Steroid compounds from the starfish *Lysastrosoma anthosticta* collected in the Sea of Japan

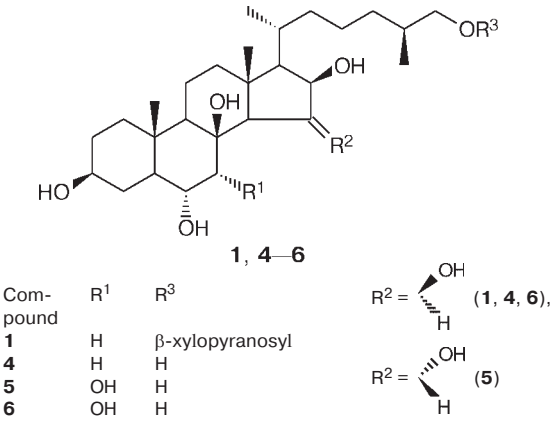
E. V. Levina, P. V. Andriyashchenko, A. I. Kalinovsky, P. S. Dmitrenok, V. A. Stonik, and N. G. Prokof´eva*

Pacific Institute of Bioorganic Chemistry, Far-Eastern Branch of the Russian Academy of Sciences, 159 prosp. 100-letiya Vladivostoka, 690022 Vladivostok, Russian Federation. Fax: +7 (423 2) 31 4050. E-mail: piboc@stl.ru

Six polyhydroxylated steroids and their derivatives were isolated from the starfish *Lysastrosoma anthosticta* collected in the Posyet Bay, Sea of Japan. These include a new glycoside of the steroid polyol, lysastroside A (**1**), which was identified as (25*S*)-26-*O*-β-D-xylopyranosyl-5α-cholestane-3β,6α,8,15β,16β,26-hexaol, and the previously known pycnopodioside C monoglycoside (**2**), marthasterone sulfate (**3**), (25*S*)-5α-cholestane-3β,6α,8,15β,16β,26-hexaol (**4**), (25*S*)-5α-cholestane-3β,6α,7α,8,15α,16β,26-heptaol (**5**), and (25*S*)-5α-cholestane-3β,6α,7α,8,15β,16β,26-heptaol (**6**). The compounds were tested for the haemolytic activity and the action on the embryogenesis of the sea urchin *Strongylocentrotus intermedius*.

Key words: starfish, *Lysastrosoma anthosticta*, polyhydroxysteroids, sulfated polyols and glycosides, ^{1}H and ^{13}C NMR spectroscopy.

Previously,[1] we studied the composition of the steroid compounds from the starfish *Lysastrosoma anthosticta* collected in the Sea of Okhotsk and demonstrated that the starfish contains known monoglycosides, pycnopodioside C and luridoside A, as the major steroid components. In addition, this steroid fraction contains marthasterone and dihydromarthasterone sulfates. We continued our research into physiologically active compounds from Far-Eastern starfishes by investigating the steroid composition of *Lysastrosoma anthosticta* collected in the Posyet Bay of the Sea of Japan. Study of the steroid composition of the same starfish species from different habitats gives the idea of the role of populational, seasonal, and geographic factors along the route of biogenesis of these metabolites.



1, **4**—**6**

Compound	R^1	R^3
1	H	β-xylopyranosyl
4	H	H
5	OH	H
6	OH	H

R^2 = (**1**, **4**, **6**),

R^2 = (**5**)

Results and Discussion

An aqueous-ethanol extract from the starfish *Lysastrosoma anthosticta* was treated according to the previously described procedures[1] including chromatography on silica gel, Amberlite XAD-2, Florisil, and Sephadex LH-60 to give several fractions containing steroid compounds. The subsequent HPLC on columns with Silasorb C_{18} and Zorbax ODS resulted in the isolation of a new glycoside, lysastroside A (**1**) and five known steroid compounds (**2**—**6**).

