CHEMISTRY OF METABOLITES OF MARINE ORGANISMS FROM INDIAN OCEAN REGION

A THESIS SUBMITTED TO THE GOA UNIVERSITY FOR THE DEGREE OF DOCTOR OF PHILOSOPHY IN MARINE SCIENCE

BY

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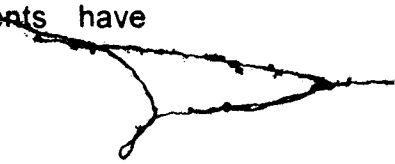
1998

DEDICATED TO

MY PARENTS

STATEMENT REQUIRED TO BE SUBMITTED UNDER ORDINANCE 0.19.8 (vi) OF THE GOA UNIVERSITY

This thesis is based entirely on, the experimental work carried out by me under the guidance of Dr. S. Y. Kamat. To the best of my knowledge, the present study is the first comprehensive work of its kind from the area mentioned. The literature concerning to the problem investigated has been surveyed and list of references is appended. Due acknowledgements have been made wherever outside facilities have been availed of.



Prabhu Dutt Mishra

CERTIFICATE

This is to certify that the thesis entitled "**CHEMISTRY OF METABOLITES OF MARINE ORGANISMS FROM INDIAN OCEAN REGION** " submitted by Mr. Prabhu Dutt Mishra for the award of the degree of Doctor of Philosophy in Marine science is based on his original studies carried out by him 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.

Place: Dona Paula,

Date: September, 1998

(Dr. S. Y. Kamat)

Research Guide

All the suggestions made be experts / evaluators have been incorporated in this copy of the Thesis

Examiner

Guide

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In last but not least, I am indebted to my elder brothers Dr. RN Mishra, Shri TD Mishra, family members and my wife Archana for their blessings and encouragements during this work.

Prabhu Dutt Mishra

September 1998

GENERAL REMARKS

1. All figure numbers, table numbers, structure numbers and references in a chapter refer to that particular chapter only .

2. Petroleum ether refers to the fraction boiling between the range 60-80 °C.

3. Silica gel used for column chromatography was of 60-120 mesh size and activated at 110 °C before use.

4. Thin layer chromatography was done on glass plates coated with 0.25 mm layer on TLC grade silica gel containing 13% $CaSo_4$ as binder. The plates were activated for 1 hour before use.

5. ^{1}H NMR and ^{13}C NMR spectra were recorded on Bruker WM 400 Mhz, FT NMR spectrometer, EIMS and FAB Mass Spectra recorded on Jeol D-300 mass spectrometer at 70 ev and IR on Shimadzu FTIR-8001, unless otherwise stated. The chemical shifts are expressed in 'δ' downfield to TMS, which is used as internal standered.

6. The known compounds reported were indentified from the comparison of their spectral data with those of similar or identical compounds reported in the literature.

7. The details regarding instruments and experimental conditions employed are given wherever possible.

8. The sponge samples have been kept in marine biology museum / taxonomy reference centre, at National Institute of Oceanography, Dona Paula, Goa.

Chapter 1

Introduction

Since ages man has been using oceans for transportation, precious stones, foods, drugs and inorganic minerals. However, recently, marine flora and fauna in terms of their biomedical potential have received increasing attention from chemists and pharmacologists. Rapid developments in biochemical studies of marine natural products emphasize the importance of the sea as a rich store house of new organic compounds, which are having interesting and useful bioactivities.

In ancient times, many remedies used to be derived from crude extract of whole or a part of plants and animals. The modern medicine has advocated the use of pure chemical substances having pharmacological properties and specific action sites. These substances were initially isolated from terrestrial organisms, but as the knowledge and skill in chemistry evolved, the drugs of synthetic origin began to appear. In the begining of this century biomedical research on several plants and microorganisms helped in introducing several antibiotics, tranquilizers etc. into the market. However, systematic investigation of marine organisms for their medicinal use is relatively new phenomenon.[1]

Man's awareness of the possible biomedical benefits from the sea is not new. Japanese, Chinese and other civilizations have been using sea products for medicinal purposes. Catalyst for the exploration of marine natural products were the isolation of nucleosides from marine sponge *Crytptotethya crypta* by Bergman in 1950 and the presence of

prostaglandin derivatives in the gorgonian *Plexaura homomalla* by Spraggins and Weinheimer. The oceans have the potential to provide important resource for discovery of new molecules as it cover approximately 70% of earth surface. It is estimated that oceans contain about 200,000 invertebrates and algal species. Research for past 15 years have shown that marine plants and invertebrates are excellent source of natural products with exceptionally diverse chemical structure. The diversity of chemical compounds is thought to be due to the extreme competition among organisms for their survival in the marine habitats.[2]

More than 6500 natural products have been reported from marine organisms over the past 10 years. These compounds encompass a wide variety of chemical structures including steroids, terpenes, alkaloids, and peptides etc., but therapeutic use of these compounds has only been briefly examined. The discovery of marine natural products with potential therapeutic agents requires a multidisciplinary team approach.

At present marine natural products research requires much more developed instrumentation techniques. These efforts do not center merely on extracting the desired substances from marine organisms, but lay emphasis on duplicating them in the laboratory. Biomedical research is inherently slow program, as it has to be time tested in order to be beneficial and safer in its final application.

The development in this field was relatively slow and this may be attributed to several factors, but the most important ones are;

1. Marine environment is not easily accessible.

2. Difficulties in collecting the marine organisms.

3. Non availability of sophisticated instrumentation facilities.

However, now, modern chromatographic techniques as well as high tech spectroscopic methods, have made it easy to work on microquantities of organic compounds obtained from marine flora and fauna. SCUBA diving, computerized closed circuit mixed gas rebreathers, remotely operated vehicles and manned submersibles have now enabled the oceanographers to reach a depth of several thousand meters. This has enormously brightened the scope of sample collection for research on marine bioactive agents.[3]

Marine life of Indian ocean is very rich and diverse. It is practically untouched as far as commercial use of marine natural products are concerned, except for the production of carrageenan, alginic acid ,agar agar etc. In order to exploit the rich source of the seas around India a research programme was initiated at National Institute of Oceanography, Goa, in collaboration with Central Drug Research Institute, Lucknow, in 1978. In 1984, this programme received tremendous boosts with the introduction of an Indo-US collaborative research project to study the

bioactive molecules from marine flora and fauna along Indian coastal region. More than 500 marine flora and fauna were evaluated for their biological activity, which included antifungal, antifertility, antiviral, hypotensive, spasmogenic, analgesic, toxic and diuretic etc. Now, this programme is further extended by Department of Ocean Development with participation of several other Indian laboratories and universities. Based on these screening results detailed chemical investigations were undertaken. A number of compounds including terpenes, steroids, esters, alcohols, lactones and glycosides and some heterocyclic compounds have been isolated from marine organisms collected from Indian coastal region for biological evaluation (briefly discussed in Chapter 2).

As a part of this study, following marine plant and animals were investigated for their chemical constituents, yielding different class of compounds, such as fatty acids, steroids, heterocyclic compounds and polyphenols .

1. *Carpophyllum plumosum.*

2. *Suberites carnosus.*

3. *Suberites vestigium.*

4. *Chrotella australiensis.*

The results obtained are described in the following chapters.

References

1. *Food and drugs from Sea*, Procceedings 1974, edited by H Webber, George D Ruggieri. Published by Marine technology society, USA.
2. Any E Wright, Peter J McCarthy, *Sea technology* (1994), August, 10.
3. *Pharmaceuticals and Sea* edited by Charles W. Jefford, Kenneth L Rinehart, Lois S Shield, 1998, 3-8. Published by Technomic publishing company.

Chapter 2

Secondary metabolites from marine organisms.

This chapter deals with literature survey, in which efforts have been made to give a brief account of bioactive and novel organic compounds isolated from marine organisms. These compounds are classified under different groups.

The majority of diseases in man are essentially caused by the entry of micro organisms into the body, where they multiply and thereby overcome the defence system. There are several marine metabolites which inhibit the growth of these organisms and thus, can be used for the cure of a disease. Apart from acting against certain micro organisms a drug should be non-toxic and specific in its action. Research on antimicrobial, antiviral and anticancer drugs are on priority for biomedical scientists, as cancer is a major cause of death and several illness are a consequence of viral infections.

A survey indicates that marine invertebrates, especially sponges have high possibility of providing compounds with bioactive properties. Ecological consideration based on field observations of biological interaction can also provide pointer towards the ultimate selection of active species. In 1978, Renihart carried out such a search in Carribean Sea. He concluded that antiviral activity was highest in Cyanophyta, Phaeophyta, Chordata and was significant in Porifera phyla.[1]

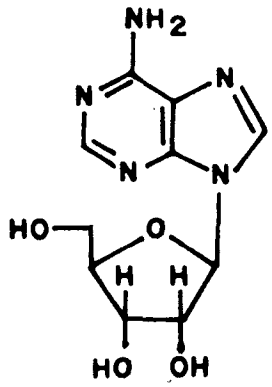
Nucleosides

Bergman discoverd the natural occurrence of nucleosides as spongothymidine **(1)**, spongosine **(2)** and spongouridine **(3)** from sponge *Cryptotethya crypta*[2]. Another compound arabinose-A(9β-D arabinofuranosyladenine) has been isolated from the Mediterranean gorgonian *Eunicella cavolini*[3] along with 3'-O-acetyl derivative. Spongouridine ara-A has been therapeutically used against *Herpes encephalitis* since 1970. 5'-Deoxy-5-iodotubercidin was isolated from the red algae *Hypnea valendiae*.[4] Dorodosine (1-Methyl isoguanosine was isolated from sponge *Tedania digitata*,[5] and synthesized in laboratory. This nucleoside exhibits pharmacological properties such as muscle relaxant, hypothermic and cardiovascular effects. The nucleoside 9β-(2-deoxy-D-ribofuranosyl) has been isolated from a Australian sponge of order Hadromerida, along with known nucleosides 9β-D-ribofuranosyl-2-methoxyadenine, thymine and 2-methoxyadenine.[6]

Steroids

Marine sponges are good source of sterols, sulphated and alkaloidal sterols were isolated from sponges, which exhibit antimicrobial activity. Halistanol **(4)** from *Halichondria mooriei*[7] and sterols from *Toxadocia zumi*[8] inhibited the growth of *Staphylococcus aureus*. 24-Ethyl cholest-3β, 5-α-diol-6-one, cholesterol and 24-ethyl-cholest 3β, 5α, 6β- triol were

isolated from sponge *spirastrella inconstans.*[9] Some unusual ketosteroids and peroxide sterols have been reported from sponge *Chrotella australiensis*[10] and *Suberites carnosus.*[11] A new hydroximino (6-hydroximino-4-en-3-one) steroid has been isolated from sponge *Cinachyrella alloclada* and *C. Apion.*[12] 24- Methyl-5α-cholest-7-enyl-3β-methoxy methyl ether, a new sterol ether has been isolated from a deep water marine sponge *Scleritoderma sp.*[13] This compound exhibited *in vitro* cytotoxicity. Ophirapstanol trisulphate **(5),** a new biologically active steroid was isolated from a deep water marine sponge *Topsentia ophiraphidites.*[14] Xestobergsterol C, a new pentacyclic steroid was isolated from Okinawan sponge *Ircinia sp.* [15] Aragusterol A, B, and C, which are novel 26,27-cyclosterols were isolated from Okinawan sponge of genus *Xestospongia.*[16] A new epoxy sterol **(9)** was isolated from the sponge *Ircinia fasciculata,* collected from Tuticorin coast.[17] Plakinamine A **(6)** and B **(7)** as antimicrobial metabolites were obtained from sponge *Plakina sp.,* these compounds inhibited the growth of *Staphylococcus aureus.*[18] A hydroxy sterol **(8)** was isolated from sponge *Dysidea* sp.[19] Two uncomman marine sterols sokotrasterol and 24, 24, 26, 26 tertramethyl cholesta-5, 22(E), 25(27) trien 3β-ol have been isolated from a marine sponge.[20] Δ^5-3β-Hydroxy-7ketosteroids, Δ^5 -3β,7α-dihydroxy sterols and Δ^5-3β,7α-dihydroxy sterols have been reported from a marine sponge *Cliona copiosa.* [21]



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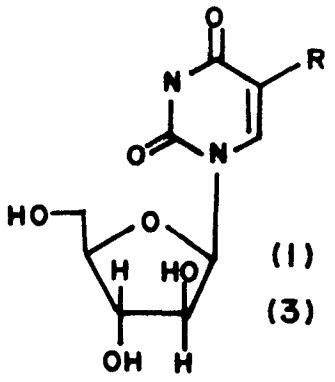

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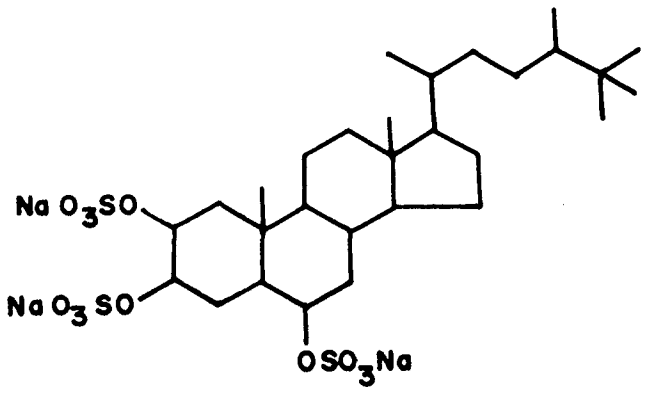

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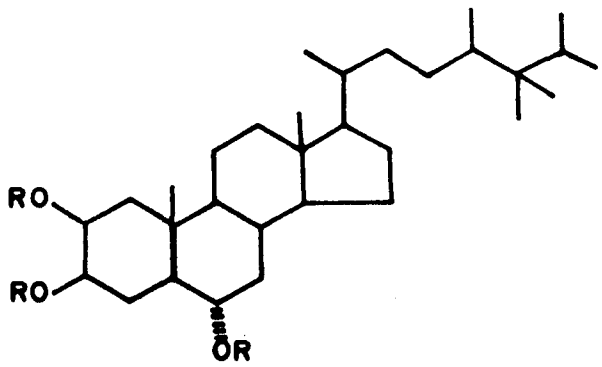

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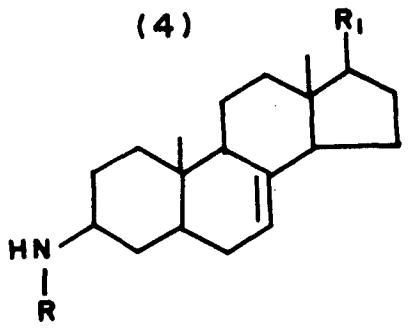

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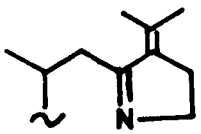

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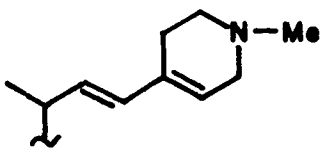

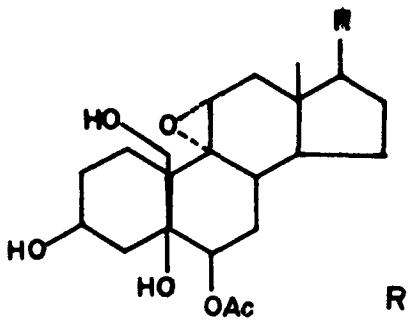

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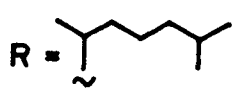
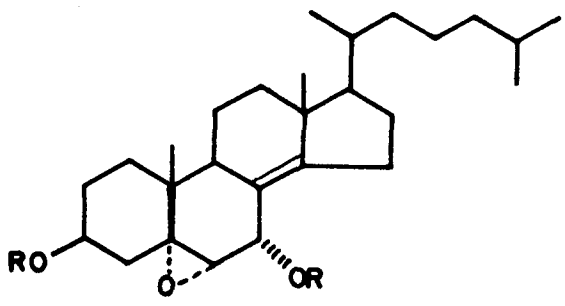




R = Ac

(9)

Secondary metabolites from marine organisms

Alkaloids

Alkaloids and other heterocyclic compounds have been obtained from sponges. Keramadine, a bromine containing alkaloid, antagonist of serotonergic receptor, has been isolated from the Okinawan sea sponge *Agelas* sp.[22] Ptilocaulin **(10)** and isoptilocaulin from a Caribbean sponge *Ptilocaulis spiculifer* exhibited antimicrobial activity.[23] Aaptamine **(11)** isolated from the sponge *Aaptos aaptos* possesses adrenoceptor blocking activity in the isolated rabbit aorta.[24] Marine sponges are also good source of many peptide alkaloids. Purealin, an enzyme activator isolated from Okinawan sponge *Psammaphysilla purea,*[25] which modulates enzymatic reaction of ATPases. Amphimedine **(12)** a fused pentacyclic yellow aromatic alkaloid from a sponge *Amphimedon* sp. has been reported as a cytotoxic agent.[26]

A new alkaloid axinellamide [5-hydroxy-(6-methyl-2,4-octadienyl)-3 pyrrolin-2-one was isolated from the marine sponge *Axinella Sp.*[27] collected along the coast of Trinidad. Two new guanine alkaloids ptilocaulin and 8β-hydroxyptilocaulin have been isolated from methanolic extract of marine sponge *Monanchora arbuscula.*[28] Nine new bromo tyrosine alkaloids, purealidins have been isolated from Okinawan sponge *Psammaplysilla purea,* [29] few of them were cytotoxic to tumor cell lines while others showed activity against epidermal growth factor receptor kinase. A novel alkaloid tetrahydroquinolizinium ion was reported from the Indo-Pacific sponge *Clathria Basilana.*[30] A new indole alkaloid

pallidin along with known compound 1,3-dimethylxanthine were isolated from the sponge *Phaphisia pallida*.[31] 5-Alkyl pyrazole-2-carboxaldehyde has been isolated from the sponge *Mycale mytilorum*. [32] Three new bromo tyrosine alkaloids, pseudoceratinines have been isolated from the sponge *Pseudoceratina verucosa*.[33] The known compounds aplysamine, purealin and purealidins were also isolated from this sponge. Three novel nitroalkyl pyridine alkaloids with antimicrofouling activity, were isolated from Okinawan marine sponge *Callyspongia sp.*[34] Lipopurealins, alkaloids were obtained from the Okinawan marine sponge *Psammaplysilla purea*.[35] Oceanapamine **(13)** isolated from Philippine sponge *Oceanpia* sp. exhibited antimicrobial property.[36] Two new β-carboline alkaloids have been isolated from the sponge *Amphimedon sp.*,[37] these compounds showed antibacterial activity against a gram positive bacterium *Sarcina lulea*. Konbamidin **(14),** a new indole alkaloid from the Okinawan marine sponge *Ircinia sp.*[38] exhibited cytotoxicity against HeLa cells *in vitro* with an IC_{50} value of 5.4 mg/ml. Hamacanthin A and B are two bioactive indole alkaloids isolated from a new species of deep water marine sponge *Hamacatha* sp.[39]

Biopolymers

Aplidiasphingosine, polyandrocarpidines, acarnidines were isolated by Rinehart. Aplidiasphingosine was isolated from *Aplidium sp.* of tunicate.[40] This compound could be considered a sphingosine derivative exhibiting antimicrobial, antiviral, and antitumor activity. Polyandrocarpidines, guanine derivatives were isolated from colonian tunicate of genus *Poluandrocarpa* and are found to be antibacterial.[41a] Acarnidines isolated from the sponge *Acarnus erithacus* showed antiviral activity.[41b]

Terpenoids

Terpenoids found in marine organisms are linear furanoterpenes, isoprenyl quinols, sesqiterpenes, diterpenes and triterpenes. Sesqiterpenes having phenolic or quinoid moiety are common in sponges. Sesqiterpenoid avarol **(15)** was isolated from sponge *Dysidea avara,* which is antimicrobial and active against HIV.[42] The sesterterpene variabilin was isolated by Faulkner in high yield from the sponges of the genus *Ircinia variabilis,*[43] which exhibited antibiotic, cytotoxicity, and antiviral properties. A series of four monoterpene cyclohexadienones were isolated from red alga *Desmia hornemanni.* Each of these compounds was tested for antitumor and antiviral activity against LK10 cells and HSV-1. Three hemiterpenoid derivatives from the marine

organisms were isolated by Howard from Californian colonial tunicate *Aplidium californicum.*[44] These were prenyl hydroquinones, which prevented the induction of some forms of leukemia, sarcoma and mammary carcinoma. Antineoplastic compound lemnalol **(16)** was isolated from the soft coral *Lemnalia tenuis.*[45] Globostellatic acids , cytotoxic isomalabaricane triterpenes were isolated from the marine sponge *Stelletta Globostellata,*[46] whereas Jaspiferals A-G, isomalabaricane type nor triterpenoids with a 3α-hydroxy group were isolated from the Okinawan marine sponge *Jaspis stellifera.*[47] A new sesterterpene 25-dihydroxy 12-epi -deacetylscalarin was isolated from sponge *Hetronema erecta.*[48] A novel sesqiterpenoid containing a benzoxazole ring was found to be a constituent of an Okinawan sponge of family *Spongiidae.*[49] Two sesquiterpene quinols, cyclorenierins A and B were isolated from sponge *Haliclona sp.*[50]

Didemnins

These are tunicate products of *Tridemnum* genus of the family *Didemnidae.*[51] These are cyclic peptides, the constituent aminoacids and their configuration were identified by a combination of mass spectrometry, GC, GC-MS and NMR and hydrolysis product from didemnin A. In addition to didemnin A, B, C a series of nor didemnins have been isolated. In this series the amino acid isostatin is replaced by

a nor isostatin unit. The didemnins are being assesed as potential antiviral, antineoplastic and immunosuppressive agents and chosen for clinical trial as antitumor medicine.

Eudistomins

The most active antiviral species detected during Rinehart's 1978 Alpha helix Caribbean expedition was the colonial tunicate *Eudistoma olivaceum*. The crude extract from all the samples inhibited the plaque formation by HSV-1 virus in monkey kidney cells with little cytotoxicity. Partitioning of extract followed by extensive reverse phase and silica gel chromatography gave the eudistomins A **(17)** -Q as yellow oils.[52] The eudistomins are β carboline derivatives.

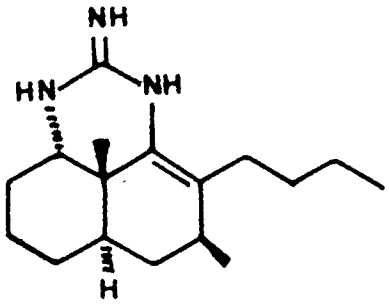
Toxins

There has been interest in metabolites of jellyfish containing toxins, which are responsible for numerous fatalities or severe injuries. There are other toxins like saxitoxin and ciguatoxin which are responsible for intoxication caused by eating various species of coral reef fishes. Kato and scheuer were the first to characterize debromoaplysiatoxin, but the absolute stereochemistry was established some what later by

Moore et. al. Initially it was found in the digestive gland of the sea hare *Stylocheilus longicauda*, but the ultimate source is blue green algae *Lyngbia majuscula* upon which the sea hare feeds. Palytoxin, the most potent marine toxin, was first isolated from the zooanthid *Palythoa toxica* by Moore and Scheur.[53] Other Palythoa sp. such as *P. tuberculosa* and *P. mammilosa* are also source of palytoxin. Palytoxin is found to be 25 times more toxic to mice than tetrodotoxin due to its effect on the cardiovascular system. Surugatoxin and neosurugatoxin have been isolated from the toxic ivory mollusk *B Japonica*,[54] neosurugatoxin inhibits the contractile response of isolated guinea pig ileum. Tetrodotoxin was detected in the fish *Deodontidae*.

Prostaglandins

Prostaglandins were isolated from gorgonians *Plexaura homomalla* and *Euplexaura depressum*. Kikuchi amada and coworkers isolated the geometric isomers of clavulone I **(19)**, II, III from Japanese soft coral *Clavularia viridis*.[55] Prostaglandins derived from marine sources are found to exhibit a variety of biological activities. Medical applications include in areas of reproductive biology, renal pathology and treatment of intestinal ulcers.



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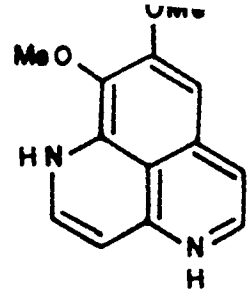

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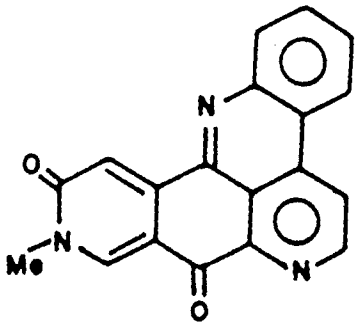

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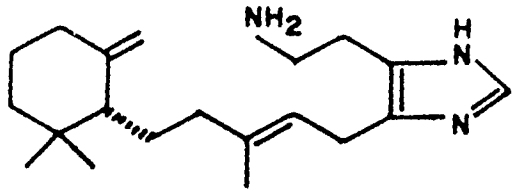

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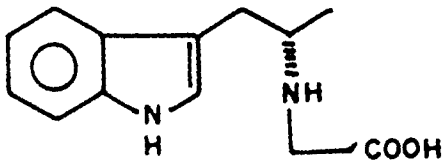

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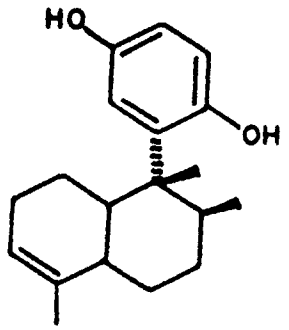

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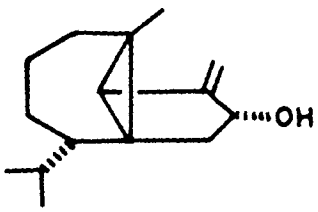

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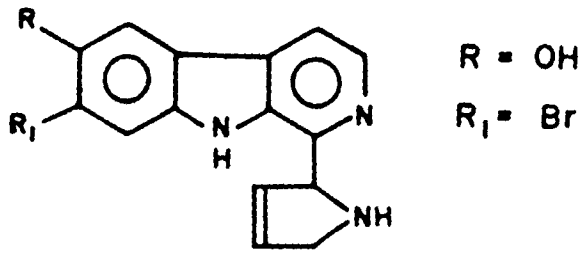

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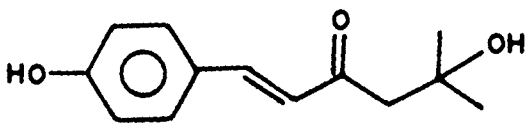

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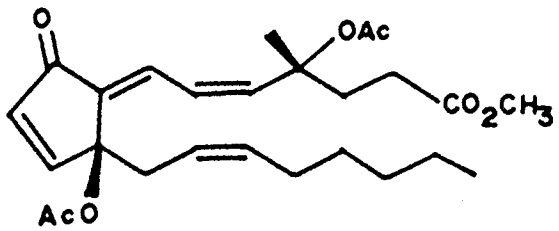

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Secondary metabolites from marine organisms

Bryostatins

In 1968, Pettit and co-workers initiated a systematic search for antineoplastic activity in marine organisms. This search had a wide geographical base and was launched as a consequence of the discovery by this group of antineoplastic compounds in terrestrial Anrthropods. They found promising activity in alcoholic extracts from several phyla, but especially from the Bryozoa. From different sp. of Bryozoa 17 related compounds, bryostatins 1-17 have been reported. Two more bryostatin A & B have been detected in sponge *Lissodendoryx isodictyalis* invaded by *Bugula neritina*.[56] The bryostatins are present at about the 10^{-6} % range in the whole animal.

Dolastatin

Dolastatins 1-9 were obtained from sea hare *Dolabella auricularia*,[57] collected from Indian ocean by Pettit and coworkers. The structure for only few of them has been proposed , since these compounds occur in very low yield and their amorphous nature precluded X-ray analysis. Dolastatin-10, a cytotoxic agent from same organism was reported and showed antineoplastic activity.[58]

Halichondrins

Halichondrins were isolated from the sponge *Halichondria Okadai*,[59] This invertebrate animal is widely distributed on the rocky Pacific shores of Japan. Crude extract produced significant *in vivo* inhibition of the growth of melanoma cells.

Phenolics

Phenol and its derivative are known to exhibit antimicrobial activity. These compounds have been derived from sponges and algae (especially brown algae). Now, tunicates are also emerging as a source of phenolics. *Dysidea herbacea*, a sponge of Indian ocean yielded a new tetrabromo diphenyl ether and 2-(2',4'-Dibromophenoxy)3,5-dibromoanisole.[60] Crotonic acid, phenyl acetic acid, 4-hydroxy phenyl acetic acid, 4-hydroxybenzaldehyde and 4-isobutyl-α-methyl benzyl alcohol and a new phenolic derivative phyeosisenone **(18)** have been isolated from sponge *Phycopsis*.[61] Oligomers of phloroglucinol isolated from brown algae exhibited antimicrobial and tannin property. Glombitza and co-workers suggested the term phlorotannin for this class of phenols. Phlorotannins were classified as fucols, phlorethols, fucophlorethols and fuhalols. These compounds have been isolated from several species of brown algae.[62]

Miscellaneous

Aplysilline A, a disulphate ester of a 1,4-diphenyl-1,3-butadiene was isolated from marine sponge *Aplysina fistularis*,[63] which was highly unstable and rapidly decomposed after final purification. This compound inhibited binding to the thrombin receptor with an IC_{50} value of 20 μg/ml. Halicylindramides have been isolated from marine sponge *Halichondria cylindrata.*[64] Halicylindramides D was antifungal against *Mortierella ramanniana* and cytotoxic against p-388 murine leukemia cells. Mauritiamine, a new antifouling oroidin dimer from marine sponge *Agelas mauritiana.*[65] inhibited larval metamorphosis of the barnacle *Balanus amphitrite* with ED_{50} value of 15mg/ml. A new C_{43} acetylenic alcohol, vasculyn was isolated by cytotoxicity guided fractionation on the Caribbean sponge *Cribrochalina vasculum.*[66] Three compounds 3,5-dibromo-4-methoxyphenyl acetonitrile, 3-brorno-4-methoxy phenyl acetonitrile and 3,5-dibromo-4- methoxy benzoic acid have been isolated from sponge *Psammaplysilla purpurea.*[67] A novel brominated benzocyclooctane derivative was isolated from a sponge *Hamigera Taragenesis,*[68] which showed activity against the micro organisms *Staphylococcus aureus*, *Candida albicans* and *Bacilus subtilis* . A new cyclic hepta peptide, hymenamide was isolated from an Okinawan sponge *Hymeriacidon* sp.[69] The salt water culture of *Aspergillus lochraceus* separated from the Indo-Pacific sponge *Jaspis sp.* has yielded two new chlorine containing polyketides.[70] A tyrosine derivative

containing iodine has been isolated from the Senegalese sponge *Ptilocaulis spiculifer.*[71] A novel sphingosine (2S,3S, 4R)-N-[2'-(R)-hydroxy tetracosanoate]-1,3,4-trihydroxy-2-amino octadeca-6-ene was isolated from the sponge *Spirastrella inconstans.*[72] Marine sponge *Plakortis* sp. yielded two new peroxides plakortolide E and plakoric acid.[73] A novel cytotoxic macrolide superstolide was reported from the deep water marine sponge *Neosiphonia superstes.*[74] Four new 3,4-dihydroxystyrene dimers isolated from a marine sponge *Jaspis sp.*[75] induce larval metamorphosis in *Ascidians.* Three new cytotoxic and antimicrobial peptides discodermins have been isolated from marine sponge *Discoderminia kiiensis.*[76] These compounds are growth inhibitor of *Candida albicans* and *Cryptococus neoformans.* Three new peroxylactone, plakortolides and a new peroxy ester, epiplakinic acid (E) methyl ester were isolated from a marine sponge *Plakinastrella onkodes.*[77] Zarzissine, a new 4,5-guanidino pyridazine compound and known p-hydroxybenzaldehyde were isolated from Mediterranean sponge *Anchinoc paupertas.*[78] These compounds exhibited cytotoxicity against human and murine tumor cell lines namely human nasopharyngeal carcinoma cells.

