Chemical Investigation of Some Selected Marine Organisms

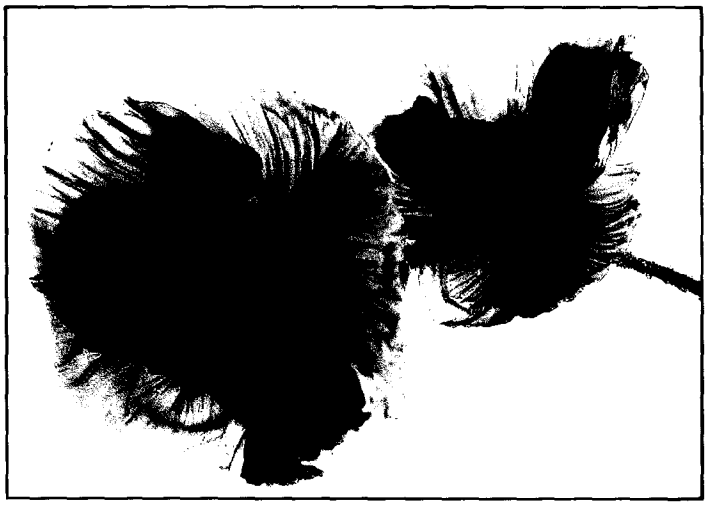
Thesis submitted to Goa University
for the degree of
Doctor of Philosophy
in
Chemistry

By
Supriya Tilvi, M.Sc.

National Institute of Oceanography
Council of Scientific and Industrial Research
Dona Paula, Goa-403 004, INDIA

2005

Dedicated
to
my parents
and
Brother



For all their love, support, encouragement and guidance,
without which my dreams wouldn't have come true.

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Ph.D Thesis

Ms. Supriya Tilvi, M.Sc.

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Declaration

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

26/09/05

Dr. C. G. Naik
(Research Guide)

Ms. Supriya Tilvi
(Candidate)

Certificate

This is to certify that the thesis entitled "Chemical investigation of some selected marine organisms", submitted by Ms. Supriya Tilvi for the award of the degree of Doctor of Philosophy in Chemistry is based on her original studies carried out by her under my supervision. The thesis or any part thereof has not been previously submitted for any other degree or diploma in any Universities or Institutions.

Dr. C. G. Naik
Research Guide
Scientist
National Institute of Oceanography
Dona Paula – 403 004, Goa

CONTENTS

Acknowledgements

Education is not preparation for life; education is life itself.

~John Dewey

The work in this thesis has been an inspiring, often exciting, sometimes challenging, but moreover an interesting experience. This work is not only the result of many years of dedicated work, but also the contributions of many people. It was a privilege to be part of a team consisting of so many talented people and I learned much from them.

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A seed can grow into a plant only if it is grown in the proper environment. With this respect, I have been very lucky and I feel deeply gratified to many people who have accompanied me. Perhaps most significant to my success was the love and support of my parents. They provided me with every opportunity to succeed in life and the encouragement I needed to succeed. Their love, concern and pride in my work were always a major source of strength to me. Their encouragement, support and personal sacrifices made an everlasting impression on my life. I am grateful to all my other loving family members for always lending their support and unconditional love as well as giving advice and a shoulder when the things seemed overwhelming.

This thesis is also dedicated to my brother, Vithal, for being my role model, and instilling in me the inspiration to set high goals and the confidence to achieve them.

Supriya Tilvi

GENERAL REMARKS

Compounds used are commercially available. All the solvents used were dried and freshly distilled. All melting points were measured on a digital melting point apparatus (Electothermal 9100) and were uncorrected.

Silica gel 60 F_{254} plates (Merck, 0.2 mm) TLC plates (aluminium sheets) were used. Silica gel (Merck, 60-120 mesh, 200-400 mesh) was used for column chromatography.

UV-Vis spectrophotometer (Shimadzu) was used to record λ_{max} (nm). Infra red spectra were taken on Shimadzu FTIR spectrophotometer. ^{1}H and ^{13}C NMR spectra including 2D experiments (COSY, TOCSY, HMQC and HMBC) were recorded on Bruker (Avance 300MHz) spectrometer using TMS as internal standard unless otherwise stated. ESI-MS/MS spectra were recorded on QSTAR XL (Applied Biosystems, Canada) mass spectrometer. Optical polarimeter (ADP220 polarimeter, Bellingham and Stanley Ltd.) was used to record optical rotation.

All figures, tables, structure numbers and references in a section refer to that particular section only.

Abbreviations

α	Alpha
Amu	Atomic mass unit
AIDS	Acquired immunodeficiency syndrome
AMD	Age related macular degeneration
br s	broad singlet
β	Beta
c	Concentration (g/100 ml)
°C	Degrees celsius
CAD	Collisionally activated dissociation
CD	Circular dichroism
$CDCl_3$	Deuterated chloroform
CD_3OD	Deuterated methanol
CFU	Colony forming units
$CHCl_3$	Chloroform
CH_2Cl_2	Dichloromethane
CID	Collision induced dissociation
cm	10^{-2} metre
COLO-205	Human colon carcinoma
COSY	Correlated spectroscopy
d	doublet
dd	Doublet of doublet
δ	NMR chemical shift (ppm)
D	Dextra rotatory isomers
1D	One dimensional
2D	Two dimensional
Da	Dalton
DCM	Dichloromethane
DEPT	Distortionless enhancement by polarisation transfer
DEHP	bis(2-ethylhexyl)phthalate
DNA	Deoxyribrose nucleic acid
E	Entegegen
ED_{50}	Effective dose 50%
EDTA	Ethylene diamine tetra acetic acid
e.g.	example given
EGF	Epidermal growth factor inhibitor kinase
ESI-MS	Electrospray ionisation-Mass spectrometry
EtOAc	Ethyl acetate
FAB	Fast atom bombardment
g	gram
GABA	γ-amino butyric acid
GC	Gas chromatography
GI_{50}	Growth inhibitory power
h	Hour
HCT-116	Human colon tumor cell line
HIV-1	Human immunodeficiency virus 1

HMBC	Hetero nuclear multiple bond correlation
HMQC	Hetero nuclear multiple quantum coherence
HPLC	High performance liquid chromatography
HR	High resolution
Hz	Hertz
H_2O	Water
I_2	Iodine
IC_{50}	Inhibitory concentration 50%
i.d.	Internal diameter
IR	Infrared spectroscopy
J	Spin-spin coupling constant [Hz]
$^2J_{CH}/^3J_{CH}$	1,2 and 1,3 carbon-proton correlations
KB cells	Human carcinoma of the nasopharynx
KBr	Potassium bromide
Kg	Kilogram
LCB	Long chain base
LINAC	Linear accelerating collision cell quadrupole
L	Laevo rotatory isomer
L-1210	Lymphocytic leukemia
LOVO	Human colon cancer cell lines
LSI-MS	Liquid secondary ionization-Mass spectrometry
m	multiplet
MALDI	Matrix assisted laser desorption ionization
MCV	Molluscum contagiosum virus topoisomerase
MeOH	Methanol
mg	Milligram
MHz	Megahertz
min	minute
MIC	Minimal inhibitory concentration
ml	10^{-3} litre
mM	10^{-3} Mol
mm	Millimeter
m.p.	melting point
m/z	mass to charge ratio (amu)
µg	10^{-6} gram
µL	10^{-6} litre
µm	10^{-6} metre
µM	10^{-6} Molar
ε	Molar absorptivity
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
MTPA	∝-methoxy-2-(trifluoromethyl)phenylacetyl chloride
nm	10^{-9} metre
NKT	Natural killer T-cell
NMR	Nuclear magnetic resonance
ODS	Octadecylsilane
$[\alpha]_D$	Optical rotation

%	Percentage
P-388	Mouse leukemia
PDA	Potato dextrose agar
PDB	Potato dextrose broth
pp1	Protein phosphatase type 1
ppm	Parts per million
q	quartet
R	Stereoisomers
ROESY	Rotational nuclear overhauser effect spectroscopy
RP	Reversed phase
RT	Room temperature
Rt	Retention time
R_f	Retention factor
s	singlet
S	Stereoisomers
SA	Sphinganine
SARS	Sudden acute respiratory syndrome
SIM	Selected ion monitoring
SKMEL-2	Human caucasian melanoma
SM	Sphingomyelin
sp.	species
t	triplet
TAG	Triacylglycerol
TFA	Trifluoroacetic acid
TK	Tyrosine kinase
TLC	Thin layer chromatography
TOCSY	Total correlated spectroscopy
TOF	Time of flight
UV	Ultra Violet
V	Volt
VEGF	Vascular endothelial growth factor
λ	Wavelength (nm)
ν	Wave number (cm^{-1})
z	Zusammen

Introduction

Nature has continuously provided mankind with a broad and structurally diverse arsenal of pharmacologically active compounds that continue to be utilised as highly effective drugs to combat a multitude of deadly diseases or as lead structures for the development of novel synthetically derived drugs that mirror their models from nature.

For centuries, extracts of terrestrial plants were used in the treatment of diseases although the nature of the compounds in the mixture was not exactly known. Later on, with the discovery of penicillin, an antibiotic, from the fungus *Penicillium notatum* by Alexander Fleming, attention was focused on microorganisms as a new source of drugs and many new families of antibiotics have resulted from these studies. At present, with the development of bacterial resistance towards the existing drugs and emergence of new diseases (e.g., AIDS, SARS) a need is felt for search of new more powerful drugs and the oceans are the last frontier which remains to be explored as possible source of new drugs.[1]

The oceans cover more than 70% of the world surface and among 36 known living phyla, 34 of them are found in marine environments with more than 300,000 plus known species of fauna and flora.[2] The rationale of searching for drugs from marine environment stems from the fact that marine plants and animals have adapted to all sorts of marine environments and these creatures are constantly under tremendous selection pressure including space competition, predation, surface fouling and reproduction. Over hundred million years of selection, it has led to the evolution and production of various secondary metabolites to offset negative effects of selection force. The outcome is that there exist vast arrays of natural compounds that could benefit human beings if these compounds could be identified and examined for its effects.[3]

1.1 The evolution of marine natural product chemistry

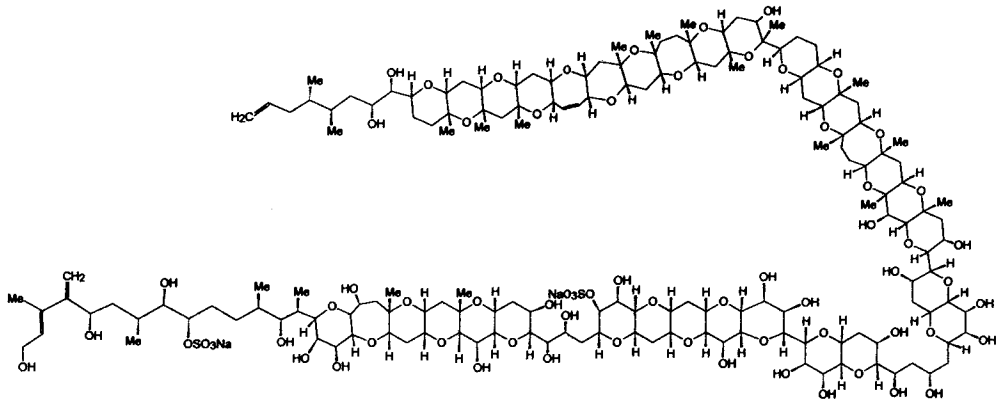
Nature has been instrumental as a source for therapeutics. Despite the fact that we live in an oceanic planet, a number of technical factors have historically hampered the evolution of a marine-based medicine. With the development of scuba diving

for the collection of the specimens and sophisticated instruments for the isolation and elucidation of structure of natural products from marine organisms, major advances have been made in the discovery of marine derived therapeutics.

Marine Natural Products Chemistry is essentially a child of the 1970's that developed rapidly during the 1980's and matured in the last decade. By 1975 there were already three parallel tracks in marine natural products chemistry: marine toxins, marine biomedicinals and marine chemical ecology. It is the integration of the three fields of study that has given marine natural products chemistry its unique character and vigour.[4]

Initially, the course of structure elucidation was incredibly complex and indirect, with the need to combine evidence from many different types of experiments. For example functionalities such as amino, ketone or aldehyde groups were recognized based on specific derivatisation of the group followed by redetermination of molecular formula; the change in molecular composition leading to the identification of the functionality.[5] Hence, only the major constituents could be identified by painstaking structure elucidation process while the compounds present in minor quantities remained uninvestigated. The technological advances over the last 50 years have seen the invention and introduction of instrumentation, such as the High performance liquid chromatography (HPLC) and Nuclear magnetic resonance spectrometer (NMR). This equipment has enabled chemists to isolate trace quantities of material, which can subsequently be used for non-destructive structural elucidation work. One noteworthy example of the application of this technology is the isolation and structure elucidation of maitotoxin **(1)** during the mid 1990's. This polyether toxin, has a molecular weight of 3422 Da, from the marine dinoflagellate, *Gambierdiscus toxicus*, and to date represents the largest molecular structure (excluding biopolymers) known to natural products chemists.[6-8] Only 8.1mg of **(1)** was initially isolated, chemically degraded and HPLC purified to produce fragments of this metabolites which were subsequently elucidated by a variety of

NMR experiments. After extensive analysis of the NMR data the polyether structure 1, was assigned to maitotoxin.



Maitotoxin (1)

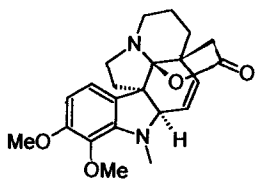
Multi-dimensional NMR technology serves as a versatile tool in the structure determination of organic compounds. However, when molecular weights exceed 1000 Da, 2D NMR spectra become complicated and less informative. In such cases, structural data derived from mass spectrometry (MS) are very informative. The current focus on analytical techniques in the pharmaceutical industry emphasizes four primary figures of merit; sensitivity, selectivity, speed and high throughput. MS provides each of these key attributes, and therefore, has been benchmarked an effective solution for pharmaceutical analysis in each stage of drug development.[9] Perhaps more enabling than the MS-based technology itself is the diverse applications of MS in conjunction with sample preparation, chromatographic separation, and informatics. It is within this context that MS has played an increasingly vital role in the pharmaceutical industry and has become the preferred analytical method for trace-mixture analysis.[10]

Recent developments in MALDI (Matrix Assisted Laser Desorption Ionization) and ESI (Electrospray Ionization) combined with TOF (Time Of Flight) mass spectrometers greatly facilitated the mass measurements of compounds over 2000 Da. CID-MS/MS (Tandem mass spectrometry) serves as a powerful and practical method for the elucidation of complicated and large organic molecules.[11]

New technologies are constantly introduced into drug development to address throughput issues and improve development cycles. This has resulted in fundamental change in the drug development paradigm. Recently, sample generating based technologies such as high throughput biomolecular screening and automated parallel synthesis have shifted the bottleneck to sample analysis-based technologies.[10]

Advances in synthetic organic chemistry are another gift for natural product chemistry. Total synthesis of complex molecules are now often reported concomitantly with their structure determination. With these powerful tools in hand, the structures of large and complicated natural products can be elucidated in a much shorter period.[12]

For over 25 years, aspidophytine **(2)** has remained an unanswered challenge for organic synthesis. Best known for its use as an anticockroach/insecticidal powder.[13] Its complex structure was not elucidated until 1973 by Yates and his groups.[14] The first total (enantioselective) synthesis of this molecule was finally completed in 1999 by E. J. Corey and co-workers and featured a rapid assembly of the aspidophytine core via a novel cascade sequence.[15] Aside from developing a breathtaking new domino sequence to assemble the aspidophytine skeleton, this work raises the standards for the concise synthesis of extremely complex alkaloids from simple starting materials.



Aspidophytine (2)

Collaboration between academic researchers and pharmacologists has resulted in great progress in finding drugs from marine animals and plants. For instance, about 300 patents on bioactive marine natural products have been issued between

1969 and 1999. So far, more than 10,000 compounds have been isolated from marine organisms.[3]

1.2 Marine bioactive potentials:

In recent years, a significant number of novel metabolites with potent pharmacological properties have been discovered from the marine organisms. Although there are only a few marine-derived products currently in the market, several robust new compounds derived from marine are now in pipeline, under clinical development. While the marine world offers an extremely rich resource for novel compounds, it also represents a great challenge that requires multidisciplinary approach to bring the marine chemical diversity up to its therapeutic potential. It is interesting to note that majority of marine natural products currently in clinical trials or under pre-clinical evaluation are produced by invertebrates such as sponges, tunicates, molluscs and bryozoans **(Table 1)**. The wealth of bioactive metabolites isolated from soft-bodied, sessile or slow moving marine invertebrates that usually lack morphological defense structures such as spines or a protective shell is no coincidence but reflects the ecological importance of these constituents for the respective invertebrates. It has been repeatedly shown that chemical defense through accumulation of toxic or distasteful natural products is an effective strategy to fight off potential predators (e.g. fishes) or to force back neighbours competing for space.[16-18]

Of the marine natural products (or analogues) that are currently under clinical development as potential anti-cancer agent is the tetrahydroisoquinolinoline alkaloid produced by the tunicate *Ecteinascidia turbinate*, Ecteinascidin 743 [ET-743, Trabectedin, Yondelis] **(3)**. It is the only compound, which has reached Phase III clinical trial.[19] It is a novel DNA interactive agent that has shown *in vivo* activity in nude mice harbouring human resistant xenografted tumors. The compound demonstrated high potency against a broad spectrum of tumour types in animal models. It has been approved as an Orphan Drug by European commission.[20,21]

The dolastatins are series of cytotoxic linear peptides isolated from the Indian Ocean sea hares *Dollabella auricularia.* Dolastatin 10 **(4)** a well known antitumour agent against prostrate cancer, metastatic melonoma, *etc.*[21] was discontinued in Phase II clinical trial due to non-consistency in result. But other dolastatins and related molecules are still under clinical development.

Bryostatin 1 **(5)** from the marine bryozoan, *Bugula neritina* was originally described as antitumor agent inhibiting the growth of murine P388 lymphocytic leukemia cells at subnanomolar concentrations.[19] A range of properties have been subsequently described including activation of T-cells, immunomodulation and stimulation of haematopoietic progenitor cells, and its molecular site of action was identified. It was found to bind to protein kinase C with high affinity, which may be the mechanistic basis for both observed anticancer and immunostimulating activities. The recent Phase I clinical trials of bryostatin 1 in combination with vincristine, Ara-C and Fludarabine provided encouraging results. It is now being tested in Phase II human clinical trials by the National Cancer Institute (NCI) under an agreement with Bristol-Mayers. Bryostatin 1 may be effective in tandem with cancer treatments that respond to taxol, such as breast, ovarian and lung cancers.[19]

Didemnin B **(6),** depsipeptide isolated from the Caribbean tunicate *Tridemnum solidum* (Didemnidae) displayed antineoplastic, antiviral and subsequently immunosuppressive activites.[22] Mechanistically, didemnin B acts at the GTP-binding protein elongation factor. This compound though toxic is useful as antiviral or immunosuppressive agent and is in Phase II clinical trials as an anticancer agent.

Aplidin [APL, Aplidine, Dehydrodidemnin B] **(7)** also a depsipeptide from the tunicate, *Aplidium albicans* is under clinical development since 1999. It induces cytotoxicity in a non-MDR/p53 dependent manner, blocks the cell cycle progression at G1 and decreases the secretion of the Vascular Endothelial growth factor (VEGF) and the expression of the VEGF-r1 receptor.[23] Its phase I clinical

studies confirmed the positive therapeutic index in patients harbouring pretreated solid tumors and lymphoma.[24] Consistent evidence of activity has been noted in pretreated neuroendocrine tumors and other tumors types. Phase II clinical trials are under way in Europe for renal and colon carcinomas. European Commission has approved aplidine for acute lymphoblastic leukemia and other trials covering renal, head and neck, and medullary thyroid are ongoing, simultaneously.[23]

KRN-7000 **(8)** is a biological response modifier that belongs to glycosphingolipids or agelasphins obtained from marine sponge *Agelas mauritianus*. It demonstrated antitumor and potential immunostimulatory activities. This compound entered Phase I clinical trials in both Asia and Europe in 2001 for cancer immunotherapy.[21] Both reports on the PK and effects on Natural Killer T-cell (NKT-cells) populations in patients have been reported from the same phase I trial. No significant adverse effects were seen, and biological effects were observed in the patients with high levels of NKT-cells. Since no objectionable antitumor responses were reported from this trial, it was felt that a preselection of patients with high natural NKT cells might give objective responses in other trials.[25]

(+)-Discodermolide **(9)**, polyketide lactone from the Caribbean sponge *Discodermia dissoluta* is a potent inhibitor of tumor cell growth and has been prepared in a 39-step synthesis by the Novartis Chemical & Analytical Development Group in Basel, Switzerland.[26] The synthetic material is now undergoing Phase I clinical trials for pancreatic cancer at the Cancer Therapy & Research Center in San Antonio, Texas.

Halichondrin B **(10)** isolated from the Japanese sponge *Halichondria okadai* is one of the most potent of the halichondrins against P-388 leukemia, B-16 melanoma and L-1210 leukemia *in vivo*. Halichondrin B, along with isohomohalichondrin B, is under Phase I clinical trials at NCI and Pharma Mar, respectively, for various types of cancers.[27]

Squalamine lactate (EVIZON™, **11**), a naturally occurring antiangiogenic steroidal compound, found in tissues of the dogfish shark is a systemically administered anti-angiogenic drug used for the treatment of choroidal neovascularization associated with age-related macular degeneration (AMD), also known as "wet" AMD. It is the first clinical drug candidate in a class of naturally occurring, pharmacologically active, small molecules known as aminosterols. EVIZON is a potent molecule with a unique multi-faceted mechanism of action that blocks the action of a number of angiogenic growth factors, including vascular endothelial growth factor (VEGF), cytoskeleton and integrin expression. Genaera has performed clinical trials evaluating EVIZON in the treatment of non-small cell lung cancer, ovarian cancer, and other adult solid tumors, and for age-related macular degeneration (AMD), the leading cause of blindness in the United States.[28]

Kahalalide F **(12)**, cyclic depsipeptide isolated from the Hawaiian mollusk, *Elysia rufescens* showed positive evidence of *in vivo* activity in human models of androgen independent prostate cancer and other solid tumors. It is now tested in phase II on patients with advance liver cancer and other tumors.[29] This compound has also been isolated from *Elysia grandifolia,* which is discussed in details in Chapter 3.

Cyclodidemniserinol trisulfate **(13)**, an inhibitor of HIV-1 integrase from the Palauan ascidian *Didemnum guttatum* is an attractive target for anti-retroviral chemotherapy.[30]

Ziconotide (Conotoxin MVIIV, Prialt) **(14)** is a 25 amino acid peptide from the venom of a predatory snail *Conus magnus.* It acts by binding to and inhibiting presynaptic calcium channels, thereby preventing neurotransmitter release.[31] Ziconotide is 50 times more potent analgesic than morphine and does not cause the adverse effects of opiates. It stops pain messages from getting through while allowing the rest of the nervous system to function normally. United States Food

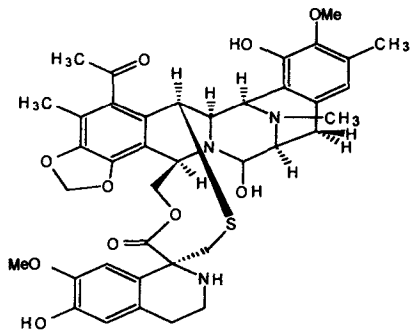
and Drug Administration (USFDA) approved ziconotide for hard-to-treat pain associated with cancer, AIDS and neuropathies in Dec. 2004.[32]

IPL-576 **(15)**, a synthetic analogue of the steroid contignasterol isolated from the sponge *Petrosia contignata*, is in Phase II trials as a leukocyte-suppressing anti-inflammatory drug from the treatment of asthma.[33,34]

Methopterosin **(16)**, diterpene glycosides so-called pseudopterosins from the extracts of Caribbean gorgonian *Pseudopterogorgia elisabethae* is currently in Phase I clinical trials as a potential new anti-inflammatory agent.[35]

Manoalide **(17)**, sesquiterpenoid isolated from the Indo-Pacific sponge *Luffariella variabilis* is a potent analgesic and anti-inflammatory agent and is far the best characterized PLA2 inhibitor from natural sources. At low concentrations, manolide inhibited calcium channels with no effect on phosphoinositide metabolism. Allergen Pharmaceuticals conducted Phase I clinical trials on manolide for the treatment of psoriasis and launched a medicinal chemistry program. Though no pharmaceutical based on manoalide has yet reached the drug stores, manolide itself is commercially available as a standard probe for PLA2 inhibition.[31]

GTS-21 **(18)**, 3-(2,4-dimethoxy benzylidene)-anabaseine, is a selective α7-nicotinic acetylcholine receptor partial agonist in clinical development at Taiho to treat Alzheimer's disease and schizophrenia.[36]



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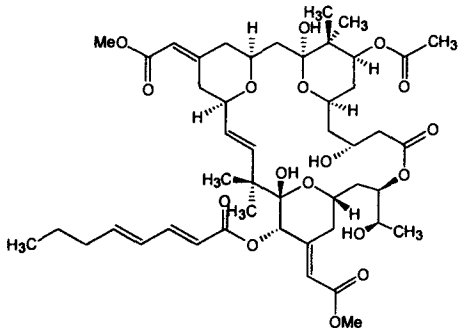
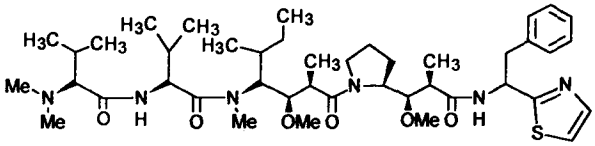
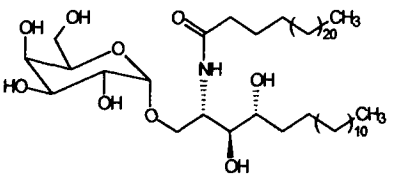


Bryostatin 1(5)



Dolastatin 10 (4)



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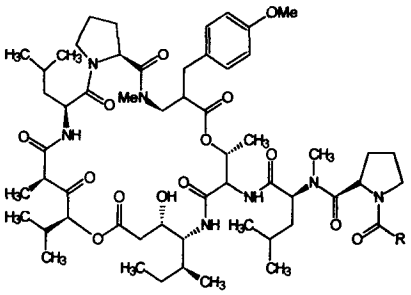
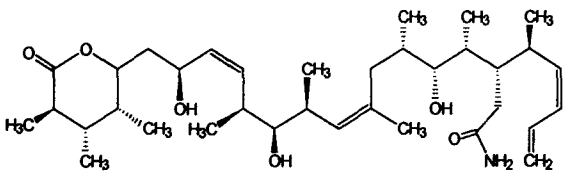
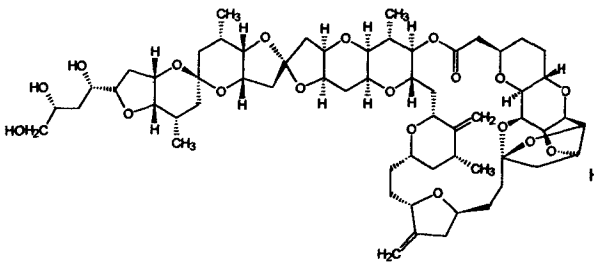


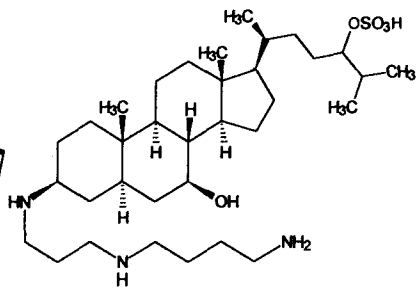
Didemnin B R = $CH(OH)CH_3$ (6)
Dihydrodidemnin B R = $COCH_3$ (7)
(Aplidine)



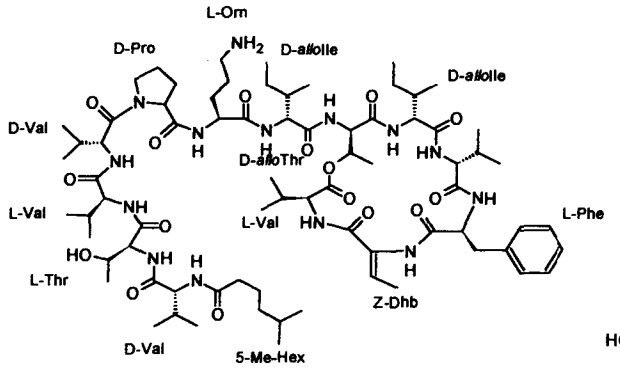
(+)-Discodermolide (9)



Halichondrin B (10)



Squalamine (11)



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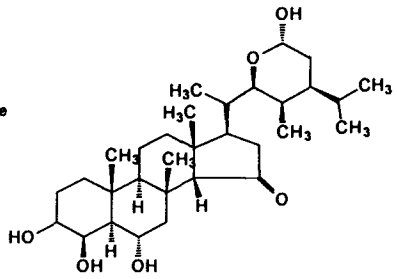
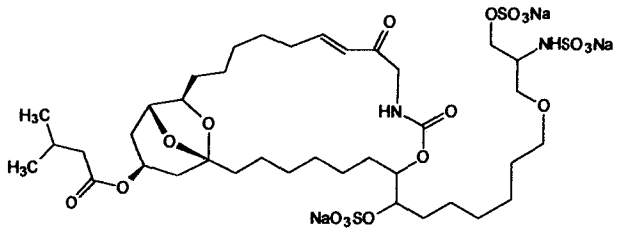
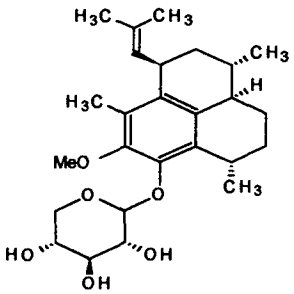


IPL-576 (15)



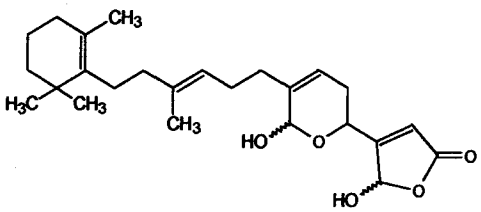
Cyclodidemniserinol trisulfate (13)



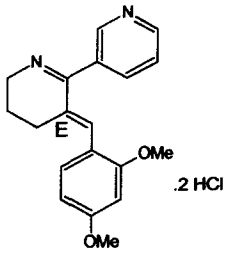
Methopterosin (16)

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

Ziconotide (14)



Manoalide (17)



GTS-21 (18)

Table 1: Selected marine natural products currently in clinical trials.

Source	Compounds	Disease area	Phase of Clinical trials
Ecteinascidia turbinata (tunicate)	Ecteinascidin 743 **(3)**	Cancer	II/III
Dolabella auricularia (sea hare)	Dolastatin 10 **(4)**	Cancer	II
Bugula neritina (bryozoan)	Bryostatin 1 **(5)**	Cancer	II
Tridídemnum solidum (tunicate)	Didemnin B **(6)**	Cancer	II
Aplidium albicans (tunicate)	Aplidine **(7)**	Cancer	I/II
Agelas mauritianus (sponge)	KRN7000 **(8)**	Cancer	I
Discodermia dissoluta (sponge)	(+)-Discodermolide **(9)**	Cancer	I
Halichondria okadai (sponge)	Halichondrin B **(10)**	Cancer	I
Squalus acanthias (shark)	Squalamine **(11)**	Cancer	II
Elysia rufescens, (mollusk)	Kahalalide F **(12)**	Cancer	II
Didemnum guttatum (tunicate)	Cyclodidemniserinol trisulfate **(13)**	HIV	II
Conus magnus (cone snail)	Ziconotide **(14)**	Pain	III
Petrosia contignata (sponge)	IPL 576 **(15)**	Inflammation/ asthma	I
Pseudopterogorgia elisabethae (soft coral)	Methopterosin **(16)**	Inflammation/ wound	I
Luffariella variabilis (sponge)	Manoalide **(17)**	Inflammation/ psoriasis	I
Amphiporus lactifloreus (marine worm)	GTS-21 **(18)**	Alzheimer/ schizophrenia	I

1.3 The supply issue

A serious obstacle to the ultimate development of most marine natural products that are currently undergoing clinical trials or that are in preclinical evaluation is the problem of supply. The concentrations of many highly active compounds in marine invertebrates are often minute, sometimes accounting for less than 10^{-6} % of the wet weight. For example, in order to obtain approximately 1 g of the promising anti-cancer agent ET-743, close to 1 metric tonne (wet weight) of the tunicate *E. turbinata* has to be harvested and extracted.[37] In other cases, such as halichondrins (e.g halichondrin B), which are powerful cytostatic polyketides of sponge origin, the ratio of biomass to yield of product is even less favourable. In

order to obtain as little as 300 mg of a mixture of two halichondrin analogues, 1 metric tonne of the sponge *Lissodendoryx* sp. had be collected and extracted.[38] This already causes considerable difficulties and delays in clinical studies where gram quantities of compounds are generally needed but will prove to be an overwhelming obstacle once one of these compounds is licensed as a drug. For example, if the halichondrins make it to the market as new anti-cancer drugs the annual need for these compounds is estimated to be in the range 1–5 kg per year, which corresponds to roughly 3,000–16,000 metric tonnes of sponge biomass per year.[38] It is obvious that such large amounts of biomass of either sponges, tunicates or other pharmacologically promising marine invertebrates can never be harvested from nature without risking extinction of the respective species. Alternative strategies for an environmentally sound and economically feasible supply of marine natural products are therefore needed.

The commercial source of choice for the pharmaceutical industry is synthesis, which allows the company to control all aspects of production. This is the best solution for relatively simple compounds but many bioactive marine natural products are extremely complex and require multi-step syntheses of heroic proportions. For these more complex molecules, it seems best to elucidate the mechanism of action and identify the pharmacophore so that simpler compounds can be synthesized. Wender's recent research on simplifying the bryostatin structure while retaining bioactivity is an excellent example of this approach.[39]

If synthesis is not economically viable, mariculture should be considered as an alternative for large-scale production of active molecules. Shallow water specimens may be transplanted and grown in sheltered waters or in artificial raceways but the successful culture of deep water specimens may require considerable research effort. *Bugula neritina*, the source of bryostatins, has been produced under controlled conditions by Cal Bio-Marine Technologies while Battershill and his colleagues in New Zealand have reported success in growing even deepwater sponges under experimental aquaculture conditions.[40] It is hoped that future developments in the field of mariculture will make it possible for

marine invertebrates to be cultured as part of community-based conservation projects in the developing nations, thereby providing an economic incentive for the restoration of coral reef environments. An attractive alternative to mariculture of sponges would be to grow sponge cells in tissue culture but research in progress suggests that this approach will be very difficult to achieve.

1.4 Future of the marine natural products:

The vast diversity of marine fauna and flora offer human beings the last frontier to explore the existence of potential drugs for use in disease treatment. The compounds that are identified so far represent only the tip of the iceberg. More manpower and funding are needed to accelerate the pace of identifying lead compounds. These efforts required concerted efforts from private sectors, governmental agencies as well as research scientists. However, most of the raw materials for use in marine natural compound research are derived from nature. It has been predicated that isolation and structural elucidation will still play a major role for the marine natural product chemist in the future, with further advances in instrumentation allowing even smaller quantities of material to be purified and structures subsequently determined.

The biological and chemical investigations of marine microorganisms have also been identified as an area that will play a major role in natural chemistry during the next century. The past five years have already seen a rapidly developing interest in the chemistry of marine organisms, and this has been reflected by an increasing number of literature reports related to microbial metabolites.[41] The importance of symbiosis in the marine environment has been acknowledged by many researchers during the past decade, who have speculated that numerous marine products are produced by a symbiotic microorganism and not invertebrate host.[42] At present most claims lack experimental support, however with the continued improvement of bacterial and fungal culturing and cell separation techniques, this area will attract future attention, resulting in the unequivocal determination of the biosynthetic origin of particular marine compounds.

Genetic engineering is also predicted to have a large impact on the study of marine natural products. With possibility of the transfer of biosynthetic genes from one organism to another, it appears that the fermentation of genetically modified microbes will be a future a source of unique and highly desirable metabolites. This is especially relevant with biomedical industries growing interest in marine natural products chemistry and the likelihood of a marine-derived drug reaching the marketplace in the near future.[17] Genetic engineering, fermentation and aquaculture methods will all have to be further developed in order to provide certain marine natural products for commercial production, since the total synthesis of particularly complex bioactive compounds will not always be possible or practical. This advancement in biotechnology may hopefully provide an acceptable means for supplying marine natural products, while at the same time preventing excess harvesting of the fragile marine environment.

Thus the future of the marine natural products chemistry looks to be very promising with the embracement of biotechnology set to enhance, and further develop this scientific discipline well into the next millennia. In concordance with development of enhanced purification techniques to obtain natural compounds at a faster pace, scientists should also engage in developing advanced aquacultural technology to provide needed large scale production facilities to offer raw materials that could be produced in the man-made controlled environment so as to minimize impacts to the nature.

India is surrounded by oceanic waters especially in near tropical or tropical zones thereby harboring innumerable genera and species of marine plants and animals. A few research groups in India are engaged in identifying lead marine metabolites with assistance from Department of Ocean Development. One of the prominent group at National Institute of Oceanography is actively engaged in pursuing research on bioactive metabolites from marine organisms from Indian ocean for the last two decades. I had an opportunity to work under the guidance of Dr. C. G. Naik, Group Leader at the Institute and the work carried out is presented here.

Thesis deals with the chemical investigations of some selected marine organisms. These include invertebrates belonging to,

Phylum Porifera:

- *Psammaplysilla purpurea*
- *Haliclona cribricutis*

Phylum Molusca:

- *Elysia grandifolia* and its algal diet *Bryopsis plumosa*, and

Marine fungi:

- *Eurotium sp.* isolated from the mangrove plant *Porteresia coarctata.*

Biotransformation of benzoxazolinone using several marine microbial strains has been studied. The investigations carried out have been divided into five chapters:

Chapter I: Literature review on cyclic peptides and cyclodepsipeptides from marine organisms for the period 1999-2004.

Chapter II: Deals with the biological screening of marine organisms and chemical investigations of two marine sponges *Psammaplysilla purpurea* and *Haliclona cribricutis.* It has been sub divided into the three sections.

Section 1: Reports the *in vitro* antimicrobial screening of methanolic extracts of some selected marine organisms from east and south coast of India.

Section 2: This section deals with chemical investigation of the sponge *Psammaplysilla purpurea.* It has been further subdivided into three sub-sections.

Section 2.1: Reviews the literature belonging to the Order Verongida.

Section 2.1: Structural elucidation of seven new bromotyrosine alkaloids purpurealidin A, B, C, D, F, G, H along with the known compounds purealidin Q, purpurealidin E, 16-debromoaplysamine-4 and purpuramine I from the sponge *P. purpurea* by using spectroscopic techniques has been described.

Section 2.3: Detection and identification of three new bromotyrosine alkaloids purpurealidin I, J and K along with the other known bromotyrosine alkaloids

using extensively electrospray ionization-tandem mass spectrometry (ESI-MS/MS) technique has been discussed here in detail.

Section 3: Chemical investigation of the sponge *Haliclona cribricutis*. This is divided into two sub-sections.

Section 3.1: Brief review on the sponge belonging to genus *Haliclona* has been discussed.

Section 3.2: Tandem mass spectrometry (ESI-MS/MS) approach for the identification of eight molecular species of ceramides from *Haliclona cribricutis* is reported here.

Chapter III: Chemical investigation of mollusk *Elysia grandifolia* and its algal diet *Bryopsis plumosa*.

Section 1: Reviews the chemistry of mollusk belonging to genus *Elysia*.

Section 2: Detection, sequencing and detailed fragmentation of two new cyclodepsipeptides kahalalide P and Q along with kahalalide D, kahalalide F, an anticancer agent and kahalalide G from the Mollusk *Elysia grandifolia* and its algal diet *Bryopsis plumosa* using ESI-QTOF MS/MS is presented.

Chapter IV: It describes chemical constituents from marine fungi *Eurotium sp.* isolated from the mangrove plant *Porteresia coarctata*.

Chapter V: Biotransformation of 2-benzoxazolinone (BOA) using different marine microorganisms is reported here.

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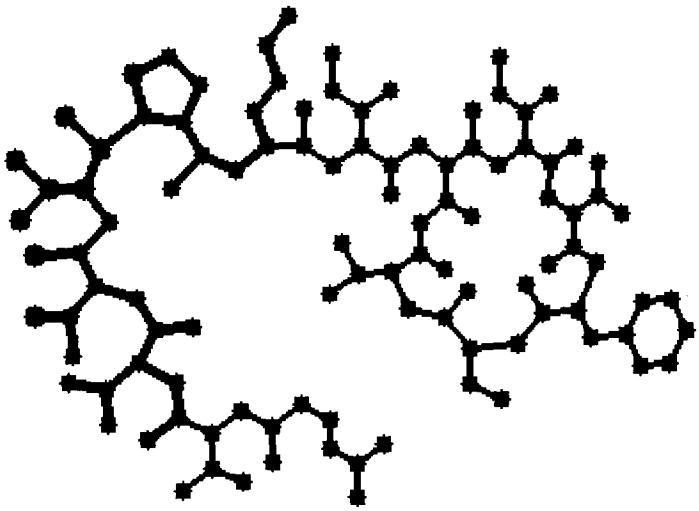
Chapter I



Marine Cyclic peptides and cyclodepsipeptides

Peptides play an important role in many biologically relevant processes and are of outstanding interest in pharmaceutical research. Recent reports show that they are common in marine environment. Our interest in these molecules originated from the detection of biologically active cyclic depsipeptides [cyclic peptides which include ester (depside) bonds as part of their backbone] in one of the organism under investigation.

Cyclic peptides and cyclic depsipeptides display a variety of biological effects, such as immunosuppressant, antibiotic, antiinflammatory or antitumoral activities. In addition, many of the cyclic depsipeptides represent useful tools for the research of biological processes involved in cellular regulation.[1]

The reduction in conformational freedom brought about by cyclization often results in higher receptor binding affinities and increase their stability *in vivo* compared to their linear counterparts leading to more promising drug candidates. Although the significance of incorporating the depside bond is not clear, but appears to be essential for biological activity, since all-amide analogues are often inactive.[2]

In the present review an attempt has been made to cover all known marine cyclic peptides and cyclodepsipeptides that have appeared in the literature from 1999 through December 2004 with special reference to compounds isolated from marine micro-organisms & phytoplanktons, sponges, green algae, mollusks and tunicates. The emphasis is laid on novel molecules together with their relevant biological activities, source organisms and country of origin. Their synthesis including those essential for revision of structure and stereochemistry have been included.

Marine microorganisms & phytoplanktons:

Marine microorganisms continue to be the subject of vigorous chemical investigation although less attention is being paid to marine bacteria as compared to studies on other microorganisms. The interest in these microorganisms

emanates from the observations that marine microorganisms or their host are a good source of novel metabolites.

Cultures of an unidentified marine bacterium MK-PNG-276A obtained from the reefs off Loloata Island, Papua New Guinea, have yielded four cyclic decapeptide antibiotics, loloatins A-D **(1-4)**, that inhibit methicillin-resistant *Staphylococcus aureus*, vancomycin resistant enterococci and drug-resistant *Streptococcus pneumoniae*.[3]

Salinamides A-E **(5-9)** are minor anti-inflammatory bicyclic depsipeptides from *Streptomyces sp.* (isolate #CNB-091) isolated from the surface of the jellyfish *Cassiopeia xamachana* from the Florida Keys.[4] The absolute configuration of the previously reported[5] **(5)** and **(6)** has been revised using chiral capillary electrophoresis of the derivatized hydrolysates. Of cytotoxic prenylated cyclic peptides, cyclomarins A-C **(10-12)** from an unidentified *Streptomyces* sp. (isolate # CNB-982) from sediment sample of Mission Bay, San Diego, only **(10)** was found to possess significant anti-inflammatory activity.[6]

The cyclic pentadepsipeptide, sansalvamide **(13)** was produced by a *Fusarium sp.* (isolate # CNL-292) grown on seagrass *Halodule wrightii* from Little San Salvador Island, Bahamas.[7] It was initially reported as having selective cytotoxicity against the COLO-205 and SKMEL-2 cell lines and found to inhibit molluscum contagiosum virus (MCV) topoisomerase.[8] Unguisins A **(14)** and B **(15)** are GABA-containing cyclic heptapeptides from the fungal culture of *Emericella unguis*.[9]

Lyngbya majuscula from Guam was a source of cytotoxic cyclic depsipeptides lyngbyastatin 2 **(16)** and norlyngbyastatin 2 **(17)**,[10] analogs of the sea hare cytotoxins dolastatin G **(18)** and nordolastatin G **(19)**[11] respectively. *Symploca hydnoides* also from Guam[12] yielded symplostatin 2 **(20)**, which is somewhat similar in structure to dolastatin 13 **(21)**, a metabolite of the sea hare *Dolabella auricularia*.[13] The structure of antillatoxin, an ichthyotoxic metabolite of *L.*

majuscula from Curacao,[14] has been revised from **(22)** to **(23)** as a result of comparison of the two structures based on total syntheses.[15-16]

N-methylsansalvamide **(24)**, a cyclic depsipeptide, was isolated from extracts of a cultured marine fungus strain CNL-619, identified as a member of the genus *Fusarium*. It showed weak *in vitro* cytotoxicity in the NCI human tumor cell line screen (GI50 8.3 μM).[17] A collection of *L. majuscula* from Palau contained the cyclic peptides dolastatin 3 **(25)**, homodolastatin 3 **(26)**, and kororamide **(27)**.[18]

Lyngbyabellin A **(28)**, a potent cytotoxic depsipeptide with unusual structural features, was isolated from a Guamanian, cyanobacterium *L. majuscula*. It was also shown to be a potent disrupter of the cellular microfilament network.[19] The first synthesis of **(28)**[20] in 58% yield by a convergent strategy has been described.[21] An analogue of **(28)**, lyngbyabellin B **(29)**, with lower cytotoxicity has been isolated from identical source and geographical location but different site of collection. The absolute configuration of lyngbyabellin B **(29)** has been ascertained by chiral HPLC analysis of degradation products and by comparison with lyngbyabellin A. Florida samples of *L. majuscula* also contained **(29)** that displayed potent toxicity towards brine shrimp and the fungus *Candida albicans*.[22]

Novel structures are being continuously reported from *L. majuscula* of Guam. Apratoxin A **(30)**, a cyclic depsipeptide of mixed peptide-polyketide origin exhibited potent cytotoxicity *in vitro* against KB and LoVo cell lines but was toxic *in vivo* to mice and was poorly tolerated.[23] *Lyngbya sp.* afforded apratoxin B **(31)** and apratoxin C **(32)** from Guam and Palau collections respectively. Apratoxin C **(32)** exhibited appreciable cytotoxicity against KB and LoVo cells, while apratoxin B **(31)** was considerably less active.[24]

A different population of *L. majuscula* from Guam was the source of two cyclic depsipeptides, pitipeptolides A **(33a)** and B **(33b)**. Both compounds exhibited weak cytotoxicity against LoVo cells and were active in the antimycobacterial diffusion susceptibility assay. Pitipeptolides A and B also stimulated elastase

activity.[25] The cyclic peptide lyngbyastatin 3 **(34)**, containing two unusual amino acid units, including 4-amino-2, 2-dimethyl- 3-oxopentanoic acid (Ibu) was also isolated from the same source. The configuration of the Ibu unit was established by acid hydrolysis and comparison with synthetic standards. Lyngbyastatin 3 **(34)** exhibited activity against KB and LoVo cell lines *in vitro*, but was poorly tolerated *in vivo* with little antitumour activity.[26]

Somamides A **(35)** and B **(36)** were isolated from mixed assemblages of the cyanobacteria *L. majuscula* and *Schizothrix sp.* from Fiji. These depsipeptides are analogous to symplostatin 2, isolated from the cyanobacterium *Symploca hydnoides*[27] and dolastatin 13, originally isolated from the sea hare *Dolabella auricularia*[28] but most likely originating from its cyanobacterial diet.[29] Cultured *Bacillus pumilus*, isolated from the surface of the ascidian *Halocynthia aurantium* from Troitza Bay in Russian waters, yielded a mixture of surfactin-like cyclic depsipeptides **(37-41)**. These lipopeptides differed from surfactin by substitution of the 4-valine by leucine and were isolated as two carboxy-terminal variants with either valine or isoleucine in the 7-position.[30]

—

Collections of *Lyngbya sp.* from various Palauan dive sites were the source of six new β-amino acid containing cyclic depsipeptides, ulongamides A-F **(42-47)**. The absolute stereochemistries of the hydroxy acid and all α-amino acid-derived units were elucidated as (*S*) by chiral HPLC analysis of hydrolysis products. Advanced Marfey's analysis of the acid hydrolysates determined the stereochemistry of 3-amino-2-methylhexanoic acid as (2*R*,3*R*) in ulongamides A-C **(42-44)** and (2*S*,3*R*) in ulongamides D-F **(45-47)**. Ulongamides A-E **(42-46)** were weakly cytotoxic against KB and LoVo cells *in vitro*, while ulongamide F **(47)** was inactive.[31]

Six cyclic depsipeptides, guineamides A-F **(48-53)**,[32] and cyclic dodecapeptide, wewakazole **(54)** which contains an unprecedented number of five-membered heterocyclic rings (six) were isolated from different collection of *Lyngbya majuscula* from Papua New Guinea. Guineamides B and C were moderately cytotoxic to a mouse neuroblastoma cell line. The absolute stereochemistry was

determined by standard methods.[33] A collection of *Lyngbya sp.* from the same site yielded cytotoxic cyclic depsipeptide, ulongapeptin **(55)**[34] while bioassay-guided fractionation led to the isolation of cytotoxic palauamide **(56)**.[35, 36]

A Madagascan collection of *L. majuscula* was the source of depsipeptides, antanapeptins A-D **(57-60)**.[37] Southern Kenyan collection of this source yielded cyclic depsipeptide, homodolastatin 16 **(61)**. It displayed moderate activity against oesophageal and cervical cancer cell lines.[38] *L. confervoides*, different species of *Lyngbya*, from Saipan in the Commonwealth of the Northern Mariana Islands was the source of a cytotoxic cyclic depsipeptide, obyanamide **(62)**.[39] An antifungal cyclododecapeptide, lobocyclamide B **(63)** has been isolated from a benthic mat of *L. confervoides* from the Bahamas. The absolute stereochemistry of **(63)** was established by a combination of chiral HPLC and Marfey's methods. Lobocyclamide B **(63)** displays antifungal activity against fluconazole-resistant *C. albicans*.[40]

The cyclic hexapeptide halolitoralin A **(64)** and tetrapeptides halolitoralins B **(65)** and C **(66)** were isolated from the fermentation broth of *Halobacillus litoralis*, which had originated from high-salt sediment from the Huanghai Sea, China. All amino acid residues were established as (*S*) by hydrolysis and subsequent Marfey's analysis. The halolitoralins A-C **(64–66)** exhibited moderate antifungal activity against *Candida albicans*, *Trichophyton rubrum* and four crop-threatening fungi, in addition to moderate activity against the human gastric tumour BGC cell line.[41] A culture of an unidentified fungus from the South China Sea yielded the cyclic tetrapeptides **(67-69)**, which are very similar to the halolitoralins B and C (*vide supra*).[42]

Marine *Fusarium sp.* isolated from the green alga *Codium fragile* subsp. *atlanticum* collected in Scottish waters yielded cyclic tetrapeptide, designated as JM47 **(70)**. This was determined to be cyclo(Ala-Ala-Aoh-Pro), where Aoh is (2*S*,9*S*)-2-amino-8-oxo-9-hydroxydecanoic acid.[43] Two cyclic thiopeptides **(71)** and **(72)**, obtained from a culture of *Bacillus cereus* isolated from the marine

sponge *Halichondria japonica*,[44] exhibited potent antibacterial activities against *Staphylococci* and *Enterococci sp.*, and were active against multiple-drug resistant strains.[45] (6*Z*)-Geometry for these compounds was implied by ROESY correlations. Culture of an exocellular extract of a *Pseudomonas sp.* associated with *Ircinia muscarum* from the Bay of Naples, Italy gave the cyclotetrapeptide **(73)**.[46]

Five novel depsipeptides, aspergillicins A-E **(74-78)**, were obtained from a culture of *Aspergillus carneus* collected from estuarine sediment in Tasmania, Australia. These aspergillicins exhibited modest cytotoxicity against *Haemonchus contortus*.[47] Two cyclic heptapeptides, scytalidamides A **(79)** and B **(80)**, have been isolated from the culture broth of another *Scytalidium sp.* derived from the surface of the green alga *Halimeda sp.* collected off the Bahamas. The absolute configurations were confirmed by standard methods including CD measurements. Both scytalidamides displayed moderate cytotoxicity to the HCT-116 cell line *in vitro*.[48]

Sponges:

A survey of sponges has provided with large number of new cyclic peptides, many of which have interesting biomedical potential. Jaspamide derivatives, jaspamides B **(81a)** and C **(81b)**, moderately active cytotoxic agents were obtained from a specimen of *Jaspis splendens* from Vanuatu[49] while the related metabolites, geodiamolides J–P **(82a-82g)** and R **(82h)**, were isolated as minor metabolites of a *Cymbastela sp.* from Papua New Guinea.[50]

Serine protease inhibitors dihydrocyclotheonamide A **(83)**, were obtained from a Japanese specimen of *Theonella swinhoei*.[51] From the same location *Theonella* was found to contain an antibacterial depsipeptide, nagahamide A **(84)**.[52] Three total syntheses of motuporin **(85)**, which is a potent inhibitor of protein phosphatase type 1 (PP1) from a Papua New Guinea specimen of *T. swinhoei*,[53] has been reported during 1999.[54-56] Two sponges *T. mirabilis* and *T. swinhoei*

yielded, two each cytotoxic and HIV-inhibitory depsipeptides papuamides A **(86a)** & B **(86b)**, and papuamides C **(87a)** & D **(87b)**, respectively.[57]

Keramamides M **(88a)** & N **(88b)**, sulfated cyclic peptides[58] and two theonellapeptolide congeners **(89a)** & **(89b)**[59] one of which had methylsulfinylacetyl group at the *N*-terminus were isolated from an Okinawan *Theonella sp.* Synthesis of the proposed structure of keramamide J **(90)**,[60] earlier isolated from *Theonella sp.*, indicated that the structure of the natural product should be re-examined.[61] An X-ray study of theonellapeptolide Id **(89c)** from *T. swinhoei*[62] revealed that the crystals were highly solvated.[63] Cyclic peptide barangamide A **(91)** has been isolated from an Indonesian specimen. [64]

A large (500 kg) collection of a *Phakellia* species from Chuuk, Micronesia, yielded the growth inhibitory phakellistatin 12 **(92)**,[65] while a Chinese collection of *Phakellia fusca* yielded the very cytotoxic phakellistatin 13 **(93)**.[66] Total synthesis of phakellistatin 11 **(94)**, isolated from *Phakellia sp.*,[67] revealed that the synthetic product is much less cytotoxic than the originally isolated sample.[68] Two distinct conformers of peptide phakellistatin 2 **(95)** from *P. carteri* were reported to be potently cytotoxic[69] A subsequent reisolation of **(95)** from the Fijian specimen of *Stylotella aurantum* and a total synthesis failed to reproduce the biological activity.[70] The less polar conformer from *Stylotella sp.* was having a similar activity to that originally reported but was found to lose activity at room temperature on standing for several weeks.[70] Isolated from the same sponge was the weakly cytotoxic octapeptide, axinellin C **(96)**.[71] Wainunuamide **(97)**, weakly cytotoxic heptapeptide, was isolated from *S. aurantium* collected in Fiji.[72]

Arenastatin-A **(98)**, a cytotoxic depsipeptide from *Dysidea arenaria*,[73] has been synthesized together with related cyanobacterial cytotoxins.[74] Due to differences in biological activity, the *cis,cis-* **(99a)** and reputed *trans,trans-* **(99b)** isomers of ceratospongamide, originally isolated from the Indonesian symbiotic pairing of the red alga *Ceratodictyon spongiosum* and the sponge *Sigmadocia symbiotica*,[75] continue to attract considerable attention from synthetic chemists. Although both

rotamers had been synthesised previously,[76-78] slight differences in the NMR spectra of the synthetic *trans,trans* isomer **(99b)** and the isolated natural product were noted. Suspecting a possible epimerisation the *trans,trans*-[D-*allo*-Ile] isomer, **(99c)** was synthesised, by two separate routes, to produce a compound that is identical in all respects to the natural isomerisation product. [79]

Hymenamide C **(100)** from *Hymeniacidon sp.*[80] has been synthesised using solid support methodology.[81] A collection of *Sidonops microspinosa* from Sulawesi, Indonesia was found to contain the HIV-inhibitory depsipeptide microspinosamide **(101)**.[82] The structures of two potent anti-inflammatory peptides, halipeptin A **(102a)** and B **(102b)**, isolated from Vanuatu, *Haliclona sp.*[83] has been revised to include sulfur, replacing the proposed oxazetidine ring with a thiazoline moiety **(103a)** and **(103b)**.[84] Halipeptin C **(103c)** was also obtained from the same sponge.[84] A Palauan specimen of the same genus yielded two iron-chelating peptides, haliclonamide A **(104a)** and B **(104b)**.[85] It also contained haliclonamides C-E **(105a-c)** which were found to repel the settlement of *Mytilus edulis* adults.[86] The previously described sulfoxide, waiakeamide **(106a)** and a new sulfone analogue **(106b)** were isolated from a *Haliclona sp.* collected in Palau. The sulfone **(106b)** was found to inhibit the settlement of larvae of the blue mussel (*Mytilus edulis galloprovincialis*).[87]

Two depsipeptides with nematocidal activity, phoriospongins A **(107a)** and B **(107b)**, were isolated from both a *Phoriospongia sp.* and *Callyspongia bilamellata* from southern Australia.[88]

The cyclic tripeptide renieramide **(108)**, isolated from a new species of *Reniera* collected in Vanuatu, showed immunomodulatory activity. Interestingly, the structure of **(108)** proved to be identical to a patented synthetic analogue of the microbial product OF4949.[80] Leucamide A **(109)**, originally isolated from the Australian sponge *Leucetta microraphis*,[89] has been synthesised.[90] Two new cyclotheonamides E4 **(110a)** and E5 **(110b)** were obtained from an *Ircinia sp.* from Japan and were found to be potent tryptase inhibitors.[91]

Three unusual cyclic peptides, kapakahines E-G (**111a-c**), have been isolated from a Micronesian collection of *Cribrochalina olemda* and reported as cytotoxic to P388 murine leukemia cells.[92] The myriastramides A-C (**112a-c**) were isolated from the Philippine collection of *Myriastra clavosa* that had previously yielded the clavosidemacrolides.[93-94]

Algae:

Algae discharges toxic substances like cyclic peptides into the water, which are hazardous to fish, molluscs and humans. But during this period there was only one report on the isolation of cyclic depsipeptide kahalalide K (**113**) from the Hawaiian species of *Bryopsis*.[95]

Mollusc:

The stereostructure of dolastatin I (**114**), a cytotoxic metabolite of a Japanese specimen of *Dolabella auricularia*,[96] was confirmed by an enantioselective synthesis.[97] The mechanism of biological action of dolastatin 11 (**115**), a cytotoxic depsipeptide isolated from the same source,[98] involves stabilisation of F-actin, which has been studied using X-ray fiber diffraction of oriented filament sols.[99]

Kulokekahilide-1 (**116**) is a moderately cytotoxic depsipeptide isolated from the mollusk, *Philinopsis speciosa* collected off Pupukea, O'ahu.[100] The absolute stereochemistry of kahalalide F, the structure (**11**) as given in introduction, which is a bioactive metabolite of the Hawaiian sacoglossan *Elysia rufescens* and its diet *Bryopsis sp.*,[101] has been determined by acid hydrolysis and hydrazinolysis, followed by chiral analysis of the fragments.[102]

Tunicate (ascidians):

Organisms from the subphylum Urochordata (Tunicata), class Ascidiacea, are a rich source of pharmacologically active peptides and hence have been studied by several research groups. These organisms are variously referred to as tunicates, ascidians and sea squirts.

The macrocyclic hexapeptide, bistratamide D (**117**) from *Lissoclinum bistratum*[103] has been synthesized using a convergent strategy using enantiomerically pure oxazole, thiazole and oxazoline segments.[105] Six new congeners of the bistratamide family cyclic hexapeptides, E-J (**118a-e**), were reported from a Tablas Island, Philippines collection of *L. bistratum*.[105] All six compounds showed weak to moderate activity towards the HCT-116 tumour cell line. A total synthesis of trunkamide A (**119**), a cyclic heptapeptide from a *Lissoclinum sp.*,[106-109] has been determined using 2DNMR data and simulated annealing methods.[110]

A full account of the total synthesis of mollamide (**120**), a cytotoxic cycloheptapeptide isolated from an Australian collection of *Didemnum molle*,[111-112] has been reported.[108] The synthesis of cytotoxic cyclic depsipeptides, tamandarin A (**121a**), and B (**121b**), isolated from a Brazilian collection of an unidentified didemnid ascidian has been accounted.[113-115] Fluorescent analogues of tamandarin A have been used to study short-term predator-prey relationships between fish and marine invertebrate larvae.[116] From the collection at Ibo Island north of Mozambique, *D. molle* yielded cycloheptapeptide, cyclodidemnamide B (**122**).[117] Cyclic hexapeptides, Didmolamides A (**123a**) and B (**123b**) containing all (*S*)-configuration amino acids were isolated from Madagascar *D. molle*.[118] Both compounds exhibited modest cytotoxicity towards a panel of tumour cell lines.

Conclusions:

Sea is a rich source of metabolites with a distribution of peptides as shown in **Fig. 1.** As evident, microorganisms including cyanobacteria (47%) are the richest sources of these compounds with *L. majuscula* (29%) being the most prolific producer. Among invertebrates sponges, are the next largest source of peptides (39%) followed by mollusks (18%) and tunicates (9%). Coelentrates, echinoderms, brown algae and bryozoans are totally devoid of cyclic peptides and cyclodepsipeptides.

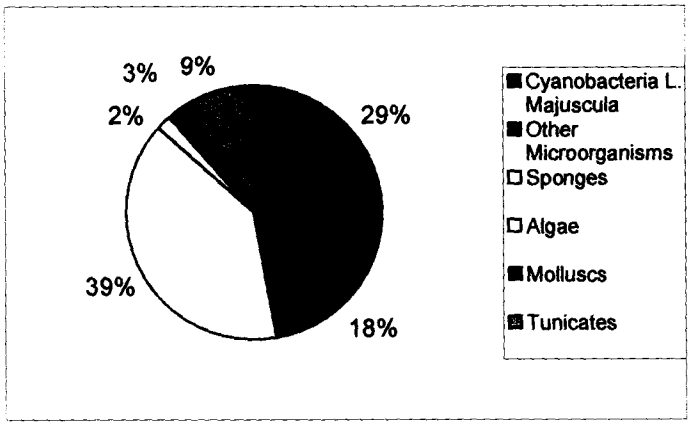
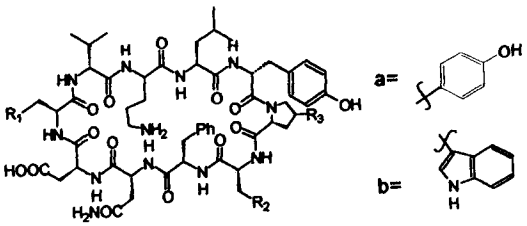
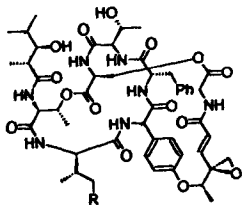


Fig. 1: Percentage of cyclic peptides and cyclodepsipeptides by phylum (1999-2004).

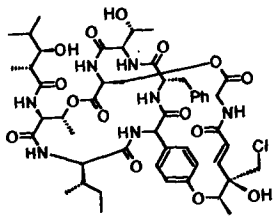
From the review it is clear that almost all the cyclic peptides and cyclodepsipetides show cytotoxicity against cancer cell lines. Some of these peptides show anti-inflammatory and antibiotic activities. Among sponges *T. swinhoei* is richest source of cyclic peptides. Further studies on this type of molecules are expected to yield lead compounds as anticancer agents.



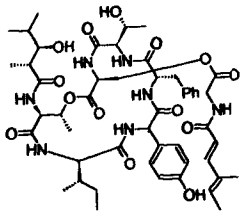
(1) R_1 = a; R_2 = Ph, R_3=H
(2) R_1 = b; R_2 = Ph, R_3=H
(3) R_1 =R_2= b; R_3 = H
(4) R_1 = b; R_2=Ph; R_3=OH



(5) R=Me
(8) R=H



(6)



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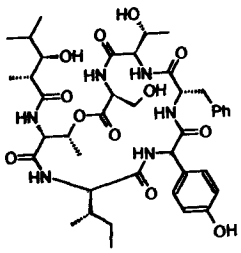

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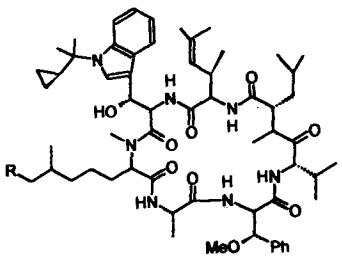
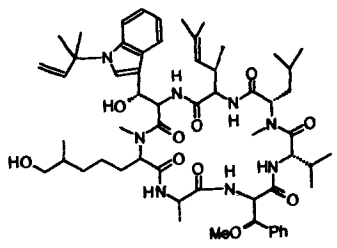


(10) R=OH
(11) R=H



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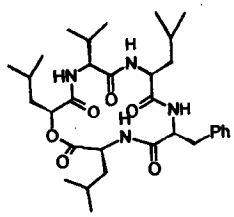

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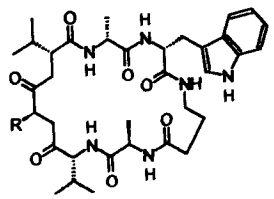
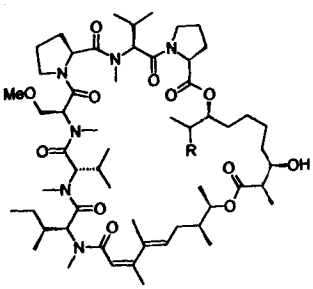
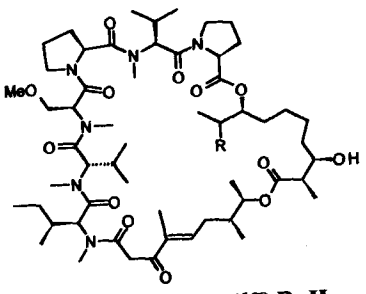


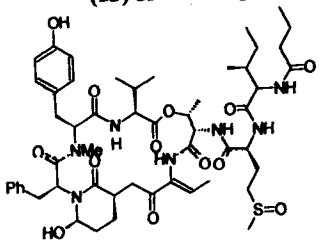
(14) R=CH_2Ph
(15) R=$CHMe_2$



(16) R=H
(18) R=Me



(17) R=H
(19) R=Me



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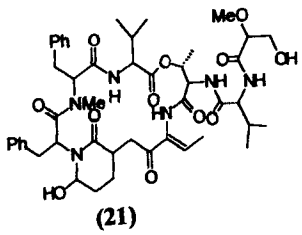

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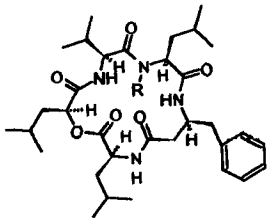
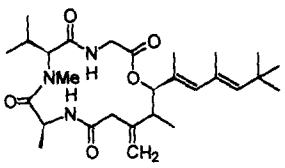


(22) R=β-Me
(23) R=α-Me



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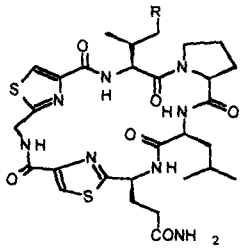
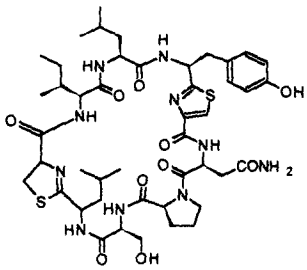


(25) R=H
(26) R=Me



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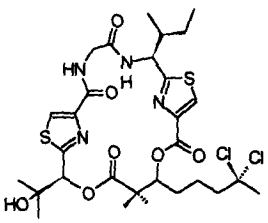

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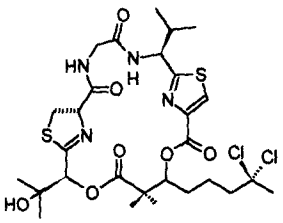

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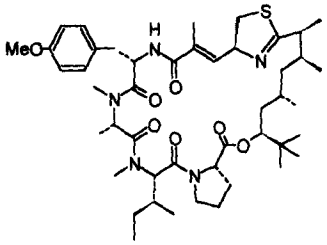

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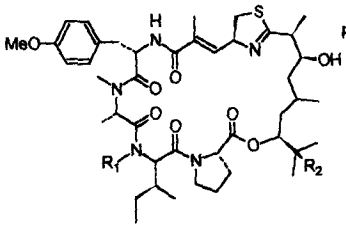
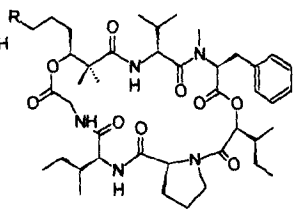
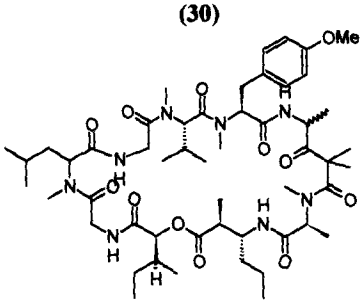


(31) R_1=H, R_2=Me
(32) R_1=Me, R_2=H



(33a) R=HC≡CH
(33b) R=HC=CH_2



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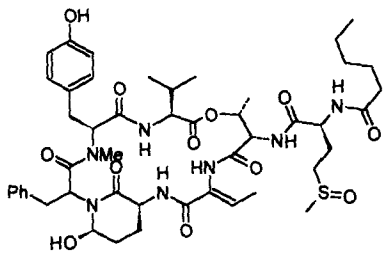

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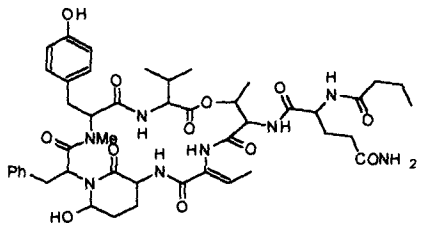

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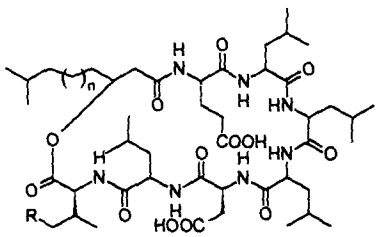
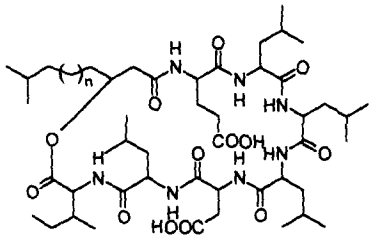
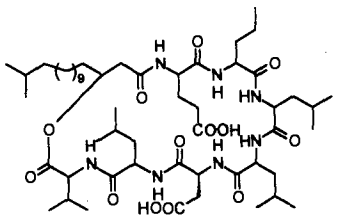


(37) R=H, n=7
(38) R=Me, n=8



(39) n=7
(40) n=9



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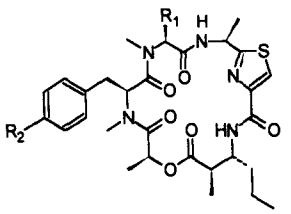
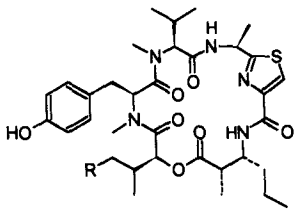
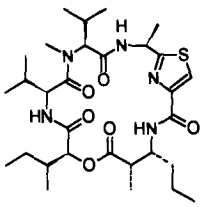


(42) R_1=CH(CH_3)$_2$, R_2=H
(43) R_1=CH(CH_3)$_2$, R_2= OH
(44) R_1=$CH_2C_6H_5$, R2=OH



(45) R=H
(46) R=Me



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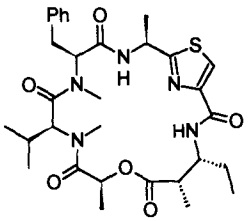

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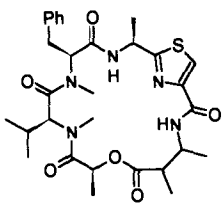
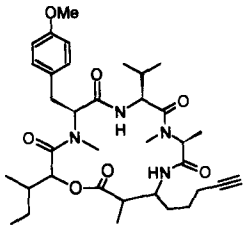


(49)



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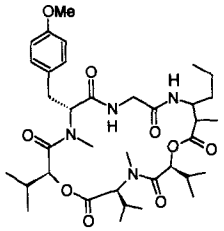
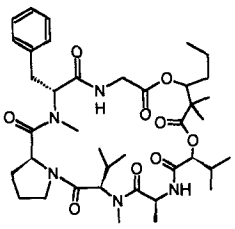


(51)



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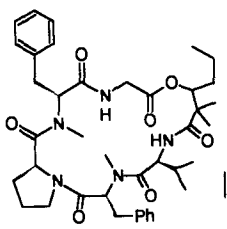

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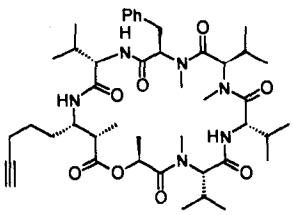

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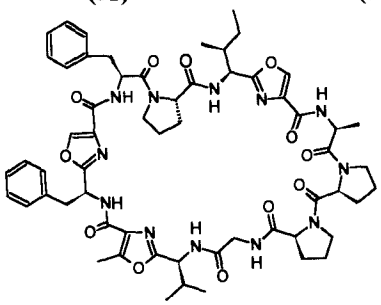

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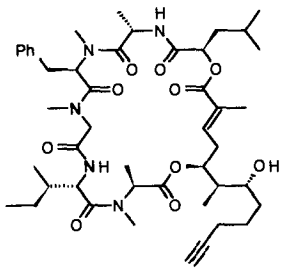

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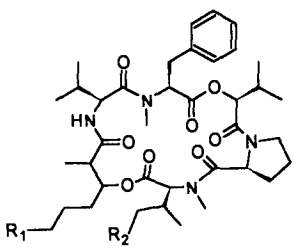
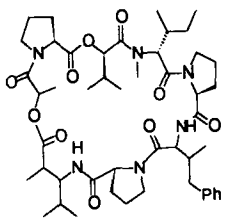


(57) R_1=C≡CH, R_2=Me
(58) R_1=HC=CH_2, R_2=Me
(59) R_1=CH_2CH_3, R_2=Me
(60) R_1=C≡CH, R_2=H



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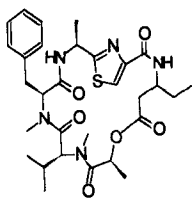

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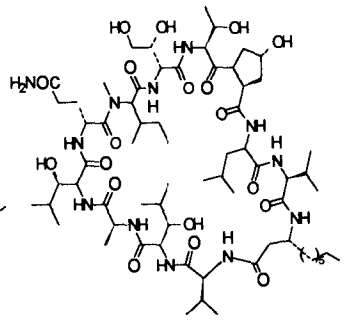
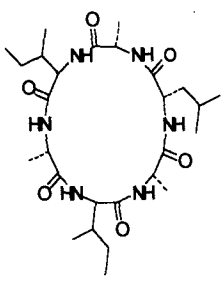


(63)



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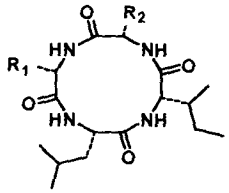
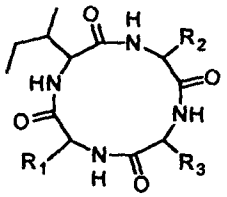
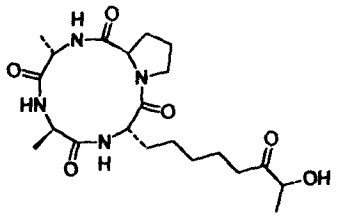


(65) R_1=$CH(CH_3)CH_2CH_3$, R_2=$CH_2CH(CH_3)_2$
(66) R_1=$CH(CH_3)CH_2CH_3$, R_2=$CH(CH_3)_2$



(67) R_1= $CH_2CH(CH_3)_2$, R_2=R_3=$CH(CH_3)_2$
(68) R_1= R_3=$CH_2CH(CH_3)_2$, R_2= $CH(CH_3)_2$
(69) R_1= R_2 = R_3= $CH(CH_3)_2$



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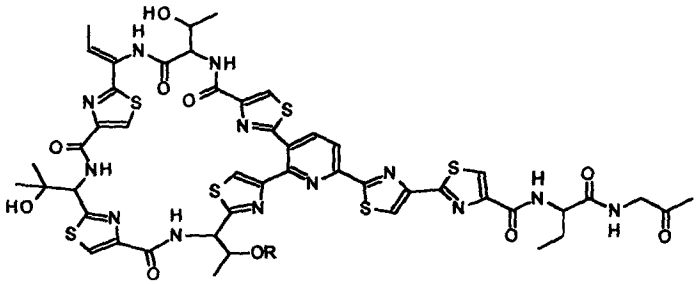
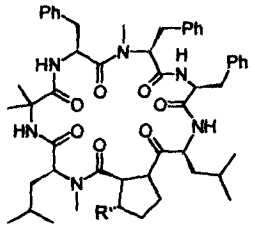
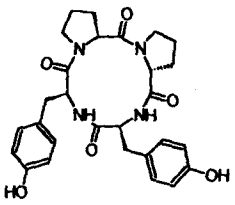


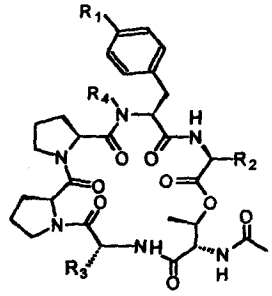
(71) R=H
(72) R=Me



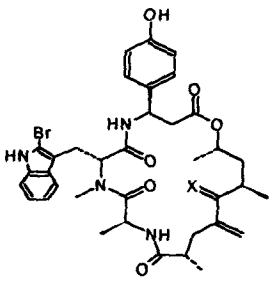
(79) R=H
(80) R=Me



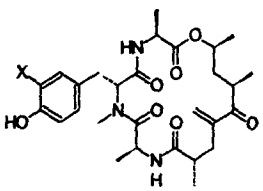
(73)



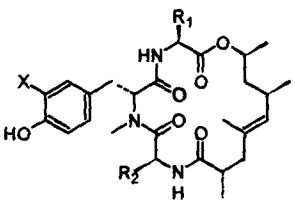
74) R_1=OMe, R_2=$CHMe_2$, R_3=  , R_4=Me
75) R_1=OMe, R_2=$CHMe_2$, R_3=$CH_2CH_2CH_3$, R_4=Me
76) R_1=H, R_2=$CHMe_2$, R_3=  , R_4=Me
77) R_1=R_4=H, R2=$CHMe_2$, R_3= 
78) R_1=OMe, R_2=  R3=  R_4=Me



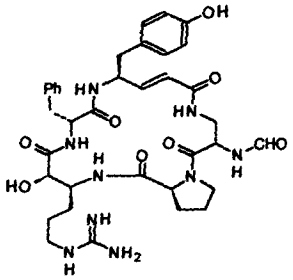
(81a) X=O
(81b) X=NOH



(82a) X=Br
(82b) X=Cl



(82c) R_1=CH_2OH; R_2=Me; X=I
(82d) R_1=CH_2OH; R_2=Me; X=Br
(82e) R_1=CH_2OH; R_2=Me; X=Cl
(82f) R_1= Me; R_2 =CH_2OH; X=I
(82g) R_1=Me; R_2=CH_2OH; X=Br
(82h) R_1=CH_2OH; R_2=H; X=I



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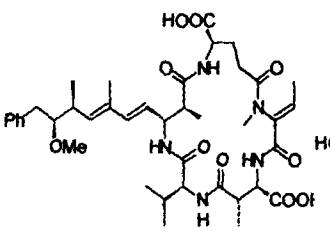

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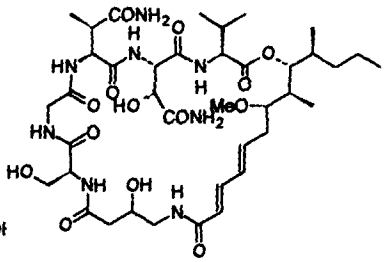

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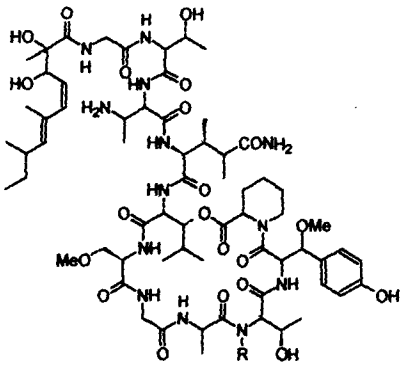
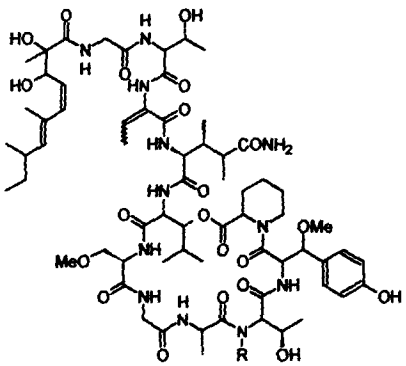
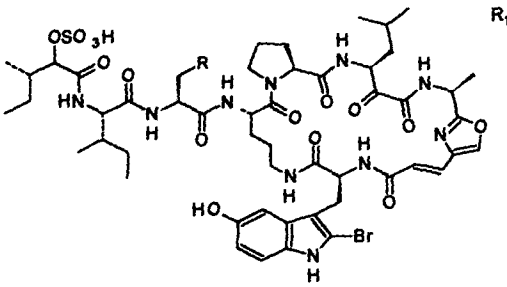


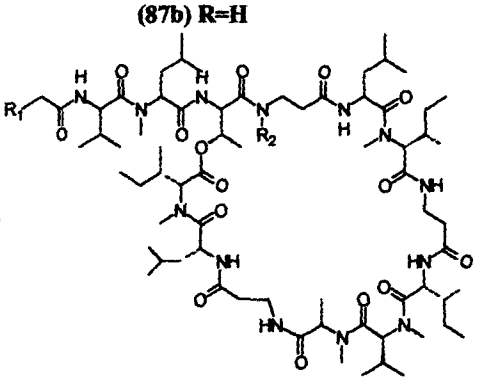
(86a) R=Me
(86b) R=H



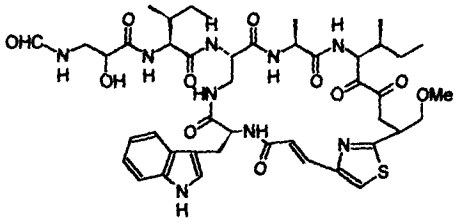
(87a) R=Me
(87b) R=H



(88a) R=Me
(88b) R=Et



(89a) R_1=SOMe, R_2=H
(89b) R_1=H, R_2=Me
(89c) R_1=OMe, R_2=H



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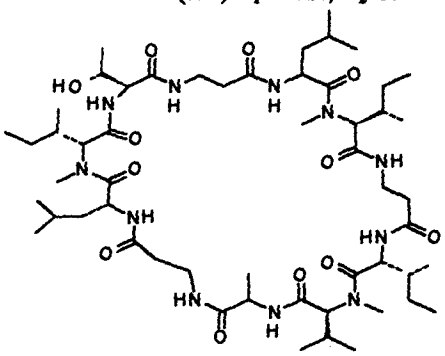

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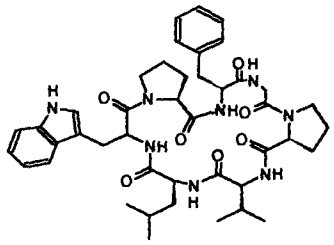

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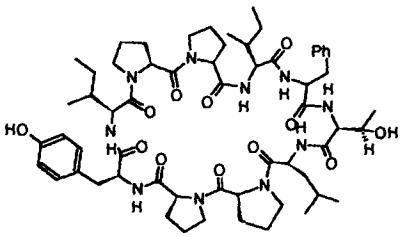

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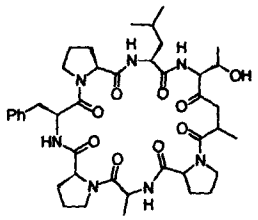

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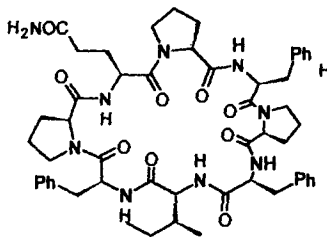
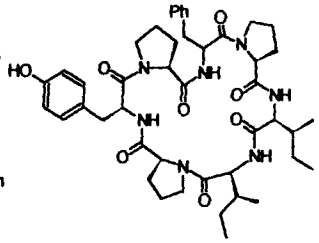
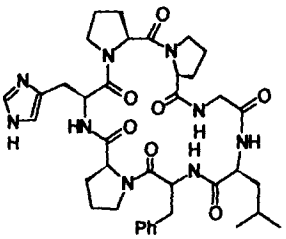


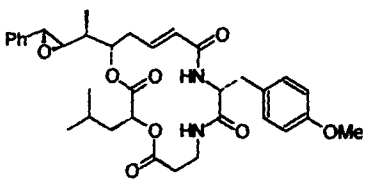
(94)



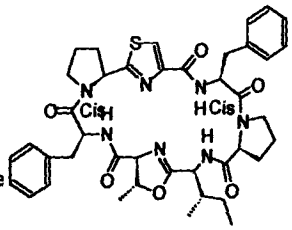
(95)



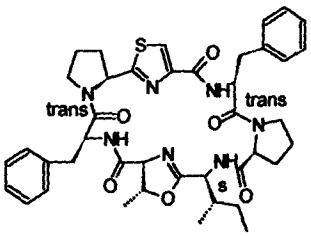
(97)



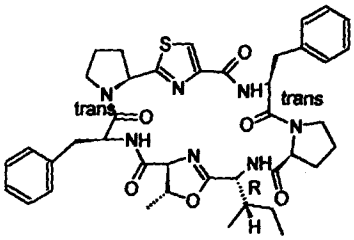
(98)



(99a)



(99b)



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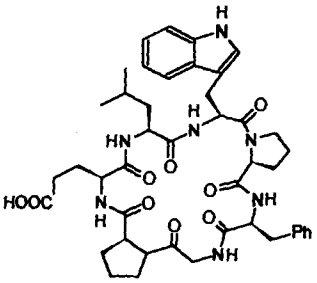

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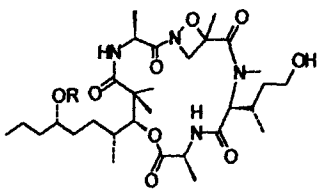
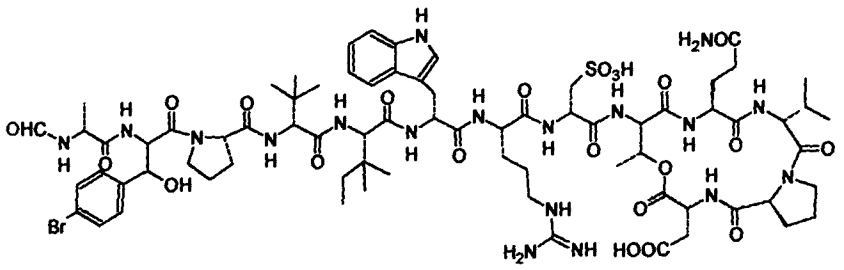


(102a) R=Me
(102b) R=H



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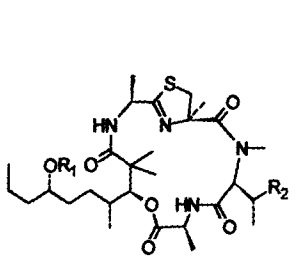
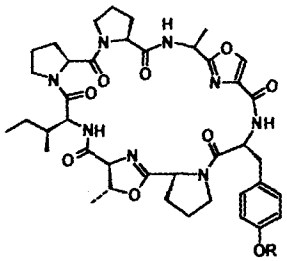
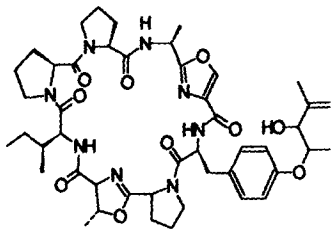


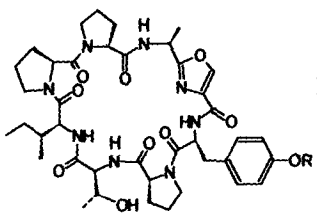
(103a) R_1=Me, R_2=CH_2CH_2OH
(103b) R_1=H, R_2=CH_2CH_2OH
(103c) R_1=H, R_2=Me



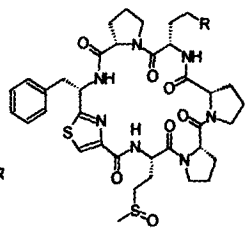
(104a) R= -CH_2-CH=$C(CH_3)_2$
(104b) R=H



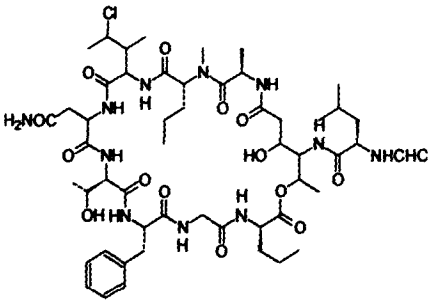
(105a)



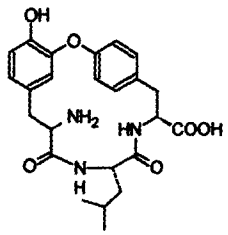
(105b) R=H
(105c) R= -CH_2-CH=$C(CH_3)_2$



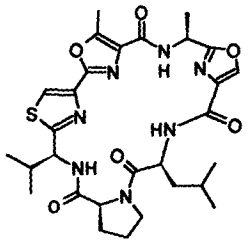
(106a) R=(SO)Me
(106b) R=(SO_2)Me



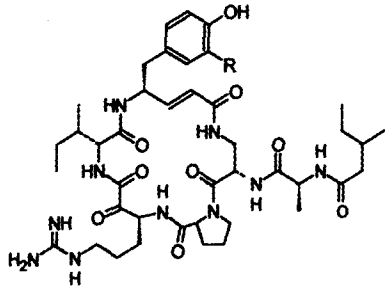
(107a) 22(R)
(107b) 22(S)



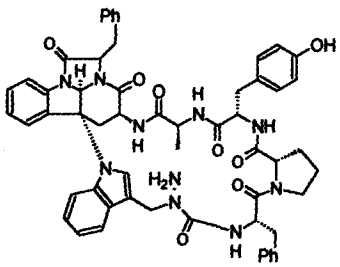
(108)



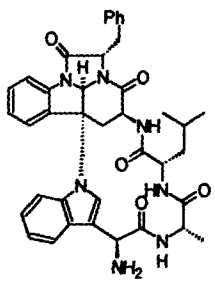
(109)



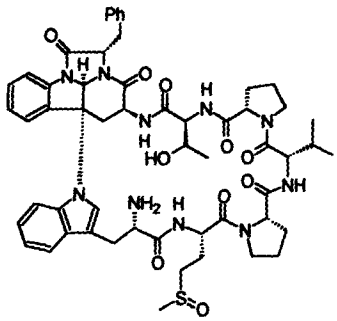
(110a) R=H
(110b) R=OH



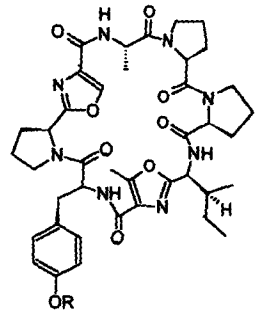
(111a)



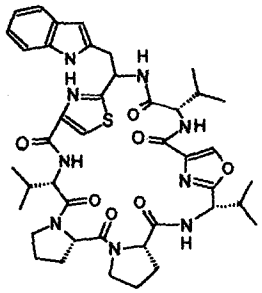
(111b)



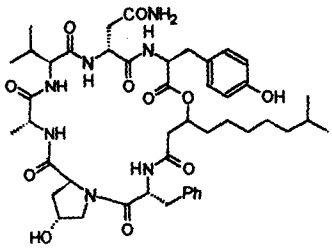
(111c)



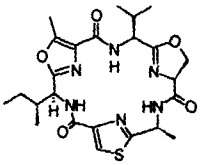
(112a) R=-$CH_2CH=C(Me)_2$
(112b) R= -CH_2-CHCl-C(Me)=CH_2



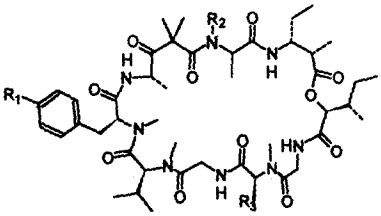
(112c)



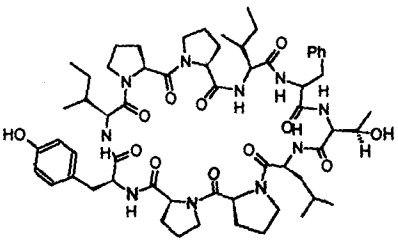
(113)



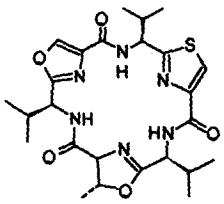
(114)



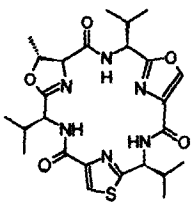
(115) R_1=-OMe, R_2=H, R_3=-$CH_2CH(CH_3)_2$



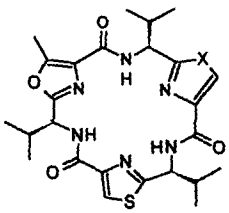
(116)



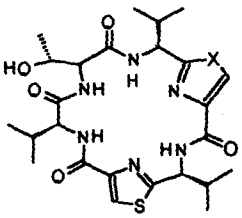
(117)



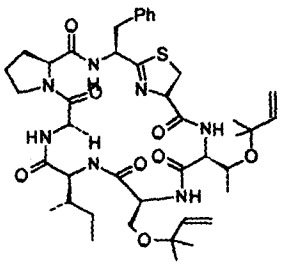
(118a)



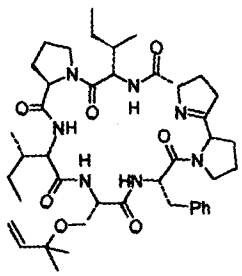
(118b) X=O
(118c) X=S



(118d) X=O
(118e) X=S



(119)



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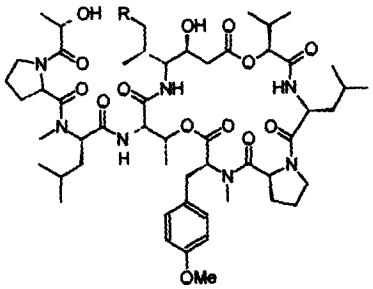
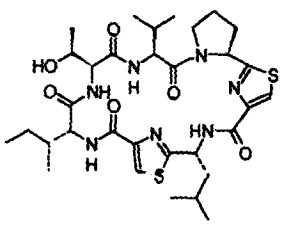
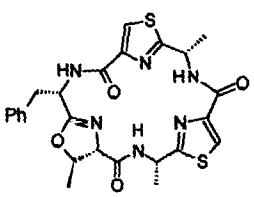


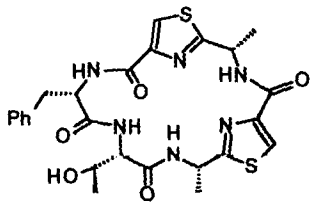
(121a) R=Me
(121b) R=H



(122)



(123a)



(123b)

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Chapter II

Chemistry of marine sponges Psammaplysilla purpurea and Haliclona cribricutis

Introduction:

In terrestrial environment, plants are the richest sources of natural products. However in marine environment, this leading position is taken by invertebrates such as sponges, molluscs, bryozoans, tunicates, *etc*. They not only produce a great number of marine natural products currently known but also show the largest chemical diversity of natural products, including alkaloids, peptides, terpenes, polyketides, *etc*. Among marine invertebrates, Porifera (sponges) remains the most prolific phylum, concerning novel pharmacologically active compounds.[1]

The work on sponge natural products was systematically started with the discovery in 1949 by Bergmann and Feeney,[2] of three nucleosides from the Caribbean sponge *Cryptotethya crypta* (Laubenfels) as lead antiviral agents.[3] Their synthetic analogs led to the development first antiviral compound Ara-A (active against Herpes virus) and anti-tumour compound Ara C (effective in acute lymphoid leukemia) now in clinical use.[4]

The fact that marine sponges are associated with microorganisms and most of the metabolites identified from them are the true metabolites of microorganism have attracted significant attention from various scientific disciplines. They also found their way into biotechnological applications having potential for drug discovery.[5]

There are approximately 8000 described species of sponges and perhaps twice as many undescribed species.[6] They are grouped into three classes, the Hexactinellida (glass sponges), the Calcarea (calcarious sponges) and the Demospongiae. The latter class is known to contain the vast majority of sponges living today.

Class Demospongiae includes approximately 4,750 species in 10 orders and of this subclass Ceractinomorpha consist of major orders. Their geographic distribution in the marine environment is from the intertidal to the abyssal zone; some species inhabit freshwater. Members of the Demospongiae are asymmetrical that can range in size from a few millimeters to over 2 meters in largest

dimension. They can form thin encrustations, lumps, finger-like growths, or urn shapes. Pigment granules in amoebocytes often make members of this class brightly colored, including bright yellow, orange, red, purple, or green.[7] Generally the spicule skeleton is associated with a system of well-developed spongin fibres, forming hymedesmoid, plumose, plumo-reticulate, reticulate or condensed axial architecture, but three orders Dictyoceratida, Dendroceratida and Verongida during their evolution have lost their siliceous spicules altogether and several genera have also lost spongin fibres. Hence, the sponges belonging to these three orders have developed chemical defense (secondary metabolites) to protect themselves in the marine environment against intensive evolutionary pressure from competitors, that threaten by overgrowth, poisoning, infection or predation. Investigations in sponge chemical ecology reveal that the secondary metabolites not only play various roles in the metabolism of the producer but also in their strategies in the given environment. The diversity of these metabolites that range from derivatives of amino acids and nucleosides to macrolides, porphyrins, terpenoids to aliphatic cyclic peroxides and sterols has been highlighted in several reviews.[8-9] There are evidences documenting the role of sponge metabolites in chemical defence against predators[10-12] and epibionts.[13-17] Several studies show that sponges are rich in terpenoids and steroids, which are thought to function in antipredation, space competition and control of epibiont overgrowth.[18] The studies on sponge chemical ecology include three different aspects; diversity of chemical compounds produced by sponges, potential functions of these metabolites in nature and finally, the strategies for their use for human benefit.[19]

In the present thesis this chapter has been divided into three sections. The first section deals with biological screening of the methanolic extract of several marine organisms. The second and third sections of this chapter deals with the isolation, purification and structural elucidation of chemical constituents of two sponges, *Psammaplysilla purpurea* and *Haliclona cribricutis* respectively belonging to Phylum Porifera, Class Demospongiae and Sub-class Ceractinomorpha.

