

Holothurins B₂, B₃, and B₄, 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 B₂ (**1**), B₃ (**2**), and B₄ (**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 B₂ (**1**), B₃ (**2**), and B₄ (**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 (¹H and ¹³C NMR, HSQC, HMBC, COSY RCT, NOESY, and MALDI TOF MS).

Results and Discussion

DEPT and ¹³C 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 ¹³C 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 C₄₁H₆₅O₁₇SNa by a pseudomolecular ion [M + Na]⁺ at *m/z* 907.3689 in HR MALDI TOF (positive ion mode), calculated for C₄₁H₆₅O₁₇SNa₂ 907.3738 *m/z*. ¹³C 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 B₂ (**1**). The ¹³C NMR spectrum of the aglycon moiety of **1** was identical to that of the tetraoside holothurin A₁ 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 B₂ is 3β-O-[β-D-quinovopyranosyl-(1→2)-4-O-sodium sulfate-β-D-xylopyranosyl]-22-hydroxyholost-9-ene-12α,17α-diol.

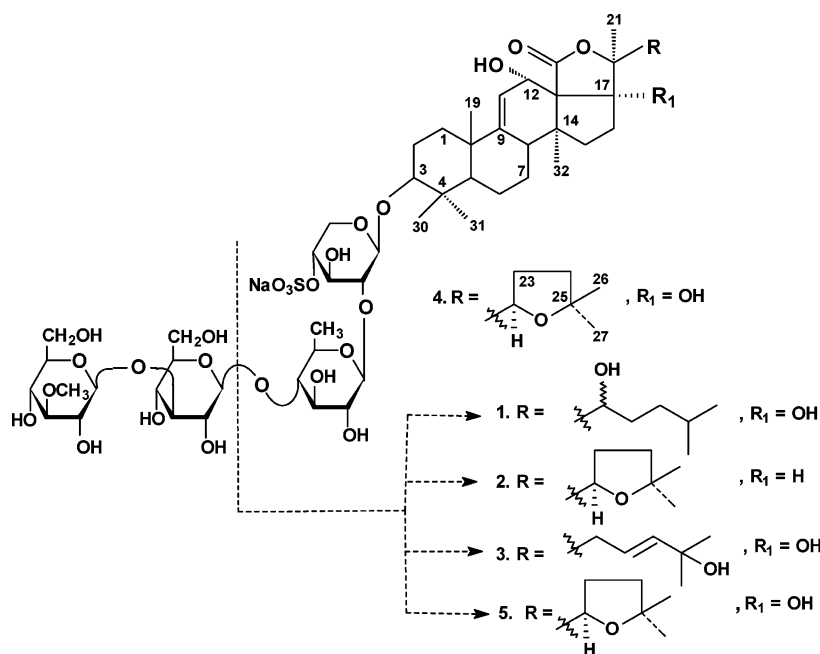
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Chart 1

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

position	$\delta_C^{a,b,c}$	δ_H mult. (<i>J</i> 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 C₅D₅N. ^b Bold – interglycosidic linkage. ^c Italic – sulfate position. ^d Recorded at 500 MHz in C₅D₅N.

The molecular formula of holothurin B₃ (**2**) was determined as C₄₁H₆₃O₁₆SNa by the pseudomolecular ion [M + Na]⁺ at 889.3569 *m/z* in the HR MALDI TOF (positive ion mode), calculated for C₄₁H₆₃O₁₆SNa₂ 889.3632 *m/z*. The NMR spectral data of the aglycon part of holothurin B₃ (**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 ¹³C 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 spectroscopy (¹H and ¹³C NMR, HSQC, HMBC, COSY RCT, NOESY). Previously, the *S*-configuration of C-22 was determined by Ilyin and collaborators using X-ray analysis⁸ of the acetate of 17-desoxy-22,25-oxidoholothurinogenin, an artificial genin obtained from the holothurin B fraction after acid hydrolysis. Therefore holothurin B₃ (**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

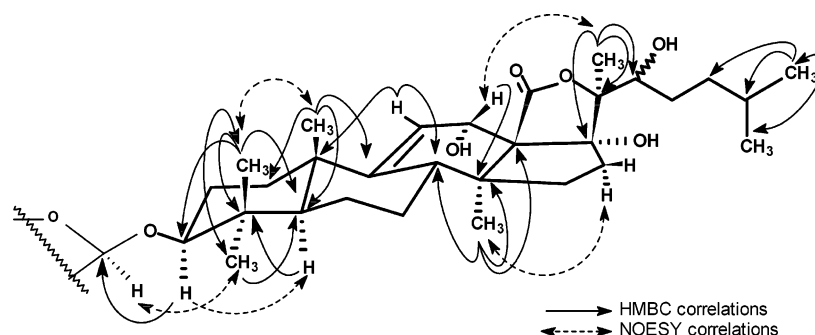
terminal monosaccharide residue) in the negative ion mode. All these data including results of 2D NMR experiments indicate that holothurin B₃ (**2**) is 3β-*O*-[β-D-quinovopyranosyl-(1→2)-4-*O*-sodium sulfate-β-D-xylopyranosyl]-22,25-epoxyholost-9-en-12α-ol.

