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Determination of H_α,H_β and H_β,C′ coupling constants in ^{13}C-labeled proteins

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SUMMARY

A sensitive method to assign H_β protons stereospecifically as well as to determine rotamer populations about χ_1 in two 3D experiments is presented. The SOFT-HCCH-COSY experiment allowed us to measure the $^3J(H_\beta,C')$ couplings, using constant time evolution of C_α in t_2 and $C_{aliphatic}$-selective decoupling during t_3. The SOFT-HCCH-E.COSY experiment allowed us to measure the $^3J(H_\alpha,H_\beta)$ couplings, using constant time evolution of C_α in t_2, a small flip angle ^1H excitation pulse in the second mixing time, and double-band-selective decoupling (aliphatic and carbonyl carbons) during t_3. The method was applied to ribonuclease T_1.

INTRODUCTION

Homo- as well as heteronuclear ^3J coupling constants yield useful information about local conformations in molecules. In biomolecules these coupling constants are often small and therefore difficult to measure. Techniques based on the E.COSY principle (Griesinger et al., 1985, 1987, 1989) have proven useful to measure such coupling constants. Magnetization is transferred from spin A to spin B without disturbing the spin levels of a third spin, C, during the mixing and during both the preceding and following evolution periods. The A,B cross peak consists of two submultiplet patterns that are displaced with respect to each other by a displacement vector $J_C=(J_{AC},J_{BC})$. Provided J_{AC} is sufficiently large, J_{BC} can be measured with an accuracy that depends not on its size but only on the signal-to-noise ratio of the spectrum. J_{AC}, which usually represents a coupling that is useless in terms of structure determination, is called the associated coupling of the coupling J_{BC}. The E.COSY concept has been used to measure proton-proton

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couplings utilizing $^2J(H,H)$ as associated coupling of $^3J(H,H)$ (Griesinger et al., 1985) in E.COSY and P.E. COSY (Griesinger et al., 1987; Müller, 1987; Bax and Marion, 1988) experiments. With $^1J(N,H)$ as associated coupling, heteronuclear long-range J(N,H) coupling constants, such as $^3J(N,H_\beta)$ and $^3J(N_i,H_{\alpha i-1})$ (Montelione et al., 1989a; Kurz et al., 1991; Schmieder et al., 1991) as well as $^3J(N\underline{H},H_\alpha)$ (Montelione and Wagner, 1989b, 1990; Wagner et al., 1991), have been measured. Likewise, using $^1J(C,H)$ as associated coupling, $^3J(C,H)$ (Bax and Freeman, 1981; Griesinger et al., 1986; Kessler et al., 1988; Kurz et al., 1991; Schmieder et al., 1991; Sattler et al., 1992) as well as $^3J(H,H)$ (Sørensen 1990; Gemmecker and Fesik, 1991; Emerson and Montelione, 1992; Griesinger and Eggenberger, 1992) coupling constants are accessible. Approaches that yield qualitative H_α,H_β (Clore et al., 1991) and H_β,C' (Grzesiek et al., 1992) coupling constants in labeled proteins have also recently been published.

In this communication a sensitive method for the quantitative determination of $^3J(H_\alpha,H_\beta)$ coupling constants using $^1J(C_\alpha,H_\alpha)$ as associated coupling in an E.COSY type method is presented. In addition, the $^1J(C_\alpha,C')$ coupling was used as associated coupling to measure $^3J(H_\beta,C')$ coupling constants. The two novel methods are applied to the protein ribonuclease T_1.

Measurement of these two types of coupling constants allows stereospecific assignment of the H_β protons as well as determination of rotamer populations about χ_1 since each staggered conformation has a unique set of small and large H_α,H_β and H_β,C' coupling constants (Fig. 1).

METHODS

The measurement of $H_\beta C'$ coupling constants is achieved by correlating C_α with H_β using C′ as undisturbed spin and decoupling H_α (Fig. 2a). The measurement of the desired H_α,H_β coupling constant is achieved by correlation of C_α with H_β, using H_α as the untouched spin and decoupling the carbonyls. Fig. 2b shows the expected multiplet structure of such an experiment.

