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Saponin and Sapogenol. XV.[1] Antifungal Glycosides from the Sea Cucumber *Stichopus japonicus* Selenka. (2). Structures of Holotoxin A and Holotoxin B

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The structure of holotoxin A, the major antifungal glycoside isolated from the Far Eastern sea cucumber *Stichopus japonicus* Selenka, has been elucidated as **4** on the bases of chemical and physicochemical evidence and enzymatic hydrolysis. Holotoxin B (**14**), a minor antifungal glycoside of the same origin, has been suggested to be the glucoside of holotoxin A (**4**) on the similar basis. Holotoxin C, another minor glycoside, has been suggested to be the similar type of glycoside as holotoxin A and B.

Holotoxin A and B are distinctive by the possession of two α-linkages of D-xylose and D-quinovose in the oligosaccharide portion. Finally, comparison of the anti-microbial activity of holotoxins with the various kinds of saponins isolated from the plant materials has been presented.

In the preceding paper,[1] we have described the isolation of three antifungal glycosides named holotoxin A (major), B, and C from the Far Eastern sea cucumber *Stichopus japonicus* Selenka and have elucidated that the structure of stichopogenin A_4, which is the genuine aglycone of holotoxin A, is expressed as **2a** rather than previously proposed **1a**.[3] In addition, another new aglycone 25-O-methyl-stichopogenin A_4 (**3**), which was obtained by mild methanolic acid hydrolysis of holotoxin A together with stichopogenin A_4 (**2a**), has been elucidated.[1]

In this paper, we wish to present the full account on the structure elucidation of holotoxin A (**4**)[4] and also wish to mention briefly on the structures of the minor glycoside holotoxin B (**14**) which corresponds to the glucoside of holotoxin A and of holotoxin C which was isolated in a tiny amount. It is interestingly pointed out that both holotoxin A and B are distinctive by the possession of two α-linkages of D-xylose and D-quinovose as compared with the hitherto known saponins originated in the vegetable kingdom.[5]

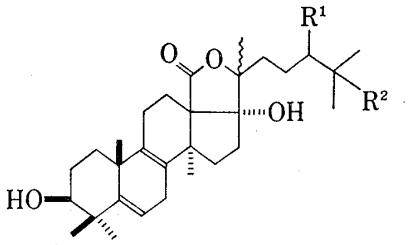
As reported previously,[1] aqueous acid hydrolysis of holotoxin A (**4**) liberated xylose, quinovose, 3-O-methyl-glucose, and glucose as the carbohydrate ingredients whereas stichopogenin A_4 (**2a**) and the mixture of stichopogenin A_2 (**2b**) and its double bond isomer (presumably Δ^{25} deriv.) were obtained from the triterpenoid portion.

Enzymatic hydrolysis of holotoxin A (**4**) with the takadiastase A preparation furnished two partial hydrolysates, **5**, mp 259—262°, and **6**, mp 274.5—277°. The circular dichroism (CD) spectrum of **5** exhibits the presence of the same chiral chromophores as in holotoxin A[1]: $\Delta^{9(11)}$, γ-lactone, and 16-CO, while the infrared (IR) spectrum of **5** shows the absorption bands of OH (3400 (br) cm^{-1}) and the overlapped broad band due to γ-lactone and 16-CO (1750 (br) cm^{-1}). Hydrolysis of **5** with aq. 2N HCl yielded the aglycone mixture as from holotoxin A[1] and xylose, quinovose, 3-O-methyl-glucose, and glucose as the sugar components. The

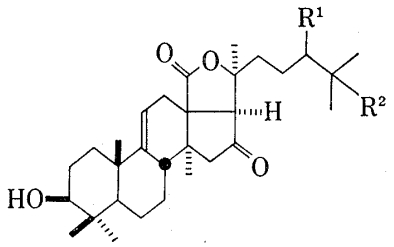
1) Part XIV: I. Kitagawa, T. Sugawara, I. Yosioka, and K. Kuriyama, *Chem. Pharm. Bull.* (Tokyo), **24**, 266 (1976).
2) Location: *133-1, Yamada-kami, Suita, Osaka, 565, Japan.*
3) G.B. Elyakov, T.A. Kuzunetsova, A.K. Dzizenko, and Y.N. Elkin, *Tetrahedron Letters*, **1969**, 1151.
4) *a*) I. Kitagawa, T. Sugawara, and I. Yosioka, *Tetrahedron Letters*, **1974**, 4111; *b*) I. Kitagawa, T. Sugawara, I. Yosioka, and K. Kuriyama, *ibid.*, **1975**, 963 (preliminary reports).
5) R. Tschesche and G. Wulff, *Fortschr. Chem. Org. Naturstoffe*, **30**, 461 (1973).

IR spectrum of another partial hydrolysate **6** shows the similar absorption bands as that of **5**: 3350 (br) and 1750 (br) cm^{-1}, and aqueous acid hydrolysis of **6** liberated the aglycone mixture as from **4** and **5** and xylose and quinovose as the monosaccharides.

On the other hand, enzymatic hydrolysis of holotoxin A (**4**) with cellulase III yielded **5** as the partial hydrolysate and only xylose was detected as the sugar component in the hydrolysate, thus holotoxin A being presumed to be a xyloside of **5**.



1a : R^1=H, R^2=OH
1b : R^1, R^2=Δ^{24}
(by Elyakov, *et al.*[3])



