Proton Nuclear Magnetic Resonance Study of Bleomycin in Aqueous Solution. Assignment of Resonances[†]

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ABSTRACT: The ^1H NMR spectrum of the glycopeptide antineoplastic antibiotic bleomycin has been examined in D_2O solution (Fourier transform nuclear magnetic resonance, 270 MHz) and in H_2O solution (correlation nuclear magnetic resonance, 250 MHz). Resonances have been assigned to specific hydrogens of the two most abundant congeners, bleomycin-A_2 (BLM-A_2) and bleomycin-B_2 (BLM-B_2), on the basis of (1) homonuclear spin decoupling, (2) comparison of the spectra of BLM-A_2, BLM-B_2, fragments of these antibiotics, and the related antibiotic phleomycin, and (3) the pH dependence of chemical shifts. Resonance assignments are presented for all the CH protons of BLM-A_2 and BLM-B_2 except for the saccharide groups, for which only the anomeric proton assignments are given. All of the NH protons have been identified with specific resonances except for the two primary amide groups, which yield four well-resolved peaks, whose specific assignment was not attempted. This study serves as a basis for future investigations of the conformation of bleomycin and its interaction with metals and nucleic acids.

The bleomycins (BLM)[1] are a group of glycopeptide antibiotics isolated from *Streptomyces verticillus* (Umezawa, 1965; Umezawa et al., 1966a). The primary structures of the two main components, bleomycin A_2 (BLM-A_2) and bleomycin B_2 (BLM-B_2), as elucidated by Umezawa and co-workers (Takita et al., 1972) are shown in Figure 1. Table I summarizes the notation by which the various residues of bleomycin are designated in this study. In order to clarify references to the sequence of residues in these molecules, we have adopted a notation (see Table I) differing from the previous one which denoted hydrolysis products by their relative order of chromatographic elution (Takita et al., 1968). BLM-A_2 and BLM-B_2, as well as other BLMs, differ from each other only in the terminal amine residue. A related group of antibiotics, the phleomycins (PLM), differ from the corresponding bleomycins by hydrogenation of one of the olefinic bonds in the bithiazole moiety (Figure 1).

Bleomycin is employed as an antineoplastic agent in the treatment of a variety of human and animal tumors. It is clinically administered in the treatment of squamous cell carcinoma, various lymphomas, and testicular carcinoma. Because of its low bone marrow toxicity (Boggs et al., 1974), bleomycin is increasingly being included in combination chemotherapy. When injected into experimental animals, bleomycin has been demonstrated to accumulate preferentially in tumor tissues which are sensitive to its pharmacological action (Ishizuka et al., 1967; Michiko et al., 1975). Both in vitro (Muller et al., 1972; Saito and Andoh, 1973) and in vivo studies (Onishi et al., 1973) have demonstrated that BLM and PLM cause strand scission of DNA, which, at least in the case

of the former antibiotic, is accompanied by the liberation of purine and pyrimidine bases.

Bleomycin and PLM associate with polyvalent metals. These antibiotics are isolated from the bacterial culture filtrate as copper salts. It is intriguing to note that the copper(II) complex of PLM, in contrast to the metal-free antibiotic, has been reported to cross-link DNA (Umezawa, 1973). Copper(II) inhibits BLM interaction with DNA. Degradation of DNA by PLM has been reported to involve a free radical mechanism involving specific polyvalent metals and oxygen (Sleigh, 1976). Iron(II) but not iron(III) has been shown to catalyze the scission of DNA by BLM (Sausville et al., 1976). Bleomycin labeled with radioisotopes such as ^{57}Co, ^{64}Cu, ^{65}Zn, ^{67}Ga, ^{99m}Tc, ^{111}In, ^{169}Yb, ^{197}Hg, and ^{203}Pb has been employed as a tumor scanning agent in the diagnosis and staging of a wide variety of solid tumors and malignant lymphomas (Monod and Rymer, 1973; Thakur, 1973; Kono et al., 1972; Renault et al., 1972). Models of the structures of BLM complexes with polyvalent cations and DNA have been proposed (Murakami et al., 1973, 1976).

The present investigation represents the initial stage of an NMR study of the solution conformation and dynamics of BLM and its derivatives and of the mechanism of their interaction with nucleic acids and metals. Prerequisite to such a program is the identification of spectral resonances with specific nuclei of the antibiotic. In this communication we present the assignment of most of the resonances in the ^1H NMR spectrum of BLM. These assignments were obtained (1) by comparison of the spectra of BLM-A_2, BLM-B_2, PLM, and various fragments of these molecules, (2) by a study of the pH dependence of chemical shifts, and (3) by homonuclear spin decoupling.

Experimental Section

Spectra of BLM in D_2O were recorded with a Bruker HX-270 spectrometer (operated in the pulse Fourier transform mode). Field frequency stabilization was accomplished by locking on the ^2H signal of the solvent. Chemical shifts were measured with respect to internal TSP (Stohler Isotopic Chemicals, Waltham, Mass.). To avoid the dynamic range problem (Redfield and Gupta, 1971; Waelder et al., 1975;

[1] Abbreviations used: BLM, bleomycin; BLM-A_2, bleomycin-A_2; BLM-B_2, bleomycin-B_2; BNX, Blenoxane (commercial bleomycin); PLM, phleomycin; TSP, $(CH_3)_3Si(CD_2)_3SO_3Na$; NMR, nuclear magnetic resonance; CM, carboxymethyl.
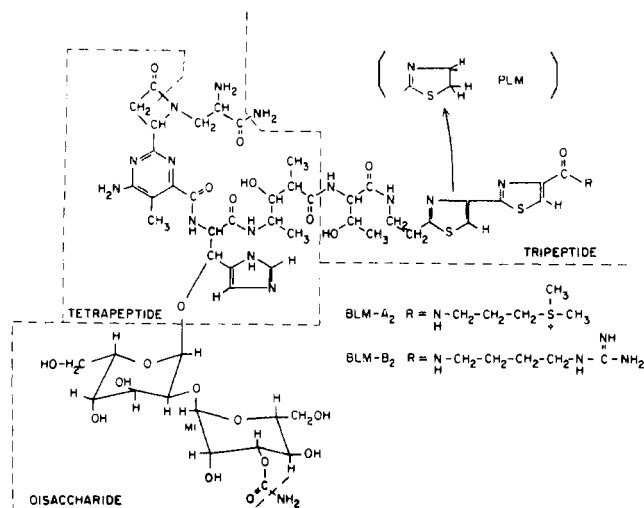
FIGURE 1: Structures of bleomycin (BLM) and phleomycin (PLM). The dotted lines indicate fragmentation of BLM by acid hydrolysis to give the tetrapeptide, the tripeptide, and the disaccharide fragments.

