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# Peptide Mode of β-Lysine Residues in Racemomycin-C[1]

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β-Lysine dipeptide was isolated from the acid hydrolysate of Racemomycin-C and identified with a synthetic sample of ε-N-(L-β-lysyl)-L-β-lysine. Similarly, β-N-dinitrophenyl-L-β-lysine and β-N,N-dimethyl-L-β-lysine were respectively isolated from the acid hydrolysate of dinitrophenylated Racemomycin-C and hexa-N-methyl Racemomycin-C and identified with authentic samples. These results confirm that β-lysine peptide sequence of Racemomycin-C is ε-peptide bond.

Racemomycin complex[3] is produced by a mutant strain[4] of *Streptomyces racemochromogenus*, and is an antibiotic with acute and delayed toxicity. The components of this complex, Racemomycin-A, B, C, and D, are characterized by the number of β-lysine in their molecules, and their antimicrobial and antiviral activities and toxicity increase as their β-lysine content increases.[5] In the present work, we confirmed the arrangement of β-lysine peptide in the structure of Racemomycin-C and obtained a suggestion relating to the biological activities and the structure of Racemomycin.

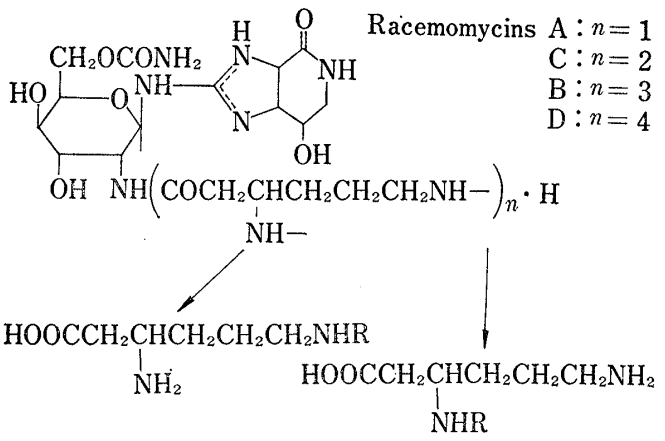


Fig. 1[6]

van Tamelen, *et al.*[6] reported the proposed structures for Streptothricin and Streptolin, and suggested that the peptide bond in β-lysyl-β-lysine moiety of Streptolin might be in ε-amino position. In their communication, however, no detailed study on the β-lysine peptide bond was reported.

In order to solve this problem, following two different methods were carried out; (1) the β-lysine peptide was isolated from the hydrolysis product of Racemomycin-C and identified with a synthetic sample, and (2) dinitrophenyl- and N,N-dimethyl-β-lysine, from the hydrolysates of dinitrophenylated and N-methylated Racemomycin-C, were also isolated and identified with synthetic samples.

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3) H. Taniyama and S. Takemura, *Yakugaku Zasshi*, **77**, 1210 (1957).
4) T. Sugai, *J. Antibiotics Ser. B*, **9**, 170 (1956).
5) H. Taniyama, Y. Sawada, and T. Kitagawa, *Chem. Pharm. Bull.* (Tokyo), **19**, 1627 (1971).
6) E.E. van Tamelen, J.R. Dyer, H.A. Whaley, and H.E. Carter, *J. Am. Chem. Soc.*, **83**, 4295 (1961).

Hydrolysis of Racemomycin-C with 4N hydrochloric acid at 99° produced β-lysine dipeptide, and that of Racemomycin-B produced β-lysine dipeptide and tripeptide. The variation of these components were traced by paper chromatography as shown in Fig. 2.

