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Accurate and Precise Measurement of Heteronuclear Long-Range Couplings by a Gradient-Enhanced Two-Dimensional Multiple-Bond Correlation Experiment[1]

Shuqun Sheng*,[2] and Herman van Halbeek†

*Department of Chemistry, Georgia State University, Atlanta, Georgia 30303-3083; and †Glycobiology Program, UCSD Cancer Center, University of California San Diego, La Jolla, California 92093-0687

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We propose a phase-sensitive gradient-enhanced two-dimensional heteronuclear multiple-bond correlation (psge-2D HMBC) experiment for speedy, accurate, and precise measurement of $^2J_{CH}$ and $^3J_{CH}$. The experiment does not suppress one-bond correlations. Rather, the value of a desired long-range J_{CH} is obtained from the pertinent cross-peak pattern in the HMBC spectrum, using the corresponding $^1J_{CH}$ correlation pattern as reference. The application of the proposed experiment is illustrated for the trisaccharide raffinose. © 1998 Academic Press

The classic heteronuclear multiple-bond correlation (HMBC) experiment (*1*) has long been recognized as a powerful method for qualitative detection of long-range heteronuclear couplings. For carbohydrates, where the only scalar couplings observable across a Sugar′(1→*x*)Sugar glycosidic bond are the transglycosidic $^3J_{C1'Hx}$ and $^3J_{H1'Cx}$, the HMBC experiment provides the key to determining primary structures (''sequencing'') based exclusively on tracing through-bond *J* couplings (*2*). Typically, a low-pass *J* filter is inserted in the HMBC experiment as a means to suppress the intensities of $^1J_{CH}$ correlations. If necessary, researchers go through great pains to further improve upon reducing the intensities of such one-bond correlation cross peaks (see, for example (*3*)).

However, quantitatively determining the absolute values of $^2J_{CH}$ and $^3J_{CH}$ coupling constants with high accuracy and precision from the HMBC cross-peak patterns is not straightforward. Cross-peak multiplets in HMBC spectra are necessarily recorded in mixed phase mode in the ^{1}H dimension, due to evolution of ^{1}H chemical shifts and homonuclear couplings during the relatively long delay period (Δ) required for heteronuclear long-range couplings to evolve (*4*). Direct extraction of heteronuclear coupling constants from the resulting nonpure absorption patterns is impossible. To work around this problem, a reference spectrum of the molecule under investigation that has the same phase behavior as the cross peaks in the HMBC spectrum is recorded. A conventional 1D ^{1}H spectrum recorded using the sequence ''pulse–delay Δ–acquire'' can serve as the reference spectrum if the desired reference peak can be isolated (*5*). A more practical method (*6, 7*) involves recording a 2D ^{1}H total correlation spectroscopy (TOCSY) reference spectrum. The TOCSY proton multiplets can be recorded to possess the same phase properties as the corresponding multiplets in the HMBC spectrum. Shifting such a TOCSY-derived multiplet by a trial $^nJ_{CH}$ value and co-adding the original and the shifted-and-inverted multiplets is followed by matching the pattern with the corresponding HMBC cross peak by a nonlinear least-squares fit. Critical to the success of this procedure is that the multiplets in the 2D TOCSY spectrum are in-phase and have pure absorption lineshapes. In order to achieve this, the antiphase dispersion signals typically created during the isotropic mixing period need to be eliminated, e.g., by flanking the mixing time with a *z* filter (*8*). Alternatively, a so-called quantitative HMBC experiment has been described (*9, 10*). As part of this experiment, either a refocused HMBC experiment is conducted to arrive at a pseudo 2D reference spectrum at $F_1 = 0$ or a full 2D one-bond heteronuclear multiple-quantum correlation (HMQC) spectrum is used for reference purposes. Coupling constants are derived from the intensity ratios of the long-range correlation peaks to the one-bond correlation peaks in the reference spectra.

