

[Chem. Pharm. Bull.]
29(7)1951—1956(1981)

Marine Natural Products. VIII.¹⁾ Bioactive Triterpene-Oligoglycosides from
the Sea Cucumber *Holothuria leucospilota* BRANDT (2).
Structure of Holothurin A

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(Received February 16, 1981)

The chemical structure of holothurin A, the major lanostane-type triterpene oligoglycoside from the Cuvierian tubules of the sea cucumber *Holothuria leucospilota* BRANDT (= *H. vagabunda* SELENKA), has been elucidated to be 3-O-[β -D-3-O-methylglucopyranosyl-(1 $\rightarrow$ 3)- β -D-glucopyranosyl-(1 $\rightarrow$ 4)- β -D-quinovopyranosyl-(1 $\rightarrow$ 2)- β -D-xylopyranosyl]-holothurigenol 4'-O-sodium sulfate (8), on the basis of chemical, physicochemical, and biochemical evidence. Holothurin A (8) corresponds to the 4''-O-[3'''-O-(β -D-3'''-O-methylglucopyranosyl)- β -D-glucopyranoside] of holothurin B (6), which is the major oligoglycoside of the body walls of the same sea cucumber.

Keywords—sea cucumber; *Holothuria leucospilota*; lanostane-type oligoglycoside; holothurin A; ¹H NMR; ¹³C NMR; CD; snail enzyme; crude naringinase; T₁

In the preceding paper,¹⁾ we reported the isolation of two lanostane-type triterpene oligoglycosides named holothurin A and holothurin B from the sea cucumber *Holothuria leucospilota* BRANDT (= *H. vagabunda* SELENKA, Japanese name: nisekuro-namako) and also described in detail evidence for the chemical structure of holothurin B (6) which is the major oligoglycoside of the body walls of the sea cucumber. As a continuation of this study, we have clarified the chemical structure of holothurin A (8), which is the major oligoglycoside of the Cuvierian tubules of the same sea cucumber. This paper deals with the details of the investigation.²⁾

Holothurin A (8), C₅₄H₈₅NaO₂₇S, isolated from the Cuvierian tubules of the sea cucumber as described in our preceding paper,¹⁾ was crystallized from aq. 95% ethanol as colorless needles of mp 228—230 °C. The ultraviolet (UV) spectrum of holothurin A shows no absorption maximum, while the infrared (IR) spectrum shows absorption bands at 3420 (br) and 1070 (br) cm⁻¹ which are ascribable to the glycosidic structure. The presence of a sulfate group in holothurin A has been suggested by the positive potassium rhodizonate test^{1,3)} and by IR absorption bands observed at 1225 and 827 cm⁻¹.^{1,4)} Furthermore, the IR spectrum suggests the presence of a γ -lactone moiety, as in holothurin B (6), in view of the presence of an absorption band at 1745 cm⁻¹ together with a negative maximum of $[\theta]_{224} - 6400$ observed in the circular dichroism (CD) spectrum.^{1,5)} The CD spectrum also suggests that holothurin A possesses a 9(11)-ene moiety in the lanostane-type carbon skeleton in view of a positive maximum of $[\theta]_{200} + 41600$.^{1,5)}

On acidic hydrolysis, holothurin A (8) liberated the same artifact aglycone, 22,25-oxidoholothurinogenin (1), as was obtained from holothurin B (6), together with equimolar amounts of xylose, quinovose, glucose, and 3-O-methylglucose. In the carbon nuclear magnetic resonance (¹³C NMR) spectrum (taken in pentadeutero(*d*₅-)pyridine) of holothurin A (8) (Table I), signals due to the aglycone carbons are observed at chemical shifts almost identical with those for the aglycone carbon signals of holothurin B (6), thus suggesting that holothurin A contains the same genuine aglycone holothurigenol (2),¹⁾ with a sugar moiety which includes a sulfate

