, C-6″, C-2″, C-4, C-5 and C-3 were evidence of the benzodihydropyranmethylene moiety linked to a lactone ring at C-4. Furthermore HMBC correlation from H-2′(6′) to C-3 established *para* di-substituted phenolic moiety at C-3. Out of two carbonyls, C-5 and C-1, δ_c 169.6 was assigned to C-5 on the basis of its HMBC correlation to the protons H-6 and H_3–5OMe.

Aspernolide B (**2**) (Rf, 0.51), more polar than **1** (Rf, 0.81) was obtained as a light brown syrup $[\alpha]_D$ + 48.27 (c 0.29, MeOH). The IR spectrum showed the presence of – OHs at 3330 cm^{-1}, ester/lactone carbonyls overlapping peaks at 1732 and 1747 cm^{-1} and 1610 and 1519 cm^{-1} for aromatic rings. Although chemical shift variations were present in the ^1H and ^{13}C NMR of **2**, they were similar to those of **1**. Significant variations in the chemical shifts were observed for the ring carrying the iso-pentyl chain wherein C-1″, C-3″, C-7″, C-8″ C-10″ and C-11″ were considerably deshielded to resonate at δ_c 128.1 ($\Delta\delta$ 4.6 ppm), 124.0 ($\Delta\delta$ 3.7 ppm), 24.2 ($\Delta\delta$ 2.1 ppm), 43.2 ($\Delta\delta$ 10.7 ppm), 28.4 ($\Delta\delta$ 1.8 ppm) and 28.5 ($\Delta\delta$ 1.9 ppm) while C-5″ and C-9″ were considerably shielded to resonate at δ_c 114.6 ($\Delta\delta$ 2.0 ppm) and 70.8 ($\Delta\delta$ 3.4 ppm) as compared to **1**, suggesting a change on the aromatic ring carrying iso-pentyl chain. Compound **2** was well distinguished from ESI–MS spectrum which showed pseudomolecular ions $[M+H]^+$ at 443.1699 (calcd. 443.1706 for $C_{24}H_{27}O_8$) and $[M+Na]^+$ at 465.1516 (calcd. 465.1525 for $C_{24}H_{26}O_8Na$) suggesting a molecular weight of 442 for the compound **2**, which was 18 units more than that of **1**. Based on these observations it was evident that **2** has open chain hydroxylated prenyl chain ortho to a phenolic –OH(C-4″).

Based on the reported feeding experiments for establishing the biosynthesis of xenofuranones A (**4**) and B (**5**) together with compound **3** (Brachmann et al., 2006; Nitta et al., 1983) and isolation of **1** and **2** from *A. terreus*, it is apparent that the structures of **1** and **2** are an extension of the biosynthesis of **3**, which is derived from p-hydroxyphenylpyruvate. The enzyme-catalysed cyclization or

