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Proton Nuclear Magnetic Resonance Study of the Structure of Bleomycin and the Zinc–Bleomycin Complex†

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ABSTRACT: ^{1}H NMR spectral studies at 360 MHz have been conducted on bleomycin, a modified bleomycin, and the zinc–bleomycin complex. In these studies, the revised structure of bleomycin has been independently verified by (1) the pD dependence of chemical shifts, (2) observation of the exchangeable resonances with $(CD_3)_2SO$ as solvent, and (3) acylation of the α-amine of the β-aminoalanine moiety. The ^{1}H NMR spectra of the zinc–bleomycin complex (D_2O as solvent) have been obtained and analyzed. Chemical shift changes larger than 0.1 ppm are observed upon metal binding for the following groups: (1) β-aminoalanine, (2) propionamide, (3) pyrimidinyl methyl, (4) β-hydroxyhistidine, (5) mannose 3′, and (6) methylvalerate. Coordination to zinc also causes large changes in vicinal coupling constants for the hydroxyhistidine α- and β-CH protons and for the vicinal coupling in the β-aminoalanine moiety, thus indicating the predominance of specific rotamers around these bonds. Titration studies of the zinc–bleomycin complex establish the pK_a of the imidazole coordinated to zinc as 11.0. The deprotonation of the zinc–imidazole also causes a further 0.4-ppm shielding of the methylvalerate α-CH resonance. This shift is in addition to the 0.6-ppm upfield shift observed upon the initial formation of the zinc–bleomycin complex. These results establish that coordination to zinc confers a conformational rigidity on bleomycin and demonstrate the existence of a heretofore unsuspected association between the imidazole and methylvalerate groups.

Bleomycin is a glycopeptide antibiotic first isolated from *Streptomyces verticillus* by Umezawa et al. (1966). It shows remarkable selectivity toward squamous cell carcinoma, various lymphomas, and testicular carcinoma. Of particular importance is the general absence of immunosuppression (Boggs et al., 1974), thus accounting for its increased use in combination chemotherapy. The structure of bleomycin was first proposed by Takita et al. (1972), and a revised structure suggested by Takita et al. (1978a) is shown in Figure 1. The drug has a high affinity for divalent transition-metal ions and is isolated as the 1:1 cupric complex (Umezawa et al., 1966; Umezawa, 1974). It is the ferrous–bleomycin complex, however, that has been implicated as the possible active species in bleomycin-directed DNA strand scission (Sausville et al., 1976; Lown & Sim, 1977).

Proton nuclear magnetic resonance (NMR) has proven to be of limited value for the study of the cupric- or ferrous–bleomycin complexes because of paramagnetic effects. Investigations into the conformational changes resulting from coordination of metal to bleomycin, however, can be studied by using zinc(II) since bleomycin will form a tightly associated complex with this diamagnetic ion. ^{1}H NMR studies (Dabrowiak et al., 1978a; Cass et al., 1978) indicate that the exchange rate between the complex and free metal is less than 1 s^{-1}, and highly resolved ^{1}H NMR spectra have been obtained (Oppenheimer, 1979).

This study is concerned with two important aspects of the structure of bleomycin. First we present data that independently verify the revised structure of the pseudo-dipeptide moiety[1] proposed by Takita et al. (1978a). Secondly, by using the chemical shift resolution available at 360 MHz, homonuclear spin decoupling, and partially relaxed Fourier transform spectroscopy, we focus on those resonances that yield chemical shift and coupling constant data important to defining the tertiary structure of bleomycin and the conformational changes associated with metal binding. Knowledge of the chemical structure of bleomycin and the conformation of its metal-containing complexes is prerequisite to understanding the molecular mechanism of this important chemotherapeutic drug.

Experimental Section

Materials. The mixture of bleomycin A2 and B2 (blenoxane) was obtained from the National Cancer Institute, and

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[1] The pyrimidinylpropionamide-β-aminoalanine substituents comprise the pseudo-dipeptide region.

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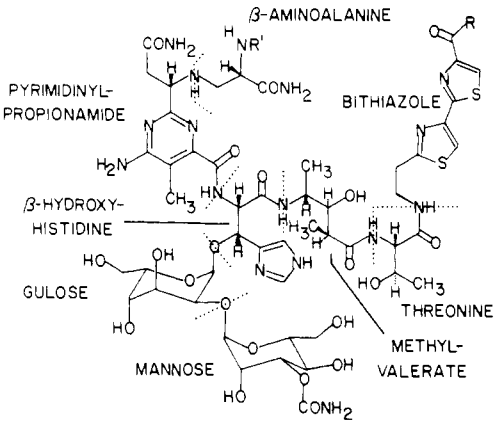


FIGURE 1: Revised structure of bleomycin: R′ = H, R = OH, bleomycinic acid; R′ = H, R = $NH(CH_2)_3S^+(CH_3)_2$, bleomycin A2; R′ = $COCH_3$, acylated bleomycin.

bleomycin A2 was isolated by a modification of the procedure of Chien et al. (1977). Bleomycinic acid was a generous gift of Dr. T. Takita. All other reagents were the highest quality available.

