Chapter 3

CHEMICAL INVESTIGATION OF
MARINE INVERTEBRATES
OCEANAPIA SP. AND LOBOPHYTUM CRASSUM
I. Introduction

i) General

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

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

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

Unfortunately since then marine natural products have not found their way to store shelves with much success. This is because compounds isolated from marine sources have failed to progress to the research stage in the past due to numerous
reasons. But, the continuous advancement of technology has brought significant number of compounds from marine sources especially invertebrates into preclinical and clinical trials since the early 1980s. The result of this led to the development of two drugs ziconotide (prialt\textsuperscript{TM}) (3) and ecteinascidin 743 (ET 743) (yondelis\textsuperscript{TM}) (4).

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

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

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

This chapter deals with isolation and characterization of bioactive metabolites from a marine sponge Oceanapia sp. and a marine soft coral Lobophytum crassum. The investigation carried out on each organism is discussed two separate sections.
Section 3.1 Secondary Metabolites from marine sponge *Oceanapia* sp.

I. Introduction to marine sponges

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

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

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

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

Species of *Oceanapia* are common, with more than 50 nominal species recorded for the Indo-west Pacific region alone, relatively abundant in both soft and hard
substrates, and widely distributed (Hooper et al., 1993). However, only few compounds have been isolated from this sponge. The majority of the compounds isolated from this sponge are alkaloids, but several non-alkaloids constituents have also been isolated. In our following discussion metabolites from *Oceanapia* sp. are divided into alkaloids and non-alkaloids.

**i) Alkaloids**

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

![Reaction](image)

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

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

A chemical investigation on Indian marine sponge resulted in isolatation 6-methoxy-2(3H)-benzoxazolinone, coixol (10). It was toxic to brine shrimp (Venkateswarlu et al., 1999).
The novel bromotyrosine-derived alkaloids 11 and 12 were isolated from an Australian Oceanapia species and were found to be inhibitors of S-conjugate amidase (Nicholas et al., 2001).

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

**ii) Non-alkaloids**

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

In an investigation on the antimicrobial constituent of a Japanese Oceanapia sp., bis-acetylene (17) was identified active principle (Matsunaga et al 2000).
Three dithiocyanates, thiocyanatins A (18), B (19), and C (20), were isolated from an Oceanapia species collected from South West Australia. These compounds had nematocidal activity and their structures were confirmed by synthesis (Capon et al., 2001). Similar, but branched dithiocyanates, a pair of isomeric thiocyanatins D1 (21) and D2 (22), and related thiocyanate-thiocarbamates, thiocyanatins E1 (23) and E2 (24), were obtained as inseparable pairs from a South Australian Oceanapia species. These were also potent nematocides. (Alam et al., 2001)

III. Results and Discussion

i) Biological materials

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

ii) Extraction and purification

Lyophilized sponge was extracted in EtOAc and solvent evaporated to give crude EtOAc extract. Two compounds 25 and 26 were purified by column chromatography by eluting with gradients of MeOH-CHCl₃, which is depicted in Scheme 3.1. A bright yellow spot seen in visible on TLC (10% MeOH-CHCl₃, v/v) was purified from EtOAc extract using preparative TLC to yield bright yellow solid (27).
iii) Structure elucidation of metabolites

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

The initial examination of EtOAc extract using ESI-MS (Fig 3.2) gave very interesting mass peaks in the region 440-500 amu. In this spectrum there were pairs of peaks, which had difference of 16 or 14 units. The difference between pairs of peaks at m/z 447 and 463, m/z 463 and 479, and m/z 479 and 495 was 16 units indicating the compound with lower mass unit in the pair lacked hydroxyl group. Similarly peaks at m/z 447 and 461, m/z 463 and 477, m/z 479 and 493 had
a difference of 14 units indicating the compound with lower molecular weight lacked a methyl group. Subsequent to chromatographic purification of EtOAc extract, only two compounds 25 and 26 were purified, which were analyzed using IR, NMR and MS. The attempts to isolate any more compounds were unsuccessful.

