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FREE AND SULFATED STEROLS OF TWO FAR-EAST *Leptasterias* STARFISH

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NMR spectroscopy, capillary GLC, and GLC—MS are used to study the composition of free and sulfated sterols from the far-east starfish Leptasterias alaskensis asiatica *(Fischer) and* L. fisheri *(Djakonov). The total free sterols of both species are shown to have similar qualitative and quantitative compositions and contain mainly* Δ^7*-sterols. Sterol sulfate fractions contain cholesterol sulfate as the main component but differ in the ratios of* Δ^5*:*Δ^0*:*Δ^7*-sterol derivatives. Possible reasons for these differences are discussed. A new steroid,* 3β*-hydroxycholest-5-en-7-one sulfate, was isolated.*

Key words: *Leptasterias alaskensis asiatica, L. fisheri*, free sterols, sulfated sterols, 3β-hydroxycholest-5-en-7-one sulfate, new steroid.

The composition, biogenesis, and metabolism of steroids in echinoderms, especially those belonging to the Asteroidea (starfish) and Holothuroidea (sea cucumber) classes are still insufficiently studied [1-5]. These echinoderms, in contrast with most other animals, contain complex compositions of free sterols in which, as a rule, Δ^7-sterols and stanols predominate whereas Δ^5-sterols are minor components or absent [2, 5-11]. It was shown that only 5α-cholest-7-en-3β-ol is biosynthesized de novo in starfish and, apparently, in certain sea cucumbers whereas other sterols are products from biotransformations of Δ^5-sterols [2, 5, 10-17].

Furthermore, a portion of ingested sterols is transformed into sulfates at the same time as the biotransformations occur in these animals [5, 18, 19]. As a rule, cholesterol is converted to the sulfate more effectively than other ingested sterols. Therefore, cholesterol sulfate is the main component of the sulfated sterols in starfish [2, 3, 18-21].

Many starfish are known to contain toxic asterosaponins that can react with cholesterol and other Δ^5-sterols of biomembranes to form ion-conductive complexes. Replacing Δ^5-sterols in starfish biomembranes by Δ^7-sterols and stanols protects these animals from the effects of their own toxins [22]. Italian researchers found that the starfish *Euretaster insignis*, which does not contain asterosaponins, has more Δ^5-sterols in the free sterols, in contrast with toxic species [23]. However, how the different content of asterosaponins affects the ratio of sulfated sterols has not yet been established.

We compared the compositions of free sterols and sterol sulfates from two related species of starfish. We determined the hemolytic activity of ethanol extracts from these animals. We discussed possible reasons for the different sterol content in the sulfate fractions as a function of starfish toxicity.

The total free sterols were isolated from alcohol extracts of *L. alaskensis asiatica* and *L. fisheri* by hydrophobic and column chromatography. These fractions were separated by HPLC into subfractions. The PMR spectra of the subfractions are practically identical for both species and showed that the main components of these mixtures are Δ^7-sterols (singlets for angular methyls CH_3-18 and CH_3-19 at δ 0.54 and 0.80 ppm, respectively, and multiplets for olefinic protons at $\delta_{H\text{-}7}$ 5.17 ppm) [1, 4, 10]. The structures of the free sterols as the acetylated derivatives were identified by comparing relative retention times (RRT) in GLC and GLC—MS results with the corresponding literature data [1, 4, 9, 11, 24]. The results are listed in Table 1.

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TABLE 1. Free and Sulfated Sterols in Starfish *Leptasterias alaskensis asiatica* and *L. fisheri*