NMR Parameters. Spectra were obtained at 360 MHz on a Bruker HXS-360 NMR spectrometer equipped with a Nicolet Technologies 1180 computer/Fourier transform system and a computer-controlled homonuclear decoupling accessory. Quadrature detection was employed, and both 16K and 32K Fourier transforms were obtained with a spectral width of 3610 Hz. Typically, 128 transients were accumulated with 2.7 s between the pulses. Equilibrium intensities were observed for all of the resonances under these conditions except those of the bithiazole moiety. Partially relaxed Fourier transform spectra were obtained by using the standard inversion/recovery pulse sequence (Vold et al., 1968) with the delay time (τ) adjusted for optimum nulling of either CH or CH_2 resonances.

Samples were prepared by twice lyophilizing the unbuffered solution of bleomycin from 99.8% D_2O and then dissolving it in 100% D_2O. The pD of the resulting solution was adjusted to the indicated values with either D_2SO_4 or NaOD. The zinc–bleomycin complex was prepared by addition of zinc sulfate dissolved in 100% D_2O to the unbuffered solution of bleomycin in 100% D_2O followed by adjustment of the pD with NaOD. The concentration of bleomycin was measured spectrophotometrically, and all NMR spectra were obtained on 5 mM solutions. The standard electrode correction has been employed, pD = meter reading + 0.4 (Glasoe & Long, 1960). The internal standard sodium 3-trimethylsilylpropionate-*2,2,3,3-d*$_4$ (TSP) was used as the aqueous standard, and Me_4Si was used for solutions in $(CD_3)_2SO$. Protonation of the TSP carboxylate, $pK_a \simeq 5$, causes a 0.017-ppm downfield shift of the $(CH_3)_3Si$ resonance, and the chemical shifts below pD 5 have been corrected for this effect.

Preparation of Acetylated Bleomycin A2. To a 25-mL round-bottom flask was added 5 mL of DEAE-cellulose (~1 mequiv, HCO_3^- form), 8 mg (~6 μmol) of bleomcyin A2 (Takita, 1979), 10 μL (0.13 mmol) of acetic anhydride, and 5 mL of water. The mixture was stirred at room temperature for 3 h and then filtered to give 72 A_{290} units of product (90% recovery). The solution was concentrated to ~1 mL under diminished pressure (bath temperature <30 °C) and applied to a column of Sephadex C-25 (1.1 × 38 cm, $HCOO^-$ form). The column was washed with 50 mL of 0.05 M ammonium formate at 25 °C and then with a linear gradient of ammonium formate (400 mL total volume, 0.05 → 0.50 M, 5-mL fractions) at a flow rate of 50 mL/h. Fractions 20–27 (43 A_{290} units) were pooled, partially desalted by sublimation (0.05 mmHg, 30 °C, ~20 h), and then applied to a column of Sephadex G-10 (1.2 × 42 cm) that had been equilibrated at 4 °C with 0.02 M ammonium bicarbonate. Washing with the same buffer (2-mL fractions) at a flow rate of 13 mL/h effected elution of the desired compound in fractions 9–14. These fractions were pooled and desalted by repeated evaporations of portions of water (bath temperature <30 °C), affording 40 A_{290} units (~50%) of N-acetylated bleomycin A2 as a glass after drying under desiccation (0.05 mmHg/25 °C) for 6 h.

Table I: Chemical Shifts[a]

	bleomycinic acid, pD 5.4	zinc-bleomycinic acid, pD 6.4	acylated bleomycin A2, pD 6.1
propionamide			
CH_2	2.723, 2.638	3.335, 2.913	2.802, 2.749
CH	3.992	4.585	4.124
β-aminoalanine			
CH_2	2.972, 2.945	3.416, 2.542	3.062, 2.980
CH	4.016	3.79 ± 0.01	4.484
hydroxyhistidine			
α	5.090	4.922	5.074
β	5.363	5.253	5.315
C2H	8.018[b]	8.016	7.919[b]
C4H	7.397[b]	7.367	7.324[b]
methylvalerate			
α-H	2.522	1.908	2.472
β-H	3.736	3.474	ND[c]
γ-H	3.898	3.674	ND
α-CH_3	1.141	1.014	ND
γ-CH_3	1.141	0.975	ND
acyl CH_3			2.057
pyrimidine CH_3	2.031	2.439	2.034
gulose C1′	5.286	5.351	5.279
mannose			
C1′	5.025	4.944	5.021
C3′	4.709	4.09 ± 0.01	4.701

[a] Values are reported in parts per million relative to internal TSP and are accurate to 0.002 ppm. [b] The chemical shifts of the imidazole protons are very sensitive to pD over the range of pD from 4.6 to 6.0. [c] ND, not determined.

