Holothurins B_2, B_3, and B_4, New Triterpene Glycosides from Mediterranean Sea Cucumbers of the Genus *Holothuria*

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Triterpene glycosides of three species of the Mediterranean sea cucumbers *Holothuria polii*, *Holothuria tubulosa*, and *Holothuria* sp. were studied. Three new monosulfated biosides, holothurins B_2 (**1**), B_3 (**2**), and B_4 (**3**), along with the previously known holothurins A (**4**) and B (**5**) were isolated from the sea cucumber *H. polii*. Triterpene glycosides belonging to holothurin A and B groups were found in *H. tubulosa*, while only one individual glycoside, holothurin A (**4**), was isolated from *Holothuria* sp. Structures of new substances were elucidated on the basis of spectral data (2D NMR and MS). The significance of holothurins as chemotaxonomic markers of the animals belonging to the genus *Holothuria* and taxonomic status of some representatives of the holothurians studied are discussed.

As a continuation of our studies on triterpene glycosides from the sea cucumbers of the genus *Holothuria*,[1−3] we carried out a comparative study on triterpene glycosides from three Mediterranean sea cucumbers, *Holothuria polii, Holothuria tubulosa*, and *Holothuria* sp. Animals belonging to the numerous genus *Holothuria* are widespread in tropical waters of the World Ocean, but a few species of this genus inhabit areas with subtropical and temperate climate, including several ones from the Mediterranean Sea. However, only glycosides from the Atlantic species *Holothuria forskali* were isolated as individual compounds and structurally studied.[4] The Mediterranean holothurians *H. polii, H. tubulosa,* and *Holothuria* sp. were collected in Capo Miseno Bay (Naples) from a depth of 10−20 m. In this paper we report glycoside compositions of these species and structures of three new minor glycosides from *H. polii*: holothurins B_2 (**1**), B_3 (**2**), and B_4 (**3**). Ethanolic extracts of *H. polii, H. tubulosa*, and *Holothuria* sp. were sequentially submitted to column chromatography on Polychrom-1 (powdered Teflon, Latvia) and silica gel. Two fractions containing monosulfated tetraosides and monosulfated biosides were obtained from *H. polii* and *H. tubulosa*, respectively. Only tetraosides were isolated from *Holothuria* sp. Tetraoside fractions from all these species predominantly consisted of the previously known substance holothurin A (**4**). HPLC on a Discovery C-18 column (Supelco) of another fraction from *H. polii* yielded four individual biosides, **1**, **2**, **3**, and **5**, while the corresponding fraction from *H. tubulosa* gave known glycoside **5** only. Structures of the isolated glycosides were elucidated on the basis of spectral data (^{1}H and ^{13}C NMR, HSQC, HMBC, COSY RCT, NOESY, and MALDI TOF MS).

Results and Discussion

DEPT and ^{13}C NMR spectral data of the carbohydrate moieties of new glycosides **1**−**3** (Table 1) were found to be identical to each other and coincident with that of the earlier known holothurin B (**5**).[5] These carbohydrate chains included xylose and quinovose residues with a sulfate group attached to C-4 of the xylose unit. The presence of two monosaccharide units was confirmed by ^{13}C NMR spectra of the substances, which exhibited the signals of anomeric protons at 105.1 and 105.7 ppm, correlated by HSQC with the corresponding anomeric protons at 4.73 ppm (d, J = 7.2 Hz) and 5.15 ppm (d, J = 7.5 Hz). The coupling constants were indicative for the β-configuration of glycosidic bonds.[6] Interglycosidic linkages in sugar chains of **1**−**3** and their bonding to aglycons were confirmed by NOESY experiments (Table 1) that showed cross-peaks between H-1 of the xylose residue and H-3 of the aglycons and between H-1 of quinovose and H-2 of the xylose residue. Analogous cross-peaks were also observed in the HMBC spectrum.

