BIOSYNTHESES OF NEW BLEOMYCINS

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Bleomycins, a group of glycopeptide antibiotics differ from one another in their terminal amine moieties. ^{14}C-Methyl-labeled 3-aminopropyl-dimethylsulfonium was confirmed to be incorporated into the amine moiety of bleomycin A_2. Addition of amines of bleomycin A_2, A_2'-a, A_2'-b, A_2'-c, A_5, B_2 and B_4 to fermentation media increased the rate of production of the bleomycin which contained the added amine. Addition of the amines of bleomycins A_2, A_2'-b, A_2'-c, A_5, B_2 and B_4 suppressed production of the other natural bleomycins almost completely. New bleomycins which contained deiminated products of amines of B_2 and B_4 were isolated from media to which these amines were added. Amines which were not found in natural bleomycins also suppressed production of natural bleomycins and caused production of new biosynthetic bleomycins which contained the added amines. The data of the production of these new bleomycins suggested a relationship of the incorporation rates to the structures of possible metabolites.

As reported in previous papers[1,2], various bleomycins are produced by *Streptomyces verticillus*, and they have been shown[3] to differ from one another in their terminal amine moieties[4,5]. These differences suggested that addition of such amines to the fermentation medium might increase the production of specific bleomycins. As briefly reviewed in a general paper[6], the amines of bleomycins A_2, A_2'-a, A_2'-b, A_5 and B_4 added to a fermentation medium were apparently incorporated into bleomycins. We now report that not only the amines of natural bleomycins but also unnatural amines are utilized in biosyntheses of bleomycins and the addition of them caused the selective production of new bleomycins.

Materials and Methods

Fermentation: The seed (15 ml) was prepared by the shake-culturing *Streptomyces verticillus* (ATCC 15003) at 27°C for 48 hours in 100 ml of the following medium in an Erlenmeyer flask of 500-ml volume: 1.0 % glucose, 1.0 % starch, 0.75 % peptone (Kyokuto Seiyaku Co., Ltd., Tokyo), 0.75 % meat extract (Mikuni Kagaku Co., Ltd., Tokyo), 0.3 % NaCl, 0.02 % Pronal ST-1 (antifoam agent, Toho Chemical Industry Co., Ltd., Tokyo), pH 7.2 before sterilization. It was inoculated into 100 ml of the following production medium and grown at 27°C for 8~10 days on a rotary shaking machine (190 r.p.m.): 6.4 % millet jelly (Asadaame Shokuhin Co., Ltd., Kanagawa-ken, Japan), 0.5 % glucose, 3.5 % soybean meal, 0.75 % corn steep liquor (Ajinomoto Co., Ltd., Tokyo), 0.3 % NaCl, 0.1 % K_2HPO_4, 0.05 % $ZnSO_4 \cdot 7H_2O$, 0.01 % $CuSO_4 \cdot 5H_2O$, 0.2 % $NaNO_3$, 0.01 % Pronal ST-1, pH 6.5 before sterilization. Unless specially noted, a neutralized aqueous solution of an amine was sterilized by Millipore filter filtration and added to the medium at the start of the culture.

Assay of antibacterial activity: Antibacterial activity was assayed by a cylinder-agar plate method using *Mycobacterium* 607 as the test organism and copper-free bleomycin A_2 sulfate

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was used as the standard. Copper-free bleomycin A_2 free base was defined to be 1,000 units/mg. Samples were diluted with M/10 phosphate buffer at pH 6.8.

The amines added to the fermentation medium: The amines shown in Table 1 were prepared by the authors as described in a previous papers[4,5], and the amines shown in Table 2 except commercially available ones were synthesized by Mr. K. TAKADA, Research Laboratory of Nippon Kayaku Co., Ltd. Radioactive 3-aminopropyl-dimethylsulfonium chloride was synthesized by S-methylation of N-benzoyl-3-methylthiopropylamine with $^{14}CH_3I$ followed by acid hydrolysis.

