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Calcium(II) and the Trivalent Lanthanide Ion Complexes of the Bleomycin Antibiotics. Potentiometric, Fluorescence, and ^{1}H NMR Studies[1]

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Abstract: The interactions of Ca(II), Tb(III), and several other lanthanides with the bleomycins have been monitored by a combination of potentiometric, fluorescence, and NMR methods. From the potentiometric titrations we conclude that on complex formation, a single proton is displaced from the α-amino group of the diaminopropionamide group of the antibiotics. The results of Tb(III) emission experiments indicate that there is energy transfer between the fluorophores of the antibiotics and the Tb(III) emission manifold, resulting in large enhancements in the emission spectrum of the Tb(III). We have made use of this enhancement to determine the binding constants for the Tb(III) complexes of the antibiotics directly. Binding constants for other lanthanide ions were determined from an analysis of competition experiments in which these ions displace Tb(III) from its complexes with the bleomycins, thus decreasing the intensity of the Tb(III) emission lines. Proton NMR experiments conducted at 400 MHz indicate that the Pr(III) complex is in fast exchange on the NMR chemical shift time scale, while the Yb(III) complex is found to be in slow exchange. The lifetimes of the various lanthanide complexes have been estimated from a comparison with the Yb(III) case. The observed Gd(III)-induced line broadenings are shown to be exchange limited, i.e., the lifetime of the complex is longer than the transverse relaxation times in the complex and hence the relaxation rates are approximately independent of the distances of protons from the metal ion binding site.

The bleomycins are a family of glycopeptide antibiotics isolated from *Streptomyces verticillus* by Umezawa and co-workers.[2] The primary structures of these molecules are shown in Figure 1.[3] The various congeners of the bleomycins differ from each other in their terminal amine moiety (R group in Figure 1). Blenoxane, the commercial form of these drugs, marketed by Bristol Laboratories, contains ca. 70% bleomycin A_2 and ca. 25% bleomycin B_2 with trace amounts of other congeners. The bleomycins have been employed as antineoplastic agents in the treatment of a wide variety of human carcinomas and lymphomas.[4] The pharmacological activity of the bleomycins appears to be related to their ability to degrade cellular DNA.[5] A number of mechanisms for this process which involve the interaction of a ferrous bleomycin complex with molecular oxygen have been suggested.[6-8] It has also been reported that other polyvalent cations (e.g. Zn(II), Cu(II)) and EDTA[10] inhibit the strand scission process.[10] Additional interest in the complexes of the bleomycins with polyvalent metals derive from the use of bleomycin labeled with a radioisotope such as ^{57}Co, ^{64}Cu, ^{65}Zn, ^{67}Ga, ^{99m}Tc, ^{111}In, ^{169}Yb, ^{197}Hg, or ^{203}Pb as a tumor-scanning agent in the detection of a wide variety of solid tumors and malignant lymphomas.[11]

Previous studies of the interactions of the bleomycins with metal ions have focused on the Zn(II),[12-15] Cu(II),[16,17] and Fe(II)[18-21] complexes. Umezawa and co-workers have proposed a square-pyramidal coordination geometry for the Cu(II) complex on the basis of UV evidence[16] and X-ray crystallographic data.[16,17] In contrast, Dabrowiak et al. have inferred a square-planar geometry for the Zn(II) and Cu(II) complexes from the results of ^{13}C NMR studies,[14] ^{1}H NMR investigations,[12] UV difference spectra,[12] and EPR studies. On the basis of our previous studies on the kinetics of dissociation of the Zn(II) complex of bleomycin, we have suggested that the coordination geometry may be tetrahedral.[22] Oppenheimer et al.[15] have suggested that the imidazole group as well as the primary and secondary amines of the diaminopropionamide moiety are ligands of Zn(II) in the Zn–bleomycin A_2 complex. We have found that the binding of Ga(III) to both bleomycin A_2 and bleomycin B_2[23] displaces a single proton from

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(2) (a) H. Umezawa, *Antimicrob. Agents Chemother.*, 1079 (1965); (b) H. Umezawa, K. Maeda, T. Takenchi, and Y. Okami, *J. Antibiotics, Ser. A*, **19**, 195 (1966).

(3) T. Takita, Y. Muraoka, T. Nakatani, A. Fujii, Y. Umezawa, H. Maganawa, and H. Umezawa, *J. Antibiotics*, **31**, 801 (1978).

(4) For a review see: S. T. Crooke and W. T. Bradner, *J. Med. (Westbury, N.Y.)*, **7**, 333 (1977).

(5) (a) W. E. G. Muller, Z. Yamazaki, H. J. Breter, and R. K. Zahn, *Eur. J. Biochem.*, **31**, 518 (1972); (b) M. Saito and T. Andoh, *Cancer Res.*, **33**, 1696 (1973); (c) T. Onishi, K. Shimada, and Y. Tokagi, *Biochem. Biophys. Acta*, **312**, 248 (1973); (d) W. E. G. Muller and R. K. Zahn, *Prog. Nucleic Acid Res. Mol. Biol.*, **2**, 21–57 (1977).

(6) J. W. Lown and S. K. Sim, *Biochem. Biophys. Res. Commun.*, **77**, 1150 (1977).

(7) E. A. Sausville, J. Peisach, and S. B. Horwitz, *Biochem. Biophys. Res. Commun.*, **73**, 814 (1977).

(8) (a) H. Murakami, H. Mori, and S. Taira, *J. Theor. Biol.*, **42**, 443 (1973); (b) H. Murakami, H. Mori, and S. Taira, *ibid.*, **59**, 1 (1976).

(9) (a) M. Takeshita, A. P. Grollman, and S. B. Horwitz, *Virology*, **69**, 453 (1976); (b) I. Shirakawa, M. Agezami, S. Ishii, and H. Umezawa, *J. Antibiotics*, **24**, 761 (1971).

