Hymenosulphate, A Novel Sterol Sulphate with Ca-Releasing Activity from the Cultured Marine Haptophyte *Hymenomonas* sp.

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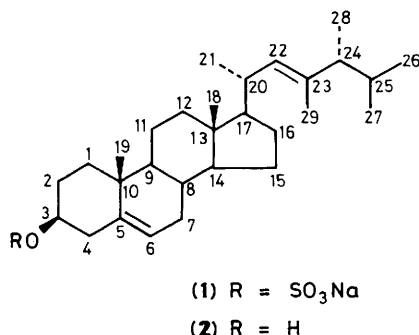
A novel sterol sulphate, hymenosulphate (1), with potent Ca-releasing activity in sarcoplasmic reticulum (SR), has been isolated from the cultured marine haptophyte *Hymenomonas* sp. and the structure elucidated on the basis of spectroscopic data and chemical reactions. This is the first isolation of a sterol sulphate from marine microalgae.

The Haptophyceae are microscopic, unicellar algae, which are widely distributed in the oceans and often constitute a major proportion of marine phytoplankton.[1] Although symbiotic or ingested marine micro-organisms are of considerable current interest as a source of compounds with useful bioactivity,[2] few studies have been performed on the chemistry and the biological activity of the metabolites of haptophycean algae.† During our research for bioactive substances from marine microalgae,[2,3] we have studied the chemical constituents of a laboratory-cultured haptophyte *Hymenomonas* sp. In this paper we report the isolation and structure elucidation of hymenosulphate (1), a new C_{29}-sterol sulphate with potent SR (sarcoplasmic reticulum) Ca-releasing activity[4] from this microalga.



Results and Discussion

The haptophyte *Hymenomonas* sp. was isolated from an unidentified Okinawan stony coral. The microalga was grown unialgally in a sea water medium enriched with Provasoli's ES supplement[2] and the cultured cells were harvested by centrifugation (*ca.* 400 g of cells from 1 000 l of culture). The extracts of the harvested cells with methanol–toluene (3:1) were partitioned between toluene and water. The aqueous layer was successively extracted with chloroform. The SR Ca-releasing activity was found in the toluene-soluble portion, which was chromatographed on a LH-20 column [MeOH–$CHCl_3$ (1:1)] followed by a silica gel column [$CHCl_3$–1-BuOH–AcOH–H_2O (1.5:6:1:1)] to give hymenosulphate (1) in 0.007% yield (wet weight). The major components of the toluene layer proved to be glycolipids such as mono- and di-galactosyldiacylglycerols[5] (3) and (4), respectively, while the chloroform layer was found to contain mainly octadecatetraenoic acid[6] (5) together with a small amount of monogalactosylmonoacylglycerol (6).[6,7]

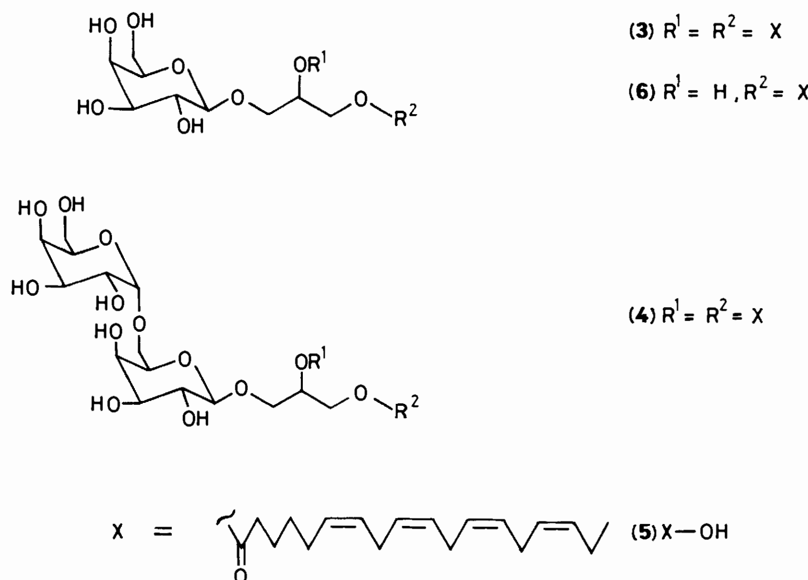
Hymenosulphate (1), m.p. 247—250 °C, $[\alpha]_D^{22}$ −23° (*c* 0.1 in MeOH), gave a quasi-molecular ion peak at *m/z* 491 (M^- − Na) in its negative fast-atom bombardment mass spectrum (f.a.b.m.s.). The ^1H and ^{13}C n.m.r. spectra of compound (1) indicated the presence of seven methyl groups and two trisubstituted double bonds. Compound (1) remained unchanged on reaction with Ac_2O–pyridine, CH_2N_2, $NaBH_4$, or KOH–MeOH. However, treatment in refluxing methanolic hydrochloric acid afforded a less polar product (2), m.p. 150—152 °C. The molecular formula of compound (2) was established to be $C_{29}H_{48}O$ by high-resolution electron impact mass spectroscopy (e.i.m.s.) [*m/z* 412.3698 (M^+), Δ −0.7 mmu]. The difference in molecular weight between compounds (1) and (2), along with the observation of the remarkable change in the polarity [R_F values on silica gel t.l.c. developed with methanol–chloroform (2:8); (1) 0.23 and (2) >0.9] suggested the presence of a sulphate group in (1), which was supported by the i.r. absorption[8] of (1) at 1 250 and 1 220 cm^{-1} and confirmed by a sodium rhodizonate test[9] as well as ion chromatography of sulphate ions liberated by solvolysis.[10] The e.i.m.s. analysis of compound (2) revealed that it was a C_{29}-sterol with an unusual C_{10} side-chain [*m/z* 271 (M^+ − $C_{10}H_{21}$); base peak]. By comparison of the ^1H n.m.r. data with those in the literature[11] this sterol (2) was identified as (24*R*)-23, 24-dimethylcholesta-(22*E*)-5,22-dien-3β-ol, which was previously obtained from some marine organisms.[†,11,12] Thus the structure of hymenosulphate ($C_{29}H_{47}NaO_4S$) was established as (1).

