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STARFISH SAPONINS, PART 18.[1] STEROIDAL GLYCOSIDE SULFATES FROM THE STARFISH *LINCKIA LAEVIGATA*[2]

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ABSTRACT.—The asterosaponins of the Pacific starfish *Linckia laevigata* have been separated into five compounds. Four of them are known: thornasteroside A (**1**), marthasteroside A_1 (**2**), ophidianoside F (**3**), and maculatoside (**4**). The fifth minor component, named laevigatoside, is new and has been characterized as 6α-*O*- { β-fucopyranosyl (1↦2)-α-arabinopyranosyl (1↦4)-[β-quinovopyranosyl (1↦2)] β-quinovopyranosyl (1↦3)-β-quinovopyranosyl } 20-hydroxy-23-oxo -5α-cholest-9(11)-en-3β-yl sodium sulfate (**5**).

The number of reported structures of asterosaponins, "sulfated steroidal glycosides," is rapidly growing. Following the structure elucidation of thornasteroside A, the major *Acanthaster planci* saponin, by Kitagawa and Kobayashi in 1978 (1), a number of novel structures have been recently described (2-10). All these compounds have common structural features. Thornasterol A, 3β,6α,20ξ-trihydroxy-5α-cholest-9(11)-en-23-one (11), has been the most widely reported steroidal aglycone. Recently we have found saponins from *Hacelia attenuata* and *Ophidiaster ophidianus* containing a lower homologue, 24-nor-thornasterol A, with the apparent extrusion of the carbon-24 atom from the standard C_8 side chain (9,12). Marthasterone, 3β,6α,dihydroxy-5α-cholesta-9(11),24(25)-dien-23-one, and its 24(25)-dihydroderivative, with the same steroidal nucleus as thornasterol A and 24-nor-thornasterol A, have been found in saponins from *Marthasterias glacialis* (8) and *Lucidia maculata* (7,10).

There is also a close resemblance in the carbohydrate portions of these asterosaponins. In all the thornasterol A- containing saponins described, a β-D-quinovopyranosyl (1↦2)-β-D-xylopyranosyl (1↦3)-β-D-quinovopyranosyl moiety is attached to C-6 of the aglycone. Further substitution (two or three more monosaccharides) is at C-4 of the xylose residue and a sulfate group is at C-3 of the aglycone (See formulae **1-4**). The carbohydrate portions of the described saponins containing marthasterone or dihydromarthasterone are remarkably akin to the carbohydrate moieties of the thornasterol A-containing saponins. The deviation is that in these saponins a glucose, also with the 3-hydroxyl group involved in glycoside linkage, has replaced the quinovose unit directly attached to the aglycone and a quinovose has replaced the branched xylose unit (8). Because of the structural similarities, the asterosaponins, which usually occur as complex mixtures, are difficult to identify based only on chromatographic data taken on their mixtures. Thus, the identification of known saponins equally requires the isolation of individual compounds. The Fabms, ^{1}H nmr, sugar analysis, and, if enough material is available, ^{13}C nmr provide unambiguous evidence for their structures.

As part of continuing work on active metabolites from starfishes, we have examined the saponins from the starfish *Linckia laevigata* L. and wish to report the results of our investigation.

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[1]For Part 17, see R. Riccio, C. Pizza, O. Squillace Greco, and L. Minale, *J. C. S. Perkin Trans I*, (in press).

