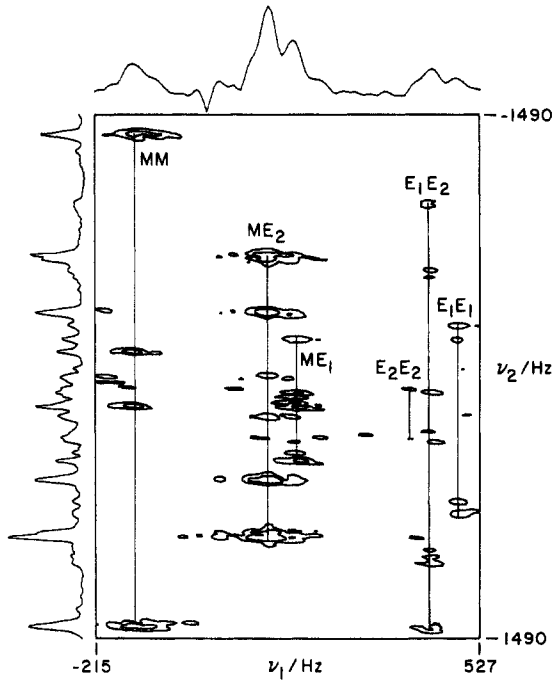

Figure 2. Part of a double-quantum vs. single-quantum spectrum of the same sample as in Figure 1, obtained by using the pulse sequence described in the text. Deuterium-decoupled FIDs, 128 × 1024 points, were recorded on a 360-MHz spectrometer with quadrature phase detection in both dimensions and a recycle delay of 5 s. The spectral width was 3.3 kHz in ν_1 and 7.35 kHz in ν_2. τ_1 was 250 μs and τ_2 4 ms. Vertical lines parallel to the single-quantum axis illustrate the six double-quantum frequencies of molecules with two protons along which the A_2- and AB-type spectra can be identified.

phase between 0 and 180°. The spin-echo delay τ_2 eliminates any contribution to the signal from the liquid crystal. The double-quantum filter time τ_1 is varied to average out all double-quantum coherences not refocused at the end of τ_1. The spectrum effectively contains only signals from two proton isotopomers, since signals from one and three proton isotopomers are eliminated by the two-quantum filter. Four or more proton molecules do not contribute due to a combination of low statistical probability of occurrence and a wide range of isomers. The COSY-type spectrum thus has 16 subspectra of the type A_2 or AB. Signals in the same spin system can easily be identified by off-diagonal correlations which form square patterns with diagonal peaks. All 16 dipole couplings could be read off this map and from a similar experiment in which a π pulse was applied in t_1. The dipole coupling constants thus determined are listed in Table I, together with a site assignment based on chemical shift.

The same information was corroborated in a second 2D-INADEQUATE-type[12] experiment. The pulse sequence used was

$$(\pi/2)_\phi - \tau_1/2 - \pi_\phi - \tau_1/2 - (\pi/2)_\phi - t_1 - (\pi/2)_x - \tau_2/2 - \pi_x - \tau_2/2 - \text{sample}$$

where ϕ is incremented by 90° while the receiver oscillates between 0 and 180°. Here two- and one-quantum signals were correlated in a two-dimensional map as shown in Figure 2. Six vertical lines were produced parallel to the one-quantum axis, corresponding to the six possible double-quantum frequencies

$$2\nu_M, \quad \nu_M + \nu_{E_1}, \quad \nu_M + \nu_{E_2}, \quad 2\nu_{E_1}, \quad 2\nu_{E_2}, \quad \nu_{E_1} + \nu_{E_2}$$

(The notation used is M = methyl protons, E_1 = C-2 methylene protons, E_2 = C-3 methylene protons.) The six slices along the one-quantum axis each contain A_2 or AB subspectra corresponding to their group type; thus there are two MM, two ME_1, two ME_2, three E_1E_1 (two shown), three E_2E_2 (one shown), and four E_1E_2 (three shown) subspectra. Subspectra are easily identified by their symmetric disposition around the central chemical shift position in ω_2. Spin systems with more than two protons in general produce two-quantum spectra lying outside the chemical-shift range, since they are dominated by dipole couplings. They may have inner lines of low intensity, which will not correspond, except by accident, to any of the six double-quantum frequencies. Different two-quantum preparation times result in different relative intensities of the subspectra. Table I includes a list of dipole coupling constants obtained with two preparation times, 250 μs and 2.5 ms. The values of D_{ij} from the COSY-type and INADEQUATE-type experiments agree rather well. It remains to assign the couplings constants to specific pairs of protons on the molecule. These couplings can be used to test various theoretical models of conformational motions for hydrocarbon chains in anisotropic environments.[13-15] The fact that D_{ij}'s for a molecule with 14 protons can be determined bodes well for the application of two-dimensional and multiple-quantum NMR to structure and motions of oriented molecules.