Section 1

Biological screening of marine organisms

Without doubt, natural products have been, and still are, the cornerstone of the health care armamentarium. Indeed, at the last estimate, 80% of the world's populations still rely on traditional medicines for their health care needs.[20] Considering prescription medicines alone, microbial and plant-derived drugs account for greater than 30% of the worldwide sales.[21] Some of the most notable include the analgesics aspirin (*Filipendula ulmaria*), morphine, codeine (*Papaver somniferum*), the malaria prophylaxis, quinine (*Cinchona pubescens*) and the cardiotonic drugs, digoxin and digitoxin (*Digitalis purpurea*).[22-23]

The popularity of drug discovery programs based on nature is associated to a number of factors. Firstly, the diversity and complexity of the chemical structures go far beyond those, which can be synthesized in a laboratory. Secondly, the molecules isolated from nature are, more often than not, small (< 1000 Da), with existing drug-like properties.[24] Added to this, as a result of evolutionary pressures, many organisms, both terrestrial and marine, have developed chemical defense mechanisms, secondary metabolites, which confer a selective advantage and often have distinct biological activities against enzymes and receptors that makes them ideal candidates for pharmacological investigation.[25]

In the search for new pharmaceuticals, a number of approaches are used by natural product researchers in the selection of candidate species. These include (a) Ethnomedical information, which refers to species used in popular medicine to treat ailments; (b) Chemotaxonomy, which involves selection of species due to promising biological activity or the presence of a particular class of molecule(s) with the desired activity, in congeneric species and (c) Random collection.[26] Generally speaking, there is little or no ethnobotanical information regarding the use of marine species for medical ailments, therefore the screening of marine organisms generally fall into the latter category, although once an interesting species/compound is discovered, chemotaxonomy can be used to select related species.

Among marine invertebrates, Porifera (sponges) are the most primitive and represent an important constitutive group of the coral reef fauna.[27] These animals are frequently exposed to intense predation and/or tissue infection by microorganisms.[28-29] However, despite being sessile and soft bodied, sponges appear to be predated only by selected groups of marine animals, such as turtles,[30] a few fish species,[31-32] nudibranchs,[33-35] sea urchins[36] and sea stars.[37] While sponges tissue and skeleton constituents appear to have little or no activity against potential predators,[38-40] sponges secondary metabolites clearly present a defensive role against predation.[40-42] Thus, sponges remain the most prolific phylum, concerning novel pharmacologically active compounds.

Bacteria and fungi are common disease-causing agents in humans, as shown by the wide clinical use of antibiotics and the undeniable impact of these "micracle drugs" on human health. Antimicrobial screening of organic extracts from marine sponges and other marine organisms is a common approach to identify compounds of biomedical importance.[43-46] This is evidenced by high incidence of bactericidal activity observed in the crude extracts of marine sponge against terrestrial pathogenic bacteria,[47-52] but a low incidence of activity against marine bacteria,[47-48,53] Only a few cases of sponges infection by exogenous microorganisms are known, presumably because of its production and/or accumulation of compounds that have antimicrobial activity.[48,53-54] Several antibiotics have so far been identified from marine sponges, a number that themselves against infections by producing and/or accumulating secondary metabolites.

Our main objective is to search for new biologically active secondary metabolites from marine sponges and algae with special reference to antimicrobial compounds. With this aim, we have screened organic extract of marine sponges and algae collected from coasts of India for antimicrobial activities (antibacterial and antifungal) against clinical isolates.

The results of screening of methanolic extracts of marine organisms collected from South-east and west coast of India are presented here. Samples were collected by scuba diving from sub tidal habitats at depths of 8 to 10 meters at different locations 2 nautical miles off the coastline of southeast coast of India. They were frozen as soon as possible and transferred to the laboratory where they were extracted in methanol. Algae were collected from Anjuna, west coast of Goa, India by hand picking during low tide.

Identification of sponges was done Dr. P. A. Thomas of Vizhingam Research Center of Central Marine Fisheries Research Insititute, Kerala, India. The species investigated in this study are summarized in **Table 1** along with the site of collection. The samples are deposited at the Taxonomic reference Center of National Institute of Oceanography, Dona-Paula, Goa.

Table 1: Marine organisms examined in this study

Serial No.	Species	Collection site
NIO 727	*Sigmadocia carnosa*	Mahabalipuram, Tamil Nadu
NIO 729	*Echinogorgia reticulata*	-do-
NIO 730	*Haliclona cribricutis*	-do-
NIO 731	*Callyspongia fibrosa*	-do-
NIO 732	*Ircinia sp.*	-do-
NIO 733	*Echinogorgia competa*	-do-
NIO 734	*Petrocia testudinaria*	-do-
NIO 735	*Psammaplysilla purpurea*	Mandapam, Tamil Nadu
NIO 738	*Chrotella australiensis*	Malvan, Maharashtra
NIO 736	*Stoechospermum marginatum* (brown alga)	Anjuna, Goa
NIO 739	*Cladophora prolifera* (green alga)	Anjuna, Goa

Bioassay for preliminary screening:

Bacterial and fungal strains:

As shown in **Table 2**, seven bacterial pathogens: one gram positive (*Staphylococcus aureus*) and six gram negative (*Escherichia coli, Pseudomonas aeruginosa, Salmonella typhi, Shigella flexineri, Klebsiella sp.* and *Vibrio cholerae*) were used for the study. The fungal pathogens used for the study included *Aspergillus fumigatus, Fusarium sp., Cryptococcus neoformans, Aspergillus niger, Rhodotorula sp., Nocardia sp.,* and *Candida albicans*. All the test strains were clinical isolates from the Department of Medical Microbiology of Goa Medical College (GMC), Goa.

In vitro biological screening was performed by disc diffusion method[55-56] for primary selection of the extracts for studying therapeutic potential as antimicrobial agents. Disc diffusion method is highly effective for rapidly growing microorganisms. The activities of the methanolic extracts are expressed by measuring the diameter of the zone of inhibition. Generally, the more susceptible the organism the bigger is the zone of inhibition. The method is essentially a qualitative or semi quantitative test indicating sensitivity or resistance of microorganisms to the test materials as well as bacteriostatic or bactericidal activity of a compound.[57] The diameters of zones of inhibition produced by the compounds were compared with the standard antibiotic (Streptomycin) and antifungal (Nystatin) agents for their antibacterial and antifungal activities. The experiment was performed in triplicates to minimize errors.

Table 3 and **4** represents the result of *in vitro* antibacterial and antifungal activity shown by the methanolic extract of marine organisms against seven bacterial pathogens.

Table 2: List of pathogens (bacteria and fungi) used in the study.

Sr. No	Microorganisms	Code No.	Strain characteristics	Diseases caused
	Bacterial pathogens			
1.	*Escherichia coli*	B1	Gram Negative	Neonative meningitis
2.	*Pseudomonas aeruginosa*	B2	Gram Negative	Urinary tract infection
3.	*Staphylococcus aureus*	B3	Gram Positive	Skin infection
4.	*Salmonella typhi*	B4	Gram Negative	Typhoid
5.	*Shigella flexineri*	B5	Gram Negative	Gastro instestinal infection
6.	*Klebsiella sp.*	B6	Gram Negative	Urinary tract infection
7.	*Vibrio cholerae*	B7	Gram Negative	Cholera
	Fungal pathogens			
1.	*Aspergillus fumigatus*	F1		Skin infection
2.	*Fusarium sp.*	F2		Skin infection
3	*Cryptococcus neoformans*	F3		Skin infection
4.	*Aspergillus niger*	F4		Skin infection
5.	*Rhodotorula sp.*	F5		Skin infection
6.	*Nocardia sp.*	F6		Skin infection
7.	*Candida albicans*	F7		Candiasis

B-bacterial pathogens, F-Fungal pathogens

Table 3: Antibacterial activity of methanolic extract of marine organisms.

Species	Microorganisms						
	B1	B2	B3	B4	B5	B6	B7
Sigmadocia carnosa	+	+	+	+	+	+	++
Echinogorgia reticulata	-	-	-	-	-	-	-
Haliclona cribricutis	-	-	+	-	+	++	-
Callyspongia fibrosa	-	-	-	-	-	+	+
Ircinia sp.	-	-	+	-	-	-	-
Echinogorgia competa	-	-	-	-	-	-	-
Petrocia testudinaria	-	-	+	-	-	+	++
Psammaplysilla purpurea	++	-	++	+	+	+	++
Chrotella australiensis	-	-	-	+	+	+	++
Stoechospermum marginatum	-	-	-	-	-	+	+
Streptomycin (Standard)	+	+	+	+	+	+	+

(-) No activity, (+) weak activity (7-10 mm), (++) good activity (10-15 mm)

Table 4: Antifungal activity of methanolic extract of marine organisms.

Species	Microorganisms						
	F1	F2	F3	F4	F5	F6	F7
Sigmadocia carnosa	+	-	+	+	+	+	+
Echinogorgia reticulata	-	-	-	-	-	+	-
Haliclona cribricutis	+	-	-	-	-	-	-
Callyspongia fibrosa	-	-	-	-	-	-	-
Ircinia sp.	-	-	-	-	-	-	-
Echinogorgia competa	-	+	-	-	-	+	-
Petrocia testudinaria	-	-	++	-	+	-	-
Psammaplysilla purpurea	-	++	+	+	-	-	-
Chrotella australiensis	+	+	-	+	+	+	++
Stoechospermum marginatum	-	-	-	-	-	-	-
Nystatin (Standard)	+	+	+	+	+	+	+

(-) No activity, (+) weak activity (7-10 mm), (++) good activity (10-15 mm)

As evident from the **Tables 3** and **4** the organic extract from *Sigmadocia carnosa* exhibited weak activity against each of the bacteria and fungi tested except, its inactivity towards *Fusarium sp.* and good bactericidal activity against *Vibrio cholerae*. Good activity against the latter is also expressed by the extracts of sponges, *Petrocia testudinaria*, *Psammaplysilla purpurea* and *Chrotella australiensis*. *Psammaplysilla purpurea* also showed good activity against *E. coli* and *S. aureus* and weakly active against *Salmonella typhi*, *Shigella flexineri* and *Klebsiella sp.* Good activity was observed against fungal strain *Fusarium sp.* and it was weakly active against *Cryptococcus neoformans* and *Aspergillus niger*. *Haliclona cribricutis* showed considerable activity against *Klebsiella species* and was weakly active against both, *Staphylococcus aureus* and *Shigella flexineri*. *Callyspongia fibrosa* was weakly effective against *V. cholerae* and *Klebsiella sp.* Equally active was the brown alga *Stoechospermum marginatum* against the last two bacteria, contrary to the observation made by De Silva *et al.* (1982).[58] These authors report bactericidal activity attributed to the spatane diterpenoid, 19-acetoxy-5,15,18-trihydroxyspata-13,16-diene. The inactivity towards *S. aureus*

observed by us could be due to the collection of the seaweed at different developmental stages of the plant.

In this present investigation, as mentioned, *Haliclona cribricutis* inhibited *Klebsiella sp.* considerably and was weakly active against *Staphylococcus aureus* and *Shigella flexineri.* It was also found to be weakly active against the fungus, *Aspergillus fumigatus.* This is not surprising as the sponge belonging to this genus and collected from different regions is reported to possess wide variety of compounds with different biological activities. Thus *Haliclona sp.* from Indonesia yielded a triterpene ketide, Halicotriol B with weak antimicrobial activity against *S. aureus* and *Bacillus subtilis.*[59] The antifungal papuamine has been reported by Baker, *et al.* (1988)[60] from a *Haliclona sp.* Fahy *et al.* (1988)[61] reports a major antimicrobial alkaloid haliclonadiamine together with antifungal papuamine from *Haliclona sps* of Palau. Antifungal aminoalcohols have been identified from a new species of *Haliclona* from Queensland.[62] Charan *et al.* (1996)[63] report antimicrobial Haliclonacyclamines. It is therefore expected that the activity found by us in the extract of *Haliclona cribricutis* could have, at least partially, been contributed by any one of the above compounds isolated from this genus. Organisms belonging to the same genus are bound to have common chemical constituents. Parameswaran *et al.* (1998)[64] reports significant anti-viral and antibacterial activities in petroleum ether and ethyl acetate fractions of *H. cribricutis* and the activity observed against *K. pneumoniae* and *Vibrio parahaemolyticus* was attributed to O-demethyl renierones. *Ircinia sp.* exhibited mild antibacterial activity only against *Staphylococcus aureus* but all the fungal strains tested were insensitive to it.

A number of cytotoxic compounds are reported from the genus *Ircinia.* These include, 73-deoxychondropsin A from an Australian *Ircinia ramosa.* Chondropsin C was found in a Philippine *Ircinia* species.[65] Moderately cytotoxic cumulated ketene irciniketene has been reported from *Ircinia selaginea* collected from Guangxi Province, China.[66] Cytotoxic Kohamaic acids A and B are known to be constituents of Okinawan *Ircinia sp.*[67] Three tricarbocyclic sesterterpenoids of the

cheilanthane class isolated from a Queensland *Ircinia species* was found to be inhibitors of MSK-1 and MAPKA-2 protein kinases.[68] Though cytotoxic compounds are reported from this genus there are no reports of any antimicrobial activity in the extracts.

In the present investigation, the green alga *Cladophora prolifera* exhibited mild bactericidal activity against *S. aureus* and *Vibrio cholerae.* This alga has been recently reported to support the growth of indicator bacteria *Escherichia coli.*[69]

Marine organisms collected from the Southeast coast of India have been shown to possess a number of biological activities. In our studies the most interesting species are that of *Sigmadocia carnosa, Haliclona cribricutis, Psammaplysilla purpurea* and *Petrocia testidinaria.* To the best of our knowledge this is the first report demonstrating the antimicrobial activity of most number of the marine organisms taken up in such a study, though some of these have been reported earlier. (Published results)*

Preliminary biological screening of marine organisms showing good activities were selected for Minimal Inhibitory Concentration (MIC). MIC is defined as the lowest concentration of that compound/extract in a medium without visible growth of the test organisms. For the test, standard serial dilution technique[57] was employed. Four dilutions with concentrations 100, 50, 25 and 12.5 μg/disc were used which are designated as 1, 2, 3 & 4 respectively. The results obtained are given in the **Table 5.**

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* Antimicrobial activity of marine organisms collected off the coast of South East India", Rodrigues Ely, Tilvi Supriya, Naik C.G., ***Journal of Experimental Marine Biology and Ecology***, 309, 121-127, 2004.

Table 5: Minimal Inhibitory Concentration (MIC) results

Strains	*Sigmadocia Carnosa* MIC (µg/disc)	*Haliclona cribricutis* MIC (µg/disc)	*Petrocia testudinaria* MIC (µg/disc)	*Psammaplysilla purpurea* MIC (µg/disc)	*Chrotella australiensis* MIC (µg/disc)
B1	+ (50)	-	-	+ (25)	-
B2	-	-	-	-	-
B3	-	+ (100)	+	++ (12.5)	-
B4	-	-	-	+	-
B5	-	-	-	+ (50)	-
B6	-	++ (50)	+	-	-
B7	+ (25)	-	++ (25)	++ (12.5)	++ (100)
Strept-omycin	+	+	+	+	+
F1	-	+ (25)	-	-	-
F2	-	-	-	++ (50)	-
F3	-	-	++ (100)	+ (100)	-
F4	+ (100)	-	-	+	-
F5	-	-	+ (100)	-	-
F6	-	-	-	-	+
F7	-	-	-	-	++ (50)
Nystatin	+	+	+	+	+

(+) Weak activity: 1-3 mm; (++) Good activity: 4-6 mm

Among the organisms tested for MIC, *Psammaplysilla purpurea* showed good activity against bacterial strains, *E. coli* (25µg/disc), *S. aureus* (12.5µg/disc), *Shigella flexineri* (50µg/disc), *V. cholerae* (12.5µg/disc) and fungal strains, *Fusarium sp.* (50µg/disc), *Cryptococcus neoformans* (100µg/disc). *Haliclona cribricutis* showed MIC 100 and 50µg/disc against *S. aureus* and *Klebsiella sp.* respectively. It also exhibited antifungal activity against *Aspergillus fumigatus* at MIC 25µg/disc. *Sigmadocia carnosa* showed activity against *E. coli*, *V. cholerae* and *Aspergillus niger* with MIC of 50, 25 and 100 µg/disc respectively. Plate 1-6 shows bioassay results.

Based on the biological screening results two sponges *Psammaplysilla purpurea* and *Haliclona cribricutis* were taken up for detailed investigations with the objective of identifying active principles and novel molecules and has been discussed in the next sections, **Section 2** and **Section 3** respectively. Furthermore,

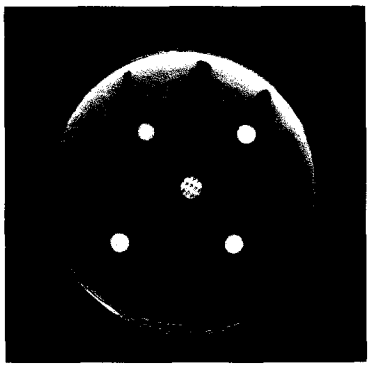

Plate 1: ***Psammaplysilla purpurea*** **against** ***Escherichia coli***

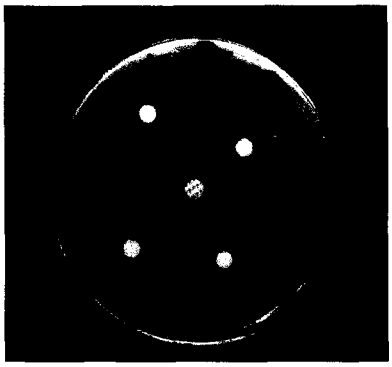

Plate 2: ***Psammaplysilla purpurea*** **against** ***Shigella flexineri***

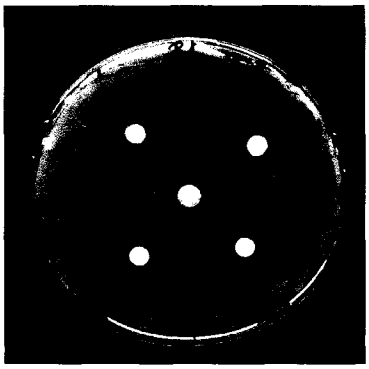

Plate 3: ***Haliclona cribricutis*** **against** ***Klebsiella sp.***

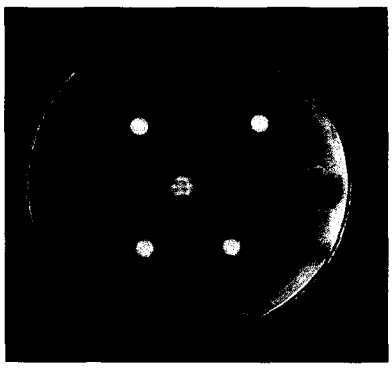

Plate 4: ***Haliclona cribricutis*** **against** ***Aspergillus fumigatus***

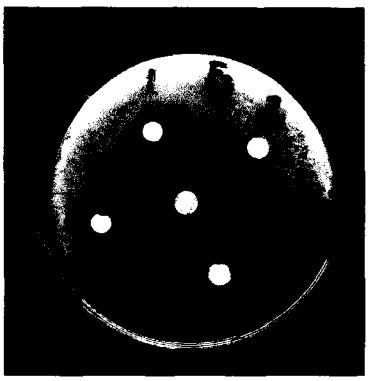

Plate 5:***Petrocia testudinaria*** **against** ***Cryptococcus neoformans***

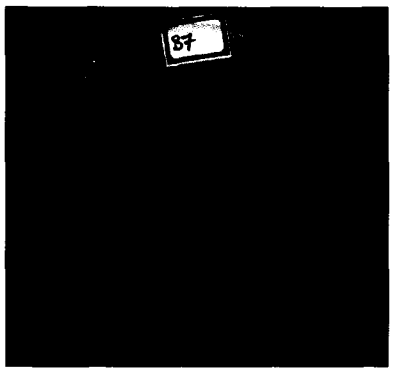

Plate 6: ***Sigmadocia carnosa*** **against** ***Vibrio cholerae***

Plates 1-6: Bioassay plates showing minimal inhibitory concentration (MIC) results

the encouraging results observed in this study indicated that the Indian coastline is a potential source of variety of marine organisms worthy of further investigation.

Experimental section:

Sample collection:

Marine organisms were collected by Scuba diving at depth range of 5-25 m, at different locations, Mahabalipuram and Mandapam, Southeast coast, India (9°16' N; 79°12' E) and Anjuna, west coast of Goa. Immediately after collection, they were freed from extragenous matter and plunged into containers containing aqueous methanol and transported to the laboratory.

Extract preparation:

The marine organism was homogenized in a blender and repeatedly extracted with aqueous methanol (3 X 500 ml) at room temperature. The combined methanolic extract was filtered and concentrated under vacuum on a rotary evaporator to get crude extract. These extracts were used for primary screening.

Preservation of test strains:

The strains obtained were preserved on plates and slants at 4°C for further studies.

Antibacterial assay:

Antibacterial activity was different tissue extracts was investigated by the standard paper disk assay method.[55-56] Whatman No.1 filter paper disk of 6 mm diameter were sterilized by autoclaving at 121°C/15min. The sterile disks were impregnated with different extracts (500μg/disc). Agar plates were surface inoculated uniformly from the broth culture of the test microorganisms. In all cases, the concentration was approximately 1.2 X 10^8 colony forming units (CFU)/ml. The impregnated disks were placed on the medium, suitably spaced apart and the plates were incubated at 37 °C for 24 h. Disk of streptomycin (10 μg/disc, Hi Media, Mumbai) was used as a positive control. The growth inhibition zones (mm) caused by the methanolic extracts of marine organisms were

examined. All the assays were carried out in triplicates. The samples showing good activity were checked for minimal inhibitory concentration (MIC) using four concentrations 100, 50, 25 and 12.5 μg/disc of the methanolic extract.

Antifungal assay:

Antifungal activity was determined using the paper disk assay method as previously described in the antibacterial assay. The sterile disks were impregnated with different extracts (500 μg/disc). The inoculum concentration was 0.5 X 10^3 - 2.8 X 10^3 CFU/ml. Nystatin (HiMedia, Mumbai) was used as a positive control at a concentration of 10 units/disc. The plates were incubated at room temperature for 24-48 h. The inhibition zones (mm) caused by the methanolic extracts of marine organisms were examined. All the assays were carried out in triplicates. The samples showing good activity were checked for minimal inhibitory concentration (MIC) using four concentrations 100, 50, 25 and 12.5 μg/disc of the methanolic extract.

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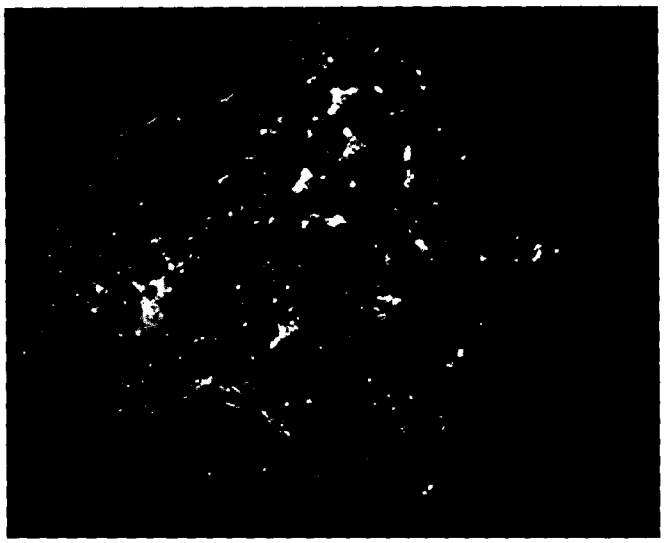
Section 2



Chemical investigation of marine sponge Psammaplysilla purpurea

2.1: Order Verongida-Review

Marine sponges (Phylum Porifera) are a rich source of secondary metabolites with novel structures and interesting biological activities.[1] In the context of the recent increased interest in the chemistry of marine organisms, the sponges, very primitive multicellular animals, have also received attention leading to the discovery of many novel molecules. Since Bergmann's pioneering work[2] in sponges, fatty acid, sterols and hundreds of different compounds have been isolated mostly in the last two decades.

A broad survey of bromo compounds is presented here as the chemical investigation on the sponge *Psammaplysilla purpurea* of the Order Verongida led to the identification of several bromotyrosine derivatives from this sponge.

Marine sponges belonging to the Order Verongida **(Table 1)** show peculiar biochemistry characterised by the production of sterols with an aplysane skeleton and of bromo compounds biogenetically related to tyrosine. These bromotyrosine derivatives are considered as distinct chemotaxonomical markers for Verongid sponges and range from simple monomeric metabolites to more complex structures. Over the past 30 years, an ever-increasing number of bromotyrosine derived secondary metabolites have been isolated from marine sponges, mainly from the Order Verongida. Here the emphasis is made on the Family Aplysinidae and Aplysinellidae.

Table 1: Systematic distribution of sponges of the Order Verongida (Phylum Porifera, Class Demospongiae, Subclass Ceractinomorpha)

Order	Family	Genera
Verongida	Aplysinidae	*Aplysina* *Verongula*
	Aplysinellidae	*Aplysinella* *Psammaplysilla* *Pseudoceratina* *Suberea*
	Ianthellidae	*Anomoanthella* *Bajalus, Ianthella*

Family Aplysinidae: This family consists of two genera *Aplysina* and *Verongula.*

Sponges of the Order Verongida have provided a series of antibiotics bromotyrosines and other closely related compounds. The first two members **1, 2** of the series were isolated from the methanolic extracts of *Verongia fistularis* (Syn. *Aplysina fistularis*) and *Verongula cauliformis* by Sharma and Barkholder.[5-7] The failure to convert **1** to **2** by reacting with methanol under various conditions allowed the authors to assume that the ketal **(2)** was a genuine natural product and not an artifact generated during the extraction. Subsequently, Audersan and Faulker[8] isolated from ethanolic extract of an undescribed species of *Verongia* the dienone **(1)** and the mixed ketal **(3)**, which was revealed to be a mixture of diastereoisomers. This suggested that the ketal was not a natural product and led the authors to propose that the dienone **(1)**, the ketal **(2)**, and the mixed ketal **(3)** may be derived from a single intermediate such as an arene oxide **(4)**, by 1,4 addition of water, methanol or ethanol during the extraction process.

From *A. fistularis forma fulva*, Gopichand *et al.*[9] reported more complex bromotyrosine metabolites fistularin-1 **(5)**, fistularin-2 **(6)**, fistularin-3 **(7)** and 5-[3,5-dibromo-4-(2-oxazolidinyl)methoxyphenyl]-2-oxazolidinone **(8)**. Two more complex brominated metabolites aerothionin **(9)** and homoaerothionin **(10)** obtained from *V. aerophoba* (syn. *A. aerophoba*) were also isolated from *V. thiona* by Moody and Thomson.[10] X-ray crystallography of the spirocyclohexadienyl isoxazole **(9)** established the relative stereochemistry of OH and O-N as *trans*. The spiro system in **(9)** and **(10)** could arise in various ways including nucleophilic attack by an oxime function on an arene oxide as shown in **(4)**.

All the verongida brominated metabolites seem obviously biosynthesized from 3,5-dibromotyrosine which itself is found in sponge proteins[11] and presumably the centered C_4N_2 and C_5N_2 chains of aerothionin and homoaerothionin seems to be derived from ornithine and lysine, respectively. But, isotopic studies failed to incorporated radioactivity from (U-^{14}C)-L-tyrosine into aerothionin, aeroplysinin-1 **(11)** and the dienone **(1)**. Inactive aerothionin was also isolated when the

animals were fed with (U-^{14}C)-L-ornithin and (CH_3-^{14}C)-methionin.[12] Aeroplysinin-1 **(11)**, the nitrile component was first isolated as the dextro-rotatory isomer from *V. aerophoba*[13] (Syn. *A. aerophoba*). Fulmor *et al.*[14] isolated the laevorotatory antipods of **(11)** from the closely related sponge *Ianthella ardis* from which they assigned the absolute configuration as (-). The absolute structure of both antipods as shown has been firmly established by two independent X-ray studies.[15-16]

V. aerophoba contained lactone, aerophobin-2 **(12)**,[17] isofistularin-3**(7)** and aerophobin-2 **(12)**.[18] Disruption of the compartmentation (by wounding), results in the conversion of isofistularin-3 **(7)** and aerophobin-2 **(12)** to dienone **(1)** under mild alkaline condition of seawater.[19] Compound **(9)** and **(10)** possesses antibacterial activity against *Alteromonas, Moraxella, Vibrio sp.* and *Photobacterium phosphoreum.*[19] They were also known to inhibit the growth of the marine microalgae *Coscinodiscus wailesii* and *Prorocentrum minimum* and act as repellent towards the marine gastropod *Littorina littorea.*[19] It is suggested that enzymatically catalyzed conversion of brominated metabolites in *V. aerophoba* represents a wound-induced defense mechanism from the marine environment.

A. insularis from Caribbean yielded two novel compounds **(20)** & **(21)** and number of known brominated metabolites **(1, 4, 7, 9-10 & 13-19)**.[20] Fendert *et al.* isolated 14-oxoaerophobin-2 **(22)** from the same source.[21] Fistularin-3 **(7)** & 1-epifistularin-3 **(23)**, has been found in the *Verongia aerophoba* collected in the Aegean Sea.[22]

A. archery showed to contain various bromotyrosine derivatives **(2, 19, 24-27)**[23] along with earlier isolated, aeroplysinin-1 **(11)**, dienone **(1)**, fistularin-3 **(7)** & oxazolidone **(9)**. Structure of **(24)** containing two units of cyclohexanone instead of cyclohexadienyl ring was assigned on the basis of spectroscopic evidence including 2D NMR experiments. It showed antifungal activity against *Cryptococcus neoformans* ATCC90113.

Patrizia *et al.*, 1997[24] reported oxahomoaerothionin **(28)** and 11-hydroxyfistularin-3 **(29)** from Mediterranean sponge *A. cavernicola.* The configuration at the chiral center has been assigned through application of the modified Mosher's method. Compound **(29)** was treated with R(-) & S(+) ∝-methoxy-2-(trifluoromethyl)-phenylacetyl (MTPA) chloride in pyridine solution at room temperature for 2hr. to give esters **(30-31).**

Evan *et al.*, 2000[25] isolated aplyzanzine A **(32)** from Indo-Pacific sponge *Aplysina sp.* aerothionin **(9)** and calafianin **(33)** from *A. gerardogreeni* were evaluated against multidrug-resistant clinical isolates of *M. tuberculosis.* But only aerothionin showed antimycobacterial acivity against four monoresistant variants of *M. tuberculosis* H37Rv (rifampin, isoniazid, ethambutol and streptomycin resistant).[26-27]

Archerine **(34)** from the Caribbean sponge *A. archeri,* showed above typical feature of Verongida bromo metabolites in having the central chain formed by two 2-aminohomohistamine residues connected through a carbon-carbon bond. Archerine **(34)** exhibited antihistamine activity on the isolated guinea pig ileum at concentrations as low as 1 μM and the capability of it to chelate zinc ions was also investigated.[28]

Family: Aplysinellidae

1) *Aplysinella*

Four bromotyrosine metabolites **(37-40)** along with psammaplin A **(35)** and bisaprasin **(36)** were isolated from *Aplysinella rhax* collected from Guam, Palau and Pohnpei. Extensive spectroscopic analysis and chemical reactions established the structures of psammaplins A1 **(37)** and A2 **(38)** as N, N-dimethylguanidium salt of psammaplin A sulfate and its bis-N, N-dimethylguanidium disulfate derivatives, respectively. These compounds exhibited moderate cytotoxicity and inhibitory activities against farnesyl protein transferase and leucine aminopeptidase.[29] Tabudravu *et al.* reported psammaplins K **(41)** and L **(42)** along with **(35)** and **(36)** from the Fijian sponge *A. rhax* during a bioassay guided

isolation protocol. Psammaplins A was found to moderately inhibit chitinase B from *Serratia marcescens*, the mode of inhibition being non-competitive. Crystallographic studies suggest that a disordered psammaplin A molecule is bound near the active site.[30]

2) *Psammaplysilla:*

Three species are known of the genus *Psammaplysilla*; 1) *Psammaplysilla purpurea,* 2) *Psammaplysilla purea* and 3) *Psammaplysilla arabica.*

Psammaplysilla purpurea:

Early studies on the sponge *P. purpurea* afforded a dibromotyrosine metabolite, 2-hydroxy-3,5-dibromo-4-methoxyphenyl acetamide **(43)**[31] characterised through interpretation of spectral data. In 1978, Ayanoglu *et al.*,[32] isolated four sterols, aplysterol **(44)**, 3β-hydroxy-26,27-bis-norcholest-5-en-24-one **(45)**, 3β-hydroxypregna-5-en-20-one **(46)** and 3β-hydroxy-5α-pregnan-20-one **(47)** along with the usual sterols in minor amounts. Three new bromotyrosine-cysteine derivatives, psammaplins B, C & D **(48-50)** including 3-bromo-4-hydroxybenzene aldehyde **(51)** and 3-bromo-4-hydroxyphenyl acetonitrile **(52)** were isolated from the sponge *P. purpurea.* Psammaplin D **(50)** showed antimicrobial and mild tyrosine kinase inhibitory activity.[33]

Psammaplin-A **(35)**, containing a disulfide bridge along with 3-bromo-4-hydroxyphenylacetonitrile **(52)** has been isolated from the cytotoxic extract of an unidentified species of *Psammaplysilla.*[34] Kim *et al.* (1999) reported that psammaplin A **(35)**, possesses antibacterial activity against methicillin-resistant gram-positive *Staphylococcus aureus* that was almost comparable to ciprofloxacin, a quinolone antibiotic currently used in the US. Furthermore, extensive mechanism of action studies completed by these investigators determined that psammaplin A did not bind to penicillin-binding proteins. It did not inhibit DNA synthesis of *S. aureus* SG 511 in a dose-dependent manner (IC_{50}=2.83 μg/ml) but inhibited the super coiling activity of DNA gyrase, similar

to the quinilones although less efficiently than ciprofloxacin.[35] 14-Debromo araplysillin (**53**) and 14-debromo prearaplysillin (**54**) together with araplysillin (**55**) has been reported from *Druinella(=Psammaplysilla) purpurea* by James *et al.*[36] Copp *et al.*[37] described cytotoxic psammaplysins A-C (**56-58**) from the same source. All these compounds possess moderate *in vitro* cytotoxicity towards the human colon tumor cell HCT116.

A macrocyclic peptides bastadin 2 (**59**) and the previously reported bastadins 5, 7 & 12 (**60-62**) were identified from Pohnpei *P. purpurea* by Carney *et al.*[38] Compound (**62**) is mildly cytotoxic against several cell lines and inhibits the enzymes topoisomerase II and dehydrofolate reductase.

Nine bromotyrosine metabolites purpuramines A-I (**63-71**) were isolated from the marine sponge *P. purpurea.*[39] All the compounds exhibited antibacterial activity against *S. aureus*. Jurek *et al.*[40] reported three cytotoxic bromotyrosine metabolites, aplysamines 3-5 (**72-74**) from the same source. In 1994, Pakrashi *et al.*[41] reported two new bromotyrosine metabolites (**75-76**) from the sponge *P. purpurea* collected from Bay of Bengal and the structures assigned on the basis of spectroscopic analysis of their acetylated products (**75a & 76a**).

Venkateswarlu *et al.*[42] identified three compounds, 3,5-dibromo-4-hydroxyphenyl acetonitrile (**77**), 3-bromo-4methoxyphenyl acetonitrile (**52**) and 3,5-dibromo-4-methoxybenzoic acid (**78**) from the *P. purpurea* collected from Tuticorin coast and subsequently Venkateswarlu and his co-workers[43-44] reported three bromotyrosine derivative (**79a & b, 80**) besides the known molakaimine (**81**), 2,6-dibromo-4-hydroxyacetamido-1,1-dimethoxy-2,5-cyclohexadiene (**2**), aerothionin (**9**), bastadin-6 (**82**), bastadin-16 (**83**). Purealidin P, purealidin G and aplysamine-2 (**84**) were also reported from the same source collected off the Mandapam coast, India.

Psammaplysilla purea:

Rotem *et al.*,[45] isolated bromotyrosine metabolite **(85)** along with the known 14-debromo prearaplysillin **(54)** from *P. purea.* Same sponge yielded two antimicrobial constituents psammaplysin A and B **(56-57)** characterized by spectroscopic analysis, which was further confirmed by single crystal X-ray diffraction studies on its acetamide acetate.[46] The FABMS of the known metabolites aerophobin-1 **(86)** and aerophobin-2 **(87)** has been discussed in detail by Cimino *et al.*[47] Nakamura *et al.* isolated purealin **(88)** from the Okinawan, *P. purea.*[48] Purealin **(88)** moderates enzymic reactions of ATPases. From the same source lipopurealins A-C **(89-91)** inhibitors of Na, K-ATPase were obtained.[49] Takido *et al.* [50] & Nakamura *et al.* [51-52] independently established purealin **(92)** and its methoxy derivative **(93)**, which activates myosin EDTA-ATPase at 0.3-30μM inhibited myosin Ca-ATPase and Na^+, K^+-ATPase.

Okinawan *P. purea* yielded cytotoxic bromotyrosine alkaloid purealidin-A **(94)**.[53] It also showed promising antileukemic activity. Later, Kobayashi[54] reported purealidins B **(95)** & C **(96)**. Of this **(95)** showed antibacterial activity and **(96)** exhibited antifungal & antineoplastic activities. Tsuda *et al.*[55-56] reported purealidin D **(97)** and purealidins E-G **(98-100)** from the same source.

Two new guanidine alkaloids, aplysillamides **(101-102)**[57] having antimicrobial activity was isolated from *P. purea.* Okinawan *P. purea* yielded lipopurealidins D-E **(103-104)** and purealidin H **(105)**.[58] The structures were elucidated on the basis of 2D NMR and FAB MS/MS data. Further, Kobayashi *et al.*[59-60] reported nine bromotyrosine alkaloids, purealidins J-R **(106-113, 19)**. Purealidins N, P and Q **(110, 112 & 113)** were cytotoxic to tumor cell lines, while purealidins J, K, P and Q **(106, 107, 112 & 113)** showed moderate inhibitory activity against epidermal growth factor (EGF) inhibitor kinase.

Psammaplysilla arabica:

Longeon *et al.* [61] isolated antimicrobial agent araplysillin-I & II **(114 & 115)**, from the sponge *P. arabica* that inhibited of Na^+/K^+-ATPase.

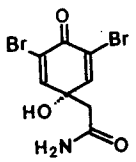
3) *Pseudoceratina*:

So far there are only few reports on the genus *Pseudoceratina*. Faulker *et al.*[62] reported two dibromotyrosine metabolites, ethyl 3,5-dibromo-4 -(3'-N,N-dimethylaminopropyloxy)cinnamate and 3,5-dibromo-4-(3'-N,N-dimethylaminopropyloxy)cinnamic acid **(116-117)** from the Caribbean sponge *Pseudoceratina crassa.* In 1993, Costantino *et al.*[63] reported five membered alkaloids, crasserides **(118-123)**, unique glycerides from the same sponge. A triterpene **(124)** & bromotyrosine metabolites **(125-128)** were isolated from the same source.[64-65]

Caribbean sponge, *Pseudoceratina sp.* yielded brominated products **(129 & 130).**[66] Benharref *et al.*[67] reported three new bromotyrosine alkaloids, pseudoceratinines A-C **(131-133)** from *P. verrucosa* in 1996. Spermidine, pseudoceratidin **(134)** with two 4,5-dibromopyrrole-2-carbamyl units[68] and an antifouling cyanoformamide derivative, pseudoceramine **(135)**[69] were reported from *P. purpurea* by Tsukamoto *et al.* Tsukamoto *et al.*[70] again isolated ceratinamides A and B **(136-137)**, which inhibits larval settlement and metamorphosis of the barnacle *Balanus amphitrite* with ED_{50} values of 0.1-0.8 μg/mL. Ceratinamine **(138)** and molakaimine **(139)** isolated from the same source was synthesized by Ryan *et al.* in 1998.[71]

Zamamistatin **(140)** exhibited significant antibacterial activity against *Rhodothalassium salexigens,* which has adhering properties and may be a valuable candidate for a novel antifouling agent.[72] Three new bromotyrosine metabolites, Tokaradines A-C **(141-143)** isolated from *P. purpurea* were found lethal to the crab *Hemigrapsus sanguineus* and acetylation of **(142)** yielded N,O–diacetyltokaradine B **(144).**[73]

In the section which follows, chemical investigation of the sponge *Psammaplysilla purpurea* has been discussed.



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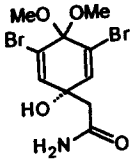

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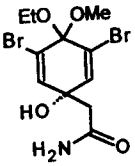

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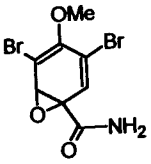

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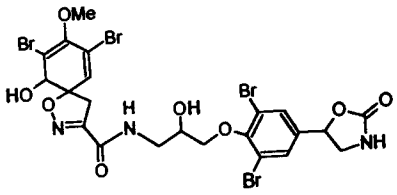

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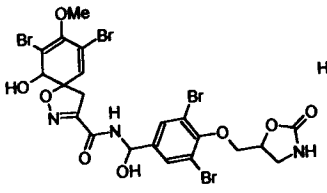

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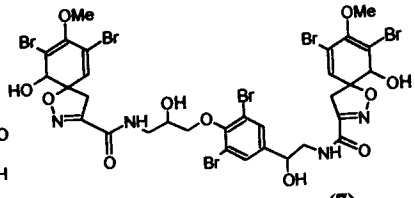

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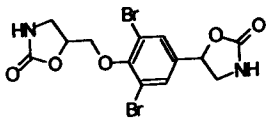

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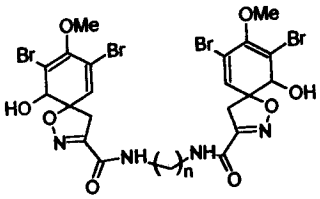

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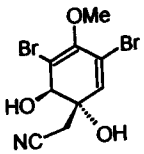

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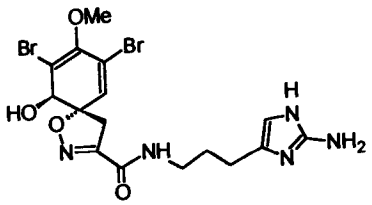

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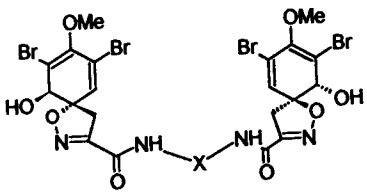

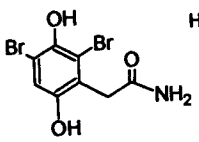

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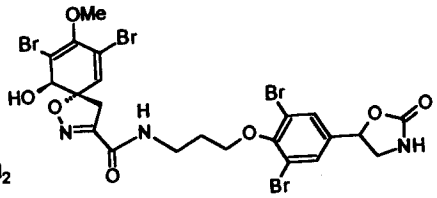

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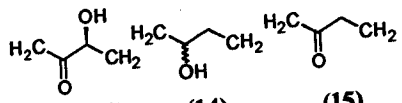

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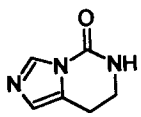

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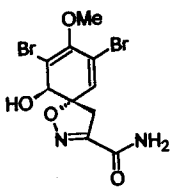

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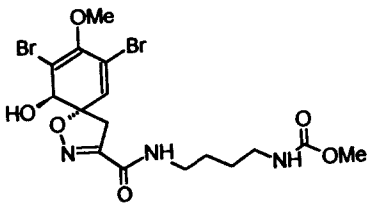

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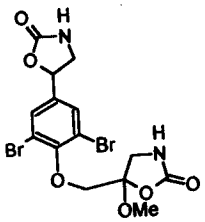

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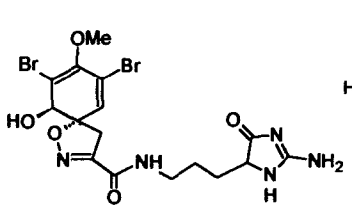

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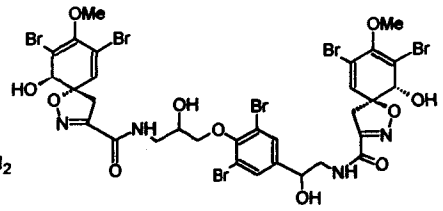

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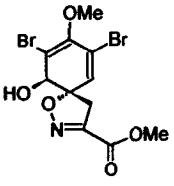

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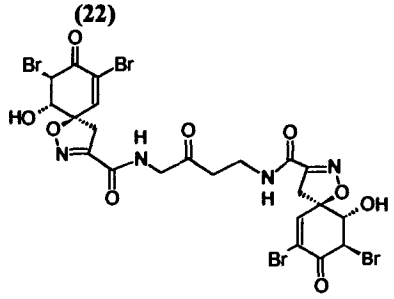

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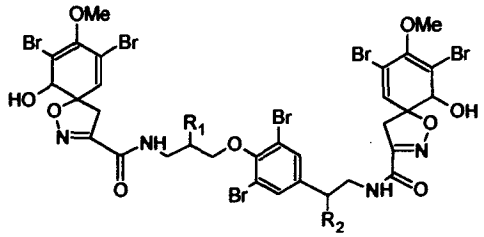

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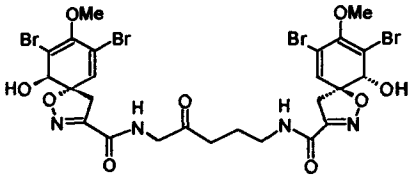

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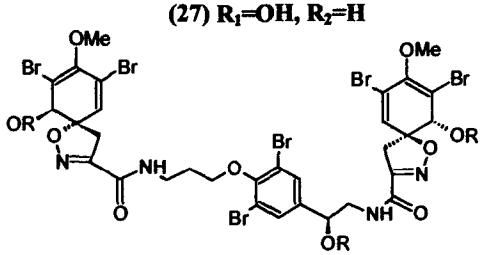

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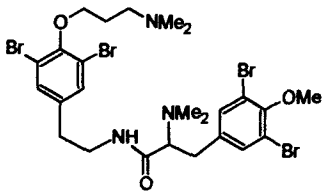

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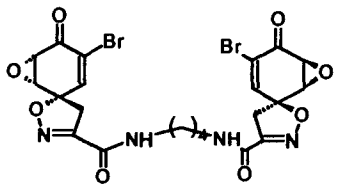

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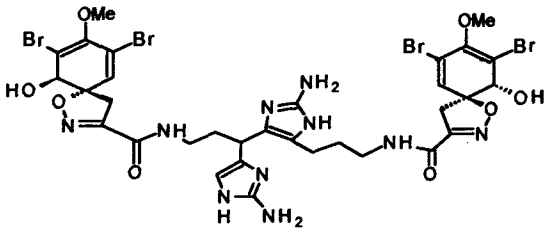

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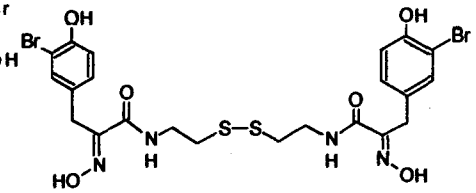

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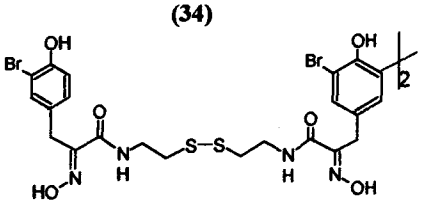

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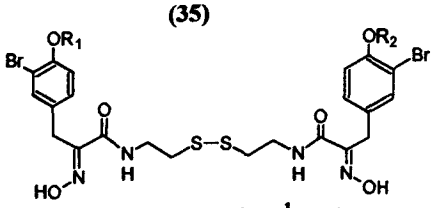
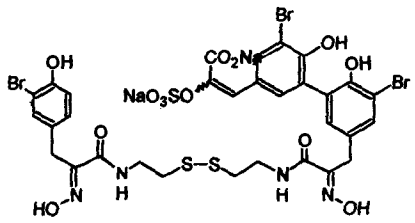


(37) R_1=H, R_2=SO_3^{-1}, n=1
(38) R_1=R_2=SO_3^-, n=2



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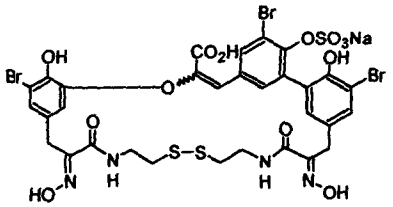

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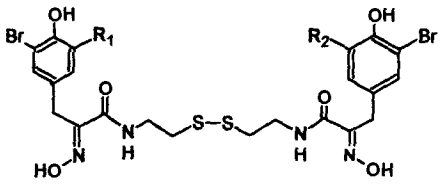
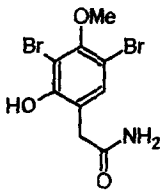


(41) R_1= H, R_2=OH
(42) R_1=OH, R_2=OH



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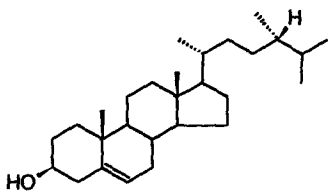

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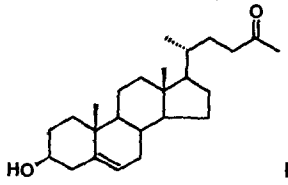

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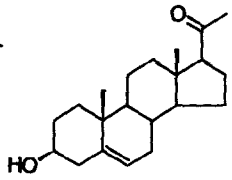

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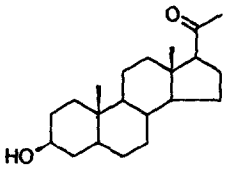
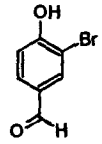


(47)

(48) R= SC
(49) R= SO_2NH_2
(50) R=$S_2CH_2NHCOOMe$



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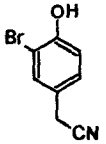

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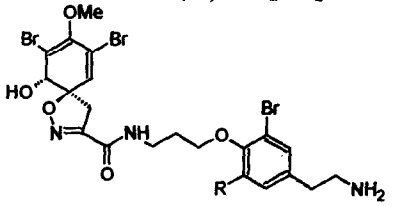
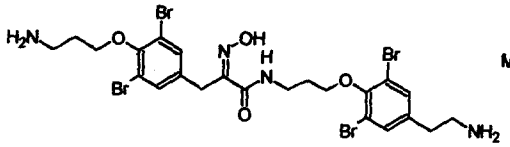


(53) R= H
(54) R=Br



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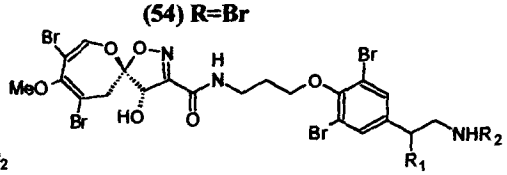
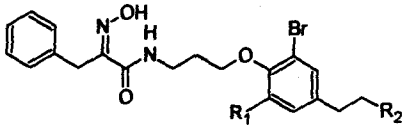
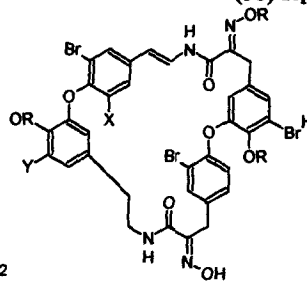


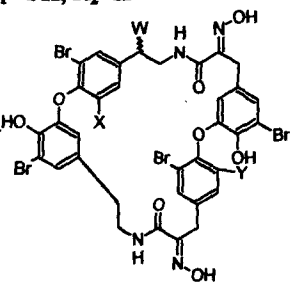
(56) R_1=OH, R_2=Me
(57) R_1=R_2=H
(58) R_1=OH, R_2=H



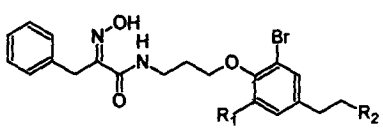
(66) R=H
(67) R=Me

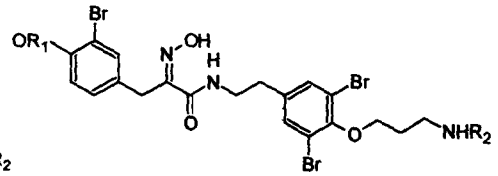


(59) X=Br, Y=Br, R=H
(60) X=H, Y=Br, R=H



(61) W=H, X=H, Y= Br
(62) W=OH, X=Br, Y=H

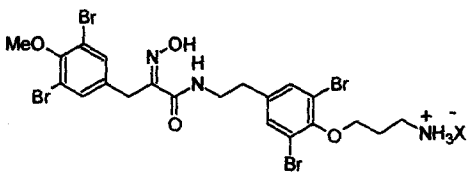




(63) R_1= Br, R_2=NH_2;
(64) R_1=H, R_2=NH_2

(68) R_1=H, R_2=H; (69) R_1=H, R_2=Me
(70) R_1=Me, R_2=H; (71) R_1=Me, R_2=Me

(65) R_1=Br, R_2=



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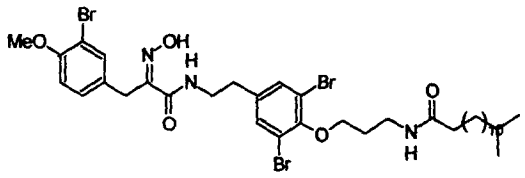
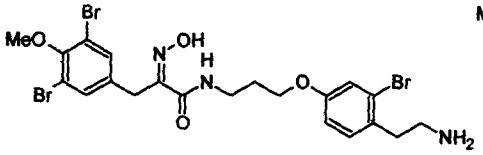
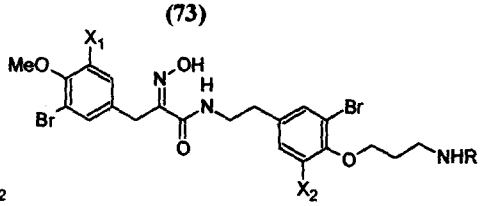


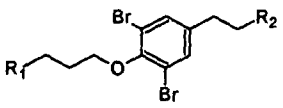
(73)

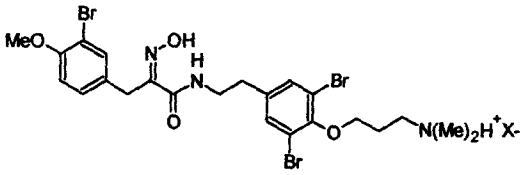


(74)



(75) R=H, X_1=X_2=H
(76) R=H, X_1=Br, X_2=H
(75a) R=Ac, X_1=X_2=H
(76a) R=Ac, X_1=Br, X_2=H

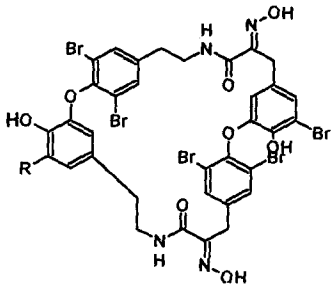




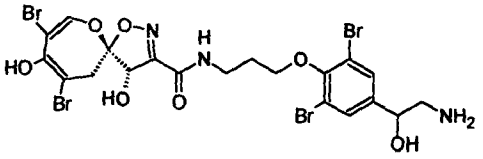
(77) R_1=Br, R_2=CH_2CN
(78) R_1=Br, R_2=COOH

(79a) R_1=$N(CH_3)_2H^+X^-$;R_2=NH_2
(79b) R_1=$N(CH_3)_2H^+X^-$;R_2=NHAc
(80) R_1= $N(CH_3)_2$; R_2=NHAc
(81) R_1=R_2=NH_2

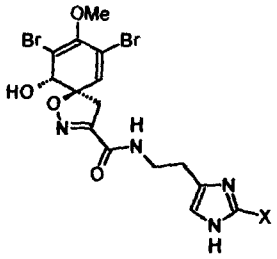
(84)



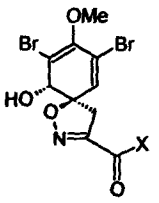
(82) R=Br
(83) R=H



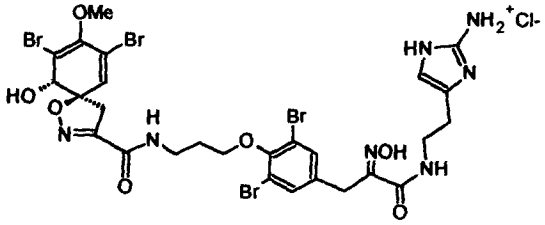
(85)



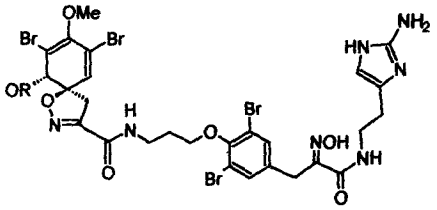
(86) X=H
(87) X=NH_2



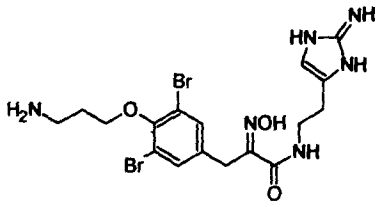
(89) X=$CO(CH_2)_{11}CH_2CH_3$
(90) X=$CO(CH_2)_{11}CH(CH_3)_2$
(91) X=$CO(CH_2)_{13}CH_2CH_3$



(88)



(92) R=Me
(93) R=H



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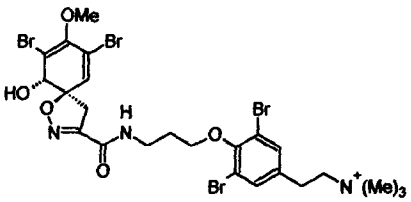

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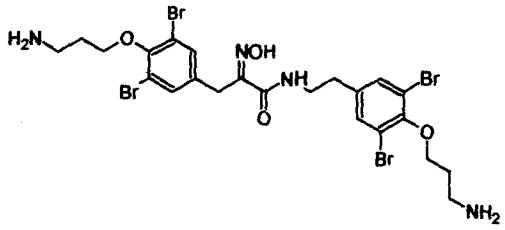

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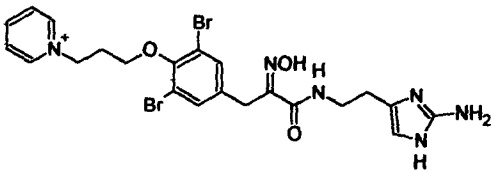

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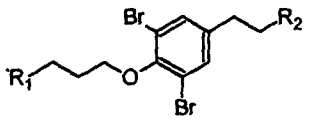
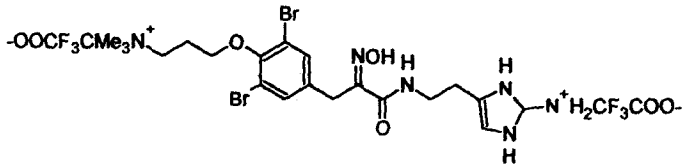


(99) R_1=$N^+H_2MeCl^-$; R_2=$NHMe_2Cl^-$
(100) R_1= $N^+HMe_2Cl^-$; R_2= NH_2MeCl^-



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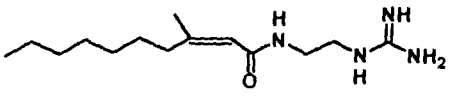

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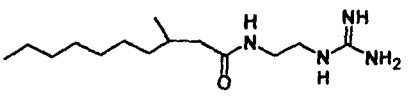

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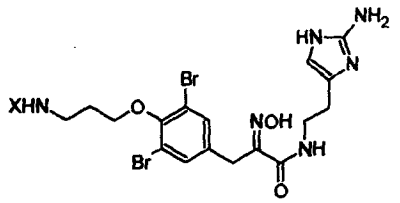
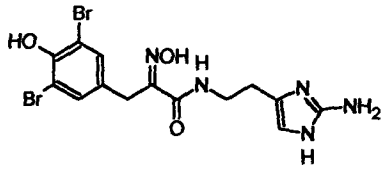


(103) X=CO(CH$_2$)$_7$CH=CH(CH$_2$)$_4$CH(CH$_3$)$_2$
(104) X=CO(CH$_2$)$_{17}$CH$_3$



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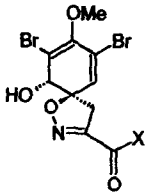
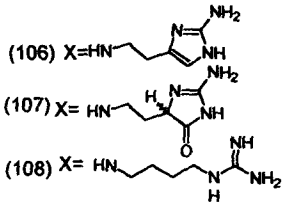
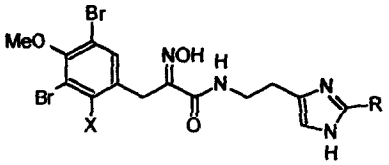
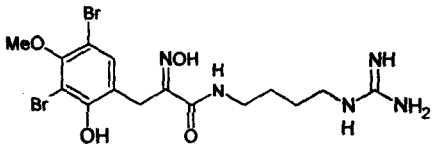


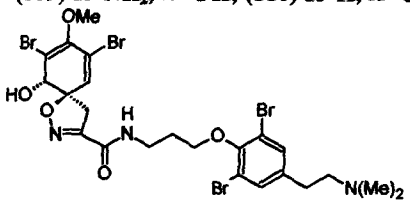




(109) R=NH$_2$, X=OH; (110) R=H, X=OH



(111)



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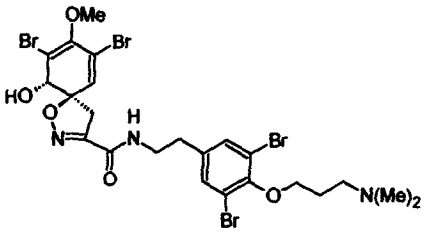

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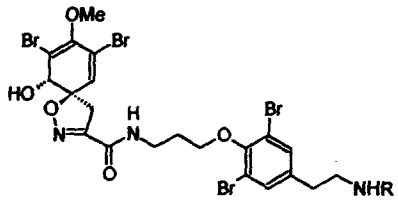
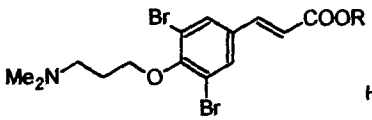
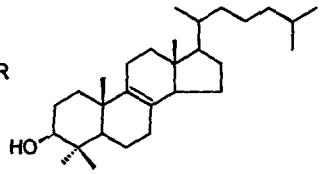


(114) R=H
(115) R=COCH$_2$(CH$_2$)$_n$CH(CH$_3$)$_2$



(116) R=Et
(117) R=H



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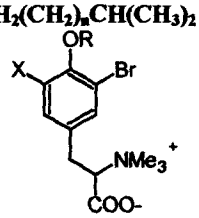
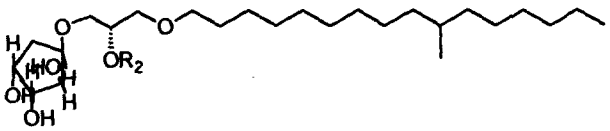
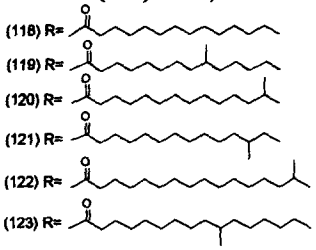
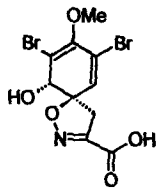


(125) R=H; X=Br
(126) R=Me; X=Br
(127) R=Me; X=H
(128) R=H; X=H







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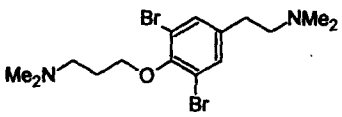

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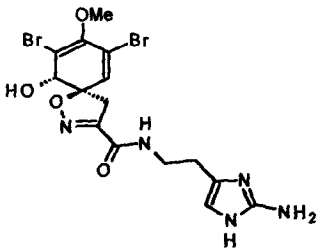

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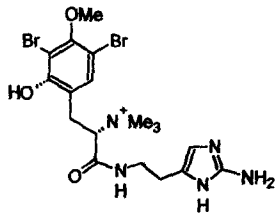

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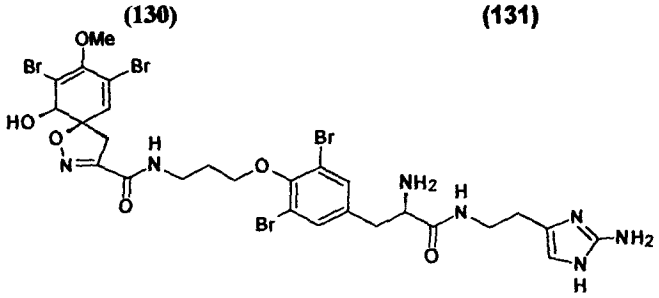

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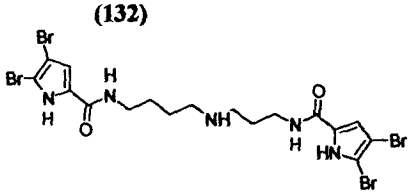

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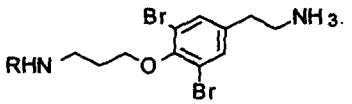

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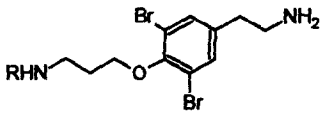
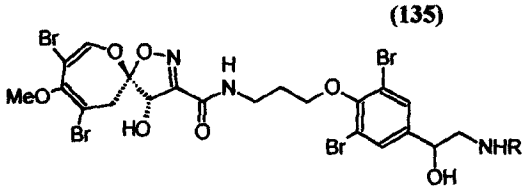
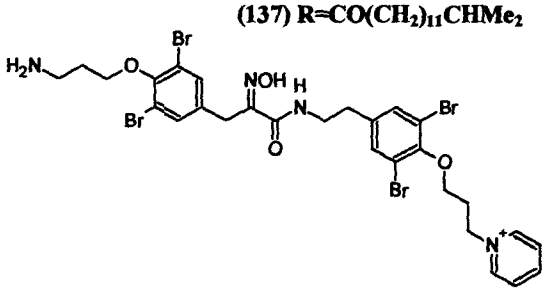


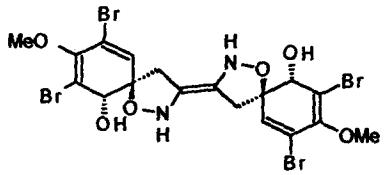
(138) R=COCN
(139) R=H



(136) R=CHO
(137) R=CO(CH$_2$)$_{11}$CHMe$_2$



(141)



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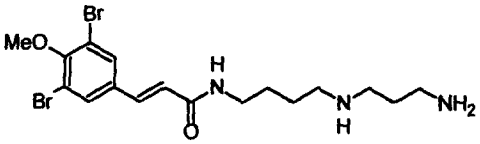
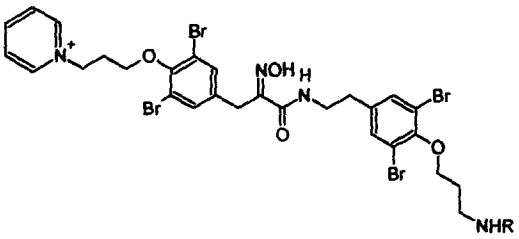


(143)



(142) R=H
(144) R=Ac

2.2: Bromotyrosine alkaloids from marine sponge *Psammaplysilla purpurea.*

Search of drugs from the sea is an active area of research all over the world. The work on bioactive substances from marine organisms in India is still in its infancy though it has gained momentum in the past one decade.

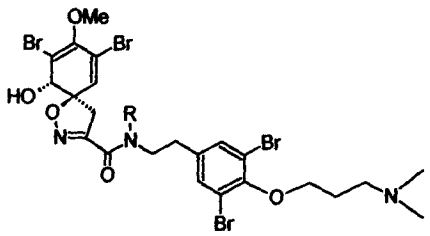
A programme in collaboration with CDRI and under PL480 funds led to the collection of baseline data on the bioactivities of flora and fauna from the organisms of Indian coast. The programme also helped in establishing a diving center at NIO. This was followed by a National programme in collaboration with CDRI and ten other laboratories funded by DOD. I had an opportunity of working under this programme "Development of potential drugs from Indian Ocean.

The work carried out on the chemistry of the sponge *Psammaplysilla purpurea* from Mandapam is presented here. This organism was selected on the basis of antimicrobial activity exhibited by its crude methanolic extract. The crude methanolic extract of sponge *P. purpurea* exhibited antibacterial activity against *E. coli, Staphylococcus aureus, Salmonella typhi, Shigella flexineri, Klebsiella sp.* & *V. cholerae* and antifungal activity against *Fusarium sp., Cryptococcus neoformans* and *Aspergillus niger* **(Chapter II, Section 1)**. Fractionation of the crude extract located the activity in ethyl acetate fraction.

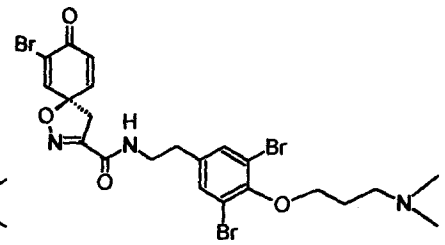
Psammaplysilla purpurea (Carter, 1880) belongs to the phylum Porifera, Class Demospongiae, Subclass Ceractinomorpha, Order Verongida, Family Aplysinellidae. It is yellow sponge when fresh, turning dark violet when it is dried characteristics of any marine organisms containing bromo compounds.

The chemical investigation of active ethyl acetate fraction of the sponge resulted in isolation of seven new bromotyrosine alkaloids purpurealidin A **(2)**, B **(3)**, C **(4)**, D **(5)**, F **(7)**, G **(8)**, H **(9)** along with the known compounds purealidin Q **(1)**, purpurealidin E **(6)**, 16-debromoaplysamine-4 **(10)** and purpuramine I **(11)**. The

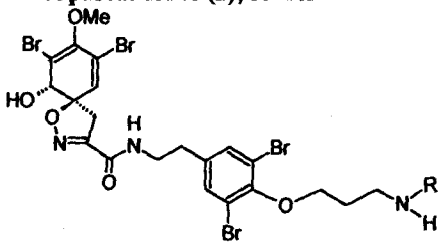
investigations concerning the elucidation of their structures are presented in this section.



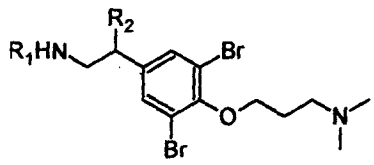
Purealidin Q (1); R=H
Purpurealidin A (2); R=Me



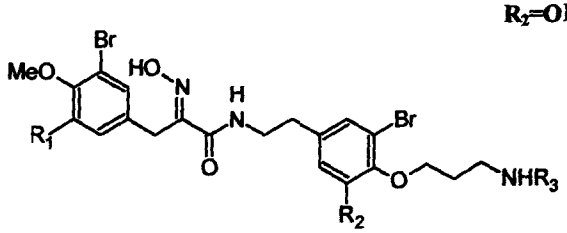
Purpurealidin B (3)



Purpurealidin C (4); R=–CO(CH$_2$)$_{11}$CH(CH$_3$)$_2$
Purpurealidin D (5); R=–CO(CH$_2$)$_{12}$CH$_2$CH$_2$CH$_3$



Purpurealidin E (6); R$_1$=R$_2$=H
Purpurealidin F (7); R$_1$=H, R$_2$=OH
Purpurealidin G (8); R$_1$=–COCH$_2$CH$_3$, R$_2$=OH



16-Debromoaplysamine-4 (9); R$_1$=Br, R$_2$=R$_3$=H
Purpurealidin H (10); R$_1$=Br, R$_2$=H, R$_3$=Me
Purpuramine I (11); R$_1$=H, R$_2$=Br, R$_3$=Me

The ethyl acetate fraction was subjected to repeated chromatography using successive sephadex LH-20 and silica gel columns to give polar compounds purealidin Q, purpurealidin A, B, C, D, E, F, G. Final purification of 16-debromo aplysamine 4 and purpuramine I was done on reversed phase ODS column (250 × 8mm i.d, flow rate 2ml/min & UV detection at λ_{max} 254nm) with H_2O:MeOH (15:85).

The elution of the ethyl acetate fraction on silica gel column with 8% MeOH:$CHCl_3$ followed by purification on gel chromatography (Sephadex LH-20) using MeOH:$CHCl_3$ (1:1) as mobile phase yielded compound (1) which was

obtained as colourless oil. The UV spectrum showed λ_{max} at 284 and 277 nm. HRMS with pseudomolecular ion peak at m/z 741.8691, 743.7871, 745.7710, 747.7762, 749.7914 in the ratio 1:4:6:4:1, was indicative of the presence of four bromine atoms in the molecule and established the molecular formula as $C_{23}H_{27}N_3O_4Br_4$ **(Fig 1.7)**. The IR **(Fig 1.1)** absorptions at 3418 and 1668 cm^{-1} implied the presence of NH/OH and secondary amide carbonyl group respectively. The ^{1}HNMR spectrum **(Fig 1.2)** showed two-proton singlet at δ_H 7.35 for a symmetrically tetrasubstituted benzene. Peak at δ_H 6.29 (s) was attributed to benzene proton. Intense proton signal observed at δ_H 2.52 (6H, s), was assigned to two N-Me groups. In addition to N-Me, the peak was evident at δ_H 3.74 (3H, s) for -OMe. Methylene protons displayed four triplets at δ_H 3.54, 2.77, 4.05, 2.92 and multiplet at δ_H 2.19.

The proton spin-spin coupling of the AB quartet at δ_H 2.98 and 3.92 could be assigned to an isolated methylene group that is adjacent to an π-electron system because of the large germinal J value of 18.3 Hz. The ^{1}H-^{1}H COSY revealed the presence of a 1,3-disubstituted propane moiety (cross-peaks of H18/H19 and H19/H20) and 1,2-disubstituted ethane moiety (cross peaks of H10/H11).

The TOCSY spectrum **(Fig 1.4)** showed the proton connectivities H10-H17, H11-H13 in addition to H10-H11. The methylene protons (H10) next to the amide nitrogen (δ_H 3.54, 2H, t) were coupled to benzyl methylene protons (δ_H 2.77, 2H, t). The spectrum also revealed that the methylene protons at δ_H 2.98, d and 3.92, d each for H7a and H7b were coupled to H5 of the dibromospirocyclohexadienyldihydroisoxazole ring moiety. TOCSY correlations are illustrated in **Fig 1a**.

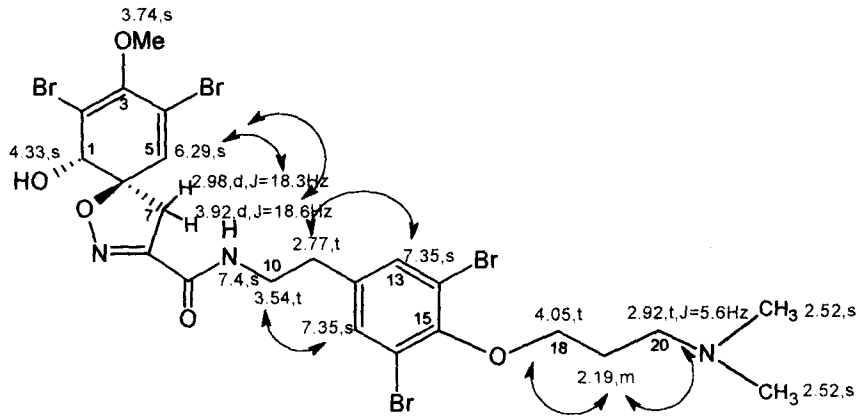


Fig 1a: TOCSY correlations of purealidin Q (1).

The ^{13}CNMR data including DEPT experiments **(Fig 1.4, Table 1)** showed two methyl signals, six methylene signals, three methine signals and nine quaternary carbon signals. Considering the intensities of the ^{13}C signals at δ_C 118.1(s) and 132.9 (d) were ascribed to two carbons each on a symmetrical benzene ring, respectively.

The protonated carbons were all assigned by heteronuclear multiple quantum coherence (HMQC) experiments **(Fig 1.5)**. Intense ^{13}C signal at δ_C 44.5 was assigned to the methyl group of dimethyl amine group. Since there were numerous quaternary carbons, extensive Heteronuclear multiple bond correlation (HMBC) experiments were necessary for determining the carbon-carbon connectivities in **1**.

The $^2J_{CH}/^3J_{CH}$ correlations obtained by the HMBC spectrum **(Fig 1.6)** are summarized in **Fig 1b**, revealing the presence of tetrasubstituted benzene and a pentasubstituted 1,3-cyclohexadiene moiety. The position of the oxygen substituents was determined to be C1, C3, C6, C15 and C18 based on the relatively low-field shifts of these carbons. The relatively high-field sp^2 carbons of C2, C4, C14 and C16 were suggestive of bromine substitution at these carbons. The HMBC correlations of -OMe/C3 and H-11/C12 indicated the connectivities between C3 and the methyl group of -OMe via ether bond and between C11 of ethyl amine and C12 of benzene ring. The HMBC correlations of H-20/NMe_2

revealed the connectivity between C20 and -NMe_2 via a nitrogen atom. The singlet at δ_H 4.33 (H1) showed the cross peaks to C2, C3, C5 and C6. The aromatic proton signal at δ_H 6.29 (H5) was correlated to C1 (δ_C 73.8), C3 (δ_C 147.5) and C4 (δ_C 121.4) in the HMBC experiments. Isolated methylene protons δ_H 2.98 and 3.92 showed correlations with C1, C5, C6, C8 and C9. The complete assignments of all the HMBC correlations are shown in **Fig 1b.**

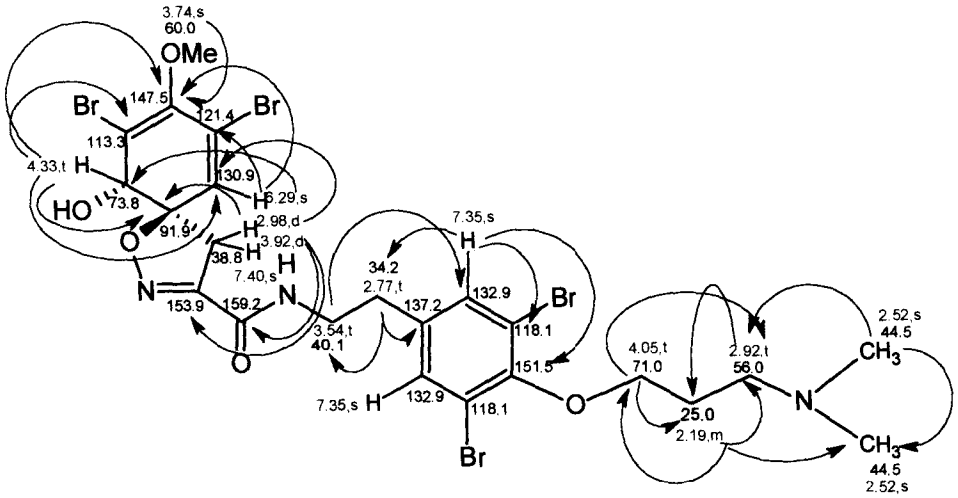


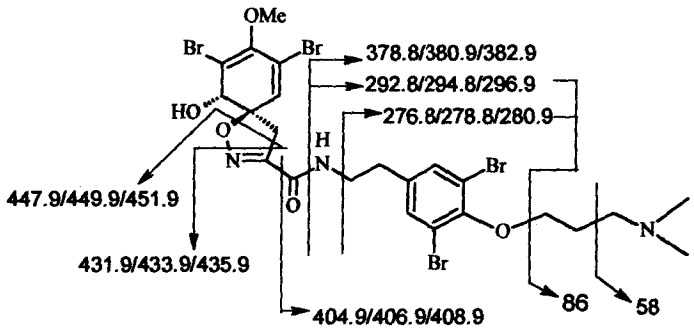
Fig 1b: HMBC correlations of purealidin Q (1).

Table 1: ^1H, ^{13}C NMR, TOCSY and HMBC of purealidin Q (1)

Carbon No.	^{13}CNMR δ_C, ppm	^{1}HNMR δ_H, ppm	TOCSY Correlations	HMBC Correlations
1	73.8,d	4.33(1H,s)		C2, C3, C5, C6
2	113.3,s			
3	147.5,s			
4	121.4,s			
5	130.9,d	6.29(1H,s)	H7	C1, C3, C4,
6	91.9,s			
7	38.8,t	Ha=2.98(1H,d, J=18.3Hz) Hb=3.92(1H,d, J=18.6Hz)	H5	C1, C5, C6, C8 C1, C5, C6, C8, C9
8	153.9,s			
9	159.2,s			
10	40.1,t	3.54(2H,t, J=12.2,6.6Hz)	H11, H13	C11
11	34.2,t	2.77(2H,t, J=12.6,6.8Hz)	H10, H17	C10
12	137.2,s			
13,17	132.9,d	7.35(2H,s)		C11, C14, C15
14,16	118.1,s			
15	151.5,s			
18	71.0,t	4.05(2H,t, J=12.0,5.6Hz)	H19	C19, C20
19	25.0,t	2.19(2H,m)	H18,H20	C18, C20
20	56.0,t	2.92(2H,t, J=5.6Hz)	H19	C18, C20
-OMe	60.0,t	3.74(3H,s)		C3
-$N(Me)_2$	44.5,q	2.52(6H,s)		C20
NH		7.40(1H,s)		

Based on the spectral data compound was identified as purealidin Q (**1**) which was previously described from the Okinawan marine sponge *Psammaplysilla purea*.[59] The stereochemistry at C1 and C6 of the spiroisoxazole ring in **1** was deduced to be *trans* from the proton chemical shift (ca. δ_H 4.05) of H1 in CD_3OD.[74] The absolute configuration was not assigned.

The ESI-MS of **1** (**Fig 1.7**) also showed pseudomolecular peaks at m/z 755.8, 757.8, 759.8, 761.8, 763.8 for the minor compound purpurealidin A (**2**), which is 14 amu higher than purealidin Q. The MS/MS of the purealidin Q (1) at m/z 745.7782 (**Fig 1.7A**) gave several fragment ion peaks as illustrated in **Scheme 1**. In case of 14 amu can be accounted for the presence of an additional methyl group either as –OMe at C-1 or –NMe at N-9. The position was established as -NMe at N-9 based on the fragmentation ion peaks. The MS/MS (**Fig 1.7B**) at m/z 759.7988 gave the product ions at m/z 418.9, 420.9, 422.9 for fragmentation at C8-C9 (**Scheme 1**) and the absence of mass peaks at m/z 404.9, 406.9, 408.9 (**Scheme 2**) as found in purealidin Q (**1**). The other fragment ions formed also support the presence of methyl group at N-9 position. The monoisotopic peaks at m/z 58 and 86 also helps in confirming the side chain to be dimethylpropylamine.



Scheme 1: Mass fragmentation of purealidin Q (1).

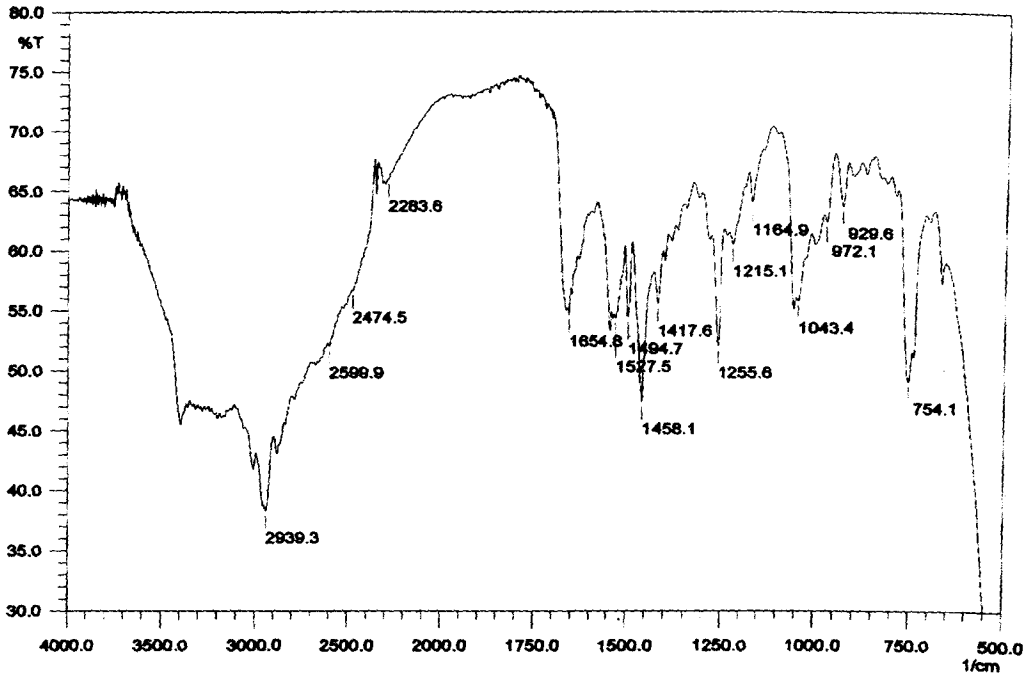


Fig 1.1: IR spectrum of purealidin Q (1).

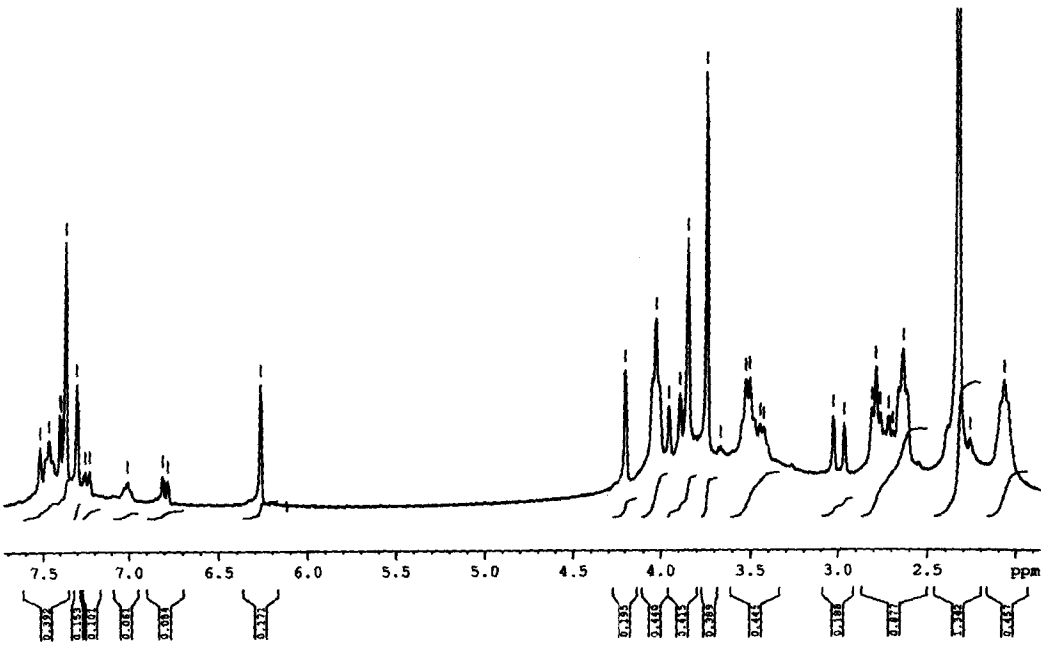


Fig 1.2: ^{1}HNMR spectrum of purealidin Q (1).

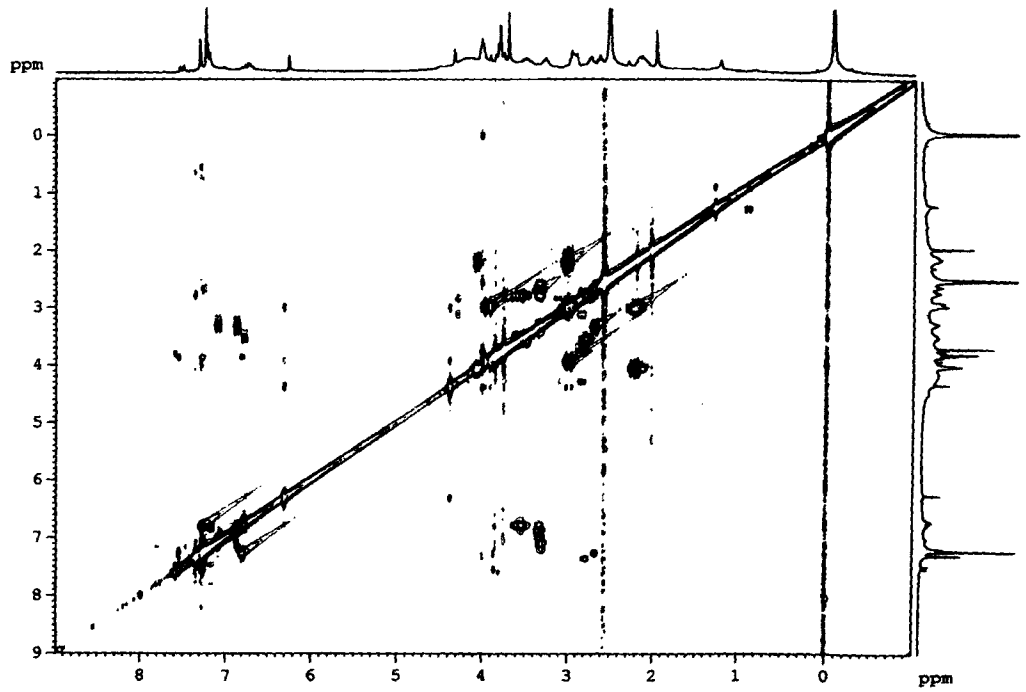


Fig 1.3: TOCSY spectrum of purealidin Q (1).

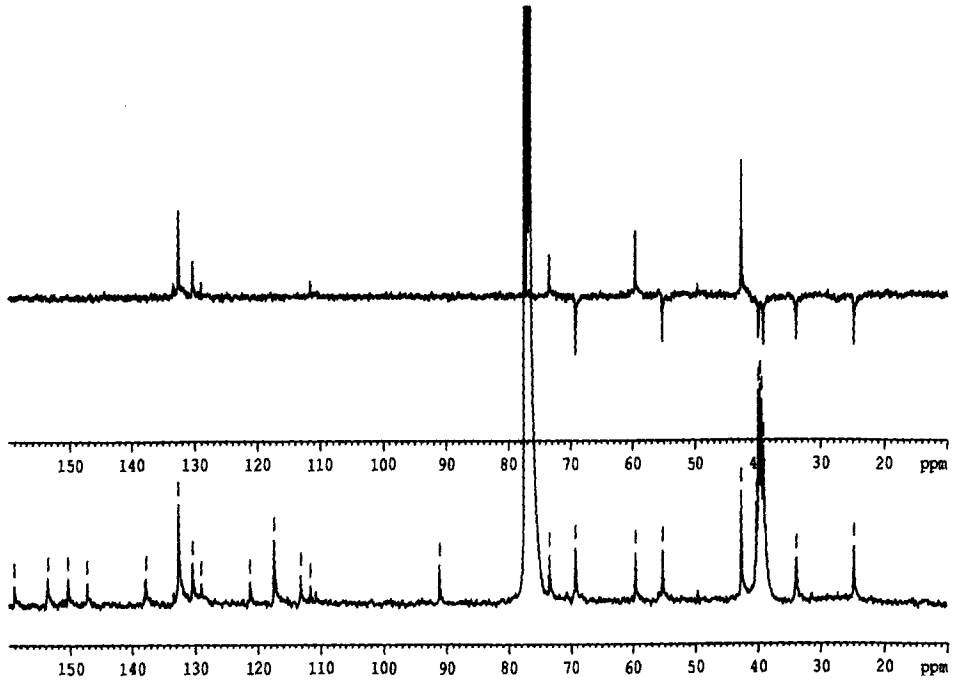


Fig 1.4: ^{13}CNMR and DEPT spectra of purealidin Q (1).

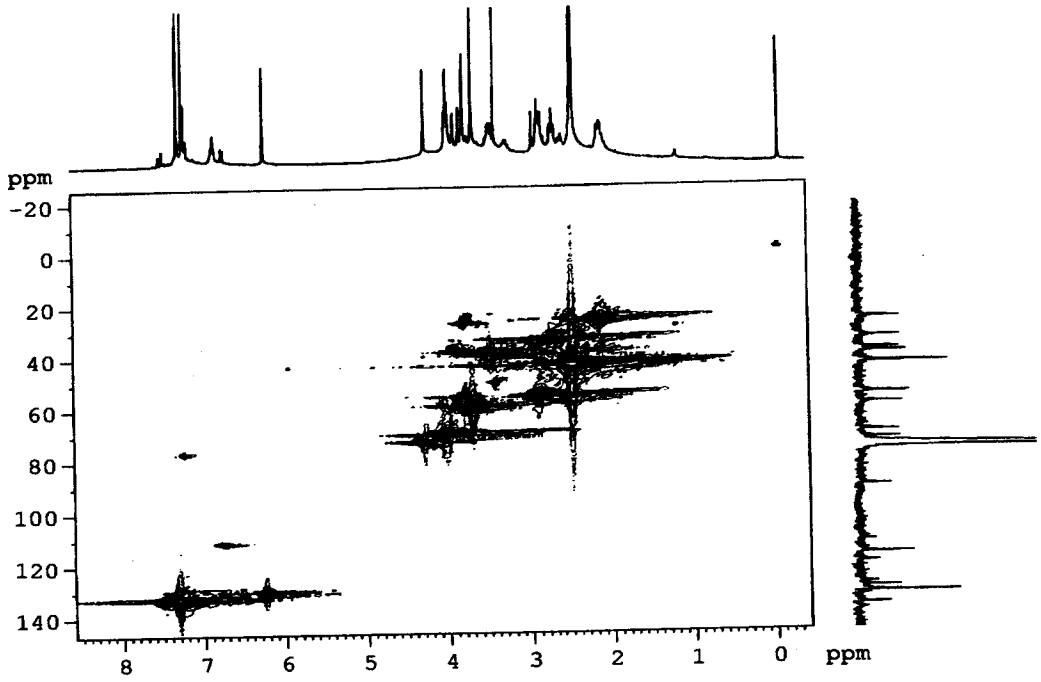


Fig 1.5: HMQC spectrum of purealidin Q (1).

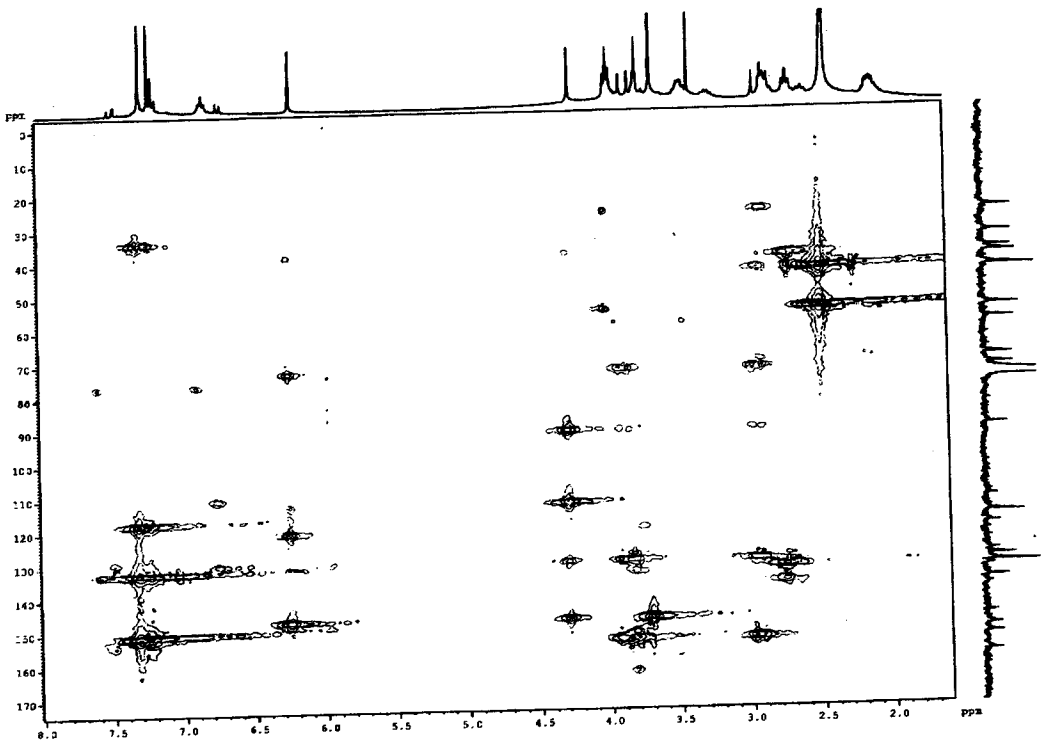


Fig 1.6: HMBC spectrum of purealidin Q (1).

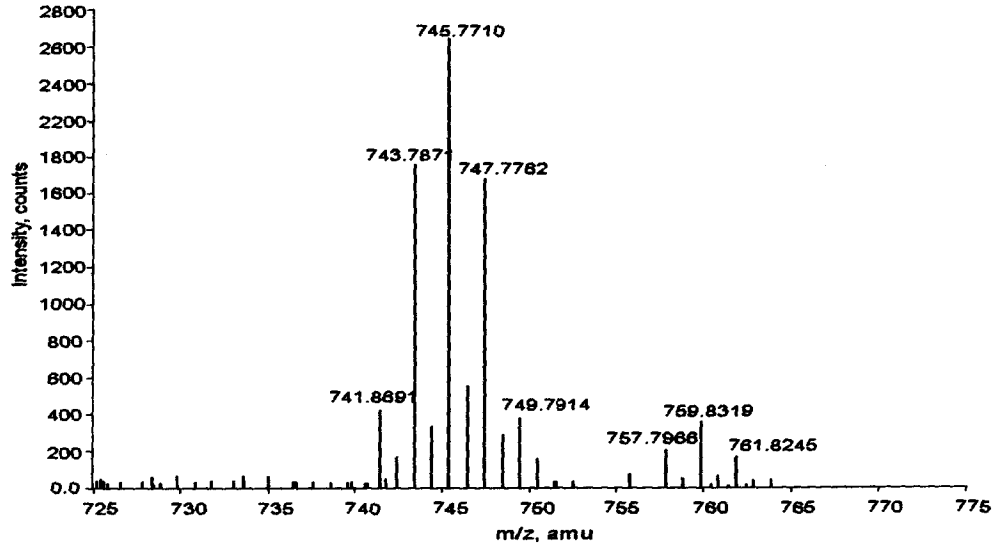


Fig 1.7: ESI-MS spectrum of purealidin Q (1).

