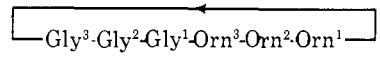
Nitrogen-15 Nuclear Magnetic Resonance Spectrum of Alumichrome. Detection by a Double Resonance Fourier Transform Technique[1]

Miguel Llinás,* William J. Horsley, and Melvin P. Klein

Contribution from the Laboratory of Chemical Biodynamics, Lawrence Berkeley Laboratory, University of California, Berkeley, California 94720. *Received April 16, 1976*

Abstract: Alumichrome, the Al^{3+} analogue of ferrichrome, a ferric cyclohexapeptide, was enriched in ^{15}N by growing the fungus *Ustilago sphaerogena* in a medium containing 99.5% ^{15}N-ammonium acetate as the sole nitrogen source. The magnitude of the amide proton–nitrogen scalar coupling constant, obtained directly from the ^1H NMR spectrum is correlated with the angular deviation from planarity at the peptide bond. The ^{15}N amide resonances were measured indirectly by heteronuclear double resonance methods. The amide ^1H NMR spectrum was obtained by Fourier transform at 220 MHz while the ^{15}N resonances were irradiated selectively with low power near 22.3 MHz. The chemical shifts of the amide ^{15}N resonances are sensitive to conformation and the responses to temperature and solvent changes provide excellent criteria for determining the spatial configuration of each –NH–CO– group within the peptide structure. An extension of the Fourier transform double resonance method, Fourier internuclear difference spectroscopy (FINDS), was tested successfully on the amide region of the unenriched peptide thus permitting detection of ^{15}N resonances at natural abundance. The results suggest a large potential for the method.

Recent direct detection of ^{15}N nuclear magnetic resonance (NMR)[2] signals in peptides[3] and proteins[4] has shown the feasibility of the technique for the study of biopolymers. Indeed, it is to be expected that in conjunction with the well established ^1H and ^{13}C NMR spectroscopies, ^{15}N studies ought to help refine the analysis of plausible solution conformations. It would thus be desirable to reach a better understanding of the features that contribute to determine the ^{15}N NMR spectrum so that the potential of the technique for the study of polypeptide structures can be more fully appreciated. For any such study, assignment of the resonances appears as a mandatory requirement so that, e.g., chemical shifts and coupling constants can be discussed in terms of known molecular geometries at particular sites. In this sense, the alumichromes (Figure 1) provide a most useful set of model compounds because their common conformation,[5] well characterized by x-ray crystallography,[6] is rigidly maintained in solution.[7] Alumichrome is the Al^{3+} analogue of ferrichrome, a ferric cyclohexapeptide whose primary sequence is



where Gly stands for glycyl and Orn for δ-*N*-acetylhydroxyornithyl residues.

The amide ^1H–^{15}N scalar couplings (J^1) are ca. 90 Hz.[8] It is thus possible to obtain a ^{15}N NMR spectrum by observing those frequencies in the ^{15}N domain which bring about a collapse of the ^{15}N splittings in the proton spectrum.[8a,9] This can be achieved most elegantly and conveniently by Fourier spectroscopy in the proton domain while simultaneously irradiating the ^{15}N resonances under low level, selective conditions. This technique is related to recent FT-INDOR experiments designed to detect ^1H–^1H and ^1H–^{13}C indirectly coupled resonances.[10]

We present a study of polypeptide amide ^{15}N NMR spectroscopy of ^{15}N-alumichrome using this Fourier double resonance technique. We also report an extension of the method devised to detect the ^{15}N resonances at natural abundance by Fourier internuclear difference spectroscopy (FINDS).

Materials and Methods

^{15}N-Ferrichrome was obtained from the growth medium of the smut fungus *Ustilago sphaerogena* as described by Garibaldi and Neilands[11] except that ^{15}N-ammonium acetate (99.2% enrichment, Analytical Supplies Development Corp., Little Falls, N.J.) was used as the sole nitrogen source. Isotopically enriched inocula (20 ml) were used to start growth of each of two 3-l. Fernbach flasks containing a total of 1.28 l. of medium. After processing the supernatant as described by Neilands,[11,12] the crude extract was fractionated in a short silica gel column.[13] In this method the peptide mixture is eluted with water-saturated organic solvents of increasing polarity (starts with 100% chloroform followed by increasing concentration steps (5% v/v) of ethanol). In our case the organic phase was equilibrated with an equal volume of water. Ferrichrome elutes in the range 80–65% chloroform:ethanol while the analogue ferrichrome A elutes in the range 60–50% of the solvent mixture. The method yields a most satisfactory resolution of the two ferric peptides as well as excellent purification. The two colored bands were identified by paper electrophoresis at pH 6.9, based on the fact that the net charge of ferrichrome is zero while ferrichrome A is, at neutrality, a 3− anion.

