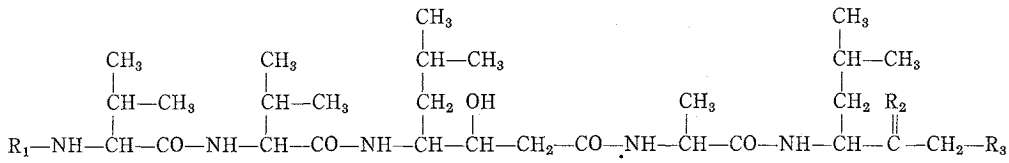
NEW PEPSTATINS, PEPSTATINS B AND C, AND PEPSTANONE A, PRODUCED BY STREPTOMYCES

Sir:

In 1970, UMEZAWA *et al.* isolated pepstatin, a specific pepsin inhibitor, from cultured broths of *Streptomyces testaceus* HAMADA *et* OKAMI and *Streptomyces argenteolus* var. *toyonakensis*[1]. Pepstatin is the N-*iso*-valeryl derivative of a pentapeptide composed of one mole of L-alanine, two moles of L-valine and two moles of 4-amino-3-hydroxy-6-methylheptanoic acid, a new naturally-occuring amino acid. The amino acid sequence (I) was determined by mass spectrometry[2]. In this communication, we report new pepsin inhibitors, pepstatins B (II) and C (III), and pepstanone A (IV), which were produced by the same pepstatin-producing *Streptomyces*. Now the name pepstatin A is assigned to the former pepstatin (I).



	R_1	R_2	R_3
I	*iso*-valeryl	$\begin{matrix}H\\OH\end{matrix}$	—COOH
II	*n*-caproyl	$\begin{matrix}H\\OH\end{matrix}$	—COOH
III	*iso*-caproyl	$\begin{matrix}H\\OH\end{matrix}$	—COOH
IV	*iso*-valeryl	=O	—H

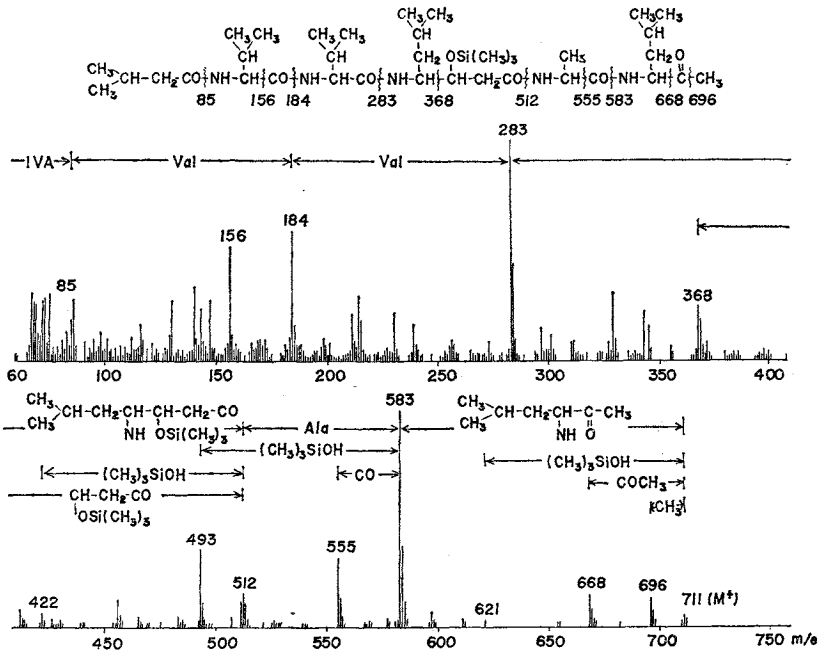
Pepstatins B and C could not be separated because of closely similar structures, as described later. At first, pepstatins B and C were thought to be a single minor component contained in the crude preparation of pepstatin. Degradation studies revealed that it was composed of two isomeric components. The new pepstatins were not separated from A by silica gel thin-layer chromatography; however, after esterification with methanol and sulfuric acid, the new pepstatins were separated from A by silica gel thin-layer chromatography. When the solvent system: chloroform, methanol and acetic acid (95 : 4 : 1) was used, the Rf-value of methyl ester of the new pepstatins was 0.39 and that of A was 0.35. The new pepstatins were preparatively isolated by Amberlite XAD-2 column chromatography using 65 % methanol as the developing solvent. Pepstatin A was eluted first, and the mixture of B and C followed.

The mixture of B and C was hydrolyzed with 20 % HCl at 105°C for 16 hours in a sealed tube. The fatty acid component was extracted with ethyl ether and esterified with ethanol/HCl. Gas chromatographic analysis indicated the presence of two components: ethyl *normal*- and *iso*-caproate. The water layer fraction of the hydrolyzate contained alanine, valine, and 4-amino-3-hydroxy-6-methylheptanoic acid in the molar ratio of 1, 2, and 2. These results suggested the presence of two new isomeric pepstatins which were named pepstatins B and C. Pepstatin B was assigned to the N-*n*-caproyl derivative of the pentapeptide, which is contained in pepstatin A, and pepstatin C was assigned to the N-*iso*-caproyl derivative. The mass spectrum of the methyl ester of isomeric mixture of B and C showed the parent peak at *m/e* 713, and the fragmentation pattern supported the sequences (II) and (III).

The mixture showed almost the same pepsin-inhibitory activity as that of pepstatin A, regardless of the composition. This suggests that pepstatins B and C have the same activity as pepstatin A.

