CHEMISTRY OF BLEOMYCIN. XXII

INTERACTION OF BLEOMYCIN WITH NUCLEIC ACIDS, PREFERENTIAL BINDING TO GUANINE BASE AND ELECTROSTATIC EFFECT OF THE TERMINAL AMINE

Sir:

The bleomycins are glycopeptide antibiotics isolated from *Streptomyces verticillus*[1]. This family of antibiotics exhibits high antitumor activities and has been used in the treatment of human neoplasms. Bleomycin (BLM) causes strand scission of DNA *in vitro* as well as *in vivo*. We have studied BLM binding to various nucleic acids using fluorescence spectroscopy technique which was introduced by CHIEN *et al.*[2]

Uncorrected fluorescence emission spectra were taken with a Hitachi fluorescence spectrometer, Model MPF-4. All fluorescence spectra were measured in 15 mM Tris-HCl buffer (pH 7.6) containing 15 mM NaCl and 1.5 mM EDTA, at 20°C, in a cuvette with a 1-cm light path. To confirm the assignment of the fluorescence emission maximum of BLMs at about 350 nm to the bithiazole chromophore[2] and to characterize the intensity of the fluorescence, the spectra (Fig. 1) of BLMs in metal-free and copper-chelated forms, copper-complex of bleomycinic acid (BLM-acid)[3,4], N-acetyl-VI-amide[5] (the smallest model compound for the bithiazole chromophore of BLM, see Fig. 2) and P-5m (a biosynthetic intermediate of BLM, unpublished, see Fig. 2) in metal-free and copper-chelated forms were taken by excitation at 290 nm. All compounds containing the bithiazole chromophore showed maxima at about 350 nm although they had different fluorescence intensity, whereas P-5m which does not contain the bithiazole chromophore did not show the maximum at 350 nm. BLMs which contained different terminal amines showed the same intensity. A metal-free BLM showed a stronger intensity than its copper-chelated form. This is probably due to quenching by copper. This was confirmed by specific interaction between the chelated copper and the bithiazole chromophore shown by ^{1}H-NMR spectroscopy. That is: a well-resolved ^{1}H-NMR spectrum of metal-free BLM A2 (0.02 M in D_2O at 30°C, Fig. 3-c) did not change significantly by addition of 10% molar amount of BLM A2 Cu-complex except for the bithiazole protons at δ

Fig. 1. Fluorescence spectra of bleomycins and their related compounds

Concentration of samples 7.5×10^{-6} M in 1.5 mM EDTA, 15 mM NaCl, 15 mM Tris-HCl (pH 7.6), excitation at 290 nm. (+Cu) means copper-complex and the others are metal-free.

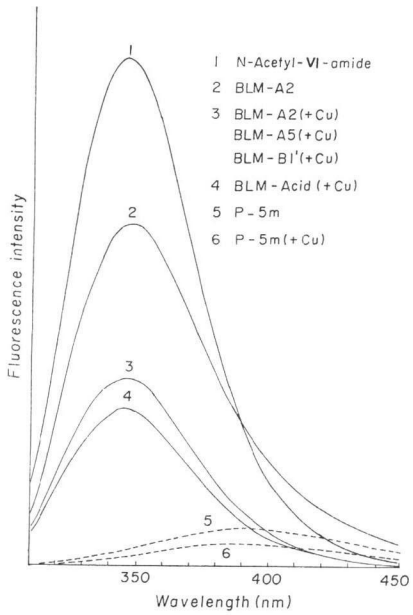
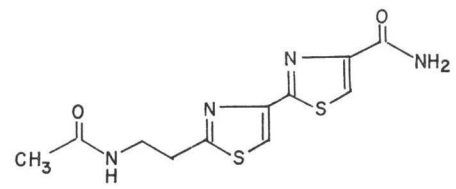


