CHAPTER-III  SECTION-II

5α-CHOLESTANE-3,6-DIONE, AN ANTIBACTERIAL SUBSTANCE FROM THE RED ALGA ACANTHOPHORA SPICIFERA
Substances from marine sources especially seaweeds have been used in folk medicine all over the world since ancient times. However, the development of modern chemical, pharmacological and engineering technology has facilitated the investigations and exploitation of hitherto untapped drug resource of the oceans. During last three decades, several bioactive compounds have been isolated from marine sources which include a wide spectrum of chemical metabolites e.g. organic acids, carbohydrates, proteins, amino acids, steroids, lipids, and enzymes.

Notable among these are prostaglandins, alginates, kainic acids, cephalosporin and fish toxins like tetradotoxin and saxitoxin. The potential use of many of these compounds as drugs has acted as an incentive for the intensification of research into these compounds and their synthetic analogues.

A great deal of chemical work has been done on Indian seaweeds during the last twenty five years. However, this work was confined mostly to the information on the mineral constituents, carbohydrates with special reference to alginates, agar and carrageenin and other organic chemicals. However, very little is known about the biomedical potential of Indian Ocean.

In order to obtain bioactive compounds from these marine sources a project was initiated at N.I.O. in collaboration
with C.D.R.I.*, Lucknow. In connection with this work, we have screened methanolic extracts of a number of marine organisms including the red alga *Acanthophora spicifera* belonging to the order Ceramiales and family *Rhodomelaceae*, for different pharmacological activities. This red alga has been reported to possess *in vitro* antimicrobial activity against *S. aureus*, *C. albicans* and *mycobacterium smegmates*\(^\text{38}\). *Acanthophora spicifera* (Vahl) Boergeesen is generally found in tropical and subtropical oceans. It was collected from the intertidal zone of Anjuna beach (Goa, west coast of India) during premonsoon period washed, air dried and powdered. About 3 kg of this powder was extracted thrice with 90% aqueous methanol. The combined extract was concentrated at reduced pressure to yield crude methanolic extract which was sent to C.D.R.I for screening.

*Acanthophora spicifera* has been extensively investigated for its mineral content\(^\text{39-41}\) but the literature on the isolation of specific organic constituents is scanty\(^\text{42}\). There being some studies on its evaluation for polysaccharides, proteins, amino acids\(^\text{43-45}\), fatty acids, lipids\(^\text{45-47}\) content. Tocopherol content has been reported by Jayasree et al\(^\text{48}\) and its ascorbic acid and dehydro ascorbic acid by Qasim and Barkati\(^\text{49}\). The screening results indicated that the crude extract exhibited promising (100%)

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antifertility activity. Follow-up study resulted in the location of the activity in petroleum ether and chloroform soluble fractions. In this section, we wish to report the results concerning the isolation and identification of a steroid, 5α-cholestane-3,6-dione (6a) from the active chloroform soluble fraction.

The marine sterols of the majority of red algae are C_{27} compounds, cholesterol (7) being the major component of most of them although in a few species desmosterol (8) has been reported to be present in substantial amount and may even be the major steroid.

Red algae also contains, though in minor amounts, C_{26}, C_{28} and C_{29} sterols and in some species the presence of Δ^{4} Cholestene-3-one (9) has been reported.

Since the active principle of Acanthophora spicifera was located in petroleum ether and CHCl_{3} soluble fractions, air dried seaweed (7 Kg) was extracted with CHCl_{3} and this extract was chromatographed over silica gel and the column eluted successively with solvents of increasing polarity. From the EtOAc-petroleum ether (10:90) eluents, a crystalline compound, m.p. 168-170° was isolated which appeared to be pure (TLC. single spot, Golden yellow colour with 2,4-dinitrophenyl hydrazine as spray reagent). Its ketonic nature was evident from the strong IR (Fig. 14) absorption at
(15a) \( \text{C}_5 = \beta \text{H}, \text{C}_6 = \beta \text{OH} \)

(15b) \( \text{C}_5 = \alpha \text{H}, \text{C}_6 = \alpha \text{OH} \)
γ max 1700 cm\(^{-1}\), there was no \(^1\)HNMR evidence for an aldehyde proton and no IR band for an hydroxyl group. It also showed in the IR spectrum the presence of \(\text{O} - \text{C-CH}_2\) functionality (γ max 1410 cm\(^{-1}\)).

