NOTES

Bu-2743E, A LEUCINE AMINOPEPTIDASE INHIBITOR, PRODUCED BY *BACILLUS CIRCULANS*

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Systematic screening of microbial fermentation products has yielded a variety of enzyme inhibitors[1,2], some of which have been found to be clinically useful as anti-ulcer agents, immunomodulators and/or anticancer agents.

In the course of testing microbial metabolites for inhibition of leucine aminopeptidase (LAP), a strain of *Bacillus circulans* (No. J725-B93) was found to produce a new compound designated as Bu-2743E, which showed potent LAP inhibitory activity with no antimicrobial activity. Bu-2743E exhibited characteristic UV absorption at 249.5 and 315 nm, and its structure was determined to be (2,3-dihydroxybenzoyl)-L-alanyl-L-threonine (Fig. 1). This paper describes fermentation, isolation, properties and structure of Bu-2743E.

Strain J725-B93 was isolated from a soil sample collected in India. It is an aerobic, Gram-negative, spore-forming bacterium, classified as belonging to the genus *Bacillus*. The morphological, cultural and physiological characteristics of strain J725-B93 are shown in Tables 1 and 2. Taxonomically diagnostic properties of strain J725-B93 are summarized as follows: 1) negative Gram-stain, 2) sporangia swollen at endospore site, 3) spore position terminal to central, 4) elliptical spores, 5) acid but no gas formation from glucose and xylose, 6) starch hydrolysis, 7) no acetoin production, 8) no indole production, and 9) moderate growth in ordinary media. On the basis of these characteristics, strain J725-B93 would appear to belong to *Bacillus circulans*, even though it differs in some respects from the *B. circulans* description, *i.e.* negative acid formation from arabinose or mannitol and negative catalase formation.

Fig. 1. Structure of Bu-2743E.

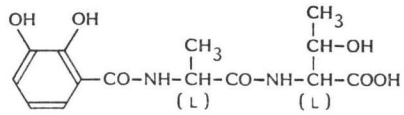


Table 1. Morphological characteristics of strain J725-B93.

Vegetative cells	
Shape	Rods. Rounded end.
Size	0.4~0.6×2.4~4.0 μm
Motility	Positive
Spores	
Shape and size	Elliptical, 0.7×1.4 μm
Distension of sporangia	Swollen at spore site
Position	Terminal or central
Gram-stain	Negative

Bu-2743E was produced in 500-ml Erlenmeyer flasks using a medium composed of 2% glycerol, 1% corn steep liquor, 1% Pharmamedia, 0.4% $CaCO_3$, 0.3% $(NH_4)_2SO_4$ and 0.003% $ZnSO_4 \cdot 7H_2O$, pH of the medium being adjusted to 7.0 before sterilization. The flasks were shaken on a rotary shaker (250 rpm) at 27°C for 4 days. The LAP-inhibitory activity in fermentation broth or extract was determined by the method of UMEZAWA *et al.*[3] using LAP of rabbit kidney origin[4] and L-leucine β-naphthylamide (Sigma Chemical Co., U.S.A.) as the substrate.

The harvested whole broth (10 liters) was acidified to pH 2.0 with 6 N HCl and extracted with butanol. The butanol extract was concentrated to an aqueous solution which was adjusted to pH 2.0 and extracted with ethyl acetate. Bu-2743E contained in the ethyl acetate extract was transferred into alkaline water (pH 8.0) and then back-extracted into ethyl acetate at pH 2.0. Evaporation of the solvent under reduced pressure afforded a crude solid of Bu-2743E (1.35 g, IC_{50}: 1.8 μg/ml). The solid was dissolved in a small amount of ethyl acetate and charged on a silica gel column (φ 4.0×70 cm) which was developed with a mixture of ethyl

Table 2. Cultural and physiological characteristics of strain J725-B93.

Temperature for growth	
Growth	20~45°C
No growth	7°C and 50°C
Acid from glucose and xylose	Positive
Acid from arabinose and mannitol	Negative
Gas from glucose, arabinose, xylose and mannitol	Negative
Acetoin from glucose	Negative
Hydrolysis of starch	Positive
Liquefaction of gelatin	Positive
Nitrite from nitrate	Positive
Indole production	Negative
Growth in 5% and 7% NaCl	Negative
Growth in 0.001% lysozyme	Positive
Decomposition of casein	Positive
Growth on Sabouraud dextrose agar	Negative
Anaerobic growth in Hugh-Leifson medium (fermentative or oxidative)	Negative (oxidative)
Catalase	Negative
Colony on BBL-trypticase soy agar (observed after incubation at 28°C for 5 days)	Two types of colony: 1) 4~6 mm in diameter. Good growth. Yellowish white. Entire, smooth, drop-like, raised and round. 2) 2~3 mm in diameter. Poor growth. Yellowish white. Hilly, wrinkled and scalloped or filiformed.

acetate - methanol (20: 1). The active fractions were combined, concentrated *in vacuo* and purified by repeated silica gel chromatography. The semi-pure solid thus obtained was further purified by Sephadex LH-20 chromatography (ϕ 2.2 × 70 cm column, developed with methanol). Evaporation of LAP-active eluates afforded a homogeneous sample of Bu-2743E (190 mg, IC_{50}: 0.5 μg/ml).

