THE CHEMISTRY OF BLEOMYCIN. IX*

THE STRUCTURES OF BLEOMYCIN AND PHLEOMYCIN**

Sir:

The results of the structural studies of bleomycin which have been reported[1,2] can be summarized in Fig. 1. In this communication, we propose the total structure of bleomycin as shown in Fig. 2.

Potentiometric titration of copper-free bleomycin B_2 hydrochloride showed the presence of three basic functions at pK'a 7.3, 4.7 and *ca.* 2.9. The guanidino group of the terminal amine moiety was out of measureable region (pK'a>11.5). The titration equivalent was 1540.

There are 7 dissociable groups in the partial structure of bleomycin B_2: terminal guanidino group, one primary and one secondary aliphatic amine, imidazole, 4-aminopyrimidine, and two carboxyl groups. The two carboxyl groups should be covered in the bleomycin molecule because a free carboxyl function could not be detected by KOSHLAND's method.[3]

Bleomycin was treated with 2,4-dinitrofluorobenzene followed by acid hydrolysis. α-DNP-β-Aminoalanine was found in the hydrolyzate, but neither di-DNP- nor β-DNP-β-aminoalanine was detected. The result suggested that the α-amino group of β-aminoalanine moiety is free and the secondary amine forms an amide in the bleomycin molecule. The conclusion was in accord with the fact that there was only one aliphatic amine function at pK'a 7.3 which can be assigned to the α-amino group.

A basic function of pK'a 4.7 can be assigned to the imidazole because the chemical shift of the imidazole-2 proton is sensitive to pH change around this pK'a value.

The UV spectrum of bleomycin A_2 is

Fig. 2. Structure of bleomycin. (R: terminal amine)

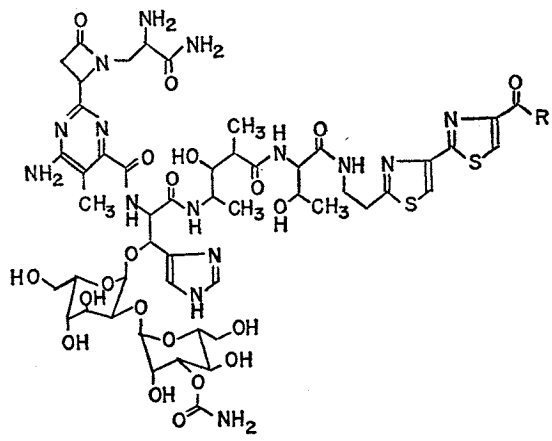

Fig. 1. Partial structure of bleomycin. (R: terminal amine)

