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# THE IRON COMPLEXES OF BLEOMYCIN AND TALLYSOMYCIN

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Summary: Using uv-visible absorption, epr, electrochemistry, and $^{13}C$ nmr, the Fe(II) and Fe(III) binding sites of the antitumor antibiotics bleomycin and tallysomycin have been located. Both drugs appear to utilize the amine-pyrimidine-imidazole region for iron binding. The ligating atoms of the drugs for Fe(II) and Fe(III) are dependent for iron and the presence of buffer ions. The ligation of the pyrimidine moiety has been determined under a variety of experimental conditions and correlated with epr observation of high and low spin forms of Fe(III). The results indicate that the displacement of some of the ligating atoms does not inhibit the action of the iron-drug complex.

## Introduction

The fungus-derived glycopeptides bleomycin (BLM) and its larger cognate tallysomycin (TLM), Figure 1, are anticancer agents (1,2). The receptor site for the drugs within the tumor cell is believed to be DNA. In vivo and in vitro experiments with BLM have shown that the molecule is capable of binding to and degrading DNA. A unique mechanism of DNA degradation by BLM has been recently proposed by Sausville et al., (3-5) who suggested that the drug exists in the cell as its iron(II) complex which is in turn bound to DNA. The ternary complex, iron-drug-DNA, is air sensitive and the bleomycin-bound Fe(II) ion is readily oxidized to Fe(III) with the production of a radical. Recent studies have shown that radicals are in fact produced in the oxidation of Fe(II)BLM to Fe(III)BLM (6,7). It has been postulated that it is a radical which leads to DNA breakage and ultimately to the death of the tumor cell. The structural similarity of TLM and BLM strongly suggests that both antibiotics function by the same biological mechanism.

The structural aspects of the ternary complexes iron-drug-DNA are under intense scrutiny. It is known from nmr, fluorescence and linear dichroism experiments (8-10) that bleomycin utilizes the 2,4' bithiazole moiety for binding to DNA. This information, coupled with our observations that the pyrimidine-imidazole-sugar regions of both cancer drugs are involved in binding Cu(II) and Zn(II) (11-13) has led to the realization that the drugs are bifunctional-one portion of the molecule, the bithiazole moiety, is used for