Extraction of bleomycins from cultured broth: Filtered broth was passed through a column of Amberlite IRC-50 (H-form). The adsorbed bleomycin was eluted with 0.5 N HCl. The eluate was adjusted to pH 6.5 with 1 N NaOH and charged on a carbon column. After washing, bleomycin was eluted with a mixture of acetone and 0.02 N HCl (1 : 1). After neutralization with Dowex 44 (OH-form), the fractions containing bleomycins were combined and evaporated under reduced pressure. A mixture of bleomycins in the crude powder thus obtained was further purified by alumina chromatography using 80 % aqueous methanol as the developing solvent and Sephadex G 25 chromatography.

Analysis of bleomycin composition: Bleomycin composition in the mixture obtained by the process described in previous paragraph was analyzed by CM-Sephadex C 25 (100 ml) column chromatography with a gradient of ammonium formate from 0.05 to 1.0 M (the total solvent volume was 1,000 ml). The eluate was taken in 5-ml fractions, and the optical density measured at 292 nm.

Identification of components: Purity of each bleomycin isolatated by CM-Sephadex column chromatography was examined by thin-layer chromatography (Silica gel G: MeOH - 10 % $AcONH_4$ - 10 % NH_4OH, 10 : 9 : 1) and paper chromatography (Toyo No. 51 filter paper, 10 % NH_4Cl)[2], and the terminal amine of each bleomycin was identified by two-dimensional paper electrophoresis and chromatography of the total acid hydrolyzate as described in a previous paper[5].

Results and Discussion

The analysis of a bleomycin mixture produced in a natural medium described in a previous section is shown in Fig. 1 b. It contains bleomycins A_1, demethyl-A_2, B_1', A_2, A_2'-a, A_2'-b, A_2'-c, B_2, A_5, B_4, A_6 and B_6. Though the total bleomycin produced in this medium was not high, we studied the biosynthesis of various bleomycins under these conditions in which bleomycin appeared at 4th or 5th day and the maximum production was obtained at 8th to 10th day. Preliminary experiments in which unlabeled 3-aminopropyl-dimethylsulfonium was added to the medium suggested the incorporation of this amine into bleomycin A_2. This was confirmed by addition of ^{14}C-methyl-labeled 3-aminopropyl-dimethylsulfonium chloride to the medium. Ninety mg (100 μCi, 2.2×10^8 dpm) of this amine was added to 100 ml of the medium at 48 hours of the shaking culture. After 6 days the cultured broth was harvested. The filtrate (140 ml) which was combined with the wash contained 152 units/ml of bleomycin and 1.5×10^8 dpm radioactivity. To this filtrate was added 780 ml of the unlabeled filtrate (114 units/ml) which was obtained by a similar shaking culture with addition of the unlabeled amine. Bleomycin in the combined filtrate was extracted by the successive application of Amberlite IRC-50 chromatography, carbon chromatography and alumina chromatography, and 62 mg (871 units/mg, 7.2×10^6 dpm) of crude bleomycin was isolated. The analysis of 7.8 mg (9.4×10^5 dpm) of the sample is shown in Fig. 1 a. Fractions 76~85 showed a major peak of radioactivity (5.7×10^5 dpm), and coincidence of this radioactivity with bleomycin A_2 was confirmed. These fractions were combined and desalted after dilution with 4.8 mg of unlabeled A_2 as carrier

to give 5.4 mg of labeled bleomycin A_2 (5.6×10^4 dpm/mg). Acid hydrolysis of the labeled A_2 gave labeled terminal amine, but the other hydrolysis products did not show any significant radioactivity. The fractions in another peak of the radioactivity in Fig. 1 a did not contain any bleomycin, because they had no absorbance at 292 nm.

Thus, it became evident that the added amine was incorporated into the terminal amine moiety of bleomycin A_2. Moreover, as seen in Fig. 1 a and 1 b, the addition of the amine of A_2 suppressed almost completely the production of the other natural bleomycins. This precursor effect was also found with the terminal amines of the other natural bleomycins except for those of A_1, demethyl-A_2 and A_6. The results are summarized in Table 1. In these experiments, the

Fig. 1. CM-Sephadex C-25 chromatography of
a) Bleomycins produced by addition of $^{14}CH_3-\overset{+}{S}(CH_3)-CH_2-CH_2-CH_2-NH_2\cdot Cl^-$
b) Control: Bleomycins produced without addition of the amine

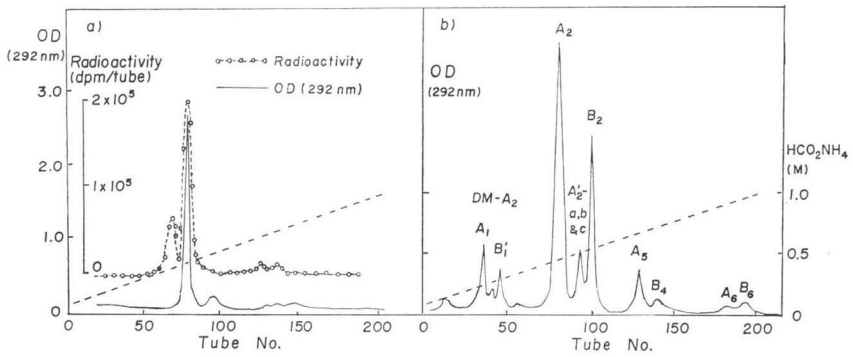

Table 1. Bleomycins produced by addition of terminal amines of natural bleomycins

Origin	Added amine: Structure	Added amine: Concentration (mg/ml)	Produced bleomycins (%)	Control* (%)
A_1	$NH_2-CH_2-CH_2-CH_2-SO-CH_3\cdot\frac{1}{2}H_2SO_4$	8.0	almost same as control	9.2
DM-A_2	$NH_2-CH_2-CH_2-CH_2-S-CH_3\cdot HCl$	5.0	almost same as control	2.4
A_2	$NH_2-CH_2-CH_2-CH_2-\overset{+}{S}(CH_3)_2\cdot Cl^-\cdot HCl$	1.0	A_2 82	54.5
A_2'-a	$NH_2-CH_2-CH_2-CH_2-CH_2-NH_2\cdot 2HCl$	4.0	A_2'-a 24	3.6
A_2'-b	$NH_2-CH_2-CH_2-CH_2-NH_2\cdot 2HCl$	2.0	A_2'-b 85	
A_2'-c	$NH_2-CH_2-CH_2-$ $\cdot 2HCl$	1.0	A_2'-c 93	
B_2	$NH_2-CH_2-CH_2-CH_2-CH_2-NH-C(=NH)-NH_2\cdot H_2SO_4$	2.0	B_2 45, a new BLM 34**	26.7
B_4	$NH_2-(CH_2)_4-NH-C(=NH)-NH-(CH_2)_4-NH-C(=NH)-NH_2\cdot\frac{3}{2}H_2SO_4$	0.53	B_4 38, a new BLM 40**	2.4
A_5	$NH_2-(CH_2)_3-NH-(CH_2)_4-NH_2\cdot 3HCl$	0.36	A_5 100	1.3
A_6	$NH_2-(CH_2)_3-NH-(CH_2)_4-NH-(CH_2)_3-NH_2\cdot 4HCl$	0.30	A_5 *ca.* 100, A_6 trace	trace

* Composition of bleomycins produced without addition of amine.
** Deiminated products of terminal guanidino groups of bleomycins B_2 and B_4, respectively.

Table 2. New bleomycins produced by addition of amines

Added amine		Content of corresponding bleomycin in the product (%)	Antibacterial activity of corresponding bleomycin (u/mg)
Structure	Concentration (mg/ml)		
(I) NH_2-$(CH_2)_2$-NH_2·2HCl	2.0	86	2,072
(II) NH_2-$(CH_2)_3$-$N(CH_3)_2$·2HCl	2.0	100	810
(III) NH_2-$(CH_2)_3$-N⟨ ⟩O·2HCl	0.1	100	592
(IV) NH_2-$(CH_2)_3$-NH-C(=NH)-NH_2·2HCl	2.0	100	2,830
(V) NH_2-$(CH_2)_3$-NH-$(CH_2)_3$-NH_2·3HCl	0.5	66(13)*	2,067
(VI) NH_2-$(CH_2)_3$-N(CH_3)-$(CH_2)_3$-NH_2·3HCl	0.5	65(35)*	1,671
(VII) NH_2-$(CH_2)_3$-NH-$(CH_2)_3$-NH-C_4H_9·3HCl	0.5	100	5,840
(VIII) NH_2-$(CH_2)_3$-NH-$(CH_2)_3$-NH-CH(CH_3)-Ph·3HCl	0.9	100	9,010

