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

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The chemical structures of two antifungal oligoglycosides, holotoxin A and B, which were isolated from the sea cucumber *Stichopus japonicus* Seneka, have been re-investigated. On the basis of chemical and physicochemical evidence, the genuine sapogenol of holotoxin A and B has been elucidated to be holotoxigenol rather than previously proposed stichopogenin A4 (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-glucopyranosyl(1→4)-β-D-glucopyranosyl[1→3]-β-D-glucopyranosyl(1→3)-β-D-glucopyranosyl]-holotoxigenol (6) and 3-O-[2-O-[3-O-methyl-β-D-glucopyranosyl(1→3)]-β-D-glucopyranosyl(1→4)-β-D-glucopyranosyl(1→3)-β-D-glucopyranosyl(1→4)-β-D-glucopyranosyl(1→3)-β-D-glucopyranosyl]-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* Seneka. As described there, 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 of holotoxin A and B. Since a considerable quantity of the holotoxin mixture have been generously provided by Mr. S. Shimada, we have re-investigated the chemical structures of holotoxins, and finally have reached a conclusion that the previously proposed structures 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.

Medium pressure column chromatography of the holotoxin mixture 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. The circular dichroism (CD)

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spectrum also shows the presence of these chromophores together with \( \Delta^9(11) \) in the sapogenol portion.\(^8\)

On methanolation under the anhydrous conditions using 0.25 m 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 stichopenogenin \( A_4 \) (3)\(^{3a,b}\) as the major sapogenol together with trace amounts of genin-3 (1) and genin-1 (2). Stichopenogenin \( A_4 \) (3) was not detected in the former methanolsates. Genin-1 (2) is a mixture (ca. 4:1) of \( \Delta^{24} \) and \( \Delta^{25} \) isomers as revealed by the proton magnetic resonance (PMR) spectrum (the ratio of signal intensities at \( \delta \) 7.07 (24-H) and 4.67 (26-H\(_2\)) = ca. 2:1). These findings have led us to doubt stichopenogenin \( A_4 \) (3)\(^{3a,b}\) as the genuine sapogenol of holotoxin A (6).

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\begin{align*}
1 \quad (\text{genin-3}) & \quad R = \begin{array}{c}
\text{OCH}_3
\end{array} \\
2 \quad (\text{genin-1}) & \quad R = \begin{array}{c}
\text{OH}
\end{array} + \Delta^{24}\text{isomer} \\
3 \quad (\text{stichopenogenin } A_4) & \quad R = \begin{array}{c}
\text{HO}
\end{array}
\end{align*}
\]

Chart 1

In order to solve the problem, the carbon magnetic resonance (CMR) spectrum of holotoxin A (6) has been examined in pentadeutero\((d_{26})\)-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 (\( \delta \) 151.6 (s)), C-11 (111.2 (d)), C-18 (175.7 (s)), C-16 (212.5 (s)), C-25 (145.5 (s)), and C-26 (110.4 (t)).\(^8\) 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\((d_{26})\)-dimethyl sulfoxide (DMSO). Therefore, it has become evident that the \( \Delta^{25} \) 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 stichopenogenin \( A_4 \) (3) and that the \( \Delta^{24} \) isomer is an artifact sapogenol. The \( \Delta^{25} \) structure in the side chain of the sapogenol part has been further substantiated by two-proton broad singlet at around \( \delta \) 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) (\textit{vide infra}). At this stage of investigation, the previous discussion on the anomic 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\(^6\) or crude hespéridinase, 6 yielded two prosapogenols 4 and 5. The less polar

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one (4), mp 281—282°, contains one mole each of xylose and quinovose as the carbohydrate ingredients. 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 (CH₃I)—DMSO—sodium hydride (NaH) yielded a hexa-O-methyl derivative (4a), the IR spectrum of which shows the formation of a methoxy-

