Chemical and Biological Modification of Bleomycin, an Antitumor Antibiotic

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Dedicated to Professor John C. Sheehan on the occasion of his sixty-fifth birthday.

Chemical and biological modifications of bleomycin, an antitumor glycopeptide antibiotic, have been achieved by three procedures analogous to the modifications of penicillin.

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Sir:

Bleomycin (BLM) is an antitumor antibiotic first reported in 1966 (1) and currently used in the treatment of squamous cell carcinoma and malignant lymphoma (2). BLM is produced by the culture of *Streptomyces verticillus* ATCC-15003. The produced BLM is a mixture of congeners, which are separated by a CM-Sephadex C-25 column chromatography. They are obtained as blue-colored amorphous powders. The BLMs are glycopeptides containing one atom of copper and are different from each other in their terminal amine moieties (Table 1) (3). Recently, we proposed the total structures of metal-free BLM (Figure 1) (4), which is obtained by treatment of the copper complex with hydrogen sulfide in methanol, and the copper-complex of BLM (Figure 2) (5). On the basis of our proposed structure, the mechanism of action of BLM can be explained on a molecular level. That is: the primary action for the antitumor activity of BLM is shown by the DNA strand scission caused by a reactive oxygen species reductively activated from molecular oxygen at the sixth coordination site of the BLM-Fe(II) complex (5-7).

Table 1

Some Natural Bleomycins

BLM	Terminal Amine	Antibacterial Activity	Content in Natural BLMs (%)
A1	$NH_2(CH_2)_3SOCH_3$	460 U/mg. (a)	9.2
A2	$NH_2(CH_2)_3S^+(CH_3)_2Cl^-$	910	54.5
B2	$NH_2(CH_2)_4NHC(=NH)NH_2$	2720	26.7
A5	$NH_2(CH_2)_3NH(CH_2)_4NH_2$	2500	2.4

(a) BLM A2 free base: 1000 U/mg. (Mycobact. 607).

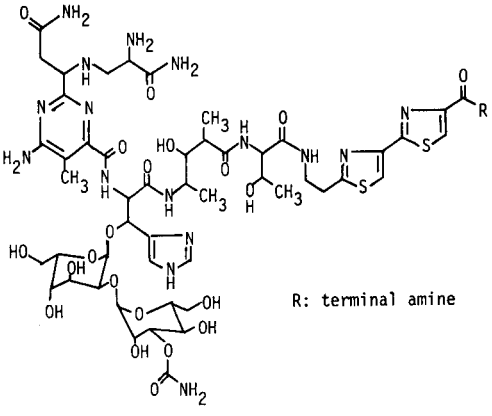

Figure 1. Total structure of bleomycin

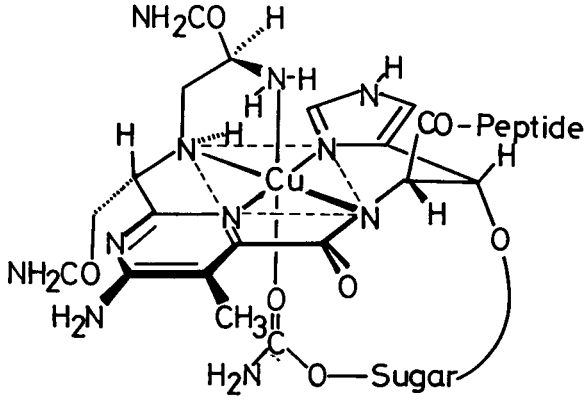

Figure 2. Structure of Cu(II)-complex of bleomycin

The modification of an antibiotic to obtain a more potent one was first successful in penicillin. Penicillin is also obtained as a mixture of congeners by fermentation. The natural penicillins are different from each other in their *N*-terminal acyl residues, in contrast to BLMs which are different in the *C*-terminal amines. As is well known, when phenylacetic acid is added to the culture medium of penicillin fermentation, penicillin G is solely produced in the cultured medium. Thus, it was established that new artificial penicillins were produced by fermentation by addition of unnatural acids. In this case, monosubstituted acetic acid is only efficiently incorporated into the acyl residue of penicillin. The second modification method is the semisynthetic one starting from 6-APA obtained by chemical and enzymatic cleavage of the amide bond of natural penicillins. We have succeeded in modification of BLM in the three procedures similar to penicillin modification.

