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Studies on Viomycin. VIII.[1] Selective Modifications of the Terminal Amino Groups of Viomycin

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In order to determine which amino function of N-terminal two amino groups is important factor for the exhibition of antimicrobial activity of viomycin, selective acetylations for both amino functions were performed. The limited acetylation gave III exclusively while, the limited benzyloxycarbonylation followed by acetylation and debenzyloxycarbonylation yielded VI. Antimicrobial activities of III and VI showed almost the same reduced activities than I but somewhat stronger activities than II. From these results both of the terminal amino functions are deduced to be important factors for the exhibition of the activities of I.

Viomycin (I), a sixteen membered cyclic tuberculostatic peptide antibiotic, possesses four chemically reactive functional groups in its structure,[3,4] and the influences of individual chemical modifications of these groups on its biological activities have been studied[1,4–6] in our laboratory. During the course of the studies, we reported[4,5] that acetylation or propionylation of the both N-terminal amino groups of β-lysine residue resulted inactivation of the antimicrobial activities of viomycin. The result suggested that either or both of the amino groups has an important role for the exhibition of the activities of the antibiotic.

In this paper we report the studies to determine which amino group in viomycin is the important factor for the exhibition of its activities by means of the selective acetylations of these two amino groups and the measurements of antimicrobial activities of the modified products.

Viomycin possesses basic pK_a' values 8.2, 10.3 and 12 (measured in water)[5] which correspond to β- and ε-amino functions of β-lysine residue and guanidine group respectively. Therefore, the selective acetylation of viomycin at the ε-amino group of β-lysine residue was first investigated, since the ε-amino group is more basic and less hindered than the β-amino group.

A reaction of viomycin with excess amounts of N-acetoxy succinimide[7] at slightly basic conditions gave diacetyl product named acetylviomycin (II),[5] while with one equivalent amount of the same reagent by the same condition used for the preparation of II furnished almost a sole product N$^{\varepsilon}$-monoacetylviomycin (III) which was isolated and purified by column chromatographies of Sephadex LH-20. Characterizations and confirmations of the structure of III are described below.

To obtain the mono acetylation product of the β-amino group of viomycin, selection of a protecting group of the ε-amino function was the important problem, since viomycin is labile toward acid and base[5] and also easilly hydrogenated by catalytic hydrogenations[3,6,8] at the

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6) T. Kitagawa, T. Miura, S. Tanaka, and H. Taniyama, *J. Antibiotics*, **26**, 528 (1973).
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8) B.W. Bycroft, D.C. Cameron, L.R. Croft, A. Hassanali-Walji, A.W. Johnson, and T. Webb, *J.C.S. Perkin* I. **1972**, 827.

