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Optimization of Three-Dimensional TROSY-Type HCCH NMR Correlation of Aromatic ^{1}H–^{13}C Groups in Proteins

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Improved methods for three-dimensional TROSY-Type HCCH correlation involving protons of negligible CSA are presented. The TROSY approach differs from the conventional approach of heteronuclear decoupling in evolution and detection periods by not mixing fast and slowly relaxing coherences and usually suppressing the former. Pervushin *et al.* (*J. Am. Chem. Soc.* 120, 6394–6400 (1998)) have proposed a 3D TROSY-type HCCH experiment where the TROSY approach is applied only in one of the ^{13}C dimensions. A new pulse sequence applying the TROSY approach in both indirect dimensions is advantageous when the TROSY effect of the carbons is large or when a relatively high resolution is required. For lower resolutions or moderate TROSY effects we show that it is possible to combine the best of both worlds, namely to suppress heteronuclear couplings without mixing fast and slowly relaxing coherences while at the same time superimpose the two components and thus have both contribute to the detected signal. That is possible using the novel technique of Spin-State-Selective Time-Proportional Phase Incrementation (S^3 TPPI). The new 3D S^3 TPPI TROSY HCCH method is demonstrated on a ^{13}C,^{15}N-labeled protein sample, RAP 18–112 (*N*-terminal domain of α_2-macroglobulin receptor associated protein), at 750 MHz and average sensitivity enhancements of 10% are obtained for the cross peaks in comparison to methods based on conventional decoupling on one of the carbons or on TROSY on both carbons.

Key Words: **multidimensional NMR; S^3 TPPI; TROSY; HCCH.**

Interference between dipole–dipole (DD) and chemical shift anisotropy (CSA) relaxation (*1*) can be exploited for enhancement of resolution and sensitivity in NMR spectroscopy of large biomolecules (*2–4*) at high fields. This effect is particularly attractive in ^{1}H–^{15}N amide groups in the backbone of proteins (*1*) and in aromatic side chains where the ^{13}C nuclei exhibit large CSA (*3*). So far the experiments of this so-called TROSY-type have focused mainly on the effect within two-spin systems. That is fine for the amide groups but for neighboring ^{1}H–^{13}C groups in aromatic spin systems there is more to be gained by optimizing the pulse sequences. The key parameters in this process are the resolution required, the ^{13}C transverse relaxation times, and the size of the TROSY effect (i.e., the difference in ^{13}C linewidths).

For the sensitivity of TROSY in ^{15}N and even more in ^{13}C applications it is important to include the native ^{15}N or ^{13}C magnetization, which enhances the TROSY and reduces the anti-TROSY resonance (*3*). This means that only a moderate TROSY effect can be sufficient to make TROSY more sensitive than the corresponding experiment employing conventional decoupling.

A crucial point when it comes to HCCH correlation in aromatic spin systems is that while it is easy to direct coherence transfer within the heteronuclear two-spin systems in question, so as to optimize transfer between transitions of long transverse relaxation times, there is no spin-state selectivity with respect to protons when transferring coherence between two neighboring ^{13}C nuclei. In other words, the TROSY coherence on one carbon gets transferred equally into TROSY and anti-TROSY resonances on a neighboring carbon, e.g., by application of a $\pi/2$ pulse or TOCSY-type mixing. Hence there is no sensitivity enhancement on the second ^{13}C nucleus corresponding to the one gained by including the native ^{13}C magnetization on the first carbon. Therefore, a larger differential relaxation effect is necessary in order for the TROSY approach on the second carbon to be more sensitive than conventional decoupling.

We can envisage three main types of techniques for TROSY-type HCCH correlations in aromatic spin systems and it is the purpose of this communication to present and compare them experimentally.

The first is the one proposed by Pervushin *et al.* (*3*) that with a few modifications is outlined in Fig. 1a. It consists of application of the TROSY approach to the first carbon and conventional decoupling on the second carbon. That is sensible for an extremely coarse resolution in the dimension of the second carbon. However, for larger proteins the often rather small spectral dispersion of the ^{1}H and ^{13}C resonances in aromatic rings can complicate the resonance assignment and call for good resolution in both ^{13}C dimensions of three-dimensional (3D) HCCH spectra. Under these circumstances conventional decoupling is detrimental to the sensitivity and it can be worthwhile to employ the TROSY approach throughout. That leads to the pulse sequence shown in Fig. 1b that is recommended in