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

Compound 26 was isolated as dextrorotatory solid, more polar than 25. It showed pseudomolecular ion [M+H]^+ at m/z at 463, which was 16 units more than
molecular weight of xestospongin C (25). More polar nature and molecular weight more by 16 units compared to 25 was suggestive of a hydroxyl group in the 26, which was confirmed from the strong absorption band at 3340 cm\(^{-1}\) IR spectrum (Fig 3.4a). In comparison to 25 an additional signal as expected due to hydroxylated carbon in the \(^{13}\)C NMR spectrum was observed at \(\delta_C\) 70.1 (C-9) (Fig 3.4b). These data is identical to a known sponge metabolite xestospongin D (26), suggesting the molecule under investigation is Xestospongin (D).

![Fig 3.3b: \(^1\)H NMR spectrum of xestospongin C (25)](image)

![Fig 3.3c: \(^{13}\)C NMR spectrum of xestospongin C (25)](image)
Both xestospongin C (25) and D (26) along with xestospongin A and B were first isolated from sponge *xestospongia exigua* (Nakagawa and Endo, 1984). They are bis-1-oxaquinolizidine alkaloids and other members of the family includes araguspongins A-J (Kobayashi et al., 1989), 3β,3’β-dimethylxestospongin C (Reddy and Faulkner, 1997), (+)-7S-Hydroxyxestospongin (Moon et al., 2002). The stereochemistry of the xestospongin/aragusponge alkaloids is complex, and it is clear that several stereostructural issues were unresolved until Baldwin’s biomimetic syntheses (Baldwin et al., 1998). The biomimetic synthesis allowed unambiguous assignments and revision of absolute configurations of 25 as 2R,2’R,5S,5’R,9S,9’R,10S,10’S, which was actually assigned exactly opposite in its first report (Nakagawa and Endo 1984), while 26 was determined to be 2R,2’R,5S,5’R,9S,9’R,10S,10’S from XRD studies (Moon et al., 2002).

Mimosamycin (27)

Compound 27 gave bright yellow fluorescent spot on TLC plate indicating the presence of chromophoric group such as quinone. It was bright yellow and
optically inactive solid. Its IR spectrum showed peaks at 1686, 1646, 1636 and 1616 cm\(^{-1}\) indicating presence of quinone carbonyls, amides and C=C bonds. There were no absorption bands for NH or OH stretching. The EIMS of 27 revealed to molecular weight to be 233 indicating presence of odd number nitrogen atoms in the molecular formula. \(^1\)H (Fig 3.5a) and \(^{13}\)C NMR (Fig 3.5b) data coupled with its molecular weight, the molecular formula of the compound was deduced to be C\(_{12}\)H\(_{11}\)NO\(_4\). Its \(^1\)H NMR gave signals for three methyls, of which signal at \(\delta_H 2.05\) was due to a vinylic methyl and signals at \(\delta_H 3.65\) and 4.40 were due NCH\(_3\) and OCH\(_3\) respectively. The additional two signals were due to highly deshielded vinylic protons at \(\delta_H 7.08\) and 8.25. The \(^{13}\)C NMR suggested the presence of two carbonyls at \(\delta_C 183.4\) and 177.2, amide carbonyl at \(\delta_C 162.7\), two vinylic methines at \(\delta_C 142.0\) and 116.6, and 4 quartenary sp\(^2\) carbons at \(\delta_C 159.4, 138.3, 133.1\) and 111.2 of which one at \(\delta_C 159.4\) is oxygenated. These data accounts for six \(\delta_C 159.4\) degrees of unsaturation, while its molecular formula requires eight. Considering the molecule to have one quinone type of ring and three carbons are accounted by methyls the remaining three carbons and a nitrogen atom should be involved six membered fused rings. Using the above data, the molecular structure of the compound was interpreted to be of mimosamycin (27), a known natural product (Parameswaran et al., 1998).

