N-ACYLATED DERIVATIVES OF A PEPTIDE OBTAINED BY ENZYMATIC DEGRADATION OF PEPSTATINS

Sir:

Naturally occurring pepstatins have been shown to possess two moles of an interesting amino acid, 4-amino-3-hydroxy-6-methylheptanoic acid (AHMHA), and one of them which exists between valine and alanine moieties is involved in their action against acid proteases, and the presence of the hydroxyl group of this amino acid is of importance.[5] Moreover, as reported by AOYAGI *et al.*[6] renin inhibitory activity of pepstatins increases with the increase of the number of carbon atoms in the acyl moiety, although the activity against pepsin and cathepsin D is not influenced by this number of carbon atoms. In addition, more water-soluble pepstatins are required for *in vivo* experiments, because these inhibitors are not soluble enough for this purpose. These have aroused our interest in coupling various kinds of acyl groups to the N-terminal of the tetrapeptide which lacks acylvalyl residue in pepstatins and which is obtained by enzymatic hydrolysis of pepstatins as described in previous papers.[7,8] In this communication we report synthesis of N-acyl derivatives of the tetrapeptide, L-valyl-4-amino-3-hydroxy-6-methylheptanoyl-L-alanyl-4-amino-3-hydroxy-6-methylheptanoic acid (Val-AHMHA-Ala-AHMHA), and their activities in inhibiting proteases.

Acetyl-Val-AHMHA-Ala-AHMHA (**I**), benzoyl-Val-AHMHA-Ala-AHMHA (**V**) and phenoxyacetyl-Val-AHMHA-Ala-AHMHA (**VI**) (Table 1) were synthesized by acylation of N-terminal of Val-AHMHA-Ala-AHMHA with acid anhydrides, and *iso*-butyryl-Val-AHMHA-Ala-AHMHA (**II**), *iso*-valeryl-Val-AHMHA-Ala-AHMHA (**III**), palmitoyl-Val-AHMHA-Ala-AHMHA (**IV**) and 2-phenoxypropionyl-Val-AHMHA-Ala-AHMHA (**VII**) (Table 1) were obtained by reaction with the corresponding acid chlorides. Val-AHMHA-Ala-AHMHA was obtained as described in the previous paper.[7]

Val-AHMHA-Ala-AHMHA (100 mg, 0.2 mmoles) in absolute methanol (4 ml) was reacted with acetic anhydride (24 mg, 0.24 mmoles) in absolute methanol (1 ml) at 5°~7°C for 20 hours. The reaction was terminated by addition of water (5 ml) and the methanol was evaporated. After acetic acid was removed by ether washing, the major product was extracted with *n*-butanol. A residue that resulted from the evaporation of the *n*-butanol was dissolved in methanol and subjected to preparative thin-layer chromatography on a silica gel plate (E. Merck Silica gel G) using chloroform-methanol-acetic acid (80:20:2). The migration of the product on the chromatogram was detected by spraying water, *i.e.* a band which was not wetted was shown by this method. Extraction of the product with methanol from the corresponding band, followed by Sephadex LH-20 (1.5 × 80 cm) gel filtration in methanol, gave a white powder of **I** (52 mg) in 48 % yield. Treatment of Val-AHMHA-Ala-AHMHA

Table 1. Properties of N-acylated tetrapeptides.

No.	N-Acylated derivatives	M.W.	m.p. (°C)	Rf-values	
				(I)	(II)
I	Acetyl-Val-AHMHA-Ala-AHMHA	544	122~124	0.09	0.05
II	*iso*-Butyryl-Val-AHMHA-Ala-AHMHA	572	125~128	0.17	0.12
III	*iso*-Valeryl-Val-AHMHA-Ala-AHMHA	586	128~132	0.19	0.15
IV	Palmitoyl-Val-AHMHA-Ala-AHMHA	740	207~210	0.34	0.17
V	Benzoyl-Val-AHMHA-Ala-AHMHA	606	123~125	0.24	0.21
VI	Phenoxyacetyl-Val-AHMHA-Ala-AHMHA	636	110~113	0.19	0.18
VII	2-Phenoxypropionyl-Val-AHMHA-Ala-AHMHA	650	103~105	0.24	0.21

* Solvents (I) Chloroform-methanol-acetic acid (100:10:2) (II) Butyl acetate-*n*-butanol-acetic acid-water (100 : 30 : 4 : 2)
Silica gel GF (E. Merck).

with 1.2 equivalent of benzoyl anhydride under the same conditions and isolation of the product by Sephadex LH-20 gel filtration in methanol afforded **V** in 26 % yield. Acylation of the peptide with 1.5 equivalent of phenoxyacetic anhydride in dioxane at room temperature for 18 hours followed by isolation of the product by removal of phenoxyacetic acid with benzene extraction, concentation of the aqueous layer to give white powder, and recrystallization in water, gave colorless crystals of **VI** in 36 % yield.

