New Steroid Glycosides from the Starfish *Asterias rathbuni*

Natalia V. Ivanchina, Alla A. Kicha, Anatoly I. Kalinovsky, Pavel S. Dmitrenok, Nina G. Prokof'eva, and Valentin A. Stonik*
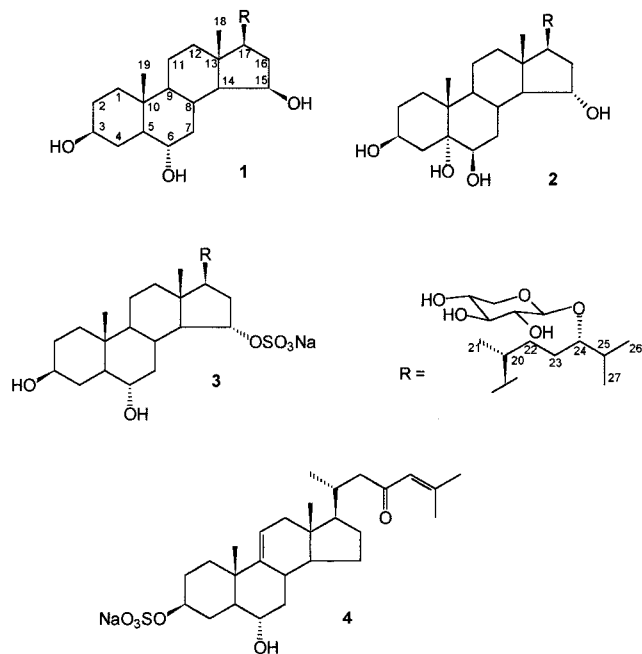
Pacific Institute of Bioorganic Chemistry of the Far East Branch of the Russian Academy of Science, Vladivostok-22, Russian Federation

Two novel steroidal 24-*O*-xylosides, designated as rathbuniosides R$_1$ (**1**) and R$_2$ (**2**), and the known amurensoside A (**3**) and 3-*O*-sulfomarthasterone (**4**) have been isolated from the starfish *Asterias rathbuni.* The structures of all the compounds were determined from their spectroscopic data, including one- and two-dimensional NMR methods. The compounds **1** and **4** inhibit the cell division of fertilized sea urchin eggs at doses of 7.0×10^{-5} and 2.9×10^{-5} M, respectively.

The steroids isolated from starfish as a rule are very complex mixtures of highly oxygenated compounds, many of which have no counterpart within the entire animal kingdom. In a continuation of our long-term search for new polyhydroxylated steroids and related natural products from starfish,[1,2] we have turned our attention to the steroid content of the starfish *Asterias rathbuni* Verrill (order Forcipulata, family Asteriidae), collected by trawling from a depth of 90 m in the Bering Sea.

The water-soluble materials from the alcoholic extracts of *A. rathbuni* were sequentially submitted to column chromatography on Amberlite XAD-2, Sephadex LH-20, Si gel, and Florisil. Final separation and isolation was achieved by reversed-phase HPLC on a Silasorb C$_{18}$ column to give the steroids **1−4**.

The pseudomolecular ion *m/z* 591.3876 [M + Na]$^+$ in the positive MALDI-TOF mass spectrum as well as the ^{13}C and ^{1}H NMR spectral data of rathbunioside R$_1$ (**1**) indicated the molecular formula C$_{32}$H$_{56}$O$_8$. The ^{13}C NMR and DEPT spectra (Table 1) showed the presence of 32 carbon atoms,

Table 1. ^{13}C NMR and DEPT Data (C$_5$D$_5$N) of Rathbuniosides R$_1$ (**1**) and R$_2$ (**2**)

