A new steroidal glycoside phrygioside A and its aglycone from the starfish *Hippasteria phrygiana*

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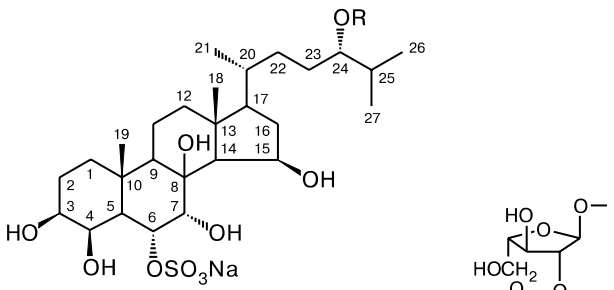
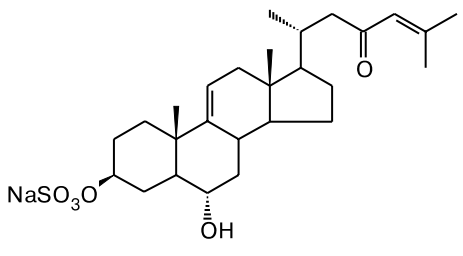
Three polyhydroxylated steroids were isolated from the Far-Eastern starfish *Hippasteria phrygiana* collected from the Sea of Okhotsk and were characterized as a new glycoside phrygioside A, *viz.*, sodium (20*R*,24*S*)-3β,4β,7α,8,15β,24-hexahydroxy-24-*O*-[3-*O*-methyl-β-D-xylopyranosyl-(1→2)-α-L-arabinofuranosyl]-5α-cholestan-6α-yl sulfate, its aglycone, and the already known marthasterone sulfate.

Key words: starfishes, *Hippasteria phrygiana*, polyhydroxysteroids, sulfated polyhydroxysteroids, glycosides, aglycones, FAB, MALDI-TOF, ^{1}H NMR, and ^{13}C NMR spectra.

As part of our continuing studies of metabolites from starfishes of the Far-Eastern seas,[1] we established the structures of steroids from the starfish *Hippasteria phrygiana* belonging to the family Goniasteridae (the order Valvatida), whose chemistry is poorly studied. One of these steroids is a bioside of polyhydroxylated steroid monosulfate (**1**). Another polar steroid (**2**) appeared to be identical to the aglycone of the above-mentioned glycoside. We also identified marthasterone sulfate (**3**), *viz.*, sodium (20*R*)-6α-hydroxy-23-oxocholest-9(11),24-dien-3β-yl sulfate, which is abundant in starfishes.[2]





Results and Discussion

The steroidal compounds were isolated from an ethanolic extract of the starfish *H. phrygiana* according to a procedure that we have used previously for related compounds.[2] This involves chromatography on Polychrom, Amberlite XAD-2, Florisil, and silica gel followed by reversed-phase HPLC on ODS-A and Diasphere-C_{18}. The compounds were identified by spectroscopic methods (NMR spectroscopy and MALDI-TOF and FAB mass spectrometry).

The ^{13}C NMR spectra (using DEPT experiments) of compound **1** contain signals for six methyl groups, nine methylene groups, twenty methine groups, and three C atoms bearing no protons. These data together with the negative-ion MALDI-TOF mass spectrum, which contains a pseudomolecular ion peak $[M - H]^-$ at *m/z* 841.48, suggest the molecular formula $C_{38}H_{65}O_{15}SO_3Na$. In the FAB-(−) mass spectrum, the peaks corresponding to successive abstraction of the *O*-methylpentose and pentose residues at *m/z* 694.73 $[(M - H) - C_6H_{10}O_4]^-$, 676.71 $[(M - H) - C_6H_{12}O_5]^-$, and 544.84 $[(M - H) - C_6H_{12}O_4 - C_5H_8O_4]^-$, respectively, suggest the presence of a disaccharide carbohydrate chain in compound **1**. An analogous fragmentation is also observed in the FAB-(+) mass spectrum of **1** (see Experimental). Acid hydrolysis of glycoside **1** afforded 3-*O*-methylxylose and arabinose, which were identified by GLC as the corresponding aldononitrile acetates and by paper chromatography using comparison with authentic compounds. Judging from the specific optical rotation of the resulting mixture of the monosaccharides, arabinose and 3-*O*-methylxylose belong to the L and D series, respectively.