Waelder and Redfield, 1977; Patt and Sykes, 1972; Benz et al., 1972; Mooberry and Krugh, 1975; Tomlinson and Hill, 1973; Jesson et al., 1973; Schaeffer, 1972; Campbell et al., 1974; Bleich and Glasel, 1975; Krishna, 1976) encountered in proton FT NMR spectroscopy, spectra of bleomycin in H_2O were recorded with a 250-MHz spectrometer operated in the correlation mode (Dadok and Sprecher, 1974; Gupta et al., 1974). For the correlation NMR experiments, the solvent water peak was used for homonuclear locking with samples contained in 5-mm (o.d.) NMR tubes (Wilmad Glass Co., Buena, N.J.).

Blenoxane (BNX), the commercial form of bleomycin, was a generous gift from Bristol Laboratories (Syracuse, N.Y.). Separation of BNX into the A_2 and B_2 components was achieved by eluting the antibiotic from a CM-Sephadex C-25 column with a linear gradient of ammonium formate (0.02–1 M) (Fuiji et al., 1973). The separated fractions were pooled and lyophilized. Excess ammonium salt was removed by lyophilizing the products from basic (pH $\sim$10) and then from acidic (pH $\sim$1.5) solutions and finally by passing the material through a Sephadex G-10 column and eluting with distilled water.

Purified BLM-A_2 was hydrolyzed in 6 N HCl to give the tetrapeptide and the tripeptide (see Figure 1) (Umezawa et al., 1966b). The disaccharide moiety was obtained by mild acid hydrolysis (0.3 N H_2SO_4) (Omoto et al., 1972).

In D_2O solution, the pH$_m$ (pH meter reading calibrated with H_2O buffers but uncorrected for the deuterium isotope effect) was adjusted with DCl and NaOD (Merck, Sharp and Dohme of Canada, Montreal, Canada).

Results

Comparison of BLM-A_2 and BLM-B_2. The 270-MHz ^{1}H NMR spectrum of BNX in D_2O solution is shown in Figure 2. Resonances have been designated by their spectral assignments (Table I), which are justified in the Discussion (vide infra). Since BNX is approximately a 3:1 mixture of BLM-A_2 and BLM-B_2, it was useful to compare its spectrum with those of its two primary components. This comparison revealed that the spectrum of BNX is essentially a weighted (3:1) superposition of the spectra of BLM-A_2 and BLM-B_2. The spectra of these two congeners are identical for most resonances, but there are a few significant differences, which will be considered in

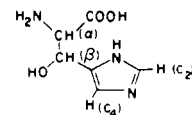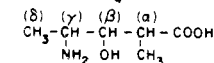TABLE I: Amino Components of BLM-A_2 and BLM-B_2.
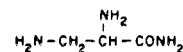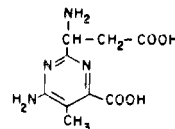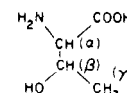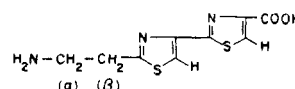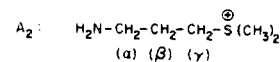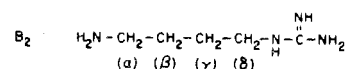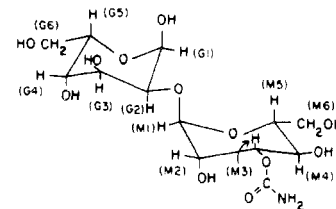
a subsequent section. Careful examination of the aromatic region of the spectrum of BNX (Figure 2A) revealed a number of weak resonances, which do not appear in the spectra of purified BLM-A_2 or BLM-B_2, and, therefore, probably originate from the trace quantities of the other congeners.

Bleomycin Fragments. As indicated in Figure 1, partial hydrolysis of BLM yields a tetrapeptide (residues I–IV), a tripeptide (residues V–VII), and a disaccharide (VIII). The spectrum of BNX (Figure 2) was compared with the spectra of the above three fragments of BLM-A_2 measured under the same experimental conditions. Some hydrolysis of the disaccharide may have occurred, but no attempt was made to purify this fragment. Except for the resonances which were subsequently shown to originate uniquely from BLM-B_2, the spectrum of BNX was found to be an approximate superposition of the spectra of the three fragments. Small displacements of the BNX resonances from their positions in the fragments were observed.

pH$_m$ Studies. Figure 3 summarizes the dependence of chemical shifts of BLM on the pH$_m$. The indicated chemical

