Antifungal Steroidal Glycosides from the Patagonian Starfish *Anasterias minuta*: Structure−Activity Correlations

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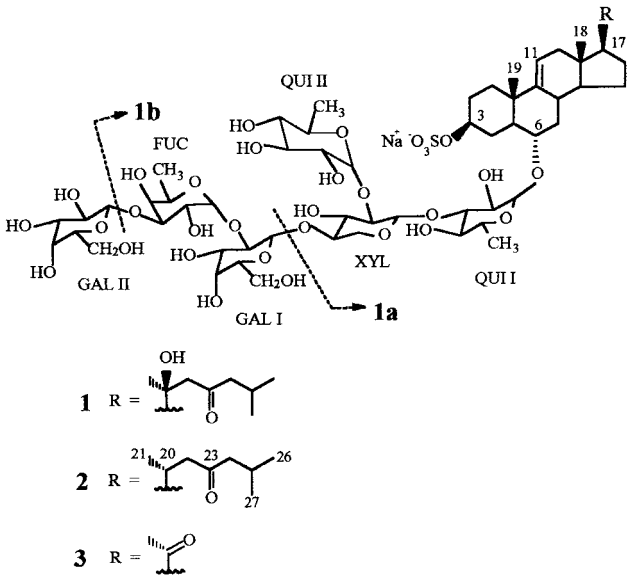
Two new sulfated steroidal hexaglycosides, anasterosides A (**2**) and B (**3**), along with the known versicoside A (**1**) have been isolated from the Patagonian starfish *Anasterias minuta*. Their structures have been elucidated by spectroscopic analysis (NMR and FABMS) and chemical transformations. Compounds **1** and **2** and the synthetic pentaglycoside **1b** derived from versicoside A showed antifungal activity against the plant pathogenic fungus *Cladosporium cucumerinum*. Desulfation of hexaglycoside **1** rendered a totally inactive saponin.

Steroidal glycosides are the predominant metabolites of starfish (phylum Echinodermata, class Asteroidea) and are responsible for their general toxicity.[1,2] Starfish extracts containing these saponins (asterosaponins) have shown a wide spectrum of biological effects: cytotoxic, hemolytic, antibacterial, antiviral, and antifungal activities.[3] However, few pharmacological studies have been carried out on the pure glycosides.

As part of our search for new bioactive compounds from echinoderms of cold waters of the South Atlantic,[4−6] we have investigated the ethanolic extract of the starfish *Anasterias minuta* (Perrier, 1875) (family Asteriidae, order Forcipulatida) collected off the Patagonian coast of Argentina. We report here the isolation and structure elucidation of two new sulfated hexaglycosides, anasterosides A (**2**) and B (**3**), together with the known versicoside A (**1**), as well as the results of the antifungal evaluation of these compounds and semisynthetic derivatives.



Results and Discussion

Bioactivity-guided fractionation of the ethanolic extract of *A. minuta* using the brine shrimp (*Artemia salina* L.) larvae mortality bioassay[7] led us to the isolation of three fractions containing steroidal sulfated glycosides that exhibited significant brine shrimp lethality. Purification of the mixture of asterosaponins by chromatography over Sephadex LH-60 and reversed-phase HPLC on Bondclone C_{18} yielded versicoside A (**1**) and the new anasterosides A (**2**) and B (**3**). The three glycosides contain the same hexasaccharide chain, but differ in the side chain of the aglycon.

The presence of galactose, fucose, xylose, and quinovose in a 2:1:1:2 ratio in glycoside **1** was deduced by acid hydrolysis with aqueous 2 N trifluoroacetic acid followed by GC analysis of the corresponding peracetylated alditols. The NMR and all of the physical constants of this compound were found to be identical in all respects to the known compound, versicoside A (**1**), which was isolated previously from the starfish *Asterias amurensis versicolor*.[8]