Results and Discussion

The ^1H NMR spectrum at 360 MHz of metal-free bleomycinic acid has been obtained, and the values of the chemical shifts for selected resonances are given in Table I. We report data for bleomycinic acid and the zinc–bleomycinic acid complex because the spectra have fewer overlapping resonances. The spectral parameters, however, do not significantly depend on the presence or absence of the basic terminal group, with the exception of the expected differences in the chemical shifts of the bithiazole resonances. The resonances have been assigned by homonuclear decoupling experiments and are consistent with those specific assignments that have been made by Chen et al. (1977).

Verification of Structure

Spectra in $(CD_3)_2SO$. The use of $(CD_3)_2SO$ as a solvent for ^1H NMR studies of bleomycin A2 allows the direct observation of the exchangeable protons without the problems of solvent absorption associated with H_2O. As shown in Figure 2, all of the exchangeable primary and secondary amide resonances can be accounted for, as well as the resonances from the imidazole N–H at 12.152 ppm (not shown), the carbamoyl $-OCONH_2$, and the pyrimidine NH_2. The latter resonances have been assigned by comparison of their chemical shifts with the corresponding proton resonances in model compounds (see

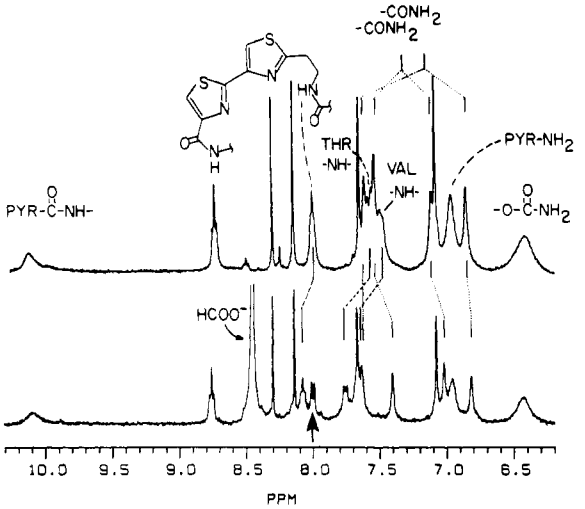


FIGURE 2: A portion of the ^1H NMR spectrum obtained in $(CD_3)_2SO$ at 360 MHz showing the resonances of the exchangeable N–H protons of bleomycin A2 (top) and acylated bleomycin A2 (bottom). Note the appearance of a new, secondary amide N–H resonance at 8.00 ppm in the bottom spectrum.

Table II: Chemical Shift[a] of Exchangeable Protons

	bleomycin A2 in $(CD_3)_2SO$	acylated bleomycin A2 in $(CD_3)_2SO$
imidazole NH	12.152	ND[d]
pyr-His amide	10.150	10.105
bithiazole amide	8.740	8.758
Thr-bithiazole amide	8.006	8.077
β-aminoalanine α-NHAc		8.000
His-Val amide	7.570	7.755
$CONH_2$ (1 H)	7.589	7.632
$CONH_2$ (1 H)	7.540	7.401
Val-Thr amide	7.483	7.649
$CONH_2$ (1 H)	7.101	7.018
pyrimidine NH_2[b]	6.972	6.957
$CONH_2$ (1 H)	6.844	6.814
carbamoyl NH_2[c]	6.426	6.426

[a] Values are reported in parts per million relative to internal Me_4Si and are accurate to 0.002 ppm. [b] Model compound: β-(4-amino-6-carboxamido-5-methylpyrimidin-2-yl)propionamide, 7.20 ppm. [c] Model compound: 3′-carbamoyl-α-methyl-D-mannose, 6.45 ppm. [d] ND, not determined.

Table II). The resonances for the primary and secondary amine protons and the hydroxyls could not be observed because of exchange broadening. Attempts at slowing the exchange rate of the amine protons by protonation (through addition of trifluoroacetic acid) lead only to decomposition of the bleomycin.

Four relatively narrow resonances are observed at 7.589, 7.540, 7.101, and 6.844 ppm (see Figure 2). This is the pattern typically expected for two primary carboxamide moieties. It arises from restricted rotation around the C–N bond which causes the individual proton resonances of each amide NH_2 group to be resolved. The observation of two carboxamide groups is in accord with the revised structure of the pseudo-dipeptide moiety and inconsistent with a β-lactam structure where only one set of carboxamido resonances would be present. The secondary amide protons have all been assigned by homonuclear spin decoupling of the adjacent CH and CH_2 resonances. We find that of all the secondary amide protons, the one associated with the pyrimidine carboxamide moiety is the most strongly deshielded. This result indicates an electron deficiency at that proton and is consistent with the

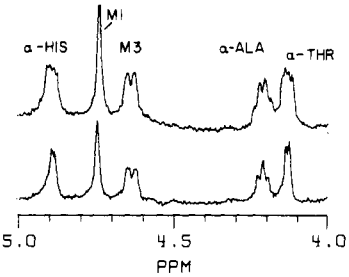
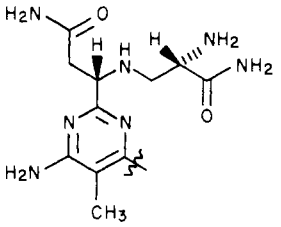


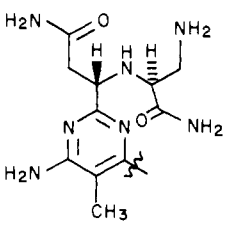
FIGURE 3: A portion of the ^1H NMR spectrum of acylated bleomycin A2 obtained in $(CD_3)_2SO$ (top) and $(CD_3)_2SO$ + 25 μL of D_2O (bottom). The addition of D_2O causes exchange of the N–H protons with deuterium and hence loss of the vicinal coupling as shown in the bottom spectrum.

effects expected for a carboxamide attached to a pyrimidine.