The \(^1\)HNMR (Fig. 15) spectrum of the compound revealed that it was a steroid but it gave no reaction with the Lieberman-Burchard test thus indicating the absence of a 3β-OH group. It displayed signals at 0.68 (3H, \(\text{H}_{18}\)) and 0.98 (3H, \(\text{H}_{19}\)) for the two tertiary methyls, a signal at 0.7 was attributed to the secondary methyl at \(\text{C}_{21}\) and a signal for six protons at 0.85 was assigned to the secondary methyls situated at the end of side chain. Moreover, the spectrum demonstrated signals for seven protons resonating between 2.1-2.6 probably alpha to the \(\text{C=O}\) group. Signals due to olefinic hydrogen were absent in the \(^1\)HNMR spectrum. The presence of two ketonic groups was evident from its \(^{13}\)CNMR (Fig. 16) spectrum which displayed signals for this functionality at 208 and 210. In view of this data, it was concluded that the compound is a saturated keto-steroid.

The mass spectrum exhibited a base peak molecular ion at m/z 400 (M)\(^+\) corresponding to molecular formula \(\text{C}_{27}\text{H}_{44}\text{O}_2\) further confirming the saturated nature of the compound and the tetracyclic carbon skeleton with two ketonic groups. The mass spectrum also showed prominent peaks at m/z 385, 371, 287, 260, 246, 244, 231, 149, 137, 123, 109, 94, 79, 69 and
The peaks at m/z 287 (loss of side chain); 260, 246, (ring D cleavage); 109 (ring C cleavage); the peaks at 149 associated with fragments at 137 and 123 and 55 were indicative of a steroid belonging to the cholestane series with the two carbonyl groups in rings A and B. Since cholesterol is a precursor of this type of compound, one of the carbonyl group was placed at C3 and the other would be either at C6 or C7. As the melting point (168-70°) of the natural product agreed well with that reported for 5α-cholestan-3,6-dione (6a) (m.p. 171-72°), and since the mass spectrum did not show peaks at m/z 135 and 178 which are characteristic of 7-ketones, the second carbonyl was placed at C6. The sterochemistry at C5 was inferred as α from the presence of a prominent peak at m/z 371 (M⁺-29). 3,6-diketo-5β-steroids are known to show peaks at (M⁺-70) and (M⁺-96). No fragments corresponding to m/z 330 (M⁺-70) and 304 (M⁺-96) were observed in the mass spectrum of the diketosteroid from A. spicifera. The natural product was, therefore identified as 3,6-diketocholestane (6). The genesis of various fragments in the mass spectrum of 5α-cholestane-3,6-dione (6a) are shown in chart-1.

A definite proof of the assigned structure came finally from its synthesis by a known procedure. Cholesterol (7) was subjected to hydroboration oxidation to yield a mixture of 5α-cholestan-3β, 6α-diol (15a, 70%) and 5β-cholestan-3β,6β-diol (15b, 16%). Column chromatography over silica
Chart - 1
gel gave $5\alpha$-cholestane-3β,6α-diol, (15a), m.p. 213° which on oxidation with Sarett's reagent afforded $5\alpha$-cholestane-3,6-dione (6a), m.p. 171°, identical in all respects (Co TLC, IR, m.p., mix m.p. and $^1$HNMR) with the natural diketone isolated from the red alga *Acanthophora spicifera*. Cholesterol (7) was subjected to dichromate oxidation by known method\textsuperscript{60} to furnish $\Delta^4$-cholestene-3,6-dione (14), which was reduced by zinc and acetic acid to give $5\alpha$-cholestan-3,6-dione (6a) and its $5\beta$ isomer (6b). A pure sample of $5\alpha$-cholestan-3,6-dione (6a) could be obtained by careful chromatography.

An interesting feature which has been observed here is the relatively low concentrations of cholesterol present in the alga. This can be explained by it being utilized in the biosynthesis of the keto steroid. Also the presence of oxygenated sterols is known to inhibit cholesterol synthesis\textsuperscript{61}. It may be pointed out that this is the first report of the isolation of $5\alpha$-cholestan-3,6-dione (6a) from *Acanthophora spicifera*. To the best of our knowledge this substance has not been isolated before from any other source.

Besides the diketone mentioned above cholesterol (7), cholest-4-en-3-one (9), $11\alpha$-hydroxy-$5\alpha$-cholestan-3,6-dione (10)\textsuperscript{62} and aurantiamide (11), its acetate (12) and
diaaurantiamide acetate (13) have also been isolated from the same source.

