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Transition-Metal Binding Site of Bleomycin A_2. A Carbon-13 Nuclear Magnetic Resonance Study of the Zinc(II) and Copper(II) Derivatives†

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ABSTRACT: The ^{13}C NMR spectra at 25.2 MHz of the Zn(II) and Cu(II) complexes of the antitumor antibiotic bleomycin A_2 are discussed. Complexation of the drug to Zn(II) causes 38 of the 52 resonance lines of bleomycin A_2 to shift to new positions. All but ten of these shifted lines have been assigned in the Zn(II) bleomycin complex. Although the specific donor sites of the drug cannot be identified from the ^{13}C NMR data, the analysis clearly shows that the pyrimidine–imidazole portion of the molecule is affected by chelation. This finding is in agreement with the previously reported metal-binding site of the antibiotic. The analysis also shows that carbon atoms which have large through-bond distances from the binding site can experience substantial chemical-shift changes upon metal binding. Complexation of the drug to Cu(II) eliminates 23 resonances from the spectrum of the molecule. All of these resonances emanate from carbon atoms which are located in the pyrimidine–imidazole portion of the drug.

The utility of ^{13}C NMR as a probe of structure is well recognized. Perhaps the best evidence of the power of this technique can be seen in its application to structural studies on large molecules (Allerhand, 1975). Where conventional ^{1}H NMR techniques yield featureless spectra made up of a large number of overlapping resonances, ^{13}C NMR spectroscopy exhibits a high degree of resolution. More often than not, each of the carbon atoms in the molecule produces a single, distinct, well-resolved resonance line. Since the position and width of the resonance line are sensitive to electronic changes which can occur within the molecular framework, the technique is well suited for studying metal–ligand interactions.

Metal complexation can affect the ^{13}C NMR spectrum of an organic molecule in a number of ways. If the metal ion is diamagnetic, the carbon resonances are generally narrow and well defined but are shifted from their original positions in the unbound molecule (Chisholm and Godleski, 1976). Most studies to date show that the magnitude of the shift is a strong function of the through-bond distance between the metal ion and the carbon atom (Stockton and Martin, 1974; Fuhr and Rabenstein, 1973; Yasui and Ama, 1975; Ama and Yasui, 1976; Fedarko, 1973). Carbon atoms remote from the metal-binding site remain unshifted or experience only a small shift upon metal complexation. Although changes in molecular conformation, as would result from metal binding, should have a significant effect on the position of a ^{13}C resonance line, this aspect of ^{13}C NMR vis-a-vis metal complexation has been largely unexplored.

The ^{13}C NMR spectrum of a complex containing a paramagnetic metal ion is different than the previously described case. For this type of compound, the observed carbon shifts are about one order of magnitude greater than those found for diamagnetic systems (Doddrell and Roberts, 1970; Anderson and Matwiyoff, 1972). In addition, the paramagnetic ion broadens the carbon resonance line. Depending on the spin–lattice relaxation time (LaMar et al., 1972) and the distance between the metal ion and the carbon atom, the resonance line may be so wide so as to go undetected in the ^{13}C NMR spectrum of the compound.

The use of ^{13}C NMR to study metal binding to large biological or biological-type molecules is just beginning. Ohnishi et al. (1972) examined the ^{13}C NMR spectrum of the potassium complexes of valinomycin and nonactin. Although the largest shifts upon complexation occurred for the carbonyl carbon atoms of these antibiotics, the carbon atom closest to the metal-binding site, other shifts occurred for carbon atoms which were far from the metal ion. The authors suggested that induced changes in conformation of the molecule as a whole were as important as direct interactions with the potassium ion in determining the chemical shifts for the complex.

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In a related study, Llinas et al. (1976) examined a series of aluminum ferrichromes using ^{13}C NMR. In addition to significant carbon-resonance shifts in the immediate vicinity of the metal-binding site, substantial shifts in the backbone portion of the molecule were observed. These shifts were attributed to strain or environmental effects rather than to inductive effects arising from the primary structure of the complex.

The copper-containing proteins azurin (Ugurbil et al., 1977) and plastocyanin (Markley et al., 1977) have recently been investigated using ^{13}C NMR. By cycling the copper oxidation state in these proteins between diamagnetic Cu(I) and paramagnetic Cu(II), it was possible to identify protein functionalities in the vicinity of the metal ion.

A good example of paramagnetic broadening and its implementation as a gage of distance between a carbon nucleus and a paramagnetic center for a biological system has recently been reported. By examining the line widths of carbon resonances emanating from a bound saccharide, Brewer et al. (1975) were able to measure the distance between the sugar and the protein-bound manganese ion of concanavalin A. Since the line widths for the various carbon resonances of the sugar changed by different amounts on binding, the orientation of the saccharide relative to the manganese-binding site was also determined.

