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

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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→3)-β-D-glucopyranosyl(1→4)-β-D-quinovopyranosyl(1→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; ^{1}H NMR; ^{13}C NMR; CD; snail enzyme; crude naringinase; T_1

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**), $C_{54}H_{85}NaO_{27}S$, 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 (^{13}C 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

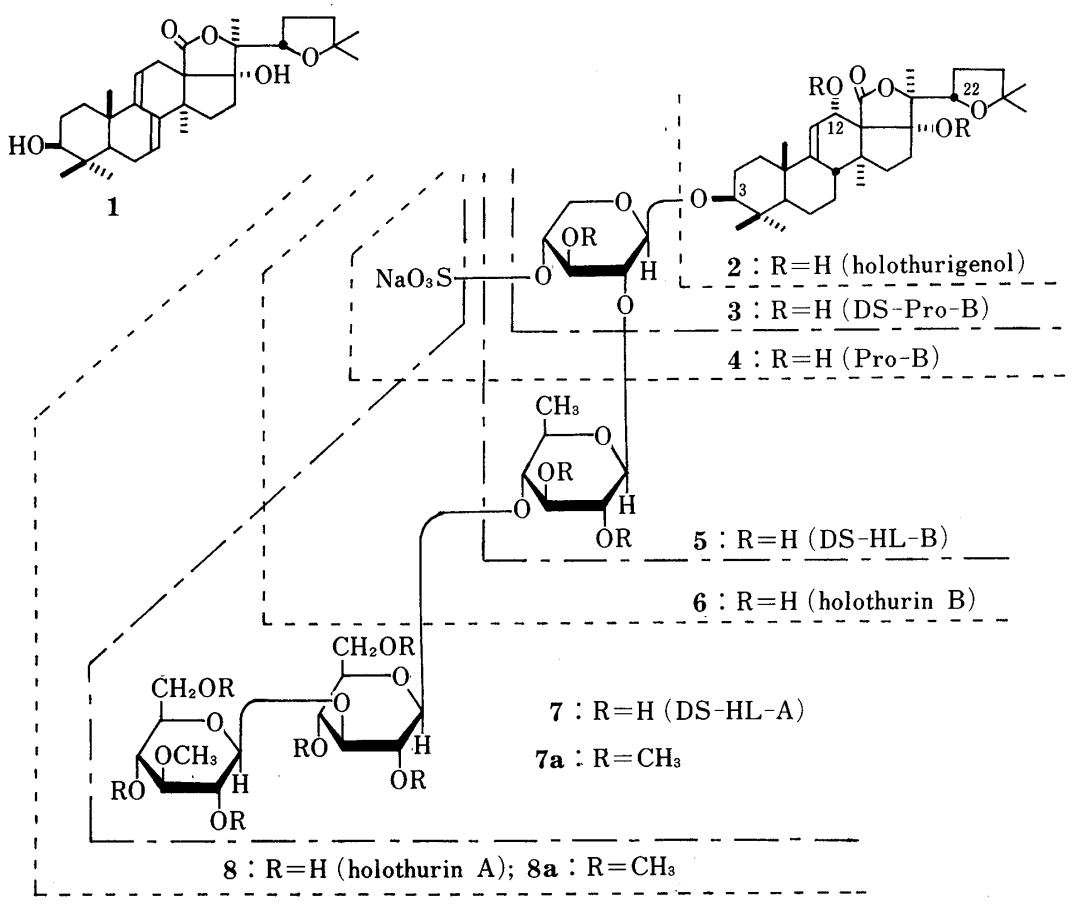

Chart 1

group attached at the 3β-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 104.6[6] (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 naringinase[8] 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 preser vation 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_5-py., δc)

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	Me β-D-xylopyranoside (OMe: 56.6)
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	Me β-D-quinovopyranoside (OMe: 56.5)
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	Me β-D-glucopyranoside (OMe: 56.7)
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	Me 3-O-Me-β-D-glucopyranoside (OMe: 56.7)
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.

:$[\theta]_{225}$ −8600 (neg. max.) and $[\theta]_{202}$ +59000 (pos. max.). Methylation of DS–HL–A with methyl iodide and a dimsyl carbanion[1] yielded a fully methylated trideca-O-methyl derivative (**7a**), whick lacks hydroxyl absorption in its IR spectrum. The ^{1}H NMR spectrum of the methyl ether exhibits four anomeric proton doublets at δ 4.19, 4.27, 4.54, and 4.57 (J=7 Hz for each), thus supporting the presence of four β-glycosidic linkages ($^4C_1$) in holothurin A (**8**), which is in good accord with the above-mentioned ^{13}C NMR analysis (Table I).

