THE REVISED STRUCTURE OF CAPREOMYCIN

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The total structures of capreomycins IA and IB were determined mainly from the results of the comparative study on the NMR spectra of capreomycins and tuberactinomycins, resulting in a revision of the formerly proposed structure. The β-lysine residue as a branched part was revealed to be linked with the β-amino group of an α, β-diaminopropionic acid residue in the cyclic pentapeptide moiety in a different manner than in tuberactinomycins.

The antibacterial peptide, capreomycin, was first isolated by HERR *et al.* from *Streptomyces capreolus* in 1960[1]. The antibiotic in practical use as an antituberculous agent was reported to be composed of four components IA, IB, IIA and IIB*. Capreomycin has been known to have similar chemical and biological characteristics to those of viomycin or tuberactinomycins, whose chemical structures had been determined unequivocally in our laboratory[2,3]. The structure of capreomycin had been proposed by BYCROFT *et al.*[4] in 1971, presumably on the basis of analogy to the tentative structure for viomycin[5]. However, this proposal has become questionable with respect to the amino acid sequence, since the formula of viomycin itself has been revised from that time due to the results of our investigations on tuberactinomycins in which viomycin was included as one congener (Fig. 1)[3] and to the X-ray analysis of viomycin by BYCROFT[6]. Hence, we started a reinvestigation to establish the correct structure of capreomycin mainly by comparison of its NMR spectra with those of the tuberactinomycins.

Isolation of Capreomycins IA and IB

In our experiments commercially available capreomycin was used. This proved to be a mixture of capreomycins IA and IB (ratio, 3: 2)**. The separation of the two components was carried out either by preparative thin-layer chromatography on Silica gel with phenol - water - 28% ammonia (30: 10: 1) as a developing solvent or by Amberlite IRC-50 column chromatography (Fig. 2).

Physicochemical Properties

Table 1 shows the physicochemical data of capreomycins IA and IB and also their amino acid compositions, which proved to be the same as reported by earlier workers[1,4].

The Amino Acid Sequence of the Cyclic Moiety

Concerning the amino acid sequence in the cyclic pentapeptide moiety in capreomycin, a detailed

* Capreomycin II lacks β-Lys residue of capreomycin I[4].

** Our estimation of the ratio of capreomycins IA and IB was different from that by BYCROFT's group (1: 2). Such differences may depend on the production lot of the antibiotics. In our sample, no capreomycin II was found in detectable amounts.

Fig. 1. Structures of tuberactinomycins including viomycin

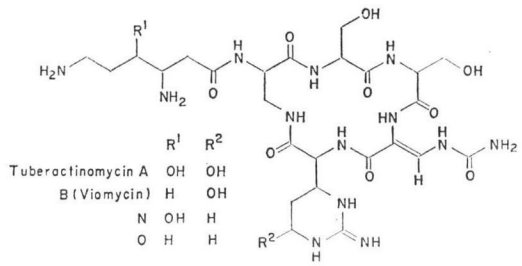
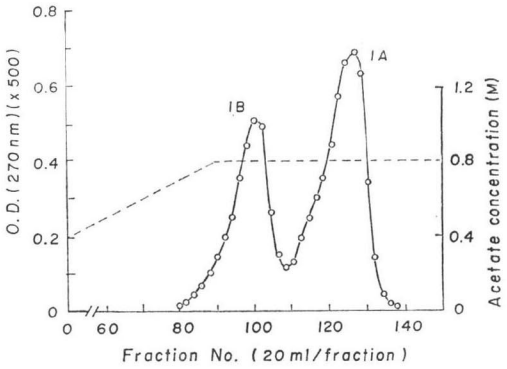


Fig. 2. Elution pattern of capreomycins on Amberlite IRC-50 column chromatography

Buffer: ammonium acetate (pH 9.0).

Column: Amberlite IRC-50 (Type I, 100~200 mesh, NH_4 form), 2.5×70 cm.