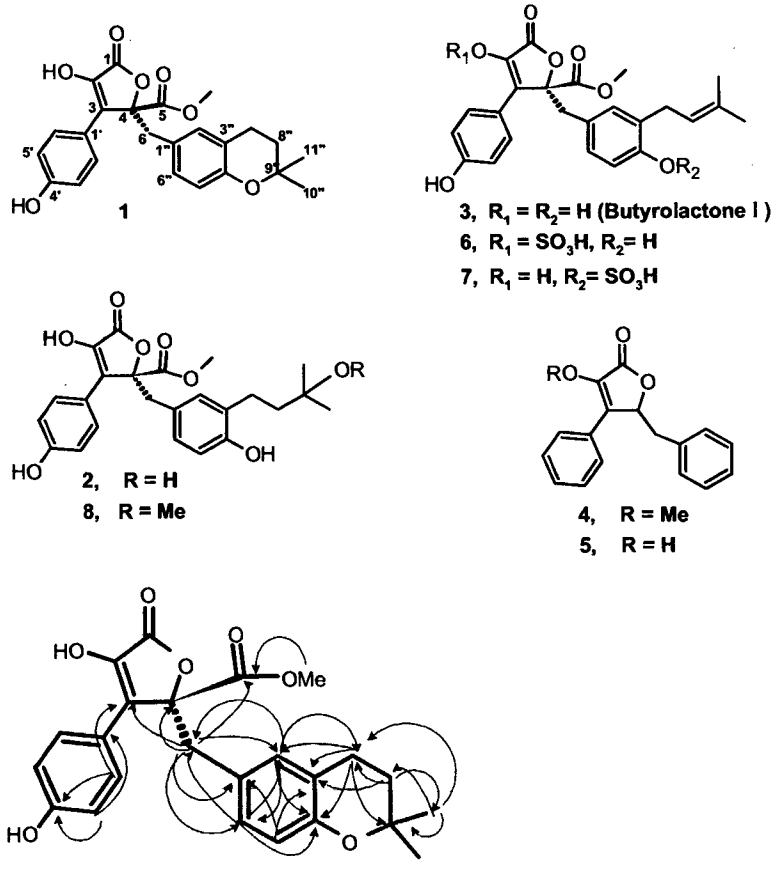
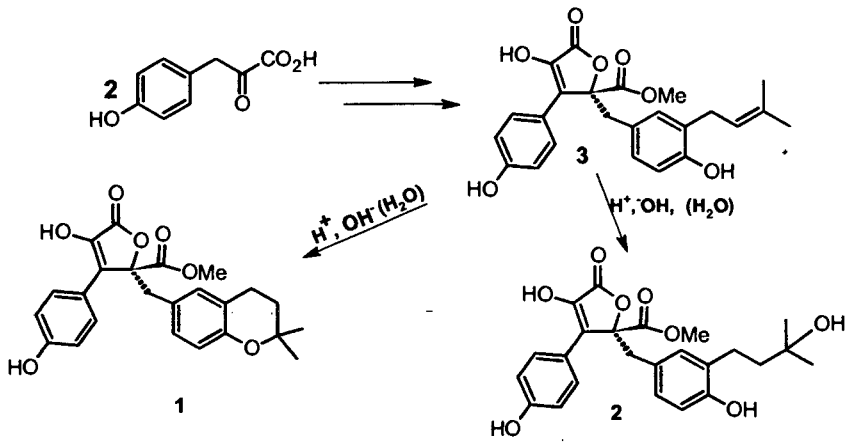


Fig. 1. HMBC correlation for 1.



Scheme 1. Biogenetic pathway of 1 and 2.

addition of water across the double bond of the prenyl chain of **3** results in the formation of **1** and **2**, respectively (Scheme 1). The last step in the biogenetic scheme was mimicked using mild acid catalysis to confirm the structure of **1**.

On heating with 1% aqueous sulphuric acid, **3** was converted to **1** as well as small amount of **2** (Scheme 2). Only 50% conversion was observed. When the same reaction was carried out using 2% conc. HCl in methanol, complete conversion of **3** was observed as indicated by TLC (90:10, $CHCl_3$/MeOH). On chromatographic separation, 75% of **1** was obtained along with minor amounts of **2** and a new product **8**. Compound **8** displayed an extra methoxy signal δ_H 3.15, *s*, δ_C 49.0 *q* in its NMR spectra compared with **2**. In the rest of the NMR spectra it was seen that C-7", C-8", C-10" and C-11" were shielded and observed at δ_c 23.2($\Delta\delta$ 1.0), 40.0 ($\Delta\delta$ 3.2), 24.0 ($\Delta\delta$ 4.4), and 24.4($\Delta\delta$ 4.1) while only methoxylated quaternary carbon C-9" was deshielded to 75.4 ($\Delta\delta$ 4.6) compared to **2**. Pseudomolecular ion peaks $[M+H]^+$ at 457.1851 and $[M+Na]^+$ at 479.1669 observed in ESI-MS indicated molecular weight of 456 for the compound. On the basis of these observations, the structure was assigned as shown in **8**. Compounds **2** and **8** are formed by Markovnikoff's addition of water and methanol respectively, across the double bond of the prenyl chain.

