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Sensitivity- and Gradient-Enhanced Hetero (ω_1) Half-Filtered TOCSY Experiment for Measuring Long-Range Heteronuclear Coupling Constants

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An enhanced version of the X(ω_1) half-filtered TOCSY experiment for measurement of long-range heteronuclear coupling constants is proposed which yields high-quality spectra with substantially increased sensitivity and resolution. The modified method features gradient-enhanced X filtering sequences, broadband homonuclear decoupling during t_1, optional $^1J_{XH}$ scaling in the F_1 domain, and gradient coherence selection in combination with the sensitivity-enhanced protocol for the TOCSY transfer. These modifications extend the applicability of the method—coupling constants can be measured accurately for natural abundance samples at low concentrations and for compounds yielding complex spectra. Computer-aided analysis of E.COSY-type multiplets is applied for the determination of heteronuclear long-range coupling constants.

Key Words: **X(ω_1) half-filtered TOCSY; gradient-enhanced X filtering; echo–antiecho selection; sensitivity enhancement; long-range heteronuclear couplings.**

INTRODUCTION

The measurement of heteronuclear long-range coupling constants at natural abundance of the heteronuclei is always a challenging task due to inherently low sensitivity. Several proton-detected long-range heterocorrelated experiments (*1*) have been proposed during the past few years aimed at improving sensitivity and reliability in the determination of such coupling constants. For example, X(ω_1) half-filtered proton–proton correlation (*2–7*) offers a straightforward method for the determination of long-range heteronuclear coupling constants from E.COSY multiplets (*8*). Using this approach coupling constants smaller than the natural linewidth of proton multiplets can be obtained from a single experiment without acquiring reference multiplets. A number of applications of the X(ω_1) half-filtered TOCSY (HETLOC) experiment for resolving structural and conformational ambiguities which cannot be eliminated solely by using the homonuclear coupling data (*2–7, 9–11*) have been published. However, there are still concerns regarding suppression of the ^{12}C-bound protons, sensitivity of the experiment, and accidental overlap of E.COSY multiplets.

It is demonstrated here that with appropriate modifications it is possible to improve upon the original HETLOC experiment (*2*) with respect to all of these problem areas. Protons bound to the magnetically nonactive heteronuclei are effectively suppressed by X filtering schemes employing pulsed field gradients (PFGs). The sensitivity of the experiment is increased by a G-BIRD^r pulse applied during the t_1 interval, giving rise to a broadband homonuclear decoupling in F_1. The evolution of one-bond heteronuclear coupling required for the generation of E.COSY multiplets is not affected by this pulse and simplified multiplets in F_1 reduce significantly the probability of overlaps. Even so, a coincidental overlap of E.COSY multiplets cannot be excluded completely and an optional scaling of the one-bond heteronuclear splitting in F_1 with an arbitrary factor may be used. Finally, the use of sensitivity-enhanced TOCSY transfer with the aid of PFGs further improves the sensitivity of the experiment. The proposed modifications shown in Fig. 1 are illustrated using a trisaccharide (**1**) (*12*) and a linear heptapeptide Tyr-D-Ala-β-iPrPhe-Asp-Val-Val-Gly (**2**).

RESULTS AND DISCUSSION

The quality of the X(ω_1) half-filtered spectra depends upon the efficiency of the applied X filtering scheme. Originally (*2–4*), elimination of signals arising from ^{12}C-bound protons was achieved by the BIRD presaturation of protons attached to ^{12}C (*13, 14*) followed by an X(ω_1) half-filter (*15*). This method is not recommended for large molecules where the negative NOEs would decrease the sensitivity of the experiment.