On methanolysis with anhydrous 2.5 N hydrogen chloride in methanol, the trideca-O-methyl ether (**7a**) liberated methyl pyranosides of 2,3,4,6-tetra-O-methylglucose, 2,4,6-tri-O-methylglucose, 2,3-di-O-methylquinovose, and 3,4-di-O-methylxylose. On the other hand, methanolysis of the dodeca-O-methyl ether (**8a**), which was prepared from holothurin A (**8**) as described for **7a**, furnished methyl pyranosides of 2,3,4,6-tetra-O-methylglucose, 2,4,6-tri-O-methylglucose, 2,3-di-O-methylquinovose, and 3-O-methylxylose.

Based on the combined evidence described above, the chemical structure of holothurin A has been elucidated to be 4''-O-[3'''-O-(β-D-3''''-O-methylglucopyranosyl)-β-D-glucopyranosyl]-holothurin B (**8**). This structure is consistent with values for the spin-lattice relaxation time (T_1) measured in d_5-pyridine for the oligosaccharide carbons of DS–HL–A (**7**). As shown in Fig. 1, the average NT_1 values (N=number of proton(s) attached to specific carbons) for skeletal carbons of individual carbohydrate units in DS–HL–A are 0.26 (for 3-O-methylglucose), 0.23 (glucose), 0.22 (quinovose), and 0.18 (xylose) sec. The NT_1 value for the non-reducing terminal 3-O-methylglucose is longest and that for the reducing terminal xylose is shortest, both being in agreement with the sequence of monosaccharide units[10] in DS–HL–A (**7**).

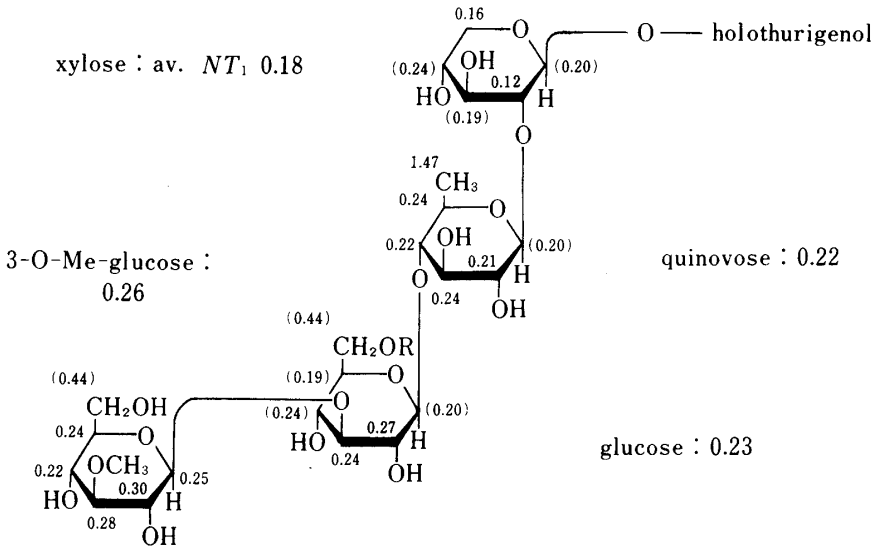

Fig. 1. NT_1 Values (sec) for DS–HL–A (**7**)[14]

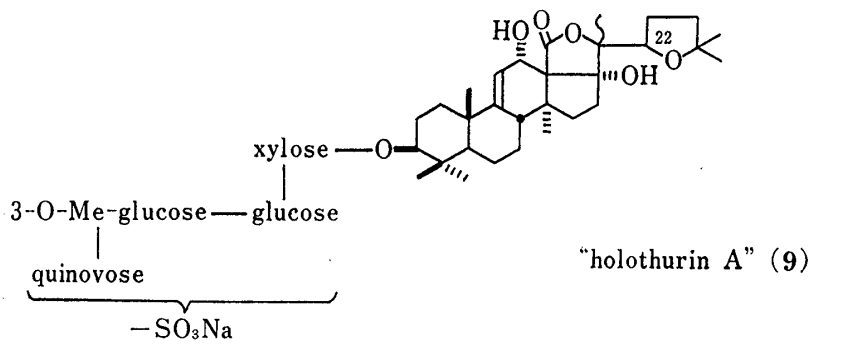

Chart 2

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 paper[1] unless otherwise specified.