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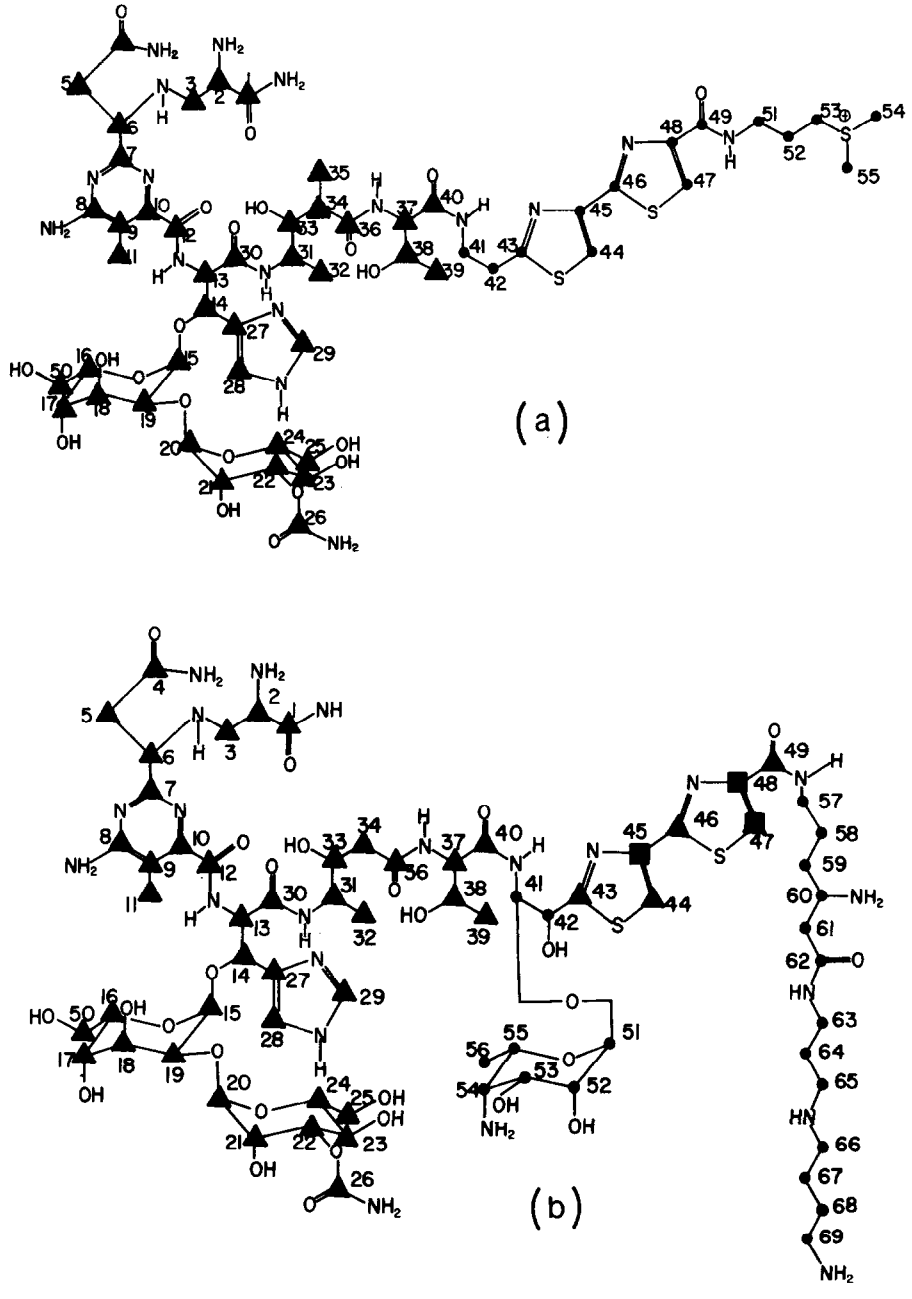


Figure 1 (a) The structure of bleomycin $A_2$. Those $^{13}C$ nmr resonance lines which are missing from the spectra of Fe(II)BLM and Fe(III)BLM are indicated by (▲). (b) The structure of tallysomycin-A. Those $^{13}C$ nmr resonances lines which are missing from the spectrum of Fe(II,III)TLM are indicated by (▲). Those resonance lines which are substantially broadened in the spectrum of Fe(III)TLM are indicated by ■ .

binding to DNA, while the amino-pyrimidine-imidazole region of the peptide is free to bind metal ions. In an effort to more clearly define the structural and mechanistic role played by iron ions in the DNA degrading process, we have examined the physical properties of Fe(II, III)BLM and Fe(II, III)TLM. This report discloses the location of the iron binding site and discusses the physical properties of the iron complexes in light of the proposed mechanism of action of both antibiotics.

## MATERIALS AND METHODS

Bleomycin $A_2 \cdot HC\ell$ and tallysomycin-A, $5HC\ell \cdot 9H_2O$ were used as obtained from Bristol Laboratories. The preparation and handling of the Fe(II)-antibiotic complexes were done in an inert atmosphere box. Unless otherwise noted, complexes were prepared by the addition of stoichiometric amounts of either $Fe(II)(C\ell O_4)_2 \cdot 6H_2O$ or $Fe(III)(C\ell O_4)_3 \cdot 6H_2O$ to the drug followed by the adjustment of the pH to a specific value. The epr as well as the nmr and absorption data were collected in the earlier described manner (11-13). The variation in the amounts of the high and low spin forms of Fe(III)BLM as a function of pH was determined using epr. Since the linewidth of the low spin epr signal was independent of pH, the peak height of the signal was used as a measure of the amount of low spin Fe(III)BLM present. Although the linewidth of the high spin species varied as a function of the pH, especially in the presence of buffers, the peak height of the signal was taken as rough measure of the amount of high spin Fe(III)BLM present in the mixture.

