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A Proposed Standard Sample for Nuclear Overhauser Effect Measurements

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ABSTRACT: A standard sample for the measurement of proton–proton nuclear Overhauser effects (NOE) is proposed. 1,5-Dichloro-2,4-dimethoxybenzene (DCDMB) shows an essentially full (50%) enhancement of the proton H-3 when the protons of the flanking methoxyl groups are saturated. The molecule is appropriate as a standard for the following reasons. The molecule is stable and easily synthesized. All the lines in the spectrum appear as singlets, so selective population transfer effects cannot obscure the NOE. The proton at position 6 provides a monitor of other relaxation mechanisms that would reduce the NOE. The NOE was verified by two complementary experiments. One is the standard technique of measuring the intensity with and without irradiation. A second method, based on spin–lattice relaxation rate experiments, provides unbiased data and gives an statistical estimate of the errors. Within experimental error, the NOE for DCDMB is 50%. © 1998 John Wiley & Sons, Ltd.

KEYWORDS: nuclear Overhauser measurements; standard sample; 1,5-dichloro-2,4-dimethoxybenzene

INTRODUCTION

The nuclear Overhauser effect (NOE) is an essential tool in NMR.[1–4] The Overhauser effect is caused by the coupled relaxation of two different spins, i.e. when the relaxation rate of one spin depends on the state of the other. One of the consequences is that saturation of one of the spins changes the intensity of the signal of the other. The most familiar case occurs when the coupled relaxation is caused by dipole–dipole interaction. Since the rate of dipolar relaxation is proportional to the inverse sixth power of distance, a combination of relaxation and NOE measurements can provide a direct measure of the distance between these two spins. This information can be crucial in the structural determination of small molecules where the stereochemistry must be established.[5,6] In peptide and protein investigations, one of the essential experiments is a multidimensional extension of the basic Overhauser effect.[7,8]

Much NOE work is qualitative or semi-quantitative. For instance, one proton of a CH_2 group may exhibit an NOE to a neighbouring spin whereas the other does not. This observation can permit differentiation of pro-*R* from pro-*S* protons.[9] In protein studies, NOESY cross peaks are classified as weak, medium or strong, but further quantitation is difficult. However, there are many situations when an exact value of the Overhauser effect would be very useful. If the correlation time is known, the value of the NOE can be used to estimate the distance between spins from T_1 measurements.[10] A reliable NOE reference material could also serve as a check that a spectrometer is functioning, and being operated, properly.

A candidate for a quantitative NOE standard is proposed in this paper. The material, 1,5-dichloro-2,4-dimethoxybenzene (DCDMB), shown in Fig. 1, gives an essentially full NOE to the proton at position 3 when the methoxyl protons are saturated. To establish the value of the NOE, two types of experiment were performed. One was the traditional, steady-state NOE experiment. The other, a spin–lattice relaxation experiment, measures the Overhauser effect in a more unbiased fashion and gives realistic error bounds on the observed value.

BASIC THEORY

The Overhauser effect can be derived from the following basic equation governing coupled spin–lattice relaxation of two spins, *I* and *S*:[11]

$$\frac{\partial}{\partial t}\begin{pmatrix} I - I_\infty \\ S - S_\infty \end{pmatrix} = -\begin{pmatrix} R_1(I) & R_1(IS) \\ R_1(IS) & R_1(S) \end{pmatrix}\begin{pmatrix} I - I_\infty \\ S - S_\infty \end{pmatrix} \quad (1)$$

where $R_1(I)$ is the spin–lattice relaxation rate of spin *I* and $R_1(S)$ is that of *S*. $R_1(IS)$ is the coupling term.

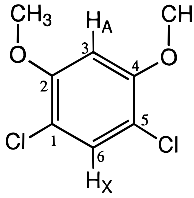

Figure 1. Structure of 1,5-dichloro-2,4-dimethoxybenzene (DCDMB).

