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Saponin and Sapogenol. XXVII.[1] Revised Structures of Holotoxin A and Holotoxin B, Two Antifungal Oligoglycosides from the Sea Cucumber *Stichopus japonicus* Selenka

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The chemical structures of two antifungal oligoglycosides holotoxin A and B, which were isolated from the sea cucumber *Stichopus japonicus* Selenka, have been reinvestigated. On the basis of chemical and physicochemical evidence, the genuine sapogencl of holotoxin A and B has been elucidated to be holotoxigenol rather than previously proposed stichopogenin A_4 (**3**), and it has been shown that the structures of holotoxin A and B should be revised as 3-O-{2-O-[3-O-methyl-β-D-glucopyranosyl(1→3)-β-D-glucopyranosyl-(1→4)-β-D-quinovopyranosyl]-4-O-[3-O-methyl-β-D-glucopyranosyl(1→3)-β-D-glucopyranosyl]-β-D-xylopyranosyl}-holotoxigenol (**6**) and 3-O-{2-O-[3-O-methyl-β-D-glucopyranosyl-(1→3)-β-D-glucopyranosyl(1→4)-β-D-quinovopyranosyl]-4-O-[β-D-glucopyranosyl(1→3)-β-D-glucopyranosyl]-β-D-xylopyranosyl}-holotoxigenol (**9**), respectively.

Keywords——sea cucumber; *Stichopus japonicus*; genuine sapogenol; holotoxigenol; lanostane-type triterpenoid; holotoxin A; holotoxin B; antifungal oligoglycoside; saponin; CMR

A few years ago, we reported the structural elucidation of two antifungal oligoglycosides (saponins) named holotoxin A and B, which were isolated from the sea cucumber *Stichopus japonicus* Selenka.[3] As described there,[3d] holotoxin A and B are noteworthy because of their distinct growth inhibitory activities against the pathogenic microorganisms, *e.g. Trichophyton sp.*, *Candida sp.*, and *Trichomonas sp.* During the course of our continuing studies in regard to relationship between the chemical structures and antifungal activities of holotoxins, we have noticed some discrepancy on the proposed structures[3] of holotoxin A and B. Since a considerable quantity of the holotoxin mixture have been generously provided by Mr. S. Shimada,[4] we have reinvestigated the chemical structures of holotoxins, and finally have reached a conclusion that the previously proposed structures[3] of holotoxin A and B should be revised as **6** and **9**, respectively. This paper deals with the details being consistent with the new structures.[5]

Medium pressure column chromatography of the holotoxin mixture[4] furnished holotoxin A (**6**) and B (**9**). Holotoxin A (**6**), mp 250—253°, shows no ultraviolet (UV) absorption maximum above 210 nm, while the infrared (IR) spectrum of **6** exhibits the absorption bands characteristic to the glycosidic structure along with the absorption bands due to a γ-lactone and a five-membered ring ketone as reported previously.[3] The circular dichroism (CD)

1) Part XXVI: I. Kitagawa and M. Kobayashi, *Chem. Pharm. Bull.* (Tokyo), **26**, 1864 (1978).
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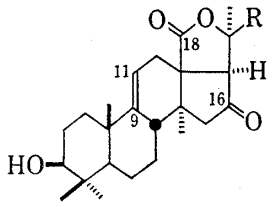
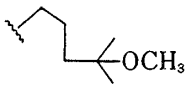
3) *a*) I. Kitagawa, T. Sugawara, I. Yosioka, and K. Kuriyama, *Tetrahedron Lett.*, **1975**, 963; *b*) *Idem, Chem. Pharm. Bull.* (Tokyo), **24**, 266 (1976); *c*) I. Kitagawa, T. Sugawara, and I. Yosioka, *Tetrahedron Lett.*, **1974**, 4111; *d*) *Idem, Chem. Pharm. Bull.* (Tokyo), **24**, 275 (1976).
4) S. Shimada, *Science*, **163**, 1462 (1969).
5) Presented at the 98th Annual Meeting of Pharmaceutical Society of Japan, Okayama, April 1978, by I. Kitagawa, H. Yamanaka, and M. Kobayashi.

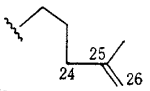
spectrum also shows the presence of these chromophores together with $\Delta^{9(11)}$ in the sapogenol portion.[3)]

On methanolysis under the anhydrous conditions using 0.25 N hydrogen chloride in methanol, holotoxin A (**6**) furnished two sapogenols: genin-3 (**1**) and genin-1 (**2**),[3a,b)] while, on acid hydrolysis with aqueous 7% sulfuric acid–methanol–benzene, **6** afforded stichopogenin A_4 (**3**)[3a,b)] as the major sapogenol together with trace amounts of genin-3 (**1**) and genin-1 (**2**). Stichopogenin A_4 (**3**) was not detected in the former methanolysates. Genin-1 (**2**) is a mixture (*ca.* 4: 1) of Δ^{24} and Δ^{25} isomers as revealed by the proton magnetic resonance (PMR) spectrum (the ratio of signal intensities at δ 5.07 (24-H) and 4.67 (26-H_2)=*ca.* 2: 1). These findings have led us to doubt stichopogenin A_4 (**3**)[3a,b)] as the genuine sapogenol of holotoxin A (**6**).



1 (genin-3) R= 

2 (genin-1)

R= 

+ Δ^{24} isomer

(holotoxigenol)

3 (stichopogenin A_4)

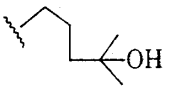
R= 

Chart 1

In order to solve the problem, the carbon magnetic resonance (CMR) spectrum of holotoxin A (**6**) has been examined in pentadeutero(d_5)-pyridine. By combination of the off-resonance method, the hetero decoupling without nuclear Overhauser effect (NOE) method,[6)] and the weak noise decoupling method (for the quaternary carbon atoms),[7)] the signals due to the following carbons have been assigned: C-9 (δ_c 151.6 (s)), C-11 (111.2 (d)), C-18 (175.7 (s)),[8)] C-16 (212.5 (s)), C-25 (145.5 (s)), and C-26 (110.4 (t)).[9)] The signals ascribable to four olefinic carbons (C-9, C-11, C-25, and C-26) are also observed in the CMR spectrum of **6** taken in hexadeutero(d_6)-dimethyl sulfoxide (DMSO). Therefore, it has become evident that the Δ^{25} isomer (now named as holotoxigenol although not isolated yet) contained in genin-1 (**2**) is the genuine sapogenol of holotoxin A (**6**) rather than previously proposed stichopogenin A_4 (**3**) and that the Δ^{24} isomer is an artifact sapogenol. The Δ^{25} structure in the side chain of the sapogenol part has been further substantiated by two-proton broad singlet at around δ 4.8 (due to 26-H_2) which is observed in the PMR spectra of the methylated derivatives of holotoxin A, B, and their prosapogenols (**4a**, **5a**, **6a**, **7a**, **8a**, and **9a**) (*vide infra*). At this stage of investigation, the previous discussion on the anomeric configurations of holotoxin A and the prosapogenols[3c,d)] has become questionable, since it has been noticed that the broad singlet due to 26-H_2 would possibly have led us to confusion in the PMR assignment of the anomeric protons.

