CHEMISTRY OF BLEOMYCIN. XXV. REDUCTIVE METHYLATION OF BLEOMYCIN, A CHEMICAL PROOF FOR THE PRESENCE OF THE FREE SECONDARY AMINE IN BLEOMYCIN

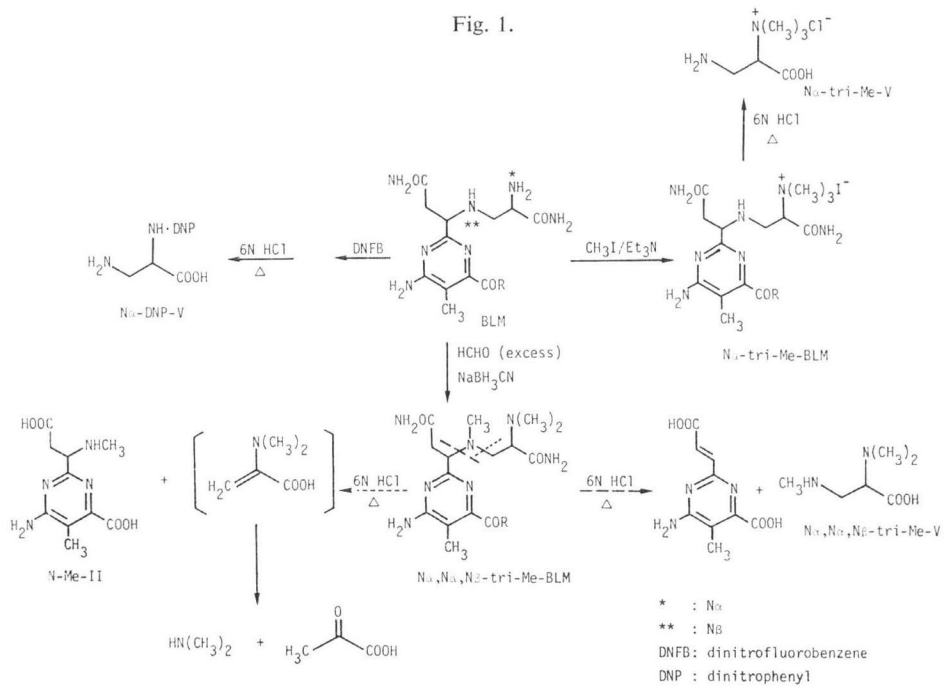
Sir:

In a previous paper, we proposed the new structure of bleomycin (abbreviated as BLM), which contains one primary and one secondary amino groups (Fig. 1)[1]. The secondary amino group in BLM, however, did not react with methyl iodide or 2,4-dinitrofluorobenzene, that is after treatment with these reagents followed by total acid hydrolysis, BLM gave Nα-trimethyl-β-aminoalanine (Nα-trimethyl-V[2]) or Nα-dinitrophenyl-β-aminoalanine (Nα-DNP-V), respectively, but neither the Nβ-methyl-derivative nor the Nβ-DNP-derivative (see Fig. 1). This lack of reactivity of the secondary amino group had led to the former β-lactam structure[3]. Therefore, we studied reactions of BLM to establish the presence of a free secondary amino group in BLM. In this communication, methylation of BLM with formaldehyde and sodium cyanoborohydride and the bioactivity of the resulting N-methyl derivatives are reported.

Methylation of aliphatic amino group with formaldehyde and sodium cyanoborohydride[4] was mild enough to apply to BLM, which generally suffers from undesired modifications such as isomerization[5] and ring closure[1]. Treatment of metal-free BLM A2 with excess formaldehyde (5 moles) and sodium cyanoborohydride in methanol at room temperature for 24 hours gave predominantly one product in a yield of 85%. The structure of this product was found by NMR and degradation studies to be Nα,Nα,Nβ-trimethyl BLM A2 (Fig. 1). The ^{1}H-NMR spectrum of Nα,Nα,Nβ-trimethyl-BLM in D_2O solution showed two N-methyl signals at δ 2.79 (3H, singlet) and 3.32 (6H, singlet), [external reference: TMS, $\delta=0$], and the ^{13}C-NMR spectrum also showed the two N-methyl signals at δ 37.9 and 41.9 (2×C), [internal reference: dioxane, $\delta=67.4$]. On complete acid hydrolysis (6 N HCl, 105°C, 18 hours), the trimethyl BLM gave 2-(1-methylamino-2-carboxyethyl)-4-amino-5-methyl-6-carboxy-pyrimidine (N-methyl-II[2]), Nα,Nα,Nβ-trimethyl-β-aminoalanine (Nα,Nα,Nβ-trimethyl-V) and dimethylamine, corresponding to II, V and ammonia formed by competitive β-elimination of BLM under the same hydrolysis conditions (Fig. 1). They were isolated by ion-exchange chromatography as described below.

