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Studies on the β-Lysine Peptide. III.[1] Synthesis of β-(L-β-Lysyl)-L-β-lysine

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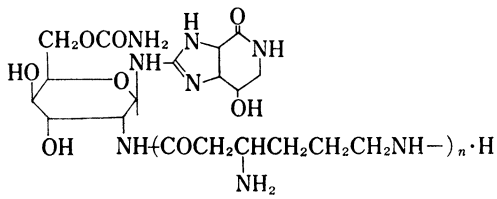
Racemomycins[3] belonging to a water-soluble, basic streptothricin group antibiotic contain a number of L-β-lysine (n) in their molecules. The antibiotics are active against both Gram positive and Gram negative bacteria, and the activities such as antimicrobial, antiviral, and toxicities to mouse increase as their β-lysine contents increase. The fact suggested that the elongation of β-lysine in their molecules increases the activities. Therefore, we have made a plan to synthesize L-β-lysine peptides. Further elucidation of the β-lysine peptide structure (β- or ε-peptide) in streptothricins ($n \geqq 2$) was needed also for their structural studies.



racemomycins A: $n=1$
B: $n=2$
C: $n=3$
D: $n=4$

Chart 1[4]

In the previous communicatlon,[1] we described the synthesis of ε-(L-β-lysyl)-L-β-lysine (II) based on the formation of L-β-lysine copper complex (IV). In this paper, we wish to describe the synthesis of β-(L-β-lysyl)-L-β-lysine (III) and the comparison of the both peptides.

The reaction mixture of L-β-lysine (I) with carbobenzoxy chloride in 1N sodium hydroxide solution afforded N,N'-dicarbobenzoxy(Cbz-) L-β-lysine (VIII) and N,N'-diCbz-L-β-lysine benzyl ester (ratio of product 5:3). The formation of the latter was unavoidable despite of examinations, and its structure was confirmed by direct synthesis from VIII. Therefore, the ester was converted to VIII by treatment with methanolic sodium hydroxide followed by purification on silica-gel column chromatography.

ε, N-Cbz-L-β-lysine (VI) was quantitatively obtained from L-β-lysine copper complex (IV) and carbobenzoxy chloride. ε, Cbz-L-β-lysine methyl ester (VII) was obtained in a pure form from VI by a treatment with thionyl chloride in absolute methanol. Combination of VII with VIII was carried out by dicyclohexylcarbodiimide (DCC) to produce a protected β-lysine dipeptide as needles, mp 176—179°, $C_{37}H_{46}O_9N_4$, which was saponified with sodium hydroxide followed by decarbobenzoxylation. Hydrogenation proceeded very slowly as monitored by paper chromatography (PPC). A dipeptide β-(L-β-lysyl)-L-β-lysine (III) was

