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Sensitivity enhancement in (HCA)CONH experiments

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Abstract

A novel sensitivity-enhancement technique is proposed for experiments which correlate protein backbone resonances and start with magnetization from $^{13}C^{\alpha}$-$^{1}H^{\alpha}$ groups. The technique is based on replenishing magnetization lost by dipole-CSA cross-correlated relaxation of the $^{13}C^{\alpha}$ spin with $^{13}C^{\alpha}$ steady state magnetization. The principle is demonstrated for the (HCA)CONH experiment, resulting in 1.6-fold sensitivity enhancement compared to the HN(CA)CO experiment. Furthermore, other versions of the (HCA)CONH experiment were evaluated, including a novel experiment with spin-locking of transverse ^{13}C-^{1}H two-spin coherence, and a cross-correlation compensated (CA)CONH experiment which starts from ^{13}C rather than ^{1}H magnetization.

Introduction

The HN(CA)CO experiment yields inter- and intraresidual connectivities between amide protons and carbonyl carbons of ^{15}N,^{13}C double-labeled proteins, complementing the HNCO experiment for sequential resonance assignments (Clubb et al., 1992). The same connectivities are generated by the (HCA)CONH experiment, but with overall improved sensitivity (Löhr and Rüterjans, 1995; Bazzo et al., 1996). Although the (HCA)CONH experiment relies on the evolution of transverse $^{13}C^{\alpha}$ magnetization with respect to the small one- and two-bond $^{13}C^{\alpha}$-^{15}N couplings, sensitivity is improved with respect to the HN(CA)CO experiment by a shorter pulse sequence with fewer magnetization transfer steps and fewer pulses. Here, we propose modifications to the (HCA)CONH experiment to further increase its sensitivity.

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Methods

Figure 1a shows the pulse sequence of the (HCA)CONH experiment with sensitivity enhancement by cross-correlation compensation ('crococo-(HCA)CONH'). Detailed descriptions of the magnetization transfer pathway have been published earlier (Löhr and Rüterjans, 1995; Bazzo et al., 1996). Briefly, $^{13}C^{\alpha}$-magnetization is excited by the initial INEPT step. The delay $2T_C$ is set to $1/^{1}J_{C\alpha C\beta}$ to refocus the evolution of $^{13}C^{\alpha}$ magnetization with respect to β carbons. In addition, the $^{13}C^{\alpha}$ magnetization dephases with respect to both the amide nitrogen and the carbonyl carbon ($^{13}C'$), the carbonyl carbon is frequency-labeled during t_1, and the $^{13}C^{\alpha}$ magnetization refocuses to be in-phase with respect to $^{1}H^{\alpha}$ and $^{13}C'$ by the end of the delay $2T_C$. At point *a*, the magnetization is transferred to ^{15}N. The pulse sequence uses common building blocks for the subsequent frequency-labeling of the nitrogen during the constant time period $2T_N$ and the following sensitivity-enhancement scheme for the detection of $^{1}H^{N}$ magnetization during t_3 (Muhandiram and Kay, 1994). The initial part of the pulse scheme is similar to that by

