Synthetic Analogues and Biosynthetic Intermediates of Bleomycin
METAL-BINDING, DIOXYGEN INTERACTION, AND IMPLICATION FOR THE ROLE OF FUNCTIONAL
GROUPS IN BLEOMYCIN ACTION MECHANISM*

(Received for publication, June 29, 1982)

Yukio Sugirač; and Tadashi Suzuki
From the Faculty of Pharmaceutical Sciences, Kyoto University, Kyoto 606, Japan

Masami Otsuka, Susumu Kobayashi, and Masaji Ohno
From the Faculty of Pharmaceutical Sciences, University of Tokyo, Hongo, Bunkyo-ku, Tokyo 113, Japan

Tomohisa Takita and Hamao Umezawa
From the Institute of Microbial Chemistry, Kamiosaki, Sinagawa-ku, Tokyo 141, Japan

In order to clarify the role of bleomycin functional groups in action mechanism, the metal-binding, dioxygen activation, and DNA cleavage of several synthetic analogues and biosynthetic intermediates of bleomycin have been investigated. The present results support that 1) the β-aminoalaninepyrimidine-β-hydroxyhistidine portion of the bleomycin molecule substantially participates in the Fe(II) and dioxygen interactions, 2) the transposition of the pyridine (or pyridine) and imidazole groups in the Fe(II)-coordination is essential for the effective binding and activation of molecular oxygen by the bleomycin ligands, and 3) the gulose-mannose moiety plays an important role as an environmental factor for the efficient dioxygen reduction and DNA cleavage, although the sugar portion does not contribute significantly to the nucleotide specificity in the DNA strand scission. Certain oligopeptides are able to mimic the metal-binding and dioxygen activation by bleomycin, but not induce the effective DNA cleavage. Probably, the bithiazole DNA interaction site of bleomycin delivers the iron/dioxygen chemistry to particularly the DNA G-C nucleotide sequences.

BLM* is an antitumor antibiotic clinically used in the treatment of squamous cell carcinoma, malignant lymphoma, and testis tumors (1). In the presence of Fe(II) ion and molecular oxygen, BLM causes DNA strand scission (2). This effect probably relates to the chemotherapeutic and toxic properties of the BLM antibiotics. At least, two characteristics are necessary for the antineoplastic action of BLM. The bithiazole and NH2-terminal residues contribute toward the binding to DNA (3). Although the β-aminoalaninepyrimidine-β-hydroxyhistidine portion appears to be capable of dioxygen activation by the chelation with ferrous ion (4), some possible transition metal-binding sites of BLM have been proposed on the basis of theoretical and spectroscopic investigations (5-7). Among them, the x-ray crystallographic analysis of biosynthetic intermediate P-3A-Cu(II) complex isolated from culture broth of BLM demonstrated the most direct evidence for the metal coordination sites (8), and also an acid hydrolysis product of the BLM-Co(III) complex was recently shown to be analogous to the structure of the P-3A-Cu(II) complex (9). However, these metal complexes are biologically inactive and do not activate molecular oxygen. The studies using depyruvamide BLM and N-acetyl BLM indicated that the α-amino group of the β-aminoalanine residue has an important effect on metal coordination, dioxygen activation, and DNA cleavage activity (10, 11). However, the role of the gulose-mannose sugar portion for the BLM action is uncertain. Herein, we wish to report the metal-binding, dioxygen interaction, and DNA strand scission of the synthetic analogues and biosynthetic intermediates of BLM, and to implicate the role of the respective functional groups in the BLM action mechanism. The BLM-related compounds as shown in Chart 1 are used in this paper.

EXPERIMENTAL PROCEDURES
Materials—The standard Fe(II) solution was prepared immediately before use from reagent-grade material, FeSO4·7H2O. Other divalent metal solutions were also made from reagent-grade metal nitrates. Deuterium oxide (99.75 atom % deuterium) and 3-(trimethylsilyl)propionic acid-d4 sodium salt were purchased from Merck, Japan. The isotope 57Fe (Fe57O4, 90.42%) was obtained from Oak Ridge National Laboratory and was reduced to metallic iron. The 2H-labeled sodium nitrite (99.2 atom %) was supplied from The British Oxygen Company. Calf thymus DNA and ethyl isocyanide were purchased from P-L Biochemicals and Sigma, respectively. All other reagents used were of commercial reagent grade. Purified BLM-A1 (R = -NH(CH2)5S(CH3)2) was prepared by Nippon Kayaku Co., Japan. The biosynthetic intermediates, P-3A and deglyco BLM-A2, were obtained from the culture broth of BLM fermentation and also from the mild acid hydrolysisate of BLM-A1 (12, 13). Recently, the total chemical syntheses of BLM-A1 and deglyco BLM-A2 were achieved by us (14, 15).

Synthesis of Model Ligands—PYML-1 (16) and its methyl ester (PYML-2) were synthesized as follows (see Scheme 1). Methyl 8-formylpyridine-2-carboxylate (1) (17) was treated with (S)-3-amino-2-[(tert-butoxycarbonyl)amino]propionamide (2) (18) in an equal molar ratio in CH3CN in the presence of an activated molecular sieve at

*This study was supported in part by a grant from the Ministry of Education, Science, and Culture, Japan. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1To whom all correspondence should be addressed at the Faculty of Pharmaceutical Sciences, Kyoto University, Kyoto 606, Japan.

2The abbreviations used are: BLM, bleomycin; PYML-1, N-[6-[(S)-2-amino-2-carbomoyl]lamine]methyl]pyridine-2-carboxamide; P-3A, pyrimidobolany1-ε-bistidyl-ε-alamine; BPN, N-tert-butyl-N-phenyltrinitrile; DMPO, 5,5-dimethyl-1-pyrroline-N-oxide; CD, circular dichroism; NHE, normal hydrogen electrode.
The role of functional groups in bleomycin action was studied.

