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The Novel Synthetic Substance MR-387C[(2S,3R)-3-amino-2-hydroxy-4-phenylbutanoyl-L-valyl-L-prolyl-L-leucine] as an Aminopeptidase M Inhibitor

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Abstract: In the course of screening for new aminopeptidase M inhibitors which were expected to be analgesic, immunopotentiating, or anti-metastatic agents, the novel synthetic substance MR-387C[(2S,3R)-3-amino-2-hydroxy-4-phenylbutanoyl-L-valyl-L-prolyl-L-leucine] (M.W. 504 daltons) was obtained. It was competitive with the substrate and had an IC_{50} value of 0.04 μg/ml (7.9×10^{-8} M) and an inhibition constant (K_i) of 3.8×10^{-8} M. This novel MR-387C was compared with various known inhibitors of aminopeptidase M. It inhibited the enzyme more strongly than any other microorganism-originated inhibitor, except probestin.
Key words: Aminopeptidase M, inhibitor, synthetic peptide.

Cell-surface metallopeptidases have specific functions that differ according to their cellular locations, including potential roles in the control of growth and differentiation in both hematopoietic and epitherial cell systems (Kenny *et al.*, 1989). Among these metallopeptidases, aminopeptidase M (EC 3.4.11.2, leucine aminopeptidase, microsomal, AP-M) plays an important role in the inactivation process of bioactive peptides, especially enkephalins, which have an analgesic function in cerebral membranes (Gros *et al.*, 1985). Recently, Saiki *et al.* (1993) reported that this enzyme may be partly involved in the activation mechanism for type IV collagenolysis to achieve tumor cell invasion. Menrad *et al.* (1993) reported that bestatin and amastatin, competitive inhibitors of AP-M, inhibit invasion of this enzyme-expressing metastatic melanoma cell line through the reconstituted basement membrane Matrigel in a dose-dependent manner. Thus, it is expected that AP-M inhibitors will act as anti-invasive agents for tumor cells, as well as acting as analgesic agents.

In the course of extensive screening for new AP-M inhibitors, the actinomycete *Streptomyces* sp. SL-387 (KCTC 0102BP) produced MR-387A[(2S,3R)-3-amino-2-hydroxy-4-phenylbutanoyl-valyl-prolyl-hydroxyproline] and MR-387B[(2S,3R)-3-amino-2-hydroxy-4-phenylbutanoyl-valyl-prolyl-proline] as novel AP-M inhibitors (Chung *et al.*, 1994). On the basis of both MR-387A and B structure, the novel synthetic peptide-like substance MR-387C[(2S,3R)-3-amino-2-hydroxy-4-phenylbutanoyl-L-valyl-L-prolyl-L-leucine] was obtained. This MR-387C was obtained by chance in searching molecules similar to MR-387A with the same molecuar weight. MR-387C inhibited AP-M more strongly than both MR-387A and B. The physico-chemical properties and biological activities of MR-387C are reported herein.

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Materials and Methods

General methods

Mass spectra were recorded on a Kratos Concept-1S spectrometer. NMR spectra were recorded on a Varian UNITY 300 (300MHz) spectrometer. UV spectra were recorded on a Shimazu UV-260 spectrophotometer, and IR spectra on a Laser Precision Analytical IFX-65S spectrophotometer. Amino acid analysis was performed on a Pharmacia LKB 4151 α amino acid autoanalyzer. HPLC analysis used a Tosoh TSK 6011 pump with a TSK 6041 detecter system.