Holothurin A (8)——An analytical sample of holothurin A (**8**) isolated from the title sea cucumber[1] was obtained by recrystallization from aq. 95% EtOH as colorless needles of mp 228—230°C, $[\alpha]_D^{18}$ −14.9° (c=0.1, H_2O). *Anal.* Calcd for $C_{54}H_{85}NaO_{27}S$: 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 ν_{max}^{KBr} cm^{-1}: 3400 (br), 1745, 1634 (w), 1060 (br); 1225, 827 (sulfate). UV (EtOH): transparent above 210 nm. CD ($c=6.67\times10^{-4}$, MeOH, 23°C): $[\theta]_{243}$ 0, $[\theta]_{224}$ −6400 (neg. max.), $[\theta]_{216}$ 0, $[\theta]_{200}$ +41600 (pos. max.). ^{13}C 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 (SiO_2 5 g, *n*-hexane–acetone=8: 1) to furnish 22,25-oxidoholothurinogenin (**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 (SiO_2 30 g, $CHCl_3$–MeOH=40: 1→$CHCl_3$–MeOH–H_2O=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 samples[1] 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 (SiO_2 40 g, $CHCl_3$–MeOH–H_2O=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 samples[1] 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, $[\alpha]_D^{22}$ −27.6° (c=0.5, MeOH). *Anal.* Calcd for $C_{54}H_{86}O_{24}\cdot2H_2O$: C, 56.23; H, 7.87. Found: C, 56.51; H, 7.82. IR ν_{max}^{KBr} cm^{-1}: 3410 (br), 1748, 1639 (w), 1068 (br). CD ($c=8.17\times10^{-4}$, MeOH, 22°C): $[\theta]_{252}$ 0, $[\theta]_{225}$ −8600 (neg. max.), $[\theta]_{217}$ 0, $[\theta]_{202}$ +59700 (pos. max.). ^{13}C 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 CH_3I (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. $Na_2S_2O_3$ 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° (c=0.2, MeOH). IR $\nu_{max}^{CCl_4}$ cm^{-1}: no OH, 1766, 1642 (w), 1086 (br). CD ($c=7.87\times10^{-4}$, MeOH, 22.5°C): $[\theta]_{253}$ 0, $[\theta]_{226}$ −7900 (neg. max.), $[\theta]_{219}$ 0, $[\theta]_{202}$ +74000 (pos. max.). ^{1}H NMR (90 MHz, $CDCl_3$, δ): 4.19, 4.27, 4.54, 4.57 (1H each, all d, J=7 Hz, anom. H×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×2 m, temp. 170°C, N_2 30 ml/min, for I, II, and III; column: 15% PEGS, 3 mm×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″; Rf 0.60, 0.70), methyl 2,3,4,6-tetra-O-methylglucopyranoside (II) (t_R 4′52″, 6′28″; Rf 0.50, 0.67), methyl 2,4,6-tri-O-methylglucopyranoside (III) (t_R 12′18″, 17′15″; Rf 0.21, 0.27), and methyl 3,4-di-O-methylxylopyranoside (IV) (t_R 5′31″, 7′08″; Rf 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] $[\alpha]_D^{13}$ −11.7° (c=0.29, MeOH). IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3450 (w),[17] 1757, 1644 (w), 1090 (br); 1238, 823 (sulfate). CD ($c=8.97\times10^{-3}$, MeOH, 22.5°C): $[\theta]_{252}$ 0, $[\theta]_{226}$ −7200 (neg. max.), $[\theta]_{218}$ 0, $[\theta]_{200}$ +51300 (pos. max.). ^{1}H NMR (90 MHz, $CDCl_3$, δ): 4.22—4.73 (4H, anom. H×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×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″; Rf 0.61, 0.69), II (t_R 1′23″, 1′50″; Rf 0.51, 0.67), III (t_R 4′27″, 6′33″; Rf 0.21, 0.27), and methyl 3-O-methylxylopyranoside (V) (t_R 5′56″, 9′03″; Rf 0.11).

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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 ^{1}H NMR signal intensities due to overlapping by other signals.
17) Probably due to hygroscopicity of the methyl ether.