Quantitative Measurement of Long Range Nuclear Overhauser Enhancement

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Measurement of nuclear Overhauser enhancement is one of the mainstays of structural studies of molecules in solution. Measurements, particularly over long distances, are often compromised by spin diffusion. A robust and accurate method for measuring nuclear Overhauser enhancements that are largely free of spin diffusion effects is presented. The effects of using imperfect radiofrequency pulses are considered and experimental data are presented for Amphotericin B. © 1999 Academic Press

Key Words: **NOE; spin diffusion; excitation sculpting; Amphotericin B.**

The measurement of nuclear Overhauser enhancement (NOE) is one of the mainstays of structural studies of molecules in solution (*1, 2*). NOEs arise when longitudinal magnetization is transferred between nuclear spins as a result of their mutual dipolar relaxation. Since the magnitude of dipolar relaxation between a pair of nuclei is inversely proportional to the sixth power of their internuclear distance, the NOE decreases rapidly with increasing internuclear distance and is generally only observed up to 4.5–5.0 Å. When several nuclei are in close proximity, magnetization can be transferred both directly from one spin to another and indirectly by way of one or more additional nuclei. This effect, which may distort the size of the measured NOE, is known as spin diffusion. Spin diffusion is often particularly severe between pairs of spins which are closer to other spins than they are to each other. While present in NOE studies of all molecules, spin diffusion effects are often most severe for molecules in the slow tumbling regime, when $\omega \tau_c > 1$. Here we describe a robust NMR experiment that combines excitation sculpting with an effective spin diffusion suppression procedure to enable NOE build-up curves to be measured largely free of spin diffusion. The effects of the nonideal behavior of radiofrequency pulses is considered and experimental data are presented for Amphotericin B.

Methods for suppressing or taking into account spin diffusion in NOE measurements have been described in the literature (*3–10*). There are two general approaches to addressing the problem. In the context of full structure determination, spin diffusion effects can be removed iteratively to first order during structure determination (*3*). It is also possible to remove the effects of spin diffusion on the NOE build-up curve experimentally (*4–10*); this approach has the advantage of not requiring a full structure determination. The most generally applicable approach to the latter, which we adopt as the basis of the method presented here, can usually only be used to measure one NOE at a time and is most commonly implemented in transient NOE measurements (*6, 7*). In experiments of this type, the nuclear spin which the NOE is to be measured from (the parent) is selectively perturbed from equilibrium. Halfway through the subsequent mixing period, this nuclear spin and the spin to which the NOE is to be measured are simultaneously inverted. While this simultaneous double inversion has no effect on the build-up of the NOE (other than a change in sign), it causes spin diffusion effects during the two halves of the mixing period to undergo mutual cancellation. Both the initial perturbation of the chosen spin from equilibrium and the subsequent double inversion during the mixing period are usually implemented using semiselective "soft" pulses. Three issues must be addressed if this experiment is to be implemented reliably: the selectivity of the soft pulse(s) used to select the parent; the intrinsic effectiveness of the spin diffusion suppression procedure, and the consequences of the nonideal behavior of the semiselective pulses used to achieve suppression.