* Content of the des-3-aminopropyl derivative

amines were added at the start of the shaking culture, because the precursor effect appeared most clearly when added at the start.

The terminal amines of bleomycins A_1 and demethyl A_2 (DM-A_2) were not incorporated even at high concentrations in the medium, and addition of these amines to fermentation media did not suppress the production of the other natural bleomycins. Addition of A_2'-b amine caused the selective production of bleomycin A_2'-b, while addition of A_2'-a amine (putrescine) did not suppress the production of the other natural bleomycins strongly. This difference between precursor effects of these amines may be due to the easier metabolism of A_2'-a amine than A_2'-b amine. A_2'-c amine (histamine) was well incorporated.

Addition of B_2 amine (agmatine) increased the production of B_2 and suppressed the production of the other natural bleomycins. However, in this case, besides bleomycin B_2, a new bleomycin which was negative ninhydrin and SAKAGUCHI reactions and less basic than B_2 was produced. From the acid-hydrolyzate of this new bleomycin, N-4-aminobutyl-urea, the deiminated product of agmatine was isolated and the terminal amine moiety of this new bleomycin was determined.

With the addition of B_4 amine, a similar result was obtained. Besides B_4, a new bleomycin which was negative in ninhydrin and SAKAGUCHI reactions and less basic than B_4 was also produced. The basicity of this bleomycin was similar to that of B_2 as judged by high-voltage paper electrophoresis[4]. Thus, the terminal amine of this bleomycin was suggested to be the deiminated product of the terminal guanidino group of B_4 amine, which is shown in Table 1.

Spermidine was very efficiently incorporated into bleomycin A_5 and addition of its 0.3 mg/ml completely suppressed the production of the other bleomycins. Addition of A_6 amine (spermine) caused the selective production of bleomycin A_5, but not A_6. It suggests that spermine is transformed into spermidine before incorporation into bleomycin.

The above results suggested that aminoalkyl derivatives of basic substances such as amine, guanidine, sulfonium *etc.* could be incorporated into the terminal amine moiety of bleomycin.

The results of testing the incorporation of such amines are shown in Table 2. 1,2-Diaminoethane (**I**) was incorporated almost to the same extent as 1,3-diaminopropane (A_2'-b amine). This suggests that 2-aminoethyl derivatives of basic compounds are incorporated as well as the 3-aminopropyl derivatives.

A strong precursor effect was observed with N-3-aminopropyl derivatives of dimethylamine (**II**), morpholine (**III**) and guanidine (**IV**). The addition of these amines caused the completely selective production of bleomycins which contained the amines added. In the case of 3-guanidopropylamine (**IV**), unlike B_2 and B_4 amines, a bleomycin having the deiminated amine was not produced.

Addition of N-(3-aminopropyl)-1, 3-diaminopropane (**V**) and its N-methyl derivative (**VI**) gave the corresponding bleomycins (66 % and 65 % respectively) and their des-3-aminopropyl derivatives (13 % and 35 % respectively). As already described, when A_6 amine was added, the product was exclusively A_5, which contained the des-3-aminopropyl derivative of A_6 amine. These results might be related. It was noticed that N′-*n*-butyl (**VII**) and N′-(1-phenylethyl) (**VIII**) derivatives of N-(3-aminopropyl)-1, 3-diaminopropane gave only the bleomycins containing these amines and did not give the related bleomycins in which the 3-aminopropyl group had been removed.

The results described above indicate that the bleomycin-producing strain is able to utilize various types of amines for biosyntheses of various bleomycins. It suggests that the same strain can produce different bleomycin mixtures depending on use of different natural nitrogen sources.

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