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Strictly, the R_1 values are the initial-slope relaxation rates following selective inversion, since the full relaxation is bi-exponential. The relaxation matrix in Eqn (1) includes dipolar interaction plus other relaxation mechanisms such as random fields and chemical shielding anisotropy. For small molecules in the extreme narrowing limit, the elements of the relaxation matrix are given by

$$\begin{aligned} R_1(I) &= R_1^{\mathrm{other}(I)} + R_1^{\mathrm{dd}} \\ R_1(IS) &= R_1^{\mathrm{dd}}/2 \\ R_1(S) &= R_1^{\mathrm{other}(S)} + R_1^{\mathrm{dd}} \end{aligned} \tag{2}$$

In the Overhauser experiment, spin S is irradiated, so that S in Eqn (1) is changed from its equilibrium value. The steady-state value of I can then be calculated by setting the derivative to zero. If the matrix elements of Eqn (1) are given by Eqn (2), then the steady-state value of I is given by

$$\frac{I - I_\infty}{I_\infty} = \frac{S_\infty - S}{I_\infty} \frac{R_1^{\mathrm{dd}}/2}{R_1^{\mathrm{other}(I)} + R_1^{\mathrm{dd}}} \tag{3}$$

If the irradiated spin is fully saturated, then $S = 0$ and Eqn (3) reduces to the usual equation for the NOE. For a homonuclear two-spin system with pure dipolar relaxation, $I_\infty = S_\infty$ and $R_1^{\mathrm{other}(I)} = 0$, and the enhancement of I is 50%. Other cases can be calculated in a similar way.

THE SAMPLE

DCDMB has a proton NMR spectrum consisting of three apparent singlets (Fig. 2). The methoxyl protons (intensity 6) are at $\delta = 3.891$, the proton (H_A) at position 3, between the two methoxyl groups, appears at $\delta = 6.516$ and the proton (H_X) at position 6 has a chemical shift of 7.337 ppm. The sample is appropriate for NOE measurements for several reasons. First, all of the signals in the system are singlets, so selective population transfer effects are not present.[12] Second, the relaxation of H_A should be dominated by dipolar interactions with the methoxyl protons. Third, any non-dipolar relaxation mechanism for H_A should also affect H_X. Such relaxation can be monitored by observing the relaxation of H_X. In the sample discussed here, the relaxation of H_X is very slow ($T_1 > 200$ s), so additional mechanisms affecting H_A can be largely ignored. On the basis of the above three criteria, saturation of the methoxyl protons in DCDMB was expected to produce a substantial NOE at H_A. The observed enhancement was nearly 50%.

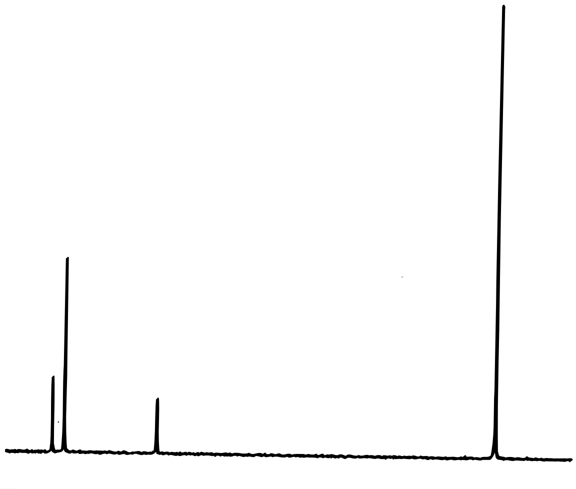

Figure 2. 300 MHz proton NMR spectrum of DCDMB. The peak at 7.23 ppm is residual chloroform in the solvent.

DCDMB is not the only spin system to give an essentially full NOE. The half-cage acetate, described by Anet and Bourn[5] in pioneering work, exhibits a *ca.* 43% NOE (degassed) for two highly proximate methine protons.[13] Isolated CH_2 groups in which the two protons are diastereotopic also show appreciable enhancements. However, the latter protons are also scalar coupled, which requires that the second-irradiating field must saturate the entire multiplet, yet not affect nearby transitions. If all the members of a multiplet in a coupled system are not equally saturated, then polarization can be transferred from one spin to another, independent of the Overhauser effect.[12] DCDMB provides no such problems.

