NOTES

MR-387A and B, New Aminopeptidase N Inhibitors, Produced by *Streptomyces neyagawaensis* SL-387

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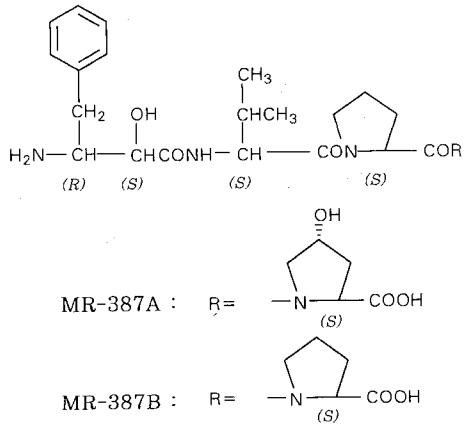
Aminopeptidase N (AP-N, or aminopeptidase M, EC 3.4.11.2) is a member of a family of membrane-bound metallopeptidases with wide distribution on the surfaces of diverse cell types.[1] This enzyme plays the part of an enzyme which inactivates enkephalins, the pentapetides with morphine-like activities in cerebral membranes.[2] In recent years, the human myeloid plasma membrane glycoprotein CD13 has been proved to be identical to AP-N,[3] and it is expressed in human myeloid leukemia cell lines[4] and malignant mesenchymal tumors.[5] SAIKI *et al.*[6] and MENRAD *et al.*[7] have found aminopeptidase N to play important roles in the invasion of metastatic tumors *in vitro*. Recently, INO *et al.*[8] also suggested that AP-N inhibitors such as bestatin and actinonin could become new drugs for cancer treatment, not as biological response modifiers (BRM), but as anti-cancer chemicals exerting direct suppressive action on the growth or metastasis of certain cancers. Thus, the specific inhibition of AP-N could be a new approach to suppress the growth or metastasis of cancer.

During the screening for new inhibitors of aminopeptidase N, we obtained new substances MR-387A and B (Fig. 1) from the culture broth of *Streptomyces neyagawaensis* SL-387. In this communication, we report the isolation, physico-chemical properties, structure elucidation and biological activities of the inhibitors.

Fig. 1. Structures of MR-387A and B.



Strain SL-387 was isolated from a soil sample collected in the province of Wolchoolsan National Park, Chullanam-do, Korea. This strain was identified by taxonomic characterization as a strain of *Streptomyces neyagawaensis*.[9] It has been deposited in the Korean Collection for Type Cultures (KCTC), Korea Research Institute of Bioscience and Biotechnology, KIST, Taejon, Korea, under the accession No. KCTC 0102BP.

One loopful of spores of strain SL-387 was inoculated into 50 ml of a production medium consisting of glucose 1.0%, soybean meal 3.0%, yeast extract 0.2%, beef extract 0.1%, K_2HPO_4 0.01%, NaCl 0.3%, $CaCO_3$ 0.3%, $MgSO_4 \cdot 7H_2O$ 0.0005%, $MnCl_2 \cdot 4H_2O$ 0.001% and $ZnCl_2 \cdot 7H_2O$ 0.001% in a 250-ml Erlenmeyer flask (pH 7.0 before sterilization). It was cultured at 28°C for 2 days on a rotary shaker (200 rpm) to obtain a seed culture. This seed culture was inoculated into the same medium (3 liters) in 5-liter jar fermenter sterilized at 120°C for 20 minutes. Fermentation was carried out for 4 days at 28°C with an agitation rate of 200 rpm and air flow rate of 1 vvm.

The isolation of MR-387 was monitored by measuring of the inhibitory activity against AP-N from microsomal membranes of porcine kidney (purchased from Sigma Co., L-0632). AP-N activity was measured by the method of UMEZAWA *et al.*[10] except for using a 96-well microplate reader (Bio-Rad Model 3550, U.S.A.). The culture filtrate (10 liters) was passed through Diaion HP-20 (1.5 liters). The column was eluted with 80% MeOH (5 liters) to give active fractions. The active eluate was concentrated under reduced pressure to remove MeOH and the resulting aqueous solution was extracted with BuOH (equal volume) in pH 2.0. The BuOH layer was concentrated under reduced pressure to obtain a crude brownish powder. The powder was loaded on a column of silica gel (× 50 w/w powder) which had been packed with a solvent mixture of BuOAc-BuOH-AcOH-H_2O (2:4:1:1), followed by developing with the same solvent mixture. The active eluate was subjected to column chromatography on a column of MCI gel CHP-20P (Mitsubishi Kasei Co., 3.0 × 20 cm) which had been packed with H_2O. The active fractions were eluted gradiently with 0~70% MeOH. The eluate fractions containing MR-387 was subjected to Sephadex LH-20 column (2.8 × 90 cm) chromatography and then developed with MeOH. The active fractions were dissolved in 22% aq. MeCN containing 0.1% trifluoroacetic acid (TFA) and applied to medium pressure liquid chromatography on a packed column of Lobar (Merck Art. 10625, 2.5 × 31 cm, RP-18) and then developed with the same solvent mixture. The two active eluates were concentrated under reduced pressure to afford a colorless powder. The