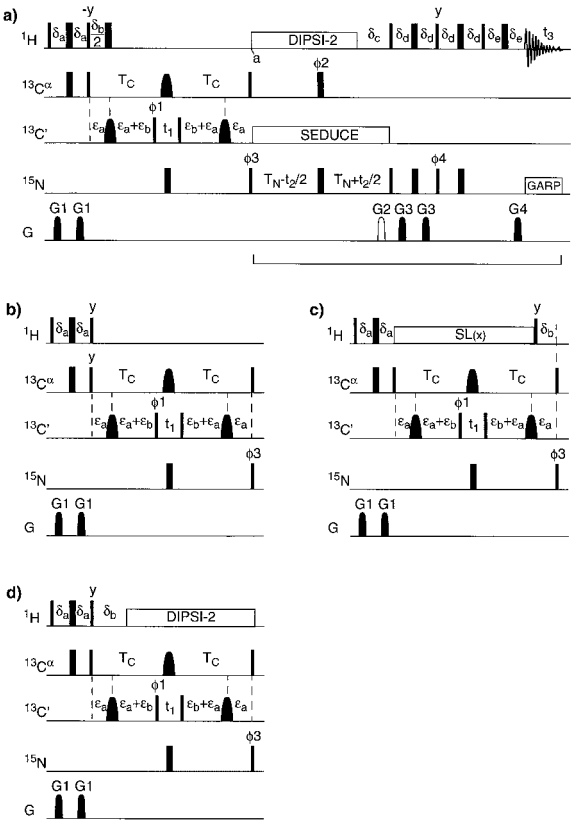

Figure 1. Pulse sequences of novel versions of the (HCA)CONH experiment. (a) Cross-correlation-compensated version, starting from ^{1}H magnetization, crococo-(HCA)CONH; (b) cross-correlation-compensated version, starting from ^{13}C magnetization, crococo-(CA)CONH; (c) spin-lock version, SL-(HCA)CONH; (d) conventional (HCA)CONH experiment with broad-band decoupling (Löhr and Rüterjans, 1995). Only the initial part of the pulse sequence is shown in (b), (c), and (d), as the rest is identical to the section identified by a bar in (a). Narrow and wide filled squares denote non-selective 90° and 180° pulses, respectively. Phases are x unless indicated differently. Delays: $\delta_a = 1.5$ ms, $\delta_b = 2.31$ ms, $\delta_c = 5.5$ ms, $\delta_d = 2.3$ ms, $\delta_e = 608$ ms, $T_C = 12$ ms, $T_N = 11$ ms, $\varepsilon_a = (T_c - \varepsilon_b - (t_1/2 - \tau_{p180}/2) - t_{p90} - t_{p180})/2$, where t_{p90} and t_{p180} denote the durations of the carbonyl 90° and 180° pulses, respectively, and τ_{p180} denotes the duration of the shaped 180°(^{13}C$^{\alpha}$) pulse, $\varepsilon_b = 9$ ms. The 180°(C′) pulses are omitted, when $t_1/2 - \tau_{p180}/2 > T_C - \tau_{p90} - \varepsilon_b - \tau_{p180}$. Gradients: G1 = 3, G2 = ±40, G3 = 2, G4 = 12.15 G/cm, all sine-shaped and with 500 μs duration except for G2 (1.5 ms). Rectangular ^{13}C-pulses were applied with 40.5 μs (90° pulse) and 36.5 μs (180° pulse) durations at 56 ppm (^{13}C$^{\alpha}$) and 174 ppm (^{13}C′) to minimize mutual excitation. Shaped pulses are 180° pulses: 350 μs RE-BURP (Geen and Freeman, 1991) for ^{13}C$^{\alpha}$ and ^{13}C$^{\beta}$, applied at 40 ppm, and 512 μs G^3 (Emsley and Bodenhausen, 1990) for ^{13}C′. Decoupling field strengths were 6.3 kHz (DIPSI-2; Shaka et al., 1988) and 1250 Hz (GARP; Shaka et al., 1985). SEDUCE decoupling (McCoy and Mueller, 1992) used 370 μs 90° Gaussian pulses. Phase cycle: ϕ_1 = x,−x; ϕ_2 = 4(x),4(−x); ϕ_3 = x,x,−x,−x; ϕ_4 = −y; receiver = x,−x,−x,x. This phase cycle is valid for Bruker instruments, while for Varian instruments any phase y should be replaced by the phase −y and vice versa (Zhu et al., 1999). The sign of ϕ_4 is inverted together with the polarity of G2 (Kay et al., 1992). ϕ_1 is incremented in the States-TPPI manner (Marion et al., 1989). The proton carrier was shifted at point a from 4 ppm to 8 ppm. Spectra were recorded at a ^{1}H frequency of 800 MHz with 22 × 58 × 640 complex points corresponding to $t_{max} = 9.7$ ms (t_1), 20.9 ms (t_2), 79.9 ms (t_3). The 180°(^{13}C′) pulses were omitted after 11 t_1 increments. The spin-lock strength in the SL-(HCA)CONH experiment was 6.3 kHz.