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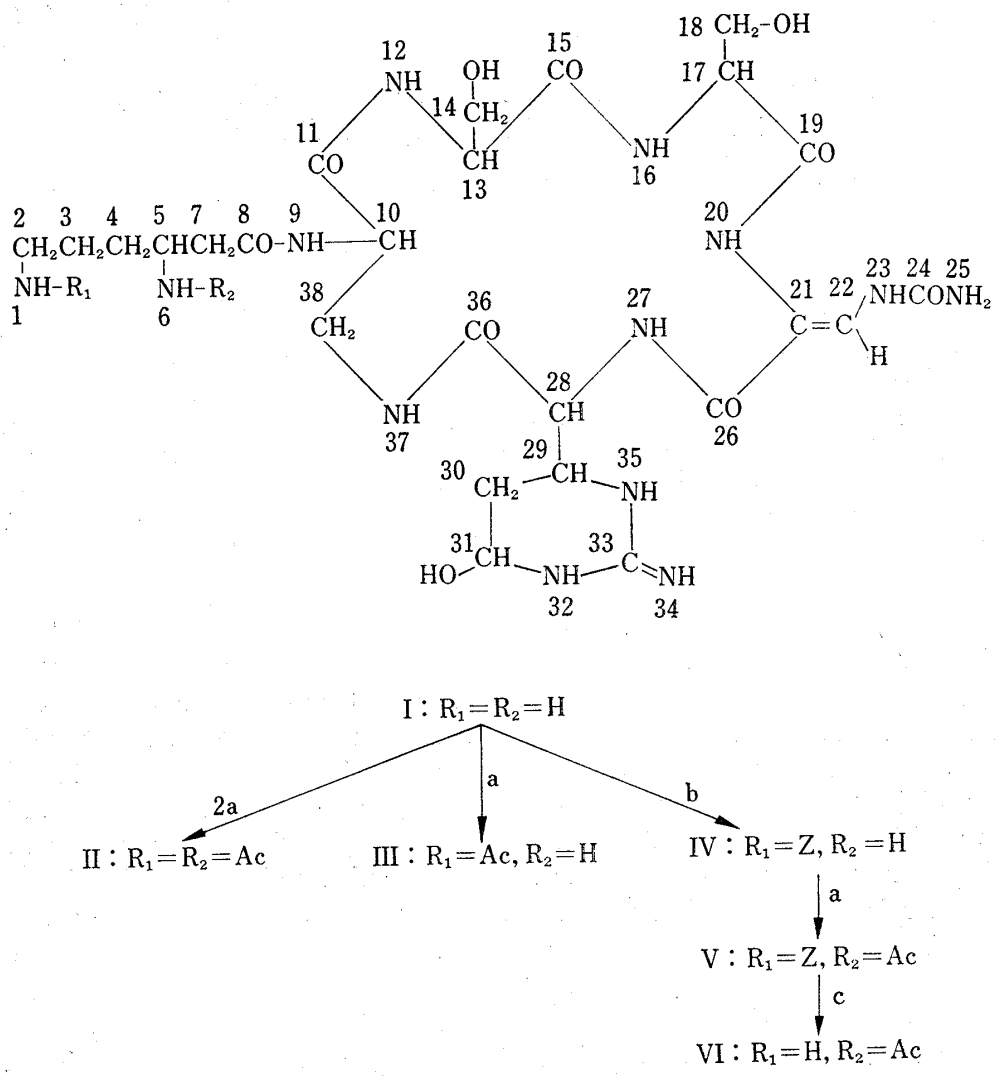

Fig. 1. Structures, Numbering and Synthetic Procedures of Viomycin and Its Acetyl Derivatives

Abbreviations: a, one equivalent amounts of N-acetoxy succinimide; b, benzyl-*p*-nitrophenyl carbonate; c, H_2/Pd

presences of Adams catalyst or palladium charcoal. Therefore, stabilities of viomycin at the conditions used for removal of protective groups were first investigated.

Among the methods investigated, a treatment of viomycin hydrochloride in 30% acetic acid solution with palladium black[9] under hydrogen atmosphere at room temperature with stirring for 20 hours resulted recoverly of the antibiotic exclusively. Thus, benzyloxycarbonyl group was selected for the protecting group.

The protection of the ε-amino group of viomycin with benzyloxycarbonyl group was achieved by reacting an equivalent amount of benzyl-*p*-nitrophenyl carbonate[10] in weakly basic aqueous tetrahydrofuran solution followed by purification procedures of Sephadex column chromatography using aqueous 30% dioxan solution as the eluent to give N^{ε}-carbobenzoxyviomycin (IV).

Acetylation of IV with N-acetoxy succinimide by the similar method described for the preparation of III yielded N^{β}-acetyl-N^{ε}-benzyloxyviomycin (V).

9) M.C. Boswell and R.R. McLaughlin, *Trans. Roy. Soc.* (Canada), **17**, 1 (1923).
10) Y. Wolman, D. Ladkany, and M. Frankel, *J. Chem. Soc.* (*C*), **1967**, 689.

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Removal of the carbobenzoxy group from V by a catalytic hydrogenation at the presence of palladium black at room temperature for three and half hours furnished N^{β}-monoacetylviomycin (VI).

Both of the mono acetylated products III and VI show positive ninhydrin, Sakaguchi and Rydon-Smith color tests like as viomycin,[5,11] The very unstable chromophoric 3-ureidodehydroalanine residue presented in viomycin also intactly remained during the synthetic procedures of the both monoacetylation products, since they have the same ultraviolet (UV) absorption maxima with original antibiotic in acidic, neutral and basic conditions.