Hymenosulphate (1) is the first sterol sulphate from marine microalgae, although several sterol sulphates have been isolated from marine sponges.[13] In the SR,[4] the Ca-releasing activity of hymenosulphate (1) was ten times more potent than that of caffeine, a well known Ca-releaser. The glycolipids (3), (4), and (6) exhibited inhibition of Na^+, K^+-ATPase activity[14] with an IC_{50} value of 2 × 10^{-5}M each.

Experimental

M.p.s were determined with a Yanagimoto micro melting point apparatus and are uncorrected. Optical rotations were measured on a JASCO DIP-360 polarimeter. I.r. spectra were obtained with a Hitachi 260-50 spectrometer. ^1H and ^{13}C N.m.r. spectra were recorded on a Bruker AM-500 spectrometer. Mass spectra were obtained with a JEOL HX-100 spectrometer. Wako C-300 silica gel (Wako Pure Chemical) was used for glass

† The composition of sterols and fatty acids in four marine haptophycean algae has been examined by g.c.–m.s. analysis: J. K. Volkman, D. J. Smith, G. Eglinton, T. E. V. Forsberg, and E. D. S. Corner, *J. Mar. Biol. Assoc. U. K.*, 1981, **61**, 509.



column chromatography. T.l.c. was carried out on Merck silica gel GF_{254}.

Isolation.—The haptophyte *Hymenomonas* sp. was obtained from an unidentified cylindrical stony coral collected at Sesoko Island, Okinawa. The isolation of the microalga was performed by shaking the coral after a rinse with sterilized sea water. The alga was mass cultured in the laboratory by the procedure previously described.[2] The alga (408 g, wet weight) harvested from the culture (1 040 l) by centrifugation was extracted with methanol–toluene (3:1; 500 ml × 3). After addition of 1M aqueous NaCl (750 ml), the mixture was extracted with toluene (250 ml × 4) and the aqueous layer was then extracted with chloroform (250 ml × 4).

The toluene-soluble fraction was evaporated under reduced pressure to give a crude residue (9.7 g), part of which (1.8 g) was subjected to gel filtration on Sephadex LH-20 (2.1 × 110 cm) eluted with chloroform–methanol (1:1). Evaporation of the fraction eluting between 130—160 ml gave a residue (620 mg), part of which (130 mg) was further separated by silica gel column chromatography (1.7 × 34 cm) with methanol–chloroform (5:95; 300 ml, 10:90; 100 ml, and 25:75, 200 ml) as eluant to afford monogalactosyldiacylglycerol (**3**) (27 mg), f.a.b.m.s. *m/z* 771 (M^+ + H) in the 160—220 ml fraction and digalactosyldiacylglycerol (**4**) (22 mg), f.a.b.m.s. *m/z* 933 (M^+ + H) in the 370—420 ml fraction. The acid part of both glycolipids was identified as being mainly octadecatetraenoic acid after alkaline hydrolysis. Galactose was also identified by g.l.c. after methanolysis and trimethylsilylation. The fraction (33 mg) eluting between 430—480 ml of the Sephadex LH-20 column described above was purified by flash chromatography on a silica gel column (1.8 × 36 cm) eluted with chloroform–butan-1-ol–acetic acid–water (1.5:6:1:1) to give, in the 120—150 ml fraction, hymenosulphate (**1**) (6 mg), m.p. 247—250 °C (from MeOH); $[\alpha]_D^{22}$ −23° (*c* 0.1 in MeOH); i.r. (KBr) 1 575, 1 410, 1 250, 1 220, 1 065, 995, 860, and 805 cm^{-1}; δ_H [$CDCl_3$–CD_3OD (1:1)] 5.39 (1 H, d, *J* 4.9 Hz, 6-H), 4.91 (1 H, d, *J* 9.6 Hz, 22-H), 4.23 (1 H, m, 3-H), 2.41 (1 H, m, 20-H), 1.53 (3 H, s, 29-H_3), 1.04 (3 H, s, 19-H_3), 0.96 (6 H, d, *J* 6.7 Hz, 21- and 28-H_3), 0.86 (3 H, d, *J* 6.6 Hz, 26-H_3), 0.80 (3 H, d, *J* 6.6 Hz, 27-H_3), and 0.74 (3 H, s, 18-H_3); δ_C [$CDCl_3$–CD_3OD (1:1)] 140.70, 135.86, 132.40, 123.00, 79.76, 57.52, 57.46, 50.92, 50.90, 42.81, 40.34, 39.76, 37.82, 37.12, 35.15, 32.54, 32.51, 31.39, 29.38, 28.47, 24.81, 22.02, 21.62, 20.98, 20.39, 19.62, 17.26, 13.45, and 12.57; f.a.b.m.s. (negative; glycerol as matrix) *m/z* 491 (M^- − Na); f.a.b.m.s. (positive; diethanolamine as matrix) *m/z* 725 (M^+ + diethanolamine × 2 + H).

