# BIOSYNTHETIC STUDIES ON PEPSTATIN

## BIOSYNTHESIS OF (3S, 4S)-4-AMINO-3-HYDROXY-6-METHYLHEPTANOIC ACID MOIETY

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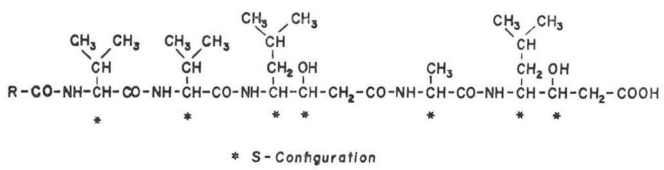
Biosynthesis of pepstatin by *Streptomyces testaceus* HAMADA *et* OKAMI was studied. U-$^{14}$C-L-Alanine and U-$^{14}$C-L-valine were incorporated into alanine and valine moieties of pepstatin respectively. U-$^{14}$C-L-Leucine, U-$^{14}$C-malonate and U-$^{14}$C-acetate were incorporated into (3S, 4S)-4-amino-3-hydroxy-6-methylheptanoic acid (AHMHA) moiety of pepstatin. Radioactivity of U-$^{14}$C-L-leucine was located almost exclusively in $C_3$-$C_8$ of AHMHA but not in $C_1$ and $C_2$ and radioactivity of U-$^{14}$C-malonate was located in $C_1$ and $C_2$. Labeled AHMHA itself was not incorporated into pepstatin. Malonate was incorporated into pepstatin at about 18 times higher rate than acetate. These results indicate that AHMHA is biosynthesized from L-leucine and malonic acid during biosynthesis of pepstatin and suggested that pepstatin biosynthesis proceeds on a multienzyme system in a similar way as in the case of fatty acid synthesis.

Pepstatin is an inhibitor of pepsin, cathepsin D, renin *etc.*[1~6], and produced by various types of Streptomycetes[1,7~9]. As shown by the structure elucidated by us (Fig. 1)[10], pepstatins are a group of pentapeptides of which N-terminuses are acylated with various fatty acids. Moreover, there are the pepstatin analogs: hydroxypepstatins[9] contain serine residue instead of alanine and pepstanones[7] contain methyl keto group instead of 2-carboxy-1-hydroxyethyl group in the C-terminus. Recently, chemical synthesis of pepstatin which contains isovaleryl group was achieved by us[11,12].

Pepstatins contain two moles of (3S, 4S)-4-amino-3-hydroxy-6-methylheptanoic acid[13] (abbreviated as AHMHA) which was first found in the acid hydrolysate. The structure of this amino acid suggested that it would be biosynthesized from L-leucine and malonic acid. On the other hand, LIPMANN[14] clarified that peptide chain elongation in biosynthesis of peptide antibiotics such as gramicidin S proceeds in a similar way as fatty acid synthesis. From this view point, we were interested especially in biosynthesis of AHMHA.

In this paper, we will report the biogenetic studies on incorporation of amino acids and malonic acid into pepstatin A.

Fig. 1. Structure of pepstatins



## Materials and Methods

### (1) $^{14}$C-Labeled Compounds

U-$^{14}$C-L-Alanine, U-$^{14}$C-L-valine, U-$^{14}$C-L-leucine, U-$^{14}$C-sodium acetate, U-$^{14}$C-sodium malonate and U-$^{14}$C-sodium pyruvate were purchased from Daiichi Pure Chemical Co.