Assay for AP-M and inhibitory activities

The inhibitory activity of MR-387C against AP-M was determined by the method of Umezawa *et al.* (1985). Porcine kidney AP-M was purchased from Sigma Chemical Co. (USA). A substrate solution was freshly prepared by dissolving 100 μl of L-leucine-p-nitroanilide stock solution (12.5 mg/ml in DMSO) in 10 ml of 0.1 mM Tris-HCl buffer (pH 7.0). The reaction mixture (total 200 μl) in a 96 well microplate contained 160 μl of substrate solution, 20 μl of water or aqueous solu-

Table 1. Physico-chemical properties of the synthetic substance

Appearance	Amorphous colorless powder
FAB-MS (m/z)	505 $(M+H)^+$
Color reaction	Ninhydrin
R_f value*	0.52
UV λ_{max} in MeOH (ε)	218(1,580), 252(156), 258(240), 264 (235), 268(192), 280(sh.96)
IR (cm^{-1}, KBr)	3200, 2960, 1620, 1520, 1440, 1400, 1260, 1100, 1030, 800

*On silica gel TLC plate (Merck Art No. 5715) with BuOH-MeOH-H_2O (4 : 1 : 2).

tion containing the test compound, and 20 μl of AP-M solution (final 1 mU). The microplate was incubated at 37℃. After 30 min the absorbance at 405 nm was measured using a microplate reader (Bio-Rad model 3550, USA). Percent inhibition was calculated by the formula (A-B)/A×100, where A is the measured value of the enzymatic reaction in the system without an inhibitor, and B is the value with an inhibitor. The IC_{50} value is the concentration of inhibitor which produced a 50% inhibition of enzyme activity.

Synthesis of MR-387C

MR-387C was automatically synthesized by the solid phase method (Stewart and Young, 1969) using an Applied Biosystems (Model 431A) peptide synthesizer on HMP-resin(4-hydroxy-methylphenoxymethyl copoly-styrene-1% divinylbenzene resin). The t-BOC-(2S,3R)-3-amino-2-hydroxy-4-phenylbutanoic acid(t-BOC-AHPA) used in peptide synthesis was purchased from Sigma Peptides and Amino acids (USA). Purification of the inhibitor resulting from a cocktail of synthesis reactions was carried out by HPLC on a Phenomenex ODS column ($5C_{18}HS$, φ10.6×250 mm, 2 ml/min, 30% MeCN-0.1% TFA). Active fractions were combined and concentrated under reduced pressure in a small volume of water, then lyophilized under a freeze dryer to give an amorphous white powder. The Rf value on silica gel TLC developed with a solvent system BuOH-MeOH-H_2O (4 : 1 : 2) was 0.52.

Hydrolysis of MR-387C

An HCl solution (6 N, 200 μl) was added to a solution of MR-387C (500 μg) and heated at 105℃ for 24 h in a sealed tube. The solution was evaporated to dryness. The evaporation was carried out several times following addition of H_2O to remove HCl.

Results

Physico-chemical properties of MR-387C

The physico-chemical properties of MR-387C are summarized in Table 1. The substance is soluble in water, methanol, and dimethylsulfoxide, but insoluble in acetone, ethylacetate, chloroform, and hexane.

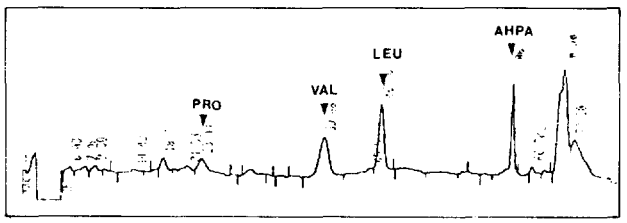

Fig. 1. Amino acid analysis of the synthetic substance.

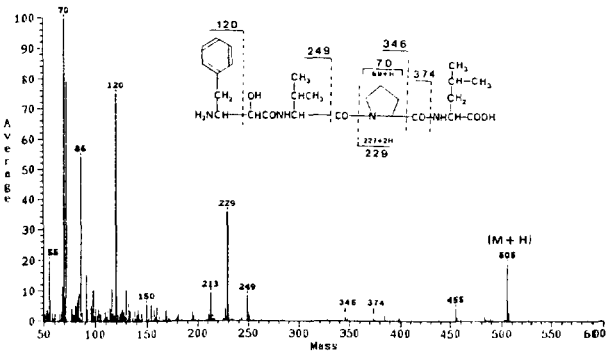

Fig. 2. FAB-MS spectrum of the synthetic substance. (Fast atom; Ar, Kinetic energy; 7 KeV).