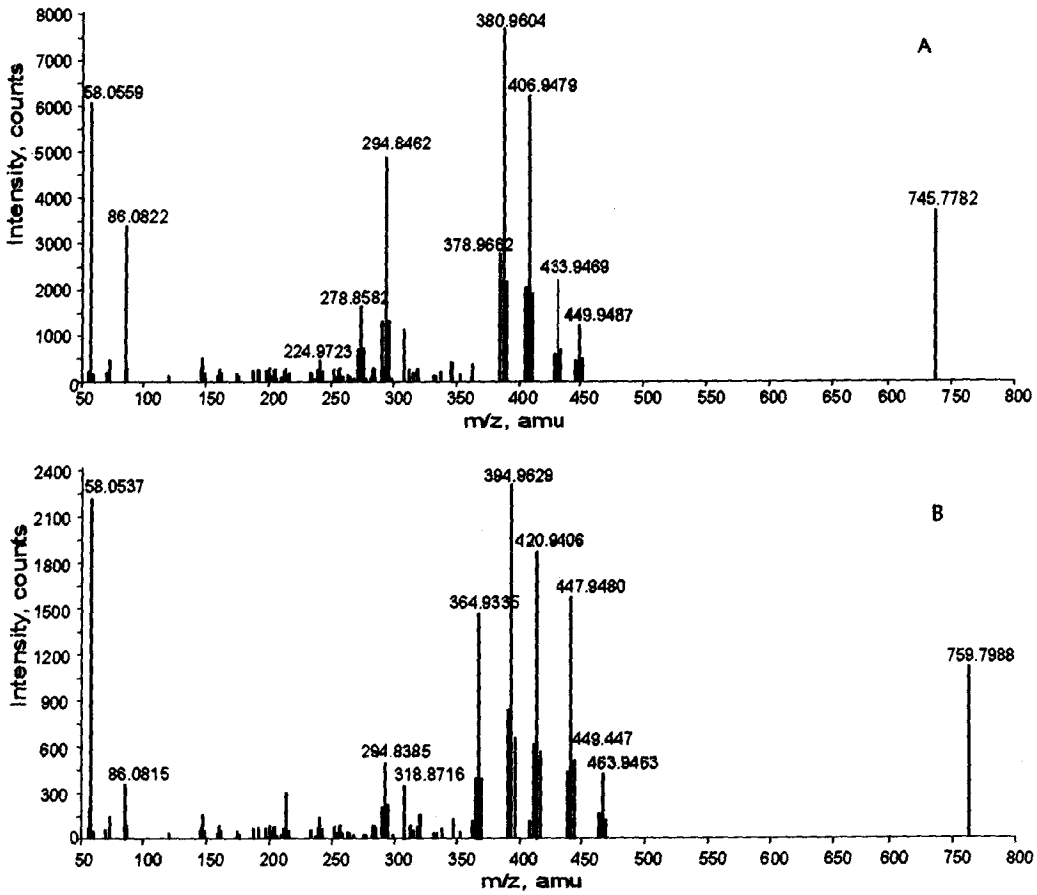
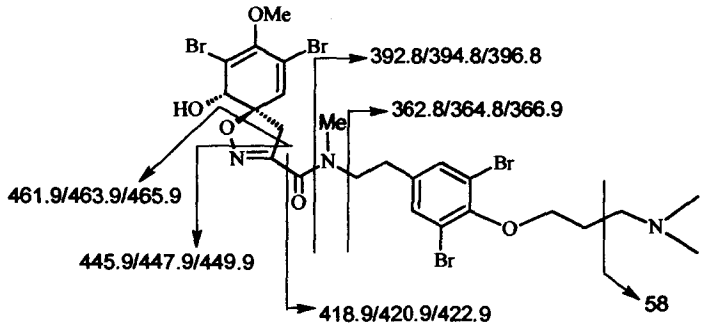


Fig 1.7: A) MS/MS spectrum at m/z 745.7782 of purealidin Q (1).
B) MS/MS spectrum at m/z 759.7988 of purpurealidin A (2).



Scheme 2: Mass fragmentation of purpurealidin A (2).

Further elution of the silica gel column yielded new bromotyrosine alkaloid purpurealidin B **(3)** which was obtained as white amorphous powder with m.p. 175.8 °C. Its UV spectrum **(Fig 2.1)** gave absorption at λ_{max} at 283nm. The IR absorption band at 3302 and 1678 cm^{-1} **(Fig 2.2)** showed presence of amine (-NH) and amide group in the molecule. The molecule contains a bromospirocyclohexadienonyldihydroisoxazole moiety of the type found in Verongida metabolites but differing in having one bromine atom and dienone ring system.[75-76] The mass spectrum of purpurealidin B showed a 1:3:3:1 quartet for the pseudomolecular ion peak $[M+H]^+$ at m/z 631.8403, 633.8185, 635.8118 and 637.8226, indicative of the presence of three bromine atoms in the molecule, which is appropriate for the molecular formula $C_{22}H_{24}N_3O_4Br_3$ **(Fig 2.8)**. The coupling pattern in proton signals at δ_H 7.27 (1H, d, J=2.5Hz), 6.87(1H, dd, J=2.1, 9.7Hz) & 6.34 (1H, d, J=9.8Hz) indicated the presence of a 2,3,6-trisubstituted aromatic moiety **(Fig 2.3, Table 2)**. The ^{13}CNMR spectrum **(Fig 2.4)** had 22 carbon signals, the multiplicities of which were assigned from a DEPT 135 experiment as two methyls, six methylenes, five methines and nine quaternary carbons.

Analysis of the proton COSY spectrum **(Fig 2.5)** showed connectivities for H1-H5, H4-H5 and H5-H1-H4 for the 2,3,6-trisubstituted aromatic moieties as illustrated in **Fig 3a**. Connectivities were also observed for H18-H19, H19-H20 and H19 with both H18 & H20.

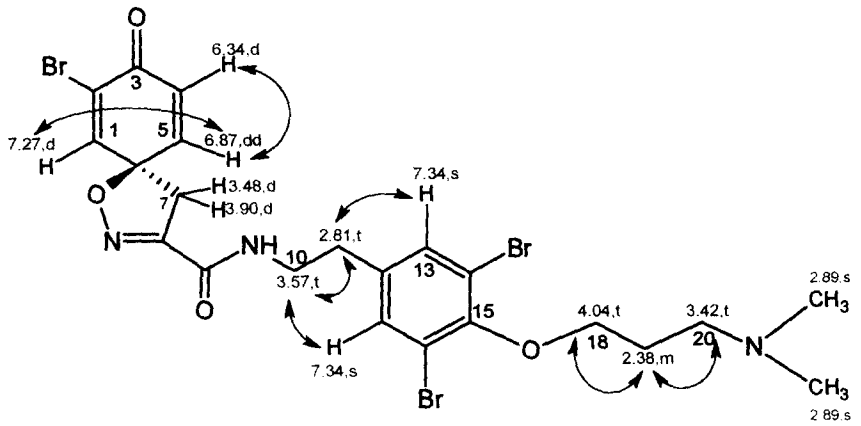


Fig 3a: COSY correlations of purpurealidin B (3).

The proton-carbon connectivities were established by the HMQC experiments as seen in **Fig 2.6**.

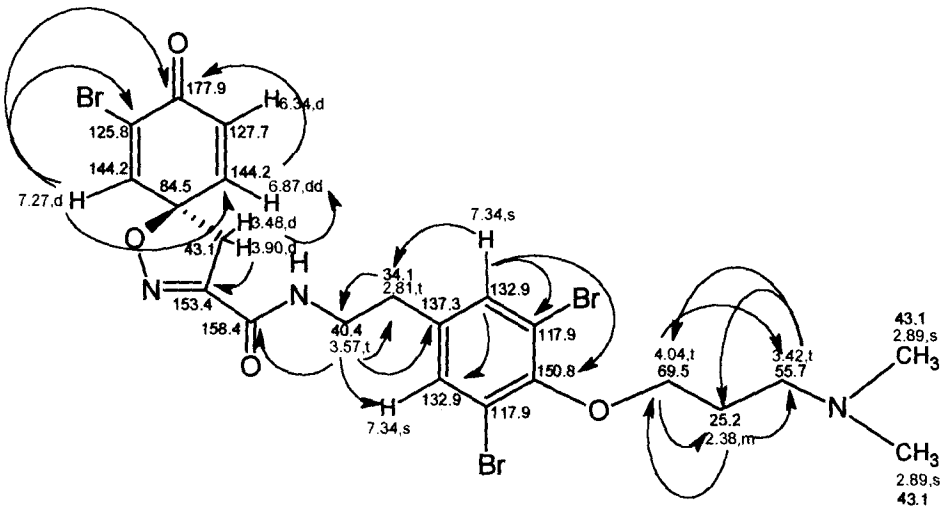
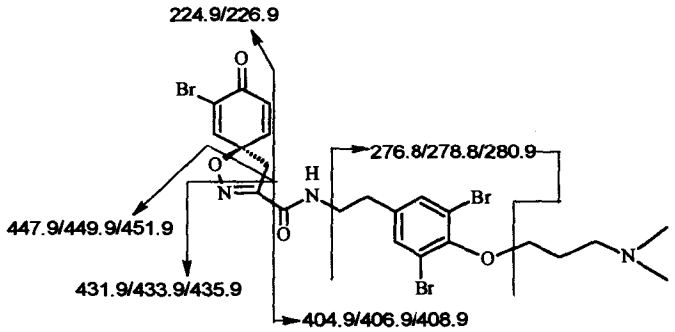


Fig 3b: HMBC correlations of purpurealidin B (3).

The HMBC experiment **(Fig 2.7)** showed connectivities of proton signal at δ_H 7.27 to C2, C3, C5; connectivities of proton signal at δ_H 6.34 to C2, C6, C5 and δ_H 6.87 to C3. The signal at δ_C 177.18 showed presence of an additional carbonyl group in the ring system. Thus, partial structure was confirmed to be monobromospirocyclohexadienoneisoxazole. The structure of the remaining part of the molecule, which is linked to the nitrogen atom of the carboxamide group at C8, was established as identical to that of purealidin Q **(1)**, based on the ^1H-^1H

connectivities. This clearly indicates presence of H10-H11 and H18-H19-H20 methylene chains. All the HMBC correlations are illustrated in **Fig 3b**. The HMBC showed proton signal at δ_H 7.34 (2H, s) connected to C11, C13, 17, C15, C14,16 for the tetrasubstituted aromatic ring. A 6H singlet at δ_H 2.89 was assigned to be a dimethylamino group. The similarity of the structure with purealidin Q (**1**) is also confirmed by pseudomolecular peaks at m/z 404.9, 406.9, 4089; 447.9, 449.9, 451.9 and 431.9, 433.9, 435.9 **(Scheme 3)** observed in the MS/MS spectrum at m/z 631.8403 **(Fig 2.9A)** and 633.8185 **(Fig 2.9B)**. Peaks at m/z 224.9 and 226.9 were observed for the monobromospirocyclohexadienone-isoxazole moiety.



Scheme 3: Mass fragmentation of purpurealidin B (3).

Table 2: ^{1}H, ^{13}CNMR, COSY and HMBC of purpurealidin B (3), in $CDCl_3$

Carbon Nos.	^{13}CNMR δ_C, ppm	^{1}H NMR δ_H, ppm	COSY	HMBC
1	144.2,d	7.27(1H,d, J=2.5Hz)	H5	C2, C3, C5
2	125.8,s			
3	177.9,s			
4	127.7,d	6.34(1H,d,J=9.8Hz)	H5	C2, C6
5	144.2,d	6.87(1H,dd, J=9.7Hz)	H1, H4	C3
6	84.5,s			
7	43.1,t	3.48(1H,d, J=18.0Hz) 3.90(1H,d, J=18.0Hz)		C5, C8
8	153.4,s			
9	158.4,s			
10	40.4,t	3.57(2H,t, J=7.0Hz)	H11	C9, C11, C12, C13, 17
11	34.1,t	2.81(2H,t, J=7.2Hz)	H10	C10
12	137.3,s			
13,17	132.9,d	7.34(2H,s)		C11, C13,17, C15, C14,16
14,16	117.9,s			
15	150.8,s			
18	69.5,t	4.04(2H,t,J=5.6Hz)	H19	C19, C20
19	25.2,t	2.38(2H,m)	H20, H18	C18, C20
20	55.7,t	3.42(2H,t, J=5.6Hz)	H19	C18, C19
-NMe_2	43.1,q	2.89(6H,s)		
-NH		7.4(s)		C8

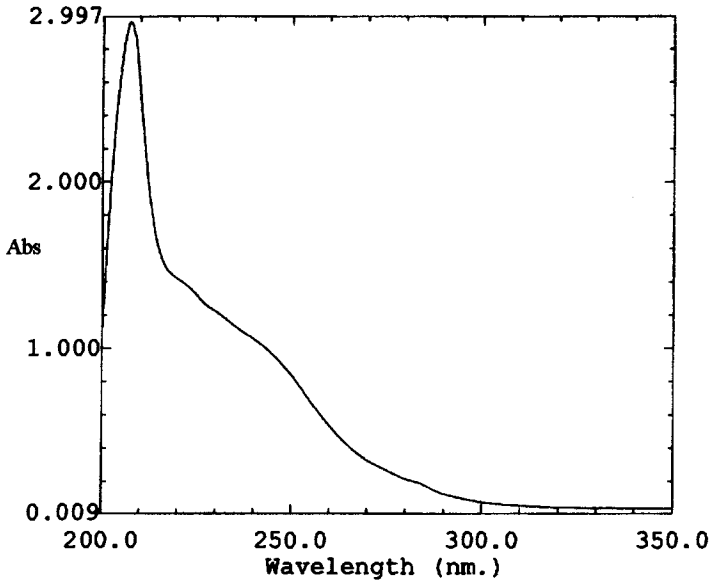


Fig 2.1: UV spectrum of purpurealidin B (3).

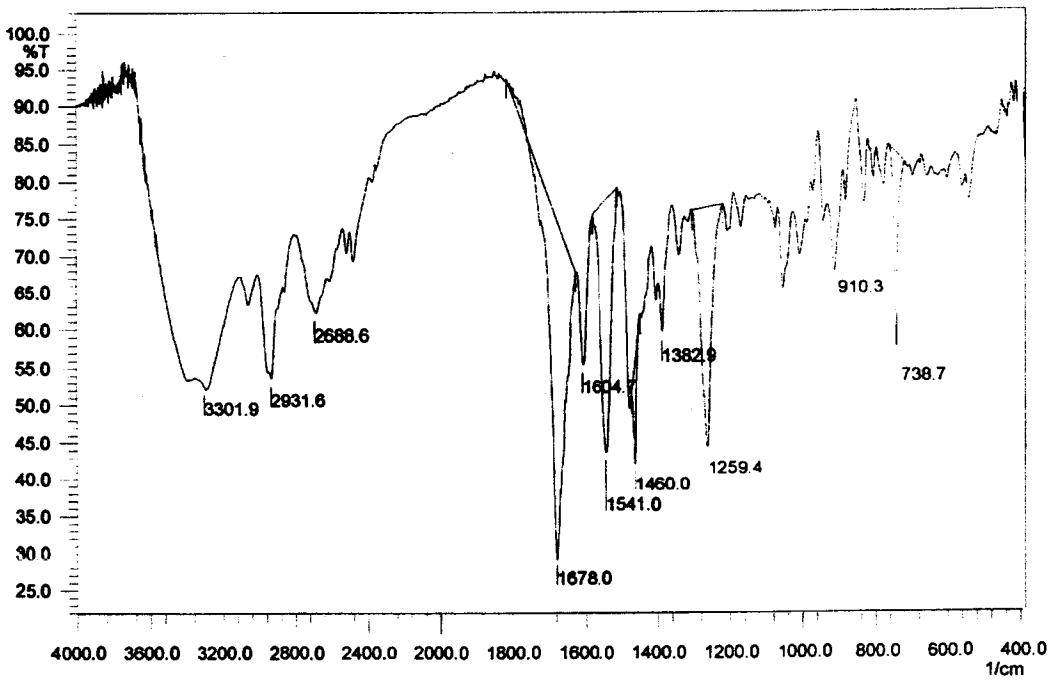


Fig 2.2: IR spectrum of purpurealidin B (3).

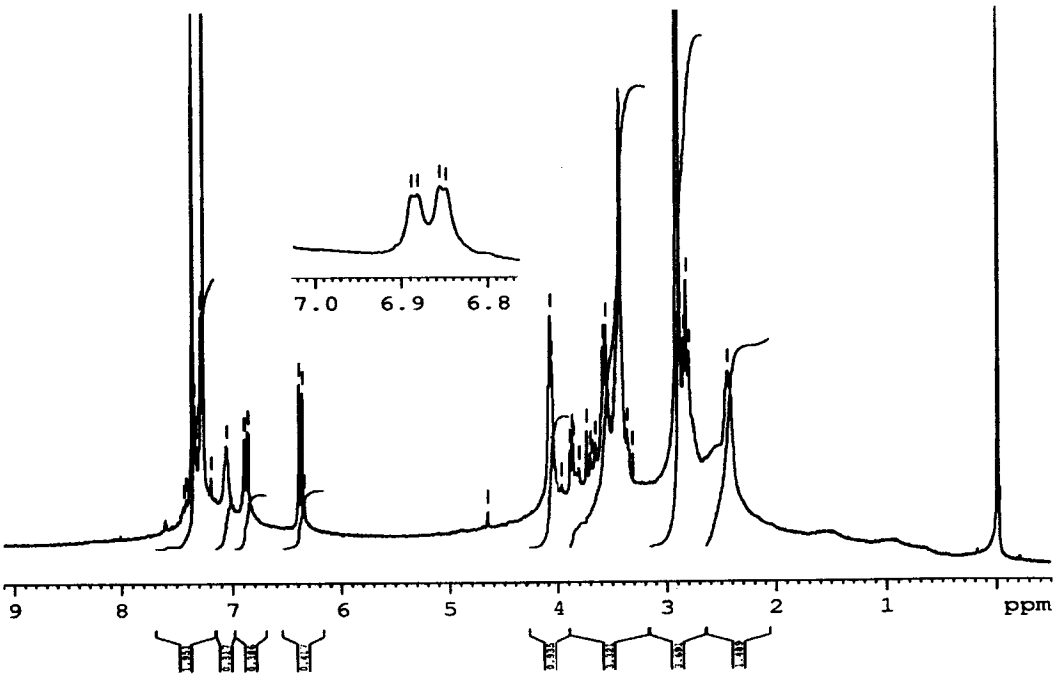


Fig 2.3: ^{1}HNMR spectrum of purpurealidin B (3).

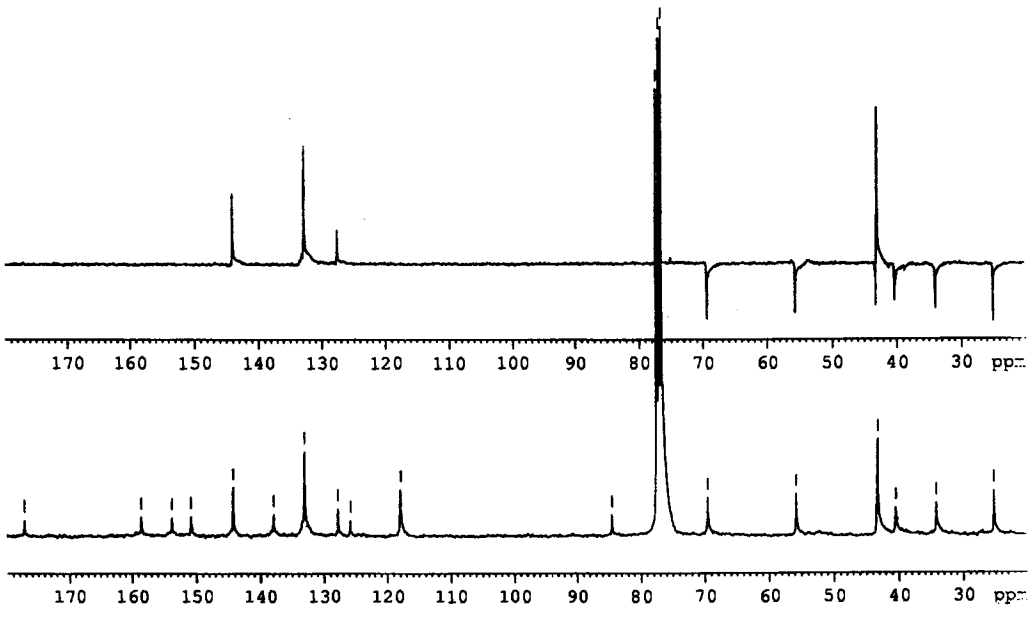


Fig 2.4: ^{13}CNMR and DEPT spectra of purpurealidin B (3).

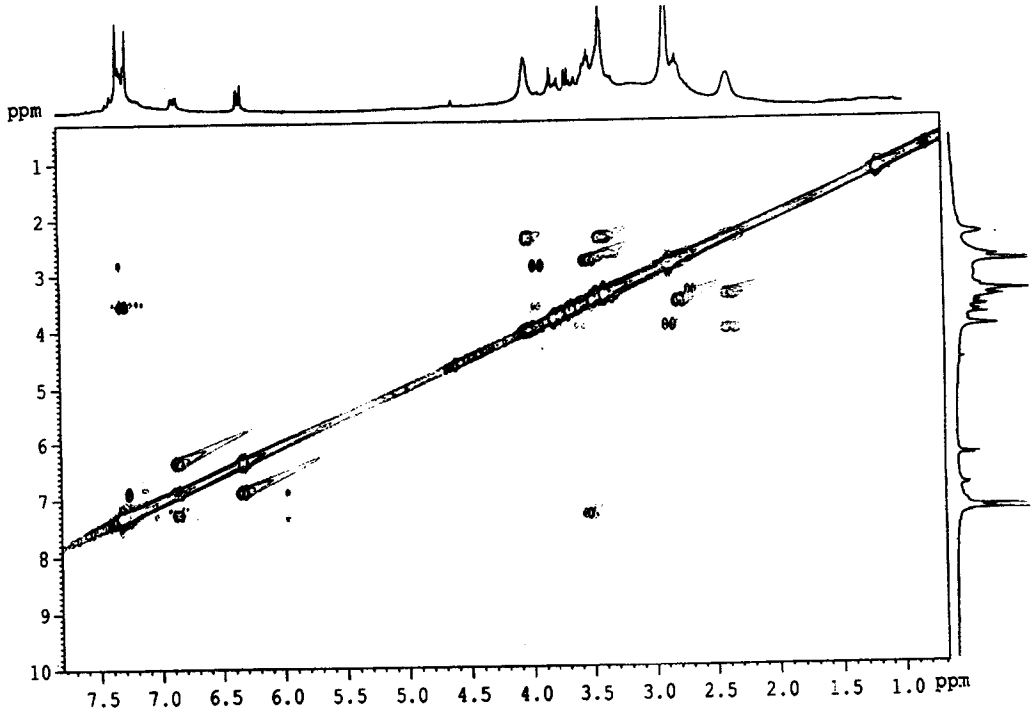


Fig 2.5: COSY spectrum of purpurealidin B (3).

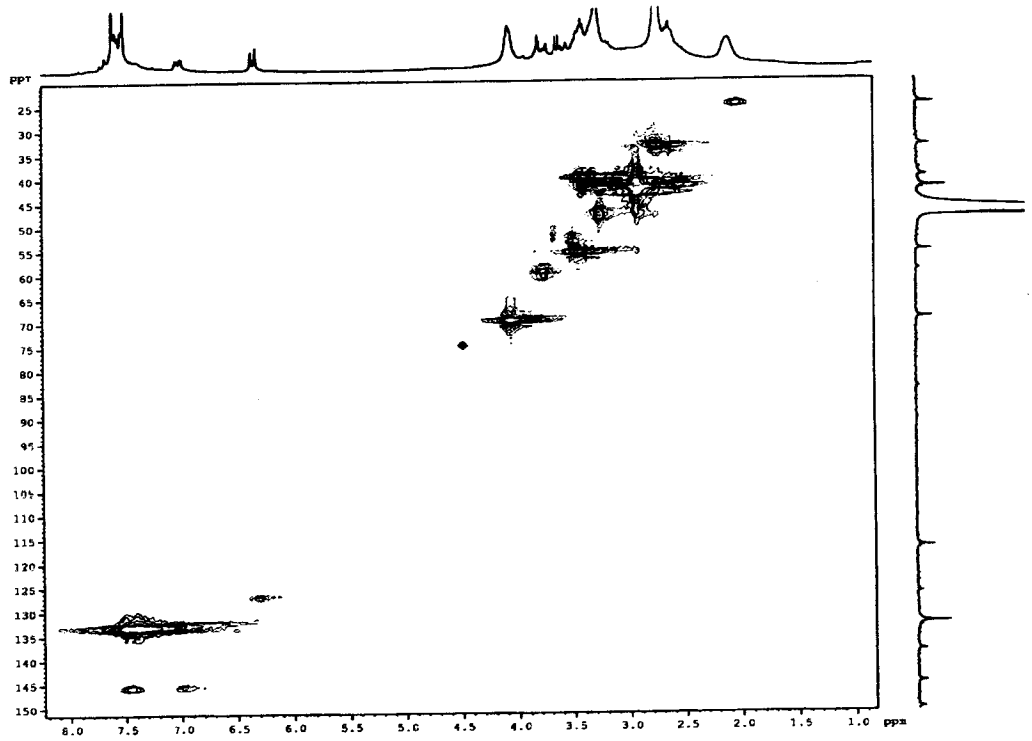


Fig 2.6: HMQC spectrum of purpurealidin B (3).

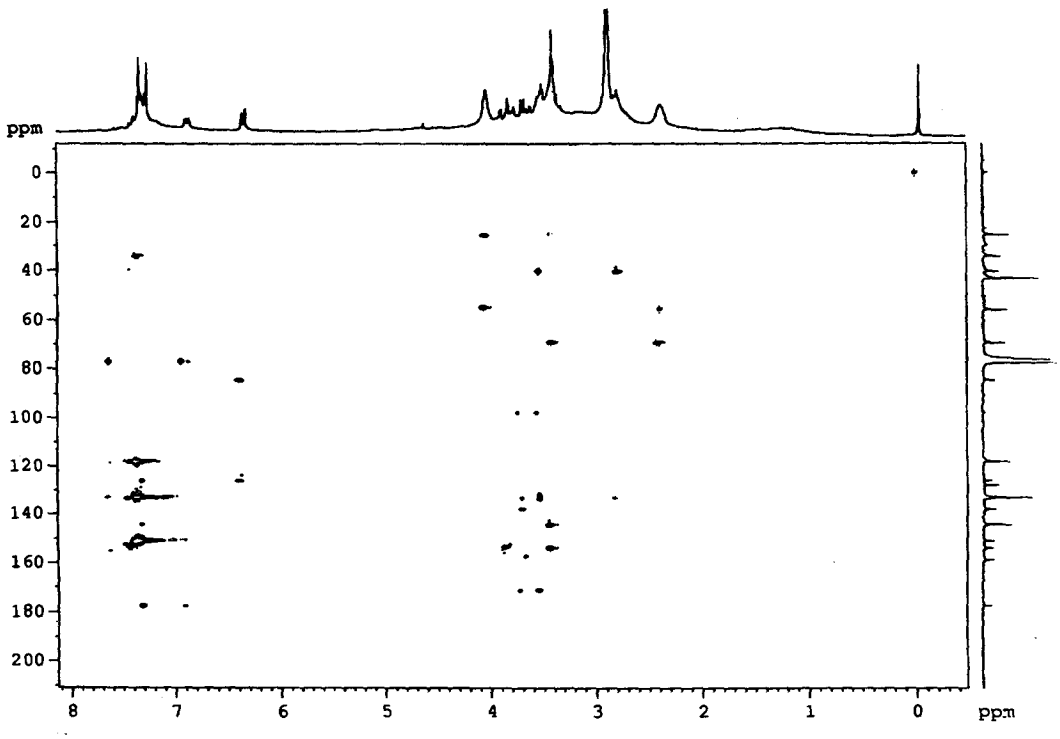


Fig 2.7: HMBC spectrum of purpurealidin B (3).

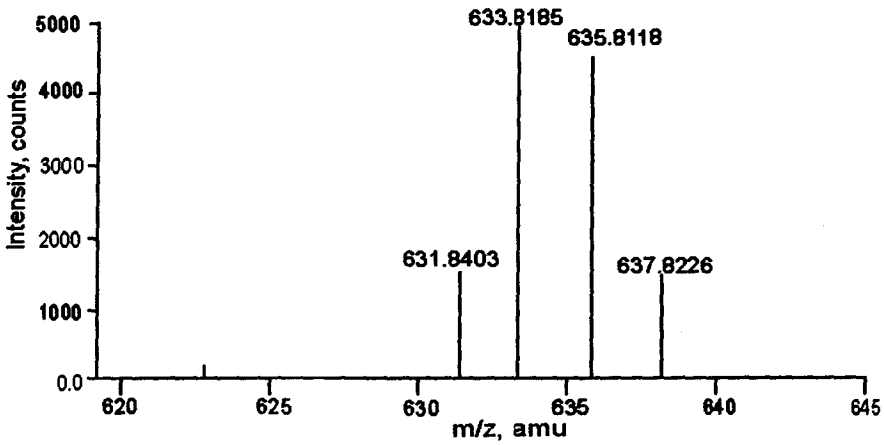


Fig 2.8: ESI-MS spectrum of purpurealidin B (3).

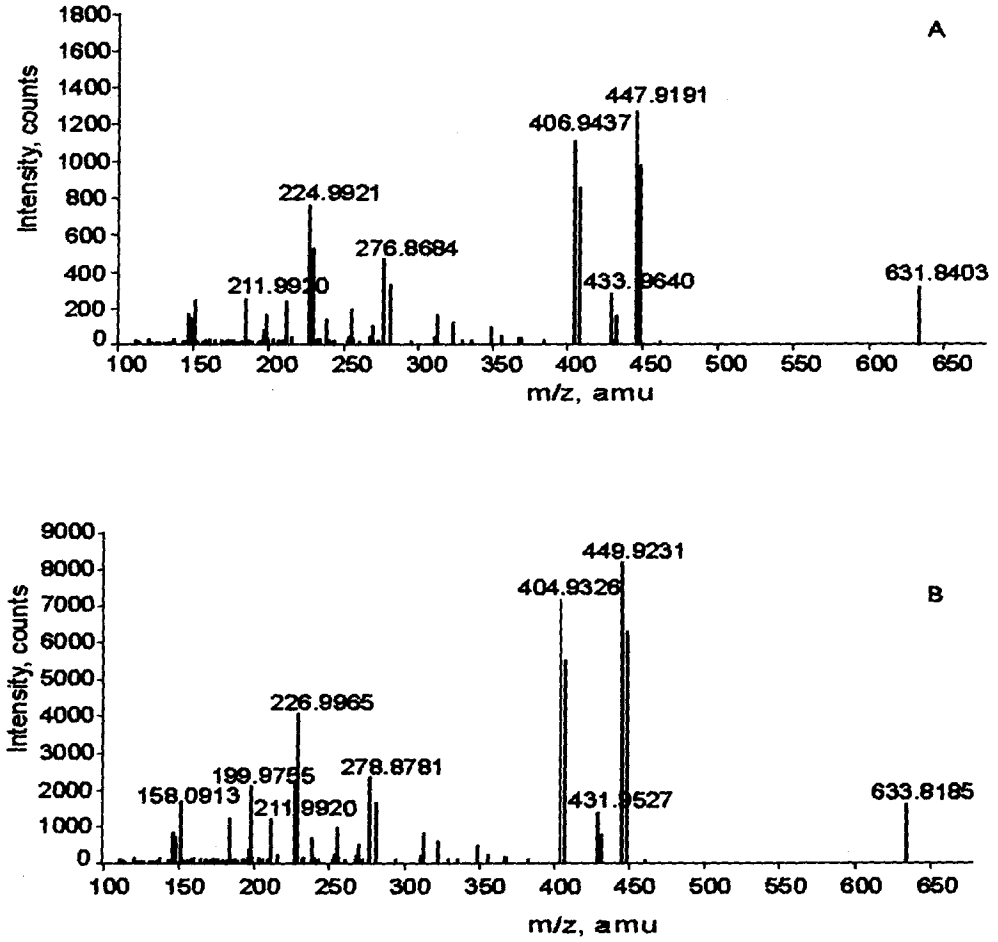


Fig 2.9: MS/MS spectrum at A) m/z 631.8403 $[M+H+2]^+$ and B) m/z 633.8185 $[M+H+4]^+$ of purpurealidin B (3).

From the less polar ethyl acetate fraction, elution of the silica gel column with 5% MeOH: $CHCl_3$ yielded an inseparable mixture of two compounds, purpurealidin C **(4)** and D **(5)** differing in the fatty chain were isolated. The nature of the side chain was confirmed by mass spectrum.

The IR spectrum **(Fig 3.1)**♦ showed absorption bands at 1660 and 1652 cm^{-1} for amide carbonyls. The intense band at 2925 cm^{-1} (C-H stretching) was indicative of long aliphatic chain. ^{1}HNMR **(Fig 3.2)**, ^{13}CNMR and DEPT experiment **(Fig 3.3)** exhibited same characteristic features as purealidin Q **(1)** except for the absence of the N, N-dimethyl resonance and presence of one additional amide proton at δ_H 5.3, carbonyl signal at δ_C 173.6 and methylene signals at δ_C 27.0-32.7 (δ_H 1.19) indicating the presence of an additional amide carbonyl group and long straight fatty chain. A doublet at δ_H 0.80 (6H, J=6.8Hz) was assigned to the isopropyl group **(Table 3)**.

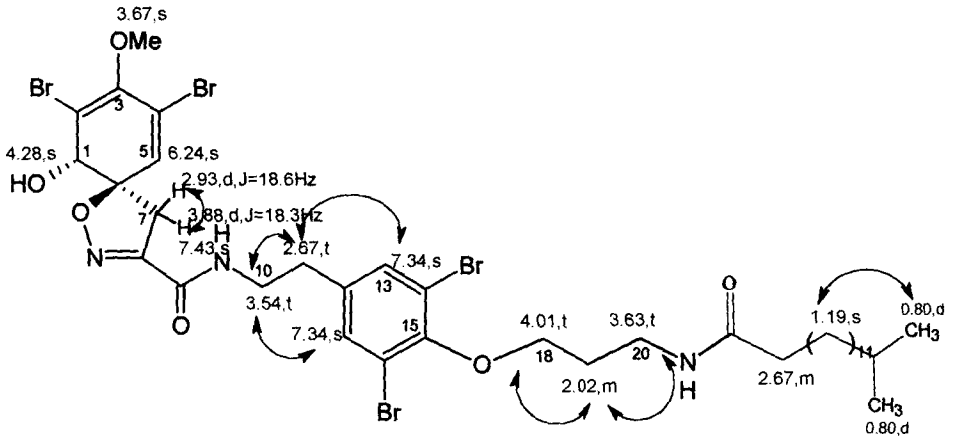


Fig 4a: COSY correlations of purpurealidin C (4).

The structure is also confirmed by COSY **(Fig 3.4)**, HMQC **(Fig 3.5)** and HMBC **(Fig 3.6)** spectral data **(Table 3)**. The only difference in the COSY of **(4)** with respect to purealidin Q **(1)** was ^{1}H-^{1}H connectivity of long fatty chain at δ_H 1.19 with the terminal methyls at δ_H 0.80. Similarly, HMBC spectrum showed

♦ All the spectra for the mixture (Purpurealidin C and D) are represented under Fig 3, from Fig 3.1 to Fig 3.7.

correlation of triplet at δ_H 3.63 (H-20) to an amide carbonyl at δ_C 173.6 (C-21). Long methylenes chain with δ_H 1.19 showed correlation with terminal methyls C34 and C35. COSY and HMBC correlations are illustrated in **Fig 4a** and **Fig 4b** respectively.

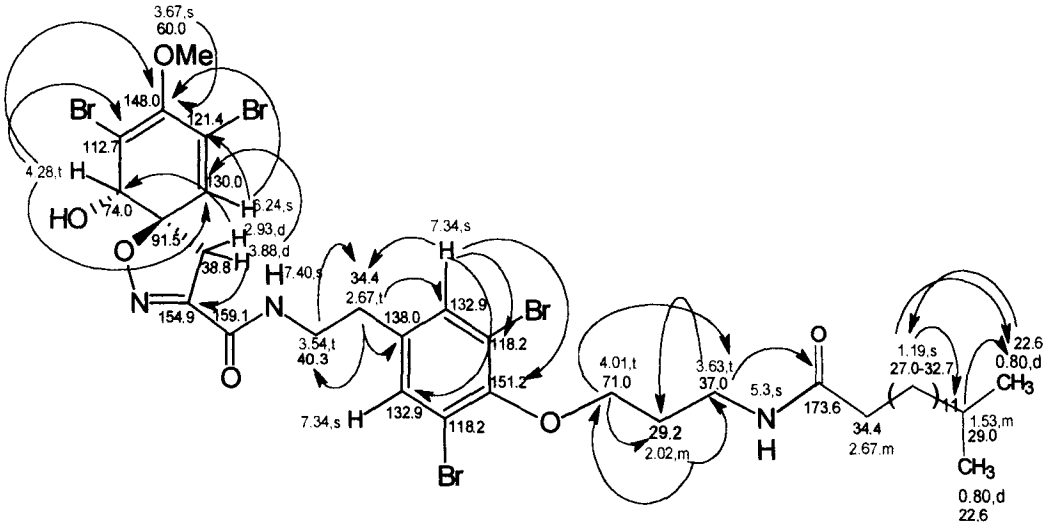
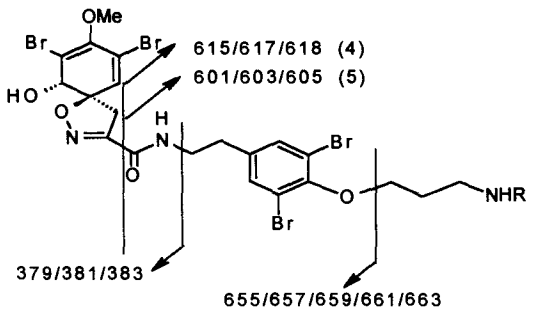


Fig 4b: HMBC correlations of purpurealidin C (4).

The molecular weight of purpurealidin C **(4)** was higher than that of purealidin Q **(1)**. The mass spectrum (ES-MS) **(Fig 3.7)** showed cluster of pseudomolecular ion peaks at m/z 938.2, 940.2, 942.2, 944.2 and 946.2 in the ratio 1:4:6:4:1. There was evidence of additional cluster of pseudomolecular ion peaks at m/z 952.2, 954.2, 956.2, 958.2 and 960.2, which were 14 units higher than **(4)** indicative of different molecule with an extra methylene group. The fragment ions peaks observed for **(4)** and **(5)** are illustrated in **Scheme 4.**



Purpurealidin C (4); R=-CO$(CH_2)_{11}CH(CH_3)_2$
Purpurealidin D (5); R=-CO$(CH_2)_{12}CH_2CH_2CH_3$

Scheme 4: Fragmentation pattern of purpurealidin C (4) and D (5).

The presence of a signal at δ_H 0.70 (t) in ^{1}HNMR spectra and of ^{13}C signal at δ_C 14.0 confirmed the presence of terminal methyl group in purpurealidin D **(5)**. The fragmentation pattern of **4** and **5** **(Scheme 4)** is different from the araplysillin-II **(115 in Section 2.1)** isolated from the *P. purpurea* [61] and agrees well with the structure assigned. The difference in the structure of araplysillin-II is that propyl amine chain is attached to isoxazole ring moiety and ethyl amine chain to C15 of the aromatic ring. The opposite is the case with the compound reported by us, which was established by studying 2D NMR experiments. Also, the fragment ion peaks at m/z 655/657/659/661/663 were absent in case of earlier reported compound araplysillin-II.

Table 3: ^{1}H, ^{13}CNMR, COSY and HMBC of purpurealidin C (4), in $CDCl_3$

Carbon Nos.	^{13}CNMR δ_H, ppm	^{1}H NMR δ_C, ppm	COSY	HMBC
1	74.0,d	4.28(1H,s)	OH	C2, C3, C5
2	112.7,s			
3	148.0,s			
4	121.4,s			
5	130.0,s	6.24(1H,s)		C3, C4
6	91.5,s			
7	38.8,t	Ha=2.93(1H,d, J=18.6Hz) Hb=3.88(1H,d, J=18.3Hz)	Hb Ha	C1, C5, C8
8	154.9,s			
9	159.1,s			
10	40.3,t	3.54(2H,t, J=13.3,6.6Hz)	H11	C11
11	34.4,t	2.67(2H,t, J=12.6,7.8Hz)	H10	C10, C12, C13,17
12	138.0,s			
13,17	132.9,d	7.34(2H,s)	H10, H11	C11, C13,17, C15, C14,16
14,16	118.2,s			
15	151.2			
18	71.0,t	4.01(2H,t, J=12.0,6.0Hz)	H19	C15, C19, C20
19	29.2,t	2.02(2H,m)	H18, H20	C18, C20
20	37.0,t	3.63(2H,t)	H19	C18, C19, C21
21	173.6,s			
22	34.4,t	2.67(2H,m)		
23-32	27.0-32.7,t	1.19(24H,s)		C34, C35
33	29.0,t	1.53(2H,m)		C25-C30
34,35	22.6,q	0.80(6H,d, J=6.8Hz)	H33	C23-C32
OH		4.51 (brs)	H1	
N9		7.43(1H,d)		
N20		5.30(1H,s)		
-OMe	60.0,q	3.67(3H,s)		C3

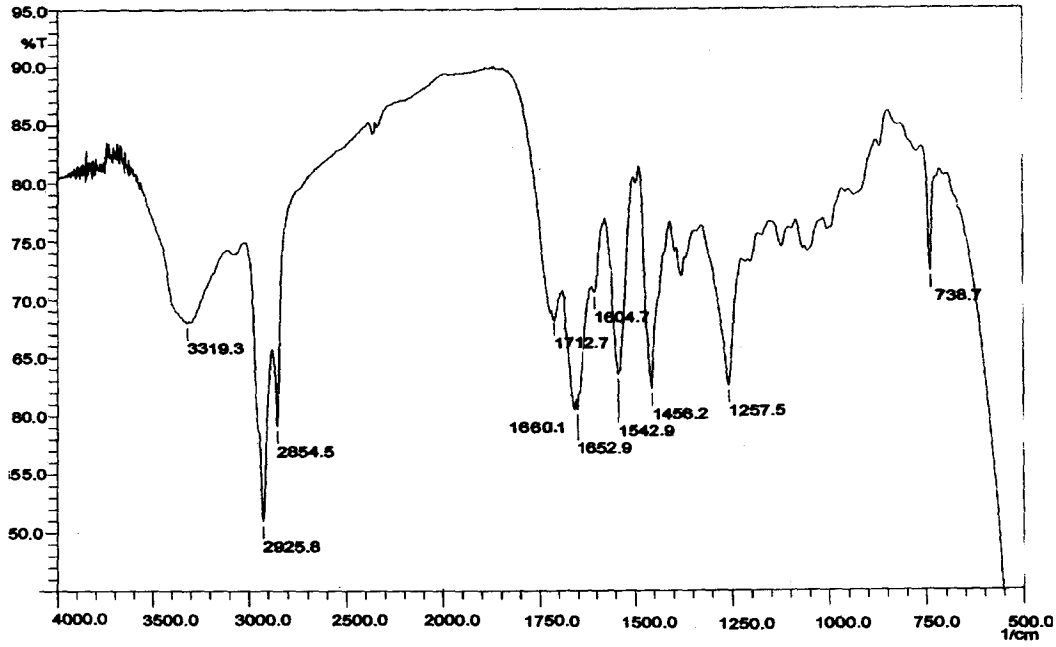


Fig 3.1: IR spectrum of purpurealidin C (4).

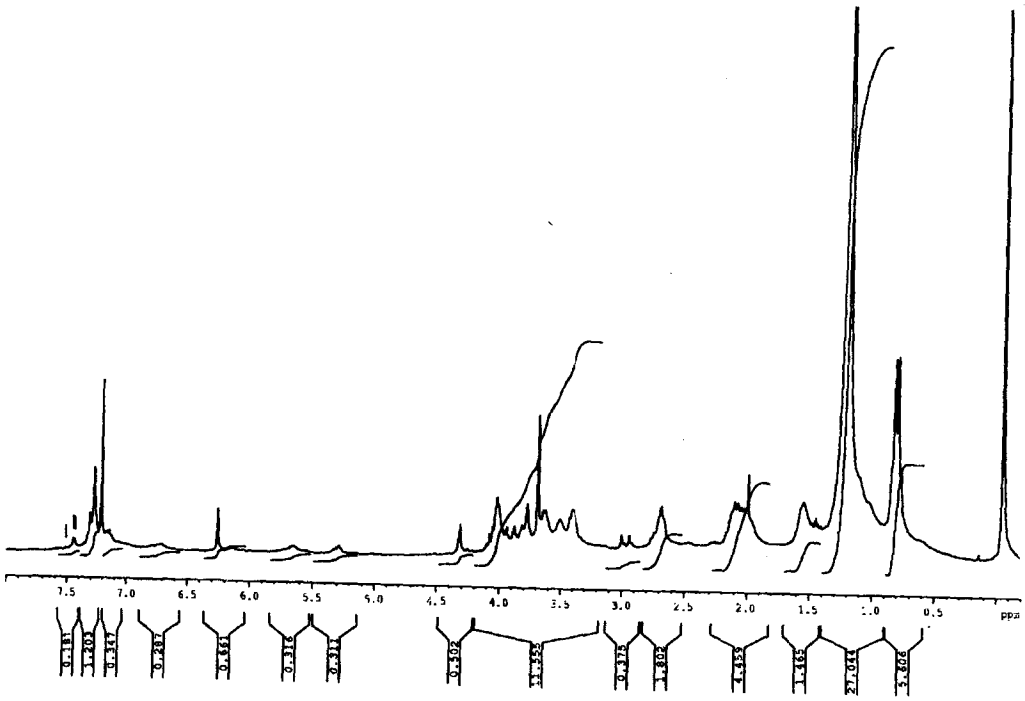


Fig 3.2: ^{1}HNMR spectrum of purpurealidin C (4).

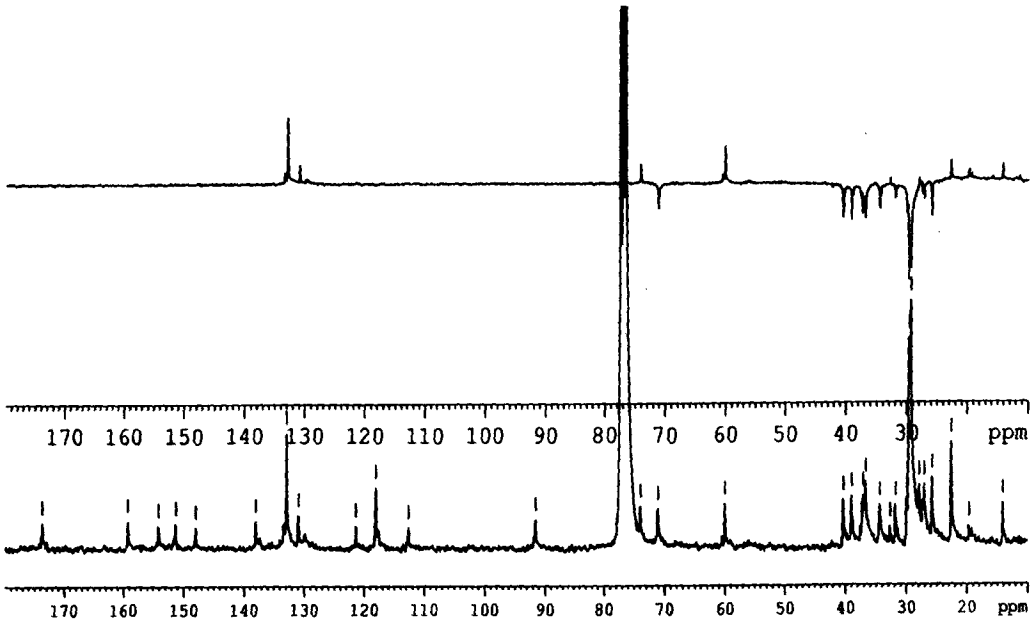


Fig 3.3: ^{13}CNMR and DEPT spectra of purpurealidin C (4).

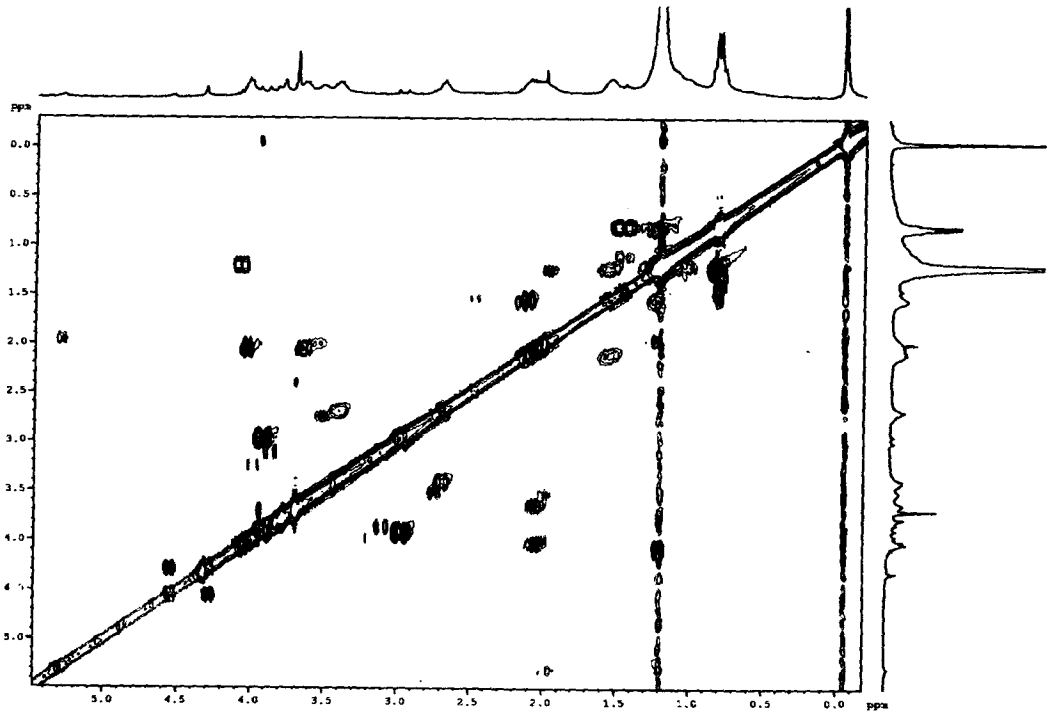


Fig 3.4: COSY spectrum of purpurealidin C (4).

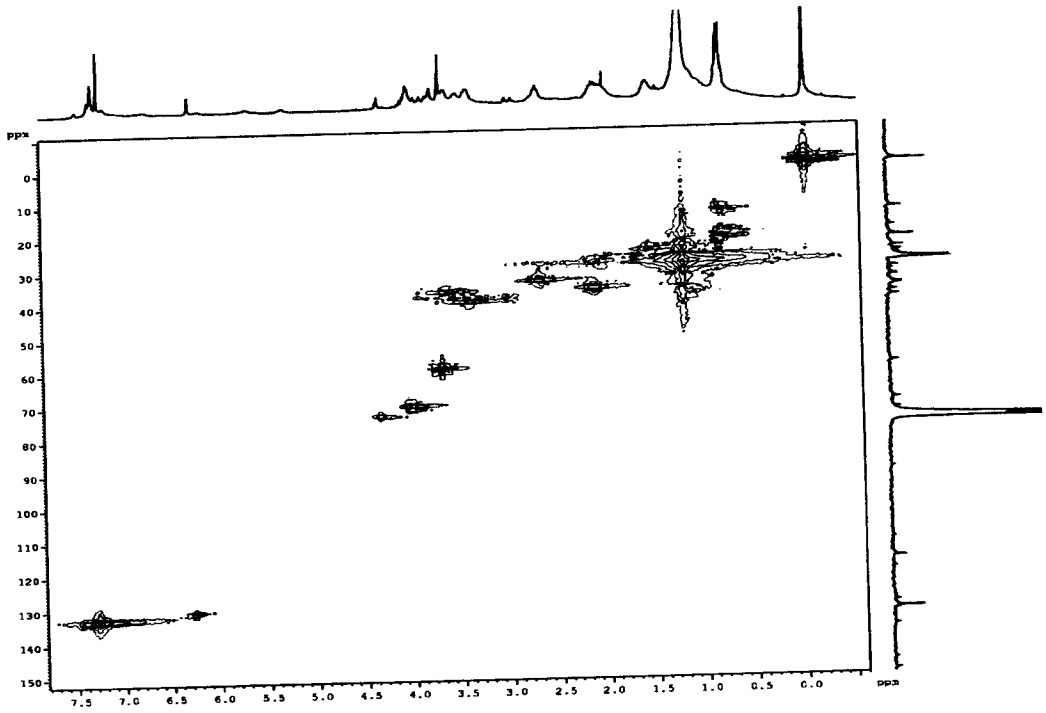


Fig 3.5: HMQC spectrum of purpurealidin C (4).

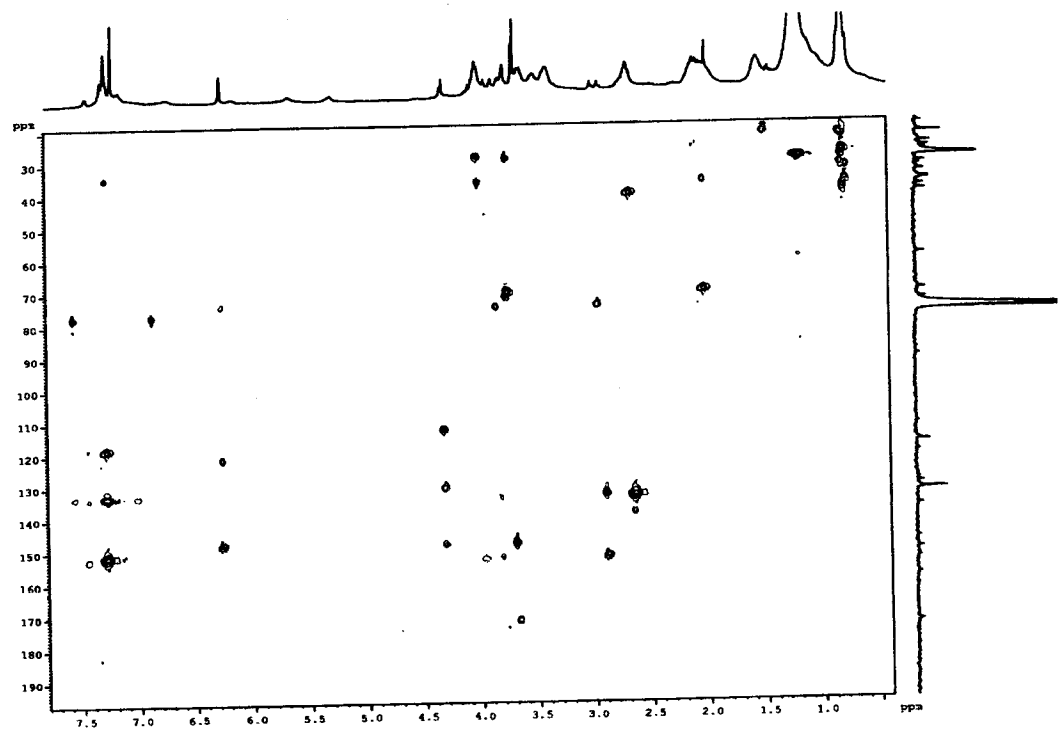


Fig 3.6: HMBC spectrum of purpurealidin C (4).

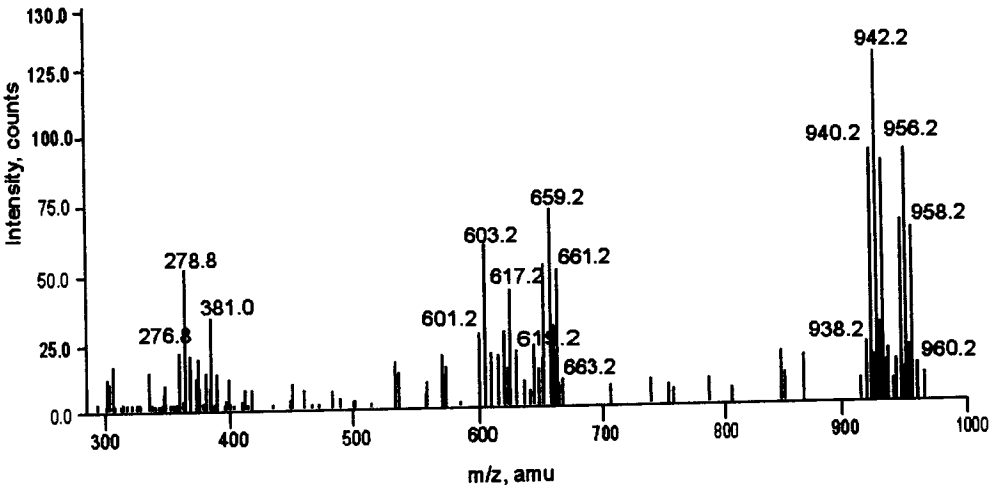
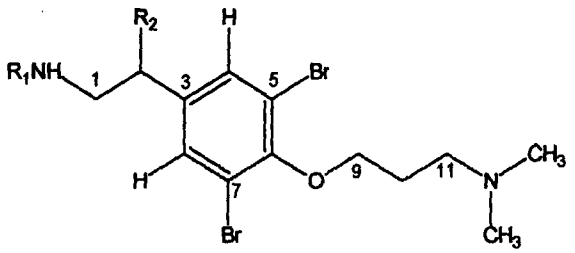


Fig 3.7: ES-MS spectrum of purpurealidin C (4).

Elution of the silica gel column with 20% MeOH in chloroform yielded yet another mixture of bromotyrosine derivatives designated as purpurealidin E (**6**), F (**7**) and G (**8**). Three bromotyrosine compounds were identified from the polar fraction. The mass spectrum (**Fig 4.6**)* of purpurealidin E (**6**) showed a pseudomolecular ion peak $[M+H]^+$ at m/z 378.9768, 380.9757, 382.9755 in the ratio 1.05:2.05:1.0, characteristic for the presence of two bromine atoms. Examination of the spectral data (^1H, ^{13}C, COSY and HMBC) led to (**6-8**) for the compounds in the mixture, which are similar to the part structure of purealidin Q (**1**). HMBC spectrum also confirmed the structure to be a part structure of purealidin Q (**1**). But the mass spectrum of (**6**) showed two more minor pseudomolecular ion peaks at m/z 394.966, 396.966, 398.961 and 449.9660, 451.9661, 453.9501 attributed to compounds purpuealidin F (**7**) and G (**8**) respectively. IR, ^1H, ^{13}CNMR & DEPT, HMQC and HMBC spectra for the mixture are shown in **Fig 4.1, 4.2, 4.3, 4.4 and 4.5** respectively. The presence of these compounds is further evidenced by the minor peak signals appeared at δ_H 3.77 (1H, m) and δ_C 59.6 that can be accounted for the hydroxy methine at C2 in 7 & 8. ^1H and ^{13}CNMR values assigned for all three compounds are given in **Table 5**. The carbonyl signal at δ_C 173.0, methylene signal at δ_C 29.2 (δ_H 1.20, s) and methyl signal at δ_C 14.0 (δ_H 0.81, t, J=7.0Hz) were indicative of the presence of an additional amide carbonyl group and ethyl groups. Compound purpurealidin E has been previously isolated by Venkateshwarlu *et al.* from the same source collected from Mandapam. The compound was purified as acetate and dimethylammonium salt. [43]



Purpurealidin E (6): R_1=R_2=H
Purpurealidin F (7): R_1=H; R_2=OH
Purpurealidin G (8): R_1=$-COCH_2CH_3$; R_2=OH

* All the spectra for the mixture (Purpurealidin E, F and G) are represented under Fig 4, from Fig 4.1 to Fig 4.6.

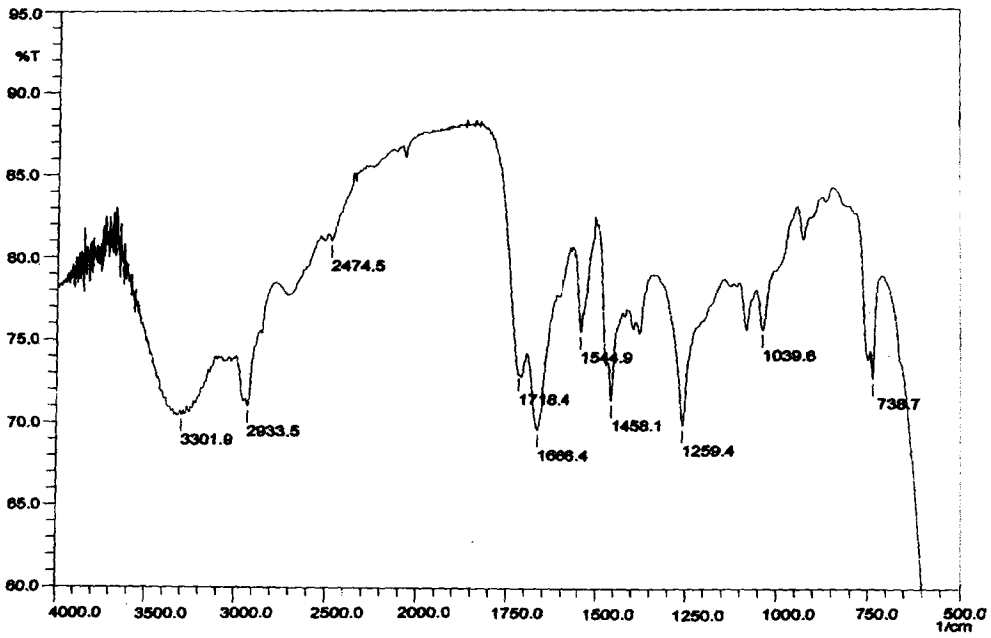


Fig 4.1: IR spectrum of purpurealidin E (8).

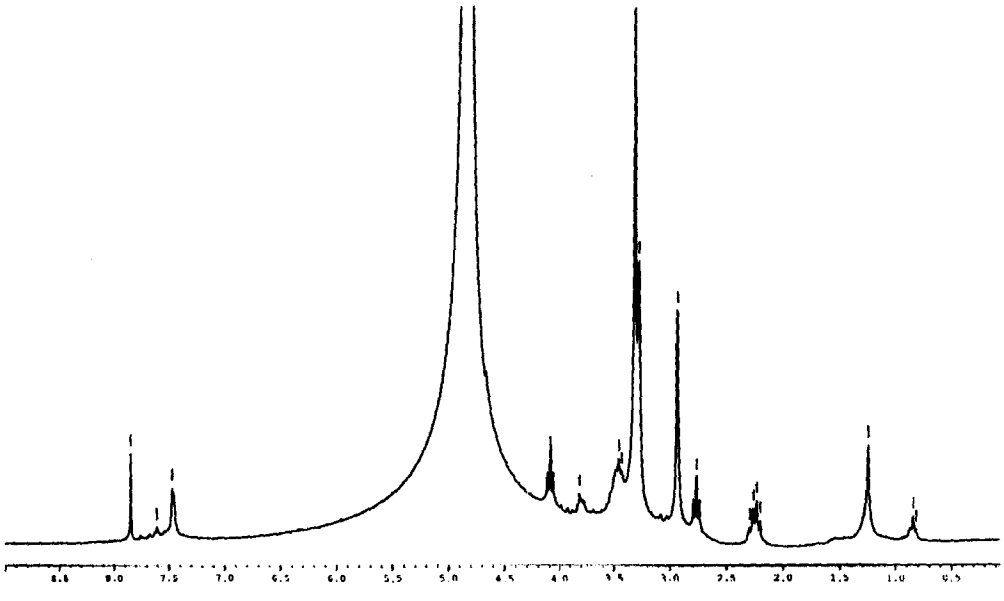


Fig 4.2: ^{1}HNMR spectrum of purpurealidin E (8).

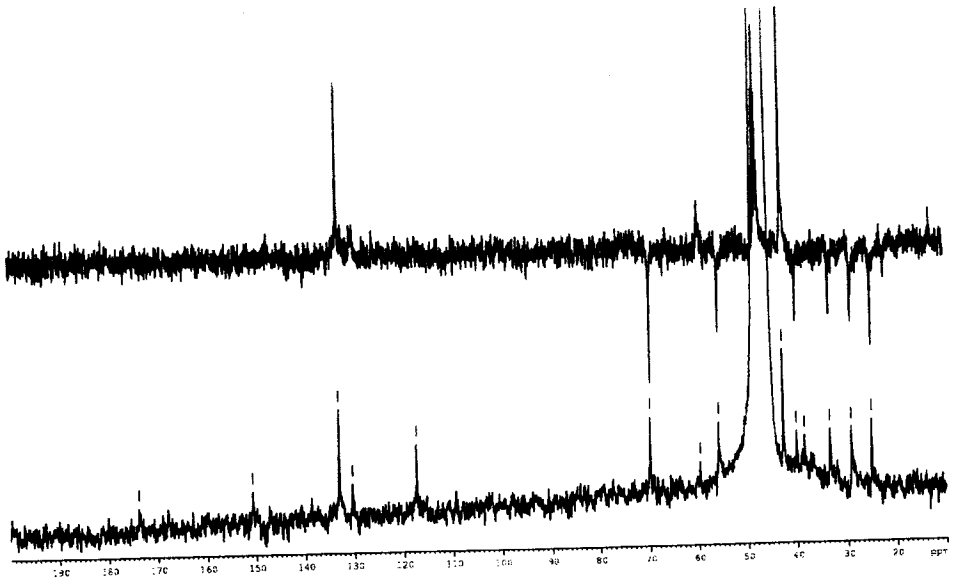


Fig 4.3: ^{13}CNMR and DEPT spectra of purpurealidin E (8).

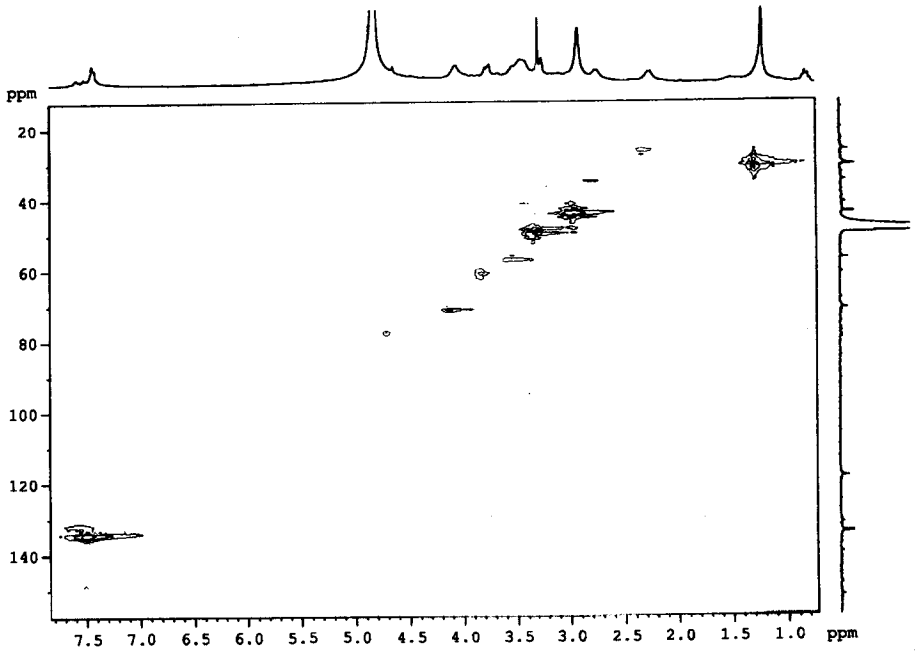


Fig 4.4: HMQC spectrum of purpurealidin E (8).

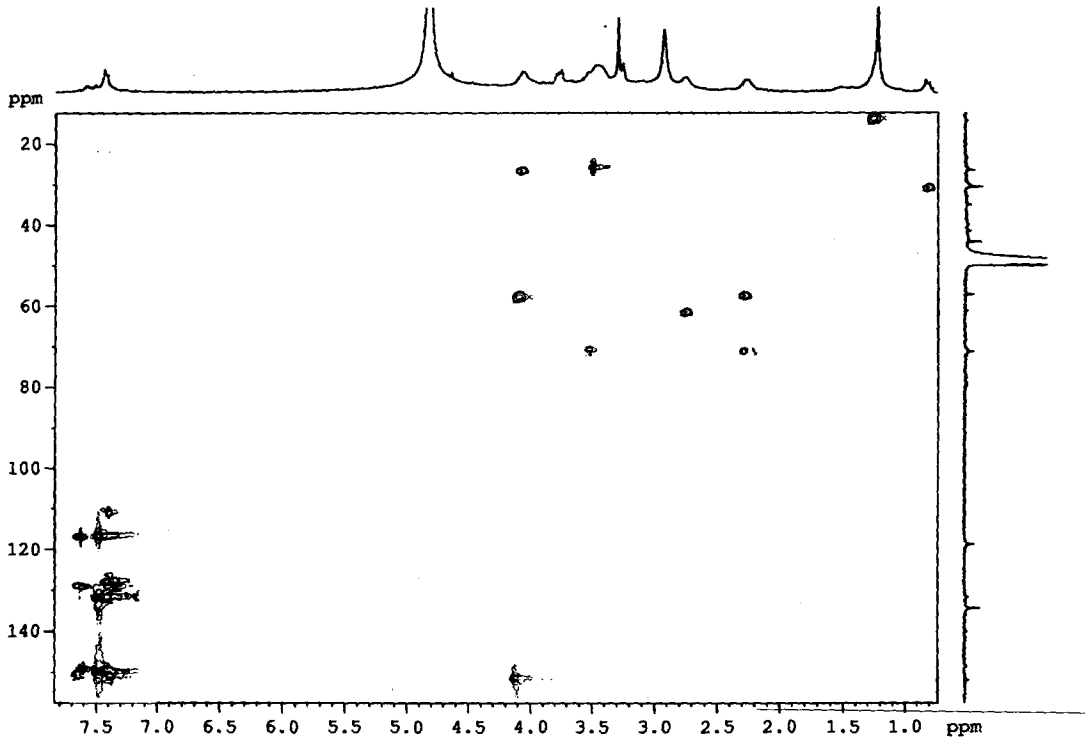


Fig 4.5: HMBC spectrum of purpurealidin E (8).

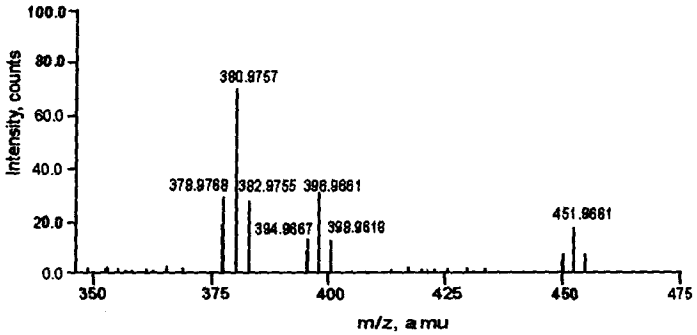


Fig 4.6: ESI-MS spectrum of purpurealidin E (8).

Table 4: ^{1}H and $^{13}CNMR$ of purpurealidin E (6), F (7) & G (8) in CD_3OD

Carbon No.	$^{13}CNMR$ (6) δ, ppm	$^{1}HNMR$ (6) δ, ppm	$^{13}CNMR$ (7) δ, ppm	$^{1}HNMR$ (7) δ, ppm	$^{13}CNMR$ (8) δ, ppm	$^{1}HNMR$ (8) δ, ppm
1	40.0, t	2.73(2H,t, J=13.2,6.6Hz)	40.0, t	2.73(2H,t, J=13.2,6.6Hz)	40.0, t	2.73(2H,t, J=13.2,6.6Hz)
2	33.6, t	3.24(2H,t)	59.6, t	3.77(1H,m)	59.6, t	3.77(1H,m)
3	130.3, s		130.3, s		130.3, s	
4,8	133.0, t	7.43(2H,s)	133.0, t	7.43(2H,s)	133.0, t	7.43(2H,s)
5,7	117.3, s		117.3, s		117.3, s	
6	150.7, s		150.7, s		150.7, s	
9	69.8, t	4.05(2H,t, J=5.6Hz)	69.8, t	4.05(2H,t, J=5.6Hz)	69.8, t	4.05(2H,t, J=5.6Hz)
10	25.0, t	2.23(2H,m)	25.0, t	2.23(2H,m)	25.0, t	2.23(2H,m)
11	55.8, t	3.44(2H,t) J=5.6Hz)	55.8, t	3.44(2H,t) J=5.6Hz)	55.8, t	3.44(2H,t) J=5.6Hz)
12,13	42.7, q	2.90(6H,s)	42.7, q	2.90(6H,s)	42.7, q	2.90(6H,s)
NH_2		7.63(br, s)				
NH				8.10(br,s)		8.10(br,s)
CO					173.0	
CH_2					29.2	1.20(2H,s)
CH_3					14.0	0.81(3H,t)

Two known compounds, 16-debromoaplysamine-4 **(9)** and purpuramine I **(11)** were also isolated. Silica gel fraction with 8% MeOH:$CHCl_3$ was subjected to reversed phase HPLC using H_2O:MeOH (15:85) as mobile phase resulting in purification of two compounds, 16-debromoaplysamine-4 **(9)** at Rt 18.4min and purpuramine I **(11)** at Rt 27.5min.

16-Debromoaplysamine-4 **(9)** obtained as colourless amorphous solid with the R_f value 0.37 in 14% MeOH:$CHCl_3$ visualised as dark pink spots with ninhydrin reagent, indicated free amine group. The mass spectrum **(Fig 5.7)** of this known compound **(9)**, revealed characteristic isotopic peaks for $[M+H]^+$ pseudo molecular ion at 619.87, 621.85, 623.84 and 625.88 in the ratio 1.05:3.1:3.06:1.0, indicating the presence of three bromine atoms in the molecule. Amide and oxime functionalities were indicated by IR absorptions at 3350, 1654.8 and 1624 cm^{-1} **(Fig 5.1)**.

The proton signals at δ_H 7.40(2H, s) and 7.33(1H, d, J=2.0Hz), 6.86(1H, d, J=8.4Hz) & 7.02(1H, dd, J=8.4,2.0Hz) in the $^{1}HNMR$ spectrum **(Fig 5.2)**

indicated the presence of tetra and trisubstituted aromatic moieties. The double of doublet at δ_H 7.02 with J value of 2 and 8.4 Hz indicated meta and ortho coupling. Signals at δ_H 2.09 (m), 2.65 (t), 3.15 (t), 3.34 (t), 3.74 (s) and 4.06 (t) were observed for six methylene protons. Singlet at δ_H 3.75 indicated presence of methoxy group (**Table 5**). The proton signals also appeared at δ_H 3.34 (t), 2.65 (t) for ethylamine and 4.06 (t), 2.09 (m), 3.15 (t) for propylamine chain. The upfield of the triplet at δ_H 3.15 suggest that it is attached to primary amine.

Its ^{13}CNMR spectrum (**Fig 5.3**) had 21 carbon signals, which were designated as one methyl, six methylenes, five methines, and nine quarternary carbons from a DEPT experiment (**Fig 5.3**). The presence of oxime and amide groups was also confirmed by ^{13}C signals at δ_C 153.7 and 165.2 respectively. The upfield ^{13}CNMR chemical shift of C7 (δ_C 27.3) suggested E configuration of the oxime as the corresponding value for (Z)-Oxime is >35ppm. It is observed that there is absence of the quaternary carbon at δ_C 91.9 and methine carbon at δ_C 73.8 as observed in purealidin Q (**1**) suggesting there is double bond formation in the aromatic ring and opening of the isoxazole ring moiety to form an oxime.

Interpretation of the COSY spectrum classified the six methylenes into three spin systems; one unit of O-CH_2-CH_2-CH_2-N (H18-H19-H20), one unit of N-CH_2-CH_2 (H10-H11) and an isolated singlet methylene (H7). The TOCSY spectrum (**Fig 5.4**) showed 1,3 shift ^{1}H-^{1}H connectivities. Methylene at H7 showed connectivity with H1 and H2 (δ_H 7.40, s, each). This reveals that methylene is attached to the tetrasubstituted aromatic rings, which consists of two bromine atoms. Methylene (H10) showed correlation with aromatic proton H13 (δ_H 7.33, d) indicating that ethylamine chain is attached to the trisubstituted aromatic ring consisting of only one bromine atom. COSY and TOCSY correlations are illustrated in **Fig 5a**. The direct proton-carbon correlation was established by HMQC experiments (**Fig 5.5**).

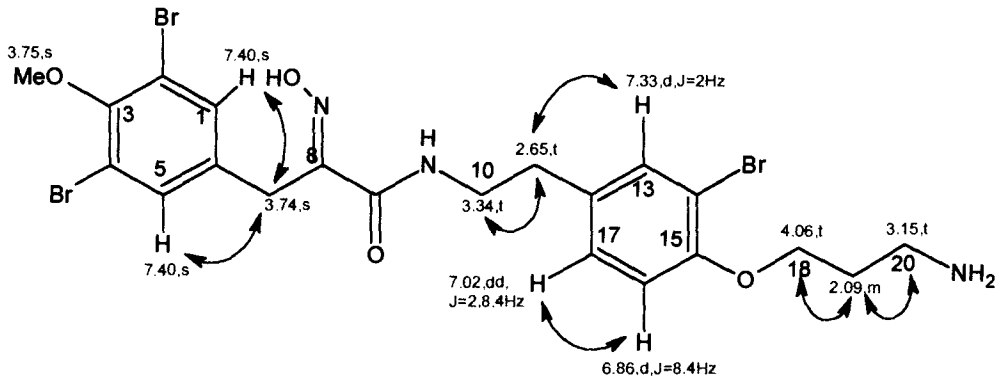


Fig 5a: TOCSY correlations of 16-debromoaplysamine-4 (9).

The HMBC spectrum **(Fig 5.6)** showed all the $^2J_{CH}/^3J_{CH}$ connectivities. Aromatic proton at δ_H 7.40 showed correlation with δ_C 118.5 (C2), 151.9 (C3), and 137.2 (C7). Methoxy group δ_H 3.75 showed correlation with δ_C 151.9 (C3). Methylene singlet at δ_H 3.74 was connected to C6 and to amide carbonyl, δ_C 165.2 (C9). Triplet at δ_H 3.34 showed correlation with amide carbonyl while δ_H 2.65 (2H, t) with aromatic ring at δ_C 134.4 (C12) and 130.1(C17). Aromatic proton at δ_H 7.33 (d, J=2Hz) displayed connection with C14 with bromine atom and C17. Doublet of doublet at δ_H 7.02 was connected with C12, C15 and C16. Another doublet at δ_H 6.86 with J=8.4Hz showed connection with C15 and C17. Propyl amine chain also showed all $^2J_{CH}/^3J_{CH}$ connectivities. All the HMBC assignments are given in **Fig 5b.**

The combined study of 1D and 2D experiments showed it to 16-debromo aplysamine-4 previously reported by Pakrashi *et al.*[77] from the same source collected from Orissa coast. He has reported four bromotyrosine alkaloids namely, aplysamine 1a-d purified as monoacetates 1a'-d'**(75, 76, 75a & 76a in Section 2.1)** on HPLC. Around the same time, Jurek *et al.*[40] obtained three similar compounds, viz., aplysamine 3-5 **(72-74 in Section 2.1)** from same organisms collected from deep waters (-40m) near Hawaii. Closer examination of the compound **(10)** isolated by us was identical to aplysamine 1d, reported by Pakrashi *et al.* and was similar to aplysamine-4 with one less Br atom, thus could

be named as 16-debromoaplysamine-4. 2D NMR studies of this compound were being reported here for the first time. The ESI-MS **(Fig 5.7)** of this compound gave an additional minor singly charged $[M+H]^+$ at m/z at 633.87, 635.87, 637.87, 639.88 for **(10)**, 14 units higher than that of the compound **(9)**. This is accounted for the methyl group at the terminal N-methyl.

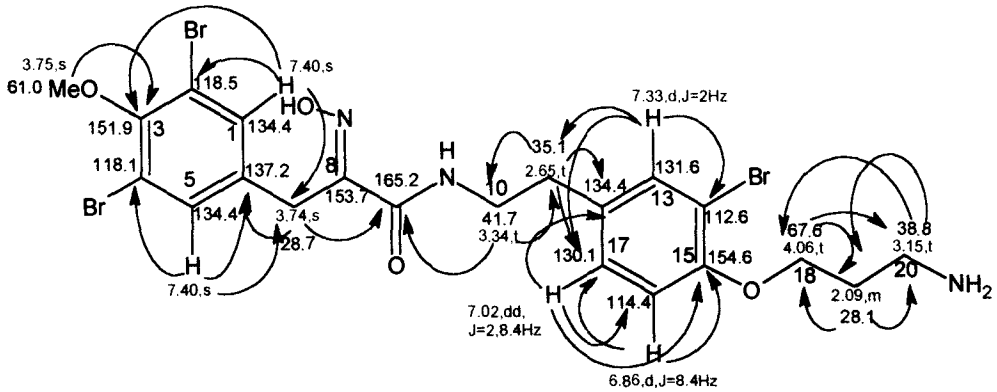


Fig 5b: HMBC of 16-debromoaplysamine-4 (9).

Table 5: ^{1}H, ^{13}CNMR, COSY, TOCSY and HMBC assignment of 16-debromoaplysamine-4 (9), CD_3OD

Carbon no.	^{13}CNMR δ, ppm	^{1}HNMR (δ, ppm)	COSY	TOCSY	HMBC
1	134.4	7.40(1H,s)			C2,4, C3, C6, C7
2	118.5				
3	151.9				
4	118.1				
5	134.4	7.40(1H,s)			C2,4, C3, C6, C7
6	137.2				
7	28.7	3.74(1H,s)		H5	C6, C9
8	153.7				
9	165.2				
10	41.7	3.34(2H,t, J=7Hz)	H11	H11	C9, C11
11	35.1	2.65(2H,t, J=7Hz)	H10	H10, H13	C10,C12,C17
12	134.4				
13	131.6	7.33(1H,d, J=2Hz)			C11,C14, C17
14	112.6				
15	154.6				
16	114.4	6.86(1H,d,J=8.4Hz)	H17	H17	C15, C17
17	130.1	7.02(1H,dd, J=2, 8.4Hz)	H16,H13	H16, H13	C12,C15,C16
18	67.6	4.06(2H,t,J=6.5Hz)			C19, C20
19	28.1	2.09(2H,m)	H18,H20	H18, H20	C18, C20
20	38.8	3.15(2H,t,J=6.8Hz)		H18	C18, C19
21	61.0	3.75(3H,s)			C3

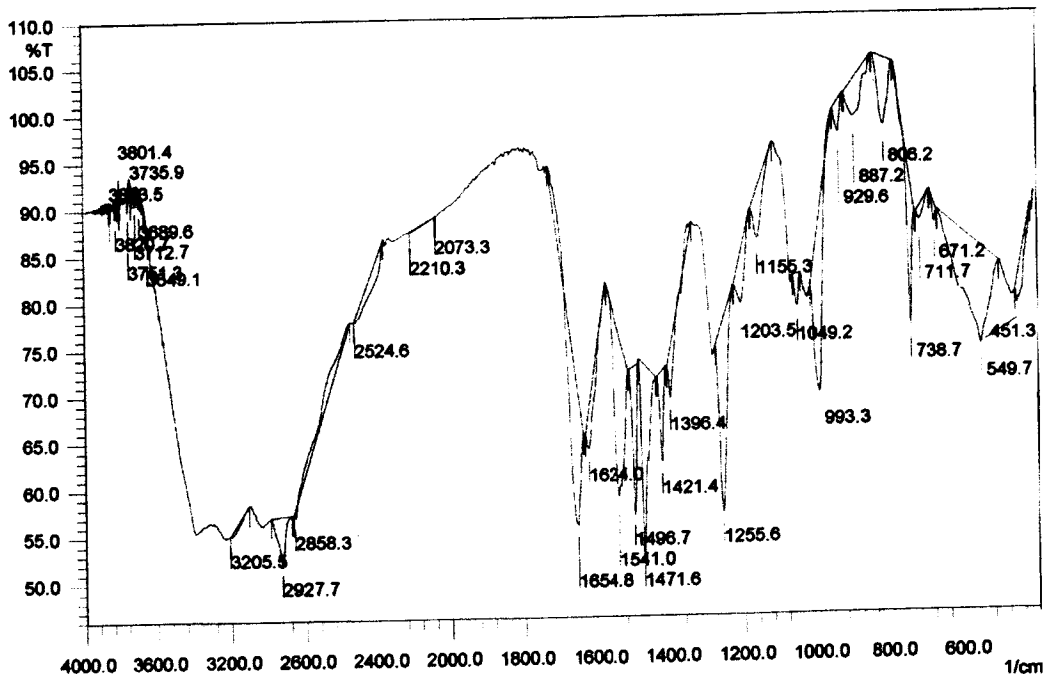


Fig 5.1: IR spectrum of 16-debromoaplysamine-4 (9).

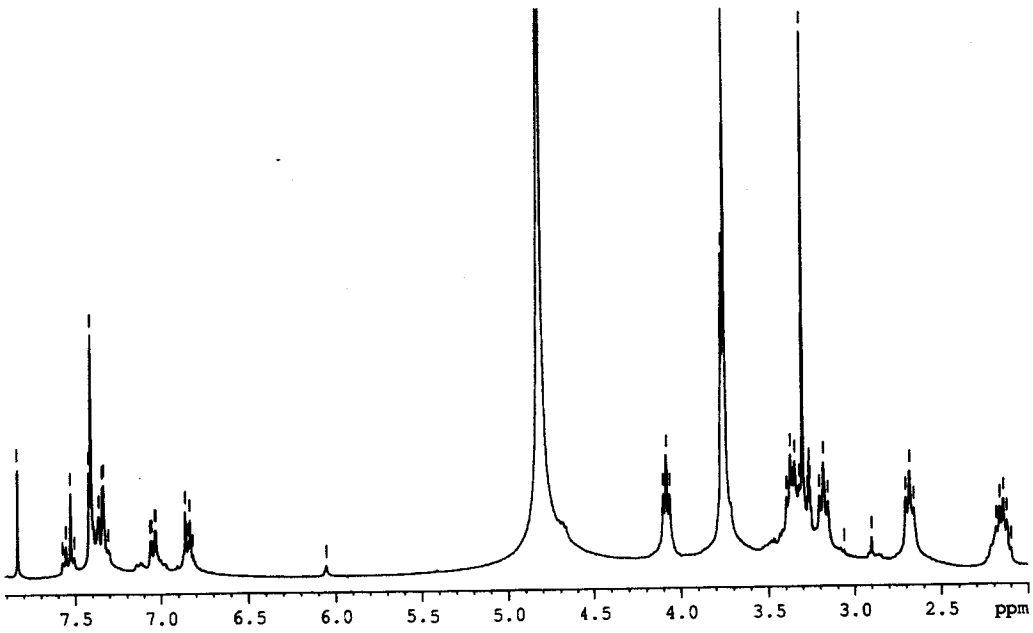


Fig 5.2: ^{1}HNMR spectrum of 16-debromoaplysamine-4 (9).

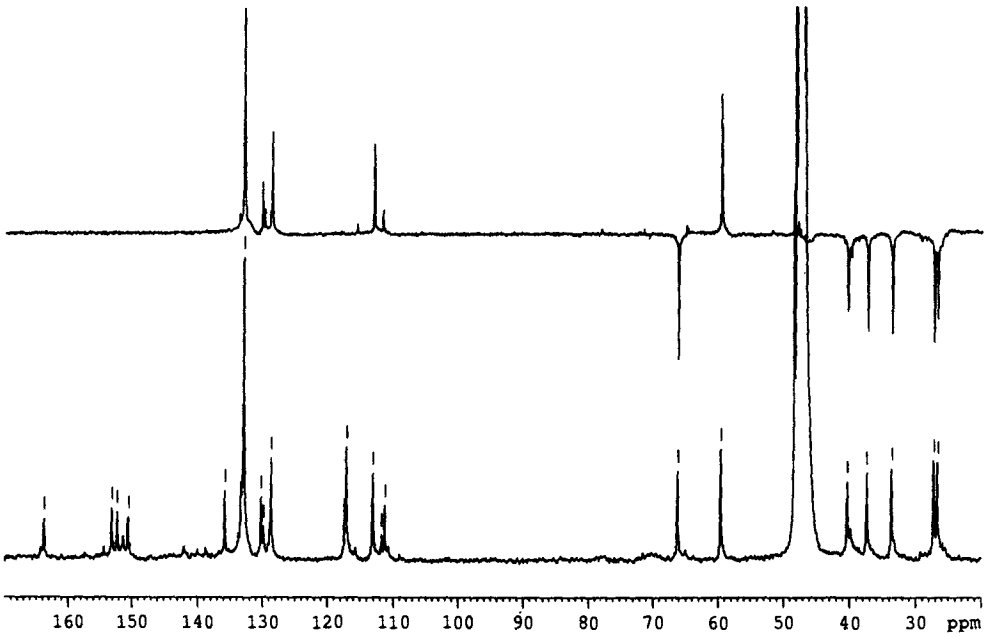


Fig 5.3: ^{13}CNMR and DEPT spectra of 16-debromoaplysamine-4 (9).

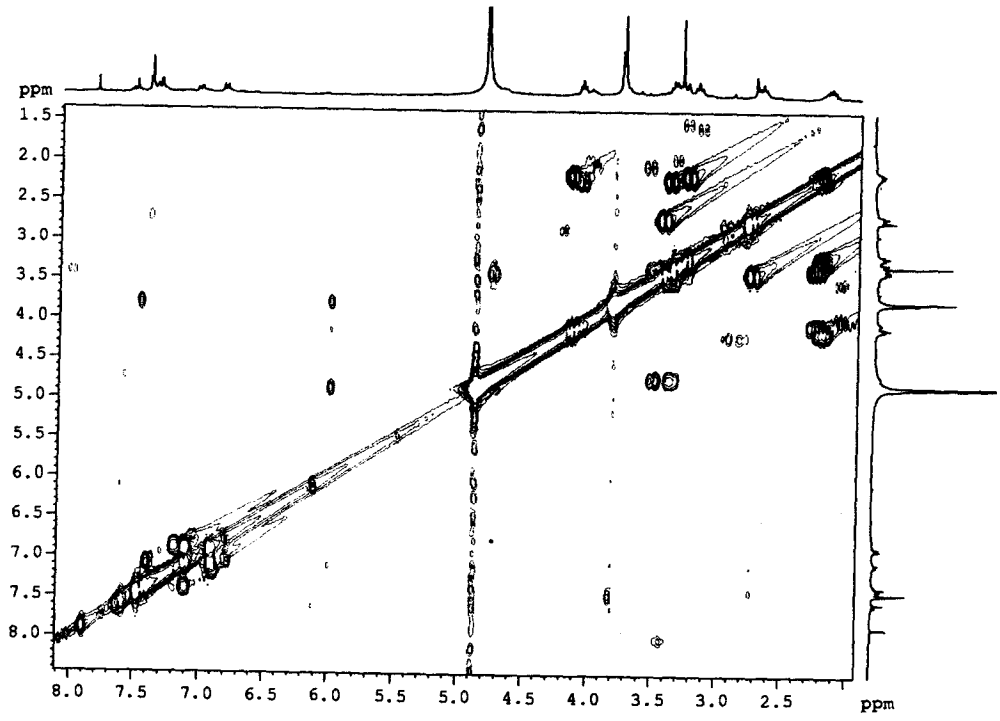


Fig 5.4: COSY spectrum of 16-debromoaplysamine-4 (9).

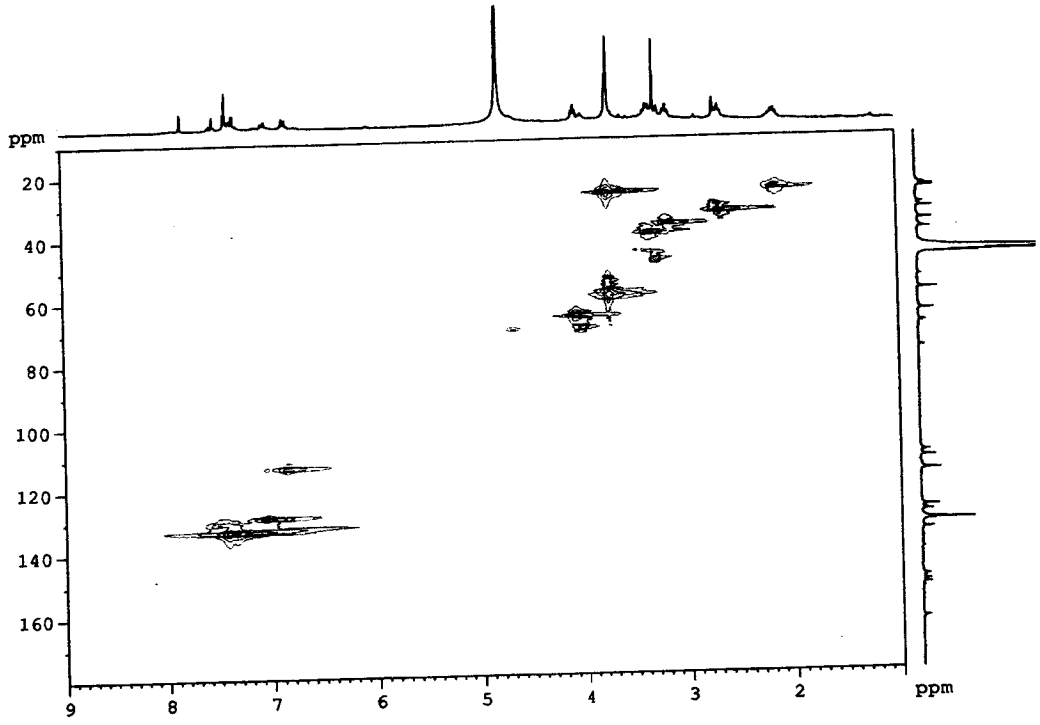


Fig 5.5: HMQC spectrum of 16-debromoaplysamine-4 (9).

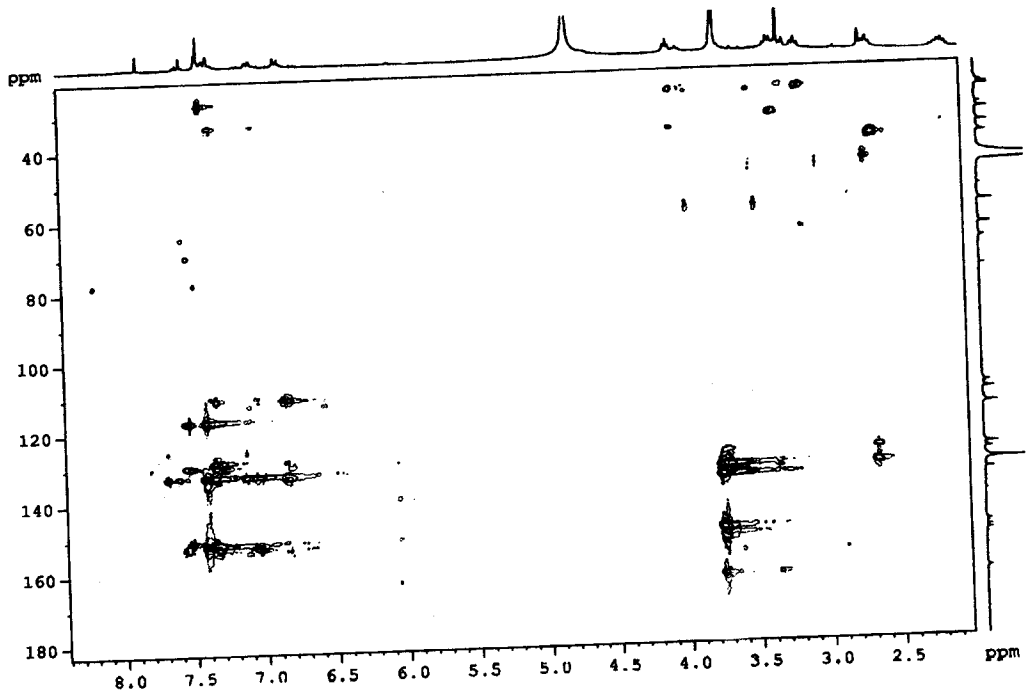


Fig 5.6: HMBC spectrum of 16-debromoaplysamine-4 (9).

The second compound, purpuramine I (**11**) was purified on RP-HPLC at Rt 27.4 min was obtained as colourless compound with R_f value 0.35 in 14% MeOH:$CHCl_3$ solvent system. It appeared light pink on strong heating with ninhydrin reagent. The R_f value was very close to the previous isolated compound (**9**). The mass spectrum of compound **11** (**Fig 6.6**) had $[M+H]^+$ pseudo molecular ion peaks at m/z 633.9605, 635.9542, 637.9490 and 639.9531 in the ratio 1:3:3:1. Its ^{13}CNMR spectrum indicated presence of 22 carbon signals in it, which were distributed as two methyls, six methylnes, five methines and nine quarternary carbons, corresponding to a molecular formula $C_{22}H_{26}O_4N_3Br_3$. Detailed examination of its ^{1}H and ^{13}CNMR spectrum (**Fig 6.1 and 6.2**) revealed this compound to be identical to that of purpuramine I previously isolated by Yagi *et al.* from the same source collected from Hachijo-jima Island.[39] The structure was further confirmed by extensive 2D NMR studies (COSY, HMQC & HMBC) which also helped in assigning all the proton and carbon signals (**Table 6**).

Interpretation of the COSY spectrum (**Fig 6.3**) and chemical shift values led to two sub unit structures, O-CH_2-CH_2-CH_2-N (H18-H19-H20) and N-CH_2-CH_2 (H10-H11) similar to that of compound (**9**). Methylene singlet at δ_H 3.84 showed COSY correlation with aromatic protons δ_H 7.49 (d, J=2.1Hz) and 7.21 (dd, J=8.1, 2.1Hz). δ_H 7.21 showed cross peak with doublet at δ_H 6.77 (J=8.4 Hz). The J=8.1, 2.1Hz of δ_H 7.21 displays ortho and meta coupling with other aromatic protons. The second aromatic proton at δ_H 7.32 (H13) showed cross peak with methylene proton (H11) at δ_H 2.70 (t) of ethylamine chain. This indicates compound (**11**) differs from the 16-debromoaplysamine-4 (**9**) in having one bromine atom in first aromatic ring while two bromine atoms in the second ring. All the ^{1}H-^{1}H connectivities are shown in **Fig 6a**.

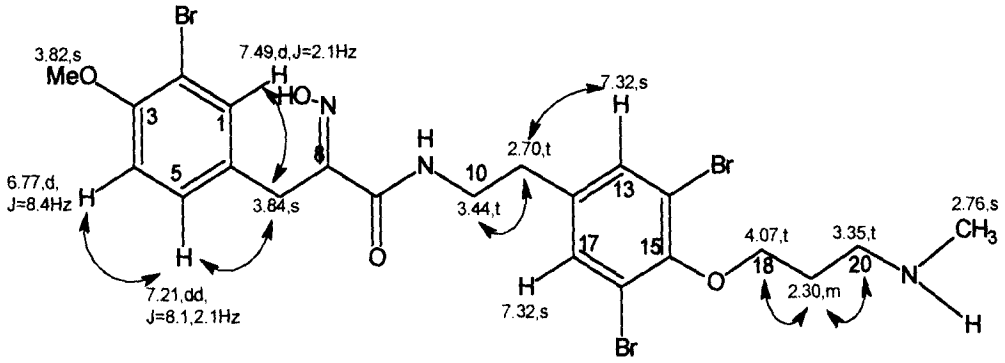


Fig 6a: COSY correlations of purpuramine I (11).

HMQC experiment (**Fig 6.4**) showed direct proton-carbon connectivities. All $^2J_{CH}/^3J_{CH}$ connectivities are established by the HMBC experiment (**Fig 6.5**) as shown in **Fig 6b**. Aromatic proton H1 at δ_H 7.49 (d, J=2Hz) showed cross peaks with carbons at δ_C 154.2 (C3), 110.2 (C2), 129.2 (C5), 130.4 (C6) and 27.8 (C7). Proton at δ_H 6.77 was correlated with C3 and C6 while δ_H 7.21 was correlated C1, C3 and C7. The connectivities confirmed that the first aromatic ring is with the single bromine atom. In the second aromatic ring the two proton singlet at δ_H 7.32 which are attached to carbons δ_C 132.8, showed cross peaks δ_C 117.6 (C14), 117.7 (C16) with bromine atoms and 150.7 (C15) with etheral oxygen. Ethylamine proton H11 at δ_H 2.70 (t) displayed HMBC cross peaks with the aromatic ring carbons 138.4 (C12) and 132.8 (C13). This revealed that ethylamine chain is attached to aromatic ring containing two bromine atoms. Methylene proton at δ_H 4.07 of the propyl amine side chain showed long range coupling with the aromatic carbon at δ_C 150.7. Presence of N-Me gave singlet at δ_H 2.76 which increased the chemical shift of the side chain methylene to δ_H 3.35 (δ_C 47.5). Based on 1D and 2D NMR studies the structure was deduced to be purpuramine I (**11**). The detailed mass fragmentation has been discussed in the next section (**Section 2.3**).