![Fig 3.5a: \(^1\)H NMR spectrum of Mimosamycin (27)](image-url)
iv) Biological activity

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

IV. Experimental

i) Biological material

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

ii) Extraction and purification of metabolites

Sponge sample (500 g) was dried in a freeze drier and extracted with EtOAc (500 ml x 4) under sonication. EtOAc extract was evaporated under vacuum. A bright yellow spot (10% MeOH-CHCl₃ TLC mobile phase) was purified from EtOAc
extract (250 mg) on preparative TLC to yield mimosamycin (2.7 mg). EtOAc (500 mg) extract was loaded on flash Si-gel column and eluted with increasing concentration MeOH (0-100%, v/v) in CHCl₃. Elution with 4% MeOH-CHCl₃ gave xestospongin C (4.8 mg). Further elution with 7% MeOH-CHCl₃ gave xestospongin D (3.9 mg).

a) Xestospongin C (25): colorless crystals, C₂₈H₅₀N₂O₂, mp (CHCl₃-MeOH); [α]²⁸ D -2.7° (c 0.29, CHCl₃); IR (KBr): νₘₐₓ 2990, 1636, 1581, 1549 cm⁻¹. (¹H NMR, CDCl₃, 300 MHz, δ_H) 4.38 (s, H-10), 3.57 (br t, J = 12.6, H-2), 3.36 (br t, J = 10.8 Hz, H-2'), 3.11 (br d, J = 7.7 Hz, H-10'), 3.09 (br d, J = 10.5, H-4, H-6′α), 2.95 (br d, J = 10.7, H-4, 2xH4'), 2.77 (br t, H-6α, J = 16.8), 2.50 (br d, H-4), 2.17 (br t, H-6′β), 1.93 (br t, H-6β), 1.17-1.69 (m, 38H). (¹³C NMR, CDCl₃, 75 MHz, δ_C) 95.7, 87.3, 75.7, 75.3, 54.2, 54.0, 52.7, 45.2, 40.5, 40.2, 35.9, 32.8, 32.5, 32.2, 31.5, 31.1, 29.3, 28.8, 28.7, 27.0, 26.4, 26.2, 25.7, 25.2, 25.0, 24.9, 24.7.

b) Xestospongin D (26): Colorless crystals, C₂₈H₅₀N₂O₃, mp 158-159°C (CHCl₃-MeOH); [α]²⁸ D +17.5° (c 0.33, CHCl₃); (¹H NMR, CDCl₃, 300 MHz, δ_H) 4.59 (s, H-10), 3.94 (br t, J = 10.3, H-2'), 3.32 (br d, J = 6.0, H-6′α), 3.07 (br d, J = 11.1, 2xH-4', H-10), 2.79 (br d, J = 11.1, H-6α), 2.25 (br d, J = 10.8, H4), 2.17 (br t, J = 8.7, H-6′β), 1.98 (br t, J = 8.7, H-6β), 1.17-1.69 (m, 38H). (¹³C NMR, CDCl₃, 75 MHz, δ_C) 95.8, 90.2, 77.2, 75.9, 70.1, 54.0, 53.9, 52.4, 45.0, 40.2, 39.4, 34.5, 34.2, 32.3, 31.8, 30.3, 30.0, 29.6, 28.7, 26.6, 25.6, 25.2, 25.0, 24.5, 22.2, 18.2.

c) Mimosamycin (27): Yellow crystalline solid, C₁₂H₁₁NO₄, mp 228-230 °C; IR (KBr): νₘₐₓ 2990, 1636, 1581, 1549 cm⁻¹. (¹H NMR, CDCl₃, 300MHz, δ_H): 2.06 (3H, s), 3.67 (3H, s), 4.17 (3H, s), 7.09 (1H, s), 8.27 (1H, s); (¹³C NMR, CDCl₃, δ_C): 9.6 (q), 38.4 (q), 61.3 (q), 111.3 (s), 116.7 (d), 133.2 (s), 138.9 (s), 142.1 (d), 159.5 (s), 162.8 (s), 177.3 (s), 183.5 (s). MS m/z (%): 233 (M⁺), 218, 205, 190, 162, 134.

