Chem. Pharm. Bull. 24(6)1324—1330(1976)

UDC 547.898.04 : 547.466.04

Studies on Viomycin. IX.[1] Amino Acid Derivatives of Viomycin[2]

TSUNEHIRO KITAGAWA, TAKAKO MIURA, CHIEKO TAKAISHI and HYOZO TANIYAMA

Faculty of Pharmaceutical Sciences, Nagasaki University[3]

(Received October 21, 1975)

The limited acylations of I on the two amino functions of β-lysine residue with protected amino acid active esters followed by de-protection by catalytic hydrogenolyses resulted N_1-amino acid acylated viomycins. While, the limited carbobenzoxylation followed by the reaction with dicarbobenzoxylysine active ester and the decarbobenzoxylation gave N_6-acylated product VI. Antimicrobial assay of the above obtained derivatives result that neutral amino acid derivatives of I have reduced potencies while, basic amino acid derivatives possess almost the similar potencies with I *in vitro* and *in vivo* tests. Thus, one of the provable reason for the importance of the two free amino functions of β-lysine residue for the exhibition of the potency of I is concluded to be due to their basicity.

Previously, we reported that both of the free amino groups of β-lysine residue in viomycin have important role for the exhibition of antimicrobial activities of viomycin.[1,4] To find out the reason for the importance of these two amino groups, we have now introduced some amino acids especially basic amino acids selectively to the positions 1 and 6. The present paper describes the syntheses and antimicrobial activities of the selectively acylated viomycins with neutral and basic amino acids, and also one of the probable reason for the neccessity of these two amino functions of β-lysine residue for the expression of antimicrobial activities of viomycin is due to their basicity.

Syntheses of Amino Acid Derivatives of Viomycin

For the protecting groups of the α-amino function of amino acids and guanidino group of arginine, carbobenzoxy group and nitro function which can removed by catalytic hydrogenation were chosen, since, parent part of viomycin has proved to be stable at the de-protecting condition.[1]

Coupling reactions of viomycin with protected amino acid have to be done as quickly and moderately as possible in aqueous solution, since viomycin is soluble in water and practically insoluble in most organic solvents and also it is very unstable toward acid and base.[4a] For this reason, active ester method of N-hydroxysuccinimide[5] was applied. Each N-hydroxysuccinimide esters of carbobenzoxy amino acids were prepared according to the method of Anderson, *et al.*[5] using dicyclohexylcarbodiimide or the mixed anhydride of ethylchlorocarbonate.[6]

The selective acylation of the N_1-amino function of β-lysine residue with carbobenzoxy amino acid active esters was performed by modified methods described in the preparation of

1) Part VIII: T. Kitagawa, T. Miura, M. Takaishi and H. Taniyama, *Chem. Pharm. Bull.* (Tokyo), **23**, 2124 (1975).
2) A part of this work was presented at the 94th Annual Meeting of Japanese Pharmaceutical Society Held at Sendai, April 6th 1974.
3) Location: *Bunkyo-machi, Nagasaki.*
4) *a)* T. Kitagawa, T. Miura and H. Taniyama, *Chem. Pharm. Bull.* (Tokyo), **20**, 2176 (1972); *b)* T. Kitagawa, T. Miura, S. Tanaka and H. Taniyama, *J. Antibiotics*, **25**, 429 (1972).
5) G.W. Anderson, J.E. Zimmerman and F.M. Callahan, *J. Am. Chem. Soc.*, **86**, 1839 (1964).
6) R.A. Boissonnas, *Helv. Chim. Acta*, **34**, 874 (1951); T. Wieland and H. Bernhard, *Ann. Chem.*, **572**, 190 (1951); J.R. Vaughan, Jr., *J. Am. Chem. Soc.*, **73**, 3547 (1951).

