NEW α-AMYLASE INHIBITOR, TRESTATINS

I. ISOLATION, CHARACTERIZATION AND BIOLOGICAL ACTIVITIES OF TRESTATINS A, B AND C

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(Received for publication March 11, 1983)

Trestatin complex which exhibited a potent inhibitory activity on various α-amylases has been isolated from the culture filtrate of *Streptomyces dimorphogenes* nov. sp. NR-320-OM7HB. Three major components, trestatins A, B and C, have been purified by adsorption and ion-exchange chromatography. Their spectral and chemical properties suggested that trestatins were novel basic oligosaccharide homologues each characterized by the possession of a trehalose moiety at the non-reducing end of the molecule.

It has been suggested that useful therapy of diabetes and obesity might be achieved by reducing the digestion of dietary starch using inhibitors of α-amylase. This could be expected to decrease hyperglycemia and hyperinsulinemia. From these viewpoints, a number of α-amylase inhibitors have been isolated from the microbial cultures.[1~14] In the course of our screening program searching for α-amylase inhibitors, we discovered a novel complex, designated trestatin* in the culture filtrate of a new Streptomyces species to which we have given the name *Streptomyces dimorphogenes* NR-320-OM7HB. The trestatin complex contained trestatins A, B and C as the major components. They have shown a remarkable inhibitory activity against various α-amylases.

The present paper describes the production by fermentation, isolation, physico-chemical characteristics and biological properties of the major components of trestatin. Their structures are reported in the subsequent paper. Mycological characteristics of the producer strain will be described elsewhere.

Results and Discussions

Fermentation

The seed culture was prepared in a medium containing 2.0% potato starch, 2.0% glucose, 2.0% soya meal, 0.5% yeast extract, 0.25% NaCl, 0.005% $ZnSO_4 \cdot 7H_2O$, 0.0005% $CuSO_4 \cdot 5H_2O$, 0.0005% $MnCl_2 \cdot 4H_2O$, 0.32% $CaCO_3$ (pH 7.0) by shaking for 48 hours at 27°C, and transferred into a 50-liter fermentor containing 25 liters of the same medium as the seed culture. Fermentation was carried out at 27°C for 43 hours with an air flow rate of 25 liters per minute under an agitation at 300 rpm. The inhibitory activity of trestatin on porcine pancreatic α-amylase was determined by the method as described in Experimental. A typical trestatin fermentation broth (pH 6.9) had an activity of 3.1×10^4 inhibitory unit (IU)/ml. The detailed process for the fermentation will be described elsewhere.

* Parts of the present work were orally presented at 218th Scientific Meeting of Japan Antibiotics Research Association, Tokyo, May 23, 1980.

Isolation of Trestatin Complex and the Major Components A, B and C

Whole harvested broth cultured in a 50-liter fermentor was adjusted to pH 7.0 with 5 N NaOH and then filtered. To the filtrate (19 liters, 3.1×10^4 IU/ml, 5.9×10^8 IU/total) was added 380 g of activated carbon. The mixture was stirred at room temperature for 20 minutes and then filtered. The carbon cake was washed with water and suspended in 10 liters of 50% aqueous acetone. The suspension was adjusted to pH 2 with 6 N HCl. After 20 minutes of stirring at 60°C, the mixture was filtered, and adjusted to pH 7.0 with 6 N NaOH and concentrated under reduced pressure to a volume of about 3 liters. The concentrate (*ca.* 3 liters, 5.6×10^8 IU/total) was passed through a column (3.5 liters) of Dowex 50 (H^+ form). The column was washed with water and eluted with 1 N NH_4OH. The eluate fractions were monitored for the α-amylase inhibitory activity. The active fractions were combined, concentrated under reduced pressure and lyophilized to give 75.4 g of brown powder (7.0×10^6 IU/g, 5.3×10^8 IU/total). The lyophilized powder was suspended in 3,770 ml of methanol and stirred at room temperature for 2 hours. The insoluble part was collected by filtration and dried to give 41.5 g of brown powder (1.1×10^7 IU/g, 4.6×10^8 IU/total). The brown powder was dissolved in 100 ml of water and applied onto a column (2 liters) of Dowex 1 (acetate form, 200~400 mesh). The column was developed with water. The active eluate was combined, concentrated under reduced pressure and lyophilized to give 22.6 g of a yellow powder (1.8×10^7 IU/g, 4.1×10^8 IU/total). The yellow powder was dissolved in 50 ml of water and applied onto a column (2.9 liters) of Dowex 50 (ammonium form, 200~400 mesh), which was developed with water. The active eluate was combined, concentrated under reduced pressure and lyophilized to give 10.6 g of trestatin complex as a pale yellow powder (3.6×10^7 IU/g, 3.8×10^8 IU/total). A typical high performance liquid chromatogram of this purified trestatin complex (Ro 09-0154) is shown in Fig. 1.

