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# SULFATED STEROIDS FROM PACIFIC BRITTLE STARS *OPHIOPHOLIS ACULEATA, OPHIURA SARSI,* AND *STEGOPHIURA BRACHIACTIS*

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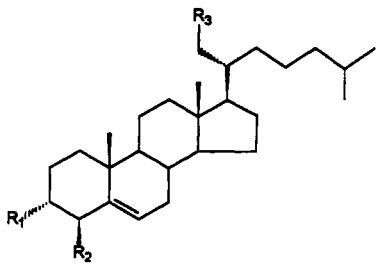
ABSTRACT.—Five new and three known sulfated steroidal polyols have been identified from the ophiuroid *Ophiopholis aculeata*. All of the new steroids possess the C-5, C-6 double bond and sulfoxy groups attached to C-3α and C-21. The structures of these new steroids were established from spectral data and chemical correlations with other steroids as (20*R*)-cholesta-5,25-diene-3α,4β,21-triol 3,21-disulfate [**1**], (20*R*)-cholesta-5,22*E*-diene-3α,4β,21-triol 3,21-disulfate [**2**], (20*R*)-24-nor-cholesta-5,22*E*-diene-2β,3α,21-triol 3,21-disulfate [**3**], (20*R*,24*R*)-cholesta-5,25-diene-2β,3α,21,24-tetrol 3,21-disulfate [**4**], and (20*R*,24*S*)-cholesta-5,25-diene-2β,3α,21,24-tetrol 3,21-disulfate [**5**]. The previously known compounds were identified as the 3,21-disulfates of (20*R*)-5α-cholestane-3α,21-diol [**6**], (20*R*)-cholest-5-ene-3α,21-diol [**7**], and (20*R*)-cholest-5-ene-3α,4β,21-triol [**8**]. Compounds **6** and **7** were also isolated from both *Ophiura sarsi* and *Stegophiura brachiactis*.

Sulfated steroidal polyols, although relatively uncommon in nature, have exhibited interesting biological activities, including potential anti-HIV properties, cytotoxic action, and inhibition of some enzymatic activities (1–3). These secondary metabolites have previously been found almost exclusively in sponges and starfish (1). Since the first publication on the isolation of physiologically active sulfated polyhydroxysteroids from ophiuroids (brittle stars) in 1985 (4), only a few papers on the same subject have been published (5,6), including our recent papers on steroids from the Far Eastern brittle stars *Ophiura sarsi* and *Ophiura leptoctenia* (7,8).

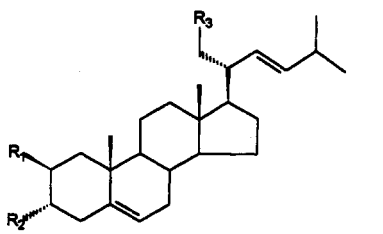
Herein we report the structural identification of new sulfated steroidal polyols [**1**–**5**] and the previously known (20*R*)-5α-cholestane-3α,21-diol disulfate [**6**], (20*R*)-cholest-5-ene-3α,21-diol disulfate [**7**], and (20*R*)-cholest-5-ene-3α,4β,21-triol 3,21-disulfate [**8**], from the Pacific brittle star *Ophiopholis aculeata* L. (phylum Echinodermata, class Ophiuroidea, family Ophiactidae), as well as the identification of the known steroid disulfates [**6** and **7**] from the Pacific brittle stars *Ophiura sarsi* Lutken and *Stegophiura brachiactis* H.L. Clark (class Ophiuroidea, family Ophiuridae).

## RESULTS AND DISCUSSION

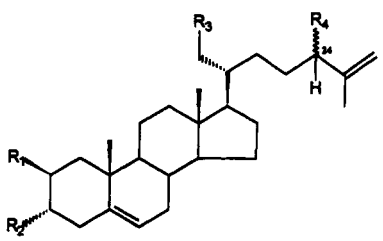
Fresh tissues of brittle stars, collected from a depth of about 200 m in different localities of the Okhotsk Sea, were extracted with EtOH, with the solvent removed, and the resulting aqueous suspensions extracted with *n*-BuOH. The extracts were chromatographed on Si gel and Polychrom-1 columns to give crude fractions of steroid sulfates. Corresponding subfractions were obtained by hplc on a prep. Zorbax ODS column, using EtOH-$H_2O$ systems (0:100→80:20). Pure samples of the sulfated polyhydroxysteroids **1**, **6**, **7**, and **8** as well as samples of steroids **2**–**5**, contaminated with other steroid impurities, were isolated from these subfractions after repeated hplc on a Silasorb SPH $C_{18}$ and Zorbax ODS analytical columns using 50% or 60% EtOH as eluent. Acetylation of **1**–**5** and **8** with $Ac_2O$ and pyridine, followed by solvolytic desulfation of the resulting products by refluxing in dioxane/pyridine mixtures and hplc purification, gave the monoacetates **1a**–**3a**, and **8a** and the diacetates **4a** and **5a**. Peracetates **1b**, **2b**, and **8b** were obtained on acetylation of the corresponding monoacetates in the usual manner and were purified by hplc.