(10) (a) K. Nagai, H. Yamaki, H. Suzuki, N. Tanaka, and H. Umezawa, *Biochem. Biophys. Acta*, **179**, 165 (1969); (b) H. Suzuki, K. Nagai, E. Akurn, H. Yamahi, N. Tanaka, and H. Umezawa *J. Antibiotics*, **23**, 473 (1970).

(11) For a review see: E. Silberstein, *Am. J. Med.*, **60**, 226 (1976).

(12) J. D. Dabrowiak, F. T. Greenaway, W. E. Longo, M. Van Husen, and S. T. Crooke, *Biochim. Biophys. Acta*, **517**, 517 (1978).

(13) A. E. G. Cass, A. Goldes, A. Allen, A. O. Hill, and C. E. McClelland, *FEBS Lett.*, **89**, 187 (1978).

(14) J. C. Dabrowiak, F. T. Greenaway, and R. Grulich, *Biochemistry*, **17**, 4090 (1978).

(15) N. J. Oppenheimer, L. O. Rodriquez, and S. M. Hecht, *Biochemistry*, **18**, 3439 (1979).

(16) T. Takita, Y. Muraoka, T. Nakatani, A. Fujii, Y. Itaka, and H. Umezawa, *J. Antibiotics*, **31**, 1073 (1978).

(17) Y. Itaka, H. Nakamura, T. Nakatani, Y. Muraoka, A. Fujii, T. Takita, and H. Umezawa, *J. Antibiotics*, **31**, 1070 (1978).

(18) (a) E. A. Sausville, J. Peisach, and S. B. Horwitz, *Biochem. Biophys. Res. Commun.*, **73**, 814 (1976); (b) E. A. Sausville, R. W. Stein, J. Peisach, and S. B. Horwitz, *Biochemistry*, **17**, 2746 (1978); (c) E. A. Sausville, R. J. Peisach, and S. B. Horwitz, *ibid.*, **17**, 2740 (1978).

(19) R. K. Gupta, J. A. Ferretti, and W. J. Caspary, *Biochem. Biophys. Res. Commun.*, **89**, 534 (1979).

(20) R. M. Burger, J. Peisach, W. E., Blumberg, and S. B. Horwitz, *J. Biol. Chem.*, *254*, 10906 (1979).

(21) N. J. Oppenheimer, L. O. Rodriquez, and S. M. Hecht, *Proc. Natl. Acad. Sci. U.S.A.*, **76**, 5616 (1979).

(22) R. E. Lenkinski and J. L. Dallas, *J. Am. Chem. Soc.*, **101**, 5902 (1979).

(23) R. E. Lenkinski, B. Peerce, J. L. Dallas, and J. D. Glickson, *J. Am. Chem. Soc.*, **102**, 131 (1980).

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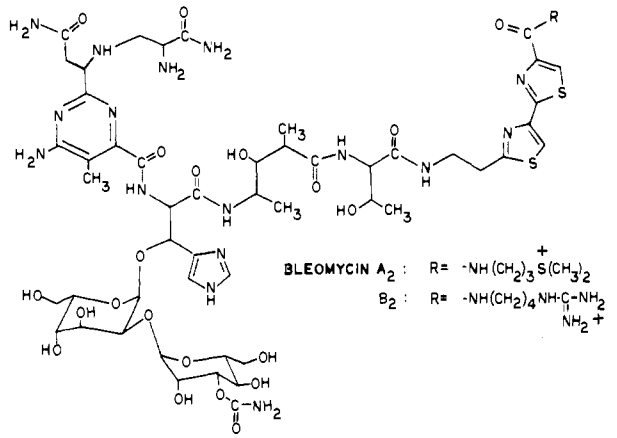

Figure 1. Primary structure of the bleomycin antibiotics.

the primary amine group of the same moiety. By analyzing paramagnetic perturbations of the ^{13}C NMR spectrum, Gupta et al.[19] have also found this amino group to be part of the Fe(II) binding site. However, the same authors found no evidence to support the participation of the imidazole in binding to Fe(II). In contrast, NMR[21] and UV[20] spectral data have also been interpreted in terms of direct coordination of the imidazole to Zn(II) and Fe(II). Thus, there is still controversy surrounding the identification and the geometry of the coordinating ligands in the bleomycins.

In the present study, we have monitored the interactions of Ca(II) and several of the trivalent lanthanide ions with the bleomycins, using a variety of methods. Interest in these complexes derives from the possible in vivo formation of Ca(II) complexes. In addition, the analysis of the dipolar shifts and relaxation rate enhancements of the lanthanide complexes, which serve as paramagnetic analogues of calcium, can often provide geometrical information about the ligands. In contrast, the analysis of paramagnetic perturbations induced by transition metals is generally complicated by the difficulty of the separation of dipolar and Fermi contact contributions.

Using potentiometric titrations, we have determined the association constants for the Ca(II) and Tb(III) complexes. The binding constants for the Tb(III) complexes were also determined from energy transfer between the antibiotics and Tb(III) emission manifold. The binding constants for the other trivalent lanthanides were obtained from an analysis of competition experiments in which these ions displaced Tb(III) from the antibiotics. In addition, we present the results of NMR investigations into the Pr(III)- and Yb(III)-induced shifts in the ^1H spectrum of the bleomycins, as well as Gd(III)-induced line broadenings.

Experimental Section

Blenoxane, a gift from Bristol Laboratories (Syracuse, New York), was separated into its component congeners by chromatography on carboxymethyl-Sephadex CM-25, using a linear gradient of 0–0.5 M NaCl. The fractions were extracted into methanol to remove the salt and passed over an R-10 Amberlite ion-exchange column using neutral H_2O as an eluant. The concentration of each Blenoxane congener was determined spectrophotometrically by using a molar absorptivity of $(1.3 \pm 0.2) \times 10^4$ M^{-1} cm^{-1} at 290 nm. The concentration of lanthanide ions in solution was determined by a complexiometric titration with EDTA, using arsenazo as indicator.