The trestatin complex (10.6 g, 3.6×10^7 IU/g) thus obtained was dissolved in 20 ml of distilled water and applied onto a column (1.4 liters) of Amberlite CG-50 (a mixed bed consisting of 3.5 parts of

Fig. 1. Typical high performance liquid chromatogram of trestatin complex.

Taken with a Waters chromatograph to analyse trestatin complex and assess purity of trestatins A, B and C under the following conditions. Column: μBondapak/carbohydrate (3.9×300 mm, Waters Associate). Carrier: CH_3CN - H_2O (63: 37). Flow rate: 4.0 ml/minute. Injection volume: 2~10 μl. Detection: UV absorption at 210 nm.

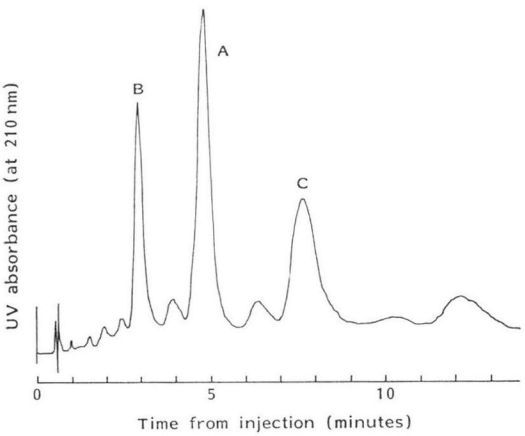


Fig. 2. Isolation procedure for trestatins.

Broth filtrate: 19 liters, 3.1×10^4 IU/ml

Yield 95% | adsorbed on charcoal, eluted with 50% aq. acetone at pH 2

Dowex 50 (H^+)

95% | eluted with 1 N NH_4OH

Treated with MeOH

87% |

Insoluble part (1.1×10^7 IU/g)

Passed through Dowex 1 (acetate)

89% |

Passed through Dowex 50 (NH_4^+)

93% |

Trestatin complex 10.6 g (3.6×10^7 IU/g)

Amberlite CG-50 (H^+/NH_4^+) eluted with H_2O

Trestatin B	Trestatin A	Trestatin C
1.4 g	2.2 g	1.0 g
1.4×10^8 IU/g	7.1×10^7 IU/g	4.9×10^7 IU/g

Table 1. Physico-chemical properties of trestatins.

		Trestatin A (Ro 09-0183)	Trestatin B (Ro 09-0184)	Trestatin C (Ro 09-0185)
Appearance		Colorless powder	Colorless powder	Colorless powder
Melting point (dec.)		221~232°C	209~219°C	230~237°C
UV spectrum		End absorption	End absorption	End absorption
$[\alpha]_D^{24}$ (*c* 1.0, H_2O)		+177°	+187°	+169.5°
pKá (in H_2O)		5.0	5.0	5.0
Titration equivalent		720	960	650
Molecular weight (osmometry)		1,470	975	1,890
Elementary analysis (%)	C	46.72	46.27	47.55
	H	7.13	7.04	7.15
	N	2.08	1.65	2.29
	O	43.28	44.69	42.66
Molecular formula		$C_{56}H_{94}N_2O_{40}$	$C_{37}H_{63}NO_{28}$	$C_{75}H_{125}N_3O_{52}$
Color reactions				
Phenol-sulfuric acid		+	+	+
Anthrone		+	+	+
Red-tetrazolium		−	−	−
SAKAGUCHI		−	−	−

Table 2. Chromatographic behaviors of trestatins.

