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Characterization of Viomycin and Its Acyl Derivatives[1]

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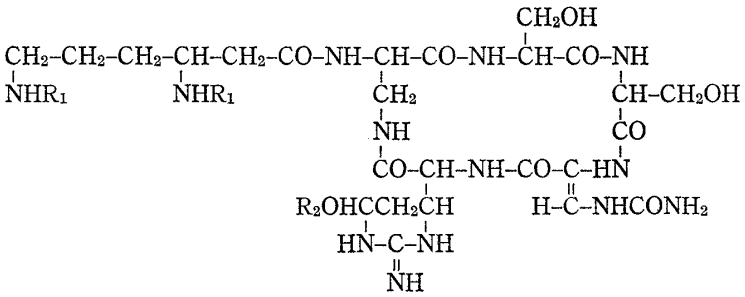
The new purification method, alkylations and acylations of tuberculostatic antibiotic viomycin were performed. The characterizations were conducted with viomycin as well as the derivatives obtained by these reactions. And also it was clarified that the alkylations on the reactive hydroxyl group of tuberactidine function had no significant influence on the antimicrobial activities while, the acylations on the N-terminal amino functions nullified the potency.

Introduction

Since the finding of the tuberculostatic antibiotic viomycin, a strongly basic polypeptide, in 1951 by Finlay, *et al.* and others,[3] numerous attempts have been made for the structural work of viomycin. These studies led our recent degradative studies[4] concluding that the structure of viomycin is formulated as (I), the formula which was speculated by Yoshioka, *et al.*[5] by extending the results obtained by X-ray crystallography of tuberactinomycin O, an antibiotic of newly found viomycin series.

In this paper detailed fundamental investigations of viomycin including the improved purification method, alkylation and acylation reactions as well as biological activities of the purified or modified products are described.



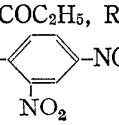
Viomycin (I): $R_1=R_2=H$
Methylviomycin (II): $R_1=H$, $R_2=CH_3$
Ethylviomycin (III): $R_1=H$, $R_2=C_2H_5$
Isopropylviomycin (IV): $R_1=H$, $R_2=CH(Me_2)_2$
Acetylviomycin (V): $R_1=COCH_3$, $R_2=H$
Propionylviomycin (VI): $R_1=COC_2H_5$, $R_2=H$
Bis-DNP-viomycin (VII): $R_1=$  $R_2=H$

Fig. 1. The Chemical Structures of Viomycin Derivatives

1) A part of this work was reported on the 7th Symposium on peptide Chemistry, Kyoto, Japan, 1969, p. 61.
2) Location: *Bunkyo-machi, Nagasaki.*
3) *a*) A. Finlay, G.L. Hobby, F.A. Hochstein, T.M. Lees, T.F. Lenert, J.A. Meens, S.Y. P'An, P.P. Rgna, J.B. Routin, B.A. Sobin, K.B. Tate, and J.H. Kane, *Am. Rev. Tuberc.*, **63**, 1 (1951); *b*) Q.R. Bartz, J. Ehrlich, J. Mold, M.A. Penner, and R.M. Smith, *ibid.*, **63**, 4 (1951).
4) T. Kitagawa, T. Miura, K. Fujiwara, and H. Taniyama, *Chem. Pharm. Bull.* (Tokyo), **20**, 2215 (1972).
5) H. Yoshioka, T. Aoki, H. Goko, K. Nakatzu, T. Noda, H. Sakakibara, A. Nagata, J. Abe, T. Wakamiya, T. Shiba, and T. Kaneko, *Tetrahedron Letters*, **1971**, 2043.

Result and Discussion

Purification Method of Viomycin

The commercial viomycin sulfate[6] was not sufficient to permit its characterizations, since it was a white hygroscopic powder containing a small amount of impurity. The sulfate when developed by circular paper chromatography[7] gave one distinct and one tailing spots with *Rf* values 0.32 and nearly 0. Purifications of the antibiotic by the repeated precipitation method,[8] column chromatographic procedures such as ion exchange resin Amberlite CG-50 or cellulose powder[9] had not resulted in sufficient and quantitative purification.

However, the dextran gel filtration methods, which the present authors successfully applied for the separation and purification of water soluble streptothricin group antibiotics,[10] were also found to be the method of choice to purify viomycin sulfate.

The hydrochloride obtained from the commercial sulfate was also purified by the same procedures.

Sephadex columns, because of their mildness in elution conditions, caused only negligible decomposition of viomycin. The results of column chromatographies, comparisons of biological activities of purified products and with commercial sulfate are summarized in Table I, in which some increases of the activities by purification are noticed.

