Communications to the Editor

NEW α-AMYLASE INHIBITOR, TRESTATINS III. STRUCTURE DETERMINATION OF NEW TRESTATIN COMPONENTS Ro 09-0766, Ro 09-0767 AND Ro 09-0768

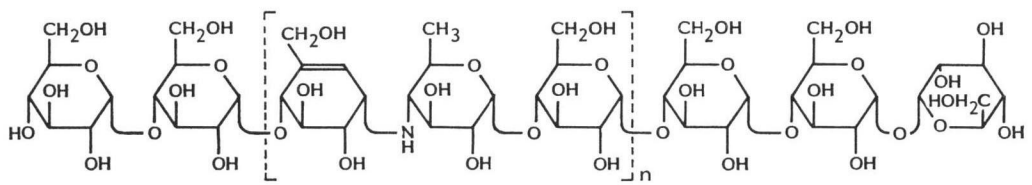
Sir:

In our previous papers[1,2], we reported the isolation, biological activities and structure elucidation of trestatins A, B and C produced by *Streptomyces dimorphogenes* NR320-OM7HB. Trestatins A, B and C were the main components of trestatin complex (Ro 09-0154), which exhibited a potent inhibitory activity on various α-amylases. Their structures were determined as **4**, **5**, and **6** on the basis of the structural analysis of mild acid hydrolyzed products. Further isolation studies on trestatin complex resulted in the purification of 3 new minor components, all possessing potent α-amylase inhibitory activities. This communication describes the isolation, characterization and structure determination of these new trestatins, Ro 09-0766, Ro 09-0767 and Ro 09-0768.

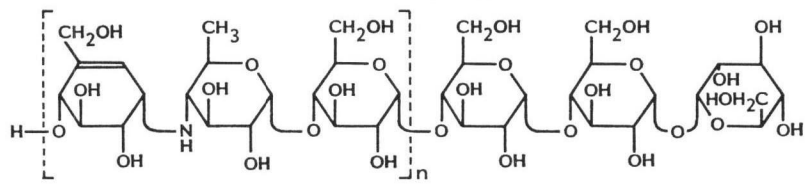
These minor components were isolated by the procedures outlined in Fig. 2. The trestatin complex (30 g, 3.5×10^7 IU/g)[1] was dissolved in 90 ml of water, applied onto a column of Amberlite CG-50 (3.1 liters, a mixed bed consisting of one part of NH_4^+ form and two parts of H^+ form, type I) and eluted with distilled water. The fractions were monitored by the α-amylase inhibitory activity[3] and HPLC[1]. Active fractions were pooled, concentrated under reduced pressure and lyophilized. Ro 09-0768 was first eluted followed by trestatin B, Ro 09-0767, trestatin A, Ro 09-0766 and trestatin C in this order. The fractions containing Ro 09-0766, Ro 09-0767 and Ro 09-0768 were further purified by gel filtration on Sephadex G-25. Each fraction thus obtained was pooled, concentrated under reduced pressure and lyophilized. Typical yields of purified products from 30 g of trestatin complex were: 315 mg for Ro 09-0766, 710 mg for Ro 09-0767 and 188 mg for Ro 09-0768.

Molar concentrations of Ro 09-0766, Ro 09-0767 and Ro 09-0768 required for a 50% inhibition of porcine pancreas α-amylase[1] were 1×10^{-8} M, 1×10^{-8} M and 1.7×10^{-8} M, respectively.

Fig. 1.



Ro 09-0766 **1** (n = 3)
Ro 09-0767 **2** (n = 2)
Ro 09-0768 **3** (n = 1)



Trestatin A **4** (n = 2)
Trestatin B **5** (n = 1)
Trestatin C **6** (n = 3)

Fig. 2. Isolation procedure for trestatins.

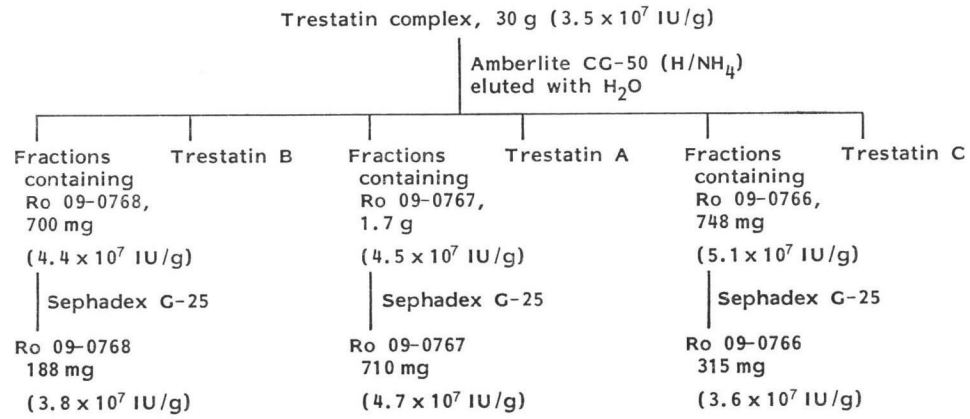


Table 1. Physico-chemical properties.

