NEW α-AMYLASE INHIBITOR, TRESTATINS

II. STRUCTURE DETERMINATION OF TRESTATINS A, B AND C

KAZUTERU YOKOSE, KIYOSHI OGAWA, YUKIKO SUZUKI,
ISAO UMEDA and YASUJI SUHARA

Department of Microbiology and Chemotherapy
Nippon Roche Research Center
200 Kajiwara, Kamakura City,
Kanagawa 247, Japan

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The structures of trestatins A ($C_{56}H_{94}N_2O_{40}$), B ($C_{37}H_{63}NO_{23}$) and C ($C_{75}H_{125}N_3O_{52}$), new basic oligosaccharides with potent inhibitory activity against various α-amylases, have been shown by spectroscopic and chemical methods to be **1**, **2** and **3**, respectively.

Trestatin complex (Ro 09-0154) which is produced by a novel streptomycete, *Streptomyces dimorphogenes* NR320-OM7HB, contains new potent α-amylase inhibitors. We reported in the previous paper[1] the isolation and characterization of three major components of trestatin complex, trestatins A, B and C, all of which are water-soluble basic oligosaccharides consisting of D-glucose and the pseudodisaccharide, dehydro-oligobiosamine[6] **4** (Fig. 1).

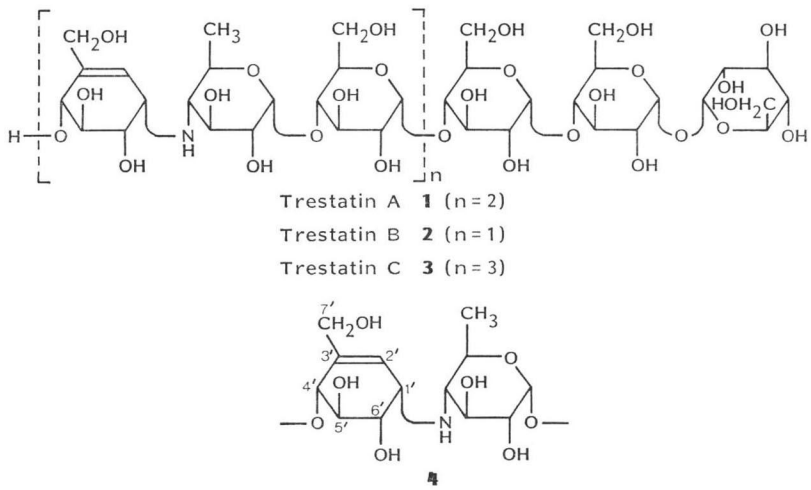
In the present paper, we describe the structural elucidation of the molecular structures of the homologous oligosaccharides, trestatins A, B and C.

Results and Discussion

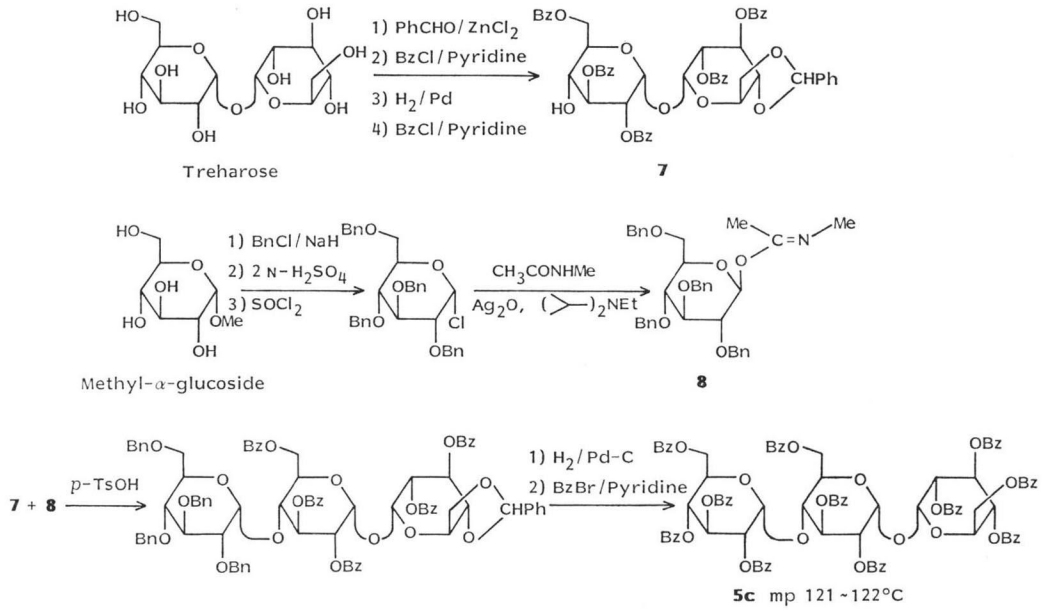
Structures of Glucotriose and Glucotetraose

In order to determine the sequences, each trestatin (A, B and C) was subjected to mild acid hydrolysis using Dowex 50 (H^+ form) as described in the previous paper[1], and partially hydrolyzed products

Fig. 1. Structures of trestatins A, B, C and pseudonitrogen disaccharide.



Scheme 1.



