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UNUSUAL SULFATED MARINE STEROIDS FROM THE OPHIUROID *OPHIODERMA LONGICAUDUM*

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Abstract—Polar extracts of the ophiuroid *Ophioderma longicaudum* contain unusual sterol sulfates together with a mixture of common 3β-hydroxysterol sulfates. The more polar compound has been shown to be 5β-cholestane-3α,4α,11β,12β,21-pentol 3,21-disulfate **1**. A second group of unusual compounds are disulfated 3α,21-dihydroxysteroids. After solvolysis to remove the sulfate groups they have been identified as: (20*R*)-5α-cholestane-3α,21-diol **4a**, (20*R*)-cholest-5-ene-3α,21-diol **5a**, (20*R*,22*E*)-cholest-5,22-diene-3α,21-diol **6a** and (20*R*)-24-methylcholest-5,24(28)-diene-3α,21-diol **7a**. Analysis of the "non-sulfated" sterol fractions has shown the presence of common 3β-hydroxy sterols.

The echinoderms can be divided into five classes: Crinoidea (sea lilies), Holothuroidea (sea cucumbers), Echinoidea (sea urchins), Ophiuroidea (brittle stars) and Asteroidea (starfishes). The study of natural products from sea cucumbers and starfishes has received in the last few years considerable attention especially because their content of toxic saponins.[1] Sea urchins too have attracted the attention of chemists especially because of their brilliant coloration.[2] On the contrary sea lilies and brittle stars have received moderate attention as compared to the three above classes. Only sporadic papers on their sterols content have appeared in literature.[3] Sea lilies, brittle stars and sea urchins contain Δ^5-sterols while sea cucumbers and starfishes contain a predominance of Δ^7-sterols. This division of echinoderms according to the sterol type is supported by distribution of the saponins, which are apparently absent from sea lilies, brittle stars and sea urchins.

As a direct consequence of our efforts to isolate biologically active compounds from starfishes[4] we had the occasion to examine some ophiuroids and we now report on the unusual sulfated steroids from the mediterranean *Ophioderma longicaudum*.

The n-butanol-soluble material from a methanol extract of brittle star (0.7 kg, wet weight) was chromatographed on Sephadex LH-20 to obtain three steroidal sulfate fractions. The more polar fraction was further purified by reverse phase HPLC to give 12 mg of **1**; the less polar fraction was further purified by droplet counter-current chromatography (DCCC) and contained the common sulfated 3β-hydroxysteroids (*ca* 40 mg); the second fraction contained the sulfated 3α,21-dihydroxysteroids (*ca* 45 mg).

5β - Cholestane - 3α,4α,11β,12β,21 - pentol 3,21 - *disulfate* **1**. $[\alpha]_D 10°$, m.p. 192–195°. Negative ion fast atom bombardment (FAB) mass spectrum exhibited molecular ion species peaks at *m/z* 649 and 633, corresponding to the monopotassium and monosodium salt of the dianion, respectively. Fragmentation peaks at *m/z* 531 and 513 were interpreted as losses of KSO_3 (+H) and $KHSO_4$ from *m/z* 649 and $NaSO_3$ (+H) and $NaHSO_4$ from *m/z* 633, respectively. The ^{13}C-NMR data, especially J-modulated spin echo, showed **1** to contain four methyl groups, nine methylene, seven methine, two quaternary carbons, four OCH and one OCH_2 (Table 1). A total of 27 carbon resonances were observed. Taken together these data indicated a disulfated pentahydroxycholestane structure with one of the five methyl groups typical of a sterol oxidized to hydroxymethylene. The ^{1}H-NMR confirmed the

Table 1. ^{13}C-NMR data for **1**, **2**, **4** and **4a**[a]

C	1[b]	2[b]	4	4a[c]
1	36.0	34.6	33.2	32.2
2	24.8 (−3.4)	28.2	27.9	29.0
3	82.3[d] (+9.3)	73.0[f]	76.5	66.6
4	75.2 (−1.4)	76.6	33.9	35.9
5	48.6	46.8	41.3	39.1
6	26.4[e]	25.4[g]	28.5	28.6
7	27.0[e]	25.8[g]	33.1	32.0
8	30.6	29.1	34.7	35.5
9	45.2	44.2	55.9	54.3
10	36.4	35.1	36.9	36.1
11	73.4	72.5[f]	21.9	20.8
12	81.2[d]	80.6	40.5	39.5
13	49.0	47.5	43.6	42.4
14	57.0	56.3	57.8	56.5
15	24.8	24.8	25.1	24.1 (−0.2)
16	29.5	28.7	29.6	28.0 (+0.3)
17	53.2	52.1	52.5	50.5 (+6.2)
18	10.5	10.2	12.8	12.4 (+0.2)
19	27.5	26.9	11.8	11.2
20	39.1 (−3.7)	42.8	40.7	42.5
21	72.3 (+7.9)	64.4	69.8	62.8
22	30.1 (−0.9)	31.0	31.1	32.2
23	23.5	23.8	24.6	24.1
24	40.7	39.4	40.7	39.5
25	29.1	28.0	29.1	27.8
26	23.0	22.6	23.0	22.6
27	23.1	22.7	23.1	22.7

[a] 62.9 MHz; the values of the chemical shifts are in CD_3OD for **1** and **4** and $CDCl_3$ for **2** and **4a**.