	A	B	C
HPLC (retention time, minutes)[1]	4.8	2.9	7.7
TLC (Rf value)[2]	0.19	0.28	0.14
HVPE (Rm value)[3]	0.66	0.49	0.71

[1] μBondapak (CH), CH_3CN - H_2O (63:37), 4.0 ml/minute, UV absorption at 210 nm.
[2] Silica gel F_{254} (Merck), $CHCl_3$ - MeOH - 25% NH_4OH - H_2O (1:4:2:1), H_2SO_4
[3] Toyo Roshi No. 51, HCOOH - AcOH - H_2O (25:75:900, pH 1.8), 3,000 V, 40 minutes, 17°C, Rm (relative mobility to alanine).

ammonium form and 6.5 parts of H^+ form, type I). The water eluate fractions were monitored by the α-amylase inhibition assay and HPLC. Trestatins B, A and C were separately eluted in this order. The fractions containing each component were combined, concentrated under reduced pressure and lyophilized. Fig. 2 shows the over-all process and the amount of isolated for trestatins A, B and C, respectively. Each trestatin could be further purified by rechromatography on Amberlite CG-50 (a mixed bed of ammonium form and H^+ form, type II), or gel filtration on Sephadex G-25, to give a colorless amorphous powder.

Physico-chemical Properties of Trestatins A, B and C

The physico-chemical properties of trestatins are summarized in Table 1. They were soluble in water and DMSO but insoluble in other common organic solvents. Chromatographic behavior in TLC, HPLC and high voltage paper electrophoresis is shown in Table 2. As examples, IR, ^1H NMR and ^{13}C NMR spectra of trestatin A are shown in Figs. 3, 4 and 5, respectively. The IR spectrum showed strong absorption maxima at 3100~3600 and 980~1180 cm^{-1}, attributable to its oligosaccharide nature. The ^1H NMR spectrum showed a methyl signal at 1.33 ppm (d, J=6.3 Hz), a methine proton at 2.47 ppm (m), –CHOH signals at around 3.4~4.2 ppm, anomeric protons at around 5.1~5.4 ppm and olefinic protons at 5.9~6.0 ppm.

Other trestatins B and C exhibited very close or almost the same spectra as those of trestatin A.

From spectroscopic and other physico-chemical properties, it was suggested that these components were basic oligosaccharide homologues possessing the same constituents in common. Upon hydrolysis

Fig. 3. IR spectrum of trestatin A (KBr).