Role of Functional Groups

NH₂, R: terminal amine

HO₂ANH₂ BLM

H₃P⁻3A

1329

q! + c₀₂R

n

H₂PYML - 1 R = H

PYML - 2 R = CH₃

CHART I

25 °C for 12 h. The resulting Schiff base (3) was hydrogenated over 5% Pd-C in MeOH, affording yellow foam (4) upon workup and chromatography on silica gel (eluted with 9:1 CH₂Cl₂-MeOH) (7: 85% yield from 5, m.p. 168-170 °C, [α]ᵢ₀ +60.1° (C, 0.1, CHCl₃)). The protective groups of 7 were removed with 30% HBr-AcOH (2.5 h at 25 °C), affording methyl ester (PYML-2) as a solid residue after workup. The methyl ester was hydrolyzed with 1 n NaOH at pH 9-10, and the solution was neutralized with 1 n HCl. After removal of the solvent, the residue was purified by Amberlite CG-50 (H⁺ form, eluted with 1% aqueous NH₃). Thus, yellow solid PYML-1 (8) was obtained in 87% yield upon usual workup (m.p. 120-122 °C, [α]ᵢ₀ +2.85° (C, 1, H₂O)).

PEML was synthesized as follows (see Scheme 2). Methyl 6-formylpyridine-2-carboxylate (1) was treated with a large excess of ethylenediamine in EtOH in the presence of an activated molecular sieve at 25 °C for 12 h. After filtration of the molecular sieve, the solution of the resulting Schiff base 9 was hydrogenated over 5% Pd-C. The crude hydrogenated product obtained by usual workup was treated with benzyl chloroformate (4 eq) in the presence of 1 n NaOH in CH₂Cl₂. Usual workup and chromatography on silica gel (eluted with 41% AcOEt-hexane) afforded crystalline tris(benzyloxycarbonyl) derivative 10 (59% from 1, m.p. 106-107 °C). The protective groups of 10 were removed with 30% HBr-AcOH. After evaporation of the solvent, the residue was purified by Diaion WK-10 (H⁺ form, eluted with 1% aqueous NH₃), affording hydroscopic yellow solid PEML (11) in quantitative yield. All new compounds were chromatographically homogeneous and gave satisfactory analytical and spectral data.

Preparation of Metal Complexes—The metal complexes of the synthetic analogues and the biosynthetic intermediates were prepared according to previously reported procedures for the BLM-metal complexes (20-22). For the electronic spectral measurements, the Fe(II) complexes with CO, C₂H₅NC, and NO were obtained anaerobically at pH 7.2 in a Thunberg cuvette equipped with a sidearm stoppered with a rubber septum. For the determinations of ESR, 'H NMR, and Mössbauer spectra, the Co(II), Fe(II), and Fe(III)-CO complexes were formed under the fully anaerobic condition which was achieved by using a vacuum line. The Fe(II)-NO complexes were prepared by addition of a few milligrams of sodium nitrite and sodium borohydride (or sodium dithionite) to the solution of the corresponding Fe(II)
complexes. Spin-trapping experiments using BPN (Aldrich) and DMPO (Aldrich) were carried out according to the previously reported procedure (23). DMPO was used after purification by filtration with charcoal.

**Physicochemical Measurements**—Electronic absorption and CD spectra were obtained on a Hitachi 330 recording spectrophotometer and a Jasco J-500AS spectropolarimeter, respectively. 220-MHz Fourier transform-1H NMR and X-band ESR spectra were recorded at 25 °C and 77 K with a Varian 220 and a JES-FE-3X spectrometer, respectively. Proton chemical shifts and \( g \)-values were determined from sodium \( 3-(\text{trimethylsilyl})\) propionate-\( d_4 \) (1 ppm) and 7,7,8,8-tetracyanoquinodimethane lithium salt (\( g \approx 2.0526 \)) respectively.

**DNA Cleavage Experiments**—Plasmid pBR 322 DNA was digested with \( \text{HindIII} \) (Takara), and the restriction fragment (396 base pairs) was isolated from a 5% polyacrylamide gel (24). Terminal phosphates were removed by treatment with bacterial alkaline phosphatase (Bethesda Research Laboratories), and the 5' ends were labeled with \( ^{32} \text{P} \) by using T4 polynucleotide kinase (Bethesda Research Laboratories) and \([\gamma-\text{P}]\text{ATP} \) (Amersham). This doubly end-labeled molecule was digested with \( \text{HindIII} \) (Takara), and the singly end-labeled 327-base pair fragment was isolated by electrophoresis on a 6% polyacrylamide gel. The restriction fragments of DNA were incubated with the BLM-related compounds, under the conditions described in the legends to the figure. Nucleotide sequences of the restriction fragments were determined as reported by Maxam and Gilbert (25). The present restriction fragment contains 327 bases and the 5' terminus corresponds to position 2845 in the pBR 322 DNA map.