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Assignment of Secondary Amide ^{15}N Resonances of Bleomycin A_2 by Two-Dimensional Multiple-Quantum ^1H-^{15}N Shift-Correlation NMR Spectroscopy

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As part of our program to delineate the solution conformation of metal and nucleic acid complexes of the bleomycins (Bleo),[1] Figure 1a, we have assigned the ^{15}N NMR resonances of Bleo A_2, the most abundant congener of these antineoplastic antibiotics.[2,3] Because isotopic enrichment of this antibiotic cannot readily be achieved, our experiments were performed at natural abundance in aqueous solution by using the recently introduced two-dimensional multiple-quantum ^1H-^{15}N shift-correlation NMR spectroscopic method.[4-10] This method is several orders of

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(1) The abbreviations used: Bleo, bleomycin; NMR, nuclear magnetic resonance; DMS, (3-aminopropyl)dimethylsulfonium salt; BIT, 2'-(2-aminoethyl)-2,4'-bithiazole-4-carboxylic acid; Thr, L-threonine; Val, 4-amino-3-hydroxy-2-methyl-*n*-valeric acid; His, L-*erythro*-β-hydroxyhistidine.

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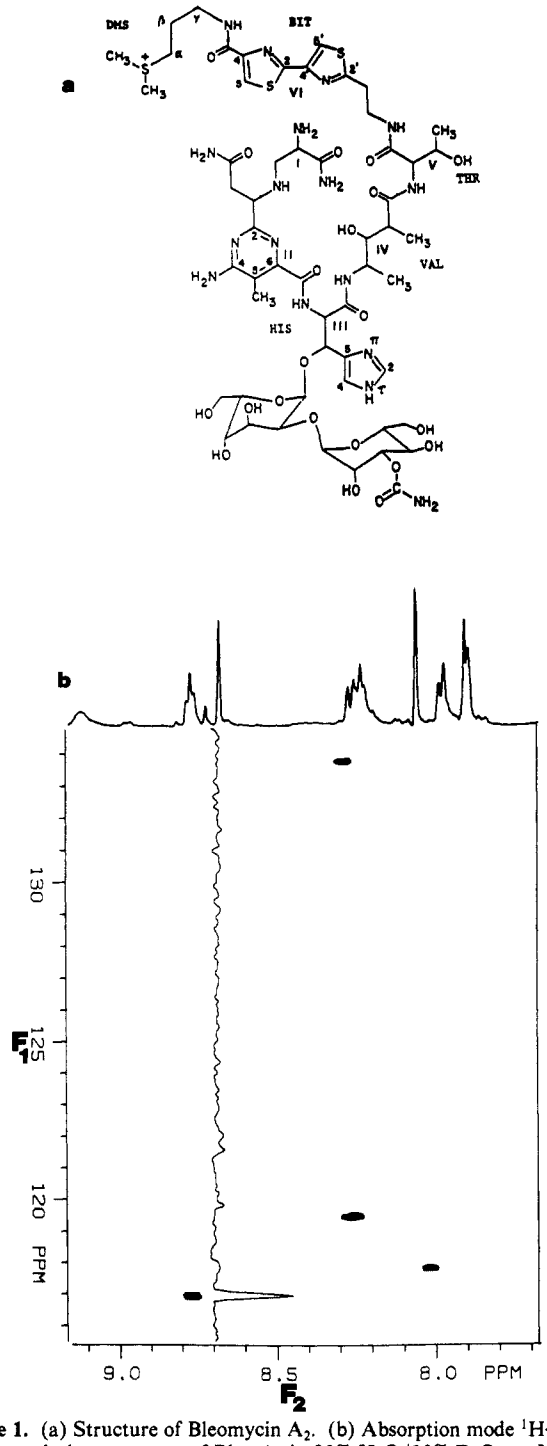

Figure 1. (a) Structure of Bleomycin A_2. (b) Absorption mode ^1H–^{15}N shift-correlation spectrum of Bleo A_2 in 80% H_2O/20% D_2O at pH 3.9 and at 20 °C. Amide region of the regular ^1H spectrum is shown along the F_2 axis of the 2D spectrum. The inset shows a F_1 cross section taken at the F_2 frequency of the DMS amide proton and displays both the high sensitivity of the data and the excellent suppression of signals from protons attached to ^{14}N.

magnitude more sensitive than ^{15}N NMR and yields definitive resonance assignments of protonated nitrogens.

