

# NATURAL AND ARTIFICIAL BLEOMYCINS: CHEMISTRY AND ANTITUMOUR ACTIVITIES

HAMAO UMEZAWA

*Institute of Microbial Chemistry, Shinagawa-Ku, Tokyo, Japan*

## ABSTRACT

The bleomycins are a group of antibiotics produced by *Streptomyces verticillus* and their therapeutic effect against the squamous cell carcinoma has been proved by clinical studies. In our laboratory, eight products, viz. bleomycin A<sub>1</sub>, demethyl-A<sub>2</sub>, A<sub>2</sub>-a, A<sub>2</sub>-b, B<sub>2</sub>, A<sub>5</sub> and B<sub>4</sub> have been isolated in the pure state. Structures of the various bleomycins have been elucidated and it has been observed that they differ from each other in the *amine* moiety. The addition of an amine, especially diamines and triamines, during fermentation, induced the production of the bleomycin containing the amine added and suppressed the production of other bleomycins. Thus, 42 artificial bleomycins were synthesized and their biological activities studied. The selective effect against squamous cell carcinoma and the mechanism of the selective activity that was shown by an enzymatic inactivation will be discussed. A partial purification of the inactivating enzyme has been achieved.

In the study started from phleomycin<sup>1,2</sup>, we found a group of antibiotics which were produced by *Streptomyces verticillus* and were differentiated from phleomycin by paper chromatography and stability in aqueous solution. Phleomycin caused irreversible renal damage but this antibiotic did not, and we named this antibiotic bleomycin<sup>3,4</sup>. Bleomycin has been confirmed to be effective against squamous cell carcinoma and it is used for treatment of this type of human cancer. Bleomycins which are obtained from the culture filtrate by cation exchange resin process, carbon chromatography and alumina chromatography are in their copper-chelated forms. Both the copper-chelated and the copper-free bleomycins are equally active and copper-free bleomycin mixture is used clinically. In the second paper, we reported bleomycins A<sub>1</sub>, A<sub>2</sub>, A<sub>3</sub>, A<sub>4</sub>, A<sub>5</sub>, A<sub>6</sub>, B<sub>1</sub>, B<sub>2</sub>, B<sub>3</sub>, B<sub>4</sub> and B<sub>5</sub>. Later, we found demethyl-A<sub>2</sub>, A<sub>2</sub>-a, A<sub>2</sub>-b, B<sub>1</sub> and B<sub>6</sub>. These bleomycins were separated by CM-Sephadex C-25 column chromatography raising the concentration of ammonium formate from 0.05M to 1.0M. An example of the chromatography is shown in *Figure 1*. All bleomycins have the maximum at 292 m $\mu$  and the absorption of the eluate at this wavelength was determined.

A<sub>1</sub> and demethyl-A<sub>2</sub> which appeared in peaks I and II respectively were separated by column chromatography of Dowex 50 resin treated with 0.2M pyridine acetate buffer at pH 4.4. The elution was carried out by raising the pH of the same buffer from pH 4.4 to 5.5. The separation of A<sub>2</sub>-a and A<sub>2</sub>-b, both of which appeared in the peak V, was accomplished by repetition of CM-Sephadex C-25 chromatography, raising the concentration of

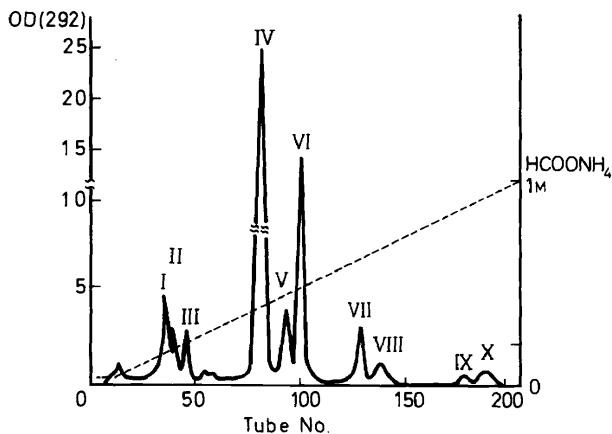


Figure 1. CM-sephadex C-25 chromatography. I: A<sub>1</sub> II: Demethyl-A<sub>2</sub> III: B<sub>1</sub> IV: A<sub>2</sub> V: A<sub>2</sub>-a and A<sub>2</sub>-b VI: B<sub>2</sub> VII: A<sub>5</sub> VIII: B<sub>4</sub> IX: A<sub>6</sub> X: B<sub>6</sub>

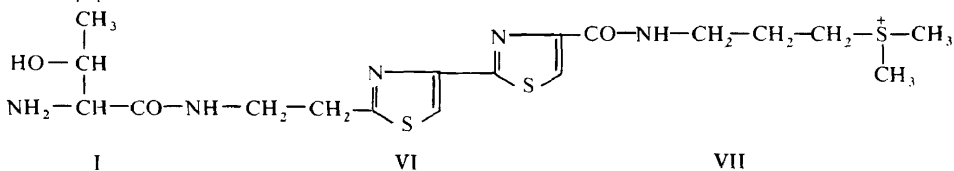
ammonium formate from 0.05M to 0.5M. One of the objects of our bleomycin study was to find the chemical differences between these natural bleomycins and to establish a method of fermentation to produce a single bleomycin. If it was successful, based on the biological activity of each bleomycin we thought that we could find more active bleomycins against squamous cell carcinoma than the present one. As we have reported in this paper, all bleomycins were different in their amine moiety and, on the basis of this observation, we were able to establish a method of fermentation to produce artificial bleomycins. By addition of an amine, especially a diamine or a triamine, to the fermentation medium bleomycin containing the amine added was selectively produced. Thus, we were able to prepare forty-two bleomycins. Now we need the fundamental basis which could be used to predict the efficiency of each bleomycin against squamous cell carcinoma in humans. In this paper, we will report the chemical differences between natural bleomycins, preparation of artificial bleomycins by fermentation and the enzymatic inactivation of bleomycins which is one of the mechanisms of the selective effect against the squamous cell carcinoma.

The study of the structure of bleomycin A<sub>2</sub>, which is the main component of bleomycin employed clinically, is now being continued by Takita, Muraoka, Omoto, Maeda and the author<sup>5-8</sup>. The results hitherto obtained can be summarized in the structures of the partial hydrolysis products shown in Figure 2.