C	**1** DEPT	**1** δ	**2** DEPT	**2** δ
1	CH$_2$	38.0	CH$_2$	32.1
2	CH$_2$	32.1	CH$_2$	33.2
3	CH	71.0	CH	67.2
4	CH$_2$	33.6	CH$_2$	41.6
5	CH	52.9	C	75.6
6	CH	68.8	C	76.1
7	CH$_2$	41.8	CH$_2$	35.9
8	CH	30.9	CH	30.9
9	CH	54.6	CH	45.9
10	C	36.6	C	39.0
11	CH$_2$	21.5	CH$_2$	21.5
12	CH$_2$	41.6	CH$_2$	42.6
13	C	42.6	C	43.9
14	CH	61.3	CH	63.2
15	CH	69.0	CH	73.0
16	CH$_2$	42.1	CH$_2$	40.8
17	CH	56.7	CH	54.2
18	CH$_3$	15.0	CH$_3$	13.6
19	CH$_3$	13.6	CH$_3$	17.1
20	CH	36.1	CH	35.8
21	CH$_3$	19.1	CH$_3$	18.8
22	CH$_2$	32.2	CH$_2$	32.3
23	CH$_2$	28.2	CH$_2$	28.2
24	CH	84.5	CH	84.4
25	CH	31.0	CH	31.0
26	CH$_3$	18.0	CH$_3$	18.1
27	CH$_3$	18.2	CH$_3$	17.9
Xyl$_p$				
1	CH	104.9	CH	104.8
2	CH	75.2	CH	75.2
3	CH	78.4	CH	78.3
4	CH	71.1	CH	71.0
5	CH$_2$	67.0	CH$_2$	67.0

including five methyl groups, nine methylenes, 16 methines, and two quaternary carbons. The signal at δ 104.9 ppm was assigned to an anomeric carbon, and the signals at δ 67.0, 68.8, 69.0, 71.0, 71.1, 75.2, 78.4, and 84.5 ppm were assigned to oxygen-bearing carbons. Along with the presence of the doublet signal at δ 4.83 (*J* = 7.5 Hz) in the ^{1}H NMR spectrum, these data were indicative of a β-pentoside fragment and tetrahydroxy-substituted cholestane nucleus. Positions and configurations of hydroxyl groups in the aglycon moiety of **1** were established by 1D NMR spin-decoupling experiments as well as ^{1}H−^{1}H COSY. The correlation between ^{1}H and ^{13}C NMR spectra was established by HMQC and HMBC data (Table 2). The chemical shifts of C-1−C-13 and H-3, H-4a, H-4e, H-5, H-6, H-7a,

* To whom correspondence should be addressed. Tel: 7 (4232) 312360. Fax: 7 (4232) 314050. E-mail: piboc@stl.ru.

Table 2. ^{1}H NMR and 2D Correlation Data (C_5D_5N) of Rathbuniosides R_1 (**1**) and R_2 (**2**)[a]

	1		**2**
	δ_H	HMBC	
2H-1	1.75 m, 1.1 m		
2H-2	2.11 m, 1.77 m		
H-3	3.99 m		4.88 m
H-4a	1.70 m		3.00 dd (11.0, 12.5)
H-4e	3.05 m		2.36 dd (5.0, 12.5)
H-5	1.45 m		
H-6	3.79 dt (3.8, 10.6)		4.23 m
H-7a	1.43 m		2.78 m
H-7e	3.05 m		2.78 m
H-8	2.25 dq (4.0, 11.2)		2.46 m
H-9	0.86 m		
2H-11	1.57 m, 1.39 m		
2H-12	2.01 m, 1.16 m		
H-14	1.00 dd (5.5, 11.2)		1.63 m
H-15	4.48 brt (~6.4)		4.25 m
H-16	2.60 td (7.5, 14.6)		2.18 m
H-16′	1.73 m		2.03 m
H-17	1.20 m		
CH$_3$-18	1.25 s	C-12, C-13, C-14, C-17	0.80 s
CH$_3$-19	0.92 s	C-1, C-5, C-9, C-10	1.72 s
H-20	1.73 m		
CH$_3$-21	1.09 d (6.7)		1.01 d (6.2)
2H-22	1.92 m, 1.31 m		
2H-23	1.88 m, 1.71 m		
H-24	3.73 m		3.68 m
H-25	2.09 m		2.045 m
CH$_3$-26	1.09 d (6.7)	C-24, C-25, C-27	1.03 d (6.8)
CH$_3$-27	1.12 d (6.7)	C-24, C-25, C-26	1.11 d (6.8)
Xyl$_p$			
H-1′	4.83 d (7.5)	C-24	4.87 d (7.4)
H-2′	4.03 t (8.0)		4.01 t (7.8)
H-3′	4.18 t (8.5)		4.17 t (8.4)
H-4′	4.23 m		4.24 m
H-5′	4.39 dd (4.9, 11.2)		4.39 dd (4.9, 11.0)
H-5″	3.75 t (11.2)		3.72 t (10.9)

[a] J (Hz) values are shown in parentheses. Assignments from 300 MHz ^{1}H–^{1}H COSY, HMQC, and HMBC data.

and H-7e coincided with those of the previously described 5α-cholestane-$3\beta,6\alpha,15\beta,16\beta,26$-pentaol from the Far Eastern starfish *Ceramaster patagonicus*.[1] On this basis we assigned the hydroxyl groups to the 3β- and 6α-positions.