The dipeptide produced by hydrolysis of Racemomycin-C for 1 hr was separated as follows. The hydrolysate was charged on a carbon column and the column was eluted with water; the dipeptide separated after elution of streptolidine, β-lysine, and streptolidyl-gulosaminide components. It was further chromatographed on a cellulose column, and purified by Sephadex G-10 column chromatography to give a single spot on paper. Then, a white powder of β-lysine dipeptide was obtained by lyophilization. Similarly, a white powder of β-lysine dipeptide and tripeptide was obtained from the hydrolysate of Racemomycin-B. Ratio of adsorption of β-lysine peptides to activated carbon and comparison of *Rf* values of β-lysine and β-lysine peptides are shown in Fig. 3. Optical rotation, infrared (IR) and nuclear magnetic resonance (NMR) spectra, and Avicel-SF thin-layer chromatography (Table I) of the dipeptide were identical with those of synthetic ε-N-(L-β-lysyl)-L-β-lysine.[7]

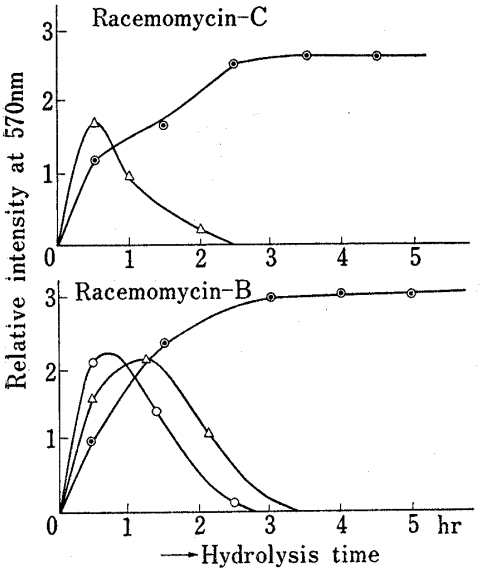


Fig. 2. Degradative Process of Racemomycins C and B

—⊙—: β-lysine
—△—: β-lysyl-β-lysine
—○—: β-lysyl-β-lysyl-β-lysine

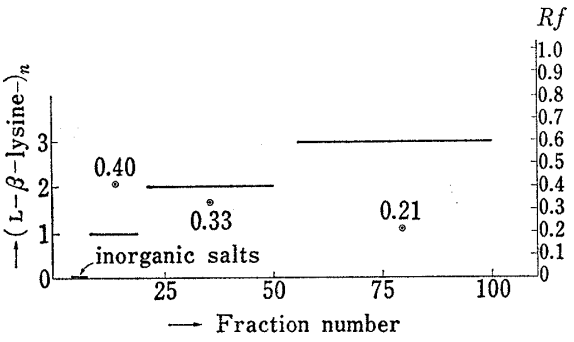


Fig. 3. Adsorption of β-Lysine and β-Lysine Peptides to Activated Carbon and Their *Rf* Values

—: fractions containing the corresponding substance
⊙: *Rf* value of paper chromatography (solvent system: BuOH: pyridine: HOAc: water: *t*-BuOH=15:10:3:12:4)

In the isolation of the dipeptide, there is a possibility of transpeptidations in some of these procedures, such as hydrolysis or purification. Therefore, the structure of β-lysine peptide moiety in Racemomycin-C was further confirmed by the second method.

Racemomycin-C, isolated by carbon column chromatography,[5] was dinitrophenylated and a crude product was hydrolyzed without purification. The hydrolysate was submitted to silica gel thin-layer chromatography comparing with three dinitrophenyl-β-lysine derivatives; β,ε-N,N′-di-dinitrophenyl-L-β-lysine, β-N-dinitrophenyl-L-β-lysine (V), and ε-N-dinitrophenyl-L-β-lysine (IV). The synthesis of compounds IV and ε-N-benzoyl-L-β-lysine (II) was described in a previous paper.[8] The compound III was prepared by dinitrophenylation of II, and V by hydrolysis of III. Elemental analyses and physical properties of III and V were in fair agreement with their structure. On thin-layer plate of silica gel, a spot of β,ε-N, N′-di-dinitrophenyl-L-β-lysine and a smaller but distinct spot corresponding to V were detected

7) H. Taniyama, Y. Sawada, K. Miyazeki, S. Tanaka, and F. Miyoshi, *Chem. Pharm. Bull.* (Tokyo), **19**, 2645 (1971).
8) H. Taniyama, Y. Sawada, and T. Kitagawa, *Chem. Pharm. Bull.* (Tokyo), **19**, 2631 (1971).