iii) Biological activity

Test cultures were grown on nutrient broth with paper disk impregnated with the extract or pure compounds. Antibacterial assay plates were incubated at 37°C, for 24 hr. Antifungal assay plates were incubated at room temperature for 24-48 hr. The plates were observed for zones of inhibition.
Section 3.2: Secondary metabolites from soft coral Lobophytum crassum

I. Introduction to soft corals and their metabolites

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

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

II. Results and discussion

i) Biological material, extraction and purification of metabolites

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

Lyophilized organism was extracted sequentially with pet ether, CHCl₃, EtOAc, n-BuOH and MeOH and the extracts were labeled as LP, LC, LB and LM (Scheme 3.2). More than 90% of the organic substances were extracted in pet ether and CHCl₃.

LP had very pleasant smell probably due to presence of terpenoids in it. It was chromatographed on Si gel by eluting with gradient of EtOAc in pet ether (0-50%, v/v), collected in 22 fractions and analyzed with TLC (10% EtOAc-P.ether, 5% H₂SO₄ sprayed and heated at 120°C) (Scheme 3.2). The substances in fractions 5-
9 (fraction A) had peculiar smell of fatty acids and were obtained in the form of wax. Its IR spectrum showed characteristic peaks of fatty esters in the region 2800-3000, 1740 and 1463 cm\(^{-1}\). They were not further analyzed.

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

Fraction 13 (fraction C) had solids with green pigments in it. TLC showed green spots on spraying with sulfuric acid and heating at 120° C. A crystalline solid
compound (29) was purified by repeated column chromatography on flash Si gel by elution with EtOAc in pet ether (0-20% v/v).

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

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

\textit{ii) Structure elucidation of purified compounds}

![Cembrenoid 1 (28)](image)

Compound 28 was obtained as sweet smelling liquid. Its IR spectra showed strong absorption bands for C-H str 2854, 2925 and 2960 cm\textsuperscript{-1} and for lactone carbonyl str at 1768 cm\textsuperscript{-1}. No other functional groups were detected from IR spectrum. Its molecular C\textsubscript{20}H\textsubscript{28}O\textsubscript{2} was deduced from the combination of 1D NMR and EI mass spectrum which showed molecular ion (M\textsuperscript{+}) at m/z 300. The molecular formula indicated it to be diterpene. Its \textsuperscript{1}H NMR (Fig 3.6a) showed downfield doublets ($J = 2.6$) at $\delta_H$ 5.70 (H-3) and 6.23 (H-16) indicating an exomethylene in conjugation with lactone carbonyl. A set of multiplets corresponding to three protons were observed between $\delta_H$ 4.86-5.07 revealing the presence of three vinylic hydrogens (H-5, H-9 and H-13). A signal due to lactonic methine (H-15a) appeared as triplet at $\delta_H$ 4.15 ($J = 9.6$Hz). Another methine (H-3a) appeared as multiplet at $\delta_H$ 2.66. Set of multiplets appeared in the region between $\delta_H$ 2.02-
2.29 for 12 hydrogen atoms (6 methylenes \textit{viz} C-4, C-7 C-8, C-11, C-12, and C-15). Three vinylic methyl singlets were observed at $\delta_H$ 1.57, 1.65 and 1.71. These signals accounted for all the 28 hydrogen atoms in the molecule. The data was equally supported by its $^{13}$C (Fig 3.6b) and DEPT NMR (Fig 3.6c). They revealed presence of a lactone carbonyl $\delta_C$ 170.4, three sp olefinic methines, three olefinic quarternaries, a exo-methylene $\delta_C$ 120.5, a oxymethine at $\delta_C$ 81.8 a methine at $\delta_C$ 44.9, six methylenes between $\delta_C$ 24.4 and 38.7, and three methyls at $\delta_C$ 15.9, 16.5 and 17.4. The molecular formula of the compound requires 7 degrees of unsturation. A lactone and 4 ene functionalities accounted for 6 degrees of unsaturation, hence compound most have another alicyclic ring, which suggested a cembranolide type of ring system as shown in the structure 28. The comparison of our NMR data with reported data for cembranolide 1 isolated from \textit{Lobophytum crassospiculatum} (Ahond et al., 1979) indicated our compound to be identical to cembranolide 1. Cembranolide 1 (28) has been later isolated as an antimalarial constituent of \textit{Lobophytum crassum} collected from Pange reef in Zanzibar (Said, 2005).