Anasteroside A (**2**) was obtained as a white amorphous powder. An examination of the ^{1}H, ^{13}C NMR and DEPT spectra of **2** suggested the presence of a steroidal aglycon with a 9(11)-double bond [δ_C 145.3 (s, C-9) and 116.2 (d, C-11); δ_H 5.19 (1H, m, H-11)], one sulfated oxomethine [δ_C 78.0 (d, C-3); δ_H 4.84 (1H, m, H-3)], one oxomethine [δ_C 79.5 (d, C-6); δ_H 3.82 (1H, m, H-6)], and one ketone carbonyl group (δ_C 211.2, C-23). Glycosidation at C-6 was supported by the downfield shift of the C-6 signal in the ^{13}C NMR spectrum with respect to the corresponding value in synthetic asterosaponin aglycons containing the 3β,6α-dihydroxy oxidation pattern.[9,10] The methyl signal at δ_C 19.0 (C-19) in anasteroside A (**2**) and in natural and synthetic aglycons supports the assignment of the α configuration at C-6 since the chemical shift of C-19 is affected by the configuration of δ carbon substituents, such as a hydroxyl group at C-6.[11] The upfield chemical shifts of C-18 (δ 11.4) and C-21 (δ 19.3) in the ^{13}C NMR spectrum of **2** with respect to those in versicoside A (**1**) (δ 13.5 (C-18) and 27.0 (C-21))[8] indicated the absence of a hydroxyl group at C-20. This was confirmed by the presence of a doublet signal at δ 0.99 (3H, J = 6.2, H_3-21) and a methyl signal at δ 0.61 (s, H_3-18) in the ^{1}H NMR spectrum of **2**. The assignments of the NMR signals associated with the aglycon moiety (Table 1) were derived from ^{1}H−^{1}H COSY

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Table 1. ^{1}H and ^{13}C NMR Data for the Aglycon Moieties of Anasterosides A (**2**) and B (**3**)

	2		**3**	
carbon	δ_C mult[a]	δ_H mult[b] (J in Hz)	δ_C mult[a]	δ_H mult[b] (J in Hz)
1	35.7 t	1.41; 1.61 m	35.8 t	1.30 m
2	29.0 t	1.85 m; 2.75 m	29.1 t	2.73 m
3	78.0 d	4.84 m	78.0 d	4.81 m
4	30.4 t	1.71 m; 3.30 m	30.5 t	3.26 m
5	49.1 d	1.48 m	48.8 d	1.46 m
6	79.5 d	3.82 m	79.6 d	3.70 m
7	41.0 t	1.25 m; 2.62 m	41.0 t	2.58 m
8	35.5 d	2.06 m	35.4 d	1.98 m
9	145.3 s		145.9 s	
10	38.0 s		38.2 s	
11	116.2 d	5.19 m	115.9 d	5.17 m
12	41.5 t	1.95 bd	40.4 t	1.98 m
13	40.9 s		42.5 s	
14	53.5 d	1.24 m	53.5 d	1.09 m
15	25.1 t		23.0 t	
16	28.3 t	1.60 m	25.4 t	
17	55.9 d	1.16 m	63.3 d	2.78 d (8.7)
18	11.4 q	0.61 s	13.0 q	0.86 s
19	19.0 q	0.95 s	19.1 q	0.51 s
20	32.2 d	2.13 m	210.0 s	
21	19.3 q	0.99 d (6.2)	31.0 q	2.17 s
22	50.1 t	2.48 bd (15.0)		
23	211.2 s			
24	52.1 t	2.31 d (7.3)		
25	24.3 d	2.20 m		
26	22.4 q	0.93 d (6.6)		
27	22.3 q	0.92 d (6.6)		

[a] Recorded at 125 MHz in pyridine-d_5/D_2O (5:1); multiplicity by DEPT. [b] Recorded at 500 MHz in pyridine-d_5/D_2O (5:1).

and HETCOR experiments. These data are very similar to those reported for (20*R*)-5α-cholest-9(11)-en-23-one-3*β*,6α-diol 3-sulfate, the aglycon of asterosaponins isolated from the starfishes *Marthasterias glacialis*,[12] *Coscinasterias tenuispina*,[13] *Luidia maculata*,[14] and *Neosmilaster georgianus*.[15]

In addition to the aglycon signals, the ^{1}H and ^{13}C NMR spectra of **2** (Table 2) show six anomeric protons at δ 4.82 (d, J = 7.5 Hz), 4.86 (d, J = 7.7 Hz), 4.90 (d, J = 7.7 Hz), 5.06 (d, J = 7.5 Hz), 5.09 (d, J = 7.3 Hz), and 5.18 (d, J = 7.7 Hz) and carbons at δ 101.7, 103.9, 104.2, 105.9, 106.1, and 106.2. The β stereochemistries at the anomeric carbons were deduced from the coupling constant values (J = 7.3−7.7 Hz). The methyl carbon signals at δ 16.8, 18.0, and 18.3 and the doublet methyl proton signals at δ 1.39 (J = 6.3 Hz), 1.58 (J = 6.0 Hz), and 1.83 (J = 6.4 Hz) indicated the presence of three 6-deoxy sugars. The presence of galactose, fucose, xylose, and quinovose in a 2:1:1:2 ratio was confirmed by acid hydrolysis with aqueous 2 N trifluoroacetic acid followed by GC analysis of the corresponding alditol peracetates.