As reported above pharmaceuticals from marine organisms have been accepted as useful drugs against various ailments . These includes compounds such as cephalosporin C, kainic acid, carrageenan, alginic acid , alginates, tetradotoxin, saxitoxin, halitoxin, tenadolide etc. Among

these Carrageenan is reported to have antiviral activity for certain influenza viruses. It also exhibits anticoagulant activity. Carrageenans are potentially useful as antiulcer agents. Alginic acid and alginates were isolated from algae such as *Laminaria*, *Fucus* and *Macrocystis*. Alginic acid continues to be of pharmaceutical value as tablet disintegrating agent. Alginates are known to exhibit anticoagulant property. Agar and alginic acid have their use in as components of tissue culture media, as adhesives, stabilizers and as emulsifiers in food products. Cephalosporin C, a marine antibiotic has been found to be active against variety of bacteria including penicilline-resistant strains and used clinically. Tetrodotoxin is commercially available and being used as muscle relaxant, pain killer in leprosy and terminal cancer. Kainic acid isolated from red alga *Digenea simplex* has been found as a useful anthelmintic and vermifuge against tapeworm *Taenia* sp., the parasitic round worm *Ascaris lumbricoides* and whip worm *Trichuris trichura*. Tenolide isolated from a sponge *Tedania sp.* Inhibits tumor cells. Halitoxin isolated from a sponge *Haliclona rubens* exhibit potent cytolytic, hemolytic and toxic properties.[79]

There are several reports of isolation and identification of organic compounds from marine organisms collected along Indian coast.

Mangrove plant *Acanthus ilicifolius* exhibiting analgesic activity yielded 2-benzoxazolinone as an active principle.[80] Red alga *Acanthophora spicifera* yielded aurantiamide along with its diasteriomer, which is a

potential intestinal stimulant and showing antifertility activity.[81] Subergorgic acid has been isolated from *Suberogorgia suberosa* [82] and also a new nitrogen containing aromatic acid 4-methyl-3(5) carboxylic acid from sponge *Tedania anhelans.*[83] Several other secondary metabolites have been isolated from marine flora and fauna, collected from Indian coastal region. Petroleum ether and chloroform extract of marine alga *Acanthophora spicifera* exhibiting antifertility activity yielded a novel steroid, 11α-hydroxy cholestane, 3, 6, dione along with some known sterols.[84] Marine alga *Dyctyota dichotama* collected from Andaman and Nicobar islands yielded large number of diterpenoids. These diterpenes are derivatives of dolabellane or derivable from it through internal cyclization.[85] Zoanthamins exhibiting anti-inflammatory and analgesic properties have been reported from *Zoanthus* species, collected from Bay of Bengal.[86] *Phyllospongia dendyi*, a marine sponge collected from Anadaman and Nicobar islands yielded furanoterpenes exhibiting antimicrobial properties.[87] A new antiviral agent 2-N-palmitoyl,4,5-dihydro,1,3,4,5-tetrahydroxy sphingosine was reported from green alga *Ulva faciata.*[88]

Scientific work in this area is progressing well resulting in more marine products as candidate for clinical trial. There are numerous developments in area of chromatographic techniques as well as sophisticated instruments to study the spectral properties of compounds. As a result number of publications related to bioactive substances from

marine organisms have been increasing since past few years. There are better prospects for marine natural products chemistry in years to come. However, collection of organisms from deep sea water, isolation of minute quantity of organic compounds and repeat collection of the marine organisms are some problems to be solved.

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Chapter 3

Phlorotannins from brown alga *Carpophyllum plumosum*.

Phlorotannins consist of polymers of 1,3,5-trihydroxy benzene (phloroglucinol) and found to occur in brown algae (Phaeophyceae). Tannins constitute 5-10 % of the dry weight of the algae, they precipitate proteins from solution and bind metal ions. It is believed that this protein binding ability is responsible for the poor availability of dietary protein in animal fodders containing brown algal meal. The phlorotannins are widely used in tannin industry.[1]

Apart from antibacterial, antialgal and antiviral activity phlorotannins are toxic to a variety of animals, such as hydroids, various worms etc. They inhibit fertilization in Echinoderm eggs. Extracts containing phlorotannins are toxic to mice, they inhibit several enzymes such as lipase, tripsin, while a few are effective antiplasmin inhibitors.[2]

Vesicles of high optical refractivity, known as physodes or fucosan granules are found in many cells of Phaeophyceae. They are also present in many *Laminariales*, *fucales*, *Ectocarpales*. Crato concluded that physodes contain phloroglucinol or its derivatives.[3]

The term phlorotannin for these phenolic polymers was coined by Sattler[4] in 1974. Prior to that, they were known as phloroglucotanoides,[5] fucosan of Kylin[6] etc. In 1969, Millardet had observed that the black colour of air dried brown algae was due to a complex oxidation product of phlorotannins.[7] Algae excrete part of their tannin into sea water, these excretion products have strong antibacterial and algicidal

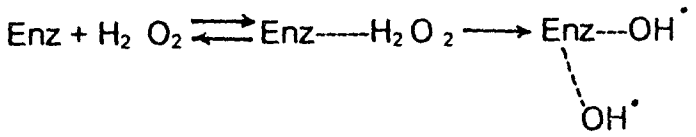
properties. Phloroglucinol was recovered after alkaline hydrolysis of polyphenol preparations of brown algae. However, it was only in year 1973 that phloroglucinol was identified as a constituent of brown algae.[1]

Phlorotannins are classified in different groups, such as fucols, phlorethols, fuhalols, etc. Fucols are dehydrooligomers consisting of phloroglucinol units linked through aryl-aryl bonds. The prefix denotes number of phloroglucinol units in one molecule like difucol, trifucol etc. Fuhalols are comprised of ether-linked phloroglucinol units with an extra hydroxyl group in one unit, making that unit vicinally trihydroxylated. As in case of fucols, prefix indicates the number of phloroglucinol unit present in molecule such as bifuhalol, trifuhalol etc. Bifuhalol was earlier discovered in Bifucaria (Glombitza and Rosener 1974)[10] and (Sattler et al. 1977).[11] The structures of bifuhalol and trifuhalol have been established by total synthesis. Phlorethols are dehydrooligomers, consisting of phloroglucinol units linked through diaryl ether bonds, the name is derived from phloroglucinol ethers. The prefix indicates the number of phloroglucinol units in a molecule, such as diphlorethol, triphlorethol etc. They are found to occur in algae belonging to the orders fucales and laminariales. In triphlorethol suffix A, B, C refers to o, p, and m oriented ether bonds respectively.[12] There are some reports of halogenated phlorotannins. The first halogenated phlorotannin monochlorotriphlorethol C, was isolated from brown alga *Laminaria ochroleuca* by Glombitza et.al in 1977.[13] Later Koch and

Gregson isolated all the possible nos. of isomeric monobromodiphlorethols in *Cystophora congesta*.[12]

Algae synthesize polyphenols by condensation of acetate units, which produce polyketides. Condensation of these chains of acetate units into aromatic rings yield phloroglucinols. However, other phenolics are derived by shikimic acid pathway via phenyl alanine or tyrosine, monohydric phenols derived from these aminoacids are usually p-hydroxy compounds. The oxidation of phloroglucinol leads to coupling reaction involving oxygen and /or carbon centers. The products from these intermolecular coupling reaction may belong to different groups, e.g. fuhalol, phlorethols, fucol and fucophlorethols. The reaction is of considerable biological importance and mediated by enzymes, most probably peroxidases which are known to be present in brown algae. Waters proposed that oxidation with high potential oxidants in acidic media is likely to form phenoxonium species favouring O-C coupling while alkaline condition with lower potential oxidants would lead to radicals preferring C-C coupling.[8]

Brown algae contain peroxidases, which oxidize iodide to iodine and bromide to bromine atoms. Subsiquently, these halogen atoms are used by these enzymes for halogenation of phenolic substances, the process involved is not well characterized but possible reaction may be summerized as,[8]



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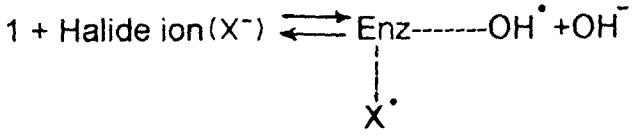

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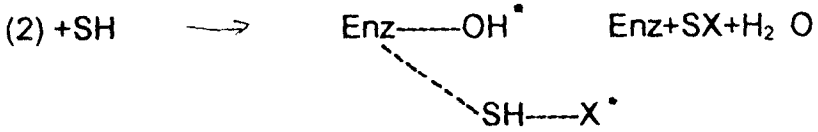


Acetylated phlorotannins exhibit molecular ion peak in EIMS and FABMS, fragments of 42 amu ($C_2 H_2 O$, ketene) are split off resulting series of ketene elimination ions extending downwards from the molecule ion, which reveals the total number of hydroxy groups present in parent molecule. Cleavage takes place preferably on the side of the ether-oxygen which is attached ortho position to an acetoxy group. The driving force for this cleavage may be assumed to be provided by an interaction between electrophilic carbon of the acetyl group and electrons of the ether oxygen, with the acetyl group being transferred to the ether-

oxygen after cleavage. MS is also very useful in detection and estimation of halogens in the molecules.

^{1}H NMR spectra were very useful in establishing the position of the acetate groups in the molecule of the phlorotannins. Methyl protons present on acetoxyl groups gave well differentiated signals between δ 1.6-2.4 in $CDCl_3$. Number of methyl groups could be determined by the integration of signal intensities. On the other hand, the aromatic protons appeared at δ 6-7 in $CDCl_3$. ^{13}C NMR spectra were also helpful to assign the nature of carbon atom present in molecules.

Ethanolic crude extract of brown alga *Carpophyllum plumosum* was extracted with pet-ether, chloroform and ethyl acetate. Chemical investigation of ethyl acetate fraction yielded 10 phlorotannins (CP-**1**-**10**) as shown in fig-1, 7, 13.

EIMS of compound **CP-1** showed molecular weight to be 252 and other fragments appeared at m/z 210, 168, 126 corresponding to elimination of the ketene units successively, thus, indicating the presence of three acetoxyl groups in the molecule. In ^{13}C NMR spectrum, signal at δ 112.8 was assigned to aromatic carbons, where as the signal at δ 151.2 was attributed to aromatic carbons to which acetoxyl groups are attached. The methyl and carbonyl carbons

associated with acetoxyl groups were evident from the presence of the signals at δ 21.0 and 168.0 respectively. In ^{1}H NMR spectrum (Table-1), a signal at δ 6.84 (3H, s) was assigned to aromatic protons H-2,6,4, where as signal at δ 2.25 (9H,s) attributed to three acetoxyl protons present at C-1,3,5

EIMS of the compound **CP-2** (Fig-2) showed molecular weight to be 286-288 (3:1) indicating the presence of chlorine atom. Ketene elimination series led to the conclusion for the presence of three acetoxyl groups in the compound. Due to the presence of halogen, methyl protons of acetoxyl groups are no more identical as in CP-1, but give two separate signals in ^{1}H NMR spectrum (Table-1,). However, two aromatic protons remained identical but shifted to lower field. A signal at δ 2.34 (6H,s) was assigned to acetoxyl protons at C-3 and C-5 and other signal at δ 2.28 (3H,s) indicated acetoxyl group at C-1. Signal appeared at δ 6.9 (2H,s) was assigned to aromatic protons H-2 and H-6. Compound CP-1 and CP-2 were identified as phloroglucinol triacetate[14] and monochlorophloroglucintriacetate respectively.

FABMS of compound **CP-3** (Fig-4) showed molecular weight to be 502. Loss of six ketene moiety indicated the presence of six acetoxyl groups in the compound. In ^{1}H NMR spectrum (Fig-3) a signal appeared at δ 6.99 (4H,s) was assigned to the aromatic protons H-3, H-5, H-3' and H-5'. Two more signal at δ 2.02 (12H,s) and 2.25 (6H, s) were assigned for

two acetoxyl groups attached to adjacent biphenyl C-C bond in each ring and two terminal acetoxyl groups respectively.

^{13}C NMR signals were assigned as 113.8 (C-3,5 and 3',5'), 115.8 (C-1 and 1'), 149.4 (C-2,6 and 2',6'), 150.6 (C-4 and 4'), 168.4 and 20.6 for acetoxyl ketone and methyl carbons respectively.[4] Compound CP-3 was identified as difucol hexaacetate and spectral data were identical with reported compound.[15]

Table-1: ^{1}H NMR data of phlorotannins (500 MHz, Chemical shift measured in $CDCl_3$, δ in ppm).

Position	**CP -1**	**CP-2**	**CP-3**
H-2,4,6	6.84 (3H,s)		
H-2,6		6.9 (2H,s)	
H-3,5,3',5'			6.99 (4H,s)
Me of Ac at			
C-1,3,5	2.25 (9H,s)		

C-3, 5 C-1 C-2,6, 2',6' C-4,4'		2.34 (6H,s) 2.28 (3H,s)	 2.02(12H,s) 2.25 (6H,s)

FABMS (Fig-5) indicated the molecular weight of compound **CP-4** to be 518, corresponding to the molecular formula as C_{24} H_{22} O_{13}. In addition, successive loss of 6 ketene units indicated the presence of six acetoxyl groups in the molecule.[5]

^{1}H NMR spectrum (Fig-6) of compound showed two signals at δ 6.9 (2H,s) and 6.7 (2H,s) for aromatic protons H-3, H-5 and H-2',H-6' respectively. Other signals at δ 2.28 (3H,s), 2.24 (6H,s) and 2.07 (6H,s) were assigned to methyl protons of acetoxyl groups at C-4, C-3', 5' and C-2, 6 respectively. An additional signal at δ 2.25 (3H,s) was evident for acetoxyl group present at C-4'. Compound CP-4 was identified as bifuhalol hexaacetate and chemical shifts were compared with reported values. [11-12]

EIMS of the compound **CP-5** showed molecular weight of compound to be 460 and subsiquent loss of ketene moiety were observed at m/z

418, 376, 334, 292, 250 indicating the presence of five acetoxyl groups in the molecule.[6] ^{1}H NMR spectrum (Fig-8) of compound showed AB_2 spin system at δ 6.63 (1H,m) and 6.57(2H,d,) with a coupling constant of 2.1 Hz indicating aromatic protons H-4' and H-2', 6' respectively. Another signal at δ 6.9 (2H,s) indicated the aromatic protons of 1-phenoxylated 2, 4, 6-triacetoxybenzene.[12] ^{1}H NMR spectrum also had signals δ 2.23 (6H,s) 2.06 (6H,s) and 2.26 (3H,s), these were assigned to acetoxyl protons attached to C-3',5' , C-2,6 and C-4 respectively. Chemical shifts in ^{1}H NMR spectrum of compound CP-5 were found to be similar to diphlorethol pentaacetate isolated by Koch et.al in 1984.[12]

FAB mass spectrum of compound **CP-6** (Fig-9) showed molecular ion peak at m/z 538-540, indicating the presence of one bromine atom in the molecule, other peaks after elimination of ketene units indicated the five acetoxyl groups in the molecule. Its ^{1}H NMR spectrum (Fig-10) was very much similar to compound CP-5, however, downfield signal at δ 7.04 (1H,s) assigned to H-5 indicated the presence of bromine in ring A. Signals δ 2.04 (3H,s) and 2.19 (3H,s) were assigned to acetoxy methyl protons at C-6 and C-2 respectively, leaving C-3 position for bromine atom. Protons present at ring B showed AB_2 system and signals appeared at δ 6.66 (1H,dd) and 6.58 (2H,d) were assigned for H-4' and H-2',6' protons respectively. Terminal acetoxyl group protons at C-4 could be recognised at δ 2.35 (3H,s). Low chemical shifts were observed for C-

5 protons as well as acetoxyl protons of ring A due to presence of halogen.[6] This compound was identified as monobromodiphlorethol.[12]

Table-2: ^1H NMR data of phlorotannins (500 MHz, Chemical shift measured in $CDCl_3$, δ in ppm).

Position	**CP-4**	**CP-5**	**CP-6**
H-3,5	6.9 (2H,s)	6.95 (2H,s)	
H-2', 6'	6.7 (2H,s)	6.57 (2H,d)	6.58 (2H,d)
H-4'		6.63 (1H,m)	6.66 (1H,dd)
H-5			7.04 (1H,s)
Me of Ac at			
C-4	2.28 (3H,s)	2.26 (3H,s)	2.35 (3H,s)
C-4'	2.25 (3H,s)		
C-2,6	2.07 (6H,s)	2.06 (6H,s)	

C-3', 5'	2.24 (6H,s)	2.23 (6H,s)	2.26 (6H,s)
C-2			2.19 (3H,s)
C-6			2.04 (3H,s)

FAB mass of compound **CP-7** (Fig-11) showed presence of bromine atom which was evident from the molecular ion peak pattern at m/z 538-540. Loss of five ketene moieties were indicated by peaks at m/z 496-498, 454-456, 412-414, 370-372, 328-330 assigned for the five acetoxyl groups in the molecule. FAB mass spectrum of this compound was found to be similar to that of compound CP-6, however, differs in ^{1}H NMR spectrum (Fig-12). Signal at δ 6.9 (2H,s) was assigned to aromatic protons of 1-phenoxylated 2, 4, 6-triacetoxybenzene. AB_2 spin system vanishes from spectrum and two signals appeared at δ 6.7(1H,d) and 6.45 (1H,d) assigned to H-4' and H-6' respectively, leaving the possibility of bromine atom at 2' or 6' in ring B. Two signals at δ 2.35 (3H,s) and 2.21 (3H,s) were assigned to acetoxy group at C-3',5'. respectively.[6] ^{1}H NMR signals at δ 2.11 (6H,s) and 2.27 (3H,s) were assigned for acetoxyl protons attached to C-2,6 and C-4 respectively. Compound CP-6 and CP-7 were isomers and identified as

3[A] bromodiphlorethol pentaacetate and 2[B] bromodiphlorethol pentaacetate respectively.[12]

FAB mass spectrum of compound **CP-8** (Fig-14) showed molecular ion peak at m/z 494-496 (3:1) indicated to be a chlorinated molecule. Ketene elimination indicated by fragment ion series suggested five acetoxyl groups in the molecule. ^{1}H NMR spectrum (Fig-15) of CP-8 showed a signal at δ 6.95 (2H,s) assigned to aromatic proton of 1-phenoxylated-2,4,6 triacetoxybenzene. Protons present in ring B were symmetrical and appeared at δ 6.68 (2H,s), leaving the C-4' position for chlorine atom. This was further supported by the signal at δ 2.32 (6H,s), which was assigned to two acetoxyl methyl protons at C-3' and C-5'. Thus, indicating the position of chlorine at C-4'. Two more signals at δ 2.29 (3H,s) and δ 2.09 (6H,s) were assigned to terminal methyl protons of acetoxyl group at C-4 two acetoxy methyls at C-2,6. This compound was identified as 4[B} chloro-diphlorethol.[16]

FABMS of compound **CP-9** showed molecular weight of the compound to be 668. Loss of seven ketene moiety indicated the presence of seven acetoxyl groups in the molecule.

^{1}H NMR of compound (Fig-16) CP-9 was similar to diphlorethol and differing only in the presence of an additional benzene ring. AB_2 system was observed at δ 6.65 (1H, m) and 6.51 (2H,d) for protons in ring C. Two signal at δ 2.13 (6H,s) and 2.06 (6H,s) were assigned to

methyl protons for two acetoxyl groups at C-2,6 in ring A and two acetoxyl groups at C-2,6 in ring B respectively. Terminal acetoxyl methyl protons showed a singlet at δ 2.28 (3H,s). A singlet at δ 2.24 (6H,s) was assigned for methyl protons of acetoxyl group of ring C. 4-Phenoxy-triacetoxy benzene moiety was evidenced by an aromatic proton peak at δ 6.9 (2H,s) and another signal at δ 6.7 (2H,s) was assigned for aromatic protons of ring B.[5] Compound CP-9 was identical with reported compound triphlorethol -A- heptaacetate.[12, 14]

Table-3: ^{1}H NMR data of phlorotannins (500 MHz, Chemical shift measured in $CDCl_3$, δ in ppm).

Position	CP-7	CP-8	CP-9	CP-10
Ring A				
H-3,5	6.9 (2H,s)	6.95 (2H,s)	6.92 (2H,s)	6.92 (2H,s)
Ring B				
H-4'	6.72 (1H,d)			
H-6'	6.45 (1H,d)			
H-2' ,6'		6.68 (2H,s)		
Ring B				

H-3, 5			6.70 (2H,s)	
H-4				6.52 (1H,d)
H-6				6.74 (1H,d)
Ring C				
H-2,6			6.51(2H,d)	6.55 (2H,d)
H-4			6.65 (1H,m)	6.67 (1H,m)
Me of Ac at				
Ring B				
C-3'	2.35 (3H,s)			
C-5'	2.21 (3H,s)			
C-3',5'		2.32(6H,s)		
Ring A				
C-2,6	2.11 (6H,s)	2.09 (6H,s)	2.13 (6H,s)	2.02 (6H,s)
C-4	2.27(3H,s)	2.29 (3H,s)	2.28 (3H,s)	2.26(3H,s)

Ring B				
C-2,6			2.06(6H,s)	
C-1				2.13 (3H,s)
C-5				2.22 (3H,s)
Ring C				
C-3,5			2.24 (6H,s)	2.23 (6H,s)

FAB mass spectrum of compound **CP-10** was similar to that of CP-9, as the molecular ion peak as well as ketene elimination series was same. This indicated compound CP-10 was similar to CP-9 or may be an isomer. However, ^{1}H NMR signals (Fig-17) showed that substitution of ring C at ring B was at ortho position where as in CP-9 it was at para position. AB and AB_2 spin system were observed in ring B and C respectively. A singlet at δ 6.92 for two proton was again assigned for 1-phenoxy- triacetoxybenzene. Methyl protons of acetoxy groups of ring B are no more identical and appear at δ 2.13 and 2.22, where as methyl protons of acetoxyl groups present in ring A and C gave two signal at δ 2.23 (6H,s) and 2.02 (6H,s) respectively. It also indicated that both the acetoxyl group in ring A and C are in same environment in their

respective ring. Terminal acetoxyl group protons of ring A showed a singlet at δ 2.26 for three protons. Compound CP-10 was identified as triphlorethol -B- heptaacetate.[16]

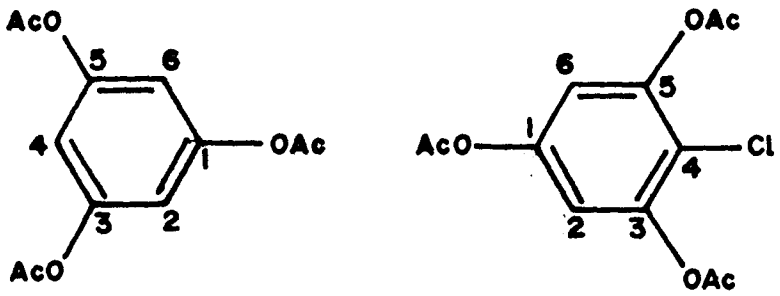
Experimental

Phlorotannins are very labile molecules. In order to prevent air oxidation and at the same time to increase their lipophilic properties, they were isolated as peracetates. Brown alga *Carpophyllum plumosum* was collected from Newzealand coast. Deep frozen alga was treated with liquid nitrogen and grinded to small pieces using grinder. The ground alga was extracted with ethanol in a homogeniser (2-3 hours) under nitrogen gas atm. The extract was filtered through filter paper and concentrated under reduced pressure on a thin layer evaporator, to yield aqueous residue. This residue was successively extracted with petroleum ether, chloroform and ethyl acetate. The ethyl acetate fraction was dried over sodium sulphate and concentrated under reduced pressure on a rotatory evaporator to dryness. The phenols obtained were acetylated {acetic anhydride: pyridine (3:2), 12 hours} from this mixture. The mixture of peracetates thus, obtained was dissolved in acetone and low molecular weight phlorotannins precipitated from their higher homologues by adding an equimolar mixture of pet-ether and ether.

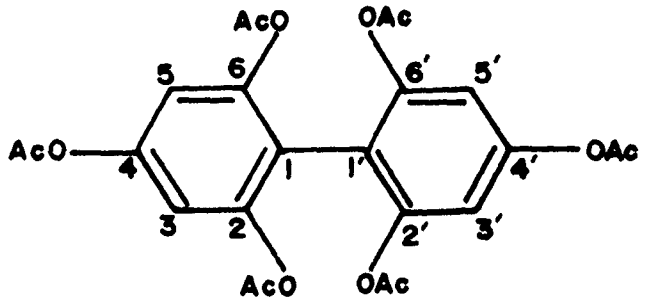
Low molecular weight phlorotannin acetates were separated using flash chromatography on silica gel (mesh 120) column with stepwise gradient elution of chloroform: n-hexane(1:1) , chloroform and chloroform: acetone (8:2). The fractions were monitored by thin layer chromatography using

chloroform: acetone (9:1) as mobile phase. The spots were visualized by spraying with vanillin-sulphuric acid- water as detecting reagent. Vanillin reacts at free ring-carbon position of 1,3 or 1,3,5-oxysubstituted benzenoid units to produce covalently bonded products, the absorption maxima of which depends on the type of reactants. Low molecular weight phlorotannins give red-orange coloured spots. Final purification of the individual compounds were achieved by high performance liquid chromatography (Fig-18-19) [Knauer (Berlin,Germany), Lichrosorb si 60, 8mm, Hexane and chloroform: ethanol gradient , 254 nm].

The structures of purified tannins were determined from their spectral data including EIMS, FABMS, ^{1}H NMR, ^{13}C NMR and UV-VIS spectroscopy. ^{1} H NMR data of individual compounds were confirmed with the published data of corresponding molecule and are cited in the discussion part of the text.



