Sensitivity- and Gradient-Enhanced Heteronuclear Coupled/Decoupled HSQC–TOCSY Experiments for Measuring Long-Range Heteronuclear Coupling Constants

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A pulsed field gradient version of the sensitivity-enhanced 2D HSQC–TOCSY experiment is proposed for measurement of long-range heteronuclear coupling constants. The coupling constants are obtained by computer-aided analysis of mixed-phase multiplets with and without the heteronuclear splitting. Generation of pure phase data is not required. Since large $^1J_{XH}$ and J_{HH} couplings are used for coherence transfer, small $^nJ_{XH}$ can be measured accurately, which could be difficult to obtain from purely heteronuclear polarization transfer experiments. © 1997 Academic Press

It has been long recognized that heteronuclear long-range coupling constants provide a valuable source of structural information due to their dependence on dihedral angles (1, 2). However, their use in structural elucidation has been hampered by the difficulties of their measurement. A large number of proton-detected methods (3) have been proposed in recent years to overcome the low sensitivity associated with heteronuclei detection and offer a sensitive and reliable way for measurement of heteronuclear coupling constants in molecules without isotopic enrichment.

Several variants of long-range HMQC/HSQC experiments which are based on either quantitative J correlation (4) or multiplet analysis (5–7) are limited by the inefficiency of coherence transfer through small $^nJ_{XH}$ couplings. In some instances, separate experiments which provide data on T_2 relaxation of protons and reference proton multiplets need to be performed. Recently, an HMQC-based experiment (8), yielding in-phase multiplets, has been proposed which eliminates the sensitivity degradation caused by cancellation of antiphase components of multiplets. However, the additional refocusing interval of this and also of the quantitative J correlation method (4) makes them sensitive to the spin–spin relaxation.

Techniques which overcome the inefficiencies of heteronuclear magnetization transfer via small long-range coupling constants make use of large one-bond heteronuclear coupling

constants in combination with homonuclear TOCSY transfer for spreading the magnetization in protonated spin systems. These methods can be classified as hetero- (9, 10) or homonuclear chemical-shift correlated techniques (11–15).

In the present article we propose a sensitivity- and gradient-enhanced variant of our earlier z-filtered HSQC–TOCSY experiment (9). Conceptually the proposed method is similar to the recently published sensitivity-enhanced 3D HSQC–TOCSY experiment (16). In our implementation (Fig. 1) sensitivity-enhancement was achieved by the use of pulsed field gradients as proposed for the HSQC experiment by Kay *et al.* (17, 18). A DIPSI-2 mixing scheme (19) preserved both orthogonal components of magnetization and was therefore suitable for sensitivity-enhanced experiments (20). A purging trim pulse (Tr_x) applied subsequent to the 2τ ($\tau = 1/4\ ^1J_{XH}$) spin-echo period serves as the first step in removing undesired signals originating from protons not bound to active heteronuclei. Alternatively, a gradient pulse inserted between the two 90° pulses when the magnetization is of the form I_zS_z (where I_z and S_z denote the z components of ^1H and X magnetization, respectively) could be applied (21).