**1** $R_1=R_3=OSO_3Na$; $R_2=OH$; $\Delta^{25}$
**1a** $R_1=R_3=OH$; $R_2=OAc$; $\Delta^{25}$
**1b** $R_1=R_2=R_3=OAc$; $\Delta^{25}$
**2** $R_1=R_3=OSO_3Na$; $R_2=OH$; $\Delta^{22}$
**2a** $R_1=R_3=OH$; $R_2=OAc$; $\Delta^{22}$
**2b** $R_1=R_2=R_3=OAc$; $\Delta^{22}$
**6** $R_1=R_3=OSO_3Na$; $R_2=H$; 5,6-dihydro
**7** $R_1=R_3=OSO_3Na$; $R_2=H$
**8** $R_1=R_3=OSO_3Na$; $R_2=OH$
**8a** $R_1=R_3=OH$; $R_2=OAc$
**8b** $R_1=R_2=R_3=OAc$



**3** $R_1=OH$; $R_2=R_3=OSO_3Na$
**3a** $R_1=OAc$; $R_2=R_3=OH$



**4** 24*R*; $R_1=R_4=OH$; $R_2=R_3=OSO_3Na$
**4a** 24*R*; $R_1=R_4=OAc$; $R_2=R_3=OH$
**5** 24*S*; $R_1=R_4=OH$; $R_2=R_3=OSO_3Na$
**5a** 24*S*; $R_1=R_4=OAc$; $R_2=R_3=OH$

Identification of the steroids **6–8** was carried out by comparison of their physical constants, chromatographic properties, and nmr spectra, and by comparison of the eims of the acetates **8a** and **8b** with those of the known sulfated steroids and derivatives (4,8).

The structure of (20*R*)-cholesta-5,25-diene-3α,4β,21-triol 3,21-disulfate [**1**] was assigned from examination of its $^{13}C$- and $^{1}H$-nmr spectra (Tables 1 and 2) and of the nmr and mass spectra of **1a** and by comparison of these spectra with those of **8** and **8a**. It was shown that **1** and **8** possess virtually identical nuclei with the same 3α,21-disulfoxy-4β-hydroxy- substitution, but differ from each other in that **1** has a C-25,C-26- double bond in the side-chain. The stereochemistry at C-20 in **1** was determined as 20*R* on the basis of the chemical shift and the form of the $CH_2$-21 signal in the $^{1}H$-nmr spectrum of **1a** (3.71 ppm, m, $W_{1/2}=11$ Hz) (4). The structure of **1** was confirmed by acetylation and hydrogenation of **1a** to give the previously described **8b**.

We were unable to purify the sulfated steroid **2** and its monoacetate **2a** from impurities of the isomeric **1** and **1a**, respectively. However, triacetate **2b** was isolated as a homogeneous compound. The $^{13}C$-nmr spectrum of **2** and the $^{1}H$-nmr spectrum of **2a** were obtained by subtraction of the spectra of the known compounds [**1** or **1a**] from the spectra of the mixtures.

The signals at 130.2 and 133.3 ppm in the $^{13}C$-nmr spectrum of **2** as well as the proton signals at 5.12 (dd, $J=14.0$ and 9.0 Hz) and 5.52 ppm (dt, $J=14.0$ and 6.5 Hz) in the $^{1}H$-nmr spectrum of **2a** indicated the presence of the C-22,C-23(*E*)- double bond (4–6). The structure of **2** as (20*R*)-cholesta-5,22*E*-diene-3α,4β,21-triol 3,21-disulfate was finally confirmed by double resonance nmr experiments on **2b**, indicating the sequence $(CH_3)_2CHCH_2CH=CHCH(R)CH_2OAc$ in the side-chain (R is a steroidal

TABLE 1. $^{13}$C-Nmr Spectra of Steroids **1–5**, **8**, and Derivatives.[a]