CP - 1 CP - 2



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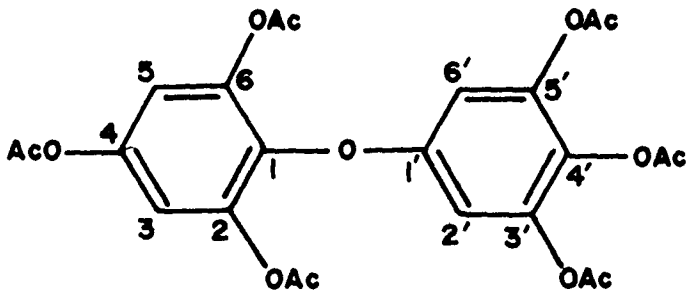
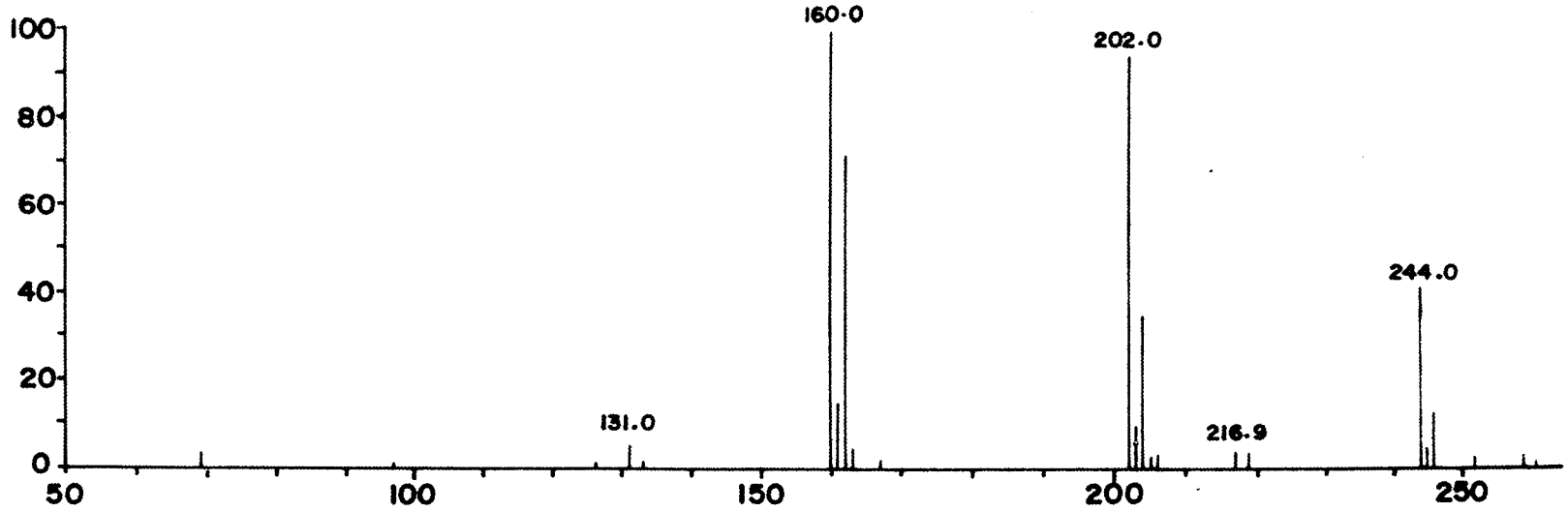


CP - 4

Fig. 1 : Phlorotannins from brown alga Carpophyllum plumosum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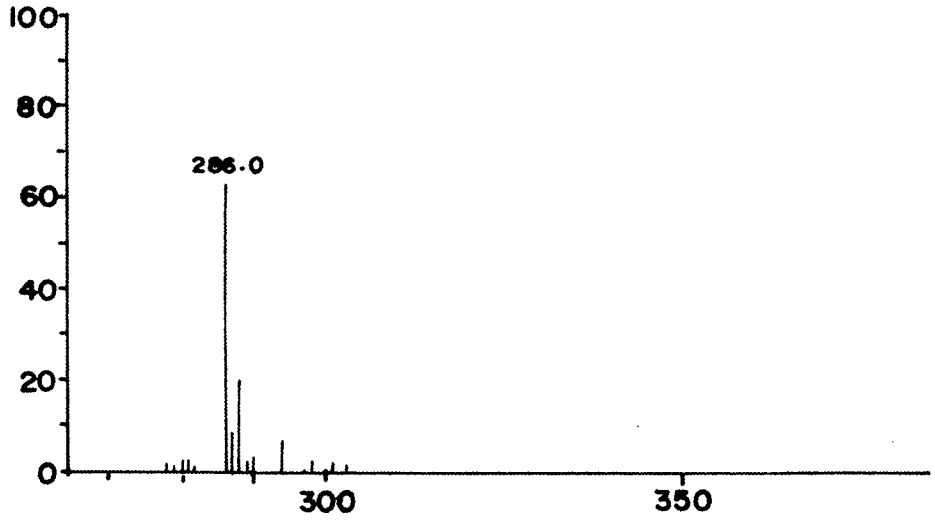
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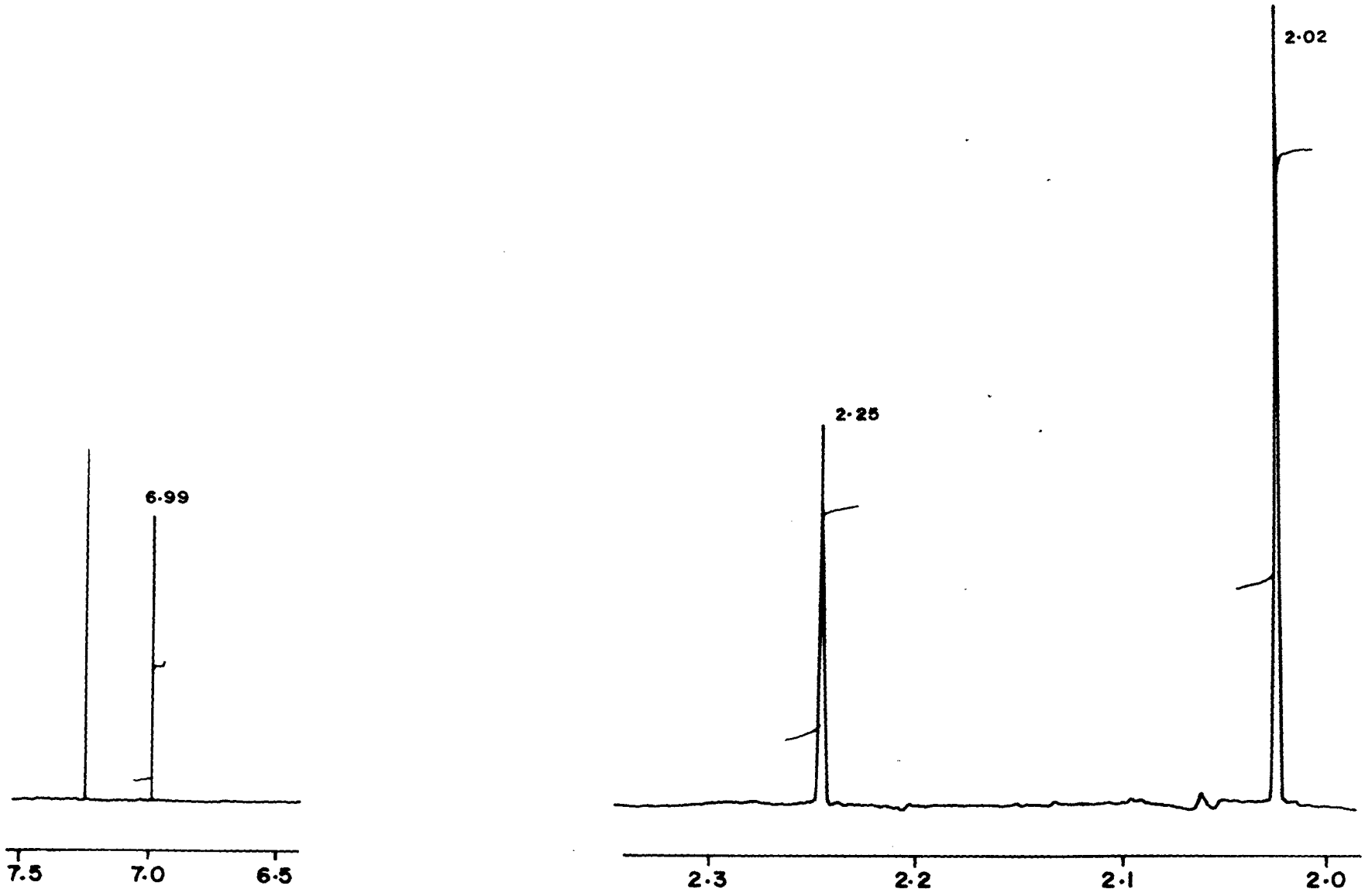


Fig. 3 ^{1}HNMR spectrum of Compound CP-3

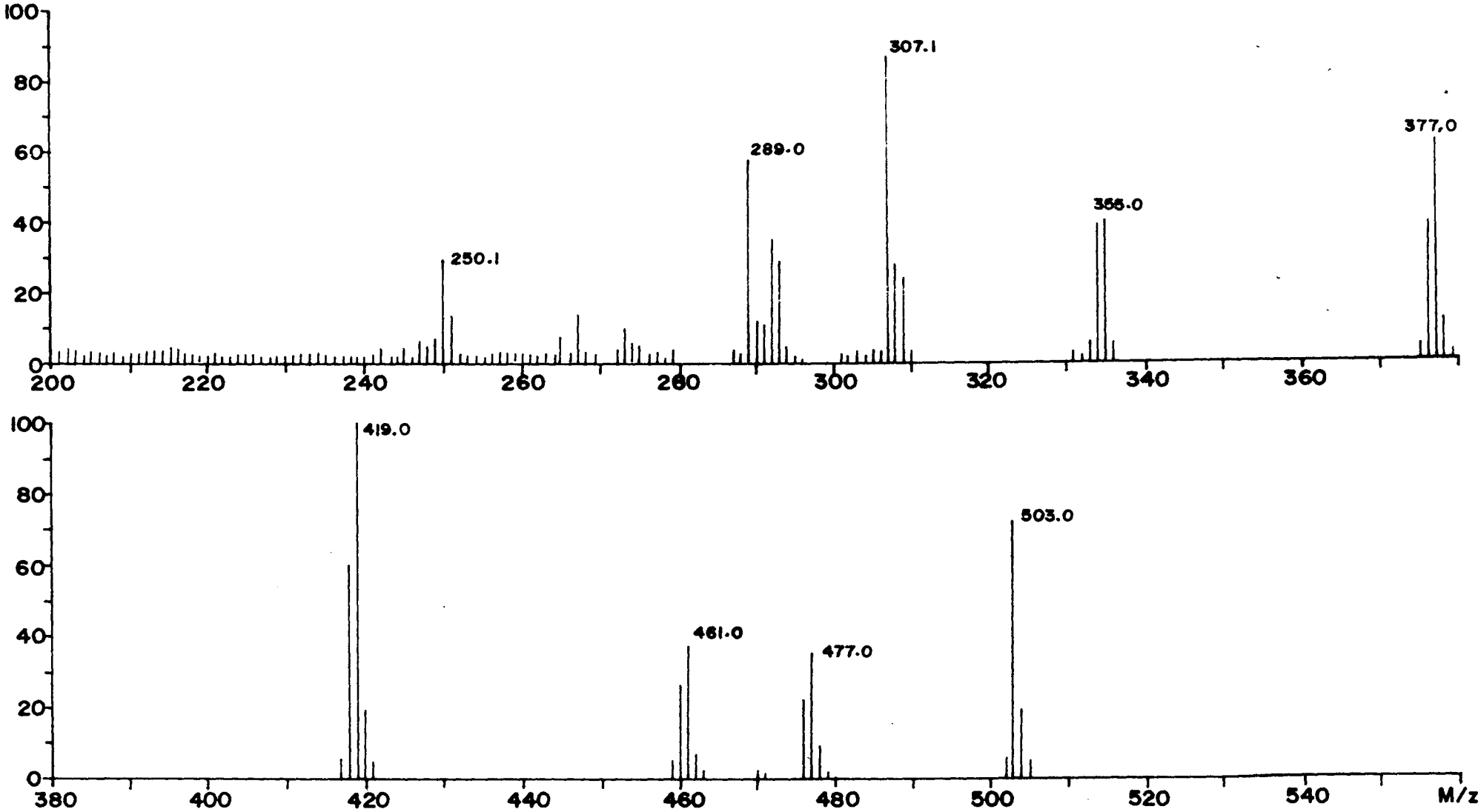


Fig. 4 FABMS of Compound CP-3

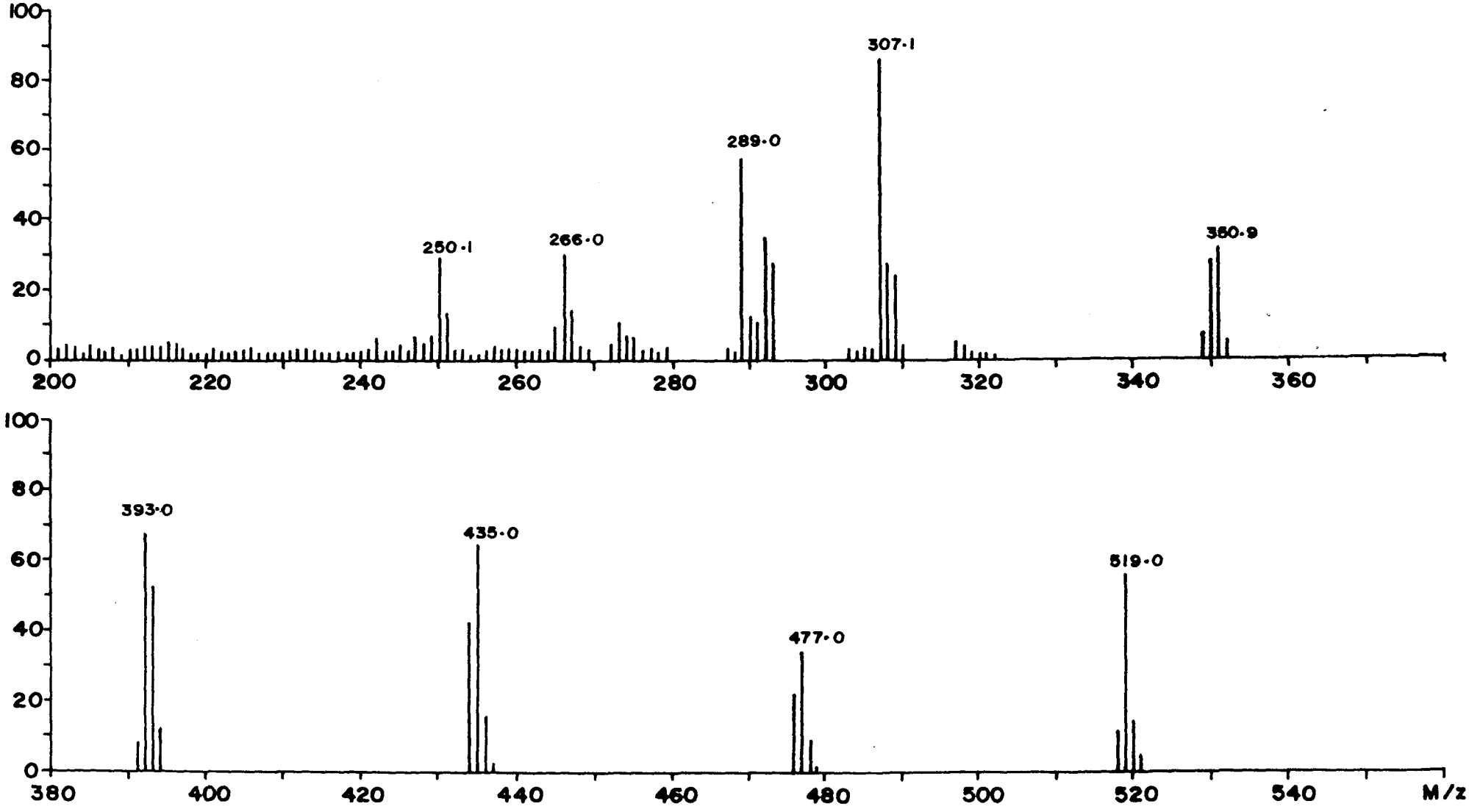


Fig. 5 FABMS of Compound CP-4

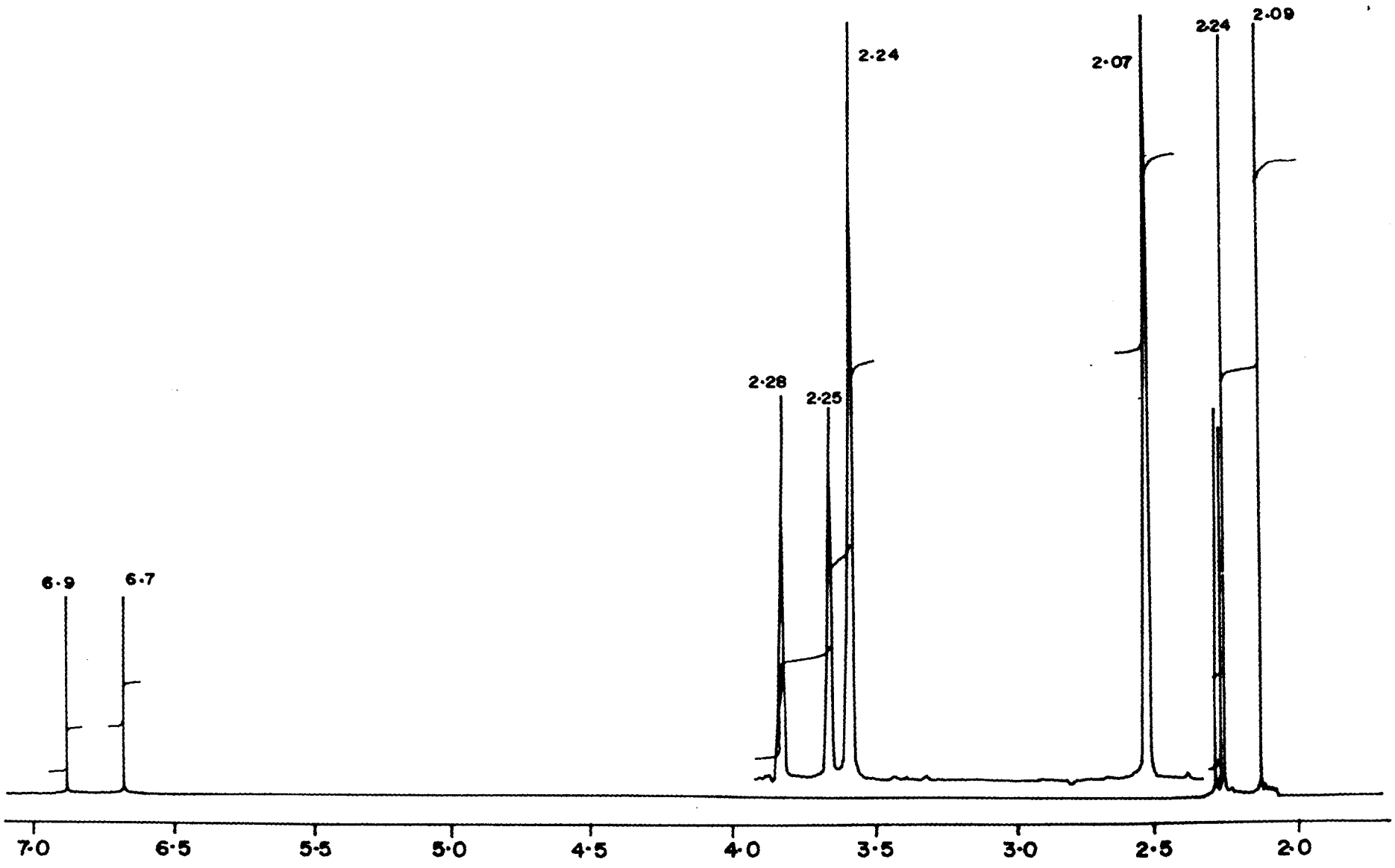
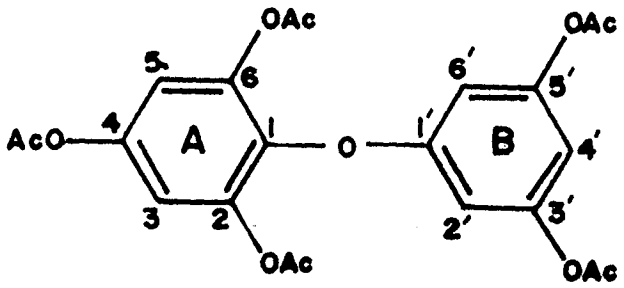
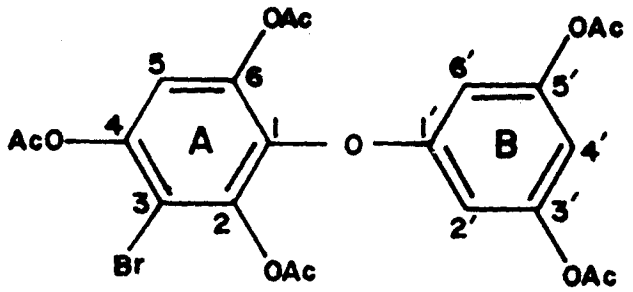


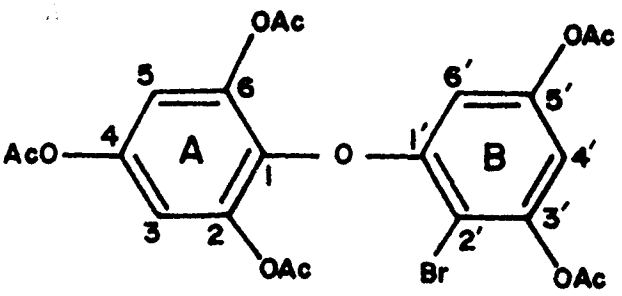
Fig. 6 ^{1}HNMR spectrum of Compound CP-4



