THE STRUCTURE OF PEPSTATIN

Sir:

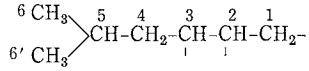
The biological properties, isolation and characterization of pepstatin, a pepsin inhibitor, were reported in the accompanying paper[1]. Here, we report on the structure of pepstatin.

Pepstatin is obtained as colorless needles, m.p. 228~229°C (dec.), $[\alpha]_D^{27}$ −90.3° (*c* 0.288, MeOH). It has the molecular formula $C_{34}H_{63}N_5O_9$ (M.W. 685.92). Found: C 59.02, H 9.27, N 10.11, O 21.41. Calcd.: C 59.53, H 9.25, N 10.21, O 20.99. The molecular formula was confirmed by mass spectrometry of the methyl ester. Found: m/e 699. Calcd.: 699. The UV spectrum of pepstatin shows only end absorption. Strong absorptions centered at 1630 cm^{-1} and 1540 cm^{-1} in the infrared spectrum suggest that pepstatin is a kind of peptide. Pepstatin shows a negative reaction for ninhydrin, but positive for RYDON-SMITH reagent. Pepstatin has no free basic function. It gives mono-methyl ester by treatment of diazomethane. The di-acetyl derivative of the methyl ester is obtained by acetic anhydride and pyridine.

Acid hydrolysis of pepstatin in 6 N hydrochloric acid at 110°C for 27 hours gave two ninhydrin-positive products and another material designated compound I. The two ninhydrin-positive products were found to be valine and alanine by thin-layer chromatography, high voltage paper electrophoresis, and their infrared spectra. Automatic amino acid analysis of the hydrolysate indicates that pepstatin contains two moles of valine (found, 1.63 mole) and one mole of alanine (found, 0.85 mole). Optical rotations of isolated valine and alanine indicate that they have all L-configurations. Found: valine, $[\alpha]_D^{20}$ +23.6° (*c* 2.96, 1 N HCl). alanine, $[\alpha]_{436}^{24}$ +30.4° (*c* 1.00, 6 N HCl).

Compound I was isolated by ion-exchange resin column chromatography (Dowex 50, pH 5.0 pyridine-acetate buffer) and obtained as colorless crystals. m. p. 199~201°C (dec.). $[\alpha]_D^{20}$ −18.9° (*c* 0.424, H_2O). It is an amphotheric compound (pKa' 3.8 and 9.7, titration equivalent 175). It has the molecular formular $C_8H_{17}NO_3$ (M.W., 175.22). Found: C 54.46, H 9.73, N 7.91, O 27.89. Calcd.: C 54.83, H 9.77, N 7.99, O 27.39. It gives a mono-methyl ester ($\nu_{C=O}$ 1735 cm^{-1}) with methanol and hydrochloric acid. The mono-O-acetyl derivative ($\nu_{C=O}$ 1745 cm^{-1}) is obtained by reaction with acetyl chloride in 6 N hydrochloric acid and acetic acid (1 : 1)[2]. From these results, compound I was deduced to be a saturated aliphatic amino acid having one each of amino, carboxy, and hydroxy groups.

The nmr spectrum of I in deuterium oxide is shown in Fig. 1. The assignment was made with aid of double resonance experiments. The result indicates the presence of the following carbon chain:



Methylene protons of C-1 couple with each other and appear at 2.93 δ (reference: external TMS), the methine proton of C-2 is at 4.46 δ, and the methine proton of C-3 at 3.74 δ. The chemical shifts suggest that a carboxy group is present at C-1, a hydroxy group at C-2, and an amino group at C-3. Then, the structure of I is presented as 4-amino-3-hydroxy-6-methylheptanoic acid. The stereochemistry of I is now under study.

The content of I in the hydrolysate of pepstatin was determined by gas chromatography[3]. Trifluoroacetyl derivatives of the methyl esters of the hydrolyzed products were analyzed. The results showed the

Fig. 1. The nmr spectrum of compound I (in D_2O, 100 MHz, reference: external TMS)