Recently, a quad G-BIRD sequence introduced by Shaka and co-workers (*16*) was applied successfully for suppres-

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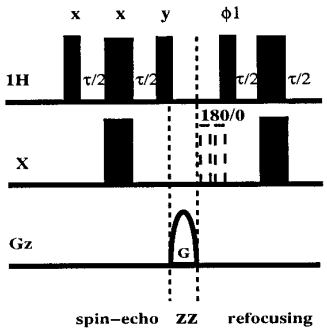
(a)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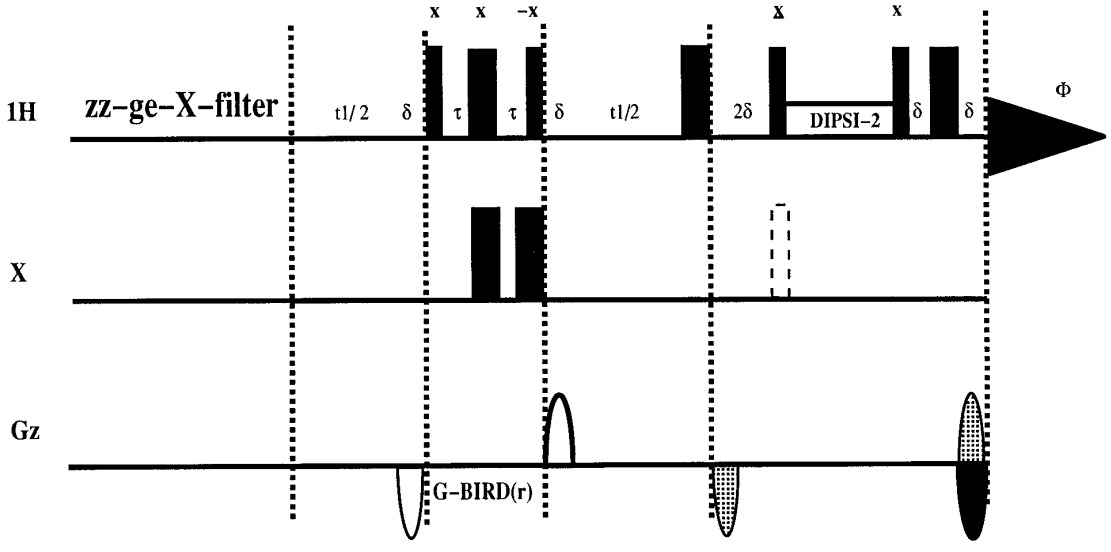
(b)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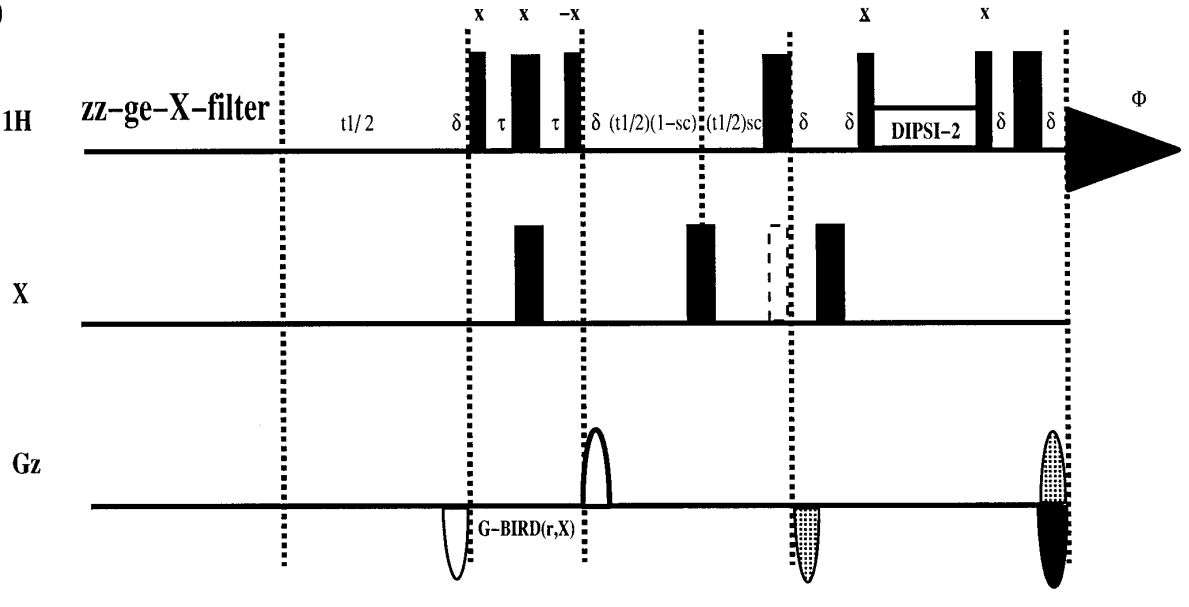
(c)