CP-5



CP-6



CP-7

Fig. 7 Phlorotannins from brown alga Carpophyllum plumosum

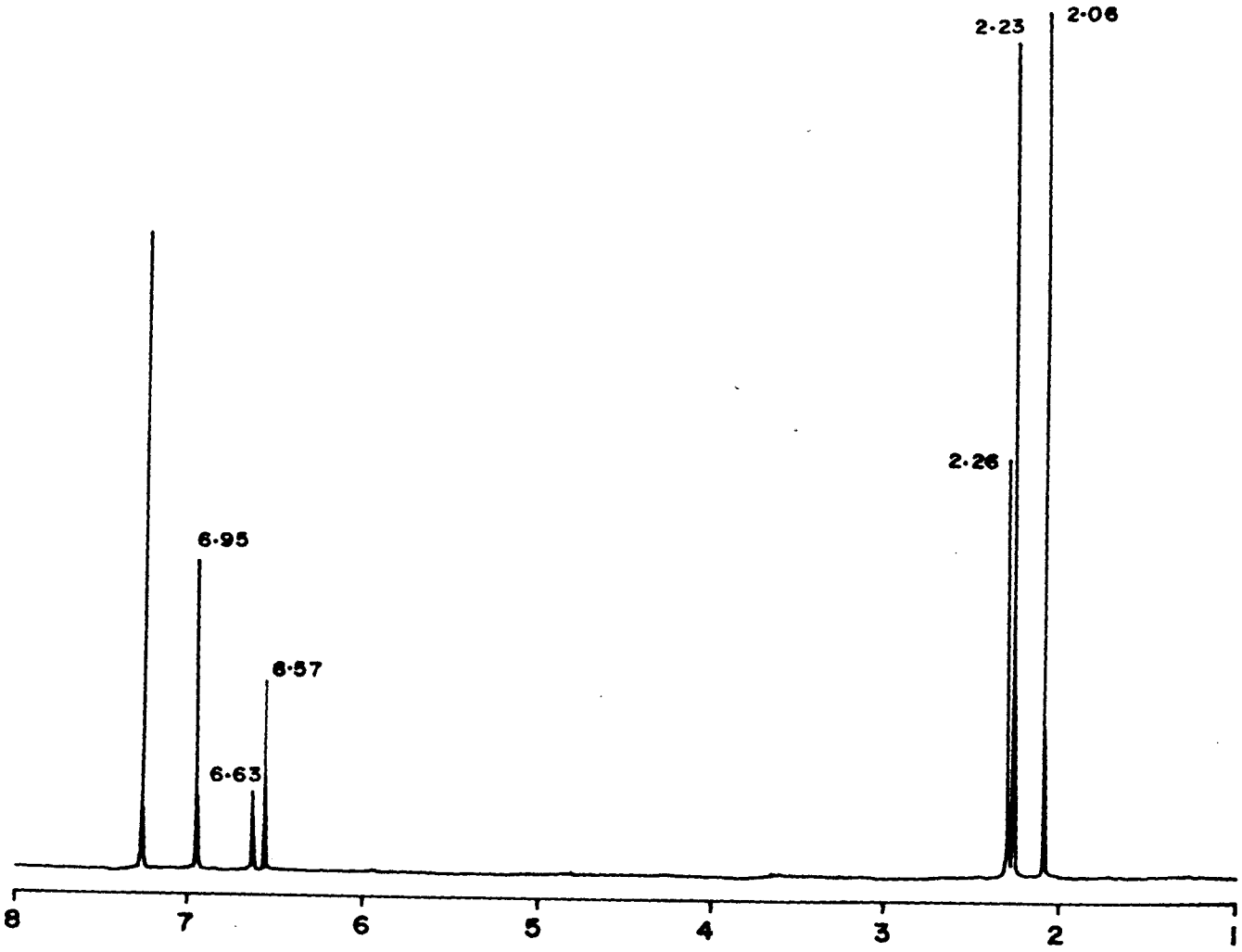


Fig. 8 ^{1}HNMR spectrum of Compound CP-5

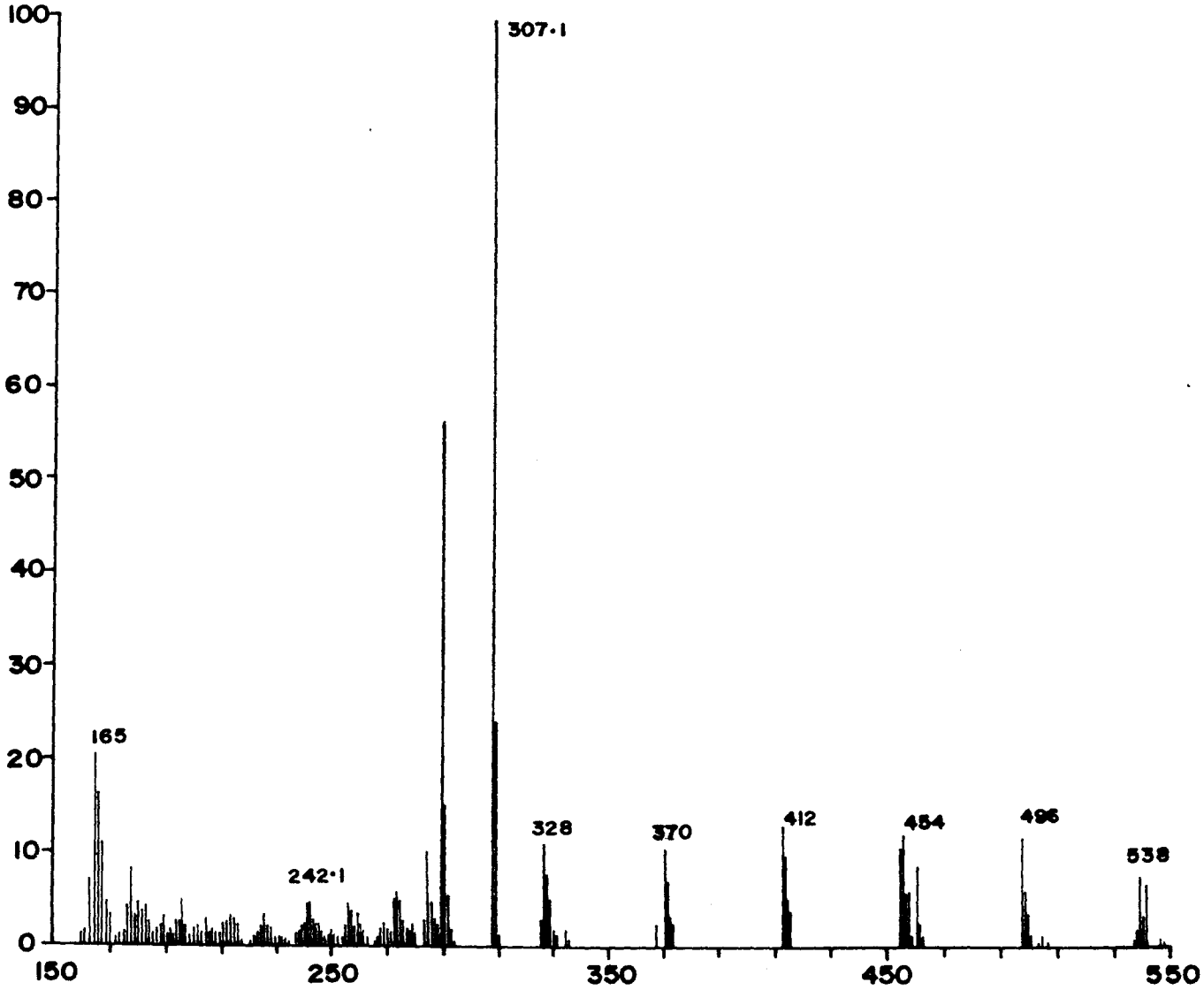


Fig. 9 FABMS of Compound CP-6

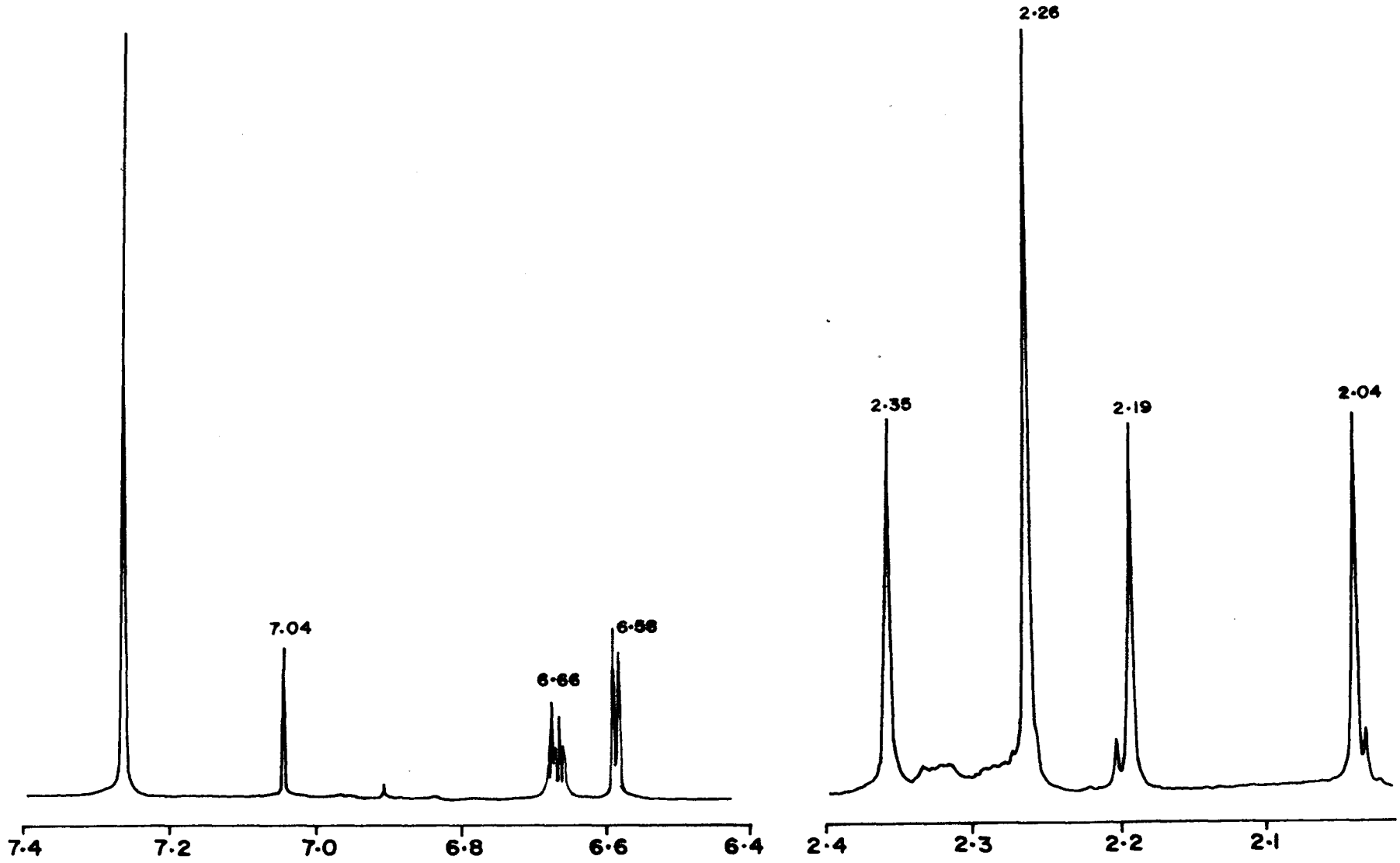


Fig. 10 ^{1}HNMR spectrum of Compound CP - 6

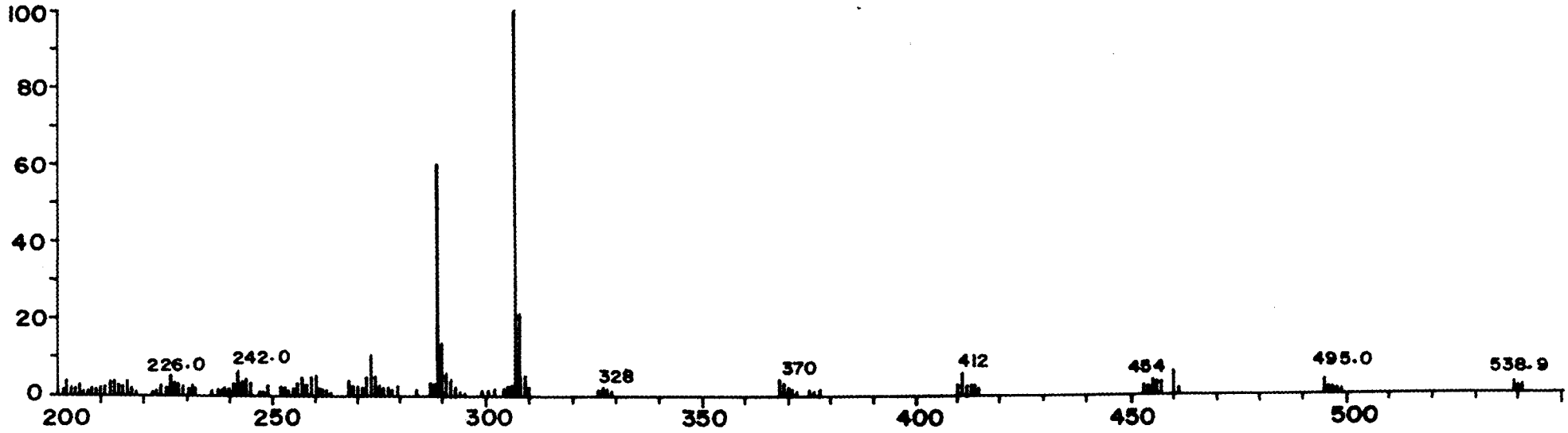


Fig. 11 FABMS of Compound CP-7

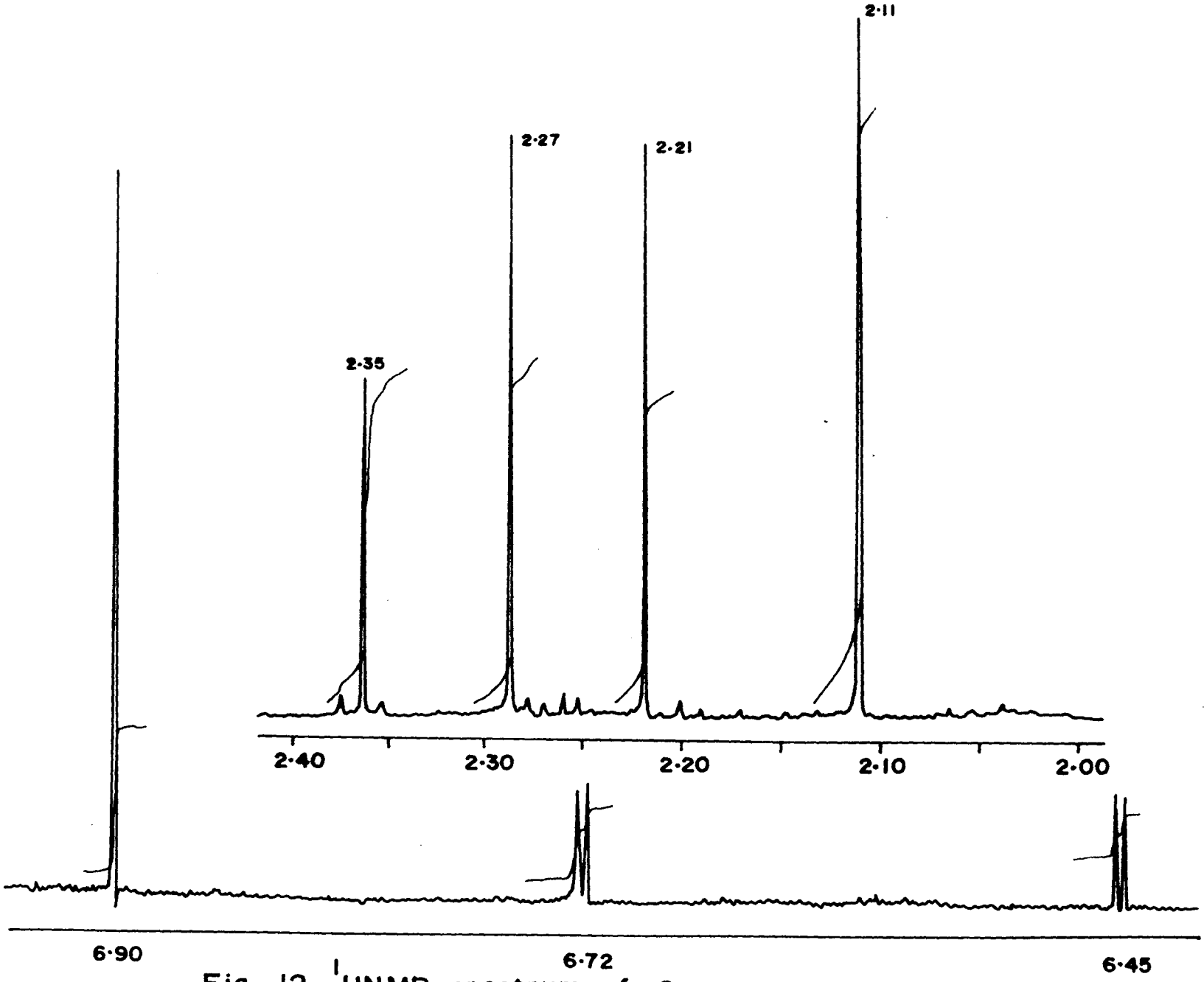
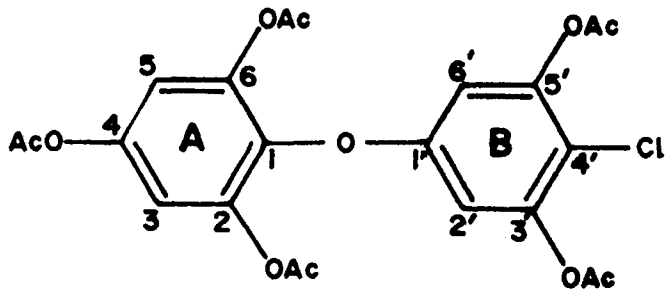
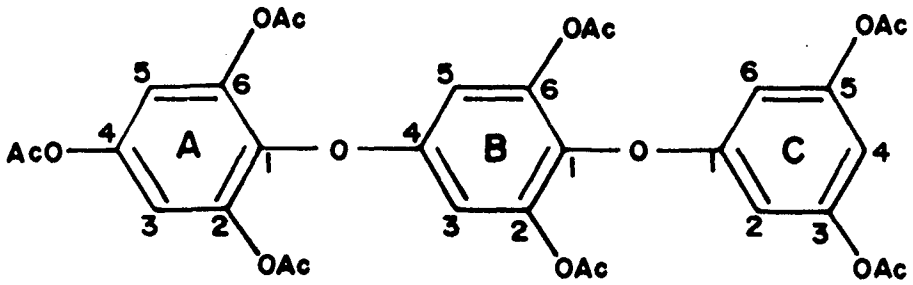


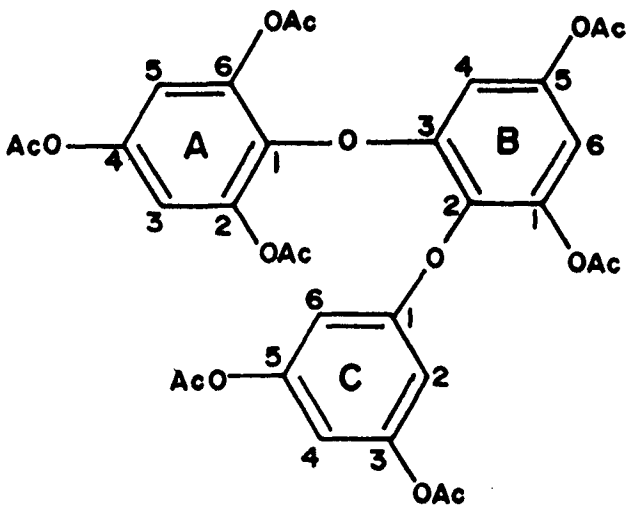
Fig. 12 ^{1}HNMR spectrum of Compound CP-7



CP - 8



CP - 9



CP - 10

Fig. 13 Phlorotannins from brown alga Carpophyllum plumosum

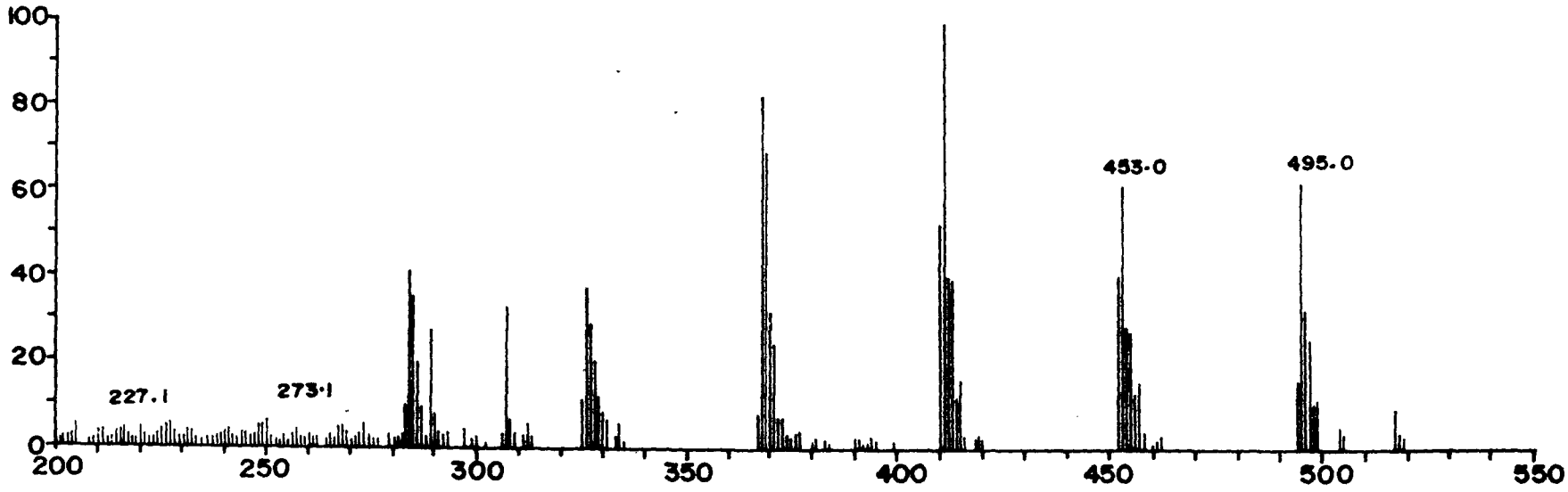


Fig. 14 FABMS of Compound CP-8

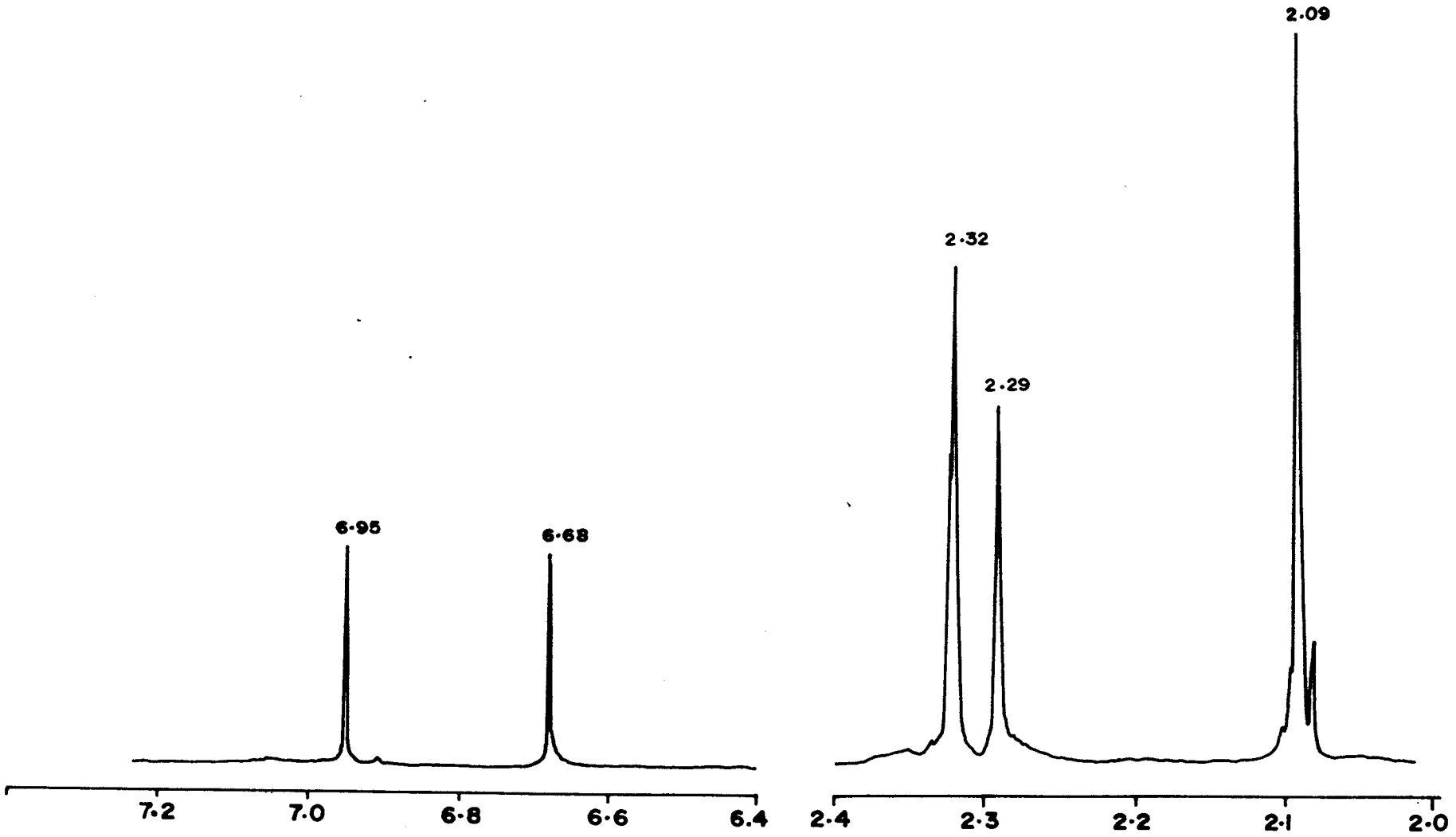


Fig. 15 ^{1}HNMR spectrum of Compound CP-8

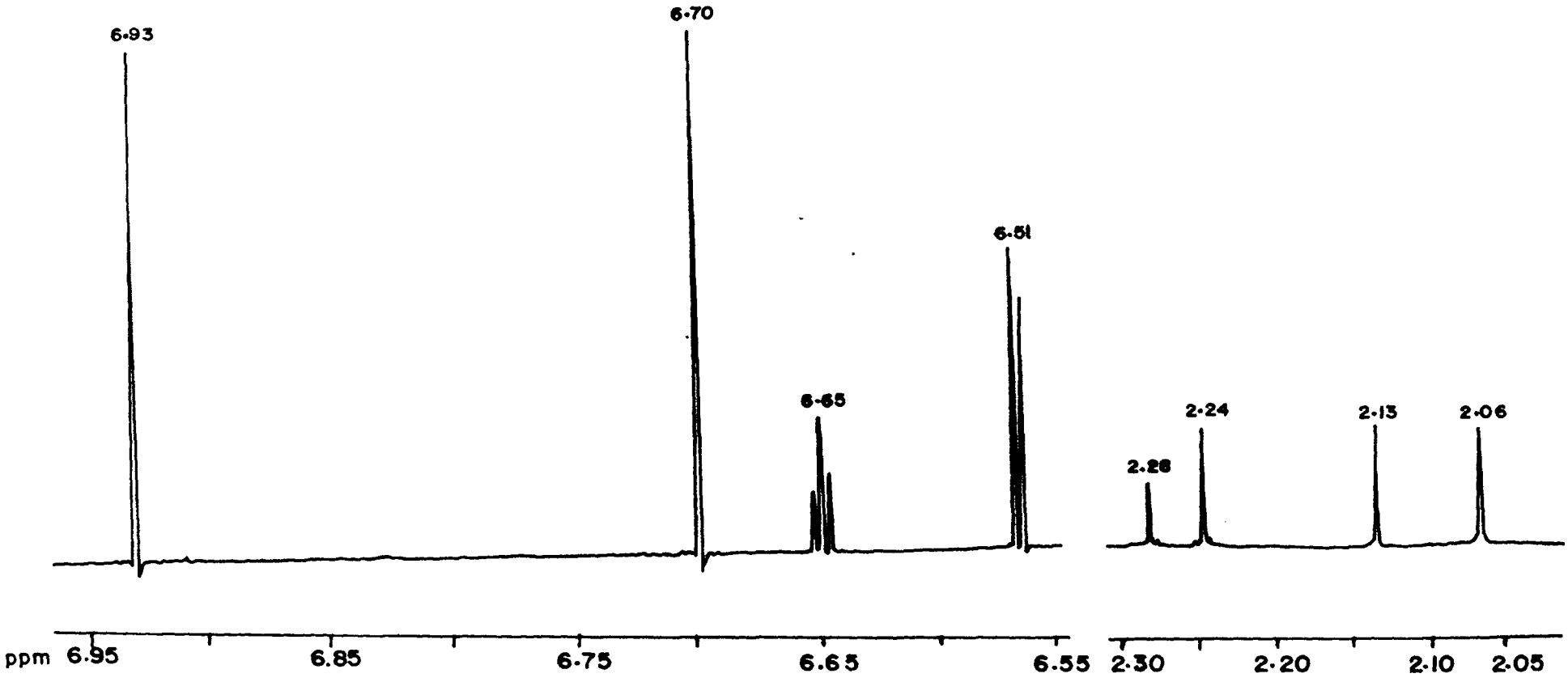


Fig. 16 ^{1}HNMR spectrum of Compound CP-9

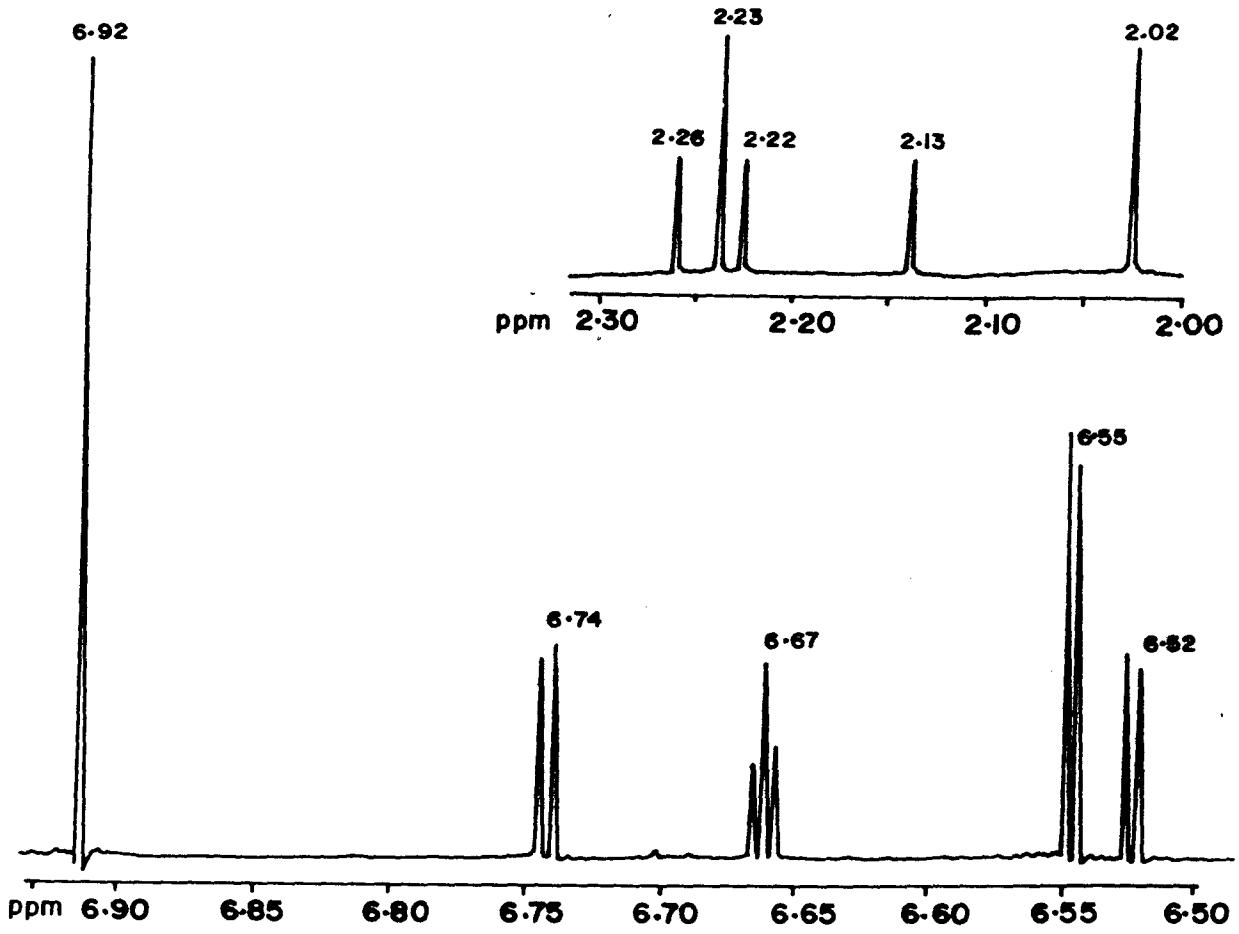


Fig. 17 ^{1}HNMR spectrum of Compound CP-10

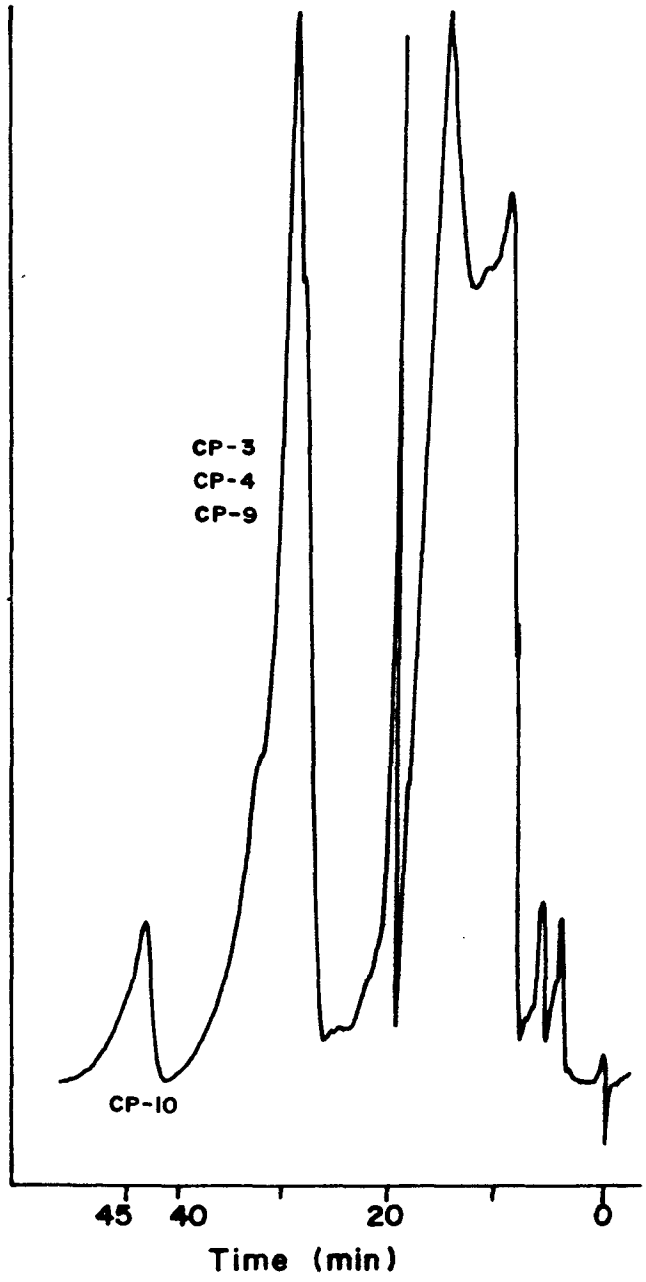


Fig. 18 HPLC chromatogram of Phlorotannins from alga Carpophyllum plumosum

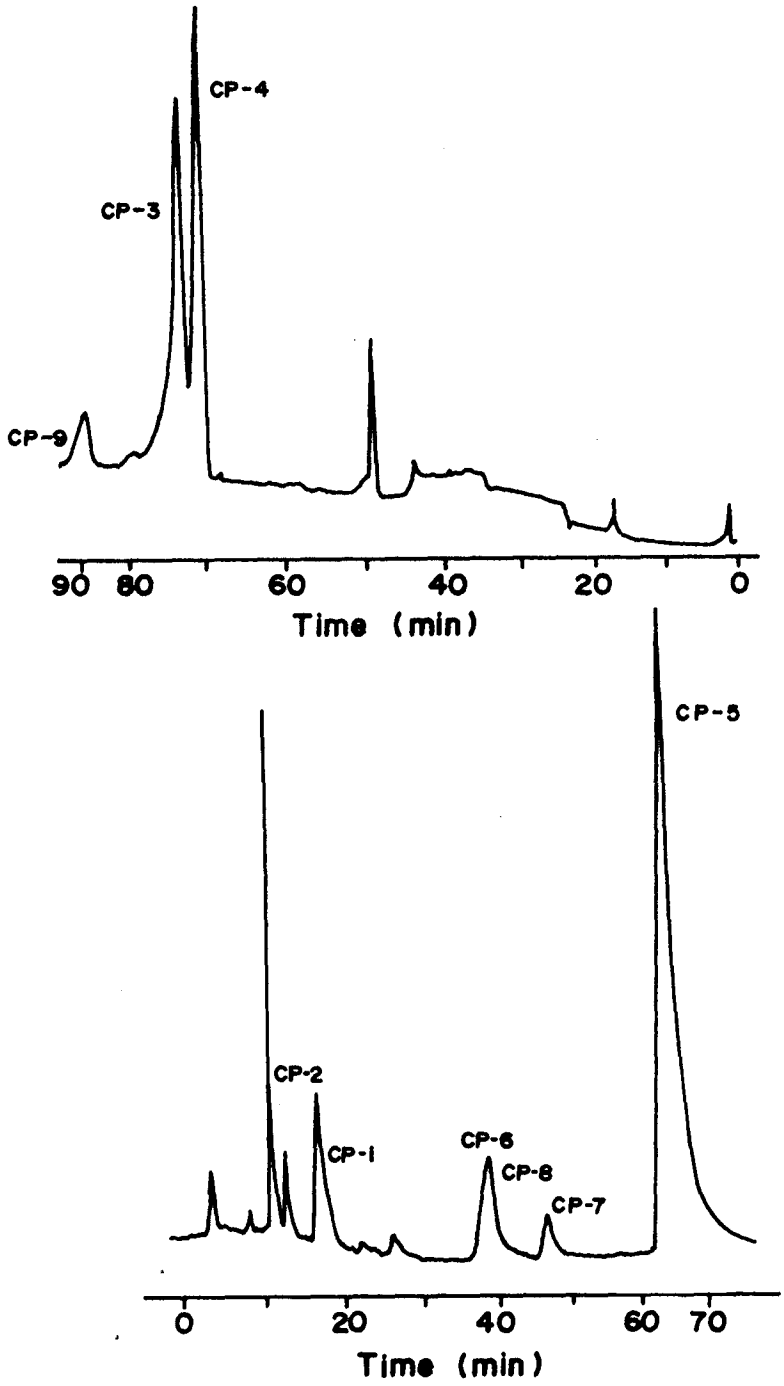


Fig. 19 HPLC chromatogram of Phlorotannins from alga Carpophyllum plumosum

CP-1: Phloroglucinol triacetate (1,3,5- triacetoxy benzol)

Molecular formula: $C_{12}H_{12}O_6$. M.P. 105-106 °C; EIMS (70 eV, 200-300 °C, Positive ions) m/z=252 $[M]^+$, 210, 168, 126; ^1H NMR ($CDCl_3$, 300 MHz) δ 6.8 (3H,s), 2.25 (9H,s).

CP-2: Monochlorophloroglucintriacetate (4-chloro-1,3,5-triacetate phloroglucinol)

Molecular formula: $C_{12}H_{11}O_6Cl$; EIMS (70 eV, 200 - 300 °C, Positive ions) m/z = 286-288 $[M]^+$, 244-246 , 202-204, 160-162; ^1H NMR ($CDCl_3$, 300 MHz) δ 6.9 (2H,s), 2.34 (6H,s), 2.28 (3H,s).

CP-3 Difucol hexaacetate (2,4,6, 2',4',6'- hexaacetoxy biphenyl)

Molecular formula:$C_{24}H_{22}O_{12}$; M. P.=177-178 °C; FABMS(Xe gun,3-nitrobenzylalcohol as matrix, M+1 positive ions) m/z 502 $[M]^+$ 460, 418, 376, 334, 292, 250; ^1H NMR ($CDCl_3$, 500 MHz) δ 6.99 (4H,s), 2.02 (12H,s), 2.25 (6H,s).

CP-4 Bifuhalol hexaacetate (2,4,6, 3',4',5'-hexaacetoxy diphenyl ether)

Mol. formula: C_{24} H_{22} O_{13}. M.P. 186-188 °C; FABMS(Xe gun,3-nitrobenzylalcohol as matrix, M+1 positive ions) m/z 518 $[M]^+$ 476, 434, 392, 350, 308, 266; ^1H NMR ($CDCl_3$, 500 MHz) δ 6.9 (2H,s) 6.7 (2H,s), 2.28 (3H,s), 2.25 (3H,s), 2.07 (6H,s), 2.24 (6H,s).

CP-5:Diphlorethol pentaacetate (2,4,6,3',5'- pentaacetoxydiphenyl ether)

Molecular formula: $C_{22}H_{20}O_{11}$; M.P. 121-123 °C; EIMS (70 eV, 200 - 300 °C, Positive ions) m/z 460 $[M]^+$ 418, 376, 334, 292, 250; ^1H NMR ($CDCl_3$, 300 MHz) δ 6.95 (2H,s), AB_2 system 6.63/6.57 (J=2.1 Hz), 2.06 (6H,s), 2.23 (6H,s), 2.26 (3H,s).

CP-6 : 3[A] Bromodiphlorethol penta acetate.

Molecular formula: $C_{22}H_{19}O_{11}Br$; FABMS(Xe gun,3-nitrobenzylalcohol as matrix , M+1 positive ions) m/z 538-540 $[M]^+$, 496-498, 454-456, 412-414, 370-372, 328-330; ^1H NMR ($CDCl_3$, 500 MHz) δ 7.04 (1H,s), 6.58 (2H,d), 6.66 (1H,dd), 2.35 (3H,s), 2.19 (3H,s), 2.04 (3H,s), 2.26 (6H,s). (J=2.0Hz)

CP-7: 2[B] Bromodiphlorethol pentaacetate

Mol. Formula and FABMS : Similar to CP-6. ^1H NMR ($CDCl_3$, 500 MHz) δ 6.9 (2H,s), 6.72 (1H,d), 6.45 (1H,d), 2.27 (3H,s), 2.11 (6H,s), 2.35 (3H,s), 2.21 (3H,s).

CP-8: 4[B] Chloro-2,4,6,3',5' pentaacetoxydiphenyl ether.

