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Investigation of the polar steroids from an Antarctic Starfish of the family *Echinasteridae*: isolation of twenty seven polyhydroxysteroids and steroidal oligoglycosides, structures and biological activities. [1]

Maria Iorizzi
Università degli Studi del Molise, Dipartimento di Scienze e Tecnologie Agro-Alimentari, Ambientali e Microbiologiche, Via Tiberio 21\A, 86100 Campobasso, Italy.

Simona De Marino, Luigi Minale* and Franco Zollo
Dipartimento di Chimica delle Sostanze Naturali, Università di Napoli Federico II, Via Domenico Montesano, 49, 80131 Napoli, Italy.

Valérie Le Bert and Christos Roussakis
Institut des Substances et Organismes de la Mer (ISOMER), Faculté de Pharmacie, 1-Rue Gaston Veil 44035 NANTES Cedex 01, France.

Abstract: An investigation of the extracts from a starfish collected in the Antarctic Sea, *Echinasteridae* family, has led to the isolation of thirteen glycosides of polyhydroxysteroids (**1-13**), fourteen polyhydroxysteroids (**14-27**), which are the subject of this report, and seven asterosaponins (penta-and hexa-saccharides), described in a previous paper. Glycosides **1-13** are composed of the same 3β,4β,6α,8,15β,26-hexahydroxysteroidal aglycone with small variations in the side chains, and a carbohydrate portion made up of one or two monosaccharide units attached at C-26, except **11**, which has a steroidal aglycone with a 24,28-dihydroxylated side chain and the monosaccharide unit attached at C-24. The structures of the polyhydroxysteroids (**14-27**) have the hydroxyl groups typically disposed on one side of the steroid nucleus, *i.e.* 3β,4β,6β (or α),8,15α (or β) and 16β and the majority of them possess a 26-hydroxyl function. A selection of fifteen compounds were tested against human non-small-cell lung carcinoma cells and found to be moderately cytotoxic. Copyright © 1996 Elsevier Science Ltd

Starfish contain steroidal glycosides which are responsible of their general toxicity. According to their chemical structures the steroidal glycosides were subdivided into three main groups: the asterosaponins, which are sulphated glycosides (usually penta- and hexa-glycosides) based on the $\Delta^{9(11)}$-3β,6α-dioxysteroidal aglycone with a sulphate at C-3 and the oligosaccharide moiety at C-6, the cyclic steroidal glycosides, only found in two species of the genus *Echinaster*, and the glycosides of polyhydroxysteroids, which consist of a polyhydroxylated steroidal aglycone linked to one or two or rarely three sugar units and can be found in both sulphated and non-sulphated form. [2] These glycosides are often accompanied by varied polyhydroxysteroids sulphated and non-sulphated. [2] Asterosaponins and glycosides of polyhydroxysteroids usually occur as complex mixtures of very similar molecules along with other polar steroids. The investigation of several species of starfish as for example *Halityle regularis*, [3] *Coscinasterias tenuispina*, [4] *Henricia laeviscula*, [5] *Culcita novaeguinea*, [6] *Oreaster reticulatus* [7] and *Henricia downeyae* [8] has required the separation of many related compounds going from the thirteen compounds in *H. regularis* up to the twenty two in *O. reticulatus*. In the course of our research for biologically active compounds from echinoderms, we have encountered a starfish collected in the Antarctic Sea and identified as belonging to the *Echinasteridae* family, most probably *Henricia* genus. From the starfish we have isolated thirty four steroid constituents (Table 1). The structures of seven cytotoxic highest molecular weight compounds "asterosaponins", have been determined. [9] Now we report the isolation and structure elucidation of the remaining twenty seven compounds, thirteen steroidal mono- and di-glycosides (**1-13**), that we

designate antarcticosides D-P, and fourteen polyhydroxylated steroids (**14-27**). Among the echinoderms this is one of the best example of the structural variety of polyhydroxysteroid constituents co-occuring in the same organism.

Table 1. Compounds isolated from the Antarctic starfish *Echinasteridae* family (from 0.8 kg fresh)

Asterosaponins*	Amount (mg)	$[\alpha]_D$ (MeOH)	m.w.	Citotoxicity** (IC_{50} in μg/ml)
antarcticoside A	10.8	+4.8°	1420	-
antarcticoside B	5.7	+6.1°	1434	-
antarcticoside C	9.6	+4.3°	1420	< 3.3
brasiliensoside	6.5	+3.9°	1274	< 3.3
24S-methylbrasiliensoside	4.9	+12.5°	1288	< 3.3
pectinioside A[a]	12.9	-6.6°	1258	7-10
24S-methylpectinioside	5.0	+12.0°	1272	< 3.3
Glicosides of polyhydroxysteroids				
antarcticoside D **1**	10.0	-12°	612	12.35
antarcticoside E **2**	7.5	-8.6°	612	-
antarcticoside F **3**	8.6	-26.0°	758	-
antarcticoside G **4**	5.9	-21.8°	730	> 30
antarcticoside H **5**	0.5	-28.0°	598	> 30
antarcticoside I **6**	7.7	-26.1°	628	inactive
antarcticoside J **7**	10.3	-4.5°	706	10.1
antarcticoside K **8**	0.4	-10.0°	626	15.08
antarcticoside L **9**	10.7	-30.7°	788	> 30
antarcticoside M **10**	1.0	-17.0°	758	-
antarcticoside N **11**	0.8	-11.0°	630	-
antarcticoside O **12**	4.0	+10.7°	736	-
antarcticoside P **13**	1.7	-15.5°	882	-
Polyhydroxylated steroids				
compound **14**	4.5	+8.0°	450	>30
compound **15**	7.0	+1.0°	450	inactive
compound **16**	60.0		452	>30
compound **17**	0.4	+5.0°	468	-
compound **18**	0.6	-6.6°	450	-
compound **19**	1.2	-0.83°	466	-
compound **20**	1.0	+16.0°	466	>30
compound **21**	1.0	+12.0°	466	>30
compound **22**	5.8		468	3.3
compound **23**	1.0	+4.0°	482	-
compound **24**	3.7	+11.2°	480	8.3
compound **25**	4.9	+12.3°	450	>30
compound **26**	1.0	-10.0°	466	-
compound **27**	1.4		526	-

*Described in the previous paper [9]

[a]Optical rotation was run in pyridina.

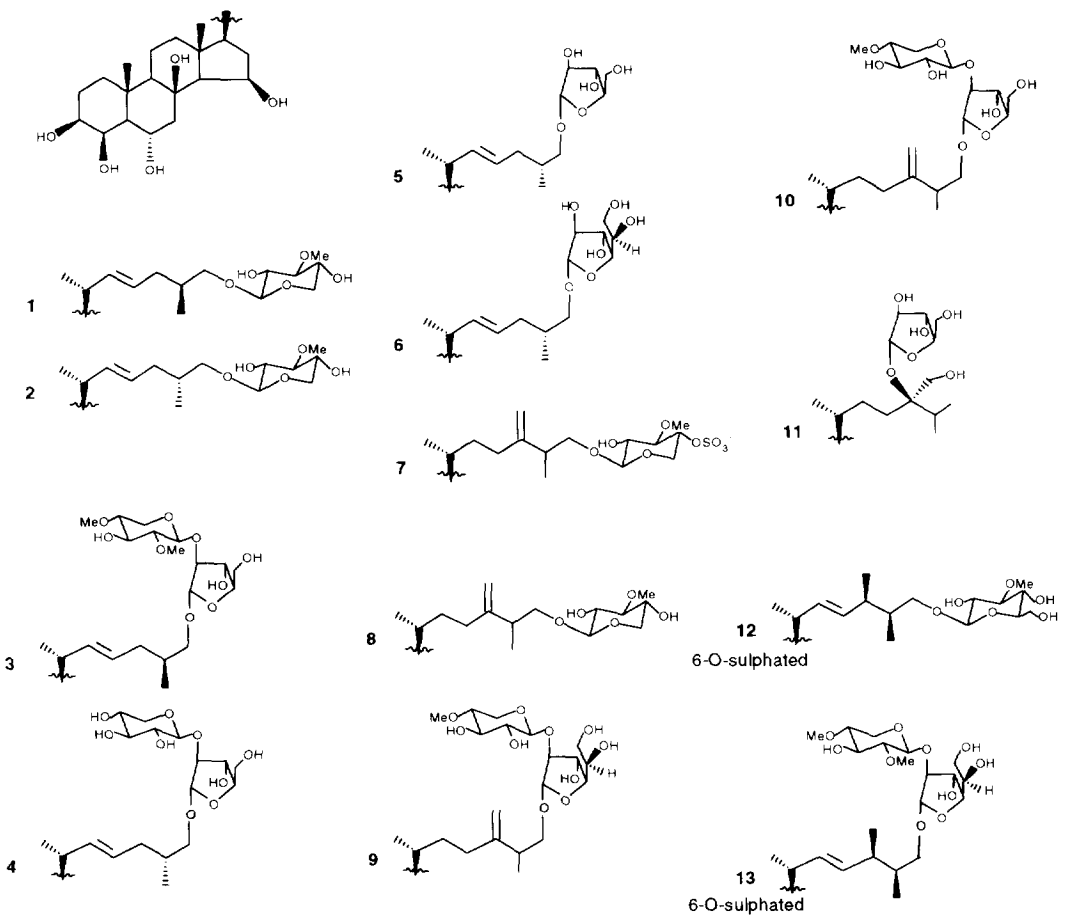
-Not tested

**Against human bronchopulmonary non-small-cell lung carcinoma cells *in vitro* (NSCLC-N6); steroids were tested at 30, 10 and 3.5 μg/ml concentrations.