The dc polarographic studies were carried out in buffered and in unbuffered aqueous solutions. Potentials were referenced against an Ag/AgCℓ saturated NaCℓ electrode. The electrochemical assignments and the apparatus used to collect the polarographic data have been previously described (14, 15).

## RESULTS AND DISCUSSION

$^{13}C$ nmr: $^{13}C$ nmr spectra of the complexes were characterized by two types of resonance lines: signals which were narrow and unshifted from their original positions and those that were noticeably broadened and in most cases shifted from their original positons in the metal-free antibiotics. Only the narrow, unshifted resonances were assigned. The $^{13}C$ nmr assignments of both antibiotics have been previously reported (16, 17).

Figure 2 shows the $^{13}C$ nmr spectra of BLM, Fe(II)BLM, and Fe(III)BLM formed by oxidizing Fe(II)BLM with molecular oxygen. Carbon atoms 41-49 and 51-55 of Fe(II)BLM were

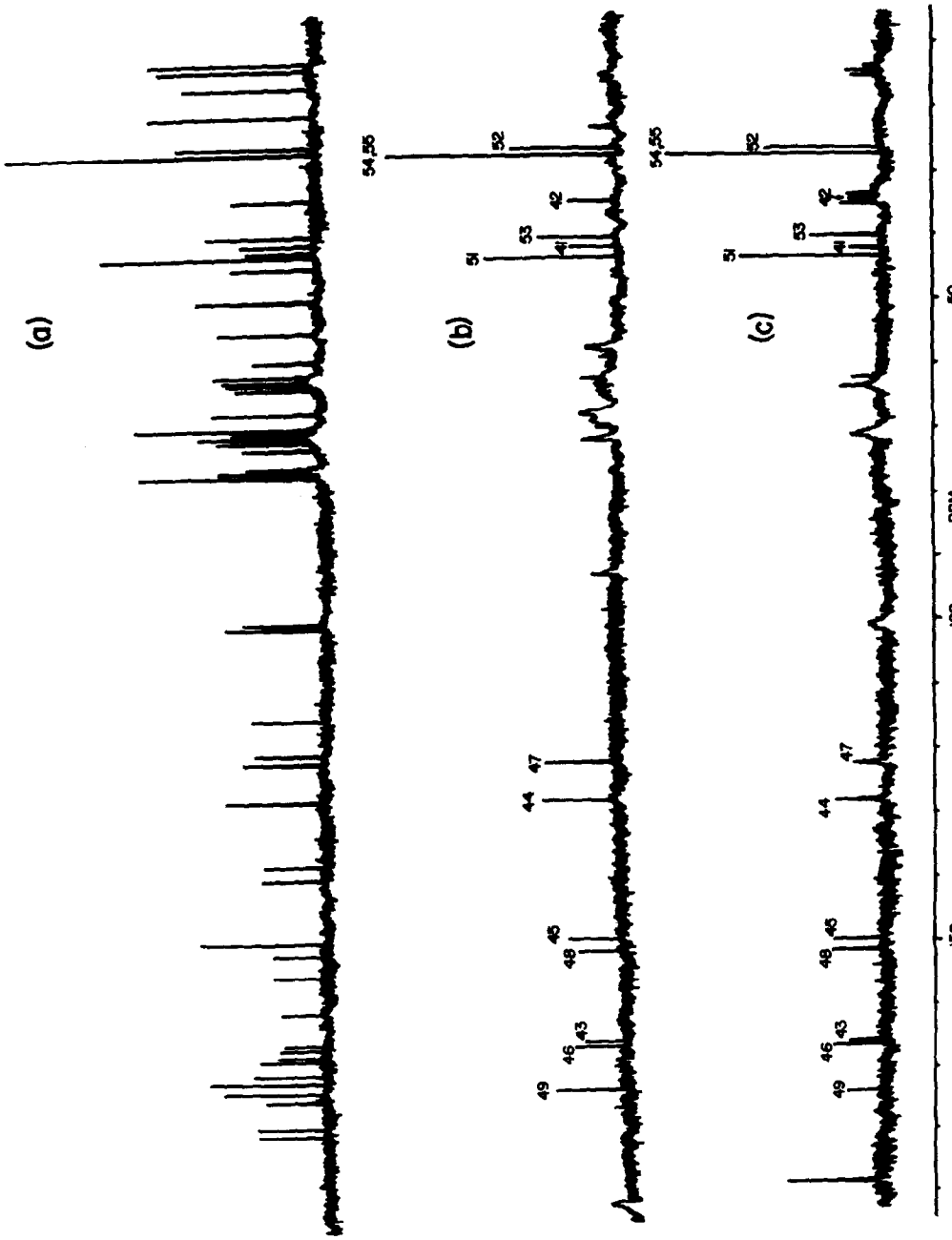


Figure 2 The $^{13}C$ nmr spectra of bleomycin-$A_2$ (a), Fe(II)BLM (b), and Fe(III)BLM (c) at pH 6.5.

observed as narrow resonance lines (See Figure 1(a) for the numbering system used). Broadened and unassigned resonances for Fe(II)BLM occurred at 23.2, 57.5, 93.0, 168.5 and 192.5 ppm. A number of broad, overlapping resonances also occurred in the sugar region of the nmr spectrum (60-75 ppm).