Fig 3.6a: $^1$H NMR of Cembranolide 1 (28)

Fig 3.6b: $^{13}$C NMR of Cembranolide 1 (28)
Compound 29 was isolated as crystalline solid and showed intense bands at 2946, and 2854 cm\(^{-1}\) and less intense bands at 1464 and 1470 cm\(^{-1}\) for C-H stretching and bending vibrations respectively. It showed band at 3320 cm\(^{-1}\) revealing presence of hydroxyl in the molecule. A weak intensity band at 1640 cm\(^{-1}\) due to C=C stretching was evidence of alkene functionality in the molecule. The \(^1\)H NMR (Fig 3.7a) spectra showed characteristics typical of sterols. A vinylic proton signal was observed as broad singlet at \(\delta_H 5.35\). A hydroxymethine signal was observed as multiplet at \(\delta_H 3.55\). Six methyls were evident from the signals at \(\delta_H 1.01\) (s, C-19), 1.00 (d, C-27), 0.91, (d, C-26), 0.85 (d, C-28), 0.77 (d, C-21) and 0.70 (s, C-18). Several multiplets were observed between 1.0-2.5 due to methylenes and methines. These data suggested compound G to be 24-methyl Cholesterol (campesterol). The compound was acetylated using anhydrous pyridine Ac\(_2\)O mixture. The acetylated compound (34) was a monoacetate which was evident from the signals at \(\delta_H 2.03\) (s, 3H) and \(\delta_C 21.4\) and 170.5 (s), which confirms that natural product is campesterol (Pouchert and Behnke 1993).
Fig 3.7c: $^1$H NMR of campesterol (29)

Fig 3.7c: $^1$H NMR of acetate of campesterol

Fig 3.7c: $^{13}$C NMR of acetate of campesterol
Compound 31 was obtained as crystalline solid which was identified as cholesterol by comparison of its TLC (10% EtOAc, v/v; sprayed with methanolic H$_2$SO$_4$) with authentic sample.

Compound 31 was obtained as crystalline solid [α]$^D_{25}$ -21.1° (c 1.3, MeOH). Its ESI-TOF-MS gave pseudomolecular ion [M+Na]$^+$ at m/z 515.4124 [M+K]$^+$ 531.3912 revealing the molecular formula of compound to be 492. From the molecular weight and NMR data the molecular formula of 31 was determined to be C$_{30}$H$_{52}$O$_5$. $^1$H $^{13}$C and DEPT NMR (CD$_3$OD) (Fig 3.8a-c) spectrum of 31 corroborated the presence of four oxygenated carbon atoms, of which two were secondary hydroxyls ($\delta_H$ 3.44 brs, $\delta_C$ 67.4, C-3 and $\delta_H$ 3.99, $\delta_C$ 75.5, C-6) one tertiary hydroxyl ($\delta_C$ 75.8) and one tertiary acetoxy ($\delta_H$ 2.14, $\delta_C$ 22.4, 170.7). The acetoxy was considered to be tertiary on the basis that two oxymethine signals ($\delta_H$ 3.99 and 3.44) were below $\delta_H$ 4. The $^1$H shifts of the C-26 and C-27 methyl groups ($\delta_H$ 1.35) in 31 pointed to a substituent at C-25. Therefore, either hydroxyl or acetoxy must be present at this carbon. The presence of 3 singlets [$\delta_H$ 0.66 (3H), 1.06 (3H), and 1.35 (6H)] for 4 methyls, and two doublets [0.85 ($J$ = 6.7) and 0.87 ($J$ = 6.33)] suggested an ergostane skeleton similar to campesterol (29). Therefore, one of the secondary hydroxyl is established at C-3. There were no signals for alkene functionality in the molecule indicating absence of any double bond such as $\Delta^5$ in 29. The two remaining oxygenated carbons were assumed to be C-5 and C-6. The second secondary hydroxyl must be present at C-6, but the third hydroxyl which tertiary must be present on C-5, if acetoxy is present in C-25 or vice versa. In HMBC (Fig 3.8d) acetate methyl and the C-28 methyl protons showed correlations to a common carbon signal (C-25, 86.0) establishing acetoxy group at C-25, hence tertiary hydroxyl has to be present at C-5. Therefore the
structure of polyhydroxy sterol 31 is 24S-24-methyl cholestan-3β,5α,6β,25-tetraol-25-O-acetate. It is a known compound, which was isolated first from soft coral species e.g., Sarcophyton elegans (Raju et al., 1992) and then from a gorgonian Junceella junea (Rubinstein et al., 1976) and very recently from sponge Callyspongia sp (Rao et al., 2010). Its optical activity and spectral characteristics are in good agreement with the literature records also confirming the stereochemistry as shown in its structure 31. The spectral properties of the triacetate (35) obtained by Ac₂O/Py treatment of 31 was also in accord for 24S-24-methylcholestan-3β,5α,6β,25-tetraol-3,6,25-O-triacetate [¹H NMR (Fig 3.10)] (Radhika et al., 2004). Recently, compound 31 has been shown to exhibit good antimalarial activity. It showed good activity against chloroquine-resistant strain of P. falciparum, but was less active against chloroquine-sensitive strain (Rao et al., 2010)
Fig 3.9c: DEPT NMR of 32

