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Gradient- and Sensitivity-Enhanced TOCSY Experiments

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A pulsed field gradient version of the sensitivity-enhanced 2D TOCSY experiment is proposed which yields high-quality spectra with improved sensitivity and a minimum of two scans per t_1 increment. For rapid acquisition of 1D TOCSY spectra, the 1D DPFGSE–TOCSY experiment was modified to include phase-encoded multiple-selective excitation followed by a simple spectral editing. Combination of these two building blocks is used in a sensitivity-enhanced 2D analog of the 3D TOCSY–TOCSY experiment which provides an efficient tool for resolving severely overlapped signals of oligomers in short experimental time. © 1998 Academic Press

Key Words: **gradient version of 2D TOCSY; sensitivity enhancement; echo–antiecho selection; 1D DPFGSE–TOCSY; multiple-selective excitation; 2D analog of 3D TOCSY–TOCSY.**

INTRODUCTION

The sensitivity-enhanced protocol with gradient echo–antiecho coherence selection (*1*) has widespread applications in HSQC-type heterocorrelated experiments (*2, 3*), in heteronuclear relaxation measurements (*4*), in doubly sensitivity-enhanced heteronuclear 3D pulse sequences (*5–8*), and more recently in HMQC-based experiments (*9*). The advantages of this approach, which include (i) increased sensitivity, (ii) clean, high-quality spectra, (iii) no sensitivity loss due to gradient coherence pathway selection, and (iv) short minimum phase cycles, clearly underline the superiority of these methods over their phase-cycled counterparts.

To the best of our knowledge, this combined sensitivity- and gradient-enhanced protocol as proposed by Kay has not yet been applied to homonuclear chemical-shift correlation experiments. In gradient-enhanced versions of COSY (*10*), multiple-quantum-filtered COSY (*11–14*), and multiple-quantum correlation (*15, 16*) experiments, coherence selection is achieved at the expense of sensitivity. The gradient pulses used for coherence selection rephase the magnetization of only one out of the two possible coherence-transfer pathways, thus leading to unavoidable sensitivity loss. This applies also to a recently proposed gradient-selected 2D TOCSY experiment (*17*). On the contrary, in the experiment proposed here, the gradient selection is combined with the sensitivity-enhanced protocol originally introduced without gradients (*18*) and an increased sensitivity is obtained as compared to that achieved with the nonenhanced phase-cycled experiment.

Similarly, there are pulsed field gradient 1D TOCSY experiments which use gradients for coherence selection (*19–22*) and those which use gradients for coherence rejection (*23–25*). The former suffer from loss of signal; the latter do not. It has been demonstrated on small molecules that by using a double pulsed field gradient spin echo (DPFGSE) sequence (*26*) for selective excitation, clean 1D TOCSY spectra with full sensitivity were obtained (*23, 24*). We propose here a simple modification of the DPFGSE module to allow for multiple-selective excitation. Several subspectra acquired simultaneously by this method are separated by a simple editing as suggested previously (*27*). This procedure employs a coding scheme based on the Hadamard matrices (*28*) and reduces the measurement time by a factor of $\sqrt{n}$ (where n is the number of selectively excited signals), as compared to the total time required for a set of experiments where the resonances are selectively excited one at a time. The in-phase absorption multiplets required for reliable measurement of coupling constants are generated by including spin locking under an adiabatically switched pulsed field gradient pulse applied before and after the isotropic mixing sequence (*29*).

We further demonstrate that, by replacing the first 90° ^{1}H excitation pulse of the above-introduced sensitivity-enhanced 2D TOCSY experiment by a multiple-selective DPFGSE excitation sequence, a 2D analog of the 3D TOCSY–TOCSY experiment is constructed. This experiment is closely related to previously reported 2D analogs of the 3D HOHAHA–COSY experiment (*30–32*) and the selective 2D TOCSY–TOCSY experiment (*33*).