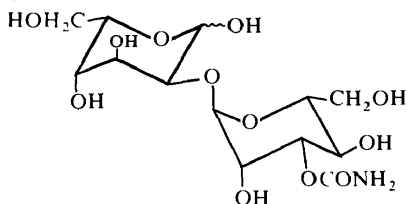
The hydrolysis of bleomycin A<sub>2</sub> in 6N hydrochloric acid at 37°C for four days gives the tripeptide S and the tripeptide A. The total acid hydrolysis of the tripeptide A in 6N hydrochloric acid gives four amino acids: β-amino-β-(4-amino-6-carboxy-5-methylpyrimidin-2-yl)-propionic acid (II), L-β-aminoalanine (V), β-hydroxyhistidine (IV) and 4-amino-3-hydroxy-2-methyl-*n*-valeric acid (III). Because of this tripeptide A was described as a tetrapeptide in previous papers. The structure of the amino acid II-V in Figure 2, which gave II and V by acid degradation, was recently determined by

NATURAL AND ARTIFICIAL BLEOMYCINS

Tripeptide S:



Sugar moiety. 2-O-(3-O-carbamoyl- $\alpha$ -D-mannopyranosyl)-L-gulopyranose



Tripeptide A:

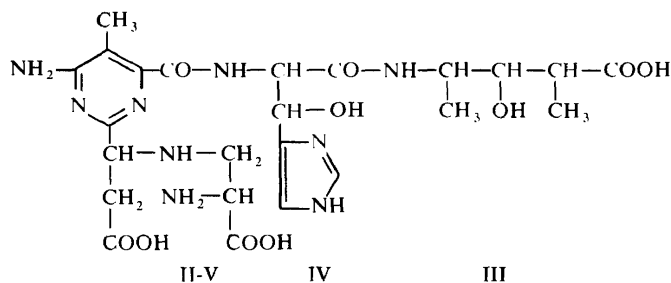


Figure 2. Partial hydrolysis products of bleomycin A<sub>2</sub> (I, II, III, IV, V, VI, VII, are the numbers assigned to the amino acids and the amine obtained by total acid hydrolysis, and were employed in previous papers.)

its synthesis from II and the methyl ester of  $\alpha$ -acetylaminoacrylic acid. As shown in Figure 2, the acid hydrolysis of the tripeptide S gives L-threonine (I), 2'-(2-aminoethyl)-2,4'-bithiazole-4-carboxylic acid (VI) and 3-amino-propyl-dimethylsulphonium (VII).

We could find a hydrolysis condition for bleomycins, that is, in 0.3N sulphuric acid at 81–83°C for 6 hours, to give the sugar moiety in the disaccharide form, as shown in Figure 2. L-Gulose and 3-O-carbamoyl-D-mannose in bleomycins can be identified by gas chromatography of the trimethylsilyl derivatives of the products obtained by reflux of bleomycins in methanol with strong acidic resin (Amberlyst 15) for 30 hours. The pattern of a gas chromatogram is shown in Figure 3. From the results of the gas chromatography of trimethylsilyl derivatives of methyl  $\alpha$ -L-gulopyranoside, methyl  $\beta$ -L-gulopyranoside, methyl 3-O-carbamoyl- $\alpha$ -D-mannopyranoside and methyl  $\alpha$ -D-mannopyranoside, each peak was assigned as follows: A is methyl  $\alpha$ -L-gulopyranoside; B, methyl  $\beta$ -L-gulopyranoside; C, methyl 3-O-carbamoyl- $\alpha$ -D-mannopyranoside; E, methyl  $\alpha$ -D-mannopyranoside.

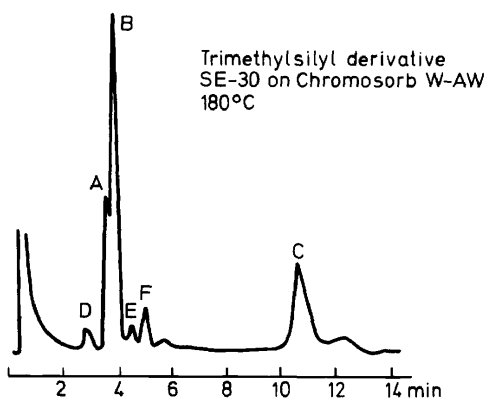


Figure 3. Gas chromatogram of sugar components of bleomycin  $A_2$

The other peaks D and F appeared together with A in the gas chromatography of trimethylsilyl methyl  $\alpha$ -D-gulopyranoside. The patterns of the gas chromatography of trimethylsilyl derivatives of the methanolysis products of natural bleomycins were the same and therefore the sugar moiety was confirmed to be the same in natural bleomycins.

Six amino acids and an amine obtained by the total hydrolysis of bleomycins can be identified by high voltage electrophoresis in formic acid-acetic acid-water (25:75:900) followed by ascending paper chromatography using *n*-propanol-pyridine-acetic acid-water (15:10:3:12). The result for bleomycin  $A_2$  is shown in Figure 4. By this method, the amine moiety was

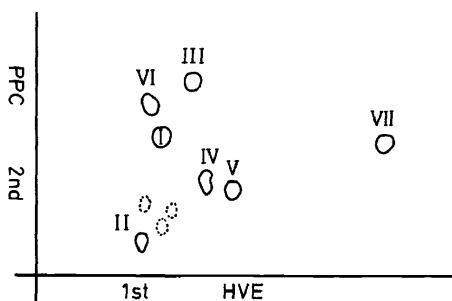
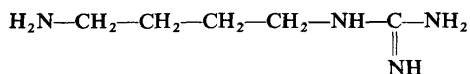


Figure 4. The high voltage electrophoresis followed by ascending paper chromatography of the hydrolysis products of bleomycin  $A_2$ . I: *L*-threonine II:  $\beta$ -amino- $\beta$ -(4-amino-6-carboxy-5-methylpyrimidin-2-yl)-propionic acid III: 4-amino-3-hydroxy-2-methyl-*n*-valeric acid IV:  $\beta$ -carboxyhistidine V: *L*- $\beta$ -aminoalanine VI: 2'-(2-aminoethyl)-2,4'-bithiazole-4-carboxylic acid VII: 3-aminopropyl-dimethylsulphonium

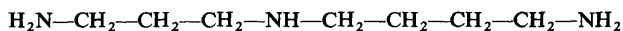
found to be different in natural bleomycins. The amines of bleomycins were isolated and their structures were studied by Fujii, Takita and the author. The bleomycins were hydrolysed in 6*N* hydrochloric acid at 105°C for 30 hours and, after evaporation to dryness, dissolved in distilled water and passed through a column of Dowex-1 in OH form. Then, the amines appeared in the effluent and were crystallized as picrates.