TABLE I. UV Absorptions of Viomycin and Its Acetyl Derivatives

	λ_{max} nm (log ε)		
	0.1N HCl	H_2O	0.1N NaOH
I	268 (4.5)	268 (4.5)	284 (4.4)
II	268 (4.4)	268 (4.4)	284 (4.3)
III	268 (4.5)	268 (4.5)	284 (4.3)
VI	268 (4.5)	268 (4.5)	285 (4.3)

Molecular formula of both products based on elementary analyses is $C_{27}H_{45}O_{11}N_{13}$.

Determinations of the positions of acetylations were made on NMR spectroscopic studies. Assigned NMR shifts of C_2, C_3, C_4 and C_7 protons of β-lysine residue in viomycin[12] and its acetyl derivatives determined on 100 MHz instrument in heavy water using sodium 4,4-dimethyl-4-silapentan sulfonate (DSS) as the internal standard are summarized in Table II.

TABLE II. Chemical Shift Values of Protons on the Carbon of β-Lysine Residue in Viomycin and Its Acetyl Derivatives

$C_{No.}$	I	II	III	VI
2	3.04 (2H, m)	3.14 (2H, m)	3.20 (2H, m)	3.0 (2H, m)
3, 4	1.78 (4H, m)	1.50 (4H, m)	1.65 (4H, m)	1.62 (4H, m)
7	2.76 (2H, m)	2.40 (2H, m)	2.74 (2H, m)	2.45 (2H, m)

The assignment were made on the basis of these proton resonance shift values of viomycin and its acetyl derivatives relative to the proton resonances of β-lysine as well as relative intensities and spin-spin decoupling experiments.

Resonances of the C_5 proton of acetyl derivatives gave multiplet patterns and overlap with resonances of $C_{3\beta}$ methylene protons which made difficult to determine the exact values. However the position of the selective acetylations are clearly determined by interpreting the assignments of the other protons of β-lysine residue of viomycin and its acetyl derivatives.

Viomycin and its N^{β}-monoacetyl derivative VI have almost the same C_2 proton resonance shift values 3.04 and 3.00 ppms (2H, m) respectively, while those of the acetyl derivatives II and III shifted to 3.14 and 3.20 ppms which are the proof for III as the selective acetylation at the N^{ε}-amino group.

11) A. Finlay, G.L. Hobby, F.A. Hochstein, J.M. Lees, T.F. Lenert, J.A. Means, S.Y. P'An, P.P. Regna, J.B. Routin, B.A. Sobin, K.B. Tate, and J.H. Kan, *Am. Rev. Tuberc.*, **63**, 1 (1951); Q.R. Bartz, J. Ehrlich, J. Mold, M.A. Penner, and R.M. Smith, *ibid.*, **63**, 4 (1951).

12) P. Viglino, C. Franconi, A. Lai, E. Brosio, and F. Conti, *Org. Magnetic Resonance*, **4**, 237 (1972); T. Kitagawa, T. Miura, and H. Taniyama, Paper contributed for the 2nd symposium of "Molecular biology of microorganisms and their pharmaceutical applications," p. 86, held at Chiba, Nov. 27th, 1974.

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The C_7 proton shift values of I and III are 2.76 and 2.74 ppms but those of II and VI are 2.40 and 2.45 ppms respectively. From these results, VI is determined to be the N^{β}-monoacetyl derivative of viomycin.

Antimicrobial Activities of Viomycin Derivatives

The antimicrobial activities of viomycin sulfate, and its acetyl derivatives against gram positive and gram negative bacteria were investigated and the results are summarized in Table III.

Table III. Antimicrobial Spectra of Viomycin and Its Acetyl Derivatives

	Minimun inhibitory concentration (mcg/ml)			
	I	II	III	VI
Staphyrococcus aureus 209P	400	>3200	>3200	>3200
Bacillus subtilis PCI 219	50	>3200	1600	1600
Saltina lutea IFO 3239	400	>3200	>3200	>3200
Mycobacterium 607	6.25	3200	800	800
Escherichia coli NIHJ	100	>3200	>3200	>3200
Proteus vulgaris OX-19	400	>3200	3200	3200
Pseudomonas aeruginosa IFO 3448	>400	>3200	>3200	>3200

Medium, Culture: Bouillon two fold dilution method (1 ml/test tube); Inoculum: 0.5 ml/test tube of 50 fold dilution of an overnight culture in the broth for test organisms except *Mycobacterium* for which two fold dilution was used by the same way; Incubation temperature and time: 37° for 40 hr.