Characterisation of trace constituents of the chloroform soluble fraction is in progress.

* Isolation and characterisation of these constituents has been carried out by a research colleague and hence the details are not given.
(6a): C₅ = αH
(6b): C₅ = βH

(7)

(9)

(12), R = COMe
(11), R = H

(13)

Constituents of red alga *Acanthophora spicifera*
EXPERIMENTAL

Preparation of the extract

Fresh *Acanthus illicifolius* pods (5 kg) were collected from the banks of Cumbarjua canal connecting the two estuaries of the river Mandovi and Zuari during the month of May. As such the pods were soaked in 90% aq. methanol at room temp. for 72 hrs. This process was repeated thrice and the combined extracts were concentrated to dryness under reduced pressure at 50°C. The concentrated extract was kept in a vacuum descicator for final drying, before it was tested for pharmacological activity.

Analgesic activity

This activity was studied in albino mice (Haffkine strain) of either sex weighing 25-30 g. Six mice fasted overnight were used per group.

Tail clip method

An artery clip with the jams covered with thin rubber tubing was applied to the base of the tail of the mouse. Only those animals which made repeated attempts to remove the clip within 15 seconds were used. Absence of any attempt to remove the clip within 30 seconds was taken as criterion of analgesia. Pethidine hydrochloride was used as a standard for
comparison. Different doses of the extract or pethidine were administered intraperitoneally (IP) 30 min before application of the clip.

**Writhing method**

An aqueous solution (v/v) of hydrochloric acid 0.5% was administered IP in the dose of 0.2 ml per mouse. The animals were observed continuously for 20 minutes for writhing syndrome. Sodium salicylate was used as a standard for comparison. Different doses of the extract or sodium salicylate were administered IP 30 minutes before the injection of HCl and the percent protective effect was noted.

**Anti-inflammatory activity**

This activity of the extract was studied in group of 6 albino rats (Haffkine strain) of either sex weighing 150 - 200 g. Oedema was induced by injection of 0.1 ml of 1% carrageenin into the hind paws of the rats according to the method of Winter et al\(^{34,35}\). The paw volume was measured before and 3 hr after the injection of carrageenin by the plethysmographic method of Buttle et al\(^{36}\). Phenylbutazone was used as a standard and saline was used as a control.

**Fractionation of the methanolic extract**

The crude methanolic extract was fractionated into petroleum ether, chloroform, n-butanol and water soluble
fractions. The follow-up investigations led us to locate the analgesic activity in chloroform soluble fraction and water soluble fraction.

**Isolation of BOA (1)**

The chloroform fraction dissolved in a minimum amount of chloroform adsorbed over silica gel, was placed on a chromatographic column containing silica in petroleum ether and eluted with progressively increasing polar mixture of petroleum ether - EtOAc. Fractions of 20 ml each were collected. Elution of the column with petroleum ether: EtOAc (90:10) yielded a slightly brownish crystalline compound which on repeated chromatography afforded colourless crystals of BOA (1), (688 mg, m.p. 138°C).

IR, \(\gamma\) max, Nujol, (Fig. 1): 3560, 2965, 1750, 1480, 1375, 1325, 1275, 1158, 1015, 960, 900, 850, 765, 750 and 710 cm\(^{-1}\)

\(^1\)HNMR: (200 MHz, \(\delta\), CDCl\(_3\)) (Fig. 2): 7.1-7.2 (4H, m), 10.1 (1H, s)

Mass spectrum: EI-MS, 70 ev (Fig. 3) - M\(^+\) 135 m/z 91, 79 and 64.

\(^13\)CNMR (200 MHz, CDCl\(_3\)) : 110.1, 110.2, 122.7, 124.2, 129.52, 143.99, and 156.2.

**Synthesis of BOA (1)**

O-aminophenol (1 g) and urea (0.6 g) were refluxed for 14 hr in dry pyridine. Usual work-up, afforded after
chromatography BOA (1, 0.98 g, m.p. 138°), identical in all respect with the natural product.

Isolation of glucosides

The aqueous fraction was chromatographed over silica gel filled in CHCl₃ and eluted with CHCl₃, MeOH: CHCl₃ and H₂O:MeOH. The MeOH:CHCl₃ eluent was rechromatographed, being eluted with increasing concentration of MeOH in CHCl₃. The fraction eluted in MeOH: CHCl₃ (20:80), gave a crystalline solid (B, 1.567 g, m.p. 214°C).