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

carbonyl (1733 cm\(^{-1}\)) and an \(\alpha,\beta\)-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: \([\eta]_{350} = 7000\) (neg. max.), \([\eta]_{365} = 7000\) (neg. max.), and \(\lambda_{max} 252\) nm (\(e=12000\)), 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 anomic protons are observed at \(\delta 4.45\) and 4.89 as doublets of \(J=7\) Hz which indicate \(\beta\) orientation (\(\text{C}_1\) conformation) of both carbohydrate ingredients in 4a. In addition, a broad singlet\(^{12}\) due to the terminal methylene at C-25 is observed at \(\delta 4.81\). 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\(^o\), 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 \(\beta\) linkage (\(\text{C}_1\) conformation) by the signals assignable to four anomic protons at \(\delta 4.10\) (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-di-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 anomic protons at \(\delta 4.10\) (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 \(\beta\) linkages in 6a (\(\text{C}_1\) conformation).\(^{13}\) The CMR spectrum (in \(d_5\)-pyridine) of holotoxin A (6) also substantiates the presence of six \(\beta\)-anomeric configurations in 6 by the signals at \(\delta\) 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-\(\beta\)-d-glucopyranosyl(1→3)-\(\beta\)-d-glucopyranosyl(1→4)-\(\beta\)-d-quinovopyranosyl]-4-O-[3-O-methyl-\(\beta\)-d-glucopyranosyl(1→3)-\(\beta\)-d-xylopyranosyl]-\(\beta\)-d-glucopyranosyl]-holotoxigenol (6).

Holotoxin B (9), mp 252—253\(^o\), 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\(^o\), is a new tetracyloside 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 (\(\gamma\)-lactone, C-16 ketone, and \(\Delta^{23}\)) as in 4.

12) The signal intensity accounts approximately (slightly less than) two protons, since a weak signal is observed at \(\delta 5.05\) which suggests minor contamination of the \(\Delta^{23}\) isomer presumably formed during the methylation procedure.

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

On complete methylation, 7 yielded the dodeca-O-methyl derivative (7a), which carries four β linkages (C1 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). Methanolation 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
these findings, the structure of the prosapogenol has been elucidated to be the linear tetracygoside (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-methyl-
glucose, and two moles of glucose. Complete methylation of 8 furnished the pentadeca-
O-methyl derivative (8a). Here again, all the anomic configurations are β (C_4 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-xlyopyranoside, thus the structure (8) being assigned to the prosapogenol.

Finally, complete methylation of holotoxin B (9) afforded the octadeca-O-methyl deriva-
tive (9a), whose six anomic configurations are β (C_4 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-xlyopyranoside, thus the struc-
ture of holotoxin B being assigned as having a glucopyranoside moiety attached to 3-OH of
the terminal glucose moiety in the prosapogenol (8). The anomic configuration of the
terminal glucopyranoside linkage has been ascertained as β by application of the Klyne’s rule:
[M]_D (9)=[M]_D (8) = -30°, [M]_D of methyl β-D-glucopyranoside = -66°, [M]_D of methyl
α-D-glucopyranoside = +307°. Based on the accumulated evidence, the structure of holotoxin B has been established as 3-O-[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-xlyopyranosyl]-holotoxigenol (9).

As one of the reasons, which are responsible for our previous conclusion on the structures
of holotoxins A and B, 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. As has been noticed in our studies on the oligoglycosidic
constituents of the starfish, 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.

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, [α]_D, UV, CD, PMR, and GLC analysis of the methanalysis products of both.
17) Since one of the coupling constants for six anomic 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 and the antifungal activities. However, identity of holotoxins used in the studies of the oligosaccharide portion is obscure from our present knowledge. Holotoxins used there were originated from the sea cucumber collected in some other places (e.g. Korea).
Experimental