2a : R^1=H, R^2=OH stichopogenin A_4
2b : R^1, R^2=Δ^{24}
3 : R^1=H, R^2=OCH_3

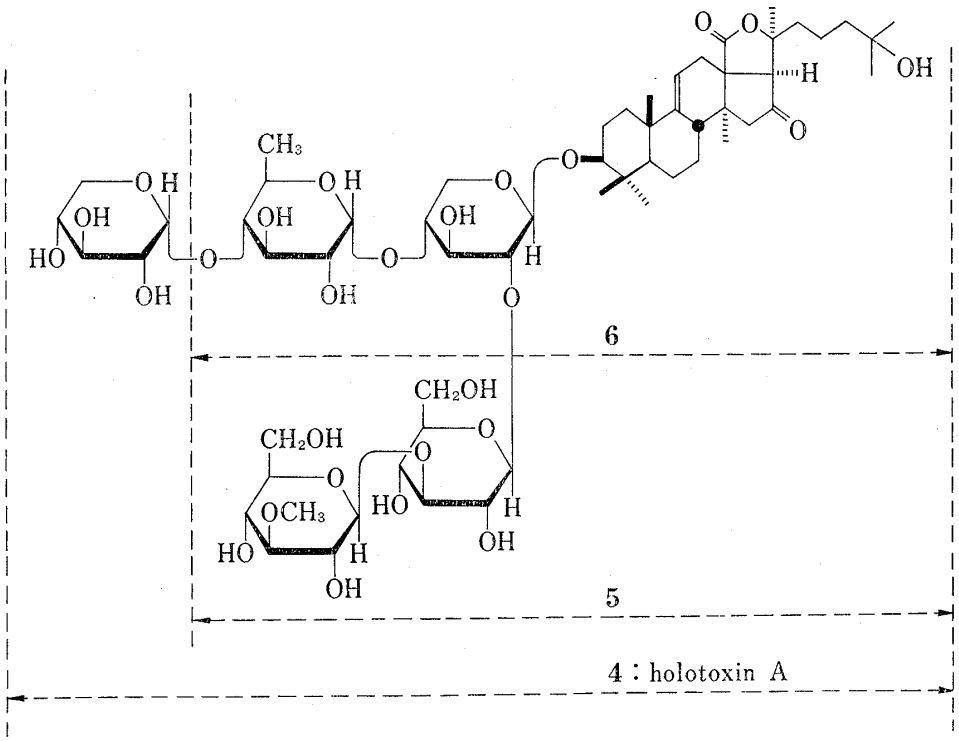


Chart 1

Methylation of **5** with CH_3I/dimethyl sulfoxide (DMSO)/NaH[6] gave the dodeca-O-methyl derivative (**8**), which exhibits the absorption bands due to 25-OH (3600 (w) cm^{-1}), COOMe (1730 cm^{-1}), and the enone (1715, 1610 cm^{-1}) but lacks the absorption bands of γ-lactone and five-membered ring CO in the IR spectrum (CCl_4). The conversion of two functions during the methylation of **5** are also shown by the CD and ultraviolet (UV) spectra of **8**: $[\theta]_{222}$+6150 (pos. max.), $[\theta]_{250}$−14500 (neg. max.), $[\theta]_{350}$−1540 (sh), and λ_{max} 252 nm (ε=6700), which, along with the above IR evidence, reveals the formation of $\Delta^{17(20)}$-16-CO and 13-COOMe on

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

dimsyl carbanion treatment of **5** through the opening of γ-lactone ring followed by dehydration.[7]

Since the retained OH in **8** is not acetylated under the ordinary conditions (Ac_2O/pyridine), the sugar moiety in **5** is considered to attach to 3-OH of the aglycone (**2a**). In the proton magnetic resonance (PMR) spectrum of **8**, are observed four anomeric proton signals as shown in Table I, and since these four monosaccharides of D-series are known to prefer the Cl conformation, the coupling constants of the anomeric H's have shown the presence of three β-linkages and one α-linkage in **8**.

Methanolysis of **8** with anhydrous 2N HCl/MeOH gave methyl 3-O-methyl-xylopyranoside, methyl 2,4,6-tri-O-methyl-glucopyranoside, methyl 2,3,4-tri-O-methyl-quinovopyranoside, and methyl 2,3,4,6-tetra-O-methyl-glucopyranoside as identified by gas liquid and thin-layer chromatography (GLC, TLC). The liberation of these two methylated glucosides has shown that 3-O-methyl-glucose in **5** is the terminal end together with quinovose. In addition, the oligosaccharide moiety in **5** has been revealed to be branched at the xylose moiety.

Methanolysis of the hexa-O-methyl derivative (**9**), which was prepared by methylation of **6** as above, furnished methyl 2,3,4-tri-O-methyl-quinovopyranoside and methyl 2,3-di-O-methyl-xylopyranoside, thus showing that quinovose attaches to 4-OH of xylose in **6**.

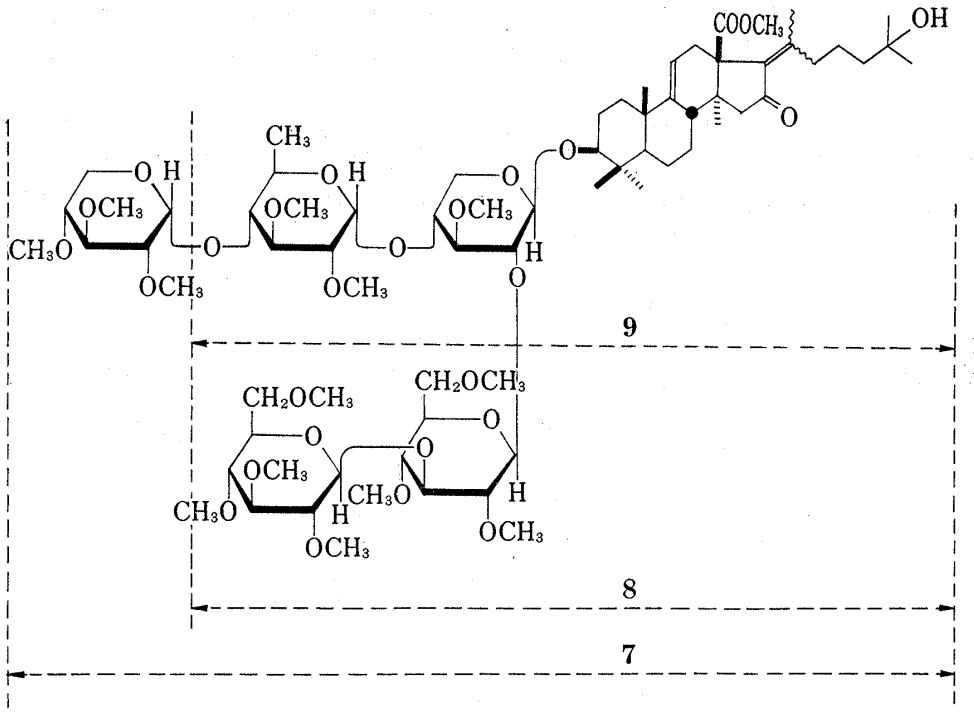


Chart 2

TABLE I. Anomeric Protons (δ Values in C_6D_6)

7	8
4.30 (1H, d, J=4 Hz)	
4.56 (1H, d, J=7 Hz)	4.60 (1H, d, J=7 Hz)
4.78 (1H, br.s)	4.81 (1H, br.s)
4.88 (1H, d, J=7 Hz)	4.93 (1H, d, J=7 Hz)
4.91 (1H, d, J=8 Hz)	4.96 (1H, d, J=7 Hz)

7) *a*) I. Rothberg, B. Tursch, and C. Djerassi, *J. Org. Chem.*, **38**, 209 (1973); *b*) G. Snatzke, "Optical Rotatory Dispersion and Circular Dichroism in Organic Chemistry," Heyden & Son Ltd., London, 1967; *c*) C. Djerassi, R. Records, E. Bunnenberg, K. Mislow, and A. Moscowitz, *J. Am. Chem. Soc.*, **84**, 870 (1962).

