CHEMISTRY OF BLEOMYCIN. XVI
EPI-BLEOMYCIN

Sir:

As previously reported[1,2], migration of the carbamoyl group from the 3- to the 2-hydroxyl group of the mannose moiety of bleomycin occurs in aqueous alcoholic solution of copper-free bleomycin in the presence of a weak base such as triethylamine until an equilibrium is established. This carbamoyl-migration product has been called *iso*-bleomycin. Under the same reaction condition, copper-chelated bleomycin does not give *iso*-bleomycin[1], but yields irreversibly another product designated *epi*-bleomycin. In this communication, the formation, isolation, chemical and biological properties and structure of *epi*-bleomycin are reported.

Preliminary experiments showed that epimerization proceeds slower than isomerization. In order to study the reaction process, liquid chromatography, as shown in Fig. 1, was used for the rapid quantitative determination of the reaction product. This analytical method is also useful for determination of the products derived by chemical and biological reactions in general, although minor modifications of the solvent system are necessary in some case.

To 1 ml of a 10% aqueous solution of copper-chelated bleomycin B_2 was added 2 ml of ethanol containing 2% triethylamine. The reaction mixture was divided into two fractions, which were kept at 25°C and 70°C, and analyzed intermittently by liquid chromatography. At both temperatures, the reaction apparently proceeded with first order. Fifty percent conversion was achieved in about 120 hours at 25°C, and in about 1.5 hour at 70°C. At 70°C in 6 hours, more than 90% of bleomycin B_2 was converted to copper-chelated *epi*-bleomycin B_2. Copper-chelated *epi*-bleomycin B_2 was preparatively isolated by CM-Sephadex C-25 column chromatography using 0.15 M sodium acetate-acetic acid buffer at pH 4.5. *Epi*-bleomycin B_2 was eluted faster than the intact bleomycin. Under these conditions *iso*-bleomycin is eluted later than bleomycin.[1] Other *epi*-bleomycins showed similar chromatographic behavior. Thus, *epi*-bleomycins A_2, B_2, A_2'-C, and B_4 were isolated in the pure state.

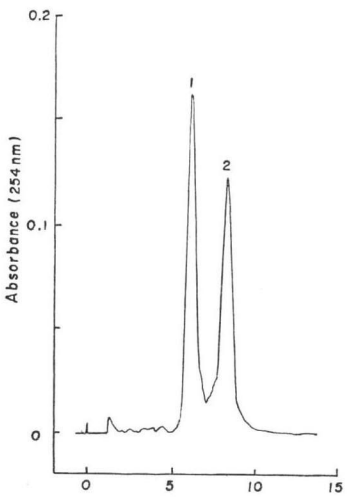
^{13}C-NMR studies of copper-free bleomycin and *epi*-bleomycin, which were obtained by treatment of copper-chelated materials with hydrogen sulfide, showed that they have the same number of carbon atoms. The results of the N- and C-terminal analyses suggested that no bond cleavage occurred during epimerization.

To test whether carbamoyl migration occurred during epimerization as in the case of *iso*-bleomycin or not, copper-free *epi*-bleomycin was methanolized using Amberlyst 15 as an acid catalyst, and the resulting methyl glycosides of the sugar components were analyzed by thin-layer chromatography after O-benzoylation[1,2]. The result indicated that gulose and 3-O-carbamoyl-mannose exist in *epi*-bleomycin molecule, but 2-O-carbamoyl-mannose does not.

Copper-chelated *epi*-bleomycin showed essentially the same UV spectrum as copper-chelated bleomycin. The CD spectra were distinctly different (Fig. 2), which suggested conversion of an asymmetric carbon atom adjacent to a chromophore.