**RESULTS**

**Visible, Circular Dichroism, and Redox Characteristics of Copper(II) Complexes**—Fig. 1 shows the visible absorption and CD spectra of the PYML-1-Cu(II) complex at pH 7.2. These electronic and CD features closely resemble those of naturally occurring BLM-Cu(II) complex which reveal an absorption maximum at 595 nm and CD extrema at 555 and 655 nm. The \( \lambda_{\text{max}} \) values of 597 and 395 nm indicate similar copper ligand fields for PYML-1 and BLM, because the magnitude of the ligand field around the central Cu(II) is reflected in the d\( _{2z^2} \)-d\( _{x^2-y^2} \) transition (26). The cyclic voltammogram of the PYML-1-Cu(II) complex exhibited a quasi-reversible 1-electron oxidation-reduction wave with an \( \text{E}_{1/2} \) value of ~319 mV versus the NHE. This redox potential also corresponds well to that (~327 mV versus NHE) of the BLM-Cu(II) complex (27). Table I summarizes the visible, CD spectral constants, and redox potentials for the Cu(II) complexes of PYML-1, PEML, P-3A, deglyco BLM, and BLM. The red shift of the \( \text{E}_{1/2} \) for the P-3A- and the deglyco BLM-Cu(II) complexes suggests a distortion from square-planar configuration of the Cu(II) site. Their Cu(II)/Cu(I) redox potentials are clearly higher than those of the Cu(II) complexes of PYML-1 and BLM. Indeed, the x-ray crystallographic result of the P-3A-Cu(II) complex demonstrated that the Cu(II) site is a distorted square-pyramidal structure with four chelate rings of 5-5-5-6 ring members coordinated by the \( \alpha \)-amino, secondary amine, pyrimidine ring, deprotonated peptide of histidine residue, and histidine imidazole nitrogens, and that the Cu(II) ion is displaced about 0.20 Å from the basal plane in the direction of the axial \( \alpha \)-amino nitrogen ligand (8). The longer wavelength shift of the d-d band and the positive shift of the \( \text{E}_{1/2} \) value for the deglyco BLM-Cu(II) complex indicate that the ligand field splitting is somewhat weakened in comparison with that in the BLM-Cu(II) complex. The high reduction potential of the PEML-Cu(II) complex is presumably attributed to the structural strain of the 5-5-5-6 chelate ring members.

**Electron Spin Resonance Features of Divalent Metal Complexes**—Fig. 2 displays the ESR spectral comparison for the Cu(II), Co(II), Co(II)-\( \text{O}_2 \), and Fe(II)-\( \text{tetracyanoquinodimethane lithium salt} \) complexes between PYML-1 and BLM. Table II summarizes the ESR parameters for the divalent metal complexes of PYML, PEML, P-3A, deglyco BLM, and BLM. As seen in Fig. 2A, the ESR spectra for the Cu(II) complexes of PYML and BLM were characterized by axially symmetric \( g \)- and A-tensor components, and both Cu(II) complexes evidently presented comparable ESR

![Scheme 2](attachment:Scheme2.png)
Role of Functional Groups in Bleomycin Action

FIG. 2. ESR spectra for Cu(I) (A), Co(I) (B), Co(II)-O2 (C), and Fe(II)-14NO (D) complexes of PYML-1 (left) and BLM (right) at 77 K.

parameters. A similar axially symmetric ESR feature was also observed for the PEML-Cu(II) complex, although the increasing $g_1$ value and the decreasing $A_z$ value are noted in this case. However, the ESR spectra for the Cu(II) complexes of P-3A and deglyco BLM showed the copper hyperfine structures with lower symmetric $g$-anisotropies, suggesting a rhombic distortion of the Cu(II) chromophore.

PYML, P-3A, and deglyco BLM formed the Co(I) complexes and their dioxygen adducts similar to those of BLM (21). These ESR features are typical of a low spin square-pyramidal Co(I) complex with the electronic configuration \[[d_{xy}, d_{yz}, d_{zx}]^5(d_{z^2})^1\] and of monooxygenated low spin Co(II)-O2 adduct complex. The PEML-Co(II) complex was detected only under the fully anaerobic condition which was achieved by using a vacuum line, and its $A_z$ value (84.3 G) was smaller than those (92-95 G) of the Co(I) complexes with PYML, P-3A, deglyco BLM, and BLM. This is due to the higher $pK_a$ of the axial $\alpha$-amino base in PEML ligand, which clearly lacks the electron-withdrawing CONH$_2$ group. Repeated and careful experiments demonstrated no formation of monooxygenated adduct for the PEML-Co(II) complex.

Under anaerobic conditions, the present six ligands formed the high spin ferrous ($S = 2$) complexes (see the following section). However, such high spin Fe(I) ESR spectra are difficult to detect at 77 K because of their short lattice times.

The nitric oxide adduct complexes were easily obtained by addition of a few milligrams of Na$\text{I}_4\text{NO}_2$ (or Na$\text{N}_2\text{NO}_2$) and sodium dithionite (or sodium borohydride) to the Fe(I) com-