The paramagnetic Fe^{3+} ion was substituted by diamagnetic Al^{3+} as already described,[14] and the aluminum peptide (^{15}N-alumichrome) was further purified by gel filtration with distilled water through a 2.5 × 100 cm column packed with a mixture of 75% Bio-Gel P2 with 25% Sephadex G10; we have observed that adding some Sephadex to the Bio-Gel stabilizes the packing properties of the latter. A final yield of 100 mg of ^{15}N-alumichrome per liter of culture medium was obtained. The final product was reextracted twice with 40 fold excess of 8-hydroxyquinoline to eliminate any residual trace of Fe^{3+}; in the process some of the Al^{3+} was lost, which gave a weak background in the ^1H NMR spectrum due to the demetallopeptide. The NMR data confirmed the identity and purity of the peptide. Commercial, spectroscopic grade solvents were used.

The experiments reported here were performed on a Varian HR-220 spectrometer extensively modified in this laboratory for Fourier and double resonance operation. Both the ^1H and ^{15}N frequencies were derived from frequency synthesizers. The 22.3 MHz power was supplied to the doubly tuned probe by a commercial amplifier. The decoupling frequency was switched from far off-resonance to on-resonance in comparing undecoupled with decoupled spectra to minimize effects due to radiofrequency heating of the system.

* Address correspondence to this author at the Department of Chemistry, Carnegie-Mellon University, Pittsburgh, Pa. 15213.

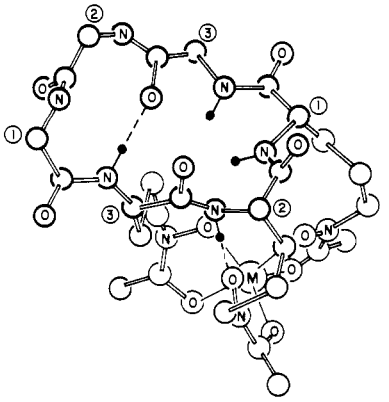


Figure 1. Conformational model of ferrichrome as determined by x-ray crystallography[6] and solution studies by NMR.[5,7] The peptide backbone is denoted by heavier lines. Intramolecular H bonds of significance are depicted by dotted lines. The metal atom, M, coordinated by the three ornithyl side chains, is represented by a larger sphere. Glycyl and ornithyl residues are numbered according to previous convention.[5-7]

Results and Discussion

Figure 2 shows the ^{1}H spectrum of the amide region of (A) natural abundance ^{14}N-alumichrome and (B) of the 99.5% ^{15}N-enriched peptide dissolved in DMSO-d_6. The aliphatic region of the spectrum (not shown) is little affected by the isotopic substitution, suggesting that the geminal and vicinal ^{1}H–^{15}N scalar couplings are small, in agreement with observations reported by other autors.[15] The J^1_{NH} couplings in ^{15}N-alumichrome are between 90 and 95 Hz. By reference to a previous identification of the amide proton resonances,[7b,14] the large doublets exhibited by the ^{15}N peptide are easily assignable to the corresponding residues (Figure 1) as indicated at the bottom of Figure 2.

Low power decoupling is manifest by a gradual convergence of the pair of resonances within each doublet to the central (i.e., the ^{14}N–^{1}H) position as the ^{15}N frequency approaches the proper resonance value, aside from any isotope shift. This allows a determination of the decoupling conditions[16] which were satisfied with $\gamma H_2/2\pi$ = 0.1263 Hz and was kept constant throughout the experiment. The ^{15}N resonance frequency was identified according to a best symmetry and collapse of the two double components as judged by maximal signal amplitude and optimal resolution of the $J^3_{H_NH_{C_\alpha}}$ multiplet structure. The spectrum shown in Figure 2C depicts the appearance of the ^{15}N–^{1}H amide FT ^{1}H NMR upon continuous wave irradiation of the ^{15}N nuclei at 22 294 938 Hz. This frequency corresponds to selective ^{15}N decoupling of the Orn^3 NH proton. The other ^{15}N–^{1}H couplings are affected according to the proximity of their ^{15}N frequencies to that of Orn^3 resulting from off-resonance decoupling. Compare, e.g., the Orn^1, Orn^2, and Orn^3 multiplets in Figure 2B with those in Figure 2C. The ^{15}N frequencies determined by this method are accurate to within ±1 Hz.

