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ARTICLE

Broadband homonuclear TOCSY with amplitude and phase-modulated RF mixing schemes

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Abstract We have explored the design of broadband scalar coupling mediated ^{13}C–^{13}C and cross-relaxation suppressed ^{1}H–^{1}H TOCSY sequences employing phase/amplitude modulated inversion pulses. Considering a variety of supercycles, pulsewidths and a RF field strength of 10 kHz, the Fourier coefficients defining the amplitude and phase modulation profiles of the 180° pulses were optimised numerically so as to obtain efficient magnetisation transfer within the desired range of resonance offsets. The coherence transfer characteristics of the mixing schemes were assessed via numerical simulations and experimental measurements and were compared with commonly used sequences based on rectangular RF pulses. The efficacies of the clean ^{1}H–^{1}H TOCSY sequences were also examined via numerical simulations for application to weakly oriented systems and sequences with efficient, broadband and clean dipolar transfer characteristics were identified. In general, the amplitude and phase modulated TOCSY sequences presented here have moderately better performance characteristics than the sequences currently employed in biomolecular NMR spectroscopy.

Keywords TOCSY · Scalar couplings · Residual dipolar couplings · Numerical design · Amplitude and phase modulated RF

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Introduction

Sequence specific resonance assignment is the critical first step in the NMR based structural characterisation of biomolecular systems (Wüthrich 1986; Ernst et al. 1987). Multidimensional experiments such as HCCH-TOCSY (Bax et al. 1990; Clore et al. 1990) and HCCONH-TOCSY (Logan et al. 1992; Grzesiek et al. 1993) involving ^{13}C–^{13}C magnetisation transfers mediated by large J_{cc} couplings have become indispensable for protein sidechain assignments. In the case of medium sized molecules, ^{15}N edited ^{1}H–^{1}H TOCSY experiment (Marion et al. 1989) based on ^{1}H–^{1}H scalar couplings is sufficient for grouping all intra-residue protons, e.g. all ^{1}H nuclei belonging to a specific aminoacid residue in a peptide/protein. A variety of multiple pulse sequences have been reported (Glaser and Quant 1996) to generate an efficient TOCSY mixing Hamiltonian in which the effects of chemical shifts are suppressed and only the isotropic scalar coupling interaction, $2\pi J_{12}\boldsymbol{I}_1 \cdot \boldsymbol{I}_2$, is retained. This leads to efficient transfer of net magnetisation through the entire scalar coupled spin network, resulting in multiple bond correlations in the homonuclear chemical shift correlation spectrum. Under the isotropic mixing Hamiltonian $2\pi J_{12}\boldsymbol{I}_1 \cdot \boldsymbol{I}_2$, magnetisation exchange can be achieved starting with any of the x, y and z magnetisation components (Braunschweiler et al. 1983; Ernst et al. 1987). However, taking into account relaxation losses during mixing (Bax et al. 1990) and spectral phase anomalies (Rance 1987) it will be advantageous to carry out such experiments via longitudinal magnetisation transfers, as implemented in several recent studies (Peti et al. 2000; Bennett et al. 2003; Bermel et al. 2006; Jordan et al. 2006).

It is well known that in ^{1}H–^{1}H TOCSY experiments, in addition to the coherent Hartmann-Hahn transfers,

incoherent transfers arising via dipolar cross-relaxation processes may take place simultaneously during the TOCSY mixing period (Glaser and Quant 1996; Griesinger and Ernst 1988). This, in turn, may result in ambiguous interpretation of such TOCSY spectra and also lead to loss in sensitivity in situations where cross-peak intensities arising from coherent and incoherent magnetisation transfer pathways are of opposite sign. In the case of small molecules the cross-relaxation rates are small and hence their effects on the TOCSY spectra are minimal. In biological macromolecules, however, cross-relaxation rates become competitive with the rate of coherent magnetisation transfer. Fortunately, clean TOCSY sequences have been developed for such systems to suppress cross-relaxation effects during the mixing period (Glaser and Quant 1996; Griesinger et al. 1988; Bearden et al. 1988; Briand and Ernst 1991; Cavanagh et al. 1992; Kadkhodaei et al. 1993; Kupce and Hiller 2001; Kramer and Glaser 2004). This makes use of the fact that incoherent magnetisation transfers result from a superposition of longitudinal (σ_l) and transverse (σ_t) cross-relaxation processes and in the case of large molecules the rates of these two cross-relaxation processes have opposite algebraic sign. For example, in a macromolecule undergoing slow, rigid-body, isotropic motions (spin diffusion limit, $\omega_o\tau_c >> 1$), the ratio of the cross-relaxation rates between two spins (σ_l/σ_t) is equal to $-1/2$. For this situation cross-relaxation effects can be cancelled out if the magnetisation trajectory during the TOCSY mixing period leads to the spins spending twice the time along the z axis compared to that in the transverse plane.

Depending on the experimental conditions such as the range of chemical shifts over which coherence transfer is to be achieved, the maximum available RF power and the extent of RF field inhomogeneities, broadband TOCSY and clean TOCSY spectra can be generated using one of the many existing RF pulse sequences (Glaser and Quant 1996). The commonly used TOCSY mixing schemes such as DIPSI (Shaka et al. 1988) and FLOPSY (Kadkhodaie et al. 1991) involve the repeated application of a basic RF pulse cycle of the type $\{RR\overline{R}\overline{R}\}$ and $\{RR\overline{R}R\overline{R}R\overline{R}R\}$, where R is a composite rectangular 180° pulse. Many of the published cross-relaxation suppressed ^{1}H–^{1}H TOCSY sequences such as clean CITY (Briand and Ernst 1991) and TOWNY (Kadkhodaei et al. 1993) are also based on rectangular RF pulses. Considering the fact that modern spectrometers have increasingly sophisticated capabilities for the fast manipulation of RF phases and amplitudes, it will be of considerable interest to examine the possibilities to implement RF pulse schemes with superior coherence transfer characteristics using amplitude and phase modulated 180° pulses. Such sequences will be useful when the experiments have to be carried out at very high Zeeman field strengths due to sensitivity and resolution considerations. In the design of TOCSY mixing schemes based on such inversion pulses, the approach to use adiabatic pulses has received considerable attention recently (Kupce et al. 1998; Peti et al. 2000; Kupce and Hiller 2001; Bennett et al. 2003). The main attractive feature in implementing adiabatic mixing schemes is that such sequences are expected to have a very good tolerance to large variations in the B_1 field strength. Although B_1 field inhomogeneities are not typically very large in modern high resolution NMR probes, such sequences are highly attractive in the context of automated biomolecular NMR spectroscopic investigations. Here, we present the design of broadband ^{13}C–^{13}C and cross-relaxation suppressed ^{1}H–^{1}H TOCSY mixing sequences using supercycles of phase/amplitude modulated 180° pulses, assuming a limited variation of the B_1 field strength.