Table I. Thin- Layer Chromatographic Comparisons of L-β-Lysine Dipeptides

| Compound | *Rf* values solvent system 1 | 2 |
|---|---|---|
| L-β-Lysine | 0.21 | 0.76 |
| β-N-L-β-Lysyl-L-β-lysine[9] | 0.18 | 0.30 |
| ε-N-L-β-Lysyl-L-β-lysine[7] | 0.26 | 0.36 |
| L-β-Lysyl dipeptide isolated from Racemomycin-C[7] | 0.25 | 0.36 |

Avicel-SF (Funakoshi Co.) detection: ninhydrin
1: phenol-$H_2O$ (3:1)
2: phenol-HOAc-$H_2O$ (6:1:2)

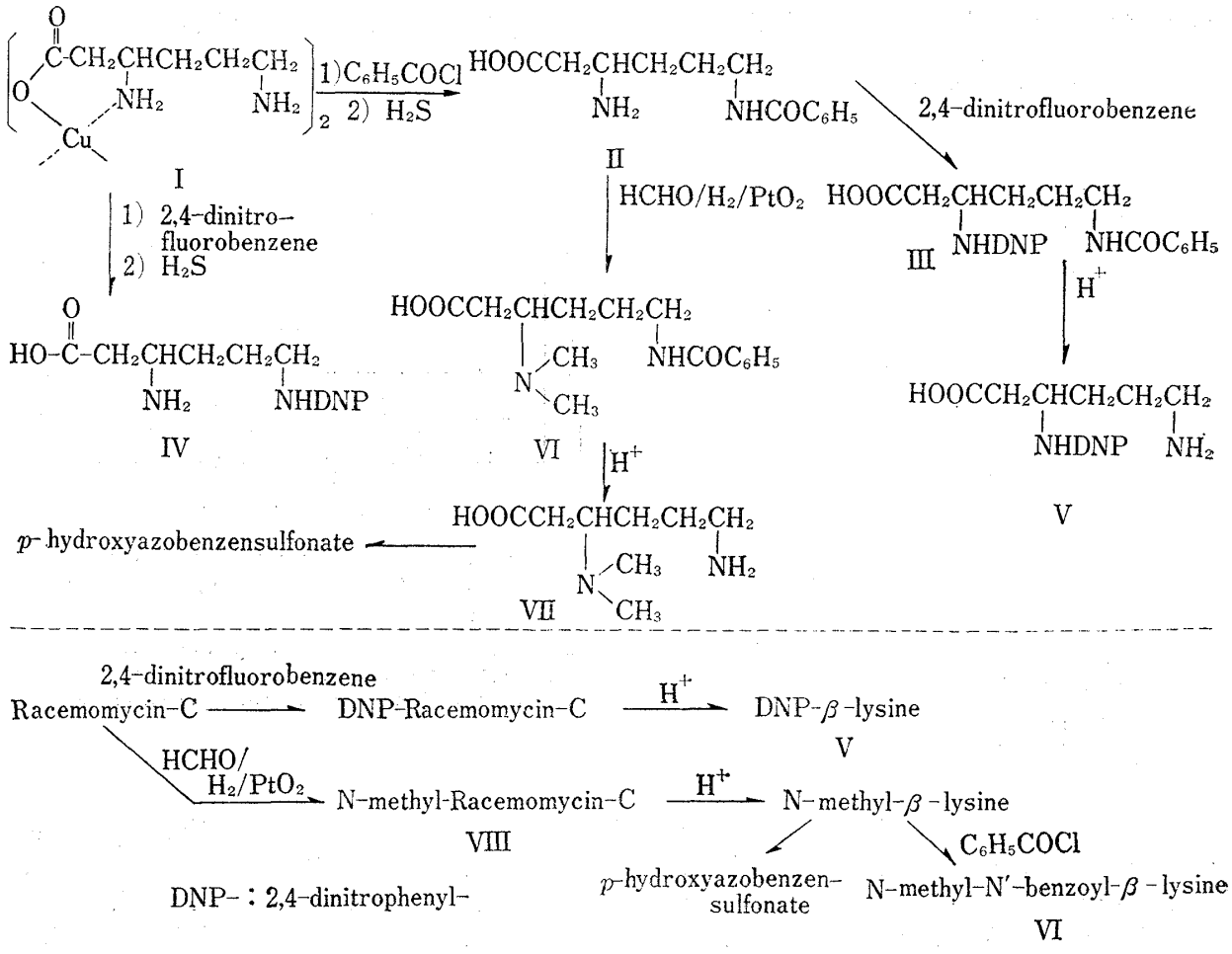


Chart 1

from dinitrophenylated Racemomycin-C hydrolysate. Three solvent systems were used for the chromatography, and a solvent system containing phenol seemed to be preferable for the identification of mono-dinitrophenyl-L-β-lysine derivatives (Table II).

It was presumed that the incomplete dinitrophenylation product might be precipitated by the use of a solvent containing water. Therefore, we ran the reaction by two steps; yellow precipitate, produced by addition of excess 2,4-dinitrofluorobnezene to Racemomycin-C in 5% sodium hydrogencarbonate solution, was treated with an additional 2,4-dinitrofluoro-

9) H. Taniyama, Y. Sawada, K. Miyazeki, and F. Miyoshi, *Chem. Pharm. Bull.* (Tokyo), **20**, 601 (1972).

Table II. Thin-Layer Chromatographic Comparison of L-β-Lysine Dinitrophenyl Derivatives

| Compound / Solvent system | *Rf* values 1 | 2 | 3 | 4 | |
|---|---|---|---|---|---|
| Acetone–MeOH–AcOEt (2:1:1) | 0.78 | 0.085 | 0.037 | 0.79 | 0.036 |
| Acetone–MeOH (2:1) | 0.70 | 0.15 | 0.08 | 0.74 | 0.10 |
| Phenol–$H_2O$ (3:1) | 0.76 | 0.70 | 0.57 | 0.77 | 0.59 |

compound: 1 β,ε-N,N′-di-DNP-L-β-lysine[8] 2 ε-N-DNP-L-β-lysine[8]
3 β-N-DNP-L-β-lysine 4 dinitrophenyl-Racemomycin-C hydrolysate

benzene in pyridine and water (1: 1). Since the color of the solution changed to black during the reaction, a column chromatography using silica gel was applied for the purification. However, dinitrophenyl derivative of Racemomycin-C was not complete enough to be submitted to elementary analysis, and was hydrolyzed with acid without further purification. On the chromatogram of the hydrolysate, a spot of streptolidine was detected and identified by *Rf* values and by the tone of color (Ninhydrin and Pauly reaction) with an authentic sample. Therefore, it seems that the cyclic guanidine group of Racemomycin-C is not react with 2, 4-dinitrofluorobenzene.