Table I. Yield of Viomycin Sulfate and Hydrochloride by Sephadex LH-20 Column Chromatographies and Their Antimicrobial Activities

	A	B	C	D	E
Rf_1 Values	0.32	0.0	0.32, 0.0	0.36	0.05
Yield %	82.5	5		82.3	4.6
Test organism					
Staphylococcus aureus Terajima	3	>30	10	3	
Escherichia coli K-12	30	>30	10	30	
Shigella flexineri 2a EW 10	10	>30	30	30	
Pseudomonas aeruginosa Tsuchijima	>30	>30	>30	>30	

A: viomycin sulfate
B: byproduct on the purification of A
C: commercial viomycin sulfate
D: viomycin hydrochloride
E: byproduct on the purification of D

Alkylation of Viomycin Hydrochloride

In 1960, Hochstein, *et al.*[11] reported the formation of methylviomycin (II) by refluxing hydrochloride of the antibiotic in methanol but the similar treatments were unsuccessful to give ethyl and propyl derivatives.

However, re-examining these methods it was found that not only methylviomycin, ethylated or propylated products were also produced when the hydrochloride was refluxed with corresponding alcohols. Thus, each of the reaction products possessed alkoxyl function which could not removed by digestion in acetone. Nuclear magnetic resonance (NMR) spectrometry provided us useful informations for the identification and quantitative analy-

6) Commercial viomycin sulfate was kindly supplied by Messrs. Chas. Pfizer and Co., Inc. The amount of the impurity increased during kept it in a refrigerator.
7) M.I. Horowitz and C.P. Schaffner, *Anal. Chem.*, **30**, 1616 (1958).
8) A.C. Finlay and B.A. Sobin, Japan. Pat., Showa 29-697, Feb. 2 (1954).
9) H. Taniyama, F. Miyoshi, and K. Kageyama, *Yakugaku Zasshi*, **82**, 87 (1962).
10) H. Taniyama, Y. Sawada, and T. Kitagawa, *J. Chromatogr.*, **56**, 360 (1971).
11) F.A. Hochstein, G. City, and R.L. Miller, U.S. Pat., 2920998 Jan. 12 (1960).

sis of these alkoxyl derivatives. For example yields of these alkylations were obtained by comparing the area of their methyl protons signals with that of characteristic 1H singlet proton in the spectrum of viomycin as well as these derivatives. Also each products when developed on Avicel SF thin-layer chromatography (TLC),[12] gave distinct spots *Rf* values 0.62 for II, 0.69 for ethylviomycin (III) and 0.77 for isopropylviomycin (IV) besides 0.58 of contaminating recovered viomycin as shown in Table II.

Table II. NMR Data, Yield and *Rf* Values of Alkylviomycin Hydrochloride

Peptide	Methyl proton (δ: ppm)[a]	Yield %	*Rf* Values
I			0.58
II	3.46 (singlet)	>90	0.62
III	1.25 (triplet, $J=8$ cps)	65	0.69
IV	1.18 (doublet, $J=10$ cps)	50	0.77

a) chemical shift in δ from DSS, solvent D_2O

The yield of ethylation or isopropylation was less than 65% while, more than 90% was observed for the methylation. The reason for these low yields for the former reactions could be attributed to the less solubilities of viomycin hydrochloride in these corresponding alcohols than in methanol and it would be responsible for the earlier assignment that these alkylated derivatives were not produced. From these results it is clarified that viomycin possesses alkoxylable hydroxyl function of carbinolamine type[13] in its molecule as expected from the nature of its constituent tuberactidine residue.[14] However these alkoxyl derivatives especially the newly obtained ones were very insoluble in organic solvents and had tendencies to regenarate viomycin hydrochloride in aqueous solution. These natures especially the later tendencies made it difficult to purify the products, and all the efforts for the purifications were proved to be unsuccessful.

Acylations of Viomycin

Acetylations of viomycin using acetic anhydride or acetylchloride in methanol or pyridine resulted in the recoveries of starting meterial, since the antibiotic was insoluble in these reagents and Schotten-Baumann type reactions provided with decompositions of the amine component. However, viomycin was exclusively converted into diacetyl or dipropionyl derivatives named acetylviomycin (V) or propionylviomycin (VI) using acetyl or propionyl esters of N-hydroxysuccinimide[15] as acylating active reagents. Thus viomycin dissolved in 50% dioxane solution was reacted at room temperature with suitable active esters with the presence of triethylamine until it showed negative ninhydrin reaction. The crude reaction products precipitated by ethanol from the condenced reaction mixtures were also successfully separated and purified by Sephadex column chromatographies.

Physico-Chemical Properties of Viomycin and Its Derivatives

Viomycin sulfate and hydrochloride or hydrochlorides of alkylated or acylated derivatives are unstable in acidic or especially basic solution and neither one of their free base was obtainable as purified form. They are all white plates but very hygroscopic. Melting points and specific rotations of them are summarized in Table III.

These compounds are all insoluble in common organic solvents. They give positive Sakaguchi,[3] Fehling,[3b] Biuret,[3b] and Rydon-Smith[16] reactions besides ninhydrin test[3] which acylviomycins are negative.

12) K. Nagasawa, H. Yoshidome, and K. Anryu, *J. Chromatogr.*, **52**, 173 (1970).
13) E. Suzuki, S. Inoue, and T. Goto, *Chem. Pharm. Bull.* (Tokyo), **16**, 933 (1968).
14) T. Wakamiya, T. Shiba, and T. Kaneko, *Tetrahedron Letters*, **1970**, 3497.
15) G.W. Anderson, J.E. Zimmerman, and F.M. Callajana, *J. Am. Chem. Soc.*, **86**, 1839 (1964).
16) H.N. Rydon and P.W.G. Smith, *Nature*, **169**, 922 (1963).