1) Part II: H. Taniyama, Y. Sawada, K. Miyazeki, S. Tanaka, and F. Miyoshi, *Chem. Pharm. Bull.* (Tokyo), **19**, 2645 (1971).
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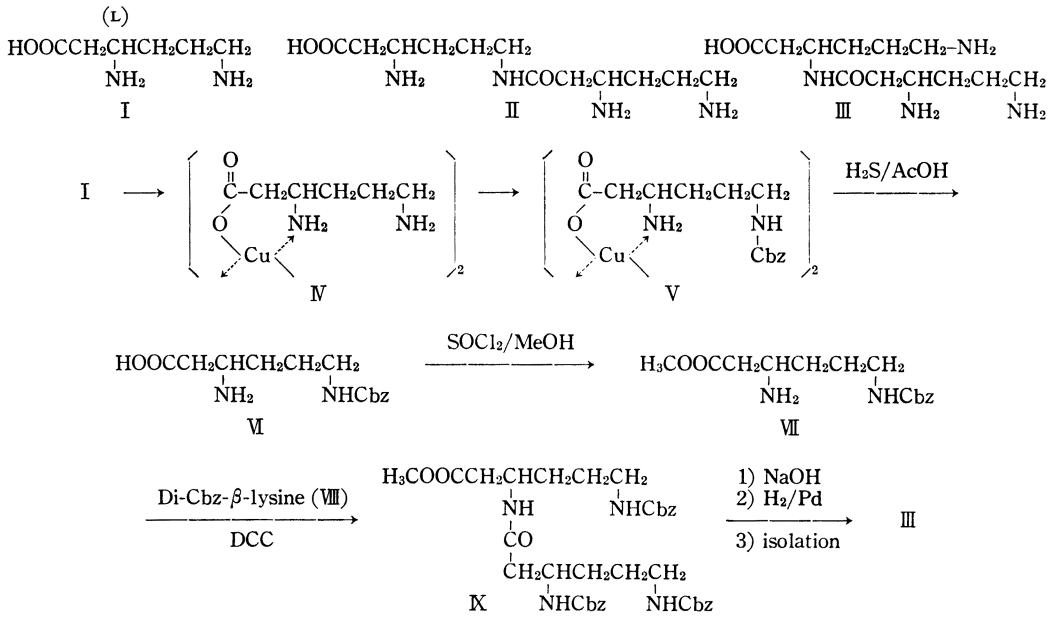
3) H. Taniyama, Y. Sawada and T. Kitagawa, *Chem. Pharm. Bull.* (Tokyo), **19**, 1627 (1971).
4) E.E. van Tamelen, J.R. Dyer, H.A. Whaley, H.E. Carter, and G.B. Whitfield, *J. Am. Chem. Soc.*, **83**, 4295 (1961).



Cbz: carbobenzoxy-

Chart 2

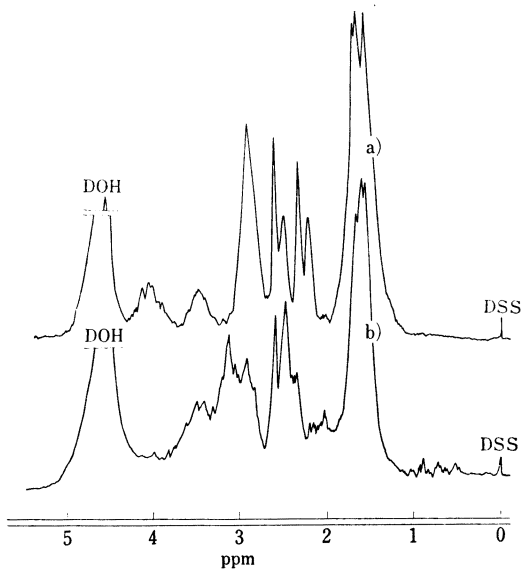


Fig. 1. NMR Spectra of L-β-Lysine Dipeptides (free bases) in D_2O

a) β-(L-β-lysyl)-L-β-lysine
b) ε-(L-β-lysyl)-L-β-lysine

obtained as powder after Sephadex LH-20 column chromatography. III was converted to the *p*-hydroxyazobenzenesulfonate to confirm the empirical formula.

Comparisons of the synthetic dipeptides were made by chromatographic techniques (Table 1) and nuclear magnetic resonance (NMR) spectra of their free bases (Fig. 1). Avicel-SF thin layer chromatography using solvent systems of phenol showed a difference of *Rf* values each other. In the NMR spectrum of III, α protons (doublet) at δ 2.24 and 2.56, and β protons (multiplet) at δ 3.5 and 4.05 were cleary analyzed. However, α protons at δ 2.43 and 2.45 and, especially, β protons at δ 3.45 of II were closely overlapping.

Reshetov, *et al.*[5] reported the synthesis of ε-(L-β-lysyl)-L-β-lysine starting from L-ornithine. However physico-chemical constants of the dipeptide were not described because of its hygroscopic, non-crystalline properties. Therefore the present work seems to constitute the first synthesis and characterization of the dipeptides.

