

## Lipid contents of the sponge *Haliclona* sp.

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Several fatty acids, sterols, batyl alcohol and its analogs and an N-acylated sphingosine (ceramide) have been isolated from the lipid fraction of the extract of the sponge *Haliclona* sp. The major sterol is found to be cholesterol (54%), followed by 24(28)-didehydroaplysterol (21.2%), 24-ethylcholesterol (3.9%), 24-methylenecholesterol,  $\Delta^{22}$ , 24-methylcholesterol and  $\Delta^{22}$ , 24-ethylcholesterol (latter three together 14.7%). HPLC analysis of the glyceryl ethers of the long chain fatty alcohols lead to the isolation of 16:0 (35.3%, chymyl alcohol), 17:0 (2.45%), 18:0 (15.6%, batyl alcohol), 24:1 (17.25%) and 26:1 (3.54%). The most polar fraction is found to be a mixture of N-acylated sphingosines from which docosoyl ester amide of a dihydroxy sphingosine has been isolated in pure form.

The drab to pale yellow coloured sponge *Haliclona* sp., ubiquitous around the world, both in intertidal and subtidal waters has been reported to contain a large number of antibacterial compounds such as renieramycins renierone, mimosamycin, etc.<sup>1,2</sup>. Besides, this extract has also been reported to be toxic to mice and extremely haemolytic towards mouse red blood cells<sup>3</sup>. The latter activity, however, is destroyed within a few days on storage at 0°C.

As part of our investigations of the marine organisms in the seas around India for potential bioactive compounds, we decided to undertake a systematic chemical investigation of the secondary metabolites of the sponge *Haliclona* sp., collected from the intertidal waters of Gujarat coast. Our preliminary investigations indicated that the crude methanolic extract possess strong anti-viral activity against *Encephalomyocarditis* virus (75%) in *in vitro* tests besides being active against a broad spectrum of Gram positive and negative bacteria<sup>2</sup>. Herein we report the results of our studies on the other lipid fractions of this extract including fatty acids, sterols, batyl alcohol and its homologs and sphingosine derivatives.

The low polar lipid fraction containing fatty acids and methyl esters were re purified on a silica gel column. The methyl esters were analysed using capillary GC (SE 30, N<sub>2</sub>, 5 ml/min, 150-200°C). The individual fatty acids were identified from a comparison of its retention time with standards. The major fatty acids

identified in this manner were 16:1 (17.8%), 16:0 (18.5%), 18:1 (17.6%) and 18:0 (18.3%). A small portion of the fraction rich in sterols was subjected to HPLC separation (ODS, 250 × 8 mm MeOH, 2 ml/min, RI detection), yielding fractions A-D. Detailed <sup>1</sup>H and <sup>13</sup>C NMR spectral analysis of fraction A (R<sub>t</sub> = 32.34 min; M<sup>+</sup> = 398, 412) indicated it to be a mixture of three  $\Delta^5$ , 3 $\beta$ -hydroxysterols. Its spectrum had multiplets at  $\delta$  5.25 and 5.18 (2H each) besides two broad singlets at  $\delta$  4.72 and 4.66 (1H each) indicative of *cis*- $\Delta^{22}$ , 24-methyl,  $\Delta^{22}$ , 24-ethyl and 24-methylene moieties in their side chains. The  $\Delta^5$ , 3 $\beta$ -hydroxy system was evident from the characteristic doublet at  $\delta$  5.4 and multiplet at  $\delta$  3.5 respectively. Its <sup>13</sup>C NMR spectrum showed the corresponding vinyl carbon peaks at  $\delta$  156.9 and 106 (exomethylene), 138.1 and 126.3 ( $\Delta^{22}$ , 24-methyl), 136.1 and 131.9 ( $\Delta^{22}$ , 24-ethyl) for the above side chain unsaturations. The peaks at  $\delta$  140.8, 121.7 and 71.4 were indicative of  $\Delta^5$ , 3 $\beta$ -hydroxy moiety in the nuclei.