Fluorescence experiments were performed on a Perkin-Elmer MPF-3 fluorimeter (uncorrected) at ambient temperature. Nuclear magnetic resonance spectra were recorded at 400 MHz on a Bruker WH-400 spectrometer operating in the FT mode. All computations were performed on an IBM-370 computer.

Results and Discussion

Proton-Release Experiments. The addition of Tb(III) to an unbuffered solution of bleomycin at pH 7.0 results in a decrease in the pH of the solution. This decrease in pH reflects the fact that the metal ion is competing with hydrogen ions for an ionizable group or groups in the drug. The concentration of hydrogen ions released per equivalent of Tb(III) added can be determined by

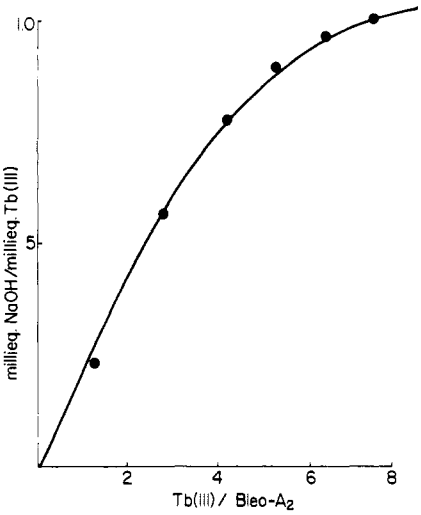

Figure 2. The number of milliequivalents of sodium hydroxide added to a solution of bleomycin A_2 (1×10^{-4} M, initial pH 7.0) as the Tb(III) to bleomycin A_2 ratio is increased.

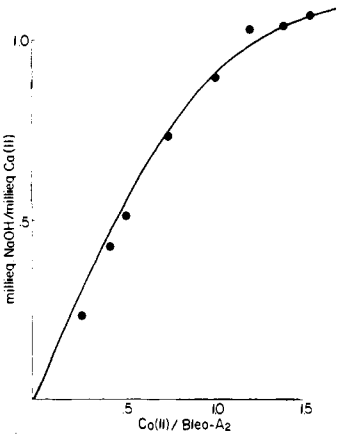

Figure 3. The number of milliequivalents of sodium hydroxide added to a solution of bleomycin A_2 (1×10^{-4} M, initial pH 7.0) as the Ca(II) to bleomycin A_2 ratio is increased.

titrating the solution to pH 7.0 after each addition of metal ion. The results of these experiments for bleomycin A_2 are shown in Figure 2. These data clearly demonstrate that the binding of Tb(III) to this congener displaces a single proton from an ionizable group in the antibiotic. We obtained similar results for the same experiments conducted with bleomycin B_2.

The addition of Ca(II) to an unbuffered solution of bleomycin A_2 at pH 7.0 also results in a decrease in the pH of the solution. The results of the titration of this solution to pH 7.0 after each addition of Ca(III) are shown in Figure 3. From these data it is clear that a single proton is being displaced from the antibiotic on binding to Ca(II). The affinity of the antibiotic for Ca(II) appears to be lower than that for Tb(III). Again, similar results were obtained for the interactions of Ca(II) with the bleomycin B_2 congener.

Proton-Displacement Experiments. Since a single proton is displaced from the bleomycins on metal binding, we can describe the competition between metal ions and protons by eq 1; further

$$[B]^3 + (K_M + K_H + H_T^+ + M_T - B_T)[B]^2 + \{K_M K_H + M_T K_H + H_T^+ K_M - B_T(K_M + K_H)\}[B] - B_T K_M K_H = 0 \quad (1)$$

details are given in a previous report,[23] where M_T, B_T, and H_T^+ refer to the total concentrations of the metal ion, the antibiotic, and protons present, respectively, K_M and K_H are the dissociation constants for the metal complex and the protonated form of bleomycin, and the brackets denote equilibrium concentrations.

Since the values of B_T and M_T are known experimentally, the solution of eq 1 requires the specification of two parameters, K_M and K_H. The fitting of experimental data to this equation is

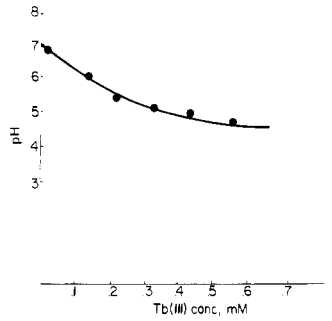

Figure 4. Variation in pH of a solution of bleomycin A_2 (1×10^{-4} M, initial pH of 7.0) in the presence of increasing concentrations of Tb(III).

simplified by the fact that the pK_a's of the three ionizable groups have been reported to be 2.9, 4.7, and 7.3 in bleomycin B_2[24] and 2.9, 5.0, and 7.7 for bleomycin A_2.[25] Our approach to the data fitting procedure was to begin our nonlinear least-squares algorithm with a value of K_H corresponding to each of the three pK_a values and then to solve eq 1.

The variation in the pH of a solution of bleomycin A_2 in the presence of various concentrations of Tb(III) is shown in Figure 4. Nonlinear least-squares minimization of these data, using our computational approach, gave values of $K_M = (3.7 \pm 0.3) \times 10^{-5}$ M ($pK_M = 4.43$) and $K_H = (6.2 \pm 0.5) \times 10^{-8}$ M ($pK_H = 7.21$). Similar experiments and analyses were carried out for bleomycin B_2. The values of K_M and K_H were found to be $(4.0 \pm 0.4) \times 10^{-5}$ M ($pK_M = 4.40$) and $(6.4 \pm 0.5) \times 10^{-8}$ M, ($pK_H = 7.19$), respectively. The rather good agreement between the values of K_M obtained for the complexes of bleomycin A_2 and bleomycin B_2 indicated that the C-terminal amine moiety (R group in Figure 1) is not involved in binding to Tb(III).

The results of similar experiments carried out on bleomycin A_2 in the presence of Ca(II) were analyzed in the same way, giving values for $K_M = (1.2 \pm 0.5) \times 10^{-4}$ M ($pK_M = 3.92$) and $K_H = (6.4 \pm 0.2) \times 10^{-8}$ M ($pK_H = 7.19$). In a similar manner, we obtained values for $K_M = (1.5 \pm 0.5) \times 10^{-4}$ M ($pK_M = 3.82$) and $K_H = (6.3 \pm 0.2) \times 10^{-8}$ M ($pK_H = 7.20$) for the Ca(II)–bleomycin B_2 complex.

It can be seen that the K_H values obtained for all four complexes (Tb(III)–bleomycin A_2, Tb(III)–bleomycin B_2, Ca(II)–bleomycin A_2, and Ca(II)–bleomycin B_2) correspond to a pK_a of 7.2 ± 0.1, implicating the α-amino group of the diaminopropionamide moiety in metal binding. In an X-ray crystallographic study of a copper(II) complex of a bleomycin metabolite, Umezawa and coworkers have found this nitrogen to be a ligand of Cu(II). As was pointed out earlier, this nitrogen has also been implicated in metal binding in the Zn(II)–bleomycin complex[15] and the Fe(II)–bleomycin complex,[19] as well as the Ga(III) complexes of bleomycin A_2 and bleomycin B_2.[23]

Fluorescence Experiments. The use of Tb(III) emission in studies of metal binding to protons has been discussed by Brittain et al.[26] In the case of bleomycin A_2, the binding of Tb(III) results in large enhancements in the emission bands of the metal ion (cf. Figure 5). These enhancements are dependent on the excitation wavelength of the experiment with a maximum enhancement occurring at ca. 300 nm. This is evident from the large band present centered at 300 nm in the excitation spectrum of Tb(III) in the presence of bleomycin A_2 (Figure 5). Since the bleomycins are fluorescent, having excitation and emission maxima at ca. 300 and 350 nm, respectively,[27] we can conclude that there is significant

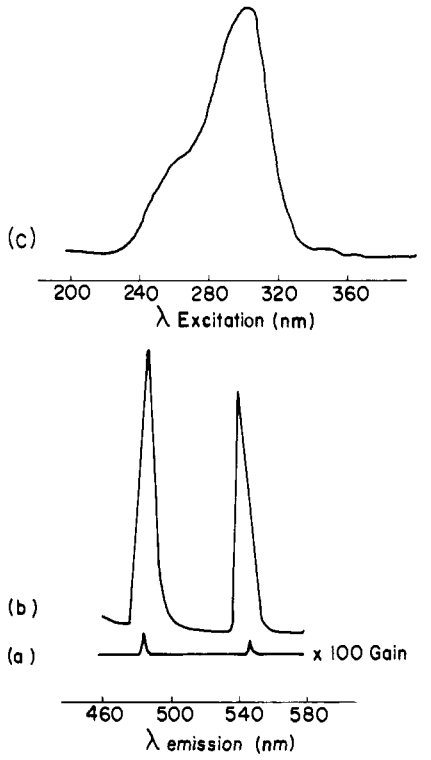

Figure 5. Fluorescence spectra of Tb(III) solutions at ca. 25 °C: (a) emission spectrum of a solution containing 1×10^{-4} M Tb(III) and 20 mM Pipes buffer at pH 6.8 (excitation of wavelength is 300 nm); (b) same as a with 1×10^{-4} M bleomycin A_2 present; (c) excitation spectrum of b monitored at 545 nm.

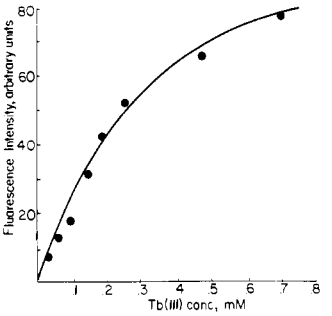

Figure 6. Variation in the intensity of the emission of Tb(III) (545 nm) as a function of Tb(III) concentration in a solution containing 2×10^{-5} M bleomycin A_2, in 20 mM Pipes at pH 6.8. The excitation wavelength was 300 nm.

energy transfer occurring between the fluorophore of the antibiotic and the Tb(III) ion.

We have made use of this large enhancement in Tb(III) emission observed in the presence of bleomycin A_2 and bleomycin B_2 in order to determine the dissociation constants for the Tb(III) complexes of these antibiotics. The variation in the intensity of the Tb(III) emission with bleomycin A_2 concentratiion is shown in Figure 6. This data was analyzed in terms of the quadratic equation (2).[23] Note that the value of K_H is known from the

$$[B]^2(K_H + [H^+]) + [B][(K_H + [H^+])K_M + K_H(M_T - B_T)] - K_H K_M B_T = 0 \quad (2)$$

results of proton displacement experiments. Also if the experiment is performed in a buffered system, the value of $[H^+]$ is kept constant. Thus, the computation of the values of [B] requires the specification of one parameter, K_M. Once the values of [B] are known, the concentrations of MB, M, and BH^+ can be calculated. If the contribution of the free Tb(III) to the fluorescence intensity is negligible, the change in the fluorescence intensity of the Tb(III), ΔF, obeys the relationship (3), where ΔF_{max} is the maximum

$$\Delta F = \Delta F_{max}[MB] \quad (3)$$

(24) T. Takita, Y. Muraoka, and H. Umezawa, *J. Antibiotics*, **25**, 210 (1972).

(25) The pK_a values for bleomycin A_2 were reported by Y. Sugiura, K. Ishizu, and K. Miyoshi, *J. Antibiotics*, **32**, 453 (1979). Similar values were obtained for blenoxane by E. S. Mooberry, J. L. Dallas, T. T. Sakai, and J. D. Glickson, *Int. J. Pept. Protein Res.*, **15**, 365 (1980).