NII-Electronic Library Service

$CH_2(CH_2)_2CHCH_2CO-NH-$ (with $NH-R_1$ at position 1 and $NH-R_2$ at position 6), attached to the viomycin cyclic peptide core

I. $R_1=R_2=H$

A → II'—V', VII': R_1=Prot. A.A.; R_2=H

B → VI': R_1=Z; R_2=2ZLys

C ↓

II: $R_1=CH_2CH_2CH_2CH_2CH(NH_2)CO-$ (with NH_2 on the first CH_2); R_2=H

III: $R_1=CH_2CH_2CH_2CH(NH_2)CO-$ (with NH_2 on the first CH_2); R_2=H

IV: $R_1=CH_2CH_2CH_2—CH(NH_2)CO-$ (with NHC(=NH)NH_2 on the first CH_2); R_2=H

V: $R_1=CH_2(NH_2)-CO-$; R_2=H

VII: $R_1=CH_2CH_2CH_2—CH(NH_2)CO-$ (with NHC(=O)NH_2 on the first CH_2); R_2=H

VI: R_1=H; $R_2=CH_2CH_2CH_2CH_2CH(NH_2)CO-$ (with NH_2 on the first CH_2)

Chart 1

abbreviation: A: carbobenzoxy amino acid succinimide ester
B: benzyl-*p*-nitrophenyl carbonate
C: hydrogenolysis
Prot. A,A, protected amino acid; Z, carbobenzoxy

N_1-acylated derivative of I as follows: A solution of an equivalent amount of each N-hydroxysuccinimide esters of carbobenzoxy amino acid in tetrahydrofuran (THF) or pyridine was added dropwise to a stirring 0.1M triethylamine-carbonate buffer solution of I (pH 8—9) with occasional additions of triethylamine (TEA) to maintain the pH. The mixture were kept stirring for one to three hours and if neccessary kept it in a refrigerator for overnight to finish the coupling. In these treatments, prolonged reaction at room temperature or a more basic condition sometimes accompanied a decomposed product which was detected by weak Sakaguchi color reaction according to the method of Weber[7] and the presence of nuclear magnetic resonance (NMR) signal at 5.4—5.5 ppm.[8]

After each reaction mixture was condenced *in vacuo* below 30°, the residue was dissolved in *ca.* 30% acetic acid solution and subjected column chromatographies on a tower of cellulose or Sephadex to isolate N_1-carbobenzoxy amino acid derivatives of I.

Removals of protective groups of the above synthesized derivatives were performed by catalytic hydrogenation over paradium black by the same method used for the removals of the protective group from N_6-acetyl-N_1-carbobenzoxyviomycin.[1] The desired amino acid derivatives are purified by Sephadex column chromatographies.

7) C.J. Weber, *J. Biol. Chem.*, **86**, 217 (1930).
8) T. Kitagawa, T. Miura and H. Taniyama, Abstracts of papers of 17th symposium on "The Chemistry of Natural Products" held at Tokyo Oct. 18th 1973, p. 245.

NII-Electronic Library Service

The other selective monoacylation product N_6-lysylviomycin (VI) was obtained by reacting N_1-carbobenzoxyviomycin[1] with N-hydroxysuccinimide ester of dicarbobenzoxylysine followed by the catalytic decarboxylations by the similar procedures used for the preparation of N_6-acetyl derivatives.

Characterizations and Confirmations of Structures of Amino Acid Derivatives of Viomycin

All of the amino acid derivatives show positive ninhydrin, Sakaguchi and Rydon-Smith color tests like as I.[4a,9]

Elemental analyses of them showed good correspondences to the each calculated values as given in Table II. They have the same ultraviolet (UV) absorption maxima with original

Table I. Physical Properties of Viomycin and Its Amino Acid Derivatives

Compound	mp (decomp.) (°C)	Rf_1	Rm	$[\alpha]_D^{20°}$ (c=1%, H_2O)
I	258	0.30	1.00	−29.5°
II	270	0.27	1.02	−27.8°
III	253	0.27	1.04	−25.6°
IV	275	0.30	1.04	−21.4°
V	275	0.32	0.95	−20.2°
VI	260	0.23	1.06	−33.3°
VII	285	0.39	1.00	−31.2°