The molecular formula of **1** was determined as $C_{41}H_{65}O_{17}SNa$ by a pseudomolecular ion $[M + Na]^+$ at *m/z* 907.3689 in HR MALDI TOF (positive ion mode), calculated for $C_{41}H_{65}O_{17}SNa_2$ 907.3738 *m/z*. ^{13}C NMR and DEPT spectra of glycoside **1** showed the signals C-1−C-18 coincident with those of the aglycon part of holothurin B (**5**)[5] and had the signals characteristic for the presence of hydroxy groups at C-12 (71.4 ppm) and C-17 (89.9 ppm) in the holostane nucleus. The first of the signals was correlated with the corresponding proton signal at 5.05 ppm (H-12β, J = 5.8)[5] by HSQC, which indicated the α-configuration of the hydroxy group (Table 2). However the signals of the side chain differed from those of **5** by the absence of the signals at 81.0 and 81.4 ppm, characteristic for a 22,25-epoxy group. Moreover, the signal of C-22 was observed at 75.2 ppm, which indicated the presence of a hydroxy group in this position of the side chain of holothurin B_2 (**1**). The ^{13}C NMR spectrum of the aglycon moiety of **1** was identical to that of the tetraoside holothurin A_1 from *H. floridana*.[7]

In confirmation of the carbohydrate chain structure, the MALDI TOF MS of the glycoside **1** exhibited a pseudomolecular ion peak at *m/z* 907 $[M + Na]^+$ in the positive ion mode and pseudomolecular ion peaks at *m/z* 861 $[M - Na]^-$ and 715 $[M - Na - Qui + H]^-$ (due to the loss of a terminal monosaccharide residue) in the negative ion mode. All these data including results of 2D NMR experiments (presented in Figure 1) indicate that holothurin B_2 is 3β-*O*-[β-D-quinovopyranosyl-(1→2)-4-*O*-sodium sulfate-β-D-xylopyranosyl]-22-hydroxyholost-9-ene-12α,17α-diol.

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Chart 1

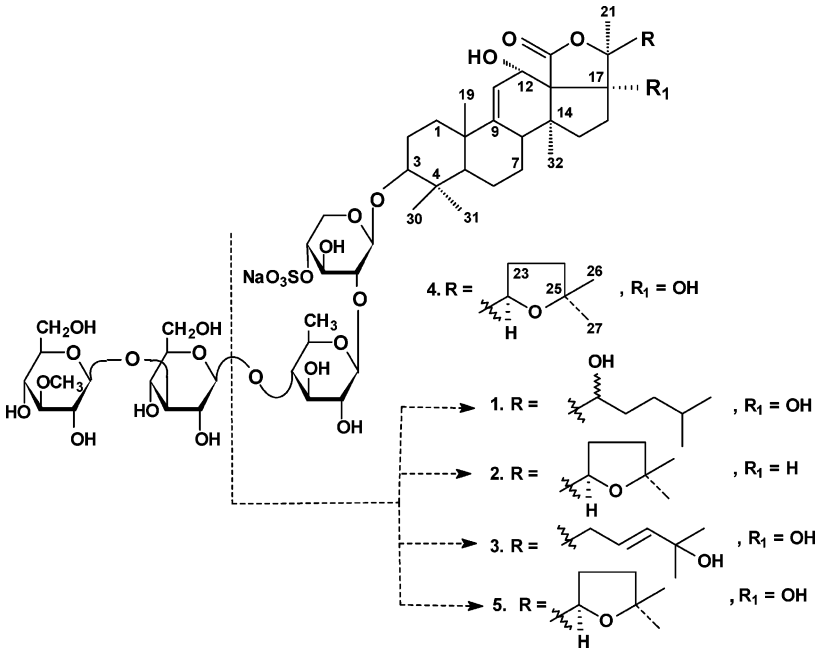

Table 1. ^{13}C and ^{1}H NMR Chemical Shifts and Selected HMBC and NOESY Correlations of Carbohydrate Moieties of Holothurines **1**–**3**