| Carbon | Compound | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | **1**[b] | **1a**[c] | **2**[d] | **3**[d] | **3a**[c] | **4a+5a**[c] | | **8**[b] | **8**[d] |
| 1 | 33.7 | 31.8 | 33.6 | 40.8 | 37.9 | 37.8 | | 33.7 | 33.6 |
| 2 | 22.5 | 24.7 | 23.2 | 70.6 | 72.7 | 72.7 | | 23.0 | 23.1 |
| 3 | 78.7 | 78.7 | 78.6 | 79.2 | 68.1 | 68.1 | | 78.9 | 79.0 |
| 4 | 76.5 | 68.1 | 76.0 | 33.8 | 35.7 | 35.7 | | 76.5 | 75.8 |
| 5 | 142.1 | 137.0 | 143.0 | 139.4 | 137.5 | 137.4 | | 142.2 | 142.9 |
| 6 | 129.4 | 132.9 | 127.5 | 121.7 | 124.6 | 124.7 | | 129.5 | 127.5 |
| 7 | 33.2 | 32.0 | 32.4 | 31.7 | 31.9 | 31.8 | | 33.2 | 32.6 |
| 8 | 33.1 | 32.2 | 32.4 | 31.5 | 31.6 | 31.6 | | 33.2 | 32.5 |
| 9 | 51.7 | 50.5 | 50.7 | 49.8 | 50.8 | 50.7 | | 51.8 | 51.0 |
| 10 | 37.3 | 36.6 | 37.0 | 36.8 | 36.9 | 36.8 | | 37.3 | 37.1 |
| 11 | 21.4 | 20.5 | 20.9 | 21.0 | 21.0 | 21.0 | | 21.5 | 21.0 |
| 12 | 40.3 | 39.3 | 39.4 | 39.0 | 39.3 | 39.3 | | 40.3 | 39.6 |
| 13 | 43.4 | 42.3 | 42.5 | 42.3 | 42.3 | 42.2 | | 43.4 | 42.9 |
| 14 | 58.3 | 56.9 | 57.4 | 57.0 | 56.8 | 56.6 | | 58.4 | 57.6 |
| 15 | 25.1 | 24.2 | 24.6 | 24.2 | 24.3 | 24.2 | | 25.1 | 24.8 |
| 16 | 28.4 | 27.2 | 28.1 | 27.5 | 27.7 | 27.6 | | 28.4 | 28.0 |
| 17 | 52.0 | 50.7 | 51.1 | 50.7 | 51.4 | 50.5 | (50.6) | 52.1 | 51.4 |
| 18 | 12.6 | 12.3 | 12.9 | 12.6 | 12.5 | 12.2 | | 12.7 | 12.7 |
| 19 | 21.6 | 20.6 | 21.7 | 22.7 | 21.4 | 21.3 | | 21.7 | 21.7 |
| 20 | 41.1 | 42.6 | 45.3 | 44.6 | 48.5 | 47.2 | | 41.2 | 40.6 |
| 21 | 69.8 | 63.1 | 71.0 | 68.7 | 64.3 | 63.0 | | 70.1 | 69.0 |
| 22 | 30.5 | 29.4 | 130.2 | 128.5 | 127.9 | 25.2 | (25.3) | 31.1 | 30.9 |
| 23 | 25.1 | 24.7 | 133.3 | 138.1 | 141.1 | 29.4 | (29.5) | 25.0 | 24.8 |
| 24 | 39.2 | 38.4 | 39.4 | — | — | 77.1 | (77.9) | 40.7 | 40.0 |
| 25 | 146.9 | 146.3 | 28.9 | 31.0 | 31.2 | 143.0 | (143.3) | 29.1 | 29.4 |
| 26 | 110.3 | 109.7 | 22.7 | 22.3 | 22.7 | 112.4 | (113.0) | 23.2 | 23.3 |
| 27 | 23.2 | 22.4 | 22.8 | 22.4 | 22.8 | 18.1 | (18.3) | 23.2 | 23.3 |
| OAc | | 21.5 | | | 21.4 | 21.2 | (21.3) | | |
| | | 169.5 | | | 169.5 | 169.9 | (170.1) | | 21.4 |
| | | | | | | | | | 169.5 |

[a]Assignments made by comparison with known compounds.
[b]Spectrum run in $CD_3OD$.
[c]Spectrum run in $CDCl_3$.
[d]Spectrum run in $C_5D_5N$.

nucleus), and by conversion of **2b** after catalytic hydrogenation into the previously described **8b** (8).

The sulfated steroid **3** was obtained only with an impurity of **8**. However its monoacetate derivative **3a** was purified as a homogeneous compound. Comparison of the nmr spectra of **3** (Tables 1 and 2) with published data for related compounds (20*R*)-cholest-5-ene-2β,3α,21-triol trisulfate (8), (25ξ)-methylcholest-5-ene-2β,3α,26-triol trisulfate (5), and 24ξ,25-dimethylcholest-5-ene-2β,3α-diol disulfate (9) indicated the presence of a C-5,C-6- double bond, a free 2β-OH and sulfoxy groups at C-3α and C-21. The final structure of (20*R*)-24-nor-cholesta-5,22*R*-diene-2β,3α,21-triol 3,21-disulfate [**3**] was assigned from $^1$H-nmr double resonance and decoupling experiments on **3a**, as previously described for 24ξ,25-dimethylcholest-5-ene-2β,3α-diol disulfate (9). These experiments revealed not only the C-5,C-6- double bond and the 2β,3α-positions of the acetoxy and hydroxy groups, respectively, but the sequence $(CH_3)_2CHCH{=}CHCH(R)CH_2OH$ (R is a steroidal nucleus) in the side-chain. The configuration at C-20 in **3a** was proposed as *R* on the basis of the similarity of chemical shifts and the form of the $CH_2$-21 signals in the $^1$H-nmr spectra of **3a** and **2a** (Table 2).

