Inhibition of Aminopeptidase N by 2-Hydroxy-3-amino-4-(*p*-nitrophenyl)butyryl Peptide Derivatives

Myung-Chul Chung, Choong-Hwan Lee, Ho-Jae Lee, Hyo-Kon Chun and Yung-Hee Kho*

Enzyme Inhibition Research Unit, Korea Research Institute of Bioscience and Biotechnology, P.O. Box 115, Yusong, Taejon 305-600, Korea

Received October 17, 1998

To investigate the inhibitory activity of 2-hydroxy-3-amino-4-phenylbutyrate-harboring aminopeptidase N inhibitors, *p*-nitro-AHPA-peptide derivatives (1 and 2) and an AHPA-peptide derivative (3) were synthesized by chain elongation from C-terminal end using DCC/HOBt as a coupling reagent. The peptides 1~3 exerted strong inhibitory activities against aminopeptidase N with IC_{50} values of 1.8, 7.3 and 24.0 μg/ml, respectively, and cytotoxicity on cancer cell lines *in vitro*.

Key words : *aminopeptidase N, inhibitor, peptides, cytotoxicity.*

Since aminopeptidase N (EC 3.4.11.2) plays an important role in invasion of metastatic tumors *in vitro*,[1,2] a number of inhibitors have been developed for medicinal application.[3] In an effort to develop AP-N inhibitors, we have isolated MR-387A and B from the culture filtrate of *Streptomyces neyagawaensis* SL-387.[4] The structures of MR-387A and B were determined to be (2S,3*R*)-2-hydroxy-3-amino-4-phenylbutyryl-L-valyl-L-prolyl-(2,4-*trans*)-L-hydroxyproline and (2S,3*R*)-2-hydroxy-3-amino-4-phenylbutyryl-L-valyl-L-prolyl-L-proline, respectively. Their inhibitory activities against AP-N exhibited IC_{50} values of nanomolar range (approximately 200 nM).[4] In a continuing effort to develop strong AP-N inhibitors, *p*-nitro-AHPA-peptide derivatives (**1** and **2**) and an AHPA-peptide derivative (**3**) were synthesized by chain elongation from C-terminal end (Fig. 1). Compared to MR-387A and B, compounds **1~3** are revealed to be more active against AP-N. In this paper, synthesis and biological activity of the compounds are reported.

Experimental Method

NMR spectroscopy was carried out on a Varian UNITY 300 spectrometer. Optical rotations were measured on a Polartronic Universal polarimeter (Schmidt and Haensch, Germany). *Boc*-(*p*-NO_2)-(2*S*,3*R*)-AHPA, *Boc*-(2*S*,3*R*)-AHPA, *Boc*-Val, Val-OBz and Pro-OBz were purchased from Sigma Chemical.

Synthesis of compounds 1~3. Synthesis of *p*-nitro-AHPA-peptide derivatives (**1** and **2**) and AHPA-peptide derivative (**3**) were synthesized by chain elongation from C-terminal end using DCC and HOBt as coupling reagents (Fig. 2). The corresponding O-benzyl amino acid (10 mmole) was dissolved in methylene chloride (20 ml) then in triethylamine (1.4 ml). The corresponding *Boc*-

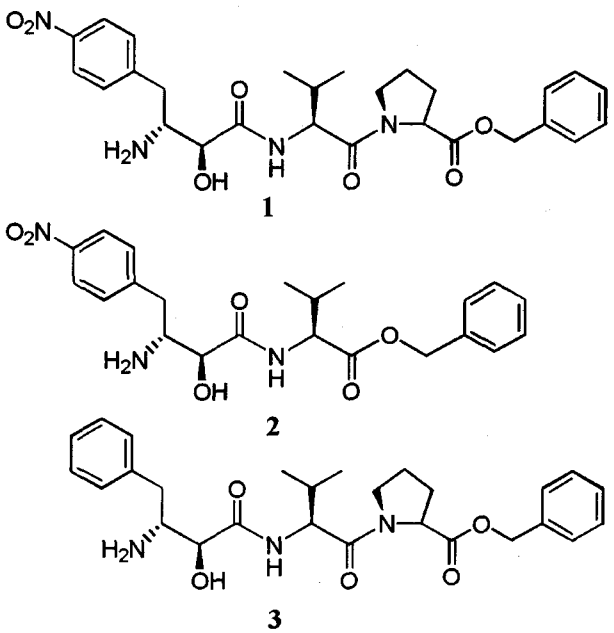

Fig. 1. Structures of 1~3.