Fig. 2. Structures of N-acetyl-VI-amide and P-5m



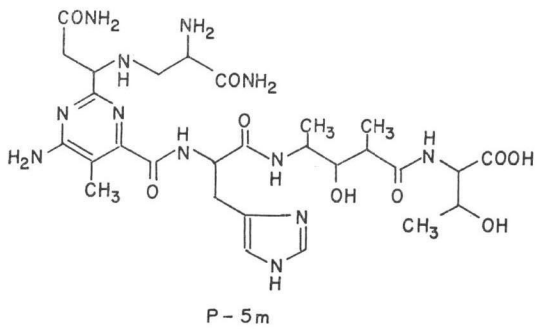
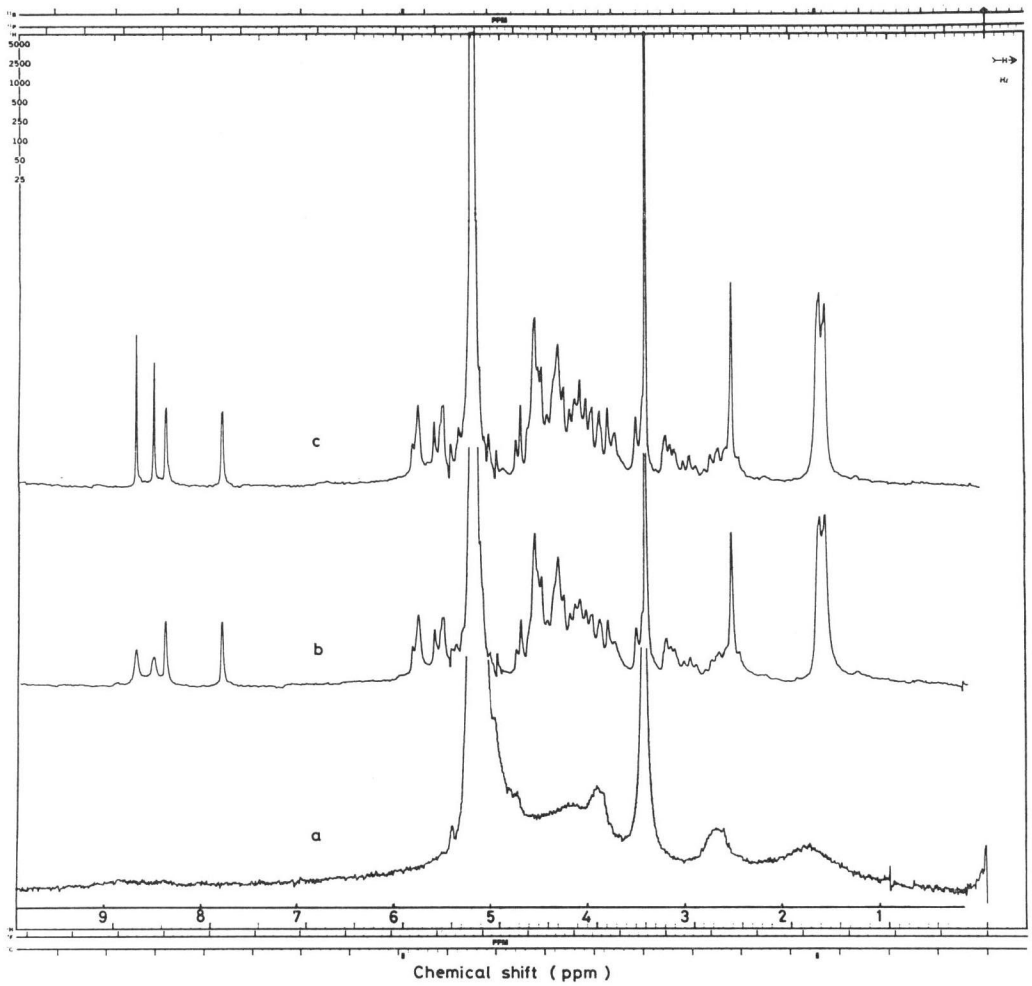


Fig. 3. 100 MHz ^{1}H-NMR spectra of metal-free BLM A2, Cu-chelated BLM A2 and their mixture at 30°C
a) Cu-chelated BLM A2 (0.02 M in D_2O)
b) metal-free BLM A2: Cu-chelated BLM A2=10: 1
c) metal-free BLM A2 (0.02 M in D_2O)
The pH was adjusted to 6.0 before dissolving in D_2O. External TMS reference.