Isolation of Holotoxin A (6) and B (9) —— The crude holotoxin mixture (1.00 g) (isolated from the sea cucumber *Stichopus japonicus* Sekena) which was collected mainly in the Inland Sea, Japan) was subjected to medium pressure column chromatography (silica gel 60; 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 CHCl₃-MeOH-H₂O=8:3:1 (lower layer)→7:3:1 (lower layer) to furnish holotoxins A (6, 300 mg) and B (9, 170 mg). Holotoxin A (6), mp 250—253°C (CHCl₃-MeOH-H₂O), [α]D⁰ +76° (c = 0.43, pyridine). *Anal. Calcd.* for C₄₅H₇₀O₉₅H₂O: C, 55.13; H, 7.60. Found: C, 54.91; H, 7.45. IR νcm⁻¹: 3400 (br), 1764 (sh), 1747, 1070 (br), 886. UV λmax nm: transparent above 210 nm. CD (c = 1.5×10⁻⁴, MeOH): [α]₁₂₅₁⁰ = 0, [α]₂₅₀ = 19000 (neg. max.), [α]₂₄₅₂ = 10000 (neg. min.). [α]₁₄₀⁰ = -24000 (neg. max.), [α]₁₆₅⁰ = -40000 (pos. max.). CMR (δ, ppm): 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-28), 105.5 (2C), 105.3, 105.0, 104.5, 103.0 (2C each) (all d, anomic C×6). CMR (δ, ppm): 212.9, 175.7, 150.9, 145.3, 111.2, 110.5 (d). Holotoxin B (9), mp 252—253°C (CHCl₃-MeOH-H₂O). [α]D⁰ = -78° (c = 0.28, pyridine). *Anal. Calcd.* for C₄₅H₇₀O₉₅H₂O: C, 55.53; H, 7.48. Found: C, 55.54; H, 7.47. IR νcm⁻¹: 3400 (br), 1760 (br), 1050 (br), 890. UV λmax nm: transparent above 210 nm. CD (c = 2.98×10⁻⁴, MeOH): [α]₃₃₅₀ = 0, [α]₃₄₅₀ = -5000 (neg. max.). [α]₁₉₅ₐₐ = 0, [α]₂₄₅ₐₐ = -12500 (neg. max.). [α]₁₉₅ₐₐ = 0, [α]₂₄₅ₐₐ = +29000 (pos. max.). [α]₂₄₅ₐₐ = +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 CHCl₃. The CHCl₃ layer was taken, washed successively with water, aq. sat. NaHCO₃, and water, and dried over MgSO₄. Evaporation of the solvent under reduced pressure gave a sapogenin 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°C (MeOH), [α]D⁰ = -92° (c = 0.96, CHCl₃). *Anal. Calcd.* for C₄₅H₇₀O₉₅H₂O: C, 74.36; H, 9.66. Found: C, 74.50; H, 9.68. IR νcm⁻¹: 3400 (OH), 1748 (γ-lactone, 16-CO). PMR (CDCl₃): δ 0.84, 0.89 (each 3H, both s, 4-(CH₃)₂), 0.99 (3H, s, 10-CH₃), 1.17 (3H, s, 14-CH₃), 1.13 (6H, s, 25-(CH₃)₂), 1.39 (3H, s, 20-CH₃), 3.16 (3H, s, OCH₃), 3.16 (1H, m, 3x-H), 5.27 (1H, m, 11-H). Genin-1 (2), PMR (CDCl₃): δ 0.84, 0.89 (each 3H, both s, 4-(CH₃)₂), 0.99 (3H, s, 10-CH₃), 1.19 (3H, s, 14-CH₃), 1.39 (3H, s, 20-CH₃), 1.56—1.66 (olefinic CH₂), 4.68, 5.07 (the intensity ratio = ca. 1:2, each m, 26-H, 24-H), 5.28 (1H, m, 11-H).

Aqueous Acid Hydrolysis of Holotoxin A (6) giving Stichogephenin A (3), Genin-3 (1), and Genin-1 (2) —— A suspension of 6 (200 mg) in aqueous 7% H₂SO₄ (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. NaHCO₃, and water, and evaporated under reduced pressure to give the residue (64 mg). Silica gel column chromatography (SiO₂ 15 g, benzene—benzene—acetone = 4:1:1) of the residue afforded genin-1 (2, 3 mg), stichogephenin A (3, 55 mg), and genin-3 (2, 1.3 mg). Genin-1 (2) was identified by TLC (benzene—MeOH=25:1, benzene—acetone=4:1) and mixed mp with the respective authentic samples. Stichogephenin A (3), mp 228—236°C (with gradual decomposition) (cryst. from MeOH), [α]D⁰ = -130° (c = 0.31, CHCl₃), IR νcm⁻¹: 3300 (br), 1755 (br). PMR (CDCl₃): δ 0.83, 0.88 (each 3H, s, 4-(CH₃)₂), 0.97 (3H, s, 10-CH₃), 1.20 (6H, s, 14-CH₃), 2.58 (3H, s, 25-(CH₃)₂), 1.30 (3H, s, 20-CH₃), 3.15 (1H, m, 3x-H), 5.27 (1H, m, 11-H).

Carbohydrate Ingredients of Holotoxin A (6), B (9), and Prosapogenins (4, 5, 7, 8) —— A mixture of 6 (9 mg) in anhydrous 2N HCl-MeOH (1.5 ml) was heated under reflux for 2 hr. The reaction mixture was neutralized with Ag₂CO₃ 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 = 5 mm; 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°, acquisition time 0.68 sec, number of data points 8192, transient time 1.5 sec, number of transient 2500—8000, TMS in d₄ as an internal standard, chemical shifts given as δ: 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 F₅₄₄ for TLC, detection by spraying with 1% Ca(SO₄)₂-10% H₂SO₄ followed by heating.

