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CHEMISTRY OF MARINE ORGANISMS OF SRI LANKA : LIPID AND TRITERPENOID CONSTITUENTS OF AN UNIDENTIFIED ALCYONACEAN AND AN UNIDENTIFIED HOLOTHURIAN

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Abstract : The major constituents of the lipid fraction of an unidentified alcyonacean is shown to be hexadecyl hexadecanoate and the sterols, gorgosterol, 23-demethylgorgosterol, brassicasterol, cholesterol and 24-methylcholesterol. The aglycone of the saponin from an unidentified holothurian species had been characterized as 22, 25-oxidoholothurinogenin.

1. Introduction

1.1 Alcyonaceae

The Order Alcyonaceae belongs to the subclass Octocorallia of the phylum Coelenterata. The animals belonging to this order are commonly known as soft corals. Soft corals are relatively abundant in Sri Lankan waters and have recently been the subject of extensive chemical investigations.[2,3] A large number of novel secondary metabolites which include sesquiterpenoids, diterpenoids and prostaglandins[11] have been isolated from soft corals. The sterols of the soft corals are particularly interesting because of the diversity of their side chains.[4] As the soft corals are known to contain unicellular algae, the zooanthellae, which are symbiotically associated with them, several studies to define the origin of the sterols have been reported.[6,12]

1.2 Holothroidea

The marine phylum Echinodermata can be divided into five classes: Crinoidea (sea lilies), Holothuroidea (sea cucumbers), Echinoidea (sea urchins), Ophiuroidea (brittle-stars) and Asteroidea (star fishes). The dried body wall of certain sea cucumber species (e.g. *Holothuria atra)* is a well known Chinese culinary delicacy traded under the name trepany or beche-de-mer. In contrast, the poisonous properties of sea cucumbers have also been known for many centuries in parts of the Pacific where mashed or chopped sea cucumbers have traditionally been used to poison fish. The

general toxicity of the sea cucumbers have been attributed to a group of compounds known as holothurins, a class of water soluble saponins. Although saponins have been isolated from several terrestrial plants, in the animal kingdom they have so far been found in Echinodermata with only sea cucumbers and star fishes having appreciable quantities. Curiously, holothurins have triterpenoid aglycones whereas star fish saponin (asterosaponin) have steroid aglycones.[4]

2. Results and Discussion

2.1 Analysis of the lipid part of the soft coral

The combined petroleum and chloroform extracts of the soft coral on flash chromatography on silica gel gave a waxy solid which on purification by repeated recrystallization afforded a t.l.c. homogeneous compound as colourless plates, m.p. 51 – 52°C. This compound had a molecular weight of 480 by mass spectrometry. The IR spectrum showed strong bands at 1730, 1240 and 1180 cm^{-1} indicating the presence of an ester carbonyl group. The ^1H N.M.R. spectrum confirmed the presence of the ester group by triplets at δ 4.00 (J = 6Hz) and 2.16 (J = 6Hz) which could be assigned to the protons of the methylene groups attached to the alkoxy oxygen and the carbonyl carbon of the ester group, respectively. The remaining broad band at δ 1.26 indicated the presence of a long alkyl chain and possible absence of other functional groups.

Treatment of this ester with lithium aluminium hydride ($LiAlH_4$) afforded a single alcohol indicating the 'symmetric' nature of the natural ester. The identity of the reduced product as l-hexadecanol (2) was established by comparing its mass spectrum with computer-recorded known mass spectra.[13] Mixed m.p. and Co-I.R. spectrum of this alcohol with an authentic sample prepared by $LiAlH_4$ reduction of hexadecanoic acid further confirmed its identity as hexadecanol. Saponification of the natural ester yielded hexadecanol. Further, the mass spectrum of the natural ester compared with that for hexadecylhexadecanoate (1).[14] The ester (1) was also synthesized from hexadecanoic acid.