The antimicrobial activities of viomycin are diminished by acetylation of either of the two terminal amino groups of β-lysine residue. III and VI possess almost the same antimicrobial spectra and somewhat more effective than II for bacteria, since III and VI show four times effective than II for acid fast strain of Mycobacterium 607.

From these results, both of the N-terminal amino groups in viomycin are proved to be important factors for the exhibitions of the antimicrobial activities of viomycin. A possible reason for the necessity of these amino groups for the exhibition of antimicrobial activities of viomycin will be reported in the following paper.

Experimental

All melting points were taken on Yanagimoto micromelting point apparatus and were uncorrected. Nuclear magnetic resonance (NMR) spectra were determined on a JEOL JMN-PS-100 instrument (100 MHz) and are given parts per million (ppm) down field shift from the internal standard DSS in heavy water and optical rotations on Yanagimoto direct recording polarimeter model OR-20 ($c=1\%$, cell length 5 cm, in H_2O). Paper partition chromatographies (PPC) were performed with Toyo filter paper No. 51 UH. Rf_1 and Rf_2 values refer to the following solvent systems, *n*-BuOH: *t*-BuOH: pyridine: AcOH: H_2O (15: 4: 10: 3: 12) and *n*-BuOH: pyridine: H_2O (5: 3: 2) respectively. Electrophoreses were performed at 430 V, 3-1.3 mA using Toyo C type instrument. *Rm* values were obtained with reference to viomycin defining the electrophoresis distance of viomycin as 1, using pyridine: AcOH: H_2O (36: 4: 964, pH 6.14) for the solvent and ninhydrin, Sakaguchi and Rydon-Smith reagents for detections: (a) ninhydrin reagent; a 0.2% solution of ninhydrin in methylcellosolve (then heated at 100° for a few minutes); (b) Sakaguchi reagent; a 0.2% solution of 8-hydroxyquinoline in acetone and then 5N sodium hydroxide solution containing 6 drops of bromine; (c) Rydon-Smith reagent[13]; (1) a 1% solution of sodium hypochloride, (2) ethanol, (3) a mixture of a solution of *o*-tolidine (32 mg) in 50 ml of 6% acetic acid solution and 50 ml of a 0.4% aqueous potassium iodide solution.

Materials——Viomycin, acetylviomycin, N-acetoxy succinimide and benzyl-*p*-nitrophenyl carbonate were prepared according to the method given in the corresponding references.

N^{ε}-Monoacetylviomycin (III)——To a stirring solution of I sulfate (0.97 g) in 0.1M triethylamine (TEA) carbonate buffer solution (10 ml, pH 9), a dioxane solution of N-acetoxy succinimide (0.16 g in 5 ml) and 0.1M TEA solution were added dropwise alternatively so that pH of the reaction mixture maintain 8—9.

13) H.N. Rydon and P.W.G. Smith, *Nature*, **169**, 922 (1952).

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After one hour, the reaction mixture was condenced *in vacuo* below 30°, and to the residue EtOH (50 ml) was added and the resulting precipitate was separated. The precipitate was dissolved in H_2O and chromatographed with Sephadex LH-20 column using H_2O as the eluent. Fractions (10 g/fract.) positive to Rydon-Smith test were developed on PPC and the same component(s) fractions were combined and lyophilized to give the following results: No. 16 (Rf_1 Ca. 0), No. 17 (Rf_1 0, 0.35), No. 18—21 (Rf_1, 0.35, 0.49). The lyophilized product of fractions No. 19—21 was further purified by the same procedures for three times to give purified III sulfate as white amorphous powder, mp 270° (decomp.), Rf_1 0.49, Rf_2 0.21, *Rm* 0.71, $[\alpha]_D^{20}$ −37.9°. *Anal.* Calcd. for $C_{27}H_{47}O_{15}N_{13}S$ ($C_{27}H_{45}O_{11}N_{13}\cdot H_2SO_4$): C, 39.27; H, 5.74; N, 22.06; S, 3.88. Found: C, 39.14; H, 5.94; N, 22.34; S, 4.20. The characteristic spectroscopic properties are given in Table I and II.