The molecular formula of anasteroside A (**2**) was determined as $C_{62}H_{101}O_{32}SNa$ (*m*/*z* 1412) from the $[M - Na]^-$ ion at *m*/*z* 1389 in the FABMS (negative ion mode). Fragment ion peaks at *m*/*z* 1242 $[M - Qui - Na]^-$ and 1226 $[M - Gal - Na]^-$ indicated the presence of galactose and quinovose as terminal units. This was further confirmed by methylation of **2** followed by acid hydrolysis and GC−MS analysis of the methylated alditol acetates. Other significant ion peaks visible at *m*/*z* 1081 [M − Gal-*O*-Fuc + H − Na]$^-$ and 917 [M − Gal-*O*-Fuc-*O*-Gal − H − Na]$^-$ corresponded to the sequential losses of galactose, fucose, and galactose units.

The position of the interglycosidic attachments was determined using a combination of ^{1}H−^{1}H COSY, relayed COSY,[16,17] and HETCOR experiments. From the ^{1}H−^{1}H COSY spectrum of **2** the anomeric proton signals at δ 5.18, 5.09, 5.06, 4.90, 4.86, and 4.82 were coupled to the adjacent proton signals at δ 4.48, 4.00, 3.95, 4.38, 4.47, and 3.96, respectively. These assignments were confirmed by the single relayed COSY spectrum on the basis of correlations of each anomeric proton signal with the corresponding H-2 and H-3 protons in each sugar unit, as evidenced by cross-peaks at δ 5.18/4.48 (H-1‴″/H-2‴″) and 5.18/4.14 (H-1‴″/H-3‴″) for the galactose terminal unit, δ 5.09/4.00 (H-1‴/H-2‴) and 5.09/4.07 (H-1‴/H-3‴) for the quinovose terminal unit, δ 5.06/3.95 (H-1″/H-2″) and 5.06/4.22 (H-1″/H-3″) for the xylose unit, δ 4.90/4.38 (H-1⁗/H-2⁗) and 4.90/4.11 (H-1⁗/H-3⁗) for the galactose unit, δ 4.86/4.47 (H-1⁗/H-2⁗) and 4.86/4.06 (H-1⁗/H-3⁗) for the fucose unit, and δ 4.82/3.96 (H-1′/H-2′) and 4.82/3.88 (H-1′/H-3′) for the quinovose unit attached to the aglycon. From the double relayed COSY spectrum, the anomeric proton signals also showed cross-peaks at δ 5.18/4.50 (H-1‴″/H-4‴″), δ 5.09/3.85 (H-1‴/H-4‴), δ 5.06/4.19 (H-1″/H-4″), δ 4.90/4.42 (H-1⁗/H-4⁗), δ 4.86/4.21 (H-1⁗/H-4⁗), and δ 4.82/3.54 (H-1′/H-4′). Analysis of ^{1}H−^{1}H COSY and relayed COSY spectra allowed us to establish the connectivity of H-4, H-5, and H-6 in each sugar unit. In particular, the correlation of the 3-linked quinovose methyl group (δ 1.58) with the proton signal at δ 3.71 (H-5′) in the ^{1}H−^{1}H COSY spectrum and the cross-peaks at δ 1.58/3.71 (H_3-6′/H-5′) and 1.58/3.54 (H_3-6′/H-4′) in the relayed COSY spectrum allowed assignments of H-4′ and H-5′ of the quinovose unit attached to the aglycon. In a similar manner, we were able to assign proton doublets at δ 1.83 (H_3-6‴) and 1.39 (H_3-6⁗) to the methyl groups of the quinovose terminal unit and the fucose unit, respectively. The ^{13}C NMR signals of each sugar moiety were assigned by means of direct H−C correlations in the HETCOR spectrum. The cross-peaks between δ_H 1.58 (H_3-6′) and δ_C 18.3 (C-6′), δ_H 1.83 (H_3-6‴) and δ_C 18.0 (C-6‴), and δ_H 1.39 (H_3-6⁗) and δ_C 16.8 (C-6⁗) were consistent with the presence of two quinovose and one fucose unit.[18]

Location of the interglycosidic linkages in the hexasaccharide chain was also deduced from the ^{13}C chemical shifts of C-3′ (δ 89.3), C-2″ (δ 83.3), C-4″(δ 78.2), C-2‴ (δ 82.9), and C-3⁗ (δ 83.8), assigned on the basis of cross-peaks at δ 3.88/89.3 (H-3′/C-3′), 3.95/83.3 (H-2″/C-2″), 4.19/78.2 (H-4″/C-4″), 4.38/82.9 (H-2⁗/C-2⁗), and 4.06/83.8 (H-3⁗/C-3⁗) in the HETCOR spectrum. The carbons involved in the interglycosidic linkages were downfield relative to shifts expected for the corresponding methyl glycopyranosides.[19] The linkage positions for the sugar units were confirmed by methylation of **2** followed by acid hydrolysis and GC−MS analysis of the partially methylated alditol acetates derived from 3-linked quinovopyranose, 2,4-linked xylopyranose, 2-linked galactopyranose, and 3-linked fucopyranose.