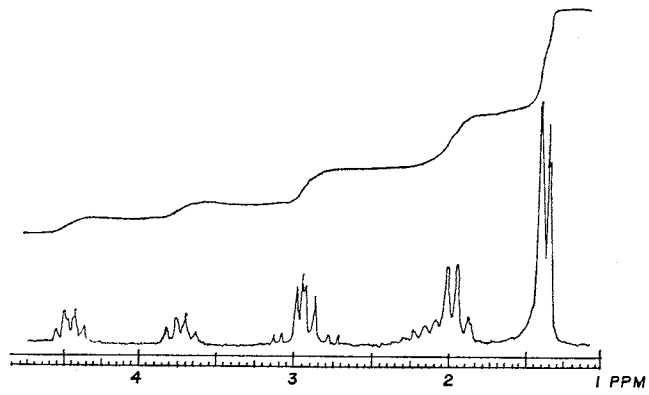
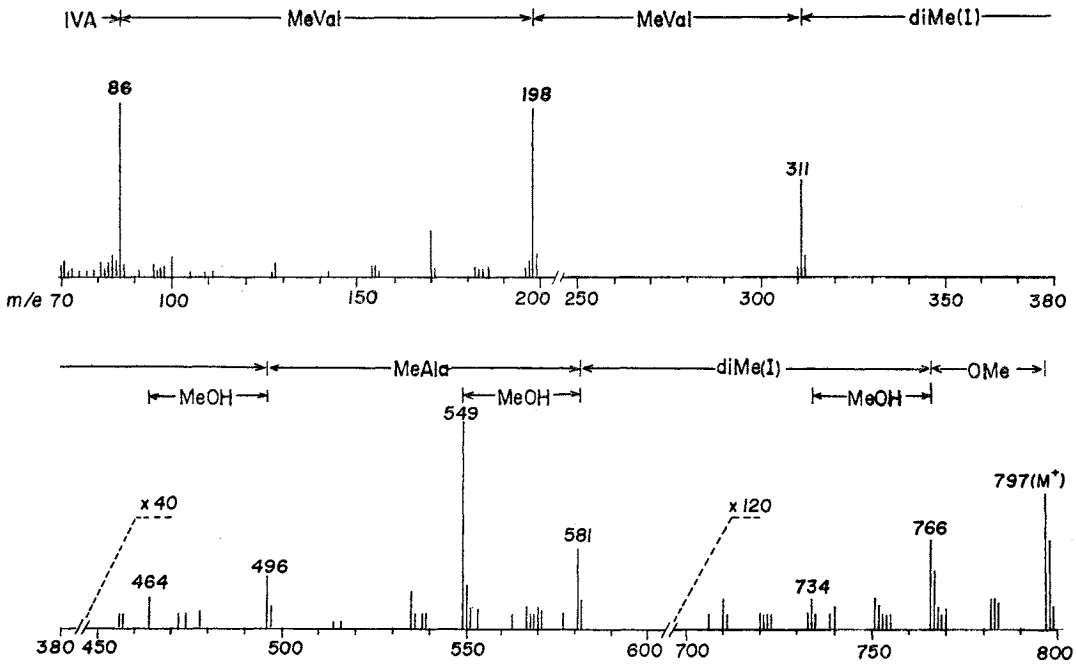


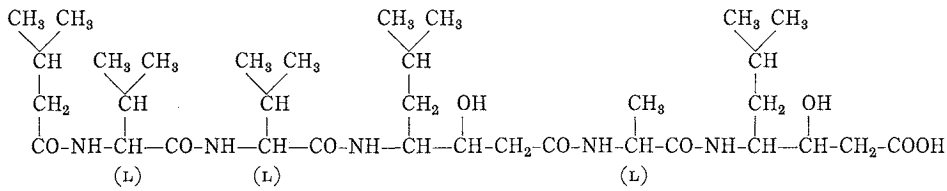
Fig. 2. The mass spectrum of permethylated pepstatin
(abbreviation, IVA : *iso*-valeric acid, diMe(I) : O, N-dimethyl compound I)



molar ratio of I to valine to be 0.69 : 1.00. Considering the nitrogen content of pepstatin molecule, and decomposition of I during acid hydrolysis (I was decomposed gradually under the condition of hydrolysis) the molar ratio of I, valine, and alanine in pepstatin should be 2 : 2 : 1.

As already mentioned, there is no free amino group in pepstatin. This suggests that the N-terminus of pepstatin is masked by a C_5-fatty acid. The acid hydrolysate of pepstatin was treated with ether, and the ether extract was neutralized with sodium hydroxide. By addition of *p*-bromophenacylbromide, a precipitate was obtained, which was crystallized from ethanol, m.p. 68°C. The mass, nmr, and infrared spectra, and the mixed melting point indicated that it was the *p*-bromophenacyl ester of *iso*-valeric acid.

From these results, it is evident that pepstatin is a pentapeptide, with the N-terminus acylated with *iso*-valeric acid. The sequence of amino acids and *iso*-valeric acid in pepstatin was determined by mass spectrometry[4]. Permethylated pepstatin was prepared[5] and used for analysis. The mass spectrum (Fig. 2) indicates clearly the following sequence :



Thus, *iso*-valeryl-L-valyl-L-valyl-4-amino-3-hydroxy-6-methylheptanoyl-L-alanyl-4-amino-3-hydroxy-6-methylheptanoic acid is proposed as the structure of pepstatin.

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