Fig. 1. Separation of bleomycin B_2 and *epi*-bleomycin B_2 by liquid chromatography

Sample:	Reaction mixture (70°C, 1.5 hours) 33 μg (see text)
Instrument:	Perkin-Elmer 1220
Column:	LiChrosorb SI 60 (5 μ)
Dimensions:	150×2.6 mm (ID)
Mobile Phase:	10% $AcONH_4$-H_2O-MeOH-EtOH (5: 19: 60: 116)
Flow Rate:	0.5 ml/min
Temperature:	60°C
Pressure:	600 psi
Detector:	UV (254 nm)



1. Bleomycin B_2. 2. Epi-bleomycin B_2

The amine components obtained by total acid hydrolysis of copper-free *epi*-bleomycin A_2 were analyzed by cellulose thin-layer chromatography, cellulose electrophoresis and in an automatic amino acid analyzer. All amine components (I~VII) present in the total acid hydrolyzate of bleomycin A_2[3] (Table 1) were found in the hydrolyzate of *epi*-bleomycin A_2. The diastereomers of components I, III[4], and IV[5] (Table 1) were not detected by automatic amino acid analysis. This means that the asymmetric carbons of these three amine components kept their configuration intact during epimerization.

As already reported component II (Table 1) is partially racemized during total acid hydrolysis[6]. The racemate of II can be easily resolved by cellulose thin-layer chromatography using 1-propanol - pyridine - acetic acid-water (15: 10: 3: 12)[6]. The thin-layer chromatograms of the hydrolyzates of bleomycin and *epi*-bleomycin were analyzed by a double-wavelength UV densitometry[7], as shown in Fig. 3. The Rf-values of the intact II (S-configuration[8], designated S-II) and its enantiomer (designated R-II) are 0.11 and 0.20, respectively. As shown in Fig. 3, in the hydrolyzate of bleomycin the peak of S-II is higher than that of R-II, while in the case of *epi*-bleomycin the peak heights are reversed. The results indicate that the configuration of the methine carbon of the β-lactam ring of *epi*-bleomycin is converted to R-configuration during epimerization.

A simple method for determination of R- or S-V (Table 1) has not been established. Therefore, V was isolated from the hydrolyzate of *epi*-bleomycin A_2 by ion-exchange column chromatography[3]. The optical rotatory dispersion spectrum indicated that it has S-configuration, although it was partially racemized during acid hydrolysis*.

From the above experimental results, it can be concluded that bleomycin and *epi*-bleomycin are different only in the configuration of the methine carbon of the β-lactam ring. The reason why copper-chelated bleomycin is epimerized in basic aqueous alcoholic solution while copper-free bleomycin is isomerized under the same conditions may be as follows. A nitrogen atom of the pyrimidine chromophore is one of the copper-ligands of bleomycin, as verified by the UV spectrum. Complex formation causes the activation of the adjacent chiral methine, which is, therefore, easily epimerized with base.

We should add a further brief comment on the copper-ligands of the bleomycin molecule. One

* Partial racemization was also observed with bleomycin A_2.

Fig. 2. CD Spectra of copper-chelated *epi*-bleomycin A_2 and copper-chelated bleomycin A_2

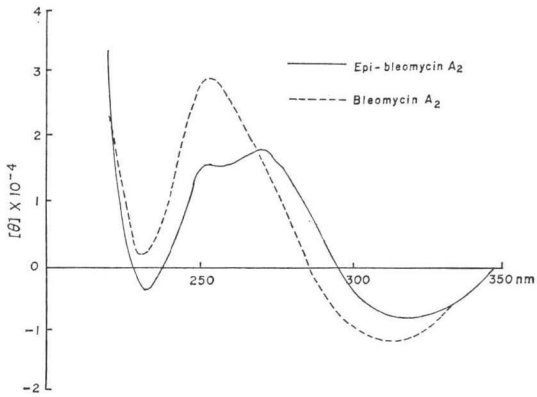
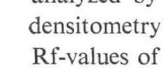
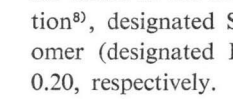
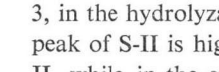
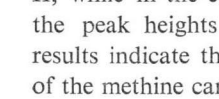
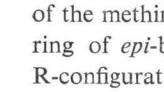
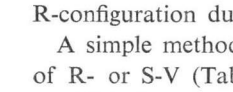
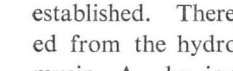


Table 1. Amine components of *epi*-bleomycin A_2 and bleomycin A_2.