The sequence of CH-6, CH$_2$-7, CH-8, CH-14, CH-15, CH$_2$-16, and CH-17 was deduced by spin-decoupling experiments and ^{1}H–^{1}H COSY and HMQC data. The assignment of these signals (Table 2) suggested the presence of a hydroxyl group at C-15. According to literature data, the coupling constant $J_{14,15}$ of 10.0 Hz and practically identical H-16′ and H-16 chemical shifts are characteristic for the steroids having a 15α-hydroxyl group.[3,4] On the contrary, the coupling constant $J_{14,15}$ of 5.5 Hz and chemical shifts of H-16 and H-16′ differing from each other by 0.8 ppm were indicated in the spectra of 15β-hydroxysteroids.[3,5] The observed proton coupling constant $J_{14,15}$ was found to be 5.5 Hz and the chemical shifts of H-16 (δ 2.60) and H-16′ (δ 1.73) differed from each other by 0.87 ppm in the ^{1}H NMR spectrum of **1** (Table 2). These data demonstrated that a hydroxyl group at C-15 has β-configuration.

Acid hydrolysis of **1** gave d-xylose (TLC, GLC, optical rotation). The spectral data also showed the presence of the β-xylopyranosyl unit in **1** (Tables 1, 2). The chemical shifts of the protons and carbons of the side chain and the coupling constants of protons of the monosaccharide residue of **1** were virtually identical with those of 24(*S*)-24-*O*-(β-xylopyranosyl)-5α-cholestane-$3\beta,6\alpha,8,15\alpha,24$-pentaol, obtained from the desulfation of helianthoside from *Heliaster*

helianthus.[6] This suggested the attachment of a β-xylopyranosyl unit to C-24 in the side chain and 24*S*-configuration in **1**. In addition, the selective irradiation of H-24 at δ 3.73 or doublets of CH$_3$-26 (δ 1.09) and CH$_3$-27 (δ 1.12) produced the same multiplet H-25 at δ 2.09 under conditions of difference decoupling, while the irradiation of H-25 transformed the doublets of CH$_3$-26 and CH$_3$-27 into singlets. Moreover, we have observed correlations between C-24 and anomeric H-1′, CH$_3$-26, and CH$_3$-27 with HMBC experiments. Thus, the structure of rathbunioside R_1 (**1**) was established as 24(*S*)-24-*O*-(β-d-xylopyranosyl)-5α-cholestane-$3\beta,6\alpha,15\beta,24$-tetraol. The $3\beta,6\alpha,15\beta,24$-tetrahydroxy substitution has been found for the first time in steroids from starfish.

The molecular formula of rathbunioside R_2 (**2**) was deduced to be $C_{32}H_{56}O_9$ on the basis of the pseudomolecular ion m/z 607.3877 [M + Na]$^+$ in the positive MALDI-TOF mass spectrum and NMR data. The ^{13}C NMR and DEPT spectra of **2** (Table 1) showed peaks for 32 carbons, including five belonging to a β-xylopyranosyl moiety closely similar to those from the spectrum of **1**. The aglycon part of **2** contained three quaternary carbons, 10 methines, nine methylenes, and five methyl groups, including four secondary (δ 67.2, 73.0, 76.1, 84.4) and one tertiary (δ 75.6) oxygen-bearing carbon. On this basis we suggested that **2** is a β-xylopyranoside of a cholestane pentaol.

The ^{1}H and ^{13}C NMR data of the steroidal nucleus in **2** (Tables 1 and 2) closely resembled those of (22*E*)-24-methyl-5α-cholest-22-en-$3\beta,5,6\beta,15\alpha,25,26$-hexaol, obtained by desulfation of the corresponding 26-sulfate from the Far Eastern starfish *Ctenodiscus crispatus*.[7] The 3β, 5α, 6β, and 15α positions of hydroxyl groups in **2** were confirmed by 1D spin-decoupling, ^{1}H–^{1}H COSY, and HMQC experiments.