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In the ^{13}C NMR spectrum of compound **1**, the signals at δ_C 104.9 and 107.4 were assigned to anomeric carbon atoms, and the signals at δ 60.7, 62.1, 66.8, 69.2, 69.4, 70.0, 71.5, 74.1, 74.5, 74.7, 77.3, 78.9, 83.1, 84.3, 87.8, and 92.3 were assigned to the carbon atoms bound to oxygen in the monosaccharide residues and the aglycone.

The positions of the signals for C(1)—C(13) and C(19) in the ^{13}C NMR spectrum (C_5D_5N) of compound **1** are identical to the corresponding values for sodium 3β,4β,7α,8,15β,16β,26-heptahydroxy-5α-cholestan-6α-yl sulfate isolated from the starfish *Patiria pectinifera*, which we have characterized earlier.[3] This suggests the 3β,4β,6α,7α,8,15β-hexahydroxy substitution and sulfation of the hydroxy group at the C(6) atom in the polycyclic moiety of the aglycone of glycoside **1**. The chemical shifts of the signals for the C atoms of the ring D, the side chain, and the monosaccharide unit, C(15)—C(27) and C(1′)—C(5′), are identical to the corresponding shifts in the spectra of the known 24-*O*-[2,4-di-*O*-methyl-β-D-xylopyranosyl-(1→2)-α-L-arabinofuranosyl]-5α-cholestane-3β,4β,6α,8,15β,24-hexaol (culcitoside C_1) from *Culcita novaeguineae*.[4] This indicates that compound **1** has the 15β-hydroxy functionality and that the α-arabinofuranosyl residue of the carbohydrate chain is linked to the hydroxy group at the C(24) atom.

The fact that the chemical shifts of the signals for the second monosaccharide residue in the NMR spectra of phrygioside **1** (C(1″)—C(5″)) are identical to those in the spectra of borealoside C from *Solaster borealis*,[5] which contains the 3-*O*-methylxylopyranose carbohydrate chain, as well as the data from FAB-(+) and FAB-(−) mass spectrometry of **1** confirm that the 3-*O*-methylxylose group is the terminal residue in the carbohydrate chain of this glycoside. The coupling constant $^3J_{1'',2''}$ (7.5 Hz) suggests the β configuration of the glycosidic bond.

The structure of glycoside **1** was also confirmed by the COSY-45, HMBC, and HSQC spectra, which allowed us to make the assignment of the signals for all carbon and hydrogen atoms in the NMR spectra (Table 1). In particular, the $^1H—^1H$ COSY-45 spectrum shows coupling in the system of the protons $H_2C(2)—HC(3)—HC(4)—HC(5)—HC(6)—HC(7)$. The multiplicities of the protons and the chemical shifts of the signals for the H(6) atom confirm the presence of the sulfo group. Two low-field protons give cross-peaks with the high-field protons. They were assigned to H(24) (δ 3.59, the signal characteristic of 24-*O*-glycosylated steroids from starfishes)[4] and H(15) (δ 5.05). The chemical shifts of the signals and the coupling constants of the protons $H_2(16)$ ($J_{15,14} \approx 4.0$ Hz, the coupling constant was measured from the cross-peak) confirm the 15β-OH configuration.