The starfish, *Echinasteridae* family, is a big yellow starfish with some red spots on the arms, collected in the Tethys Bay during the Italian-Antarctic expedition in January 1991. The starfish was extracted with water

and then with acetone; from the aqueous extract the asterosaponins, the steroidal glycosides and the polar steroids were recovered by passing it throught a column of Amberlite, washing out salts with distilled water, and eluting the absorbed material with methanol. The acetone extracts were solvent partitioned between water and *n*-butanol. The combined methanol eluate and *n*-butanol soluble material, were separated by sequential application of gel permeation on Sephadex LH-60, DCCC and HPLC as described in refers 2.

THE STEROIDAL GLYCOSIDES-ANTARCTICOSIDES D-M (1-13).



Compounds **1-6**.-NMR spectral data indicated that compounds **1-6**, antarcticosides D-I, possess identical steroidal aglycones, but differ in the saccharide moiety linked at C-26, or, as in the case **1-2**, only in the stereochemistry at C-25.

The FAB mass spectrum of antarcticoside D (**1**) showed a pseudomolecular ion at m/z 611 [M - H]$^-$ accompained by a fragment ion at m/z 465 (loss of 146 mass units), which, along with the presence of a methoxy singlet at δ 3.65, suggested that **1** contained a methoxylated pentose (146 mass units) moiety.

Examination of the ^{1}H and ^{13}C NMR (Tables 2, 3) spectra and comparison with known compounds [2,6,10] established the 3β,4β,6α,8,15β–hydroxylation pattern in a 5α-cholestane skeleton.

Table 2. Selected NMR data of the steroidal nucleus of compounds **1-11***

position	δ_H	δ_C
H-3	3.47 m	73.5
H-4	4.30 brs	68.9
H-6	4.19 dt (10.0, 3.5)	64.6
H-7β	2.48 dd (12.0, 4.0)	49.8
H-15	4.46 t (5.9)	70.9
H_2-16	2.40 m-1.05 m	42.3
H_3-18	1.30 s	16.3
H_3-19	1.19 s	16.8

*The data here reported have been extracted for the spectra of **9**

Table 3. NMR data of the steroidal side chain of compounds **1**, **3**, **9***, **11** and **12***.

	1		**3**		**9**		**11**		**12**	
position	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C
21	1.03 d (7.0)	21.0	1.03 d (6.5)	20.9	0.99 d (7.0)	18.8	0.98 d (6.8)	18.9	1.02 d (6.5)	21.0
22	5.26 dd (14.0, 7.0)	139.7	5.27 dd (14.0, 6.5)	139.6	-	35.4	-	29.7	5.27 dd (15.0, 7.8)	137.1
23	5.35 dt (14.0, 5.5)	126.8	5.39 dt (14.0, 6.5)	126.7	-	32.6	-	29.6	5.21 dd (15.0, 7.8)	133.5
24	2.12 m	37.4	-	37.4	-	154.0	-	84.0	2.17 m	40.9
26	3.72 dd (10.5, 5.0) 3.32 dd (10.5, 6.0)	75.5	3.58 dd 3.21 dd	73.4	3.56 dd (9.7, 7.6) 3.48 dd (9.7, 7.6)	72.9	0.96 d (7.0)	17.0	3.98 dd (10.8, 6.5) 3.28 dd	74.6
27	0.94 d (7.0)	17.1	0.95 d (6.5)	17.2	1.10 d (7.0)	17.6	0.96 d (7.0)	18.0	0.97 d (6.5)	17.2
28	-	-	-	-	4.81 brs	109.0	3.72 d (12.5) 3.66 d (12.5)	66.0	0.96 d (6.5)	14.4

*^{1}H assignments aided by COSY and HOHAHA experiments.
The coupling constants are given in Hz and are enclosed in parentheses.

The ^{1}H NMR spectrum also showed signals for the side chain: two methyl doublets at 0.94 (J= 7.0 Hz) and 1.03 (J= 7.0 Hz), two olefinic protons at δ 5.26 dd (J= 14.0, 7.0 Hz) and 5.35 dt (J= 14.0, 5.5 Hz), two double doublets at δ 3.72 (J= 10.5, 5.5 Hz) and 3.32 (J= 10.5, 6.0 Hz) for a CH_2OH group. Those data indicated a Δ^{22E}26-hydroxycholestane side chain, already found in polyhydroxysteroids from the starfish *Dermasterias imbricata*.[11] In addition to the steroid moiety, the ^{1}H NMR spectrum showed one methoxyl singlet at δ 3.65 and four methine protons at δ 4.20 d (J= 7.0 Hz), 3.25 dd (J= 9.0, 7.0 Hz), 3.05 t (J= 9.0 Hz), and 3.46 m, which couple with their neighbors in that order. The last methine proton is further coupled to methylene protons at δ 3.20 t (J= 10.0 Hz) and 3.85 dd (J= 10.0, 5.0 Hz). The coupling constants indicated that the molecule bears a moiety equivalent to a β-xylopyranoside. The upfield shift of H-3 to 3.05 ppm (3.30 in β-xylopyranosides) [12] indicated a 3-O-methyl-β-xylopyranosyl unit, as confirmed by ^{13}C NMR spectrum, ($\delta_{C\text{-}3}$: 87.5 in **1** *vs.* 77.9 in β-xylopyranoside),[12] which also established the sugar moiety to be linked at C-26 of the steroid ($\delta_{C\text{-}26}$: 75.5 ppm in

1 *vs.* 68.6 ppm in the free steroid [11]). Thus antarcticoside D can be defined as 26-O-(3-O-methyl-β-xylopyranosyl)-5α-cholest-22E-ene-3β,4β,6α,8,15β,26-hexaol (**1**).

The second major compound, antarcticoside E (**2**), FABMS, m/z 611 and 465, showed ^{1}H and ^{13}C NMR spectra virtually superimposable on those of **1** except for the signals of the 26-methylene protons, which in **2** were observed closer at δ 3.61 dd (J= 10.5, 6.0 Hz) and 3.40 dd (J= 10.5, 5.0 Hz); in the ^{13}C NMR spectra small differences ranging from 0.1 to 0.3 ppm were also observed for side chain carbon signals. This could be interpreted that **1** and **2** are epimers at C-25. It has been shown [2,13] that (25R)-and (25S)-26-hydroxysteroids can be differentiated by the ^{1}H NMR of their (+)-R and (-)-S-α-(trifluoromethyl)phenylacetic acid (MTPA, Mosher's reagent). [14] In the spectra of the (+)-MTPA ester of saturated side chain 26-hydroxysteroids, the 26-protons of the 25S-isomer appear as signals resonanting much closer that in 25R-isomer, and the reverse in the (-)-MTPA ester. Thus we have treated with hydrochloric acid in methanol both **1** and **2**, and obtained the corresponding 5α-cholesta-8,14,22-triene-3β,4β,6α,26-tetraols **1a** and **2a**, dehydration at C-8 and C-15 being also occurred. Treatment with (-)-α-methoxy-α-(trifluoromethyl)phenylacetyl chloride of both epimeric alcohols afforded the corresponding 3,6,26-tri-(-)-MTPA esters. The 26-methylene proton signals of the (-)-MTPA ester of **1a** (deriving from **1**) appeared as a dd at δ 4.13 and 4.30, while in the spectrum of the (-)-MTPA ester of **2a** (deriving from **2**) they appeared closer at δ 4.17 and 4.23. On this basis we assign the 25S configuration to **1** like many 26-hydroxysteroids isolated from starfishes, and the more rarely found 25R configuration to **2**. We never encountered both epimers in the same starfish species, but once in the ophiuroid *Ophioracna incrassata*. [15]

In addition to the signals ascribable to the steroid, the ^{1}H and ^{13}C NMR spectra of antarcticoside F (**3**) showed signals virtually superimposable on those assigned to the 2,4-di-O-methyl-β-D-xylopyranosyl-(1→2)-α-L-arabinofuranosyl unit in the spectra of halityloside E [3] and culcitoside C3. [6] The sequence of the disaccharide unit was confirmed by the FAB mass spectrum showing a pseudomolecular ion at m/z 757 $[M - H]^-$ and fragment ions at 597 and 465, corresponding to the sequential loss of a dimethoxylated pentose (160 mass units) and a pentose (132 mass units). The configuration of C-25 was deduced from NMR data of 3,6,26(-)-MTPA ester of 5α-cholesta-8,14,22-triene-3β,4β,6α,26-tetraol **3a**, derived from **3** on acid hydrolysis. The 26-methylene proton signals were observed resonanting at δ 4.13 dd and 4.30 dd, suggesting the 25S-configuration.