Table III. Melting Points and Specific Rotations of Viomycin Derivatives

	mp (decomp.) (lit.)	$[\alpha]^{25°}$ (c=1%, H_2O) (lit.)
I sulfate	266° (280°,[3a] 252°[3b])	−29.5° (−32°[3a])
I hydrochloride	270° (265—268°)[a]	−16.7°
V hydrochloride	265°	−33°
VI hydrochloride	245°	−19°

a) A.C. Finlay and B.A. Sobin, Japanese Pat. Showa 29—697 Feb. 9th 1954.

The ultraviolet (UV) spectra of viomycin and its acyl derivatives show the same characteristic absorptions as cited in Table IV.

The infrared (IR) spectra of their hydrochlorides are very similar as shown in Fig. 2.

Table IV. UV Absorptions of Viomycin Derivatives

Derivative	$\lambda_{max}^{m\mu}$ (log ε)		
	in H_2O	in 0.1N HCl	in 0.1N NaOH
I sulfate	268 (4.4)	268 (4.4)	285 (4.2)
I hydrochloride	268 (4.5)	268 (4.4)	285 (4.3)
V hydrochloride	268 (4.4)	268 (4.4)	285 (4.3)
VI hydrochloride	268 (4.4)	268 (4.4)	285 (4.3)

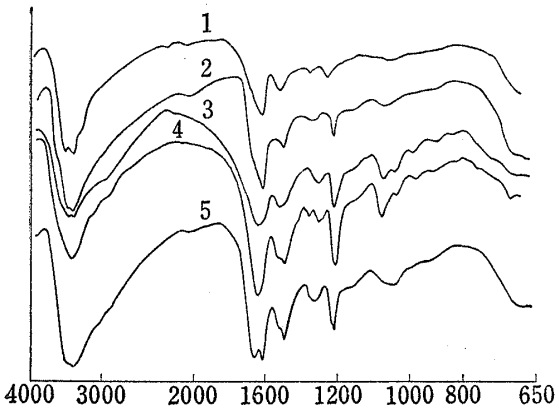


Fig. 2. Infrared Spectra of Viomycin Derivatives Hydrochloride in KBr

1: ethylviomycin 2: methylviomycin 3: propionylviomycin 4: acetylviomycin 5: viomycin

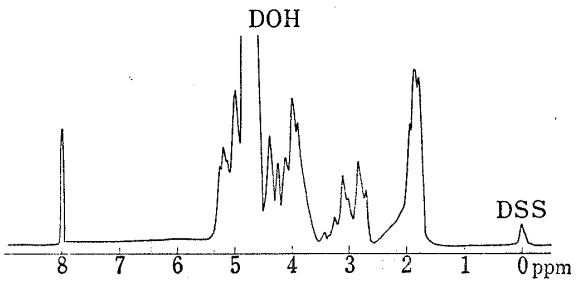


Fig. 3. NMR Spectrum of Viomycin Sulfate in D_2O

The NMR spectrum of viomycin sulfate is given in Fig. 3. Similar profiles of NMR spectrum were obtained for its alkyl and acyl derivatives except for their substituents. The spectral signals of the alkyl substituents are already cited in Table II, and those data for acyl derivatives are δ, 1.95 ppm (6H, singlet) for acetyl methyl protons and δ, 1.2 ppm (6H, triplet, J=8 cps) for propionyl methyl protons. The characteristic absorptions of δ, 8.0 ppm (1H, singlet) for a proton of its chromophore group[17] and δ, 5.2 ppm (1H, triplet, J= 3 cps) for a proton on the carbon bearing the hydroxyl and guanidyl[14] were observed in every spectrum.

The empirical formula by elementary analysis for these compounds are summarized in Table V.

17) B.W. Bycroft, D. Cameron, A. Hassanali-Walji, and A.W. Johnson, *Tetrahedron Letters*, **1969**, 2539.

Table V. Elemental Analysis of Viomycin Derivatives

Derivative	Formula		Anal. C	H	N	S	Cl
I Sulfate	$C_{25}H_{43}O_{10}N_{13}\cdot 3/2H_2SO_4\cdot 4H_2O$	Calcd.:	33.18	5.75	20.13	5.31	
		Found:	33.09	6.09	20.35	5.59	
I Hydrochloride	$C_{25}H_{43}O_{10}N_{13}\cdot 3HCl\cdot 2H_2O$	Calcd.:	36.14	5.90	21.92		12.83
		Found:	35.85	6.38	21.73		13.04
V Hydrochloride	$C_{29}H_{47}O_{12}N_{13}\cdot HCl\cdot 3H_2O$	Calcd.:	40.51	6.28	21.18		4.14
		Found:	40.89	6.32	21.26		4.13

Complete hydrolyzates of every viomycin derivatives with constant boiling hydrochloric acid were submitted to an automatic amino acid analyser with a column of strongly acidic ion exchange resin. They gave the same chromatographic results which typical analysis is cited in Fig. 4 for basic and Fig. 5 for neutral amino acids. To determine the molar ratios of the constituents, the mixtures of serine, α,β-diaminopropionic acid and β-lysine in the ratios 1:1:1 (A), 2:1:1 (B), and 3:1:1 (C) were also supplied to the analyser after acid hydrolysis. Table VI summarized the calculated relative intensity of the peaks in reference to β-lysine for the individual compounds.

