BENASTATINS A AND B, NEW INHIBITORS OF GLUTATHIONE *S*-TRANSFERASE, PRODUCED BY *Streptomyces* sp. MI384-DF12

I. TAXONOMY, PRODUCTION, ISOLATION, PHYSICO-CHEMICAL PROPERTIES AND BIOLOGICAL ACTIVITIES

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Benastatins have been isolated as part of a program designed to find microorganism-produced inhibitors of glutathione *S*-transferase from *Streptomyces* sp. MI384-DF12. They were purified by chromatography of reversed-phase silica gel, silica gel and Capcell Pak C_{18} (HPLC) followed by solvent extraction and then isolated as yellow powders. Benastatins A and B have the molecular formulae, $C_{30}H_{28}O_7$ and $C_{30}H_{30}O_7$, respectively. They were competitive with 3,4-dichloronitrobenzene as the substrate, and the inhibition constants (*Ki*) of benastatins A and B were 5.0×10^{-6} and 3.7×10^{-6}, respectively.

Glutathione *S*-transferase (EC 2.5.1.18) (GST) catalyses the transfer of the glutathionyl group to an electrophilic acceptor and is distributed in a wide variety of mammalian cells as a multi-functional enzyme[1]. It is known that some isozymes of GST play an important role in the drug detoxication process of drug-resistant tumor cells against anti-tumor agents. Furthermore, GST also participates in the biosynthesis of leukotrienes and prostaglandins. Thus, a potent inhibitor of this enzyme is of considerable interest: Firstly, it has been proposed that inhibition of GST could overcome the resistance to some antineoplastic drugs[2]. Secondly, modulation of the biosynthesis of eicosanoids by inhibition of GST could be of potential therapeutic benefit in the treatment of related disorders[3].

In the course of screening for an inhibitor of GST, we discovered benastatins A and B in the culture broth of *Streptomyces* sp. MI384-DF12. In this communication we report the taxonomy, production, isolation, physico-chemical properties and biological activities.

Materials and Methods

Chemicals

Chemicals employed were as follows: Silanised silica gel 60, Silica gel 60 and TLC-plate Silica gel F_{254} (0.25 mm thickness) from E. Merck, Darmstadt, FRG; YMC GEL (ODS-A60-200/60) from Yamamura Chemical Laboratories Co., Ltd., Kyoto, Japan; packed column of Capcell Pak C_{18} from Shiseido Co., Tokyo, Japan; glutathione, reduced form (GSH) from Wako Pure Chemical Industries, Ltd., Osaka, Japan; 3,4-dichloronitrobenzene (DCNB) from Tokyo Kasei Kogyo Co., Ltd., Tokyo, Japan. All other chemicals were of analytical grade.

Enzymes

GST was prepared from rat liver as described by WU *et al.*[4]. Partially purified enzyme was used in

this assay.

Microorganism

Strain MI384-DF12 was isolated from a soil sample collected in Suginami-ku, Tokyo and has been deposited in the Fermentation Research Institute, Agency of Industrial Science and Technology, Ministry of International Trade and Industry, Tsukuba, Japan under the accession No. FERM P-11270.

Taxonomic Characterization

Morphological and physiological properties of the strain were examined according to SHIRLING and GOTTLIEB[5]; several other tests were also used.

Production of Benastatins A and B

The strain MI384-DF12 was inoculated into 110 ml of a seed medium consisting of galactose 2.0%, dextrin 2.0%, Bacto Soytone 1.0%, corn steep liquor 0.5%, $(NH_4)_2SO_4$ 0.2% and $CaCO_3$ 0.2% (pH 7.4) in a 500-ml Erlenmeyer flask, and cultured at 30°C for 3 days on a rotary shaker (180 rpm). Two ml of this seed culture were inoculated into 110 ml of the production medium consisting of glycerol 2.0%, soy bean meal (Ajinomoto Co., Inc.) 1.5%, K_2HPO_4 0.1% and $CoCl_2 \cdot 6H_2O$ 0.0005% (pH 6.2 adjusted with 1 M KH_2PO_4 before sterilization) in a 500-ml Erlenmeyer flask and cultured at 27°C for 4 days on a rotary shaker (180 rpm).