21) The spectrum was initially taken with d₅-DMF as the internal standard and the δ 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 x 2 m; column temp. 140°C; N₂ 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 (590 mg) in a solution of takadiastase A preparation (AcOH-AcONa buffer solution, pH 5.1, 200 ml) was kept stirring at 31°C for 6 days. After addition of n-ButOH (200 ml) and warming for a while, the total mixture was centrifuged to collect the n-ButOH layer. The resulting precipitate was also collected and washed with n-ButOH and a small amount of MeOH and the washings were combined with the n-ButOH layer. The combined organic solution was evaporated under reduced pressure to give the n-ButOH extract (2.85 g). The extractive (2.85 g) was successively subjected to column chromatography (silica gel 70-230 mesh, 60 g, CHCl₃-MeOH-H₂O; 7:3:1 (lower layer) and to medium pressure column chromatography (silica gel 80 g, CHCl₃-MeOH-H₂O; 15:3:1 (lower layer) -10:3:1 (lower layer), the other conditions as above) to furnish crude 4 (80 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).

**Anal. Calcd. for C₄₉H₅₈O₂₂: C, 65.92; H, 8.37. Found: C, 65.97; H, 8.65. IR υmax cm⁻¹: 3400 (br), 1765, 1754 (sh), 1707 (br), 886. CD (c=1.3×10⁻⁴, MeOH): [θ]₂₅₀ 0, [θ]₃₄₀ -17000 (neg. max.).**

**Methylation of 4 Followed by Methanolysis** — To a solution of 4 (30 mg) in DMSO (4 ml) was added dimethyl carbanion solution (3 mol) and the total solution was kept stirring at 15°C for 1.5 hr under N₂ atmosphere. After addition of CH₃I (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. Na₂SO₄ and water successively, dried over MgSO₄ 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, [α]₂₅° -125° (ε=0.23, CHCl₃), IR υmax cm⁻¹: 1733 (COOCH), 1718, 1610 (eneone), 1650 (w), 1088 (br), 890. UV λmax nm: 232 (ε=12000). CD (c=1.6×10⁻⁴, hexane): [θ]₃₄₀ 0, [θ]₁₅₀ -7000 (neg. max.), [θ]₁₂₀ -1500 (neg. min.), [θ]₁₃₁₂ -70000 (neg. max.), [θ]₁₃₃₈ 0, [θ]₁₃₅ +30000. PMR (d₄-benzene): δ 4.45 (1H, d, J=7), 4.89 (1H, d, J=7) (anomeric H x2), 4.81 (ca. 2H, br s, 26-H₂), 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 Ag₂CO₃, 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 x 2 m, column temp. 180°C, N₂ flow rate 50 ml/min) and TLC (benzene-acetone = 3:1) to identify with methyl 2,3,4-tri-O-methyl-quinovopyranoside (1) (Rf = 0.32', 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 (4 ml) was added dimethyl carbanion solution (2 mol) and the total solution was kept stirring for 1.5 hr as above and treated with CH₃I (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, [α]₂₅° -46° (ε=0.67, CHCl₃). **Anal. Calcd. for C₄₉H₅₈O₂₂: C, 62.98; H, 8.62. Found: C, 63.15; H, 8.68. IR υmax cm⁻¹: 1732 (COOCH), 1717, 1612 (eneone), 1648 (w), 1100 (br), 886. UV λmax nm: 232 (ε=12000). CD (c=1.57×10⁻⁴, hexane): [θ]₃₄₀ 0, [θ]₁₆₀ -4500 (neg. max.), [θ]₁₈₀ -2000 (neg. min.), [θ]₁₂₀ -58000 (neg. max.), [θ]₁₃₁₂ 0, [θ]₁₃₃₈ +18000 (pos. max.), [θ]₁₃₅ +25000. PMR (d₄-benzene): δ 4.10 (1H, d, J=7), 4.60 (1H, d, J=7), 4.95 (1H, d, J=7). 4.97 (1H, d, J=7) (anomeric H x2), 4.81 (ca. 2H, br s, 26-H₂), 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 hr, treated as for methanolation of 4a. The following products were identified: 1 (Rf = 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 x 2 m, column temp. 170°C, N₂ 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% PEOS on Chromosorb WAW 80-100 mesh, 3 mm x 2 m, column temp. 170°C, N₂ flow rate 30 ml/min); 1 (Rf' = 0.63), 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) was added crude hesperidinase (Tanabe Pharm. Co., Lot No. N-30, 3729, No. 12)
10 mg) and the total mixture was kept stirred at 38°C for 3 days. The reaction mixture was extracted with n-BuOH, and the n-BuOH extract was evaporated under reduced pressure to give a residue, which was identified as 4 and 5 by TLC (CHCl₃-MeOH-H₂O = 7:3:1, lower layer).

