4-Methylenesterols from Theonella swinhoei sponge are natural pregnane-X-receptor agonists and farnesoid-X-receptor antagonists that modulate innate immunity

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Bioactive Asterosaponins from the Starfish *Luidia quinaria* and *Psilaster cassiope*. Isolation and Structure Characterization by Two-Dimensional NMR Spectroscopy

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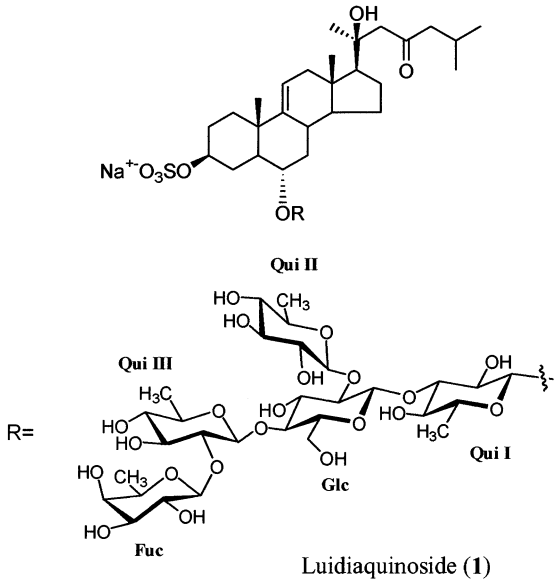
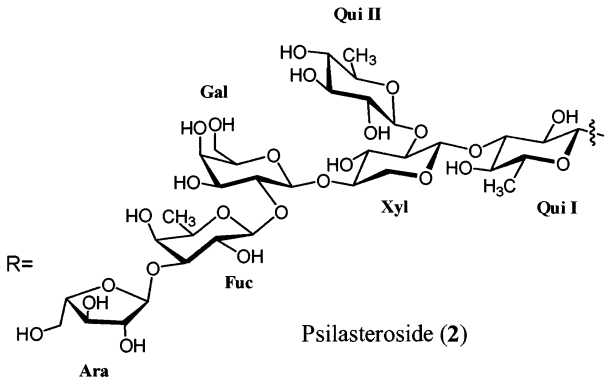
An investigation of the polar extracts from two starfish, *Luidia quinaria* and *Psilaster cassiope*, led to the isolation of five sulfated "asterosaponins". Two of them, named luidiaquinoside (**1**) and psilasteroside (**2**), are new compounds. Luidiaquinoside (**1**) contains a novel pentasaccharide chain composed of D-glucose, D-quinovose, and D-fucose, with the D-glucose unit being the branching point. Psilasteroside (**2**) contains a hexasaccharide chain in which an arabinose residue is detectable in the furanose form. Both of these compounds possess a $\Delta^{9(11)}$,3β,6α-dihydroxysteroidal nucleus with a 20-hydroxy, 23-oxo functionality. The structures of the new asterosaponins were elucidated by a combination of NMR techniques including ^{1}H−^{1}H (COSY, TOCSY, and ROESY) and ^{1}H−^{13}C (HMQC and HMBC) spectroscopy, ESIMS and HRFABMS spectrometry, and GC analyses. The new asterosaponins show marginal in vitro cytotoxicity against RBL-2H3 (rat basophilic leukemia) cell lines.

Steroidal oligoglycosides are broadly distributed among starfish, and a number of monographs have been devoted, entirely or in part, to steroidal oligoglycosides and polyhydroxysteroids with published structures, distribution, and biological activities.[1−7] Continuing our work on biologically active compounds from echinoderms, we have analyzed the polar extracts of the starfish *Luidia quinaria* von Martens (order Paxillosida, family Astropectinidae) collected at Sendai (Japan) and *Psilaster cassiope* Sladen (order Paxillosida, family Astropectinidae) collected from offshore of the northern Gulf of Mexico. Our investigation of the MeOH extracts led to the isolation of two new asterosaponins, designated as luidiaquinoside (**1**) and psilasteroside (**2**). In this paper we describe the isolation, structural elucidation, and biological activity of these two saponins, each containing different oligosaccharide moieties linked to C-6 of the same typical (20*S*)-20-hydroxy-23-oxo-5α-cholest-9(11)-en-3β-yl sulfate steroidal nucleus. The complete structural assignments of the oligosaccharide portion have been accomplished exclusively on the basis of two-dimensional proton−proton and proton−carbon chemical shift correlation spectroscopy.