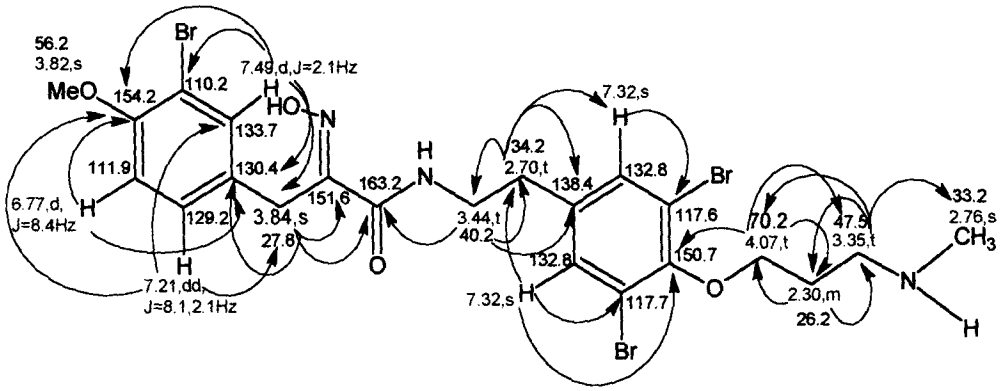


Fig 6b: HMBC correlations of purpuramine I (11).

Table 6: ^{1}H, $^{13}CNMR$, COSY and HMBC assignments of purpuramine I (11) in CD_3OD.

Carbon no.	$^{13}CNMR$ δ_C, ppm	$^{1}HNMR$ δ_H, ppm	COSY	HMBC
1	133.7	7.49(1H,d, J=2.1Hz)	H7	C2, C3, C5, C6, C7
2	110.2			
3	154.2			
4	111.9	6.77(1H,d, J=8.4Hz)	H5	C3, C6
5	129.2	7.21(1H,dd, J=8.1,2.1Hz)	H4, H7	C1, C3, C7
6	130.4			
7	27.8	3.84(2H,s)	H1, H4	C6, C8, C9
8	151.6			
9	163.2			
10	40.2	3.44(2H,t, J=3Hz)	H11	C9, C11, C12
11	34.2	2.70(2H,t, J=3, 7.2Hz)	H10, H13	C10, C12, C13
12	138.4			
13	132.8	7.32(1H,s)		C11, C14, 16 C15
14	117.6			
15	150.7			
16	117.7			
17	132.8	7.32(1H,s)		C11, C14, 16 C15
18	70.2	4.07(2H,t, J=2.4,10.6Hz)	H19	C15, C19, C20
19	26.2	2.30(2H,m, J=7.0,13.2Hz)	H18, H20	C18, C20
20	47.5	3.35(2H,t, J=7.5, 14.7 Hz)	H19	C18, C19, C21
21	33.2	2.76(3H,s)		C20
22	56.2	3.82(3H,s)		

All the compounds were characterized by 1D and 2D NMR experiments. Since these compounds are structurally related, it would have been difficult to characterize them only by 1D experiments. Hence, 2D NMR experiments were performed on the molecules and these proved to be extremely useful in

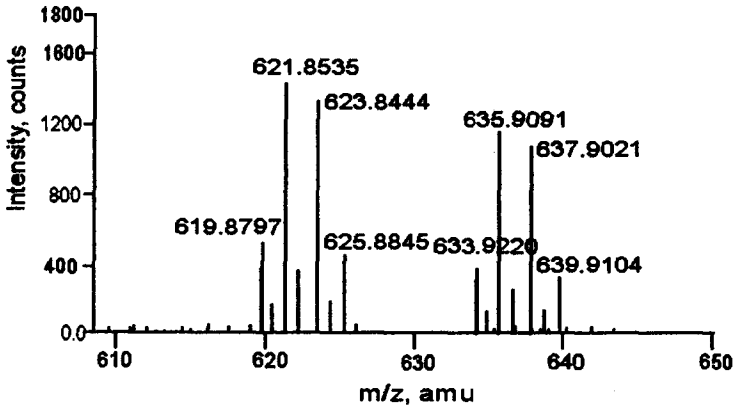


Fig 5.7: ESI-MS spectrum of 16-debromoaplysamine-4 (9).

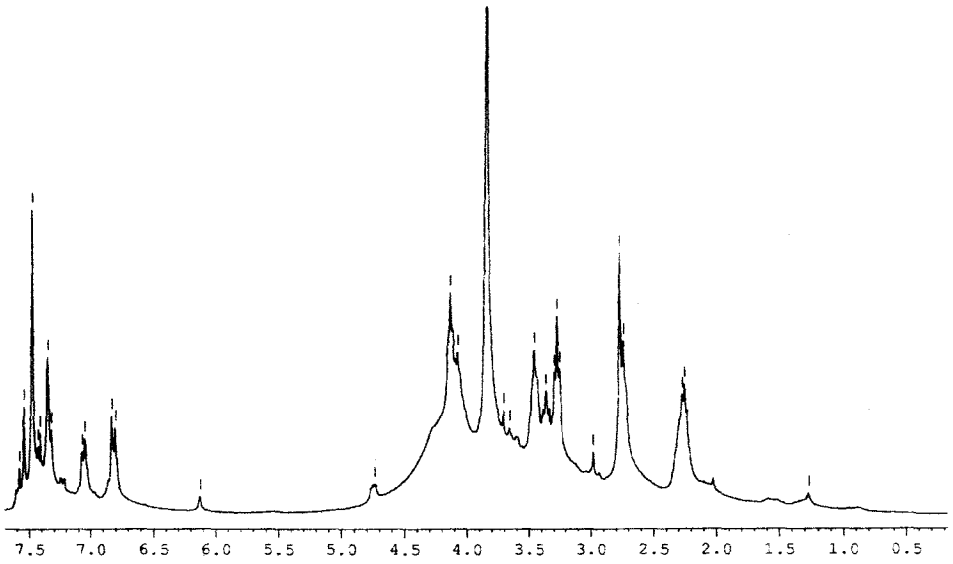


Fig 6.1: ^{1}HNMR spectrum of purpuramine I (11).

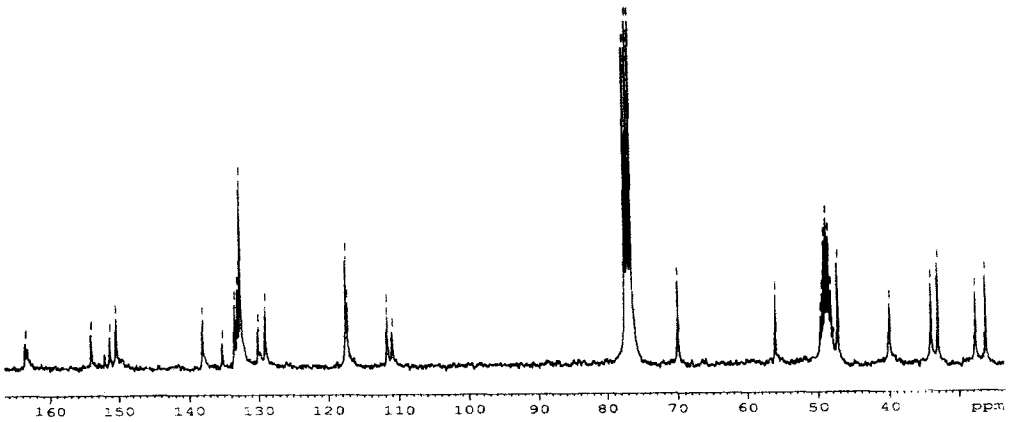


Fig 6.2: ^{13}CNMR spectrum of purpuramine I (11).

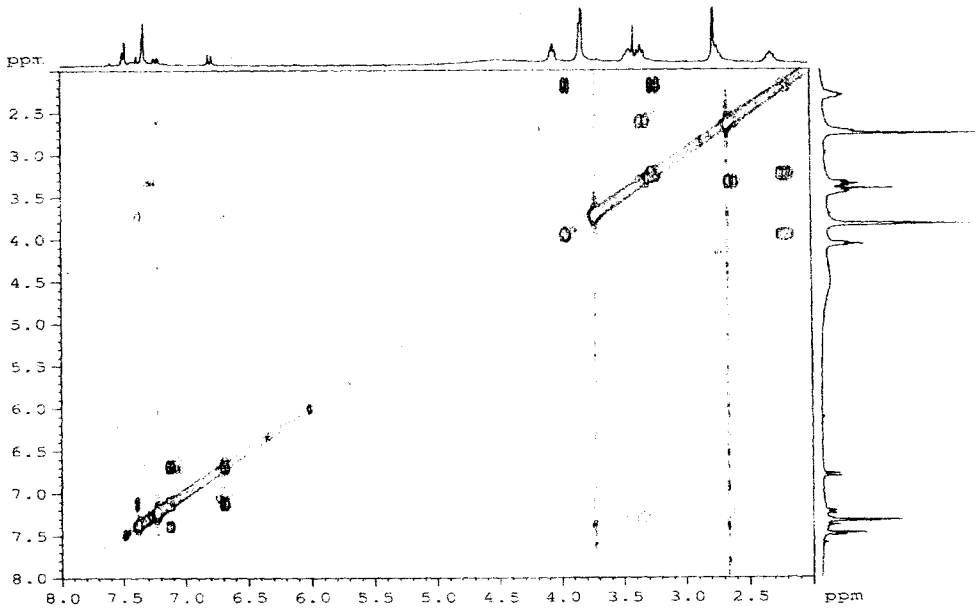


Fig 6.3: COSY spectrum of purpuramine I (11).

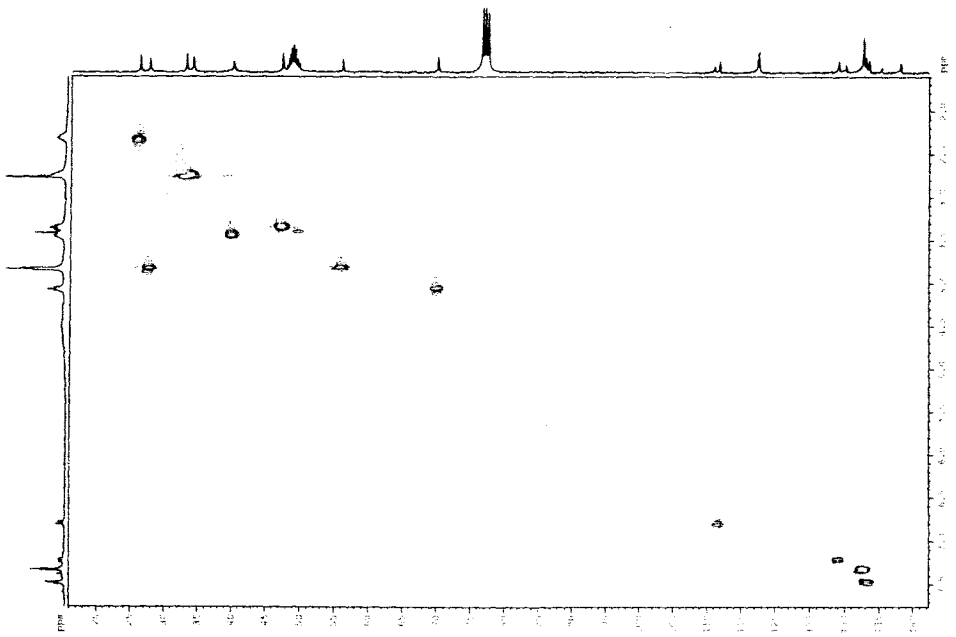


Fig 6.4: HMQC spectrum of purpuramine I (11).

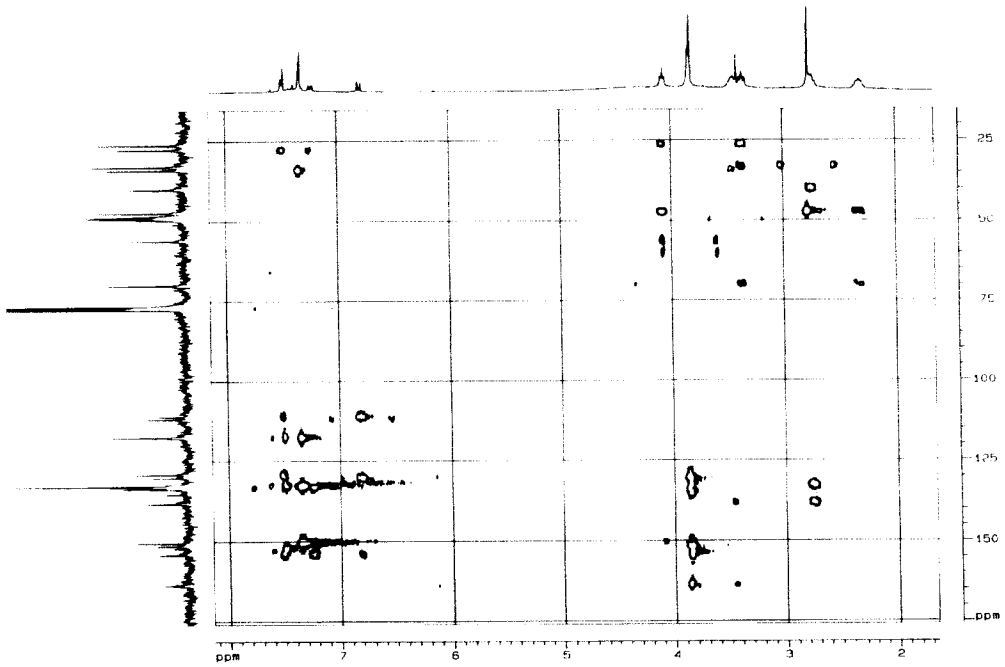


Fig 6.5: HMBC spectrum of purpuramine I (11).

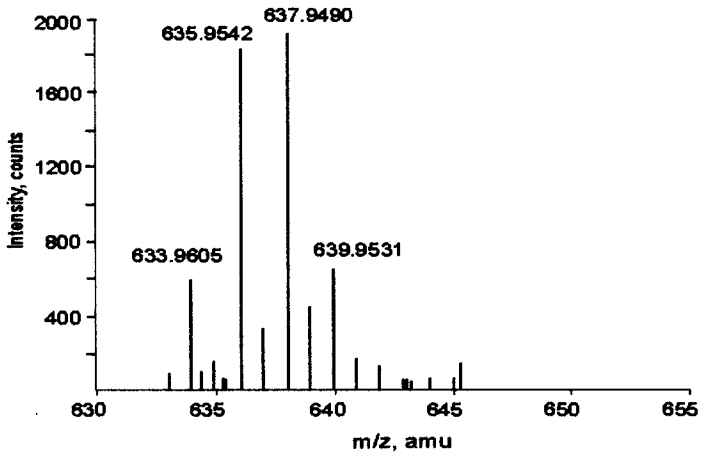


Fig 6.6: ESI-MS spectrum of purpuramine I (11).

characterizing them. A probable biogenetic pathway for these molecules has been proposed and discussed in **Section 2.3.**

***In vitro* antimicrobial screening of the bromotyrosine compounds:**

Compounds **1, 3, 9** and **11** were evaluated for their antimicrobial activity **(Table 6)** against bacterial strains *E. coli, Pseudomonas aeruginosa, Staphylococcus aureus, Salmonella typhi, Shigella flexineri, Klebsiella sp.* and *Vibrio cholerae* and fungal strains *Aspergillus fumigatus, Fusarium sp., Cryptococcus neoformans, Aspergillus niger, Rhodotorula sp., Nocardia sp., and Candida albicans.* The compounds did not show any activity against bacterial strains *Klebsiella sp., Pseudomonas aeruginosa* and all fungal strains. Purealidin Q **(1)** showed good activity against *Salmonella typhi.* It was previously reported to show cytotoxic activity against tumor cell lines and moderate inhibitory against epidermal growth factor (EGF) kinase.[10] Purpurealidin B **(3)** showed good activity against *E. coli, S. aureus, V. cholerae* and weak activity against *Shigella flexineri.* 16-debromo-aplysamine-4 **(9)** showed moderate activity against *Salmonella typhi* and very weak acivity against *E. coli, S. aureus* and *V. cholerae.* Moderate acivity against *S. aureus* was confirmed for purpuramine I **(11)** according to the previous studies. It also showed moderate activity against *E. coli* and *V. cholerae* (Published results)[♣].

Table 6: Effect of compounds 1, 3, 9 & 11 on growth of microbial strains (MIC in µg/ml)

Compounds	*E. Coli*	*S. aureus*	*Salmonella typhi*	*Shigella flexineri*	*Vibrio cholerae*
Purealidin Q (1)	-	-	>25	-	-
Purpurealidin B (3)	>12	10	-	100	25
16-Debromo aplysamine 4 (9)	250	200	>50	-	100
Purpuramine I (11)	100	50	-	-	100
Streptomycin (Standard)	10	10	10	10	10

Good Activity: 10-25µg/ml; Moderate Activity: 26-100µg/ml; Weak Activity: >100µg/ml

♣ New bromotyrosine alkaloids from the marine sponge *Psammaplysilla purpurea*, Supriya Tilvi, Celina Rodrigues, C. G. Naik, P. S. Parameswaran and Solimabi Wahidullah, ***Tetrahedron*** 60 (2004) 10207–10215.

EXPERIMENTAL SECTION:

General experimental procedures:

Column chromatographies were carried out using Silica gel (60-120 mesh, Qualigens), gel filtrations were carried out using Sephadex LH20 17-0090-01 Pharmacia Biotech). Fractions were monitored on TLC using alumina-backed sheets (Si gel 60 F254, 0.25mm thick) with visualization under UV (254nm) and ninhydrin spray reagent. All analytical reverse phase HPLC (Chromspher 5 C18 column 250x 10mm, MeOH/H_2O 85/15) were performed with a P4000 pump (Spectra system) equipped with a UV2000 detector (Spectra system).

UV spectra were recorded in MeOH, using a Shimadzu UV-Vis 2401PC Spectrophotometer and IR spectra were recorded on a Shimadzu FT-IR 8201PC Spectrophotometer. Optical rotations were recorded in MeOH using Optical Polarimeter ADP220 (Bellingham Stanley ltd.).

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

NMR (^1H, ^{13}C, COSY, HMQC & HMBC) experiments were performed on Bruker (Avance 300) spectrometer with TMS as internal standard. Spectra were obtained either in $CDCl_3$ (δ_H 7.27; δ_C 76.3 ppm) or CD_3OD (δ_H 3.46; δ_C 40.3 ppm) solvents.

Animal material:

The animals were collected by scuba diving at a depth of 8-10m from Mandapam (9° 16' N; 79° 12' E), Tamil Nadu, India and identified by Dr. P. A. Thomas of Vizhingam Research Center of Central Marine Fisheries Research Institute, Kerala, India. A voucher specimen is deposited at the National Institute of Oceanography, Dona Paula, Goa, India.

Extraction and Isolation:

The frozen sponge (250g, dry weight) was extracted with Methanol (1L × 3) and concentrated under vacuo to obtain crude extract. The residue (10.5gm) was partitioned between ethyl acetate (400ml × 3). The ethyl acetate fraction was

concentrated under vaccum to give crude residue (5gm). The ethyl acetate soluble fraction was subjected to column chromatography on Sephadex LH-20 with Chloroform:Methanol (1:1). The eluted fractions were monitored on the TLC using different polarities of mobile phase. Second fraction was found to be rich in spots when detected with I_2 vapour, 5% methanolic sulphuric acid and ninhydrin reagent. This fraction was further chromatographed on silica gel (Qualigens silica gel 60-120 mesh) column using chloroform with increasing amounts of methanol as eluent. The sub fractions eluted with 5, 8, 10 and 20% were purified separately. The fraction eluted with 8% MeOH (1.5g) was further purified by repeated gel chromatography (Sephadex LH20) columns using Chloroform:Methanol (1:1) to get purealidin Q **(1)** (200mg) and purpurealidin B **(3)** (800mg). On TLC, purealidin Q **(1)** purpurealidin B **(3)** showed dark brown spots with I_2 vapour and 5% methanolic sulphuric acid. The compounds were also visible under UV 254 nm wavelength. From the non-polar fraction (5% MeOH:$CHCl_3$) mixture of two compounds, purpurealidin C **(4)** & D **(5)** (400mg) were isolated using repeated silica gel chromatography. The fractions eluted with 10% MeOH were purified on reverse phase HPLC using Chromspher 5 C18 column; 250x 10mm, MeOH/H_2O 85/15, flow rate 2ml/min & UV detection at λ_{max} 254nm) which afforded 16-debromoaplysamine-4 **(9)** (Rt 18.4min) (20mg) and purpuramine I **(11)** (Rt 27.5 min) (25mg). Compound **9** showed dark spot with ninhydrin reagent while compound **(11)** gave light pink on strong heating. The most polar fractions with 20% MeOH:$CHCl_3$ were subjected to silica gel column eluted with increasing amounts of methanol in chloroform to yield mixture of three compounds, purpurealidin E **(6)**, F **(7)** & G **(8)** (300mg).

Purealidin Q (1): Colorless oil, UV (MeOH) λ_{max} 277 nm (ϵ 1700), 284nm (ϵ 1400); $[\alpha]_D^{28}$ +9.5 (c 0.2, MeOH); IR (neat) ν_{max} 3418, 2939, 1654, 1527, 1494, 1458, 1255, 1043, 929, 754 cm^{-1}; ^1H and ^{13}CNMR (CD_3OD+$CDCl_3$, 300MHz) See **Table 1**; HRMS: m/z (relative heights) 741.8691(450), 743.7871(1780), 745.7710(2600), 747.7762(1700), 749.7914(420) [1.07:4.23:6.2:4.0:1.0][a],

[a] Real ratios of pseudomolecular ion peaks

$[M+H]^+$, found 741.8691 $C_{23}H_{27}N_3O_5Br_4$ requires 741.8764; $[M+H-Br]^+$ 662.9,664.9,666.9,668.9; $[M+H+Br+CH_3]^+$ 647.8,649.8,651.8,653.8; 404.8,406.8,408.8; 378.9,380.9,382.9; 348.9, 350.8, 352.8; 58; 86.

Purpurealidin A (2): Colorless oil, UV (MeOH) λ_{max} 277 nm (ϵ 1700), 284nm (ϵ 1400); $[\alpha]_D^{28}$ +9.5 (c 0.2, MeOH) ; IR (neat) ν_{max} 3418, 2922, 1668, 1537, 1458.1, 1254, 1051, 920, 737 cm^{-1}; ^1H and ^{13}C (CD_3OD+$CDCl_3$, 300MHz) See **Table 1**; HRMS: m/z (relative heights) 755.8(55), 757.8(210), 759.8(310), 761.8(200), 763.8(50) [1.1:4.2:6.2:4.0:1.0][a], $[M+H]^+$, found 755.8819 $C_{24}H_{29}N_3O_5Br_4$ requires 755.8920.

Purpurealidin B (3): White amorphous solid, mp 175.8 °C; UV (MeOH) λ_{max} 283 (1320); IR (KBr pellet) ν_{max} 3302, 2932, 2689, 1678, 1605, 1541, 1460,1383, 1259, 910 and 739 cm^{-1}. ^1H and ^{13}CNMR ($CDCl_3$, 300MHz) See **Table 2**; HRMS: m/z (relative heights) 631.8403(1600), 633.8185(4700), 635.8118(4600), 637.8226(1500) [1.06:3.13:3.03:1.0][a], $[M+H]^+$, found 631.840 $C_{22}H_{24}N_3O_4Br_3$ requires 631.9396; 404.9, 406.9, 40 8.9; 376.9, 378.9, 380.9; 224.9, 226.9.

Purpurealidin C (4): Colorless oil, UV (MeOH) λ_{max} 282(10,000), 218(2500), $[\alpha]_D^{28}$ +158.5 (c 0.2, $CHCl_3$); IR (KBr pellet) ν_{max} 3319, 2925, 2854, 1712, 1660, 1652, 1605, 1456, 1257, 739 cm^{-1}, ^1H and ^{13}C ($CDCl_3$, 300MHz) See **Table 3**; ES-MS: m/z (relative heights) 938.2(22), 940.2(85), 942.2(125), 944.2(80), 946.2(20) [1.1:4.2:6.2:4.0:1.0][a], $[M+H]^+$, found 938.05 $C_{36}H_{51}N_3O_6Br_4$ requires 938.0591; 615, 617, 619; 379, 381, 383.

Purpurealidin D (5): Colorless oil, UV (MeOH) λ_{max} 282(10,000), 218(2500), IR (KBr pellet) ν_{max} 3319, 2925, 2854, 1660, 1605, 1456, 1257, 739cm^{-1}, ^1H ($CDCl_3$, 300MHz) δ_H 7.43 (1H,s, 9-NH), 7.34 (2H,s, H-13,17), 6.24 (1H, s, H13,17), 6.24 (1H,s, H-5), 5.40 (1H, s, 20-NH), 4.28 (1H,s, H-1), 4.01 (2H,t, J=12.0, 6.0 Hz, H-18), 3.88 (1H, d, J=18.3 Hz, H7-b), 3.67 (3H, s, -OCH_3), 3.63 (2H,t, H-20), 3.54 (2H, t , J=13.2, 6.6 Hz), 2.98 (1H, d, J=18.6 Hz, H7a), 2.67 (2H,m, H-22), 2.06

(2H,m, H-19), 1.53 (2H,m, H-35), 1.19(24H, s, H23-24) and 0.70 (3H, t, H-36). ^{13}CNMR ($CDCl_3$, 300MHz) δ_C 173.6 (s, C-21), 159.1 (s, C-9), 154.9 (s, C-8), 151.2 (s, C-15), 148.0 (s, C-3), 138.0 (s, C-12), 132.9 (d, C-13,17), 130.0 (s, C-5), 121.4 (s, C-4), 118.2 (s, C14,16), 112.7 (s, C-2), 91.5 (s, C-6), 74.0 (d, C-1), 71.0 (t, C-18), 60.0 (q, -OCH_3), 40.3 (t, C-10), 37.0 (t, C-20), 38.8 (t, C-7), 34.3 (t, C-11), 34.4 (t, C-22), 27.0-32.7 (t, C-23-34), 29.0 (d, C-35) and 14.0 (q, C-36); ES-MS: m/z (relative heights) 952.2(16), 954.2(65), 956.2(95), 958.2(60), 960.2(15) [1.06:4.3:6.3:4.0:1.0][a], $[M+H]^+$, found 952.2 $C_{37}H_{53}N_3O_6Br_4$ requires 952.2; 655, 657, 659, 661, 662; 601, 603, 605; 379, 381, 383.

Purpurealidin E (6): Colourless oil, UV (MeOH) λ_{max} 282 (ϵ 950), 277 (ϵ 925); IR (neat) ν_{max} 3302, 2933, 1666, 1545, 1458, 1259, 1039, 739cm^{-1}, ^{1}H and ^{13}CNMR (CD_3OD, 300MHz) See **Table 5,** ESI-MS: m/z (relative heights) 378.9768(37), 380.9757(72), 382.9755(35) [1.05:2.05:1.0][a], $[M+H]^+$, found 378.9768 $C_{13}H_{20}N_2OBr_2$ requires 378.9943.

Purpurealidin F (7): Colourless oil, UV (MeOH) λ_{max} 282 (ϵ 950), 277 (ϵ 925); IR (neat) ν_{max} 3302, 2933, 1666, 1545, 1458, 1259, 1039, 739cm^{-1}, ^{1}H and ^{13}CNMR (CD_3OD, 300MHz) See **Table 5**, HRMS: m/z (relative heights) 394.9667(16), 396.9661(32), 398.9618(15) [1.06:2.1:1.0][a], $[M+H]^+$, found 394.9667 $C_{13}H_{20}N_2O_2Br_2$ requires 394.9970.

Purpurealidin G (8): Colorless oil, UV (MeOH) λ_{max} 282 (ϵ 950), 277 (ϵ 925); IR (neat) ν_{max} 3302, 2933, 1666, 1545, 1458, 1259, 1039, 739cm^{-1}, ^{1}H and ^{13}CNMR (CD_3OD, 300MHz) See **Table 5,** HRMS: m/z (relative heights) 451.9661(10), 453.9501(20), 455.9502(10) [1:2:1], $[M+H]^+$, $C_{16}H_{24}N_2O_3Br_2$

16-Debromoaplysamine-4 (9): Colorless amorphous solid (MeOH): m.p. 124-126°C; UV (MeOH) λ_{max} 218 nm (ϵ 12675), 280nm (ϵ 2675); IR (KBr pellet) ν_{max} 3350, 3205, 2958, 1655, 1624, 1541, 1497, 1472, 1421, 1256, 1203, 1049, 993 and 739 cm^{-1}; ^{1}H & ^{13}CNMR (CD_3OD, 300MHz): See **Table 5,** HRMS: m/z

(relative heights) 619.8797(525), 621.8535(1550), 623.8444(1530), 625.8845(500) [1.05:3.1:3.06:1.0][a], $[M+H]^+$, found 619.8797 $C_{21}H_{24}N_3O_4Br_3$ requires 619.9396.

Purpurealidin H (10): Colorless amorphous solid (MeOH): UV (MeOH) λ_{max} 218 nm (ϵ 12675), 280nm (ϵ 2675); IR (KBr pellet) ν_{max} 3350, 3205, 2958, 1655, 1624, 1541, 1497, 1472, 1421, 1256, 1203, 1049, 993 and 739 cm^{-1}; ^1H (CD_3OD, 300MHz) δ_H 7.4 (2H, s, H-1, 5), 7.33 (1H, d, J=2.0Hz, H-13), 7.02 (1H, dd, J=2.0, 8.4 Hz, H-17), 6.86 (1H, d, J=8.4 Hz, H-16), 4.06 (2H, t, J=6.5 Hz, H-18), 3.75 (3H, s, $-OCH_3$), 3.74(1H, s, H-7), 3.34 (2H, t, J=7Hz, H-10), 3.15(2H, t, J=6.8 Hz, H-20), 2.764 (3H, s, $-NCH_3$), 2.65 (2H, s, J=7.0Hz, H-11), 2.09 (2H, m, H-19); ^{13}CNMR(CD_3OD, 300MHz) δ165.2 (s, C-9), 154.6 (s, C-3), 153.7 (s, C-8), 151.9 (s, C-15), 137.2 (s, C-12), 134.5 (s, C-6), 134.4(d, C-1, 5), 134.4(d, C-13), 130.2(d, C-17), 118.5 (s, C-2, 4), 114.4(d, C-16), 112.6 (s, C-14), 67.6 (t, C-18), 61.0 (q, $-OCH_3$), 41.7 (t, C-10), 38.8 (t, C-20), 35.1 (t, C-11), 28.7 (t, C-7), 28.1 (t, C-19), 27.615 (q, $-NCH_3$); HRMS: m/z (relative heights) 633.9220(420), 635.9091(1250), 637.9021(1220), 639.9104(400) [1.05:3.12:3.05:1.0][a], $[M+H]^+$, found 633.9220 $C_{22}H_{26}N_3O_4Br_3$ requires 633.9550.

Purpuramine I (11): a colorless amorphous solid, IR (KBr pellet) ν_{max} 3398.3, 2927.7, 1660.6, 1627.6, 153.3, 1458.1, 1494.7, 1396.4, 1255.6, 1203.5, 1109.0, 1055.0, 1031.8, 995.2 and 738.7 Cm^{-1}; ^1H & ^{13}CNMR: See **Table 6;** HRMS m/z peaks at m/z 633.9605, 635.9542, 637.9490, 639.9531 [1:3.32:3.30:1.1][a] $[M+H]^+$.

Antibacterial and antifungal assays: As described in **Chapter II, Section 1.**

2.3: Detection and identification of bromotyrosine alkaloids from *Psammaplysilla purpurea* using electrospray ionization-tandem mass spectrometry (ESI-MS/MS).

The interest in studying novel structures and functions of molecules in the biological systems has created an impetus for the development of efficient, effective and information-rich methods and techniques of analysis. One of the most common techniques used for studying these interactions are soft ionization mass spectrometry, specifically electrospray ionization-mass spectrometry (ESI-MS) that has shown the greatest development in the last several years.

NMR had indicated the presence of bromotyrosine alkaloids in the sponge *Psammaplysilla purpurea.* With a view to detect similar compounds and study their fragmentation pattern a repeat collection of the same sponge was analysed by tandem mass spectrometry (ESI-MS/MS).

The technique is known to be very sensitive and rapid. It was observed that, the repeat collection of sponge was devoid of many of the compounds identified from first collection but studies did result in identification of three new bromotyrosine alkaloids purpurealidin I **(12)**, J **(16)** and K **(17)**. Their identification was exclusively based on the fragmentation pattern observed under ESI-MS/MS conditions. This section is initiated with a brief introduction on tandem ESI-MS technique.

Tandem mass spectrometry (ESI-MS/MS):

Mass spectrometry (MS) is an important analytical tool in biological and biochemical research. The speed, accuracy and sensitivity is unmatched by conventional analytical techniques. Since its beginnings about 100 years ago, 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, the characterization of new elements,

quantitative gas analysis, stable isotope labeling, fast identification of trace pollutants and drugs and the characterization of molecular structure.

Tandem mass spectrometry (MS/MS) is at the heart of most of modern mass spectrometric investigations of complex mixtures.[74-76] The combination of the newer soft ionization techniques *i.e.* electrospray ionization MS (ESI-MS) and matrix assisted laser desorption ionization MS (MALDI-MS) with collision-induced dissociation is what gives tandem MS its power in the analysis of mixtures. Both 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 in many cases these masses can be measured with accuracies better than 0.01%. Applications 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.[77-78]

In our studies, we have used QSTAR XL-MS/MS Quadrupole TOF 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 precusor 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.

For known compounds, mass spectra can be used much like fingerprints. A match is extremely strong evidence that the compounds are identical. For unknowns, results from a mass spectrum often provide significant information that can help elucidate the structure of an organic compound. Analysis of a mass spectrum can provide a formula and considerable information about structure. As fragment ions are produced, the challenge is to put the pieces of a puzzle together to form the whole structure.

Recently, electrospray ionization (ESI) quadrupole ion-trap tandem mass spectrometry (MS/MS) was utilized to characterize a class of complex oligosaccharide antibiotics (everninomicins) that include SCH 27899, everninomicin-D, amino everninomicin (SCH 27900), and SCH 49088 (containing a hydroxylamino-ether sugar).[79] Eighteen cerebrosides were detected in a mixture from the sea cucumber *Holothuria coronopertusa* by combination of LSIMS as ionization mode and high-energy MS/MS. The method appeared to be a very powerful for the rapid analysis of unresolved mixtures of complex molecules.[80]

Tandem mass spectra of lithiated adducts of triacylglycerol (TAG) species obtained by ESI-MS with low-energy collisionally activated dissociation (CAD) on a triple stage quadrupole instrument was described by Fong-Fu Hsu and John Turk in 1999.[81] Similarly in 2001, they described characterization of glycosphingolipids as their lithiated adducts by using same techniques.[82] Glycosphingolipids from bovine erythrocytes, mouse kidney and fetal calf brain were characterized by matrix-assisted laser desorption/ionisation time-of-flight

(MALDI-TOF) MS, liquid secondary ionization mass spectrometry (LSIMS), and tandem mass spectrometry (MS/MS).[83] The cyclic peptides were analyzed directly from the extract of seeds of *Linum utitatissimum* by ESI-MS and neutral loss ESI-MS/MS technique. The sequences of the unreported cyclic peptides were proposed on the basis of CID experiments.[84]

We have therefore examined whether ESI-MS/MS is similarly useful in determining the structures of bromotyrosine alkaloids from the extract of *P. purpurea.*

As we know mass spectrometer separates positively charged species according to their mass to charge ratios, isotopes are separated and detected as distinct species. This fact often allows immediate recognition of the presence of certain elements such as bromine and chlorine. As bromine has two essentially equally abundant isotopes (79 and 81), the parent peak region of mass spectra of compounds that contain one bromine atom have two peaks of the same intensity separated by two mass units. For chlorine (75.5% chlorine-35 and 24.5% chlorine-37), the ratio is about 3:1. So the characteristics halogen isotope pattern can be used as the valuable structural indicators in characterizing halogenated compounds.

A small piece of the frozen sponge *Psammaplysilla purpurea* was extracted with methanol and concentrated under vacuum to obtain the crude extract, which was then fractionated with ethyl acetate. The residue was used for the ESI-MS/MS analysis.

Ion peaks shown in the full scan ESI-MS spectra:

Tandem mass spectrometric experiments were conducted with the Q-TOF mass analyzer for the detailed structure elucidation. The positive ion ESI mass spectrum of ethyl acetate fraction of sponge *P. Purpurea* in methanol obtained by direct infusion showed several clusters of pseudomolecular ion peaks. The spectrum clearly indicates its isotopic patterns consistent with the presence of bromine

atoms in the molecules. The mass spectrum acquired in full scan (mass range of 350-800 amu) is illustrated in **Fig 1.** The cluster of pseudomolecular ion peaks dominates in the region m/z 500-800. **Fig 1A** and **B** shows the expansion of ESI-MS spectrum in the range m/z 700-755 and m/z 620-655 amu repectively. The ratios of the cluster indicate presence of either two, three or four bromine atoms in the molecule. The real ratios with the relative abundance and number of bromine atoms of all the pseudomolecular ions are given in the **Table 1.** Peak at m/z 745.8182 represents the base peak The MS/MS of each pseudomolecular peak gave the fragment ions for the particular molecular ions.

Table 1: Illustrates isotopic ion peaks with their real ratios and relative abundance observed in the ESI-MS

No.	Isotopic ion peaks, $[M+H]^+$	Real ratios	No. of Br atoms	Relative Abundance (%)
1	741.8264, 743.8201, 745.8182 *, 747.8148, 749.8147	1: 4.76: 7.36: 4.48: 1	4	100
2	727.8147, 729.8068, 731.8046, 733.8152, 735.7991	1.6: 4.8: 6.2: 4.0: 1	4	15.79
3	619.8924, 621.8897, 623.8861, 625.8888	1: 3.28: 3.2: 1.14	3	47.37
4	633.9051, 635.9027, 637.9023, 639.9006	1: 3.32: 3.30: 1.1	3	65.79
5	647.9252, 649.9187, 651.9178, 653.9186	1: 3.3: 3.34: 1.03	3	78.95
6	697.7960, 699.7986, 701.7961, 703.7927, 705.7954	1: 4.5: 7.5: 4.6: 1.16	4	23.68
7	555.9670, 557.9912, 559.9908	1.1: 2: 1	2	18.42
8	527.9449, 529.9444, 531.9403	1: 2.1: 1.14	2	31.58

*Base Peak at m/z 745.8123

Comparing the ESI-MS spectrum (**Fig 1**) with the bromotyrosines isolated in the previous study (**Section 2.2**) we found that the cluster of pseudomolecular ion peaks at m/z 741.8264, 743.8201, 745.8182, 747.8148 and 749.8147 in the ratio 1:4:6:4:1 correspond to purealidin Q (**1**) and m/z 633.9051, 635.9027, 637.9023 and 639.9006 (1:3:3:1) correspond to purpuramine I (**11**). They are present in the repeat collection with the relative abundance of 100 and 67%. The number

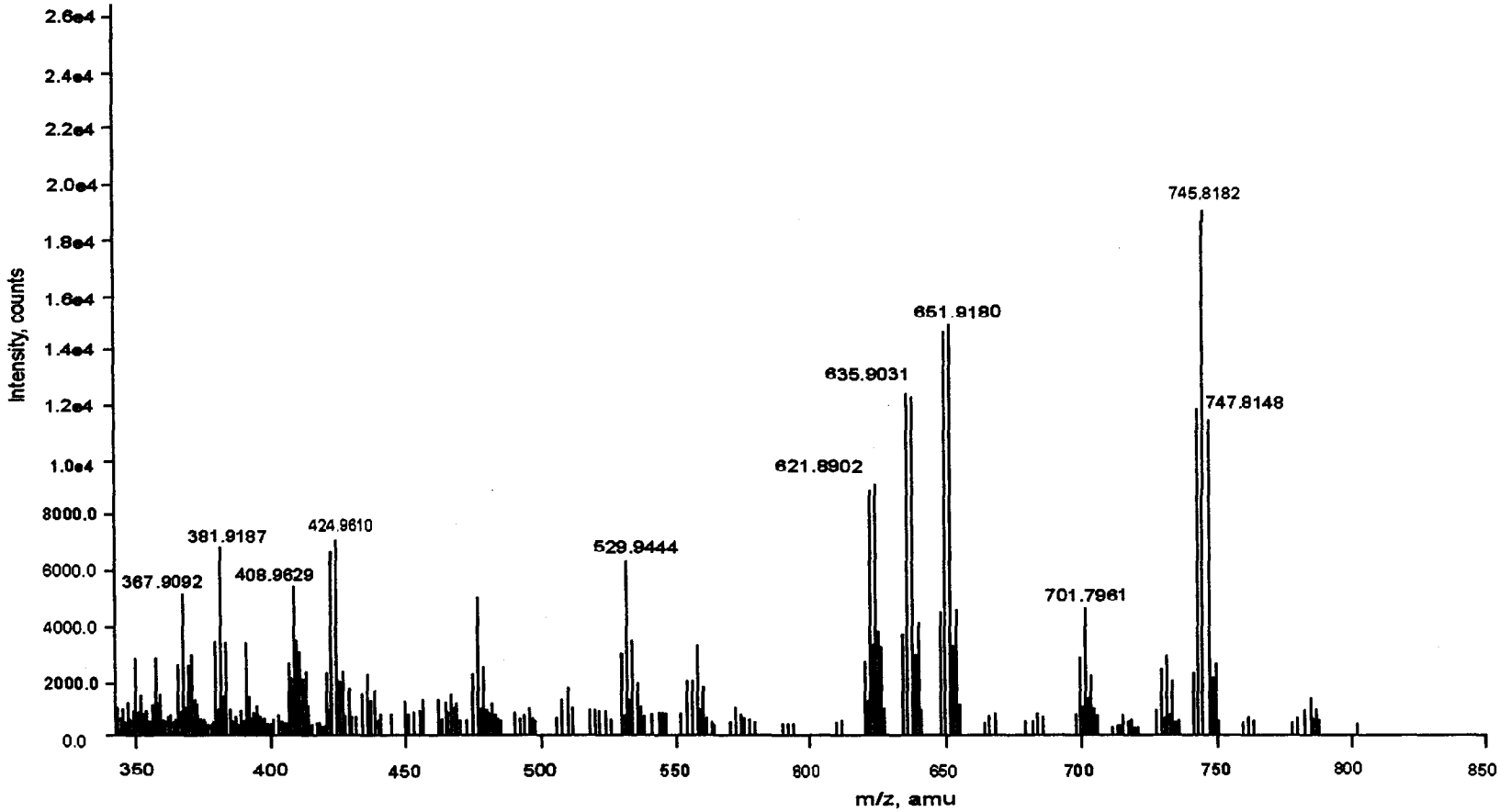
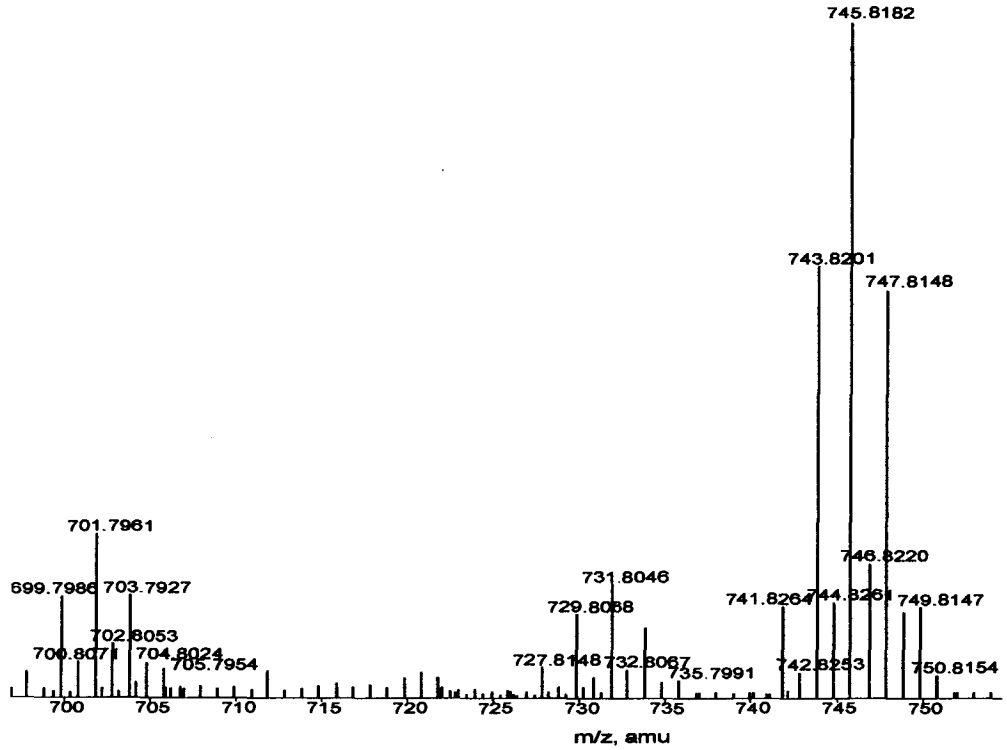
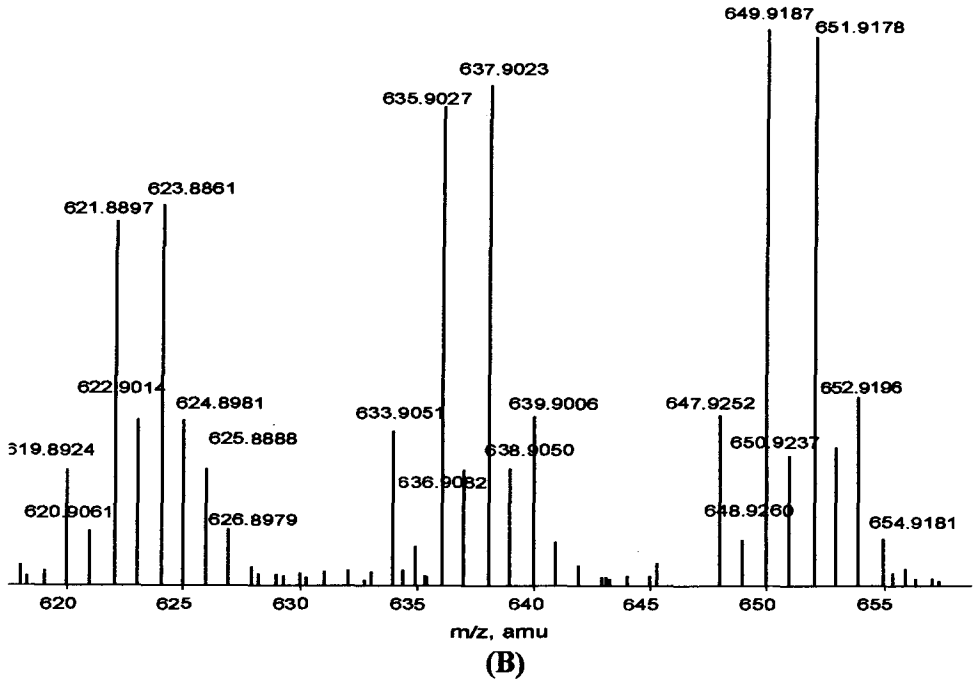


Fig 1: ESI-MS of ethyl acetate fraction of *Psammaplysilla purpurea.*



(A)



(B)

Fig 1: Expansion of ESI-MS spectrum in the range A) m/z 700-755 amu and B) m/z 620-655 amu

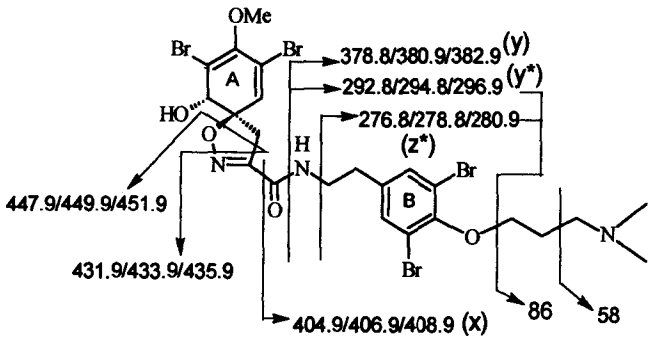
captions for purealidin Q **(1)** and purpuramine I **(11)** used here are same as described in the **Section 2.2** while for the newly identified compounds under this section are numbered from **12** onwards. The fragmentation pattern for **(1)** and **(11)** has been studied previously and are taken as the reference in identifying new brominated molecules.

From the literature survey and previous chemical investigation carried out the same source, described in the previous section **(Section 2.2)** we know that sponge *Psammaplysilla purpurea* is rich source of bromotyrosine alkaloids and chemical modification occurring both in the side chain and the aromatic ring of the brominated tyrosine precursors, give rise to a broad range of biosynthetically related compounds. As mentioned earlier to identify more new bromotyrosine molecules, pseudomolecular ion peaks were subjected to MS/MS. The major fragment ion peaks formed are labeled. The first aromatic ring with isoxazole ring is designated as 'A' and second aromatic ring with ethylamine chain is designated as 'B'. The common fragment ions formed towards the 'B' ring or side chain are represented by x, y and z type of ions while fragment ion observed towards ring 'A' is represented by 'a' type ion. The cleavage between oxime and amide carbonyl is represented as 'x'; between amide carbonyl and amine as 'y'; and between amine and alkyl carbon as 'z' ions. Fragment ions x, y and z with the loss of side chain is represented by an astrick (*). The detailed identification of new molecules based on the fragment ions observed has been discussed under this section.

1) MS/MS at m/z 741.8, 743.8, 745.8, 747.8 and 749.8 of purealidin Q (1):

The set of pseudomolecular ion at m/z 741.8, 743.8, 745.8, 747.8 and 749.8 (1:4:6:4:1) were subjected to CID at collision energy of 40V. The detailed fragmentation pattern has been also discussed in the **Section 2.2.** This is used as the reference for the identification of the other bromo compounds. The MS/MS of each of the pseudomolecular ion peaks were carried out. **Fig 2A** shows the MS/MS at m/z 745.8123 $[M+H+4]^+$. The fragment ions 'x' are observed at m/z 404.9, 406.9 and 408.9. The 'y' type ions were found to be dominant fragment

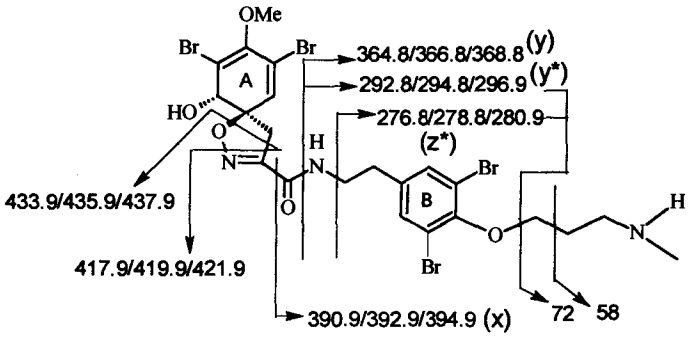
ions at m/z 378.8, 380.9 and 382.9. The loss of the side chain (-86 amu) gave y* fragment ions at m/z 292.9, 294.9 and 296.9. Fragment ion 'z' was not observed instead z* were observed at m/z 276.8, 278.8 and 280.8. These fragment ions are in the ratio 1:2:1 confirming two Br atoms on aromatic ring 'B'. The side chain, dimethyl propyl amine gives three fragment ions at m/z 86, 78 and 58. In addition peaks were also observed at m/z 447.9, 449.9, 451.9 and 431.9, 433.9, 435.9 for cleavage between oxygen and nitrogen of the dihydroisoxazole ring moiety respectively. Schematic fragmentation pattern is illustrated in **Scheme 1**.



Scheme 1: Mass fragmentation of purealidin Q (1).

2) MS/MS at m/z 727.8, 729.8, 731.8, 733.8 and 735.8 of purpurealidin I (12): The CID of another set of cluster ions at m/z 727.8, 729.8, 731.8, 733.8 and 735.8 (1:4:6:4:1) were carried out. The molecule is less by 14 amu than purealidin Q (**1**). The CID spectrum of $[M+H+4]^+$ is shown in **Fig 2B.** On comparing **Fig 2B** with the spectrum **Fig 2A** of (**1**) shows that y* and z* fragment ions identical with purealidin Q (**1**) evidencing two bromine atoms on ring B are intact. The other fragment ions observed were less than 14 amu. Fragment ion 'x' gave peaks at 390.9, 392.9, 394.9 and 'y' at m/z 364.8, 366.8, 368.8. This revealed change in the side chain. The removal of one methyl group from the dimethyl amine chain fits well with fragment ions observed. Absence of peak at m/z 86 and intense peak at m/z 72 also confirms absence of dimethyl amine on side chain but presence of methylamine on the side chain. Proposed structure with the fragmentation pattern is illustrated in **Scheme 2.** Cleavage of oxygen and nitrogen in the dihydroisoxazole ring gives peaks at m/z 433.9, 435.9, 437.9 and 417.9, 419.9,

421.9 respectively. Peaks at m/z 305.9, 307.9 and 309.9 were due to $[b\text{-}58]^+$. This represent a new bromotyrosine alkaloid and was designated as purpurealidin I **(12)**.



Scheme 2: Mass fragmentation of purpurealidin I (12).

3) MS/MS at 633.9, 635.9, 637.9 and 639.9 of purpuramine I (11):

The MS/MS of each of pseudomolecular ion peaks at m/z 633.9, 635.9, 637.9 and 639.9 (1:3:3:1) were carried out. These peaks correspond to purpuramine I **(11)**[39] isolated from the earlier collection. The fragmentation pathway of this compound has been discussed here in the details. The MS/MS spectrum at m/z 635.9 $[M+H+2]^+$ and 637.9 $[M+H+4]^+$ are shown in **Fig 3A** and **3B** which gives all the isotopic peaks. The fragment ions x, y and z* with same values as purpurealidin I **(12)** were observed in the spectrum **Fig 3**. Intense peaks at m/z 72 also confirmed side chain with methylamine. The spectrum showed fragment ion 'a' peaks at m/z 198 and 200 towards the ring A containing only one bromine atom. The fragmentation pattern is illustrated in **Scheme 3.** It can be noted that in case of MS/MS at m/z 635.9 **(Fig 3A)** m/z 198 peak is more intense than peak at m/z 200 and vice versa in case of MS/MS at m/z 637.9 **(Fig 3B)**. This is due to the percentage of two different isotopes of Br atom (Br^{79} and Br^{81}) present in the molecule. The fragment ion 'a' contains only one bromine atom, which can be present either as Br^{79} or Br^{81} in the molecule. The percentage of Br^{79} is more when MS/MS is carried out at m/z 635.9, which increases the intensity of the peak at m/z 198. While during MS/MS at m/z 637.9 the percentage of Br^{81} is more increasing the intensity of the peak at m/z 200. Similar change in the intensity of the isotopic peaks are observed in other fragment ions like x, y, and z*.

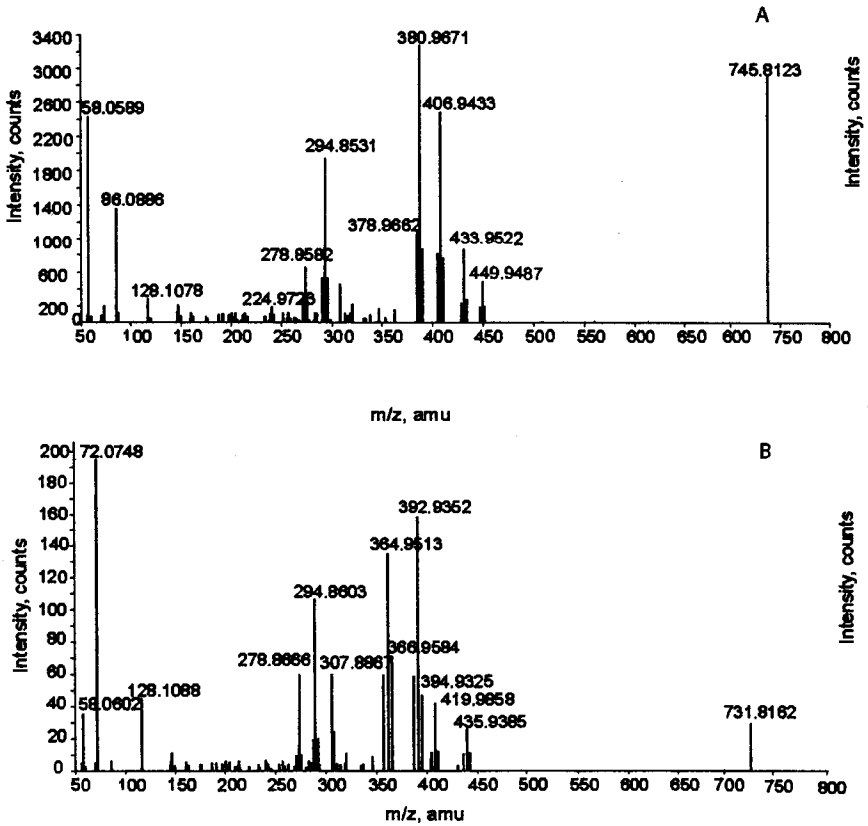


Fig 2: A) MS/MS at m/z 745.8123 of purealidin Q (1)
B) MS/MS at m/z 745.8123 of purpurealidin I (12)

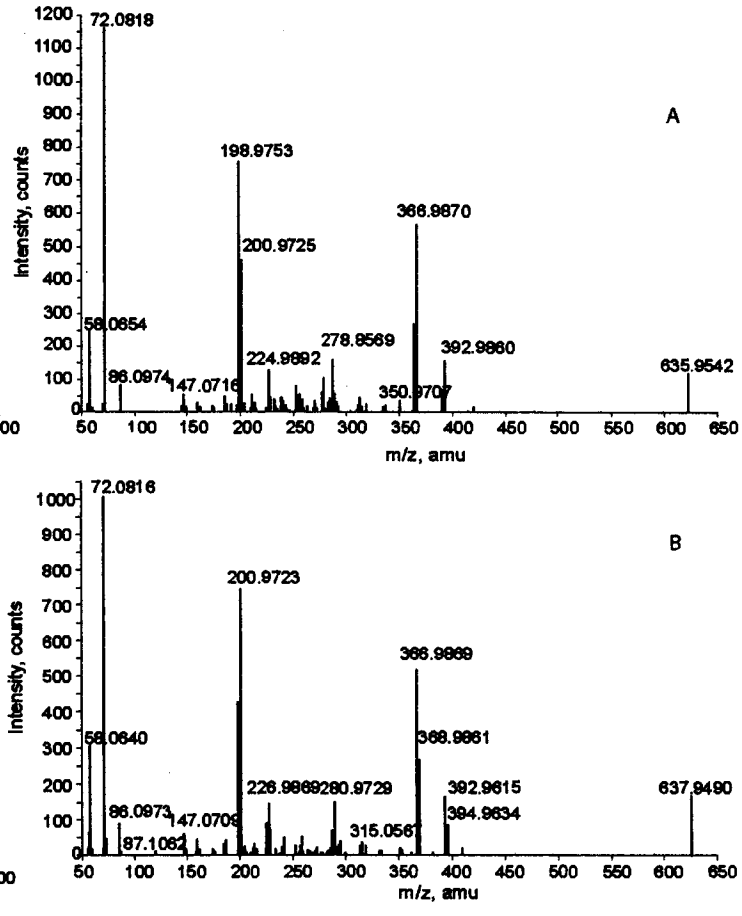
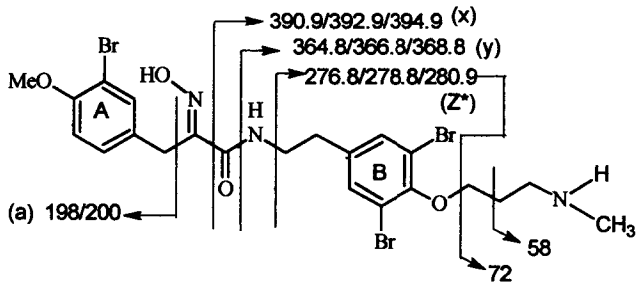


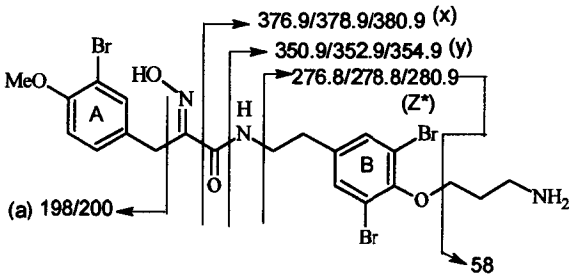
Fig 3: A) MS/MS at m/z 635.8642 of purpuramine I (11)
B) MS/MS at m/z 637.8490 of purpuramine I (11)



Scheme 3: Mass fragmentation of purpuramine I (11).

4) MS/MS at 619.9, 621.9, 623.9 and 625.9 of purpuramine H (13):

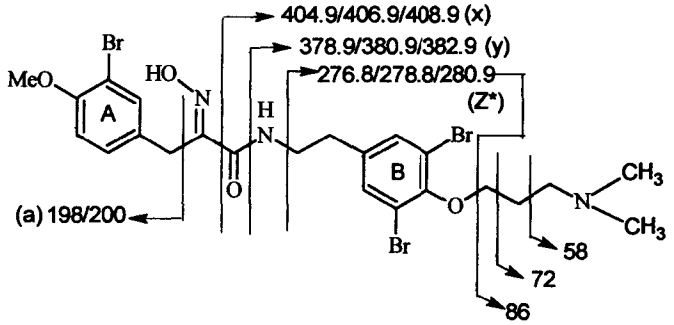
The ESI-MS at m/z 619.9, 621.9, 623.9 and 625.9 in the ratio 1:3:3:1 indicates three bromine atoms in the molecules. The MS/MS of each isotopic molecular ion peaks were carried out. The MS/MS spectrum at m/z 621.9 and 623.9 are shown in **Fig 4A** and **4B** respectively. Intense fragment ion at 58 indicates absence of methyl groups amine group on the side chain. The fragment ion at m/z 606.9 in the case **Fig 4B** for loss of NH_3 also revealed the presence of primary amine. Fragment ions 'x' at m/z 198, 200 and z* at m/z at 276.8, 278.8, 280.8 were identical with purpuramine I **(11)** revealing that ring A and ring B are with one and two bromine atoms respectively. The fragment ions x and y showed peaks at m/z 376.9, 378.9, 380.9 and 350.9, 352.9, 354.9 respectively. The decrease by 14 amu confirms presence of free amine in the side chain. The structure was proposed according to the fragment ions formed and was found to be identical to already known compound purpuramine H.[39] The schematic fragmentation pattern is illustrated in **Scheme 4.**



Scheme 4: Mass fragmentation of purpuramine H (13).

5) MS/MS at 647.9, 649.9, 651.9 and 653.9 of aplysamine 2 (14):

The CID at m/z 647.9, 649.9, 651.9 and 653.9, 14 amu greater than purpuramine I **(11)** was carried out. The MS/MS spectrum at m/z 649.9 $[M+H+2]^+$ and 651.9 $[M+H+4]^+$ are shown in **Fig 5A & B** respectively. The fragment ions x, y and z* were identical to purealidin Q **(1)** suggesting identical part structure. Peaks at m/z 86, 72 and 58 showed presence of dimethyl amine. The fragment ion 'a' at m/z 198, 200 was similar to that of purpuramine H **(13)**. By arranging the fragments, the structure **(14)** was proposed which was identical to aplysamine 2.[41] The schematic fragmentation pattern is shown in **Scheme 5**.



Scheme 5: Mass fragmentation of aplysamine 2 (14).

6) MS/MS at 697.79, 699.79, 701.79, 702.79 and 703.79 of aplysamine 3 (15):

The five peaks at m/z 697.79, 699.79, 701.79, 702.79 and 703.79 in the ratio 1:4:6:4:1 is for the presence of four bromine atoms. The CID of each of the isotopic peak was taken. **Fig 6** shows MS/MS at m/z 701.9 $[M+H+4]^+$. Fragment ions x, y, z* were identical with those observed in purpuramine H **(13)**. The absence of fragment at m/z 198 and 200 indicated change in the aromatic ring A. If the two bromines are placed on the ring A, it fits the molecular formula $C_{21}H_{23}O_4N_3Br_4$. Based on the fragmentation pattern the structure was identified aplysamine 3[41] **(15)**. **Scheme 6** illustrates all the fragment ions formed in the molecule.

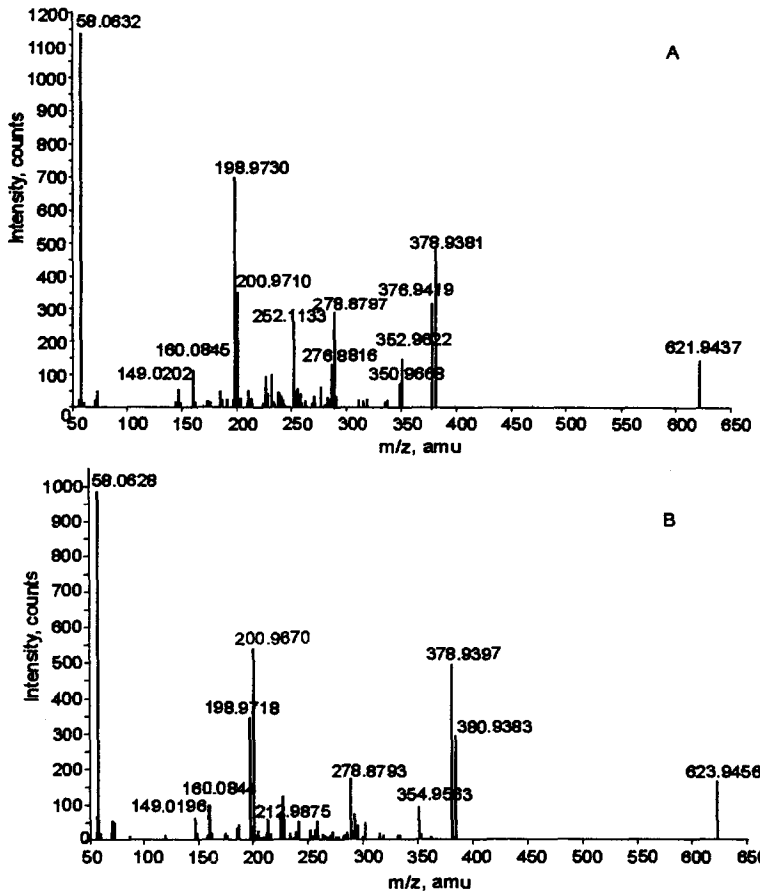


Fig 4: A) MS/MS at m/z 621.9437 of purpuramine H (13)
B) MS/MS at m/z 623.9456 of purpuramine H (13)

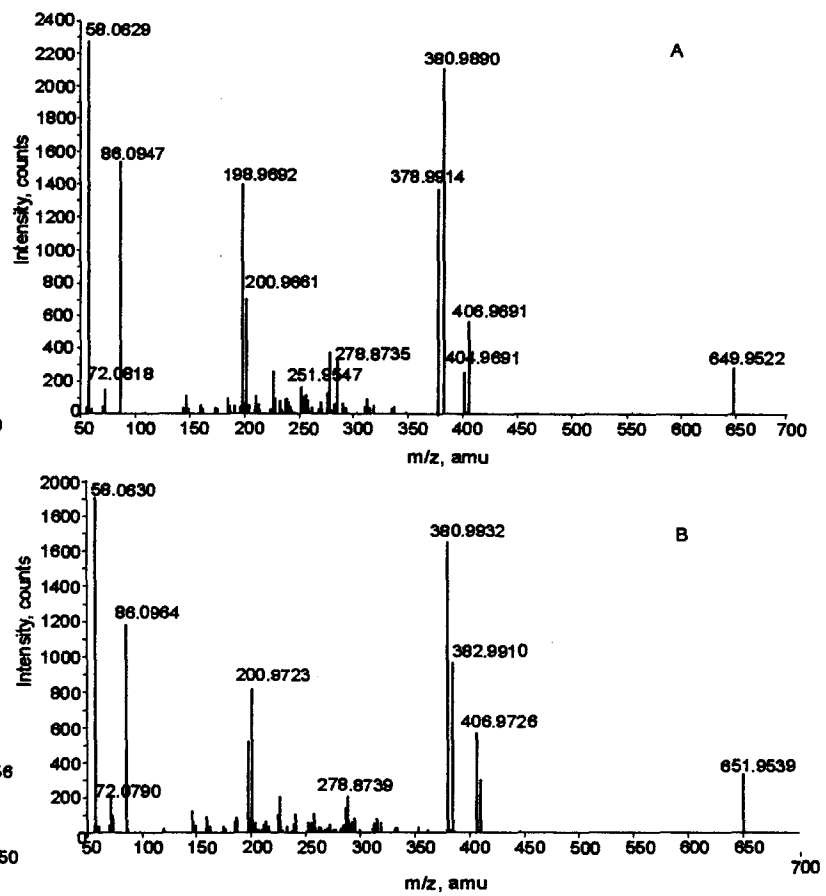
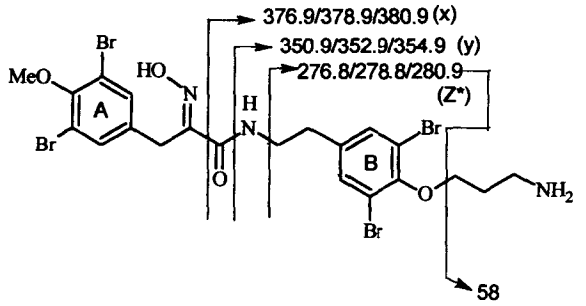


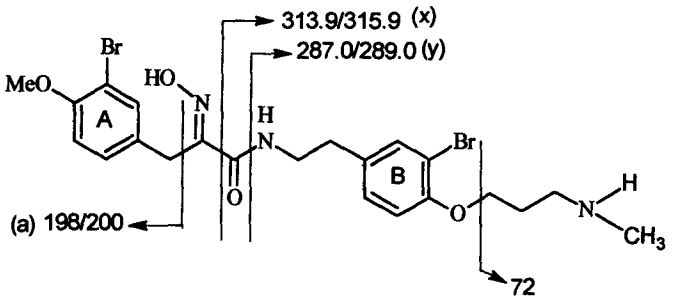
Fig 5: A) MS/MS at m/z 649.9522 of aplysamine 2 (14)
B) MS/MS at m/z 651.9539 of aplysamine 2 (14)



Scheme 6: Mass Fragmentation of aplysamine 3 (15).

7) MS/MS at 555.96, 557.99 and 559.99 of purpurealidin J (16):

The MS/MS of peaks at m/z 555.96, 557.99 and 559.99 (1:2:1) indicated presence of only two bromine atoms in the molecule. The MS/MS spectrum at m/z 557.9 $[M+H+2]^+$ **(Fig 7)** shows fragment ion 'a' at m/z 198 and 200 for ring A with one bromine atom. The fragment ions 'x' and 'y' showed peaks at 313.9, 315.9 and 287, 289 respectively. The ratio is approximately 1:1 suggesting only one bromine atom on ring B. Thus, structure assigned was similar to purpuramine I **(11)** with one less bromine on C16. The intense peak at m/z 72 also confirmed side chain with methylamine. The proposed structure with schematic fragmentation is given in **Scheme 7.** The compound is designated as purpurealidin J **(16).**

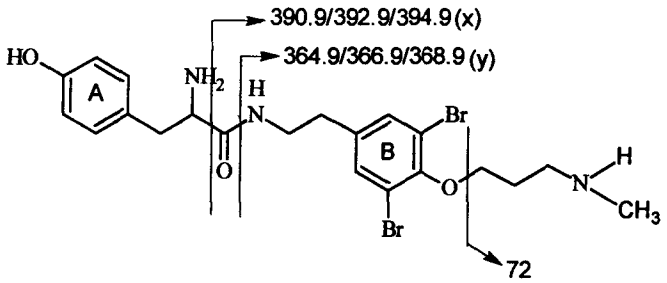


Scheme 7: Mass Fragmentation of purpurealidin J (16).

8) MS/MS at 527.94, 529.94 and 531.94 of purpurealidin K (17):

The fragment ions formed during the MS/MS of pseudomolecular molecular ion peaks at m/z 529.94, 531.94 and 533.94 (1:2:1) were totally different from rest of all. **Fig 8** shows MS/MS spectrum at m/z 529.9 $[M+H+2]^+$. Fragment ion peaks

'x' and 'y' were observed at m/z 390.9, 392.9, 394.9 and m/z 364.9, 366.9, 368.9 respectively as that of purpuramine I **(11)** revealing identical part structure. Two bromines atoms were already accounted. Peak at m/z 513.9 was observed for loss of NH_3 accounting for free amine group. Thus, oxime group could be modified to amine group. Putting tyrosine as part structure with part structure of purpuramine I **(11)**, new structure of purpurealidin K **(17)** was proposed. The fragmentation pattern is explained in **Scheme 8.**



Scheme 8: Mass Fragmentation of purpurealidin K (17).

Besides the five known compounds, purealidin Q **(1)**, purpuramine H **(13)**, purpuramine I **(11)**, aplysamine 2 **(14)**, aplysamine 3 **(15)**, three new bromotyrosine alkaloids, purpurealidin I **(12)**, J **(16)** and K **(17)** were identified based on the fragmentation pattern in the repeat collection. We demonstrated here that the detailed fragmentation patterns obtained from ESI-MS/MS studies are valuable for structural elucidation of other related bromotyrosine derivatives. Several characteristic fragment ions are observed to be associated with changes on certain functional groups in the bromotyrosine. Thus, ESI-MS/MS provides an effective means to characterize these compounds and their derivative. Such methods ideally are fast and simple, keeping the number of steps in the analytical process at a minimum. This also helped in determining the structure of the compounds using minute concentrations of analytes in complex biologically relevant samples.

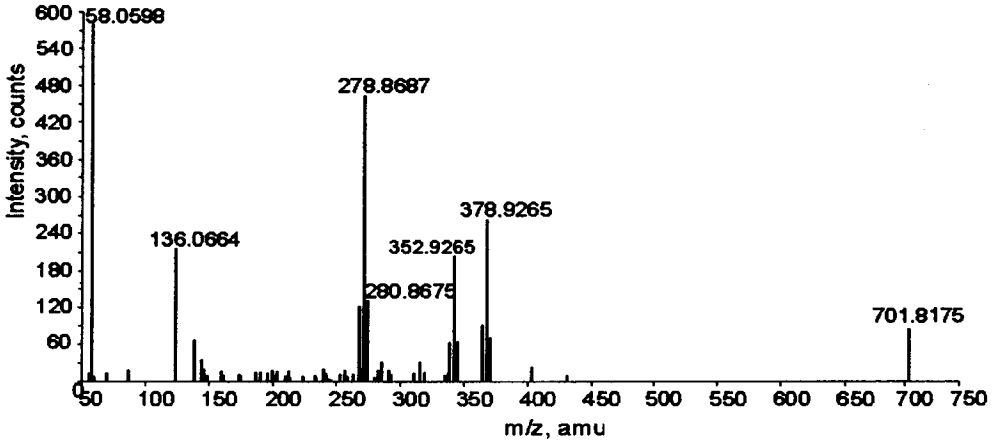


Fig 6: MS/MS at m/z 701.8175 of aplysamine 3 (15).

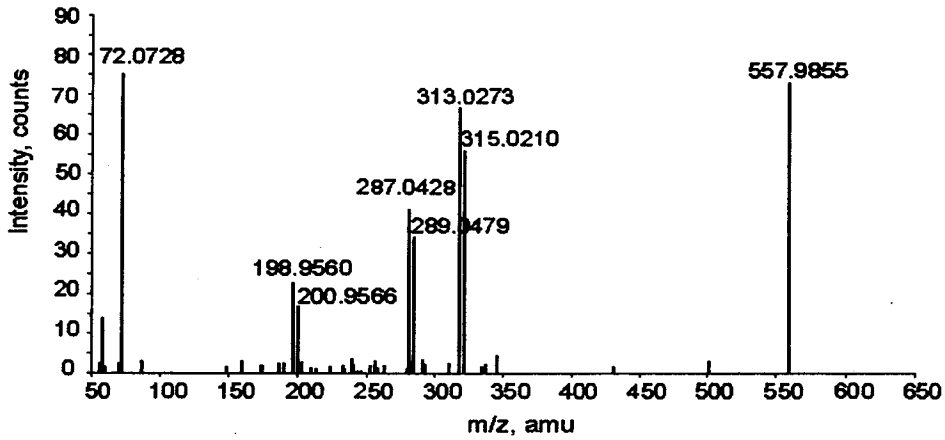


Fig 7: MS/MS at m/z 557.9855 of purpurealidin J (16).

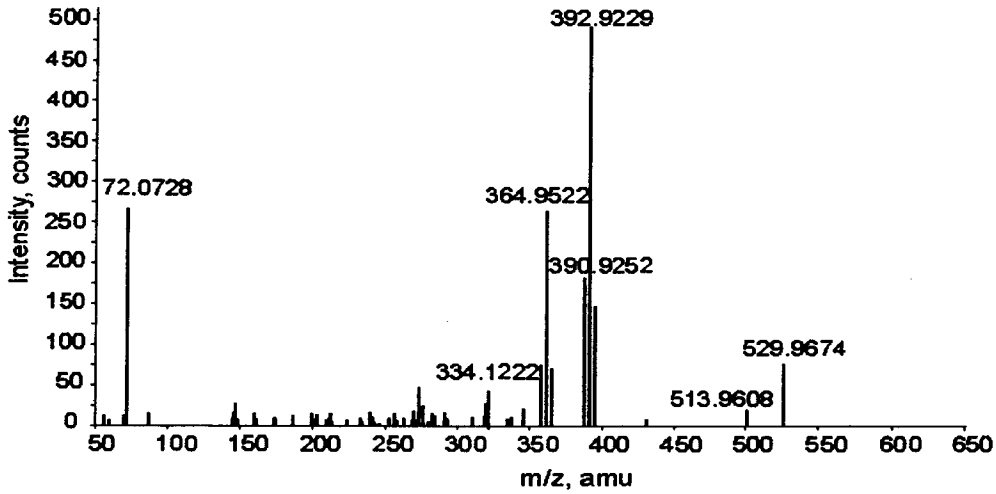
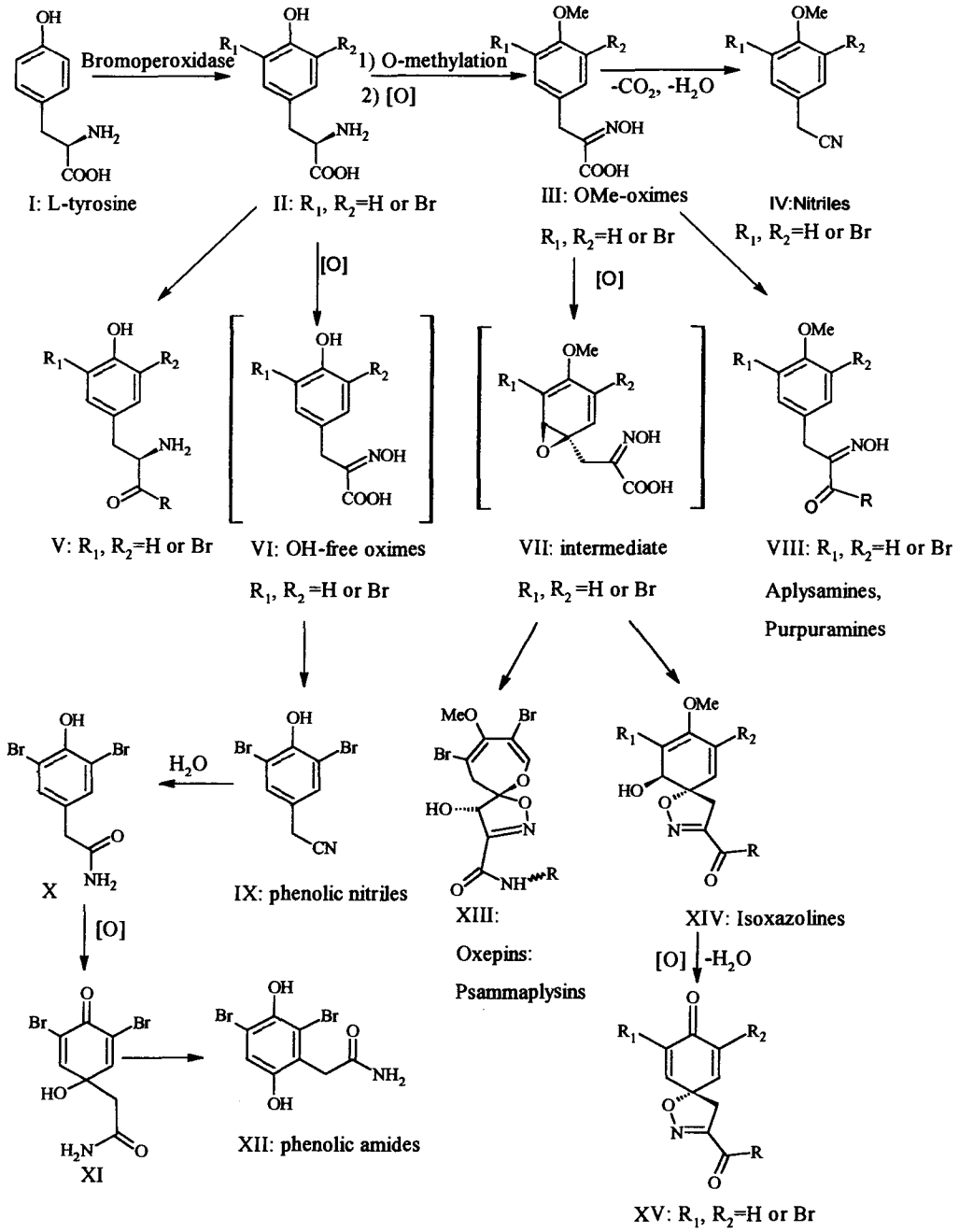


Fig 8: MS/MS at m/z 529.9674 of purpurealidin K (17).

Biogenetic pathway:

Biosynthetic pathway has been proposed for bromotyrosine alkaloids. The earlier studies on biosynthesis showed that the metabolic pathway employed by Verongida sponges **(Scheme 9)** involves a bromination of the tyrosine **(I)** using bromoperoxidase enzymes as the first step to form brominated tyrosine **(II)**.[85-86] Brominated tyrosine **(II)** can directly form **(V)** e.g purpurealidin K or can follow further two different pathways: A) oxidation of the amine to an oxime **(VI)** or B) O-methylation of the tyrosine followed by oxidation of the amine to an oxime **(III)** which can form compounds like purpuramines and aplysamines **(VIII)**. The first pathway (A) can give phenolic nitriles **(IX)** and amides **(XII)**. The second pathway (B) can follow two different biosynthetic routes: (C) dehydration and decarboxylation to give the O-methylated nitriles **(IV)**, (D) direct epoxide formation **(VII)** which leads to isoxazoline rings **(XIII)** giving metabolites like aerothionin, homoaerothionin, purealdin Q, purpurealidin A, J *etc* or oxepins **(XIII)** giving the psammaplysins. Isoxazoline ring **(XIV)** after oxidation followed by dehydration can lead to **(XV)**, molecules like purpurealidin B.



Scheme 8: Proposed biogenetic pathway for bromotyrosine alkaloids.

Experimental section:

Animal material:

The animals were collected by scuba diving at a depth of 8-10m from Mandapam, Tamil Nadu, India, and identified by Dr. P. A. Thomas of Vizhingam Research Center of Central Marine Fisheries Research Institute, Kerala, India. A voucher specimen (NIO759) is deposited at the National Institute of Oceanography, Dona Paula, Goa, India.

Extraction and fractionation:

A small piece of freeze dried sponge was finely ground in mortar and pastle and extracted three times with MeOH:$CHCl_3$ (1:1) using sonicator. The extract was filtered and concentrated under vacuo to obtain crude extract. The crude extract was further partitioned with ethyl acetate, which yielded 0.35gm of ethyl acetate fraction and this was used for MS analysis.

ESI-QTOF MS/MS spectrometry: The mass spectrometer used was a QTOF-XL MS/MS Applied Biosystem instrument (Canada) equipped with Analyst software application. The instrument was operated in positive ionization mode. The sample dissolved in MeOH:$CHCl_3$ (99.5:0.5) containing traces of 0.1% TFA was directly infused at a constant flow rate of 10μL/min into the ion spray source using integrated syringe pump. The ESI-MS/MS setting is given in **Table 8**. The MS/MS products were produced by collision-induced dissociation (CID) of selected precursor ions at collision energy between 25-40V and mass analyzed using TOF analyzer of the instrument.

Table 8: ESI-MS/MS settings

Parameters	Settings
Duration	
Ion Source	Ion spray
Ion source Gas 1 (GS1)	20 psi
Ion Source Gas 2 (GS2)	0
Curtain Gas (CUR) N_2	20 psi
Ion Spray Voltage (IS)	5500 V
Declustering potential (DP)	60 V
Focusing Potential (FP)	300 V
Declustering potential 2(DP2)	15 V
Collision Energy (CE)	25-40 V
Collision Gas (CAD)	3 psi
Ion Release Delay (IRD)	6 psi
Q1 Resolution: Unit Resolution	
Ion Energy (IEI)	1.5 V
DC Quad Lens Horizontal Focus (GR)	7.2
DC Quad Lens Vertical Focus (TFO)	12.2
DC Quad Lens Steering (TST)	0.7
MCP (CEM)	2250 V

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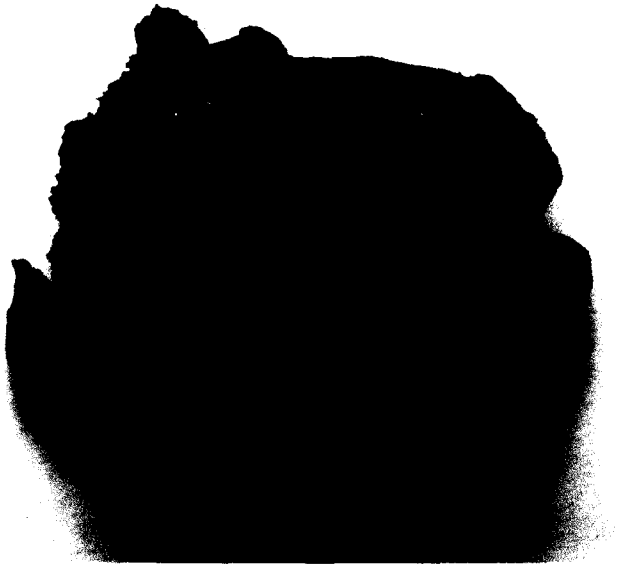
Section 3



Chemical investigation of marine sponge Haliclona cribricutis

3.1: Genus *Haliclona*-Review

Sponges belonging to Sub class Ceratinomorpha, Order Haplosclerida, Family Haliclonidae and Genus *Haliclona* (Dendy, 1922, also classified by taxonomist as *Reniera*) are well known to contain chemically diverse secondary metabolites with interesting biological activities, *e.g.,* antifungal and cytotoxic activity.[1] Here mention may be made of anti-tumor isoquinoline alkaloids, renieramycins, reported from various blue sponges of genus *Haliclona;* saframycins from actinomycetes and ecteinascidin from tunicates.

I had an opportunity to work with the sponge *Haliclona cribricutis* from Mandapam. Chemical investigation of this benthic organism led to the detection of ceramides. But before going into the details of chemical studies of present investigation a brief review of the metabolites reported from genus *Haliclona* has been presented.

Manzamine A **(1)**, an antitumor alkaloid from an unidentified *Haliclona sp.* shows significant inhibition of P388 mouse leukemia with IC_{50} 0.07 μg/ml. Manzamine A is found to have broad spectrum of activity against human tumour cell lines. Increased interest in this molecule led to its assessment as an effective anti-malarial, an anti-inflammatory agent, an insecticide and a fungicide. It is also bactericidal inhibiting *Mycobacterium tuberculosis.*[2a-b]

Haliclona sp. collected from Thailand furnished a novel triterpenoidal pentacyclic sulfated hydroquinone, phuklona sulfate **(2)**.[3] South African *H. tulearensis,* yielded cytotoxic alkaloid, halitulin **(3)**.[4] Adociasulfates 1-6 **(4-9)** were obtained as inhibitors of kinesin motor proteins from a Palauan *Haliclona* (aka *Adocia*).[5] A presumably new species of *Haliclona* from Queensland contained four unsaturated aminoalcohols **(10-13)** with antifungal properties.[6] The absolute stereochemistries of the salicylihalamides A **(14)** and B **(15)** from *Haliclona sp.*[7] have been revised following a re-interpretation of Mosher ester derivatives[8] and enantioselective syntheses of both enantiomers of each.[9-10] A Vanuatuan,

Haliclona sp. was found to contain the *in vitro* antitumour macrolide haliclamide **(16)**.[11] The structure **(17)** assigned to (-)-Haliclorensin, from *H. tulearensis*,[12] was found to be spectroscopically non-identical with the natural product on the basis of its synthesis by two independent groups.[13,14]

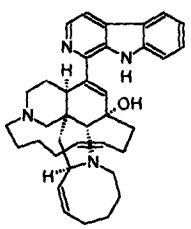
The *Reniera sp.* from Palau contained two potent but unstable antimicrobial alkaloids, renieramycins E **(18)** and F **(19)**.[15] Philippines *Haliclona sp.* contained 1-hydroxymethyl-7-methoxyisoquinolin-6-ol **(20)**.[16] The structure of renieramycin H, previously described from *H. cribricutis* as **(21)**[17] has been revised to **(22)** on the basis of spectral comparison to synthetic model compounds.[18] Subsequently, structure **(22)** was found to be identical to cribrostatin 4 on the basis of X-ray analysis.[19] Hence, the trivial name renieramycin H must be retained. The symbiotic sponge *H. cymaeformis* and the associated red alga *Ceratodictyon spongiosum* from Philippines were found to contain *p*-sulfooxyphenylpyruvic acid **(23)** and its phenol congener **(24)**.[20]

Three chlorinated polyacetylenes **(25-27)** were identified from the Californian *H. lunisimilis*.[21] Halitulin **(28)** from a South African, *H. tulearensis*,[23] has been synthesised, establishing the stereochemistry at C-15 as (S).[24] Many of the polyacetylenic compounds from marine sponges exhibits cytotoxicity. A *Haliclona sp.* from Palau yielded three relatively simple acetylenic metabolites **(29-31)**.[25] (+)-Papuamine, the antipode of the natural product **(32)** from *Haliclona sp.*[26] has been synthesized by a stereoselective route.[27] In an accompanying paper, the syntheses of both (-)-papuamine **(32)** and (-)-haliclonadiamine **(33)**, with a common origin from *Haliclona sp.*[28] are also reported.[29]

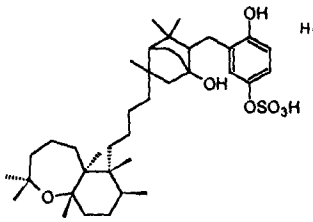
Haliclonacyclamines A **(34)** and B **(35)** were reported from Great Barrier Reef *Haliclona sp.* and the structure of **(34)** was determined by X-ray analysis.[30] The macrolides salicylhalamides A **(36)** and B **(37)**, isolated from a Western Australian *Haliclona sp.*, are considered to be members of a potentially important new class of antitumour agents.[31] The synthesis of an alkaloid haliclonadiamine **(38)**, from Palauan *Haliclona sp.*[32] featured a diastereoselective and

enantioselective hydrogenation of a racemic β-keto ester.[33] The anticholinesterase activity of 3-alkylpyridinium polymers **(39)** from *Reniera* (=*Haliclona*) *sarai* from the Adriatic Sea was associated with two fractions of molecular weight 5520 and 18900 Da.[34] The total synthesis of haliclamine A **(40)**, from *Haliclona sp.*,[35] was achieved *via* stepwise inter- and intramolecular *N*-alkylations of 3-alkylpyridine intermediates.[36] From a Malaysian *Haliclona sp.* Haliclostanone sulfate **(41)**, an unusual polyhydroxylated sterol sulfate was isolated.[37]

Hirsch and Kashman report halicerebroside A **(42)**[38] from *Haliclona* of Red Sea. *Haliclona sp.* from Gujarat coast in India furnished a known ceramide[39] **(43).** An antifouling ceramide **(44)** against the macroalgae was isolated from *H. koremella* collected in Palau.[40] Chemical investigation of the Arctic sponge *H. viscosa* led to the isolation of a new trimeric 3-alkyl pyridinium alkaloid (viscosamine) **(45).**[41]



(1)



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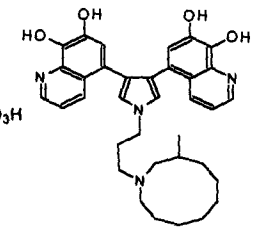

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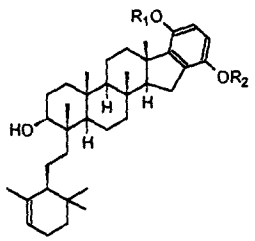
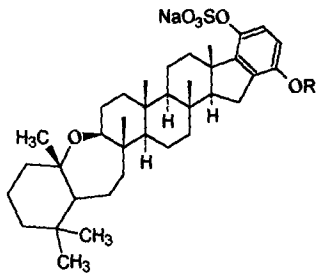
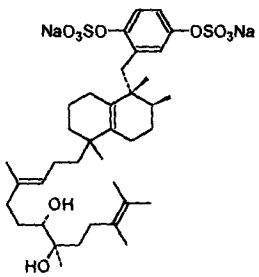


(4) R_1=R_2=SO_3Na
(8) R_1=SO_3Na, R_2=H



(5) R=SO_3Na; (9) R=H



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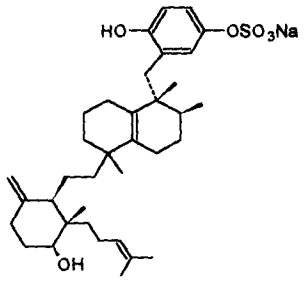

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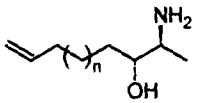
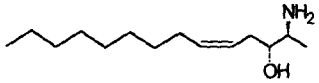


(10) n=7
(11) n=5



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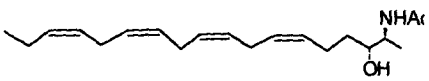

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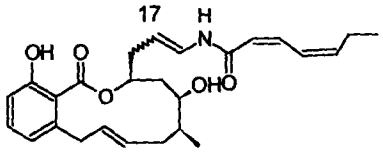
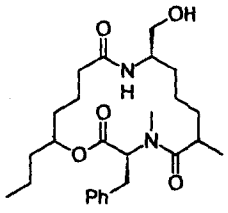


(14) (17E)
(15) (17Z)



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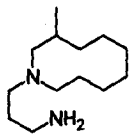

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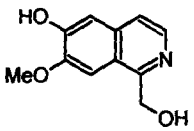

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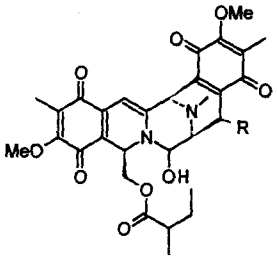
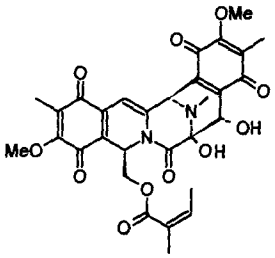


(18) R=OCH_3
(19) R=H



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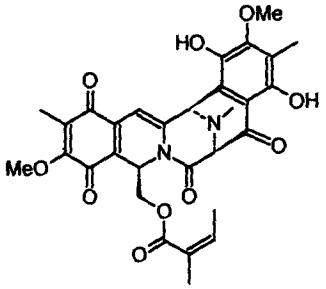

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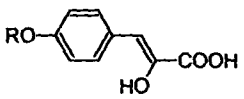
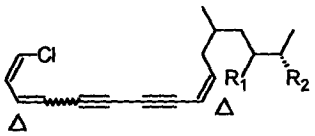
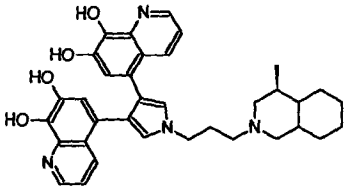


(23) R=SO_3H
(24) R=H



(25) R_1=OH, R_2=H, (3Z), Δ saturated
(26) R_1=H, R_2=OAc, (3E)
(27) R_1=OAc, R_2=H, (3E), Δ saturated



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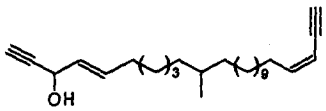

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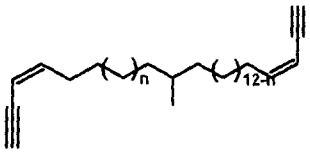

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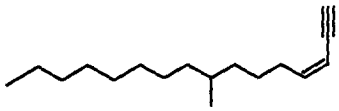

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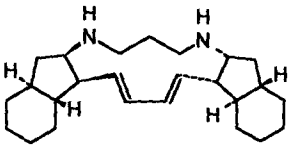

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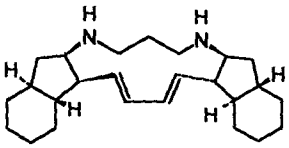

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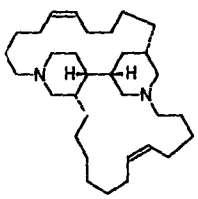

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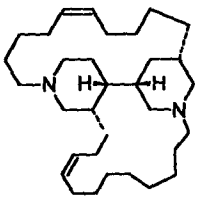

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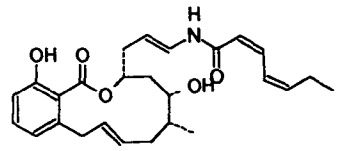

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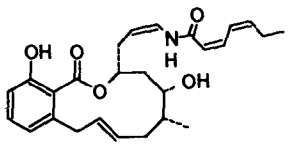

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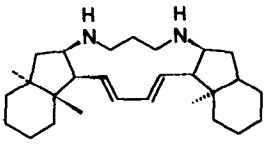

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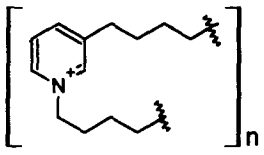

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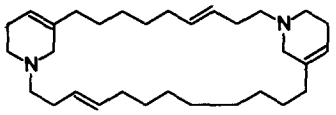

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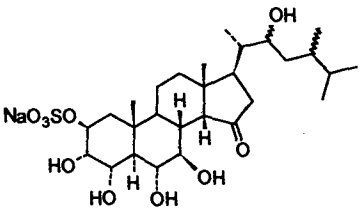

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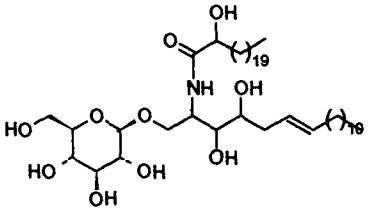

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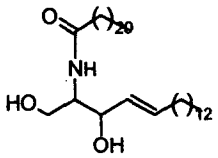

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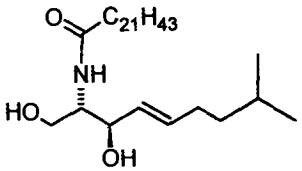

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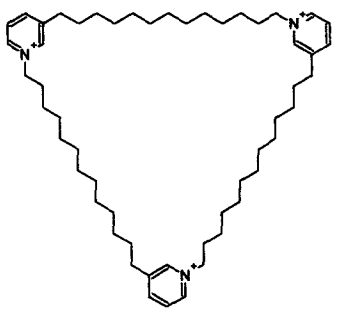


(45)

In addition to these metabolites, *Haliclona sp.* contains peptides like halipeptin A-C, haliclonamide A-E and waiakeamide, which have been discussed in **Chapter 1.**

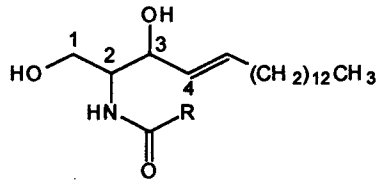
Identification and characterization of eight molecular species of ceramide from the sponge *Haliclona cribricutis* using tandem mass spectrometry (ESI-MS/MS) has been discussed in the **Section 3.2.**

3.2: Tandem mass spectrometry (ESI-MS/MS) approach for the identification of eight molecular species of ceramide from *Haliclona cribricutis.*

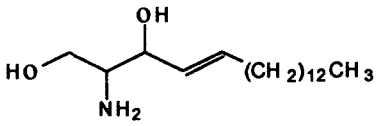
As described in **Section 1** screening of the *Haliclona cribricutis* showed it to be antiviral, bactericidal and fungicidal and was therefore taken up for further investigations leading to the isolation and identification of ceramides from this source.