## NATURAL AND ARTIFICIAL BLEOMYCINS

The amine from B<sub>2</sub>:



The amine from A<sub>5</sub>:



The amine from A<sub>6</sub>:

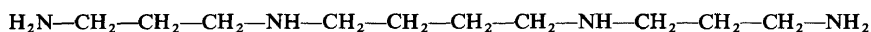


Figure 5

The amine of B<sub>2</sub> was obtained as the dipicrate: m.pt 238–239°C, C<sub>5</sub>H<sub>14</sub>N<sub>4</sub>·2(C<sub>6</sub>H<sub>3</sub>N<sub>3</sub>O<sub>7</sub>). The guanidine group was shown by Sakaguchi reaction and the amino group by positive ninhydrin and one mole of Van Slyke nitrogen. The result of the elemental analysis conforms to the formula in Figure 5. Two middle methylene groups were shown by the multiplet signals of four protons at 2.18δ and the methylene groups adjacent to amino and guanidine groups were shown by the triplet of pairs of protons at 3.52δ and at 3.71δ (in D<sub>2</sub>O) respectively. The identity with agmatine was confirmed by comparison with an authentic sample.

The amine of bleomycin A<sub>5</sub> was first crystallized as the tripicrate and was converted to the trihydrochloride, m.pt 247–248°C, C<sub>7</sub>H<sub>19</sub>N<sub>3</sub>·3HCl. The result of the elemental analysis, positive ninhydrin, two moles of Van Slyke nitrogen and n.m.r. data obtained by the decoupling, suggested that this amine was spermidine. The identity with spermidine was proved by comparison with an authentic sample.

The amine of A<sub>6</sub> was crystallized as the tetrapicrate, m.pt 249–250°C (dec), C<sub>10</sub>H<sub>26</sub>N<sub>4</sub>·4(C<sub>6</sub>H<sub>3</sub>N<sub>3</sub>O<sub>7</sub>). The amine of A<sub>5</sub> was spermidine and, therefore, from the result of elemental analysis we suspected the amine of A<sub>6</sub> to be spermine. The identity with spermine was confirmed by comparison of melting point, behaviour in high voltage electrophoresis, thin layer chromatography in several solvents, infra-red spectrum and gas chromatography with an authentic sample. The amine of B<sub>4</sub> was first crystallized as the picrate, C<sub>10</sub>H<sub>25</sub>N<sub>7</sub>·3(C<sub>6</sub>H<sub>3</sub>N<sub>3</sub>O<sub>6</sub>), m.pt 194–195°C, decomposition at 203°C, and it was converted to its sulphate by the procedure using Dowex-1 resin in OH form, C<sub>10</sub>H<sub>25</sub>N<sub>7</sub>·1.5 H<sub>2</sub>SO<sub>4</sub>, m.pt 270–272°C (dec). The molecular weight 377 was obtained by titration (pK<sub>a</sub> 10.2 and > 12). It was positive in ninhydrin and Sakaguchi and gave one mole of Van Slyke nitrogen. Coupling of *bcfg* with *a* and *deh* protons (Figure 6) was shown by irradiating to *bcfg*: *a* and *deh* signals turned to a broad singlet. The *N*-monoacetyl derivative was crystallized as the dipicrate, m.pt 173–173.5°C, C<sub>10</sub>H<sub>24</sub>N<sub>7</sub>·CH<sub>3</sub>CO·2(C<sub>6</sub>H<sub>3</sub>N<sub>3</sub>O<sub>7</sub>). In this monoacetate the signal of *a* in the previous compound shifted to lower field and was assigned to the methylene adjacent to the acetylated amino group. The treatment of the B<sub>4</sub> sulphate in 1N sodium hydroxide at 105°C for 45 hours gave a urea derivative (Figure 6), putrescine and ammonia. The urea derivative was crystallized as its dipicrate,



NATURAL AND ARTIFICIAL BLEOMYCINS

$C_9H_{22}N_4 \cdot 2(C_6H_3N_3O_7)$  and it was converted to its crystalline dihydrochloride, m.pt  $171^\circ C$  (dec),  $C_9H_{22}N_4O \cdot 2HCl$ . The bands at  $1647\text{ cm}^{-1}$  and  $1582\text{ cm}^{-1}$  indicated the presence of the urea group. Further hydrolysis in 1N sodium hydroxide at  $105^\circ C$  gave putrescine (1,4-diaminobutane). The urea compound was resistant to treatment in 6N hydrochloric acid at  $105^\circ C$  for 24 hours. Thus, for the urea compound, *N,N'*-bis-4-aminobutylurea was proposed. Thus, we propose 1-(4-aminobutyl)-3-(4-guanidinobutyl)-guanidine for the amine of  $B_4$ .

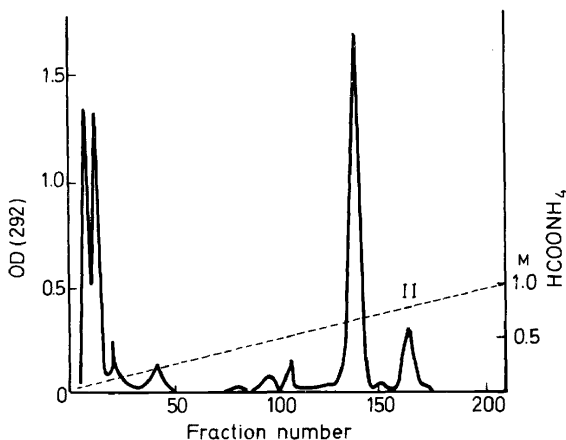
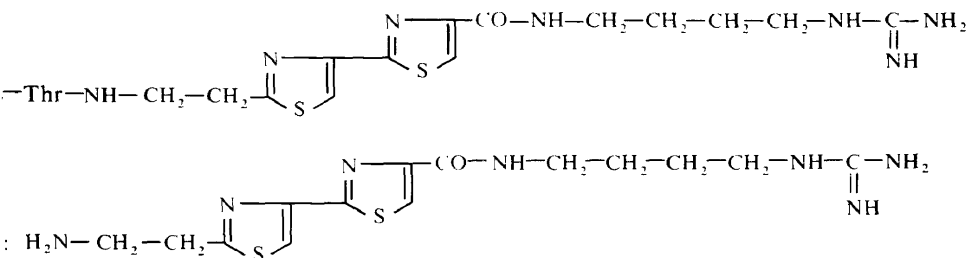
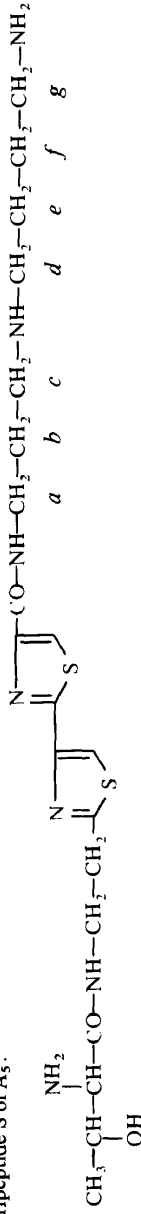