The method we propose here uses a phase-sensitive gradient-enhanced (psge) 2D HMBC pulse sequence, without suppression of one-bond correlations. Indeed, the one-bond correlation peaks are essential because they provide the necessary references for the phase properties of the multiple-bond correlations. Thus, values for the long-range couplings

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[2] To whom correspondence should be addressed: Department of Chemistry, Rutgers University, 610 Taylor Road, Piscataway, NJ 08854-8087.

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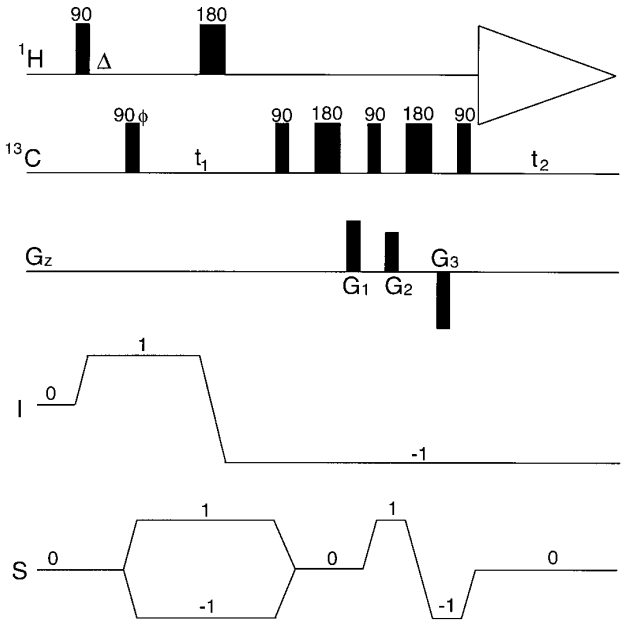

FIG. 1. Pulse sequence of the phase-sensitive gradient-enhanced 2D HMBC experiment along with the selection of coherence transfer pathways (I = ^{1}H, S = ^{13}C).

can be extracted by applying the shift method introduced by Titman *et al.* (*6*). No separate, additional NMR experiment needs to be conducted to obtain an appropriate reference spectrum. Also, the reference peaks here have truly the same phase properties as the multiple-bond correlation peaks, as they are collected during the very same experiment.

The pulse sequence of the psge-2D HMBC experiment along with the selection of the coherence transfer pathways is shown in Fig. 1. To obtain pure-absorption lineshapes in the t_1 dimension, signals ought to be amplitude-modulated in that dimension, which requires that both the $p = 1$ and the $p = -1$ coherence pathways are retained. At least three different methods have been proposed to achieve this goal. In the switched acquisition time (SWAT) method, the two coherence pathways are alternately rephased and sampled during acquisition (*11*). Alternatively, absorption-mode spectra can be obtained by repeating the experiment twice, once to select the $p = 1$ coherence pathway and once to select the $p = -1$ coherence pathway, followed by combining the two experiments (*12*). A third approach is to avoid the use of gradient pulses during the evolution period (*13–15*). The pulse sequence that we propose here uses the third approach to achieve pure-absorptive lineshapes in the t_1 dimension. It allows the recording of phase-sensitive HMBC spectra with a single scan per t_1 increment. The rephasing condition for the desired coherence transfer pathway is the gradient combination of G_1:G_2:G_3 = 4:3:−5 (see Fig. 1).

To test the proposed method, we recorded a psge-2D HMBC spectrum of raffinose, Gal$p\alpha$(1 → 6)Glc$p\alpha$(1 ↔ 2)βFru$f$. A sample of the trisaccharide was dissolved in $D_2O/(CD_3)_2CO$ (4:1, v/v) at a concentration of 60 mM; the sample temperature was 25°C, and the sample was not spun. The experiment was conducted on a Varian Unity*plus* 600 instrument (600 MHz for ^{1}H) running under VNMR software version 5.1A, and equipped with a 5-mm inverse-detection (ID) probe with pulsed magnetic field z gradients. Further experimental details are included in the legend to Fig. 2.