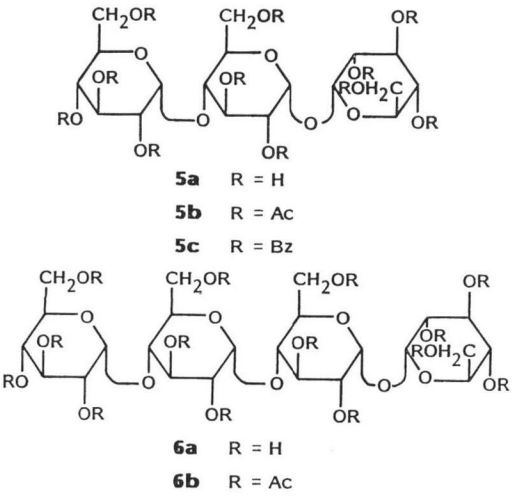
were separated into neutral and basic fragments. HPLC analysis of the neutral fragments from each trestatin showed identical chromatograms which revealed the presence of glucose, maltose, α,α-trehalose, glucotriose (**5a**) and glucotetraose (**6a**). Acetylation of the neutral fragments with acetic anhydride in pyridine followed by chromatography on silica gel gave peracetates of D-glucose, D-maltose, α,α-D-trehalose, glucotriose (**5a**) and glucotetraose (**6a**). Each peracetate of the glucotriose and the glucotetraose obtained from trestatins A, B and C showed identical physico-chemical properties indicating that trestatins A, B and C contained the same partial structure.

The glucotriose peracetate (**5b**) showed the highest peak at m/z 906 $(M-AcOH)^+$ in the EI-MS. The ^1H NMR spectrum of **5b** in $CDCl_3$ showed no signal assignable to $-\overset{O}{\overset{|}{C}}H-OAc$ at δ 5.6~6.5, indicating that **5b** contained α,α-trehalose moiety. The ^{13}C NMR spectrum of **5b** showed anomeric carbons at δ 95.7 and 91.8 ($\times 2$) assignable to α-(1→4) and α,α-(1↔1) linkages, respectively[2]. This spectral evidence indicated that the triose **5a** was 4-*O*-α-D-glucopyranosyl-α,α-trehalose, which was confirmed by the chemical synthesis of perbenzoate **5c** starting from α,α-trehalose and methyl-α-D-glucoside as shown in Scheme 1[3].

The glucotetraose peracetate (**6b**) showed a molecular ion peak at m/z 1,255 (MH^+ for glucotetraose peracetate, $C_{52}H_{70}O_{35}$) in its FD-MS. The ^1H NMR spectrum of **6b** was similar to that of **5b**, also indicating the absence of $-\overset{O}{\overset{|}{C}}H-OAc$. The ^{13}C NMR spectrum of **6b** in $CDCl_3$ showed at least 22 carbon signals attributable to sugar skeleton, of which four anomeric carbons were observed at δ 95.6 ($\times 2$) and 91.8 ($\times 2$) assignable to α-(1→4) and α,α-(1↔1) linkages, respectively. From these results, the tetraose **6a** was indicated to be 4-*O*-α-maltosyl-α,α-trehalose.

These assignments were supported by GC-MS analysis of methylated alditol acetates obtained from each trestatin according to the method of LINDBERG[4], which revealed the acetates to be 1,5-di-*O*-acetyl-2,

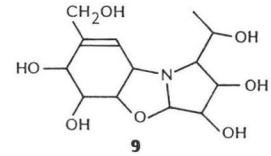
Fig. 2.



5a R = H
5b R = Ac
5c R = Bz

6a R = H
6b R = Ac

Fig. 3.



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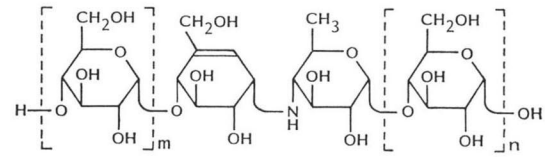


10 m = 0, n = 1
11 m = 0, n = 2
12 m = 1, n = 1

3,4,6-tetra-*O*-methyl glucitol and 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methyl glucitol. This fact indicated that only the 1 and/or 4 positions of glucose were involved in the glycosidic linkages in trestatins A, B and C. Furthermore, the ^{13}C NMR spectra of trestatins A, B and C indicated that the glucosidic linkages in trestatins were either α-(1→4) or α,α-(1↔1) (Table 1)[5,6]. Thus, the structure of glucotetraose **6a** was determined as 4-*O*-α-maltosyl-α,α-trehalose (Fig. 2).

On the other hand, glucopentaose and glucohexaose were never detected in the hydrolysate of trestatins, suggesting that glucotetraose **6a** was the largest common neutral oligosaccharide moiety in trestatins A, B and C.

Structure of Trestatin B

As described in the previous paper[1], trestatin B was composed of 4 mol of D-glucose and 1 mol of **4**. Therefore, the determination of glycosidic linkage between **4** and **6a** permitted the structure elucidation of trestatin B. This linkage was considered to be α-(1→4) based on the following evidence: 1) GC-MS analysis on the methylated alditol acetates as described above, 2) the anomeric carbons of trestatin B were observed at δ 94.0, 94.2 (α,α-1↔1), 100.4 (α-1→4), 100.5 (α-1→4) and 100.8 (α-1→4 of **4**)[5,6]. The ^{13}C spin-lattice relaxation times (T_1's) of these anomeric carbons measured at 50°C in 0.16 M D_2O solution were found to be 0.21, 0.42, 0.16, 0.16 and 0.25, respectively. Since carbons of terminal monosaccharide unit of straight chain oligosaccharides were reported to have much longer spin-lattice relaxation time than those of the inner units in the ^{13}C NMR spectroscopy[7], location of the trehalose moiety was deduced to be at the terminal. Thus, the structure **2** was assigned to trestatin B.