x x –x x x
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t1/2 δ τ τ δ (t1/2)(1–sc) (t1/2)sc δ δ DIPSI–2 δ δ
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Gz
G–BIRD(r,X)

sion of remote protons in the X(ω_1) half-filtered TOCSY experiment (*7*) without the use of the classical X filter. This filtering scheme is rather complex and its overall length, typically 30 ms, might become a source of signal losses due to the T_2 relaxation. From this perspective it is preferable to use a double G-BIRD sequence; the number of pulses and delays is only half that of the quad G-BIRD pulse. The use of the double G-BIRD pulse on its own is not sufficient since the signals from remote protons are reduced only to the level of satellite signals (*16*) and additional measures must be taken to eliminate them completely. In our sequence, such an additional suppression element is present in the form of a single G-BIRD^r pulse (*17*) in the middle of the t_1 interval. Although applied primarily for a different purpose, this pulse attenuates the magnetization of remote protons to the level where the inclusion of the classical X filter becomes unnecessary.

The filtration scheme which performed best in our experiments is a combination of the *zz* filter (*18, 19*) and the X filter (*15*) without the initial BIRD pulse (Fig. 1a). Typically, a 20-fold decrease in the intensity of signals of remote protons was observed in a one-scan X filter experiment. The remaining magnetization of remote protons not eliminated by the PFG was removed by phase cycling of the X filter. Further improvement was observed when the two-scan phase cycle of the X filter was extended to four steps, including a phase inversion of the 90° ^{1}H excitation pulse ($\phi 1$) prior to the τ refocusing period together with an inversion of the receiver phase.

The *zz*-ge X filter (typically 7 ms) is half as long as the double G-BIRD pulse and contains less pulses, suggesting a superior performance for small and large molecules. For medium-size molecules we did not observe a significant difference in the performance between the two methods, although, it needs to be emphasized that the additional single G-BIRD^r pulse applied in the middle of t_1 is required for the first method, while the *zz*-ge X filter is sufficient on its own. As a generally applicable method the *zz*-ge X filter is recommended and only examples using this method are presented below.

The second modification, incorporation of a broadband homonuclear decoupling during the t_1 period, serves multiple purposes and results in higher sensitivity of the experiment, better resolution, and improved suppression of remote protons. A BIRD^r pulse (*17*) surrounded by two gradient pulses of opposite signs is applied in the middle of the t_1 interval (Fig. 1b). We refer to this pulse as a G-BIRD^r pulse, the superscript r indicating that only remote protons and neither directly attached protons nor the X nuclei are inverted by this pulse. The outcome of this pulse is effective refocusing of homonuclear coupling evolution of directly attached protons, whereas the heteronuclear one-bond couplings continue to evolve. As a result simplified E.COSY cross peaks with higher intensities and resolution are obtained which are split only by the one-bond heteronuclear coupling constants in F_1 as is illustrated on comparison of partial ^{13}C(ω_1)-filtered TOCSY spectra of **1** in Fig. 2.

The chemical-shift evolution of the directly attached protons is not affected by the G-BIRD^r pulse; their magnetization is dephased by the first gradient and rephased by the second gradient of the opposite sign. On the other hand, the magnetization of remote protons is inverted by the G-BIRD^r pulse and therefore continues to dephase due to the second PFG. This additional suppression of remote protons improves the quality of the spectra regardless of the suppression scheme chosen; however, as discussed above, it is necessary when the initial double G-BIRD is used. Proton chemical shift evolution and the evolution of one-bond heteronuclear coupling during the PFGs is eliminated by a 180° ^{1}H pulse applied after the t_1 period.