[b] Assignments made by comparison to 5β-cholestan-3α-ol,[5] substituent parameter considerations[7,9] and chemical shift comparison from **1** to **2**; in parentheses the sulfation shifts.

[c] The shifts at nuclear carbons C-1 through C-16 and C-18 and C-19 were very similar with those published for 5α-cholestan-3α-ol;[5] in parentheses the deviations of more than ±0.1 ppm from the reference compound; + denotes upfield shift in **4a** and − downfield shifts.

[d] Distinguished by selective decoupling.

[e–g] Signals within a column may be reversed.

presence of four methyl groups: δ 0.90 (s, 18-H), 0.92 (6H, d, J = 6.5 Hz, 26- and 27-H) and 1.19 (s, 19-H). Although one methyl doublet was absent, a B portion of an ABX system at δ 4.07 (dd, J = 9 and 5.5 Hz, the A portion overlaps under a complex 3H signal at δ *ca* 4.19) was observed, which was ascribable to a $O_3SO—H_2C—$(21) group. Hydroxylation at C-26 or C-27 could be ruled out at this stage primarily on the basis of the presence in the ^{13}C-NMR spectrum of the typical signals for C-26, C-27 at 23.0 and 23.1 ppm.[5] The ^{1}H-NMR spectrum of **1** also contained a doublet at δ 3.28 (1H, J = 3.5 Hz) and a triplet at δ 3.99 (1H, J = 3.5 Hz), coupled each to the other as proved by decoupling. These NMR data lead to a structural element with vicinal, secondary OH groups—one adjacent to a C bearing one proton and the other to one without a proton. There is only one way such a fragment can be put into a steroid skeleton, i.e. 11, 12. The downfield shift and narrowing of the resonance signal at δ 3.99 are consistent for an equatorial proton, while the upfield shift of the signal at δ 3.28 is consistent for an axial proton. Thus the 11β,12β-dihydroxy moiety in **1** could be established. The remaining two OCH signals overlap at δ *ca* 4.19 together with the second arm of the AB system. Of special note were the chemical shifts of the angular Me carbons at 10.5 and 27.0 ppm. The lowfield shielding of one of them was strongly indicative for the *cis*-A/B ring fusion.[6] In addition since the relative rigidity of the steroid skeleton is such that the C-18 shielding may be expected to be essentially unaffected by a change of the A/B ring fusion, the shift of 10.5 ppm agreed with that we expected for a C-18 carbon in a structural environment such as in **1** on the basis of the substituent effects that have been published for hydroxy steroids.[7]

Solvolysis of **1** afforded the pentol **2**, m.p. 126–128°. In the electron impact high resolution mass spectrum the ion observed at highest mass (*m/z* 434.3389) corresponded to loss of water from the molecular formula $C_{27}H_{48}O_5$ (calc for $C_{27}H_{46}O_4$ 434.3395). An intense peak at *m/z* 287 was interpreted as loss of a hydroxylated C_8 side chain and two molecules of water. In the ^{1}H-NMR of **2** the resonance frequencies of 11-H and 12-H remained essentially unshifted at δ 4.08 (t, J = 3.0 Hz, 11-H) and 3.32 (d, J = 3.0 Hz, 12-H) relative to **1**, while the remaining hydroxy and hydroxymethylene signals moved upfield and overlap at δ 3.61 (2H, 3- and 21-H) and 3.90 (2H, 4- and 21-H). Treatment of **2** with *p*-bromobenzoyl chloride in pyridine gave a di(*p*-bromobenzoate **3**, *m/z* 616–618 ($M^+ - BrC_6H_4CO_2H$), which provided for an apparent first-order spectrum in the downfield region with four resolved bands. In addition to the signals at δ 3.30 (dd, J = 3.0 and 5 Hz, 12-H, transformed in a sharp doublet, J = 3.0 Hz, on D_2O exchange) and 4.03 (t, J = 3.0 Hz) assigned to 12- and 11-H, respectively, the spectrum ($CDCl_3$) contained a narrow signal at δ 4.14 (1H), a doublet at δ 4.50 (2H, J = 5.5 Hz, 21-H) and a broad doublet at 4.99 (J = 11 Hz). Irradiation at δ 4.14 simplified the broad doublet at δ 4.99 into a sharp double doublet with J = 11.0 and 4.5 Hz, while irradiation of the δ 4.99 signal transformed the former into a doublet (J = 4.5 Hz). This experiment lead to a structural element with vicinal secondary hydroxy and benzoyloxy groups the former adjacent to a C bearing one proton and the other to one bearing two protons. There is only one way such a fragment can be put into a steroidal skeleton, i.e. 3,4. The downfield shift and narrowing of the resonance signal at δ 4.14 are consistent for an equatorial proton (hydroxymethine), while the large coupling constants associated with the signal at δ 4.99 are consistent for an axial proton (benzoyloxymethine). Thus, assuming the 5β-cholestane skeleton for our steroid the 3α,4α-dihydroxy moiety in **2** could be established. Further evidence for the structural assignment with two *cis* 1,2 (vicinal) diol groups was provided by the formation of a *bis*-acetonide from **2**. The shift of the 19-methyl protons (δ 1.18) is in agreement with the *cis*-A/B ring fusion (calc[8] 1.19; for the alternative 5α - cholestane - 3β,4β,11β,12β,21 - pentol structure, calc[8] 19-H : 1.33) as does the shift of the 19-Me carbon (26.9 ppm). In 5β - cholestan - 3α - ol the 19-Me group resonates at 24.3 ppm. Given this Me resonance as a model and the effect of the 11β-OH group (+3.2 ppm) (the remaining OH groups would be expected to give very small shifts),[7] the expected chemical shift for the 19-Me carbon should be around 27.5 ppm. Otherwise in 5α-cholestan-3β,4β-diol the 19-Me group resonates at 14.7 ppm[9] and the expected chemical shift for the alternative 5α-cholestan-3β,4β,11β,12β,21-pentol structure should be 17.9 (i.e. 14.7 + 3.2 ppm), which is far off from our value.