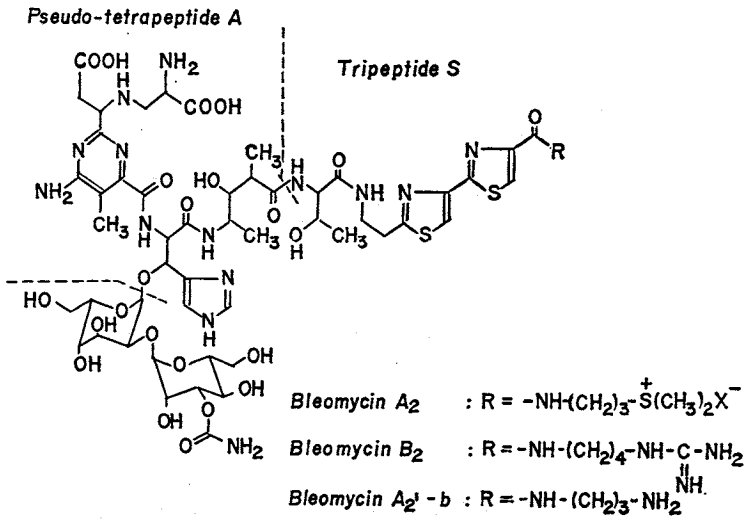

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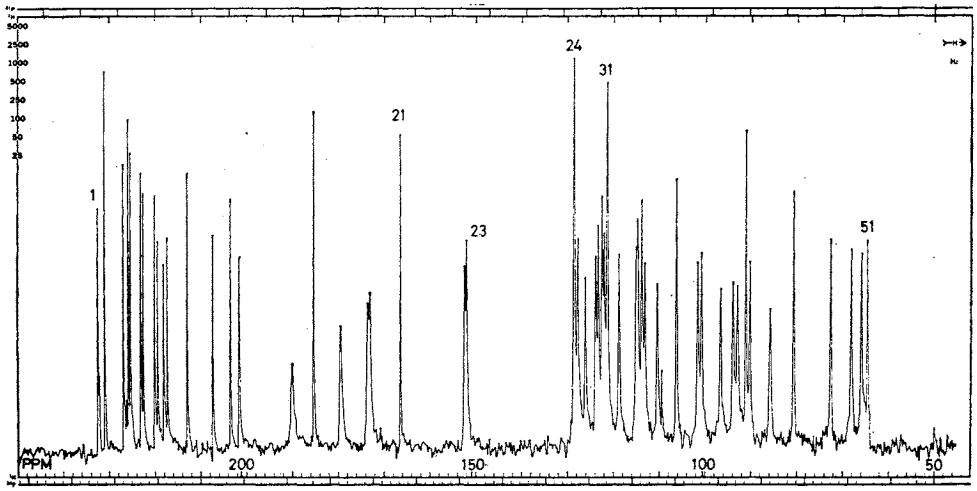
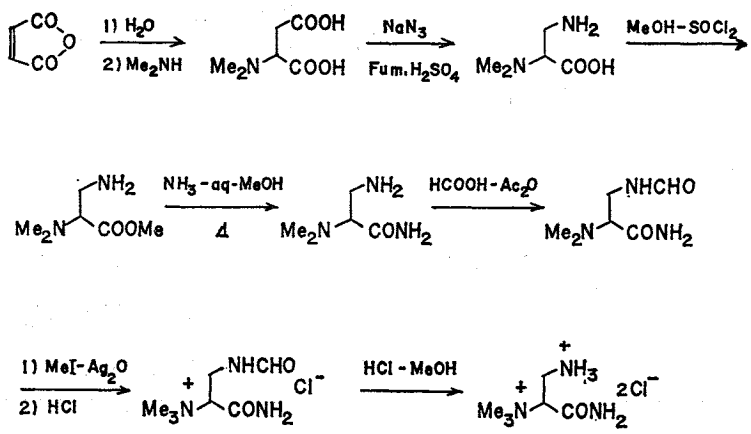
Fig. 3. ^{13}C-PFT-NMR spectrum of bleomycin A_2'-b. (in D_2O, external reference CH_2I_2, 25.2 MHz, 36,000 scans)



essentially the same as that calculated from the UV spectra of pseudo-tetrapeptide A and tripeptide S (Fig. 1)[1]. Therefore, the basic function of 4-aminopyrimidine moiety in bleomycin should be free and account for the weak base of pK'a *ca.* 2.9.

The elemental analysis of copper-free bleomycin B_2 hydrochloride supported the molecular formula $C_{55}H_{81}N_{19}O_{21}S_2 \cdot 3HCl$ (MW 1517.9). [Found: C 43.26, H 5.79, N 17.48, O 22.90, S 4.10. Calcd.: C 43.52, H 5.58, N 17.53, O 22.13, S 4.22]. The carbon number (C_{55}) of bleomycin B_2 was confirmed by ^{13}C-PFT-NMR spectrum. Bleomycin A_2'-b, which contained 1,3-diaminopropane as the terminal amine and thus had the lowest carbon number of the bleomycins, was used as a sample for ^{13}C-PFT-NMR spectrometry (Fig. 3). There were 51 well-resolved signals, of which two (24 th and 31 th signals from low field) were composed of two superimposed signals, judged from the intensity and line width. Thus, the spectrum indicated the presence of 53 carbons in bleomycin A_2'-b molecule, corresponding to 55 carbons in bleomycin B_2. There are 55 carbons already in the partial structure of bleomycin B_2 (Fig. 1). By proton NMR studies, all non-exchangeable protons of bleomycin (55 protons for B_2) are shown in this partial structure. There are 19 nitrogens in the molecular formula of bleomycin B_2,