^{13}C NMR and DEPT spectra showed that the molecule of compound **1** contains 32 carbon atoms including the C atoms of 4 methyl, 11 methylene, and 14 methine groups and 3 quaternary C atoms (Table 1). The MALDI-TOF positive-ion mass spectrum of compound **1** exhibits a pseudo-molecular ion peak with m/z 623 $[M + Na]^+$, indicating that the molecular formula is $C_{32}H_{56}O_{10}$. Acid hydrolysis of **1** gave D-xylose, which was identified by GLC and paper chromatography (PC) and by comparison of the physical parameters of the hydrolysis product with those of an authentic sample. The chemical shifts of the anomeric carbon atom and the spin-spin coupling constants of the anomeric proton in the ^{1}H NMR spectrum (Table 1) indicate that the glycoside bond in **1** has a β-configuration. The positions of signals for C(1)—C(21) and C(3)H, C(6)H, C(7)H_2, C(14)H, C(15)H, C(16)H, C(18)H_3, C(19)H_3, and C(21)H_3 and the spin-spin coupling constants of these protons in the ^{13}C and ^{1}H NMR spectra of compound **1** coincided with

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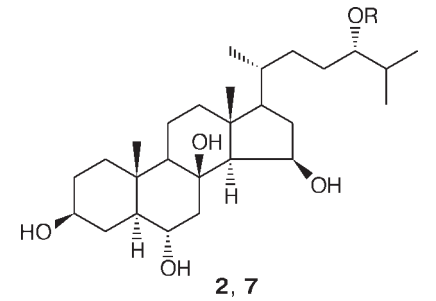
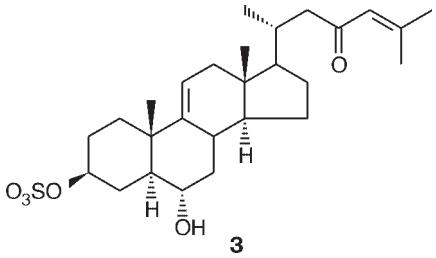


2, **7**



3

R = β-glucopyranosyl-6′-sulfate (**2**), β-xylopyranosyl (**7**)

the corresponding values for previously described 24-methyl-5α-cholest-24(28)-ene-3β,6α,8,15β,16β,26-hexaol from the starfish *Dermasterias imbricata*,[2] indicating that the polycyclic fragments in these molecules are identical and that the carbohydrate residue is located in the side chain of **1**. In particular, the signals at δ 67.6, 71.1, 72.1, 72.8, and 77.2 in the ^{13}C NMR spectrum of glycoside **1** were assigned to the methine C atoms at the OH groups of the pentahydroxycholestane steroid nucleus. The signal of the anomeric carbon atom (δ 105.4) and the signals at δ 66.9, 71.1, 74.9, and 77.9 were ascribed to the carbon atoms of the monosaccharide residue. The signals of protons and side-chain C atoms of **1** coincided with the corresponding values in the spectra of two glycosides from starfishes having identical side chains, namely, coscinasteroside E (*Coscinasterias tenuispina*[3]) and 15-sulfate-(25*S*)-26-*O*-β-D-xylopyranosyl-5α-cholestane-3β,5α,6β,15α,26-hexaol (*Myxoderma platyacanthum*[4]). The slight difference between the chemical shifts of C(27) (18.9 ppm for coscinasteroside E and 17.5 ppm for the second reference compound and glycoside **1**) with complete coincidence of the other signals is, evidently, due to the different steric influence of the functional groups of ring D.

To confirm the structure of glycoside **1**, HMQC correlation experiments between the ^1H and ^{13}C signals were carried out, and the positions of the hydroxy groups were identified from the ^1H—^1H COSY data (Fig. 1).

As a result, it was found that glycoside **1** is (25*S*)-26-*O*-β-D-xylopyranosyl-5α-cholestane-3β,6α,8,15β,16β,26-hexaol. Lysastroside A is one of the three currently known monoglycosides from starfishes whose molecules contain a (25*S*)-26-*O*-β-xylopyranose fragment. The *S*-configuration of the C(25) atom was established by comparison of the NMR spectra with the spectra of 26-hydroxy steroid derivatives with known configurations at C(25).[3,4]

Table 1. NMR spectra (δ, CD_3OD) of lysastroside A (**1**)