Fig 3.9d: HMBC NMR of 32

Fig 3.10: $^1$H NMR of 35
The other two compounds 32 and 33 also appear to be polyhydroxy sterols from their NMR spectra but the exact positions of hydroxyls have not been yet determined. **Fig 3.11** is $^{13}$C NMR spectra of 32.

**III. Experimental**

**i) Biological material**

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

**ii) Extraction of metabolites from the soft coral**

The organism (2 Kg approx.) was lyophilized and cut into small pieces. It was sequentially extracted by sonicating with petroleum ether (300 ml x 5), CHCl$_3$ (300 ml x 4), n-BuOH (300 ml x 3) and MeOH (200 ml x 3). Organic extracts were evaporated under vacuum to yield concentrated extracts petroleum ether (3.8 g), CHCl$_3$ (2.3 g), n-BuOH (430 mg), and MeOH (470 mg).

**iii) Purification of metabolites from Petroleum ether extract**

Petroleum ether extract was chromatographed on Si gel column by eluting with increasing volumes of EtOAc in petroleum ether (0-50% v/v) and collected in 22 fractions. Each of these fractions were analyzed with TLC and IR and similar fractions were pooled together to make 5 fractions. TLC was developed in mixture of EtOAc-pet ether (10%, v/v), sprayed with H$_2$SO$_4$-MeOH (5%, v/v) and heated.
to 120°C to develop spots. On the basis of TLC, fractions 5-9, 10-11, 12, 16-17 and 18-20 were pooled, solvent evaporated. These fractions were labeled as A, B, C, D and E. Fraction B (65 mg) was purified on flash Si gel by eluting with EtOAc- petroleum ether (1-10%, v/v) to yield pure compound 28 (13 mg). Fraction C (85 mg) was flash chromatographed on Si gel using EtOAc- petroleum ether (0-12%, v/v) to give pure campesterol (29) (60 mg).