Table VI. Relative Intensity of the Peaks in Amino Acid Analysis

Derivative	I	II	III	IV	V
I	1.49	0.52	1.0	0.15	0.58
V	1.50	0.47	1.0	0.26	0.60
Sample A	1.0	0.50	1.0		
B	1.50	0.56	1.0		
C	1.70	0.57	1.0		

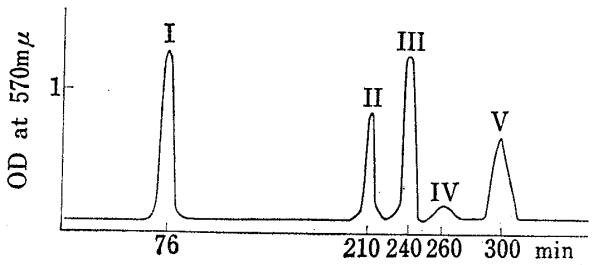


Fig. 4. Basic Amino Acids Analysis of Viomycin Hydrolysates with 6N Hydrolysates with 6N Hydrochloric Acid

I: serine
II: α,β-diaminopropionic acid
III: β-lysine
IV: viomycidine
V: ammonia

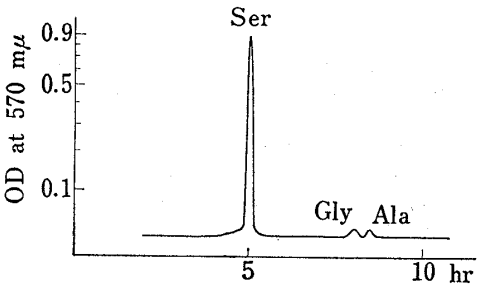


Fig. 5. Neutral Amino Acid Analysis of Viomycin Hydrolysates with 6 N Hydrochloric Acid

From these results the molar ratios of serine, α,β-diaminopropionic acid and β-lysine in viomycin as well as acylviomycins are determined as 2:1:1, whereas that of the other component viomycidine,[18] an artifact derived from the true constituent tuberactidine residue,[14] was very small and quantitative analysis was not obtained. Besides these amino acids a small amount of glycine or alanine and one equivalent amount of urea were found in their hydro-

18) G. Buchi and J.A. Raleigh, *J. Org. Chem.*, **36**, 873 (1971).

lysates. However, amount of these two amino acids were increased remarkably with alkaline hydrolysis using barium hydroxide solution. The detection of glycine in the hydrolysate of viomycin was already reported[19] but the formaiton of alanine had not been known.

Glycine and alanine were also identified on gas chromatography, the method which is widely used for the identification of amino acids.[20] Thus the above mentioned alkaline hydrolysate of viomycin was esterified with ethanol and hydrochloric acid and the resulting ester derivatives were reacted with trifluoroacetic anhydride. The reaction products when submitted to a gas chromatography, showed the peaks of N-trifluoroacetyl glycine ethylester and *N*-trifluoroacetylalanine ethylester. However, the amount of glycine or alanine found in the acid hydrolysate of viomycin is very small comparing with that of serine suggests that neither one of these amino acids is a constituent but an artifact formed during the hydrolysis.

Viomycin possesses pK_a' values 8.2, 10.3, and 12 (measured in water), from which presences of three basic but no acidic functions were infered in its molecule. Thus, viomycin was reacted with bis-2,4-dinitrofluorobenzene in the sodium bicarbonate solution to give bis-DNP derivative of viomycin as a yellow amorphous powder. On acid hydrolysis the DNP derivative gave all of the amino acids obtained from acid hydrolysate of viomycin except for β-lysine: bis-DNP-β-lysine, mp 200—201°,[21] was obtained and was identical with authentic sample synthesized from β-lysine obtained from acid hydrolysate of racemomycin group antibiotics[22] by measuring mixed melting point and the comparisons of their IR spectra. The conclusion that the N-terminus of viomycin is β-lysine was also confirmed by the dancyl procedure.[23] However, the C-terminal residue was not observed in viomycin by Akabori's method[24] or Matsuo's procedure[25] as expected.

Judging from these above mentioned results, acetylviomycin and propionylviomycin are concluded to be the acylated products of viomycin at the N-terminal β-lysine residue without affecting the other reactive parts such as the chromophore group or tuberactidine residue.

Antimicrobial Activities of Viomycin Derivatives

The antimicrobial activities of viomycin sulfate, the alkylated or the acylated derivatives of viomycin aganist gram positive and gram negative bacteria were investigated and the results are summarized in Table VII.