5) L.I. Rostovtseva, P.D. Reshetov and A.S. Khokhlov, Zn. *Obshch. Khim.*, (1969), **39** (1), 96 (Russ), *cf. C.A.* **70**, 106860r (1969).

The synthetic β-(L-β-lysyl)-L-β-lysine were inactive against *Escherichia coli* and *Staphylococcus aureus* at a concentration of 1000 μg/ml by agar plate method.

TABLE I. Thin-Layer Chromatography of L-β-Lysine Dipeptides

Compounds	*Rf* values solvent systems		
	I	II	III
L-β-Lysine	0.39	0.21	0.76
β-(L-β-Lysyl)-L-β-lysine	0.32	0.18	0.30
ε-(L-β-Lysyl)-L-β-lysine	0.34	0.26	0.36

conditions: Avicel SF thin layer (Funakoshi Co.), room temp., 26°, room moisture 70%
detection: ninhydrin and sodium hypochloride-*o*-tolidine-potassium iodide reagents
solvent systems: I: *n*-PrOH-pyridine-HOAc-H_2O (15: 10: 3: 13)
II: phenol-H_2O (3: 1)
III: phenol-HOAc-H_2O (6: 1: 2)

Experimental

L-β-Lysine (I)——L-β-Lysine (I) used was isolated from hydrolysates of racemomycin complex under the same condition reported in the previous paper.[4]

DiCbz-L-β-lysine——To a solution containing 3 g (0.02 mole) of L-β-lysine in 1N NaOH (10 ml) CbzCl (9 g, eq. mole) was slowly added at 0°. The reaction mixture was stirred for three hours with adjusting to weak alkaline with 1N NaOH until it became to negative ninhydrin test. The reaction mixture was extracted with ether (20 ml×3). Aqueous solution was acidified with HCl and then extracted with EtOAc (20 ml×3). Condensation of the EtOAc fractions gave white crystals (VIII), mp 152—153°, yield 2.5 g, $[\alpha]_D^{24} = -9 \pm 3°$ (c=0.33 in MeOH). *Anal.* Calcd. for $C_{22}H_{26}O_6N_2$: C, 61.35; H, 6.28; N, 6.76%. Found: C, 61.55; H, 6.40; N, 6.90%. IR ν_{max}^{KBr} (cm^{-1}): 3430, 3380, 3250, 3120, 2980, 1725, 1690, 1650, 1545, 1453, 1440, 1410, 1355, 1305, 1272, 1240, 1220, 1197, 1167, 1140, 1090, 1067, 1025, 1003, 975, 960, 907, 895, 855, 775, 735, 692. UV λ_{max}^{EtOH} nm: 258.5 (ε 530).

DiCbz-L-β-lysine Benzyl Ester——Ether fractions extracted above gave white needles of diCbz-β-lysine benzyl ester, mp 113—114°, yield 1.5 g. *Anal.* Calcd. for $C_{29}H_{32}O_6N_2$: C, 69.05; H, 6.35; N, 5.56%. Found: C, 69.06; H, 6.35; N, 5.36%. IR ν_{max}^{KBr} (cm^{-1}): 3440, 3380, 3120, 2960, 1722, 1685, 1540, 1510, 1450, 1378, 1320, 1296, 1280, 1250, 1210, 1165, 1130, 1104, 1065, 1020, 982, 965, 937, 905, 855, 820, 780, 757, 742, 696. UV λ_{max}^{EtOH} nm: 258.5 (ε 720). It was identified with an authentic sample by IR spectra and mixed melting point.

DiCbz-β-lysine Benzyl Ester from VIII——An abs. benzene solution (100 ml) of VIII (0.1 g, 0.00024 mole) and *p*-toluenesulfonic acid (0.04 g, eq. mole) was refluxed in presence of abs. benzyl alcohol (5 ml) at 110—120° for 3 hr. Reaction mixture was concentrated and recrystallized from ether-pet. ether. Acetone-ether-pet. ether was used for recrystallization. Yield 0.1 g, mp 113—114°. *Anal.* Found: C, 69.35; H, 6.47; N, 5.26%.