The mapped ^{15}n nmr spectrum of the alumichrome amide region, in DMso d_6, is diagrammed in Figure 3 which also shows the effect of the J^1_{NH} couplings on the nitrogen lines as derived from the directly measured splittings of the proton resonances (Figure 2B). The resonance frequencies ($f^{15N}_{TMS=220}$) are given by reference to internal TMS and correspond to the static magnetic field at which the TMS protons resonate at exactly 220 MHz, i.e., δ (TMS) ≡ 0. The ^{15}N frequency values are calculated by multiplying 220 MHz by the experimental Γ, where $\Gamma \equiv (^{15}\text{N freq})/(^1H_{TMS}\text{ freq})$ is a parameter independent of field strength. For convenience the chemical shifts in Figure 3 are also referred, in ppm, to the nitrogen frequency of ^{15}N-urea dissolved in DMSO-d_6. Having a ^{15}N standard is advantageous in that it permits locating the

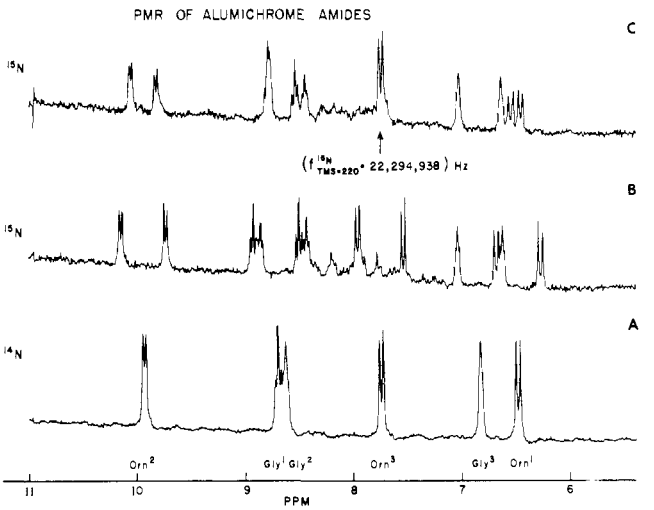


Figure 2. Fourier transform ^{1}H NMR spectra of the alumichrome amide region at 220 MHz. The peptides were dissolved in DMSO-d_6 and the spectra, recorded at ~70 °C, are referred to internal TMS. (A) Natural abundance ^{14}N-alumichrome, (B) 99.5% ^{15}N-enriched alumichrome. (C) same as (B) except that simultaneous continuous wave irradiation was applied at 22 294 938 Hz; the latter frequency ($f^{15N}_{TMS=220}$) corresponds to the Orn^3 ^{15}N resonance in a static magnetic field where the TMS ^{1}H NMR occurs at exactly 220 MHz (see text).

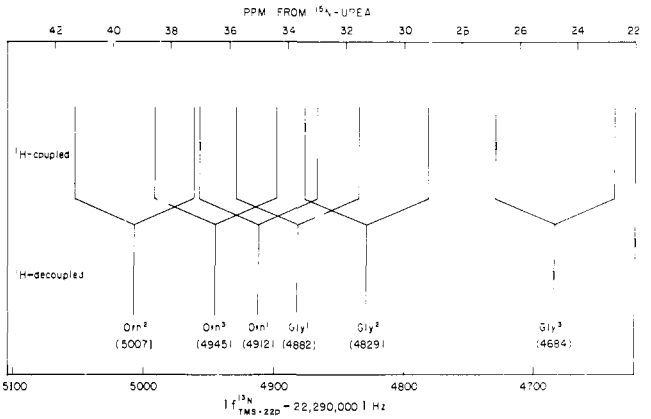


Figure 3. The ^{15}N NMR spectrum of alumichrome at 22.3 MHz. The ^{15}N peptide was dissolved in DMSO-d_6 and the spectrum, taken at 19 °C, was mapped by DR-FT techniques as indicated in Figure 2C. The chemical shifts are given in ppm (top) by reference to ^{15}N-urea dissolved in DMSO-d_6 at the same temperature. The frequency scale ($f^{15N}_{TMS=220}$, bottom) refers the ^{15}N resonances to the TMS ^{1}H NMR at 220 MHz, determined as shown in Figure 2. The multiplet structure results from J^1 coupling between ^{15}N and ^{1}H and was measured from the ^{1}H spectrum (Figure 2B).

