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Choosing a Spin-Lock Transmitter Position Which Minimizes HOHAHA Distortions of ROESY Spectra. Observation of a Correlation-Time Dependence of Frequency Offset

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A technique for reliably selecting a transmitter position for rotating-frame NOE experiments which minimize scalar coupling artifacts is described. The technique makes use of the COSY spectrum of the molecule in question. The method is illustrated by application to thiostrepton, an antibiotic of molecular weight 1665. For an individual compound, the range of transmitter settings over which HOHAHA distortions are observed is found to depend on the molecular correlation time of the compound under study. The origin of this effect is discussed in terms of rotating-frame relaxation during the spin-lock period.

Rotating-frame Overhauser experiments [CAMELSPIN (*1*) or ROESY (*2*)] have been increasingly used in recent years to probe the conformation of molecules of mid-range molecular weight, i.e., from 800–2000 Da (*3–5*). However, quantitation of transverse Overhauser effects has proven to be difficult for two primary reasons: (1) ROESY cross-peak intensities are dependent on transmitter offset from the interacting proton peaks and (2) cross-peak intensities can suffer severe distortion whenever scalar-coupled peaks are present in the spectrum (*6–10*). The quantitative effect of transmitter offset on ROESY peak intensities has been previously addressed, both experimentally and theoretically, (*6*) and is easily corrected. However, the distortion of ROESY peaks due to scalar-coupling effects, although well understood, is difficult to correct with simple theoretical expressions. There is little dispute that spectra should be collected to minimize coupling artifacts. Recent publications from Hwang *et al.* (*11*) demonstrate an approach which uses altered spin-locking pulse sequences to allow ROESY-like transfers while reducing or eliminating HOHAHA-induced peak-intensity variation. However, this approach can involve a compromise in transfer efficiency. If one chooses to rely on more-conventional ROESY experiments, previous advice simply recommends ''careful'' placement of the transmitter frequency or to run the experiment at two or more frequencies (*7–10*). In this paper, we propose a systematic method for choosing a transmitter position which will minimize distortion of ROESY peaks due to HOHAHA artifacts. In addition, we report the observation of a molecular-correlation-time dependence of the range of transmitter positions over which such HOHAHA effects occur and provide some theoretical justification for the observation.

Scalar-coupling interference with ROESY cross peaks manifests itself either (1) as COSY-like antiphase absorptions or (2) as in-phase distortions usually associated with HOHAHA effects (*6*). Because of the antiphase character of the COSY-like distortions, they have a minimal effect on the cross-peak volume integral. However, the effect of in-phase contaminations is to reduce the absolute value of the cross-peak integral leading to errors when these integrals are used for internuclear distance determinations. For this reason, it is essential to eliminate or to minimize HOHAHA effects in ROESY spectra. An example of a spectrum containing numerous such HOHAHA distortions is shown in Fig. 1A for the antibiotic thiostrepton. A pulsed-RF spin-lock sequence similar to that described by Griesinger and Ernst (*6*) was used to generate this and all ROESY spectra described in this paper.

HOHAHA distortions are at a maximum when the magnitude of the effective field is the same at the two peaks, i.e., when Hartmann–Hahn conditions are satisfied. For weaker RF fields such as those used in ROESY, a perfect Hartmann–Hahn match occurs only when the spin-lock transmitter is at the midpoint of the two peaks. Thus, HOHAHA effects can be minimized if a spin-lock transmitter position can be found which is not exactly at the midpoint of *any* two coupled resonances.

Many of the molecules for which ROESY is useful have crowded proton NMR spectra. This complicates the *a priori* selection of a transmitter position which avoids the midpoint of all coupled resonances. We now describe a simple procedure to choose such a position by judicious use of the information available in the COSY spectrum of the molecule. Since a COSY (or DQF-COSY) spectrum of such complex