Cembrenoid 1 (28)- Colourless liquid, IR (KBr) \( \nu_{\text{max}} \) 2960, 2925, 2854, 1768, 1661, 1437, 1281, 1120, 947, 942, 833 cm\(^{-1}\). \(^1\)H NMR (300 MHz, CDCl\(_3\)) \( \delta \) 6.23 (d, J = 2.7, 1H, H-16), 5.70 (d, J = 2.1, 1H, H-16), 4.86-5.07 (m, H-5, H-9-H-13), 4.15 (t, J = 9.6 Hz, 1H, H-15a), 2.02-2.29 (H-4, H-7, H-8, H-11, H-12, H-15), 1.75, (s, 3H), 1.65 (s, 3H), 1.57 (s, 3H). \(^{13}\)C NMR (75 MHz, CDCl\(_3\)) 170.4 (C-2), 139.6 (C-3) 137.1 (C-6), 133.4 (C-14), 129.5 (C-10), 128.1 (C-5), 124.5 (C-9), 122.0 (C-16), 120.5 (C-13), 81.8 (C-15-), 44.9 (C-15), 38.7 (C-11), 38.1 (C-12), 33.7 (C-7), 24.48 (C-8), 24.41 (C-4), 17.4 (C-17), 16.5 (C-19) 15.9 (C-19).

Campesterol- White solid IR (KBr) \( \nu_{\text{max}} \) 3320, 2946, 2854, 1470, 1464 cm\(^{-1}\). \(^1\)H NMR (300 MHz, CDCl\(_3\)) 5.35 (m, 1H, H-6), 3.35 (m, 1H, H-3), 1.01 (s, 3H, H-19), 1.00 (d, 3H H-27), 0.91 (d, 3H, H-26), 0.85 (d, 3H, H-28), 0.77, (d, 3H, H-21), 0.70 (s, 3H, H-18). 1.20- 2.35 (sets of merged multiplets for rest of the methines and methylenes).

iv) Purification of CHCl\(_3\) extract

CHCl\(_3\) extract (200 mg) was fractionated on Si gel column by eluting with MeOH-CHCl\(_3\) (0-40%, v/v). Fraction 3 was purified on flash Si gel column by eluting with MeOH-CHCl\(_3\) (3-7%, v/v) to yield pure compound 31 (51 mg). Fraction 4 was purified on flash Si gel column by eluting with MeOH-CHCl\(_3\) (5-10%, v/v) to yield 32 (7 mg). Fraction 5 was purified on flash Si gel column by eluting with MeOH-CHCl\(_3\) (5-15%, v/v) to yield pure sterol 33 (11 mg).

Polyyhydroxy sterol (31): Colourless needles (MeOH-CHCl\(_3\)) m.p >200°C [\(\alpha\)]\(_D\)-25 19.9° (c 2.1, MeOH); FTIR (KBr): \( \nu_{\text{max}} \) 3512, 2992, 1732, 1418, 1262, 935 cm\(^{-1}\); \(^1\)H NMR (300 MHz, CD\(_2\)OD) \( \delta \) 3.44 (m, 1H, 3β-H), 3.99 (br s, 1H, 6β -H), 0.66 (s, 3H, 18-H), 1.06 (s, 3H, 19-H), 0.85 (d, \( J = 5.4 \)Hz, 3H, 21-H), 1.35 (s, 6H, 26,27-H), 0.87 (d, \( J = 6.33 \) Hz, 3H, 28 H), 1.98 (s, 3H, OCOCH\(_3\)), several
multiplets in the region 0.5-2.0. $^{13}$C NMR (75 MHz, CDCl$_3$): 30.3 (C-1), 22.3 (C-2), 67.0 (C-3), 38.0 (C-4), 75.4 (C-5), 75.8 (C-6), 34.5 (C-7), 34.0 (C-8), 41.9 (C-9), 36.1 (C-10), 21.0 (C-11), 40.0 (C-12), 45.4 (C-13), 55.8 (C-14), 24.0 (C-15), 28.0 (C-16), 42.6 (C-17) 12.0 (C-18), 16.5 (C-19), 36.1 (C-20), 18.8 (C-21), 32.1 (C-22), 23.1 (C-23), 42.6 (C-24), 86.0 (C-25), 22.7 (C-26), 27.6 (C-27), 14.3 (C-28), 22.3 (OCOCH$_3$), 170.7 (OCOCH$_3$) ESIMS [M+Na]$^+$ m/z 515.4214, [M+K]$^+$ m/z 531.3912.

References:


