CHEMISTRY OF BLEOMYCIN. XIX REVISED STRUCTURES OF BLEOMYCIN AND PHLEOMYCIN

Sir:

During studies on the biosynthesis of bleomycin (BLM), we have isolated several peptides structurally related to BLM (NAKATANI *et al.* unpublished). One of them, designated P-3A, was isolated as a crystalline Cu(II)-complex. Acid hydrolysis of metal-free P-3A gave β-aminoalanine (amine component V[1] of BLM), 2-(1-amino-2-carboxyethyl)-6-aminopyrimidine-4-carboxylic acid (a demethyl analogue of amine component II[2] of BLM) (Fig. 1), histidine and alanine. The ^{13}C-NMR spectrum of P-3A showed six signals, of which the chemical shifts (bold letters in Table 1) were very similar to those[3] assigned to the six carbon atoms in the substituent including the β-lactam ring at the 2-position of the pyrimidine ring of our proposed structure of BLM (Fig. 2)[4]. As will be reported by IITAKA *et al.*[11], the result of the X-ray crystallographic analysis of P-3A Cu(II)-complex indicated that a β-lactam ring was not present in P-3A, although the structure of the carboxy part with an asterisk in Fig. 1 was not determined. Therefore, we reinvestigated the structure of the β-lactam part of BLM.

The most probable structure of the substituent at the 2-position of the pyrimidine ring of P-3A was assumed to be the same as that of the triamide of the pseudodipeptide[5] (Fig. 1), which was prepared by ammonolysis of the trimethylester of pseudodipeptide. The elemental analysis of the triamide was: Found: C, 38.25; H, 6.01; N, 29.30; Cl, 9.21. Calcd. for $C_{12}H_{20}N_8O_3 \cdot H_2O \cdot HCl$: C, 38.05; H, 6.12; N, 29.58; Cl, 9.36.

Table 1. ^{13}C-NMR chemical shifts of BLM A2′-c, P-3A and pseudodipeptide triamide

Assignment		BLM A2′-c*	P-3A	Pseudodipeptide triamide
	CO (side)	**177.0**	**176.9**	**176.9**
	CO (ring)	168.5	169.3	172.1
	2	166.1	166.0	166.5
	4 (-NH_2)	165.5	165.8	165.1
II	6	153.0	154.9	155.8
	5	113.1	103.2	111.2
	CH	**53.3**	**53.2**	**53.2**
	CH_2	**41.0**	**40.9**	**41.1**
	CH_3	11.7		11.9
	CO	**171.9**	**171.5**	**171.9**
V	CH	**60.5**	**60.8**	**60.8**
	CH_2	**47.8**	**47.9**	**47.8**
	CO		172.5	
	2		135.5	
	4		130.0	
His	5		118.9	
	CH		53.5	
	CH_2		28.6	
	CO		180.8	
Ala	CH		52.1	
	CH_3		17.9	

* There is a slight chemical shift difference from the former value[3]. It is due to corrected adjustment of the data processing (internal reference: dioxane as δ 67.4). In BLM A2′-c, chemical shifts of the pseudodipeptide moiety are only shown in this table.

The ^{13}C-NMR chemical shifts of six carbon atoms in the 2-substituent on the pyrimidine ring of pseudodipeptide triamide (see Table 1) suggested

Fig. 1. Structures of pseudodipeptide and its competitive β-elimination degradation products, **II** and **V**, under a total acid hydrolysis condition.

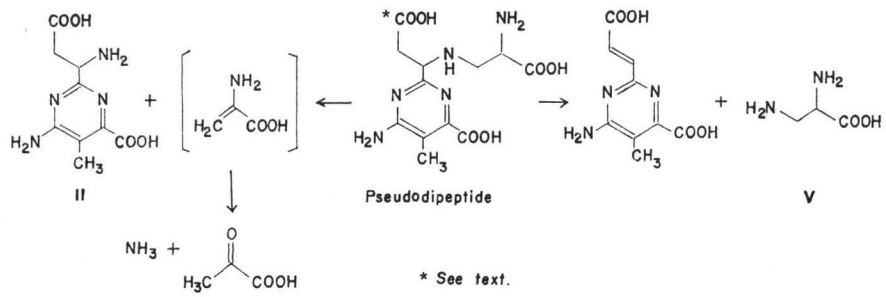


Fig. 2. Total structure of bleomycin (1972)

