STUDIES ON THE INACTIVATION AND REGENERATION OF STREPTOTHRICIN

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Partial hydrolysis of streptothricin was most readily carried out in dilute acid solution. The hydrolysate had no antimicrobial activity, and its toxicity was six times that of streptothricin. From this inactive substance the original active antibiotic was regenerated with dicyclohexylcarbodiimide.

Inactivation of Streptothricin

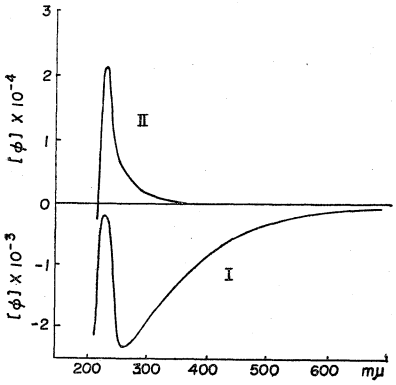
Racemomycin A (RM-A, I)[1,2] is a water-soluble, basic antibiotic which inhibits Gram-positive and negative bacteria, mycobacteria, and some viruses and fungi. It was identified as streptothricin[2] which is unstable and is easily changed to an inactive substance with mineral acids or especially with alkali. Racemomycin A, when dissolved in 1 N hydrochloric acid and allowed to stand over 12 hours at room temperature, produced quantitatively racemomycinic A acid (RM-A acid, II). The derivative, easily detectable from its lower Rf value on paper chromatography, was purified by a column chromatograms on Sephadex LH-20. The physico-chemical properties of II are summarized in Table 1. II was formulated as $C_{19}H_{36}O_9N_8$ from

Table 1. The physico-chemical properties of RM-A (I), and RM-A acid (II)

Items	RM-A(I)·HCl	RM-A acid(II)·HCl
Color test	positive: ninhydrin(purple), PAULY, biuret, ELSON-MORGAN	positive: ninhydrin(brownish), PAULY, biuret, ELSON-MORGAN
m.p.	above 220°C (decomp.)	above 235°C (decomp.)
P.P.C.: (Rf value)	0.33	0.20
P.E.: (Rf value)	1.00	0.87
Anal.	$C_{19}H_{34}O_8N_8 \cdot 3HCl \cdot H_2O$ (MW=629.5) Calcd.: C 36.22, H 6.20, N 17.79, Cl 16.92 % Found: C 36.07, H 5.95, N 17.51, Cl 17.05 %	$C_{19}H_{36}O_9N_8 \cdot 4HCl \cdot 2H_2O$ (MW=702) Calcd.: C 32.47, H 6.26, N 15.95, Cl 20.22 % Found: C 32.73, H 6.35, N 15.54, Cl 21.27 %
pKa′ values	7.20, 8.50, 10.20 (MW=603)	2.70, 7.0, 8.50, 9.65, 10.15 (MW=656)
VAN-SLYKE amine (30 min.)	2.07 mol	2.98 mol
$[\alpha]_D^{20}$	−50° (*c* 1, H_2O)	+4.7° (*c* 2, H_2O)
$IR_{\nu c=0}$	1710 cm^{-1} due to a carbamoyl group	1720 cm^{-1} due to a carboxylic acid and 1710 cm^{-1} due to a carbamoyl group
Toxicity in mouse	LD_{50}: 300 mg/kg (i.v.)	LD_{50}: below 50 mg/kg (i.v.)
Amino acid analysis	streptolidine, β-lysine, ammonia, aminosugar (1.00: 1.13: 0.46: 0.082)	streptolidine, β-lysine, ammonia, aminosugar (1.00: 1.21: 0.38: 0.091)

P.E. : Paper electrophoresis. P.P.C. : Paper chromatography.

Fig. 1. The ORD curves of RM-A (I)·HCl and RM-A acid (II)·HCl



elemental analysis and a molecular weight determination by titration. It was optically inactive at wavelengths above 400 mμ, but the ORD curve in water was different from that of I over the whole wavelength range as shown in Fig. 1.

Table 2. Antimicrobial activity of RM-A and its derivatives.
Sample: RM-A·HCl (I), RM-A acid·HCl (II), the regenerated substance·HCl (III).

