1D Determination of Long-Range Heteronuclear Coupling Constants by Gradient Enhanced SIMBA

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Two ^1H-detected one-dimensional gradient enhanced experiments and corresponding reference experiments are proposed for the measurement of heteronuclear long-range $^nJ_{CH}$ coupling constants in organic molecules. These experiments use inverse detection and selective carbon excitation, provide excellent suppression of protons not coupled to ^{13}C, and are able to measure couplings from regions that are crowded in both the proton and carbon frequency ranges. The experiments are applied to determine the long-range $^nJ_{CH}$ couplings in the sucrose molecule, including coupling across the glycosidic linkage. These pulse sequences are shown to provide accurate and efficient measurements of coupling constants that would be difficult or time-consuming to measure by other techniques. © 1998 Academic Press

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A wide variety of 1D, 2D, and 3D methods for the determination of long-range heteronuclear coupling constants have been proposed (*1–10*), and many of these have been recently reviewed (*1*). These experiments fall into three fundamental categories: those in which $^nJ_{AX}$ is measured from the splitting observed in the X (usually ^{13}C) or A (usually ^1H) multiplet (type I), those in which $^nJ_{AX}$ is determined from an E.COSY type cross peak pattern (type II), and those in which $^nJ_{AX}$ is determined from signal intensity (type III). In cases where both the A and X 1D spectra are extremely crowded, 2D (or 3D) techniques are usually necessary, and the type I HMBC-based experiment proposed by Titman, Neuhaus, and Keeler (*2*) or the type III HMBC-based experiment proposed by Zhu, Renwick, and Bax (*7*) is relatively straightforward. However, in cases where the A and X 1D spectra are not overcrowded and only a few coupling constants are needed, selective 1D experiments can provide a more efficient alternative to long 2D experiments. For the measurement of ^{13}C and ^1H couplings, it is advantageous to use an ^1H-observed ''inverse'' 1D experiment with selective excitation of a ^{13}C resonance, since one obtains the increased sensitivity and shorter relaxation time of ^1H along with the greater spread in ^{13}C frequencies that facilitates selectivity. To date, only the HMBC-based SIMBA pulse

sequence (*3*) incorporates all of these advantages, although it suffers from the well-known problem of insufficient suppression of ^1H signals not coupled to ^{13}C; also, as shown by Titman *et al.* (*2*), the lack of good ^1H reference spectra for comparison with the SIMBA spectra.

In Fig. 1 we show two new variations of the SIMBA pulse sequence: gradient enhanced SIMBA (ge-SIMBA) and refocused gradient enhanced SIMBA (ge-RESIMBA). These experiments allow for type I determination of $^nJ_{CH}$ for protons that are long-range coupled to the selectively excited ^{13}C resonance, as well as excellent suppression of ^1H signals not coupled to that carbon. Both experiments require a specific reference spectrum in which the signal does not contain the long-range heteronuclear coupling of interest but is otherwise identical. These sequences can provide rapid and efficient coupling constant measurements for selectively chosen ^{13}C resonances, and can in some cases be performed in a matter of minutes.

In ge-SIMBA (Fig. 1a), a low pass J-filter is first used to suppress direct $^1J_{CH}$ couplings (*11*). This is followed by the Δ_2 delay and hard ^{13}C $\pi/2$ pulse, which create heteronuclear double-quantum coherence (symbolized in product operator notation by $2 I_+S_+$, where $I = {}^1H$, and $S = {}^{13}C$), which is converted to heteronuclear zero-quantum coherence ($2 I_-S_+$) by a hard ^1H π pulse. A shaped selective ^{13}C $\pi/2$ pulse converts the zero-quantum coherence to ^1H coherence antiphase with respect to the selectively hit ^{13}C ($2 I_-S_Z$). A medium strength ^1H spinlock pulse during the selective ^{13}C pulse effectively ''freezes'' the ^1H precession (*12*), and also greatly prolongs the allowable duration and selectivity of the pulse. We also find that sinc-function-shaped ^{13}C pulses with three maxima, when compared to Gaussian pulses, Hermite pulses, rectangular pulses, or sinc pulses with more or fewer maxima, give the most ''rectangular'' excitation profiles as determined by direct observation of ^{13}C, with relatively constant phase and amplitude throughout most of the excitation region. This aids in uniform excitation of the carbon resonance. Most important is the combination of gradient selection and phase cycling used to select the pathways shown in Fig. 1b.