| Table II | ESR parameters for divalent metal complexes of PYML, PEML, P-3A, deglyco BLM, and BLM |
|----------|----------------------------------------------------------------------------------------------------------------------------------|
| Complex  | $g_1$ (g$_0$) | $g_2$ (g$_0$) | $A_z$ G | $A_z$ G | Nitrogen-hyperfine splitting (line) |
| PYML-1-Cu(II) | 2.206 | 2.048 | 179.4 | 13 | 3 |
| PYML-1-Co(II) | 2.022 | 2.255 | 92.5 | 3 |
| PYML-1-Co(II)-O2 | 2.093 | 2.005 | 22.5 | 3 |
| PYML-1-Co(Fe) | No ESR signals |
| PYML-1-Co(II)-14NO | 2.009 | 2.036 1.972 | 25.6 | 3 |
| PYML-1-Co(II)-14NO | 2.009 | 2.036 1.972 | 35.6 | 3 |
| PYML-1-Co(II)-14NO + DNA | 2.009 | 2.036 1.972 | 25.6 | 3 |
| PYML-2-Cu(II) | 2.204 | 2.052 | 179.8 | 3 |
| PYML-2-Co(II) | 2.029 | 2.052 | 92.0 | 3 |
| PYML-2-Co(II)-O2 | 2.093 | 2.005 | 22.5 | 3 |
| PYML-2-Co(Fe) | No ESR signals |
| PYML-2-Co(II)-14NO | 2.009 | 2.036 1.970 | 25.5 | 3 |
| P-3A-Cu(II) | 2.277 | 2.090 | 170.8 | 3 |
| P-3A-Co(II) | 2.018 | 2.260 | 84.3 | 3 |
| P-3A-Co(II)-O2 | Not detected |
| P-3A-Co(Fe) | No ESR signals |
| P-3A-Co(II)-14NO | 2.148 | 2.072 | 167.3 | 3 |
| P-3A-Co(II)-O2 | 2.072 | 2.275 | 93.8 | 3 |
| P-3A-Co(II)-Fe(II) | 2.102 | 2.007 | 22.4 | 3 |
| P-3A-Co(II)-Fe(II)-14NO | 2.007 | 2.038 1.989 | 24.8 | 3 |
| P-3A-Co(II)-Fe(II)-14NO | 2.007 | 2.038 1.989 | 35.0 | 3 |
| P-3A-Co(II)-Fe(II)-14NO + DNA | 2.007 | 2.038 1.989 | 24.8 | 3 |
| Deglyco BLM-Cu(II) | 2.214 | 2.131 2.077 | 167.0 | 3 |
| Deglyco BLM-Co(II) | 2.027 | 2.277 | 95.0 | 3 |
| Deglyco BLM-Co(II)-O2 | 2.100 | 2.009 | 22.5 | 3 |
| Deglyco BLM-Co(Fe) | No ESR signals |
| Deglyco BLM-Co(II)-14NO | 2.214 | 2.131 2.077 | 167.0 | 3 |
| Deglyco BLM-Co(II)-14NO | 2.027 | 2.277 | 95.0 | 3 |
| Deglyco BLM-Co(II)-14NO + DNA | 2.100 | 2.009 | 22.5 | 3 |
| Deglyco BLM-Co(II)-Fe(II) | No ESR signals |
| Deglyco BLM-Co(II)-Fe(II)-14NO | 2.007 | 2.038 1.989 | 24.8 | 3 |
| Deglyco BLM-Co(II)-Fe(II)-14NO | 2.007 | 2.038 1.989 | 33.0 | 3 |
| Deglyco BLM-Co(II)-Fe(II)-14NO + DNA | 2.007 | 2.038 1.989 | 25.1 | 3 |
| BLM-Cu(II) | 2.211 | 2.055 | 183.0 | 3 |
| BLM-Co(II) | 2.025 | 2.272 | 92.5 | 3 |
| BLM-Co(II)-O2 | 2.098 | 2.007 | 20.2 | 3 |
| BLM-Co(II)-Fe(II) | No ESR signals |
| BLM-Co(II)-Fe(II)-14NO | 2.008 | 2.041 1.976 | 23.6 | 3 |
| BLM-Co(II)-Fe(II)-14NO | 2.008 | 2.040 1.976 | 31.6 | 2 |
| BLM-Co(II)-Fe(II)-14NO + DNA | 2.006 | 2.060 1.982 | 24.0 | 3 |

a The complex was detected only under the fully anaerobic condition which was achieved by using a vacuum line.
plexes of PYML, P-3A, deglyco BLM, and BLM. In contrast, the PEML-Fe(II)-NO complex was not detected even under the fully anaerobic condition. These ESR features exhibited rhombic symmetry with a triplet $^{14}$N (or doublet $^{15}$N) hyperfine splitting in the central $g$ signal, and are typical of the six-coordination type (4). The changes of the nitrogen-hyperfine splitting lines and the $A_N$ values by the substitution of $^{14}$NO by $^{15}$NO were in accord with the nuclear spin and magnetogyric ratio of $^{14}$N ($I = 1$ and $\gamma_N = 1.934$) and $^{15}$N ($I = \frac{1}{2}$ and $\gamma_N = -2.712$) nuclei.

Visible, Proton Magnetic Resonance, and Mössbauer Spectra of Iron(II) Complexes—Except for the PEML-Fe(II) complex, the Fe(II) complexes of PYML-1, P-3A, deglyco BLM, and BLM reacted with carbon monoxide, ethyl isocyanide, and nitric oxide to form these adduct complexes. As shown in Table III, the visible absorption spectra of the Fe(II) complexes with CO, C$_2$H$_5$NC, and NO differ markedly from that of the original Fe(II) complex. These dioxygen analogous adducts characterized by their large extinction coefficients which are due to iron-ligand charge transfer transition, and the absorption maxima were shifted to a longer wavelength in the order C$_2$H$_5$NC $>$ NO $>$ CO.

Fig. 3 illustrates the hyperfine shifted proton resonances of the PYML-1-Fe(II) complex obtained at 220 MHz and 20 °C. The PYML-1-Fe(II) complex showed large paramagnetic shifts of approximately 13 proton signals, as a result of contact and pseudo-contact effects by the central high spin Fe(II) ion. Upon carbon monoxide binding to the PYML-1-Fe(II) complex, these paramagnetic shifted protons disappeared. As summarized in Table IV, the present $^1$H NMR results indicate that 1) high spin Fe(II) ion ($S = 2$) and diamagnetic Fe(II) ion ($S = 0$) are present in the PYML-1-Fe(II) complex and its CO adduct, respectively; 2) $^1$H NMR behavior between the Fe(II) complexes of PYML-1 and BLM is remarkably similar; and 3) the gulose-mannose sugar protons are involved in the numerous paramagnetic resonances of the BLM-Fe(II) complex. Indeed, we observed that the magnitude of the chemical shifts in the deglyco BLM-Fe(II) complex is comparable to that in the BLM-Fe(II) complex, but the resonance lines of the former are fewer than those of the latter (28). Therefore, it is reasonably supposed that the sugar moiety of BLM is located near the Fe(II)-coordination site spatially and that the sugar protons experience the paramagnetic effect of the central Fe(II) ion.