peptide nitrogen resonances in a ^{15}N spectrometer. However, in absolute terms, TMS affords an internal reference that is more satisfactory than any J^1 coupled ^{15}N–^{1}H system whose NMR frequencies are strongly temperature and solvent dependent.[9]

Plots of the ^{15}N chemical shift vs. temperature for each residue are given in Figure 4 which shows that the temperature dependenceis linear with coefficients ranging from 0 to −18 × 10^{-3} ppm/°C. The ppm scale at the various temperatures is referred to a hypothetical, temperature independent signal at 22 294 131.4 Hz, which is the ^{15}N-urea resonance frequency ($f^{15N}_{TMS=220}$) at 19 °C. On going from DMSO to TFE significant shifts, ranging from −91 to +109 Hz, are observed in the amide ^{15}N resonances (Figure 5). As shown below, the ^{15}N temperature coefficients and solvent effects reveal further conformational aspects of the individual -HN–CO- groups.

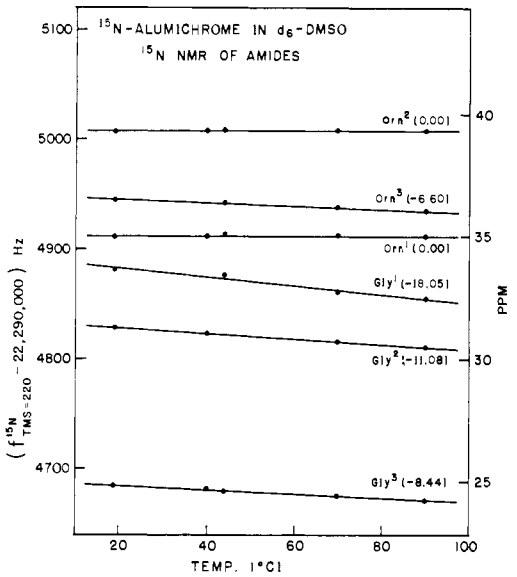


Figure 4. Temperature dependence of the ^{15}N chemical shift of the alumichrome amides. The ordinate scales refer the resonances to 22 294 131.4 Hz which is the urea ^{15}N resonance at 19 °C (ppm scale, right) and to the TMS ^{1}H NMR at 220 MHz ($f^{15}N_{TMS=220}$ frequency scale in Hz, left). The slopes of the lines are given in parentheses in units of 10^{-3} ppm/°C (i.e., $-11.08 = -11.08 \times 10^{-3}$ ppm/°C).

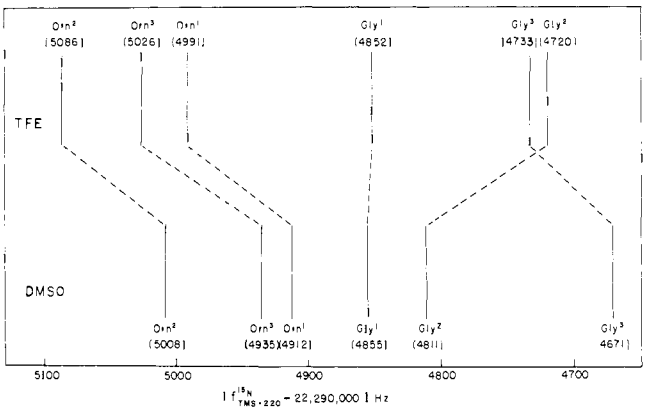
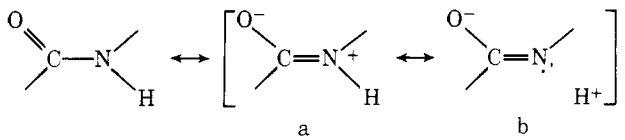


Figure 5. The mapped ^{15}N NMR spectrum of alumichrome for the ^{15}N peptide dissolved in DMSO and in TFE at 69.7 °C. The frequency scales are referred to the internal TMS ^{1}H NMR at 220 MHz and the exact resonance frequencies are indicated in parentheses.

The spectra in Figure 2 show that the alumichrome amide protons exhibit narrower lines in the ^{15}N than in the ^{14}N peptide. Other investigations by the authors[17] indicate a measurable dipolar effect of the nitrogen spin on the proton spin–lattice relaxation.

