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Characterization of Racemomycins[1]

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The characterization was conducted on racemomycin (abbreviated as RM) A, B, C, and minor D components isolated in pure form by column chromatographies. RM-A was identified with streptothricin. Amino acid analysis of the acid hydrolysates revealed the presence of 1,2,3 and 4 β-lysine residues in RM-A, C, B, and D components respectively. And also it was apparent that the biological activities such as antimicrobial, antiviral and acute toxicity were stronger in order of the number of β-lysine residues in a molecule.

Introduction

Since the finding of the water-soluble, basic antibiotic streptothricin by Waksman and Woodruff[3] in 1942 from a culture broth of *Spreptomyces lavendulae*, a number of other antibiotics of this group have been discovered.

Racemomycin, also one of this group, was separated from a broth culture of a mutant strain of *S. racemochromogenus*.[4,5] The crude preparation was found to be a mixture of four biologically active components. However, the column chromatographic methods for separation of individual RM components such as ion exchange resin Amberlite IRA-400,[5] cellulose,[6] and carboxymethylcellulose[7-9] have not resulted in sufficient purification to permit further characterizations. In this paper, we describe our results of the quantitative isolation and characterizations of the racemomycin components.

Result and Discussion

Separation and Purification Methods of Racemomycins

The RM complex, prepared as reported previously,[6] was a white powder containing about 30% (w/w) inorganic substances. The complex, when developed by circular paper chromatography as reported by Horowitz[10] gave distinct spots with *Rf* values of 0.33, 0.24, 0.18, and 0.11. These spots were shown to correspond to RM-A, C, B, and D respectively according to their mobilities on paper using the Peterson–Reinecke solvent system.[11] The lowermost spot was found to be a new minor component and provisionally named RM-D.

Khokhlov[7-9] showed in their studies on polymycin that carboxy methyl cellose (CMC) resin was applicable to the isolation of antibiotics of the streptothricin group. An improvement of their methods was achieved by introducing a volatile buffer, pyridine–acetate, as an elution solvent so that the desalting procedure of the eluates in later steps could be omit-

1) It was reported in the 6th Symposium on Peptide Chemistry Fukuoka, Japan, 1968, p.61.
2) Location: *Bunkyo-machi 1-14, Nagasaki-shi.*
3) S.A. Waksman and H.B. Woodruff, *Proc. Soc. Explt. Biol. Med.*, **49**, 207 (1942).
4) T. Sugai, *J. Antibiotics*, **9**, **Ser B**, 170 (1956).
5) H. Taniyama and S. Takemura, *J. Pharm. Soc. Japan*, **77**, 1210 (1957).
6) H. Taniyama, F. Miyoshi and K. Kageyama, *J. Pharm. Soc. Japan*, **82**, 87 (1962).
7) P.D. Reshetov and A.S. Khokhlov, *Antibiotiki*, **9**, 197 (1964).
8) A.S. Khokhlov and P.D. Reshetlov, *J. Chromatog.*, **14**, 495 (1964).
9) O.I. Voronina, K.I. Shutova, I.I. Tovarova and A.S. Khokhlov, *Antibiotiki*, **14**, 1063 (1969).
10) M.I. Horowitz and C.P. Schaffner, *Anal. Chem.*, **30**, 1616 (1958).
11) D.H. Peterson and L.H. Reinecke, *J. Am. Chem. Soc.*, **72**, 3598 (1950).

ted. Also our procedures enabled the application of much larger amounts of sample to the column. In CMC column chromatography, good separation of the components was dependent on factors such as the amount of sample embedded in the resin (in relation to the diameter and the length of the column) and the concentration of pyridine in the elution solvent. Separation was reproducible in a number of experiments. It is possible to point out some defects in the method. For example, too much volume in the eluted solution required a prolonged time in the succeeding concentration procedure which caused concurrent decomposition of the active component.

