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STARFISH SAPONINS, 19.[1] A NOVEL STEROIDAL GLYCOSIDE SULFATE FROM THE STARFISHES *PROTOREASTER NODOSUS* AND *PENTACERASTER ALVEOLATUS*[2]

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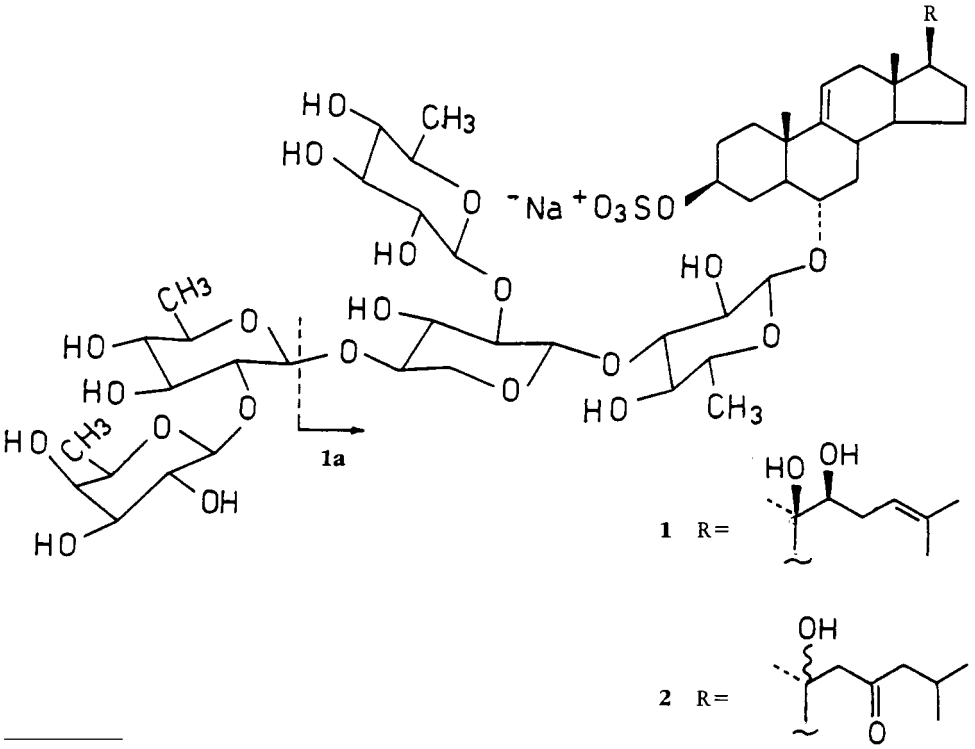
ABSTRACT.—A new sulfated asterosaponin (**1**), containing the unprecedented (20*R*,22*S*)-5α-cholesta-9(11), 24(25)-diene-3β,6α 20,22-tetraol aglycone, has been isolated from the Pacific starfishes *Protoreaster nodosus* and *Pentaceraster alveolatus*. The sulfate residue is at C-3 and the oligosaccharide chain, β-D-fucopyranosyl(1→2)-β-D-quinovopyranosyl(1→4) [-β-D-quinovopyranosyl(1→2)]-β-D-xylopyranosyl (1→3)-β-D-quinovopyranosyl, is at C-6.

We have reported previously the occurrence of a novel type of steroidal glycoside, nodososide, (24*S*)-24-*O*-[2-*O*-methyl-β-D-xylopyranosyl-(1→2)-α-L-arabino-furanosyl]-5α-cholestane-3β,5,6β,8,15α,24-hexol, along with several polyhydroxylated sterols, from the Pacific starfish *Protoreaster nodosus* L. (order Phanerozonia, family Oreasteridae) (1-3). We have now examined the more polar components and have isolated a new sulfated asterosaponin (**1**), which we named protoreasteroside, along with



[1]For Part 18, see *J. Nat. Prod.*, **48**, 97 1985).