Bleomycin A_2, a generous gift from Dr. Ted Sakai, was isolated from Blenoxane (Bristol Laboratories, Syracuse, NY) by ion-exchange chromatography with triethylammonium bicarbonate[11] and was converted to the chloride salt by addition of hydrochloric acid. The NMR experiments were performed on a Nicolet-500 spectrometer using a Cryomagnet Systems probe equipped with a broad-band (^{15}N to ^{31}P) decoupling coil outside the ^1H observe coil.[12] The study was conducted using a 5-mm sample tube with a 70 mM solution of Bleo A_2 in 80% H_2O/20% D_2O at pH 3.9. The ^{15}N 90° pulse width was 210 μs. For every t_1 value, 128 scans were recorded with 1-s recycling time. The experiment was repeated for 180 t_1 increments of 300 μs each, resulting in a total data accumulation time of 6.5 h. The ^1H rf carrier was positioned at 8.40 ppm, and a weak rf field (460 Hz) was employed. The 90° and 180° pulse widths of 545 and 1090 μs corresponded to a null at the position of the H_2O resonance. Because the 180° pulse affects the amide protons but not the Cα protons (which resonate near the H_2O resonance), modulations due to homonuclear J coupling did not occur and a purely absorptive 2D spectrum could be recorded.[13] Griffey et al.[7] used a 90°–τ–90° sequence for spin inversion with τ adjusted to minimize excitation of water instead of a single long 180° pulse used in the present experiment. The advantage of this 1–1 sequence is that an approximately twice as wide ^1H region can be covered; a disadvantage, at least on our spectrometer, is less effective suppression of protons coupled to ^{14}N. Data for odd- and even-numbered scans were stored in separate locations and processed in the standard manner[14-16] to yield a 2D absorption mode spectrum. To improve the sensitivity by a factor of 2, ^{15}N decoupling was employed during data acquisition in the t_1 dimension. Provided adequate rf bandpass filters are used between the probe and the ^{15}N power amplifier, ^{15}N decoupling does not affect the suppression of signals from protons not coupled to ^{15}N.

Table I. Assignment of Secondary Amide Resonances of Bleomycin A_2 by the Double-Quantum ^1H–^{15}N Shift-Correlation Technique[a]

assignments	^{15}N chem shifts,[b] ppm	^1H chem shifts,[c] ppm
Thr	117.9	8.01
BIT	119.4	8.25
Val	133.8	8.29
DMS	116.9	8.77
His[d]	120.0	9.14

[a] In 80% H_2O/20% D_2O at pH. 3.9 and at 20 °C. [b] ^{15}N Chemical shifts are reported with reference to external NH_3. [c] ^1H Chemical shifts are with respect to external TSP. [d] At pH 1.24 and at 30 °C.

Figure 1b shows the multiple-quantum natural-abundance ^1H–^{15}N shift-correlation spectrum of Bleo A_2. The ^{15}N assignments directly follow from the previously determined chemical shifts of amide ^1H resonances.[17,18] The ^{15}N chemical shifts of all five secondary amide resonances are summarized in Table I. The His amide resonance is not observed in this spectrum due to exchange broadening of the resonance of the directly bonded proton at this pH (3.9).[17] The base-catalyzed exchange rate of this proton is enhanced because of proximity to nearby cationic and electron-withdrawing groups. Since the chemical shifts of the Val and BIT N*H* resonances are not assigned unambiguously at pH 3.9, distinction of the corresponding ^{15}N resonances is not possible. Exchange of the His amide N*H* is slow on the ^1H chemical shift time scale at pH 1.24, and the Val and BIT amide

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NH resonances have been assigned at this pH.[17] Performance of the multiple-quantum correlation experiment at this pH allows completion of the amide ^{15}N assignments.

Our study demonstrates that all the amide ^{15}N resonances of Bleo can be detected and assigned in aqueous solution at natural abundance within a reasonable amount of time by the two-dimensional multiple-quantum method. We are currently employing this method to characterize complexes of this antibiotic with metals and nucleic acids.

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Assignment of Proton Amide Resonances of T4 Lysozyme by ^{13}C and ^{15}N Multiple Isotopic Labeling

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The unambiguous resolution and assignment of resonances from specific protons is the major limitation in ^1H NMR studies of proteins.[1] Heteronuclear double-resonance spectroscopy of samples labeled with stable isotopes such as ^{13}C and ^{15}N offers one solution to the problem.[2-4] This methodology has been used to identify the signals from specific imino protons in transfer RNA and amide protons in peptides. When introduced into proteins, a heteroatomic label can be used to edit a complex proton NMR spectrum into a subset of resonances from a particular functional group.[5,6] We have observed the peaks from the amide protons of the five phenylalanines in T4 lysozyme labeled with (^{15}N)-phenylalanine but could not assign the signals to specific amino acids based solely on the chemical shifts.[5,7]

We now demonstrate a general method which permits the observation and assignment of the ^1H, ^{13}C, and ^{15}N signals from any amide unit. It is possible to uniquely ^{13}C–^{15}N co-label specific peptide bonds of the sequence AB by biosynthetically incorporating a (^{13}C)carbonyl-labeled amino acid A and an (^{15}N)amino-labeled amino acid B into a protein.[8] Often, only one such sequence will occur in the protein. The assignments of the doubly labeled peptide