Consequently, β-lysine peptide of Racemomycin-C must be bonded to ε-amino position on the basis of the fact that β-*N*-dinitrophenyl-L-β-lysine (V) was obtained from dinitrophenyl-Racemomycin-C by acid hydrolysis. In addition, we carried out the following procedure because the complete purification of dinitrophenyl-Racemomycin-C and the isolation of mono-N-dinitrophenyl-L-β-lysine was unsuccessful by the above method.

N-Methylated Racemomycin-C (VIII) was obtained by the reductive methylation[10] of Racemomycin-C over platinum dioxide in presence of formaldehyde. VIII has no antimicrobial activity and has a formula of hexa-N-methyl-Racemomycin-C. Elemental analysis of the reineckate and N-methyl values (6x N-Me) estimated by NMR spectrum of VIII satisfied the formula. Streptolidine, N,N-dimethyl-L-β-lysine, and N,N,N′,N′-tetramethyl-L-β-lysine were obtained by acid hydrolysis of VIII followed by column chromatography over Amberlite CG-120 ($H^+$). Streptolidine and N,N-dimethyl-β-lysine gave a positive Ninhydrin test, but N,N,N′,N′-tetramethyl-β-lysine was negative to the test. N,N-Dimethyl-L-β-lysine is very hygroscopic that it was benzoylated. The resulting white powder was identified with a synthetic sample VI, prepared by methylation of II, by IR and NMR spectra. It is apparent that dimethyl-β-lysine derived from Racemomycin-C must be β-N,N-dimethyl-L-β-lysine (VII). The following consideration of NMR spectra (Fig. 4) also supports this conclusion. The signal at 3.07 ppm (triplet) in the spectrum of dimethyl-β-lysine (VII: free base) is due to two protons of ε-methylene. Benzoylation of VII shifts the signal by about 0.37 ppm to a lower field, and hence, the ε-methylene protons of benzoyl compound (VI: free base) was observed at