### (2) Fermentation

*Streptomyces testaceus* HAMADA *et* OKAMI A-536 was cultured for a week at 27°C on a slant agar medium consisting of soluble starch 1.0%, glucose 0.5%, glycerol 0.3%, peptone 0.3%, yeast extract (purchased from Oriental Yeast Co., Tokyo) 0.2%, agar 2.0% (pH 7.2). It was shake-cultured in 50 ml of a medium in 500 ml volume flask consisting of soybean meal (S-3 meat) 2.0%, glucose 2.0%, yeast extract (Daigo Eiyo Kagaku Co.) 0.1%, $K_2HPO_4$ 0.05%, $MgSO_4 \cdot 7H_2O$ 0.05% (pH 7.2) at 27°C for 48 hours on a rotary shaker (180 r.p.m.). One milliliter of the cultured broth thus prepared was inoculated into 50 ml of a medium consisting of glucose 1.0%, starch 1.0%, peptone (Polypeptone) 0.75%, meat extract 0.75%, $MgSO_4 \cdot 7H_2O$ 0.05%, $K_2HPO_4$ 0.1%, $CuSO_4 \cdot 5H_2O$ 0.0007%, $FeSO_4 \cdot 7H_2O$ 0.0001%, $MnCl_2 \cdot 4H_2O$ 0.0008%, $ZnSO_4 \cdot 7H_2O$ 0.0002% (pH 7.4) and shake-cultured at 27°C. After 21 hours on a rotary shaker (180 r.p.m.) labeled compounds were added, and 24 hours thereafter, the fermentation was stopped. Under this culture condition, pepstatin A which contains isovaleryl group was the dominant type.

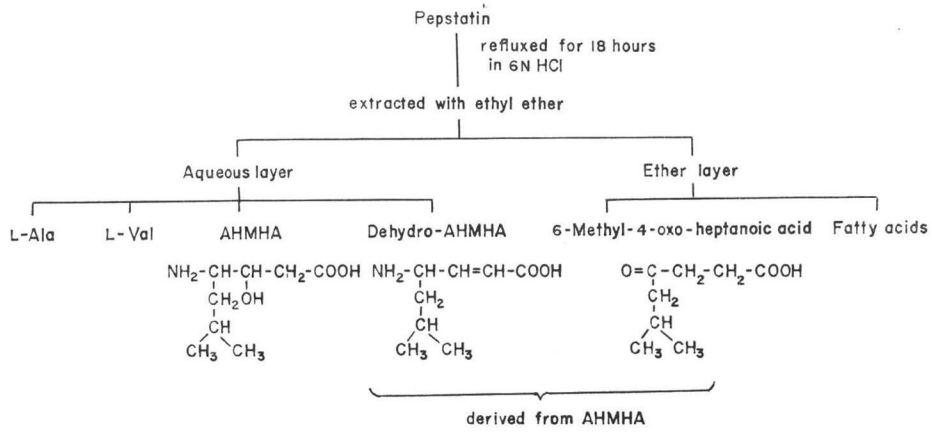
### (3) Extraction of Pepstatins and Isolation of Pepstatin A

The cultured broth (100 ml) was extracted with 50 ml of *n*-butanol three times, and the combined butanol extract was washed with 40 ml of water. The washed extract was dried under reduced pressure, and then subjected to silica gel column chromatography (Silicic acid AR, 100 mesh, Mallinckrodt, 2×30 cm) using chloroform - methanol - acetic acid (95 : 4 : 1). The bioactive eluate was dried under reduced pressure to give crystalline pepstatin A.

### (4) Determination of Pepstatins

To 1.0 ml of 0.6% casein (Wako Chemical Co., Osaka) dissolved in 0.08 M lactic acid (pH 2.2), 0.7 ml of 0.02 M KCl-HCl buffer (pH 2.0) and 0.2 ml of the same buffer containing a test material were added. After 3 minutes incubation at 37°C, 0.1 ml of 0.02 M KCl-HCl buffer (pH 2.0) containing 4 $\mu$g of pepsin was added and the reaction mixture was incubated for 30 minutes at 37°C. Two milliliter of 1.7 M perchloric acid was added and the mixture was kept for 1 hour at room temperature and then centrifuged. The optical density of the acid-soluble fraction thus prepared was measured at 280 nm. From the standard curve of percent inhibition *versus* pepstatin A concentration, total amount of pepstatins in a test material was determined.

Fig. 2. Separation of acid hydrolysis products of pepstatins



(5) Separation of Acid Hydrolysis Products of Pepstatin A

The separation procedure of the hydrolysis products is illustrated in Fig. 2. One milligram of radioactive pepstatin A was dissolved in 1 ml of 6 N HCl and refluxed for 18 hours. The hydrolyzate was extracted with 1 ml of ethyl ether three times. The combined ether extract was washed twice with 0.5 ml of water. The washed ether extract contained fatty acid of the N-terminus of pepstatin and 6-methyl-4-oxo-heptanoic acid derived from AHMHA. The ether was evaporated and the residue was dried under reduced pressure after neutralization with ammonia. Radioactivity of the dried material was counted.

