

Observation of Long-Range Small-Molecule NOEs Using a Neoteric Sensitivity Enhancement Scheme

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Received August 12, 1999; revised September 1, 1999

A new method to increase the sensitivity of the 1D transient NOE experiment for molecules in the positive NOE regime is presented. This method, the reverse NOE, simply replaces the conventional relaxation delay between scans. Transient positive NOE enhancements from all other spins are used to accelerate the recovery of the target resonance toward its equilibrium intensity. In favorable cases, the intensity of the target peak at the start of an experiment can actually be increased beyond its equilibrium value. There is also a sensitivity enhancement in the rapid pulsing regime, where recovery is always incomplete. This sensitivity enhancement is illustrated with the one-dimensional double pulsed field gradient spin echo NOE experiment to observe a “fourth-order” NOE. Sensitivity gains of 30% are demonstrated. © 1999

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Key Words: excitation sculpting; nuclear Overhauser effect; NMR; pulsed field gradient; solution structure determination; sensitivity enhancement.

Internuclear distances can be inferred from measurements of transient nuclear Overhauser enhancements (NOEs) and are routinely used in molecular structure elucidation (1). However, for small molecules in nonviscous solutions, the cross relaxation rates are slow, making the positive transient NOEs very weak, and their detection and quantitation difficult. For these cases, we describe a new method that yields a significant increase in sensitivity. Positive NOE enhancements from ¹H to ¹H are used to accelerate the recovery of the target during the relaxation delay between scans. Using this sensitivity enhancement on the target, the double pulsed field gradient spin echo (DPFGSE) (2–4) for selective irradiation, and broadband radio frequency (RF) inversion pulses (5, 6) in the mixing time to suppress *T*₁ relaxation (7) has allowed us to observe a quartic NOE. That is, the enhancement is relayed through three intervening spins and shows an initial increase that is quartic in the mixing time. This sensitivity enhancement method, which we call the reverse NOE, is broadly applicable to experiments where the NOEs are positive. The extension of the idea to band-selective multidimensional experiments is easily accomplished by replacing the customary equilibrium delay with the

reverse NOE in the pulse sequences already described in the literature, like XS-NOESY and XS-TOCSY (8).

Our focus is on the measurement of a potentially very tiny transient NOE from a well-resolved target spin to a rather distant spin in a small molecule. Generally speaking, in small molecules it is difficult to observe NOEs between protons greater than 5 Å apart. Part of the difficulty in conventional difference spectroscopy lies in the inevitable subtraction errors that can swamp the NOE. These problems are overcome by using the DPGSE-NOE sequence (4, 7), in which the stray unwanted magnetization is on the same order of magnitude as a typical NOE, making its subtraction about 100 times easier. The other problem is intrinsic sensitivity: A small NOE may take a prohibitively long time to emerge from the noise, resulting in long experiment times, especially if a full buildup curve is desired. Sensitivity can be improved, with no trade-off, by the reverse NOE method described here.

Because small molecules give a positive NOE, inversion or saturation of one spin tends to increase the *z*-magnetization on a spatially proximate spin. It follows that, rather than standing idly by during the interscan (or relaxation) delay, it might be possible to increase the magnetization of the target spin by disturbing some other spatially proximate spin, either saturating or inverting it. For example, complete saturation of one spin of an isolated homonuclear pair described by the Solomon equation (9) gives a potentially quite large steady-state enhancement of 50% when the relaxation mechanism is entirely dipolar. Thus, one could attempt to irradiate some nearby spin during the entire relaxation delay, to try to capitalize on this enhancement. Of course, one would need to know in advance which spin to irradiate—information that may or may not be available. An alternative would be to try to saturate *all* the other spin multiplets during the relaxation delay by using a modulated or time-shared RF field. The problem with this approach is that it is essential that the target multiplet remain sensibly unperturbed by the direct effect of the field, a circumstance which, while not proven to be impossible, is unlikely to be easy to engineer for arbitrary spectra in the limit of saturating RF.