Sulfated steroids **4** and **5** were isolated by hplc as an inseparable mixture. Acetylation of the mixture followed by solvolysis also gave an inseparable mixture of **4a** and **5a**. The identity of the polycyclic moieties in **3a**, **4a**, and **5a** was established by comparison

TABLE 2. $^1$H-Nmr Spectral Data ($W_{1/2}$ and $J$ in Hz) of Steroids **1–3** and **8**, and Steroid Acetates **1a**, **1b**, **2a**, **2b**, **3a**, **4a**, and **5a**, **8a** and **8b** (250 MHz).

| Proton | Compound | | | | | |
|---|---|---|---|---|---|---|
| | **1**[a] | **1a**[b] | **1b**[b] | **2a**[b] | **2b**[b] | **3**[a] |
| H-2 | | | | | | 4.10 m ($W_{1/2}$=6) |
| H-3 | 4.52 m ($W_{1/2}$=6) | 3.90 m ($W_{1/2}$=5) | 4.88 m ($W_{1/2}$=4.5) | 3.90 m ($W_{1/2}$=5) | 4.88 m ($W_{1/2}$=4.5) | 4.46 m ($W_{1/2}$=7) |
| H-4 | 4.17 m ($W_{1/2}$=6) | 5.09 m ($W_{1/2}$=6) | 5.15 m ($W_{1/2}$=6) | 5.09 m ($W_{1/2}$=6) | 5.15 m ($W_{1/2}$=6) | 2.85 m (ax) |
| H-6 | 5.62 m | 5.87 m | 5.80 m | 5.87 m | 5.80 m | 5.33 m |
| H-18 | 0.76 s | 0.70 s | 0.70 s | 0.72 s | 0.73 s | 0.76 s |
| H-19 | 1.21 s | 1.13 s | 1.11 s | 1.13 s | 1.11 s | 1.17 s |
| H-21 | 4.00 dd (1H) ($J$=9.0 and 5.0)<br>4.19 m (1H) | 3.71 m ($W_{1/2}$=11) | 4.01 dd (1H) ($J$=10.5 and 5.5)<br>4.20 dd (1H) ($J$=10.5 and 3.0) | 3.22 t (1H) ($J$=10.0)<br>3.78 m (1H) | 3.92 dd (1H) ($J$=10.0 and 8.0)<br>4.20 dd (1H) ($J$=10.0 and 4.0) | 3.91 dd (1H) ($J$=9.0 and 7.0)<br>4.16 dd (1H) ($J$=9.0 and 4.0) |
| H-22,23 | | | | 5.52 dt (1H) ($J$=14.0 and 6.5)<br>5.12 dd (1H) ($J$=14.0 and 9.0) | 5.37 dt (1H) ($J$=14.0 and 7.0)<br>5.15 dd (1H) ($J$=14.0 9.0) | 5.42 dd (1H) ($J$=15.0 and 6.0)<br>5.26 dd (1H) ($J$=15.0 and 9.0) |
| H-24 | | | | | | |
| H-26 | 4.68 s (2H) | 4.67 br s (1H)<br>4.70 br s (1H) | 4.66 br s (1H)<br>4.70 br s (1H) | 0.88 d (3H) ($J$=6.3) | 0.87 d (3H) ($J$=6.3) | 0.96 d (3H) ($J$=6.5) |
| H-27 | 1.72 s (3H) | 1.72 s (3H) | 1.71 s (3H) | 0.88 d (3H) ($J$=6.3) | 0.87 d (3H) ($J$=6.3) | 0.96 d (3H) ($J$=6.5) |
| OAc | | 2.04 s | 2.02 s<br>2.03 s<br>2.04 s | 2.04 s | 2.02 s<br>2.03 s<br>2.04 s | |

[a]Spectrum run in $CD_3OD$.
[b]Spectrum run in $CDCl_3$.
[c]Spectrum run in $C_5D_5N$.
[d]Includes $CH_3$-27 signal.