In the ^{15}N spectrum the Orn resonances occur at fields lower than those at which the Gly signals appear indicating the importance of side chain substituent effects (Figure 3). The corresponding amide proton resonances are, by comparison, less dependent on the type of residue to which they belong and reflect mainly the extent of H bonding in which they participate.[7a] However, while the amide ^{1}H spectrum spans an approximate 4 ppm range, the ^{15}N resonances span ~15 ppm, suggesting a major sensitivity of the nitrogen chemical shift to structural features. Indeed, the relative order of the ^{15}N resonances within each class of residue does reflect the relative order in the ^{1}H spectrum. Thus, on going from low to high magnetic fields, both the ^{1}H and ^{15}N resonances occur in the order Orn^2 < Orn^3 < Orn^1 and Gly^1 < Gly^2 < Gly^3. This indicates a direct influence of the amide H bonding which in case of the ornithyl residues is stronger for Orn^2 than for Orn^3, Orn^1 not being H bonded.[5] Consistently, in case of the glycyl amides, the order of H bond strength is Gly^1 ≳ Gly^2 > Gly^3 where Gly^1 and Gly^2 are H bonded to the solvent while Gly^3 is unexposed.

Elsewhere[7a] we have shown that the amide ^{1}H NMR can occur at somewhat lower fields when the carbonyl becomes H bonded by protic solvents. The increased deshielding of the NH proton was attributed to stabilization of resonant structure IIb upon increase in the Brönsted acidity of the solvent. Since protic acids are poor bases, C=O protonation will favor resonant structure IIa over IIb which implies a larger deshielding ought to be expected for the ^{15}N than for the ^{1}H nuclei.



structure I structure II

In alumichrome there are four internal amides (Gly^3, Orn^1, Orn^2, and Orn^3), i.e., amides whose hydrogen atoms are protected from the solvent but whose peptide-linked carbonyls are exposed (Figure 1). On going from DMSO to TFE (pK_{a_2} = 12.4) their ^{15}N resonances move to lower felds by 62 Hz (Gly^3), 79 Hz (Orn^1), 78 Hz (Orn^2), and 91 Hz (Orn^3) consistent with a stronger solvent-to-carbonyl H-bonding effect at the amide dipole. Gly^2 is the only residue whose amide H atom is external with an internally linked carbonyl (Gly^3). Hence, on going from DMSO ($pK_{a_1} \sim 0$) to TFE (estimated $pK_{a_1} \sim -8.2$[7a]) the Gly^2 NH senses a net loss of amide-to-solvent H-bond deshielding effect because of a solvent basicity decrease; at the same time, the amide does not gain any amide dipole stabilization because of the inward (away from solvent) orientation of the Gly^3 C=O. These effects, when combined, suggest that for an amide whose C=O and NH are exposed the shielding gained because of a weaker solvent basicity may be opposed by the shielding lost because of a stronger C=O protonation. The Gly^2-CO-NH-Gly^1 amide is in such a configuration, its net shift of −3 Hz being in close agreement with the small difference between the average low field shift of the internal NH's (Gly^3, Orn^{1,2,3}, average shift = 77.5 Hz) and the high field shift of the exposed Gly^2 NH (shift = −91 Hz). This indicates that the ^{15}N deshielding effect due to carbonyl protonation is comparable to the effect of direct H bonding of the amide to a suitable acceptor. Saitô, Tanaka, and Nukada[18] have observed similar direct and indirect H-bonding deshielding effects on the ^{14}N resonances of simple amides and have interpreted them on the basis of electronic shifts from the NH to the C=O. Such solvent effects on the nitrogen resonances add another criterion to be considered when determining the plausibility of particular conformations for a polypeptide in solution. At the same time, these results reinforce the view of electronic lability for the peptide bond,[7a,19] intimately related to its potential catalytic reactivity.[20]

As shown in Figure 4, the ^{15}N resonances shift with temperature in a fashion similar to that encountered for the ^{1}H signals.[5,14] Two residues exhibit temperature independence and these are the two whose NH hydrogen atoms are the most protected from the solvent either because of strong intramolecular H bonding (Orn^2) or because of serious steric shielding (Orn^1). In contrast, the two residues which have the NH exposed exhibit the largest slopes: -18.05×10^{-3} ppm/°C (Gly^1) and -11.08×10^{-3} ppm/°C (Gly^2), the difference between the two reflecting the fact that while the Gly^2 NH is linked to an internal C=O, the Gly^2-CO-NH-Gly^1 peptide unit is completely exposed. The two residues whose NH participate in a type of β-sheet structure, namely Gly^3 and Orn^3,