On the basis of these data we conclude that the oligosaccharide part of **2** is identical to the sugar chain of versicoside A (**1**). The six carbohydrate units are of the D-series, as determined by GC analysis of the mixture of 1-[(*S*)-*N*-acetyl-(2-hydroxypropylamino)]-1-deoxyalditol acetate derivatives, as described previously.[6]

On the basis of the data discussed above, the structure of anasteroside A (**2**) was determined as sodium 6α-*O*-{*β*-D-galactopyranosyl-(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}-5α-cholest-9(11)-en-23-one-3*β*-yl-sulfate.

The molecular formula of anasteroside B (**3**) was deduced to be $C_{56}H_{89}O_{32}SNa$ on the basis of the pseudomolecular ion at *m*/*z* 1305 $[M - Na]^-$ in the FABMS (negative ion

Table 2. ^{1}H and ^{13}C NMR Data for the Sugar Moieties of Anasterosides A (**2**) and B (**3**)

		2 and **3**				**2** and **3**	
	carbon	δ_C[a]	δ_H[b] (*J* in Hz)		carbon	δ_C[a]	δ_H[b] (*J* in Hz)
Qui I	1′	104.2	4.82 d (7.5)	Gal I	1′′′′	101.7	4.90 d (7.7)
	2′	73.9	3.96 m		2′′′′	82.9	4.38 m
	3′	89.3	3.88 m		3′′′′	74.5	4.11 m
	4′	74.1	3.54 m		4′′′′	69.0	4.42 m
	5′	73.8	3.71 m		5′′′′	76.8	4.03 m
	6′	18.3	1.58 d (6.0)		6′′′′	61.9	4.37 m; 4.26 m
Xyl	1′′	103.9	5.06 d (7.5)	Fuc	1′′′′′	106.2	4.86 d (7.7)
	2′′	83.3	3.95 m		2′′′′′	72.3	4.47 m
	3′′	75.2	4.22 m		3′′′′′	83.8	4.06 m
	4′′	78.2	4.19 m		4′′′′′	71.5	4.21 m
	5′′	64.1	3.77 m; 4.40 m		5′′′′′	71.3	3.58 m
					6′′′′′	16.8	1.39 d (6.3)
Qui II	1′′′	105.9	5.09 d (7.3)	Gal II	1′′′′′′	106.1	5.18 d (7.7)
	2′′′	76.1	4.00 m		2′′′′′′	72.7	4.48 m
	3′′′	76.7	4.07 m		3′′′′′′	74.6	4.14 m
	4′′′	75.5	3.85 m		4′′′′′′	69.7	4.50 m
	5′′′	71.8	3.71 m		5′′′′′′	76.7	4.08 m
	6′′′	18.0	1.83 d (6.4)		6′′′′′′	61.7	4.41 m; 4.23 m

[a] Recorded at 125 MHz in pyridine-d_5/D_2O (5:1). [b] Recorded at 500 MHz in pyridine-d_5/D_2O (5:1).

mode), which also showed fragment ions at *m/z* 996 [M − Gal-*O*-Fuc − Na]$^-$, 832 [M − Gal-*O*-Fuc-*O*-Gal − 2H − Na]$^-$, and 558 [M − Gal-*O*-Fuc-*O*-Gal-*O*-Xyl-(*O*-Qui) + 2H − Na]$^-$, corresponding to the sequential losses of galactose, fucose, galactose, quinovose, and xylose units. The fragment ion at *m/z* 410 corresponded to the pseudomolecular ion of the aglycon. The presence of galactose, fucose, xylose, and quinovose in a 2:1:1:2 ratio was established by acid hydrolysis with aqueous 2 N trifluoroacetic acid followed by GC analysis of the corresponding alditol peracetates. Analysis of ^{1}H and ^{13}C NMR data (Table 2) showed that anasteroside B (**3**) has the same hexasaccharide chain as anasteroside A (**2**).