In the current study use of electrospray tandem mass spectrometry (ESI-MS/MS) has been made for the analysis of eight molecular species of ceramide from the lipid fraction of marine sponge *Haliclona cribricutis* without chromatographic purification. The eight molecular species identified are N-eicosanoic (20:0), N-hencicosanoic (21:0), N-docosanoic (22:0), N-tricosanoic (23:0), N-tetrasanoic (24:0), N-pentasanoic (25:0), N-hexasanoic (26:0) and N-heptasanoic (27:0) sphingosine. Of these N-hencicosanoyl (N21:0) to N-hexasanoyl (N26:0) Octadecasphing-4 (E)-enine are being reported for the first time from this source.

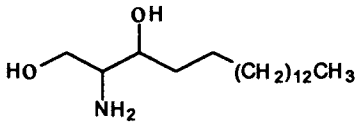
Ceramide is known as an important precursor **(Fig 1)** common to the biosynthesis of glucosphingolipids (cerebrosides, gangliosides, sulfatides and globosides) and sphingomyelin[42] and are emerging as important messengers for various cellular processes including cell cycle arrest, differentiation, apoptosis, senescence, and immune responses.[43-49] Chemically ceramides are *N*-acylated sphingoids **(I)**, consisting of a long-chain aliphatic amino alcohol referred to as long-chain base (LCB), which are covalently linked to a fatty acyl chain via an amide linkage. Usually, the major LCB is a sphingosine (sphing-4-enine) **(II)** with smaller amounts of dihydrosphingosine (sphinganine) **(III)**; sometimes phytosphingosine (4-hydroxysphinganine) **(IV)** may also be present. The fatty acids of naturally occurring ceramides range in chain length from C16 to C26, and may contain one or more double bonds/or hydroxyl substituents at C-2.



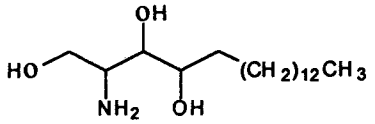
(I) Ceramide(N-acyl-sphing-4-enine)



(II) Sphingosine (sphing-4-enine) (d18:1)



(III) dihydrosphingosine(sphinganine) (d18:0)



(IV) Phytosphingosine (4-hydroxysphinganine) (t18:0)

Sphingolipids are present in all eukaryotic organisms, however depending on their source they differ in their sphingoid base composition; while in the animal kingdom the major is sphingosine and the minor is dihydrosphingosine. Phytosphingosine is the major sphingosine base of the plant kingdom including fungus.[50] The head groups define the various sphingolipid classes, with a hydroxyl group found in ceramides, phosphorylcholine in sphingomyelin (SM) and glycoside with simple (e.g. Glucosylceramide) to complex carbohydrates moieties in the various glycosphingolipids. The metabolic and synthetic pathway for ceramide is given in the **Fig 1.**[51] It shows that intracellular ceramide can be formed either by *de novo* synthesis, or through the SMase-dependent catabolism of SM, in various separate cellular compartments. Increased ceramide leading to cytotoxicty can come from *de novo* synthesis due to stimulation of serine palmitoyltransferase and/or dihydroceramide synthase, or by degradation of sphingomyelins via spingomyelinases. Metabolism of ceramide by glycosylation or acylation, appear to 'shunt' ceramide into less toxic forms, as does catabolism via ceramidase. Phosphorylation of sphingosine derived from ceramide stimulates pro-life metabolic pathways and acts to oppose certain cytotoxic actions of ceramides. Several anticancer agents, including the cytotoxic retinoid, fenretinide (4-HPR), have been shown to act, at least in part, by increasing tumor cell ceramide via *de novo* synthesis. Phase I trials of ceramide metabolism inhibitors in combination with 4-HPR and with other cytotoxic agents are in development. This offers a novel approach to cancer chemotherapy.[51]

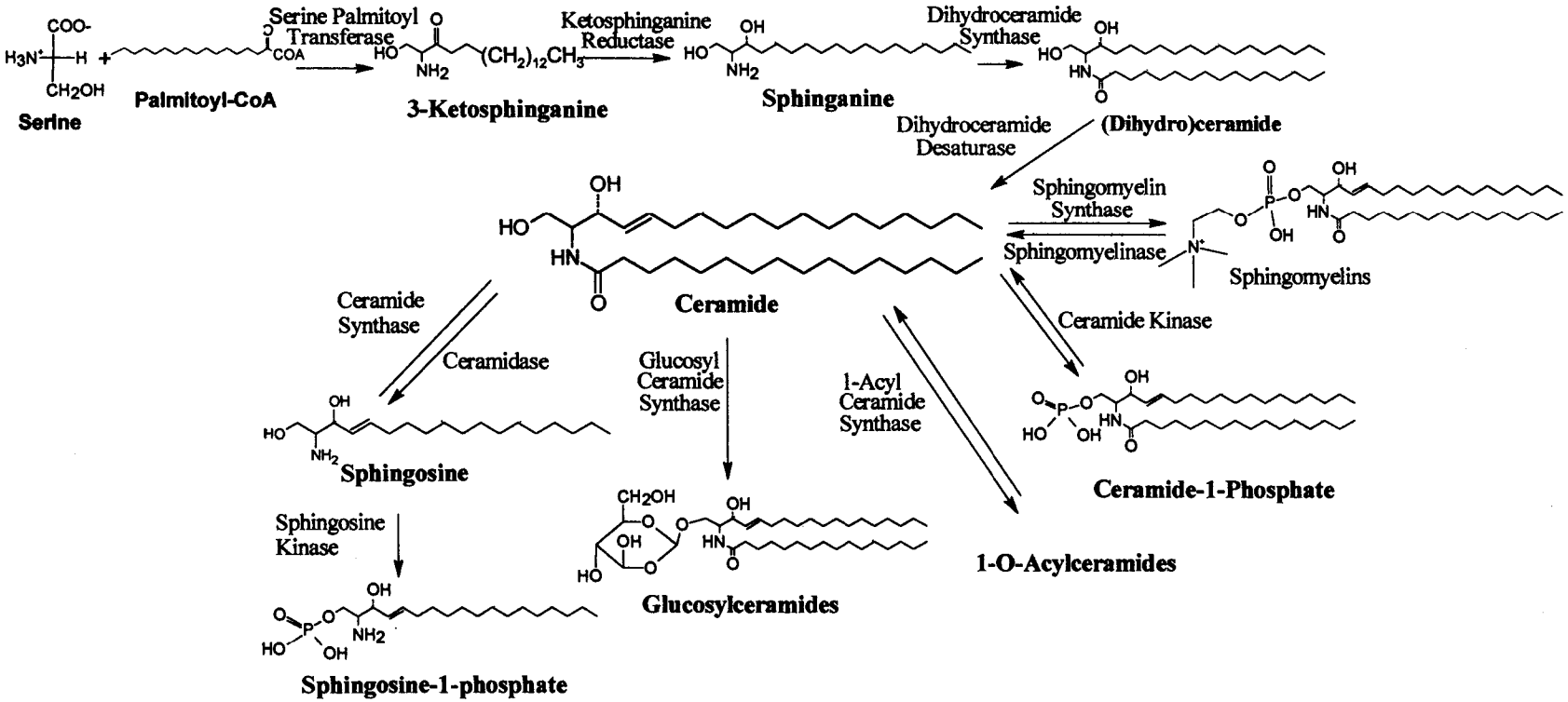


Fig 1: Major synthetic and metabolic pathway for ceramide.

In a review by Norman Radin, eight approaches have been described of killing tumors by ceramide-induced apoptosis. To aid this approaches, the article catalogs many drugs that can act on the different aspects of ceramide metabolism.[52]

Over the decades sphingolipids were first proposed to act as second messengers in intracellular signaling pathways. However, ceramide has now taken center stage and appears to fulfill many of the criteria to be defined as a true secondary messenger, in as much as transient changes in ceramide levels are detected in response to a variety of extracellular signals in a variety of physiological situations.[53]

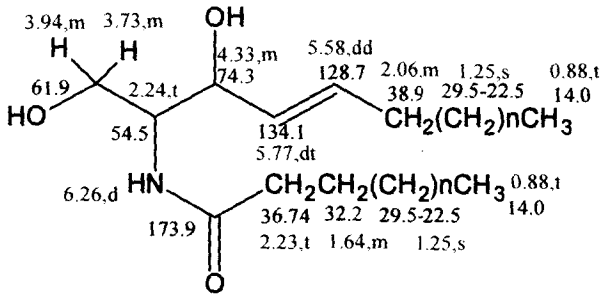
Ceramide-1-phosphate (Cer-1-P), which is formed by the action of ceramide kinase, is emerging as a novel bioactive sphingolipid. Recent work has demonstrated that Cer-1-P is a novel regulator of cell activation.[54] There is increasing evidence suggesting that Cer-1-P is implicated in the regulation of vital cellular processes, such as cell proliferation,[55-56] apoptosis,[57] phagocytosis,[58] and inflammation.[59]

The beneficial effect of topical application of ceramides on skin and hair appearance and performance has led to their introduction into premium high-class products referred to as "cosmeceuticals". An increasing number of products containing ceramides or ceramides analogs have come into the market.[60] Thus, because of their multiple physiological functions they have gained considerable attention.[61-64] With this brief introduction on ceramides, the work carried out on *Haliclona cribricutis*, is now being presented here.

The $CHCl_3$ soluble fraction (2gm) of sponge *H. cribricutis* was chromatographed on a silica gel column using gradient elution of ethyl acetate in petroleum ether. The fractions with similar TLC profile (R_f 0.172) in ethyl acetate: petroleum ether (35:65) were further subjected to gel chromatography using Sephadex LH-20 in MeOH:$CHCl_3$ (1:1) as mobile phase. This yielded a fraction **(1)**, homogenous on TLC. IR and NMR spectra were also indicative of purity of **(1)**.

The amorphous solid displayed a broad IR **(Fig 2)** absorption band at 3100-3500 cm^{-1} for NH and OH groups. The intense peaks at 2918.1 and 2850.6 cm^{-1} indicated long aliphatic chain. Presence of the bands at 1630.0, 1618.2 (Amide I) and 1467.7, 1550.7 (Amide II) were suggestive of amide group.

The ^{1}HNMR spectrum **(Fig 3, Table 1)** showed signals at δ_H 6.26 (1H, d, J=7.2 Hz) for secondary amide proton. The signals at δ_H 5.77 (1H, dt, J=15.4, 6.4Hz, H-4) and 5.58 (1H, dd, J=15.4, 6.7Hz, H-5) were assigned to disubstituted double bond. Multiplets at δ_H 4.33 (1H, m, H-3), 3.94 (2H, m, 1-H, H_A-1) and 3.73(1H, m, H_B-1) were suggestive of methines and methylene protons bearing hydroxyl group respectively. A signal at δ_H 2.24 (2H, t, J=7.1Hz, H-2) is attributed to the methine proton bearing amide group and δ_H 2.06 (2H, m, H-6) for methylene proton next to the double bond. The above data shows presence of the system – CO-NH-CH(CH_2OH)-CH(OH)-CH=CH-$(CH_2)_n$-CH_2-. ^{1}HNMR spectrum also displayed signals at δ_H 2.23 (2H, t, J=7.6Hz) for –CO-CH_2-CH_2-, 1.64 (2H, m) for CO-CH_2-CH_2-CH_2- and 1.25 (br s) for several methylene protons of a long chain fatty alkyl moiety. Methyl protons of two terminal ethyl groups gave triplet at δ_H 0.88 (6H, t, J=6.85Hz).



(1)

The ^{13}CNMR **(Fig 4)** spectrum showed a quarternary carbon signal at δ_C 173.9 for the presence of a carbonyl group. The doublets at δ_C 134.1 and 128.7 were assigned to two olefinic carbon atoms. The signals at δ_C 61.9 (t), 54.5 (d) and 74.3(d) confirmed the presence of the system -CH(CH_2OH)-CH(OH). The

aliphatic chain was confirmed by triplets ranging from δ_C 22.6-32.2 and terminal methyl at δ_C 14.0(q). The IR and NMR data was indicative of the molecule belonging to the class of ceramides and also well in agreement with reported values.[65]

Table 1: ^{1}H and ^{13}CNMR values of compound 1 in $CDCl_3$

Carbon No.	^{1}HNMR δ_H, ppm	^{13}CNMR δ_C, ppm
1	3.94(2H, m, 1-H, H_A-1) 3.73(1H, m, H_B-1)	61.9 (t)
2	2.24 (2H, t, J=7.1Hz)	54.5 (d)
3	4.33 (1H, m)	74.3 (d)
4	5.77(1H, dt, J=15.4, 6.4Hz)	134.1(d)
5	5.58 (1H, dd, J=15.4, 6.7Hz)	128.7(d)
6	2.06 (2H, m)	
1'		173.9 (s)
2'	2.23 (2H, t, J = 7.6 Hz)	
3'	1.64 (2H, m)	
$(CH_2)_n$	1.25 (br s)	22.6-32.2 (t)
$2xCH_3$	0.88 (6H, t, J = 6.85 Hz)	14.0 (q)
NH	6.26 (1H, d, J=7.2 Hz)	

Though the TLC, IR and NMR indicated the compound to be pure its ESI-MS/MS was suggestive of it being a mixture of ceramides. Thus the mixture contained minimum of eight similar molecules which were identified based on tandem MS/MS of each pseudomolecular ion $[M+H]^+$.

This second messengers requires sensitive and specific analytical method to detect individual ceramide species and to differentiate between them. Different analytical methods have been used for the separation and differentiating the individual ceramide molecular species. These included GC-MS,[66] FAB-MS[65, 67-69] and more recently ESI and MALDI[70] ionization techniques. Electrospray ionization (ESI) offers several advantages over the FAB-MS/MS technique, including lower background signals because of the absence of matrix ions, the long lasting and stable primary ion currents, the ease of sampling and compatibility with liquid chromatography. Informative patterns are described by Lee *et al.* employing LC/ESI-MS in both positive and negative ionization modes to identify the

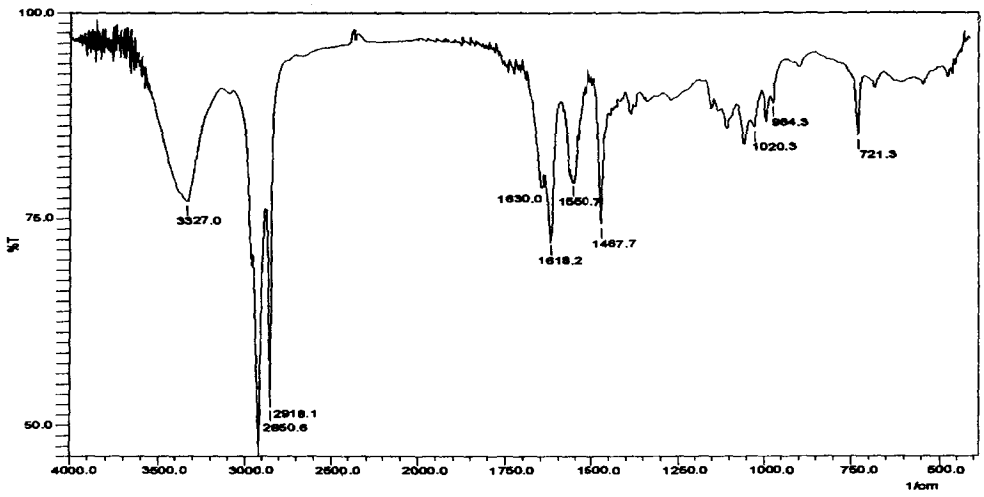


Fig 2: IR spectrum of ceramides

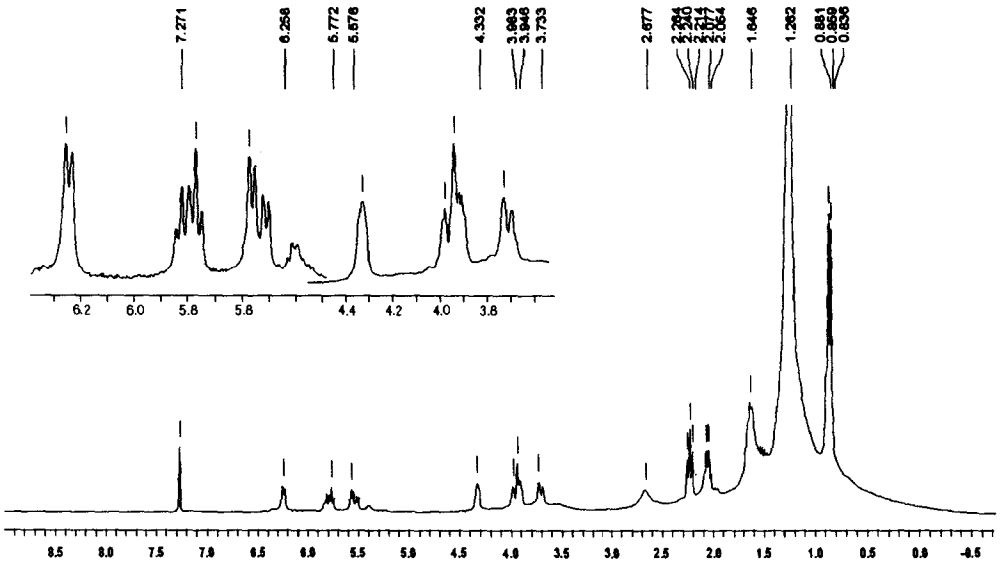


Fig 3: ^{1}HNMR spectrum of ceramides

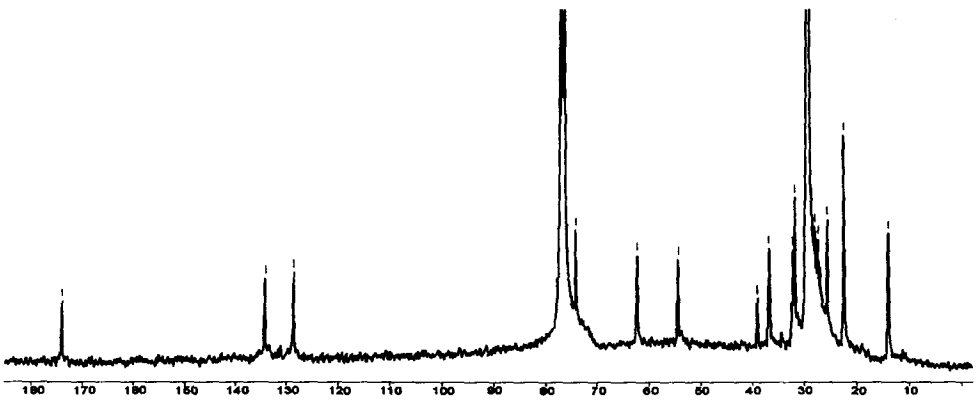


Fig 4: ^{13}CNMR spectrum of ceramides

structures of both sphingoid base and N-acyl chains of ceramides and also of an impurity in cosmetics.[71] Kerwin *et al* (1997) reports ceramide profiling in a commercial preparation of bovine brain using this technique.[72] LC-ESI-MS in combination with the selected ion monitoring (SIM) mode is a very sensitive and useful method for the determination of sphingosine (SO) and sphinganine (SA) in cell cultures.[73]

ESI-MS/MS fragmentation of molecular species of ceramide:

The ESI-MS/MS was carried out on QTOF-XL MS/MS, Applied Biosystem instrument (Canada). The instrument was operated in positive ionization mode. The sample dissolved in MeOH:$CHCl_3$ (99.5:0.5) containing traces of 0.1% TFA were directly infused at a constant flow rate of 10μl/min into the Ion spray source using integrated syringe pump.

The electrospray ionization mass spectrum (ESI-MS) exhibited several molecular $[M+H]^+$ ions at m/z 594.7731, 608.7684, 622.7798, 636.8062, 650.8289, 664.8407, 678.8644 and 691.8769 **(Fig 5)** and relatively less intense in the region 960-1100 amu. A comparison of signal intensities indicates that the mixture contains substantial amount of ceramides with molecular masses 691.8769 (100%), 622.7789 (86.9%) and 636.8062 (76.1%) as shown in **Table 2.**

The MS/MS experiment of each molecular ions $[M+H]^+$ were carried out at collision energy of 40V. The **Fig 5'a-h** shows MS/MS at m/z 594.7731, 608.7684, 622.7798, 636.8062, 650.8289, 664.8407, 678.8644 and 691.8769 respectively. All the molecular ions showed some common fragment ions. The fragment ion corresponding to cleavage between the carbon and nitrogen bond of the sphingosine base are observed at m/z 282.33 (X) and with loss of an additional neutral water molecule resulted in the abundant ion at m/z 264.32 $[X'=X-H_2O]^+$. The cleavage between the amide bond resulted in the fragment ion at m/z 296.35 (Y) and m/z 278.33 $[Y'=Y-H_2O]^+$ for the loss of water molecule. It also showed product ions at m/z 252.31 (Z) due to loss of 31 amu from the sphingosine base **(Scheme 1)**.[74-79]

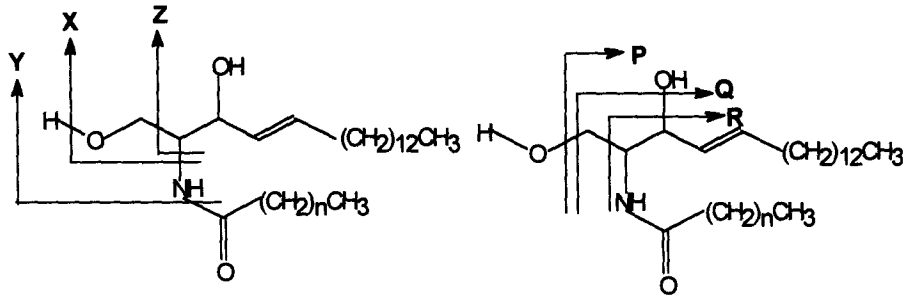
Table 2: The relative abundance and fragment ion peaks for molecular species of ceramide in the ESI-MS/MS spectrum of *Haliclona cribricutis*.

Long Chain Base	$[M+H]^+$	Relative intensity (%)	$[M+H+DHEP]^+$	Relative intensity (%)	P	Q	R
C20:0	594.7731	0.8	985.1335	0.1	576.6974	558.6857	546.6858
C21:0	608.7684	9.2	999.1561	0.6	590.7074	572.6957	560.6968
C22:0	622.7798	86.9	1013.1820	7.6	604.7165	586.6988	574.6961
C23:0	636.8062	76.1	1027.1971	7.6	618.7366	600.7356	588.7353
C24:0	650.8289	43.8	1041.2176	3.8	632.7584	614.7357	602.7368
C25:0	664.8405	15.4	1055.2394	1.1	646.7738	628.7422	610.7174
C26:0	678.8644	16.9	1069.2573	1.0	660.7929	642.7680	630.7840
C27:0	691.8769[b]	100	1083.2759	0.5	674.7829	656.7780	644.7940

[b]Base Peak at m/z 691.8769 amu.

The series of product ions, X, X', Y, Y' and Z provided the information about the molecular weights of the sphingoid and the fatty acid residues. It was indicative that the ceramide is with the common d 18:1 sphingosine moiety and differing only in the long chain base (C20:0-C27:0). It also yielded the abundant product ion corresponding to loss of one water molecule $[M+H-H_2O]^+$ designated as "P". The product ions Q and R were observed for $[M+H-2(H_2O)]^+$ and $[M+H-49]^+$ respectively **(Scheme 1 & Table 2)**. Thus, based on the above observation the ceramides were identified to be N-eicosanoic (20:0), N-hencicosanoic (21:0), N-docosanoic (22:0), N-tricosanoic (23:0), N-tetrasanoic (24:0), N-pentasanoic (25:0), N-hexasanoic (26:0) and N-heptasanoic (27:0) sphingosine.

The less intense peaks in the range 960-1140 amu **(Fig 6)** were attributed to the bis (2-ethyl-hexyl) phthalate adducts of the ceramides $[M+H+DEHP]^+$ based on the MS/MS of this individual peaks which is explained in detail below. Comparing with the $[M+H]^+$, DEHP adducts were found to be almost negligible with less then 8 % **(Table 2)**.



n =18-25 (C20:0 – C27:0)
X = 282.33; **X'**= **X**-H_2O = 264.32
Y = 296.35; **Y'**= **Y**- H_2O = 278.33
Z = 252.31

Scheme 1: ESI-MS/MS fragmentation of molecular species of ceramide.

The MS/MS of the phthalate adducts $[M+H+DEHP]^+$ at m/z 985.1335, 999.1561, 1013.1820, 1027.1971, 1041.2176, 1055.2394, 1069.2573 and 1083.2759 (**Fig 6'a-h**) showed presence of corresponding $[M+H]^+$ and peaks due to loss of water molecules $[M+H-H_2O]^+$ and $[M+H-2(H_2O)]^+$. In addition it showed common fragment ion at m/z 391.38, which indicated the adduct formation of ceramide with bis-(2-ethyl hexyl) phthalate (DEHP). The literature shows that DEHP adducts results from the plasticizer contamination most probably from the cap of bottle of solvents or plastic washing bottles etc.[80]

In conclusion, identification of individual ceramides (fatty acid amides of sphingoid base) present in a complex mixture would not have been possible by FAB-MS. Thus as evident, ESI-MS/MS is a very sensitive and useful method for the determination of molecular species of ceramide and has several advantages compared to other methods. Product ion peaks at m/z at 252.31, 264.32, 278.33, 282.33 and 296.35 in the spectra of all the compounds, confirmed the sphingosine as being d 18:1 and the heterogeneity as being in the acyl group. Based on the relative intensity of the peak signals, ceramides with C27:0, C22:0, C23:0 were found in substantial amount. Product ion peak at m/z 391.38 showed presence of contaminant bis-(2-ethyl-hexyl) phthalate (DEHP). The presence of such contaminants forming adduction with analyte molecule causes error in a mass

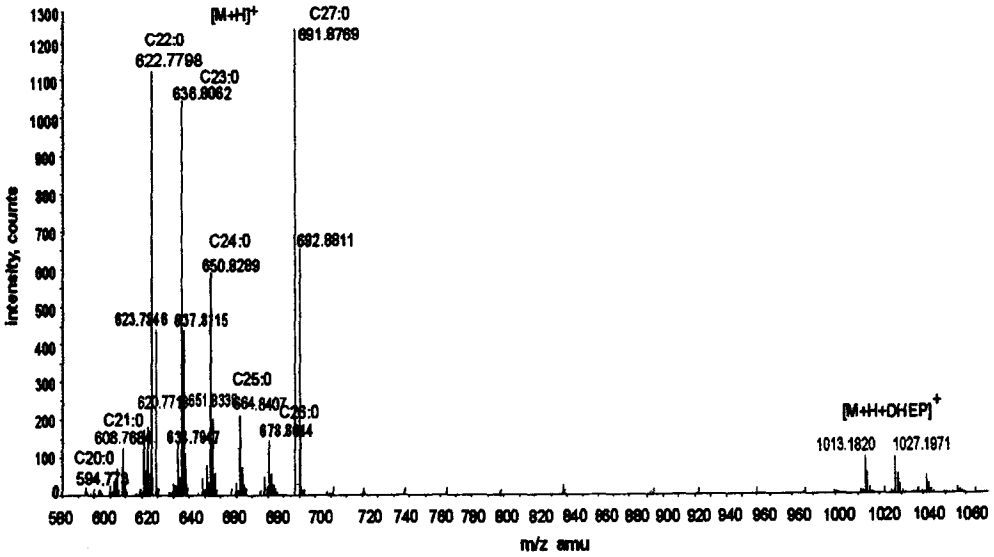


Fig 5: The ESI-MS spectra of the ceramides showing full scan from m/z 580 to 1100 amu

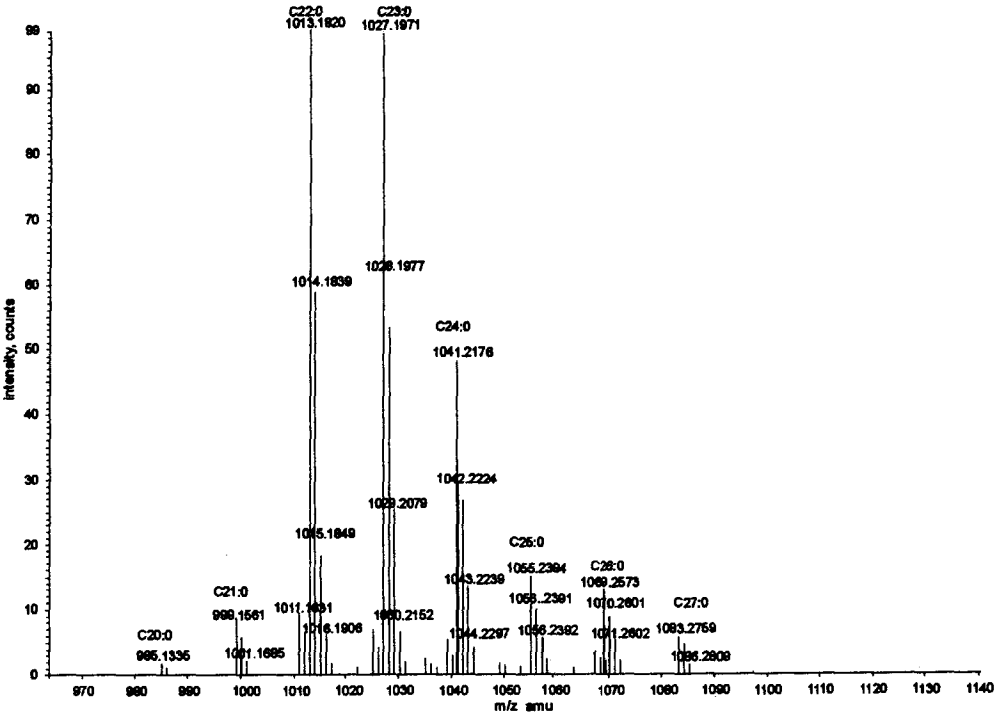


Fig 6: The ESI-MS spectra of the ceramides showing only $[M+H+DHEP]^+$ from m/z 960 to 1140 amu

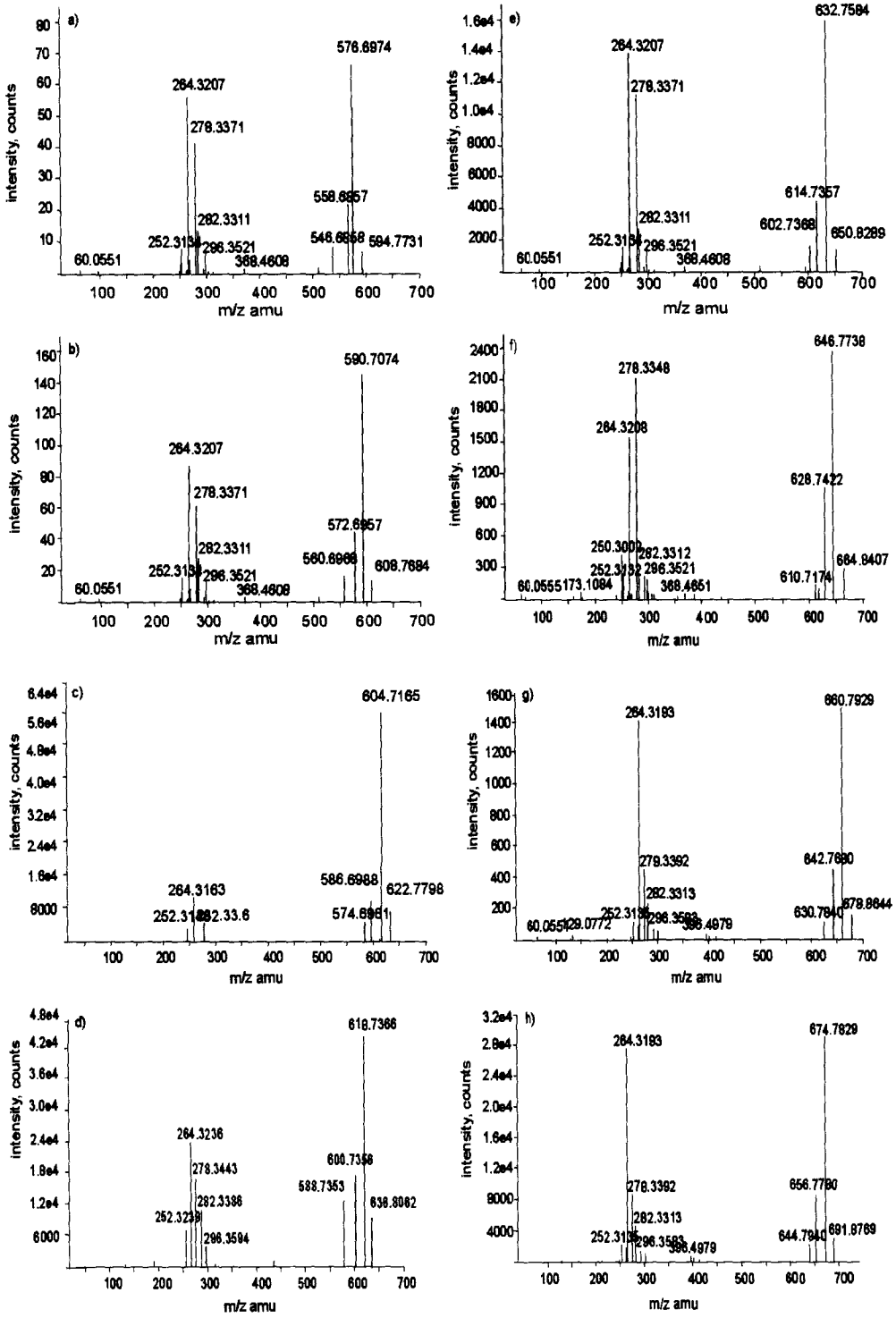


Fig 5′: MS/MS spectra of the $[M+H]^+$ at m/z a) 594.7; b) 608.7; c) 622.8; d) 636.8; e) 650.8; f) 664.8; g) 679.8 and h) 691.8

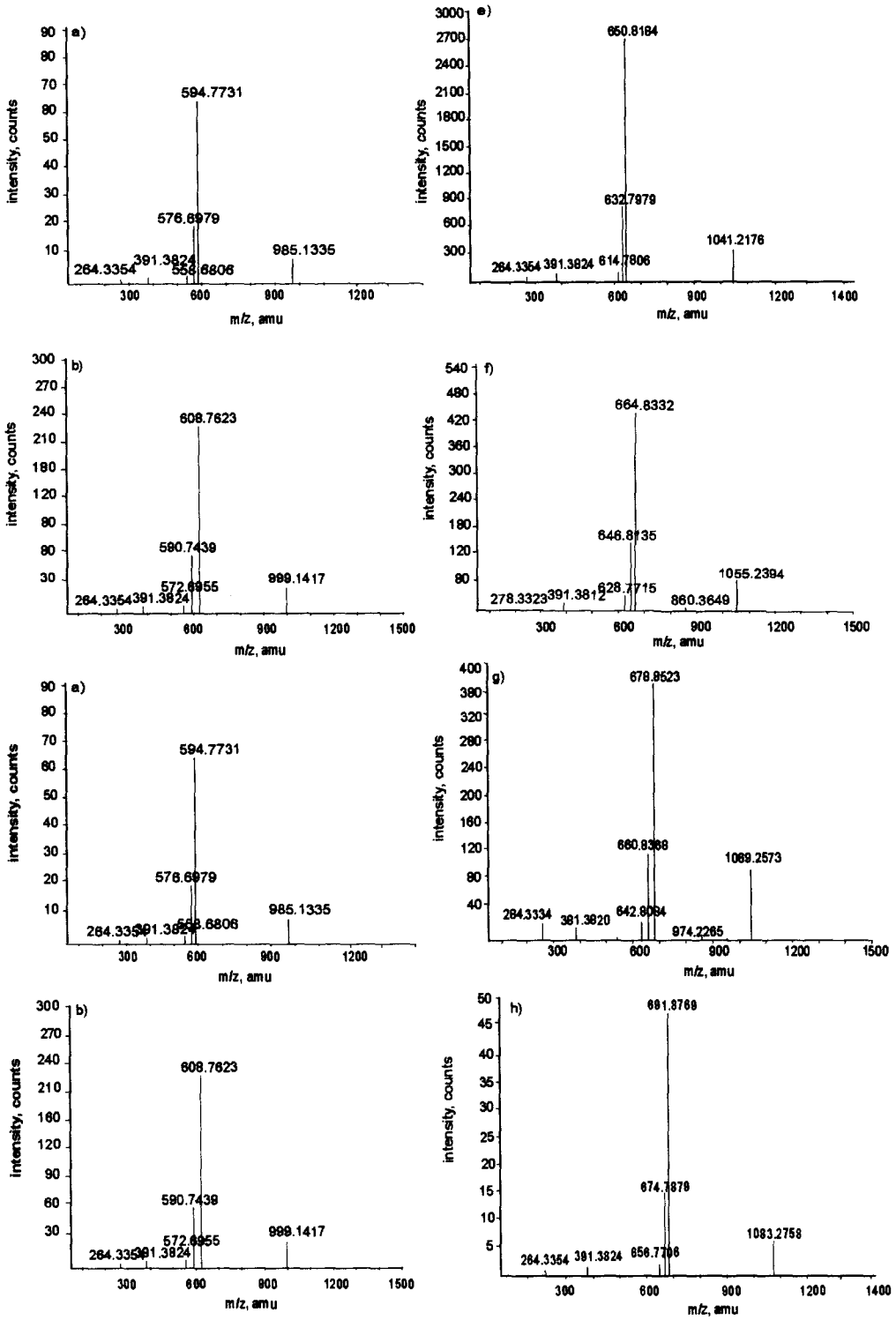


Fig 6′: MS/MS spectra of the $[M+H+DHEP]^+$ at m/z a) 985.2; b) 999.2; c) 1013.2; d) 1027.2; e) 1041.2; f) 1055.2; g) 1069.2 and h) 1083.2

spectrometer. So the permanent solution must be found for avoiding the error. It also demonstrates the identification of ceramides using minimum chromatographic separation and with minimum concentration of sample. (Published results)♦

EXPERIMENTAL SECTION:

Material: The sponge was collected by scuba diving at a depth of 8-10 m from south east coast of India in March, 2003. The sample was identified by Dr. P A Thomas, Vizhingam Research Center of Central Marine Fisheries Research Institute, Kerala, India. A voucher specimen (NIO 730) is deposited at National Institute of Oceanography, Dona-Paula Goa, India.

Extraction, fractionation and purification:

The frozen sponge (500 gm) was extracted with MeOH (2 Lit) under sonication and concentrated under vacuum to obtain 6gm of crude extract. The extract was partitioned with $CHCl_3$ and the soluble fraction (2gm) was chromatographed on a silica gel (Kieselgel 60, 230-400 mesh E. Merck) column using hexane containing increasing amounts of ethyl acetate as eluent. The fractions were monitored by thin-layer chromatography (TLC), alumina-backed sheets (Si gel 60 F_{254}, 0.25mm thick) using ethyl acetate:petroleum ether (35:65) as developing solvent with 5% methanolic sulphuric acid as spraying agent. The fractions showing similar TLC profile with R_f 0.172 were combined and concentrated. It was further purified by gel chromatography using Sephadex LH-20 17-0090-01 Pharmacia Biotech to get the fraction rich in sphingolipids.

Sample preparation:

Infra red spectra were taken, as KBr pellet, on Shimadzu 820PC FTIR spectrometer. The NMR spectra of the sample were obtained in $CDCl_3$ (δ_H 7.271

♦ Tandem mass spectrometric approach for determining structure of molecular species of Ceramide in the Marine Sponge *Haliclona cribricutis*, Supriya Tilvi, Mahesh Majik and C. G. Naik, ***European Journal of Mass Spectrometry***, 2005, 11(3), 345-352.

and δ_C 76.349-77.344 ppm) on Bruker (Avance, 300MHz) instrument with TMS as the internal standard. The ESI-MS analysis was carried out using QTOF-XL MS/MS, Applied Biosystem instrument (Canada). The instrument was operated in positive ionization mode. The sample dissolved in MeOH:$CHCl_3$ (99.5:0.5) containing traces of 0.1% TFA were directly infused at a constant flow rate of 10μL/min into the Ion spray source using integrated syringe pump as described in **Section 2.3**.

Compound 1: white amorphous solid (10mg), R_f 0.172 in ethyl acetate:petroleum ether (35:65); IR (KBr pellet): ν_{max} 3500-3100, 2918.1, 2850.6, 1630.0, 1618.2, 1467.7, 1550.7 and 1020.3 cm^{-1}; ^1H & ^{13}C NMR ($CDCl_3$): **Table 1**.

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Chapter III

Chemical investigation of mollusk Elysia grandifolia and its algal diet Bryopsis plumosa

Section 1

Genus Elysia-Review

Secondary metabolites can play important role as chemical messengers in marine and terrestrial environments. A variety of sessile and slow-moving invertebrates are organized in marine communities where, in analogy with terrestrial habitats, the interactions are mediated by chemical compounds.[1] Thousands of biologically active natural products have been identified from these organisms and, in last few years, marine benthos has been the subject of many chemical ecological studies.[2]

Ophisthobranch mollusks represent an ideal model for studying chemo-ecological interactions.[3-5] Despite an apparent vulnerability, these invertebrates defend themselves by sophisticated mechanisms that involve both behavioural stratergies and use of chemical compounds.[1,3,4] A large number of secondary metabolites, usually with interesting biological properties, have been isolated from ophisthobranchs.[3-5]

Most of these products show a wide chemical variety and are usually derived from the organisms preyed on by the ophisthobranchs. Accordingly, the structure elucidation of these products has often been involved in proving prey-predator relationships even in the absence of direct field observations. In some instances the molluscs are known to biotransform or biosynthesize *de novo* their own chemicals. In these cases, the chemical characteristics of secondary metabolites have sometimes been used as chemo-taxonomical markers.[6] Ophisthobranch mollusks are, in fact, known to possess a great variety of chemicals. Most of them are associated with specific ecological function including defence, inter-and intraspecific communications.

Despite a large amount of work reported on the chemistry of marine ophisthobranchs, little is known about the ophisthobranchs from the Indian Sea. We have carried out chemical investigation of the mollusk *Elysia grandifolia* (Kelaart, 1858) belonging to the Order Sacoglossans and found preying upon the seaweed *Bryopsis plumosa*. Before going into the details of chemical investigation a brief review on the genus *Elysia* is presented here.

Ophisthobranchs [Mollusca:Gastropoda] are either herbivorous (feeding on algae) or carnivorous (feeding mainly on sponges, tunicates, soft corals or other mollusks).[7] Sacoglossans are herbivorous marine mollusks with the ability to sequester from their algal diet functioning chloroplasts,[5,8] which then may participate in the biosynthesis of secondary metabolites.[9] The sacoglossan of genus *Elysia* are represented in Hawaii by several species.[10] Among them are *E. degeneri* and *E. rufescens*. *E. degeneri* is known to feed on *Udotea sp.* a green alga from which antifeedant diterpene aldehydes have been isolated.[1] *E. rufescens* feeding on the green alga *Bryopsis sp.* contain a series of difficulty separable depsipeptides ranging from a C_{31} tripeptide to a C_{75} tridecapeptides known as kahalalides (named after the collection site, the Kahala district on O'ahu).

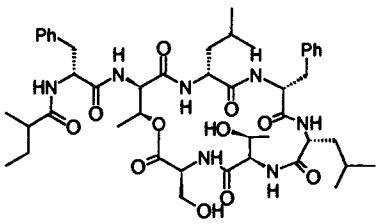
Hamann *et al.* in May 1993 isolated six cyclic depsipeptide kahalalide A-F **(1-6)** ranging from a C_{31} tripeptide to a C_{75} tridecapeptide from *E. rufescens* collected from Black point, O'ahu[11,12] and from its algal diet *Bryopsis sp.* kahalalide F (KF) along with its acyclic analogs kahalalide G (KG) **(7)** was isolated. Previous collection during spring 1991 Hamann *et al.* had isolated only kahalalide F **(6)** from the ethanolic extract. Based on the high-resolution FAB-MS provided a molecular formula of $C_{75}H_{124}N_{14}O_{16}$ ($[M+H]^+$ m/z 1477.9408). Amino acid analysis by GC-MS on Chirasil-Val column revealed 12 amino acids two units of D-alloisoleucine, L-orthinine, D-proline, L-threonine, D-allothreonine, three units of D-valine, two units of L-valine and L-phenylalanine accounting for $C_{64}H_{107}N_{13}O_{14}$.[11] The remaining molecular formula was accounted for the side fatty chain dehydroaminobutric acid. The side fatty chain dehydroaminobutric acid-containing peptides are known to be isolated from a terrestrial blue-green alga[13] and from herbivorous marine mollusk.[14] But it was isolated for the first time from a macroalga. In kahalalide A-F **(1-6)** all constituent amino acids were of common occurrence except for the rare dehydroaminobutric acid in KF and KG. Structures and the absolute configuration of most amino acids were determined by spectral methods. Kahalalide F **(6)** exhibited antifungal activity against fungal strains *Aspergillus oryzae*, *Penicillium notatum*, *Trichophyton mentagrophytes* and *Candida albicans*. Additionally, it also showed slight immunosuppressive

activity in a mixed lymphocyte reaction assay (MLR) with an IC_{50} of 3μg/ml, and with lymphocyte viability (LcV) IC_{50} of 23μg /ml.[11]

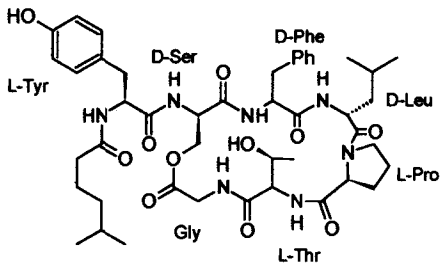
Gilles Goetz *et al.*, 1999[15] succeeded in assigning the exact position of the five valine and two threonine amino acids residues, represented D- and L-enantiomers, in KF by extensive hydrolytic trials, a combination of acid hydrolysis and hydrozinolysis. Isabelle *et al.* revised the stereochemistry of the amino acids in kahalalide F by series of degradation reactions (hydrolysis, ozonolysis, Edman degradation and Marfey derivatization), yielding smaller fragments of the molecule. The results of these reactions agreed with the structure originally proposed by Goetz, Yoshida and Scheuer except for opposite stereochemistry of valine, which seems to be responsible for the antitumor activity of kahalalide F.[16]

With a suitable combination of soluble and polymeric groups and coupling reagents, Lopez-Macia *et al.* succeeded in synthesizing cyclodepsipeptide, kahalalide B **(2)**.[17]

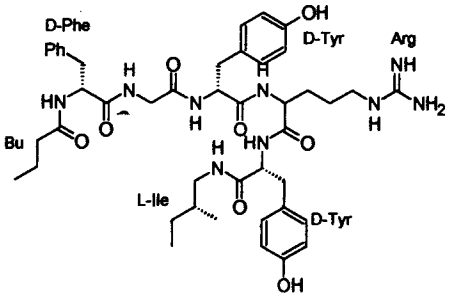
Further analysis of the *E. rufescens* revealed two acyclic peptides, kahalalide H **(8)** and kahalalide J **(9)** structurally related to KF in having four amino acids (leucine, phenylalanine, serine and valine). Both contain aspartic acid and 4-hydroxy proline residue with additional lysine being present in kahalalide J **(9)** only. They have in common a beta-hydroxy fatty acid, 3-hydroxy-9-methyl decanoic acid, previously encountered in kahalalide E **(5)**.[12,18] Kahalalide K **(10)**, a cyclic depsipeptide, from the Hawaiian green alga *Bryopsis sp.* was determined to possess a new array of three L-and three D-amino acids, including a 3-hydroxy-9-methyldecanoic acid that had been previously reported in kahalalides E, H and J.[19] A cyclic depsipeptide, kahalalide O **(11)** was reported from *E. ornata* and its algal diet B*ryopsis sp.* The structure was elucidated using NMR and MS spectral data while the stereochemistry of the amino acid residues was determined by chiral HPLC and Marfey analyses.[20]



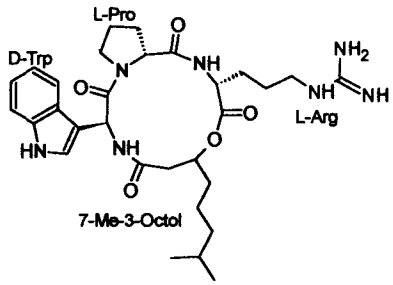
(1)


HO
L-Tyr
D-Ser
D-Phe
Ph
D-Leu
L-Pro
Gly
L-Thr

(2)


D-Phe
Ph
OH
D-Tyr
Arg
Bu
L-Ile
D-Tyr
OH

(3)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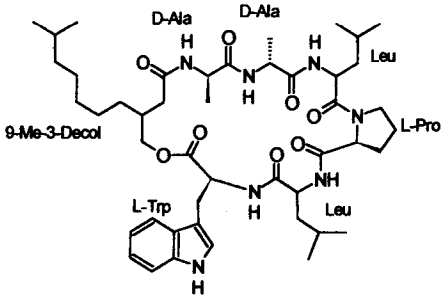
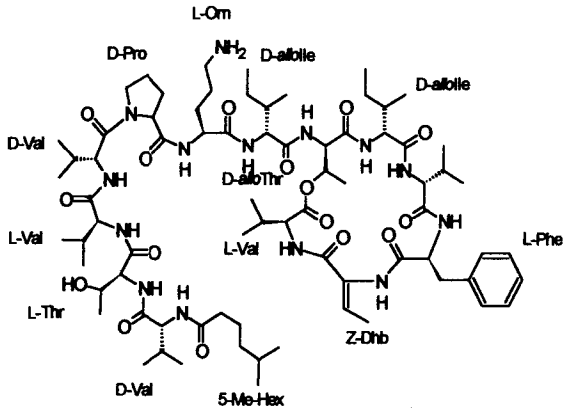
D-Trp
L-Arg
7-Me-3-Octol

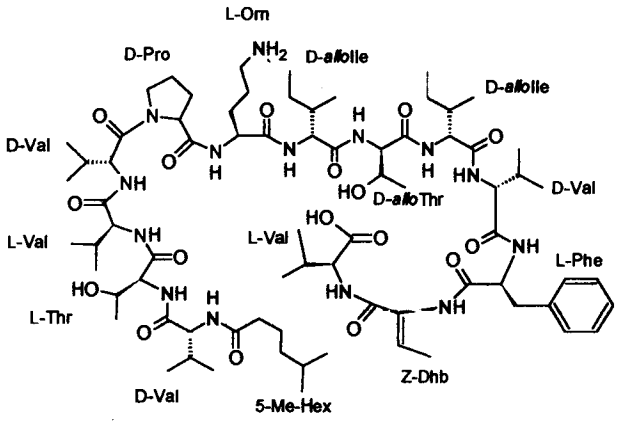
(4)


D-Ala
D-Ala
Leu
9-Me-3-Decol
L-Pro
L-Trp
Leu

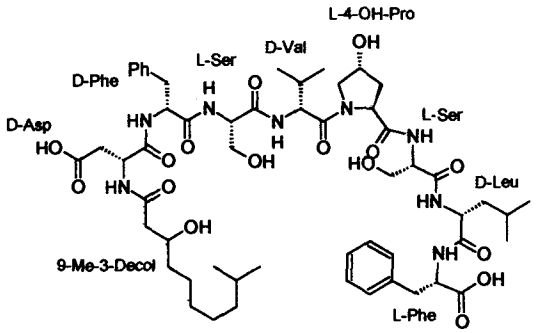
(5)


L-Orn
D-Pro
D-alloIle
D-alloIle
D-Val
D-alloThr
L-Val
L-Val
L-Phe
L-Thr
Z-Dhb
D-Val
5-Me-Hex

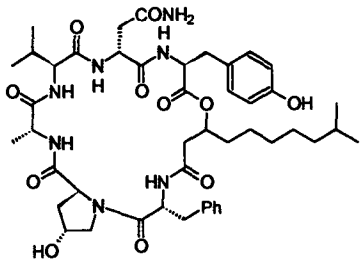
(6)


L-Orn
D-Pro
D-alloIle
D-alloIle
D-Val
D-alloThr
D-Val
L-Val
L-Val
L-Phe
L-Thr
Z-Dhb
D-Val
5-Me-Hex

(7)



(8)



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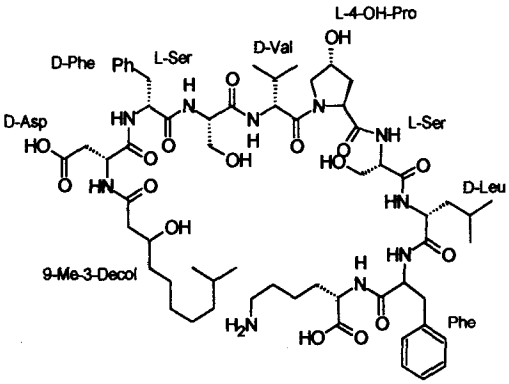
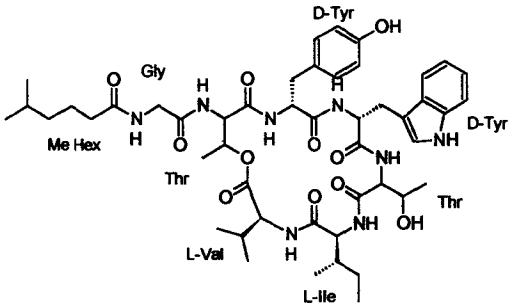


(9)



(11)

Kahalalides showed wide range of activities. Kahalalide A (1) exhibited anti-TB activity[21] and antimalarial activity against *Plasmodium falciparum.* Kahalalide E (5) was selective in activity against *Herpes simplex* II *virus* (HSV II).[12] Among all the peptides isolated KF is the largest and most active exhibiting significant bioactivity against tumor cell lines while its acyclic analogue KG was inactive.

KF is active both *in vitro* as well as *in vivo* against prostate cancer and in particular androgen-independent prostate cancer, breast cancer, colon cancer, non-small cell lung cancer and ovarian cancer.[22] It showed activity against some of pathogenic microorganisms that cause the opportunistic infection of HIV/AIDS. Its mode of action has been studied[23] and is currently in phase II clinical trials in Europe[24-25] It is cytotoxic against dedifferentitated and mesenchymal chodrosarcomas, CHSAs, and osteosarcomas, OSAs.[22] The toxicity of KF on murine hematopoietic stem cell in patients (Phase II clinical trial) did not show

any toxic effect in either short-term or long-term repopulating cells up to 10 μM.[25] It has entered into Phase II trials for the treatment of patients with severe psoriasis.[26]

In the present thesis kahalalides has been identified from the mollusk *Elysia grandifolia* and its algal diet *Bryopsis plumosa* and this has been discussed in section, which follows.

Section 2

Peptides from mollusk Elysia grandifolia and its algal diet Bryopsis plumosa

NIO in collaboration with Institute of Biomolecular Chemistry, Naples, Italy has been working on the chemistry of Indian ophistobranch secondary metabolites. The aim of this study was mainly to establish prey-predator relationships, their defensive mechanism and chemo-taxonomy. Our main interest was to know their chemical defense as the defensive metabolites being toxic to the predators are expected to exhibit antitumor activity. During the course of these investigations we studied secondary metabolites from the Indian ophistobranch from Malvan, *Elysia grandifolia* (Muller) that grows in association with its algal diet *Bryopsis plumosa.*

The crude extracts of the *Elysia grandifolia* collected from Malvan, India exhibited promising antifungal activity against food spoilage fungal strains (*Aspergillus fresenii, Aspergillus niger, Aspergillus japonicus*).[27] It is evident from the literature survey, as described in the previous section, that the opisthobranch belonging to genus *Elysia* contain toxic metabolites known as kahalalides. Because of the multiple activities kahalalides attracted attention and the work on the isolation of new representatives of this genus is in progress. The kahalalide F (KF), a metabolite from *Elysia rufescens* first isolated by Hamann and Scheuer (1993) is an anticancer agent[28] under Phase II clinical trial against non-small cell lung cancer (NSCLC) and in melanoma being developed at Pharma Mar. These trials began on the strength of Phase I results when this compound showed a very favourable safety profile and clinical benefit in non-small cell lung cancer (NSCLC) and melanoma as well as in a broad spectrum of tumour types.[29] Since these compounds are typically administered at very low doses, considerable demands are placed upon the sensitivity of analytical methods used to characterize their pharmacokinetic behaviour in humans.

Though the cyclic depsipeptides from genus *Elysia* are diverse they possess similar physiochemical properties, making their separation and analysis difficult. Therefore, a need was felt to develop a reliable analytical technique for rapid detection and identification of peptides from genus *Elysia.* Tandem mass spectrometry was found to be the best analytical method suitable for

characterization of trace amount of peptides, present in the biological system, using peptide sequencing.

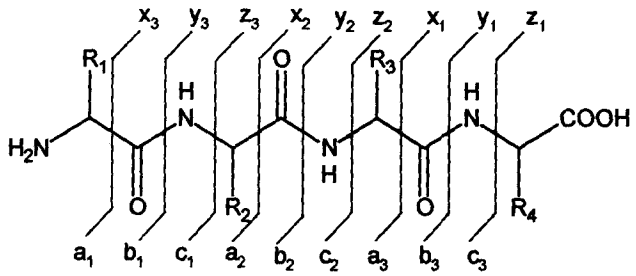
In our studies ESI-MS/MS technique yielded two new cyclodepsipeptides, kahalalide P **(4)** and Q **(5)** present in trace quantities along with the known kahalalide D **(1)**, F **(2)**, and G **(3)** from *Elysia grandifolia.* Its algal diet *Bryopsis plumosa* contained only kahalalide F. Sequences of the new peptides were elucidated on the basis of collision-induced dissociation (CID) experiments and comparison with fragmentation of the known compound kahalalide F **(2)**.

Tandem mass spectrometry for peptide sequencing:

Collision-induced dissociation (CID) (also called "collisionally activated dissociation") is at the heart of most of modern mass spectrometric investigation of complex mixtures.[30-32] CID involves activation of a precursor ion via collisions with a target gas and may produce charged and neutral fragments. The nature of the fragment ions, as well as their intensities, are often indicative of the structure of the precursor ion and thus can yield useful information for the identification of unknown analytes, as well as providing a useful screening technique for different classes of analytes.

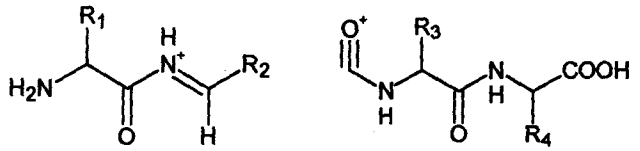
With the advent of soft ionization techniques such as electrospray ionization (ESI)[33-35] and matrix-assisted laser desorption-ionization (MALDI)[36-37] which efficiently ionize by protonation a wide variety of peptides and proteins, tandem mass spectrometry[30,38] has become a method of significant importance for the sequencing of peptides through CID studies.

As a result, the main types of fragmentation reactions occurring are well-established,[39-41] at least in a phenomenological sense and are outlines in **Scheme 1.**



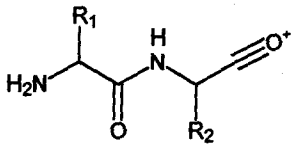
Scheme 1: Peptide Fragmentation

A major mode of fragmentation in many cases involves cleavage of an amide bond in the protonated peptides. When the charge is retained by the C-terminus fragment migration of a labile hydrogen from the N-terminus neutral fragment occurs to form a protonated amino acids (y_1) or peptide (y_n).[42-44] When the charge remains on the N-terminus fragment, a neutral amino acid or peptide is eliminated and b_n ions are formed.

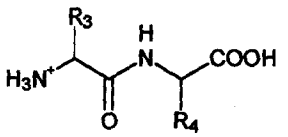


a_2

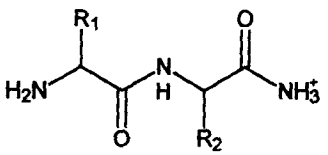
x_2



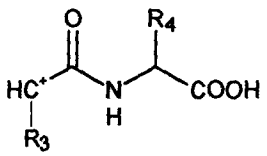
b_2



y_2



c_2



z_2

The appearance of peptides' CID spectra, especially those of $[M+H]^+$ ions, are highly dependant on the collision energy.[42] The CID spectra of peptides recorded at low collision energy (<100eV) tend to show abundant fragment ions formed by cleavage of the peptide bonds. In low energy CID, a peptide carrying a positive

charge fragments, mainly along its backbone, generating predominantly a, b and y ions. In addition, peaks due to loss of ammonia (-17 Da) denoted a*, b* and y* and water (-18 Da) denoted a^0, b^0 and y^0 are also observed. While high-energy CID (KeV) spectra often contain fragment ions formed by other backbone x, c, z and also side-chain cleavages are observed.[45-46] In case of the cyclic depsipeptides, the fragmentation pattern is different. Das and co-workers have suggested that the protonation of ester oxygen opens the ring to form a linear peptide with the C-terminal carboxylic acid that fragments sequentially.[47]

For an ideal fragmentation process the sequence of a peptide could be simply determined by converting the mass differences of consecutive ions in a spectrum to the corresponding amino acids. This ideal situation would occur if the fragmentation process could be controlled so that each peptide was cleaved between every two consecutive amino acids.[48-49]

Tandem Mass spectrometric analysis for peptides from mollusk *Elysia grandifolia* and its algal diet *Bryopsis plumosa*:

The peptide rich fractions, identified on the basis of TLC from the ethyl acetate fraction were studied from the mollusk *Elysia grandifolia* and its algal diet *Bryopsis plumosa* using tandem mass spectrometry (ESI-MS/MS) which resulted in the identification of two new depsipeptides kahalalide P **(4)** and Q **(5)** along with the known peptides, kahalalide D **(1)**, F **(2)** & G **(3)**. The results have been presented in this section in terms of the type of fragment ions observed in the ESI-MS/MS. The sequence of the two new cyclic depsipeptides were proposed on the basis of the CID experiments and homology with known peptide kahalalide F **(2)**, supported by the fragmentation pattern.

The mass spectrometer used was a QTOF-XL MS/MS Applied Biosystem instrument (Canada). The instrument was operated in positive ionization mode. The samples dissolved in 1:1 MeOH: 0.1% TFA were directly infused at a constant flow rate of 10μL/min into the ion spray source using integrated syringe pump. Full-scan data acquisition was performed, scanning from m/z 100 to m/z

2000 in profile mode and using a cycle time of 1 sec. The MS/MS products were produced by collision-induced dissociation (CID) of selected precursor ions and mass analyzed using TOF analyzer of the instrument. Collision energy applied was between 25-40 V.

Parent ion scan (ESI-MS) for the detection of peptides:

The ESI-MS of the ethyl acetate fraction of the *E. grandifolia* showed several molecular ion peaks **(Fig 1)**. Closer examination of the peaks revealed the presence of both singly (+1) and doubly (+2) charged ions. As seen in the expanded spectra **(Fig. 1a)** the isotopic peaks that differ by one mass unit are singly charged ions (+1) and isotopic peaks that differ by 0.5 mass units apart are doubly charged ions (+2) **(Fig 1b).** The ethyl acetate fraction of its algal diet *B. plumosa* was also subjected to ESI-MS, which showed only few molecular ion peaks as compared to *Elysia.* The **Fig 2** shows full scan of ESI-MS of *B. plumosa* from 100-2000 amu.

The theroretical monoisotopic molecular weight of the peptides in various forms of +1 and +2 charges at m/z 596.3554, 1463.9706, 1477.9346, 1492.0016 and 748.0010 amu was calculated. Based on the theoretical calculations singly and doubly charged ion peaks including sodium and potassium adducts (shown in bold) for five peptides could be detected in the full scan spectra **(Table 1)**. The literature reveals that $[M+H]^+$ at m/z 596.3554, 1477.9346, and doubly charged ion at m/z 748.0010 corresponds to earlier reported cyclodepsipeptides kahalalide D **(1)**, F **(2)** and linear peptide kahalalide G **(3)** respectively from *E. rufescens* and its algal diet *Bryopsis sp.*[12] The two $[M+H]^+$ peaks at m/z 1463.9706 and 1492.0016 have not been reported so far.

Table 1: Theoretical monoisotopic molecular weights of peptides in various forms of +1 and +2 charges. Molecular ion peaks of the peptides detected in ESI-MS experiment of *Elysia grandifolia* is shown in bold.

$[M+H]^+$	$[M+Na]^+$	$[M+K]^+$	$[M+Na+K]^+$	$[M+2H]^{+2}$	$[M+H+Na]^{+2}$	$[M+H+K]^{+2}$	$[M+2Na]^{+2}$	$[M+Na+K]^{+2}$	$[M+2K]^{+2}$
596.45	618.3	634.3	657.3	-	-	-	-	-	-
1463.97	**1485.9**	1501.9	1524.9	**732.45**	**743.45**	**751.45**	754.45	762.45	770.45
1477.93	**1499.9**	1515.9	1538.9	**739.46**	**750.46**	**758.46**	**761.46**	769.46	777.46
1492.00	1515.0	1530.0	1553.0	746.00	757.50	765.50	768.50	776.50	784.50
1497.0	1519.0	1558.0	**1536.0**	**748.00**	-	-	-	-	-

Relative abundance of the molecular ion peaks:

The relative abundance of singly as well as doubly charged is given in **Table 2**. The base peak in the ESI mass spectrum of *Elysia* was the singly charged ion at m/z 596.3554 amu that corresponds to kahalalide D **(1)**. The table reveals kahalalide D as the major (100%) cyclodepsipeptide followed by kahalalide F **(2)** with 40.33%. The other two new peptides designated as kahalalide P **(4)** (13.56%) and Q **(5)** (0.76%) are present in trace quantities.

Comparison of the ESI-MS of both the sources reveals that *B. plumosa* is dominated by only one peptide kahalalide F while *E. grandifolia* contains five peptides. To characterise two new peptides and to study the fragmentation pattern of these cyclodepsipeptides, MS/MS of all the molecular ion peaks were carried out.

Table 2: Relative abundance of the singly and doubly charged molecular ions in the ESI Mass spectra of *Elysia grandifolia* and *Bryopsis plumosa*. The ion abundance (%) was normalized to the base peak[a] (100%) in the spectrum.

Elysia grandifolia				*Bryopsis plumosa*	
+1 charged molecular ions	Relative abundance (%)	+2 charged molecular ions	Relative Abundance (%)	+1 and +2 charged molecular ions	Relative Abundance (%)
596.3554[a]	100.00	750.4661	93.88	1477.9140	68.75
1477.9356	40.33	739.4820	69.54	739.4798	75.00
1463.9706	13.56	748.0010	31.30	750.4561	100.00
1499.9835	2.78	743.46	24.34		
1491.9876	0.70	758.4820	17.38		
		732.4854	15.30		
		766.4703	4.87		

[a]Base Peak at m/z 596.3554 amu

[a]Base Peak at m/z 750.4561amu

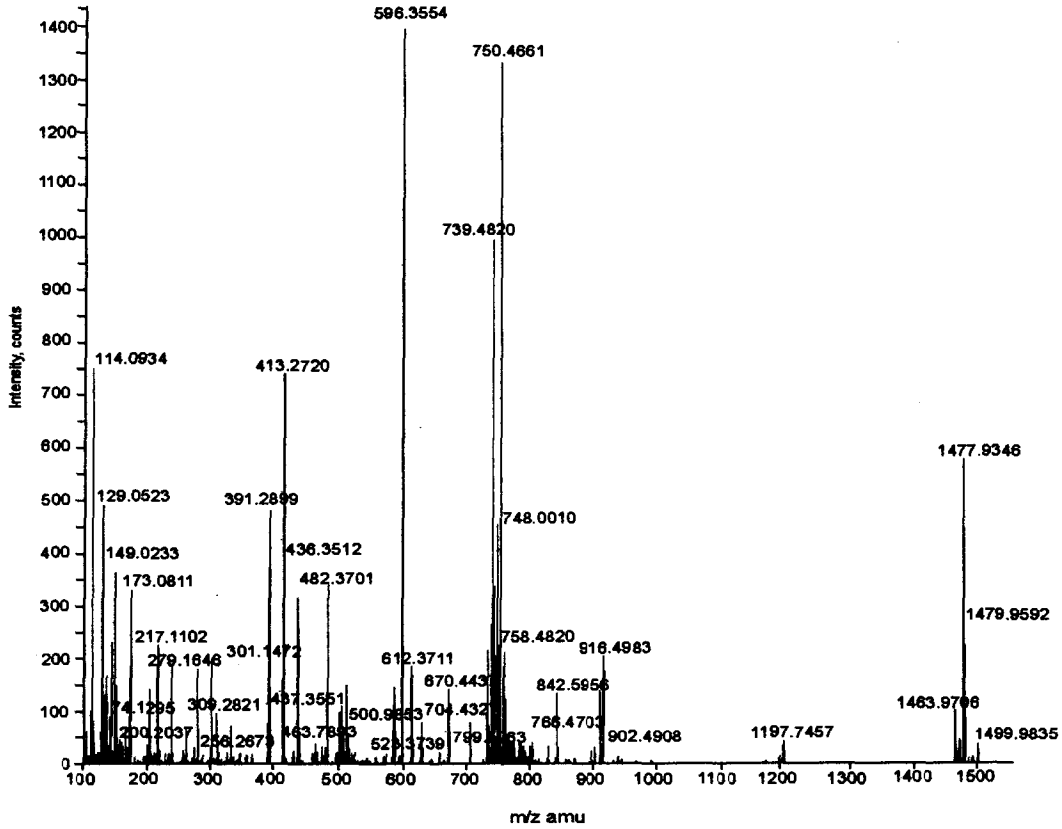


Fig 1: Full scan of ESI-MS of peptide rich fraction of *Elysia grandifolia*

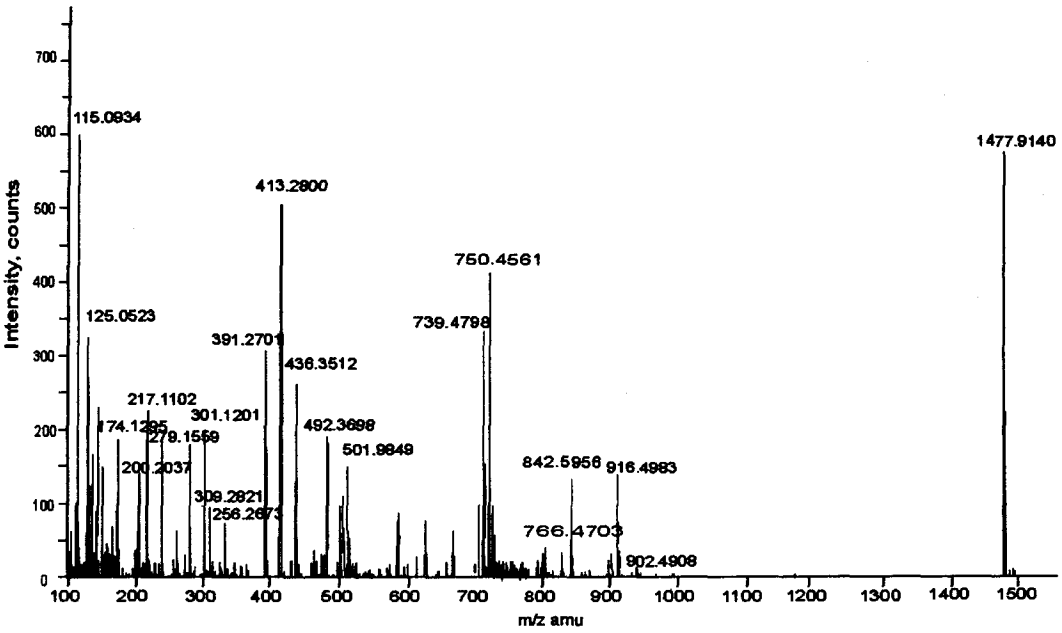


Fig 2: Full scan of ESI-MS of peptide rich fraction of *Bryopsis plumosa*

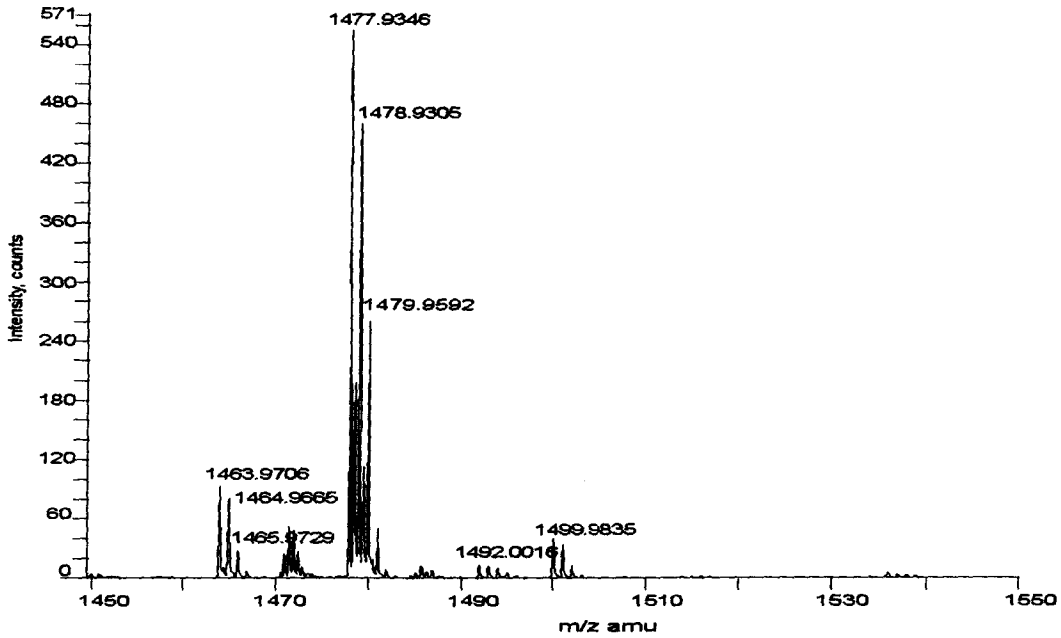


Fig 1a: Expansion of only singly charged molecular ion from *Elysia grandifolia*

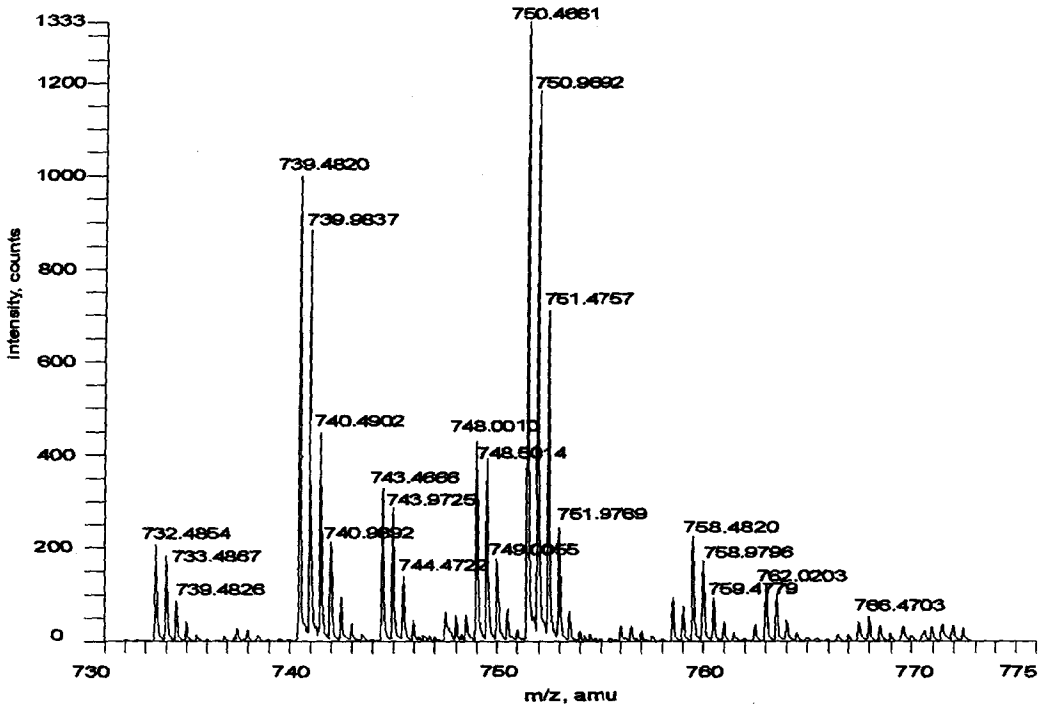


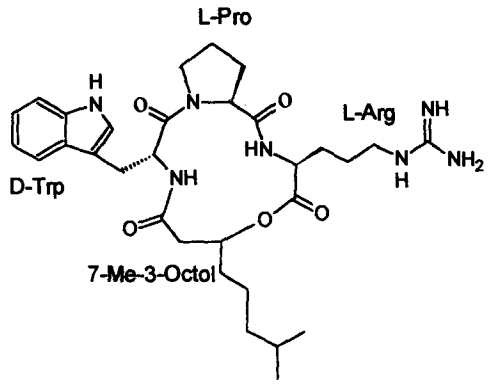
Fig 1b: Expansion of only doubly charged molecular ion from *Elysia grandifolia*

The fragment ion peaks are labeled according to the nomenclature proposed by Roepstorff and Fohlman, subsequently modified by Johnson *et al.*[39] To retrieve a sequence from the mass spectrometric data of the new peptides we applied the criteria that the mass difference between two adjacent peaks should precisely fit the mass of an amino acid residue. Initially the MS/MS of the protonated molecular ion of all the peptides has been discussed. Depending upon the fragmentation pattern the sequence for the new peptides has been assigned. Also, the fragmentation pathway for sodium and potassium adducts has been discussed.

Collision induced dissociation (CID) or MS/MS of each molecular ion peaks:

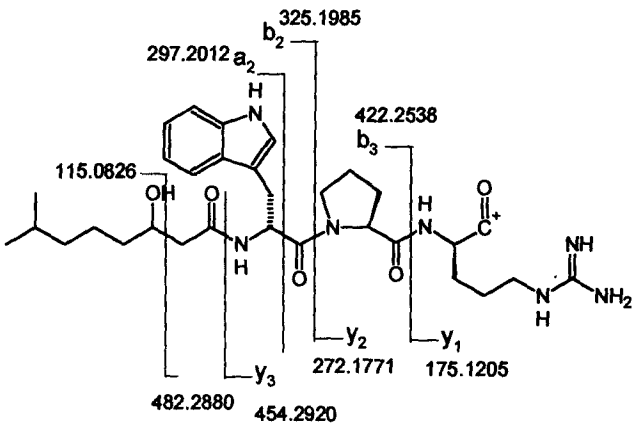
1) MS/MS at m/z 596.3722 of kahalalide D (1):

The **Fig 3** shows the MS/MS spectrum of a protonated peptide at m/z 596.3722 which corresponds to the $[M+H]^+$ of the kahalalide D for the molecular formula $C_{31}H_{44}N_7O_5$ (Calculated mass 596.3560 amu) earlier isolated from the *E. rufescens*.[12] In this case the $[M+H]^+$ at m/z 596.3722 was selected which is base peak and subjected to CID. The kahalalide D **(1)** is the smallest of the kahalalides isolated from the *Elysia* and is made up of three amino acids, L-arginine, L-proline and D-tryptophan. The β-hydroxy group of its fatty acid component, 3-hydroxy-7-Methyloctanoic acid (7-Me-3-octol), furnishes the ester linkages of the depsipeptide cycle.



Kahalalide D (1)

During CID with collision energy of 25V the protonated lactone oxygen opens the ring to form the linear acylium ion and the amino acid residues were sequentially deleted as shown in **Scheme 1**. The side fatty chain with the loss of water gives peak at m/z 140.0807 designated as b_1^0. The corresponding y_3 fragment ion is observed at m/z 454.2920. The fragment ions b_2 and y_2 were significantly observed at m/z 325.1925 and 272.1771 for the cleavage between D-tryptophan and N-terminal of the L-Proline. The peaks were also observed at the C-terminal of the proline residue b_3 and y_1 at m/z 422.2538 and 175.1205 respectively. The peak at m/z 579.346 is due to loss of ammonia while m/z 568.3784 is for loss of CO. The combined loss of CO and NH_3 gives peak at m/z 551.3499. Peaks at m/z 187.0908 and 159.0918 were observed for the D-tryptophan and its immonium ion respectively. The detailed fragment ions are given in the **Table 3**. It is observed that in case of kahalalide D (**1**) the protonation of ester oxygen opens the ring to form a linear peptide and the amino acids are deleted sequentially. Similar case was also observed by Das and co-workers who proposed that protonation of an ester oxygen opens the ring to form a linear peptide with the C-terminal carboxylic acid that fragmented to furnish the amino acid sequence.[47]



Scheme 1: Fragmentation pattern of kahalalide D (1)

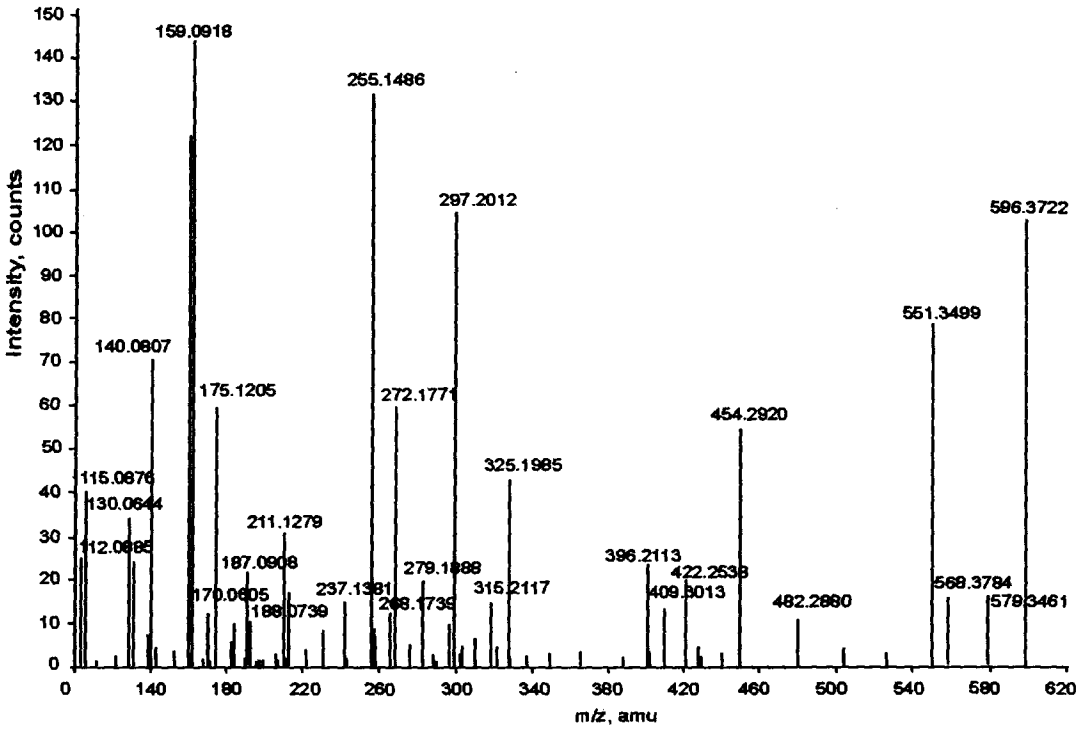


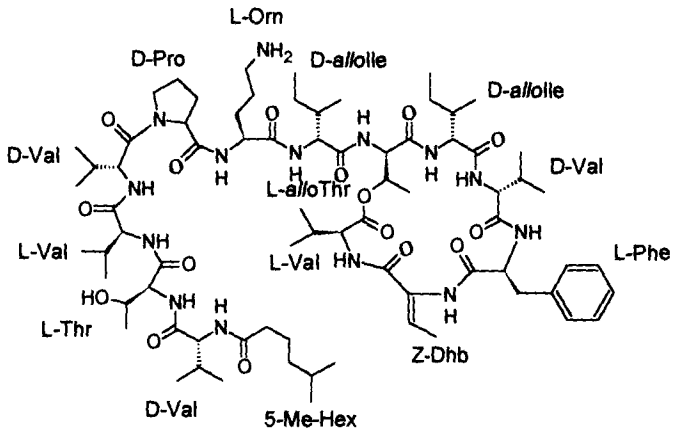
Fig 3: MS/MS spectrum at m/z 596.3722 $[M+H]^+$ of kahalalide D (1)

Table 3: Assignment of fragment ions observed from the MS/MS spectra of kahalalide D (1)

Fragment ions of m/z 596.3722	m/z	Fragment ions of m/z 596.3722	m/z
a	115.0826	$[y_3\text{-}NH_3]^+$	158.0
b_1^0	140.0807	$[M\text{-}CO]^+$	568.3784
x	482.2880	$[M\text{-}(CO+NH_2)]^+$	551.3499
y_3	454.2920	$[M\text{-}NH_2]^+$	579.346
a_2	297.2012	$[L\text{-}Trp]^+$	187.0908
b_2	325.1985	L-Trp*	159.0918
y_2	272.1771	$[L\text{-}Arg + L\text{-}Pro]^+$	255.1486
b_3	422.2538		
y_1	175.1205		

2) MS/MS at m/z 1477.9346 $[M+H]^+$ and 739.4820 $[M+2H]^{+2}$ of kahalalide F:

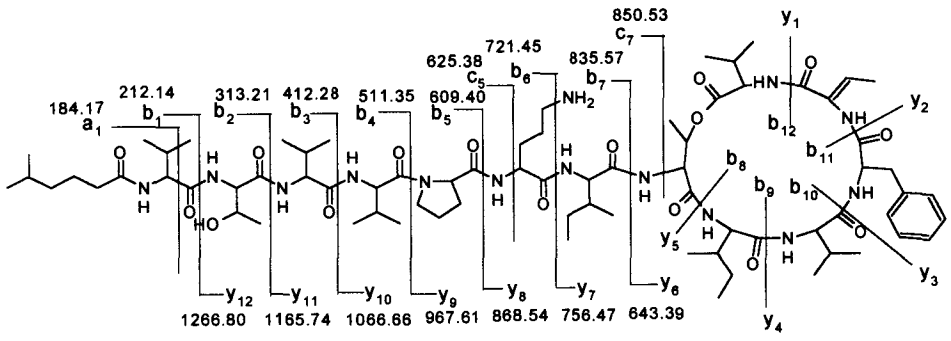
Kahalalide F **(2)**, an anticancer agent is a tridecapeptides with the molecular mass 1476.8467 composed of linear region with short-chain fatty acid (5-methylhexanoic acid) conjugated to the N-terminus and a macrolidic region. The 12 amino acids present are two units of D-alloisoleucine, L-orthinine, D-proline, L-threonine, D-allothreonine, three units of D-valine, two units of L-valine and L-phenylalanine.



Kahalalide F (2)

The MS/MS spectrum at m/z 1477.9346 **(Fig 4a)** showed intense series of 'b' and 'y' type fragment ions in the linear region. The **Fig 4a** (m/z 1477.9346) shows the

fragment at m/z 212.1449 consistent with D-val-(5-methylhexanoic acid), designated as b_1 ion, and results from the cleavage of the amide bond between the D-valine and L-threonine residue in the linear region of the molecule. The corresponding y_{12} ion was observed at m/z 1266.8085. Similarly, the fragments at m/z 313.2144, 412.2842, 511.3546, 609.4066, 721.4731, and 835.5762 were assigned as b_2, b_3, b_4, b_5, b_6 and b_7 respectively. The corresponding y ions were observed at m/z 1165.7494 (y_{11}), 1066.6857 (y_{10}), 967.6124 (y_9), 868.5424 (y_8), 756.4777 (y_7), and 643.3934 (y_6). The y_9 ion due to the cleavage of the amide between the D-val and D-pro was most prominent. This is because proline residue contains a tertiary nitrogen atom and is thus more basic than other residues, and hence more likely to be protonated, thereby leading to enhance peptide-bond cleavage.[50] The above fragment ion observed confirms the peptide sequence 5-Me Hex-DVal-LThr-LVal-DVal-DPro- LOrn-D*allo*Ile-, containing seven amino acids with short fatty chain. In the earlier studies by E. Stokis *et al.*[51] the quantitative analysis of KF in human plasma done during a Phase I clinical trial, in patients with androgen refractory prostate cancer using high performance liquid chromatography (HPLC) coupled to positive electrospray ionization tandem mass spectrometry (ESI-MS/MS), is reported. The present study reports only b_1 to b_4 fragment ion and y_9 (969) and y_{10} (1068). In this study it is observed that there is sequential loss of amino acid residues up to isoleucine (b_7 & y_6) in the linear region. Fragment ions y_1 to y_5 and its corresponding b_8 to b_{12} were not detected, which suggests that there is no further sequential loss of amino acids in the cyclic region of KF **(Scheme 2)**.



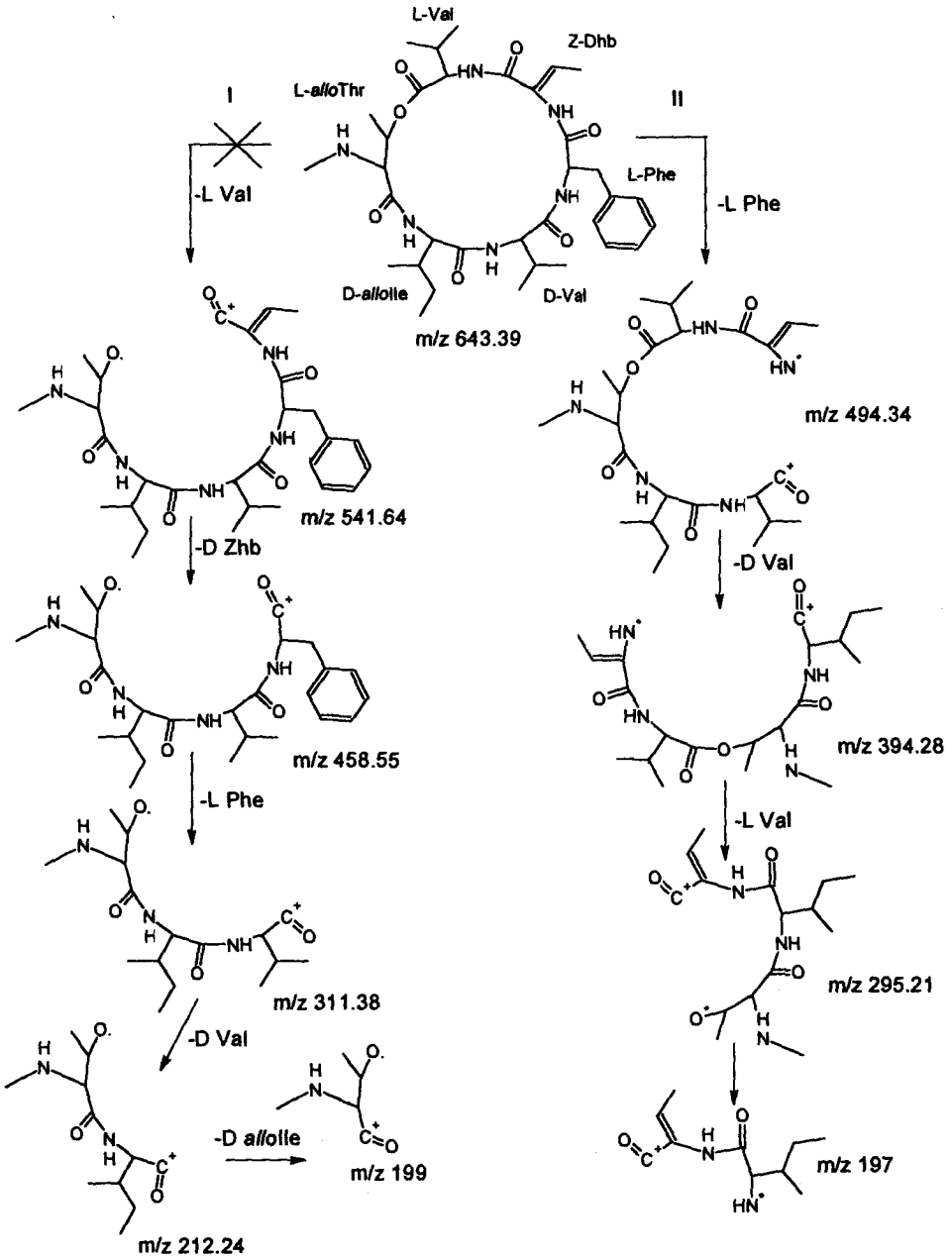
Scheme 2: Fragmentation pattern of kahalalide F (2) in the linear region.

In addition to b and y fragment ions 'a' and 'c' type of fragment ions were also observed. Fragment ions at m/z 184.1705, 693.4758, 625.3814 and 850.5354 were assigned to a_1, a_6, c_5 and c_7 respectively. A summary of the CID fragmentation of kahalalide F is presented in **Table 4**. Peaks at m/z 1469.9568 and 1460.9655 were observed due to the loss of H_2O and NH_3. The fragmentation pathway for the loss of amino acid residues in the cyclic region of KF is discussed below.

Fragmentation pathway in the cyclic region of kahalalide F (2):

For any cyclic peptides the first dissociation step involves the opening of the cyclopeptide ring by cleavage of the either lactone group or one of the amide bonds. In most cases the basic site gets protonated first resulting in the weakening of the C-N or C-O bond.

In case of KF, two pathways of the ring opening were considered which are designated as 'I' and 'II'. Pathway 'I' is for the ring opening at the lactone oxygen with the first cleavage of L-Val, which would result in the formation of fragment ion at m/z 541.64, which is not observed in the spectra. Similarly the sequential loss of other amino acid residues D-Zhb, Phe, Val, Thr would result in the fragment ions at m/z 558, 311, 212 and 199 respectively which are also not observed in the spectra. This indicates that ring opening at the lactone oxygen i.e. pathway 'I' is not favored. But instead pathway 'II' is favored with the initial loss L-Phe giving peak at m/z 494.3450 $[y_6\text{-L-Phe}]^+$ designated as 'A'. This loss can be attributed to N-C ring opening. The second loss of D-Val gave peak at m/z 394.2727 $[\text{A-D-Val}]^+$ designated as 'B'. This complex formed undergoes internal cyclization and with the loss L-Val gives very intense peak at m/z 295.2055 $[\text{B-L-Val}]^+$ which is designated as 'C'. The peak at m/z 197 is due to the cleavage of ester bond of L-*allo*-Thr and Z-Dhb designated as 'D'. The pathway proposed for the loss of the amino acids in the macrolidic region is outlined in **Scheme 3**. The electron donating aromatic ring makes the phenylalanine group more basic residue, and hence it gets protonated and not lactone oxygen. Thus, the ring opens at amide bond of the phenylalanine group. Also, **Scheme 3** shows that loss of amino acid residues from the cyclic region is not sequential.



Scheme 3: The proposed fragmentation pathway for loss of amino acid residues from the cyclic region of kahalalide F (2).

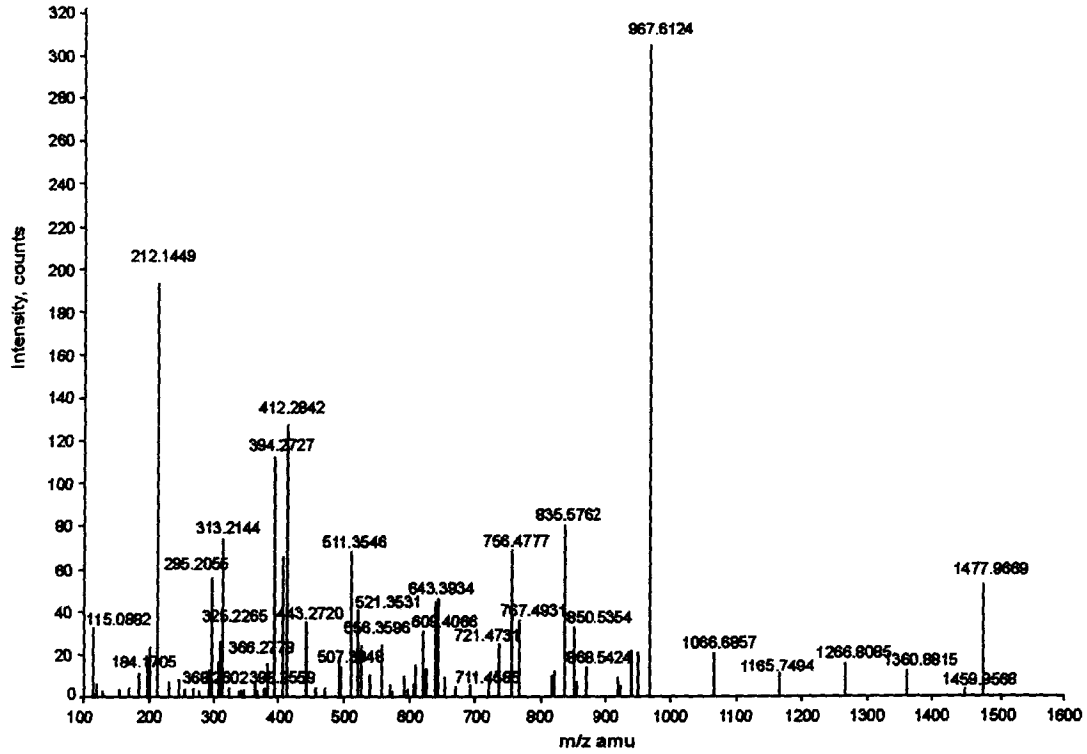


Fig 4a: MS/MS spectrum at m/z 1477.9669 $[M+H]^+$ of kahalalide F (2)

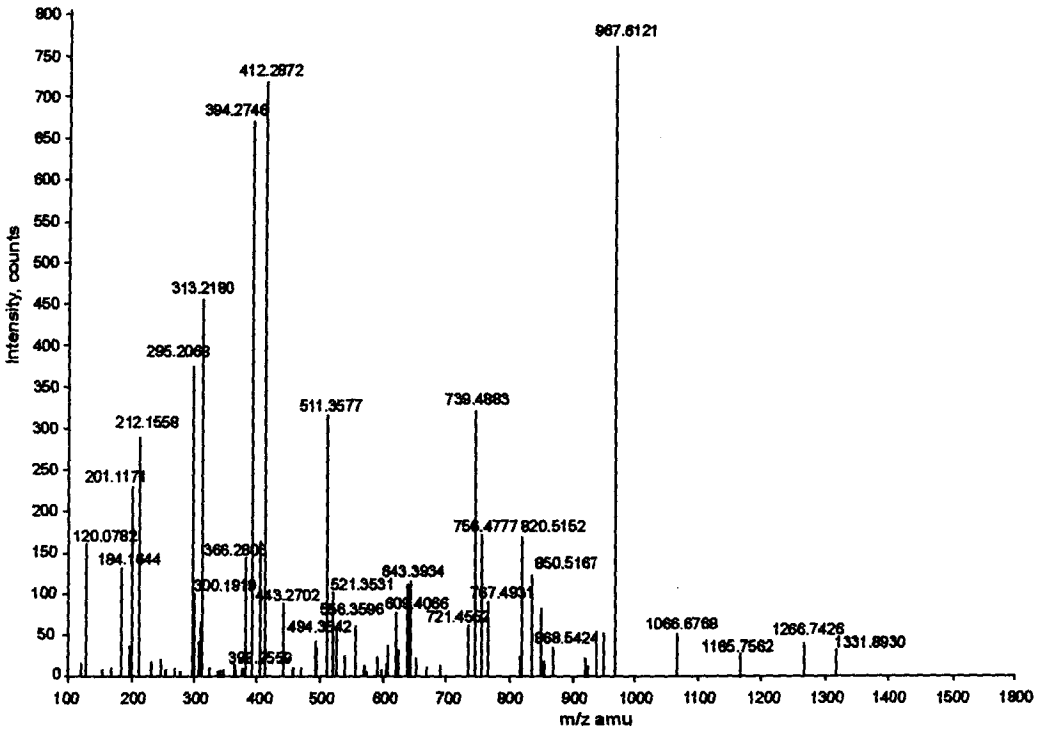


Fig 4b: MS/MS spectrum at m/z 739.4937 $[M+2H]^{+2}$ of kahalalide F (2)

The tandem mass spectra of doubly charged ion at m/z 739.4883 shown in **Fig 4b** are remarkably similar to **Fig 4a**. In addition there were peaks observed at m/z 1331.8930 and 1378.7 which are accounted for elimination of phenylalanine and valine residue from the parent molecule. Fragment ion peaks b_5, b_7, y_7 and y_8 could not be detected or they may be less intense.