Molecular formula: $C_{22}H_{19}O_{11}Cl$. FABMS(Xe gun,3-nitrobenzylalcohol as matrix, M+1 positive ions) m/z 494-496 $[M]^+$) 452-454, 410-412, 368-370.

Molecular formula: C_{22} H_{19} O_{11} Cl. FABMS(Xe gun,3-nitrobenzylalcohol as matrix, M+1 positive ions) m/z 494-496 $[M]^+$, 452-454, 410-412, 368-370, 326-328, 284-286; ^{1}H NMR ($CDCl_3$, 500 MHz) δ 6.95 (2H,s), 6.68 (2H,s), 2.09 (6H,s), 2.29 (3H,s), 2.32 (6H,s).

CP-9:Triphlorethol-A-heptaacetate (1,5-diacetoxy-2-(3,5-diacetoxyphenoxy) 5- (2,4,6-triacetoxyphenoxy)benzol.

Molecular formula: C_{32} H_{28} O_{16}. M.P. 138-139 °C; FABMS(Xe gun,3-nitrobenzylalcohol as matrix, M+1 positive ions) m/z 668 $[M]^+$, 626, 584, 542, 500, 458, 416, 374; ^{1}H NMR ($CDCl_3$, 500 MHz) δ 6.95 (2H,s), 2.13 (6H,s), 2.28 (3H,s), 6.70 (2H,s), 2.06 (6H,s), AB_2 system 6.65, (1H,m) /6.55 (2H,d) J=2.0 Hz; 2.24 (6H,s).

CP-10: Triphlorethol-B-heptaacetate (1,5-diacetoxy-2-(3,5 diacetoxyphenoxy) -3-(2,4,6-triacetoxyphenoxy) benzol.

Molecular formula and FABMS: similar to CP-9. ^{1}H NMR ($CDCl_3$, 500 MHz) δ 6.92 (2H,s), 2.26 (3H,s), 2.02 (6H,s) AB system 6.52 (1H,d)/ 6.74 (1H,d), 2.13 (3H,s), 2.22 (3H,s) AB_2 system 6.67 (1H,m)/6.55 (2H,d), J=2.0 Hz, 2.23 (6H,s).

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Chapter 4

Lipid constituents of the sponge *Suberites carnosus*

Sponges, grouped under the subkingdom Parazoa with its phylum Porifera are well distributed in both intertidal and subtidal including deeper zones of ocean. Sponges have no triploblastic or diploblastic system. They are devoid of mouth, intestine and nervous system. There is cellular democracy among cells, as they can change their position, function and structure as per necessity. The minute organisms carried by water currents are used as food by them. It has been shown that various kind of microorganisms are associated with sponges. It is also possible that some of constituents reported from sponges might have come from these micro organisms.

In early sixties only major sterols from invertebrates were isolated and identified. Many animals were reported to contain new sterols. Some trivial names were given to these sterols such as magakesterol, shakosterol meritristerol, most probably named after source or place of collection. During those days workers were dealing with complex mixtures, because, crystallization was the only method available for purification. Development of improved separation methods and modern spectroscopic techniques have now made possible the purification and structure determination of even minor sterols from their complex mixture. Natural sterols are derived by a series of chemical transformations from two parent triterpenes, lanosterol and cycloartenol. These terpenoids in turn are biosynthesized through mevalonic acid pathway.

In the animal kingdom the sponges are known to contain variety of sterols. Earlier studies of Henz and Doree revealed that sponges contained sterols other than cholesterol, though the stucture of cholesterol was not known. This interest was maintained by Heilbron with his work on marine algal sterols. Investigations of Bergmann in 1962 are the basis for present day studies of marine invertebrate sterols. Marine sponge *Axinella canabina* yielded several compounds related to $C_{27\text{-}29}$, Δ^7, $\Delta^{8(9)}$ and Δ^5 sterols and sterol peroxides.[1] Sheikh and Djerassi isolated a series of 5α, 8α sterol peroxides from *Tethya aurantia*.[2] There are some reports on the secondary metabolites of genus *Suberites*. Ethanolic extract of *Suberites inconstans* had cytotoxic effect on HeLa cells.[3] Isolation and purification of suberitine, a toxic protien from *Suberites domuncula* was reported by Cariello L in 1980.[4] Blue carotene has been reported from *Suberites domuncula*.[5] A new carotenoid isorenieradieinstine was isolated from sponge *Suberites Sericeus*.[6] Several sterols have been reported from the sponge *Aaptos aaptos*, *Suberites domuncula* and *Suberites carnosus*,[7] which include 24-methyl, 24-ethyl cholesterol and 5-cholestane etc. There are also reports on isolation of C_{26} to C_{30} sterols and minor amount of cholest 7-en-3β-ol and 24-ethyl cholestanol from these sponges.

Marine sponge *Suberites carnosus* was collected from the intertidal region of Malvan (Maharastra). This sponge belongs to order *Hadromerida* and family *Suberitidae*. White colour sponges could be recognized by specules seen on the surface and body covered with mud. Methanolic extract exhibited cytotoxic, diuretic and antihistaminic activities during study on its pharmacological properties. Sponges were thoroughly washed with water and immediately soaked in methanol. Solvent was decanted off, filtered and concentrated under vacuum. The lipid solubles were extracted with pet.ether and chloroform. Chemical investigation of chloroform fraction of the sponge yielded sterols, (22E)-24R-methyl-cholest-4, 8(9), 22 (23) - trien-3α -7β -diol **(1),** (22E) 24R- methyl- ergost-6, 22(23)-dien-5α - 8α -epidioxy- 3β-ol **(2),** (22E)-ergost-6, 22(23), 24(28)-trien-5α -8α- epidioxy - 3β -ol **(3)** along with ergosterol **(4),** an ester (**5**), and fattyacid **(6)** .

EI mass spectrum of **compound (1)** showed molecular weight to be 412 indicating the molecular formula C_{28} H_{44} O_2. Other fragment ions appeared at m/z 394 (C_{28} H_{42} O) and 376 (C_{28} H_{40}) corresponding to two successive loss of 18 mass units from molecular ion peak, which indicated the loss of two hydroxyl groups from the molecule. ^{1}H NMR spectrum of compound (1) was found to be similar to that of cholesterol. Signals at δ 0.85 (3H, d, J=6.8Hz) and 0.83 (3H, d, J=6.8Hz) assigned

to H-26/27 protons. Two signals appeared at δ 0.61 (3H,s) and 1.12 (3H,s) corresponding to H-18 and H-19 methyl protons respectively. Furthermore, two signals observed at δ 0.91 (3H,d, J = 6.6 Hz) and 1.01 (3H, d, J = 6.6 Hz) were assigned to H-28 and H-21 methyl protons respectively. The stereochemistry at C-24 methyl group was established by comparison of ^{1}H NMR data of similar compound, which indicated 24 (R) configuration.[8] Signal at δ 3.5 (1H,m) was quite narrow as would be expected for H-3 proton. Two signal at δ 4.09 (1H, m) and δ 5.19 (2H,m) were assigned to H-7 and $\Delta^{22(23)}$ protons respectively. The absence of additional olefenic proton in ^{1}H NMR spectrum limited the possible position of the third double bond to $\Delta^{8(9)}$ or $\Delta^{8(14)}$. On the basis of mass fragmentation pattern the third double bond was placed at C- 8(9) position (Fig-2). IR spectrum of compound exhibited bands at 3382, 1034, 1658 and 970 cm^{-1} indicating the presence of hydroxyl group and carbon-carbon double bond in the molecule. The structure of compound **(1)** was established as (22E)-24R- methyl - cholest-4, 8(9), 22 (23) - trien-3α -7β-diol (Fig-1).

EI mass spectrum of **compound (2)** showed molecular weight to be 428, fragment ions were observed at m/z 410, due to loss of H_2O. Other peaks at m/z 271 253 and 211 indicated that compound was a cholesterol derivative. Furthermore, ion at 396 indicated the loss of 32 mass units from molecular ion peak, possibly due to loss of oxygen

molecule from the compound. ^{1}H NMR spectrum (Fig-3) of compound (2) showed signals at δ 0.81 (3H,s) and 0.88 (3H,s), these were attributed to H-18 and H-19 methyl protons. Four signals at δ 0.83 (3H, d, J = 6.8 Hz), 0.82 (3H, d, J = 6.8Hz), 0.92 (3H,d J = 6.4 Hz) and 1.01 (3H, d, J = 6.4 Hz) were assigned to H-26, H-27, H-28 and H-21 secondary methyl protons respectively. Signals exhibiting characteristic eight line pattern centered at δ 5.18 (2H, m) was assigned for H-22 and H-23 protons with J = 7.5 Hz (coupling H-22/20 and H-23/24) and J = 15Hz (coupling H-22/23) indicating 22(23) a trans double bond. The ^{1}H NMR signals at δ 6.24 (1H, d, J = 8.5 Hz) and 6.5(1H, d, J=8.5 Hz) were assigned to H-6 and H-7 respectively. The downfield shift of the H-3 carbinol proton at δ 3.96 as compared to ergosterol (δ 3.6) indicated the presence of more electronegative group in the vicinity of C-3 position. The stereochemistry of peroxide and methyl group were assigned on the basis of ^{1}H NMR data reported in literature for similar compounds.[13] IR spectrum of compound displayed characteristic bands at 3525 (OH group), 968 (C=C) and 1180 cm^{-1} (peroxide group). Compound (2) was identified as (22E) 24R-Methyl- ergost-6, 22(23)-dien-5α-8α -epidioxy 3β-ol (fig-1). The structure was established by comparison of the spectral data of compound with reported values in the literature for similar molecules.[9-11] Further evidence for this structure was provided by its ^{13}C NMR data, which showed signals at δ 135.4, 135.2, 132.3 and 130.7. These signals were assigned to

unsaturated carbon C-22 and C-23, C-6 and C-7 respectively. C-3 signal was observed at δ 66.4, whereas, signals at δ [illegible] and [illegible] were assigned to C-5 and C-8.

Stereochemistry of 24-methyl group in compound **(1)** and **(2)** was established after comparison of chemical shift of H-21, H-22-23, H-26, H-27, H-28 of Δ^E, 24α and Δ^E, 24β steroids (Table-1)[12]

Table-1: ^{1}H NMR chemical shift of Δ^E, 24α and Δ^E, 24β steroids, compound (1) and (2).

H-No	Δ^E, 24β	Δ^E, 24α	(1)	(2)
21-H	1.02	1.00	1.01	1.01
22,23-H	5.21	5.16	5.19	5.18
26-H	0.85	0.84	0.85	0.83
27-H	0.83	0.82	0.83	0.82
28-H	0.92	0.91	0.91	0.92

Compound (2) has previously been detected in some sponges,[13-14] tunicates,[15-16] fungi,[17-18] and lichens.[13] However , peroxide sterols are not reported from the sponge of *Suberites* sp.

EI mass spectrum of compound **(3)** showed molecular weight to be 426, which is 2 mass units less than Compound (2), possibly due to presence of additional double bond in the molecule. Fragment ion at m/z 394 (426-O_2) indicated loss of oxygen molecule. ^{1}H NMR spectrum showed singlets at δ 4.69 and 4.71 indicating exomethylene protons. The signals at δ 6.3 and 6.6 were assigned to H-6 and H-7 protons. Signal at δ 1.2 (3H,s) was assigned to H-21 methyl protons. Compound (3) was identified as (22E)-ergost-6, 22(23), 24(28)-trien-5α - 8α - epidioxy - 3β-ol (Fig-1). The mass spectrum of compound also indicated trace amount of ergosterol **(4)** as evidenced by presence of fragment ions at m/z 159, 158, 157 and 143 which are typical of $\Delta^{5,7}$ ring structure in sterols.[19-20]

Literature survey indicated that steroids 24-methyl-5-cholest-22-ene-3β-ol, cholesterol, 24-melthyl-5 -cholestane-3β-ol, 24-methyl-cholest-5-ene-3β-ol, 24-ethyl cholest-5-ene-3-ol and 24-methylidene cholesterol were isolated from the sponge *Suberites carnosus*.[21-22] Substitution at C-24 seems to be predominant in sterols of family *Suberitidae*.[23]

EI mass spectrum of compound **(5)** showed molecular weight to be 312, analysed for $C_{20}H_{40}O_2$. Base peak appeared at m/z 74, which is characteristic of straight chain methyl esters and arises by Mc Lafferty rearrangement. Other fragments of the general formula $[(CH_2)_n COOCH_3]^+$ indicated by m/z 87, 101, 129, 143, 157, 171, 185, 199, 213, 227, 270 and 298. High resolution ^1H NMR spectrum showed a signal at δ 0.89 (3H, t, J = 9.0 Hz) indicating a methyl group. Another signal at δ 1.28 (32H, brs) was assigned to 16 methylene units in the molecule. A two proton multiplet centered at δ 1.63 was assigned to protons in the acid portion of the molecule, whereas, a singlet at δ 3.68 (3H) for methyl group in alcohol portion. IR spectrum showed band at 1745 cm^{-1}. In the view of above evidences, compound (5) was characterised as methyl nonadecanoate.

Molecular formula of the compound **(6)** was assigned as $C_{19}H_{38}O_2$ based on EI mass spectrum, which showed molecular ion peak at m/z 298. IR spectrum of compound was characteristic of carboxylic acid with absorption at 3000-2851, 1710, 1435, 1290 and 940 cm^{-1}. A uniform difference of 14 mass units in fragmentation pattern of the molecule indicated the presence of long aliphatic chain. The absence of $[M-15]^+$ ion peak indicated a straight chain skeleton. Compound (6) was characterised as nonadecanoic acid. Its methyl ester was found to be identical with compound (5) in all respect.

Experimental

The sponge *Suberites carnosus*, was collected in the month of March from the intertidal region of Malvan, West coast of India. Animals were stored in MeOH and transported to laboratory. The solvent was decanted after a week and the process of extraction repeated twice. The decants were concentrated under reduced pressure yielding dark brown residue. This concentrated extract was suspended in 20% aq. MeOH and successively extracted with $CHCl_3$ and n-butanol. The residue of $CHCl_3$ fraction (7 gms) was chromatographed over silica gel column using gradient pet.ether-ethyl acetate mixture as eluent yielding fractions 1,2,3. Repeated silica gel column chromatography of Fraction 1 gave compound **(5)** whereas compound **(2)**, **(4)** & **(6)** were obtained from fraction 2. Compound **(1)** was isolated from fraction 3.

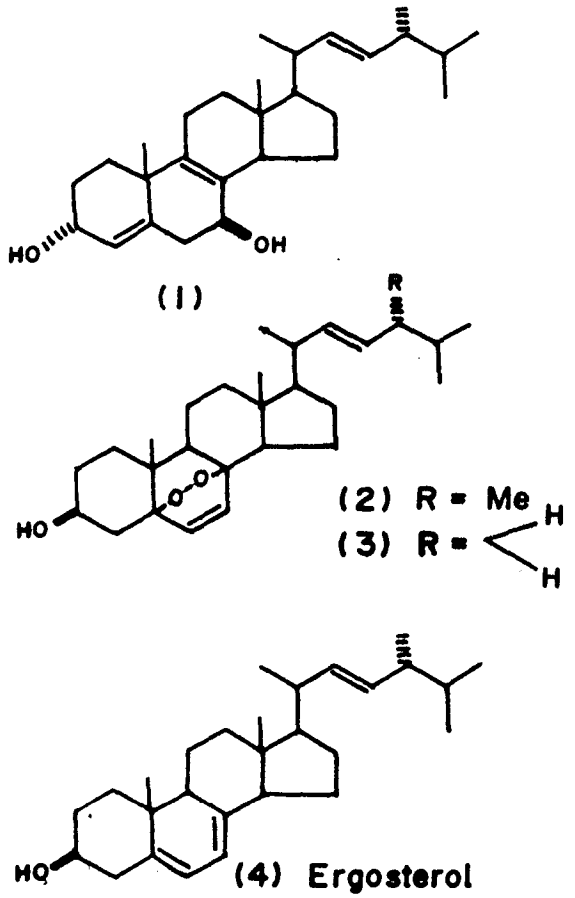
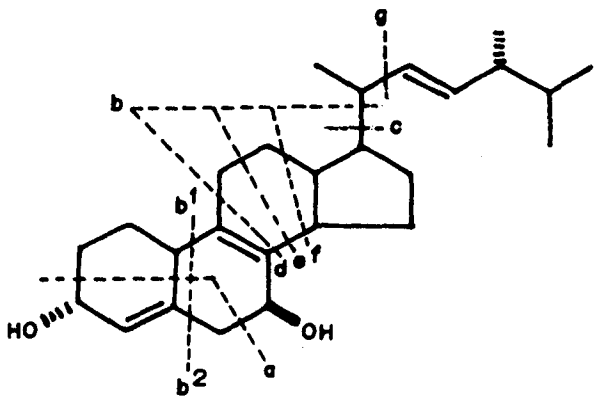


Fig. 1 Sterols isolated from the sponge Suberites carnosus



m/e	Probable structure
412	M^+
394	$M^+ - H_2O$
376	$M^+ - 2H_2O$
315	g
287	c (M^+ – SC)
109	d
95	e
81	f
69	a
55	b_1 & b_2

Fig. 2 EIMS fragmentation pattern of Compound (1)

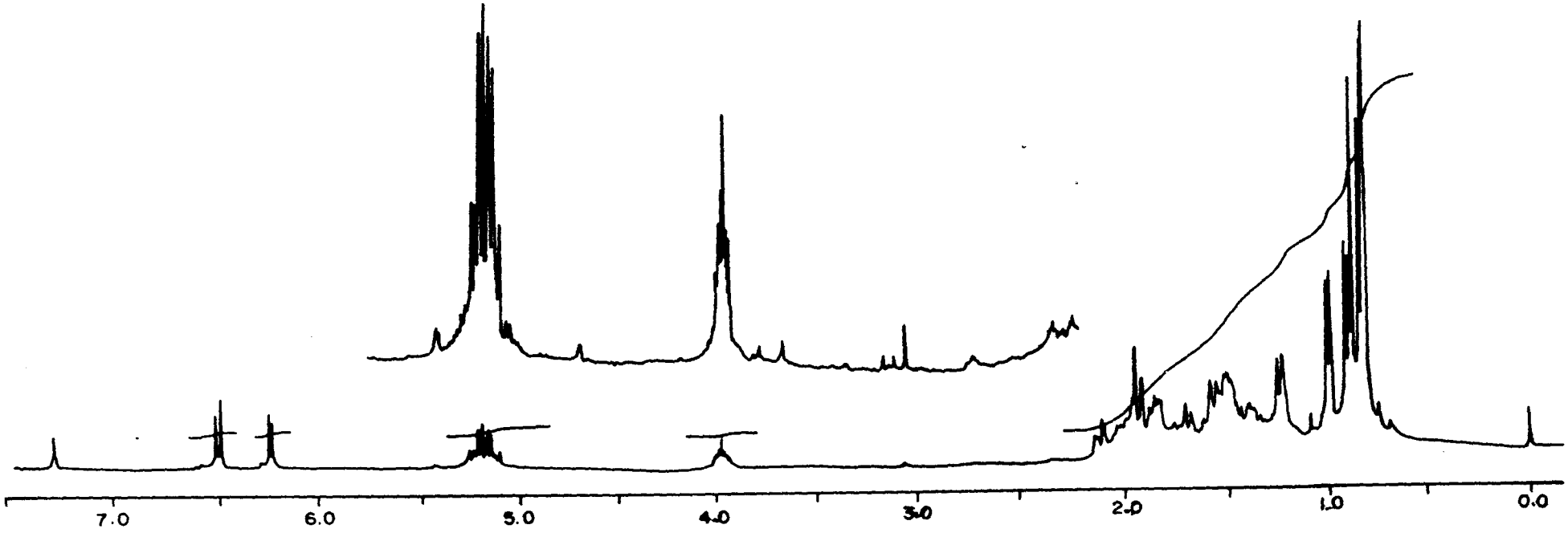


Fig. 3 ^{1}HNMR specrum of Compound (2)

(22E)-24R-methyl-cholest-4, 8(9), 22(23)-trien-3α-7β-diol (1).

Solid (5 mg); M.P. 155-157°C; IR (KBr, cm^{-1}) : 3382, 2953, 2871, 1658, 1456, 1380, 1237, 1161, 1034, 970 and 868 cm^{-1} ; ^1H NMR (400 MHz, $CDCl_3$) : δ 0.61 (3H, s, H-18), 0.85 (3H, d, J=6.8 Hz, H- 26-27), 0.83 (3H, d, J=6.8 Hz, H-27-26), 0.91 (3H, d, J=6.6 Hz, H-28), 1.01(3H, d, J=6.6Hz, H-21), 3.5(1H, t, H-3), 4.09 (1H, m, H-7), 5.19 (2H,m, H-22/23), 5.35 (1H, d, H-4); EIMS: M/z 412 $[M]^+$, 394 $[M-H_2O]^+$, 376 $[M-H_2O]^+$, 287$[M-SC]^+$, 269$[M- (SC + H_2O]^+$, 251 $[M-(SC+2H_2O]^+$.

(22E) 24R- Methyl-ergost-6, 22(23)-dien-5α-8α-epidioxy 3β-ol (2).

Solid (15 mg); M.P. 157-158 °C; IR (KBr, cm^{-1}) : 3525, 2872, 2955, 1460, 1380, 1180, 1074, 1043, 1028, 968, 935, 858, 779 and 275 cm^{-1} . ^1H NMR (400 MHz CDCl3): δ 0.81(3H, s, H-18), 0.88 (3H, s, H-19), 0.83 (3H, d, J=6.8 Hz, H-26-27), 0.82 (3H, d, J=6.8 Hz, H-27-26), 0.92 (3H, d, J=6.4Hz, H-28), 1.01 (3H,d,J=6.4Hz, H-21), 3.96 (1H, m, H-3), 5.18 (2H,m, H-22-23), 6.24 (1H, d, J = 8.5Hz, H-6), 6.5 (1H, d, J = 8.5Hz, H-7), ^{13}C NMR ($CDCl_3$) : δ 135.4, 135.2, 132.37, 130.75, 66.4, 56.3, 51.7, 51.2, 44.59, 42.8, 39.6, 39.4, 37.0, 34.76, 33.09, 30.17, 28.57, 25.33, 23.42, 20.88, 20.65, 19.91, 19.62, 18.16, 17.55, 12.87 and 12.33; EIMS : M/z 428 $[M]^+$, 410 $[M- H_2O]^+$, 303 $[M-SC (C_9H_{17})]^+$, 285 $[M-(SC+H_2O]^+$,

271 [M- (SC + O_2)$^+$, 253 [M-(SC + H_2O + O_2)]$^+$, 211 [253-ring D fusion (42)]$^+$.

(22E) 24-Methylene-ergost-6, 22(23), 24(28)-trien-5α-8α-epidioxy-3β-ol (3).

Solid (10 mg); M.P. 160-162 °C; IR (KBr, cm^{-1}) : 3428, 2752, 2953, 1460, 1380, 1170, 1076 cm^{-1}. ^{1}H NMR (400 MHz CDCl3): δ 0.80(3H, s, H-18), 0.89 (3H, s, H-19), 0.86 (3H, d, J=6.8 Hz, H-26-27), 0.87 (3H, d, J=6.8 Hz, H-27-26), 4.69 and 4.71 (1H each, s, H-28). 1.20 (3H,d,J=6.4Hz, H-21), 3.94 (1H, m, H-3), 5.2 (2H, m, H-22/23), 6.3 (1H, d, J = 8.5Hz, H-6), 6.6 (1H, d, J = 8.5Hz, H-7). EIMS: m/z 426, 271, 253, 211.

Methyl**Nonadecanoate (5).**

liquid, IR (KBr, cm^{-1}) : 2941, 2850, 1745, 1460, 1435, 1363, 1248, 1146, and 1170 cm^{-1}; ^{1}H NMR (CDCl3) : δ 0.89(3H, t, J = 9.0Hz), 1.28 (32H, brs, -CH2 groups), 1.63(2H, m), 3.68(3H, s). EIMS : M/z 312 [M]$^+$, 298, 270, 227, 213, 199, 185, 171, 157, 143, 129, 101, 87 and 74 (base peak).

Nonadecanoic acid (6).

Solid, m.p. 125-127 °C. IR (KBr, cm-1) : 3000, 2851, 1710, 1460, 1435, 1412, 1375, 1290, 940 and 721 CM^{-1}. EIMS : M/z 298, 284, 270, 256, 242, 228, 213, 199, 185, 171, 157, 143, 129, 115, 111, 97, 83, 73, 65, 55 and 44.

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Chapter 5

Chemical constituents of the sponge *Suberites vestigium*

In continuation of studies, on the secondary metabolites of marine organisms of Indian ocean region, black sponge *Suberites vestigium* was collected from Mandapam coast in South India by SCUBA diving. This sponge belongs to order *Hadromerida* and family *Suberitidae*. The methanolic extract exhibited *in vitro* anti histaminic activity. In view of above activity it was decided to carry out chemical investigation of this sponge. Literature survey, on compounds isolated from sponge of *Suberites* sp. have been described in chapter 4, which shows that marine sponge *Suberites vestigium* has not been investigated for its chemical constituents. Crude extract was fractionated with Pet.ether, chloroform, and n-butanol. Four sterols and a heteroaromatic acid were isolated from chloroform and n-butanol fraction respectively. They are cholesterol **(1),** 24-methylene cholesterol **(2),** 24-ethyl-cholest-5,25-dien-3β-ol or clerosterol **(3),** 24-ethyl-cholest-7-en-3β-ol **(4),** batyl alcohol **(5)**, and 4-methyl pyrazole-3(5) carboxylic acid **(6)**. Structure of these compounds were finalised from their spectral data including IR, NMR, and EIMS. Fatty acids from this sponge were analysed by GCMS as their methyl esters. Fourteen major saturated and unsaturated fatty acids were identified ranging from C_{12} to C_{22}.

The molecular weight of **compound (1)** was determined to be 386. Other peaks appeared at m/z 371, 368, 353,273, 231. The molecular ion at m/z 386 and other peaks indicated cholest-5-en-3-ol nucleus.[1] Further structural evidences for compound (1) were provided by ^1H

NMR spectrum. Signals at δ 5.35 (1H, s) 3.53 (1H, m), 0.67 (3H, s) and 1.01 (3H, s) were assigned for H-6, H-3, H-18 and H-19 respectively. The signals at δ 140.86 (C-5), 121.30 (C-6), 71.87 (C-3), 11.90 (C-11) and 18.79 (C-19) in the ^{13}C NMR indicated the presence of 3β-hydroxy Δ^5-nucleus. Side chain (C_8H_{17}) was saturated as indicated by mass spectrum. IR spectrum exhibited bands at 3418, 2944, 2420, 1640, 1456, 1376, 1122 and 1042 cm^{-1}. These spectral values are identical with that of cholesterol (Fig-1).

The EIMS of **Compound (2)** showed the molecular weight to be 398, which is 12 mass units more than compound (1). ^1H NMR of compound (2) was similar to that of (1) except for two broad singlets at δ 4.62 and 4.70 assigned to exomethylene group. Characteristic fragment ion at m/z 314 [M^+-84] corresponding to a MacLafferty rearrangement ion which must have generated by either $\Lambda^{24(28)}$ or $\Lambda^{25(27)}$ double bond in the molecule.[2-4] Presence of a triplet of low intensity at m/z 299, 300 and 301 in the EIMS is characteristic of 24(28) C=C double bond.[5] These spectral values were compared with that of reported compound 24-methylene cholesterol. Thus, compound (2) was identified a 24-methylene cholesterol (Fig-1).

EIMS of **compound (3)** showed molecular weight to be 412, which is 14 mass units more than compound (2). ^1H NMR spectrum of compound (3)

showed signals at δ 3.6 (1H,m) and δ 5.35 (1H,m), these spectral values were characteristic of double bond at C-5 and 3β-hydroxy sterols.[6] Other signals were observed at δ 0.68 (3H, s) 1.02 (3H, s) assigned to H-18 and H-19 protons respectively. The ^{1}H NMR spectrum also had a signals at δ 1.62 (3H, s) attributed to H-26/27 protons. Chemical shift of H-26/27 to downfield may be due to its proximity with the double bond. Signals appeared at δ 0.85 (3H,d) and δ 0.89 (3H, t) were assigned to H-21 and H-29 methyl protons. Two broad singlets at δ 4.76 and 4.62 indicated exomethylene protons Δ^{25}. A prominent peak at m/z 314 in EIMS is consistent with a double bond in the side chain.[3-4] These results indicated that the compound **(3)** was 24-ethyl-cholest-5, 25-dien-3β-ol or clerosterol (Fig-1).[7]

EIMS of **compound (4)** showed the molecular weight to be 414, which is 2 mass units more than compound **(3)**. EIMS spectrum also indicated a saturated side chain and a double bond in the nucleus. ^{1}H NMR spectrum of the compound showed a singlet at δ 5.17 probably for a vinyl proton. The C=C double bond at C-7 position was indicated by high field resonance for the H -18 and H-19 methyl protons at δ 0.57 and δ 0.68 respectively.[8] The methyl proton signal appeared as triplet at δ 0.85 was assigned for ethyl group attached to C-24 in the side chain. Comparison of chemical shift of H-21, H-26, H-27, and H-29 proton signals with those of 24R and 24S isomers indicated the

stereochemistry of this compound is probably 24R (Table-1).[9] Structure of the **compound (4)** was established as 24R-ethyl -cholest-7-ene-3β-ol (Fig-1).

Table-1: ^{1}H NMR chemical shift of 24α & 24β-ethyl cholesterols, Compound (4).

H-No	24α-Ethyl	24β-Ethyl	(4)
21-H	0.92	0.91	0.92
26-H	0.82	0.83	0.84
27-H	0.80	0.81	-
29-H	0.85	0.84	0.85

The ^{1}H NMR spectrum of **compound (5)** showed signals at δ 0.87 (3H. t), δ 1.25 [$(CH2)_n$] and 3.4-3.9 (m). ^{13}C NMR of compound showed signals at δ 72.44. 71.84,70.49 and 64.27 for four oxygenated carbons in the molecule. Methylene protons of the aliphatic chain showed signal between δ 39.4-18.8 and a signal at δ 13.9 for the terminal methyl group. Compound (5) was identified as batyl alcohol, furthermore its higher homologue was indicated by fragmentation pattern of the mass spectrum of the mixture which showed compounds with molecular weight 344 and 358.