FIGURE 2: The 270-MHz ^{1}H FT-NMR spectra of BNX in D_2O (A) at pH_m 1.25 and 30 ± 1 °C and (B-E) at pH_m 5.0 at 65 ± 1 °C.
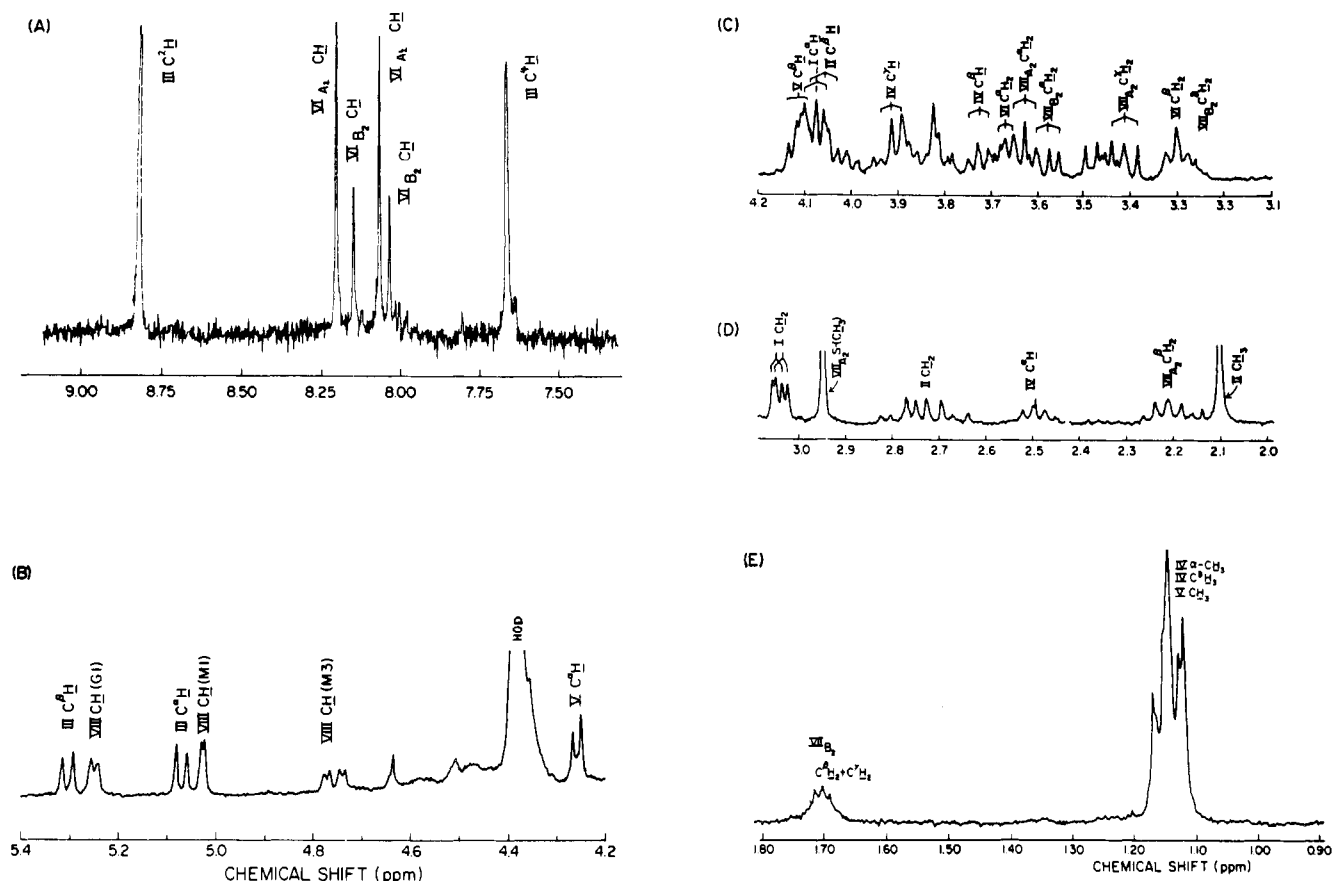
shifts correspond to the "center of gravity" of each multiplet. No attempt was made to precisely measure the chemical shift by computer analysis of the spin-coupling patterns. In the complex region of overlapping resonances (about δ 3-4 ppm), it was difficult to identify positions of some of the individual resonances. Figure 3B shows only the resonances in this spectral region which changed with pH_m and a few prominent pH_m invariant peaks.

Spectral changes associated with acid-base titration were reversible up to pH_m ~10. Above this pH_m irreversible changes occurred, which are believed to be associated with hydrolysis of the β-lactam group. The most prominent change was a decrease in the intensity of the three-proton singlet at δ 2.06 ppm and the concomitant appearance of a singlet at δ 1.98 ppm.

Spin-Decoupling Experiments in D_2O. The results of homonuclear decoupling experiments performed on BNX in D_2O solution are summarized in Figure 4. Weak coupling to aromatic resonances, which showed at most a splitting of less than 1 Hz, was not examined. These low-field resonances (δ 7-9 ppm) are not included in this spectrum.

Spectra in H_2O. Correlation ^{1}H NMR spectra (250 MHz) of BNX at pH 0.87 and 4.70 (28 °C) are compared in Figure 5. In addition to resonances of the aromatic CH hydrogens, which were previously detected in D_2O solution (Figure 2), these spectra contain resonances of NH hydrogens. In D_2O solution, the latter protons are replaced by deuterium and therefore are not observed at ^{1}H resonance frequencies. As in the spectra measured in D_2O solution, two of the aromatic resonances shift to high field with increasing pH. Chemical shifts, vicinal coupling constants, and spin-decoupling data are summarized in Table II. Increasing the pH to 4.70 broadened out the multiplet structure in the doublet at extreme low field

and sharpened the resonances of all the NH singlets. The broad peak near δ 6.5 ppm resolved into three relatively sharp resonances. Increasing the temperature to 45 °C (at pH 4.09) revealed a distinct doublet NH peak at δ 7.87 ppm (J = 6.7 Hz), which could not be resolved at lower temperatures (Figure 5A, ~δ 8.1 ppm).

Discussion

Comparison of the Spectra of BLM-A_2 and BLM-B_2. Spectral analysis of BNX was greatly simplified by the exact coincidence of almost all the resolved resonances originating from protons which are chemically identical in the two principal congeners, BLM-A_2 and BLM-B_2. Differences between the spectra of these two congeners were limited to resonances originating from the chemically distinct basic groups at the C terminus of the peptide chain (i.e., residue VII), and from the adjacent bithiazole containing residue VI. Except for their C-terminal moieties, these congeners must have very similar conformations. This observation greatly facilitated the assignment of resonances and permitted most of the studies to be performed with the commercial mixture (BNX) rather than with the individual congeners, whose purification was time consuming.