Sample: 5.00 g



examination of its NMR spectra in comparison with those of the tuberactinomycins revealed that the structure must be changed to that shown in Fig. 3 as already reported[7]. Therefore, the only remaining problem for an elucidation of the total structure of capreomycin should be determination of the mode of linkage of the branched part, *i.e.*, β-Lys to the cyclic moiety which has two free amino groups at α-position of A_2pr^1 and β-position of A_2pr^3.†

Table 1. Characterization of capreomycins

		Capreomycin IA		Capreomycin IB	
Rf*		0.25		0.29	
m.p. (°C)		246~248 (dec.)		253~255 (dec.)	
$[\alpha]_D^{22}$ (c 0.5, H_2O)		−21.9°		−44.6°	
UV λ_{max} (nm)(ε)	0.1 N HCl	269 (24,000)		268 (22,700)	
	H_2O	268 (23,900)		268 (22,300)	
	0.1 N NaOH	289 (15,900)		290 (14,400)	
		$C_{25}H_{48}N_{14}O_8Cl_4 \cdot \frac{1}{2}EtOH$**		$C_{25}H_{48}N_{14}O_7Cl_4 \cdot \frac{1}{2}EtOH \cdot \frac{1}{2}H_2O$**	
		Found	Calcd.	Found	Calcd.
Elemental analysis (%)	C	37.11	37.28	37.87	37.60
	H	6.11	6.14	6.38	6.31
	N	23.61	23.41	23.76	23.61
	Cl	17.20	16.93	16.73	17.07
Amino acid composition*** (molar ratio)		Ser (1), A_2pr (2), Cpd (1), Uda (1), β-Lys (1)		Ala (1), A_2pr (2), Cpd (1), Uda (1), β-Lys (1)	

* Tlc (Silica gel, phenol - water - 28% ammonia, 30: 10: 1)

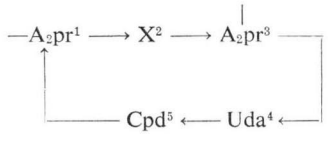
** Presence of ethanol in the sample was shown in NMR spectrum.

*** Ser, Ala, A_2pr and Cpd were identified by amino acid analysis, Uda by NMR and UV spectra. β-Lys showing the same retention time as ammonia in amino acid analysis was identified by NMR spectra. All component amino acids are of L-configurations.

Abbreviations; A_2pr: α, β-diaminopropionic acid, Cpd: capreomycidine, Uda: β-ureidodehydroalanine, β-Lys: β-lysine.

† The numbering of the residue in tuberactinomycin started from the branched amino acid.[7] It should be noted that a numbering of positions of amino acid residues in cyclic part of capreomycin is different from that used in tuberactinomycin family which was considered to be cyclization product of chain-like hexapeptide with all α-peptide bonds.

Fig. 3. The structures of cyclic moieties in capreomycins



	X
Capreomycin IA	Ser
IB	Ala

Fig. 4. The total structures of capreomycins

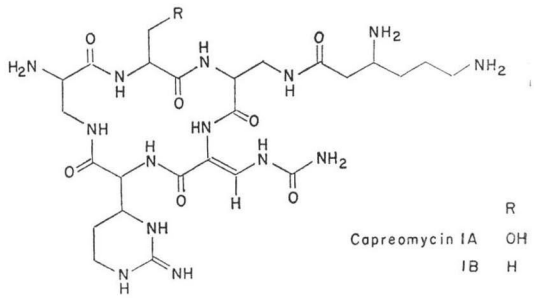


Table 2. Amino acid analysis and paper electrophoresis of hydrolyzate of DNP-capreomycin