Bazzo et al. (1996). The important difference is that the pulses of the INEPT step are not phase cycled. As a result, magnetization lost by cross-correlated relaxation is replenished by steady-state ^{13}C magnetization.

The effect is readily described in terms of Cartesian product operators (Sørensen et al., 1983). The initial INEPT step simultaneously excites ^{13}C$^{\alpha}$ in-phase magnetization, C_y, and ^{13}C$^{\alpha}$ antiphase magnetization, $2C_yH_z$, which originate from steady-state ^{13}C$^{\alpha}$ and ^{1}H$^{\alpha}$ magnetization respectively. In the absence of cross-correlated relaxation, the pulse sequence selects the term $2C_yH_z$, while the term C_y would not lead to observable magnetization. In the presence of cross-correlated relaxation, however, one of the ^{13}C$^{\alpha}$ doublet components would relax more rapidly during the delay $2T_C$, which can be described as a partial conversion of $2C_yH_z$ to C_y and vice versa. Therefore, cross-correlated relaxation of the starting term $2C_yH_z$ can be compensated by the provision of new antiphase coherence which originates from C_y by the same cross-correlation effect. The compensation is complete when steady-state ^{13}C$^{\alpha}$ and ^{1}H$^{\alpha}$ magnetizations are identical and the pulse phases of the INEPT step are adjusted for constructive interference of the more slowly relaxing doublet component of the ^{13}C$^{\alpha}$ resonance (Pervushin et al., 1998; Weigelt, 1998).

For proteins in the slow tumbling regime, steady-state ^{13}C$^{\alpha}$ magnetization can indeed be as large as the ^{13}C$^{\alpha}$ magnetization transferred by INEPT from excited ^{1}H$^{\alpha}$ magnetization, due to the shorter non-selective T_1(^{13}C) relaxation times (Pervushin et al., 1998). With the phase cycle of Figure 1 and in the case of equal amounts of in-phase and antiphase magnetization, the effective ^{13}C$^{\alpha}$ relaxation rate during $2T_C$ is given by the relaxation rate of the low-field doublet component, which usually relaxes more slowly than the high-field component (Tjandra and Bax, 1998). In contrast to TROSY experiments (Pervushin et al., 1998), the cross-correlation compensation scheme does not rely on suppression of half