TLC solvent system MeOH:CHCl₃ (40:60). Spots were visualized with iodine vapour and vanilline: H₂SO₄. Surprisingly when a fluorescent TLC plate was used an additional compound was obtained which could be visualized only with fluorescent TLC plate and under short wavelength (254 nm) uv radiation, as purplish spot on greenish background.

The solid B on DCI indicated it to be a mixture of two major compounds. Separated by careful column chromatography over silica gel using gradually increasing concentration of MeOH: CHCl₃. The less polar was designated as B₁ (m.p. 158-60°C, with decomp.) and the more polar one as B₂ (m.p. 200°C).
Compound-B₁, (5)

UV MeOH, λ max 255, 286 nm.

IR, ν max, Nujol (Fig. 8): 3600, 2900, 1640, 1610, 1510, 1440, 1320, 1290, 1230, 1060, 1025, 1000 and 860 cm⁻¹

¹HNMR (200 MHz, D₂O, δ) (Fig. 9): 7.4 (1H, bs), 7.1 (3H, m), 5.9 (1H, s), 4.8 (1H, d, J = 7.7 Hz), 3.1-3.9 (6H, m).


¹³CNMR: (200 MHz, D₂O) (Fig. 10): 174, 164.6, 142.5, 127.6, 126.5, 120.4, 119.2, 104.6, 98, 79.01, 78.18, 75.5, 71.9 and 63.3.

Compound-B₂, (4)

UV MeOH, λ max 255, 280 nm.

IR, ν max, nujol (Fig. 11): 1660, 1680, 1600, 1455, 1375, 1300, 1270, 1210, 1150, 1130, 1070, 1020, 980, 880, 840 and 830 cm⁻¹

¹HNMR (200 MHz, D₂O, δ) (Fig 12): 7.3 (1H, bs), 6.95 (3H, m), 5.83 (1H, s), 4.55 (1H, d, J = 7.7 Hz), 3.0-3.8 (6H, m).

CNMR (200 MHz, D₂O) (Fig. 13): 143.1, 110, 128.1, 126.4, 119.962, 116.764, 104.518, 79.021, 78.140, 75.519, 71.875, 63.291 and 63.130.

Preparation of Δ⁴-cholestene-3,6-dione (14) from (7)

Commercial cholesterol (7, 12.5 g, 0.0325 mol) was dissolved in benzene (112.5 ml) by warming the solution and AcOH (112.5 ml) was added. The solution was cooled to 20°C. To this solution was added cold dichromate solution [(112.5 ml), prepared by dissolving Na₂Cr₂O₇ (32 g) in AcOH] whereupon thick orange paste of cholesteryl chromate (C₂₇H₄₅O₂) CrO₂ separates. It was kept in a refrigerator for 40-48 hr, the temperature soon dropped to 0°C and the dichromate dissolved in few hr.

The resulting brown solution was diluted with H₂O (250 ml) and extracted in petroleum ether (4 x 50 ml) and was washed well with H₂O. The combined organic layer was extracted with Claisen's alkali (4 x 50 ml) (Claisen's alkali extracts enedione as yellow enolate). The aqueous extract was neutralized by HCl (36%) in cold. The neutral solution was then extracted in ether (4 x 50 ml). The combined ethereal solution was washed with Na₂CO₃ solution (5%, 2 x 50 ml) followed by saturated NaCl solution (2 x 20 ml), dried and evaporated to dryness, to give yellow solid (6.5 g, 40%). This was recrystallised in boiling MeOH (50 ml) when Δ⁴-
cholestene-3,6-dione separates as thin yellow plates (m.p. 124-125°C, lit. 125°C\(^6\)).

**Preparation of 5α-cholestane-3,6-dione (6a) from (14)**

A mixture of \(\Delta^4\)-cholestene-3,6-dione (14, 3 g) and Zn dust (30 g) in glacial AcOH (350 ml) was refluxed for 18 hr. The mixture was diluted with benzene and filtered, the Zn was repeatedly washed with benzene. The filtrate was washed with \(\text{H}_2\text{O}\), dried and evaporated in vacuo. The product was analysed by TLC and was found to consist of a 3:1 mixture of 5α-cholestane-3,6-dione (6a) and 5β-cholestane-3,6-dione (6b). The products were separated by column chromatography (silica gel).