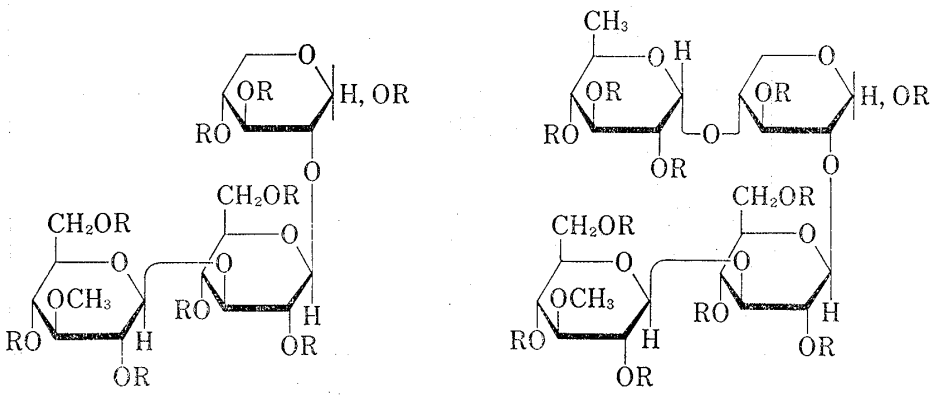
It has become evident on the above described bases that two partial hydrolysates obtained from holotoxin A are formulated respectively as **5** and **6** except the anomeric configurations.

Methylation of holotoxin A (**4**) with CH_3I/DMSO/NaH afforded the tetradeca-O-methyl derivative (**7**), the IR spectrum (CCl_4) of which shows the retention of free 25-OH (3500 (w) cm^{-1}) and the formation of COOMe (1730 cm^{-1}) and $\Delta^{17(20)}$-16-CO (1710, 1610 cm^{-1}) as in **8** and **9**. The PMR spectrum of **7** shows the signals due to 11-H (δ 5.40, m) and five anomeric H's, among the latter one additional anomeric H as compared with those in **8** (Table I, δ 4.30, d, J=4 Hz) is attributable to the anomeric H of terminal α-xyloside (Cl form). The assignment of α-linkage has been further supported by application of the Klyne's empirical rule[8]: $[M]_D$ of **4**−$[M]_D$ of **5**=+238°; $[M]_D$ of methyl α-D-xylopyranoside=+249°; $[M]_D$ of methyl β-D-xylopyranoside=−107°.[9]

Methanolysis of **7** with anhydrous 10% HCl/MeOH furnished methyl 3-O-methyl-xylopyranoside, methyl 2,3-di-O-methyl-quinovopyranoside, methyl 2,4,6-tri-O-methyl-glucopyranoside, methyl 2,3,4-tri-O-methyl-xylopyranoside, and methyl 2,3,4,6-tetra-O-methyl-glucopyranoside as identified by GLC and TLC. Comparison of the methanolysis products obtained from **7** and **8** has led to a conclusion that terminal xylose in holotoxin A is connected with α-linkage to 4-OH of quinovose in **5** and consequently holotoxin A is expressed as **4** except the other four anomeric configurations.

Next, in order to clarify all the anomeric configurations in holotoxin A, acetolysis of holotoxin A with $Ac_2O/ZnCl_2$[10] was undertaken and nonaacetyl-trisaccharide (**10**) and undeca-acetyl-tetrasaccharide (**11**) were isolated.

The IR spectrum of **10** shows no OH absorption band while the PMR spectrum reveals the presence of one OMe and nine AcO's in **10**. Treatment of **10** with CH_3I/DMSO/NaH afforded the deca-O-methyl-trisaccharide (**12**), which possesses no OH as shown by the IR



10 : R=Ac
12 : R=CH_3

11 : R=Ac
13 : R=CH_3

Chart 3

TABLE II. Anomeric Protons (δ Values in $CDCl_3$)

12	**13**
	4.22 (1H, br.s)
4.27 (2H, d, J=8 Hz)	4.28 (2H, d, J=8 Hz)
4.57 (1H, d, J=7 Hz)	4.58 (1H, d, J=7 Hz)

8) W. Klyne, *Biochem. J.*, **47**, xli (1950).
9) M. Kimura, M. Tohma, and I. Yoshizawa, *Chem. Pharm. Bull.* (Tokyo), **16**, 1228 (1968).
10) R.D. Guthrie and J.F. McCarthy, *Advan. Carbohyd. Chem.*, **22**, 11 (1967).

spectrum. The PMR spectrum of **12** shows the presence of three anomeric H's on the β-linkage as judged from the J values (Table II). Methanolysis of **12** with anhydrous 10% HCl/MeOH yielded methyl 2,3,4,6-tetra-O-methyl-glucopyranoside, methyl 2,4,6-tri-O-methyl-glucopyranoside, and methyl 3,4-di-O-methyl-xylopyranoside, thus the structure of nona-acetyl-trisaccharide being formulated as **10.**

The IR spectrum of undecaacetyl-tetrasaccharide (**11**) also shows no OH absorption band while the PMR spectrum of **11** shows the signals ascribable to one quinovose-Me (3H, d, J=6 Hz), one OMe (3H, s), and eleven AcO's. Methylation of **11** as for **10** gave the dodeca-O-methyl-tetrasaccharide (**13**), which carries no free OH as shown by the IR spectrum. The PMR spectrum of **13** exhibits the signals due to four anomeric H's, among which the broad singlet at δ 4.22 is the additional one as compared with three anomeric H's of **12** and is assignable to anomeric H on the terminal α-quinovopyranoside linkage in connection with the following methanolysis of **13.** Treatment of **13** with anhydrous 10% HCl/MeOH 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, thus the structure of undecaacetyl-tetrasaccharide being formulated as **11** including three anomeric configurations.

Consequently, the full structure of holotoxin A is now expressed as **4.** The present work seems to be the first elucidation of the sea cucumber saponins.

Holotoxin B (**14**),[1] mp 236—239°, shows no UV absorption maximum above 210 nm, but shows the strong IR absorption band at 3360 (br) and 1070 (br) cm^{-1} due to OH and C–O–C along with the broad absorption band at 1750 cm^{-1} which is ascribable to γ-lactone and five-membered ring CO since holotoxin B gives the CD spectrum being indicative of the presence of similar chiral chromophores as in holotoxin A (**4**): $[\theta]_{205}$+14200 (pos. max.), $[\theta]_{232}$ −10300 (neg. max.), and $[\theta]_{307.5}$−12600 (neg. max.).

Aqueous acid hydrolysis of holotoxin B furnished the same aglycone mixture as from holotoxin A (**4**): *i.e.* stichopogenin A_4 (**2a**) and the mixture of stichopogenin A_2 (**2b**) and its isomer, while the carbohydrate ingredients identified were xylose, quinovose, 3-O-methyl-glucose, and glucose.

Enzymatic hydrolysis of holotoxin B with β-glucosidase yielded holotoxin A (**4**) and glucose whereas hydrolysis using cellulase III afforded holotoxin A (**4**), above-mentioned hydrolysate **5**, xylose, and glucose. On the other hand, enzymatic hydrolysis of holotoxin B with crude naringinase or crude takadiastase A produced holotoxin A (**4**) together with **5** and **6.** These findings suggest that holotoxin B is a glucoside of holotoxin A (**4**).

Methylation of holotoxin B with CH_3I/DMSO/NaH afforded the heptadeca-O-methyl derivative (**15**), which possesses newly formed functions such as COOMe (1732 cm^{-1}) and $\Delta^{17(20)}$-16-CO: 1718, 1610 cm^{-1}; $[\theta]_{218}$+8200 (pos. max.), $[\theta]_{250}$−26300 (neg. max.), $[\theta]_{360}$−2200 (neg. max.); λ_{max} 253 nm (ε=6200),[7] as in **7**, **8**, and **9** (*vide supra*).

Methanolysis of **15** with anhydrous 5% HCl/MeOH liberated methyl 3-O-methyl-xylopyranoside, methyl 2,4,6-tri-O-methyl-glucopyranoside, methyl 2,3-di-O-methyl-quinovopyranoside, methyl 2,3,4,6-tetra-O-methyl-glucopyranoside[11] and an unidentified methyl di-O-methyl-xylopyranoside.[12] It follows therefore that the terminal glucose moiety in holotoxin B connects to the terminal xyloside moiety in holotoxin A (**4**).

11) These four methylated monosaccharides are same as those obtained by methanolysis of **7**, but the peak intensity of methyl 2,3,4,6-tetra-O-methyl-glucopyranoside liberated from **15** is increased as compared with that from **7**, thus suggesting that an additional glucose moiety in holotoxin B is present as another terminal end.

12) As for the location of terminal glucose in holotoxin B, 4-OH of terminal xylose in holotoxin A (**4**) is most likely, however, since methyl 2,3-di-O-methyl-xylopyranoside and methyl 2,3-di-O-methyl-quinovopyranoside were so far not distinguished from each other by GLC and TLC, the location of the glucose moiety is still uncertain.

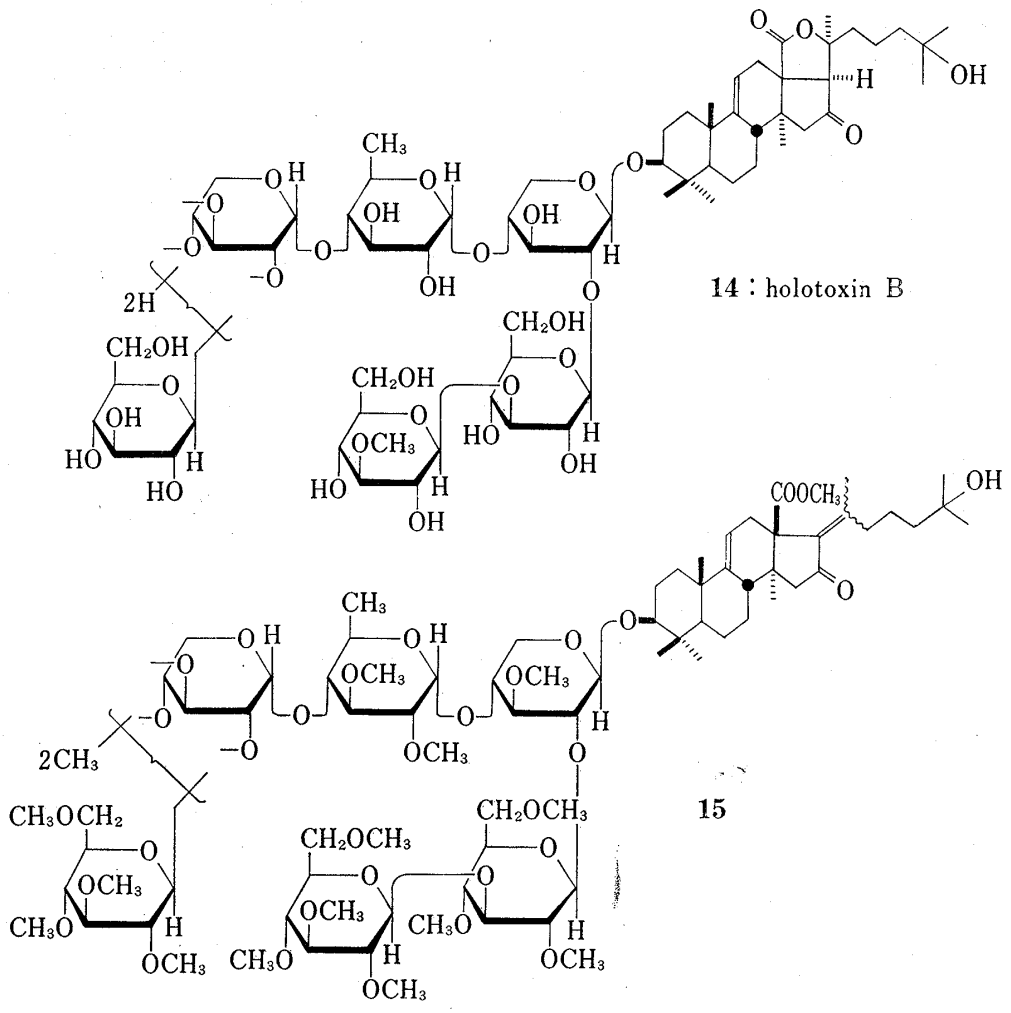


Chart 4

Finally, application of the Klyne's rule has shown the β-linkage of the terminal glucoside moiety: $[M]_D$ of **14**—$[M]_D$ of **4**=$-79°$; $[M]_D$ of methyl β-D-glucopyranoside=$-66°$; $[M]_D$ of methyl α-D-glucopyranoside=$+307°$,[13] thus the total structure of holotoxin B and the methylated derivative being expressed as **14** and **15** in which the location of terminal glucose moiety is still uncertain. Due to shortage of the material, we have been unable to procure the definite evidence on this point.

Holotoxin C, another minor glycoside isolated only in tiny quantity, possesses the similar physicochemical properties (UV, IR, and CD) as holotoxin A (**4**) and B (**14**). On aqueous acid hydrolysis, it furnished the same aglycone mixture (**2a**, **2b**, and the isomer) and sugar ingredients (xylose, quinovose, 3-O-methyl-glucose, and glucose) as from **4** and **14**. It has been suggested therefore that the structure of holotoxin C is closely related to those of holotoxin A and B, however, the structure has not yet been elucidated due to lack of the material.

Since Shimada reported[14] the antifungal activity of holotoxin, which was the mixture of holotoxin A, B, and C, we have examined[15] the growth inhibitory activity of holotoxin A, B, and C together with some vegetable saponins isolated in this laboratory against the various species of microorganisms and the results are given in Table III.

13) T. Kawasaki and T. Yamauchi, *Chem. Pharm. Bull.* (Tokyo), **10**, 703 (1962).
14) S. Shimada, *Science*, **163**, 1462 (1969).
15) By courtesy of the Res. Lab. of Ohtsuka Pharm. Co.

In 1965, Tschesche and Wulff suggested that the antifungal activities of various kinds of steroidal and triterpenoidal saponins were irrelevant to their hemolytic indices and their formation of insoluble precipitates with cholesterol, but were related with their structures of oligosaccharide portions comprised four or five monosaccharide moieties.[16] Later on, Wolters[17] and Imai, *et al.*[18] also suggested the importance of the oligosaccharide portion in saponin for the antifungal activity.