The aqueous layer was dried under reduced pressure and the residue was dissolved in 0.1 ml of water. Ten microliters of this solution was analyzed by paper chromatography using *n*-propanol - 28 % ammonia (8 : 2). Rf values in this system of alanine, valine, AHMHA and *trans*-4(s)-amino-6-methyl-2-heptenoic acid (dehydration product of AHMHA, abbreviated as dehydro-AHMHA) were 0.33, 0.47, 0.67 and 0.72 respectively. Each portion of the chromatogram was excised and extracted with 1 ml of water and the radioactivity was counted.

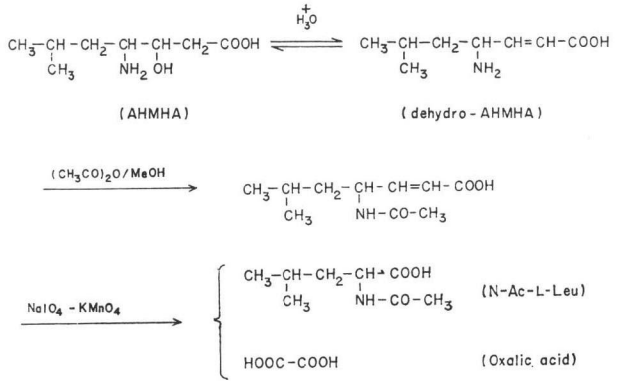
(6) Preparation of Radioactive AHMHA

Forty two milligrams of pepstatin A (142.6 $\mu$Ci/mmole) labeled with U-$^{14}$C-L-leucine was dissolved in 6 ml of 6 N HCl and refluxed for 14 hours. The hydrolyzate was dried after washing with ethyl ether. The residue was adsorbed on Dowex 50×8 ($H^+$-form, 50~100 mesh, 45 ml) and then eluted with 1 N $NH_4OH$. Ninhydrin-positive fractions were collected and dried. The dried material was applied to a column of Dowex 50×8 (pretreated with 0.1 M pyridine acetate buffer (pH 5.0), 200~400 mesh, 100 ml, 2.3×35 cm) and eluted with 0.1 M pyridine acetate buffer (pH 5.0). The eluate was fractionated into 3-g aliquots. The mixture of alanine and valine was eluted in fractions 16~28, AHMHA was eluted in fractions 111~173, and dehydro-AHMHA was eluted in fractions 174~245. Fifteen milligrams of radioactive AHMHA (76.1 $\mu$Ci/mmole) and 4 mg of dehydro-AHMHA were obtained.

(7) Degradation of AHMHA

Degradation procedure of AHMHA is illustrated in Fig. 3. Seven milligrams of dehydro-AHMHA, which was derived from AHMHA by dehydration with conc. HCl, was dissolved in 3 ml of dry methanol and a few drops of acetic anhydride were added at 0°C. After stirring for 17 hours at room temperature, the reaction mixture was dried under reduced pressure to yield the N-acetyl derivative. It was dissolved in 3 ml of an oxidizing reagent containing 0.18 M sodium periodate and 0.025 M potassium permanganate in 1 ml of 1 M sodium carbonate solution. The reaction mixture was stirred for 27 hours at room temperature, and then sodium metabisulfite was added until effervescence ceased. The solution was acidified to pH 1.5 with 1 N HCl and extracted three times with 5 ml of *n*-butanol. The combined butanol solution was washed with 2 ml of water, and then evaporated to dryness. The residue was subjected to thin-

Fig. 3 Degradation of AHMHA



layer silica gel (DC-Fertigplatten Kieselgel $F_{254}$, Merck) chromatography developed with *n*-butanol - butyl acetate - acetic acid - water (4 : 4 : 1 : 1). In this chromatography N-acetyl-L-leucine showed Rf value 0.65 and oxalic acid 0.05 as detected with 0.2% of bromphenol blue aqueous solution. Each fraction was extracted with 1 ml of water and then subjected to the radioactivity measurement.