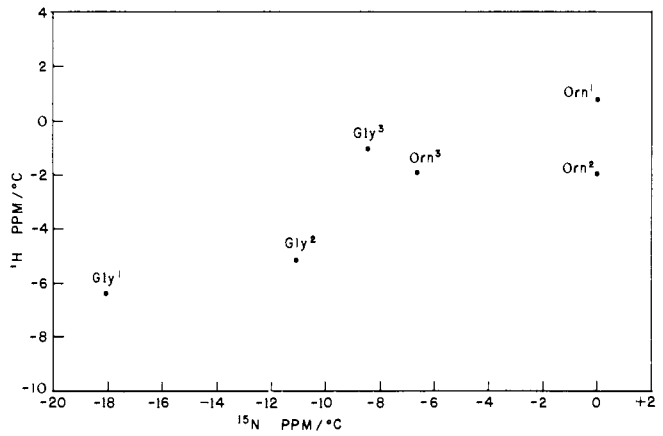


Figure 6. The temperature coefficients of the amide ^1H resonances[14] vs. those of ^{15}N resonances (Figure 4) for the peptide dissolved in DMSO-d_6. The units for ordinate and abscissa are 10^3 ppm/°C.

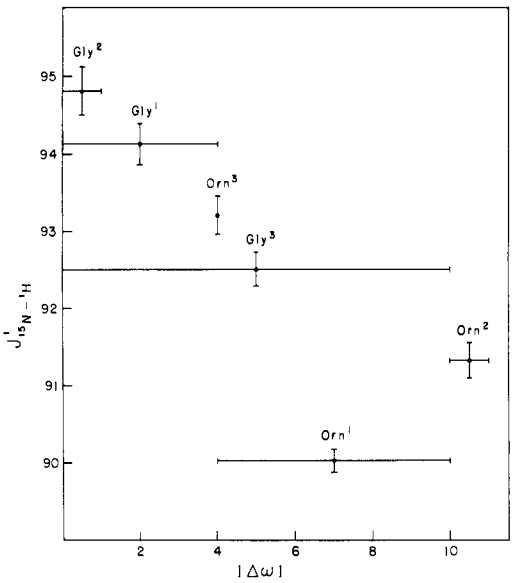


Figure 7. Plot of the ^{15}N-^1H scalar coupling (in Hz units) vs. the deviation from peptide bond planarity ($\Delta\omega = 180 - |\omega|$). The horizontal bars span $\Delta\omega$ ranges limited by two independent crystallographic determinations.[6a,b] The vertical bars denote standard deviations of the ^1H NMR determined J^1 mean values.

exhibit similar temperature coefficients, -8.44×10^{-3} and -6.60×10^{-3} ppm/°C, respectively. The lesser slope of the Orn³ nitrogen possibly result from the relatively stronger H bond in which this residue participates.[5]

For comparative purposes, the temperature coefficients of the amide ^1H resonances are plotted vs. those of the ^{15}N resonances for the peptide dissolved in DMSO-d_6 (Figure 6). Although the two sets of slope values appear to be somewhat correlated, it should be noted that coefficients larger than zero, exhibited by the proton, are not observed for the nitrogen nucleus. Furthermore, the range of slopes is about threefold larger for ^{15}N than for ^1H.

The ^{15}N-^1H scalar spin-spin interaction ought to reflect the extent of amide planarity as the magnitude of the J^1_{NH} coupling is dependent on the s character of the intervening orbital.[21] On going to a nonplanar amide configuration, the nitrogen valence electrons shift from a partial sp^2 to an sp^3 hybridization and J^1_{NH} should decrease. Figure 7 depicts the experimentally determined J^1_{NH} couplings vs. the crystallographic[6] angular deviation from planarity $\Delta\omega = 180 - |\omega|$. For diketopiperazine[15] ($\Delta\omega = 180°$), $J^1_{NH} \sim 90$ Hz and for secondary amines[21] $J^1_{NH} \sim 67$ Hz. Hence a cyclic dependence of J^1_{NH} on $\Delta\omega$ is suggested starting with $J^1_{NH} \sim 95$ Hz for $\Delta\omega = 0°$ (Figure 7), going through a minimum, $J^1_{NH} \sim 67$ Hz at $\Delta\omega = 90°$, and increasing back to $J^1_{NH} \sim 90$ Hz at $\Delta\omega = 180°$, consistent with the observation that the nitrogen-proton scalar interaction is stronger for trans than for cis amides.[21] The trend suggested by Figure 7 should be of immediate practical use in the NMR analysis of polypeptide conformation as the assumption of peptide planarity can at best lead to only approximate models.[22]

