NOTES

Phebestin, a New Inhibitor of Aminopeptidase N, Produced by *Streptomyces* sp. MJ716-m3

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Aminopeptidase N [AP-N, EC 3.4.11.2] is a Zn^{2+}-dependent ectoenzyme that is anchored in the plasma membrane *via* a hydrophobic domain adjacent to a small cytoplasmic region at the NH_2 terminus[1,2]. This enzyme is widely expressed by the brush border of the small intestine, synaptic cells of the central nervous system, and hematopoietic cells of myeloid lineage[3]. It has been postulated that AP-N performs multiple functions to regulate the action of hormones and neurotransmitters by inactivating such peptides at the cell surface[4,5]. Recently, this enzyme has been proved to be identical to the human myeloid plasma membrane glycoprotein CD13 which is expressed on human normal and malignant hematopoietic cells, and human malignant melanoma cells, but not normal melanocytes[6,7]. It has been indicated that AP-N is involved in the process to degrade and invade the extracellular matrix by tumor cells[7~9]. Furthermore, it has been demonstrated that the inhibition of AP-N by enzyme inhibitors or antibodies against AP-N is correlated with growth suppression of human tumor cells[10]. Thus, it is expected that the specific AP-N inhibitor may become a drug to suppress the metastasis or growth of certain cancers.

We have already reported actinonin[11], probestin[12] and leuhistin[13] as specific inhibitors of AP-N. We continued the screening for new AP-N inhibitors and discovered phebestin (Fig. 1) from the culture of *Streptomyces* sp. MJ716-m3. In this communication, we report the production, isolation, physico-chemical properties, structure determination and biological activities of the inhibitor.

The strain MJ716-m3 was inoculated into 110 ml of a production medium consisting of Bacto Soytone 1.0%, galactose 2.0%, corn steep liquor 0.5%, dextrin 2.0%, glycerol 1.0%, $(NH_4)_2SO_4$ 0.2% and $CaCO_3$ 0.2% (pH 7.4 before sterilization) in a 500-ml Erlenmeyer flask, and cultured at 27°C for 3 days on a rotary shaker (180 rpm) to obtain a seed culture. Two ml of this seed culture were inoculated into 110 ml of the same medium in a 500-ml Erlenmeyer flask and cultured for 3 days under the same conditions.

The isolation of phebestin was followed by measuring the inhibitory activity against AP-N from hog kidney (purchased from Boehringer Mannheim GmbH, FRG) as reported previously[11]. The culture filtrate (9.8 liters) was passed through a column of Diaion HP-20 (1 liter), which was washed with water and eluted with 50% aqueous acetone. The active fractions were concentrated under reduced pressure to remove acetone. The resulting solution was adjusted to pH 2.0 with HCl, and then extracted with equal volume of BuOH. The BuOH layer was concentrated under reduced pressure to give a brownish powder. The powder was placed on a column of silica gel (3.8 × 52 cm) packed with a solvent mixture of BuOAc - BuOH - AcOH - H_2O (6:4:1:1) and was eluted with the same solvent mixture. The active fractions were concentrated under reduced pressure to give a light brownish powder. The powder was dissolved in 0.1 M pyridine-AcOH buffer (pH 3.0), and subjected to a column of Dowex 50WX8 (1.3 × 26 cm) equilibrated with the same buffer and chromatographed with a liner gradient from 0.1 M pyridine-AcOH (pH 3.0) to 2.0 M pyridine-AcOH (pH 4.75). The active fractions were concentrated under reduced pressure to give a yellowish powder. The powder was purified by reversed phase HPLC, performed by a GILSON's system equipped with a Waters 991J photodiode array detector. It was applied to a reversed phase HPLC column; Shiseido CAPCELL PAK C18 SG120 20 × 250 mm which was equilibrated with a solvent mixture of MeCN - 0.1% TFA (6 : 19) and a flow rate of 8 ml/minute, and was eluted with the same solvent. The active fractions were concentrated and lyophilized to give a slightly yellow powder. This powder was dissolved in MeOH, and chromatographed on a column of Sephadex LH20 (1.5 × 91 cm) with MeOH. The active fractions were concentrated under reduced pressure to give phebestin as a colorless powder (15.6 mg).

The physico-chemical properties of phebestin are summarized in Table 1. Phebestin is soluble in water, methanol and dimethyl sulfoxide, but insoluble in chloroform and ethyl acetate. It gives positive color reactions with ninhydrin, Greig-Leaback and phoshomolybdate. The UV absorption spectrum showed a maxi-

Fig. 1. Structure of phebestin.

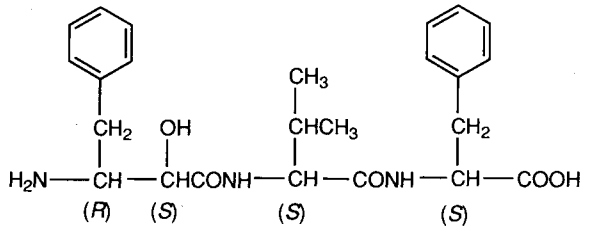

Table 1. Physico-chemical properties of phebestin.