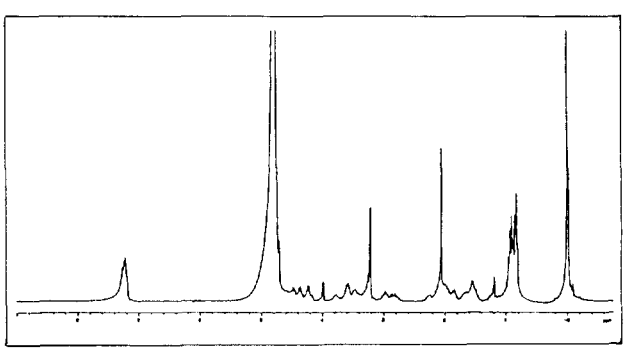

Fig. 3. ^{1}H-NMR spectrum of the synthetic substance at 300 MHz in CD_3OD.

Identification of MR-387C

Identification of the structure was carried out by amino acid analysis, FAB-MS, and ^{1}H-NMR spectroscopy. Amino acid analysis of the acid hydrolysate of the compound revealed a molar ratio of AHPA : Val : Pro : Leu of 1 : 1 : 1 : 1 (Fig. 1). The molecular weight of the compound was determined 505 (M+H) by the FAB-MS spectrum (Fig. 2). The assignment of protons by ^{1}H-NMR (Fig. 3) and ^{1}H-^{1}H COSY data of the compound is as follows: chemical shift (ppm) from TMS in CD_3OD at 300 MHz, AHPA(2-CH, 3.98d, J=2.4; 3-CH, 3.57m; 4-CH_2, 2.97dd, 2.83dd, J=8.1, 13.8; Ph-o,m,p, 7.28m); Val(α-CH, 4.21m; β-CH, 2.10m; CH_3,

Table 2. Inhibitory activities of various inhibitors against aminopeptidase M

Inhibitors	IC_{50} (μg/ml)	K_i	Reference
Actinonin	0.40	1.7×10^{-7}	Umezawa *et al.*, 1985
Bestatin	6.20	4.0×10^{-6}	Umezawa *et al.*, 1976
Leuhistin	0.20	2.3×10^{-7}	Aoyagi *et al.*, 1991
Amastatin	0.58	1.6×10^{-6}	Aoyagi *et al.*, 1978
Probestin	0.03	1.9×10^{-8}	Aoyagi *et al.*, 1990
Synthetic	0.04	3.8×10^{-8}	this experiment

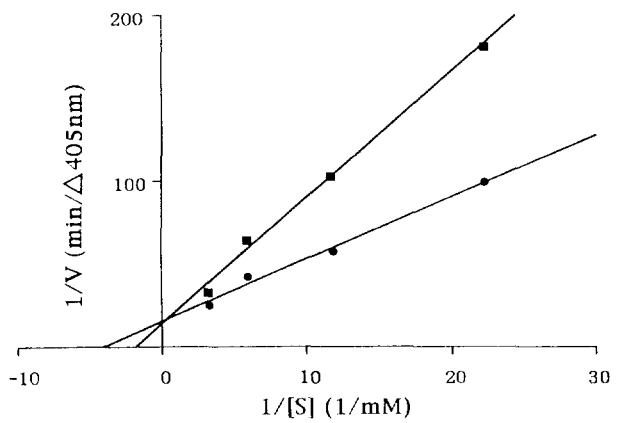

Fig. 4. Lineweaber-Burk plot of inhibition of AP-M by synthetic substance. ●–●: [I]=0 M, ■–■: [I]=49.8 nM.

0.85d, J=6.6; CH_3, 0.82d, J=6.6); Pro(α-CH, 0.45m; β-CH_2, 1.86m, 2.22m; γ-CH_2, ca. 1.93m, 2.02m; δ-CH_2, ca. 3.44m, 3.75m); and Leu(α-CH, 4.37m; β-CH_2, 1.52 m, 1.58m; γ-CH, 1.67m; CH_3, 0.88d, J=6.5; CH_3, 0.92 d, J=6.5).