Acylation of Bleomycin A2. Although the revised structure proposed for bleomycin is consistent with the experimental data available, the anomalously low pK_a value of 2.7 assigned to the secondary amine has prompted our investigation of alternative bleomycin structures. One alternative structure that most nearly accounts for the experimental results involves attachment of the β-aminoalanine amide through the α-amino rather than the β-amino group. This structure was ostensibly excluded by the earlier report (Takita et al., 1972) that successive treatments of bleomycin B2 with 2,4-dinitrofluorobenzene and acid gave α-DNP-β-aminoalanine rather than β-DNP-β-aminoalanine. It was thought, however, that a carboxamide adjacent to the secondary amine, as shown in the alternative structure, could account for the low pK_a. This possibility suggested the need for an independent verification of the mode of attachment for the β-aminoalanine moiety. The approach we chose was to acylate bleomycin A2 selectively on the primary amine according to the procedure of Takita (1979). The resulting secondary amide proton could then be



revised structure



alternative structure

observed by ^1H NMR, and the presence of either an adjacent CH or CH_2 group, as determined by homonuclear spin decoupling, would then unequivocally establish the structure.

The ^1H NMR spectra of bleomycin A2 and acylated bleomycin A2 in $(CD_3)_2SO$ are compared in Figure 2. The spectrum of acylated bleomycin A2 clearly shows the presence of a new doublet at 8.00 ppm in addition to the other previously assigned exchangeable protons. This doublet has been unambiguously assigned through homonuclear spin decoupling as arising from a secondary amide that is vicinal to the methine proton of the β-aminoalanine moiety (the β-aminoalanine CH resonance also shows a substantial downfield shift ~0.5 ppm relative to bleomcyin A2). Addition of 20 μL of D_2O to the solution in $(CD_3)_2SO$ results in the disappearance of the 8.1-Hz vicinal coupling to the α proton of the β-aminoalanine moiety as shown in Figure 3. These experiments conclusively exclude the alternative structure and are fully consistent with the revised structure proposed for bleomycin.

pH Studies. The chemical shifts of the resonances of the β proton of the hydroxyhistidine and the α and γ protons of the methylvalerate moiety are sensitive to the protonation of

Table III: Coupling Constants[a]

		bleomycinic acid pD 5.4		Zn-bleomycinic acid pD 6.4		acylated bleomycin A2 pD 6.1	
	J	Hz	rotamer population[b]	Hz	rotamer population[b]	Hz	rotamer population
propionamide $CHCH_2$	^3J	5.5	<10% gg	6.3	<10% gg	5.5	<10% gg
	^3J	8.9		8.4		8.1	
	^2J	−14.8[c]		−16.0		−15.2	
β-aminoalanine $CHCH_2$	^3J	5.4	10% gg	2.0	>90% gg	4.9	10% gg
	^3J	7.7		3.1		8.1	
	^2J	−13.5		−13.1		−13.8	
hydroxyhistidine	$^3J_{\alpha-\beta}$	6.0	40% trans	2.7	<10% trans	6.0	40% trans
methylvalerate	$^3J_{\alpha-\beta}$	6.0 ± 0.3	38–45% trans	5.9 ± 0.3	36–43% trans		
	$^3J_{\beta-\gamma}$	6.0 ± 0.3		5.9 ± 0.3			
threonine	$J_{\alpha-\beta}$	4.7	25% trans	4.9	28% trans	5.0	30% trans
gulose	$J_{1'-2'}$	3.6		4.3		3.4	
mannose	$J_{1'-2'}$	1.5 ± 0.3		1-1.5		1.6	

[a] Coupling constants are accurate to within ±0.2 Hz unless otherwise noted. [b] Rotamer populations have been calculated by using the parameter J_{trans} = 11 Hz and J_{gauche} = 2.5 Hz; gg designates the gauche-gauche rotamer. [c] The corresponding coupling constants for a representative β-lactam are ^3J = 4.9 and 2.5 Hz and ^2J = −14.5 Hz (Wong et al., 1977).

the imidazole, pK_a = 4.7, as shown in Figure 4. Note that the α proton of the methylvalerate moiety shifts by over 0.1 ppm. Through-bond effects can be ruled out as an explanation for this shift since the closer α proton of the β-hydroxyhistidine moiety shows a much smaller change. The observed sensitivity of the methylvalerate α-CH must, therefore, be due either to through-space interactions with the imidazole or to conformational changes. The latter are less likely since only minor variations are observed in the vicinal coupling constants for the methylvalerate resonances over this range of pD. These results suggest that the imidazole and methylvalerate moieties, at least for a significant portion of the time, are in close proximity and preclude the *predominance* of structures wherein the methylvalerate moiety is extended away from the hydroxyhistidine group.