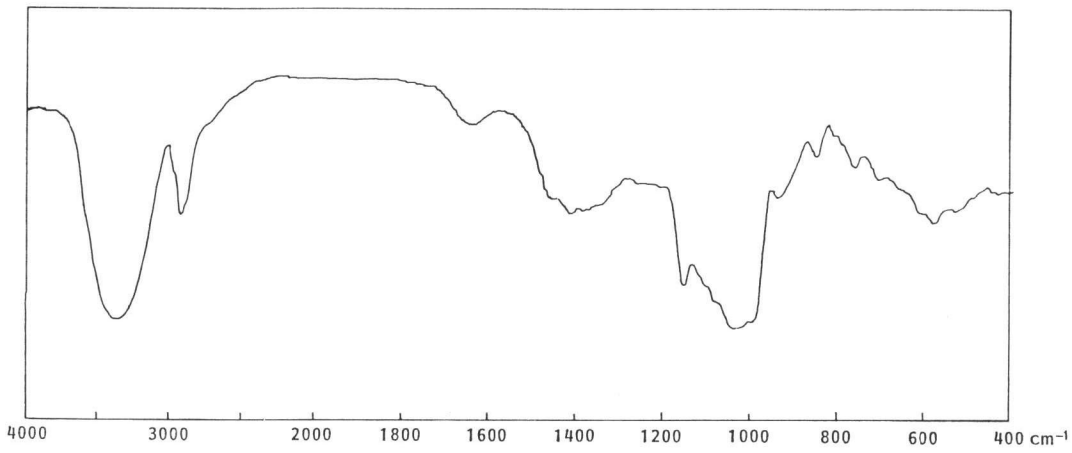
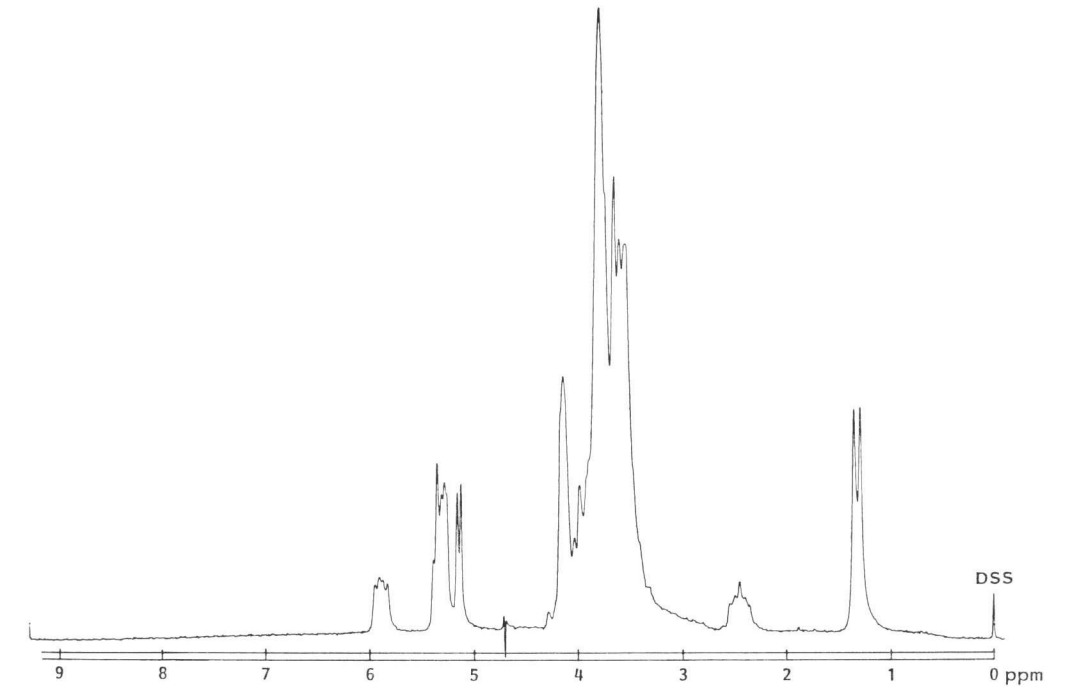


Fig. 4. ^{1}H NMR spectrum (100 MHz) of trestatin A (D_2O, WEFT).



(4 N HCl, 80°C, 3 hours), trestatins A, B or C gave D-glucose and a basic product **1** which had *pKá* of 3.9 and molecular formula of $C_{13}H_{21}NO_7$ determined by high resolution mass spectroscopy, ^{13}C NMR spectroscopy and titration. All analytical and spectroscopic data of **1** were identical with the published data for a tricyclic compound reported by FROMMER *et al.* except ^{13}C NMR data.[15,16] This structure was confirmed by the detailed studies of ^{1}H NMR spectrum of its hexaacetate (Fig. 7) and mass spectrum of **1** which revealed a fragmentation pattern consistent with the tricyclic structure (Fig. 8). Finally, **1** was identified by a direct comparison.

On the other hand, mild acid hydrolysis of trestatins A, B and C using Dowex 50 (H^+ form) as the

Fig. 5. ^{13}C NMR spectrum (25 MHz) of trestatin A (D_2O).