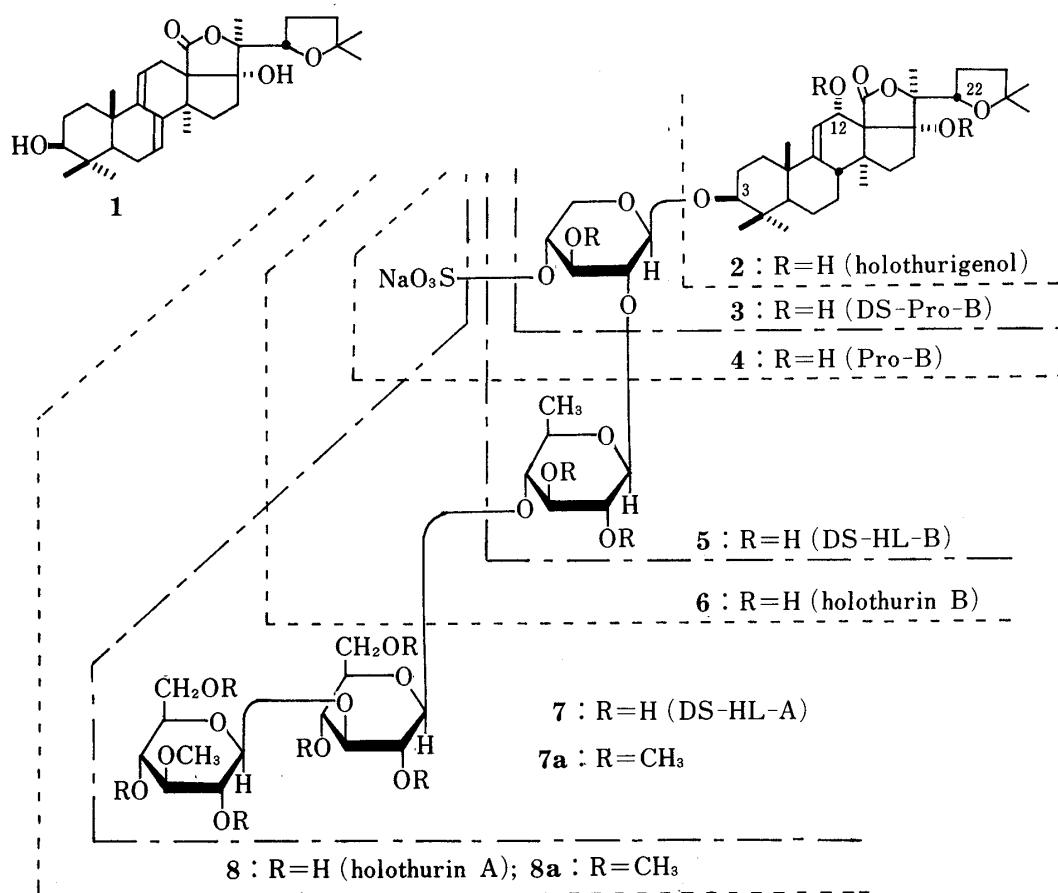


Chart 1

group attached at the 3 β -OH group, as holothurin B. The ^{13}C NMR spectrum also indicates β -orientation of the four glycosidic linkages of the above-mentioned monosaccharide moieties in holothurin A in view of the signals observed at δ 105.7, 105.2, 105.1, and 104.6⁶⁾ (Table I).

In order to elucidate the structure of the oligosaccharide moiety, holothurin A (8) was subjected to enzymic hydrolysis using snail enzyme^{1,7)} to furnish two prosapogenols, DS-Pro-B (3)¹⁾ (major) and Pro-B (4)¹⁾ (trace), together with holothurin B (6). On the other hand, enzymic hydrolysis of holothurin A with crude naringinase⁸⁾ yielded Pro-B (4) and holothurin B (6). Therefore, it has become evident so far that holothurin A is a tetraglycoside comprising holothurin B (6), glucose, and 3-O-methylglucose.