Table III. Minimum Inhibitory Concentration (μg/ml)

	Holotoxin A (**4**)	Holotoxin B (**14**)	Holotoxin C	Saponins[a]
Trichophyton rubrum	0.78	0.78	6.25	>100
Trichophyton mentagrophytes	1.56	1.56	12.5	>100
Microsporum gypseum	3.12	1.56	12.5	>100
Candida albicans	6.25	6.25	25.0	>100
Candida utilis	3.12	3.12	12.5	*b*)
Torula utilis	3.12	3.12	12.5	*b*)
Aspergillus oryzae	6.25	12.5	25.0	*b*)
Penicillium chrysogenum	3.12	6.25	12.5	*b*)
Trichomonas vaginalis	3.12	1.56	3.12	*b*)

a) The following saponins were examined: a) Japanese horse-chestnuts saponin[19]; b) jegosaponin[20]; c) desacyl-jegosaponin[21]; d) tea seeds saponin[22]; e) sakurasō-saponin[23,24a]; f) soyasaponin[24]; g) Misaponin[25]; h) kurinsō-saponin[23,24a]; i) ziyu-glycoside I[26]
b) Not examined.

As shown in Table III, the growth inhibitory activities of holotoxins are significant as compared with those of the listed vegetable saponins, and the structure of the carbohydrate moieties including the anomeric configurations seems to be one of the important requisites for the activities. The problem will be a subject of future investigation.

Experimental[27]

Enzymatic Hydrolysis of Holotoxin A (4)——i) Takadiastase A Preparation: Crude takadiastase A powder (60 g, Sankyo Co.) was treated with dist. water (420 ml) for 3 hr under ice-cooling, and the aqueous extract was added with small amount of Celite 535 with stirring and filtered. The filtrate was then added with aq. 1M $Ca(OAc)_2$ (102 ml) and adjusted to pH 7.0 with aq. 5N NaOH to give a suspension, which was filtered with the aid of Celite 535 again. To the ice-cooled filtrate, was added dropwise cold acetone up to 40% concentration by volume, and the precipitate collected with a centrifuge was dissolved in the AcOH–AcONa buffer (pH 5.1, 200 ml) and was used as the takadiastase A preparation in the following experiments.

ii) Hydrolysis of Holotoxin A (**4**) with Takadiastase A Preparation: To a suspension of **4** (200 mg) in water (20 ml) was added the takadiastase A preparation (20 ml) and the total mixture was kept gentle stirring

16) R. Tschesche and G. Wulff, *Z. Naturforschung*, **20b**, 543 (1965).
17) B. Wolters, *Planta Medica*, **14**, 392 (1966).
18) S. Imai, S. Fujioka, E. Murata, M. Goto, T. Kawasaki, and T. Yamauchi, *Ann. Rept. Takeda Res. Lab.*, **26**, 76 (1967).
19) I. Yosioka, A. Matsuda, K. Imai, T. Nishimura, and I. Kitagawa, *Chem. Pharm. Bull.* (Tokyo), **19**, 1200 (1971).
20) I. Yosioka, S. Saijoh, and I. Kitagawa, *Chem. Pharm. Bull.* (Tokyo), **20**, 564 (1972).
21) I. Kitagawa, Y. Imakura, T. Hayashi, and I. Yosioka, *Chem. Pharm. Bull.* (Tokyo), **22**, 1675, 3009 (1974).
22) *a*) R. Tschesche, A. Weber, and G. Wulff, *Ann.*, **721**, 209 (1969); *b*) I. Yosioka, T. Nishimura, A. Matsuda, and I. Kitagawa, *Chem. Pharm. Bull.* (Tokyo), **18**, 1610, 1621 (1970); *ibid.*, **19**, 1186 (1971).
23) I. Kitagawa, A. Matsuda, and I. Yosioka, *Chem. Pharm. Bull.* (Tokyo), **20**, 2226 (1972).
24) *a*) I. Kitagawa, M. Yoshikawa, Y. Imakura, and I. Yosioka, *Chem. Pharm. Bull.* (Tokyo), **22**, 1339 (1974); *b*) I. Kitagawa, M. Yoshikawa, and I. Yosioka, *ibid.*, **22**, 3010 (1974).
25) I. Yosioka, A. Inada, and I. Kitagawa, *Tetrahedron*, **30**, 707 (1974); *b*) *Idem*, *Chem. Pharm. Bull.* (Tokyo), **23**, 2268 (1975).
26) I. Yosioka, T. Sugawara, A. Ohsuka, and I. Kitagawa, *Chem. Pharm. Bull.* (Tokyo), **19**, 1700 (1971).
27) Instruments used in the experimental section and the experimental conditions for chromatography were same as in the preceding paper.[1]

at 32° for 4 days. The reaction mixture was treated with *n*-BuOH (50 ml), heated on a boiling water-bath for 10 min, and after cooling, filtered with the aid of small amount of Celite 535. The precipitate was washed twice with hot *n*-BuOH (50 ml each) and the filtrate and washings were combined and concentrated under reduced pressure to give a residue (1 g). The residue (1 g) was chromatographed on a silica gel (Merck, 80 g) column eluting with $CHCl_3$–MeOH–H_2O (7: 3: 1, lower layer; each fraction: 50 ml). A product (20 mg) obtained from fr. 4—6 was recrystallized from MeOH to give the partial hydrolysate **6** (10 mg), mp 274.5—277°, $[\alpha]_D^{24}$ −8° (c=0.3, MeOH). *Anal.* Calcd. for $C_{41}H_{64}O_{13}\cdot 2H_2O$: C, 61.47; H, 8.56. Found: C, 61.41; H, 7.81. IR ν_{max}^{KBr} cm^{-1}: 3350 (br), 1750 (br), 1070 (br). Fr. 7—13 gave another product (60 mg) which was recrystallized from MeOH to give the partial hydrolysate **5** (50 mg), mp 259—262°, $[\alpha]_D^{29}$ −85° (c=0.4, pyridine). *Anal.* Calcd. for $C_{54}H_{86}O_{23}$: C, 58.82; H, 7.80. Found: C, 58.57; H, 7.96. IR ν_{max}^{KBr} cm^{-1}: 3400 (br), 1750 (br), 1060 (br). CD ($c=1.07\times10^{-4}$, MeOH): $[\theta]_{340}$ 0, $[\theta]_{305}$ −8600 (neg. max.), $[\theta]_{262}$ −1200 (neg. min.), $[\theta]_{233}$ −13500 (neg. max.), $[\theta]_{216}$ 0, $[\theta]_{205}$ +19400 (pos. max.). From the fractions after fr. 14 was recovered holotoxin A (50 mg).

iii) Hydrolysis of Holotoxin A (**4**) with Cellulase III: To a suspension of **4** (7 mg) in dist. water (10 ml) was added commercial cellulase III (5 mg, Worthington Biochemical Corp.) and the total mixture was kept gentle stirring at 32° for 24 hr, treated with small amount of MeOH, boiled for a while, and filtered with the aid of Celite 535. The collected precipitate was washed with hot MeOH and the combined filtrate and washings were evaporated to dryness under reduced pressure to give a residue. The residue was then treated with small amount of dist. water to separate the insoluble portion (5 mg) and the soluble portion (1 mg). Recrystallization of the insoluble portion (5 mg) from MeOH gave colorless crystals of mp 258—259°, which were identified with **5** by mixed mp and TLC ($CHCl_3$–MeOH–H_2O=7: 3: 1, lower layer). The soluble portion was subjected to PPC (Toyo Filter Paper, no. 50, iso-PrOH–*n*-BuOH–H_2O=7: 1: 2, developing for 20 hr, detection with aniline hydrogen phthalate), and only xylose was identified. The residue of the soluble portion was dissolved in pyridine and treated as usual with Me_3SiCl/hexamethyldisilazane and evaporated to dryness to give a product, which was extracted with *n*-hexane and subjected to GLC (3% SE-30 on chromosorb W, 1 m×3 mm; column temp.: 168°; N_2 flow rate: 40 ml/min) and xylose was identified.