RDCs provide valuable structural information (Bax et al. 2001) and can be effectively used for establishing correlation between distant nuclear spins. Similar to the clean TOCSY mixing schemes based on ^{1}H-^{1}H scalar couplings, cross-relaxation compensated TOCSY sequences based solely on residual dipolar couplings (RDC) of weakly oriented molecules have been reported recently (Kramer and Glaser 2004). The dipolar transfer efficiencies of our clean ^{1}H–^{1}H TOCSY sequences have also been assessed via numerical simulations. In general, the performance characteristics of the sequences reported here have been compared with some of the best commonly used TOCSY sequences. The results from these investigations are presented below.

Numerical and experimental procedures

For the design of TOCSY and clean TOCSY sequences, we have considered a homonuclear system consisting of two spin 1/2 nuclei with the rotating-frame Hamiltonian during the application of an RF pulse given by:

$$H = 2\pi\delta_1 I_{1z} + 2\pi\delta_2 I_{2z} + 2\pi J_{12} I_1 \cdot I_2 + \omega_1(t)\{(I_{1x} + I_{2x})\cos\phi(t) + (I_{1y} + I_{2y})\sin\phi(t)\}.$$

The amplitude $\{\omega_1(t)\}$ and phase modulation profile $\{\phi(t)\}$ of the pulses are expressed as Fourier series (Geen and Freeman 1991; Paepe et al. 2003). Initial studies were carried out considering both cosine and sine Fourier series. However, it turned out that good broadband TOCSY sequences could be obtained by using only the cosine series and, hence, the sequences reported here were generated by expressing the modulation profiles as:

$$\phi(t) = \Sigma a_n \cos(n\omega t),$$

$$\{\nu_1(t)\}_{CC} = \left|C(1 - \{b_0 + \sum b_n \cos(n\omega t)\})\right| \text{ or}$$

$$\{v_1(t)\}_{\mathrm{HH}} = \left|\mathrm{C}\{b_0 + \sum b_n \cos(n\omega t)\}\right|,$$

where $\omega = 2\pi/t_p$ is the modulation frequency, t_p is the pulse duration and C is a constant, set to 10,000 Hz in our case. The Fourier coefficients that play a critical role in determining the performance of the sequence were numerically optimised. $\{v_1(t)\}_{\mathrm{CC}}$ and $\{v_1(t)\}_{\mathrm{HH}}$, respectively, represent the modulation profiles used in generating the ^{13}C–^{13}C TOCSY and ^{1}H–^{1}H clean TOCSY sequences reported here. In the numerical optimisation approach, B_1 field inhomogeneities were neglected and the amount of z magnetisation transferred to the second spin $<I_{2z}>$ is calculated at $\tau_{\mathrm{mix}} = 1/(2J_{12})$ {or at $\tau_{\mathrm{mix}} = 1/(D_{12})$, in the dipolar case}, starting with $<I_{1z}> = 1$ and $<I_{2z}> = 0$ at zero mixing time. In order to obtain efficient magnetisation transfers over the desired range of resonance offsets δ_1 and δ_2 the Fourier coefficients are optimised via the downhill simplex method of Nelder and Mead (Press et al. 1985) by minimising the error function

$$\varepsilon(a_0, a_n, b_0, b_n) = \sum_{\delta_1,\delta_2} \varepsilon_J(a_0, a_n, b_0, b_n, \delta_1, \delta_2) + \sum_{\delta_1,\delta_2} \varepsilon_{\mathrm{eer}}(a_0, a_n, b_0, b_n, \delta_1, \delta_2) + \varepsilon_{\mathrm{rms}}.$$

$\varepsilon_{\mathrm{rms}}$ is a penalty function that is employed to avoid the root mean square of the RF field strength, B_1(rms), exceeding a pre-set value, e.g. 10 kHz in our case. During optimisation, the value of $\varepsilon_{\mathrm{rms}}$ was either set to zero or to a large value, depending on whether the calculated value of B_1(rms) for a given amplitude modulation profile was found, respectively, to be less than or greater than the desired upper limit of B_1(rms). The error term ε_J is set to $[1 - <I_{2z}>]$. The error term $\varepsilon_{\mathrm{ecr}}(\propto |\bar{\sigma}|)$ was used only in the design of clean TOCSY sequences. This facilitates the optimisation of the amplitude and phase modulation profiles of the RF pulses such that the resultant magnetisation trajectories lead to an effective cross-relaxation rate, $\overline{\sigma}$, of zero. In the calculation of ε (a_0, a_n, b_0, b_n), the values of ε_J and $\varepsilon_{\mathrm{ecr}}$ were calculated over a representative number of (δ_1, δ_2) values. The effective cross-relaxation rate during the TOCSY mixing period is calculated via the invariant-trajectory formalism (Griesinger and Ernst 1988; Briand and Ernst 1991) by projecting the time-dependent magnetisation onto the transverse and longitudinal axes:

$$\overline{\sigma} = \sigma_t/\tau \int_0^{\tau} (m_{1x}(t)m_{2x}(t) + m_{1y}(t)m_{2y}(t)) + \sigma_l/\sigma_t(m_{1z}(t)m_{2z}(t))\, dt,$$

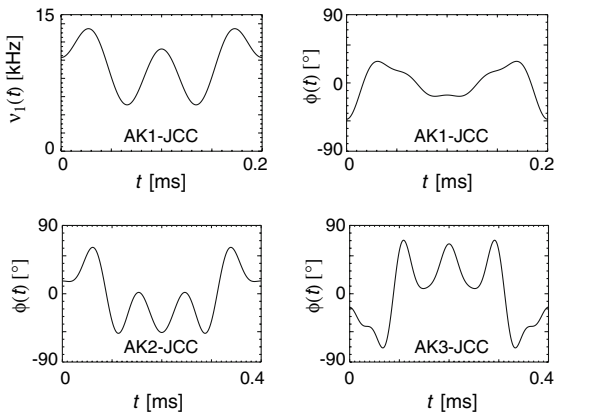
where the integral is taken over one period of the multiple pulse sequence.