An alternative that is simple to implement and requires no prior knowledge about the spin system is to invert all the spins

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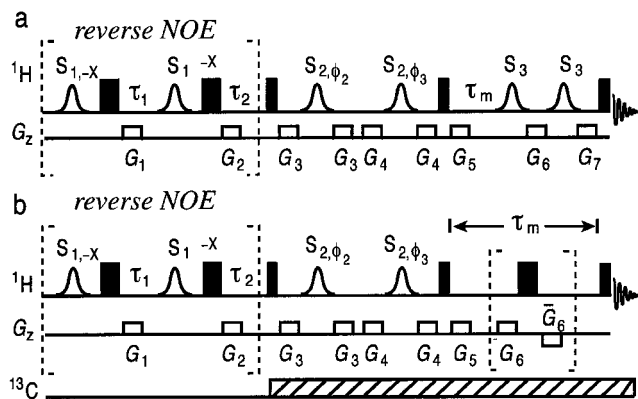


FIG. 1. (a) The DPGFSE-NOE experiment with the equilibrium delay replaced with the reverse NOE shown in brackets. S_1 is 20 ms selective 180° pulse to the target. The delays τ_1 and τ_2 are set for optimal sensitivity to the target. Thin and thick rectangles are 90° and 180° pulses, respectively. All pulses have phase x unless noted otherwise. S_2 and S_3 were 180° selective Son2 pulses (7) with lengths 37 and 40 ms, respectively. The S_3 selective pulses at the end of the mixing time were used to get of antiphase contributions to the NOEs in coupled spin systems (7). The basic phase cycling is $\phi_1 = x, y, -x, -y, \phi_2 = 8x, 8y, 8(-x), 8(-y)$, and $Rx = 2(x, -x, x, -x), 2(-x, x, -x, x)$. G_3 and G_4 were each 200 μs long with strengths of $G_3 = 7 \text{ G cm}^{-1}$ and $G_4 = 3 \text{ G cm}^{-1}$, with a 100 μs recovery delay following each gradient. The other gradients were each 1 ms long with strengths $G_1 = 0.5 \text{ G cm}^{-1}$, $G_2 = 0.8 \text{ G cm}^{-1}$, $G_5 = 4.2 \text{ G cm}^{-1}$, $G_6 = 250 \text{ G cm}^{-1}$, and $G_7 = 5.5 \text{ G cm}^{-1}$. (b) Broadband RF inversion pulses sandwiched between antiphase gradients (one set shown in brackets) are placed in the mixing time to suppress unwanted T_1 relaxation in long mixing times. The timing ratio for four 180° sweeps used in an 8 s mixing time to observe the quartic NOE was 16–15–25–25–9. ^{13}C decoupling was performed to avoid NOEs from irradiation of ^{13}C satellite peaks. All spectra were recorded on a 500 MHz Varian UnityPlus spectrometer.

except the target. This profile is easy to obtain using a combination of a soft 180° pulse to the target followed immediately by a broadband 180° pulse across the entire spectrum. As a soft 180° pulse is used in the DPGFSE sequence, and a broadband 180° pulse is used as a nulling pulse during the mixing time, these waveforms can simply be reused for the reverse NOE. This sensitivity enhancement mechanism thus uses the transient, rather than the steady-state, NOE, but uses the entire available pool of cross-relaxing magnetization to enhance the target. The net increase in target magnetization is larger than one might have expected.

Figure 1 shows the modified transient NOE pulse sequence, with the reverse NOE pulse segment in brackets. Every peak but the target is inverted at the start of the equilibrium delay with a 180° selective pulse to the target followed immediately by a 180° pulse to return the target back to the $+z$ axis while simultaneously inverting everything else. A small pulsed field gradient then purges any residual transverse magnetization. During the delay that follows, transient NOEs accelerate the recovery of the target. Under favorable conditions, the target magnetization can increase beyond its equilibrium intensity, offsetting any magnetization lost by transverse relaxation during the DPGFSE. Near 100% inversion of the target can be

achieved at the start of the mixing time, even with interscan delays that are quite a bit shorter than $3T_1$, leading to the largest measurable NOE signals. Of course the partner spins are not fully inverted by the soft–hard 180° combination, as they are usually recovering from saturation after the final 90° read pulse from the previous scan; they also relax toward equilibrium, lowering the cross-relaxation rate with time. We thus found it useful to repeat the soft–hard 180° combination, after an empirically optimized delay. More than two inversions does not lead to noticeably better results, and eventually magnetization losses from the repeated manipulation of the target overwhelm the gains from the NOEs, leading to a loss of sensitivity.