The pD dependence of the chemical shifts for the resonances of the pseudo-dipeptide moiety is shown in Figure 5. No significant change in chemical shift is observed for the pyrimidine group from pD 1.9 to 9.9. This result is consistent with the report by Takita et al. (1978a) that the pK_a of the pyrimidine is 1.2 or less. The observed pD-dependent shifts of the methine and methylene proton resonances in the pseudo-dipeptide indicate the presence of two functionalities with pK_a's of 2.7 and 7.8. The protonation at pD 7.8 clearly affects only the chemical shifts of the β-aminoalanine protons, with the α-CH proton showing the largest change. In contrast, the pyrimidinylpropionamide moiety has only a negligible shift over this pD range. The results are consistent with protonation occurring predominantly on the primary amine. Diprotonation, on the other hand, appears to affect both sets of protons nearly equally as reflected by the similar pD dependence of the chemical shifts. Interestingly, the changes in pD do not cause any concomitantly large changes in the vicinal coupling constants, indicating that protonation or diprotonation occurs *without major alterations in rotamer populations around these bonds*. In contrast, the geminal coupling constants for the methylene protons increase at low pD. It should be noted that protonation and diprotonation of diamines are generally expected to show both electrostatic and hydrogen-bonding interactions between the amino groups. In this case it would appear that monoprotonation involves only the primary amine whereas diprotonation shows a participation of both amino groups.

The anomalously low pK_a of the secondary amine [assigned as 2.7 by Takita et al. (1978a)], its remarkable lack of reactivity [by inference from Takita et al. (1972)], and the absence of interaction between the amino groups upon monoprotonation are all consistent with stabilization of the secondary amine by hydrogen bonding to the many potential donor and acceptor groups that are in close proximity. It should be noted, however, that the calculated rotamer populations argue against the *predominance* of any single hydrogen-bonded network among the many possibilities since the gauche-trans and trans-gauche rotational isomers are both present (see Table III).

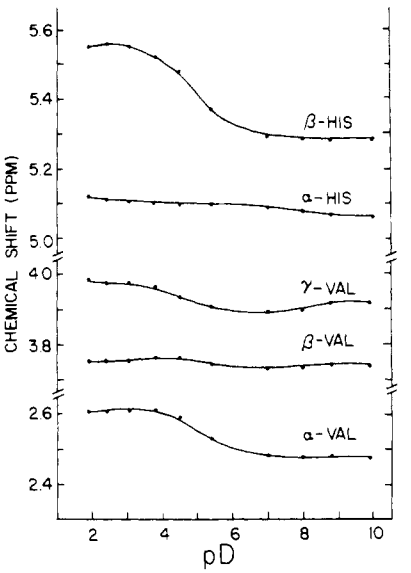


FIGURE 4: The pD dependence of the chemical shift of the hydroxyhistidine and methylvalerate methine resonances in bleomycin A2. Note that the 0.12-ppm shift of the methylvalerate α proton clearly parallels the titration of the imidazole group.

Zinc-Bleomycin Complex

Chemical Shift Changes Induced by Zinc Binding. Zinc, a diamagnetic ion, does not cause line broadening or other NMR phenomena usually associated with ligand–paramagnetic ion interactions; hence, well-resolved spectra can be obtained. Changes in the chemical shifts of groups coordinated to zinc will result from a combination of two separate effects.

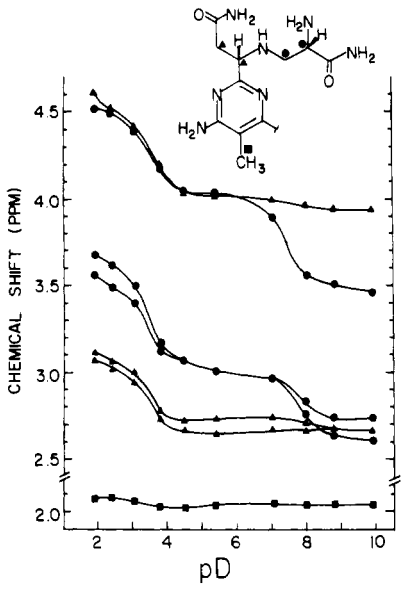


FIGURE 5: The pD dependence of the chemical shifts for the pseudo-dipeptide moiety of bleomycin A2. The top two curves (starting at 4.5 ppm, pD 2) show the pD dependence of the CH resonances. The dependence of their corresponding CH_2 resonances are shown by the middle curves. The pyrimidine methyl group (bottom curve) shows no large pD dependence, consistent with a pK_a of <1.2 for the pyrimidine.