Table II. Elemental Analyses of Viomycin Derivatives

	Formula		*Anal.* C	H	N	S
II	$C_{39}H_{73}O_{20}N_{15}$ ($C_{25}H_{42}O_{10}N_{13}\cdot C_6H_{13}ON_2\cdot 4CH_3CO_2H\cdot H_2O$)	Calcd.	43.69	6.86	19.60	
		Found	43.65	7.01	19.30	
III	$C_{30}H_{61}O_{21}N_{15}S_2$ ($C_{25}H_{42}O_{10}N_{13}\cdot C_5H_{11}ON_2\cdot 2H_2SO_4\cdot 2H_2O$)	Calcd.	34.91	5.95	20.36	6.21
		Found	34.95	6.08	20.40	6.09
IV	$C_{39}H_{75}O_{21}N_{17}$ ($C_{25}H_{42}O_{10}N_{13}\cdot C_6H_{13}ON_4\cdot 4CH_3CO_2H\cdot 2H_2O$)	Calcd.	41.89	6.76	21.30	
		Found	42.01	7.09	21.48	
V	$C_{27}H_{49}O_{17}S_{1.5}$ ($C_{25}H_{42}O_{10}N_{13}\cdot C_2H_4ON\cdot 3/2H_2SO_4$)	Calcd.	36.44	5.55	22.04	5.40
		Found	36.51	5.70	21.00	5.27
VI	$C_{39}H_{73}O_{20}N_{15}$ ($C_{25}H_{42}O_{10}N_{13}\cdot C_6H_{13}ON_2\cdot 4CH_3CO_2H\cdot H_2O$)	Calcd.	43.69	6.86	19.60	
		Found	43.84	7.07	19.23	

Table III. UV Absorptions of Viomycin Derivatives

Derivative	$\lambda_{max}^{m\mu}$ ($\log \varepsilon$) in 0.1N HCl	in H_2O	in 0.1N NaOH
I	268(4.4)	268(4.4)	284(4.2)
II	268(4.3)	268(4.3)	282(4.1)
III	268(4.4)	268(4.4)	282(4.2)
IV	268(4.2)	268(4.2)	282(4.0)
V	268(4.3)	268(4.3)	282(4.1)
VI	268(4.4)	268(4.4)	283(4.2)
VII	268(4.2)	268(4.2)	282(4.0)

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

NII-Electronic Library Service

Table IV. Spectroscopic Sakaguchi Tests of Viomycin Derivatives at 510 mμ According to the Method of Weber[7]

	I	II	III	IV	V	VI	VII
log ε	3.41	3.41	3.79	4.40	3.34	3.45	3.24

antibiotic in acidic, neutral and basic conditions, showing the very unstable chromophoric 3-ureidodehydroalanine residue[10] is intact during the synthetic procedures.

Quantitative Sakaguchi colorimetric measurement of the newly synthesized derivatives according to the method of Weber[7] indicated that all of them possess almost the same molar absorption coefficiency with parent antibiotic except N_1-arginylviomycin (IV) which showed a large ε value owing to the other guanidino group in arginine residue. Also every derivatives showed NMR signals of C_{31} protons at *ca.* 5.18 ppm as shown in Table V and no ptoton resonance peak at 5.4—5.6.[8] Therefore, these artificials are proved to possess an intact tuberactidine residue.

Table V. NMR Data of Viomycin Derivatives (δ value: ppm from DSS in D_2O)

	C_2–H_2	$C_{3,4}$–H_4	C_7–H_2	C_{31}–H
I	3.04	1.78	2.76	5.18
II	3.21	1.64	2.76	5.18
III	3.28	1.68	2.74	5.18
IV	3.21	1.64	2.68	5.16
V	3.26	1.66	2.72	5.18
VI	3.00	1.62	2.50	5.18
VII	3.22	1.62	2.70	5.17

The location of the each aminoacylations were confirmed by NMR spectroscopic studies. Assigned NMR shift values of C_2, C_3, C_4, C_7, and C_{31} proton resonances of β-lysine residue in I and its acylated derivative determined on 100 MHz instrument in heavy water using sodium 4,4-dimethyl-4-silapentane sulfonate (DSS) as the internal standard are summarized in Table V.

As reported already,[1] acetylation of N_1-amino group resulted C_2 proton shift values about 3.20 ppm while, those of free amino groups possess the values of *ca.* 3.04 ppms. All of the compounds except VI possess the C_2 proton shift values of 3.21—3.28 ppm and determined as N_1-acylated compounds. The compound VI having the C_7 proton shift value of 2.50 ppm[1] was assigned as N_6-lysyl derivative.