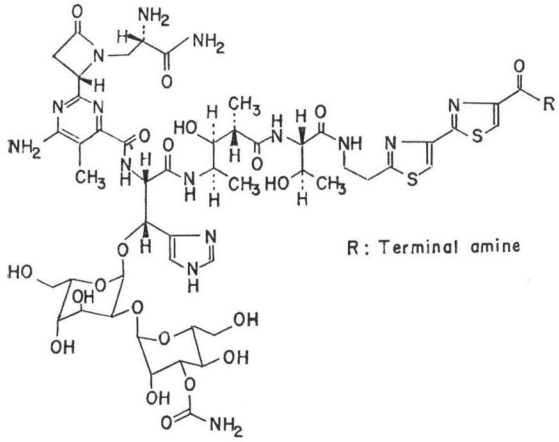
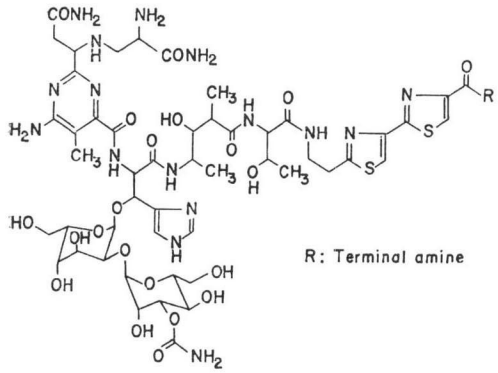


Fig. 4. Revised structure of bleomycin



that the structures of the substituents at the 2-position of the pyrimidine ring of BLM and P-3A should be the same as that of the pseudodipeptide triamide.

As to the total structure of BLM shown in Fig. 2, details of the structural elucidation were described in our previous papers[4,6]. The work is summarized here. We established an assembly of all degradation products of BLM as shown in Fig. 3. Although elemental analysis and field desorption mass spectrometry did not give a definite molecular formula and molecular weight for BLM, the presence of 50 carbon atoms in BLM except for the terminal amine moiety was shown by ^{13}C-NMR analysis[8]. This carbon number was the same as that in the structure shown in Fig. 3. The elemental analysis suggested the presence of one more nitrogen atom which should be added to the structure shown in Fig. 3. For example, the number of the nitrogen atom corresponding to the 55 carbon atoms in

Fig. 3. An assembly of degradation products of bleomycin

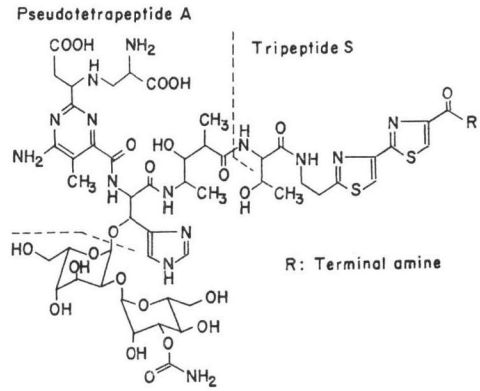
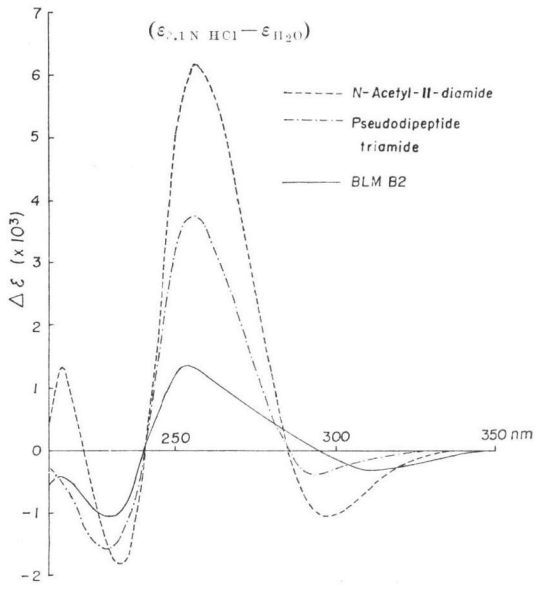


Fig. 5. Difference UV spectra of N-acetyl-II-diamide, pseudodipeptide triamide and BLM B2