Further elution of the above column afforded a sterol mixture constituting about 0.02% of dry weight of the concentrate from soxhlet extraction. The sterol mixture was silylated and subjected to gas chromatographic analysis. The sterols present were identified as brassicasterol (3a). 24-methylcholesterol (3b), gorgosterol (3c), cholesterol (3d), and 23-demethylgorgosterol (3e)(Fig. 1). Subsequent gas chromatographic-mass spectral (GC-MS) analysis confirmed the above findings (see Table 2). Relative concentrations of the sterols in the column fraction calculated from gas chromatographic trace were: gorgosterol (40%); 24-methylcholesterol (45%); brassicasterol (10%); cholesterol (5%), 23-demethylgorgosterol (trace).

Table 1. ^{1}H N.M.R. spectral data[a] (δ in $CDCl_3$ at 60 MHz) of 22, 25-oxidoholothurinogenin (4) and its 3-acetate (5)

Compound	3–H	7–H 11–H	22–H	C–4 $(Me)_2$	C–14 Me	C–10 Me	C–20 $(Me)_2$	C–25 Me
(4)	3.26 m	5.51 m 5.20 m	4.25 m	0.85 s 1.00 s	1.17 s	1.20 s	1.38 s	1.26 s
(5)	4.56 bt	5.40 m	4.20 m	0.91 s 0.99 s	1.13 s	1.23 s	1.26 s 1.28 s	1.33 s

[a]abbreviations: s, singlet; bt, broad triplet; m, multiplet

Cholesterol (3d) is the most common steroid found in marine organisms. Brassicasterol (3a) and 24-methylcholesterol (3b) have been reported to be present in some sponges, coelentrates, molluscs, crustaceans and echinoderms.[8] Gorgosterol (3c) first isolated from the gorgonian *Plexaura flexuosa* occurs in many coelentrates. The uncommon steroid 23-demethylgorgosterol (3e) which was initially obtained from the gorgonian *Gorgonia flabellum*[9] has been found in some alcyonacean species.[10]

Table 2. G.C.-M.S. data[a] of sterols isolated from the soft coral

Sterol	R.R.T./min[b]	Mass fragmentation (m/z)
Brassicasterol (3a)	1.17	398(M^+), 380($M^+ - H_2O$), 355, 300, 273(M^+ –side chain), 271, 255($M^+ - H_2O$- side chain).
24-methylcholesterol (3b)[c]	1.35	400(M^+), 367, 273(M^+-side chain), 301
Gorgosterol (3c)	2.43	426(M^+), 314, 300, 299, 271
Cholesterol (3d)	1.00	386(M^+), 372, 301, 273, 255
23-demethylgorgosterol (3e)	1.79	M.S. not recorded.

[a]Using Varian MAT–44(3% OV–17 column, 260°C)

[b]Relative Retention Time (R.R.T.) for cholesterol = 1.00

[c] Stereochemistry of C 24–Me was not determined.

2.2 22,25-Oxidoholothurinogenin

Hot methanolic extract of the dried holothurian was hydrolysed with HCl in methanol-water under reflux conditions. The reaction mixture was filtered and the precipitate was chromatographed over silica gel to yield a white solid which when recrystallised from chloroform-methanol yielded colourless needles. The ^{1}H N.M.R. spectrum (60 MHz) of this compound showed seven tertiary methyl singlets in the region δ 0.85 – 1.38. This along with the molecular ion peak observed at m/z 484 in its mass spectrum suggested that the compound could be a triterpenoid. The presence of a lactone carbonyl absorption band at 1750 cm^{-1} in the I.R. spectrum, and two olefinic hydrogens at δ 5.51 and 5.20 in its ^{1}H N.M.R. spectrum suggested it to be a holothurinogenin. Comparison of physical data of 4 and its acetylated product (5) with those reported from *Actinophyta agrossert*[5] confirmed the triterpenoid to be 22,25-oxidoholothurinogenin (4).