We have next subjected holotoxin A (**6**) to the enzymatic hydrolysis, in order to reexamine the structure of the oligosaccharide portion. On hydrolysis using takadiastase A preparation[3d)] or crude hesperidinase, **6** yielded two prosapogenols **4** and **5**. The less polar

6) R. Freanana, K.G. Pachler, and G.N. LaMar, *J. Chem. Phys.*, **55**, 4586 (1971).
7) I.H. Sadler, *J. Chem. Soc. Chem. Commun.*, **1973**, 809.
8) I. Kitagawa, T. Nishino, T. Matsuno, H. Akutsu, and Y. Kyogoku, *Tetrahedron Lett.*, **1978**, 985.
9) J.B. Stothers, "Carbon-13 NMR Spectroscopy," Academic Press, New York, 1972, p. 433.

one (**4**), mp 281—282°, contains one mole each of xylose and quinovose as the carbohydrate ingredients.[10] The IR spectrum of **4** shows the absorption bands at 3400 (br, hydroxyl), 1765 (br, γ-lactone), and 1754 (br, C-16 ketone), while the CD spectrum of **4** shows the presence of the same chromophores as in parent holotoxin A.

Methylation of **4** with methyl iodide (CH_3I)–DMSO–sodium hydride (NaH)[11] yielded a hexa-O-methyl derivative (**4a**), the IR spectrum of which shows the formation of a methoxy-

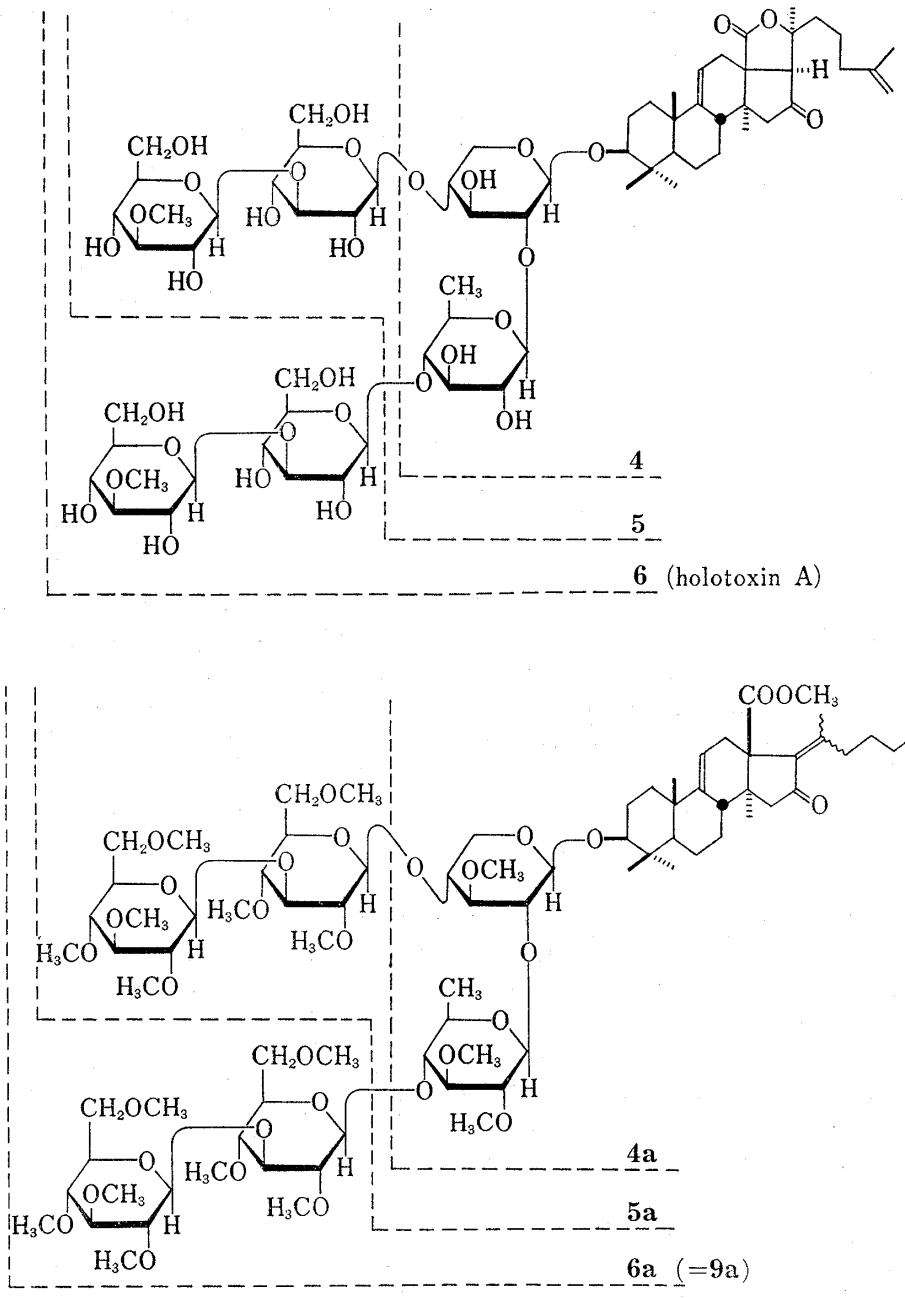


Chart 2

10) The monosaccharide ingredients of saponins and the prosapogenols were elucidated by gas–liquid chromatographic (GLC) analysis of the trimethylsilylated (TMS) derivatives of the methanolysis products.

11) S. Hakomori, *J. Biochem.* (Tokyo), **55**, 205 (1964).

carbonyl (1733 cm^{-1}) and an α,β-unsaturated ketone (1718, 1610 cm^{-1}) and the absence of free hydroxyl. The CD and UV spectra of **4a**, respectively, suggest the presence of an enone chromophore: $[\theta]_{350}$ −7000 (neg. max.), $[\theta]_{252}$ −70000 (neg. max.), and λ_{max} 252 nm (ε=12000), thus the modification in the sapogenol part of **4a** being ascertained as discussed before.[3c,d] In the PMR spectrum of **4a**, the signals ascribable to two anomeric protons are observed at δ 4.45 and 4.89 as doublets of J=7 Hz which indicate β orientation ($^4C_1$ conformation) of both carbohydrate ingredients in **4a**. In addition, a broad singlet[12] due to the terminal methylene at C-25 is observed at δ 4.81. Methanolysis of **4a** with anhydrous 2.5 N hydrogen chloride in methanol liberated methyl 2,3,4-tri-O-methyl-quinovopyranoside and methyl 3,4-di-O-methyl-xylopyranoside. Based on the foregoing evidence, the structure of **4** has been ascertained.

Another prosapogenol (**5**), mp 274—276°, contains one mole each of xylose, quinovose, glucose, and 3-O-methyl-glucose.[10] Here again, the CD spectrum of **5** shows the presence of the same chromophores as in **4** and holotoxin A (**6**). The PMR spectrum of the completely methylated dodeca-O-methyl derivative (**5a**) demonstrates the presence of four β linkage ($^4C_1$ conformation) by the signals assignable to four anomeric protons at δ 4.10 (d, J=8 Hz), 4.60 (d, J=7 Hz), 4.95 (d, J=7 Hz), and 4.97 (d, J=8 Hz). On methanolysis, **5a** liberated methyl 2,3,4-tri-O-methyl-quinovopyranoside, methyl 2,3,4,6-tetra-O-methyl-glucopyranoside, methyl 2,4,6-tri-O-methyl-glucopyranoside, and methyl 3-O-methyl-xylopyranoside. Therefore, the structure of **5** has become unequivocal.