Although an ^1H multiplet observed in the ge-SIMBA experiment is pure antiphase with respect to the selectively excited
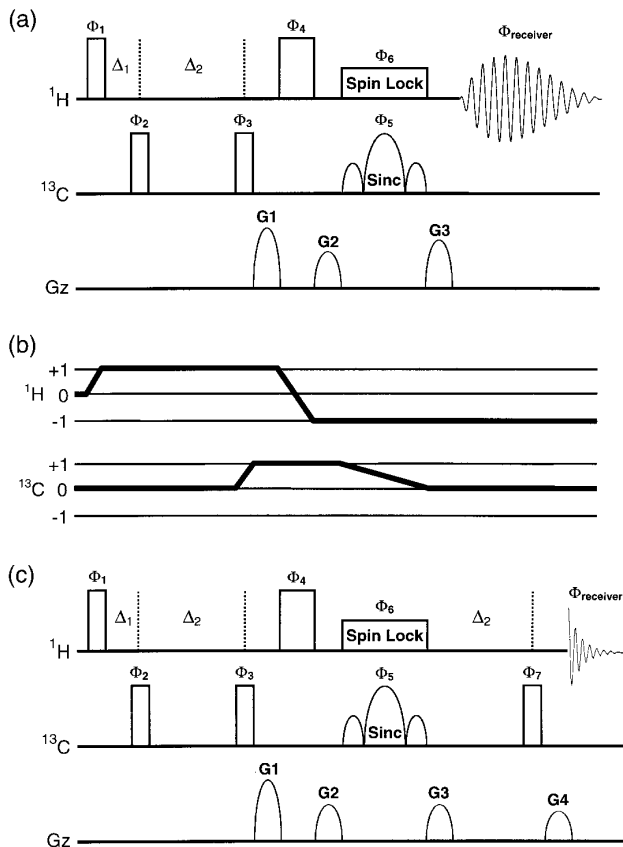
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FIG. 1. (a) Gradient-enhanced SIMBA pulse sequence (see text for discussion). Narrow rectangles represent $\pi/2$ pulses and the thicker rectangle represents a π pulse. Delay Δ_1 is $1/(2\ ^1J_{CH})$, and delay Δ_2 is set to approximately $1/(2\ ^nJ_{LR})$. The relative strengths of the sine-shaped gradients are G1:G2:G3 = 5:3:4. The phase cycle is $\Phi_1 = x$, $\Phi_2 = \{x, x, -x, -x\}$, $\Phi_3 = \{x, -x, x, -x, -y, y, -y, y\}$, $\Phi_4 = x$, $\Phi_5 = \{x, x, x, x, x, x, x, x, x, -x, -x, -x, -x, -x, -x, -x\}$, $\Phi_6 = x$, $\Phi_{\text{Receiver}} = \{x, -x, x, -x, y, -y, y, -y, -x, x, -x, x, -y, y, -y, y\}$. (b) Coherence transfer pathways for both the ge-SIMBA experiment and the ge-RESIMBA experiment. (c) Refocused ge-SIMBA experiment (ge-RESIMBA). Delays are the same as for the ge-SIMBA experiment. Relative gradient strengths are G1:G2:G3:G4 = 8:4:4:3. The phase cycle is the same as for ge-SIMBA, with the addition of $\Phi_7 = x$.