Figure 2 is a control experiment that just compares the spectrum obtained with a 4 s relaxation delay followed by a 90° read pulse, with the reverse NOE sensitivity-enhanced spectrum. A sample of 6(5*H*)-phenanthridinone, **1**, in $\text{DMSO-}d_6$ was used. The target was the H6 proton and the acquisition time was 1.828 s. The recycle time, 5.828 s, is ~ 4.5 times the T_1 of H6 ($T_1 = 1.27 \text{ s}$). Therefore, H6 is within about one percent of its full equilibrium intensity. The integrated signal of H6 using the reverse NOE, Fig. 2b, is 28% larger. The duration of the control and reverse NOE experiments is identical; i.e., the time for the RF pulses and gradients was subtracted from the delays τ_1 and τ_2 so that the total time between scans is exactly the same for the spectra in Fig. 2. Averaged over an ensemble of possible targets, a 20% sensitivity enhancement was observed. Note that the reverse NOE also conveniently functions as a suppression technique. If the desired target is close in frequency to another peak, the delays τ_1 and τ_2 can be optimized to start the experiment with the problematic peak passing through a null. The target can then be irradiated with a shorter, less selective 180° pulse in the DPGFSE sequence, minimizing magnetization loss. Addition-

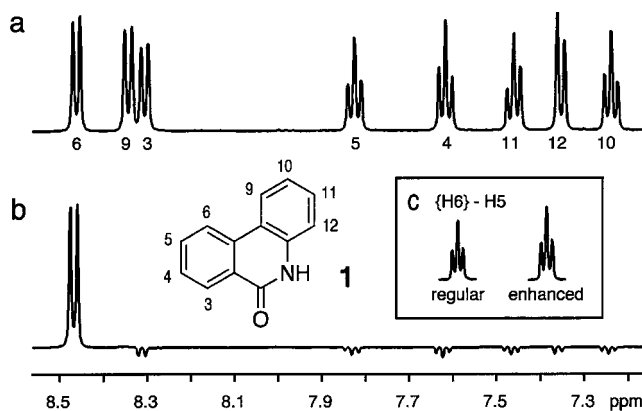


FIG. 2. (a) The spectrum of 6(5*H*)-phenanthridinone, **1**, with a relaxation delay of 4 s. The NH proton at 11.6 ppm is not shown. (b) A 28% sensitivity increase was obtained for H6 using the inverse NOE with $\tau_1 = 3.1 \text{ s}$ and $\tau_2 = 0.9 \text{ s}$. The reverse NOE was optimized to suppress H9 while maintaining sensitivity enhancement to H6. (c) Using the DPGFSE-NOE experiment in Fig. 1a, a 28% larger NOE {H6}-H5 is observed at $\tau_m = 325 \text{ ms}$.

ally, note that the selective 180° pulse in the reverse NOE need not be highly selective, as accidental perturbation of other spins leads to a decrease in the reverse NOE enhancement, but nothing worse. Magnetization loss from relaxation during a very long selective 180° pulse to the target is, of course, counterproductive. Finally, internuclear distances can be obtained accurately using the enhanced spectra, as all NOEs are referenced to the integral of the target multiplet.

Second- and higher-order NOEs are not routinely used in small molecule structure determination owing to their inherent weak signal intensities and geometry-dependent multiple pathways for magnetization transfer. However, linear arrangements of spins promote mostly the single "forward" relayed pathway, allowing chains of spins to be identified in a single experiment with an extended mixing time. The longer mixing time could also allow unwanted magnetization from T_1 relaxation to build the resonances back to their full equilibrium intensity and hence lead to subtraction artifacts. Broadband 180° RF sweeps systematically placed in the mixing time are used to suppress this T_1 relaxation, with negligible effect on the NOE buildups. This technique has been discussed in detail by Stott *et al.* (7).