Figure 7. CM-sephadex C-25 chromatography of partial hydrolysate of bleomycin  $B_2$ .



To obtain the tripeptides containing the amine from bleomycins  $A_1$ , demethyl  $A_2$ ,  $A_2$ ,  $B_2$ ,  $A_5$ ,  $B_4$  and  $A_6$ , hydrolysis in concentrated hydrochloric acid (12N), at  $27^\circ C$  for 2-3 days, was the most suitable condition. The hydrolysate was evaporated to dryness and was subjected to column chromatography on CM-Sephadex C-25 which was treated with 0.05M ammonium formate. The chromatogram was developed, raising the concentration of ammonium formate from 0.05M to 1.0M continuously. The optical density of the eluate at  $292\text{ m}\mu$  was determined for each fraction. The pattern of the hydrolysate of  $B_2$  is shown in Figure 7. The penultimate peak corresponds to the tripeptide consisting of L-threonine (I), the bithiazole amino acid (VI) and the amine, and the last one corresponds to the dipeptide consisting of the bithiazole amino acid and the amine.

The tripeptide S of A<sub>5</sub>:



n.m.r. in D<sub>2</sub>O:

a: 3.98 (t); b: 2.50 (q); c: 3.51 (t);  
 d, g: 3.59 (t); e, f: 2.24 (q)

The amine of deamino-A<sub>5</sub>:



A<sub>5</sub>-*Serratia marcescens*-A<sub>2</sub>-b

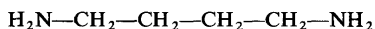
Figure 8

NATURAL AND ARTIFICIAL BLEOMYCINS

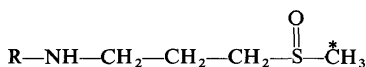
Figure 7 indicates that the tripeptide or the dipeptide of B<sub>2</sub> containing the amine appears in the later fraction. In the case of B<sub>4</sub> containing the more basic amine, it was necessary to use 2M ammonium formate for elution of the tri- and dipeptides.

Here we describe the isolation of the tripeptide from bleomycin A<sub>5</sub>. The tripeptide appeared in the fractions eluted with 0.91–1.0M ammonium formate and crystallized from aqueous ethanol as the trihydrochloride, m.pt 196–197°C (dec), C<sub>20</sub>H<sub>33</sub>N<sub>7</sub>O<sub>3</sub>S<sub>2</sub>·3HCl. The dipeptide consisting of the bithiazole amino acid and spermidine was obtained from a peak which appeared in the eluate with 1.0M ammonium formate. The total hydrolysis of the tripeptide gave L-threonine (I), the bithiazole amino acid (VI) and spermidine. The hydrolysis of the tripeptide in 6N hydrochloric acid at 105°C for 1 hour gave a dipeptide which was separated by high voltage paper electrophoresis (R<sub>m</sub> 0.56, taking L-alanine as 1.0). The amino terminal was shown to be L-threonine and total hydrolysis gave L-threonine and the bithiazole amino acid. Thus, the structure of the tripeptide obtained by partial hydrolysis of A<sub>5</sub> is shown in Figure 8. However, it must be determined which amino group of spermidine forms the amide bond with the carboxyl group of the bithiazole amino acid. The coupling of the middle methylene protons of the trimethylene group with methylene adjacent to the amide was shown by irradiating to 2.50δ. The triplet at 3.98δ became the singlet. In spermidine, the signal of this methylene appeared at 3.70δ. The shift to 3.98δ suggests that the amino group adjacent to this methylene participates in binding with the bithiazole amino acid. Bleomycin A<sub>5</sub> was deaminated by treatment with sodium nitrite, deaminobleomycin A<sub>5</sub> was hydrolysed in 6N HCl at 105°C for 30 hours and deaminospermidine was crystallised as the picrate, m.pt 166–167°C, C<sub>7</sub>H<sub>18</sub>N<sub>2</sub>O(C<sub>6</sub>H<sub>3</sub>N<sub>3</sub>O<sub>7</sub>)<sub>2</sub>. This deaminospermidine was compared with N-(3-hydroxypropyl)-1,4-diaminobutane and 4-(3-aminopropyl-amino)-1-butanol by infra-red spectrum, melting point, behaviour on high voltage electrophoresis and thin layer chromatography, and the identity with 4-(3-aminopropyl)-1-butanol was confirmed. Thus, the amino group of spermidine binding with the carboxyl group of the bithiazole amino acid was determined. Bleomycin A<sub>5</sub> (50 mg, copper-free) was converted to another bleomycin by shaking with dried cells (50 mg) of *Serratia marcescens* in phosphate buffer at pH 6.8 for three days. After this reaction, this bleomycin was purified by CM-Sephadex C-25 chromatography, raising the concentration of ammonium formate from 0.05M to 1.0M. The amine part of this bleomycin was found to be 1,3-diaminopropane.

The amine of A<sub>2</sub>-a:



Bleomycin A<sub>1</sub>:



\* 3.20 δ (s)

The amine of A<sub>2</sub>-b:



Bleomycin demethyl-A<sub>2</sub>:



\* 2.50 δ (s)

Figure 9

Bleomycin A<sub>2</sub>-a and A<sub>2</sub>-b were hydrolysed and the amines were separated and identified as 1,4-diaminobutane and 1,3-diaminopropane respectively.