In a previous report, we examined the Zn(II) and Cu(II) complexes of the antitumor antibiotic bleomycin A_2 (BL-A_2) (Figure 1a) (Dabrowiak et al., 1978). On the basis of visible absorption, ^{1}H NMR, and ESR studies, these two metal ions were assigned the same bleomycin binding site. The proposed structure of the Cu(II)-binding site is shown in Figure 1b. Since the primary structure and stereochemistry of bleomycin A_2 are known, as well as its complete ^{13}C NMR assignment, the molecule is an ideal candidate for studying metal–peptide interactions using ^{13}C NMR. The ^{13}C NMR spectral analysis of the Zn(II) and Cu(II) derivatives of bleomycin A_2 forms the basis of this report.

Experimental Section

Materials. The bleomycin A_2 sulfate was supplied by Bristol Laboratories, Syracuse, N.Y. 13206.

Methods. NMR Spectra. ^{13}C spectra were recorded at 25.2 MHz using a Varian XL-100 spectrometer operating in the pulsed FT mode. The samples were run in 12-mm tubes using an acquisition time of 0.8 s and a 20-μs pulse width.

Sample Preparation. Bleomycin A_2 sulfate, 150 mg, was dissolved in 2.0 mL of D_2O. The pH_m of the solution (where pH_m is the pH meter reading calibrated with H_2O buffers but uncorrected for the deuterium isotope effect) was adjusted to 6.5 using NaOD. Except for minor differences, discussed in the text, the ^{13}C NMR spectrum of this solution compared favorably with that earlier published (Naganawa et al., 1977). The bleomycin complexes were prepared by adding microliter quantities of M(II)$(ClO_4)_2$ solutions in D_2O (1.0 M) (where M is Zn or Cu) to a 10^{-2} M solution of bleomycin A_2 in D_2O. Before recording the spectrum, the pH_m of the solution containing the complex was adjusted to 6.5 by the addition of NaOD.

Results

The ^{13}C NMR Spectrum of Zn(II)-BL-A_2. The ^{13}C NMR spectrum of Zn(II)-BL-A_2, a composite spectrum of an equimolar mixture of metal-free bleomycin A_2 and its zinc complex, and the spectrum of metal free BL-A_2 are shown in Figures 2–6. For the purpose of analysis, the NMR spectrum is divided into five regions with 1 at highest field.

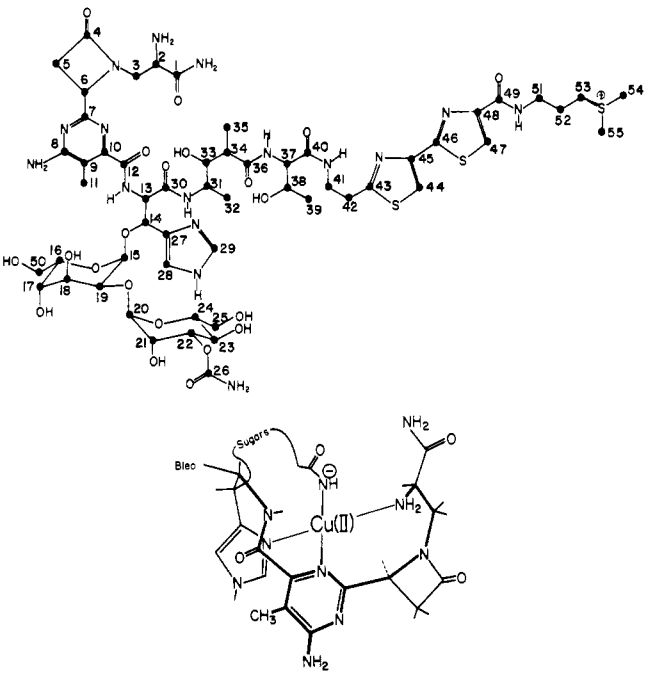

FIGURE 1: (a, top) The structure of bleomycin A_2. (b, bottom) The Cu(II) [Zn(II)] binding site of bleomycin A_2.

Region 1 (Figure 2). This region contains 14 resonance lines. Resonances 11, 34, 35, 39, 41, 42, and 51–55 either remain unshifted or sustain only a small shift (~0.1 ppm) upon complexation of the antibiotic to Zn(II). Resonances 3, 5, 31, and 32 experience significant shifts upon zinc binding.

Region 2 (Figure 3). Region 2 contains 15 closely spaced lines accounting for 17 carbon atoms. The resonances which occur in this region are due to carbon atoms 2, 6, 13, 14, 16–19, 21–25, 33, 37, 38, and 50. Complexation to the zinc ion again yields 15 resonance lines. All but four of these resonance lines (signals at 61.8, 63.6, 69.8, and 72.9 ppm), corresponding to carbon atoms 37, 25, 16, 38, and 18, appear to shift by more than 0.1 ppm upon metal binding.

Region 3 (Figure 4). Both bleomycin A_2 and its zinc complex give rise to seven resonances in this region. The observed carbon resonances are due to carbon atoms 9, 15, 20, 27, 28, 44, and 47. Except for resonances 44 and 47, the positions of the lines are all affected by zinc binding.

