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Studies on Viomycin. VII.[1] Oxidative Modifications of Viomycin[2]

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As an extension of the investigations on structure-activity relationships of viomycin (VM), a tuberculostatic antibiotic, chemical modifications of its chromophore group by oxidative reactions were studied.

Oxidations with $KMnO_4$ gave normal oxidation products named oxoviomycin and its derivative while, with bromine, gave abnormal products V, VI and VIII, those of which were characterized by chemical and physical analyses. Antimicrobial potencies of both oxidation products of VM showed the nullified activities, the results of which were agreeable from the predictions that D-form or dehydro derivative of amino acid residue is important factor for their potencies of original antibiotics.

Introduction

After the structural elucidation of tuberculostatic antibiotic viomycin,[4–6] investigations of the structure–activity relationships of the antibiotic by means of chemical modifications of its reactive functions have been studied in our laboratory.[1,7,8]

It is well known that most peptide antibiotics are cyclic and contain D-form amino acid constituent (s).[9] The recent hypotheses suggested that dehydroamino acid is supposed to be a precursor of D-form amino acid.[10] Taking account of the above mentioned evidences and hypotheses, one could expect that D-form or dehydro amino acid (s) is an important factor for the expression of antimicrobial activity of the antibiotic. Since viomycin is a cyclic peptide and all of its constitutive amino acids are L-form[11] except 3-ureidodehydroalanine residue,[12] the characteristic chromophore group of the antibiotic, modifications of this dehydroamino acid moiety and investigation of modified products might give us usefull informations from the view point of structure–activity relationships especially since, no systematic studies on chemicall modifications in this residue have been reported so far.

However, in the preceeding paper we reported that on contradictorily to the above mentioned expectation that the chromophore group might be an important factor for the antimicro-

1) Part VI: T. Kitagawa, T. Miura, S. Tanaka, and H. Taniyama, *J. Antibiotics*, **26**, 528 (1973).
2) A part of this work was presented at the 88th Annual Meeting of Pharmaceutical Society of Japan, Tokyo, April 5, 1968.
3) Location: *Bunkyo-machi, Nagasaki.*
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. Nakatsu, T. Noda, H. Sakakibara, A. Nagata, J. Abe, T. Wakamiya, T. Shiba, and T. Kaneko, *Tetrahedron Letters*, **1971**, 2043.
6) B.W. Bycroft, *J. C. S. Chem. Comm.*, 660 (1972).
7) T. Kitagawa, T. Miura, S. Tanaka, and H. Taniyama, *J. Antibiotics*, **25**, 429 (1972).
8) T. Kitagawa, T. Miura, and H. Taniyama, *Chem. Pharm. Bull.* (Tokyo), **20**, 2176 (1972).
9) M. Bodanszky and D. Perlman, *Science*, **163**, 352 (1969).
10) B.W. Bycroft, *Nature*, **224**, 595 (1969).
11) T.H. Haskell, S.A. Fusari, R.P. Frohardt, and Q.R. Bartz, *J. Am. Chem. Soc.*, **74**, 599 (1952); H.E. Carter, W.R. Hearn, and E.M. Taylor, *ibid.*, **74**, 3704 (1952); E.E. van Tamelen and E.E. Smissman, *ibid.*, **74**, 3713 (1952).
12) B.W. Bycroft, D. Cameron, L.R. Croft, A. Hassanali-Walji, A.W. Johnson, and T. Webb, *Tetrahedron Letters*, **1968**, 5901; *J. C. S. Perkin I.*, **1972**, 827.

bial activity of viomycin, the tetrahydro derivative in which the chromophore group was destructively hydrogenated to alanine residue, still possessed half to one forth potency of original antibiotic. The result prompted us further investigations about modifications of its chromophore group and in this paper we report the oxidative studies.