The signals of the carbons and protons of the side chain and xylose residue in the spectra of **2** were practically identical to those of **1**. Only the signal of H-1′ resonated at δ 4.87 in the spectrum of **2** compared to δ 4.83 in **1**. This was probably due to the difference between substitutions in the steroidal nucleus of both glycosides. These data showed the presence of the 24-*O*-β-d-xylopyranosyl unit attached to position 24 of the steroidal aglycon with 24*S* configuration at C-24. The position of a xylopyranosyl unit at C-24 was confirmed by NOE enhancement of H-24 (δ 3.68) after irradiation of H-1′ (δ 4.87). On the basis of the data discussed above, the structure of rathbunioside R_2 (**2**) was determined as 24(*S*)-24-*O*-(β-d-xylopyranosyl)-5α-cholestane-$3\beta,5,6\beta,15\alpha,24$-pentaol.

The positive MALDI-TOF mass spectrum of the glycoside **3** showed a pseudomolecular cation peak at m/z 693 [M + Na]$^+$, and the negative MALDI-TOF mass spectrum of **3** exhibited a pseudomolecular anion peak at m/z 647 [M − Na]$^-$. The ^{1}H and ^{13}C NMR spectra of **3** were identical with those of amurensoside A from *Asterias amurensis*.[8] On this basis, **3** was identified as amurensoside A.

Steroid **4** showed a pseudomolecular anion peak at m/z 493 [M − Na]$^-$ in the negative MALDI-TOF mass spectrum. The NMR spectra were very similar to those of an aglycon moiety of marthasteroside B from *Marthasterias glacialis*.[9] However, there were differences in the ^{13}C NMR resonances of C-5, C-6, and C-7, suggesting that a carbohydrate chain at C-6 is absent in **4**. The observed signals of H-3 (δ 4.21) and H-6 (δ 3.51) in the ^{1}H NMR spectrum of **4** were identical to those of 3-*O*-sulfotornasterol A from *Aphelasterias japonica*.[10] This suggested the presence of an O-sulfate at the 3β-position and a nonglycosylated 6α-hydroxyl group in the steroid **4**. Spin-decoupling experi-

ments confirmed the structure of the side chain of **4**. The selective irradiation of H-24 (δ 6.17) produced the collapse of CH$_3$-26 (δ 1.91) and CH$_3$-27 (δ 2.11). Thus, compound **4** is a genuine aglycon of marthasteroside B and was identified as 3-*O*-sulfomarthasterone.

Compounds **1** and **4** showed 100% inhibition of cell division of the fertilized eggs of the sea urchin *Strongylocentrotus intermedius* at doses of 7.0×10^{-5} and 2.9×10^{-5} M, respectively. Steroids **2** and **3** demonstrated the lesser cytostatic effects, with IC$_{100}$ 1.7×10^{-4} and 1.9×10^{-4} M, respectively.

Experimental Section

General Experimental Procedures. Optical rotations were determined on a Perkin-Elmer 141 polarimeter. The ^{1}H and ^{13}C NMR spectra were recorded on a Bruker AC-250 spectrometer at 250 and 62.9 MHz, respectively, and on a Bruker DPX 300 spectrometer at 300 and 75 MHz, respectively, with tetramethylsilane as an internal standard. MALDI-TOF mass spectra were recorded on a Bruker Biflex III laser-desorption mass spectrometer coupled with delayed extraction using an N$_2$ laser (337 nm). Samples of compound were dissolved in MeOH (1 mg/mL). Aliquots (1 μL) were analyzed. 2,5-Dihydroxybenzoic acid (DHB) was used as the matrix. GLC analysis was carried out using a Tsvet-110 apparatus, with a glass column (0.3 $\times$ 150 cm) containing 1.5% QF-1 and Chromatone N-HMDS as the stationary solid phase. The following experimental conditions were used: carrier gas, Ar at 60 mL/min, column temperature from 165 to 225 °C, 4 °C /min. HPLC separations were conducted on a column with Silasorb C$_{18}$ (13 μ, 250 $\times$ 9.4 mm) using a DuPont 8800 chromatograph equipped with a differential refractometer.

