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The Total Structure of Viomycin by Sequential Analysis[1)]

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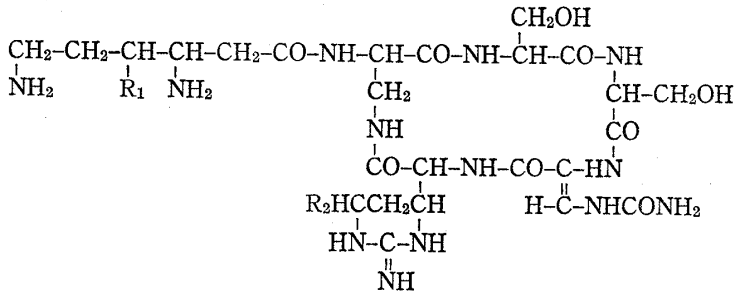
The novel partial hydrolysis of peptide bonds by using N→O acyl migrative reactions followed by the methanolysis of the resulting ester linkages were studied to give the successful application with tetrahydro derivative of viomycin, a tuberculostatic antibiotic. Also, the partial hydrolysis of perhydroacetylviomycin yielded two peptide fragments. The sequential analysis of these fragments by Edman-dansyl procedures and the end group analysis concluded that the primary structure of viomycin is formulated as XXV. The sequence is well compatible to the chemical formula I, the one of recently proposed two structures.

The tuberculostatic antibiotics, viomycin produced by *Streptomyces puniceus* and *St. floridae*,[3)] capreomycins by *St. capreolus*[4)] and tuberactinomycins by *S. griseoverticillatus* var. *tuberacticus*,[5)] possess similar antimicrobial activities.[6)] Structural works on viomycin as well as these two groups of antibiotics have been pursued in several laboratories. Thus, Shiba and Abe group[7)] presented the formula I, II, III, and IV for viomycin and tuberactinomycins A, N, and O respectively while, Bycroft, *et al.*[8)] proposed the formulas V, VI, and VII for viomycin and capreomycins IA and IB respectively. Consequentially, two different primary structures were assigned for these two groups which resulted incapable conclusions for the structure of viomycin.

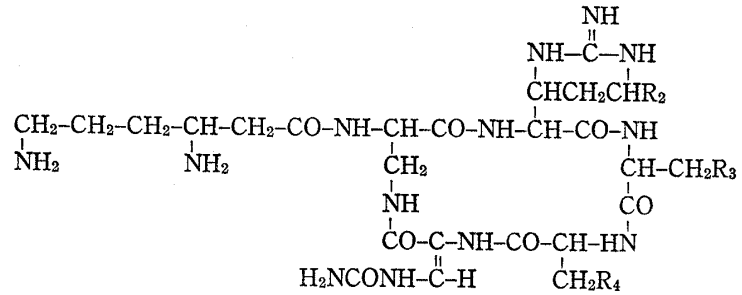
Independent from these studies, chemical studies of viomycin has been performed in our laboratory and the amino acid sequence was determined by using the new improved method of selective partial hydrolysis of peptides. This paper deales with the details of novel investigations for the determination of the total structure of viomycin.

Acetylviomycin (VIII),[1)] a diacetylated product of viomycin (I) at the N-terminal β-lysine residue, was hydrogenated in acetic acid solution with the presence of Adams catalyst to give tetrahydroacetylviomycin (IX) with liberation of urea unit. Also, IX was obtainable by acetylating tetrahydroviomycin (X)[9)] with N-acetoxysuccinimide.[1)] The compound IX showed only an end absorption in its ultraviolet (UV) spectrum. On acid hydrolysis, X

1) This forms Part V of "Studies on Viomycin," Part IV: T. Kitagawa, T. Miura, and H. Taniyama, *Chem. Pharm. Bull.* (Tokyo), **20**, 2176 (1972).
2) Location: *Bunkyo-machi, Nagasaki.*
3) *a*) A.J. Finlay, G.L. Hobby, F.A. Hochstein, T.N. 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, *Amer. Rev. Tuberc.*, **63**, 1 (1951); *b*) Q.R. Bartz, J. Ehrlich, J.D. Mold, M.A. Penner, and R.M. Smith, *ibid.*, **63**, 4 (1951).
4) E.B. Herr, Jr., M.E. Haney, G.E. Pitterger, and C.E. Higgins, *Proc. Indian Acad. Sci.*, **96**, 134 (1960).
5) T. Ando, R. Izumi, H. Sakakibara, T. Take, K. Hayano, and J. Abe, *J. Antibiotics*, **21**, 681 (1968); T. Ando, K. Matsuura, R. Izumi, T. Noda, T. Take, A. Nagata, and J. Abe, *ibid.*, **24**, 680 (1971).
6) T. Kitagawa, T. Miura, S. Tanaka, and H. Taniyama, *J. Antibiotics*, **25**, 429 (1972).
7) H. Yoshioka, T. Aoki, H. Goko, K. Nakatsu, T. Noda, H. Sakakibara, T. Take, A. Nagata, J. Abe, T. Wakamiya, H. Shiba, and T. Kaneko, *Tetrahedron Letters*, **1971**, 2043.
8) B.W. Bycroft, D. Cammeron, L.R. Croft, A. Hassanali-Walji, A.W. Johnson, and T. Webb, *Experientia*, **27**, 501 (1971); *idem*, *Nature*, **231**, 301 (1971).
9) T. Kitagawa, T. Miura, and H. Taniyama, in press.



I viomycin: $R_1=H$, $R_2=OH$ III tuberactinomycin N: $R_1=OH$, $R_2=H$
II tuberactinomycin A: $R_1=R_2=OH$ IV tuberactinomycin O: $R_1=R_2=H$



V viomycin: $R_2=R_3=R_4=OH$
VI capreomycin IA: $R_2=H$, $R_3=OH$, $R_4=NH_2$
VII capreomycin IB: $R_2=R_3=H$, $R_4=NH_2$

Fig. 1. Chemical Structures of Antibiotics

yielded amino acids, serine, α,β-diaminopropionic acid (dpr), β-lysine, viomycidine[10] and alanine[9,12] in ratios 2:1:1:1:1 respectively while, the hydrolysate of I contained the same constituent amino acids except alanine. Therefore, during the reduction its chromophore group was converted to alanine residue with liberation of urea unit. However, the other reducible tuberactidine residue was still remained intactly.[9] Since IX showed a positive Sakaguchi reaction and it formed methylated derivative, as like as I[1,13] when the hydrochloride of IX was refluxed in methanol. The nuclear magnetic resonance (NMR) spectrum of IX was characterized by the absence of the low field proton δ 8.0 ppm and the presence of δ 5.2 ppm proton and newly formed methyl proton at 1.45 ppm as shown in Table I.

