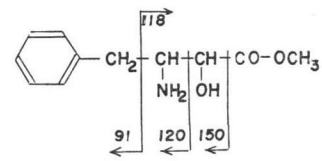
THE STRUCTURE OF BESTATIN

Sir:

In the preceding paper[1], the isolation and physicochemical properties of bestatin, a new aminopeptidase B inhibitor produced by an actinomycetes, were described. In this communication, we report the structure determination of bestatin which is [(2S, 3R)-3-amino-2-hydroxy-4-phenylbutanoyl]-L-leucine.

Bestatin is obtained as colorless fine needles, m.p. 233~236°C. The molecular formula was established as $C_{16}H_{24}N_2O_4$ (M.W. 308) by elemental analysis and mass spectrometry. Anal. Calcd.: C, 62.32; H, 7.82; N, 9.08; O, 20.75. Found: C, 61.86; H, 7.79; N, 8.61; O, 21.06. M^+ *m/e* 308. The ultraviolet absorption spectrum suggested the presence of a phenyl chromophore [λ_{max}^{MeOH} nm (ε): 248(104), 253(139), 259(172), 265(132) and 268 (shoulder)], and the infrared absorption spectrum suggested the presence of an amide bond (1640 and 1545 cm^{-1} in KBr disc). Bestatin is an amphoteric compound, that is, it affords crystalline monohydrochloride and crystalline monosodium salts. It gives a positive ninhydrin reaction. Potentiometric titration showed the existence of single amino (pKa 8.1) and carboxyl (pKa 3.1) groups, with a titration equivalent of 310. Bestatin gives a monomethyl ester (M^+ *m/e* 322) upon treatment with methanol-HCl, an N-acetyl derivative (M^+ *m/e* 350) by treatment with acetic anhydride-NaOH, and the diacetyl methyl ester (M^+ *m/e* 406) by treatment of the methyl ester with acetic anhydride in pyridine. These results indicate that bestatin has single free amino, hydroxyl and carboxyl groups. Acid hydrolysis of bestatin with 6 N HCl at 105°C for 16 hours yields two ninhydrin-positive products. They are separated by sulfonic acid resin (Dowex 50W×8) column chromatography with linear gradient elution between 0.2 M pyridine acetate buffer at pH 3.0 and 1.0 M pyridine acetate buffer at pH 4.75. The fast eluted substance is identical with L-leucine, $[\alpha]_D^{22}$ +12.9° (*c* 0.778, 1 N HCl). The late eluted substance (**I**) is a new amino acid.