Fig. 4 shows the $^{57}$Fe-Mössbauer spectra of the PYML-1-Fe(II) complex and its CO adduct at 110 K in zero magnetic field, which are characterized by a single quadrupole doublet. The quadrupole splitting ($\Delta E_Q = 3.00$ mm/s) and the isomer shift ($\delta_{es} = +1.05$ mm/s) of the PYML-1-Fe(II) complex are remarkably close to those of the BLM-Fe(II) complex (see Table IV) (22). The Mössbauer parameters are typical of a high spin ferrous ion. The PYML-1-Fe(II)-CO complex has the Mössbauer characteristics ($\Delta E_Q = 0.51$ and $\delta_{es} = +0.18$ mm/s) which are similar to the BLM-Fe(II)-CO complex (22) and consistent with a S = 0 ferrous assignment.

**Dioxygen Activation by Iron(II) Complexes (Spin Trapping)—** From the viewpoint of dioxygen activation, the PYML-1 ligand was investigated and compared with BLM (4). The ESR spin trapping experiments using BPN and DMPO at pH 6.9 evidently revealed that hydroxyl radicals are generated from the PYML-1-Fe(II)-O$_2$ complex system. The ESR pattern and the parameters were as follows: BPN spin adduct (triplet of doublet, $g = 2.0057$, and $a^\perp = 15.3$ G) and DMPO spin adduct (quartet, $g = 2.0056$, and $a^\perp = 9.8$ G). In contrast with the corresponding PYML-1-Fe(II)-O$_2$ complex system, the CO introduction strongly interfered with dioxygen activation by the PYML-1-Fe(II) complex (see Fig. 5). Carbon monoxide is in competition for dioxygen for interaction with the PYML-1-Fe(II) complex and is a typical O$_2$ antagonist, just as with the BLM-Fe(II) complex. Table V summarizes the relative spin concentration of hydroxyl radical BPN spin adduct in the Fe(II) complex systems of BLM, deglyco BLM, P-3A, PYML-1, and PEML. Here, it is of

---

**Table III**

| Complex                  | $\lambda_{max}$ $^a$ | $\delta$ $^a$ |
|--------------------------|-----------------------|---------------|
| PYML-1-Fe(II)            | 465 (300)             |               |
| PYML-1-Fe(II)-CO         | 390 (2000)            |               |
| PYML-1-Fe(II)-C$_2$H$_5$NC | 490 (1800)          |               |
| PYML-1-Fe(II)-NO         | 470 (1650)            |               |
| PEML-Fe(II)              | 470 (300)             |               |
| PEML-Fe(II)-CO*          | Not detected          |               |
| PEML-Fe(II)-C$_2$H$_5$NC* | Not detected         |               |
| PEML-Fe(II)-NO*          | Not detected          |               |
| F-3A-Fe(II)              | 470 (320)             |               |
| P-3A-Fe(II)-CO           | 385 (2300)            |               |
| P-3A-Fe(II)-C$_2$H$_5$NC | 490 (2000)            |               |
| P-3A-Fe(II)-NO           | 470 (1800)            |               |
| Deglyco BLM-Fe(II)       | 472 (300)             |               |
| Deglyco BLM-Fe(II)-CO    | 380 (2800)            |               |
| Deglyco BLM-Fe(II)-C$_2$H$_5$NC | 495 (2700)   |               |
| Deglyco BLM-Fe(II)-NO    | 485 (1950)            |               |
| BLM-Fe(II)               | 476 (380)             |               |
| BLM-Fe(II)-CO            | 390 (3000)            |               |
| BLM-Fe(II)-C$_2$H$_5$NC  | 495 (2700)            |               |
| BLM-Fe(II)-NO            | 470 (2300)            |               |

$^a$ Values in parentheses are $g$.

$^a$ The formation of these dioxygen analogous adducts was not observed even under the fully anaerobic condition.
Role of Functional Groups in Bleomycin Action

special importance to note that 1) the radical spin concentrations of the PYML-1 (or P-3A)-Fe(II) and the deglyco BLM-Fe(II) complex systems were estimated to be approximately 20 and 40% of that of the corresponding BLM-Fe(II) complex system, respectively, and 2) the dioxygen activation ability of the PML-Fe(II) complex system was negligibly small in comparison with that of the corresponding BLM system. The result corresponds well to the recent observation (29) in which the deglyco BLM-Fe(II) complex system gave about half as much [\(^{1}\text{H}\)Ithymin release from PM-2 DNA as the BLM-Fe(II) complex system. Therefore, it is inferred that the gulose-mannose sugar portion and the bithiazole-containing tripeptide S moiety contribute to the more effective dioxygen activation by the BLM ligand.

DNA Cleavage Reaction of Iron(II) Complex Systems—In the DNA strand scission, the nucleotide sequence specificity of the deglyco BLM-Fe(II) complex system was determined and compared with that of the corresponding BLM system. Cleavage of double-stranded restriction fragments of plasmid pBR 322 DNA, 327 nucleotides in length, is illustrated in Fig. 6. Although the BLM-Fe(II) complex system preferentially attacked GC(5' → 3') sequences, other GT(5' → 3'), GG(5' → 3'), and GA(5' → 3') sequences were also cleaved. The preferential pyrimidine base releasing is consistent with the observation of Takeshita et al. (30) who used bacteriophage \(\phi\)X174 DNA fragments. Of special interest is the fact that the deglyco BLM-Fe(II) and the BLM-Fe(II) complex systems revealed almost identical patterns of the cleavage in the DNA fragments labeled at the 5' terminus, although the DNA cleavage ability of the deglyco BLM system was somewhat less active than that of the BLM system (see Figs. 6 and 7). The present result strongly demonstrates that the gulose-mannose sugar moiety does not give an alteration for the nucleotide sequence specificity in the DNA cleavage reaction by the BLM antibiotics. Under the same experimental condition, on the other hand, the PYML-1-Fe(II) complex system showed significantly less activity.