Appearance	Colorless powder
MP (°C)	190~192
Molecular formula	$C_{24}H_{31}N_3O_5$
FAB-MS (positive, *m/z*)	442 $(M+H)^+$
Elemental analysis	
Calcd for	
$C_{24}H_{31}N_3O_5 \cdot H_2O$:	C 60.36, H 6.97, N 8.80
Found:	C 60.63, H 6.71, N 8.74
$[\alpha]_D^{27}$ (*c* 0.54, AcOH)	−12.6°
UV λ_{max}^{MeOH} nm (ε)	259 (441)
IR ν_{max}^{KBr} cm^{-1}	3380, 3230, 2970, 1670, 1520, 1390, 1270, 1210, 1120
Rf value	
I[a]	0.70
II[b]	0.34
Color reaction	Ninhydrin, Greig-Leaback, Phosphomolybdate
Solubility	Soluble: H_2O, MeOH, DMSO

[a] On silica gel TLC plate (Merck Art. No. 5715) with BuOH-AcOH-H_2O (4:1:1).
[b] On ODS silica gel TLC plate (Merck, Art No. 15389) with CH_3CN-buffered solution of 5% CH_3COOK and 1% citric acid monohydrate (7:13).

mum at 259 (ε 441). The IR spectrum suggested the presence of a peptide bond (1670 and 1520 cm^{-1}).

The molecular formula of phebestin was established as $C_{24}H_{31}N_3O_5$ by FAB-MS and elemental analysis. The amino acid analysis of the acid hydrolysate of phebestin suggested the amino acid constitution was Val (1), Phe (1), *threo*-3-amino-2-hydroxy-phenylbutanoic acid (AHPA, 1)[14,15]. The ^1H and ^{13}C NMR data on phebestin are shown in Table 2. The assignments of proton and carbon signals were determined by the ^1H-^1H COSY, HMBC, HSQC and DEPT experiments. These data were consistent with the amino acid residues which were detected by the amino acid analysis.

The configuration of the each amino acid residue was examined by the procedure described by NISHIKIORI *et al.*[16]. The amino acid mixture obtained by acid hydrolysis of phebestin was derivatized to L-phenylalanyl dipeptides, and compared by HPLC (CAPCELL PAK C18) with the reference compounds. Judging from the elution time observed, the existence of L-Val, L-Phe and (2*S*,3*R*)-AHPA was confirmed. The amino acid sequence of phebestin was determined by the analyses of FAB-MS and HMBC spectra. In the HMBC spectrum of phebestin, the amide proton at δ_H 8.16 ppm (Phe) is coupled to amide carbonyl carbon at δ_C 170.39 ppm (Val) and a methine proton at δ_H 4.12 ppm (Val) is coupled to amide carbonyl carbon at δ_C 171.92 ppm (AHPA). Consequently, the structure of phebestin was determined to be (2*S*,3*R*)-3-amino-2-hydroxy-4-phenylbutanoyl-L-valyl-L-phenylalanine.

The inhibitory activities of phebestin and various inhibitors of aminopeptidases are shown in Table 3. Phebestin inhibits not only AP-N but also AP-A and AP-B. It is competitive with the substrate L-leucine-β-naphthylamide. The *Ki* value of phebestin was 2.95×10^{-8} M. It has no antimicrobial activity at 100 μg/ml. It has low toxicity; there were no deaths after intraperitoneal injection of mice with 100 mg/kg of phebestin.

Table 2. ^1H and ^{13}C NMR data for phebestin in DMSO-d_6.

Assignment		^1H[a]	^{13}C[b]
AHPA	CO		171.92
	2-CH	3.81 d (*J*=2.7)	71.04
	3-CH	3.25 dt (*J*=2.7, 7.3)	54.96
	4-CH_2	2.61 dd (*J*=7.3, 13.4), 2.80 dd (*J*=7.3, 13.4)	38.52
	Ph-*i*	7.1~7.3	138.59
	Ph-*o*		129.30
	Ph-*m*		128.32[c]
	Ph-*p*		126.21[d]
	NH_2		
Val	CO		170.39
	α-CH	4.12 dd (*J*=6.4, 8.8)	57.32
	β-CH	1.98 m	30.79
	γ-CH_3	0.76 d (*J*=6.7)	19.22
	CH_3	0.80 d (*J*=6.7)	17.82
	NH	7.80 d (*J*=7.9)	
Phe	CO		172.91
	α-CH	4.31 m	53.99
	β-CH_2	2.84 dd (*J*=8.8, 13.7), 3.03 dd (*J*=5.2, 13.7)	36.93
	Ph-*i*	7.1~7.3	138.03
	Ph-*o*		129.09
	Ph-*m*		127.97[c]
	Ph-*p*		126.14[d]
	NH	8.16 d (*J*=7.6)	

[a] 500 MHz; δ in ppm, *J* in Hz.
[b] 125 MHz; δ in ppm.
[c,d] Assignment could be interchanged.

Table 3. Inhibitory activities of various inhibitors.

	IC_{50} (μg/ml)		
	AP-N	AP-A	AP-B
Phebestin	0.18	9.0	9.0
Leuhistin	0.20	10	13
Probestin	0.030	>100	37
Actinonin	0.40	>100	>100
Amastatin	0.58	0.54	>100
Ubenimex*	6.2	>100	0.05

* Bestatin.

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