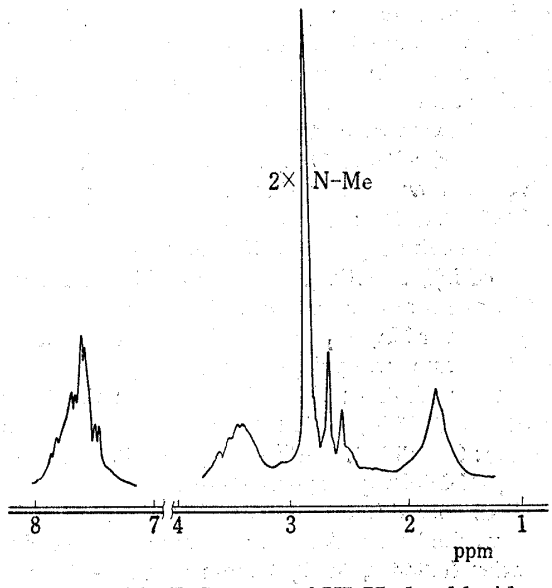


Fig. 4. NMR Spectra of VI Hydrochloride ($D_2O$, External Standard: Trimethylsilan)

10) P. Claes and H. Vanderhaeghe, *Bull. Soc. Chim. Belges*, **78**, 561 (1969).

3.44 ppm. In the case of hydrochloride of VI, such a shift was not clear, and the signal of the free base was found to overlap with two α-methylene protons at 2.50 ppm (doublet, 2H) from the measurement of signal intensity. Consequently, it is confirmed that the peptide mode of β-lysine residues in Racemomycin-C, one of streptothricin group antibiotics, is an ε-peptide bond, and it can therefore be assumed that the modes in other antibiotics of this group, Racemomycin-B, Racemomycin-D, *etc.*, are similar to it.

Although the role of β-lysine peptide component to the biological activities of this group of antibiotics is not yet clear, we are studing the synthesis of antibiotic derivatives and their antimicrobial activities, which will be reported in future.

## Experimental

**Hydrolysis of Racemomycins C and B**——A solution of Racemomycin-C (0.05 g) dissolved in 5 ml of 4N HCl was heated at 99°, and a quantitative volume of the hydrolysate was spotted on paper (Toyo-Roshi No. 51 UH type) by using a microsyringe. The paper was developed with a solvent system of BuOH–pyridine–HOAc–$H_2O$–*t*-BuOH (15:10:3:12:4).[11] Color was developed by Ninhydrin reagent and colorimetric determination of the paper strips was carried out. The hydrolysate of Racemomycin-B was treated by the same method.

**Isolation of β-Lysine Dipeptide from Racemomycin-C**[7]——A solution of Racemomycin-C sulfate (1.0 g) dissolved in 50 ml of 4N HCl was heated at 99° for 1 hr. The hydrolysate was concentrated *in vacuo* at 30—40°, charged on a carbon column (2.8×17 cm), and the column was eluted with water. Fractions were collected at 5 g each. From this chromatographic column, streptolidine and β-lysine were eluted in the fraction No. 4—7 and β-lysine dipeptide in the fraction No. 21—50. The dipeptide fraction was concentrated *in vacuo*, charged on a cellulose column (2×21 cm), eluted with avove solvent system and only the fractions of the dipeptide were collected. The dipeptide was purified by chromatography on Sephadex G-10 column (2×150 cm) using $H_2O$. The pure fraction was lyophilized to give β-lysine dipeptide acetate in a yield of about 0.06 g.