Antarcticoside G (**4**) also had the same steroid aglycone as **1-3**. The saccharide chain was composed of a xylopyranosyl and an α-arabinofuranosyl unit, as shown by NMR data (Table 4).

Table 4. Assignments of the NMR signals (CD_3OD) of β-xylopyranosyl-(1→2)-α-arabinofuranosyl unit in **4**.

	β-xylopyranosyl		α-arabinofuranosyl	
positon	^{1}H	^{13}C	^{1}H	^{13}C
H-1'	4.39 d (7.6)	104.8	5.00 brs	108.0
H-2'	3.22 t (11.5)	74.8	4.08 brd (4.0)	91.7
H-3'	3.33 t (9.0)	77.0	4.04 m	77.7
H-4'	3.50 m	71.0	3.93 m	83.7
H-5'	3.88 dd (12.0, 5.6)	67.0	3.80 dd (12.0, 3.0)	62.4
	3.18 dd (12.0, 4.5)		3.63 dd (12.0, 4.8)	

In addition to the quasi-molecular ion at m/z 729 $[M - H]^-$, the FABMS gave fragment ions at m/z 597 and 465 corresponding to the consecutive loss of two pentose (132 mass units) residues. The ^{13}C NMR signals of C-1 and C-2 of the arabinosyl unit, which are shifted to 108.0 and 91.7 ppm (109.6 and 83.7 ppm in unsubstituted arabinofuranosides, *e.g.* **5**; glycosidation shift), established xylose linked to C-2 of arabinose, a common structural feature in steroidal glycosides from starfishes. [2] The ^{13}C NMR signal of C-26 of the aglycone, shifted dowfield to 73.4 ppm, established the sugar moiety to be linked there. In 26-O-glycosides, the 26-methylene proton signals are sensitive to the stereochemistry at C-25; in the epimeric xylopyranosides **1** and **2** they resonated at δ 3.32 dd-3.72 dd in **1** (25S) and much closer at δ 3.40 dd-3.61 dd in **2** (25R). In the spectrum of **4** the 26-methylene proton signals were observed at δ 3.28 dd-3.52 dd closer than in the closely related (25S)-26-O-2,4-di-O-methyl-β-xylopyranosyl-(1→2)-α-arabinofuranoside **3**, δ 3.21 dd-3.60 dd. A small but significative difference was also observed for the carbon shift of C-26, *i.e.* 73.6 in **4** *vs.* 73.4 in **3**. On these arguments we propose for **4** the 25R-configuration.

Antarcticoside H (**5**), FABMS, m/z 597 $[M - H]^-$ and m/z 465 $[M - H - 132]^-$, is the 26-O-arabinofuranoside of the same aglycone as **1-4**, as shown by NMR data (Tables 2, 3, 5). The 25R-configuration is proposed on the basis of the chemical shifts of the 26-methylene protons at δ 3.28 dd-3.54 dd.

Antarcticoside I (**6**) gave a FABMS pseudomolecular ion peak at m/z 627 $[M - H]^-$, thirty mass units higher than **5** (m/z 597), and a fragment ion at m/z 465 corresponding to the loss of an hexose unit (162 mass units) from $[M - H]^-$. On acid methanolysis **6** liberated methyl galactosides (GLC after silylation). The ^{13}C NMR shifts for the sugar residue match those of methyl β-D-galactofuranoside.[16] In the ^1H NMR spectrum, the sugar protons gave rise to six signals, that were correlated by sequential decoupling (Table 5). The chemical shifts and coupling constants were consistent with a furanoside structure. The chemical shifts of the 26-methylene protons at δ 3.30 dd and 3.54 dd pointed for a 25R-configuration as in **4** and **5**. Antarcticoside I (**6**) along with the antarcticosides **9** and **13**, described below, are the fourth example of galactofuranosides found in the starfishes after indicoside A,[17] crossasterosides P1 and P2,[18] and oreasteroside F.[7]

Table 5. Assignments of the NMR signals (CD_3OD) of α-arabinofuranosyl moiety in **5** and β-galactofuranosyl moiety in **6**.

	α-arabinofuranosyl in **5**		β-galactofuranosyl in **6**	
positon	^1H	^{13}C	^1H	^{13}C
H-1'	4.92 brs	109.6	4.86 brs	109.6
H-2'	3.99 dd (4.5, 2.0)	83.7	3.98 dd (5.8, 1.5)	83.5
H-3'	3.86 dd (7.5, 4.5)	78.8	4.04 dd (5.8, 3.0)	79.0
H-4'	3.94 m	85.2	3.94 dd (6.2, 3.0)	84.4
H-5'	3.78 dd (12.5, 3.0)	63.0	3.74 m	72.6
	3.65 dd (12.5, 5.2)			
H-6'			3.65 d (6.0)	64.8

The coupling constants are given in Hz and are enclosed in parentheses.

Compounds **7-10**.-Antarcticosides J-M (**7-10**) possess identical steroid aglycones and differ in the saccharide moiety linked at C-26. Examination of the ^1H NMR spectral data conclusively showed that they all have the same 3β,4β,6α,8,15β-pentaol structure as in **1-6**. The side chain was composed of two methyl doublets, one exomethylene, two methylenes and one hydroxymethylene, reminiscent of a 26-hydroxy-24-methylenecholestane side chain,[6] which was confirmed by ^{13}C NMR data of the two major constituents **7** and **9** (Table 3). The chemical shift of the C-26 oxygenated methylene at 74.9 ppm in **7** and 72.9 ppm in **9** *v.s.* 67.6 observed in 24-methylene-26-hydroxy steroids[11] confirmed the location of the sugar moiety at C-26.

The FABMS of the more polar constituent **7**, antarcticoside J, gave an anion peak at m/z 705 $[M^-]$. In addition to the steroid moiety, ^1H and ^{13}C NMR spectra showed signals that match those of 3-O-methyl-β-xylopyranosyl-4-O-sulphated residue, found in scoparioside C.[19] Upon solvolysis, **7** afforded a less polar desulphated derivative **7a**, m/z 627 $[M + H]^+$ identical (^1H, FABMS, HPLC) with the minor antarcticoside K (**8**). Upfield shift of the C-4 proton of the xylopyranosyl moiety from δ 4.28 m in **7** to δ 3.56 m in **8** confirmed that C-4 bears the sulphate in **7**. The high field shift observed for H-3' at δ 3.05 in the spectrum of the desulphated **8** *vs.* δ 3.30 in steroid xylopyranosides confirmed the 3-O-methylxylopyranosyl unit. The stereochemistry at C-25 remains unassigned. Acid hydrolysis to remove the saccharide moiety also resulted in the migration of the double bond to C-24 (25), whereas attempts to remove the saccharide moiety by enzymic hydrolysis (glycosidic mixture from *Charonia lampas*) were unsuccessful.

The FABMS of antarcticoside L (**9**) gave a pseudomolecular ion peak at m/z 787 $[M - H]^-$ accompained by fragment ion peaks at m/z 641 and 465, corresponding to the consecutive loss of a methoxylated pentose unit (146 mass units) and an hexose unit (162 mass units). The hexose was identified as galactose by acid methanolysis affording methyl galactosides (GLC after silylation). The COSY and HOHAHA spectra allowed us to assign all ^1H NMR signals of the sugar moiety, thereby allowing identification of a 4-O-methyl-β-xylopyranosyl and a α-galactofuranosyl moieties (Table 6). The ^{13}C NMR spectrum confirmed the sequence, the presence of a 4-O-methylxylopyranosyl unit ($\delta_{C\text{-}4}$: 80.8 *vs.* 71.0 in β-xylopyranoside: *cfr.* **4**), and established the

interglycosidic linkage to be 4-OMexyl$_p$-(1→2)-gal$_f$ ($\delta_{C\text{-}1}$ and $\delta_{C\text{-}2}$ of Gal$_f$: 107.7 and 91.7 ppm respectively, *vs.* 109.6 and 83.3 ppm, in galactofuranosides; see **6**; glycosidation shift).

The minor compound of this series, antarcticoside M (**10**), FABMS, m/z 781 $[M + Na]^+$, has a saccharide portion composed of an α-arabinofuranosyl and a 4-O-methyl-β-xylopyranosyl units, as shown by ^1H NMR spectrum. Previously we isolated two glycosides, culcitoside C4 and C5, from the starfish *Culcita novaeguinea*,[6] both having a 4-O-methyl-β-xylopyranosyl-(1→2)-arabinofuranosyl disaccharide chain linked to the steroidal aglycones. The ^1H NMR shifts for the sugar residues in antarcticoside M (**10**) match those of both culcitosides, and on this basis we propose the structure **10**.

Table 6. Assignments of NMR signals (CD_3OD) of 4-O-methyl-β-xylopyranosyl-(1→2)-β-galactofuranosyl moiety in **9*** and 2,4-di-O-methyl-β-xylopyranosyl-(1→2)-β-galactofuranosyl in **13**.