In contrast with ^{13}C-^1H experiments, the NOE will, in general, algebraically reduce the signal amplitude when observing the nitrogen nucleus under simultaneous proton irradiation.[3,4b] Since the nitrogen-proton dipolar interaction is expected to dominate the amide ^{15}N spin-lattice relaxation, the NOE may seriously decrease the signal/noise ratio in the direct ^{15}N detection experiment.[23] When observing ^{15}N-coupled ^1H resonances, the NOE signal loss is insignificant and a net amplification of the ^1H intensity results, upon ^{15}N decoupling, simply because of the J^1 doublet collapse. Taking these facts into consideration and remembering that $\gamma_{^1H}/\gamma_{^{15}N} \sim 10$, there is a 2×10^3-fold sensitivity gain vis-à-vis direct ^{15}N detection, when using the double irradiation technique.[24]

Such amplification, added to the experimental time saving provided by the indirect DR-FT technique, should allow for ^{15}N detection even under conditions of natural isotopic abundance (0.37%). Our strategyemploys pairs of free induction decay signals from protons so that odd members of the pairs are coadded to computer memory in the absence of perturbing fields at the heteronuclear frequency while even numbered members of the pairs are cosubtracted from memory while the perturbing field is present. The nature of the difference spectrum acquired following Fourier transformation of these pairs depends on the frequency, amplitude, and type and depth of modulation of the heteronuclear radiofrequency field, H_2. If H_2 is of sufficient amplitude and is noise modulated over a bandwidth sufficient to encompass the entire heteronuclear spectrum, one obtains a difference spectrum in which the protons normally coupled to the heteronuclei appear upright while the corresponding decoupled lines appear inverted. Results are exemplified in Figure 8 where the amide ^1H signals connected to ^{15}N are perturbed at 22.3018 MHz and appear in spectrum (B) but cancel in the control experiment (A). The experiment (B) is repeated in (C) for the natural abundance ^{15}N peptide and with a considerably larger number of scans.

The sensitivity attainable thus far has been limited by the stability of the spectrometer field. The short term drift in the superconducting magnet *cum* environment is compensated for by the small time interval between pulse pairs (Figure 8, A and B). Over an extended period, such as is required to obtain spectra from dilute samples (Figure 8C), we have obtained useful difference spectra but the excellent cancellation of resonances not coupled to the ^{15}N is slowly replaced by difference features related only by the field drift. Such drift, of course, will not occur with field-frequency stabilized spectrometers. It should be noted that noise irradiation is not necessary. If H_2 is unmodulated and of moderately strong amplitude, the heteronuclear lines will respond, as shown by Figure 2C, according to the proximity of F_2 to their coupled nitrogens. Indeed, this affords a convenient way of assigning the resonances in an unknown case.[25]

It is thus possible to map the entire ^{15}N spectrum of peptides, or in fact any other molecules exhibiting coupling to protons, by this Fourier internuclear difference spectroscopy. Since the number of proton spectrometers far exceeds those designed for other nuclei, the method permits these proton spectrometers to have their utility extended into new and important regimes.

