/ 20

Journal of Biomolecular Structure & Dynamics, ISSN 0739-1102
Volume 8, Issue Number 2 (1990), ©Adenine Press (1990).

Two-Dimensional NMR Study of Bleomycin and Its Zinc(II) Complex: Reassignment of ^{13}C Resonances

Daniel Williamson,[1,†] Ian J. McLennan,[2,‡]
Ad Bax,[1] Michael P. Gamcsik,[2,*] and Jerry D. Glickson[2]
[1]Laboratory of Chemical Physics, NIDDK
National Institutes of Health
Bethesda, Maryland 20892 USA

[2]Division of NMR Research
Department of Radiology and Radiological Science
The Johns Hopkins University School of Medicine
Baltimore, Maryland 21205 USA

Abstract

Two-dimensional NMR experiments--one bond ^{1}H-^{13}C correlation spectroscopy and heteronuclear multiple bond correlation spectroscopy, both performed in the reverse detection mode--have been employed to unambiguously assign all of the ^{13}C resonances of the antibiotic bleomycin and its zinc(II) complex. Previous ^{1}H resonance assignments of bleomycin (Chen et al. (1977) Biochemistry 16, 2731-2738) were confirmed on the basis of homonuclear Hartmann-Hahn and homonuclear COSY experiments. The ^{13}C assignments differ substantially from those previously obtained by other investigators (Naganawa et al., (1977) J. Antibiot. 30, 388-396; Dabrowiak et al., (1978) Biochemistry 17, 4090-4096) but are in agreement with those reported by Akkerman et al.(1988) (Magn. Reson. Chem. 26, 793-802). The more recent study employed similar two-dimensional correlation experiments (performed in the direct detection mode) in conjunction with attached proton tests. Their study often required model compound data to identify carbonyls adjacent to aliphatic moieties. Previous ^{13}C NMR studies of the structure, pH titration, and molecular dynamics of bleomycin and its zinc complex have been reinterpreted in terms of the revised assignments.

Introduction

The bleomycins (BLEO), secreted by *Streptomyces verticillus*, are a group of antibiotics employed in cancer chemotherapy that differ only in the structure of the C-terminal residue (Figure 1). The antineoplastic activity of these antibiotics has been attributed to their ability to facilitate the degradation of DNA in the presence of oxygen and a metal cofactor, Fe(II) or possibly Cu(I) (1). The Zn(II)-BLEO complex has been

---

[†] Current Address: Brigham and Womens Hospital, 75 Francis Street, Boston, MA 02145.

[‡] Current Address: Merck Sharpe & Dohme Research Laboratories, PO Box 2000, Rahway, NJ 07065.

[*] Author to whom correspondence should be addressed.

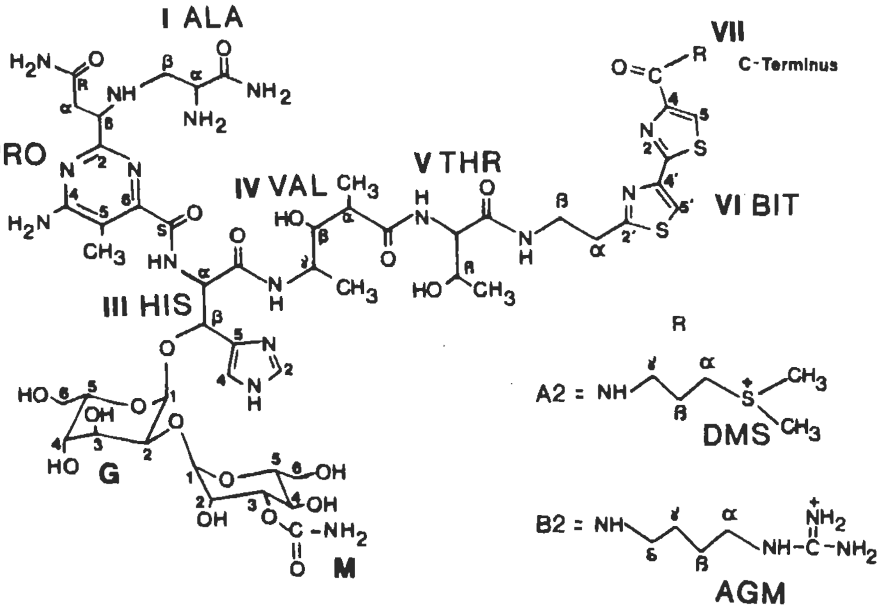

Figure 1: The structure of bleomycin A_2 and B_2. Abbreviations used are: I ALA, β-aminoalaninamide; II PRO, pyrimidyl propionamide; III HIS, β-hydroxyhistidine; IV VAL, methylvalerate; V THR, threonine; VI BIT, bithiazole; VII (B_2) AGM, agmatine; VII (A_2) DMS, γ-aminopropyldimethylsulfonium; M, mannose; G, gulose;

employed as a stable diamagnetic analog of Fe(II)-BLEO (2). Comparative NMR studies of the free antibiotic and its complexes with metals and nucleic acids have been directed at identifying the metal ligands (2-6), DNA binding sites (7-11), molecular conformation and dynamics (2,3,12-14), and pH titration (15,16) properties of this antibiotic. The ultimate objective of these investigations is the delineation of the mechanism of antineoplastic activity of this agent.

Critical to the achievement of this goal is the correct identification of resonances of the ^{1}H and ^{13}C spectra of the antibiotic. The original assignments of the ^{13}C resonances of free BLEO were based on comparative studies of fragments and analogs of the antibiotic and on several one-dimensional selective ^{1}H-^{13}C decoupling experiments (4). A partial assignment of the ^{13}C resonances of the Zn(II) complex was obtained by adopting the assignments of the free antibiotic proposed by Naganawa et al. (4) and by comparing spectra of the free and complexed antibiotic on the assumption that proximal resonances originate from the same carbons (5). These procedures involve a number of assumptions which may be in error. With the availability of two-dimensional NMR techniques that can map through-bond connectivity, it is now possible to obtain rigorous ^{13}C assignments. Employing these methods, we have found that a substantial number of the previously published assignments were in error and that previously unassigned resonances can now be unambiguously identified. Application of these methods required confirmation of the ^{1}H assignments (13,15) which we have accomplished by the homonuclear Hartmann-Hahn (HO HAHA) technique (17). Our assignments confirm those recently reported by Akker-

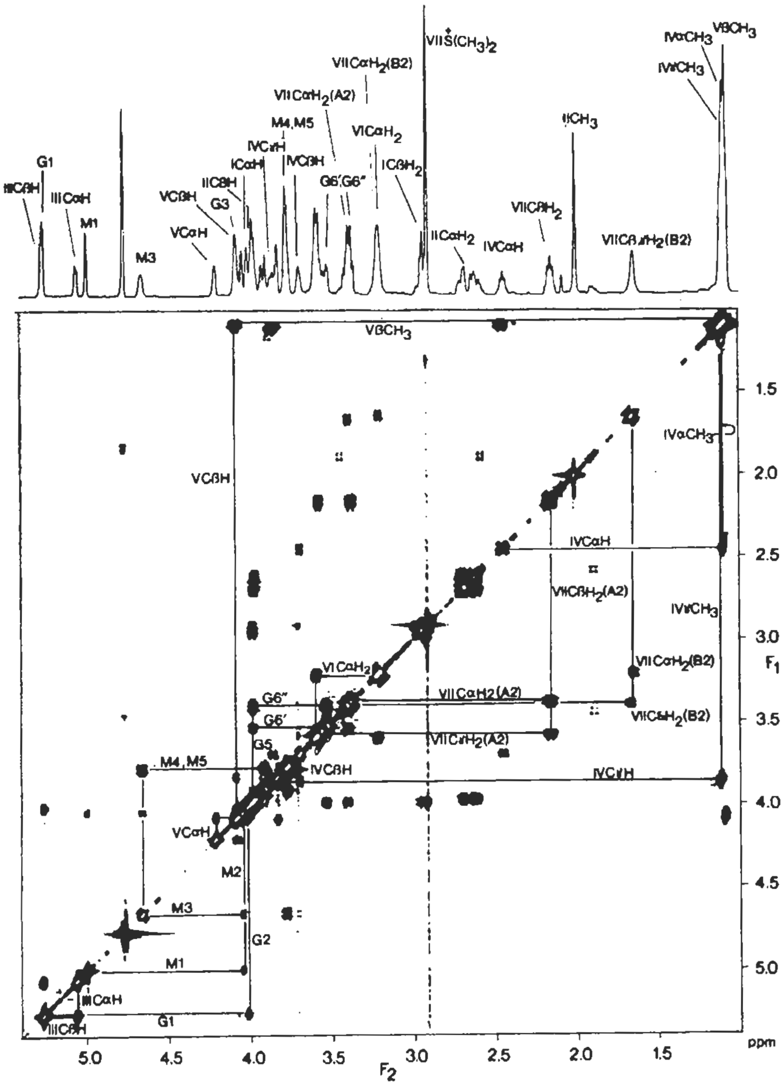

Figure 2: Absorption mode COSY (P. COSY) spectrum of free BNX, recorded on a Bruker AM 500 spectrometer. The spectrum results from a 800×1024 data matrix. Total measuring time was 2 h.

man et al. (18) but do not require the use of model compound data to assign carbonyl resonances nor other assumptions that were employed to identify other resonances.

