Polar Steroidal Compounds from the Far Eastern Starfish *Henricia leviuscula*

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Received September 28, 2005

Five new polar steroids, polyhydroxysterols **2**−**5** and the glycoside leviusculoside J (**7**), were isolated, along with the previously known compounds **1**, **6**, **8**, and **9**, from the alcoholic extract of the Far Eastern starfish *Henricia leviuscula*. The structures of novel compounds were elucidated by interpretation of spectral data (mainly 2D-NMR), and the stereochemistry of chiral centers in the side chain of sterols **2** and **3** was determined by using *J*-based configuration analysis and the modified Mosher's method. Steroids **1**, **3**, **6**, **7**, and **9** showed moderate hemolytic activity in the mouse erythrocytes assay.

Starfishes contain a great number of polar natural products, especially steroids,[1,2] that are generally present as very complex mixtures of highly oxygenated compounds, many of which have no counterpart in the entire animal kingdom. In a continuation of our investigation on the polar steroidal constituents from Far Eastern starfishes,[3,4] we have examined the alcoholic extract of the starfish *Henricia leviuscula* (Stimpson) (Spinulosida, Echinasteridae) collected from the Sea of Okhotsk.

Results and Discussion

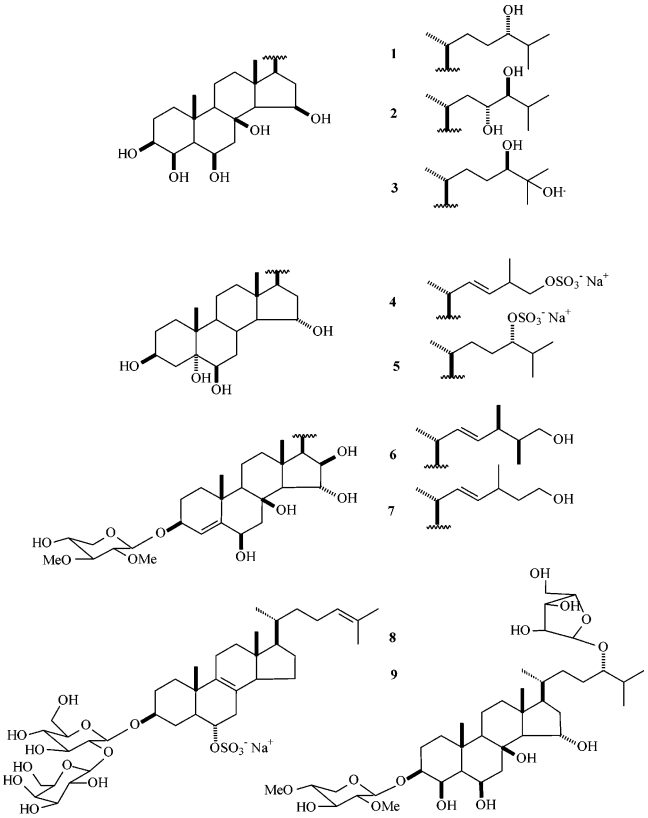
The water-soluble material from the EtOH extracts of *H. leviuscula* was sequentially subjected to column chromatography on Amberlite XAD-2, Sephadex LH-20, and Si gel. Final separation of steroidal fractions and isolation of individual compounds were achieved by reversed-phase HPLC on a Zorbax ODS and YMC-Pack ODS-A columns to give five polyhydroxysteroids (**1**−**5**) and four glycosides of polyhydroxysteroids (compounds **6**−**9**).

Polyhydroxysteroids **2**−**5** and steroidal glycoside **7**, named leviusculoside J, were unprecedented molecules. The known compounds were identified as (24*S*)-5α-cholestane-3*β*,4*β*,6*β*,8,15*β*,-24-hexaol (**1**), henricioside H_2 (**6**),[5] and laeviuscolosides A (**8**) and G (**9**).[6]

Polyhydroxysteroid **1** was identified as 5α-cholestane-3*β*,4*β*,6*β*,8,-15*β*,24-hexaol by comparison of its MS and NMR data with those reported.[5] This compound was earlier isolated from the starfish *Henricia derjugini*.[5] The 24*S* stereochemistry in the isolated compound was determined on the basis of the identity of the ^{1}H NMR spectrum of 3,24-di-*R*-(+)-MTPA ester of **1** with those of hexaol from *H. derjugini*.[7]

Compounds **2** and **3** exhibited the same molecular formula, $C_{27}H_{48}O_7$, as deduced by both HR (+)-MALDI-TOF MS (pseudomolecular peaks at *m*/*z* 507.3272 $[M + Na]^+$ for **2** and at *m*/*z* 507.3267 $[M + Na]^+$ for **3**) and ^{13}C NMR spectra (Tables 1 and 2). The (+)-LSI MS of **2** and **3** were very similar, but the (−)-LSI MS of these compounds differed in the peaks indicating the losses of the corresponding fragments from side chains of these steroids (see Experimental Section).

