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Suppression of Diagonal Peaks in Three-Dimensional Protein NMR TROSY-Type HCCH Correlation Experiments

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A novel method for suppression of ^{13}C–^{13}C diagonal peaks without sensitivity loss in three-dimensional HCCH TROSY-type NMR correlation experiments involving aromatic side chains in proteins (Pervushin *et al., J. Am. Chem. Soc.* 120, 6394–6400 (1998)) is presented. The key element is a spin-state-selective filter in the ^{13}C–^{13}C mixing sequence with the dual effect of selecting the TROSY resonance in the preceding evolution period and interchanging TROSY and anti-TROSY resonances. The cross peaks are invariant to this filter but diagonal peak coherence gets concentrated on the anti-TROSY transition so that it can be eliminated by a $^{13}C \rightarrow {}^{1}H$ TROSY transfer element. The new method is demonstrated using a ^{13}C,^{15}N-labeled protein sample, RAP 18-112 (N-terminal domain of α_2-macroglobulin receptor associated protein), at 750 MHz. © 2000 Academic Press

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Diagonal peak suppression has been a subject of long-standing interest in multidimensional NMR. It can easily be achieved when the two spins to be correlated are of different isotopes or are attached to different isotopes like, e.g., proton correlation in $\{(^1H^{13}C)$–$(^1H^{15}N)\}$ systems. However, it was only recently that effective means of diagonal peak suppression in ^1H NOESY of $\{(^1H^{15}N)$–$(^1H^{15}N)\}$ systems were introduced (*1–4*). These methods employ the TROSY approach (*5*) and hence are particularly attractive for large molecules at high fields.

In this Communication, we show that virtually perfect diagonal peak suppression can be achieved in another important experiment in protein NMR, namely HCCH correlation in aromatic side chains. Pervushin *et al.* (*6*) showed that by incorporating TROSY-type evolution the sensitivity of this experiment could be improved by a factor 4–10 while a later paper improved the sensitivity further by a theoretical factor $\sqrt{2}$ (*7*). (Note that the sensitivity enhancement factor 2 (ignoring relaxation and pulse imperfections) in Ref. (*7*) only holds when heteronuclear gradient echoes are included in both experiments. Otherwise, the factor is $\sqrt{2}$ and the average experimental gain only about 22%.) That is very handy as the assignment of the aromatic spin systems is of critical importance for high-quality structures and often represents a severe bottleneck. However, this bottleneck is not only a matter of sensitivity but also the fact that there usually is a rather small spectral dispersion of the ^1H and ^{13}C resonances in aromatic rings, which complicates the assignment. Due to this narrow spectral range of resonances, cross peaks tend to be quite close to the ^{13}C–^{13}C diagonal so it would be a further enhancement of this technique if the uninformative diagonal peaks could be suppressed. We show that this is indeed possible and even at zero penalty with respect to cross peak sensitivity compared to the earlier TROSY–HCCH experiments (*6, 8*).

In analogy to diagonal peak suppression in TROSY–NOESY experiments the novel approach for diagonal peak suppression in three-dimensional (3D) HCCH TROSY-type ^1H–^{13}C correlation is based on a combination of control and lack of control of coherence transfer processes in the relevant spin systems. While it is easy to control coherence transfer within the ^1H–^{13}C two-spin systems there is no spin-state selectivity when transferring coherence between the ^{13}C nuclei of ^1H–^{13}C systems via ^{13}C–^{13}C couplings by the usual methods of a $\pi/2$ pulse or a multipulse sequence.

Three-dimensional TROSY HCCH experiments start by directing most of the available magnetization into the TROSY resonance of the first ^{13}C spin by exploiting both the native ^1H and ^{13}C magnetizations (*6*). The polarization of the anti-TROSY resonance is often small relative to the TROSY resonance so that suppression of it can be unnecessary (*6*). On the other hand, it is an integral part of our scheme for diagonal peak suppression to also suppress the anti-TROSY resonance (vide infra). This feature is crucial as these anti-TROSY contributions otherwise would lead to diagonal signals.