The ^{1}H and ^{13}C NMR data (Table 1) of the aglycon moieties of **2** and **3** indicated that the glycosides differ only in the side chain. The presence of a methyl singlet at δ 2.17 ppm in the ^{1}H NMR spectrum of **3** and the signals at δ 210.0 and 31.0 in the ^{13}C NMR spectrum confirmed the presence of asterone (3β,6α-dihydroxypregn-9(11)-en-20-one) as the aglycon of glycoside **3**, identical to that of forbeside E3 from *Asterias forbeside*.[20]

Hence, the structure of anasteroside B (**3**) was determined as sodium 6α-*O*-{β-D-galactopyranosyl-(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}-5α-pregn-9(11)-en-20-one-3β-yl-sulfate.

Although a number of saponins containing asterone as aglycon have been reported in the literature, some authors question whether these glycosides are true natural saponins[2] since asterone is an artifact easily obtained by retroaldol cleavage of glycosides containing aglycones with 20-hydroxy-23-oxo side chains. These types of asterosaponins have rendered asterone glycosides by treatment of their alkaline solutions under reflux[21] or hydrothermolysis in water/dioxane at 140 °C.[22]

Nevertheless, the mild extraction and processing conditions employed in this work and our previous experience in the isolation from the starfish *Cosmasterias lurida*[23] of five steroidal glycosides containing 3β,6α,20*S*-trihydroxy-5α-cholest-9(11)-en-23-one as the aglycon, with no detection of the corresponding asterone glycosides, suggest that anasteroside B (**3**) is a naturally occurring asterosaponin and not an artifact.

Since some asterosaponins have been reported to show antifungal properties,[3] hexaglycosides **1**−**3** were evaluated by a bioautographic technique[24] for their antifungal activity against the plant pathogenic fungus *Cladosporium cucumerinum*. To evaluate the influence of the oligosaccharide moiety on the antifungal activity of these hexaglycosides, versicoside A (**1**), the major saponin isolated from *A. minuta*, was enzymatically hydrolyzed with *Charonia lampas* glycosidase mixture to give the triglycoside forbeside H (**1a**) and the pentaglycoside thornasteroside A (**1b**). Hexaglycosides **1** and **2** and pentaglycoside **1b** were active in a concentration-dependent manner (Figure 1). The compounds were found to be less active than benomyl, a commercially available fungicide, which showed an inhibition zone of 20 mm at a concentration of 10 μg/spot. Anasteroside A (**2**), lacking the hydroxyl group at C-20, was the most active compound, while anasteroside B (**3**), containing the same hexaglycoside chain as **1** and **2** but with a shorter side chain, was inactive at all the tested concentrations. On the other hand, the enzymatic hydrolysis products of saponin **1**, the pentaglycoside **1b**, and the triglycoside **1a** showed a marked difference in their antifungal activity. While pentaglycoside **1b** is active, the triglycoside **1a** shows no antifungal activity in all concentration ranges. Desulfation of versicoside A (**1**) by solvolysis in dioxane/pyridine (1:1) rendered a totally inactive saponin. These results suggest that the side chain in the steroidal aglycon moiety, together with the structure of the sugar portion and the presence of a sulfate group at C-3, may play an important role in the antifungal activity of these steroidal saponins.

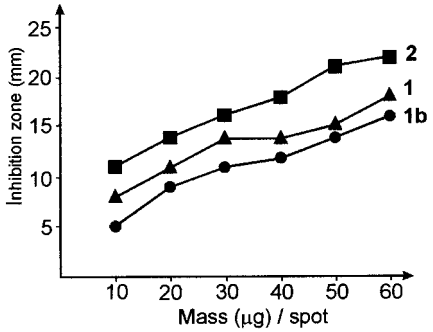

Figure 1. Dose−response curves for antifungal activity of versicoside A (**1**), anasteroside A (**2**), and thornasteroside A (**1b**) against *C. cucumerinum*.

Experimental Section

General Experimental Procedures. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker AM 500 spectrometer. The FABMS (positive and negative ion modes) were obtained on a VG-ZAB mass spectrometer, on a glycerol matrix. Optical rotations were measured on a Perkin-Elmer 343 polarimeter. IR spectra were recorded on a Nicolet Magna-550 FT-IR spectrometer. Preparative HPLC was carried out on an SP liquid chromatograph equipped with a Spectra Series P100 solvent delivery system, a Rheodyne manual injector, and a refractive index detector using a C_{18} Bondclone 10μ column (30 cm × 21.2 mm i.d.). TLC was performed on precoated Si gel F254 (*n*-BuOH/HOAc/H_2O (12:3:5)) and C_{18} reversed-phase plates (65% MeOH/H_2O). GC was performed on a Hewlett-Packard 5890A chromatograph equipped with a flame-ionization detector, an SP-2330 column (25 m × 0.2 mm i.d.) (for analysis of peracetylated alditols), and an ULTRA-2 column (50 m × 0.2 mm i.d.) (for analysis of 1-[(*S*)-*N*-acetyl-(2-hydroxypropylamino)]-1-deoxyalditol acetate derivatives). GC−MS was performed on a TRIO-2 VG mass spectrometer coupled to a Hewlett-Packard 5890A chromatograph.