Acid Hydrolysis of Partial Hydrolysates 5 and 6——i) A solution of **5** (5 mg) in aq. 2N HCl (1 ml) was heated on a boiling water-bath for 3 hr. After cooling, the precipitate collected by filtration was washed with water and subjected to TLC ($CHCl_3$–MeOH=50: 1) to identify with **2a** and the mixture of **2b** and its isomer. The filtrate and washings were combined and neutralized with Ag_2CO_3 and the supernatant obtained with a centrifuge was evaporated to dryness under reduced pressure to give a residue which was subjected to PPC (Toyo Filter Paper, no. 50, iso-PrOH–*n*-BuOH–H_2O=7: 1: 2, developing for 19 hr, detection with aniline hydrogen phthalate) and three spots of quinovose +3-O-methyl-glucose (Rf=0.50), xylose (Rf=0.45), and glucose (Rf=0.39) were identified. The TMS derivative was also prepared from the residue as above and subjected to GLC (2% SE-52 on chromosorb W, 2 m×3 mm; column temp.: 140°; N_2 flow rate: 45 ml/min) to identify with xylose, quinovose, 3-O-methyl-glucose, and glucose. ii) A solution of **6** (4 mg) in aq. 2N HCl (2 ml) was heated on a boiling water-bath for 2 hr and extracted with $CHCl_3$. The $CHCl_3$ extract was washed with water, neutralized, and evaporated under reduced pressure to give a product (1 mg), which was identified with **2a** and the mixture of **2b** and its isomer by TLC ($CHCl_3$–MeOH=50: 1). The aqueous layer was neutralized with resin Dowex 44 (OH^- form) and evaporated under reduced pressure to give another product which was subjected to PPC and GLC as above and xylose and quinovose were identified.

Methylation of Holotoxin A (4) followed by Methanolysis——i) A solution of **4** (25 mg) in the dimsyl carbanion solution (2 ml), which was prepared from NaH (500 mg, washed with dry *n*-hexane beforehand) and DMSO (15 ml), was kept stirring under N_2 atmosphere for 1 hr, treated with CH_3I (1 ml), and kept stirring for further 2 hr. The reaction mixture was poured into ice-water and extracted with EtOAc three times. The combined EtOAc extract was washed with water, dried, and evaporated to give the tetradeca-O-methyl derivative (**7**) (18 mg), amorphous, $[\alpha]_D^{26}$ +1° (c=0.7, $CHCl_3$). *Anal.* Calcd. for $C_{72}H_{120}O_{27}$: C, 61.02; H, 8.41. Found: C, 61.32; H, 8.20. IR $\nu_{max}^{CCl_4}$ cm^{-1}: 3500 (w), 1730, 1710, 1610. PMR (90 MHz): as given in Table I. ii) A solution of **7** (2 mg) in anhydrous 10% HCl/MeOH (1 ml) was refluxed for 1 hr, diluted with small amount of MeOH, neutralized with Ag_2CO_3, and filtered to remove the precipitate. The filtrate was analyzed by TLC (benzene–acetone=3: 1; $CHCl_3$–MeOH=20: 1) and by GLC (10% SE-30 on chromosorb W, 2 m×3 mm; column temp.: 180°; N_2 flow rate: 11 ml/min) to identify with Me 2,3,4-tri-O-methyl-xylopyranoside (1′20″, 1′35″), Me 2,3,4,6-tetra-O-methyl-glucopyranoside (3′10″, 3′40″), Me 2,3-di-O-methyl-quinovopyranoside (1′45″, 2′00″), Me 2,4,6-tri-O-methyl-glucopyranoside (3′40″, 4′30″), and Me 3-O-methyl-xylopyranoside (1′55″, 2′05″).

Methylation of 5 followed by Methanolysis——i) To a solution of **5** (26 mg) in DMSO (2 ml) was added the dimsyl carbanion solution (2 ml) (prepared from 2 g of NaH and 35 ml of DMSO as above) and the solution was kept stirring under N_2 atmosphere for 15 min, treated with CH_3I (1 ml), kept stirring for further 1.5 hr, and poured into ice-water. The mixture was then extracted with ether (30 ml each) three times and ordinary work-up of the ether extract gave the dodeca-O-methyl derivative (**8**) (25 mg), amorphous, $[\alpha]_D^{11}$ −85° (c=2.5, $CHCl_3$). *Anal.* Calcd. for $C_{65}H_{108}O_{23}$: C, 62.05; H, 8.59. Found: C, 62.35; H, 8.26. IR $\nu_{max}^{CCl_4}$ cm^{-1}: 3600 (w), 1730, 1715, 1610. UV $\lambda_{max}^{n\text{-hexane}}$ nm (ε): 252 (6700). CD ($c=2.58\times10^{-4}$, *n*-hexane): $[\theta]_{400}$ 0, $[\theta]_{350}$ −1540 (neg. max.), $[\theta]_{294}$ −500 (neg. min.), $[\theta]_{250}$ −14500 (neg. max.), $[\theta]_{231}$ 0, $[\theta]_{222}$ +6150 (pos. max.). PMR (90 MHz):

as given in Table I. ii) A solution of **8** (10 mg) in anhydrous 2N HCl/MeOH (3 ml) was heated under reflux for 1.5 hr and neutralized with Ag_2CO_3. The supernatant obtained with a centrifuge was analyzed by TLC (benzene–acetone=3: 1; $CHCl_3$–MeOH=6: 1) and by GLC (3% SE-30 on chromosorb W, 2 m×3 mm; column temp.: 160°; N_2 flow rate: 40 ml/min and 15% PEGS on Uniport B, 1 m×3 mm; column temp.: 190°; N_2 flow rate: 40 ml/min) to identify with Me 2,3,4,6-tetra-O-methyl-glucopyranoside, Me 2,3,4-tri-O-methyl-quinovopyranoside, Me 2,4,6-tri-O-methyl-glucopyranoside, and Me 3-O-methyl-xylopyranoside.