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major amounts of the known thornasteroside A (4). It has a novel steroid aglycone which is akin to the aglycones of the previous sulfated saponins (Figure 1). The new aglycone has the same steroidal nucleus with an hydroxyl function at C-22 instead of the common keto group at C-23. The carbohydrate moiety turned out to be identical with that of maculatoside (**2**) and acanthaglycoside A from *Luidia maculata* (9,10) and *Acanthaster planci* (7), respectively. It is attached at C-6 and the sulfate group is at C-3, which is a general feature of asterosaponins.

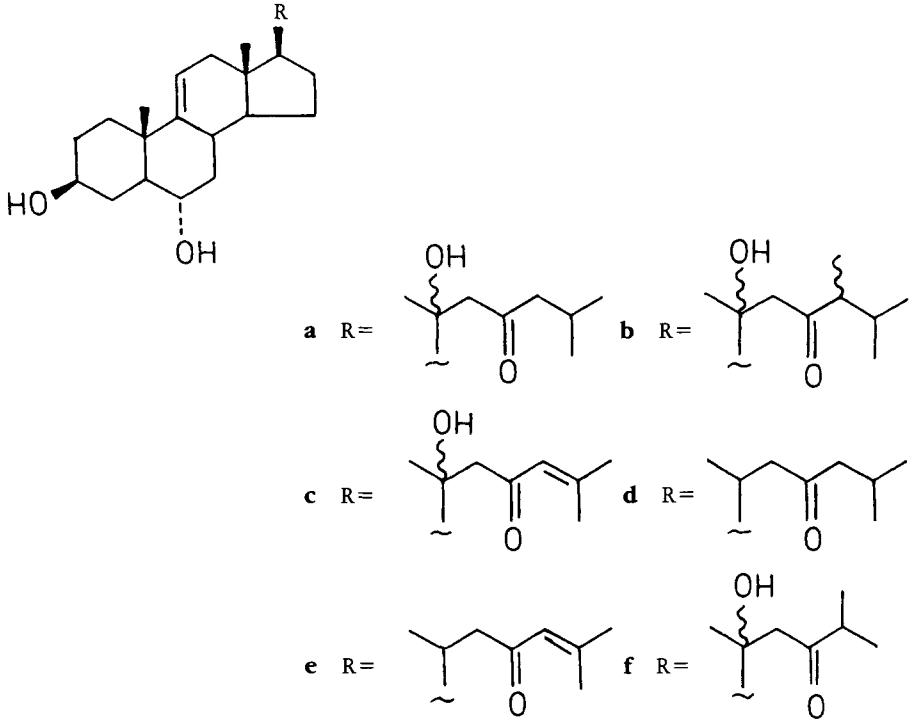

FIGURE 1. Reported structures of the genuine aglycone of sulfated asterosaponins: (**a**, thornasterol A, the most widely reported aglycone (5); **b**, the 24-methyl analogue from *Acanthaster planci* (4,5); **c**, from a minor saponin of *A. planci* (6); **d** and **e**, dihydromarthasterone and marthasterone first described from *Marthasterias glacialis* (7); **f**, 24-nor-thornasterol A, recently discovered from *Ophidiaster ophidianus* and *Hacelia attenuata* (8).

Continuing with our investigation of biologically active steroidal glycosides from echinoderms, we have been working on the extractives of the Pacific starfish *Pentaceraster alveolatus* Perrier from the family Oreasteridae and have isolated one minor asterosaponin identical with **1** along with major amounts of the known thornasteroside A. *P. alveolatus* has also given nodososide, the polyhydroxylated sterol previously isolated from *P. nodosus* (2,3) and novel steroidal glycosides, which will be the subject of a next report.

This present study adds further data which indicate a relationship between taxonomy and asterosaponin content.

EXPERIMENTAL

INSTRUMENTAL.—For instruments used, see Riccio, *et al.* (11).