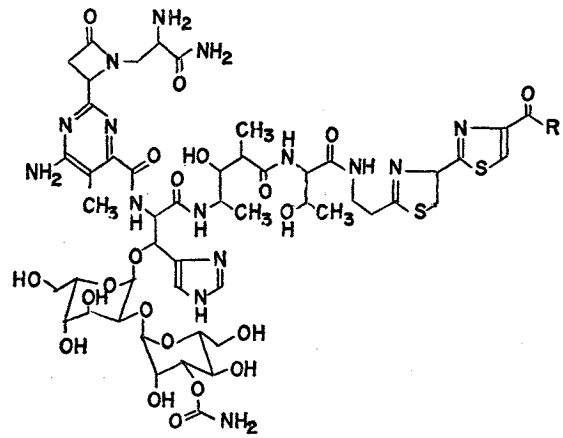
Fig. 4. Synthesis of β-aminoalanine-α-betaine amide.



but 18 nitrogens are present in the partial structure.

Therefore, to establish the total structure, one more nitrogen must be added to the partial structure, but no carbon and no non-exchangeable proton can be added. By addition of one nitrogen, two carboxyl groups and the secondary amino group must be transformed to non-dissociable functions. To satisfy these conditions, only two possible structures remained: one of the two carboxyl groups forms an amide with the missing nitrogen and the other carboxyl group forms a β-lactam with the secondary amine which is in the β-position to both carboxyl groups.

Fig. 5. Structure of phleomycin (R : terminal amine).



Generally, β-lactam and amide bonds are more susceptible to acid hydrolysis than a peptide bond. We thought if an acid-stable bulky group were introduced into the free primary amino group, the neighboring postulated β-lactam or amide bond would become more resistant to acid hydrolysis. Bleomycin was methylated with methyl iodide and triethylamine in aqueous alcohol.*** Total acid hydrolysis gave all amine components except β-aminoalanine. Instead, β-aminoalanine-α-betaine was present in the hydrolyzate. The methylated bleomycin was refluxed for 18 hours in 1 N HCl solution. The hydrolyzate contained a basic substance other than β-aminoalanine-α-betaine. This new compound was isolated by resin chromatography. The molecular formula of the dihydrochloride $C_6H_{17}N_3OCl_2$ was established by elemental analysis. [Calcd.: C 33.04, H 7.86, N 19.26. Found: C 33.22, H 8.09, N 18.97]. Vigorous acid hydrolysis (6 N HCl, 105°C, 24 hours) gave β-aminoalanine-α-betaine. These results suggested that the basic substance was β-aminoalanine-α-betaine amide. It was synthesized by the scheme shown in Fig. 4 and identity with the natural material was confirmed.

Thus, the carboxyl group of the β-aminoalanine moiety exists as an amide in the bleomycin molecule, leading to the only possible structure which is shown in Fig. 2.

An unfused β-lactam generally shows IR absorption at 1730~1760 cm^{-1} [4]. However, it has been shown that in pachystermine B[5] which is a steroidal alkaloid with an unfused β-lactam, the β-lactam absorption appears at 1718 cm^{-1}. This bathochromic shift is caused by intramolecular hydrogen bonding. This kind of intramolecular hydrogen bonding could occur in the proposed bleomycin structure. In bleomycin, there is a strong and broad absorption centered at 1650 cm^{-1} (amide I band) with a shoulder at 1720 cm^{-1}. Thus, the proposed structure agrees with the IR spectrum.

The ^{13}C-NMR spectrum not only gave the exact total carbon number, but also gave us other useful information to support the structure. The 21 signals from low field indicated the presence of 21 sp_2-carbons in the proposed structure. Among the signals which can be assigned to sp_3-carbons, the two lowest signals (22 nd and 23 rd signals from low field can be assinged to the C-1 carbons of gulose and 3-O-carbamoyl-mannose.

The structural relation between bleomycin and phleomycin has already been established by oxidative transformation of phleomycin D_1 to bleomycin B_2 [6]. Thus, the structure of phleomycin is shown in Fig. 5.

Tomohisa Takita
Yasuhiko Muraoka*
Takeo Yoshioka

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Akio Fujii*
Kenji Maeda
Hamao Umezawa

Institute of Microbial Chemistry,
Kamiosaki, Shinagawa-ku,
Tokyo, Japan
* Research Laboratory
Nippon Kayaku Co., Ltd.,
Shimo, Kita-ku, Tokyo, Japan

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