This structure was further confirmed by analyzing the structure of the basic fragments **9**, **10** and **11** obtained by mild acid hydrolysis of trestatin B using Dowex 50. **9** and **10** were identified as the tricyclic compound and the pseudotrisaccharide (Fig. 3), respectively, which had already been isolated and characterized[1]. The ^1H NMR spectrum of **11** showed a close similarity to that of **10** except for an additional anomeric proton at δ 5.38 (d, J=3.7 Hz, 1 H), indicating that **11** was a congener of **10** and consisted of 2 mol of glucose and 1 mol of **4**. This indication was supported by the FD-MS of **11**, which exhibited molecular ion at m/z 646 (MH^+ for pseudotetrasaccharide **11**, $C_{25}H_{43}NO_{18}$) and 668 $(M+Na)^+$. Since **6a** had already been isolated from trestatin B and the ^1H NMR spectrum of **11** showed the presence

Table 1. ^{13}C NMR chemical shifts[a] (in ppm) of trestatins A, B, C and hydrolyzed products in D_2O with dioxane as an internal standard (67.4 ppm) at 25.05 MHz.

		A	B	C	10	11	12	14	15	18	13	9
C=CH	Terminal unit	139.8	139.9	139.9	139.9	139.9		139.8	139.8	139.8	139.8	139.3
	Inner unit	137.4		137.4*			137.4	137.3	137.2	137.3*	137.4	
C=*CH*	Inner unit	126.9		127.0*			126.9	127.1	127.1	127.2*	124.6	122.8
	Terminal unit	124.4	124.5	124.4	124.6	124.6		124.6	124.6	124.6	124.4	
C-1	Pseudodisaccharide moiety **4**	100.9*	100.8	100.7*	100.7 100.6	100.8	100.7	100.7*	100.7*	100.8*	100.9 94.3	95.2
	α-1,4(Glc)	100.5 100.4	100.5 100.4	100.5*		100.4 100.3			100.4			
	**	98.5		98.3*			98.5	98.4	98.3	98.4*	98.9	
	α,α-1,1 terminal (Glc) inner	94.2 94.0	94.2 94.0	94.2 94.0								
	*** β				96.6	96.6	96.6	96.6	96.6	96.6		
	α				92.7	92.8	92.7	92.7	92.7	92.8		
H–C–O		78.0 ~ 70.4	78.0 ~ 70.4	77.9 ~ 70.3	78.3 ~ 70.4	78.0 ~ 70.4	78.2 ~ 70.4	78.1 ~ 70.4	77.9 ~ 70.4	78.1 ~ 70.4	78.1 ~ 66.7	77.5 ~ 68.3
-N-C	Terminal unit	65.8	65.7	65.7	65.7	65.8		65.8	65.8	65.8	70.4	71.2
	Inner unit	65.0		65.0*			65.0	65.1	65.0	65.1*	65.8	
CH_2OH	Inner unit	62.8		62.9*			62.9	62.9	62.9	62.9*	63.5	63.0
	Terminal unit	62.4	62.5	62.4	62.4	62.5		62.5	62.4	62.5	62.5	
C-6 (Glc)		61.4*	61.4*	61.4*	61.6, 61.5	61.5, 61.4	61.4*	61.4*	61.4*	61.4*	61.4	
C-N-	Terminal unit	56.8	56.8	56.8	56.8	56.9		56.9	56.8	56.9	62.5	62.8
	Inner unit	55.9		55.9*			55.8	55.9	55.8	55.9*	56.9	
CH_3-		18.2*	18.2	18.2*	18.2	18.2	18.2	18.2*	18.2*	18.3*	18.2, 20.1	20.6

* Doubly or more intense signal.

** C-1 resonance of Glc linked to allylic position (C-4′) of **4** through α-linkage.

*** Reducing end unit of Glc.

[a] Carbon signal assignments were mainly based on the corresponding data for some related oligosaccharide[5] and on selective proton decoupling and off-resonance decoupling experiments and ^{13}C spin-lattice relaxation times (T_1's).

of an anomeric proton of reducing end unit of glucose at δ 4.64 (d, J=7.8 Hz, 0.6 H) and 5.21 (d, J=3.7 Hz, 0.4 H) assignable to β and α anomer respectively, the pseudotetrasaccharide structure was assigned to **11** (Fig. 3). This structure was also supported by ^{13}C NMR spectrum of **11** (Table 1), confirming the proposed structure **2** for trestatin B.