^{13}C–^{13}C and ^{1}H–^{1}H scalar coupling strengths of 35 Hz and 10 Hz, respectively, 180° pulses with durations in the range of 100–700 μs and an upper limit of 10 kHz (rms) for the RF field strength were considered, with no restrictions imposed on the peak RF field strength. Unless mentioned otherwise, computer programs were written in-house and simulations were typically carried out for a Zeeman field strength corresponding to a ^{1}H resonance frequency of 750 MHz (~17.5 T). The 180° pulses were divided into 100 slices of equal duration. Using well known phase cycles such as m4, m8, m16 (Levitt et al. 1983), xy16 (Gullion et al. 1990), t5, t7 and t9 (Tycko et al. 1985), appropriate supercycling of the inversion pulses were generally carried out for generating mixing sequences. However, optimisation of the supercycles were also attempted in order to achieve further improvements of the TOCSY performance. Initially, optimisation runs were carried out starting with Fourier coefficients corresponding to different amplitude and phase modulated inversion pulses constructed, via a simulated annealing procedure (Kirkpatrick et al. 1983), considering only a single spin 1/2 system. However, during the course of this study it became apparent that it is possible to much more successfully generate TOCSY and clean TOCSY sequences by setting the starting Fourier coefficients to zero. Typically, it was possible to generate sequences with satisfactory performance characteristics with a limited number of Fourier coefficients in the range of 5–8. Increasing the number of coefficients did not lead to significant improvements. RF pulse sequences were tested experimentally via the HCCH-TOCSY or ^{15}N edited ^{1}H–^{1}H TOCSY experiment, using a ~1 mM (^{13}C,^{15}N)-labelled sample of a 154 amino acid heat stable protein from Methanobacterium thermoautotrophicum (in D_2O at pH 7.0 and 318 K; Carella 2008), and/or a 94 a.a. RNA-binding domain of the hnRNP C1 protein (in H_2O at pH 5.5 and 293 K; Wittekind et al. 1992). Experiments were carried out on a 750 MHz Varian INOVA NMR spectrometer equipped with pulse field gradient accessories, waveform generators and a triple resonance probe. The States procedure was used for quadrature detection in the indirect dimension (States et al. 1982). Proton chemical shifts were referenced to the H_2O signal relative to DSS and ^{13}C chemical shifts were referenced to external dioxane/H_2O. The transfer efficiency of the clean TOCSY sequences was evaluated for application to residual dipolar couplings by neglecting the scalar coupling interaction between the two spins and considering, instead, the dipolar coupling term $H_D = 2\pi D_{12}[3I_{1z}I_{2z} - \boldsymbol{I}_1 \cdot \boldsymbol{I}_2]$ in the Hamiltonian, with $D_{12} = 10$ Hz.

Results and discussion

The phase/amplitude modulation profiles of the inversion pulses with the corresponding Fourier coefficients and the supercycles employed for the ^{13}C–^{13}C TOCSY mixing sequences (AKn-JCC) reported here are shown in Fig. 1. The magnetisation transfer characteristics of the different RF mixing schemes, as a function of the resonance offsets of the two spins, are presented in Fig. 2. The performance characteristics of the FLOPSY sequence that has the best coherence transfer characteristics, in particular along the antidiagonal, compared to many of the commonly used ^{13}C–^{13}C TOCSY mixing sequences (Peti et al. 2000), is also given. Moderate improvements with respect to FLOPSY (Fig. 2a) can be realised with the sequences reported here at large offsets along the antidiagonal, where $\delta_1 = -\delta_2$ without sacrificing much of the efficacy of the magnetisation transfers along the diagonal, e.g. the AK1-JCC sequence leads to ~20% higher transfer efficiency at $(\delta_1,\ \delta_2) = (-5$ kHz, $+5$ kHz). For amino acid residues such as threonine, where the C^β and C^γ carbon chemical shifts differ by ~50 ppm, such improved magnetisation transfer characteristics for large chemical shift differences should be beneficial at high Zeeman field strengths. Considering a system of two spin 1/2 nuclei and starting with $\langle I_{1z}\rangle = 1$ at zero mixing time, the amount of polarisation transferred to the second spin at $\tau_{mix} = 1/(2J_{12})$ was examined as a function of the chemical shift difference between the two nuclei. Based on such simulations the effects of B_1 inhomogeneities on the magnetisation transfer characteristics were examined. Although RF field inhomogeneities were not explicitly taken into account in the local optimisation procedure, the performance characteristics of the RF pulse sequences are found to be not significantly affected by minor variations (±5%) in the B_1 field strength (data not shown). The magnetisation transfer characteristics of the computer optimised mixing sequences were also assessed in a three spin system. These three spin simulations were carried out using the SIMPSON program (Bak et al. 2000) starting either with C^α or C^β polarisation. Typical C^α, C^β and C^γ carbon chemical shifts of Threonine were considered and the carrier was kept at 45 ppm. Here again, slightly improved $C^\beta \rightarrow C^\gamma$ and $C^\alpha \rightarrow C^\gamma$ cross-peak intensities are seen (also at 900 MHz) with the new mixing sequences reported here compared to FLOPSY (data not shown).

The phase and amplitude modulation profiles of the inversion pulses, Fourier coefficients and the supercycles employed in the ^1H–^1H TOCSY mixing sequences, with suppression of cross-relaxation effects in the spin diffusion limit, developed here are presented in Fig. 3. Figure 4 shows the scalar coupling mediated magnetisation transfer characteristics of our AK1-JHH (Fig. 4a) and AK2-JHH (Fig. 4b) mixing schemes. For comparison, the performance characteristics of the clean CITY (Fig. 4c) sequence is also given. The corresponding effective cross-relaxation rate plots are depicted in Fig. 5. Cross-relaxation rates are given in units of transverse cross-relaxation rate constant, with positive contours indicating that transverse cross-relaxation contributes more than longitudinal cross-relaxation. It is worth mentioning that in assessing the relative performance of different cross-relaxation compensated TOCSY sequences one has to consider not only the efficiency with which cross-relaxation effects are suppressed but also the bandwidth over which coherent magnetisation transfer can be effectively achieved, with ^1H RF field strengths that can be safely employed over long mixing times in typical high resolution NMR probes. Coupled with satisfactory suppression of cross-relaxation effects, the sequences reported here exhibit good broadband coherence transfer characteristics. The performance of the phase and amplitude modulated RF pulse sequences are also found to be unaffected by minor variations in the B_1 field strength (data not shown).