	Compound **9***				Compound **13**			
position	4-O-methyl-β-xylopyranosyl		α-galactofuranosyl		2,4-di-O-methyl-β-xylopyranosyl		β-galactofuranosyl	
	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C
H-1	4.39 d (7.6)	104.7	5.05 s	107.7	4.48 d (8.7)	104.7	4.98 brs	108.2
H-2	3.23 t	75.0	4.08 m	91.7	2.92 t (9.0)	84.8	4.11 brd	91.6
H-3	3.40 t (8.6)	76.7	4.18 dd (7.6, 4.0)	77.3	3.42 t (9.8)	77.7	4.18 dd (8.7, 3.7)	76.5
H-4	3.21 t	80.8	3.92 dd (7.8, 3.4)	82.9	3.22 m	80.9	3.87 dd (8.7, 3.5)	82.9
H-5	3.14 m 4.05 m	64.7	3.75 m	72.2	3.15 t (12.0) 4.04 dd (12.0, 5.2)	64.4	3.72 m	72.1
H-6		57.8	3.65 d (6.3)	64.5			3.65 d (6.3)	64.8
OMe					3.50 s	59.1	3.50 s	59.1
OMe					3.61 s	61.2	3.61 s	61.2

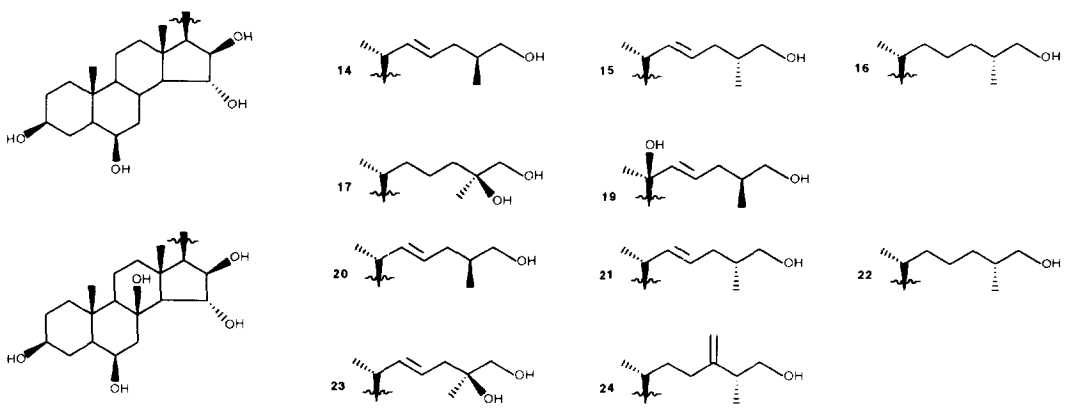
*^1H assignments aided by COSY and HOHAHA experiments.

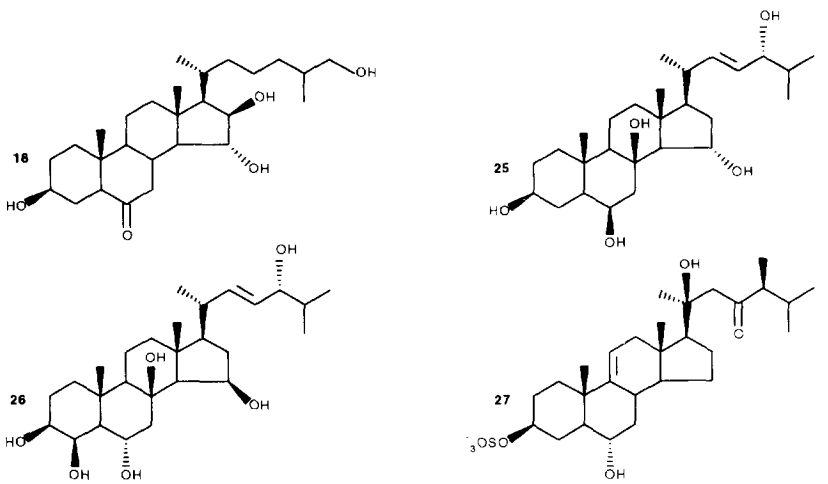
Compound **11**.-The ^1H and ^{13}C NMR spectra readily implied that antarcticoside N (**11**) had a steroidal nucleus identical with that of the previous compounds. The side chain (Table 3) was composed of three methyls, two methylenes, one methine, one oxygenated quaternary carbon and one CH_2-OH grouping, reminiscent of a 24-hydroxymethyl-24-hydroxycholestane side chain, recently encountered in a polyhydroxylated steroid isolated from the starfish *Luidia clathrata*,[20] which was confirmed by ^{13}C NMR data. In addition to the steroid, ^1H and ^{13}C NMR spectra showed signals for an α-arabinofuranosyl unit. This was also supported by FABMS, m/z 629 $[M - H]^-$ and 497 (loss of a pentose unit). The ^{13}C NMR signal of the side chain oxygenated quaternary carbon, which is shifted downfield to 84.0 ppm (77.1 ppm in 24-hydroxymethyl-24-hydroxycholesterol) [20] indicated the location of the arabinose at C-24. Further support is given by the ^{13}C NMR signal for the CH_2-OH grouping at δ 66.0, virtually unshifted relative to the reference compound. [20] The 24R configuration was assigned to **11** after enzymic hydrolysis with a glycosidase mixture from *Charonia lampas* (37°, 3 days) to remove the sugar and treatment of the resulting steroid aglycone **11a**, with (+)- and (-)-MTPA chloride. The H_2-28 signals appeared as two well separated doublets at δ 4.20 and 4.32 in the spectrum of 3,6,28-tri-(+)-MTPA ester and closer, δ 4.22 and 4.26, in the spectrum of the 3,6,28-tri (-)-MTPA ester, in agreement with the spectral behaviour of the 28-MTPA esters of the model (24R) 24-hydroxy-24-(hydroxymethyl)cholesterol. [20] On these arguments we assign the 24R configuration to **11**.

Compounds **12-13**.-The remaining antarcticosides O (**12**) and P (**13**) are sulphated compounds. The ^{1}H and ^{13}C NMR spectra readily implied that **12**, FABMS, m/z 735 [M$^-$], had a 3β,4β,6α,8,15β-pentahydroxysteroid nucleus sulphated at C-6; the signals for H-6 and C-6 were found shifted dowfield to δ_H 4.90 dt and δ_C 74.3 ppm relative to the previous non-sulphated **1-11** shown in Table 2. This was also supported by solvolysis using dioxane/pyridine, affording desulphated **12a**, FABMS m/z 655 [M - H]$^-$, whose ^{1}H NMR spectral data for the steroid nucleus was identical with those of the previous **1-11**. The structure of the side chain of antarcticoside O (**12**) was elucidated by analysis of 2D NMR data (Table 3). The COSY spectrum allowed construction of the side chain Δ^{22},24-methyl-26-oxygenated, already encountered in starfish derived steroid constituents. [2,5,21].The 3-O-methyl-β-glucopyranosyl unit was also evident from the COSY cross peaks and analysis of the coupling constants (see Experimental). This was also supported by FABMS spectrum, m/z 735 [M$^-$] and 559 [M - H -176]$^-$, which indicated that **12** had a methoxylated hexose unit. The ^{13}C NMR spectrum and comparison with known starfish derived Δ^{22},24-methyl-26-hydroxy steroids, [5,21] and with methyl 3-O-methyl-β-glucopyranoside, [22] confirmed the structure **12**, except for the stereochemistry at C-24 and C-25, which was then assigned as 24R,25S. Acid hydrolysis gave 24-methylcholest 8,14-22-triene-3β,4β,6α,26-tetraol **12b** whose NMR data, δ H_2-26: 3.31-3.61 dd, δ H_3-27,28: 0.92 d, 0.97 d, were indicative for a *threo* stereochemistry at C-24, C-25,[2,23] the same signals being observed at δ 3.34 dd-3.53 dd, 0.87 d and 1.02 d in the spectrum of the *erythro* model steroid. [2,23] Assignment of the S-absolute configuration at C-25, and hence R to C-24, was made by observing the shape of the C-26 methylene proton signals in the ^{1}H NMR spectrum of the 3,6,26-(-)-MTPA ester derivative, δ 4.13 dd-4.42 dd, close to that found in the spectrum of the 26-(-)-MTPA ester 24R,25S *threo* model steroid, δ 4.13 dd-4.38 dd, and away from that found in the 24S,25R-isomer, δ 4.19-4.31 dd. [23]

The ^{1}H and ^{13}C NMR spectra of antarcticoside P (**13**) readily indicated that **13** had a steroid sulphate aglycone identical with that of **12**. The FAB mass spectrum, m/z 881 [MSO_3^-], m/z 721 (loss of 160 mass units) and 559 (loss of 162 mass units from m/z 721), along with the presence of two methoxyl singlets at δ 3.50 s and 3.61 s, indicated that **13** contained a di-methoxylated pentose unit linked to an hexose one. The structure of the saccharide was elucidated by analysis of 2D NMR data. COSY spectrum revealed two spin systems, which could be attributed to known monosaccharide units, 2,4-di-O-methyl-β-xylopyranose, and α-galactofuranose. Analysis of ^{13}C NMR spectrum disclosed the signal for C-2 of the galactose to be shifted dowfield to 91.6 ppm (*vs.* 83.5 ppm in β-galactofuranosides, *e.g.* **6**). Thus, the terminal 2,4-di-O-methyl-β-xylopyranose is linked at C-2 of galactofuranose.

THE POLYHYDROXYLATED STEROIDS 14-27.