Detailed comparison of spectral data of steroidal nuclei of these compounds with those of hexaol **1** clearly showed that both **2** and **3** have the 3*β*,4*β*,6*β*,8,15*β*-pentahydroxysteroidal nucleus, the same as **1**, and differ from each other only in the side-chain substitution pattern.



The side chain of steroid **2** exhibited three methyl, one methylene, and four methine groups, two of which linked to oxygen (signals at *δ* 73.2 and 81.4, Table 2). ^{1}H−^{1}H COSY, HSQC, and HMBC experiments led to the assignment of all proton and carbon values (Table 2). In particular, the presence in the ^{1}H NMR spectrum of three 3H doublets at *δ* 0.92 (J = 6.8 Hz), 0.94 (J = 6.8 Hz), and 1.06 (J = 6.6 Hz), assigned to CH_3-27, CH_3-26, and CH_3-21, respectively, of a double doublet at *δ* 3.11 (H-24, J = 4.9, 6.4 Hz), and of a multiplet at *δ* 3.55 (H-23) indicated a 23,24-dihydroxycholestane side-chain moiety for compound **2**. The relative configuration at chiral centers C-23 and C-24 was determined by *J*-based configuration analysis.[8] Spin-coupling constants $^3J_{H,H}$ and $^{2,3}J_{C,H}$ were measured from the ^{1}H NMR spectrum and by hetero-half-filtered TOCSY (HETLOC) experiments, respectively. Unfortunately, the small magnetization transfer observed in TOCSY experiment hampered the accurate measure-

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10.1021/np050373j CCC: $33.50
Published on Web 01/13/2006

Table 1. ^{1}H and ^{13} NMR Data (CD_3OD) for the Steroidal Nucleus of Compounds **1**−**3**, **4**/**5**, and **6**/**7**[a]

	1−**3**			**4**/**5**			**6**/**7**	
carbon	δ_C mult[b]	δ_H[c] mult	HMBC	δ_C mult[b]	δ_H[c] mult	HMBC	δ_C mult[b]	δ_H[c] mult
1	40.9 (CH_2)	1.00 m, 1.73 m		31.7 (CH_2)	1.48 m, 1.77 m		39.7 (CH_2)	1.29 m, 1.78 m
2	26.6 (CH_2)	*ax* 1.90 m *eq* 1.63 m		33.6 (CH_2)	*ax* 1.75 m *eq* 1.50 m		27.9 (CH_2)	*ax* 1.97 m *eq* 1.75 m
3	73.2 (CH)	3.50 ddd (3.7, 5.0, 11.9)		68.4 (CH)	4.00 m		77.5 (CH)	4.20 m
4	77.4 (CH)	4.08 brs		41.5 (CH_2)	*ax* 2.05 dd (11.2, 13.0) *eq* 1.54 m		126.9 (CH)	5.67 brs
5	51.0 (CH)	1.27 t (2.1)		76.6 (qC)			148.5 (qC)	
6	76.4 (CH)	4.26 m		76.4 (CH)	3.50 brs		76.4 (CH)	4.31 t (3.0)
7	44.1 (CH_2)	*ax* 1.66 dd (3.3, 14.3) *eq* 2.41 dd (3.3, 14.1)		35.3 (CH_2)	1.85 m		44.4 (CH_2)	*ax* 1.48 m *eq* 2.56 dd (3.1, 14.5)
8	79.1 (qC)			30.8 (CH)	1.28 m		76.2 (qC)	
9	57.4 (CH)	1.00 m		46.6 (CH)	1.17 m		57.9 (CH)	1.04 m
10	36.9 (qC)			39.3 (qC)			37.7 (qC)	
11	19.3 (CH_2)	*ax* 1.92 m *eq* 1.52 m		22.2 (CH_2)	1.35 m		19.5 (CH_2)	1.46 m, 1.87 m
12	43.1 (CH_2)	*ax* 1.20 m *eq* 2.04 m		41.7 (CH_2)	1.23 m		43.0 (CH_2)	1.20 m, 1.96 m
13	44.3 (qC)			45.0 (qC)			45.0 (qC)	
14	61.8 (CH)	1.00 d (5.4)	C-13, C-18	63.6 (CH)	1.12 m		63.7 (CH)	1.03 m
15	71.4 (CH)	4.41 ddd (1.9, 5.5, 7.4)		74.3 (CH)	3.85 dt (3.0, 9.4)		80.3 (CH)	4.16 dd (2.6, 8.1)
16	42.0 (CH_2)	1.45 m, 2.40 m		41.8 (CH_2)	1.76 m, 1.90 m		83.3 (CH)	3.88 dd (2.7, 7.4)
17	57.9 (CH)	1.09 m		54.9 (CH)	1.41 m		61.0 (CH)	1.25 m
18	16.5 (CH_3)	1.28 s	C-12, C-13, C-14, C-17	13.8 (CH_3)	0.73 s	C-12, C-13, C-14, C-17	16.9 (CH_3)	1.15 s
19	18.7 (CH_3)	1.44 s	C-1, C-5, C-9, C-10	17.4 (CH_3)	1.16 s	C-1, C-5, C-9, C-10	22.7 (CH_3)	1.37 s

