Supplementary Material Available: Spectroscopic data (^{1}H NMR, IR, EIMS, and HRMS) are given for compounds **1-15** (4 pages). Ordering information is given on any current masthead page.

Accurate Measurements of Homonuclear H^N-H^α Coupling Constants in Polypeptides Using Heteronuclear 2D NMR Experiments†

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We have developed a heteronuclear 2D NMR method for accurate measurements of homonuclear H^N-H^α coupling constants from nonoverlapping cross peak components. This is achieved by separating along ω_1 the two multiplet components, which characterize the two orientations of the H^α spin, utilizing the large coupling $^1J(C^\alpha$-$H^\alpha)$. Protein structure determination in solution by NMR depends on collecting a large number of conformational parameters, most importantly NOE distance constraints. Vicinal coupling constants can provide local structural information complementary to NOE data. However, they are difficult to measure accurately with conventional techniques and have therefore been of less value in protein structure determinations. We have concentrated recently on developing new methods for measuring accurately conformation-dependent coupling constants in polypeptides.

Of special interest are $^3J(H^N$-$H^\alpha)$ coupling constants, which provide constraints on the backbone dihedral angle ϕ. Because these coupling constants are often small compared to protein ^{1}H line widths, the corresponding multiplet components of 2D NMR cross peaks overlap, and the apparent splittings are significantly smaller or larger than the true coupling constants when measured from inphase or antiphase cross peaks, respectively.[1] This overlap of multiplet components can sometimes be avoided by homonuclear ECOSY,[2] COSY-45,[3] or frequency-selective COSY[4] experiments, which provide cancellation of some multiplet components and simplify the cross peak pattern. This requires, however, that the two active coupling partners of the cross peak are each coupled to a third nucleus (passive spin). Accurate measurements of coupling constants from ECOSY-like experiments require also that at least one active coupling is significantly larger than the line width. In considering only proton-proton spin coupling, these requirements prohibit the application of such methods in measurements of $^3J(H^N$-$H^\alpha)$ coupling constants in all amino acid spin systems except for glycine. This restriction is overcome, however, when one considers heteronuclear H^N-^{15}N-H^α or H^N-$^{13}C^\alpha$-H^α spin systems in which heteronuclear ECOSY-like effects can be generated.