Bu-2743E was isolated as a white amorphous solid. Bu-2743E exhibited a single spot at Rf 0.23 on a silica gel TLC plate developed with ethyl acetate - methanol - water (10: 3: 1). Bu-2743E is readily soluble in methanol, ethanol, butanol, acetone, ethyl acetate, dimethyl sulfoxide and alkaline water but insoluble in benzene, *n*-hexane and acidic and neutral water. It gives a positive response with ferric chloride and ELSON-MORGAN reagents, while ninhydrin, SAKAGUCHI and EHRLICH tests are negative. Bu-2743E is optically active, $[\alpha]_D^{25}$ +6° (*c* 1.0, MeOH) and mp 147~152°C. The molecular formula of Bu-2743E was determined to be $C_{14}H_{18}N_2O_7$ based on the elemental analysis (Calcd for $C_{14}H_{18}N_2O_7$: C 51.53, H 5.56, N 8.56. Found: C 51.33, H 6.04, N 7.45) and mass spectrometry of its methyl ester (M^+: *m/z* 340). The UV spectrum of Bu-2743E showed absorption maxima at 213 nm (ε 20,400), 249.5 (8,700) and 315 (3,200) in ethanol and 0.01 N HCl - ethanol. The spectra underwent bathochromic shift to 224 nm (ε 14,400), 238 (sh, 12,700), 262 (sh, 7,900) and 328 (3,900) in 0.01 N NaOH - ethanol. The IR spectrum showed absorption bands for carbonyl (1725 cm^{-1}), amide (1670, 1640 and 1550 cm^{-1}) and NH and/or OH (3300 cm^{-1}) groups. The NMR spectrum of Bu-2743E (60 MHz, CD_3OD) indicated the presence of two methyl (δ 1.20 ppm, 3H, d, J=6.6 Hz and 1.49 ppm, 3H, d, J=7.0 Hz), three methine (δ 4.1~5.1 ppm, 3H, m) and three aromatic protons (δ 6.64 ppm, 1H, t, J=7.5 Hz; 6.91 ppm, 1H, dd, J=2.0 & 7.5 Hz and 7.26 ppm, 1H, dd, J=2.0 & 7.5 Hz).

Upon acid hydrolysis (6 N HCl, 110°C, 16 hours), Bu-2743E was cleaved into a chromophoric substance and two amino acids. An acidic chromophore was extracted with ether from the hydrolysate and identified as 2,3-dihydroxybenzoic acid by UV (λ_{max}^{EtOH} 220, 247.5 and 318 nm), IR and TLC. The amino acid moieties were separated by Dowex 50WX4 chromatography with gradient HCl elution. One amino acid eluted with 0.2 N HCl was identified as L-alanine by TLC, IR, NMR and optical rotational value, $[\alpha]_D^{25}$ +13° (*c* 0.85, 5 N HCl). The other amino acid obtained by 0.3 N HCl elution was identified

Table 3. LAP inhibitory activity of Bu-2743E and bestatin.

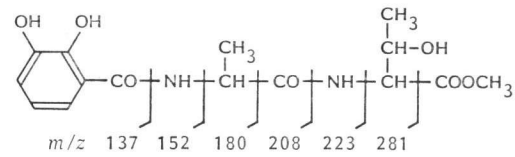
Method	Enzyme source	Substrate	IC_{50} (μg/ml)	
			Bu-2743E	Bestatin
A	Rabbit kidney[4]	L-Leucine β-naphthylamide	0.51	0.0048
B	Porcine kidney (Sigma)	L-Leucine β-naphthylamide	0.51	0.0048
C	Rabbit kidney[4]	L-Leucine β-diethylanilide	11.3	0.22

as L-threonine, $[\alpha]_D^{25}$ $-15°$ (c 1.25, 5 N HCl).

The linkage of the three constituents of Bu-2743E was elucidated by mass spectrometric analysis on its methyl ester which was prepared by treatment of Bu-2743E with diazomethane at room temperature. The methyl ester showed an ester carbonyl band at 1740 cm^{-1} in the IR and a singlet methyl signal at δ 3.59 in the NMR. It showed no LAP inhibitory activity. The mass spectrum of Bu-2743E methyl ester exhibited the molecular ion peak at m/z 340 and prominent ion peaks due to cleavages of amide linkage. These fragment ions together with other diagnostic ions established the structure of Bu-2743E methyl ester as shown in Fig. 2. Thus, Bu-2743E is (2,3-dihydroxybenzoyl)-L-alanyl-L-threonine.

The LAP inhibitory activity of Bu-2743E was evaluated by three assay systems (Methods A, B and C; see Table 3) using different enzymes or substrates. The results are shown in Table 3 along with those of bestatin which was tested as a reference compound. Bu-2743E was approximately 1/50~1/100 as active as bestatin in terms of IC_{50} values when tested by these methods.

Fig. 2. Structure and mass spectrum of Bu-2743E methyl ester.



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