^{13}C resonance, it also has antiphase components from passively coupled protons, such as $4I_{1+}I_{2z}S_z$ (2). For this reason, a special reference ^{1}H spectrum must be obtained, which has the same gradient durations, proton pulses (including the spin-lock), and delays as the ge-SIMBA experiment. The only differences between this experiment and the ge-SIMBA experiment are that the ^{13}C power is set to zero (in practice 120 dB attenuation) and the gradient strengths and phase cycle are changed to select only the ^{1}H part of the phase coherence pathways shown in Figure 1b. Only a few scans are required for the reference spectrum, since ^{13}C-coupled protons are not selected. The reference ^{1}H multiplet obtained in this way may be "antiphase-doubleted" in the same manner as described by Titman *et al.* (2) to match the corresponding ge-SIMBA multiplet. A computer algorithm is used to carry out the doubleting

process, in which the reference signal is added to a negative copy of itself (180° out of phase) to simulate the effect of the antiphase coupling. The offset between the two copies is optimized to match the ge-SIMBA multiplet. The antiphase-doubleting process is equivalent to convolution of the reference signal with an antiphase delta-function doublet.

The less sensitive ge-RESIMBA experiment (Fig. 1c) is useful when overlapping proton resonances in the reference spectrum prevent simulations of the ge-SIMBA data. The ge-RESIMBA experiment selects the same coherence pathways as the ge-SIMBA experiment, but yields spectra that are in-phase with respect to the selectively excited ^{13}C because of the addition of a long-range refocusing delay after the selective ^{13}C pulse. After this delay, an additional hard ^{13}C $\pi/2$ purge pulse is used to remove any remaining signal that is antiphase with respect to ^{13}C. For an active heteronuclear coupling with two weak passive homonuclear couplings, ge-RESIMBA produces a mixture of terms that are all in-phase with respect to the long-range heteronuclear coupling, but are antiphase with respect to the homonuclear couplings (e.g., $2I_{1+}I_{2z}$, $2I_{1+}I_{3z}$, and $4I_{1+}I_{2z}I_{3z}$). Because of terms like these, a special reference ^{1}H spectrum must again be obtained. In this case the in-phase nature of the heteronuclear signal allows the reference spectrum for ge-RESIMBA to be obtained with the same pulse sequence, including all ^{13}C pulses, but with the addition of ^{13}C decoupling. This allows for selective ^{13}C "long-range filtering" of the protons, so that only the protons long-range coupled to the selectively excited ^{13}C resonance are observed in the reference spectrum. The resulting spectrum is then "doubleted" in an in-phase (additive) manner, with all other aspects of the processing the same as for ge-SIMBA. The fourth gradient shown in Fig. 1c does not change the coherence pathway selected by ge-RESIMBA, but was added because it improved the quality of the ge-RESIMBA signals.

All ge-SIMBA experiments were performed at 298 K on a Bruker AMX2-500 spectrometer operating in the quadrature mode at a proton frequency of 500.13 MHz, using a 5 mm ^{1}H–^{13}C–^{15}N triple resonance single (z) axis gradient probe, and a maximum gradient strength of about 60 G/cm. Because of the suppression of non-^{13}C coupled protons afforded by gradient selection of the ^{1}H–^{13}C heteronuclear phase coherence pathway, it was possible to use the highest possible receiver gain for maximum sensitivity.

We use a nonlinear regression Levenburg–Marquardt algorithm (13, 14) to carry out the "doubleting" processes described earlier. Nonlinear fits of reference spectra to ge-SIMBA and ge-RESIMBA were performed using a computer program based on the MINPACK functions LMDER and LMDIF (14).

The selective ^{13}C pulses used for this work were generated with Bruker xShape computer software. For ^{13}C resonances that do not require extreme selectivity, we use 50-ms sinc-shaped pulses (with three maxima) that have uniform excitation bandwidths of about 140 Hz. In cases where more selec-

tivity is required, a 120-ms sinc pulse with a nearly rectangular excitation bandwidth of about 50 Hz is used. For the latter case, small transmitter offsets (up to ± 15 Hz) can also be used to ensure that undesired ^{13}C resonances are not hit. The box-shaped excitation profile of the sinc pulse allows it to be easily maneuvered onto the desired carbon resonance by small changes in this offset. In this way, we find that we can easily selectively pulse ^{13}C resonances which have neighbors as close as 25 Hz. Calibration of selective pulses takes only a few minutes on a concentrated sample, although it is always necessary to obtain a ^{13}C spectrum of the actual sample (or 2D HMQC or HMBC spectrum) in order to identify the target offsets.