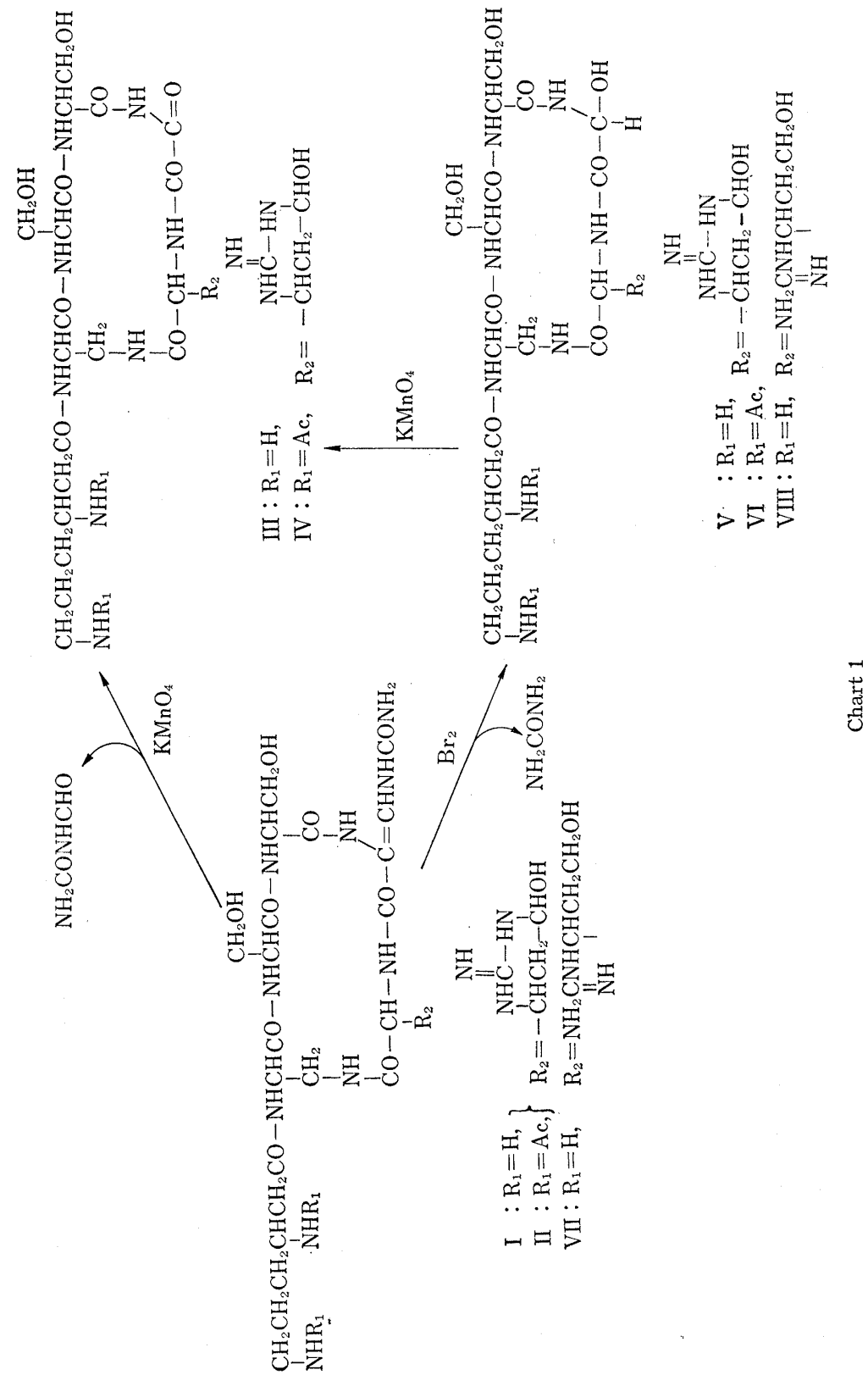

Chart 1

Oxidations and Characterizations

Oxidative reaction of viomycin was reported by Bycroft, *et al.*[12] who proposed the structure of the chromophore group of the antibiotic as 3-ureidodehydroalanine[13] residue. But no peptidic oxidation product was isolated by them.

Independently from the studies of Bycroft, *et al.*, we had found[2] that viomycin readily consume oxydants such as potassium permanganate, hydrogen peroxide, bromine or N-bromosuccinimide with concomitant disappearance of its characteristic ultraviolet (UV) absorption and liberating urea derivatives. Each one of these oxidation products was isolated by means of an ion exchange resin chromatography. All peptides isolated here possessed the same *Rf* and *Rm* values on paper chromatographies and paper electrophoresis and they contained the same amino acids constituents, L-serine, L-α,β-diaminopropionic acid, L-β-lysine and viomycidine,[14] an artifact derived from true constituent tuberactidine[15] in ratio of 2: 1: 1: 1. All of these amino acids were found in the acid hydrolysate of viomycin[8,11] with the same ratio.