Holotoxin A (**6**), which comprises one mole each of xylose and quinovose, and two moles each of glucose and 3-O-methyl-glucose, yielded the octadeca-O-methyl derivative (**6a**) on complete methylation. The PMR spectrum of **6a** shows the signals ascribable to six anomeric protons at δ 4.10 (1H, d, J=8 Hz), 4.33 (1H, d, J=8 Hz), 4.55 (1H, br. d, J=5 Hz), 4.91 (2H, d, J=7 Hz), and 4.94 (1H, d, J=7 Hz), all of which indicate the presence of six β linkages in **6a** ($^4C_1$ conformation).[13] The CMR spectrum (in d_5-pyridine) of holotoxin A (**6**) also substantiates the presence of six β-anomeric configurations in **6** by the signals at δ_c 103.0 (d, 1C), 104.5 (d, 1C), 105.0 (d, 1C), 105.3 (d, 1C), 105.5 (d, 2C).[14]

On methanolysis, **6a** furnished methyl 2,3,4,6-tetra-O-methyl-glucopyranoside, methyl 2,4,6-tri-O-methyl-glucopyranoside, methyl 2,3-di-O-methyl-quinovopyranoside, and methyl 3-O-methyl-xylopyranoside.

Consequently, the structure of holotoxin A has been established as 3-O-{2-O-[3-O-methyl-β-D-glucopyranosyl(1→3)-β-D-glucopyranosyl(1→4)-β-D-quinovopyranosyl]-4-O-[3-O-methyl-β-D-glucopyranosyl(1→3)-β-D-glucopyranosyl]-β-D-xylopyranosyl}-holotoxigenol (**6**).

Holotoxin B (**9**), mp 252—253°, shows the similar spectroscopic properties as holotoxin A (**6**),[3c,d] and contains one mole each of xylose, quinovose, and 3-O-methyl-glucose, and three moles of glucose.[10] On enzymatic hydrolysis with crude hesperidinase, **9** furnished two prosapogenols (**4** and **7**). The less polar prosapogenol has been found to be identical with the one (**4**) obtained above from holotoxin A (**6**) by thin-layer chromatography (TLC), mixed mp, and $[\alpha]_D$, thus the genuine sapogenol of holotoxin B (**9**) has been demonstrated to be holotoxigenol as in holotoxin A (**6**).

The more polar prosapogenol (**7**), mp 273—276°, is a new tetraglycoside containing one mole each of xylose, quinovose, glucose, and 3-O-methyl-glucose.[10] The CD spectrum of **7** shows preservation of the same chromophores (γ-lactone, C-16 ketone, and $\Delta^{9(11)}$) as in **4**.

12) The signal intensity accounts approximately (slightly less than) two protons, since a weak signal is observed at δ 5.05 which suggests minor contamination of the Δ^{24} isomer presumably formed during the methylation procedure.

13) The β orientation of all six anomeric carbons in **6a** has been further supported by its identity with another octadeca-O-methyl derivative prepared from holotoxin B (**9**) as described later.

14) R.U. Lemieux and S. Koto, *Tetrahedron*, **30**, 1933 (1974).

On complete methylation, **7** yielded the dodeca-O-methyl derivative (**7a**), which carries four β linkages ($^4C_1$ conformation) as revealed by the PMR signals at δ 4.35 (d, J=8 Hz), 4.44 (d, J=7 Hz), 4.91 (d, J=7 Hz), and 4.98 (d, J=8 Hz). Methanolysis of **7a** liberated methyl 2,3,4,6-tetra-O-methyl-glucopyranoside, methyl 2,4,6-tri-O-methyl-glucopyranoside, methyl 2,3-di-O-methyl-quinovopyranoside, and methyl 3,4-di-O-methyl-xylopyranoside. Based on

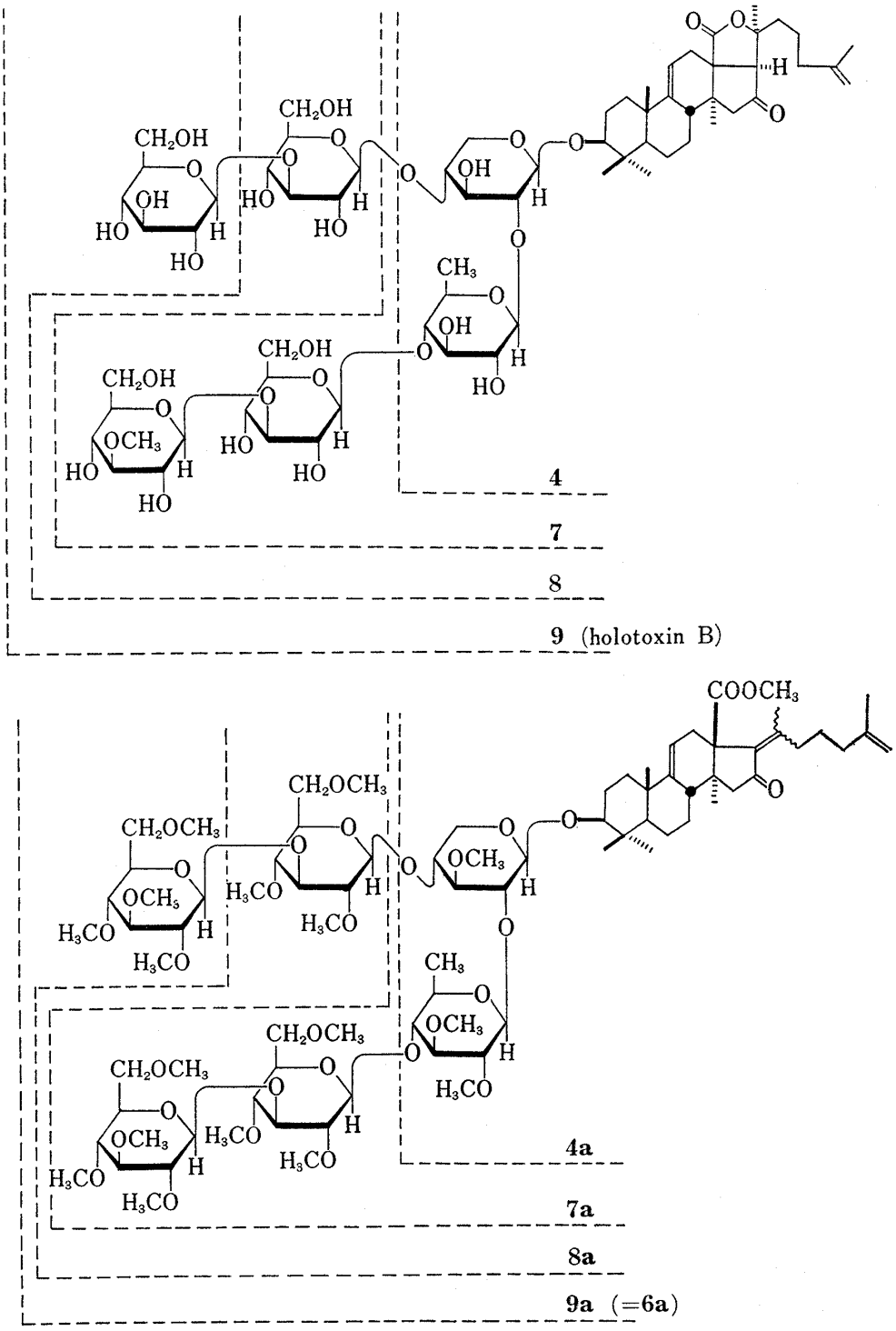


Chart 3

these findings, the structure of the prosapogenol has been elucidated to be the linear tetraglycoside (**7**).