Test organisms	Sample, MIC (mcg/ml)		
	I	II	III
Bacillus subtilis PCI-219	3.12	>2,000	3~1.5
Micrococcus luteus ATCC-398	3	2,000~1,500	3
Staphylococcus aureus FDA 209P	25	>2,000	25
Escherichia coli NIHJ	12.5	>2,000	12
Proteus vulgaris OX-19	25~12.5	>2,000	25
Serratia marcescens IAM-1223	70~50	>2,000	50

Table 3. Toxicity of RM-A acid (II)·HCl

mg/kg	Died/Tested							
	0	1 day	2 days	3 days	4 days	5 days	6 days	7 days
25	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
50	3/5	3/5	3/5	3/5	3/5	3/5	3/5	3/5
100	5/5	5/5	5/5	3/5	5/5	5/5	5/5	5/5

II was active against some bacteria at a concentration of over 2,000 mcg/mg as shown in Table 2. Toxicity tests showed that injection of 50 mg/kg was lethal for 3 out of 5 mice in a few minutes. Therefore, the acute toxicity of the compound appears to have increase approximately six-fold over that of I. Observations for a week showed no delayed toxicity (Table 3).

Regeneration of the Active Compound

II was treated with dicyclohexylcarbodiimide (DCC)[8] in a water-pyridine-dioxane mixture for 2 hours at 48°C with stirring. Paper chromatography of the reactant gave the result shown in Plate 1. The new zone at Rf 0.33 (middle spot in the reaction mixture) exhibited inhibitory activity against *Staphylococcus aureus* by bioautography, but the highest spot, due to a dicyclohecylurea derivative, had no inhibitory activity. The substances were separated by cellulose column chromatography, the active substance was further purified on a Sephadex G-10 column and then converted to its hydrochloride. It was identified as racemomycin A hydrochloride

Plate 1. Paper chromatography of the reaction mixture obtained by treating racemomycin-A acid with dicyclohexylcarbodiimide.

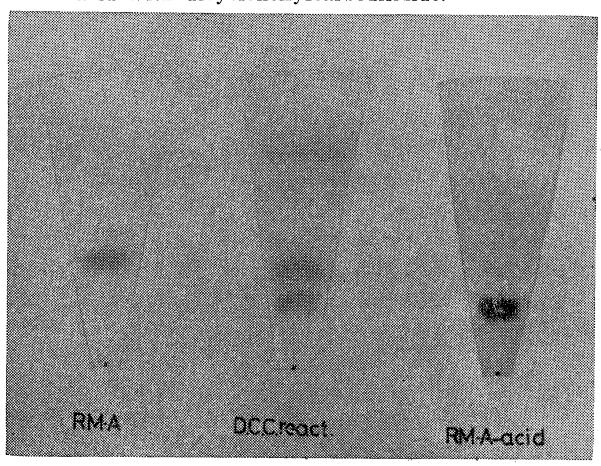


Fig. 2. IR spectrum of RM-A·HCl (A), RM-A acid ·HCl (B), and regenerated substance·HCl (C) in KBr.

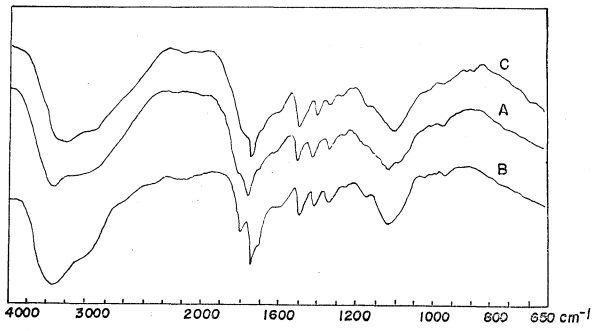
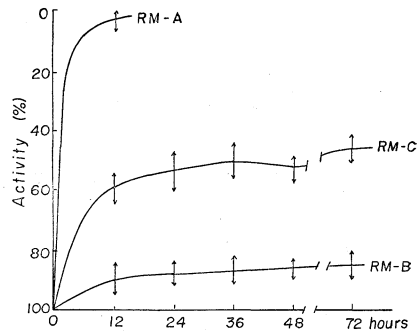


Fig. 3. Decomposition rate of racemomycin components (*E. coli*).



from its physico-chemical properties and the results of amino acid analysis. The antimicrobial activity of the regenerated substance is shown in Table 2.

Discussion

It is clear that the substance regenerated by DCC has the same structure as the original racemomycin A. The specific recombination of a carboxyl group with one of the three primary amino groups is of interest since it bears on the conformation of the active state of streptothricin. The ease with which the streptothricin group of antibiotics are partially hydrolyzed in dilute acid and lose their antimicrobial activity has been reported by an American group[4] as well as ourselves[1], but the implications have not been discussed in detail.

We have also obtained the similar reversible results to the structural study of an antibiotic SF-701[5,6], which belongs to the streptothricin group and involves one mole of sarcosine. This could be explained by the structure presented by VAN TAMELEN *et al.*[4] In the structure proposed for streptolidine by CARTER *et al.*[7], 4-(1-hydroxy-2-aminoethyl)-2-aminoimidazoline-5-carboxylic acid, the lactam ring, which is under a strong strain due to a *trans* configuration[8] at positions 4 and 5 would easily be converted to the more stable, open-chain form, however, the six-membered lactam ring could be specifically reformed by DCC in yields of about 60 %.