The use of carbon is known to allow the application of relatively large amounts of sample in a batch with respect to the amount of adsorbent, as well as ease in handling. Concomitant decoloration with separation and good reproducibility in experiments were also stressed. Yet, clear-cut separation of the above antibiotics by using an activated carbon column has not been previously successfully accomplished. In procedures using active carbon, strong absorption of antibiotics to the carrier was observed. To reduce it, the carbon was pre-treated in distilled water containing a small volume of methanol. However, a small amount of RM-C and B was observed in the rinse with 50% methanol following an overall elution. With no pre-treatment with aqueous methanol, about a 5% reduction in the total recovery was observed, although the recovery for RM-A was quantitative throughout the experiments.

It is known that the RM components can be molecular-sieved in dextrangel column by the number of β-lysine residues in their molecules.[12] The application of this method gave a good results.[13] Sephadex columns, because of their mildness in elution conditions, caused only neglibible decomposition of the RM components.

The individual applications of the three methods disclosed the relative amounts of components RM-A, B, and C, and the presence of a new minor component D. Their application suggested a manner for the characterization of other streptothricin group antibiotics,[14] for example, the determination of molecular weight to Sephadex column, and the adsorption rate on the carbon surface.

Physico-Chemical Properties of Racemomycins

RM components are water-soluble, basic antibiotics and are unstable in acidic or especially in basic solutions. They are white plate but very hygroscopic. Melting points are indefinite with decomposition above 210°.[15] Their salts and free bases are insoluble in com-

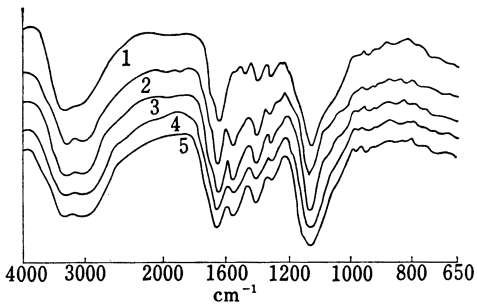

Fig. 1. Infrared Spectrum of Racemomycins Sulfate and Streptothricin Sulfate in KBr

1: RM-A 2: RM-C 3: RM-B
4: RM-D 5: streptothricin

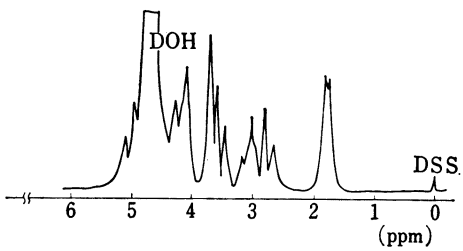

Fig. 2. NMR Spectrum of Racemomycin A Sulfate in D_2O

12) P.D. Reshetov, Z.A. Jegorov and A.S. Khokhlov, *Khim. Prirodn. Soedin. Akad. Nauk. Uz. SSR.*, **2**, 117 (1965).
13) H. Taniyama, Y. Sawasa and T. Kitagawa, *J. Chromatog.*, **56**, 360 (1971).
14) It will be published later.
15) Melting points are untrustworthy in their characterization, and therfore, was not corrected.

mon organic solvents. They give positive ninhydrin, Elson–Morgan, Pauly, Fehling and biuret reactions. The ultraviolet (UV) spectrum of components in water show end absorption. The infrared (IR) spectrum of the sulfates are very similar as shown in Fig. 1. The nuclear magnetic resonance (NMR) spectrum[16] of the RM-A sulfate was shown in Fig. 2. Similar absorption profiles of NMR spectrum were obtained for other components except for the increase of protons on β-lysine residues.

The specific rotations[17] for the sulfates were compared as shown in Table I. The empirical formula by elemental analysis for the components are summarized in Table II.

