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Isolation, Structure Elucidation, and Biological Activity of the Steroid Oligoglycosides and Polyhydroxysteroids from the Antarctic Starfish *Acodontaster conspicuus*

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A total of 19 steroids, of which 13 steroidal oligoglycosides (nine new and four known) and six polyhydroxylated steroids (four new and two known), has been isolated from the Antarctic starfish *Acodontaster conspicuus*. The mixture is dominated by glycosides composed of steroidal aglycons having the hydroxyl groups typically disposed on one side of the tetracyclic nucleus, i.e., 3β,4β,6α,8,15β-, with some having a sulfate at C-6, and differing in the side chains and/or in the disaccharide moieties that are usually attached at C-26, with some at C-28 and C-29. Those compounds are accompanied by minute amounts of glycosides with a $\Delta^{8(14)}$-double bond in the steroid, which is a structural feature not previously found among polyhydroxysteroids derived from starfish. Small amounts of six related unglycosidated polyhydroxysteroids and three higher-molecular-weight asterosaponins complete the composition of the mixture. The structures of the new compounds were determined by interpretation of their spectral data and by comparison with spectral data of known compounds. Eighteen of these compounds were evaluated for their ability to inhibit growth in Antarctic marine bacteria isolated from either the water column or the surfaces of benthic marine invertebrates. Of these compounds, 50% were active against at least one Antarctic marine bacterium. This suggests that these compounds may play an important role in deterring microbial fouling.

As part of a project on the chemical ecology of Antarctic marine invertebrates and in a continuation of our search for new biologically active compounds from echinoderms, we have examined the extracts of the Antarctic starfish *Acodontaster conspicuus* (Koehler, 1920; family *Odontasteridae*, order Valvatida), from which a mixture of 19 compounds has been separated into four groups of compounds. The first group consisted of three known "asterosaponins"(**1**−**3**), the components with the highest molecular weight, made up of a pentasaccharide chain linked at C-6 of a $\Delta^{9(11)}$-3β,6α-dihydroxysteroid-3-sulfated aglycon. The second group was made up of nine steroidal diglycosides (**4**−**12**), of which eight (**5**−**12**) are new and are designated acodontasterosides A−H. The third group consisted of a single new monoglycoside, acodontasteroside I (**13**). The fourth

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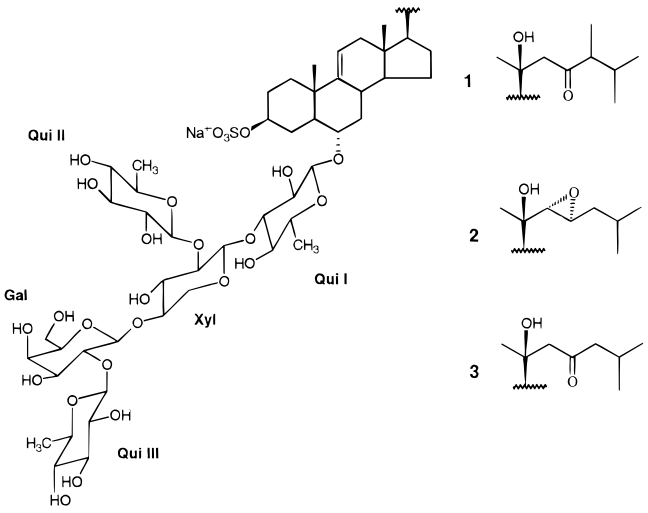


group was composed of six polyhydroxylated steroids (**14**−**19**), of which four are new (**16**−**19**).

The major glycosides are characterizated by a common 3β,4β,6α,8,15β-pentahydroxysteroid, four (**4**−**7**) having a sulfate at C-6, which is also a common feature among starfish-derived steroid glycosides.[1] They differ in the side chain and for the saccharide moieties, which are linked at C-26, with some at C-28 or C-29. Two minor components (**12** and **13**) possess steroid aglycons with a $\Delta^{8(14)}$-double bond, a rare feature in marine steroids, previously found in the sponges *Theonella swinhoe*[2,3] and *Pellina semitubulosa*.[4] In agreement with a common distribution of the polyhydroxysteroid constituents found in starfishes,[1] in the antarctic species *Acodontaster conspicuus*, the steroid oligoglycosides are accompanied by minor amounts of related polyhydroxysteroids, two of which also have a $\Delta^{8(14)}$-double bond.

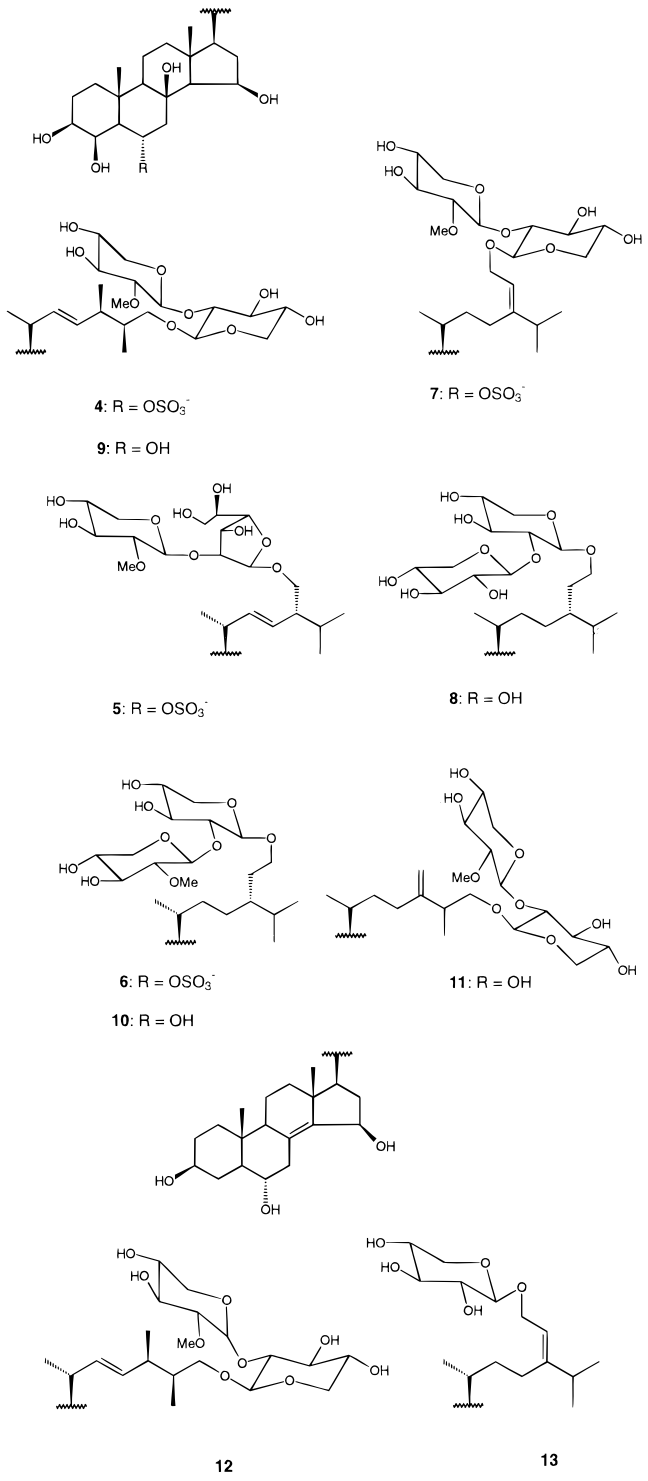
In this paper we describe the isolation of the steroidal metabolites, the structural elucidation of the new constituents, and also report the biological activities associated with the isolated metabolites.