1. New Biosynthetic BLMs (8).

We found that when 3-aminopropyldimethylsulfonium chloride, the terminal amine of BLM A2, was added to the culture medium of BLM fermentation, BLM A2 was solely produced in the cultured medium. The result suggested

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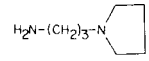
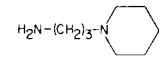
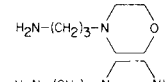
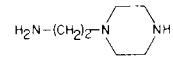
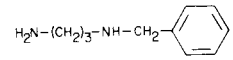
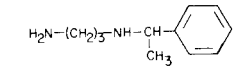
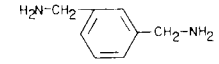
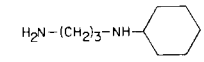
Table 2

Amines Well-Incorporated into the Terminal Amine Moiety of BLM and Antibacterial Activity of the New Biosynthesized BLMs

$H_2N\text{-}CH_2\text{-}CH(CH_3)\text{-}NH_2$	2173 (a)		1066
$H_2N\text{-}(CH_2)_3\text{-}NH\text{-}CH_3$	860		1257
$H_2N\text{-}(CH_2)_3N(CH_3)_2$	869		592
$H_2N\text{-}(CH_2)_3\text{-}\overset{+}{N}(CH_3)_3X^-$	633		799
$H_2N\text{-}(CH_2)_3\text{-}NH\text{-}(CH_2)_3\text{-}N(CH_3)_2$	1200		
$H_2N\text{-}(CH_2)_3\text{-}NH(CH_3)\text{-}(CH_2)_3\text{-}NH_2$	1671		1986
$H_2N\text{-}(CH_2)_3\text{-}NH\text{-}CH(CH_3)\text{-}(CH_2)_2\text{-}NH_2$	1820		2400
$H_2N\text{-}(CH_2)_3\text{-}NH\text{-}(CH_2)_5\text{-}OH$	1330		2546
$H_2N\text{-}(CH_2)_3\text{-}NH\text{-}(CH_2)_5\text{-}OCH_3$	1335		5355

(a) BLM A2 free base: 1000 U/mg.

that some unnatural alkyl amine could be incorporated into the terminal amine moiety of BLM and the new artificial BLM would be produced by the BLM fermentation. This was actually realized. The well-incorporated amines and the anti-microbial activity of the resulting new biosynthetic BLMs are shown in Table 2. Generally speaking, these amines showing good incorporation had the following properties: first, they had two or more basic functional groups; and second, at least one of them was a primary alkyl amine which connects with the other part of BLM by an amide linkage.

2. Bleomycinic Acid, The Starting Material for Semi-Synthetic BLM.

2.1. Enzymatic Hydrolysis of BLM B2 (9,10).

The BLM molecule consists of two parts: a part common to all BLMs, which was named bleomycinic acid, and a terminal amine, which is specific to each individual BLM. If bleomycinic acid could be obtained, semi-synthetic BLMs can be easily prepared by chemical coupling with an amine, because there exists only one free carboxy function in bleomycinic acid. During the structural study of BLM, we found that the amide bond between bleomycinic acid and the terminal amine is the most resistant to acid hydrolysis among the amide bonds present in BLM. Therefore, we first attempted to obtain bleomycinic acid by enzymatic hydrolysis of BLM.

Microbial enzymes were examined on an appropriate hydrolase using copper-chelated BLM B2 as a substrate. Bleomycin B2 was chosen since after cleavage into bleomycinic acid and agmatine, the terminal amine of BLM B2, the latter could be easily detected by the Sakaguchi reaction on thin layer chromatograms. Moreover, BLM B2 is readily available because it is the second major component of natural BLMs. Washed mycelia of various bacteria, actinomycetes and fungi were tested as enzymes sources. Bleomycin B2 and the mycelium were incubated in *p*H 7.5 phosphate buffer at 37° and 15 hours, and the antibacterial activity against *Mycobacterium smegmatis* 607 of the reaction mixture was then assayed. The anti-*Mycobacterium* activity of bleomycinic acid was supposed to be very weak from the known structure-activity relationships of biosynthetic BLMs. Those reaction mixture with reduced anti-*Mycobacterium* activity were further tested for liberation of agmatine. From this screening process a strain of *Fusarium anguioides* was selected. A massive amount of BLM B2 was digested with the crude enzyme preparation from this microorganism and the remaining fragment after liberation of agmatine was isolated from the reaction mixture. Structural studies indicated that it was the expected bleomycinic acid. The structure was confirmed by synthesis of BLM B2 from bleomycinic acid and agmatine with water-soluble carbodiimide as a coupling reagent. The anti-*Mycobacterium* activity of bleomycinic acid was very weak as expected (159 unit/mg., about 5% of that of BLM B2).