Luidiaquinoside (**1**)



Psilasteroside (**2**)

Results and Discussion

The freeze-dried specimens of *Luidia quinaria*, collected at Sendai (Japan), were extracted with H_2O, and the asterosaponins were recovered from the aqueous extract by passing it through a column of Amberlite XAD-2, washing out salts with distilled H_2O, and eluting the absorbed material with MeOH. The MeOH extract from Amberlite was subjected to gel permeation on Sephadex

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Table 1. NMR Data for the Steroidal Aglycone of Asterosaponins **1** and **2** (500 MHz, CD_3OD)[a]

position	δ_H	δ_C
1	1.76, 1.48	36.9
2	2.29, 1.68	29.5
3	4.22 m ($W_{1/2}$ = 22.0 Hz)	79.7
4	2.62, 1.34	31.2
5	1.29	50.2
6	3.60	81.0
7	2.42, 0.99	41.9
8	2.12 m	36.6
9		146.6
10		39.6
11	5.37 br d (J = 5.5 Hz)	117.8
12	2.27, 2.10	43.3
13		43.2
14	1.33	54.5
15		23.4
16	1.92	24.3
17	1.68	60.5
18	0.81 s	13.7
19	1.02 s	19.6
20		74.6
21	1.37 s	26.6
22	2.65 ABq (J = 15.0 Hz)	55.2
23		214.0
24	2.41 d (J = 7.5)	54.7
25	2.12	25.6
26	0.93 d (J = 6.6)	23.0
27	0.94 d (J = 6.6)	23.0

[a] From COSY, TOCSY, and HMQC experiments.

LH-60, droplet counter current chromatography (DCCC), and reversed-phase HPLC to give four pure saponins. Solasteroside A,[8] luidiaglycoside D,[9] and thornasteroside A[10] were identified by direct comparison (^{1}H NMR and ESI-MS) with authentic samples.

Luidiaquinoside (**1**) is a novel pentaglycoside containing the typical (20*S*)-20-hydroxy-23-oxo-5α-cholest-9(11)-en-3β-yl sulfate steroidal nucleus with an unusual glucose unit placed as a branching point in the oligosaccharide chain. The only previously known asterosaponin containing a branched glucose unit is labidiasteroside A from the starfish *Labidiaster annulatus*.[11]

Luidiaquinoside (**1**) analyzed for $C_{57}H_{93}O_{28}SNa$ by combined HRFABMS and ^{13}C NMR analyses. The ESIMS spectrum of **1** showed a molecular anion at m/z = 1257 $[MSO_3]^-$ and a fragment ion at m/z = 1157 $[MSO_3 - 100]^-$ due to the loss of the side chain. Significant fragments were also detectable at m/z = 1011 (1157 − 146), m/z = 865 (1011 − 146), ascribable to the sequential losses of two deoxyhexose units, and m/z = 557, corresponding to the loss of additional hexose and deoxyhexose units.

Luidiaquinoside (**1**) contained five sugar residues. The D-configuration of fucose, quinovose, and glucose moieties was assigned after hydrolysis of **1** with 1 N HCl. The hydrolysate was trimethylsilated, and GC retention times of each sugar were compared with those of authentic samples prepared in the same manner (Table 3).[12] In this way, the sugar moieties of **1** were determined to be D-fucose, D-quinovose, and D-glucose in the ratio 1:3:1. The ^{1}H and ^{13}C NMR spectra (Table 1) of the intact saponin revealed signals due to the common thornasterol A 3β-sulfated aglycone and also confirmed the attachment of the oligosaccharide chain at the C-6 position.[3]

The ^{1}H NMR revealed the presence of five well-resolved doublets in the downfield region, ascribable to the anomeric protons [δ_H 4.42, 4.50, 4.51, 4.58, and 4.63] that correlated in the HMQC experiment with carbons at δ_C 104.8, 107.1, 102.7, 106.6, and 104.1, respectively (Table 2). The large vicinal coupling constants ($^3J_{H-1/H-2}$ = 7.2−8.1 Hz) of each anomeric proton indicated a *trans*-diaxial orientation with respect to their coupling partners (β-configuration). The presence of four 6-deoxy-sugar moieties was supported in the proton spectrum by four methyl doublets in the region between δ_H 1.28 and 1.38.