Table I. NMR Data of Viomycin Derivatives

Compound	Proton (δ: ppm)[a]		
I	8.0 (1H, s),[b]	5.2 (1H, t, J=3 cps)[c]	
VIII	8.0 (1H, s),	5.2 (1H, t, J=3 cps),	1.95 (6H, s)[d]
IX		5.2 (1H, t, J=3 cps),	1.95 (6H, s), 1.45 (3H, d, J=8 cps)[e]
XII			1.95 (6H, s), 1.45 (3H, d, J=8 cps)

a) chemical shift in δ from DSS, solvent D_2O
b) a proton on the chromophore
c) a proton on the carbone bearing hydroxyl and guanidine groups
d) methyl protons of acetyl group
e) methyl protons of alanine residue

10) An artifact derived from the true constituent tuberactidine[11]: G. Buchi and J.A. Raleigh, *J. Org. Chem.*, **36**, 837 (1971).
11) T. Wakamiya, T. Shiba, T. Kaneko, H. Sakakibara, T. Take, and J. Abe, *Tetrahedron Letters*, **1970**, 3497.
12) B.W. Bycroft, D. Cameron, L.R. Croft, A. Hassali-Walji, A.W. Johnson, and T. Webb, *Tetrahedron Letters*, **1968**, 5901.
13) F.A. Hochstein, G. City, and R.L. Miller, U.S. Pat., 2920998, Jan. 12 (1960).

Under the more drastic conditions, this tuberactidine residue could be converted to capreomycidine moiety.[9,12] Thus IX gradually absorbed one equivalent of hydrogen in 1N hydrochloric acid solution with the presences of Adams catalyst and paradium on charcoal to give perhydroacetylviomycin (XII). On complete acid hydrolysis XII gave serine, dpr, β-lysine, alanine and capreomycidine (in ratios 2:1:1:1:1 respectively). It showed a positive Rydon–Smith reaction but negative to ninhydrin and Sakaguchi tests and possessed no methoxylable hydroxyl function.

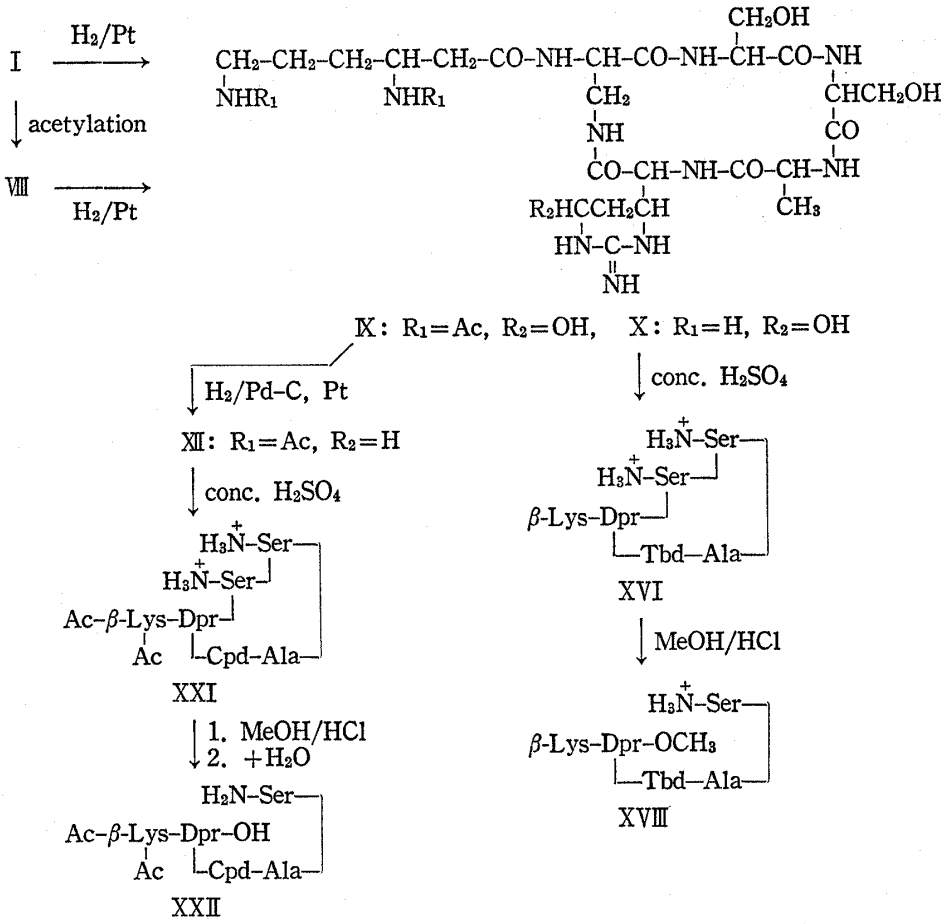


Chart 1

The partial hydrolysis of viomycin as well as its hydrogenated derivatives were attempted using N→O acyl migration reactions.[14] Since they contain two equivalents of hydroxy amino acid serine residues in their molecule, it is expected to produce N→O acyl migrated products by the treatment of concentrated sulfuric acid.[15] Therefore, viomycin was initially treated with concentrated sulfuric acid at room temperature. Thus, we were able to obtaine a peptide named isoviomycin (XIII). Though, viomycin showed a positive Sakaguchi reaction, XIII obtained here was negative. The UV spectrum of XIII showed the maximum shifted to 239 mμ. Therefore, both reactive residues in viomycin namely tuberactidine moiety which shows a positive Sakaguchi test and the chromophore group which exhibited the absorption maximum at 268 mμ were reacted besides the desired migration reaction. The compound XIII was very unstable and easily decomposed to give several compounds difficult to purify and the only isolable products were serine, β-lysine and 2-aminopyrimidine-5-sulfonic acid (XIV). The latter product is supposed to be a sulfonation product of 2-aminopyrimidine (XV) which presumably resulted from the tuberactidine part of this molecule as suggested

14) D.F. Elliott, *Biochem. J.*, **50**, 542 (1959).
15) K. Iwai, *Nippon Kagaku Zasshi*, **80**, 1066 (1959); **81**, 1320 (1960); **82**, 1088 (1961).

by Bycroft, *et al.*[16]

Isotetrahydroviomycin (XVI) was also obtained from X by the treatment of concentrated sulfuric acid. This compound showed positive to ninhydrin and Rydon–Smith tests and negative to Sakaguchi reaction. The total acid hydrolysate of XVI contained amino acids, serine, alanine, dpr and β-lysine in ratios 2:1:1:1 respectively, besides ammonia and degradation product(s) of tuberactidine, the product(s) which was not characterized yet. Despite of this undesired side reaction on the constituent tuberactidine residue, XVI was paper chromatographically and paper electrophoretically a pure compound. For the selective hydrolysis of the ester linkage, XVI was subjected to various hydrolytic conditions, since the treatment with 6N hydrochloric acid[15] failed to work. For this purpose, an ester exchange reaction with methanol at the presence of hydrochloric acid was found to be the method of choice and gave the best results.