Although the introduction of homonuclear decoupling significantly increases resolution in F_1, coincidental overlap of E.COSY multiplets may still occur, precluding evaluation of long-range coupling constants. To overcome this limitation, we have implemented scaling of $^1J_{XH}$ coupling constants in F_1 by an arbitrary scaling factor (*20*). The pulse sequence including the $^1J_{XH}$ scaling is shown in Fig. 1c. Here, the second 180° X pulse of the G-BIRD^r sequence is shifted and applied during the second half of the t_1 period. Its actual position within the second $t_1/2$ interval depends on the desired scaling of the heteronuclear coupling with respect to the chemical-shift evolution. The residual splitting is determined by the effective time of the heteronuclear coupling evolution during t_1 which is, using the notation of Fig. 1c, equal to $t_1 * sc$, where sc stands for the scaling factor. The 180° ^{1}H pulse applied at the end of the t_1 period eliminates the proton chemical-shift evolution during the pulsed field gradients. A 180° X pulse is needed in the middle of

FIG. 1. Pulse sequences for the sensitivity- and gradient-enhanced X(ω_1) half-filtered TOCSY experiments including G-BIRD^r for broadband homonuclear decoupling in F_1. Thin and thick bars represent 90° and 180° pulses, respectively; $\tau = 1/(2^1J_{XH})$. (a) *zz* gradient-enhanced X filter. This block precedes both (b) and (c) pulse sequences. Details of phase cycling are discussed in the text. (b) Modified HETLOC pulse sequence. Echo–antiecho signals are obtained by alternately inverting the amplitude of the last gradient pulse and the phase of the 90° ^{1}H pulse applied prior to the DIPSI-2 sequence (*27*) for consecutive FIDs. Sine bell-shaped *z*-gradient pulses of duration of 1 ms and 4 G/cm were applied for coherence selection. The purging gradients of the same duration (1 ms) were 2–2.5 times stronger. Proton chemical-shift evolution and the evolution of $^1J_{XH}$ during PFGs is eliminated by a 180° ^{1}H pulse applied after the t_1 and a subsequent delay of 2δ, where δ (1.2 ms) is equal to the duration of PFG and the recovery delay. (c) Pulse sequence incorporating scaling of the $^1J_{XH}$ coupling constants. Details of the pulse sequence and the phase cycling are given in the text. Note that by applying the dashed 180° X pulse the tilting of E.COSY cross peaks can be reversed.

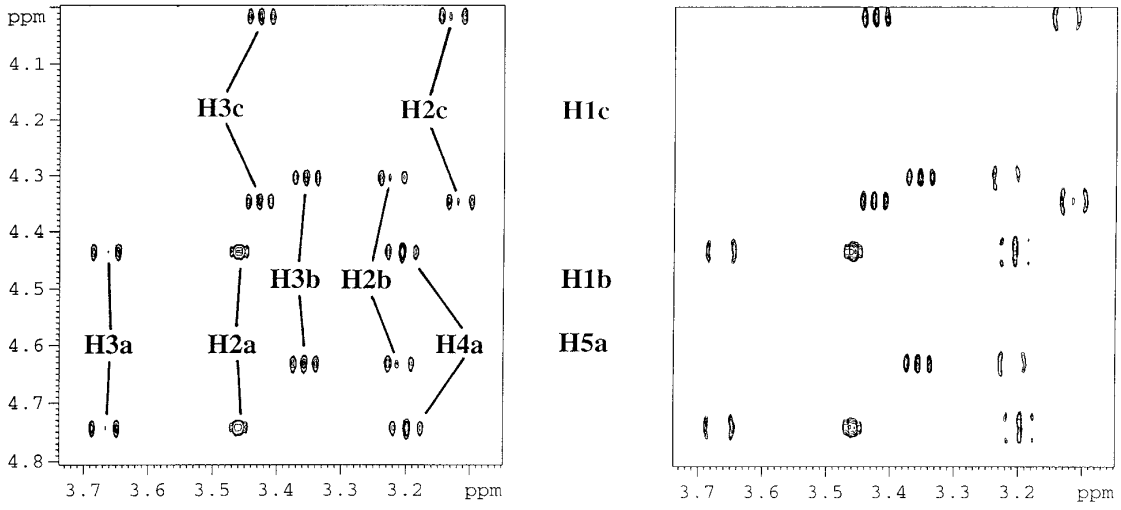

FIG. 2. A comparison of $^{13}C(\omega_1)$ half-filtered TOCSY spectra of **1** (6.5 mg/0.5 ml D_2O) acquired using the pulse sequence of Fig. 1b (left) and the original $X(\omega_1)$ half-filtered TOCSY experiment (right). Forty-eight transients were accumulated for each of 256 t_1 increments with a relaxation delay of 1.8 s; τ delay was set to 3.3 ms; isotropic mixing time was 52 ms. All PFGs were 1 ms and followed by a 200-μs delay (δ = 1.2 ms). The spectral width was 1400 Hz in both dimensions; 1024 complex data points were acquired in F_2.

the consecutive 2δ interval for removal of the evolution of the heteronuclear one-bond coupling constants. In the nonscaled experiment (Fig. 1b), the evolution of $^1J_{CH}$ coupling constants during the 2δ interval is removed solely by a 180° ^{1}H pulse.