We chose 0.304 molal sucrose in D_2O as a test sample to illustrate the advantages and limitations of the ge-SIMBA sequences. Sucrose contains crowded ^1H and ^{13}C regions that are challenging in terms of ^{13}C selectivity, ^1H resonance overlap, and strong ^1H–^1H J-coupling. In terms of applicability of these experiments to oligo- and polysaccharides, the three-bond proton–carbon coupling across the glycosidic linkage is of particular interest (15). The structure and chemical shift data for sucrose are shown in Fig. 2a, and its proton spectrum is shown in Fig. 2b.

Figure 2c shows an example of a full ge-SIMBA spectrum for sucrose, with the selective pulse set for the quaternary carbon (C8) at 104.4 ppm. In this case the Δ_2 delay was 50 ms, which was close to optimum for H1 and H7. Extremely small ge-SIMBA signals are also observed with this delay for H11, H10, and H2. Note the excellent suppression of uncoupled ^1H signals in ge-SIMBA, as compared to the proton spectrum in Fig. 2b, a feature that is also observed in ge-RESIMBA spectra. This virtually complete suppression is achieved through the combination of gradient selection and phase cycle selection of the desired phase coherence pathway.

The values for the four observable ^2J and ^3J couplings to the H-1 proton in sucrose were determined by ge-SIMBA, and were compared to those determined by a ^{13}C-observed technique, selective J-resolved spectroscopy (Sel-J-Res) (9). This is a two-dimensional technique that uses a selective ^1H π pulse. We use a 25-ms Gaussian-shaped ^1H π pulse split into two-half Gaussian pulses with the hard ^{13}C π pulse applied between the two halves. The antiphase fits for the ge-SIMBA data are shown in Fig. 3. The results of the comparison between coupling constants determined by ge-SIMBA and Sel-J-Res are shown in Table 1 and indicate that the long-range coupling constants obtained by both methods are similar. The four ge-SIMBA experiments were run in less than one-fourth of the time required for the single Sel-J-Res on the H-1 proton, required no selective proton pulses, and elucidated four times as much long-range coupling information (since four different carbons were selected). Both 50- and 120-ms pulses were used in the ge-SIMBA experiments, and the long-range coupling delay Δ_2 was set to either 60 or 160 ms to maximize sensitivity for different long-range couplings. A reference spectrum was
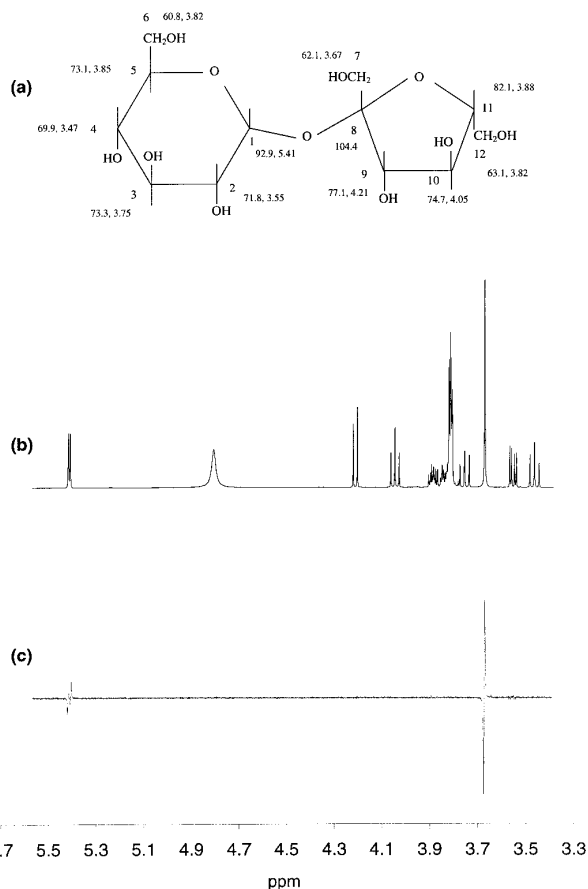

