Communications to the editor

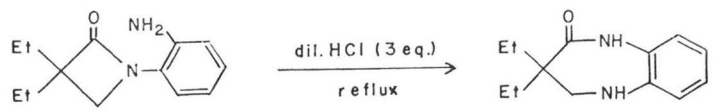
CHEMISTRY OF BLEOMYCIN. XVII* CHEMICAL PROOF FOR THE β-LACTAM OF BLEOMYCIN

Sir:

In 1972, we proposed a total structure for bleomycin[1] which contained a monocyclic β-lactam. The IR spectrum of bleomycin is not typical for unfused β-lactams, which usually absorb at 1730~1760 cm^{-1} [2]. Therefore, we have studied the chemical modification of bleomycin to prove the presence of a β-lactam. In this communication, the acidic degradation *via* ring-expansion of the β-lactam, which is chemical evidence for the existence of the β-lactam of bleomycin, is reported.

Every effort to get a small fragment containing the intact β-lactam failed because of degradation. Therefore, we attempted to obtain a derivative which can be formed only if a β-lactam is present. NICOLAUS *et al.*[3] reported that the synthetic β-lactam (**1**), for which the IR absorption (1715 cm^{-1}) was also exceptional, was transformed to the seven-membered ring derivative (**2**) under acidic conditions (see Scheme 1). The structural relation between the β-lactam and the free amino group of bleomycin is the same as in **1**. Therefore, we tried to prepare the seven-membered ring derivative of bleomycin, although there are functional groups which are apt to be easily hydrolyzed under acidic conditions. Refluxing copper-free bleomycin A2′-c[4] in 10% aqueous acetic acid for about five hours gave several degradation products. The major product (**3**) was isolated by CM-Sephadex chromatography (0.1~0.5 M NaCl linear gradient elution) followed by silica gel

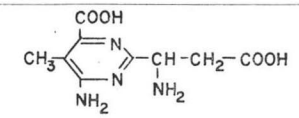
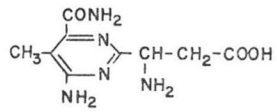
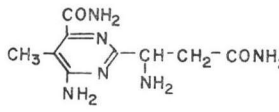
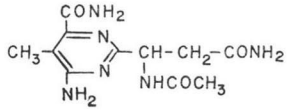
Scheme 1.



1 1715 cm^{-1}

2 1670 cm^{-1} (yield 73.8%)

Table 1. The pKa values of compound II and its derivatives

	4-Aminopyrimid.	Aliph.-NH_2	Aliph.-COOH	Ring-COOH
II 	<1.5	9.0	3.8	2.7
II-a 	<1.5	8.9	3.3	—
II-b 	<1.5	7.7	—	—
II-c 	2.7	—	—	—

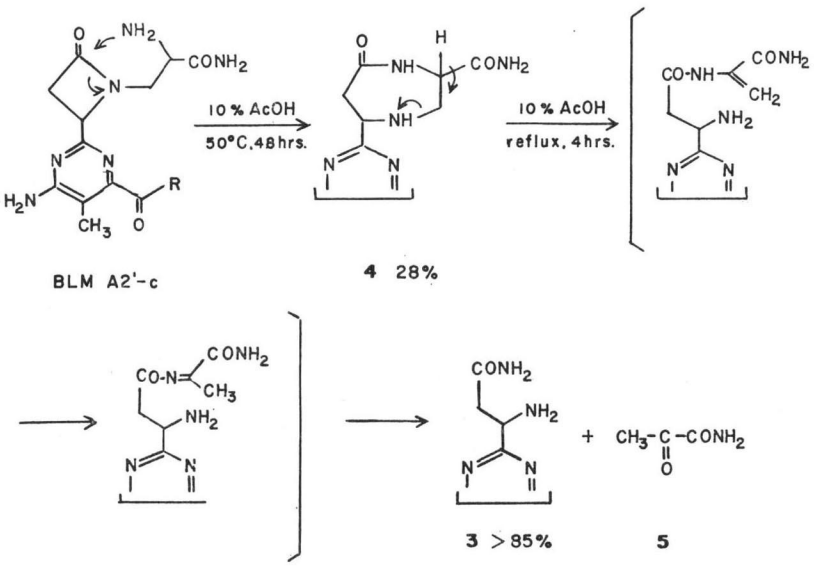
* Chemistry of bleomycin. XVI, Reference 5)

chromatography[5] (Merck prepacked silica gel 60 column, 10% $AcONH_4$-H_2O-MeOH-EtOH = 5 : 19 : 60 : 116) after copper-complex formation.