Atom	Assignment (DEPT)	δ_C	δ_H (*J*/Hz)
1	CH_2	39.3	
2	CH_2	31.4	
3	CH	72.1	3.46 m
4	CH_2	32.2	
5	CH	53.8	
6	CH	67.6	3.68 m
7	CH_2	49.7	2.38 (dd, *J* = 4, *J* = 12.5)
8	C	77.2	
9	CH	57.4	
10	C	37.9	
11	CH_2	19.4	
12	CH_2	43.4	
13	C	44.5	
14	CH	61.0	1.02 (d, *J* = 6)
15	CH	71.1	4.36 (dd, *J* = 6, *J* = 7.5)
16	CH	72.8	4.23 (t, *J* = 6.7)
17	CH	63.1	
18	CH_3	17.6	1.23 s
19	CH_3	14.0	0.99 s
20	CH	30.9	
21	CH_3	18.4	0.97 (d, *J* = 6.1)
22	CH_2	37.0	
23	CH_2	24.6	
24	CH_2	35.2	
25	CH	34.8	
26	CH_2	76.3	3.60 (dd, *J* = 11, *J* = 6); 3.41 (dd, *J* = 11, *J* = 7.5)
27	CH_3	17.5	0.93 (d, *J* = 6.8)
1′	CH	105.4	4.14 (d, *J* = 7.5)
2′	CH	74.9	3.12 (m, *J* = 7.4, *J* = 9.1)
3′	CH	77.9	3.27 m
4′	CH	71.1	3.43 m
5′	CH_2	66.9	3.80 (dd, *J* = 5.2, *J* = 11.2); 3.13 (dd, *J* = 11.2, *J* = 10.3)

Note. Determined using DEPT, COSY, and HMQC experiments. The spin-spin coupling constants (Hz) are given in parentheses.

Comparison of the NMR spectra and physical parameters of compound **2** (see Experimental) with the corresponding data for the reference compound isolated in our previous study from the starfishes *Distolasterias elegans*[6] and the *Lysastrosoma anthosticta* (the latter collected in the sea of Okhotsk)[1] showed that compound **2** is pycnopodioside C (6′-sulfate-(24*S*)-24-*O*-β-D-glucopyranosyl-5α-cholestane-3β,6α,8,15β,24-pentaol Na salt), which is widely distributed both in tropical[5] and in Far Eastern starfish species.

Comparison of the spectral characteristics of steroid **3** with the corresponding data for marthasterone sulfate, which we isolated previously from the starfish *Lysastrosoma*

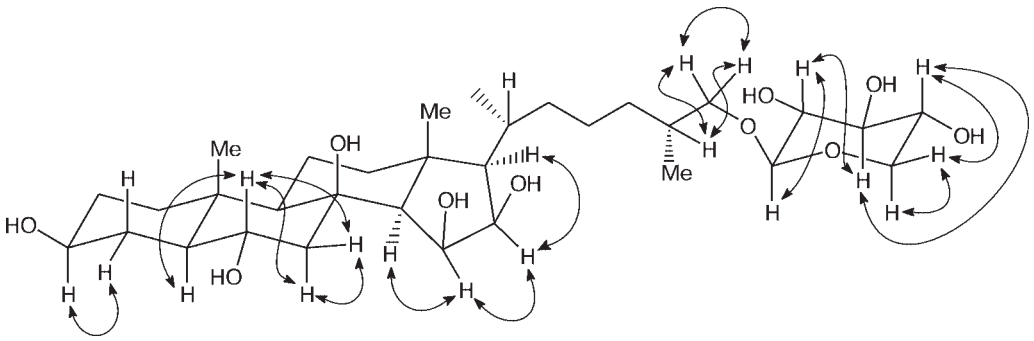


Fig. 1. Some $^1H—^1H$ COSY correlations for lysastroside A.

anthosticta[1] showed that they completely coincide; thus, steroid **3** was identified as 3-sulfate-3β,6α-dihydroxy-5α-cholesta-9(11),24-dien-23-one.

Comparison of the spectral characteristics of steroid **4** and lysastroside **1** demonstrated good agreement between the NMR chemical shifts and spin-spin coupling constants of the protons and the C atoms of the polycyclic fragment of the molecule. The spectra differed only in the signals for C(26) and by the absence of signals corresponding to a monosaccharide residue in the spectrum of steroid **4**. In addition, the NMR spectra of compound **4** were close to those of the hexaol isolated previously from the starfish *Halytile regularis*,[7] which was not, however, fully characterized. Subsequently, this compound was isolated by our research group from the starfish *Crossaster papposus*.[7] Comparison of the physical characteristics and the spectra of **4** with the corresponding data for the reference compound (see Experimental) confirmed that compound **4** was (25*S*)-5α-cholestane-3β,6α,8,15β,16β,26-hexaol.