During the structure elucidation of **3**, compound **1** has been reported as the product of its reaction with ethanolic HCl (Kiriyama et al., 1977). In the present investigation, the same reaction in methanolic HCl yielded besides **1**, two additional products **2** and **8**. Co-metabolite **3** used in the above reaction was determined to be 4*R* configured by comparison of specific rotation data $[\alpha]_D$ + 84.32° with the previously reported result (Kiriyama et al., 1977). The absolute configurations of **1** and **2**, therefore could be deduced to be also 4*R* based on the biosynthetic grounds and similarity of the specific rotations $[\alpha]_D$ + 88.73° for **1** and +48.27° for **2**. Further confirmation of the absolute stereochemistry of **1** and **2** results from the fact that the natural **1** and **2** and those obtained as products of the acid-catalyzed reaction had identical spectral data and specific rotations $[\alpha]_D$ + 88.73° for **1** and +48.27° for **2**. Therefore, we conclude that the compounds **1** and **2** also have 4*R*-configuration.

Butyrolactone I (**3**), a metabolite of *A. terreus* var. *africans* IFO 8355 discovered in 1977, seems to be a common metabolite of this fungus (Kiriyama et al., 1977; Rao et al., 2000; Niu et al., 2008; Schimmel and Parsons, 1999). To our best knowledge, this is the first report of **3** and its derivatives from marine-derived fungus. Aspernolides B (**2**) and C (**3**) were unstable and were converted to **1** on long standing. Aspernolide C (**1**) when tested against five cell lines displayed weak cytotoxicity against H460, ACHN, Calu, Panc1 and HCT116 cell lines (IC_{50} > 88, >103, >147, >130, >121 μM, respectively).

3. Conclusion

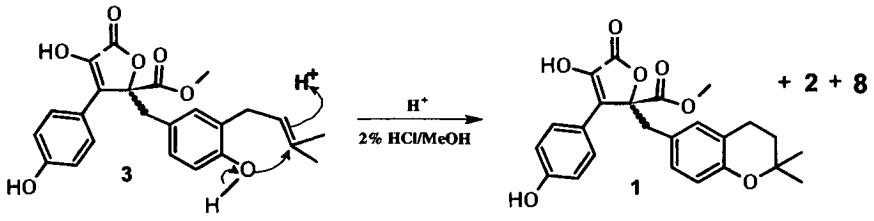
A. terreus is known to be a producer of the cholesterol lowering agent lovastatin (mevinoline) (Alberts et al., 1980) and many other important secondary metabolites such as terreineol (Macedo et al., 2004), terreulactone A (Kim et al., 2002), terrein, terreic acid and aspulvinones. The list of metabolites also includes butyrolactone I (**3**), a specific inhibitor of cdk1/cyclin B and cdk2/cyclin A (Fischer and Lane, 2000) as potent as roscovitine, a drug currently undergoing phase IIb clinical evaluation.[1] Compound **3** exhibits antiproliferative activity against colon and pancreatic carcinoma, human lung cancer (Nishio et al., 1996) and prostatic cancer (Suzuki et al., 1999). Computer aided molecular modeling using automated docking methods and molecular dynamics simulations studies (Brana et al., 2004) have proven the importance of the alkenyl (prenyl) side chain in the molecule and explains why these butenolides lacking the alkenyl side chain do not maintain antitumor activity. After considering the potential of **3** as a cdk inhibitor it can be suggested that these marine natural products **1** and **2** or synthetically modified natural products such as **8**, which retain the alkenyl side chain, could be tested in future to explore as potential cdk inhibitors.