	Ro 09-0766	Ro 09-0767	Ro 09-0768
Appearance	Colorless powder	Colorless powder	Colorless powder
Mp (dec)	232~239°C	223~233°C	213~220°C
UV spectrum	End absorption	End absorption	End absorption
$[\alpha]_D^{24}$ (*c* 1.0, H_2O)	+168°	+172°	+184°
FAB-MS *m/z*	2,224 (MH^+)	1,759 (MH^+)	1,294 (MH^+)
Molecular formula	$C_{87}H_{145}N_3O_{62}$	$C_{83}H_{114}N_2O_{50}$	$C_{49}H_{83}NO_{38}$
Elemental analysis Calcd for	$C_{87}H_{145}N_3O_{62}\cdot 12H_2O$	$C_{83}H_{114}N_2O_{50}\cdot 8H_2O$	$C_{49}H_{83}NO_{38}\cdot 6H_2O$
C	42.80	42.90	41.97
H	6.98	6.88	6.83
N	1.72	1.47	1.00
Found C	42.86	42.86	41.52
H	7.50	7.25	7.11
N	1.81	1.55	1.10
Color reactions			
Phenol - sulfuric acid	+	+	+
Anthrone	+	+	+
Red-tetrazolium	−	−	−
TLC (Rf value)[a]	0.1	0.13	0.16
HVPE (Rm value)[b]	0.63	0.58	0.48
HPLC (retention time)[c]	9.3	6.3	4.3

[a] Silica gel F_{254} (Merck): $CHCl_3$ - MeOH - 25% NH_4OH - H_2O (1: 4: 2: 1), H_2SO_4.
[b] Toyo Roshi No. 51: HCOOH - AcOH - H_2O (25: 75: 900, pH 1.8), 3,000 V/40 minutes/12°C, Rm (relative mobility to alanine).
[c] μBondapak (CH): CH_3CN - H_2O (62: 38) 4.0 ml/minute, UV absorption at 210 nm.

Physico-chemical properties of Ro 09-0766, Ro 09-0767 and Ro 09-0768 are summarized in Table 1. The IR, ^1H NMR and ^{13}C NMR spectra of these compounds very closely resembled each other and also resembled those of trestatins A, B and C. Their IR spectra showed strong absorption maxima at 3100~3600 and 980~1180 cm^{-1}. The ^1H NMR spectra showed methyl signals at δ 1.33, CH–NH at δ 2.47, CH–OH at around δ 3.4~4.2, anomeric protons at around δ 5.17~

Table 2. ^{1}H NMR data for Ro 09-0766, Ro 09-0767 and Ro 09-0768.

	Ro 09-0766	Ro 09-0767	Ro 09-0768
$-CH_3$	1.33 (d, J=5.9 Hz, 9H)	1.32 (d, J=6.1 Hz, 6H)	1.34 (d, J=6.1 Hz, 3H)
$-\overset{|}{C}H-N$	2.47 (m, 3H)	2.47 (m, 2H)	2.49 (m, 1H)
$-\overset{|}{C}H-OH$	3.4~4.2	3.4~4.2	3.4~4.2
$O-\overset{|}{C}H-O$	5.18 (d, J=3.4 Hz, 2H) 5.2~5.4 (9H)	5.17 (d, J=3.4 Hz, 2H) 5.2~5.4 (7H)	5.19 (d, J=3.7 Hz, 2H) 5.2~5.4 (5H)
C=CH	5.98 (d, J=3.7 Hz, 3H)	5.97 (d, J=3.7 Hz, 2H)	5.99 (d, J=3.7 Hz, 1H)

Spectra were recorded at 100 MHz in D_2O.
Chemical shifts and coupling constants are given in ppm (δ value from external TMS) and Hz, respectively.

Fig. 3. Structure of pseudodisaccharide.