[a] *J* (Hz) values are shown in parentheses. Assignments from 300 MHz ^{1}H−^{1}H COSY and HSQC data. [b] Multiplicity by DEPT. [c] "*ax*" and "*eq*" refer to axial and equatorial orientations, respectively.

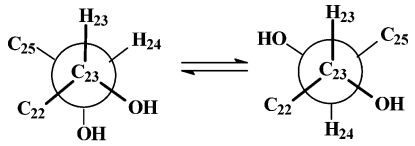

Figure 1. B-2/B-3 rotamers for the C-23/C-24 diol system of compound **2**.

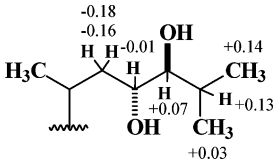

Figure 2. $\Delta\delta^{SR}$ sign distribution in di-MTPA esters and absolute configuration of the side chain of **2**.

ment of the $^2J_{C-25,H-23}$. The medium value of $^3J_{H-23,H-24} = 6.4$ Hz, $^3J_{C-22,H-24} = 3.5$ Hz, and $^2J_{C-24,H-23} = -2.0$ Hz indicated that the favored conformer should be an intermediate between two major rotamers with H/H anti and gauche orientation of hydroxyl groups. The large value for $^2J_{C-23,H-24} = -4.3$ Hz showed that H-24 is gauche-oriented with respect to oxygen attached to C-23. Of the six possible pairs of alternating rotamers arising from *erythro*- and *threo*-configurations, only one pair satisfies all of these data (Figure 1). On this basis the C-23/C-24 system should be the alternating B-2/B-3 rotamers with *erythro*-orientation of hydroxyl groups. The absence of ROE effect between H-22 and H-25 protons as well as the presence of ROE effect between H-22 and H-24 protons further supported the *erythro*-configuration.[8]

To determine the absolute configuration at chiral centers C-23 and C-24, we attempted to prepare *S*- and *R*-MTPA monoesters in the side chain of **2** and applied the modified Mosher's method [9] to these derivatives. However, only 23,24-di-*R*-(+)- and 23,24-di-*S*-(−)-MTPA esters were obtained. In accordance with the very recent publication of Riguera's group,[10] we undertook the analysis of NMR spectra of di-MTPA esters to determine the absolute configuration of the two vicinal carbinol carbons. The negative value of $\Delta\delta^{SR}$ for H_2-22 and H-23 and positive value of $\Delta\delta^{SR}$ for H-24, H-25, CH_3-26, and CH_3-27 (Figure 2) indicated 23*R*,24*S*-configurations in **2**.[10] On the basis of all the above-mentioned data, the structure of **2** was established as (23*R*,24*S*)-5α-cholestane-3β,4β,6β,8,15β,-23,24-heptaol.

Analysis of ^{13}C NMR and DEPT spectra of compound **3** revealed the presence of eight signals attributable to carbons in the side chain, including three methyls, two methylenes, two methines, and one quaternary carbon (Table 2). Analogously with steroid **2**, two carbons bearing oxygen were present in the side chain, resonating at δ 80.6 (d, C-24) and 73.9 (s, C-25) (Table 2). The ^{1}H NMR spectrum of **3** contained a 3H doublet at δ 0.95 (CH_3-21, $J = 6.5$ Hz), two 3H singlets at δ 1.11 and 1.15 (CH_3-27 and CH_3-26), and a double doublet at δ 3.15 (H-24, 1H, $J = 1.6$, 8.3 Hz). Analysis of the 2D NMR data (^{1}H−^{1}H COSY, HSQC, HMBC) led to the assignment of all proton and carbon resonances (Table 2) and indicated that **3** contains a rare 24,25-dihydroxylated side chain.