RESULTS AND DISCUSSION

Gradient- and Sensitivity-Enhanced 2D TOCSY Experiment

A sensitivity-enhanced variant of a 2D TOCSY experiment based on preserving both orthogonal transverse magne-

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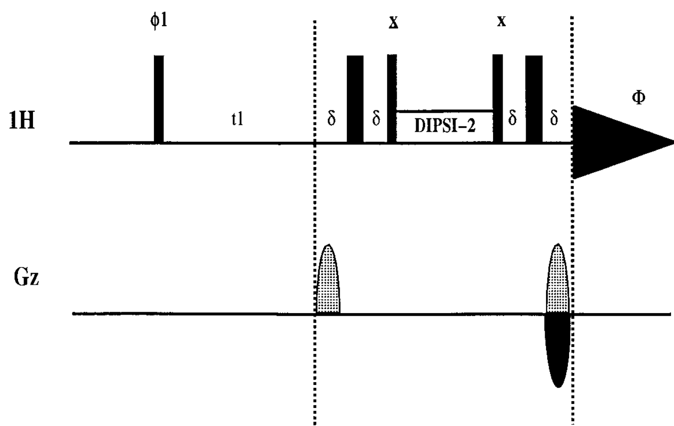
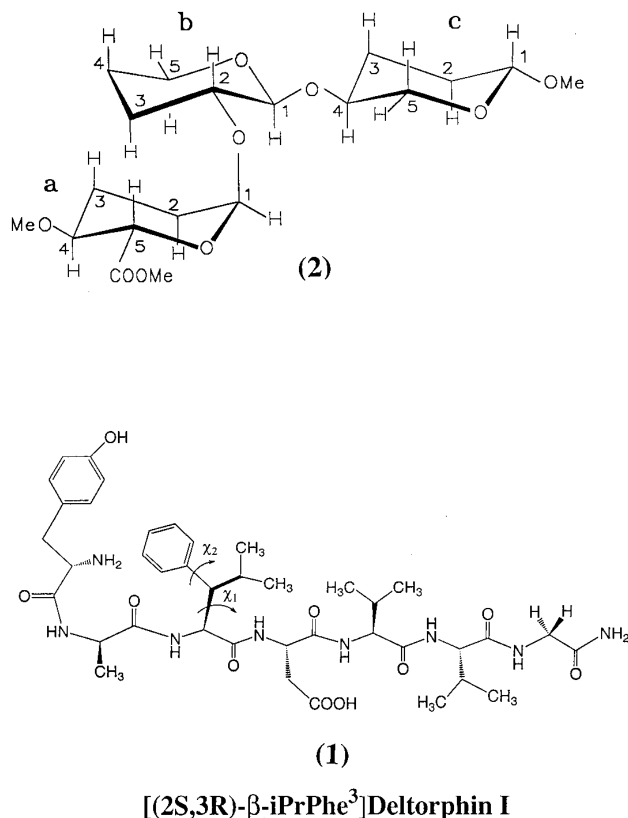


FIG. 1. Pulse sequence for gradient- and sensitivity-enhanced 2D TOCSY experiment. Thin and thick bars represent 90° and 180° pulses, respectively. Echo–antiecho signals are obtained by alternatively inverting the amplitude of the last gradient pulse and the phase of the 90° ^{1}H pulse applied prior to the DIPSI-2 sequence for consecutive FIDs. Sine bell-shaped z-gradient pulses of duration of 1 ms and 5 G/cm were applied for coherence selection $\phi 1 = x$ and $\Phi = x$.