Methylation of 6 followed by Methanolysis——i) A solution of **6** (5 mg) in the dimsyl carbanion solution (1 ml) (prepared from 250 mg of NaH and 6 ml of DMSO) was kept stirring under N_2 atmosphere for 1 hr, treated with CH_3I (1 ml), and stirred for further 2 hr. The reaction mixture was poured into ice-water and extracted with EtOAc three times, and the EtOAc extract was treated in the usual manner to give the hexa-O-methyl derivative (**9**) (3 mg), which was dissolved in anhydrous 10% HCl/MeOH (2 ml) and heated under reflux for 30 min. The reaction mixture was neutralized with Ag_2CO_3 and filtered. The product obtained from the filtrate was subjected to TLC (benzene–acetone=3: 1) and to GLC (10% SE-30 on chromosorb W, 2 m×3 mm; column temp.: 180°; N_2 flow rate: 11 ml/min) and Me 2,3,4-tri-O-methyl-quinovopyranoside (1′00″, 1′10″) and Me 2,3-di-O-methyl-xylopyranoside (1′45″, 2′00″) were identified.

Acetolysis of Holotoxin A (**4**)——To a solution of **4** (150 mg) in Ac_2O (12 ml) was added $ZnCl_2$ (150 mg, molten beforehand) and the mixture was heated at 90° for 1.5 hr, poured into ice-water, neutralized with $NaHCO_3$ powder, and extracted with ether (150 ml each) three times. Working-up of the ether extract in the usual manner gave a product (200 mg), which was purified by preparative TLC (benzene–EtOH=35: 5) to afford nonaacetyl-trisaccharide (**10**) (60 mg) and undecaacetyl-tetrasaccharide (**11**) (15 mg). **10**, amorphous. *Anal.* Calcd. for $C_{36}H_{50}O_{24}$: C, 49.87; H, 5.67. Found: C, 49.93; H, 5.80. IR $\nu_{max}^{CHCl_3}$ cm^{-1}: no OH, 1750 (br). PMR (90 MHz): 2.00, 2.06 (total 27H, 9 AcO's), 3.37 (3H, s, OMe). **11**, amorphous. *Anal.* Calcd. for $C_{46}H_{64}O_{30}$: C, 50.37; H, 5.67. Found: C, 50.45; H, 5.78. IR $\nu_{max}^{CHCl_3}$ cm^{-1}: no OH, 1750 (br). PMR (90 MHz): 1.29 (3H, d, J=7 Hz, quinovose-Me), 2.02, 2.06 (total 33H, 11 AcO's), 3.34 (3H, s, OMe).

Methylation of 10 followed by Methanolysis——i) To a solution of **10** (20 mg) in DMSO (3 ml) was added the dimsyl carbanion solution (1 ml) (prepared from 250 mg of NaH and 6 ml of DMSO) and the total solution was kept stirring under N_2 atmosphere for 1 hr, treated with CH_3I (1 ml), stirred for further 2 hr, poured into ice-water and extracted with EtOAc three times. The product (15 mg), obtained from the EtOAc extract after usual work-up, was purified by preparative TLC (benzene–acetone=3: 1) to afford the deca-O-methyl-trisaccharide (**12**) (13 mg), amorphous, $[\alpha]_D^{28}$ +2° (c=0.3, $CHCl_3$). IR $\nu_{max}^{CCl_4}$ cm^{-1}: no OH. PMR (90 MHz): as given in Table II. ii) A solution of **12** (2 mg) in anhydrous 10% HCl/MeOH (2 ml) was refluxed for 30 min, diluted with small amount of MeOH, neutralized with Ag_2CO_3, and filtered. The product obtained from the filtrate was analyzed by TLC (benzene–acetone=3: 1; $CHCl_3$–MeOH=50: 1) and by GLC (10% SE-30 on chromosorb W, 2 m×3 mm; column temp.: 180°; N_2 flow rate: 11 ml/min) to identify with Me 2,3,4,6-tetra-O-methyl-glucopyranoside (3′10″, 3′40″), Me 2,4,6-tri-O-methyl-glucopyranoside (3′40″, 4′30″), and Me 3,4-di-O-methyl-xylopyranoside (1′55″, 2′00″).

Methylation of 11 followed by Methanolysis——i) A solution of **11** (10 mg) in DMSO (3 ml) was treated with the dimsyl carbanion solution (1 ml, prepared as above) and the total solution was kept stirring under N_2 atmosphere for 1 hr, treated with CH_3I (1 ml), stirred for further 2 hr, and poured into ice-water. The product (8 mg) obtained as above was purified by preparative TLC (benzene–acetone=3: 1) to afford the dodeca-O-methyl-tetrasaccharide (**13**) (7 mg), amorphous, $[\alpha]_D^{31}$ +4° (c=0.2, $CHCl_3$). IR $\nu_{max}^{CCl_4}$ cm^{-1}: no OH. PMR (90 MHz): as given in Table II. ii) A solution of **13** (2 mg) in anhydrous 10% HCl/MeOH (2 ml) was refluxed for 30 min, diluted with small amount of MeOH, neutralized with Ag_2CO_3, and filtered. The product obtained from the filtrate was analyzed by TLC and GLC (as for **12**) to identify with Me 2,3,4-tri-O-methyl-quinovopyranoside (1′00″, 1′10″), Me 2,3,4,6-tetra-O-methyl-glucopyranoside (3′10″, 3′40″), Me 2,4,6-tri-O-methyl-glucopyranoside (3′40″, 4′30″), and Me 3-O-methyl-xylopyranoside (1′55″, 2′05″).

Isolation of Holotoxin B (**14**) **and Holotoxin C**——Fr. 19—20 containing holotoxin B, which was obtained by separation of crude holotoxin by droplet countercurrent chromatography in the preceding paper,[1] was recrystallized from $CHCl_3$–MeOH–H_2O to give holotoxin B (**14**), mp 236—239°, $[\alpha]_D^{29}$ −52° (c=0.4, pyridine). *Anal.* Calcd. for $C_{65}H_{104}O_{33}\cdot 2H_2O$: C, 53.18; H, 7.44. Found: C, 53.49; H, 7.56. IR ν_{max}^{KBr} cm^{-1}: 3360 (br), 1750 (br), 1070 (br). UV λ_{max}^{MeOH} nm: transparent above 210 nm. CD (c=5.9×10^{-4}, MeOH): $[\theta]_{340}$ 0, $[\theta]_{307.5}$ −12600 (neg. max.), $[\theta]_{262}$ −1100 (neg. min.), $[\theta]_{232}$ −10300 (neg. max.), $[\theta]_{215}$ 0, $[\theta]_{205}$ +14200 (pos. max.). Fr. 21—22 gave holotoxin C (amorphous, trace amount). IR ν_{max}^{KBr} cm^{-1}: 3380 (br), 1755 (br), 1070 (br). UV λ_{max}^{MeOH} nm: transparent above 210 nm. CD (MeOH):[28] 200 (pos.), 232 (neg. max.), 260 (neg. min.), 305 (neg. max.) nm.

Acid Hydrolysis of Holotoxin B (**14**)——A solution of **14** (115 mg) in aq. 2N HCl (10 ml) was heated on a boiling water-bath for 3 hr and the reaction mixture was centrifuged to give a precipitate (20 mg) and an aqueous supernatant. Purification of the precipitate by preparative TLC ($CHCl_3$–MeOH=50: 1) followed by recrystallization from MeOH furnished genin-1 (15 mg) and genin-2 (2 mg).