Materials and Methods

NMR spectra were recorded on Nicolet NT-270 and NT-500 spectrometers, both equipped with Cryomagnet Systems reverse detection heteronuclear probes (19), and on Bruker AM 600 and Bruker AM 360 spectrometers. The ^{1}H-^{1}H chemical shift correlation spectroscopy (COSY) experiments were performed at 500 MHz and 360

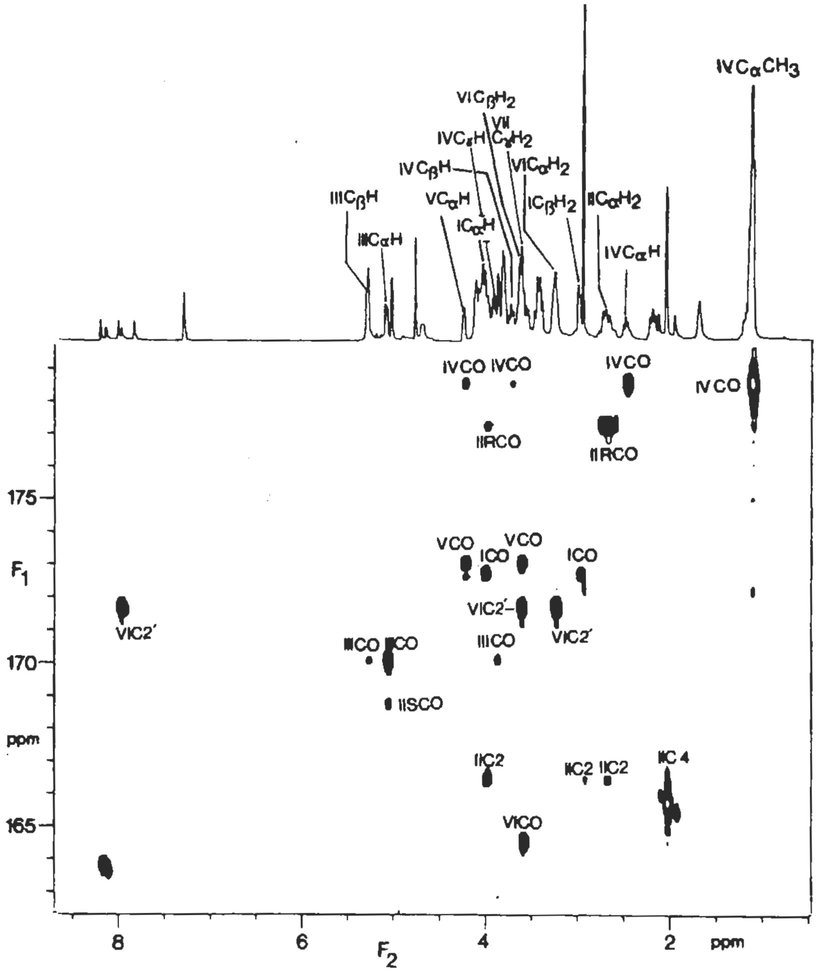

Figure 3a: The mixed mode (F_1 absorption, F_2 absolute value) HMBC spectrum of free BNX, recorded at 270 MHz. The spectrum results from a 2×400×512 data matrix, acquired in the hypercomplex format. Acquisition times were 25 and 110 ms in the t_1 and t_2 dimensions, respectively. The total measuring time was 28 h. Zero filling was used to yield a digital resolution of 15 (F_1) and 4.5 (F_2) Hz. The ^{13}C 90° pulse width was 40 μs. Rejection of one-bond correlations was optimized for a coupling constant of 135 Hz.

MHz. The COSY at 500 MHz was measured in the absorption mode using the purged COSY (P.COSY) method of Marion and Bax (20). One-bond ^1H-^{13}C multiple quantum correlation (HMQC) (21) spectra were recorded at 500 MHz. Heteronuclear multiple bond correlation (HMBC) (22) experiments were performed at 270 MHz and at 600 MHz (23). These newer instruments were superior to the NT-500 spectrometer with respect to reproducibility and consequently produce less t_1-noise (24). HMQC spectra were measured in the absorption mode (25); HMBC spectra at 600 MHz were recorded in the absorption mode using the time proportional phase incrementation (TPPI) method (26).

The commercial preparation, Blenoxane (BNX; Bristol Laboratories, Syracuse,

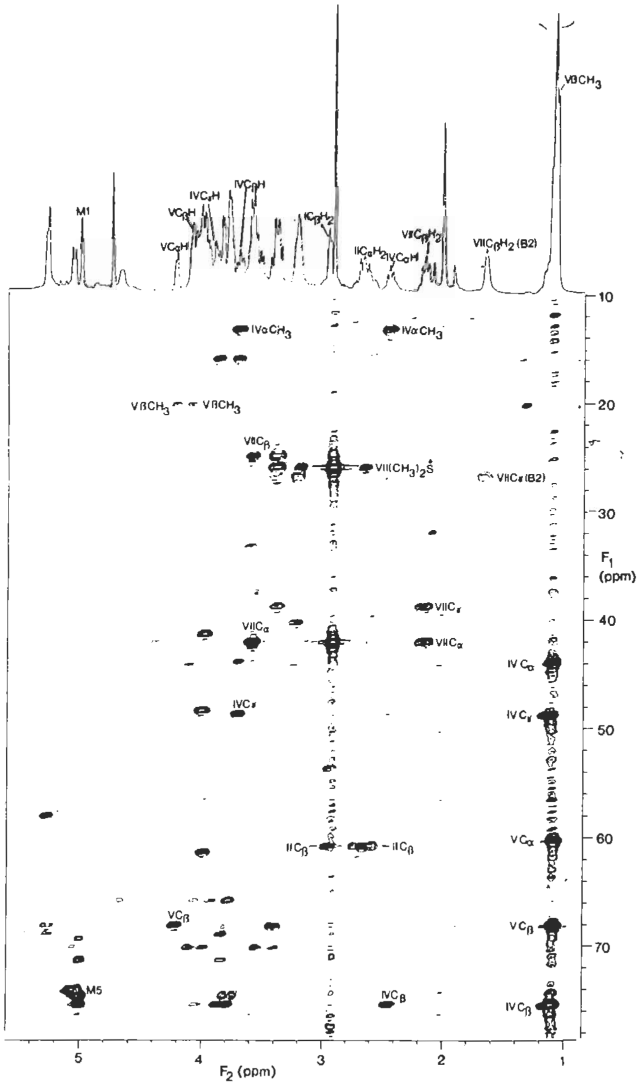

Figure 3b An HMBC spectrum of 25 mM free BNX acquired under the same conditions as Figure 3a.

NY), consisting of approximately a 70/30 mole/mole mixture of bleomycin A_2/bleomycin B_2 was employed without further purification. A 20 mM solution of the zinc(II)-BNX complex was prepared by dissolving BNX in D_2O (99.96%, Aldrich Chemical Co.) and adding one equivalent of $ZnSO_4$ (J.T. Baker Co.). The pH of the solution (meter readings uncorrected for isotope effects) was adjusted with 0.10 M NaOD (Merck, Sharpe, & Dohme, Montreal) to 6.6. All experiments were carried out at 27°C. Proton chemical shifts are referred to the methyl resonance (0 ppm for ^1H) of sodium 2,2,3,3[$^2H_4$]-3-trimethylsilylpropionate (TSP). Carbon chemical shifts are referenced to external tetramethylsilane (TMS).

Results and Discussion

Spectral Analysis of Free Bleomycin

The procedure used for assignment of the ^{13}C and ^{1}H resonances of free BNX relies on using the HMBC experiment for a stepwise correlation of the various residues via long range ^{1}H-^{13}C couplings. When this information is combined with the HMQC and COSY spectra, straightforward assignment of spectral resonances can be achieved.

We start with the proton resonances of the methylvalerate (VAL; IV) residue, which constitute a unique A_3MPTX_3 spin system manifested in the COSY spectrum (Figure 2). The proton peaks of the two VAL methyl groups are found at 1.15 and 1.16 ppm, whereas the remaining methyl proton resonance at 1.14 ppm is assigned to the threonine (THR; V) residue. The low field region of the HMBC spectrum (Figure 3a) shows coupling between a carbonyl carbon resonance at 178.59 ppm and the 1.15 ppm methyl proton resonance. This carbonyl resonance also couples to a single proton resonance at 2.47 ppm, which in turn couples to the 1.15 ppm methyl proton resonance (Figure 2). In unconjugated systems only two- and three-bond connec-

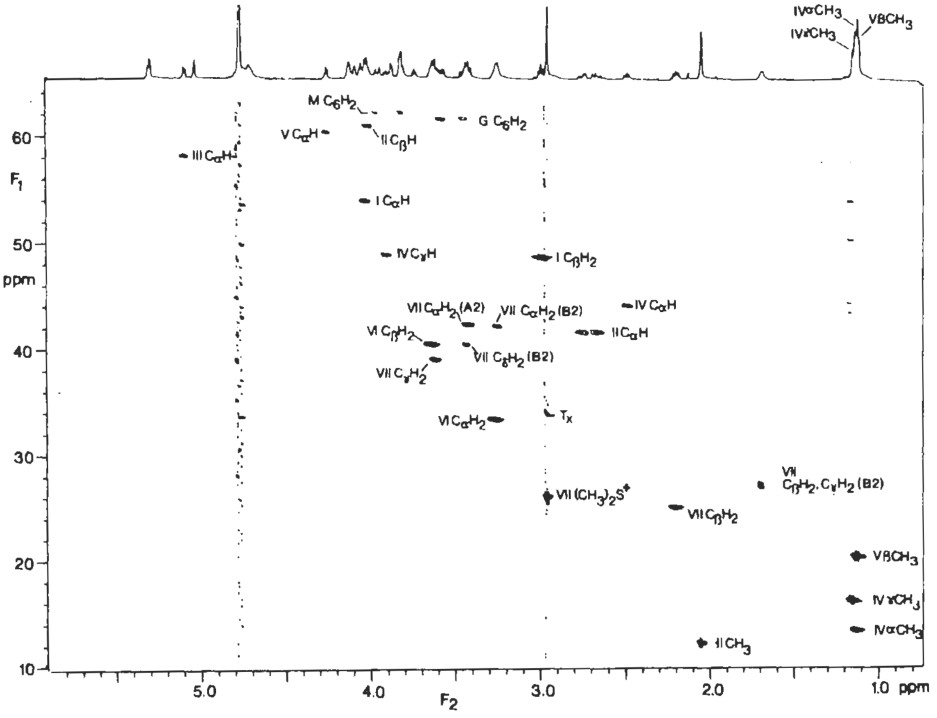

Figure 4: ^{1}H-^{13}C correlation spectrum of free BNX, recorded at 500 MHz ^{1}H frequency using the HMQC pulse sequence. The spectrum results from a 2×400×1024 data matrix, acquired in the hypercomplex format. Acquisition times were 32 and 102 ms in the t_1 and t_2 dimensions, respectively. The total measuring time was 6.3 h. Gaussian line broadening was used to avoid truncation, and data were zero filled to yield a digital resolution of 15 and 5 Hz in the F_1 and F_2 dimensions, respectively.