Fe(III)BLM exhibited narrow resonance lines for carbon atoms 41-49 and 51-55. Broad unassignable resonances were observed at 64 and 71 ppm but the sugar region was simpler than was observed for Fe(II)BLM. In addition, broad and apparently shifted resonances were found at 14, 34, and 101 ppm. The $^{13}C$ nmr spectrum of Fe(III)BLM also contained a number of weak but narrow resonances at 14.2, 15.2, 33.9, 34.2 and 188.5 ppm. These resonances have not been previously observed in the spectrum of the drug or any of its metalloderivatives and they may be due to bleomycin fragments which occur upon oxidation of Fe(II)BLM (18). The resonances which were missing or otherwise unassignable for Fe(II)BLM and Fe(III)BLM are shown in Figure 1(a).

For Fe(II)TLM, carbon atoms 41, 42, 45, 47, 48 and 51-69 were observed as narrow resonance lines. After air oxidation of Fe(II)TLM to Fe(III)TLM, changes in the spectrum were the broadening of resonance lines due to carbon atoms 45, 47 and 48. In both Fe(II)TLM and Fe(III)TLM a few very broad unassignable resonances were observed in the sugar region of the spectrum (60-75 ppm). Figure 1(b) shows the carbon resonances which were missing for Fe(II)TLM and Fe(III)TLM.

The $^{13}C$ nmr data (Fig 1) clearly show that the pyrimidine-imidazole-sugar regions of both antibiotics are utilized for binding the cations Fe(II) and Fe(III) and that both the Fe(II) and Fe(III) complexes are paramagnetic. These results are similar to those earlier obtained for Cu(II)BLM (11). Although the bithiazole is an important group for DNA binding (8-10), the $^{13}C$ nmr data for the iron complexes graphically show that the nitrogen-sulfur heterocycle of BLM is not involved in metal binding. Its resonance lines are narrow and unshifted and are not affected by the presence of the paramagnetic cations.