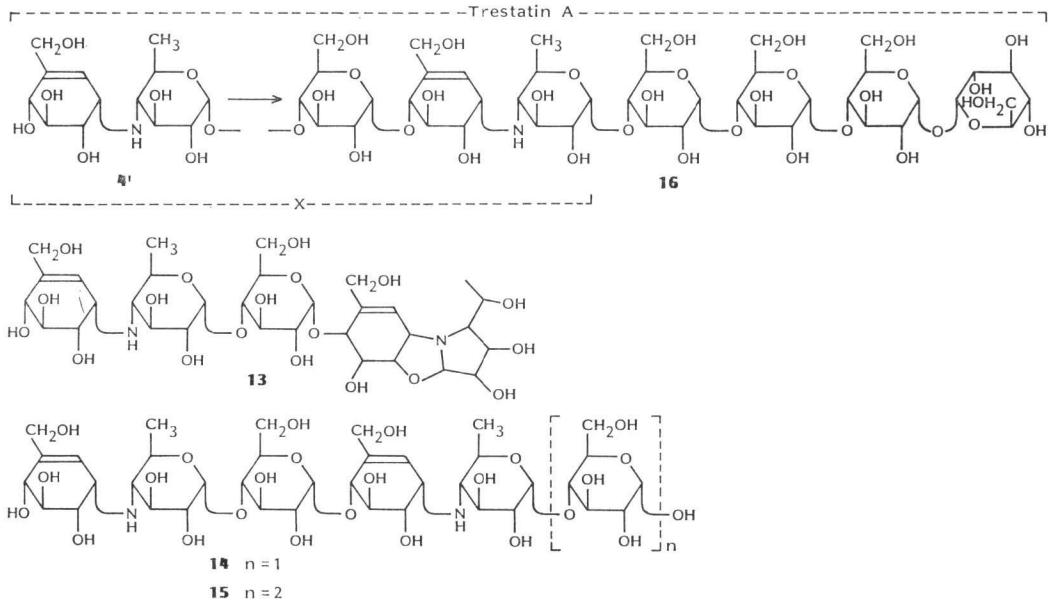
Structure of Trestatin A

Trestatin A was composed of 5 mol of D-glucose and 2 mol of **4**[1]. Mild acid hydrolysis of trestatin A gave D-glucose, D-maltose, α,α-trehalose, glucotriose (**5a**), glucotetraose (**6a**), the tricyclic compound **9**, the pseudotrisaccharide (**10**) and the pseudotetrasaccharide (**11**) in addition to four basic fragments **12**~**15** characteristic of trestatin A.

Fragment **12** showed an almost identical ^{1}H NMR spectrum with that of **11**, indicating that **12** was a sequential isomer of **11**. Comparison of ^{13}C NMR spectrum of **12** with that of **11** showed that resonances of olefinic carbons of **12** were displaced 2.5 ppm upfield (at δ 137.4) and 2.3 ppm downfield (at δ 126.9) from those of **11** (Table 1), being consistent with the β and γ shift of the olefinic carbons caused by glycosidation at the allylic position[8]. Since carbon resonance of the hydroxymethyl at the allylic position in the cyclitol unit (C-7′ of moiety **4** of **12**) was observed at almost the same position (δ 62.9) as that of **11**, the site of the glycosidation was suggested to be at the allylic secondary alcohol (C-4′ of moiety **4**). This was supported by the hydrogenolysis (H_2/Pd-C) of **12**, which gave glucose. The presence of a reducing glucose unit in **12** was demonstrated by the ^{1}H NMR which showed anomeric proton signals at δ 4.64 (d, J=7.8 Hz, 0.6 H) and 5.22 (d, J=3.9 Hz, 0.4 H). The spectrum also indicated that all glycosidic linkages in **12** were α: δ 5.32 (d, J=2.4 Hz, 1 H) and 5.37 (d, J=3.4 Hz, 1 H). From these results the pseudotetrasaccharide structure containing **4** as an inner unit was assigned to **12** (Fig. 3).

The above results coupled with the previous finding[1] that trestatin A was composed of 5 mol of glucose and 2 mol of **4** indicated that trestatin A should be composed of the partial structures **16** and **4′** (Fig. 4). The glycosidic linkage between **16** and **4′** was suggested to be α-(1→4) based on the results of

Fig. 4.



GC-MS analysis on methylated alditol acetate mentioned above and ^{13}C NMR data of trestatin A (Table 1)[5,6]. The linking position was finally clarified by analyzing the structure of the degradation product **13**.

Fragment **13** showed quite different ^{1}H and ^{13}C NMR spectra from those of **10**~**12**. The ^{1}H NMR spectrum showed the presence of the tricyclic compound **9**, the pseudodisaccharide **4** and glucose as the structural constituents[1,9]: δ 1.33 (d, J=6.1 Hz, 6 H, CH-CH_3), 2.56 (m, 1 H, N-CH of **4**), 2.99 (t, J=7.3 Hz, 1H, N-CH of **9**), 4.79 (d, J=3.7 Hz, 1H, N-CH-O), 5.20 (d, J=3.4 Hz, 1H, O-CH-O), 5.28 (d, J=3.0 Hz, 1H, O-CH-O) and 5.88 (d, J=3.4 Hz, 2H, C=CH). Upon mild acid hydrolysis with Dowex 50 (H^+ form) at 80°C for 2 hours, **13** yielded the tricyclic compound **9** and the pseudotrisaccharide **10**, being consistent with the above NMR evidence. These results indicated that **13** was originally composed of 1 mol of glucose and 2 mol of **4** and that one of **4** located at the reducing end was recyclized into the tricyclic structure under the hydrolysis conditions. This finding coupled with the foregoing results allowed an unequivocal assignment of the linking position of **4'** to **16** as shown in Fig. 4 *i.e.* 4-position of the left side glucose moiety of **16**, not to the trehalose moiety. This in turn led to the structural assignment of trestatin A as shown in Fig. 1. Fragment **13** was apparently considered to be derived from part X of trestatin A as shown in Fig. 4.