The sulfate groups are assigned to C-3 and C-21 in **1** by consideration of chemical shift and coupling patterns in comparison with ^{1}H-NMR data for the

Table 2. 250 MHz ^{1}H-NMR ($CDCl_3$; shifts are δ values; J in Hz) of **4a–7a**

	4a	**5a**	**6a**	**7a**
3-H	4.06 (m)	4.03 (m)	4.03 (m)	4.03 (m)
6-H	—	5.43 (d)	5.43 (d)	5.43 (d)
		J = 5.5	J = 5.5	J = 5.5
21-H	3.70 (broad, s)	3.71 (broad, s)	3.65–3.85 (broad)	3.72 (broad, s)
22,23-H	—	—	5.10 (dd)	—
			J = 16.0, 8.5	
			5.53 (dt)	
			J = 16.0, 7.5	
28-H				4.75 (s)–4.70 (s)
18-H	0.68 (s)	0.71 (s)	0.74 (s)	0.72 (s)
19-H	0.79 (s)	1.02 (s)	1.02 (s)	1.02 (s)
26,27-H	0.88 (6H, d)	0.88 (6H, d)	0.90 (d)–0.89 (d)	1.04 (6H, d)
	J = 7.0	J = 6.5	J = 6.5 J = 6.5	J = 7.0

desulfated derivative **2**, as did comparison of the ^{13}C-NMR spectra (Table 1). Assignments of C signals in the spectra of **1** and **2** have been made by using 5β-cholestane-3α-ol as reference compound,[5] substituent effects that have been published for hydroxysteroids[7] and the additivity relationships that have been published for dihydroxysteroids.[9]

We would note that shift values for the C in the side chain of **2** were similar with those of the same side chain observed for the diol **4a** (Table 1). The major deviation was observed for C-21 64.4 in **2** vs 62.7 in **4a** and could be ascribable to the interaction with 12β-OH group in **2**. The shift values for the C in the side chain reported for a group of 26-hydroxysteroids[10] were far off from our results, thus giving further support for the location of the primary OH group at C-21.

The configuration 20*R* (natural configuration) to **1** is proposed by analogy with the diols **4a–7a**, whose stereochemistry at C-20 was suggested by comparison of their spectral data with those of synthetic (20*R*)- and (20*S*)-cholest-5-en-3β,21-diol (see below).

The sulfated 3α,21-dihydroxysteroids. This mixture was fractionated by HPLC to give four main compounds, **6**, **7**, **5** and **4**, eluted in that order (Table 3). The ^{1}H-NMR spectra of all four compounds contained signals at δ 3.98 (dd, J = 9.5 and 5.3 Hz) and 4.22 (dd, J = 9.5 and 3.8 Hz), reminiscent of those observed in the spectrum of the mixture of disulfated 3β,21-dihydroxysteroids from the starfish *Euretaster insignis*[11] and assigned to protons at C-21. A narrow signal at δ 4.64 (3β-H) was also observed in all spectra. Solvolysis of each compound gave the corresponding diol and the analysis of the spectral properties was pursued on the desulfated materials (^{1}H-NMR in Table 2).