(26) H. G. Brittain, F. S. Richardson, and R. B. Martin, *J. Am. Chem. Soc.*, **98**, 8255 (1976).

(27) M. Chien, A. P. Grollman, and S. B. Horwitz, *Biochemistry*, **16**, 3641 (1977).

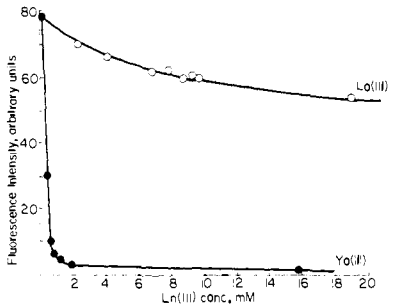

Figure 7. Variation in the intensity of emission of Tb(III) (545 nm) in a solution containing 2×10^{-5} M bleomycin A_2, 1×10^{-4} M Tb(III), 20 mM Pipes at pH 6.8, and varying concentrations of the other lanthanide ions as indicated. The excitation wavelength was 300 nm.

Table I. Association Constants[a] and Estimated Lifetimes[b] of Various Lanthanide Complexes of Bleomycin A_2

lanthanide ion	K_a, M^{-1}	τ, s
La(III)	$(2.1 \pm 1.35) \times 10^2$	0.0008
Pr(III)	$(1.6 \pm 1) \times 10^3$	0.006
Nd(III)	$(4 \pm 1.2) \times 10^3$	0.02
Eu(III)	$(2.0 \pm 1.0) \times 10^4$	0.08
Gd(III)	$(2.2 + 1.0) \times 10^4$	0.09
Tb(III)	$(2.5 \pm 1.0) \times 10^4$	0.1
Dy(III)	$(4.5 \pm 1.5) \times 10^4$	0.2
Ho(III)	$(6.3 \pm 2) \times 10^4$	0.25
Er(III)	$(1 \pm 1) \times 10^5$	0.4
Yb(III)	$(1.0 \pm 1.0) \times 10^6$	4[c]

[a] These constants were determined from an anlysis of the displacement of Tb(III) from bleomycin A_2. [b] Lifetimes were estimated from the association constants under the assumption of a constant rate of metal complexation for all of the lanthanides. [c] Estimated from transfer of saturation experiments.

increase in fluorescence intensity and all other symbols have been defined previously.

The data in Figure 6 were fit to eq 2 and 3; the best-fit value of K_M obtained in this manner was $(4.0 \pm 1.0) \times 10^{-5}$ M. The experiments and analyses were repeated for the B_2 congener and a value for K_M of $(5.0 \pm 1.0) \times 10^{-5}$ M was obtained. Note the good agreement between these values for K_M and those obtained from an analysis of the proton-displacement experiments conducted with Tb(III). This confirms the consistency of the two approaches.

The competition of another metal ion both with protons and Tb(III) can be taken into account by including the equilibrium (4), where N and NB refer to the second metal ion and its complex, respectively. The inclusion of this equilibrium yields the cubic equation (6) in [B]. If K_M and K_H are known from a previous

$$N + B \rightleftarrows NB \quad (4)$$

$$K_N = [N][B]/[NB] \quad (5)$$

$$[B]^3(K_H + [H^+]) + [B]^2(K_H + H^+)(K_M + K_N) + K_H(M_T + N_T - R_T) - B_T K_H K_M K_N = 0 \quad (6)$$

experiment, only K_N remains unknown. Therefore, eq 6 provides a basis for the analysis of Tb(III) fluorescence data taken in the presence of a competing ion. The variation in the fluorscence intensities of a solution containing Tb(III), bleomycin A_2, and various concentrations of La(III) is shown in Figure 7. For the purposes of comparison, the results of a similar experiment using Yb(III) are also shown in Figure 7. Note that the binding of Yb(III) to bleomycin is much stronger than that observed for La(III). These experiments were repeated with several other trivalent lanthanide ions. The data obtained were analyzed in terms of eq 3 and 6, and the results of these analyses are summarized in Table I.

The variation in the logarithm of the association constant with the ionic radius of the lanthanide ion is shown in Figure 8. Similar trends have been observed for the binding of lanthanide ions to NTA, EDTA,[28a] and trypsin.[28b] The increase in the binding

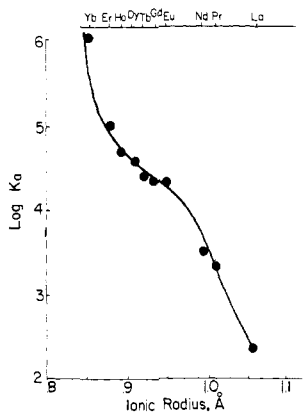

Figure 8. Variation in the logarithm of the association constants for the lanthanide ion complexes of trivalent bleomycin A_2 with the ionic radius of the lanthanide ion.

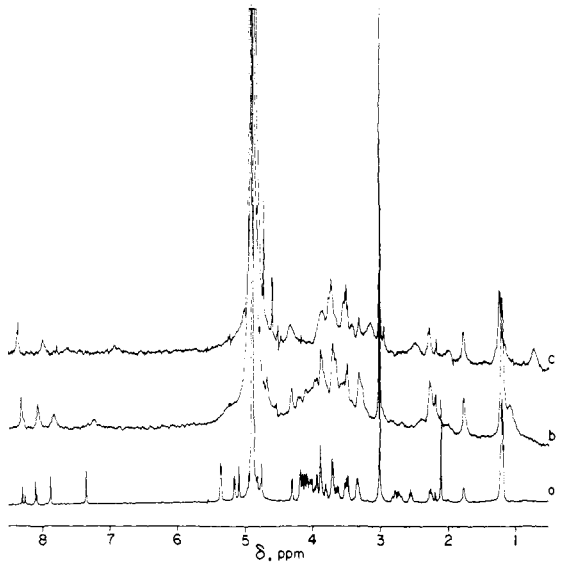

Figure 9. The 400-MHz ^1H NMR spectrum of a solution containing 10 mM Blenoxane at pH 6.8 with (a) no Pr(III), (b) 10 mM Pr(III) present, and (c) 20 mM Pr(III) present. Each spectrum required 64 accumulations.