Table I. The Specific Rotations of Racemomycins and Streptothricin

A ntibiotics (sulfate)	$[\alpha]_D$	Temperature (°C)
RM-A	−53	20
RM-C	−35	17
RM-B	−26.5	17
RM–D	−17	20
Streptothricin	−51	25

Table II. Elemental Analysis of Racemomycin Components

Antibiotics	Formula	Mol. wt		Analysis (%) C	H	N	S	Cl	H_2O
RM-A sulfate	$C_{19}H_{34}O_8N_8\cdot 3/2H_2SO_4\cdot 3H_2O$	703	Calcd:	32.43	6.12	15.93	6.83		7.68
		630 (titration)	Found:	32.65	6.15	16.21	7.02		7.00
RM-A hydrochloride	$C_{19}H_{34}O_8N_8\cdot 3HCl\cdot H_2O$	629.5	Calcd:	36.22	6.20	17.79		16.92	2.85
		603 (titration)	Found:	36.07	5.95	17.51		17.05	2.60
RM-C sulfate	$C_{25}H_{46}O_9N_{10}\cdot 2H_2SO_4\cdot 4H_2O$	898	Calcd:	33.41	6.46	15.59	7.13		8.62
			Found:	33.56	6.34	15.87	7.26		7.90
RM-C hydrochloride	$C_{25}H_{46}O_9N_{10}\cdot 4HCl\cdot 2H_2O$	812	Calcd:	36.95	6.40	17.24		17.49	4.43
			Found:	37.18	6.66	16.98		17.77	4.0
RM-B sulfate	$C_{31}H_{58}O_{10}N_{12}\cdot 5/2H_2SO_4\cdot 5H_2O$	1093	Calcd:	34.03	6.68	15.37	7.32		8.23
			Found:	34.32	6.38	15.11	7.11		8.0
RM-B hydrochloride	$C_{31}H_{58}O_{10}N_{12}\cdot 5HCl\cdot 3H_2O$	994.5	Calcd:	37.41	6.94	16.89		17.84	5.43
			Found:	37.57	7.01	16.62		17.91	4.9

Complete hydrolysates of the components with hydrochloric acid were submitted to an automatic amino acid analyser[18] with a column of strongly acidic ion exchange resin. The results of such analysis on the streptothricin group antibiotics had been reported by Khokhlov, *et al.*[19] Instead of 0.7 N sodium citrate buffer used in their studies, 0.35 N buffer was adopted here. They reported that the peak appearing next to the ammonia peak was that of streptolidine (roseonine).[20] However, in our study, that peak was likely due to an unknown substance, and the one which they attributed to anhydro-D-gulosamine appeared to be that for streptolidine. These observations were also applied in the characterization of a new streptothricin-like antibiotic E-749-C.[21] The chromatographic results are shown

16) NMR spectrum were recorded at 60 MHz with a Hitachi-Perkin H-60 spectrometer.
17) Specific rotations were measured by a Yanagimoto Direct Recording Polarimeter Model OR-20 (C, 1 cell length: 1 cm, H_2O).
18) Hitachi KLA-III type analyser was used.
19) C.A. Egorov, P.D. Reshetov and A.S. Khokhlov, *J. Chromatog.*, **19**, 214 (1965).
20) H.E. Carter, C.C. Sweeley, E.E. Daniels, J.E. McNary, C.P. Schaffner, C.A. West, E.E. van Tamelen, J.R. Dyer and H.A. Whaley, *J. Am. Chem. Soc.*, **83**, 4296 (1961).
21) J. Shoji, S. Kozuki, M. Ebata and H. Otuka, *J. Antibiotics*, **21**, 509 (1968).

in Fig. 3. Table III summarizes the calculated relative intensity of the peaks in reference to streptolidine (peak 2) for individual components. It should be noted the relative increase in quantity of β-lysine (peak 3) for RM-C, B, and D, compared with RM-A. To confirm the accuracy of the measurements, the mixtures of streptolidine and β-lysine in the molar ratios of 1:1 to 1:3 were supplied to the analyser after hydrolysis with acid and results were in good agreement with the above. As a whole, it was found that the presence of 1, 2, 3, and 4 β-lysine residues were associated with RM-A,C,B, and D components respectively.