A long range NOE is usually relatively small compared to its parent peak, typically <1%, and consequently it is important that the initial perturbation of the parent peak from equilibrium not also perturb any other multiplets, since these may give rise to NOEs of their own. Furthermore, if the peak an NOE is being measured to is itself perturbed its value will be distorted. This is frequently a problem when the parent peak is perturbed with a single semiselective pulse since all pulse shapes developed to date perturb the magnetization away from the nominal chosen excitation band to some extent. This problem can be largely overcome using excitation sculpting (*11–14*). This technique comprises the preparation period of the pulse sequence given in Fig. 1. Excitation sculpting works by exciting all of the nuclear magnetization into the *xy*-plane and then using magnetic field gradient pulses to dephase all that which is not refocused by the two semiselective Gaussian shaped pulses. Since two semiselective pulses are used, the fraction of
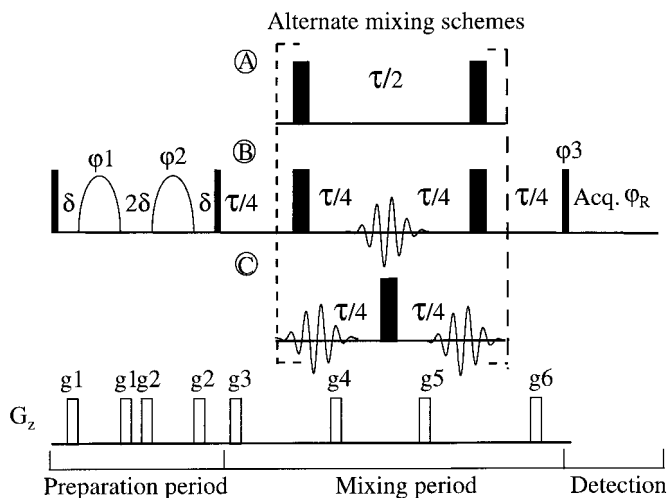
FIG. 1. Pulse sequence for transient NOE spectroscopy. Solid narrow and wide rectangles represent nonselective 90° and 180° pulses, respectively; all other pulses are semiselective. The two semiselective pulses in the preparation period are calibrated to select the multiplet NOEs are to be measured from. The bracketed mixing scheme designated A is used to measure NOE build-up without spin diffusion suppression; schemes B and C utilize one and two semiselective pulses, respectively, to suppress the contribution of spin diffusion to the NOE build-up curve. The suppression pulses are cosinusoidally modulated at half the difference in frequencies of the two multiplets between which the NOE is to be measured between (18, 19). Gaussian modulated selective pulses are used in the preparation period (11–14) and pulse shapes designed to give a "top hat" inversion profile such as the $q3$ gaussian cascades (19, 20) are most effective in the mixing period. Cross-relaxation is considered to occcur 47% of the time $q3$ pulses are applied (21). While only one magnetic field gradient pulse is required between two rf pulses, for the sake of simplicity a single scheme is given here that works for all three mixing periods. Phase cycling: $\phi1 = x, y, -x, -y$; $\phi2 = y$; $\phi3 = 4*(x), 4*(-x)$; $\phi R = x, -x, x, -x, -x, x, -x, x$.

the magnetization refocused at a given offset will be the square of that refocused with a single pulse, thus resulting in much cleaner selection. The selected magnetization is subsequently returned to the z-axis. While the parent peak must be resolved in the 1D spectrum, this need not be true of the NOE peak. For spin diffusion to be effectively suppressed, it is only necessary that the semiselective 180° pulse(s) used to invert the NOE and its parent peak in the mixing period not substantially invert any other peaks present in the transient NOE spectrum. Following Keeler (14) we also introduce nonselective 180° pulses into the mixing period as indicated in Fig. 1 to prevent the magnetization dephased during the preparation period from recovering. The cross-relaxation rate σ_{IS} responsible for the NOE between two nuclei I and S can be extracted by fitting the data with the standard transient NOE equation (2),

$$S(\tau) = -(\sigma_{IS}/D)(e^{-(R'+D)\tau} - e^{-(R'-D)\tau}), \qquad [1]$$

where $D = [(\tfrac{1}{4})(R_I - R_S)^2 + \sigma_{IS}^2]^{1/2}$, $R' = (\tfrac{1}{2})(R_I + R_S)$, and R_I and R_S are the longitudinal relaxation rates of the two nuclei. The spin diffusion suppression procedure utilised here

relies on the selective inversion of the parent and chosen NOE peaks half way through the mixing period. The technique makes the assumption that the effects of spin diffusion during the two periods are roughly the same, except for their signs, and can consequently be made to cancel out. The less longitudinal relaxation that has occurred during this period, the better this approximation will be. The effect of spin diffusion on the calculated NOE rate are explored in the results of a simulation study presented in Fig. 2A. The error in the cross relaxation rate calculated from the NOE build-up curve has been determined for a pair of nuclei at a fixed distance from each other but experiencing differing amounts of spin diffusion. The amount of spin diffusion they experience has been varied by varying the coordinates of a third spin that is equidistant from the first two. As might be expected, introducing a single inversion pulse into the mixing period decreases the error in the calculated cross relaxation rate relative to the corresponding experiment with no suppression. However, as the amount of spin diffusion (and the longitudinal relaxation rates) increase, the effectiveness of suppression decreases. The effectiveness of suppression can be improved by introducing an additional semiselective inversion pulse into the mixing period (10) as indicated in Fig. 1. The improvement in suppression is reflected in a corresponding reduction in error in Fig. 2A. In principle it is possible to further improve suppression of the effects of spin diffusion by using more inversion pulses; in practice the time constraints that this imposes on the minimum mixing time that can be used (the sum of the pulse lengths) and the consequences of nonideal behavior of the pulses may make this impractical.