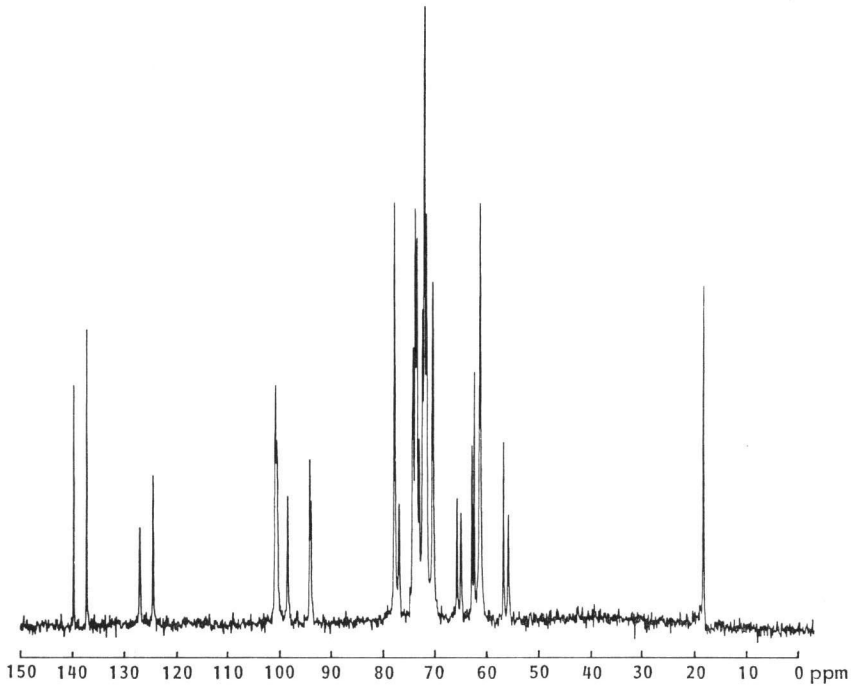
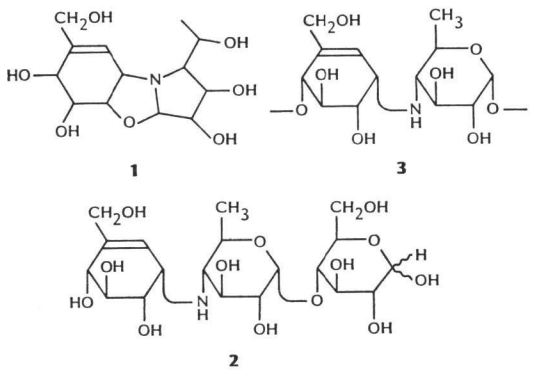


Fig. 6. Structures of tricyclic compound **1**, pseudotrisaccharide **2** and pseudodisaccharide **3**.



catalyst at 80°C for 5 hours gave another basic product, which was identified as **2** by a direct comparison. Since it is known that the tricyclic compound **1** is derived from the pseudodisaccharide, dehydro-oligobiosamine moiety[1,8] of **2** during hydrolysis[3,8], it was obvious that trestatins were composed of D-glucose and the pseudodisaccharide, dehydro-oligobiosamine **3**. These observations indicated that trestatins were closely related to BAYe4609[3]. But, mild acid hydrolysis of trestatins using Dowex 50 (H^+ form) also gave α,α-D-trehalose in each case. The isolation of trehalose revealed that trestatins were non-reducing oligosaccharides, which is consistent with negative color reaction of red tetrazolium.

This fact differentiates trestatins from other known oligosaccharide α-amylase inhibitors, such as BAYe4609,[3] S-Al,[5] TAI-I, -II[7] and oligostatin[8], since all of them lacked trehalose and possessed reducing properties. Furthermore, all known α-amylase inhibitors characterized as oligosaccharides were monoacidic bases, whereas trestatins A and C were diacidic and triacidic bases (Table 1), respectively. These properties indicated that trestatins were new basic oligosaccharide homologues having inhibitory activity on α-amylase.