Results and Discussion

Specimens of *Acodontaster conspicuus* were collected in November 1993, from McMurdo Sound, Antarctica. The freeze-dried material was extracted with H_2O and then with Me_2CO. The asterosaponins, the steroidal glycosides, and the polyhydroxylated steroids were recovered from the aqueous extract by passing it through a column of Amberlite, washing out salts with distilled H_2O, and eluting the absorbed material with MeOH. The Me_2CO extract was partitioned between H_2O and Et_2O, and the aqueous fraction was then extracted with 1-butanol. The MeOH eluate from the Amberlite column and the 1-butanol-soluble material from the Me_2CO extract were combined and fractionated by sequential application of gel permeation on Sephadex LH-60, DCCC, and HPLC.

The known asterosaponins (**1**−**3**) were identified by direct comparison (^{1}H NMR and FABMS) with authentic samples isolated from *Asterias amurensis*.[5,6]

Halityloside I (**4**), first isolated as a minor component among the glycosides from *Halityle regularis*,[7] is the second most abundant steroid constituent isolated from the extracts of *Acodontaster conspicuus*. The structure was elucidated by analysis of ^{1}H-NMR, ^{13}C-NMR, and FABMS spectra and by comparison with an authentic



sample. The stereochemistry at C-24 and C-25 is suggested by analogy with the co-occurring steroid **18**, whose 24*R*,25*S* configuration has been determined (see below).

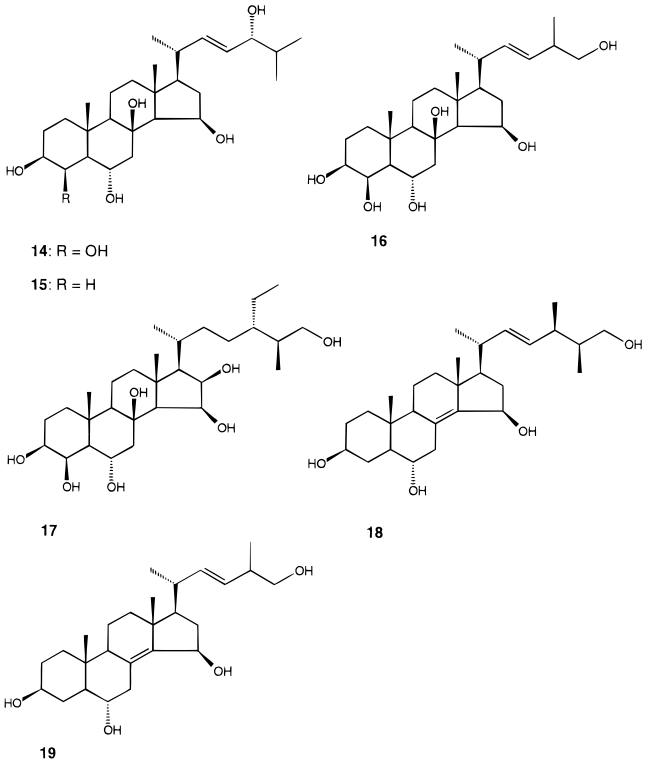
NMR data indicated that acodontasterosides A−C (**5**−**7**) possess tetracyclic steroidal nuclei identical with that of halityloside I (**4**) (Table 1). Thus, it remained to determine the structures of the side-chains and of the saccharide moieties.

The FABMS of **5** gave a molecular anion peak at *m*/*z* 867 [MSO_3^-] accompanied by fragment ion peaks at *m*/*z* 721 and 559, corresponding to the sequential loss of a methoxylated pentose unit (146 mass units) and a hexose unit (162 mass units). The hexose was identified

Table 1. NMR Data of The Steroidal Nucleus of Compounds **5**, **11**, and **17**[a]

	5[b]		**11**[c]		**17**	
position	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C
1		39.5		39.5		39.8
2		26.5		26.0		26.3
3	3.50 m	72.7	3.47 m	73.5	3.45 m	73.6
4	4.33 br s	68.9	4.29 br s	68.9	4.29 br s	69.2
5		56.0		57.1		57.3
6	4.95 dt[d]	74.5	4.19 dt (10.0, 3.5)	64.6	4.22 dt (10.2, 3.5)	64.9
7	2.74 dd (12.2, 4.0)	47.6	2.48 dd (12.0, 4.1)	49.8	2.50 dd (12.0, 3.5)	49.8
8		77.5		77.2		77.2
9		57.6		58.2		58.6
10		38.7		38.0		38.1
11		19.1		19.0		19.1
12		43.7		43.2		43.6
13		44.2		44.3		44.4
14		62.7		62.5		61.3
15	4.44 t (5.5)	71.1	4.47 t (5.5)	70.9	4.40 t (5.6)	71.4
16	2.40 m	42.6	2.40 m	42.3	4.25 t (5.6)	72.9
17		58.1		57.6		63.4
18	1.31 s	16.7	1.29 s	16.3	1.27 s	18.0
19	1.26 s	17.0	1.19 s	16.8	1.19 s	17.1

[a] The coupling constants are given in Hz and are enclosed in parentheses. [b] The data reported for compound **5** are identical with those of compounds **4**, **6**, and **7**. [c] The data reported for compound **11** are identical with those of compounds **8**, **9**, **10**, **14**, and **16**. [d] Overlapped with solvent signal.



as galactose by acid methanolysis affording methyl galactosides (GLC after silylation). An accurate analysis of the 500-MHz ^{1}H-NMR spectrum and comparison with known compounds such as the crossasterosides P_1 and P_2 having 2-*O*-MeXyl$_p$-(1→2)-Gal$_f$ saccharide[8] and with antarcticoside L with a 4-*O*-MeXyl$_p$-(1→2)-Gal$_f$ saccharide,[9] allowed us to assign all the signals of the sugar moiety. This identified the presence of a 2-*O*-methyl-*β*-xylopyranosyl and *β*-galactofuranosyl moieties (Table 2). The ^{13}C-NMR spectrum confirmed the sequence and the presence of 2-*O*-methyl-*β*-xylopyranosyl (δ_{C-2}: 84.8 vs. 75.0 in *β*-xylopyranoside, cf. **13**) and *β*-galactofuranosyl units, and established the intergly-

Table 2. Assignments of the NMR Signals (CD_3OD) of the 2-*O*-Methyl-*β*-xylopyranosyl-(1→2)-*β*-galactofuranosyl Unit in **5**[a]