EXTRACTION, SAPONINS ISOLATION AND FRACTIONATION.—*P. alveolatus:* The animals (6 kg), collected in November 1983, off Nouméa and identified by the zoologists of Centre ORSTOM where a voucher specimen is deposited, were chopped and extracted with H_2O at room temperature. The extracts were filtered and lyophilized to give 130 g of material. This material was redissolved in H_2O, clarified by

centrifugation, and passed through a column of Amberlite XAD-2 (1 kg). This column was washed with H_2O (2 liter) and then MeOH (1.5 liter). The MeOH eluates were dried on a rotary evaporator to give 2.4 g of 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 eluent. The flow rate was 30 ml/h. The eluants were collected in 10 ml fractions and monitored by tlc on silica precoated glass sheets (Merck) with *n*-BuOH-HOAc-H_2O (12:3:5); detection with ceric sulfate/H_2SO_4.

Fractions 41-59 contained the crude saponins (0.53 g); fractions 60-86 contained polyhydroxylated sterols and nonsulfated steroidal glycosides (1.076 g). Fractionation of the saponins 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; the flow rate was 24 ml/h; the eluants were collected in 7-ml fractions and monitored by tlc) to give four main fractions: 80-102 (mg 37), 103-112 (mg 18), 113-125 (mg 22), and 126-136 (mg 17). These fractions were then separated by hplc on a C_{18} μ-bondapack column (30 cm×7.8 mm i.d.) with MeOH-H_2O (9:11). The flow rate was 5 ml/min. Fractions 80-102 and 103-112 contained protoreasteroside (**1**, mg 2.7), eluted after 12 min, along with other material with longer retention time (ca. 28 min); fractions 113-125 and 126-136 mainly contained thornasteroside A (14.3 mg), eluted after 18 min.

P. nodosus: The saponins were recovered from the MeOH extract of the lyophilized animals (collected in March, 1981, off Nouméa, 2 kg dry weight, and identified by the zoologists of Centre ORSTOM where a voucher specimen is deposited) from which we also isolated nodososide and polyhydroxylated sterols (2,3). The MeOH extract was washed with 30% MeOH in $CHCl_3$; the $CHCl_3$-MeOH extracts were used for the isolation of the less polar compounds, and the residue was used for the isolation of the saponins through the steps described above to give 19 mg of protoreasteroside (**1**) along with 31 mg of thornasteroside A and minor amounts of other materials.

PHYSICAL DATA OF PROTOREASTEROSIDE (**1**).—$[\alpha]_D$ +3.8° (*c* 1, MeOH); Fabms see Figure 2; ^{13}C nmr see Tables 1 and 3, ^1H nmr in CD_3OD δ (aglycone) 0.85 (3H, s, 18-H_3), 1.02 (3H, s, 19-H_3), 1.25 (3H, s, 21-H_3), 1.67 and 1.74 (each 3H, s, 26- and 27-H_3), 4.22 (1H, m, $W_{1/2}$=22Hz, 3α-H), 5.28 (1H, t, *J*=6.5 Hz, 25-H), and 5.38 (1H, broad d, *J*=5.5 Hz, 11-H); δ (sugar) 1.29 (6H), 1.32 (3H), and 1.40 (3H) (each d, *J*=5.2, 6.1, and 6.2 Hz, 5-CH_3 of quinovose and fucose), 4.14 (1H, dd, *J*=12 and 4 Hz, 5-He of xylose), 4.42, 4.43, 4.51, and 4.55 (2H) (each d, *J*=6.5, 6.3, 7.4, and 7.5; anomeric-H's); in ($^2H_5$)-pyridine δ 0.94 (s, 19-H_3), 1.10 (s, 18-H_3), 1.64 (s, 21-H_3), 1.45, 1.52, 1.62, and 1.72 (each d, *J*=6.2, 6.3, 6.9, and 6.8 Hz, 5-CH_3 of quinovose and fucose); sugar analysis: quinovose (3 times), xylose, and fucose.

METHANOLYSIS OF GLYCOSIDES **1** AND **1a**, SUGAR ANALYSIS.—Methanolysis of each glycoside **1** and **1a** (0.2-1 mg) and subsequent gc analysis of the silylated sugar compounds was carried out at 140° on a 25 mt SE-30 capillary column as previously described (11). The identification was based on co-chromatography with standards.