The enzyme was highly purified by ammonium sulfate precipitation, DEAE-Sephadex chromatography, gel filtration with Sephadex G-100, *etc.*. The enzyme was active

Table 3

The Substrate Specificity of Acylagmatine Amidohydrolase from *F. anguioides*

Substrate	Km (*M*)	V max (*μM*/min./mg.)
Bleomycin B2	8.0×10^{-4}	0.029
Bleomycin A2 (a)	—	—
Bleomycin A2'-b (a)	—	—
Bleomycin B4 (a)	—	—
Bleomycin X (a,b)	—	—
acetylagmaine	13.3×10^{-4}	0.168
propionylagmatine	20.0×10^{-4}	0.242
benzoylagmatine	2.2×10^{-4}	0.279
benzoylarginine (a)	—	—

(a) Not to be hydrolyzed. (b) Artificial bleomycin (terminal amine: NH_2-$(CH_2)_3$-NH-C(=NH)-NH_2).

specifically on BLM B2 among the natural and biosynthetic BLMs. This suggested that the enzyme has an affinity for agmatine, but not the bleomycinic acid moiety. A study on substrate specificity revealed that the enzyme is a new acylagmatine amidohydrolase (Table 3). Before this study such an enzyme was unknown in nature. Bleomycin B2 was chosen as the substrate from other reasons in this study, but this choice was the crucial factor leading to successful results.

2.2. Selective chemical cleavage of BLM A2 (11).

For this chemical cleavage, BLM A2, the main component of natural BLM, was chosen as the starting material because it has a reactive sulfonium group terminus and iminolactonization driven by the leaving of dimethylsulfide can be expected (Figure 3). This cyclization was not successful, but in this study it was observed that methylchloride but not dimethylsulfide could be easily removed by pyrolysis to afford BLM demethyl-A2. If BLM demethyl-A2 were available as the starting material, it would be expected that iminolactonization would occur by treatment with cyanogen bromide, since the leaving of methylthiocyanate is much easier than that of dimethylsulfide. This idea was suggested by the selective cleavage of the methionyl peptide bond by cyanogen bromide, though this involves the carboxy side while the present purpose is scission at the amino side of decarboxymethionine. Transformation of BLM A2 into BLM demethyl-A2 was achieved in over 85% yield by treatment with sodium thiocyanate at 120° for 2 hours in methanol solution in a pressure vessel. Thus, it became feasible to use BLM demethyl A-2 as starting material for chemical transformation.

Bleomycin demethyl-A2 was dissolved in 1% trifluoroacetic acid and reacted with excess cyanogen bromide at room temperature overnight. The main product was

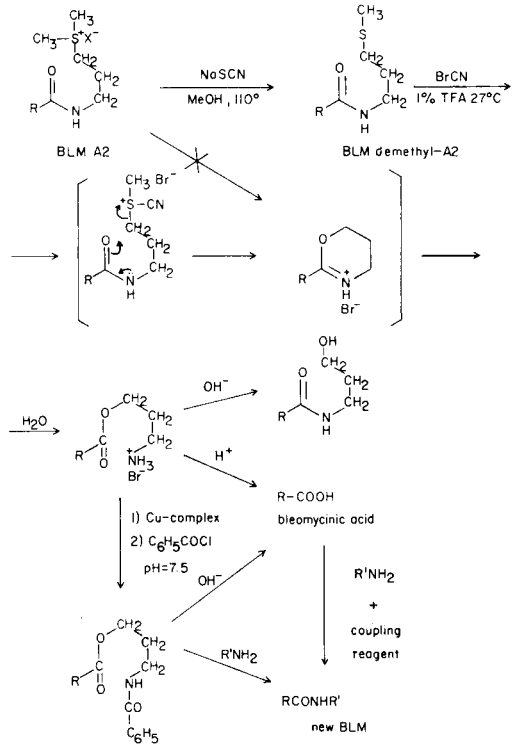


Figure 3. Chemical transformation of bleomycin A2

isolated by CM-Sephadex C-25 column chromatography in 75% yield. This product was not the expected iminolactone, but rather the 3-aminopropyl ester of bleomycinic acid, the hydrolysis product of the former (Figure 3).