The COSY-45 spectrum of compound **1** also shows coupling between the protons of the carbohydrate chain. The HMBC correlations H(1′)/C(24) and H(1″)/C(2′) support the fact that the α-L-arabinofuranosyl residue is linked to O(24) of the aglycone and is glycosylated with the 3-*O*-methyl-β-D-xylopyranosyl group at position 2 (see Table 1). We assigned the (*R*) configuration to the C(20) center of compound **1** based on the chemical shift of the protons $H_3C(21)$ (CD_3OD, δ 0.93) taking into account the results of the earlier study of a series of model steroids[6] where it was demonstrated that the signal for the protons $H_3C(21)$ in the 20*S* isomer (δ 0.83) is shifted upfield compared to the corresponding signal in the spectrum of the 20*R* isomer by approximately 0.1 ppm. Finally, the chemical shifts of the signals for the protons and C atoms in the side chain of compound **1** (CD_3OD) appeared to be identical to the corresponding shifts in the spectra of borealoside C,[5] for which the stereochemistry of the chiral centers of the side chain has been established earlier. Hence, taking into account the aforesaid, we assigned the structure of sodium (20*R*,24*S*)-3β,4β,7α,8,15β,24-hexahydroxy-24-*O*-[3-*O*-methyl-β-D-xylopyranosyl-(1→2)-α-L-arabinofuranosyl]-5α-cholestan-6α-yl sulfate (**1**) to phrygioside A.

The positive-ion MALDI-TOF mass spectrum of steroid **2** shows a pseudomolecular ion peak $[M + Na]^+$ at *m/z* 585, which suggests the molecular formula $C_{27}H_{46}O_7SO_3Na$. According to the ^{13}C NMR spectroscopic data (see Table 1), molecule **2** contains 27 C atoms, including carbon atoms of five methyl groups, seven methylene groups, twelve methine groups, and three C atoms bearing no protons.

The chemical shifts of the signals for the C(1)—C(13) and C(19) atoms in the NMR spectrum (CD_3OD) of compound **2** are virtually the same as those in the spectrum of sodium (25*S*)-3β,4β,7α,8,15α,16β,26-heptahydroxy-5α-cholestan-6α-yl sulfate from the starfish *Oreaster reticulatus*,[7] which confirms that the corresponding fragments in both compounds are identical. Sulfation of the hydroxy group at the C(6) atom leads to a downfield shift of the signal for H(6) by 0.7 ppm (δ 5.05) compared to the chemical shift of the analogous proton (δ 4.32) in the spectrum of nonsulfated 5α-cholestan-3β,4β,6α,7α,8,15β,16β,26-octaol from *Asterina pectinifera*.[8] A comparison of their ^{13}C NMR spectra also revealed the characteristic shifts associated with the α- and β-effects of sulfation of the hydroxy group at the C(6) atom.[7] The chemical shifts of the C(15)—C(27) and C(18) atoms in the spectrum of compound **2** are identical to the corresponding chemical shifts for (24*S*)-5α-cholestane-3β,6α,8,15β,24-pentaol from *Comophia watsoni*.[9] Based on these data, we suggested that compound **2** is the aglycone of phrygioside A (**1**) and has the structure of sodium (20*R*,24*S*)-3β,4β,7α,8,15β,24-hexahydroxy-5α-cholestan-6α-yl sulfate.

Table 1. Parameters of the NMR spectra (δ, *J*/Hz) of phrygioside A (**1**) and steroid (**2**)