In reality, the semiselective pulses within the mixing period often do not achieve complete inversion of the target nuclei. With modern pulse shapes, which are designed to invert a band of frequencies efficiently and exhibit a "top hat" excitation function, this is largely due to relaxation effects. While magnetization that is unaffected by a given selective inversion pulse will decay due to longitudinal relaxation during the pulse, magnetization that is perturbed will be attenuated due to transverse relaxation as well since it will spend part of its time in the xy-plane. For molecules in the slow tumbling regime $T_1 > T_2$, and consequently the inverted magnetization is likely to suffer greater relaxation losses. The minimum length of the selective inversion pulse is dictated by the necessity of only inverting the two spins involved in the NOE of interest in the transient NOE spectrum. The effects of incomplete inversion on the effectiveness of spin diffusion suppression are investigated in Fig. 2B. The conditions used for the simulations were the same as for Fig. 2A except that 20% of the magnetization was "lost" upon inversion. While a comparison of Figs. 2A and 2B shows that incomplete inversion does reduce the efficiency of spin diffusion suppression, it is also clear that the procedure is still effective, and as with ideal pulses that two inversion pulses are better than one.

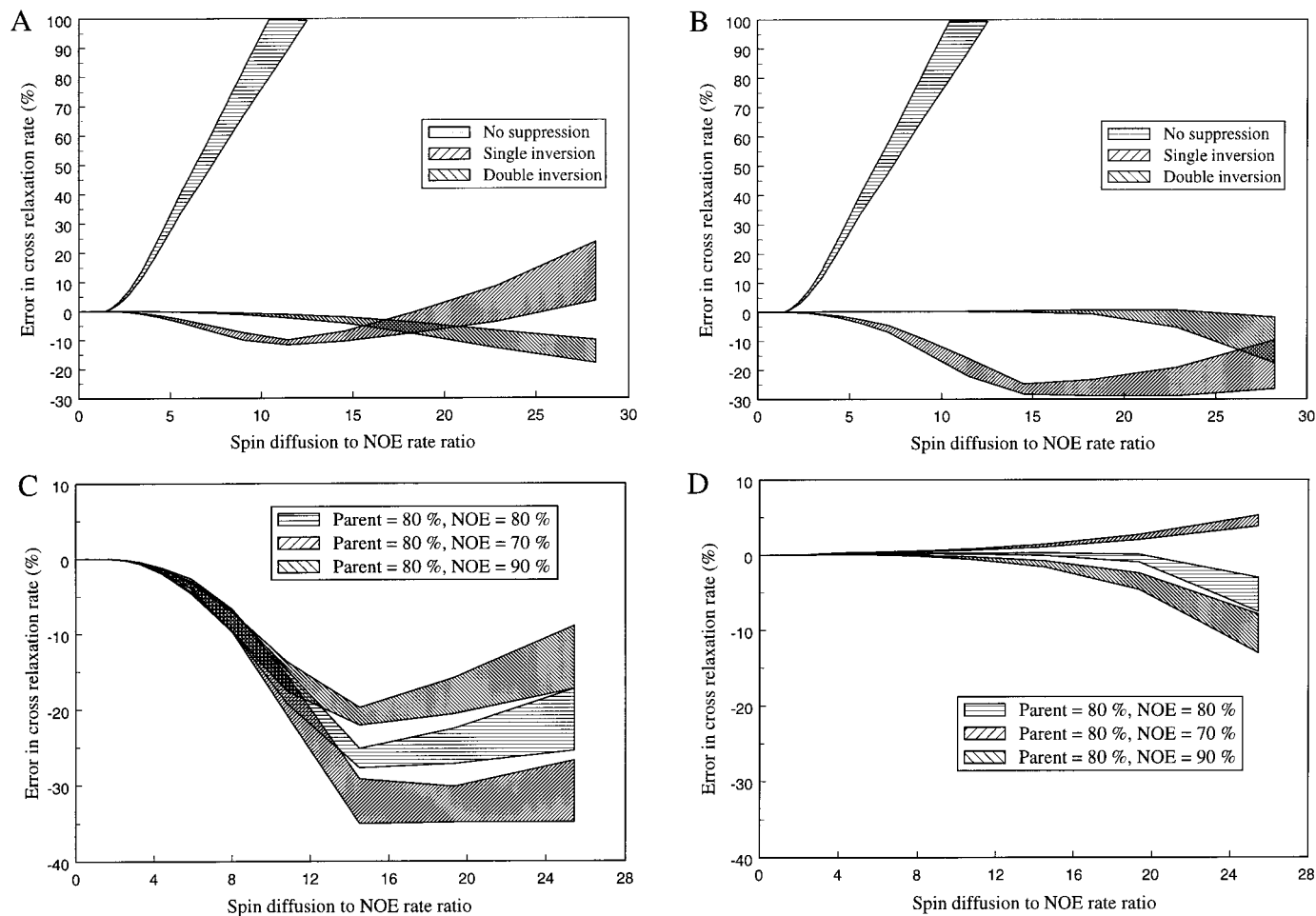Variations in relaxation rates between spins may also give