The fact that the streptothricins such as racemomycins C and B[2] have two and three β-lysine moieties in the molecules suggests that opening the lactam ring is made more difficult by increased basicity due to amino groups on the β-lysine moieties under the same conditions (Fig. 3).

Experimental

pKa′ values were obtained by titration with alkali. Amino acids in acid hydrolysates were analysed[2] with a Hitachi KLA-3 type apparatus using a 0.9×20 cm column and 0.35 M sodium citrate buffer (pH 5.28). Antimicrobial tests were carried out by the agar dilution streak method. Microorganisms such as *Bacillus subtilis*, *Micrococcus luteus* and *Serratia marcescens* were incubated at 27°C. A solution of the antibiotic was administered to mice (female, DDN strain, weighing 18~20 g, 5 animals in each group) by intravenous injection. Some mice died from choking immediately after the injection. Rf values by paper chromatography were obtained on Toyo Roshi No. 51 UH paper with the solvent system *n*-butanol - pyridine - acetic acid - water - *tert.*-butanol (15 : 10 : 3 : 12 : 4). Paper electrophoresis was carried out with a Toyo-C type (500 V, 3~5 mA) instrument using Toyo Roshi No. 51 paper for 3~5 hours and a solvent system of pyridine - acetic acid - water (5 : 0.2 : 95, pH 6.5).

ORD curves of RM-A and its preparations

A Jasco ORD/UV-5 spectrophotometer was used in D_2O. The results were as follows:

	Cotton effect 1 st extremum (mμ)	Back ground rotation
RM-A (I)	226~228 (200°) 0.1 %, Cell length: 10, 1 mm	negative
RM-A acid (II)	222~223 (22560°) 0.1 %, 10, 1 mm	positive

Inactivation of RM-A

RM-A hydrochloride was dissolved in ten volumes of 3 N hydrochloric acid and the solution was evaporated to dryness after a few hours. The conversion of RM-A to RM-A acid was monitored by paper chromatography. Zones were detected by ninhydrin and by assay on agar seeded with *E. coli*. The product, an amorphous, white powder, showed an MIC value of 1,500 mcg/ml against *Staphylococcus albus*.

Inactivation of RM-C and RM-B

RM-C and RM-B were dissolved in ten volumes of 3 N hydrochloric acid at room temperature, and the activity remaining at the end of each hour was monitored by an agar plate assay with *E. coli*.

Regeneration of I from II

To a solution of 330 mg (5×10^{-4} mole) of RM-A acid hydrochloride in 10 ml of water - pyridine - dioxane (7 : 3 : 3), 100 mg (*ca.* 5×10^{-4} mole) of DCC in 1.5 ml of dioxane was added with stirring at room temperature. After warming at 48°C for 2 hours with stirring, the reaction mixture was diluted with 20 ml of water, and dicyclohexylurea liberated removed by filtration. The filtrate was concentrated to dryness. The residue showed activities of 100 mcg/ml against only *D. pneumoniae* Type I.

Isolation and purification of the regenerated substance

The crude reaction product (300 mg) was purified on a cellulose column (2×40 cm) using the solvent system mentioned above; 5 g of the eluate was collected in each tube. An active substance was eluted in fractions 20~26. The active solution was washed with ether, and the water layer was concentrated to 1 ml. The concentrated solution was applied to a column of Sephadex G-10 (2×150 cm), eluted with water, and active fraction was lyophilized to a white powder which was found to be pure by paper chromatography (detection by ninhydrin). The powder was converted to the hydrochloride with 0.3 N HCl and had an Rf of 0.33. Bioautography against *S. aureus* gave Rf 0.32. Paper electrophoresis: Mobility, 1.00~RM-A; yield, 180 mg; purity, *ca.* 85 % by bioassay; $[\alpha]_D^{20}$ −43° (*c* 1, H_2O); m.p. above 220°C (decomp.); IR spectrum, lack of absorption at 1,720 cm^{-1} due to carboxylic acid; amino acid analysis of acid hydrolysate; streptolidine - β-lysine - ammonia - amino sugar (1.00 : 1.23 : 0.88 : 0.005).

RM-A acid (II) did not form a reineckate salt and not adsorbed on active carbon. But I and the regenerated active substance (III) formed a product obtained as a rose-colored, insoluble powder from cool water. Their reineckates decolored at 145°C, and decomposed at 260°C. When the hydrochloride of the regenerated substance was also dissolved in 1 N HCl and allowed to stand for 12 hours at room temperature, 60~70 % of original activity against *E. coli* was lost, and Rf values decreased to 0.20, corresponding to RM-A acid (II). From the later fractions eluted from a cellulose column, RM-A acid (II) was obtained.

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