Table 2. NMR Data for the Oligosaccharide Moiety of Luidiaquinoside (**1**) and Psilasteroside (**2**) (500 MHz, CD_3OD)[a]

luidiaquinoside (**1**)			psilasteroside (**2**)		
Qui I	δ_H[b]	δ_C	Qui I	δ_H[b]	δ_C
1	4.42 d (7.2)	104.8	1	4.42 d (7.0)	104.7
2	3.37	74.7	2	3.36	74.6
3	3.38	90.5[c]	3	3.37	90.4[c]
4	3.10	74.6	4	3.07 t (8.8)	74.8
5	3.40	73.0	5	3.39	72.6
6	1.30 d (6.8)	17.5	6	1.29 d (6.0)	18.2
Glc			Xyl		
1	4.63 d (7.9)	104.1	1	4.57 d (6.1)	104.9
2	3.51	84.8[c]	2	3.51	84.3[c]
3	3.77	76.0	3	3.76	75.9
4	3.56	79.4[c]	4	3.77	79.3[c]
5	3.50	76.2	5	4.15 dd (11.7, 4.4), 3.45	64.5
6	4.00	61.9			
Qui II			Qui II		
1	4.58 d (7.2)	106.6	1	4.58 d (6.1)	106.6
2	3.32	76.4	2	3.33	76.6
3	3.35	77.2	3	3.36	77.0
4	3.15	76.3	4	3.15 t (8.8)	76.1
5	3.37	73.7	5	3.38	74.6
6	1.38 d (6.8)	18.2	6	1.39 d (6.5)	17.9
Qui III			Gal		
1	4.51 d (7.9)	102.7	1	4.52 d (6.6)	102.6
2	3.45	84.2[c]	2	3.71	82.8[c]
3	3.55	78.0	3	3.70	74.7
4	3.09	76.2	4	3.87	69.9
5	3.43	73.5	5	3.60	76.8
6	1.32 d (6.8)	17.9	6	3.81, 3.70	62.5
Fuc			Fuc		
1	4.50 d (8.1)	107.1	1	4.47 d (7.5)	106.8
2	3.56	74.0	2	3.68	72.6
3	3.52	74.8	3	3.62	81.6[c]
4	3.61	73.0	4	3.80	72.9
5	3.65	72.4	5	3.70	72.2
6	1.28 d (6.8)	17.0	6	1.32 d (6.0)	16.9
			Ara(f)		
			1	5.21 br s	111.1
			2	4.12	82.9
			3	3.87	78.9
			4	4.07	86.7
			5	3.75, 3.66	63.3

[a] From COSY, TOCSY, HMQC, and HMBC experiments. [b] Coupling constants (in Hz) are given in parentheses. [c] Glycosidated carbons.

The COSY experiment allowed the sequential assignment of most of the resonances for each sugar ring, starting from the anomeric signals. Complete assignments were achieved by a combination of COSY and 2D TOCSY results. Indeed, the 2D TOCSY experiment clearly showed correlation signals for the H-1 to H-5 spin system of quinovose and for the H-1 to H_2-6 spin system of glucose, while the magnetization transfer of the TOCSY experiment stopped at H-4 of fucose due to the very small J coupling between H-4 and H-5. We also observed a broad signal at δ_H 4.00, ascribable by the TOCSY experiment to H_2-6 of glucose, which has never before been found in the reference compounds.[3]

The HMQC experiment correlated all proton resonances with those of their corresponding carbons. Data from the above experiment determined the position of interglycosidic linkages by comparison of carbon chemical shifts with those of the corresponding methyl quinovopyranoside, methyl glucopyranoside, and methyl fucopyranoside and by taking

Table 3. Retention Times for Authentic Samples and Compounds **1** and **2**

authentic samples		luidiaquinoside (**1**)		psilasteroside (**2**)	
sugar	retention time	sugar	retention time	sugar	retention time
D-arabinose	8.82, 9.73				
L-arabinose	8.90, 9.78			L-arabinose	8.92, 9.80
D-fucose	9.88, 10.80	D-fucose	9.90, 10.80	D-fucose	9.88, 10.83
L-fucose	9.80, 10.75				
D-xylose	10.98, 12.00			D-xylose	10.98, 12.00
L-xylose	11.00, 12.05				
D-quinovose	11.65, 12.70	D-quinovose	11.65, 12.68	D-quinovose	11.68, 12.72
D-galactose	13.98, 14.95			D-galactose	13.98, 14.97
L-galactose	13.75, 14.75				
D-glucose	14.70	D-glucose	14.67		
L-glucose	14.65				

into account the known effects of glycosidation on ^{13}C chemical shifts. On the basis of these considerations, the fucose and quinovose units were located as terminal sugars as suggested by the absence of any ^{13}C glycosidation shift.