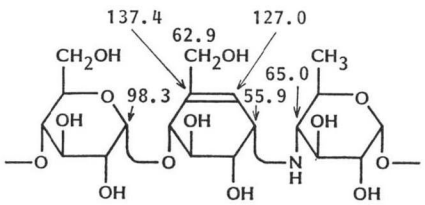
Fragments **14** and **15** were determined to be pseudohexasaccharide and pseudoheptasaccharide, respectively, by analyzing the ^{13}C NMR spectra (Table 1). The structure of trestatin A was thus determined to be **1** as shown in Fig. 1.

Structure of Trestatin C

Mild acid hydrolysis of trestatin C using Dowex 50 (H^+ form) gave D-glucose, D-maltose, α,α-trehalose, glucotriose (**5a**), glucotetraose (**6a**) and basic fragments **9**, **10**, **11**, **12**, **13** and **14** in addition to **18** characteristic to trestatin C.

The ^{1}H NMR spectrum of **18** revealed the presence of 3 mol of the pseudodisaccharide **4** and 3 mol of glucose as the structural constituents; olefinic protons at δ 5.85~6.0 (3 H) and anomeric protons at δ 4.64 (d, J=7.8 Hz, 0.6 H), 5.22 (d, J=4 Hz, 0.4 H) and 5.3~5.4 (5 H). This was supported by the FAB-MS of **18**, which exhibited molecular ion peak at m/z 1,414 (MH^+ for **18**, $C_{57}H_{95}N_3O_{37}$).

Fig. 5. ^{13}C NMR chemical shifts (in ppm) of partial structure of **17** in D_2O.



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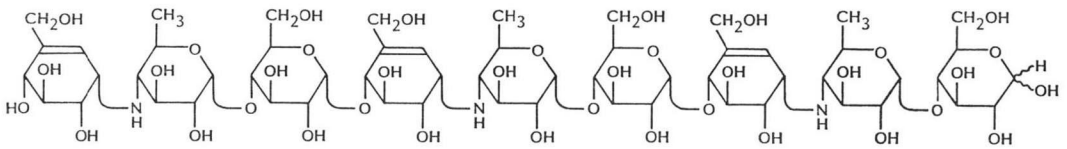
The ^{13}C NMR spectrum (Table 1) of **18** showed a close similarity to that of **14** except for the intensity of carbon resonances at δ 55.9, 62.9, 65.1, 98.4, 127.2 and 137.3 assignable to the glucosyl pseudodisaccharide moiety **17** (Fig. 5). These signals were approximately twice as intensive as the corresponding signals at δ 56.9, 62.5, 65.8,

Fig. 6.



18

124.6 and 139.8 attributable to the terminal pseudodisaccharide moiety **4**, respectively. This indicated that **18** possessed one more inner unit of the glucosyl pseudodisaccharide moiety **17** than did **14**. Therefore the pseudononasaccharide structure **18** was assigned to this fragment (Fig. 6).

Since trestatin C was composed of 6 mol of glucose and 3 mol of **4**[1], the isolation of fragments **6a**, **11** and **18** from trestatin C excluded all possible structures for trestatin C but **3**. This was also confirmed by the following NMR evidence; all carbon resonances of trestatin C were observed at almost the same chemical shifts as those of trestatin A, and the only difference was that the carbon resonances of trestatin C at δ 55.9, 62.9, 65.0, 98.3, 127.0 and 137.4 attributable to moiety **17** were approximately twice as intensive as the corresponding signals at δ 56.8, 62.4, 65.7, 124.4 and 139.9 attributable to the terminal pseudodisaccharide moiety **4**. The structure of trestatin C was thus determined to be a higher homologue **3** of trestatins A and B as shown in Fig. 1.

Experimental

Mass spectra were measured with a Hitachi RMU-6M spectrometer and a Jeol JMS-DX300 mass spectrometer. NMR spectra were recorded with a Jeol FX-100 spectrometer; ^{1}H chemical shifts are given in ppm from internal 3-(trimethylsilyl)propanesulfonic acid sodium salt or Me_4Si and ^{13}C chemical shifts are given in ppm with dioxane (67.4 ppm) or Me_4Si as an internal standard. T_1 values were measured using the inversion recovery method. Optical rotations were measured on a Perkin-Elmer Polarimeter. Gas chromatography was carried out on a Shimadzu model GC-4CM gas chromatograph and GC-MS analysis on a Hitachi RMU-6M spectrometer.

Hydrolysis of Trestatin C and Isolation of Peracetates of D-Glucose, D-Maltose, α,α-Trehalose, **5a** and **6a**