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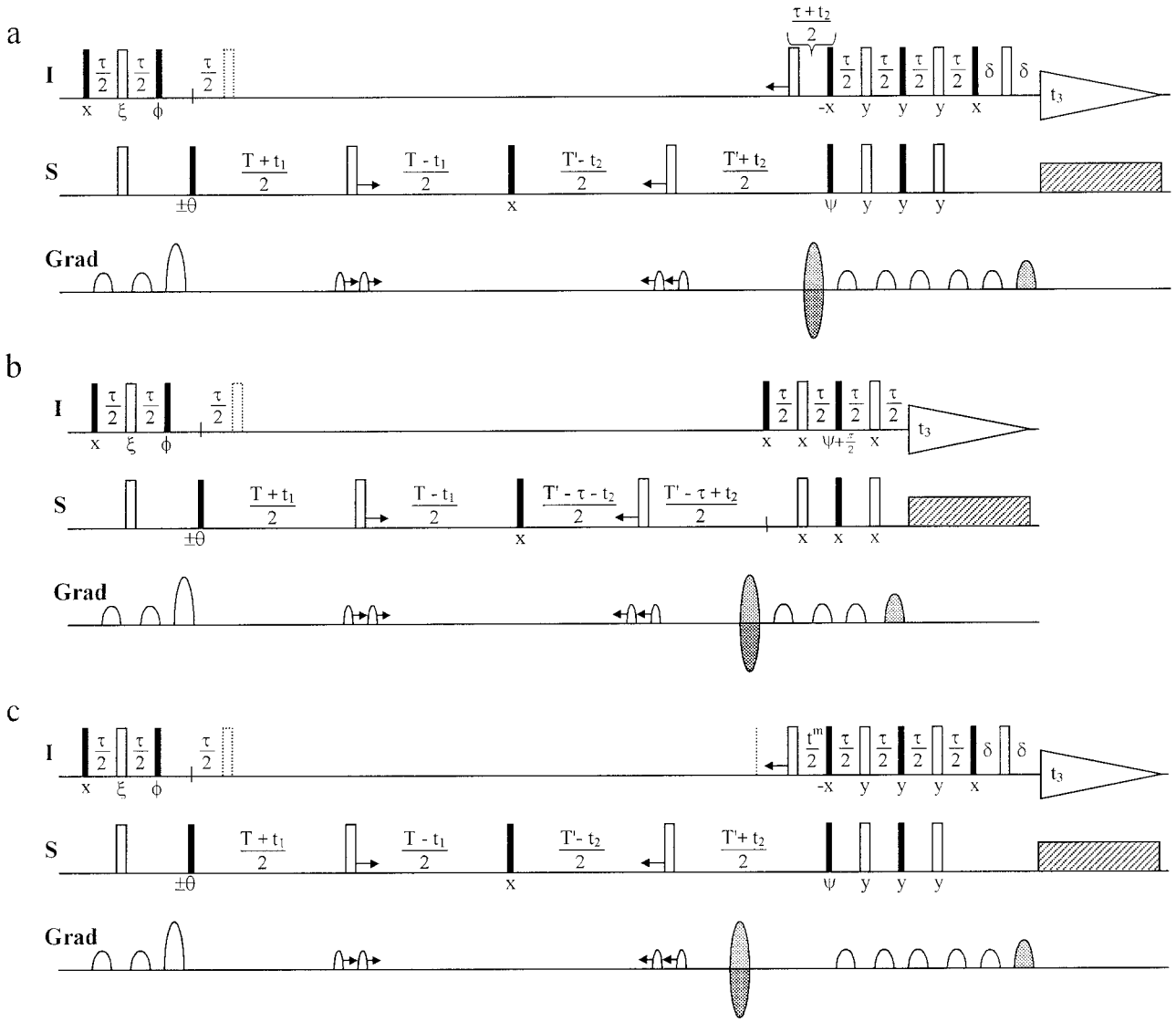

FIG. 1. Pulse sequences for 3D TROSY HCCH correlation. Filled and open bars represent $\pi/2$ and π pulses, respectively. $\tau = (2J_{CH})^{-1}$; $T = T' = \frac{3}{4}(J_{CC})^{-1}$; $t^m/2 = \{\frac{1}{2}(t_2 + \tau)\}\mathrm{mod}(\tau)$; δ = gradient delay. The receiver reference phase is incremented by π at the discontinuities of t^m. In order to include the native S spin magnetization in the TROSY resonance, the phase ξ must be x on our Bruker DRX 600 instrument while it must be y on our Varian Unity Inova spectrometers. In combination with the shaded pulsed field gradients, echo and antiecho data sets are on the Varian instruments recorded with $\psi = -x$ and x, respectively, while for the sequence in (b) it would be reversed on the Bruker instrument. The phase ϕ is $-y$ and θ is x in the scans where the dotted ^{1}H π pulse of the two-step S^3 filter selecting the TROSY resonance is included and otherwise $\phi = y$ and $\theta = -y$ (on the Varian spectrometers). The individual steps of the S^3 filter are coadded at constant receiver phase. In addition to the S^3 filter the only phase cycle is 0, π of the first S spin $\pi/2$ pulse along with alternating receiver phase. (a) 3D TROSY HCCH pulse sequence with conventional decoupling in t_2, (b) 3D TROSY HCCH pulse sequence with TROSY $S \rightarrow I$ mixing, and (c) 3D S^3 TPPI TROSY HCCH for superimposing TROSY and anti-TROSY resonances in t_2.