For example, in the aromatic region of the spectrum (δ 7.5-9.0 ppm), four prominent resonances are observed in the spectrum of BLM-A_2 and BLM-B_2 (Figure 2A). The two outer resonances have identical chemical shifts (at corresponding pH_ms) in the two congeners and must therefore originate from the imidazole group common to BLM-A_2 and BLM-B_2. This assignment is supported by additional evidence (vide infra). The two central resonances of BLM-A_2 are slightly displaced from those of BLM-B_2 so that in the spec-
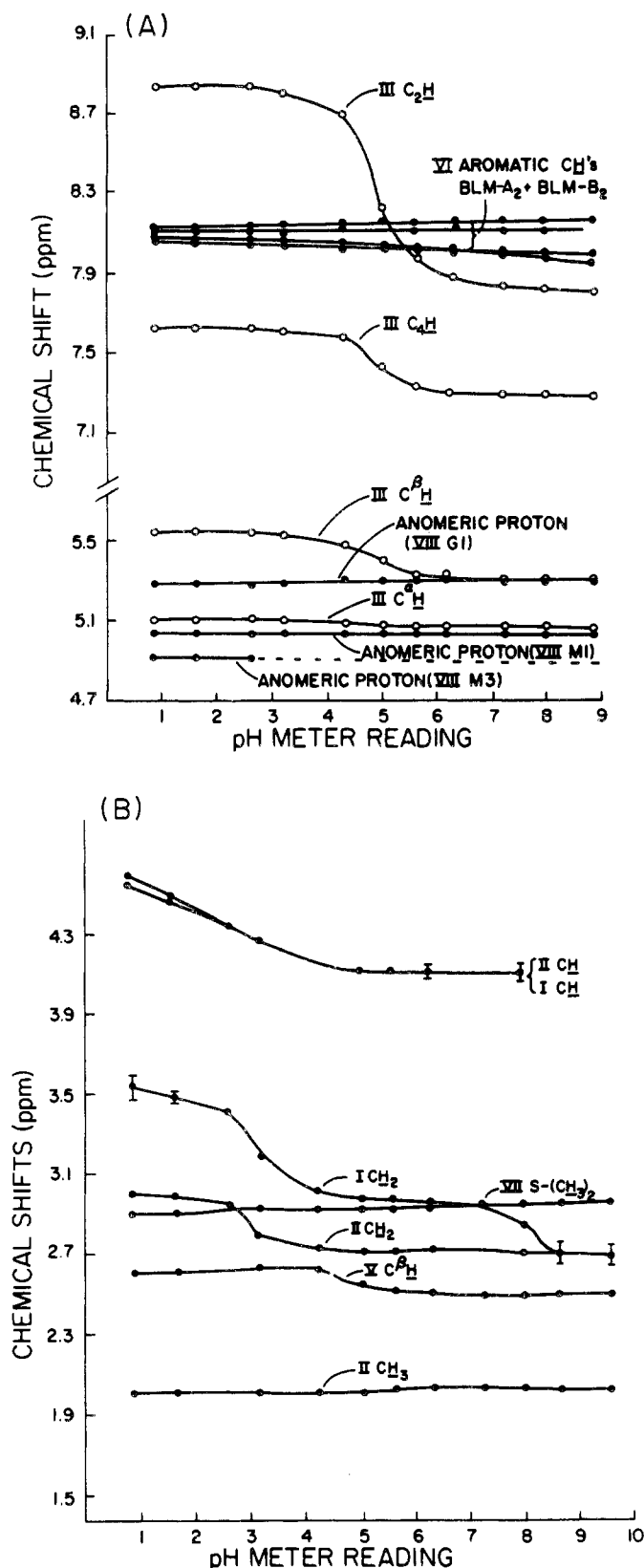
FIGURE 3: pH_m dependence of the proton resonances of BNX in D_2O at $30 \pm 1\ °C$.

FIGURE 4: Summary of spin-coupling experiments for BNX (pH_m 1.5) in the spectral region δ 1–6 ppm at $30 \pm 1\ °C$ (270 MHz, FT NMR). Irradiated peaks are indicated by the tails and the collapsed peaks are indicated by the heads of the arrow. Peaks joined only by a line have been decoupled in both directions.

different magnetic environments in the two congeners because of their proximity to the chemically distinct terminal amine residue.

A singlet of six proton intensity appears at δ 2.94 ppm in the spectrum of BLM-A_2, but is absent from the spectrum of BLM-B_2. In the spectrum of BNX (Figure 2D), this peak can therefore be designated as originating from the BLM-A_2 congener. Similar comparisons lead to the association of the peak at δ 2.22 ppm with BLM-A_2 (Figure 2D) and the peak at δ 1.69 ppm with BLM-B_2 (Figure 2E). In the region of closely overlapping resonances (Figure 2C), comparison of the BLM-A_2 and BLM-B_2 spectra is more difficult. Except for this region, where small differences between the spectra of BLM-A_2 and BLM-B_2 may exist, and except for the differences already indicated (vide supra), the spectra of the two congeners in D_2O solution are identical and a single peak is observed in the spectrum of BNX for the corresponding resonances of the two components.

Similar conclusions were drawn from spectra measured in H_2O solution (Figure 5). Comparison of spectra of BLM-A_2 and BLM-B_2 permitted identification of resonances unique to BLM-A_2 (the triplet at δ 8.83 ppm) and BLM-B_2 (the triplet at δ 8.64 ppm). As expected these resonances have relative intensities of 3:1, respectively, in the spectrum of BNX. In Figure 5B, the weak peak at δ 7.2 ppm and the broad resonance at δ 6.7 ppm occur only in the spectra of BLM-B_2 and are attributed to NH protons of the guanidino group of the agmatine residue (residue VII and BLM-B_2) (vide infra). Except for these differences and the differences between the aromatic CH resonances, which have already been considered, the spectra of BLM-A_2 and BLM-B_2 in H_2O are identical.