Regions 4 and 5 (Figures 5 and 6). Regions 4 and 5 contain a total of 16 resonance lines all emanating from sp^2 carbon atoms of bleomycin A_2. Resonances 40, 43, 45, 46, 48, and 49, due to carbon atoms in the region of the bithiazole residue, are unperturbed by metal binding, as are resonances 4, 12, and 30. Resonances 1, 7, 8, 10, 26, 29, and 36 experience significant shifts upon metal ligation.

All of the atoms which experience shifts upon zinc binding to the antibiotic are shown in Figure 7a.

The Copper(II)-Bleomycin Complex. The binding of Cu(II) to the antibiotic causes nearly half of the carbon resonance lines to disappear from the ^{13}C NMR spectrum of Cu(II)-BL-A_2.

Carbon atoms 1–6, 8–10, 12–14, 26–30, 32, 33, 35, and 36, which include the pyrimidine residue, the imidazole group, and the carbon backbone of the binding site, are absent in the spectrum of the complex. The carbon resonances which are missing in the ^{13}C NMR spectrum of Cu(II)-BL-A_2 are shown in Figure 7b.

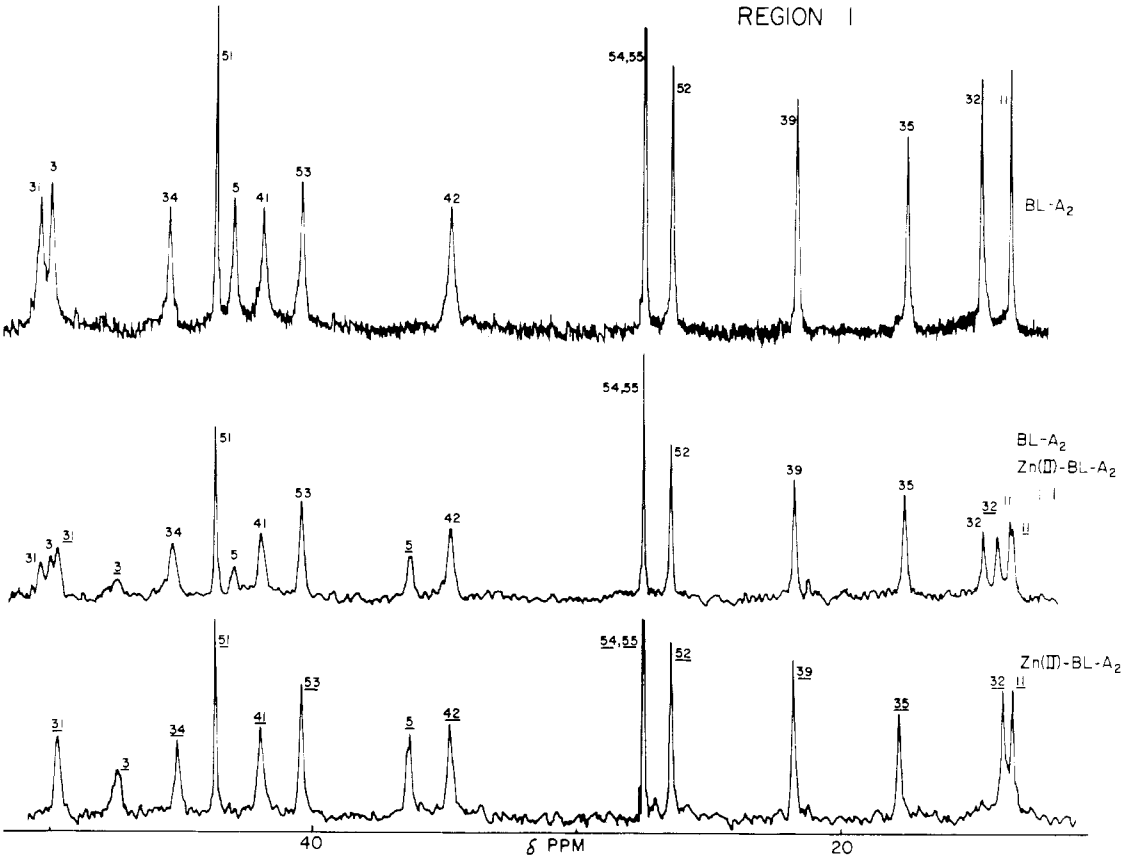

FIGURE 2: Figures 2–6 give the ^{13}C NMR spectra of bleomycin A_2 (BL-A_2), of a 1:1 mixture of BL-A_2 and its zinc complex, and of pure Zn(II)-BL-A_2. Refer to Figure 1 for the carbon indexing system.