The chloroform-soluble fraction, after evaporation, gave a residue (0.25 g), part of which (68 mg) was separated by silica gel column chromatography (1.7 × 25 cm) with methanol–chloroform (1:9) as eluant to give octadecatetraenoic acid (**5**) (34 mg) in the 40—60 ml fraction and monogalactosylmonoacylglycerol[6] (**6**) (3 mg), f.a.b.m.s. *m/z* 513 (M^+ + H) in the 190—270 ml fraction.

Acid Hydrolysis of Hymenosulphate (**1**).—Hymenosulphate (**1**) (3 mg) was heated in 2M HCl (2 ml) and MeOH (2 ml) under reflux for 1 h. After cooling, the reaction mixture was extracted with chloroform (5 ml × 3). The combined chloroform layers were purified by silica gel column chromatography (0.7 × 7 cm) with methanol–chloroform (4:96) as eluant to give (24*R*)-23,24-dimethylcholesta-(22*E*)-5,22-dien-3β-ol (**2**) (1 mg), m.p. 150—152 °C (lit.,[11] 156—158.5 °C); $[\alpha]_D^{22}$ −50° (*c* 0.5 in $CHCl_3$) (lit.,[12] $[\alpha]_D$ −53.7°); δ_H($CDCl_3$) 5.35 (1 H, m, 6-H), 4.89 (1 H, d, *J* 9.6 Hz, 22-H), 3.53 (1 H, m, 3-H), 1.51 (3 H, s, 29-H_3), 1.02 (3 H, s, 19-H_3), 0.94 (6 H, d, *J* 6.8 Hz, 21- and 28-H_3), 0.85 (3 H, d, *J* 6.5 Hz, 26-H_3), 0.79 (3 H, d, *J* 6.7 Hz, 27-H_3), and 0.72 (3 H, s, 18-H_3); e.i.m.s. *m/z* (relative intensity, %) 412 (M^+), (94), 394 (95), 369 (18), 351 (22), 341 (14), 323 (13), 300 (83), 282 (25), 271 (100), 255 (82), and 253 (36) (Found: M^+, 412.3698. Calc. for $C_{29}H_{48}O$: *M*, 412.3705).

Detection of Sulphate Ions by Ion Chromatography.—Hymenosulphate (**1**) (1.1 mg) was dissolved in pyridine–dioxane (1:1) (1 ml) and the solution was heated at 120 °C for 4 h. The solution was evaporated, the residue was suspended in water, and the suspension was loaded on a SEP-PAK C_{18} cartridge column (Waters Associates). The cartridge column was first washed with water which eluted SO_4^{2-}, then with MeOH which yielded desulphated sterol. Quantitative determination of SO_4^{2-} was carried out by h.p.l.c. on a YMC-Pack AM-314 (ODS) [Yamamura Chemical, 7 × 300 mm] with an aqueous solution containing 1.0 mM-tetrabutylammonium hydroxide and 0.8 mM-benzene-1,3,5-tricarboxylic acid (flow rate 1.0 ml min^{-1}). Elution of the ions was monitored at 280 nm. A calibration curve prepared with Na_2SO_4 was used for quantitative determination. The sulphate ion was detected at R_t 4.4 min. This h.p.l.c. analysis showed that 0.73 mol of SO_4^{2-} was liberated from one mole of compound (**1**) by solvolysis.

Biological Assay.—The extravesicular Ca^{2+} concentration in sarcoplasmic reticulum was monitored with a Ca^{2+} electrode prepared by the method of Tsien and Rink with modifications.[4] Na^{+}, K^{+}-ATPase assay was carried out as previously described.[14]

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