8.49 and 8.68 (external TMS reference)[6] which broadened significantly (Fig. 3-b). The broadened signals were sharpened by elevation of the measurement temperature due to increased exchange rate. Incidentally, BLM A2 Cu-complex gave an unresolved broad ^{1}H-NMR spectrum except for the S-dimethyl signal (δ 3.42) of the terminal amine due to paramagnetic effect of the copper (II) (Fig. 3-a).

The difference of fluorescence intensity between N-acetyl-VI-amide and metal-free BLM (Fig. 1) suggested intramolecular quenching in metal-free BLM.

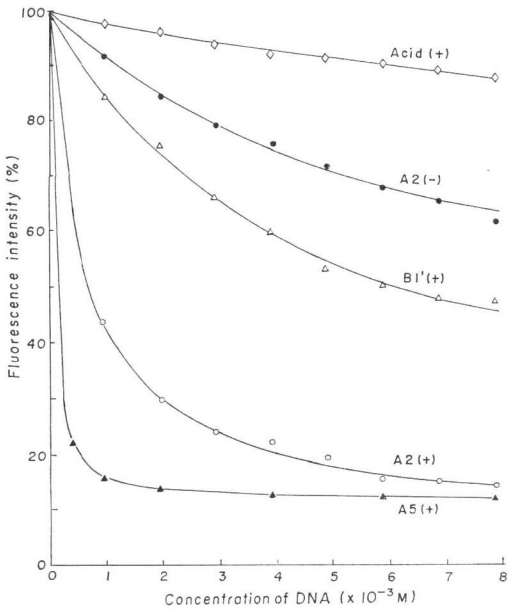
Association constant (K) and maximum binding site (n) of BLMs to nucleic acids were calculated as follows[7]: If each binding site of nucleic acids interacts with each BLM molecule with an equal affinity, the association constant, K, can be expressed by

$$K=\frac{[Bb]}{(n[P]-[Bb])[Bf]} \quad (1)$$

where $[Bb]$, $[Bf]$ and $[P]$ represent the concentration of bound BLM, free BLM and nucleic acid phosphate; n is the maximum number of binding sites per phosphate of nucleic acid. The equa-

Fig. 4. Fluorescence quenching at 350 nm of bleomycins by addition of various amount of calf thymus DNA

Concentration of bleomycins 7.5×10^{-6} M, excitation at 310 nm. (+) means copper-complex and (−) means metal-free.



tion (1) can be rearranged to the following:

$$\frac{1}{[Bf]}=K\left(n\frac{[P]}{[Bb]}-1\right) \quad (2)$$

$[Bb]$ and $[Bf]$ were calculated from equation (3) and (4) as follows:

$$[Bb]=\frac{Fo-F}{Fo-Ff}[B] \quad (3)$$

$$[Bf]=\frac{F-Ff}{Fo-Ff}[B] \quad (4)$$

where $[B]$ represents the total concentration of BLM, Fo, F and Ff are the observed fluorescence of BLM in the absence, in the presence of an experimental amount and in the presence of an extreme excess amount of nucleic acid.