	2-*O*-methyl-*β*-xylopyranosyl		*β*-galactofuranosyl	
position	^{1}H	^{13}C	^{1}H	^{13}C
H-1′	4.49 d (7.3)[b]	104.6	5.00 s	108.0
H-2′	2.88 dd (9.7, 7.3)	84.8	4.11 m	91.7
H-3′	3.34[c]	77.6	4.18 dd (7.6, 4.0)	77.3
H-4′	3.50 m	71.0	3.88 dd (7.8, 3.4)	83.0
H-5′	3.88 dd (10.5, 4.7) 3.20 dd (10.5, 7.9)	67.1	3.74 m	72.2
H-6′			3.65 d (6.3)	64.8
OMe	3.61 s	61.3		

[a] ^{1}H assignments were aided by COSY experiments. [b] The coupling constants are given in Hz and are enclosed in parentheses. [c] Overlapped with solvent signal.

cosidic linkage to be 2-*O*-MeXyl$_p$-(1→2)-Gal$_f$ (δ_{C-1} and δ_{C-2} of Gal$_f$: 108.0 and 91.7 vs. 109.6 and 83.3 ppm in *β*-galactofuranosides[9]). The ^{1}H-NMR spectrum (Table 3) also contained olefinic signals at δ 5.27 (2H, AB part of an ABXY system with J_{AB} = 16 Hz), two 1H double doublets at δ 3.61 (J = 10.0, 6.5 Hz) and 3.50 (J = 10.0, 5.0 Hz), this latter signal partially overlapped with H-4′ of the xylosyl residue, and three methyl doublets at δ 1.05, 0.93, and 0.88. These data corresponded to a Δ^{22E}-24-methylcholestanol side chain in which one of the methyl groups has been oxidized to an oxymethylene. A comparison of the NMR data of **5** (Table 3) with those of steroid-containing Δ^{22E}-24-methyl-21-hydroxy,[10] Δ^{22E}-24-methyl-26-hydroxy,[11] and Δ^{22E}-24-(hydroxymethyl)[12] side chains allowed assignment of the 24-(hydroxymethyl) structure instead of the alternative ones. The ^{13}C-NMR spectrum of **5** (Table 4) gave support to this assignment and also indicated the disaccharide moiety to be linked to C-28 [δ_{C-28}: 70.9 vs. 64.1−64.3 ppm in the model 24*S*- and 24*R*-(hydroxymethyl)steroids,[12] respectively]. The configuration at C-24 of acodontasteroside A (**5**) was then assigned after acid treatment with 2 M HCl−MeOH to yield 24-methyl-5α-cholesta-8(9),14,22E-triene-3*β*,4*β*,6α,28-tetrol (see Experimental Section), which was converted to its 3,6,28-tri-(−)-MTPA derivative.[13] In the ^{1}H-NMR spectrum (CD_3OD) of the latter, the resonances of the C-28 protons, δ 4.44 dd (J = 10.5, 5.5 Hz) and 4.24 (partially overlapped with other signals) were in good agreement with the shifts of the 28-(−)-MTPA derivative of (22*E*,24*R*)-3α,5-cyclo-6*β*-methoxy-5α-ergost-22-en-28-ol, but significantly different from those of the (−)-MTPA ester of the corresponding 24*S*-isomer (δ 4.32 br s, 2H),[12] thus establishing the stereochemistry in acodontasteroside A (**5**) to be 24*R*. Thus, the structure of acodontasteroside A can be defined as (22*E*,24*R*)-28-*O*-[2-*O*-methyl-*β*-xylopyranosyl-(1→2)-*β*-galactofuranosyl]-24-methyl-5α-cholest-22-ene-3*β*,4*β*,6α,8,15*β*,28-hexol, 6-sulfate (**5**).

The FABMS of acodontasteroside B (**6**) gave a molecular anion peak at *m*/*z* 853 [MSO_3^-] accompanied by fragment ions at *m*/*z* 707 and 575 due to the sequential loss of a methoxylated pentose (146 mass units) and a pentose (132 mass units). Analysis of ^{1}H- and ^{13}C-NMR spectra (Tables 1, 3, 4, and 5) immediately indicated the presence of the common 2-*O*-methyl-*β*-xylopyranosyl-(1→2)-*β*-xylopyranosyl disaccharide moiety, already found in glycosides from starfishes[1] (e.g., halitylosides from *Halityle regularis*[7]) as well as the presence of the 24-(*β*-hydroxyethyl)cholestane side chain, previously encountered in steroids from starfishes[1] including halityloside A and B, major constituents of *Halityle regu-*

Table 3. Selected ^{1}H-NMR Signals (δ_H) of the Steroidal Side Chains of Compounds **4**−**19**

position	**4**, **9**, **12**[a]	**5**	**6**, **8**, **10**[c]	**7**, **13**[d]	**11**	**14**, **15**[e]	**16**, **19**[f]	**17**	**18**
H-20	2.19 m	2.17 m	1.83 m	2.21 m	1.59 m	2.22 m	2.18 m	1.74 m	2.19 m
H_3-21	1.07 d (6.5)	1.05 d (6.8)	0.97 d (7.0)	1.02 d (7.0)	0.99 d (7.0)	1.04 d (7.0)	1.03 d (6.8)	0.98 (6.8)	1.06 d (7.0)
H-22	5.30 dd[b]	5.27 dd[b]				5.45 dd (15.0, 7.5)	5.30 dd[b]		5.31 dd (15.2, 8.0)
H-23	5.30 dd[b]	5.27 dd[b]				5.37 dd (15.0, 7.0)	5.30 dd[b]		5.27 dd (15.2, 8.5)
H-24						3.68 t (7.0)			
H-25				2.30 m	2.42 m	1.62 m	3.43 dd (10.5, 6.0) 3.30 dd[g]		
H_2-26	3.82 dd (10.2, 6.0) 3.32 dd (10.2, 7.0)				3.63 t (8.4) 3.57 dd (8.4, 5.3)			3.56 dd (11.0, 6.2) 3.40 dd[g]	3.60 dd (10.5, 6.0) 3.30 dd[g]
H_3-26		0.88 d (7.0)	0.90 d (6.8)	1.07 d (7.0)		0.94 d (6.5)	1.00 d (6.8)		
H_3-27	0.95 d (7.0)	0.93 d (7.0)	0.87 d (6.8)	1.06 d (7.0)	1.12 d (7.0)	0.88 d (7.0)		0.91 d (6.8)	0.92 d (7.0)
H_2-28		3.61 dd (10.0, 6.5) 3.50 dd (10.0, 5.0)		5.36 t (7.0)	4.80 br d				0.97 d (7.0)
H_3-28	0.97 d (7.0)								
H_2-29				4.32 dd (10.5, 7.0) 4.19 dd (10.5, 6.8)				0.92 t (7.0)	

[a] The chemical shifts of compounds **9** and **12** are virtually identical with those of **4**. [b] Overlapped each other. [c] The chemical shifts of compounds **8** and **10** are virtually identical with those of **6**. [d] The chemical shifts of compound **13** are virtually identical with those of **7**. [e] The chemical shifts of compound **15** are virtually identical with those of **14**. [f] The chemical shifts of compound **19** are virtually identical with those of **16**. [g] Overlapped with solvent signal. The coupling constants are given in Hz and are enclosed in parentheses.