Isolation of Benastatins A and B

The culture broth was filtered and separated into the mycelial cake and the culture filtrate. The mycelial cake was extracted with methanol; the extract was filtered and concentrated *in vacuo* to an aqueous solution. The solution was combined with the culture filtrate and extracted with an equal volume of ethyl acetate. The active extract was concentrated to dryness under reduced pressure. The dried material was chromatographed on a column of silanised silica gel (× 50 w/w powder) with a linear gradient 40 to 100% aq MeOH and on a column of YMC GEL (× 100 w/w powder) with the same linear gradient, successively. The active fractions were collected and evaporated to give a brownish powder. The powder was suspended in a solvent mixture of $CHCl_3$ - MeOH (90 : 10), charged to a silica gel column (× 100 w/w powder), and eluted with $CHCl_3$ - MeOH (85 : 15). The eluate was concentrated under reduced pressure to give a yellowish powder containing benastatins A and B. The crude powder was further purified by a reversed-phase HPLC using a Capcell Pak C_{18} column (2.0 × 25 cm, flow rate 8 ml/minute, using GILSON's system) with a solvent mixture of CH_3CN - H_2O - AcOH (78 : 22 : 1). The active fractions to appear first are composed of benastatin B, and the active fractions coming next comprise benastatin A. Each eluate was evaporated to dryness to obtain a yellow powder.

Assay for Glutathione *S*-Transferase (GST) and Inhibitory Activity

GST activity was measured by a modification of the method of HABIG *et al.*[6]. The reaction mixture (total 2.0 ml) consisted of 85 mM potassium phosphate buffer (pH 7.4), 4.5 mM GSH, 0.5 mM DCNB, GST and water or aqueous solution containing the test compound. The enzyme reaction was started by the addition of the enzyme, following incubation at 37°C for 30 minutes. The conjugate was determined spectrophotometrically at 345 nm, corrections were made for chemical reactivity.

The percent inhibition was calculated by the formula $(A-B)/A \times 100$, where A is the conjugate by the enzyme in the system without an inhibitor and B is that with an inhibitor. IC_{50} value shows the concentration of inhibitor at 50% inhibition of enzyme activity.

Physico-chemical Properties

Melting points were taken using a Yanaco MP-S3 apparatus and are uncorrected. UV spectra were recorded on a Hitachi U-3210 spectrophotometer, and IR spectra on a Hitachi 260-10 spectrophotometer. Mass spectra were obtained on a Hitachi M-80H mass spectrometer and a Jeol JMS-SX 102 mass spectrometer. X-ray micro analysis was performed by a Kevex 7500 X-ray micro analyser equipped with a Hitachi S-510 scanning electron microscope.

Results and Discussion

Taxonomic Characterization of the Producing Strain

Strain MI384-DF12 had branched substrate mycelia developing aerial hyphae which usually extended straightforwards and had spiral spore chains with more than 20 spores per chain. It was characteristic that this strain had a pseudosporangium with a diameter of 1.5 to 6 μm. No whirl-formation as well as no sporangia were obserbed. Spores ranged about 0.5~0.6 by 0.7~0.8 μm in size and their surfaces were smooth. The color of vegetative growth was light brown to dark brown, although, in certain media it was pink. There were many cases where no adherence of aerial hyphae was observed, although white aerial hyphae was observed in ISP-media 2, 3, 4 and 5. Soluble pigments were faintly tinged with pink to brown. Melanoid pigments were positive, the protein-decomposing action was of a middle strength, and the starch hydrolysis was of a middle degree. The whole-cell hydrolysate of the strain showed that it contained LL-diaminopimelic acid.

Based on its characteristics, strain MI384-DF12 is considered to belong to the genus *Streptomyces*. Among the known species of *Streptomyces*, *Streptomyces paradoxus* and *Streptomyces vitaminophilus* are recognized to be similar to the strain MI384-DF12 having pseudosporangia. *S. vitaminophilus* is distinguished from strain MI384-DF12 by its vitamin requirements. The comparison to *S. paradoxus* is remained and the results will be described in another paper. Therefore, the strain MI384-DF12 was designated as *Streptomyces* sp. MI384-DF12.