powder was further purified by a reversed phase HPLC using a Phenomenex Bondclone $10C_{18}$ column (7.6 × 300 mm, flow rate 2.0 ml/minute, Tosoh HPLC system equipped with a TSK-6011 pump and a TSK-6041 detector) with a solvent mixture of 35% MeOH. The active fractions were collected and concentrated under reduced pressure to remove MeOH. The concentrated solutions were lyophilized under freeze-dryer system to give MR-387A (3.6 mg) and B (2.0 mg) as a colorless powder.

The physico-chemical properties of MR-387 are summarized in Table 1. The MW and formula of

Table 1. Physico-chemical properties of MR-387A and B.

	MR-387A	MR-387B
Appearance	Amorphous white powder	Amorphous white powder
FAB-MS (*m/z*)	505 $(M+H)^+$	489 $(M+H)^+$
Molecular formula	$C_{25}H_{36}N_4O_7$	$C_{25}H_{36}N_4O_6$
HRFAB-MS $(M+H)^+$		
Found:	505.2672	489.2711
Calcd:	505.2622	489.2713
CD λ_{ext} nm($\Delta\varepsilon$) in MeOH	258 (−0.03), 245 (−0.09), 230 (−0.38), 222 (0), 217 (+0.21), 209 (0), 207 (−0.04)	270 (+0.22), 261 (+0.04), 240 (0), 229 (−0.12), 220 (0), 210 (−0.27), 204 (0)
UV λ_{max}^{MeOH} nm (ε)	253 (204), 258 (210), 263 (195), 268 (184), 280 (sh. 143)	252 (203), 258 (213), 263 (206), 267 (sh. 180), 280 (sh. 141)
IR ν_{max}^{KBr} cm^{-1}	3400, 2964, 1670, 1535, 1457, 1203, 1141	3421, 2965, 1675, 1529, 1452, 1205, 1137
Rf value*	0.35	0.42
Color reaction	Ninhydrin	Ninhydrin
Solubility		
Soluble:	H_2O, MeOH, EtOH, DMSO	H_2O, MeOH, EtOH, DMSO
Slightly soluble:	BuOH	BuOH
Insoluble:	$CHCl_3$, Hexane, EtOAc	$CHCl_3$, Hexane, EtOAc

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

Table 2. ^1H and ^{13}C NMR data for MR-387A and B in D_2O.