Aromatic Resonances. The aromatic region of the 270-MHz spectrum of BNX in D_2O solution (pH_m 1.5) is shown in Figure 2A. The resonances at δ 7.65 and 8.82 ppm have been assigned to the C_2H and C_4H protons of the imidazole group of β-hydroxyhistidine(III) for the following reasons: (1) As noted above, these resonances coincide in the spectra of BLM-A_2 and BLM-B_2 indicating that they originate from hydrogens common to both molecules and far removed from the amine terminus. (2) Consistent with this assignment, the imidazole peaks appear in the spectrum of the tetrapeptide (residues I–IV) but do not appear in the spectrum of the tri-

trum of BNX there appear four central aromatic resonances—two originating from BLM-A_2 at δ 8.22 and 8.07 ppm and two from BLM-B_2 at δ 8.17 and 8.04 ppm with an intensity ratio of BLM-A_2:BLM-B_2 peaks of about 3:1, as expected from the proportions of these congeners in BNX. These resonances are attributed to the bithiazole protons, which experience slightly
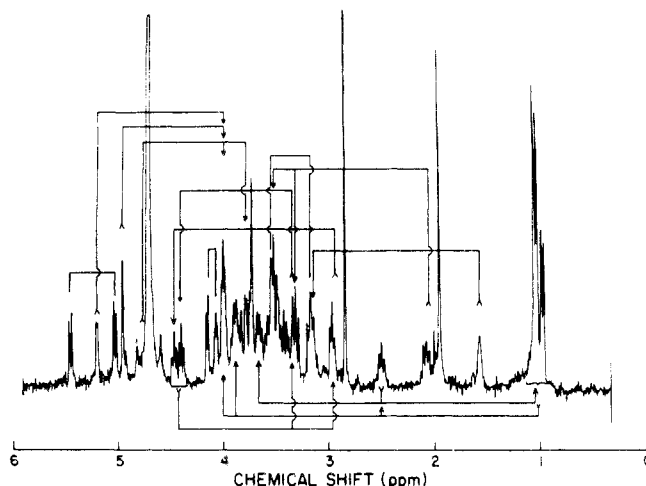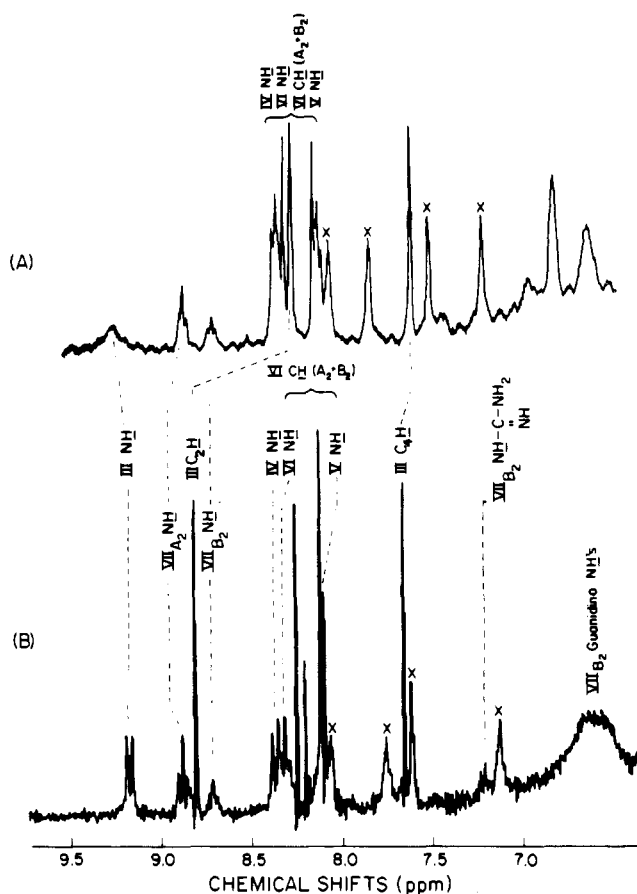
FIGURE 5: Spectral region δ 6.5-9.5 of BNX in H_2O recorded in the correlation mode at 250 MHz at 30 ± 1 °C at (A) pH 4.85; (B) pH 1.24. Resonances marked "x" arise from unassigned primary amide protons.

TABLE II: Assignments of Proton Resonances in the "NH" Region of BNX by Decoupling Experiments in H_2O.

Assignments	Chemical shifts[a]	Coupling constant (Hz)	Peak coupled to spectral region[a]
III NH	9.14	7.8	5.10
IV NH	8.33	8.5	3.95
V NH	~8.1		4.27
VI NH	8.26	5.8	3.62
VII-A$_2$ NH	8.83	7.0	3.61
VII-B$_2$ NH	8.64	5.6	3.6?
VII-B$_2$ NHC(=NH$_2$$^+$) NH$_2$	7.2	~5	
VII-B$_2$ guanidino NH	~6.7		
Amide NH	8.05		
Amide NH	7.75		
Amide NH	7.61		
Amide NH	7.20		

[a] ppm from internal TSP, chemical shifts measured at pH 1.24 (30 ± 1 °C).

peak and one small peak) originate from the thiazole group closer to residue VII and the upper field peaks correspond to the other thiazole CH protons.

The δ 4-6-ppm Region. This region of the spectrum, shown in Figure 2B, contains peaks from aliphatic CH protons which resonate to low field from the residual solvent HDO peak. The AB pattern (J = 6.85 Hz) centered at δ 5.08 and 5.31 ppm must emanate from the C$^\alpha$H and C$^\beta$H hydrogens of hydroxyhistidine (residue III) since these are the only adjacent methine hydrogens in the molecule that are not vicinal to other protons. Coupling of these nuclei was demonstrated by a double resonance experiment (Figure 4). Consistent with this assignment is the observation of the lower field doublet in the spectrum of the tetrapeptide fragment (residue I-IV) but not in spectra of the other fragments. The higher field doublet in the spectrum of the tetrapeptide was obscured by the intense solvent absorption. In the spectrum of BNX (Figure 2B), the lower field doublet exhibits a considerable high-field shift with a pK_a of 5.4, whereas the pH$_m$ dependence of the other doublet is much less (Figure 3A). This argues for the assignment of the low-field doublet to the C$^\beta$H hydrogen since this proton is closer to the titratable imidazole group. The demonstrated coupling of the high-field multiplet to an NH doublet (Figure 5, Table II) confirms this assignment. These spectral identifications agree with those of Omoto et al. (1972).