position	δ_C[a,b,c]	δ_H mult. (*J* in Hz)[d]	HMBC	NOESY
Xyl (1→C-3)				
1	105.1	4.73 d (7.2)	C-3	H-3, H-31, H-3,5 Xyl
2	83.1	4.08 m	C-1 Qui, C-3Xyl	H-1 Qui
3	75.4	4.34 t (8.9)	C-4Xyl, C-2Xyl	H-1,5 Xyl
4	75.9	5.15 m	C-2 Xyl	
5	64.0	3.76 m	C-1 Xyl	H-1,3 Xyl
		4.80 dd (5.4; 11.7)		
Qui (1→2Xyl)				
1	105.6	5.15 d (7.5)	C-2 Xyl	H-2 Xyl, H-3,5 Qui
2	76.7	4.06 m	C-3 Qui	
3	77.5	4.15 t (8.8)	C-4 Qui	H-1,5 Qui
4	76.5	3.72 t (9.1)	C-3.5 Qui	
5	73.2	3.78 m		H-1,3 Qui
6	18.4	1.66 d (6.0)	C-4,5 Qui	H-30

[a] Recorded at 125.77 MHz in C_5D_5N. [b] Bold − interglycosidic linkage. [c] Italic − sulfate position. [d] Recorded at 500 MHz in C_5D_5N.

The molecular formula of holothurin B_3 (**2**) was determined as $C_{41}H_{63}O_{16}SNa$ by the pseudomolecular ion $[M + Na]^+$ at 889.3569 *m/z* in the HR MALDI TOF (positive ion mode), calculated for $C_{41}H_{63}O_{16}SNa_2$ 889.3632 *m/z*. The NMR spectral data of the aglycon part of holothurin B_3 (**2**) (Table 2) were very close to those of the known holothurin B (**5**),[5] but the signals of C-16 and C-17 were shifted upfield to 23.5 and 47.5 ppm, correspondingly, due to the absence of a hydroxy group at C-17. The absence of a hydroxyl group at C-17 was also confirmed by the upfield shift of the signal of C-20 to 83.5 ppm. On the basis of the ^{13}C NMR spectrum of the aglycon part of **2**, it was concluded that this aglycon was identical to that of holothurinosides C and D from the holothurian *H. forskali*.[4] This structure was confirmed by extensive NMR sperctroscopy (^{1}H and ^{13}C NMR, HSQC, HMBC, COSY RCT, NOESY). Previously, the *S*-configuration of C-22 was determined by Ilyin and collaborators using X-ray analysis[8] of the acetate of 17-desoxy-22,25-oxidoholothurinogenin, an artificial genin obtained from the holothurin B fraction after acid hydrolysis. Therefore holothurin B_3 (**2**) had the same configuration of C-22.

The MALDI TOF spectrum of glycoside **2** exhibited a pseudomolecular ion at *m/z* 889 $[M + Na]^+$ in the positive ion mode and pseudomolecular ion peaks at *m/z* 843 $[M - Na]^-$ and 697 $[M - Na - Qui + H]^-$ (due to the loss of a terminal monosaccharide residue) in the negative ion mode. All these data including results of 2D NMR experiments indicate that holothurin B_3 (**2**) is 3*β*-*O*-[*β*-D-quinovopyranosyl-(1→2)-4-*O*-sodium sulfate-*β*-D-xylopyranosyl]-22,25-epoxyholost-9-en-12α-ol.

The molecular formula of holothurin B_4 (**3**) was determined as $C_{41}H_{63}O_{17}SNa$ by the pseudomolecular ion $[M + Na]^+$ at 905.3632 *m/z* in the HR MALDI TOF (positive ion mode), calculated for $C_{41}H_{63}O_{17}SNa_2$ 905.3581 *m/z*. The tetracyclic triterpene nucleus moiety of holothurin B_4 (**3**) was found by extensive NMR spectroscopy (^{1}H and ^{13}C NMR, HSQC, HMBC, COSY RCT, NOESY) to be similar to that of holothurin B_2 (**1**), and signals C-1–C-18 were coincident in their spectra (Table 2). However, signals of the side chain were distinguished from those in the spectra of **1**. There were the signals at 120.6, 143.6, and 69.5 ppm in the ^{13}C NMR spectrum of holothurin B_4 (**3**) that suggest the presence of a 23(24) double bond and a hydroxy group at C-25. The same side chains were earlier found in some glycosides of animals belonging to the order Dendrochirotida, namely, *Eupentacta fraudatrix* (cucumarioside G_4),[9] *Psolus eximius* (eximisoside A),[10] and *Cucumaria frondosa* (frondoside A_2-3).[11] 25-Hydroxydehydroechinoside A was isolated from *Actinopyga flammea*[12] (Holothuriidae, Aspidochirotida), but there was no decisive proof of a double-