In the succeeding ^{13}C–^{13}C coherence transfer process, part of the coherence gets transferred to a neighboring carbon but inevitably some of it remains on the first carbon and that represents a potential diagonal peak contribution. This contribution is still on the TROSY resonance but in the cross peaks of the neighboring carbon the coherence is distributed equally on the TROSY and anti-TROSY resonances. This means that a ^1H π pulse simultaneous with the ^{13}C–^{13}C coherence transfer process leaves the cross peaks invariant but in the diagonal

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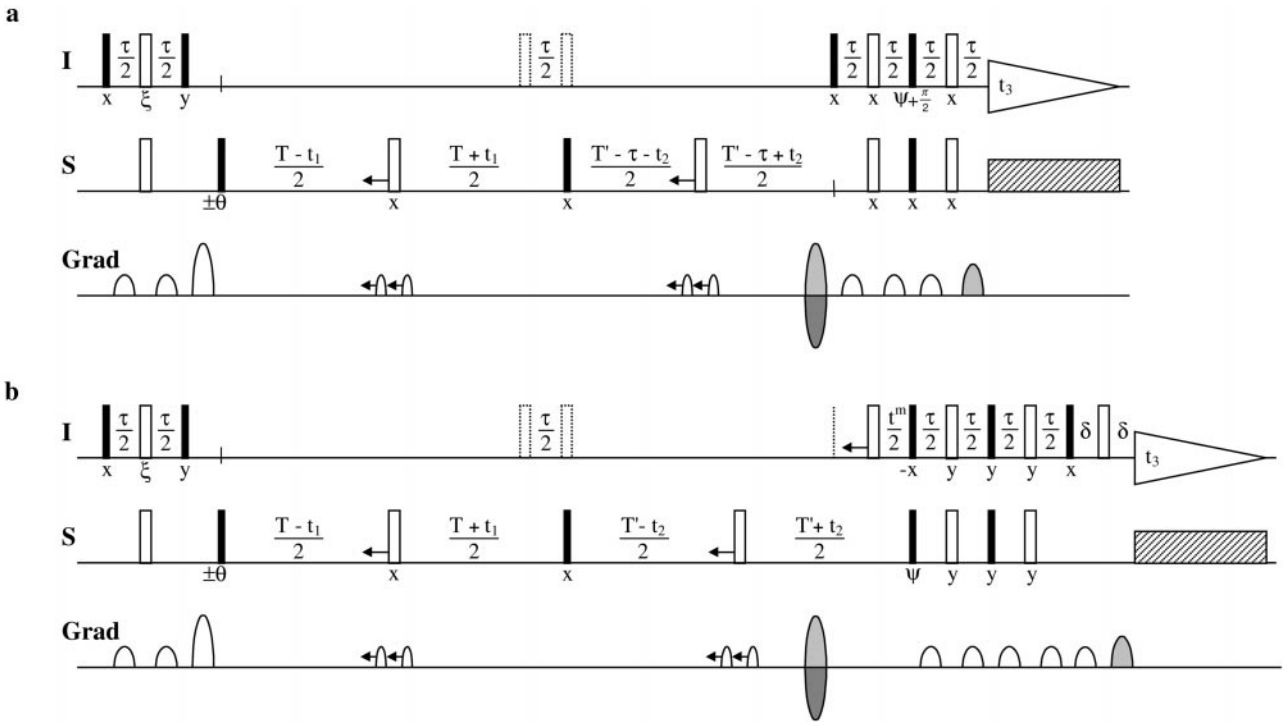

FIG. 1. Pulse sequences for 3D TROSY HCCH correlation with diagonal peak suppression. Filled and open bars represent $\pi/2$ and π pulses, respectively. $\tau = (2J_{CH})^{-1}$; $T \approx T' \approx (2n + 1)/4(J_{CC})^{-1}$; $t^m/2 = \{\frac{1}{2}(t_2 + \tau)\}\mathrm{mod}(\tau)$; δ = gradient delay. The receiver reference phase is incremented by π at the discontinuities of t^m (*10*). 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 (a) it would be reversed on the Bruker instrument. The dotted π pulse of the two-step (data coadded at constant receiver phase) S^3 filter is combined with $\theta = x$ and $\theta = y$ when it is at its left and right positions, respectively. (a) 3D TROSY HCCH pulse sequence with TROSY S $\rightarrow$ I mixing. (b) 3D S^3 TPPI TROSY HCCH for superimposing TROSY and anti-TROSY resonances in t_2. The relative gradient strengths used are 1.998 and 1.25 for the shaded gradients, 0.5 for those in the evolution periods, 1 for those in the preparation sequence, 0.75 for those in the final mixing sequence, and 10 for the purge gradient before the first $\pi/2$ *S*-spin pulse. 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.

peaks transfers the TROSY coherence to the anti-TROSY resonance.