In principle, it is possible to determine both types of coupling constants in a single experiment

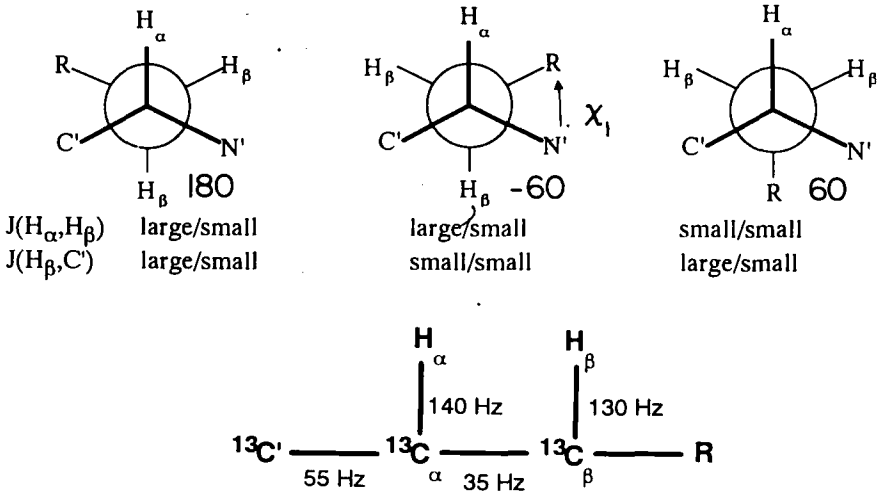

Fig. 1. The staggered conformations about the side-chain angle χ_1 of an L-amino acid with two H_β protons. The ^1J coupling topology about the C_α–C_β bond is shown underneath.

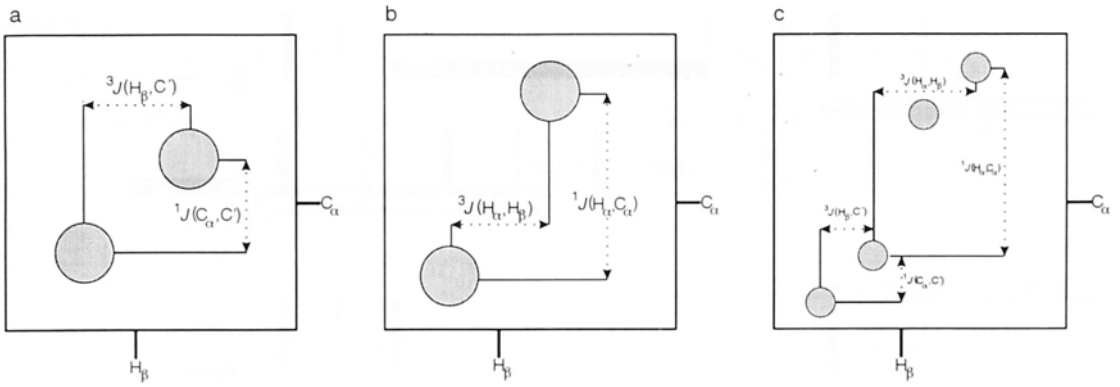

Fig. 2. Schematic multiplet pattern obtained by correlation of C_α with H_β with full decoupling except for the couplings to C′ (a) and H_α (b). If the couplings to both spins H_α and C′ are allowed to evolve, pattern (c) arises. The expected displacement vectors together with their components are indicated.

in which H_α and C′ are used simultaneously as undisturbed spins during the $C_\alpha \rightarrow H_\beta$ coherence transfer (Fig. 2c) in twice the measurement time. However, two experiments that each generate a doublet are at least $\sqrt{2}$ times more sensitive than one experiment that generates two doublets in the same measurement time.