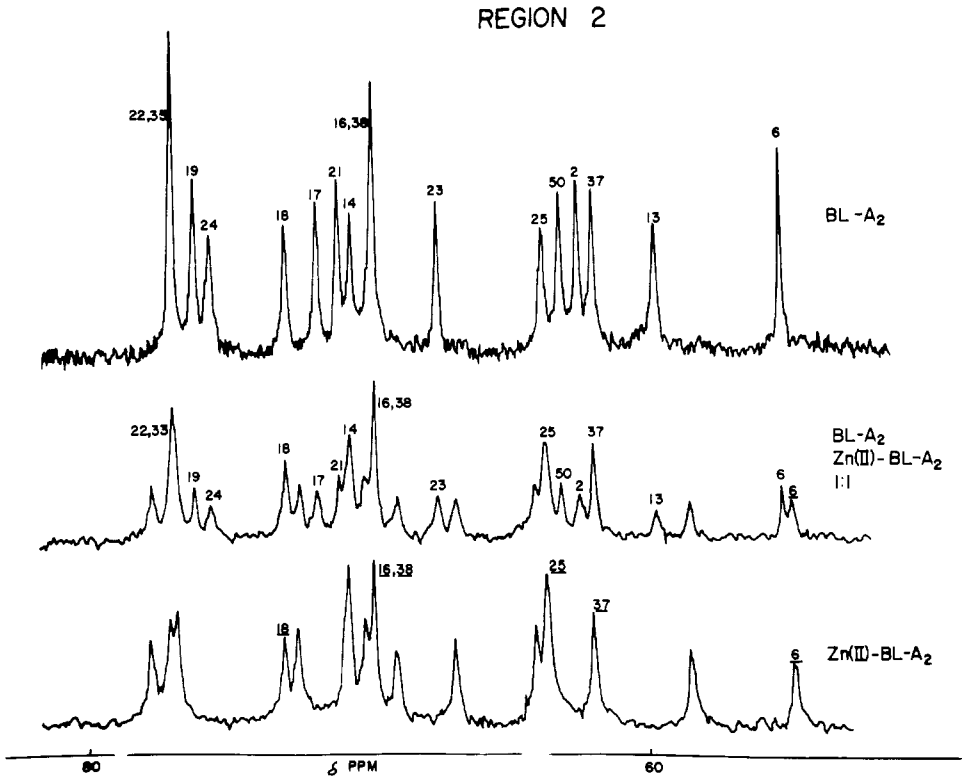

FIGURE 3: See the caption to Figure 2.

Discussion

The ^{13}C NMR Spectrum of Bleomycin A_2. Figures 2–6 show the ^{13}C NMR spectrum of bleomycin A_2. The specific carbon assignments are labeled on the figures and the chemical-shift data are collected in Table I. Using a series of bleomycin fragments and intact but different bleomycins, Naganawa et al. (1977) were able to completely assign all of the carbon resonances of bleomycin A_2. The 55 carbon atoms

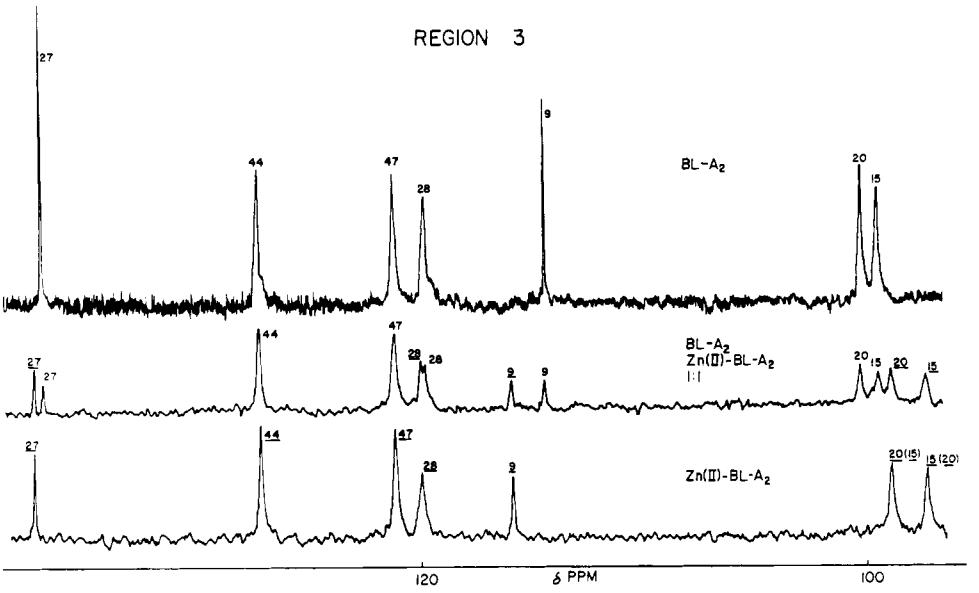

FIGURE 4: See the caption to Figure 2.