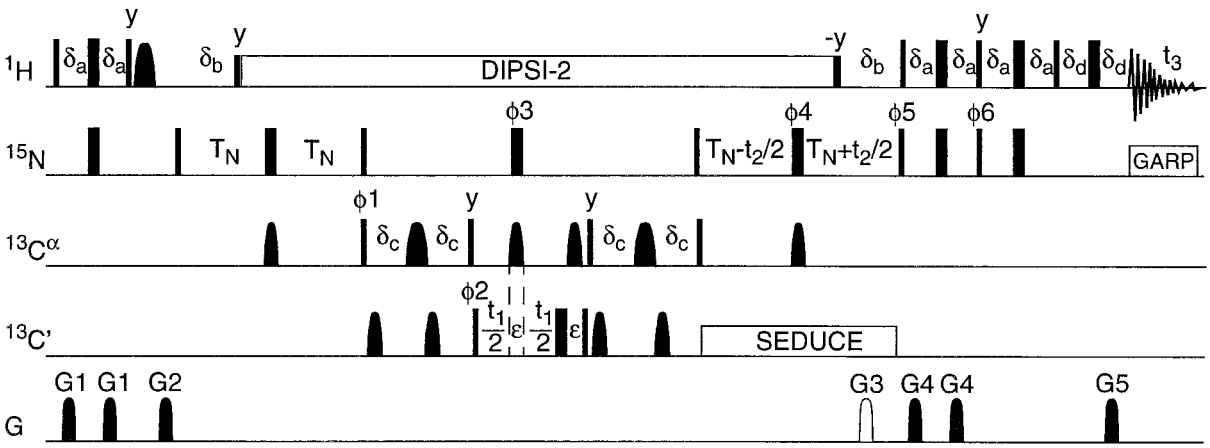

Figure 2. Pulse sequence used to record reference HN(CA)CO spectra. The experiment includes water flip-back, sensitivity enhancement, and $^{13}C^{\alpha}$ selective pulses to prevent evolution under $^1J_{C^{\alpha}C^{\beta}}$ couplings during the delays δ_c (Matsuo et al., 1996). Narrow and wide filled squares denote non-selective 90° and 180° pulses, respectively. Phases are x unless indicated differently. Delays: $\delta_a = 2.4$ ms, $\delta_b = 5.5$ ms, $\delta_c = 3.775$ ms, $\delta_d = 608$ μs, $T_N = 11$ ms. The delay ϵ is of the same duration as the shaped $^{13}C^{\alpha}$ pulse with which it coincides; together with the rectangular 180°($^{13}C'$) pulse, any chemical shift evolution is refocused for $t_1 = 0$. Gradients: G1 = −1.5, G2 = 15, G3 = ±40, G4 = −3.5, G5 = 12.15 G/cm, all sine-shaped and with 500 μs duration except for G2 and G3. G2 and G3 were 1.5 ms long and followed by recovery delays of 0.2 and 0.4 ms, respectively. Rectangular ^{13}C-pulses were applied with 39.8 μs (90° pulse) and 35.9 μs (180° pulse) durations at 54 ppm ($^{13}C^{\alpha}$) and 174 ppm ($^{13}C'$) to minimize mutual excitation. The shaped ^{1}H pulse was a 90° water flip-back pulse of 2 ms duration with an amplitude following the central lobe of a sinc-function. Shaped ^{13}C pulses were 180° G^3 (Emsley and Bodenhausen, 1990) pulses, where wide and narrow shapes represent pulses of 450 μs and 256 μs duration, respectively. Decoupling field strengths were 6.25 kHz (DIPSI-2) and 1250 Hz (GARP). SEDUCE decoupling used 370 μs 90° Gaussian pulses. Phase cycle: ϕ_1 = 8(x),8(−x); ϕ_2 = x,−x; $\phi_3 = \phi_4$ = 4(x),4(−x); ϕ_5 = x,x,−x,−x, ϕ_6 = −y,−y,y,y, receiver = 2(x,−x,−x,x),2(−x,x,x,−x). The sign of ϕ_6 was inverted together with the polarity of G3 (Kay et al., 1992). ϕ_1 was incremented in the States-TPPI manner. The proton carrier was placed at the water resonance. Spectra were recorded at a ^{1}H frequency of 800 MHz with 22 × 58 × 1024 complex points corresponding to t_{max} = 9.7 ms (t_1), 20.9 ms (t_2), 79.9 ms (t_3).

of the $^{13}C^{\alpha}$ magnetization. Denoting the steady-state magnetizations of $^1H^{\alpha}$ and $^{13}C^{\alpha}$ as M_H and M_C respectively, the starting magnetization of a TROSY experiment would be $0.5(M_H + M_C)$. In the absence of cross-correlation effects, the starting magnetizations of the crococo-(HCA)CONH (Figure 1a) and crococo-(CA)CONH (Figure 1b) experiments are M_H and M_C, respectively, while cross-correlation effects contribute additional magnetization from the other nuclear species (M_C and M_H, respectively). In the limit where cross-correlation effects completely relax one of the $^{13}C^{\alpha}$ doublet components, the effective starting magnetization thus becomes $0.5(M_H + M_C)$ as in TROSY.