The SOFT-HCCH-COSY experiment

The pulse sequence used to measure C′,H_β coupling constants is an $C_{aliphatic}$-selective 3D H,C-COSY-CT-C-Relayed-C,H-COSY (Fig. 3a). We will use the abbreviation SOFT-HCCH-COSY for this experiment. The carbonyl spins are not disturbed in the sequence in which carbon pulses that excite only the aliphatic region are used. After a refocusing period of $(2J(H_\alpha C_\alpha))^{-1}$, the protons are decoupled during $2(\tau+\tau'-\Delta-\Delta')$ with GARP (Shaka et al., 1985) following a 90° purging pulse. Selective 180° and 90° pulses on aliphatic carbons were implemented by G3 and G4 Gaussian cascades (Emsley and Bodenhausen, 1989, 1990) of 250 μs and 400 μs, respectively. Decoupling during t_3 was achieved with an MLEV 16 expansion of G3 pulses (Eggenberger et al., 1992; McCoy and Müller, 1992a,b) of 500 μs length. The decoupling performance of a MLEV expansion of G3 pulses is demonstrated experimentally in Fig. 4a. The scaling of the heteronuclear coupling as a function of offset frequency shows that a decoupling bandwidth of ± 4500 Hz $= 1.65$ $\langle \gamma B_1/2\pi \rangle$ can be achieved with such an expansion, whereas the coupling in the spectral window between 20 kHz and 22 kHz downfield from the carbon transmitter frequency (C′ region) is not reduced to a measurable extent. The theoretical value of scaling of heteronuclear coupling is 0.985.

Using a constant time evolution period, only the C′,C_α coupling evolves during t_2. The C′,C_α coupling is not well resolved for $t_2^{max} = 14$ ms. This is because less than half a period of the oscillation which is caused by the C_α,C′ coupling of about 55 Hz is acquired. Therefore, linear prediction of the mirror-imaged FIDs was performed to enhance resolution of ω_2 (Zhu and Bax, 1990). After t_1 and t_3 Fourier transformation, the complex FID in t_2 was mirror-imaged (126 to 250 points). Then 225 complex points were linearly predicted. Squared-cosine weighting and Fourier transformation were applied to the FID after discarding the mirror-image points.