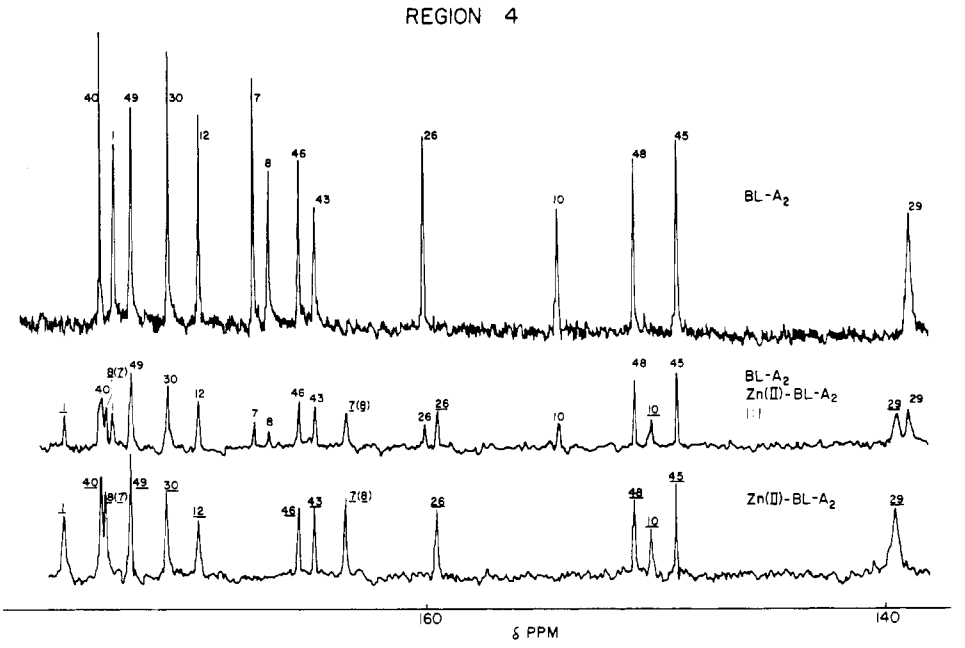

FIGURE 5: See the caption to Figure 2.

of the glycolpeptide produce 52 distinct resonance lines. In region 1, the sulfonium methyl carbons, C-54 and -55, are degenerate and yield a single resonance at 27.4 ppm. Two additional accidental degeneracies occur in region 2. Carbon atoms 16 and 38 resonate at 69.8 ppm, while atoms 22 and 33 produce a composite signal at 77.0 ppm. Each of the remaining resonances is associated with a single carbon atom of the drug. The chemical-shift values which appear in Table I are, in general, 1.8–2.0-ppm higher than those published earlier (Naganawa et al., 1977). Since different standards were used, dioxane vs. TSP, this discrepancy is not unexpected. Carbon atom 27, however, of the imidazole residue is significantly outside of this range, being 2.6-ppm higher than previously reported. This shift is probably due to slight difference in pH between the two experiments. It is known that the proton resonances of the imidazole residue are sensitive to pH changes (Chen et al., 1977). Our experiments show that the chemical shifts of several carbon resonances are also sensitive to pH and that C-27 is significantly more sensitive to pH changes below 6.5 than is any other carbon resonance of BL-A_2.

The ^{13}C NMR Spectrum of Zn(II)-BL-A_2. The center spectrum of Figures 2–6 shows that the zinc complex is in slow exchange on the NMR time scale, and resonances for bound and unbound bleomycin are observed. Since the system is in slow exchange, it is not possible to unequivocally assign the carbon resonances for the zinc complex. However, all but ten resonances in the zinc-bleomycin complex have been tentatively assigned. The unassigned resonances are located in region 2 (Figure 3) which contains 15 closely spaced lines with two degenerate sets of resonances. Ten of the 12 sugar resonances appear in this region of the spectrum.

The following criteria were used for making the assignments: (1) carbon signals which appear to be unaffected by metal complexation (unshifted) carry the same assignment as the

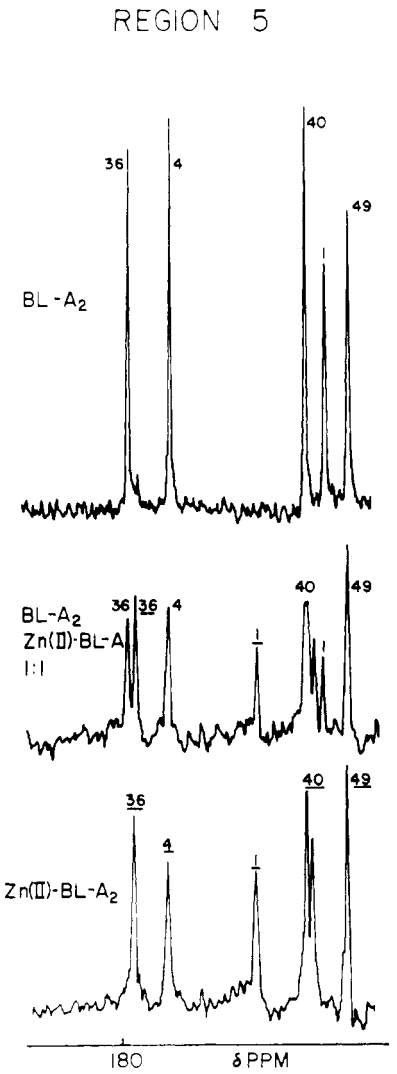

FIGURE 6: See the caption to Figure 2.