From these results every amino acid derivatives of I are deduced to be the desired acylated product on β-lysine residue without affecting the other reactive part of viomycin such as the chromophoric group or tuberactidine residue.

Antimicrobial Activities

The antimicrobial activities of I sulfate and its amino acid derivatives against gram positive and gram negative bacteria were investigated. The obtained minimum inhibitory concentration (MIC) values determined by the two hold tube dilution method are summarized in Table VI.

10) B.W. Bycroft, D. Cameron, L.R. Croft, A. Hassanali-Walji, A.W. Johnson and T. Webb, *J.C.S. Perkin I.*, **1972**, 827.

NII-Electronic Library Service

Table VI. Antimicrobial Spectra of Viomycin and Its Derivatives

		Minimum inhibitory concentration (mcg/ml)						
		I	II	III	IV	V	VI	VII
A	*Escherichia coli*	10	30	100	30	500	100	100
	Staphylococcus aureus Terajima	30	10	30	10	500	30	100
	Pseudomonas aeruginosa Tsuchijima	500	>100	>500	1000	>500	>100	1000
	Shigella flexneri 2a EW-10	100	100	>100	30	>500	100	500
B	*Mycobacterium tuberculosis* $H_{37}Rv$	3	3	3	3	30	3	10
	M. tuberculosis INH, PAS, SM-r$H_{37}Rv$	3	3	10	3		3	
	M. tuberculosis Kurono	3	3	3	3		3	

method: Bouillon dilution method
culture: A; Bouillon pH 7.0, 37°, 48 hr, B; Kirchner medium containing 0.2% bovine albumin, pH 7.0, 37°, 21 days
abbreviations: INH, isonicotinic acid hydrazide; PAS, *p*-amino salicylic acid; SM, streptomycin; r-, resistance

Concerning the modification products of N_1-amino group of I, basic amino acid derivatives such as lysyl, arginyl and ornithyl derivatives possess almost the same MIC values with viomycin against the tested bacteria, while, the neutral amino acid derivatives possess very reduced potencies against gram positive and gram negative bacteria and only one third to one tenth activities against acid fast strain of *Mycobacterium tuberculosis.*

Modification products on the N_6-amino group of viomycin, the acetyl derivative showed almost nullified antimicrobial activities,[1] while, a basic amino acid lysyl derivative again shows the similar potencies with those of viomycin and N_1-lysylviomycin. Thus, it is concluded that acylations of N_1- and N_6-amino functions with basic amino acid residues maintain almost the same biological activities with the original antibiotic but the derivatives of neutral amino acids or acetyl residue results inactivation of the potency.

Judging from the results obtained above, the reason for neccessity of free amino groups of β-lysine residue for the potency of viomycin could be deduced for their basicity.

Besides *in vitro* tests, *in vivo* activity of N_1-lysylviomycin was investigated to compare the original antibiotic, using female mice and the results are summarized in Table VII.

Table VII. Antituberculostatic Activity of N_1-Lysylviomycin *in Vivo*. Efficacy against *M. tuberulosis* Kurono Infection in Mice

Sample	Route	Dose (mg/kg/dose)	Survival Total	Symptom positive[a] Survival	Efficacy
II	sc	100	6/6	0/6	+
	sc	50	6/6	2/6	+
	sc	25	5/6	5/5	−
I	sc	100	6/6	0/6	+
	sc	50	6/6	0/6	+
	sc	25	6/6	2/6	+
	sc	12.5	5/5	5/5	−
Control	untreated		12/12	12/12	−

a) Symptoms: emaciation and fur ruffling
mice: ddY, female mice weighing 18 to 20 g
infection: intravenous infection with 0.5 ml of a bacterial suspension (O.D.=1.0 at 525 nm) in modified Kirchner medium without albumin per mouse (LD_{50} was about 1)
medication: once a day for 20 days from the next day of infection
observation of mortality: 21 days

II shows almost the same activity against *M. tuberculosis* in *in vitro* test, while about half efficacy is observed with *in vivo* test compared with I.