Compound (6) was isolated as crystalline solid, soluble in methanol and sparingly soluble in chloroform. EIMS of the compound showed its molecular weight to be 126, corresponding to the molecular formula $C_5H_6N_2O_2$. ^1H NMR spectrum of this compound (Table-2) showed signals at δ 12.2 and 13.0, which indicated -COOH and -NH proton respectively (deuterium exchangeable). A singlet at δ 7.3 (1H) indicated it to be a vinyl proton, a methyl group on sp^2 carbon was indicated by a singlet at δ 1.92. The DEPT experiment (Fig-2) also indicated the presence of one methyl, one methine and three quaternary carbons. which could be correlated with its ^{13}C NMR and ^1H NMR spectrum. COSY spectrum of compound (Fig-3) showed long range coupling between methyl and vinyl protons. IR spectrum showed bands at 3218, 3000, 2570 3062, 1648 and 1606 cm^{-1} (Fig-4) indicating the presence of corboxylic group , C=C, and N-H bond in the molecule. These data indicated that compound (6) is either imidazole or pyrazole derivative. The former one is ruled out after comparing ^1H and ^{13}C NMR spectral data with that of similar compound. Thus compound (6) was identified as 4-methyl 3(5) pyrazole carboxylic acid. 4-Methyl-3(5) -carboxylic acid was also reported as novel compound from sponge *Tedania anhelans.*[10]

Table -2: ^{1}H NMR and ^{13}C NMR spectral data of compound (6)[a].

Position	δ H	δ C	DEPT
2 -NH	12.2 (brs)		
3		153	C
4		108	C
5	7.3 (1H, s)	137	CH
6-COOH	13.0 (brs)	166	C
7	1.92 (3H, s)	12.2	CH_3

[a] 400 MHz, ^{1}H NMR and ^{13}C NMR, Pyridine-d_5 referenced to solvent signals at δ 7.2 and δ 8.7 ppm.

Pet.ether fraction of the sponge was refluxed with methanolic KOH for 2-3 hours. The residue after removal of solvent was dissolved in water and extracted with hexane. The aqueous solution was acidified with cold dilute HCl. The liberated fatty acids were extracted with ether, dried in vacuum and converted to their methyl ester by treating with diazomethane. These methyl ester derivatives were subjected to GC-MS analysis (Table-3).

Table-3: Fatty acid composition of marine sponge *Suberites vestigium* analysed by GCMS as their methyl esters.

GC-MS ((Rt., min.)	Structure	$[M]^+$	% Composition
31.90	12:1	198	1.4
33.66	12:0	200	10.6
34. 30	13:1	212	1.3
34.46	13:0	214	4.1
35.76	14:0	228	3.4
35.90	15:0	242	16.1
36.80	14:1	226	11.0
38.06	15:1	240	4.5
38.23	16:1	254	3.3
38.43	16:3	250	1.1
39.60	16:0	256	0.8
40.53	17:1	268	1.0
41.26	17:0	270	8.0
41.46	17:5	260	0.9
42..66	17:1	268	21.0
42.96	19:3	292	2.0
43.40	19:4	290	1.2
43.70	18:3	278	3.4

Besides serving as an efficient energy storage, many of fatty acids are useful for normal physiological functioning and proper growth of the organisms. It is well known for many years that saturated fatty acids raise the blood cholesterol level. Unsaturated fatty acids, not only lower the blood cholesterol level, but also ensure a more favorable ratio of HDL (high density lipoproteins) to LDL (low density lipoproteins). Unsaturated fatty acids lower blood cholesterol level by regulating cholesterol synthesis as well as its elimination from the body. Some of these, especially arachidonic acid and homolinolenic acid also get converted in *in vivo* into useful prostaglandins.

The marine sponges have also furnished several saturated, unsaturated, methoxy and methyl branched, as well as phospholipid bound fatty acids. Several fatty acid derived metabolites of sponges exhibit high order of biological activities.[11-12] Acanthifolicin, an episulphide containing acid from sponge *Pandaros acanthifolium* exhibit high order of activity. Eleven straight chain unsaturated poly acetylenic bromoacids from the sponge *Spongia hispida* were shown to inhibit HIV protease, a critical enzyme in the replication of human immuno deficiency virus.[13] The origin of various fatty acids in sponges are probably via *de novo*, biosynthesis, dietary intake, incorporation from symbionts with or without further modifications.

Experimental

The sponge samples were collected from Mandapam coast in South India by SCUBA diving. Animals were extracted with methanol. The solvent was drained off and concentrated under reduced pressure. Methanolic extract was suspended in 20% aq. methanol and extracted with organic solvents i.e. pet.ether, chloroform, and n-butanol. Fatty acid composition of sponge was analysed from pet-ether fraction. The chloroform fraction was chromatographed on silica gel column using gradient of ethyl acetate- pet-ether system, yielding fractions rich in sterols. These fractions were monitored on TLC using 5-10% ethyl acetate in pet.ether, spots were visualised by iodine vapour. Individual sterols were purified using HPLC, (Spectra physics, SP-8800, ODS, 250 X 8 mm, RI, MeOH, 1ml/min) which yielded sterols **1-4**. Silica gel column chromatography of more polar fraction resulted in isolation of a fraction rich in glyceryl ethers **(5)**.

Butanol solubles were chromatgraphed over XAD-2 resin and eluted with water methanol mixture in different proportion followed by repeated column chromatigraphy over silica gel and eluted with chloroform-methanol (8:2) which yielded a white coloured crystalline compound **(6)**.

GC-MS data were obtained on a Shimadzu QP-2000 instrument at 70 eV and 250 °C. GC column ULBON HR-1 equivalent to OV-1, fused silica capillary 0.25mm 50 M with film thickness -0.25 micron. Initial temperature was 100 °C for 6 minutes and then heated at the rate of 10 °C per minute to 250 °C. Carrier gas helium, flow 2 ml /min.

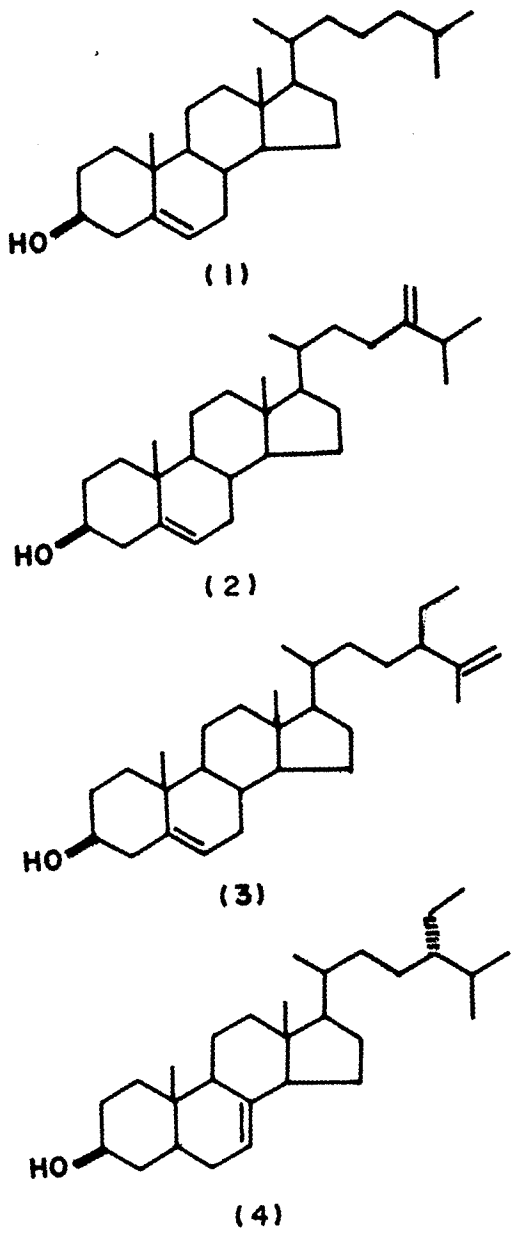

Fig. 1 Sterols isolated from the sponge Suberites vestigium

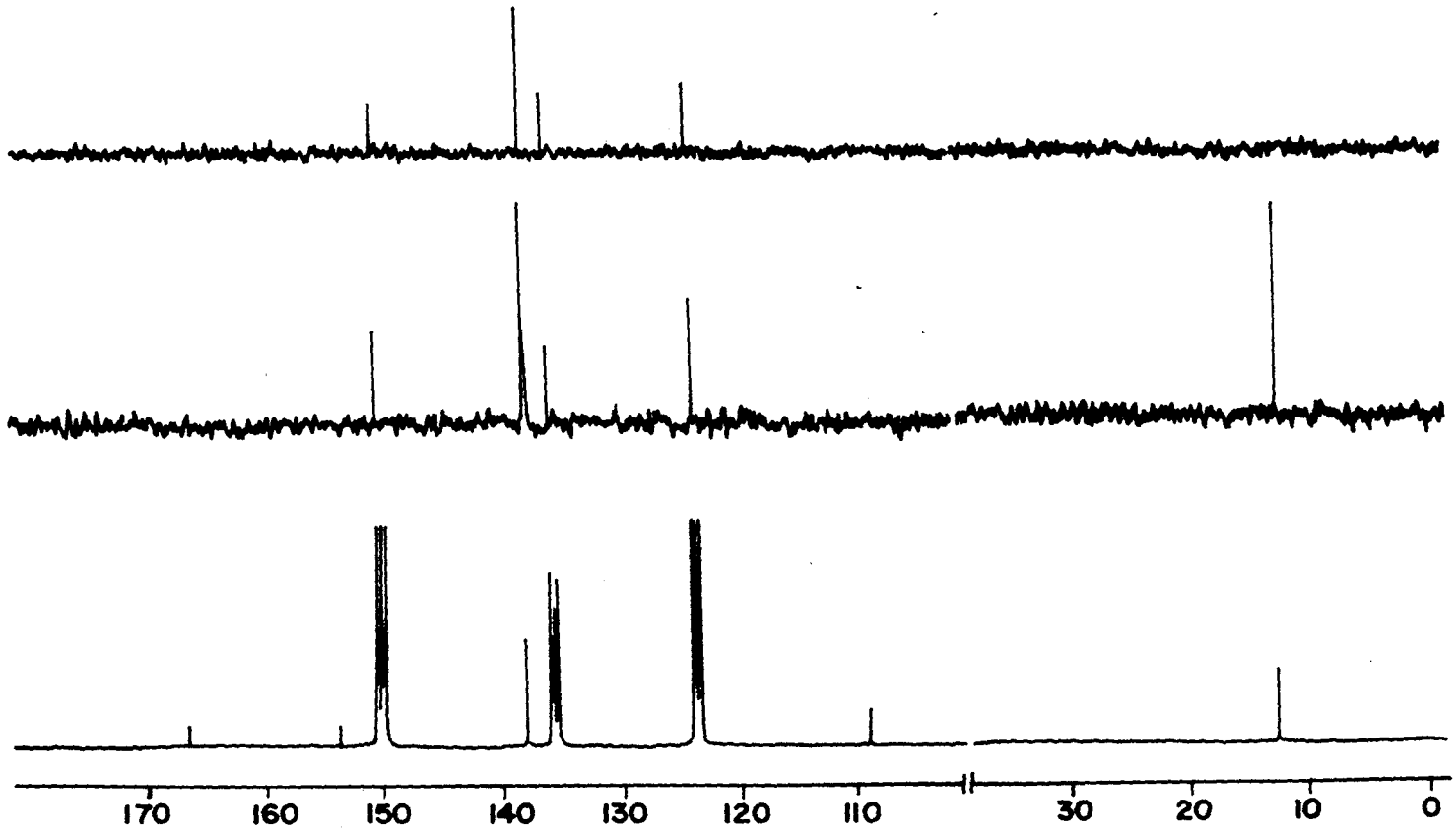

Fig. 2 ^{13}CNMR spectrum of 4 - Methyl - 3(5) Pyrazole Carboxylic acid (6)

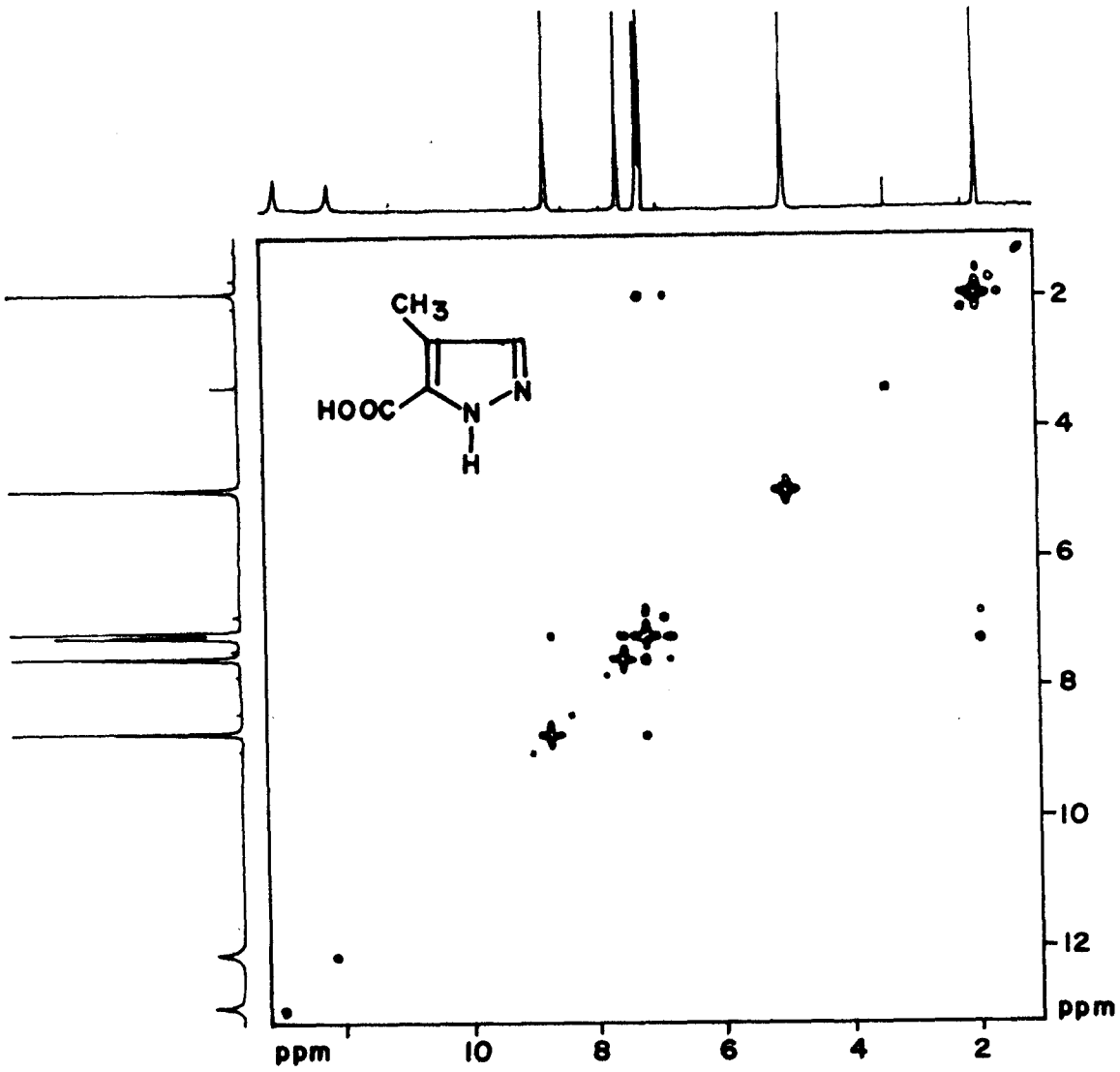

Fig. 3 COSY spectrum of
4 - Methyl - 3(5) Pyrazole Carboxylic acid (6)

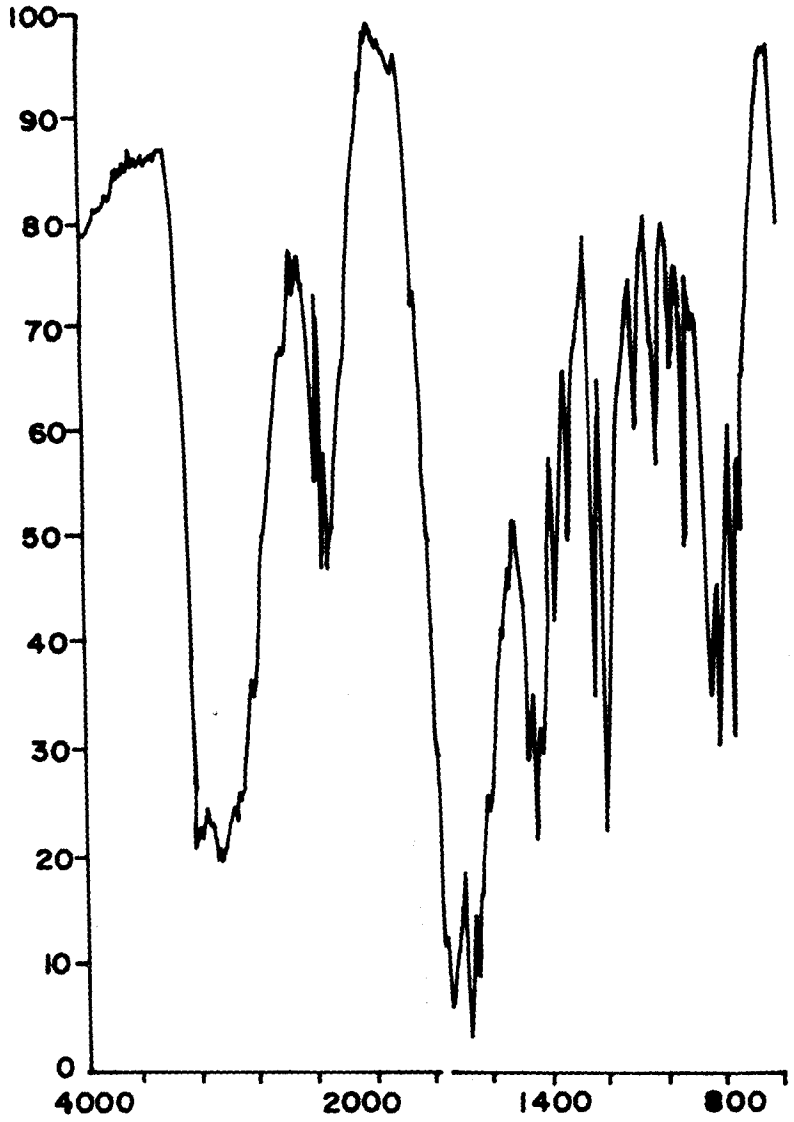

Fig. 4 IR (KBr) spectrum of
4 - Methyl - 3(5) Pyrazole Carboxylic acid (6)

Cholesterol (1).

^{1}H NMR ($CDCl_3$) : δ 5.35 (1H,brd, 6-H), 3.5 (m, 3-H), 1.01 (3H,s,19-H), 0.91 (3H, d, 21-H), 0.85 (6H, d, 26-27-H) and 0.67 (3H, s, 18-H). EIMS: m/z (Int.): 386 $[M]^+$, 371 $[M-15]^+$, 368 $[M-H_2O]^+$ (14.0), 353 $[M-H2O-CH_3]^+$ (11.8), 301 $[M-C_6H_{13}]^+$ (15), 273 $[M-SC]^+$ (10.4), 271 $[M-SC-2H]^+$ (11.9), 255 $[M-SC-H_2O]^+$ (22), 231 $[M-SC-C_3H_6]^+$ (12.4), 213 $[M-SC-C_3H_6-H_2O]^+$ (18.9). IR: 3418, 2944, 2420, 1640,1456,1376, 1122 and 1042 cm^{-1}.

24-methylene-cholesterol (2).

^{1}H NMR ($CDCl_3$): δ 5.36 (1H,brd, 6-H), 3.5 (m, 3-H), 1.01 (3h,s,19-H), 0.90 (3H, d, 21-H), 0.84 (6H, d, 26-27-H) and 0.68 (3H, s, 18-H), 4.7 and 4.6 (brs, 2H, 28-H). EIMS: 398 $[M]^+$, 314, 273, 255,231 etc.

24-Ethyl-cholest-5, 25-dien-3β-ol (3).

^{1}H NMR ($CDCl_3$): δ 5.35 (1H,brd, 6-H), 3.6 (m, 3-H), 1.02 (3H,s,19-H), 0.85 (3H, d, 21-H), 1.62(3H,s, 26-27-H) and 0.89 (3H, t, 29-H), 0.68

(3H,s, 18-H), 4.7 and 4.6 (brs, 26/27-H). EIMS: m/z 412 $[M]^+$ 329 $[M\text{-}C_6H_{11}]^+$ 287 $[M\text{- }SC]^+$, 298 [M- part of side chain $C_8H_{15}]^+$, 314 [M-part of the side chain $C_7H_{14}]^+$, 271 [M- side chain +2H]$^+$.

24-ethyl -cholest-7-ene-3β-ol (4).

^{1}H NMR ($CDCl_3$) δ 3.5 (m, 3-H), 5.17 (brs, 1H, 7-H), 0.68 (3H,s,19-H), 0.92 (3H, d, 21-H), 0.84 (6H, d, 26/27-H) and 0.57 (3H, s, 18-H) 0.85 (3H, t, 29-H) . EIMS : 414 $[M]^+$.

4-methyl-pyrazole-3(5) carboxylic acid (5).

Molecular formula: $C_5H_6N_2O_2$, Crystalline solid (10-12 mg). M. P. 300-302 °C, ^{1}H NMR and ^{13}CN MR (Table-1). EIMS: m/z 126 $[M]^+$,125, 110, 98, 83 and 55. IR (KBr pellet): 3218, 3000-2570, 3062,1648, 1606 cm^{-1}.

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Chapter 6

Chemical constituents of the sponge *Chrotella australiensis*.

The sponge *Chrotella australiensis* belongs to phylum *Porifera* and order *Hadromerida*. This sponge is yellow coloured and round in shape. It was collected from the intertidal region of Anjuna beach, Goa. Methanolic extract of this sponge exhibited antiviral activity during pharmacological screening of marine organisms. It prompted to investigate this sponge for its chemical constituents. Literature survey indicates that sponge *Chrotella australiensis* has not been investigated for its chemical constituents. Crude extract was partitioned against pet-ether, chloroform and n-butanol. Chloroform fraction yielded two ketosteroids (**1-2**) and three common sterols (**3-5**), whereas. n-butanol fraction yielded a nucleoside (**6**). Fatty acid constituents from pet-ether fraction were analysed by GCMS. Ten saturated and unsaturated fatty acids were identified (Table-2).

EIMS spectrum of **compound(1)** showed the molecular weight to be 384, which is two mass units less than cholesterol. ^{1}H NMR spectrum (Fig-3), showed chemical shifts for H-18, H-21, H-26/27, which are similar to that of cholesterol. However, signals at δ 5.75 (1H, s) and δ 1.19 (3H, s) indicated its structure to be cholest 4-en-3-one[1]. Downfield signal at δ 1.19 was assigned to H-19. Base peak in EIMS appeared at m/z 124 (B ring cleavage), which is assigned for Δ^1 and Δ^4-3-ones and has its origin in the greater specificity of charge stabilisation in the conjugated chromophore. The most abundant ion m/z 124 in the mass

spectrum is generated by the same bond rupture as the m/z 122 ion of Δ^1 isomer but differs in that two hydrogen atoms, which are gained by the charged fragment (Fig-4).[1] ^{13}C NMR spectrum of compound showed signals at δ 198.8, 170.9, and 123,6 suggesting a ketone group and unsaturation in the compound. A ketone group was further supported by absorptions at 1680, 1620 and 1273 cm^{-1} in IR spectrum (Fig-2). These data established the structure of compound (1) as cholest- 4-ene-3-one (Fig-1).

EIMS of **compound (2)** showed molecular ion peak at m/z 412, which is 28 mass units more than compound (1), probably due to additional ethyl group present in the side chain. Other important fragments were at m/z 124, 149 and 229 indicating the presence of 4-ene-3-one moiety in the molecule. ^1H NMR spectrum was similar to that of compound (1). The additional peak at δ 0.85 (3H, t, J=7.4) is attributed to that of an ethyl group , presumably at C-24. Comparison of chemical shifts of H-21, H-26, H-27, and H-29 signals of this compound with those of 24(R) and 24 (S) isomers suggested that stereochemistry of this compound is 24(R) (Chapter 5, Table-1). Compound (2) was identified as 24(R) -ethyl-cholest-4-ene-3-one (Fig-1).[2]

EIMS of **Compound (3)** showed molecular ion peak at m/z 398, which is 12 mass units more than cholesterol. ^{1}H NMR spectrum showed signals at δ 5.3 (1H,m) and two broad singlet at δ 4.76 and 4.62, these signals were assigned for H-6 and exomethylene group respectively. EIMS of compound showed a triplet at m/z 299, 300 and 301, which is characteristic of 24 (28) double bond. Spectral data of this compound was found to be identical with 24-methylene cholesterol isolated from sponge *Suberites vestigium* (Chapter 5).

EIMS of **compound (4)** revealed its molecular weight to be 384, which is two mass units less than cholesterol, indicating loss of two hydrogen atoms. The fragment ions at 273 $[M\text{-}Sc]^+$, 366 $[M\text{-}H_2O]^+$, 351 $[M\text{-}H_2O\text{-}CH_3]^+$ indicated the presence of hydroxy group and C_8H_{15} side chain in the molecule. These data in conjunction with the ^{1}H NMR data [signals at δ 5.2 (2H,m), 3.6 (1H,m) and 0.70 (3H,s) for 22-23-H, H-3, and H-18 respectively] suggested that compound (4) was cholest-5,22-dien-3β-ol (Fig-1).

EIMS of **Compound (5)** showed molecular weight to be 412, which is 28 mass units more than compound (4). ^{1}H NMR spectrum showed signal at δ 0.85 (3H,t), which was assigned to ethyl group presumably attached to C-24.[5] Other signals at δ 5.12 (m, 2H), 3.6(1H, m) and 0.71 (3H, s) were assigned to 22-23-H , H-3, and H-18 respectively. From these

results the structure of this compound was finalised as 24-ethyl cholest-5,22 dien-3β-ol (Fig-1).[3]

4-ene-3- one steroids occur rarely in nature. They were first reported from sponge *Stelleta clarella* along with other sterols.[5] Subsequently Kokke *et.al* isolated three such compounds from dinoflagellate *Procystis lunula* [6] and Parameswaran *et.al* reported similar compounds from sponge *Ircinia ramosa*.[4] 4-en-3-one steroids have also been reported from the red alga *Gracilaria textori*. The co-occurrence of 4-ene-3-keto steroids and corresponding 5-ene-3-hydroxy sterols is of particular significance because it suggests that the sponge *Chrotella australiensis* contains 3-hydroxy dehydrogenase and Δ^4 isomerase, which are essential for conversion of sterols to steroidal hormones.

Compound **(6)** was isolated as white amorphous solid by repeated chromatography over silica column FAB mass revealed its molecular weight to be 267 (Fig-7), corresponding to the molecular formula C_{10} H_{13} N_5 O_4 indicating the compound to be a nucleoside . The fragment ion at m/z 135 (70 %) and 132 could be from adenine and pentose sugar moiety respectively. ^{13}C NMR and DEPT spectra (Fig-6) of compound (6) revealed the presence of ten carbon atoms, which indicated one methylene, six methines and three quaternary carbons in the molecule. ^1H NMR spectrum revealed presence of 6 protons, characteristic of C_5

sugar moiety between δ 5.8-3.6. ^{1}H-^{1}H COSY spectrum helped in assigning these coupled protons. Besides, the ^{1}H NMR spectrum had three more downfield signals. Among these, the broad signal at δ 7.33 (2H, brs) was assigned to NH_2 group of the purine. The other two protons signals at δ 8.3 (1H, s) and 8.1 (1H,s) were assigned to that of H-2 and H-8 of purine group.[7] Chemical shift of the anomeric carbon of the sugar moiety at δ 87.8 (d) in ^{13}C NMR spectrum indicated it to be as N-β glycoside.[7] The other carbons of sugar were found at δ 85.8 (d), 73.4 (d), 70.6 (d) and 61.6 (t). These were very much in agreement with those of N-β-riboside. Interpretation of ^{1}H NMR (Table-1) and COSY spectrum (Fig-5) in DMSO-d_6 further indicated the presence of the molecule of two sub structures. A ribose molecule was evident by analysis of the COSY spectrum, which showed a contiguously coupled spin system from H-1' through H-5'. This is first report of nucleoside molecule from sponge *Chrotella australiensis*. Earlier, Searle and Molinski had reported isolation of spongosine, which is 2-methoxy analog of this compound and its 2-deoxy derivative from an Australian sponge belonging to order Hadromerida. From these results, the structure of this compound was finalised as 9β-D-ribofuranosyl adenine.

Table-1: ^{1}H NMR and ^{13}C NMR data of compound (6).[a]

Position	δ H	δ C/DEPT
2	8.3 (1H,s)	152.0(CH)
4		149.3(C)
5		119.3(C)
6		156.1(C)
6-NH_2	7.33 (2H br s)	
8	8.1(1H s)	139.8(CH)
1'	5.8 (1H d)	87.8(CH)
2'	4.60 (1H m)	73.4(CH)
3'	4.12 (1H m)	70.6(CH)
4'	3.95 (1H m)	85.8(CH)
5'	3.64 (2H,m)	61.6(CH_2)

[a] 300 MHz, ^{1}H and ^{13}C NMR, DMSO-d_6 referenced to solvent signals at δ 2.49 and 39.5 ppm.

Nucleosides are common components of the polymeric nucleic acids and known to be present in marine organisms. Several purine

nucleosides have been isolated from marine sponges. For examples, Adenosine has been isolated from marine sponge *Dasychaline cyathine,* [8] its transport, formation and interaction in different tissues, and its involvement in the pathophysiology of the renal changes observed in various types of renal insufficiency has been discussed.[9-11] Isoguanosine was isolated from marine nudibranch mollusk *Dialulula sandiegenes.*[11] An unusual marine nucleoside isolated from marine nudibranch mollusk *Doris veruucosa*[12] was characterised as 9-[5'-deoxy-5-(methylthio)-B-D-xylofuranosyl]adenosine. Several analogues of this nucleoside were developed and evaluated for biological activity.[13] Arsenic containing nucleoside include 5'-deoxy-5'dimethyl arsinyl adenosine from giant clam *Tridacna maxima.*[14] The arsenic containing compounds are probably formed in marine organisms by reduction and oxidative methylation of absorbed oceanic arsenate in two stages by reduction to dimethyl arsenic acid followed by oxidative adenosylation to give the nucleoside.[15]

Pet-ether fraction was processed as described in Chapter 5 to obtain methyl ester of fatty acids. This ester mixture was analysed by GCMS (Table-2).

Table-2: Fatty acid constituents of marine sponge *Chrotella australiensis* analysed by GCMS as their methyl esters.

GC- MS (Rt., min.)	Structure	$[M]^+$	% Composition
31.83	12:1	198	3.1
33.13	13:1	212	12.6
33.33	13:0	214	1.8
33.53	17:0	270	1.1
34.13	16:0	256	1.0
35:60		-	2.0
35.96	-	-	8.6
36.53	17:1	268	22.0
37.40	18:0	284	6.4
37.80	-	-	5.4
38.00	-	-	4.2
38.53	-	-	1.0
40.3	-	-	4.8
40.5	19:1	296	8.4
41.3	19:0	298	8.6
42.56	-	-	1.1
43.33	20:0	312	8.0

Experimental

Sponge samples were collected from Anjuna (Goa), west coast of India, stored in methanol and transported to laboratory. The solvent was drained off and concentrated under reduced pressure, this process was repeated three times. The crude methanolic extract was suspended in 20 % aq. Methanol and extracted successively with chloroform, ethyl acetate and n-butanol. The chloroform fraction was chromatographed over silica gel and eluted with gradient mixture of ethyl acetate in petroleum ether, which yielded steroid mixture. The above mixture was purified on HPLC (Spectra physics, SP-8800, ODS, 250-8 mm, RI, MeOH, 1ml/min).