FIG. 2. Simulations of the effects of spin diffusion on the accuracy of cross-relaxation rates measured from NOE build-up curves for the three alternative mixing schemes given in Fig. 1, (A) assuming perfect inversion pulses and (B) assuming that 20% of the magnetization is lost upon inversion. The effects of different inversion efficiencies for the parent and NOE peaks are investigated in (C) for the single and (D) for the double inversion mixing schemes, where inversion efficiencies are as indicated. The bands given in each case correspond to the 95% confidence limits. Numerical simulations were carried out at 400 MHz for an NOE between two nuclei 4 Å apart using a correlation time of 1 ns and using a third spin at a variable but equal distance from the first two to generate spin diffusion. The data were fitted out to twice the time taken for the NOE to reach its maximum intensity. The spin diffusion to NOE rate ratio is defined as the cross-relaxation rate between either of the first two and the third spin divided by the cross-relaxation rate between the first two spins.

rise to variations in inversion efficiency. The effects of variations in inversion efficiency between parent and NOE peaks for the single and double inversion sequences are investigated in Figs. 2C and 2D respectively. In each case the parent peak is assumed to be inverted with an efficiency of 80% while the inversion efficiency of the NOE is varied between 70%, 80%, and 90%. Any difference in inversion efficiency between the two spins has two consequences: the efficiency of spin diffusion suppression will be affected and the NOE build-up curve will be scaled by the ratio of the NOE to parent peak inversion efficiencies (assuming that the NOE is determined from the ratio of the integrals of the NOE at a given time to the parent peak at zero mixing time). The latter has been corrected for in the figure. While the effectiveness of spin diffusion suppression clearly varies with the inversion efficiency of the NOE

peak for both mixing schemes the double inversion sequence continues to produce better results.

Magnetic field gradient pulses (*15, 16*) applied during the mixing period serve to eliminate any unwanted components of transverse magnetization that may result from nonideal behavior of the pulses.