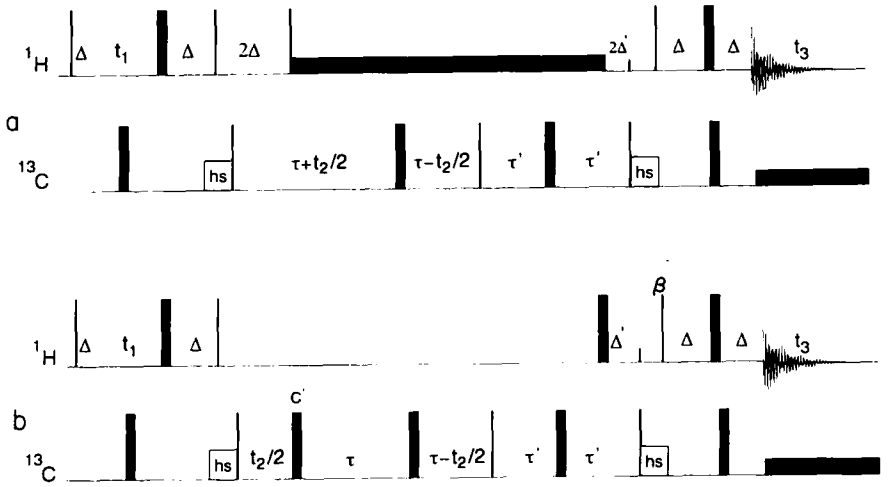

Fig. 3. (a) $C_{aliphatic}$-selective 3D H,C-COSY-CT-C-Relayed-C,H-COSY sequence to measure $^3J(C',H_\beta)$ coupling constants, $\Delta = 1.7$ ms, $\tau = 7$ ms, $\tau' = 3.05$ ms, $\Delta' = 0.95$ ms. The homospoil pulses in between heteronuclear polarization transfer had a duration of 3 ms and 2 ms, respectively. The recovery time was 3 ms; 8 scans were performed per t_1,t_2 experiment, and 180°-phase shifts were applied for the initial 90°(^{1}H) pulse and the initial 90°(^{13}C) pulse. TPPI was used for t_1, and RSH for t_2; 74 real, 126 complex, and 1k complex points were recorded in t_1, t_2, and t_3; G3 pulses had a duration of 250 μs; G4 pulses had a duration of 400 μs, and the second carbon 90° pulse was a time-inverted G4. Decoupling during $2(\tau+\tau'-\Delta-\Delta')$ was achieved with GARP with a 90°(^{1}H) of 200 μs. $C_{aliphatic}$ decoupling during acquisition was achieved by MLEV 16 expansion of G3 according to $A = G3_x$, $B = G3_{-x}$: AABBBAABBBAAABBA; (b) $C_{aliphatic}$-selective 3D H,C-COSY-CT-C-Relayed-C,H-β-COSY sequence to measure $^3J(H_\alpha,H_\beta)$ coupling constants. Same parameters as (a), $\beta = 36°$ in order to achieve suppression of the undesired versus desired multiplet components by a factor of 10. Decoupling was achieved by expanding an amplitude-modulated G3 pulse $G3^{ampl}(t) = G3(t) + \cos(\Delta\Omega t)\ G3(t)$ according to: AA(A)BB(A)BA(B)AB(B) (MLEV−8/4). AB(B) means: On resonance a $G3_x = A$ is followed by a $G3_{-x} = B$ pulse, and during this on-resonance $G3_xG3_{-x}$ pulse the off-resonance pulse (B) $= \cos(\Delta\Omega t)\ G3(t)$ with phase $-x$ is applied. The off-resonance G3 pulse is twice as long as the on-resonance G3 pulse. Four shapes AA(A), AB(A), BA(A) and BB(A) are sufficient to program the decoupling sequence on the Bruker AMX.

The SOFT-HCCH-E.COSY experiment

The pulse sequence for the measurement of H_α,H_β coupling constants is a $C_{aliphatic}$-selective 3D H,C-COSY-CT-C-Relayed-C,H-β-COSY with C′ decoupling during t_2 (Fig. 3b). The abbreviation SOFT-HCCH-E.COSY is used. All carbon pulses are selective for aliphatic carbons except when indicated otherwise. The undesired C_α,C′ coupling is refocused in t_2. The C_α,H_α coupling is in antiphase in ω_2. The constant time delay 2τ is set to $(2J(C_\alpha,C_\beta))^{-1} = 14$ ms to achieve maximum transfer from the C_α to C_β and to remove the $^1J(C_\alpha,C_\beta)$ coupling in ω_2. The refocusing delay, $2\tau'$, is set to $(5J(C,C))^{-1}$ to allow detection of C_β connected to no, one or two γ carbons. The 180° and 90° pulses selective for aliphatic carbons were implemented as in the former sequence. Since all carbon pulses were derived from the same frequency source, the 180° pulse selective for C′ was implemented as G3, with a phase gradient of 20 kHz and a duration of 250 μs. For the same reason, the decoupling from aliphatic as well as carbonyl carbons during t_3 was achieved by simultaneously applying MLEV-8 and MLEV-4 expanded G3 cascades to the aliphatic and the carbonyl resonances, respectively (see Fig. 4 for details). The mean rf amplitude on C′ was set to 0.5 of the amplitude applied to the aliphatic carbons to avoid Hartmann–Hahn transfer between carbonyl and