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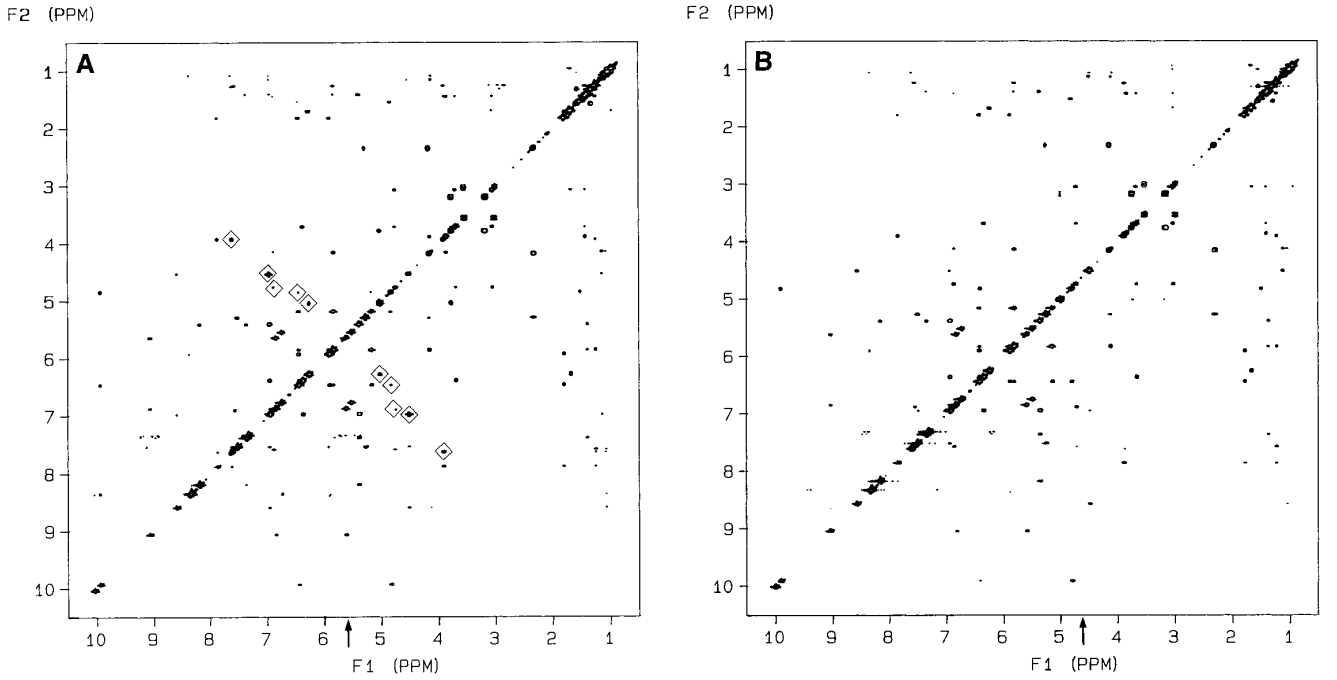

FIG. 1. ROESY spectra of thiostrepton, molecular weight 1665, under conditions giving rise to (A) substantial HOHAHA distortions and (B) negligible HOHAHA distortions. The only difference in experimental conditions for the two spectra is the position of the transmitter which is marked by the arrow in each spectrum. Each spectrum is 2048 × 512 complex points (zero-filled to 2048 in F_1), 4514.7 Hz in both F_1 and F_2, and was collected with 32 scans per t_1 value, a mixing time of 400 ms and a spin-locking field of 2.3 kHz. In spectrum (A), the carrier was deliberately set close to the midpoints of several pairs of coupled peaks. HOHAHA-distorted peaks, which are marked with diamonds in spectrum (A), are opposite in phase from the remainder of the cross peaks in spectrum (A) (except for those peaks arising from chemical exchange), or are markedly reduced in intensity when compared to spectrum (B). In spectrum (B), the transmitter position is selected by the procedure described in the text to be at least 100 Hz away from the midpoint of any pair of coupled resonances.

molecules will generally have been taken in the course of resonance assignments, this should not represent an additional experiment.

The 400 MHz COSY spectrum of thiostrepton is shown in Fig. 2. To find a transmitter position which is not at the midpoint of any two coupled resonances, we take advantage of the fact that, in a COSY spectrum, cross peaks between coupled resonances lie on a line perpendicular to the diagonal of the spectrum at a transmitter position exactly halfway between the resonances. Setting the transmitter at a position where cross peaks are abundant perpendicular to the diagonal, therefore, would be expected to give rise to a ROESY spectrum with substantial HOHAHA distortions. Figure 1A shows an example of a ROESY spectrum in which the spin-lock transmitter position (marked ''A'' in the COSY spectrum, Fig. 2) has been deliberately chosen to generate distortions. The position marked ''B'' in the COSY spectrum corresponds to a transmitter setting where the region perpendicular to the diagonal is free of cross peaks. The ROESY spectrum obtained for this transmitter position is shown in Fig. 1B and appears to be free of artifacts.

This technique is particularly well suited for peptides since NH, CHα, and CHβ protons are usually in different spectral regions. This may be less frequently true for ribose rings in nucleic acids or carbohydrate-containing molecules in general.

The onset of HOHAHA distortions of ROESY spectra is not catastrophic, but occurs over a band of transmitter settings (*8*). Such ''bandwidths'' are indicated around positions A and B in Fig. 2. From previous work, it is known that this bandwidth is a function of RF power level and coupling constant, J (*6–8*). In this work, we report that the width of the HOHAHA distortion onset appears also to be a function of molecular size, becoming narrower with increasing molecular correlation time.