3. Experimental

3.1 Investigation of the soft coral

The unidentified soft coral collected at Nilaveli, on the East coast of Sri Lanka was stored in methanol. Methanol was decanted and the residue (dry wt. 1 kg) was extracted successively and exhaustively with hot light petroleum, hot chloroform and hot methanol. The light petroleum (29 g, 3%) and the chloroform (3.5 g, 0.35%) extracts were combined as there was no significant difference in their behaviour on T.L.C.

Flash chromatography of the above combined extract (10 g) over silica gel (T.L.C. grade 250 g) with chloroform containing increasing amounts of methanol furnished three fractions; hexadecyl hexadecanoate (1), a mixture of sterols and a fraction which mainly consisted of a diterpenoid (by mass spectroscopy). Further characterization of the last fraction was not possible due to lack of sufficient material.

3.1.1 Hexadecyl hexadecanoate (1)

The crude fraction containing (1) was recrystallised from light petroleum to obtain colourless plates (0.519 g; 0.17% from dry weight) m.p. 51 – 52°C (lit.[15] 51°C); I.R. ν_{max} (KBr) 2820, 2900, 1725, 1460, 1180, 725 cm^{-1}; ^{1}H N.M.R. δ (CCl_4, 60MHz) 4.00 (2H,t, J =6.0Hz), 2.16 (2H, t, J = 7.8Hz), 1.26 (methylene envelope); M.S. m/z (rel. int.) 480 (M^+, 18%) and 257 (1.0%).

3.1.2 Lithium Aluminium Hydride Reduction of (1) *to obtain hexadecanol* (2)

To an ice-cold solution of hexadecyl hexadecanoate (25 mg) in dry THF

(5ml), $LiAlH_4$ (5 mg) was added portion-wise. Usual work-up afforded the reduced product which was purified by preparative T.L.C. and recrystallised from acetone to yield hexadecanol (2) (21 mg; 34%) m.p. 53 – 54° (lit.[7] m.p. 49°C); IR ν_{max} (KBr) 3340, 2830, 2900, 1480 cm^{-1}; ^1H N.M.R. δ (CCl_4, 60MHz) 3.23(2H,t, J = 6Hz), 1.26 (methylene envelope).

3.3.3 Saponification of hexadecyl hexadecanoate (1) *to obtain hexadecanol* (2)

A mixture of the ester (62 mg) and methanolic KOH (7%, 1 ml) was refluxed for 3h. The reaction mixture was concentrated under *vacuo* and the residue was partitioned between ether and water. The ether layer was washed with water (3 x 10 ml). Evaporation of ether yielded a crude solid which was purified by preparative T.L.C. Recrystallisation from acetone yielded hexadecanol (35 mg, 58%) m.p. 53 – 54°C (lit.[7] 49°C); I.R. ν_{max} (KBr) 3340, 2830, 2900, 1480 cm^{-1}; ^1H N.M.R. δ (CCl_4, 60 MHz) 3.23 (2H,t, J = 6Hz), 1.26 (methylene envelope); M.S. m/z (rel. int.) 224 ($M^+ - H_2O$; 0.9%), 196, 182, 168, 154, 111, 97, 83, 69, 55, 43.

3.1.4 Synthesis of hexadecyl hexadecanoate (1)

Hexadecanoic acid (960 mg) was dissolved in dry ether (10 ml). $LiAlH_4$ (15 mg) was added portion-wise. The mixture was heated under reflux for 2h. The reaction mixture was cooled, acidified with dilute hydrochloric acid and extracted into chloroform (50 ml x 2). Evaporation of chloroform yielded a white solid (225 mg). The crude product (225 mg) and hexadecanoic acid (220 mg) were dissolved in acetone (20 ml), and conc. H_2SO_4 (1 ml) in acetone (5 ml) was slowly run down to the acetone solution along the wall of the flask. After heating under reflux for 4h the reaction mixture was worked-up in usual manner. Subsequent purification by preparative TLC and recrystallization from acetone afforded pure compound (1) (200 mg, 50%) m.p. 51–52°C; IR ν_{max} (KBr) (cm^{-1}) 2900, 2820, 1730, 1180, 760–720.

