MANNOSTATINS A AND B[†]: NEW INHIBITORS OF α-D-MANNOSIDASE, PRODUCED BY *STREPTOVERTICILLIUM VERTICILLUS* VAR. *QUINTUM* ME3-AG3: TAXONOMY, PRODUCTION, ISOLATION, PHYSICO-CHEMICAL PROPERTIES AND BIOLOGICAL ACTIVITIES

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Mannostatins have been isolated as part of a program designed to find microorganism-produced inhibitors of α-D-mannosidase from *Streptoverticillium verticillus* var. *quintum*. They were purified by sequential use of active carbon and Dowex resins and then isolated as colorless powders. Mannostatins A and B have the molecular formula, $C_6H_{13}NO_3S$ and $C_6H_{13}NO_4S$, respectively. They were competitive with the substrate, and the inhibition constants (Ki) of mannostatins A and B were 4.8×10^{-8} M.

In recent years, it has become clear that glycoconjugates present on the surface of mammalian cells have an important functional role.[1,2] Various phenomena such as immunological effects, inflammation, cell fusion, complement fixation, transformation into cancer cells, metastasis of cancer cells and viral infection, are being recognized as involving glycoconjugates on the cell surfaces. Extensive studies are proceeding on this topic.[3~5]

We have previously reported the discovery, from microorganisms, of panosialin[6] and siastatin[7] which inhibit sialidase, and pyridindolol,[8] isoflavonoid[9,10] and *p*-hydroxyphenylacetaldoxime (HPAAO),[11] which inhibit β-galactosidase. Specific enzyme inhibitors such as these are known to be useful in elucidation of various physiological phenomena.

In the course of screening for an inhibitor of α-D-mannosidase, we discovered mannostatins A and B as specific inhibitors. They were isolated from the culture broth of *Streptoverticillium verticillus* var. *quintum*. In this communication we report the taxonomy, production, isolation, physico-chemical properties and biological activities.

Materials and Methods

Chemicals

Chemicals employed were as follows: Active carbon (chromatography) from Wako Pure Chemical Industries, Ltd., Osaka, Japan; Dowex 50W and Dowex 1 from Muromachi Kagaku Kogyo Kaisha, Ltd., Tokyo, Japan; *p*-Nitrophenyl α-D-mannopyranoside (NP-Man) from Seikagaku Kogyo Co., Ltd., Tokyo, Japan. All other chemicals were of analytical grade.

† Mannostatins A and B were presented as mannostatin and mannostatin S-oxide in Jpn. Kokai 76445 ('86), Apr. 18, 1986=Eur. Pat. Appl. 175,291, Mar. 26, 1986 [CA105: 23100h, 1986]. The strain was deposited in Fermentation Research Institute, Agency of Industrial Science and Technology, Japan (Kogyo Gijutsuin Hakko Kenkyusho) and the deposit No. is FERM P-7299.

Enzymes

α-D-Mannosidase (EC 3.2.1.24) was prepared from epididymides of adult rats as described by LEVVY *et al.*[12] Partialy purified enzyme was used in this assay.

Microorganism

Strain ME3-AG3 was isolated from a soil sample collected in Kiso-gun, Nagano Prefecture, Japan and has been deposited in the Fermentation Research Institute, Agency of Industrial Science and Technology, Ministry of International Trade and Industry, Tsukuba-shi, Japan, under the accesion No. FERM P-7299.

Taxonomic Characterization

Morphological and physiological properties of the strain were examined according to SHIRLING and GOTTLIEB;[13] several other tests were also used.

Production of Mannostatins A and B

The strain ME3-AG3 was inoculated into 110 ml of a production medium consisting of glycerol 2.0%, Polypeptone 1.5%, yeast extract 0.3%, and $CaCO_3$ 0.3% (pH 7.2) in a 500-ml Erlenmeyer flask, and cultured at 27°C for 3 days on a rotary shaker (180 rpm). One ml of the above seed culture was transferred to 110 ml of the same medium in a 500-ml Erlenmeyer flask and cultured for 4 days under the same conditions.

Isolation of Mannostatins A and B

The flow diagram for the isolation of mannostatins A and B is shown in Chart 1. The inhibitors were present in the culture filtrate which was separated from the mycelium by centrifugation. The supernatant fraction was percolated through a column packed with active carbon (1% volume) for decolorization. The activated carbon was washed with 0.2 M formic acid (2-fold volume), and the washings combined with the decolorized filtrate. The combined liquid was adsorbed on Dowex 50W-X2 (H^+), and eluted with 0.5 M HCl. The eluate was adjusted to pH 10 with 1 M NaOH, and then poured into a column packed with active carbon (1% volume). The adsorbed mannostatins A and B were eluted with 0.2 M formic acid. The active fractions were adsorbed on Dowex 50WX8 (H^+ type), which was washed with water and eluted with 0.3 M HCl. The active fractions to appear first (Fraction A) are composed of mannostatin B, and the active fractions coming next comprise mannostatin A (Fraction B).

