Sulfated steroid compounds from the starfish *Aphelasterias japonica* of the Kuril population

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Four sulfated steroid compounds were isolated from the Kuril population of the starfish *Aphelasterias japonica*. The structure of a new glycoside, aphelasteroside D, was elucidated as (24*R*)-29-*O*-[2-*O*-sulfo-β-D-glucopyranosyl]-24-ethyl-5α-cholestane-3β,5,6β,8β,15α,29-hexol sodium salt. Other three substances were identified as the known pycnopodioside C, 3-*O*-sulfo-24,25-dihydromarthasterone, and 3-*O*-sulfothornasterol A, the latter compound being isolated as a tyrammonium salt for the first time.

Key words: starfish *Aphelasterias japonica*, glycosides, polyhydroxysteroid, asterosaponin aglycons.

Unlike other echinoderms, starfishes are characterized by a wide diversity of highly oxygenated steroid compounds. They usually contain polyhydroxysteroids, polyhydroxysteroid mono- and biosides, and also toxic steroid oligoglycosides named asterosaponins. Oxygenated steroids can occur in both free and sulfated forms in starfishes.[1] In continuation of our studies of steroid metabolites of the Far Easten starfishes,[2,3] we investigated the steroid composition of the starfish *Aphelasterias japonica* Bell (Forcipulata order, Asteriidae family) collected in the Sea of Okhotsk at the Kuril coast.

Results and Discussion

A new glycoside named aphelasteroside D (**1**) and also the known compounds, *viz.,* pycnopodioside C (**2**), 3-*O*-sulfo-24,25-dihydromarthasterone (**3**) as the sodium salt, and 3-*O*-sulfothornasterol A (**4**) as the tyrammonium salt, were isolated by column chromatography on Polychrom-1, Sephadex LH-20, silica gel, and Florisil and also by HPLC on Octadecyl-Si100 from the water—ethanol extract of the Kuril population of the starfish *A. japonica*.

The ^{13}C NMR and DEPT spectra of glycoside **1** showed the presence of 35 carbon atoms including the C atoms of five methyl groups, twelve methylene groups, fourteen methine groups, and four H-free C atoms (Table 1). The signal at δ 102.0 was assigned to the anomeric C atom of the monosaccharide residue, and the signals at δ 62.4, 67.1, 69.0 (×2), 71.5, 75.5, 76.5, 77.7, 77.9, 78.1, and 80.4 were assigned to the O-linked C atoms. The ^1H NMR spectrum of compound **1** contained the doublet signal of the anomeric proton of the monosaccharide residue at δ 5.04 (see Table 1). These data allowed us to assume the presence of a hexahydroxystigmastane aglycon and the β-hexose residue in the molecule of **1**. The structure of **1** was established by two-dimensional NMR spectroscopy including HMQC and $^1H—^1H$ COSY experiments. The chemical shifts and the corresponding coupling constants of protons and also the chemical shifts of C atoms in the NMR spectra of **1** virtually coincided with the analogous values in the spectrum of leptasteroside L (**1a**) from the starfish *Leptasterias polaris acervata*,[4] except for some signals of the monosaccharide residue. Thus the signals of HC(1′), HC(2′), and HC(3′) in the spectrum of **1** were observed at δ 5.04, 5.14, and 4.51 (*cf.* with those in the spectrum of leptasteroside L at δ 4.90, 4.07, and 4.27, respectively), and the signals of C(1′), C(2′), and C(3′) were observed at δ 102.0, 80.4, and 78.1 (*cf.* with those in the spectrum of leptasteroside L at δ 104.6, 75.1, and 77.7, respectively). Considering the differences in the chemical shifts of these atoms of the two substances and the literature data,[5] we assumed glycoside **1** to differ from leptasteroside L only in the presence of the sulfo group at the C(2) atom of the glucopyranose residue. The MALDI-TOF mass spectrum with registration of positive ions for compound **1** contained the pseudomolecular peak with *m/z* 783 $[M + Na]^+$, which corroborated the presence of the sulfo group and Na^+ as a counterion in the molecule of **1**. The MALDI-TOF mass spectrum with registration of negatively charged ions contained the pseudomolecular peak with *m/z* 737 $[M - Na]^-$, which also corroborated the presence of the sulfo group in the molecule of **1**. The molecular formula $C_{35}H_{61}O_{14}SNa$ was assigned to compound **1** based on the analysis of its NMR spectra and MALDI-TOF mass spectra. Mild solvolysis of glycoside **1** by heating in a 1 : 1 dioxane—pyridine mixture at 100 °C yielded **1a**, which was identified as leptasteroside L by direct comparison with the sample at our disposal (TLC, $[\alpha]_D$, ^1H NMR). We