BLM B2, which was most easily purified among natural BLMs and contained agmatine (N=4) as the terminal amine, was found to be 19 by repeated elemental analyses (There are 14 nitrogens in Fig. 3 except for the terminal amine). The missing nitrogen was confirmed to be added to the structure of Fig. 3 as an amide of the α-aminocarboxylic acid moiety[4]. In the terminal group analysis of BLM, only α-DNP-V, but neither β- nor di-DNP-V, was isolated by SANGER's DNP-method and no free carboxy group was detected by KOSHLAND's glycine condensation method in BLM[4]. Potentiometric titration of copper-free BLM B2 showed the

presence of four measurable basic functional groups at pKa > 11.5, 7.4, 4.7 and 2.7. The guanidino group at the terminal amine moiety was assigned to pKa > 11.5, α-amino group of V to 7.4, the imidazole of β-hydroxyhistidine to 4.7, which was confirmed by pH-dependence of the ^{1}H- and ^{13}C-NMR chemical shifts[3,7], and the 4-aminopyrimidine to 2.7. The latter assignment was apparently confirmed by the fact that among II [2-(1-amino-2-carboxyethyl)-6-amino-5-methylpyrimidine-4-carboxylic acid, see Fig. 1] and its three derivatives, only when the amino group of the side chain of II was masked by acetylation, did the basicity of the 4-aminopyrimidine become strong enough to be measurable by potentiometric titration, of which the pKa-value was 2.7[6]. Moreover, the secondary amine nitrogen shown in Fig. 3 was suggested to be masked in BLM molecule by application of SANGER's DNP-method and by lack of methylation with methyliodide-triethylamine[4]. Therefore, it was only possible in BLM that the secondary amine in Fig. 3 was acylated with a remaining free carboxy group at the β-position. Thus, the total structure of BLM shown in Fig. 2 was proposed.

If BLM has the structure shown in Fig. 4 as suggested by the ^{13}C-NMR study of the triamide of pseudodipeptide, the secondary amine is free in BLM and the pKa 2.7 of BLM B2 should be assigned to this secondary amine, although its basicity is extremely weak as an aliphatic amine. Therefore, the dissociation constants of the pyrimidine chromophores were reinvestigated by UV spectrometry. The pKa-value of N-acetyl-II-diamide was 2.7 (measured by potentiometric titration)[6], indicating that in 0.1 N HCl solution (pH = 1.0) about 98% of the pyrimidine is protonated. From the difference UV absorption (Fig. 5) at 256 nm between 0.1 N HCl and aqueous solution of this compound, the difference molecular extinction coefficient between the protonated and intact pyrimidine chromophore was calculated as $\Delta\varepsilon 6300$. In Fig. 5, the difference UV spectra of pseudodipeptide triamide and BLM B2 are also shown. The pH-dependent difference of UV absorption of BLM B2 was slightly different from N-acetyl-II-diamide and pseudodipeptide triamide, although the UV absorption of the bithiazole part, another chromophore of BLM, remained unchanged between the aqueous and 0.1 N HCl solution, which was confirmed by UV spectrum of tripeptide S (see Fig. 3), and no decomposition of BLM in 0.1 N HCl during the UV measurement was confirmed by antimicrobial assay. From the difference UV absorption at 256 nm, the pKa-value of the pyrimidine chromophore of pseudodipeptide triamide was calculated to be 1.2 and that of BLM B2 was estimated to be less than 1.0 from the difference UV absorption in the 0.1 N HCl solution. These results indicated that pKa 2.7 of BLM should be assigned to the secondary amine. The pKa-value of the secondary amine of pseudodipeptide triamide was found to be 3.4 by potentiometric titration. Both of the pKa-values of the secondary amines were extremely weak for aliphatic amines. It can be explained by the effect of the neighboring primary amino group and masking of the lone paired-electron of the secondary amine by intramolecular 6-membered ring hydrogen bonding with a carboxamide proton. The pKa-value of the secondary amine of BLM B2 was significantly lower than that of pseudodipeptide triamide. This may be due to the presence of an additional stronger basic functional group, the imidazole of β-hydroxyhistidine moiety, of which the pKa-value was 4.7. Prevention of dinitrophenylation and methylation of the secondary amine by SANGER's DNA-method and with methyliodide-triethylamine, respectively, also may be explained by its very weak nucleophilicity and steric hindrance.

Although the chemical verification for the existence of a new carboxamide group in BLM has not yet been successful and elemental analysis has always shown a somewhat small nitrogen content, the ^{13}C-NMR studies of pseudodipeptide triamide, P-3A and BLM shown in Table 1 and studies of the dissociation constants of the pyrimidine and secondary amino groups by UV spectrometry and potentiometric titration indicated that the structure of BLM should be revised as shown in Fig. 4. Diazepine ring formation described in a previous study[6] can be explained by ring closure but not by ring expansion of a β-lactam ring.

Phleomycins have been converted to BLMs by manganese dioxide oxidation of the thiazoline moiety[4,8]. Thus, their structures should be revised to the ring-open structures as BLMs. And the structures of the β-lactam moiety of other bleomycin-phleomycin group antibiotics, YA-56X[9] and tallysomycins[10], also should be

revised, because their studies on this part are dependent on our previous structures.

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