constant with the decrease in ionic radius probably reflects the fact that there is a large increase in entropy upon complex formation. For example, in the case of the EDTA complexes of the lanthanides, the enthalpies of formation are relatively small and negative, while the entropies of complexation fall between 60 and 80 cal deg^{-1} mol^{-1}.[29]

NMR Experiments. The effects of the presence of Pr(III) on the ^1H NMR spectrum of Blenoxane are shown in Figure 9. The fact that the magnitudes of the chemical shift perturbations induced by Pr(III) increase with increasing metal ion concentration indicates that the antibiotic is in fast exchange on the ^1H NMR chemical shift time scale between its free and complexed states. The chemical shift perturbations in the presence of paramagnetic ions can be expressed as

$$\Delta = \Delta_{CF} + \Delta_C + \Delta_D \quad (7)$$

where Δ_{CF} is the complex formation shift and is diamagnetic in origin, Δ_C is the shift arising from the Fermi contact interaction, and Δ_D is the shift arising from the dipolar interaction. In the present system Δ_{CF} is negligible since the spectrum of Blenoxane is unaffected by the presence of a saturating concentration of

(28) (a) J. Reuben, "Handbook on the Physics and Chemistry of the Rare Earths", Vol. 4, North-Holland Publishing Co., Amsterdam, 1979, p 515. (b) M. Epstein, A. Levitzki, and J. Reuben, *Biochemistry*, **13**, 1777 (1974).
(29) C. S. G. Phillips and R. J. P. Williams, "Inorganic Chemistry", Vol. 2, Oxford University Press, New York, 1966, p 117.

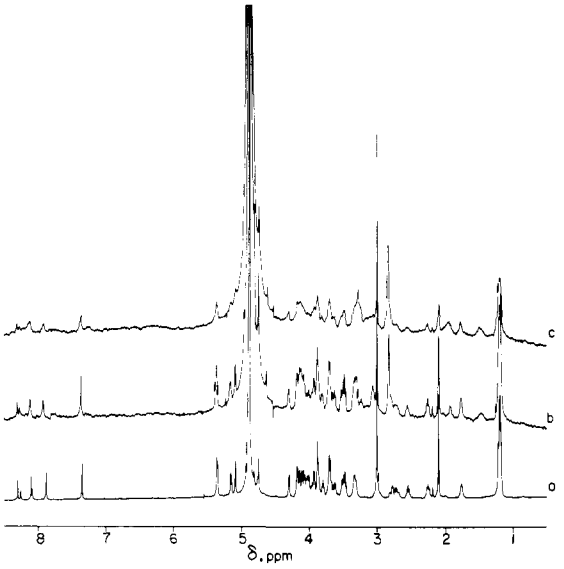

Figure 10. The 400-MHz ^{1}H NMR spectrum of a solution containing 10 mM Blenoxane at pH 6.8 with (a) no Yb(III), (b) 5 mM Yb(III) present, and (c) 10 mM Yb(III) present. Each spectrum required 64 accumulations.

La(III). For most of the trivalent lanthanide ions (including Gd(III)) the contribution of Δ_{CF} is also negligible. The dipolar shift is given by eq 8,[30] where K_1 and K_2 are constants related to

$$\Delta_D = K_1[(3\cos^2\theta - 1)/r^3] + K_2(\sin^2\theta\cos 2\phi/r^3) \quad (8)$$

the elements of the magnetic susceptibility tensor of the metal ion and r, θ, and ϕ are the spherical polar coordinates of the nucleus relative to the principal magnetic axis system of the metal ion. From eq 8, it is clear that the dipolar shifts are stereospecific.

In the spectra shown in Figure 9, perturbations are observed in the resonances arising from several groups in the antibiotic. The histidine $C^{\alpha}H$, $C^{\beta}H$, and aromatic resonances are all shifted upfield in the presence of Pr(III). It is also clear that the anomeric resonances of the dissacharide moiety are shifted upfield. The resonances of the methylene group and the methyl group of the pyrimidine moiety are all shifted downfield by Pr(III). It is apparent that for the methyl resonance this shift is accompanied by significant line broadening. The resonances associated with the $C^{\alpha}H$ of hydroxyvaleric acid portion of the antibiotic as well as one of the three methyl groups (ca. 1.1 ppm) are shifted upfield by Pr(III). On the basis of these observations we suggest that all of the groups whose resonances are perturbed by Pr(III) are involved in, or are in close proximity to, the site of metal ion complexation. The hydroxyvaleric acid moiety has been implicated as a possible ligand in the Zn(II) complex[15] as well as in a diamagnetic Fe(II) carbon monoxide complex[21] of the bleomycins. We have reached a similar conclusion based on results obtained in a study of the paramagnetic Fe(II) complex of bleomycin A_2 (unpublished results).

The effects of the presence of Yb(III) on the ^{1}H NMR spectra are shown in Figure 10. Note that in these spectra, the successive additions of Yb(III) bring about perturbations in the intensities of the resonances of the antibiotic concomitant with the appearance of several new peaks, indicating that the bleomycin is in slow exchange between its free and complexed states. We have employed transfer of magnetization techniques[23,31] to assign the peak at ca. 2.8 ppm to the methyl groups of the dimethylsulfonium moiety of the Yb(III)–bleomycin complex. From the chemical shift separation and the extent of transfer of saturation we estimate that the lifetime of the complex is ca. 4 s at 25 °C.