the case of very large TROSY effects and more so if high resolution is required in the F_2 dimension.

As mentioned above, this sequence has the drawback that half the original magnetization on the TROSY resonance of the first carbon does not contribute to the detected signal, which represents a loss in case of moderate TROSY effects. The pulse sequence in Fig. 1c retains the anti-TROSY contribution and also the TROSY enhancement by applying Spin-State-Selective TPPI (S^3 TPPI) (*5*). By S^3 TPPI the two ^{13}C magnetization components corresponding to the directly attached proton being in the α and β states, respectively, are given different TPPIs so that their effective precession frequencies are the decoupled ^{13}C chemical shift. This is achieved in a delay τ of the second constant time period with transverse ^{13}C magnetization where a ^{1}H π pulse is moved to the left at the same speed as the ^{13}C π pulse. It starts in the middle of the τ delay

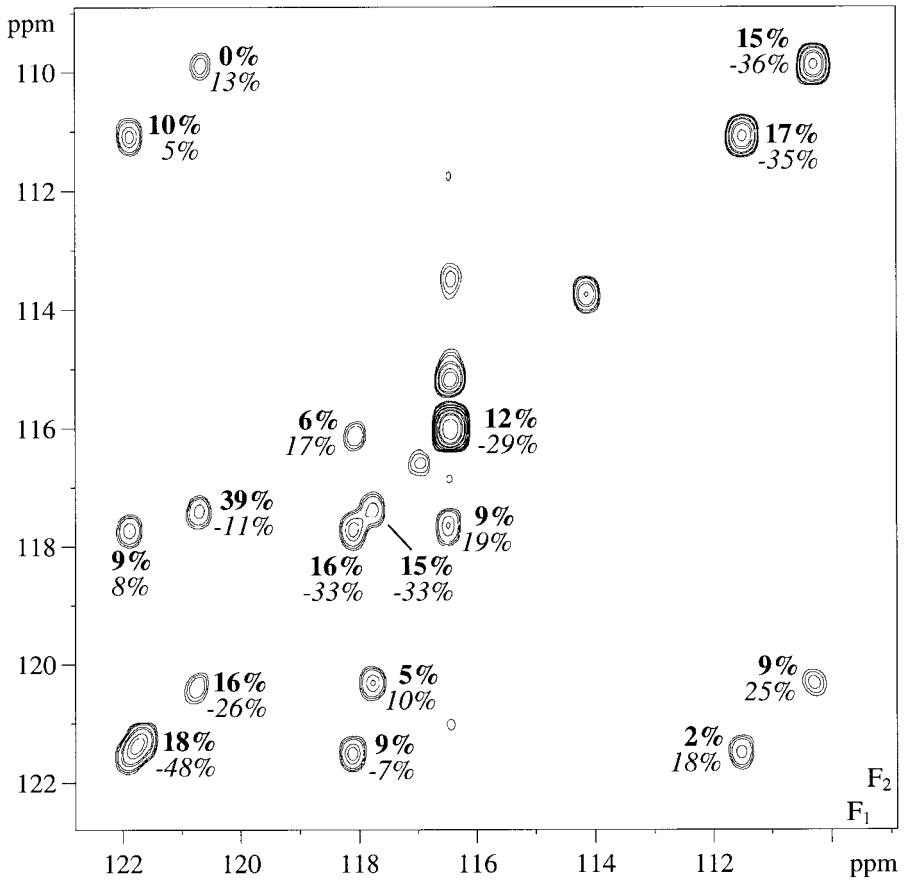

FIG. 2. Excerpt from the ^{13}C–^{13}C COSY-type F_1/F_2 projection of the aromatic region of the 3D S^3 TPPI TROSY HCCH spectrum of ^{15}N,^{13}C-labeled RAP 18–112 (90% H_2O/10% D_2O, 25°C, pH 6.4) recorded with the sequence in Fig. 1c on a Varian Unity Inova 750 MHz spectrometer. Parameters: relaxation delay 1.5 s with water presaturation, $T = T' = 13.2$ ms; $\tau = 3.21$ ms; t_1(max) = 12.12 ms; t_2(max) = 9.04 ms; 4 scans. GARP was used for ^{13}C decoupling in t_3. Data matrices of 128 × 96 × 2048 points covering 5200 × 5200 × 10000 Hz were zero-filled to 1024 × 1024 × 4096 prior to Fourier transformation and the window functions were cosine in all three dimensions. The numbers next to the signals indicate the sensitivity enhancement of S^3 TPPI TROSY HCCH relative to the corresponding signals from the spectra recorded with the sequence in Fig. 1a (bold) and 1b (italic), respectively, and identical parameters.

and when it reaches the left end it is restarted at the right end and the receiver reference phase incremented by π, i.e., $t^m/2 = \{\frac{1}{2}(t_2 + \tau)\}\mathrm{mod}(\tau)$. Because both components are transferred to the attached proton, a planar mixing sequence (*6–8*) rather than Double Spin-State-Selective Coherence Transfer (S^3CT) (*4*, *9*) is applied.

S^3 TPPI in the pulse sequence in Fig. 1c can be understood as a modification of the conventional scheme for heteronuclear decoupling in constant time evolution periods as, e.g., in the t_2 period of the sequence in Fig. 1a. The fact that the I and S π pulses move in parallel ensures heteronuclear decoupling. It starts out in the same way in the t_2 S^3 TPPI sequence in Fig. 1c until the π^I pulse reaches the dotted line. By increasing the distance between the two π pulses by $\tau = (2J)^{-1}$ at this point there is an additional J evolution of 2τ which leaves the S-spin magnetization invariant apart from a sign change. This sign change is taken into account by a π phase