Pepstanone A was found as a minor active component in a crude pepstatin preparation. On silica gel thin-layer chromatogram deve-

Fig. 1. The mass spectrum of TMS-pepstanone A.



loped with chloroform, methanol, and acetic acid (92.5 : 6 : 1.5), pepstanone A gave an Rf value 0.45, while pepstatins gave 0.15 detected by RYDON-SMITH reagent. Pepstanone A was isolated by silica gel column chromatography using the same solvent. After crystallization with methanol, fine needles of pepstanone A was obtained. It showed 78~86 % of the pepsin-inhibitory activity of pepstatin A.

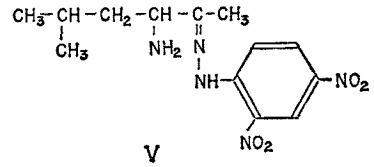
The new compound melted at 263~265°C. The molecular formula was established as $C_{33}H_{61}O_7N_5$ (M.W. 639), [Found: C 61.99, H 9.81, N 11.03. Calcd.: C 61.94, H 9.61, N 10.95]. The molecular weight was confirmed by mass spectrometry, [M^+, *m/e* 639]. Pepstanone A gave positive reaction with RYDON-SMITH and BRADY (2,4-dinitrophenylhydrazine) reagents, but was negative to ninhydrin. Potentiometric titration, electrophoretic behavior, and color reactions suggested that there is no free carboxyl or amino group. The UV [$\lambda_{max.}^{MeOH}$ 280 nm (ε 114)] and IR [$\nu_{C=O}$ 1715 cm^{-1}] absorptions suggested the presence of a keto-group, which accounts for the positive BRADY reaction.

Pepstanone A was hydrolyzed with 6 N HCl at 105°C for 15 hours. The amino acid analysis indicated the presence of one mole of alanine, two moles of valine, and one mole of 4-amino-3-hydroxy-6-methylheptanoic acid. The presence of *iso*-valeric acid was confirmed by gas chromatography of the hydrolyzate after esterification of the acidic ether extract. However, there could not be found a BRADY positive substance in the hydrolyzate.

The 2,4-dinitrophenylhydrazone of pepstanone A was obtained as yellow needles, m.p. 268~270°C. It has still 37 % of the activity of pepstatin. The results of the elemental analysis agreed with the formula $C_{39}H_{65}O_{10}N_9$ for the hydrazone. [Found: C 56.86, H 8.01, N 14.63. Calcd. for $C_{39}H_{65}O_{10}N_9$ (M.W. 819): C 57.13, H 7.99, N 15.37]. The UV absorption maximum appeared at 358 nm (ε 19,800) in methanol.

Acid hydrolysis of the hydrazone yielded a yellow substance (**V**), which showed a positive ninhydrin reaction. Compound **V** was isolated by silica gel column chromatography using chloroform and methanol (9 : 1) and was crystallized with mixed solvent of ethyl acetate and methanol, m.p. 202~206°C, $\lambda_{max.}^{MeOH}$ 350 nm (ε 18,300). The molecular formula was established as $C_{13}H_{19}O_4N_5 \cdot 2HCl$, [Found: C 41.26, H 5.30, N 18.34, Cl 18.37. Calcd.: C 40.85, H 5.54, N 18.32, Cl 18.55].

The mass spectrum showed the parent peak at *m/e* 309 ($C_{13}H_{19}O_4N_5$) and the base peak at *m/e* 252 ($M-C_4H_9$). The NMR spectrum was taken in DMSO-d_6 solution as internal TMS reference, [0.98 (δ) (3H, doublet, J=5.5 Hz), 1.00 (3H, doublet, J=5.5 Hz), 1.6~1.8 (3H, multiplet), 2.21 (3H, singlet), 4.18 (1H, triplet, J=6.5 Hz), 8.2~9.1 (3H, aromatic H), 8~9.5 (broad NH)].



V

From the above results structure V was deduced. Then the C-terminus of pepstanone A was suggested to be 3-amino-5-methyl-hexanone-2. The amino acid sequence in pepstanone A was expected to be the same as that of pepstatins. The mass spectra of pepstanone A and the O-trimethylsilyl derivative (Fig. 1) unambiguously supported the sequence (IV). Biogenetically pepstanone A can be derived from pepstatin A by oxidative decarboxylation of the C-terminus. Gas chromatographic analysis of crude pepstanone obtained from cultured broth on casein medium indicated the production of pepstanone B containing an *n*-caproyl group and C containing an *iso*-caproyl group.

The other pepstatin variant, in which R is acetyl, was recently reported by MURAO *et al.*[3,4]

Naturally, the presence of the corresponding pepstanone can be expected in the cultured medium.

Table 1. Composition of pepstatins A, B and C and pepstanones

Culture medium	Pepstatin A	Pepstatin B	Pepstatin C	Pepstanones
Peptone medium*	74.1 %	6.3 %	12.0 %	7.6 %
Casein medium*	23.5	61.0	10.0	5.5**

* Peptone medium: 1.0% glucose, 1.0% starch, 0.75% peptone, 0.75% meat extract, 0.3% NaCl, 0.1% $MgSO_4 \cdot 7H_2O$, 0.1% K_2HPO_4.
Casein medium: 5.5% glucose, 2.0% soybean oil, 5.0% milk casein, 4.5% skimmed milk, 0.35% NaCl, 0.15% $MgSO_4 \cdot 7H_2O$, 0.15% K_2HPO_4.

** The gas chromatographic analysis of the fatty acid component suggested the presence of pepstanones B and C.

The content of pepstatins A, B and C can be analyzed by gas chromatography of the fatty acid component. The content of pepstanones can be analyzed by UV absorption at 360 nm after isolation of the 2,4-dinitrophenylhydrazone. The results of the analysis of pepstatins and pepstanones produced in two kinds of media are shown in Table 1. It is noticed that the production of each pepstatin differed remarkably depending on the culture medium.

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