The scaling of the heteronuclear one-bond coupling constant is illustrated in Fig. 3 where overlaps between low- and high-field satellites of the H4a/H1a and H2a/H1a cross peaks of the nonscaled experiment (Fig. 3a) are removed by using a scaling factor of $\frac{1}{3}$ in the *J*-scaled experiment (Fig. 3b). The E.COSY multiplets became completely separated, allowing accurate measurement of displacement for the corresponding multiplet components. This was achieved by a computer-aided analysis shown in Fig. 4 using the H2a/H1a cross peaks.

There are cases where solely reversing the tilt of E.COSY

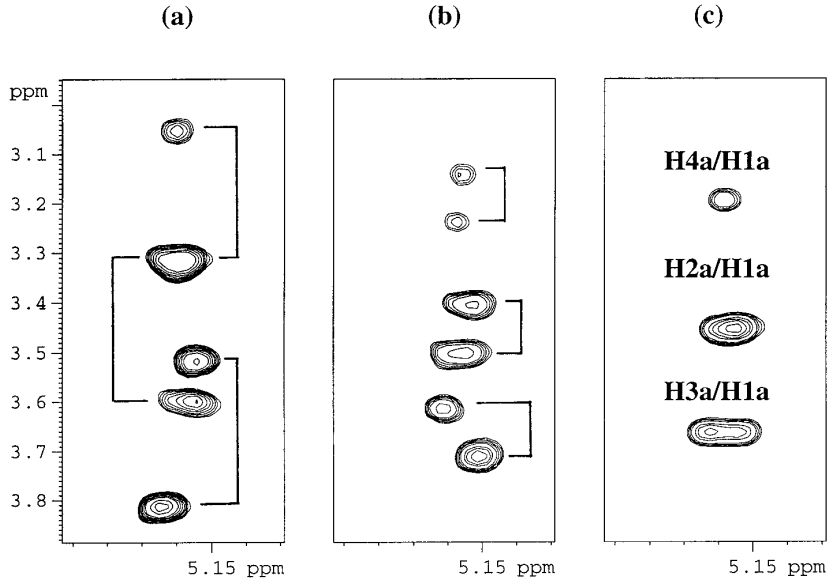

FIG. 3. Expanded regions of $^{13}C(\omega_1)$ half-filtered TOCSY spectra of **1.** (a) A partial spectrum acquired using the pulse sequence of Fig. 1b and parameters given in Fig. 2. Spectra (b) and (c) were acquired using the pulse sequence of Fig. 1c and sc equal to $\frac{1}{3}$ and 0, respectively. Note that the tilting of E. COSY cross peaks is reversed in spectrum (b) compared to (a).