tivities can give rise to significant correlations in the HMBC spectrum; therefore, the VAL methyl proton resonance at 1.15 ppm is assigned to the Cα methyl group, and the 2.47 ppm resonance originates from the Cα proton of the VAL residue. As depicted in Figure 3a, a correlation between the 178.59 ppm resonance and VAL CβH (assigned at 3.72 ppm, Figure 2) confirms this assignment. Of course, the CγH and $C\gamma CH_3$ proton resonances at 3.86 and 1.16 ppm, respectively, can then be assigned from the COSY spectrum (Figure 2). Corresponding correlations to the ^{13}C resonances are found at 48.6 ppm (HMBC, Figure 3b) and 15.76 ppm (HMQC, Figure 4), respectively. Assignment of the methyl ^{13}C resonances were interchanged in a previous study (4). Chemical shifts are given in Table I.

Starting with the VAL assignments, spectral analysis can be continued in both directions of the peptide backbone. For example, the VAL carbonyl carbon resonance at 178.59 ppm also shows a correlation to the CαH proton resonance of THR at 4.24 ppm (Figure 3a), which shows connectivity to the CβH proton resonance of THR at 4.09 ppm in the COSY spectrum (Figure 2). The THR methyl proton resonance (1.14 ppm) correlates with both the THR Cα and Cβ ^{13}C resonances at 60.22 and 68.12 ppm in Figure 3b, which also can be identified from the HMQC spectrum of Figure 4 (only the Cα correlation is shown in Figure 4). The THR CαH peak at 4.24 ppm does not overlap with any other proton resonances, and the ^{13}C resonance to which it correlates (173.04 ppm in Figure 3a) arises from the THR carbonyl.

The THR carbonyl carbon resonance shows connectivity to the $C\beta H_2$ proton of the bithiazole (BIT; VI) moiety resonating at 3.62 ppm (Figure 3a). The $C\alpha H_2$ proton peaks of the BIT residue are recognized at 3.25 ppm in the COSY spectrum (Figure 2) but overlap with the $C\alpha H_2$ resonance of the agmatine (AGM; VII) residue at 3.25 ppm. Correlations between these overlapping ^{1}H resonances and ^{13}C resonances at 33.06 and 41.95 ppm are observed in the HMQC spectrum (Figure 4).

In the conventional 1-D ^{13}C spectrum (data not shown), the 41.95 ppm signal has much lower intensity compared to the one at 33.06 ppm and is therefore assigned to the AGM residue, present in 30% of the molecules (BLEO-B_2) in the Blenoxane mixture. The carbon resonance at 33.06 ppm therefore can be assigned to the Cα of BIT. Both $C\alpha H_2$ and $C\beta H_2$ BIT proton resonances show connectivity in the HMBC spectrum to a resonance at 171.65 ppm, which must be the C2′ ring carbon (data not shown). In the HMBC spectrum (Figure 5) the C5′ protons of the A_2 and B_2 congeners also show a coupling to this carbon. Consequently, the protons resonating at 7.95 and 7.98 ppm can be identified as the C5′ ring protons which allows assignment of the corresponding ^{13}C resonances from the HMQC spectrum (not shown). The remaining set of resonances at 8.19 and 8.11 ppm with relative intensities of 70/30 must correspond to the C5 ring protons of BLEO-A_2 and BLEO-B_2, respectively. These proton resonances show HMBC connectivities to carbon resonances at 163.74 and 163.54 ppm, respectively, which are assigned to the corresponding C2 carbons of the thiazole ring proximal to the C-terminus. A correlation from the resonances of the BIT C5′H proton to the C2 carbon of the adjacent ring is also observed (Figure 5), confirming the aromatic assignments. It should be emphasized that by tracing through-bond connectivities, this method allows unambiguous dis-

Table I
A Summary of the ^{13}C Chemical Shifts for Free Bleomycin

	Residue	^1H (δ ppm)	^{13}C (δ ppm)	T_1 (ms) (Ref. 12)
	CO		172.52	
I	CαH	4.00	53.59*	232
(ALA)	$C\beta H_2$	2.98	48.19	
	RCO		177.30*	
	CβH	3.98	60.74*	180
	$C\alpha H_2$	2.65,2.75	41.25	
	CH_3	2.08	11.93	
II	C2		166.40	
(PRO)	C4		165.72 or 153.13	
	C5		113.38	
	C6		153.13 or 165.72	
	SCO		168.78*	
	CO		170.09	
	CαH	5.08	57.98	
III	CβH	5.30	74.02*	
(HIS)	C5		135.88*	
	C4H	7.28	118.67*	
	C2H	7.82	138.02	
	CO		178.59	
	αCH_3	1.15	13.08*	1206
IV	CαH	2.47	43.74	
(VAL)	CβH	3.72	75.42	
	γCH_3	1.16	15.76*	1029
	CγH	3.86	48.60	
	CO		173.04	
V	CαH	4.24	60.22	
(THR)	CβH	4.09	68.12	
	βCH_3	1.14	19.99	
	CO		164.45*	
	C2		163.74	
	C5H	8.19(A2) 8.11(B2)	126.12*	
	C4		149.85	
VI	C4′		147.98	
(BIT)	C5′H	7.98(A2) 7.95(B2)	120.08*	
	C2′		171.65*	
	$C\alpha H_2$	3.25	33.06	
	$C\beta H_2$	3.62	40.14	
	$C\alpha H_2$	3.38	41.95	
VII	$C\beta H_2$	2.18	24.72	
(DMS)	$C\gamma H_2$	3.56	38.69	
(A2)	$(CH_3)_2$	2.94	25.73	
	$C\alpha H_2$	3.25	41.95	
	$C\beta H_2$	1.70	26.95 or 26.55	

Table I

continued

VII (AGM) (B2)	CγH$_2$	1.70	26.55 or 26.95
	CδH$_2$	3.44	40.15
	GN		157.05
G (Gulose)	1′	5.28	98.46
	2′	4.01	71.31*
	3′	4.12	68.89*
	4′	3.86	70.16
	5′	3.98	68.12*
	6′	3.54	
	6″	3.43	61.37
M (Mannose)	1′	5.03	99.22
	2′	4.04	69.39
	3′	4.65	75.42
	4′	3.82	65.79
	5′	3.82	74.63
	6′	3.94	
	6″	3.82	61.99
	CO		159.00

The numbers with asterisks refer to the chemical shifts which have been reassigned with respect to the work of Naganawa et al. (4).

tinction between resonances of the two thiazole groups.

The DMS fragment is assigned starting with the unique six proton singlet dimethyl sulfonium resonance in the ^{1}H spectrum at 2.94 ppm. The dimethyl proton resonance shows satellites to their carbon resonance at 25.73 ppm in Figure 3b, as well as a multiple-bond correlation to the CαH$_2$ carbon resonance at 41.94 ppm. By referring to the HMQC spectrum (Figure 4), we can assign the DMS CαH$_2$ protons to the resonance at 3.38 ppm. Using the COSY spectrum (Figure 2) and the HMQC spectrum (Figure 4), the remaining CβH$_2$ and CγH$_2$ protons (2.18 and 3.56 ppm, respectively) and carbons (24.72 and 38.69 ppm, respectively) are easily assigned. Finally, correlation of the carbonyl resonance at 164.45 ppm to the DMS CγH$_2$ proton resonances at 3.56 ppm (Figure 3a) allows its assignment as the BIT carbonyl.

The terminal AGM resonances are assigned starting with the CδH$_2$ proton resonances at 3.44 ppm. This assignment is made from the HOHAHA ^{1}H spectrum of Zn(II)-BNX complex in H$_2$O, which showed a correlation between the amide NH proton resonance and the CδH$_2$ proton resonance (data not shown). Since the AGM proton resonances are unperturbed by zinc complexation, it is reasonable to ascribe the 3.44 ppm resonance in the spectrum of free BNX to the CδH$_2$ protons of AGM. Indeed this resonance shows a COSY correlation to the resonance at 1.70 ppm (Figure 2), which represents the CβH$_2$ and CγH$_2$ protons of AGM as confirmed by their absence from the spectrum of purified BLEO-A$_2$. The CδH$_2$ protons are also assigned from the COSY data in Figure 2. The CαH$_2$ and CδH$_2$ carbons are assigned by inspection of the HMQC spectrum (Figure 4), but the CβH$_2$ and CγH$_2$ carbon peaks cannot be distinguished due to the degeneracy of their proton chemical shifts.