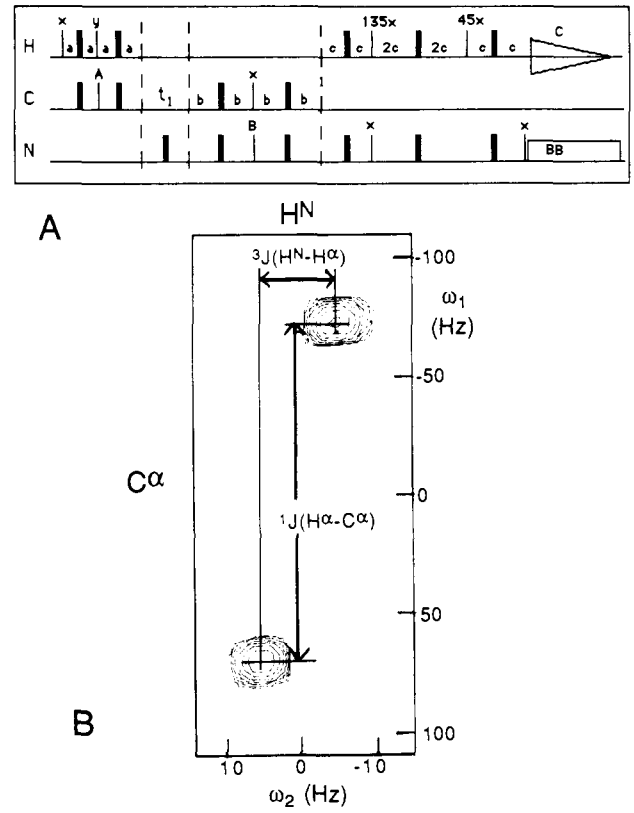

Figure 1. (A) Pulse sequence of the H^α-$C^\alpha(\omega_1)$-N-selective-$H^N(\omega_2)$ heteronuclear RELAY for measurements of homonuclear $^3J(H^\alpha$-$H^N)$ coupling constants. The delays are tuned in the following way: $a = (4^1J_{H\alpha\text{-}C\alpha})^{-1}$, $b = (4^1J_{N\text{-}C\alpha})^{-1}$, and $c = (4^1J_{NH})^{-1}$. The phase cycles used were as follows: A, x, $-x$; B, x, x, $-x$, $-x$; C, x, $-x$, $-x$, x. Time-proportional 90° incrementation of phase A provided quadrature detection in ω_1. Water suppression can be performed by preirradiation of the solvent signal. (B) Intraresidue tyrosine heteronuclear RELAY cross peak of Ac-Asn-Pro-(^{15}N)Tyr-NHMe between C^α and H^N. The heteronuclear coupling $^1J(H^\alpha$-$C^\alpha)$ is along ω_1, and the homonuclear coupling constant $^3J(H^\alpha$-$H^N)$ can be measured along ω_2. The sample was prepared at 30 mM concentration in dimethyl-d_6-sulfoxide (Cambridge Isotopes). These data were recorded on a General Electric GN-500 spectrometer with a custom-built triple resonance probe and a modified transciever board (details available on request). Nine hundred t_1 values were recorded over 16 h. The final digital resolution after zero filling is 6.1 Hz/pt in ω_1 and 1.6 Hz/pt in ω_2.

Recently, we have described an approach which provides accurate measurements of long-range heteronuclear ^{15}N-^{1}H coupling constants from homonuclear 2D and 3D spectra of ^{15}N-enriched polypeptides.[5] These experiments rely on the analysis of homonuclear cross peak patterns between protons coupled to a common ^{15}N nucleus, which is not pulsed in the experiment. An analogous strategy can be used to measure $^3J(H^N$-$H^\alpha)$ coupling constants accurately in heteronuclear correlation experiments using pulse schemes which selectively excite either amide or α-proton resonances. One of several possible pulse sequences is described here. It can be denoted as H^α-$C^\alpha(\omega_1)$-N-selective-$H^N(\omega_2)$ heteronuclear RELAY. This is a short notation of the pulse sequence given in Figure 1A. It uses refocussed INEPT[6] polarization transfer from H^α to C^α. During the evolution period, the carbon coherence evolves decoupled from nitrogens but coupled to protons. This provides the desired large splitting ($^1J_{CH}$ = 140 Hz) of the cross peaks along the ω_1 axis which prevents an overlap of the two multiplet components. The remainder of the experiment

† Basic ideas leading to this experiment were presented at the UCLA symposium *Frontiers of NMR Molecular Biology*; Jan. 12-19, 1989; Park City, UT.

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is relayed coherence transfer to the amide proton without applying an effective pulse to the C^α proton. Following the evolution period, carbon coherence is transferred to the ^{15}N with a refocussed INEPT sequence ($^1J_{CN}$ = 11 Hz). Subsequently, the nitrogen coherence is defocussed with respect to the coupling to the amide proton and then transferred to the amide proton using a proton 90° TANGO[7] pulse which is selective for protons directly coupled to ^{15}N and does not flip α-protons. Subsequently, the proton coherence which is antiphase with respect to nitrogen is refocussed and detected during t_2 with broadband nitrogen decoupling. A purge pulse at the nitrogen frequency prior to detection converts incompletely refocussed antiphase proton magnetization into multiple quantum coherence which is not detectable.

To test this technique we have applied it to the terminally blocked tripeptide Ac-Asn-Pro-[^{15}N]Tyr-NHMe. The synthesis and characterization of this peptide has been described elsewhere.[8] The coupling constant $^3J(H^\alpha-H^N)$ for Tyr 3 can readily be measured from a 1D spectrum and compared with the result of the new technique. Its value is 8.9 ± 0.2 Hz. In this sample, ^{13}C is at natural abundance. The resulting cross peak between the α-carbon (along ω_1) and the amide proton (along ω_2) of tyrosine is shown in Figure 1B. The splitting along ω_1 (140 Hz) is due to the coupling between H^α and C^α, and the splitting along ω_2 of 9.7 ± 0.8 Hz is due to the coupling between H^α and H^N, the quantity to be measured. As expected, this is slightly larger than the value obtained from the inphase doublet in the 1D experiment. The precision of these $^3J(H^\alpha-H^N)$ measurements is determined by the digital resolution of the data in the ω_2 dimension (1.6 Hz). It could even be improved by determination of the first moments (centers of mass) of the two cross peak components.