**Preparation of 5α-cholestan-3β,6α,6δ-diol (15a) from (7)**

Diborane was generated by adding a solution of NaBH\(_4\) (1.8 g, 47.7 mmol) in diglyme (90 cc) to a solution of BF\(_3\), Et\(_2\)O (15 g, 105.6 mmol) in diglyme (60 cc), under inert atmosphere (N\(_2\)). The diborane gas was passed into a solution of commercial cholesterol (7, 3 g) in dry THF (120 cc) during 3 hr by means of slow stream of N\(_2\). After an additional hr at room temperature, H\(_2\)O (20 cc) was added to destroy excess diborane. To the alkyl borane thus generated was added NaOH (3N, 25 ml) followed by dropwise addition of H\(_2\)O\(_2\) (30%, 25 ml) during 30 min maintaining the temperature throughout at 35°C. After an additional stirring for 1 hr, the reaction
mixture was saturated with NaCl (2 g) and the organic layer separated. The aqueous layer was extracted with benzene (3 x 25 ml). The combined organic layer was washed with H2O and dried, solvent evaporated in vacuo. The product was purified on column chromatography (silica gel), yielded $5\alpha$-cholestan-3$\beta$, 6$\alpha$-diol (15a, m.p. 215-217°C lit. m.p. 213-150°C, 70%) and $5\beta$-cholestan-3$\beta$, 6$\beta$-diol (15b, m.p. 197-199°C, lit 198-200°C, 16%).

IR, ν max, nujol (15a): 3350, 2960, 2910, 1475, 1385, 1175, 1065, 1050 and 965. cm$^{-1}$

Preparation of $5\alpha$-cholestane-3,6-dione (6a) from (15a)

$\text{CrO}_3$ (4 g) was added to dry pyridine (100 ml) slowly and with stirring till a solid complex was obtained. Then the compound (15a, 2 g) in pyridine (20 ml) was added dropwise while stirring and the mixture left overnight, poured in cold H2O, filtered and extracted with benzene (3 x 50 ml). The combined extracts were washed with dil HCl and then with H2O. Dried and removal of solvent furnished $5\alpha$-cholestan-3,6-dione (6a, m.p. 170°C, lit m.p. 173°C), yield (1.5 g).

IR, ν max, KBr (Fig. 14): 3000, 1730, 1485, 1445, 1405, 1395, 1360, 1350, 1320, 1270, 1250, 1175, 1130, 1100, 1035 and 1000. cm$^{-1}$
$^1$H NMR (90 MHz, CDCl$_3$, $\delta$) (Fig. 15): 0.68(3H, C$_{18}$), 0.7(3H, C$_{21}$), 0.85(6H, d), 0.98(3H, C$_{19}$), 2.1-2.6(7H).

$^{13}$C NMR (100 MHz, CDCl$_3$) (Fig. 16): 211, 209, 57.6, 56.7, 56, 53.6, 46.6, 43, 41, 39, 38.1, 38, 37, 36.9, 36.1, 35.7, 28, 24, 23.8, 22.7, 22.5, 21.7, 18.7, 12.5 and 12.

Mass spectrum : (EI-MS, 70 ev): (M$^+$ 400, m/z, 385, 371, 287, 260, 246, 244, 231, 149, 137, 123, 109, 94, 79, 69 and 55.
Fig. 1 IR Spectrum of BOA (I)
Fig. 2  $^1$HNMR spectrum of BOA (1)
Fig. 3 Mass Spectrum of BOA (1)
Fig. 4 $^{13}$CNMR Spectrum of BOA$^\circ$(1)
Fig. 5 $\text{NH}_3$–DCI mass spectrum of fraction–B
Fig. 6 ND$_3$-DCI mass spectrum of fraction B
Fig. 7 CH$_4$-DCI mass spectrum of fraction -B
Fig. 8  IR Spectrum of (5)
Fig. 9 $^1$HNMR Spectrum of (5)
Fig. 10 $^{13}$C NMR Spectrum of (5)
Fig. 11 IR Spectrum of (4)
Fig.-12 \textsuperscript{1}HNMR Spectrum of (4)
Fig. 13 $^{13}$CNMR Spectrum of (4)
Fig. 14 IR Spectrum of (6a)
Fig. 15 $^1$HNMR spectrum of (6a)
Fig. 16 $^{13}$CNMR spectrum of (6a)
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