(20R) - 5α - *Cholestane* - 3α,21 - *diol* **4a**. The high resolution MS (*m/z* 404.3653) indicated a molecular formula $C_{27}H_{48}O_2$ corresponding to a C_{27} dihydroxylated steroid. The ^{1}H-NMR spectrum contained signals for four of the five Me groups typical of a sterol: δ 0.69 (s, 18-H), 0.79 (s, 19-H) and 0.88 (6H, d, J = 7 Hz, 26- and 27-H). Although one Me doublet was absent, a broad singlet at δ 3.70 was observed, which was ascribable[11,12] to a hydrolated C-21 group. The ^{1}H-NMR also contained a 1H narrow signal ($W_{1/2}$ = 9 Hz) at δ 4.06, typical for an equatorial proton. This suggested that the compound might be 3α-OH, 5α-stanol or 3β-OH, 5β-stanol (coprostanol). There was a good agreement between the observed values of the shifts of the angular Me groups (0.68 and 0.79) and the reported ones (0.68; 0.78) for 5α-cholestan-3α-ol.[13] The reported values (0.63; 0.93) for coprostanol (5β-cholestan-3β-ol)[14] were far off from our values. The ^{13}C-NMR spectrum (Table 1) confirmed that it was 5α-cholestane-3α,21-diol **4a**. The shifts of the nuclear carbons were virtually identical with those published for 5α-cholestan-3α-ol[5] except C-17 which is shifted to higher field from its assignment in the reference compound (50.5 vs 56.2 ppm), reflecting the presence of the C-21 OH group. The ^{13}C-NMR spectrum of the corresponding sulfated diol **4** is described in Table 1 and confirmed the presence of sulfate at C-3 and C-21. The stereochemistry at C-20 is suggested to be 20*R* (natural configuration) by comparison of ^{1}H-NMR spectral data of **4a** with those of (20*R*)- and (20*S*) - cholest - 5 - en - 3β,21 - diol.[12] The (20*R*)-isomer is reported[12] to exhibit a resonance at δ 3.70 as singlet ($C\underline{H}_2OH$), while the (20*S*)-isomer shows a multiplet centered at δ 3.62. Our value of 3.70 ppm (broad singlet) suggested that the configuration at C-20 is *R* in **4a**.

(20R) - *Cholest* - 5 - *en* - 3α,21 - *diol* **5a**. The high resolution MS showed a molecular ion at *m/z* 402.3493 ($C_{27}H_{46}O_2$) and an intense peak at *m/z* 384 (loss of H_2O), which suggested the presence of a Δ^5-double bond. This was confirmed by ^{1}H-NMR spectrum, which contained one olefinic proton signal at δ 5.43 (d, J = 5.5 Hz). The spectrum also showed the 3β-proton signal at δ 4.03 ($W_{1/2}$ = 8 Hz) and the 19-Me protons signal downfield shifted to 1.02 ppm relative to **4a**, as expected for the introduction of a Δ^5-double bond. The signal for the 21-hydroxymethylene protons remained almost unshifted at δ 3.71 (broad singlet).

(20R,22E) - *Cholesta* - 5,22 - *diene* - 3α,21 - *diol* **6a**. High resolution MS showed the molecular ion peak at *m/z* 400.3337 ($C_{27}H_{44}O_2$), corresponding to a C_{27} dihydroxysteroid with two degrees of unsaturation. The base peak in the MS appeared at *m/z* 271 (loss of the side chain +2H), consistent with the presence of a hydroxylated C_8 side chain containing one degree of unsaturation,[15] the other unsaturation being in the nucleus. The ^{1}H-NMR spectrum included two well separated olefinic protons at δ 5.10 (dd, J = 16.0 and 8.5 Hz, 22-H) and 5.52 (dt, J = 7.5 and 16.0 Hz, 23-H), which indicated a Δ^{22}-*trans*-double bond. The spectrum also displayed the Δ^5-3α-ol signals with a doublet (J = 5.5 Hz) at δ 5.43 (6-H) and a broad singlet at δ 4.03 ($W_{1/2}$ = 9 Hz, 3β-H). In the Me region two singlets at δ 0.74 and 1.02 were in agreement with the expected values for C-18 and C-19 angular Me protons. The remaining signals in the spectrum were observed at δ 0.90–0.89 (6H, overlapping doublets, J = 6.5 Hz) and 3.75 (2H, broad signal) and assigned to isopropyl methyl, and 21-hydroxymethylene protons, respect-

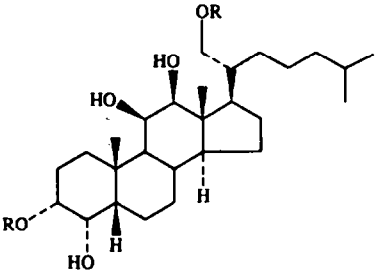
Table 3. Composition of disulfated 3α,21-dihydroxysteroids of *Ophioderma longicaudum*

Sterol	Mobility[a] HPLC (min)	(%)
(20*R*)-cholestane-3α,21-diol **4**	13.8	31
(20*R*)-cholest-5-ene-3α,21-diol **5**	11.8	39
(22*E*,20*R*)-cholesta-5,22-diene-3α,21-diol **6**[b]	10.6	9
(20*R*)-24-methylcholesta-5,24(28)-diene-3α,21-diol **7**	9.6	21

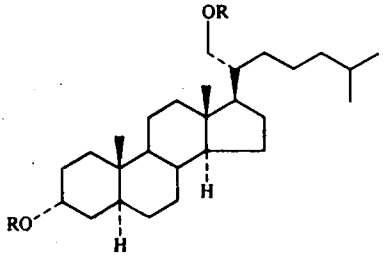
[a] On a μ-Bondapak C-18 column (7.8 mm × 30 cm) and methanol–water 45:55 as eluent.
[b] The stereochemistry at C-20 is probable.

ively. The 20*R* stereochemistry is tentatively assigned by analogy.