Table VII. Antimicrobial Activities of Viomycin Derivatives

Test organism	I	II	III	V	VI
Staphylococcus aureus Terajima	6.2	25	25	>400	400
Bacillus subtilis NRRL 3014	3.1	6.2	6.2	>400	400
B. subtilis K-02	12.5	25	25	>400	>400
Proteus valgalis OX-19	25	100	100	>400	>400
Escherichia coli NIHJ	50	100	100	>400	>400
Mycobacterium 607	1.6	1.6	1.6	>400	400
Pseudomonas aeruginosa Tsuchijima	100	400	400	>400	>400

19) J.R. Dyer, H.B. Hayes, E.G. Miller, Jr, and R.F. Nasser, *J. Am. Chem. Soc.*, **86**, 5363.
20) W.M. Lamkin and C.W. Gehrke, *Anal. Chem.*, **37**, 383 (1965); F. Weygand, B. Kolbe, A. Prox, M.A. Tilak, and I. Tomida, *Z. Physiol. Chem.*, **322**, 38 (1960).
21) T. Goto, Y. Hirata, S. Hosoya, and N. Komatsu, *Bull. Chem. Soc. Japan*, **30**, 729 (1957).
22) H. Taniyama, Y. Sawada, and T. Kitagawa, *Chem. Pharm. Bull.* (Tokyo), **19**, 1627 (1971).
23) B.S. Hartley and V. Massey, *Biochem. Biophs. Acta*, **21**, 58 (1958); W.R. Gray and B.S. Hartley, *Biochem. J.*, **89**, 379 (1963).
24) S. Akabori, K. Ohno, and K. Narita, *Bull. Chem. Soc., Japan*, **25**, 24 (1952).
25) H. Matsuo, Y. Fujimoto, and T. Matsuno, *Biochem. Biophys. Res. Comm.*, **22**, 69 (1966).

From these results it was concluded that acylations of free amino groups of β-lysine residue nullified the activity of mother molecule while the hydroxyl function of tuberactidine moiety could be alkylated without resulting in any significant change of the activity.

Experimental

IR spectra were determined on a Japan Spectroscopic DS-301 spectrophotometer, UV spectra on a Hitachi EPS-2, and NMR spectra on a Hitachi Perkin-Elmer H-60 (60 MHz) spectrometers. Hitachi KLA-3 type amino acid analyser with 9 mm × 50 cm column was used for basic amino acids using the eluent of 0.7M citrate buffer solution[26] (pH at 5.25 ± 0.02) instead of using the standard 0.35M citrate buffer solution[27] (pH 5.25 ± 0.02) for this analyser except for neutral amino acids. For neutral amino acid analysis 9 mm × 150 cm column was used with the standard 0.2M citrate buffer solution (pH, 3.25 ± 0.02) and gas chromatography on Shimazu GC-1C type spectrometer with diethylene glycol iso-phthalate on Shimalite column (0.3 cm × 2.5 m) using following operating conditions: the carrier gas (N_2) and (H_2) flow rates through the column at room temperature were about 24 ml and 30 ml per minute respectively and the temperatures 150° for column, 150° for detector and 220° for injector. Paper chromatographies were determined on Toyo filter paper No. 51 UH (diam. 31.5 cm) by circular ascending method and Rf_1 values refer to the system of *n*-BuOH, *t*-BuOH, pyridine, AcOH, H_2O (15: 4:10: 3: 12, solvent 1) and Rf_2 values for the system of *t*-BuOH, AcOH, H_2O (2:1:1). TLC were carried out on silicagel (Kiesel gel G, Merk) with Rf_{11}, Rf_{12} and Rf_{13} values refer to the systems of the solvents 1, methanol: ethylacetate (3:1) and phenol: ethanol: H_2O (15: 4: 1) respectively, and on Avicel with Rf_{14} values refering to the solvent 1. Electrophoresis was determined on a Toyo C type (500 V, 3—5 mA) instrument for 3 hr, with Toyo filter paper No. 51 and pyridine: HOAc: H_2O (5: 0.2: 95) (pH 6.5) buffer solution. *Rm* values were obtained with reference to viomycin defining the electrophoresis distance of viomycin as 1, under the above conditions. Ninhydrin, Sakaguchi or Rydon–Smith reagents were used from the detections in these tests.

Materials——Glycine, L-alanine and L-serine were bought from Wako Pure Chemical Industries Ltd. L-α,β-Diaminopropionic acid was synthesized from L-aspartic acid by Schmidt reaction.[28] L-β-Lysine was obtained from acid hydrolysate of racemomycin. Hydrochloric acid; analytical grade of Wako Pure Chemical Industries Ltd. was used.