tization components evolving during t_1 has been proposed by Cavanagh and Rance (*18*). A simple modification of the original TOCSY experiment (*34, 35*), including 90° ^{1}H pulses at the beginning and end of the mixing period and a simple phase cycling, allows both the sine- and the cosine-modulated components to contribute to the acquired signal. These components are separated during subsequent data processing, leading to a phase-sensitive spectrum with $\sqrt{2}$ improvement in sensitivity compared to the nonenhanced experiment. In analogy with the gradient- and sensitivity-enhanced heteronuclear correlated experiment (*1–3*), it is possible to introduce gradient pulses for coherence selection in the sensitivity-enhanced TOCSY experiment. The pulse sequence of the proposed sensitivity-enhanced TOCSY experiment, including coherence selection by gradients, is shown in Fig. 1. Both gradients are placed within spin-echo intervals so that the chemical-shift evolution, which occurs during gradients, is refocused. At the end of the first spin echo and before the DIPSI-2 mixing period a 90° ^{1}H pulse rotates one of the orthogonal transverse magnetization components to the z axis, while the other (in-phase with the applied pulse) is unaffected. Since the DIPSI mixing sequences were designed to produce an isotropic mixing Hamiltonian in the presence of couplings (*36*) they are ideally suited to the sensitivity-enhanced TOCSY experiment. During the DIPSI-2 mixing period both orthogonal components participate, independently and simultaneously, in the coherent magnetization transfer, preserving both cosine- and sine-modulated signals. Finally, the last 90° ^{1}H pulse restores both components to the transverse plane, and after rephasing the desired coherences by the second gradient pulse, both components are detected. Since a z filter or spin locking combined with a gradient pulse (*29*) applied after the mixing sequence would destroy one of the two magnetization components, their application is not allowed. Accordingly, some phase distortion of multiplets is expected due to the nonsuppressed zero-quantum coherences. The echo–antiecho signals are obtained by inverting the amplitude of the last gradient pulse (shaded and filled, respectively, in Fig. 1) and the phase of the 90° ^{1}H pulse preceding the mixing period from one FID to another. The phase inversion of the 90° ^{1}H pulse combined with simultaneous inversion of the rephasing gradient pulse allows the sine- and cosine-modulated signal components to be separated by adding or subtracting the corresponding data sets. Note that applying the echo–antiecho transformation protocol of the standard Bruker software gives rise to the desired phase-sensitive spectrum without any additional data manipulation. The processing of these spectra is therefore somewhat simpler than that of the original phase-cycled sensitivity-enhanced experiment. Finally, since the suppression of axial signals is achieved by gradients, the minimum number of scans is reduced from 4 to 2 in respective experiments. Because of the presence of gradients, the spectra are also cleaner.

To demonstrate the sensitivity gain of the proposed gradient-enhanced TOCSY experiment, two TOCSY spectra were recorded on a heptapeptide (deltorphin I analog, **1,** Scheme



(1)