By end groups analyses, β-lysine residue was proved to be the N-terminus as like as Viomycin,[16,17] and serine moiety as free carboxyl group with all of these peptides. Therefore all of them are not cyclic peptides though I is cyclic.[4–6] Each one of them possessed almost the same spectroscopic properties such as infrared (IR) and nuclear magnetic resonance (NMR) spectra. Therefore, it seemed that they were the same compound but the definitive conclusion was very difficult to obtain, since characterizations of each oxidation product were unsuccessfull owing to their unstabilities. Indeed, as will be shown below, the C-terminal serine residue was proved to be archifactorily formed during the purification procedures.

Using the more mild purification procedures which we found to be applicable to viomycin,[8] oxidation products obtained by potassium permanganate and bromine were isolated and it was proved that these products were obviously different. Thus, characterizations were conducted for the both reaction products.

Then, viomycin (I) sulfate and acetylviomycin (II) hydrochloride[8] were reacted with potassium permanganate solution untill their characteristic UV absorptions were completely disappeared. After removal of precipitated manganese dioxide, the filtrate was lyophilized. Extraction of the residue with ethanol gave formylurea which was identical with authentic sample by comparison of their IR spectra and mixed melting points. The same product was obtained by Bycroft, *et al.*[12]

When the residue was subjected to Sephadex column chromatography using water as the eluent, peptides, named oxoviomycin (III) and oxoacetylviomycin (IV) were isolated as purified form, respectively. On acid hydrolysis, III and IV gave the same amino acids obtained from acid hydrolysate of I. End groups analyses were performed and it was found that β-lysine is the N-terminus of III but there is no free carboxyl function for both products. Therefore, both oxo derivatives are cyclic peptides as like as I. The molecular formula based on elementary analyses were $C_{23}H_{39}O_{10}N_{11}$ for III and $C_{27}H_{43}O_{12}N_{11}$ for IV. Both III and IV were positive to Sakaguchi test.

In conclusion, the permanganate oxidations were conducted on the double bond of their chromophore to produce carbonyl function and formylurea without affecting the other reactive functions such as the N-terminal amino groups or tuberactidine moiety. Spectroscopic data for the both derivatives were quite agreeable with these formulation.

13) Bycroft, *et al.*[12] presented the name of ureidodehydroserine to the residue but Yoshioka, *et al.*[5] reviced the name as ureidodehydroalanine.

14) J.R. Dyer, H.B. Hayes, E.G. Miller, Jr., and R.F. Nassar, *J. Am. Chem. Soc.*, **86**, 5363 (1964); G. Büchi, and J.A. Raleigh, *J. Org. Chem.*, **36**, 873 (1971).

15) T. Wakamiya, T. Shiba, T. Kaneko, H. Sakakibara, T. Take, and J. Abe, *Tetrahedron Letters*, **1970**, 3497; *Bull. Chem. Soc. Japan*, **46**, 949 (1973).

16) J.R. Dyer, C.K. Kellog, R.F. Nassar, and W.E. Streetman, *Tetrahedron Letters*, **1965**, 585.

17) T. Kitagawa, T. Miura, Y. Sawada, T. Ozasa, and H. Taniyama, *Tetrahedron Letters*, **1968**, 109.

On the other hand, bromination of I or II gave abnormal reaction products. Thus, I-sulfate or II-hydrochloride were reacted with one equivalent molar bromine at room temperature untill UV absorptions at 268 mμ were lost. After lyophilization of each reaction mixtures, urea was identified from the both residues. The same Sephadex column chromatographic purification procedures as mentioned already, yielded peptides named broxoviomycin (V) and broxoacetylviomycin (VI) respectively.

By end groups analyses, β-lysine residue was proved to be the N-terminus of V as like as I, while VI has no N-terminal residue. Again, both peptides have no free carboxyl function. The evidence was different from the earlier experiments. Color reactions of both compounds V and VI showed positive tests for Sakaguchi, Fehling and ninhydrin the latest of which was negative for VI.