NII-Electronic Library Service

Experimental

All melting points were taken on Yanagimoto micromelting point apparatus and were uncorrected. NMR spectra were determined on a JEOL JNM-PS-100 type instrument (100 MHz) and are given in part per million (ppm) down field shift from the internal standard DSS in heavy water and optical rotations (O.D.) on Yanagimoto direct recoring 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 values refer to the *n*-BuOH: *t*-BuOH: pyridine: AcOH: H_2O (15: 4: 10: 3: 12). Electrophoreses were performed at 430 V, 1.3—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. Physico-chemical properties as well as the results of elemental analyses of the modified viomycins are summarized in Table I—V.

Materials——N-(and N'-) protected amino acids used are the products of Protein Research Foundations, Mino Osaka, Japan. Viomycin,[4a)] N_1-carbobenzoxyviomycin[1)], dicarbobenzoxyornithine[11)] and N-hydroxysuccinimide esters of carbobenzoxyglycine[5)] and dicarbobenzoxylysine[12)] were prepared according to the methods given in the corresponding references.

N_1-Lysylviomycin (II)——Sulfate of I (2 g) was dissolved in 0.1 M TEA buffer (30 ml, pH 9.0) containing dioxan (8.5 ml). To the stirring and ice cooling above solution, pyridine solution of N-hydroxysuccinimide ester of dicarbobenzoxylysine (0.98 g, in 6 ml) was added dropwise with occasional addition of 0.5 M Na_2CO_3 solution to maintain its pH at 8.5—9.5. After stirring for one hr, the mixture was condenced *in vacuo* and the residue was chromatographed on cellulose powder column (2.5 × 20 cm) using the solvent system *n*-BuOH: AcOH: pyridine: H_2O: *t*-BuOH (15: 3: 10: 12: 4) as an eluent. The fractions (3 g/fract.) positive to Rydon-Smith test (No. 8—40) were pooled and condenced to dryness *in vacuo* to give N_1-dicarbobenzoxylysylviomycin (Rf_1 0.71, positive to ninhydrin, Sakaguchi and Rydon-Smith tests). The product was dissolved in 30% AcOH (20 ml) and the solution was stirred for 5 hr under hydrogen atmosphere with the presence of paradium black[13)] which was prepared from 1% $PdCl_2$ solution (20 ml). After catalyst was removed by filtration and the filtrate was condenced *in vacuo*, the residue was chromatographed with Sephadex LH-20 column (2 × 150 cm) using H_2O as the eluent. Fractions positive to ninhydrin test (No. 19—22, 10 g/fract.) were pooled and lyophilized to give II as white amorphous, (150 mg), positive to ninhydrin, Rydon-Smith and Sakaguchi test, IR ν_{max}^{KBr} cm^{-1}: 3360, 3240, 1660, 1540 (broad), 1400, 1220, 1150.

N_1-Ornithylviomycin (III)——To a cooling THF solution (20 ml, $-15°$) containing TEA 0.2 g and 0.8 g of dicarbobenzoxyornithine, THF solution (0.22 g in 5 ml) was added with vigorous stirring. After 5 min the reaction mixture was removed into ice bath and a solution of N-hydroxysuccinimide (0.23 g) in THF (5 ml) was added to the solution dropwise during 30 min with stirring. Then, above solution was added dropwise to a solution of I sulfate (1.68 g) in H_2O (30 ml) containing 0.4 g of TEA with occasional further addition of TEA to maintain pH of the mixture at about 9. After addition of the solution, the ice bath was taken off and the mixture was kept stirring for 1 hr at room temperature and then kept it in a refrigerator for overnight. After neutralization with AcOH, the solution was condenced *in vacuo* and the residue was chromatographed with the tower of Sephadex LH-20 (2 × 150 cm) using 5% AcOH as an eluent. Fractions (8 g/tube) positive to Rydon-Smith test (No.38—43) were pooled and condenced to give almost purified N_1-dicarbobenzoxyornithylviomycin (Rf_1 0.78). The product, without further purification, was catalytically decarbobenzoxylated and purified by the similar prcedures used for the preparation of II to give III as white amorphous (240 mg), positive to ninhydrin, Sakaguchi and Rydon-Smith tests, IR ν_{max}^{KBr} cm^{-1}: 3360, 3120, 1660, 1540 (broad), 1395, 1220, 1100.