A suspension of XVI in methanol saturated with hydrogene chloride was refluxed for 6 hr. The reaction mixture became a homogeneous solution which contained three ninhydrin and Rydon–Smith positively tested products, two of which were separated and purified by column chromatographies on Sephadex LH-20 using methanol as an eluent but the other trace amount of minor component was unsuccessful to be isolated. One of the products was determined as β-lysine methyl ester (XVII) by comparisons of its *Rf* and *Rm* values, with those of synthetic sample and the mass spectrum of the acetyl derivatives as shown in Table II and Fig. 2.

Table II. Comparison of the Properties of Degradative and Synthetic β-Lysine Methyl Ester

	XVII	Synthetic β–Lys–OCH_3
Rf_1	0.56	0.56
Rm_1	1.62	1.62
IR ν_{max}^{KBr} cm^{-1} (CO)	1726 (shoulder)	1726 (shoulder)
	1620 (s)	1620 (s)
Amino acid *anal.*	β–Lys, NH_3	β–Lys, NH_3
Rf_{12} of DNS derv. after the hydrolysis	0.83	0.83

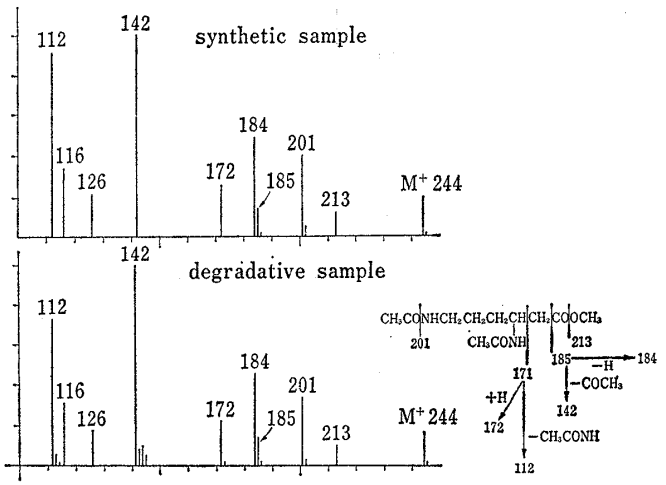


Fig. 2. Mass Spectra[a] of XVII

a) All peak heights are relative to that of *m/e* 142.

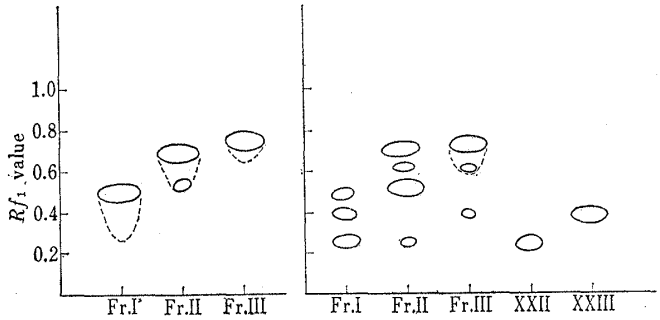
The main reaction product named T-1-M peptide (XVIII) was obtained with a yield of 50%. The complete hydrolysate of XVIII contained serine, dpr, alanine and β-lysine in ratios 1:1:1:1 besides ammonia and decomposed product(s) of tuberactidine. β-Lysine and serine residues were determined as N-terminal amino acids by Sanger's method[17] and dansyl procedures.[18] But just as expected, there is no free carboxyl function. The selective hydrolysis of XVIII at methyl ester bond

16) B.W. Bycroft, D. Cammeron, L.R. Croft, A.W. Johnson, and T. Webb, *Tetrahedron Letters*, **1968**, 2925.
17) F. Sanger, *Biochem. J.*, **39**, 507 (1945).
18) B.S. Hartley and V. Massey, *Biochim. Biophys. Acta*, **21**, 58 (1958); W.R. Gray, and B.S. Hartley, *Biochem. J.*, **89**, 379 (1963).

with 2N methanolic sodium hydroxide solution at room temperature resulted a peptide named T-1-A (XIX) having dpr residue as the C-terminus.

The sequential analysis by Edman-dancyl procedures[18,19] were attempted to XVIII. When XVIII was reacted with phenylisothiocyanate, phenylthiocarbamyl derivative of the peptide (XX) was obtained. However, XX was very insoluble to organic solvents such as trifluoroacetic acid or acetic acid containing hydrochloric acid[18] and so the sequential analysis was unsuccessful for this peptide.

To avoid difficulties such as the decomposition of tuberactidine residue or insolubilities of phenylthiocarbamyl derivative, the selective partial hydrolysis of XII was investigated. When XII was treated with concentrated sulfuric acid for a week at room temperature, isoperhydroacetylviomycin (XXI) which showed positive to ninhydrin and Rydon-Smith reactions was obtained. The acid hydrolysate of XXI contained the same constituents with those of XII. Consequentially, it is obvious that only the desired migration reaction had took place without an undesired side reaction. The selective hydrolysis by the ester exchange reaction was also performed. The reaction product was separated with combinations of column chromatographies on Sephadex gel filtration method using water as the eluent and on cellulose powder with the solvent 1 and then further purified by preparative paperchromatography and paper electrophoresis to give two peptide fragments named P-1-A and P-2-A. However, the *Rf* values of these fragments were different from those of product obtained by the first column chromatography on Sephadex LH-20, therefore these fragments were artifactory formed during these purification procedures. The chromatographed product of the first column on Sephadex LH-20 showed some changes on *Rf* values after lyophilization compared with those values of just after the chromatography. To confirm the changes on *Rf* values, the lyophilized products were kept in the solvent systems, methanol, ammonia and pyridine (200:1:1) and the changes of *Rf* values were followed on time course and the getting results as well as *Rf* values of purified peptide fragments were summarized in Fig. 3 and Fig. 4.



