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Improved Cross Peak Detection in Two-Dimensional Proton NMR Spectra Using Excitation Sculpting

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In two-dimensional homonuclear experiments, reliable detection of very small cross peaks is sometimes hampered by t_1-noise from larger diagonal peaks which have the same F_2 frequency. This t_1-noise can be selectively removed by recording partial 2D spectra using the double pulsed field gradient spin echo (DPFGSE). Resonances not selected by the DPFGSE do not contribute any t_1-noise to the resulting spectrum. The absence of t_1-noise from the diagonal peaks render the small cross peaks at the same F_2 frequency observable. The success of this technique is shown by partial 2D NOESY and TOCSY spectra.

Key Words: **Excitation sculpting; NOESY; TOCSY; t_1-noise; DPFGSE; partial 2D NMR.**

Interferometric experiments like two-dimensional (2D) NMR are commonly plagued by additional noise-like signals, generally called ''t_1-noise,'' which appear as streaks or ridges in the interferometric or F_1 dimension. Sources for t_1-noise include phase noise in the frequency synthesizer (*1*), instrumental instabilities in the field/frequency lock and radiofrequency (RF) pulses, sample spinning, and scan-to-scan variation in the steady-state longitudinal magnetization during the experiment when the repetition rate is optimized for sensitivity (*2*). This additional ''noise'' is different in character from the thermal noise and is typically proportional to the maximum signal amplitude that is present at each F_2 frequency on any given single transient, whether or not this signal is eventually attenuated by phase cycling or difference spectroscopy. In natural abundance heteronuclear experiments like HMQC (*3, 4*) and HMBC (*5*) the unwanted parent proton signals from the carbon-12 isotopomers can lead to large noisy ridges at these frequencies even though a subtraction step is used to eliminate the parent signal and reinforce the proton signals from carbon-13 isotopomers. Pulsed field gradient (PFG) methods (*6, 7*) are able to improve these experiments by greatly attenuating unwanted signals *before* they ever reach the receiver (*8–11*), thereby diminishing the maximum signal amplitude ever present, and with it the associated interferometric noise.

While conventional PFG methods can eliminate unwanted signals, they do not intrinsically remove t_1-noise from the spectrum: there is still interferometric noise on each resonance, and it is still roughly proportional to the amplitude of the signal itself. In heteronuclear experiments, this signal is just the desired cross peak correlating the chemical shifts of the carbon-13 (or nitrogen-15) and the protons, and so typical contours show an *apparently* t_1-noise-free 2D spectrum, or may run into the true thermal noise floor for typical samples. However, in homonuclear experiments like NOESY (*12*) and TOCSY (*13*) there are inevitably diagonal peaks in the 2D spectrum, and they are sometimes much larger than the cross peaks at the same F_2 frequency. In these cases, using PFGs does essentially nothing to improve the detectability of the cross peak and, depending on the spectrometer design and PFG reproducibility, may actually make matters worse. In this communication it is shown that very small cross peaks can be reliably detected by recording *partial* 2D spectra in which the corresponding diagonal peak is never detected by the receiver.

Semi-selective 2D spectra have been obtained using a variety of selective pulse schemes (*14–16*). The main advantage of such spectra has been a reduction in the overall experiment time because a smaller number of t_1 increments need to be used if a selective pulse restricts the possible range of F_1 frequencies. Thus, either very high resolution spectra over a limited bandwidth in F_1 or very rapid lower resolution spectra can be obtained; some cross peak multiplet simplification is also possible. However, these spectra are no fundamental improvement with respect to the detection of very small cross peaks, because the unwanted axial peaks from magnetization left on the z-axis after the selective pulse must be removed by differ-

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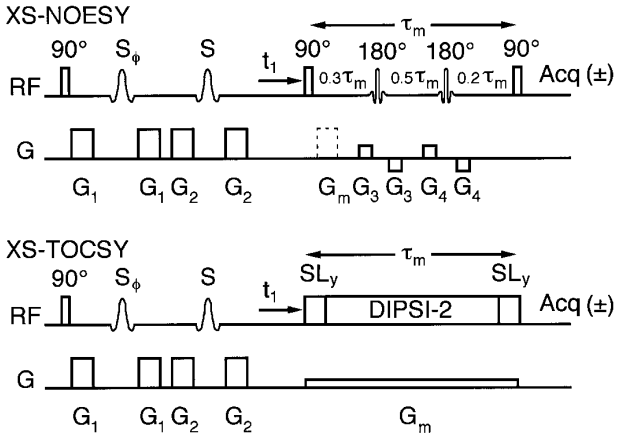

FIG. 1. The excitation sculpting (XS) NOESY and TOCSY sequences. The shaped pulse *S* is typically a frequency-modulated (FM) pulse which inverts spins in a particular frequency band but not outside, as this is most convenient for a Fourier experiment with minimum time increments. However, *S* can also be chosen to excite multiple bands if desired. The basic phase cycle is $\phi = 0°, 90°$ with subtraction of alternate transients. All other pulses are phase $+x$ unless indicated otherwise. The actual timing of the broadband nulling pulses in XS-NOESY depends on the ratio of the mixing time to a typical T_1 relaxation time. The ratios shown are appropriate for $\tau_m \sim T_1$.

ence spectroscopy and will contribute to t_1-noise in the usual way.