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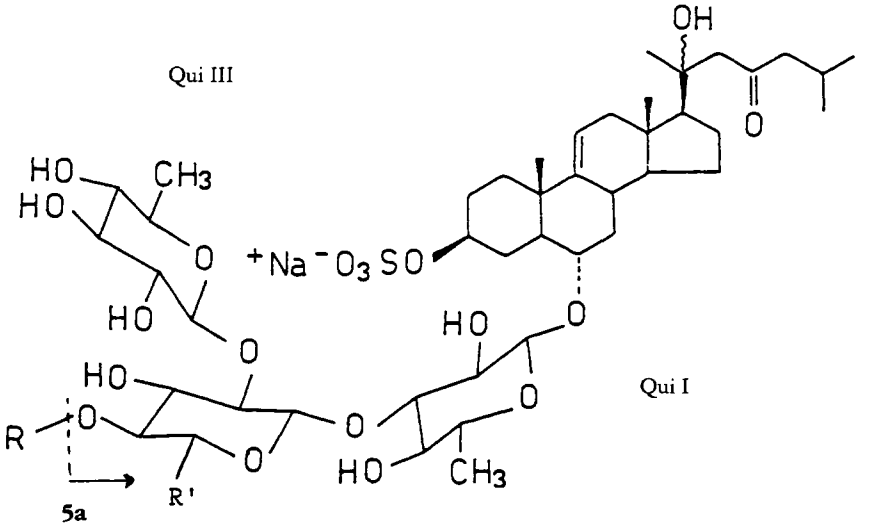
The separation of the saponins from the aqueous extracts followed the steps described previously (13). Compounds **1-4** were known and identified by comparison (Fabms, ^{1}H nmr, and ^{13}C nmr) with authentic samples and sugar analysis. The spectral data of thornasteroside A (**1**), marthasteroside A_1 (**2**), ophidianoside F (**3**), and maculatoside (**4**) are described in references 10, 8, 9, and 10, respectively. The fifth minor compound (**5**) is new and has been named laevigatoside.



1 R′=H, R=β-D-fucopyranosyl (1↦2)-β-D-galactopyranosyl: thornasteroside A.
2 R′=H, R=β-D-fucopyranosyl (1↦3)-β-D-fucopyranosyl (1↦2)-β-D-galactopyranosyl marthasteroside A_1.
3 R′=H, R=β-D-fucopyranosyl (1↦2)-β-D-xylopyranosyl: ophidianoside F.
4 R′=H, R=β-D-fucopyranosyl (1↦2)-β-D-quinovopyranosyl: maculatoside.
5 R′=CH_3, R=β-D-fucopyranosyl (1↦2)-α-L-arabinopyranosyl: laevigatoside.

On acid methanolysis, laevigatoside (**5**) liberated methyl quinovosides, methyl arabinosides, and methyl fucosides in the ratio 3:1:1. The aglycone of laevigatoside is thornasteryl A 3-sulfate. The ^{1}H-nmr spectrum of the intact saponin showed signals for the aglycone protons (see Experimental section) which were virtually identical to the signals that we had previously seen for the thornasterol A-containing saponins (13). The ^{13}C-nmr spectrum (see Experimental section) substantiated the identification of the aglycone and confirmed the C-3 attachment of the sulfate and C-6 attachment of the oligosaccharide. All the aglycone carbon signals were within ±0.1 ppm with the signals that we had previously seen for the thornasterol A-containing saponins (13). Fabms gave molecular ion species at *m/z* 1251 (M_{Na}+H), 1267 (M_k+H), and 1273 (M_{Na}+Na) corresponding to the expected molecular weight of 1250 for a pentaglycoside of thornasteryl A sodium sulfate.

Permethylation of laevigatoside and methanolysis of the methylated material gave permethylated methyl quinovosides and permethylated methyl fucosides. These data placed one quinovose and one fucose as the terminal monosaccharides and indicated that the carbohydrate moiety contains one branching point. The ^{13}C-nmr and ^{1}H-nmr data have suggested the glycosidic linkages as shown in **5**. The shifts of the anomeric carbons by ^{13}C nmr (Table 1)) and the coupling constants of the anomeric protons at δ 4.44 (2H, J=7.5 Hz), 4.57 (3H, J=7.8 Hz) by ^{1}H nmr have suggested that all the linkages are β (α for the arabinopyranose unit). In the ^{13}C-nmr spectrum one glycosidated carbon signal appeared shifted downfield to 91.1 ppm. The same signal was observed in