Table 2. ^{13}C and ^{1}H NMR Chemical Shifts of Holothurin B_2 (**1**), B_3 (**2**), and B_4 (**3**) Aglycon Moieties

	1	**1**	**2**	**2**	**3**	**3**
atom	δ_C[a]	δ_H mult. (J in Hz)[b]	δ_C[a]	δ_H mult. (J in Hz)[b]	δ_C[a]	δ_H mult. (J in Hz)[b]
1	36.2[c]	1.84 m; 1.40 m	36.3	1.86 m; 1.45 m	36.2	1.83 m; 1.42 m
2	26.8	2.11 m; 1.93 m	26.9	1.93 m; 2.12 m	26.8	1.93 m; 2.12 m
3	88.5	3.16 dd (4.2; 11.6)	88.6	3.16 dd (4.0; 11.5)	88.5	3.17 dd (4.0; 11.5)
4	39.8		39.9		39.8	
5	52.5	1.02 m	52.6	1.01 m	52.5	1.02 m
6	21.0	1.56 m; 1.77 m	21.1	1.57 m; 1.75 m	21.0	1.57 m; 1.75 m
7	28.2	1.78 m	28.6	1.45 m; 1.78 m	28.1	1.50 m; 1.79 m
8	40.7	3.42 m	39.9	3.41 m	40.6	3.36 dd (4.5; 10.5)
9	153.5		153.0		154.0	
10	39.5		39.4		39.5	
11	115.4	5.65 dd (1.5; 5.8)	115.9	5.74 dd (1.6; 5.7)	115.3	5.61 brd (5.2)
12	71.3	5.05 d (5.8)	68.0	4.57 brd (5.7)	71.1	4.96 m[f]
13	59.0		63.6		58.3	
14	45.8		46.1		46.2	
15	36.7	1.49 m; 1.87 m	37.2	1.67 m	36.4	1.42 m; 1.83 m
16	35.4	2.52 m; 3.45 m	23.5	2.07 m; 2.37 m	35.7	2.34 m; 2.75 m
17	89.9[d]		47.5	3.41 dd (2.9; 11.0)	89.2[e]	
18	174.5		177.3		174.5	
19	22.3	1.40 s	22.3	1.41 s	22.4	1.37 s
20	87.2[d]		83.5		86.6[e]	
21	19.3	1.90 s	20.6	1.62 s	23.0	1.77 s
22	75.2	4.18 dd (2.0; 9.8)	80.1	4.24 t (7.2)	41.2	2.74 m
23	30.7	1.86 m; 1.93 m	27.2	1.98 m	120.6	6.13 dt (15.5; 7.0)
24	36.2[c]	1.37 m; 1.84 m	38.4	1.64 m	143.6	6.03 d (15.5)
25	28.2	1.58 m	81.0		69.5	
26	22.7	0.89 s	28.4	1.25 s	30.3	1.54 s
27	22.4	0.88 s	27.2	1.21 s	30.3	1.55 s
30	16.5	1.14 s	16.5	1.14 s	16.5	1.14 s
31	27.9	1.29 s	27.9	1.29 s	27.9	1.29 s
32	20.1	1.74 s	21.9	1.30 s	19.8	1.67 s

[a] Recorded at 125.77 MHz in C_5D_5N. [b] Recorded at 500 MHz in C_5D_5N. [c,d,e] Signals are interchangeable. [f] Partly overlapped by the signal of H_2O.