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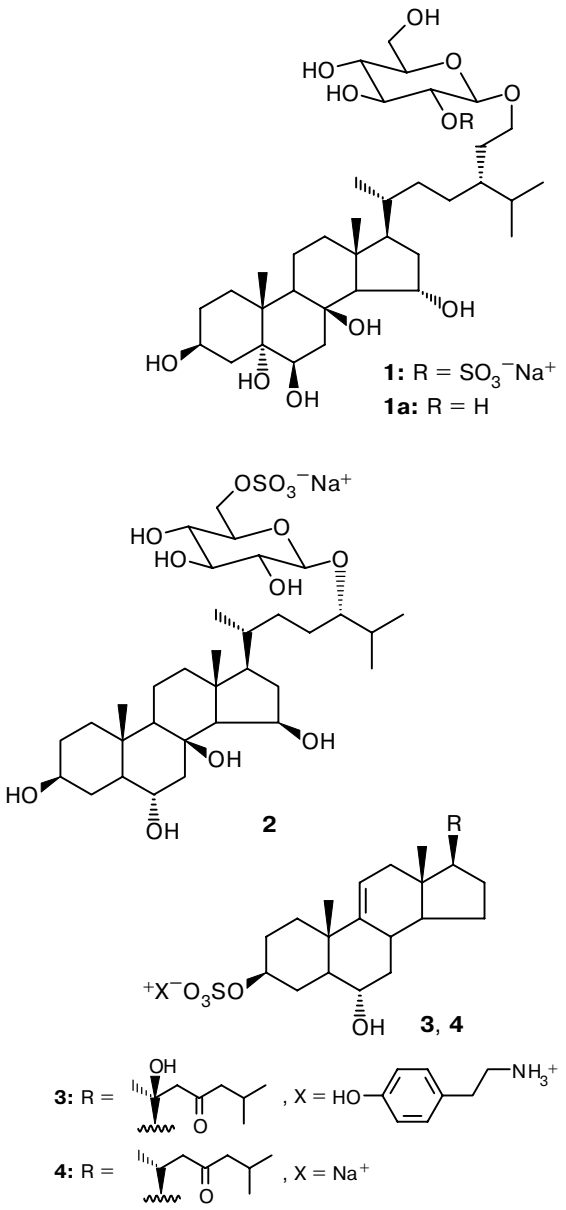

assumed the C(24) asymmetric center in the molecule of **1** to have the *R* configuration and the residue of sulfated glucopyranose to belong to the D-series, as this had previously been established for **1a**. Thus, aphelasteroside D (**1**) has the structure of (24*R*)-29-*O*-[2-sulfo-β-D-glucopyranosyl]-24-ethyl-5α-cholestane-3β,5,6β,8β,15α,29-hexol sodium salt. This is the first case of identification, in starfishes, of glycosides sulfated at the C(2) atom of the glucopyranose residue.

The MALDI-TOF mass spectrum (registration of negative ions) of glycoside **2** contained the pseudomolecular peak with m/z 693 $[M - Na]^-$. The ^1H and ^{13}C NMR spectra of **2** agreed well with the literature data for pycnopodioside C from the starfish *Pycnopodia helianthoides*.[6] Thus, glycoside **2** was identified as pycnopodioside C.