The smallest chemical shift perturbation induced by Pr(III) was found to be ca. 30 Hz for one of the methyl resonances centered at 1.1 ppm. From this chemical shift separation we estimate that the lifetime of the Pr(III)–bleomycin complex is less than or equal to 7×10^{-3} s. Table I contains estimates for the lifetimes of the lanthanide complexes for which association constants were determined fluorimetrically. These estimates were obtained under the assumption that the rate of metal ligation with the bleomycins is constant through the lanthanide series. This assumption is supported by kinetic data for other systems such as oxalate[32] and murexide.[33]

We have also determined the line widths of the aromatic resonances of the bleomycins in the presence of increasing concentrations of Gd(III). When the chemical shift induced by Gd(III) is negligible, the concentration dependence of the line broadening in the absence of outer sphere relaxation is given by eq 9,[34] where

$$1/T_{2p} = P_M/(T_{2M} + \tau_m) \quad (9)$$

P_M is the fraction of ligand bound to the metal ion, $1/T_{2M}$ is the transverse relaxation rate of the resonance in its complexed state, and τ_m is the lifetime of the ligand in its complexed form. If the relaxation enhancement is dipolar in origin, $1/T_{2M}$ is given by eq 10,[35] where ω_I and ω_S are the nuclear and electronic Larmor

$$1/T_{2M} = \frac{\gamma_I^2\beta^2 S(S+1)g^2}{15r^6}\left[4\tau_c + \frac{3\tau_c}{1+\omega_I^2\tau_c^2} + \frac{13\tau_c}{1+\omega_S^2\tau_c^2}\right] \quad (10)$$

frequencies related to the resonance frequency ν by $\omega_I = 2\pi\nu$ and $\omega_S = 660\omega_I$, γ_I is the magnetogyric ratio of the nucleus being observed, β is the Bohr magneton, S is the total electron spin, and r is the distance between the nucleus and the paramagnetic ion. The correlation time τ_c in the above equation is defined in eq 11,

$$1/\tau_c = 1/\tau_s + 1/\tau_r + 1/\tau_m \quad (11)$$

where τ_r is the rotational correlation time of the complex, τ_s is the electron-spin relaxation time of the paramagnetic species, and τ_m is the mean residence time of the ligand in the metal complex.

We have shown previously that at high spectrometer frequencies τ_s becomes ca. 1×10^{-7} s.[36] From Table I, τ_m is ca. 0.08 s for Gd(III). Krishna et al. have reported a value of $\tau_r = 4 \times 10^{-10}$ s for bleomycin A_2 from an analysis of ^{13}C spin–lattice relaxation measurements.[37] Therefore τ_c is approximately equal to τ_r.

Using this value for τ_c in eq 10, we can compute $1/T_{2M}$ as a function of r, the metal–nuclear distance. At a distance of 8 Å which is the approximate radius of the bleomycin molecule[37] T_{2M} would be ca. 5×10^{-4} s. By comparison, τ_m is ca. 0.08 s (cf. Table I). Since T_{2M} will be smaller for protons that are closer than 8 Å, we can conclude that for most of the protons in bleomycin $\tau_m \gg T_{2M}$.[38] Therefore, eq 9 can be rewritten as eq 12. Since τ_m

$$1/T_{2p} \approx P_M/\tau_m \quad (12)$$

is a characteristic of the molecule, this result implies that all of the protons which are closer than 8 Å will be broadened equally, and a plot of $1/T_{2p}$ vs. P_M should yield a slope of $1/\tau_m$. The slopes of our experimental plots of $1/T_{2p}$ vs. P_M yielded straight lines with nearly equal slopes of ca. 20 s^{-1} for all four of the aromatic protons of bleomycin, confirming the fact that $\tau_m > T_{2M}$ for these nuclei.

(30) B. Bleaney, *J. Magn. Reson.*, **8**, 91 (1972).
(31) R. K. Gupta and A. G. Redfield, *Biochem. Biophys. Res. Commun.*, **41**, 273 (1970).
(32) A. J. Graffeo and J. L. Bear, *J. Inorg. Nucl. Chem.*, **30**, 1577 (1968).
(33) G. Geier, *Ber. Bursenges. Phys. Chem.*, 69, 617 (1965).
(34) (a) T. J. Swift and R. E. Connick, *J. Chem. Phys.*, **37**, 307 (1962); (b) Z. Luz and S. Meiboom, *J. Chem. Phys.*, **40**, 2686 (1964).
(35) (a) I. Solomon, *Phys. Rev.*, 99, 559 (1955); (b) N. Bloembergen, *J. Chem. Phys.*, **27**, 572 (1957).
(36) R. E. Lenkinski, J. L. Dallas, and J. D. Glickson, *J. Am. Chem. Soc.*, **101**, 3971 (1979).
(37) N. R. Krishna, J. L. Dallas, E. S. Mooberry, T. T. Sakai, R. C. Allen, G. C. Levy, and J. D. Glickson, *Biochem. Biophys. Res. Commun.*, **85**, 363 (1978).
(38) Note that T_{2M} depends on an inverse sixth power of the distance; therefore, a proton which is 4 Å from the metal ion would have a T_{2M} which is a factor of ca. 50 shorter than the T_{2M} for a proton which is 8 Å from the metal ion.

Conclusions

(1) On the basis of potentiometric studies, we conclude that a single proton is displaced from the primary amine moiety of the diaminopropionamide portion of the bleomycin molecule on binding Ca(II) and Tb(III).

(2) The binding of the lanthanides occurs with a large entropy of complexation suggesting that both the metal ions and the antibiotic are desolvated on complex formation.

(3) The complexes of bleomycin with the lanthanides are relatively long lived. These results, taken together with our previous studies on the Zn(II) and Ga(III) complexes of these antibiotics, indicate that these metal complexes are kinetically stable. This suggests that the interpretation of Gd^{3+}-induced relaxation rate enhancements in terms of intermolecular distances may not be possible.