	Amino acid analysis* (Retention time, min)		Paper electrophoresis** (Migration distance toward cathode, cm)	
	Capreomycin IA	Capreomycin IB	Capreomycin IA	Capreomycin IB
Hydrolyzate of DNP-capreomycin	29.0(N^{α}-DNP-A_2pr) 31.5(NH_3) 41.5(A_2pr) 57.0(Cpd)	29.0(N^{α}-DNP-A_2pr) 31.5(NH_3) 41.5(A_2pr) 57.0(Cpd)	9.3(N^{α}-DNP-A_2pr) 12.2(Ser) 14.3(Cpd) 15.5(A_2pr)	9.3(N^{α}-DNP-A_2pr) 13.5(Ala) 14.3(Cpd) 15.5(A_2pr)
N^{α}-DNP-A_2pr	29.0		9.3	
N^{β}-DNP-A_2pr	35.0		8.5	

* Column of 9 × 100 mm and 0.35 N sodium citrate buffer were used.
** A buffer of formic acid - acetic acid - water (50: 150: 800) was used at 0.80 mA/cm for 130 min.

The Total Structures of the Capreomycins

In order to elucidate the position of the branched part, capreomycin was 2,4-dinitrophenylated and subsequently hydrolyzed with 6 N HCl. In the hydrolyzate of each of capreomycins IA and IB, N^{α}-dinitrophenyl-α,β-diaminopropionic acid (N^{α}-DNP-A_2pr) was detected by means of paper electrophoresis as well as amino acid analysis (see experimental) in variance with the results of BYCROFT[4]. Therefore, it was concluded that the α-amino group of A_2pr^1 must be free and accordingly, the β-amino group of A_2pr^3 should be linked to the β-Lys residue of the branched part. Although this conclusion was rather surprising especially in comparison with the structure of the tuberactinomycins, reexamination of the NMR spectra in more detail, in addition to the observations already reported[7], supported strongly our assumption.

a) An α-amide resonance of A_2pr^1, which was observed at about δ 8.5 (doublet) in the spectra of tuberactinomycins, disappeared in NMR spectra of capreomycins just as in the case of tuberactinamine N lacking the branched part[8] with a free α-amino group in A_2pr^1.

b) A β-amide proton of A_2pr^3 was observed as a triplet or a broad singlet at about δ 8.2, suggesting the presence of an amide linkage there.

c) An α-methine signal of A_2pr of tuberactinamine N appears at about 0.3 ppm higher field (δ 4.40) than those of tuberactinomycins (about δ 4.70). In the case of capreomycins, the signals were observed at δ 4.3~4.5 in correspondence with that of tuberactinamine N.

Additional evidence for the sequence, H-A_2pr^1-Ser^2- was obtained by EDMAN-dansyl analysis of

reduced capreomycin IA. Thus, capreomycin IA was first hydrogenolyzed in the presence of PtO_2 catalyst in N HCl to give [Ala^4]-capreomycin IA with removal of urea. The cyclic peptide thus obtained was degraded by EDMAN's procedure. The product was dansylated and then hydrolyzed with 6 N HCl. In the hydrolyzate, N-dansylserine was clearly detected by thin-layer chromatography. This indicates that the antibiotic possesses one free α-amino group, presumably of A_2pr^1 residue and an adjacent amino acid could be Ser. The results seems to exclude a possibility of an H-A_2pr^3-Ala^4- sequence, because this would give Ala as the adjacent amino acid.

Consequently, the total structures of capreomycins can be assigned as shown in Fig. 4. This newly proposed formula was recently confirmed by our total syntheses of capreomycins IA and IB[9]. Furthermore, a so-called pseudocapreomycin IB of the tuberactinomycin type where a β-Lys residue is linked to α-amino group of A_2pr^1 and the β-amino group of A_2pr is free, was synthesized in a separate experiment.* This compound was found to be quite different from natural capreomycin IB.

Such structural variation in similar peptide antibiotics, tuberactinomycin and capreomycin, seems to be very interesting in view of their biosyntheses.

Experimental

Melting points were not corrected. The NMR spectra were recorded on a Varian Associates XL-100-15 Spectrometer, and 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) was used as internal reference. The specific rotation was measured with a Perkin-Elmer 141 Polarimeter. UV spectra were measured on a Hitachi 124 Spectrophotometer. Amino acids were analyzed with a Hitachi KLA-5 amino acid analyzer. Thin-layer chromatography was carried out by the ascending method on silica gel G. Paper electrophoresis was carried out on Toyo Roshi No. 51 paper.