PPC of just after the chromatography

PPC of lyophilized products after 3 days and purified fragments

Fig. 3. Changes on Rf_1 Values of the Methanolysis Products of XXI after the Chromatography on Sephadex LH-20

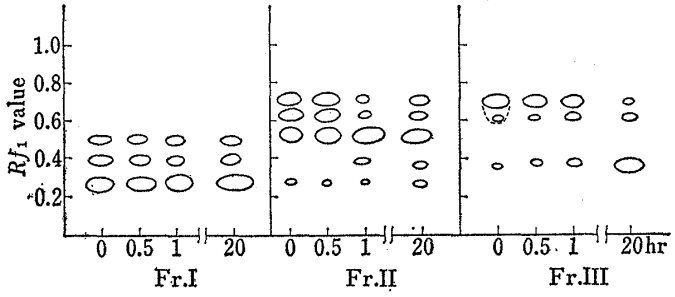


Fig. 4. Periodic Changes on Rf_1 Values of the Fractions in Methanol–Pyridine–28% Ammonia (200:1:1)

The reason for the change during the purification procedures was attributed to the hydrolysis of the methyl ester bond from the results of amino acid analysis and end groups determinations of peptide fragments P-1-A (XXII) of the main product and P-2-A (XXIII) of the minor one. Both fragments showed positive to ninhydrin and Rydon-Smith tests. Amino acid analysis provided that both peptides contained the same amino acids constituents with those found in the acid hydrolysate of XXI except for one equivalent of serine had

19) B.S. Hartley, *Biochem. J.*, **119**, 805 (1970).

disappeared in P-1-A. End group analysis by dansyl method[18] and dansyl-hydrazinolysis procedures revealed serine moiety as the N-terminus and dpr residue as the C-terminus. To confirm the C-terminal analysis, the main product XXII was converted to dansyl (DNS) derivative (XXIV) which could be purified with preparative thin-layer chromatography on silica gel using the solvent system of chloroform–ethanol–acetic acid (38: 4: 3) and then column chromatography on DEAE Sephadex A-25, since dansylated product of XXII contained a very small but almost negligible amount of DNS amino acids which contaminated amino acids were not detected by ninhydrin reaction on PPC. Acid hydrolysate of XXIV contained DNS-serine. The C-terminal determination of ^3H labeling method[20] was applied for XXIV and it was also found that dpr residue was the C-terminus.

When the Edman-dansyl procedures were applied for these XXII and XXIII, following results as summarized in Table III and IV were obtained.

Table III. Amino Acid Components, End Groups and Color Reactions of Peptides

Peptide	Molecular formula	Ser	Dpr	UDA	Ala	Tbd	Cpd	β-Lys	Sa	Nin	R.S
I	$C_{25}H_{43}O_{10}N_{13}$[a]	2	1	1		1		1N	+	+	+
VIII[1]	$C_{29}H_{47}O_{12}N_{13}$[a]	2	1	1		1		1	+	−	+
IX	$C_{28}H_{47}O_{11}N_{11}$[a]	2	1		1	1		1	+	−	+
X[9]	$C_{24}H_{43}O_9N_{11}$[a]	2	1		1	1		1N	+	+	+
XII	$C_{28}H_{47}O_{10}N_{11}$[b]	2	1		1		1	1	−	−	+
XVI		2	1		1	1?		1	−	+	+
XVII		1N	1CH		1	1?		1N	−	+	+
XXI		2	1		1		1	1	−	+	+
XXII	$C_{25}H_{43}O_9N_{10}$[b]	1N	1C		1		1	1	−	+	+
XXIII	$C_{28}H_{49}O_{11}N_{11}$[b]	2N	1C		1		1	1	−	+	+

Ser: L-serine, Dpr: L-α,β-diaminopropionic acid,[21] Tbd: L-tuberactidine,[11] Cpd: L-capreomycidine,[12] UDA: 3-ureidodehydroalanine,[12] Ala: alanine,[9,12] β-Lys: L-β-lysine,[1] N: N-terminus, C: C-terminus, CH: C-terminus after the hydrolysis, Sa: Sakaguchi test, Nin: ninhydrin test, R.S: Rydon-Smith test, ?: unidentified. *a*) empirical formula by elemental analysis, *b*) empirical formula by amino acid analysis

Table IV. The Amino Acid Sequence of Fragments

XXII	Ser–Ala–Cpd Ac–β-Lys–Dpr Ac	X	β-Lys–Dpr–Ser–Ser Tbd–Ala
XXIII	Ser–Ser–Ala–Cpd Ac–β-Lys–Dpr Ac	XII	Ac–β-Lys–Dpr–Ser–Ser Cpd–Ala
		XXV	β-Lys–Dpr–Ser–Ser Tbd–UDA

Arrows pointing to the right indicate sequences determined by the Edman-dansyl procedure.[19] C-Terminal amino acid (arrow to the left) was determined by DNS method after hydrazinolysis or ^3H labeling method.[20]

From these results, one can conclude that the amino acid sequence of viomycin is formulated as XXV.

The sequence XXV is well compatible with the formula I.[22] Thus, the total structure of viomycin is determined by the sequential analysis of the degradation products of viomycin obtained by the novel partial hydrolysis.

20) H. Matsuo, *Protein Nucleic Acid Enzyme*, **14**, 523, 589 (1969).
21) T. Kitagawa, T. Ozasa, and H. Taniyama, *Yakugaku Zasshi*, **89**, 285 (1969).
22) We had proposed another structure,[23] based on the sequence of peptide III.[23b] However, three times trials to isolate this peptide again was unsuccessful. From the data obtained here, we invalidate earlier proposal.
23) *a*) T. Kitagawa, T. Miura, T. Sawada, and H. Taniyama, *Progress in Antimicrobial and Anticancer Chemoth.*, **II**, 1027 (1970); *b*) T. Kitagawa, Y. Sawada, T. Miura, T. Ozasa, and H. Taniyama, *Tetrahedron Letters*, **1968**, 109.

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 was used. Melting points were uncorrected. On paper chromatography (Toyo filter paper No. 51 UH, diam. 31.5 cm, by circular ascending method), *Rf* values refer to the following solvent systems; Rf_1, *n*-BuOH–*t*-BuOH–pyridine–AcOH–H_2O (15: 4: 10: 3: 12, solvent 1), Rf_2, *t*-BuOH–AcOH–H_2O (2:1:1) and on thin-layer chromatography; Rf_{11}, solvent 1, Rf_{12}, $CHCl_3$–benzyl alcohol–AcOH (70: 30: 3), Rf_{13}, $CHCl_3$–CH_3OH–AcOH (75: 20: 5), Rf_{14}, AcOEt–iso-PrOH–28% NH_3 (45: 35: 20), Rf_{15}, $CHCl_3$–EtOH–AcOH (38: 4: 3). On paper electrophoresis (Toyo C type instrument, 500 V, 3—5 mA, 3 hr with Toyo filter paper No. 51) *Rm* values refer to the following buffer solution and difining the electrophoresis distance of viomycin as 1; Rm_1, pyridine–AcOH–H_2O (5: 0.2: 95, pH at 6.3), Rm_2, 0.025M triethyamine–CO_2 (pH at 8.0). Ninhydrin, Sakaguchi[24] or Rydon–Smith[25] reagents were used for the detections in these test.