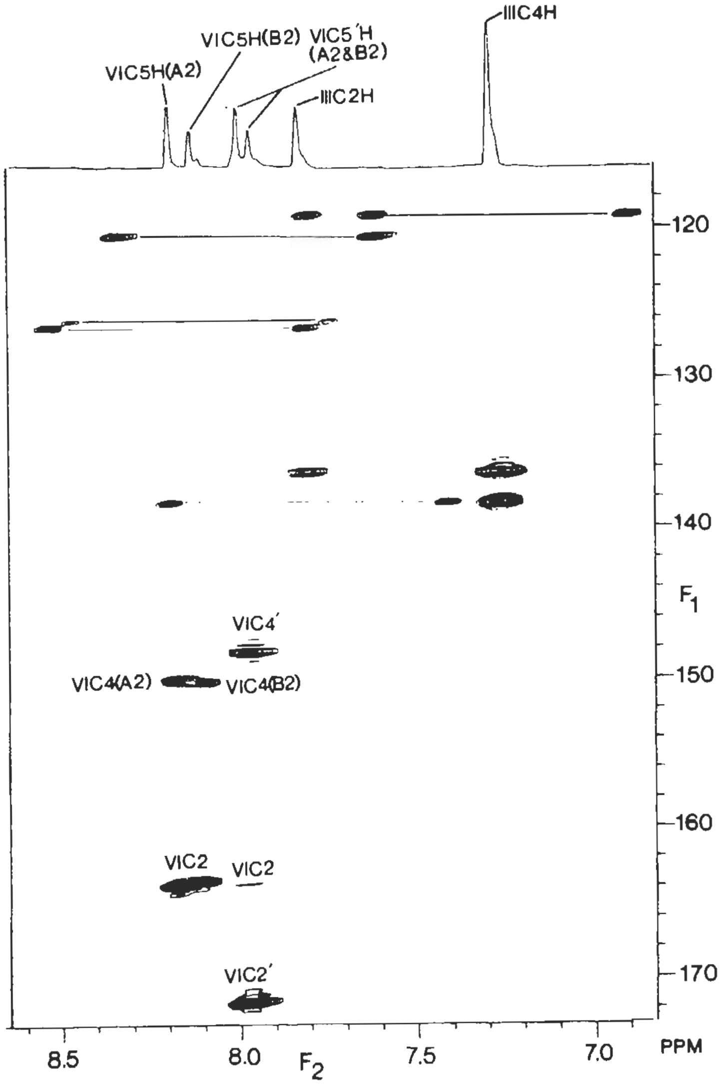

Figure 5: An HMBC spectrum of 25 mM free BNX acquired under the same conditions as Figure 3. Horizontal lines connect incompletely suppressed one-bond ^{1}H-^{13}C satellites.

As the only unassigned low intensity resonance (30%) in the 1-D carbon spectrum, the guanidino carbon assignment is made by exclusion at 157.05 ppm. This assignment is consistent with the unique chemical shift expected for the resonance of the only guanidino moiety in the molecule. According to the HMBC spectrum, the BIT carbonyl of BLEO-B_2 resonating at 165.11 ppm is correlated to the $C\delta H_2$ protons. This low intensity correlation does not show up in the contour levels depicted in Figure 3a.

Assignments of the remaining residues are obtained in a similar manner starting with the VAL residue. The CγH proton resonance of VAL at 3.86 ppm, exhibits a correlation to a carbonyl carbon resonating at 170.09 ppm, and similar correlations are observed for the protons peaks at 5.08 and 5.30 ppm (Figure 3a). These protons form the only AX system in the molecule, corresponding to the Cα and Cβ protons of the β-hydroxyhistidine (HIS; III), respectively, confirming the assignment of the 170.09 ppm resonance to the HIS carbonyl. The HIS CβH proton shows a correlation to the gulose C1 carbon resonating at 98.46 ppm (outside the regions shown in Figure 3a,b), and the HIS CαH proton (5.08 ppm) shows a correlation to a carbonyl resonating at 168.78 ppm, which is assigned to the pyrimidyl propionamide (PRO; II) S-carbonyl (Figure 3a). Assignment of the imidazole ring protons is straightforward: Both HIS CαH and CβH proton resonances show a correlation to the 135.88 ppm peak (not shown), assigning the latter to the C5 carbon. The CβH proton resonance also is correlated to a carbon resonating at 118.67 ppm (not shown), which originates from the imidazole C4. A correlation between the C4 carbon and the C4H proton resonance (7.28 ppm) is observed in the HMQC spectrum (not shown). The remaining unassigned low field singlet must correspond to the C2H proton resonance (7.82 ppm), which exhibits an HMQC correlation to the corresponding carbon resonance at 138.02 ppm.

The ring methyl carbon resonance of PRO at 11.93 ppm is assigned on the basis of a one-bond correlation to the methyl protons resonating at 2.08 ppm (Figure 4), the only unassigned methyl peak. A multiple bond correlation (HMBC) from the methyl proton peak to the aromatic carbon resonances at 165.72 (Figure 3a), 113.38 (not shown), and 153.13 ppm (not shown) identifies these peaks with the C4, C5, and C6 carbons, respectively, although there is some ambiguity with regard to the C4 and C6 carbon assignments. Specific assignments are based on comparisons with model compounds. The large chemical shift differences between these carbons ascertain the correctness of these assignments. The remaining PRO and β-aminoalanine (ALA; I) resonances are both ABX proton spin systems. The PRO resonances at 2.65, 2.75, and 3.98 ppm are identified on the basis of their HMBC correlations to the resonances of the C2 aromatic ring carbon at 166.40 ppm and a carbonyl at 177.30 ppm (RCO; Figure 3a). The $C\alpha H_2$ carbon resonance at 41.25 ppm is unambiguously assigned from the HMQC spectrum (Figure 4). Since the CβH proton peak overlaps with two other resonances, the ^{13}C assignment (to a carbon resonance at 60.74 ppm) requires HMBC correlation to the $C\alpha H_2$ proton resonances (Figure 3b).

The remaining ABX system resonating at 2.98 and 4.00 ppm corresponds to the ALA residue. This is confirmed by an HMBC correlation between the ALA $C\beta H_2$ proton resonating at 2.98 ppm and the PRO Cβ carbon at 60.74 ppm (Figure 3b). HMBC correlations between the ALA $C\beta H_2$ (2.98 ppm) and CαH (4.00 ppm) proton peaks and a carbon resonating at 172.52 ppm allow assignment of this carbon to the ALA carbonyl (Figure 3a). The ALA CαH (53.59 ppm) and $C\beta H_2$ (48.19 ppm) carbon resonances can be assigned from the HMQC spectrum (Figure 4).

The only remaining assignments are those of the disaccharide unit. The anomeric gulose carbon (G1) resonance at 98.46 ppm was identified earlier on the basis of

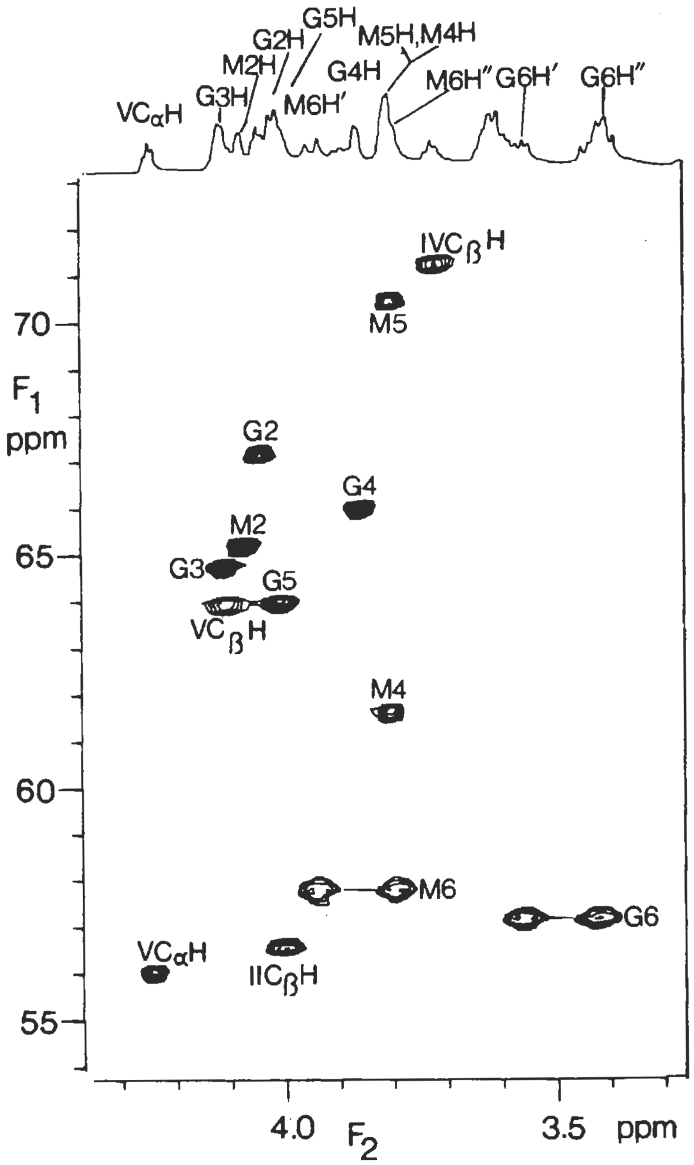

Figure 6: An HMQC spectrum of 25 mM free BNX acquired under the same conditions as Figure 4.