Assgnment		MR-387A		MR-387B	
		^1H[a]	^{13}C[b]	^1H[a]	^{13}C[b]
AHPA	CO		171.2[c]		172.1[e]
	2-CH	4.11 (d, J=3.9)	69.5	4.25 (d, J=5.1)	70.6
	3-CH	3.65 (m)	56.8	3.81 (m)	55.6
	4-CH_2	2.82 (dd, J=7.8, 13.5)	36.7	2.96 (dd, J=8.1, 13.8)	35.9
		2.94 (dd, J=7.8, 13.5)		3.09 (dd, J=8.1, 13.8)	
	Ph-*i*		133.4		136.8
	Ph-*o*	*ca.* 7.33 (m)	129.0[d]	*ca.* 7.33 (m)	130.2[f]
	Ph-*m*		129.2[d]		130.3[f]
	Ph-*p*		127.8		127.8
Val	CO		171.9[c]		172.3[e]
	α-CH	4.39 (d, J=6.9)	54.5	4.38 (d, J=3.6)	53.6
	β-CH	2.08 (m)	29.7	2.08 (m)	30.7
	γ-CH_3	0.92 (d, J=6.6)	18.1	0.98 (d, J=6.6)	18.4
	CH_3	0.97 (d, J=6.6)	17.3	0.93 (d, J=6.6)	19.2
Pro[1]	CO		172.3[c]		173.2[e]
	α-CH	4.63 (m)	58.8	4.66 (m)	57.8
	β-CH_2	1.94 (m), 2.28 (m)	27.8	1.94 (m), 2.34 (m)	29.0
	γ-CH_2	*ca.* 1.98 (m), 2.05 (m)	23.3	*ca.* 1.98 (m), 2.01 (m)	25.4
	δ-CH_2	*ca.* 3.56 (m), 3.88 (m)	48.2	*ca.* 3.65 (m), 3.89 (m)	48.4
Hyp	CO		172.7[c]		
	α-CH	4.30 (t, J=7.8)	58.8	—	—
	β-CH_2	*ca.* 1.98 (m), 2.22 (m)	38.3	—	—
	γ-CH	4.51 (m)	69.1	—	—
	δ-CH_2	3.74 (m)	50.4	—	—
Pro[2]	CO	—	—		177.8
	α-CH	—	—	4.36 (m)	59.9
	β-CH_2	—	—	*ca.* 1.98 (m), 2.28 (m)	30.1
	γ-CH_2	—	—	*ca.* 1.98 (m), 2.01 (m)	25.7
	δ-CH_2	—	—	*ca.* 3.65 (m), 3.89 (m)	48.4

[a] 300 MHz; δ in ppm, J in Hz.
[b] 125 MHz; δ in ppm.
[c,d,e,f] Assignment could be interchanged.

Fig. 2. The ^1H-^1H COSY and the selected 2-D ROESY data of MR-387A at $-10°C$ in CD_3OH (pH 3.6).

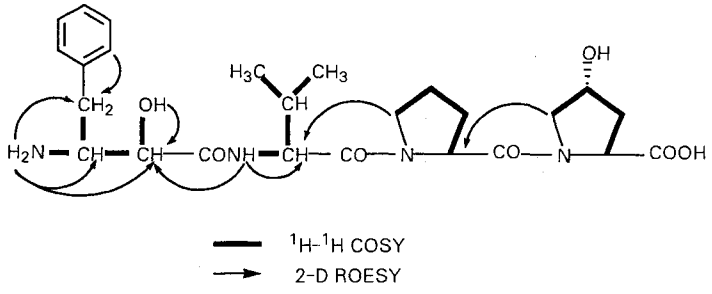


Table 3. Inhibition of aminopeptidases by MR-387A and B.

Target enzyme	Enzyme source	Substrate	Enzyme activity (nmole/hour/2 × 10^5 cells)	IC_{50} (μM)	
				MR-387A	MR-387B
AP-N	Porcine kidney microsome	L-Leu-*p*NA	104.2*	0.198	0.164
AP-N	Human fibrosarcoma HT1080	L-Leu-*p*NA	37.6	0.218	0.201
AP-N	Human myelogenous leukemia K562	L-Leu-*p*NA	36.8	17.0	4.6
AP-B	Human myelogenous leukemia K562	L-Lys-*p*NA	21.8	0.651	0.260

* nmole/hour/test, L-Leu-*p*NA; L-leucine-*p*-nitroanilide, L-Lys-*p*NA; L-lysine-*p*-nitroanilide. Cells were cultured for 48 hours, and 200 μM substrate was added, followed by incubation at 37°C for 1 hour. Enzyme activity was detected spectrophotometrically by monitoring the increase in optical density at 405 nm.

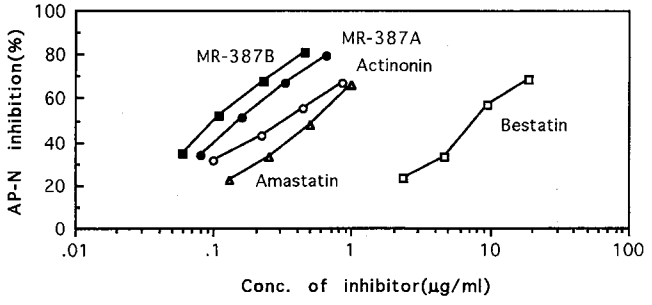
MR-387A and B were determined to be $C_{25}H_{36}N_4O_7$ (MW 504.26) and $C_{25}H_{36}N_4O_6$ (MW 488.27), respectively by electrospray ionization (ESI)-MS and HRFAB-MS. The UV spectra of these compounds suggested the presence of a phenyl group in both molecules. The IR (KBr) spectra of MR-387A and B showed the absorption of peptide bonds (near 1670 and 1530 cm^{-1}). A positive color reaction with ninhydrin reagent suggested the presence of an amino group in both molecules. Amino acid analysis of the acid hydrolysates of the compounds suggested that the amino acid constitutions are valine (Val, 1), proline (Pro, 1), hydroxyproline (Hyp, 1) and *threo*-3-amino-2-hydroxy-4-phenylbutanoic acid (AHPA, 1)[11] for MR-387A, and Val(1), Pro(2) and AHPA(1) for MR-387B, respectively.