In the following experiments, the emission intensity at 350 nm was measured by excitation at 310 nm to minimize the absorption by nucleic acids. The fluorescence intensity of BLM-nucleic acid mixture was corrected by subtracting the fluorescence of blank solution (without BLM), and the fluorescence was measured immediately after mixing BLM and nucleic acid. Addition of calf thymus DNA to BLM quenches the fluorescence of BLM without changing the emission maximum wave length. The effect of various concentrations of the DNA on the fluorescence of BLMs was studied (Fig. 4). From these fluorescence quenching curves, we obtained $[Bb]$ and

Table 1. Association constant (K) and maximum binding site (n) of bleomycins to calf thymus DNA

Bleomycins	K	n
BLM A5(+Cu)	3.0×10^5	0.071
BLM A2(+Cu)	2.3×10^5	0.013
BLM A2(−Cu)	3.2×10^5	0.0024
BLM B1'(+Cu)	2.1×10^5	0.0034

Table 2. Fluorescence quenching of BLM A5 (+Cu) by addition of nucleic acids

Nucleic acids	Quenching(%)
5'-pA	0
5'-pU	0
5'-pG	4
5'-pC	0
5'-pdG	5
dG (deoxyguanosine)	0
G (guanosine)	0
Calf thymus DNA	56
Denatured calf thymus DNA*	65
poly(dG-dC)	64
poly dG·poly dC	61
poly(dA-dT)	31
poly dA·poly dT	0
poly G·poly C	51
poly A·poly U	35
poly I·poly C	18
$d(pTpG)_{6-9}$	29
$d(pCpA)_{6-9}$	13
$d(pTpG)_{6-9}+d(pCpA)_{6-9}$	32
poly A	39
poly U	14
Poly G	77
poly C	0
poly dA	18
poly dT	20
poly dG	80
poly dC	0

* hyperchromicity 31%

Concentrations of BLM A5(+Cu) and nucleic acids are 0.075×10^{-4} M and 1.22×10^{-4} M (P), respectively. Excitation at 310 nm, emission at 350 nm.

Fig. 5.

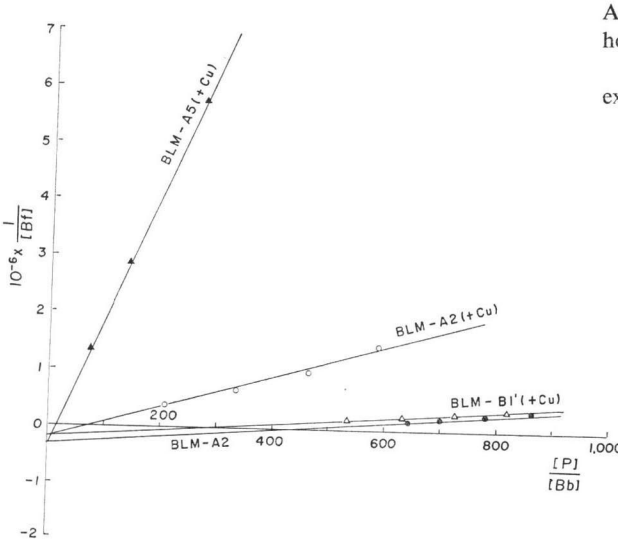
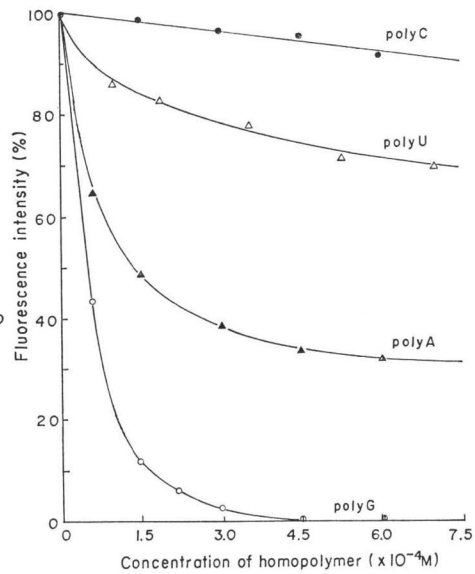


Fig. 6. Fluorescence quenching at 350 nm of BLM A5 (+Cu) by addition of various amount of ribohomopolymers

Concentration of BLM A5 (+Cu) 5.3×10^{-6} M, excitation at 310 nm.