By a detailed interpretation of the HMQC data for **1**, we observed that C-2 (δ_C 84.8) and C-4 (δ_C 79.4) of the glucose unit were shifted downfield (β-effect) by 8.8 and 7.6 ppm, respectively, as expected for a glycosidation shift. Conversely, C-3 (δ_C 76.0) and C-5 (δ_C 76.2) were shifted upfield (γ-effect) by 1.8 and 2.2 ppm with respect to the reference data for glucose.[13] These data allowed us to establish the presence of a 2,4-glycosidated β-D-glucopyranosyl unit. We observed that in the ^1H NMR spectrum of **1** the H_2-6 signal of glucose is shifted downfield (δ_H 4.00 br s) and is clearly detectable.

In a nonsubstituted glucose unit the H_2-6 are generally observed at δ_H 3.68 dd−3.91 dd.[13] This signal is an excellent lead for the detection of 4-*O*-substituted glucose. The same behavior is detectable for the H-5eq (δ_H 4.14 vs δ_H 3.92) of a 4-*O*-substituted xylose in the spectra of many asterosaponins with a β-D-quinovopyranosyl moiety attached to C-6 of the aglycone.[3]

Proton-detected multiple bond heteronuclear correlation (HMBC) and ROESY experiments allowed us to establish the position of the glycosidic linkages. The anomeric proton of quinovose I at δ_H 4.42 exhibited a long-range correlation with C-6 of the aglycone (δ_C 81.0), whereas the H-1 of fucose (δ_H 4.50) showed a correlation with C-2 of quinovose III (δ_C 84.2).

Significant cross-peaks were also detected in the HMBC spectrum for the disubstituted glucose: H-1 of quinovose II (δ_H 4.58) with C-2 of glucose (δ_C 84.8) and H-1 of quinovose III (δ_H 4.51) with C-4 of glucose (δ_C 79.4), confirming the position of the branching points. This represents a singular exception to the general pattern encountered in asterosaponins. The quinovose II can be located at the branched glucose because the ROESY experiment showed cross-peak correlations between the H-1 of quinovose II (δ_H 4.58) with H-2 (δ_H 3.51), H-3 (δ_H 3.77), and H-5 (δ_H 3.50) of the 2,4-disubstituted glucose unit. The interglycosidic ROEs between the anomeric protons and the sugar-ring protons beyond the glycosidic linkage support these assignments. Therefore, the structure of luidiaquinoside (**1**) was elucidated as sodium (20*S*)-6α-*O*-{β-D-fucopyranosyl-(1→2)-β-D-quinovopyranosyl-(1→4)-[β-D-quinovopyranosyl-(1→2)]-β-D-glucopyranosyl-(1→3)-β-D-quinovopyranosyl}-20-hydroxy-23-oxo-5α-cholest-9(11)-en-3β-yl-sulfate.

Specimens of *Psilaster cassiope* were collected offshore of the northern Gulf of Mexico. The freeze-dried material was extracted with MeOH and then with Me_2CO. The Me_2CO extract was partitioned between H_2O and Et_2O, and the aqueous fraction was then extracted with *n*-BuOH. The MeOH and *n*-BuOH extracts were combined and fractionated by sequential application of gel permeation on Sephadex LH-60 and HPLC to give one pure saponin named psilasteroside (**2**).