The amino acid sequence of MR-387C was confirmed by the FAB-MS spectrum (Fig. 2). In the spectrum of the compound the parent peak (m/z 505, M+H) can be recognized. A mass difference of 130 between m/z 504 and m/z 374 corresponds to a loss of leucine from the C-terminus. A peak at m/z 229 was regarded as being derived from elimination of Pro-Leu. A peak at m/z 120 suggested that the N-terminal amino acid is AHPA (Suda *et al.*, 1976). From these results the amino acid sequence of the compound was confirmed to be AHPA-Val-Pro-Leu.

Biological Properties of MR-387C

The inhibitory activities of this synthetic compound and various AP-M inhibitors are shown in Table 2. The synthetic substance is a competitive inhibitor (Fig. 4). The K_i and IC_{50} values of the compound are 3.8×10^{-8} M and 0.04 μg/ml (7.9×10^{-8} M), respectively. The inhibitory activity was stronger than any other inhibitor of microbial origin, except probestin.

Discussion

Strong inhibition of AP-M requires a 2(S)-hydroxyl group in the N-terminal amino acid derivatives of its inhibitors. The 2(S)-hydroxyl group is responsible for chelation of zinc atoms at the active site of the enzyme (Nishizawa *et al.*, 1977). In order to inhibit leucine aminopeptidase, hydrophobic amino acids such as L-leucine and L-valine as the second residue from the amino end, are better than hydrophilic amino acids (Tobe *et al.*, 1982). In addition, the size of the inhibitor is important in inhibitory activity to AP-M. Rich *et al.* (1984) reported that amastatin[(2S,3R)-3-amino-2-hydroxy-5-methylhexanoyl (AHMHA)-L-valyl-L-valyl-L-aspartic acid] (Aoyagi *et al.*, 1978), a tetrapeptide, is a 100-fold stronger inhibitor of AP-M than bestatin[(2S,3 R)-AHPA-L-leucine] (Umezawa *et al.*, 1976) which is a dipeptide. They suggested that AP-M binds tri- and tetrapeptide inhibitors more strongly than dipeptide inhibitors, and that the number of amino acids in the inhibitor affects the binding tightness between the inhibitor and AP-M. The tetrapeptide MR-387C was, therefore, designed having both a 2(S)-hydroxy group in the N-terminal amino acid, and L-valine as the second residue from the amino end, on the basis of the structures of MR-387A and B isolated from the culture filtrate of *Streptomyces* sp. SL-387. The tetrapeptide MR-387C[(2S,3R)-AHPA-Val-Pro-Leu] strongly inhibited AP-M, which supports the idea that increasing the peptide chain length of the inhibitor produces more a potent inhibitor as a consequence of a slower binding process. In addition, the C-terminal amino acid of the tetrapeptide may be an important factor in the activity of inhibitors against AP-M. Probestin[(2S,3R)-AHPA-Leu-Pro-Pro] (Aoyagi *et al.*, 1990) and amastatin have proline and aspartic acid in the C-terminus, respectively. However, probestin inhibits AP-M more strongly than does amastatin (the inhibition constant, K_i is 1.9×10^{-8} M in probestin and 1.6×10^{-6} M in amastatin). MR-387 C with leucine in the C-terminus also inhibits AP-M more strongly than does amastatin.

Amastatin, bestatin, actinonin (Umezawa *et al.*, 19 85), probestin, and leuhistin (Aoyagi *et al.*, 1991) are specific inhibitors isolated from culture broths of microorganisms. In the course of searching for AP-M inhibitors from soil microorganisms, the new AP-M inhibitors MR-387A[(2S,3R)-AHPA-Val-Pro-Hyp] and B[(2S, 3R)-AHPA-Val-Pro-Pro], from the culture broth of *Streptomyces* sp. SL-387 (KCTC0102BP)(Chung *et al.*, 1994) were discovered. On the basis of the MR-387 structure, the novel MR-387C, (2S,3R)-AHPA-Val-Pro-Leu was designed. This specific inhibitor is expected to contribute greatly of studies on the various peptide

processing and disease processes of AP-M.

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