Acid hydrolysate of the trimethyl BLM was

Fig. 1.



charged onto a column packed with Dowex 1 (acetate form). N-Methyl-II and 2′-(2-aminoethyl)-2,4′-bithiazole-4-carboxylic acid, an amino acid component of BLM[6], were adsorbed on the resin and the other products passed through the column. After elution of the thiazole amino acid with 0.1 M acetic acid, N-methyl-II was eluted with 1 M acetic acid. Nα,Nα,Nβ-Trimethyl-V, which was contained in the effluent, was isolated by chromatography on a Dowex 50 column developed with a pyridine-acetic acid buffer.

N-Methyl-II showed the same UV spectrum as II. The ^{1}H-NMR spectrum of N-methyl-II in D_2O showed an N-methyl signal at δ 3.29 (3H, singlet) in addition to the other signals of II. The chemical shifts of the ^{13}C-NMR spectrum of N-methyl-II are shown in Table 1 in comparison with those of II[7]. The N-methyl signal appeared at δ 32.6 and the significant shift of the methine carbon signal (δ 49.5→δ 57.7) by the N-methylation indicated that the methyl group attached to the aliphatic amino group of II, which stemmed from the secondary amine of BLM.

Nα,Nα,Nβ-Trimethyl-V was crystallized from water and ethanol, m.p. 166°C (decomp.). In its ^{1}H-NMR spectrum, the N-methyl signals appeared at δ 3.35 (3H, singlet) and 3.43 (6H, singlet) and the methylene and methine signals showed an ABX splitting pattern at δ *ca.* 4.1 and 4.55, respectively. The structure was confirmed by direct comparison with an authentic sample, synthesized from β-benzylaminoalanine[8] by methylation with formaldehyde and sodium cyanoborohydride followed by reductive removal of the benzyl group.

Dimethylamine in the hydrolysate was converted to its dansyl derivative. This was identified by comparison with an authentic sample by silica gel thin layer chromatography (Rf= 0.84, isopropyl ether; Rf=0.88, ethyl acetate-cyclohexane=3:2).

When 1.5 molar amount of formaldehyde was used for the reductive methylation of BLM, three products were obtained. They were separated by chromatography on a CM-Sephadex C-25 column developed with a linear gradient of sodium chloride at pH 4.5 after copper-complex formation[9]. The structures were determined by NMR and degradation studies as described above. The first eluate was found to contain Nα,Nα-dimethyl-BLM, the second one was Nα,Nα,Nβ-trimethyl-BLM, and the third to contain Nα-methyl-BLM. The ratio of the isolated products was about 8:9:39. Nβ-Methyl-BLM and Nα,Nβ-dimethyl-BLM could not be found in the methylation products of BLM. This suggests that the methylation of the secondary amine of BLM takes place less readily than the second methylation of the primary amine.

Table 1. Chemical shifts of ^{13}C-NMR spectra of amine component II[2] of BLM and its N-methyl derivative.

Assignment		II*	N-Me-II**
Carboxy (side)		172.8	173.2
Carboxy (ring)		166.6	166.9
Pyrimidine	4 (–NH_2)	165.1	166.4
	2	157.1	156.5
	6	146.0	149.3
	5	114.5	112.8
CH		49.5	57.7
CH_2		36.9	36.3
CH_3		12.3	12.0
N–CH_3		—	32.6