(20R)-24-*Methylcholesta*-5,24(28)-*dien*-3α,21-*diol* **7a**. The high resolution MS exhibited a molecular ion peak at *m/z* 414.3496 ($C_{28}H_{47}O_2$) corresponding to a C_{28} dihydroxylated steroid with two degrees of unsaturation, and showed loss of the side chain (+2H) (*m/z* 271) indicating[15] the additional oxygen function and one degree of unsaturation in the C_9 side chain, the other unsaturation being in the nucleus. The peak at *m/z* 330 (McLafferty rearrangement) suggested a side chain with a 24(28) double bond.[16] The ^{1}H-NMR spectrum included the expected olefinic protons at δ 4.75 and 4.70 and the isopropyl methyl doublet at δ 1.04. The absence of the absorption of the C-21 Me group, together with the presence of a 2H signal at δ 3.72 indicated that the compound possess a primary OH function at C-21. The spectrum displayed the Δ^5-3α-ol signals with a doublet ($J \simeq 5.5$ Hz) at δ 5.43 (6-H) and a broad singlet ($W_{1/2} = 9$ Hz) at δ 4.03 (3β-H). In the Me region two singlets at δ 0.72 and 1.02 are in agreement with the expected values for C-18 and C-19 Me protons. Thus the structure of the minor component of the mixture is (20*R*)-24-methylcholesta-5,24(28)-diene-3α,21-diol **7a**.



1 R = $SO_3^-Na^+$
2 R = H
3 R = *p*-bromo benzoyl



4	R = $SO_3^-Na^+$	**4a**	R = H
5	Δ^5, R = $SO_3^-Na^+$	**5a**	R = H
6	$\Delta^{5,22E}$, R = $SO_3^-Na^+$	**6a**	R = H
7	24-Me, $\Delta^{5,24(28)}$, R = $SO_3^-Na^+$	**7a**	R = H

The 3β-hydroxysterol sulfates. The ^{1}H-NMR spectrum of this mixture clearly indicated a mixture of sterol sulfates (7-line multiplet at δ 4.20). Solvolysis of this material to remove sulfate group gave a mixture of 3β-sterols which was partitioned by HPLC on a Partisil ODS-2 column into nine fractions, which were each analyzed by EI mass spectrometry and 250 MHz ^{1}H-NMR spectroscopy. All are known sterols and are listed in Table 3 together with their relative retention times (r.r.t.) in HPLC and their relative percentage. Cholesterol was the major constituent of the mixture. The assignment of the configuration of the 24-Me group in the side chain with Δ^{22}-double bond has been made by comparing the ^{1}H-NMR spectra of the two epimers **13** and **14** and in particular the chemical shift of the C-21 Me protons which occurs at higher field in the spectrum of the 24*S* epimer [**13**, δ 1.00] than in the spectrum of the 24*R* epimer [**14**, δ 1.03].[17,18] The 24*S* configuration assigned to **9** is based on the chemical shift of the C-21 Me protons (δ 1.00) identical with that of **13**.

The free 3β-hydroxysterols. Table 4 lists the nine sterols isolated from the ophiuroid *Ophioderma longicaudum*. All are known compounds and their identification was achieved with the aid of r.r.t.s in HPLC, MS and 250 MHz ^{1}H-NMR spectroscopy. The assignment of the 24*R* (α) configuration to the minor component **12** was based on the accurate analysis of its ^{1}H-NMR in the Me doublets region.[19]

The composition of the free sterol in Table 4 corresponds to that reported by Voogt[20] apart from some minor discrepancies. Compound **9** has been

Table 4. Composition of 3β-hydroxysteroids of *Ophioderma longicaudum*

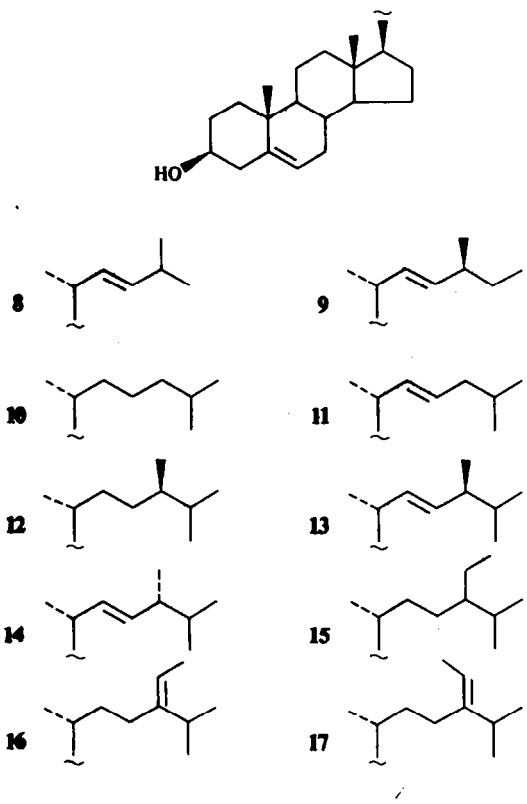
Sterol	Mobility[a] HPLC (r.r.t)	Free sterols (%)	Sulfated sterols (%)
(22*E*)-24-norcholesta-5,22-dien-3β-ol **8**	0.67	0.6	trace
(22*E*,24*S*)-27-norcholesta-5,22-dien-3β-ol **9**	0.72	7.5	1.5
(22*E*)-cholesta-5,22-dien-3β-ol **11**	0.74	7.7	4.3
(22*E*,24*S*)-24-methylcholesta-5,22-dien-3β-ol **13**	0.82	26.8	5.4
(22*E*,24*R*)-24-methylcholesta-5,22-dien-3β-ol **14**	0.87	2.9	2.5
(24*Z*)-24-ethylcholesta-5,24(28)-dien-3β-ol **16**[b]	0.92	3.9	1.0
(24*E*)-24-ethylcholesta-5,24(28)-dien-3β-ol **17**[b]	0.92	1.3	1.1
Cholest-5-en-3β-ol **10**	1.00	20.3	62.4
(24*R*)-24-methylcholest-5-en-3β-ol **12**	1.08	4.6	5.1
24-ethylcholest-5-en-3β-ol **15**	1.12	18.4	16.7