MS/MS of $[M+Na]^+$ at m/z 1499.9488, $[M+H+Na]^{2+}$ at m/z 750.5001 and $[M+H+K]^{2+}$ at m/z 758.4938 of kahalalide F (2):

In addition to the protonated molecular ion $[M+H]^+$, CID was also obtained for the sodium and potassium adducts of kahalalide F. As shown in **Table 2,** kahalalide F **(2)** displayed sodium adducts $[M+Na]^+$ at m/z 1499.9499 **(Fig 4c)** and $[M+H+Na]^{+2}$ at m/z 750.5001 **(Fig 4d).** The potassium adduct $[M+H+K]^{+2}$ were observed at m/z 758.4938 **(Fig 4e).** The MS fragmentation pattern for the protonated and alkali metal (Na/K) catalyzed molecular ion peaks differs significantly from the protonated molecular ion. The metal ion Na or K affects the fragmentation pattern depending upon the location of the metal ion in the molecule.

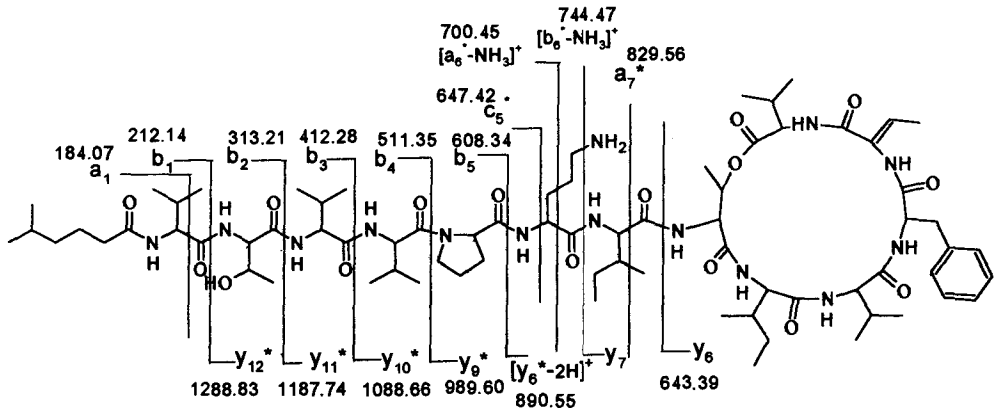
Tandem mass spectrometry of alkali metal cationized peptides has been extensively studied by Gross,[52-53] Adams,[54-55] Russell[56-57]and Tang.[58] Russell[57] suggested that the sodium ion interacted with the amino terminus of the amide nitrogen in a small tyrosine containing peptide. Gross[52-53]and Tang[58] proposed that metal cation binding occurs at the carbonyl oxygen of the C-terminus and the adjoining amino acid residue; upon collisional activation, rearrangement of the alkali metal cationized peptide and subsequent expulsion of the C-terminal residue ensues. Gross[52] also presented a strategy for the determination of amino acid sequences of cyclodepsipeptide antibiotics by a highly specific sodium ion interaction with the backbone lactone opening the depsipeptide ring to form a linear acylium ion or isomeric equivalent and fragmentation is charge-driven deleting sequentially amino acid residue from the C-terminus of the acylium ion. Das and co-workers also proposed that protonation of an ester oxygen opens the

ring to form a linear peptide with the C-terminal carboxylic acid that fragmented to furnish the amino acid sequence as explained above in case of kahalalide D.[47]

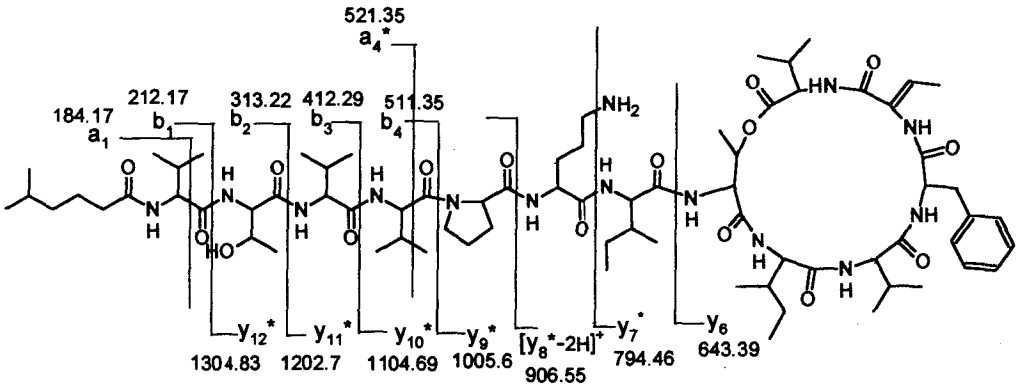
In the MS/MS of the $[M+Na]^+$ at m/z 1499.9 of kahalalide F **(2)** intense series of fragment ion with Na attached to the y and b ions were observed. The fragment ion peak with metal ion is denoted by astrick (*), which is illustrated in the **Table 4** and **Scheme 4 (a)**. The fragment ion peaks from b_1 to b_5 were exactly matching with protonated molecular ion. Its corresponding y ions, y_{12}* (1288.8340), y_{11}* (1187.7437), y_{10}* (1088.6007), y_9* (989.6007) and y_8* (890.5561) were ionized by sodium ion attachment. It also shows peak at 1271.8135 for $[y_{12}*\text{-}NH_3]^+$. Peaks at m/z 505.3441, 647.4226, 700.4534, 744.4786 and 829.5623 were attributed to the sodium attached fragment ions a_4*, c_5*, $[a_6*\text{-}NH_3]^+$, $[b_6*\text{-}NH_3]^+$ and a_7* respectively. No changes in the fragment ions of the cyclic region were observed. It was identical to the fragments of $[M+H]^+$ ion. This indicated that Na is attached either to amide nitrogen or amide carbonyl oxygen of the linear chain Val-Pro-Orn-Ile. The Na does not bind to the ester oxygen of the cyclic ring, which opens the ring to give linear peptide as described by Gross.[59] This suggests that the ester linkage is stronger than any other amide bond in the linear as well as cyclic part of the molecule and hence the new fragmentation pattern was observed. **Scheme 4a** summarizes the fragmentation pattern for the sodium adduct. Combined loss of CO and NH_2 from sodiated molecular ion gives peak at m/z 1455.9123 $[M+Na\text{-}(CO+NH_2)]^+$. Similarly it showed peaks at m/z 1382.8553 and 1356.8642 for $[M+Na\text{-}(H_2O+Val)]^+$ and $[M+Na\text{-}(CO+NH_2+Val)]^+$ respectively. Doubly charged ion $[M+H+Na]^{+2}$ at m/z 750.5001 **(Fig 4d)** displayed similar peaks.

Similar fragmentation pattern was observed during CID of the potassium adduct $[M+H+K]^{+2}$ at m/z 758. 4938 **(Fig 4e)**. Fragment ion peaks from b_1 to b_4 were identical to that of protonated molecular ion but the y fragment ions were ionized by potassium. The y* fragment ion peaks were observed at m/z 1304.8310 (y_{12}*), 1202.7 (y_{11}*), 1104.6974 (y_{10}*), 1005.6 (y_9*), 90.5515 $(y_8*\text{-}2H)^+$ and 794.46 (y_7*) but the y_6 fragment is observed at m/z 643.3958 without addition of K. No change was observed in the fragmentation pattern of the cyclic ring. This indicated that K

is not attached to the lactone oxygen of the cyclic region but may be attached to the amide nitrogen or carbonyl oxygen in the linear chain of the molecule. The fragmentation pattern is illustrated in **Scheme 4b**.



(a)



(b)

Scheme 4: Fragmentation pattern for a) sodium adduct $[M+Na]^+$ at m/z 1499.9499 and b) potassium adduct $[M+H+K]^{+2}$ at m/z 758.4938 of KF (2).

Table 4: Assignment of fragment ions observed from the MS/MS spectra of cyclic depsipeptide kahalalides F (2)

Fragment ions	$[M+H]^+$ 1477.9669	$[M+2H]^{2+}$ 739.4883	Fragment ions	$[M+Na]^+$ 1499.9488	$[M+H+Na]^{2+}$ 750.5001	$[M+H+K]^{+2}$ 758.4938
a_1	184.1705	184.1646	a_1	184.0729	184.1745	184.1757
b_1	212.1449	212.1612	b_1	212.1444	212.1716	212.1709
y_{12}	1266.8085	1266.7426	y_{12}*	1288.8340	1288.8334	1304.8310
			$[y_{12}$*$-NH_3]^+$	1271.8135		
b_2	313.2144	313.2178	b_2	313.2119	313.2249	313.2255
y_{11}	1165.7494	1165.7562	y_{11}*	1187.7437	1187.7263	1202.7
b_3	412.2842	412.2876	b_3	412.2836	412.2975	412.2982
y_{10}	1066.6857	1066.6768	y_{10}*	1088.6648	1088.7041	1104.6974
a_4			a_4*	505.3441	505.3588	521.3500
b_4	511.3546	511.3550	b_4	511.3497	511.3708	
y_9	967.6124	967.6123	y_9*	989.6007		1005.6188
b_5	609.4066		b_5	608.3460	608.3637	-
c_5	625.3814		c_5*	647.4226	-	-
y_8	868.5424		$[y_8$*$-2H]^+$	890.5561	890.5554	906.5515
a_6	693.4758		$[a_6$*$-NH_3]^+$	700.4534	-	-
b_6	721.4731	721.4552	$[b_6$*$-NH_3]^+$	744.4786		-
y_7	756.4777		y_7	785.5398		794.4641
			a_7*	829.5628		
b_7	835.5762		b_7		-	-
c_7	850.5354	850.5167	c_7		-	-
y_6	643.3934	643.3940	y_6	643.3958	643.3940	643.3944
			$[M-Na]^+$			1477.9899
$[M-H_2O]^+$	1459.9568		$[M+Na-H_2O]^+$			-
$[M-Phe]^+$		1331.8930	$[M+Na-Phe]^+$		-	1368.9282
$[M-Val]^+$		1378.7	$[M+Na-Val]^+$	1400.8870		1416.9251
$A=[y_6$-LPhe-$2H]^+$	494.3450	494.3458	$A=[y_6$-LPhe-$2H]^+$	494.3167	494.3167	494.3167
$B=[A$-DVal$]^+$	394.2727	394.2746	$B=[A$-DVal$]^+$	394.2679	394.2857	394.2880
$C=[B$-LVal$]^+$	295.2055	295.2068	$C=[B$-LVal$]^+$	295.2095	295.2135	295.2140
D=[Ile+ ZDhb]$^+$		201.1171	D=[D*allo*Ile+ ZDhb]$^+$		201.1306	-
$[M-NH_3]^+$	1460.9655		$[M+Na-NH_3]^+$			1461.0342
$[M-CO+NH_2]^+$	-		$[M+Na-CO+NH_2]^+$	1455.9123		-
$[M-(H_2O+$ Val$)]^+$	-		$[M+Na-(H_2O+$ Val$)]^+$	1382.8553		1398.9311
$[M-(CO+NH_2+$ Val$)]^+$	-		$[M+Na-(CO+NH_2+$ Val$)]^+$	1356.8642		1372.8765
Val*	72.0860	72.0723	Val*		-	-
Phe*		120.0800	Phe*		120.0853	120.0857

* Na adduct

Val * and Phe * are for immonium ions

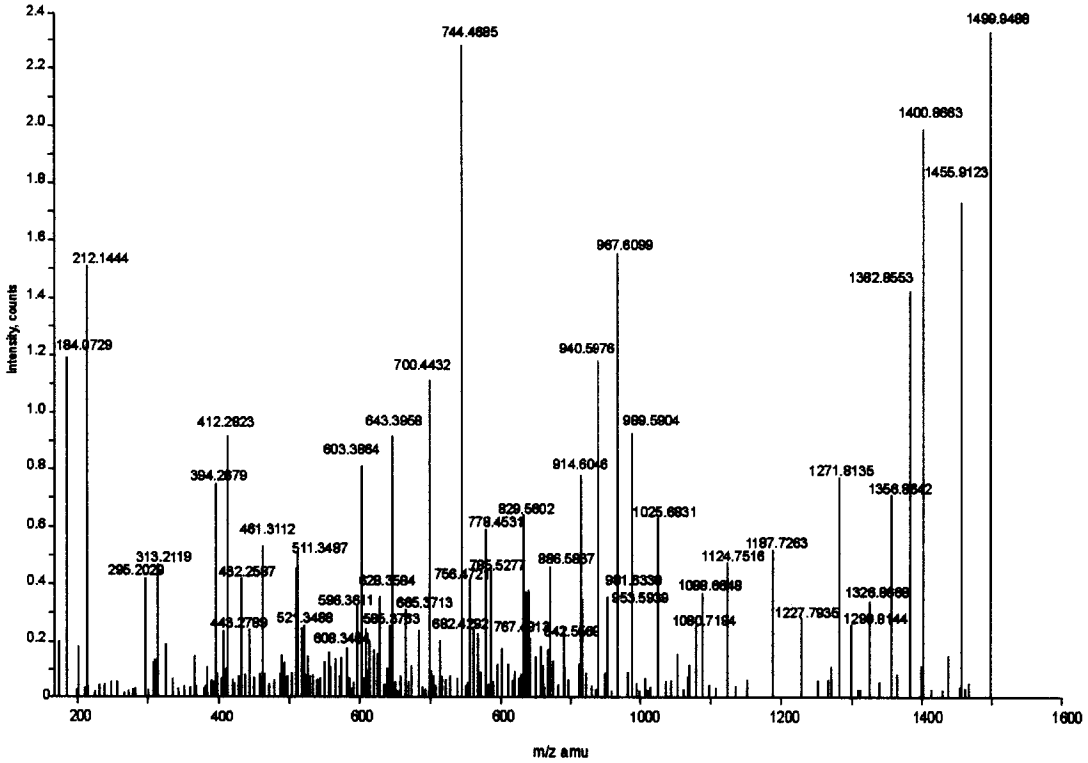


Fig 4c: MS/MS spectrum at m/z 1499.9488 $[M+Na]^+$ of kahalalide F (2)

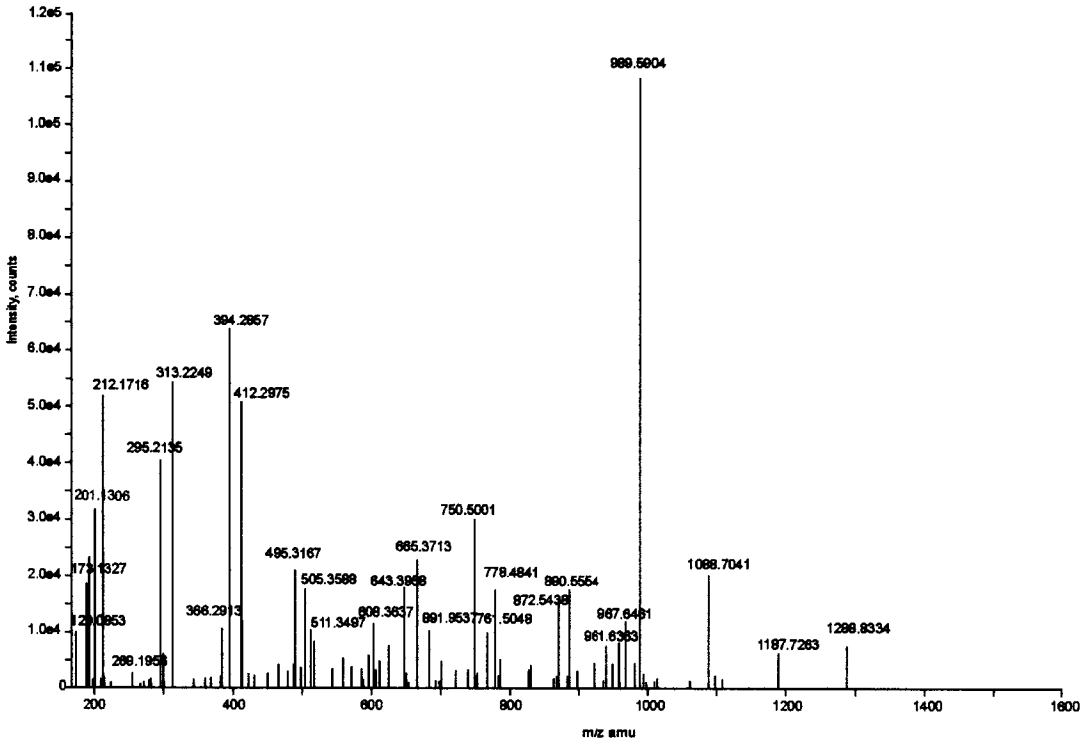


Fig 4d: MS/MS spectrum at m/z 750.5001 $[M+Na+H]^{+2}$ of kahalalide F (2)

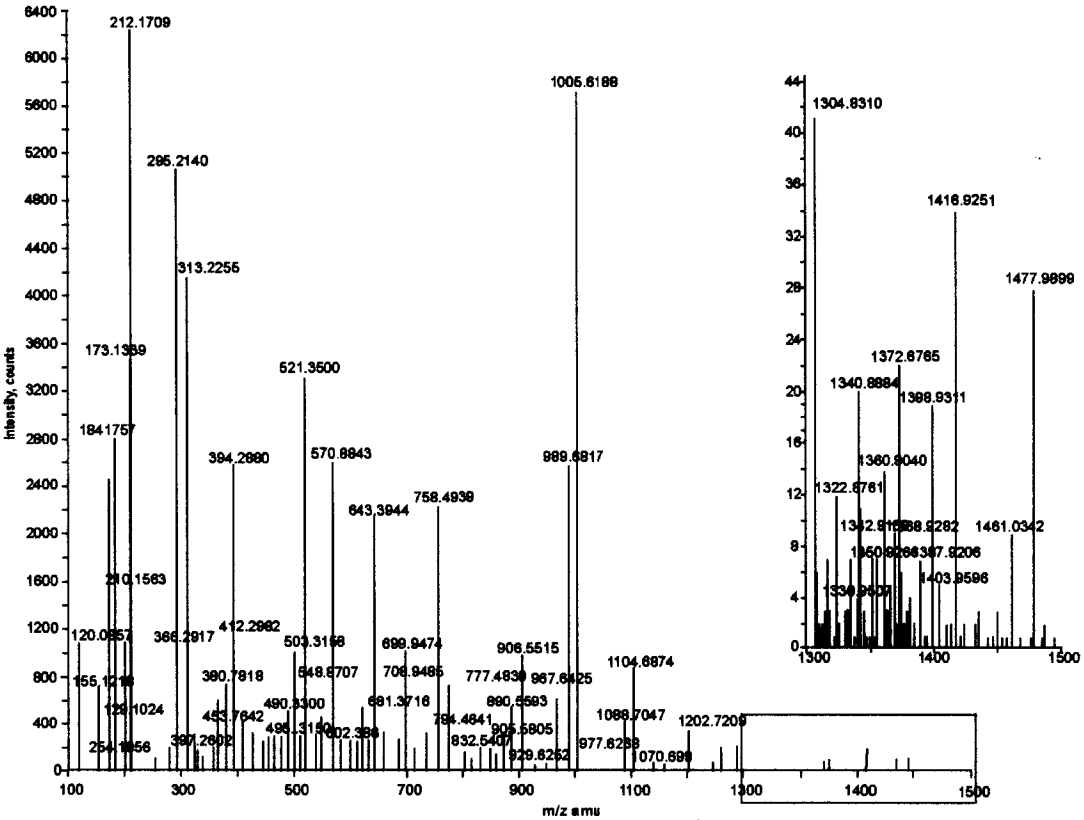


Fig 4e: MS/MS spectra at m/z 758.4938 $[M+K+H]^{+2}$ of kahalalide F (2)

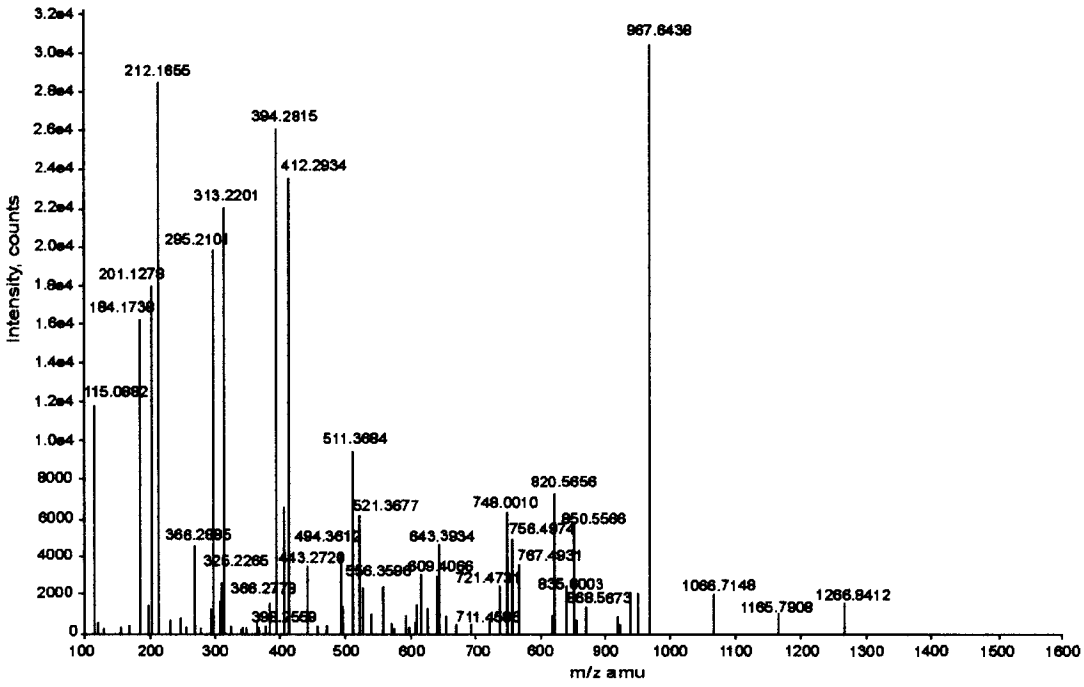
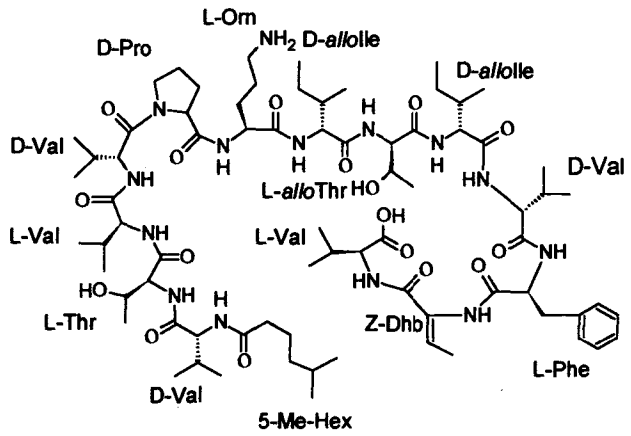


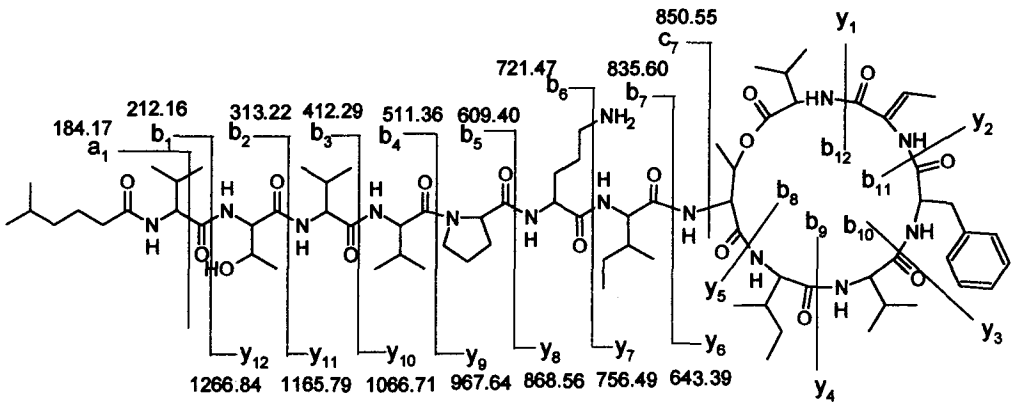
Fig 5: MS/MS spectrum at m/z 748.0010 $[M+2H]^{+2}$ of kahalalide G (3)

3) Mass fragmentation of kahalalide G (3):

The doubly charged molecular ion at m/z 748.0010 for $[M+2H]^{2+}$ corresponds to the molecular mass 1496 which is for linear peptide kahalalide G **(3)**.[12] The MS/MS experiment of doubly charged ion m/z 748.00 was carried out at 25V, which showed identical b and y fragment ions as that of kahalalide F **(2)**. This suggests that there is initial loss of water from the molecule, which results in the cyclisation of the peptide via an ester bond between the threonine (sixth residue from the C-terminal) and carboxylic group to form cyclic depsipeptide KF **(2)** **(Fig 5 and Table 5)**. Thus, fragmentation pattern is identical to that of **(2)**. Some of the fragments b_5 and y_5 were not observed **(Scheme 5)**.



Kahalalide G (3)



Scheme 5: Fragmentation pattern of $[M+2H]^{2+}$ at m/z 748.00 of kahalalide G (3) after cyclisation.

4) Elucidation of the peptide sequence of new cyclodepsipeptides kahalalide P (4) and Q (5):

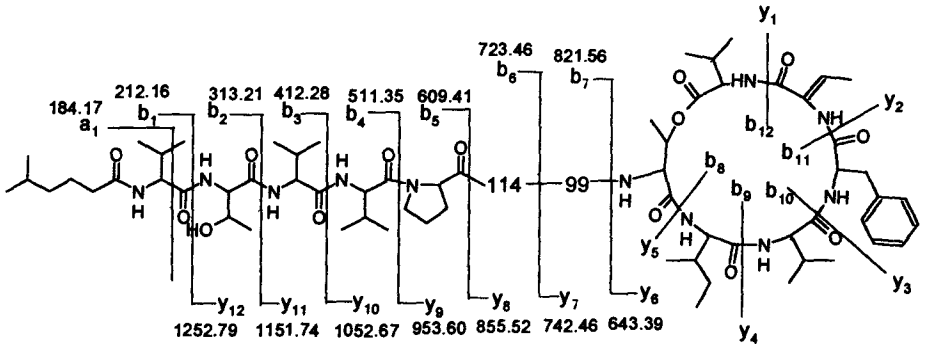
In addition to kahalalide D (1), F (2) and G (3), two new cyclodepsipeptides, kahalalide P (4) and Q (5) were detected showing molecular ion peaks at m/z 1463.9541 and 1491.9876, 14 Da less and 14 Da more than kahalalide F respectively.

4.1) MS/MS at m/z 1463.9541 $[M+H]^+$, 732.4723 $[M+2H]^{2+}$ and 743.4778 $[M+H+Na]^{2+}$ of kahalalide P (4):

The CID spectra of $[M+H]^+$ at m/z 1463.9541 **(Fig 6a)**, $[M+2H]^{2+}$ at m/z 732.4723 **(Fig 6b)** and sodium adduct $[M+H+Na]^{2+}$ at m/z 743.4778 **(Fig 6c)** of kahalalide P **(4)** were studied. Molecular ion $[M+H]^+$ showed peaks at m/z 212.1447 (b_1) 313.2171 (b_2), 412.2843 (b_3), 511.3579 (b_4) and 609.4121 (b_5) identical to those observed in case of kahalalide F. The corresponding y ions were observed at 1252.7944 (y_{12}), 1151.7452 (y_{11}), 1052.6714 (y_{10}), 953.6054(y_9) and 855.5289 (y_8). The fragment ion y_6 at m/z 643.3909 (y_6) was also observed which reveals identical cyclic part as that of KF. This indicated that part of the amino acid sequence in the linear region is identical with 5-Me-Hex-D-Val-L-Thr-L-Val-D-Val- peptide sequence. Since the cyclic region is also identical, the change in the peptide sequence should be in the linear region of the molecule.

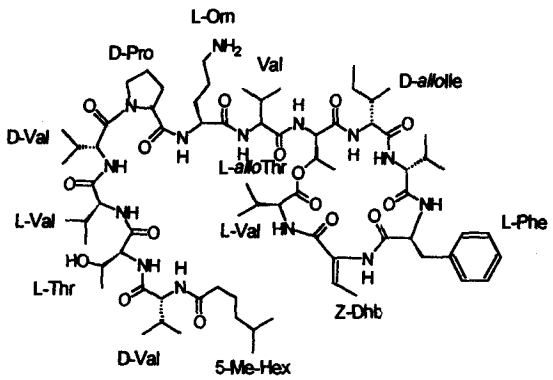
We know that the addition of b and the corresponding y ions gives the molecular mass of the parent molecule. Same theory was applied here to get the total sequence of the molecule. The peak value at m/z 821.5634 and 643.3 (y_6) gives the total of 1464 **(Table 5)**. Thus, peak at m/z 821.5634 should correspond to b_7. Similarly, peaks at m/z 723.4637 and 742.4637 adds up to give total mass of 1463.9 showing that they are either b or y fragment ions. The difference between the 609.4121 (b_5) & 723.4 is 114 and the difference between the 855.5289 (y_8) and 742.4637 is 114, which corresponds to the ornithine residue. Thus, the peak at m/z 723.4675 and 742.4637 was fixed as b_6 and y_7 fragment ions respectively. The mass difference between the 821.5634 (b_6) and 723.4675 (b_7) is 99, which could be accounted for the amino acid valine instead of D-*allo*Ile. Also, the

difference between y_7 and y_6 was exactly 99 amu confirming the presence of valine residue **(Scheme 6)**.



Scheme 6: Fragmentation pattern of $[M+H]^+$ at m/z 1463.9541 of kahalalide P

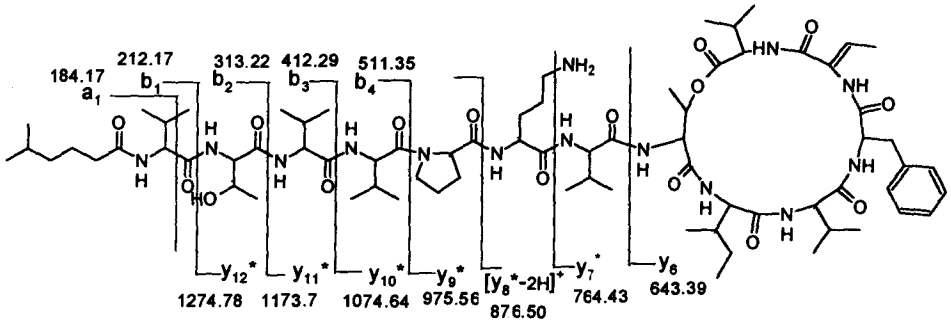
This allowed the accurate identification of the new cyclodepsipeptide kahalalide P **(4)** from the complex mixture of peptides. The proposed structure of **(4)** is given below. According to the nomenclature by Sorin and Florine[60] the one line representation of peptide sequence of kahalalide P is given as cyclo-[LVal-(5MeHex-DVal-LThr-LVal-DVal-LPro-LOrn-Val)L*allo*Thr-DIle-DVal-LPhe-ZDhb]. The stereochemistry of newly added valine is not assigned. The CID of doubly charged ion at m/z 732.4723 also showed similar peaks but were less intense. The peak at 1316.8 was assigned to the loss of phenylalanine from the molecule.



Kahalalide P (4)

In the CID of doubly charged sodiated molecular ion $[M+H+Na]^{2+}$ at m/z 743.4778, **(Fig 6a)** metal Na was found attached to y fragment ions from y_{12}* to y_7* in the linear peptide chain. The y_6 fragment ion is devoid of Na **(Scheme 7)**.

This confirms that Na is bound either to nitrogen or oxygen of valine residue attached to cyclic ring. Immonium ion of phenylalanine is observe at m/z 120. The cyclic ring shows fragment ions identical to those found in $[M+H]^+$.



Scheme 7: Fragmentation pattern of sodium adduct $[M+H+Na]^{2+}$ at m/z 743.4778 of kahalalide P (4).

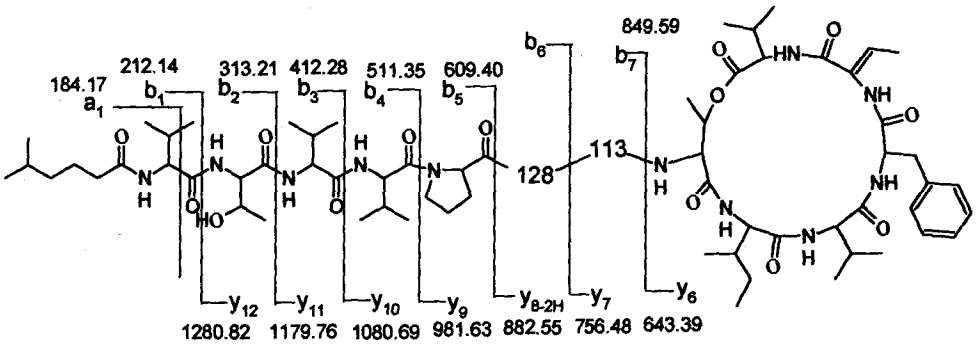
Table 5: Assignment of fragment ions for cyclic depsipeptides, kahalalide G & P.

Fragment ions	Kahalalide-G (3)	Kahalalide P (4)			
	$[M+H]^{2+}$	$[M+H]^+$	$[M+H]^{2+}$		$[M+Na]^{2+}$
	748.0010	1463.9541	732.4723		743.4778
a_1	184.1738			a_1	184.1679
b_1	212.1655	212.1447	212.1640	b_1	212.1600
y_{12}	1266.8412	1252.7944	1252.7418	y_{12}*	1274.7826
b_2	313.2201	313.2171	313.2097	b_2	313.2173
y_{11}	1165.7908	1151.7452	1151.6663	y_{11}*	1173.7
b_3	412.2934	412.2843	412.2844	b_3	412.2827
y_{10}	1066.7146	1052.6714	1052.6339	y_{10}*	1074.6487
b_4	511.3684	511.3579	511.3509	b_4	511.3616
y_9	967.6438	953.6054	953.5745	y_9*	975.5659
b_5	609.4066	609.4121		$[b_5\text{-}2H]^+$	606.3925
c_5	-	625.3814	626.9051	c_5	-
y_8	868.5673	855.5289		$[y_8\text{*-}2H]^+$	876.5036
b_6	721.4731	723.4675	723.4665	b_6	721.4498
c_6	-	-		c_6	-
y_7	756.4974	742.4637		y_7*	764.4381
b_7	835.6003	821.5634		b_7	-
c_7	850.5566	-		c_7	-
y_6	643.3934	643.3909		y_6	
$[M\text{-}Phe]^+$	-		1316.8	$[M\text{-}Phe]^+$	-
$[M\text{-}Val]^+$	-	-		$[M^*\text{-}Val]^+$	1386.9010
A=$[y_6\text{-}LPhe\text{-}2H]^+$	494.3612	-		A=$[y_6\text{-}LPhe\text{-}2H]^+$	494.3116
B=$[A\text{-}Val]^+$	394.2815	394.2715	394.2744	B=$[A\text{-}Val]^+$	394.2794
C=$[B\text{-}Val]^+$	295.2101	295.2031	295.2130	C=$[B\text{-}Val]^+$	295.2075
D=[*Dallo*Ile+ ZDhb]$^+$	-	-		D=[*Dallo*Ile+ ZDhb]$^+$	-
Val*	-	72	72.0770	Val*	72.779
Phe*	-	-		Phe*	120.0816

* Na adduct; Val * and Phe * are for immonium ions

4.2) MS/MS of $[M+H]^+$ at m/z 1491.9876 of kahalalide Q (5):

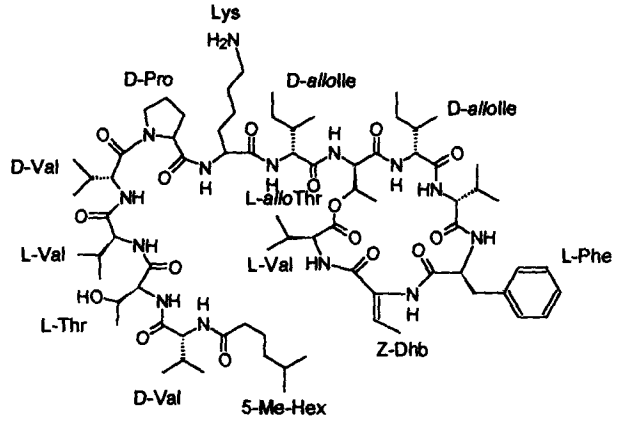
In a similar fashion the MS/MS of the $[M+H]^+$ at m/z 1491.9876 **(Fig 7 and Table 6)** showed identical fragment ions b_1 to b_5 indicating linear peptide chain 5-Me Hex-DVal-LThr-LVal-DVal-D-Pro- and the y_6 peak at m/z 643.4 showed there is no change in the cyclic ring. This indicated that 14 units extra should be present in the linear region of the molecule. The b_7 ion for corresponding y_6 ion peak (m/z 643.3946) was observed at m/z 849.5965 giving giving molecular mass of 1492. The mass difference between 643.3946 (y_7) and newly observed peak at m/z 756.4822 was 113, which is accounted for the presence of D-*allo*Ile. Thus, peak at m/z 756.4822 was assigned for y_7 fragment ion. The corresponding b_6 fragment ion could not be detected in the spectrum. The sum of the peak value at m/z 882.5582 and 609.3977 $[b_5\text{-}2H]^+$ fragment ions gives total of 1492. This indicated peak at m/z 882.5582 corresponds to $[y_8\text{-}2H]^+$ fragment ion. The peak m/z 864.5491 was attributed for loss of ammonia $[y_8\text{-}NH_3]^+$. The mass difference between $[y_8\text{-}2H]^+$ (882.5582) and y_7 (756.4822) give 128, which can be fitted by placing amino acid, lysine in the linear region of the molecule. Peak at m/z 226.1566 was observed for $[Pro+Lys]^+$ which was not observed in the previous peptides.



Scheme 8: Fragmentation pattern of $[M+H]^+$ at m/z 1491.9876 of kahalalide Q (5)

This completes the peptide sequence of kahalalide Q **(5)** and the one line representation is given as cyclo-[Val-(5-MeHex-DVal-LThr-LVal-DVal-DPro-Lys-D*allo*Ile)Thr-Ile-Val-Phe-Dhb] with the cyclic residue. The stereochemistry was not assigned to the lysine residue. Peak at m/z 1374.8921 was assigned to

combined loss of water and valine $[M-(H_2O+Val)]^+$. The proposed structure of kahalalide Q (**5**) is given below.



Kahalalide Q (5)

Table 6: Assignment of fragment ions observed in cyclic depsipeptide kahalalide Q.

	Kahalalide Q (**5**)		
Fragment ions	$[M+H]^+$ m/z 1491.9876	Fragment ions	$[M+H]^+$ m/z 1491.9876
a_1	184.0732	y_7	756.4822
b_1	212.1510	b_7	849.5965
y_{12}	1280.8282	c_7	-
b_2	313.2130	y_6	643.3946
y_{11}	1179.7604	$[M-Phe]^+$	-
b_3	412.2840	$[M-D\ Val]^+$	
y_{10}	1080.6975	$A=[y_6\text{-L Phe-2H}]^+$	-
b_4	511.3550	$B=[A\text{-DVal}]^+$	394.2760
y_9	981.6323	$C=[B\text{-L Val}]^+$	295.2034
b_5	609.3977	D=[D*allo*Ile+ZDhb]$^+$	197.1285
y_8	882.5586	$[M-(H_2O+ Val)]^+$	1374.8921
$[y_8\text{-}NH_3]^+$	864.5491	$[Pro+Lys]^+$	226.1566
b_6			

Based on the fragment ions observed in the CID spectra and by comparison with the KF fragmentation pattern the sequence of amino acids in the two new cyclic depsipeptide, kahalalide P (**4**) and Q (**5**) were determined. The change in structure and the fragment ions formed in all the peptides are summarized in **Table 7**. Comparing the fragmentaion pattern of all the cyclodepsipeptides it is seen that the y_7 fragment at m/z 643 is common to all the peptides showing identical cyclic ring in kahalalide P (**4**) and Q (**5**).

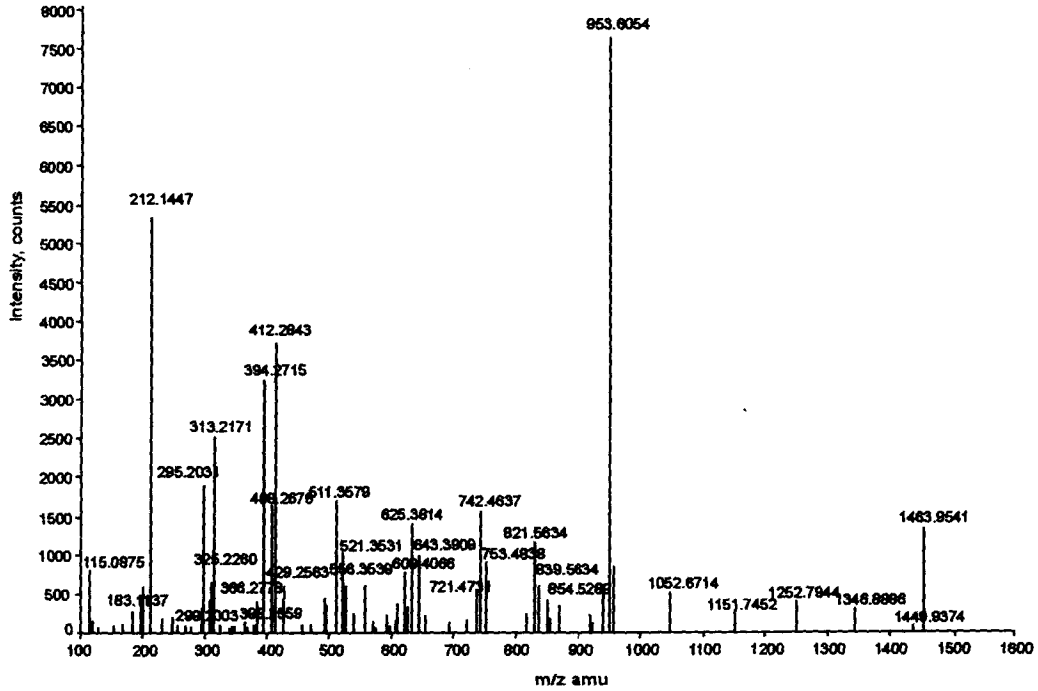


Fig 6a: MS/MS spectrum at m/z 1463.9541 $[M+H]^+$ of kahalalide P (4)

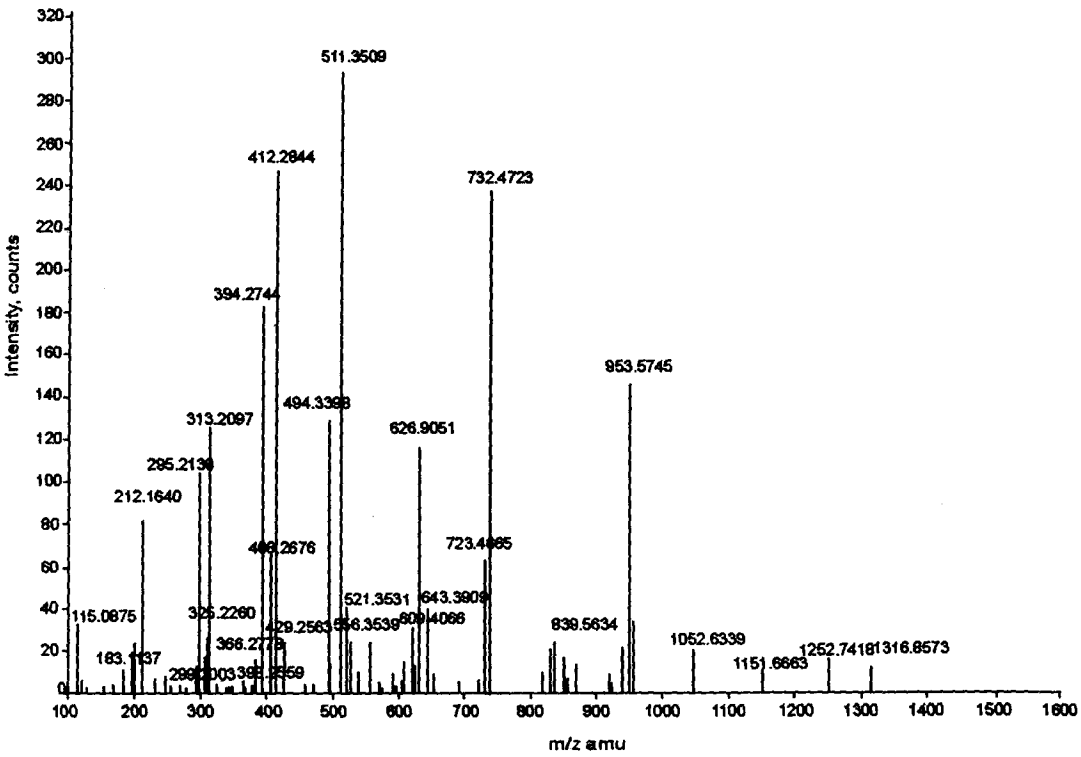


Fig 6b: MS/MS spectrum at m/z 732.4723 $[M+2H]^{2+}$ of kahalalide P (4)

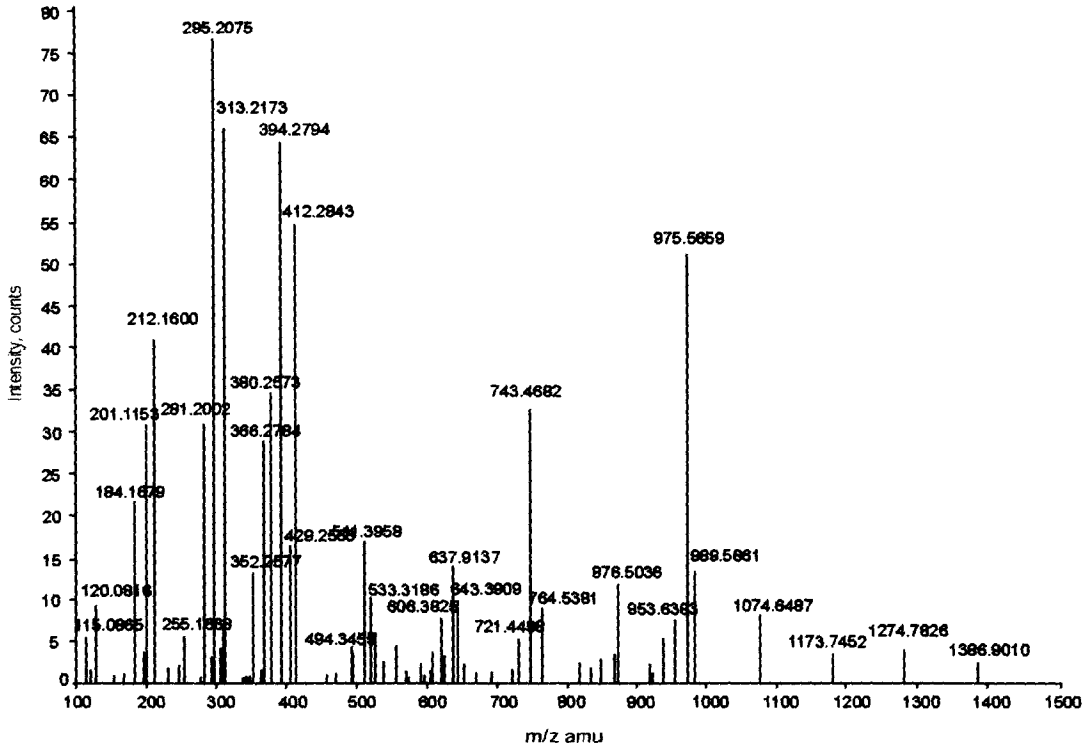


Fig 6c: MS/MS spectrum at m/z 743.4778 $[M+Na+H]^{+2}$ of kahalalide P (4)

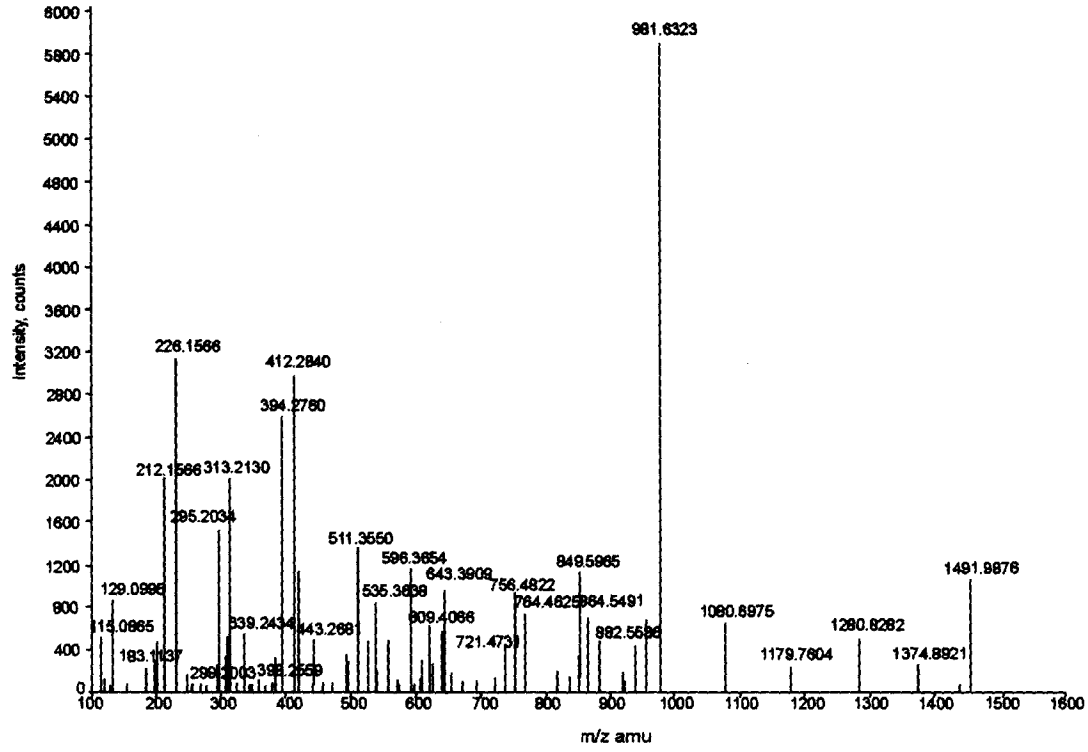


Fig 7: MS/MS spectrum at m/z 1491.9876 $[M+H]^{+}$ of kahalalide Q (5)

Table 7: Conclusion of fragmentation and sequence of the linear chain deduced from +1 and +2 charged fragments.

Parent Fragment	**Found peaks and assigned sequence**	
	b type fragments (m/z)	**y type fragments (m/z)**
Kahalalide F **(2)** 1477.9 $C_{75}H_{124}N_{14}O_{16}$	b_1 b_2 b_3 b_4 b_5 b_6 b_7 5MeHex-Val- Thr- Val- Val- Pro- **Orn-** **Ile-** R 212 313 412 511 609 **723** **835**	y_{12} y_{11} y_{10} y_9 y_8 y_7 y_6 5MeHex- Val- Thr- Val- Val- Pro- **Orn-** **Ile-** R 1266 1165 1066 967 868 756 643
Kahalalide G **(3)** 1496.9 $C_{75}H_{126}N_{14}O_{17}$	5MeHex-Val- Thr- Val- Val- Pro- **Orn-** **Ile-** R' 212 313 412 511 609 **723** **835**	5MeHex- Val- Thr- Val- Val- Pro- **Orn-** **Ile-** R' 1266 1165 1066 967 868 756 643
Kahalalide P **(4)** 1463.9 $C_{74}H_{122}N_{14}O_{16}$	5MeHex-Val- Thr- Val- Val- Pro- **Orn-Val-** R 212 313 412 511 609 **723** **821**	5MeHex- Val- Thr- Val- Val- Pro- **Orn-** **Val-** R 1252 1151 1052 953 $-NH_3$ 742 643 839
Kahalalide Q **(5)** 1492.0 $C_{76}H_{126}N_{14}O_{16}$	5MeHex-Val- Thr- Val- Val- Pro- **Lys-** **Ile-** R 212 313 412 511 609 **764** **849**	5MeHex- Val- Thr- Val- Val- Pro- **Lys-** **Ile-** R 1280 1179 1080 981 882 756 643 $-NH_3$ 864

R-Cyclo (Thr-Ile-Val-Phe-Dhb-Val)

R'-Thr-Ile-Val-Phe-Dhb-Val-OH

Conclusion:

Two new cyclodepsipeptides, kahalalide P (**4**) & Q (**5**) along with the three known compounds kahalalide D (**1**), F (**2**) & G (**3**) were detected and their sequences were established on the basis of spectra. The unreported peptides are close analogues of reported depsipeptide kahalalide F. In case of kahalalide P, amino acid isoleucine is replaced by valine. While in kahalalide Q, ornithine is replaced by lysine. The presence of amino acid lysine was previously reported in kahalalide J.[18] ESI-MS/MS experiments provided data that was difficult to obtain by the NMR technique, because of the low concentration. The mentioned method requires about ten thousand times less raw material than that of NMR based procedure and all experiments including extraction, takes only a few days. On the other hand, basic limitation of mass spectrometric procedure is to determine the configuration of amino acid residues. The present study also indicates that the loss of amino acid residues from depsipeptide is not always sequential.

KF exhibits significant bioactivity and is currently in Phase II clinical trials in Europe. Hence it elicits maximum interest chemically. The two new cyclodepsipeptides, kahalalide P and Q are identified, chemically similar to kahalalide F and may exhibit similar bioactivity. Since the synthetic methods are well known for KF, the new cyclodepsipeptides can be prepared to check for the biological activity. As these compounds are typically administered at very low doses, considerable demands are placed upon the sensitivity of analytical methods to characterize their pharmacokinetic behaviour in humans. Thus, ESI-MS/MS is the detection method of choice for the analysis of cyclodepsipeptides.

Experimental Section:

Material: The mollusk *Elysia grandifolia* and green seaweed *Bryopsis plumosa* were collected from Malvan (16° 50'N, 17° 35'E), east coast of Goa, India.

Extraction and fractionation of mollusk *Elysia grandifolia*:

The mollusk *Elysia grandifolia* was finely ground and extracted with MeOH (1L). The extract was filtered and concentrated under vacuum to obtain crude extract, which was further fractionated with ethyl acetate. The ethyl acetate fraction was subjected to gel chromatography (Sephadex LH-20) using methanol as mobile phase. The fractions obtained were monitored on thin layer chromatography (TLC), alumina backed sheets (Si gel 60 F254, 0.25mm thick) using Butanol:Acetic Acid:Water (5:1:4) mobile phase and sprayed with ninhydrin reagent. The fraction rich in peptides were used for the MS analysis.

Extraction and fractionation of green algae *Bryopsis plumosa*:

Immediately after collection, seaweed was freed from extragenous matter and extracted with methanol. The fractionation and purification procedure were carried out in the same manner as mentioned above.

ESI-QTOF MS/MS spectrometry: The mass spectrometer used was a QTOF-XL MS/MS Applied Biosystem instrument (Canada). The instrument was operated in positive ionization mode. The sample was dissolved in 1:1 MeOH:0.1% TFA. The ESI-MS/MS operating parameters have been discussed in details in **Chapter 2 section 2.3**.

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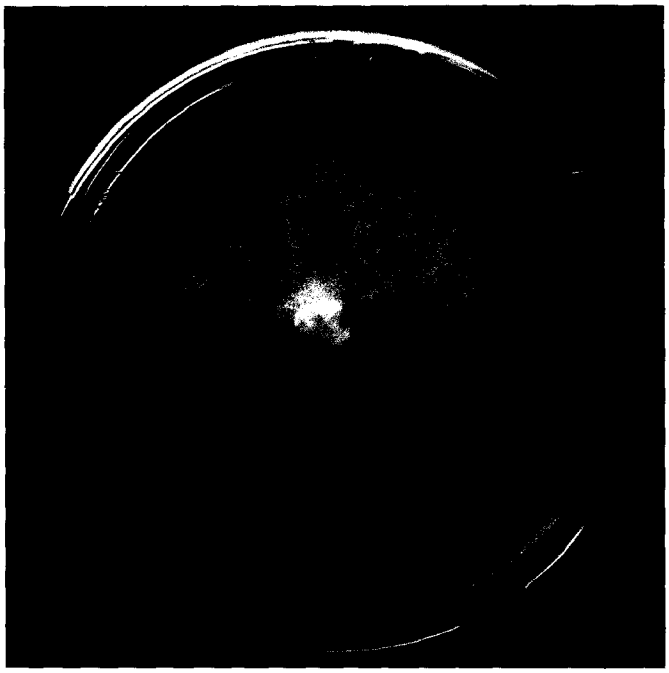
Chapter IV



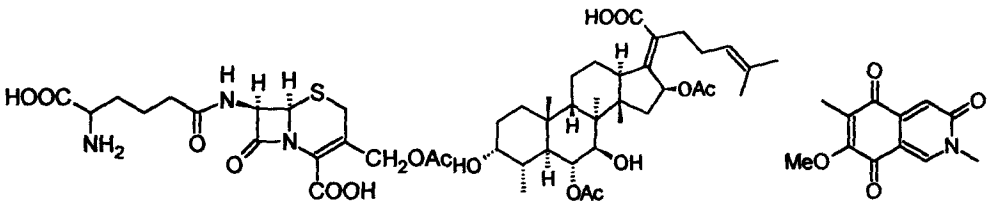
Chemical constituents from marine fungi Eurotium sp.

Introduction:

Marine natural products have complex structures. Hence the only reasonable way of supply is their isolation from the producing organisms. Organisms that could easily be cultured in large amounts would thus be ideal. Such organisms might be marine microorganisms. The fact that marine microorganisms are easily cultured and that they had long been neglected by many marine natural product chemists, has led to an increased research effort in this area during the past one decade. Research has been focused mainly on marine bacteria, fungi and micro algae as reflected by the number of natural products described from each group of organisms.[1-2]

The world oceans do represent a broad and diverse microbial resource of huge dimension but about which we know relatively little. It is estimated that less than 5% of marine bacterial and fungal species are known. The importance of terrestrial bacteria and fungi as sources of valuable bioactive metabolites has been very well established for more than half a century. As a result, over 120 of the most important medicines in use today (penicillins, cyclosporin A, adriamycine, etc.) are obtained from terrestrial microorganisms. At first sight thus, the expectable enormous biodiversity of marine microorganisms might have been the reason for the interest in their study. An additional possible explanation should be that marine microorganisms constituted the ultimate "inviolated" frontier for the search of marine natural products. But although valid, these were not the true starting reasons. Hence, when, how and why did such studies start? The isolation by Brotzu, in the late forties, of the antibiotics cephalosporins C **(I)** and P1 **(II)**, together with other metabolites, from the fungi *Cephalosporium acremonium* cultivated from seawater collected near a sewage outlet off the coast of Sardinia[3] seems well to be the first conclusive work in this area, but it remained an isolated fact and the marine ancestry of such compounds was even claimed to be "dubious".[4] Undoubtedly more important was the suspicion that a number of metabolites obtained from algae and invertebrates could be produced by associated microorganisms. Indeed, it has been frequently suggested, but seldom demonstrated, that microorganisms should be in some instances the true producers

of a number of secondary marine metabolites. Dibromotyrosines from *Aplysia* sponges, halogenated metabolites from *Dysidea sp,* macrolactones and sulfur containing compound were claimed to be probably produced by associated organisms. Aryl carotenoids in sponges were suspected to have originated from inhabiting bacteria.[5] Similarly, it was stated that "there is strong circumstantial evidence that the alkaloids from a species of the genus *Reniera* may be fabricated by a symbiotic microorganism",[6] since mimosamycin **(III)** obtained from the sponges *Reniera sp.*[7] and *Xestospongia sp.*[8] had previously been isolated from the fungi *Streptomyces lavendulae* No. 314.[9]



(I) **(II)** **(III)**

The advantages of the investigation of microorganisms as compared to macroorganisms are obvious: biotechnological fermentations are possible without ecological exploitation, compounds can be reisolated after recultivation in large amounts which is nearly impossible for marine macroorganisms.

Although the occurrence of fungi in the sea has been known since the middle of the 18th century,[10] they received broader interest only during the last six decades. The work of Barghoorn & Linder (1944) provided the first key to marine fungi and stimulated further research in this area.[11]

Substrates used for the isolation of marine-derived fungi for chemical investigations are very diverse. Besides marine sponges, predominantly algae,[12-15] crab shell,[16] fish,[17] mangroves,[18] a sea hare[19] a tunicate[20] and sediment samples[21] were used. So far more than 4000 fungal metabolites are described[22] and 5000-7000 taxonomic species have been studied with respect to their chemistry[23] In 1995 Hawksworth estimated the probable number of existing fungi to be 1.5 million with only 71,000 being described so far. Apparently, the majority of fungi inhabiting the world have not yet been described. This implicates fungi to

represent an enormous source for natural products with diverse chemical structures and 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.[24] Even if the natural function of secondary metabolites often is unknown, it is assumed that they play an important role in chemical defense and communication.[25] Many of them have been suggested to act as pheromones, antifeedants or repellents, and as regulators in the development of organism.[26] Gloer (1995) suggested that the biosynthesis of secondary metabolites does not occur randomly but is correlated with ecological factors.[27] Most fungi studied to date have been isolated from soil and were proven to have a high creativity index, i.e. new and interesting secondary metabolites could be isolated. Genera such as *Aspergillus*, *Penicillium*, *Acremonium*, *Fusarium*, all typical soil isolates, are known for their ability to synthesize diverse chemical structures. Some relatively unexplored fungal groups derived from such ecosystems are fresh-water fungi, marine fungi and endophytic fungi.[24]

Open ocean waters are nutrient-deficient, but coastal waters, in particular mangrove areas, have an abundant supply of all kinds of plant materials for colonization. Studies revealed that mangrove fungi are the second largest group among the marine fungi.[28] Chemical investigations of mangrove associated fungi in search of biologically active natural products began only few years ago hence there is still an enormous task ahead.

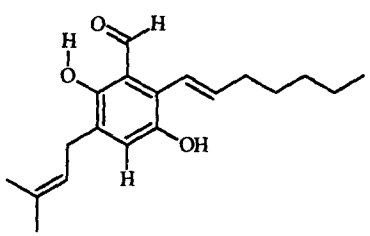
This chapter deals with the chemistry of metabolites from marine fungi *Eurotium sp.* (NIO FMB#001) isolated from leaves of mangrove plant *Porteresia coarctata* (Roxb).

Eurotium sp. belongs to Phylum Ascomycota; Class Ascomycetes; Order Eurotiales and Family Trichocomaceae. They are common and are most closely related to another genus *Aspergillus*. This fungus, frequently encountered in tropical and subtropical regions, is reported from soils, dried or concentrated food products, leather goods, cotton, seeds, and such other dried products. Species of

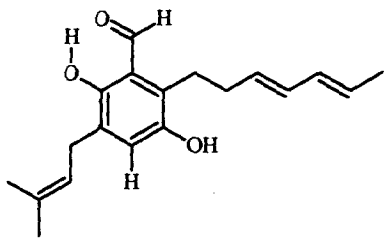
Eurotium grow best in dry situations and are usually cultivated on media high in sucrose or glycerine. They are common in homes, stored grains and rodent dwellings.

The chemical investigation of this fungus resulted in isolation of two antioxidants, tetrahydroauroglaucin **(1)**, isodihydroauroglaucin **(2)**, cyclic peptide (diketopiperazine) echinulin **(3)** and four anthraquinone derivatives, physcion **(4)**, erythroglaucin **(5)**, alaternine **(6)** & catenarin **(7)**. These compounds have been isolated from ethyl acetate soluble extract of fungal mycelia.

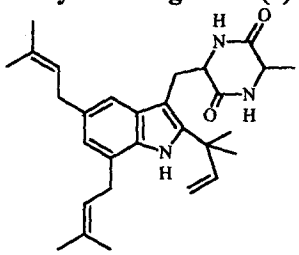
The ethyl acetate fraction was subjected to Sephadex LH-20 (gel permeation chromatography) using MeOH:$CHCl_3$ (1:1) as eluent. Based on TLC profile of eluted fractions four subfractions were obtained. Preliminary NMR data of these fractions indicated that fraction 3 (F-III) is rich in aromatic oxygenated compounds. Fraction- III was further subjected to gel chromatography using Sephadex LH-20 with MeOH:$CHCl_3$ (1:1) as mobile phase which resulted into two fractions F-IIIA and F-IIIB. Extensive silica gel chromatography of each of these fractions yielded compounds 1-7.



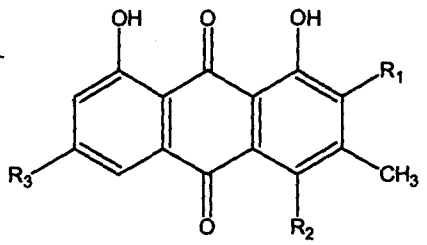
Tetrahydroauroglaucin (1)



Isodihydroauroglaucin (2)



Echinulin (3)



(4) Physcion R_1=R_2=H, R_3=OCH_3
(5) Erythroglaucin R_1=H, R_2=OH, R_3=OCH_3
(6) Alaternin R_1= R_3=OH, R_2=H
(7) Catenarin R_1=H, R_2=R_3=OH

During the elution of silica gel column of fraction F-IIIB with 4% ethyl acetate: petroleum ether, compound **1** was obtained as yellow crystals with m.p. 70°C. The UV spectrum **(Fig 1.1)** showed absorption at λ_{max} 395.5 nm indicating extended conjugation. Its mass spectrum **(Fig 1.8)** displayed strong $[M+H]^+$ and $[M+Na]^+$ peaks at m/z 303.1840 and 325.1655 respectively in accord with the molecular formula of $C_{19}H_{26}O_3$ indicating seven degrees of unsaturation. The IR spectrum **(Fig 1.2)** with absorption at ν_{max} 3265, 2924, 1622, 1606, 1581, 1437, 1489, 983, 958.6, 889, 704 cm^{-1} suggested the presence of hydroxyl, hydrogen bonded conjugated carbonyl groups and aromaticity in the molecule.

The ^{1}HNMR spectrum ($CDCl_3$, 300MHz) **(Fig 1.3)** featured D_2O exchangeable proton signals at δ_H 11.72 (1H, s) and δ_H 5.23 (1H, brs) assigned to one bonded and non-bonded hydroxyl groups. The downfield signal of one of the phenolic protons (δ_H 11.72) as well as the shift in aldehyde absorption frequency in IR spectrum (1622 cm^{-1}) indicated ortho-hydroxy benzaldehyde moiety. The presence of an aldehyde group was further confirmed by the proton signal at δ_H 10.09 (1H, s) and carbon signal at δ_C 196.3 (d) in its NMR spectra. Aromatic proton signal at δ_H 7.02 (1H, s) indicated benzene ring to be penta-substituted. The remaining signals were assigned to the protons of 1-heptenyl and 3-methyl-2-butenyl moieties present in the molecule. Double bond protons were evident as doublets at δ_H 6.48 and doublet of triplet at δ_H 5.97 with the coupling constant J=16.15 Hz indicating its stereochemistry as E. Triplets at δ_H 0.92 (3H, J=6.76Hz), multiplets at δ_H 1.34, 1.36, 1.52 and quartet at δ_H 2.32 were attributed to the terminal methyl, methylenes and for allylic methylene groups. Two singlets at δ_H 1.64 (3H), 1.694 (3H), doublets at δ_H 3.25 and triplets at δ_H 5.22 indicated presence of a dimethyl allyl group (3-methyl-2 -butenyl).

The ^{13}CNMR spectrum showed 19 carbon signals. The multiciplicity of these as evidenced from DEPT experiment **(Fig 1.4)** was found to be three methyls (δ_C 13.9, 17.7, 25.7), five methylenes (δ_C 22.4, 27.2, 28.7, 31.4, 33.4), five methines (δ_C 120.1, 121.0, 125.1, 142.6, 196.3) and six quaternary carbons (δ_C 124.6,

130.1, 133.8, 144.6, 155.1). Among, these signals δ_C 144.6, 155.1 were attributed to the phenolic carbons and δ_C 117.5 for aromatic carbon with formyl group.

The structure of this molecule was finalized as tetrahydroauroglaucin [2-(1E-heptenyl)-3,6-dihydroxy-5-(3-methyl-2-butenyl)-benzaldehyde] from the above data well in agreement with the literature values[29] reported for the compound from *Aspergillus ruber*. It might be mentioned here that only IR, UV and ^{1}HNMR is reported for this compound. The structure was further confirmed by extensive 2DNMR studies including TOCSY **(Fig 1.5 & Fig 1a)**, HMQC **(Fig 1.6)** and HMBC **(Fig 1.7)**, which helped in assignment of all proton and carbon signals **(Table 1)**.

Proton carbon chemical shift correlations were established by HMQC experiments **(Table 1)**. The cross peaks from the HMQC spectrum **(Fig 1.6)** identified the sets of directly bonded carbons and hydrogens. ^{1}H-^{1}H connectivities of aliphatic moieties, 3-methyl-2-butenyl and 1-heptenyl were established by TOCSY spectrum. Methylene protons at δ_H 3.31(H1) showed correlation with methine proton at δ_H 5.29 (H2′) and aromatic proton at δ_H 7.02 (H4). The methyls at δ_H 1.66 (H4′) and δ_H 1.76 (H5′) were in turn correlated with the methylene proton signal at δ_H 3.31(H1′). The terminal methyl at δ_H 0.92 (H7″) showed connectivity with the methylene at δ_H 1.34 (H6″), which was again linked to the methylene at δ_H 1.36 (H5″). This methylene at δ_H 1.34 (H6″) showed connectivity with methylene at δ_H 1.52 (H4″) that was in turn correlated with methylene at δ_H 2.32 (H3″). The quartet at δ_H 2.32 (H3″) was coupled with another methine at δ_H 5.97 (H2″), which in turn is correlated with methine signal at δ_H 6.48 (H1″). Thus, TOCSY spectrum revealed the presence of the 3-methyl-2-butenyl and 1-heptenyl units. These subunits were linked together with the help of long-range correlation HMBC experiments as shown in **Fig 1b.**

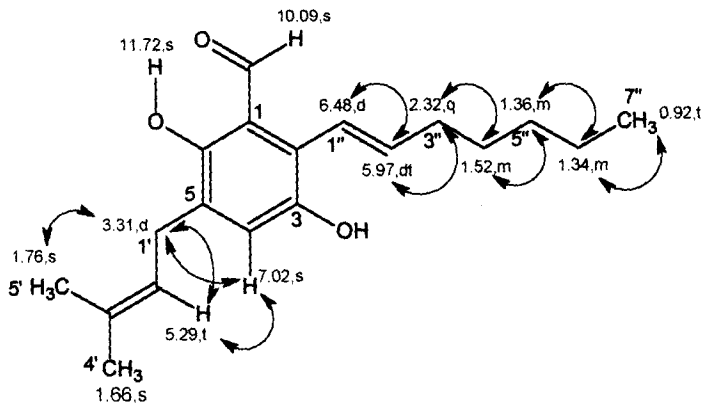


Fig 1a: TOCSY correlations of tetrahydroauroglaucin (1).

The HMBC **(Fig 1.7)** showed proton signal at δ_H 6.48 was connected to δ_C 117.1(C1), 124.6(C2), 144.6(C3) of the aromatic ring and δ_C 33.4(C3″) of 1-heptenyl side chain. Based on this the position of the 1-heptenyl was confirmed at δ_C 124.6(C2). Aromatic proton at δ_H 7.02 was connected to δ_C 124.6(C2), 144.6(C3), 130.2(C5), 155.1(C6) of aromatic ring and 27.2(C1′) of 3-methyl-2-butenyl side chain. Doublet at δ_H 3.31 showed connectivities with δ_C 125.1(C4), 130.2(C5), 155.1(C6) and 121.0(C2′), 133.8(C3′) of the side chain. This confirmed that the side chain 3-methyl-2-butenyl is connected at C5 position. Aldehydic proton was linked to δ_C 117.1(C1), 130.2(C5) and 155.1(C6). All the connectivities were in agreement with the derived structure. The mass spectrum in addition to the molecular ion peaks showed peaks at m/z 282 $[M+H+Na-43]^+$ for loss of propyl group.

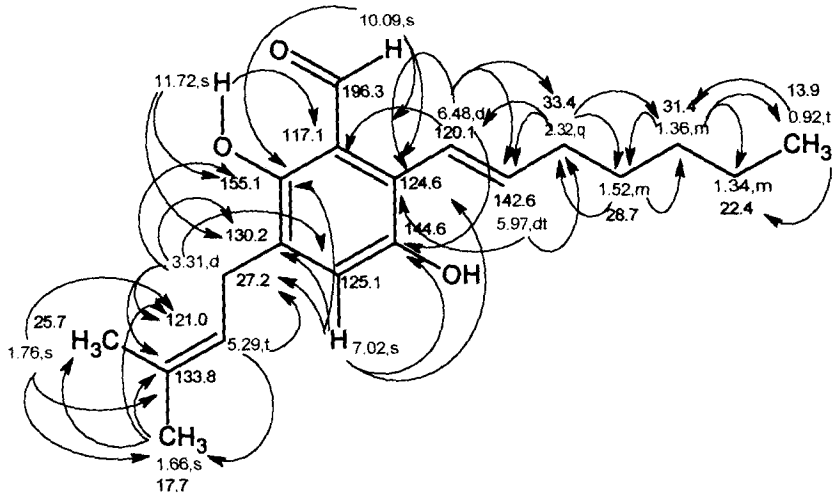


Fig 1b: HMBC correlations of tetrahydroauroglaucin (1).

Table 1: ^{1}H, $^{13}CNMR$, TOCSY and HMBC correlations of tetrahydroauroglaucin (1) in $CDCl_3$

Carbon no.	$^{13}CNMR$ δ_H, ppm	$^{1}HNMR$ δ_H, ppm	TOCSY	HMBC correlations
CHO	196.3	10.09(1H,s)		C1, C5, C6
1	117.1			
2	124.6			
3	144.6			
4	125.1	7.02 (1H,s)	H1′, H2′	C1, C2, C3, C5, C6, C1′
5	130.2			
6	155.1			
1′	27.2	3.31 (2H,d, J= 7.44Hz)	H4, H2′, H4′, H5′,	C4, C5, C6, C2′, C3′
2′	121.0	5.29(1H,t, J=7.73Hz)	H1′	C1′, C4′
3′	133.8			
4′	17.7	1.66 (3H,s)	H1′	C2′, C3′, C5′
5′	25.7	1.76 (3H,s)	H1′	C2′, C3′, C4′
1″	120.1	6.48(1H,d, J=16.15Hz)	H2″	C1, C2, C3, C3″
2″	142.6	5.97(1H,dt, J=16.12,6.8Hz)	H1″, H3″	C2, C3″
3″	33.4	2.32 (2H,q, J=7.02,6.79Hz)	H2″, H4″	C1″, C2″, C4″, C5″
4″	28.7	1.52 (2H,m)	H3″, H5″	C3″, C5″
5″	31.4	1.36(2H,m)	H4″, H6″	C4″, C6″, C7″
6″	22.4	1.34 (2H,m)	H5″, H7″	C4″, C5″, C7″
7″	13.9	0.92 (3H,t, J=6.76Hz)	H6″	C5″, C6″
1-OH		11.72 (1H,s)		C1, C5, C6
3-OH		5.23(1H,s, exchanged with D_2O)		

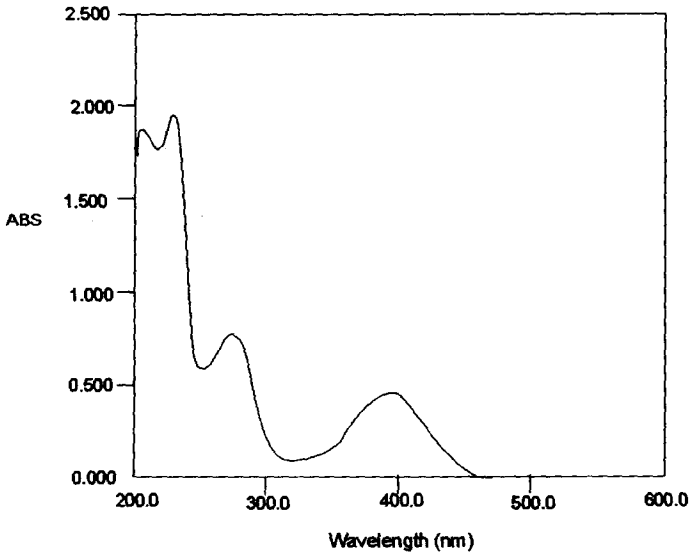


Fig 1.1: UV spectrum of tetrahydroauroglaucin (1)

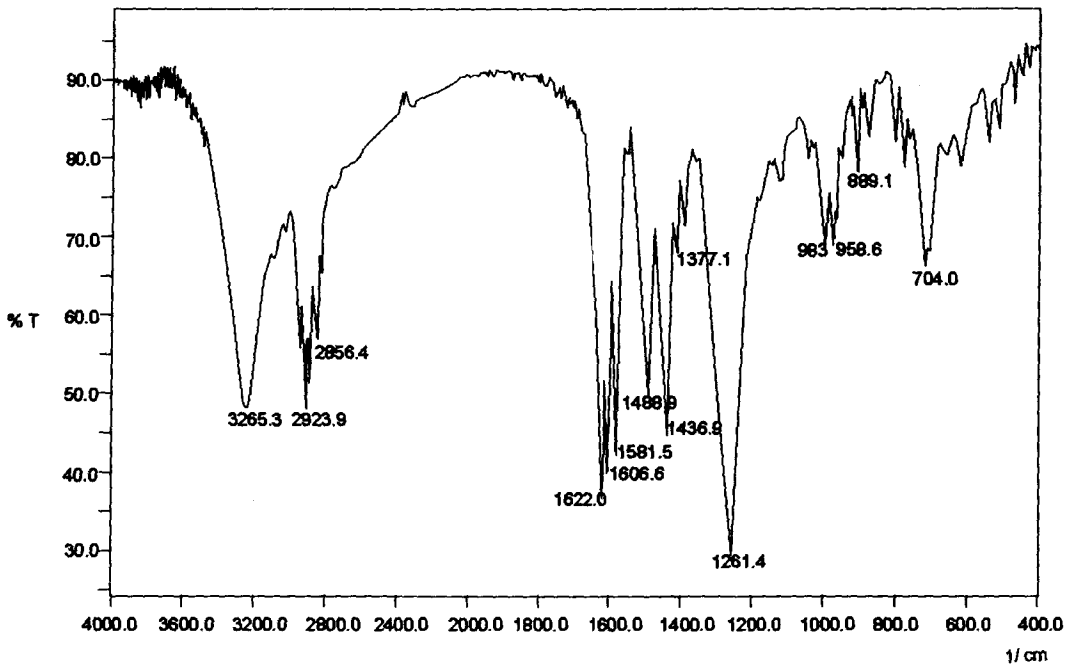


Fig 1.2: IR spectrum of tetrahydroauroglaucin (1)

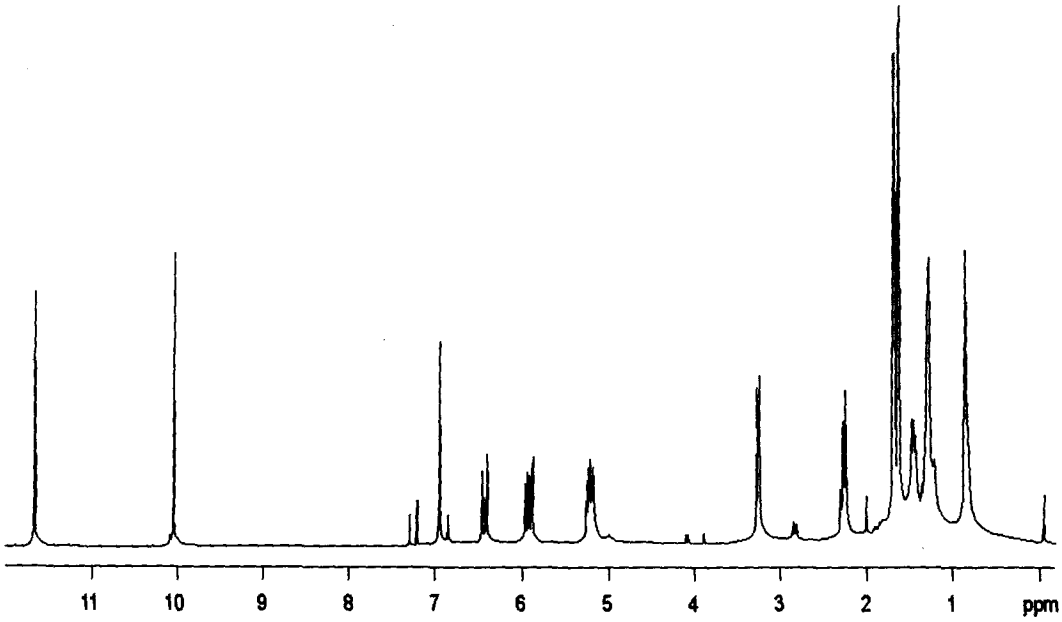


Fig 1.3: ^{1}HNMR spectrum of tetrahydroauroglaucin (1)

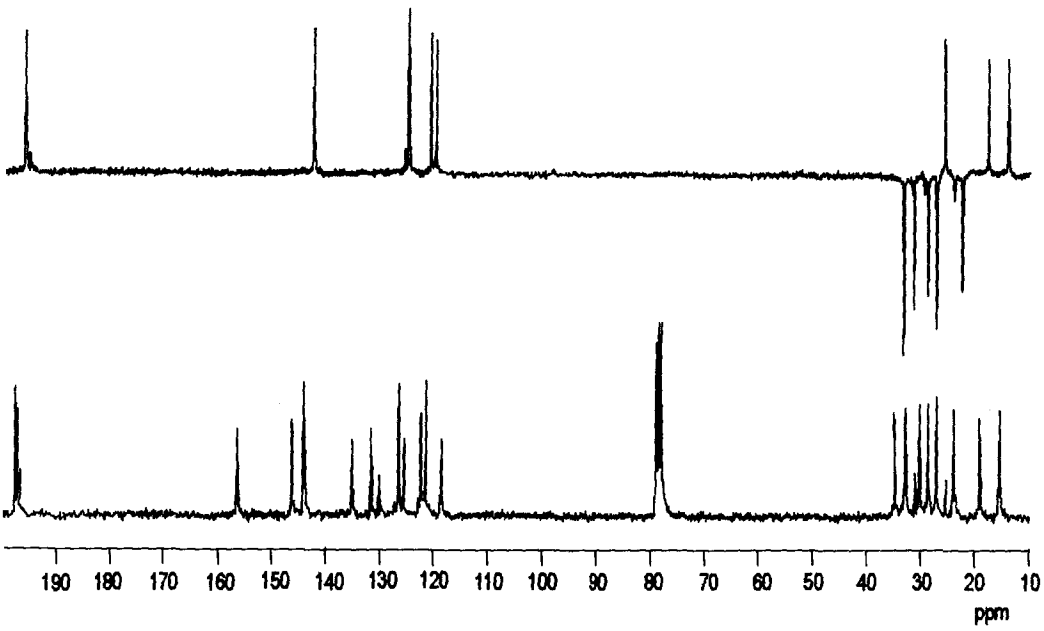


Fig 1.4: ^{13}CNMR and DEPT spectra of tetrahydroauroglaucin (1)

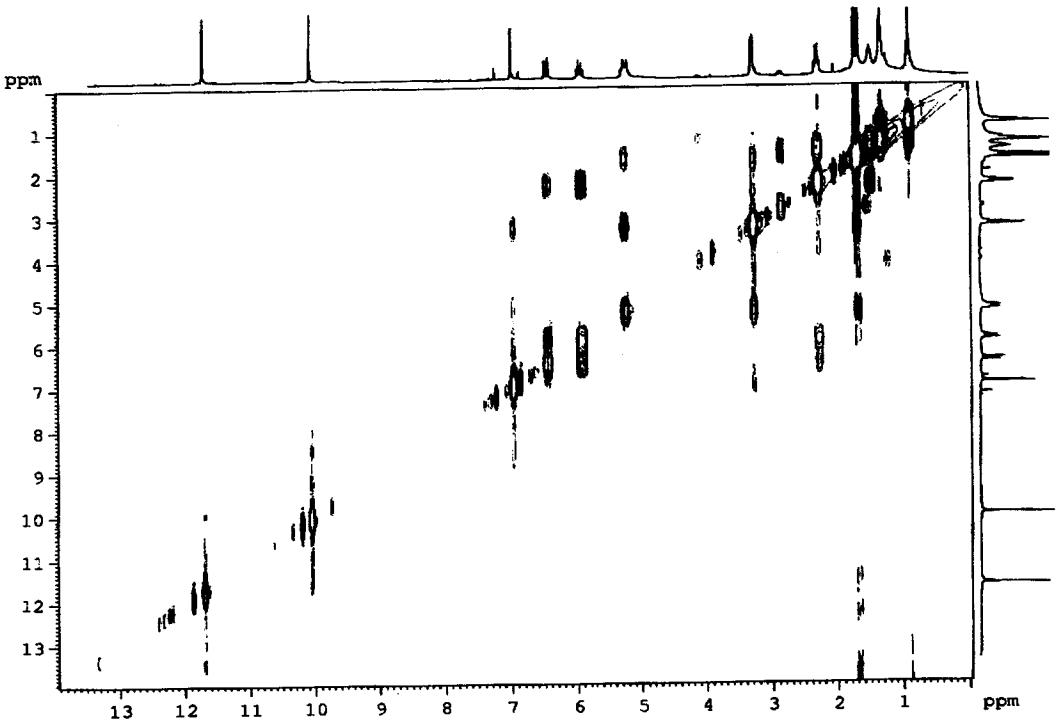


Fig 1.5: TOCSY spectrum of tetrahydroauroglaucin (1)

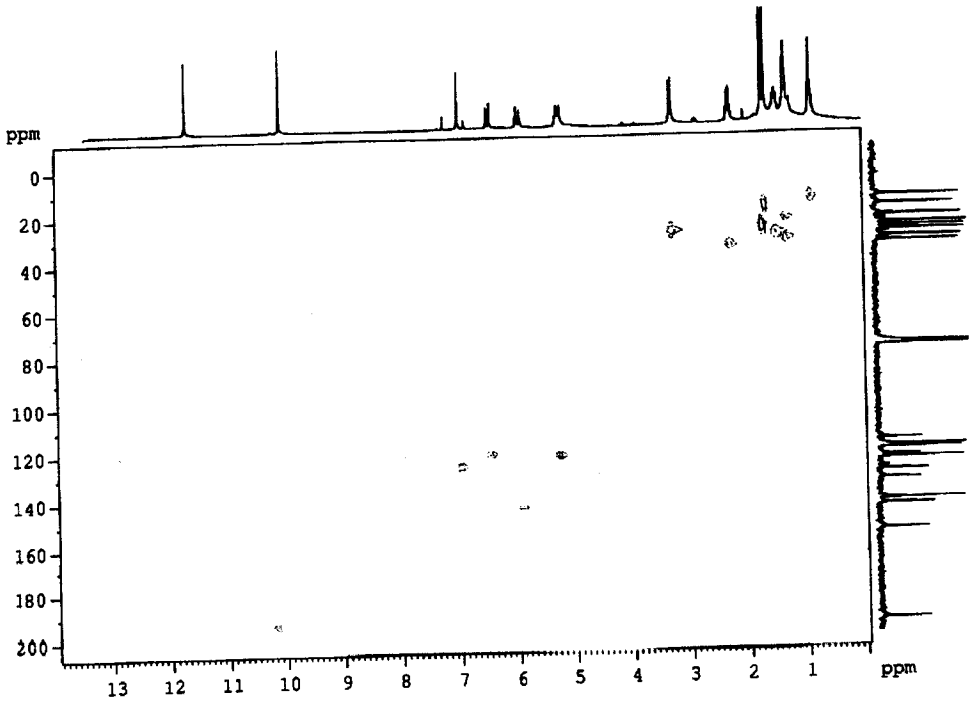


Fig 1.6: HMQC spectrum of tetrahydroauroglaucin (1)

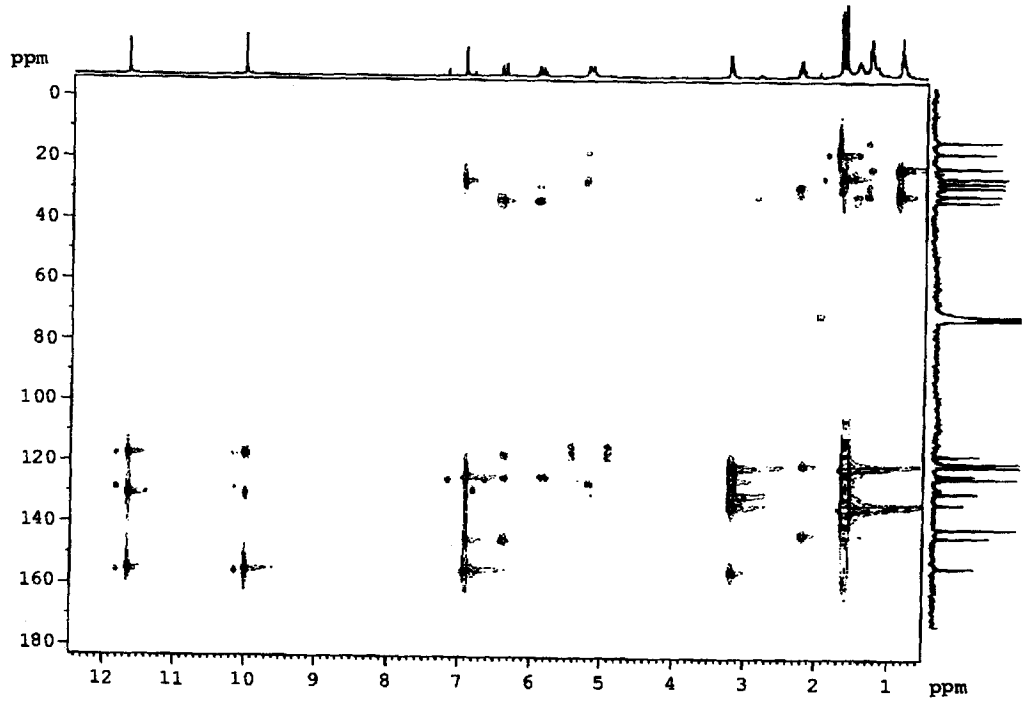


Fig 1.7: HMBC spectrum of tetrahydroauroglaucin (1)

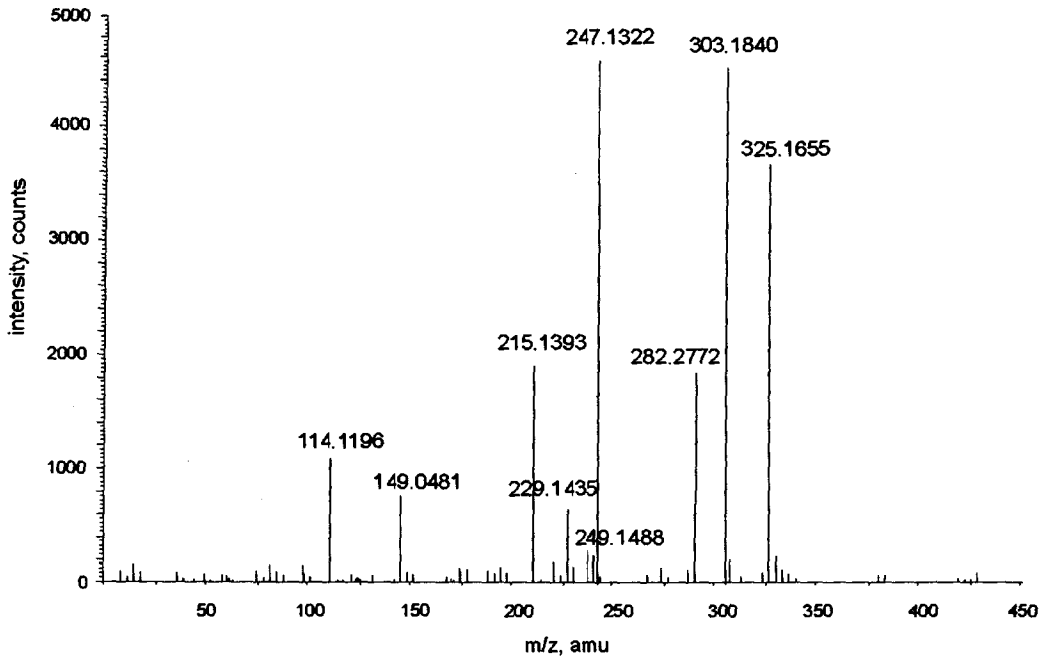


Fig 1.8: ESI-MS spectrum of tetrahydroauroglaucin (1)

Compound **(2)**, isodihydroauroglaucin, was obtained as orange crystals (m.p. 114 °C), on elution of silica gel column with 20% ethyl acetate: petroleum ether. Its UV spectrum **(Fig 2.1)** showed absorption bands at λ_{max} 202.6, 223.2, 249.6, 265.0 & 285 nm. IR spectrum **(Fig 2.2)** displayed bands at ν_{max} 3417, 2925, 2854, 1643, 1434, 1296, 1128, 993, and 761 cm^{-1} for the presence of hydroxyl, hydrogen bonded conjugated carbonyl groups and aromaticity.

The mass spectrum **(Fig 2.8)** showed strong molecular ions at m/z, 301 $[M+H]^+$, 323 $[M+Na]^+$ and m/z 339 $[M+K]^+$ indicating its molecular weight to be 300, differing from compound **1** by two mass units. The molecular formula was determined to be $C_{19}H_{24}O_3$. Its ^{1}HNMR spectrum **(Fig 2.3)** differed from that of **(1)** in the terminal methyl group of C7 chain appearing as a doublet (δ_H 1.78, J=7Hz) and presence of two conjugated double bonds in the chain. This is also confirmed by ^{13}CNMR and DEPT spectra **(Fig 2.4)** where a downfield shift of δ_C 13.9 (t) to signal at δ_C 17.9 (t) is observed. This indicated that the side chain of 1-heptenyl in (1) is replaced by 3,5-heptadienyl side chain. The multiplets at δ_H 5.62 (2H), 5.99 (2H) and ^{13}C values at δ_C 131.8, 128.8, 129.3 & 131.1 were attributed to the presence of two double bonds. The structure of this compound was finalized as 2-(3E,5E-heptadienyl)-3,6-dihydroxy-5-(3-methyl-2-butenyl)-benzaldehyde **(2)** from the above data. The stereochemistry of these double bonds was assigned as E, in agreement with the structure of isodihydroauroglaucin reported earlier for the compound from *Aspergillus ruber*.[29]

Extensive COSY **(Fig 2.5)**, HMQC **(Fig 2.6)** and HMBC **(Fig 2.7)** correlation studies **(Table 2)** confirmed the above structure. The COSY spectrum **(Fig 2.4)** showed doublet at δ_H 1.78 was coupled with multiplet at δ_H 5.62, which in turn coupled with δ_H 5.99 (H7″-H6″-H5″). The methylene signal at δ_H 2.37 (H2″) was coupled with methylene at δ_H 2.81 (H1″) and methine proton at δ_H 5.62 (H3″). Thus, COSY spectrum showed double bonds to be connected to the terminal methyl group and not in conjugation with the aromatic ring. This is also evident in the IR spectrum **(Fig 2.2)** which showed shift in absorption from 1622 to 1643

cm^{-1} because of less conjugation. The olefinic proton at δ_H 5.27 showed a strong COSY correlation to a methyl proton resonating at δ_H 1.73 and methylene resonating at δ_H 3.34. Methylene proton at δ_H 3.34 in turn shows crosspeak with methyl proton at δ_H 1.83. This confirms the side chain as 3-methyl-2-butenyl **(Fig 2a)**.

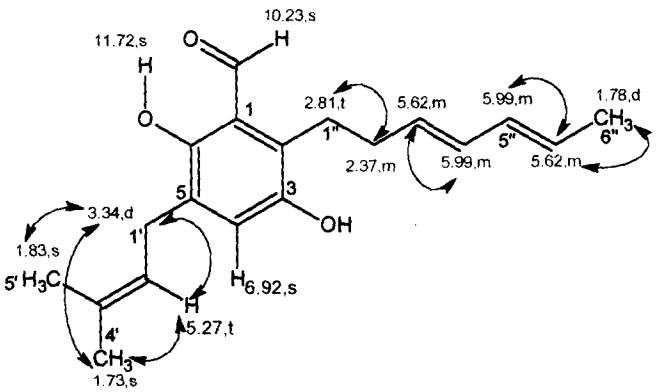


Fig 2a: COSY correlations of isodihydroauroglaucin (2).

The HMBC correlation of the methylene protons (δ_H 2.81) with aromatic carbons at δ_C 117.2 (C1), 127.4 (C2) and 145.1 (C3) further supported that the aromatic ring is not in conjugation with the double bond. Similarly the aromatic proton at δ_H 6.92 showed $^2J_{CH}$/ $^3J_{CH}$ correlations to δ_C 145.1 (C3), 127.4 (C2) and 155.7 (C6). All the HMBC proton-carbon connectivities are shown in **Fig 2b.**

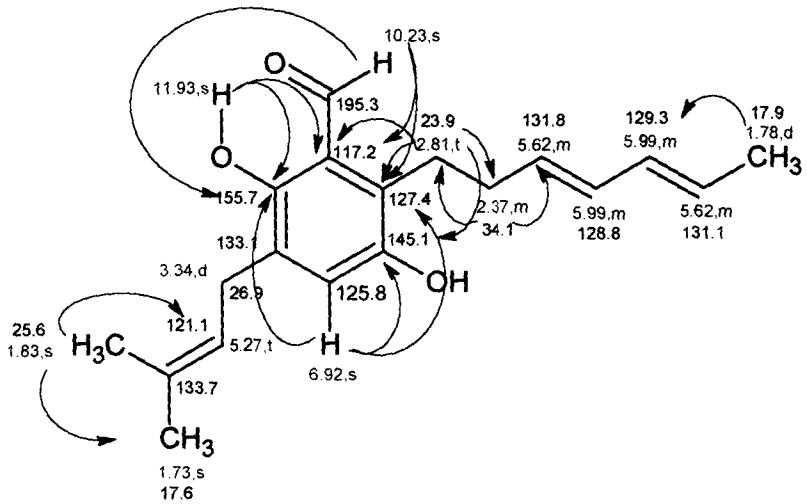


Fig 2b: HMBC correlations of isodihydroauroglaucin (2).