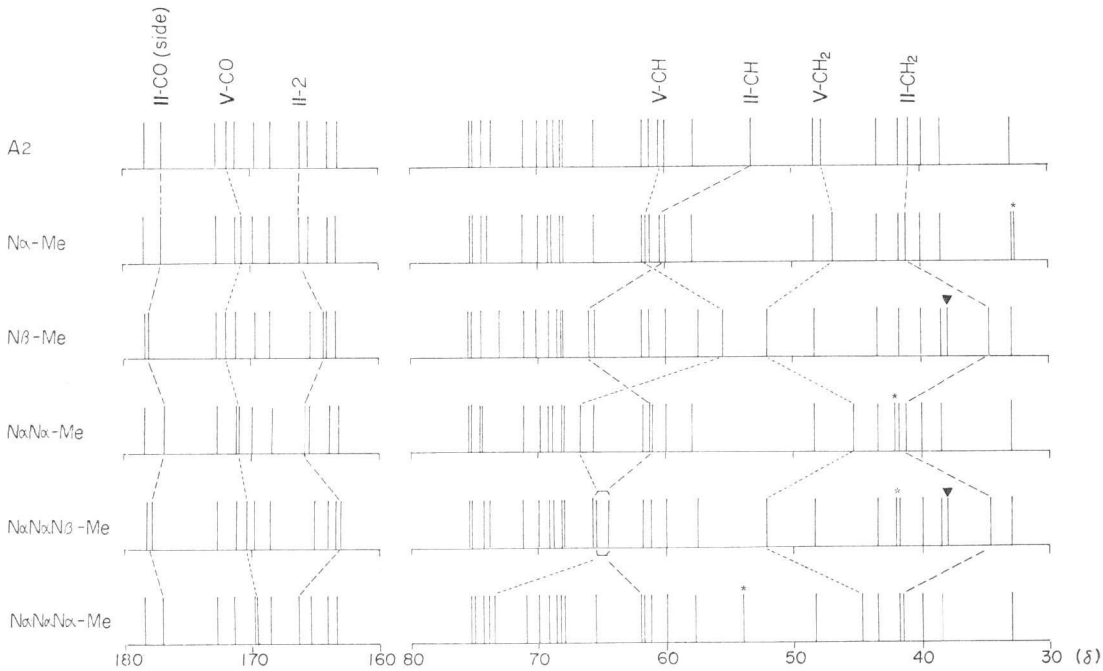
* δ-value, (from reference 7)

** The assignment was confirmed by selective long-range C-H decoupling.

Nβ-Methyl-BLM was prepared by the following procedure: The primary amino group of BLM A2 was protected by *t*-butoxycarbonyl group with S-*t*-butoxycarbonyl-4,6-dimethyl-2-mercaptopyrimidine[10]. The butoxycarbonyl derivative of BLM was reductively methylated and, thereafter, the protective group was removed by treatment with 50% trifluoroacetic acid at room temperature for 1 hour to give Nβ-methyl-BLM in almost quantitative yield.

^{13}C-NMR chemical shifts of five N-methyl derivatives of BLM A2:Nα- and Nβ-monomethyl, Nα,Nα-dimethyl and Nα,Nα,Nβ- and Nα,Nα,Nα-trimethyl derivatives, are shown in Fig. 2 together with those of the original BLM A2. The 6 signals between δ 10 and 30 and the 12 signals between δ 80 and 160 are omitted in Fig. 2, because these chemical shifts are almost the same among these six compounds. In Fig. 2, the newly introduced methyl signals are marked with * for Nα-methyl and with ▼ for Nβ-methyl. Significant shifts of the signals were observed only in the carbons adjacent to the introduced N-methyl groups with some regularity[11]. This

Fig. 2. ^{13}C-NMR chemical shifts of BLM A2 and its N-methyl derivatives.



means that methylation of BLM A2 with formaldehyde and sodium cyanoborohydride occurred only in the free amines without any change in the other part of the molecule.

The validity of the new amide structure of BLM[1] has been recently confirmed most directly by the ^{15}N-NMR spectroscopic study[12], and in this paper the presence of the free secondary amino group in BLM was confirmed by chemical modification. The free secondary amine, of which the pKa-value is 2.7[1], has also been confirmed by pH-dependence of the ^{13}C-NMR chemical shifts of the vicinal carbons to the secondary amine*.

The bioactivity of BLM has been found to be due to reactive oxygen radicals formed at the sixth coordination site of its square-pyramidal Fe(II)-complex[13,14], in which the Nα-nitrogen occupies the apical coordination site and the Nβ-nitrogen occupies one of the in-plane coordination sites. The effect of methylation of these nitrogen donors on the bioactivity was examined by testing the antibacterial activity of the N-methyl derivatives against *Mycobacterium smegmatis* 607 and the growth inhibition of HeLa cells (Table 2). Among the five N-methyl derivatives, only the Nα-monomethyl derivative showed activities comparable to the parent BLM A2 and the other four showed only very weak or no activities. It is remarkable that the Nα-monomethyl derivative did not undergo the action of BLM hydrolase, which inactivates BLM by hydrolysis of the amide bond vicinal to the primary amino (Nα) group[15,16].

Table 2. Bioactivity of bleomycin A2 and its N-methyl derivatives.

Bleomycin A2 and its N-methyl derivatives	Antimicrobial activity (units/mg)	HeLa cell ID_{50} (mcg/ml)
A2	1,000	0.73
Nα-Me-A2	423	0.81
Nβ-Me-A2	51	inactive
Nα,Nα-diMe-A2	57	inactive
Nα,Nα,Nβ-triMe-A2	0	inactive
Nα,Nα,Nα-triMe-A2	0	inactive

* measured by cup-assay method with *Mycobacterium smegmatis* 607 as the test organism (reference: BLM A2 1,000 u/mg).