HMBC correlation to the HIS-CβH hydrogen (not shown). The anomeric proton peak (G1H) at 5.28 correlates with the G1 carbon resonance. The mannose anomeric carbon (M1) resonance at 99.22 ppm is identified from the HMQC spectrum (not shown), which shows correlation of this peak to the remaining anomeric proton resonance (M1H) at 5.03 ppm. All remaining gulose protons are identified on the basis of the COSY spectrum (Figure 2), and correlation to their corresponding ^{13}C resonances is straightforward on the basis of the HMQC spectrum (Figure 6). Assignment of the mannose proton resonances, M1H through M4H, is accomplished in a similar manner. Assignment of M1, M2, and M3 carbons is subsequently made on the basis of the HMQC spectrum (Figure 6). Assigning the M4 carbon resonance is more difficult, because two other unassigned proton peaks overlap with the M4H proton resonance at 3.82 ppm (Figure 6). One of these resonances clearly corresponds to an M6 methylene proton, with the other methylene proton resonating at 3.94 ppm. This indicates that the only remaining unassigned proton resonance, M5H, must overlap with that of M4H, explaining the absence of a M4H-M5H correlation in the COSY spectrum (Figure 2). Carbons M4 and M5 are distinguished on the basis of the HMBC spectrum (Figure 3b); the M1H proton resonance shows a correlation to the ^{13}C resonance at 74.63 ppm and not to that at 65.79 ppm, permitting the assignment of the 74.63 ppm resonance to the M5 carbon (three-bond correlation *via* the ring oxygen; Figure 3b). An HMQC spectrum of the sugar region, which facilitates the sugar assignments, is shown in Figure 6. In earlier work (4), the G3 carbon was misassigned to the HIS CβH carbon, and the G2 carbon was misassigned as the G3 carbon.

Reinterpretation of Previous ^{13}C NMR Studies of Bleomycin

Eleven of the fifty-nine ^{13}C assignments of free BLEO (A_2 and B_2) made by Naganawa et al. (4) have been revised (Table I). The previous ^{13}C assignments were used in investigations of the pH titration behavior (16) and rotational correlation times (12) of BLEO and must now be reinterpreted in terms of the revised assignments.

pH Titration of BLEO

Studies of amines and amino acids demonstrate that the carbon resonances generally exhibit low-field (positive) dissociation shifts, with the shift of the Cα carbon being much smaller (and sometimes slightly negative) than that of the Cβ carbon; the carbonyl carbon resonances also exhibit large low-field shifts (27-30). The BLEO dissociation shifts associated with titration of the secondary and primary amino groups are more consistent with the anticipated "β-effect" when the revised assignments are employed.

Thus, for the secondary amino group (pK_a 2.98) the shifts for the ALA residue are 0.32±0.03, 1.56±0.07, and 2.24±0.06 ppm for carbons that are one, two, and three bonds removed from the titrated nitrogen (i.e., the Cβ, Cα, and carbonyl carbons), respectively. For the PRO residue the adjacent carbon (CβH) exhibits a shift of 0.63±0.02 ppm, whereas the $C\alpha H_2$ and RCO groups exhibit shifts of 3.71±0.03 and 2.02±0.09, respectively. The pyrimidine C2′ carbon, which is β to the titrated nitrogen, also exhibits a larger shift (6.09±0.01 ppm) than the carbon that is one bond closer to the secondary amino group.

Titration of the primary amino group (pK_a 7.38) shifts the Cα and Cβ carbon resonances by 2.15±0.69 and 5.82±1.23 ppm, respectively, and the carbonyl resonance by 10.02±1.42 ppm. The resonances of the carbons of the PRO residue exhibit diminished shifts consistent with their greater distance from the titration site (0.72±0.42 and 0.28±0.10 for the CβH and $C\alpha H_2$ carbon resonances, respectively). It is noteworthy that the anomalously large shift of the M5 carbon resonance (0.91±0.15 ppm) is retained in the revised assignments, indicating that there is a very long range

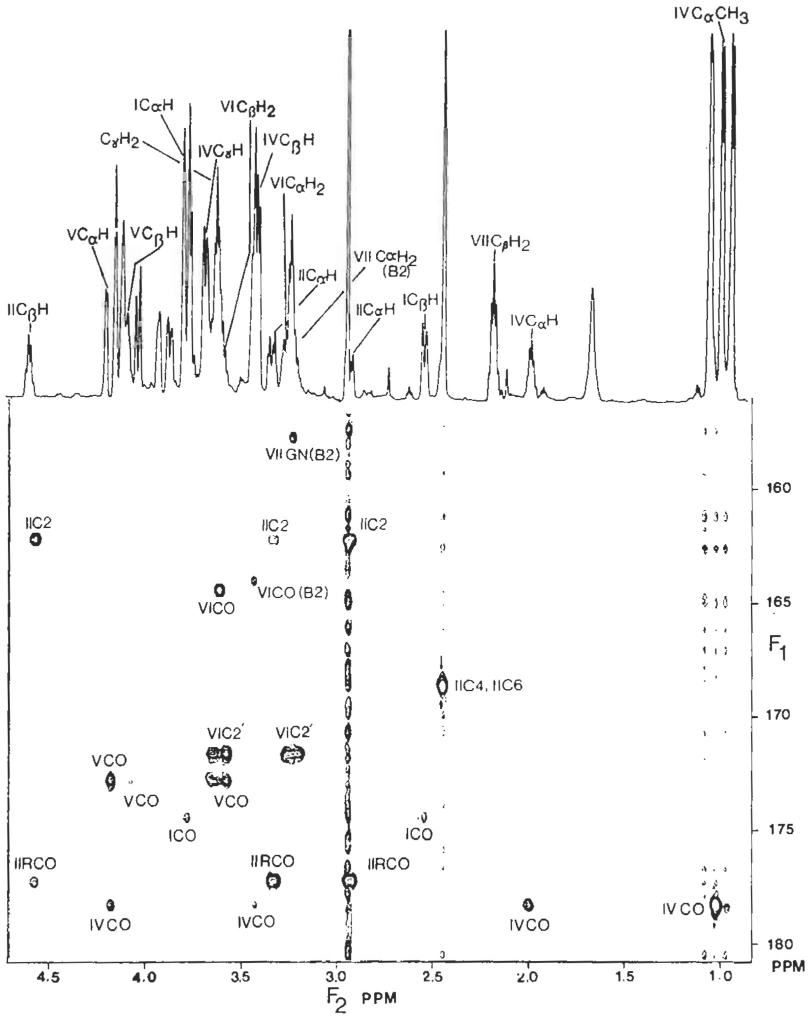

Figure 7: Mixed mode (F_1 absorption F_2 absolute value) HMBC spectra of 25 mM Zn(II)-BNX recorded at 600 MHz. The spectra result from a 1024×1024 data matrix, acquired in the TPPI mode. Acquisition times were 16 and 102 ms in the t_1 and t_2 dimensions, respectively. The total measuring time was 6 h. Zero filling yielded a digital resolution of 30 (F_1) and 5 (F_2) Hz. The ^{13}C 90° pulse width was 12 μs. No rejection of one-bond correlations was employed.

interaction between the primary amino group and the mannose ring.

Relaxation Times

Krishna et al. (12) estimated the rotational correlation times of BLEO-A_2 from measurements of the ^{13}C T_1s of specific carbon atoms. The relaxation times that have been modified by the revised resonance assignments are listed in Table I. Most of the changes are small (< 10%) and hence do not substantially modify the analysis of molecular dynamics. The most significant modifications are those associated

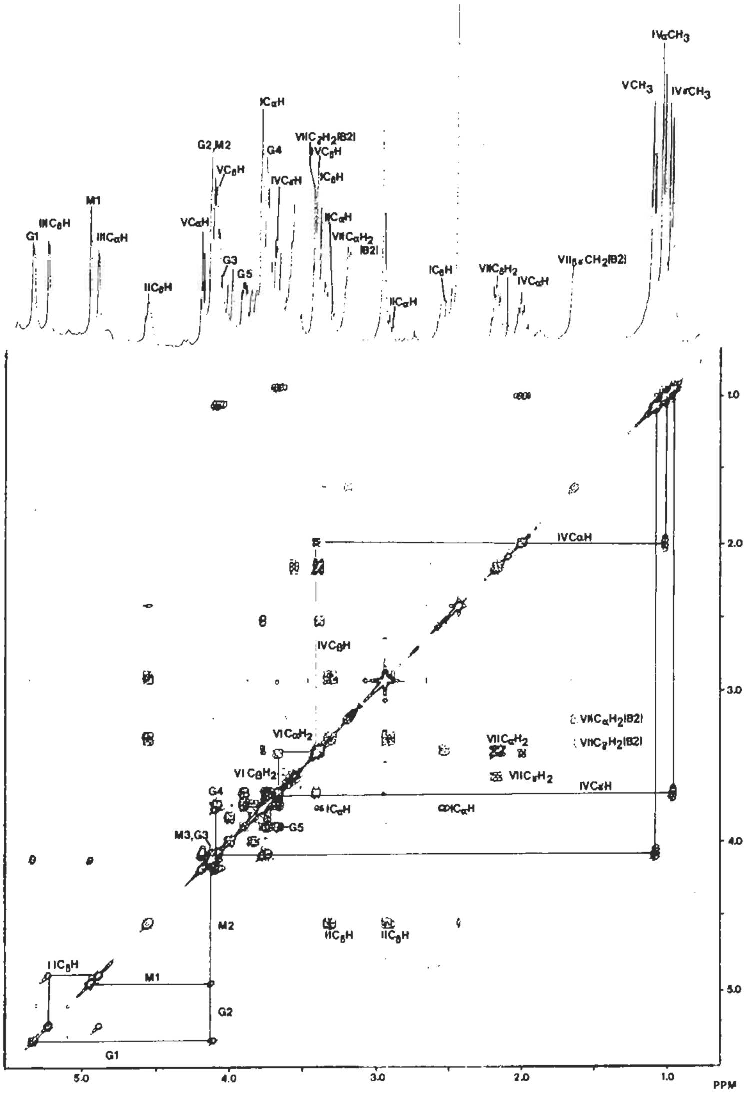

Figure 8: A ^{1}H COSY spectrum of 20 mM Zn(II)-BNX acquired at 360 MHz. In both dimensions the digital resolution was 2.1 Hz, and sine bell multiplication and symmetrization were applied.

with the inter-change of the ALA CαH and PRO CβH carbon resonances.