On the other hand, another new prosapogenol (**8**) was obtained by enzymatic hydrolysis of holotoxin B (**9**) using crude β-glucosidase (almond emulsin). The prosapogenol (**8**), mp 281—284°, is a pentaglycoside comprising one mole each of xylose, quinovose, and 3-O-methylglucose, and two moles of glucose.[10] Complete methylation of **8** furnished the pentadeca-O-methyl derivative (**8a**). Here again, all the anomeric configurations are β ($^4C_1$ conformation) as demonstrated by the five PMR doublets observed at δ 4.13 (J=7 Hz), 4.35 (J=7 Hz), 4.53 (J=7 Hz), 4.91 (J=7 Hz), and 4.97 (J=7 Hz).

On methanolysis, **8a** liberated methyl 2,3,4,6-tetra-O-methyl-glucopyranoside, methyl 2,4,6-tri-O-methyl-glucopyranoside, methyl 2,3-di-O-methyl-quinovopyranoside, and methyl 3-O-methyl-xylopyranoside, thus the structure (**8**) being assigned to the prosapogenol.

Finally, complete methylation of holotoxin B (**9**) afforded the octadeca-O-methyl derivative (**9a**),[13,15] whose six anomeric configurations are β ($^4C_1$ conformation) as shown by the PMR signals at δ 4.12 (1H, d, J=8 Hz), 4.35 (1H, d, J=8 Hz), 4.55 (1H, br. d, J=5 Hz), 4.92 (2H, d, J=7 Hz), and 4.95 (1H, d, J=7 Hz). On methanolysis, **9a** liberated methyl 2,3,4,6-tetra-O-methyl-glucopyranoside, methyl 2,4,6-tri-O-methyl-glucopyranoside, methyl 2,3-di-O-methyl-quinovopyranoside, and methyl 3-O-methyl-xylopyranoside, thus the structure of holotoxin B being assigned as having a glucopyranoside moiety attached to 3-OH of the terminal glucose moiety in the prosapogenol (**8**). The anomeric configuration of the terminal glucopyranoside linkage has been ascertained as β by application of the Klyne's rule: $[M]_D$ (**9**)−$[M]_D$ (**8**)= −39°, $[M]_D$ of methyl β-D-glucopyranoside= −66°, $[M]_D$ of methyl α-D-glucopyranoside= +307°.[16,17]

Based on the accumulated evidence, the structure of holotoxin B has been established as 3-O-{2-O-[3-O-methyl-β-D-glucopyranosyl(1→3)-β-D-glucopyranosyl(1→4)-β-D-quinovopyranosyl]-4-O-[β-D-glucopyranosyl(1→3)-β-D-glucopyranosyl]-β-D-xylopyranosyl}-holotoxigenol (**9**).

As one of the reasons, which are responsible for our previous conclusion on the structures of holotoxin A and B,[3c,d] we presume that holotoxins used in our previous studies were not completely homogeneous. Due to shortage of the material at that time, holotoxins isolated from the sea cucumber collected in the different area were erroneously believed to be identical on the basis of simple TLC comparison.[18] As has been noticed in our studies on the oligoglycosidic constituents of the starfish,[1,19] if the habitat of the marine animal differs (although the species is identical), the carbohydrate ingredients in the oligoglycosides produced by the animal would possibly vary while the sapogenol constituents unchange. This would bring about the contamination of the closely related saponins in the starting material. However, this problem should be a subject of the future investigation on the oligoglycosides produced by the marine animal.

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15) The octadeca-O-methyl derivative (**9a**) obtained here was found to be identical with the above mentioned octadeca-O-methyl derivative (**6a**) of holotoxin A (**6**) by TLC, $[\alpha]_D$, UV, CD, PMR, and GLC analysis of the methanolysis products of both.

16) T. Kawasaki and T. Yamauchi, *Chem. Pharm. Bull.* (Tokyo), **10**, 703 (1962).

17) Since one of the coupling constants for six anomeric protons of **9a** (although unspecified) is rather small (5 Hz), comparison of the molecular rotation has been carried out.

18) Holotoxin A and B used in the present study are identical in all respects with those used for the studies of the aglycone part[3a,b] and the antifungal activities.[3d] However, identity of holotoxins used in the studies of the oligosaccharide portion[3c,d] is obscure from our present knowledge. Holotoxins used there were originated from the sea cucumber collected in some other places (*e.g.* Korea).

19) *a*) I. Kitagawa, M. Kobayashi, T. Sugawara, and I. Yosioka, *Tetrahedron Lett.*, **1975**, 967; *b*) I. Kitagawa, M. Kobayashi, and T. Sugawara, *Chem. Pharm. Bull.* (Tokyo), **26**, 1852 (1978); *c*) I. Kitagawa and M. Kobayashi, *Tetrahedron Lett.*, **1977**, 859.

Experimental[20]

Isolation of Holotoxin A (6) and B (9)——The crude holotoxin mixture (1.00 g) (isolated from the sea cucumber *Stichopus japonicus* SELENKA which was collected mainly in the Inland Sea, Japan[4]) was subjected to medium pressure column chromatography (silica gel 80 g; preparation of column at 5 kg/cm²; elution at 3 kg/cm²; column size 2.5×60 cm, flow rate 50 ml/hr; developing solvent $CHCl_3$–MeOH–H_2O=8:3:1 (lower layer)→7:3:1 (lower layer)) to furnish holotoxin A (6, 300 mg) and B (9, 170 mg). Holotoxin A (6), mp 250—253° ($CHCl_3$–MeOH–H_2O), $[\alpha]_D^{23}$ −76° (c=0.43, pyridine). *Anal.* Calcd. for $C_{67}H_{106}O_{32}\cdot 2H_2O$: C, 55.13; H, 7.60. Found: C, 54.91; H, 7.45. IR ν_{max}^{KBr} cm⁻¹: 3400 (br), 1764 (sh), 1747, 1070 (br), 886. UV λ_{max}^{MeOH} nm: transparent above 210 nm. CD ($c=5.15\times10^{-2}$, MeOH): $[\theta]_{340}$ 0, $[\theta]_{305}$ −19000 (neg. max.), $[\theta]_{265}$ −1000 (neg. min.), $[\theta]_{234}$ −24000 (neg. max.), $[\theta]_{215}$ 0, $[\theta]_{200}$ +49000 (pos. max.), $[\theta]_{197}$ +40000!. CMR (d_5-pyridine): 212.5 (s, C-16), 175.7 (s, C-18), 151.6 (s, C-9), 145.5 (s, C-25), 111.2 (d, C-11), 110.4 (t, C-26), 105.5 (2C), 105.3, 105.0, 104.5, 103.0 (1C each) (all d, anomeric C×6). CMR (d_6-DMSO): 212.9, 175.7, 150.9, 145.3, 111.2, 110.5.[21] Holotoxin B (9), mp 252—253° ($CHCl_3$–MeOH–H_2O), $[\alpha]_D^{23}$ −78° (c=0.28, pyridine). *Anal.* Calcd. for $C_{66}H_{104}O_{32}\cdot H_2O$: C, 55.53; H, 7.48. Found: C, 55.54; H, 7.47. IR ν_{max}^{KBr} cm⁻¹: 3400 (br), 1750 (br), 1050 (br), 890. UV λ_{max}^{MeOH} nm: transparent above 210 nm. CD ($c=2.98\times10^{-2}$, MeOH): $[\theta]_{340}$ 0, $[\theta]_{305}$ −15000 (neg. max.), $[\theta]_{268}$ 0, $[\theta]_{230}$ −12500 (neg. max.), $[\theta]_{220}$ 0, $[\theta]_{201}$ +29000 (pos. max.), $[\theta]_{198}$ +25000!.