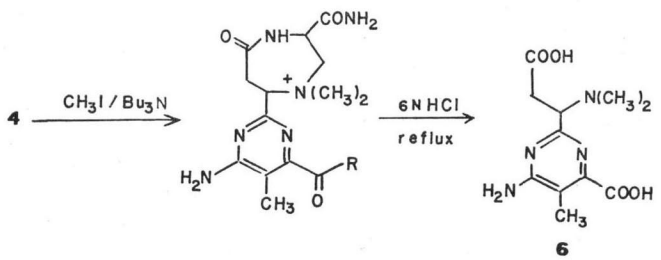
The chromatographic behavior of **3** was similar to the starting material, but it had no biological activity. The total acid hydrolysis of **3** in 6 N HCl gave the same amine components as bleomycin A2′-c[6,7] except for β-aminoalanine. In the ^{13}C-NMR spectrum of **3** three signals assigned to the carbons of the β-aminoalanine moiety were missing. Potentiometric titration indicated the presence of three dissociable groups (pKa 4.7, 6.6 and 7.6) in **3** while there were four dissociable groups [pKa 2.7 (4-aminopyrimidine), 4.7 (imidazole of hydroxyhistidine), 6.5 (imidazole of terminal histamine) and 7.5 (α-amino group of β-aminoalanine)] in bleomycin A2′-c. The UV spectra were the same for neutral (H_2O), acidic (0.05 N HCl) and alkaline (0.05 N NaOH) solutions of **3** and were essentially the same as those of bleomycin A2′-c in neutral and alkaline solutions. The UV spectrum of bleomycin A2′-c in acidic solution was different from the others due to protonation of the 4-aminopyrimidine chromophore. These results suggested that the basicity of the 4-aminopyrimidine of **3** is reduced by the increase of the basicity of the nitrogen atom contained in the β-lactam ring of bleomycin. In order to confirm this fact, derivatives of β-amino-β-(4-amino-6-carboxy-5-methylpyrimidin-2-yl)-propionic acid (designated as amine component II of bleomycin)[6,8] were prepared and their pKa-values were measured (Table 1).

The N-acetyl-dicarboxamide of II (II-c in Table 1) is a model compound for the basicity of the 4-aminopyrimidine moiety of bleomycin.

Scheme 2.



Scheme 3.



Its pKa-value was 2.7, the same as that of bleomycin A2′-c, while those of II, II-a and II-b (see Table 1) were reduced by the neighboring free amino functions and less than 1.5. The pKa-value of the amino group of II-b was 7.7, which corresponds to pKa 7.6 of **3**. These results suggested that **3** has the structure shown in Scheme 2.

Formation of **3** can be interpreted only by the presence of the β-lactam in bleomycin. A reaction mechanism is shown in Scheme 2, which suggests that the expected seven-membered ring derivative (**4**) is formed during the degradation. The isolation of pyruvamide (**5**) from the reaction mixture as its 2,4-dinitrophenylhydrazone is confirmatory evidence.

Since **4** was expected to degradate to **3**, we investigated reaction conditions to yield **4**. The behavior on ion-exchange resin chromatography of **4** could be anticipated from the presumed basicity of **4**: the imino group of **4** should be less basic than the amino group (pKa 7.6) of **3**, because of the presence of the neighboring carboxamide group, and **4** should be eluted faster than **3** in CM-Sephadex chromatography developed with pH 6.8 phosphate buffer containing 1/60 M NaH_2PO_4, 1/60 M Na_2HPO_4 and 1/10 M NaCl. The basicity of the 4-amino-pyrimidine group of **4** also should be reduced by the presence of the neighboring free imino group, and **4** is expected to be eluted faster than the starting material in SP-Sephadex chromatography developed with pH 2.5 citrate buffer containing 1/60 M 3Na-citrate, 8/60 M citric acid and 4/10 M NaCl. Using these chromatographic analyses, we found that the expected **4** is formed in 28% yield by warming in a 50°C oil bath for 48 hours in a 10% aqueous acetic acid solution. Compound **4** was isolated by CM- and SP-Sephadex chromatography as described above. The total acid hydrolysis of **4** in 6 N HCl gave the same amine components as bleomycin A2′-c. The ^{13}C-NMR spectrum of **4** showed the presence of all carbons of A2′-c. Potentiometric titration showed that there are three dissociable groups: pKa 4.5 (imidazole of hydroxyhistidine), 5.6 (a newly formed basic group) and 7.1 (imidazole of terminal histamine).