Psilasteroside (**2**) is a new hexaglycoside, and an examination of its ^1H and ^{13}C NMR spectra revealed signals due to aglycon protons identical with those observed in luidiaquinoside (**1**), containing the same typical $\Delta^{9(11)}$,3β,6α-dihydroxysteroidal nucleus with a 23-oxo function and 20-hydroxy group in the side chain (Table 1). It contains a hexasaccharide moiety with an arabinofuranosyl residue, contrary to what is usually found in all previously isolated asterosaponins in which the sugar residues are in their pyranose form.[14−16]

The molecular formula of psilasteroside (**2**) was determined as $C_{61}H_{99}O_{32}SNa$ by ^{13}C NMR as well as from HRFABMS. The ESIMS spectrum of **2** gave a molecular anion at m/z = 1375 $[MSO_3]^-$ with a fragment at m/z = 1275 (loss of the side chain). The next fragmentation was obtained by ESI MS/MS experiments, with the following sugar losses: m/z = 1143 $[1275 - 132]^-$ loss of arabinose or xylose; m/z = 997 $[1143 - 146]^-$ loss of a deoxyhexose; m/z = 835 $[997 - 162]^-$ loss of a hexose; m/z = 689 $[835 - 146]^-$ loss of a deoxyhexose; m/z = 557 $[689 - 132]^-$ loss of an arabinose or xylose unit. The assignments of the signals attributable to the various sugar units were achieved by the application of several types of two-dimensional NMR experiments including COSY, TOCSY, ROESY, HMBC, HMQC, and the HMQC-TOCSY technique.[17,18]

Psilasteroside (**2**) contained six sugar residues. The D-configuration of quinovose, xylose, galactose, and fucose and the L-configuration of arabinose moieties were assigned after hydrolysis of **2** with 1 N HCl. The hydrolysate was trimethylsilated, and GC retention times of each sugar were compared with those of the authentic samples prepared in the same manner[12] (Table 3). In this way the sugar moieties of **2** were determined to be L-arabinose, D-fucose, D-quinovose, D-xylose, and D-galactose in the ratio 1:1:2:1:1.

The ^1H NMR spectrum showed signals for six anomeric protons at δ_H 4.58 (1H, d, J = 6.1 Hz, Qui II), 4.57 (1H, d, J = 6.1 Hz, Xyl), 4.52 (1H, d, J = 6.6 Hz, Gal), 4.47 (1H, d, J = 7.5 Hz, Fuc), 4.42 (1H, d, J = 7.0 Hz, Qui I), each a doublet, and δ_H 5.21 [1H, br s, Ara(f)] broad singlet, which were correlated in the HMQC experiment with the corresponding carbons at δ_C 106.6, 104.9, 102.6, 106.8, 104.7, and 111.1, respectively (Table 2).

The COSY experiment allowed the sequential assignment of most of the resonances for each sugar ring, starting from the anomeric signals. ^1H and ^{13}C NMR spectra indicated that five sugar residues are in their pyranose forms, and the glycosidic linkages are β-oriented. The β-configuration of the five sugars in the pyranose form was

fully defined from the chemical shift and the coupling constant of each anomeric proton. The presence of a monosaccharide in the furanose form was suggested by the chemical shift of the anomeric proton (δ_H 5.21 br s, δ_C 111.1) and arose from the data of the HMQC-TOCSY experiment.

A double doublet at δ_H 4.15 (J = 11.7, 4.4 Hz) due to the 5-Heq of a xylose unit revealed the presence of a 2,4-glycosidated xylopyranose, typical for many asterosaponins in which the xylose represents the branching point. The ^{13}C NMR of the trisaccharide chain is almost superimposable with that of myxodermoside A[19] from *Myxoderma platyacanthum*. A 2-glycosidated galactopyranosyl unit and a 3-glycosidated fucopyranosyl unit were also identified by analysis of the ^{13}C NMR data. Because the arabinofuranose was not affected by any ^{13}C glycosidation shift, with respect to the reference data,[20] it was located as the terminal unit. The ^{13}C NMR data (Table 2) established the linkage to be Ara (1→3)-Fuc. The C-3 glycosidic linkage is evidenced by the downfield shift exhibited by C-3 of fucose (δ_C 81.6; δ_C 75.0 in β-D-methylfucoside)[21] and the relatively high field of C-2 and C-4.

Finally, direct support of the attachment points of each monosaccharide came from the results of a HMBC experiment which showed correlation peaks between H-1 of arabinose (δ_H 5.21) and C-3 of fucose (δ_C 81.6) and between H-1 of fucose (δ_H 4.47) and C-2 of galactose (δ_C 82.8). The ROESY experiment clarified the interglycosidic linkage between galactose and xylose because it showed a cross-peak between H-1 Gal (δ_H 4.52) and H-4 Xyl (δ_H 3.77). Hence, the structure of psilasteroside (**2**) was defined as (20*S*)-6α-*O*-{α-L-arabinofuranosyl-(1→3)-β-D-fucopyranosyl-(1→2)-β-D-galactopyranosyl-(1→4)-[β-D-quinovopyranosyl-(1→2)]-β-D-xylopyranosyl-(1→3)-β-D-quinovopyranosyl}-20-hydroxy-23-oxo-5α-cholest-9(11)-en-3β-yl-sulfate.