On the basis of spin–spin coupling constants and comparisons with derivatives of gulose and 3-O-carbamoylmannose, Omoto et al. also assigned the remaining three resonances in this spectral region to the two anomeric protons of the disaccharide and to the M3 proton (Table I) of 3-O-carbamoylmannose (Figure 2B). Consistent with these assignments, homonuclear doublet resonance experiments (Figure 4) indicate coupling of these resonances to multiplets near δ 4 ppm, which, by comparison with the spectrum of the disaccharide, originate from other hexose protons.

Methyl Resonances. Absorptions originating from methyl protons are readily identified by their characteristic coupling patterns and intensities. The singlet at δ 2.94 ppm (Figure 2D) is attributed to the dimethylsulfonium protons of residue VII of BLM-A$_2$ on the basis of its six proton intensity and its presence in the spectrum of BLM-A$_2$ but not in the spectrum of BLM-B$_2$ (vide supra). Also supporting this assignment is

peptide (residues V–VII) or the disaccharide (residue VIII). (3) The pH$_m$ dependence of the chemical shifts of these peaks (Figure 3A) is characteristic of imidazole protons, but the pK_a (uncorrected for the deuterium isotope effect) is displaced from the normal range (6–7) to 5.4, in agreement with potentiometric titration data on the β-hydroxyhistidine fragment, which exhibits a pK_a of 5.5 in H_2O (Takita et al., 1971). This decrease in pK_a is attributed to the inductive effect of the proximal oxygen. In accordance with data on histidine residues, the lower field resonance which exhibits the greater pH$_m$ shift is identified with the C$_2$H proton, and the upper field resonance with the C$_4$H hydrogen.

The remaining aromatic resonances (Figure 2A) originate from the bithiazole groups of BLM-A$_2$ and BLM-B$_2$ (vide supra). This assignment is consistent with the appearance of these resonances in the spectrum of the tripeptide (residues V–VII), but not in the spectra of the tetrapeptide (residues I–IV) or the disaccharide (residue VIII). Being far removed from any group which is titrated between pH$_m$ 1 and 8, the chemical shifts of these resonances are essentially unaltered by changes in pH$_m$ (Figure 3A). On the basis of the relative intensity ratio of these resonances and their chemical shifts, the two larger peaks are identified with BLM-A$_2$ and the weaker peaks with corresponding hydrogens of BLM-B$_2$ (vide supra). The spectrum of PLM measured under the same conditions as the spectra of the BLMs exhibits only three aromatic CH peaks, two approximately coincident with the imidazole CH peaks of BNX and one at δ 8.17 ppm, which is coincident with the lower field set of the BLM bithiazole CH peaks. Consequently, the two lower field bithiazole peaks (one large

the occurrence of this peak in the spectrum of the tripeptide fragment of BLM-A$_2$ (residues V–VII) and its absence in the tetrapeptide fragment (residues I–IV).

The only remaining singlet methyl resonance at δ 2.06 ppm (Figure 2D) originates from the pyrimidine residue (II). In the free amino acid, this methyl resonance occurs at δ 2.41 ppm (Muraoka et al., 1970). The appearance of this resonance in the spectrum of the tetrapeptide fragment (residues I–IV) supports this assignment.

The methyl groups of threonine and the methylvaleric acid derivative (residue IV) overlap at δ 1.18–1.19 ppm (Figure 2E). In random-coil polypeptides, the threonine methyl resonance occurs at δ 1.23 ppm (McDonald and Phillips, 1969). Takita et al. (1968) report that the methyl resonances of free 4-amino-3-hydroxy-2-methyl-n-valeric acid occur at δ 1.26 ppm (doublet, $J = 7$ Hz) and δ 1.30 ppm (doublet, $J = 7$ Hz). This assignment is supported by the appearance of the threonine methyl doublet in the tripeptide fragment (residues V–VII) and the appearance of the two methyl doublets of methylvaleric acid in the spectrum of the tetrapeptide (residues I–IV).

The δ 2.0–4.2 ppm Region. Double irradiation of the threonine and methylvaleric acid methyl resonances at δ 1.18–1.19 ppm decouples three resonances: at δ 2.49, 3.91, and 4.12 ppm, respectively (Figure 4). Figures 2C and 2D show this region of the spectrum on an expanded scale. The decoupled resonances originate from the three methine protons vicinal to methyl groups—the C$^\alpha$H and C$^\gamma$H hydrogens of methylvaleric acid (residue IV) and the C$^\beta$H hydrogen of threonine (residue V). If allowance is made for a small temperature variation of chemical shifts, the experiments in H$_2$O indicate that the δ 3.91 ppm resonance is coupled to a doublet NH resonance at δ 8.33 ppm (in H$_2$O at 30 °C; Figure 5B and Table II). The resonance at δ 3.91 ppm (Figure 2C) must therefore originate from the C$^\gamma$H hydrogen of the methylvaleric acid residue (IV), which is the only methine hydrogen vicinal to both methyl protons and to an NH proton. The δ 4.12 ppm resonance is coupled to a doublet at δ 4.27 ppm, which is coupled to an NH doublet at δ 8.1 ppm (in H$_2$O at 30 °C); the δ 4.27 ppm peak must therefore originate from the threonine C$^\alpha$H proton. The spin-coupling pattern (doublet), NH coupling, and chemical shift (δ 4.27 ppm) all support this assignment. The δ 4.12 ppm peak is therefore associated with the threonine C$^\beta$H. Since two of the three methine resonances coupled to the δ 1.18–1.19 ppm methyls have now been assigned, the remaining methine resonance (at δ 2.49 ppm) which exhibits coupling to a methyl group must originate from the C$^\alpha$H hydrogen of residue IV (the methylvaleric acid residue). This peak is coupled to a triplet (doublet of doublets) at δ 3.73 Hz (Figures 2C and 4), which is assigned to the C$^\beta$H of methylvaleric acid residue (IV).