Purification of Viomycin (I) Sulfate——An aqueous solution (3 ml) of commercial viomycin sulfate (2 g) was passed through a column of Sephadex LH-20 (2.0 × 150 cm) and the column was eluted with distilled water. The ninhydrin positive fractions (No. 11—17, 10 g/fract.) were developed on PPC and the same component (s) fractions were combined and lyophilized to give the following results: Fractions No. 11—12, 0.06 g (3.0%), Rf_1, 0. Fractions No. 13—14, 0.1 g (5%) Rf_1, 0.0 and 0.32. Fractions No. 15—17, 1.65 g (82.5%), Rf_1, 0.32. For the elemental analysis the same procedures were repeated at least three times to give purified I sulfate, mp 266° (decomp.), $[\alpha]_D^{18}$ −29.5° (*c*=1%, H_2O), UV λ_{max}^{nm} (log ε): 268 (4.4) in H_2O or 0.1N HCl; 285 (4.2) in 0.1N NaOH. IR ν_{max}^{KBr} cm^{-1}: 3060, 2915, 1650, 1490, 1320, 1220, 1100. NMR δ, ppm; 8.0 (1H, s, C<u>H</u>=N), 5.20 (1H, t, *J*=3 cps, C<u>H</u>OH-N). Rf_1, 0.32. Rf_2, 0.01. Rf_{11}, 0.10. Rf_{12}, 0.65. Rf_{13}, 0.58. *Rm*, 1.0.

Viomycin (I) Hydrochloride——To an aqueous solution (10 ml) of commercial viomycin sulfate, equivalent amount of barium chloride solution was added and the resulting precipitated $BaSO_4$ was removed by centrifuge. The supernatant was condensed to dryness *in vacuo* below 40°. The residue chromatographed on Sephadex LH-20 tower (2 × 150 cm, 7 g/Fract.) using H_2O as the eluent and ninhydrin reagent for detections. Fractions No. 27—29 contained the impurity, 0.14 g, Rf_1, 0, as a white amorphous powder. Fractions No. 30—38 contained I hydrochloride (2.48 g, 83%) mp 270° (decomp.), $[\alpha]_D^{18}$ −16.6° (*c*=1%, H_2O) Rf_1, 0.36. *Rm*=1.0. White hygroscopic plate. UV λ_{max}^{nm} (log ε); 268 (4.5, H_2O), 268 (4.4, 0.1N HCl), 285 (4.3, 0.1N NaOH). IR ν_{max}^{KBr} cm^{-1}; 3340, 3115, 2980, 1650, 1485, 1220. The elementary analysis is cited in Table V.

Acetylviomycin Hydrochloride (V)——To an aqueous solution (4 ml) of viomycin sulfate (2.9 g) a dioxane solution containing excess N-acetoxysuccinimide (1 g) was added and the mixture was kept basic with occasional titration of triethylamine with stirring at room temperature until it showed the negative ninhydrin test. After the reaction an equivalent amount of $BaCl_2$ was added to the mixture and the resulting precipitated $BaSO_4$ was removed by centrifuge and the supernatant was condenced to dryness *in vacuo* below 30°. The residue was titrated and washed with methanol. The resulting precipitated crude acetylviomycin hydrochloride was separated by centrifuge and washed with ether and then dried over P_2O_5 in a desiccator. The crude acetylviomycin (3.0 g) thus obtained was purified by Sephadex column chromatographys to

26) Preliminary experiment showed better analyses were obtained with this eluent than using the standard solution.

27) "Instructuin for KLA-3 type amino acid analyser" Hitachi Ltd. Tokyo.

28) T. Kitagawa, T. Ozasa, and H. Taniyama, *Yakugaku Zasshi*, **80**, 285 (1969).

give 1.98 g of V, mp 265° (decomp.), $[\alpha]_D^{18}$ −33° (c=1%, H_2O). UV λ_{max}^{nm} (log ε); 268 (4.4, in H_2O), 268 (4.4, in 0.1N HCl), 285 (4.2, in 0.1N NaOH). IR ν_{max}^{KBr} cm^{-1}: 3360, 3140, 2970, 1705 (shoulder), 1655, 1495, 1220, 1070, 995. NMR δ ppm; 8.0 (1H, s, CH=N), 5.20 (1H, t, J=3 cps, CHOH–N), 1.95 (6H, s, $2COCH_3$). Colorless plate. Rf_1, 0.61, Rf_2, 0.84, Rf_{11}, 0.23, Rm, 0.58. The color reactions: positive to Sakaguchi, Rydon–Smith and negative to ninhydrin. The elementary analysis data are cited in the Table V.

Propionylviomycin Hydrochloride (VI)——To an aqueous solution of (20 ml) of I hydrochloride (2 g), a dioxan solution of N-propionyloxysuccinimide[29] (0.8 g) was added and the mixture was kept basic with occational addition of triethylamine under stirring at room temperature untill it showed a negative ninhydrin reaction. The mixture was then condensed *in vacuo* and the resulting sirup was washed with ethanol and then chromatographed on Sephadex LH-20 column (2×150 cm) using H_2O as the eluent, to give VI (1.2 g, yield, 55%), colorless plate, mp 245° (decomp.), $[\alpha]_D^{18}$ −19° (c=1%, H_2O), Rf_1, 0.70. Rf_{11}, 0.42. Rm, 0.58. IR ν_{max}^{KBr} cm^{-1}: 1630, 1500. NMR δ ppm; 8.0 (1H, s), 5.15 (1H, t, J=3 cps), 2.2 (2H, q, J=8 cps), 1.1 (3H, t, J=8 cps). UV λ_{max}^{nm} (log, ε): 268 (4.4 in H_2O or 0.1N HCl), 285 (4.2 in 1N NaOH). The color reactions: Positive to Sakaguchi, Rydon–Smith and negative to ninhydrin.