The amino acid mixtures obtained by acid hydrolysis of MR-387A and B were L-phenylalanylated by the procedure of YOSHIDA *et al.*[12] The resulting dipeptides were compared by HPLC (Cosmosil $5C_{18}$) with reference compounds. As a result, the existance of L-valine, L-4-hydroxyproline and (2*S*,3*R*)-AHPA were confirmed. The absolute configurations of the proline residues were determined by HPTLC on a Merck CHIR pre-coated plate eluting with MeOH - H_2O - MeCN (1 : 1 : 4) as L.

The ^1H and ^{13}C NMR data on MR-387A and B are presented in Table 2. The assignment of proton and carbon signals was determined by the ^1H-^1H COSY (Fig. 2), the total shift correlation spectroscopy (TOCSY) and the DEPT. These data supported the presence of the amino acids which were detected by the amino acid analysis. The amino acid sequence of MR-387A was determined by the FAB-MS fragmentation study and the two-dimensional rotating-frame NOE spectroscopy (ROESY) data (Fig. 2) at $-10°C$ in CD_3OH. At the condition of $-10°C$ in CD_3OH (pH 3.6), the protons of NH_2 (δ 8.02 ppm, m) and OH (δ 6.91 ppm, d) of AHPA and NH (δ 8.05 ppm, d) of Val were detected. However, as the temperature was raised, these peaks gradually disappeared. As shown in Fig. 2, NOEs between 2-CH of AHPA and HN of valine, between α-CH of valine and δ-CH_2 of proline, and between α-CH of proline and δ-CH_2 of hydroxyproline were detected, suggesting that the amino acid sequence of MR-387A is AHPA-Val-Pro-Hyp. The linkage between Pro and Hyp was confirmed to be *trans* by the presence of NOE between α-CH of proline and δ-CH_2 of hydroxyproline. The relative configuration of 4-hydroxyproline was deduced by using vicinal ^1H-^1H coupling constants and some clues such as 1 : 2 : 1 triplet (J = 7.8 Hz) of 2-H and band overlapping of 4-H in *N*-protected Hyp as 2,4-*trans*.[13,14] The structure of MR-387A was determined to be (2*S*,3*R*)-3-amino-2-hydroxy-4-phenylbutanoyl-L-valyl-L-prolyl-(2,4-*trans*)-L-4-hydroxyproline. Furthermore, the structure of MR-387B was confirmed by NMR, FAB-MS fragmentation studies and the chemical synthesis to be (2*S*,3*R*)-3-amino-2-hydroxy-4-phenylbutanoyl-L-valyl-L-prolyl-L-proline. MR-387A and B are related to probestin isolated from the culture filtrate of *Streptomyces azureus*.[15] The difference is that the second amino acid leucine and the C-terminal proline are replaced by valine and 4-hydroxyproline, respectively for MR-387A. Replacement of leucine in probestin by valine is the only difference between probestin and MR-387B.

The compounds were tested for inhibition of some cell

Fig. 3. Comparison of inhibitory activities of various AP-N inhibitors against porcine kidney AP-N.



surface ectoenzymes. As shown in Table 3, MR-387A and B inhibited not only AP-N from porcine kidney microsomes (Sigma, L-0632) but also AP-N of human fibrosarcoma HT1080 and human myelogenous leukemia K562 cell lines. These compounds also inhibited AP-B of human myelogenous leukemia K562 cell line with IC_{50} values in the nanomolar range. Fig. 3 showed the comparison of the inhibitory activities of MR-387 with some inhibitors of aminopeptidases. MR-387A and B were competitive with the substrate L-leucine-*p*-nitroanilide. These inhibitors had no antimicrobial activity at 100 μg/ml.

Acknowledgments

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