![Fig. 4. \(^{57}\text{Fe}\) Mössbauer spectra of PYML-1-Fe(II) (A) and PYML-1-Fe(II)-CO (B) complexes at 110 K.](image)

![Fig. 5. ESR spin trapping by PYML-1-Fe(II) complex systems in the presence of BPN.](image)

![Table V. Spin concentration of hydroxyl radicals from iron(II) complex systems of PYML-1, PML, P-3A, deglyco BLM, and BLM.](table)

\[
\begin{array}{|c|c|}
\hline
\text{Complex} & \text{Relative spin concentration} \\
\hline
\text{BLM-Fe(II)} & 100 \\
\text{Deglyco BLM-Fe(II)} & 43 \\
\text{P-3A-Fe(II)} & 22 \\
\text{PYML-1-Fe(II)} & 18 \\
\text{PEML-Fe(II)} & <2 \\
\hline
\end{array}
\]
Although the role of metal ions in the BLM action mechanism has been actively studied, the controversial information for the transition metal-binding sites of BLM has been obtained. The coordination of the sugar carbamoyl group for Cu(II) (31) and Fe(II) (6) complexes of BLM, and the bindings of the diaminopropanamide and the β-aminopropanamide groups for Co(III) complex (32) have been proposed. Recent ‘H nuclear relaxation study of the BLM-Mn(II) complex also suggested the bithiazole group as a metal ligand (7). The present study provides the most reliable evidence for the proposed metal-binding sites (4, 5) in which 1) the β-aminoalaine-pyrimidine-β-hydroxyhistidine region of the BLM molecule is substantially important for the Fe(II), Co(II), and Cu(II) interactions and 2) the guanosine and the methylyvaleroyl moieties in BLM are not necessarily participating as direct ligands toward the metal coordination. This result is consistent with the structural assignment by x-ray analyses for the P-3A-Cu(II) complex (8) and the Co(III) complex of pseudo-tetrapeptide A of BLM (33). The synthetic ligand PYML-1 includes 1) simplification of the pyrimidine nucleus of BLM to pyridine nucleus, 2) use of a simplified side chain, \([[(S)-2-amino-2-carbamoylethyl]amino}\) methyl group, and 5) use of histidine for β-hydroxyhistidine of BLM. Nevertheless, the physicochemical properties of the PYML-1-divalent metal complexes are remarkably similar to those of the corresponding BLM metal complexes. Certainly, PYML-1 is a simplified and valuable analogue which corresponds to the amine-pyrimidine-imidazole portion of BLM.

As seen between PYML and PEML, the substitution of the imidazole group by the amino group gives significant influence on dioxygen-binding and -activation by the Fe(II) complexes. In contrast with PYML, P-3A, deglyco BLM, and BLM ligands, the Co(II)-O₂, Fe(II)-CO, Fe(II)-C₅H₅NC, and Fe(II)-NO adduct complexes of PEML were not observed by all means. Therefore, the presence of the imidazole group, in particular the transposition of pyrimidine (or pyridine) and imidazole groups in the Fe(II)-coordination, appears to be essential for the effective binding and activation of molecular oxygen by the Fe(II) complexes of BLM-related ligands. We previously reported that 1) the spectroscopic and crystal field parameters of the BLM-iron complexes with CO, C₂H₅NC, NO, N₃, OH⁻, and CH₃NH⁺ are similar to those of the corresponding hemoprotein complexes, except for the CN⁻ adducts and 2) the iron ligand donors in BLM are arranged in a rigid square-pyramidal configuration with a 5-5-5-6 ring member, as seen in the case of heme (22). Probably, the aromatic nitrogen-containing and electron-rich structure formed by the coordination of pyrimidine (or pyridine) and imidazole nitrogen ligands trans to each other, contributes to the same consequence on iron electronic state as found in the hemoproteins.

The remarkable similarity of the divalent metal complexes between deglyco BLM and BLM definitely indicates that the
carbamoyl group of the sugar portion in BLM does not directly coordinate to the metal ions. It is well known that the addition of \( \text{H}_2\text{O}_2 \) induces the conversion from the inactive BLM-Fe(III)-OH complex (\( g_1 = 1.893, g_2 = 2.185, \) and \( g_3 = 2.434 \)) to the active BLM-Fe(III)-O\(^2-\) complex (\( g_1 = 1.937, g_2 = 2.171, \) and \( g_3 = 2.254 \)) for the DNA cleavage (34–36).