Production and Isolation of Benastatins A and B

The strain of *Streptomyces* sp. MI384-DF12 was cultured in Erlenmeyer flasks at 27°C for 4 days on a rotary shaker. The time course of the production is shown in Fig. 1. The maximum peak of benastatin production in the flasks was obtained at 4 days. As shown in Fig. 2, calcium salts of benastatins A and B were isolated from the culture filtrate (30 liters), and the total yields were 2.25 g and 262 mg, respectively. The purity of each preparation was confirmed by TLC and HPLC.

Physico-chemical Properties of Benastatins A and B

The physico-chemical properties of benastatins calcium salts are summarized in Table 1. The IR spectra of benastatins indicated the presence of a carboxylate anion (A: 1605 and 1395 cm^{-1}; B: 1608 and 1375 cm^{-1}). We surveyed the nature of the cation in the benastatins by using an X-ray micro analyser. The result of this analysis showed that benastatins were in the form of their calcium salts, although the origin of the calcium is still not known.

The molecular weights and formulae of benastatins A and B were determined to be $C_{30}H_{28}O_7$ (MW 500) and $C_{30}H_{30}O_7$ (MW 502), respectively, by FAB-MS, HREI-MS and elemental analysis after EtOAc extraction (pH 2) to remove the salt. These compounds are soluble in dimethyl

Fig. 1. Time course of benastatins production by *Streptomyces* sp. MI384-DF12.

● Inhibition, △ pH, ▲ glycerol.

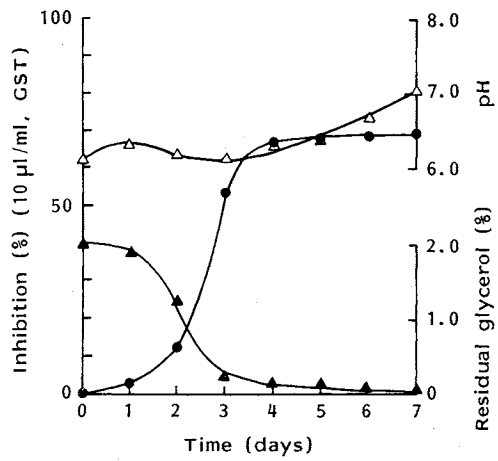

Fig. 2. Isolation of benastatins A and B.

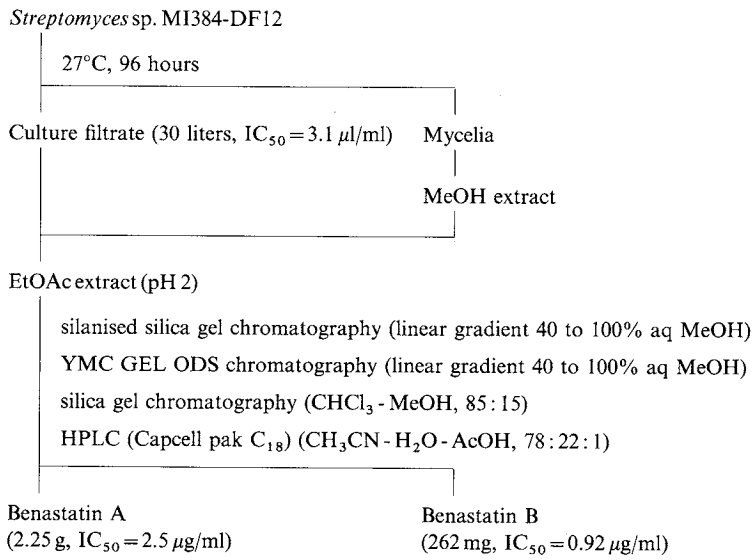

Table 1. Physico-chemical properties of benastatins calcium salts.