* H. NAGANAWA, T. TAKITA and H. UMEZAWA: unpublished, the same finding by J. D. GLICKSON (private communication).

Acknowledgement

Thanks are due to Dr. A. MATSUDA and his staff, Nippon Kayaku Co., Ltd., for biological tests.

TAKEYO FUKUOKA
YASUHIKO MURAOKA
AKIO FUJII
HIROSHI NAGANAWA*
TOMOHISA TAKITA*
HAMAO UMEZAWA*

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

(Received October 25, 1979)

References

1) TAKITA, T.; Y. MURAOKA, T. NAKATANI, A. FUJII, Y. UMEZAWA, H. NAGANAWA & H. UMEZAWA: Chemistry of bleomycin. XIX. Revised structures of bleomycin and phleomycin. J. Antibiotics 31: 801~804, 1978
2) TAKITA, T.; Y. MURAOKA, K. MAEDA & H. UMEZAWA: Chemical studies on bleomycin. I. The acid hydrolysis products of bleomycin A2. J. Antibiotics 21: 79~80, 1968
3) TAKITA, T.; Y. MURAOKA, T. YOSHIOKA, A. FUJII, K. MAEDA & H. UMEZAWA: Chemistry of bleomycin. IX. The structures of bleomycin and phleomycin. J. Antibiotics 25: 755~758, 1972
4) BORCH, R. F. & A. I. HASSID: A new method for the methylation of amines. J. Org. Chem. 37: 1673~1674, 1972
5) NAKAYAMA, Y.; M. KUNISHIMA, S. OMOTO, T. TAKITA & H. UMEZAWA: Chemistry of bleomycin. XII. *Iso*-Bleomycin A2, a product of carbamoyl group migration. J. Antibiotics 26: 400~402, 1973
6) KOYAMA, G.; H. NAKAMURA, Y. MURAOKA, T. TAKITA, K. MAEDA, H. UMEZAWA & Y. IITAKA: Chemistry of bleomycin. II. The molecular and crystal structure of a sulfur-containing chromophoric amino acid. Tetrahedron Lett. 1968: 4635~4638, 1968
7) NAGANAWA, H.; Y. MURAOKA, T. TAKITA & H. UMEZAWA: Chemistry of bleomycin. XVIII. Carbon-13 NMR studies. J. Antibiotics 30: 388~396, 1977
8) FU, S. C. J. & J. P. GREENSTEIN: Saturation of acetyldehydroalanine with benzylamine. J. Am. Chem. Soc. 77: 4412~4413, 1955
9) MURAOKA, Y.: Liquid chromatography of bleomycin. Bleomycin: Chemical, Biochemical and Biological Aspects. Springer-Verlag, New York, in press
10) NAGASAWA, T.; K. KUROIWA, K. NARITA & Y. ISOWA: New agents for *t*-butyloxycarbonylation and *p*-methoxybenzyloxycarbonylation of amino acids. Bull. Chem. Soc. Jap. 46: 1269~1272, 1973
11) LEVY, G. C. & G. L. NELSON: Carbon-13 Nuclear Magnetic Resonance for Organic Chemists. pp. 51~53, Wiley-Interscience, New York, 1972
12) NAGANAWA, H.; T. TAKITA, H. UMEZAWA & W. E. HULL: Chemistry of bleomycin. XXIII. Natural abundance ^{15}N-NMR spectroscopic evidence for the structure of bleomycin. J. Antibiotics 32: 539~541, 1979
13) TAKITA, T.; Y. MURAOKA, T. NAKATANI, A. FUJII, Y. IITAKA & H. UMEZAWA: Chemistry of bleomycin. XXI. Metal-complex of bleomycin and its implication for the mechanism of bleomycin action. J. Antibiotics 31: 1073~1077, 1978
14) SUGIURA, Y. & T. KIKUCHI: Formation of superoxide and hydroxy radicals in iron(II)-bleomycin-oxygen system: Electron spin resonance detection by spin trapping. J. Antibiotics 31: 1310~1312, 1978
15) UMEZAWA, H.; S. HORI, T. SAWA, T. YOSHIOKA & T. TAKEUCHI: A bleomycin-inactivating enzyme in mouse liver. J. Antibiotics 27: 419~424, 1974
16) SUGIURA, Y.; Y. MURAOKA, A. FUJII, T. TAKITA & H. UMEZAWA: Chemistry of bleomycin. XXIV. Deamidobleomycin from viewpoint of metal coordination and oxygen activation. J. Antibiotics 32: 756~758, 1979