The absolute configuration at C-24 was determined using the modified Mosher's method.[9] Analysis of ^{1}H NMR spectra of *R*-(+)-MTPA and *S*-(−)-MTPA esters of **3** (see Experimental Section) showed a positive value of $\Delta\delta^{RS}$ for both H_2-23 and CH_3-21 protons and a negative value of $\Delta\delta^{RS}$ for both CH_3-27 and CH_3-26 protons, indicating the *R*-configuration at C-24.[9] On the basis of the data discussed above, the structure of **3** was thus established as (24*R*)-5α-cholestane-3β,4β,6β,8,15β,24,25-heptaol.

Compound **4** had the molecular formula $C_{26}H_{43}O_8SNa$ as deduced by the HR (+)-MALDI-TOF MS spectrum, displaying the pseudomolecular peak at *m*/*z* 561.2427 [M + Na]$^+$. The ^{13}C NMR

Table 2. ^{1}H and ^{13} NMR Data (CD_3OD) for Side Chains of Compounds **2**−**5** and **7**[a]

	2			**3**			**4**		**5**		**7**	
carbon	δ_C mult[b]	δ_H mult	HMBC	δ_C mult[b]	δ_H mult	HMBC	δ_C	δ_H mult	δ_C mult[b]	δ_H mult	δ_C mult[b]	δ_H mult
20	35.6 (CH)	1.74 m		36.6 (CH)	1.59 m		41.0	2.03 m	36.7 (CH)	1.40 m	31.4 (CH)	2.49 m
21	20.8 (CH_3)	1.06 d (6.6)	C-20, C-22, C-17	19.1 (CH_3)	0.95 d (6.5)	C-20, C-22, C-17	21.1	1.01 d (6.6)	19.1 (CH_3)	0.94 d (6.4)	20.6 (CH_3)	1.01 d (6.6)
22	40.5 (CH_2)	1.09 m, 1.85 m		34.4 (CH_2)	1.78 m		138.2	5.33 dd (8.3, 15.4)	32.4 (CH_2)	1.07 m, 1.57 m	136.6 (CH)	5.49 dd (7.7, 15.4)
23	73.2 (CH)	3.55 m		28.8 (CH_2)	1.13 m, 1.73 m		130.6	5.26 dd (6.9, 15.4)	28.0 (CH_2)	1.55 m, 1.71 m	135.2 (CH)	5.33 dd (7.7, 15.4)
24	81.4 (CH)	3.11 dd (4.9, 6.4)	C-22, C-23, C-26, C-27	80.6 (CH)	3.15 dd (1.6, 8.3)				86.0 (CH)	4.12 m	35.5 (CH)	2.20 m
25	30.4 (CH)	1.88 m		73.9 (qC)			37.8	2.45 m	31.7 (CH)	2.00 m	40.9 (CH_2)	1.50 m
26	20.4 (CH_3)	0.94 d (6.8)	C-24, C-25, C-27	35.3 (CH_3)	1.15 s	C-24, C-25, C-27	73.6	3.74 dd (7.6, 9.3), 3.87 m	18.6 (CH_3)	0.95 d (6.9)	61.4 (CH_2)	3.56 m (2H)
27	17.1 (CH_3)	0.92 d (6.7)	C-24, C-25, C-26	25.8 (CH_3)	1.11 s	C-24, C-25, C-26	17.5	1.03 d (6.7)	17.8 (CH_3)	0.92 d (6.9)		
28											31.7 (CH_3)	0.98 d (6.8)

[a] *J* (Hz) values are shown in parentheses. Assignments from 300 MHz ^{1}H−^{1}H COSY and HSQC data. [b] Multiplicity by DEPT.

spectrum of **4** showed signals due to 26 carbon atoms, including five oxygen-bearing carbons resonating at δ 68.4, 73.6, 74.3, 76.4, and 76.6 and two olefinic carbons resonating at δ 130.6 and 138.2. ^{1}H and ^{13}C NMR data of the steroidal nucleus of **4** (Table 1) closely resembled those of known steroids previously isolated from starfishes *Myxoderma platiacanthum*[11] and *Luidia clathrata*[12] and exhibiting a 3β,5α,6β,15α-tetrahydroxy-substitution pattern. Therefore, the same steroidal nucleus was suggested for compound **4**, implying that the remaining functional features, which are a sulfate residue and a double bond, should be located in the side chain. In fact, the ^{1}H NMR spectrum of **4** showed, for the side chain, signals attributable to olefinic protons [δ 5.26 (H-23, 1H, dd, J = 6.9, 15.4) and 5.35 (H-22, 1H, dd, J = 8.3, 15.4)], two methyl groups [δ 1.01 (H_3-21, 3H, d, J = 6.6) and 1.03 (H_3-28, 3H, d, J = 6.7)], and a methylene group linked to the sulfate function [H_2-26, δ 3.74 (dd, J = 7.6, 9.3) and 3.87 (m)]. These data suggested a 26-*O*-sulfated-Δ^{22E}-24-*nor*-cholestane side-chain structure. This assumption was confirmed by comparison of the spectral data of the side chain of **4** with those of the steroid with a 26-hydroxy-Δ^{22E}-24-*nor*-cholestane side chain previously reported from the starfish *Acodontaster conspicuous*.[13] In compound **4**, due to the well-known sulfatation effect, proton and carbon resonances of C-26 were observed at lower field with respect to those of the literature compound,[13] while the signal of C-24 was detected at higher field. The structure of **4** was thus established as the 26-*O*-sulfate derivative of Δ^{22E}-24-*nor*-5α-cholestane-3β,5α,6β,15α,26-pentaol. Recently, **4** was found by us also in the Far Eastern starfish *Ctenodiscus crispatus*.