**Methylation of 6 followed by Methanalysis**—To a solution of 6 (30 mg) in DMSO (1 ml) was added dimethyl carbonate solution (3 ml) and the total solution was kept stirred for 1 hr as above and treated with CH₃I (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, [α]D₂⁰ = -46° (c = 0.48, CHCl₃). Anal. Calcd. for C₃₆H₇₁O₂₂: C, 60.49; H, 8.44. Found: C, 60.64; H, 8.57. IR νmax cm⁻¹: 1735, 1715, 1615, 1105, 886 (w). UV λmax nm: 232 (ε = 9500). CD (c = 0.95 x 10⁻⁴, hexane): [α]₁₀₀₀ = 0, [α]₂₀₀₀ = -1000 (neg. max.), [α]₃₀₀₀ = -45000 (neg. max.), [α]₄₀₀₀ = 0, [α]₅₀₀₀ = 15000!. PMR (d₄-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 x 6), 4.79 (ca. 2H, br, s, 26-H₂), 5.38 (1H, m, 11-H).

A solution of 6a (5 mg) in anhydrous 2% HCl-MeOH (1.5 ml) was heated under reflux for 2 hr and worked up as above. The products (5 mg) was identified by GLC (15% NPGS, 3 mm x 2 cm, column temp. 170°C, N₂ flow rate 30 ml/min) with III (t₁₄ = 6.40, 9.11') and methyl 2,3-diol-O-methyl-quinovopyranoside (VI) (6.10', 7.52') by GLC (15% PEPS, 3 mm x 1 cm, column temp. 180°C, N₂ flow rate 35 ml/min) with IV (8.15', 9.02') and V (8.12', 12.27') and by TLC (benzene-acetone = 3:2) with III (Rf = 0.60, 0.71), VI (0.40, 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 stirred at 38°C 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 filtrate which was purified by column chromatography (silica gel 1.5 g, benzene—benzene-acetone = 10:1) to furnish the dodeca-O-methyl derivative (7a, 40 mg). 7a, amorphous, [α]D₂⁰ = -79° (c = 0.35, CHCl₃). Anal. Calcd. for C₃₈H₇₈O₁₁: C, 62.98; H, 8.82. Found: C, 62.66; H, 8.37. IR νmax cm⁻¹: 1732, 1718, 1685 (w), 1609, 1100 (m), 887. UV λmax nm: 253 (ε = 9600). CD (c = 3.15 x 10⁻⁴, hexane): [α]₁₂₀₀ = 0, [α]₁₆₀₀ = -5000 (neg. max.), [α]₂₄₀₀ = -1000 (neg. min.), [α]₃₂₀₀ = -10000 (neg. max.), [α]₄₄₀₀ = -16000!.

**Methylation of 7 followed by Methanalysis**—To a solution of 7 (50 mg) in DMSO (4 ml) was added dimethyl carbonate solution (5 ml) and the total solution was kept stirred for 1.5 hr as above. After addition of CH₃I (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 1.5 g, benzene—benzene-acetone—10:1:1) to furnish the dodeca-O-methyl derivative (8a, 40 mg). 8a, amorphous, [α]D₂⁰ = -66° (c = 0.42, CHCl₃). Anal. Calcd. for C₃₆H₇₁O₂₂: C, 61.56; H, 7.56. Found: C, 61.61; H, 7.82. IR νmax cm⁻¹: 1732, 1718, 1685 (w), 1610, 1100 (br), 886. UV λmax nm: 253 (ε = 8200). CD (c = 2.85 x 10⁻⁴, hexane): [α]₁₂₀₀ = 0, [α]₁₆₀₀ = 0 (neg. max.), [α]₂₄₀₀ = -1000 (neg. min.), [α]₃₂₀₀ = -43000 (neg. max.), [α]₄₄₀₀ = 1700 (pos. max.).