The final suppression of unwanted coherences is achieved by the subsequent gradient pulses. A four-step phase cycle includes phase inversion of the first 90° X pulse (ϕ_1) with simultaneous inversion of the receiver phase in the first two transients. During the third and fourth transients the phase inversion of both ϕ_2 and ϕ_3 followed by the receiver is combined with the gradient coherence selection. Possible overlap between direct and relay peaks can be avoided by eliminating the intense one-bond correlation peaks which is achieved by inserting an additional spin-echo before the acquisition as shown in Fig. 1. The last 180° X pulse, applied on alternate scans as a result of a pair of appropriately phased consecutive 90° pulses, alters the sign of the antiphase magnetization of directly attached protons. By keeping the re-
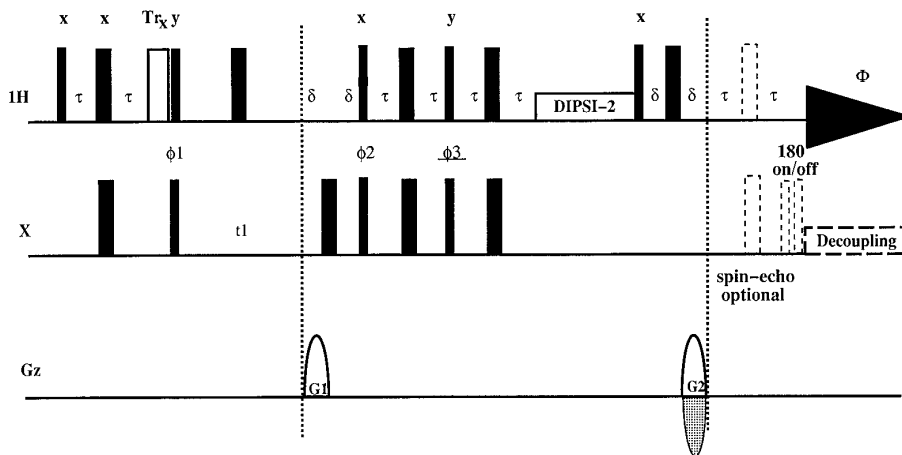
FIG. 1. Pulse sequence of sensitivity- and gradient-enhanced 2D HSQC–TOCSY experiment including optional direct peak suppression. Thin and thick bars represent 90° and 180° pulses, respectively. Sine bell-shaped z gradient pulses of duration of 1 ms and G1:G2 = γ_H: γ_X were applied for echo–antiecho coherence selection. A trim pulse (Tr_x) of 2 ms was included after the 2τ ($\tau = 1/4\,^1J_{XH}$) period to reduce ^1H–(^{12}C) magnetization. Details of phase cycling are given in the text: $\phi_1 = x - x$; $\phi_2 = x\,x\,-x\,-x$; $\phi_3 = y\,y\,-y\,-y$; $\Phi = x\,-x\,-x\,x$. The echo–antiecho signals were obtained by alternatively inverting the amplitude of G2 and the phase, ϕ_3, for consecutive FIDs.

ceiver phase constant, the corresponding direct correlation signals are canceled out. Accordingly, the direct peak suppression requires a minimum eight-step phase cycling scheme.

The sensitivity enhancement achieved by this approach relative to a nongradient, nonenhanced experiment depends on the efficiency of proton–carbon and carbon–proton magnetization transfers in the sensitivity-enhanced experiment. For detection of CH_2 and CH_3 correlations the first refocusing interval after the t_1 period needs to be adjusted (22) which results in reduced sensitivity improvement compared to the theoretically predicted maximum enhancement of $\sqrt{2}$ for CH correlations only. In addition, magnetization losses due to T_2 relaxation and/or translational diffusion might further decrease the sensitivity. Typically, in our experience, a 1.2–1.4-fold sensitivity improvement was achieved with our enhanced method for CH carbons compared to the earlier (23) phase-cycled, nonenhanced experiment.

Heteronuclear coupling constants are evaluated using the FIDS method (10) from heteronuclear coupled and decoupled in-phase multiplets acquired in two separate experiments. One of the advantages of this approach is that it does not require a homonuclear experiment for obtaining reference multiplets, as these are provided by another heteronuclear experiment with X decoupling. This procedure does not require the pure absorption phase multiplets used earlier (9), and therefore sensitivity losses associated with the use of the z filter are eliminated.

The main limitation of the proposed method is that only long-range coupling constants between protonated hetero-

nuclei and other protons can be measured if a homonuclear TOCSY transfer is possible between the proton(s) attached to the heteronuclei and nearby protons. Otherwise, the NOE, ROE, or the recently introduced off-resonance ROE (24, 25) mixing schemes must be used in place of the isotropic mixing sequence in Fig. 1. Unfortunately, in this case, the sensitivity is much lower and depends on the efficiency of the cross-relaxation. Note that such an HSQC–(off-resonance)-ROESY experiment takes full advantage of the sensitivity-enhanced protocol by preserving both orthogonal components during the mixing process. This is not possible for the standard NOE and ROE mixing schemes.