shift of the receiver reference phase, but heteronuclear decoupling is still warranted by continuing to move the two π pulses in parallel with the greater distance between them. When the π^I pulse again reaches the dotted line the procedure of increasing the interpulse distance by τ and the receiver phase by π is repeated. The points where the interpulse distance is increased by τ represent discontinuities in the signal envelope function, the extent of which depends on the difference in transverse relaxation times of the two doublet components. For large differences when this can become intolerable the sequence in Fig. 1b is the one of choice.

All three pulse sequences in Fig. 1 include a simple Spin-State-Selective (S^3) filter that selects the TROSY resonance and eliminates the anti-TROSY resonance of the first carbon. It consists of a ^1H π pulse applied in alternate scans along with appropriate phase settings of the ^1H and ^{13}C $\pi/2$ pulses preceding the t_1 period. Stronger filters are available but because the anti-TROSY resonance is of low intensity in

aromatic $^{1}H–^{13}C$ systems (*3*) it will in most cases be sufficient. When the anti-TROSY resonance intensity on the first carbon is not negligible, S^3 TPPI could also be applied in t_1 instead of the S^3 filter in order to superimpose both components.

An experimental comparison of the three pulse sequences in Fig. 1 was carried out on a Varian Unity Inova 750 MHz spectrometer using a $^{13}C,^{15}N$-labeled protein, RAP 18–112 (N-terminal domain of α_2-macroglobulin receptor associated protein) (*10*). Figure 2 shows an excerpt from the two-dimensional (2D) $^{13}C–^{13}C$ COSY-type projection of the 3D spectrum recorded with the 3D S^3 TPPI TROSY HCCH pulse sequence in Fig. 1c. Next to the peaks are indicated the sensitivity enhancements of S^3 TPPI TROSY HCCH relative to the sequences in Figs. 1a (boldface) and 1b (italic), respectively, all recorded and processed under identical conditions. These numbers were obtained by adding up sections above a threshold intensity (about 25% of the smallest cross peak) around the peaks, so that the number of coadded sections was the same for the peaks compared but varied across the spectrum. The average sensitivity enhancements for the cross peaks in the S^3 TPPI TROSY HCCH spectrum compared to the spectra recorded with the sequences in Figs. 1a and 1b were in both cases 10%. An interesting feature is that the ratio of diagonal to cross peaks is least favorable in the all-TROSY spectrum.

In conclusion, we have presented improved methods for TROSY-type HCCH correlation in aromatic side chains of proteins. For large TROSY effects the anti-TROSY coherence of the second carbon should be eliminated and no form of ^{1}H decoupling employed. In contrast, for moderate TROSY effects, the sensitivity is enhanced when the TROSY and anti-TROSY coherences are superimposed by S^3 TPPI.

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