The FAB mass spectrum of the steroids **14** and **15** gave a pseudomolecular ion peak at m/z 451 $[M + H]^+$ corresponding to a cholestene-pentaol. Examination of ^1H and ^{13}C NMR spectra (Table 7) readily indicated the presence in both compounds of the 3β,6β,15α,16β,26-hydroxy structure already encountered in a steroid from the starfish *Hacelia attenuata*, [24] and of a Δ^{22E}-double bond. Small differences observed in the shifts of the side chain carbons were observed in the spectra of **14** and **15**, which could be interpreted as due to the presence of both 25R and 25S-epimers. This was supported by treatment of both **14** and **15** with (+)-MTPA chloride, followed by NMR analysis. The 26-methylene proton signals were observed closer at δ 4.16 dd and 4.22 dd in the spectrum of the 3,15,26-tri (+)-MTPA ester of **14** and at δ 4.11 dd and 4.26 dd in the spectrum of the 3,15,26-tri-(+)-MTPA ester of **15**. Thus, **14** is the 25S and **15** the 25R-isomer. [2,13]

Steroid **16**, FABMS, m/z 453 $[M + H]^+$, is the 22(23)-dihydroderivative of **15**. The 25R stereochemistry was assigned as in the case of **14** and **15**. The 25S-isomer was described from the starfish *Hacelia attenuata*. [24]

Steroid **17** had the same steroid nucleus of **16** with a dihydroxylated side chain, which was implied by ^1H NMR and FAB mass spectrum [m/z 469 $(M + H)^+$]. The determination of the structure of the side chain was straightforward: the 26-methylene protons resonated as a 2H singlet at δ 3.38 and the 27-methyl protons were observed downfield shifted to δ 1.15 s. This implied the presence of an additional hydroxyl group at C-25. 25S stereochemistry was inferred from the NMR spectrum of the 26-(-)-MTPA derivative, δ H_2-26 4.19 brs; in the spectrum of the 25R-isomer they are expected to resonate as two well separated signals. [25]

Steroid **18**, a very minor component, is the 6-keto analogous of **16**, FABMS, m/z 451 $[M + H]^+$. The 6-keto structure received support from the presence of a methylene protons signal at δ 2.55 dd and 2.28 brt for H_2-7 and of a methine signal at δ 2.41 dd for H-5 in the ^1H NMR spectrum with the contextual absence of the hydroxymethine proton signal assigned at H-6 in **16**. In agreement with the presence of the ketone group at C-6 the signal for the 19-methyl protons was observed highfield shifted to δ 0.79 *vs.* 1.09 in **16**.

Steroid **19** is the 20-hydroxyderivative of the previous **14**, which was implied by NMR data and FAB mass spectrum [m/z 467 $(M + H)^+$]. In the ^1H NMR spectrum the 21-methyl protons signal appeared as a singlet at δ 1.42, and one of the olefinic protons was a sharp doublet at δ 5.64 (J= 14.0 Hz). The 20-hydroxy structure received support from the presence of a quaternary carbon signal at 77.5 ppm in the ^{13}C NMR spectrum (Table 7) along with the 20-methyl carbon signal shifted downfield to 28.7 ppm. The 20S configuration is proposed on the basis of the chemical shift of the 21-methyl (δ 1.42) and comparison with (20S)- and (20R)-hydroxycholesterol: δ 1.28 and 1.13 respectively. [26] 25S stereochemistry was inferred as in the case **14** and **15**.

Steroids **20** and **21** are the 8β-hydroxyderivatives of the previous **14** and **15**, respectively (Table 7). Hydroxylation at C-8 is a common feature among the starfish derived steroids. [2] In particular the 3β,6β,8β,15α,16β-hydroxylation pattern has been found in steroids from *Spherodiscus placenta*, [27] *Hacelia attenuata* [24] and *Culcita novaeguinea*. [6] Stereochemistry at C-25 was inferred as in the case of **14** and **15**, and

confirmed by hydrogenation of **21** and treatment of the resulting 22(23)-dihydroderivative (**21a**) with (+)-MTPA chloride; H_2-26 signals were observed at d 4.26 dd and 4.13 dd in close agreement with the (25R) 26-hydroxysteroids. [2,13]

Table 7. Selected ^1H and ^{13}C NMR signals of steroids **14**, **19**, **20**, **23** and **25**.

	14		**19**		**20**		**23**		**25**
	^1H	^{13}C	^1H	^{13}C	^1H	^{13}C	^1H	^{13}C	^1H
position									
1	-	39.7	-	39.8	-	41.2	-	41.4	-
2	-	32.0	-	32.2	-	31.5	-	31.6	-
3	3.57 m	72.3	3.57 m	72.5	3.62 m	72.2	3.57 m	72.4	3.62 m
4	-	36.2	-	36.3	-	36.2	-	36.3	-
5	-	48.9	-	48.9	-	48.8	-	49.6	-
6	3.79 brs	72.3	3.78 brs	72.6	3.89 brs	73.9	3.89 brs	74.2	3.88 brs
7	-	40.5	-	40.6	2.45 dd (12.0, 2.0)	45.2	2.45 dd (12.0, 2.0)	45.4	2.41 dd (12.0, 2.0)
8	-	31.1	-	30.7	-	76.7	-	76.9	-
9	-	55.7	-	55.8	-	57.0	-	57.2	-
10	-	36.7	-	36.6	-	36.5	-	36.7	-
11	-	21.7	-	21.8	-	19.5	-	19.6	-
12	-	41.7	-	42.1	-	42.9	-	42.9	-
13	-	44.5	-	41.2	-	45.0	-	45.5	-
14	-	61.0	-	61.7	-	63.6	-	63.6	-
15	3.92 dd (6.5, 2.0)	84.2	3.91 dd (10.5, 3.0)	85.1	4.17 dd (12.0, 2.0)	80.1	4.17 dd (12.0, 2.0)	80.3	4.29 dt (10.0, 3.0)
16	3.78 dd (6.5, 2.0)	83.2	4.18 dd (8.5, 3.0)	82.9	3.92 dd (6.5, 2.5)	83.2	3.93 dd (6.5, 2.5)	83.3	-
17	-	60.2	-	60.1	-	60.8	-	60.9	-
18	0.98 s	15.0	1.04 s	16.9	1.18 s	16.7	1.18 s	16.9	1.02 s
19	1.07 s	16.1	1.09 s	16.3	1.20 s	15.7	1.20 s	15.8	1.19 s
20	2.58 m	34.7	-	77.5	2.56 m	34.4	-	34.8	-
21	1.09 d (6.5)	20.6	1.42 s	28.7	1.06 d (6.5)	20.4	1.06 d (6.5)	20.6	1.04 d (6.5)
22	5.57 dd	139.4	5.64 d (14.0)	141.9	5.55 dd	139.3	5.57 brd	141.4	5.47 dd (16.0, 7.5)
23	5.52 dt	127.5	5.50 dt (14.0, 6.8)	125.6	5.52 dt	127.5	5.57 brd	124.8	5.37 dt (16.0, 7.0)
24	-	37.7	-	37.3	-	37.7	-	42.9	-
25	-	37.1	-	37.2	-	37.1	-	73.0	-
26	3.43 dd (8.0, 6.5) 3.38 dd	68.0	3.44 dd (10.5, 5.8) 3.38 dd	68.0	3.44 dd (8.0, 6.5) 3.37 dd	67.9	3.38 s	70.0	0.88 d (6.5)
27	0.93 d (6.5)	16.8	0.92 d (7.0)	16.7	0.92 d (7.0)	16.8	1.15 s	24.0	0.94 d (6.5)

The coupling constants are given in Hz and are enclosed in parentheses.

Steroid **22** is the 25R isomer of the known (25S) cholestane-3β,6β,8β,15α,16β,26-hexaol previously described. [27] 25R stereochemistry was inferred as in the case of **16**.

Steroid **23** had the same steroidal nucleus of **20-22**, and a Δ^{22E}25,26-dihydroxy cholestane side chain, which was implied by NMR data (Table 7) and FAB mass spectrum, m/z 505 $[M + Na]^+$. The 25S stereochemistry is proposed by analogy with **17**.

The ^{1}H and ^{13}C NMR spectra readily implied that steroid **24** had a steroid nucleus identical with that of the previous **20-23** and a 24-methylene-26-hydroxycholestane side chain. This steroid was isolated from the starfish *Sphaerodiscus placenta* [27] and at that time the 25R configuration was assigned on the basis of the positive Cotton effect of the CD curve of the derived 24-keto compound. [27] We have now converted **24** into the diastereoisomeric (+)- and (-)-MTPA esters, which showed signals of the 26-methylene protons more separated, δ_H 4.23 dd-4.43 dd, in the spectrum of the (+)-MTPA ester than in that of the (-)-MTPA ester, δ_H 4.26 dd-4.39 dd, thus confirming the 25R configuration.