[(2S,3R)-β-iPrPhe³]Deltorphin I

SCHEME 1

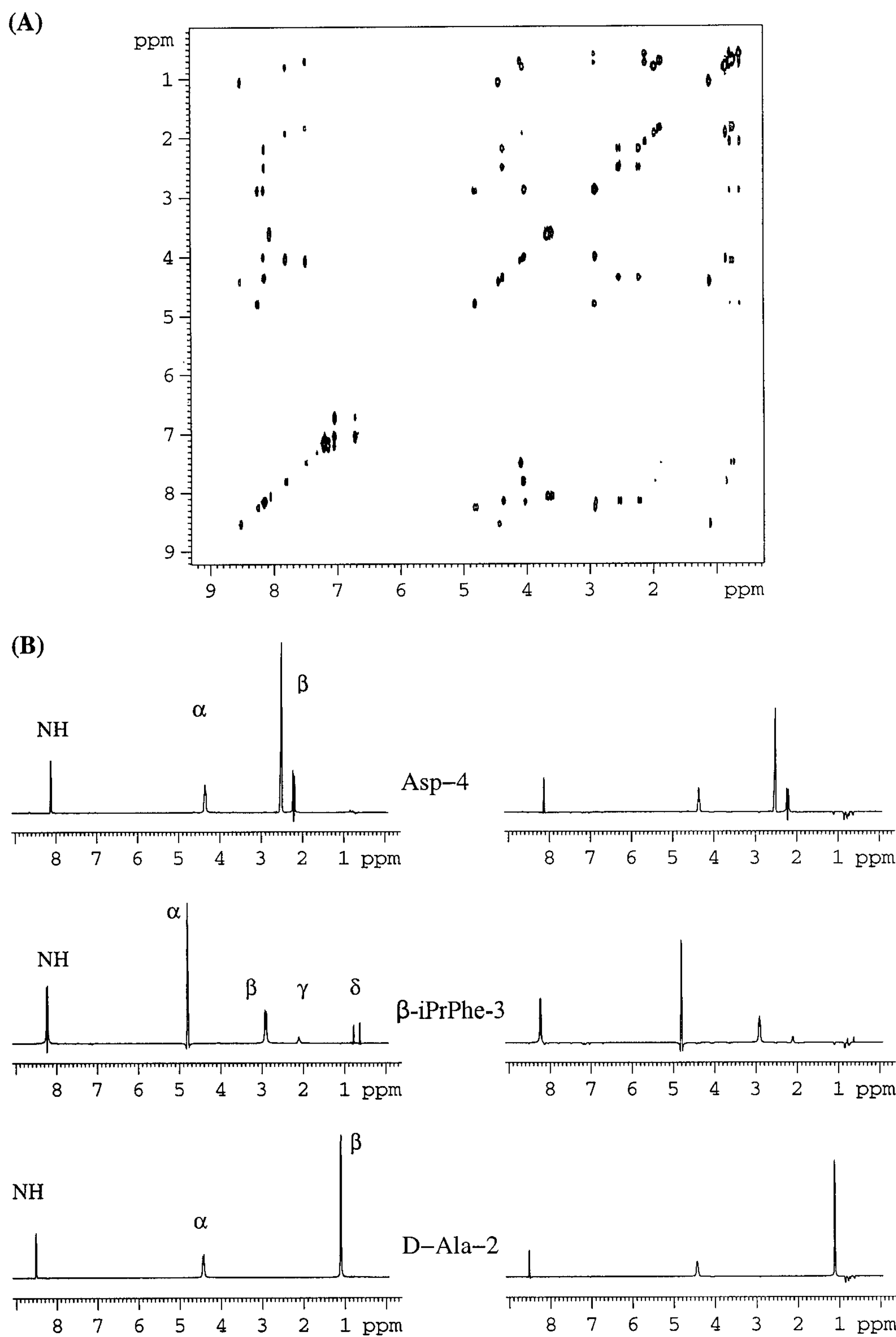


FIG. 2. (A) 2D TOCSY spectrum of **1** (0.08 M solution in DMSO-d_6) recorded using the pulse sequence of Fig. 1. Eight transients were accumulated for each of 256 experiments with a relaxation delay of 2.0 s; isotropic mixing time was 52 ms. All PFGs were 1 ms, 5 G/cm and followed by a recovery delay of 200 μs (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 standard Bruker software was applied for transformation. (B) Selected F_2 cross sections taken from (A) (left) and the nongradient, unenhanced TOCSY spectrum (right) recorded during the same total experiment time.

1): one with the standard (i.e., nongradient, unenhanced) TOCSY sequence, and the other with the sensitivity- and gradient-enhanced sequence, both during the same total experimental time. A representative TOCSY spectrum acquired on **1** applying the proposed sequence is shown in Fig. 2A. For sensitivity comparison, Fig. 2B shows F_2 cross sections (right) from the spectrum recorded with the standard sequence, including an eight-step phase cycle for suppression