*Corresponding author
Phone: 82-42-860-4350; Fax: 82-42-860-4595
E-mail: yhkho@kribb4680.kribb.re.kr

Abbreviations: AHPA, 2-hydroxy-3-amino-4-phenylbutyrate; AP-N, aminopeptidase N; DCC, dicyclohexylcarbodiimide; HOBt, 1-hydroxybenzotriazole; TFA, trifluroacetic acid.

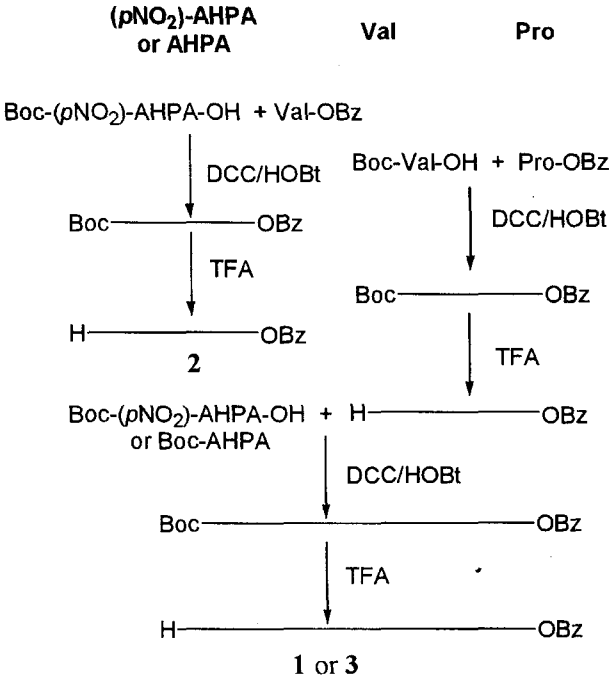

Fig. 2. Synthetic scheme of aminopeptidase N inhibitors (1~3) by chain elongation from C-terminal.

amino acid (10 mmole) and HOBt (12 mmole) were added at 0°C into the solution, followed by the addition of DCC (12 mmole). The reaction mixture was stirred at 0°C for 1 hour then at room temperature overnight. The resulting dicyclohexylurea was filtered. The filtrate was concentrated to dryness and was dissolved in benzene then filtered. It was reconcentrated and purified by silica gel chromatography with a solvent system (chloroform : methanol=20 : 1). The *Boc* group was removed by the addition of TFA (5 ml) for 20 min at room temperature. Di- and tripeptide benzylesters were synthesized as described above and were purified by HPLC with gradient elution from 20 to 60% acetonitrile containing 0.1% TFA (column, YMC-ODS-A, ø 4.5 ×250 mm; detector: UV 210 nm).

H-(*p*-NO$_2$)-AHPA-Val-Pro-OBz (1). ^{1}H NMR (δ, ppm) in CD_3OD : 0.96~1.00 (6H, *dd*), 1.91~2.11 (4H, *m*), 2.25 (1H, *m*), 3.05 (1H, *m*), 3.23 (1H, *dd*), 3.68 (1H, *m*), 3.82 (1H, *m*), 3.91 (1H, *m*), 4.10 (1H, *d*), 4.40 (1H, *d*), 4.46 (1H, *dd*), 5.13 (2H, *s*), 7.28~7.35 (5H, *m*), 7.55 (2H, *d*) and 8.23 (2H, *d*); FAB-MS (in glycerol) : 543 $(M+H)^+$; R_f 0.80 (BuOH-AcOH-H_2O=4 : 1 :); $[\alpha]_D^{22}$ -9.6° (*c* 1.46, MeOH).