Several other features were implemented in the experiments of Figure 1 to maximize sensitivity. These include a short semi-selective 180°($^{13}C^{\alpha}$) refocusing pulse to excite both $^{13}C^{\alpha}$ and $^{13}C^{\beta}$ resonances with near 100% efficiency, without affecting the carbonyl resonances. As the flanks of the excitation profile of this pulse were outside the range of $^{13}C^{\alpha}$ and $^{13}C^{\beta}$ resonances, sensitivity losses by incomplete excitation were minimized. Furthermore, sensitivity was maximized by frequency-labeling the carbonyl carbon by the use of a conventionally incremented evolution time t_1 rather than a constant time experiment. This minimizes transverse relaxation of the carbonyl carbon, which can be pronounced at high magnetic fields due to CSA. As the delay $2T_C$ is tuned to $1/J_{C^{\alpha}C^{\beta}} \approx 24$ ms, $1/^1J_{C^{\alpha}C'} \approx 18$ ms and the duration of the selective 180°($^{13}C'$) pulses is about 0.5 ms, the maximum t_1 value seems limited to about 5 ms. For improved resolution, t_{1max} was extended to almost 10 ms by omitting the 180°($^{13}C'$) pulses for $T_C - t_1/2 < 2.5$ ms and allowing somewhat shorter $^1J_{C^{\alpha}C'}$ evolution delays than $1/(2^1J_{C^{\alpha}C'})$ for the longer t_1 values. For the t_{1max} value used, incomplete refocusing and defocusing of the $^{13}C^{\alpha}$-$^{13}C'$ couplings reduced the signal intensity of the last FID by not more than 12%.

Water flip-back (Grzesiek and Bax, 1993) could readily be incorporated into the pulse schemes of Figure 1, but practical tests showed no significant gains in sensitivity. Therefore, the ^{1}H carrier frequency was placed at the amide protons instead of the α protons during the second half of the pulse sequence to minimize signal loss by off-resonance effects during DIPSI-2 decoupling (Figure 1), which become noticeable at a ^{1}H NMR frequency of 800 MHz.

Results and discussion

$^{13}C/^{15}N$ double-labeled ubiquitin and the FMN-binding protein from *Desulfovibrio vulgaris* (FMN-bp) (Liepinsh et al., 1997) were used to compare the sensitivity of the crococo-(HCA)CONH experiment with that of alternative versions. These included (a) a version with $^{13}C^{\alpha}$ steady-state magnetization as the main starting magnetization ('crococo-(CA)CONH'; Figure 1b), which uses the initial INEPT step merely to supply $2C_xH_z$ magnetization for compensation of cross-correlated relaxation of C_x during $2T_C$; (b) a spin-locked version, 'SL-(HCA)CONH', where $^{13}C^{\alpha}$ relaxation is retarded by locking $2C_yH_x$ in transverse two-spin coherence (Figure 1c; Grzesiek and Bax, 1995; Larsson et al., 1999); and (c) the original HN(CA)CO experiment with water flip-back (Grzesiek and Bax, 1993), sensitivity-enhancement scheme (Kay et al., 1992), and $^{13}C^{\alpha}$ selective 180° pulses with a band width of about 20 ppm to refocus $^{13}C^{\alpha}$-$^{13}C^{\beta}$ couplings (Figure 2) (Matsuo et al., 1996). All experiments were recorded under identical conditions, using the same recording times and the same maximum evolution times in all dimensions, and were processed with identical parameters.

For ubiquitin at pH 5.3 and 30 °C, the (HCA)CONH experiments of Figures 1a and 1c performed similarly well, with 1.6- to 1.7-fold higher average cross peak intensities compared to the HN(CA)CO spectrum. The same comparison performed with FMN-bp yielded enhancement factors of 1.6 and 2.0 for the experiments of Figures 1a and 1c, respectively. Projections of the 3D spectra are shown in Figure 3. Clearly, the sensitivity advantage of (HCA)CONH experiments over the HN(CA)CO experiment is maintained for the longer rotational correlation time of FMN-bp (11.6 ns versus 3.2 ns for ubiquitin under the conditions used).