Excitation sculpting (*17, 18*) is a method we have recently introduced, and it differs from other selective excitation methods in that signals not actively selected by the pulse sequence are dephased by PFGs and so do not contribute any signal intensity. Band-selective excitation sculpting was demonstrated by Stott *et al.* (*18*). Using two 25-ms hyperbolic secant 180° pulses (truncated at 26.7% amplitude) in the double pulsed field gradient spin echo (DPFGSE), excitation profiles of various bandwidths were demonstrated. These excitation profiles were demonstrably superior to a 50-ms E-BURP1 selective pulse (*19*). The excellent phase and amplitude properties of the excitation sculpting technique make it perfect for use in multi-dimensional NMR experiments, and the very high reject ratio for out-of-band signals means that a tight spectral width in F_1 and a minimum number of increments can be used. These advantages were quickly appreciated by others (*20, 21*). Homonuclear decoupling between the target band and the corresponding cross peaks in the F_1 dimension was cleverly added into the 2D excitation sculpting technique by Krishnamurthy (*22, 23*). In addition, the complete suppression of unwanted diagonal peaks in homonuclear experiments that can be achieved with excitation sculpting makes it possible to detect cross peaks that just cannot be reliably seen in conventional or even semi-selective 2D spectra using other methods.

Figure 1 shows the pulse sequence timing diagrams for the sequences we propose. A double pulsed field gradient spin echo is used to select a desired band (or bands) of signals, the *target spins*, and dephase all other magnetization. Except for homonuclear spin–spin coupling between spins *within* the excitation band, all coupling and chemical shift interactions of the targets are refocused, leading to excellent phase characteristics and flat 2D baseplanes that are easily contoured. This target magnetization is allowed to evolve and then transferred by either a NOESY or TOCSY step to the desired *destination spins*. To detect cross peaks reliably, the target and destination spins should be disjoint sets, and all unwanted magnetization that could appear on the destination spins should be minimized *before* any phase cycling. In the NOESY experiment this is achieved by using broadband 180° pulses in the mixing time to null out T_1 relaxation of the destination spins while maintaining the cross relaxation responsible for the Overhauser buildup (*24*). In the TOCSY experiment a weak continuous gradient in conjunction with short spin locking segments bracketing the DIPSI-2 (*25*) mixing sequence serves to dephase unwanted magnetization and to partially dephase residual zero-quantum co-

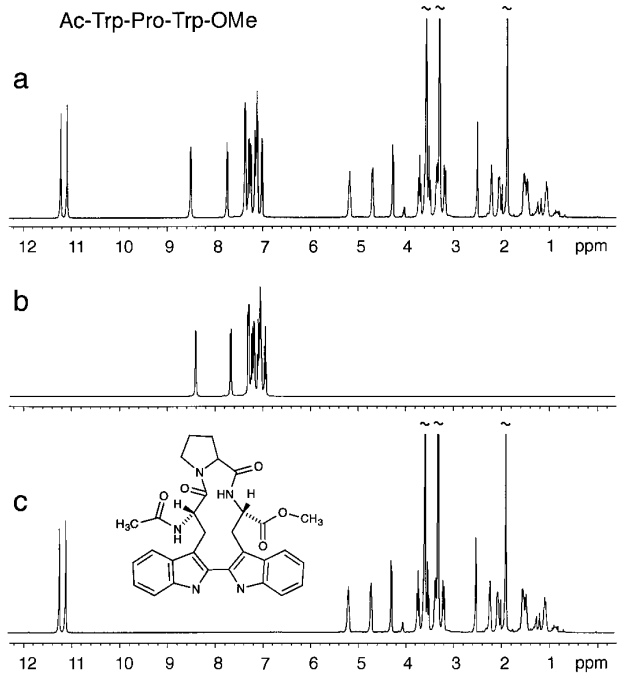

FIG. 2. (a) The 1D reference spectrum of the linked ditryptophan molecule shown. (b) Use of a DPFGSE sequence to select a band of resonances. The shaped pulse *S* was a 4.1-ms FM pulse (*24*). The gradients were each 1 ms long with strengths $G_1 = 7$ G cm^{-1} and $G_2 = 3$ G cm^{-1}. (c) Selection of the complementary set of resonances using a complementary inversion sequence. Here, *S* is composed of the same selective 180° pulse used in (b) immediately followed by a 192-μs FM broadband inversion pulse (*24*). All other parameters are the same as those in (b).