**Isolation of β-Lysine Tripeptide-like Component from Racemomycin-B**——A solution of Racemomycin-B sulfate (0.5 g) dissolved in 10 ml of 4N HCl was heated at 100° for 1 hr. The hydrolysate was concentrated *in vacuo* at 30—40°, charged on a carbon column (3×15 cm), and the column was eluted with $H_2O$. After 5 g each of the fractions were collected, the fraction Nos. 53—100 was concentrated and purified as described for β-lysine dipeptide, affording tripeptide-like white powder of mp 220° (decomp.), yield 0.05 g. $[\alpha]_D^{25}$ +15° ($c$=1.0, $H_2O$). The powder gave similar IR and NMR spectra to β-lysine dipeptide,[7] and automatic amino acid analysis of the acid hydrolysate gave only β-lysine peak.

**Dinitrophenylation of Racemomycin-C**——After Racemomycin-C sulfate (0.2 g) was dissolved in 2 ml of $H_2O$ and made weakly basic with 5% $NaHCO_3$, 5% ethanol solution of 2,4-dinitrofluorobenzene (DNFB) (4 ml) was added and the mixture was stirred for 12 hr. The solution was washed three times with ether concentrated *in vacuo*, and the product was dissolved in 4 ml of pyridine-$H_2O$ (1:1). To this mixture was further added 5% DNFB (2 ml) and the mixture was stirred for 12 hr. The resulting black solution was washed twice with ether concentrated *in vacuo*, and charged on a silica gel column (2×7 cm). The column was eluted with AcOEt and then MeOH. The yellow eluate with MeOH was collected and concentrated to give a yellow powder of mp 230° (decomp.), yield 0.15 g. Thin-layer chromatography (silica-gel, Merck Co.): *Rf* 0.11 (AcOEt: acetone=2:1), UV $\lambda_{max}^{DMFA}$ nm ($\varepsilon$): 360 ($4.7\times10^4$), UV $\lambda_{min}^{DMFA}$ nm ($\varepsilon$): 305 ($2.6\times10^3$).

**β-N-Dinitrophenyl-ε-N′-benzoyl-L-β-lysine (III)**——ε-*N*-Benzoyl-L-β-lysine (II) (0.2 g) was dissolved in 4 ml of 5% $NaHCO_3$, a solution of 5% DNFB (2 ml) was added, and the mixture was allowed to stand for 24 hr at room temperature. The mixture was acidified with 1N HCl and then extracted with AcOEt. After drying over $Na_2SO_4$ the solution was concentrated to dryness. Recrystallization of the product from ether–cyclohexane gave yellow granular crystals of mp 98—102°, yield 0.12 g (72%). *Anal.* Calcd. for $C_{19}H_{20}O_8N_4$: C, 54.80; H, 4.84; N, 13.46. Found: C, 54.98; H, 4.89; N, 13.16. IR $\nu_{max}^{KBr}$ $cm^{-1}$: 3330, 3200, 2930, 1720, 1618, 1588, 1520, 1425, 1335, 1290, 1150, 920, 835, 746, 717, 650. UV $\lambda_{max}^{MeOH}$ nm (log $\varepsilon$): 350 (4.25), UV $\lambda_{min}^{MeOH}$ nm (log $\varepsilon$): 290 (3.38). $[\alpha]_D^{17}$ −70.3° ($c$=0.833, MeOH).

**β-N-Dinitrophenyl-L-β-lysine (V)**——A solution of 0.1 g of β-*N*-dinitrophenyl-ε-N-benzoyl-L-β-lysine (III) dissolved in 30 ml of 5N HCl and 5 ml of BuOH was heated at 100° for 20 hr. When cooled the solution was washed with AcOEt and concentrated *in vacuo* to dryness. Recrystallization of the resulting yellow powder (0.044 g, 52.5%) from acetone–ether afforded yellow needles, mp 174—178° (decomp.), positive to Ninhydrin test. *Anal.* Calcd. for $C_{12}H_{18}O_7N_4\cdot HCl$: C, 39.29; H, 5.18; N, 15.28; Cl, 9.68. Found: C, 39.42; H, 5.09; N, 15.02; Cl, 9.41. IR $\nu_{max}^{KBr}$ $cm^{-1}$: 3300, 3100, 2910, 1705, 1625, 1583, 1500, 1410, 1335, 1250, 1190, 1137, 1057, 833, 827, 743, 690. UV $\lambda_{max}^{0.5N\,HCl}$ nm ($\varepsilon$): 363 ($1.2\times10^4$). $[\alpha]_D^{20}$ −50.9° ($c$=0.58, MeOH). NMR