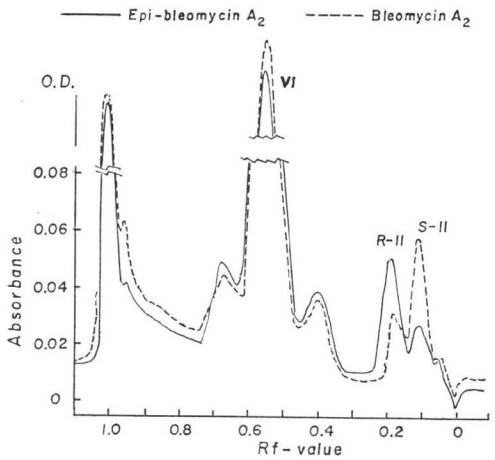
Amine component		Number of asymmetric carbon	Stereochemistry	
			Epi-bleomycin A_2	Bleomycin A_2
I		2	2S, 3R	2S, 3R[8]
II		1	R*	S*[7]
III		3	2S, 3S, 4R	2S, 3S, 4R[10]
IV		2	2S, 3R	2S, 3R[11]
V		1	S*	S*[3]
VI		0		
VII		0		

* Partially racemized during acid hydrolysis

Fig. 3. Densitogram of TLC of hydrolyzates of *epi*-bleomycin A_2 and bleomycin A_2

Precoated Avicel SF, 1-propanol - pyridine - acetic acid - water (15: 10: 3: 12).

Detection: Hitachi 356 double-wavelength spectrophotometer with TLC accessories, $\lambda_1=350$ nm, $\lambda_2=290$ nm.



ligand is the nitrogen atom of the imidazole of IV, as can be deduced from the fact that the pKa value of 4.7 due to the imidazole of copper-free bleomycin (B_2) disappears in the copper-complex and copper-free bleomycin gives positive PAULI reaction but copper-complex does not. Another ligand is the α-amino group of V. The reactivity of this amino function is markedly reduced by copper-chelate formation[9] and the pKa value 7.3 due to this amino group of the copper-free bleomycin disappears in the copper-complex. The other ligand would be the carbamoyl group, as suggested by lack of migration of this group in the copper-complex.

The antimicrobial activity of *epi*-bleomycins was compared with that of the intact bleomycins (Table 2). The antimicrobial activity against *Mycobacterium* 607 of *epi*-bleomycins was about 10~20% that of the original ones. But the anti-*B. subtilis* activity was much less than the anti-*Mycobacterium* activity. Concentrations of *epi*-bleomycin A_2 and B_2 required for fifty percent growth inhibition of HeLa cell (ID_{50}) were 9.8 and 8.8 μg/ml, while the ID_{50}s of the normal compounds were 2.8 and 2.2 μg/ml, respectively.

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Table 2. Antimicrobial activity of *epi*-bleomycins and intact bleomycins.

Bleomycin	*Mycobacterium* 607	*Bacillus subtilis*
epi-A_2 (+Cu)	93 u/mg*	3 u/mg*
A_2 (+Cu)	910	910
epi-B_2 (+Cu)	584	15
B_2 (+Cu)	2,720	1,460
epi-A_2'-C(+Cu)	69	trace
A_2'-C (+Cu)	847	548
epi-B_4 (+Cu)	675	175
B_4 (+Cu)	6,350	14,500

* Cup-assay method, bleomycin A_2 (−Cu) free base as the standard: 1,000 u/mg.

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