N_1-Arginylviomycin (IV)——N^{α}-Carbobenzoxy-N^G-nitroarginine (0.88 g) was converted to its N-hydroxysuccinimide ester and the ester was reacted with I followed by deprotective groups by catalytic hydrogenolysis by the similar procedures used for preparation of II yielded IV as white amorphous (220 mg), positive to ninhydrin, Sakaguchi and Rydon-Smith tests, IR ν_{max}^{KBr} cm^{-1}: 3380, 1630, 1520 (broad), 1220; very soluble in water, insoluble in CH_3OH, dioxan.

N_1-Glycylviomycin (V)——N-Hydroxysuccinimide ester of carbobenzoxyglycine (0.68 g) was reacted with I by the similar method used for the preparation of II. An intermediate N_1-carbobenzoxyglycylviomycin was purified by column chromatography of Sephadex LH-20 tower (2 × 150 cm) with eluents of first with 5% AcOH solution (90 ml) followed by H_2O, instead of using cellulose powder chromatography as used for the preparation method of II. Tubes containing N_1-carbobenzoxyglycylviomycin (Rf_1 0.73) were pooled, condenced to dryness and then decarboxylated by the same procedures as II gave V as white amorphous (230 mg), positive to ninhydrin, Sakaguchi and Rydon-Smith tests, IR ν_{max}^{KBr} cm^{-1}: 3400, 3260, 1665, 1500, 1325, 1225.

11) R.L.M. Synge, *Biochem. J.*, **42**, 99 (1948).
12) R.A. Boissonnas, S. Guttmann, R.L. Huguenin, P.A. Jaquenoud and Ed. Sandrin, *Helv. Chim. Acta*, **41**, 1867 (1958).
13) R. Willstätter and E. Waldschmidt-Leitz, *Ber.*, 54, 113 (1921).

NII-Electronic Library Service

N_6-Lysylviomycin (VI)——A solution of N_1-carbobenzoxyviomycin (1.8 g) in 13% THF (22 ml) was added to a solution of N-hydroxysuccinimide ester of dicarbobenzoxylysine (0.78 g) in 90% THF solution (20 ml) and pH of the reaction mixture was kept at ca. 8 with occasional addition of TEA under stirring at room temperature for 5 hr. After the mixture was neutralized with AcOH and condenced *in vacuo*, the resulting sirupy residue was (chromatographed on cellulose powder column (2.5×53 cm) using the solvent system *n*-BuOH: pyridine: H_2O (5: 3: 2). Fractions (5 g/tube) No. 3—16 contained N_1-carbobenzoxy N_6-dicarbobenzoxylysylviomycin (white amorphous, 1.6 g, Rf_1 0.71). The product on catalytic hydrogenation and working up by the same procedures as II yielded VI as white amorphous (190 mg), IR ν_{max}^{KBr} cm^{-1}: 3360, 3120, 1660, 1540 (broad), 1395, 1220, 1150; positive to ninhydrin, Sakaguchi and Rydon-Smith tests.

N_1-Citrullylviomycin (VII)[14]——Carbobenzoxycitrulline (1 g) was converted to its N-hydroxysuccinimide ester by the same procedure used for III. Condensation of the ester with I followed by the decarbobenzoxylation and purification by the similar procedure used for III gave VII as white amorphous (100 mg), positive to ninhydrin, Sakaguchi and Rydon-Smith tests, IR ν_{max}^{KBr} cm^{-1}: 3240, 3060, 2920, 1650, 1510 (broad), 1400, 1325, 1220, 1155.

Acknowledgements The authors express their deep gratitude to Dr. Shimizu of Dainippon Pharmaceutical Co. Ltd. for the antimicrobial assay of viomycin derivatives. Thanks are also due to Mrs. Mazume and Mr. Inada of the Central Analyses Room of this Faculty for their microanalyses and spectral measurements. A part of microbial assay *in vitro* was performed by Miss M. Takaishi of our laboratory to whom they are also grateful.

14) VII obtained above was PPC and electrophoretically a pure compound. Physico-chemical properties as shown in Tables indicate VII is also a pure compound. Although elemental analyses of VII has not done.

NII-Electronic Library Service