The observation that the bithiazole resonances of Fe(II)TLM and Fe(III)TLM are affected by the presence of the metal ion is similar to that earlier found for Cu(II)TLM (13). This effect is probably due to the basic structural differences which exist between the two antibiotics. The β-lysine-spermidine group and/or the L-talose moiety of TLM apparently force the bithiazole function to occupy a different spatial environment relative to the iron ion than is occupied by the bithiazole moiety of the analogous iron bleomycins.

Electrochemistry: Both BLM and TLM exhibit an irreversible reduction wave at -1.22V, which has been previously assigned to the two electron reduction of the 4-amino pyrimidine of the antibiotics (15). This wave (-1.22V) is absent from the dc polarograms of Fe(II, III)BLM and Fe(II, III) TLM in the pH range 4-9 in unbuffered aqueous media. Outside of this pH range in unbuffered media the pyrimidine reduction for the compounds can be observed. At pH 7 and 8 in phosphate buffer, the pyrimidine wave of both Fe(III)BLM and Fe(III)TLM can be observed whereas the pyrimidine wave of Fe(II)BLM and Fe(II)TLM is missing. We have previously observed (15) that the binding of metal ions to the 4-amino pyrimidine moiety of BLM and TLM dramatically shifts the reduction potential of the heterocycle out of the polarographic window of observation. We conclude that the 4-amino pyrimidine is not bound to Fe(II, III) at low and high pH in unbuffered media and it is not bound to Fe(III) at pH 7-8 in phosphate buffer. Upon returning the pH to the range 4-9, the pyrimidine rebinds to the metal ion.

EPR and Absorption Spectra: EPR results show (19-21) that Fe(III)BLM and Fe(III)TLM are low spin, S = ½, systems between pH 4 and 9 which is the same pH range over which the electrochemical results indicate that the 4-amino pyrimidine is bound to Fe(III). Further evidence that the pyrimidine is bound in the iron derivatives is found in the uv visible difference spectra shown in Figure 3. All of the complexes studied exhibited a pyrimidine $\pi \rightarrow \pi^*$ electronic transition which was shifted from its original position in bleomycin and tallysomycin. This band occurs at ~230 nm in the free drugs and shifts to ~250 nm in their metalloderivatives (12). In addition, the iron complexes exhibit a second strong envelope at ~300 nm. Since transitions due to two chromophores present in the antibiotics, the $n \rightarrow \pi^*$ of the pyrimidine and the $\pi \rightarrow \pi^*$ of the bithiazole moiety, overlap at 290 nm, the origin of the band at ~300 nm in the difference spectra of the metalloderivatives is difficult to determine. However, in view of the fact that the $^{13}C$ NMR data for Fe(II, III) BLM clearly shows that the bithiazole is not a metal ligating site, we have assigned the 300 nm band which occurs in the difference spectra of both drugs to the shifted $n \rightarrow \pi$ transition of the pyrimidine moiety.

At pH values outside the range 4-9 in unbuffered solution epr shows that the major species is high spin (S = 5/2) Fe(III) in a rhombic crystal field (g = 4.3). The high spin species is in a reversible pH-dependent equilibrium with the low spin Fe(III) species, although at low pH the rate of conversion of the high spin species to the