FIG. 2. (a) The structure of the sucrose molecule. Directly attached protons share the carbon numbering scheme. The ^{13}C and ^1H chemical shifts are indicated for each carbon and its directly attached proton(s). (b) The proton spectrum of 0.3 molal sucrose in D_2O. (c) The ge-SIMBA spectrum of 0.3 molal sucrose in D_2O, with the selective ^{13}C pulse set on-resonance for the carbon at 104.4 ppm (C-8). Two strong antiphase long-range coupled proton signals are observed with this Δ_2 delay of 50 ms. Weaker signals, which can be made more intense by suitable adjustment of Δ_2, are also observed. A 50 ms sinc pulse was used for selective ^{13}C excitation.

therefore run for each ge-SIMBA experiment that required different timings. This takes only a few minutes because of the high sensitivity of the ge-SIMBA reference experiment. A measure of experimental uncertainty is also included in Table 1. Each ge-SIMBA experiment on a given carbon was repeated four times, and the spectra obtained were processed and submitted to the fitting procedures separately. The average value for the four runs is reported for each of the coupling constants as $^nJ_{CH} \pm \sigma$, where σ is the standard deviation of the four values.

All of the detectable heteronuclear ^2J, ^3J, and ^4J values for sucrose that could be quantitated are reported in Table 2. In several cases, we were able to obtain useful data with as few as 128 transients (in about 2 min.) with the ge-SIMBA experiment. Although ge-SIMBA and ge-RESIMBA experiments yielded some signals for long-range coupled ^1H resonances that are not reported in Table 2, the coupling constants for
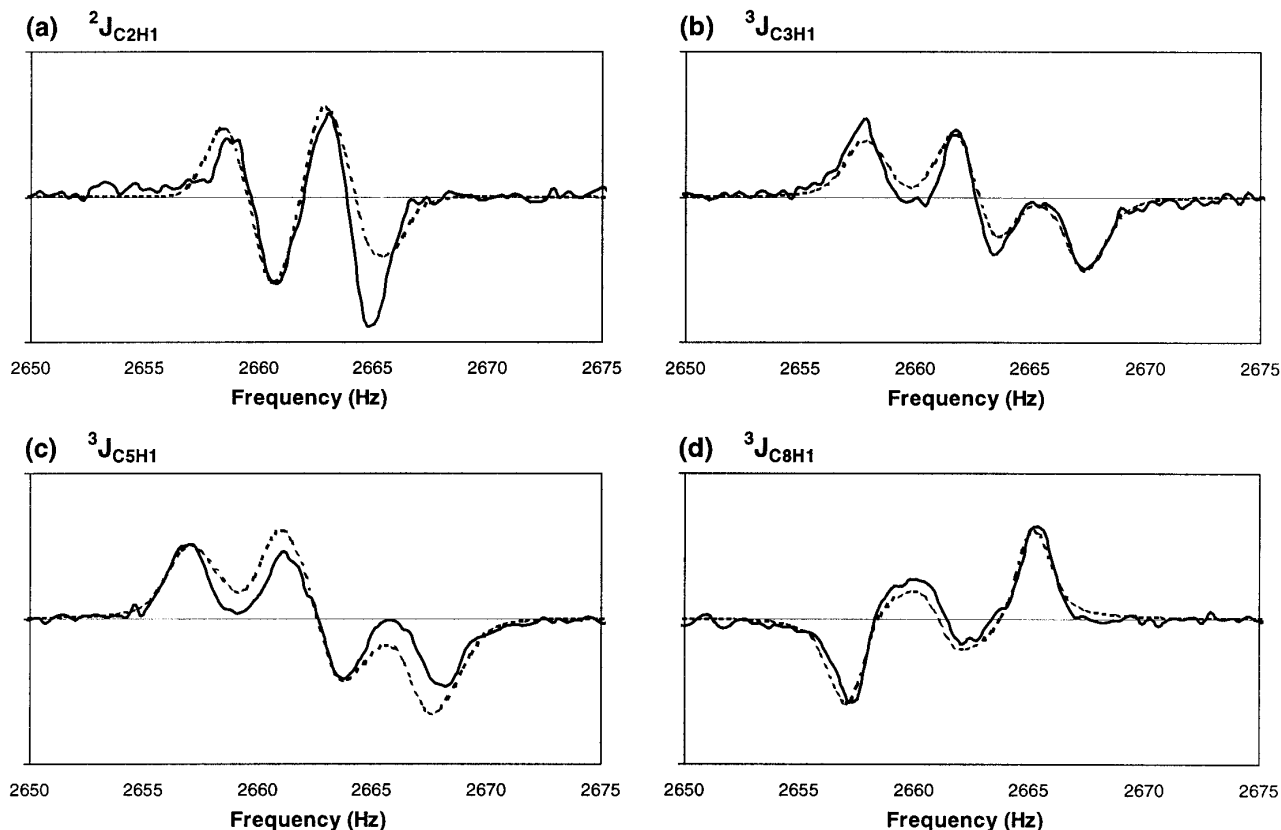
FIG. 3. Antiphase doubleted fits of ge-SIMBA data for the sucrose glycosidic proton (H-1). Solid lines represent experimental ge-SIMBA data, and dotted lines represent ''doubleted'' reference spectra. See Table 1 for the long-range coupling constants obtained from these fits.