After A<sub>2</sub> had been kept in the air for a year, chromatographic analysis indicated the appearance of A<sub>1</sub> and demethyl-A<sub>2</sub>. The methyl signal appeared at 3.20δ in the n.m.r. spectrum of A<sub>1</sub> and at 2.50δ for demethyl-A<sub>2</sub>. We confirmed the methyl signal of dimethylsulphoxide at 3.20δ and the methyl signal of dimethylsulphide at 2.50δ. Thus, these n.m.r. data suggested the amines of A<sub>1</sub> and demethyl-A<sub>2</sub> as those shown in Figure 9. Recently we succeeded in obtaining A<sub>2</sub> from demethyl-A<sub>2</sub> by the reaction with methyl iodide and we were able to obtain A<sub>1</sub> by treatment of demethyl-A<sub>2</sub> with hydrogen peroxide at room temperature for 30 minutes. Pyrolysis of A<sub>2</sub> at 100°C for several hours gave demethyl-A<sub>2</sub>. Thus, the amine parts of A<sub>1</sub> and demethyl-A<sub>2</sub> were determined.

The differences in the amine moieties of natural bleomycins suggested to us that addition of an amine to the fermentation medium would induce the preferential production of the bleomycin containing the amine added. We thought also that it would be possible to produce artificial bleomycins by adding amines which were not in natural bleomycins. In fact, we have obtained the results expected.

The one methyl carbon ( $2 \times 10^8$  cpm)-labelled 3-aminopropyldimethylsulphonium (90 mg) was added to one hundred millilitres medium at 48h of the shaking culture of *S. verticillus* and it was further shake-cultured for six days. The bleomycin was extracted by IRC-50(H) column chromatography, carbon chromatography and alumina chromatography and the bleomycin mixture obtained was analysed by CM-Sephadex C-25 column chromatography. As shown in Figure 10, if the result is compared with that in Figure 1, bleomycin A<sub>2</sub> (the total counts  $6.9 \times 10^6$  cpm) was preferentially produced. Another radioactive peak shows no absorption at 292 mμ and

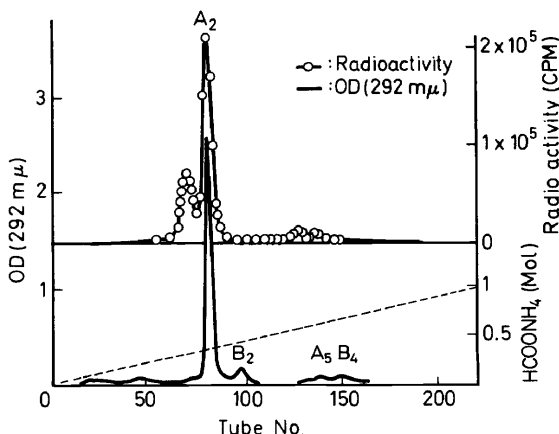
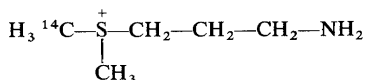


Figure 10. CM-sephadex C-25 chromatography of bleomycins produced by addition of



NATURAL AND ARTIFICIAL BLEOMYCINS

Bleomycins produced

Amines added to media

$\text{H}_2\text{N}-\text{CH}_2-\text{CH}_2-\text{CH}_2-\text{CH}_2-\text{SO}-\text{CH}_3$ $\text{H}_2\text{N}-\text{CH}_2-\text{CH}_2-\text{CH}_2-\text{CH}_2-\text{S}-\text{CH}_3$ $\text{CH}_3$	8 mg/ml 5 mg/ml	Same as the control† Same as the control
$\text{H}_2\text{N}-\text{CH}_2-\text{CH}_2-\text{CH}_2-\text{S}^+$ $\text{CH}_3$	1 mg/ml	81.7% was A <sub>2</sub>
$\text{H}_2\text{N}-\text{CH}_2-\text{CH}_2-\text{CH}_2-\text{CH}_2-\text{CH}_2-\text{NH}_2$ $\text{H}_2\text{N}-\text{CH}_2-\text{CH}_2-\text{CH}_2-\text{CH}_2-\text{NH}_2$ $\text{H}_2\text{N}-\text{CH}_2-\text{CH}_2-\text{CH}_2-\text{CH}_2-\text{NH}_2$ $\text{H}_2\text{N}-\text{CH}_2-\text{CH}_2-\text{CH}_2-\text{CH}_2-\text{NH}-\text{C}(=\text{NH})-\text{NH}_2$	4 mg/ml 2 mg/ml 2 mg/ml	Per cent of A <sub>2</sub> -a was increased to 23.5% Per cent of A <sub>2</sub> -b was increased to 84.5% B <sub>2</sub> was 45%; B <sub>1</sub> was 34.4%
$\text{H}_2\text{N}-\text{CH}_2-\text{CH}_2-\text{CH}_2-\text{CH}_2-\text{NH}-\text{C}(=\text{NH})-\text{NH}_2$ $\text{H}_2\text{N}-\text{CH}_2-\text{CH}_2-\text{CH}_2-\text{CH}_2-\text{NH}-\text{C}(=\text{NH})-\text{NH}_2$	0.53 mg/ml	B <sub>4</sub> was increased to 38.3% A new bleomycin was about 40% B <sub>6</sub> was increased. A <sub>5</sub> was 100% A <sub>5</sub> was 100%
Spermidine Spermine	0.36 mg/ml 0.5 mg/ml	

† Control is the fermentation without addition of the amine. Amount of each bleomycin produced: A<sub>1</sub> 9.19%; demethyl-A<sub>2</sub> 2.36%; A<sub>2</sub> 54.53%; sum of A<sub>2</sub>-a and A<sub>2</sub>-b 3.63%; B<sub>2</sub> 26.66%; A<sub>3</sub> 1.25%; B<sub>4</sub> 2.39%; A<sub>4</sub> trace; B<sub>6</sub> trace.