(8) Measurement of Radioactivity

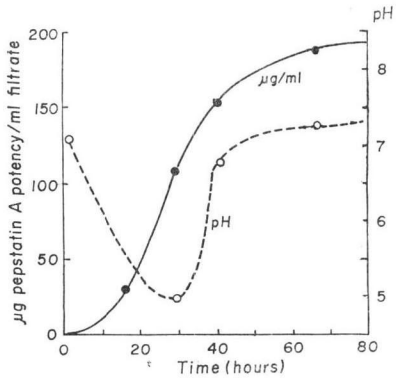
One milliliter of a test solution was added to 5 ml of scintillation fluid (BRAY's cocktail) consisting of 4 g of PPO (2, 5-diphenyloxazole), 0.2 g of dimethyl POPOP {1, 4-bis [2-(4-methyl-5-phenyloxazolyl] benzene}, 60 g of naphthalene, 100 ml of methanol, 20 ml of ethylene glycol and 880 ml of dioxane. The radioactivity was measured in a Beckman LS-250 liquid scintilation counter.

## Results and Discussion

Production of pepstatins and pH change of a culture filtrate during shaking culture are shown in Fig. 4. Pepstatins appeared in the cultured liquid at about 10 hours and reached the maximum at about 70 hours. From these results, the time of addition of labeled compounds was set at 21 hours and the harvest time at 45 hours after incubation.

A significant amount of the produced pepstatins remained in the mycelium. Therefore, pepstatins were extracted from the whole broth with *n*-butanol.

Fig. 4. Time course of pepstatin production




The results of testing incorporation of labeled compounds into pepstatins are shown in Table 1. Incorporations of U-$^{14}$C-L-valine and U-$^{14}$C-L-leucine were much higher than that of U-$^{14}$C-L-alanine. U-$^{14}$C-Sodium acetate and U-$^{14}$C-sodium pyruvate were less incorporated than the above-described amino acids and U-$^{14}$C-sodium malonate.

Distribution of radioactivity in pepstatin A molecule biosynthesized by addition of the

Table 1. Incorporation of $^{14}$C-compounds into pepstatins

| $^{14}$C-Compounds added | | | Pepstatins isolated | | Dilution $\left(\frac{a}{b}\right)\times10^{-3}$ | Incorporation rate* (%) |
|---|---|---|---|---|---|---|
| Compound | Amount added (μCi) | Specific activity (mCi/mmole) (a) | Amount (μmole) | Specific activity (μCi/mmole) (b) | | |
| U-$^{14}$C-L-Alanine | 40 | 130 | 58.3 | 30.8 | 4.2 | 8.2 |
| U-$^{14}$C-L-Valine | 40 | 233 | 84.7 | 155.7 | 1.5 | 55.4 |
| U-$^{14}$C-L-Leucine | 40 | 251 | 63.4 | 142.6 | 1.8 | 51.8 |
| 〃 | 0.5 | 251 | 56.4 | 2.2 | 114.1 | 53.0 |
| 〃 | 73.3 | 282 | 38.6 | 163.8 | 1.7 | 16.7 |
| U-$^{14}$C-Sodium malonate | 40 | 9.5 | 59.2 | 43.8 | 0.2 | 13.8 |
| U-$^{14}$C-Sodium acetate | 40 | 49.8 | 50.3 | 13.5 | 3.7 | 3.2 |
| U-$^{14}$C-Sodium pyruvate | 50 | 29.5 | 25.8 | 9.4 | 3.1 | 2.2 |

* calculated at the step of *n*-butanol extraction

Table 2. Distribution of radioactivity in pepstatin A

| Source of radioactivity | Distribution % of radioactivity in pepstatin | | | |
|---|---|---|---|---|
| | L-Alanine | L-Valine | AHMHA[1] | Ether ext.[2] |
| U-$^{14}$C-L-Alanine | 79.2 | 7.2 | 12.6 | 1.0 |
| U-$^{14}$C-L-Valine | 0.8 | 85.7 | 13.1 | 0.4 |
| U-$^{14}$C-L-Leucine | 0.5 | 1.2 | 94.5[3] | 3.9 |
| U-$^{14}$C-Sodium malonate | 1.2 | 1.6 | 91.4[4] | 5.8 |
| U-$^{14}$C-Sodium acetate | 6.1 | 6.9 | 84.7 | 2.4 |
| U-$^{14}$C-Sodium pyruvate | 27.4 | 24.3 | 46.8 | 1.5 |