Based on the previous assignments, the ALA Cα carbon had an anomalously short spin-lattice relaxation time (180 msec compared to 240 msec for the adjacent ALA Cβ carbon), and the PRO Cβ carbon exhibited a surprisingly long T_1 for a branched carbon (232 msec *vs.* 216 msec for the adjacent Cα carbon). The revised assignments reflect the reduction in mobility of the PRO Cβ carbon anticipated for a sterically hindered carbon (T_1 = 180 msec). This relaxation time is comparable to 163 msec, the average relaxation time of the backbone carbon atoms from the central residues of BLEO-A_2, which should exhibit little or no segmental motion. The revised T_1 of

Table II

^{1}H and ^{13} Assignments for the Zn(II)-Bleomycin Complex

	Residue	^{1}H(δ ppm)	^{13}C(δ ppm)
I (ALA)	CO	----	174.52
	CαH	3.80	53.19*
	CβH$_2$	2.55, 3.42	45.67
II (PRO)	RCO	----	177.29
	CβH	4.58	56.82
	CβH$_2$	2.94, 3.35	34.63
	CH$_3$	2.46	11.84
	C2	----	162.34
	C4	----	149.12 or 168.72
	C5	----	114.86
	C6	----	149.12 or 168.72
	SCO	----	170.03
III (HIS)	CO	----	172.68
	CαH	4.90	61.78*
	CβH	5.24	71.32*
	C5	----	136.20
	C4H	7.37	118.86
	C2H	8.09	138.53
IV (VAL)	CO	----	178.33
	αCH$_3$	1.03	12.90*
	CαH	1.99	43.78
	CβH	3.47	75.45*
	γCH$_3$	0.97	15.54
	CγH	3.69	47.97
V (THR)	CO	----	172.90
	CαH	4.19	60.18
	CβH	4.09	68.13*
	βCH$_3$	1.08	20.01
VI (BIT)	CO	----	164.47(A2),164.03(B2)*
	C2	----	163.77(A2),163.56(B2)
	C5H	8.19(A2),8.11(B2)	126.04(A2),125.74(B2)*
	C4	----	149.84(A2),150.08(B2)
	C4′	----	147.97
	C5′H	8.02(A2),7.97(B2)	120.10*
	C2′	----	171.70(A2),171.60(B2)*
	CαH$_2$	3.23	33.08
	CβH$_2$	3.58	40.29
VII (DMS) (A2)	CαH$_2$	3.41	41.95
	CβH$_2$	2.18	24.73
	CγH$_2$	3.62	38.71*
	(CH$_3$)$_2$	2.95	25.74
VII	CαH$_2$	3.23	41.90*
	CβH$_2$	1.68	26.5*
	CγH$_3$	1.68	26.95*

Table II
continued

(AGM)	$C\delta H_2$	3.39	40.09*
(B2)	GN	----	157.82*
	1′	5.28	96.36
	2′	4.13	69.10*
	3′	4.09	67.23*
G	4′	3.79	70.81*
(Gulose)	5′	3.91	68.46*
	6′	3.79	
	6″	3.66	62.35*
	1′	4.94	97.89
	2′	4.13	68.89*
	3′	4.09	76.19*
M	4′	3.76	65.16*
(Mannose)	5′	3.79	75.45*
	6′	4.02	
	6″	3.86	61.96
	CO	----	158.45

Numbers with asterisks refer to ^{13}C chemical shifts which have been either assigned for the first time or reassigned in this paper and reference 18.

the ALA CαH carbon (232 msec) is consistent with an increased extent of segmental motion in aliphatic carbons which approach terminal ends of aliphatic chains.

Assignment of Zinc-Bleomycin

Assignment of the zinc-BNX complexes was accomplished in a similar fashion as for the free antibiotic. The assignment of the proton resonances begins with identification of the unique VAL spin system in the COSY spectrum (Figure 8). The HMBC spectra show a correlation from the central upfield methyl proton (1.03 ppm) resonance to a carbonyl resonating at 178.33 ppm (Figure 7). Since the VAL αCH_3 is the only methyl group with significant multiple bond correlation to a carbonyl, the methyl proton and carbonyl resonances are assigned to VAL αCH_3 and VAL CO respectively. The VAL αCH_3 (1.03 ppm) resonance shows a COSY cross-peak to a multiplet at 1.99 ppm which must be the VAL CαH proton resonance (Figure 8). Inspection of the HMBC (Figure 7) shows a correlation of the VAL CαH hydrogen peak to the VAL CO resonance as expected. The VAL αCH_3 and CαH carbons are assigned to resonances at 12.90 and 43.78 ppm, respectively, from the HMQC spectrum (not shown) and are included in the summary of Zn(II)-BNX chemical shifts in Table II. The VAL CO resonance (178.33 ppm) also shows a multiple bond correlation to a lone doublet at 4.19 ppm and a weak multiple bond correlation to a doublet at 3.47 ppm (Figure 7). These two multiplets originate from the THR CαH and VAL CβH protons, respectively, since the doublet at 4.19 ppm also shows a multiple bond correlation to the resonance at 172.90 ppm, which must derive from the THR carbonyl. The VAL CβH proton resonance (3.47 ppm) should show no HMBC to low-field carbon resonances other than the VAL carbonyl resonance. Since the VAL CβH peaks overlap with several other proton resonances, assignment of the corres-

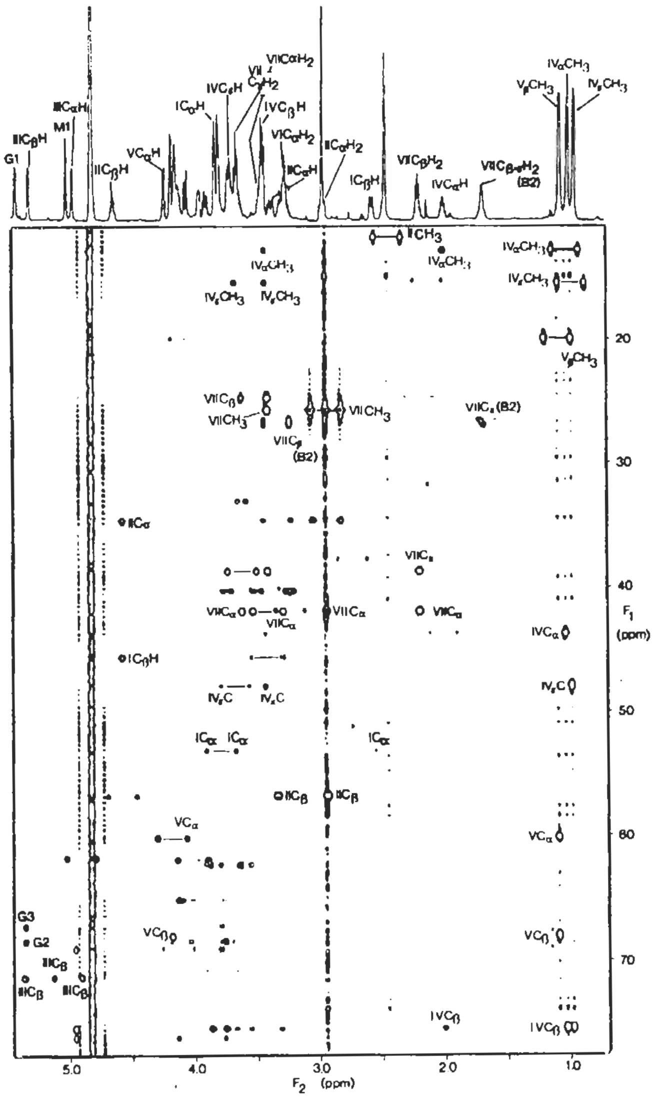

Figure 9: An HMBC spectrum of the aliphatic region of 20 mM Zn(II)-BNX measured under the same conditions as Figure 7. One-bond correlations appear as satellites and are connected by a horizontal line.

ponding carbon resonance requires observation of a multiple bond correlation to the VAL αCH_3, CαH, and γCH_3 proton resonances. The VAL αCH_3 and CαH proton peaks (1.03 and 1.99 ppm, respectively) exhibit multiple bond correlations to a carbon resonance at 75.45 ppm, which is assigned to the VAL CβH carbon (Figure 9). The assignment of the highest field methyl proton resonance (0.97 ppm) to VAL γCH_3 is accomplished on the basis of its strong multiple bond correlation to the VAL CβH carbon resonance at 75.45 ppm. The COSY spectrum then allows assignment of the VAL CγH proton peak at 3.69 ppm (Figure 8). Assignment of the γCH_3 carbon resonance is easily made by referring to the HMQC data (not shown). Multiple bond correlations of the γCH_3 protons (0.97 ppm) to a carbon resonance at 47.97

ppm allows unambiguous assignment of this peak to the VAL CγH carbon (Figure 9). A multiple bond correlation of the αCH_3 proton doublet (1.03 ppm) to the carbon peak at 43.78 ppm (Figure 9) confirms the previous assignment of this peak to the CαH carbon.

The only remaining high-field methyl resonance at 1.08 ppm may be assigned as the THR βCH_3. Its carbon is assigned by one-bond carbon-proton correlation to the peak at 20.01 ppm (which appear as satellites in Figure 9). The previously assigned THR CαH proton peak at 4.19 ppm shows a multiple bond correlation to the THR βCH_3 carbon resonance at 20.01 ppm, and another correlation to the carbon resonance at 68.13 ppm, which must orignate from the THR CβH carbon (Figure 9). The THR βCH_3 proton resonance at 1.08 ppm exhibits an HMBC to the THR CβH carbon resonance (68.13 ppm) and to a peak at 60.18 ppm, which must originate from the THR CαH carbon.