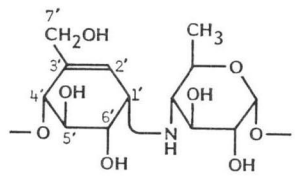


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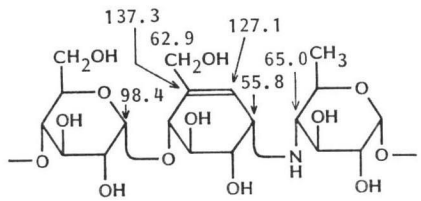
Fig. 4. Carbon-13 chemical shifts (in ppm) of partial structure **8** in D_2O.



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5.4 and olefinic protons at δ 5.98 in common (Table 2). These spectroscopic and other physico-chemical data indicated that Ro 09-0766, Ro 09-0767 and Ro 09-0768 were basic oligosaccharide homologues comprising the same constituents; glucose and the pseudodisaccharide, dehydro-oligobiosamine **7** (Fig. 3), as those of trestatins A, B and C[1,4]. The molar ratio of glucose and pseudodisaccharide **7** were determined as shown in Table 3 by the comparison of the intensity of anomeric protons *versus* olefinic protons in the ^{1}H NMR spectra (Table 2)[1].

These molar ratios were supported by fast atom bombardment mass spectra of Ro 09-0766, Ro 09-0767 and Ro 09-0768 which exhibited molecular ion peak at m/z 2,224 (MH^+ for $C_{87}H_{145}N_3O_{62}$), 1,759 (MH^+ for $C_{65}H_{114}N_2O_{50}$) and 1,294 (MH^+ for $C_{49}H_{88}NO_{38}$), respectively (Fig. 5). The ^{13}C NMR spectra (Table 4) revealed the presence of trehalose moieties at δ 94.0 and 94.2 and glucosyl pseudodisaccharide moieties **8** (Fig. 4) at around δ 55.8, 62.9, 65.0, 98.4, 127.1 and 137.3. Signals at δ 56.8, 62.4, 65.8, 124.4 and 139.8 which were characteristic of a terminal pseudodisaccharide moiety were not recognized in common[2]. Upon hydrogenolysis (H_2/Pd-C), Ro 09-0766, Ro 09-0767 and Ro 09-0768 each gave maltose in common. These results suggested Ro 09-0766, Ro 09-0767 and Ro 09-0768 to be maltosyl trestatin C(**1**), maltosyl trestatin A(**2**) and maltosyl trestatin B(**3**), respectively (Fig. 1). These structures were confirmed by enzymatic degradation; Ro 09-0766, Ro 09-0767 and Ro 09-0768 were treated with β-amylase from barley (Sigma) in acetate buffer pH 4.9 at 27°C for 20 hours. Reaction mixtures were subjected to gel chromatography on Sephadex G-10 which gave maltose and trestatin C; maltose and trestatin A; and maltose and trestatin B, respectively. Maltose and trestatins A, B and C were identified by direct comparison (TLC, HPLC, $[\alpha]_D$, ^{1}H NMR and ^{13}C NMR). Thus, the structures of trestatin minor components; Ro 09-0766, Ro 09-0767 and Ro 09-0768 were determined to be as shown in Fig. 1.

Table 3. Molar ratio of glucose and pseudodisaccharide **7**.

	Ro 09-0766	Ro 09-0767	Ro 09-0768
Glucose	8	7	6
7	3	2	1

Fig. 5. Fast atom bombardment mass spectra of Ro 09-0767 and Ro 09-0768.