Their molecular formula based on elementary analyses were $C_{23}H_{41}O_{10}N_{11}$ for V and $C_{27}H_{45}O_{12}N_{11}$ for VI. When the molecular formula of I ($C_{25}H_{43}O_{10}N_{13}$)[8,12] and II ($C_{29}H_{47}O_{12}N_{13}$)[8] are taking account, two carbon unit are deminished during the reaction. Therefore it is obvious that besides urea, one carbon unit was missed, and also treatment of I and II with bromine did not give normal addition product on the double bond of dehydroalanine residue.

In spite of possessing one methoxylable hydroxyl function on tuberactidine residue of I[8,18] or II,[8] V and VI possessed three methoxylable hydroxyl functions when refluxed with methanol.

Dihydroviomycin (VII),[1,19] a sodium borohydride reduction product of viomycin, which still retaining the same chromophoric property but having dihydroviomycidine[20] residue instead of tuberactidine, failled to form a methoxyl derivative. While, broxodihydroviomycin

Table I. Physico-Chemical Properties of Viomycin Derivatives

	I	III	V
Color test: positive	nin. R-S. Saka.	nin. R-S. Saka.	nin. R-S. Saka.
mp (decomp.)	266°[8]	>300°	270°
Rf_1	0.23	0.25	0.27
Rf_2	0.059	0.048	0.033
Rm	1.0	0.90	0.85
UV: λ_{max}^{nm} (log ε)	268(4.4, H_2O)	end absorption	end absorption
IR: ν_{max}^{KBr} cm^{-1}	1650, 1495	1650, 1505	1650, 1510
NMR: τ ppm from DSS	2.0(1H, s), 4.83(1H, t, J=3Hz)	4.80(1H, t, J=3Hz)	4.80(1H, t, J=3Hz) 4.70(*ca.* 0.2H, s)
$[\alpha]_D^{20°}$ (c=1%, H_2O)	−29.5°[8]	−15.8°	−32°
Moleculer formula	$C_{25}H_{43}O_{10}N_{13}\cdot 3/2H_2SO_4$	$C_{23}H_{39}O_{10}N_{11}\cdot H_2SO_4$	$C_{23}H_{41}O_{10}N_{11}\cdot H_2SO_4$

abbreviations; I: viomycin sulfate, III: oxoviomycin sulfate, V: broxoviomycin sulfate
nin.: ninhydrin, R-S.: Rydon-Smith, Saka.: Sakaguchi.

Table II. NMR Absortpions of Methoxy Methyl Protons of the Oxidation Products

	τ ppm from DSS		
Methyloxoviomycin		6.50	
Methylbroxoviomycin	6.23	6.60	6.66
Methylbroxodihydroviomycin	6.34		6.73

18) F.A. Hochstein, and R.L. Miller, U.S. Patent 2920998 Jan. 12th, (1960) [*Chem. Abs.*, **54**, 10246 (1960)].
19) T. Takita and K. Maeda, *J. Antibiotics*, **21**, 512 (1968).
20) T. Takita and K. Maeda, *J. Antibiotics*, **22**, 39 (1969).

(VIII) obtained from VII by the reaction of one molar bromine, also yielded its dimethoxyl derivative by the same treatment.

Therefore, it is obvious that during the treatment with bromine, two methoxylable hydroxyl function was introduced for these V, VI and VIII.

On acid hydrolysis, V, VI or VIII gave the same amino acids in the same ratio obtained from acid hydrolysate of I, II or VII. Besides, a fairly amount of glycine was observed in every hydrolysates of these broxo derivatives, despite the acid hydrolysate of I,[8,14] II or VII contains a trace amount of glycine.

Judging from these results it was concluded that the reactive tuberactidine residue was intact and only the reactive chromophore group was transformed to a residue which could form glycine on acid hydrolysis during the treatment with bromine.

Acetylviomycin II forms monohydrochloride[8] on the strongly basic tuberactidine residue, while, IV and VI did not form hydrochloride or hydrobromide even at the presence of these acids. Therefore, both of them must have strongly acidic and basic functions in its molecule which forms betain structure. As suggested in Chart 2, a contribution of betain form could be excluded hydrochloric acid at the lyophilization condition.