Trestatin C (510 mg) was dissolved in 60 ml of water containing 10 ml of Dowex 50 (H^+ form, 200~400 mesh) and heated at 80°C for 5 hours with stirring as described in the previous paper[1]. The resin was collected by filtration and washed with water. The combined filtrate was concentrated under reduced pressure and lyophilized to give 205 mg of neutral fragments. The neutral fragments (195 mg) were acetylated with acetic anhydride (2 ml) and pyridine (2 ml) at room temperature. The reaction mixture was evaporated to dryness and chromatographed on a 1.4×44 cm column of silica gel in benzene - methyl ethyl ketone mixture giving α,β-mixture (4.4: 5.6) of D-glucose peracetate (135 mg); $[\alpha]_D^{25}$ +41° (*c* 1, $CHCl_3$), [Ref.[12]) α, $[\alpha]_D$ +101.6° ($CHCl_3$); β, $[\alpha]_D$ +3.8° (*c* 7, $CHCl_3$)], β-D-maltose peracetate (9.2 mg); $[\alpha]_D^{25}$ +68° (*c* 0.5, $CHCl_3$) [Ref.[12]) $[\alpha]_D$ +62.6° ($CHCl_3$)], α,β-mixture of D-maltose peracetate (14 mg), α,α-D-trehalose peracetate (85 mg); $[\alpha]_D^{23}$ +163.5° (*c* 1, $CHCl_3$) [Ref.[12]) $[\alpha]_D$ +162.3° (*c* 10, $CHCl_3$)], **5b** (94 mg) and **6b** (19.5 mg).

5b: $[\alpha]_D^{25}$ +134.5° (*c* 1, $CHCl_3$); MS *m/z* (relative intensity) 906 $(M-AcOH)^+$, 619 (36), 559 (20), 331 (98), 169 (100); ^{13}C NMR ($CDCl_3$) δ 20.7~21.0 (acetyl methyl), 61.6, 61.8, 62.6, 68.1 (×2), 68.5, 68.8, 69.2, 70.0 (×2), 70.1, 70.4, 72.4, 73.0, 91.8 (×2), 95.7, 169.4~172.8 (acetyl carbonyl).

6b: $[\alpha]_D^{25}$ +130° (*c* 1, $CHCl_3$); FD-MS *m/z* 1,255 (MH^+); ^{13}C NMR ($CDCl_3$) δ 20.6~20.8 (acetyl methyl), 61.4, 61.8, 62.4, 62.7, 67.9, 68.1, 68.5 (×2), 68.7, 69.1, 69.4, 69.5, 69.8, 70.1 (×2), 70.4, 71.5, 72.3, 72.6, 73.7, 91.8 (×2), 95.6 (×2), 169.4~170.7 (acetyl carbonyl).

Trestatins A (500 mg) and B (250 mg) were treated in a same manner to give the same neutral fragments as those of trestatin C.

HPLC Analysis of Neutral Fragments

It was carried out with a Waters chromatograph under the following conditions: column, μBondapak/carbohydrate (3.9×300 mm, Waters Associate); carrier, CH_3CN - H_2O (73: 27 or 80: 20); flow rate, 4.0 ml/minute; detector, differential refractometer; retention times (CH_3CN - H_2O, 73: 27); **5a**, 3.4 minutes and **6a**, 4.9 minutes.

Protected Trehalose **7**

4,6,4′,6′-*O*-Dibenzylidene-2,2′,3,3′-tetra-*O*-benzoyl-D-trehalose (1.0 g)[13] in methanol - chloroform

(2: 3, 100 ml) was hydrogenated at room temperature with palladium black (0.05 g) for 20 minutes. After filtration, the filtrate was evaporated to dryness and the residue was applied onto a 3×30 cm column of silica gel. Elution with chloroform - ethyl acetate mixture (20: 1) yielded starting material (0.1 g), 2,2′,3,3′-tetra-*O*-benzoyl-D-trehalose (0.2 g) and 4,6-*O*-benzylidene-2,2′,3,3′-tetra-*O*-benzoyl-D-trehalose as a colorless powder (0.2 g): mp 234~235°C; $[\alpha]_D^{25}$ +248° (*c* 1, CH_2Cl_2 - MeOH, 1: 1); FDMS *m/z* 847 (MH^+); ^1H NMR (DMSO-d_6) δ 2.90, 3.16 (AB quartet, J=12 Hz, 2 H, H-6′), 3.3~4.2 (m), 5.10 (dd, J=4 and 10 Hz, 1 H), 5.38 (dd, J=4 and 10 Hz, 1 H), 5.52 (3 H), 5.78 (t, J=10 Hz, 1 H), 5.88 (t, J=10 Hz, 1 H), 7.0~8.0 (25 H); IR (KBr) 3420, 3330 (OH), 1720 cm^{-1} (ester C=O).

A solution of 4,6-*O*-benzylidene-2,2′,3,3′-tetra-*O*-benzoyl-D-trehalose (0.2 g) in dry pyridine (2 ml) was treated at 0°C with benzoyl chloride (1.0 molar equiv.) overnight. The solution was concentrated *in vacuo*. The residue was dissolved in methylene chloride and washed twice with 5% solution of sodium bicarbonate. The organic layer was dried over magnesium sulfate. Solvent was removed under reduced pressure and the residue was crystallized from ethyl acetate - *n*-hexane to give **7** as a colorless powder (0.14 g). mp 201~202°C, $[\alpha]_D^{25}$ +244° (*c* 1, CH_2Cl_2 - MeOH, 1: 1), FDMS *m/z* 951 (MH^+); ^1H NMR (DMSO-d_6) δ 3.5~4.2 (8 H), 5.2~6.0 (8 H), 7.0~8.0 (m, 30 H); IR (KBr) 3400 (OH), 1720 cm^{-1} (ester C=O).