**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 emulsion, G8625, Sigma Co., 60 mg) and kept stirring at 37°C 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, CHCl₃-MeOH-H₂O = 10:1—CHCl₃-MeOH-H₂O = 8:3:1, lower layer) to furnish 8 (145 mg) and 9 (25 mg, recovered). 8, mp 281-284°C (CHCl₃-MeOH), [α]D₂⁰ = -85° (c = 0.48, pyridine). Anal. Calcd. for C₃₆H₇₁O₂₂: C, 56.95; H, 7.65. Found: C, 57.06; H, 7.71. IR νmax cm⁻¹: 3400 (br), 1760 (br), 1070 (br), 890. CD (c = 1.0 x 10⁻¹, MeOH): [α]₁₆₀₀ = 0, [α]₂₄₀₀ = -15000 (neg. max.), [α]₃₂₀₀ = -10000 (neg. min.), [α]₄₄₀₀ = -15000 (neg. max.), [α]₅₀₀₀ = +10000!

**Methylation of 8 followed by Methanalysis**—A solution of 8 (60 mg) in DMSO (3 ml) was treated with dimethyl carbonate solution (6 ml) and kept stirring for 1.5 hr as above. After methylation with CH₃I (3 ml) and stirring for additional 3.5 hr, the reaction mixture was worked up as above to give the AcOEt extractive (90 mg), which was purified by column chromatography (silica gel 5 g, benzene—benzene-acetone = 3:1) to furnish the pentadeca-O-methyl derivative (8a, 65 mg). 8a, amorphous, [α]D₂⁰ = -66° (c = 0.42, CHCl₃). Anal. Calcd. for C₃₆H₇₁O₂₂: C, 61.56; H, 8.52. Found: C, 61.61; H, 8.87. IR νmax cm⁻¹: 1732, 1718, 1650 (w), 1610, 1100 (br), 886. UV λmax nm: 253 (ε = 8200). CD (c = 2.85 x 10⁻⁴, hexane): [α]₁₂₀₀ = 0, [α]₁₆₀₀ = -4000 (neg. max.), [α]₂₄₀₀ = -1000 (neg. min.), [α]₃₂₀₀ = -43000 (neg. max.), [α]₄₄₀₀ = 1700 (pos. max.). PMR
(d₄-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 x 5), 4.80 (ca. 2H, br.s, 26-H₂), 5.40 (1H, m, 11-H).

A solution of 8a (3 mg) in anhydrous 2.5 x 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 x 2 m, column temp. 170°C, N₂ flow rate 40 ml/min) with III (δ₈ = 3.57°, 5.17°) and VI (3.49°, 4.37°), by GLC (15% PEGS, 3 mm x 1 m, column temp. 170°C, N₂ 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 Methanalysis—A solution of 9 (200 mg) in DMSO (3 ml) was treated with dimethyl carbanion solution (14 ml) and kept stirring for 1 hr. The reaction mixture was then treated with CH₃I (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, [x]_ø = -42° (c=0.83, CHCl₃). Anal. Calcd. for C₆₈H₁₂₆O₂₈: C, 60.49; H, 8.44. Found: C, 60.40; H, 8.54. IR ν max cm⁻¹: 1730, 1715, 1610. 1105 (br), 888 (w). UV λ max nm: 251 (ε=9900). CD (c=3.05 x 10⁻³, hexane): [θ]₂₄₀° 0, [θ]₃₅₀° -5000 (neg. max.), [θ]₄₃₅° -1000 (neg. min.), [θ]₅₂₅° -46000 (neg. max.), [θ]₆₀₀° 0, [θ]₇₃₅° +21000. PMR (d₄-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 x 6), 4.80 (ca. 2H, br.s, 26-H₂), 5.40 (1H, m, 11-H).

A solution of 9a (10 mg) in anhydrous 2 x HCl-MeOH (1.5 ml) was heated under reflux for 1.5 hr and worked up as above. The products were identified with III (δ₈ = 5.28°, 7.24°) and VI (5.07°, 6.27°) by GLC (15% NPGS, 3 mm x 2 m, column temp. 170°C, N₂ flow rate 35 ml/min), with IV (7.00°, 10.13°) and V (9.11°, 14.06°) by GLC (15% PEGS, 3 mm x 1 m, column temp. 168°C, N₂ 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=8:2).

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