these could not be determined in the fitting algorithm. This is due to a limitation of the ge-SIMBA experiment that is observed when the long-range J value is similar to the linewidth. Since the ge-SIMBA signal is antiphase, a small coupling constant ($J < 1.5$ Hz in this case) will cause antiphase cancellation and attenuate the signal, making the data more difficult to fit. Nevertheless, ge-SIMBA allows for the reliable identification of such ^{1}H resonances, which is in itself useful.

A feature of both ge-SIMBA and ge-RESIMBA that compares favorably with Sel-J-Res or with nonselective 2D HMBC-derived experiments (2, 7) is the high selectivity avail-

TABLE 1
Comparison of Sel-J-Res and ge-SIMBA Values for Selected Sucrose Couplings

Coupling constant	Fitted ge-SIMBA value (Hz)[a]	Sel-J-Res value (Hz)
$^3J_{C8H1}$	3.92 ± 0.03	3.9
$^3J_{C5H1}$	6.56 ± 0.09	6.6
$^3J_{C3H1}$	5.47 ± 0.11	5.5
$^2J_{C2H1}$	1.75 ± 0.03^b	2.0

[a] The ge-SIMBA values reported are the averages of four independent experiments and fit calculations for each of the four coupling constants. The $^nJ_{CH}$ values are reported as $^nJ_{CH} \pm \sigma$, where σ is the standard deviation obtained from the four measurements.

[b] Antiphase cancellation may have affected this result (see text).

TABLE 2
Long-Range Sucrose Couplings Measured via ge-SIMBA and/or ge-RESIMBA

Coupling constant	Fitted ge-SIMBA value (Hz)	Method[a]
$^2J_{C2H1}$	1.8	1
$^2J_{C3H4}$	4.3	1
$^2J_{C4H3}$	4.5	1
$^2J_{C5H4}$	4.2	1
$^2J_{C8H7}$	3.2	1,2
$^3J_{C3H1}$	5.5	1
$^3J_{C5H1}$	6.6	1
$^3J_{C6H4}$	3.5	1
$^3J_{C7H9}$	2.1	1
$^3J_{C8H1}$	3.9	1,2
$^3J_{C8H11}$	2.0	2
$^3J_{C9H7}$	3.2	1
$^4J_{C12H9}$	1.1	1

[a] The method(s) used to obtain the value of the long-range coupling constant: 1 denotes ge-SIMBA, 2 denotes ge-RESIMBA.