1-*O*-(*N*-Methyl)acetimidyl-2,3,4,6-tetra-*O*-benzyl-β-D-glucopyranose (**8**)[8]

A dry benzene solution (5 ml) of 2,3,4,6-tetra-*O*-benzyl-α-D-glucopyranosyl chloride (1.2 g)[10] was stirred for 12 hours at room temperature in the presence of *N*-methylacetamide (0.16 g), silver oxide (1.5 g), diisopropylethylamine (0.28 g) and powdered 4-Å molecular sieves (0.2 g). After filtration, the filtrate was evaporated to dryness to give **8** as a colorless syrup (1.1 g, 88%): $[\alpha]_D^{20}$ +28.6° (*c* 1.5, $CHCl_3$).

Glucotriose Perbenzoate **5c**

A solution of protected trehalose **7** (0.1 g) in dry benzene (3 ml) was treated at room temperature with **8** (0.062 g) and *p*-toluenesulfonic acid (0.018 g) under rigorously anhydrous conditions. After stirring for 6 days, the solution was concentrated and diluted with ethyl acetate (20 ml). The ethyl acetate solution was washed with water, dried and evaporated to a syrup (0.06 g). This was chromatographed on silica gel in benzene - ethyl acetate mixture (2: 1) giving an amorphous powder of protected triose (0.035 g). The protected triose in ethanol (10 ml) was hydrogenated with 10% Pd-C (20 mg) for 20 hours at room temperature. After filtration, the filtrate was evaporated to dryness, the residue was treated with benzoyl bromide (1 ml) and pyridine (1 ml) overnight at room temperature. Purification by preparative TLC (silica gel, ethyl acetate - *n*-hexane, 1: 2, Rf 0.5) yielded **5c** as colorless crystals (21 mg), mp 121~122°C, $[\alpha]_D^{21}$ +217° (*c* 0.7, $CHCl_3$) which was identical in all respects with glucotriose perbenzoate derived from partially hydrolyzed product of trestatins.

Methylated Alditol Acetate Analysis of Trestatins

Trestatin A (3.5 mg) was methylated with methylsulfinyl carbanion and methyl iodide by HAKOMORI's method[11]. The permethylated trestatin was hydrolyzed with 2 N HCl - MeOH (2 ml) at 100°C for 4 hours. The solution was neutralized with Dowex 44, filtered and concentrated to dryness. The residue was hydrolyzed again with 0.5 N H_2SO_4 at 100°C for 4 hours and neutralized with Dowex 44 and filtered. The filtrate was treated with 5 mg of sodium borohydride at room temperature for 2 hours. The excess reagent was decomposed by Dowex 50 and the solution was evaporated to dryness. The residue was acetylated with acetic anhydride (0.5 ml) and pyridine (0.5 ml). The reaction mixture was evaporated to dryness and then dissolved in acetone (0.3 ml) to be analyzed by GC-MS. Similar procedures were applied to reference samples. GC-MS analysis was carried out on a Hitachi RMU-6M spectrometer using a column (ϕ 3 mm×100 cm) packed with 3% ECNSS-M on Chromosorb WAW-DMCS. Helium was used as a carrier gas (60 ml/minute). The column temperature was 170°C. Retention times and fragmentation patterns of the methylated alditol acetates derived from trestatin A were identical with 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl glucitol and 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methyl glucitol; retention times, 1.1 and 2.8 minutes, respectively.

Analysis of trestatins B and C under the same condition gave the same result.

Hydrolysis of Trestatin C and Isolation of **9**, **10**, **11**, **12**, **13**, **14** and **18**

Trestatin C (510 mg) was hydrolyzed with Dowex 50 as described above. After neutral fragments were removed by filtration, the resin was treated with 1% NH_4OH to give basic fragments (330 mg). These were chromatographed on a 1.4×34 cm column of Amberlite CG-50 (a mixed bed consisting of 1 part of ammonium form and 2 parts of H^+ form) in water to give a pale yellow powder **9** (46 mg), a colorless powder of **10** (120 mg), a colorless powder of **12** (6 mg), a mixture of **10**, **11**, **12** and **13** (85 mg), a colorless powder of **14** (44 mg) and a colorless powder of **18** (10 mg). The mixture of **10**, **11**, **12** and **13** was further chromatographed on a 0.9×105 cm column of Amberlite CG-50 (a mixed bed consisting of 1 part of ammonium form and 4 parts of H^+ form) in water, giving a colorless powder of **11** (5 mg) and a colorless powder of **13** (4.2 mg).

11: FD-MS *m/z* 646 (MH^+), 668 $(M+Na)^+$; $[\alpha]_D^{26}$ +154° (*c* 0.4, H_2O); ^1H NMR (D_2O) δ 1.33 (d, *J*=6.3 Hz, 3 H, CH–CH_3), 2.46 (m, 1 H, N–CH), 3.1~4.2 (O–CH, O–CH_2, N–CH), 4.64 (d, *J*=7.8 Hz, 0.6 H, O–CH–O), 5.21 (d, *J*=3.7 Hz, 0.4 H, O–CH–O), 5.30 (d, *J*=2.4 Hz, 1 H, O–CH–O), 5.38 (d, *J*=3.7 Hz, 1 H, O–CH–O), 5.89 (d, *J*=4.9 Hz, 1 H, C=CH); ^{13}C NMR, see Table 1.