The molecular formula of compound **5** was deduced to be $C_{27}H_{47}O_8SNa$ on the basis of a quasi-molecular ion peak at *m*/*z* 577.2757 $[M + Na]^+$ in the HR (+)-MALDI-TOF MS and further supported by both LSI MS and ^{13}C NMR spectra.

^{13}C NMR and DEPT spectra of **5** showed 27 carbon signals, including those of five methyl groups, nine methylenes, 10 methines, and three quaternary carbons. Five signals at δ 68.3, 76.4, 76.6, 74.2, and 86.0 were attributed to oxygen-bearing carbons. The NMR data of the steroidal nucleus were very similar to those of **4**, suggesting the presence of the same steroidal structure exhibiting 3β,5α,6β,15α-tetrahydroxy-substitution. The signals of the side chain of **5** were identical with those of known steroids having 24-*O*-sulfated (24*S*)-hydroxycholestane side chains, previously reported from the starfishes *Astropecten scoparius*[14] and *Distolasterias elegans*.[15] On the basis of these data, the structure of **5** was determined as the 24-*O*-sulfate of (24*S*)-5α-cholestane-3β,5α,6β,15α,24-pentaol. Analysis of ^{1}H−^{1}H COSY and HSQC spectra allowed assigning all proton and carbon resonances (Tables 1 and 2).

Glycoside **6** was identified as henricioside H_2, already reported from *Henricia derjugini*.[5] The 24*R*, 25*S* stereochemistry was also confirmed as previously determined.[7]

In the HR (+)-MALDI-TOF MS of leviusculoside J (**7**) the quasi-molecular ion peak was observed at *m*/*z* 647.3725 $[M + Na]^+$, which along with ^{13}C NMR and LSI MS data gave the molecular formula $C_{34}H_{56}O_{10}$. The (+)-LSI mass spectrum of this steroid exhibited fragmentation peaks corresponding to the loss of a di-*O*-methylpentose residue (see Experimental Section).

Detailed analysis of NMR spectra of glycoside **7** (Tables 1 and 2) indicated that it was closely related to henricioside H_2 (**6**). In particular, **7** exhibited the Δ^4-3β,6β,8,15α,16β-pentahydroxy-steroidal nucleus bearing a 2,3-di-*O*-methyl-β-xylopyranosyl residue at C-3, the same as glycoside **6**. On the other side, comparison of the spectral data of **7** with the corresponding data for desulfated 26-norechinasteroside A from the starfish *Henricia downeyae*[16] showed that leviusculoside J has a Δ^{22E}-27-nor-24-methyl-26-hydroxy side chain and differs from desulfated 26-nor-echinasteroside A only in a sugar residue. Thus, the structure of new leviusculoside J (**7**) was established as 3-*O*-β-D-(2,3-di-*O*-methyl-

β-xylopyranosyl)-22*E*-27-*nor*-24-methyl-5α-cholesta-4,22-diene-3β,6β,8,15α,16β,26-hexaol.

Compounds **8** and **9** were identified as laeviuscolosides A and G by comparison of their spectral data (^{1}H and ^{13}C NMR, MS) and optical rotation values with those previously reported in the literature for the glycosides isolated from the starfish *Henricia laeviuscola*.[6]

The novel polar steroids described here contain rare structural features. In fact, it is interesting to note that the 23,24-dihydroxy fragment in the side chain of **2** and the 24,25-dihydroxy fragment in the side chain of **3** as well as the sulfated shortened side chain of **4** are reported here for the first time in polar steroids from starfishes.