Table 1. ^1H and ^{13}C NMR spectra of aphelasteroside D (**1**) (C_5D_5N)

Atom	Assignment (DEPT)	δ (*J*/Hz)	
		δ_C	δ_H
1	CH_2	31.6	2.32 (m); 2.15 (m)
2	CH_2	34.1	2.33 (m); 1.69 (m)
3	CH	67.1	4.95 (m)
4	CH_2	42.2	2.98 (t, *J* = 12.1); 2.32 (m)
5	C	75.5	—
6	CH	77.7	4.33 (m)
7	CH_2	40.7	3.05 (br.d, *J* = 14.4); 3.18 (br.d, *J* = 14.4)
8	C	76.5	—
9	CH	48.4	2.20 (m)
10	C	38.9	—
11	CH_2	19.1	—
12	CH_2	42.2	1.37 (m)
13	C	44.5	—
14	CH	66.0	1.76 (d, *J* = 9.5)
15	CH	69.0	4.97 (m)
16	CH_2	41.6	2.13 (m), 2.08 (m)
17	CH	54.8	1.48 (m)
18	Me	15.4	1.34 (s)
19	Me	17.93	1.86 (s)
20	CH	35.54	1.40 (m)
21	Me	18.69	0.95 (d, *J* = 6.0)
22	CH_2	33.63	1.40 (m); 1.02 (m)
23	CH_2	27.13	1.02 (m)
24	CH	41.09	1.20 (m)
25	CH	29.5	1.63 (m)
26	Me	19.3	0.77 (d, *J* = 5.8)
27	Me	18.7	0.75 (d, *J* = 5.8)
28	CH_2	31.0	1.85 (m); 1.60 (m)
29	CH_2	69.0	4.21 (m); 3.80 (m)
1′	CH	102.0	5.04 (d, *J* = 7.8)
2′	CH	80.4	5.14 (dd, *J* = 7.8, 8.8)
3′	CH	78.1	4.51 (t, *J* = 8.8)
4′	CH	71.5	4.22 (t, *J* = 9.4)
5′	CH	77.9	3.95 (m)
6′	CH_2	62.4	4.53 (dd, *J* = 5.5, 11.8); 4.33 (dd, *J* = 2.7, 11.8)

The MALDI-TOF mass spectrum (registration of negative ions) of compound **3** contained the pseudomolecular peak with m/z 511 $[M - cation]^-$. Comparison of ^1H and ^{13}C NMR spectral data and chromatographic (TLC) behavior of steroid **3** with those for the specimens previously isolated from the population of *A. japonica* collected in the Posyet Bay of the Sea of Japan and from the starfish *Lethasterias nanimensis chelifera*[3,7] allowed us to assign the structure of 3-*O*-sulfothornasterol A to this compound. In addition to the signals of the steroid moiety, the ^1H and ^{13}C NMR spectra of this compound also contained additional signals, which corresponded to tyramine according to the literature data.[8] Thus, the ^1H NMR spectrum (CD_3OD) contained two doublets of aromatic protons at δ 7.08 (2 H, *J* = 8.7 Hz) and 6.76 (2 H, *J* = 8.7 Hz) and two triplets of methylene protons at δ 3.10 (2 H, *J* = 7.6 Hz) and 2.84 (2 H, *J* = 7.6 Hz). The ^{13}C NMR spectrum

(CD_3OD) contained the signals of C atoms corresponding to tyramine at δ 157.7 (C(1′)), 130.8 (C(2′), C(6′)), 128.5 (C(4′)), 116.9 (C(3′), C(5′)), 42.8 (—CH_2—N<), and 33.8 (Ar—CH_2<). The MALDI-TOF mass spectrum of steroid **3** obtained by registration of positive ions contained the peak of the tyrammonium cation with *m/z* 138. The data mentioned above allowed us to conclude that compound **3** is the tyrammonium salt of 3-*O*-sulfothornasterol A. Starfish sulfated polyhydroxysteroids usually occur in the form of sodium salts. Only one case where the tyrammonium salt of steroidal pentol monoside, asterosaponin P_1, was isolated from the starfish *Asterina pectinifera* has been reported so far.[8]