Atom C, group	**1** (C_5D_5N)		HMBC	**2** (CD_3OD)	
	δ_C (m)	δ_H		δ_C (m)	δ_H
1	38.9 (CH_2)	1.13, 1.84 (both m)		39.5 (CH_2)	
2	26.8 (CH_2)	2.16 (m, H_{ax}); 1.80 (m, H_{eq})		26.6 (CH_2)	1.97 (m, H_{ax}); 2.13 (m, H_{eq})
3	71.5 (CH)	3.86 (m)		73.2 (CH)	3.53 (m)
4	69.2 (CH)	5.12 (m)		69.4 (CH)	4.26 (br.s, $W_{1/2}$ = 9)
5	46.2 (CH)	2.49 (dd, *J* = 11.9, *J* = 2.0)		46.7 (CH)	1.76 (dd, *J* = 11.5, *J* = 2.5)
6	74.7 (CH)	6.01 (dd, *J* = 11.9, *J* = 2.5)		76.6 (CH)*	5.09 (dd, *J* = 11.5, *J* = 2.5)
7	74.1 (CH)	5.24 (d, *J* = 2.5)		75.1 (CH)	4.30 (d, *J* = 3.0)
8	78.9 (C)			79.4 (C)	
9	50.4 (CH)	1.62 (m)		51.2 (CH)	
10	37.6 (C)			38.4 (C)	
11	18.2 (CH_2)	1.55, 2.11 (both m)		18.9 (CH_2)	
12	42.0 (CH_2)	1.26, 2.07 (both m)		42.9 (CH_2)	
13	43.3 (C)			44.2 (C)	
14	55.6 (CH)	2.02 (m)		56.4 (CH)*	1.41 (d, *J* = 5.5)
15	69.4 (CH)	5.05 (m)		71.0 (CH)	4.81 (td, *J* = 3.3, *J* = 10.0)
16	42.3 (CH_2)	2.62, 1.73 (both m)		42.7 (CH_2)	1.95, 2.4 (both m)
17	56.9 (CH)	1.10 (m)		57.9 (CH)	1.00 (m)
18	16.3 (CH_3)	1.62 (s)	C(12), C(13), C(14), C(17)	16.4* (CH_3)	1.26 (s)
19	16.8 (CH_3)	1.76 (s)	C(1), C(5), C(9), C(10)	16.8* (CH_3)	1.23 (s)
20	35.4 (CH)	1.65 (m)		36.5 (CH)	1.52 (m)
21	18.7 (CH_3)	1.02 (d, *J* = 6.2)	C(17), C(20), C(22)	19.5 (CH_3)	0.94 (d, *J* = 7.0)
22	31.8 (CH_2)	1.20, 1.86 (both m)		33.3 (CH_2)	
23	27.9 (CH_2)	1.55, 1.73 (both m)		31.5 (CH_2)	1.53, 1.20 (both m)
24	83.1 (CH)	3.59 (m)		78.1 (CH)	3.57 (m)
25	30.4 (CH)	1.97 (m)		34.5 (CH)	
26	17.9* (CH_3)	0.95 (d, *J* = 6.9)	C(24), C(25)	17.5 (CH_3)	0.98 (d, *J* = 6.8)
27	18.0* (CH_3)	0.99 (d, *J* = 6.9)	C(24), C(25)	19.5 (CH_3)	0.98 (d, *J* = 6.8)
OMe	60.7 (CH_3)	3.90 (s)			
1′	107.4 (CH)	5.65 (br.s)	C(24), C(2′)		
2′	92.3 (CH)	4.90 (br.s)			
3′	77.3 (CH)	4.91 (m)	C(3′), C(4′)		
4′	84.3 (CH)	4.73 (m)			
5′	62.1 (CH_2)	4.37 (dd, *J* = 3.0, *J* = 12.3); 4.21 (m)			
1″	104.9 (CH)	5.07 (d, *J* = 7.5)	C(2′)		
2″	74.5 (CH)	3.96 (t, *J* = 8.5)			
3″	87.8 (CH)	3.60 (m)	C(2″), C(4″)		
4″	70.0 (CH)	4.14 (m)			
5″	66.8 (CH_2)	3.60 (m); 4.24 (dd, *J* = 5.4, *J* = 11.0)	C(4″)		

* Assignments may be interchangable.

The structure of **2** was completely confirmed by 2D NMR spectroscopy (HMBC, HSQC, and COSY-45) analogously to compound **1** (see Table 1).

A comparison of the spectroscopic characteristics of compound **3** with the corresponding data for marthasterone sulfate, which we have isolated earlier from the starfish *Lysastrosoma anthosticta*,[2] demonstrated that they are completely identical. Hence, we identified steroid **3** as sodium (20*R*)-6α-hydroxy-23-oxocholesta-9(11),24-dien-3β-yl sulfate.