Mixing scheme	a_1	a_2	a_3	a_4	a_5	a_6	a_7	a_8
AK1-JCC (t9m8)	3.4	- 24.1	-13.1	-7.7	-6.1	-	-	-

Mixing scheme	b_0	b_1	b_2	b_3	b_4	b_5	b_6	b_7
AK1-JCC (t9m8)	0.037	-0.231	-0.130	0.278	0.017	-	-	-

Mixing scheme	a_1	a_2	a_3	a_4	a_5	a_6	a_7	a_8
AK2-JCC (M12)	35.1	3.5	-15.8	- 26.6	14.6	5.9	-	-
AK3-JCC (m12)	- 45.6	- 6.7	15.9	31.2	-16.3	-9.7	4.2	9.1

Fig. 1 The modulation profiles of the 180° pulses of the mixing schemes AKn-JCC. The Fourier coefficients (a_0[°], a_n[°], b_0, b_n) and the supercycling employed are also given. The amplitude and phase modulated AK1-JCC sequence uses the t9m8 phasing scheme (t9: 0°, 15°, 180°, 165°, 270°, 165°, 180°, 15°, 0°; m8: 0°, 0°, 180°, 180°, 180°, 180°, 0°, 0°) and has an rms field strength of 10 kHz. The phase modulated AK2-JCC scheme uses an optimised 12 step phase cycle (M12: 343.2°, 159.5°, 181.7°, 45.0°, 125.1°, 217.5°, 73.9°, 123.5°, 359.4°, 287.5°, 98.0°, 252.1°) and the phase modulated AK3-JCC sequence employs the m12 phase cycle (m12: 0°, 0°, 180°, 180°, 0°, 180°, 180°, 0°, 180°, 180°, 0°, 0°)

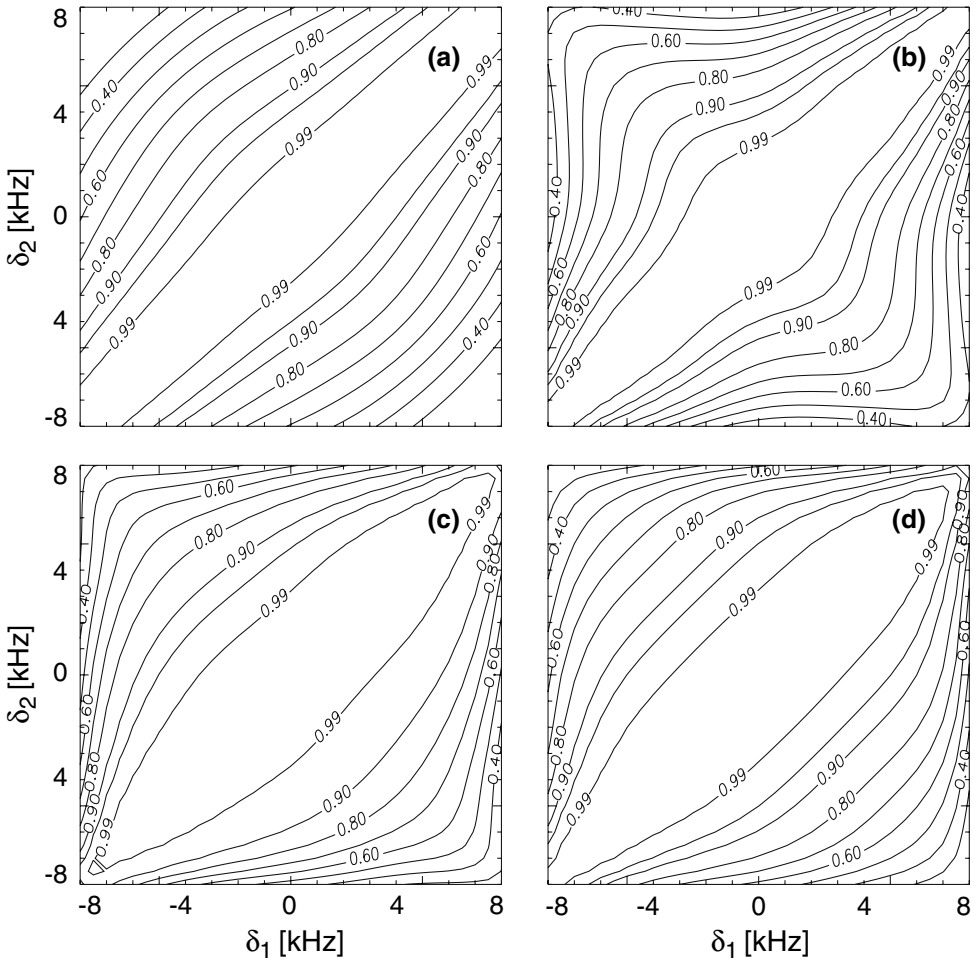


Fig. 2 Contour plots of the polarisation transferred to the second spin at $\tau_{mix} \sim 1/(2J_{12})$, as a function of the resonance offsets of the two spins and starting with $\langle I_{1z} \rangle = 1$ at zero mixing time. A J_{12} value of 35 Hz and mixing times of (**a**) 14.14 ms (FLOPSY-16), (**b**) 14.4 ms (AK1-JCC) and (**c**, **d**) 14.4 ms (AK2-JCC, AK3-JCC) were employed. A ^{13}C RF field strength of 10 kHz was employed in generating the plots **a**, **c** and **d**

The dipolar transfer characteristics of the different amplitude and phase modulated clean TOCSY sequences were evaluated via numerical simulations. During the course of the design of ^{1}H–^{1}H TOCSY sequences, in addition to the AK1-JHH and AK2-JHH schemes, a variety of other sequences exhibiting good suppression of cross-relaxation effects were also found. Although, compared to the AK1-JHH and AK2-JHH schemes, these sequences did not exhibit superior coherence transfer characteristics for the scalar coupling case, the dipolar transfer characteristics of such sequences were also examined. Figure 6 shows the dipolar magnetisation transfer characteristics of the AK1-DHH, AK1-JHH and DIPSI-2rc-xy4 (Kramer and Glaser 2004) mixing schemes. The effective cross-relaxation rate plots for the AK1-DHH and DIPSI-2rc-xy4 are given in Fig. 7. Although the AKn-JCC sequences can also be employed in principle for ^{1}H–^{1}H TOCSY mixing, as shown in Fig. 7c for the representative AK2-JCC mixing scheme, the corresponding effective cross-relaxation rate plots were found to be far from satisfactory. It is apparent, that the AK1-DHH and AK1-JHH sequences exhibit efficient broadband dipolar transfer characteristics, coupled with good suppression of cross-relaxation effects. It is expected that clean TOCSY sequences with broadband magnetisation transfer characteristics will be especially useful for the study of weakly oriented molecules, as it is possible to envisage situations where ^{1}H nuclei resonating at the extremes of the proton spectral range, e.g. the methyl and amide protons in a protein, can be spatially proximal, e.g. due to the protein fold. In general, sequences with broadband magnetisation transfer characteristics will typically be required only at high Zeeman field strengths where one expects large resonance offsets for the scalar/dipolar coupled nuclei. In situations where the experiments are carried out at low fields, satisfactory performance can be successfully realised by making use of one of the many existing rectangular pulse based mixing sequences.