Butanol fraction was chromatographed over silica gel (mesh 60-120) column and eluted with increasing concentration of methanol in chloroform. Fractions of 50 ml each were collected and monitored on thin layer chromatography using the solvent system 2-propanol:ammonia:water (9:.5:.5). The TLC spots were visualised by UV light and iodine. Fractions eluted in range of 15-20 % methanol in chloroform showed UV active spots on TLC. These fractions were mixed together and purified on silica gel, after removal of solvent. The mixture was triturated in methanol and applied on sephadex LH-20 column. The column was eluted with methanol. An amorphous, white solid was obtained from 2-3 fractions. This was finally purified on preparative TLC, developing with 2-propanol: ammonia :water (9:.5:.5) to give 10 mg

of **compound (6).** Pet-ether fraction was processed as discussed in chapter 5 to obtain methyl esters of fatty acids. These derivatives were analysed by GCMS on Shimadzu QP-2000 instrument.

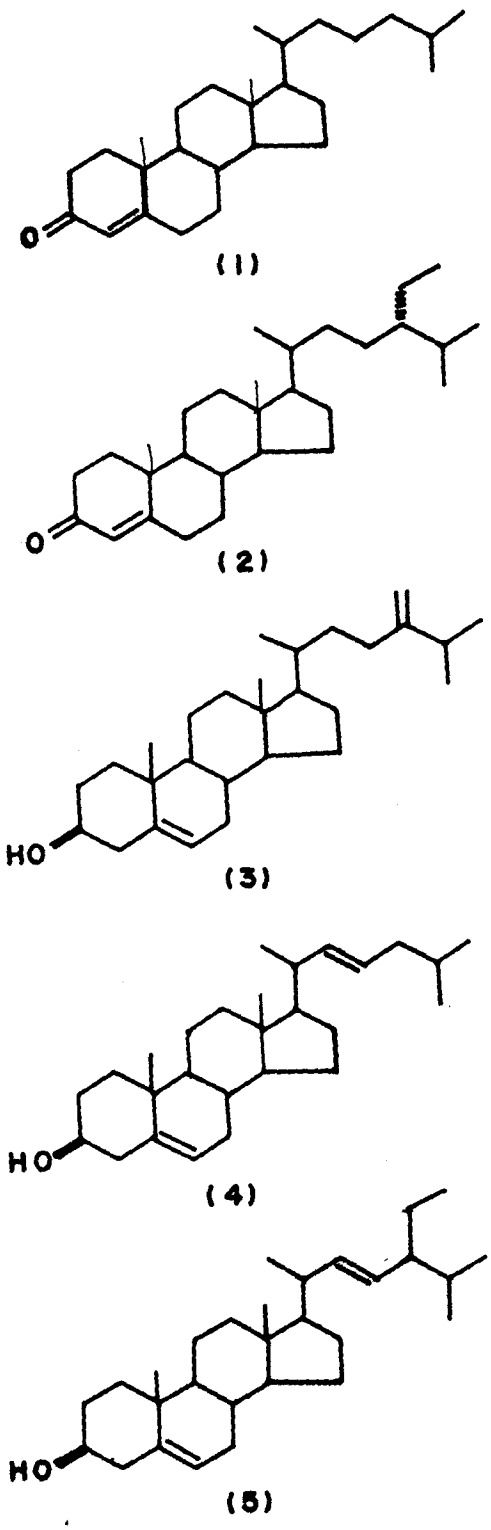


Fig. 1 Steroids isolated from the sponge Chrotella australiensis

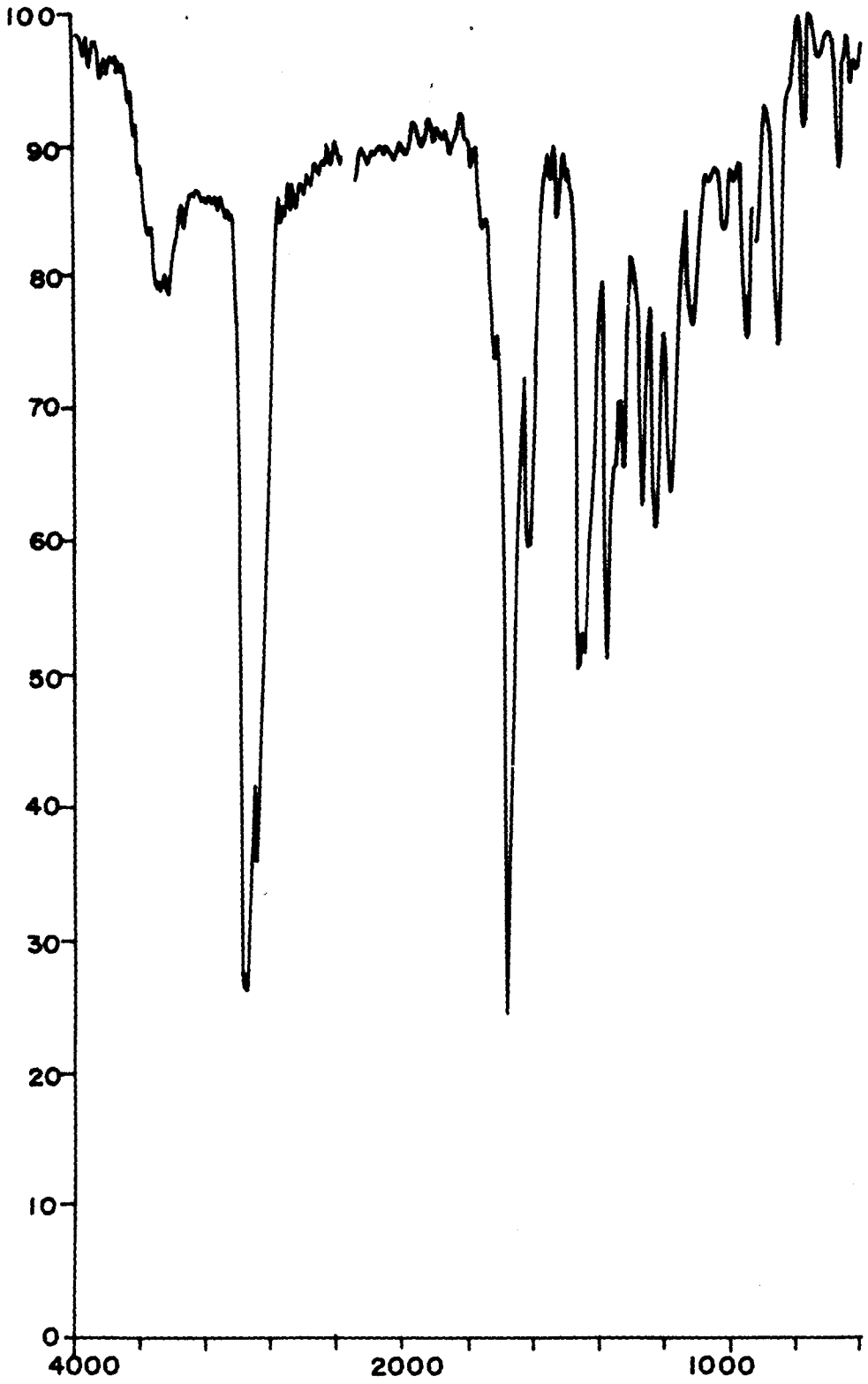


Fig. 2 IR (K Br) spectrum of Compound (1)

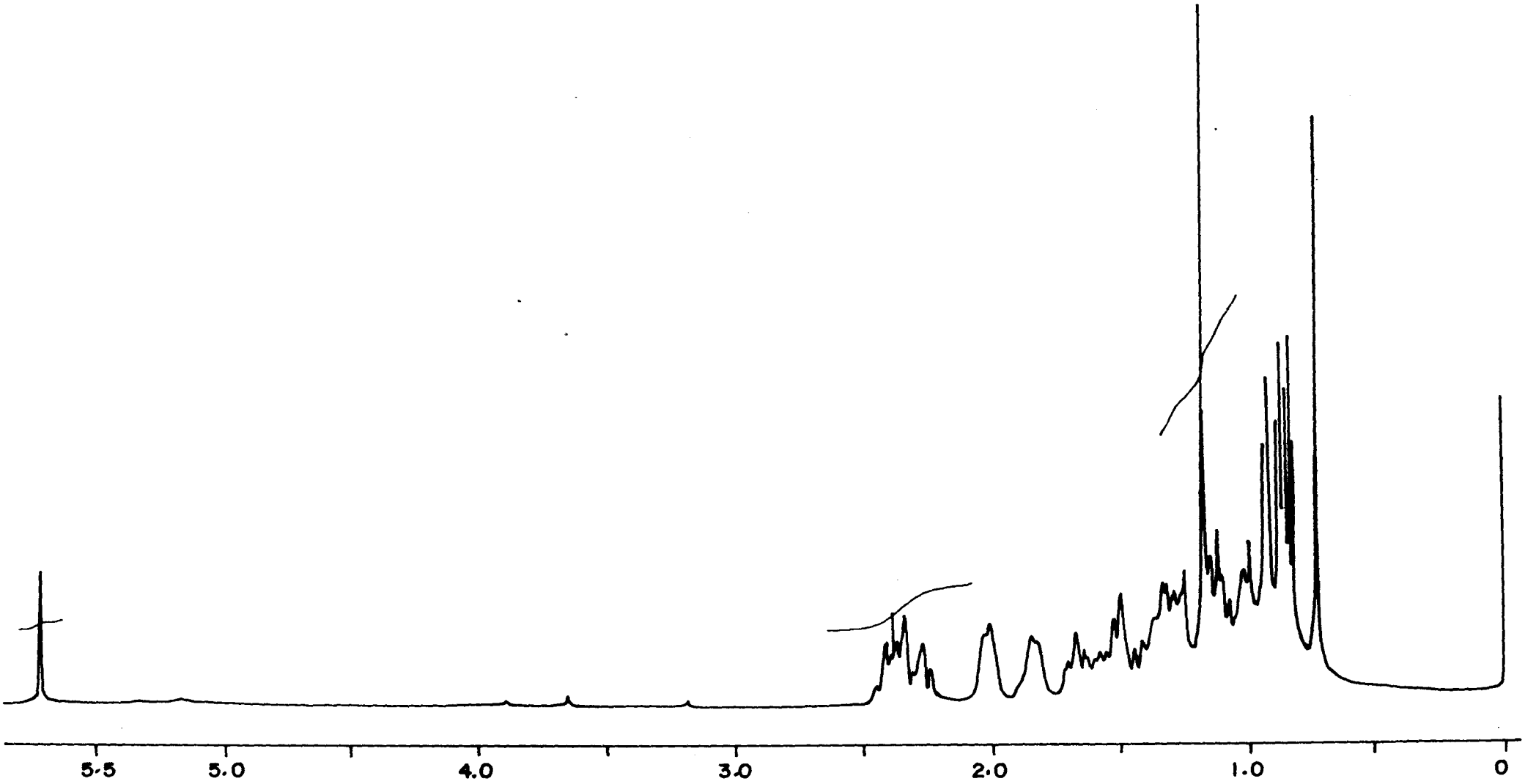


Fig. 3 ^{1}HNMR spectrum of Compound (1)

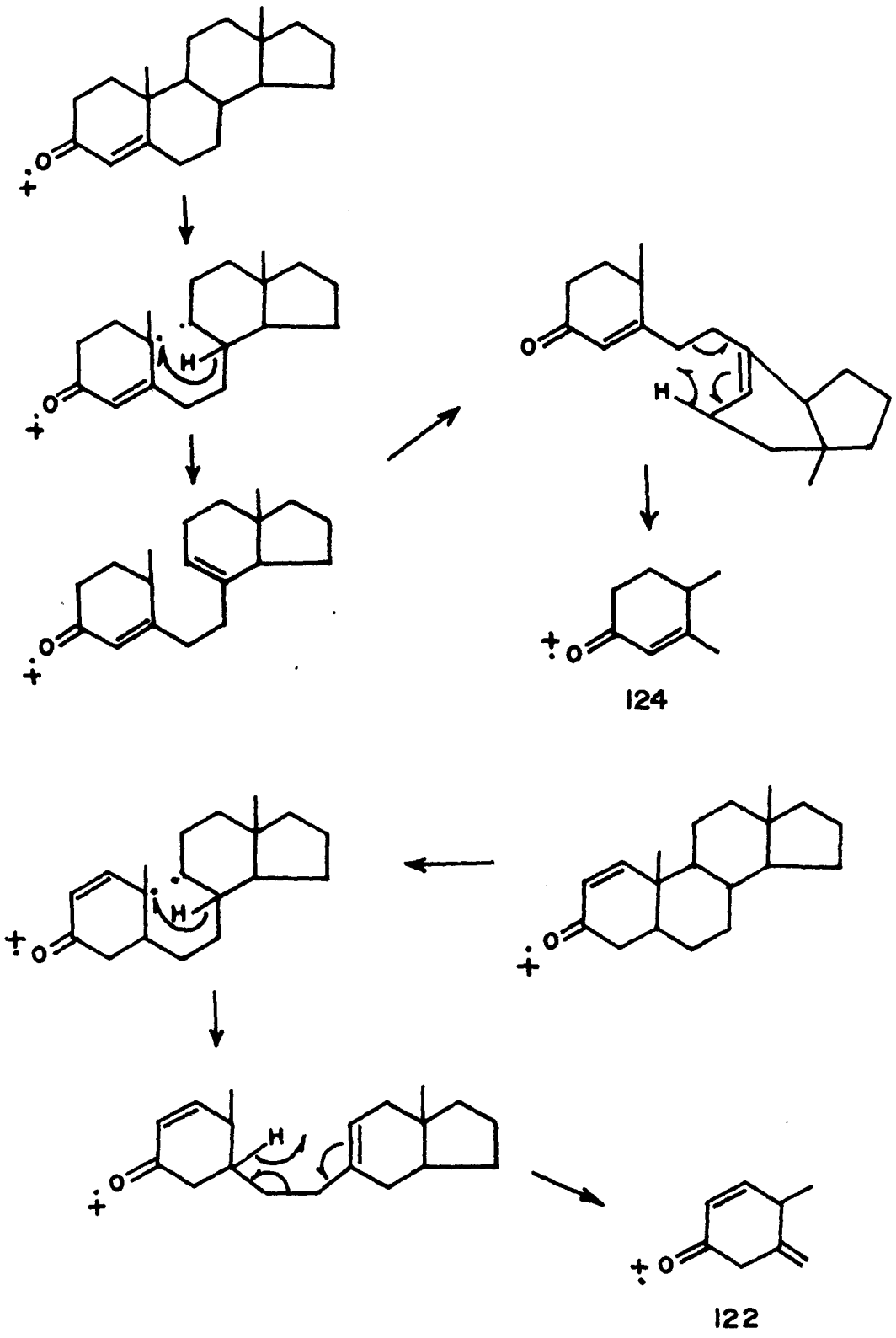
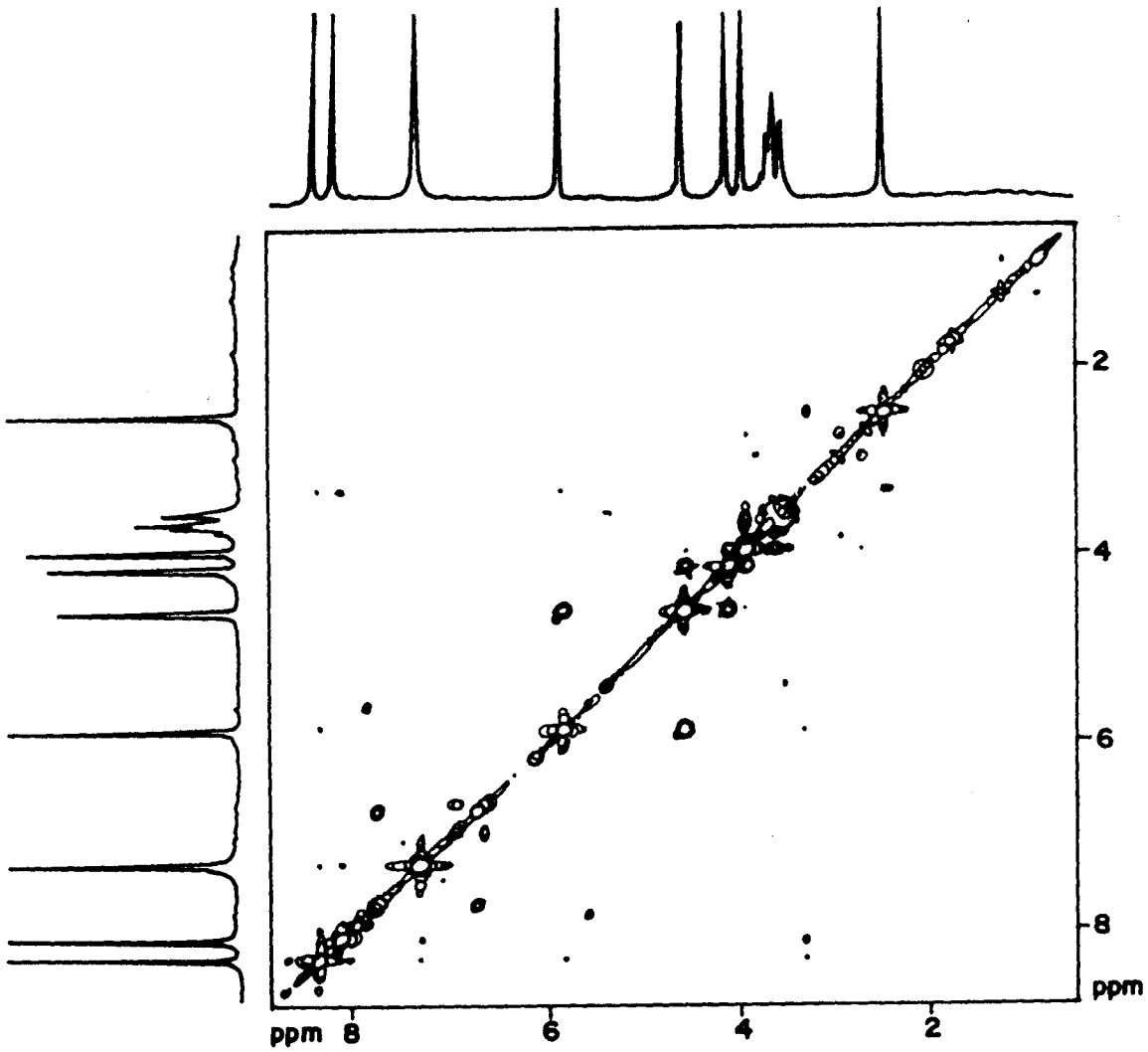


Fig. 4 Mass fragmentation pattern of
Cholest-4-ene-3-one & cholest-1-ene-3-one
type nucleus



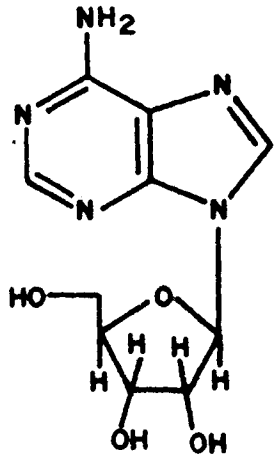


Fig. 5 COSY spectrum of 9β-D-ribofuranosyladenine

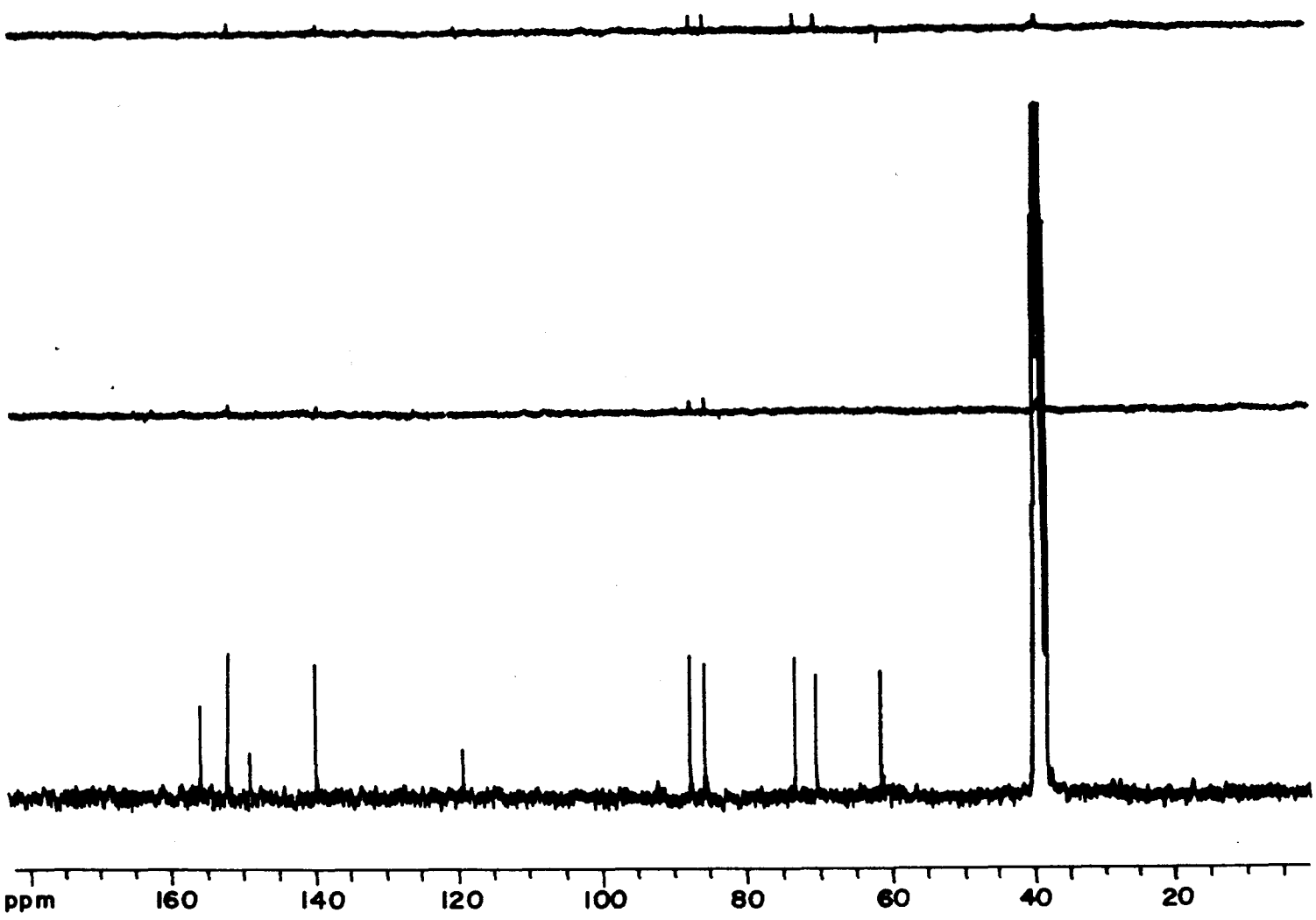


Fig. 6 ^{13}CNMR spectrum of Compound (6)

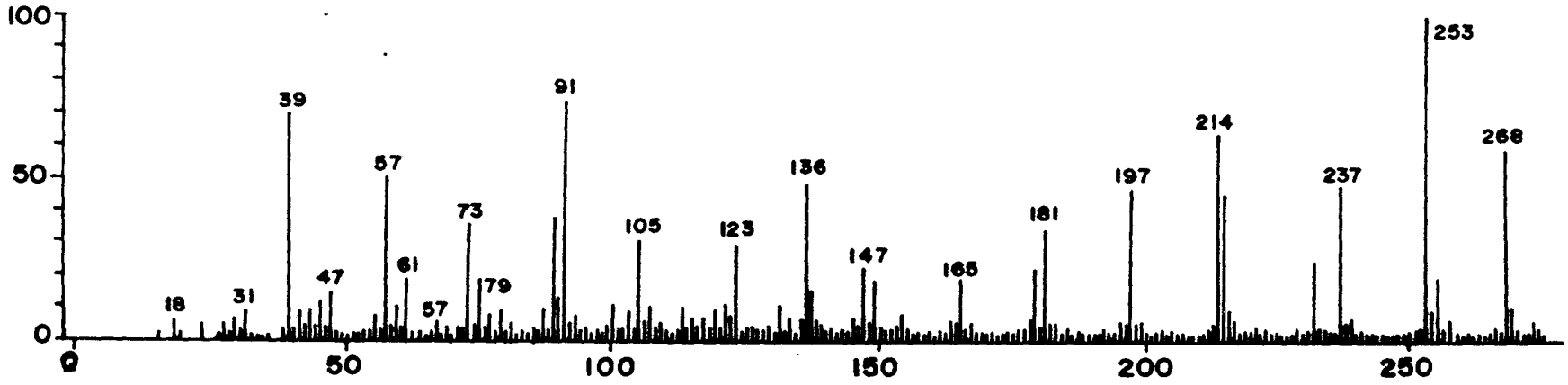


Fig. 7 FABMS of Compound (6)

Cholest-4-en-3-one (1).

^{1}H NMR ($CDCl_3$): δ 5.75 (1H, s, 4-H), 1.19 (3H, s, 19-H), 0.90 (3H, d, 21-H), 0.85 (6H, d, J=6.4, 26-27-H), 0.71(3H, s, 18-H). EIMS: 384 $[M]^+$, 271 $[M\text{-}SC]^+$, 229 $[M\text{-}SC\text{-}Ketene]^+$, 187 $[M\text{-}SC\text{-}84(C_5H_8O)]^+$ 149 (cleavage at ring B), 124. IR (KBr): 2930, 2905, 2840, 1680, 1620 and 1273 cm^{-1}.

24-ethyl-cholest-4-en-3-one (2).

^{1}H NMR ($CDCl_3$): δ 5.75 (1H, s, 4-H), 1.18 (3H, s, 19-H), 0.91 (3H, d, 21-H), 0.82 (6H, d, J=6.8, 26-27-H), 0.71(3H, s, 18-H), 0.85 (3H, t, J=7.4). EIMS: 412, 271, 229 ,149, 124 etc.

24-Methylene-cholesterol (3).

^{1}H NMR ($CDCl_3$): δ 5.35 (1H,brd, 6-H), 3.45(m, 3-H), 1.01 (3H,s,19-H), 0.89 (3H, d, 21-H), 0.84 (6H, d, 26-27-H) and 0.68 (3H, s, 18-H), 4.5 and 4.6 (brs, 2H, 28-H). EIMS: 396, 299, 300, 30, 272, 229, 149 etc.

Cholest-5,22-dien-3β-ol (4).

^{1}H NMR ($CDCl_3$): δ 5.12 (2H, m, 22-23-H), 5.2 (1H, brs, 6-H) 3.6 (1H,m, 3-H), 1.01 (3H,s,19-H), 0.91 (3H, d, 21-H), 0.85 (6H, d, 26/27-H) and 0.70 (3H, s, 18-H). EIMS: 384 $[M]^+$, 366, 371, 353,273, 231 etc.

24-Ethyl-cholest-5,22(23) dien-3β-ol (5).

^{1}H NMR ($CDCl_3$): δ 5.30 (1H, brd, 6-H), 3.6 (1H,m, 3-H), 1.02 (3H,s,19-H), 0.98 (3H d, 21-H), 0.85 (3H, t, 29-H) 0.81 (6H, m, 26/27-H) 0.71 (3H,s,18-H), 5.2 (2H, m, 22/23-H). EIMS: 412, 399, 396, 327, 299, 272, etc.

9β-D-ribofuranosyl adenine (6).

Molecular formula: C_{10} H_{13} N_5 O_4, white amorphous solid, M. P. 170-172 °C, UV (MeOH): λmax 254 (ε 2800), ^{1}H NMR and ^{13}C NMR (Table-1). FABMS: m/z 267 $[M]^+$, 252, 236, 213, 196, 178, 164 , 135, 132.

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Summary

This thesis has been divided in six chapters. Chapter 1 is devoted to give an introduction to marine natural products chemistry. In chapter 2, efforts have been made for extensive literature survey of bioactive as well as novel organic compounds isolated from marine organisms. These compounds are classified in different groups such as, nucleosides, steroids, terpenoids, toxins, polyphenols etc.

Chapter 3 deals with isolation of phlorotannins from brown alga *Carpophyllum plumosum*, low molecular weight phlorotannins obtained from ethyl acetate fraction, yielded 10 halogenated and non halogenated phloroglucinol derivatives. Structure of these compounds were identified based on spectral studies.

In chapter 4, lipid constituents of marine sponge *Suberites carnosus* has been discussed. Spectral studies of a new sterol and two sterol peroxides have been described in detail. Sterol peroxide has been reported for the first time from this sponge.

Chapter 5 deals with chemical investigation of marine sponge *Suberites vestigium*. Chloroform fraction of this sponge yielded C-27 to C-29 mono and di unsaturated sterols, whereas, butanol fraction gave a novel heteroaromatic acid. Pet.ether fraction yielded fattyacids ranging from C-12 to C-18.

Chapter 6 deals with chemical investigation of marine sponge *Chrotella australiensis*. This sponge yielded ketosteroids and a nucleoside from

chloroform and butanol fraction respectively. Several fatty acids ranging from C-12 to C-20 have been identified from this sponge.

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Lipid constituents of marine sponge *Suberites carnosus*

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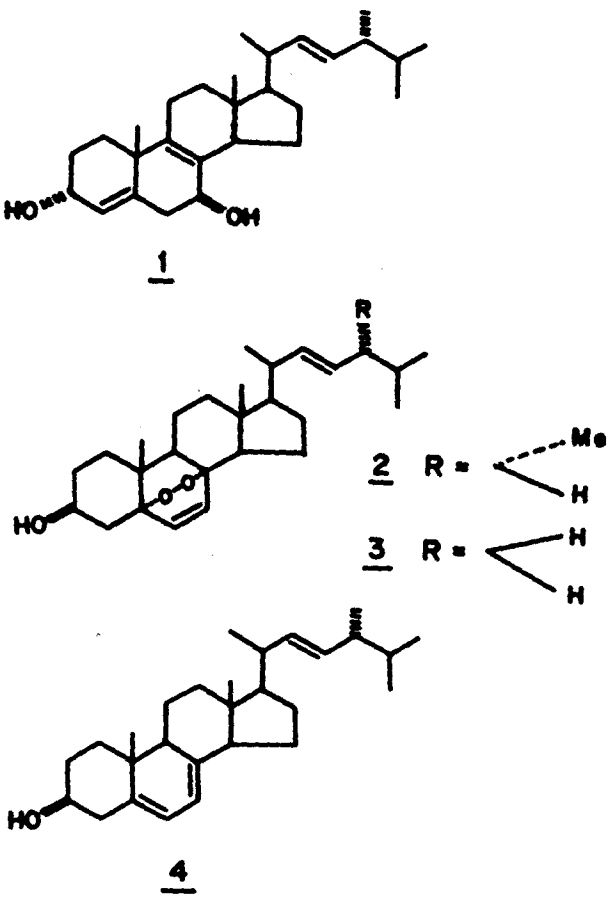
Sterols (22*E*) - 24α - methyl-cholest - 4, 8(9), 22 (23) - triene - 3 α, 7β - diol (**1**) and (22*E*)-24α-methyl-ergost-6, 22(23)-diene-5α, 8α-epidioxy-3β-ol (**2**), a fatty acid nonadecanoic acid and a fatty ester methyl nonadecanoate have been isolated from the sponge *Suberites carnosus*. Minor amounts of (22*E*)-ergost-6, 22(23), 24(28)-triene-5α, 8α-epidioxy-3β-ol (**3**) and ergosterol (**4**) are also present. The structures have been elucidated on the basis of spectral data.