Tetrahydroacetylviomycin (IX)——a) The Catalytic Hydrogenation of Acetylviomycin (VIII)[1]: To a solution of VIII (1.01 g) in 50% acetic acid (20 ml), Adams catalyst (500 mg) was suspended and the mixture was stirred under atmosphere of hydrogen until it showed only end absorption in UV region for two days. During the reaction two equivalents of hydrogen were absorbed. After the catalyst was filtered off, the filtrate was condensed *in vacuo* below 30° and the residue was chromatographed on Sephadex LH-20 (3×200 cm) using water as the eluent. The fractions (10 g/fract.) positive to Rydon-Smith test were developed on PPC. Thus from the fraction No. 50—52, a single component (20 mg, Rf_1, 0.27) was obtained. The fract. No. 53—54 contained three compounds (158 mg, Rf_1, 0.27, 0.55, 0.65) and from the fractions No. 55—64, IX (658 mg, Rf_1, 0.65) was obtained after lyophilization. IX: colorless amorphous powder, very soluble in H_2O, unsoluble in common organic solvents, yield, 65%: mp 237—240° (decomp.), $[\alpha]_D^{25°}$ −3.4° (c=1%, H_2O): UV, end absorption: IR ν_{max}^{KBr} cm^{-1}: 3280, 3050, 2935, 1655, 1522, 1435, 1040: NMR δ ppm in D_2O: 5.2 (1H, t, J=3 cps, C<u>H</u>OH-N), 1.90 (6H, s, CO<u>CH</u>$_3$), 1.44 (3H, d, J=8 cps, CH-C<u>H</u>$_3$). The color reactions: Positive to Sakaguchi test (λ_{max}^{KBr} 500 mμ, ε, 3700; *cf.* ε of viomycin 3500).[26] Rf_1, 0.65, Rf_2, 0.92, Rf_{11}, 0.38, Rm_1, 0.62, Rm_2, 0.82. *Anal.* Calcd. for $C_{28}H_{47}O_{11}N_{11}\cdot CH_3CO_2H\cdot 4H_2O$: C, 42.70; H, 6.81; N, 18.36. Found: C, 42.80; H, 6.62; N, 18.46. Amino acid analysis: ser (2.1), ala (1.0), dpr (0.95), β-lys (0.80), vio (0.1).

b) Acetylation of Tetrahydroviomycin (X)[9]: To a solution of X (1 g) in 50% dimethylformamide (DMF) (5 ml), N-acetoxysuccinimide (0.45 g) in 50% DMF (5 ml) was added and the solution was kept slightly basic with triethylamine at room temperature under mechanical stirring until it showed a negative ninhydrin reaction. After the mixture was washed with ether (100 ml×2) the aqueous layer was condensed to dryness *in vacuo* below 30°. The residue was chromatographed on the column of Sephadex LH-20 (3×95 cm) using 10% methanolic solution as the eluent. The fractions (No. 19—25, 18 g/fract.) which were positive to Sakaguchi tests were collected and lyophilized and the product (1 g) was again chromatographed on a column of cellulose powder (2×95 cm) using the solvent 1 as the eluent. The fractions (5 g/fract.) No. 11—31 contained IX (Rf_1, 0.65) and the fracts. No. 32—43 contained a mixture of IX and a compound (Rf_1, 0.65 and 0.55). The fractions No. 11—31 were combined, washed with ether to remove organic solvents and the aqueous layer was lyophilized to give IX (0.5 g). The results of amino acid analysis, *Rf* values and NMR spectrum of IX were found to be the same as those obtained from the procedure a.

The Methylation of Tetrahydroacetylviomycin (IX)——A solution of IX hydrochloride (100 mg) in MeOH (100 ml) was heated under reflux for 48 hr. After evaporation, the residue was dissolved in MeOH (100 ml) and was again refluxed for 24 hr. The solvent was removed and the residue was washed with acetone and then dried in a desiccator over NaOH and P_2O_5 to give IX. The NMR spectrum of IX showed the presence of methoxyl group: δ ppm, 3.45 (3H, s, OC<u>H</u>$_3$), 1.95 (6H, s, COC<u>H</u>$_3$).

Perhydroacetylviomycin (XII)——To a solution of X (300 mg) in 3% hydrochloric acid, 10% paradium on charcoal (0.3 g) was suspended and the suspension was stirred under atmosphere of hydrogen at room temperature. A small part (0.1 ml) of the reaction mixture was occasionally developed by Sakaguchi reagent and the development was measured by the absorbances at 500 mμ according to the method of Weber.[24] Whenever the mixture showed a large ε values for the test, was added Adams catalyst (0.2 g) and the catalytic hydrogenation was continued until it showed the ε values less than 200 for ten days. After the catalyst was filtered off the filtrate was made neutral with basic type ion exchange resin Amberlite CG-4B and was condensed to dryness *in vacuo*. The residue was chromatographed on a tower of Sephadex LH-20 (2×150 cm) using water as the eluent. The fractions (6 g/fract.) positive to Rydon-Smith tests (No. 35—43)

24) J.B. Jepson and I. Smith, *Nature*, **172**, 1100 (1953).
25) H.N. Rydon and P.W.G. Smith, *Nature*, **169**, 922 (1963).
26) The quantitative analysis of Sakaguchi color test with the absorbance at 500 mμ was determined according to the method of C.J. Weber, *J. Biol. Chem.*, **86**, 217 (1930).

were developed on PPC. The fracts. No. 35—40 showed Rf_1 value 0.65 but strongly tailing were observed presumably due to decompositions during the reaction (150 mg). The fracts. No. 41—43 showed a single spot and on lyophilization 120 mg of XII was obtained. However, XII showed the same Rf values with IX (Rf_1, 0.65, Rf_{11}, 0.38) and almost the same Rm values (Rm_1, 0.63. Rm_2, 0.89). The differences were result of amino acid analysis: ser (1.92), ala (1.0), dpr (0.7), β-lys (1.0), cpd (0.82), and the behavior toward Sakaguchi reaction, the paper test was almost negative but the absorbance at 500 mμ according to Weber's method, ε value was 100 from which less than 3% of the contamination was calculated.[9] The properties of XII: colorless hygroscopic powder, very soluble in H_2O, a little soluble in CH_3OH. $[\alpha]_D^{27°}$ −17.4° (c=0.83%, H_2O). UV: end absorption. IR ν_{max}^{KBr} cm^{-1}: 3340, 3140, 2970, 1660, 1545, 1530, 1440. NMR δ ppm in D_2O: 1.90 (6H, s, $COC\underline{H}_3$), 1.43 (3H, d, J=8 cps, $CH\text{-}C\underline{H}_3$). Amino acid analysis, Rf and Rm values and the color reactions were already described.