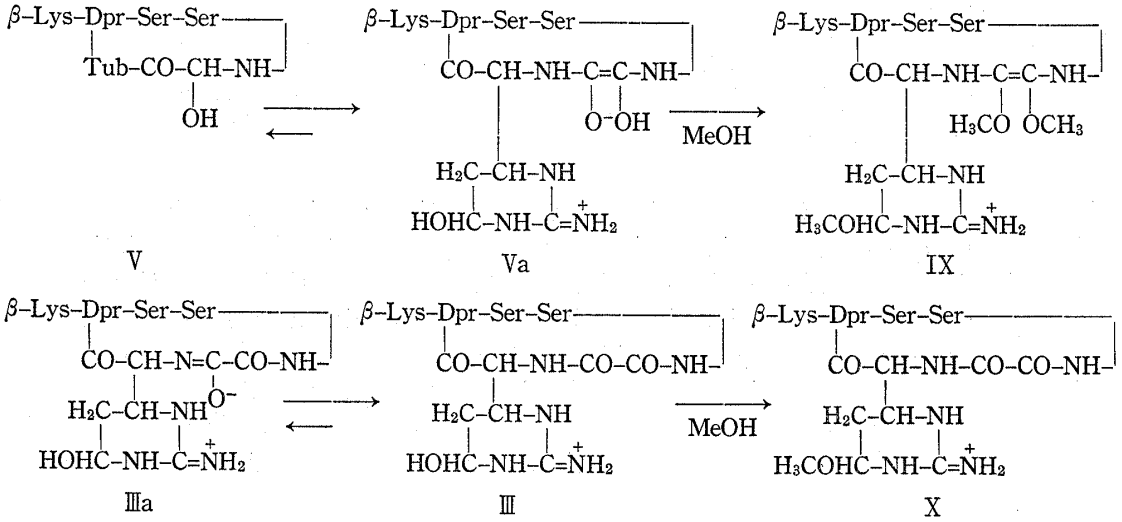

Chart 2

Spectroscopic investigations of the broxo derivatives with IR spectra have not given any significant information but with NMR spectra, a proton having τ value at 2 ppm (1H, s, a hydrogen on the chromophore group) of I,[8,16] II[8] and VII[1] was missing while, a hydrogen at τ 4.80 (1H, t, J=3 Hz, a proton on a carbon bearing hydroxyl function of tuberactidine residue)[8,15] was observed with both IV and VI. Noted to be was that all spectra of V, VI and VIII exhibited new absorptions at 4.70 (a hydrogen on a carbon bearing newly formed methoxylable hydroxyl function). However, these new absorptions are very weak when compared with those of 4.80 ppm proton, owing to contributions of the betain forms as suggested in Chart 2.

Probably, the betain structure (Va) mainly contribute to the tautomeric mixture of the broxo derivative, since when refluxed in methanol, the betain form was converted to the trimethoxylated derivative as shown in Table II and Chart 2.

Judging from above mentioned results, respective structural formula of V, VI and VIII were deduced for broxoviomycin, broxoacetylviomycin and broxodihydroviomycin respectively.

For further confirmation of these structures, V was oxidized with potassium permanganate and oxoviomycin III was obtained as expected. Thus, the structure of abnormal reaction product of viomycin with bromine was confirmed as V.

Antimicrobial Activities

The antimicrobial activities of viomycin and its oxidation products aganist gram positive and negative microorganisms were investigated and the obtained results are summarized in Table III.

Table III. Antimicrobial Spectra of Viomycin and Its Oxidation Product

	Minimum inhibitory concentration (mcg/ml)		
	I	III	V
Staphylococcus aureus 209p	200	>1600	>1600
S. aureus Terajima	50	>1600	1600
Bacillus subtilis PCI 219	3.1		1600
Proteus vulgalis OX 19	25	>1600	1600
Escherichia coli NIHJ	100	>1600	>1600
Pseudomonas aeruginosa IFO 3448	100	>1600	1600
Mycobacterium 607	1.6		800

I: viomycin sulfate; III: oxoviomycin sulfate; V: broxoviomycin sulfate
medium, culture: bouillon dilution method, nutrient bouillon (pH 7.0, 1 ml/test tube), inoculum: 0.5 ml of $1:10^2$ dilution of an overnight culture in the broth per test tube; incubation temperature and time: 37° for 48 hours.