(1) Binding of zinc to heteroatoms will cause *downfield* shifts of the adjacent proton resonances. The shifts will be of similar magnitude as those resulting from protonation at that site. (2) Conformational changes will fix certain spatial interactions among the substituents. The consequences of the new orientations on the chemical shift of the neighboring proton resonances can be either shielding or deshielding. These phenomena have a relatively short range so that the general area of the metal binding site will be reflected by altered chemical shifts of the groups involved.

The chemical shifts of a number of resonances in bleomycinic acid are compared in Table I to the corresponding resonances in the 1:1 complex with zinc. The largest changes in chemical shifts occur for the proton resonances of the following substituents: pyrimidinylpropionamide, β-aminoalanine, pyrimidine methyl, β-hydroxyhistidine, methylvalerate, and the mannose 3′ proton (the site of the carbamoyl group). The chemical shifts induced in the remaining resonances are less than 0.1 ppm.[2] The sensitivity of the resonances to zinc binding is shown schematically in Figure 6. Except for the methylvalerate, these results are consistent with the groups implicated by Umezawa (1973), Dabrowiak et al. (1978b), Iitaka et al. (1978), and Oppenheimer (1979) as constituting the metal binding site and specifically exclude participation of the bithiazole moiety.

As discussed above, coordination of ligands to zinc is expected to cause a deshielding of the adjacent proton resonances because of the localized positive charge. The shift should be of a similar magnitude as protonation at that site. Therefore, the observation of strongly shielded resonances indicates that metal binding causes the juxtaposition of the affected protons with anisotropic environments arising from such sources as carbonyls, heteroatoms, or the aromatic imidazole or pyrimidine rings.

[2] The differences must be considered as only a qualitative indication of the shift induced by zinc binding since for bleomycinic acid at pD 5.4 the imidazole and secondary amine are both unprotonated, whereas in the zinc complex they can be considered as "protonated" because of coordination to the zinc dication.

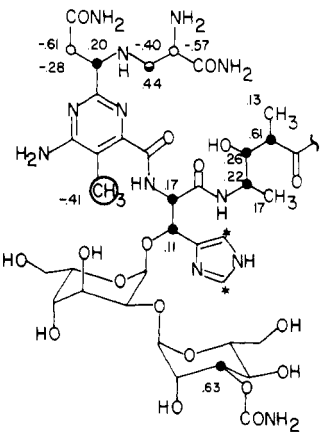


FIGURE 6: Schematic representation of the chemical shift changes, in parts per million, induced by formation of the 1:1 zinc–bleomycinic acid complex. Positive numbers indicate upfield shifts upon coordination to zinc and are shown by closed circles (●). Negative values are designated by open circles (○), and changes in chemical shift of less than 0.1 ppm are not shown. Because of their sensitivity to pD in the uncomplexed bleomycinic acid, no values are given for the imidazole resonances (designated by an asterisk).

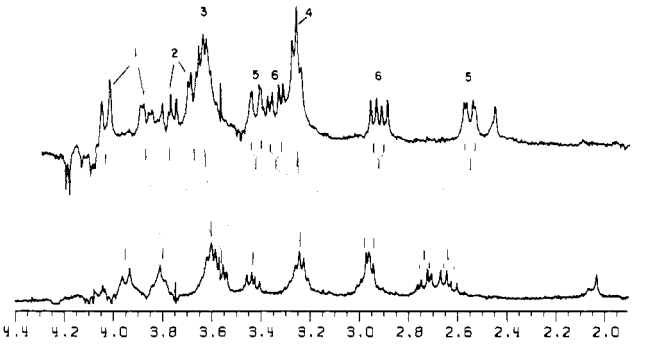


FIGURE 7: Comparison of the partially relaxed Fourier transform spectra of the CH_2 resonances of bleomycinic acid and the 1:1 zinc–bleomycin complex (τ = 0.55 s). Resonances 1 and 2 correspond to the 6′-methylene protons of the sugar moieties, 3 and 4 to the two methylenes adjacent to the bithiazole; resonances 5 are assigned to the methylene of the β-aminoalanine moiety, and 6 to the methylene of the propionamide moeity.

The most dramatic changes in chemical shift occur in the pseudo-dipeptide. The methylene protons for bleomycinic acid and the zinc complex are readily observed in the partially relaxed Fourier transform spectrum shown in Figure 7. The two methylenes of the β-aminoalanine and pyrimidinylpropionamide, designated 5 and 6, respectively, show a very large change in chemical shift upon complexation with zinc. The specific assignments of these resonances are based on homonuclear spin decoupling and analysis of the geminal coupling constants. Although the vicinal coupling constants of the propionamide and β-aminoalanine groups in metal-free bleomycin do not show a significant pD dependence, the geminal coupling constants of the methylene protons are sensitive to changes in pD. At pD 7 the values are −12.6 and −14.8 Hz for the β-aminoalanine and propionamide methylenes, respectively, whereas they change to −13.6 and −16.2 Hz below pD 2.4 when both amines are protonated. The values observed for these geminal coupling constants in the zinc–bleomycinic acid complex are −13.1 and −16.0 Hz. Since the coordination of zinc to groups in bleomycin is expected to be analogous to their protonation, we therefore assign the methylene with the smaller coupling constant (no. 5) to the β-aminoalanine group and the remaining methylene (no. 6) with the larger coupling constant to the propionamide. In addition, methylene no. 5 also shows large changes in the