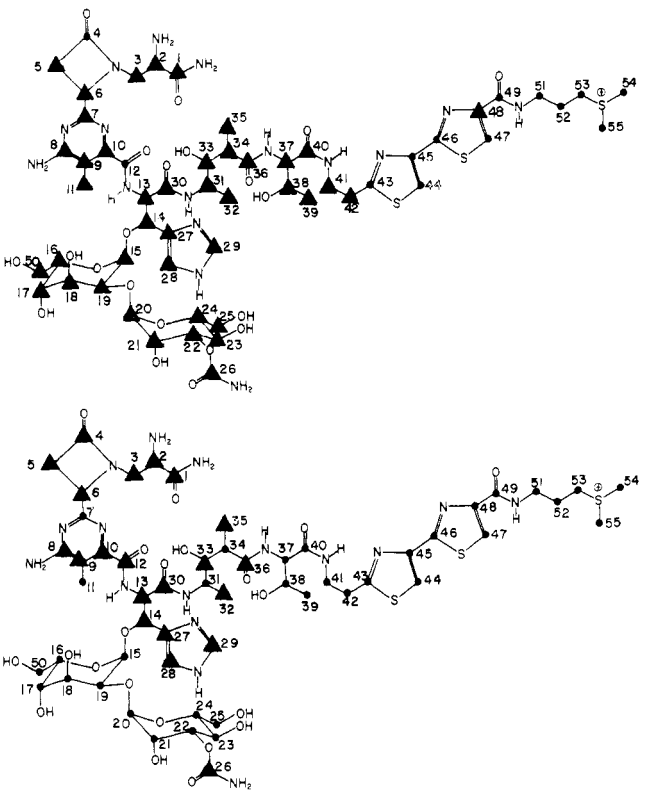

FIGURE 7: (a, top) The carbon resonances which shift when Zn(II) binds to bleomycin are indicated (▲) on the structure of bleomycin A_2. (b, bottom) The carbon resonances which are missing in the ^{13}C NMR spectrum of Cu(II)-BL-A_2 are indicated (▲) on the structure of the drug.

corresponding signal in metal-free bleomycin; (2) for carbon atoms which experience a shift upon complexation, the shift was assumed to be small (i.e., $\lesssim 5$ ppm). Previous studies suggest that metal complexation to diamagnetic metal ions should result in small carbon-resonance shifts (Stockton and Martin, 1974; Fuhr and Rabenstein, 1973; Yasui and Ama, 1975; Ama and Yasui, 1976; Fedarko, 1973); (3) both negative and positive shifts upon complexation are possible.

Resonances 11, 34, 35, 39, 41, 42, and 51–55 which occur in region 1 in the metal-free bleomycin either remain unshifted or sustain only a small shift upon formation of the zinc complex. These resonances have the same assignments for both compounds. The remaining peaks have been assigned by minimizing the shift. This allows the unambiguous assignment of resonances 5 and 32.

Owing to the fact that region 2 contains 15 closely spaced lines accounting for 17 carbon atoms of the drug, only six assignments were made. Carbon atom 6, which resonates in a relatively isolated area of the spectrum, shifts upfield (0.3 ppm) upon complexation. The remaining assignments were made on the basis that the unshifted peaks retain the same assignments as those determined for BL-A_2.

All of the lines which occur in region 3 (Figure 4) for the zinc–bleomycin complex have been assigned. Both anomeric carbon atoms, C-15 and -20, move upfield upon complexation. Tentative assignments have been made on the basis that the shifts are approximately equal, rather than the alternative assignment where the shift of resonance 20 is five times that of resonance 15.

All 16 resonances which occur in regions 4 and 5 (Figures 5 and 6) have been assigned. The assignment of the pyrimidine carbon atoms, 7 and 8, in the zinc complex is considered very tentative. More definitive statements on the assignment of those resonances must await further study on the effects of metal binding to simple nitrogen heterocyclic compounds.

Figure 7a and Table I show that the carbon resonances assigned to atoms 36–49 and 51–55 either experience very small shifts (~0.1 ppm) or are unshifted upon complexation of bleomycin A_2 to zinc(II). However, those carbon resonances emanating from the pyrimidine–imidazole portion of the drug, carbon atoms 1–35 and atom 50, are affected by the presence of the zinc ion. Thus, the ^{13}C NMR data are in general agreement with the proposed transition-metal binding site shown in Figure 1b (Dabrowiak et al., 1978).

In view of the number of different factors which control the position of the ^{13}C resonance line, using the chemical-shift data to determine the site of metal binding is difficult. However, some observations concerning the shifts and the binding site can be made. Of the two heterocyclic ring systems which experience shifts upon metal binding, the imidazole residue and the pyrimidine moiety, the latter is most affected by the presence of zinc ion (Table I). Even though both residues are metal-ligating groups (Figure 1b), the shifts sustained by the four carbon atoms of the pyrimidine are, in general, about an order of magnitude greater than those found for the imidazole group. Since similar trends have been observed upon protonation of these heterocycles (Reynolds et al., 1973; Pugmire and Grant, 1968), the transmission of electronic effects whether

they originate from protonation or from complexation appears to be mainly via the π system of the molecule. The pyrimidine moiety with its enhanced aromatic character should be better suited than the imidazole group for this type of transmission.