Fraction B (R<sub>t</sub> = 36.8 min, M<sup>+</sup> = 412) had an exomethylene group in the side chain as evident from the broad singlet (2H) at  $\delta$  4.67 and the corresponding peaks at  $\delta$  155.3 (s) and 107.8 (t) in its <sup>13</sup>C NMR and DEPT spectra. Besides this, the above spectra also indicated the presence of 5 methyl groups in the nucleus. Presence of a methyl triplet at  $\delta$  0.83 indicated that one of the methyl groups is part of an ethyl system. The position of the exomethylene group was finally fixed at C-24, leaving C-26 as possible site for further chain elongation based on the strong McLafferty peak at 314[M - C<sub>7</sub>H<sub>14</sub>]<sup>+</sup>, as expected for  $\Delta^{24}$ -sterols. This was further supported by the fragment ions at m/z 396[M - CH<sub>3</sub> - H]<sup>+</sup> and 382[M - C<sub>2</sub>H<sub>5</sub> - H]<sup>+</sup> in its EIMS. The peaks at m/z 271, 255, 229 and 213 confirmed the cholesterol-type ring structure of this compound. Based on the above results, the compound was identified as 24-methylene, 26-methylcholesterol (24, 28-didehydroaplysterol). Fraction C (R<sub>t</sub> 42.4 min, M<sup>+</sup> = 386) was found to be cholesterol from its <sup>1</sup>H NMR and mass spectral data. Similarly, fraction D (R<sub>t</sub> = 45.4, M<sup>+</sup> = 414) was identified as 24-ethylcholesterol from its spectral data.

According to De Rosa *et al.*, aplystane sterols are restricted to sponges belonging to the order *Verongidaea*<sup>4</sup>. Later, Bergquist *et al.*, reported 24, 28-didehydroaplysterol from *Haliclona* sp.<sup>5</sup>. Our finding of the same sterol from a similar species from a different habitat further refutes De Rosa's claim. Yet another

school of thought questions the very origin of these sterols. Thus recent biosynthetic studies using radiolabelled acetate, methionine, mevalonate, etc., have shown that the sponge *Verongia aerophoba* is incapable of biosynthesising sterols of aplystane skeleton<sup>6</sup>. Based on this study, it has been suggested that probably these sterols are of planktonic origin.

The next fraction was found to be a mixture of glyceryl ethers of long chain alcohols. This was collected separately and a portion of it was purified on HPLC (ODS, MeOH, 1 ml/min) yielding 5 major compounds. All of them had almost identical <sup>1</sup>H and <sup>13</sup>C NMR spectra, similar to that of batyl alcohol. Thus presence of four oxygenated carbons, in the region 64-72 ppm, three of which are CH<sub>2</sub>'s while the fourth is a CH, besides the bunch of methylene signals around 29 ppm and the lone CH<sub>3</sub> signal at 14 ppm were all characteristic of batyl alcohol and its analogs. The first three compounds (R<sub>f</sub>s = 17.7, 20.3 and 22.6 mins) were saturated and were identified as glyceryl ethers of 16:0, 17:0 and 18:0 long chain fatty alcohols from their EIMS results (M<sup>+</sup> = 316, 330 and 344 respectively). <sup>1</sup>H NMR spectra of the remaining two compounds (R<sub>f</sub>s = 36.5 and 50.3 min respectively) also had identical features. In addition they also had signals at δ 5.36 (2H, t) and 2.0 (4H, m) indicating the presence of additional *cis* double bonds in the aliphatic chain. This was confirmed by their <sup>13</sup>C NMR signals at δ 129.85 and 27.16 (vinyl and allyl carbons). A *trans* double bond would have shifted the allylic carbon signal further downfield to 32 ppm. Their molecular weights were determined to be 426 and 454 by EIMS suggesting the fatty chains to be 24:1 and 26:1 respectively.