Phrygioside A (**1**) can be related to glycosylated steroids containing the 3β,4β,6α,8,15β-pentahydroxycholestane fragment sulfated at the O(6) atom. Most of these steroids were isolated from the Antarctic starfish *Acodontaster conspicuous*.[10] The isolation of the new compounds added to a knowledge of the chemical diversity of

secondary metabolites and various biosynthesis and metabolism processes of natural steroids occurring in starfishes.

Experimental

The ^1H and ^{13}C NMR spectra were recorded on Bruker AC-250 (250 MHz for ^1H and 62.9 MHz for ^{13}C) and Bruker DPX-300 (300.13 MHz for ^1H and 75.5 MHz for ^{13}C) spectrometers with tetramethylsilane as the internal standard. The optical rotation was measured on a Perkin—Elmer 343 polarimeter. The MALDI-TOF mass spectra were obtained on a Bruker Biflex III matrix-assisted laser ionization/desorption mass spectrometer (N_2 laser, 337 nm). A sample was dissolved in methanol (10 mg mL^{-1}), and an aliquot (1 μL) was analyzed using α-cyanohydroxycinnamic acid as the matrix. The sodium content was determined on a Nippon Jarrel Ash AA-780 atomic-absorption flame-emission spectrophotometer. Studies by HPLC were carried out on a DuPont Model 8800 chromatograph equipped with a refractometer detector and columns packed with Diasphere-110-C_{18} (5 μm, 4×250 mm) and YMC-Pack ODS-A (5 μm, 12 nm, 10×250 mm). The melting points were determined on a Leica VMTG hot-stage apparatus. The TLC analysis was carried out on Sorbfil plates with a layer of silica gel CTX-1A (5—17 μm, Krasnodar, Russia) fixed on aluminum foil using the 5 : 1 : 1 BuOH—EtOAc—H_2O system. Column chromatography was performed on silica gel L (80—100 and 200—250 mesh, Chemapol, Czech Republic), Polychrom (Russia), and Florisil (200—250 mesh, Merck, Germany).

Samples of the starfish *Hippasteria phrygiana* were trawled up from a depth of 100—200 m in the Sea of Okhotsk (Kuril Islands) in August 2002 during the expedition No. 27 of the research ship "Akademik Oparin" and were identified by Prof. V. S. Levin (Pacific Institute of Bioorganic Chemistry of the Far-Eastern Branch of the Russian Academy of Sciences).

Extraction and isolation of total fractions. Samples of the starfish *H. phrygiana* (0.108 kg) were milled and exhaustively extracted with 95% ethanol at room temperature for 3 days. The combined ethanolic extracts were concentrated *in vacuo* to a crude oily residue, which was chromatographed on a column (6×25 cm) with silica gel (80—100 mesh) using the chloroform—ethanol system (75 : 25 → 50 : 50). The following two fractions of polyhydroxysteroids were eluted as the polarity of the eluent was increased: less polar fraction **I** (0.55 g) containing the known sulfate **3** and steroid **2** and more polar fraction **II** (0.98 g) containing compound **1**.

Isolation of steroid derivatives 1, 2, and 3. Fraction **I** was dissolved in water and passed through a column (3×12 cm) with Polychrom and eluted with water and 50% aqueous ethanol. The aqueous-ethanolic eluate was concentrated *in vacuo* to a brown resinous residue (0.41 g), which was successively chromatographed on columns with Florisil (1.5×20 cm, 200—300 mesh) using the $CHCl_3$—EtOH system (9 : 1 → 6 : 4). The resulting fractions were chromatographed on SiO_2 (1.3×15) with the $CHCl_3$—EtOH system (7.5 : 2.5 → 7 : 3). The fraction containing steroid **2** with minor impurities (5.2 mg) was purified by HPLC on a column with Diasphere-110-C_{18} using the MeOH—H_2O mixture (75 : 25) as the eluent. Sulfate **2** was obtained in a yield of 2.1 mg (0.0019 % of the starfish weight). Steroid **3** was isolated by HPLC using the same system on a column with Diaspher-110-C_{18}; the yield of marthasterone sulfate **3** was 6.2 mg (0.0057%).