Earlier, Minale's group[6] reported the isolation of 12 steroidal glycosides from the same starfish, *Henricia leviuscula*, collected off the Gulf of California (Pacific Ocean). (In accordance with Lambert[18] *H. leviuscula* is the correct name of the starfish.) However, only compounds **8** and **9** have been found in both collections. This result confirmed our hypothesis about a possible strong dependence of steroid compositions of starfish on ecological factors, for instance on the animals' diet.[19]

When tested for hemolytic activity using suspensions of mouse erythrocytes at 37 °C, compounds **1**, **3**, **6**, **7**, and **9** showed hemolytic activity to mouse erythrocytes with HC_{50} values of 5.3×10^{-5}, 2.1×10^{-4}, 1.2×10^{-4}, 8.0×10^{-5}, and 1.3×10^{-4} M, respectively. The comparison of hemolytic activity of compounds **1** and **3** at 40 °C showed that at the same concentrations of 1.0×10^{-4} M the steroid **1** induced 100% hemolysis of erythrocytes during 5 min, but **3** did not induce hemolysis within this time. We observed 70% and 100% hemolysis of erythrocytes for compounds **6** and **7**, respectively, at a concentration of 8.0×10^{-5} M at 40 °C during 5 min.

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 DPX 300 spectrometer at 300 and 75.5 MHz, respectively, and on a Bruker DRX 500 spectrometer at 500 and 125.8 MHz, respectively, using tetramethylsilane as the internal standard. The hetero-half-filtered TOCSY (HETLOC) experiments were performed on a Bruker Avance DRX 750 spectrometer. MALDI-TOF mass spectra were recorded on a Bruker Biflex III laser-desorption mass spectrometer coupled with delayed extraction using a N_2 laser (337 nm). Mass spectral samples were dissolved in MeOH (1 mg/mL), and 1 μL aliquots were analyzed using an α-cyano-4-hydroxycinnamic acid (CCA) matrix. LSI mass spectra were recorded on an AMD-604S mass spectrometer (AMD, Germany) with an accelerating voltage of 8 keV and energy of Cs^+ ions of 10−12 keV. For recording the mass spectra, a sample was dissolved in MeOH (10 mg/mL) and an aliquot (1 μL) was analyzed using glycerol (Sigma) as the matrix. HPLC separations were conducted on Zorbax ODS (13 μm, 250 × 9.4 mm) and YMS-Pack ODS-2 (5 μm, 250 × 10 mm) columns 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), and Si gel L (40/100 μm, Chemapol, Praha, Czech Republic). Si gel plates (4.5 × 6.0 cm, 5−17 μm, Sorbfil, Russia) were used for thin-layer chromatography.

Animal Material. Specimens of *Henricia leviuscula* (Stimpson) (Spinulosida, Echinasteridae) were collected by dredging in August 1999 at a depth of 100 m near Onekotan Island (Kuril Islands) in the Sea of Okhotsk (research vessel *Akademik Oparin*, 23th scientific cruise). Species identification was carried out by Dr. S. Sh. Dautov (Institute of Marine Biology, Far East Branch of the Russian Academy of Science, Vladivostok, Russia). A voucher specimen [no. 023-38] is on deposit at the marine specimen collection of the Pacific Institute of Bioorganic Chemistry, Vladivostok, Russia.

Extraction and Isolation. The fresh animals (2.22 kg) were chopped and extracted twice with EtOH at 20 °C. The water/ethanol layer was evaporated, and the residue was dissolved in H_2O (1 L). The H_2O-soluble fraction was passed through an Amberlite XAD-2 column (7.5 × 18 cm) and eluted with distilled H_2O until a negative chloride ion reaction was obtained, followed by elution with EtOH. The combined EtOH eluate was evaporated to give a brownish material (9.3 g) that was chromatographed on a Sephadex LH-20 column (3 × 50 cm) with EtOH/H_2O (2:1). The subfractions containing mixtures of polyhydroxylated steroids were purified by chromatography on a Si gel column (4 × 13 cm) using $CHCl_3$/EtOH (8:1 → 1:3). Fractions were analyzed by TLC on Si gel using the eluent system BuOH/EtOH/H_2O (4:1:2) and detected by spraying with H_2SO_4 followed by heating at 100 °C.

HPLC of these fractions on a Zorbax ODS column (13 μm, 250 × 9.4 mm, 1.5 mL/min) with EtOH/H_2O (70:30) as the eluent system yielded pure **8** (1 mg, R_f 0.45) and a subfraction containing **9**, with EtOH/H_2O (80:20) and then EtOH/H_2O (60:40), yielding subfractions containing **1**−**3** and **6**−**7**, and with EtOH/H_2O (45:55), yielding subfractions containing **4** and **5**. Repeated HPLC of the subfractions on a YMS-Pack ODS-2 column (5 μm, 250 × 10 mm, 1.5 mL/min) with EtOH/H_2O (55:45) as the eluent system yielded pure **1** (9 mg, R_f 0.78), **2** (4 mg, R_f 0.80), **3** (8 mg, R_f 0.71), **4** (1.2 mg, R_f 0.65), and **5** (1 mg, R_f 0.63), with EtOH/H_2O (60:40) as the eluent system, yielding pure **6** (11 mg, R_f 0.85) and **7** (3.5 mg, R_f 0.83), and with EtOH/H_2O (65:35), yielding pure **9** (7.3 mg, R_f 0.70).