Steroid **25**. The FAB mass spectrum showed a pseudomolecular ion at m/z 473 $[M + Na]^+$ corresponding to a cholestene-pentaol. Examination of ^{1}H NMR spectrum indicated the structure of 5α-cholest-22E-ene-3β,6β,8,15α,24-pentaol (Table 7), already found in *Coscinasterias tenuispina* [4] as 15-sulphated derivative. The 24R configuration could be proposed on the basis of chemical shift of H-24 signal at δ 3.71 in comparison with the 24R and 24S model compounds, δ H-24: 3.71 and 3.68, respectively. [12,28] Indeed a greater confidence in the stereochemical assignment can be had by relying on ^{1}H NMR spectral data of (+)- and (-)-MTPA derivatives; [2a] a major differences were observed for the isopropyl methyl signals, shifted upfield to δ 0.87-0.90 in the (+)-MTPA ester of the (22E,24R)-6β-methoxy-3α,5-cyclo-5α-cholest-22-en-24-ol model compound and shifted downfield to δ 0.95-0.98 in (+)-MTPA ester of the (22E, 24S) isomer. [2] Thus, the steroid **25** was converted into the diastereoisomeric (+)- and (-)-MTPA esters, which showed the signals of the isopropyl methyls significantly upfield shifted (δ 0.82-0.84) in the NMR spectrum of (+)-MTPA ester and downfield (δ 0.95-0.98) in that of (-)-MTPA, thus establishing the 24R configuration in the steroid **25**.

Steroid **26** had a molecular weight 16 mass units higher than **25**. The ^{1}H NMR data indicated that **26** had the same side chain as **25** and a 3β,4β,6α,8,15β-pentahydroxylated steroid nucleus already found in a steroid firstly isolated from *Gomophia watsoni* [29] and then from *Culcita novaeguinea*. [6] The 24R stereochemistry was inferred as in the case of **25**.

The more polar steroid **27** FABMS, m/z 525 $[MSO_3^-]$ is the 24S methyl thornasterol A (=thornasterol B) 3-sulphated, steroid aglycone of the asterosaponin acanthaglycoside F, isolated from *Acanthaster placi*. [30] The 24S stereochemistry was inferred from the negative Cotton maximum, $[\theta]_{280}$ -400, in the CD spectrum as compared with that of the diacetyl derivatives of (20R, 24R)-thornasterol B and (20S, 24S)-thornasterol B, which are reported as $[\theta]_{280}$ -5720 and $[\theta]_{277}$ -631, respectively. [31]

BIOLOGICAL ACTIVITY

The cytotoxic activities *in vitro* of a selection of fifteen steroids and steroid glycosides were tested against human bronchopulmunary non-small-cell-lung-carcinoma cells (NSCLC-N6) and the results are reported in Table 1 along with data of the asterosaponins described in the previous paper. [9] The data clearly show that the sulphated penta- and hexa-glycosides (asterosaponins) are more active than the other groups of the related steroids, parallelling previous investigations. [2,32] Among the steroid glycosides those having a 3-OMe-xylopyranosyl moiety are more active; of the eight polyhydroxylated steroids tested, only 2 compounds, **22** and **24**, showed an IC_{50}< 30 μg/ml. Steroids **14**, **15** and **16** revealed to have a cytostatic effect upon NSCLC-N6 cells as was determined by photo microscopic examination and flow cytometric analysis. Actually the cell cycle analysis of NSCLC-N6 cells treated with 30 μg/ml of **14** confirmed a blockage in phase G1 and this was true for all cells in an independent manner to their DNA content. In fact, the NSCLC-N6 cell line is polyploidic, with cells figuring 2C, 4C and 8C nuclei. On the other hand, the sulphated asterosaponins pectinioside A [2,9] and antarcticoside C [9] showed a cytostatic activity on NSCLC-N6 cells due most likely to the same mechanism as the steroid **14**, but associated to other toxic effects *in vitro*. The flow cytometric analysis of NSCLC-N6 cells treated 72 h long with 5 μg/ml of saponins pectinioside A and antarcticoside C revealed important cell decomposition (IC_{50}< 3 μg/ml) and cell blockage in phase G1. These data corrobate the interest of studing **14**, **15** as well as **16** rather than saponins since the toxic side effects *in vivo* which still represents the major obstacle to the application of all actual treatments in clinical chemotherapy could by avoided.

EXPERIMENTAL SECTION

General Methods.

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; mass spectra, VG AUTOSPEC instruments (Cs^+ ions bombardment) with fab source [in glycerol or glycerol-thioglycerol (3:1) matrix]; optical rotation were measured on a Perkin-Elmer 141 polarimeter operating at 589 nm; 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 x 8 mm i.d.; flow rate 5 ml min^{-1}) or (30 cm x 3.9 mm i.d.; flow rate 2 ml min^{-1}) Waters Model 6000 A or 510 pump equipped with U6K injector and a differential refractometer, model 401; DCCC, DCC-A apparatus manufactured by Tokyo Rikakikai Co., equipped with 250 tubes.

Animal Collection.

The animal, (only one organism was collected), yellow with some red spots on the arms, was collected at the Tethys Bay in January 1991, in the course of the Italian-Antarctic expedition, a voucher specimen is kept at Istituto per la Chimica di Molecole di Interesse Biologico del CNR, Arco Felice, Napoli under the ref. number MOR 108; identification was done by Professor Michel Jangoux, Université Libre de Bruxelles, Laboratorie de Biologie Marine, Bruxelles. Professor Jangoux has supposed *Henricia* as the most probable genus.

Extraction and Isolation.

The animal (0.8 Kg), was cut into small pieces and soaked in water for 5h. The aqueous extracts were centrifuged and passed through a column of Amberlite XAD-2 (700 g). The column was washed with distilled water (1 l) and then eluted with methanol (4 l). The methanol eluate was taken to dryness to give the glassy material (6.6 g). The remaining solid mass, after extraction with water, 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 which was combined with the above MeOH eluate from Amberlite XAD-2 column and chromatographted 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 27-51 (1.03 g) mainly contained the asterosaponins, which were the subject of the preceding paper and contained the known brasiliensoside and pectinioside A and the new hexaglycosides anctarcticoside A, B, C with 24-methylbrasiliensoside and 24-methylpectinioside A.[9] Fractions 52-62 (0.50 g) contained steroidal glycoside sulphates and fractions 63-100 (1.2 g) contained mixed glycosides and polyhydroxysteroids. Fractionation of the 63-100 fractions (1.2 g) was pursued by DCCC 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). Each of the above fractions was then submitted to HPLC with MeOH-H_2O (7:3), or MeOH-H_2O (75:25), on C_{18} column (30 cm x 3.9 mm i.d. or 30 cm x 8 mm i.d.) to give pure compounds. Fractions 52-62 (0.5 g) eluted from the column of Sephadex LH-60, containing the more polar compounds were combined with the first fractions 10-24 (100 mg) derived from the DCCC separation, and submitted again 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 11-39 (30 mg) contained the asterosaponins mixture; fractions 40-56 (40 mg) contained mainly compound **13** and fractions 75-105 (48 mg) contained compounds **7** and **12**. Each fraction was purified by HPLC on a C_{18} column with MeOH-H_2O (55:45) to give pure compounds. The subsequent DCCC fractions contained a complex mixture of sulphated material, difficult to separate. Rotations are in Table 1; FABMS data in the text; most of ^{1}H and ^{13}C NMR data are in Tables 2-7. All NMR spectra were taken in methanol-d_4.

Additional physical data:

1: ^{1}H NMR (sugar): 4.20 (1H, d, J=7.0 Hz., H-1'), 3.85 (1H, dd, J=10.0, 5.0 Hz, H-5'eq), 3.65 (3H, s, OCH_3), 3.56 (1H, m, H-4'), 3.25 (1H, dd, J=9.0, 7.0 Hz, H-2'), 3.20 (1H, t, J=10.0 Hz, H-5'ax),3.05 (1H, t, J=9.0 Hz, H-3'); ^{13}C NMR (sugar): 105.3 (C-1'), 87.5 (C-3'), 74.5 (C-2'), 70.9 (C-4'), 66.8 (C-5'), 60.7 (OCH_3).

2: ^{1}H and ^{13}C NMR virtually identical with the data reported for **1** except for steroid side chain: 5.34 (!H, dt, J=14.0, 5.5 Hz, H-23), 5.28 (1H, dd, J=14.0, 7.0 Hz, H-22), 3.61 (1H, dd, J=10.5, 6.0 Hz, H-26), 3.40 (1H,dd,

J=10.5, 5.0 Hz, H'-26), 1.02 (3H, d, J=7.0 Hz, H_3-21), 0.93 (3H, d, J=7.0 Hz, H_3-27); 139.6 (C-22), 126.6 (C-23), 75.4 (C-26), 37.3 (C-24), 34.9 (C-25), 20.9 (C-21), 17.0 (C-27).

3: ^{1}H NMR (sugar): 4.99 (1H, br s, H-1'), 4.46 (1H, d, J=7.6 Hz, H-1''), 4.10 (1H, br d, H-2'), 4.06 (1H, m, H-3'), 4.00 (1H, dd, J=10.6, 4.0 Hz, H-5''), 3.91 (1H, m, H-4'), 3.81 (1H, dd, J=12.5, 3.0 Hz, H-5'), 3.65 (3H, s, OCH_3), 3.64 (1H, dd, J=12.5, 4.8 Hz, H-5'), 3.50 (3H, .s, OCH_3), 3.42 (1H, t, J=9.0 Hz, H-3''), 3.20 (1H, m, H-4''), 3.15 (1H, t, J=10.6 Hz, H-5''), 2.89 (1H, t, J=9.0 Hz, H-2''); ^{13}C NMR (sugar): 108.1 (C-1'), 104.6 (C-1''), 91.4 (C-2'), 84.7 (C-2''), 84.0 (C-4'), 80.7 (C-4''), 77.5 (C-3'), 76.5 (C-3''), 64.2 (C-5''), 62.5 (C-5'), 61.0 (OCH_3), 58.9 (OCH_3).