Under mild alkaline hydrolysis conditions, the carbonyl group of the ester bond, which was formed by the treatment of cyanogen bromide, shifted readily to the newly formed terminal amino group to form a stable amide. Under mild acid hydrolysis conditions, bleomycinic acid was obtained in about 50% yield, but formation of an appreciable amount of by-products, which were formed by acid hydrolysis at other weak bonds and were not easily separable from bleomycinic acid, could not be avoided. To block the *O* to *N* acyl migration in alkali, selective acylation of the terminal amino group was studied. The primary amino group present originally in BLM was protected by copper-chelation and *N*-benzoylation was carried out with benzoyl chloride at *p*H 7.5. The desired mono-*N*-benzoyl derivative was obtained in over 90% yield. Mild alkaline hydrolysis (0.025*N* potassium hydroxide at 0°) of it afforded bleomycinic acid in over 90% yield. This, selective chemical cleavage of the terminal amide bond of BLM A2 was achieved by participation of the terminal dimethylsulfonium group through successive elimination of methylthiocyanate.

From the standpoint of preparation of semi-synthetic BLM, bleomycinic acid was not necessarily needed. Semi-

synthetic BLM was derived directly from the intermediate, the *N*-benzoylaminopropyl ester of bleomycinic acid, by aminolysis with a specific amine in various yields depending on the amine used, some of which gave over 80% yield.

The study on chemical transformation of BLM was motivated by establishment of an enzymatic method for preparation of bleomycinic acid. However, the chemical transformation method became much superior to the enzymatic method after examination of reaction conditions in each reaction step and by finding a new direct route to semi-synthetic BLM from the intermediate.

BLM clinically used today is a mixture of natural BLMs (BLM A2 *ca.* 60%, BLM B2 *ca.* 30%, and others). The most serious adverse effect of the present BLM is pulmonary fibrosis. Distribution of BLM parenterally administered is affected by the terminal amine, and the affinity between BLM and DNA is also dependent on the electric charge of the terminal amine (12). We have already prepared more than 300 new BLMs by the above-mentioned procedures. Among them, pepleomycin, of which the terminal amine is 3-(*S*-1-phenylethylamino)-propylamine, has been selected as the second generation BLM (2), which shows less lung toxicity and higher anticancer effect than the present BLM clinically used.

REFERENCES AND NOTES

(1) H. Umezawa, K. Maeda, T. Takeuchi and Y. Okami, *J. Antibiot.*, **19A**, 200 (1966).

(2) H. Umezawa, *GANN Monogr. Cancer Res.*, No. 19, 9 (1976).

(3) A. Fujii, T. Takita, K. Maeda and H. Umezawa, *J. Antibiot.*, **26**, 396 (1973).

(4) T. Takita, Y. Muraoka, T. Nakatani, A. Fujii, Y. Umezawa, H. Naganawa and H. Umezawa, *ibid.*, **31**, 801 (1978).

(5) T. Takita, Y. Muraoka, T. Nakatani, A. Fujii, Y. Iitaka and H. Umezawa, *ibid.*, **31**, 1073 (1978).

(6) Y. Sugiura, and T. Kikuchi, *ibid.*, **31**, 1310 (1978).

(7) E. A. Sausville, J. Peisach and S. B. Horwitz, *Biochem. Biophys. Res. Commun.*, **73**, 814 (1976).

(8) A. Fujii, T. Takita, N. Shimada and H. Umezawa, *J. Antibiot.*, **27**, 73 (1974).

(9) H. Umezawa, Y. Takahashi, A. Fujii, T. Saino, T. Shirai and T. Takita, *ibid.*, **26**, 117 (1973).

(10) Y. Takahashi, T. Shirai and S. Ishii, *J. Biochem.*, **77**, 828 (1975).

(11) T Takita, A. Fujii, T. Fukuoka and H. Umezawa, *J. Antibiot.*, **26**, 252 (1973).

(12) H. Kasai, H. Naganawa, T. Takita and H. Umezawa, *ibid.*, **31**, 1316 (1978).