11) Unless otherwise stated, all paper and cllulose column chromatographies were carried out by this method.

($D_2O$) $\delta$: 1.88 (m, 4H), 2.89 (d, $J$=7.0 Hz, 2H), 3.08 (t, $J$=7.0 Hz, 2H), 3.72 (m, 1H), 7.22 (d, $J$=10 Hz, 1H), 8.26 (d, $J$=10 Hz, 3 Hz, 1H), 8.95 (d, $J$=3 Hz, 1H).

**Hydrolysis of Dinitrophenylated Racemomycin-C**——Suspension of dinitrophenylated Racemomycin-C in 50 volumes of 6N HCl was hydrolyzed at 120° for 24 hr. The solution was concentrated, then the hydrolysate was dissolved in acetone–$H_2O$ (1:1), and the solution was submitted to paper and thin-layer chromatographies (Table 1). The spot of streptolidine was detected at *Rf* 0.29 (Ninhydrin and Pauly reaction) on the paper but no $\beta$-lysine was detected.

**N-Methylated Racemomycin-C (VIII)**——A mixture of Racemomycin-C sulfate (1.0 g), $H_2O$ (10 ml), 40% HCHO (5 ml), and $PtO_2$ (0.2 g) was hydrogenated for 50 hr. The mixture was filtered and the filtrate was concentrated *in vacuo*. Reprecipitation of N-methylated Racemomycin-C from $H_2O$–acetone was repeated 3 times to give a white powder, mp above 300° (decomp.), yield 1.1 g. NMR ($D_2O$) $\delta$: 2.80—2.87 (18H, N-Me). Reineckate, prepared in $H_2O$, was recrystallized twice from $H_2O$ to red crystals, whose mp was not clear. *Anal.* Calcd. for $C_{31}H_{58}O_9N_{10}\cdot 4[Cr(NH_3)_2(SCN)_4]\cdot H_2O$ (mol. wt.=2058): C, 27.40; H, 4.37; N, 24.10; Cr, 10.16. Found: C, 27.70; H, 4.63; N, 23.74; Cr, 10.56. mol. wt.=1978 ($c$=1.03, tetrahydrofuran by osmometry).

**Isolation of $\beta$-N,N-Dimethyl-L-$\beta$-lysine (VII)**——A solution of N-methylated Racemomycin-C (VIII) in 6N HCl (100 ml) was heated in a sealed tube at 110—120° for 12 hr. The solution was concentrated and submitted to column chromatography (3.0×21 cm) over Amberlite CG-120 ($H^+$), which was pre-equilibrated at pH 4.7 with 0.2M HOAc–pyridine. The same buffer was used for elution (flow rate, 3 ml/min; each fraction, 20 g). Streptolidine (*Rf* 0.29 on paper) was eluted in fraction No. 90—105 and $\beta$-N,N-dimethyl-L-$\beta$-lysine (VII) (*Rf* 0.36 on paper; positive to Ninhydrin test) in No. 110—132, $\beta$-N,N-$\varepsilon$-N′,N′-tetramethyl-L-$\beta$-lysine in No. 140—160. The fraction No. 110—132 was concentrated and dried to give 0.06 g of hygroscopic white powder, NMR ($D_2O$: free base) $\delta$: 1.80 (m, 4H), 2.58 (d, $J$=7.0 Hz, 2H), 2.85 (s, 6H), 3.07 (t, $J$=7.0 Hz, 2H), 3.55 (m, 1H). 4-Hydroxyazobenzene-4′-sulfonate of VII, prepared in $H_2O$, was recrystallized three times from $H_2O$ to give orange prisms of 220—223°, yield 0.08 g. IR $\nu_{max}^{KBr}$ $cm^{-1}$: 1715 (C=O). *Anal.* Calcd. for $C_8H_{18}O_2N_2\cdot C_{24}H_{20}O_8N_4S_2$: C, 52.60; H, 5.20; N, 11.50; S, 8.77. Found: C, 52.77; H, 5.27; N, 11.24; S, 8.45. $[\alpha]_D^{28}$ +7.3° ($c$=1.03, MeOH).