In agreement with the numerical simulations, the experimental performance of the amplitude and phase modulated mixing schemes was found to be satisfactory and representative data are given in Figs. 8, 9, 10. Figure 8a shows a conventional 2D ^{13}C–^{1}H HSQC correlation spectrum of the 154 a.a. protein. The 2D HCCH-TOCSY spectrum obtained with the sequence AK1-JCC is

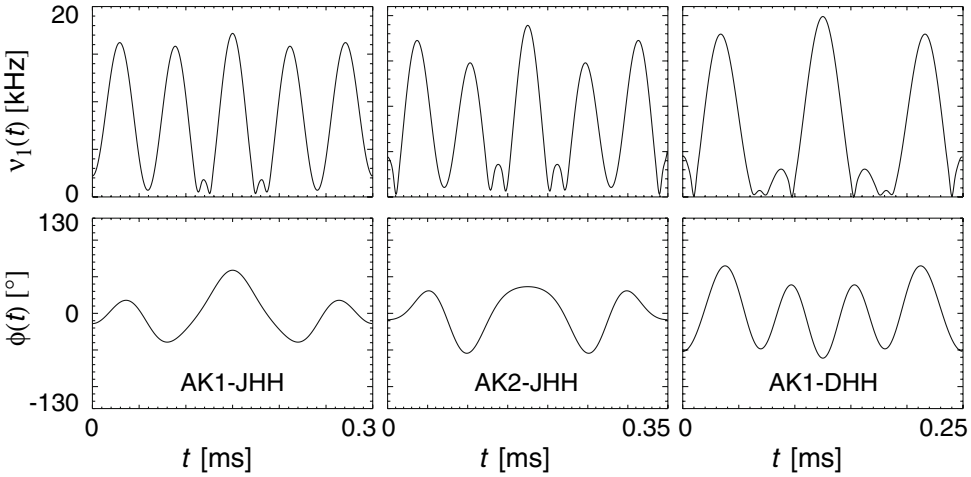


Fig. 3 The modulation profiles of the 180° pulses of the mixing schemes AK1-JHH (**a**), AK2-JHH (**b**) and AK1- DHH (**c**). The Fourier coefficients and the supercycling employed are also given. The amplitude and phase modulated AK1-JHH, AK2-JHH and AK1-DHH sequences employ, respectively, the xy16, t5xy16 and xy16 supercycles

Mixing scheme	a_1	a_2	a_3	a_4	a_5	a_6
AK1-JHH (t5xy16)	-11.0	28.4	-20.8	-7.6	-4.3	1.6
AK2-JHH (xy16)	-2.3	24.8	-26.6	-14.4	6.624	3.24
AK1-DHH (xy16)	8.9	-10.9	-20.34	-45.5	15.8	-

Mixing scheme	b_0	b_1	b_2	b_3	b_4	b_5
AK1-JHH (t5xy16)	0.808	0.095	0.033	-0.035	0.124	-0.808
AK2-JHH (xy16)	-0.744	-0.026	0.038	0.0512	-0.014	0.086
AK1-DHH (xy16)	0.193	0.940	-0.628	-0.171	-0.785	-

shown in Fig. 8b. The expanded plots of the region shown in Fig. 8b and obtained from HCCH-TOCSY spectra generated employing the AK1-JCC or FLOPSY mixing schemes are shown in Fig. 9. The cross-peaks arising from the $C^{\beta} \rightarrow C^{\gamma}$ magnetisation transfer in the different threonine residues are also indicated in Fig. 9b. 1D cross sections taken at a few representative $C^{\beta} \rightarrow C^{\gamma}$ cross-peaks are also given in Fig. 9. As expected from the numerical simulations, cross-peaks with marginally higher intensities are observed with the amplitude and phase modulated scheme. The other mixing sequences given in Fig. 1 were also found to lead to experimental spectra of comparable quality to that obtained using the FLOPSY sequence. Under significant RF field miscalibrations (>>10 kHz), however, the phase modulated sequences reported here were found to lead to spectra of much superior quality compared to that of the FLOPSY sequence (data not shown). The experimental spectra presented above were obtained with a mixing time of ~14 ms and keeping the ^{13}C carrier at 45 ppm. In addition, spectra were also generated with slightly larger mixing times, keeping the ^{13}C carrier at 35 ppm and also with the hnRNP C1 protein sample. In general it is observed that the mixing schemes reported here can be effectively employed for achieving efficient broadband ^{13}C–^{13}C polarisation transfers.

The ^{15}N edited clean ^{1}H–^{1}H TOCSY spectrum of the hnRNP C1 protein sample generated with the amplitude and phase modulated sequence AK1-JHH is shown in Fig. 10a. In addition to the intense cross-peaks arising from direct $H^{\alpha} \rightarrow H^{N}$ magnetisation transfers, cross-peaks arising via relayed magnetisation transfers from the side chain protons can also be clearly seen in the spectrum 10a collected using a mixing time of 48 ms. As expected, all the cross-peaks observed in the amide region arise only from geminal proton pairs of side-chain amino groups, possibly through both chemical exchange and scalar coupling mediated magnetisation transfers. Some of these NH_2 cross-peaks and the resonances arising from a few well resolved backbone amide protons are indicated in spectrum 10a. From a comparison with the ^{15}N filtered NOE spectrum collected at the same mixing time, it can be seen that magnetisation transfers through dipolar cross-relaxation processes have been efficiently suppressed by the clean TOCSY mixing sequence (data not shown). The quality of clean TOCSY spectra generated using different amplitude and phase modulated mixing schemes were found to be comparable (data not shown). The spectral region indicated in Fig. 10a and taken from data acquired using the clean TOCSY sequence AK1-JHH, clean CITY-xy16 and the TOCSY sequence AK2-JCC are shown, respectively, in