This ^{1}H π pulse is conveniently realized as part of a spin-state-selective (S^3) filter selecting the TROSY resonance in the preceding t_1 evolution period as shown in the pulse sequence in Fig. 1a. The S^3 filter is similar to the S^3P technique (*9*) and consists of a two-step cycle of experiments with the ^{1}H π pulse placed at two different positions separated by $(4J_{CH})^{-1}$ and combined with appropriate setting of θ. Because the diagonal contributions are concentrated in the anti-TROSY resonances in the t_2 evolution period, a TROSY coherence transfer between ^{13}C in the t_2 evolution period and ^{1}H for acquisition in t_3 (Fig. 1a) will eliminate diagonal peaks.

However, unless the TROSY effect is very large it can for the sensitivity of the cross peaks be worthwhile also to retain the 50% of the magnetization on the anti-TROSY resonances in the t_2 evolution period. That is done in the sequence of Fig. 1b, where Spin-State-Selective TPPI (S^3 TPPI) (*8, 10*) is used to superimpose TROSY and anti-TROSY coherences without mixing them as conventional decoupling would do. In this sequence the diagonal is not eliminated but only suppressed due to faster relaxation of the selected diagonal anti-TROSY coherences in t_2.

The new method for diagonal peak suppression was tested on a ^{13}C,^{15}N-labeled protein sample, RAP 18-112 (N-terminal domain of α_2-macroglobulin receptor associated protein) (*11*), using a Varian Unity Inova 750 MHz spectrometer. For illustration, the 3D spectra were projected onto the ^{13}C–^{13}C 2D plane with the results shown in Fig. 2. The two spectra were recorded with the scheme in Fig. 1a (right) with and (left) without diagonal peak suppression; the latter included an S^3 filter placed in the beginning of the first evolution period to suppress the anti-TROSY coherence in the t_1 period (*6, 8*). As is evident from the 1D sections shown, a very high degree of diagonal peak suppression is achieved by the new method at no cost in cross-peak intensity. An interesting feature is seen for the large signal around 115.5 ppm from a Histidine residue exhibiting truncation wiggles in the normal spectrum. In contrast, this signal is reduced to 6% of its original amplitude in the spectrum with the diagonal peaks suppressed. The residual