When hydrogen peroxide (1–10 mM) was added to the deglyco BLM-Fe(III)-OH complex (1 mM) which shows the typical low-spin ferric ESR signals at \( g_1 = 1.887, g_2 = 2.180, \) and \( g_3 = 2.432 \), it quite similarly changed to the ESR spectrum with \( g_1 = 1.973, g_2 = 2.171, \) and \( g_3 = 2.254, \) whereas, the decreasing dioxygen-activation ability of the deglyco BLM-Fe(II) complex system suggests that the guanine-mannose moiety is situated near the Fe(II)-coordination site and plays an important role as the environmental factor in the efficient dioxygen activation, just as pivalamidophenyl groups in picket-fence porphyrins (37, 38). The previous ESR studies demonstrated that the environment of in-plane ligands with the pyrimidine and imidazole groups in the BLM-Fe(II)-NO complex is altered by the binding of DNA (39, 40). Antholine et al. (41) observed by \(^1\)H NMR study that the binding to poly(dA-dT)-poly(dA-dT) changes the environment of the imidazole and pyrimidine rings of the BLM-Fe(II)-Co complex but does not have a similar effect on these rings when the iron is absent. As shown in the BLM-Fe(II)-NO complex, the binding of DNA to the deglyco BLM-Fe(II)-NO complex also induced a greater separation of the \( g_1 \) and \( g_2 \) absorptions in comparison with the original ESR spectrum of the deglyco BLM-Fe(II)-NO complex (see Table II). Herein, it is of interest that the difference \( g_1 - g_2 \) (2.060–2.041) and \( g_1 - g_3 \) (1.976–1.962) values of the BLM complexes are larger than those \( g_1 - g_2 \) (2.046–2.038) and \( g_1 - g_3 \) (1.976–1.963) of the deglyco BLM complexes. A weak interaction such as hydrogen bond between the sugar group of BLM and DNA phosphate group may be responsible for the larger \( g \) separations in the BLM complex. As clearly demonstrated in the DNA cleavage by the deglyco BLM-Fe(II) complex system, however, the absence of the guanine-mannose portion in BLM ligand gives no noticeable effect on the nucleotide sequence specificity. Therefore, the sugar moiety does not contribute significantly to the specificity of DNA binding, which is mainly due to the interaction with the bithiazole group (42). Recently, the binding specificity of the four BLM analogues, BLM-B, BLM-A, BLM-B, and peplomycin, was compared, and the result also shows no significant contribution of the terminal amine side chains to the DNA nucleotide specificity (43). On the other hand, it has been noted that the somewhat altered specificity of tallowyosmicin must be attributed to the presence of the additional amino sugar (43). The present guanine-pyrimidine (5' → 3') specificity of BLM represented by \( G \rightarrow C \) sequences is consistent with the observations by D'Andere and Haseltine (44) and Takeshita et al. (30, 43) who used lactose operon Pl J3 and bacteriophage \( \phi X 174 \) DNA fragments, respectively. The synthetic analog PYML-1-Fe(II) complex showed the effective dioxygen activation, but was remarkably less active than the corresponding BLM complex in the DNA cleavage reaction. The DNA binding molecule to deliver a metal ion to the site of the DNA helix where activated molecular oxygen attacks the DNA is required for the efficient DNA strand scission. The examples involve the 1,10-phenanthroline-cuprous complex (45, 46) and the methidiumpropyl-EDTA-ferrous complex (47) which result in the DNA cleavage under the presence of oxygen. On the other hand, the BLM-Cu(I) complex has been reported to produce oxygen radicals under aerobic conditions (48) and to form a carbon monoxide adduct (49). However, the BLM-Cu(I) complex did not cleave the present pBR 322 DNA fragment in the presence of 2-mercaptoethanol (or sodium dithionite). This phenomenon is probably attributed to the low redox potential of the BLM-copper complex. Indeed, the BLM-iron complex has the much higher redox potential (\( E_{\text{ox}} = +0.15 \text{ V versus NHE} \) (60).

In summary, 1) the \( 8\)-aminoalanine-pyrimidine-\( \beta\)-hydroxyhistidine portion of BLM molecule is substantially important for the Fe(II) and dioxygen interactions, and indeed the synthetic analog PYML is able to mimic the metal binding and dioxygen reduction by BLM ligand, 2) the pyrimidine group of BLM can be probably replaced by the pyridine group without loss of its function of this group, but the substitution of the imidazole by the amino groups shows the significant decrease of the effective O\(^2-\) binding and activation, 3) although the guanine-mannose group does not contribute essentially to the nucleotide specificity in the DNA cleavage, the sugar portion plays an important role as the environmental factor in the efficient oxygen activation and DNA cleavage, and 4) the guanine-mannose, methylvalerate, and bithiazole moieties in BLM are not clearly participating as direct ligands toward the Fe(II) binding. We believe that the present results promise good hope for the molecular design of a synthetic compound with the function of selective DNA base cleavage.

Acknowledgments—We are grateful to Prof. H. Tanaka for kind encouragement, Prof. M. Takamani and Dr. H. Sugisaki for pertinent advice of DNA cleavage experiments, Drs. S. Ogawa and I. Morishima for \(^1\)H NMR measurements, and Dr. K. Watanabe for \(^{57}\)Fe-Mossbauer measurements.

REFERENCES

1. Umezawa, H. (1977) *Lloydia* (Cinci) 40, 67–81
2. Sauseville, E. A., Stein, R. W., Peisach, J., and Horwitz, S. B. (1978) *Biochemistry* 17, 2746–2754
3. Kasa, N., Naganawa, H., Takita, T., and Umezawa, H. (1978) *J. Antibiot.* (Tokyo) 31, 1316–1320
4. Sugiyama, T. (1980) *J. Am. Chem. Soc.* 102, 5208–5215
5. Takita, T., Muraoka, Y., Nakatani, T., Fujii, A., Iitaka, Y., and Umezawa, H. (1978) *J. Antibiot.* (Tokyo) 31, 1073–1076
6. Oppenheimer, N. J., Rodriguez, L. O., and Hecht, S. M. (1979) *Proc. Natl. Acad. Sci. U. S. A.* 76, 5616–5620
7. Sheridan, R. P., and Gupta, R. K. (1981) *J. Biol. Chem.* 256, 1242–1247
8. Itaka, Y., Nakamura, H., Nakatani, T., Muraoka, Y., Fujii, A., Takita, T., and Umezawa, H. (1978) *J. Antibiot.* (Tokyo) 31, 1070–1072
9. Dabrowiak, J. C., and Tsukayama, M. (1981) *J. Am. Chem. Soc.* 103, 7543–7580
10. Sugiyama, T. (1979) *Biochem. Biophys. Res. Commun.* 88, 913–918
11. Oppenheimer, N. J., Rodriguez, L. O., and Hecht, S. M. (1980) *Biochemistry* 19, 4096–4103
12. Fujii, A. (1979) in *Bleomycin* (Hecht, S. M., ed) pp. 75–91, Springer-Verlag, New York
13. Muraoka, Y., Suzuki, M., Fujii, A., Umezawa, Y., Naganawa, H., Takita, T., and Umezawa, H. (1981) *J. Antibiot.* (Tokyo) 34, 353–357
14. Takita, T., Umezawa, Y., Saito, S., Morishima, H., Naganawa, H., Umezawa, H., Tsuchiya, T., Miyake, S., Kageyama, S., Umegawa, S., Muraoka, Y., Suzuki, M., Osuka, M., Narita, M., Kobayashi, S., and Ohno, M. (1982) *Tetrahedron Lett.* 23, 521–524
15. Saito, S., Umezawa, Y., Morishima, H., Takita, T., Umezawa, H., Narita, M., Osuka, M., Kobayashi, S., and Ohno, M. (1982) *Tetrahedron Lett.* 23, 525–528
16. Ohno, M., Yohida, M., Kobayashi, S., Ohno, M., Sugiyama, T., Takita, T., and Umezawa, H. (1981) *J. Am. Chem. Soc.* 103, 6986–6988
17. Mathes, W., Sauerbirmich, W., and Klein, T. (1953) *Chem. Ber.* 86, 584–588
18. Umezawa, Y., Morishima, H., Saito, S., Takita, T., Umezawa, H., Kobayashi, S., Osuka, M., Narita, M., and Ohno, M. (1980) *J. Am. Chem. Soc.* 102, 6630–6631
19. Greenstein, J. P., and Winitz, M. (1961) in *Chemistry of the*
Role of Functional Groups in Bleomycin Action