thornasteroside A (10) and in the other thornasterol A containing saponins (4,8,13). This was assigned to C-3 of the quinovose unit directly attached to the aglycone. In the ^{1}H-nmr spectrum one methyl doublet of 6-deoxysugar units appeared shifted downfield to δ 1.45 (J=5.5 Hz). The same signal was observed in marthasteroside B and C and assigned to 5-methyl protons of the 2,4-disubstituted quinovose unit (8). This downfield shift is interpreted as due to the substitution at C-4. From the ^{13}C-nmr signals, we have substracted those due to the terminal quinovose, fucose, and 3-substituted quinovose units on comparing the spectrum with those of the appropriate methyl β-monoglycosides (14) and those reported for similar glycosides (3,4,8). The remaining signals are only consistent with a 2,4-disubstituted β-quinovopranose and a 2-substituted α-arabinopyranose. The location of the linkage (1↦2) to the arabinose was mainly based on the appearance in the spectrum of a signal at 68.2 ppm which may only be assigned to C-4 of arabinose. This not only excludes a glycosidation at C-4 but also eliminates a substitution at C-3 because in that case one would expect a strong upfield shift (ca. 4.0 ppm) for C-4 (15). The C-2 glycosidic linkage is evidenced by the fact that C-2 carbon of arabinose is shifted downfield by 10.2 ppm (β-effect) to 82.0 ppm and C-1 carbon is upfield shifted by 2.4 ppm (γ-effect) to 102.7 ppm (in methyl α-*L*-arabinopyranoside; C-1: 105.1, C-2: 71.8; C-3: 73.4; C-4: 69.4; C-5: 67.3) (14).

Enzymic hydrolysis with *Charonia lampas* glycosidase mixture yielded a trisaccharide (**5a**). Acid hydrolysis of **5a** yielded quinovose as the sole sugar component. The ^{1}H nmr showed three doublets at δ 1.30, 1.32, and 1.40 for the methyl protons of the quinovose units. The absence of the methyl doublet downfield shifted to around δ 1.45 in the ^{1}H nmr suggests that the substitution at C-4 has been removed on passing from **5** to **5a**. Thus, the saccharide chain of the prosapogenol (**5a**) is β-quinovopyranosyl (1↦2)-β-quinovopyranosyl (1↦3)-β-quinovopyranosyl and consequently that of laevigatoside (**5**) is β-fucopyranosyl (1↦2)-α-arabinopyranosyl (1↦4)-[β-quinovopyranosyl (1↦2)]-β-quinovopyranosyl (1↦3)-β-quinovopyranosyl. The carbohydrate structure of laevigatoside is similar with the carbohydrate moieties of the above saponins. The deviations are that in **5** a quinovose unit has replaced the branched xylose unit, and an arabinopyranose unit is attached at C-4 of the branched quinovose.

The glycosidase mixture of *Charonia lampas* easily removed the monosaccharide residues linked at C-4 of the branched unit leaving a triglycoside which was more resistant to further enzymic cleavages. This behavior appears to be common in asterosaponins (4,9,10), and it has been supposed (8) that the cleavage of the remaining linkages was inhibited by the spatial arrangement assumed by the triglycoside portion as shown in formulae **1-5**. The scarcity of material did not permit the configuration of the monosaccharides to be established. We prefer the D-configuration for the quinovose

TABLE 1. ^{13}C-nmr Shifts[a] of Sugar Carbon Atoms of Compound **5**. (The Signals Due to the Aglycone Have Been Reported in the Experimental Section)

Sugar Carbon Atoms	Qui I	Qui II	Qui III	Ara	Fuc
1	105.2	103.9	104.9	102.7[b]	106.8
2	73.9	82.3	75.4	82.0[b]	71.8
3	91.1[b]	75.2	76.7	73.4	75.1
4	74.6	84.8	76.2	68.2[b]	73.4
5	72.5	73.7	73.6	66.1	71.9
6	18.3	17.9	18.5		17.1

[a]Spectrum was run in d_5-pyridine (8.8 mg/0.4 ml) at room temperature. Assignments have been made by comparing the spectrum with those of the appropriate methyl-β-monoglycopyranosides (14) and using the assignments reported for similar glycosides (3,4,8).

[b]Pertinent shifts discussed in the text.

and fucose by analogy with the other asterosaponins. The L-configuration of arabinose is preferred because L-arabinose occurs in nodososide, α-24-*O*-glycosidated steroid, from the starfish *Protoreaster nodosus* (16) and luzonicoside, a steroidal cyclic glycoside, also originating from a starfish, the Pacific *Echinaster luzonicus* (17).