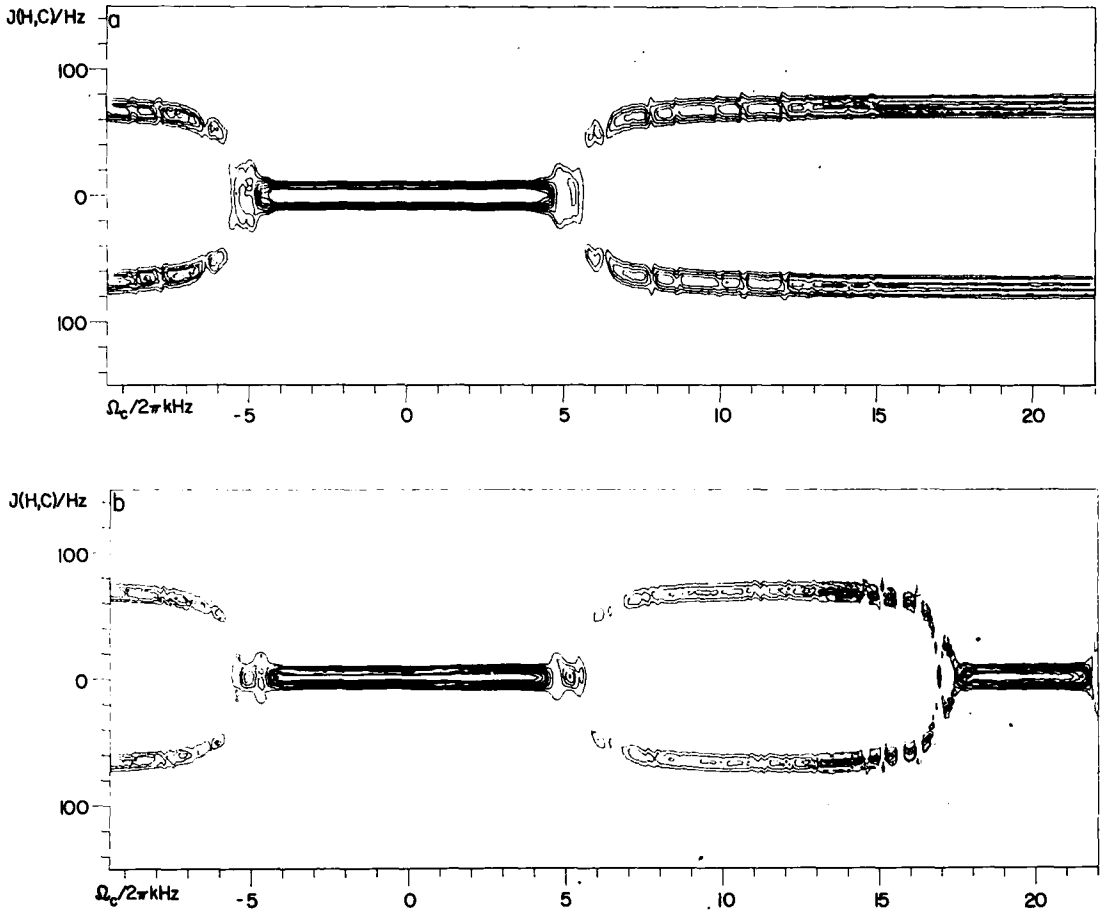

Fig. 4. (a) Experimental decoupling efficiency demonstrated for the H_α resonance of glutamic acid in D_2O for MLEV 16 (G3) with 500 μs G3 pulse duration (mean $\gamma B_1/2\pi = 2.75$ kHz). The plot was obtained by increasing the carbon frequency in steps of 100 Hz from 9.5 kHz upfield of C_α to 22 kHz downfield of C_α. Decoupling in a range between ± 4500 Hz = 1.65 $\langle \gamma B_1/2\pi \rangle$ on resonance is observed. No decoupling is detected from 20 kHz downfield. (b) Experimental decoupling efficiency for the H_α resonance of glutamic acid in D_2O for MLEV – 8/4 (amplitude-modulated G3) with 500 μs on-resonance G3 pulse duration. The plot was obtained by increasing the carbon frequency in steps of 100 Hz from 5 kHz upfield of C_α to 22 kHz downfield of C_α. Decoupling with a width of 9 kHz on resonance and with a width of 4 kHz at 20 kHz downfield from the carbon frequency source is observed.

aliphatic resonances. Therefore, the MLEV 8 cycle for the aliphatic and the MLEV 4 cycle for the carbonyl carbons have identical durations. The experimental decoupling performance as a function of offset is shown in Fig. 4b.

The proposed sequence for determination of H_α,H_β couplings is broad-band for the protons in contrast to that proposed by Gemmecker and Fesik (1991). The INEPT transfer and the constant time segment in t_2 provide higher sensitivity and less multiplet structure than the sequence of Emerson and Montelione (1992). A further improvement is expected if ^{15}N decoupling is performed throughout the sequence.