When holothurin A (8) was subjected to solvolysis with dioxane and pyridine,^{1,9)} a crystalline desulfated derivative DS-HL-A (7) was obtained. The preservation of the γ -lactone and the 9(11)-ene moieties in DS-HL-A is unequivocal on the basis of the CD spectrum^{1,5)}

TABLE I. ^{13}C -NMR Data for Holothurin A (8), B (6), and DS-HL-A (7) (25.05 MHz, d_6 -py., δ c)

Carbon	Holothurin ¹⁾ B (6)	DS-HL-A ^{a)} (7)	Holothurin ^{a)} A (8)
1	35.7 (t) ^{b)}	35.7 (t)	35.5 (t)
2	27.4 (t) ^{c)}	27.4 (t) ^{c)}	27.4 (t) ^{c)}
3	88.7 (d)	88.7 (d)	88.7 (d)
4	40.0 (s)	40.1 (s)	40.0 (s)
5	52.6 (d)	53.1 (d)	52.9 (d)
6	20.3 (t)	20.3 (t)	20.3 (t)
7	28.1 (t) ^{c)}	28.1 (t) ^{c)}	28.2 (t) ^{c)}

Carbon	Holothurin ¹⁾ B (6)	DS-HL-A ^{a)} (7)	Holothurin ^{a)} A (8)
8	40.9(d)	41.1(d)	41.0(d)
9	153.6(s)	153.9(s)	153.7(s)
10	39.7(s)	39.8(s)	39.7(s)
11	115.5(d)	115.6(d)	115.6(d)
12	71.4(d)	71.7(d) ^{c)}	71.7(d) ^{c)}
13	58.7(s)	59.2(s)	59.0(s)
14	45.9(s)	46.0(s)	46.0(s)
15	27.1(t)	27.1(t)	27.2(t)
16	38.4(t)	38.6(t)	38.6(t)
17	89.7(s)	89.8(s)	89.7(s)
18	174.4(s)	174.2(s)	174.3(s)
19	18.8(q)	18.9(q)	19.0(q)
20	86.5(s)	86.5(s)	86.5(s)
21	22.5(q)	22.5(q)	22.5(q)
22	80.6(d)	80.8(d)	80.7(d)
23	36.4(t)	36.6(t)	36.6(t)
24	28.7(t) ^{c)}	28.7(t) ^{c)}	28.7(t) ^{c)}
25	81.3(s)	81.3(s)	81.3(s)
26	28.7(q) ^{c,d)}	28.7(q) ^{c,d)}	28.7(q) ^{c,d)}
27	28.1(q) ^{c,d)}	28.1(q) ^{c,d)}	28.2(q) ^{c,d)}
28	21.2(q)	21.3(q)	21.4(q)
29	27.4(q) ^{c)}	27.4(q) ^{c)}	27.4(q) ^{c)}
30	16.7(q)	16.8(q)	16.7(q)

Carbon	DS-HL-A ^{a)} (7)	Holothurin ^{a)} A (8)	Me glycopyranoside
1'	105.5	105.7	106.0
2'	83.9	83.1	74.6
3'	77.8	76.7 ^{c)}	78.1
4'	70.9	74.9	70.9
5'	66.4	63.8	67.0
1''	105.4 ^{d)}	105.2 ^{d)}	105.3
2''	76.0	76.0	76.6
3''	76.3	76.7 ^{c)}	78.0
4''	87.1	86.5	77.2
5''	71.7 ^{c)}	71.7 ^{c)}	73.8
6''	18.2	17.9	18.5
1'''	105.3 ^{d)}	105.1 ^{d)}	105.5
2'''	73.6	73.9	74.9
3'''	88.4	88.1	78.3
4'''	70.9	70.8	71.4
5'''	77.8	77.3	78.2
6'''	62.5	62.2	62.7
1''''	104.6	104.6	105.5
2''''	74.9	74.9	74.6
3''''	87.8	87.7	88.2
4''''	70.0	69.5	70.7
5''''	78.1	78.0	78.0
6''''	62.5	61.9	62.4
3''''OMe	60.4	60.5	60.8

a) Measured at 60°C for 8 and at 70°C for 7.

b) Abbreviations given in parentheses denote signal patterns observed in the off-resonance experiments.

c) The two-carbon intensities of overlapping signals were confirmed by the hetero-decoupling method without NOE.

d) Assignments may be interchangeable in the same vertical column.

