BENADROSTIN, NEW INHIBITOR OF POLY(ADP-RIBOSE) SYNTHETASE, PRODUCED BY ACTINOMYCETES

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

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(Received for publication February 23, 1988)

Benadrostin, a new inhibitor of poly(ADP-ribose) synthetase was discovered in the fermentation broth of *Streptomyces flavovirens* MH499-O'F1. It was purified by chromatography followed by solvent extraction and then isolated as colorless prisms. Benadrostin has the molecular formula of $C_8H_5NO_4$. It was competitive with the substrate, and the inhibition constant (*Ki*) was 34 μM.

Poly(ADP-ribose) synthetase is a chromatin-bound enzyme, which synthesizes a protein-bound homopolymer, poly(ADP-ribose), from nicotinamide adenine dinucleotide (NAD). Various nuclear proteins, including histones and non-histone proteins, have been reported as acceptors of this polymer.[1~3] In association with this, there is clear evidence that poly(ADP-ribose) biosynthesis is required for efficient DNA repair.[4] Also it was suggested that this enzyme plays an important role in various pathological conditions, such as systemic lupus erythematodes, myasthenia gravis,[5] and diabetes mellitus and that the activities of this enzyme are somehow related to the function of B lymphocytes.[6] Thus a potent inhibitor of this enzyme could be useful as a therapeutic agent or as a research tool.

In the course of screening for an inhibitor of poly(ADP-ribose) synthetase, we discovered benadrostin as a specific inhibitor. Benadrostin was isolated from the culture broth of *Streptomyces flavovirens* MH499-O'F1. In this communication we report the taxonomy, production, isolation, physico-chemical properties and biological activities.

Materials and Methods

Chemicals

Chemicals employed were as follows: Wako gel C-200 and dithiothreitol (DTT) from Wako Pure Chemical Industries, Ltd., Osaka, Japan; protamine sulfate (salmon), β-nicotinamide adenine dinucleotide (β-NAD), DNA (calf thymus) and histone (calf thymus) from Sigma Chemical Co., St. Louis, U.S.A.; [*adenine-U*-^{14}C]β-NAD and Aquasol-II from New England Nuclear, Boston, U.S.A. All other chemicals were of analytical grade.

Enzyme

Poly(ADP-ribose) synthetase (EC 2.4.99.-) was prepared from calf thymus as described by ITO *et al.*[7] Partially purified enzyme was used in this assay (61.8 nmol/minute/mg).

Microorganism

Strain MH499-O'F1 was isolated from a soil sample collected on the premises of the Institute of Microbial Chemistry, Shinagawa-ku, Tokyo 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 accession number FERM-P8658.

Taxonomic Characterization

Morphological and physiological properties of the strain were examined according to SHIRLING and GOTTLIEB;[6] several other tests were also used.

Production of Benadrostin

The strain MH499-O'F1 was inoculated into 110 ml of a production medium consisted of glycerol 1.5%, Pharmamedia 1.5%, NaCl 0.3% and L-asparagine (pH 7.4) in a 500-ml Erlenmeyer flask, and cultured at 27°C for 2 days on a rotary shaker (180 rpm). Two ml of the above seed culture was transferred to 110 ml of the same medium in a 500-ml Erlenmeyer flask and cultured for 5 days under the same condition.

Isolation of Benadrostin

The culture broth was filtered and the filtrate was adsorbed on an Amberlite XAD-4 column (10% filtrate), which was washed with water and eluted with 50% Me_2CO ($\times 3$ column) to give active fractions. The active eluate was concentrated under reduced pressure. The solution containing benadrostin was adjusted to pH 2.0 with HCl, and benadrostin was extracted with equal volume of EtOAc. Benadrostin was transferred into an equal volume of water (pH 9.0, NaOH), and then transferred into an equal volume of EtOAc (pH 2.0, HCl). The extract was concentrated to give a crude brownish powder. A solution of this crude powder in $CHCl_3$ was passed through a $CHCl_3$-filled column of silica gel ($\times 50$ w/w powder). Successive elution with $CHCl_3$ - MeOH (20 : 1) gave a yellowish powder of benadrostin. The yellowish powder was subjected to a Sephadex LH-20 column (1.6×100 cm) chromatography developed with MeOH to afford a colorless powder, homogeneous by silica gel TLC developed with $CHCl_3$ - MeOH (10 : 1). The powder was crystallized from $CHCl_3$ to yield colorless crystals of benadrostin.

Measurement of Enzyme Activities

Poly(ADP-ribose) synthetase activity was measured by a modification of the method of ITO *et al.*[7] The reaction mixture (total 50 μl) consisting [*adenine-U*-^{14}C]β-NAD 0.43 μM, $MgCl_2$ 10 mM, DTT 1 mM, DNA 100 μg, histone 100 μg, Tris-HCl buffer 100 mM (pH 8.0) and 5 μl of poly(ADP-ribose) synthetase with or without inhibitors was incubated for 10 minutes at 25°C. The reaction was terminated by the addition of 3 ml of 10% TCA and the poly(ADP-ribose) formed was collected on a Millipore filter (0.45 μm). The filter was washed three times with 5 ml of 5% TCA. The radioactivity of the dried filter was counted with a scintillator (Aquasol-II, 5 ml).

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

The mp was measured with a micro melting point apparatus MP-S3 (Yanagimoto Seisakusyo Co., Japan) and was uncorrected. Electron impact mass spectrometry (EI-MS) and high resolution mass spectrometry (HR-MS) were carried out on a Hitachi M-80H mass spectrometer. The optical rotation was determined with a Perkin-Elmer 241 polarimeter using a micro-cell (light path 10 cm).

Results and Discussion

Taxonomic Characterization of the Producing Strain

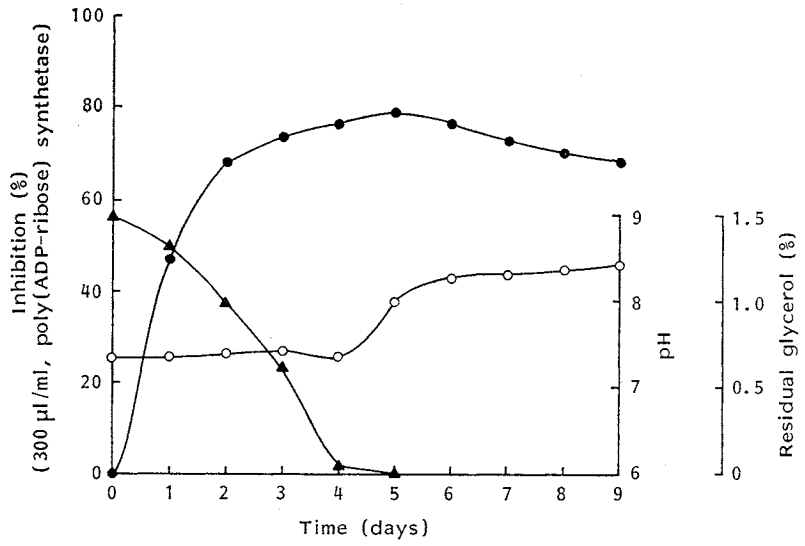
Strain MH499-O'F1 produces aerial mycelia forming straight chains of spores with more than 30 spores per chain. The spores are 0.4~0.6 by 0.6~0.8 μm in size with smooth surface. Aerial mass

Table 1. Comparison of taxonomic characteristics of strain MH499-O'F1 and *Streptomyces flavovirens*.

	MH499-O'F1	*S. flavovirens* IMC S-0230 (ISP 5062)
Spore chain morphology	Straight	Straight
Spore surface	Smooth	Smooth
Aerial mass color	White to light gray	White to light gray
Color of vegetative growth	Colorless to pale yellow, pale yellowish brown	Pale yellow to pale yellowish brown
Soluble pigment	None to yellow, yellowish brown	Yellow
Melanin formation	Negative	Negative
Hydrolysis of starch	Positive	Positive
Coagulation of skim milk	Positive	Positive
Peptonization of skim milk	Positive	Positive
Liquefaction of gelatin (20%, 20°C)	Positive	Negative (positive)[10]
Liquefaction of glucose-peptone-gelatin	Positive	Positive
Nitrate reduction	Negative or positive	Negative
Carbon utilization		
D-Glucose	+	+
L-Arabinose	±	+
D-Xylose	+	+
D-Fructose	+	+
Sucrose	−	−
Inositol	−	−
L-Rhamnose	−	+
Raffinose	−	−
D-Mannitol	+	+

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

Fig. 1. Time course of benadrostin production by *Streptomyces flavovirens* MH499-O'F1.
● Inhibition, ○ pH, ▲ glycerol.



color of the colony is white to light gray. Color of vegetative growth is colorless to pale yellow or pale yellowish brown. Melanoid pigments are not formed. The whole-cell hydrolysate of the strain showed that it contained LL-diaminopimelic acid. Based on its characteristics, strain MH499-O'F1

Fig. 2. Isolation of benadrostin.

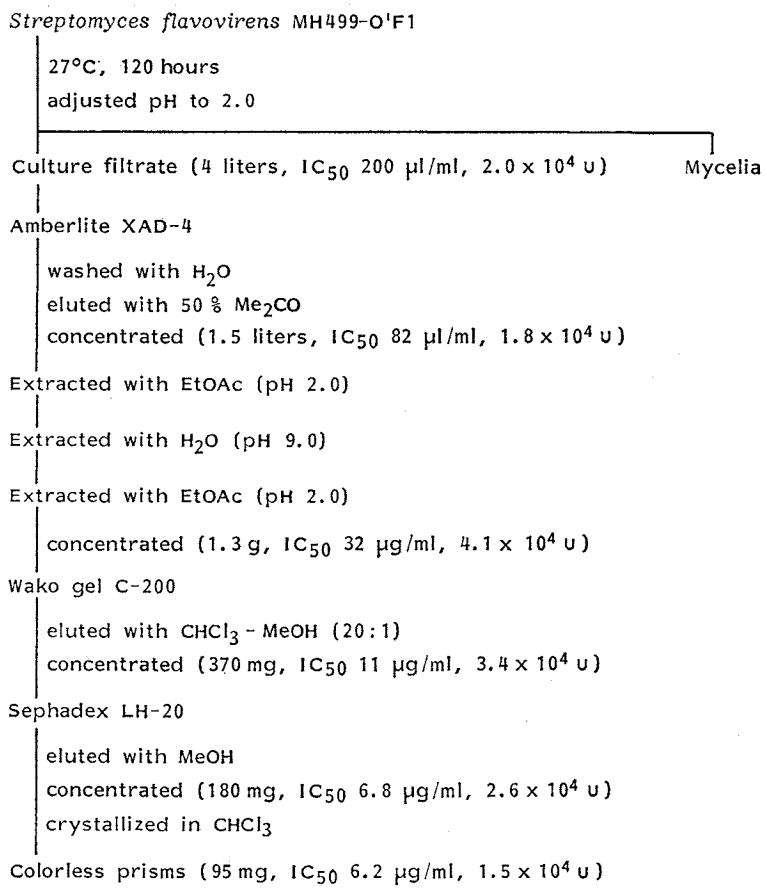


Table 2. Physico-chemical properties of benadrostin.

Appearance	Colorless prisms
MP	190~192°C
EI-MS (*m/z*)	179
Molecular formula	$C_8H_5NO_4$
Elemental analysis	
Calcd:	C 53.64, H 2.81, N 7.82.
Found:	C 53.42, H 2.73, N 7.59.
$[\alpha]_D^{20}$ (*c* 1.0, MeOH)	0°
Rf* value on TLC	
Benzene - EtOAc (1:1)	0.48
$CHCl_3$ - MeOH (10:1)	0.30

* Silica gel TLC plate, Merck Art. No. 5715.

Table 3. Inhibitory effects of benadrostin and other inhibitors on poly(ADP-ribose) synthetase.

	IC_{50} (µM)
Benadrostin	35
Benzamide	8.3
Nicotinamide	74

designate the strain MH499-O'F1 as a new species. Therefore, the strain is considered to be a member of *S. flavovirens*.

is considered to belong to the genus *Streptomyces*. Among the known species of *Streptomyces*, *S. flavovirens* is recognized to be similar to the strain MH-499-O'F1 except for the utilization of L-rhamnose as shown in Table 1. This and other minor differences are not sufficient to

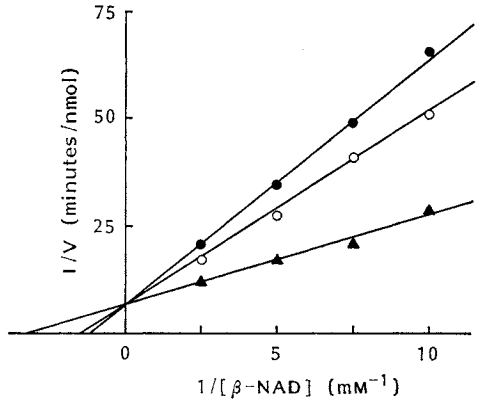
Production and Isolation of Benadrostin

The strain of *S. flavovirens* was cultured in Erlenmeyer flasks at 27°C for 5 days on a rotary shaker.

The time course of the production is shown in Fig. 1. The maximum peak of benadrostin production in the flasks was obtained at 5 days, thereafter the production slowly decreased with a pH change to alkaline. The flow diagram for the isolation is shown in Fig. 2. The yield of pure benadrostin was 95 mg from 4 liters of culture filtrate.

Fig. 3. Lineweaver-Burk plot of inhibition of poly(ADP-ribose) synthetase by benadrostin.

● I=10 μg/ml, ○ I=5 μg/ml, ▲ I=0 μg/ml.



Physico-chemical Properties of Benadrostin

The physico-chemical properties of benadrostin are summarized in Table 2. The molecular weight and formula of benadrostin was determined by EI-MS, HR-MS and elemental analysis. Benadrostin is soluble in acetone, chloroform, ethyl acetate and methanol, but insoluble in water and benzene. The spot on silica gel TLC plates is visualized by potassium permanganate or GIBBS reagent.

Determination of the structure of benadrostin will be described in the following paper.[9]

Biological Activities of Benadrostin

The inhibitory activities of benadrostin, benzamide and nicotinamide are shown in Table 3. They showed IC_{50} value of 35 μM, 8.3 μM, 74 μM against poly(ADP-ribose) synthetase, respectively. As shown in Fig. 3, inhibition of benadrostin against poly(ADP-ribose) synthetase is competitive with the substrate, and the *Ki* and *Km* values were 3.4×10^{-5} M and 3.1×10^{-4} M, respectively. Benadrostin at 100 μg per ml had no antimicrobial activity. It has low toxicity; no deaths after intravenous injection of 250 mg/kg to mice.

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