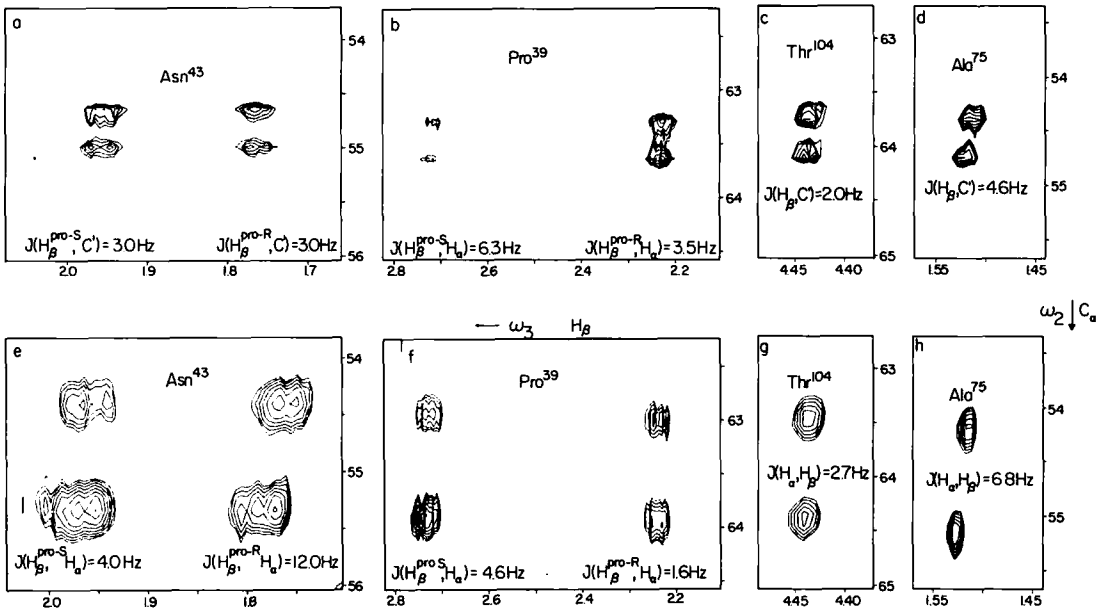

Fig. 5. ω_2,ω_3-Slices through the SOFT-HCCH-COSY experiment showing the cross peaks of Asn^{43} (a), Pro^{39} (b), Thr^{104} (c), and Ala^{75} (d). ω_2,ω_3-Slices through the SOFT-HCCH-E.COSY experiment showing the cross peaks of Asn^{43} (e), Pro^{39} (f), Thr^{104} (g), and Ala^{75} (h). The extracted coupling constants are indicated. They have an accuracy of ± 0.8 Hz.

RESULTS AND DISCUSSION

The two experimental methods were applied to 1.5 mM ribonuclease T_1 in D_2O. The C_α,H_β cross peaks of four representative amino acids (Asn^{43}, Pro^{39}, Thr^{104}, Ala^{75}) in the SOFT-HCCH-COSY experiment are shown in Figs. 5a–d and for the SOFT-HCCH-E.COSY experiment in Figs. 5e–h. These 2D excerpts are taken as ω_1 slices at the chemical shifts of the respective H_α resonances. The splitting of the multiplet in two components shifted by $\mathbf{J}(C')$ (Figs. 5a–d) and $\mathbf{J}(H_\alpha)$ (Figs. 5e–h) is clearly seen. The coupling constants for these residues which include Glu^{28} (not shown) were found by shifting the traces obtained by summation over the resonances in ω_1 and ω_2 (Table 1) with respect to each other and maximizing the overlap of the traces. The overlap de-

TABLE 1
^3J COUPLING CONSTANTS IN Hz AND χ_1 ANGLES DERIVED

Residue	$H_\alpha H_\beta^{pro\text{-}R}$	$H_\alpha H_\beta^{pro\text{-}S}$	$C'H_\beta^{pro\text{-}R}$	$C'H_\beta^{pro\text{-}S}$	χ_1
Thr^{104}	2.7		2.0		60
Pro^{39}	1.6	4.6	3.5	6.3	50
Glu^{28}	9.5	1.5	1.0	1.0	−60
Asn^{43}	12.0	4.0	3.0	3.0	−60
Ala^{75}	6.8		4.6		

creased notably when the shift was mis-set by one point in ω_3. This amounts to an accuracy of ± 0.8 Hz for the coupling constants.

These values indicate predominance of one staggered conformation for Asn^{43}, Glu^{28}, and Thr^{104}. For Ala^{75}, the expected averaged coupling constants were obtained. For Pro^{39}, deviations from the staggered conformations were expected. χ_1 was determined to be approximately $+50°$ by using the modified Karplus equation introduced by Haasnoot et al. (1981) for a quantitative analysis of the homonuclear couplings and a qualitative analysis of the heteronuclear couplings.