of the corresponding $^{13}$C-nmr spectra (Table 1). Signals of side-chain carbons (besides the C-20 and C-21 signals) in the $^{13}$C-nmr spectra of the sum of **4a** and **5a** looked like "doublets," with differences in the chemical shifts ranging from 0.1–0.8 ppm, which are characteristic of epimeric steroids (10). Groups of signals at 112.4, 113.0 ppm and 143.0, 143.3 ppm in the $^{13}$C-nmr spectrum of a mixture of **4a** and **5a** were indicative of the presence of the C-25,C-26- double bond (6). The singlet of $CH_3$-27 at 1.73 ppm in the corresponding $^1$H-nmr spectrum confirmed the presence of this double bond, and the double doublets of $CH_2$-21 centered at 3.65 and 3.74 ppm proved the 20*R*-configuration in **4a** and **5a**. The "doublet" with components at 77.1 and 77.9 ppm in the $^{13}$C-nmr spectrum led to the assumption of the presence of a hydroxyl group at C-24. The downfield shift of the CH′-26 and CH″-26 signals in comparison with the corresponding $^{13}$C-nmr signals of **1a** (Table 2), the triplet character of the $^1$H-nmr signal at 5.14 ppm (CH-24), and nOe difference nmr experiments with the signals at 4.95 ppm (CH″-26) and at 1.73 ppm ($CH_3$-27) being enhanced after irradiation of the triplet at 5.14 ppm, established the structures (20*R*,24*R*)- and (20*R*,24*S*)-cholesta-5,25-diene-2β,3α,21,24-tetrol 3,21-disulfate, respectively, for **4** and **5**. The appearance of two approximately equal $CH_3$ signals at 2.05 and 2.06 ppm for acetoxyl groups in the spectrum of the mixture of **4a** and **5a** indicated that equal quantities of the 24*R*- and 24*S*- epimers were present.

Sulfated steroidal polyols from the Mediterranean brittle star *Ophioderma longicaudum* (1), from *Ophiocoma dentata, Ophiarthrum elegans,* and *Ophiorachna incrassata*, collected off

TABLE 2. Continued.

| Compound | | | | | | |
|---|---|---|---|---|---|---|
| **3**[c] | **3a**[b] | **4a+5a**[b] | **8**[a] | **8**[c] | **8a**[b] | **8b**[b] |
| 4.85 m ($W_{1/2}$=6) | 4.91 m ($W_{1/2}$=6) | 4.90 m | | | | |
| 5.47 m ($W_{1/2}$=6) | 3.85 m ($W_{1/2}$=6) | 3.85 m ($W_{1/2}$=6) | 4.52 m ($W_{1/2}$=6) | 5.49 m | 3.90 m ($W_{1/2}$=5) | 4.88 m ($W_{1/2}$=4.5) |
| 3.30 m (ax) | 2.85 m (ax) | 2.85 m (ax) | 4.17 m ($W_{1/2}$=6) | 4.94 m | 5.09 m ($W_{1/2}$=6) | 5.15 m ($W_{1/2}$=5) |
| 5.38 m | 5.48 m | 5.49 m | 5.62 m | 5.69 m | 5.87 m | 5.81 m |
| 0.70 s | 0.71 s | 0.70 s | 0.76 s | 0.70 s | 0.70 s | 0.70 s |
| 1.43 s | 1.11 s | 1.11 s | 1.21 s | 1.50 s | 1.13 s | 1.11 s |
| 4.38 dd (1H) ($J$=9.0 and 6.5) | 3.21 t (1H) ($J$=10.0) | 3.65 dd (1H) ($J$=11.0 and 4.0) | 4.00 dd (1H) ($J$=9.0 and 5.0) | 4.43 m (1H) | 3.70 m ($W_{1/2}$=8) | 4.01 dd (1H) ($J$=11.0 and 5.5) |
| 4.64 dd (1H) ($J$=9.0 and 4.0) | 3.78 dd (1H) ($J$=10.0 and 4.0) | 3.74 dd (1H) ($J$=11.0 and 3.0) | 4.19 m (1H) | 4.64 m (1H) | | 4.18 dd (1H) ($J$=11.0 and 3.3) |
| 5.48 m | 5.53 dd (1H) ($J$=14.5 and 6.5) | | | | | |
| | 5.07 dd (1H) ($J$=14.5 and 8.5) | | | | | |
| | | 5.14 t ($J$=6.5) | | | | |
| 0.95 d (3H) ($J$=6.5) | 0.99 d (3H) ($J$=6.5) | 4.90 m (1H) | 0.89 d[d] ($J$=7.0) | 0.85 d[d] ($J$=7.0) | 0.87 d[d] ($J$=6.5) | 0.87 d[d] ($J$=6.5) |
| 0.95 d (3H) ($J$=6.5) | 0.99 d (3H) ($J$=6.5) | 4.95 br s (1H) | | | | |
| | | 1.73 s (3H) | | | | |
| | 2.07 s | 2.05 s | | | 2.04 s | 2.02 s |
| | | 2.06 s | | | | 2.03 s |
| | | 2.07 s | | | | 2.04 s |

Noumea (New Caledonia) (5), as well as from *Ophiolepis superba,* collected at Okinawa, Japan (6), consist mainly of 3α,21-disulfates with saturated polycyclic moieties, having either a trans- or a cis-A/B ring fusion. Steroids which have been identified by us from Northern Pacific species of brittle stars all contain sulfates at the C-3α- and C-21 positions, and also have a C-5,C-6- double bond; some of them additionally have a C-25,C-26- double bond. These double bonds have been found only sporadically in metabolites of the Mediterranean and tropical brittle stars (4–6). Hydroxylation at C-24, commonly encountered among polyhydroxysteroids from starfish (1), was discovered for the first time from brittle star steroids after our identification of **4** and **5**.

## EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—The $^1$H- and $^{13}$C-nmr spectra were recorded on a Bruker WM-250 spectrometer at 250 and 62.9 MHz, with TMS as internal standard. Mps were determined on a Boethius apparatus. Optical rotations were determined on a Perkin-Elmer 141 apparatus. Eims were measured on an LKB-9000S spectrometer (ionizing energy 70 eV). Negative-ion fabms were measured on an LKB 9091 spectrometer. Hplc separations were conducted with a Du Pont 8800 chromatograph equipped with an refractive index detector. The column lplc was performed using Si gel L (Chemapol, former Czechoslovakia) and Polychrome-1 (Olayna, Latvia).

ANIMAL MATERIAL.—Specimens of ophiuroids were collected on the seventh scientific cruise of the research vessel "Akademic Oparin" in July 1988, using Sigsby trawl equipment (2×0.4 m). *Ophiopholis aculeata* was collected from Kashevarov's bank (−200 m), and *Ophiura sarsi* and *Stegophiura brachiactis* from near Paramushir Island (−225 m). Voucher specimens are on deposit in the marine specimen collection of the Zoological Institute, Russian Academy of Sciences, St. Petersburg, Russia.

EXTRACTION AND ISOLATION.—Fresh animals were immediately extracted with EtOH. Extracts of *O. aculeata* (20 kg, wet wt), *O. sarsi* (20 kg, wet wt), *S. brachiactis* (0.5 kg, wet wt) were concentrated *in vacuo* at 50° with periodical additions of *n*-BuOH. The remaining aqueous suspensions were extracted with *n*-BuOH. Dark oils, obtained after evaporation of corresponding *n*-BuOH extracts, were chromatographed on columns with Si gel L, 100/400 μm, using the following solvent systems: $CHCl_3$ (A); $CHCl_3$-EtOH (4:1) (B); $CHCl_3$-EtOH (1:1) (C); and $CHCl_3$-EtOH-$H_2O$ (100:100:17) (D). Eluates with solvent system D were evaporated and repeatedly chromatographed on columns with Si gel L, 40–100 μm, using solvent systems B, C, and D. Eluates with system D were evaporated *in vacuo*. The resulting crude fractions of steroidal sulfates were purified by hplc on a preparative Zorbax-ODS column, 21.2 mm×25 cm, with elution by $H_2O$, 10% EtOH, 20% EtOH, 30% EtOH, 40% EtOH, 50% EtOH, 60% EtOH, 70% EtOH, and 80% EtOH (400 ml each). Subfraction 1 (40% EtOH) contained **1–3** and **8**. Subfraction 2 (30% EtOH) contained **4** and **5**. These components were obtained only from *O. aculeata*. Subfraction 3 (50% EtOH) contained **6** and **7**. A subfraction containing these compounds was obtained from all three species investigated.

*Hplc separation of subfractions* **1–3**.—Subfraction 1 (260 mg) was subjected to hplc on a Silasorb SPH $C_{18}$ column, 4 mm×25 cm, with 50% EtOH as eluent at a flow rate of 0.8 ml/min. As a result steroid **8** (54 mg) was obtained along with mixtures of **1** and **2** (58 mg), and **3** and **8** (67 mg). Steroid **1** (28 mg) was purified by hplc of **1** and **2** on a Zorbax CN column, 4.6 mm×25 cm, with 30% EtOH as eluent at a flow rate of 1 ml/min. Repeated hplc gave 20 mg of **2** (70% purity) and 35 mg of **3** (70% purity). Hplc of subfraction 2 (88 mg) on a Silasorb SPH $C_{18}$ column, 4 mm×25 cm, with 40% EtOH as eluent at a flow rate of 0.8 ml/min, yielded a (1:1) mixture of **4** and **5** (72 mg). Subfractions 3 from *O. aculeata* (58 mg), *O. sarsi* (43 mg), and *S. brachiactis* (35 mg) were individually separated by hplc on a Zorbax ODS column, 4.6 mm×25 cm with 60% EtOH as eluent, at a flow rate of 1 ml/min, to give **6** and **7** (20 and 18 mg in the first case, 15 and 13 mg in the second case, and 10 and 12 mg in the third case, respectively). The mp and $[\alpha]^{20}D$ values for the known **6** and **7** as well as the $^{13}C$-nmr spectrum of **7** are reported here for the first time.

*(20R)-Cholesta-5,25-diene-3α,4β,21-triol 3,21-disulfate* [**1**].—Colorless amorphous solid; $[\alpha]^{20}D$ −40° ($c$=0.1, $H_2O$); $^{13}C$- and $^1H$-nmr spectra, see Tables 1 and 2, respectively; fabms *m/z* $[M-Na]^-$ 597, $[M-2Na+H]^-$ 575.