The MALDI-TOF mass spectrum of **4** (registration of positive ions) contained pseudomolecular peaks with *m/z* 541 $[M + Na]^+$ and 557 $[M + K]^+$. The MALDI-TOF mass spectrum of **4** obtained by registration of negative ions contained the pseudomolecular peak with *m/z* 495 $[M - Na]^-$. The ^1H and ^{13}C NMR spectra of compound **4** are similar to those for the aglycon of the steroid tetroside, santiagoside, from the starfish *Neosmilaster georgianus*.[9] The upfield shifts of the signals for the CH(6) proton at δ 3.84 and for the C(6) atom at δ 68.2 by 0.06 and 12.0 ppm, respectively, as compared to the corresponding signals in the spectrum of santiagoside, proved the presence of the carbohydrate chain at C(6) in the molecule of **4**. The structure of compound **4** was also confirmed by DEPT and HMQC spectra. Steroid **4** was established to be the sodium salt of 3-*O*-sulfo-24,25-dihydromarthasterone, which is the native aglycon of santiagoside and some other starfish steroid oligosides.[1,9]

The steroid composition of the starfish *A. japonica* was previously studied twice. The E. Finamore group had isolated five compounds, three of them belonging to free and xylosylated pentols and hexols, from this starfish of the population collected in the Mutsu bay of the Sea of Japan.[10] We had isolated six compounds structurally similar to asterosaponins with the 3-*O*-sulfo-3β,6α-dihydroxy-$\Delta^{9,(11)}$-steroidal fragment, three of them being glycosylated at C(6), from the population of *A. japonica* collected in the Posyet Bay of the Sea of Japan.[3] Thornasterol A sulfate was the only common compound dominating in all three populations, being isolated from the Kuril population as the tyrammonium salt. Thus, comparison of three various populations of *A. japonica* demonstrated the marked difference in steroid composition. On this basis, we can assume that the starfish steroid composition probably depends on their area where different nutrition sources and other ecological factors play an essential role. We cannot also rule out the dependence of the steroid composition on the season cycles of vital activity of these animals.

Certain low-molecular metabolites of marine animals are known to possess taxonomic specificity and can be detected irrespective of the place of collection and season factors. For example, it was established that triterpenoid glycosides from sea cucumbers can be considered as taxonomic markers at the level of subfamily, genus, and sometimes species.[11] However, comparison of the steroid composition of various populations of *A. japonica* shows that polyhydroxysteroids and related compounds cannot serve as taxonomic markers for this starfish.

Experimental

^1H and ^{13}C NMR spectra were registered on Bruker WM-250 (^1H, 250 MHz; ^{13}C, 62.9 MHz) and Bruker DPX 300 (^1H, 300 MHz; ^{13}C, 75 MHz) spectrometers with Me_4Si as the internal standard. The optical rotation was measured on a Perkin—Elmer 141 polarimeter. MALDI-TOF mass spectra were obtained on a Biflex III (Bruker, Germany, N_2-laser, 337 nm) instrument. A sample was dissolved in MeOH (1 mg mL^{-1}) and a 1-μL aliquot was analyzed using 2,5-dihydroxybenzoic acid as a matrix. HPLC was performed on a DuPont Model 8800 chromatograph equipped with a refractometer as a detector using an Octadecyl-Si100 (5 μm, 250×4.6 mm) column.