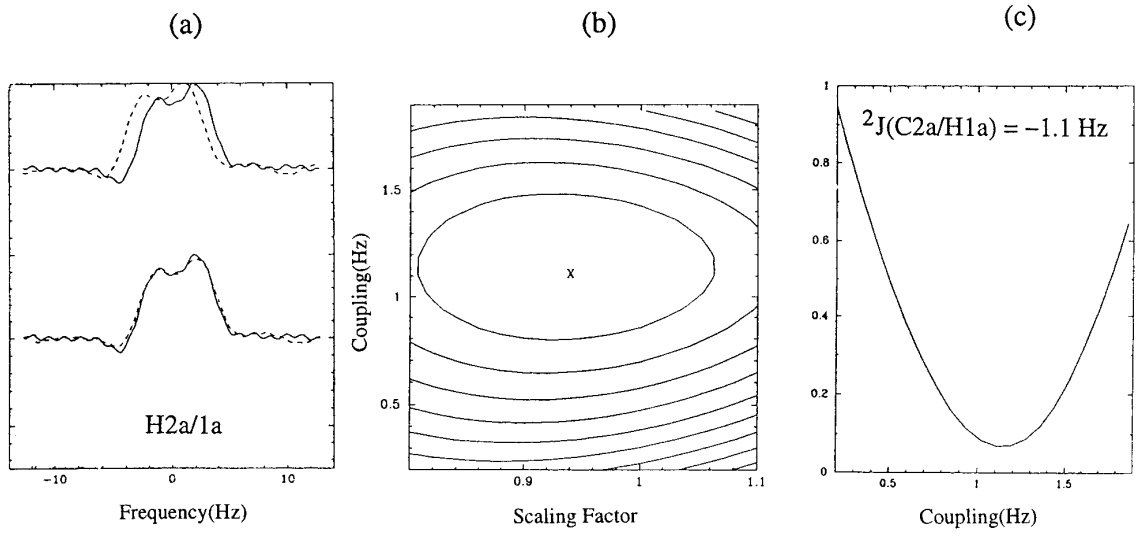

FIG. 4. Computer-aided analysis of the *J*-scaled E.COSY H2a/H1a cross peak of Fig. 3b. Individual traces were extracted from the 2D spectrum shown in Fig. 3b and the digital resolution was increased in the F_2 domain by inverse Fourier transformation, zero-filling, and back-transformation of one-dimensional spectra. (a) Low- and high-field H-2 satellites of the H2a/H1a cross peak (upper trace); one of the spectra is shifted and compared to the other one (lower trace). The displacement corresponding to the best match of multiplets provides the coupling constant. (b) Contour maps of sums of the squares of differences between the two components of the multiplet as a function of the coupling constant and the scaling factor. (c) The *J* profile at the optimum scaling factor extracted from the map given in (b).

multiplets is sufficient to remove some of the overlaps. Note that this can be achieved by applying an additional 180° X pulse (dashed line in the pulse sequences of Figs. 1b and 1c). For a special case of sc = 0, the second 180° X pulse of the pulse sequence of Fig. 1c can be discarded and a sequence with a G-BIRD^{r,X} pulse in the middle of the t_1 period is obtained. The inversion of remote protons and of heteronuclei by this pulse results in refocusing of both homonuclear and heteronuclear one-bond coupling evolution during t_1, yielding cross peaks fully decoupled in F_1 (Fig. 3c). The collapsed cross peaks are no longer of the E.COSY type; nevertheless they contain the heteronuclear long-range coupling in the form of an additional in-phase splitting in F_2. In the presence of several homonuclear couplings such multiplets can be too complicated for a straightforward extraction of $^nJ_{CH}$ values. In these circumstances another experiment needs to be acquired using the X nucleus decoupling during the acquisition. The heteronuclear splitting can then be determined by comparing the coupled and decoupled multiplets as proposed previously (*12, 21*). This method which requires two separate experiments is an option when severe overlap cannot be removed by a *J*-scaled experiment.

As a final source of improved sensitivity, we have implemented a sensitivity-enhanced TOCSY transfer for spreading the magnetization along the coupled spins. Although originally introduced without PFGs (*22*), we have used pulsed field gradients for coherence selection by analogy with Kay's implementation of the sensitivity-enhanced heteronuclear experiments (*23, 24*).

In order to demonstrate the sensitivity enhancement of the proposed method in comparison with the original X(ω_1) half-filtered TOCSY experiment, selected F_2 traces through the 2D spectra of the peptide **2** are displayed in Fig. 5. All experiments were recorded using identical repetition times. The traces on the right are taken from the spectrum acquired by the original X(ω_1)-filtered TOCSY experiment and serve as a reference. The traces in the middle and on the left correspond to the gradient- and sensitivity-enhanced spectra acquired using the pulse sequence of Fig. 1b without and with the BIRD^r pulse in the middle of t_1, respectively. The superior sensitivity improvement observed in the latter spectrum arises from the cumulative contribution of the sensitivity-enhanced TOCSY transfer and the broadband homonuclear decoupling during the t_1 period. The absolute increase of the signal intensities depends on the complexity of proton multiplets and the digital resolution in the F_1 dimension. Typical values were between 1.5 and 2.5.