Assignment of resonances of the BIT residue is initiated by searching for multiple bond correlations to the previously assigned THR carbonyl resonance at 172.90 ppm. An HMBC to the proton multiplet at 3.58 ppm is identified with the BIT $C\beta H_2$ protons (Figure 7). Due to overlap of proton resonances, the assignment of the BIT $C\alpha H_2$ proton resonance to the multiplet at 3.23 ppm is made from the COSY spectrum (Figure 8), and HMBC data (see below). Also, the overlap of the BIT proton resonances with other multiplets necessitates assignment of the $C\beta H_2$ carbon by other methods. This is accomplished by first analyzing the aromatic region of the spectrum.

The aromatic region of the proton spectrum of Zn(II)-BNX reveals six well resolved singlets, originating from the HIS and BIT residues. The BIT C5 and C5′ resonances occur as two pairs with approximate relative intensities of 0.7 to 0.3 protons (relative to degenerate BNX resonances), corresponding to the A_2 and B_2 congeners, respectively (Figure 10). The BIT C2 is readily assigned since it is the only carbon expected to exhibit long-range coupling to both C5H and C5′H protons. Such a multiple bond correlation exists at 163.77 ppm, identifying BIT C2 of the BLEO-A_2 (Figure 10). The correlation for the corresponding resonances of BLEO-B_2 occurs at 163.56 ppm. The correlations are stronger for the pair of proton resonances downfield, which are tentatively assigned to the C5H protons of BLEO-A_2 and -B_2 (8.19 and 8.11 ppm, respectively) because stronger correlation is expected for nuclei in the same thiazole ring. The upfield BIT aromatic resonances are therefore assigned to the C5′H proton of BLEO-A_2 at 8.02 ppm, and to the C5′H proton of BLEO-B_2 at 7.97 ppm. These assignments will be confirmed (see below). Since the one-bond proton-carbon correlations in the HMBC spectrum (Figure 10) are incompletely suppressed in this region, the remaining aromatic carbon assignments were made based upon the C5H and C5′H proton assignments (Table II).

The C2′ carbons resonating at 171.70 and 171.60 ppm for the A_2 and B_2 congeners, respectively, were identified by HMBC with the corresponding upfield thiazole proton resonances (tentatively assigned to the C5′H hydrogens) (Figure 10). If the C2′ carbon is correctly assigned, it should exhibit a multiple bond correlation to the aliphatic proton region to the BIT $C\alpha H_2$ proton resonances Indeed, the C2′ carbon shows multiple bond correlations to the BIT $C\beta H_2$ protons at 3.58 ppm and to a multiplet at 3.23 ppm (Figure 7), which must be the BIT $C\alpha H_2$ proton resonance. This confirms

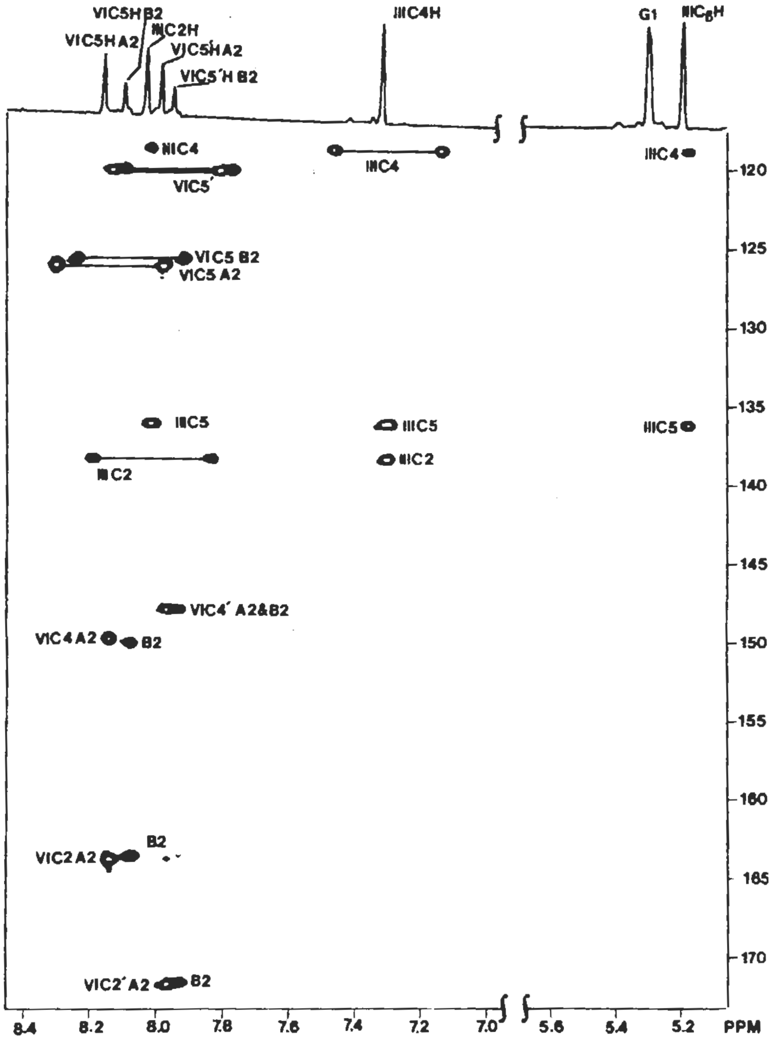

Figure 10: An HMBC spectrum of the aromatic region of 20 mM Zn(II)-BNX measured under the same conditions as Figure 7.

the assignments of all the hydrogen and carbon BIT resonances except for the CβH$_2$ and carbonyl carbons, whose assignments are delineated below.

Assignment of the terminal amines of the BLEO-A$_2$ and -B$_2$ is easily accomplished since they both have characteristic proton resonances. The DMS residue of the A$_2$ congener has a dimethyl singlet at 2.95 ppm (the only six proton singlet). Inspection of the HMBC data allows assignment of the dimethylsulfonium moiety to the carbon resonance at 25.74 ppm, on the basis of the incompletely suppressed satellites (Figure 9). The DMS methyl protons also show a strong HMBC to the carbon resonating at 41.95 ppm, which is assigned to the DMS CαH$_2$ moiety (Figure 9). The proton multiplet at 3.41 ppm is attributed to the DMS CαH$_2$ group on the basis of its multiple bond correlation to the DMS methyl carbon resonance (25.74 ppm) (Figure 9). The use of a similar stepwise correlation of protons and carbons via COSY and HMBC data allows assignment of the remaining DMS resonances (Table II). Since only the DMS $C\gamma H2$ proton peaks (3.62 ppm) overlap with the BIT $C\beta H_2$ resonance (3.58 ppm), and have now been independently assigned, the HMQC unambiguously identifies the BIT $C\beta H_2$ carbon by exclusion.

As mentioned earlier, the AGM residue of BLEO-B_2 has a proton multiplet at 1.68 ppm (readily identified by its absence from the spectrum of BLEO-A_2) originating from the overlapping resonances of the $C\beta H_2$ and $C\gamma H_2$ protons. The COSY spectrum (Figure 8) identifies two methylene resonances at 3.23 and 3.39 ppm which are coupled to this peak and must originate from the $C\alpha H_2$ and $C\delta H_2$ protons, respectively. The 3.25 ppm peak was assigned to the $C\alpha H_2$ protons by observation of the correlation of this resonance to the guanidino amino proton peak in a HOHAHA spectrum of BNX in H_2O (data not shown). The HMBC map (Figure 9) allows presumptive assignment of the $C\beta H_2$ and $C\gamma H_2$ carbons to resonances at 26.50 and 26.95 ppm, respectively, since the $C\delta H_2$ protons at 3.39 ppm show only one multiple bond correlation to the most downfield carbon of the $C\beta H_2$, $C\gamma H_2$ pair. This is based on the assumption that the $C\delta H_2$-$C\gamma$ coupling is greater than the $C\delta H_2$-$C\beta$ coupling. Assignment of the guanidino carbon to the resonance at 157.82 ppm is made by multiple bond correlations from the AGM $C\alpha H_2$ proton resonances at 3.23 ppm (Figure 7). This experiment also allows assignment of the AGM $C\alpha H_2$ carbon to the resonance at 41.90, and removes any ambiguity caused by overlap of the proton resonance of this methylene group with the BIT $C\alpha H_2$ proton resonance at 3.23 ppm. The HMBC experiment reveals multiple bond correlation between the AGM $C\alpha H_2$ protons and the guanidino carbon as well as the partially suppressed correlation to the directly bonded $C\alpha H_2$ carbon (not shown). Correlation between the BIT $C\alpha H_2$ resonances and the directly bonded carbons is also indicated in the HMBC experiment (through partially suppressed single bond correlations; data not shown). Since resonances of the DMS $C\alpha H_2$ (3.41 ppm) and AGM $C\delta H_2$ (3.39 ppm) protons overlap with each other and with one of the ALA $C\beta H_2$ proton resonances, the assignment of the AGM $C\delta H_2$ carbon must be made by exclusion after carbon resonances originating from these other groups have been identified. The DMS $C\alpha H_2$ carbon at 41.95 ppm was assigned from HMBC data, and the ALA $C\beta H_2$ carbon is assigned to a resonance at 45.67 ppm by inspection of the HMQC data (not shown). The correlation between one of the ALA $C\beta H_2$ protons at 2.55 ppm to the ALA $C\alpha H$ carbon at 53.19 ppm is evident in the HMBC spectrum in Figure 9. Assignment of these ALA resonances requires distinguishing between the two AMX proton coupling patterns that originate from the ALA and PRO groups. The basis for distinguishing between these hydrogen resonances is described below. The assignment of the ALA βCH_2 carbon subsequently allows unambiguous assignment of the AGM $C\delta H_2$ carbon to the resonance at 40.09 ppm by exclusion as described above.