Compound 2: $C_{27}H_{48}O_7$; amorphous powder; $[\alpha]_D$ −5.0 (*c* 0.18; MeOH); ^{1}H and ^{13}C NMR data of steroid nucleus, see Table 1; ^{1}H and ^{13}C NMR data of side chain, see Table 2; LSIMS(+) *m/z* 507 $[M + Na]^+$ and 485 $[M + H]^+$; LSIMS(−) *m/z* 483 $[M - H]^-$, 465 $[M - H - H_2O]^-$, and 409 $[M - H - C_4H_9OH]^-$; HR MALDI-TOF(+) *m/z* 507.3272 $[M + Na]^+$ (calcd for $C_{27}H_{48}O_7Na$, 507.3293).

Compound 3: $C_{27}H_{48}O_7$; amorphous powder; $[\alpha]_D$ −2.4 (*c* 0.3; MeOH); ^{1}H and ^{13}C NMR data of steroid nucleus, see Table 1; ^{1}H and ^{13}C NMR data of side chain, see Table 2; LSIMS(+) *m/z* 507 $[M + Na]^+$ and 485 $[M + H]^+$; LSIMS(−) *m/z* 483 $[M - H]^-$, 465 $[M - H - H_2O]^-$, and 423 $[M - H - C_3H_7OH]^-$; HR MALDI-TOF(+) *m/z* 507.3267 $[M + Na]^+$ (calcd for $C_{27}H_{48}O_7Na$, 507.3293).

Compound 4: $C_{26}H_{43}O_8SNa$; amorphous powder; $[\alpha]_D$ +2.0 (*c* 0.1; MeOH); ^{1}H and ^{13}C NMR data of steroid nucleus, see Table 1; ^{1}H and ^{13}C NMR data of side chain, see Table 2; LSIMS(+) *m/z* 539 $[M + Na]^+$, 517 $[M - Na + 2H]^+$, 499 $[M - Na + 2H - H_2O]^+$, and 481 $[M - Na + 2H - 2H_2O]^+$; LSIMS(−) *m/z* 515 $[M - Na]^-$, 497 $[M - Na - H_2O]^-$, 97 $[HSO_4]^-$, and 80 $[SO_3]^-$; HR MALDI-TOF(+) *m/z* 561.2427 $[M + Na]^+$ (calcd for $C_{26}H_{43}O_8SNa_2$, 561.2474).

Compound 5: $C_{27}H_{47}O_8SNa$; amorphous powder; $[\alpha]_D$ +34.0 (*c* 0.1; MeOH); ^{1}H and ^{13}C NMR data of steroid nucleus, see Table 1; ^{1}H and ^{13}C NMR data of side chain, see Table 2; LSIMS(+) *m/z* 577 $[M + Na]^+$, 555 $[M + H]^+$, and 457 $[M - HSO_4]^+$; LSIMS(−) *m/z* 531 $[M - Na]^-$, 513 $[M - Na - H_2O]^-$, 97 $[HSO_4]^-$, and 80 $[SO_3]^-$; HR MALDI-TOF(+) *m/z* 577.2757 $[M + Na]^+$ (calcd for $C_{27}H_{47}O_8SNa_2$, 577.2787).

Leviusculoside J (7): amorphous powder; $[\alpha]_D$ −12.0° (*c* 0.1; MeOH); ^{1}H and ^{13}C NMR data of steroid nucleus, see Table 1; ^{1}H and ^{13}C NMR data of side chain, see Table 2; MALDI-TOF(+) *m/z* 647 $[M + Na]^+$; LSIMS(+) *m/z* 647 $[M + Na]^+$, 447 $[M - C_7H_{14}O_5 + H]^+$, 429 $[M - C_7H_{14}O_5 - H_2O + H]^+$, 411 $[M - C_7H_{14}O_5 - 2H_2O + H]^+$, 393 $[M - C_7H_{14}O_5 - 3H_2O + H]^+$; LSIMS(−) *m/z* 623 $[M - H]^-$; HR MALDI-TOF(+) *m/z* 647.3725 $[M + Na]^+$ (calcd for $C_{34}H_{56}O_{10}Na$, 647.3771).