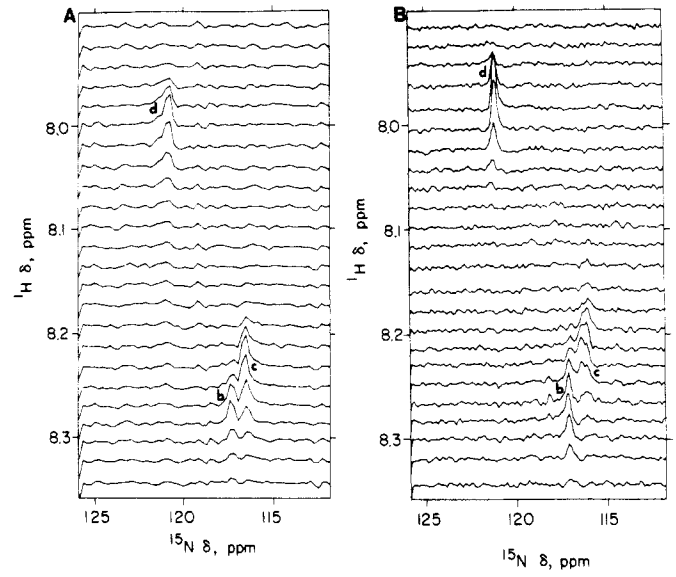

Figure 1. Forbidden echo map[5] of the correlated ^1H and ^{15}N chemical shifts for (A) (^{15}N)phenylalanine-enriched T4 lysozyme and (B) (1-^{13}C)leucine/(^{15}N)phenylalanine T4 lysozyme. Samples contained 30–40 mg of protein mL^{-1} in 100 mM sodium phosphate buffer, pH 6.5, with 500 mM NaCl, 1 mM $MgSO_4$, 1 mM 2-mercaptoethanol, and 10% deuterium oxide. The spectra were recorded with a 500-MHz spectrometer equipped with a probe (Cryomagnetics, Inc., Indianapolis, IN) tuned to both ^1H and ^{15}N. The jump–return pulse sequence was used for selective 90° and 180° pulses.[10] A total of 832 and 800 scans, respectively, were acquired for 128 values of t_1. The values of t_1 were incremented in 600- and 1200-μs steps, giving ^{15}N sweep widths of 1666 and 833 Hz, respectively. Sets of 1000 × 128 points were transformed and the section of the maps near 8.3 ppm (^1H) are presented. The preparation periods were 3.5 ms. The temperature was maintained at 15 ± 1 °C for both spectra.

Table I. Correlated Proton and Nitrogen Chemical Shifts with Proton Relaxation Times for Phenylalanine Amide Protons in T4 Lysozyme

amide	peak[a]	exchange kinetics[b]	^1H, ppm[c]	^{15}N, ppm[d]	^1H T_1, ms
Val 103–Phe 104	a	slow	9.35	121.2	300 ± 30
Thr 152–Phe 153	b	slow	8.29	117.3	290 ± 40[e]
Leu 66–Phe 67	c	slow	8.27	116.3	290 ± 40[e]
Gly 113–Phe 114	d	fast	8.07	121.1	290 ± 30
Ile 3–Phe 4	e	fast	7.74	120.4	420 ± 50

[a] The peak designation is that from ref 5. [b] The H/D exchange kinetics were measured at 4 °C and pD 5.5. Fast refers to exchange half-times of hours and slow refers to times of weeks.[5] [c] ±0.02 ppm. [d] ±0.2 ppm; referenced to ammonia at 25 °C. [e] The T_1 relaxation times could not be distinguished clearly for these overlapping signals.

^1H and ^{15}N resonances are accomplished by observation of the ^{13}C–^{15}N scalar coupling using detection of proton resonances by ^1H–^{15}N forbidden echo spectroscopy.[5,7,9] Assignment of the ^{13}C resonance can be accomplished by direct observation of the ^{13}C–^{15}N scalar coupling in the ^{13}C NMR spectrum of the same labeled protein.

We have applied this approach to three (^{15}N)phenylalanine-labeled T4 lysozyme samples which also contained (^{13}C)-carbonyl-labeled leucine, valine, or glycine. This permitted us to unambiguously assign the signals from three of the five phenylalanine amide protons. We have assigned the remaining two peptide ^1H–^{15}N resonances on the basis of their hydrogen exchange properties.

Samples of T4 lysozyme containing the ^{15}N–^{13}C double label were produced from a high expression plasmid in a derivative of the *E. coli* strain RR1 auxotropic for phenylalanine (PheA), leucine, and valine (IlvC) grown on a defined medium including

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(1) Abreviations: NMR, nuclear magnetic resonance; 2D, two dimensional.

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