VIII from DiCbz-β-lysine Benzyl Ester——A solution of diCbz-β-lysine benzyl ester (1.5 g) in MeOH (30 ml) and 1N NaOH (6 ml) was allowed to stand overnight at room temperature. Reaction mixture was extracted with ether (30 ml×3), and aq. layer was acidified with HCl, and extracted with EtOAc to give white crystals. It was identified with VIII by melting point and IR spectra. Yield 35%.

VI from IV and V——L-β-Lysine copper complex (IV) was prepared by the same method reported in the previous paper.[6] IV (3 g, 0.008 mole) was dissolved in water (50 ml) and mixed with 2N NaOH (6 ml). To the solution was added CbzCl (5 g) in limited amounts at 0° for 70 min. with adjusting to weak alkaline with 1N NaOH. The precipitate corresponding to V obtained as blue powder was filtered and washed with ether followed with water. Yield *ca.* 5 g. The compound (V, 5 g) in 150 ml of HOAc was decomposed with H_2S. The filtrate containing VI was concentrated to dryness to give an oily substance. The substance was chromatographed with a cellulose column (2.5×43 cm) using solvent system of *n*-BuOH-pyridine-HOAc-water-*tert.*-BuOH (15: 10: 3: 12: 4). *Rf* value of VI was 0.78 by comparing with that of β-lysine (*Rf* 0.36). The earlier fractions was concentrated to a small volume and precipitated with acetone. Reprecipitation with acetone gave white powder in the yield of 1.4 g, mp 202—205° for the free base. *Anal.* Calcd. for $C_{14}H_{20}O_4N_2$: C, 60.00; H, 7.14; N, 10.00%. Found: C, 60.13; H, 7.14; N, 9.94%. The free base was converted to its hydrochloride with ten fold volume of 1N HCl; $[\alpha]_D^{25} = +17 \pm 2°$ (c=1 in H_2O). IR ν_{max}^{KBr}

6) H. Taniyama, Y. Sawada and T. Kitagawa, *Chem. Pharm. Bull.* (Tokyo), **19**, 2631 (1971).

(cm^{-1}): 3490, 2980, 1720, 1700, 1610, 1520, 1450, 1410, 1260, 1130, 1000, 775, 740, 695. NMR (in D_2O) δ: 1.6 m. 4H, 2.70 d. $J=6.0$ Hz 2H, 3.11 seems to be a triplet 2H, 3.57 m. 1H, 5.02 singlet 2H, 7.34. singlet 5H; UV $\lambda_{max}^{H_2O}$ nm: 258 (ε 250).

IX from VII——To a solution of compound VI (0.7 g) in 10 ml of abs. MeOH was added $SOCl_2$ (0.5 ml) with cooling, and stirred for 12 hr. The solution was evaporated to dryness to give ε-Cbz-β-lysine methyl ester (VII) in the yield of 0.7 g, TLC: *Rf* 0.75 on silica-gel with MeOH (detection with serium sulfate). Needles of mp 73—78°. *Anal.* Calcd. for $C_{15}H_{23}O_4N_2Cl$: C, 54.46; H, 7.01; N, 8.47; Cl, 10.72%. Found: C, 54.23; H, 6.81; N, 8.31; Cl, 11.15%. IR ν_{max}^{KBr} (cm^{-1}): 3420, 2950, 1715, 1685, 1595, 1583, 1520, 1438, 1390, 1334, 1260, 1235, 1135, 1000, 975, 905, 775, 740, 695.