The carbon resonance due to the methyl group of the pyrimidine moiety (C-11) experiences very little shift upon zinc binding. However, the proton resonance of this group is dramatically affected by complexation. ^{1}H NMR studies show that the protons of carbon atom 11 shift 0.4 ppm to lower field upon ligation—the largest shift of any of the proton resonances (Dabrowiak et al., 1978). Apparently, the diamagnetic and paramagnetic contributions to the chemical shift cancel for the ^{13}C case but not for the ^{1}H case.

Six of the carbonyl carbon resonances (C-4, -12, -30, -36, -40, and -49) are essentially unaffected by zinc binding. However, carbonyl carbon atoms 1 and 26 shift 2.1 and 0.6 ppm, respectively, upon complexation. These shifts (along with the shift for carbon atom 3) provide strong supportive evidence that the α-amino group of the α-aminocarboxamide moiety (C-1 to -3) and the carbamoyl function are metal-ligating groups.

The shifts observed for the sugar moieties, particularly the anomeric carbon atoms (15 and 20), are also significant. It is possible that in the process of binding the metal ion the conformations of one or both of the sugars are altered. Conformational changes in carbohydrates are known to give rise to significant ^{13}C NMR chemical-shift changes (Strothers, 1972).

C-31 to -35 shift by small but significant amounts upon zinc complexation. No metal-binding site lies near this region of the carbon backbone, and the shifts must be due largely to conformation effects. The shift of C-5 upon complexation is remarkably large, considering that the nearest ligand atoms are four bonds away [the amino group and N(1) of the pyrimidine moiety]. Models show that complexation fixes the position of C-5 relative to that of the pyrimidine moiety. The change in the ring-current contribution associated with the heterocycle may be responsible for the large observed shift of that carbon atom.

The Copper(II)–Bleomycin Complex. Figure 7b shows that nearly half of the carbon resonances of bleomycin A_2 disappear when copper(II) binds to the drug. Since the affected resonances lie in the pyrimidine–imidazole portion of the drug, the copper data complement the earlier discussed zinc results. In addition, C-26 and -1, which are two and three atoms respectively, removed from the metal ion, are also missing. Most of the sugar resonances as well as the nonmetal-bound "tail" of the drug are either only slightly broadened or are not notably affected by the presence of the paramagnetic ion. Molecular models show that those carbon atoms which are more than about 5 Å from the copper ion give observable resonance lines.

Although the ^{13}C NMR chemical-shift data are not in themselves diagnostic of the specific donor atoms utilized by bleomycin in binding metal ions, they provide graphic evidence as to what region of the molecule is affected by chelation. While the major influence on the position of a carbon resonance line appears to be the distance of the carbon atom from the metal center, the bleomycin system shows that conformational changes which can occur within a large molecule upon metal binding are also significant. The results of this study underscore the importance of understanding the origins of chemical shifts for small molecules as a prerequisite to the full utilization of ^{13}C NMR as a probe of chelation phenomena in large molecular systems.

TABLE I: ^{13}C NMR Data for BLA_2 and Zn(II)–BLA_2.[a]

region	BLA_2 δ	BLA_2 assignment	Zn(II)–BLA_2 δ	Zn(II)–BLA_2 assignment	$\Delta\delta$[b]
1	13.6	11	13.5	11	−0.1
	14.7	32	13.9	32	−0.8
	17.5	35	17.8	35	+0.3
	21.7	39	21.8	39	+0.1
	26.4	52	26.4	52	0
	27.4	54, 55	27.4	54, 55	0
	34.8	42	34.7	42	−0.1
	40.4	53	36.2	5	−6.7
	41.8	41	40.4	53	0
	42.9	5	41.9	41	+0.1
	43.6	51	43.6	51	0
	45.4	34	45.1	34	−0.3
	49.8	3	47.3	3	−2.5
	50.2	31	49.6	31	−0.6
2	55.2	6	54.9	6	−0.3
	59.7	13	58.5	?	
	61.8	37	61.9	37	+0.1
	62.4	2	63.6	25	0
	63.0	50	64.0	?	
	63.6	25	66.8	?	
	67.4	23	68.9	?	
	69.8	16, 38	69.7	16, 38	−0.1
	70.6	14	70.1	?	
	71.0	21	70.6	?	
	71.8	17	72.5	?	
	72.9	18	73.0	18	+0.1
	75.7	24	76.8	?	
	76.2	19	77.1	?	
	77.0	22, 33	77.8	?	
3	100.1	15	98.0	15 (20)	−2.1 (−2.8)
	100.8	20	99.5	20 (15)	−1.3 (−0.6)
	114.9	9	116.5	9	+1.6
	120.3	28	120.5	28	+0.2
	121.7	47	121.7	47	0
	127.7	44	127.7	44	0
	137.3	27	137.7	27	+0.4
4	139.5	29	140.1	29	+0.6
	149.5	45	149.5	45	0
	151.4	48	150.6	10	−4.1
	154.7	10	151.3	48	−0.1
	160.5	26	159.9	26	−0.6
	165.2	43	163.9	7 (8)	−3.9 (−3.3)
	165.9	46	165.2	43	0
	167.2	8	165.9	46	0
	167.8	7	170.2	12	0
	170.2	12	171.6	30	+0.1
	171.5	30	173.1	49	0
	173.1	49	174.2	8 (7)	+7.2 (+6.4)
	173.9	1	174.4	40	−0.1
	174.5	40	176.0	1	+2.1
5	178.7	4	178.7	4	0
	180.0	36	179.8	36	−0.2