The two proton resonance centered at δ 2.20 ppm appears only in the spectrum of BLM-A$_2$ (Figure 2D); it is present in the spectrum of the tripeptide (residues V–VII) and not in the spectra of the other fragments. Consequently, this peak originates from a CH_2 of the terminal base (residue VII) of BLM-A$_2$. This peak is coupled to two other methylene residues—one centered at δ 3.41 ppm (Figures 2D and 4) and the other at δ 3.63 ppm. The latter methylene peak couples to an NH triplet at δ 8.83 ppm (Figure 5 and Table II) and must therefore originate from the C$^\alpha$H$_2$ of residue VII. The δ 2.20 and 3.41 ppm peaks are therefore assigned to the C$^\beta$H$_2$ and C$^\gamma$H$_2$, respectively, of this residue.

Except for its C$^\alpha$H$_2$ group, the agmatine residue (B$_2$, VII) is formally identical with the side chain of arginine. The res-

onances of agmatine are therefore expected to occur close to the corresponding resonances of this amino acid. In NMR spectra of random-coil proteins and peptides, C$^\beta$H$_2$ and C$^\gamma$H$_2$ peaks of arginine occur close together at δ 1.66 and 1.84 ppm, respectively (McDonald and Phillips, 1969). In the spectrum of BNX, a single resonance occurs in this region at δ 1.70 ppm (Figure 2E). Since this resonance appears only in the spectrum of BLM-B$_2$ and not of BLM-A$_2$, it is associated with aliphatic hydrogens unique to the B$_2$ congener—i.e., the methylene hydrogens of agmatine. The integrated intensity of this peak corresponds to four BLM-B$_2$ protons (taking into account an approximate 3:1 ratio of BLM-A$_2$:BLM-B$_2$). Consequently, this peak is assigned to overlapping resonances of the C$^\beta$H$_2$ and C$^\gamma$H$_2$ protons of agmatine. This resonance is coupled to a resonance at δ ~3.3 ppm (Figure 4) which forms a small shoulder on the high-field side of the strong triplet peak at δ 3.30 ppm (Figure 2C). Since the arginine C$^\delta$H$_2$ peak occurs at δ 3.20 ppm in random-coil peptides, the δ 3.3 ppm peak of BNX is attributed to the C$^\delta$H$_2$ hydrogens of agmatine. The BLM-B$_2$ C$^\beta$H$_2$ + C$^\gamma$H$_2$ resonance is also coupled to a peak at δ 3.63 ppm (Figure 2C), which in turn is coupled to an NH triplet (in H$_2$O at 30 °C, Figure 5, Table II) at δ 8.64 ppm. The δ 3.63 ppm peak must therefore originate from the C$^\alpha$H$_2$ protons of agmatine.

The only remaining triplet NH peak in the spectrum of BNX (in H$_2$O at 30 °C) occurs at δ 8.26 ppm (Figure 5B) and is coupled to a resonance at δ 3.62 ppm. In the spectrum of BNX in D$_2$O at 65 °C (Figure 2C), this resonance occurs at δ 3.67 ppm. Reference to Figure 1 indicates that the only remaining methylene vicinal to an NH proton originates from residue VI. The δ 3.62 ppm peak is therefore ascribed to the C$^\alpha$H$_2$ group of residue VI, and the δ 3.30 ppm two proton triplet, to which it couples, is assigned to the C$^\beta$H$_2$ group of this residue.

In the spectrum of BNX, in D$_2$O at pH$_m$ 5.0 and 65 °C (Figure 2D), there are two multiplets, each with two proton intensity, and each exhibiting a spin-spin splitting pattern characteristic of the AB portion of an ABX spectrum, which are centered at δ 2.73 and 3.04 ppm. The X portions of these resonances resonate between δ 4.0 and 4.2 ppm but are obscured by other overlapping resonances. At pH$_m$ 1.25 these resonances move to low field and are clearly discerned as two triplets each of one proton intensity at δ 4.60 and 4.55 ppm in Figure 4. The δ 3.45 ppm peak couples to the low-field triplet (δ 4.60 ppm) and the δ 3.05 ppm peak to the upper field triplet (δ 4.55 ppm). Each pattern therefore corresponds to an XYCH-CH$_2$Z moiety, which is present in residues I and II. The upper field eight-line methylene peak shifts to high field with a pK_a of about 2.9 (Figure 3B), the reported pK_a of the pyrimidine amino group of residue II (Umezawa, 1973). The lower field four-line methylene peak also exhibits a spectral shift with a pK_a of 2.9 but, in addition, exhibits a shift between pH$_m$ 7 and 9. Since the pK_a of the α-amino group of residue I has been reported as 7.3 (Umezawa, 1973), the lower field quartet is assigned to residue I and the upper field octet to residue II.

This completes the assignment of all the detectable CH resonances of BNX except for the higher field resonances associated with the disaccharide moiety. Because of the complexity of the latter peaks and their overlap with other resonances, no attempt was made to identify these peaks.

NH Resonances. The lowest field NH doublet (Figure 5B) is identified with the hydroxyhistidine peptide NH proton by demonstration of its coupling to the C$^\alpha$H proton of this residue (Table II) (vide supra). The hydroxyhistidine NH proton ex-

TABLE III: Assignments of CH Resonances of BNX.