To a solution of VII (0.7 g, 0.0024 mole) and VIII (0.9 g, eq. mole) in dry pyridine (10 ml), abs. dioxane (10 ml) and Et_3N (0.1 ml) was added DCC (0.5 g, 0.0024 mole) in dioxane ($3\frac{1}{4}$ml) at 0° with stirring. After 6 hr, DCC (0.3 g, 0.00097 mole) in dioxane (2 ml) was further added at 0°. Stirring was carried out for 12 hr to give negative ninhydrin test. Dicyclohexylurea was filtered off and the filtrate was concentrated to dryness. The product was dissolved in MeOH (20 ml) and ether (10 ml). The solution was evaporated to a small volume and allowed to stand overnight to give white crystals (IX). Recrystallization was carried out three times from MeOH–ether, affording white crystals, yield 0.4 g. *Anal.* Calcd. for $C_{37}H_{46}O_9N_4$: C, 64.35; H, 6.67; N, 8.12%. Found: C, 64.27; H, 6.73; N, 8.38%. IR ν_{max}^{KBr} (cm^{-1}): 3380, 3120, 2980, 1730, 1690, 1640, 1540, 1450, 1380, 1265, 1210, 1170, 1125, 1065, 1007, 775, 745, 695. UV λ_{max}^{MeOH} nm: 259 (ε 780).

Formation and Isolation of III——Compound IX (1.1 g) was dissolved in MeOH (40 ml) at 60° and saponified by 1N NaOH (5 ml) at room temperature overnight. The reaction mixture was concentrated to dryness and dissolved in HOAc (20 ml) and water (15 ml). The resulting solution was hydrogenated on Pd (60 mg). After 24 hr, the reaction mixture was added with water (10 ml) and the same weight of catalyst, and further hydrogenated for 12 hr. The reaction mixture was filtered and the filtrate was concentrated *in vacuo*. An aqueous solution (50 ml) of the concentrate was extracted with EtOAc (20 ml×3). The aqueous layer was concentrated to a small volume, charged to a column (2.5×140 cm) of Sephadex LH-20 (Pharmacia brand), and eluted with water. It was fractionated 10 g in each tube. Fr. No. 37—41 gave only one spot which has lower *Rf* value than that of L-β-lysine on a paper chromatography. Detection: ninhydrin and sodium hypochloride–*o*-tolidine–potassium iodide reagents. NMR (D_2O) of free base δ: 2.6 m. 8H, 2.24 d. $J=7.0$ Hz 2H, 2.56 d. $J=7.0$ Hz 2H, 2.93 m. 4H, 3.5 m. 1H, and 4.05 m. 1H. III was converted to the *p*-hydroxyazobenzenesulfonate from aqueous solution. mp 230° with decomp. *Anal.* Calcd. for $C_{48}H_{62}O_{18}N_{10}S_3$: C, 49.56; H, 5.34; N, 12.05; S, 8.26%. Found: C, 49.37; H, 5.42; N, 11.91; S, 8.04%. III was also converted to its hydrochloride by 1N HCl followed by lyophilization to give hygroscopic white powder, mp 218—225° with decomp., $[\alpha]_D^{26}=+6°$ ($c=2.2$ in H_2O), IR ν_{max}^{KBr} (cm^{-1}): 3460, 3080, 1715, 1640, 1550, 1480, 1395, 1260, 1215, 1175, 1125, 940, 745. Mol. wt. of III hydrochloride was obtained by Sephadex G-10 column (2×150 cm) as near 400 by comparison with racemomycin A hydrochloride (mol. wt. 630) and β-lysine hydrochloride (mol. wt. 219). Also III was hydrolyzed with 6N HCl at 120° for 6 hr to produce only β-lysine (by PPC and automatic amino acid analysis).

Paper Chromatography——Paper chromatography was carried out by Toyo-Roshi No. 51 UH paper by using a solvent system of *n*-BuOH–pyridine–HOAc–H_2O–*t*-BuOH (15: 10: 3: 12: 4). As *Rf* values were affected by moisture in air, β-lysine was used together as a standard.

Antimicrobial Activity——Agar plates of *Escherichia coli* NIHJ JC-2 and *Staphylococcus aureus* FDA-209P JC-1 were used. Disc papers at a concentration of 1000 μg/ml of II and III hydrochlorides were checked by culture at 35° for 24 hr. They were inactive to both microorganisms.