The last fraction from the original silica gel fraction was found to be a mixture of N-acylated sphingosines (ceramides). Careful separation of this mixture on another silica gel column using hexane-CHCl<sub>3</sub>-MeOH systems yielded about 50 mg of a pure compound having strong IR absorptions at 3450 and 1630 cm<sup>-1</sup> indicating the presence of hydroxyl and amide groups in the molecule. Its <sup>1</sup>H NMR spectrum suggested the presence of amide (δ 6.4, 1H, d, J = 7.2 Hz, CONH), *trans* double bond [δ 5.7 (1H, dt, J = 15.4 & 6.4 Hz) and 5.5 (1H, dd, J = 15.4 & 6.7 Hz)], and at least three oxygen or nitrogen-bearing carbons [δ 4.2 (1H), 3.8 (2H) and 3.65 (1H)]. This was further supported by its <sup>13</sup>C NMR and DEPT spectra [175.4 (NHCO), 134.4 (d), 129.7 (d), 73.68 (d), 61.92 (t), 54.94 (d) etc.]. These data and extensive decoupling studies starting from the amide signal at δ 6.4, established the structure as dihydroxysphingosine. The absolute stereochemistry has been tentatively assigned as D-erythro from biogenetic considerations.

This was confirmed by ASIS experiment, using Py-d<sub>5</sub> as the solvent. This led to a downfield shift of the NH signal to 8.25 ppm, merging of the vinyl signals into a complex triplet at 6 ppm besides further spreading of the four proton signals attached to the hydroxyl and amide-bearing carbons between 4 and 5 ppm (see experimental). FABMS revealed the molecular weight of the compound to be 691, corresponding to the molecular formula C<sub>45</sub>H<sub>48</sub>NO<sub>3</sub>. The strong McLafferty peak at m/z 411 and 393 (411-H<sub>2</sub>O) suggested the acyl group to be C<sub>22:0</sub>. This was confirmed by the alkaline hydrolysis of the compound followed by separation of the acidic component from the mixture, yielding C<sub>22:0</sub> acid as the sole product as shown by its <sup>1</sup>H NMR and CIMS data. The structure of the compound was thus deduced to be *n*-docosoyl sphingosine.

N-acylated sphingosines are reported to be active against *Semiliki forest virus* (SFV)<sup>7</sup> and *Encephalomyocarditis virus* (EMCV)<sup>8</sup> both *in vivo* and *in vitro* tests. The strong anti-viral properties of the crude methanolic extract of this sponge against EMCV (*in vitro*) is also attributable to the sphingosine derivatives present in the extract.

### Experimental

HPLC separation was carried out on a Spectraphysics Model 8800 fitted with a RI detector and ODS column (5 μm, 250 × 8 mm) using 100% MeOH as mobile phase. The IR spectra were recorded on a PE FTIR Model 1650. <sup>1</sup>H NMR and <sup>13</sup>C NMR were recorded on a Bruker WM 200 MHz instrument. CIMS was obtained on a Biospect mass spectrometer using CH<sub>4</sub> as the reagent gas at a source temperature of 180°C. EIMS was recorded on a Finnigan MAT-90 spectrometer at 70 eV and at a probe temperature of 150°C and source temperature of 250°C.

### Animal material

The sponge *Haliclona* sp. was collected from the Gujarat coast and a voucher specimen is kept in the animal museum at NIO, Goa.

### Extraction and isolation of compounds

The animals, collected from the intertidal regions of Gujarat coast in Western India, were immediately stored in MeOH and transported to the laboratory; where the solvent was drained off, filtered and concentrated to yield about 300g of crude extract. The extract was resuspended in 10% aq. MeOH and successively extracted with hexane (110g), EtOAc (20g), and *n*-BuOH (80g). The hexane fraction, rich in lipids, was chromatographed over silica gel using hexane-EtOAc solvent systems, yielding five main fractions, viz., methyl esters of fatty acids and free fatty

acids (25 g), sterols (20 g), fraction rich in aromatic alkaloids, batyl alcohol and its homologs and finally a very polar fraction eluting with 80-100% EtOAc, rich in *N*-acylated sphingosines (5 g). The methyl esters of fatty acids were reperfired over another silica column and analysed using HP 5880 A model GC (SE 30 capillary column, 12.5 meters long, carrier gas N<sub>2</sub>). The individual compounds were identified from a comparison of its retention time with that of known standards.

The sterol fraction was purified on HPLC (ODS, 5 µm, 250 × 8 mm, RI detector) with MeOH (2 ml/min) as eluant resulting in the isolation of various compounds. The polar mixture, rich in batyl alcohol was further purified on HPLC (ODS, MeOH, 2 ml/min, RI detector) yielding five major compounds, which were identified as C<sub>16:0</sub>, C<sub>17:0</sub>, C<sub>19:0</sub>, C<sub>24:1</sub> and C<sub>26:1</sub> fatty alcohol-glycerol ethers characterized from their spectral data.