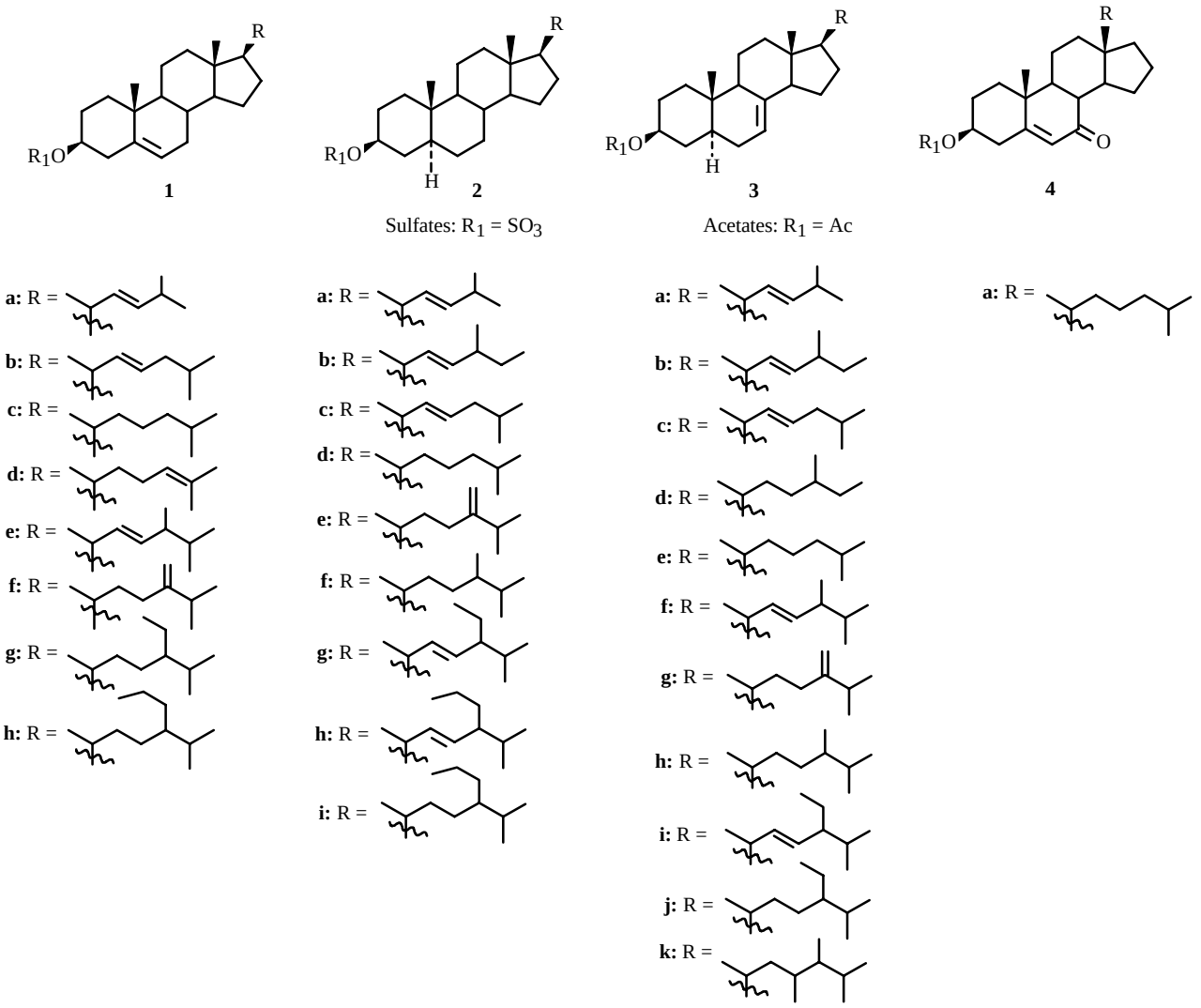
Sterols	RRT*	Composition, %				Mass spectrum, *m/z*
		L. alaskensis		*L. fisheri*		
		Sterols	Sulfates	Sterols	Sulfates	
24-Norcholesta-5,22-dien-3β-ol (**1a**)	0.69	Tr.	0.2	Tr.	0.15	352, 255, 213,
24-Nor-5α-cholest-22-en-3β-ol (**2a**)	0.71	-	0.2	-	0.6	414, 344, 315, 284, 257
24-Nor-5α-cholesta-7,22-dien-3β-ol (**3a**)	0.78	Tr.	0.3	3.74	1.0	412, 342, 313, 288, 255, 228
27-Nor-24-methyl-5α-cholest-22-en-3β-ol (**2b**)	0.91	Tr.	-	-	-	428, 368, 344, 315, 284, 257, 229, 215
Cholesta-5,22-dien-3β-ol (**1b**)	0.92	-	0.6	Tr.	0.3	366, 282, 255, 213
5α-Cholest-22-en-3β-ol (**2c**)	0.94	-	1.0	-	1.65	428, 413, 344, 316, 315,257, 215
27-Nor-24-methyl-5α-cholesta-7,22-dien-3β-ol (**3b**)	0.97	2.1	0.2	1.7	Tr.	426, 342, 313, 288, 255, 229, 213
Cholest-5-en-3β-ol (**1c**)	1.0	2.3	53.4	1.7	37.8	368, 260, 255, 247, 228, 213
5α-Cholestan-3β-ol (**2d**)	1.025	1.0	24.3	0.97	28.2	430, 415, 370, 355, 276, 275, 257, 230
5α-Cholesta-7,22-dien-3β-ol (**3c**)	1.045	9.43	-	9.0	1.5	426, 342, 315, 313, 288, 255, 229, 213
27-Nor-24-methyl-5α-cholest-7-en-3β-ol (**3d**)	1.06	1.35	0.35	1.64	0.4	428
Cholesta-5,24-dien-3-ol (**1d**)	1.09	-	-	-	Tr.	426
24-Methylcholesta-5,22-dien-3β-ol (**1e**)	1.10	Tr.	-	-	0.1	380, 365, 282, 255, 228, 213
5α-Cholest-7-en-3β-ol (**3e**)	1.12	53.12	11.2	51.05	20.0	428, 413, 368, 313, 288, 255, 229, 213
24-Methyl-5α-cholesta-7,22-dien-3β-ol (**3f**)	1.23	9.41	0.4	8.81	1.7	440, 425, 397, 380, 342, 313, 288, 255
24-Methylcholesta-5,24(28)-dien-3β-ol (**1f**)	1.24	0.41	-	-	-	380, 365, 296, 283, 255, 253
24-Methyl-5α-cholestan-3β-ol (**2f**)	1.24	-	0.45	Tr.	-	444
24-Methyl-5α-cholest-24(28)-en-3β-ol (**2e**)	1.27	-	-	-	0.6	358, 343, 315, 255, 215
24-Ethyl-5α-cholest-22-en-3β-ol (**2g**)	1.38	-	1.3	-	-	456
24-methyl-5α-cholesta-7,24(28)-dien-3β-ol (**3g**)	1.39	19.11	-	19.18	3.65	440, 380, 356, 296, 288, 255, 229, 213
24-Methyl-5α-cholest-7-en-3β-ol (**3h**)	1.40	-	0.2	-	0.2	442, 427, 288, 255, 229,213
Unidentified sterol	1.42	-	0.15	-	0.15	-
24-Ethyl-5α-cholesta-7,22-dien-3β-ol (**3i**)	1.49	-	0.3	-	-	454, 313, 255, 213
24-Ethylcholest-5-en-3β-ol (**1g**)	1.50	Tr.	-	-	-	396, 381, 255, 213
23,24-Dimethyl-5α-cholest-7-en-3β-ol (**3k**)	1.66	Tr.	-	Tr.	-	456, 441, 396, 381, 288, 255, 229, 213
24-Ethyl-5α-cholest-7-en-3β-ol (**3j**)	1.69	1.46	0.3	1.65	0.5	456, 396, 288, 255, 229,213
24-Propyl-5α-cholest-22-en-3β-ol (**2h**)	1.75	-	-	-	0.2	470
3β-Hydroxycholest-5-en-7-one (**4a**)	1.77	-	4.3	-	0.5	382, 367, 269
24-Propylcholest-5-en-3β-ol (**1h**)	1.78	-	Tr.	-	Tr.	410
24-Propyl-5α-cholestan-3β-ol (**2i**)	1.84	-	Tr.	-	-	472