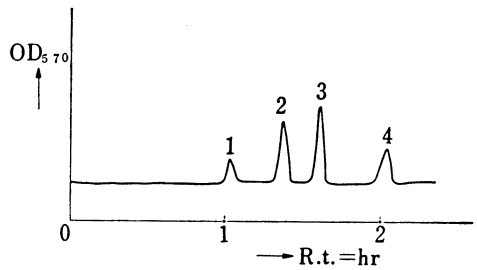

Fig. 3. Amino Acid Anialysis of Racemomycin Hydrolysates

1: amino sugar moiety R.t. 1.06′
2: streptolidine 1.24′
3: β-lysine 1.46′
4: ammonia 2.02′

Table III. Relative Intensity of the Peaks in Fig. 3

Antibiotics	Peak No.			
	1	2	3	4
RM-A	0.082	1.00	1.13	0.46
RM-C	0.044	1.00	1.53	0.40
RM-B	0.098	1.00	1.85	0.65
RM-D	—	1.00	2.35	0.55
Streptothricin	0.067	1.00	1.20	0.47

In these RM components, RM-A was identified with streptothricin,[22] purified by Sephadex LH-20 column, in amino acid analysis and in specific rotation. The electrometric titrations in water indicated pK_a′ values of 7.33, 8.45, and 10.00 for streptothricin,[23] whereas those for RM-A were 7.20, 8.50, and 10.20 respectively. From the viewpoints such as the IR spectrum, and NMR spectrum, RM-A was identical to streptothricin.[23] Therefore, these results permitted proposal of structure, presented by van Tamelen,[23] for RM-A and other RM components was differentiated by the number of β-lysine residues based on the structure. In our structural studies for RM-A,[24] a few obscure parts on structural feature need further elucidations. It will be presented later with respect to the bond-mode of the components.

Antimicrobial, Antiviral Activities of Racemomycins

RM components purified above were found active against Gram positive and Gram negative bacteria as shown in Table IV. The antimicrobial activity sometimes were reduced to one fifth to one tenth of the original, although no decomposition was observed by paper chromatographic examinations. To avoid such deviations, samples were maintained in complete dryness.

Germanova, *et al.*[25] reported the inhibitory effects of nine streptothricins on the development of influenza viruses and bacteriophages. Having reported[6] the strong toxicities of the

22) Prepared from the cultured broth in 1948 by the Upjohn Co. (USA).
23) It was reported the pK_a′ values of 7.1, 8,2 and 10.1 by E.E. van Tamelen, J.R. Dyer, H.A. Whaley and H.E. Carter, *J. Am. Chem. Soc.*, **83**, 4295 (1961).
24) H. Taniyama and F. Miyoshi, *Chem. Pharm. Bull.* (Tokyo), **10**, 156 (1962).
25) K.I. Germanova, T.Y. Goncharovskaya, I.D. Delova, S.A. Ilunskaya, A.A. Melnikova, T.P. Oreshnikova, P.D. Reshetov, S.D. Rudaya, Z.T. Sinitsina, N.K. Solovieva and A.S. Khokhlov, *Antibiotiki*, **10**, 117 (1965).

racemomycins to mice if administered repeatedly, we did not performed the *in vivo* type experiments using RM components. Instead, the shaking culture method by Shimizu[26] was applied. The results were summarized in Table V. The inhibition concentrations against the influenza PR-8 strain was 30 μg/ml with RM-A and C and 10 μg/ml with RM-B. Possibly, as in the case of antimicrobial activities, there was some correlation between the antiviral activity and the number of β-lysine residues in the RM components.

TABLE IV. Antimicrobial Spectrum of Racemomycins (Sulfate)