Table 4. Selected ^{13}C-NMR Signals of the Steroidal Side Chains of Compounds **4**−**7**, **11**, **14**, **16**−**18**

carbon	**4**[a]	**5**	**6**[b]	**7**[c]	**11**	**14**[d]	**16**	**17**	**18**
20	39.6	41.4	36.6	36.7	35.9	40.2	40.8	31.5	40.5
21	21.7	21.1	19.0	19.0	18.8	20.7	20.7	18.7	21.7
22	136.6	140.3	34.8	36.9	35.5	139.3	137.7	35.1	136.6
23	133.9	128.0	28.1	27.2	32.6	130.5	131.3	28.5	133.9
24	39.4	50.2	41.9	152.5	154.0	79.3	40.4	43.8	39.4
25	41.9	29.6	30.5	35.9	40.6	34.1	68.3	38.9	41.9
26	66.5	21.4	19.1	22.5	74.6	18.8	17.1	66.9	66.5
27	16.8	19.0	20.0	22.5	17.7	18.7		13.8	16.8
28	14.1	70.9	31.7	119.2	109.1			25.9	14.1
29			69.5	66.5				12.6	

[a] The chemical shifts of the the side chain carbons of compound **4** are virtually identical with those of **9** and **12**. [b] The chemical shifts of the the side chain carbons of compound **6** are virtually identical with those of **10**. [c] The chemical shifts of the the side chain carbons of compound **7** are virtually identical with those of **13**. [d] The chemical shifts of the the side chain carbons of compound **14** are virtually identical with those of **15**.

Table 5. Assignments of the NMR Signals (CD_3OD) of 2-*O*-Methyl-β-xylopyranosyl-(1→2)-β-xylopyranosyl Unit in **4**, **6**, **7**, **9**−**12**[a,b]

	2-*O*-methyl-β-xylopyranosyl		β-xylopyranosyl	
position	^{1}H	^{13}C	^{1}H	^{13}C
H-1′	4.74 d (7.3)	104.4	4.37 d (6.9)	103.4
H-2′	2.95 dd (9.7, 7.3)	84.8	3.48[c]	81.1
H-3′	3.42 t (9.7)	77.5	3.26 t (9.0)	76.8
H-4′	3.52 m	70.9	3.52[c]	71.0
H-5′	3.91 dd (10.5, 4.7) 3.19 dd (10.5, 7.9)	66.5	3.89 dd (10.5, 4.7) 3.20 t (10.5)	66.3
OMe	3.64 s	60.7		

[a] ^{1}H assignments were aided by COSY experiments. The coupling constants are given in Hz and are enclosed in parentheses. [b] Data extracted from the spectra of **11**. [c] Overlapped with other signals.

laris.[7] The ^{13}C-NMR data also indicated the attachment of the disaccharide moiety at C-29. The 24*R* configuration was assigned on the basis of the chemical-shift difference between the C-26 and C-27 resonances, δ 19.1 and 20.0 ppm ($\Delta\delta$ 0.9 ppm) and comparison with those in the ^{13}C-NMR spectra of the 24*R* (18.6 and 19.7 ppm; $\Delta\delta$ 1.1 ppm) and 24*S* (19.3 and 19.2 ppm; $\Delta\delta$ 0.1 ppm) model steroids.[14]

Acodontasteroside C (**7**), is the 24(28)-dehydro derivative of **6**. In FABMS it gave a molecular ion peak at *m*/*z* 851 [MSO_3^-] and fragments at *m*/*z* 705 and 573 (loss of 146 and 132 mass units). The signal of the allylic methine proton at C-25 appeared at δ 2.30 (m) and the *E* stereochemistry of the 24(28)-double bond was assigned based on relative shielding arguments between the *E* and *Z* isomers, fucosterol and isofucosterol.[15] This assignment was supported by the ^{13}C-NMR signals for C-25 (downfield shifted to 35.9 ppm) (Table 4) when compared with the corresponding data for fucosterol (*E* isomer, C-25: 34.8 ppm) and isofucosterol (*Z* isomer, C-25: 28.6 ppm).[16]

NMR data (Table 1) of acodontasterosides D−G (**8**−**11**) indicated that these compounds possess the same nuclear 3β,4β,6α,8,15β-hydroxylation pattern and differ in their side chains and in their saccharide moieties. Glycosides **9** and **10** are simply desulfated halityloside I (**4**) and acodontasteroside B (**6**), respectively, as conclusively shown by direct comparison of the NMR spectral data of **9** and **10** with those of the desulfated **4** and **6**, respectively prepared by solvolysis in pyridine−dioxane 1:1 at 140 °C for 2 h.

Acodontasteroside D (**8**), FABMS, *m*/*z* 759 [M − H]$^-$, and fragment peaks at *m*/*z* 627 and 495 corresponding to the sequential loss of two pentose units, is related to **10** by the lack of the methyl group at position 2 of the terminal xylose, as confirmed by ^{1}H- and ^{13}C-NMR data (see Experimental Section).

Acodontasteroside G (**11**), FABMS, *m*/*z* 757 [M − H]$^-$, 611 (loss of a methoxylated pentose), 479 (loss of a pentose), is isomeric with **9** by having the 26-hydroxy-24-methylenecholestane side chain instead of the Δ^{22}-26-hydroxy-24-methylcholestane side chain, as implied by the NMR spectra of **11**, which showed signals for two

methyls (δ 0.99 d, 18.8 ppm; δ 1.12 d, 17.7 ppm), one exomethylene (4.80 br d, 109.1 ppm and 150.0 ppm), one oxymethylene (δ 3.57 dd−3.63 t, 74.6 ppm), two methylenes (δ_C 32.6 and 35.5 ppm), and one methine (δ 2.42, 40.6 ppm). The chemical shift of the C-26 oxygenated methylene at 74.6 ppm confirmed the location of the disaccharide moiety in that position.