As mentioned briefly in our preceding paper,¹⁾ Chanley *et al.* proposed a partial structure 9 for "holothurin A" which was isolated from the Bahamean sea cucumber *Actinopyga agassizi*¹¹⁾ and Hashimoto *et al.* suggested the probable identity of "holothurin A" from *A. agassizi* with holothurin A from *H. leucospilota*.¹²⁾ However, as is apparent from our present investigation, the partial structure 9 is inconsistent with the structure of holothurin A (8). Recently, we obtained the Cuvierian tubules of *A. agassizi* and elucidated the chemical composition of "holothurin A" as comprising holothurin A (8) and a new oligoglycoside in a ratio of approximately 1:3;¹³⁾ the details will be reported in a forthcoming paper.

We examined some antimicrobial activities of holothurin A (8), holothurin B (6), and their prosapogenols (3, 4, 5, 7) and the artifact aglycone (1). The moderate activities found for 6, 7, and 8 will be reported in a future paper.

Experimental

The instruments used to obtain physical data and the experimental conditions for chromatography were the same as in our preceding paper¹⁾ unless otherwise specified.

Holothurin A (8)—An analytical sample of holothurin A (8) isolated from the title sea cucumber¹⁾ was obtained by recrystallization from aq. 95% EtOH as colorless needles of mp 228–230°C, $[\alpha]_D^{25} -14.9^\circ$ ($c=0.1$, H₂O). *Anal.* Calcd for C₅₄H₈₅NaO₂₇S: C, 53.10; H, 7.03; Na, 1.88; S, 2.62. Found: C, 52.92; H, 7.00; Na, 1.84; S, 2.78. IR $\nu_{\text{max}}^{\text{KBr}}$ cm⁻¹: 3400 (br), 1745, 1634 (w), 1060 (br); 1225, 827 (sulfate). UV (EtOH): transparent above 210 nm. CD ($c=6.67 \times 10^{-4}$, MeOH, 23°C): $[\theta]_{243} 0$, $[\theta]_{224} -6400$ (neg. max.), $[\theta]_{216} 0$, $[\theta]_{200} +41600$ (pos. max.). ¹³C NMR: Table I. Holothurin A gave a yellow spot on the paper at *Rf* 0.56 in the potassium rhodizonate test.^{1,3)}

Acidic Hydrolysis of Holothurin A (8)—A solution of 8 (200 mg) in aq. 3 N HCl (16 ml) was heated in a boiling water-bath for 2 h. After cooling, the reaction mixture was centrifuged to separate a precipitate (56 mg), which was purified by column chromatography (SiO₂ 5 g, *n*-hexane–acetone=8:1) to furnish 22,25-oxidoholothuringenin (1, 38 mg), mp 301°C (AcOEt).¹⁾ The supernatant was neutralized with Amberlite IRA-400 (OH⁻) and evaporated to dryness under reduced pressure to yield a residue which was examined by PPC, TLC, and GLC as described in the preceding paper.¹⁾ Quinovose, 3-O-methylglucose, xylose, and glucose were identified in a ratio of 1:1:1:1.

Hydrolysis of Holothurin A (8) with Snail Enzyme—A glycosidase mixture (300 mg), which was prepared from snail as described in the preceding paper,^{1,7)} was added to a mixture of 8 (352 mg) in AcOH–AcONa buffer (pH 5.2, 200 ml) and the whole was incubated with gentle stirring at 40°C for 14 days. After treatment with *n*-BuOH (10 ml), the reaction mixture was heated in a boiling water-bath for 15 min and treated with Celite 535 (20 g) with stirring, then filtered. The filtrate was extracted with *n*-BuOH five times (50 ml each) and removal of the solvent from the *n*-BuOH extract under reduced pressure gave a residue which was purified by column chromatography (SiO₂ 30 g, CHCl₃–MeOH=40:1→CHCl₃–MeOH–H₂O=7:3:1, lower phase) to furnish DS-Pro-B (3, 105 mg), holothurin B (6, 43 mg), and Pro-B (4, 3 mg), which were identical with the corresponding authentic samples¹⁾ as judged by mixed mp determination, TLC, and IR spectroscopy.