Methanolysis of Holotoxin A (6) giving Genin-3 (1) and Genin-1 (2)——A mixture of 6 (237 mg) in anhydrous 0.25 N HCl–MeOH (10 ml) was heated under reflux for 2.5 hr. The resulting solution was poured into water and extracted with $CHCl_3$. The $CHCl_3$ layer was taken, washed successively with water, aq. sat. $NaHCO_3$, and water, and dried over $MgSO_4$. Evaporation of the solvent under reduced pressure gave a sapogenol mixture which was purified by column chromatography (silica gel 4 g, benzene→benzene–acetone (40:1) as the eluants) to furnish genin-3 (1, 54 mg) and genin-1 (2, 19 mg). Genin-3 (1), mp 240—243° (MeOH), $[\alpha]_D^{12}$ −92° (c=0.96, $CHCl_3$). *Anal.* Calcd. for $C_{31}H_{48}O_5$: C, 74.36; H, 9.66. Found: C, 74.50; H, 9.68. IR $\nu_{max}^{CHCl_3}$ cm⁻¹: 3460 (OH), 1748 (γ-lactone, 16-CO). PMR ($CDCl_3$) δ: 0.84, 0.89 (each 3H, both s, 4-$(CH_3)_2$), 0.99 (3H, s, 10-CH_3), 1.17 (3H, s, 14-CH_3), 1.13 (6H, s, 25-$(CH_3)_2$), 1.39 (3H, s, 20-CH_3), 3.16 (3H, s, OCH_3), 3.16 (1H, m, 3α-H), 5.27 (1H, m, 11-H). Genin-1 (2), PMR ($CDCl_3$) δ: 0.84, 0.89 (3H, each, both s, 4-$(CH_3)_2$), 0.99 (3H, s, 10-CH_3), 1.19 (3H, s, 14-CH_3), 1.39 (3H, s, 20-CH_3), 1.56—1.66 (olefinic CH_3), 4.68, 5.07 (the intensity ratio=*ca.* 1:2, each m, 26-H_2, 24-H), 5.28 (1H, m, 11-H).

Aqueous Acid Hydrolysis of Holotoxin A (6) giving Stichopogenin A_4 (3), Genin-3 (1), and Genin-1 (2)——A suspension of 6 (200 mg) in aqueous 7% H_2SO_4 (30 ml)–MeOH (8 ml)–benzene (20 ml) was heated under reflux with stirring. After every 10 hr, the benzene layer was replaced with fresh benzene and the total mixture was kept refluxing for totally 40 hr. The combined benzene layer was washed successively with water, aq. $NaHCO_3$, and water, and evaporated under reduced pressure to give the residue (64 mg). Silica gel column chromatography (SiO_2 15 g, benzene→benzene–acetone=4:1) of the residue afforded genin-1 (2, 3 mg), stichopogenin A_4 (3, 55 mg), and genin-3 (1, 3 mg). Genin-1 (2) was identified by TLC (benzene–MeOH=25:1, benzene–acetone=4:1) and genin-3 (1), mp 242—246° (MeOH), was identified by TLC (benzene–MeOH=25:1, benzene–acetone=4:1) and mixed mp with the respective authentic samples.[3a,b] Stichopogenin A_4 (3), mp 228—236° (with gradual decomposition) (cryst. from MeOH), $[\alpha]_D^{21}$ −130° (c=0.31, $CHCl_3$), IR ν_{max}^{KBr} cm⁻¹: 3300 (br), 1755 (br). PMR ($CDCl_3$) δ: 0.83, 0.88 (each 3H, s, 4-$(CH_3)_2$), 0.97 (3H, s, 10-CH_3), 1.20 (9H, s, 14-CH_3, 25-$(CH_3)_2$), 1.39 (3H, s, 20-CH_3), 3.15 (1H, m, 3α-H), 5.27 (1H, m, 11-H).

Carbohydrate Ingredients of Holotoxin A (6), B (9), and Prosapogenols (4, 5, 7, 8)——A mixture of 6 (9 mg) in anhydrous 2 N HCl–MeOH (1.5 ml) was heated under reflux for 2 hr. The reaction mixture was neutralized with Ag_2CO_3 and filtered. The filtrate was evaporated under reduced pressure to give the product

20) The following instruments were used for obtaining the physical data. Melting points: Yanagimoto Micro-melting point Apparatus and recorded uncorrected; Specific rotations: JASCO DIP-181 Digital Polarimeter, l=0.5 dm); IR spectra (Hitachi IR Spectrometer EPI-G3); UV spectra (Shimadzu MPS-50L Spectrophotometer); CD spectra (JASCO UV/ORD-5 Spectropolarimeter, c=g/100 ml); PMR spectra (Hitachi R-22 (90 MHz) NMR Spectrometer, TMS as an internal standard); CMR spectra (JEOL JNM-FX 100 (25.05 MHz) NMR Spectrometer, at 90°, 0.14 mM/ml, with a micro cell fitted in a 10 mm ϕ tube, spectral width 6 kHz, pulse flipping angle 45°, aquisition time 0.68 sec, number of data points 8192, transient time 1.5 sec, number of transient 2500—8000, TMS in d_5-pyridine as an internal standard, chemical shifts given as δ_c: transient time in the hetero decoupling without NOE method 3.0 sec). Signal multiplicities in PMR and CMR: s=singlet, d=doublet, t=triplet, br.s=broad singlet, and br.d=broad doublet, coupling constants (J values) given in Hz.

Chromatography was carried out as follows unless specified otherwise: Hitachi Gas Chromatograph model 063 with FID for GLC; Merck Kieselgel 60 230—400 mesh for ordinary column chromatography; Merck Kieselgel H nach Stahl Type 60 for medium pressure column chromatography; Pre-Coated TLC, Merck Kieselgel 60 F_{254} for TLC, detection by spraying with 1% $Ce(SO_4)_2$–10% H_2SO_4 followed by heating.