Genin-1, mp 217—222°, IR ν_{max}^{KBr} cm^{-1}: 3450, 1760 (br), UV λ_{max}^{EtOH} nm: transparent above 210 nm, was identified with genin-1 (the mixture of stichopogenin A_2 (**2b**) and the isomer), which was obtained from holotoxin

28) Molecular ellipticity is obscure due to unknown molecular size.

A (**4**),[1] by mixed mp, IR (KBr), and TLC. Genin-2, mp 240—242°, IR ν_{max}^{KBr} cm^{-1}: 3400, 1750 (br), was identified with stichopogenin A_4 (**2a**) by mixed mp, TLC, and IR (KBr).

The aqueous supernatant was neutralized with resin Dowex 44 (OH^- form), treated with active charcoal, and filtered. The combined filtrate and washings of the charcoal were evaporated to dryness under reduced pressure to give a residue, which was analyzed by PPC (Toyo Filter Paper, no. 50, iso-PrOH–*n*-BuOH–H_2O=7:1:2, developing for 19 hr, detection with aniline hydrogen phthalate) to identify with quinovose +3-O-methyl-glucose (Rf=0.65), xylose (Rf=0.45), and glucose (Rf=0.40), and also analyzed by TLC using cellulose MN 300 (phenol–H_2O=100:40, detection as above) to identify with 3-O-methyl-glucose (Rf=0.80), quinovose (Rf=0.70), xylose (Rf=0.50), and glucose (Rf=0.45). The residue was also converted to the tetramethylsilyl (TMS) derivative in the usual manner and analyzed by GLC (3% SE-30 on chromosorb W, 1 m×3 mm; column temp.: 180°; N_2 flow rate: 40 ml/min) to identify with xylose, quinovose, 3-O-methyl-glucose, and glucose.

Acid Hydrolysis of Holotoxin C——A solution of holotoxin C (5 mg) in aq. 2N HCl (2 ml) was heated on a boiling water-bath for 2 hr and extracted with $CHCl_3$. The $CHCl_3$ extractive, after ordinary work-up, was analyzed by TLC ($CHCl_3$–MeOH=50:1) to identify with genin-1 (*vide supra*) and **2a**. The aqueous layer was treated with Ag_2CO_3 and the product obtained from the supernatant was converted to the TMS derivative and analyzed by GLC as above to identify with xylose, quinovose, 3-O-methyl-glucose, and glucose.

Enzymatic Hydrolysis of Holotoxin B (14)——i) A suspension of **14** (30 mg) in dist. water (20 ml) was treated with β-glucosidase (30 mg, Sigma Chemical Co.) and the total mixture was kept gentle stirring at 40° for 24 hr, added with small amount of *n*–BuOH, and heated on a boiling water-bath for 15 min. After cooling, the mixture was filtered with the aid of Celite 535 and the Celite was washed with *n*–BuOH. The combined filtrate and washings were evaporated to dryness under reduced pressure to give a residue which was extracted with MeOH. The extractive (40 mg) thus obtained was treated with small amount of MeOH to separate the soluble portion (5 mg) and the insoluble portion (30 mg). Recrystallization of the soluble portion (5 mg) from MeOH gave colorless crystals of mp 246—250°, which were identified with holotoxin A (**4**) by mixed mp and TLC ($CHCl_3$–MeOH–H_2O=7:3:1, lower layer). The insoluble portion was treated with water and the soluble portion was identified with glucose by PPC (Toyo Filter Paper no. 50, iso-PrOH–*n*-BuOH–H_2O=7:1:2, developing for 14 hr, detection as above). ii) To a suspension of **14** (5 mg) in dist. water (5 ml) was added cellulase III (5 mg) and the total mixture was kept gentle stirring at 40° for 48 hr, diluted with small amount of MeOH, and heated on a boiling water-bath for 20 min. After cooling, the mixture was filtered with the aid of Celite 535, and the Celite was washed with hot aq. MeOH. Evaporation of the combined filtrate and washings under reduced pressure gave a residue (3 mg), which was treated with water to afford the soluble and insoluble portions. The insoluble portion was analyzed by TLC ($CHCl_3$–MeOH–H_2O=7:3:1, lower layer) to identify with holotoxin A (**4**) and **5**. The soluble portion was subjected to PPC (as above) to identify with xylose and glucose. iii) A solution of **14** (2 mg) in dist. water (10 ml) was treated with crude naringinase (5 mg, Tanabe Pharm. Co., Lot no. N-1-6) or crude takadiastase A (5 mg, Sankyo Co.) at 38° with gentle stirring for 12 hr. Each reaction mixture was treated with *n*–BuOH, heated on a boiling water-bath for 15 min, and evaporated to dryness under reduced pressure. Each residue was extracted with *n*–BuOH repeatedly and the extractive was analyzed by TLC ($CHCl_3$–MeOH–H_2O=7:3:1, lower layer) to identify with holotoxin A (**4**), **5**, and **6**.

Methylation of Holotoxin B (14) followed by Methanolysis——i) A solution of **14** (10 mg) in the dimsyl carbanion solution (2 ml) (prepared from 350 mg of NaH and 15 ml of DMSO) was kept stirring at room temperature under N_2 atmosphere for 30 min, treated with CH_3I (0.5 ml), stirred for further 1 hr, poured into ice-water, and extracted with EtOAc. Working-up of the EtOAc extract in the usual manner gave the heptadeca-O-methyl derivative (**15**) (8 mg), amorphous, $[\alpha]_D^{19}$ −54° (c=0.4, $CHCl_3$). IR $\nu_{max}^{CCl_4}$ cm^{-1}: 3500 (w), 1732, 1718, 1610. UV $\lambda_{max}^{n\text{-hexane}}$ nm (ε): 253 (6200). CD ($c=2.41\times10^{-4}$, *n*–hexane): $[\theta]_{420}$ 0, $[\theta]_{360}$ −2200 (neg. max.), $[\theta]_{304}$ −1100 (neg. min.), $[\theta]_{250}$ −26300 (neg. max.), $[\theta]_{230}$ 0, $[\theta]_{218}$ +8200 (pos. max.). ii) A solution of **15** (5 mg) in anhydrous 5% HCl/MeOH (2 ml) was heated under reflux for 30 min, diluted with small amount of MeOH, neutralized with Ag_2CO_3, and centrifuged. A product obtained from the supernatant was analyzed by TLC (benzene–acetone=3:1; $CHCl_3$–MeOH=10:1) and by GLC (15% NPGS on chromosorb WAW, 2 m×3 mm; column temp.: 160°; N_2 flow rate: 40 ml/min) to identify with Me 2,3,4,6-tetra-O-methyl-glucopyranoside (6′55″, 9′30″, *vide supra*), Me 2,4,6-tri-O-methyl-glucopyranoside (20′00″, 26′50″), Me 2,3-di-O-methyl-quinovopyranoside (6′19″, 8′06″), and Me 3-O-methyl-xylopyranoside (19′00″). An unidentified Me di-O-methyl-xylopyranoside (4′15″, 9′15″, *vide supra*) was also detected.

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