Animal Material. Specimens of *A. minuta* (family Asteriidae, order Forcipulatida) were collected in February 1998 off the Golfo San Jorge near Comodoro Rivadavia, on the Argentine Patagonian coast. The organisms were identified by Dr. Alejandro Tablado of the Museo de Ciencias Naturales "Bernardino Rivadavia", Buenos Aires, Argentina, where a voucher specimen is preserved (MACN No. 34118).

Extraction and Isolation. The starfish (8 kg wet weight) were defrosted, cut into small pieces, homogenized in EtOH, and centrifuged. The EtOH extract was evaporated, and the aqueous residue was partitioned between H_2O and cyclohexane. The aqueous residue was passed through an Amberlite XAD-2 column (1 kg) and eluted with distilled water (until a negative reaction of chloride was observed) followed by MeOH. The MeOH eluate was evaporated under reduced pressure to give a glassy material (8 g) toxic to the brine shrimp *Artemia salina* (LC_{50}: 540 ppm). The MeOH extract was subjected to vacuum-dry column chromatography on Davisil C-18 reversed-phase (35−75μ) using H_2O, H_2O/MeOH mixtures with increasing amounts of MeOH, and finally MeOH as eluents. All the fractions eluted were evaluated for their lethality to *A. salina*. Fractions eluted with 50% (LC_{50}: 280 ppm), 60% (LC_{50}: 160 ppm), and 70% MeOH (LC_{50}: 80 ppm) contained the sulfated steroidal hexaglycosides. These fractions were combined and chromatographed on a Sephadex LH-60 column (80 cm × 4 cm i.d., 100 g) with MeOH/H_2O (2:1) as eluent. Fractions containing the crude asterosaponins were finally submitted to repeated reversed-phase HPLC (ODS, MeOH/H_2O 60%) to give the pure glycosides **1** (57 mg), **2** (19 mg), and **3** (7 mg).

Anasteroside A (2): white amorphous powder, mp 222−224 °C, $[\alpha]^{20}_D$ +13.6° (*c* 0.3, DMSO); IR (KBr) ν_{max} 3400, 1705, 1650, 1240, 1210, 1060; ^{1}H and ^{13}C NMR, see Tables 1 and 2; FABMS (negative ion mode) *m/z* 1389 [M − Na]$^-$, 1242 [M − Qui − Na]$^-$, 1226 [M − Gal − Na]$^-$, 1210 [M − Gal-*O* − Na]$^-$, 1081 [M − Gal-*O*-Fuc + H − Na]$^-$, 1063 [M − Gal-*O*-Fuc-*O* − H − Na]$^-$, 917 [M − Gal-*O*-Fuc-*O*-Gal − H − Na]$^-$, 640 [M − Gal-*O*-Fuc-*O*-Gal-*O*-Xyl-(*O*-Qui) − Na]$^-$, 495 [aglycon − Na]$^-$.

Anasteroside B (3): white amorphous powder, mp 228−231 °C, $[\alpha]^{20}_D$ −8.9° (*c* 0.3, DMSO); IR (KBr) ν_{max} 3400, 1700, 1645, 1240, 1210, 1065; ^{1}H and ^{13}C NMR, see Tables 1 and 2; FABMS (negative ion mode) *m/z* 1305 [M − Na]$^-$, 1158 [M − Qui − Na]$^-$, 1142 [M − Gal − Na]$^-$, 1126 [M − Gal-*O* − Na]$^-$, 996 [M − Gal-*O*-Fuc − Na]$^-$, 979 [M − Gal-*O*-Fuc-*O* − H − Na]$^-$, 832 [M − Gal-*O*-Fuc-*O*-Gal − 2H − Na]$^-$, 558 [M − Gal-*O*-Fuc-*O*-Gal-*O*-Xyl-(*O*-Qui) + 2H − Na]$^-$, 410 [aglycon − Na]$^-$.