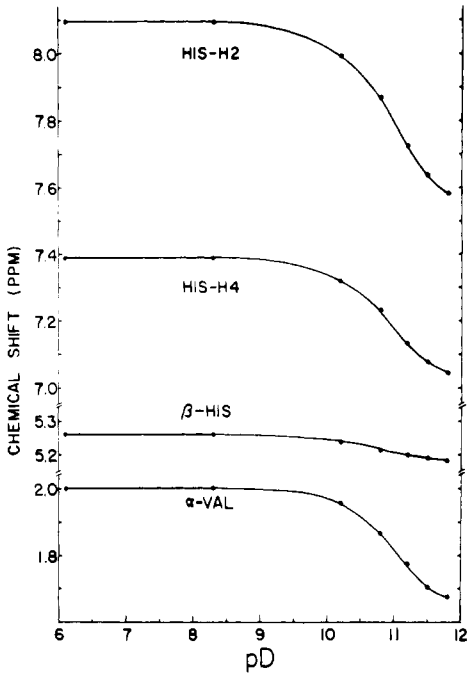


FIGURE 8: The pD dependence of the chemical shifts for the hydroxyhistidine H2, H4, and β-CH resonances and the valerate α-CH in the zinc–bleomycin complex. No other chemical shift changes greater than 0.1 ppm were observed over this range of pD.

vicinal coupling constants, whereas the changes for methylene no. 6 are negligible (see Table III), a result reflecting the participation of the primary and secondary amines as ligands to zinc.

These data are consistent with the results of potentiometric titration studies (Umezawa, 1973; Kunishima et al., 1976) that show no groups titrating in the pH range 4–10, thus implicating the imidazole and both the primary and secondary amines as ligands to the divalent metal ion. We have further investigated the ^{1}H NMR spectra of zinc–bleomycin at high pD since imidazole–metal complexes are known to have pK_a's of 11 or above (Carlson & Brown, 1966). The pD dependence of the chemical shifts for zinc–bleomycin is presented in Figure 8. The strong deshielding of the imidazole resonances observed at high pD establishes a pK_a of ~11.0 for this complex. The deprotonation of the imidazole does not cause loss of zinc since the observed changes in shifts are freely reversible. Furthermore, the resonances for the propionamide, β-aminoalanine, and pyrimidine methyl, which were strongly affected by metal binding, are all unaffected at high pD. There is also no indication of any base-catalyzed chemical modification occurring during the course of the experiments. The chemical shift of the mannose 1′ resonance is invariant, thus precluding formation of isobleomycin since a substantial change in chemical shift would be expected to result from the migration of the carbamoyl moiety to the 2′-hydroxyl. In addition, the absence of exchange of the propionamide CH with deuterium at high pD establishes that epimerization to epibleomycin has not occurred to any appreciable extent. These results demonstrate that the imidazole is coordinated to the zinc and that it is protonated at physiological pH.

The strong shielding of the valerate α-CH resonance upon formation of the zinc–bleomycin complex (see Figure 6) is anomalous if viewed in terms of the ligands currently proposed (Iitaka et al., 1978; Takita et al., 1978b). This sensitivity is further underscored by the results of the titration studies of zinc–bleomycin which show a further 0.4-ppm upfield shift for the valerate α-CH resonance with increasing pD. As can be seen in Figure 8, the shift of this proton is comparable to that of the imidazole H4 resonance. Deprotonation of the imidazole increases the electron density in the ring and thus enhances the aromatic ring current shielding. Therefore, the 0.4-ppm upfield shift of the valerate α-CH could be explained if that proton were centered directly above the imidazole ring and in van der Waals contact.

The unexpected sensitivity of the valerate α-CH resonance to the influence of the imidazole ring indicates that it is at least in some way strongly associated with the imidazole–metal complex and even suggests the possibility that the valerate moiety could be a ligand of the metal. The current model of metal coordination to bleomycin proposed by Takita et al. (1978b) is based on the putative biosynthetic intermediate of bleomycin designated P-3A (Iitaka et al., 1978). This model, however, cannot explain the results observed for the methylvalerate group. Therefore, it would seem that P-3A may represent a less than perfect model for extrapolation to the "active iron(II)–bleomycin complex", especially since P-3A lacks both sugar moieties (hence the carbamoyl ligand), the pyrimidine methyl group, the methylvalerate moiety, and the remainder of that portion of the molecule including the threonine, bithiazole, and the basic terminal groups. Investigations are currently in progress to define the role of the methylvalerate moiety in maintaining the tertiary structure of metal–bleomycin complexes.