(4) The perturbations induced by Pr(III) in the ^1H spectrum of the antibiotic indicate that the valeric acid group, the dissaccharide, as well as the histidine, are in close proximity to the site of metal complexation. Further structural studies based on these Pr(III) results are in progress in our laboratories.

Structural Studies of Ristocetin A (Ristomycin A): Carbohydrate–Aglycone Linkages

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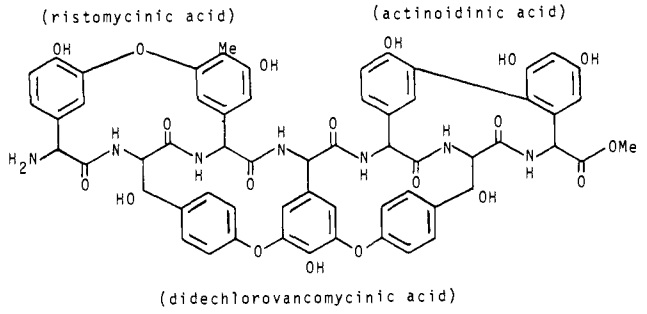
Abstract: The structure of the carbohydrate component of the antibiotic ristocetin A has been elucidated. The amino sugar L-ristosamine has been identified along with previously isolated D-glucose, D-mannose, D-arabinose, and L-rhamnose. Acid-catalyzed acetolysis of the antibiotic yielded acetylated ristotetrose along with mono-, di-, and trisaccharides derived from it. Base hydrolysis of O-methylated antibiotic in the presence of $NaBH_4$ has shown that the O-α-D-arabinofuranosyl-(1→2)-*O*-α-D-mannopyranosyl-(1→2)-*O*-[α-L-rhamnopyranosyl-(1→6)]-β-D-glucopyranosyl residue is attached to the phenolic hydroxyl group of didechlorovancomycinic acid and the L-ristosaminyl residue to one of the two alcoholic hydroxyl groups in that amino acid. Controlled acid hydrolysis of the antibiotic yielded a partial hydrolysis product which still contained ristosamine; ^{13}C NMR spectroscopy indicated an α linkage for that sugar. An additional mannose residue is attached to actinoidinic acid. The phenolic hydroxyl group to which it is attached was identified unambiguously by ^1H NMR measurements on a degradation product and by an independent synthesis of that compound; the anomeric configuration of mannose was not established. Further evidence is presented supporting the belief that ristocetin A and ristomycin A are identical. The structural data now available permit conclusions to be drawn concerning the mechanism of action of antibiotics in this class, particularly with regard to differences in structural specificity observed in the binding of small aliphatic peptides to vancomycin and ristocetin.

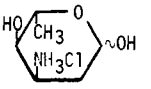
The glycopeptides ristocetin and ristomycin, produced by *Nocardia lurida*[1] and *Proactinomyces fructiferi* var. *Ristomycini*,[2] respectively, belong to the vancomycin group of antibiotics. Both antibiotics contain two biologically active components, A and B, of which the A form predominates.[3,4] Mounting evidence, both chemical and biological, indicates that the two antibiotics are identical. Studies in this laboratory and elsewhere have established that the peptide moieties of both ristocetin A[5,6] and ristomycin A[7] contain ristomycinic acid, actinoidinic acid, and didechlorovancomycinic acid. Structure **1** has been proposed for this peptide



1

by analogy with the known structure of vancomycin;[5,6] N-terminal analysis of ristocetin A and ristomycin A supports this proposal.[8]

Studies of ristomycin A have shown that it contains 2 mol of D-mannose and 1 mol each of D-glucose, D-arabinose, L-rhamnose,[9,10] and the amino sugar L-ristosamine (**2**).[11] The



2

structure of **2** was established by Sztaricskai via independent synthesis.[12–14] He has also inferred the presence of a tetra-

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(1) Grundy, W. E.; Sinclair, A. C.; Theriault, R. J.; Goldstein, A. W.; Rickher, C. J.; Warren, H. B., Jr.; Oliver, T. J.; Sylvester, J. C. *Antibiot. Annu.* **1956–1957**, 687.
(2) Brazhnikova, M. G.; Lomakina, N. N.; Sztaricskai, F.; Puskás, M.; Makleit, S.; Bognár, R. *Kem. Kozl.* **1967**, *27*, 143.
(3) Philip, J. E.; Schenck, J. R.; Hargie, M. P. *Antibiot. Annu.* **1956–1957**, 699.
(4) Lavrova, M. F.; Lomakina, N. N.; Babenko, G. A.; Brazhnikova, M. G. *Antibiotiki (Moscow)* **1964**, *9*, 885.
(5) Harris, C. M.; Kibby, J. J.; Fehlner, J. R.; Raabe, A. B.; Barber, T. A.; Harris, T. M. *J. Am. Chem. Soc.* **1979**, *101*, 437.
(6) Williams, D. H.; Rajananda, V.; Kalman, J. R. *J. Chem. Soc., Perkin Trans. 1* **1979**, 787.
(7) Sztaricskai, F.; Harris, C. M.; Harris, T. M. *J. Antibiot.* **1979**, *32*, 446.
(8) Harris, C. M.; Harris, T. M. *Tetrahedron Lett.* **1979**, 3905.
(9) Brazhnikova, M. G.; Lomakina, N. N.; Muravyeva, L. I. *Int. Congr. Chemother., Proc., 5th* **1967**, 759.
(10) Lomakina, N. N.; Muravyeva, L. I.; Puskás, M. *Antibiotiki (Moscow)* **1968**, *13*, 867.
(11) Bognár, R.; Sztaricskai, F.; Munk, M. E.; Tamás, J. *J. Org. Chem.* **1974**, *39*, 2971.

0002-7863/80/1502-7093$01.00/0