H-(*p*-NO$_2$)-AHPA-Val-OBz (2). ^{1}H NMR (δ, ppm) in CD_3OD : 0.91~0.95 (6H, *dd*), 2.20 (1H, *m*), 2.80 (1H, *dd*), 3.02 (1H, *dd*), 3.93 (1H, *d*), 4.37 (1H, *d*), 5.16 (2H, *dd*), 7.28~7.35 (5H, *m*), 7.47 (2H, *d*) and 8.17 (2H, *d*); FAB-MS (in glycerol): 446 $(M+H)^+$; R_f 0.73 (BuOH-AcOH-H_2O=4 : 1 : 1); $[\alpha]_D^{22}$ -53.0° (c 0.60, MeOH).

H-AHPA-Val-Pro-OBz (3). ^{1}H NMR (δ, ppm) in CD_3OD: 0.96~1.01 (6H, *dd*), 1.91~2.11 (3H, *m*), 2.23 (1H, *m*), 2.90 (1H, *dd*), 3.08 (1H, *dd*), 3.63~3.77 (2H, *m*), 3.91 (1H, *m*), 4.10 (1H, *d*), 4.39 (1H, *d*), 4.52 (1H, *dd*), 5.13 (2H, *s*) and 7.25~7.38 (10H, *m*); FAB-MS (in glycerol) : 498 $(M+H)^+$; R_f 0.77 (BuOH-AcOH-H_2O=4 : 1 : 1); $[\alpha]_D^{22}$ -86.2° (c 1.19, MeOH).

Biological Activity. The activity of AP-N from porcine kidney microsome (Sigma L-0632) was spectrophotometrically measured at 405 nm on a Biorad microplate reader (Model 3550) using synthetic substrate L-leucine-*p*-nitroanilide.[4] For melanoma B16F1 AP-N assay, the cell line was cultured for 48 h, before 200 μM of L-leucine-*p*-nitroanilide as a substrate and the test compound were added. The mixture was then incubated at 37°C for one h. Enzyme activity was measured by observing the increase in optical density at 405 nm.[5] The *in vitro* cytotoxicity assay against the panels of human cancer cell lines was conducted according to the National Cancer Institute protocol.[6] Briefly, various human cancer cells were precultured in 96-well plates (3×10^3 cells/well) with 180 μl of RPMI 1640 containing 5% fetal bovine serum for 24 h. This was then added to the diluted sample solution to a final volume of 200 μl and cultured under 5% CO_2 at 37°C for 48 h. After fixing with 10% trichloroacetic acid, the cells were stained with 0.4% sulforhoamine. The dye was extracted from the stained cells with 10 mM tris(hydroxymethyl) aminomethane solution. The absorbance of the extract was read at 570 nm.

Results and Discussion

The di- and tripeptides (**2** and **1**) containing *p*-nitro-AHPA were synthesized using optically pure *Boc*-(2*S*, 3*R*)-*p*-nitro-AHPA by the stepwise procedure outlined in Fig. 2. The tripeptide (**3**) without *para*-substitution of a nitro group in AHPA was also synthesized by the method described above. The peptides **1** and **2** exerted strong inhibitory activities against aminopeptidase N with IC_{50} values of 1.8 (3.3 nM) and 7.3 ng/ml (16.4 nM), respectively. Compared to MR-387A and B, AP-N inhibitors of microbial origin with IC_{50} of about 0.1 μg/ml (200 nM), **1** and **2** were revealed to be more active (60 and 12

Table 1. Inhibition of aminopeptidase N from porcine kidney microsome and melanoma B16F1 cell surface.

Compound	IC_{50} (ng/ml)	
	Porcine kidney	Melanoma B16F1[a]
1	1.8	0.85
2	7.3	0.25
3	24.0	3.80
Bestatin[b]	7000	>50
Actinonin[b]	400	7.3

[a]Cell lines were cultured for 48 h, before 200 μM of L-leucine-*p*-nitroanilide as a substrate and test compound (200 μl final volume) were added, and the mixture was incubated at 37°C for 1 h. Enzyme activity was evaluated by the increase in optical density at 405 nm.

[b]Bestatin and actinomin were purchased from Sigma Chemical (USA).