The cytotoxicity of compounds **1** and **2** was evaluated on RBL-2H3 (rat basophilic leukemia) and C6 (rat glioma) cell lines. The effect on cell viability was evaluated after 24 h by measuring the IC_{50} of each compound tested. Our results indicated that compounds **1** and **2** were marginally cytotoxic against RBL2H3 cells (IC_{50}'s 31.3 and 5.4 μg/mL, respectively), whereas they were without effect on C6 cell lines.

Experimental Section

General Experimental Procedures. Optical rotations were measured on a Perkin-Elmer 243 B polarimeter with a sodium lamp (589 nm) and a 1 dm microcell. NMR spectra were recorded on a Bruker AMX-500 spectrometer equipped with a Bruker X32 computer, using the UXNMR software package. ^{1}H and ^{13}C NMR spectra were recorded at 500.13 and 125.76 MHz, respectively. Chemical shifts are referenced to residual CHD_2OD (3.31 ppm) in CD_3OD; ^{13}C chemical shifts are referenced to the solvent (CD_3OD, 49.0 ppm). Mass spectra were recorded with a Thermofinnigan LCQ instrument equipped with an ESI source. HRFABMS spectra were recorded in a glycerol matrix on a VG PROSPEC instrument. GC analyses were performed on a L-Chirasil-Val column (0.32 mm × 25 m). HPLC was performed on a Waters HPLC system equipped with a model 6000A pump, U6K injector, and a model 401 differential refractometer and using a reversed-phase C_{18} μ-Bondapak column (30 cm × 3.9 mm i.d.; flow rate 2 mL min^{-1}); DCCC was performed on a DCC-A apparatus manufactured by Tokyo Rikakikai Co., equipped with 250 tubes.

Animal Material. *Luidia quinaria* were collected at Sendai (Japan), while *Psilaster cassiope* were collected from the offshore waters of the northern Gulf of Mexico.

Extraction and Isolation. The freeze-dried animals (200 g) of *L. quinaria* were cut into small pieces and soaked in H_2O for 4 h, and the aqueous extracts were centrifuged and passed through a column of Amberlite XAD-2 (500 g). The column was washed with distilled H_2O (5 L) and then eluted with MeOH (8 L). The MeOH eluate was taken to dryness to give a glassy material (1.3 g). The MeOH extract was subjected to gel permeation on Sephadex LH-60 (4 × 80 cm) with MeOH/H_2O (2:1) as eluent. Fractions (7 mL) were collected and analyzed by TLC on SiO_2 in *n*-BuOH/AcOH/H_2O (12:3:5) and $CHCl_3$/MeOH/H_2O (80:18:2).

Fractions 45−66 (700 mg) mainly contained the asterosaponins. The crude asterosaponins fraction was submitted to DCCC with *n*-BuOH/Me_2CO/H_2O (3:1:5) [descending mode, flow rate 14 mL/h] to give the unresolved saponins (126 mg) in fractions 24−48. These fractions were then separated by reversed-phase HPLC (C_{18} μ-Bondapak 30 cm × 3.9 mm i.d.) with MeOH/H_2O (45:55) as eluent, to give pure saponins luidiaquinoside (**1**) (2.5 mg), luidiaglycoside D (2.0 mg), solasteroside A (2.5 mg), and thornasteroside A (5.2 mg).

The freeze-dried animals (164 g) of *P. cassiope* were cut into small pieces and extracted with MeOH (3 × 3 L) at room temperature. The MeOH extract was taken to dryness to give a glassy material (1.5 g). The remaining solid mass was extracted with Me_2CO (2 × 2 L), and the Me_2CO extracts were combined, evaporated under vacuum, and partitioned between H_2O and Et_2O. The aqueous residue was then extracted with *n*-BuOH. Evaporation of the *n*-BuOH extract afforded (0.75 g) a glassy material that was combined with the above MeOH extract and purified by chromatography on Sephadex LH-60 (4 × 80 cm) with MeOH/H_2O (2:1) as eluent.