The active Fractions A and B were adjusted to pH 5 with Dowex 1X2 (OH^-) and poured upon a column packed with active carbon. After washing with water, the column was eluted with 0.2 M formic acid. The active fractions were combined, adjusted to pH 7.0 with Dowex 1X2 (OH^- type), and filterd. The filtrate was layered onto a column of Dowex 1X8 (CO_3^{2-}) that has been equilibrated with 0.05 M carbonate buffer (pH 10), and then eluted with the same buffer. The active fractions were poured onto a column packed with active carbon, and after washing with water, the column was eluted with 0.2 M HCl. The active fractions were combined, adjusted to pH 5.0 with Dowex 1X2 (OH^- type) and filtered. The filtrate was evaporated to dryness to obtain a colorless syrup.

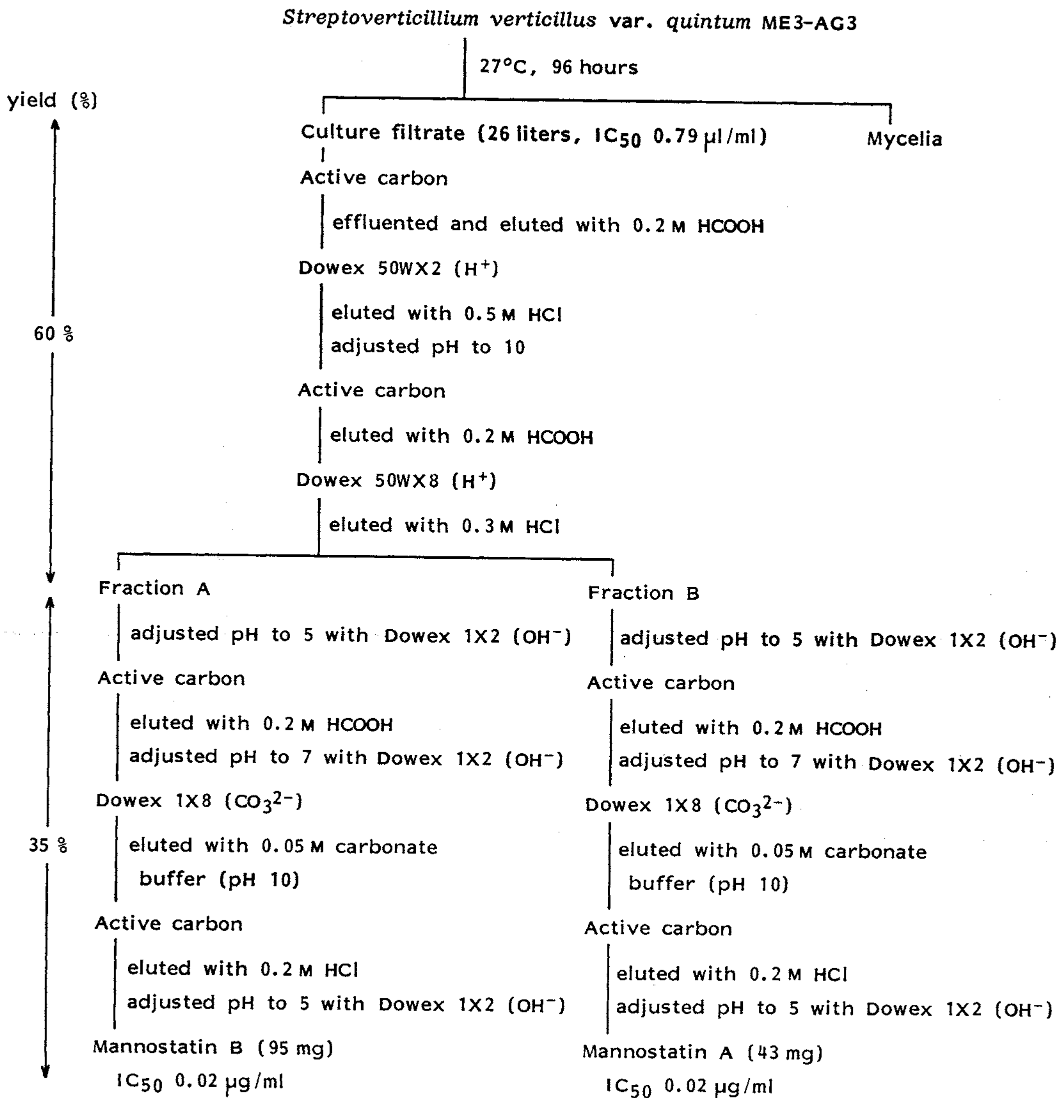
The resulting mannostatins A and B showed a single spot at the same position on high voltage paper electrophoresis (HVPE) at 3,500 V (formic acid - acetic acid - water, 25 : 75 : 900, pH 1.9).