Hydrolysis of Holothurin A (8) with Crude Naringinase—A solution of 8 (500 mg) in water (200 ml) was treated with crude naringinase (1 g)⁸⁾ and the whole was incubated with gentle stirring at 34°C for 4 days. After addition of *n*-BuOH (200 ml) and water (100 ml), the whole mixture was heated in a boiling water-bath for 20 min and filtered. The filtrate was taken in a separatory funnel and the *n*-BuOH phase was separated. The aqueous phase was extracted with *n*-BuOH three times (50 ml each) and the combined *n*-BuOH phase was evaporated to dryness under reduced pressure. Column chromatography (SiO₂ 40 g, CHCl₃–MeOH–H₂O=7:3:1, lower phase) of the residue furnished Pro-B (4, 254 mg) and holothurin B (6, 71 mg), which were identical with the corresponding authentic samples¹⁾ as described above.

Solvolysis of Holothurin A (8) giving DS-HL-A (7)—A solution of 8 (500 mg) in pyridine (5 ml) was heated under reflux for 5 min. The solution was then treated with dioxane (5 ml) and heated under reflux again for 3 h. After cooling, the reaction mixture was filtered and a residue, which was obtained after removal of the solvent from the filtrate under reduced pressure, was crystallized from MeOH to furnish DS-HL-A (7, 307 mg). An analytical sample of 7 was prepared by recrystallization from 80% EtOH as colorless needles of mp 231–233°C, $[\alpha]_D^{25} -27.6^\circ$ ($c=0.5$, MeOH). *Anal.* Calcd for C₅₄H₈₈O₂₄·2H₂O: C, 56.23; H, 7.87. Found: C, 56.51; H, 7.82. IR $\nu_{\text{max}}^{\text{KBr}}$ cm⁻¹: 3410 (br), 1748, 1639 (w), 1068 (br). CD ($c=8.17 \times 10^{-4}$, MeOH, 22°C): $[\theta]_{252} 0$, $[\theta]_{225} -8600$ (neg. max.), $[\theta]_{217} 0$, $[\theta]_{202} +59700$ (pos. max.). ¹³C NMR: Table I.

Methylation of DS-HL-A (7) followed by Methanolysis—A solution of 7 (140 mg) in DMSO (3 ml) was treated with a dimsyl carbanion–DMSO solution (8.2 ml) (prepared from 1 g of NaH and 17.5 ml of DMSO) and the whole was kept stirring under a nitrogen atmosphere at 18°C for 1 h. The reaction mixture was then treated with CH₃I (3.6 ml) under ice-cooling and was kept stirring at 18°C in the dark under a nitrogen atmosphere for a further 3 h. The mixture was poured into ice-water and the whole was extracted with AcOEt four times (80 ml each). The AcOEt extract was separated, washed with sat. aq. Na₂S₂O₃ and worked

up in the usual manner. The product, obtained by evaporation of the solvent under reduced pressure, was purified by column chromatography (SiO_2 10 g, benzene–acetone=15:1) to furnish the trideca-O-methyl derivative (**7a**). **7a**, amorphous,¹⁵ $[\alpha]_D^{17} -13.0^\circ$ ($c=0.2$, MeOH). IR $\nu_{\text{max}}^{\text{CHCl}_3}$ cm^{-1} : no OH, 1766, 1642 (w), 1086 (br). CD ($c=7.87 \times 10^{-4}$, MeOH, 22.5°C): $[\theta]_{253} 0$, $[\theta]_{226} -7900$ (neg. max.), $[\theta]_{219} 0$, $[\theta]_{202} +74000$ (pos. max.). ^1H NMR (90 MHz, CDCl_3 , δ): 4.19, 4.27, 4.54, 4.57 (1H each, all d, $J=7$ Hz, anom. H $\times 4$).¹⁶