Test organisms	A	C	B	D
Staphylococcus aureus TERAJIMA	1	0.3	0.3	—
Staphylococcus album	25	12.5	1.56	—
Staphylococcus epidermidis	10	1	1	—
Diplococcus pneumoniae Type I	0.78	25	6.25	—
Staphylococcus SMST-R	100	100	100	—
Bacillus subtilis PCI-219	3.12	3.12	0.39	—
Bacillus subtillis ATCC-6633	3.12	1.56	0.39	0.39
Bacillus anthrasis No. 119	100	25	12.5	—
Salmonella typhi O-901-W	3.12	6.25	1.56	—
Escherichia coli K-12	3	3	3	3
Aerobacter aerogenes	100	12.5	3.12	—
Pseudomonas aeruginosa IAM-1007	3.12	6.25	1.56	—
Pseudomonas aeruginosa THUCHIJIMA	100	100	100	—
Sarcina lutea	1.56	1.56	<0.19	—
Mycobacterium tuberculosis $H_{37}Rv$	50—10	50	25	—
Mycobacterium phlei	1	1	1	—
Mycobacterium avium	1	1	1	—
Shigella sonnei	10	10	10	—
Proteus vulgalis	100	30	100	—
Aspergillus fumigatus	>30	>30	>30	—
Aspergillus ferreus	>30	>30	>30	—
Nocarbia asteroides	>30	>30	>30	—
Cryptococuccus neoformans	1	0.1	0.03	—
Microsporum gypseum	30	30	10	—
Trichophyton interdigitale	3	10	10	—
Trichophyton asteroides	>50	>50	>50	—
Fpidermophyton floccosum	10	3	3	—
Sporotrichum schenchii	3	1	3	—
Candida albicans ATCC-10257	3	0.3	0.1	—
Trichophyton mentagrophytes	3	10	3	—
Trichomonas vaginalis 4F	100	100	100	—

TABLE V. Antiviral Activities of Racemomycins

Antibiotics (sulfate)	Items	Concentration (μg/ml)					
		300	100	30	10	3	control
RM-A	HA titer[a]-$\log_2$	<1.0	<1.0	3.5	6.0	6.3	6.5
	cytotoxicity	±	±	—	—	—	—
RM-C	HA titer[a]-$\log_2$	<1.0	<1.0	<1.0	5.0	5.5	7.0
	cytotoxicity	—	—	±	—	—	—
RM-B	HA titer$\log_2$	1.0	1.0	1.0	2.0	5.5	7.0
	cytotoxicity	—	±	—	—	—	—

a) hemagglutinin titer

26) M. Shimizu, K. Fujimoto, S. Ohse, Y. Yoshimura and J. Uno, *Chemotherapy*, **12**, 84 (1964).

Inhibitory concentration of RM-A to phage MS-2 resulted that 1 μg/ml did not significantly affect the phage development, while 3 μg/ml blocked the host bacterial growth to give up the following studies.

Toxicity of Racemomycins

RM complex, like most streptothricins, exhibited both acute and delayed toxicities. Purified RM components were expected to be toxic, and were examined by intravenous injection to mice. The results are shown in Table VI.

Table VI. Toxicity of Racemomycins

Antibiotics (sulfate)	LD_{50} (mg/kg)
RM-A	300
RM-C	26
RM-B	below 10
Crude-RM	12

Increase antimicrobial activity and also stronger toxicity of RM components were correlated to the number of β-lysine residues in them, or in other words, to the proportionally enhanced basicity of β-lysine peptide in the molecule. Although, they may reach a maximum at a certain number of β-lysine residues in a molecule, that awaits further study.

Experimental

Paper chromatography was carried out by paper (Toyo-Roshi No. 51 UH) with a solvent system of *n*-BuOH: pyridine: HOAc: H_2O: *t*-BuOH (15:10:3:12:4). Because of the poor reproducibility in *Rf* values for the components in our experiments, RM-A was always included as a reference standard. The purity of these components were also established bioautographically on agar layered plates using *B. subtilis* as a bacterial indicator in addition to ninhydrin detection. The eluted fractions containing pure components were pooled and concentrated *in vacuo* at a temperature below 50° and then lyophilized.

Isolation by CMC Column Chromatography——An example of the chromatographic results are presented in Table VII.