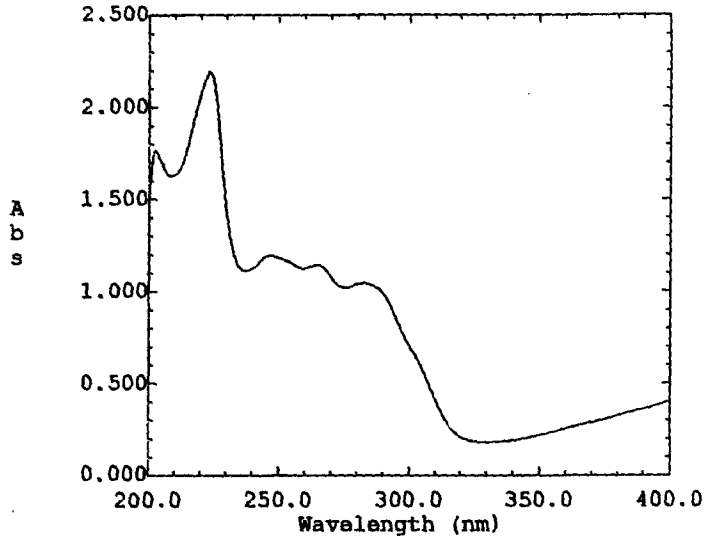


Fig 2.1: UV spectrum of isodihydroauroglaucin (2)

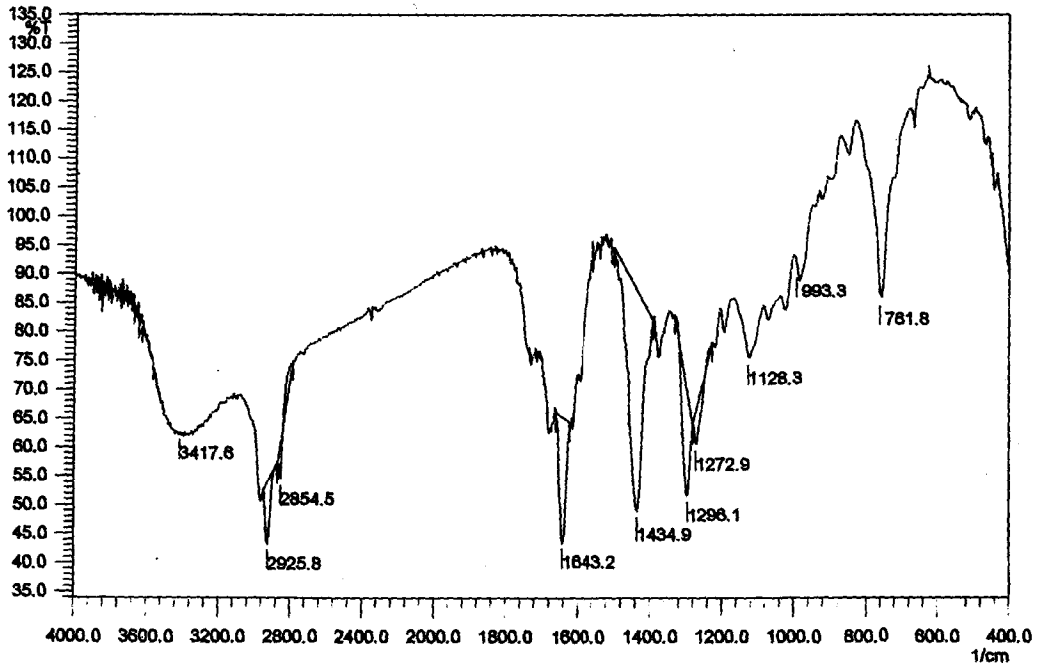


Fig 2.2: IR spectrum of isodihydroauroglaucin (2)

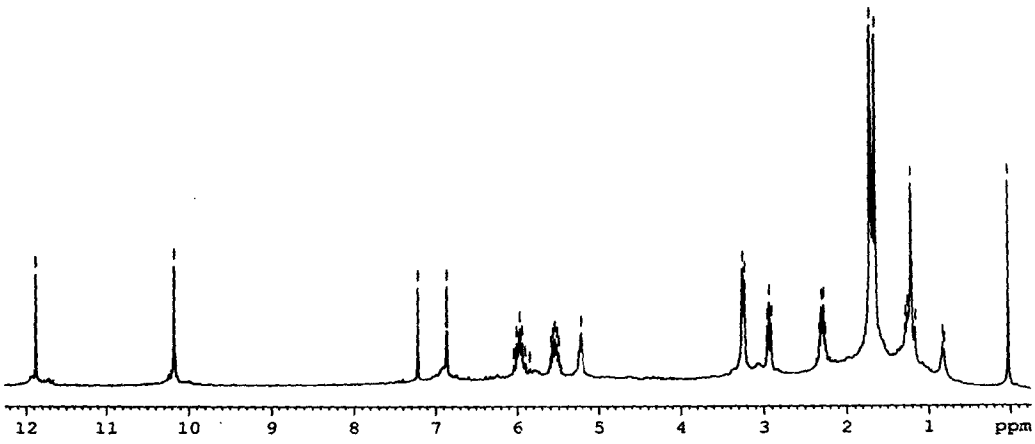


Fig 2.3: ^{1}HNMR spectrum of isodihydroauroglaucin (2)

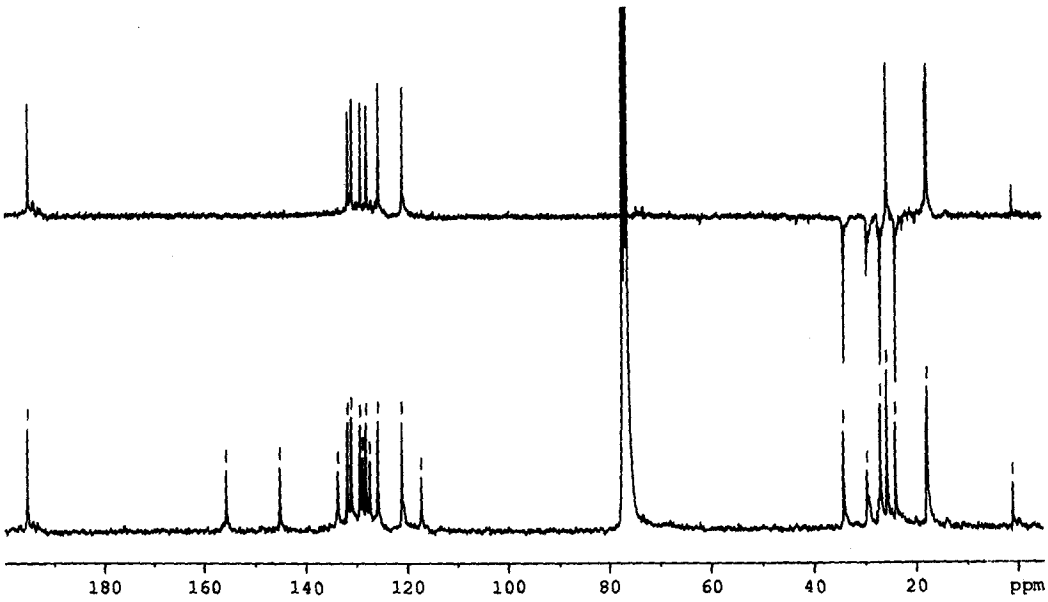


Fig 2.4: ^{13}CNMR and DEPT spectra of isodihydroauroglaucin (2)

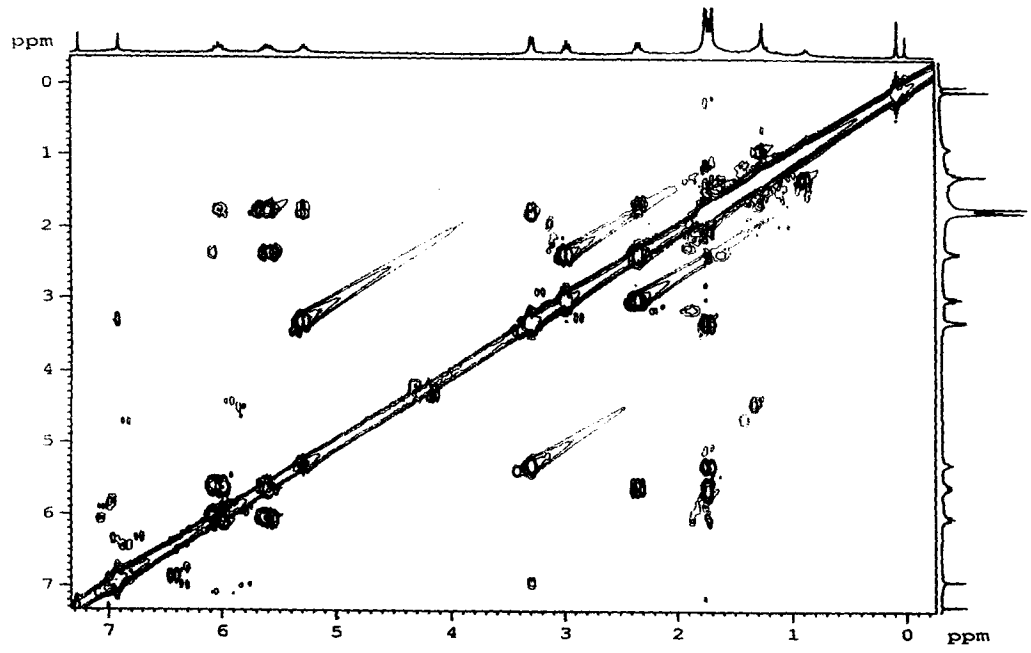


Fig 2.5: COSY spectrum of isodihydroauroglaucin (2)

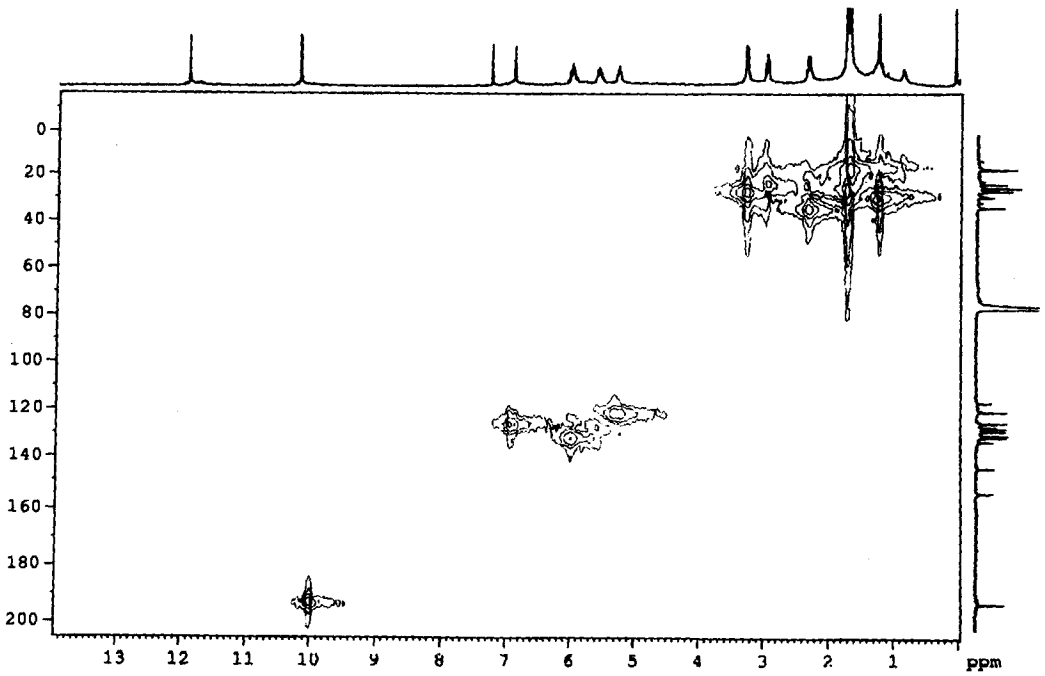


Fig 2.6: HMQC spectrum of isodihydroauroglaucin (2)

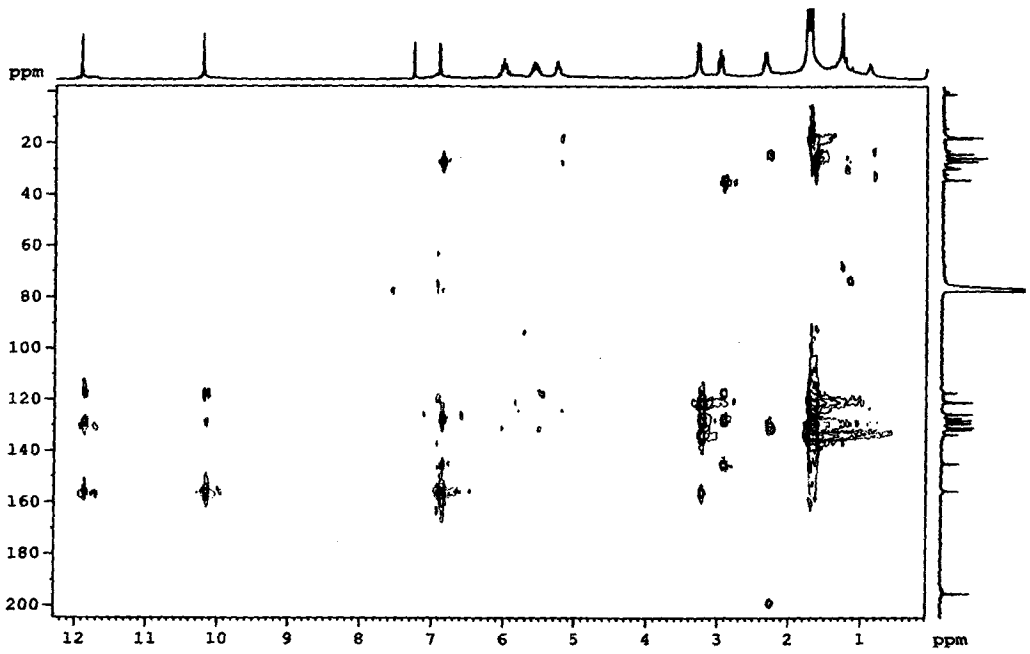


Fig 2.7: HMBC spectrum of isodihydroauroglaucin (2)

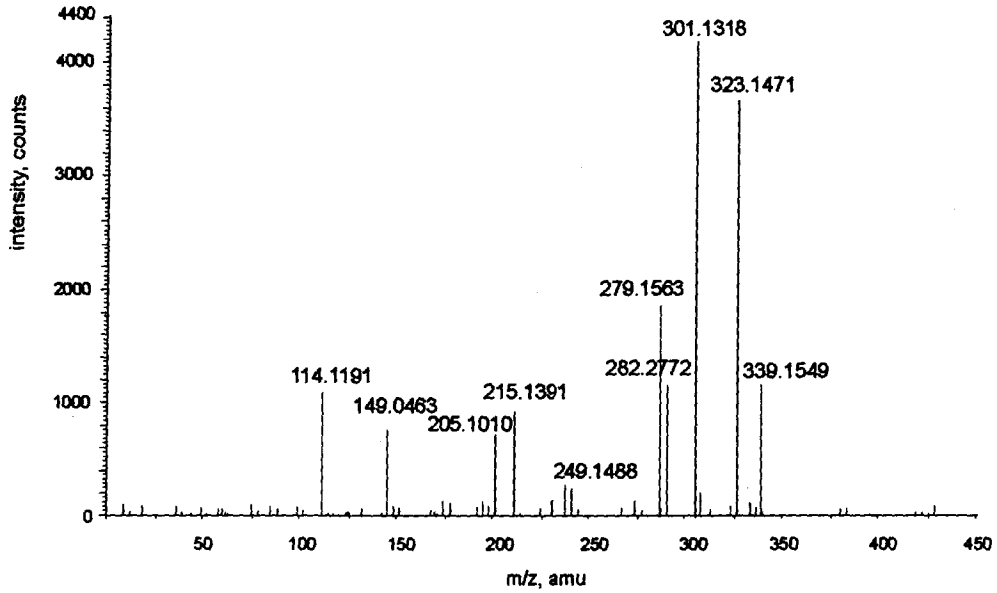


Fig 2.8: ESI-MS spectrum of isodihydroauroglaucin (2)

Table 2: ^{1}H, $^{13}CNMR$, COSY and HMBC correlations of isodihydroauroglaucin (2)

Carbon no.	^{13}C NMR δ, ppm	^{1}H NMR δ, ppm	COSY	HMBC correlations
CHO	195.3	10.23 (1H,s)		C1, C2, C6
1	117.2			
2	127.4			
3	145.1			
4	125.8	6.92 (1H,s)		C2, C3, C6
5	133.1			
6	155.7			
1′	26.9	3.34 (2H,d, J=7.18Hz)	H2′	C2′, C3′, C6
2′	121.1	5.27 (1H,t, J=6.09Hz)	H1′, H4′, H5′	----------
3′	133.7			
4′	17.6	1.73 (3H,s)	H1′	C2′, C3′, C7″
5′	25.6	1.83 (3H,s)	H1′	C2′, C3′, C4′
1″	23.9	2.81(2H,t, J=7.30Hz)	H2″	C1, C2, C3, C2″
2″	34.1	2.37(2H,m)	H1″	C1″, C6″
3″	131.8	5.62(1H,m)	H4″	----------
4″	128.8	5.99(1H,m)	H3″	----------
5″	129.3	5.99(1H,m)	H6″	----------
6″	131.1	5.62(1H,m)	H5″	----------
7″	17.9	1.78(3H,d, J=7.0H)		C5″
OH	-------	11.93(1H,s)		C1, C2, C6, C4″
		4.70(1H,s, exchanged with D_2O)		

Compounds **1** & **2** are typical fungal metabolites known to possess anti-oxidant properties and exhibit synergism with tocopherol.[30] They were initially isolated from terrestrial fungi belonging to the genus *Aspergillus* and *Eurotium*. This is the first report of the isolation of these compounds from a marine fungus, also belonging to the genus *Eurotium*. Earlier the structures of these compounds were established exclusively on the basis of IR and 1D NMR studies particularly ^{1}HNMR. The present study not only confirmed the structures of these two compounds but also helped in assigning all the proton and carbon signals unambiguously based on extensive 2DNMR experiments mentioned above.

The compound **(3)** was obtained from fraction, F-IIIA by elution of silica column with 5% methanol in chloroform. The mass spectrum of **(3)** showed molecular ion $[M+H]^+$ at m/z 462, $[M+Na]^+$ at m/z 484 in the positive mode and m/z $[M-H]^-$ m/z

460 in the negative mode. The molecular formula was calculated to be $C_{29}H_{38}N_3O_2$. A close inspection of the ^1H **(Fig 3.2),** $^{13}CNMR$ & DEPT spectra **(Fig 3.4)** and HMQC **(Fig 3.6)** experiments disclosed signals for 29 carbons. These included one secondary methyl (14-Me), two tertiary methyls (C2′ and C3′), four sp^3 hybridised methyls (C4″, C5″, C4‴ and C5‴), one sp^3 methylene (C1″, C1‴, C10), one sp^2 hybridised methylene (C5′), one sp^3 hybridised methine (C14), five sp^2 methines (C5, C6, C2″, C2‴ and C4′), one sp^3 quaternary carbon (C1′), ten sp^2 quaternary carbons including amide carbonyls (C2, C3, C4, C6, C8, C9, C1′, C3″, C3‴, C12 and C15). The presence of amide groups were inferred from signals at δ_C 168.34 and 167.73 from its $^{13}CNMR$ spectra ($CDCl_3$), sharp and strong IR **(Fig 3.1)** absorptions at 3350, 1664 and 1678 cm^{-1}, further confirmed by the presence of two D_2O exchangeable protons at δ_H 5.62 and 5.94 **(Fig 3.3).**

The coupling of the doublet signal for H14 at δ_H 1.48 (J=7Hz) to the signal for CH_3 at δ_H 4.04 (1H, br q, J=7Hz) in the COSY experiment **(Fig 3.5)** indicated presence of the amino acid alanine moiety in the molecule. Amine signal at δ_H 5.62 (NH) is coupled with δ_H 4.34 (dd) which is inturn coupled with two hydrogens each of methylene groups at δ_H 3.13 (dd) and 3.63 (dd). In the HMBC experiment **(Fig 3.7)** singlet at δ_H 5.62 (NH) showed connectivities to amide carbonyl δ_C 168.34 and the methine C16 at δ_C 54.60. A doublet at δ_H 1.48 showed HMBC connectivity to amide carbonyl at δ_C 167.73. Combining COSY and HMBC experiments showed the presence of 2,5-piperazinedione moiety with an extra methyl group **(Table 3).**

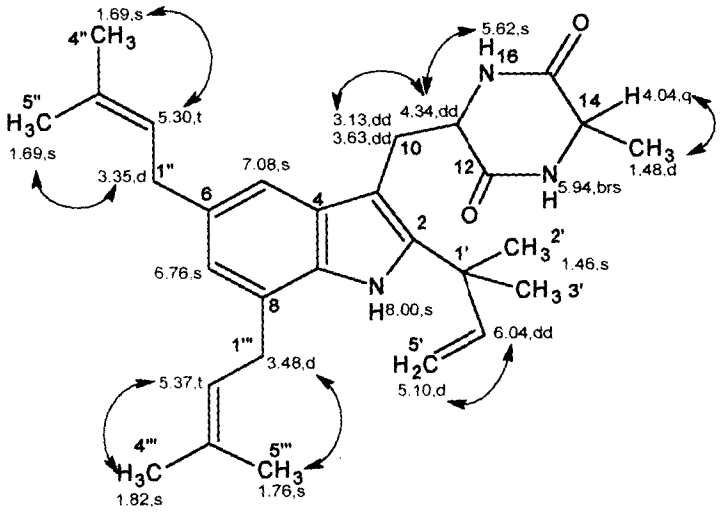


Fig 3a: COSY correlations of echinulin (3).

The presence of 1,1-dimethyl-2-propenyl moiety in the molecule was established by HMBC correlation of all the proton signals at δ_H 1.46 (s, 6H, H2′ & H3′), 6.04 (dd, 1H, J=10.5, 17.6 Hz) and 5.10 (d, 2H, J=11.0, 5.11 Hz) to δ_C 38.90. COSY showed direct coupling of δ_H 6.04 (dd) to 5.10 (d). Singlet at δ_H 1.69 (6H) due to isopropylidene methyls (H4″ and H5″) showed coupling to olefinic proton triplet at δ_H 5.30 and methylene doublet at δ_H 3.35 in the ^{1}H-^{1}H COSY experiment. The COSY correlations are illustrated in **Fig 3a**. The presence of 1,1-dimethyl-2-propenyl moiety was further confirmed by HMBC correlation of δ_H 1.69 with quaternary carbon at δ_C 133.81; δ_H 3.35 inturn showed connectivity to methylene at δ_C 34.50 (δ_H 3.35, d). Similarly, the presence of yet another side chain in the molecule identified as 3-methyl-butenyl was evident from the COSY coupling of singlets at δ_H 1.82 (3H) and 1.76 (3H) to δ_H 5.37 (t) and 3.48 (d) respectively. It also exhibited HMBC correlation with quaternary carbon at δ_C 132.9. Thus these three-prenyl groups accounted for six methyl groups. The HMBC correlations are shown in **Fig 3b**.

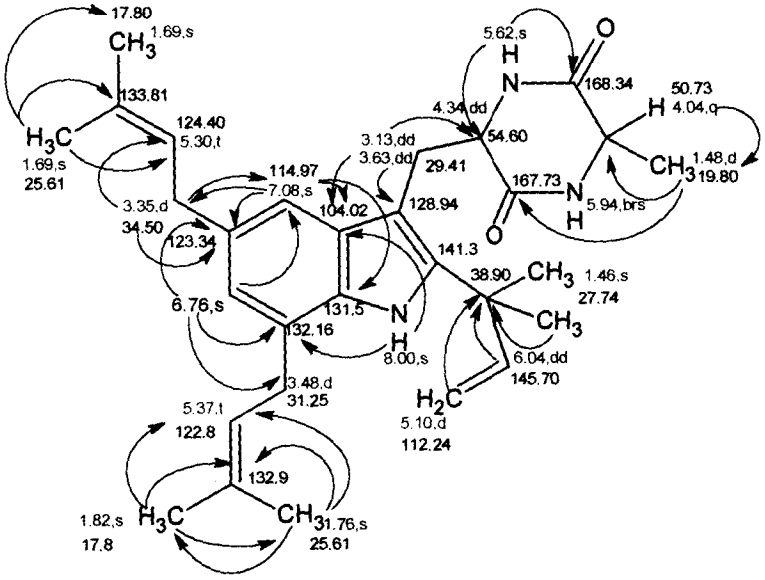


Fig 3b: HMBC correlations of echinulin (3).

The linkage of the three prenyl groups and 2,5-piperazinedione to indole moiety was established by their HMBC connectivities to respective carbons thus establishing the structure of the molecule as **(3)** suggestive of tryptophan-alanine derived cyclic peptide. Literature indicated the data is identical to that reported for echinulin, a metabolite identified from several terrestrial fungi and plants.[31]

In analogy with similar compounds reported earlier[32] and as expected in natural L-amino acids the stereochemistry of compound **(3)** was assigned to be SS. This is the first report of the echinulin from the marine fungi *Eurotium sp.*

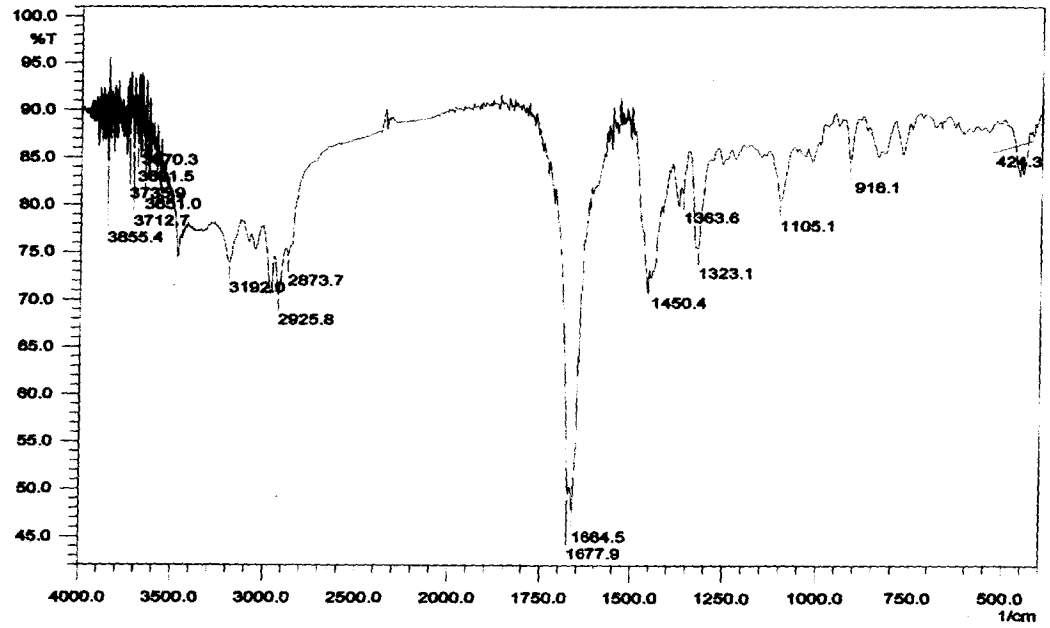


Fig 3.1: IR spectrum of echinulin (3)

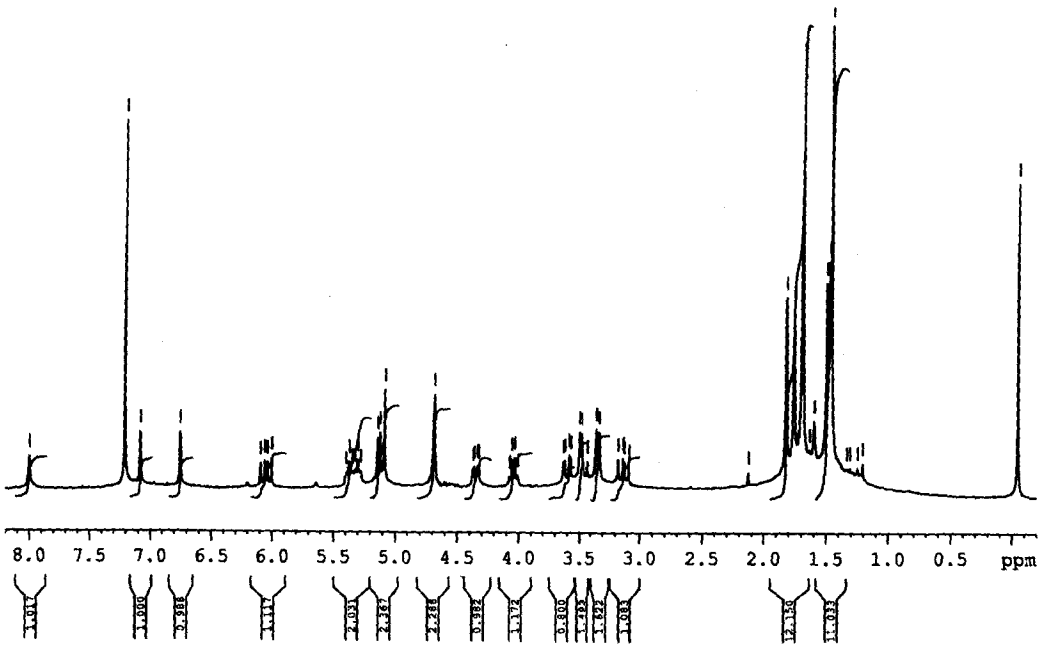


Fig 3.2: ^{1}HNMR spectrum of echinulin (3)

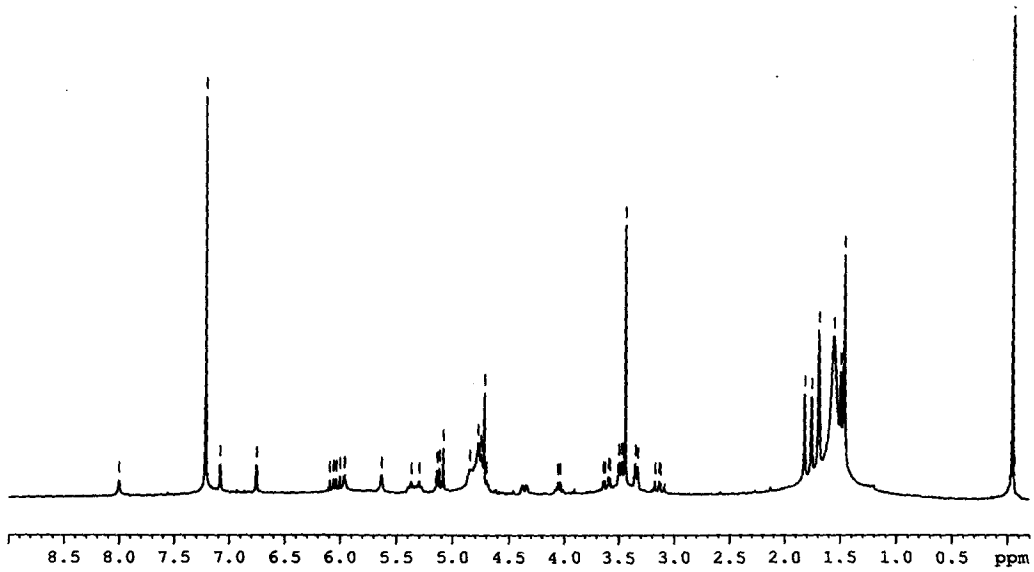


Fig 3.3: ^{1}HNMR (D_2O exchange) spectrum of echinulin (3)

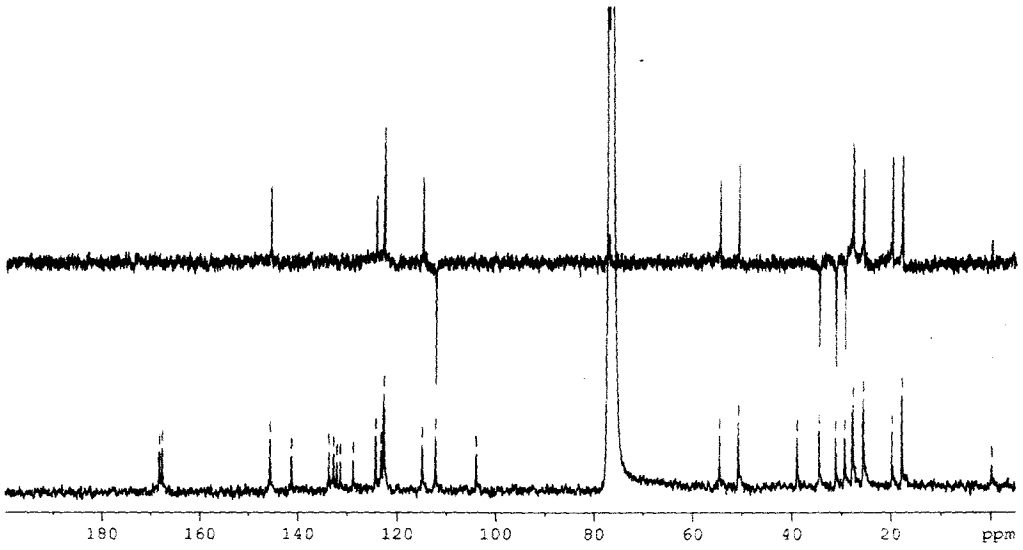


Fig 3.4: ^{13}CNMR and DEPT spectra of echinulin (3)

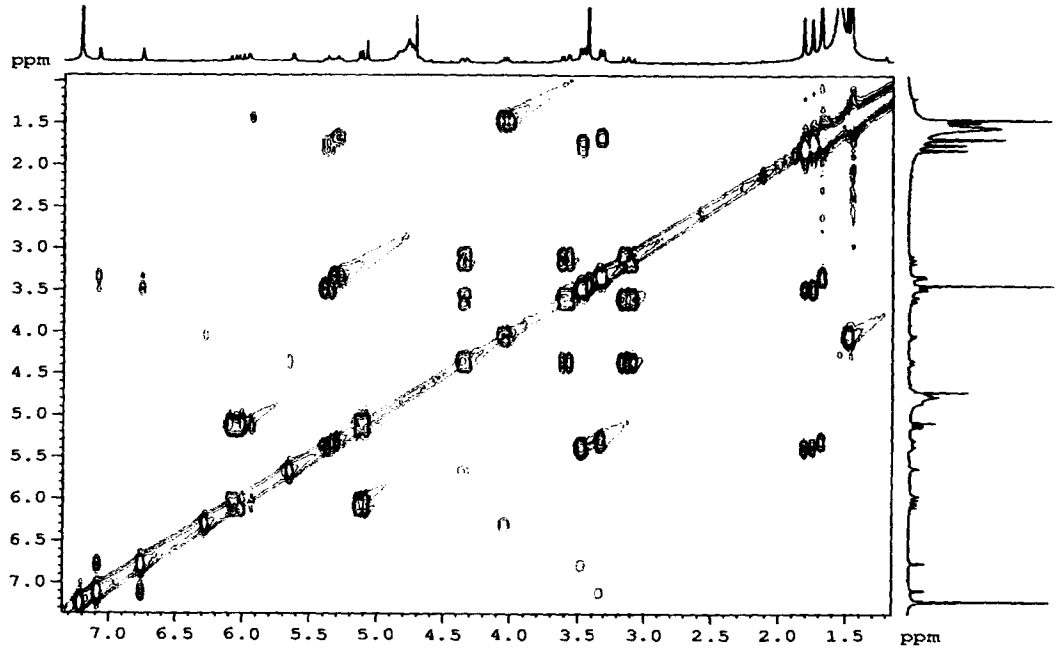


Fig 3.5: COSY spectrum of echinulin (3)

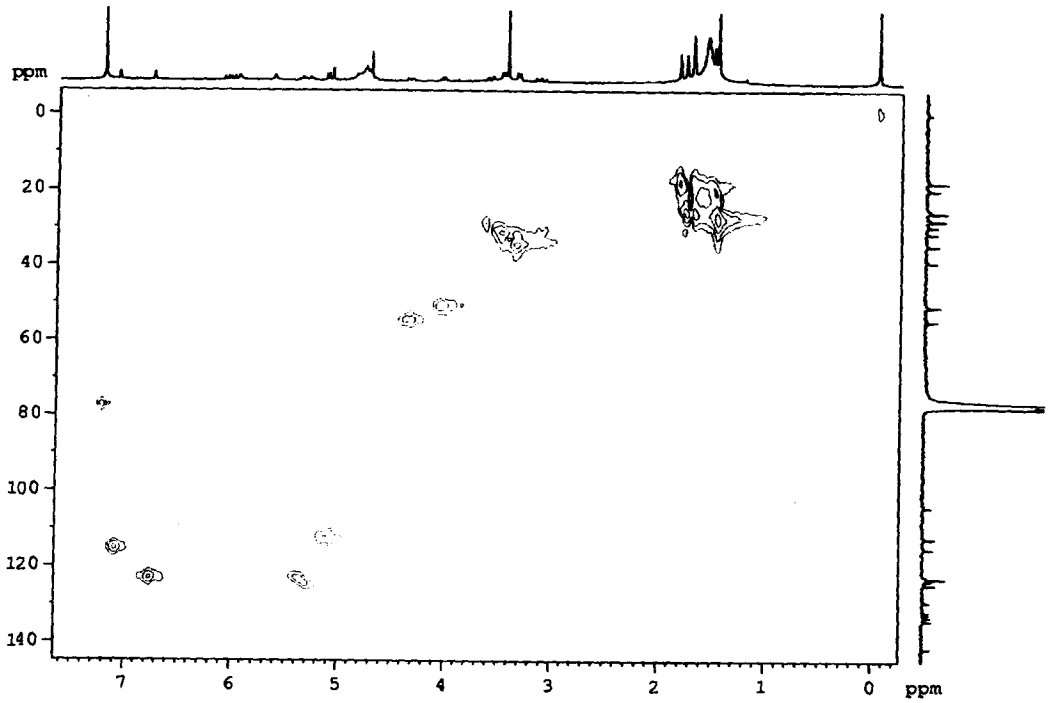


Fig 3.6: HMQC spectrum of echinulin (3)

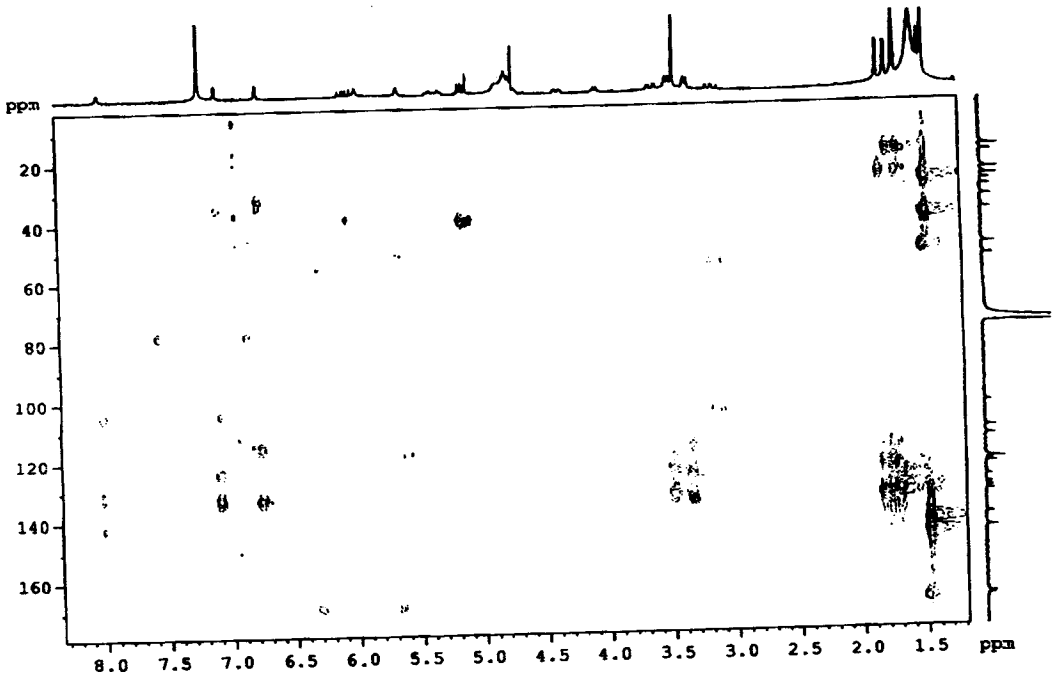


Fig 3.7: HMBC spectrum of echinulin (3)

Table 3: ^{1}H, ^{13}C NMR, COSY and HMBC correlations of echinulin (3)

Carbon No	^{13}CNMR δ_H, ppm	^{1}H NMR δ_H, ppm	COSY	HMBC	Carbon No	^{13}CNMR δ_H, ppm	^{1}H NMR δ_H, ppm	COSY	HMBC
1- NH	---	8.00,s		C2, C4, C8	16 -NH	---	5.62	H11	
2	141.27				1′	38.90			
3	128.94				2′	27.74	1.46,s		C1′, C3′
4	104.02				3′	27.74	1.46,s		C1′, C2′
5	114.97	7.08,s		C1″, C4, C6, C9	4′	145.69	6.04(dd,J=10.5, 17.6 Hz)	H5′	C1′
6	123.33				5′	112.24	5.10(d,J=11Hz) 5.11(d,J=17Hz)	H4′	C1′
7	122.78	6.76(s)		C1‴,C5, C6, C8	1″	34.50	3.35(d,J=7.1 Hz)	H5″	C2″, C5, C6
8	132.16				2″	124.39	5.30(t)	H4″	
9	131.5				3″	133.81			
10	29.41	3.13(dd,J =11.7,14. 7Hz); 3.63(dd,J =14.7,3.6 Hz)	H11	C3, C4, C11	4″	17.80	1.69(s)	H2″	C2″,C3″, C5″
11	54.57	4.34 (dd,J=2.4, 11.6 Hz)	H10, 16-NH		5″	25.61	1.69(s)	H1″	C2″,C3″, C4‴
12	168.34				1‴	31.25	3.48(d,J=7.3Hz)	H5‴	C2‴, C8, C9
13,NH	---	5.94		C12, C14	2‴	122.78	5.37(brt,J=7.4Hz)	H4‴	
14-H	50.72	4.04(q)	14-Me	14-Me	3‴	132.85			
14-Me	19.80	1.48(d, J=7Hz)	H14	C14, C15	4‴	17.80	1.82(s)	H2‴	C2‴,C3‴, C5‴
15	167.73				5‴	25.61	1.76(s)	H1‴	C2‴,C3‴, C4‴

Purification of F-IIIB fraction on silica column with gradient elution of ethyl acetate in petroleum ether resulted in the isolation of two anthraquinone derivatives, physcion **(4)**, erythroglaucin **(5)** while polar fraction of F-IIIA yielded two more anthraquinone derivatives, alaternin **(6)** and caternine **(7)**.

Among these, the most abundant anthraquinone was orange colored crystalline solid, physcion **(4)** eluted with 15% ethyl acetate:petroleum ether (m.p.207 °C). The UV-Visible absorption of **(4)** at λ_{max} 437 nm and IR **(Fig 4.1)** absorptions at 1678 and 1629 cm^{-1} suggested it to be an anthraquinone structure. The ^{1}H **(Fig 4.2)** and $^{13}CNMR$ **(Fig 4.3)** spectrum in $CDCl_3$ indicated presence of one -OMe (δ_H 3.88 s / δ_C 55.97 q), one methyl on an aromatic ring (δ_H 2.39,s / δ_C 22.04,q), four aromatic protons in the region δ_H 6.6 to 7.6 and two phenolic hydroxyl groups (δ_H 12.06,s & 12.27,s). Its $^{13}CNMR$ and DEPT 135 **(Fig 4.3)** spectra indicated presence of 16 carbon atoms assigned to 2-methyls, 4-methines and 10-quarternary carbons **(Table 4)**. The carbon signals at δ_C 181.89 and 190.69 were indicative of the presence of quinone moiety in the molecule. The structure was further confirmed using 2D NMR experiments such as COSY **(Fig 4.4)**, HMQC **(Fig 4.5)** and HMBC **(Fig 4.6)**, which also led to the unambiguous assignment of all the NMR signals **(Table 4)**. The mass spectrum **(Fig 4.7)** showed molecular ion peaks at $[M+H]^+$ at m/z 285 for the molecular formula $C_{16}H_{12}O_5$.

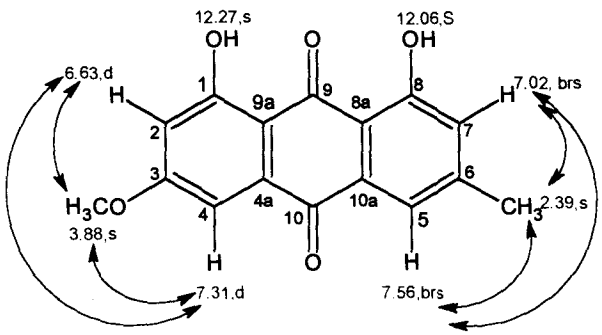


Fig 4a: COSY correlations of physcion (4).

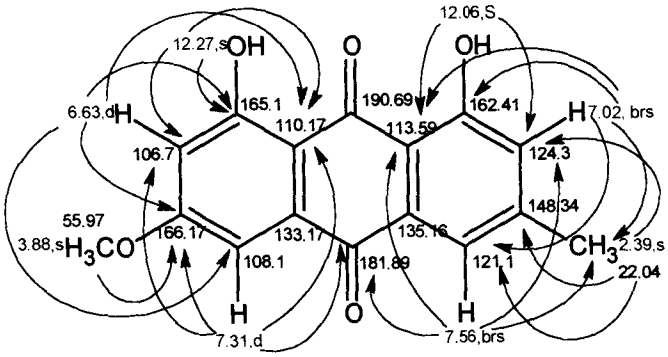


Fig 4b: HMBC correlations of physcion (4).

Table 4: ^{1}H, $^{13}CNMR$ and HMBC correlations of physcion (4), in $CDCl_3$

Carbon No.	$^{13}CNMR$ δ_C, ppm	$^{1}HNMR$ δ_H, ppm	HMBC Correlarions
1	165.1(s)	12.27(OH)	C1, C2, C9a
2	106.7(d)	6.63(d, J=2Hz)	C1, C3, C4, C9a
3	166.17(s)		
4	108.1(d)	7.31(d, J=2Hz)	C2, C3, C9a, C10
4a	133.17 (s)		
5	121.10(d)	7.56(brs)	C7, C8a, C10, C11
6	148.34(s)		
7	124.3 (d)	7.02(brs)	C5, C8, C8a, C11
8	162.41(s)	12.06(OH)	C7, C8, C8a
8a	113.59(s)		
9	190.69(s)		
9a	110.17(s)		
10	181.89(s)		
10a	135.16(s)		
11-Me	22.04(q)	2.39(s)	C5, C6, C7
-OMe	55.97(q)	3.88(s)	C3

Based on the spectral data and comparison with the ^{1}H and $^{13}CNMR$ values reported in the literature indicated the compound to be physcion **(4)**, the anthraquinone pigment isolated from several plants and fungi.[33] Physcion is widespread but only as a trace constituent in the genus *Cortinarius*, *Xanthoria* and *Dermocybe*.[34-35] Physcion and erythroglaucin has been reported to display antifungal activities against *Aspergillus niger, Doratomyces stemonitis, Trichoderma viridae* and *Penicillium verucosum*. It is also known to exhibit antibacterial acticity against *Pseudomonas fluorescens, Pseudomonas glicinea* and *Pseudomonas phaseolicola*.[36]

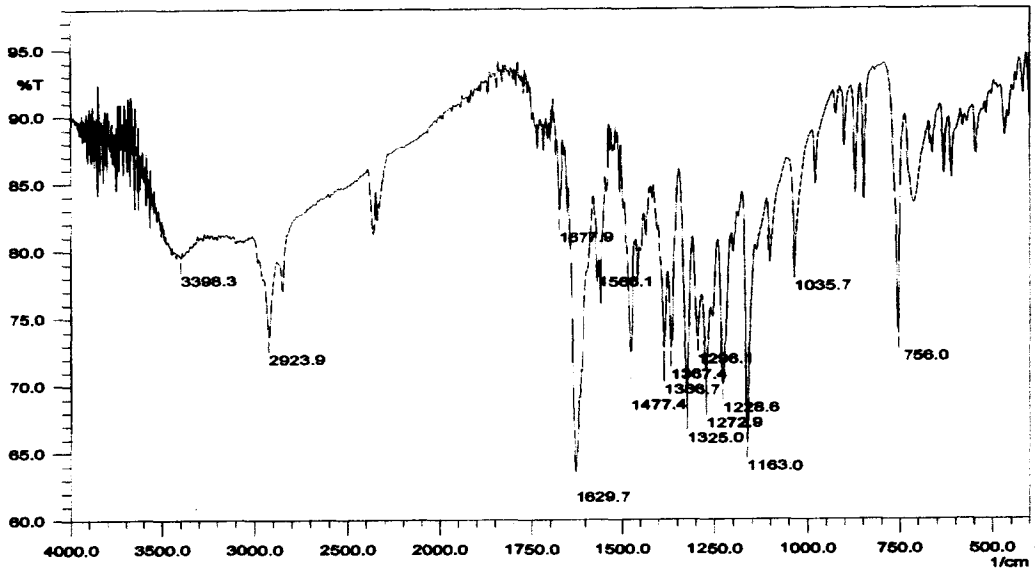


Fig 4.1: IR spectrum of physcion (4)

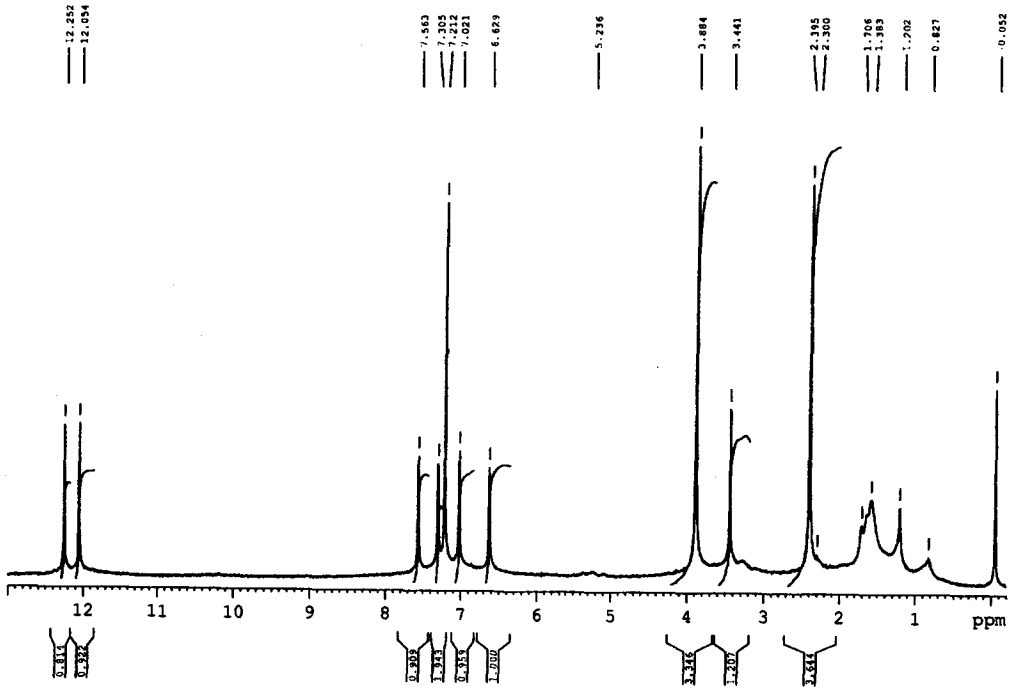


Fig 4.2: ^{1}HNMR spectrum of physcion (4)

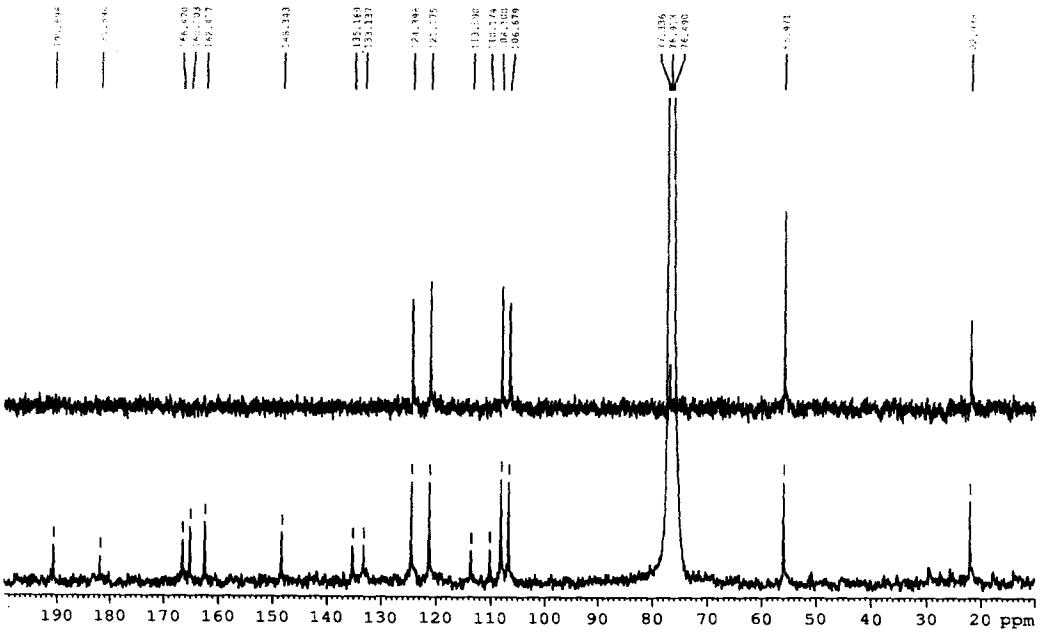


Fig 4.3: ^{13}CNMR and DEPT spectra of physcion (4)

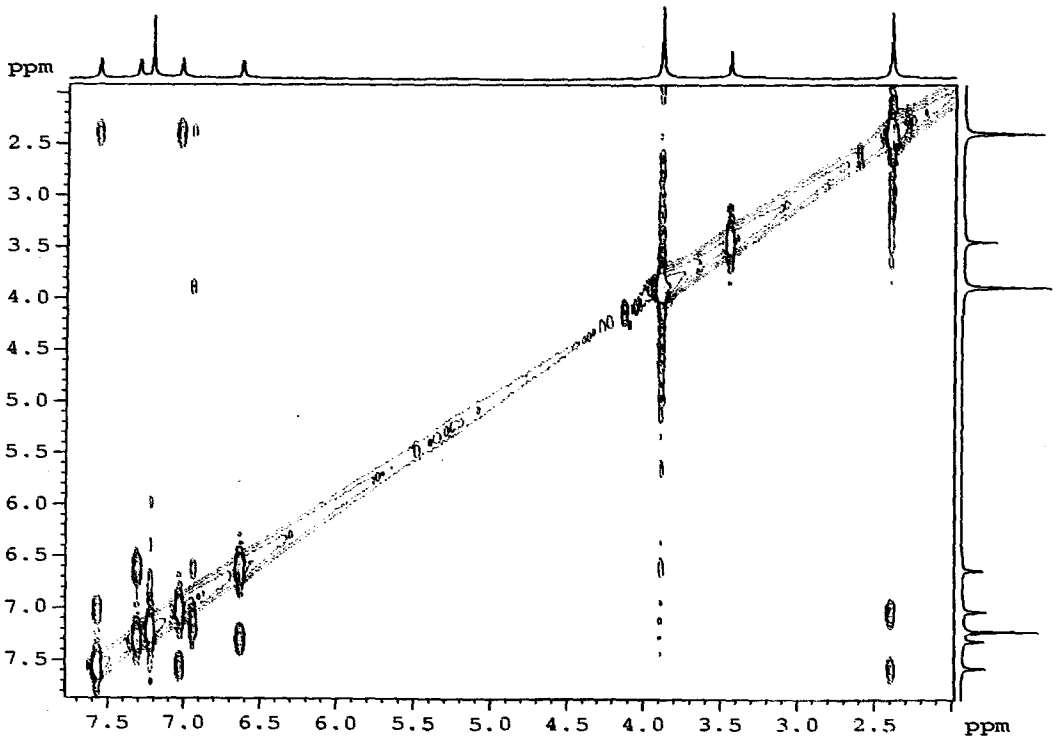


Fig 4.4: COSY spectrum of physcion (4)

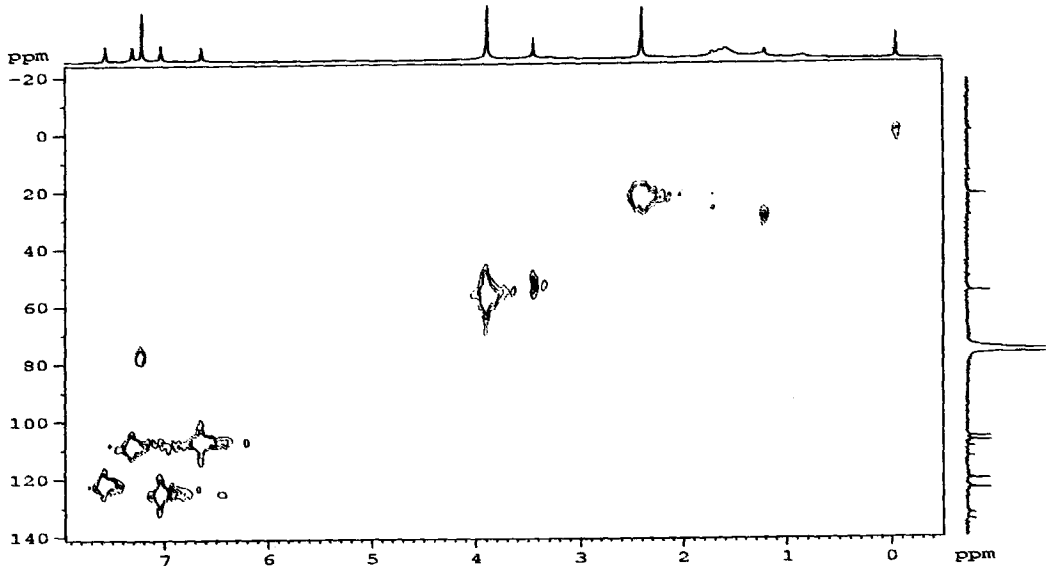


Fig 4.5: HMQC spectrum of physcion (4)

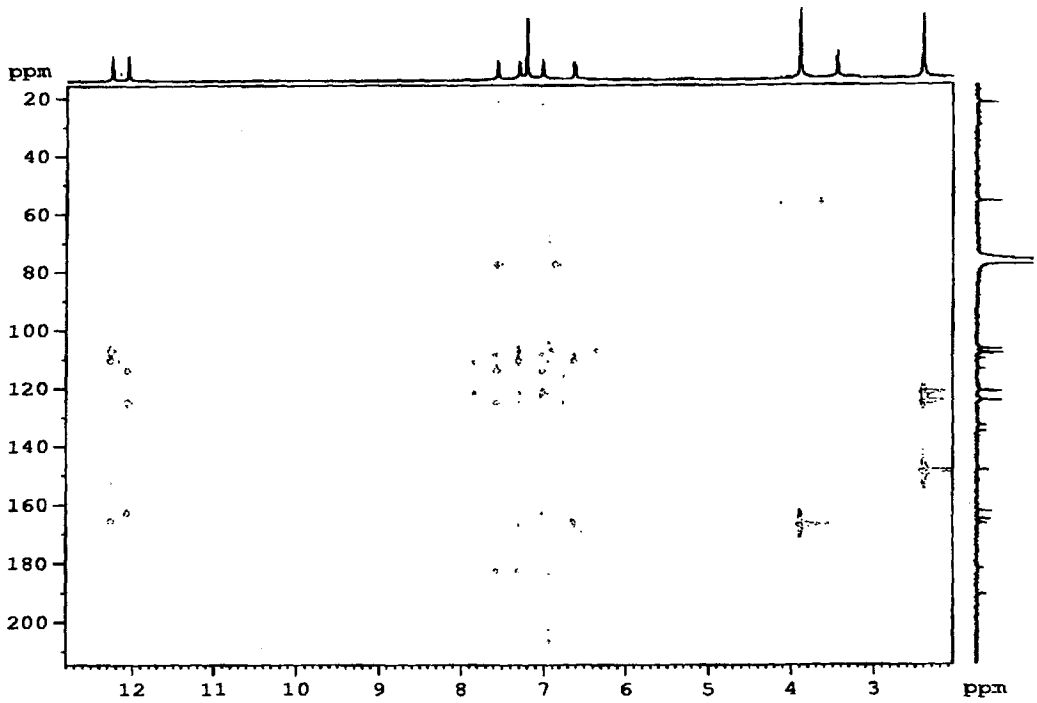
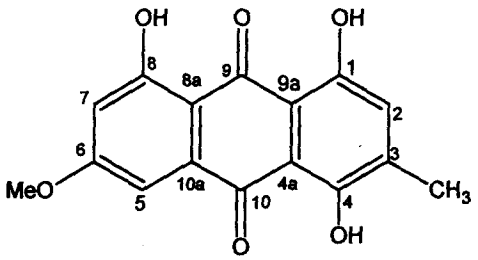


Fig 4.6: HMBC spectrum of physcion (4)

Erythroglaucin **(5)**, less polar than physcion, but produced in quantities just sufficient for recording IR and ^{1}HNMR spectra. The IR spectrum of compound **5**, had peaks at 3400, 1645, 1434, 1301, 1272 and 772cm^{-1}. Its ^{1}HNMR **(Fig 5.1)** spectrum ($CDCl_3$) was similar to that of physicon **(4)**, but for the absence of the proton signal at δ_H 7.56 (H4 in physcion) and the appearance of an additional hydroxyl proton signal at δ_H 13.32 **(Table 5)**. The structure of this compound was finalized as 4-hydroxy physcion (fluoroglaucin) **(5)** on the basis of above data.



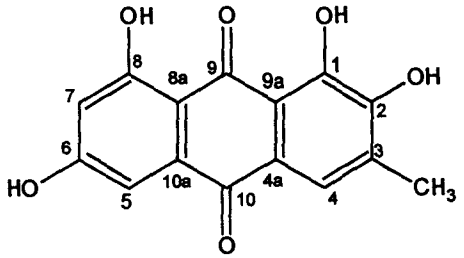
Erythroglaucin (5)

Table 5: ^{1}HNMR values of physcion (4) and erythroglaucin (5), in $CDCl_3$

Proton No.	Physcion **(4)** δ_H, ppm	Erythroglaucin **(5)** δ_H, ppm
1-OH	12.06 (s)	12.31 (s)
2-H / OH	7.02(brs)	7.08(brs)
3-Me	2.39 (s)	2.31 (s)
4-H / OH	7.56 (brs)	13.32 (s)
5-H	7.31 (2Hz)	7.36 (2Hz)
6-OH/ O Me	3.88(s)	3.90 (s)
7-H	6.63 (2Hz)	6.65 (2Hz)
8-OH	12.27	12.40 (s)

The more polar fraction of F-IIIA was insoluble in chloroform, but freely miscible with methanol. Its ^{1}HNMR spectrum indicated it to be a 2:1 mixture of two anthraquinone derivatives. Careful purification of this mixture over silica gel column yielded the more abundant compound **6** in pure form $[M+H]^+$=m/z 287. The ^{1}HNMR spectrum **(Fig 6.1)** of compound **6** recorded in d_4-MeOH displayed three aromatic proton signals at δ_H 7.37 (1H, brs), 6.95 (1H,d, J=2Hz), and 6.40 (1H, d, J=2Hz) and one methyl on aromatic ring at δ_H 2.38 (3H,s). Its ^{13}CNMR

spectrum (**Fig 6.2**) was similar to that of physcion **4**, except for the absence of the carbon signal at δ_C 124.3(d) and the appearance of a new signal at δ_C 158.33(s), indicating hydroxylation of that carbon atom (**Table 6**). The structure of this compound was finalized as 2-hydroxy emodin (alaternin) **6** from the HMBC spectrum (**Fig 6.3**).

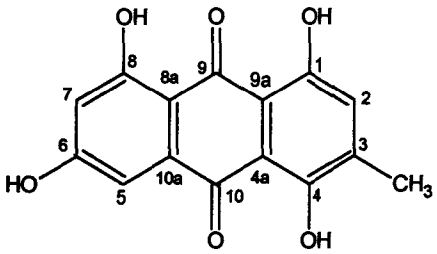


Alaternin (6)

Table 6: ^{1}H, ^{13}C NMR and HMBC correlations of alaternin (6), d_4-MeOH

Carbon No.	^{13}CNMR δ_C, ppm	^{1}HNMR δ_H, ppm	HMBC Correlations
1	167.88 (s)		
2	158.33 (s)		
3	144.14 (s)		
4	120.48 (d)	7.47 (brs)	C8a, C10, C10a, C11
4a	129.68 (s)		
5	108.75 (d)	7.06 (J=2Hz)	C4, C10
6	165.78 (s)		
7	107.64 (d)	6.48 (J=2Hz)	C1, C4
8	165.04 (s)		
8a	111.58 (s)		
9	189.73 (s)		
9a	113.58 (s)		
10	180.78 (s)		
10a	134.72 (s)		
3-Me	18.80 (q)	2.38,s	C5, C6, C10a

Compound **7**, $[M+H]^+$=m/z 287, was evidently isomeric with compound **6**. The only difference in its ^{1}HNMR spectrum (**Fig 7.1**) was the absence of the proton signal at δ_H 7.37 and the presence of an additional signal at δ_H 6.82, indicating the shift of the hydroxyl group from C2 to C4 position. The vinyl methyl signal now appeared slightly upfield, at δ_H 2.15 (**Table 7**). The structure of this compound was confirmed to be 4-hydroxy emodin (catenarin) from these results.



Catenarin (7)

Table 7: ^{1}HNMR values of alaternin (6) and catenarin (7) in CD_3OD

Proton No.	Alaternine δ_H, ppm	Catenarin δ_H, ppm
1-OH	---	---
2-H / OH	---	6.82 (brs)
3-Me	2.38 (s)	2.15 (s)
4-H / OH	7.37 (brs)	---
5-H	6.95 (2Hz)	6.92 (2Hz)
6-OH/ O Me	---	---
7-H	6.40 (2Hz)	6.36 (2Hz)
8-OH	---	---

Natural anthraquinones are distinguished by a large structural variety, a wide range of biological activity and low toxicity. They possess astringent, anti-inflammatory, antiviral, moderate antitumour and antibacterial effect. [37]

Though the compounds are known from terrestrial origin, they are being reported for the first time from marine source. Further, the data reported, was insufficient for unambiguous assignment of proton and carbon values. In the present work, the assignment has been made entirely on the basis of 2D experiments.

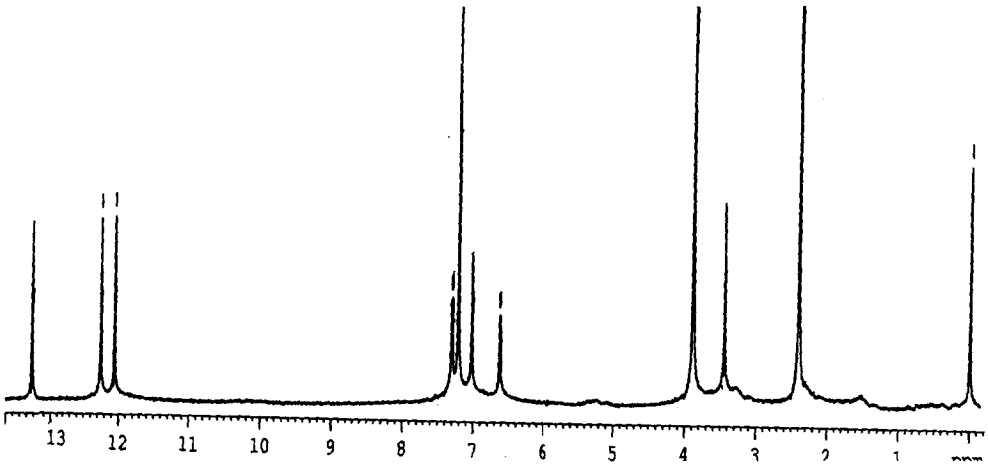


Fig 5.1: ^{1}HNMR spectrum of erythroglaucin (5)

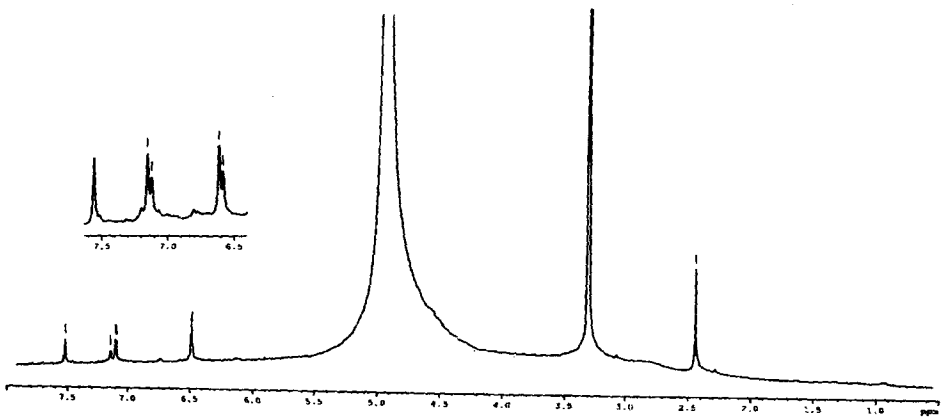


Fig 6.1: ^{1}HNMR spectrum of alaternin (6)

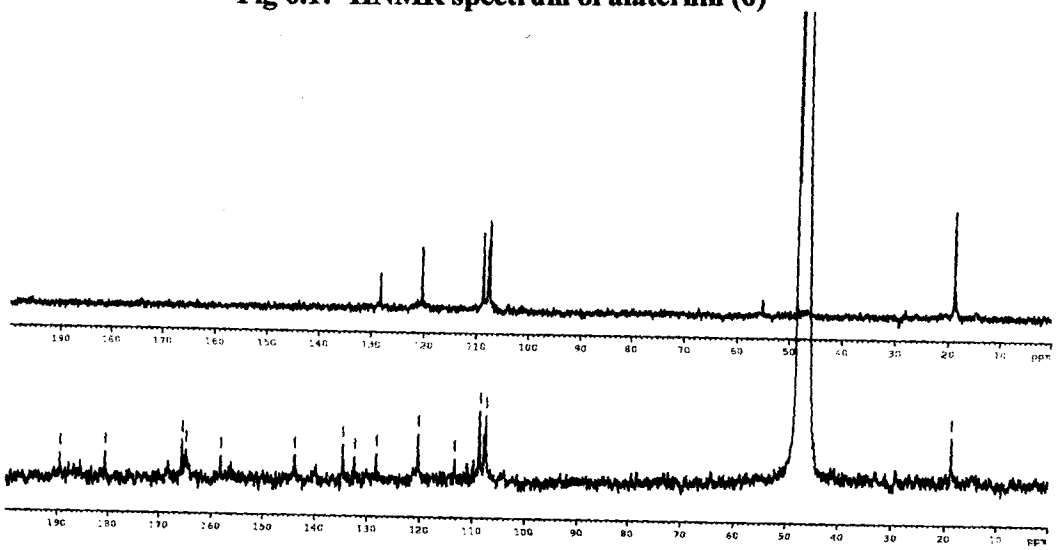


Fig 6.2: ^{13}CNMR and DEPT spectra of alaternin (6)

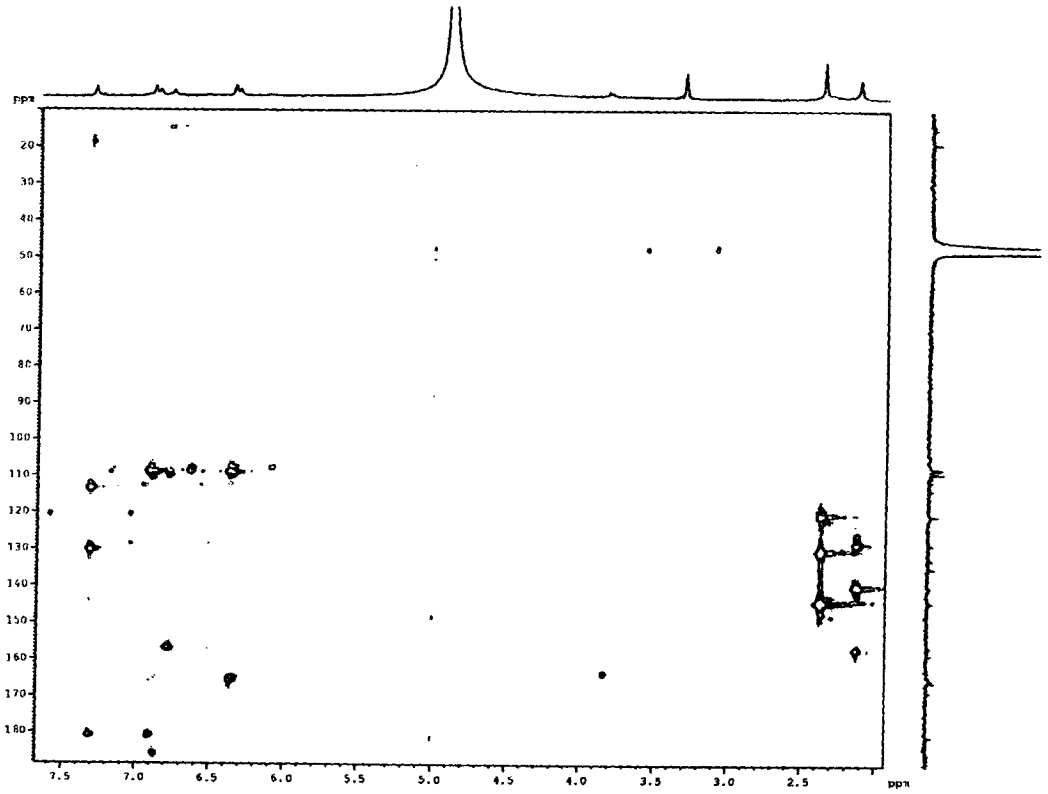


Fig 6.3: HMBC spectrum of alaternin (6)

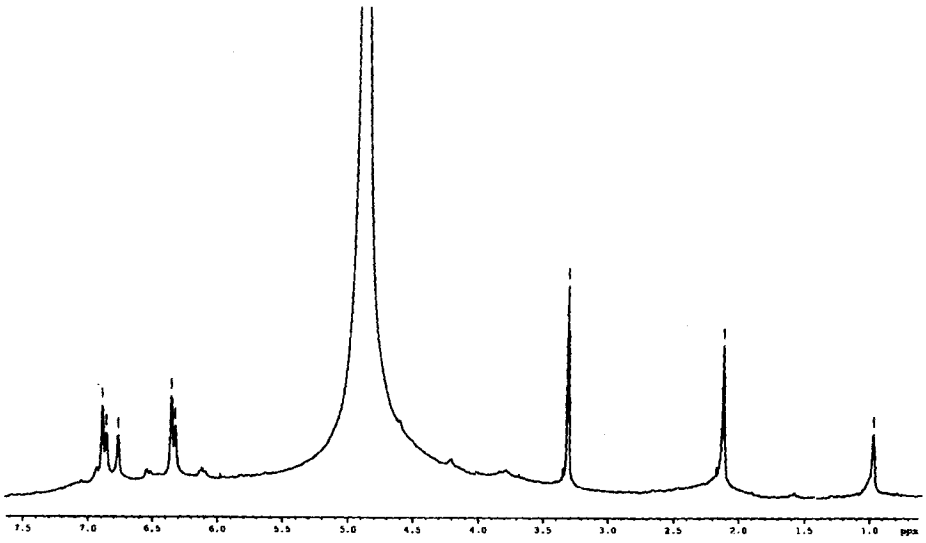


Fig 7.1: ^{1}HNMR spectrum of catenarin (7)

Experimental section:

Collection of marine flora for isolation of associated fungi:

The fungus *Eurotium sp.* (NIO FMB #001) was obtained from the leaves of mangrove plant *Porteresia coarctata* (Roxb.). The mangrove plant was collected from chorao Island along the Mandovi estuary of Goa. Leaves of the mangrove plant were collected and transported to the laboratory in sterile polythene bags. In the laboratory, the leaves were rinsed with sterile seawater to remove adhered particles and detritus material. The leaves were next kept in a moist chamber, using known standard techniques, for 2 weeks, to allow the fungi to grow and sporulate. Repeated subculturing resulted in pure fungal culture isolate.

Optimization of growth condition and mass culturing of *Eurotium sp.*:

The growth conditions of the fungal isolate were optimized and the culture was grown on potato dextrose agar (PDA) slants (HiMedia Laboratories Ltd.) for 10 days. After proper mycelial growth, the cultured mycelia were transferred into 500ml Erlenmeyer flasks containing 100ml potato dextrose broth (PDB). PDB was prepared in seawater: distilled water (1:1). The flasks were incubated under shaker condition (240 rpm) for about 4-5 days. At the end of 5 days, content of the flask was used as inoculum for mass culture. The cultured mycelia were then transferred to 5 lit. flasks, each containing 1 litre of broth, same liquid culture medium PDB and incubated for 15 days at room temperature (28-30 °C).

Extraction and fractionation of the fungal mycelia:

At the end of 15 days, fungal mat was filtered, washed with water and extracted with acetone. The acetone extract was combined and concentrated under vacuum. The acetone extract was fractionated using ethyl acetate, which yielded 5gms of ethyl acetate fraction.

Purification of the metabolites:

The ethyl acetate fraction (5gm), after removal of the solvent in vacuum, was subjected to gel chromatography using sephadex LH-20 in MeOH:$CHCl_3$ (1:1) as a mobile phase. This resulted in four sub fractions F-I, F-II, F-III & F-IV. Among

these four fractions, proton NMR of fraction F-III (0.2752 gm) showing many signals for oxygenated molecules was taken up for further purification. Fraction, F-III was again subjected to gel chromatography using mobile phase MeOH:$CHCl_3$(1:1). The column did not give much purification. The combination of the fractions collected based on the TLC profile resulted into only two fractions A (0.0481 gm) and B (1.1630 gm) (i.e. F-IIIA and F-IIIB). Each of these fractions was then subjected to series silica gel column chromatography. The gradient elution of F-IIIA with methanol in chloroform resulted in purification of three compounds while elution of F-IIIB with ethyl acetate in petroleum ether resulted in purification of four compounds. Yellow compound, tetrahydroauroglaucin **(1)** (0.145gm) was non-polar eluted with 4% ethyl acetate: petroleum ether with the R_f 0.7. The next two compound physcion **(4)** (0.157 gm) and erythroglaucin **(5)** (56.5 mg) were eluted with 15 % ethyl acetate:petroleum ether followed by dark orange coloured compound, isodihydroaurogalucin **(2)** (0.136 gm) with 20% ethyl acetate:petroleum ether. The gradient elution of F-IIIA on silica gel column with 5% methanol:chloroform yielded echinulin **(3)** (0.163 gm) with R_f value 0.5. Further elution with 25% resulted in purification of two polar compounds alaternin **(6)** (30mg) and catenarin **(7)** (10mg).

Tetrahydroauroglaucin (1) [2-(1E-heptenyl)-3,6-dihydroxy-5-(3-methyl-2-butenyl)-benzaldehyde]: Yellow crystals; Molecular formula: $C_{19}H_{26}O_3$; m.p. 70 °C; UV ($CHCl_3$) λ_{max} (ε_{max}) 205.00 (ε 4,100), 229.5 (ε 4,590), 274.5 (ε 5,490) and 395.5 nm (ε 7,190); IR (KBr pellet): ν_{max} 3265, 2924, 1622, 1606, 1581, 1437, 1489, 983, 958.6, 889, 704 cm^{-1}; ^{1}HNMR & ^{13}CNMR ($CDCl_3$): **Table 1**; ESI-MS: m/z 303.1840 $[M+H]^+$, 325.1655 $[M+Na]^+$, 282.2772 $[M+Na-43]^+$, 247, 245, 229, 215, 149, 114.

Isodihydroauroglaucin (2) [2-(3,5-heptadienyl)-3,6-dihydroxy-5-(3-methyl-2-butenyl)-benzaldehyde]: Orange crystal; Molecular formula: $C_{19}H_{24}O_3$; m.p. 114-115 °C; UV ($CHCl_3$) λ_{max} (ε_{max}): 202.6 (ε 4052), 223.2 (sh, ε (s), 249.6, 265.0, 285; IR (KBr pellet): ν_{max} 3417, 2925, 2854, 1643, 1434, 1296, 1128, 993, and

761 cm^{-1}; ^{1}HNMR & ^{13}CNMR: ($CDCl_3$): **Table 2**; ESI-MS: m/z 301.1351 $[M+H]^+$, 323.1471 $[M+Na]^+$, 299, 231, 215, 149 and 114.

Echinulin (3) (2,5-Piperazinedione, 3-[2-(1,1-dimethyl-2-propenyl)-5,7-bis(3-methyl-2-butenyl)-1H-indol-3-yl]methyl]-6-methyl-(3S,6S)):obtained as white crystals; Molecular formula: $C_{29}H_{39}N_3O_2$; m.p. 209 °C; UV ($CHCl_3$) λ_{max} (ε_{max}) 382 (7640), 344.6 (6892), 337.0 (6740), 279.4 (5588), 240.6 (4812), 231 (4620), 204 (4080) and 199 (3980) nm; IR (KBr pellet):ν_{max} 3350, 3192, 2925.8, 2873.7, 1678, 1450, 1323.1, 1363.6, 1105.1 and 916 cm^{-1}; ^{1}H & ^{13}CNMR ($CDCl_3$): **Table 3.**

Physcion (4): Orange crystals in MeOH, Molecular formula: $C_{16}H_{12}O_5$; m.p. 207-209 °C; UV: λ_{max} 437.2, 286, 281, 266, 239, 217; IR (KBr pellet):ν_{max} 3398.3, 2923.9, 1677.9, 1629.7, 1566.1, 1477.4, 1386.7, 1367.4, 1296.1, 1272.9, 1228.6, 1163.0, 1035.7, 756.0; ^{1}H & ^{13}CNMR ($CDCl_3$): **Table 4.**

Erythroglaucin (Fluroglaucin) (5): Red needles ($CHCl_3$); Molecular formula: $C_{16}H_{12}O_6$; m.p. 204-206 °C; UV ($CHCl_3$): λ_{max} 223, 256, 308, 466, 478, 493, 513, 522; IR (KBr pellet): ν_{max} 2945, 2840, 1645, 1605, 1595, 1435, 1160; ^{1}HNMR ($CDCl_3$): **Table 5.**

Alaternin (6) (2-hydroxy emodin): Molecular formula: $C_{15}H_{10}O_6$; ^{1}HNMR & ^{13}CNMR (CD_3OD): **Table 6.**

Catenarin (7) (1,4,6,8-Tetrahydroxy-3-methylanthraquinone): Molecular formula: $C_{15}H_{10}O_6$, ^{1}HNMR (CD_3OD): **Table 7.**

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Chapter V

Biotransformation of 2-benzoxazolinone using different microbial strains

For centuries, even prior to the advent of alchemy, man was totally dependent on natural products in many different forms for treatment of human maladies. As medical science progressed, these were slowly replaced by purified extracted principles, and now by pure synthetic organic compounds including their analogs. Chemical synthesis involves multiple reagents, which are expensive. Also the different conditions of the reaction to be carried out might be drastic or need standardizations or the yields obtained may be low. Hence, presently chemists have diverted their attention towards microbial enzymatic transformations (biotransformations) to obtain novel active principles. This approach possesses more advantages over conventional chemical reactions by a) regio- and stereo-selectivity, b) mild conditions (e.g. 30°C and pH 7), c) most reactions can be undertaken in water (although organic solvents may be used as well), d) they are environmentally benign (i.e. they can be easily disposed of), e) yields attained are high, f) autoxidation of relatively unstable molecules is minimum and so on.

Enzymes have been generally accepted as superior catalysts in organic synthesis. Microorganisms in particular have been regarded as treasure sources of useful enzymes. In designing a microbial transformation process, one of the most important points is to find a suitable enzyme for the reaction of interest. Various kinds of novel extracellular enzymes for specific transformations have been discovered in microorganisms and their potential characteristics revealed.[1] Before going into the details of the actual work carried out a brief account of evolution of the technique has been described.

The discovery of penicillin and its first industrial production in 1941-42 opened the doors to microbial production of drugs by exploitation of the manifold biosynthetic capabilities of microorganisms to produce antibiotics or more recently other pharmacologically active substances. The selective use of individual enzymatic transformation stages with microorganisms in chemical production pathways, in particular, by biotransformations of steroids in 1950 expanded the field of biotechnological production of pharmaceuticals.[2] Initially microbial transformation of drugs, particularly steroids and antibiotics were

performed in an effort to obtain more active or less toxic substances.[2-6] These studies paved a way to the initiation of so called "Microbial models of mammalian metabolism"[7-9] in mid 1970's and "the use of microorganisms for the study of drug metabolism"[10] in mid 1980's.

The applications of these enzymes and microorganisms as biocatalyst in synthetic organic chemistry have greatly increased in the last three decades. So far, the wide range of biotransformation reactions that have been studied using biocatalysis include hydrolysis of esters, amides, lactones, lactams, acid anhydrides, epoxides and nitriles; oxidation/reduction of alkenes, aromatics, alcohols, aldehydes and ketones, sulfides and sulfoxides; addition/elimination of H_2O, NH_3, HCN; halogenation and dehalogenation of alkenes, alkynes, aromatic compounds; alkylation and dealkylation; carboxylation and decarboxylation; isomerisation; acyloin, aldol reactions and Michael additions.[11]

The fungi, which are eukaryotic, are the most commonly utilized microorganisms in the biotransformation studies. The use of bacteria (prokaryotic) has been limited to actinomycetes strains (*Streptomyces, Nocardia, Actinoplanes*), which seem to contain enzyme equipment very similar to that of fungi. Other bacterial groups (like *Pseudomonas*) have been occasionally employed, but their use is limited by the fact that they generally consume the xenobiotic substance as a carbon and/or nitrogen source. The use of unicellular algae for biotransformation studies is also reported.[12-13]

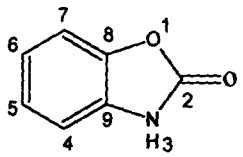
This chapter gives the details of the biotransformation of 2-benzoxazolinone (BOA) using various fungal strains. The aim of the present study was to find the type of the enzymes produced by different fungal strain under study and to characterize the biotransformed product of BOA. Its antimicrobial activity as compared to the parent molecule was also studied.

2-Benzoxazolinone (BOA) and its derivative 6-methoxy-2-benzoxazolinone (MBOA) are alleochemicals (i.e. they inhibit the growth of bacteria and fungi)

that commonly occur in cereals such as corn, rye and wheat and are considered to be an important factor in plant-parasite interaction.[14] These compounds are found to possess various biological activities including anti-convulsant,[15] antipyretic,[16] analgesic,[17-22] cardiotonic,[16,23] anti-ulcer,[24] anti-neoplastic,[25] anti-microbial[26-31] and leismanicidal activity.[32]

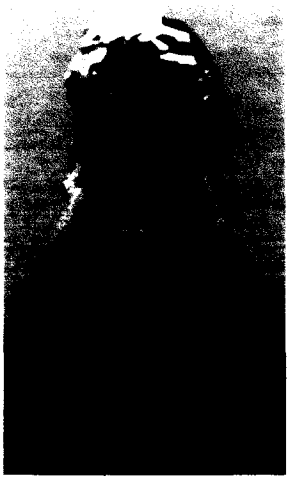
Biotransformation of 2-benzoxazolinone using five fungal strains:

BOA (1) used in the present investigation was obtained by the method of Varma *et al.*[33]



2-Benzoxazolinone (BOA) (1)

In the preliminary studies, four fungal strains *Aspergillus niger, Aspergillus ochraceous* and *Fusarium semitectum* obtained as isolates from the decayed leaves of mangrove plant and *Trichoderma pseudokoningii* an associated microorganism from sea cucumber were selected for the biotransformation of BOA. 100 ml of 50% Potato Dextrose Broth (1:1 sea water: distilled water) were inoculated from slants with the five fungal strains *A. niger, A. ochraceous, F. semitectum, T. pseudokoningii* and *C. lunata* in 500 ml Erlenmeyer flasks **(Fig 1)**. The flasks were incubated at room temperature on a flask shaker (240 rpm) for 4 days. After 4 days, 100 mg of BOA dissolved in 5 ml of ethanol was added aseptically to all the 5 flasks. All the flasks were incubated for 14 days on a rotary shaker. At the end of incubation, culture filtrates were extracted with ethyl acetate (3 X 100 ml). The ethyl acetate fraction of each broth with the substrate BOA was monitored on TLC using methanol:chloroform (5:95) solvent system. The spots were visualised under UV at 254nm as shown in **Fig 2.** TLC of the ethyl acetate fraction from four strains, *A. niger, A. ochraceous, T. pseudokoningii* and *C. lunata* indicated that the enzymes from these microorganisms had no effect on BOA. Complete transformation was observed with *F. semitectum.*

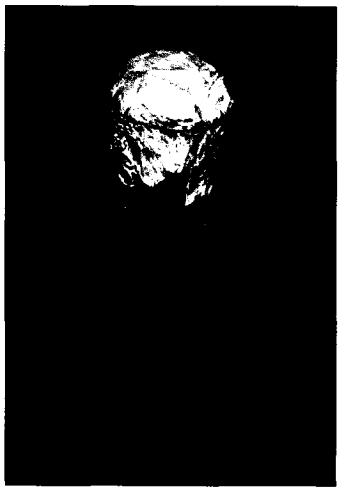


Curvularia lunata

Fusarium semitectum

Aspergillus niger

Trichoderma pseudokoningii



Aspergillus ochraceous

Fig 1: Flasks showing four fungal strain culture in 50% PDB

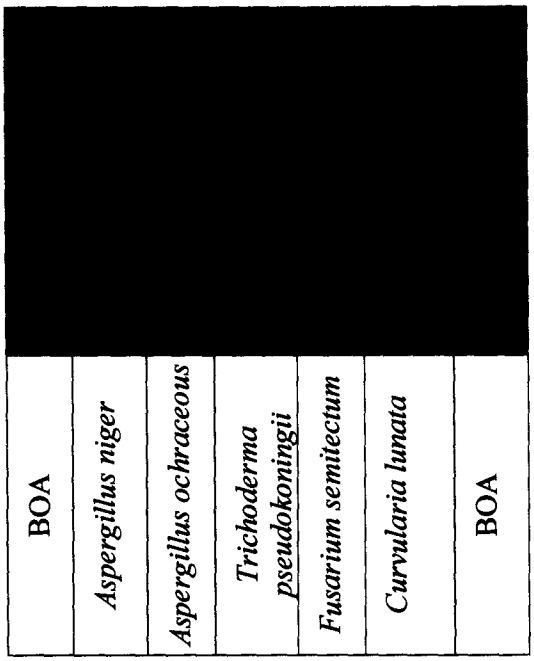


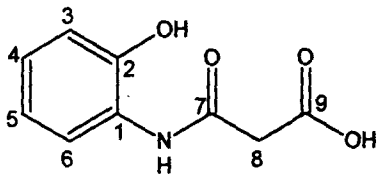
Fig 2: Monitoring the decomposition of BOA using TLC.

Optimization of time for biotransformation of BOA (1):

The incubation time is one of the most important parameters to be considered in order to have a high yield of a biotransformation product. To evaluate this parameter, 100mg of BOA dissolved in ethanol was added to six conical flasks containing a grown culture of *Fusarium semitectum* in 100ml of PDB (1:1 sea water: distilled water). Extraction of broth was carried out after every 12 hours and the samples were analysed by TLC. The complete bioconversion of the substrate was observed within 24hrs. The colour of the medium also changed from brown to red. In order to choose the best solvent for product recovery, extraction was carried out with chloroform and ethyl acetate. Chloroform fraction removed most of the pigments while the ethyl acetate fraction, after single silica gel chromatographic purification, yielded pure biotransformed product with R_f 0.5 in 10% MeOH: $CHCl_3$ as solvent system for TLC.

Structure elucidation of the biotransformed product:

The product of conversion of BOA was identified as **(2)** based on IR, ^{1}H and $^{13}CNMR$ data. Its structure was established to be N-(2-hydroxyphenyl) malonamic acid (HPMA) **(2)** on the basis of comparison of spectral data with the reported values.



N-(2-hydroxyphenyl) malonamic acid (HPMA) (2)

The UV spectrum of **(2)** when compared with that of substrate BOA **(1)** provided first evidence of change in structure of starting material after bioconversion **(Fig 3)**.

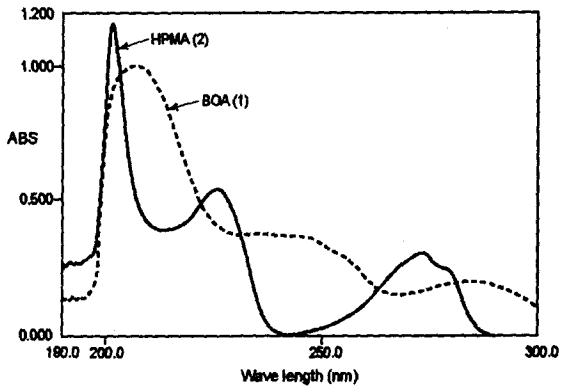


Fig 3: UV spectra of 2-benzoxazolinone (1) and N-(2-hydroxyphenyl) malonamic acid (2).

The broad IR band at 3369.4 and 1712 cm^{-1} established the presence of a carboxylic group and the band at 1651 cm^{-1} was of the amide carbonyl group **(Fig 5a)**. In the aromatic region of the $^{1}HNMR$ **(Fig 5b)** and $^{13}CNMR$ **(Fig 5c)** spectra of **(2)**, the chemical shifts and splitting patterns of protons were characteristic of a 1, 2-disubstituted benzene derivative which were similar to those of substrate **(1)**. The electron-donating substitution of the C2 hydroxyl group caused the higher

shielding effect observed at H3 and H5. Therefore, a triplet at δ_H6.77 with a coupling constant J=8.0Hz was assigned to the signal of H5, while a doublet at δ_H 6.87 was assigned to H3. The proton at C6 was observed at δ_H7.89, the lowest chemical shift, because of the deshielding effect of the electron-withdrawing substitution of the C1 amide. The ^{13}CNMR spectra showed two signals at 164.74 and 169.80 ppm that are assigned to the carbonyl groups. The methylenic functionality at δ_C 43.20 in the ^{13}CNMR spectra and the two proton singlet at δ_H 3.65 in the ^{1}HNMR spectrum suggested presence of the methylene groups. The spectral data confirmed the structure to be N-(ortho-hydroxy phenyl)malonamic acid (HPMA). The comparison of the ^{1}H and ^{13}C NMR of BOA **(1)** and HPMA **(2)** are given in the **Table 1.** IR, ^{1}HNMR and ^{13}CNMR spectra of BOA is shown in **Fig 4a, 4b and 4c** respectively.

Table 1: ^{1}H NMR and ^{13}C NMR of BOA (1), $CDCl_3$[a] and HPMA (2), d_6-DMSO[b]

Carbon No.	^{1}HNMR (1) δ_H, ppm	^{13}CNMR (1) δ_C, ppm	^{1}HNMR (2) δ_H, ppm	^{13}CNMR (2) δ_C, ppm
1			-	126.23
2		156.21	-	147.32
3		-	6.87 d (J=8.2 Hz)	115.28
4	7.09-7.23 m	110.16	6.91 t (J=7.8 Hz)	124.44
5		110.16	6.77 t (J=8.0 Hz)	118.95
6		124.19	7.89 d (J=8.0 Hz)	121.45
7		122.73	-	164.74
8		143.95	3.65, s	43.20
9		129.46	-	169.80
NH	9.88 brs		9.51 brs	
2-OH			9.83 brs	
9-OH			11.14 brs	

[a]$CDCl_3$: δ_H 7.26 ppm, δ_C 77.0 ppm; [b] d_6-DMSO: δ_H 2.5 ppm , δ_C 39.5 ppm

The literature also reports the biotransformation of BOA to HPMA using fungal strains *Gaumannomyces gravminis* var. *tritici*, *G. graminis* var. *graminis*,[34] *Fusarium moniliiforme*,[35] *F. sambucinum*[36] and *F. verticillioides*.[37] Thus, it appears that a variety of grass pathogens may use common mechanisms for

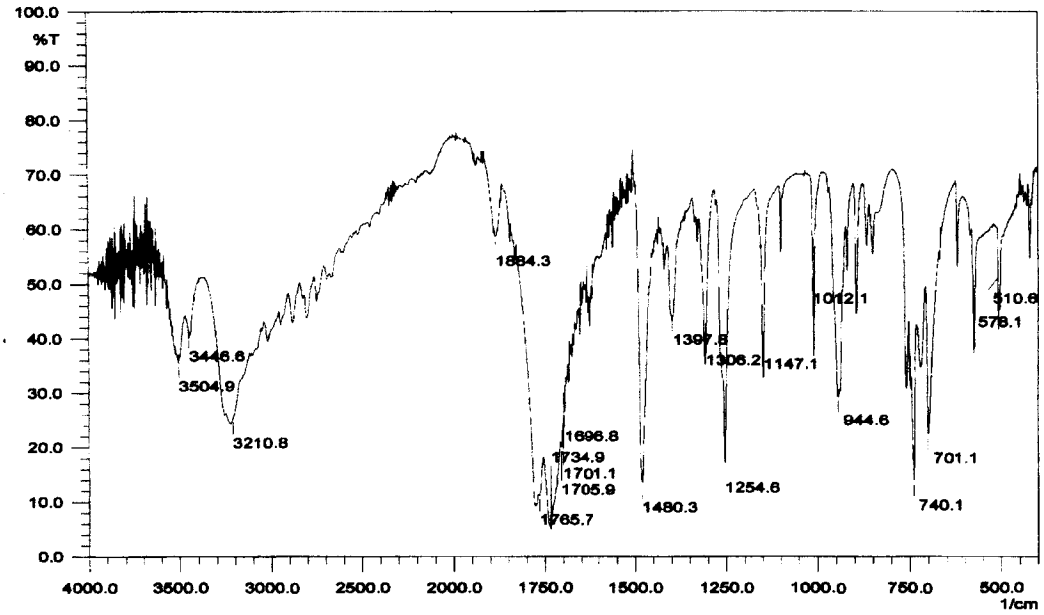


Fig 4a: IR spectrum of 2-benzoxazolinone (1).