The T_2 relaxation times of the $^{13}C'$ resonances of FMN-bp were measured by a spin-echo experiment and were found to be on average 20 ms. A 20% higher overall sensitivity would thus be expected for non-constant time versus constant time experiments, if the maximum $^{13}C'$ evolution time is about 10 ms as for the spectra of Figure 3. Spectra recorded with the constant time scheme of Löhr and Rüterjans (1995) were indeed less sensitive than the (HCA)CONH spectra of Figure 3 (data not shown).

Cross-correlated relaxation in the crococo-(HCA)CONH experiment was optimally compensated by the phase settings shown in Figure 1a, yielding on average 30% more sensitivity for FMN-bp compared to an experiment where the phase of the third ^1H pulse was y rather than −y. Although the experiment allows $^{13}C^{\alpha}$ to relax by scalar relaxation of the second kind during $2T_C$ (Abragam, 1961; Grzesiek and Bax, 1992), a scheme with averaging of the $^{13}C^{\alpha}$ relaxation rates by broadband ^1H decoupling (Figure 1d) was found to be about 25% less sensitive (data not shown).

At longer rotational correlation times, experiments may become attractive that focus on ^{13}C rather than ^1H steady state magnetization as starting magnetization. For ubiquitin, the (CA)CONH experiment of Figure 1b was almost 45% less sensitive than the ex-

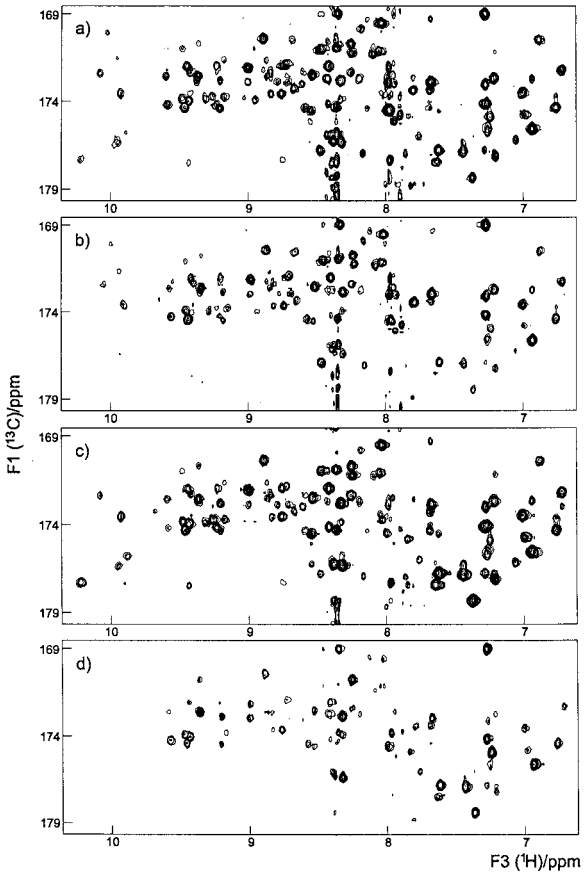

Figure 3. (HCA)CONH, (CA)CONH and HN(CA)CO spectra recorded with a ca. 2 mM solution of FMN-bp in 95% H_2O/5% D_2O at 303 K, pH 6.8, on a Bruker DRX 800 NMR spectrometer. Skyline projections of the 3D spectra onto the $^{13}C'$-^1H plane are shown. Each spectrum was recorded in about 21 h, using 16 transients per increment, an interscan relaxation delay of 0.8 s and the experimental parameters of Figures 1 and 2. Identical sweep widths, data matrix sizes, and processing parameters were used. (a) crococo-(HCA)CONH (Figure 1a); (b) crococo-(CA)CONH (Figure 1b); (c) SL-(HCA)CONH (Figure 1c); (d) HN(CA)CO (Figure 2). Positive and negative contours were drawn without distinction, with a spacing factor of 1.5.

periment of Figure 1a, whereas sensitivity was only 15% lower for FMN-bp.