NOE MEASUREMENTS

Normally, NOEs are determined by comparing a reference spectrum with one in which a spin is irradiated. Careful integration[14] is needed. This yields good data quickly, but there are no cross-checks in this method and no really good method of estimating errors or biases. For the purpose of validation of a standard sample, a modified spin–lattice relaxation time experiment, shown schematically in Fig. 3, is proposed as a check of the steady-state NOE procedure.

In the modified T_1-NOE experiment, the methoxyl protons are irradiated until a steady state is achieved. The system is then allowed to relax for some time, τ, and the z-magnetization is sampled with a $\pi/2$ pulse. This experiment is repeated for a series of τ values. The irradiation power must be sufficiently large so that the

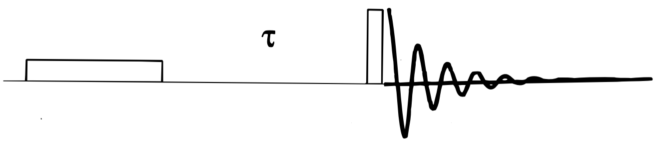

Figure 3. Pulse sequence for the combined T_1 and NOE experiment. The methoxyl protons are irradiated until the system reaches the steady state. Then the decoupler is shut off, relaxation occurs during time τ and the z-magnetizations for all the spins are measured using a non-selective $\pi/2$ pulse.

methoxyl protons are fully saturated but not so strong as to perturb H_A. The residual water signal is useful for monitoring this power level. Since the residual water and DCDMB are both dilute, there is no real chance of an intermolecular Overhauser effect. The chemical shift difference between the water and the methoxyl resonance is smaller than that between H_A and the methoxyl signal, so off-resonance irradiation will affect the water more than H_A. Provided that the water signal (or some other reference line in the same chemical shift range) does not vary as a function of τ, off-resonance effects can be assumed to be unimportant. None of the other parameters in this experiment is critical. The irradiation time should be longer than $5T_1$ but is otherwise not important.[15] The observation pulse can be of any value since there are no flip angle effects on singlets.[16]

This experiment illustrates the principle that the initial conditions in a relaxation experiment are useful parameters.[17] The quantities that govern the course of relaxation are the initial state, the equilibrium state and the rate matrix. The NOE is the ratio of the initial (irradiated) state to the equilibrium state in this experiment, and the rate matrix provides an important cross-check.

There are many relaxation experiments that could also be employed, developed for studying chemical exchange and cross-relaxation.[18] The transient NOE of H_A is observed after the methoxyl protons are inverted. For a two-spin system, this transient NOE is large if the steady-state NOE is likewise substantial. However, when a single spin is relaxed by n equivalent spins ($n = 6$ in this case), the steady-state NOE is the same, but the transient NOE is reduced *ca.* $1/n$. For DCDMB, this transient NOE would be too small to be measured accurately.

The formal solution to the differential Eqn (1) is

$$\begin{pmatrix} I(t) - I(\infty) \\ S(t) - S(\infty) \end{pmatrix} = \exp(-Rt) \begin{pmatrix} I(0) - I(\infty) \\ S(0) - S(\infty) \end{pmatrix} \quad (4)$$

where R is the relaxation matrix in Eqn (1) and 'exp' means the exponential of a matrix. The program CIFIT[18] was modified to fit the present experiment. Not only the relaxation matrix elements but also the intensities at $\tau = 0$ and those at $\tau = \infty$ are used as fitting parameters. These intensities provide a 'best fit' value of the Overhauser enhancement along with realistic error estimates,[19] and the relaxation matrix elements validate the data.

EXPERIMENTAL

1,5-Dichloro-2,4-dimethoxybenzene (Fig. 1, DCDMB)

Dimethyl sulfate (Aldrich) (7.6 g), 4,6-dichlororesorcinol (Aldrich) (1.8 g), NaOH (Fisher) (1.6 g) and the phase-transfer catalyst benzyltriethylammonium chloride (Aldrich) (0.23 g) were placed in a 250 ml round-bottomed flask containing 50 ml of water and 50 ml of methylene chloride, and the mixture was stirred overnight at room temperature. The aqueous layer was extracted with 50 ml of methylene chloride and the extract added to the methylene chloride layer. The combined methylene chloride solutions were successively washed with 3 M ammonia solution, 10% NaOH and salt water. The resulting methylene chloride solution was dried over anhydrous Na_2SO_4 and concentrated in a rotary evaporator. The resulting product was recrystallized from hexane and afforded 1.9 g of white needles.