5: ^{1}H NMR (sugar): 4.92 (1H, br s, H-1'), 3.99 (1H, dd, J=4.5, 2.0 Hz, H-2'), 3.94 (1H, m, H-4'), 3.86 (1H, dd, J=7.5, 4.5 Hz, H-3'), 3.78 (1H, dd, J=12.5, 3.0 Hz, H-5'), 3.65 (1H, dd, J=12.5, 5.2 Hz, H-5'); ^{13}C NMR (sugar): 109.6 (C-1'), 85.2 (C-4'), 83.7 (C-2'), 78.8 (C-3'), 63.0 (C-5').

7: ^{1}H NMR (sugar): 4.28 (1H, m, H-4'), 4.27 (1H, d, J07.5 Hz, H-1'), 4.25 (1H, dd, H-5', overlapped with other signals), 3.65 (3H, s, OCH_3), 3.35 (1H, t, H-3', overlapped with solvent signal), 3.30 (1H, t, J=9.0 Hz, H-2'), 3.22 (1H, t, J=10.5 Hz, H-5'); ^{13}C NMR (sugar): 104.6 (C-1'), 85.0 (C-3'), 77.1 (C-4'), 74.1 (C-2'), 64.7 (C-5'), 60.7 (OCH_3).

10: ^{1}H NMR of the side chain identical to the data reported for **9**; ^{1}H NMR (sugar): 5.02 (1H, d, J=15.0 Hz, H-1'), 4.37 (1H, d, J=7.5 Hz, H-1''), 4.07 (1h, dd, J=15.0, 4.0 Hz, H-2'), 4.05 (1H, dd, H-5'', overlapped with other signals), 4.03 (1H, m, H-3'), 4.00 (1H, m, H-4'), 3.81 (1H, dd, J=11.2, 3.0 Hz, H-5'), 3.67 (1H, dd, J=11.2, 4.2 Hz, H-5'), 3.50 (3H, s, OCH_3), 3.40 (1H, t, J=9.0 Hz, H-3''), 3.23 (1H, m, H-4''), 3.18 (1H, dd, J=7.5, 9.0 Hz, H-2''), 3.14 (1H, t, H-5'' overlapped with other signals).

11: ^{1}H and ^{13}C NMR (sugar): 5.31 (1H, br d, J=2.0 Hz, H-1'), 4.03 (1H, m, H-4'), 3.98 (1H; dd, J=4.5 2.0 Hz, H-2'), 3.86 (1H, dd J=7.5, 4.5 Hz, H-3'), 3.77 (1H, dd, J=12.5, 3.0 Hz, H-5'), 3.66 (1H, dd, J=12.5, 5.2 Hz, H-5'); 103.5 (C-1'), 85.0 (C-4'), 84.5 (C-2'), 78.1 (C-3'), 62.9 (C-5').

12: ^{1}H and ^{13}C NMR of sugar: 4.25 (1H, d, J=7.8 Hz, H-1'), 3.88 (1H, dd, J=12.0, 2.5 Hz, H-6'), 3.70 (1H, dd, J=12.0, 5.6 Hz, H-6'), 3.66 (3H, s, OCH_3), 3.38 (1H, t, J=9.0 Hz, H-4'), 3.29 (1H, m, H-5'), 3.28 (1H, t, H-2' overlapped with other signals); 104.7 (C-1'), 87.8 (C-3'), 77.3 (C-5'), 74.8 (C-2'), 70.8 (C-4'), 62.5 (C-6'), 60.9 (OCH_3).

13: ^{1}H and ^{13}C NMR of side chain virtually identical with the data reported for **12**.

15: ^{1}H NMR of side chain: 5.58 (1H, dd, H-22 overlapped with H-23), 5.52 (1H, dt, H-23 overlapped with H-22), 3.46 (1H, dd, J=8.0, 6.5 Hz, H-26), 3.37 (1H, dd, H'-26 overlapped with solvent signal), 2.58 (1H, m, H-20), 1.09 (3H, d, J=6.5 Hz, H_3-21), 0.93 (3H, d, J=6.5 Hz, H_3-27); 139.6 (C-22), 127.5 (C-23), 68.0 (C-26), 37.7 (C-24), 37.4 (C-25), 34.7 (C-20), 20.7 (C-21), 16.9 (C-27).

16: ^{1}H NMR of steroidal nucleus virtually identical with the data reported for **14**; ^{1}H NMR of side chain: 3.44 (1H, dd, J=8.0, 6.5 Hz, H-26), 3.36 (1H, dd, H'-26 partially overlapped with solvent signal), 0.98 (3H, d, J=6.8 Hz, H_3-21), 0.93 (3H, d, J=7.0 Hz, H_3-27).

17: ^{1}H NMR: 4.00 (1H, dd, J=8.5, 3.0 Hz, H-16), 3.79 (1H,dd, J=10.5, 3.0 Hz, H-15), 3.77 (1H, br s, H-6), 3.57 (1H, m, H-3), 3.38 (2H, s, H_2-26), 1.15 (3H, s, H_3-27), 1.07 (3H, s, H_3-19), 1.00 (3H, d, J=7.0 Hz, H_3-21), 0.95 (3H, s, H_3-18).

18: ^{1}H NMR: 4.01 (1H, dd, J=7.6, 2.5 Hz, H-16), 3.73 (1H, dd, J=10.3, 2.5 Hz, H-15), 3.53 (1H, m, H-3), 3.44 (1H, dd, J=10.5, 5.8 Hz, H-26), 3.30 (1H, H'-26 partially overlapped with solvent signal), 2.55 (1H, dd, J=13.7, 4.7 Hz, H-7), 2.28 (1H, br t, J=13.7, 3.5 Hz, H-7), 1.00 (3H, d, J=6.8 Hz, H_3-27), 0.94 (3H, d, J=6.8 Hz, H_3-21), 0.92 (3H, s, H_3-18), 0.79 (3H, s, H_3-19).

20: FABMS (+ve ion): m/z 467 $[M+H]^+$.

21: ^{1}H and ^{13}C NMR of side chain: 5.55 (1H, dd, H-22 overlapped with H-23), 5.53 (1H, dt, H-23 overlapped with H-22), 3.45 (1H, dd, J=8.0, 6.5 Hz, H-26), 3.36 (1H, dd, H'-26 partially overlapped with solvent signal), 2.55 (1H, m, H-20), 1.06 (3H, d, J=6.5 Hz, H_3-21), 0.92 (3H, d, J=6.5 Hz, H_3-27); 139.4 (C-22), 127.4 (C-23), 67.8 (C-26), 37.5 (C-24), 37.2 (C-25), 34.3 (C-20), 20.3 (C-21), 16.8 (C-27). FABMS (+ve ion): m/z 467 $[M+H]^+$.

22: ^{1}H NMR of steroidal nucleus virtually identical with those reported for **20**; ^{1}H NMR of side chain virtually identical with those reported for **16**; FABMS (+ve ion): m/z 469 $[M+H]^+$.

24:^{1}H NMR of steroidal nucleus identical to those reported for **20**; ^{1}H NMR of side chain: 4.83-4.77 (each 1H, br s, H_2-28), 3.61 (1H, dd, H-26 overlapped with other signals), 3.41 (1H, dd, J=8.5, 6.0 Hz, H'-26), 1.07 (3H, d, J=7.0 Hz, H_3-27), 0.99 (3H, d, J=6.8 Hz, H_3-21).

26: ^{1}H NMR: 4.42 (1H, t, J=5.0 Hz, H-15), 4.29 (1H, br s, H-4), 4.20 (1H, dt, J=10.0, 3.5 Hz, H-6), 3.47 (1H, m, H-3), 2.47 (1H, dd, J=12.0, 4.0 Hz, H-7), 1.32 (3H, s, H_3-18), 1.19 (3H, s, H_3-19); FABMS (+ve ion): m/z 489 $[M+Na]^+$.
27: ^{1}H NMR: 5.37 (1H, br d, J=5.5 Hz, H-11), 4.24 (1H, m, H-3), 3.54 (1H, dt, J=10.0, 3.5 Hz, H-6), 2.77-2.60 (each 1H, d, J=15.0 Hz, H_2-22), 2.40 (1H, m, H-24), 1.39 (3H, s, H_3-21), 1.02 (3H, d, J=7.5 Hz, H_3-28), 1.00 (3H, s, H_3-19), 0.95-0.89 (each 3H, d, J=6.8 Hz, H_3-26 and H_3-27), 0.82 (3H, s, H_3-18).