[a] Refer to Figure 1 for carbon assignments. [b] (+) Downfield shift upon complexation; (−) Upfield shift upon complexation.

Acknowledgments

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Metabolism of Prostacyclin in Rat†

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ABSTRACT: Following a single intravenous administration of [11-^{3}H]prostacyclin in rat, 77% of the administered dose was excreted within 3 days with 33% in urine and 44% in feces. Urinary metabolites were accumulated by chronic intravenous infusions of [11-^{3}H]prostacyclin for 14 days. The drug was extensively metabolized and the structures of seven metabolites were elucidated by combined gas chromatography and mass spectrometry. The urinary products include the dinor and 19-hydroxy dinor derivatives of 6-keto-$PGF_{1\alpha}$ and 13,14-dihydro-6,15-diketo-$PGF_{1\alpha}$, ω-hydroxy and ω-carboxyl dinor derivatives of dihydro-6,15-diketo-$PGF_{1\alpha}$, and a dihydrodiketotetranordicarboxylic acid. The metabolic pathways of PGI_2 in rat are similar to that of $PGF_{2\alpha}$.

Prostacyclin (PGI_2) is a labile molecule generated enzymatically from prostaglandin endoperoxide in mammalian blood vessel walls (Gryglewski et al., 1976; Moncada et al., 1976). The synthetase enzyme for PGI_2 was found to occur in many tissues and organs (Sun et al., 1977). The compound is a powerful vasodilator in many vesicular beds. It has been suggested (Moncada & Vane, 1977) that PGI_2 plays a crucial role in the hemostasis of the cardiovascular systems.

In neutral or acidic aqueous medium, PGI_2 is rapidly hydrolyzed to 6-keto-$PGF_{1\alpha}$. The half-life of PGI_2 in physiological pH was estimated to be 10.5 min at 25 °C (Cho & Allen, 1978), and, therefore, it is intuitive that PGI_2 must be hydrolyzed to 6-keto-$PGF_{1\alpha}$ after its release. This is true under most in vitro conditions where 6-keto-$PGF_{1\alpha}$ represents the only stable terminal product from PGI_2. However, under in vivo conditions where there are other metabolic processes involved, whether 6-keto-$PGF_{1\alpha}$ represents the initial metabolic products of PGI_2 remained to be proven.

We have previously shown that PGI_2 can be rapidly oxidized in vitro by 15-hydroxyprostaglandin dehydrogenase of the lung (McGuire & Sun, 1978) and blood vessel (Sun et al., 1978) to the corresponding 15-keto product. Under the same condition 6-keto-$PGF_{1\alpha}$, however, showed little reactivity. Pace-Asciak et al. (1977) recently identified two principal urinary excretion products from 6-keto-$PGF_{1\alpha}$ treated rats. Both still possess the allylic alcohol group at C-15 which indicates they have not been oxidized by PGDH. No information is yet available for the metabolism of prostacyclin itself in whole animals.

Therefore, the present study was designed to isolate and identify major urinary metabolites of PGI_2 in the rat. These results should give an indication as to which compounds should be measured in order to monitor endogenous prostacyclin production.

Materials and Methods

[11-^{3}H]$PGF_{2\alpha}$ and [11-^{3}H]$PGF_{2\alpha}$ methyl ester were kindly synthesized by Dr. D. R. Morton and J. P. McGrath. The specific activity was 79 Ci/mol. PGI_2, 6-keto-$PGF_{1\alpha}$, and 5-iodo-PGI_1 methyl ester were provided by members of the Experimental Chemistry unit of The Upjohn Co.

Rats prepared with chronic indwelling venous cannulas (Weeks, 1972) and the special cold solution reservoir device (Weeks, 1978) for long-term IV infusion of PGI_2 were kindly provided by Dr. J. R. Weeks of the Upjohn Co.

[11-^{3}H]PGI_2 Na salt was prepared by a modification of the procedure described by Johnson et al. (1977). One milligram of [11-^{3}H]$PGF_{2\alpha}$ methyl ester was mixed with 0.5 mL of an aqueous solution containing 0.5 mg of Na_2CO_3 and 1.5 mg of KI. Three milligrams of solid iodine was added and the mixture was stirred overnight in an ice bath. The reaction was then quenched with approximately 5 mg of Na_2SO_3, diluted with 1 mL of saturated salt solution, and extracted three times with

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