[*Bf*] using equation (3) and (4), respectively. The equation (2) means that the plots of 1/[*Bf*] against [*P*]/[*Bb*] should be linear; the intercepts on 1/[*Bf*] axis and [*P*]/[*Bb*] axis represent $-K$ and $1/n$, respectively. Graphical applications of 1/[*Bf*] against [*P*]/[*Bb*] for these BLMs gave straight lines as shown in Fig. 5, from which K and n were obtained. The K- and n-values for BLM-acid could not be estimated due to the low quenching rate. The results are shown in Table 1. The association constants (K) of these BLMs were similar ($2.1\sim3.2\times10^5$), whereas the maximum numbers of binding sites (n) were different. The difference of the n-values among copper-chelated BLMs B1′, A2 and A5[8] suggests that the increase of the binding sites of DNA can be due to the positive charge at the terminal amine although the positive charge is not an absolute requirement for the binding as shown by the binding of BLM B1′. The charges at the terminal amine moieties of BLM-acid, BLMs B1′, A2 and A5 neutral pH are -1, 0, $+1$ and $+2$, respectively. Electrostatic interaction between the positive charge at the terminal amine of BLM and the negative charge of phosphate of DNA should facilitate BLM binding to DNA. Both copper-chelated and metal-free BLMs interacted with DNA, although the former did not cause strand scission of DNA.[9] The structure of copper-chelated BLM was recently presented by us[10]. Both the bithiazole chromophore and the terminal amine are not involved in the copper-chelation. It has also been shown that they are not involved in the reactive site of BLM to cleave DNA[10]. CHIEN *et al.*[2] suggested from their ^{1}H-NMR study that BLM binds to DNA most tightly with these two groups. These results suggest that the binding site of BLM to DNA is independent from the reaction site of BLM, leading to strand scission of DNA.

Table 3. Association constant (K) and maximum binding site (n) of BLM A5(+Cu) to ribohomopolymers

Ribohomopolymer	K	n
poly G	5.7×10^6	0.038
poly A	1.0×10^6	0.043
poly U	1.0×10^5	0.077

To investigate the binding specificity of BLM to nucleic acids, we compared the quenching rates of copper-chelated BLM A5 by addition of the same quantity of various nucleic acids (Table 2). Copper-chelated BLM A5 was chosen because of the highest sensitivity to the quenching (see Fig. 4) and no damage to nucleic acids[9]. Based on these quenching rates, one can roughly estimate the binding strength of BLM to nucleic acids. As shown in Table 2, 5′-mononucleosides except for 5′-GMP did not show any interaction with

BLM. 5′-GMP showed a weak effect but deoxyguanosine and guanosine did not show the effect. Double-stranded DNAs containing G-C base pairs [poly(dG–dC), poly dG•poly dC] had stronger interaction with BLM than those containing A-T base pairs [poly(dA–dT), poly dA•poly dT]. Heat-denatured calf thymus DNA showed almost the same interaction as the intact double-stranded DNA. Among eight ribo- and deoxyribo-homopolymers poly G and poly dG showed the strongest interaction. It was noticed that BLM bound to RNA, although as well known, BLM does not react with RNA.

To establish the relation between these quenching rates and association constants, we studied the fluorescence quenching of copper-chelated BLM A5 by using various amounts of ribohomopolymers, poly A, poly U, poly G and poly C (Fig. 6). Application of equations (2), (3), (4) gave K- and n-values for the binding of BLM A5 to the homopolymers (Fig. 6 and Table 3). The K- and n-values for poly C could not be estimated due to the low quenching rate. This result showed that K-value for poly G is 5.7 times larger than poly A and 57 times larger than poly U, although the n-values are in the same order. From Tables 1 and 3, it is deduced that the K-value is mainly dependent on nucleic acids and the n-value on BLMs.

From all the above-described experimental results, it can be concluded that the binding site of BLM to DNA is independent from the reaction site of BLM leading to strand scission of DNA. The bithiazole part of BLM preferentially binds to guanine base in nucleic acids, and the positive charge at the terminal amine facilitates BLM binding to nucleic acids.

Acknowledgements

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