21) The spectrum was initially taken with d_6-DMSO as the internal standard and the δ_c values were obtained by conversion in terms of TMS as the internal standard.

which was trimethylsilylated with N,O-bis(trimethylsilyl)trifluoroacetamide (0.2 ml) in pyridine (0.2 ml) and the resulting TMS derivatives were quantitatively analyzed by GLC (2% silicon SE-52 on Chromosorb WAWDMCS 80—100 mesh; 3 mm×2 m; column temp. 140°; N_2 flow rate 35 ml/min) to identify with methyl xylopyranoside (**a**) (7′44″), methyl quinovopyranoside (**b**) (9′20″), methyl 3-O-methyl-glucopyranoside (**c**) (11′08″), and methyl glucopyranoside (**d**) (22′36″). As for the standards, 10 mg each of xylose, quinovose, glucose, and 3-O-methyl-glucose were treated similarly. In the similar manner, **9** (10 mg), **4** (2 mg), **5** (2 mg), **7** (4 mg), and **8** (3 mg) were treated and analyzed quantitatively by GLC. The comparatively integrated areas (given in the parentheses) of the GLC peaks were as follows: **6**: **a** (33), **b** (36), **c** (90), **d** (89); **9**: **a** (25), **b** (31), **c** (48), **d** (119); **4**: **a** (42), **b** (26); **5**: **a** (18), **b** (19), **c** (20), **d** (17); **7**: **a** (31), **b** (33), **c** (28), **d** (21); **8**: **a** (53), **b** (59), **c** (68), **d** (121).

Enzymatic Hydrolysis of Holotoxin A (6) with Takadiastase A Preparation——A mixture of **6** (950 mg) in a solution of takadiastase A preparation (AcOH–AcONa buffer solution, pH 5.1, 200 ml)[3d] was kept stirring at 31° for 6 days. After addition of *n*-BuOH (200 ml) and warming for a while, the total mixture was centrifuged to collect the *n*-BuOH layer. The resulting precipitate was also collected and washed with *n*-BuOH and a small amount of MeOH and the washings were combined with the *n*-BuOH layer. The combined organic solution was evaporated under reduced pressure to give the *n*-BuOH extractive (2.85 g). The extractive (2.8 g) was successively subjected to column chromatography (silica gel 70—230 mesh, 60 g, $CHCl_3$–MeOH–H_2O=7:3:1 (lower layer)) and to medium pressure column chromatography (silica gel 80 g, $CHCl_3$–MeOH–H_2O=13:3:1 (lower layer)→10:3:1 (lower layer), the other conditions as above) to furnish crude **4** (60 mg), crude **5** (223 mg), and crude **6** (510 mg recovered). Recrystallization from MeOH gave the pure samples of **4** (28 mg) and **5** (56 mg). **4**, mp 281—282° (MeOH), $[\alpha]_D^{18}$ −113° (c=0.15, pyridine). *Anal.* Calcd. for $C_{41}H_{62}O_{13}$: C, 65.92; H, 8.37. Found: C, 65.97; H, 8.63. IR ν_{max}^{KBr} cm^{-1}: 3400 (br), 1765, 1754 (sh), 1070 (br), 886. CD ($c=3.1\times10^{-2}$, MeOH): $[\theta]_{340}$ 0, $[\theta]_{304}$ −17000 (neg. max.), $[\theta]_{262}$ 0, $[\theta]_{234}$ −21000 (neg. max.), $[\theta]_{213}$ 0, $[\theta]_{210}$ +13000!. **5**, mp 274—276° (MeOH), $[\alpha]_D^{25}$ −99° (c=0.53, pyridine). *Anal.* Calcd. for $C_{54}H_{84}O_{22}\cdot H_2O$: C, 58.78; H, 7.85. Found: C, 58.82; H, 7.91. IR ν_{max}^{KBr} cm^{-1}: 3400 (br), 1750 (br), 1050 (br), 885. CD ($c=5.38\times10^{-2}$, MeOH): $[\theta]_{345}$ 0, $[\theta]_{304}$ −18000 (neg. max.), $[\theta]_{264}$ −2000 (neg. min.), $[\theta]_{234}$ −25000 (neg. max.), $[\theta]_{214}$ 0, $[\theta]_{210}$ +12000!.

Methylation of 4 followed by Methanolysis——To a solution of **4** (30 mg) in DMSO (4 ml) was added dimsyl carbanion solution (3 ml)[1] and the total solution was kept stirring at 15° for 1.5 hr under N_2 atmosphere. After addition of CH_3I (1.5 ml) and stirring for additional 1.5 hr, the reaction mixture was poured into ice-water and extracted with AcOEt. The AcOEt layer was taken, washed with aq. $Na_2S_2O_3$ and water successively, dried over $MgSO_4$, and evaporated under reduced pressure to give the residue (33 mg). Column chromatography (silica gel 2 g, benzene→benzene–acetone=10:1) of the product furnished the hexa-O-methyl derivative (**4a**, 18 mg). **4a**, amorphous, $[\alpha]_D^{18}$ −125° (c=0.23, $CHCl_3$), IR $\nu_{max}^{CCl_4}$ cm^{-1}: 1733 ($COOCH_3$), 1718, 1610 (enone), 1650 (w), 1088 (br), 890. UV λ_{max}^{hexane} nm: 252 (ε=12000). CD ($c=1.62\times10^{-2}$, hexane): $[\theta]_{400}$ 0, $[\theta]_{350}$ −7000 (neg. max.), $[\theta]_{290}$ −1500 (neg. min.), $[\theta]_{252}$ −70000 (neg. max.), $[\theta]_{232}$ 0, $[\theta]_{216}$ +30000!. PMR (d_6-benzene) δ: 4.45 (1H, d, J=7), 4.89 (1H, d, J=7) (anomeric H×2), 4.81 (*ca.* 2H, br.s, 26-H_2), 5.05 (weak, m, 24-H), 5.38 (1H, m, 11-H).

A solution of **4a** (5 mg) in anhydrous 2.5 N HCl–MeOH (1 ml) was heated under reflux for 1 hr. After neutralization with Ag_2CO_3, the precipitate was removed by filtration. The product obtained from the filtrate was subjected to GLC (15% NPGS on Chromosorb WAW (80—100 mesh), 3 mm×2 m, column temp. 160°, N_2 flow rate 30 ml/min) and TLC (benzene–acetone=3:1) to identify with methyl 2,3,4-tri-O-methyl-quinovopyranoside (I) (t_R=2′32″, 3′17″; Rf=0.60, 0.70) and methyl 3,4-di-O-methyl-xylopyranoside (II) (7′45″, 8′58″; 0.25, 0.27).

Methylation of 5 followed by Methanolysis——To a solution of **5** (25 mg) in DMSO (1 ml) was added dimsyl carbanion solution (2 ml) and the total solution was kept stirring for 1.5 hr as above and treated with CH_3I (1.5 ml). After additional stirring for 3 hr and working up as above, the AcOEt extractive was purified by repeated precipitation from acetone–water to give the dodeca-O-methyl derivative (**5a**, 23 mg). **5a**, amorphous, $[\alpha]_D^{18}$ −46° (c=0.67, $CHCl_3$). *Anal.* Calcd. for $C_{65}H_{106}O_{22}$: C, 62.98; H, 8.62. Found: C, 63.15; H, 8.68. IR $\nu_{max}^{CCl_4}$ cm^{-1}: 1732 ($COOCH_3$), 1717, 1612 (enone), 1648 (w), 1100 (br), 886. UV λ_{max}^{hexane} nm: 253 (ε=10000). CD ($c=1.57\times10^{-2}$, hexane): $[\theta]_{400}$ 0, $[\theta]_{350}$ −4500 (neg. max.), $[\theta]_{290}$ −2000 (neg. min.), $[\theta]_{253}$ −58000 (neg. max.), $[\theta]_{233}$ 0, $[\theta]_{220}$ +18000 (pos. max.), $[\theta]_{210}$ +25000!. PMR (d_6-benzene) δ: 4.10 (1H, d, J=8), 4.60 (1H, d, J=7), 4.95 (1H, d, J=7), 4.97 (1H, d, J=8) (anomeric H×4), 4.81 (*ca.* 2H, br.s, 26-H_2), 5.40 (1H, m, 11-H).