To 0.06 g of dimethyl-$\beta$-lysine (VII) in 10 ml of 5% $NaHCO_3$, 12 drops of $C_6H_5CH_2Cl$ was added and the mixture was stirred for 12 hr. The solution was acdified with 0.1N HCl, washed three times with 10 ml of ether, and concentrated *in vacuo*. The residue was chromatographed over a cellulose column (1.7×60 cm) using BuOH saturated $H_2O$. The pure fraction was collected, washed with ether, and concentrated *in vacuo* to give VI as a hygroscopic white powder, mp 185—195° (decomp.), yield 0.03 g. NMR ($D_2O$: free base) $\delta$: 1.80 (m, 4H), 2.90 (s, broad, 8H), 3.44 (t, $J$=7.0 Hz, 2H), 3.70 (m, 1H), 7.4—7.8 (m, 5H). UV $\lambda_{max}^{EtOH}$ nm ($\varepsilon$): 238 ($9.1\times10^3$).

The free base of VI was dissolved in 0.1 N HCl and lyophilized to give the hydrochloride as a white powder, which was identified with the synthetic sample from IR and NMR spectra.

**$\beta$-N,N-Dimethyl-$\varepsilon$-N′-benzoyl-L-$\beta$-lysine (VI)**——A mixture of $\varepsilon$-benzoyl-L-$\beta$-lysine (II, 0.1 g), $H_2O$ (1 ml), 40% HCHO (1 ml), and $PtO_2$ (0.05 g) was hydrogenated. After filtration, the solvent was evaporated to dryness, the residue was dissolved in BuOH saturated $H_2O$, and the solution was chromatographed on a cellulose column (4.8×30 cm). The pure fraction was concentrated *in vacuo* to dryness, and the residue was washed with ether to give hygroscopic white powder of 185—195° (decomp.); yield 0.075 g. UV $\lambda_{max}^{EtOH}$ nm ($\varepsilon$): 238 ($9.08\times10^3$). Hydrochloride: IR $\nu_{max}^{KBr}$ $cm^{-1}$: 3300, 2640, 1718, 1628, 1542, 1410, 1400, 1300, 1170, 830, 800, 700. NMR ($D_2O$) $\delta$: 1.66 (m, 4H), 2.50 (d, $J$=7.0 Hz, 2H), 2.76 (s, 6H), 3.1—3.5 (m, 3H), 7.3—7.7 (m, 5H), $[\alpha]_D^{24}$ +40.4° ($c$=0.6, MeOH).

VI was hydrolyzed in 6N HCl to give $\beta$-N,N-dimethyl-L-$\beta$-lysine (VII) as a hygroscopic colorless powder. *p*-Hydroxyazobenzene-*p*′-sulfonate of VII: mp 220—225°. *Anal.* Found: C, 52.36; H, 5.32; N, 11.27.

**Antimicrobial Activity of N-Methylated Racemomycin-C (VIII)**——Minimum inhibitory concentration (MIC) of VIII was determined by an agar dilution method. Compoumd VIII was inactive against *M. Tuberculosis* $H_{37}$Rv, *C. albicans* ATCC, *Tr. mentagrophytes*, *Tr. vaginalis*, *S. flexneri* 2a EW 10, and *P. aeruginosa* Tsuchijima, at a concentration of 30 $\mu$/ml and inactive against *S. aureus* Terajima and *E. coli* K-12 at 200 $\mu$/ml.

**Acknowledgement** This study was aided by a grant-in-Aid for Scientific Research from the Ministry of Education.