Viomycin possesses four chemically reactive functions: primary amino groups of the N-terminal amino groups of β-lysine residue, hydroxyl functions of serine moieties, chromophoric 3-ureidodehydroalanine residue and the reactive tuberactidine constituent.

From the expectation mentioned already, chemical modifications of the chromophore group seemed very important, and the evidences that both oxo and broxo viomycins showed nullified activities support the importance of dehydroamino acid moiety for the potency of viomycin. However, reasons for explaining the potencies of tetrahydroviomycin still possessing a half to one forth of parent antibiotic was remained to need further investigations.

Experimental

All melting points are uncorrected. IR spectra were determined from KBr discs with Japan Spectroscopic DS-301 spectrophotometer, NMR spectra on JEOL JNM-PS-100 spectrometer at 100 MHz in D_2O using DSS as internal standard, and optical rotations on Yanagimoto Direct Recording polarimeter Model OR-20 ($c=1\%$, cell length: 5 cm, H_2O). The purity of preparations were determined using the Hitachi KLA-3 type amino acid analyser and by paper chromatography with Toyo filter paper No. 51 UH. Rf_1 values refer to the following solvent system, *n*-BuOH: *t*-BuOH: pyridine: AcOH: H_2O (15: 4: 10: 3: 12) and on thin-layer chromatography on silicagel (Kieselgel G, Merck) Rf_2 values refer to the solvent system C_6H_5OH: H_2O: conc. NH_4OH (28%) (30: 10: 0.6). Electrophoresis was determined at 500 V, 3—5 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 (5: 0.2: 95, pH at 6.3) and ninhydrin, Sakaguchi and Rydon-Smith reagents for the detections. Abbreviations used are Ser, Dpr, β-Lys, Vio and Gly for serine, α,β-diaminopropionic acid, β-lysine, viomycidine and glycine respectively. The end groups analyses had done by the method previously reported.[4]

Acetylviomycin,[8] dihydroviomycin[1] and tetrahydroviomycin[1,4] were prepared according to the methods previously reported.

Oxidation of Viomycin with $KMnO_4$——a) Isolation with Ion Exchange Resin Amberlite CG-120 Column Chromatography: To a 20% solution of I-sulfate (1 g, 1.1 mmoles) was added 0.4M $KMnO_4$ solution at room temperature, until its characteristic UV absorption at 268 nm was disappeared. (8.75 ml, 3.5 mmoles of $KMnO_4$ was used). After filtration of precipitated MnO_2, the filtrate was condensed *in vacuo* below 35°. The residue was extracted with boiling ethanol (2 ml × 4) and the alcoholic layer was condensed to dryness. Crystallization of the residue with dry EtOH gave N-formylurea (19 mg, yield 22%, mp 135—140°). Further crystallization furnished white needles mp 163—165° and mixed mp with urea showed depression (95—

108°) while, mixed mp with authentic formylurea, prepared according to the method of Maynert[21] (mp 166—168°) showed no depression. IR spectra of the both formylurea were identical.

The unextracted residue with alcohol was chromatographed on ion exchange resin Amberlite CG-120 column (H^+ form, 3×50 cm). The column was washed with H_2O (1 liter) and eluted with 2N HCl solution. Fractions positive to Rydon-Smith test were checked with PPC and the same component fractions were pooled, lyophilized to give a crude peptide (*ca.* 0.1 g) $[\alpha]_D^{19°}$ −34°, mp>300°, Rf_1 0.26, *Rm* 0.85, positive to Sakaguchi, ninhydrin and Rydon-Smith tests, negative to Phenylhydrazine (for urea)[22] and Beilstein tests. Amino acid ratio in the acid hydrolysate: Ser 1.88, Dpr 1.0, β-Lys 1.0, Vio 0.1. End groups anal. N-term, β-Lys: C-term, Ser.