Table 2. Artificial bleomycins† and their activities against microorganisms and ascites form of Ehrlich carcinoma

The amine moiety	u/mg against <i>Mycob. 607</i> †	u/mg against <i>B. subtilis</i> ‡	ED <sub>50</sub> against Ehrlich carcinoma mg/kg/day	Therapeutic index
$-\text{HN}-\text{CH}_2-\text{CH}_2-\text{CH}_2-\text{CH}_2-\text{NH}-\text{CH}_3$ $-\text{HN}-\text{CH}_2-\text{CH}_2-\text{CH}_2-\text{NH}-\text{CH}_2-\text{CH}_2-\text{O}-\text{CH}_3$	860 1330	1070 1110	0.19 0.19	13.6 39.5
$-\text{HN}-\text{CH}_2-\text{CH}_2-\text{CH}_2-\text{NH}-\text{CH}(\text{CH}_3)-\text{C}_6\text{H}_5$	5030	1660	0.19	59.2
$-\text{HN}-\text{CH}_2-\text{CH}_2-\text{CH}_2-\text{N}(\text{CH}_3)-\text{CH}_2-\text{CH}_2-\text{NH}_2$ $-\text{HN}-\text{CH}_2-\text{CH}_2-\text{CH}_2-\text{NH}-\text{CH}_2-\text{CH}_2-\text{N}(\text{CH}_3)_2$	1670 1620	6830 9600	0.19 0.19	49.3 54.3
$-\text{HN}-\text{CH}_2-\text{CH}_2-\text{CH}_2-\text{NH}-\text{CH}_2-\text{CH}_2-\text{NH}-\text{C}(=\text{NH})-\text{NH}_2$ $\text{CH}_2-\text{CH}_2-\text{CH}_2-\text{CH}_3$	5840	18700	0.19	59.2
$-\text{HN}-\text{CH}_2-\text{CH}_2-\text{CH}_2-\text{NH}-\text{CH}_2-\text{CH}_2-\text{NH}-\text{C}_6\text{H}_5$	10700	23000	<0.19	>57.6

† Copper-chelated form. ‡ A<sub>1</sub> (910 u/mg) is used as the standard.

therefore it is not a bleomycin. Among the other two small radioactive peaks, peak IV may be a new bleomycin because it was also detected by absorption at 292 m $\mu$ .

The bleomycins produced by addition of an amine to the fermentation medium were analysed by CM-Sephadex C-25 column chromatography. The results are summarized in *Table 1*. Addition of 3-aminopropyl-methylsulphoxide or 3-aminopropyl-methylsulphide did not increase the production of the bleomycins containing these amines. The chromatographic patterns were almost similar to those of bleomycins produced without addition of an amine. Addition of 3-aminopropyl-dimethylsulphonium increased the production of A<sub>2</sub>. Addition of putrescine increased the production of the corresponding bleomycin. However, the precursor effect of 1,3-diaminopropane was more marked than putrescine. It can be thought that putrescine is more easily metabolized than 1,3-diaminopropane. Addition of agmatine increased the production of B<sub>2</sub> and B<sub>1</sub>'. This result suggests that bleomycin B<sub>1</sub>' might contain the amine which could be produced from agmatine. Addition of the amine of B<sub>4</sub> increased B<sub>4</sub> markedly because, without addition of the amine, content of B<sub>4</sub> among bleomycins produced was 2.39 per cent and was increased to 45 per cent by addition of the amine. B<sub>6</sub> seemed to be slightly increased by addition of the amine of B<sub>4</sub>. Another unidentified bleomycin was produced by addition of the amine of B<sub>4</sub>, and its proportion amongst bleomycins was about 40 per cent. This bleomycin appeared in fractions corresponding to B<sub>2</sub> during the CM-Sephadex C-25 chromatography but it was negative in Sakaguchi reaction. It is thought to contain the amine which might be produced by metabolism of the amine of the material B<sub>4</sub>.

By addition of spermidine at 360  $\mu$ g/ml to the medium, production of bleomycins other than bleomycin A<sub>5</sub> was almost completely suppressed and, in the medium, A<sub>5</sub> almost alone was produced. Addition of spermine did not cause the production of A<sub>6</sub> but A<sub>5</sub> was produced. It can be thought that spermine was converted to spermidine before incorporation into the bleomycin. Thus, we found that the addition of an amine increases the proportion of the bleomycin containing that amine added amongst the bleomycins produced and, in the case when the amine is metabolized to another amine, the bleomycin containing the latter is produced.

Ethylamine added to the medium is taken into the bleomycin and thirty per cent of the bleomycins produced was the bleomycin containing this amine. Diaminoethane, 1,3-diaminopropane and their *N*-alkyl or *N*-amino-alkyl derivatives were more effective precursors and the bleomycins containing these amines were exclusively produced in the fermentation media. More than forty-two bleomycins have been prepared by Nakayama and others, in the Research Laboratories of Nippon Kayaku Co., and their activities have been studied by Matsuda and others in that company. The activities against *Mycobacterium 607* and *B. subtilis*, 50 per cent effective daily dose for ten days and therapeutic indices against the ascites form of Ehrlich carcinoma of seven artificial bleomycins, in the copper-chelated state, are shown in *Table 2*.

In general, bleomycins containing diamines are less effective against microorganisms than those containing triamines. However, in general, a

NATURAL AND ARTIFICIAL BLEOMYCINS

50 per cent effective dose against the ascites form of Ehrlich carcinoma was 0.19–0.39 mg/kg. The 50 per cent lethal daily dose for 10 days varied and the therapeutic indices were generally higher in bleomycins containing triamines than in those containing diamines. It is hard to find a rule among the structure-activity relationships from the present biological data. Besides the fundamental pharmacological data and the data of testing the effect against experimental animal tumours, information on the mechanism of action and the mechanism of the selective effect against squamous cell carcinoma would be useful.

As we have reported<sup>9-11</sup>, bleomycins react with DNA and cause strand scission. This reaction is promoted by a sulphhydryl compound, hydrogen peroxide, ascorbic acid etc. Recently, Ono *et al.*, Cancer Research Institute, Tokyo, reported the inhibition of ligase at a lower concentration of bleomycin than that shown by DNA strand scission, though the possibility still remained that the effect on the ligase was due to DNA strand scission. The selective effect of bleomycin on squamous cell carcinoma has been shown by Ichikawa *et al.*, testing the effect on squamous cell carcinoma and sarcoma of mice induced by 20-methylcholanthrene. Bleomycin A<sub>2</sub> was effective on this squamous cell carcinoma but not on the sarcoma. We found that bleomycin A<sub>2</sub> is taken by this carcinoma at 4 times higher concentration than by the sarcoma, and in the carcinoma 60 per cent of bleomycin A<sub>2</sub> remained in the active form after one hour of the injection, but none of the active form was detected in the sarcoma. Thus, the selective effect on the squamous cell carcinoma was shown to be due to the high concentration of bleomycin in this tumour and the low activity of this tumour to inactivate bleomycin. Rapid inactivation of bleomycin in liver, kidney etc., other than in lung and skin, was shown by our experiments, testing the distribution of <sup>3</sup>H-bleomycin among organs. Recently we found an enzyme in tissues to inactivate bleomycin. The distribution of this enzyme must be one mechanism of the selective effect.