It was found that compound **5** is (25*S*)-5α-cholestane-3β,6α,7α,8,15α,16β,26-heptaol: the spectra of this compound coincided with the spectra of the heptaol isolated[8] from the starfish *Protoreaster nodosus* and the product that we isolated later[9] from the starfish *Patiria pectinifera* (see Experimental).

Compound **6** was identified by comparison of its spectroscopic characteristics with the corresponding data for (25*S*)-5α-cholestane-3β,6α,7α,8,15β,16β,26-heptaol isolated from the starfish *Pycnopodia helianthoides*.[5] The spectra of these substances were fully identical. To confirm the structure of **6**, $^1H—^{13}C$ HMQC correlation experiments were carried out.

We determined the minimum concentrations (C_{min}) of the isolated compounds that stop the division of the fertilized eggs of the sea-urchin *Strongylocentrotus intermedius* at the first-division stage. Glycoside **1** displayed a moderate cytostatic activity ($C_{min} = 8 \cdot 10^{-5}$ mol L^{-1}). The same activity was found for glycosides containing a sulfo group in the monosaccharide residue (pycnopodioside C (**2**) and luridoside A (**8**) from *Lysastrosoma anthosticta*, a starfish collected in the Sea of Okhotsk).[1] Compounds **2** and **8** inhibited eggs division when present at a concentration of $7 \cdot 10^{-5}$ mol L^{-1}. For pycnopodioside A (**7**), which differs from **8** by the absence of a sulfo group, $C_{min} = 6.3 \cdot 10^{-5}$ mol L^{-1}.

A study of the haemolytic activity of the isolated glycosides in relation to suspensions of mouse blood erythrocytes showed that the concentrations and the kinetic parameters of haemolysis (delay time and the number of erythrocytes lysed per unit time) depend on the numbers of hydroxy and sulfate groups. Glycosides **2**, **7**, and **8** cause haemolysis when the concentration is $3.5 \cdot 10^{-5}$ mol L^{-1}: the delay times are 165, 75, and 270 s, and the haemolysis rates are 0.007, 0.012, and 0.004 s^{-1}, respectively.

The cytostatic activity of hexaol **4** ($C_{min} = 2.6 \cdot 10^{-5}$ mol L^{-1}) proved to be three times as high as that of the corresponding glycoside **1** and higher than those of heptaol **5** or **6** ($5.0 \cdot 10^{-5}$ mol L^{-1}); this is in line with the data on the influence of additional OH groups on the membranotropic activity.[10,11] Previously,[11] it was shown that C_{min} for marthasterone sulfate is $2.9 \cdot 10^{-5}$ mol L^{-1}. Thus, the cytostatic action of polyols and their glycosides on sea-urchin embryos depends appreciably on the arrangement of the hydroxy groups and the presence of sulfate residues.

The composition of the steroid fraction from the starfishes collected in the Sea of Japan differs substantially from the composition of this fraction from the same species of starfish taken from the Sea of Okhotsk. Compounds common to both of them are **2** and **3**, which are the major components in the starfishes from the Sea of Okhotsk (in which their content is 0.00026 and 0.00016%). Recently,[5] we isolated (in 0.00015% yield) and identified one more known glycoside, namely, pycnopodioside A ((24*S*)-24-*O*-β-D-xylopyranosyl-5α-cholestane-3β,6α,8,15β,24-pentaol (**7**)) from the starfishes collected in the Sea of Okhotsk; previously, this compound was found in the starfish *Pycnopodia helianthoides*, but it was not detected in the starfishes occurring in the Sea of Japan, which contained, however, an unusually high amount of pycnopodioside C (**2**) (0.0005% of the animal weight)

and a minor amount of compound **3** (0.00004%). The main components found in this steroid mixture were nonsulfated polyhydroxysteroids **4**—**6** (0.00019—0.0003% of the animal weight), which were not detected at all in the *L. anthosticta* from the Sea of Okhotsk.