Amino Acids (Greenstein, J. P., and Winitz, M., eds) Vol. 2, p. 1061, Wiley, New York

20. Sugiura, Y., Ishizu, K., and Miyoshi, K. (1979) J. Antibiot. (Tokyo) 32, 455-461
21. Sugiura, Y. (1980) J. Am. Chem. Soc. 102, 5216-5221
22. Sugiura, Y., Suzuki, T., Kawabe, H., Tanaka, H., and Watanabe, K. (1982) Biochim. Biophys. Acta 716, 38-44
23. Sugiura, Y., and Kikuchi, T. (1978) J. Antibiot. (Tokyo) 31, 1310-1312
24. Boliver, F., and Backman, K. (1979) Methods Enzymol. 68, 245-267
25. Maxam, A. M., and Gilbert, W. (1977) Proc. Natl. Acad. Sci. U. S. A. 74, 560-564
26. Schlaffer, H. L., and Gliemann, G. (1969) in Basic Principles of Ligand Field Theory, pp. 1-171, Wiley, London
27. Ishizu, K., Murata, S., Miyoshi, K., Sugiura, Y., Takita, T., and Umezawa, H. (1981) J. Antibiot. (Tokyo) 34, 994-1000
28. Sugiura, Y., Suzuki, T., Muraoka, Y., Umezawa, Y., Takita, T., and Umezawa, H. (1981) J. Antibiot. (Tokyo) 34, 1232-1236
29. Oppenheimer, N. J., Chang, C., Chang, L.-H., Ehrenfeld, G., Rodriguez, L. O., and Hecht, S. M. (1982) J. Biol. Chem. 257, 1699-1909
30. Takeshita, M., Grollman, A. P., Ohtsubo, E., and Ohtsubo, H. (1978) Proc. Natl. Acad. Sci. U. S. A. 75, 5983-5987
31. Bereman, R. D., and Winkler, M. E. (1980) J. Inorg. Biochem. 13, 95-104
32. Vos, C. M., Westera, G., and Shipper, D. (1980) J. Inorg. Biochem. 13, 165-177
33. Tsukayama, M., Randall, C., Santillo, F. S., and Dabrowiak, J. C. (1981) J. Am. Chem. Soc. 103, 458-460
34. Kuramochi, H., Takahashi, K., Takita, T., and Umezawa, H. (1981) J. Antibiot. (Tokyo) 34, 576-582
35. Burger, R. M., Peisach, J., and Horwitz, S. B. (1981) J. Biol. Chem. 256, 11636-11644
36. Sugiura, Y., Suzuki, T., Kuwahara, J., and Tanaka, H. (1982) Biochem. Biophys. Res. Commun. 105, 1511-1518
37. Collman, J. P., Brauman, J. L., Doxsee, K. M., Halbert, T. R., and Suslick, K. S. (1978) Proc. Natl. Acad. Sci. U. S. A. 75, 564-568
38. Collman, J. P., Brauman, J. L., and Doxsee, K. M. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 6035-6039
39. Sugiura, Y., and Ishizu, K. (1979) J. Inorg. Biochem. 11, 171-180
40. Antholine, W. E., and Petering, D. H. (1979) Biochem. Biophys. Res. Commun. 91, 529-533
41. Antholine, W. E., Petering, D. H., Saryan, L. A., and Brown, C. E. (1981) Proc. Natl. Acad. Sci. U. S. A. 78, 7517-7520
42. Povirk, L. F., Hogan, M., and Dattagupta, N. (1979) Biochemistry 18, 96-101
43. Takeshita, M., Kappen, L. S., Grollman, A. P., Eisenberg, M., and Goldberg, I. H. (1981) Biochemistry 20, 7599-7606
44. D'Andere, A. D., and Haseltine, W. A. (1978) Proc. Natl. Acad. Sci. U. S. A. 75, 3608-3612
45. Sigman, D. S., Graham, D. R., D'Aurora, V., and Stern, A. M. (1979) J. Biol. Chem. 254, 12269-12272
46. Marshall, L. E., Graham, D. R., Reich, K. A., and Sigman, D. S. (1981) Biochemistry 20, 244-250
47. Hertberg, R. P., and Dervan, P. B. (1982) J. Am. Chem. Soc. 104, 313-315
48. Sugiura, Y. (1979) Biochem. Biophys. Res. Commun. 96, 375-383
49. Oppenheimer, N. J., Chang, C., Rodriguez, L. O., and Hecht, S. M. (1981) J. Biol. Chem. 256, 1514-1517
50. Melynk, D. L., Horwitz, S. B., and Peisach, J. (1981) Biochemistry 20, 5327-5331