The psge-2D HMBC data were processed with the Felix software package, version 95 (BioSym, San Diego, CA) running on a Silicon Graphics Indy Computer. No multiplication by any window function and no zero-filling in t_2 were applied before Fourier transformation. A cosine-squared window function was applied in the t_1 dimension; the first data point was multiplied by 0.5 to reduce t_1 noise. No zero-filling was applied in t_1. The final resolution in the ^{13}C dimension was 19.5 Hz/pt. Fourier transformation in both dimensions was followed by phasing in F_1. The pair of resulting F_1 cross sections that contain the $^3J_{CH}$ and $^1J_{CH}$ correlation multiplets for a particular proton was selected. Each of these spectra was subjected to Hilbert transformation (to create the imaginary part of the spectral slice), followed by inverse Fourier transformation and zero-filling to 16K real points, resulting in a digital resolution of 0.11 Hz/pt.

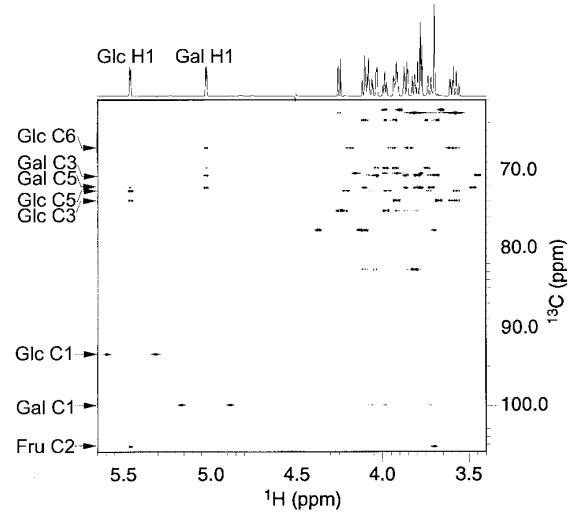

FIG. 2. The psge-2D HMBC spectrum of a 60 mM raffinose sample in $D_2O/(CD_3)_2CO$ (4:1, v/v), recorded at 600 MHz and 25°C. The delay Δ (see Fig. 1) was set to 45 ms and the acquisition times t_2 and t_1 were 1.13 s (spectral width 1805 Hz, 4K complex data points) and 25.6 ms (spectral width 10,000 Hz, 512 real data points), respectively. The relaxation delay was 1 s; eight scans were accumulated per t_1 increment. Squared gradient pulses of 1.5 ms duration were used; z gradients G_1, G_2, and G_3 were 15, 11.25, and 18.75 G/cm, respectively. Phase ϕ was incremented to accomplish quadrature detection in t_1 dimension via the States–TPPI method (*24*). The total acquisition time for the psge-2D HMBC experiment on raffinose was approximately 5 h. A 1D ^{1}H NMR spectrum of raffinose is displayed on top of the 2D HMBC spectrum.

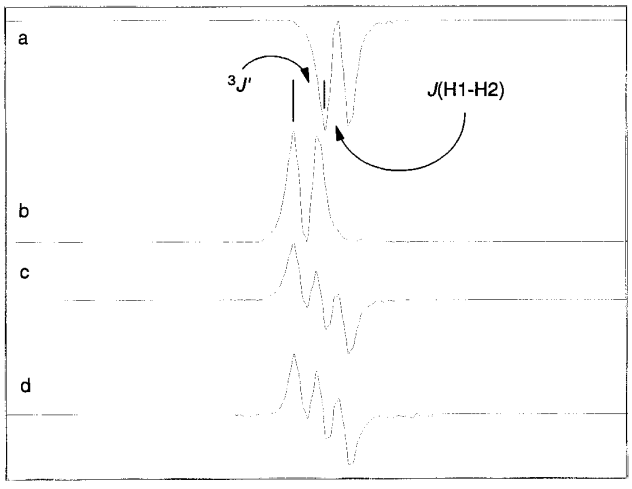

FIG. 3. J fitting as performed for raffinose Gal $^3J_{H1C5}$. (a) Experimental $^1J_{CH}$ trace for Gal H1. (b) Spectrum obtained by inverting and shifting the spectrum in (a) by a trial J'. (c) Spectrum obtained by co-adding (a) and (b). (d) Experimental $^3J_{CH}$ trace for Gal H5.