Table VII. Isolation by CMC Column Chromatography

Pyridine (M)	Total volume of buffer (L)	Ninhydrin (+) Fr. No.	Identity by P.P.C.	Yield (HOAc salt) (%)
0.05	3.0	—	—	—
0.10	6.5	250— 275	by-products	14
0.20	6.0	640— 880	RM-A	39
0.30	6.0	925—1170	RM-C	14
0.40	8.0	1225—1555	RM-B	26
0.50	7.0	1627—1922	RM-D	7

Column size, 8×20 cm. Adsorbent, H-form of CMC (Brown Co.) buffered overnight with 0.05M pyridine acetate buffer (pH 5.0). Potency, 0.77 meq/g. Amount of antibiotic mixture, 5.0 g as sulfate in 3.0 liter buffer. Elution rate, 3—4 ml/min. Temp., toom remp. Fraction volume, 20 g. Eluent, started with 0.05M pyridine acetate buffer (pH 5.0), followed by stepwise increase in the pyridine concentration.

Isolation by Carbon Chromatography——Column size, 6×22 cm. Adsorbent, activated carbon (Shirasagi-brand). Amount of antibiotic mixture, 10 g, as sulfate in 50 ml of H_2O. Fraction volume, 20 g. Eluent, 5% methanol–water. Elution rate, 20 ml/min. In a chromatographic condition, inorganic salts and metabolic by-products were eluted in the fractions 21—50, RM-A in 151—210, RM-C in 221—250, RM-B in 244—320 and RM-D in 310—400. All the components were recovered as sulfates in yields of 28% for RM-A. 11% for RM-C and 25% for RM-B and D together.

Isolation by Dextran-gel Filtration——An example of a successful application of Sephadex chromatography to the isolation of RM components is following. Column size, 2×300 cm. Adsorbent, Sephadex LH-20 (Pharmacia Co.). Amount of antibiotic mixture, 2.0 g as sulfate in 4 ml of H_2O. Fraction volume, 8 g. Eluent, 10% methanol–water. All the components were obtained as sulfates in yields of 30% for RM-A (Fr. 58—60), 13% for RM-C (Fr. 55—56), 24% for RM-B (Fr. 52—54) and 7% for RM-D (Fr. 50—51) respectively.

Purification of Streptothricin——Streptothricin sulfate,[22] 500 mg, $[\alpha]_D^{25}=-33°$, was submitted to a Sephadex LH-20 column (2×300 cm), and eluted with 10% methanol solution. Intact streptothricin (*Rf*= 0.32) was enriched in the later fractions, there the first fractions obtained a compound with *Rf* 0.19 which was bioautographically inactive to *B. subtilis* and gave a brownish color development with ninhydrin reagent and was estimated as a partial hydrolysate[23] of streptothricin. The same spot of *Rf* 0.19 was also observed in case of RM-A acid[6] converted from RM-A by treatment with diluted mineral acid. Some mixed fractions with both compounds were rechromatographed. By lyophylization, pure streptothricin sulfate was obtained in yield of 65%. Overall recovery was near 95%.

Elemental Analysis——For analysis, RM components were dried with P_2O_5 under a reduced pressure of 3 mmHg for more than 12 hr at room temp. Moisture combined was calculated from the loss of the weight by further drying the samples at 60—100° for 24 hr under reduced pressure. Enough pure sample for analysis was not obtained for the minor component D.

Hydrolysis of Racemomycins——Pure antibiotic (1 mg) was dissolved in 2 ml of 6N HCl, placed and sealed in a tube and heated at 110—120° for 24 hr. The hydrolysate was evapolated to dryness to remove excessive acid, and then was redissolved in 1 ml of water. One-half portion was used for amino acid analysis, the remaining portion for paper chromatography. In this hydrolysis condition, most of amino sugar moieties were decomposed.

Amino Acid Analysis——One-half ml of the hydrolysate was applied to the automatic amino acid analyser. The condition of operation; Column: 0.9×20 cm, Resin: Amberlite CG-120 type III, Buffer: 0.35N sodium citrate (pH 5.28), Column temp.: 50°.

Retention time (R.t) was expressed as hour and minutes.

Paper Chromatography of RM Hydrolysates——The hydrolysates were spotted on circular paper, and developed with the solvent above described. *Rf* values; streptolidine: 0.30 (positive Pauly test, brownish color response to ninhydrin reagent), β-lysine: 0.36, amino sugar moiety: 0.51 (Elson–Morgan test).