(a) Fit of $^3J_{C8H11}$

Frequency (Hz)

(b) Fit of $^3J_{C8H1}$

Frequency (Hz)

(c) Reference for $^3J_{C8H11}$

Frequency (Hz)

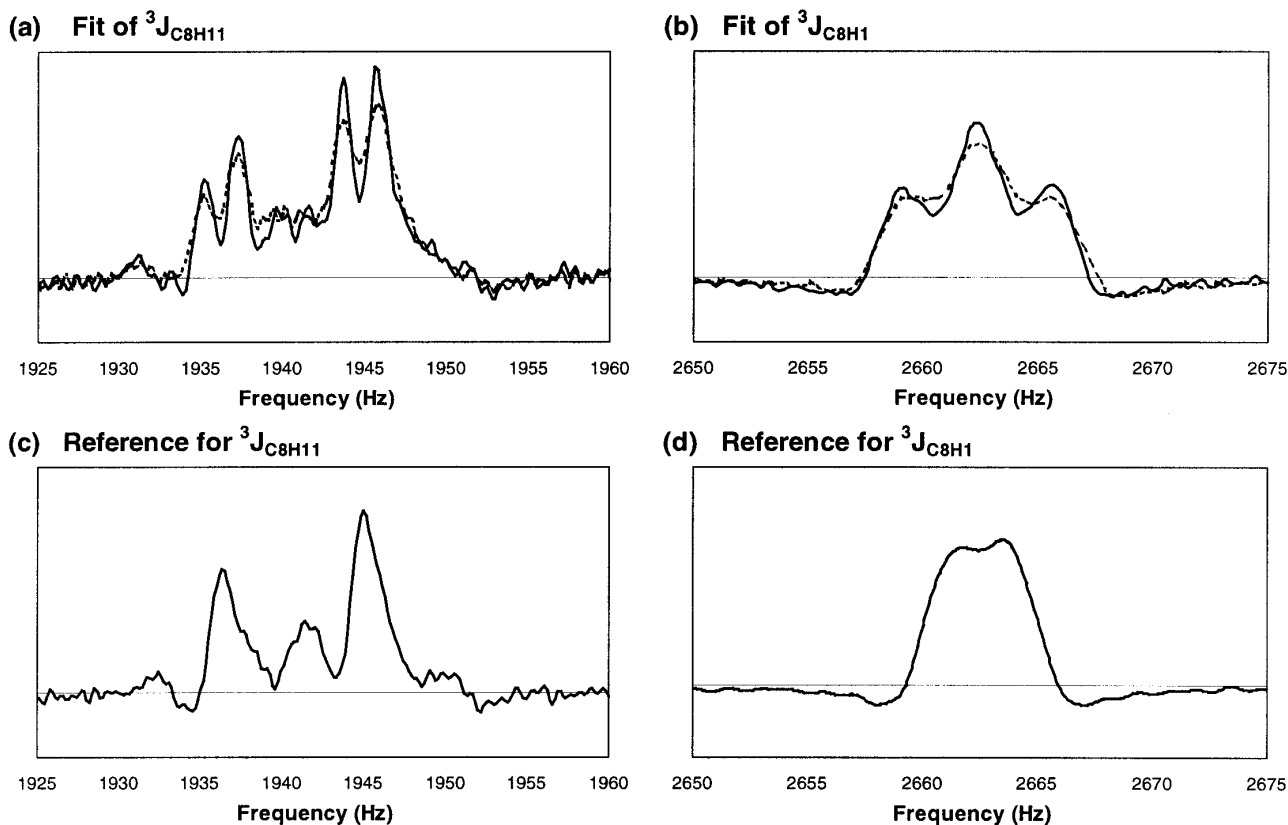**(d) Reference for $^3J_{C8H1}$**

Frequency (Hz)

FIG. 4. (a, b) Examples of in-phase doubleted fits of ge-RESIMBA data for sucrose. Solid lines represent experimental ge-RESIMBA data, and dotted lines represent ''doubleted'' reference spectra. See Table 2 for the long-range coupling constants obtained from these fits. (c, d) The ge-RESIMBA reference spectra that were in-phase doubleted to obtain the dotted lines shown in Figures 4a and 4b, respectively.