[a] On a Whatman Partisil M 9 10/50 ODS-2 column on pure methanol as eluent, standard cholesterol.

[b] Not resolved; their relative ratios have been determined by integration of the NMR signals for H-28, δ 5.13 (*Z*-isomer) and 5.18 (*E*-isomer).

regarded by the author as 22-*cis*-cholest-5,22-dien-3β-ol. This is misleading common in the past, when occelasterol **9** had not yet been isolated and characterized.[21] Furthermore, the author described the occurrence of 24-ethylcholest-5,22-dien-3β-ol, not detected in the present investigation, and he did not report the corresponding 22-saturated analog **15**.

This study adds further evidence that steryl sulfates are characteristic of the phylum of Echinodermata, even it is not yet known why echinoderms produce such relatively large amounts of steryl sulfate.



EXPERIMENTAL

The following instruments were used: NMR, Bruker WM-250; MS, Kratos MS 902 spectrometer equipped with kratos FAB source, the negative ion spectrum of **1** was obtained by dissolving the sample in a glycerol matrix and placing it on a Cu tip prior to bombardment with Xe atoms of 2–6 kV; AEI MS-30 apparatus at 70 eV for low resolution EI mass spectra; AEI MS-902 apparatus for high resolution EI mass spectra; HPLC, 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.

The ^{1}H-NMR spectra of free sterols were run in $CDCl_3$, those of sulfated sterols in CD_3OD. The chemical shifts are reported in ppm on the δ scale, J values are reported in hertz.

Extraction, sterol sulfates isolation and fractionation of the desulfated sterols. The animals (60 specimens, 0.7 kg fresh animals), collected in 1983 in the Bay of Naples, were extracted twice with MeOH (4 l), the slurry filtered, and the solvent removed to yield a deep-yellow semi-solid. The crude extract was partitioned between EtOAc and water to obtain an EtOAc extract which was dried on a rotary evaporator to yield a yellow gum (0.54 g). The aqueous fraction was then extracted with n-BuOH (3 × 500 ml) to obtain an n-BuOH extract which was dried on a rotary evaporator to yield a yellow solid.

The n-BuOH extract (3.7 g) was applied in two runs to a column (2 × 60 cm) of Sephadex LH-20 using MeOH as eluent. Fractions of 20 ml were collected and checked by TLC on silica gel n-BuOH–AcOH–water, 60:15:25. Fractions 36–45 contained free sterols (380 mg); fractions 46–72 contained 3β-hydroxysterol sulfates (207 mg); fractions 73–100 contained the mixture of 3α,21-dihydroxysteroid sulfates (45 mg) and finally fractions 102–116 contained the pentol disulfated **1**.

The crude 3β-hydroxysterol sulfates were further purified by DCCC (the solvent system was $CHCl_3$–MeOH–water, 7:13:8 in which the stationary phase consisted of the lower phase; ascending mode; flow 24 ml hr^{-1}; fractions of 8 ml were collected) to provide 40 mg of purified material. This material was solvolyzed in dioxane (0.5)–pyridine (0.5 ml) at 120° for 6 hr and after cooling, water (5 ml) was added and the soln was extracted twice with n-BuOH. The organic layer was washed with water and evaporated under vacuum to give the free sterols mixture (22 mg). The 3β-OH sterols were then fractionated by HPLC on an ODS-2 column (Whatman Partisil M9 10/50 ODS-2, 50 cm × 10 mm i.d.) with methanol into nine fractions which were each analyzed by mass spectrometry and ^{1}H-NMR spectroscopy. All fractions but one contained almost pure compounds, which were all known sterols (Table 4).

The crude 3α,21-dihydroxysteroid disulfates, were fractionated by HPLC on a μ-Bondapak C_{18} column (7.8 mm × 30 cm using 45% methanol in water) into four fractions which were each analyzed by ^{1}H-NMR.

(20R)-5α-*Cholestane*-3α,21-*diyl sodium sulfate* **4**. ^{1}H-NMR (CD_3OD) δ 0.75 (s, 18-H), 0.85 (s, 19-H), 0.91 (d, 6H, J = 7 Hz, 26- and 27-H), 3.97 (dd, 1H, J = 9.5 and 5.3 Hz, 21-H), 4.22 (dd, 1H, J = 9.5 and 3.8 Hz, 21-H), 4.64 (m, $W_{1/2}$ = 8 Hz, 1H, 3β-H). ^{13}C-NMR in Table 1.