As described above, the trestatins consisted of D-glucose and the pseudodisaccharide **3**. Titration and molecular weight data (Table 1) indicated that trestatins A, B and C contained 2, 1 and 3 mol of **3**,

Fig. 7. ^{1}H NMR spectrum of hexaacetate of tricyclic compound **1** in $CDCl_3$ containing 0.7 mol equivalent of $Eu(fod)_3$.

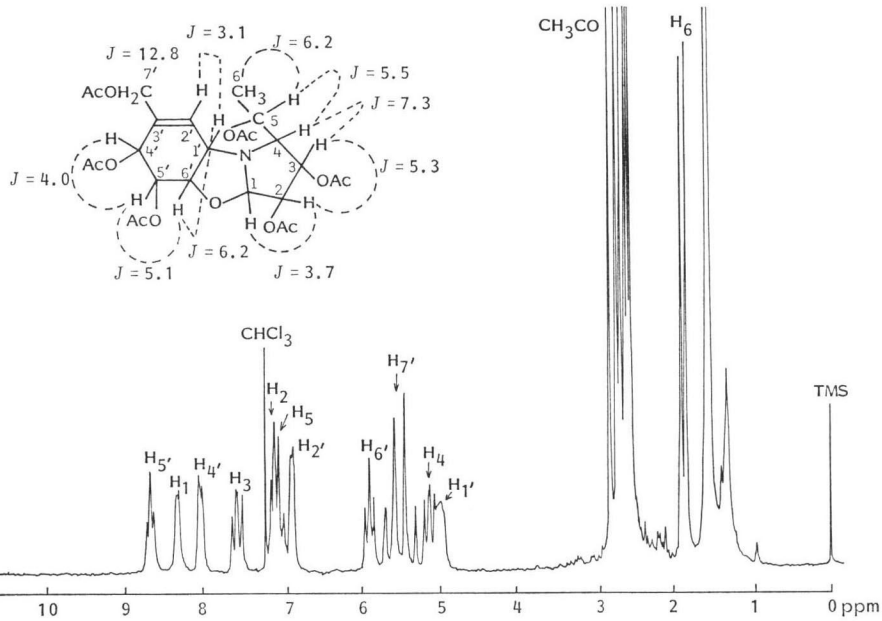


Fig. 8. Mass spectrum of tricyclic compound **1**.