Assignments		Chemical shifts[a]	
		pH$_m$ 1.5 (30 ± 1 °C)	pH$_m$ 5.0 (65 ± 1 °C)
I	C$^\alpha H$	4.55	~4.1
	CH_2	3.45	3.04
II	CH_2	3.05	2.73
	C$^\beta H$	4.6	~4.1
	CH_3	2.06	2.06
III	C$^\alpha H^b$	5.10	5.08
	C$^\beta H^b$	5.55	5.31
	C$_2H$	7.65	7.42
	C$_4H$	8.82	7.90
IV	C$^\alpha H$	2.48	2.49
	C$^\beta H$	3.72	3.73
	C$^\gamma H$	3.90	3.91
	C$^\delta H_3$	1.18–1.19	1.18–1.19
V	C$^\alpha H$	4.27	4.27
	C$^\beta H$	4.12	4.12
	CH_3	1.18–1.19	1.18–1.19
VI	C$^\alpha H_2$	3.62	3.67
	C$^\beta H_2$	3.27	3.30
	aromatic CH (no. 1) (A$_2$)	8.22	8.21
	(B$_2$)	8.17	8.17
	aromatic CH (no. 2) (A$_2$)	8.07	8.06
	(B$_2$)	8.04	8.04
VIIA$_2$	C$^\alpha H_2$	3.61	3.63
	C$^\beta H_2$	2.10	2.20
	C$^\gamma H_2$	3.40	3.41
	S-(CH_3)$_2$	2.94	2.94
B$_2$	C$^\alpha H_2$	~3.6	3.63
	C$^\beta H_2$ \} C$^\gamma H_2$ \}	\{1.68\}	\{1.70\}
	C$^\delta H_2$	~3.3	~3.3
VIII	G1^b	5.21	5.25
	M1^b	4.95	5.05
	M3^b	~4.8	4.72

[a] ppm from internal TSP. [b] Consistent with the assignment of Omoto and co-workers (Omoto et al., 1972).

changes more rapidly with the solvent than does any other amide NH. This is indicated by broadening of this resonance by pH$_m$ 4.70 (Figure 5A); all other amide NH's retain their spin-coupling multiplet structure at this pH$_m$. The rapid exchange of this proton and the extreme low-field position of its resonance are attributed to its proximity to positive charges on the imidazole group and the pyrimidine amino group. By stabilizing the anionic transition state (Berger et al., 1959), positive charges increase the rate of base-catalyzed protolysis (Glickson et al., 1976).

Double resonance experiments described above demonstrate that the two triplets at δ 8.83 and 8.64 ppm originate from the NH's of the terminal bases of BLM-A$_2$ and BLM-B$_2$, respectively (Table II). The similarities in the chemical shifts of these resonances and their relative intensities (about 3:1) support this assignment.

Decoupling (vide supra) also identifies the doublet at δ 8.33 ppm with the NH of residue IV (Table II). The adjacent resonance at δ 8.26, the only remaining NH triplet, must originate from residue VI. This assignment is confirmed by double resonance experiments (vide supra). Similarly, the remaining NH doublet at δ 8.00 ppm is ascribed to the peptide proton of the threonine residue (again confirmed by the previously described double irradiation experiments). At 30 °C this resonance

overlaps with other peaks, but at 45 °C it moves to high field and is readily discerned.

The four single proton resonances at δ 7.2, 7.6, 7.8, and 8.1 ppm (Figure 5) originate from primary amide protons of the terminal amide group of residue I and from the carbamoyl group of the mannose derivative. Distinction between these resonances was not possible with the available data.

Comparison with spectra of polyarginine (Glickson and Phillips, unpublished data) indicates that the weak resonance at δ 7.2 ppm is associated with the unique guanidino NH—C($=$NH$_2^+$)—NH$_2$ hydrogen of residue VII of BLM-B$_2$. The remaining guanidino protons contribute to the broad peak at δ 6.7 ppm (Figure 5B), which also contains amino protons (of residue I). The chemical shift of the guanidino protons agrees with the value of δ 6.7 ppm observed for this peak in the spectrum of angiotensin II (Glickson et al., 1973). At higher pH$_m$ this broad resonance is resolved into three resonances (Figure 5A), whose assignment is left to a future study.

Summary

A firm basis has been presented for the assignment of all of the CH resonances of BLM-A$_2$ and BLM-B$_2$ except for the saccharide resonances (other than the anomeric and 3-H resonance of carbamoylmannose). These assignments are summarized in Table III, which also indicates assignments previously made by researchers in Japan. Of the NH resonances, all the peptide and secondary amide peaks have been unambiguously identified (Table II). The four primary amide peaks have been identified, but assignment of these peaks to individual hydrogens has not yet been accomplished. Similarly, distinction between amino (of residue I) and guanidino protons (of the agmatine residue) is yet to be achieved. The spectral assignments derived in this investigation are currently being employed in our laboratory in studies of the structure and dynamics of bleomycin complexes with polyvalent metals and nucleic acids. Assignments of ^{13}C and ^{15}N resonances of this glycopeptide have also been undertaken.

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Phosphorus-31 Nuclear Magnetic Resonance of Dihydroxyacetone Phosphate in the Presence of Triosephosphate Isomerase. The Question of Nonproductive Binding of the Substrate Hydrate[†]

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ABSTRACT: The degree of hydration of dihydroxyacetone phosphate and its binding to triosephosphate isomerase has been studied by ^{31}P NMR. The resonance of the free keto form of dihydroxyacetone phosphate, which is the form of the substrate handled by the enzyme, is broadened in the presence of enzyme, but the resonance of the hydrate is unaffected. The hydrate does not therefore bind nonproductively to the enzyme, and this fact renders further correction of the steady-state kinetic parameters unnecessary.

M any enzyme substrates exist in solution as an equilibrium mixture of two or more readily interconvertible forms of which only one is directly handled by the enzyme. For instance, substrates containing carbonyl groups may be partially hydrated (e.g., CO_2), partially enolized (e.g., acetoacetate), or exist as epimers (e.g., the α and β forms of aldoses and ketoses). While from kinetic experiments one may often determine which form of the substrate is the "active" species, it is more difficult to discover if the "inactive" form (which will be a structural analogue of the true substrate) is a competitive inhibitor. If this were true, the kinetic parameters measured on the basis of the total substrate would be in error. The purpose of this paper is to investigate the possibility of such nonpro-