The proposed method for measurement of long-range heteronuclear coupling constants is demonstrated on a model trisaccharide (**1**) with its monosaccharide residue A, B, and C. The 2D heteronuclear decoupled HSQC-TOCSY spectrum of **1** obtained by using the pulse sequence given in Fig. 1 including the direct peak suppression is shown in Fig. 2a. To demonstrate the sensitivity improvement achieved by the proposed method in comparison with the phase-cycled variant, selected F_2 traces are shown in Fig. 2b for both methods. The traces on the left are extracted from the sensitivity-enhanced experiment, while those on the right are taken from the phase-cycled spectrum recorded under similar experimental conditions relying on phase cycling for the coherence selection. Figure 3 shows the expansions of the C1c carbon traces extracted from the ^{13}C-coupled (solid) and ^{13}C-decoupled (dashed) spectra, respectively. Since the propagation of proton magnetization is identical in both experiments, the
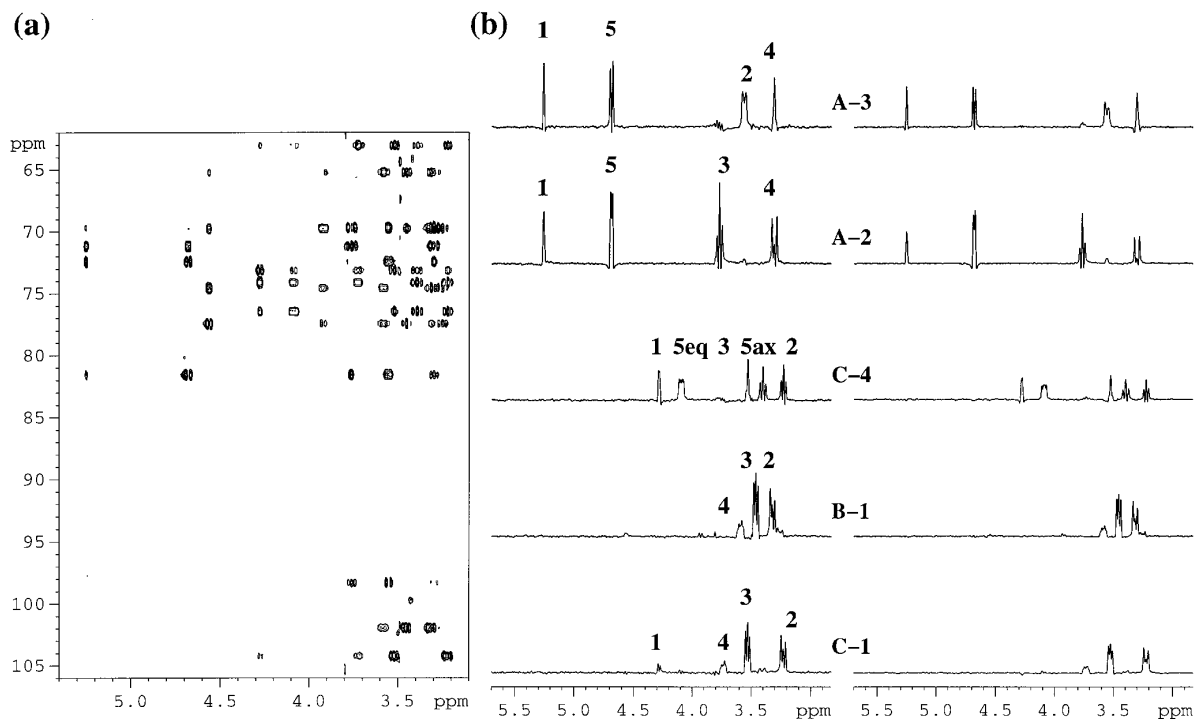
(a)

(b)

FIG. 2. (a) Heteronuclear decoupled HSQC–TOCSY spectrum of **1** (6.5 mg/0.5 mL D$_2$O) acquired on a Bruker Avance DRX-500 spectrometer using the pulse sequence given in Fig. 1 including the direct peak suppression. Sixty-four scans were accumulated for each of 256 experiments; a relaxation delay of 1.6 s was allowed between transients; τ delay was set to 1.65 ms; isotropic mixing time was 52 ms. (b) Selected F_2 traces taken from the sensitivity-enhanced experiment (left) and the phase-cycled spectrum recorded under similar experimental conditions (right).

only difference between the corresponding coupled and decoupled multiplets is in the heteronuclear splitting. Therefore, the mixed phase multiplets can be analyzed without the need to generate pure absorption phase multiplets. The coupled multiplets are reconstructed by adding two identical decoupled multiplets which have been shifted up- and downfield from their chemical shifts by half the

FIG. 3. Expansions of the C1c carbon traces extracted from the ^{13}C-coupled (solid) and ^{13}C-decoupled (dashed) spectra, respectively.

coupling constant. The reconstructed multiplets are then compared to the experimental coupled multiplets (*8, 10*). Only the absolute value of coupling constants is determined by this method.

Two representative examples of the computer-aided analysis of ^{13}C-coupled/decoupled multiplets are shown in Fig. 4. In order to enhance the accuracy of coupling constant calculation, the digital resolution in the t_2 acquisition dimension (2.19 Hz/Pt) was increased by inverse Fourier transformation, zerofilling, and back-transformation of selected traces, yielding the final digital resolution of 0.27 Hz/Pt. The large two-bond coupling, $^2J_{\text{C1cH2c}} = -6.0$ Hz, and the much smaller three-bond coupling, $^3J_{\text{C1cH3c}} = 1.1$ Hz, are obtained with an estimated precision of ± 0.27 Hz which is deduced from inspection of the graphs in Fig. 4c. The two bond proton–carbon coupling constants in aliphatic systems are negative (*1*), hence the negative sign of the $^2J_{\text{C1cH2c}}$ coupling constant.