Table 3. Inactivation of various bleomycins by homogenates of various organs of mice

Bleomycins†	Lung	Skin	Remained per cent		
			Liver	Spleen	Kidney
A <sub>2</sub>	88.3	72.3	29.5	47.3	25.5
A <sub>5</sub>	74.2	61.5	28.7	41.5	35.6
B <sub>2</sub>	40.2	34.2	6.8	18.4	5.0
B <sub>4</sub>	61.3	46.7	16	27.7	16.3

† All copper-free

Inactivation of bleomycin A<sub>2</sub>, B<sub>2</sub>, A<sub>5</sub> and B<sub>4</sub> by homogenates of liver, kidney, spleen, lung and skin of mice is shown in Table 3. Each organ was homogenized with phosphate buffer (pH 6.8, 1/15M) of 3 times the weight of the organ, centrifuged at 900 *g* and bleomycin was added at 1000 µg/ml. After 30 minutes at 37°C, the residual bleomycin was determined by the paper disc method. The faster inactivation in liver, kidney and spleen, than in lung and skin, conforms with the results obtained by injection of <sup>3</sup>H-bleomycin A<sub>2</sub>. In the case when <sup>3</sup>H-bleomycin A<sub>2</sub> was injected and after one hour bleomycin A<sub>2</sub> in organs was determined by the radioactivity and

the antibacterial activity; the presence of inactivated bleomycin A<sub>2</sub> in all organs was shown by the radioactivity but the bleomycin A<sub>2</sub> shown by the antibacterial activity was found only in the lung, skin and kidney. The bleomycin A<sub>2</sub> in kidney is due to the urine containing this antibiotic at a high concentration. Thus, the rapid inactivation in organs, except lung and skin, *in vivo* was shown by the distribution of <sup>3</sup>H-bleomycin A<sub>2</sub>. Therefore, the mechanism of inactivation by organ homogenates must give useful information on the mechanism of the selective activity.

Table 4. Partial purification of the enzyme to inactivate bleomycin

	Total protein mg	Total unit	Specific act.	Yield %	Fold
Crude ext. 10000 G <sup>1</sup>	1960	1984	1.01	100	1.0
105000 G <sup>2</sup>	1393	1991	1.43	100	1.4
Protamine sulphate <sup>3</sup>	1400	1911	1.36	96.3	1.36
Ammonium sulphate 35-60%	531	1715	3.2	86.4	3.2
Sephadex G-25 <sup>4</sup>	314	850	2.7	42.9	2.7
DEAE cellulose <sup>5</sup>	70	810	11.6	40.8	11.5

<sup>1</sup> 98 ml. <sup>2</sup> 81 ml. <sup>3</sup> Protamine (55.7 mg) was added and centrifuged at 10000 *g* for 20 minutes. <sup>4</sup> Solvent: 1/15 M phosphate buffer at pH 7.2. <sup>5</sup> The elution was carried out by raising the concentration of sodium chloride in 5 mM phosphate buffer at pH 7.2 from 0 to 0.15 M.

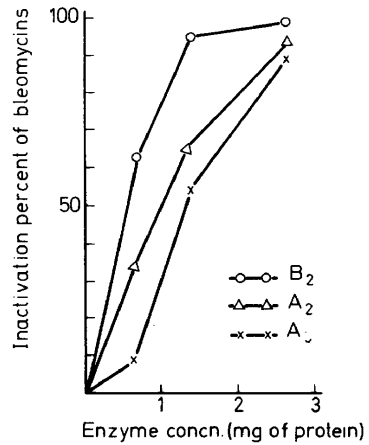
We could isolate an enzyme from various organs to inactivate bleomycins. Tentatively we defined a unit of the enzyme activity as follows: the enzyme was incubated with 100-250 µg/ml of bleomycin B<sub>2</sub> in 1/15M phosphate buffer at pH 7.2 for 60 minutes, and the enzyme activity to inactivate 1 µg of bleomycin B<sub>2</sub> per minute was defined as one unit. Starting from 23 g of mice liver which was homogenized with 70 ml of 1/15M phosphate buffer, 70 mg, as protein, of the partially purified enzyme was prepared by the procedure shown in Table 4.

The optimum pH of the enzyme was 7-7.5, and the optimum temperature was around 37°C. This enzyme, purified by DEAE cellulose chromatography, was very unstable. Therefore, the enzyme obtained by Sephadex G-25 chromatography was employed for the experiments. The standard condition was as follows: 1.2 mg of the enzyme, as protein, was incubated with 100 µg/ml of a bleomycin and after 60 minutes the bleomycin, inactivated, was determined by the paper disc method using *B. subtilis*. As shown in Figure 11, B<sub>2</sub> was more rapidly inactivated than A<sub>2</sub> and A<sub>5</sub>.

After the inactivation, ammonia was determined by the Conway micro-diffusion method followed by titration or by spectrometry after ninhydrin reaction. Then, one mole of ammonia was confirmed to be released during the inactivation. We attempted to isolate the inactivated bleomycin B<sub>2</sub> to find the chemical difference from bleomycin B<sub>2</sub>. Two hundred mg of bleomycin B<sub>2</sub> was incubated with 40 ml of the enzyme solution (1600 mg as protein) and 60 ml of 1/15M phosphate buffer for one hour at 37°C. 100 ml of methanol was added and the supernatant was concentrated to 20 ml; the inactivated and the residual bleomycins were adsorbed on IRC-50 resin in H<sup>+</sup> form and eluted with 0.05N hydrochloric acid-acetone (1:1). After neutralization with IR-45 resin in OH<sup>-</sup> form, the eluate was evaporated

NATURAL AND ARTIFICIAL BLEOMYCINS

Figure 11. Inactivation of bleomycins B<sub>2</sub>, A<sub>2</sub> and A<sub>5</sub>. Reaction mixture: 100 µg/ml of bleomycin, 1/15M phosphate buffer at pH 7.2 and mg of enzyme indicated. Incubation: at 37°C for 60 minutes



*in vacuo* to dryness. The powder was dissolved in 50 ml of distilled water and passed through a CM-Sephadex C-25 column (240 ml) and the inactivated and the residual bleomycins were eluted by raising the concentration of ammonium formate from 0.05M to 1.0M. The pattern of the elution is shown in Figure 12. The eluate was treated by carbon chromatography using 0.02M hydrochloric acid-*n*-propanol (1:1) to remove ammonium formate and the eluate was neutralized with IR-45 resin and evaporated to dryness, yielding 132 mg of the inactivated bleomycin B<sub>2</sub>.