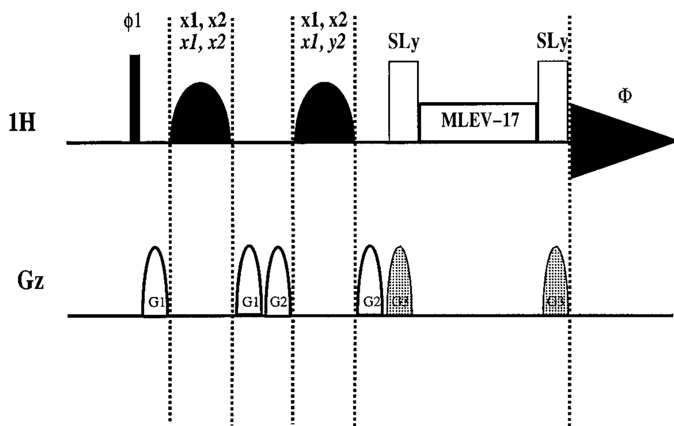


FIG. 3. Pulse sequence for multiple-selective, gradient-enhanced 1D TOCSY experiment. All PFGs were sine bell-shaped and followed by a 200-μs recovery delay. Phase-modulated multiple-frequency-shifted Gaussian pulses of 35 ms were used for selective inversion. The phase sequence of selective pulses (where the indices of phases refer to proton 1 and proton 2) corresponds to in-pase/antiphase double-site excitation. The selective pulses are surrounded by gradient pulses of different magnitudes (5 and 8 G/cm, respectively). The gradient pulses (G3) used for ZQC suppression were stronger (10–12 G/cm) and 2–3 ms of duration. A phase inversion of the initial 90° ^{1}H excitation pulse ($\phi 1$) was followed by the receiver phase: $\phi 1 = x\ -x$ and $\Phi = x\ -x$.

of artifacts and TPPI for quadrature detection. The traces on the left correspond to the same cross sections taken from the gradient-enhanced spectrum. The spectra are normalized so that the noise level is the same in each spectrum. In our experience, a sensitivity improvement with a factor of 1.2–1.4 was obtained with the proposed gradient-enhanced sequence. The diffusion-related losses occurring during the mixing time of the gradient-enhanced experiment can be minimized by using short gradient times and low gradient strengths. Note that inclusion of a WATERGATE sequence (*37*) before acquisition provides efficient solvent suppression for H_2O samples.

Multiple-Selective, Gradient-Enhanced Excitation

For instances when several 1D TOCSY spectra are recorded, the overall efficiency of the experiment can be increased by a multiple-selective excitation followed by a linear combination of the recorded data (*27*). In the past, simple, multiple-selective inversion of selected spins consisting of either consecutive (*27, 38*) or simultaneous (*39–41*) excitation has been used in these experiments. At present we propose to use a modified DPFGSE sequence (*26*) incorporating multiple-selective excitation which is achieved by phase-modulated multiple-frequency-shifted pulses (*22, 42, 43*) (Fig. 3).

To achieve phase encoding of signals belonging to different proton spin networks, a proper phase combination of multiple-selective pulses is required. For example, in the case of double-selective excitation, two data sets are recorded: one with in-phase excitation of the selected multiplets, and the other with antiphase excitation of one of the multiplets. In-phase excitation is achieved by applying the selective pulses with the same phase at both frequencies (e.g., phase x in Fig. 3). Introducing a 90° phase difference for one of the resonances between the two echo periods (e.g., phase x in the first echo, phase y in the second echo at one of the excitation sites) will give rise to 180° phase difference of signals belonging to the different coupled spin systems. Simple addition and subtraction of the two data sets separate the resonances belonging to the different spin systems. The outlined procedure can be easily extended for three, four, or more excitation sites by applying the acquisition/processing scheme of the Hadamard spectroscopy. Since the signals of the selected spin systems are accumulated during the entire experiment into each subspectrum, a multiple sensitivity gain is obtained compared to the monoselective experiments. When generation of pure-phase multi-