A solution of **5a** (3 mg) in anhydrous 2.5 N HCl–MeOH (1.5 ml) was heated under reflux for 1.5 hr, treated as for methanolysis of **4a**. The following products were identified: I (t_R=1′38″, 2′05″) and methyl 2,3,4,6-tetra-O-methyl-glucopyranoside (III) (3′57″, 5′18″) by GLC (15% NPGS on Chromosorb WAW 80—100 mesh, 3 mm×2 m, column temp. 170°, N_2 flow rate 40 ml/min); methyl 2,4,6-tri-O-methyl-glucopyranoside (IV) (5′45″, 8′32″) and methyl 3-O-methyl-xylopyranoside (V) (7′41″, 11′55″) by GLC (15% PEGS on Chromosorb WAW 80—100 mesh, 3 mm×1 m, column temp. 170°, N_2 flow rate 30 ml/min); I (Rf=0.65), III (0.50, 0.68), IV (0.20, 0.25), and V (0.10) by TLC (benzene–acetone=3:1).

Enzymatic Hydrolysis of Holotoxin A (6) with Crude Hesperidinase——To a solution of **6** (5 mg) in AcOH–AcONa buffer solution (pH 5.0, 2 ml) was added crude hesperidinase (Tanabe Pharm. Co., Lot No. N-30,

10 mg) and the total mixture was kept stirring at 38° for 3 days. The reaction mixture was extracted with *n*-BuOH, and the *n*-BuOH extract was evaporated under reduced pressure to give the product, which was identified with **4** and **5** by TLC ($CHCl_3$–MeOH–H_2O=7: 3: 1, lower layer).

Methylation of 6 followed by Methanolysis——To a solution of **6** (30 mg) in DMSO (1 ml) was added dimsyl carbanion solution (3 ml) and the total solution was kept stirring for 1 hr as above and treated with CH_3I (2 ml). After stirring for additional 2 hr, the reaction mixture was worked up as above and the AcOEt extractive thus obtained was purified by column chromatography (silica gel 1.5 g, benzene→benzene–acetone 4: 1) to furnish the octadeca-O-methyl derivative (**6a**, 17 mg). **6a**, amorphous, $[\alpha]_D^{18}$ −46° (c=0.48, $CHCl_3$). *Anal.* Calcd. for $C_{83}H_{138}O_{32}$: C, 60.49; H, 8.44. Found: C, 60.64; H, 8.57. IR $\nu_{max}^{CCl_4}$ cm^{-1}: 1733, 1715, 1615, 1105, 886 (w). UV λ_{max}^{hexane} nm: 252 (ε=9500). CD ($c=3.95\times10^{-2}$, hexane): $[\theta]_{400}$ 0, $[\theta]_{350}$ −4000 (neg. max.), $[\theta]_{290}$ 0, $[\theta]_{254}$ −45000 (neg. max.), $[\theta]_{232}$ 0, $[\theta]_{222}$ +15000!. PMR (d_6-benzene) δ: 4.10 (1H, d, J=8), 4.33 (1H, d, J=8), 4.55 (1H, br.d, J=5), 4.91 (2H, d, J=7), 4.94 (1H, d, J=7) (anomeric H×6), 4.79 (*ca.* 2H, br.s, 26-H_2), 5.38 (1H, m, 11-H).

A solution of **6a** (5 mg) in anhydrous 2 N HCl–MeOH (1.5 ml) was heated under reflux for 2 hr and worked up as above. The products were identified by GLC (15% NPGS, 3 mm×2 m, column temp. 170°, N_2 flow rate 30 ml/min) with III (t_R=6′40″, 9′11″) and methyl 2,3-di-O-methyl-quinovopyranoside (VI) (6′10″, 7′52″), by GLC (15% PEGS, 3 mm×1 m, column temp. 180°, N_2 flow rate 35 ml/min) with IV (6′15″, 9′02″) and V (8′12″, 12′27″), and by TLC (benzene–acetone=3: 2) with III (Rf=0.60, 0.71), VI (0.46, 0.54), IV (0.30, 0.44), and V (0.20).

Enzymatic Hydrolysis of Holotoxin B (9) with Crude Hesperidinase——A solution of **9** (50 mg) in AcOH–AcONa buffer solution (pH 5.0, 30 ml) was treated with crude hesperidinase (200 mg) and kept stirring at 38° for one day. After addition of a small amount of *n*-BuOH and warming for a while, the total mixture was filtered. The filtrate was extracted with *n*-BuOH and the *n*-BuOH extract was evaporated under reduced pressure to give the product (45 mg) which was purified by column chromatography (silica gel 5 g, $CHCl_3$–MeOH=10: 1→$CHCl_3$–MeOH–H_2O=13: 3: 1 (lower layer)) to furnish **4** (11 mg), **7** (17 mg), and crude **9** (6 mg, recovered). **4**, mp 278—281° (MeOH), $[\alpha]_D^{13}$ −124° (c=0.26, pyridine) was identified with the one (**4**) obtained above from holotoxin A (**6**) by TLC, mixed mp (277—279°), and $[\alpha]_D$. **7**, mp 273—276° (acetone–H_2O), $[\alpha]_D^{20}$ −88° (c=0.99, pyridine). *Anal.* Calcd. for $C_{54}H_{84}O_{22}\cdot H_2O$: C, 58.78; H, 7.85. Found: C, 58.81; H, 8.04. IR ν_{max}^{KBr} cm^{-1}: 3400 (br), 1760 (br), 1070 (br), 888. CD ($c=1.51\times10^{-1}$, MeOH): $[\theta]_{350}$ 0, $[\theta]_{305}$ −12500 (neg. max.), $[\theta]_{260}$ −1000 (neg. min.), $[\theta]_{232}$ −12000 (neg. max.), $[\theta]_{213}$ 0, $[\theta]_{210}$ +6000!.

Methylation of 7 followed by Methanolysis——To a solution of **7** (50 mg) in DMSO (4 ml) was added dimsyl carbanion solution (5 ml), and the total solution was kept stirring for 1.5 hr as above. After addition of CH_3I (2.5 ml) and stirring for additional 2 hr, the reaction mixture was worked up as above. The AcOEt extractive (80 mg) thus obtained was purified by column chromatography (silica gel 5 g, benzene→benzene–acetone=10: 1) to furnish the dodeca-O-methyl derivative (**7a**, 40 mg). **7a**, amorphous, $[\alpha]_D^{16}$ −79° (c=0.35, $CHCl_3$). *Anal.* Calcd. for $C_{65}H_{106}O_{22}$: C, 62.98; H, 8.62. Found: C, 62.66; H, 8.37. IR $\nu_{max}^{CCl_4}$ cm^{-1}: 1732, 1718, 1650 (w), 1609, 1100 (br), 887. UV λ_{max}^{hexane} nm: 253 (ε=9600). CD ($c=3.15\times10^{-2}$, hexane): $[\theta]_{394}$ 0, $[\theta]_{350}$ −5000 (neg. max.), $[\theta]_{290}$ −1000 (neg. min.), $[\theta]_{252}$ −47000 (neg. max.), $[\theta]_{230}$ 0, $[\theta]_{210}$ +16000!. PMR (d_6-benzene) δ: 4.35 (1H, d, J=8), 4.44 (1H, d, J=7), 4.91 (1H, d, J=7), 4.98 (1H, d, J=8) (anomeric H×4), 4.80 (*ca.* 2H, br.s, 26-H_2), 5.38 (1H, m, 11-H).