Isoviomycin (XIII)——Sulfate of I (1 g) was added slowly during 10 min to the ice cooling solution of 30% fuming H_2SO_4 (20 ml) and the reaction mixture was kept at 0—5° for two days. The reaction mixture showed dark brownish color. To the mixture dry ether (200 ml) was added and the resulting precipitate was collected by centrifuge and washed with dry ether for several times giving a pale yellow crude XIII as sulfate, mp above 300°, yield 1 g, $[\alpha]_D^{12°}$ −54° (c=1%, H_2O), very hygroscopic, positive to ninhydrin and phenylhydrazin[27] tests but negaive to Sakaguchi reaction. A single spot on PPC, Rf_1, 0.27 (a small amount of tailing was observed) and paper electrophoresis, Rm_1, 0.27.

The Isolation of 2-Amino-5-pyrimidinesulfonic Acid (XIV)——The crude XIII sulfate (1 g) in H_2O (5 ml) was chromatographed on a column of Sephadex LH-20 (2.5×100 cm) using 5% MeOH–3% AcOH solution as the eluent with eluting speed of 7 ml/10 min. Individual ninhydrin positive fractions (No. 20—32, 7 ml/fract.) possessed at least three components detected by PPC.[28] The fractions No. 48—64 showed UV absorption at 239 mμ. The fractions No. 20—32 were combined and lyophilized, and the residue was rechromatographed on several columns such as Sephadex G-10, carboxymethyl cellulose and ion exchange resin of strongly acidic type Amberlite CG-120 (H form) respectively, but any procedure was unsuccessful to get a purified peptide and only liberations of serine, dpr and β-lysine were detected on PPC and paper electrophoresis. The fractions No. 48—64 were combined and the resulting solution was condensed under reduced pressure to give crude XIV. Recrystallization from H_2O gave white prism, mp 303—305° (decomp., lit. 305—307°,[29] 326—328°[30]). UV $\lambda_{max}^{H_2O}$: 239 mμ (log ε, 4.58). *Anal.* Calcd. for $C_4H_5O_3N_3S\cdot 1/4H_2O$: C, 26.81; H, 3.09; N, 23.40. Found: C, 27.03; H, 3.30; N, 23.01. Mass Spectrum m/e: 175. NMR, δ ppm in D_2O: 8.81 (s).

Isotetrahydroviomycin (XVI)——To the ice cooling conc. H_2SO_4 (3 ml), IX hydrochloride (250 mg) was added and the resulting solution was kept at room temperature for two days. Then, dry ether (50 ml) was added and the resulting precipitate was collected by centrifuge and washed with dry ether to give XVI; yield, quantitative, white and very hygroscopic powder, mp above 300°, Rf_1, 0.33: Rm_1, 0.78; IR ν_{max}^{KBr} cm^{-1}: 3350 (broad, NH, OH), 1735 (shoulder, CO-O), 1660 (CO-NH): positive to ninhydrin and Rydon-Smith reactions, a negative to Sakaguchi test: amino acid analysis, ser (2.05), ala (0.98), dpr (1.0), β-lys (1.17), vio (0.1).[31]

Attempts for the Selective Hydrolysis of the Ester Bonds in Isotetrahydroviomycin (XVI) with 6N Hydrochloric Acid——Fifty mg of XVI was dissolved in 6N HCl (3 ml) and the solution was kept at room temp. for 16 hr. The reaction mixture was diluted with 100 ml of water and was lyophilized. The lyophilized product showed at least five ninhydrin positive spots on PPC including all of the constituents amino acids and was found to be difficult to isolate the desired peptide fragment after column chromatographies on Sephadex LH-20 and preparative PPC or paper electrophoresis.

The Selective Methanolysis of Isotetrahydroviomycin (XVI)——A suspension of XVI (100 mg) in dry MeOH (20 ml) was bubbled with HCl gas until it was almost saturated. The mixture was then refluxed for 6 hr. During the reaction the suspension became a clear solution. After the reaction, the mixture was condensed to 1 ml *in vacuo*, and the residue was chromatographed on a column of Sephadex LH-20 (2×150 cm) which was previously swelled with MeOH, using MeOH as the eluent. Fractions (10 g/fract.) positive to ninhydrin tests were developed on PPC. Thus, the fract. No. 29 contained two spots (Rf_1, 0.22, 0.35) (5 mg), the fract. No. 30—31, a single compound (Rf_1, 0.35) (40 mg), the fract. No. 32, two spots (Rf_1, 0.35, 0.56) (20 mg) and the fract. No. 33—34, one spot (Rf_1, 0.56) (5 mg). A compound obtained from the fract. No. 33—34 which showed the Rf_1 value 0.56 was a strongly positive to ninhydrin and a weakly positive to Rydon–Smith test and on acid hydrolysis it gave ammonia and β-lysine which was identified on PPC and with amino acid analyser. For comparison β-lysine methyl ester was synthesized from β-lysine by re-

27) The test was performed according to the method of F. Fiegle, "Spot Test in Organic Analysis," 7th Edition, Elsvier Public Co., London, 1966, p. 390.
28) Isoviomycin showed decompositions even on standing its aq. solution at room temp. overnight.
29) W.T. Caldwell and G.E. Jaffe, *J. Am. Chem. Soc.*, **81**, 5166 (1959).
30) W.T. Caldwell, W. Fiddler, and N.N. Santora, *J. Med. Chem.*, **6**, 58 (1963).
31) The retention time was the same with that of viomycidine.

fluxing the amino acid in methanol which was saturated with HCl. Both synthetic sample and the natural one showed the same Rf_1 and Rm_1 values, 0.56 and 1.62 respectively. On acetylations by acetic anhydride in methanol, both compounds (1 mg respectively) yielded diacetyl β-lysine methylester and their mass spectra showed the same fragment patterns as cited in Fig. 2.