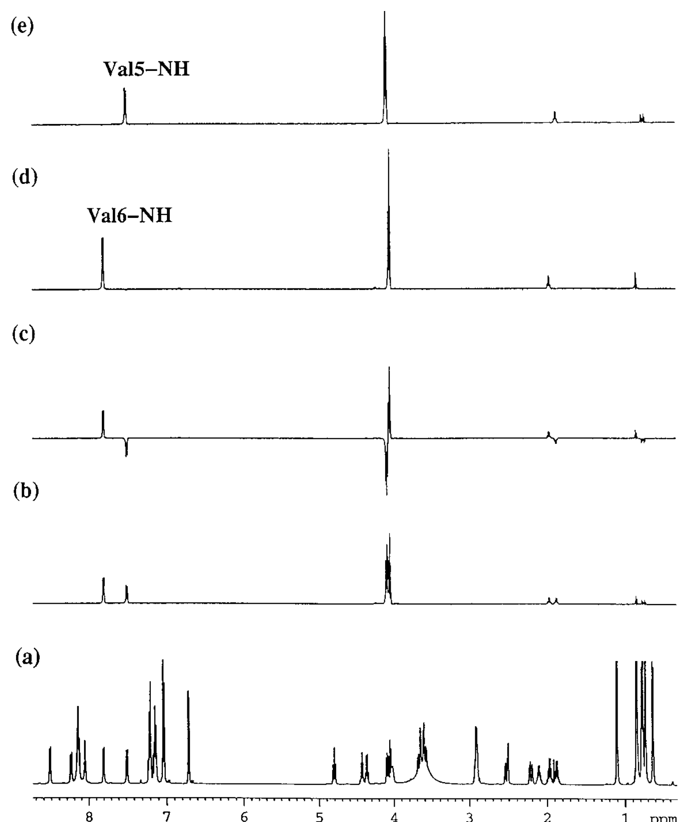


FIG. 4. Double-selective TOCSY spectra of **1** recorded using the pulse sequence of Fig. 3. (a) ^{1}H NMR spectrum. (b) Double-selective TOCSY spectrum with in-phase excitation of NH resonances of Val^5 and Val^6. (c) Double-selective TOCSY spectrum with antiphase excitation of the same resonances. (d) Subspectrum of Val^6 obtained by adding (b) and (c). (e) Subspectrum of Val^5 obtained by subtracting (b) and (c). For (b) and (c), 32 transients were accumulated with a relaxation delay of 2 s; isotropic mixing was 52 ms.