Analysis of Coupling Constants. The bleomycin molecule has potentially many degrees of torsional freedom around the backbone linkages. Thus, measurement of the vicinal coupling constants for the appropriate resonances along the backbone can provide information concerning the dihedral angles at these sites (Karplus, 1959, 1963). Furthermore, estimates of the population distribution of various rotational isomers around these bonds can be made by using the analysis of Blackburn et al. (1970).

The corresponding coupling constants for resonances in bleomycinic acid and the zinc–bleomycinic acid complex are compared in Table III, and the rotamer populations have been calculated and are also listed in Table III. We have used 10–12 Hz as an estimate for the value of the trans coupling constant and 2.5 Hz for the gauche coupling constant. The analysis is limited, however, when only a single vicinal coupling constant is available. In that case, only the relative populations of gauche and trans forms can be calculated.

There are two important sets of vicinal coupling constants that change upon complexation with zinc: the coupling between the α proton and methylene protons of the β-aminoalanine moiety and the vicinal protons in the β-hydroxyhistidine moiety. Based on conformational analysis, both of these residues change from mixtures of rotamer populations in the free bleomycinic acid to predominantly (>90%) gauche populations in the complex with zinc as shown in Figures 9 and 10. In contrast, the resonances of the methylvalerate and the pyrimidinylpropionamide moieties show only small changes in coupling constants upon zinc binding, although large changes in chemical shift are observed. The absence of large, zinc-mediated changes in coupling constants for the methylvalerate resonances does not necessarily preclude its participation as a ligand. The presence of more than one rotamer indicated by the coupling constant analysis does not absolutely require free (360°) rotation around the bonds. The results just indicate that there is rapid interconversion between the orientation of the vicinal protons, which can be accomplished either through conformer interconversions or complete rotation.

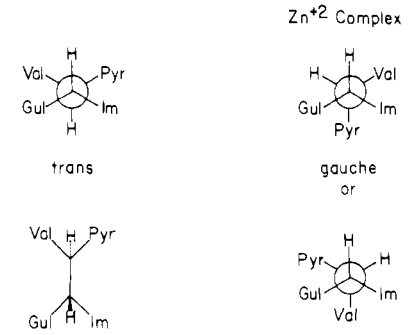


FIGURE 9: The predominant rotamer populations for the β-hydroxyhistidine moiety. Metal-free bleomycin favors a trans rotamer, whereas complexation with zinc results in an exclusively gauche rotamer.

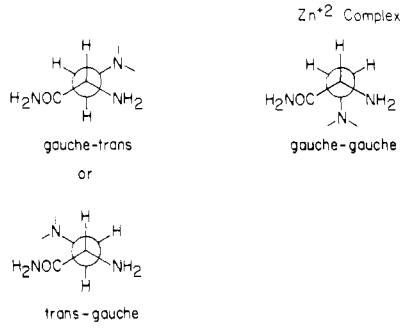


FIGURE 10: The predominant rotamer populations for the β-aminoalanine moiety. In metal-free bleomycin both the gauche-trans and trans-gauche rotamers are nearly equally populated, whereas the population of the gauche-gauche rotamer is <10%. In the zinc–bleomycin complex, the gauche-gauche rotamer is favored exclusively (>90%).

Both chemical shift and coupling parameters for the remaining resonances of the side chain, including the bithiazole moiety, change little upon metal binding. It should also be noted that although the data listed in Tables I and III are for bleomycinic acid, the results are identical for either pure bleomycin A2 or the mixture of bleomycin A2 and B2. The basic terminal group does not appear to have any significant influence on the zinc-binding domain of bleomycin.

The preferred geometry of the β-aminoalanine moiety observed in the zinc–bleomycinic acid complex juxtaposes the secondary amine nitrogen with both the primary amine and the carboxamide group of the β-aminoalanine moiety. This conformation represents an orientation that would be expected if some or all of these nitrogens were involved with coordination to the zinc. The β-hydroxyhistidine moiety likewise shows a preference for the gauche conformation (see Figure 9), consistent with maintaining a close proximity between the methylvalerate, pyrimidine, and imidazole moieties. The other gauche conformation, in which the pyrimidine and imidazole moieties are trans, can probably be ruled out since it would not permit simultaneous binding of zinc to both of these moieties. The rotamer in which the α and β protons are trans can be excluded because of the expected 10–12-Hz vicinal coupling constant. It should be noted from Figure 9 that although the trans rotamer allows the juxtaposition of the imidazole and pyrimidine moieties, it places the methylvalerate trans to the imidazole. Thus, the absence of the trans rotamer is further evidence for the close association of the methylvalerate and imidazole moieties.

In conclusion, we have observed a previously unsuspected close association or coordination of the methylvalerate group to the zinc–imidazole moieties in the binary complex. The significance of this interaction for the maintenance of the active conformation of bleomycin, however, remains to be ascertained. Future work will focus on the role of the methylvalerate moiety in bleomycin activity and the conformational effects resulting from chemical modifications of bleomycin and their correlation with altered biological properties.

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