A product showing Rf_1 value 0.35 was a peptide named T-1-M (XVIII): colorless plate, soluble in methanol and H_2O. $[\alpha]_D^{29°}$ +6.33° (c=0.9%, H_2O): positive to ninhydrin and Rydon-Smith reactions, a negative to Sakaguchi test. IR ν_{max}^{KBr} cm^{-1} 3400, (broad, NH and OH), 1730 (shoulder, CO-OCH_3), 1660 (CO-NH): amino acid analysis, ser (1.0), ala (1.0), dpr (0.99), β-lys (1.01), decomposed product of tuberactidine (0.1).[31]

The N-Terminal Analysis of T-1-M Peptide (XVI)——a) By Modified Sanger Method: The method was essentially the same as described in the general method. Two mg of XVI was subjected to the method and the hydrolysate of DNP derivative of XVI gave the following amino acid constituent: ser (0.20), ala (0.98), dpr (1.0), β-lys (0.18), vio (0.1).[31]

b) By Dansyl Method: The method for the isolation and the determination of DNS-amino acids in the hydrolysate of DNS derivative of XVI was essentially the same as described in the general procedure. The hydrolysate was proved to contain DNS-β-lysine, Rf_{12}, 0.69 and DNS-serine, Rf_{12}, 0.36.

The C-Terminal Analysis of XVI——The method for the determination of the C-terminus was essentially the same as described in the procedure of dansyl hydrazine method. No DNS amino acid was detected.

The Selective Ester Hydrolysis of T-1-M Peptide (XVI)——A solution of XVI (100 μg) in MeOH (0.1 ml), 1N NaOH (20 μl) was added and the solution was kept at room temperature for 2 hr. The solution was neutralized with 1N HCl (20 μl) and then evaporated. The hydrolysate showed a single spot on PPC, Rf_1, 0.29 and on paper electrophoresis, Rm_1, 0.98. To the residue dry NH_2NH_2 (0.1 ml) was added and then sealed in a tube. After heated at 105° for 5 hr the tube was opened and the excess reagent was evaporated over P_2O_5 and H_2SO_4 at 1 mmHg. To the residue 0.1 ml of water and 0.1 ml of benzaldehyde were added and the mixture was shaken for 30 min and then washed with ether (1 ml×3) and the aqueous layer was evaporated *in vacuo*. The residue was dissolved in 0.1 ml of water, and 20 μl of the solution was dansylated to give DNS-dpr, Rf_{12}, 0.70: Rf_{13}, 0.68, and was identical with the authentic sample synthesized from dpr[21] by the end duplicated method.[32]

Isoperhydroacetylviomycin (XXI)——To the ice cooling conc. H_2SO_4 (1 ml), XII (100 mg) was added and the solution was kept in a desiccator at room temp. for a week. Dry ether was added and the resulting precipitate was separated by centrifuge, washed with ether and then dryed in a desiccator to give colorless amorphous crude XXI, mp 190—200° (decomp.). A single spot on PPC and TLC, Rf_1, 0.30 and Rf_{11}, 0.47, but two spots on paper electrophoresis, Rm_1, 0.53 and 0.65: positive to ninhydrin and Rydon-Smith tests. $[\alpha]_D^{25°}$ −6.4° (c=1%, H_2O). Yield, quantitative. Amino acid anal.: ser (1.6), ala (1.1), dpr (1.1), β-lys (0.97), cpd (0.84).

Isolations of P-1-A (XXII) and P-2-A (XXIII) from Isoperhydroacetylviomycin (XXI) by the Selective Methanolysis——A solution of above obtained XXI (95 mg) in 6 ml of MeOH, saturated with HCl, was refluxed for 5 hr. The solution was condensed by bubbling N_2 gas to 1 ml and dry ether (10 ml) was added. The resulting precipitate was collected by centrifuge and was chromatographed on a column of Sephadex LH-20 (2×150 cm) using water as the eluent (10 g/fr.). Noticeable differences on Rf_1 values of the fractions of just after the chromatography (Fr. I, No. 19—26; Fr. II, No. 27—30; Fr. III, No. 31—33) and their lyophilized products (kept at room temp. for 3 days before lyophilization) were observed (see Fig. 3). Changes on Rf_1 values of the products in the solvent system of MeOH–28% NH_3–pyridine (200:1:1) was cited in Fig. 4. All fractions were combined. After removal of the solvent, the residue (57 mg) was chromatographed on a tower of cellulose powder (1×90 cm, solvent 1 as the eluent, 2 g/fr.) and fractions No. 6—20 contained at least five components, No. 24—44 contained a single component Rf_1 value 0.39 (3 mg) and No. 66—93 contained another single substance, Rf_1, 0.25 (10 mg). The compound (Rf_1, 0.39) was further purified by preparative PPC and paper electrophoresis to give P-2-A peptide (XXIII) (2 mg), Rf_1, 0.39, Rf_{11}, 0.23, Rm_1, 0.43; single spot on PPC, TLC and paper electrophoresis, positive to ninhydrin and Rydon-Smith tests. Amino acid anal.: ser (1.7), ala (1.0), dpr (0.8), β-lys (0.9), cpd (0.8). End group anal.: by the dansyl method, serine residue was the N-terminus (DNS-ser, Rf_{12}, 0.47, Rf_{15}, 0.27, by the duplicated TLC method) and by the dansyl-hydrazine method, dpr residue was the C-terminus (DNS-dpr, Rf_{12}, 0.62, Rf_{13}, 0.55).

Three mg of the other product (Rf_1, 0.25, 10 mg) (XXII) was also purified by preparative PPC and paper electrophoresis to give P-1-A peptide, Rf_1, 0.25, Rf_{11}, 0.2, Rm_1, 0.86. Single spot on PPC and paper electrophoresis, amino acid analysis: ser (1.07), ala (1.0), dpr (0.8), β-lys (0.8), cpd (1.1). End group anal.: by the dansyl method, serine residue was the N-terminus (DNS-ser, Rf_{12}, 0.47, Rf_{15}, 0.27) and by the dansyl-hydrazin method, dpr moiety was the C-terminus (DNS-dpr, Rf_{12}, 0.62, Rf_{13}, 0.55).

Dansyl Derivative of XXII (XXIV) and Its C-Terminal Analysis——One mg of XXII was dissolved in 0.1M triethylamine–CO_2 buffer (pH at 8.7, 1 ml) and the solution of 5% DNS-Cl in acetone was added. After incubated at 37° for 1 hr the solvents were removed *in vacuo* over NaOH and P_2O_5. The residue was dis-

32) Z. Tamura and T. Nakajima, "Tanpakushitsu Kakusan Koso," Vol. 12, Kyoritsu Publishing Co., Tokyo, 1967, p. 729.

solved in 50% acetone solution (50 μl) and was spotted on silica gel (Kieselgel G, Merck) plate (3×7 cm) and was developed with the solvent systems $CHCl_3$–EtOH–AcOH (38: 4: 3). A greenish yellow fluorescent band at starting point just below the band of DNS-OH (blue fluorescence, Rf_{15}, 0.1) was scraped together. From the absorbent silicagel, DNS-OH of contamine, was extracted by CH_3OH–pyridine–28%NH_3 (200:1:1) and the remaining DNS-peptide was extracted by acetone–H_2O–pyridine–AcOH (50: 50: 1: 3). After removal of the solvent, the residue was dissolved in 50% CH_3OH and the solution was chromatographed on a tower of DEAE Sephadex A-25 (Pharmacia) (0.5×5 cm) to separate the contaminated inorganic material and DNS-OH. The fraction of 50% MeOH contained no fluorescent product (5 ml) and the effluent of 50% MeOH–2%AcOH (5 ml) contained a purified XXIV: Rf_1, 0.85, Rf_{11}, 0.6, Rf_{12}, 0, and Rf_{14}, 0.69. The acid hydrolysate of XXIV possessed DNS-serine.