b) Isolation of Oxoviomycin (III): To a solution of I-sulfate (2 g, 2.2 mmoles) in 5 ml of H_2O, was added 0.4M $KMnO_4$ solution dropwise until its UV absorption at 268 nm was disappeared (17.5 ml, 7 mmoles). After filtration of the precipitated MnO_2, the filtrate was condensed to dryness *in vacuo* and the residue was chromatographed on Sephadex LH-20 tower (2×150 cm) using water as an eluent. Fractions (10 g/fraction) positive to ninhydrin test (No. 16—28) were checked with PPC and the same component(s) fractions were pooled, lyophilized to give No. 16 (Rf_1 0, 4.3 mg), No. 17—18 (two spots, Rf_1 0 and 0.25), No. 19—24 (single spot, Rf_1 0.24, 848 mg). The product of No. 19—24 was repeated by the same purification procedure to give purified III-sulfateas colorless amorphous powder, hygroscopic, mp above 300°, $[\alpha]_D^{25°}$ −15.8°, UV: end absorption, IR (KBr) cm^{-1}: 3400—3300 (broad), 3120, 2980, 1650, 1505. NMR: τ 4.80 (1H, t, J=3 Hz). *Anal.* Calcd. for $C_{23}H_{51}O_{19}N_{11}S$: C, 33.78; H, 6.29; N, 18.84; S, 3.92. Found: C, 33.82; H, 6.45; N, 18.41; S, 4.31. Amino acid compositions: Ser 1.86, Dpr 1.20, β-Lys 1.0, Vio 0.26. End groups anal.: N-term. β-Lys, C-term. non.

Oxidation of I with H_2O_2, N-Bromosuccinimide (NBS) or Br_2——Aqueous solution of I-sulfate was oxidized with these reagents and the each product was isolated by the similar procedures used for oxidation of I with $KMnO_4$ of the procedure a. The characteristic chromophore group easily decomposed by these oxidants. Peptides isolated showed almost the same amino acid analysis (Ser: β-Lys: Dpr: Vio=2: 1: 1: 1) and end groups analyses (N-term., β-Lys, C-term., Ser). They possessed the same Rf_1 0.26 and *Rm* 0.85.

Oxoacetylviomycin (IV)——a) Oxidation of II: To a solution of II-hydrochloride (1 g) in 10 ml of H_2O, was added 0.4M $KMnO_4$ solution dropwise until UV absorption at 268 nm was disappeared (7 ml). By working up the similar manner described for purification of III (procedure b), IV was obtained as colorless amorphous powder, mp 221—224° (decomp.), positive to Rydon-Smith and Sakaguchi and negative to ninhydrin and Beilstein tests, $[\alpha]_D^{20°}$ −6°, Rf_1 0.46, *Rm* 0.27, IR (KBr) cm^{-1}: 3230—3250 (NH, OH), 2920, 1650. NMR: τ 8.06 (6H, s, $-COC\underline{H}_3$). *Anal.* Calcd. for $C_{27}H_{51}O_{16}N_{11}$: C, 41.27; H, 6.54; N, 19.61. Found: C, 41.49; H, 6.60; N, 19.63. Amino acid compositions: Ser 1.90, Dpr 1.12, β-Lys 1.0, Vio 0.26. End groups analyses: No C- and N-terminus.

b) Acetylation of III: To a solution of III-sulfate (0.68 g) in 4 ml of H_2O, was added 1% dimethylformamide solution of N-acetoxysuccinimide (4 ml)[23] and the mixture was made basic with occational addition of NEt_3 under stirring at room temperature, until the mixture showed negative to ninhydrin reagent (10 hr). After, removal of the solvent *in vacuo* below 40°, the residue was chromatographed on Sephadex LH-20 column (2×150 cm) using H_2O as developer. From fractions 14—17 (10 g/fraction), IV was obtained and identified by comparison of mixed mp, *Rf* and *Rm* values as well as its IR and NMR spectra with those of the sample obtained by procedure a.