EXPERIMENTAL

INSTRUMENTAL.—The following instruments were used: nmr, Bruker WM-250; ms, Kratos MS 902 mass spectrometer equipped with Kratos FAB source; hplc, Waters Model 6000A pump equipped with a U6K injector and a differential refractometer, model 401 detector; glc, Carlo Erba Fractovap 2900 capillary column; DCCC, DCCC apparatus manufactured by Büchi equipped with 300 tubes. The Fab-mass spectra were obtained by dissolving the samples in a glycerol matrix and placing them on a copper probe tip prior to bombardment with Xe atoms of energy 2-6 KV. The ^{1}H nmr of the oligoglycosides was run in CD_3OD and the ^{13}C nmr was run in d_5-pyridine.

EXTRACTION, SAPONIN ISOLATION, AND FRACTIONATION.—The animals [*L. laevigata*, 5 kg], collected in 1983 off Nouméa, New Caledonia and identified by the zoologists of the Centre ORSTOM, were chopped and extracted with H_2O (5.liter) for 3 h at room temperature. The extracts were filtered and lyophilized to give 350 g of material. This material was redissolved in H_2O (1.5 liter), clarified by centrifugation and passed through a column of Amberlite XAD-2 (1 kg). This column was washed with H_2O (3 bed volumes) and then MeOH (2 bed volumes). The MeOH eluates were dried on a rotary evaporator to give 2.33 g of a glassy material, which was then chromatographed on a column of Sephadex LH-60 (4×80 cm; 100 g) using MeOH-H_2O (2:1) as the eluent. The saponins were eluted in the first fractions to give 0.79 g of material. Fractionation was continued by droplet counter-current chromatography (dccc) with *n*-BuOH-Me_2CO-H_2O (3:1:5) (ascending mode; the lower phase was used as stationary phase; flow 24 ml/h; 6-ml fractions were collected and analyzed by tlc on silica with *n*-BuOH-HOAc-H_2O, 12:3:5) to give four main fractions. Fractions 82-108 (27 mg) contained mainly **4** and smaller amounts of the novel laevigatoside (**5**); fractions 109-163 (56 mg) contained major amounts of **5** together with **1**, **3**, and **4**; fractions 164-202 (110 mg) mainly contained thornasteroside (**1**) in admixture with **3**, and fractions 203-239 (91 mg) still contained thornasteroside A (**1**) as major component in admixture with **2**. All fractions were finally separated by hplc on a C_{18} μ-bondapack column (30 cm x 7.8 mm i.d.) with MeOH-H_2O (9:11). The saponins were dissolved in water (*ca* 0.5 ml/100 mg) and the solution added to an equal volume of saturated NaCl solution. This solution was applied to the column (*ca.* 30 mg saponin mixture for each injection). The total yield of each saponin was: thornasteroside A (**1**), 88 mg; marthasteroside A_1 (**2**), 26 mg; ophidianoside F (**3**), 18 mg; maculatoside (**4**), 18 mg and the novel laevigatoside (**5**), 8.8 mg.

PHYSICAL DATA OF LAEVIGATOSIDE (**5**).—[α]D +4.2° (*c* 0.8, MeOH); Fabms, *m/z* 1273 (M_{Na}+Na), 1267 (M_K+H), 1251 (M_{Na}+H), 1153 (1273-$NaHSO_4$), 1007 (1153-146), 875 (1007-132), 755-739 (pentasaccharide cation), 609-593 (tetrasaccharide cation), 463-447-431 (trisaccharide cation, branching point); ^{1}H nmr, δ (aglycone) 0.81 (3H, s, 18-H_3), 0.93 (3H, d, J=6.6 Hz, 26- or 27-H_3), 0.94 (3H, d, J=6.6 Hz, 27- or 26-H_3), 1.02 (3H, s, 19-H_3), 1.37 (3H, s, 21-H_3), 2.41 (2H, d, J=6.8 Hz, 24-H_2), 2.61 (2H, ABq, J=16.0 Hz, 22-H_2), 4.22 (1H, m, 3α-H), 5.37 (1H, broad d, J=5.5 Hz, 11-H); δ (sugar) 1.29, 1.31, 1.41 and 1.45 (each 3H, d, J=5.7, 6.0, 6.2 and 5.5 Hz, 5-CH_3 of quinovose and fucose units), 4.44 (2H, d, J=7.5 Hz, anomeric-H's), and 4.57 (3H, d, J=7.8 Hz, anomeric-H's); ^{13}C nmr, aglycone carbons, C-1: 36.0, C-2: 29.5, C-3: 77.6, C-4: 30.0, C-5: 49.4, C-6: 80.7, C-7: 41.6, C-8: 35.3, C-9: 145.6, C-10: 38.3, C-11: 116.7, C-12: 42.4, C-13: 41.6, C-14: 54.0, C-15: 23.1, C-16: 25.1, C-17: 59.6, C-18: 13.6, C-19: 19.3, C-20: 73.7, C-21: 27.0, C-22: 54.9, C-23: 211.8, C-24: 53.7, C-25: 24.4, C-26: 22.6, C-27: 22.5 ppm; the shifts for the sugar carbons are in Table 1; sugar analysis: quinovose (x3), arabinose and fucose.