*(20R)-5α-Cholestane-3α,21-diol disulfate* [**6**].—Colorless crystals (MeOH); mp 200–202°; $[\alpha]^{20}D$ +10° ($c$=0.1, EtOH); $^{13}C$- and $^1H$-nmr spectra are identical with those reported (4); fabms *m/z* $[M-Na]^-$ 585, $[M-2Na+H]^-$ 563, $[M-NaSO_3+H-Na]^-$ 483, $[M-NaHCO_4-Na]^-$ 465.

*(20R)-Cholest-5-ene-3α,21-diol disulfate* [**7**].—Colorless crystals (MeOH); mp 176–178°; $[\alpha]^{20}D$ −11° ($c$=0.1, EtOH); $^{13}C$ nmr ($CD_3OD$) δ 34.3 (C-1), 27.8 (C-2), 76.9 (C-3), 38.3 (C-4), 139.6 (C-5), 123.1 (C-6), 33.3 (C-7), 33.1 (C-8), 51.5 (C-9), 38.0 (C-10), 21.9 (C-11), 40.3 (C-12), 43.4 (C-13), 58.2 (C-14), 25.2 (C-15), 29.1 (C-16), 52.0 (C-17), 12.6 (C-18), 19.5 (C-19), 41.3 (C-20), 69.7 (C-21), 31.1 (C-22), 24.8 (C-23), 40.7 (C-24), 28.5 (C-25), 23.0 (C-26), 23.2 (C-27). These assignments were made by comparison with model compounds. The $^1H$-nmr spectrum was identical with that reported by Riccio *et al.* (4); fabms *m/z* $[M-Na]^-$ 583, $[M-2Na+H]^-$ 561, $[M-NaSO_3+H-Na]^-$ 481, $[M-NaHSO_4-Na]^-$ 463.

*(20R)-Cholest-5-ene-3α,4β,21-triol 3,21-disulfate* [**8**].—Colorless crystals (MeOH); mp 190–191°; $[\alpha]^{20}D$ −17° ($c$=0.1, MeOH) [lit. (8) mp 189–190.5°; $[\alpha]^{20}D$ −16° ($c$=0.1, MeOH)]; $^{13}C$- and $^1H$-nmr spectra, see Tables 1 and 2, respectively; fabms *m/z* $[M-Na]^-$ 599, $[M-2Na+H]^-$ 577.

The acetylation of the steroid isolates with $Ac_2O$ and pyridine followed by solvolysis with dioxane-pyridine (1:1) afforded the various monoacetates, which were further purified by hplc. Acetate **1a** (14 mg) was obtained from a Zorbax CN column, 4.6 mm×25 cm, with 65% MeOH as eluent at a flow rate of 1 ml/min; acetates **3a** (10 mg) and **8a** (12 mg) were obtained from a Zorbax ODS column, 4.6 mm×25 cm, with 80% EtOH as eluent at a flow rate of 1 ml/min; a mixture of the steroidal diacetates **4a** and **5a** (30 mg) was obtained after purification on a Silasorb SPH $C_{18}$ column, 4.6 mm×25 cm (67% EtOH as eluent, flow rate 1 ml/min).

The mixture of the steroidal monoacetates **1a** and **2a** (14 mg) was acetylated with $Ac_2O$ and pyridine to give triacetates **1b** (5 mg) and **2b** (7 mg), which were separated by hplc on a Zorbax ODS column, 4.6 mm×25 cm (85% EtOH as eluent, flow rate 1 ml/min).

*(20R)-Cholesta-5,25-diene-3α,4β,21-triol 4-acetate* [**1a**].—Colorless amorphous solid; $[\alpha]^{20}D$ −75° ($c$=0.2, MeOH); $^{13}C$- and $^1H$-nmr spectra, see Tables 1 and 2, respectively; eims *m/z* $[M]^+$ 458 (5), $[M-H_2O]^+$ 440 (15), $[M-AcOH]^+$ 398 (100), $[398-CH_3]^+$ 383 (7), $[398-H_2O]^+$ 380 (13), $[380-CH_3]^+$ 365 (6), $[398-R-2H]^+$ 269 (46), 229 (22), where R is the side-chain.

*(20R)-24-Nor-cholesta-5,22E-diene-2β,3α,21-triol 2-acetate* [**3a**].—Colorless amorphous substance; $[\alpha]^{20}D$ −27° ($c$=0.2, EtOH); $^{13}C$- and $^1H$-nmr spectra, see Tables 1 and 2, respectively; eims *m/z* $[M-H_2O]^+$ 426 (13), $[M-AcOH]^+$ 384 (33), $[384-H_2O]^+$ 366 (15), $[384-CH_2OH]^+$ 353 (29), $[366-CH_2OH]^+$

335 (11), $[M-R-2H]^+$ 329 (68), 313 $[M-R-H_2O]^+$ (75), $[329-AcOH]^+$ 269 (100); $[313-AcOH]^+$ 253 (26), where R is the side-chain.