Previously,[12] it was shown that some groups of secondary metabolites including steroid compounds can serve as chemotaxonomic markers for various classes of echinoderms. Sulfated and nonsulfated mono- and diglycosylated polyhydroxy steroids are frequently encountered in starfishes. These include compounds **1**, **2**, **7**, and **8**, which we isolated from two collections of *L. anthosticta*. A study of the occurrence of these compounds did not reveal any taxonomic specificity; they are highly diverse in structure and form complex mixtures; in the opinion of some researchers,[13] this is indicative of the diversity of transformation pathways of their biogenetic precursors, food sterols. We showed that fractions of not only glycosides but also other steroid compounds found in the starfishes of different habitats differ substantially in both the quantitative and qualitative aspects. It appears likely that some environmental factors such as the nutrition spectrum and the temperature of water have a pronounced influence on the routes of biochemical transformation of sterols not only into various glycosides but also into free or sulfated polyhydroxysteroids.

Experimental

^{1}H and ^{13}C NMR spectra were recorded on Bruker AC-250 and Bruker DPX-300 spectrometers with working frequencies for protons of 250 and 300 MHz, respectively, using Me_4Si as the internal standard. Optical rotation was measured on a Perkin—Elmer 141 polarimeter. The mass spectra were run on a Bruker Biflex III mass spectrometer (Germany) with laser ionization/desorption (N_2 laser, 337 nm) (MALDI TOF). The sample was dissolved in MeOH (10 mg mL^{-1}), and a 1-μL aliquot was analyzed using α-cyanohydroxycinnamic acid as the matrix. HPLC was carried out on a Du Pont Model 8800 chromatograph (with refractometer as the detector) on columns with Zorbax ODS (5 μm, 250×4.6 mm) and Silasorb C_{18} (13 μm, 250×9.4 mm). Melting points were measured on a Boetius hot stage. Thin layer chromatography (TLC) was carried out on Sorbfil plates with a layer of silica gel STKh-1A (5—17 μm, Russia, Krasnodar) fixed on a foil. Preparative column chromatography was performed on silica gel L (80—100 and 200—250 mesh, Chemapol, Czechia) and Florisil (200—250 mesh).

Samples of the starfish *Lysastrosoma anthosticta* were collected in August 1999 in the Posyet Bay of the Sea of Japan by the diving method from depths of 5—10 m and identified by V. S. Levin (KamchatNIRO, Petropavlovsk-Kamchatski).

Bioassays. The haemolytic activity of glycosides **2**, **7**, and **8** relative to a suspension of mouse blood erythrocytes and the cytostatic activity of compounds **1**—**6** relative to the fertilized eggs of the sea-urchin *Strongylocentrotus intermedius* were determined by the methods described previously.[10,11]

Extraction and isolation of overall fractions. Crushed starfishes (animal weight 15 kg) were extracted exhaustively by 95% EtOH at ~20 °C. The combined ethanolic extract was concentrated *in vacuo* to a wet gum-like residue, which was extracted twice with Bu^{n}OH. The dry residue (90 g) remaining after evaporation of butanol was chromatographed on a column (6×25 cm) with silica gel (80—100 mesh) in the $CHCl_3$—EtOH system (100 : 0 → 45 : 55). The increase in the eluent polarity gave three successive fractions of polyhydroxy steroids, namely, a less polar fraction (**I**) containing polyhydroxy steroids **4**—**6** (TLC, BuOH—EtOAc—H_2O, 5 : 1 : 1, R_f 0.60—0.65), a more polar fraction (**II**) consisting of glycoside **1** with sterol sulfate impurities (TLC, R_f 0.45—0.50), and a polar fraction (**III**) consisting of compounds **2** and **3** (TLC, R_f 0.45—0.50).