Test samples were prepared by Wilmad Glass (Buena, NJ, USA) for evaluation purposes in the following manner. Approximately 1 mg of DCDMB was dissolved in 0.6 ml of $CDCl_3$. The sample was placed in an NMR tube (506-PP), subjected to six freeze–pump–thaw cycles and then sealed under vacuum. Proton NMR spectra were obtained (i) at 300 MHz on a Bruker AC300 spectrometer equipped with a 5 mm, four-nucleus probe and (ii) at 400 MHz on a Varian VXR-400S spectrometer equipped with a 5 mm, $^1H/^{13}C$ switchable probe. The $\pi/2$ pulse for protons was 9 μs on the AC300 and 20 μs on the VXR-400S. The probe temperatures on the AC300 were set to 300 K and controlled to within ±0.2 K using a Bruker BVT2000 variable-temperature regulator; those on the VXR-400S were set to 298 K and controlled to within ±0.1 K.

Steady-state NOE experiments at 400 MHz

Preliminary experiments were conducted to determine the minimum power level at which the six methyl protons were saturated. The spin–lattice relaxation time of H_A was also measured using the inversion–recovery method[20,21] and found to be 4.5 s. Presaturation times of 30 s ($>5T_1$) were employed to ensure that enhancements of H_A were fully developed. Sixty-four transients each were collected on-resonance (3.891 ppm, OCH_3) and off-resonance (9.15 ppm). The second-irradiating frequency was gated off during both the $\pi/2$ pulse and signal acquisition. The FIDs were subjected to a 1 Hz line broadening before Fourier transformation and spectral baselines were corrected prior to integration of the H_A signals.

Modified T_1-NOE experiments at 300 MHz

Spin–lattice relaxation rates were estimated using the saturation–recovery method.[22] For NOE measurements, the spectrometer 'decoupler power' parameter in Bruker's DISNMR program was set to 20L, which corresponds to a precession frequency around the decoupler field of 100 Hz. The FIDs were subjected to a 5 Hz line broadening prior to Fourier transformation. The baselines of the resulting spectra were corrected and the height of each peak above the corrected baseline was recorded.

RESULTS

Steady-state NOE experiments

Twenty-five experiments were carried out with presaturation times of 45 s, five with 30 s and five with 60 s and the observed signal enhancements of H_A were found to be identical within the limits of experimental error ($\pm 1\%$). The average $H_A\{OCH_3\}$ NOE was 49.1% and the median was 49.0%.

Modified T_1-NOE experiments

Because the relaxation of H_X was very slow, the saturation–recovery method was used to avoid lengthy equilibration times after FID acquisition. These experiments demonstrated that H_X had a $1/T_1$ of *ca.* 0.004 s^{-1} ($T_1 \approx 250$ s). The NOE experiment yielded the following values: for H_A, $I_\infty = 1.54 \pm 0.02$, $I_{\text{steady-state}} - I_\infty = 0.71 \pm 0.02$, and for the methoxyl protons, $S_\infty = 10.03 \pm 0.02$, $S_{\text{steady-state}} - S_\infty = -9.25 \pm 0.02$. This gives an NOE of 0.49 with an error estimated, as previously described, of *ca.* ± 0.05 (95% confidence limit, estimated by the profiling procedure). Repetition of the experiment produced NOE values in the range 0.45–0.51.

These results suggest that large NOEs cannot be measured as precisely by the modified T_1 method as with the steady-state experiment. However, the technique represents a valuable cross-check, because it is much less susceptible to systematic errors and biases. More importantly, two complementary experimental techniques have been shown to yield essentially the same full enhancement for proton A upon irradiation of the methoxyl protons in DCDMB. This molecule provides a valuable reference sample to test and calibrate the nuclear Overhauser effect.

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