(20R)-*Cholest-5-en*-3α,21-*diyl sodium sulfate* **5**. ^{1}H-NMR (CD_3OD) δ 0.78 (s, 18-H), 0.91 (d, 6H, J = 7 Hz, 26- and 27-H), 1.06 (s, 19-H), 3.97 (dd, 1H, J = 9.5 and 5.3 Hz, 21-H), 4.22 (dd, 1H, J = 9.5 and 3.8 Hz, 21-H), 4.64 (m, $W_{1/2}$ = 8 Hz, 1H, 3β-H), 5.34 (bd, J = 5.5 Hz, 6-H).

(22E,20R)-*Cholesta-5,22-diene*-3α,21-*diyl sodium sulfate* **6**. ^{1}H-NMR (CD_3OD) δ 0.78 (s, 18-H), 0.91 and 0.92 (each d, J = 7 Hz, H-26 and H-27), 1.06 (s, 19-H), 3.97 (dd, 1H, J = 9.5 and 5.3 Hz, 21-H), 4.22 (dd, 1H, J = 9.5 and 3.8 Hz, 21-H), 4.64 (m, $W_{1/2}$ = 8 Hz, 1H, 3β-H), 5.30 (dd, J = 16 and 10 Hz, 22-H), 5.34 (bd, J = 5.5 Hz, 6-H), 5.43 (dt, J = 16 and 7.5 Hz, 23-H).

(20R)-24-*Methylcholesta-5,24(28)-diene*-3α,21-*diyl sodium sulfate* **7**. ^{1}H-NMR (CD_3OD) δ 0.79 (s, 18-H), 1.06 (s, 19-H), 1.07 (d's, 6H, J = 7 Hz, 26- and 27-H), 3.97 (dd, 1H, J = 9.5 and 5.3 Hz, 21-H), 4.22 (dd, 1H, J = 9.5 and 3.8 Hz, 21-H), 4.64 (m, $W_{1/2}$ = 8 Hz, 1H, 3β-H), 4.70 and 4.75 (each s, 28-H), 5.34 (bd, J = 5.5 Hz, 6-H). Each compound was then solvolyzed as described above.

The fractions containing the pentol disulfate were further purified by HPLC on a Waters C_{18} μ-Bondapak column (7.8 mm × 30 cm) with MeOH–H_2O, 1:1 to provide 12 mg of **1**, which was crystallyzed from MeOH in the presence of $CHCl_3$, m.p. 192–195°, $[\alpha]_D$ = 10.8° (*c*, 0.9; MeOH); FAB–MS (−ve), *m/z* 649 (62), 633 (29), 531 (54), 513 (43), 369 (16), 289 (19), 233 (100); ^{1}H-NMR δ 0.90 (s, 18-H), 0.93 (d, J = 6.5 Hz, together 6H), 1.19 (s, 3H, 19-H), 3.28 (d, 1H, J = 3 Hz, 12-H), 3.99 (t, 1H, J = 3 Hz, 11-H), 4.08 (dd, 1H, J = 9, 6 Hz, 21-H), 4.19 (m, 3H, 3-, 4- and 21-H); ^{13}C-NMR in Table 1.

Compound **1** (mg 11) was solvolyzed as above to give the *pentol* **2**, which was purified by HPLC on an ODS column (Whatman Partisil ODS M9 10/25; 25 cm × 10 mm i.d.) with MeOH–H_2O, 78:22 (8 mg) and then crystallized from MeOH–hexane, m.p. 125–128°.

Physical data of the pentol **2** *and the* 3α,21-*dihydroxysteroids.* The ^{1}H-NMR spectra of the 3α,21-dihydroxysteroids are in Table 2; the ^{13}C-NMR spectra of the pentol **2** and the diol **4a** are in Table 1.

5β-*Cholestane*-3α,4α,11β,12β,21-*pentol* **2**, m.p. 125–128° (from MeOH–hexane); EI high reduction MS, *m/z* 434.3389 ($M^+ - H_2O$, $C_{27}H_{46}O_4$ requires M, 434.3395); EI MS, *m/z* 434 ($M^+ - H_2O$, 90%), 416 ($M^+ - 2H_2O$, 63), 404 ($M^+ - H_2O - CH_2O$, 50), 401 (416 − Me, 25), 386 (404 − H_2O, 100),

371 (20), 368 (404 − $2H_2O$, 22), 287 (M^+ − side chain − $2H_2O$, 60); ^{1}H-NMR, δ 0.88 (d, 6H, J = 6.5 Hz, 26- and 27-H), 0.90 (s, 3H, 18-H), 1.18 (s, 3H, 19-H), 3.32 (d, 1H, J = 3 Hz, 12-H), 3.60 (m from which emerged a dd with J = 12 and 5 Hz, 2H, 3- and 21-H), 3.90 (m from which emerged a dd with J = 12 and 3 Hz, 2H, 4- and 21-H), 4.08 (t, 1H, J = 3 Hz, 11-H).