	Benastatin A	Benastatin B
Appearance	Yellow powder	Yellow powder
MP	170~173°C (dec.)	210~212°C (dec.)
FAB-MS (*m/z*, Neg.)	499 $(M-\frac{1}{2}Ca)^-$	501 $(M-\frac{1}{2}Ca)^-$
MW	519	521
HREI-MS (*m/z*)		
Found:	456.1921 $(M-\frac{1}{2}Ca-CO_2+H)^+$	
Calcd:	456.1937 for $C_{29}H_{28}O_5$	
Elemental analysis		
Found:	C 69.22, H 6.10, O 24.55	C 70.51, H 6.29, O 23.50
Calcd:	C 69.49, H 5.83, O 24.68 for $C_{30}H_{28}O_7 \cdot H_2O$	C 70.44, H 6.11, O 23.46 for $C_{30}H_{30}O_7 \cdot \frac{1}{2}H_2O$
Molecular formula	$C_{30}H_{27}O_7 \cdot \frac{1}{2}Ca$	$C_{30}H_{29}O_7 \cdot \frac{1}{2}Ca$
UV λ_{max}^{EtOH} nm (log ε)	202 (4.54), 250 (4.39), 267 (4.32), 285 (4.30), 310 (4.26), 391 (4.46)	208 (4.24), 227 (4.23), 290 (3.58), 398 (4.10)
IR ν_{max}^{KBr} cm^{-1}	3430, 2955, 1630, 1605, 1500, 1480, 1395, 1380, 1295, 1182, 1160, 1045	3410, 2947, 1608, 1475, 1464, 1432, 1375, 1290, 1255, 1195, 1156, 1035
Rf value on TLC	0.37 ($CHCl_3$ - MeOH, 4 : 1, silica gel)	0.44 ($CHCl_3$ - MeOH, 4 : 1, silica gel)
Color reaction	Mo-H_2SO_4, $FeCl_3$	Mo-H_2SO_4, $FeCl_3$
Solubility	Soluble: DMSO, MeOH, Me_2CO, EtOAc Insoluble: H_2O	Soluble: DMSO, MeOH, Me_2CO, EtOAc Insoluble: H_2O

sulfoxide, sparingly soluble in methanol, acetone and ethyl acetate, but insoluble in water.

The chemical structure of benastatins A and B were found to be 8,13-dihydro-1,7,9,11-tetrahydroxy-13-dimethyl-8-oxo-3-pentyl-benzo[*a*]naphthacene-2-carboxylic acid and 5,6,8,13-tetrahydro-1,7,9,11-tetrahydroxy-13-dimethyl-8-oxo-3-pentyl-benzo[*a*]naphthacene-2-carboxylic acid, respectively. The determination process will be reported in the following paper[7].

Fig. 3. Lineweaver-Burk plots of inhibition of GST by benastatin A.

● I=5.0 μg/ml, ■ I=2.5 μg/ml, ▲ I=1.25 μg/ml, △ I=0 μg/ml.

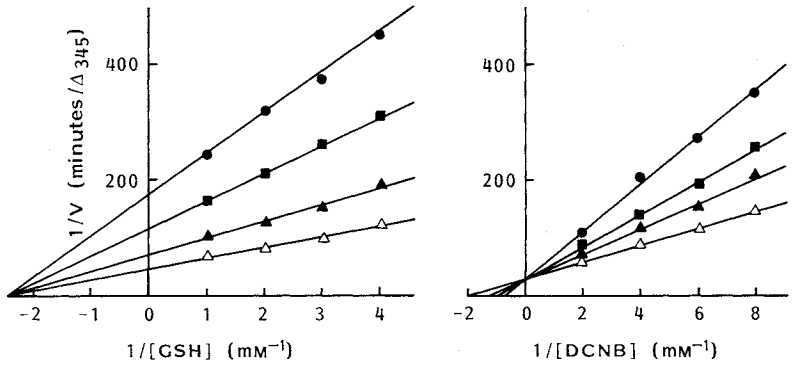

Fig. 4. Lineweaver-Burk plots of inhibition of GST by benastatin B.

● I=2.0 μg/ml, ■ I=1.0 μg/ml, ▲ I=0.5 μg/ml, △ I=0 μg/ml.