4. Experimental

4.1. General experimental procedures

Sephadex LH-20 (Pharmacia) and silica gel (60–120 mesh, Qualigens) were used for column chromatography. Culture media Czapek agar and potato dextrose broth were procured from Himedia Ltd., Mumbai. Solvents of laboratory reagent grade used for column chromatography were purchased from a local supplier and were distilled prior to use. Petroleum ether of boiling range 60–80 °C was used for column chromatography. Precoated kiesegel 60 F_{254} TLC plates were used for analytical TLC. A mixture of methanol and chloroform (10:90, v/v) was used as mobile phase for TLC analysis. Compounds were visualized as intense rose coloured spots on spraying with methanolic sulphuric acid

[1] www.cyclacel.com/cyc/investors/news/pressreleases/2006/2006-06-29/.



Scheme 2. Conversion of butyrolactone I (3) to aspernolide A (1).

(95:5, v/v) followed by heating at 120 °C. UV and IR spectra were recorded on Shimadzu UV-2401 PC and Shimadzu FTIR-8201 PC spectrometers. Optical rotations were measured on optical polarimeter ADP220 (Bellingham & Stanley Ltd.). NMR (^{1}H, ^{13}C, DEPT, HSQC and HMBC) data were obtained on Bruker Avance 300 and Bruker Avance 500 spectrometer with TMS as internal standard. EI-MS and HRESITOFMS were recorded on Shimadzu 2010 and QSTARXL MS/MS, Applied Biosystems, Switzerland.

4.2. Fungal isolation, identification and cultivation

Soft coral *Sinularia kavarattiensis* was collected by scuba diving at a depth of 8–10 m from the coast of Mandapam, Tamil Nadu, India in May 2004. After washing the soft coral with sterile sea water, fungus *A. terreus* was isolated as an epiphyte using Czapek agar containing (g/l) $NaNO_3$ (2.0), $MgSO_4{\cdot}7H_2O$ (5.0) $FeSO_4{\cdot}7H_2O$ (0.1), KH_2PO_4 (1.0), KCl (0.5), agar (3.0) sucrose (30.0) at pH 5.5 prepared in seawater supplemented with penicillin benzyl sodium salt (0.02) to avoid any bacterial growth. After 6–7 days sand brown, velvety colonies were observed. The strain was identified as *A. terreus* from the morphological features of conidiophores by Dr. Sanjay K. Singh, mycologist, Agharkar Research Institute, Pune, India. A voucher specimen of the fungus is deposited at National Institute of Oceanography, Dona Paula, Goa, India. Stock cultures of the fungus, maintained at −20 °C preserved with 20% glycerol was used to inoculate 500 ml of seed medium in an Erlenmeyer flask (4 L) containing 24 g/l of potato dextrose broth in seawater. It was then cultured at 27 ± 2 °C on a rotary shaker at 200 rpm. The flask was incubated for 72 h and used as a first stage inoculum. The same medium (1 L) was made in 10 Erlenmayer flasks (4 L) and inoculated with 5% of first stage inoculum. The flasks were incubated for 21 days at 27 ± 2 °C on a rotary shaker at 200 rpm for 10 h/day.

4.3. Extraction and isolation of metabolites

Twenty-one days old fermentation broth (10 L) was separated from fungal mat and concentrated to a volume of 1 L under reduced pressure. The broth was extracted first with chloroform (200 ml X 4) followed by ethyl acetate (200 ml X 4). The chloroform and ethyl acetate layers were separately concentrated under reduced pressure to yield chloroform extract (470 mg) and ethyl acetate extract (430 mg). The ethyl acetate extract was chromatographed over Sephadex LH-20 using MeOH–$CHCl_3$ (1:1) to yield pure crystalline compound, terrein (92 mg) (Dunn et al., 1975) and a fraction containing an intense rose coloured spot. This fraction was flash chromatographed over silica gel using gradient elution of MeOH–$CHCl_3$ (5:95–20:80) to yield **2** (7.2 mg; Rf, 0.51). The chloroform extract was repeatedly chromatographed over Sephadex LH-20 using MeOH–$CHCl_3$ (1:1) and 100% MeOH, which yielded a pure yellow coloured compound, physcion (17 mg) (Bachmann et al., 1979). Other fractions giving two prominent rose coloured spots on TLC were further purified on a silica gel column using gradient elution with MeOH–$CHCl_3$ (0:100–20:80) to afford **3** (32.0 mg; Rf, 0.64) and another nearly pure compound which was purified over Sephadex LH-20 using MeOH–$CHCl_3$–Pet. ether (40:40:20) to yield **1** (26.3 mg; Rf, 0.81).