Acid Hydrolysis of 1−3. Each glycoside (3 mg) was heated in a screwcap vial with 2 N trifluoroacetic acid (1 mL) at 120 °C for 2 h. The aglycon was extracted with CH_2Cl_2, and the aqueous residue was evaporated under reduced pressure. Each sugar mixture was treated with 0.5 M NH_3 (0.5 mL) and $NaBH_4$ (4 mg) at room temperature for 18 h. After acidification with 1 M AcOH, each reaction mixture was treated with MeOH (0.5 mL) and evaporated under reduced pressure. Each alditol mixture was peracetylated with Ac_2O (0.5 mL) and pyridine (0.5 mL) at 100 °C for 45 min. The reaction mixtures were cooled and poured into $CHCl_3$/H_2O (1:1), and the aqueous phases were extracted with $CHCl_3$. The combined chloroform extracts were washed with H_2O (0.5 mL), saturated $NaHCO_3$ solution (0.5 mL), and H_2O (0.5 mL) and evaporated to dryness under nitrogen. Each mixture of peracetylated alditols was analyzed by GC using standard peracetylated alditols as reference samples.

Desulfation of Versicoside A (1). A solution of glycoside **1** (2 mg) in pyridine (0.2 mL) and dioxane (0.2 mL) was heated at 130 °C for 2 h. After evaporation to dryness, the reaction mixture was passed through a C_{18} cartridge, washed with water, and eluted with MeOH to give the desulfated glycoside (0.9 mg).

6α-*O*-{*β*-D-galactopyranosyl-(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}-5α-cholest-9(11)-en-23-one-3*β*-ol: ^{1}H NMR data were identical with those reported by Itakura et al.;[8] FABMS (positive ion mode) *m/z* 1349 [M + Na]$^+$.

Enzymatic Hydrolysis of Versicoside A (1). Saponin **1** (9 mg) in 1 mL of citrate buffer (pH 4.5) was incubated with a glycosidase mixture (8 mg) of *Charonia lampas* (Shikagaku Kogyo) at 37 °C for 24 h. The reaction mixture was passed through a C_{18} cartridge, washed with water, and eluted with MeOH. The mixture was purified by reversed-phase HPLC on a semipreparative C_{18} Bondclone column (CH_3CN/H_2O 65%) to give the trisaccharide **1a** (3.4 mg) and the pentasaccharide **1b** (1.1 mg).

Forbeside H (1a): amorphous powder, $[\alpha]^{20}_D$ −3.7° (*c* 0.05, H_2O); NMR data were identical with those reported by Findlay et al.;[25] FABMS (negative ion mode) *m/z* 935 [M − Na]$^-$.

Thornasteroside A (1b): amorphous powder, $[\alpha]^{20}_D$ −2.3° (*c* 0.09, H_2O); NMR data were identical with those reported by Itakura et al.;[8] FABMS (positive ion mode) *m/z* 1289 [M + Na]$^+$, 1143 [M − Fuc + Na]$^+$, 981 [M − Fuc-*O*-Gal + Na]$^+$.

Methylation of Glycoside 2 Followed by Hydrolysis. A solution of **2** (4 mg) in anhydrous DMSO (1 mL) was treated with NaOH (48 mg) and stirred at room temperature for 20 min. The reaction mixture was treated with CH_3I (0.3 mL) and stirred for a further 30 min. After addition of water (4 mL) the mixture was extracted with $CHCl_3$ (5 mL) and evaporated to dryness under nitrogen. The permethylated glycoside was heated in a screwcap vial with 2 N trifluoroacetic acid (0.7 mL) at 120 °C for 1.5 h. After extraction with $CHCl_3$, the aqueous residue was evaporated and further coevaporated with H_2O (2 × 0.5 mL) and MeOH (0.5 mL). The sugar mixture was treated with 0.5 M NH_3 (0.5 mL) and $NaBH_4$ (4 mg) at room temperature for 18 h. After acidification with 1 M AcOH, the reaction mixture was treated with MeOH (0.5 mL) and evaporated under reduced pressure. The alditol mixture was acetylated with Ac_2O (0.35 mL) and pyridine (0.35 mL) at 100 °C for 45 min. The reaction mixture was cooled and poured into $CHCl_3$−H_2O (1:1) and the aqueous phase extracted with $CHCl_3$. The combined chloroform extracts were washed with H_2O (0.5 mL), saturated $NaHCO_3$ solution (0.5 mL), and H_2O (0.5 mL) and evaporated to dryness under nitrogen. The mixture of methylated alditol acetates was analyzed by GC−MS. The following carbohydrates could be detected: 3-linked quinovose (1,3,5-tri-*O*-acetyl-2,4-di-*O*-methylquinovitol; t_R = 10.5 min; *m/z* (%) 233 (1.0), 173 (0.7), 131 (6.4), 117 (13), 43 (100) or t_R = 11.1 min; *m/z* (%) 233 (0.6), 173 (1.3), 131 (6.4), 117 (14.3), 43 (100)); 2,4-linked xylose (1,4,5-tri-*O*-acetyl-2,4-di-*O*-methylxylitol; t_R = 16.6 min; *m/z* (%) 189 (4.6), 129 (23.8), 87 (17.8), 71 (3.1), 43 (100)); terminal quinovose (1,5-di-*O*-acetyl-2,3,4-tri-*O*-methylquinovitol; t_R = 7.2 min; *m/z* (%) 175 (1), 161 (1.4), 131 (6.9), 117 (8.4), 101 (11.5), 89 (8.3), 43 (100)); 2-linked galactose (1,2,5-tri-*O*-acetyl-3,4-di-*O*-methylgalactitol; t_R = 15.2 min; *m/z* (%) 205 (0.7), 189 (4.1), 161 (7.8), 145 (3), 129 (29), 101 (5), 99 (7.9), 87 (15.2), 71 (5.6), 45 (29.4), 43 (100)); 3-linked fucose (1,3,5-tri-*O*-acetyl-2,4-di-*O*-methylfucitol; t_R = 10.5; *m/z* (%) 233 (1.0), 173 (0.7), 131 (6.4), 117 (13), 43 (100) or t_R = 11.1 min; *m/z* (%) 233 (0.6), 173 (1.3), 131 (6.4), 117