The molecular formula of holothurin B₄ (**3**) was determined as C₄₁H₆₃O₁₇SNa by the pseudomolecular ion [M + Na]⁺ at 905.3632 *m/z* in the HR MALDI TOF (positive ion mode), calculated for C₄₁H₆₃O₁₇SNa₂ 905.3581 *m/z*. The tetracyclic triterpene nucleus moiety of holothurin B₄ (**3**) was found by extensive NMR spectroscopy (¹H and ¹³C NMR, HSQC, HMBC, COSY RCT, NOESY) to be similar to that of holothurin B₂ (**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 ¹³C NMR spectrum of holothurin B₄ (**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 Dendrochirotrida, namely, *Eupentacta fraudatrix* (cucumarioside G₄),⁹ *Psolus eximius* (eximioside A),¹⁰ and *Cucumaria frondosa* (frondoside A₂-3).¹¹ 25-Hydroxydehydroechinoside A was isolated from *Actinopyga flammea*¹² (Holothuriidae, Aspidochirotrida), but there was no decisive proof of a double-

Table 2. ^{13}C and ^1H NMR Chemical Shifts of Holothurin B₂ (1), B₃ (2), and B₄ (3) Aglycon Moieties

atom	1 δ_{C}^a	1 δ_{H} mult. (<i>J</i> in Hz) ^b	2 δ_{C}^a	2 δ_{H} mult. (<i>J</i> in Hz) ^b	3 δ_{C}^a	3 δ_{H} mult. (<i>J</i> 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 $\text{C}_5\text{D}_5\text{N}$. ^b Recorded at 500 MHz in $\text{C}_5\text{D}_5\text{N}$. ^{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 B₂ (1).

bond position, and authors defined the ambiguous location of the double bond at 22(23) or 23(24) on the basis of the ^1H 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 $[\text{M} + \text{Na}]^+$ in the positive ion mode and pseudomolecular ion peaks at m/z 859 $[\text{M} - \text{Na}]^-$ and 713 $[\text{M} - \text{Na} - \text{Qui} + \text{H}]^-$ (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 B₄ is 3 β -O- $[\beta$ -D-quinovopyranosyl-(1 \rightarrow 2)-4-O-sodium sulfate- β -D-xylopyranosyl]-25-hydroxyholosta-9,23-diene-12 α ,17 α -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/¹³C) and a Bruker DPX 300 spectrometer at 300/75 MHz (¹H/¹³C) in C₅D₅N 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 C₁₈ (4.6 × 250) column.

Animal Material. The sea cucumbers *Holothuria polii*, *Holothuria tubulosa*, and *Holothuria* sp. (family Holothuriidae; order Aspidochirotrida) 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 H₂O and then the fraction containing glycosides with 50% acetone. The latter fraction was twice chromatographed on silica gel columns eluting with CHCl₃–EtOH–H₂O (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 C₁₈ (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 holothurin B group. The last fraction was separated by HPLC as described above to give 17 mg of holothurin B (5), 9 mg of holothurin B₂ (1), 3 mg of holothurin B₃ (2), and 3 mg of holothurin B₄ (3).

Holothurin B₂ (1): mp 243 °C; [α]_D²⁰ –7° (c 0.1, pyridine); ¹³C and ¹H NMR data, see Tables 1 and 2; MALDI TOF MS (positive ion mode) *m/z* (rel int) 907 (C₄₁H₆₅O₁₇SNa₂ [M + Na]⁺, 1); MALDI TOF MS (negative ion mode) *m/z* (rel int) 861 (C₄₁H₆₅O₁₇S [M – Na][–], 1), 715 ([M – Na – Qui + H][–], 0.07).

Holothurin B₃ (2): mp 245 °C; [α]_D²⁰ –18° (c 0.1, pyridine); ¹³C and ¹H NMR data, see Tables 1 and 2; MALDI TOF MS (positive ion mode) *m/z* (rel int) 889 (C₄₁H₆₅O₁₆SNa₂ [M + Na]⁺, 1); MALDI TOF MS (negative ion mode) *m/z* (rel int) 843 (C₄₁H₆₃O₁₆S [M – Na][–], 1), 697 ([M – Na – Qui + H][–], 0.07).

Holothurin B₄ (3): mp 232–233 °C; [α]_D²⁰ –12° (c 0.1, pyridine); ¹³C and ¹H NMR data, see Tables 1 and 2; MALDI TOF MS (positive ion mode) *m/z* (rel int) 905 (C₄₁H₆₃O₁₇SNa₂ [M + Na]⁺, 1); MALDI TOF MS (negative ion mode) *m/z* (rel int) 859 (C₄₁H₆₃O₁₇S [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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