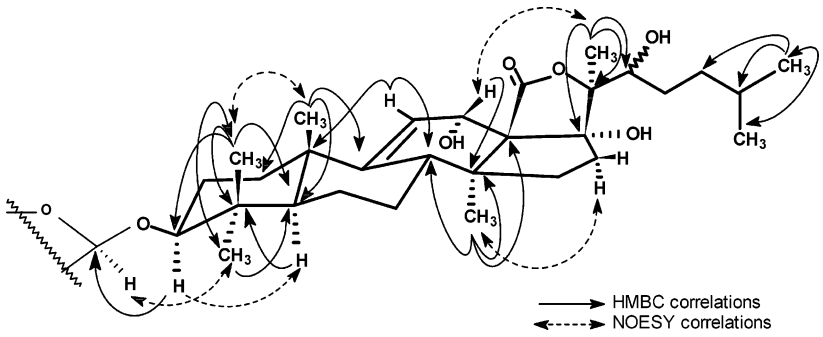

Figure 1. Selected HMBC and NOESY correlations of the aglycon moiety of holothurin B_2 (**1**).

bond position, and authors defined the ambiguous location of the double bond at 22(23) or 23(24) on the basis of the ^{1}H NMR spectrum. The presence of a 23(24) double bond and a C-25 hydroxy group in the side chain was confirmed by HMBC experiments, namely, by cross-peaks H-23 (6.13 ppm)/C-22, C-24, C-25; H-24 (6.03 ppm)/C-22, C-23, C-25; H-26 (1.54 ppm)/C-24; and H-27 (1.55 ppm)/C-24.

The MALDI TOF of glycoside **3** exhibited a pseudomolecular ion at *m/z* 905 $[M + Na]^+$ in the positive ion mode and pseudomolecular ion peaks at *m/z* 859 $[M - Na]^-$ and 713 $[M - Na - Qui + H]^-$ (due to the loss of a terminal monosaccharide residue) in the negative ion mode. All these data including results of 2D NMR experiments indicate that holothurin B_4 is 3*β*-*O*-[*β*-D-quinovopyranosyl-(1→2)-4-*O*-sodium sulfate-*β*-D-xylopyranosyl]-25-hydroxyholosta-9,23-diene-12α,17α-diol.

Glycosides **1**−**3** isolated from *H. polii* are new variants of holothurins. Isolation of these compounds gives new information concerning the diversity of the biochemical transformation of side chains in glycosides from animals of the genus *Holothuria*. Holothurins, isolated by Sobotka and co-laborators,[13] from *Actinopyga agassizi* in 1959 were among the first marine natural products. These compounds are characterized by bioside or tetraoside carbohydrate chains sulfated at C-4 of xylose and holostane type aglycons with a 12-α-hydroxy-9(11)-ene fragment. Later it has been shown that these glycosides are chemotaxonomic markers of species belonging to the genera *Holothuria, Actinopyga*, and *Pearsonothuria,* widespread in tropical waters.[1−3,14] Independently of locality and season of collections, holothurins were found in all the studied animals of these genera. Some glycosidic fractions from different collections of animals contained only biosides (belonging to holothurin B series) or only tetraosides (belonging to holothurin A series); other ones gave mixtures of biosides and tetraosides. *H. forskali* was a single exclusion and contained not holothurins, but related holothurinosides with up to five monosaccharide units in the absence of a sulfate group. Taking into account this difference in the chemical composition and some morphological peculiarities of this species, such as unusual reduction of the microskeleton, we assumed that *H. forskali* should be separated from other representatives of the genus into a new genus. This suggestion requires further confirmation by detailed mor-

phological and genetic investigations. According to the obtained chemical information, there are no doubts of the taxonomic status of the species studied belonging to the genus *Holothuria*.

Experimental Section

General Experimental Procedures. All melting points were determined with a Kofler-Thermogenerate apparatus. Specific rotation was measured on a Perkin-Elmer 343 polarimeter. NMR spectra were recorded on a AMX Bruker 500 spectrometer at 500.12/125.67 MHz ($^1H/^{13}C$) and a Bruker DPX 300 spectrometer at 300/75 MHz ($^1H/^{13}C$) in C_5D_5N with TMS as an internal reference ($\delta = 0$). The MALDI TOF MS (positive and negative ion modes) were recorded using a Bruker apparatus, model BIFLEX III, with impulse extraction of ions, on an α-cyano-4-hydroxycinnamic acid matrix. HPLC was performed using an Agilent 1100 chromatograph equipped with a differential refractometer on a Discovery C_{18} (4.6 × 250) column.