METHYLATION OF (**1**) FOLLOWED BY METHANOLYSIS, TERMINAL SUGARS.—Protoreasteroside (**1**) (1 mg) was methylated; the methylated material was methanolyzed and the permethylated sugars analyzed by gc as described by Riccio *et al.* (11). Gc peaks co-eluted with those of methyl 2,3,4-tri-*O*-methyl fucosides and methyl 2,3,4-tri-*O*-methylquinovosides standards.

ENZYMIC HYDROLYSIS OF PROTOREASTEROSIDE (**1**), PROSAPOGENOL (**1a**).—Saponin **1** (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 Si gel in *n*-BuOH-HOAc-H_2O (12:3:5). After the disappearance of the starting material, the reaction mixture was extracted with *n*-BuOH. The *n*-BuOH layer was evaporated to dryness under reduced pressure to give a residue which was purified by hplc C_{18} μ-bondapack column (30 cm×9 mm i.d.), 52% MeOH in H_2O, flow rate 6 ml/min, refractometer detector, to give the trisaccharide **1a**. ^1H nmr δ (aglycone) 0.85 (s, 18-H_3), 1.03 (s, 19-H_3), 1.25 (s, 21-H_3), 1.67 and 1.76 (each s, 26 and 27-H_3), 4.20 (m, 3α-H), 5.28 (t, *J*=6.7 Hz, 25-H), and 5.37 (broad d, *J*=5.5 Hz, 11-H); δ (sugar) 1.30 and 1.39 (each d, *J*=6.2 and 5.9 Hz, 5-CH_3 of quinovose), 3.92 (dd, *J*=12 and 4 Hz, 5-He of xylose), 4.42, 4.55, and 4.56 (each d, *J*=7.5 Hz, anomeric-H's); sugar analysis: quinovose (twice) and xylose.

BIS-ACETONIDE FORMATION FROM **1**.—Saponin **1** (2.5 mg) in dry Me_2CO (0.5 ml) containing p-TsOH (3 mg) was stirred at room temperature for 20 h. The mixture was neutralized with $BaCO_3$, centrifuged, and the supernatant evaporated to dryness under reduced pressure to give the corresponding 20,22 and T-fucosyl-3,4-bis-acetonide, tlc-SiO_2 in *n*-BuOH-HOAc-H_2O (12:3:5) Rf 0.7 *versus* 0.4 for the starting material; ^1H nmr in CD_3OD, δ 0.78 (s, 18-H_3), 1.01 (s, 19-H_3), 1.37 (s, 21-H_3), 1.32 and 1.47 (each s, O-C(CH_3)$_2$-O, 20,22-acetonide), and 1.36 and 1.52 (each s, O-C(CH_3)$_2$-O, T-fucosyl-3,4-acetonide); in methyl 3,4-di-O-isopropylidene-D-fucopyranoside, the O-C(CH_3)$_2$-O singlets are observed

at δ 1.36 and 1.50 ppm. During the reaction, removal of the sulfate group has also occurred (disappearance of the 3-H signal at δ 4.20).

RESULTS AND DISCUSSION

The extraction of *P. nodosus* and *P. alveolatus* and separation of the polar compounds followed the steps described previously (12).

On acid methanolysis, protoreasteroside (**1**) liberated methyl quinovosides, methyl xylosides, and methyl fucosides in the ratio 3:1:1. The aglycone was degraded to intractable material. Thus, the elucidation of its structure was pursued on the intact saponin.

Fabms (Figure 2) of protoreasteroside gave molecular ion species at *m/z* 1289 (M_{Na}+K), 1273 (M_{Na}+Na), 1267 (M_K+H), and 1251 (M_{Na}+H) from which the molecular weight (mw) of 1250 dalton for the new saponin (sodium salt, **1**) could be readily derived. The glycosyl residue accounts for 717 m.u. (fragments at *m/z* 739, 755, and 757 in the spectrum) out of 1250 mw, leaving 533 m.u. for the aglycone. Assuming the presence of a sodium sulfate residue, the mw of the free aglycone is 432. The ^{1}H nmr contained two vinyl methyl signals at δ 1.74 s and 1.67 s and one olefinic proton signal at δ 5.28 (t, J=6.5 Hz) indicative for a terminal isopropylidene group. The ^{1}H nmr also contained signals at δ 0.85 (3H, s, 18-H_3), 1.02 (3H, s, 19-H_3), 1.25 (3H, s, 21-H_3), 4.22 (1H, m, W½=22Hz, 3α-H), and 5.38 (1H, broad d, J=5.5 Hz, 11-H) reminiscent of the spectra of asterosaponins containing a 20-hydroxysteroidal aglycone (10). We have noted small deviations of the shifts for 18- (0.85 *vs* 0.81) and 21-H_3 (1.25 *vs* 1.37) on passing from **1** to the thornasterol A- containing saponin