As a demonstration of the current limits of detection, we explored long-range NOEs in a sample of 7-methylbenzo[a]pyrene, **2**, in DMSO- d_6 . As the desired signals were expected to be very weak, several extra precautions were necessary. First, the sample was carefully purified by recrystallization from boiling benzene, followed by column chromatography using a 4:1 benzene/pentane solution, followed by another recrystallization from hot methanol by adding water dropwise and cooling gradually over several hours. After each step a high S/N 1D spectrum showed fewer and smaller impurity peaks. Even a 1% impurity could, if it provided a hidden resonance under the target, be misleading. By the same token, accidentally targeting one of the carbon-13 satellites from an adjacent multiplet could be confusing. To avoid this possibility, we decoupled the carbon-13 spins during the experiment. An alternative could be to use a BIRD sequence (10) to invert the proton resonances from all the carbon-13 isotopomers on every other scan, and co-add the results.

Figure 3a shows the quartic NOE, {H10}-H2, of 7-methylbenzo[a]pyrene, obtained using the pulse sequence in Fig. 1b. Using an almost ridiculously long mixing time of 8000 ms, the disturbance of H10 is relayed all the way to H2. With this long mixing time there is little magnetization left on the target resonance, so it appears to be of the same order as the stronger NOEs. The alternating pattern of algebraic signs and the complete buildup curves (data not shown) confirm the pathway H10 \rightarrow H11 \rightarrow H12 \rightarrow H1 \rightarrow H2. The linear distance between H10 and H2 is approximately 8.4 Å (measured from Biosym Macromolecule) and the measured enhancement was about 0.002%. Excellent suppression of the DMSO- d_6 solvent peak and peaks not in the cross relaxation pathway of H10 was achieved. Clearly this kind of cleanly measured data would be

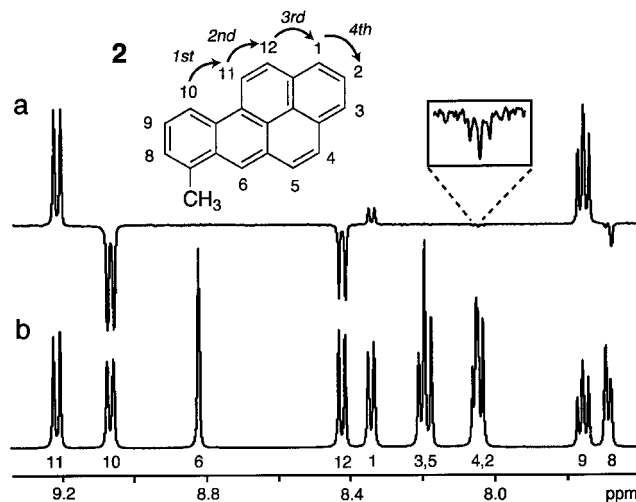


FIG. 3. (a) Observation of a quartic NOE, {H10}-H2, with an 8 s mixing time using a sample of 7-methylbenzo[a]pyrene, **2**, degassed three times and sealed under nitrogen. The pulse sequence in Fig. 1b with four broadband RF 180° sweeps in the mixing time was used. S_2 and S_3 were 74 ms son2 selective 180° pulses (7). The extremely long mixing time means that the target magnetization has decayed to within less than a percent of equilibrium, so that the target peak, rather than appearing as an intense inverted multiplet, is about the same order as the stronger NOEs. The spectrum was obtained with a total of 14,914 transients, over a weekend. The quartic NOE is a very small fraction of the residual inverted target, with an estimated enhancement, referred to the inverted target at zero mixing time, of only 0.002%. (b) The spectrum of **2** obtained with a 90° read pulse using 32 scans. The sample concentration was approximately 500 mM.

amenable to detailed analysis using a complete relaxation matrix treatment (11) although we have not explored this avenue yet.

The reverse NOE is general enough that it can be incorporated into almost any selective or semi-selective experiment in which the NOEs are in the positive regime. It simply replaces the relaxation delay between scans to improve sensitivity without increasing the total experimental time. When instrumental time is limited the reverse NOE can be used in a shortened relaxation delay to accelerate the recovery of the target, giving spectra of similar sensitivity to those obtained with a longer relaxation delay.

ACKNOWLEDGMENTS

This work was supported by the National Science Foundation, CHE-9900422. Q.V. acknowledges partial support from NIH Synthesis and Structure of Biological Macromolecules Training Grant T32 GM 07311-23.

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