General Preparation Methods of Alkoxyviomycins——A suspension of viomycin hydrochloride (0.1 g) in corresponding abs. alcohol (MeOH, EtOH or iso-PrOH) was heated under refluxing for 48 hr. The reaction mixture was condensed to dryness *in vacuo* and again the alcohol was added and the mixture was heated under reflux for 2 or 3 days. After the reaction, unsoluble precipitation was filtered off and the filtrate was condensed to dryness *in vacuo*. The residue was washed with aceton and then dried over P_2O_5 in a evacuated desiccator. To confirm the presence of alkoxyl function a small amount of pyridine was added to the individual product before kept it in the desiccator. The alkoxyl contents were determined by their NMR spectral signals of methyl protons in reference to 8 ppm 1H proton. The crude methylviomycin when dissolved in water and then lyophilized showed decrease in the content of methoxyl.

Methylviomycin Hydrochloride (II)——Colorless hygroscopic plate, Rf_1, 0.30. Rf_{14}, 0.62. Rm, 0.98. IR ν_{max}^{KBr} cm^{-1}; 3320, 3050, (shoulder) 1665 (shoulder), 1623, 1500, 1226. NMR δ ppm: 8.0 (1H, s, CH–N), 5.2 (1H, t, J=3 cps), 3.46 (3H, s, OCH_3).

Ethylviomycin Hydrochloride (III)——Colorless hygroscopic powder, Rf_1, 0.40. Rf_{14}, 0.69. Rm, 0.98. IR ν_{max}^{KBr} cm^{-1}: 3420, 3325, 1670, 1635, 1615, 1505, 1225. NMR δ ppm: 8.0 (1H, s, CH–N), 5.20 (1H, t, J=3 cps), 1.25 ($3\times3/2$ H,[30] t, OCH_2CH_3).

Isopropylviomycin (IV)——Colorless hygroscopic powder, Rf_{14}, 0.77. Rm, 0.95. IR ν_{max}^{KBr} cm^{-1}: 3330, 1660, 1480, 1355, 1310, 1220, 1045. NMR δ ppm: 8.0 (1H, s, CH=N), 5.20 (1H, t, J=3 cps), 1.18 (6/2H,[30] d, J=10 cps, isopropyl methyl proton).

Elemental Analysis——For analysis, individual viomycin derivatives was dried over P_2O_5 *in vacuo* at 1 mmHg for more than 24 hr at room temperature.

Hydrolysis of Viomycin Derivatives——The derivative (1.5 mg) was dissolved in 1 ml of 6N HCl, placed and sealed in a tube and heated at 110—120° for 20 hr. The hydrolysate was evaporated to dryness to remove excessive acid and then was re-dissolved in 1.5 ml of water. One third portions were used for basic and neutral amino acid analysis respectively, the remaining portion for paper chromatography.

Hydrolysis of Viomycin with Base——Viomycin sulfate (1 g) in 10 ml of 3N $Ba(OH)_2$ was refluxed for 6 hr. After cooling precipitated $Ba(OH)_2$ was filtered off and the filtrate was condensed to 5 ml. An equivalent amount of sulfuric acid was added and the precipitated $BaSO_4$ was removed by centrifuge and the supernatant was condensed to dryness *in vacuo* to give pale yellow powder (0.2 g). This product showed presences of glycine and alanine besides serine, dapa, β-lysine and viomycidine on amino acid analysis and PPC. The hydrolysate (0.2 g) was esterified with abs. EtOH (20 ml) and dry HCl under refluxing for overnight. To the reaction mixture abs. benzene (100 ml) was added and the solution was condensed under reduced pressure and the residue was dried up in a desiccator. The resulting ethyl ester deri-

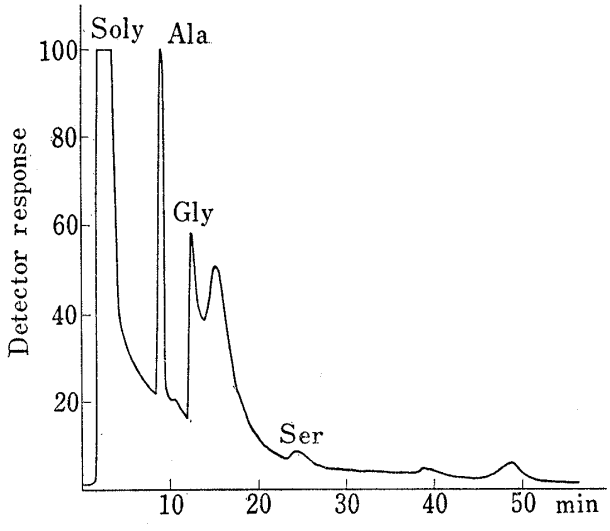


Fig. 6. Gas Chromatography of N-Trifluoroacetyl Amino Acids Ethyl Esters of Viomycin Hydrolysates.