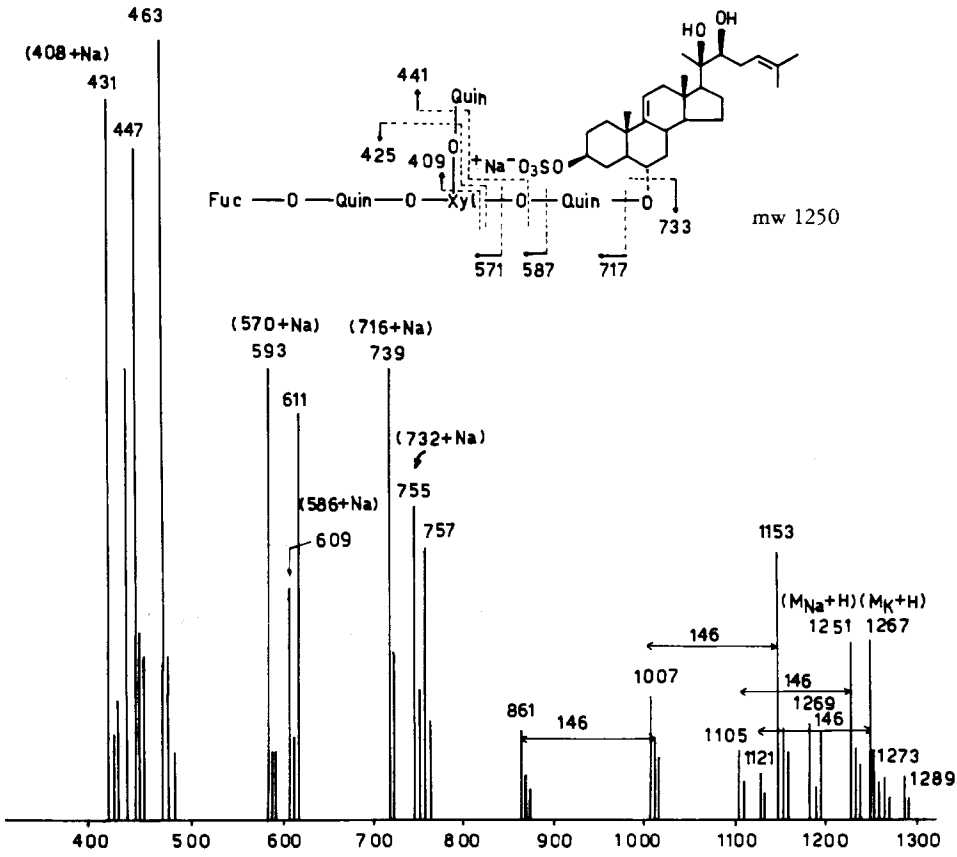

FIGURE 2. The Fabms of protoreasteroside (**1**).

(**2**). Comparison of these data with the ^{1}H nmr and Fab-ms data for maculatoside (**2**) (10) strongly supported the proposed structure for the new aglycone in **1** as did comparison of the ^{13}C-nmr spectra (Table 1).