Assay for α-D-Mannosidase and Inhibitory Activity

The activity of α-D-mannosidase was determined by colorimetrically measuring the amount of *p*-nitrophenol that has been liberated when NP-Man as a substrate was hydrolyzed with α-D-mannosidase from the rat epididymis as described by LEVVY *et al.*[12] The reaction mixture (total 0.5 ml) contained 0.05 ml of 0.04 M NP-Man, 0.25 ml of 0.1 M acetate buffer (pH 4.5) and 0.15 ml of water or an aqueous solution containing the test compound. The mixture was incubated at 37°C for 3 minutes, and 0.05 ml of α-D-mannosidase was added. After 20 minutes of the reaction, 2.0 ml of 0.4 M glycine - sodium hydroxide buffer (pH 10.5) was added and the absorbance of the liberated nitrophenol measured at 400 nm.

The percent inhibition was calculated by the formula $(A-B)/A \times 100$, where A is the liberated

Chart 1. Isolation of mannostatins A and B.



nitrophenol by the enzyme in the system without an inhibitor and B is that with an inhibitor. The IC_{50} value is the concentration of inhibitor at 50% inhibition of enzyme activity.

Physico-chemical Properties of Mannostatins A and B

MP's were measured by micro melting point apparatus MP-S3 (Yanagimoto Seisakusho Co., Japan) and were uncorrected. UV spectra were recorded on a Beckman DU-8 spectrophotometer, and IR spectra on a Hitachi 260-10 spectrophotometer. Optical rotations were measured with a Perkin-Elmer 241 polarimeter. High resolution (HR)-MS was carried out on a Hitachi M-80H mass spectrometer.

Results and Discussion

Taxonomic Characterization of the Producing Strain

The taxonomic characteristics of the strain ME3-AG3, from which it was identified as *S. verticillus*

Table 1. Comparison of taxonomic characteristics of strain ME3-AG3 and *Streptoverticillium verticillus* var. *quintum*.

	ME3-AG3	*S. verticillus* var. *quintum*
Spore surface	Smooth	Smooth
Aerial mass color	White to light gray	White to light gray
Color of vegetative growth	Pale yellow to yellowish brown	Pale yellow to dark yellow
Soluble pigment	None to yellowish brown	None
Melanin formation	Negative	Negative
Hydrolysis of starch	Negative	Negative
Coagulation of skim milk	Positive	Positive
Peptonization of skim milk	Positive	Positive
Liquefaction of gelatin (20%, 20°C)	Negative	Negative
Liquefaction of glucose-peptone-gelatin	Positive	Positive
Carbon utilization		
D-Glucose	+	+
L-Arabinose	−	−
D-Xylose	−	−
D-Fructose	−	−
Sucrose	−	−
Inositol	−	−
L-Rhamnose	−	−
Raffinose	±	±
D-Mannitol	−	−

+, Utilization; ±, doubtful utilization; −, no utilization.

var. *quintum* ME3-AG3, are described below. Microscopically, substrate mycelia were branched and extended aerial hyphae had whirls. On the aerial hyphae, no spirals were observed. Chains of mature spores included those having more than 10 spores. The spores ranged in size from 0.4×0.8 μm to 0.7×1.1 μm, and their surfaces were smooth. The color of vegetative growth was pale brown to yellowish brown or pale yellowish brown. Melanoid pigments were not formed. The whole-cell hydrolysate of the strain showed that it contained LL-diaminopimelic acid. Based on its characteristics, strain ME3-AG3 is considered to belong to the genus *Streptoverticillium*. As shown in Table 1, we concluded that the strain ME3-AG3 should be classified as a strain of *S. verticillus* var. *quintum*.

Production and Isolation of Mannostatins A and B

The strain of ME3-AG3 was cultured in Erlenmeyer flasks at 27°C for 4 days on a rotary shaker. The maximum peak of mannostatin production in the flasks was obtained at 4 days, thereafter the production slowly decreased with a pH change to alkaline. The flow diagram for the isolation is shown in Chart 1. The yields of pure mannostatins A and B were 43 and 95 mg, respectively, from 26 liters of culture filtrate.