CONCLUSIONS

In summary, we propose a sensitivity- and gradient-enhanced version of the hetero (ω_1) half-filtered TOCSY experiment which incorporates the *zz* gradient-enhanced X filter, homonuclear broadband decoupling in F_1, and gradient-supported coherence selection during the sensitivity-enhanced TOCSY step. The proposed method yields high-quality spectra with increased sensitivity and resolution. The proton-decoupled E.COSY multiplets, with large splitting in F_1 due to $^1J_{XH}$ and small displacement in F_2 due to $^nJ_{XH}$, allow accurate measurement of coupling constants smaller

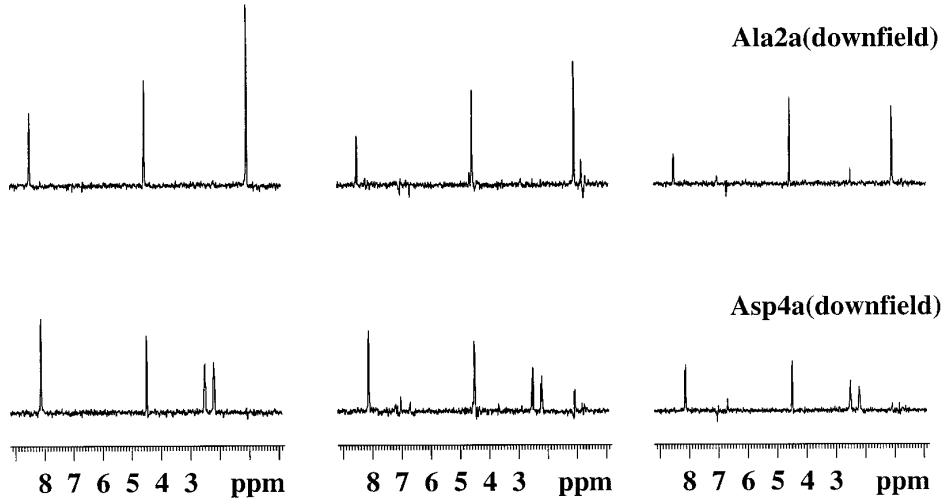

FIG. 5. Selected F_2 traces taken from spectra of **2** (0.08 M solution in DMSO-d_6) acquired using the original X(ω_1) half-filtered TOCSY experiment (right); the gradient- and sensitivity-enhanced experiment of Fig. 1b without the homonuclear decoupling during t_1 (middle); and with the decoupling (left). Sixteen transients were accumulated for each of 512 increments with a relaxation delay of 1.8 s; τ delay was set to 3.6 ms; isotropic mixing time was 52 ms. All PFGs were 1 ms and followed by a 200-μs delay (δ = 1.2 ms). The spectral width was 5480 Hz in both dimensions; 2048 complex data points were acquired in F_2. Zero-filling in F_1 and a squared cosine window function in both F_1 and F_2 were applied prior to Fourier transformation. The echo–antiecho protocol of the standard Bruker software was applied for transformation.

than the natural linewidths. The accidental overlap of E.COSY multiplets in F_1 is removed by an optional scaling of $^1J_{CH}$ coupling constants in the F_1 domain. Complete decoupling of $^1J_{CH}$ in F_1 is also possible; however, two experiments, with and without the ^{13}C decoupling, are generally needed for determination of coupling constants in these circumstances. The methods discussed so far are applicable to protonated heteroatoms with extensive proton spin coupling networks. For isolated protons cross-relaxation-based mixing schemes, e.g., NOE, ROE, or the recently introduced off-resonance ROE mixing processes (*25*), can replace the TOCSY transfer. In general, these methods are less sensitive and other techniques using the evolution of long-range coupling constants (*12, 26*) are necessary.

EXPERIMENTAL

All experiments were performed on a Bruker Avance DRX-500 spectrometer equipped with a 5-mm triple-resonance probe (^{1}H/^{13}C/^{15}N) and actively shielded *z*-gradient coil. ^{1}H and ^{13}C 90° pulses were 10 and 13 μs, respectively. For TOCSY transfer, the RF power was attenuated to provide a 90° ^{1}H pulse of 30 μs. All other experimental parameters are given in the figure legends.

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