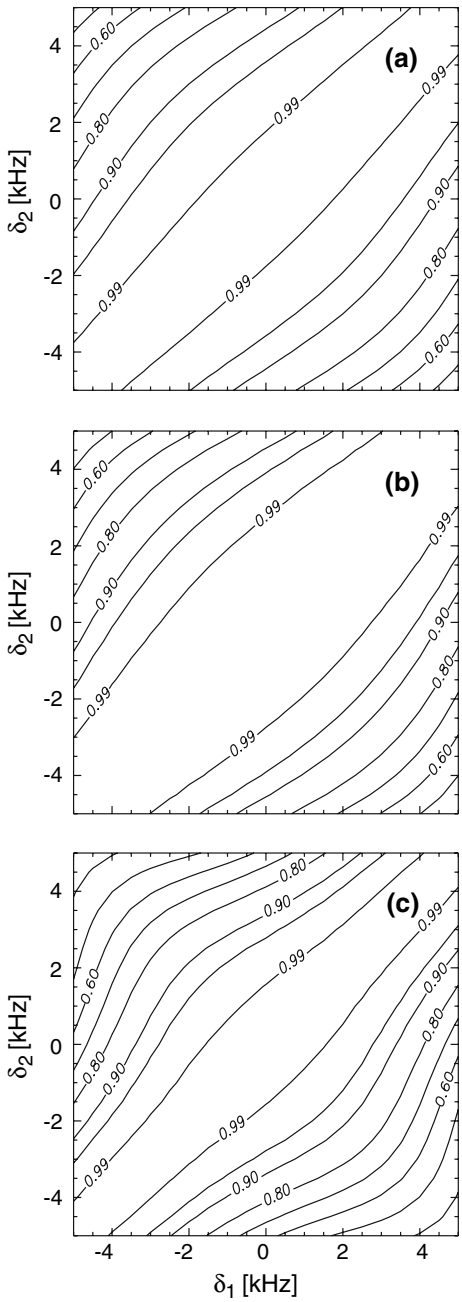


Fig. 4 Contour plots of the polarisation transferred to the second spin at $\tau_{mix} \sim 1/(2J_{12})$, as a function of the resonance offsets of the two spins and starting with $\langle I_{1z} \rangle = 1$ at zero mixing time. A J_{12} value of 10 Hz was employed in generating the plots for (**a**) AK1-JHH (**b**) AK2-JHH and (**c**) clean CITY-xy16 mixing schemes

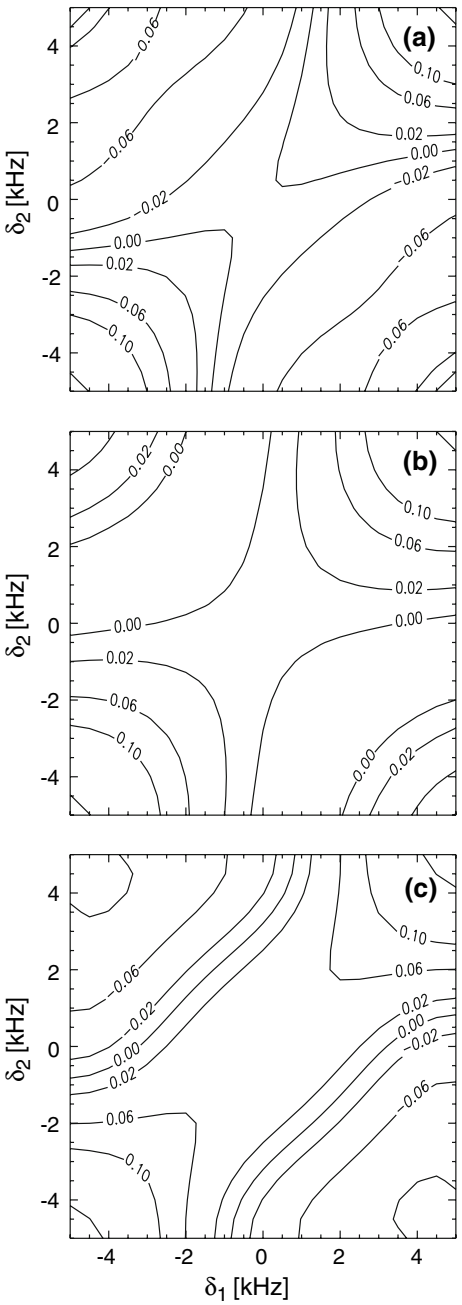


Fig. 5 The effective cross-relaxation rate plots for (**a**) AK1-JHH (**b**) AK2-JHH and (**c**) clean CITY sequences, generated assuming $J_{12} = 0$

Fig. 10b, c and d. The cross-relaxation compensated sequences AK1-JHH and clean CITY-xy16 can be clearly seen, as expected from the corresponding effective cross-relaxation plots, to lead to spectra of superior quality, with signal intensities observed in spectrum 10b marginally better than that in Fig. 10c.

In conclusion, we have presented in this study an approach for designing mixing schemes for achieving efficient, broadband ^{13}C–^{13}C longitudinal magnetisation exchange via J_{cc} couplings and for scalar/dipolar coupling mediated clean ^{1}H–^{1}H TOCSY transfers. Unlike the case with adiabatic amplitude and phase modulated pulses, the present study shows that it is possible to achieve satisfactory TOCSY performance using non-adiabatic 180° pulses with large durations. While we have taken recourse to a local optimisation procedure to save computational time, it is conceivable that improved mixing schemes can be

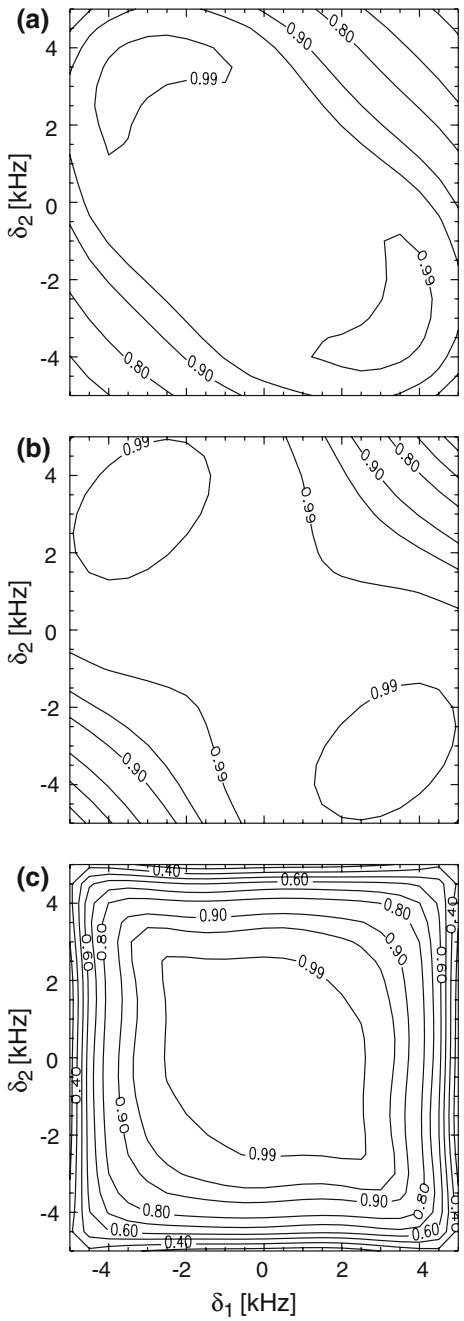


Fig. 6 Contour plots of the polarisation transferred to the second spin at $\tau_{mix} \sim 1/(D_{12})$, as a function of the resonance offsets of the two spins and starting with $<I_{1z}> = 1$ at zero mixing time. A D_{12} value of 10 Hz was employed in generating the plots for (**a**) AK1-DHH (**b**) AK1-JHH and (**c**) DIPSI-2rc-xy4 mixing schemes