Physico-chemical Properties of Mannostatins A and B

The molecular weight and formula of mannostatins A and B were determined to be $C_6H_{13}NO_3S$ (MW 179) and $C_6H_{13}NO_4S$ (MW 195), respectively, by HR-MS and elementary analysis of their tetraacetates. These compounds are highly soluble in water, and sparingly soluble in acetone, chloroform and benzene. The physico-chemical properties of mannostatins A and B or their tetraacetate are shown in Table 2. The structure of mannostatins A and B were determined as 4-amino-5-methylthio-1,2,3-cyclopentanetriol and 4-amino-5-methylsulfinyl-1,2,3-cyclopentanetriol.[14]

Table 2. Physico-chemical properties of mannostatins A and B or their tetraacetate.

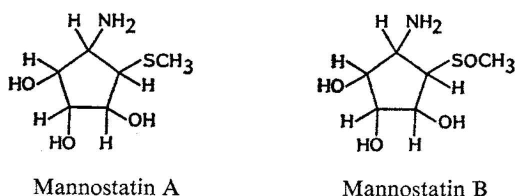
	A	B
Appearance (tetraacetate)	White needles	White needles
MP (tetraacetate) (°C, dec)	121	146
Molecular formula	$C_6H_{13}NO_3S$	$C_6H_{13}NO_4S$
(tetraacetate)	($C_{14}H_{21}NO_7S$)	($C_{14}H_{21}NO_8S$)
Elementary analysis (tetraacetate)		
Calcd:	C 48.40, H 6.09, N 4.03, S 9.23	C 46.27, H 5.82, N 3.85, S 8.82
Found:	C 48.86, H 5.95, N 3.99, S 9.28	C 46.41, H 5.80, N 3.80, S 8.87
TLC[a] (Rf value) (BuOH - AcOH - H_2O, 3 : 1 : 1)	0.43	0.23
HVPE[b]	1.07	1.07
Color reaction (positive)	Ninhydrin, Ehrlich, $KMnO_4$	Ninhydrin, Ehrlich, $KMnO_4$

[a] Silica gel TLC plate: Merck Art. No. 5715.
[b] Formic acid - acetic acid - water, 25 : 75 : 900, pH 1.8, 600 V, 30 minutes.

Table 3. Inhibitory activity of various inhibitors against glycosidases.

Inhibitor	IC_{50} (μg/ml)		
	Sialidase[a]	β-D-Galactosidase[b]	α-D-Mannosidase[c]
Siastatin A	0.35	>100	>100
Siastatin B	3	>100	>100
Pyridindolol	>100	1.8	>100
Isoflavonoid	>100	1.2	>100
HPAAO	>100	0.015	>100
Mannostatin A	>100	>100	0.02
Mannostatin B	>100	>100	0.02

[a] *Clostridium perfringens*, [b] bovine liver, [c] rat epididymis.



Mannostatin A Mannostatin B

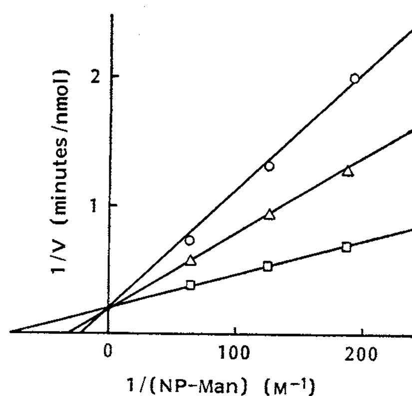


Fig. 1. Lineweaver-Burk plot of inhibition of α-D-mannosidase by mannostatin B.

○ I=32 ng/ml, △ I=16 ng/ml, □ I=0 ng/ml.

Biological Activities of Mannostatins A and B

Mannosidase inhibitors have been found to be produced by a variety of microorganisms and higher plants. Many kinds of polyhydroxylated alkaloids which structually resemble monosaccharides, have been reported such as nojirimycin,[15] nojirimycin B,[16] swainsonine[17] and 1,5-dideoxy-1,5-imino-D-mannitol.[18] These compounds have been the focus of intensive investigations because of their interesting biological activities.

The inhibitory activities of siastatins A and B, pyridindolol, isoflavonoid, HPAAO, and man-

nostatins A and B are shown in Table 3. Neither siastatins A and B, isoflavonoid, HPAAO, nor pyridindolol inhibited α-D-mannosidase. As shown in Fig. 1, inhibition of mannostatins A and B against α-D-mannosidase is competitive with the substrate, and both *Ki* values were 4.8×10^{-8} M. It was reported that mannostatins A and B inhibited the O_2^- generation from rice leaf tissue stimulated by blast fungus proteoglucomannan with pretreatment of concanavalin A or rice leaf lectin.[19] Mannostatins A and B at 100 μg per ml had no antimicrobial activity. They have low toxicity; no deaths were seen after intravenous injection of 250 mg/kg to mice.

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