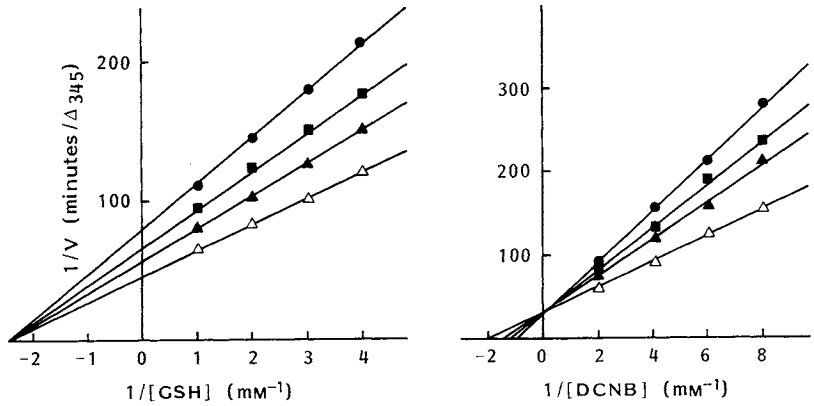

Table 2. Antimicrobial activities of benastatins A and B.

Test organisms	MIC (μg/ml)		Test organisms	MIC (μg/ml)	
	Benastatin A	Benastatin B		Benastatin A	Benastatin B
Staphylococcus aureus Smith	3.12	3.12	*Pseudomonas aeruginosa* A3	>50	50
S. aureus MS 9610	3.12	3.12	*Klebsiella pneumoniae* PCI602	>50	>50
S. aureus No. 5 (MRSA)[a]	3.12	3.12	*Mycobacterium smegmatis* ATCC 607	50	12.5
S. aureus No. 17 (MRSA)[a]	3.12	3.12	*Candida tropicalis* F-1	>50	>50
Micrococcus luteus FDA16	3.12	3.12	*Saccharomyces cerevisiae* F-7	>100	>50
Bacillus subtilis NRRL B-558	3.12	3.12	*Cryptococcus neoformans* F-10	50	50
Bacillus cereus ATCC 10702	3.12	3.12	*Cochliobolus miyabeanus*	>25	>25
Corynebacterium bovis 1810	3.12	3.12	*Pyricularia oryzae*	50	50
Escherichia coli NIHJ	100	100	*Aspergillus niger* F-16	>50	>100
Shigella dysenteriae JS11910	>50	>50			
Salmonella typhi T-63	>50	>50			
Proteus rettgeri GN311	50	50			

[a] MRSA: Methicillin-resistant *Staphylococcus aureus*.

Biological Activities of Benastatins A and B

As shown in Figs. 3 and 4, inhibitions of benastatins A and B against GST are competitive with DCNB, and noncompetitive with GSH. Both *Ki* values are 5.0×10^{-6} and 3.7×10^{-6} M with DCNB, respectively, and 3.5×10^{-6} and 4.2×10^{-6} M with GSH, respectively.

Benastatins A and B have an activity against Gram-positive bacteria including methicillin-resistant strains, but show little activity against Gram-negative bacteria and fungi. The antimicrobial activities of benastatins are shown in Table 2. Benastatins A and B have low toxicity; there were no deaths after ip injection of mice with 100 mg/kg.

References

1) KETTERER, B.: Detoxication reactions of glutathione and glutathioine transferase. Xenobiotica 16: 957~973, 1986
2) SATO, K.: Glutathine S-transferase as markers of preneoplasia and neoplasia. Adv. Cancer Res. 52: 205~255, 1989
3) LEUNG, K. H.: Selective inhibition of leukotriene C_4 synthesis in human neutrophils by ethacrynic acid. Biochem. Biophys. Res. Commun. 137: 195~200, 1986
4) WU, C. & K. P. MATHEWS: Indomethacin inhibition of glutathione S-transferases. Biochem. Biophys. Res. Commun. 112: 980~985, 1983
5) SHIRLING, E. B. & D. GOTTLIEB: Methods of characterization of *Streptomyces* species. Int. J. Syst. Bacteriol. 16: 313~340, 1966
6) HABIG, W. H.; M. J. PABST & W. B. JACOBY: Glutathione S-transferases. The first step in mercapturic acid formation. J. Biol. Chem. 249: 7130~7139, 1974
7) AOYAMA, T.; H. NAGANAWA, Y. MURAOKA, H. NAKAMURA, T. AOYAGI, T. TAKEUCHI & Y. IITAKA: Benastatins A and B, new inhibitors of glutathione *S*-transferase, produced by *Streptomyces* sp. MI384-DF12. II. Structure determination of benastatins A and B. J. Antibiotics 45: 1391~1396, 1992