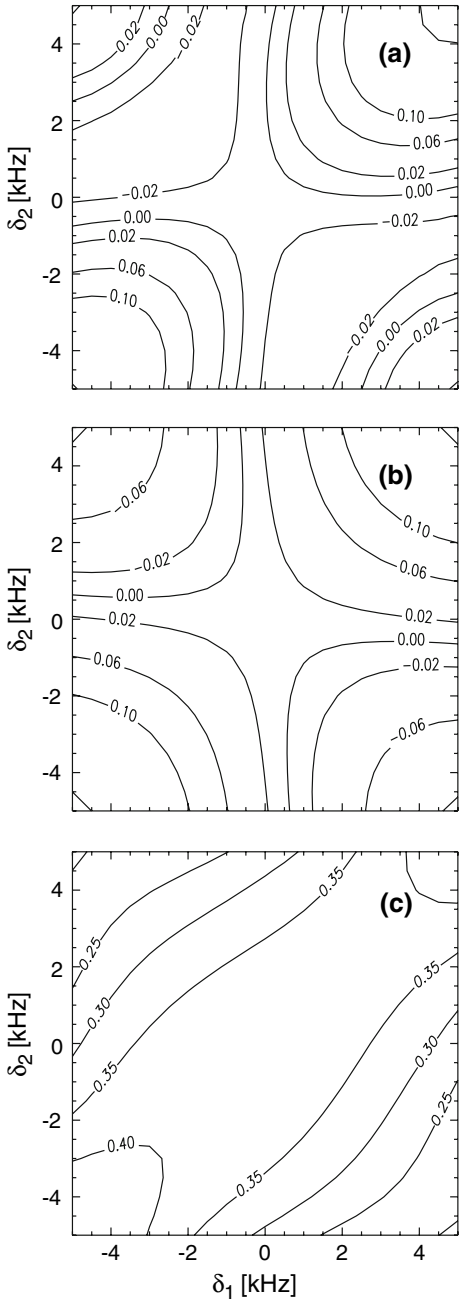


Fig. 7 The effective cross-relaxation rate plots for (**a**) AK1-DHH (**b**) DIPSI-2rc-xy4 and (**c**) AK2-JCC sequences, generated assuming $J_{12} = 0$

developed by a better scanning of the parameter space via global optimisation techniques such as simulated annealing (Kirkpatrick et al. 1983) and genetic algorithms (Forrest 1993). Although the clean TOCSY mixing schemes reported here were developed considering the spin diffusion limit, $(\sigma_l/\sigma_t) = -1/2$, it should be equally possible to extend the approach to other motional regimes. The optimisation procedure can also be extended to develop mixing schemes that are tailor made as per experimental requirements; e.g. with a different RF field strength and/or with much larger B_1 field inhomogeneities. The methodology outlined here is presently being extended for developing RF pulse sequences for broadband heteronuclear coherence transfers (Glaser and Quant 1996). In the context of MAS solid state NMR studies of isotopically labelled biological systems, it is also conceivable that the

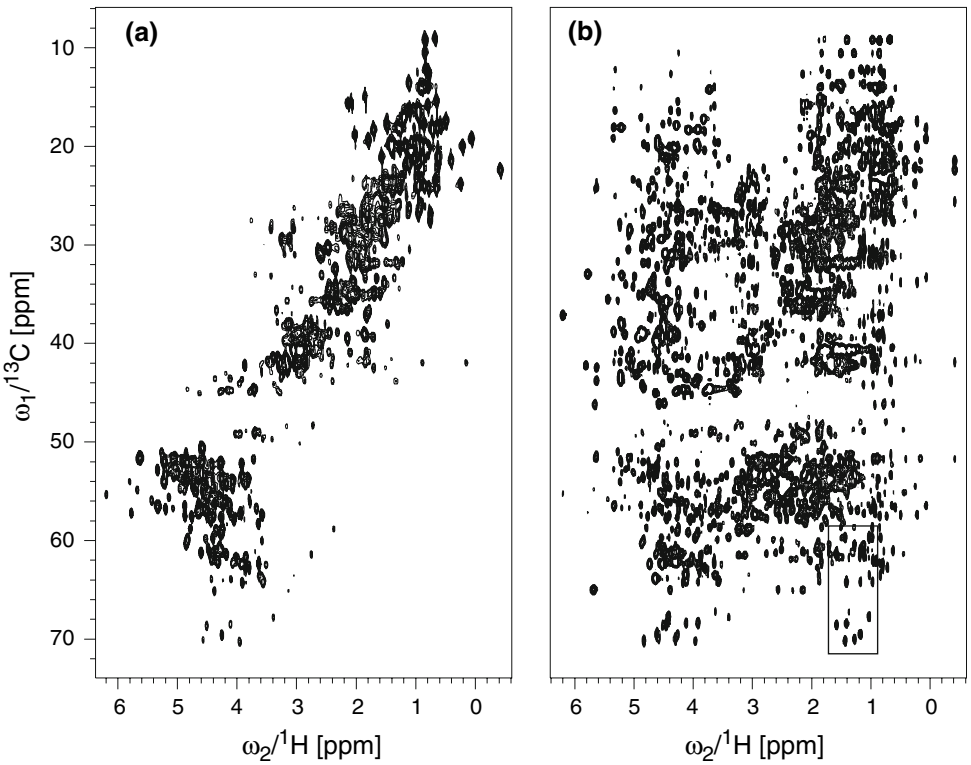


Fig. 8 (**a**) ^{13}C–^{1}H HSQC spectrum of the 154 a.a. heat stable protein from Methanobacterium thermoautotrophicum. (**b**) 2D-^{13}C–^{1}H HCCH-TOCSY spectrum of the 154 a.a. heat stable protein obtained with the AK1 sequence employing a mixing time of 14.4 ms, ^{13}C mixing RF field strength of 10 kHz, recycle time of 2 s, 16 scans per t_1 increment, ^{13}C spectral width of 15 kHz and 128 increments in the indirect ^{13}C dimension. The ^{13}C carrier was kept at 45 ppm