The FABMS of acodontasteroside H (**12**) gave a pseudomolecular ion peak at *m/z* 723 $[M - H]^-$ and fragments at *m/z* 577 (loss of a methoxylated pentose) and *m/z* 445 (further loss of pentose). Analysis of the NMR spectra indicated the presence of the common 2-*O*-methyl-β-xylopyranosyl-(1→2)-β-xylopyranosyl disaccharide moiety (cf. **6**), and of a Δ^{22E}-24-methyl-26-hydroxycholestane side chain. In addition to the signals assignable to the above structural units, the ^{13}C-NMR spectrum and DEPT measurements contained 19 more signals, including three hydroxymethine (70.2, 70.6, and 71.7 ppm) and two quaternary olefinic carbons (133.0 and 147.0 ppm) consistent with a trihydroxylated tetracyclic steroidal nucleus having a tetrasubstituted double bond. In the ^{1}H-NMR spectrum one hydroxymethine signal had the chemical shift, δ 3.52, and the shape (7-lines multiplet, $W_{1/2}$ = 20 Hz) typical of a 3α-proton of 5α-H 3β-hydroxysteroid.[17] A second hydroxyl group was located at C-6α on the basis of the shape of the signal at δ 3.40 dt (J = 10.5, 5.1 Hz) typical for an equatorial proton flanked by a methine at δ 1.20 m and an allylic methylene at δ 3.11 (dd, J = 12.9, 5.1 Hz; H-7β equatorial) and δ 1.65 (br d, J = 12.9 Hz; H-7α axial), as established by a ^{1}H−^{1}H COSY experiment. In the COSY spectrum the broad triplet at δ 4.62 due to the remaining hydromethine proton showed a distinct long-range coupling to the H-7α broad doublet (δ 1.65), thus suggesting the placement of the hydroxyl group at C-15β and the double bond at position 8(14). These structural assignments were supported by a COSY spectrum, which showed the signal at δ 4.62 (H-15) to be coupled only to one methylene at δ 2.20 m and 1.40 m (H_2-16) in addition to the homoallylic H-7 proton. Further support was provided by a comparison of the ^{1}H-NMR spectral data of **12** (see Experimental Section) with those reported for a $\Delta^{8(14)}$-3β,15β-dihydroxysteroid: δ_{H-15}: 4.65, δ CH_3-18: 1.01, and δ CH_3-19: 0.61,[3] the chemical shifts reported for the alternative $\Delta^{8(14)}$-3β,15α-dihydroxystructure, δ_{H-15}: 5.02, δ CH_3-18: 0.83, and δ CH_3-19: 0.62 ppm, were very different from our values.

Acodontasteroside I (**13**), has the common β-xylopyranosyl unit attached to a 24(28) dehydro-24-(β-hydroxyethyl)-cholestane side chain as well as the $\Delta^{8(14)}$-3β,6α,15β-trihydroxysteroidal tetracyclic nucleus as in **12**. The identification was implied by ^{1}H-NMR, ^{13}C-NMR, and FABMS [*m/z* 591 $(M - H)^-$ and fragments at *m/z* 459 corresponding to the loss of a pentose unit (132 mass units)]. The chemical shift of the carbon 29 at δ 66.5 established the attachment of the β-xylosyl unit in that position.[18]

The steroids **14** and **15** were previously isolated from an Antarctic starfish of the family *Echinasteridae*[9] and from the starfish *Coscinasterias tenuispina*,[19] respectively, and identified by direct comparison (^{1}H-NMR, ^{13}C-NMR, and FABMS).

Steroid **16** had the same steroid nucleus as **14** with a Δ^{22E}-26,27-bisnor-24-methyl-25-hydroxycholestane side chain, which was implied by ^{1}H-NMR, ^{13}C-NMR, and FABMS [*m/z* 451 $(M - H)^-$]. The determination of the structure of the side chain was straightforward: the 25-methylene protons resonated as two dd at δ 3.43 (J = 10.5, 6.0 Hz) and 3.30 (overlapped with the methanol signal) (δ_C 68.3 ppm), two methyl doublets were observed shifted downfield to δ 1.03 and 1.00 (δ_C 17.1 and 20.7 ppm), and the olefinic protons resonated as a 2H complex signal at δ 5.30 (δ_C 131.3 and 137.7 ppm). The *E* configuration was assigned on the basis of ^{13}C-NMR shift of C-20 at 40.8 ppm (value for the *Z* isomer would be expected highfield shifted to ca. 35.0 ppm).[20] Oxidized shortened side chains have already been found in polyhydroxysteroids from the starfishes *Hacelia attenuata*[21] and *Myxoderma platyacanthum*.[22] It has been suggested that marine C-26-sterols (i.e., 24-methyl-26,27-bisnor) originate from phytoplankton;[23] thus, the occurrence of **16** may be of some interest as an indicator of the capability of the starfish to oxidize dietary sterols.

Steroid **17** had the 3β,4β,6α,8,15β,16β-hexahydroxysteroidal tetracyclic nucleus already encountered in some starfish-derived steroids and steroid glycosides[1,24] and a hydroxylated C-10 side chain, which was implied by its FABMS, *m/z* 511 $[M - H]^-$. The ^{1}H-NMR spectrum contained signals for a hydroxymethylene grouping and two double doublets at δ 3.56 (J = 11.0, 6.2 Hz) and 3.40 (partially overlapped with CD_2HOD signal), and also showed two methyl doublets at δ 0.98 and 0.91 and one methyl triplet at δ 0.92. These data were consistent with a 24-ethyl-26-hydroxycholestane side chain. This conclusion was supported by ^{13}C-NMR data (Table 4); the chemical shifts from C-23 to C-29 are virtually identical with those reported for the 24-ethyl-21,26-dihydroxylated,21-sulfated side chain encountered in a sulfated steroid from the ophiuroid *Ophiolepis superba*.[25] The stereochemistry at C-24 and C-25 was assigned by comparison of the spectral data with those of stereoselectively synthesized model compounds.[26] The ^{13}C-NMR spectrum and especially the chemical shift of C-28 permitted assignment of the relative stereochemistry (*threo* model 24*R*,25*S*/24*S*,25*R*, δ_C-28: 23.6−23.7 and *erythro* model: 24*R*,25*R*/24*S*,25*S*, δ_C-28: 25.2−24.9 ppm), and allowed the *erythro* stereochemistry to be assigned to **17**, δ_C-28: 25.9 ppm. The absolute configuration was then derived by ^{1}H-NMR analysis of the *S*(−)-MTPA derivative. In the (−)-MTPA esters of each pair, with the same relative stereochemistry, the C-26 methylene protons of the 25*R*-isomer appeared as signals resonating much more closely, usually a broad doublet, than in the 25*S*-isomer, which usually appeared as two double doublets separated by 0.2 ppm or more. The opposite is true for the (+)-MTPA esters. The ^{1}H-NMR of the 3,6,26-tri-*O*-(−)-MTPA ester of **17** showed the 26-methylene protons as two double doublets at 4.15 and 4.39 ppm, and accordingly we assigned the 24*S*,25*S* configuration to the steroid **17**.

The ^{1}H and ^{13}C spectra of steroids **18** and **19** readily implied that both compounds had a steroidal nucleus identical with that of the previous **12** and **13**. The ^{1}H-NMR of steroid **18**, FABMS, *m/z* 445 $[M - H]^-$, also contained three methyl doublets at δ 1.06, 0.97, and 0.92, two olefinic signals at 5.27 dd (J = 15.2, 8.5 Hz) and 5.31 dd (J = 15.2, 8.0 Hz), and two 1H double doublets at δ 3.60 dd (J = 10.5, 6.0 Hz) and 3.30 partially overlapped with the CD_2HOD signal, consistent with a Δ^{22E}-24-methyl-26-hydroxycholestane side

chain. Direct comparison of the spectral data (^{1}H- and ^{13}C-NMR, see Experimental Section and Tables 3 and 4) with those of the stereoselectively synthetized model compounds[11] supported this conclusion, and also allowed the 24*R*,25*S* configuration to be assigned. In the ^{1}H-NMR spectra of the *threo* and *erythro* isomers, major differences relate to the shifts of the protons at C-26, C-27, and C-28[11] and our values are very close to those of *threo* isomers. Treatment of **18** with (−)-MTPA chloride and measurement of the ^{1}H-NMR spectrum of the resulting 3,15,26-tri-*O*-(−)-MTPA ester showed two well-separated double doublets at δ 4.06 and 4.44, in agreement with a 25*S* configuration. The 26-proton resonances are expected to be much closer, usually a broad doublet, in the case of the 25*R* configuration.[11] Thus, we assigned the 24*R*,25*S* configuration to the steroid **18**. The side chain of the steroid **19**, FABMS, *m*/*z* 417 $[M - H]^-$, was shown to be identical with that of **16**.