Table 2. ***In vitro*** **cytotoxicity of 1~3 on human cancer cell lines.**

Cell lines	Source	ED_{50} (μg/ml)[a]			
		1	2	3	adriamycin
WE620	colon	11.11	27.14	26.52	0.340
HCT15	colon	22.40	4.12	2.50	0.540
PC-3	prostate	22.76	17.61	16.16	0.570
NCI-H23	lung	1.18	0.30	<0.30	0.150
A549	lung	11.91	11.20	4.79	0.230
MOLT-4F	leukemia	8.34	6.34	4.22	0.049
MIA-Paca-2	pancreas	4.72	6.03	1.49	0.220
LOX-IMVI	melanoma	8.38	7.08	6.37	0.089

[a]ED_{50} value against each cell lines, which was defined as a concentration that caused 50% inhibition of the cell growth *in vitro*.

times, respectively) against AP-N from porcine kidney microsome. Also **1** and **2** strongly inhibited AP-N of melanoma B16 F1 cell surface with IC_{50} of 0.85 (1.6 nM) and 0.25 ng/ml (0.6 nM), respectively (Table 1). Peptides **3** and bestatin[7] without para substitution of a nitro group in AHPA as well as actinonin, an AP-N inhibitor of fungal origin,[8] were shown to be weaker than **1** and **2**. These results coincided with the result that *p*-hydroxyubenimex was more active than ubenimex (bestatin) against aminopeptidase B.[9]

In the *in vitro* cytotoxicity assay, peptides **1~3** had a strong cytotoxicity against human cancer cell lines exhibiting strong cytotoxicity against the growth of NCI-H 23, MOLT-4F, MIA-Paca-2 and LOX-IMVI with ED_{50} values ranging from 0.30 to 8.34 μg/ml (Table 2).

Acknowledgments. This research was made partially as a result of the G7 project supported by the Ministry of Science and Technology of Korea. We are indebted to the research group of Dr. Hwan-Mook Kim of the Korea Research Institute of Bioscience and Biotechnology for cytotoxicity assay.

References

1. Menrad, A., Speicher, D., Wacker, J. and Herlyn, M. (1993) Biochemical and functional characterization of aminopeptidase N expressed by human melanoma cell. *Cancer Res.* **53**, 1450-1455.
2. Fujii, H., Nakajima, M., Saiki, I., Yoneda, J., Azuma, I. and Tsuruo, T. (1995) Human melanoma invasion and metastasis enhancement by high expression of aminopeptidase N/CD13. *Clin. Exp. Metastasis* **13**, 337-344.
3. Aoyagi, T. (1989) Protease inhibitors and biological control. In *Bioactive metabolites from microorganisms,* Bushell M. E. and Grafe U. (eds.) *Progress in Industrial Microbiology* vol. **27**, pp. 403-418, Elsevier, Amsterdam.
4. Chung, M. C., Chun, H. K., Han, K. H., Lee, H. J., Lee, C. H. and Kho, Y. H. (1996) MR-387A and B, new aminopeptidase N inhibitors, produced by *Streptomyces neyagawaensis* SL-387. *J. Antibiotics* **49**, 99-102.
5. Chung, M. C., Lee, H. J., Chun, H. K., Lee, C. H., Kim, S. I. and Kho, Y. H. (1996) Bestatin analogue from *Streptomyces neyagawaensis* SL-387. *Biosci. Biochem. Biotechnol.* **60**, 898-900.
6. Skehan, P., Storeng, R., Scudiero, D., Monks, A., McMahon J., Vostica, D., Warren, J. T., Bokesch, H., Kenny, S. and Boyd, M. R. (1990) New colorimetric cytotoxicity assay for anticancer-drug screening. *J. Natl. Cancer Inst.* **82**, 1107-1112.
7. Umezawa, H., Aoyagi, T., Suda, H., Hamada, M. and Takeuchi, T. (1976) Bestatin, an inhibitor of aminopeptidase B, produced by actinomycetes. *J. Antibiotics* **29**, 97-99.
8. Umezawa, H., Aoyagi, T., Tanaka, T., Suda, H., Okuyama, A., Naganawa, H., Hamada, M. and Takeuchi, T. (1985) Production of actinonin, an inhibitor of aminopeptidase M, by actinomycetes. *J. Antibiotics* **38**, 1629-1630.
9. Saino, T., Seya, K., Nishizawa, R., Takata, T., Aoyagi, T. and Umezawa, H. (1987) Synthesis of *p*-hydroxyubenimex. *J. Antibiotics* **40**, 1165-1169.