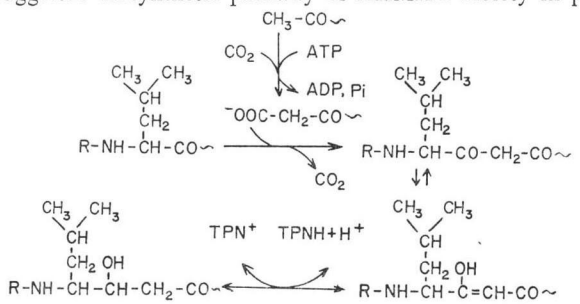
1) Dehydro AHMHA is included.
2) 6-Methyl-4-oxo-heptanoic acid derived from AHMHA and terminal fatty acids are contained.
3) After degradation 96% in N-acetyl leucine part and 4% in oxalic acid part.
4) After degradation 14% in N-acetyl leucine part and 86% in oxalic acid part.

Table 3. Incorporation of AHMHA and leucine diluted with AHMHA into pepstatins

| $^{14}$C-Compounds added | | | Pepstatins isolated | | Incorporation rate* (%) |
|---|---|---|---|---|---|
| Compounds | Amount added ($\mu$Ci) | Specific activity (mCi/mmole) | Amount ($\mu$mole) | Specific activity ($\mu$Ci/mmole) | |
| (3, 4, 5, 6, 7, 8)-$^{14}$C-AHMHA | 0.5 | 0.0761 | 30.0 | 0 | 0 |
| U-$^{14}$C-L-Leucine (control) | 0.5 | 0.0761 | 32.2 | 1.97 | 37.4 |
| U-$^{14}$C-L-Leucine diluted with cold AHMHA (100 eq.) | 30.0 | 2.5 | 59.8 | 118 | 46.1 |
| U-$^{14}$C-L-Leucine (control) | 30.0 | 251 | 47.8 | 116 | 34.9 |

* calculated at the step of *n*-butanol extraction

Fig. 5. Suggested biosynthetic pathway of AHMHA moiety in pepstatin




labeled compounds was studied by chemical degradation and the results are shown in Table 2. Labeled alanine and valine were mainly incorporated into alanine and valine moieties in pepstatin A respectively. In the case of addition of labeled alanine, the radioactivity was also distributed slightly into valine and AHMHA moieties. This randamization might be caused *via* pyruvate, which is derived from alanine by oxidative deamination. In fact, U-$^{14}$C-sodium pyruvate was incorporated into alanine, valine and AHMHA moieties.

U-$^{14}$C-L-Leucine, U-$^{14}$C-sodium malonate and U-$^{14}$C-sodium acetate were incorporated into pepstatin A at the rates of 51.8%, 13.8%, 3.2% respectively and the radioactivities were mainly located at AHMHA moiety. Degradation of AHMHA indicated that the radioactivity

of AHMHA labeled with U-$^{14}$C-L-leucine is mainly present in $C_3$-$C_8$, while the radioactivity labeled with U-$^{14}$C-sodium malonate is mainly present in the $C_1$ and $C_2$. As already described, the incorporation rate of malonate was about 18 times higher than that of acetate. These data indicate that AHMHA is synthesized from L-leucine and malonic acid.

The question remained whether pre-formed AHMHA is incorporated into pepstatin or AHMHA is synthesized by stepwise incorporation of L-leucine and malonic acid during formation of pepstatins. As shown in Table 3, U-$^{14}$C-L-leucine was incorporated into pepstatins at the rate of 37.4%, but even trace of labeled AHMHA was not incorporated under the same condition. Furthermore, incorporation of labeled leucine was not affected by addition of one hundred equivalent amount of cold AHMHA. These results indicate that AHMHA itself is not incorporated into pepstatin, but is formed from L-leucine and malonic acid during the process of pepstatin peptide synthesis (Fig. 5).

In general, peptide antibiotics are synthesized through a different process from protein synthesis. LIPMANN[14] pointed out that peptide antibiotics such as gramicidin S and tyrocidine are biosynthesized by multienzyme systems in a similar way as fatty acid synthesis. The data described above for biosynthesis of AHMHA suggest that pepstatin would be synthesized in a similar manner to biosynthesis of such peptide antibiotics.

Acknowledgement

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