TABLE 1. Comparison of ^{13}C-nmr Data for Aglycone Carbons of Maculatoside (**2**) (10) and **1**[a,b]

	2	1		2	1
C-1	35.9	35.9	C-15	23.2	22.6
C-2	29.3	29.2	C-16	25.0	25.3
C-3	78.3	78.3	C-17	59.6	55.1
C-4	30.6	30.6	C-18	12.3	13.4
C-5	49.2	49.2	C-19	19.1	19.2
C-6	80.5	80.0	C-20	74.1	76.4
C-7	41.3	41.2	C-21	26.9	21.5
C-8	35.3	35.4	C-22	54.7	77.8
C-9	145.5	145.6	C-23	211.9	29.6
C-10	38.2	38.2	C-24	53.8	124.2
C-11	116.5	116.6	C-25	24.3	131.8
C-12	42.4	42.6	C-26	22.5	17.4
C-13	41.5	41.6	C-27	22.3	25.6
C-14	53.9	54.0			

[a]The spectrum of **1** was run for solution in d_5-pyridine in the presence of few drops of D_2O to improve the solubility. The spectrum of maculatoside (**2**) was run in d_5-pyridine.

[b]Assignments of the side chain carbons in **1** were based upon comparison with published spectra of 5α-Cholestane-3β,20,22-triols (13) and published data on the isooctene side-chain resonances (14).

The sulfate group is clearly assigned to C-3 by consideration of chemical shift and coupling patterns in comparison with ^{1}H-nmr data for related sulfated asterosaponins (12), and confirmed by ^{13}C-nmr data (Table 1). ^{13}C nmr similarly established the oligosaccharide moiety to be located at C-6. The stereochemistry of the hydroxyl groups at C-20 and C-22 was defined by comparison of spectral data of **1** with those of the four stereoisomers of 5α-cholestane-3β,20,22-triol with respect to C-20 and C-22, synthesized by Hikino *et al.* (13). The ^{13}C-nmr spectra of the four stereoisomers are very similar, but the carbon-21 occurs at higher field in the spectra of (20*R*,22*R*) and (20*R*,22*S*) triols (21.1 and 21.9 ppm, respectively) than in the spectra of the (20*S*,22S) and (20*S*,22*R*) triols (23.8 and 24.2 ppm, respectively). Our value of 21.5 ppm compared well with those of (20*R*)-isomers and indicated that the configuration at C-20 is *R* in **1**. The configuration 22*S* was then assigned on the basis of ^{1}H-nmr data (Table 2). The C-21 methyl hydrogen signal is displayed at 1.60 ppm in the spectrum (in d_5-pyridine) of (20*R*,22*S*)-triol (*erythro* isomer) and at 1.51 ppm in that of (20*R*,22*R*)-triol, (*threo* isomer). Our value of 1.64 ppm suggested that the configuration at C-22 is *S* in **1**.

This assignment was corroborated by the chemical shift of the C-21 methyl hydro-

TABLE 2. ^{1}H-nmr Shifts of the C-21 Methyl Protons of the Model Triols (13) and Saponin **1**

	in d_5-pyridine	20,22-acetonides[a]
Saponin **1**	1.64	1.37 (CD_3OD)
Cholestane 3β,20*R*,22*S*-triol	1.60	1.34 ($CDCl_3$)
Cholestane 3β,20*R*,22*R*-triol	1.51	1.12 ($CDCl_3$)
Cholestane 3β,20*S*,22*S*-triol	1.32	1.08 ($CDCl_3$)
Cholestane 3β,20*S*,22*R*-triol	1.44	1.32 ($CDCl_3$)

[a]The acetonides of the triol models were characterized as 3-acetates.

gen signal of the corresponding 20,22-acetonide. Our value of 1.37 ppm compared well to that of (20*R*,22*S*)-triol (*erythro*-isomer methyl *trans* to the adjacent alkyl chain), δ 1.34 ppm, which occurs at lower field by 0.22 ppm relative to that of the corresponding *threo*-isomer (20*R*,22*R*) (Table 2).

Permethylation of protoreasteroside and methanolysis of the methylated material gave permethylated methyl quinovosides and permethylated methyl fucosides. These data placed one fucose and one quinovose as the terminal monosaccharides and indicated that the carbohydrate moiety contains one branching point. The fragmentation pattern observed in the Fabms (Figure 2) is closely reminiscent of those observed in spectra of previously isolated asterosaponins (6,10,15,16) and the triplet at *m/z* 463, 447, and 431 suggests the branched sugar (xylose) to be located on the second monosaccharide starting from the aglycone.