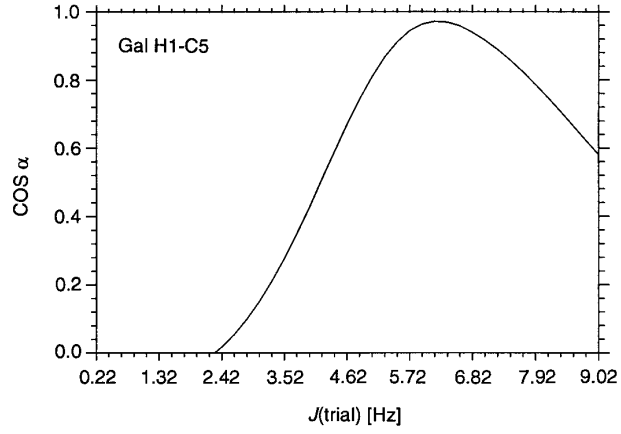

FIG. 4. Quality assessment of the fit of experimental and reconstructed psge-2D HMBC cross-peak multiplets of Gal H1-C5 in raffinose. For definition of cos α, see text.

After Fourier transformation, the baseline of the signals was carefully adjusted applying the ''flat'' routine in Felix, and any unwanted signals in the traces were zeroed.

The selected $^1J_{CH}$ multiplet was then inverted, shifted by $0.5 \times {}^3J(\text{trial})$, and added to its parent multiplet which was shifted in the opposite direction by $0.5 \times {}^3J(\text{trial})$. The resulting multiplet was fitted to the experimental $^3J_{CH}$ multiplet. These calculations were performed by an in-house written Fortran software routine. This program calculates cos α as a function of **T**. The best fit is reached when

$$\cos \alpha = \frac{\mathbf{E} \cdot \mathbf{T}}{|\mathbf{E}||\mathbf{T}|}$$

reaches its maximum (*16*). Herein, **E** is the vector corresponding to the experimental $^3J_{CH}$ multiplet, and **T** is the trial vector reconstructed from the reference multiplet by the procedure outlined above. The center of the experimental multiplet may be different from that of the trial multiplet due to the isotope shift effect. For this reason the center of the trial multiplet is shifted systematically and for each multiplet center position cos α is calculated. The position yielding the maximum value for cos α is considered the center of the multiplet.

The resulting psge-2D HMBC spectrum of raffinose is shown in Fig. 2. The ^{1}H and ^{13}C assignments of raffinose have been published (*17, 18*). The procedure for obtaining the value of a particular long-range coupling constant in raffinose is graphically illustrated for Gal $^3J_{H1C5}$. Figure 3 shows the doublet taken from the Gal $^1J_{H1C1}$ trace of the psge-2D HMBC spectrum, and the subsequent reconstruction of the $^3J_{H1C5}$ multiplet. The dependence of cos α on the value of the trial coupling J' for Gal $^3J_{H1C5}$ is shown in Fig. 4. The $^3J_{H1C5}$ coupling constant obtained by the fitting method is 6.17 Hz. This value is precise to within two times the digital resolution of the 1D traces processed as described above ($\pm$0.22 Hz), seeing as the signal-to-noise ratio of the long-range cross peak is sufficiently high. Figure 5 shows the