The C-terminal determination by isotope labeling method was applied for XXIV (*ca.* 0.3 mg) by the procedure described below and the isotope labeled hydrolysate was submitted on paper electrophoresis (10×40 cm) (500 V, 5 mA, for 3 hr) using the buffer solution (pyridine–AcOH–H_2O, 5: 0.2: 95, pH at 6.3). Basic amino acids of electrophoresis distance 6.5—7.5 cm (neutral amino acids serine and alanine, the distance, 1-3 cm) possessed isotope activity which was detected by measuring the activity of extracts of paper (1×1 cm, a side part of the paper was cut) by liquid scintillation counter (Aloka LSC-601 type instrument). The active part was cut and extracted with 1% AcOH and the extract was further subjected to a paper electrophoresis (3×40 cm) using the buffer solution of 0.025M triethylamine–CO_2 (pH at 8.0, 600 V, 3 mA, for 3 hr) and the active band was found at distance of 0—1 cm from the starting point (authentic dpr was 0.5 cm, cpd, 4 cm, and β-lys, 10 cm respectively by the same condition). The active part of the paper was cut and was extracted with 1% AcOH. The extract was dansylated to give DNS-dpr (Rf_{12}, 0.62, end duplicated method).

General Method for the Determination of N-Terminal Residue——a) By Modified Sanger's Method[17]: The individual peptide (2—5 mg) was dissolved in 2% $NaHCO_3$ solution and to the solution, 5% ethanolic solution of 2,4-dinitrofluorobenzene (DNFB) (1 ml) was added and the mixture was kept in the dark overnight with mechanical stirring at room temperature. After removal of residual DNFB with ether, the aqueous solution was acidified with 1N HCl and the resulting precipitate was collected and washed with water and ether and then was hydrolysed with 6N HCl (2 ml) in a sealed tube at 115° for 20 hr. The excessive acid was removed completely *in vacuo* and to the residue, H_2O (5 ml) was added and the layer was extracted with ethylacetate. The aqueous layer (1 ml) was submitted to an automatic amino acid analyser and the organic layer was tested on TLC using Kieselgel G (Merck) and the solvent system, AcOEt–MeOH (3:1, Rf_{15}) and also with PPC.

b) By the Dansyl Procedure: The individual peptide (1—50 nM) was taken in a stoppered tube and was lyophilized. The residue was dissolved in 10 μl of 0.1M triethylamine bicarbonate buffer solution[33] (pH, 8.5—9) and then 10 μl of 2% (w/v) solution of 1-dimethylaminonaphthalene-5-sulphonyl chloride (DNS-Cl) in acetone was added to the solution forming a single phase reaction mixture. The mixture was incubated for 1 hr at 37°. After evaporation the residue was dissolved in 10 μl of 50% acetone and subjected a preparative chromatography on TLC plate (Kieselgel G Merck, 3×7 cm) using solvent systems, $CHCl_3$–CH_3OH–AcOH (75: 20: 5, solvent A) or $CHCl_3$–benzyl alcohol–AcOH (70: 30: 3, solvent B). The DNS-peptide fraction mostly remained at starting point was scraped together and from the absorbent silica gel it was extracted with the solvent C (acetone–H_2O–pyridine–AcOH, 50: 50: 1: 3). The extract was evaporated in a small tube, added with 0.1 ml of 6N HCl and the tube was sealed and then incubated at 95° for 20 hr. The tube was opened and evaporated over NaOH and P_2O_5 *in vacuo*. The hydrolysate was dissolved in 10 μl of 50% pyridine and 2 μl of the sample was spotted on the TLC plate with 5—7 mm width. The authentic DNS amino acid prepared according to Tamura, *et al.*[32] was placed aside with 5—7 mm width which one end (*ca.* 1 mm) was duplicated. When the chromatogram was developed with the solvent A or B (for DNS-ser, DNS-ala, DNS-dpr, DNS-β-lys) or the solvent D (AcOEt–iso-PrOH–28% NH_3, 9: 7: 4, Rf_{14}, for DNS-cpd), the same DNS-amino acid in the hydrolysate and authentic sample showed a single line while, different DNS-amino acids even if their *Rf* values were very similar, their lines were separated.

General Method for the C-Terminal Analysis——a) Dansylhydrazine Method: A peptide (20—100 nM) and abs. NH_2NH_2 (0.1 ml) were placed and sealed in a small tube and then incubated at 105° for five hr. After cooling the tube was opened and the excessive reagent was removed *in vacuo* over H_2SO_4, P_2O_5 and NaOH. To the residue 0.1 ml of H_2O and 0.1 ml of benzaldehyde were added and the mixture was shaken for 30 min and then was washed with ether (0.8 ml×3). The aqueous layer was evaporated *in vacuo* over NaOH and P_2O_5 and the residue were dissolved in 10 μl of triethylamine–CO_2 buffer (pH at 8.7) and 10 μl of 2% DNS-Cl solution of acetone. After the homogeneous reaction mixture was incubated at 37° for 1 hr, the reaction product in the solution was directly compared with the corresponding authentic sample of DNS-amino acid on TLC plate by the same procedure described already.

b) Isotope Labeling Method: A peptide (1 mg) was dissolved in a mixture of ^3H_2O (0.1 ml, *ca.* 100 mCi) and pyridine (0.2 ml). After cooling with ice, 0.05 ml of acetic anhydride was added and the whole was kept at room temperature for 3 hr. After evaporation *in vacuo* at 40° and complete removal of the washable isotope, the residue was hydrolyzed in constant boiling HCl for 20 hr. The isotope labeled amino acid was separated and identified by paper electrophoresis using Toyo C type instrument (500 V, 3—5 mA,

for 3 hr) with the buffer solution (pyridine–AcOH–H_2O, 5: 0.2: 95, pH at 6.3 or 0.025M triethylamine–CO_2, pH at 8.0). For further confirmation, the radio active spot was cut and extracted with 1% AcOH. The extract was dansylated by the method described above and the DNS amino acid was identified on the TLC.