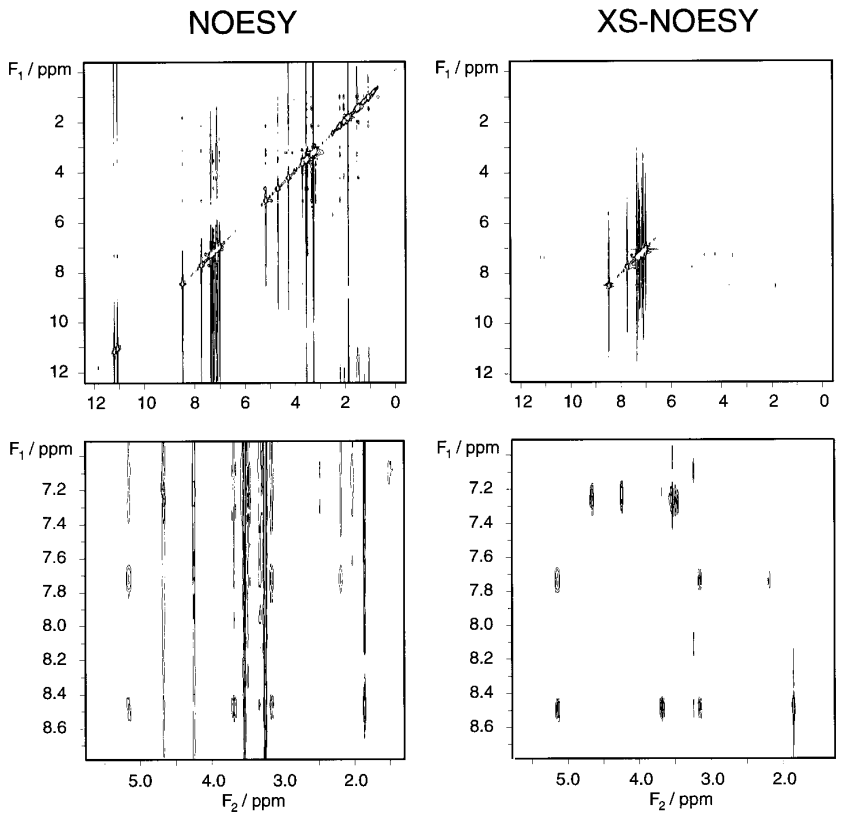

FIG. 3. Comparison of NOESY and XS-NOESY on the molecule shown in Fig. 2. In the NOESY experiment a homospoil gradient was used during τ_m (400 ms). The observed t_1-noise varies with the amplitude of the signal along the diagonal. In XS-NOESY it is far easier to discern the weak positive NOEs typical of a small molecule. Note that along the target resonances, where there are diagonal peaks, the t_1-noise appears to be similar in the two methods.

herence, as discussed by Davis *et al.* (26). Maintenance of the weak gradient during the DIPSI-2 mixing sequence is useful to eliminate radiation damping effects that may occur if strong resonances like H_2O are present (*27*). While it might be expected that such a gradient would need to be turned on and off slowly, to avoid dephasing the transverse magnetization, in fact it is possible to initiate the weak gradient slightly after the start of the mixing sequence and terminate it slightly before the end: very little magnetization is lost with a gradient that causes a ~1-kHz line broadening in a 5-kHz RF field, even with a nominally rectangular temporal PFG profile.

Figure 2 shows the ability of the excitation sculpting technique to select certain resonances in the proton spectrum of the linked ditryptophan molecule shown. The full spectrum (a) is easily edited into subspectra containing only the resonances in a narrow band around the aromatic region (b) or the complementary resonances (c). Note the razor-flat baseline and excellent phase properties of the subspectra, and the very high suppression of unwanted signals. Figure 3 shows contour plots of the conventional 2D NOESY and XS-NOESY spectra. The ditryptophan molecule has weak positive NOEs, which are quite difficult to discern in the 2D NOESY spectrum. In part this is due to t_1-noise and in part to difficulties adjusting the phase of the diagonal peaks to be in pure absorption. Even very small phase errors can bring in the dispersion-mode tails of these strong lines and make it very tedious to try to discern the cross peaks. In contrast, these weak features stand out cleanly in the XS-NOESY spectrum, where the corresponding diagonal peaks are eliminated from the F_1 traces.