Isolation of compounds 1, 4—6. Fraction **I** was dissolved in water and passed through a column with Polychrome (5×20 cm), the column being washed with water and a 50% aqueous EtOH. The aqueous-ethanol eluate was concentrated *in vacuo* to give a brown gum-like residue (0.7 g) containing a wet mixture of nonsulfated steroids. Chromatography of this mixture on a column with Sephadex LH-60 (40×1.5 cm) in the $CHCl_3$—EtOH system (4 : 1) yielded a dry residue (0.39 g), which was chromatographed on a column with Florisil (1.5×20 cm, 60—100 mesh) in the $CHCl_3$—EtOH system (6 : 1 → 5 : 1). The resulting compounds were separated by HPLC on a column with Zorbax ODS (13 μm, 9.4 mm × 25 cm, 1 mL min^{-1}) in the EtOH—water system (75 : 25). This gave 45 mg of steroid **4**, 51 mg of **5**, and 30 mg of polyol **6**. The more polar fraction **II** was purified on a column with Polychrome, as described above. The eluate in aqueous ethanol was concentrated and chromatographed on columns with silica gel (2×23 cm, 200—250 mesh) in the $CHCl_3$—EtOH system (6 : 1) and Florisil (60—100 mesh) in the same system. The fraction containing lysastroside A (**1**) was subjected to HPLC on a column with Zorbax ODS (13 μm, 9.4 mm × 25 cm, 1 mL min^{-1}) in the EtOH—water system (70 : 30) to give 8 mg of compound **1**.

Isolation of compounds 2 and 3. Fraction **III** containing sulfates **2** and **3** was passed successively through columns with Polychrom, silica gel, and Florisil in the systems described previously[1] to give 85 mg of glycoside **2** and 6 mg of marthasterone sulfate **3**.

Lysastroside A, (25*S*)-26-O-β-D-xylopyranosyl-5α-cholestane-3β,6α,8,15β,16β,26-hexaol (1), $C_{32}H_{56}O_{10}$, amorphous, $[\alpha]^{20}_D$ +5.6 (*c* 1.2, EtOH), isolated in 0.00005% yield with respect to the animal weight. MALDI-TOF MS (+): *m/z* 623 $[M + Na]^+$ (100). The ^{1}H and ^{13}C NMR spectra (MeOH) are given in Table 1. Interpretation of the COSY spectrum is given in Fig. 1.

Pycnopodioside C (2), $C_{33}H_{57}NaO_{13}S$, colorless crystals, m.p. 178—179 °C (from MeOH), $[\alpha]^{20}_D$ +4.5 (*c* 2.5, EtOH), isolated in 0.0005% yield. MALDI TOF MS (+): *m/z* 739 $[M + Na]^+$ (100). The ^{1}H and ^{13}C NMR spectra (MeOH) are identical with those reported in the literature.[5]

Marthasterone sulfate (3), $C_{27}H_{41}NaO_6S$, was isolated in 0.00004% yield; the spectra and parameters of the compound were identical with those that we reported previously.[1]

(25*S*)-5α-Cholestane-3β,6α,8,15β,16β,26-hexaol (4), $C_{27}H_{48}O_6$, colorless crystals, m.p. 261—263 °C (from MeOH), $[\alpha]^{20}_D$ +37.8 (*c* 2.5, EtOH), isolated in 0.0003% yield; the spectroscopic data and parameters of the product were identical with those described for the hexaol isolated from *Crossaster papposus*.[7]

(25*S*)-5α-Cholestane-3β,6α,7α,8,15α,16β,26-heptaol (5), $C_{27}H_{48}O_7$, isolated in 0.00034% yield, colorless crystals, m.p. 255—258 °C (from EtOH), $[\alpha]^{20}_D$ +23.8 (*c* 1.5, MeOH). The NMR spectra (CD_3OD) are identical with those reported in the literature.[8]

(25*S*)-5α-Cholestane-3β,6α,7α,8,15β,16β,26-heptaol (6), $C_{27}H_{48}O_7$, isolated in 0.00019% yield, amorphous, $[\alpha]^{20}_D$ +14 (*c* 0.8, MeOH); the spectroscopic data (CD_3OD) are identical with the published data[5] and confirmed by the data of HMQC experiments.

Pycnopodioside A (7) $C_{32}H_{56}O_9$ was isolated in 0.00015% yield from the starfishes collected in the Sea of Okhotsk,[1] amorphous, $[\alpha]^{20}_D$ +3.1 (*c* 2.5, EtOH). The NMR spectra (CD_3OD) are identical with published data.[5]

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