Fraction **II** (0.98 g) was passed through Polychrom and consecutively purified on a column with Florisil using the $CHCl_3$—EtOH system (6 : 4 → 1 : 9) and on a column with YMC-Pack ODS-A using the EtOH—H_2O mixture (43 : 57) as the eluent. Glycoside **1** was obtained in a yield of 7.4 mg (0.0068%).

Sodium (20*R*,24*S*)-3β,4β,7α,8,15β,24-hexahydroxy-24-*O*-[3-*O*-methyl-β-D-xylopyranosyl-(1→2)-α-L-arabinofuranosyl]-5α-cholestan-6α-yl sulfate (phrygioside A) (1), $C_{38}H_{65}O_{15}SO_3Na$, colorless crystals, m.p. 129—131 °C (from MeOH), $[\alpha]_D^{20}$ −10.9 (*c* 0.33, EtOH). MS FAB-(+), *m/z* (I_{rel} (%)): 887.38 $[M_{Na} + Na]^+$ (70), 767.46 $[(M_{Na} + Na) - NaHSO_4]^+$ (100), 723.35 $[(M_{Na} + Na) - C_6H_{12}O_5]^+$ (40), 621.25 $[767.46 - C_6H_{10}O_4]^+$, 489.29 $[621.25 - C_5H_8O_4]^+$ (10), 471.14 $[489.29 - H_2O]^+$ (17). The ^1H and ^{13}C NMR spectra (CD_3OD) of **1** were used for comparing with the published data[5] and are mentioned in the text. The ^1H and ^{13}C NMR spectra (C_5D_5N) are given in Table 1.

Sodium (20*R*,24*S*)-3β,4β,7α,8,15β,24-hexahydroxy-5α-cholestan-6α-yl sulfate (2), $C_{27}H_{46}O_7SO_3Na$, an amorphous compound, $[\alpha]_D^{20}$ +14.1 (*c* 0.25, EtOH). MS MALDI-TOF-(+) (I_{rel} (%)): *m/z* 585 $[M + Na]^+$ (100). The ^1H and ^{13}C NMR spectra (CD_3OD) are given in Table 1.

Sodium (20*R*)-6α-hydroxy-23-oxocholesta-9(11),24-dien-3β-yl sulfate (3), $C_{27}H_{41}NaO_6S$, colorless crystals, m.p. 126—128 °C (from MeOH—EtOAc), $[\alpha]_D^{20}$ +2.6 (*c* 0.12, EtOH). The ^1H and ^{13}C NMR spectra (CD_3OD, C_5D_5N) were identical to those documented for marthasterone sulfate from the starfish *Lysastrosoma anthosticta*.[2]

Hydrolysis of glycoside 1 and identification of monosaccharides. Glycoside (2 mg) was dissolved in 2 *M* HCl (2 mL) and heated at 100 °C for 2 h. 3-*O*-Methyl-D-xylose and L-arabinose were identified in the hydrolyzate by GLC (aldononitrile peracetates) and paper chromatography (*n*-butanol—pyridine—water, 6 : 4 : 40). For a mixture of monosaccharides prepared from **1**, $[\alpha]_D^{20}$ +57.5 (*c* 0.08, H_2O); for an equimolar mixture of the authentic samples $[\alpha]_D^{20}$ +53.4 (*c* 0.35, H_2O) (*cf.* lit. data[11]: $[\alpha]_D^{20}$ +61 (3-*O*-methyl-D-xylose + L-arabinose), $[\alpha]_D^{20}$ −44 (3-*O*-methyl-D-xylose + D-arabinose)).

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