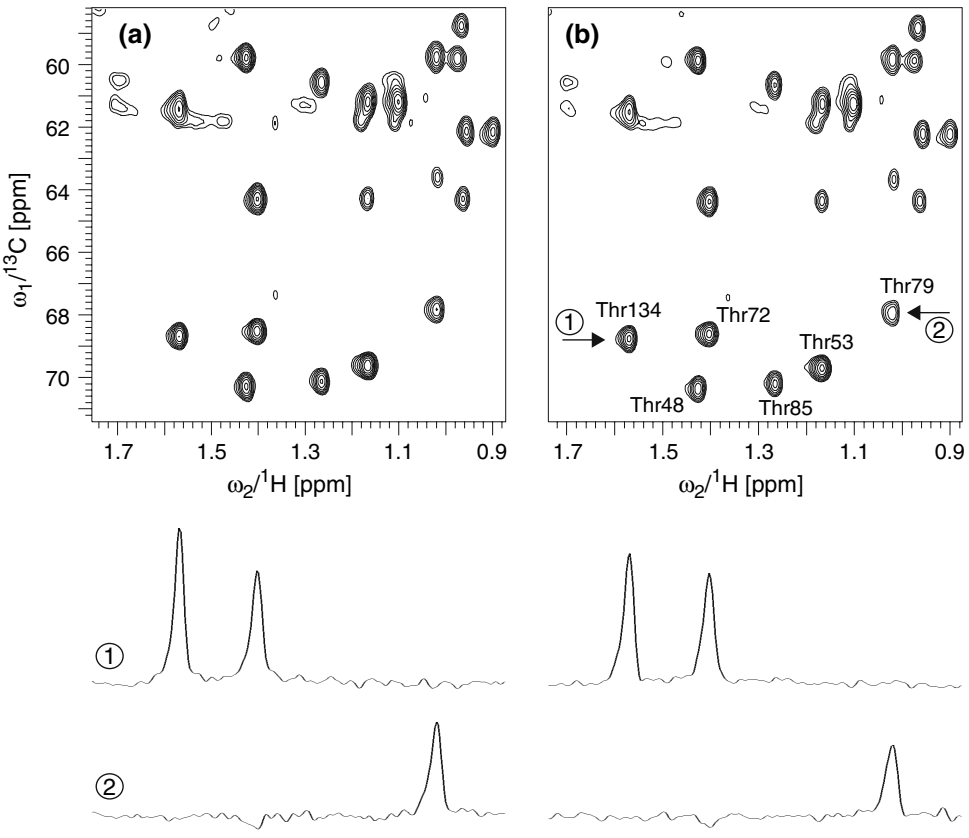


Fig. 9 Expanded plot of the spectral region indicated in Fig. 8b. HCCH-TOCSY spectra were generated with AK1-JCC (**a**) and FLOPSY-m16 (**b**) sequences employing mixing times of 14.4 and 14.14 ms, respectively. In generating the FLOPSY data, the rectangular RF pulses constituting the mixing sequence were applied with an RF field strength of 10 kHz and divided into multiple slices of 200 ns duration. The corresponding ω_2 cross sections taken at cross-peaks arising from $C^{\beta} \rightarrow C^{\gamma}$ magnetisation transfers are also given below

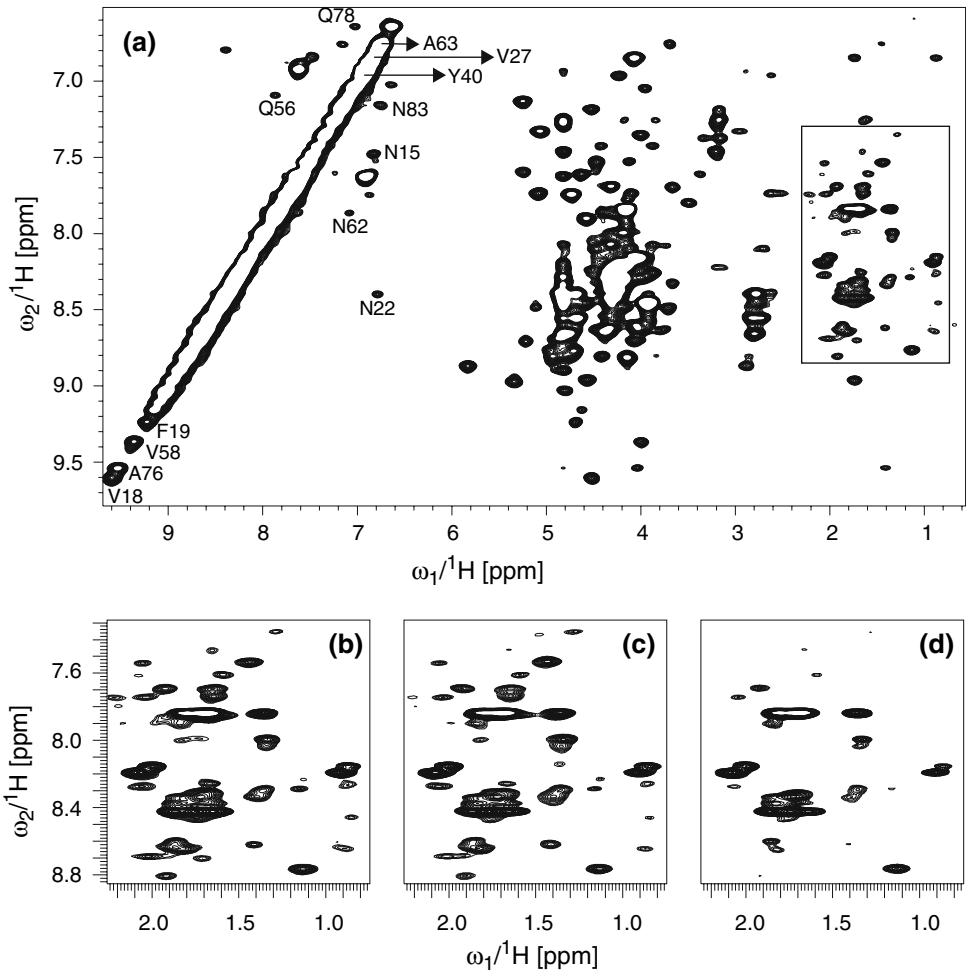


Fig. 10 (**a**) ^{15}N edited clean ^{1}H–^{1}H TOCSY spectrum of the hnRNP C1 protein sample generated with the amplitude and phase modulated sequence AK1-JHH, employing a mixing time of 48 ms, recycle time of 1 s, 32 scans per t_1 increment, ^{1}H spectral width in the indirect dimension of 10 kHz and 128 increments in the indirect ^{1}H dimension. The ^{1}H carrier was kept on resonance with H_2O. Spectra b, c and d represent the expanded plot of the region indicated in Fig. 10a and generated, respectively, using the mixing sequences AK1-JHH ($\tau_{mix} = 48$ ms), clean CITY-xy16 ($\tau_{mix} = 48.384$ ms) and the AK2-JCC ($\tau_{mix} = 48$ ms)

method outlined here can be applied for designing symmetry-based homo- and hetero-nuclear dipolar recoupling schemes (Levitt 2002).

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