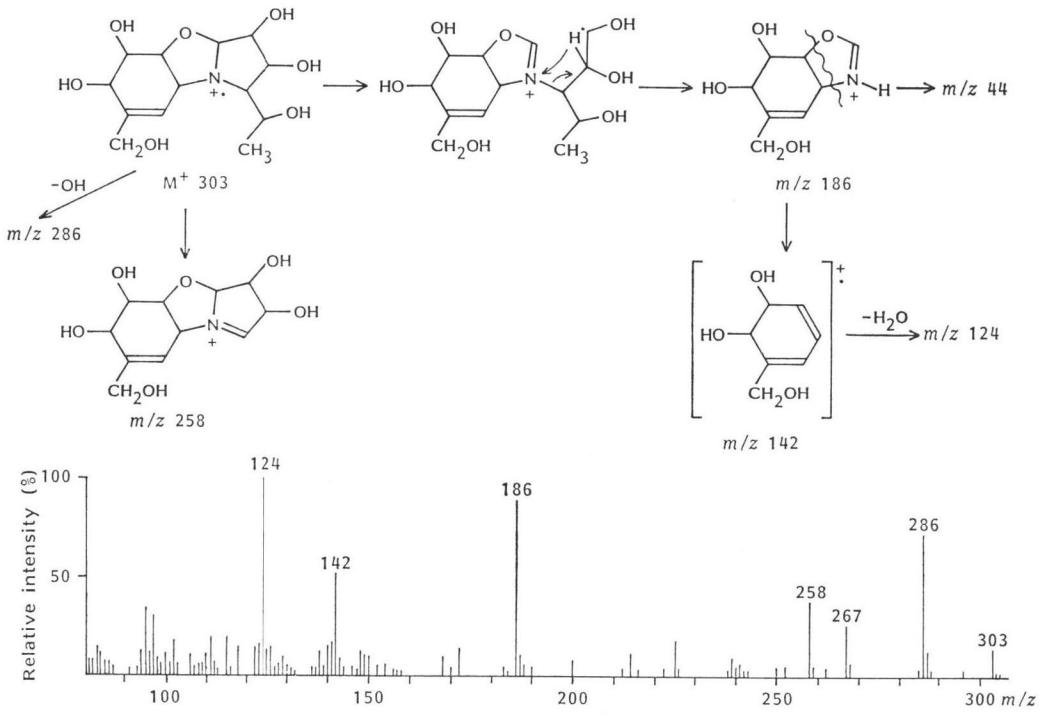


Table 3. Molar concentrations of trestatins A, B and C required for a 50% inhibition of various α-amylases.

	Porcine pancreas (M)	*A. oryzae* (M)	*B. subtilis* (M)
Trestatin A	8.1×10^{-9}	1.1×10^{-6}	2.1×10^{-5}
Trestatin B	6.1×10^{-7}	3.1×10^{-5}	5.4×10^{-5}
Trestatin C	8.8×10^{-9}	1.3×10^{-6}	1.8×10^{-6}

respectively. By subtracting the number of olefinic protons derived from **3** from that of anomeric protons in each trestatin (A, 7H; B, 5H; C, 9H) in the ^{1}H NMR spectrum, D-glucose content in trestatins A, B and C were determined to be 5, 4 and 6 mol, respectively. From these results, the molecular formulae of trestatins A, B and C were determined as shown in Table 1.

Biological Properties

Trestatins A, B and C showed a strong inhibition of pancreatic α-amylase as summarized in Table 3. Trestatins also inhibited α-amylase from *Bacillus subtilis* and *Aspergillus oryzae* and amylo-α-1,4-α-1, 6-glucosidase of *Aspergillus niger*. They were inactive against β-amylase of sweet potato, sucrase of yeast, maltase of yeast and β-glucosidase of sweet almond.

The oral administration of 500 mg/kg of each trestatin to mice did not show any toxic symptoms. None of the trestatins exhibited antimicrobial activity (MIC >100 μg/ml), when tested on 26 strains including *Escherichia coli,* Citrobacter, Klebsiella, Pseudomonas, Proteus, Serratia, Staphylococcus, Bacillus, Micrococcus, Candida, Mycobacterium and others.

Experimental

Strains

Streptomyces strain NR-320-OM7HB isolated from Australian soil in 1975 and named *S. dimorphogenes* sp. nov. was used as the producer throughout the experiment. Taxonomical characteristics will be described elsewhere and the strain was deposited to FRI (No. 3664).

Assay Methods for the α-Amylase Inhibition[17]

Amylase inhibitory activity was determined in the following manner: 0.1 ml of α-amylase from porcine pancreas (Boehringer) containing approximately 2 AU/ml was added to 0.6 ml phosphate buffer (20 mM pH 6.9, 10 mM NaCl) containing different amounts of inhibitor. After equilibration at 37°C for 5 minutes, the reaction was started by addition of 0.5 ml 4% starch solution (prepared from soluble starch, Merck 1252, by heating in boiling water and kept under continuous stirring at 37°C). After 5 minutes incubation at 37°C, 2 ml of the dinitrosalicylate reagent prepared according to BERNFELD[17] were added. The mixture was then heated for 5 minutes at 95°C (water bath) and subsequently, in an ice bath, diluted with 10 ml H_2O. The resulting color was measured at 540 nm in a Shimadzu double-beam spectrophotometer UV-150-02. Reagent blanks, enzyme controls without inhibitor and maltose standards were run simultaneously. 1 inhibitory unit (1 IU) is the amount of inhibitor which leads to 50% inhibition of 2.0 AU.