A solution of **7a** (7 mg) in anhydrous 2.5 N HCl–MeOH (1.5 ml) was heated under reflux for 1.5 hr and worked up as above. The products thus obtained were identified by GLC (15% NPGS, 3 mm×2 m, column temp. 170°, N_2 flow rate 35 ml/min) with III (t_R=5′16″, 7′08″) and VI (4′53″, 6′07″), by GLC (15% PEGS, 3 mm×1 m, column temp. 160°, N_2 flow rate 35 ml/min) with II (3′45″) and IV (7′10″, 11′00″), and by TLC (benzene–acetone=3: 1) with III (Rf=0.43, 0.55), VI (0.30, 0.37), II (0.31), and IV (0.17, 0.28).

Enzymatic Hydrolysis of Holotoxin B (9) with β-Glucosidase——A solution of **9** (220 mg) in aqueous AcOH (pH 5.0, 220 ml) was treated with β-glucosidase (almond emulsin, G8625, Sigma Co., 60 mg) and kept stirring at 37° for 3 days. The *n*-BuOH extractive (180 mg), which was obtained by working up as for above-described hydrolysis with crude hesperidinase, was subjected to column chromatography (silica gel 15 g, $CHCl_3$–MeOH=10: 1→$CHCl_3$–MeOH–H_2O=8: 3: 1, lower layer) to furnish **8** (145 mg) and **9** (25 mg, recovered). **8**, mp 281—284° ($CHCl_3$–MeOH), $[\alpha]_D^{18}$ −85° (c=0.48, pyridine). *Anal.* Calcd. for $C_{60}H_{94}O_{27}\cdot H_2O$: C, 56.95; H, 7.65. Found: C, 57.06; H, 7.71. IR ν_{max}^{KBr} cm^{-1}: 3400 (br), 1760 (br), 1070 (br), 890. CD ($c=1.0\times10^{-1}$, MeOH): $[\theta]_{340}$ 0, $[\theta]_{305}$ −15000 (neg. max.), $[\theta]_{260}$ −1000 (neg. min.), $[\theta]_{233}$ −15000 (neg. max.), $[\theta]_{214}$ 0, $[\theta]_{210}$ +10000!.

Methylation of 8 followed by Methanolysis——A solution of **8** (60 mg) in DMSO (3 ml) was treated with dimsyl carbanion solution (6 ml) and kept stirring for 1.5 hr as above. After methylation with CH_3I (3 ml) and stirring for additional 2.5 hr, the reaction mixture was worked up as above to give the AcOEt extractive (90 mg), which was purified by column chromatography (silica gel 3 g, benzene→benzene–acetone=3: 1) to furnish the pentadeca-O-methyl derivative (**8a**, 65 mg). **8a**, amorphous, $[\alpha]_D^{16}$ −66° (c=0.42, $CHCl_3$). *Anal.* Calcd. for $C_{74}H_{122}O_{27}$: C, 61.56; H, 8.52. Found: C, 61.61; H, 8.67. IR $\nu_{max}^{CCl_4}$ cm^{-1}: 1732, 1718, 1650 (w), 1610, 1100 (br), 886. UV λ_{max}^{hexane} nm: 253 (ε=8200). CD ($c=2.85\times10^{-2}$, hexane): $[\theta]_{400}$ 0, $[\theta]_{350}$ −4000 (neg. max.), $[\theta]_{290}$ −1000 (neg. min.), $[\theta]_{252}$ −43000 (neg. max.), $[\theta]_{233}$ 0, $[\theta]_{226}$ +17000 (pos. max.). PMR

(d_6-benzene) δ: 4.13 (1H, d, J=7), 4.35 (1H, d, J=7), 4.53 (1H, d, J=7), 4.91 (1H, d, J=7), 4.97 (1H, d, J=7) (anomeric H×5), 4.80 (*ca.* 2H, br.s, 26-H_2), 5.40 (1H, m, 11-H).

A solution of **8a** (3 mg) in anhydrous 2.5 N HCl–MeOH (1.5 ml) was heated under reflux for 1.5 hr. The products obtained by working up as above were identified by GLC (15% NPGS, 3 mm×2 m, column temp. 170°, N_2 flow rate 40 ml/min) with III (t_R=3′57″, 5′17″) and VI (3′43″, 4′37″), by GLC (15% PEGS, 3 mm×1 m, column temp. 170°, N_2 flow rate 30 ml/min) with IV (5′45″, 8′32″) and V (7′41″, 11′55″), and by TLC (benzene–acetone=2:1) with III (Rf=0.70, 0.80), VI (0.45, 0.60), IV (0.30, 0.40), and V (0.20).

Methylation of Holotoxin B (9) followed by Methanolysis——A solution of **9** (200 mg) in DMSO (3 ml) was treated with dimsyl carbanion solution (14 ml) and kept stirring for 1 hr. The reaction mixture was then treated with CH_3I (8 ml), kept stirring for additional 2 hr, and worked up as above. The AcOEt extractive (239 mg) thus obtained was purified by column chromatography (silica gel 6 g, benzene–acetone=20:3) to furnish the octadeca-O-methyl derivative (**9a**, 155 mg). **9a**, amorphous, $[\alpha]_D^{20}$ −42° (c=0.83, $CHCl_3$). *Anal.* Calcd. for $C_{83}H_{138}O_{32}$: C, 60.49; H, 8.44. Found: C, 60.40; H, 8.54. IR $\nu_{max}^{CCl_4}$ cm^{-1}: 1730, 1715, 1610. 1105 (br), 888 (w). UV λ_{max}^{hexane} nm: 251 (ε=9000). CD ($c=3.05\times10^{-2}$, hexane): $[\theta]_{400}$ 0, $[\theta]_{350}$ −5000 (neg. max.), $[\theta]_{290}$ −1000 (neg. min.), $[\theta]_{253}$ −46000 (neg. max.), $[\theta]_{232}$ 0, $[\theta]_{222}$ +21000!. PMR (d_6-benzene) δ: 4.12 (1H, d, J=8), 4.35 (1H, d, J=8), 4.55 (1H, br.d, J=5), 4.92 (2H, d, J=7), 4.95 (1H, d, J=7) (anomeric H×6), 4.80 (*ca.* 2H, br.s, 26-H_2), 5.40 (1H, m, 11-H).

A solution of **9a** (10 mg) in anhydrous 2 N HCl–MeOH (1.5 ml) was heated under reflux for 1.5 hr and worked up as above. The products were identified with III (t_R=5′28″, 7′24″) and VI (5′07″, 6′27″) by GLC (15% NPGS, 3 mm×2 m, column temp. 170°, N_2 flow rate 35 ml/min), with IV (7′00″, 10′13″) and V (9′11″, 14′06″) by GLC (15% PEGS, 3 mm×1 m, column temp. 168°, N_2 flow rate 35 ml/min), and with III (Rf=0.60, 0.71), VI (0.46, 0.54), IV (0.30, 0.44), and V (0.20) by TLC (benzene–acetone=3:2).

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