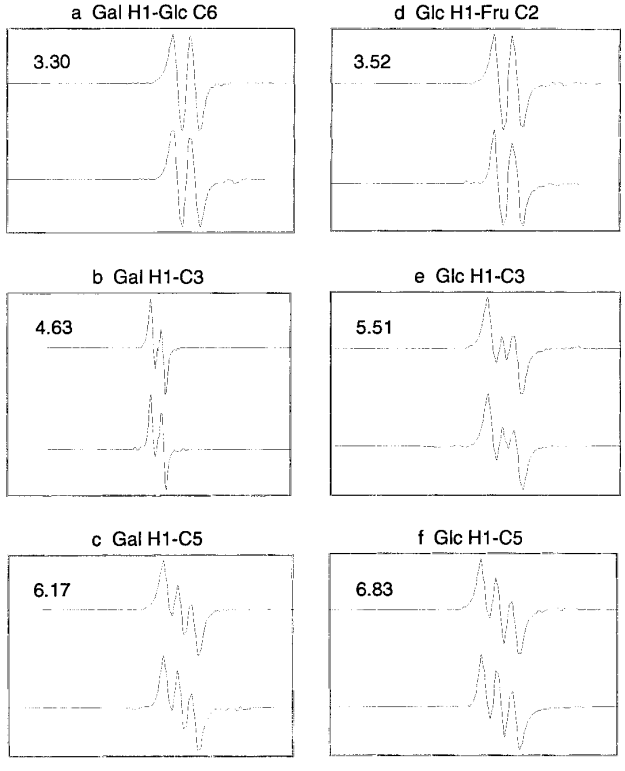

FIG. 5. Long-range J-couplings ($\pm$0.22 Hz) for raffinose, resulting from the fit of experimental (bottom traces) and reconstructed (top traces) psge-2D HMBC cross-peak multiplets.

matching patterns of five other $^3J_{CH}$ multiplets for raffinose, including those for the interglycosidic couplings $^3J_{GalH1\text{-}GlcC6}$ and $^3J_{GlcH1\text{-}FruC2}$.

Because of our long-standing interest in the solution conformation of sialyl-$\alpha(2 \rightarrow 6)$-lactose, i.e., Neu5Ac$\alpha(2 \rightarrow 6)$Gal$p\beta(1 \rightarrow 4)$Glc$p$ (*19*), we applied the psge-2D HMBC experiment to this trisaccharide and compared the results of our method with those of quantitative HMBC experiments performed on the same molecule (*10*). Our results show that the psge-2D HMBC method presented provides generally more accurate values for the sought-after couplings in a shorter amount of time (*20*). The need for recording a separate reference spectrum is circumvented, while at the same time the precision of the $^{2,3}J_{CH}$ values is significantly improved over those of the quantitative HMBC experiment.

The proposed psge-2D HMBC method is unhindered by severe overlap in the ^{1}H spectrum; the vast majority of the $^{2,3}J_{CH}$ and $^1J_{CH}$ cross peaks of interest are found isolated in the 2D spectrum. As the latter generally holds true for cross peaks corresponding to protons (H1′, Hx) and carbons (C1′, Cx) involved in $(1 \rightarrow x)$ glycosidic bonds, the psge-2D HMBC experiment should be particularly attractive to carbohydrate researchers interested in speedy and accurate measurement of transglycosidic $^3J_{CH}$ in ^{13}C natural abundance oligo- and polysaccharides. With regard to possible pitfalls, we realize that the lineshape (width and intensity) of the $^{2,3}J_{CH}$ multiplet could be different from that (those) of the $^1J_{CH}$ multiplet used for reference purposes, due to the differences in relaxation mechanisms (T_2 and T_1, respectively) they experience, as being part of ^{1}H–^{12}C and ^{1}H–^{13}C spin systems. However, in practice these differences are negligible for a molecule the size of raffinose. The cos α values obtained for raffinose were all greater than 0.9 which implies that the lineshapes for the experimental and the trial multiplets are not significantly different. Anticipating that the line width of the cross-peak signals may pose an upper bound to the size of molecules that can successfully be analyzed by the proposed psge-2D HMBC method, we extended our work to 30% uniformly ^{13}C-enriched glucuronoxylomannan polysaccharides of MW $>$ 50,000 (compare (*21*)). The results obtained thus far (data not shown) are encouraging in that interglycosidic $^3J_{CH}$ couplings in these polymers were measured successfully. It should be mentioned that the sensitivity of the proposed experiment can be improved by up to $\sqrt{2}$ if the sensitivity enhancement method developed by Rance and co-workers is employed (*22, 23*).

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