The antimicrobial activity of each of the isolated compounds, except **17**, was determined as described in the Experimental section against the four strains of bacteria McM18.1, McM13.3, McM32.2, and McM11.5 isolated from the H_2O column or surfaces of macroinvertebrates. None of the asterosaponins **1**−**3** was active against any of the bacteria, but acodontasterosides D (**8**), E (**9**), F (**10**), and I (**13**) and steroids **15** and **18** all showed activity against McM13.3 and McM32.2; acodontasteroside G (**11**) showed activity against McM32.2 strain; acodontasteroside H (**12**) showed activity against McM11.5; and steroid **19** was active against McM13.3. These results suggested that these compounds may play an ecological role in preventing microbial fouling in the body wall surfaces of *Acodontaster conspicuus*.

Experimental Section

General Experimental Procedures: NMR spectra, Bruker AMX-500 (^{1}H at 500 MHz, ^{13}C at 125 MHz), δ (ppm), *J* in Hz, spectra referred to CHD_2OD signal at 3.34 ppm and central carbon CD_3OD signal at 49.0 ppm; MS, VG AUTOSPEC instruments (Cs^+ ions bombardment) with FAB source [in glycerol or glycerol−thioglycerol (3:1) matrix]; optical rotation Perkin-Elmer 141 polarimeter; GLC, Carlo Erba Fractovap 2900 for capillary column (SPB-1, 25 m, 150 °C; helium carrier flow 10 mL min^{-1}); reversed-phase HPLC, C_{18} μ-Bondapak column (30 cm × 8 mm i.d.; flow rate 5 mL min^{-1}) and C_{18} μ-Bondapak column (30 cm × 3.9 mm i.d.; flow rate 2 mL min^{-1}), Waters Model 6000 A pump equipped with U6K injector and a differential refractometer, model 401; DCCC, DCC-A apparatus manufactured by Tokyo Rikakikai Co., equipped with 250 tubes.

Antimicrobial Assay. Four strains of psychrotrophic marine bacteria were isolated in McMurdo Sound, Antarctica (77.5°S, 166°E) in October 1996, and used to assay antimicrobial bioactivity of the purified compounds isolated from *A. conspicuus*. Strain McM18.1 was isolated on Difco Marine Agar 2216 (Difco Laboratories) (DMA) from surface swabs of the sea urchin *Odontaster validus* and formed white, mucoid colonies. Strain McM13.3 was isolated on DMA from surface swabs of the sponge *Dendrilla membranosa* and formed orange, mucoid colonies. Strain McM32.2 was isolated on DMA from surface swabs of the sponge *Leucetta leptorhapsis* and formed yellow, mucoid colonies. Strain McM11.5 was isolated on Oppenheimer-Zobell Reduced Media Agar[27] by filtration from the water column and formed red, mucoid colonies. All four strains were sensitive to the antibiotics tetracycline and chloramphenicol. McM18.1 differed from the other in being resistant to penicillin. All were capable of growth at both −1.0 °C and 20 °C. The voucher specimens of the bacterial strains tested are available in the Department of Chemistry at Florida Institute of Technology, Melbourne, FL.

Paper disks (BBL Microbiology Systems 31039) containing 0.1 mg of each compound were prepared by placing 20 μL of a 5mg/mL solution in 100% MeOH onto each disk. For bioassays, the bacteria were spread onto the media from which they were isolated. Pure compound-containing disks were placed onto the culture plates and the cells allowed to grow for 48 h (McM18.1, McM13.3, McM32.2) or 72 h (McM11.5) at 20 (±1) °C. Antimicrobial activity was defined as visible inhibition of cell growth in a region surrounding the paper disk. Disks prepared with only the MeOH solvent did not inhibit bacterial growth.

Animal Material. The animals, *Acodontaster conspicuus*, 240 g (fresh), were collected in November 1993, from the McMurdo Sound (Cape Armitage), Antarctica, at a depth of 24−30 m. The voucher specimens of *Acodontaster conspicuus* are available in the Department of Chemistry at Florida Institute of Technology, Melbourne, FL.

Extraction and Isolation. The freeze-dried animals were cut into small pieces and soaked in H_2O for 5 h. The aqueous extracts were centrifuged and passed through a column of Amberlite XAD-2 (500 g). The column was washed with distilled H_2O (6 L) and then eluted with MeOH (8 L). The MeOH eluate was taken to dryness to give the glassy material (4.3 g). The remaining solid mass, after extraction with H_2O, was then re-extracted with Me_2CO (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 extracts afforded 2.0 g of a glassy material that was combined with the above MeOH eluate from Amberlite XAD-2 column and chromatographed on a column of 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 125−150 (0.8 g) mainly contained the asterosaponins. The crude asterosaponins fraction was submitted to DCCC with *n*-BuOH−Me_2CO−H_2O (3:1:5) [descending mode; the upper phase was used as the stationary phase; flow rate 14 mL/h; fraction (7 mL) were collected] to give three main fractions: 109−119 (20 mg), 120−148 (38 mg), and 149−153 (5 mg). The flow rate was 5 mL/min. Fractions 109−119 contained asteroside C **1**. The fractions 120−148 contained asteroside C **1**, asteroside A **2**, and glycoside B_2 **3**. Fractions 149−153 contained a small amount of **3**. The first fractions 9−100 (250 mg) contained a mixture of UV-active compounds, and the last fractions 154−170 (200 mg) were composed of a mixture of sulfated compounds. These fractions were then separated by reversed-phase HPLC (C_{18} μ-Bondapak 30 cm × 7.8 mm i.d.) with MeOH−H_2O (1:1) as the eluent, to give pure

Table 6. DCCC Fractionation[a] of the Mixture of Steroid Glycosides and Polyhydroxysteroids (Fractions 156−225 from Sephadex LH-60)

fractions no.	amounts (mg)	compounds
12−35	540	mixture of sulfated compounds
36−40	34	8
41−47	66	11
48−53	45	11, 9, 10
54−58	25	9, 10
59−71	13	16, 12
72−81	19	16, 13
82−100	4.5	14
101−104	7.4	19, 17
105−109	6	19
110−118	7.8	15
119−136	28	18

[a] Solvent system $CHCl_3$−MeOH−H_2O (7:13:8), ascending mode, 250 tubes, 4 mL fractions collected.

saponins: **1** (7.6 mg), **2** (5.2 mg), **3** (5.6 mg). Fractions 151−155 (200 mg) contained steroidal glycoside sulfates, and fractions 156−225 (1.85 g) contained mixed glycosides and polyhydroxysteroids.