Fractions (7 mL) were collected and analyzed by TLC on SiO_2 in *n*-BuOH/AcOH/H_2O (12:3:5) and $CHCl_3$/MeOH/H_2O (80:18:2). Fractions 34−63 (490 mg) mainly contained the asterosaponins. The crude asterosaponins fraction was separated by reversed-phase HPLC (C_{18} μ-Bondapak, 30 cm × 3.9 mm i.d.) with MeOH/H_2O (45:55) as eluent, to give pure saponin psilasteroside (**2**) (8.0 mg).

Luidiaquinoside (1): white amorphous powder; $[\alpha]_D$ +3.9 (*c* 0.13, MeOH); ^{1}H and ^{13}C NMR data (500 MHz, CD_3OD) of the steroidal aglycone, Table 1, and those of the oligosaccharide moiety, Table 2; HRFABMS *m/z* 1303.5356 $[MNa + Na]^+$ (calcd for $C_{57}H_{93}O_{28}SNa_2$, 1303.5370).

Psilasteroside (2): white amorphous powder; $[\alpha]_D$ +4.1 (*c* 0.54, MeOH); ^{1}H and ^{13}C NMR data (500 MHz, CD_3OD) of the steroidal aglycone, Table 1, and those of the oligosaccharide moiety, Table 2; HRFABMS *m/z* 1421.5650 $[MNa + Na]^+$ (calcd for $C_{61}H_{99}O_{32}SNa_2$, 1421.5636).

Acid Hydrolysis of Saponins 1 and 2. A solution (0.7 mg each) of saponins in 1 N HCl (0.25 mL) was stirred at 80 °C for 4 h. After cooling, the solution was concentrated by blowing with N_2. The residue was dissolved in 1-(trimethylsilyl)-imidazole and pyridine (0.1 mL), and the solution was stirred at 60 °C for 5 min. After drying the solution with a stream of N_2, the residue was separated by water and CH_2Cl_2 (1 mL, v/v = 1:1). The CH_2Cl_2 layer was analyzed by GC using an L-Chirasil-Val column (0.32 mm × 25 m). Temperatures of the injector and detector were 200 °C for both. A temperature gradient system was used for the oven; the initial temperature was maintained at 100 °C for 1 min and then raised to 180 °C at the rate of 5 °C/min. Peaks of the hydrolysate of **1** were detected at 9.90, 10.80, 11.65, 12.68, and 14.67 min. Peaks of the hydrolysate of **2** were detected at 8.92, 9.80, 9.88, 10.83, 10.98, 11.68, 12.00, 12.72, 13.98, and 14.97 min. Retention times for authentic samples after being treated simultaneously with 1-(trimethylsilyl)imidazole in pyridine are shown in Table 3. Co-injection of the hydrolysate **1** with the authentic silated D-fucose, D-quinovose, and D-glucose gave single peaks at 9.90, 10.82, 11.68, 12.70, and 14.72, respectively. Co-injection of the hydrolysate **2** with the authentic silated l-arabinose, D-fucose, D-xylose, D-quinovose, and D-galactose gave single peaks at 8.92, 9.78, 9.88, 10.82, 10.98, 12.02, 11.66, 12.70, 14.0, and 14.98, respectively.

Cytotoxicity Tests. C6 rat glioma and RBL 2H3 cells (3.5 × 10^3 cells) were plated in 96-well plates in 50 μL of Dulbecco's Modified Eagle's Medium (DMEM; Biowhittaker) and allowed to adhere at 37 °C in 5% CO_2/air for 2 h. Thereafter, 50 μL of

1:4 (v/v) serial dilution of the test compounds were added, and the cells were incubated for 24 h. Cell viability was assessed through an MTT conversion assay.[22] Briefly, after 24 h, 25 μL of MTT (5 mg/mL) was added, and the cells were incubated for an additional 3 h. After this time, the cells were lysed and the dark blue crystals solubilized with 100 μL of a solution containing 50% (v/v) *N*,*N*-dimethylformamide and 20% (w/v) SDS with an adjusted pH of 4.5.[23] The optical density (OD) of each well was measured with a microplate spectrophotometer (Titerteck Multiskan MCC/340) equipped with a 620 nm filter. The viability of each cell line was calculated as % dead cells = 100 − (OD treated/OD control) × 100.

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