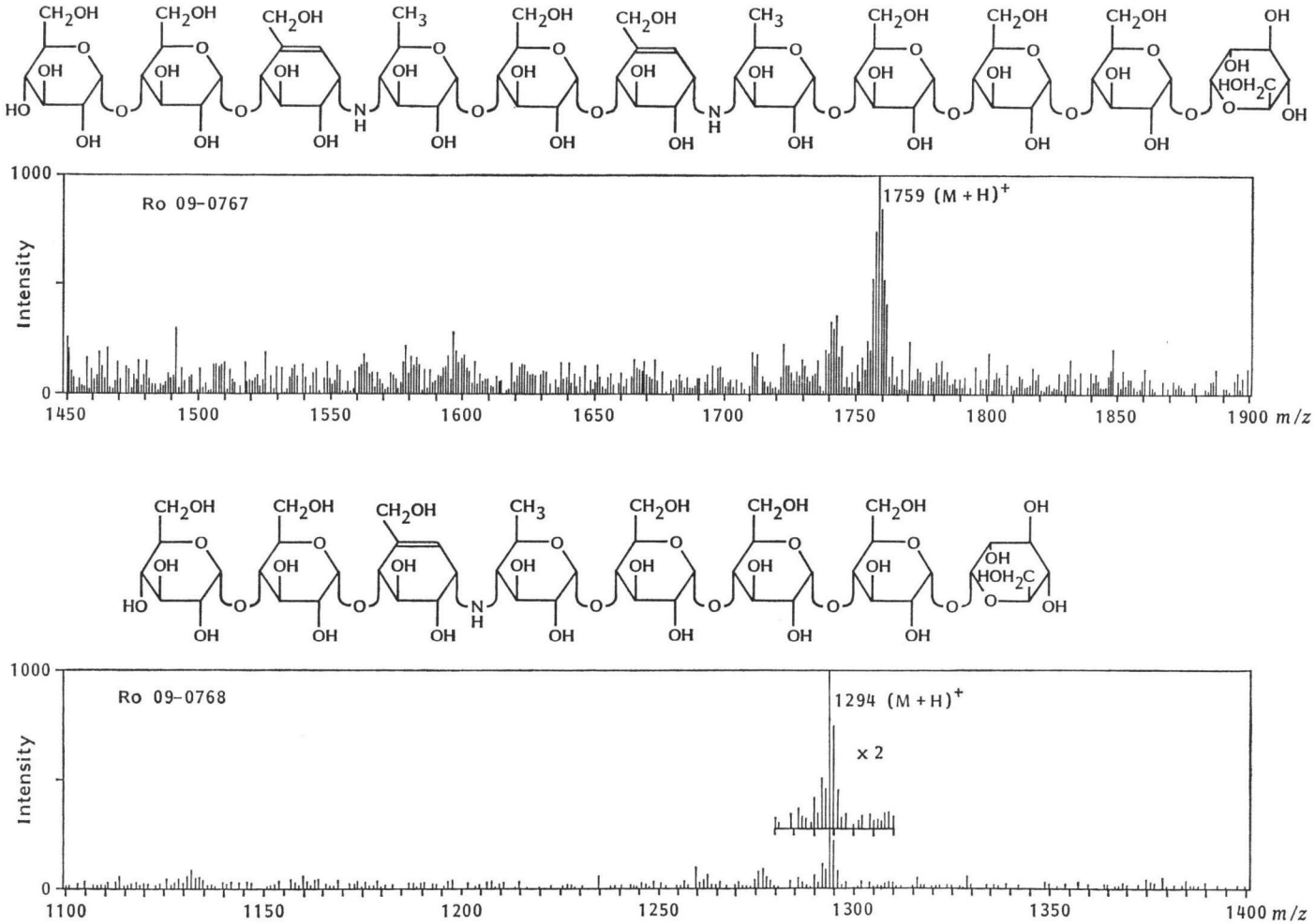

Table 4. Carbon-13 chemical shifts (in ppm) of trestatins A, B, C and Ro 09-0766, Ro 09-0767 and Ro 09-0768 in D_2O with dioxane as an internal standard (67.4 ppm) at 25.05 MHz.

		Trestatin A	Trestatin B	Trestatin C	Ro 09-0766	Ro 09-0767	Ro 09-0768
C=CH	Terminal unit	139.8	139.9	139.9			
	Inner unit	137.4		137.4*	137.3*	137.4*	137.2
C=CH	Inner unit	126.9		127.0*	127.1*	127.0*	127.1
	Terminal unit	124.4	124.5	124.4			
C-1	Pseudodisaccharide moiety **7**	100.9*	100.8	100.7*	100.7*	100.7*	100.7
	α-1,4 (Glucose)	100.5	100.5	100.5*	100.5*	100.5*	100.5*
		100.4	100.4				
	**	98.5		98.3*	98.4*	98.4*	98.4
	α,α-1,1 terminal (Glucose) inner	94.2	94.2	94.2	94.2	94.2	94.2
		94.0	94.0	94.0	94.0	94.0	94.0
H–C–O		78.0 ≀ 70.4	78.0 ≀ 70.4	77.9 ≀ 70.3	78.0 ≀ 70.4	77.9 ≀ 70.4	78.0 ≀ 70.2
-N-C	Terminal unit	65.8	65.7	65.7			
	Inner unit	65.0		65.0*	65.0*	65.1*	65.0
CH_2OH	Inner unit	62.8		62.9*	62.9*	62.9*	62.8
	Terminal unit	62.4	62.5	62.4			
C-6 (Glucose)		61.4*	61.4*	61.4*	61.4*	61.4*	61.4*
C-N-	Terminal unit	56.8	56.8	56.8			
	Inner unit	55.9		55.9*	55.8*	55.9*	55.8
CH_3–		18.2*	18.2	18.2*	18.2*	18.3*	18.2

* Doubly or more intense signal.

** C-1 Resonance of glucose linked to allylic position (C-4′) of **7** through α-linkage.

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