DCCC fractionation of the mixture of steroid glycosides and polyhydroxysteroids was pursued using $CHCl_3$−MeOH−H_2O (7:13:8) in the ascending mode (the lower phase was the stationary phase). Fraction (6 mL each) were collected and monitored by TLC on SiO_2 with $CHCl_3$−MeOH−H_2O (80:18:2) and the results are summarized in Table 6.

The DCCC fractions were then submitted to HPLC with MeOH−H_2O (7:3), or MeOH−H_2O (75:25) on C_{18} column (30 cm × 3.9 mm i.d. or 30 cm × 8 mm i.d.) to give the pure compounds.

Fractions 151−155 (200 mg) eluted from the column of Sephadex LH-60 contained more polar compounds. This material was combined with the first fractions 12−35 (540 mg) derived from the above DCCC separation (see Table 6), and submitted to DCCC using *n*-BuOH−Me_2CO−H_2O (3:1:5) in the ascending mode (the lower phase was the stationary phase; flow rate 12 mL/h; 6 mL fractions were collected and monitored by TLC). Fractions 32−47 (20 mg) were a mixture of "asterosaponins"; fractions 48−69 (50 mg) contained major amounts of tryptophan; fractions 70−92 (45 mg) contained mainly compound **4**, and fractions 93−155 (198 mg) contained compounds **4**, **5**, **6** and **7**. Each fraction was purified by HPLC on a C_{18} column with MeOH−H_2O (1:1) to give pure compounds.

Asterosaponin (1): 7.6 mg; FABMS (negative ion) *m*/*z* 1257 $[MSO_3]^-$; $[\alpha]_D$ −2.0° (MeOH, *c* 1).

Asterosaponin (2): 5.2 mg; FABMS (negative ion) *m*/*z* 1243 $[MSO_3]^-$; $[\alpha]_D$ +6.0° (MeOH, *c* 1**)**.

Asterosaponin (3): 5.6 mg; FABMS (negative ion) *m*/*z* 1243 $[MSO_3]^-$; $[\alpha]_D$ +4.0° (MeOH, *c* 1).

Halityloside I (4): 19.2 mg; FABMS (negative ion) *m*/*z* 859 $[MSO_3]^-$; ^1H and ^{13}C NMR (steroidal nucleus) in Table 1; ^1H and ^{13}C NMR (saccharide portion) in Table 5; ^1H and ^{13}C NMR (side chain) in Tables 3 and 4.

Acodontasteroside A (5): 14.5 mg; FABMS (negative ion) in the text; $[\alpha]_D$ −8.0° (MeOH, *c* 1); ^1H and ^{13}C NMR (steroidal nucleus) in Table 1; ^1H and ^{13}C NMR (saccharide portion) in Table 2; ^1H and ^{13}C NMR (side chain) in Tables 3 and 4.

Acodontasteroside B (6): 7.8 mg; FABMS (negative ion) in the text; $[\alpha]_D$ +10.0° (MeOH, *c* 1); ^1H and ^{13}C NMR (steroidal nucleus) in Table 1; ^1H and ^{13}C NMR (saccharide portion) in Table 5; ^1H and ^{13}C NMR (side chain) in Tables 3 and 4.

Acodontasteroside C (7): 9.5 mg; FABMS (negative ion) in the text; $[\alpha]_D$ −11.1° (MeOH, *c* 1); ^1H and ^{13}C NMR (steroidal nucleus) in Table 1; ^1H and ^{13}C NMR (side chain) in Tables 3 and 4; ^1H and ^{13}C NMR (saccharide portion) in Table 5.

Acodontasteroside D (8): 6.0 mg; FABMS (negative ion) in the text; $[\alpha]_D$ +8.0° (MeOH, *c* 1); ^1H and ^{13}C NMR (steroidal nucleus) in Table 1; ^1H and ^{13}C NMR (side chain) in Tables 3 and 4; ^1H NMR (sugar): 4.50 (1H, d, J = 6.6 Hz, H-1″), 4.36 (1H, d, J = 6.6 Hz, H-1′), 3.90−3.88 (each 1H, dd, H-5′ and H-5″ overlapped), 3.57 (1H, m, H-4′), 3.48−3.40−3.38 (each 1H, H-4″, H-3′ and H-2′ overlapped), 3.30−3.26−3.22 (each 1H, H-3″, H-5″and H-2″ overlapped), 3.16 (1H, H-5″ overlapped with other signals); ^{13}C NMR (sugar): 106.1 (C-1″), 103.7 (C-1′), 83.4 (C-2′), 77.4 (C-3″), 77.1 (C-3′), 75.6 (C-2″), 71.1 (C-4″), 70.9 (C-4′), 67.2 (C-5′), 66.5 (C-5″).

Acodontasteroside E (9): 5.4 mg; FABMS (negative ion) *m*/*z* 769 $[M - H]^-$; ^1H and ^{13}C NMR (steroidal nucleus) in Table 1; ^1H and ^{13}C NMR (side chain) in Tables 3 and 4; ^1H and ^{13}C NMR (saccharide portion) in Table 5.

Acodontasteroside F (10): 5.1 mg; FABMS (negative ion) *m*/*z* 773 $[M - H]^-$; $[\alpha]_D$ −8.3° (MeOH, *c* 1); ^1H and ^{13}C NMR (steroidal nucleus) in Table 1; ^1H and ^{13}C NMR (side chain) in Tables 3 and 4; ^1H and ^{13}C NMR (saccharide portion) in Table 5.

Acodontasteroside G (11): 90 mg; FABMS (negative ion) in the text; $[\alpha]_D$ −14.4° (MeOH, *c* 1); ^1H and ^{13}C NMR (steroidal nucleus) in Table 1; ^1H and ^{13}C NMR (side chain) in Tables 3 and 4; ^1H and ^{13}C NMR (saccharide portion) in Table 5.

Acodontasteroside H (12): 0.6 mg; FABMS (negative ion) in the text; $[\alpha]_D$ −21.7° (MeOH, *c* 1); ^1H NMR (steroidal nucleus): 4.62 (1H, t, J = 7.6 Hz, H-15), 3.52 (1H, m, H-3), 3.40 (1H, dt, J = 10.5 and 5.1 Hz, H-6), 3.11 (1H, dd, J = 12.9, 5.1 Hz, H-7β), 2.20 (1H, m, H-16), 1.95 (1H, dd, H-9), 1.65 (1H, br d, J = 12.9 Hz, H-7α), 1.41 (1H, m, H′-16), 1.06 (3H, s, H_3-18), 0.79 (3H, s, H_3-19); ^{13}C NMR (steroidal nucleus) 147 (C-14), 133 (C-8), 71.7 (C-3), 70.5 (C-15), 70.2 (C-6), 55.5 (C-17), 52.3 (C-9), 50.3 (C-5), 44.3 (C-13), 40.0 (C-16), 39.5 (C-7), 38.7 (C-1), 38.1 (C-12), 37.9 (C-10), 33.4 (C-4), 31.9 (C-2), 20.7 (C-11), 19.4 (C-18), 13.9 (C-19); (^1H and ^{13}C NMR (side chain) in Tables 3 and 4; ^1H and ^{13}C NMR (saccharide portion) in Table 5.