We have shown that the quantitative measurement of H_α,H_β and H_β,C′ coupling constants is possible for a ^{13}C-labeled protein. This method allows stereospecific assignment of diastereotopic protons as well as the determination of rotamer populations about the important backbone angle χ_1. We expect that conformational dynamics around the C_α–C_β bond and possible deviations from the staggered conformations will be detectable by these methods in conjunction with the measurement of $^3J(H_\beta,N)$ coupling constants and NOEs.

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REFERENCES

Bax, A. and Freeman, R. (1981) *J. Magn. Reson.*, **45**, 177–181.

Bax, A. and Marion, D. (1988) *J. Magn. Reson.*, **80**, 528–533.

Clore, G.M., Bax, A. and Gronenborn, A.M. (1991) *J. Biomol. NMR.*, **1**, 13–22.

Eggenberger, U., Karimi-Nejad, Y., Rüterjans, H. and Griesinger, C. (1992), Poster presentation at the 33rd ENC, Asilomar, CA, USA.

Emerson, D. and Montelione, G. (1992) *J. Am. Chem. Soc.*, **114**, 354–356.

Emsley, L. and Bodenhausen, G. (1989) *J. Magn. Reson.*, **82**, 211–221.

Emsley, L. and Bodenhausen, G. (1990) *Chem. Phys. Lett.*, **165**, 469–476.

Gemmecker, G. and Fesik, S.W. (1991) *J. Magn. Reson.*, **95**, 208–213.

Griesinger, C., Sørensen, O.W. and Ernst, R.R. (1985) *J. Am. Chem. Soc.*, **107**, 6394–6396.

Griesinger, C., Sørensen, O.W. and Ernst, R.R. (1986) *J. Chem. Phys.*, **85**, 6837–6852.

Griesinger, C., Sørensen, O.W. and Ernst, R.R. (1987) *J. Magn. Reson.*, **75**, 474–492.

Griesinger, C. and Eggenberger, U. (1992) *J. Magn. Reson.*, **97**, 426–434.

Grzesiek, S., Ikura, M., Clore, G.M., Gronenborn, A.M. and Bax, A. (1992) *J. Magn. Reson.*, **96**, 215–221.

Haasnoot, C.A.G., de Leeuw, F.A.A.M., de Leeuw, H.P.M. and Altona, C. (1981) *Biopolymers*, **20**, 1211–1245.

Kessler, H., Anders, U. and Gemmecker, G. (1988) *J. Magn. Reson.*, **78**, 382–388.

Kurz, M., Schmieder, P. and Kessler, H. (1991) *Angew. Chem.*, **103**, 1341–1342; *Angew. Chem. Int. Ed. Engl.*, **30**, 1329–1331.

McCoy, M. and Müller, L. (1992a), Poster presentation at the 33rd ENC, Asilomar, CA, USA.

McCoy, M. and Müller, L. (1992b) *J. Am. Chem. Soc.*, **114**, 2108–2112.

Montelione, G.T., Winkler, M.E., Rauenbühler, P. and Wagner, G. (1989a) *J. Magn. Reson.*, **82**, 198–204.

Montelione, G. and Wagner, G. (1989b) *J. Am. Chem. Soc.*, **111**, 5474–5475.

Montelione, G. and Wagner, G. (1990) *J. Magn. Reson.*, **87**, 183–188.
Müller, L. (1987) *J. Magn. Reson.*, **72**, 191–196.
Sattler, M., Schwalbe, H. and Griesinger, C. (1992), *J. Am. Chem. Soc.*, **114**, 1126–1127
Schmieder, P., Kurz, M. and Kessler, H. (1991) *J. Biomol. NMR.*, **1**, 403–420.
Shaka, A.J., Barker, P.B. and Freeman, R. (1985) *J. Magn. Reson.*, **64**, 547–552.
Sørensen, O.W. (1990) *J. Magn. Reson.*, **90**, 433–438.
Wagner, G., Schmieder, P. and Thanabal, V. (1991), *J. Magn. Reson.*, **93**, 436–440.
Zhu, G. and Bax, A. (1990) *J. Magn. Reson.*, **90**, 405–410.