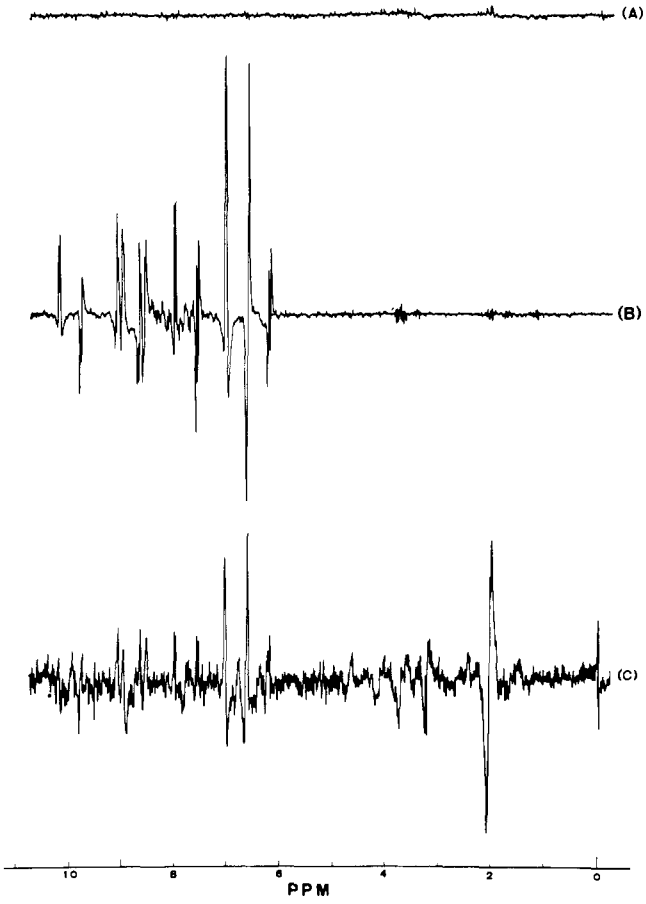


Figure 8. Natural abundance indirect ^{15}N detection by noise-irradiation Fourier internuclear difference ^{1}H NMR spectroscopy at 220 MHz. The 0.15 M alumichrome solutions were dissolved in DMSO-d_6 and the spectra were acquired at ~45 °C. (A) Difference spectrum of 99.5% ^{15}N-enriched alumichrome without ^{15}N decoupling; (B) same as (A) except that noise, centered within the amide ^{15}N frequency range, was applied during the negative accumulation; (C) same as (B) except that the peptide contains the natural isotopic composition (0.365% ^{15}N). (A) and (B) represent 300 passes each, while 69 178 free inductions were averaged in (C). Five watts of 300 Hz bandwidth noise were always applied, in (A) centered off-resonance at 22.3940 MHz while in (B) and (C) the noise was centered at 22.2967 MHz (off-resonance, add) and 22.3018 MHz (resonance, subtract). By switching the frequency from far off-resonance to on-resonance, the sample temperature reaches steady state after a few minutes of irradiation and does not fluctuate between pulse pairs. The aliphatic region, between 6 ppm and TMS, cancels in the control experiments (A) and (B) but, due to field drifts, exhibits some intensity in spectrum (C), accumulated for several hours. The doublet character of the ^{15}N-coupled proton resonances, apparent in (B) between about 6 ppm and 11 ppm (see also Figure 2B), is clearly manifest in (C).

The modifications required are simple, straightforward, and exist already in many NMR laboratories: an auxiliary frequency source, double tuning of the probe, a means for frequency switching of this source if sample heating from the decoupling field results, and logical elements in the FT computer system permitting alternate addition and subtraction of time domain data coherent with either frequency switching or enable-disable switching of the heteronuclear irradiation. Our experiments employed a simple flip-flop triggered by the end-of-sweep signal from the computer to either change the state of a balanced mixer switch or shift the frequency of the source. The sign inversion was obtained by driving another balanced mixer as a 180° phase shifter in the proton transmitter channel.

There are many quantitative details of this technique which we do not presently understand, for example, the variation in intensity and the phases of the difference lines. These, and other aspects, are under study.

The results presented here show clearly the convenience of the DR-FT technique in order to map the ^{15}N spectrum of peptides. Furthermore, the detection and assignment of the ^{15}N resonances suggest two immediate applications: (a) identification of ^{13}C resonances in the ^{13}C NMR spectrum by ^{15}N–^{13}C DR-FT, and (b) peptide sequencing by combined DR-FT in the ^{1}H, ^{13}C, and ^{15}N frequency ranges.

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References and Notes

(1) The results contained in this paper have been reported at the VIth International Conference on Magnetic Resonance in Biological Systems, Kandersteg, 1974, the XVIth Experimental NMR Conference, Asilomar, 1975, and the IIIrd International Meeting on NMR Spectroscopy, St. Andrews, 1975.
(2) Abbreviations: ^{13}C NMR, ^{13}C nuclear magnetic resonance; DMSO, dimethyl sulfoxide; DR-FT, double resonance-Fourier Transform; INDOR, internuclear double resonance; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; ^{1}H NMR, proton magnetic resonance; ppm, parts per million; TFE, trifluoroethanol; TMS, tetramethylsilane.
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