Low-pressure column liquid chromatography was performed using Amberlite XAD-2 (20−80 mesh, Sigma, Chemical Co), Sephadex LH-20 (Sigma, Chemical Co), Si gel L (40/100 μm, Chemapol, Praha, Czech Republic), and Florisil (100−200 mesh, Koch-Light Laboratories Ltd., U.K.). Si gel plates, 4.5 $\times$ 6.0 cm (5−17 μ, Sorbfil, Russia), were used for thin-layer chromatography.

Animal Material. Specimens of *Asterias rathbuni* were collected by dredging in August 1991 at a depth of 90 m near Unimak Island in the Bering Sea (research vessel *Akademik Oparin*, 14th scientific cruise). Species identification was carried out by Dr. A. V. Smirnov (Zoological Institute of the Russian Academy of Science, St. Petersburg, Russia). A voucher specimen [no. 014-56(24)] is on deposit at the collection of the Zoological Institute, St. Petersburg, Russia.

Extraction and Isolation. The lyophilized animals (1.9 kg) were chopped and extracted twice with EtOH. The combined extracts were evaporated, and the residue was dissolved in H$_2$O (3 L). The H$_2$O-soluble fraction was passed through an Amberlite XAD-2 column (6 $\times$ 20 cm) and eluted with distilled H$_2$O until a negative chloride ion reaction was obtained, followed by elution with MeOH. The combined MeOH eluate was evaporated to give a brownish material (15.7 g) that was chromatographed on a Sephadex LH-20 column (3 $\times$ 50 cm) with MeOH/H$_2$O (2:1). Two subfractions containing mixtures of polyhydroxylated steroids were purified by chromatography on a Si gel column (4 $\times$ 15 cm) using CHCl$_3$/EtOH (8:1 → 1:2), and each subfraction was chromatographed on Florisil columns (1.7 $\times$ 7 cm) using the system CHCl$_3$/EtOH (8:1 → 2:1). Fractions were analyzed by TLC on Si gel using the eluent system CHCl$_3$/MeOH/H$_2$O (30:15:2) and detected by spraying with H$_2$SO$_4$.

HPLC of the obtaining fractions on a Silasorb C$_{18}$ column (13 μ, 250 $\times$ 9.4 mm, 3 mL/min) with MeOH/H$_2$O (70:30) as the eluent system yielded **1** (25 mg, R_f 0.7), **2** (3 mg, R_f 0.58), **3** (4.5 mg, R_f 0.4), and **4** (15 mg, R_f 0.65).

Rathbunioside R$_1$ (1): C$_{32}$H$_{56}$O$_8$; amorphous powder; [α]$_D$ $-0.2°$ (*c* 1.6, MeOH); ^{1}H and ^{13}C NMR data, see Tables 1 and 2; MALDI-TOF(+) *m/z* 591.3876 [M + Na]$^+$ (calcd for C$_{32}$H$_{56}$O$_8$Na, 591.3867).

Acid Hydrolysis of 1. A solution of glycoside **1** (9 mg) in aqueous 2 N CF$_3$COOH (3 mL) was heated at 100 °C for 2 h in a sealed ampule. The reaction mixture was evaporated in vacuo. The obtained residue was dissolved in water and extracted with CHCl$_3$ twice. Xylose was identified in the aqueous layer by TLC on Si gel in BuOH/EtOH/H$_2$O (4:1:2) and by GLC as aldononitrile peracetate using standard aldononitrile peracetate of xylose as a reference sample. The d-configuration of xylose was assigned by the observed optical rotation [α]$_D$ $+9.5°$ (*c* 0.5, H$_2$O).

Rathbunioside R$_2$ (2): C$_{32}$H$_{56}$O$_9$; amorphous powder; [α]$_D$ $+7.7°$ (*c* 0.3, MeOH); ^{1}H and ^{13}C NMR data, see Tables 1 and 2; MALDI-TOF(+) *m/z* 607.3877 [M + Na]$^+$ (calcd for C$_{32}$H$_{56}$O$_9$Na, 607.3817).

Amurensoside A (3): amorphous powder; [α]$_D$ $+13.1°$ (*c* 0.45, MeOH); ^{1}H and ^{13}C NMR data were identical with those for amurensoside A from *Asterias amurensis*;[8] MALDI-TOF(+) *m/z* 693 [M + Na]$^+$; MALDI-TOF(−) *m/z* 647 [M − Na]$^-$.