The assignment was verified by measurement of the pKa-values [pKa 4.5, 5.6 and >12 (terminal agmatine)[7]] of compound-4-equivalent derived from bleomycin B2. The UV spectrum of **4** was the same as that of **3** and remained unchanged in H_2O, 0.05 N HCl and 0.05 N NaOH solutions. Compound **4** gave **3** and **5** when refluxed in a 10% aqueous acetic acid solution. Treatment of **4** with methyliodide in the presence of tri-*n*-butylamine followed by acid hydrolysis gave β-dimethylamino-β-(4-amino-6-carboxy-5-methylpyrimidin-2-yl)-propionic acid (**6**) (Scheme 3). The structure **6** was confirmed by mass spectrometry [*m/e* 223(M−Me_2NH), 179, 135], ^{1}H-NMR spectrometry [2.57 (3H-singlet), 3.38 (6H-singlet), 3.54 (2H-doublet, J = 6.5 Hz) and 5.02 δ(1H-triplet, J = 6.5 Hz) in D_2O, external TMS reference] and UV spectrometry: ($\lambda_{max}^{H_2O}$ 234 and 274 nm, cf. $\lambda_{max}^{H_2O}$ of II: 234 and 274 nm[6]). The isolation of **6** from the acid hydrolysate of methylated **4** means that the nitrogen atom contained in the β-lactam in bleomycin exists as a free imino group (pKa 5.6) in **4**. Thus, the transformation to **4** was found to be caused by the acyl migration of the β-lactam to the α-N of the β-aminoalanine moiety of bleomycin. Comparison of the ^{13}C-NMR spectrum of **4** with that of bleomycin A2′-c indicated that only the signals assigned to the carbons relevant to the ring expansion shifted significantly, which will be discussed in detail in a separate paper.

Thus, the structure of **4** was established as the seven-membered ring derivative shown in Scheme 2. These transformations are chemical evidence for the existence of the β-lactam in bleomycin.

YASUHIKO MURAOKA
AKIO FUJII
*TAKEO YOSHIOKA
*TOMOHISA TAKITA
*HAMAO UMEZAWA

Research Laboratory, Nippon Kayaku Co., Ltd., Shimo, Kita-ku, Tokyo 115, Japan
*Institute of Microbial Chemistry, Kamiosaki, Shinagawa-ku, Tokyo 141, Japan

(Received November 12, 1976)

References

1) TAKITA, T; Y. MURAOKA, T. YOSHIOKA, A. FUJII, K. MAEDA & H. UMEZAWA: The chemistry of bleomycin. IX. The structure of bleomycin and phleomycin. J. Antibiotics 25: 755~758, 1972

2) BELLAMY, L. J.: The infra-red spectra of complex molecules. p. 214. John Wiley & Sons, Inc., New York, 1958
3) NICOLAUS, B. J. R.; E. BELLASIO, G. PAGANI, MARIANI & E. TESTA: Über eine neue allgemeien Synthese von 3,3-disubstituierten 2,3,4,5-tetrahydro-1H-(benzo[b]1,4-diazepin)-2-onen durch Ringerweiterung 3,3-disubstituierter 1-(*O*-Aminophenyl)-azetidine-2-one. Helv. Chim. Acta 48: 1867~1885, 1965
4) FUJII, A; T. TAKITA, K. MAEDA & H. UMEZAWA: New components of bleomycin. J. Antibiotics 26: 396~397, 1973
5) MURAOKA, Y.; H. KOBAYASHI, A. FUJII, M. KUNISHIMA, T. FUJII, Y. NAKAYAMA, T. TAKITA & H. UMEZAWA: Chemistry of bleomycin. XVI. *Epi*-bleomycin. J. Antibiotics 29: 853~856, 1976
6) TAKITA, T.; Y. MURAOKA, K. MAEDA & H. UMEZAWA: Chemical studies on bleomycins. I. The acid hydrolysis products of bleomycin A2. J. Antibiotics 21: 79~80, 1968
7) FUJII, A.; T. TAKITA, K. MAEDA & H. UMEZAWA: Chemistry of bleomycin. XI. The structures of the terminal amines. J. Antibiotics 26: 398~399, 1973
8) MURAOKA, Y.; T. TAKITA, K. MAEDA & H. UMEZAWA: Chemistry of bleomycin. IV. The structure of amine component II of bleomycin A2. J. Antibiotics 23: 252~253, 1970