The pulse sequence given in Fig. 1 is demonstrated using amphotericin B (*17*) in Figs. 3 and 4. The effectiveness of spin diffusion suppression can be assessed by comparing spectra obtained with the three versions of the experiment at a given mixing time, Fig. 3. The data in Fig. 3A shows the unsuppressed transient NOE spectrum for H35. While incorporating a single selective inversion pulse into the mixing period clearly reduces the intensity of NOEs to peaks other than H37, Fig. 3B, the fact that most are still clearly present with significant
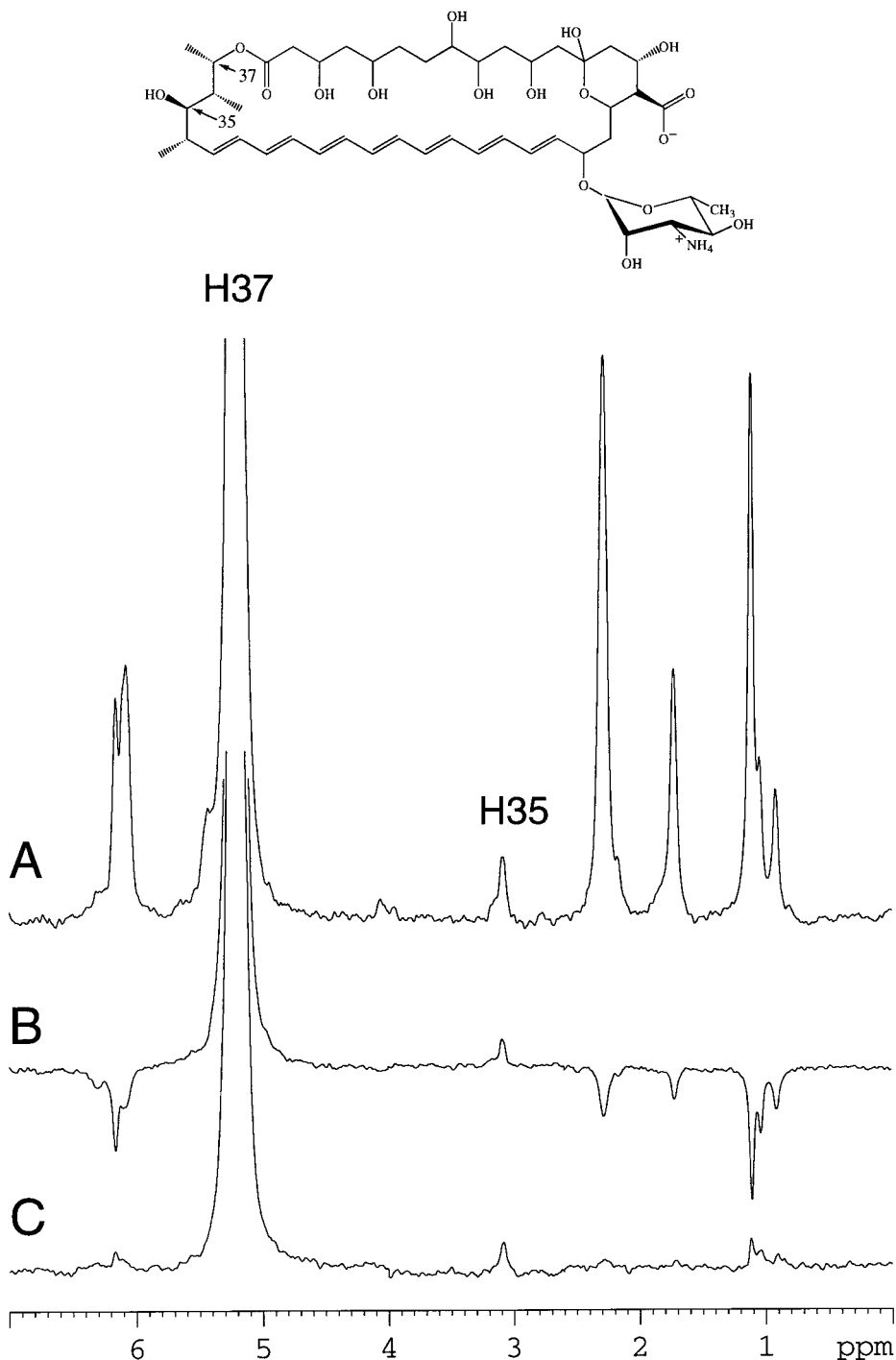
FIG. 3. 400 MHz ^{1}H NMR spectrum for 20 mM amphotericin B in DMSO. (A) Unsuppressed, (B) singly suppressed, and (C) doubly suppressed transient NOE spectra obtained using the pulse sequence given in Fig. 1 for a mixing time of 190 ms. Semiselective pulses used in the preparation period were 20 ms Gaussians, and those used in the mixing period were 30 ms $q3$ Gaussian cascades. The spin diffusion suppression pulses were cosinusoidally modulated at 630.5 Hz. Gradient values $g1-g6$ were 25, 35, 42.5, 6.5, 5, and 4 G cm^{-1}, respectively; all gradient pulses were applied for 1 ms. All data were acquired on a Bruker DRX-400 spectrometer.

intensity indicates the limited efficiency of the procedure. Introducing the second selective inversion pulse, Fig. 3C, clearly substantially improves the effectiveness of suppression.