able in the carbon frequency domain. In the case of sucrose, selective excitation was easily achieved using the 120-ms sinc pulse for the carbons at 73.1 (C-5) and 73.3 ppm (C-3), despite the fact that these resonances are less than 25 Hz apart. The smaller frequency range in the proton domain would make this kind of selectivity difficult in a Sel-J-Res experiment. In a 2D HMBC-derived experiment, resolution of the signals for these carbons would require a large number of increments in the t_1 domain, and a significantly longer experiment time. For example, an overnight HMBC experiment on the same sucrose sample with 400 t_1 increments (zero-filled to 1024 points) was unable to resolve the antiphase doublets for C-3 and C-5, making a fit difficult.

The less sensitive ge-RESIMBA experiment was used to verify some of the preceding coupling constants and to determine some others that could not be obtained with ge-SIMBA. The ge-RESIMBA experiment is the method of choice when overlapping proton resonances prevent the use of the ge-SIMBA reference experiment in the fitting algorithm. An example of such a situation is the sucrose $^3J_{C8H11}$ coupling constant, which requires a reference signal to be taken from the region of 3.88 ppm, a crowded area in the sucrose spectrum.

H11 is partially overlapped by H5 and H6 at 3.85 and 3.82 ppm as well as by its ''partially strong'' coupling partner of H12 at 3.82 ppm. The ge-RESIMBA reference spectrum's selectivity overcomes these problems because it only contains the ^1H signal from H11, the only proton in this spectral region that is long-range coupled to the selectively excited ^{13}C. The $^3J_{C8H11}$ coupling constant was determined to be 2.0 Hz as shown in Fig. 4a. The glycosidic $^3J_{C8H1}$ coupling, which was previously determined by ge-SIMBA (Table 1), was confirmed to be 3.9 Hz by ge-RESIMBA, as shown in Fig. 4b. Because they are in-phase with respect to the active heteronuclear coupling, ge-RESIMBA spectra do not experience antiphase cancellation effects in their ^{13}C-decoupled reference spectra, as is illustrated for the $^3J_{C8H11}$ and $^3J_{C8H1}$ cases in Fig. 4c and d. Antiphase effects of homonuclear ^1H-^1H couplings are the same for the actual ge-RESIMBA and the reference. However, the data for small couplings is still difficult to handle, since the small couplings appear as line broadening.

Relaxation during the additional refocusing period significantly reduces the sensitivity of the ge-RESIMBA experiment and its reference experiment as compared to ge-SIMBA and its reference. It is important to recognize that the ge-RESIMBA

reference experiment takes as long as the ge-RESIMBA experiment itself and suffers the same sensitivity losses due to relaxation. In practice, the ge-RESIMBA experiment also requires more careful optimization of the Δ_2 delays than the ge-SIMBA experiment. In addition, increased ^{13}C selectivity requires a longer selective pulse and allows more relaxation for both the ge-SIMBA and ge-RESIMBA experiments, thereby attenuating the observed signal. The loss in sensitivity due to selective pulses is similar to the loss of sensitivity during longer t_1 periods in the 2D HMBC-derived experiments, in that both are necessary for frequency selectivity. The Sel-J-Res 2D experiment is rarely limited by ^{13}C resolution, but slow relaxation of directly observed ^{13}C requires much longer experiment times.

A protocol for the use of these experiments can be suggested based on the amount of crowding observed in the normal proton spectrum. The more sensitive ge-SIMBA experiment, with its much more sensitive reference experiment, is used to measure all couplings that show a resolved proton resonance. The reference for ge-SIMBA only needs to be run once for each set of timings used in the experiment. Sucrose, for example, required only three reference spectra, corresponding to the three different combinations of selective pulse duration and long-range coupling delay that were used. When the problem of overlapping proton resonances exists in the reference spectrum, the ge-RESIMBA experiment can be used to obtain the coupling constant.

In conclusion, two gradient-enhanced 1D experiments and two corresponding reference experiments have been proposed. These experiments have been shown to successfully measure long-range J_{CH} coupling constants with significantly greater efficiency than previous methods.

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