*Relative (to cholesterol acetate) retention time of sterols (as acetylated derivatives).

We identified 18 free sterols. Both species contain >90% of Δ^7-sterols and only 1-3% of Δ^5-sterols. The main steroids for *L. alaskensis asiatica* and *L. fisheri* are 5α-cholest-7-en-3β-ol (**3e**) (53 and 51%, respectively) and 24-methyl-5α-cholesta-7,24(28)-dien-3β-ol (**3g**) (~19% for both species) (Table 1).

The ^{13}C and ^{1}H NMR spectra of sulfated sterols obtained from *L. alaskensis asiatica* and *L. fisheri* are consistent with the presence of a sulfate on C-3 ($\delta_{C\text{-}3}$ 78 ppm instead of that characteristic of free sterols at $\delta_{C\text{-}3}$ 71.7 ppm) and saturated ($\delta_{Me\text{-}18}$ 0.67 and $\delta_{Me\text{-}19}$ 0.82 ppm), 5(6)-unsaturated ($\delta_{Me\text{-}18}$ 0.71 and $\delta_{Me\text{-}19}$ 1.03 ppm), and 7(8)-unsaturated ($\delta_{Me\text{-}18}$ 0.56 and $\delta_{Me\text{-}19}$ 0.83 ppm) sterols. Acetylated subfractions were prepared after solvolytic removal of the sulfate [25] and were analyzed as described above. We identified 26 sterols (Table 1). The main component was cholesterol sulfate (**1c**) for *L. alaskensis asiatica* and *L. fisheri* (53.4 and 37.8%, respectively). Large quantities of 5α-cholestan-3β-ol sulfate (**2d**) (24.3 and 28.2%, respectively) and 5α-cholest-7-en-3β-ol sulfate (**3e**) (11.2 and 20.0%, respectively, Table 1) were also found.