Acodontasteroside I (13): 1.2 mg; FABMS (negative ion) in the text; $[\alpha]_D$ −20.8° (MeOH, *c* 1); ^1H and ^{13}C NMR data (steroidal nucleus) are identical with those reported for **12**; ^1H and ^{13}C NMR (side chain) in Table 3 and 4; ^1H NMR (sugar): 4.25 (1H, d, J = 7.6 Hz, H-1′), 3.89 (1H, dd, J = 10.0, 5.0 Hz, H-5′eq), 3.50 (1H, m, H-4′), 3.30 (1H, t, J = 9.5 Hz, H-3′), 3.20 (1H, dd, J = 9.5, 7.6 Hz, H-2′), 3.16 (1H, t, J = 10.0 Hz, H-5′ax); ^{13}C NMR (sugar): 103.8 (C-1′), 78.0 (C-3′), 75.0 (C-2′), 71.0 (C-4′), 67.0 (C-5′).

Compound (14): 1.8 mg; FABMS (negative ion) *m*/*z* 465 $[M - H]^-$; $[\alpha]_D$ +4.2° (MeOH, *c* 1); ^1H and ^{13}C NMR (steroidal nucleus) in Table 1; ^1H and ^{13}C NMR (side chain) in Tables 3 and 4.

Compound (15): 1.8 mg; FABMS (negative ion) *m*/*z* 449 $[M - H]^-$; $[\alpha]_D$ +8.7° (MeOH, *c* 1); ^1H and ^{13}C NMR (side chain) in Tables 3 and 4.

Compound (16): 7.3 mg; FABMS (negative ion) in the text; $[\alpha]_D$ +8.6° (MeOH, *c* 1); ^{1}H and ^{13}C NMR (steroidal nucleus) in Table 1; ^{1}H and ^{13}C NMR (side chain) in Tables 3 and 4.

Compound (17): 0.9 mg; FABMS (negative ion) in the text; $[\alpha]_D$ +6.6° (MeOH, *c* 1); ^{1}H and ^{13}C NMR (steroidal nucleus) in Table 1; ^{1}H and ^{13}C NMR (side chain) in Tables 3 and 4;

3,6,15-Tri-(−)-MTPA Ester of 17. The steroid **17** (0.7 mg) was treated with freshly distilled (−)-methoxytrifluoromethylphenylacetyl chloride (2 mL) prepared from (−)-(*S*)-MTPA acid, in dry pyridine (0.2 mL) for 1 h at room temperature. After removal of solvent, the product was analyzed by ^{1}H NMR: ^{1}H NMR (CD_3OD) 5.67 (1H, dt, J = 10.0 Hz, H-6), 5.02 (1H, m, H-3α), 4.32 (1H, t, J = 5.6 Hz, H-15α), 4.25 (1H, br s, H-4α), 4.20 (1H, t, J = 5.6 Hz, H-16α), 2.73 (1H, dd, J = 12.0, 3.5 Hz, H-7*β*), 1.30 (3H, s, H_3-19), 1.27 (3H, s, H_3-18).

Compound (18): 2.3 mg; FABMS (negative ion) in the text; $[\alpha]_D$ −2.5° (MeOH, *c* 1); ^{1}H and ^{13}C NMR data (steroidal nucleus) are identical with those reported for **12**; ^{1}H and ^{13}C NMR (side chain) in Tables 3 and 4.

3,15-Di-(−)-MTPA Ester of 18. The steroid **18** (1 mg) was treated with freshly distilled (−)-methoxytrifluoromethylphenylacetyl chloride (2 mL) in identical manner as steroid **17**; ^{1}H NMR (CD_3OD) 5.89 (1H, t, J = 7.6 Hz, H-15α), 5.30 (1H, dd, J = 15.2, 8.0 Hz, H-22), 5.22 (1H, dd, J = 15.2, 8.5 Hz, H-23), 4.99 (1H, m, H-3α), 3.07 (1H, dd, J = 12.8, 5.0 Hz, H-7*β*).

Compound (19): 0.7 mg; FABMS (negative ion) in the text; $[\alpha]_D$ −15.7° (MeOH, *c* 1); ^{1}H and ^{13}C NMR data (steroidal nucleus) are identical with those reported for **12**; ^{1}H and ^{13}C NMR (side chain) in Tables 3 and 4.

Acid Treatment of 5 to Give 24-Methyl-5α-cholesta-8(9),14,22*E*-triene-3*β*,4*β*,6α,28-tetrol. A solution of **5** (1.7 mg) in 2 M HCl−MeOH (0.5 mL) was heated at 80 °C in a stoppered reaction vial. After 2 h, TLC analysis [SiO_2 with $CHCl_3$−MeOH−H_2O (80:18:2)] showed that the starting material had disappeared and was replaced by a UV-active spot. The reaction mixture was cooled, neutralized with Ag_2CO_3, and centrifuged, and the supernatant was taken to dryness under N_2. The residue was purified by HPLC [C_{18} *μ*-Bondapak (30 cm × 3.9 mm i.d.), MeOH−H_2O (75:25)] to give the $\Delta^{8(9),14}$ dienesteroid; ^{1}H NMR (CD_3OD) 5.38 (1H, br s, H-15), 4.30 (1H, br s, H-4), 4.21 (1H, dt, J = 10.0, 3.5 Hz, H-6), 3.47 (1H, m, H-3), 2.61 (1H, dd, J = 12.0, 4.1 Hz, H-7), 1.24 (3H, s, H_3-19), 0.87 (3H, s, H_3-18).

3,6,28-Tri-(−)-MTPA Ester of the 24-Methyl-5α-chotesta-8(9),14,22*E*-triene-3*β*,4*β*,6α,28-tetrol. The triene formed from **(5)**, as described above, was treated with freshly distilled (−)-methoxytrifluoromethylphenylacetyl chloride (2 mL), in identical manner as steroid **17**; ^{1}H NMR (CD_3OD) 5.57 (1H, m, H-6), 4.95 (1H, m, H-3), 4.44 (1H, dd, J = 10.5, 5.5 Hz, H-28), 4.24 (1H, dd, H-28, partially overlapped with other signals).

Solvolysis of Acodontasteroside A (5), B (6) and C (7). A solution (2 mg each) of the glycosides in a mixture of pyridine (100 *μ*L) and dioxane (100 *μ*L) was heated at 140 °C for 2 h in stoppered reaction vial. The residue was evaporated to dryness and purified by HPLC (C_{18} *μ*-Bondapak column 30 cm × 3.8 mm i.d.) with MeOH−H_2O (75:25) as eluent.

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