Column chromatography was carried out on Polychrom-1 (Biolar, Latvia), Sephadex LH-20 (Sigma, USA), Silica gel L 40/100 μm (Chemapol, Czech Republic), and Florisil (100—200 mesh, Koch-Light Laboratories, UK). TLC was performed on glass (4.5×6.0 cm) plates precoated with Sorbfil (5—17 μm, Russian Federation).

Starfish samples were collected in August 1999 in the Sea of Okhotsk near the Onekotan Isle (the Kuril Islands) from a depth of 105 m and identified by S. Sh. Dautov (Institute of Sea Biology, Far East Branch of the Russian Academy of Sciences, Vladivostok).

Isolation of compounds 1—4. Disintegrated starfish (580 g) were twice extracted with 70% EtOH (3 mL g^{-1}) with heating on a water bath, and the insoluble material was removed by centrifugation. Lipids were removed from the supernatant by extraction with benzene (1 mL per 3 mL of the supernatant). The water—ethanol layer was concentrated *in vacuo*, and the residue was dissolved in 0.5 L of water and applied onto a Polychrom-1 (7×35 cm) column. The column was washed with water up to the absence of Cl^- ions in the eluate and then with 50% EtOH. The ethanolic eluate was concentrated. The resulting overall fraction of steroid compounds (1.5 g) was sequentially chromatographed on a Sephadex LH-20 (3×40 cm) column in EtOH—H_2O (2 : 1) and on a Silica gel (4×18 cm) column in $CHCl_3$—EtOH (8 : 1 → 1 : 1). As the polarity of the eluent was increased, we obtained fractions containing compounds **3+4** (R_f 0.89), **2** (R_f 0.55), and **1** (R_f 0.39) (TLC, $CHCl_3$—MeOH, 2 : 1). The fractions containing compounds **1** and **2** were purified by HPLC (elution with 46% EtOH) to yield 3 mg of **1** and 2 mg of **2**. The fraction containing the mixture of **3+4** was separated by HPLC (elution with 55% EtOH). The enriched fraction containing steroid **3** was additionally purified on a Florisil (1.5×5 cm) column in $CHCl_3$—EtOH (8 : 1 → 5 : 1). The enriched fraction containing steroid **4** was additionally purified by HPLC (elution with 46% EtOH) to yield 8 mg of **3** and 2.5 mg of **4**.

Aphelasteroside D (1), $C_{35}H_{61}O_{14}S_1Na_1$, amorphous; $[\alpha]_D$ +8.9 (*c* 0.3, MeOH), ^1H and ^{13}C spectra are given in Table 1; MALDI-TOF-(+) MS, *m/z*: 783 $[M + Na]^+$; MALDI-TOF-(−) MS, *m/z*: 737 $[M - Na]^-$.

Desulfation of glycoside 1. Compound **1** (1.5 mg) was heated with 2 mL of dioxane—pyridine (1 : 1) at 100 °C for 4 h. The reaction mixture was concentrated *in vacuo*, and the dry

residue was chromatographed on a Florisil (1.5×3 cm) column in $CHCl_3$—EtOH (4 : 1) to yield 1 mg of **1a**, which was identified by direct comparison (TLC, ^{1}H NMR, $[\alpha]_D$ −2.0 (*c* 0.1, MeOH)) with the sample of leptasteroside A previously isolated from the starfish *L. polaris acervata* ($[\alpha]_D$ −3.3 (*c* 0.15, MeOH)).[4]

Pycnopodioside C (2), amorphous; $[\alpha]_D$ +6.3 (*c* 0.2, MeOH). ^{1}H and ^{13}C spectra are identical with those reported previously;[6] MALDI-TOF-(−) MS, *m/z*: 693 $[M - Na]^-$.

3-*O*-Sulfothornasterol A, tyrammonium salt (3), amorphous; $[\alpha]_D$ −3.3 (*c* 0.3, MeOH). The ^{1}H and ^{13}C spectra of the steroid moiety are similar to those reported previously;[7,10] MALDI-TOF-(−) MS, *m/z*: 511 $[M - Na]^-$.