Separation of RM Hydrolysates——RM complex (5.0 g, purity 70%) were hydrolysed with 6N HCl for 12 hr at 120° in a sealed tube. For higher yields of the amino sugar component, milder conditions with 3N HCl, and 5 hr at 100°, were applied. The hydrolysate was adsorbed on Amberlite CG–120 (H form, column size: 2.7×13 cm) previously equilibrated with a 0.2 M HOAc–pyridine (pH 4.70) buffer. Elution was carried out at a rate of 3 ml/min with the same buffer except that the concentration of acetic acid was increased stepwisely. D-Gulosamine was eluted at the 0.2M acetic acid concentration, streptolidine at 0.3M and β-lysine at 0.4M. All are very hygroscopic. D-Gulosamine HCl: white powder, mp above 152° (decomp.), $[\alpha]_D^{20}=+6°$ (5 min)→+3° (4 hr). Streptolidine HCl: white plate, mp above 152° (decomp.), $[\alpha]_D^{25}=+47°$. β-Lysine HCl: colorless crystalline, mp 153—155° (decomp.), $[\alpha]_D^{20}=+19°$.

Formation of Streptolidine and β-Lysine Picrate——Streptolidine and β-lysine acetate salts isolated by the above procedures, were dissolved in water, to which methanol solution saturated with picric acid was added respectively. After a few days, the yellow precipitate of the picrates was filtered off, and recrystalized in water–methanol. Streptolidine picrate: mp above 245° (decomp.), $E_{1cm}^{1\%}=540$ (λ_{max} 360 mμ, H_2O). *Anal.* Calcd. for $C_6H_{12}O_3N_4\cdot 2C_6H_3O_7N_3$: C, 33.43; H, 2.78; N, 21.67%. Found: C, 33.37; H, 2.93; N, 21.89%. β-Lysine picrate: mp above 204° (decomp.), $E_{1cm}^{1\%}=556$ (λ_{max} 360 mμ, H_2O). *Anal.* Calcd. for $C_6H_{14}O_2N_2\cdot 2C_6H_3O_7N_3$: C, 35.77; H, 3.34; N, 18.54%. Found: C, 35.97; H, 3.31; N, 18.50%.

Tests on Decomposition of Amino Acids——To test the extent of decomposition of amino acids in the hydrolysis condition of antibiotics previously employed, 1:1 (by mol ratio) to 1:3 mixture of streptolidine and β-lysine picrate was subjected to hydrolysis with 6N HCl at 120°. After 0.5, 1,2,3,6, and 12 hr, samples were withdrawn and analysed for residual amino acids and newborned ammonia. No measureable decomposition of amino acids was observed. These compounds were shown stable to such drastic acid treatment.

Antimicrobial Activity and Antiviral Activity——MIC was determined by an agar dilution method. Most bacteria were cultured in brain heart infusion broth (Difco) at 37° for 2 to 3 days, except that mycobacteria were cultured in the broth with 1% glucose for 3 weeks. Fungi were grown in Sabouraud's broth (Difco), for about a week at 30°, *Candida* for 2 days at 37°, while *Trichomonas* was cultured in Asami's medium containing 5% calf serum. The results shown in Table IV are the average of 2 to 3 experiments because of their deviation.

Influenza virus PR-8, Egg Infective Dose 50% (EID_{50})=$10^{-8.8}$—$10^{-9.3}$, was used, and incubation was done at 37° for 20 hr under continuous shaking.

Toxicity Measurement——One to two percent solutions of each antibiotic in saline were administered to mice by intravenous injection (male, IRC strain, weighing 20±0.5 g, 6 animals in each test group). The drugs were given by a single injection. After 14 days observation, LD_{50} were calculated according to the Behrens–Kaerber method. Most mice suffered from extreme excitement, projection of the eyes, or choking

by injection shock, although freed from them in 30 minutes. Some mice died of chocking right after the injection.

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