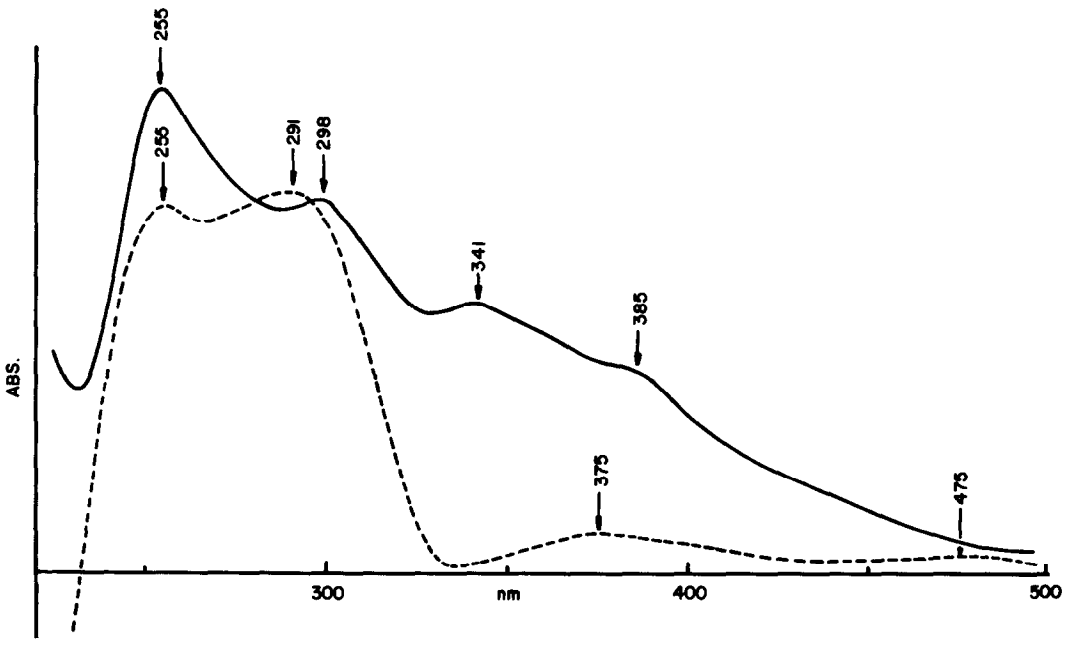


Figure 3 The difference spectrum, Fe(II)BLM vs. BLM (----) and Fe(III)BLM vs. BLM (——), in water at pH 7.0.

low spin species on raising the pH is much less than the reverse process. Furthermore at pH, 7.0 and 8.0 in either a phosphate or a borate buffer the high spin esr signal of the metal complexes dominates. A pH study using phosphate buffers shows that the loss of the low spin signal and the increase in the high spin signal for Fe(III)BLM-TLM can be correlated with the presence of the buffer species $H_2PO_4^-$. It is possible that the buffer species is binding to the metal, displacing one of the original nitrogen ligating atoms, thereby reducing the crystal field and yielding a high spin Fe(III) complex. At pH values >10, where $H_2PO_4^-$ is in low concentration and $HPO_4^{-2}$ predominates, the esr spectra of Fe(III)BLM-TLM are nearly identical to those obtained in water at pH, 10.0. Similar counter ion effects were observed for borate buffers.

Synthesizing the Fe(III)BLM-TLM complexes by anerobically combining $Fe(II)(C\ell O_4)_2$ and the drugs in a pH 7 or 8 phosphate buffer followed by air oxidation of the resulting Fe(II)-drug complex yields transient low spin Fe(III)BLM-TLM complexes (g = 2.25, 2.17, 1.94) which are rapidly and irreversibly transformed to second low-spin Fe(III) species (g = 2.41, 2.18, 1.90) which contain bound pyrimidine functions. If the oxidized solutions are allowed to stand at room temperature for a few hours, esr and visible absorp-

tion spectra show that a redistribution of the spin states occurs; high spin Fe(III) again forms at the expense of low spin Fe(III). Electrochemistry, however, shows that the pyrimidine is still bound to the metal ion. Thus, our results show that Fe(III)BLM-TLM is high spin when the pyrimidine moiety is not bound to the metal, but that some high spin forms also exist where the pyrimidine is bound to the cation. These observations, when viewed in the light that almost all of the DNA degrading experiments with the two antibiotics have been carried out in buffered media, underscore the importance of carefully defining the metal ligating properties of the drugs. Because in vitro DNA-cleavage and radical formation both occur in a wide range of buffer solutions it appears that the displacement of some of the ligating atoms does not inhibit the action of the iron-drug complex.

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