Animal Material. The sea cucumbers *Holothuria polii, Holothuria tubulosa,* and *Holohuria* sp. (family Holothuriidae; order Aspidochirotida) were collected in Capo Miseno Bay (Mediterranean Sea, Italy) in November 2003 using scuba apparatus at a depth of 10–20 m. Voucher specimens of the sea cucumbers are on deposit in the collection of the Zoological Institute, the Russian Academy of Sciences.

Extraction and Isolation. *Holothuria tubulosa*. Body walls of the sea cucumbers were cut into very small pieces and extracted twice with refluxing ethanol (dry weigh of the residue 487 g). The combined extracts were concentrated to dryness and chromatographed on a Polychrom-1 column (powdered Teflon, Biolar, Latvia), eluting first inorganic salts and polar impurities with H_2O and then the fraction containing glycosides with 50% acetone. The latter fraction was twice chromatographed on silica gel columns eluting with $CHCl_3$–EtOH–H_2O (100:100:17 and 100:75:10) solvent systems to give 226 mg of individual holothurin A (**4**) and 10 mg of individual on the TLC fraction of glycosides belonging to the holothurin B group. The last fraction was submitted for HPLC on a Discovery C_{18} (4 × 250) column with 70% MeOH as mobile phase to give 2.5 mg of individual holothurin B (**5**).

***Holothuria* sp.** Glycosides from this sea cucumber (dry weight of animals 27 g) were isolated by Teflon and silica gel chromatography as described above to give 8 mg of holothurin A (**4**).

Holothuria polii. Glycosides from this sea cucumber (dry weight of animals 110 g) were isolated by Teflon and silica gel chromatography as described above to give 75 mg of individual holothurin A (**4**) and 51 mg of individual on the TLC fraction of glycosides belonging to the holothurtin B group. The last fraction was separated by HPLC as described above to give 17 mg of holothurin B (**5**), 9 mg of holothurin B_2 (**1**), 3 mg of holothurin B_3 (**2**), and 3 mg of holothurin B_4 (**3**).

Holothurin B_2 (1): mp 243 °C; $[\alpha]^{20}_D$ −7° (*c* 0.1, pyridine); ^{13}C and ^1H NMR data, see Tables 1 and 2; MALDI TOF MS (positive ion mode) *m/z* (rel int) 907 ($C_{41}H_{65}O_{17}SNa_2$ $[M + Na]^+$, 1); MALDI TOF MS (negative ion mode) *m/z* (rel int) 861 ($C_{41}H_{65}O_{17}S$ $[M - Na]^-$, 1), 715 ($[M - Na - Qui + H]^-$, 0.07).

Holothurin B_3 (2): mp 245 °C; $[\alpha]^{20}_D$ −18° (*c* 0.1, pyridine); ^{13}C and ^1H NMR data, see Tables 1 and 2; MALDI TOF MS (positive ion mode) *m/z* (rel int) 889 ($C_{41}H_{63}O_{16}SNa_2$ $[M + Na]^+$, 1); MALDI TOF MS (negative ion mode) *m/z* (rel int) 843 ($C_{41}H_{63}O_{16}S$ $[M - Na]^-$, 1), 697 ($[M - Na - Qui + H]^-$, 0.07).

Holothurin B_4 (3): mp 232–233 °C; $[\alpha]^{20}_D$ −12° (*c* 0.1, pyridine); ^{13}C and ^1H NMR data, see Tables 1 and 2; MALDI TOF MS (positive ion mode) *m/z* (rel int) 905 ($C_{41}H_{63}O_{17}SNa_2$ $[M + Na]^+$, 1); MALDI TOF MS (negative ion mode) *m/z* (rel int) 859 ($C_{41}H_{63}O_{17}S$ $[M - Na]^-$, 1), 713 ($[M - Na - Qui + H]^-$, 0.08).

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Supporting Information Available: This material is available free of charge via the Internet at http://pubs.acs.org.

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