Careful silica gel column chromatography of the most polar fraction yielded ~50 mg of a pure sphingosine derivative, m.p. 70°C. IR (neat): 3450, 2860, 1630, 1540, 1460, 1380 and 1040 cm<sup>-1</sup>; <sup>1</sup>H NMR (CDCl<sub>3</sub>): δ 6.4 (1H, d, J = 7.2 Hz), 5.70 (1H, dt, J = 15.4, 6.4 Hz), 5.46 (1H, dd, J = 15.4, 6.7 Hz), 4.2 (1H, m, 3-H), 3.8 (2H, m, 1-H, 2-H), 3.65 (1H, m, 1-H), 2.2 (2H, t, J = 7.1 Hz), 2.0 (2H, m), 1.6-1.0 (70 H, m), 0.88 (6H, t, J = 6.85 Hz, terminal methyls); <sup>1</sup>H NMR (Py-d<sub>5</sub>): δ 8.3 (1H, d, J = 8.09 Hz, NHCO), 6.0 (2H, m, 4 & 5-H), 4.83 (1H, t, J = 6.05 Hz, 3-H), 4.70 (1H, m, 2-H), 4.43 (1H, dd, J = 10.75, 5.1 Hz, 1-H), 4.28 (1H, dd, J = 10.75, 4.2 Hz, 1-H), 2.45 (2H, t, J = 7.5 Hz, COCH<sub>2</sub>), 2.07 (2H, m, 6-H), 1.9-1.0 (70H, m), 0.87 (6H, t, J = 4 Hz); <sup>13</sup>C NMR (CDCl<sub>3</sub>): 175.4 (s), 134.4 (d), 129.7 (d), 73.68 (d), 61.92 (t), 54.94 (d), 39.09 (t), 36.65 (t), 32.31 (t), 31.9 (t), 29.66, 29.6, 29.5, 29.37, 29.3, 29.2, 27.9, 25.75, 22.6, 22.5 (all triplets) and 13.9 (q); <sup>13</sup>C NMR (Py-d<sub>5</sub>): δ 172.86 (s), 131.74 (d), 131.64 (d), 72.76 (d), 61.59 (t), 56.25 (d), 36.27, 32.12, 31.52, 29.39, 29.16, 29.0, 25.81, 22.32, 22.17 (all triplets) and 13.65 (q); FAB MS: 691 [M<sup>+</sup>, weak], 644 [M - 18 - 29]<sup>+</sup>, 631 [M - 18 - 42]<sup>+</sup>, 602 [M - 18 - 71]<sup>+</sup>, 588 [M - 18 - 83]<sup>+</sup>, 574 [M - 18 - 97]<sup>+</sup>, 560

[M - 2 × 18 - 83]<sup>+</sup>, 504 [M - 2 × 18 - 139]<sup>+</sup>, 490 [M - 2 × 18 - 153]<sup>+</sup>, 448 [M - 2 × 18 - 195]<sup>+</sup>, 421 [M - 2 × 18 - 222]<sup>+</sup>, 411 {[M - 280]<sup>+</sup>, McLafferty fragment} and 393 [M - 18 - 280]<sup>+</sup>.

#### Hydrolysis of the ceramide

A small portion (10 mg) of the sphingosine derivative was refluxed in a water bath with 5% KOH (2 ml) for 6 hr. The reaction mixture was then cooled, diluted with H<sub>2</sub>O (5 ml) and acidified with 2 N HCl. The free acid was extracted with hexane (3 × 5 ml). The organic layers were then combined, dried and concentrated; <sup>1</sup>H NMR (CDCl<sub>3</sub>): δ 2.2 (2H, t, J = 7 Hz), 1.2-1.0 (38 H, m) and 0.85 (3H, t, J = 6.5 Hz); CIMS (CH<sub>4</sub>): m/z 341 [M + H]<sup>+</sup>. The <sup>1</sup>H NMR spectrum conclusively proved the long chain and saturated nature of the acid, its molecular formula being deduced from the mass spectral data.

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