3-*O*-Sulfomarthasterone (4): amorphous powder; [α]$_D$ $+4.2°$ (*c* 0.65, MeOH); ^{1}H NMR (CD$_3$OD) δ 0.68 (3H, s, H$_3$-18), 0.91 (3H, d, *J* = 6.2 Hz, H$_3$-21), 0.98 (3H, s, H$_3$-19), 1.91 (3H, d, *J* = 1.2 Hz, H$_3$-26), 2.11 (3H, d, *J* = 1.2 Hz, H$_3$-27), 3.51 (1H, dt, *J* = 4.2, 10.8 Hz, H-6), 4.21 (1H, m, H-3), 5.34 (1H, d, *J* = 5.7 Hz, H-11), 6.17 (1H, brs, H-24); ^{13}C NMR (C$_5$D$_5$N) δ 36.0 (C-1), 29.2 (C-2), 78.3 (C-3), 30.9 (C-4), 50.4 (C-5), 68.2 (C-6), 41.7 (C-7), 35.8 (C-8), 146.0 (C-9), 38.1 (C-10), 116.2 (C-11), 41.1 (C-12), 41.1 (C-13), 53.6 (C-14), 25.3 (C-15), 28.5 (C-16), 56.4 (C-17), 11.5 (C-18), 19.1 (C-19), 32.9 (C-20), 19.5 (C-21), 51.4 (C-22), 200.4 (C-23), 124.7 (C-24), 153.8 (C-25), 20.3 (C-26), 27.1 (C-27); MALDI-TOF(−) *m/z* 493 [M − Na]$^-$.

Bioassay. Compounds **1**−**4** were tested for the inhibition of cell division of the fertilized eggs of the sea urchin *Strongylocentrotus intermedius* as previously described.[11]

References and Notes

(1) Kicha, A. A.; Kalinovsky, A. I.; Stonik, V. A. *Russ. Chem. Bull.* **1997**, *46*, 186−191.
(2) Ivanchina, N. V.; Kicha, A. A.; Kalinovsky, A. I.; Dmitrenok, P. S.; Stonik, V. A.; Riguera, R.; Jimenéz, C. *J. Nat. Prod.* **2000**, *63*, 1178−1181.
(3) Kicha, A. A.; Kalinovsky, A. I.; Gorbach, N. V.; Stonik, V. A. *Khim. Prir. Soedin.* **1993**, *2*, 249−253.
(4) Kicha, A. A.; Kalinovsky, A. I. *Khim. Prir. Soedin.* **1993**, *4*, 619−620.
(5) Kicha, A. A.; Kalinovsky, A. I.; Stonik, V. A. *Khim. Prir. Soedin.* **1993**, *2*, 257−260.
(6) Vazquez, M. J.; Quinoa, E.; Riguera, R.; San Martin, A. *Can. J. Chem.* **1993**, *71*, 1147−1151.
(7) Kicha, A. A.; Kalinovsky, A. I.; Ivanchina, N. V.; El'kin, Yu. N.; Stonik, V. A. *Russ. Chem. Bull.* **1994**, *43*, 1726−1730.
(8) Riccio, R.; Iorizzi, M.; Minale, L.; Oshima, Y.; Yasumoto, T. *J. Chem. Soc., Perkin Trans. 1* **1988**, 1337−1347.
(9) Minale, L.; Riccio, R.; Zollo, F. In *Progress in the Chemistry of Organic Natural Products*; Herz, W., Kirby, G. W., Moore, R. E., Steglich, W., Tamm, Ch., Eds.; Springer-Verlag: Wien−New York, 1993; Vol. 62, pp 75−308.
(10) Finamore, E.; Zollo, F.; Minale, L.; Yasumoto, T. *J. Nat. Prod.* **1992**, *55*, 279−282.
(11) Kuznetsova, T. A.; Prokof'eva, N. G.; Chaikina, E. L.; Afiyatullov, Sh. Sh. In *Biodiversity and Allelopathy: from Organisms to Ecosystems in the Pacific*; Chou, C. H., Waller, G. R., Reinhardt, C., Eds.; Academia Sinica: Taipei, 1999; pp 193−197.