Calculations: % inhibition$=$(Ec$-$Ei/Ec)$\times$100

Ec=extinction of enzyme control without inhibitor

Ei=extinction of inhibited sample

(all extinctions corrected for blank values containing no enzyme)

1 IU$=$($I_{50}\times$2.0/AU)

I_{50}=amount of inhibitor which leads to 50% inhibition in the assay system

AU=amylase units in the assay

1 AU is the amount of enzyme which catalyzes under the above conditions the formation of reducing sugars equivalent to 1 μmol maltose per minute.

Analytical Procedure

Melting points were determined with a Yamato melting point apparatus MP-21 and were uncor-

rected. IR absorption spectrum with a Hitachi EPI-G3 spectrophotometer (KBr pellets) and mass spectra with a Hitachi RMU-6M spectrometer and Jeol JMS-DX300 mass spectrometer. ^{1}H NMR and ^{13}C 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 and ^{13}C chemical shifts are given in ppm with dioxane (67.4 ppm) as an internal standard. Optical rotations were measured on a Perkin-Elmer polarimeter. Titration was carried out with a Metrohm Herisan Potentiograph E536.

Hydrolysis of Trestatin and Isolation of **1** and **2**

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. Dowex 50 was collected by filtration and washed with water. Combined filtrate was concentrated under reduced pressure and lyophilized to give 205 mg of neutral fragments. Dowex 50 was treated with 1% NH_4OH (350 ml) to give basic fragments (330 mg) and these were chromatographed on a 1.4×34 cm column of Amberlite CG-50 (a mixed bed consisting of 3.3 parts of ammonium form and 6.7 parts of H^+ form) in water giving a pale yellow powder of **1** (46 mg), a colorless powder of **2** (120 mg) and the other basic fragments.

1: $C_{13}H_{21}NO_7$; MS *m/z* 303 (M^+); $[\alpha]_D^{27}$ +1° (*c* 1, H_2O); *pKá* (in H_2O) 3.9; ^{1}H NMR (D_2O) δ 1.33 (d, J=6.6 Hz, 3 H, CH–CH_3), 2.99 (dd, J=6.4 Hz, 7.1 Hz, 1 H, N–CH), 3.8 (broad, 1 H, N–CH), 3.9~4.5 (8 H, O–CH, O–CH_2), 4.92 (d, J=3.9 Hz, 1 H, O–CH–N), 5.77 (d, J=3 Hz, 1 H, C=CH); ^{13}C NMR (D_2O) δ 20.6, 62.8, 63.0, 68.3 (×2), 70.4, 71.2, 76.7, 77.0, 77.5, 95.2, 122.8, 139.3.

2: $[\alpha]_D^{25}$ +153.5° (*c* 1, H_2O); IR ν_{max}^{KBr} cm^{-1} 3350, 2920, 1300~1500, 1150, 980~1120, 930, 845, 760; ^{1}H NMR (D_2O) δ 1.33 (d, J=6.1 Hz, 3 H, CH–CH_3), 2.46 (m, 1 H, N–CH), 3.2~4.2 (O–CH, O–CH_2, N–CH), 4.63 (d, J=8 Hz, 0.6 H, O–CH–O), 5.21 (d, J=3.9 Hz, 0.4 H, O–CH–O), 5.31 (d, J=2.9 Hz, 1 H, O–CH–O), 5.89 (d, J=4.9 Hz, 1 H, C=CH); ^{13}C NMR (D_2O) δ 18.2, 56.8, 61.5, 61.6, 62.4, 65.7, 70.4, 70.9, 71.6, 72.0, 73.5, 73.6, 73.8, 74.1, 74.8, 75.4, 77.0, 78.1, 78.3, 92.7, 96.6, 100.6, 100.7, 124.6, 139.9.

Hydrolysis of Trestatins A and B and Isolation of **1** and **2**

Trestatins A (500 mg) and B (250 mg) were hydrolyzed under the same condition as mentioned above to give **1** (A 38 mg, B 29 mg) and **2** (A 140 mg, B 61 mg), respectively.

Acknowledgment

We are grateful to late Dr. P. BUCHSCHACHER of F. Hoffmann-La Roche & Co. Ltd., Switzerland, for providing us authentic samples of tricyclic compound and pseudotrisaccharide.

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