On enzymatic hydrolysis with *Charonia lampas* glycosidase mixture, **1** gave one major compound, prosapogenol (**1a**). Acid hydrolysis of **1a** yielded quinovose and xylose in the ration 2:1. The ^{1}H-nmr spectrum contained anomeric proton doublets at δ 4.56 (2H, J=7.5 Hz) and 4.42 (1H, J=7.5 Hz; xylose unit), two 3H doublets at δ 1.30 (J=6.0 Hz) and 1.39 (J=6 Hz) for the methyl protons of the quinovose units and a double doublet (J=11.5 and 4.5 Hz) at δ 3.92 due to 5-He of xylose unit. The chemical shifts and coupling constants of these signals, which appear outside the bulk of overlapping resonances, are virtually superimposable with the corresponding signals at 6α-*O*-[β-D-quinovopyranosyl (1↦2)-β-D-xylopyranosyl (1↦3)-β-D-quinovopyranosyl]-20-hydroxy-23-oxo-5α-cholest-9(11)-en-3β-yl sodium sulfate, prosapogenol obtained from maculatoside (**2**) (10), and previously isolated saponins (15), by using the same glycosidase mixture. The location of the linkage (1↦3) to the quinovose is confirmed by the appearance in the ^{13}C-nmr spectrum of **1** of a glycosidated carbon signal shifted downfield to 89.8 ppm. The same signal was observed in previously isolated saponins (6,10,15) and assigned to C-3 of quinovose directly attached to the aglycone. The remaining monosaccharides in **1** are attached at C-4 of the branched xylose, as indicated by the downfield signal at δ 4.16 (dd, J=11.0 and 4 Hz) for the equatorial proton at C-5 of a 4-*O*-substituted xylopyranose (15) and confirmed by the signals at 78.3 and 64.2 ppm for C-4 and C-5 of a 4-*O*-substituted xylopyranose (17).

Further, the appearance in the ^{13}C-nmr spectrum of **1** of one anomeric carbon signal of relatively highfield (δ C 101.2) and one glycosidated carbon signal at δ C 84.0 indicated the (1↦2) linkage T-fucose-quinovose. Thus, the saccharide chain of the novel asterosaponin can be defined as β-D-fucopyranosyl (1↦2)-β-D-quinovopyranosyl (1↦4)-[β-D-quinovopyranosyl (1↦2)]-β-D-xylopyranosyl (1↦3)-β-D-quinovopyranosyl, which is identical to that assigned to maculatoside (9,10) and acanthaglycoside A (6). Comparison of the ^{13}C-nmr data for **1** and maculatoside (**2**) has confirmed that the saccharide chain is identical in both compounds (Table 3). In **1** the con-

TABLE 3. Comparison of ^{13}C-nmr Data for Sugar Carbons of Maculatoside (**2**) (10) (in parenthesis) and **1**[a]

Sugar Carbon Atoms	Qui I	Xyl	Qui II	Qui III	Fuc
1	104.4 (105.0)	104.0 (104.1)	105.2 (105.0)	101.2 (101.1)	106.2 (106.2)
2	74.1 (74.1)	82.7 (82.0)	75.6 (75.5)	84.0 (84.7)	71.8 (71.9)
3	89.8 (90.0)	75.1 (75.3)	77.0 (76.7)	75.9 (75.9)	74.7 (74.8)
4	74.3 (74.5)	78.3 (78.5)	76.0 (76.1)	77.3 (77.4)	72.8 (73.0)
5	72.3 (72.5)	64.2 (64.2)	73.3 (73.8)	73.4 (73.6)	71.8 (71.9)
6	17.8 (17.9)		17.8 (18.1)	18.2 (18.4)	16.9 (17.1)

[a]See footnote (a) of Table 1. Small differences in the two spectra can be attributed to the difference of the solvents.

figurations of the sugars have not been proved. We prefer the common D-configuration by analogy with the previous saponins.

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