During the course of our continuing investigation on the isolation of bioactive substances from marine organisms the methanolic extract of the marine desmosponge. *Suberites carnosus* belonging to the order Hadromerida, family Suberitidae, exhibited cytotoxic, diuretic and antihistaminic activities.

The alcoholic extract of the sponge *Suberites carnosus* was fractionated with chloroform and *n*-butanol. The chloroform fraction was chromatographed over a silica gel column by gradual elution with pet. ether-ethyl acetate. This afforded four compounds, which include sterols **1** and **2**, fatty acid **6** and fatty ester **5** in decreasing order of elution.

Compound **1** was obtained as a solid. Its IR spectrum exhibited absorption due to hydroxyl group (3382 and 1034 cm^{-1}), carbon-carbon double bond (1658, 970 cm^{-1}). EI mass spectrum of **1** gave a molecular ion peak at m/z 412 in agreement with the formula $C_{28}H_{44}O_2$. Further analysis of mass spectrum showed diagnostic peaks at m/z 394 ($C_{28}H_{42}O$) and m/z 376 ($C_{28}H_{40}$) corresponding to two successive losses of 18 amu from molecular ion peak, thus indicating the presence of two hydroxyl groups. Its ^1H NMR spectrum was suggestive of a sterol nucleus. These data indicated that compound **1** had C_{28} triene sterol with two hydroxyl groups.



^1H NMR spectrum of compound **1** supported the existence of a trisubstituted double bond (δ5.3, a doublet) as well as a terminal isopropyl group in the side chain [δ 0.83 (3H, d, J=6.8 Hz, $C_{26/27}$-Me), δ0.85 (3H, d, J=6.8 Hz, $C_{27/26}$-Me)], also present were two singlets at δ0.61 and 1.12 corresponding to C_{18} and C_{19} methyl protons respectively, two doublets integrating for 3H appeared at δ 0.91 (J = 6.6 Hz) and 1.02 (J=6.6 Hz) due to C_{28} and C_{21} methyl protons respectively. The nonequivalence of C_{28} methyl and one of the isopropyl methyl groups suggested 24*R* configuration[1]. Signals due to protons attached to carbon bearing −OH group were observed at δ 4.09 and 3.5. Signal at δ 3.5 (triplet in appear-

ance) was quite narrow as would be expected for an equatorial proton (3α-OH) and the other signal at 4.09 had a complexity (quintet) usually seen for β-carbinol (7β-OH). This compound also exhibited a two-proton multiplet characteristic of $\Delta^{22(23)}$ at δ5.2. A second double bond was placed at C_4 as the olefinic proton at δ5.3 appeared as a doublet. The absence of any additional olefinic proton in ^{1}H NMR limited the possible position of the third double bond to $\Delta^{8(9)}$ or $\Delta^{8(14)}$. Based on mass fragmentation pattern (**Figure 1**) the third double bond was placed at $\Delta^{8(9)}$ position, thus establishing the structure as (22*E*)-24α methyl-cholest-4, 8(9), 22(23)-triene-3α, 7β-diol.

The mass spectrum of **2** gave a molecular ion peak at m/z 428 corresponding to $C_{28}H_{44}O_3$ and indicating the presence of six double bond equivalents; four were assigned to the four rings of carbocyclic nucleus and remaining two to unsaturated bonds. Its IR spectrum displayed characteristic bands at 3525 (−OH group), 968 (a trans C=C) and 1180 cm^{-1} (peroxide group), the presence of this function was supported by the loss of oxygen from the molecular ion peak in the mass spectrum (m/z 396).

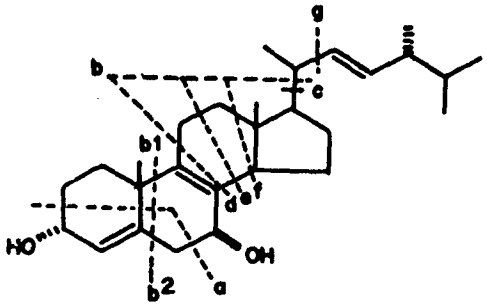
The EI mass spectrum in addition to $M^{+\cdot}$ showed diagnostic peaks at m/z 410 ($M^{+\cdot}-H_2O$), 303 [$M^{+\cdot}$ − SC (C_9H_{17})], 285[$M^{+\cdot}$ − (SC+H_2O)], 271 [$M^{+\cdot}$−(SC+O_2)], 253 [$M^{+\cdot}$−(SC+H_2O+O_2)], 211 [253 − ring D fusion (42)].

These fragments suggested that the compound was a C_{28} sterol possessing a hydroxyl group and two double bonds, one in the nucleus and one in the side chain.

The ^{1}H NMR spectrum of **2** displayed C_{18} and C_{19} methyl protons at δ 0.81 and δ 0.88 as singlets and four doublets integrating 3H each at δ0.82 (J=6.8 Hz), 0.83 (J=6.8 Hz), 0.94 (J=6.4 Hz) and δ1.02 (J=6.4 Hz) were due to C_{26}, C_{27}, C_{28} and C_{21} secondary methyl protons respectively. The nonequivalence of the C_{28} methyl and one of the isopropyl methyl groups and a characteristic downfield shift in the doublet resonance for the protons of C_{27} methyl group was indicative of 24*R* methyl configuration. The downfield shift of the C-3 carbinol proton at δ3.96 as compared to ergosterol (δ3.6) indicated some disturbance in the vicinity of C-3 position. ^{1}H NMR exhibited characteristic eight line pattern centered at δ5.18 (2H,m) for C_{22} and C_{23} protons with J=7.5 Hz (coupling between 22-20 H and 23-24 H) and J=15 Hz (coupling between 22-23 H, *trans*) indicating a $\Delta^{22(23)}$ double bond. The signals at δ 6.24 (1H, d, J=8.5 Hz) and 6.5 (1H, d, J=8.5 Hz) were assigned to the protons of nuclear double bond; the peak at m/z 396 in the mass spectrum and the signals at δ 6.24 and δ 6.5 in ^{1}H NMR spectrum were characteristic of ergosterol peroxide. These data suggested the structure of (22*E*)-24α-methyl-ergost-6, 22(23)-diene-5α-8α-epidioxy-3β-ol for compound **2**.

The structure was confirmed by comparison of the spectral data with literature data[2-4]. Further evidence for structure was provided by ^{13}C NMR data which showed signals at δ135.4, 135.2, 132.3 and 130.7 which were assigned to unsaturated carbon C_{22} and C_{23}, C_6 and C_7 respectively. Signal due to C_3 carbinol carbon was observed at δ66.4; signals at δ51.7 and 51.2 were due to C_5 and C_8 attached to endoperoxide function.

Compound **2** has previously been detected in some sponges[5,6], tunicates[7,8], fungi[9,10] and lichens[5] but no ^{13}C NMR data were reported. The mass spectrum of the compound also indicated trace amount of ergosterol **4** as evidenced by the presence of fragment ions at m/z 159, 158, 157 and 143 which are typical of $\Delta^{5,7}$ ring structure in sterols[11,12]. The mass spectrum of **2** also suggested the presence of yet another ergosterol derivative, compound **3**, with $M^{+\cdot}$ 426 and an important ion



m/z	Probable structure
412	M^+
394	$M^+ - H_2O$
376	$M^+ - 2H_2O$
315	g
287	c (M^+ − SC)
109	d
95	e
81	f
69	a
55	b_1 & b_2

Figure 1—Fragmentation of compound **1**

at m/z 394 (426-O_2) indicative of peroxy linkage. The presence of the peroxide function was further supported by the signals in the ^1H NMR spectrum at δ4.69 and 4.71 as a broad singlet, and a doublet at δ6.3 and 6.6 due to $\Delta^{24(28)}$ and Δ^6 double bond respectively. The presence of C_{21}-methyl proton was also evident at δ1.21. This led us to assign the structure of the steroid with M^+ 426 tentatively as (22*E*)-ergost-6, 22(23), 24(28)-triene-5α-8α-epidioxy-3β-ol.

Compound **5** was identified as aliphatic ester, methyl ester of nonadecanoic acid. It was obtained as liquid; IR spectrum showed bands at 1745, 1466, 1435, 1364, 1248, 1196 and 1170 cm^{-1}. High resolution ^1H NMR spectrum showed signals for a terminal CH_3 group at δ0.89 (3H, t, J=9.0 Hz), a signal for 16 methylene units at δ1.28 (32H, brs), a two-proton multiplet centered at δ1.63 was assigned to β protons in the acid portion of the molecule and a sharp singlet at δ3.68 (3H) for CH_3 group in alcohol portion.

Its mass spectrum showed M^+ at m/z 312 ($C_{20}H_{40}O_2$), a base peak at m/z 74 which is characteristic of linear chain methyl esters which arises by McLafferty rearrangement. Besides, there were also fragments of the general formula $[(CH_2)_nCOOCH_3]^+$ indicated by the fragment ions at m/z 87, 101, 129, 143, 157, 171, 185, 199, 213, 227, 270 and 298. In view of above evidence compound **5** was characterised as methyl nonadecanoate.

Compound **6** was an aliphatic acid, nonadecanoic acid, obtained as a colourless solid; molecular formula was assigned as $C_{19}H_{38}O_2$ based on EI mass spectrum (M^+ 298). Its IR spectrum was characteristic of carboxylic acid with absorptions at 3000-2851, 1710, 1435, 1290 and 940 cm^{-1} indicative of dimeric carboxcylic acid; also bands at 1412 cm^{-1} (CH_2 adjacent to $-COOH$ group) and 721 cm^{-1} of n-alkyl chain were observed. A uniform difference of 14 amu in fragmentation pattern of the molecule confirmed the presence of a long aliphatic chain. The absence of $[M-15]^+$ ion peak indicated a linear chain skeleton[13]. Compound **6** thus was characterised as nonadecanoic acid. Its methyl ester was found to be identical with compound **5** in all respects.

Though literature indicates the steroids 24-methyl-5α-cholest-22-en-3β-ol, cholesterol, 24-methyl-5α-cholestan-3β-ol, 24-methyl-cholest-5-en-3β-ol, 24-ethylcholest-5-en-3β-ol and 24-methylidenecholesterol to be the constituents of the sponge *Suberites carnosus*[14,15], but no mention has been made on the isolation of the steroids reported in this paper. To our knowledge this is the first report of natural occurrence of compound **1**. Further, substitution at C_{24} seems to be predominant in sterols of the family Suberitidae[16].

Experimental Section

General. Compounds were purified by repeated silica gel column chromatography and its purity was checked on TLC using different solvent systems. Melting points were determined on a Toshniwal melting point apparatus and are uncorrected. ^1H NMR spectra (TMS, $CDCl_3$) were recorded on a Bruker WM 400 MHz, FT NMR Spectrometer. IR spectra were taken on a Shimadzu FTIR-8001 (KBr pellet or neat sample). Mass spectra were recorded on a Jeol D-300 mass spectrometer at 70 eV. Column chromatography was performed over silica gel (60-120 mesh) and TLC on silica gel G (13% $CaSO_4$ as binder).

Extraction and isolation of the compounds. The sponge *Suberites carnosus*, collected in March 1994 from Malvan, West Coast of India, cut into small pieces, stored in methanol and transported to laboratory. The alcohol was decanted after a week and this process repeated twice. The decants were concentrated under reduced pressure to obtain a dark brown residue. This concentrated extract was resuspended in 10% aq. MeOH and successively partitioned with $CHCl_3$ and *n*-butanol. The residue from $CHCl_3$ fraction (7 g) was chromatographed over silica gel (200 g) using pet. ether-ethyl acetate as eluent yielding fractions 1, 2 and 3. Fraction 1 gave Compound **5** whereas compounds **2**, **3** and **6** were obtained from fraction 2, Compound **1** was isolated from fraction 3.

Compound 1: Solid (5 mg) m.p. 155-60°; IR (KBr, cm^{-1}): 3382, 2953, 2871, 1658, 1456, 1380, 1237, 1161, 1034, 970 and 868; 400 MHz ^1H NMR ($CDCl_3$): δ0.61 (3H, s, 18-CH_3), 0.83 (3H, d, J=6.8 Hz, $C_{26,27}$-CH_3), 0.85 (3H, d, J=6.8 Hz, $C_{27,26}$-CH_3), 0.91 (3H, d, J=6.6 Hz, 28-CH_3), 1.02 (3H, d, J=6.6 Hz, 21-CH_3), 3.5 (1H, t, H-3), 4.09 (1H, m, H-7), 5.2 (2H, m, $H_{22/23}$), 5.35 (1H, d, H-4); EIMS: m/z 412 (M^+), 394 (M^+-H_2O), 376 (M^+-2H_2O), 287 (M^+-SC), 269 [$M^+(SC+H_2O)$], 251 [$M^+-(SC+2H_2O)$].

Compound 2: Solid (15 mg) m.p. 157-62°; IR (KBr, cm^{-1}): 3525, 2872, 2955, 1460, 1380, 1180, 1074, 1043, 1028, 968, 935, 858, 779 and 275 cm^{-1}; 400 MHz ^1H NMR ($CDCl_3$): δ 0.81 (3H, s, 18-CH_3), 0.88 (3H, s, 19-CH_3); 0.82 (3H, d, J=6.8 Hz, $C_{26,27}$-CH_3), 0.83 (3H, d, J=6.8 Hz, $C_{27,26}$-CH_3), 0.94 (3H, d, J=6.4 Hz, C_{28}-CH_3),

1.02 (3H, d, J=6.4 Hz, C_{21}-CH_3), 3.96 (1H, m, 3β-H), 5.18 (2H, m, $H_{22/23}$), 6.24 (1H, d, J=8.5 Hz, 6-H), 6.5 (1H, d, J=8.5 Hz, 7-H); ^{13}C NMR ($CDCl_3$): δ135.4, 135.2, 132.37, 130.75, 66.4, 56.3, 51.7, 51.2, 44.59, 42.8, 39.6, 39.4, 37.0, 34.76, 33.09, 30.17, 28.57, 25.33, 23.42, 20.88, 20.65, 19.91, 19.62, 18.16, 17.55, 12.87 and 12.33; EIMS: m/z 428 ($M^{+\cdot}$) 410, 396, 377, 363, 337, 285, 253, 211, 185, 152, 145, 125, 107, 95, 81, 69 and 55.

Compound 5: Liquid, IR (KBr, cm^{-1}): 2941, 2850, 1745, 1460, 1435, 1363, 1248, 1146, and 1170; ^1H NMR ($CDCl_3$): δ0.89 (3H, t, J=9.0 Hz), 1.28 (32H, brs, $-CH_2$ groups), 1.63 (2H, m), 3.68 (3H, s); EIMS: m/z 312 ($M^{+\cdot}$), 298, 270, 227, 213, 199, 185, 171, 157, 143, 129, 101, 87 and 74 (base peak).

Compound 6: Solid, m.p. 125-30°; IR (KBr, cm^{-1}): 3000, 2851, 1710, 1460, 1435, 1412, 1375, 1290, 940 and 721, EIMS: m/z 298 ($M^{+\cdot}$), 284, 270, 256, 242, 228, 213, 199, 185, 171, 157, 143, 129, 115, 111, 97, 83, 73, 65, 55 and 44.

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Note

Steroids from marine sponges *Suberites vestigium* and *Chrotella australiensis*[+]

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The sponges *Suberites vestigium* and *Chrotella australiensis* have been examined for steroids. Both the sponges contain C_{27-29} mono and diunsaturated sterols, in addition sponge *C. australiensis* contains cholest-4-ene-3-one and 24-ethyl cholest-4-ene-3-one. Batyl alcohol and its higher homologue have also been identified in *S. vestigium*. This is first report of steroids from these sponges.

In continuation of our investigation on bioactive metabolites from marine organisms, we report herein the isolation and identification of steroids from the sponges *Suberites vestigium* and *Chrotella australiensis*. The crude methanolic extract of *S. vestigium* exhibited *in vitro* antihistaminic activity and *C. australiensis* was found to be anti-viral. The sponges were extracted with methanol and crude extract fractionated with chloroform, ethyl acetate and butanol. Chloroform fraction of *S. vestigium* yielded sterols **1-4** and glyceryl ethers, *n*-batyl alcohol and its higher homologue whereas the chloroform fraction of *C. australiensis* gave steroids **5-10**. Characterisation of the constituents of these sponges is based on their spectral data and comparison with the literature values. These sponges are hitherto unexamined for their chemical constituents.

Compound **1**: Its ^{1}H NMR spectra showed a multiplet at δ 3.6 and a broad singlet at δ 5.35, the spectrum further displayed signals for two secondary methyls at δ 0.68 (3H, s, C-18) and 1.02 (3H, s, C-19) characteristic of 5-ene- 3β- hydroxysterols[1], a secondary methyl at δ 0.85 (3H, d, C-21) and a primary methyl at δ 0.79 (3H, t, C-29). It also showed a tertiary methyl at 1.62 (3H, s, C-26) and broad singlets at δ 4.7 and 4.62 for methylene protons.

The mass spectrum of **1** displayed besides M^+ at 412, fragments at m/z 329 [M-C_6H_{11}], 273 [M-Sc] and 301 [M^+-Sc+2H]. A single molecular ion peak at m/z 412 and a strong fragment at 271 indicated the presence of two double bonds, one being in the nucleus and the other in the side chain. A prominent peak at m/z 314 is consistent with $\Delta^{25(26)}$ double bond. The presence of common cholesterol nucleus was evident from the diagnostic ions at m/z 273, 255, 231 and 213. All the data led us to conclude that the compound **1** is 24-ethyl-cholest-5, 25(27)-diene-3β-ol or clerosterol[2].

Compound **2**: M^+ 414; it contains cholestane nucleus with saturated side chain and the nuclear double bond at 7-position as evident from ^{1}H NMR spectra which showed characteristic broad singlet at δ 5.17 for H-7 and the high field resonances for C-18 and C-19 methyl protons at δ 0.57 and 0.68 respectively[3]. The structure of **2** was established as 24-ethyl-cholest-7-ene-3β- ol.

Sterols **3** and **4** with molecular ion peak at 386 and 398 were characterised as cholesterol and 24-methylene-cholesterol respectively, from the comparison of their spectral data (EIMS and ^{1}H NMR) with literature values[4].

Chromatography of more polar materials present in the chloroform fraction resulted in the isolation of glyceryl ethers. The ^{1}H NMR spectrum was suggestive of the presence of batyl alcohol and its homologue which exhibited signals at δ 0.87 (3H, t), 1.25 [$(CH_2)_n$] and 3.4-3.9 (m). ^{13}C NMR spectra showed signals at δ 72.44, 71.8, 70.48 and 64.27 for four carbons directly attached to oxygen in the molecule, at δ 39.4-18.8 for the methylenes of aliphatic chain and at δ 13.9 for the terminal methyl of the aliphatic chain. The presence of batyl alcohol and its homologue was further confirmed by fragmentation pattern of the mass spectrum of the mixture which showed molecular ion peaks at M^+ 344 and 358.

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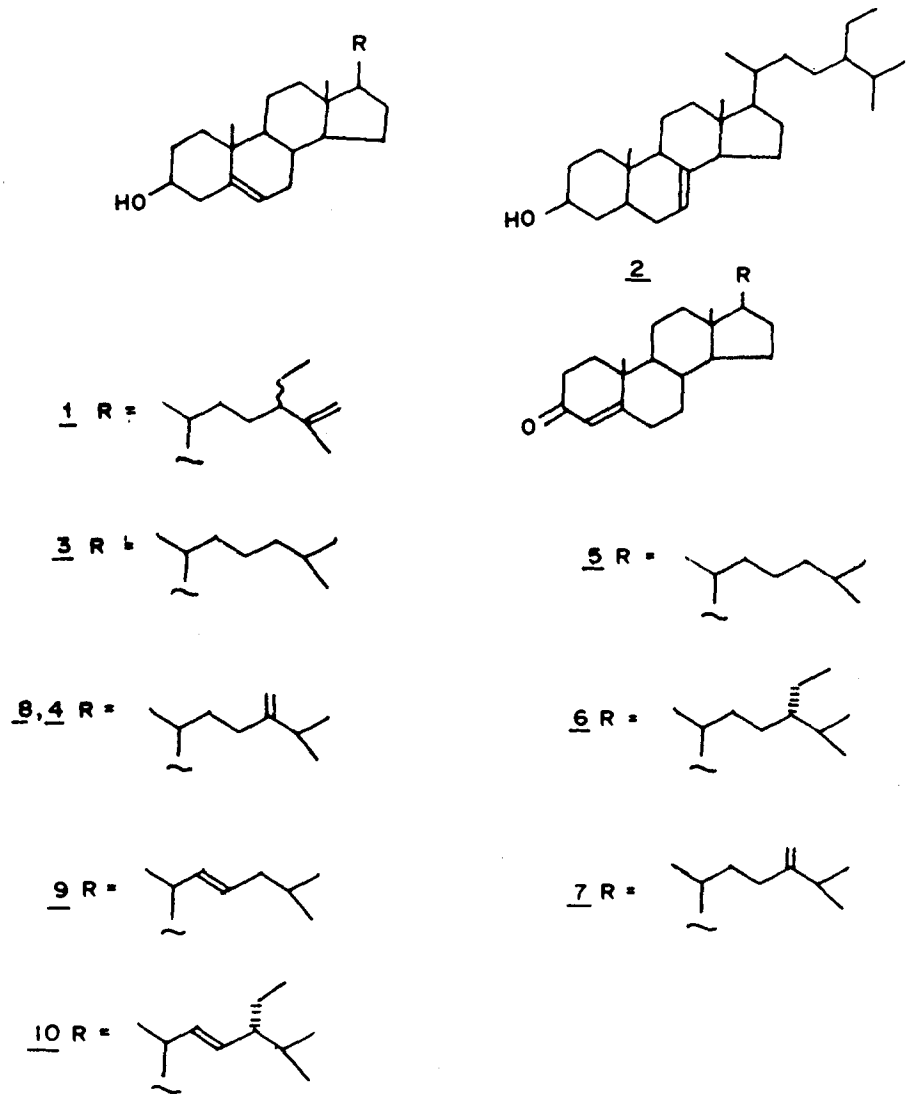

The chloroform solubles of the sponge *C. australiensis* yielded Δ^4-3-keto-steroids **5-7** and some common sterols **8-10**. A prominent peak and often base peak usually occurs at m/z 124 (B- ring cleavage), other important peaks are (M^+-42) due to loss of ketone and (M^+-42-side chain). The presence of Δ^4-3-ketene moiety was further evidenced by the presence of signals at δ 198.89, 170.99 and 123.64 in ^{13}C NMR spectrum as well as absorptions at 1728, 1620, 1273 cm^{-1} in the IR spectra. ^1H NMR spectra depicted olefinic protons at δ 5.7 (4-H) and 1.20 (19-CH_3). These data established the presence of nuclear Δ^4- 3-ketone moiety and suggested strongly that the compound **5**, with M^+ 384 and characteristic peaks at m/z 342 and 229, was cholest- 4-ene-3-one and the compound **6**, with M^+ 412 and peaks at m/z 370 and 229, was identified as 24-ethyl-cholest-4-ene-3-one, the 24*S* configuration followed from the triplet in the ^1H NMR of C-29 methyl at δ 0.858 (J=7.4). This fraction also contained 24-methylene-cholest-4-ene-3-one **7**, as evident from the mass spectrum of the compound with M^+ 396 and fragments at m/z 271 and 124.

Δ^4-3-one steroids are of rare occurrence and were first reported in the sponge *Stelleta clarella* along with other sterols[5]. Subsequently, Kokke *et al.*[6] also isolated three such compounds from the dinoflagellate *Procystis lunula* and Parameshwaran *et al.*[7] reported from sponge *Ircinia ramosa*. Δ^4-ene-3-one steroids have also been reported from the red alga *Gracilaria textorii*[8].

Compound **8** exhibited molecular ion peak at m/z 398 and a triplet at m/z 299, 300 and 301, characteristic of Δ^{24} double bond. Its ^1H NMR depicted

olefinic protons at δ 5.3 (6-H) and two broad singlets at δ 4.76 and 4.62 for methylene protons establishing the structure of **8** as 24-methylenecholesterol.

The mass spectrum of compound **9** showed in addition to the molecular ion peak at 384, the usual ion corresponding to the loss of side chain at m/z 273, 366 due to loss of H_2O from the molecule and at 371 [M^+-H_2O-CH_3]. These data in conjunction with the ^1H NMR signals at δ 5.4, 3.6 and 0.67 for protons at 22, 3 and 19 positions respectively, suggested the structure of **9** as 22-*trans*-cholesta-5,22-diene-3β-ol.

Compound **10** was identified as 22-*trans*-24*S*-ethylcholesta-5,22- diene-3β-ol on the basis of its mass spectrum which showed molecular ion peak at M^+ 412 and prominent fragments at m/z 397, 394, 351, 300, 273 and 271. The 24*S* configuration followed from the triplet at δ 0.853 from its ^1H NMR spectrum[9].

The occurrence of Δ^4-3-keto steroids and the corresponding Δ^5-3β-hydroxy sterols is of particular significance because it suggests that the sponge *C. australiensis* contains 3β-hydroxydehydrogenase, Δ^5-isomerase and Δ^4- reduction system. These enzymes are essential for conversion of sterols to steroid hormones.

Experimental

^1H NMR spectra were recorded on Bruker WM 400 and 300 MHz (chloroform, TMS) FTNMR spectrometer; mass spectra on Jeol D300 mass spectrometer at 70 eV; and IR spectra on Shimadzu FTIR-8001 (KBr pellets). Compounds were repeatedly purified over silica gel (60-120 mesh) column and their purity was checked on TLC using different solvent systems. Final purification was done on HPLC using ODS-5μ column with MeOH as solvent at the rate of 1 ml/min.

Collection, extraction and isolation

The sponges *S. vestigium* and *C. australiensis* were collected from Mandapam and Goa coast respectively, stored in methanol and transported to laboratory. The solvent was drained off and concentrated under reduced pressure. The crude methanolic extract was suspended in 20% aq. methanol and extracted with chloroform, ethyl acetate and *n*-butanol. The chloroform fraction was chromatographed over silica gel using increasing amount of ethyl acetate in pet. ether.

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A heteroaromatic acid from marine sponge *Suberites vestigium*†

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4-Methyl- pyrazole-3(5)-carboxylic acid has been isolated from the butanol fraction of marine sponge, *Suberites vestigium* for the first time. The methanol extract of the sponge exhibits *in vitro* antihistaminic activity. Pyrazole derivatives as synthetic products are widely used as medicine, however, organic compounds containing pyrazole nucleus have not been reported from marine flora and fauna. Structure elucidation of the compound is based on spectral evidences.

In continuation of our work on bioactive metabolites from marine organisms, crude methanolic extract of marine sponge *Suberites vestigium* belonging to the order Hadromerida and family Suberitidae exhibited *in vitro* anti histaminic property. The search of active principle led us to isolate 4-methyl-pyrazole-3(5)-carboxylic acid from butanol fraction. To the knowledge of authors this is the first record of its natural occurrence, though pyrrole carboxaldehyde analogues have been reported from marine sponges[1] and soft corals[2,3]. The acid was obtained as colourless crystals. The UV and IR spectra was characteristic of an amino acid nucleus, [λ_{max} (MeOH), 265 (ε 3200), and ν_{max} (KBr) 3218-2818 cm^{-1}]. From NMR and mass spectra the elemental composition was determined as $C_5H_6N_2O_2$. All five carbons of the compound could be accounted for in the ^{13}CNMR spectrum and the chemical shifts were entirely compatible with a pyrazole type skeleton. This was further confirmed by the DEPT experiments revealing the presence of three quarternary, a methine and a methyl carbons. ^{1}HNMR of compound showed a methyl group on a sp^2 carbon at δ 1.92 and a vinyl proton at δ 7.3 as singlets. The two labile protons due to COOH and NH groups were evident at δ 12.2 and 13.0 (Deuterium exchangeable) respectively. Upfield resonance of methyl carbon indicated that methyl group is in proximity with COOH group. Long range coupling of vinyl proton with methyl protons ruled out the possibility of imidazole carboxylic acid and confirms the rigid structure of the molecule in COSY spectrum (Figure-1) as 4-methyl-pyrazole-3(5)- carboxylic acid 4.

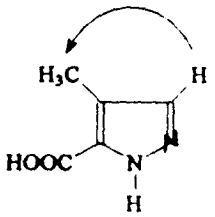

Figure 1 — 4-Methylpyrazole-3 (5)-carboxylic acid.

Experimental Section

The 10-15 Kg of sponge was collected from Mandapam, west coast of India, and extracted with methanol; the decants were filtered and concentrated by distillation under reduced pressure. The crude extract was suspended in aq. methanol (20%) and partitioned with pet. ether, chloroform, butanol. Butanol fraction was chromatographed over XAD-2 resin and eluted with water-methanol mixture in different proportions followed by repeated column chromatography over silica gel and elution with chloroform- methanol (8:2) to yield a colourless crystalline compound. NMR spectra were recorded on Bruker WM 400 MHz, FTNMR spectrometer, IR on Shimadzu FTIR- 8001 (KBr Pellet) and mass spectra on Jeol D-300 mass spectrometer at 70 ev.

4-Methyl-pyrazole-3(5)-carboxylic acid, Crystalline compound, m.p., 310-12 °C; UV (methanol): λ_{max} 265 (ε 3200); IR (KBr): 3218, 3062, 2818, 1760, 1674, 1648, 1556, 1474, 1380,

†NIO contribution No. 2560

1208, 842, 810 and 764 cm^{-1}; ^{1}HNMR (Pyridine-d_5, 400 MHz): δ 1.92 (3H, s), 12.2 (1H, bs), 13.0 (1H, bs), 7.3 (1H, s); ^{13}CNMR and DEPT: δ 12.2 (CH_3), 108 (C), 137 (CH), 153 (C), 166 (COOH); EIMS (m/z): 126 [M^+], 125, 112, 76, 83, 78, 55.

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