3.1.5 Gas Chromatographic-Mass Spectral analysis of the Sterol mixture

The above sterol mixture obtained as a T.L.C. homogeneous colourless crystalline solid was subjected to G.C.–M.S. analysis. The results of this analysis are depicted in Table 2.

3.2 Investigation of the Holothurian

The holothurian collected at Negombo, on the West coast of Sri Lanka was sun-dried and the dried material (397 g) was extracted exhaustively with hot light petroleum and then with hot methanol to yield 4.5 g (1.0%) and 20 g (5%) of extracts, respectively.

3.2.1 Isolation of 22,25-oxidoholothurinogenin (4)

Total methanol extract (20 g) in aqueous methanol (50%, 200 ml) was heated under reflux while a solution of dil. HCl (60%, 200 ml) was run down to the reaction mixture during 0.5 hours. Heating was continued for further 8 hours. The resulting precipitate (1.73 g) was filtered and chromatographed over T. L. C. grade silica gel to obtain pure 22, 25- oxidoholothurinogenin (4). Recrystallization from chloroform-methanol yielded needles (103 mg, 0.05%), m.p. 310 – 312°C (lit.[5] 315 – 316°C); $(\alpha)_D$ –22.5° (lit.[5] – 22.2°); I.R. ν_{max} (KBr) 3500 and 1750 cm^{-1}; see Table 2 for ^1H N.M.R. data.

3.2.2 22,25-Oxidoholothurinogenin-3-acetate (5)

22,25-Oxidoholothurinogenin (25 mg) was dissolved in dry pyridine (5 ml), acetic anhydride (2 ml) was added dropwise to the solution, and the reaction mixture was heated under reflux for 3 hours. Usual work-up of this mixture, yielded a white solid which on crystallization from chloroform-methanol yielded colourless needles of 22, 25-oxidoholothurinogenin-3-acetete (5); m.p. 288 – 289°C (lit.[5] 289 – 290°C); $(\alpha)_D$ + 6.7° (lit[5]+ 6.5°); I.R. ν_{max} (CCl_4) 3565 and 1770 cm^{-1}.

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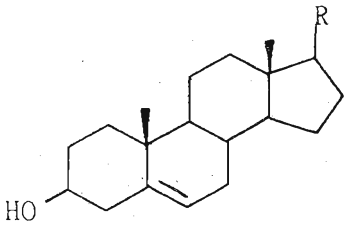

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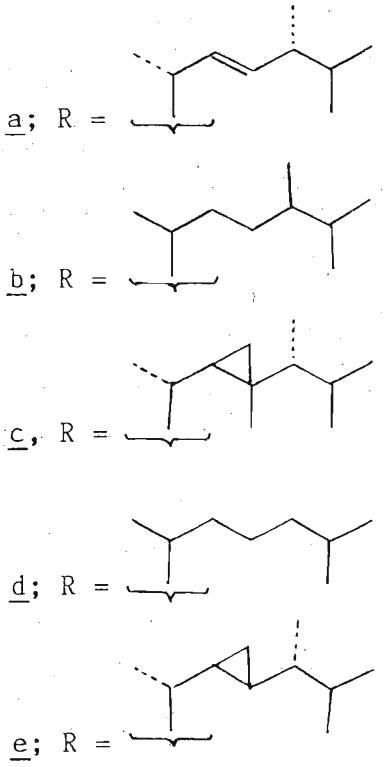
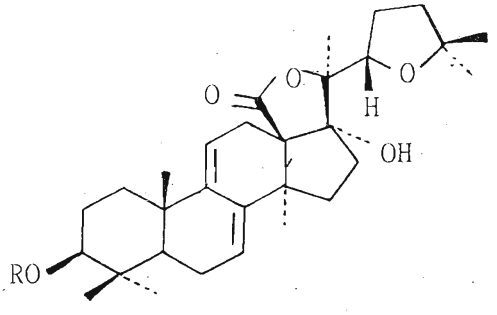


Fig. 1 Structures of sterols from the soft coral



(4); R = H

(5); R = Ac

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