N$^{\varepsilon}$-Carbobenzoxyviomycin (IV)——To an ice cooling solution of 2% TEA (20 ml) containing I sulfate (1.65 g) was added 75% dioxane solution of benzyl-*p*-nitrophenyl carbonate (0.55 g in 10 ml) with stirring. The reaction mixture was kept at pH 8 with occasional addition of TEA for 10 hr under stirring at room temperature. The solution was made acidic with 0.5N AcOH (pH 3.5) and washed with ether for three times to remove the resulting *p*-nitrophenol. After neutralization with TEA, the solution was condenced to dryness *in vacuo*, and the residue subjected column chromatography on Sephadex LH-20 tower using 25% dioxane solution as the eluent. Fractions (5 g/fract.) No. 24—40 were pooled, lyophilized and the residue was again chromatographed by the same procedure except the eluent was changed for H_2O and IV was obtained by lyophilization of pooled fractions No. 20—42 as white amorphous, mp 245° (decomp. colorized at 160°) Rf_1 0.63, Rf_2 0.25, yield 0.8 g. UV absorption: λ_{max}^{268nm} 22000 (in H_2O).

N$^{\beta}$-Acetyl-N$^{\varepsilon}$-carbobenzoxyviomycin (V)——To an ice cooling and stirring solution of IV (0.53 g) in 9 ml of H_2O and 3 ml of tetrahydrofuran which pH was maintained at 7.5—8 by occasional addition of aqueous TEA, was added dropwise a solution of N-acetoxy succinimide (0.09 g) in 10 ml of tetrahydrofuran. After 20 min the reaction mixture was neutralized with AcOH and lyophilized and the residue was subjected chromatography on cellulose powder column (2.5×50 cm) using the solvent system *n*-BuOH: pyridine: H_2O (5: 3: 2) as the eluent giving following results; Fractions (5 g/fract.) No. 7—18 (Rf_2 0.64). Condensation of the combined fractions No. 7—20 *in vacuo* below 30° gave V as white amorphous (0.345 g), Rf_1 0.72, Rf_2 0.64. Positive to Sakaguchi, Rydon-Smith and a negative to ninhydrin tests. NMR: δ, ppm from TMS: 8.07 (1H, s, C_{22}–H), 7.35 (5H, s, C_6H_5–), 5.15 (1H, t, C_{31}–H), 5.09 (2H, s, $C_6H_5CH_2$–), 3.19 (2H, m, C_2–H_2), 2.52 (2H, m, C_7–H_2); determined as CD_3COOD solution.

N$^{\beta}$-Monoacetylviomycin (VI)——To a 30% AcOH solution of V (0.37 g in 20 ml) was added palladium black (0.2 g) and the solution was stirred vigorously under hydrogen atmosphere at room temperature for 3.5 hr. After removal of the catalyst by filtration the solution was condenced to dryness *in vacuo* and the residue chromatographed on Sephadex LH-20 column (2×130 cm) with water. The fractions (5 g/fract.) positive to ninhydrin test (No. 43—48) were pooled and lyophilized to give VI (0.2 g) as amorphous powder mp 259° (decomp.) $[\alpha]_D^{20}$ −48.8°, Rf_1 0.38, Rf_2 0.15, *Rm* 0.78. *Anal.* Calcd. for $C_{31}H_{59}O_{18}N_{13}$ ($C_{27}H_{45}O_{11}N_{13}\cdot 2CH_3COOH\cdot 3H_2O$): C, 41.28; H, 6.59; N, 20.19. Found: C, 41.41; H, 6.31; N, 20.39. The characteristic spectroscopic properties are given in Table I and II.

Stability Test of Viomycin under Debenzyloxycarbonyl Condition——Aqueous 30% acetic acid solution (10 ml) containing I hydrochloride (20 mg) and palladium black (50 mg) was stirred for 20 hr at room temperature under hydrogen atmosphere. The reaction mixture showed positive to ninhydrin, Sakaguchi and Rydon-Smith tests and contained only I (Rf_1 0.31, *Rm* 1.0): UV absorptions λ_{max} mμ (log ε), 268 (4.31 in H_2O), 268 (4.31 in 0.1N HCl), 285 (4.15 in 0.1N NaOH), and no reduced product such as tetrahydroviomycin[3,6] (Rf_1 0.43, *Rm* 1.09) or perhydroviomycin[3,6] (Rf_1 0.41, *Rm* 1.05) was detected.

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