Isolation of Capreomycins IA and IB

The commercially available capreomycin (Capastat sulfate, Eli Lilly and Co., Ltd.) (3.00 g) was dissolved in 0.4 M ammonium acetate buffer (pH 9.0) and applied to a column chromatography of Amberlite IRC-50 (Type I, 100~200 mesh, 2.5×75 cm, equilibrated with the above buffer). Elution was carried out gradiently with 900 ml of 0.4 M buffer to 900 ml of 0.8 M buffer (pH 9.0) and then additional 1,200 ml of the latter. Fractions containing each component were separately collected. After neutralization and dilution with water, they were applied to Amberlite IRC-50 column (Type I, 100~200 mesh, pyridinium form, 1.8×20 cm). Each component of capreomycin was eluted with N acetic acid. Fractions with positive ninhydrin reaction were combined, neutralized with pyridine and evaporated *in vacuo*. To a solution of an oily residue thus obtained in a small amount of 3 N HCl, ethanol was added to precipitate capreomycin hydrochloride, yield, capreomycin IA: 1.41 g, capreomycin IB: 0.81 g. Precipitation was repeated from water-methanol-ethanol for analysis. The sample thus obtained was lyophilized from water to remove ethanol for measurement of NMR spectrum.

2,4-Dinitrophenylation of Capreomycin and Detection of DNP-amino Acid in the Acid Hydrolyzate of DNP-capreomycin

To an aqueous solution (10 ml) of capreomycin IA or IB hydrochloride (10 mg), sodium hydrogencarbonate (200 mg) and a solution of 2,4-dinitrofluorobenzene (0.20 ml) in ethanol (20 ml) were added. The mixture was stirred at room temperature for 5 hours in the dark. DNP-capreomycin precipitated was collected by filtration and washed with water, ethanol and then ether successively, yield, DNP-capreomycin IA: 12 mg, DNP-capreomycin IB: 9 mg.

The products were hydrolyzed with constant boiling HCl at 110°C for 10 hours in a degassed sealed tubes. The hydrolyzates were lyophilized, dissolved again in water and extracted with ethyl acetate. The aqueous layer was evaporated *in vacuo* and redissolved in 0.2 M sodium citrate buffer (pH 2.2).

* Results of the synthesis of pseudocapreomycin and another capreomycin analogs will be reported elsewhere.

The solutions were applied to an amino acid analyser and examined by paper electrophoresis (Table 2).

Catalytic Reduction of Capreomycin IA and EDMAN-Dansyl Analysis

Capreomycin IA hydrochloride (50 mg) was hydrogenolyzed in the presence of PtO_2 catalyst (15 mg) in N HCl (10 ml) for 48 hours. After the catalyst was filtered off, the filtrate was concentrated *in vacuo* and ethanol was added to the residue to give a white precipitate, yield 42.9 mg. This product gave a single spot both in thin-layer chromatography (Rf 0.35: phenol - water - 28% ammonia, 30: 10: 1) and in paper electrophoresis (migration distance toward the cathode, 10.7 cm; cf. capreomycin IA, 10.0 cm; 0.7 mA/cm, acetic acid - pyridine - water, 4: 30: 966, 90 min). Amino acid analysis of the acid hydrolyzate (in molar ratio): A_2pr (1.87), Cpd (1.12), Ser (1.00), Ala (0.93), the retention time of β-Lys could not be distinguished from that of ammonia. EDMAN-dansyl analysis was carried out in the usual manner[10,11]. Dansyl Ser was identified by thin-layer chromatography (Rf 0.52, *n*-butanol - acetic acid - water, 4: 1: 2; Rf 0.68, phenol - water - 28% ammonia, 30: 10: 1).

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