METHANOLYSIS OF SAPONINS AND SUGAR ANALYSIS.—Methanolysis of each glycoside (0.2-1 mg) and subsequent glc analysis of the silylated sugar compounds was carried out at 140° on a 25 mt SE-30 capillary column as previously described (13). The identification was based on co-chromatography with standards.

METHYLATION OF LAEVIGATOSIDE FOLLOWED BY METHANOLYSIS: TERMINAL SUGARS.—Laevigatoside (**5**) (1 mg) in 0.2 ml dry DMF was added under nitrogen to a stirred mixture of NaH (20 mg) in dry DMF (0.2 ml). The mixture was stirred for 2 h and then CH_3I (0.1 ml) was added. The reaction mixture was kept for 4 h at room temperature. The excess of NaH was destroyed by a few drops of MeOH, and, after addition of H_2O, the mixture was extracted with $CHCl_3$. The organic layer was washed with H_2O and evaporated under vacuum. The residue in anhydrous 2 N HCl in MeOH (0.3 ml) was heated at 80° in a stoppered reaction vial for 8 h. After cooling, the mixture was concentrated under a stream of nitrogen and

was used for gc analysis (SE-30 25 mt, 87°, hydrogen carrier, flow 10 ml min^{-1}). Gc peaks co-eluted with those of methyl 2,3,4-tri-*O*-methylfucoside and methyl 2,3,4-tri-*O*-methylquinovoside standards.

ENZYMIC HYDROLYSIS OF LAEVIGATOSIDE (**5**): PROSAPOGENOL (**5a**).—Saponin (**5**) (5 mg) in 0.5 ml of citrate buffer (pH 4.5) was incubated with 5 mg of glycosidase mixture of *Charonia lampas* (Scikagaku Kogyo) at 40° for 3 days. The reaction was followed by tlc on SiO_2 in *n*-BuOH-HOAc-H_2O (12:3:5). After the disappearance of the starting material, the reaction mixture was passed through a C-18 Sep-pack cartridge which was eventually washed with 1 ml of H_2O and eluted with MeOH (3 ml). The MeOH eluate was evaporated to dryness under reduced pressure to give a residue which was purified by hplc C_{18} μ-bondapack column (30 cm×4 mm i.d.), 52% MeOH in H_2O, flow rate 2 ml/min^{-1}, refractometer detector to give the trisaccharide **5a**, Fabms *m/z* 995 (M_{Na}+Na), ^{1}H nmr δ (aglycone) 0.82 (3H, s, 18-H_3), 0.93 and 0.94 (6H, each d, J=6.6 and 6.0 Hz, 26- and 27-H_3), 1.02 (3H, s, 19-H_3), 1.37 (3H, s, 21-H_3), 2.41 (2H, d, J=7.0 Hz, 24-H_2), 2.64 (2H, ABq, J=16.0 Hz, 22-H_2), 4.23 (1H, m, 3α-H), 5.37 (1H, d, J=5.5 Hz, 11-H); δ (sugars) 1.30, 1.32, 1.40 (each 3H, d, J=6.0, 6.9, 5.5 Hz; 5-CH_3 of quinovose units), 4.43, 4.53, 4.55 (each 1H, d, J=7.2, 7.5, 7.5 Hz, anomeric-H's).

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