12: $[\alpha]_D^{25}$ +148° (*c* 0.5, H_2O); ^1H NMR (D_2O) δ 1.33 (d, *J*=6.3 Hz, 3 H, CH–CH_3), 2.46 (m, 1 H, N–CH), 3.1~4.2 (O–CH, O–CH_2, N–CH), 4.64 (d, *J*=7.8 Hz, 0.6 H, O–CH–O), 5.22 (d, *J*=3.9 Hz, 0.4 H, O–CH–O), 5.32 (d, *J*=2.4 Hz, 1 H, O–CH–O), 5.37 (d, *J*=3.4 Hz, 1 H, O–CH–O), 5.97 (d, *J*=4.5 Hz, 1 H, C=CH). ^{13}C NMR, see Table 1.

13: $[\alpha]_D^{24}$ +103.3° (*c* 0.3, H_2O); ^1H NMR (D_2O) δ 1.33 (d, *J*=6.1 Hz, 6 H, CH–CH_3), 2.56 (m, 1 H, N–CH), 2.99 (t, *J*=7.3 Hz, 1 H, N–CH), 3.4~4.5 (O–CH, O–CH_2, N–CH), 4.79 (d, *J*=3.7 Hz, 1 H, N–CH–O), 5.20 (d, *J*=3.4 Hz, 1 H, O–CH–O), 5.28 (d, *J*=3.0 Hz, 1 H, O–CH–O), 5.88 (d, *J*=3.4 Hz, 2 H, C=CH); ^{13}C NMR see Table 1.

14: $[\alpha]_D^{24}$ +153.5° (*c* 1, H_2O); FAB-MS *m/z* 949 (MH^+ for **14**, $C_{38}H_{64}N_2O_{25}$); ^1H NMR (D_2O) δ 1.33 (d, *J*=6.1 Hz, 6 H, CH–CH_3), 2.46 (m, 2 H, N–CH), 3.1~4.2 (O–CH, O–CH_2, N–CH), 4.63 (d, *J*=7.8 Hz, 0.6 H, O–CH–O), 5.21 (d, *J*=3.9 Hz, 0.4 H, O–CH–O), 5.3~5.4 (3 H, O–CH–O), 5.88 (d, *J*=4 Hz, 1 H, C=CH), 5.95 (d, *J*=4 Hz, 1 H, C=CH). ^{13}C NMR, see Table 1.

18: $[\alpha]_D^{24}$ +157° (*c* 0.3, H_2O); FAB-MS *m/z* 1,414 (MH^+ for **18**, $C_{57}H_{95}N_3O_{37}$); ^1H NMR (D_2O) δ 1.33 (d, *J*=6.1 Hz, 9 H, CH–CH_3), 2.46 (m, 3H, N–CH), 3.1~4.2 (O–CH, O–CH_2, N–CH), 4.64 (d, *J*=7.8 Hz, 0.6 H, O–CH–O), 5.22 (d, *J*=4 Hz, 0.4 H, O–CH–O), 5.3~5.4 (5 H, O–CH–O), 5.85~6.0 (3 H, C=CH). ^{13}C NMR, see Table 1.

Hydrolysis of Trestatin B and Isolation of **9**, **10** and **11**

Trestatin B (250 mg) was hydrolyzed under the same condition as mentioned above to give basic fragments (122 mg). These were chromatographed on a column of silica gel with ethyl acetate - methanol - water mixture followed by chromatography on a 0.9×79 cm column of Amberlite CG-50 (a mixed bed consisting of 1 part of ammonium form and 4 parts of H^+ form) in water giving **9** (29 mg), **10** (61 mg) and **11** (13 mg).

Hydrolysis of Trestatin A and Isolation of **9**, **10**, **11**, **12**, **13**, **14** and **15**

Trestatin A (500 mg) was hydrolyzed under the same condition as mentioned above to give **9** (38 mg), **10** (140 mg), **11** (13 mg), **12** (10 mg), **13** (10 mg), **14** (62 mg) and **15** (15 mg).

15: $[\alpha]_D^{26}$ +150° (*c* 0.5, H_2O); FAB-MS *m/z* 1,111 (MH^+ for **15**, $C_{44}H_{74}N_2O_{30}$); ^1H NMR (D_2O) δ 1.33 (d, *J*=6.4 Hz, 6 H, CH–CH_3), 2.46 (m, 2 H, N–CH), 3.1~4.2 (O–CH, O–CH_2, N–CH), 4.64 (d, *J*=8 Hz, 0.6 H, O–CH–O), 5.21 (d, *J*=3.9 Hz, 0.4 H, O–CH–O), 5.3~5.4 (4 H, O–CH–O), 5.88 (d, *J*=4 Hz, 1 H, C=CH), 5.96 (d, *J*=4 Hz, 1 H, C=CH). ^{13}C NMR, see Table 1.

Hydrogenolysis of **12**

A solution of **12** (1 mg) in 50% methanol (1 ml) was hydrogenated with 10% Pd-C (0.6 mg) at atmospheric pressure for 4 hours at room temperature. After removal of Pd-C, the solution was evaporated to dryness. The resulting residue was silylated by hexamethyldisilazane (0.1 ml) and trimethylchlorosilane (0.1 ml) in pyridine (0.2 ml), and then subjected to GC under the following conditions: column, 5% silicone SE-30 on chromosorb WAW-DMCS 3 mm×200 cm; column temperature 195°C; carrier gas N_2; flow rate 40 ml/minute; retention times 12.2 and 17.4 minutes. Identified as glucose by comparison with an authentic sample.

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