(14.3), 43 (100)); terminal galactose (1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylgalactitol; $t_R = 11.7$ min; *m/z* (%) 205 (1.9), 161 (4.5), 145 (6.3), 129 (7.3), 117 (11), 101 (14.4), 87 (6.5), 71 (5.5), 45 (29.8), 43 (100)).

Determination of the Absolute Configuration of the Carbohydrate Subunits. A solution of **2** (3 mg) in 2 N trifluoroacetic acid (0.5 mL) was heated at 120 °C for 2 h. After extracting with EtOAc, the H_2O layer was concentrated to furnish the monosaccharides mixture. Then, the following solutions were added: (a) 1:8 (*S*)-1-amino-2-propanol in MeOH (20 μL), (b) 1:4 glacial AcOH/MeOH (17 μL), and (c) 3% Na[BH_3CN] in MeOH (13 μL), and the mixture was allowed to react at 65 °C for 1.5 h. After cooling, 3 M aqueous CF_3CO_2H was added dropwise until the pH dropped to pH 1−2. The mixture was evaporated and further coevaporated with H_2O (2 × 0.5 mL) and MeOH (0.5 mL). The residue was acetylated with Ac_2O (0.5 mL) and pyridine (0.5 mL) at 100 °C for 0.75 h. After cooling, the derivatives were extracted with $CHCl_3$/H_2O (1:1) (2 × 1 mL). The chloroform extracts were washed with saturated $NaHCO_3$ solution (0.5 mL) and H_2O (2 × 0.5 mL) and evaporated. The mixture of 1-[(*S*)-*N*-acetyl-(2-hydroxypropylamino)]-1-deoxyalditol acetate derivatives of the monosaccharides was identified by co-GC analysis with standard sugar derivatives prepared under the same conditions. The derivatives of D-fucose, D-quinovose, D-xylose, and D-galactose were detected with t_R (min) of 28.54, 28.92, 29.67, and 41.13, respectively.

***Artemia salina* Bioassay.** Geometric dilutions of the MeOH extract and the fractions obtained by purification of this extract by vacuum-dry column chromatography on Davisil C-18 reversed phase were freshly prepared from 10 mg/mL stock solutions in an appropriate solvent. Aliquots of these solutions (0.5 mL) were added to vials containing 10 shrimp/vial in marine water [3.8% (wt/vol) marine salts in distilled water], and the volume was adjusted to 5 mL/vial. The percentage of larvae mortality was determined after exposure to the MeOH extract and its purification fractions for 24 h at 25 °C. The LC_{50} for each fraction tested were calculated with data from four independent experiments by using the standard procedure of probit analysis.

Sample Preparation for *C. cucumerinum* Bioautographic Assay. Geometric dilutions were obtained from freshly prepared stock solutions of isolated, synthetic, and reference compounds at concentrations of 1.6−3.4 mg/mL in an appropriate solvent. Of these solutions, 10 μL was applied on the TLC plates using graduated capillaries.

Bioautographic Assay. After application of the samples on a Si gel 60 F_{254} Al sheet plate (Merck), the plate was sprayed with a suspension of *C. cucumerinum* in a nutritive medium and incubated 2−3 days in a glass box with a moist atmosphere. Clear inhibition zones appeared against a dark gray background. Benomyl was used as reference compound.

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