The spin-locked version of Figure 1c had the highest sensitivity for the observable cross peaks for both ubiquitin and FMN-bp, but the spin lock, required to prevent dephasing of the $^{1}H^{\alpha}$ magnetization under homonuclear J_{HH} couplings (Grzesiek and Bax, 1995), strongly suppressed most correlations of glycines as noted earlier for the closely related CTSL-HCANH experiment (Larsson et al., 1999). Consequently, significantly more cross peaks were observed in the crococo-(HCA)CONH spectrum.

Conclusions

In conclusion, cross-correlation compensation provides a generally applicable sensitivity enhancement scheme for larger proteins, which can be applied to all experiments starting from $^{13}C^{1}H$ groups. For $^{15}N/^{13}C$ double-labeled proteins, the (HCA)CONH experiment with the improvements proposed here should definitely supersede the HN(CA)CO experiment. As an additional benefit, carbonyl frequencies of glycine residues are readily identified by the sign of their cross peaks, which is opposite to that of the other amino acids (Löhr and Rüterjans, 1995).

Acknowledgements

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References

Abragam, A. (1961) *Principles of Nuclear Magnetism*, Clarendon Press, Oxford.
Bazzo, R., Cicero, D.O. and Barbato, G. (1996) *J. Magn. Reson.*, **B110**, 65–68.
Clubb, R.T., Thanabal, V. and Wagner, G. (1992) *J. Magn. Reson.*, **97**, 213–217.
Emsley, L. and Bodenhausen, G. (1990) *Chem. Phys. Lett.*, **165**, 469–476.
Geen, H. and Freeman, R. (1991) *J. Magn. Reson.*, **93**, 93–141.
Grzesiek, S. and Bax, A. (1992) *J. Magn. Reson.*, **96**, 432–440.
Grzesiek, S. and Bax, A. (1993) *J. Am. Chem. Soc.*, **115**, 12593–12594.
Grzesiek, S. and Bax, A. (1995) *J. Biomol. NMR*, **6**, 335–339.
Kay, L.E., Keifer, P. and Saarinen, T. (1992) *J. Am. Chem. Soc.*, **114**, 10663–10665.
Larsson, G., Wijmenga, S.S. and Schleucher, J. (1999) *J. Biomol. NMR*, **14**, 169–174.
Liepinsh, E., Kitamura, M., Murakami, T., Nakaya, T. and Otting, G. (1997) *Nat. Struct. Biol.*, **4**, 975–979.
Löhr, F. and Rüterjans, H. (1995) *J. Biomol. NMR*, **6**, 189–197.
Marion, D., Ikura, M., Tschudin, R. and Bax, A. (1989) *J. Magn. Reson.*, **89**, 496–514.
Matsuo, H., Kupce, E., Li, H. and Wagner, G. (1996) *J. Magn. Reson.*, **B111**, 194–198.
McCoy, M.A. and Mueller, L. (1992) *J. Magn. Reson.*, **98**, 674–679.
Muhandiram, D.R. and Kay, L.E. (1994) *J. Magn. Reson.*, **B103**, 203–216.
Pervushin, K., Riek, R., Wider, G. and Wüthrich, K. (1998) *J. Am. Chem. Soc.*, **120**, 6394–6400.
Shaka, A.J., Barker, P.B. and Freeman, R. (1985) *J. Magn. Reson.*, **52**, 335–338.
Shaka, A.J., Lee, C.J. and Pines, A. (1988) *J. Magn. Reson.*, **77**, 274–293.
Sørensen, O.W., Eich, G.W., Levitt, M.H., Bodenhausen, G. and Ernst, R.R. (1983) *Prog. NMR Spectrosc.*, **16**, 163–192.
Tjandra, N. and Bax, A. (1997) *J. Am. Chem. Soc.*, **119**, 9576–9577.
Weigelt, J. (1998) *J. Am. Chem. Soc.*, **120**, 10778–10779.
Zhu, G., Kong, X.M. and Sze, K.H. (1999) *J. Biomol. NMR*, **13**, 77–81.