Val-AHMHA-Ala-AHMHA (100 mg, 0.2 mmoles) dissolved in water (4 ml), the pH being adjusted to 8.5 with 1 N NaOH, was acylated by dropwise addition of *iso*-valeryl chloride (120 mg, 1 mmole) in acetone (2 ml) at 5°~7°C for 5 hours. The pH of the reaction mixture was maintained at 8.5 with NaOH using a pH-stat during the acylation. After free *iso*-valeric acid was removed by ether extraction at pH 1.8, the product was extracted with *n*-butanol. A residue that resulted from evaporation of *n*-butanol was dissolved in a mixture of methanol and water, and treated with Dowex 50 (×8) (10 ml) to remove unreacted peptide. Evaporation of methanol and water gave 69 mg of **III** in 59 % yield. **II** was obtained by acylating the peptide with *iso*-butyryl chloride similarly, in 65 % yield. Treatment of the peptide with 5 equivalents of palmitoyl chloride at room temperature for 5 hours, followed by isolation of the major product by preparative thin-layer chromatography on a silica gel plate with a solvent of chloroform-methanol (10:1), and Sephadex LH-20 gel filtration in methanol, gave **IV** in 40.8 % yield. Similarly treatment of the peptide with one equivalent of 2-phenoxypropionyl chloride at room temperature for 4 hours and isolation of the product by Sephadex LH-20 gel filtration in methanol provided **VII** in 75.7 % yield.

The structures of the derivatives were confirmed by NMR and gas chromatography after acid hydrolysis. Molecular weights, melting points and Rf values in thin-layer chromatography are summarized in Table 1. Table 2 shows the inhibitory activities (ID_{50}) of these newly obtained compounds, Val-AHMHA-Ala-AHMHA and pepstatin A against pepsin, cathepsin D and renin. These activities were tested by the methods described by AOYAGI[3,6] *et al.* Acetyl-Val-AHMHA-Ala-AHMHA at the concentration of 0.031 μg/ml decreased the pepsin activity to 50 % of the original (ID_{50} 0.031 μg/ml). *Iso*-butyryl, benzoyl, phenoxyacetyl and 2-phenoxypropionyl derivatives showed the following ID_{50} values: 0.021, 0.031, 0.02 and 0.02 μg/ml, respectively. *Iso*-valeryl-Val- AHMHA - Ala - AHMHA had the ID_{50} of 0.01 μg/ml which is same as that of pepstatin A (*iso*-Val-Val-AHMHA-Ala-AHMHA). Palmitoyl derivative has a lower inhibitory activity, *i.e.*, the ID_{50} of 0.45 μg/ml. Namely, all the N-acylated derivatives of the peptide obtained by enzymatic hydrolysis of pepstatins, showed almost equal level of anti-pepsin activity. They were in the same level of the activity as pepstatin A except N-palmitoyl peptide. Cathepsin D-inhibitory activities of the deri-

Table 2. Inhibitory activities of Val-AHMHA-Ala-AHMHA, its N-acylated derivatives and pepstatin A against pepsin, cathepsin D and renin.

Compounds	ID_{50}(mcg/ml)		
	Pepsin	Cathepsin D	Renin
Val-AHMHA-Ala-AHMHA	10	6.5	>250
Acetyl-Val-AHMHA-Ala-AHMHA	0.031	0.42	>250
iso-Butyryl-Val-AHMHA-Ala-AHMHA	0.021	0.28	>250
iso-Valeryl-Val-AHMHA-Ala-AHMHA	0.01	0.05	>250
Palmitoyl-Val-AHMHA-Ala-AHMHA	0.45	1.1	>250
Benzoyl-Val-AHMHA-Ala-AHMHA	0.031	0.05	>250
Phenoxyacetyl-Val-AHMHA-Ala-AHMHA	0.02	0.008	31
2-Phenoxypropionyl-Val-AHMHA-Ala-AHMHA	0.02	0.01	
iso-Valeryl-Val-Val-AHMHA-Ala-AHMHA[6] (pepstatin A)	0.01	0.011	4.5

Table 3. Acute toxicities of Val-AHMHA-Ala-AHMHA and its N-acylated derivatives (Intraperitoneal injection into mice.)