(20R)-5α-*Cholestane*-3α,21-*diol* **4a**, EI high resolution MS, *m/z* 404.3653 (M^+, $C_{27}H_{48}O_2$ requires M, 404.3654); EI MS, *m/z* 404 (M^+, 72%), 389 (28), 386 (66), 371 (40), 279 (20), 273 (80), 265 (44), 233 (90), 215 (100).

(20R)-*Cholest*-5-*ene*-3α,21-*diol* **5a**, EI high resolution MS, *m/z* 402.3493 (M^+, $C_{27}H_{46}O_2$ requires M, 402.3497); EI MS, *m/z* 402 (M^+, 30), 384 (100), 369 (30), 351 (16), 273 (18), 255 (25), 213 (90).

(22E,20R)-*Cholesta*-5,22-*diene*-3α,21-*diol* **6a**, EI high resolution MS, *m/z* 400.3337 (M^+, $C_{27}H_{44}O_2$ requires M, 400.3341); EI MS, *m/z* 400 (M^+, 11%), 382 (20), 367 (8), 271 (100), 255 (12), 253 (12), 213 (10), 211 (5).

(20R)-24-*Methylcholesta*-5,24(28)-*diene*-3α,21-*diol* **7a**, EI high resolution MS, *m/z* 414.3496 (M^+, $C_{28}H_{46}O_2$ requires M, 414.3497); EI MS, *m/z* 414 (M^+, 10%), 400 (57), 396 (20), 385 (60), 382 (30), 367 (15), 330 (8), 273 (95), 271 (100), 213 (50), 211 (20).

p-*Bromobenzoylation of* **2**: *dibenzoate* **3**. The mixture of **2** (3 mg) and 6.5 mg (4.5 equiv) *p*-bromobenzoyl chloride in 0.2 ml of dry pyridine was heated at 60° overnight. The mixture was diluted with water and extracted with $CHCl_3$. The $CHCl_3$ extract was washed with 0.1 N $NaHCO_3$, water and dried. Evaporation afforded a residue which was purified by silica gel chromatography (a Pasteur pipet filled with a slurry of silica gel) with $CHCl_3$ and $CHCl_3$–MeOH, 98:2 to give in the $CHCl_3$ eluates the *dibenzoate* **3** (1.2 mg; R_f 0.8 in $CHCl_3$–MeOH, 95:5), which was characterized solely from the EI MS, *m/z* 616–618 (M^+ − $BrC_6H_4CO_2H$) and ^{1}H-NMR, after D_2O exchange, 0.87 (d, 6H, J = 6 Hz, 26- and 27-H), 0.89 (s, 3H, 18-H), 1.20 (s, 3H, 19-H), 3.30 (d, 1H, J = 3 Hz, 12-H), 4.03 (t, 1H, J = 3 Hz, 11-H), 4.14 (t, J = 3 Hz, 4-H), 4.50 (d, 2H, J = 5.5 Hz, 21-H), 4.98 (broad d, 1H, J = 10.5 Hz, 3-H), 7.58, 7.61, 7.88 and 7.91 (each d, 8H, J = 7.0 Hz, Ar—H).

Bis-acetonide formation of **2**. Pentol **2** (2.0 mg) in dry acetone (0.5 ml) containing *p*-TsOH (3 mg) was stirred at room temp for 4 hr. The mixture was neutralized with $BaCO_3$, centrifuged, and the supernatant evaporated to dryness to give the 3,4-,11,12-*bis*-acetonide, which was characterized solely from EI MS, *m/z* 532 (M^+, 1%), 517 ($M^+ - CH_3$, 4), 474 ($M^+ - CH_3COCH_3$, 8), 457 (12), 444 (3), 416 ($M^+ - 2CH_3COCH_3$, 22), 399 (100), 381 (100); and ^{1}H-NMR (CD_3OD), δ 0.81 (s, 3H, 18-H), 0.92 (d, 6H, J = 6.5 Hz, 26- and 27-H), 1.21 (s, 3H, 19-H), 1.28, 1.36, 1.48 and 1.50 (s, each 3H, —$OC(CH_3)_2$—O), 3.72 (2H, AB part of an ABX system, J_{AB} = 11 Hz, $J_{AX} = J_{BX}$ = 4 Hz, 21-H), 3.81 (d, 1H, J = 6 Hz, 12-H), 4.12–4.21 (m, 2H together, 3- and 4-H), 4.41 (t, J = 6 Hz, 11-H); the spectrum in $CDCl_3$ was less resolved.

Free sterols isolation and fractionation. The fractions 36–45 (175 mg) from chromatography on Sephadex LH-20 of the n-BuOH extract and the EtOAc extract (570 mg) were combined and chromatographed on a silica gel 70 g, 70–230 mesh, kieselgel 60 column in light petroleum, b.p. 40–70° and increasing amounts of ethylether to provide the crude sterol mixture (118 mg). This material was crystallized from EtOH and fractionated on ODS-2 column with MeOH (9 mg/100 μl $CHCl_3$ for each injection) to give nine fractions, which were each analyzed by mass spectrometry and ^{1}H-NMR spectroscopy.

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