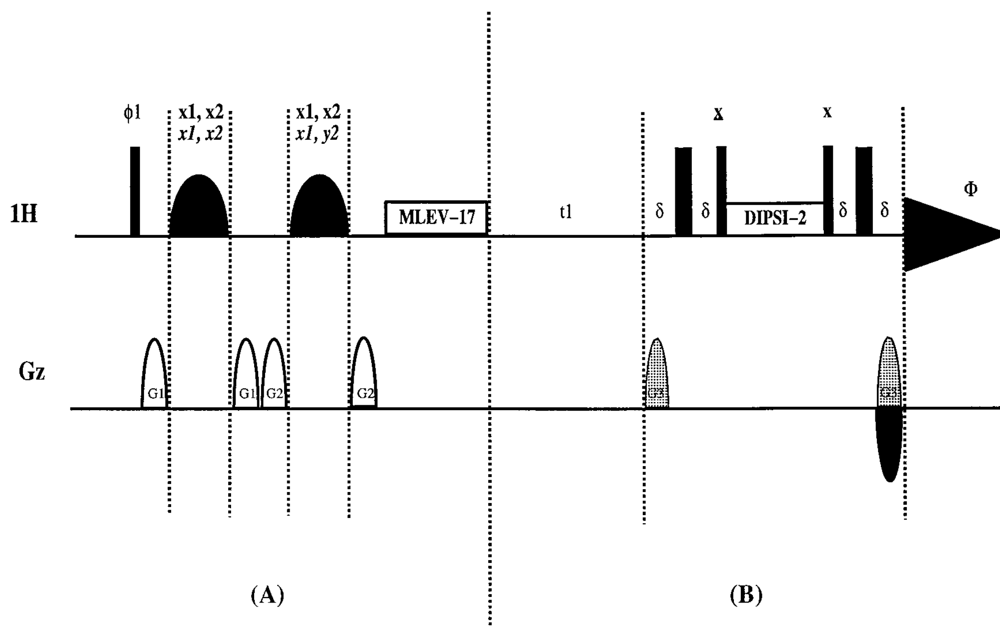


FIG. 5. Pulse sequence for multiple-selective 2D TOCSY–TOCSY experiment composed from two building blocks. (A) Multiple-selective excitation 1D TOCSY sequence; (B) gradient-enhanced 2D TOCSY sequence. $\phi 1 = x\ -x$ and $\Phi = x\ -x$.

plets is required, e.g., for coupling constant measurement, a spin-lock pulse and an adiabatically switched pulsed field gradient pulse (*29*) are simultaneously applied before and after the mixing sequence (Fig. 3) to dephase zero-quantum coherences (ZQC) which create distorted lineshapes. Note that the MLEV-17 (*35*) sequence used in the present study can be replaced by other pulse trains (e.g., WALTZ (*44*), DIPSI (*36*)).

The double-selective TOCSY spectra of **1** with phase-encoded double-site excitation (Fig. 4) were recorded using the pulse sequence of Fig. 3 with ZQC suppression. Two spectra were recorded for each pair of the excited NH resonances: one with in-phase and the other with antiphase excitation. Adding and subtracting the corresponding spectra, as shown in Fig. 4, yield the individual subspectra of the selected spin systems. Note that the α signals of Val^5 and Val^6 residues severely overlapped in the double-selective spectra become separated in the edited subspectra. The quality of the spectra clearly demonstrates the high performance of the proposed DPFGSE multiple-selective excitation sequence.

Multiple-Selective 2D TOCSY–TOCSY

With the efficient multiple-selective excitation sequence and the enhanced 2D TOCSY experiment at hand, it is straightforward to design a multiple-selective sensitivity-enhanced 2D TOCSY–TOCSY experiment by replacing the nonselective 90° ^{1}H excitation pulse of the gradient-enhanced TOCSY experiment with the multiple-selective excitation 1D TOCSY scheme presented above (Fig. 5). The resulting experiment benefits from features of both its building components, namely (i) increased sensitivity due to the enhanced 2D TOCSY sequence, (ii) efficient spectral editing due to the phase-encoded multiple-selective excitation, and (iii) substantially reduced experiment time due to the additive impact of (i) and (ii).

The proposed experiment is demonstrated on a model trisaccharide **2** (Scheme 1). The anomeric protons of a and b residues (H1a, H1b) were excited simultaneously using the in-phase and antiphase excitation in two 2D experiments. The individual 2D TOCSY–TOCSY subspectra of residues a and b obtained by appropriate editing (adding and subtracting of the recorded data sets) are given in Fig. 6.

Once the magnetization is spread by the first TOCSY transfer along the entire spin system, it is sufficient for the assignment to apply only a short mixing time for the second TOCSY transfer (*33*). During this time, magnetization is mostly propagated between directly coupled proton spin pairs; relay peaks arising from two-step transfers appear with much reduced intensities and a longer-range transfer is virtually nonexistent. The direct correlation peaks present in the 2D TOCSY–TOCSY subspectra of individual residues allow an unambiguous assignment of resonances.