A solution of **7a** (5 mg) in 2.5 N HCl–MeOH (2 ml) was heated under reflux for 30 min. After cooling, the mixture was neutralized with Ag_2CO_3 and removal of the solvent from the filtrate with a stream of nitrogen gave a residue which was analyzed by GLC (column: 15% NPGS, 3 mm $\times$ 2 m, temp. 170°C , N_2 30 ml/min, for I, II, and III; column: 15% PEGS, 3 mm $\times$ 1 m, temp. 140°C , N_2 30 ml/min, for IV) and TLC (benzene–acetone=3:1). The products were identified as methyl 2,3-di-O-methylquinovopyranoside (I) (t_R 4'30", 5'38"; R_f 0.60, 0.70), methyl 2,3,4,6-tetra-O-methylglucopyranoside (II) (t_R 4'52", 6'28"; R_f 0.50, 0.67), methyl 2,4,6-tri-O-methylglucopyranoside (III) (t_R 12'18", 17'15"; R_f 0.21, 0.27), and methyl 3,4-di-O-methylxylopyranoside (IV) (t_R 5'31", 7'08"; R_f 0.24, 0.26).

Methylation of Holothurin A (8) followed by Methanolysis—A solution of **8** (200 mg) in DMSO (3 ml) was treated with a dimsyl carbanion–DMSO solution (15 ml) (prepared from 1 g of NaH and 17.5 ml of DMSO) and the whole mixture was kept stirring at 15°C under a nitrogen atmosphere for 1 h. Under ice-cooling, the mixture was treated with CH_3I (7 ml) and the whole mixture was kept stirring at 15°C in the dark under a nitrogen atmosphere for 3 h. The reaction mixture was poured into ice-water (200 ml) and extracted with *n*-BuOH five times (50 ml each). Work-up of the *n*-BuOH extract in the usual manner followed by removal of the solvent under reduced pressure gave a product which was purified by column chromatography (SiO_2 60 g, CHCl_3 –MeOH– H_2O =12:3:1, lower phase) to furnish the dodeca-O-methyl derivative (**8a**, 101 mg). **8a**, amorphous,¹⁵ $[\alpha]_D^{18} -11.7^\circ$ ($c=0.29$, MeOH). IR $\nu_{\text{max}}^{\text{CHCl}_3}$ cm^{-1} : 3450 (w),¹⁷ 1757, 1644 (w), 1090 (br); 1238, 823 (sulfate). CD ($c=8.97 \times 10^{-3}$, MeOH, 22.5°C): $[\theta]_{252} 0$, $[\theta]_{226} -7200$ (neg. max.), $[\theta]_{218} 0$, $[\theta]_{200} +51300$ (pos. max.). ^1H NMR (90 MHz, CDCl_3 , δ): 4.22–4.73 (4H, anom. H $\times 4$).¹⁶

Methanolysis of **8a** (5 mg) with 2.5 N HCl–MeOH (2 ml) was carried out for 40 min as described for **7a**. The product was analyzed by GLC (column: 15% PEGS, 3 mm $\times$ 1 m, temp. 170°C , N_2 30 ml/min) and TLC (benzene–acetone=3:1). The products were identified as I (t_R 1'29", 1'55"; R_f 0.61, 0.69), II (t_R 1'23", 1'50"; R_f 0.51, 0.67), III (t_R 4'27", 6'33"; R_f 0.21, 0.27), and methyl 3-O-methylxylopyranoside (V) (t_R 5'56", 9'03"; R_f 0.11).

Acknowledgement The authors are grateful to Prof. T. Matsuno of Kyoto College of Pharmacy for a generous gift of authentic holothurin A. They are also indebted to Tanabe Pharm. Co. for supplying crude naringinase and to Dainippon Pharm. Co. for elemental analyses.

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- 14) T_1 values in parentheses are apparent ones for the nuclei whose signals could not be obtained separately.
- 15) Although satisfactory analytical values could not be obtained, these methyl ethers were subsequently subjected to methanolysis after confirmation of their purities by TLC.
- 16) The number of methoxyls of these methyl ethers could not be confirmed by measuring their ^1H NMR signal intensities due to overlapping by other signals.
- 17) Probably due to hygroscopicity of the methyl ether.