Figure 4 shows a comparison of phase-sensitive 2D TOCSY and XS-TOCSY applied to a solution of 1-dodecanol. A relatively long mixing time of 195 ms was used in an attempt to drive magnetization from one end of the alkyl chain to the other. In the conventional 2D TOCSY, many of the cross peaks are hard to discern, while in the XS-TOCSY spectrum, in which only the $-CH_2OH$ multiplet was excited, there is a clear and unambiguous cross peak to the methyl group at the other end of the molecule. We have verified its authenticity by con-

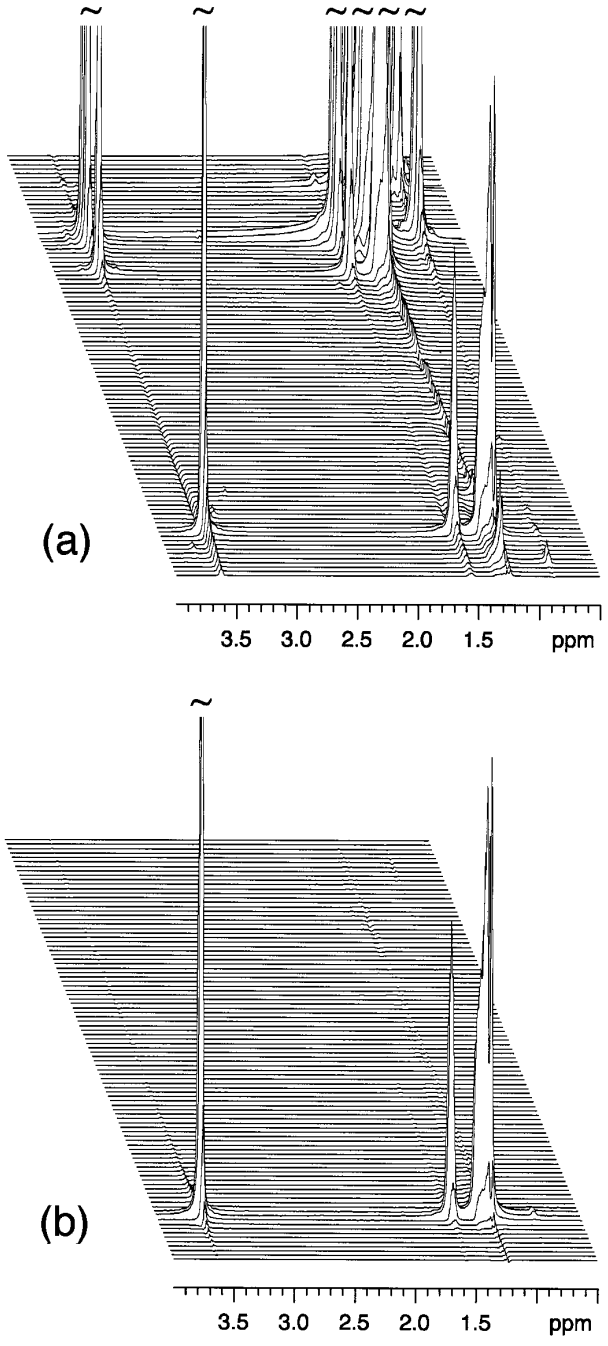

FIG. 4. Comparison of TOCSY (a) and XS-TOCSY (b) on 1-dodecanol. A very long mixing time τ_m = 195 ms was used in an attempt to transfer magnetization down the entire aliphatic chain, from the $-CH_2OH$ group to the methyl protons. A very small but unequivocal peak at the methyl resonance in the XS-TOCSY spectrum shows that magnetization can be transferred down the entire twelve-carbon chain. This peak is hard to discern in the TOCSY spectrum because it is of the order of the t_1-noise from the strong methyl groups. In the XS-TOCSY, *S* was a 37-ms FM pulse (*24*), and the duration and strengths of the PFGs were the same as those listed in Fig. 2b.

ducting the magnetization transfer *from* the methyl to the terminal methylene, by observing the time dependence of the transfer along the chain, and by control experiments in which no target is selected and no cross peaks are seen.

These spectra show that the excitation sculpting technique is a powerful addition to 2D spectroscopy. While we have kept spectral widths identical in these spectra for clarity of presentation, it is possible to reduce the number of increments greatly in the XS spectra and use a correspondingly smaller spectral width in F_1, making them more time efficient as well as more informative than conventional 2D spectra. It is also possible to use a further DPFGSE sequence in place of the simple 90° read pulse, to focus on only the cross peak frequencies, and improve the dynamic range even further.

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