CONCLUSIONS

In summary, we propose a gradient version of the sensitivity-enhanced 2D TOCSY experiment for obtaining high-quality spectra in minimum time. With the echo–antiecho

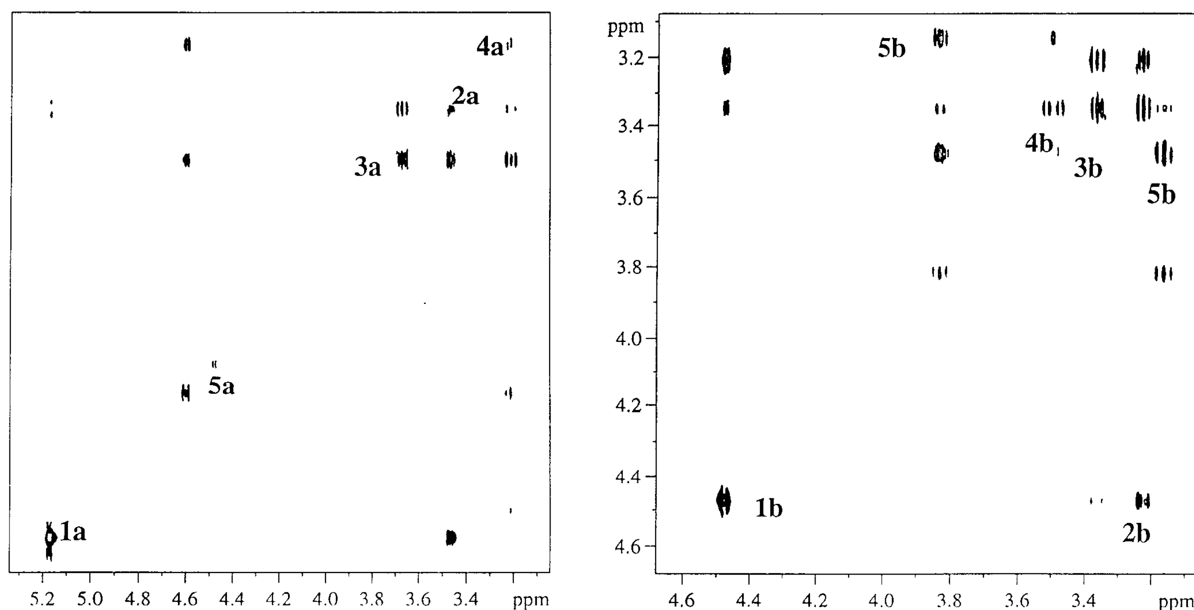


FIG. 6. 2D TOCSY–TOCSY subspectra of residues a (left) and b (right) of **2** (6.5 mg/0.5 ml D_2O). Two 2D experiments were performed using the pulse sequence of Fig. 5, one with in-phase and the other with antiphase excitation of H1a and H1b resonances. Sixteen transients were accumulated for each of 160 experiments with a relaxation delay of 1.8 s; isotropic mixing times of the first and second TOCSY transfer were 88 and 20 ms, respectively. The spectral width was 1400 Hz in both dimensions; 1024 complex data points were acquired in F_2.

procedure being a standard option of commercial NMR software packages, processing of these spectra is straightforward. The 1D TOCSY experiment modified to include phase-encoded multiple-selective excitation followed by simple spectral editing offers multiple gain in spectrometer time, and provides individual subspectra of high quality. Finally, concatenation of the two building blocks, multiple-selective excitation and the sensitivity-enhanced TOCSY scheme, results in an improved selective 2D TOCSY–TOCSY experiment. This experiment provides a useful tool for the analysis of spectra of isolated spin systems when severe overlap of resonances hampers the analysis based on solely nonselective methods. We believe that the proposed TOCSY experiments can find applications in the study of oligomers (e.g., carbohydrates, peptides) with isolated spin systems.

EXPERIMENTAL

All experiments were performed at 300 K on a Bruker Avance DRX-500 spectrometer equipped with a 5-mm triple-resonance probe ($^1H/^{13}C/^{15}N$) and an actively shielded z-gradient coil. The ^1H 90° pulse was 10 μs. For TOCSY transfer, the RF power was attenuated to provide a 90° ^1H pulse of 30 μs. Multiple-selective inversion was achieved by a phase-modulated multiple-frequency-shifted Gaussian pulse of 35 ms. All other experimental parameters are given in the figure legends.

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