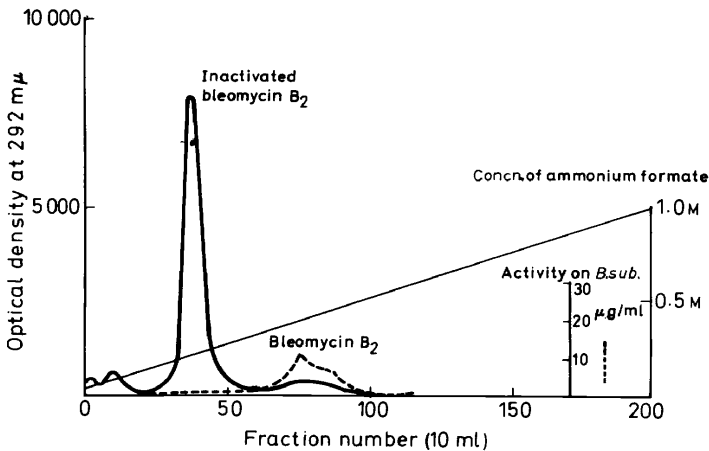


Figure 12. Separation of inactivated bleomycin B<sub>2</sub> and bleomycin B<sub>2</sub> by CM-Sephadex C-25

The antibacterial activity of the inactivated B<sub>2</sub> is shown in Table 5, compared with bleomycin B<sub>2</sub>. Except for the effect against *Mycobacterium 607* and *Salmonella enteritidis*, its solution at 100 µg/ml showed no antibacterial activity. It caused 50 per cent inhibition of Yoshida rat sarcoma cells at 200 µg/ml and bleomycin B<sub>2</sub> showed the inhibition at 2.5 µg/ml.

Table 5. Biological activity of bleomycin B<sub>2</sub> and the inactivated bleomycin B<sub>2</sub>.

## (a) Antimicrobial activity

Microorganisms	Minimum inhibition concentration (µg/ml)	
	Bleomycin B <sub>2</sub>	Inactivated bleomycin B <sub>2</sub>
<i>Alcalig. faecalis</i> IAM 105	0.05	> 100
<i>Bac. subtilis</i> PCI 219	0.39	> 100
<i>E. coli</i> K-12	0.39	> 100
<i>Mycob. smegmatis</i> ATCC 607	0.4	6.25
<i>Microc. flavus</i> FDA 16	12.5	> 100
<i>Sal. enteritidis</i> 1891	0.05	3.12
<i>Staph. aureus</i> FDA 209 P	3.12	> 100

(b) 50 per cent growth inhibition against Yoshida rat sarcoma cells *in vitro*

Bleomycin B <sub>2</sub>	Inactivated bleomycin B <sub>2</sub>
2.5 µg/ml	200 µg/ml

The difference between the inactivated B<sub>2</sub> and bleomycin B<sub>2</sub> was found in the slightly lower basicity shown by high voltage electrophoresis. Hydrolysis in 6N hydrochloric acid for 24 hours gave all the degradation products obtained from bleomycin B<sub>2</sub>. As already described, during the enzymatic inactivation, one mole of ammonia was released. Therefore, the sugar moiety was tested by gas chromatography of the trimethylsilyl derivatives of the methanolysis products. The carbamoyl group was also confirmed in the inactivated bleomycin B<sub>2</sub>. These data suggest that ammonia would be released from carboxyl amide. However, we have not yet obtained direct proof of a free carboxyl group in the inactivated bleomycin.

*In vivo*, bleomycin A<sub>5</sub> is rapidly inactivated, but in tissue homogenates it is much more resistant than bleomycin B<sub>2</sub>. This suggests the presence of another enzyme reducing the activity of A<sub>5</sub>. However, the enzyme that was described above is thought to have a role in selective toxicity because this enzyme is strong in liver, kidney, and spleen and weak in skin and lung and inactivates all the bleomycins tested.

## REFERENCES

- <sup>1</sup> K. Maeda, H. Kosaka, K. Yagishita and H. Umezawa. *J. Antibiotics (Tokyo)* **9A**, 82 (1956).
- <sup>2</sup> T. Ikekawa, F. Iwami, H. Hiranaka and H. Umezawa. *J. Antibiotics (Tokyo)* **17A**, 194 (1964).
- <sup>3</sup> H. Umezawa, K. Maeda, T. Takeuchi and Y. Okami. *J. Antibiotics (Tokyo)* **19A**, 200 (1966).
- <sup>4</sup> H. Umezawa, Y. Suhara, T. Takita and K. Maeda. *J. Antibiotics (Tokyo)* **19A**, 210 (1966).
- <sup>5</sup> T. Takita, Y. Muraoka, K. Maeda and H. Umezawa. *J. Antibiotics (Tokyo)* **21**, 79 (1968).
- <sup>6</sup> G. Koyama, H. Nakamura, T. Muraoka, T. Takita, K. Maeda and H. Umezawa. *Tetrahedron Letters* **44**, 4635 (1968).
- <sup>7</sup> T. Takita, K. Maeda, H. Umezawa, S. Omoto and S. Umezawa. *J. Antibiotics (Tokyo)* **22**, 237 (1969).
- <sup>8</sup> Y. Muraoka, T. Takita, K. Maeda and H. Umezawa. *J. Antibiotics (Tokyo)* **23**, 252 (1970).
- <sup>9</sup> K. Nagai, H. Suzuki, N. Tanaka and H. Umezawa. *J. Antibiotics (Tokyo)* **22**, 624 (1969).
- <sup>10</sup> H. Suzuki, K. Nagai, H. Yamaki, N. Tanaka and H. Umezawa. *J. Antibiotics (Tokyo)* **22**, 446 (1969).
- <sup>11</sup> T. Terashima, M. Yasukawa and H. Umezawa. *Gann* **61**, 513 (1970).