4.3.1. Aspernolide A (1)

White sticky solid; $[\alpha]_D^{28}$ + 88.73° (c 0.58, $CHCl_3$); UV (MeOH) λ_{max} nm : 303, 240; IR (NaCl) ν_{max} cm^{-1} 3340, 3024, 2970, 2935, 1737, 1732, 1610, 1519, 1498, 1436, 1386, 1261, 1182, 1122, 1068, 1037, 948, 839, 754; For NMR data see Table 1; HRESITOFMS $[M+Na]^+$ *m/z* 447.1433 (calcd. 447.1420 for $C_{24}H_{24}O_7Na$), $[2M+Na]^+$ *m/z* 871.2959 (calcd. 871.2942 for $C_{48}H_{48}O_{14}Na$); EIMS *m/z*(%): M^+ 424(2.7), 380(37.8), 348(35.1), 320(6.8), 293(13.5), 265(8.1), 237(10.8), 218(5.4), 205(6.8) 189(6.8), 175(100), 157(8.1), 145(10.8), 131(16.2), 119(13.5), 107(5.4), 91(24.3), 77(3.5), 69(12.1), 44(37.8).

4.3.2. Aspernolide B (2)

Light brown syrup; $[\alpha]_D^{28}$ + 48.27° (c 0.29, MeOH); UV (MeOH) λ_{max} nm : 303, 240; IR (NaCl) ν_{max} cm^{-1} 3380, 3024, 2975, 2933, 1745, 1610, 1519, 1442, 1386, 1182, 1070, 1037, 838, 762; For ^{1}H and ^{13}C NMR spectroscopic data see Table 2; HRESITOFMS: $[M+H]^+$ *m/z* 443.1699 (calcd. 443.1706 for $C_{24}H_{27}O_8$), $[M+Na]^+$ *m/z* 465.1516 (calcd. 465.1525 for $C_{24}H_{26}O_8Na$); EIMS *m/z*(%): $[M-CO_2]^+$ 398(13), 380(70), 348(100), 333(18), 320(15), 293(40), 205(26), 249(10), 237(23), 218(17), 205(25), 188(16), 175(77), 145(13), 131(41), 119(23), 107(15), 91(35), 77(17), 69(20), 59(33), 43(23), 41(11).

Table 1
NMR spectroscopic data of aspernolide A (**1**) (500 MHz, $CDCl_3$).

Carbon No.	δ_C, mult.	δ_H, mult., *J*(Hz)	HMBC
1	169.3 *s*		
2	137.2 *s*		
3	128.8 *s*		
4	86.1 *s*		
5	169.6 *s*		
6	38.6 *t*	3.39, *d* (15.0), 3.59, *d*, (15.0)	C4, C1″, C6″, C3, C2″, C5
1′	122.2 *s*		
2′(6′)	129.5 *d*	7.56, *d* (8.7)	C4′, C3
3′(5′)	115.9 *d*	6.86, *d* (8.7)	C1′, C4′
4′	156.4 *s*		
1″	123.5 *s*		
2″	131.4 *d*	6.53, *s*	C7″, C6, C6″, C4″
3″	120.3 *s*		
4″	152.9 *s*		
5″	116.6 *d*	6.47, *s*	C1″, C3″
6″	129.0 *d*	6.47, *s*	C6, C2″, C4″
7″	22.1 *t*	2.53 *m*	C8″, C9″, C2″, C3″, C4″
8″	32.5 *t*	1.66, *t* (6.5)	C3″, C7″, C9″, C10″(11″)
9″	74.2 *s*		
10″(11″)	26.6 *q*	1.21, *s*	C7″, C8″, C9″
5-OMe	53.4 *q*	3.72, *s*	C5

Table 2
^1H and ^{13}C NMR spectroscopic data of compounds **2** and **3** (300 MHz, $CDCl_3$+ 2drops CD_3OD).