A mixture of the epimers (20*R*,24*R*,*S*)-cholesta-5,25-dien-2β,3α,21,24-tetrol 2,24-diacetates [**4a** and **5a**], colorless amorphous residue, exhibited: $[\alpha]^{20}D$ −18° (*c*=0.1, MeOH); $^{13}C$- and $^1H$-nmr spectra, see Tables 1 and 2, respectively; eims *m/z* $[M-H_2O]^+$ 498 (11), $[M-AcOH]^+$ 456 (100), $[498-AcOH]^+$ 438 (40), $[456-AcOH]^+$ 396 (93), $[396-H_2O]^+$ 378 (37), $[378-CH_3]^+$ 363 (27), $[M-R-2H]^+$ 329 (80), $[329-AcOH]^+$ 269 (80), 213 (40), 211 (57), where R is the side-chain.

*(20R)-Cholest-5-ene-3α,4β,21-triol 4-acetate* [**8a**].—Colorless crystals (MeOH); mp 145–146°; $[\alpha]^{20}D$ −7° (*c*=0.2, $CHCl_3$) [lit. (8) mp 145–147°; $[\alpha]^{20}D$ −6° (*c*=0.2, $CHCl_3$)]; $^{13}C$- and $^1H$-nmr spectra, see Tables 1 and 2, respectively; eims *m/z* $[M-H_2O]^+$ 442 (21), $[M-AcOH]^+$ 400 (100), $[400-CH_3]^+$ 385 (13), $[400-H_2O]^+$ 382 (17), $[382-CH_3]^+$ 367 (17), $[M-R]^+$ 331 (8), $[M-R-2H]^+$ 329 (12), $[M-R-H_2O]^+$ 313 (60), $[M-R-AcOH]^+$ 271 (29), $[271-2H]^+$ 269 (27), 263 (12), 255 (15), 253 (13), 244 (27), 229 (54), 227 (13), 223 (10), 215 (15), 213 (31), 211 (21), where R is the side-chain.

*(20R)-Cholesta-5,25-diene-3α,4β,21-triol 3,4,21-triacetate* [**1b**].—Colorless amorphous substance; $[\alpha]^{20}D$ −103° (*c*=0.2, EtOH); $^1H$-nmr spectrum, see Table 2; eims *m/z* $[M-AcOH]^+$ 482 (21), $[482-CH_2CO]^+$ 440 (100), $[482-AcOH]^+$ 422 (18), $[422-CH_2CO]^+$ 380 (12), $[380-CH_3]^+$ 365 (7), $[422-AcOH]^+$ 362 (5), 341 (11), $[M-R-AcOH]^+$ 313 (96), $[313-CH_2CO-CH_3]^+$ 256 (70), 213 (47), 211 (21), where R is the side-chain.

*(20R)-Cholesta-5,22-diene-3α,4β,21-triol 3,4,21-triacetate* [**2b**].—Colorless amorphous substance; $[\alpha]^{20}D$ −125° (*c*=0.2, EtOH); $^1H$-nmr spectrum, see Table 2; eims *m/z* $[M-AcOH]^+$ 482 (60), $[482-CH_2CO]^+$ 440 (100), $[482-AcOH]^+$ 422 (32), $[422-CH_2CO]^+$ 380 (14), $[422-AcOH]^+$ 362 (9), $[482-R-2H]^+$ 311 (4), $[440-R-2H]^+$ 269 (7), where R is the side-chain.

HYDROGENATION OF THE TRIACETATES **1b** AND **2b**.—The steroidal triacetates **1b** and **2b** were hydrogenated in MeOH, using Adams catalyst, for 5 h. As a result, the known steroidal triacetate **8b** was obtained from both **1b** and **2b**.

*(20R)-Cholest-5-ene-3α,4β,21-triol 3,4,21-triacetate* [**8b**].—Colorless crystals (MeOH); mp 82–83°; $[\alpha]^{20}D$ −29° (*c*=0.1, $CHCl_3$) [lit. (8) mp 80–82°; $[\alpha]^{20}D$ −30° (*c*=0.2, $CHCl_3$)]; $^1H$-nmr spectrum, see Table 2; eims *m/z* $[M-AcOH]^+$ 484 (15), $[484-CH_2CO]^+$ 442 (100), $[484-AcOH]^+$ 424 (4), $[442-AcOH]^+$ 382 (3), $[382-CH_3]^+$ 367 (4), 341 (13), $[M-R-AcOH]^+$ 313 (13); $[313-CH_2CO-H]^+$ 270 (6), $[M-R-2AcOH]^+$ 253 (6), 223 (19); 211 (6); 205 (15), where R is the side-chain.

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