The build-up curve for the NOE from H35 to H37 measured without spin diffusion suppression and with suppression using one and two inversion pulses during the mixing period is given
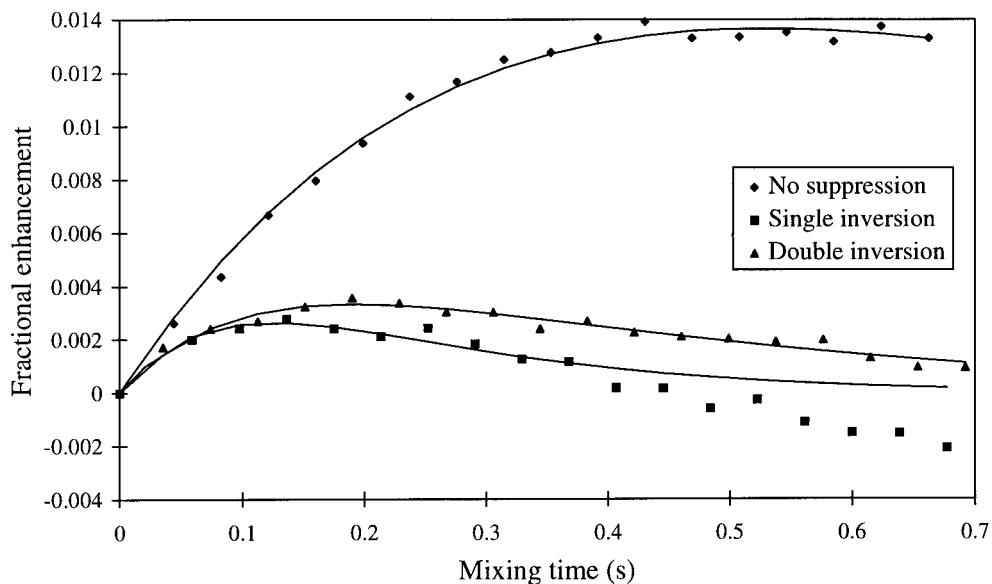
FIG. 4. 400 MHz ^{1}H NMR data for 20 mM amphotericin B in DMSO obtained using the pulse sequence given in Fig. 1 with the same parameters as for Fig. 3. The build-up curves presented are for the NOE from H37 to H35. All data are phased to be positive for ease of comparison. The solid lines correspond to the best fit for all of the date using Eq. [1] except for the single inversion data which were fitted up to 0.36 s.

in Fig. 4. The inversion pulses were found to have an efficiency of 85% for both H35 and H37, within experimental error (i.e., 15% of the magnetization was lost upon inversion). The increasing effectiveness of spin diffusion suppression as the number of inversion pulses is increased is reflected in the build-up curves. The inadequacy of using a single inversion pulse is clearly indicated by the apparent change in sign of the NOE at 0.5 s; the cross-relaxation rate obtained by fitting these data with Eq.[1] also varies with the time over which they are fitted. Fitting all of the data yields cross relaxation rates of 0.035 ± 0.002 s^{-1} for no suppression, 0.037 ± 0.026 s^{-1} for suppression with a single inversion pulse, and 0.025 ± 0.005 s^{-1} for suppression with two inversion pulses. The rate for the single inversion suppression changes to 0.029 ± 0.008 s^{-1} if only the first 0.36 s of the data are fitted. Spin diffusion suppression with two inversion pulses is clearly to be preferred since it yields a cross relaxation rate which is independent of the mixing times used and, since the data can be fitted to longer mixing times, more precise.

We note that sensitivity will be lost in these experiments as a result of both the efficiency of the inversion pulses used in the mixing period and the length of the preparation sequence. As noted above, the inversion pulse used to suppress spin diffusion were found to have an efficiency of 85%, and the efficiency of the mixing period was found to be 57%. In both cases the key determining factor is the length of the selective pulses used which depends on the resolution required and will consequently vary from case to case.

In conclusion, the pulse sequence presented in Fig. 1 combining excitation sculpting with the use of two semiselective inversion pulses to suppress spin diffusion enables reliable data to be acquired for long range transient NOEs. The procedure is effective even if the semiselective inversion pulses do not behave ideally.

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