In conclusion, the sensitivity- and gradient-enhanced HSQC–TOCSY experiment is proposed for determination of long-range heteronuclear coupling constants. The coupling constants are obtained by comparison of mixed-phase multiplets with and without the heteronuclear splitting, and
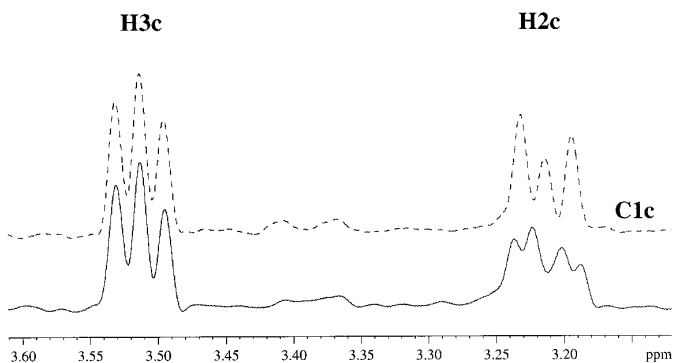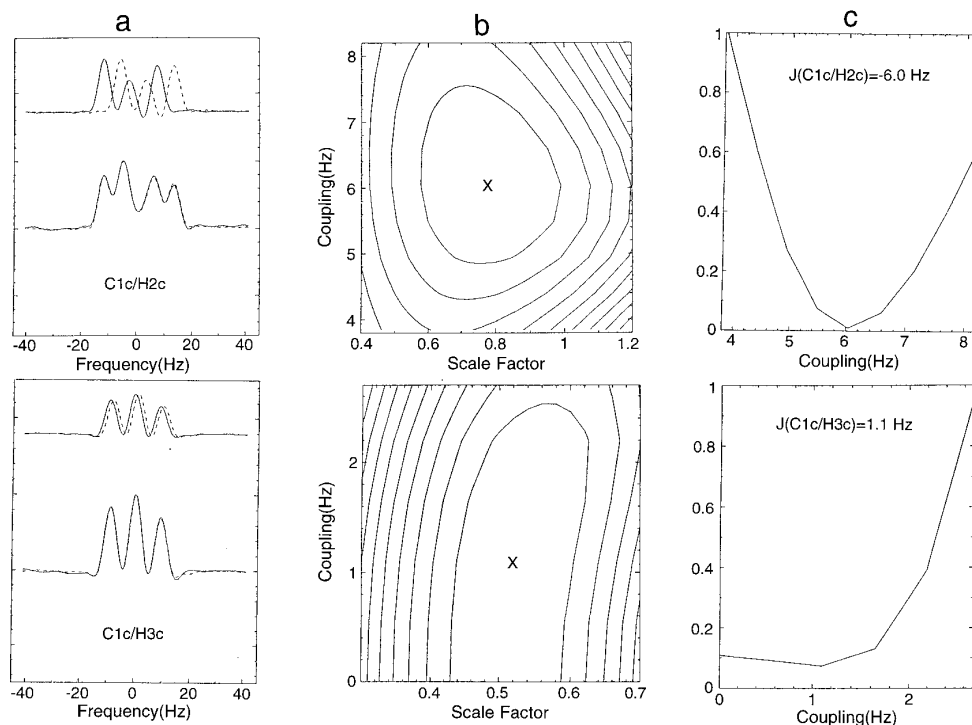
FIG. 4. Computer-aided analysis of ^{13}C-coupled/decoupled H2c and H3c multiplets. (a) Two identical shifted decoupled multiplets, both shown on the upper trace, are added and their sum, shown on the lower trace as a dashed line, is compared to the experimental coupled multiplet (solid line, lower trace). Displacement of decoupled multiplets provides the coupling constant. This occurs in steps that are twice the digital resolution (2×0.27 Hz). (b) Contour maps of sums of the squares of differences between the synthesized and experimental multiplets from (a) as a function of the coupling constant and the scaling factor. (c) J profiles at the optimum scaling factor extracted from the maps of (b).

the generation of pure phase data is not required. Artifact-free spectra with improved sensitivity are obtained. Since large $^1J_{XH}$ and J_{HH} couplings are used for coherence transfer, small $^nJ_{XH}$ can be measured accurately, which could be difficult to obtain from purely heteronuclear polarization transfer experiments.

ACKNOWLEDGMENTS

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