MTPA Esters of Compounds. The compounds were treated with *S*-(+)- and *R*-(−)-α-methoxy-α-(trifluoromethyl)phenylacetyl (MTPA) chloride (10 μL) in dry pyridine (200 μL) for 1 h at room temperature. After removal of the solvent, the products were purified on a Si gel column (1 × 4 cm) using $CHCl_3$ → $CHCl_3$/EtOH (10:1).

23,24-Di-*R*-(+)-MTPA ester of 2: selected signals of the side chain ^{1}H NMR (CD_3OD, 500 MHz) δ 0.80 (3H, d, *J* = 6.7 Hz, H_3-27), 1.04 (3H, d, *J* = 6.8 Hz, H_3-26), 1.40 (1H, m, H-22), 1.75 (1H, m, H-22′), 1.85 (1H, m, H-25), 5.08 m (1H, dd, *J* = 6.4, 7.5 Hz, H-24), 5.35 (1H, m, H-23).

23,24-Di-*S*-(−)-MTPA ester of 2: selected signals of the side chain ^{1}H NMR (CD_3OD, 500 MHz) δ 0.94 (3H, d, *J* = 6.7 Hz, H_3-27), 1.07 (3H, d, *J* = 6.8 Hz, H_3-26), 1.22 (1H, m, H-22), 1.59 (1H, m, H-22′), 1.98 (1H, m, H-25), 5.15 m (1H, dd, *J* = 6.4, 7.5 Hz, H-24), 5.34 (1H, m, H-23).

3,15,24-Tri-*R*-(+)-MTPA ester of 3: ^{1}H NMR (CD_3OD, 500 MHz) δ 0.80 (3H, s, H_3-18), 0.89 (3H, d, *J* = 6.5 Hz, H_3-21), 1.08 (3H, s, H_3-27), 1.12 (3H, s, H_3-26), 1.17 (1H, d, *J* = 5.5 Hz, H-14), 1.33 (1H, m, H-16), 1.34 (1H, m, H-23), 1.36 (1H, t, *J* = 2.8 Hz, H-5), 1.44

(3H, s, H_3-19), 1.70 (1H, dd, J = 3.3, 14.3 Hz, H-7a), 1.80 (1H, m, H-2e), 1.94 (1H, m, H-23′), 2.15 (1H, m, H-2a), 2.18 (1H, dd, J = 3.3, 14.1 Hz, H-7e), 2.49 (1H, m, H-16′), 4.21 (1H, m, H-6), 4.27 (1H, brs, H-4), 4.93 (1H, m, H-24), 4.95 (1H, m, H-3), 5.46 (1H, ddd, J = 1.9, 5.4, 7.3 Hz, H-15).

3,24-Di-*S*-(−)-MTPA ester of 3: ^{1}H NMR (CD_3OD, 500 MHz) δ 0.86 (3H, d, J = 6.5 Hz, H_3-21), 0.99 (1H, d, J = 5.5 Hz, H-14), 1.16 (3H, s, H_3-27), 1.20 (3H, s, H_3-26), 1.23 (3H, s, H_3-18), 1.27 (1H, m, H-16), 1.33 (1H, m, H-23), 1.39 (1H, t, J = 2.8 Hz, H-5), 1.46 (3H, s, H_3-19), 1.65 (1H, m, H-2e), 1.70 (1H, dd, J = 3.3, 14.3 Hz, H-7a), 1.80 (1H, m, H-23′), 2.05 (1H, m, H-2a), 2.08 (1H, m, H-16′), 2.40 (1H, dd, J = 3.3, 14.1 Hz, H-7e), 4.26 (1H, m, H-6), 4.37 (1H, ddd, J = 1.9, 5.4, 7.3 Hz, H-15), 4.41 (1H, brs, H-4), 4.95 (1H, m, H-3), 4.96 (1H, m, H-24).

Bioassay. Hemolytic activity was determined as previously reported.[20]

Acknowledgment. This study was carried out in the framework of the bilateral project between Pacific Institute of Bioorganic Chemistry of the Russian Academy of Sciences (Russia) and Institute of Biomolecular Chemistry, Consiglio Nazionale delle Ricerche (Italy). The research described in this publication was made possible in part by the Program of Presidium of RAS "Molecular and Cell Biology", Grant No. 05-04-48246 from the RFBR, Grant No. S.Sch.-725.2003.4 from the President of RF, a grant from "Russian Science Support Foundation", and Grant Nos. 05-III-Ã-05-040 and 05-II-CM-05-001 from FEB RAS. The authors thank Suntory Institute for Bioorganic Research for allowing the use of the Bruker DMX-750 NMR spectrometer.

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NP050373J