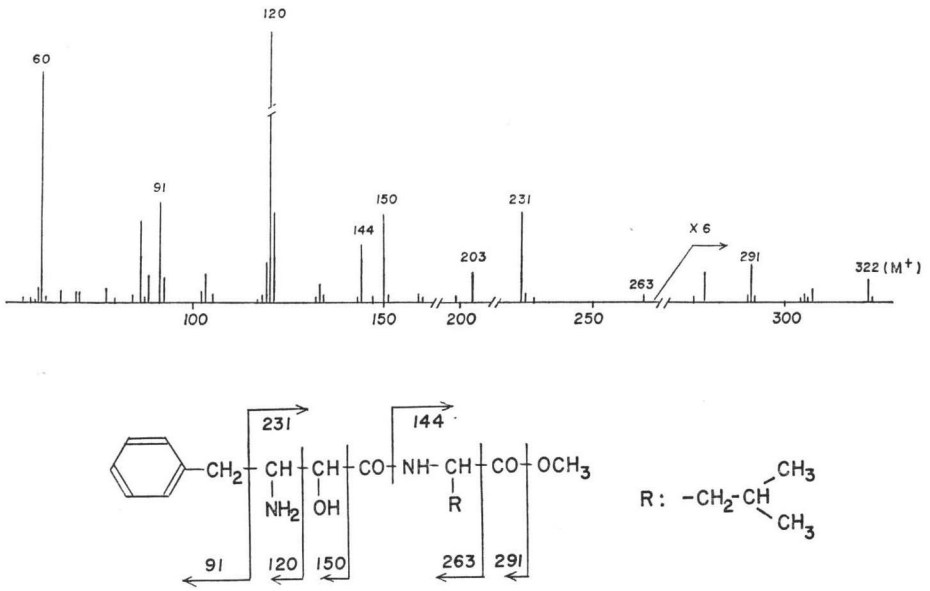
This amino acid is obtained as colorless needles from water, m.p. 219~221°C, $[\alpha]_D^{22}$ + 27.9° (*c* 0.717, 1 N HCl). It has the molecular formula $C_{10}H_{13}NO_3$ (M.W. 195). Anal. Calcd.: C, 61.53; H, 6.71; N, 7.17; O, 24.59. Found: C, 61.23; H, 6.60; N, 7.04; O, 24.50. Potentiometric titration indicates the presence of single amino (pKa 8.6) and carboxyl (pKa 2.5) groups with a titration equivalent of 212. The pKa value of the carboxyl function suggests that **I** is not an α-amino-β-hydroxy-carboxylic acid, but β-amino-α-hydroxy-carboxylic acid. The PMR spectrum of **I**-hydrochloride in deuteromethanol indicates the presence of a phenyl group (5 protons centered at δ 7.30) and a carbon chain: $-CH_2-CH-CH-$ [2 protons at δ 3.06 (doublet, J=8.0 Hz), 1 proton at δ 3.80 (double triplets, J=8.0 and 3.0 Hz), and 1 proton at δ 4.11 (doublet, J=3.0 Hz)]. The mass spectrum of **I**-methyl ester shows significant peaks at *m/e* 210 $(M+1)^+$, 150, 120, 118 and 91. These fragmentation patterns can arise only from methyl 3-amino-2-hydroxy-4-phenylbutanoate.



The absolute configuration at C_3 of **I** was determined by the following procedure. The N-acetyl derivative of **I** was prepared by acetylation with acetic anhydride under pH control at 8.0 with 1 N NaOH. Oxidation of the N-acetyl derivative with potassium permanganate affords N-acetyl-D-phenylalanine, m.p. 170~171°C, $[\alpha]_D^{22}$ +40.0° (*c* 0.1, methanol). Thus, the absolute configuration of C_3 of **I** was determined to be R.

Recently, SHIBA *et al.*[2] reported that the relative configuration of α-amino-β-hydroxy acids can be determined by PMR spectrometry of their oxazolidone derivatives, for which coupling constants of the vicinal methine protons of the oxazolidones are distinctly different in the *threo* (5.0±1.0 Hz) and *erythro* (9.6±0.6 Hz) isomers. This was applied to an oxazolidone derivative of **I**, though **I** is not an α-amino-β-hydroxy acid, but a β-amino-α-hydroxy acid. The oxazolidone of **I** was prepared by alkali treatment of the N-benzyloxycarbonyl derivative of **I**. The coupling

Fig. 1. Mass spectrum of bestatin methyl ester hydrochloride



constant of the vicinal methine protons of the oxazolidone was 4.0 Hz, while that of the diastereoisomer, which was synthesized starting from D-phenylalanine, was 9.0 Hz. These results suggested that the configuration of **I** should be *threo*. Thus, the absolute configuration of **I** is 2S, 3R. This conclusion was confirmed by X-ray crystallographic analysis of the I-methyl ester hydrobromide.[3]

The chemical shift of the C_3 methine proton of the **I** moiety (δ 4.25) of N-acetyl bestatin is significantly lower than that (δ 3.75) of bestatin hydrochloride, which indicates that the amino group of **I** is free in bestatin molecule. Finally, the amino acid sequence of bestatin was determined by mass spectrometric analysis of bestatin methyl ester (Fig. 1) as above.

Formation of fragment ions of *m/e* 120, 144 and 150 can be explained only by the structure [3-amino-2-hydroxy-4-phenylbutanoyl]-leucine methyl ester. Thus, the structure of bestatin is [(2S, 3R)-3-amino-2-hydroxy-4-phenylbutanoyl]-L-leucine.

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(Received September 19, 1975)

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