The ethanol extract of *L. fisheri* causes 100% hemolysis of mouse-blood erythrocytes at 100 μg/mL whereas that from *L. alaskensis asiatica* exhibits no hemolytic activity. Therefore, *L. fisheri* contains much more toxic asterosaponins than *L. alaskensis asiatica*.



The ratio Δ^5-sterols:stanols: Δ^7-sterols in *L. alaskensis asiatica* sulfates is approximately 4:2:1; in the more toxic *L. fisheri*, 1.3:1.1:1.0 (Table 1). We propose that the ratio of sulfated sterols in starfish depends on the level of their toxicity and is explained by the adaptation of the animals to hemolytic asterosaponins. Free sterols are more effectively sulfated in starfish with more asterosaponins, involving not only Δ^5- but also Δ^0- and Δ^7-compounds.

The acetylated subfractions obtained from both species contain the acetate of steroid **4a** (Scheme). Its mass spectrum (382 $[M - AcOH]^+$, 269 $[M - AcOH - \text{side chain}]^+$, 161 *m*/*z*) corresponds with that of cholesta-3,5-dien-7-one [26]. However, the GLC retention time is much longer than cholesta-3,5-dien-7-one. The mass spectrum of the corresponding desulfated derivative has peaks (400 $[M]^+$, 385 $[M - CH_3]^+$, 382 $[M - H_2O]^+$, 367, 287, 269, 245 *m*/*z*) that are characteristic of 3β-hydroxycholest-5-en-7-one [27]. Thus, we identified 3β-hydroxycholest-5-en-7-one sulfate (**4a**, Scheme), which is apparently formed in these animals via oxidation of cholesterol sulfate. This compound has not previously been observed in starfish.

EXPERIMENTAL

NMR spectra (^1H and ^{13}C) were recorded on a Bruker WM-250 spectrometer in C_5D_5N (sulfated sterols) and $CDCl_3$ (sterols) with TMS as internal standard.

HPLC was performed on a duPont 8800 chromatograph equipped with a refractive-index detector.

GLC of sterol acetate mixtures was carried out in a Perkin—Elmer Sigma 2000 chromatograph with a CBP capillary column at 290°C and He carrier gas at 2 mL/min.

GLC—MS was performed in a Hewlett Packard HP 5973 MSD/HP instrument using an HP-5 MS column with 5% phenylmethylsiloxane (30.0 m × 25 μm × 0.25 μ), 280°C, He- carrier gas, and 69.9 eV ionization potential.

TLC was performed on glass plates (6×9 cm) with L5/40 μ silica gel (Chemapol, Czech Rep.) with gypsum ($CaSO_4$) using the solvent systems $CHCl_3$—CH_3OH—H_2O (15:7.5:1) for sterol sulfates and C_6H_6—$(CH_3)_2CO$ (1.5:1) for free sterols.

We used polychrom-1 (Olaina, Latvia) and L 40/100 μ (Chemapol, Czech Rep.) for column chromatography.

Starfish were collected by diving to 15-20 m off the shore of Paramushir Island (Kuril Islands) in August 1992 during the 16th scientific cruise of the ship Academician Oparin. Dr. A. V. Adrianov and Acad. O. G. Kusakin (Institute of Marine Biology, FED, RAS) identified the species.

The collected *L. alaskensis* (6 kg) and *L. fisheri* (5 kg) were ground and extracted with ethanol. Hydrophobic chromatography on polychrom [28] and column chromatography over silica gel of the ethanol extracts using hexane—ethylacetate (10:1) isolated the total free sterols (0.015 and 0.010% yields of dry mass). These fractions were separated into subfractions using HPLC on an Altex Ultrasphere silica-gel column (25 cm × 10 mm) using hexane—ethylacetate (5:1). The resulting products were identified by NMR spectroscopy, by capillary GLC as the acetates [pyridine—acetic anhydride (1:1) for 12 h at room temperature], and by GLC—MS [11].

Sulfated sterols were isolated by column chromatography over polychrom and silica gel as described earlier [25] (0.005 and 0.004% yields). Then, the sulfate was removed in pyridine—dioxane (1:1) at 100°C for 1 h. Lipid impurities were removed by boiling with 2% NaOMe in MeOH (1 h) [11]. The resulting free sterols were purified over a silica-gel column and by HPLC and analyzed as described above.

The sterol sulfate fractions of both speices afforded in parallel with the sterols desulfated 3β-hydroxycholest-5-en-7-one (**4a**) as the acetate. The structure of this compound was established by comparing GLC RRT and mass spectra with the corresponding literature values for cholesta-3,5-dien-7-one [26] and 3β-hydroxycholest-5-en-7-one isolated from the sponge *Cliona capiosa* [27].

The hemolytic activity was determined using a suspension (1%) of mouse-blood erythrocytes according to the literature [29].

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