Compounds	LD_{50} (mg/kg)	LD_0 (mg/kg)	LD_{100} (mg/kg)
Val-AHMHA-Ala-AHMHA		$\geqslant$5,000	
Acetyl-Val-AHMHA-Ala-AHMHA		$\geqslant$4,000	
iso-Valeryl-Val-AHMHA-Ala-AHMHA		$\geqslant$3,000	
Benzoyl-Val-AHMHA-Ala-AHMHA	1,350	1,200	1,600
Phenoxyacetyl-Val-AHMHA-Ala-AHMHA	1,500	1,400	1,600
2-Phenoxypropionyl-Val-AHMHA-Ala-AHMHA	2,100	1,800	2,400

vatives were 10~40 times lower than of pepstatin A (ID_{50} 0.011 μg/ml), but still these new compounds were potent inhibitors. As for renin, however, none of them except N-phenoxyacetyl peptide showed 50 % inhibition at the concentration of 250 μg/ml. These N-acylated tetrapeptides have very low acute toxicity as given in Table 3. The tetrapeptide, Val-AHMHA-Ala-AHMHA, did not display signs of to toxicity in mice by the intraperitoneal injection of 5,000 mg/kg. Intraperitoneal injection of 4,000 mg/kg or 3,000 mg/kg of acetyl - Val - AHMHA - Ala-AHMHA or *iso*-valeryl-Val-AHMHA-Ala-AHMHA, respectively, did not cause any death in mice. LD_{50} values of benzoyl-Val-AHMHA-Ala-AHMHA, phenoxyacetyl-Val-AHMHA-Ala-AHMHA and 2-phenoxypropionyl-Val-AHMHA-Ala-AHMHA were 1,350, 1,500 and 2,100 mg/kg, respectively, by the same administration route. Among these products, it is noteworthy that the phenoxyacetyl and phenoxypropionyl compounds showed a strong inhibition of cathepsin D (Table 2) and were much more water-soluble than pepstatins.

YOSHIYUKI MATSUSHITA
HIROSHI TONE
SENJI HORI
YOSHIAKI YAGI
AKIRA TAKAMATSU

Central Research Laboratories,
Sanraku-Ocean Co., Ltd.
9-1, Johnan 4-chome,
Fujisawa, Japan

HAJIME MORISHIMA
TAKAAKI AOYAGI
TOMIO TAKEUCHI
HAMAO UMEZAWA

Institute of Microbial Chemistry
3-14-23, Kamiosaki, Shinagawa-ku,
Tokyo, Japan

(Received September 16, 1975)

References

1) UMEZAWA, H.; T. AOYAGI, H. MORISHIMA, M. MATSUZAKI, M. HAMADA & T. TAKEUCHI: Pepstatin, a new pepsin inhibitor produced by actinomycetes. J. Antibiotics 23: 259~262, 1970
2) MIYANO. T.; M. TOMIYASU, H. IIZUKA, S. TOMISAKA, T. TAKEUCHI, T. AOYAGI & H. UMEZAWA: New pepstatins, pepstatins B and C, and pepstanone A, produced by streptomyces. J. Antibiotics 25: 489~491, 1972
3) AOYAGI, T.; Y. YAGISAWA, M. KUMAGAI, M. HAMADA, H. MORISHIMA, T. TAKEUCHI & H. UMEZAWA: New pepstatins Bu, Pr and Ac produced by streptomyces. J. Antibiotics 26: 539~540, 1973
4) MORISHIMA, H.; T. TAKITA, T. AOYAGI, T. TAKEUCHI & H. UMEZAWA: The structure of pepstatin. J. Antibiotics 23: 263~265, 1970
5) AOYAGI, T.; S. KUNIMOTO, H. MORISHIMA, T. TAKEUCHI & H. UMEZAWA: Effect of pepstatin on acid proteases. J. Antibiotics 24: 687~694, 1971
6) AOYAGI, T.; H. MORISHIMA, R. NISHIZAWA, S. KUNIMOTO, T. TAKEUCHI & H. UMEZAWA: Biological activity of pepstatins, pepstanone A and partial peptides on pepsin, cathepsin D and renin. J. Antibiotics 25: 689~694, 1972
7) TONE, H.; N. SHIBAMOTO, Y. MATSUSHITA, T. INUI, A. TAKAMATSU, T. AOYAGI, T. TAKEUCHI & H. UMEZAWA: Enzymatic degradation of pepstatin A to a new tetrapeptide. J. Antibiotics 28: 1009~1011, 1975
8) TONE, H.; Y. MATSUSHITA, Y. YAGI, A. TAKAMATSU, T. AOYAGI, T. TAKEUCHI & H. UMEZAWA: Purification and properties of pepstatin hydrolase from *Bacillus sphaericus*. J. Antibiotics 28: 1012~1015, 1975