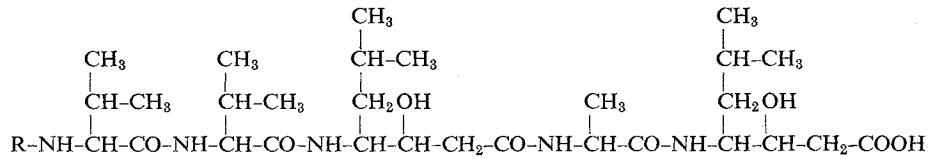
NEW PEPSTATINS, PEPSTATINS BU, PR AND AC PRODUCED BY *STREPTOMYCES*

Sir:

As previously reported[1~7], specific inhibitors of acid proteases termed pepstatins (R-L-valyl-L-valyl-4(s)-amino-3(s)-hydroxy-6-methyl-heptanoyl-L-alanyl-4(s)-amino-3(s)-hydroxy-6-methylheptanoic acid) have been obtained from cultured broth of actinomycetes; *i.e.* pepstatin A, R = isovaleryl; B, R = *n*-caproyl; C, R = *iso*-caproyl; D, R = *n*-heptanoyl; E, R = *iso*-heptanoyl; F, *R* = *anteiso*-heptanoyl;; G, R = *n*-capryl; and H, R = *iso*-capryl. We have also isolated pepstanones, compounds thought to be derived from the pepstatins by oxidative decarboxylation.

In this communication, we report the additional pepstatins Bu, Pr and Ac in which R is butyl, propionyl or acetyl, respectively. These pepstatins were isolated from a culture broth of *Streptomyces parvisporogenes* strain No. MD494-A1. Their structures are as follows:



Pepstatin	R
Bu	$CH_3 \cdot CH_2 \cdot CH_2 \cdot CO-$
Pr	$CH_3 \cdot CH_2 \cdot CO-$
Ac	$CH_3 \cdot CO-$

Pepstatin Ac has been reported by S. MURAO *et al.*[8] in their study of an antipeptic substance present in a culture filtrate of strain EF-44-201 of *Streptomyces naniwaensis*.

Strain MD494-A1 was isolated from a soil sample collected in Okusawa, Setagaya-ku, Tokyo, Japan. Its cultural characteristics can be summarized as follows: it belongs to a chromogenic type of streptomyces; it forms whorls but no spirals; the surface of the spore is smooth; the strain forms pale yellow to pale yellowish brown to yellowish brown growth and white to light brownish gray or yellowish gray aerial mycelium; it hydrolyzes starch and protein at a moderate rate; it utilizes glucose and inositol producing abundant growth but does not utilize D-xylose, sucrose, L-rhamnose, raffinose and D-mannitol; it shows no or slight growth in PRIDHAM-GOTTLIEB's media containing L-arabinose and D-fructose. Strain MD494-A1 is most closely related to *Streptoverticillium parvisporogenes*[9,10] LOCCI, BALDACCI and PETROLINI BALDAN, a species also identified as *Streptomyces parvisporogenes*.

Pepstatins Bu, Pr and Ac were produced by rotary shaking culture of strain MD494-A1 in media containing various carbon and nitrogen sources. A typical medium contained 1.0 % glucose, 1.0 % starch, 0.75 % Polypeptone, 0.75 % meat extract, 0.3 % NaCl, 0.1 % $MgSO_4 \cdot 7H_2O$, 0.1 % K_2HPO_4, 0.0007 % $CuSO_4 \cdot 5H_2O$, 0.0001 % $FeSO_4 \cdot 7H_2O$, 0.0008 % $MnCl_2 \cdot 4H_2O$ and 0.0002 % $ZnSO_4 \cdot 7H_2O$. Production of the sum of pepstatins Bu, Pr and Ac reached the maximum after 60~70 hours of incubation.

Pepstatins Bu, Pr and Ac were adsorbed by activated carbon and eluted with methanol at pH 7.0. The methanol extract was concentrated under reduced pressure to a yellow brownish powder. The powder was subjected to carbon column chromatography, using 70 % propanol. The active fraction was evaporated under reduced pressure at 40°C to a white powder. Pepstatins at the powder stage were further treated by Amberlite XAD-2 column chromatography using 60 % methanol as the developing solvent. Pepstatin Ac was separated from pepstatins Bu and Pr by silica gel column chromatography, using chloroform-methanol-acetic acid (96:6:2). The Rf-value of pepstatin Ac by silica gel thin-layer chromatography using *n*-butanol-pyridine-acetic acid-water (100:1:1:1) was 0.40 and that of pepstatin A was 0.65. The spots were detected by RYDON-SMITH reagent. Pepstatins Bu and Pr were separated by silica gel column chromatography using *n*-butanol-pyridine-acetic acid-water (100:1:1:

Table 1. The inhibitory activity of various pepstatins against pepsin, cathepsin D and renin

Acyl groups	Molecular weight	Inhibitory activity (ID_{50})		
		Pepsin ($\times 10^{-8}$ M)	Cathepsin D ($\times 10^{-9}$ M)	Renin ($\times 10^{-6}$ M)
Ac Acetyl	643	1.5	9.3	24.9
Pr Propionyl	657	1.5	9.1	15.2
Bu *n*-Butyryl	671	1.4	8.9	9.7
A *iso*-Valeryl	685	1.4	8.8	6.6
B *n*-Caproyl	699	1.4	9.3	4.3
F *anteiso*-Heptanoyl	713	1.4	9.1	2.5
G *n*-Capryl	727	1.4	8.9	1.7

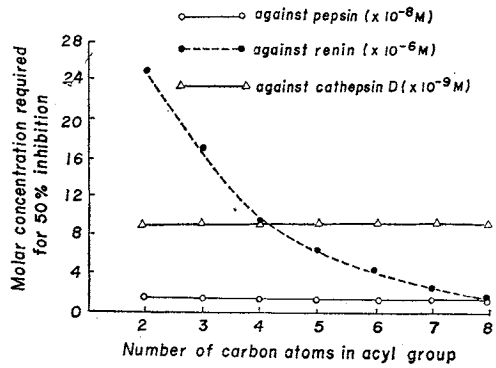
The inhibitory activity against pepsin and renin were carried out as described[6]. The inhibitory activity against cathepsin D was determined as follows: One and half ml of 1% hemoglobin solution, 0.4 ml of 0.05 M acetate buffer (pH 3.2) with or without an inhibitor and 0.1 ml of enzyme solution (protein: 0.68~2.5 mg) were mixed. After incubation at 37°C for 30 minutes, 1.0 ml of 9% trichloroacetic acid (TCA) was added and the mixture was kept for 1 hour at room-temperature. It was then centrifuged and the extinction of the acid-soluble fraction was read at 280 mμ.

1). Pepstatin Bu was eluted earlier than was pepstatin Pr. The Rf-value of pepstatin Bu by silica gel thin-layer chromatography as described above was 0.61 and pepstatin Pr 0.51.

Pepstatins Bu, Pr and Ac were hydrolyzed with 20% HCl at 105°C for 16 hours in a sealed tube. The fatty acid components were extracted with ethyl ether and esterified with diazomethane. Gas chromatographic analysis indicated the presence of methyl normal butyrate in pepstatin Bu, methyl propionate in pepstatin Pr and methyl acetate in pepstatin Ac. The aqueous layer of the hydrolyzate contained alanine, valine, and 4(s)-amino-3(s)-hydroxy-6-methyl heptanoic acid in the molar ratio of 1, 2 and 2. The mass spectra of the methyl esters of pepstatins Bu, Pr and Ac showed the molecular peaks at *m/e* 685, *m/e* 671 and *m/e* 657, respectively, and the same amino acid sequences as in pepstatin A was shown by the fragmentation patterns. Pepstatins Bu, Pr and Ac were obtained as colorless crystalline powders: Pepstatin Bu: 220~223°C (dec.); Anal. calcd. for $C_{33}H_{61}O_9N_5$: C 58.99, H 9.15, O 21.43, N 10.42; found: C 58.92, H 9.36, O 21.60, N 10.02. Pepstatin Pr: 227~228°C (dec.); Anal. calcd. for $C_{32}H_{59}O_9N_5$: C 58.42, H 9.04, O 21.88, N 10.64; found: C 58.62, H. 9.45, O 21.38, N 10.43. Pepstatin Ac: 223~225°C (dec.); Anal. calcd. for $C_{31}H_{57}O_9N_5$: C 57.83, H 8.92, O 22.36, N 10.87; found: C 57.53, H 9.36, O 22.87, N 10.63.

The activities of pepstatins Bu, Pr and Ac in the inhibition of pepsin[1,3,4,6], cathepsin D[5,6] and renin[6] are shown in Table 1. The concentrations of various pepstatins for 50% inhibition of pepsin, cathepsin D and renin are shown in Fig. 1. As shown in Table 1 and Fig. 1, pepstatins Bu, Pr and Ac are almost equally active against pepsin and cathepsin D as are pepstatins A, B, F and G. In contrast, the activities of the pepstatins against renin were dependent on the number of carbon atoms in the fatty acid moiety; the activity increased as the number of carbon atoms increased.

Fig. 1. Concentrations of various pepstatins for 50% inhibition of pepsin, cathepsin D and renin



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