METHANOLYSIS OF GLYCOSIDES: SUGAR ANALYSIS.
A solution of the glycosides **6** (1 mg) and **9** (1 mg) in anhydrous 2 M HCl in MeOH (0.5 ml) was heated at 80° in a stoppered reaction vial for 8 h. After having cooled, the reaction mixture was neutralized with Ag_2CO_3 and centrifuged and the supernatant was evaporated to dryness under N_2. The residue was trimethylsilylated with trisyl Z (Pierce Chemical Co.) for 15 min at room temperature. GLC analysis at 150° C using 25 m column of SPB-1 (helium carrier, flow 10 ml min^{-1} gave peaks which co-eluted with those of silylated methylxyloside and methylgalactoside.

SOLVOLYSIS OF THE SULPHATED COMPOUNDS 7, 12 and 13.
A solution of **7** (1.9 mg), **12** (1.6 mg) and **13** (1.8 mg) in pyridine (0.5 ml) and dioxane (0.5 ml) were heated at 150°C for 2 h in a stoppered reaction vials. After the solutions were cooled, the mixture were evaporated to dryness and then purified by HPLC on a μ-Bondapak C_{18} column (30 cm x 3.9 mm i.d.) with MeOH:H_2O (7:3), ϕ 2ml/min to give pure **7a**, **12a** and **13a**.
^{1}H NMR (sugar) identical with data reported for 3-O-methylxylose Table 2 in **1**.
^{1}H NMR of steroidal nucleus of **12a** and **13a** is identical to the data reported for **1**-**11**.
FABMS **13a** (-ve ion) m/z 801 $[M-H]^-$

ENZYMATIC HYDROLISYS OF THE GLYCOSIDE 11.
Glycoside **11** (0.8 mg) in a citrate buffer (1 mL, PH 4.5) was incubated with glycosidase mixture (1.2 mg) of *Charonia lampas* (Scikagaku Kogyo) at 38° for 3 days. The reaction mixture was passed throught a Sep-Pak C-18 cartridge, washed with H_2O and eluted with MeOH. The reaction mixture was evaporated to dryness and purified by hplc [C_{18}-μ-Bondapak (30 cm x 3.9 mm i.d.), MeOH-H_2O (7:3)] to give **11a** the 3β,4β,6α,8,15β,24-hydroxymethyl cholestane,24-heptaol. ^{1}H NMR (CD_3OD) of the side chain: δ 0.94 (each 3-H, d, J=6.8 Hz, H_3-26 and H_3-27), 0.98 (3-H, d, J=7.0 Hz, H_3-21) and 3.48-3.51 (each d, J=11.3 Hz, H_2-28); FABMS (-ve ion) m/z 497 $[M-H]^-$

HYDROLYSIS OF THE GLYCOSIDES 1, 2 , 3 and 12 to give 1a, 2a, 3a and 12b.
A solution of **1** (1.2 mg), **2** (1.9 mg), **3** (2 mg) and **12** (2.4 mg) in anhydrous 2 M HCl in MeOH (0.5 ml) was heated at 80° in a stoppered reaction vial. After 1h, TLC analysis [SiO_2 with $CHCl_3$-MeOH-H_2O (80:18:2)] showed that the starting materials have disappeared and was replaced by a uv-active spots. The reaction mixture was cooled, neutralized with Ag_2CO_3, and centrifuged, and the supernatants were taken to dryness under N_2. The residues were purified by HPLC [C_{18}-μ-Bondapak (30 cm x 3.9 mm i.d.), MeOH-H_2O (75:25)] to give **1a** (25S) 5α-cholesta-8,14,22-triene-3β,4β,6α,26-tetraol, **2a** (25R) 5α-cholesta-8,14,22-triene-3β,4β,6α,26-tetraol, **3a** (25R) 5α-cholesta-8,14,22-triene-3β,4β,6α,26-tetraol and **12b** (24R,25S) 24-methylcholest-8,14,22-triene-3β,4β,6α,26-tetraol, respectively.

^{1}H NMR of steroidal nucleus of **1a**, **2a**, **3a** and **12b**: 5.38 (1H, br s, H-15), 4.30 (1H, br s, H-4), 4.21 (1H, dt, J=10.7, 4.2 Hz, H-6), 3.50 (1H, m, H-3), 2.61 (1H, dd, J=13.0, 5.0 Hz, H-7), 1.24 (3H, s, H_3-19), 0.87 (3H, s, H_3-18).
^{1}H NMR of side chain of **1a** and **3a** are identical to the data reported for **20**.
^{1}H NMR of side chain of **2a** is identical to the data reported for **21**.
^{1}H NMR of side chain of **12b**: 5.33 (1H, dd, J=15.0, 7.8 Hz, H-22), 5.28 (1H, dd, J=15.0, 7.8 Hz, H-23), 3.61 (1H, dd, J=10.8, 6.5 Hz, H-26), 3.31 (1H, dd, H'-26 partially overlapped with solvent signal), 1.07 (3H, d, J=6.5 Hz, H_3-21), 0.97 (3H, d, J=6.5 Hz, H_3-28), 0.92 (3H, d, J=6.5 Hz, H_3-27).

HYDROGENATION OF STEROID 21.

Steroid **21** (2 mg) was hydrogenated at atmospheric pressure over 10% Pt/C in 1ml of MeOH for 24h. Removal of the catalyst by filtration and evaporation of the solvent gave the saturated compound **21a**, 5α cholestane3β,6β,8,15α,16β,26-hexaol.

^{1}H NMR of side chain: 3.43 (1H, dd, J=10.0, 6.5 Hz, H-26), 3.34 (1H, dd, H'-26 overlapped with solvent signal), 0.96 (3H, d, J=6.5 Hz, H_3-21), 0.93 (3H, d, J=6.5 Hz, H_3-27).

MTPA Esters.

Each of the following steroids (0.5-1 mg): 5α-cholesta-8,14,22-triene-3β,4β,6α,26-tetraols (**1a** and **2a**), 5α-cholesta-8,14,22-triene-3β,4β,6α,26-tetraol (**3a**), 24-methylcholestane-3β,4β,6α,8β,15β,24,28-heptaol (**11a**), 24-methylcholest-8,14,22-triene-3β,4β,6α,26-tetraol (**12b**), **14**, **15**, **16**, **17**, **19**, **20**, **21**; 5α-cholestane-3β, 6β,8β,15α,16β,26-hexaol (**21a**), **22**, **24**, **25**, was treated with freshly distilled (+) and/or (-)-methoxytrifluoromethylphenylacetyl chloride (2μl), prepared from (+)-(R)-MTPA and (-)-(S)-MTPA acid in dry pyridine (40 μl) for 1h at room temperature. After removal of solvent, the product was analyzed by ^{1}H NMR and the prominent data are summarized in Table 8.

Table 8. Selected ^{1}H NMR signals for side chain in MTPA esters of glycosides **1a**, **2a**, **3a**, **11a**, **12b** and polyhydroxysteroids **14**, **15**, **16**, **17**, **19**, **20**, **21**, **22**, **24**, **25**.

MTPA esters	H_2-26	H_3-26, H_3-27	H_2-28
1a (3,26-di-O-(-)-MTPA)	4.30 dd-4.13 dd	-	-
2a (3,26-di-O-(-)-MTPA)	4.17 dd-4.23 dd	-	-
3a (3,6,26-tri-O-(-)-MTPA)	4.13 dd-4.30 dd	-	-
11a (3,6,28-tri-O-(+)-MTPA)	-	-	4.32 d-4.20 d
11a (3,6,28-tri-O-(-)-MTPA)	-	-	4.22 d-4.26 d
12b (3,6,26-tri-O-(-)-MTPA)	4.42 dd-4.13 dd	-	-
14 (3,15,26-tri-O-(+)-MTPA)	4.16 dd-4.22 dd	-	-
15 (3,15,26-tri-O-(+)-MTPA)	4.11 dd-4.26 dd	-	-
16 (3,15,26-tri-O-(+)-MTPA)	4.13 dd-4.27 dd	-	-
16 (3,15,26-tri-O-(-)-MTPA)	4.18 dd-4.23 dd	-	-
17 (3,15,26-tri-O-(-)-MTPA)	4.19 brs	-	-
19 (3,15,26-tri-O-(-)-MTPA)	4.28 dd-4.10 dd	-	-
20 (3,15,26-tri-O-(+)-MTPA)	4.22 dd-4.16 dd	-	-
20 (3,15,26-tri-O-(-)-MTPA)	4.12 dd-4.28 dd	-	-
21 (3,15,26-tri-O-(+)-MTPA)	4.10 dd-4.26 dd	-	-
21 (3,15,26-tri-O-(-)-MTPA)	4.18 dd-4.21 dd	-	-
21a (3,15,26-tri-O-(+)-MTPA)	4.26 dd-4.13 dd	-	-
22 (3,15,26-tri-O-(-)-MTPA)	4.18 dd-4.23 dd	-	-
24 (3,6,15-tri-O-(+)-MTPA)	4.23 dd-4.43 dd	-	-
24 (3,6,15-tri-O-(-)-MTPA)	4.26 dd-4.39 dd	-	-
25 (3,6,15,24-tetra-O-(+)-MTPA)	-	0.82 d-0.84 d	-
25 (3,6,24-tri-O-(-)-MTPA)	-	0.95 d-0.98 d	-

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