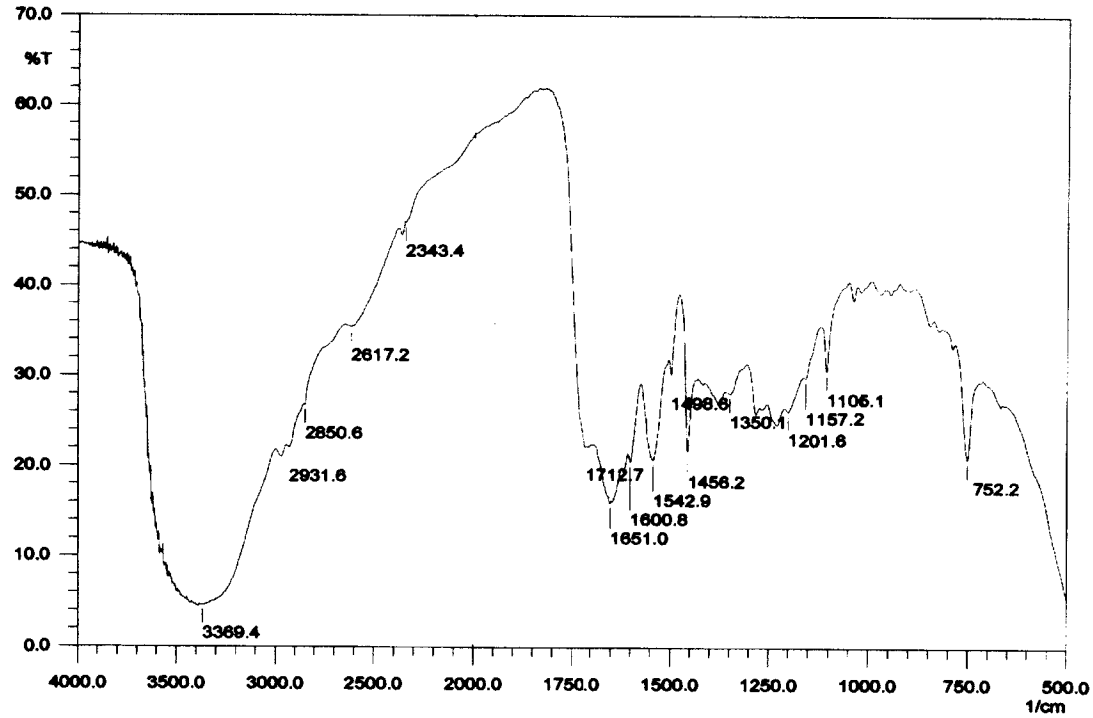


Fig 5a: IR spectrum of N-(2-hydroxyphenyl)malonamic acid (2).

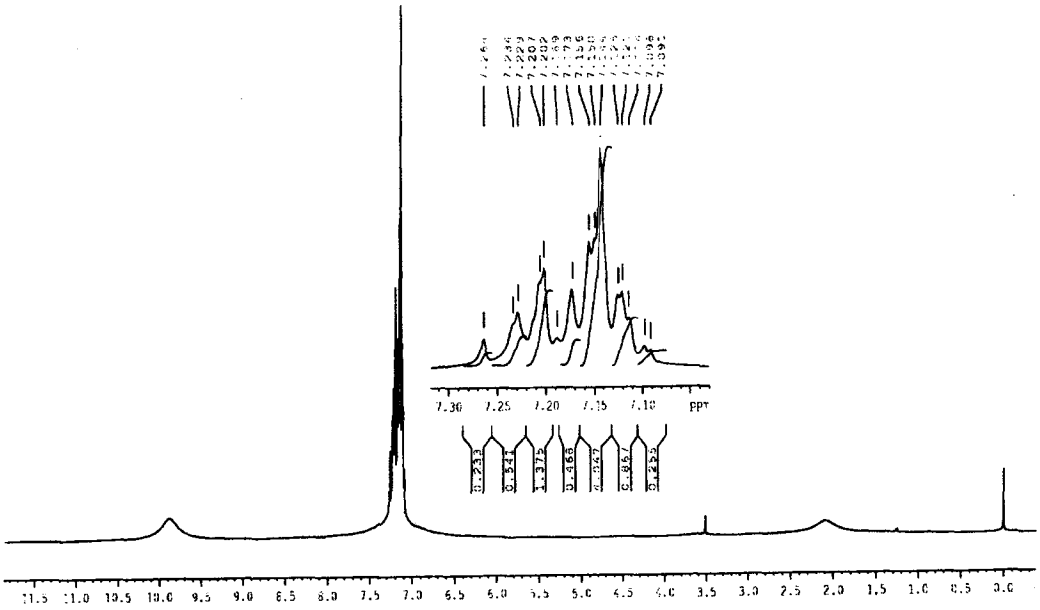


Fig 4b: ^{1}H NMR spectrum of 2-benzoxazolinone (1).

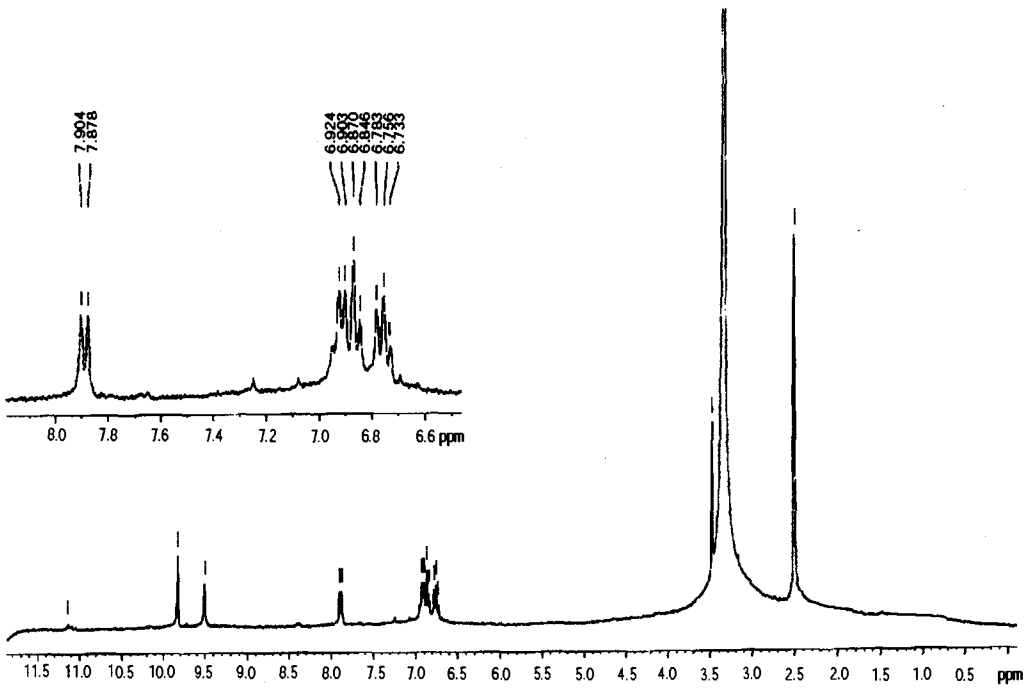


Fig 5b: ^{1}HNMR spectrum of N-(2-hydroxyphenyl)malonamic acid (2).

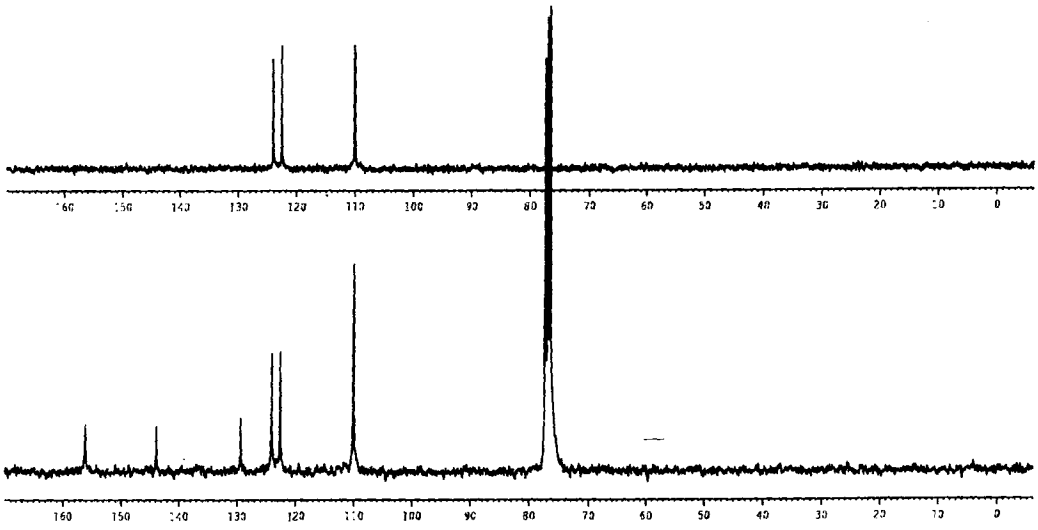


Fig 4c: ^{13}CNMR & DEPT spectra of 2-benzoxazolinone (1).

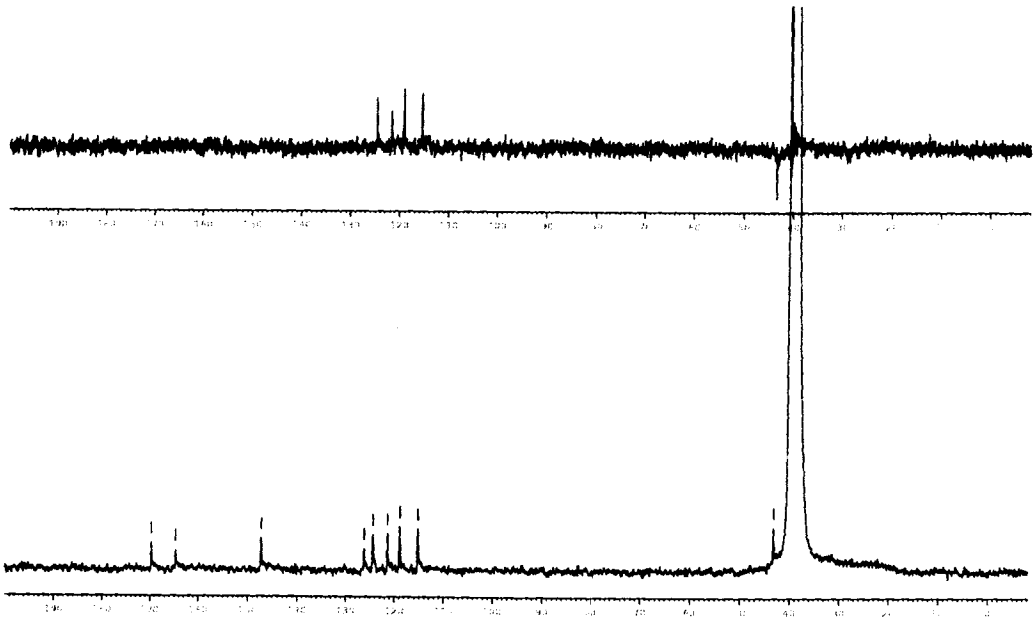
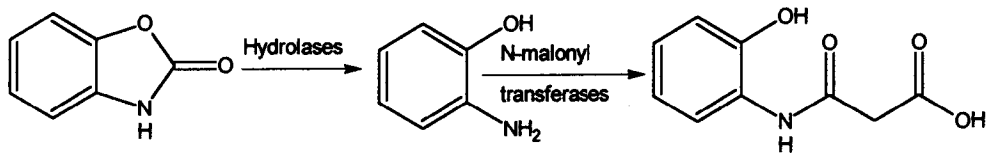


Fig 5c: ^{13}CNMR & DEPT spectra of N-(2-hydroxyphenyl)malonamic acid (2).

detoxification of benzoxazolinone. In other words all these microorganisms must be producing a common enzyme.

The metabolism of BOA involves the cleavage of the NH-C=O bond, leading to the formation of an o-aminophenol intermediate. The aminophenol is then converted to the corresponding malonamic acid by an enzyme N-malonyl transferase. This suggests *Fusarium semitectum* contains the enzyme hydrolases and N-malonyl transferase. Thus, BOA and its different derivatives can be easily bioconverted to different malonamic acid derivatives using fungal strain belonging to genus *Fusarium*. The product recovery is almost 98%.



BOA (1) **o-Amino phenol** **HPMA (2)**

β-Dicarbonyl derivatives belonging to the malonamic acid family are very important compounds having interesting pharmacological properties, including antihypertensive,[38] sedative and anticonvulsants,[39] anti-inflammatory,[40] analgesic [41,42] and central nervous system-stimulating activities.[43] The classical preparation of N-symmetrically substituted malonamides involves the high-temperature condensation of diethyl malonate derivatives with amines.[44] Other methods for the preparation of malonamides and malonamic acid esters start from Meldrum's acid[45] or from malonamic acid derivatives such as malonyl chloride,[46] malonyl monoacyl chloride[47] or malonamic acid monoethyl ester, which is activated through its conversion into the corresponding acyl chloride or via its reaction with DCC, carbonyl diheterocycles or BOP.[48] Thus, this biotransformation also describes a simple biosynthetic approach for the production of N-(2-hydroxyphenyl) malonamic acids (HPMA) with starting substrate as 2-Benzoxazolinone (BOA). As mentioned earlier 2-Benzoxazolinone (BOA) and its derivative 6-methoxy-2-benzoxazolinone (MBOA) are alleochemicals that commonly occur in cereals such as corn, rye and wheat and are considered to be

an important factor in plant-parasite interactions[49] that is they inhibit the growth of bacteria and fungi. Thus, from the above study and the literature survey it is known that fungi belonging to the genus *Fusarium* are able to degrade benzoxazolinone compounds to products like HPMA, which are less inhibitory to fungal growth.[50]

Biological screening of BOA (1) and HPMA (2) against bacterial and fungal strains:

BOA (1) and HPMA (2) using four different concentrations (100, 50, 25 and 12.5 µg/disc) were evaluated for *in vitro* antimicrobial activity against seven bacterial and seven fungal strains. Streptomycin and Nystatin were used as standard antibacterial and antifungal agents respectively. Results from antimicrobial screening tests are shown in **Table 2**. BOA inhibited the growth of *S. aureus*, *Klebsiella sp.* and *Vibrio cholerae*. It also showed antifungal activity against only one strain *Rhodotorula sp.* The biotransformed product was totally inactive at the concentration tested against bacterial as well as fungal strains.

Table 2: *In vitro* antimicrobial activity of BOA (1) and HPMA (2)

Sr. No	Microorganisms	Code No.	BOA (1) MIC (µg/disc)				HPMA (2) MIC (µg/disc)				Streptomycin
			1 (100)	2 (50)	3 (25)	4 (12.5)	5 (100)	6 (50)	7 (25)	8 (12.5)	100(µg/disc)
	Bacterial pathogens										+
1.	*Escherichia coli* (Gram -ve)	B1	-	-	-	-	-	-	-	-	+
2.	*Pseudomonas aeruginosa* (Gram -ve)	B2	-	-	-	-	-	-	-	-	+
3.	*Staphylococcus aureus* (Gram +ve)	B3	+	+	+	-	-	-	-	-	+
4.	*Salmonella typhi* (Gram –ve)	B4	-	-	-	-	-	-	-	-	+
5.	*Shigella flexineri* (Gram –ve)	B5	-	-	-	-	-	-	-	-	+
6.	*Klebsiella sp.* (Gram -ve)	B6	+	+	+	-	-	-	-	-	+
7.	*Vibrio cholerae* (Gram -ve)	B7	+	+	-		-	-	-	-	+
	Fungal pathogens										Nystatin 100units/disc
1.	*Aspergillus fumigatus*	F1	-	-	-	-	-	-	-	-	+
2.	*Fusarium sp.*	F2	-	-	-	-	-	-	-	-	+
3.	*Cryptococcus neoformans*	F3	-	-	-	-	-	-	-	-	+
4.	*Aspergillus niger*	F4	-	-	-	-	-	-	-	-	+
5.	*Rhodotorula sp.*	F5	+	+	-	-	-	-	-	-	+
6.	*Nocardia sp.*	F6	-	-	-	-	-	-	-	-	+
7.	*Candida albicans*	F7	-	-	-	-	-	-	-	-	+

(+) Active1-3mm; (-) Inactive;
(MIC) Minimal inhibitory concentration; Zone diameter (mm)

Experimental section:

Synthesis of 2-benzoxazolinone (BOA): o-aminophenol (1.0g) and urea (0.6g) were refluxed for 14 hrs. in dry pyridine.[21] After refluxing the reaction mixture was cooled and extracted with chloroform. The chloroform fraction was washed with 5% hydrochloric acid and finally with water to remove pyridine. The chloroform fraction was concentrated and purified on silica gel column chromatography. Elution of silica gel column with 15% ethyl acetate in petroleum ether afforded BOA **(1)** (0.98g).

Isolation of Fungal strains: Fungal strains *Aspergillus niger, Aspergillus ochraceous, Fusarium semitectum* and *Curvularia lunata* were isolated from the decayed leaves of mangrove plants while *Trichoderma pseudokoningii* was obtained as an association isolated from sea cucumber collected from Sinquerim, Goa, India. The above cultures were maintained on Potato Dextrose Agar (Hi Media, Mumbai) slants prepared in 1:1 sea water: distilled water. The isolates were identified by Dr. Alka Pandey, Agharkar Research Institute, Pune.

Culture conditions: Cultured mycelia of the five fungal strains used for this study were transferred into 500ml flasks containing 100ml potato dextrose broth (PDB) (Hi Media, Mumbai). PDB was prepared in 1:1 seawater: distilled water. The flasks were incubated at room temperature on a rotary shaker (240 rpm) for about 48 hrs prior to addition of BOA. 100 mg of BOA, dissolved in 5ml of ethanol was added to each flask and incubated for 14 days.

Extraction and isolation of biotransformed product: The mycelium was separated from broth by filtration. The broth was partitioned with Ethyl acetate. The organic phase was reduced to dryness in vacuo. The residue thus obtained was chromatographed on a column containing silica gel and eluted with $CHCl_3$:MeOH. After a highly pigmented (golden-yellow) band was eluted from the column, fractions

with similar composition were combined and evaporated that yielded pure biotransformed product.

2-Benzoxazolinone (BOA) (1): White crystalline solid; m.p. 140 °C; UV (MeOH): 273 (ε 610), 225 (ε 1083), 201 (ε 2312), 192 (ε 302); IR (KBr pellet) ν_{max} 3504.9, 3210.8, 1765.7, 1705.9, 1701.1, 1480.3, 1397.8, 1306.2, 1254.6, 1147.1, 1012.1, 944.6, 740.1 and 701.1 cm^{-1}; ^{1}HNMR and ^{13}CNMR ($CDCl_3$): **Table 1.**

N- (ortho-hydroxy phenyl)malonamic acid (HPMA) (2): Yellow crystalline solid, UV (MeOH): 284 (ε 397), 244 (ε 2000), 234 (ε 751), 207 (ε 2005); IR (KBr pellet) ν_{max} 3369.4, 1712.7, 1651.0, 1600.8, 1542.9, 1456.9, 1350.1, 1201.6, 1105.1 and 752.2 cm^{-1}; ^{1}HNMR and ^{13}CNMR (*d6*-DMSO): **Table 1**.

Antibacterial and antifungal assays: Antibacterial activity of BOA **(1)** and biotransformed product HPMA **(2)** was investigated by the standard paper disk assay method as described in **Chapter II, Section 1**.

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Summary

The marine environment has yielded unique secondary metabolites different from the terrestrial environment. Most natural products from the sea are novel and many possess potent biological activities. With the interest in search of such secondary metabolites, chemical investigations of some selected marine organisms were carried out. The compounds were characterized based on the spectral studies including UV, IR, NMR (^{1}H, ^{13}C, 1D & 2D) and MS.

With the aim to search for new biologically active secondary metabolites, biological screening of organic extracts of marine sponges and algae, collected from coasts of India, were studied for antimicrobial (antibacterial and antifungal) activity against clinical isolates. During this study sponges, *Psammaplysilla purpurea* and *Haliclona cribricutis* exhibited promising activity against most of the test strains and was hence selected for detailed chemical investigation.

Chemical studies of sponge *Psammaplysilla purpurea* yielded seven new bromotyrosine alkaloids purpurealidin A, B, C, D, F, G and H along with the known compounds purealidin Q, purpurealidin E, 16-debromoaplysamine-4 and purpuramine I. The structures of these compounds were elucidated using 1D and 2D NMR spectroscopic techniques. *In vitro* antimicrobial screening of purealidin Q showed good activity against *Salmonella typhi.* It was previously reported to show cytotoxic activity against tumor cell lines. Purpurealidin B showed good activity against *E. Coli, S. aureus, V. cholerae* and weak activity against *Shigella flexineri.* 16-Debromoaplysamine-4 was moderatly active against *Salmonella typhi* and very weak activity against *E. coli, S. aureus* and *V. cholerae.* An extensive effort has been made to identify additional bromotyrosine alkaloids from the second collection of the *Psammaplysilla purpurea* using electrospray ionisation-tandem mass spectrometry (ESI-MS/MS). Three new bromotyrosine alkaloids, purpurealidin I, J and K along with the four known aplysamine 2-3 and purpuramine H-I were identified.

Tandem mass spectrometry (ESI-MS/MS) approach was also used for the identification of eight molecular species of ceramides from *Haliclona cribricutis.*

Chemical investigation of mollusk *Elysia grandifolia* and its algal diet *Bryopsis plumosa* resulted in the detection of two new cyclodepsipeptides kahalalide P and Q along with kahalalide D, kahalalide F (anticancer agent under clinical development) and kahalalide G. The study was carried out using ESI-QTOF MS/MS in positive ionization mode. The sequences of the new peptides were proposed based on the mass fragmentation pattern. The two new cyclodepsipeptides, kahalalide P and Q identified are chemically similar to that of kahalalide F and by analogy expected to exhibit similar bioactivity. The fragmentation pathway showed that, not always there is sequential loss of amino acid residues from the cyclic depsipeptides. The tandem mass spectrometric study of these compounds has been discussed in details for the first time.

Microorganisms continue to be a valuable source for novel drug discoveries. Untapped fungi from unusual habitats are the focus in screening for novel bioactive secondary metabolites. The chemical investigation of the marine fungus *Eurotium sp.* resulted in isolation of two antioxidants, tetrahydroauroglaucin, isodihydroauroglaucin, cyclic peptide (diketopiperazine) echinulin and four anthraquinone derivatives; physcion, erythroglaucin, alaternine & catenarin. These compounds have been isolated from ethyl acetate soluble extract of fungal mycelia. Though the compounds are known from terrestrial origin, they are being reported for the first time from marine source. Further, the data reported, was insufficient for unambiguous assignment of proton and carbon values. In the present work, the assignment has been made entirely on the basis of 2D experiments.

Biotransformation of 2-benzoxazolinone (BOA) an alleochemical, using different marine microorganisms is also discussed. The complete biotransformation of BOA to N-(2-hydroxyphenyl) malonamic acid (HPMA) was observed with the fungal strain *Fusarium semitectum.* The structure of HPMA was elucidated using UV, IR and NMR studies.

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Antimicrobial activity of marine organisms collected off the coast of South East India

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Abstract

In vitro antimicrobial screening of nine marine sponges (Porifera) and two seaweeds, collected from south east coast of India, against selected clinical isolates of bacteria and fungi was conducted in this study. Methanolic extracts of all the marine organisms demonstrated activity against one or more of microbes tested. *Sigmadocia carnosa* was the most active exhibiting a broad spectrum antimicrobial activity against each of the microbe tested with the exception of *Fusarium* species. Contrary to this, the genus *Echinogorgia* did not show any detectable bactericidal activity but, *Echninogorgia reticulata* was weakly fungicidal against *Rhodotorula* species and *E. compecta* against *Fusarium* and *Nocardia* species. Considerable antibacterial activity was exhibited by *Haliclona cribricutis* and *Chrotella australiensis* against *Klebsiella* species and *Vibrio cholerae*, respectively. *Petrocia testudinaria* showed equally good activity against the bacterium *V. chlorae* and the fungus *Cryptococcus neoformans*. The sponges *Callyspongia fibrosa*, *Ircinia* species and the seaweed *Stoecheospermum margilatum* are totally inactive against fungi. The extracts showing good antimicrobial activity are undergoing further analysis to identify the active constituents.

Keywords: Antibacterial; Antifungal; Marine sponges

1. Introduction

Marine organisms are a rich source of structurally novel and biologically active metabolites. So far, many chemically unique compounds of marine origin with different

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biologically activity have been isolated and a number of them are under investigation and/or are being developed as new pharmaceuticals (Faulkner, 2000a,b; Da Rocha et al., 2001; Schwartsmann et al., 2001). Here mention may be made of Aurantosides (*Siliquariaspongia aponica* and *Homophynia conferta*) and Spongistatin 1 (*Hyrtios erecta*) commercially available antifungal agents discovered from marine sponges.

Screening of organic extracts from marine sponges and other marine organisms is a common approach to identify compounds of biomedical importance. Notable exception to this is the study performed by Mebs et al. (1985) and Kristina et al. (1997) where aqueous extracts from sponge species were tested for hemolytic hemagglutinating, cytotoxic, antimicrobial, anticholesterase and lethal activities.

In this report, we describe the screening of methanolic extracts of nine marine sponges and two macroalgae, collected from the coastline of India for antibacterial and antifungal activities. This study is part of a programme on screening of marine organisms for a variety of biological activities, with the aim of identifying novel with interesting and potentially useful therapeutic activities.

2. Materials and methods

2.1. Sampling and identification

Samples were collected by scuba diving from sub tidal habitats at depths of 8 to 10 m at different locations 2 nautical miles off the coastline of southeast India. They were frozen as soon as possible and transferred to the laboratory where they were extracted in methanol.

Algae were collected from Anjuna, west coast of Goa, India by hand picking during low tide.

Identification was carried out by Dr. P.A. Thomas of Vizhingam Research Center of Central Marine Fisheries Research Insititute, Kerala, India. The species investigated in this study are listed in Table 1. The samples are deposited at the National institute of Oceanography, Dona-Paula, Goa.

Table 1
Marine organisms examined in this study

Serial no.	Species
Nio 727	*Sigmadocia carnosa*
Nio 729	*Echinogorgia reticulata*
Nio 730	*Haliclona cribricutis*
Nio 731	*Callyspongia fibrosa*
Nio 732	*Ircinia sps*
Nio 733	*Echinogorgia competa*
Nio 734	*Petrocia testudinria*
Nio 738	*Chrotella australiensis*
Nio 736	*Stoechospermum marginatum* (brown alga)
Nio 739	*Cladophora prolifera* (green alga)

2.2. Extract preparation

The marine organism was homogenized in a blender with little water and extracted with MeOH (2 × 1 l.) at room temperature. The combined methanolic extract was filtered and concentrated under vacuum on a rotary evapotator at low temperature to get crude methanolic extract.

2.3. Antibacterial assay

Antibacterial activity was determined against *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Salmonella thyphi*, *Shigella flexineri*, *Klebsiella*, and *Vibrio cholerae* using the paper disk assay method (El-Masry et al., 2000). Whatman No. 1 filter paper disk of 6-mm diameter was sterilized by autoclaving for 15 min at 121 °C. The sterile disks were impregnated with different extracts (500 μg/ml). Agar plates were surface inoculated uniformly from the broth culture of the tested microorganisms. In all cases, the concentration was approximately 1.2×10^8 CFU/ml. The impregnated disks were placed on the medium suitably spaced apart and the plates were incubated at 37 °C for 24 h. Disk of Streptomycin (400 μg/ml) was used as a positive control. The diameter (mm) of the growth inhibition halos caused by the methanolic extracts of marine organisms was examined. All the assays were carried out in duplicate.

2.4. Antifungal assay

Antifungal activity was determined against of *Aspergillus fumigatus*, *Fusarium* sp., *Cryptococcus neofromans*, *Aspergillus niger*, *Rhodotorula* sp., *Norcardia* sp., and *Candida albicans* using the paper disk assay method as previously described in the antibacterial assay (see Section 2.3). The sterile disks were impregnated with different extracts (1.5 mg/ml). The inoculum concentration was 0.5×10^3–2.8×10^3 CFU/ml. Nistatin was used as a positive control at a concentration of 3 mg/ml. The plates were incubated at 24 °C for 18 h. The diameter (mm) of the growth inhibition halos caused by the methanolic extracts of marine organisms was examined. All the assays were carried out in duplicate.

3. Results and discussion

Table 2 shows the result of the in vitro testing of extracts against pathogenic bacteria. As evident from Tables 2 and 3, the organic extract from *Sigmadocia carnosa* exhibited weak activity against each of the bacteria and fungi tested except, its inactivity towards *Fusarium* species and good bactericidal activity against *V. cholerae*. Good activity against the latter is also expressed by the extracts of sponges, *Petrocia testudinria* and *Chrotella australiensis*. *Haliclona cribricutis* showed considerable activity against Klebsiella species and was weakly active against both, *S. aureus* and *S. flexineri*. *Callyspongia fibrosa* was weakly effective against *V. cholerae* and *Klebsiella* species. Equally active was the brown

Table 2
Antibacterial activity of methanolic extract of marine organisms

Species	Microorganisms						
	Escherichia coli	*Pseudomonas aeruginosa*	*Staphylococcus aureus*	*Salmonella typhi*	*Shigella flexineri*	*Klebsiella* sp.	*Vibrio cholerae*
Sigmadocia carnosa	+	+	+	+	+	+	++
Echinogorgia reticulata	–	–	–	–	–	–	–
Haliclona cribricutis	–	–	+	–	+	++	–
Callyspongia fibrosa	–	–	–	–	–	+	+
Ircinia sp.	–	–	+	–	–	–	–
Echinogorgia competa	–	–	–	–	–	–	–
Petrocia testudinria	–	–	+	–	–	+	++
Chrotella australiensis	–	–	–	+	+	+	++
Stoechospermum marginatum	–	–	–	–	–	+	+
Cladophora prolifera	–	–	+	–	–	–	+

(–) No activity, (+) weak activity (7–10-mm halo), (++) good activity (10–15-mm halo).

alga *Stoechospermum marginatum* against the last two bacteia, contrary to the observation made by De Silva et al. (1982). These authors report bactericidal activity attributed to the spatane diterpenoid, 19-acetoxy-5, 15,18-trihydroxy spata-13, 16-diene. The inactivity towards *S. aureus* observed by us could be due to the collection of the seaweed at different developmental stages of the plant.

In this present investigation, as mentioned *H. cribricutis* inhibited *Klebsiella* species considerably and was weakly active against *S. aureus* and *S. flexineri*. It was also found to be weakly active against the fungus, *A. fumigatus*. This is not surprising as the sponge belonging to this genus and collected from different regions is reported to possess wide

Table 3
Antifungal activity of methanolic extract of marine organisms

Species	Microorganisms						
	Aspergillus fumigatus	*Fusarium* sp.	*Cryptococcus neoformans*	*Aspergillus niger*	*Rhodotorula* sp.	*Norcardia* sp.	*Candida albicans*
Sigmadocia carnosa	+	–	+	+	+	+	+
Echinogorgia reticulata	–	–	–	–	–	+	–
Haliclona cribricutis	+	–	–	–	–	–	–
Callyspongia fibrosa	–	–	–	–	–	–	–
Ircinia sp.	–	–	–	–	–	–	–
Echinogorgia competa	–	+	–	–	–	+	–
Petrocia testudinria	–	–	++	–	+	–	–
Chrotella australiensis	+	+	–	+	+	+	++
Stoechospermum marginatum	–	–	–	–	–	–	–
Cladophora prolifera	–	–	–	+	–	–	–

(–) No activity, (+) weak activity (7–10-mm halo), (++) good activity (10–15-mm halo).

variety of compounds with different biological activities. Thus, *Haliclona* sp. from Indonesia yielded a triterpene ketide, Halicotriol B with weak antimicrobial activity against *S. aureus* and *Bacillus subtilis* (Crews and Harrison, 2000). The antifungal papuamine has been reported by Baker et al. (1988) from an *Haliclona* sp. Fahy et al. (1988) report a major antimicrobial alkaloid haliclonadiamine together with antifungal papuamine from *Haliclona* sp. of Palau. Antifungal aminoalcohols have been identified from a new species of *Haliclona* from Queensland (Clark et al., 2001). Charan et al. (1996) report antimicrobial Haliclonacyclamines. It is therefore expected that the activity found by us in the extract of *H. cribricutis* could have, at least partially, been contributed by any one of the above compounds isolated from this genus. Organisms belonging to the same genus are bound to have common chemical constituents. Parameswarn et al. (1992) report significant anti-viral and antibacterial activities in petroleum ether and ethyl acetate fractions of *H. cribricutis* and the activity observed against *K. pneumoniae* and *Vibrio parahaemolyticus* was attributed to *o*-demethyl renierones. *Ircinia* sp. exhibited mild antibacterial activity only against *S. aureus* but all the fungal strains tested were insensitive to it.

A number of cytotoxic compounds are reported from this genus. These include 73-deoxychondropsin A from an Australian *Ircinia ramosa*. Chondropsin C was found in a Philippine *Ircinia* species (Rashid et al., 2001). Moderately cytotoxic cumulated ketene irciniketene has been reported from *Ircinia selaginea* collected from Guangxi Province, China (Yan et al., 2001). Cytotoxic Kohamaic acids A and B are known to be constituents of *Ircinia* species from Okinawa (Kokubo et al., 2001). Three tricarbocyclic sesterterpenoids of the cheilanthane class isolated from a Queensland *Ircinia* species were found to be inhibitors of MSK-1 and MAPKA-2 protein kinases (Buchanan et al., 2001). Though cytotoxic compounds are reported from this genus, there are no reports of any antimicrobial activity in the extracts.

In the present investigation, the green alga *Cladophora prolifera* exhibited mild bactericidal activity against *S. aureus* and *V. cholerae*. This alga has been recently reported to support the growth of indicator bacteria *E. coli* (Muruleedharan et al., 2003).

4. Conclusion

Marine organisms collected from the Southeast coast of India have been shown to possess a number of biological activities. In our studies, the most interesting species are that of *S. carnosa, H. cribricutis* and *Petrocia testidinria*. To the best of our knowledge, this is the first report demonstrating the antimicrobial activity of most of the marine organisms taken up in this study, with few exceptions.

These organisms are currently undergoing detailed investigations with the objective of isolating biologically active molecules along with the search for novel compounds. Furthermore, the encouraging biological activities seen in this study show that the Indian coastline is a potential source of variety of marine organisms worthy of further investigation.

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New bromotyrosine alkaloids from the marine sponge *Psammaplysilla purpurea*

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Abstract—Seven new bromotyrosine alkaloids Purpurealidin A, B, C, D, F, G, H and the known compounds Purealidin Q, Purpurealidin E, 16-Debromoaplysamine-4 and Purpuramine I have been isolated from the marine sponge *Psammaplysilla purpurea*. Their structure was elucidated on the basis of detailed 1D, 2D NMR and MS spectroscopic data. Purpurealidin B, 16-Debromoaplysamine-4 and Purpuramine I exhibited in vitro antimicrobial activities against *E. coli*, *S. aureus*, and *V. cholerae*. In addition, Purpurealidin B and 16-Debromoaplysamine-4 were also active against *Shigella flexineri* and *Salmonella typhi* while Purealidin Q was bactericidal only against *Salmonella typhi*.

1. Introduction

Marine sponges of the order Verongida are characterized by their ability to synthesize brominated tyrosine derivatives, many of which possess potent antimicrobial and cytotoxic activities. Chemical modification occurs both in the side chain and the aromatic ring of the brominated tyrosine precursors, giving rise to a broad range of biosynthetically related compounds.[1] Purealin,[2–4] Lipopurealin A–E,[5–6] Purealidin A–S,[6–11] Psammaplysin A–B,[12] Purpuramine A–J,[11,13] Aplysamines 2-5,[14] Macrocyclic peptides Bastadins[15] etc. have been previously reported from the sponge *Psammaplysilla* sp. In our earlier communication we reported the isolation of known compounds 16-Debromoaplysamine-4[9,16] and Purpuramine I.[13,16] The present paper deals with the isolation, structures and in vitro bioactivity of bromotyrosine metabolites Purpurealidin A–D and F–H along with Purealidin Q[10] and Purpurealidin E.[17]

2. Results and discussions

The animals were collected by scuba diving at a depth of 8–10 m from Mandapam, Tamil Nadu, India. A voucher specimen is deposited at the National Institute of Oceanography, Dona Paula Goa, India. The frozen sponge (250 g, dry weight) was extracted with Methanol (1 L×3) and concentrated under vacuo to obtain 10 g of crude extract. Successive chromatography of the crude MeOH extract on Silica gel, Sephadex LH-20 and a reverse phase column yielded 11 compounds (see Fig. 1). The structures and complete assignment of the ^{1}H and ^{13}C NMR spectra for the new compounds was determined based on extensive 1D and 2D NMR spectroscopic studies.

Compound **1**, was obtained as colourless oil. HRMS showed pseudomolecular ion peak at *m/z* 741.8, 743.8, 745.8, 747.8, 749.8 in the ratio 1.07:4.23:6.2:4.0:1.0, which indicated the presence of four bromine atoms in the molecule and established the molecular formula as $C_{23}H_{27}N_3O_4Br_4$. It was identified as Purealidin Q previously described from the Okinawan marine sponge *Psammaplysilla purea*, by comparison with the spectral data (UV, IR, 1D and 2D NMR) reported in the literature (see Table 1).[10] The stereochemistry at C1 and C6 of the spiroisoxazole ring in **1** was deduced to be *trans* from the proton chemical shift (ca. δ 4.05) of H-1 in CD_3OD.[18] The absolute configuration was not assigned. The HRMS of **1** also showed pseudomolecular peaks at *m/z* 755.8, 757.8, 759.8, 761.8, 763.8 for the minor compound Purpurealidin A (**2**) (see Table 1), which is 14 amu higher than Purealidin Q. This can be accounted from the presence of an additional methyl group either as –OMe at C-1 or –NMe at N-9. The position was established as –NMe at N-9 based on the fragmentation ion peaks. The MS/MS at *m/z* 755.8, 759.8 and 763.8 gave the product ions at *m/z* 418.9, 420.9, 422.9 for fragmentation at C8–C9 (Scheme 1) and the absence of mass peaks at *m/z* 404.9, 406.9, 408.9 (Scheme 2) as found in Purealidin Q.

Keywords: Antimicrobial activity; Bromotyrosine alkaloids; Marine sponge; *Psammaplysilla purpurea*; Purpurealidin.
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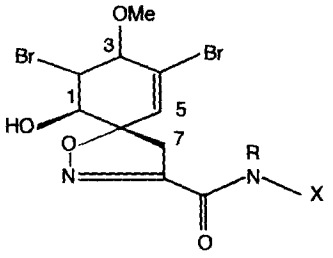
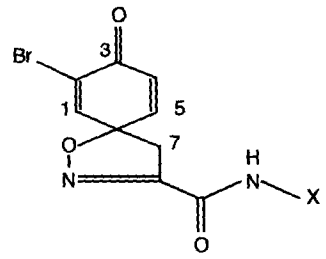
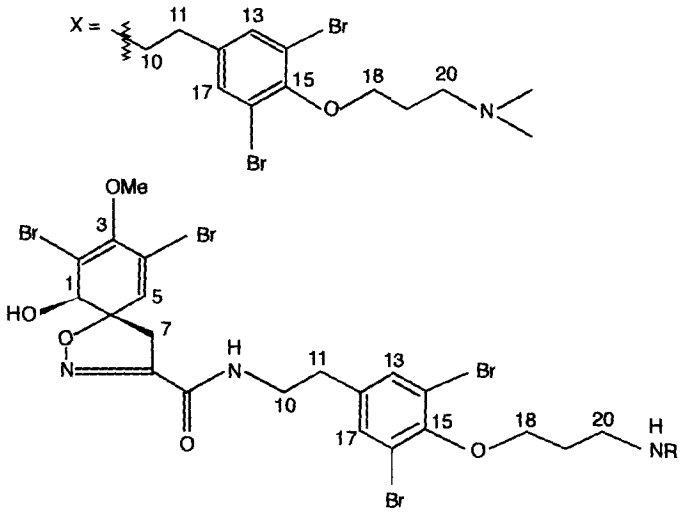


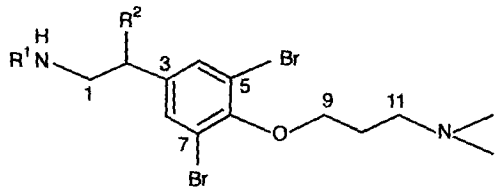
Purealidin Q (1) R=H
Purpurealidin A (2) R=Me



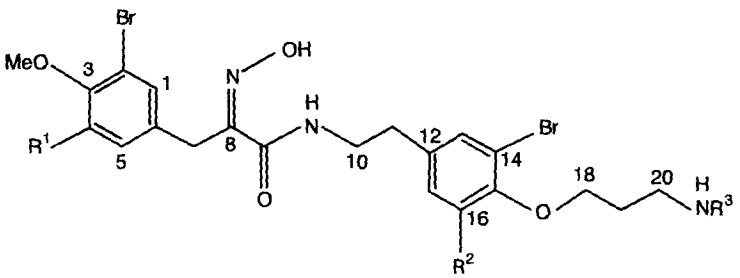
Purpurealidin B (3)



Purpurealidin C (4) R=-$CO(CH_2)_{11}CH(CH_3)_2$
Purpurealidin C (5) R=-$CO(CH_2)_{12}CH_2CH_2CH_3$



Purpurealidin E (6) R^1=H, R^2=H
Purpurealidin F (7) R^1=H, R^2=OH
Purpurealidin G (8) R^1=-$COCH_2CH_3$, R^2=OH

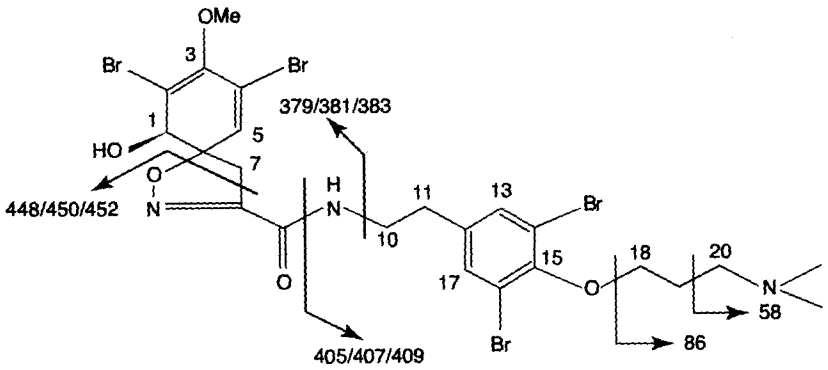


16-Debromoaplysamine-4 (9) R^1=Br, R^2=R^3=H
Purpurealidin H (10) R^1=Br, R^2=H, R^3=Me
Purpurealidin I (11) R^1=H, R^2=Br, R^3=Me

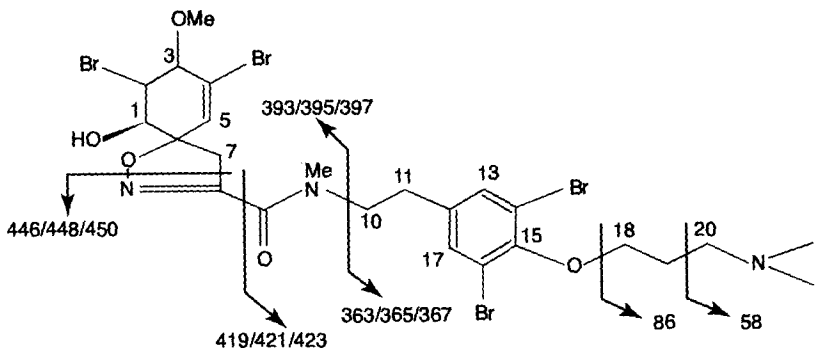
Figure 1. Structures of compounds 1–11 from the sponge *Psammaplysilla purpurea*.

Table 1. ^{1}H, ^{13}C NMR of Purealidin Q (**1**) and Purpurealidin A (**2**), in CD_3OD

Carbon Nos.	1		2	
	^{13}C NMR	^{1}H NMR	^{13}C NMR	^{1}H NMR
1	130.9, d	6.29 (1H, s)	130.9, d	6.29 (1H, s)
2	121.4, s		121.4, s	
3	147.5, s		147.5, s	
4	113.3, s		113.3, s	
5	73.8, d	4.33 (1H, s)	73.8, d	4.33 (1H, s)
6	91.9, s		91.9, s	
7	38.8, t	2.98 (1H, d, J=18.3 Hz)	38.8, t	2.98 (1H, d, J=18.3 Hz)
		3.92 (1H, d, J=18.6 Hz)		3.92 (1H, d, J=18.6 Hz)
8	153.9, s		153.9, s	
9	159.2, s		159.2, s	
10	40.1, t	3.54 (2H, t, J=12.2, 6.6 Hz)	40.1, t	3.54 (2H, t, J=12.2, 6.6 Hz)
11	34.2, t	2.77 (2H, t, J=12.6, 6.8 Hz)	34.2, t	2.77 (2H, t, J=12.6, 6.8 Hz)
12	137.2, s		137.2, s	
13,17	132.9, d	7.35 (2H, s)	132.9, d	7.35 (2H, s)
14,16	118.1, s		118.1, s	
15	151.5, s		151.5, s	
18	71.0, t	4.05 (2H, t, J=12.0, 5.6 Hz)	71.0, t	4.05 (2H, t, J=12.0, 5.6 Hz)
19	27.0, t	2.19 (2H, m)	27.0, t	2.19 (2H, m)
20	56.0, t	2.92 (2H, t, J=5.6 Hz)	56.0, t	2.92 (2H, t, J=5.6 Hz)
$-OCH_3$	60.0, q	3.74 (3H,s)	60.0, q	3.74 (3H, s)
$-N(CH_3)_2$	44.5, q	2.89 (6H, s)	44.5, q	2.89 (6H, s)
–NH		7.40 (1H, s)		
$-N-CH_3$			39.3, t	3.4 (3H, s)



Scheme 1. Fragmentation patterns of Purealidin Q (**1**).



Scheme 2. Fragmentation patterns of Purpurealidin A (**2**).

The monoisotopic peaks at *m/z* 58, 86 also help in confirming the side chain to be dimethylpropylamine.

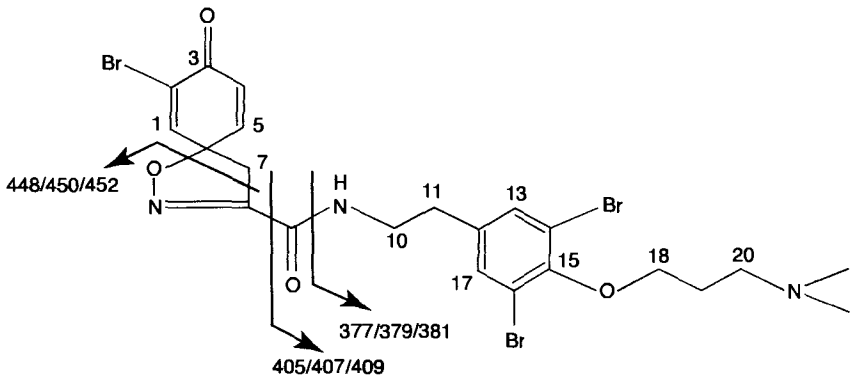
The Purpurealidin B (**3**) contains a dibromospirocyclohexadienonyldihydroisoxazole moiety of the type found in Verongida metabolites but differing in having one bromine atom and dienone ring system.[19,20] The mass spectrum of Purpurealidin B showed a 1.06:3.13:3.06:1.0 quartet for the pseudomolecular ion peak $[M+H]^+$ at *m/z* 631.8, 633.8, 635.8, 637.8, indicative of the presence of three bromine

Table 2. ^{1}H, ^{13}C NMR and COSY of Purpurealidin B (**3**), in $CDCl_3$

Carbon Nos.	^{13}C NMR	^{1}H NMR	COSY	HMBC
1	144.2, d	7.27 (1H, d, J=2.2 Hz)	H5	C2, C3, C5
2	125.8, s			
3	177.9, s			
4	127.7, d	6.34 (1H, d, J=9.8 Hz)	H5	C2, C6
5	144.2, d	6.87 (1H, dd, J=2.2, 9.8 Hz)	H1, H4	C3
6	84.5, s			
7	43.1, t	3.48 (1H, d, J=18.0 Hz)		
		3.90 (1H, d, J=18.0 Hz)		C5, C8
8	153.4, s			
9	158.4, s			
10	40.4, t	3.57 (2H, t, J=7.0 Hz)	H11	C9, C11, C12, C13, 17
11	34.1, t	2.81 (2H, t, J=7.2 Hz)	H10	C10
12	137.3, s			
13,17	132.9, d	7.34 (2H, s)		C11, C13,17, C15, C14,16
14,16	117.9, s			
15	150.8, s			
18	69.5, t	4.04 (2H, t, J=5.6 Hz)	H19	C19, C20
19	25.2, t	2.38 (2H, m)	H20, H18	C18, C20
20	55.7, t	3.42 (2H, t, J=5.6 Hz)	H19	C18, C19
-NH	7.4		C8	
$-N(CH_3)_2$	43.1, q	2.89(6H, s)		

atoms in the molecule, which is appropriate for the molecular formula $C_{22}H_{24}N_3O_4Br_3$. The ^{13}C NMR spectrum had 22 carbon signals, the multiplicities of which were assigned from a DEPT 135 experiment as two methyls, six methylenes, five methines, and nine quarternary carbons. The coupling pattern in proton signals at δ_H 7.27 (1H, d, J= 2.2 Hz), 6.87 (1H, dd, J=2.2, 9.8 Hz) and 6.34 (1H, d, J= 9.8 Hz) indicated the presence of a 2,3,6-trisubstituted aromatic moiety (see Table 2). Analysis of the proton COSY spectrum showed connectivities for H1–H5, H4–H5 and H5–H1–H4 for the 2, 3, 6-trisubstituted aromatic moieties. The HMBC experiment showed that the proton signal at δ_H 7.27 is connected to C2, C3, C5 and δ_H 6.34 to C2, C6, C5 and δ_H 6.87 to C3. The presence of signal at δ_C 177.18 in the ^{13}C NMR spectrum shows presence of a ketone in the ring system. Thus, the partial structure was confirmed to be monobromospirocyclohexadienoneisoxazole. The structure of the remaining part of the molecule, which is linked to the nitrogen atom of the carboxamide group at C-8, was similar to that of Purealidin Q, which was established by inspection of 1H–1H connectivities. This clearly indicates presence of H10–H11 and also the H18–H19–H20 methylene chain. The HMBC showed a proton signal at δ_H 7.34 (2H, s) connected to C11, C13, 17, C15, C14, 16 for the tetrasubstituted aromatic ring. A 6H singlet at δ_H 2.89 was assigned to be a dimethylamino group. This is also confirmed by pseudomolecular peaks at *m/z* 405, 407, 409, and 448, 450, 452 (Scheme 3).

Purpurealidin C (**4**) and D (**5**) exhibited the same characteristic features as Purealidin Q (**2**) except for one additional amide proton at δ_H 5.3, the carbonyl signal at δ_C 173.6, and methylene signals at δ_C 27.0–32.7 (δ_H 1.19) indicative of the presence of an additional amide carbonyl group and long straight fatty chain. A doublet at δ_H 0.80 (6H, J=6.8 Hz) was assigned to the isopropyl group. The structure is also confirmed by ^{1}H, ^{13}C, COSY and HMBC spectral data (see Table 3). The molecular weight of Purpurealidin C (**4**) was higher than that of Purealidin Q (**2**). The low resolution mass spectrum showed pseudomolecular ion peaks at *m/z* 938.0, 940.0, 942.0, 944.0, 946.0. The mass spectrum showed additional pseudomolecular ion peaks at *m/z* 952.0, 954.0, 956.0, 958.0, 960.0, which are 14 units higher than (**4**) indicative of an extra methylene group. The presence of a signal at δ_H 0.70 (t) and ^{13}C signal at δ_C 14.0 suggested for terminal methyl group in **5** (see Table 4). The



Scheme 3. Fragmentation patterns of Purpurealidin B (**3**).

Table 3. ^{1}H, ^{13}C NMR, COSY and HMBC of Purpurealidin C (**4**), in $CDCl_3$

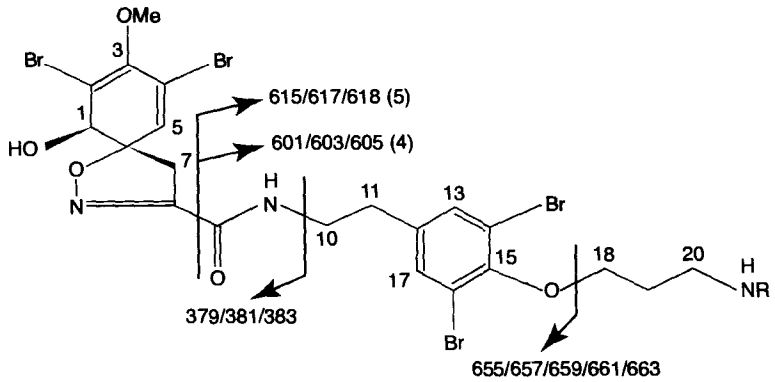
Carbon Nos.	^{13}C NMR	^{1}H NMR	COSY	HMBC
1	74.0, d	4.28 (1H, s)		C3, C2, C5
2	112.7, s			
3	148.0, s			
4	121.4, s			
5	130.0, s	6.24 (1H, s)		C4, C3
6	91.5, s			
7	38.8, t	Ha=2.93 (1H, d, J=18.6 Hz)	Hb	C5, C1, C8
		Hb=3.88 (1H, d, J=18.3 Hz)	Ha	
8	154.9, s			
9	159.1, s			
10	40.3, t	3.54 (2H, t, J=13.2, 6.6 Hz)	H11	C11
11	34.4, t	2.67 (2H, t, J=12.6, 7.8 Hz)	H10	C10, C12, C13
17				
12	138.0, s			
13,17	132.9, d	7.34 (2H, s)		C11, C13,17, C15, C14,16
14,16	118.2, s			
15	151.2, s			
18	71.0, t	4.01 (2H, t, J=12.0, 6.0 Hz)	H19	C15, C19, C20
19	29.2, t	2.06 (2H, m)	H18, H20	C18, C20
20	37.0, t	3.63 (2H, t)	H19	C18, C19
21	173.6, s			
22	34.4, t	2.67(2H, m)		
23–32	27.0–32.	1.19 (24H, s)		
	7, t			
33	29.0, d	1.53 (2H, m)		C34,35
34,35	22.6, q	0.80 (6H, d, J=6.8 Hz)	H33	
–N-9		7.43 (1H, d)		
–N-20		5.30 (1H, s)		
–OCH3	60.0, q	3.67 (3H, s)		C3

fragmentation pattern of **4** and **5** (Scheme 4) is different from the Arapplysillin II isolated from the *Psammaplysilla purpurea*[21] and agrees well with the structure assigned.

The mass spectrum of Purpurealidin E (**6**) showed a pseudomolecular ion peak $[M+H]^+$ at *m/z* 378.9768, 380.9757, 382.97 in the ratio 1.05:2.05:1.0, characteristic for the presence of two bromine atoms. Examination of the ^{1}H and ^{13}C and COSY showed that the structure is similar to the part structure of Purealidin Q. In addition, the mass spectrum of (**6**) showed minor pseudomolecular ion peaks at *m/z* 394.9, 396.9, 398.9 and 451.0, 453.0, 455.0 compounds Purpuealidin F (**7**) and G (**8**). The ^{1}H NMR signal at 3.77 (1H, m) and δ_C 59.6 is accounted for the hydroxy methine at

Table 4. ^{1}H, ^{13}C NMR, COSY and HMBC of Purpurealidin D (**5**), in $CDCl_3$

Carbon Nos.	^{13}C NMR	^{1}H NMR	COSY	HMBC
1	74.0, d	4.28 (1H, s)		C3, C2, C5
2	112.7, s			
3	148.0, s			
4	121.4, s			
5	130.0, s	6.24 (1H, s)		C4, C3
6	91.5, s			
7	38.8, t	Ha=2.93 (1H, d, J=18.6 Hz)	Hb	C5, C1, C8
		Hb=3.88 (1H, d, J=18.3 Hz)	Ha	
8	154.9, s			
9	159.1, s			
10	40.3, t	3.54 (2H, t, J=13.2, 6.6 Hz)	H11	C11
11	34.4, t	2.67 (2H, t, J=12.6, 7.8 Hz)	H10	C10, C12, C13, 17
12	138.0, s			
13,17	132.9, d	7.34(2H, s)		C11, C13,17, C15, C14,16
14,16	118.2, s			
15	151.2, s			
18	71.0, t	4.01 (2H, t, J=12.0, 6.0 Hz)	H19	C15, C19, C20
19	29.2, t	2.06 (2H, m)	H18, H20	C18, C20
20	37.0, t	3.63 (2H, t)	H19	C18, C19
21	173.6, s			
22	34.4, t	2.67 (2H, m)		
23–34	27.0–32.	1.19 (24H, s)		
	7, t)			
35	29.0, d	1.53 (2H, m)		C35
36	14.0, q	0.70 (3H, t)	H35	C34
–N-9		7.43 (1H, s)		
–N-20		5.40 (1H, s)		
–OCH3	60.0, q	3.67 (3H, s)		C3



Scheme 4. Fragmentation patterns of Purpurealidin C (4) and D (5).

C2 in 7 and 8 (see Table 5). The carbonyl signal at δ_C 173.0, methylene signal at δ_C 29.2 (δ_H 1.20, s) and methyl signal at δ_C 14.0 (δ_H 0.81, t, J=7.0 Hz) were indicative of the presence of an additional amide carbonyl group and ethyl groups.

The mass spectrum of the known compound that we have reported earlier, 16-Debromo aplysamine-4 (**9**), revealed characteristic isotope peaks for $[M+H]^+$ pseudo molecular ion at 619.8, 621.8, 623.8 and 625.8 in the ratio 1.05:3.1:3.06:1.0, indicating the presence of three bromine atoms in the molecule. Its ^{13}C NMR spectrum had 21 carbon signals, which were designated as one methyl, six methylenes, five methines, and nine quarternary carbons from a DEPT135 experiment. The signals at 7.52 (1H, s) and 7.40 (1H,s) and 7.33 (1H, d, J=2.0 Hz), 6.86 (1H, d, J=8.4 Hz) and 7.02 (1H, dd, J=8.4, 2.0 Hz) in the ^1H NMR spectrum indicated the presence of tetra and 1,2,4-trisubstituted aromatic moieties. The IR absorptions at 3350, 1655, and 1624 cm^{-1} and ^{13}C NMR signals at 163.8 and 152.3 ppm were indicative of amide and oxime groups. The exchangeable proton signals at δ_H 11.40 (2H, br m), 8.70 (1H, br s,) and 7.90 (1H, br s,) in the ^1H NMR spectrum indicated the presence of NH_2, NH and OH groups. The presence of a primary amine in the molecule is also confirmed by the positive ninhydrin test. The above results, as well as the assumption that this compound is a derivative of aplysamine/purpuramine, indicated its molecular formula to be $C_{21}H_{24}Br_3N_3O_4$. The upfield ^{13}C NMR chemical shift of C-7 (27.3 ppm) suggested *E* configuration of the oxime as the corresponding value for (Z)-oxime is > 35 ppm.[1] It also shows additional singly charged $[M+H]^+$ at *m/z* at 633.8, 635.8, 637.8, 639.8 for (**10**), 14 units higher than that of the compound **9**. This is accounted for the methyl group at the terminal *N*-methyl.

Compounds **1**, **3**, **9** and **11** were evaluated for their antimicrobial activity (see Table 6) against *E. coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Salmonella typhi*, *Shigella flexineri*, *Klebsiella* sp and *V. cholerae* bacterial strains and fungal strains of *Aspergillus fumigatus*, *Fusarium* sp, *Cryptococcus neoformans*, *Aspergillus niger*, *Rhodotorula* sp, *Norcardia* sp, and *Candida albicans*. The compounds did not show any activity against bacterial strains *Klebsiella* sp, *Pseudomonas aeruginosa* and all fungal strains. Purealidin Q (**1**) showed good activity against *Salmonella typhi*. It was previously reported to show cytotoxic activity against tumor cell lines and moderate inhibitory against epidermal growth factor (EGF) kinase.[10] Purpurealidin B (**3**) showed good activity against *E. coli*, *S. aureus*, *V. cholerae* and weak activity against *Shigella flexineri*. 16-Debromo aplysamine-4 (**9**) showed moderate activity against *Salmonella typhi* and very weak acivity against *E. coli*, *Staphylococcus aureus* and *V. cholerae*.

Table 5. ^1H, ^{13}C NMR and COSY of Purpurealidin E (**6**), F (**7**), G (**8**) in CD_3OD

Carbon Nos.	6		7		8	
	^{13}C NMR	^1H NMR	^{13}C NMR	^1H NMR	^{13}C NMR	^1H NMR
1	40.0, t	2.73 (2H, t, J=13.2, 6.6 Hz)	40.0, t	2.73 (2H, t, J=13.2, 6.6 Hz)	40.0, t	2.73 (2H, t, J=13.2, 6.6 Hz)
2	33.6, t	3.24 (2H, t)	59.6, t	3.77 (1H, m)	59.6, t	3.77 (1H, m)
3	130.3, s		130.3, s		130.3, s	
4,8	133.0, t	7.43 (2H,s)	133.0, t	7.43 (2H, s)	133.0, t	7.43 (2H, s)
5,7	117.3, s		117.3, s		117.32, s	
6	150.7, s		150.7, s		150.72, s	
9	69.8, t	4.05 (2H, t, J=5.6 Hz)	69.8, t	4.05 (2H, t, J=5.6 Hz)	69.8, t	4.05 (2H, t, J=5.6 Hz)
10	25.0, t	2.23 (2H, m)	25.0, t	2.23 (2H, m)	25.0, t	2.23 (2H, m)
11	55.8, t	3.44 (2H, t)	55.8, t	3.44 (2H, t)	55.8, t	3.44 (2H, t, J=5.6 Hz)
		J=5.6 Hz)		J=5.6 Hz)		J=5.6 Hz)
12,13	42.7, q	2.90 (6H, s)	42.7, q	2.90 (6H, s)	42.7, q	2.90 (6H, s)
NH_2		7.63 (br, s)				
NH				8.10 (br, s)		8.10 (br, s)
CO					173.0	
CH_2					29.2	1.20 (2H, s)
CH_3					14.0	0.81 (3H, t, J=7.0 Hz)

Table 6. Effect of compounds **1**, **3**, **9** and **11** on growth of microbial strains (MIC in μg/ml)

Compounds	*E. Coli*	*S. aureus*	*Salmonella typhi*	*Shigella flexineri*	*Vibrio cholarae*
Purealidin Q (**1**)	—	—	>25	—	—
Purpurealidin B (**3**)	>12	10	—	100	25
16-Debromo aplysamine 4 (**9**)	250	200	>50	—	100
Purpuramine I (**11**)	100	50	—	—	100
Streptomycin	10	10	10	10	10

Good activity: 10–25 μg/ml. Moderate activity: 26–100 μg/ml. Weak activity: >100 μg/ml.

Moderate acivity against *S. aureus* was confirmed for Purpuramine I (**11**) according to the previous studies. It also showed moderate activity against *E. coli* and *V. cholerae*.

3. Experimental

3.1. General experimental procedures

Column chromatographies were carried out using Silica gel (60–120 mesh, Qualigens), gel filtrations were carried out using Sephadex LH20 17-0090-01 Pharmacia Biotech). Fractions were monitored on TLC using alumina-backed sheets (Si gel 60 F254, 0.25 mm thick) with visualization under UV (254 nm) and Ninhydrin spray reagent. All analytical reverse phase HPLC (Chromspher 5 C18 column 250×10 mm, MeOH/H2O 85/15) were performed with a P4000 pump (Spectra system) equipped with a UV2000 detector (spectra system).

UV spectra were recorded in MeOH, using a Shimadzu UV–Vis 2401PC Spectrophotometer, and IR spectra were recorded on a Shimadzu FT-IR 8201PC Spectrophotometer. Optical rotations were recorded in MeOH using Optical Polarimeter ADP220 (Bellingham Stanley Ltd).

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

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

3.2. Animal material

The animals were collected by scuba diving at a depth of 8–10 m from Mandapam, Tamil Nadu, India, and identified by Dr. P. A. Thomas of Vizhingam Research Center of Central Marine Fisheries Research Institute, Kerala, India. A voucher specimen is deposited at the National Institute of Oceanography, Dona Paula Goa, India.

3.3. Extraction and isolation

The frozen sponge (250 g, dry weight) was extracted with methanol (1 L×3) and concentrated under vacuo to obtain 10 g of crude extract. The extract showed antimicrobial activity against pathological strains, which was chromatographed on silica gel (Qualigens silica gel 60–120 mesh) column using dichloromethane with increasing amounts of methanol as eluent. The fractions eluted with 8, 10 and 20% were purified separately. The fraction eluted with 8% MeOH (1.5 g) was further purified by repeated[1] gel chromatography (Sephadex LH20) columns using chloroform/methanol (1:1) to get Purealidin Q (200 mg), Purpurealidin B (800 mg) and Purpurealidin C and D (400 mg). The fractions eluted with 10% MeOH were purified on reverse phase HPLC using Chromspher 5 C18 column 250×10 mm^2, MeOH/H_2O 85/15, flow rate 2 mL/min and UV detection at λ_{max} 254 nm) which afforded 16-Debromo aplysamine-4, Purpurealidin-H (R_t 18.4 min) (20 mg) and Purpuramine I (R_t 27.5 min) (25 mg). The fractions eluted with 20% were subjected to silica gel column eluted with increasing amounts of methanol in dichloromethane to yield mixture of Purpurealidin E, F, G (300 mg).

3.3.1. Purealidin Q (1). Colorless oil, UV (MeOH) λ_{max} 277 nm (ε 1700), 284 nm (ε 1400); $[\alpha]_D^{28}=+9.5$ (*c* 0.2, MeOH); IR (neat) ν_{max} 3418, 2922, 1668, 1537, 1458.1, 1254, 1051, 920, 737 cm^{-1}; ^{1}H and ^{13}C recorded in CD_3OD see Table 1; HRMS: *m/z* (relative heights) 741.8691(450), 743.8871(1780), 745.8710(2600), 747.8762(1700), 749.7914(420) [1.07:4.23:6.2:4.0:1.0],† $[M+H]^+$, found 741.8691 $C_{23}H_{27}N_3O_5Br_4$ requires 741.8764; $[M+H-Br]^+$ 662.9, 664.9, 666.9, 668.9; $[M+H+Br+CH_3]^+$ 647.8, 649.8, 651.8, 653.8; 404.8, 406.8, 408.8; 378.9, 380.9, 382.9; 348.9, 350.8, 352.8; 58; 86.

3.3.2. Purpurealidin A (2). Colorless oil, UV (MeOH) λ_{max} 277 nm (ε 1700), 284 nm (ε 1400); $[\alpha]_D^{28}=+9.5$ (*c* 0.2, MeOH); IR (neat) ν_{max} 3418, 2922, 1668, 1537, 1458.1, 1254, 1051, 920, 737 cm^{-1}; ^{1}H and ^{13}C recorded in CD_3OD see Table 1; HRMS: *m/z* (relative heights) 755.8819(55), 757.8799(210), 759.8810(310), 761.8820(200), 763.8(50) [1.1:4.2:6.2:4.0:1.0],† $[M+H]^+$, found 755.8819 $C_{24}H_{29}N_3O_5Br_4$ requires 755.8920.

3.3.3. Purpurealidin B (3). White amorphous solid, mp 175.8 °C; UV (MeOH) λ_{max} 283 (1320); IR (KBr pellet) ν_{max} 3302, 2932, 2689, 1678, 1605, 1541, 1460, 1383, 1259, 910 and 739 cm^{-1}. ^{1}H and ^{13}C recorded in $CDCl_3$ see Table 2; HRMS: *m/z* (relative heights) 631.8403(1600), 633.8185(4700), 635.8118(4600), 637.8226(1500) [1.06:3.13:3.03:1.0],† $[M+H]^+$, found 631.8403 $C_{22}H_{24}N_3O_4Br_3$ requires 631.8396; 404.9, 406.9, 408.9; 376.9, 378.9, 380.9; 224.9, 226.9.

3.3.4. Purpurealidin C (4). Colorless oil, UV (MeOH) λ_{max} 282(10,000), 218(2500), $[\alpha]_D^{28}=+158.5$ (*c* 0.2, $CHCl_3$); IR (KBr pellet) ν_{max} 3319, 2925, 2854, 1660, 1605, 1456, 1257, 739 cm^{-1}, ^{1}H and ^{13}C recorded in $CDCl_3$ see Table 3 ESI-MS: *m/z* (relative heights) 938.0(22), 940.0(85), 942.0(125), 944.0(80), 946.0(20) [1.1:4.2:6.2:4.0:1.0],† $[M+H]^+$,

† Real ratios of the pseudomolecular ion peaks.

found 938.05 $C_{36}H_{51}N_3O_6Br_4$ requires 938.0591; 615, 617, 619; 379, 381, 383.

3.3.5. Purpurealidin D (5).[2] Colorless oil, UV (MeOH) λ_{max} 282(10,000), 218(2500); IR (KBr pellet) ν_{max} 3319, 2925, 2854, 1660, 1605, 1456, 1257, 739 cm^{-1}, ^1H ($CDCl_3$, 300 MHz) see Table 4 ESI-MS: *m/z* (relative heights) 952.0(16), 954.0(65), 956.0(95), 958.0(60), 960.0(15) [1.06:4.3:6.3:4.0:1.0],† $[M+H]^+$, found 952.07 $C_{37}H_{53}N_3O_6Br_4$ requires 952.0747; 655, 657, 659, 661, 662; 601, 603, 605; 379, 381, 383.

3.3.6. Purpurealidin E (6). Colourless oil, UV (MeOH) λ_{max} 282 (ε 950), 277 (ε 925); IR (neat) ν_{max} 3302, 2933, 1666, 1545, 1458, 1259, 1039, 739 cm^{-1}, ^1H and ^{13}C recorded in $CDCl_3$ see Table 5; HRMS: *m/z* (relative heights) 378.9768(37), 380.9757(72), 382.97(35) [1.05:2.05:1.0],† $[M+H]^+$, found 378.9768 $C_{13}H_{20}N_2OBr_2$ requires 378.9943.

3.3.7. Purpurealidin F (7). Colourless oil, UV (MeOH) λ_{max} 282 (ε 950), 277 (ε 925); IR (neat) ν_{max} 3302, 2933, 1666, 1545, 1458, 1259, 1039, 739 cm^{-1}, ^1H and ^{13}C recorded in $CDCl_3$ see Table 5; HRMS: *m/z* (relative heights) 394.9667(16), 396.9661(32), 398.9618(15) [1.06:2.1:1.0],† $[M+H]^+$, found 394.9667 $C_{13}H_{20}N_2O_2Br_2$ requires 394.9970.

3.3.8. Purpurealidin G (8). Colorless oil, UV (MeOH) λ_{max} 282 (ε 950), 277 (ε 925); IR (neat) ν_{max} 3302, 2933, 1666, 1545, 1458, 1259, 1039, 739 cm^{-1}, ^1H and ^{13}C recorded in $CDCl_3$ see Table 5; HRMS: *m/z* (relative heights) 451.0220(10), 453.0210(20), 455.0301(10) [1:2:1], $[M+H]^+$, found 451.0220 $C_{16}H_{24}N_2O_3Br_2$ requires 452.0232.

3.3.9. 16-Debromo aplysamine-4 (9). Colorless amorphous solid (MeOH): mp 124–126 °C; UV (MeOH) λ_{max} 218 nm (ε 12675), 280 nm (ε 2675); IR (KBr pellet) ν_{max} 3350, 3205, 2958, 1655, 1624, 1541, 1497, 1472, 1421, 1256, 1203, 1049, 993 and 739 cm^{-1}; ^1H (CD_3OD, 300 MHz) δ_H 11.40 (2H, br m, $-NH_2$), 8.70 (1H, brs, –NH), 7.90 (1H, brs, –OH), 7.4 (2H, s, H-1, 5), 7.33 (1H, d, J=2.0 Hz, H-13), 7.02 (1H, dd, J=2.0, 8.4 Hz, H-17), 6.86 (1H, d, J=8.4 Hz, H-16), 4.06 (2H, t, J=6.5 Hz, H-18), 3.75 (3H, s, $-OCH_3$), 3.74 (1H, s, H-7), 3.34 (2H, t, J=7.0 Hz, H-10), 3.15 (2H, t, J=6.8 Hz, H-20), 2.65 (2H, s, J=7.0 Hz, H-11), 2.09 (2H, m, H-19); ^{13}C NMR(CD_3OD, 300 MHz) (165.2 (s, C-9), 154.6 (s, C-3), 153.7 (s, C-8), 151.9 (s, C-15), 137.2 (s, C-12), 134.5 (s, C-6), 134.4 (d, C-1, 5), 134.4 (d, C-13), 130.2 (d, C-17), 118.5 (s, C-2, 4), 114.4 (d, C-16), 112.6 (s, C-14), 67.6 (t, C-18), 61.0 (q, -OCH_3), 41.7 (t, C-10), 38.8 (t, C-20), 35.1 (t, C-11), 28.7 (t, C-7), 28.1 (t, C-19); HRMS: *m/z* (relative heights) 619.8797 (525), 621.8535 (1550), 623.8444 (1530), 625.8845 (500) [1.05:3.1:3.06:1.0],† $[M+H]^+$, found 619.8797 $C_{21}H_{24}N_3O_4Br_3$ requires 619.9396.

3.3.10. Purpurealidin H (10). Colorless amorphous solid (MeOH); UV (MeOH) λ_{max} 218 nm (ε 12675), 280 nm (ε 2675); IR (KBr pellet) ν_{max} 3350, 3205, 2958, 1655, 1624, 1541, 1497, 1472, 1421, 1256, 1203, 1049, 993 and 739 cm^{-1}; ^1H (CD_3OD, 300 MHz) δ_H 11.40 (2H, br m, $-NH_2$), 8.70 (1H, brs, –NH), 7.90 (1H, brs, –OH), 7.4 (2H, s, H-1, 5), 7.33 (1H, d, J=2.0 Hz, H-13), 7.02 (1H, dd, J=2.0, 8.4 Hz, H-17), 6.86 (1H, d, J=8.4 Hz, H-16), 4.06 (2H, t, J=6.5 Hz, H-18), 3.75 (3H, s, $-OCH_3$), 3.74 (1H, s, H-7), 3.34 (2H, t, J=7.0 Hz, H-10), 3.15 (2H, t, J=6.8 Hz, H-20), 2.764 (3H, s, $-NCH_3$), 2.65 (2H, s, J=7.0 Hz, H-11), 2.09 (2H, m, H-19); ^{13}C NMR (CD_3OD, 300 MHz) δ 165.2 (s, C-9), 154.6 (s, C-3), 153.7 (s, C-8), 151.9 (s, C-15), 137.2 (s, C-12), 134.5 (s, C-6), 134.4 (d, C-1, 5), 134.4 (d, C-13), 130.2 (d, C-17), 118.5 (s, C-2, 4), 114.4 (d, C-16), 112.6 (s, C-14), 67.6 (t, C-18), 61.0 (q, $-OCH_3$), 41.7 (t, C-10), 38.8 (t, C-20), 35.1 (t, C-11), 28.7 (t, C-7), 28.1 (t, C-19), 27.615 (q, $-NCH_3$); HRMS: *m/z* (relative heights) 633.9220(420), 635.9091(1250), 637.9021(1220), 639.9104(400) [1.05:3.12:3.05:1.0],† $[M+H]^+$, found 633.9220 $C_{22}H_{26}N_3O_4Br_3$ requires 633.9550.

3.4. Antibacterial assays

Antibacterial activity was determined against *E. coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Salmonella typhi*, *Shigella flexineri*, *Klebsiella* sp. and *V. cholerae* using the paper disk assay method. The paper disk impregnated with the sample was placed on agar plate containing bacterium and the plates were incubated for 24 h at 37 °C, and observed for zone of inhibition halos. Streptomycin was used as a positive control.

3.5. Antifungal assays

Antifungal activity was determined against strains of *Aspergillus fumigatus*, *Fusarium* sps, *Cryptococcus neofromans*, *Aspergillus niger*, *Rhodotorula* sp., *Norcardia* sp., and *Candida albicans*. The paper disk impregnated with the sample was placed on agar plate containing fungus and plates were incubated for 18 h at 24 °C. *Nystatin* was used as a positive control.

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A tandem mass spectrometric approach for determining the structure of molecular species of ceramide in the marine sponge, *Haliclona cribricutis*

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Ceramides are important intracellular second messengers that play a role in the regulation of cell growth, differentiation and programmed cell death. Analysis of these second messengers requires a sensitive and specific analytical method to detect individual ceramide species and to differentiate between them. Eight molecular species of ceramide were identified from the marine sponge *Haliclona cribricutis* using electrospray ionization tandem mass spectrometry (ESI-MS/MS). From this marine sponge, *N*-hencicosanoyl (N21:0) to *N*-hexasanoyl (N26:0) octadecasphing-4 (E)-enine have been reported for the first time. The ESI-MS spectra gave several strong protonated $[M+H]^+$ molecular ions with the corresponding bis (2-ethyl hexyl) phthalate adduct $[M+H+DHEP]^+$. The collision-induced dissociation on ceramides at *m/z* 622.7337, 636.7645, 650.7789, 664.7925 and 678.8130 conducted at low-collision energy produced product ions with good characteristic at *m/z* 252.31, 264.32, 278.33, 282.33 and 296.35 for d18:1 sphingosine, regardless of the length of the fatty chain. The MS/MS of the phthalate adduct $[M+H+DHEP]^+$ at *m/z* 1013.1820, 1027.1971, 1041.2176, 1055.2394 and 1069.2573 also yielded characterizing product ions for sphingosine and confirmed the molecular ion at *m/z* 391 for bis (2-ethyl hexyl) phthalate. The major ions in the $[M+H]^+$ and $[M+H+DHEP]^+$ were due to neutral loss of $[M+H-H_2O]^+$ and $[M+H-(H_2O)_2]^+$.

Keywords: ceramides, sphingolipids, *Haliclona cribricutis*, electrospray ionization mass spectrometry, marine sponge

Introduction

Sphingolipids were first proposed to act as second messengers in intracellular signaling pathways and it was thought that sphingolipid breakdown products, such as sphingosine, were the key players in sphingolipid-mediated signaling pathways. However, ceramide has now taken center stage and appears to fulfill many of the criteria to be defined as a true second messenger, in as much as transient changes in ceramide levels are detected in response to a variety of physiological situations. Ceramides are important intracellular messengers that play a role in the regulation of cell growth, differentiation and programmed cell death. Because of their multiple physiological functions, they have gained considerable attention.[1–5]

Different analytical methods have been used for separating and differentiating between the individual ceramide molecular species. These included gas chromatography/mass spectrometry (GC/MS),[6] fast atom bombardment (FAB) MS[7–10] and, more recently, electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI)[11] techniques. ESI offers several advantages over the FAB tandem (MS/MS) technique, including lower background signals because of the absence of matrix ions, the long lasting and stable primary ion currents, the ease of sampling and compatibility with liquid chromatography (LC). Informative patterns are described by Lee *et al.* employing LC/ESI-MS in both positive- and negative-ionization modes to identify the structures of both sphingoid base and *N*-acyl chains of ceramides and also of an impurity in cosmetics.[12] Kerwin *et al.* reports ceramide profiling in a commercial preparation of bovine brain using this technique.[13] LC/ESI-MS, in combination with the selected ion monitoring (SIM) mode, is a very sensitive and useful method for the determination of sphingosine (SO) and sphinganine (SA) in cell cultures.[14]

As part of our research programme on the biologically active molecules from a marine source, screening of the *Haliclona cribricutis* showed it to be antiviral, bactericidal and fungicidal. Earlier investigations of this coelentrate from this laboratory resulted in a mixture of *N*-acylated sphingosines from which only the docosoyl ester amide of a dihydroxy sphingosine was isolated and identified.[7]

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Methanol extract of *Haliclona cribricutis* had also yielded alkaloids mimosamycin, 4-hydroxy-mimosamycin, 1,4-dihydroxy mimosamycin, renieramycin-H and renieramycin-I[15] and the bioactive metabolites, demethyl renierone A and its dimer B.[16]

In the current study, we report the use of ESI-MS/MS for the analysis of eight molecular species of ceramide from the marine sponge, *Haliclona cribricutis*, with minimal chromatographic purification.

Experimental

Material

The sponge was collected by scuba diving at a depth of 8–10 M on the south east coast of India in March 2003. The sample was identified by Dr P.A. Thomas, Vizhingam Research Center of Central Marine Fisheries Research Institute, Kerala, India. A voucher specimen (NIO 730) is deposited at the National Institute of Oceanography, Dona-Paula Goa, India.

Extraction and fractionation

The frozen sponge (500 g) was extracted with MeOH (2 L) under sonication and concentrated under vacuum to obtain 6 g of crude extract. The crude extract exhibited *in vitro* antimicrobial activity against *S. aureus, Shigella flexineri* and *Klebsiella sp.* bacterial strains and the fungal strain, *Aspergillus fumigatus*.[17] The extract was partitioned with $CHCl_3$ and the soluble fraction (2 g) was chromatographed on a silica gel (Kieselgel 60, 230–400 mesh, E. Merck) column using hexane containing increasing amounts of ethyl acetate as eluent. The fractions were monitored by thin-layer chromatography (TLC), alumina-backed sheets (Si gel 60 F_{254}, 0.25 mm thick) using ethyl acetate : hexane (35 : 65) as developing solvent with 5% methanolic sulphuric acid as spraying agent. The fractions, showing similar TLC profile to Rf 0.172, were combined and concentrated. It was further purified by gel chromatography using Sephadex LH-20 17-0090-01, Pharmacia Biotech, to get the fraction rich in sphingolipids.

Sample preparation

Infrared (IR) spectra were taken, as KBr pellets, on a Shimadzu 820PC Fourier transform infrared (FT-IR) spectrometer. The nuclear magnetic resonance (NMR) spectra of the sample were obtained in $CDCl_3$ on a Bruker (Avance, 300 MHz) instrument with TMS as the internal standard.

ESI-quadrupole time-of-flight (QToF) MS/MS spectrometry

The mass spectrometer used was a QTOF-XL MS/MS Applied Biosystems' instrument (Canada). The instrument was operated in positive-ionization mode. The sample, dissolved in MeOH : $CHCl_3$ (99.5 : 0.5) containing traces of 0.1% TFA, were directly infused at a constant flow rate of 10 μL min^{-1} into the ion spray source using an integrated syringe pump. Full-scan data acquisition was performed, scanning from *m/z* 100 to *m/z* 1200 in profile mode and using a cycle time of 1 s. The MS/MS products were produced by collision-induced dissociation (CID) of selected precursor ions at collision energy between 15–40 V and mass analyzed using the instrument's ToF analyzer.

Results and discussion

The IR spectrum of the sphingolipid fraction showed absorption bands at 3327.0, 2918.1, 2850.6, 1630.0, 1618.2, 1467.7, 1550.7 and 1020.3 cm^{-1}. The ^1H NMR spectrum was typical of ceramide with signals at δ6.26 (1H, d, J = 7.2 Hz) for the secondary amide, 5.77(1H, dt, J = 15.4, 6.4 Hz), 5.58 (1H, dd, J = 15.4, 6.7 Hz) for the di-substituted double bond, 4.33 (1H, m, 3–H), 3.94 (2H, m, 1–H, 2–H), 3.73 (1H, m, 1–H) for methines and methylene protons bearing a hydroxyl group, 2.24(2H, t, J = 7.1 Hz) and 2.06 (2H, m) for methine protons bearing amide and hydroxyl groups, respectively. The polymethylene chain with a terminal methyl group is evident from the signal at δ1.6–1.0 [$(CH_2)_n$, m] and 0.86 (3H, t, J = 6.85 Hz, terminal methyl). The ^{13}C of the fraction was also in good agreement with the ^{13}C NMR of ceramide [7] with the signals at δ173.9(s), 134.1(d), 128.7(d), 74.3(d), 61.9 (t), 54.5 (d), 39.0 (t), 36.7 (t), 32.2(t), 31.8, 29.6, 29.3, 27.9, 27.3, 27.0, 25.7, 22.6 (all triplets) and 14.0 (q).

ESI-MS/MS fragmentation of molecular species of ceramide

ESI-MS exhibited several molecular $[M+H]^+$ ions at *m/z* 594.7731, 608.7684, 622.7798, 636.8062, 650.8289, 664.8407, 678.8644 and 691.8769 for isolated ceramide species and relatively less intense $[M+H+DEHP]^+$ in the region 960–1100 amu [Figure 1(a)]. Figure 1(b) shows only $[M+H+DEHP]^+$ of the ESI-MS at 960 to 1140 amu. These were attributed to the bis (2-ethyl-hexyl) phthalate adducts of the ceramides $[M+H+DEHP]^+$. The literature shows that DEHP adducts result from plasticizer contamination, most probably from the cap of a bottle of solvent or plastic washing bottles etc.[18] A comparison of signal intensities indicates that the mixture contains a substantial amount of ceramides with molecular masses 691.8769 (100%), 622.7789 (86.9%) and 636.8062 (76.1%) while the DEHP adducts are almost negligible with less then 8% (Table 1).

The MS/MS experiment of each $[M+H]^+$ molecular ion was carried out at a collision energy of 40 V. Figure 2 shows MS/MS at *m/z* 622.7798, 636.8062, 650.8289, 664.8407 and 678.8644. All the molecular ions showed some common fragment ions. The fragment ion corresponding to cleavage between the carbon and nitrogen bond of the sphingosine base are observed at *m/z* 282.33 (X) and, with the loss of an additional neutral water molecule, resulted in the abundant ion at *m/z* 264.32 $[X' = X - H_2O]^+$. The cleavage between the amide bonds resulted in the fragment ion at *m/z* 296.35 (Y) and *m/z* 278.33 $[Y' = Y - H_2O]^+$ for the loss of the water molecule. It also showed product ions at *m/z* 252.31 (Z) due to the

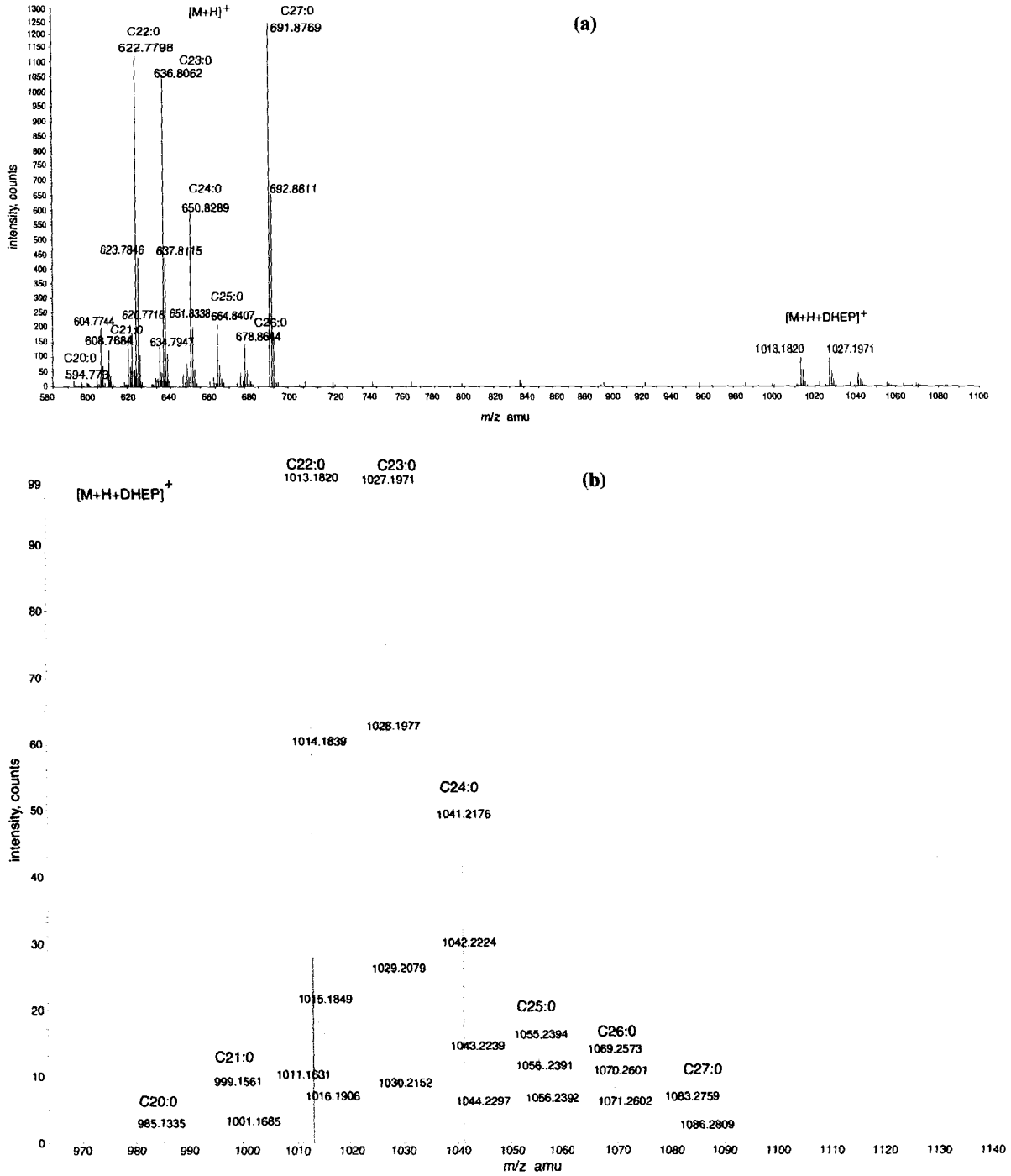


Figure . (a) The ESI-MS spectra of the ceramides showing the full scan from *m/z* 580 to 1100 and (b) the ESI-MS spectra of the ceramides showing only [M + H + DHEP] from *m/z* 860 to 1150.

loss of 31 amu from the sphingosine base (Scheme 1).[19–24] The series of product ions, X, X′, Y, Y′ and Z, provided information about the molecular weights of the sphingoid and the fatty acid residues. It was indicative that the ceramide was similar to the d 18 : 1 sphingosine moiety, differing only in the long chain base (C20:0–C27:0). It also yielded the abundant

Table 1. The ESI-MS/MS for the molecular species of ceramide showing fragment ion peaks. The relative abundance of the molecular ion peaks in the ESI mass spectra of *Haliclona cribricutis*; amounts injected 10 µL; solvent, MeOH:$CHCl_3$ (99.5:0.5) containing traces of 0.1% TFA; the ion abundance (%) was normalized to the base peak[a] (100%) in the spectrum.

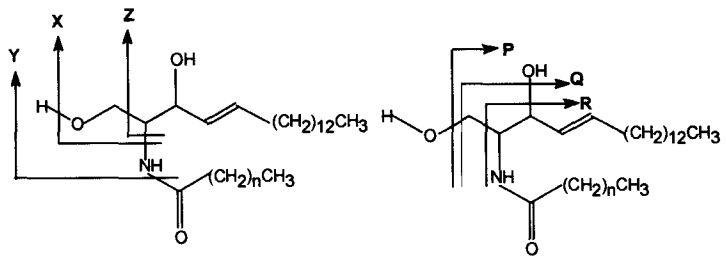
Long chain base	$[M+H]^+$	Relative intensity (%)	$[M+H+DHEP]^+$	Relative intensity (%)	P	Q	R
C20:0	594.7731	0.8	985.1335	0.1	576.69	558.68	546.68
C21:0	608.7684	9.2	999.1561	0.6	590.70	572.69	560.69
C22:0	622.7798	86.9	1013.1820	7.6	604.71	586.69	574.69
C23:0	636.8062	76.1	1027.1971	7.6	618.73	600.72	588.71
C24:0	650.8289	43.8	1041.2176	3.8	632.75	614.73	602.73
C25:0	664.8405	15.4	1055.2394	1.1	646.77	628.74	610.75
C26:0	678.8644	16.9	1069.2573	1.0	660.78	642.76	630.78
C27:0	691.8769[a]	100	1083.2759	0.5	674.78	656.77	644.79

[a]Base peak at *m/z* 691.8769amu

$[M+H-H_2O]^+$ product ion, corresponding to the loss of one water molecule, designated as "P". The product ions, Q and R, were observed for $[M+H-2(H_2O)]^+$ and $[M+H-49]^+$, respectively (Scheme 1 and Table 1). Thus, based on the above observation, the ceramides were identified as *N*-eicosanoic (20:0), *N*-hencicosanoic (21:0), *N*-docosanoic (22:0), *N*-tricosanoic (23:0), *N*-tetrasanoic (24:0), *N*-pentasanoic (25:0), *N*-hexasanoic (26:0) and *N*-heptasanoic (27:0) sphingosine.

The MS/MS of the $[M+H+DEHP]^+$ phthalate adducts at *m/z* 999.1561, 1013.1820, 1027.1971, 1041.2176, 1055.2394, 1069.2573 and 1083.2759 were carried out. The MS/MS of $[M+H+DEHP]^+$ at *m/z* 1013.1820, 1027.1971, 1041.2176, 1055.2394 and 1069.2573 (Figure 3) showed corresponding $[M+H]^+$ and peaks due to the loss of $[M+H-H_2O]^+$ and $[M+H-2(H_2O)]^+$ water molecules. In addition, it showed a common fragment ion at *m/z* 391.38, which indicated the adduct formation of ceramide with bis (2-ethyl hexyl) phthalate (DEHP).

Sphingolipids are well known constituents of the genus *Haliclona*. Richelle-Maurer *et al.*[10] report the presence of large amounts of a new antimicrobial sphingosine derivative, (2R, 3R, 7Z)-2-aminotetradec-7-ene-1, 3-diol, in *Haliclona vansoesti*. Several ceramides with saturated C_{20}–C_{23} have been isolated from *Haliclona tenuiramosa*.[8] *N*-docosanoyl-d-erthro- (2S, 3R)-16-Methyl-heptadecasphing-4 (E)-enine (C_{22} ceramide), possessing antifouling activity against macroalgae, has been identified from the sponge *Haliclona koremella*.[9] Earlier chemical investigation from our laboratory on *Haliclona cribricutis* yielded docosyl ester amides of a dihydroxy sphingosine as a major ceramide from this source.[7]



n =18-25 (C20:0 – C27:0)

X = 282.33; X'= X-H_2O = 264.32
Y = 296.35; Y'= Y- H_2O = 278.33
Z = 252.31

Scheme 1. ESI-MS/MS Fragentation of molecular species of ceramide with sphingosine.

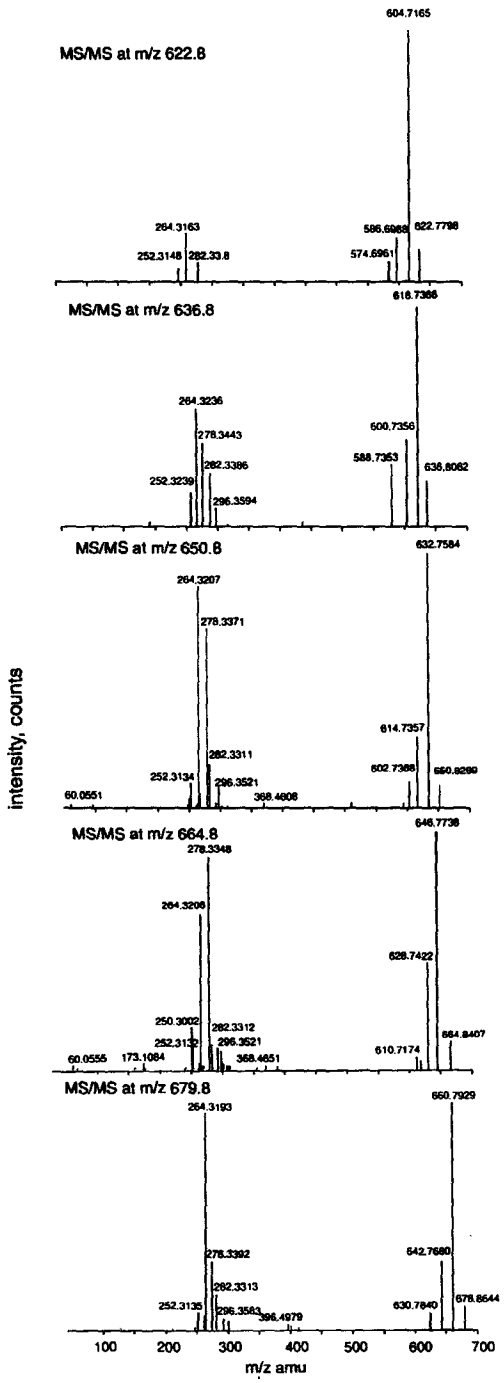


Figure 2. The MS/MS of the M + H]⁺ at *m/z* 622.7, 636.8, 650.8, 664.8 and 678.8.

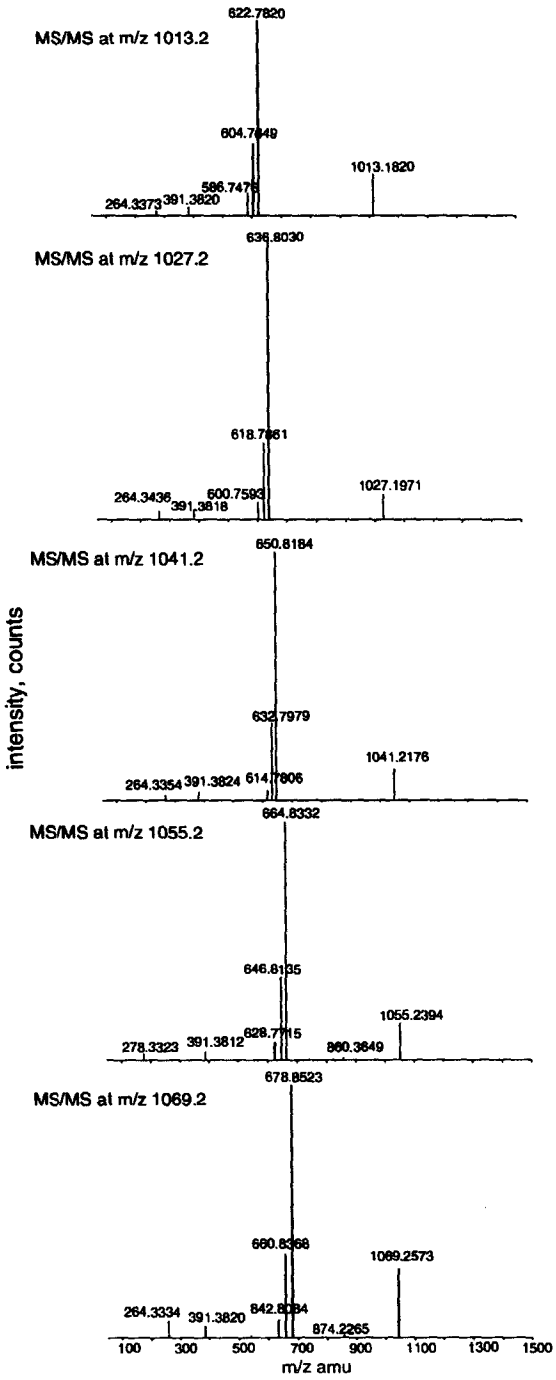


Figure 3. The MS/MS spectrum of the [M + H + DHEP]⁺ at *m/z* 1013.2, 1027.2, 1055.2 and 1069.2.

Conclusion

In conclusion, identification of individual ceramides (fatty acid amides of sphingoid base) present in a complex mixture would not have been possible by FAB-MS. Thus, as is evident, ESI-MS/MS is a very sensitive and useful method for the determination of molecular species of ceramide and has several advantages compared with other methods.

Product ion peaks at *m/z* at 252.31, 264.32, 278.33, 282.33 and 296.35 in the spectra of all the compounds confirmed the sphingosine as being d18:1 and the heterogeneity as being in the acyl group. Based on the relative intensity of the peak signals, ceramides with C27:0, C22:0, C23:0 were found in substantial amounts. Product ion peaks at *m/z* 391.38 showed the presence of a contaminant, bis (2-ethyl-hexyl) phthalate (DEHP). The presence of such contaminants forming adduction with analyte molecules causes errors in a mass spectrometer. So, a permanant solution must be found for avoiding the errors. It also demonstrates the identification of ceramides using minimum chromatographic separation and with minimum concentration of sample.

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