The assignment procedure now extends into the HIS residue. Inspection of the HMBC data shows a correlation from the VAL $C\gamma H$ proton resonance at 3.69 ppm to a carbon resonating at 172.68 ppm which must originate from the HIS carbonyl (data not shown). The HIS carbonyl carbon resonance also shows an HMBC to a proton resonating at 4.90 ppm, which must derive from the HIS CαH proton. This proton resonance is correlated to another resonating at 5.24 ppm in the COSY spectrum, allowing its assignment as the HIS CβH hydrogen (Figure 8). The assignments are continued by noting that the HIS CαH proton resonance at 4.90 ppm also shows HMBC to an as yet unidentified carbon resonance at 170.03 ppm, which must originate from the pyrimidine carbonyl (PRO SCO)(data not shown). Both the HIS CαH and CβH proton resonances exhibit multiple bond correlations to a carbon resonating at 136.20 ppm, which can be assigned as the HIS C5 carbon (not shown). This assignment is confirmed by multiple bond correlations of the two HIS aromatic protons (at 8.09 ppm and 7.37 ppm) to the same carbon (Figure 10). The HIS CβH resonance at 5.24 ppm also shows an HMBC to a carbon resonance at 118.86 ppm, which also corresponds to the ^{13}C satellites of the upfield aromatic resonance (Figure 10). This carbon resonance is assigned to the HIS C4, which now allows assignment of the proton resonance at 7.37 ppm to HIS C4H. The HIS C2H carbon at 138.53 ppm exhibits an HMBC to the HIS C4H at 7.37 ppm, and a one-bond correlation to the HIS C2H proton resonance at 8.09 ppm.

Assignment of the pyrimidine propionamide moiety (PRO) proceeds from identification of the PRO CH_3 proton resonance (2.46 ppm) with ^{13}C satellites at 11.84 ppm (Figure 9). Identification of the aromatic C4, C5, and C6 carbons is made by observation of multiple bond correlations from the PRO CH_3 proton resonance at 2.46 ppm to resonances at 114.86 (not shown), 149.12 (not shown), and 168.72 ppm (Figure 7); however, specific assignments cannot be made on the basis of single or multiple bond correlations. As in the study of Akkerman et al. (18) these resonances have been assigned on the basis of the model compound data of Naganawa et al. (4).

Except for the disaccharide resonances, the only unassigned proton resonances in the COSY spectrum are the two AMX systems originating from the PRO and ALA CH-CH_2 protons. The methylene carbon resonances at 34.63 and 45.67 ppm, are easily identified in each system on the basis of mutual correlation to the same methine proton resonance at 4.58 ppm in the HMBC spectrum (Figure 9). The corresponding CH carbon resonances are also easily identified via HMQC and HMBC spectra. Assignment of the proton and carbon resonances to the correct residue requires long-range correlation of the PRO CαH proton resonance to the aromatic PRO C2 carbon resonance. The PRO C2 carbon resonance at 162.34 ppm was assigned on the basis of an HMBC to the proton resonating at 4.58 ppm, which has been previously identified as the PRO CβH proton and multiple bond correlations to the PRO CαH_2 protons at 2.94 and 3.35 ppm (Figure 7). This permits complete assignment of the PRO and ALA systems. The PRO RCO at 177.29 ppm and ALA CO at 174.52 ppm are both easily assigned by HMBC to the CH_2 proton resonances of their respective residues (cf., Figure 7).

The assignment of the disaccharide moiety starts with the identification of the anomeric proton resonances at 5.28 and 4.94 ppm for the gulose (G) and mannose (M) residues, respectively. The HMBC spectrum of this region (not shown) confirms these assignments as well as the respective ^{13}C assignments. An HMBC from the GH1′ carbon resonance to the CβH proton resonance of HIS at 5.24 ppm (data not shown) unambiguously identifies the anomeric carbon of gulose. This carbon resonance is correlated to the 5.28 ppm proton resonance in the HMBC spectrum (data not shown). The ^{13}C assignments of the disaccharide are facilitated by the analysis of the ^1H-^1H correlations. The homonuclear COSY indicates that the correlation pattern for free BNX (Figure 2) is similar to the correlation pattern for Zn(II)-BNX (Figure 8) with several exceptions. The G1′ proton resonance at 5.28 ppm is completely resolved in the case of Zn(II)-BNX, and the relative positions of the M1′ at and HIS CβH proton resonances are reversed in the case of the Zn(II)-BNX complexes (cf., Figures 2 and 8). There is also a substantial shift in the M3 proton resonance (4.65 ppm in free BNX to 4.09 ppm in Zn(II)-BNX); this is also reflected in the ^{13}C spectrum (75.42 to 76.19 ppm, respectively; data not shown). The remaining disaccharide resonances are assigned by the same methods for free BNX and are summarized in Table II.

Conclusions

This completes the assignment of ^1H and ^{13}C resonances of both free BNX and the Zn(II)-BNX complexes. With the exception of the PRO C4, C5, and C6 carbon assignments, all the assignments are based on through-bond connectivities demonstrated by appropriate two-dimensional NMR experiments. The assignment procedures described in this paper eliminate the ambiguities associated with ^{13}C assignments based on arbitrary assumptions about ^1H and ^{13}C complex formation shifts. Our findings confirm the assignments of Akkerman et al. (18) by similar but not identical methods. The data reported in this paper will be of use in the studies of BLEO interactions with nucleic acids and with other metals.

Acknowledgments

We thank Rolf Tschudin for technical support and Dr. William Bradner of Bristol-Myers Company for the generous gift of Blenoxane. This research was supported by NIH Grant CA39958 to J.D.G.

References

1. Hecht, S.M., In *Bleomycin: Chemical, Biochemical, and Biological Aspects*, Springer-Verlag, New York (1979).
2. Oppenheimer, N.J., Rodriguez, L.O. and Hecht, S.M., *Biochemistry 18*, 3439-3445 (1979).
3. Oppenheimer, N.J., Rodriguez, L.O. and Hecht, S.M., *Proc. Natl. Acad. Sci. USA 76*, 5616-5620 (1979).
4. Naganawa, H., Muraoka, Y., Takita, T. and Umezawa, H., *J. Antibiot. 30*, 388-396 (1977).
5. Dabrowiak, J.C., Greenaway, F.T. and Grulich, R., *Biochemistry 17*, 4090-4096 (1978).
6. Pillai, R.P., Lenkinski, R.E., Sakai, T.T., Geckle, J.M., Krishna, N.R. and Glickson, J.D., *Biochem. Biophys. Res. Commun. 96*, 341-349 (1980).
7. Chien, M., Grollman, A.P. and Horwitz, S.B., *Biochemistry 16*, 3641-3647 (1977).
8. Chen, D.M., Sakai, T.T., Glickson, J.D. and Patel, D.J., *Biochem. Biophys. Res. Commun. 92*, 197-205 (1980).
9. Pillai, R.P., Krishna, N.R., Sakai, T.T. and Glickson, J.D. *Biochem. Biophys. Res. Commun. 97*, 220-278 (1980).
10. Glickson, J.D., Pillai, R. and Sakai, T.T., *Proc. Natl. Acad. Sci. USA 78*, 2967-2971 (1981).
11. Booth, T.E., Sakai, T.T. and Glickson, J.D., *Biochemistry 22*, 4211-4217 (1983).

12. Krishna, N.R., Dallas, J.L., Mooberry, E.S., Sakai, T.T., Allen, R.C., Levy, G.C. and Glickson, J.D., *Biochem. Biophys. Res. Commun. 85*, 363-370 (1978).
13. Haasnoot, C.A., Pandit, U.K., Kruk, C. and Hilbers, C.W., *J. Biomol. Struct. Dyn. 2*, 449-467 (1984).
14. Akkerman, M.A.J., Haasnoot, C.A.G. and Hilbers, C.W., *Eur. J. Biochem. 173*, 211-225 (1988).
15. Chen, D.M., Hawkins, B.L. and Glickson, J.D. (1977) *Biochemistry 16*, 2731-2738.
16. Mooberry, E.S., Dallas, J.L., Sakai, T.T. and Glickson, J.D., *Int. J. Peptide Res. 15*, 365-376 (1980).
17. Davis, D.G. and Bax, A., *J. Am. Chem. Soc. 107*, 2820-2821 (1985).
18. Akkerman, M.A.J., Haasnoot, C.A.G., Pandit, U.K., and Hilbers, C.W., *Magn. Reson. Chem. 26*, 793-802 (1988).
19. Cryomagnet Systems Inc., 4101 Cashard Ave., Indianapolis, IN 46203.
20. Marion, D. and Bax, A., *J. Magn. Reson. 80*, 528-533 (1988).
21. Bax, A. and Subramanian, S., *J. Magn. Reson. 67*, 565-569 (1986).
22. Bax, A. and Summers, M.F., *J. Amer. Chem. Soc. 108*, 2093-2094 (1986).
23. Bax, A. and Marion, D., *J. Magn. Reson. 78*, 186-191 (1988).
24. Mehlkopf, A.F., Korbee, D. and Tiggelman, T.A., *J. Magn. Reson. 58*, 315-323 (1984).
25. States, D.J., Haberkorn, R.A. and Ruben, D.J. (1982) *J. Magn. Reson. 48*, 286-292.
26. Marion, D. and Wuthrich, K., *Biochem. Biophys. Res. Commun. 113*, 967-974 (1983).
27. Stothers, J.B., *Carbon-13 NMR Spectroscopy*, pp. 144-155, Academic Press, New York (1972).
28. Deslauriers, R. and Smith, I.C.P., In *Topics in Carbon-13 NMR Spectroscopy*, pp. 1-80, Wiley & Sons, New York (1976).
29. Maciel, G.E. and Dorn, H.C., *J. Magn. Reson. 24*, 251-258 (1976).
30. Rabenstein, D.L. and Sayer, T.L., *J. Magn. Reson. 24*, 27-39 (1976).

Date Received: June 12, 1990

Communicated by the Editor R.H. Sarma