Carbon No.	Aspernolide B (**2**) δ_C, mult.	δ_H, mult., *J* (Hz)	Aspernolide C (**3**) δ_C, mult.	δ_H, mult., *J* (Hz)
1	169.2 *s*		169.3 *s*	
2	137.9 *s*		137.9 *s*	
3	128.3 *s*		128.2 *s*	
4	85.6 *s*		85.7 *s*	
5	170.1 *s*		170.1 *s*	
6	38.3 *t*	3.46, *s*	38.4 *t*	3.43, *s*
1′	121.6 *s*		121.8 *s*	
2′(6′)	129.1 *d*	7.55, *d* (8.7)	129.2 *d*	7.54, *d* (8.7)
3′(5′)	115.6 *d*	6.87, *d* (8.7)	115.0 *d*	6.85, *d* (8.7)
4′	157.5 *s*		157.5 *s*	
1″	128.1 *s*		128.1 *s*	
2″	131.7 *d*	6.41, *d* (1.8)	131.8 *d*	6.40, *d* (1.8)
3″	124.0 *s*		124.0 *s*	
4″	153.2 *s*		153.4 *s*	
5″	114.6 *d*	6.52, *d* (8.7)	114.4 *d*	6.49, *d* (8.1)
6″	128.6 *d*	6.55, *dd* (8.7, 1.8)	128.9 *d*	6.54, *dd* (8.1, 1.8)
7″	24.2 *t*	2.37, *m*	23.2 *t*	2.35, *m*
8″	43.2 *t*	1.53, *t* (7.8)	40.0 *t*	1.54, *t* (7.8)
9″	70.8 *s*		75.4 *s*	
10″	28.4 *q*	1.20, *s*	24.0 *q*	1.12, *s*
11″	28.5 *q*	1.19, *s*	24.4 *q*	1.12, *s*
12″	–		49.0 *q*	3.15, *s*
5-OMe	53.5 *q*	3.76, *s*	53.3 *q*	3.74, *s*

4.3.3. Butyrolactone I (3)

White powder; $[\alpha]_D^{28.8} + 84.32°$ (c 0.617, MeOH); and $[\alpha]_D^{28.8} + 87.56°$ (c 0.617, $CHCl_3$); ^1H NMR and ^{13}C NMR data were in agreement with the literature (Rao et al., 2000); HRESITOFMS: $[M + H]^+$ *m/z* 424.1516 (calcd. 424.1522 for $C_{24}H_{25}O_7$).

4.3.4. Aspernolide C (8)

Light brown syrup; for ^1H and ^{13}C NMR spectroscopic data see Table 2; HRESITOFMS: $[M + H]^+$ *m/z* 457.1851 (calcd. 457.1862 for $C_{25}H_{29}O_8$) and $[M + Na]^+$ *m/z* 479.1669 (calcd. 479.1682 for $C_{25}H_{28}O_8Na$).

4.4. Conversion of butyrolactone I (3) to aspernolides A (1), B (2) and C (8)

Butyrolactone I (**3**), (76.9 mg) was dissolved in MeOH (10 ml) containing conc. HCl (0.2 ml). The mixture was stirred at rt approximately for 2 h. or until complete conversion of **3** as indicated by TLC. The solvent was removed under vacuum and the resulting residue was separated on a flash Si-gel column using gradient elution of MeOH–$CHCl_3$ (0:100–20:80) to yield in order of increasing polarity **1** (58.6 mg, 75%), **8** (8.8 mg, 10.4%) and **2** (6.9 mg, 8.3%).

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