3-*O*-Sulfo-24,25-dihydromarthasterone, sodium salt (4), amorphous; $[\alpha]_D$ +4.0 (*c* 0.2, MeOH). ^{1}H NMR (C_5D_5N), δ: 0.63 (s, 3 H, Me(18)); 0.91 (d, 3 H, Me(26), *J* = 7 Hz); 0.92 (d, 3 H, Me(27), *J* = 7 Hz); 0.97 (s, 3 H, Me(19)); 1.05 (d, 3 H, Me(21), *J* = 7 Hz); 3.45 (m, 1 H, $H_aC(4)$); 3.84 (m, 1 H, HC(6)); 4.79* (m, 1 H, HC(3)); 5.27* (m, 1 H, HC(11)). ^{13}C NMR (C_5D_5N), δ: 36.0 (C(1)); 29.7 (C(2)); 31.0 (C(4)); 50.5 (C(5)); 68.2 (C(6)); 43.3 (C(7)); 35.8 (C(8)); 146.1 (C(9)); 38.1 (C(10)); 116.2 (C(11)); 41.7 (C(12)); 41.1 (C(13)); 53.6 (C(14)); 25.3 (C(15)); 28.5 (C(16)); 56.1 (C(17)); 11.5 (C(18)); 19.2 (C(19)); 32.3 (C(20)); 19.5 (C(21)); 50.1 (C(22)); 209.9 (C(23)); 52.2 C(24); 24.4 (C(25)); 22.5 (C(26)); 22.4 (C(27)). MALDI-TOF-(+) MS, *m/z*: 541 $[M + Na]^+$, 557 $[M + K]^+$. MALDI-TOF-(−) MS, *m/z*: 495 $[M - Na]^-$.

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* The position of the signals in the spectrum was refined by heating the sample to 70 °C, which resulted in the upfield shift of the H_2O signal.

References

1. L. Minale, R. Riccio, and F. Zollo, in *Progress in the Chemistry of Organic Natural Products*, Eds. W. Herz, G.W. Kirby, R.E. Moore, W. Steglich, and Ch. Tamm, Wien—New York, Springer Verlag, 1993, **62**, 234 pp.
2. A. A. Kicha, A. I. Kalinovsky, N. V. Ivanchina, and V. A. Stonik, *J. Nat. Prod.*, 1999, **62**, 279.
3. N. V. Ivanchina, A. A. Kicha, A. I. Kalinovsky, P. S. Dmitrenok, V. A. Stonik, R. Riguera, and C. Jimenez, *J. Nat. Prod.*, 2000, **63**, 1178.
4. A. A. Kicha, A. I. Kalinovsky, and V. A. Stonik, *Izv. Akad. Nauk, Ser. Khim.*, 1995, 1164 [*Russ. Chem. Bull.*, 1995, **44**, 1125 (Engl. Transl.)].
5. A. Rashid, W. Macke, I. J. Colquhoun, and D. Lamba, *Can. J. Chem.*, 1990, **68**, 1122.
6. I. Bruno, L. Minale, and R. Riccio, *J. Nat. Prod.*, 1989, **52**, 1022.
7. A. A. Kicha, A. I. Kalinovsky, and V. A. Stonik, *Khim. Prir. Soedin.*, 1991, 520 [*Chem. Nat. Compd.*, 1991 (Engl. Transl.)].
8. M. Iorizzi, L. Minale, and R. Riccio, *Gazz. Chim. Ital.*, 1990, **120**, 147.
9. M. J. Vazquez, E. Quinoa, R. Riguera, A. San Martin, and J. Darias, *Tetrahedron*, 1992, **48**, 6739.
10. E. Finamore, F. Zollo, L. Minale, and T. Yasumoto, *J. Nat. Prod.*, 1992, **55**, 767.
11. V. A. Stonik, V. I. Kalinin, and S. A. Avilov, *J. Nat. Toxins*, 1999, **2**, 235.

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