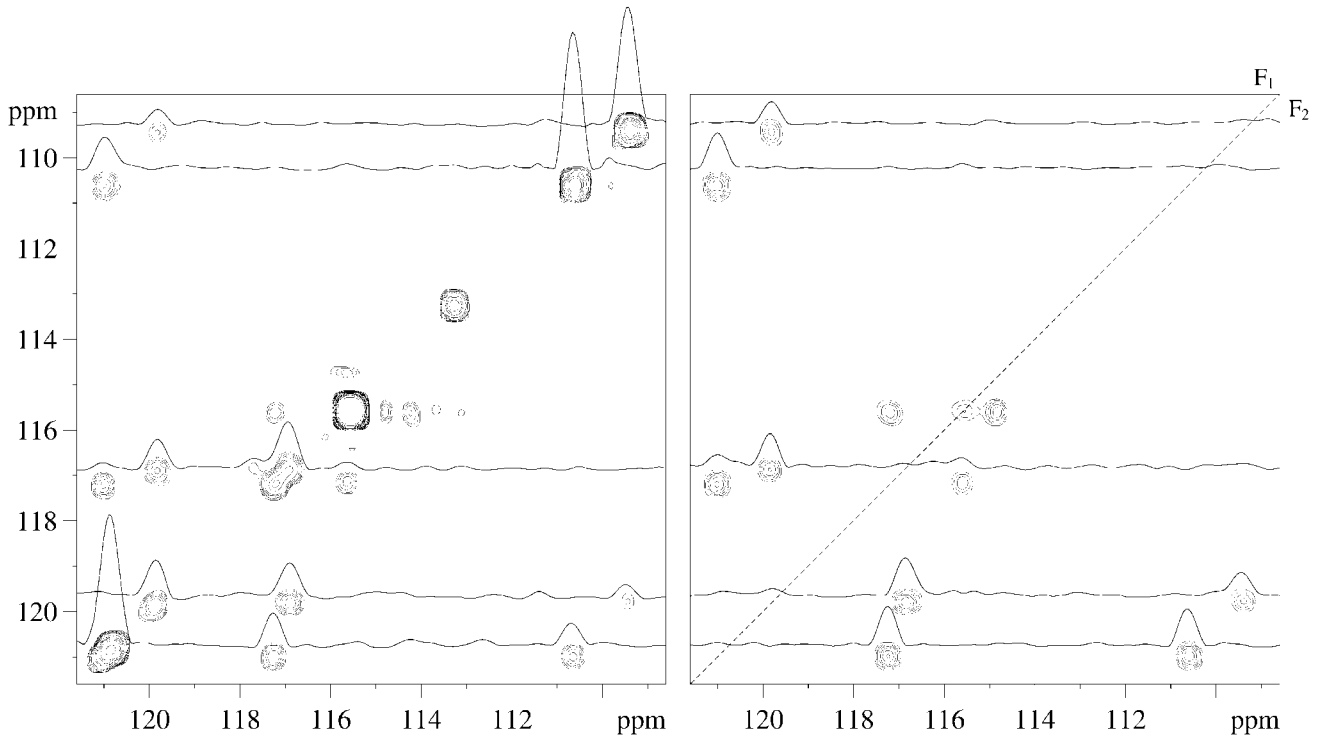

FIG. 2. Excerpts from the ^{13}C–^{13}C COSY-type F_1/F_2 projection of the aromatic region of 3D TROSY HCCH spectra (positive levels only) of ^{15}N, ^{13}C-labeled RAP 18-112 (90% H_2O/10%D_2O, 25°C, pH 6.4) recorded with the pulse sequence in Fig. 1a on a Varian Unity Inova 750 MHz spectrometer. The spectrum on the left was recorded without the diagonal peak suppression scheme but with an S^3 filter selecting the TROSY coherence in t_1 while the spectrum on the right included diagonal peak suppression. Parameters for both experiments: relaxation delay 1.5 s with water presaturation, $T = T' = 13.2$ ms; $\tau = 3.21$ ms; $t_1(\max) = 9.81$ ms; $t_2(\max) = 9.04$ ms; 8 scans. GARP was used for ^{13}C decoupling in t_3. Data matrices of $104 \times 96 \times 2048$ points covering $5200 \times 5200 \times 10000$ Hz were zero-filled to $512 \times 512 \times 1024$ prior to Fourier transformation and the window function was cosine in all three dimensions. Representative sections along the F_1 dimension are shown in the spectra. The dashed line in the spectrum on the right indicates the diagonal.

on the very diagonal is caused by imperfection of the central π^H pulse while the component at slightly lower F_1 frequency is the positive part of a dispersive peak centered $J^{His} = 202$ Hz lower than the diagonal in F_1 and representing J cross talk. (The τ delays were set to $(2 \times 155\ \text{Hz})^{-1}$.) Another negative absorptive signal J cross talk signal displaced J in F_2 is not plotted. These artifacts could be reduced by a composite pulse and more elaborate higher order J filters but that was not considered because it would prolong the pulse sequence and because the artifacts can be identified by their displacements from the diagonal.

Both the original (*6*, *8*) and the new experiments with diagonal peak suppression are susceptible to strong coupling effects between the ^{13}C spins in the two constant-time evolution periods. However, simulations show that even for strong coupling the new techniques offer a high degree of diagonal peak suppression and allow identification of cross peaks otherwise obscured by the diagonal.

Although it was not necessary in the present application we should mention the possibility to compensate the S^3 filter for the difference in transverse relaxation times between the TROSY and anti-TROSY coherences. The intensity of the anti-TROSY coherence to be suppressed is enhanced by a factor

$$\exp\left\{\frac{\tau}{2}\left(\frac{1}{T_2^{\text{anti-TROSY}}} - \frac{1}{T_2^{\text{TROSY}}}\right)\right\}$$

in the experiment with the dotted π^H pulse at its left position compared to the one with this pulse at its right position. Such corrections are unnecessary when the intensity of the anti-TROSY coherence is low or when the TROSY effect is small. For very large TROSY effects (i.e., very fast relaxation of the anti-TROSY coherence) it is sufficient to only perform the experiment with the dotted π^H pulse at its right position.

In conclusion, we have introduced an efficient method for ^{13}C–^{13}C diagonal peak suppression without sensitivity loss for cross peaks in 3D HCCH TROSY-type NMR correlation experiments involving aromatic side chains in proteins. TROSY boosts the sensitivity of this crucial technique and diagonal peak suppression alleviates the complication associated with a

rather small spectral dispersion of the ^{1}H and ^{13}C resonances in aromatic rings.

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