An alternative experiment with a longer sequence but fewer pulses would use $C^\alpha-H^N$ long-range coherence transfer and could be called $H^\alpha-C^\alpha(\omega_1)$-selective-$H^N(\omega_2)$ heteronuclear long-range COSY. An experiment symmetric to that of Figure 1A is $H^N-N(\omega_1)-C^\alpha$-selective-$H^\alpha(\omega_2)$ heteronuclear RELAY. The latter sequence has the disadvantage that the cross peaks of interest are at the ω_2 position of the α-proton which sometimes overlaps with the t_1 noise of residual water. However these two experiments are complementary in the sense that overlapping cross peaks in $C^\alpha-H^N$ RELAY (Figure 1A) may be resolved in the symmetric $N-H^\alpha$ RELAY.

The pulse sequence of Figure 1A can be easily expanded into a 3D experiment by adding a second evolution period. There are different choices: (i) After the first 90°(H) pulse a proton evolution period may be inserted. This experiment is an $H^\alpha-(\omega_1)-C^\alpha(\omega_2)-N-H^N(\omega_3)$ 3D COSY. (ii) A nitrogen evolution period could be inserted after the nitrogen magnetization has refocussed to inphase magnetization (i.e., between the delays b and c of Figure 1A); this is a $H^\alpha-C^\alpha(\omega_1)-N(\omega_2)-H^N(\omega_3)$ 3D COSY.

In all of these experiments, attention has been focussed on *intraresidue* heteronuclear coherence transfer from the α-carbon (C^α_i) to the amide proton (H^N_i) or from the amide nitrogen (N_i) to the C^α proton(H^α_i). By appropriate tuning of the delay b (Figure 1A), these same heteronuclear relay experiments can be used to develop *interresidue* cross peaks due to sequential connectivities C^α_{i-1} to H^N_i or H^α_i to N_{i+1}, as will be demonstrated in a subsequent publication.[9]

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Hyperconjugative Distortions and the Cyclopentyl Cation Structure

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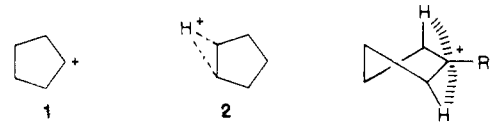
The structure of the cyclopentyl cation is intriguing. The first NMR studies of this species, one of the few secondary carbenium ions which can be observed in nonnucleophilic (superacid) media, revealed fluxional behavior.[1-4] Due to extremely rapid 1,2-hydride shifts, only one-line ^1H and ^{13}C NMR signals could be observed even at low temperatures in solution. Under these conditions, the energy difference between the "classical" (**1**) and the hydrogen-



bridged (**2**) structures must be less than 3 kcal/mol.[5] Nevertheless early analyses of the NMR chemical shifts[1,2,6] as well as the ESCA spectrum[7] clearly showed **1** to be favored over **2**. This conclusion was confirmed by the solid-state (CPMAS) NMR observations of Myhre, Yannoni et al.[8] who were able to freeze out and observe the individual carbon signals in the solid state at 70 K. The ^{13}C resonances were broadened, but the chemical shifts (given in Table I) agreed reasonably well with earlier estimates based on static models.[2] But this data tells us nothing about the detailed geometry of **1**, which might be planar (C_{2v}) or might adopt "envelope" (C_s) or twisted (C_2) symmetries. Strong experimental evidence for the last conformation is provided by Forsyth et al.'s isotopic perturbation results on the related tertiary 1-methylcyclopentyl cation (**3**, R = CH_3).[9] The Boston group concluded that partial H-bridging was involved and proposed that this possibility "should be explored further in higher level calculations which would probably predict a twisted structure as the minimum energy conformation for cyclopentyl cations". We now verify this prescient suggestion.

Low level ab initio calculations on the cyclopentyl cation establish the planar C_{2v} form of **1** to be 2-3 kcal/mol less stable than the twisted (C_2) global minimum (Table II). No stationary

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