29) Henkel und Cie. G.m.b.H., *Fr. Demande*, 2, 013, 139 (Cl. CO7c, Cl1d), 27 Mar. (1970) [*C.A.*, **73**, 132259h].
30) From which the yields of alkylations were determined.

vatives (0.1 g) were dissolved in 2 ml of $(CF_3CO)_2O$ and then refluxed for 10 min. The crude product of trifluoroacetylated amino acid ethyl esters were gas chromatographed to find presences of trifluoroacetylated glycine and alanine ethyl esters with comparisons of synthetic specimens as cited in Fig. 6.

For comparison each trifluoroacetylated glycine, alanine and serine ethyl esters was synthesized by the same procedures.

Bis-2,4-dinitrophenylviomycin (VII)——To a solution of viomycin sulfate (2 g) in 0.8N $NaHCO_3$ (50 ml), 5% ethanolic solution of 2,4-dinitrofluorobenzene (DNFB) (40 ml) was added. The reaction mixture was kept in the dark under mechanical stirring overnight at room temp. The resulting yellow precipitate was collected by filtration and the filtrate was washed well with water, EtOH and ether suceessively giving yellow amorphous VII, yield, 2.2 g, mp above 240°, $[\alpha]_D^{21}$ −26° (c=1%, in dimethylformamide), UV λ_{max} nm (log ε): 362 (4.36) (in dimethylformamide).

Bis-2,4-dinitrophenyl-β-lysine (VIII)——a) From the hydrolysis of the DNP derivative of viomycin. A suspension of the DNP derivative (0.7 g) in 6N HCl (70 ml) was refluxed for 8 hr. The remaining precipitate was collected by filtration to give yellow powder (0.1 g) which on titration and recrystallization from acetone–MeOH afforded VIII as yellow needles, mp 200—201°, UV λ_{max}^{DMFA} nm (log ε): 363 (4.6). IR ν_{max}^{KBr} cm^{-1}: 1735 (COOH), 1340 and 1520 (NO_2); Rf_1; 1.0. Rf_2, 1.0. Rf_{11}, 0.82. Rf_{13}, 0.25. *Anal.* Calcd. for $C_{18}H_{18}O_{10}N_6$: C, 45.19; H, 3.79; N, 17.57. Found: C, 45.55; H, 3.67; N, 17.41.

The filtrate was washed with EtOAc and the aqueous layer was condensed to dryness *in vacuo*. The residue showed the presences of serine, α,β-diaminopropionic acid and viomycidine but an absence of β-lysine on PPC and paper electrophoresis. Also amino acid analysis of the residue (1 mg) gave the same result.

b) From β-lysine obtained from an acid hydrolysate of racemomycin. To a solution of β-lysine (20 mg) in 0.8N $NaHCO_3$ (2 ml), 5% ethanolic solution of DNFB (1 ml) was added and the mixture was kept in the dark with mechanical stirring overnight at room temp. The solution was washed with ether and the aqueous solution was acidified with 1N HCl. The resulting precipitate was filtered and crystallized from aceton–MeOH furnished yellow needles, mp 196—198°. The mixed mp with the sample obtained by the procedure (a) showed no depression and the IR spectra of both products were identical.

Determination of C-Terminal Residue——C-Terminal residues were determined by the Akabori's hydrazinolysis method and Matsuo's ^3H isotope labeling method as followes.

a) **Hydrazinolysis Method**——A solution of 2—4 mg of antibiotic in 0.8 ml of anhydrous hydrazine was heated in a sealed tube at 105° for 5 hr. After the excessive hydrazine was evaporated over sulfuric acid in an evacuated desiccator, the residue was dissolved in 0.5 ml of H_2O and shaken with 0.5 ml of bezaldehyde. The reaction mixture was washed with AcOEt and the aqueous layer was amino acid analysed on PPC and automatic amino acid analyser.

b) **^3H labeling Method**——A solution of an antibiotic (1—2 mg) in Ac_2O (1 ml) was brought to gentle boiling for 30 min. The mixture was allowed to cool to room temp. and evaporated *in vacuo* below 40° to dryness. The residue was treated with one drop of pyridine and 0.05 ml of ^3H_2O (*ca.* 50 mCi) for 6 hr at room temp. After the evaporation *in vacuo* at 40°, addition of H_2O (1 ml), followed by evaporation was repeated for several times to remove completely the washable radioisotope. The residue was hydrolyzed with 6N HCl for 12 hr at 115°. The excessive acid was removed *in vacuo* and a small portion of the residue was subjected to paper electrophoresis using the conditions described already. The locations corresponding to positive ninhydrin test were cut and the radioactivities of them were measured by 2π Gas Flower Counter (Aloca FC-LE) using Q gas (He, isobutane, 99.05: 0.95) as flower gas and 1350 V as voltage.