Broxoviomycin (V)——To a solution of I-sulfate (2 g, 2.2 mmoles) in 10 ml of H_2O, was added dropwise with aqueous solution of bromine until color of Br_2 did not disappeared (9 ml, 2.43 mmoles). The solution showed only end absorption in UV region. After removal of the solvent, the residue was chromatographed on Sephadex LH-20 column using H_2O as an eluent. Fractions (7 g/fraction) No. 22—27 contained single component 1.21 g (61%). Repeated chromatographies by the same procedure gave purified V-sulfate as colorless amorphous powder, hygroscopic, positive to ninhydrin, Sakaguchi and Rydon-Smith tests, mp 270° (decomp.), $[\alpha]_D^{20°}$ −32°, Rf_1 0.27, Rf_2 0.033, *Rm* 0.85. IR (KBr) cm^{-1}: 3350 (broad), 3140, 2990, 1650, 1510. NMR: τ 4.70 (s, C$\underline{H}$(OH)–N), 4.80 (1H, t, J=3 Hz, C$\underline{H}$(OH)–N). *Anal.* Calcd. for $C_{23}H_{47}O_{16}N_{11}S$: C, 36.07; H, 6.19; N, 20.12; S, 4.19. Found: C, 35.80; H, 6.56; N, 20.19; S, 4.50. Amino acid compositions: Ser 1.99, Dpr 0.98, β-Lys 1.0, Vio 0.2, Gly 0.4. End groups analyses: N-terminus, β-Lys; C-terminus, non.

Broxoacetylviomycin (VI)——II-Hydrochloride (2 g) was reacted with aqueous solution of Br_2. From the reaction mixture, VI was isolated and purified by the procedure applied to purification of V-sulfate; colorless amorphous powder (1.2 g), positive to Sakaguchi and Rydon-Smith and negative to ninhydrin tests. Br^- and SO_4^{--} ions were negative ($AgNO_3$, Beilstein and $Ba(OH)_2$ tests), UV: end absorption, IR (KBr) cm^{-1}: 3270, 3060, 2915, 1635, 1520. NMR: τ 7.95 (6H, s, $-COCH_3$), 4.80 (1H, t, J=3 Hz, C$\underline{H}$(OH)–N), 4.70 (s, CO–C$\underline{H}$(OH)–N). *Anal.* Calcd. for $C_{27}H_{53}O_{16}N_{11}$: C, 41.16; H, 6.78; N, 19.56. Found: C, 41.33;

21) E.W. Maynert and E. Washburn, *J. Org. Chem.*, **15**, 259 (1950).
22) F. Fiegle, "Spot Test in Organic Analysis", 7th Edition, Elsvier Publishing Co., London, 1966, p. 390.
23) G.W. Anderson, J.E. Zimmerman, and F.M. Callajana, *J. Am. Chem. Soc.*, **86**, 1839 (1964).

H, 6.25; N, 19.35. Amino acid compositions: Ser 2.15, Gly 0.96, Dpr 1.0, β-Lys 1.07, Vio 0.2. End groups analyses, No N- and C-terminus.

Broxodihydroviomycin (VIII)——To a solution of VII-sulfate (0.5 g) in 10 ml of H_2O, was added aqueous solution of Br_2 until the UV absorption was disappeared. After lyophilization, the residue was chromatographed on Sephadex LH-20 tower (2×150 cm) using H_2O as an eluent. Fractions (10 g/fraction) No. 19—22 were combined, lyophilized to give 350 mg of VIII-sulfate as colorless amorphous powder, mp 262—267° (decomp.), $[\alpha]_D^{20°}$ +6° (c=0.5%, H_2O), Rf_1 0.26, Rm 0.84, color tests: positive to ninhydrin, Sakaguchi, and Rydon-Smith. IR (KBr) cm^{-1}: 3340, 1650, 1525. NMR: τ 4.75 (s, CH(OH)-N). Amino acid compositions: Ser 2.0, Gly 0.78, Dpr 1.0, β-Lys 1.01, 2Hvio 0.4.

General Preparation Methods of Alkoxy Derivatives——A suspension of viomycin derivatives hydrochloride (0.1 g) in abs. MeOH was heated under refluxing for 48 hr. The reaction mixture was condensed to dryness *in vacuo* and again MeOH was added and the mixture was heated under reflux for 2 or 3 days. After removal of the solvent, the residue was washed with acetone and then dried over P_2O_5 in a evacuated desiccator. To confirm the presence of methoxy function, one drop of pyridine was added to the individual product before kept it in the desiccator. The methoxy contents were determined by their NMR spectral signals of methyl protons. Results of methoxy protons absorption of methyl derivatives in NMR spectra were sum marized in Table II.

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