This molecular-correlation-time dependence is established by means of a set of 1D ROESY measurements carried out as a function of transmitter setting for a series of peptides of molecular weight 412–1665 Da, each containing an alanine or threonine residue (see Fig. 4 for a listing of the compounds used). For each compound, the alanine or threonine methyl peak was inverted with a selective 180° pulse followed by a hard 90° pulse and application of a low-power spin lock. The spin lock was applied either by low-power CW irradiation or by timeshared techniques (*6*). For all compounds, the methyl–methine coupling constant was essentially the same (~7 Hz), the spin-lock power used was 2.3 kHz, and the spin-lock time was 400 ms. The difference

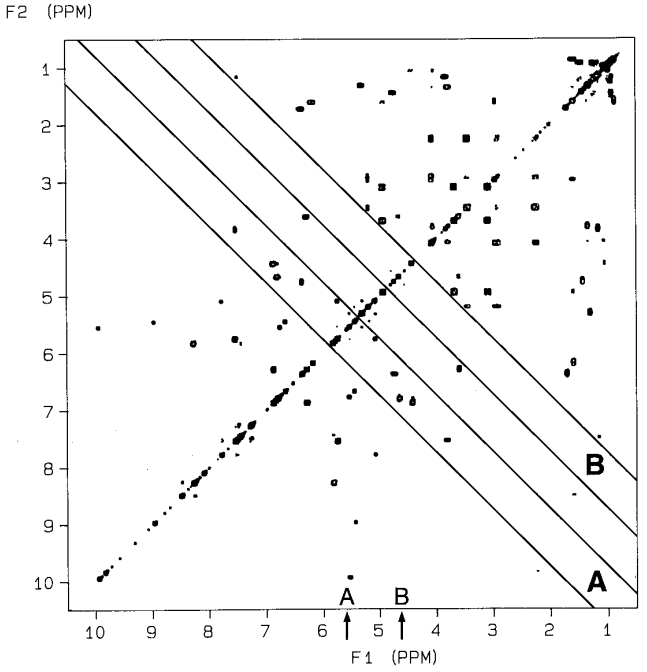

FIG. 2. COSY spectrum of thiostrepton showing bands perpendicular to the diagonal containing substantial cross-peak signal intensity (marked ''A'' on the spectrum) and no cross-peak signal intensity (marked ''B''). This spectrum consists of 2048 × 512 complex points (zero-filled to 2048 in F_1), is 4514.7 Hz in both F_1 and F_2, and was collected with 16 scans per t_1 value.

ROE peak height of the adjacent methine proton was monitored as a function of transmitter setting. At most settings, a constant ROE is observed. However, the peak height of the methine proton was observed to change dramatically as a transmitter position midway between the methyl and methine resonances was approached. Figure 3 shows the observed difference intensity for a small peptide (*t*Boc–Phe–Ala–OBn, 412 Da) and for thiostrepton (1665 Da). Figure 4 is a plot of the ''bandwidth'' at half-height of the HOHAHA onset, determined from plots such as that in Fig. 3 for each of the eight peptides, as a function of molecular weight. As can be seen from Fig. 4, the width of the HOHAHA onset decreases with increasing molecular correlation time. At very large molecular correlation times, this experimentally observed decrease in the onset bandwidth is expected to plateau as resonance widths increase.

It is clear that the distortions observed here are due to HOHAHA effects since the distorted peaks are in phase with the inverted peak. The origin of HOHAHA-type peaks and their loss of intensity with frequency offset is well understood and has been previously discussed (*6*). What is less clear is the origin of the dependence on molecular size.

The observation of a molecular-correlation-time dependence naturally suggests an association with spin relaxation. This suggestion is supported by the fact that offset experiments conducted with shorter spin-lock periods show a diminished dependence on molecular correlation time. The offset dependence would therefore appear to arise from differences in relaxation properties of modes responsible for HOHAHA transfers and modes excited when a Hartmann–Hahn match is not fulfilled.

It is possible to gain some qualitative insight into the phenomenon using a set of equations recently put forth to evaluate the relative efficiencies of coherence transfer via scalar coupling in laboratory versus rotating-frame experiments (*12*). While the formulas in this paper are not strictly applicable to our case, they do predict, qualitatively, changes in relaxation behavior as one moves off match. Using this lead, we describe rotating-frame transverse magnetization in the presence of an RF field in terms of modes written as linear combinations of product operators for a pair of spin-$\frac{1}{2}$ nuclei, $\langle I_k^+ - I_l^+ \rangle$, $\langle 2I_k{}^+ \mathrm{I}_{lz} - 2l_{kz}I_l^+ \rangle$, $\langle I_k^+ + I_l^+ \rangle$, and $\langle 2I_k^+ I_{lz} + 2I_{kz}I_l^+ \rangle$. At perfect Hartmann–Hahn match, the last two are constants of motion and the first two interconvert at a frequency, J_{kl}. This oscillation is essentially that of a

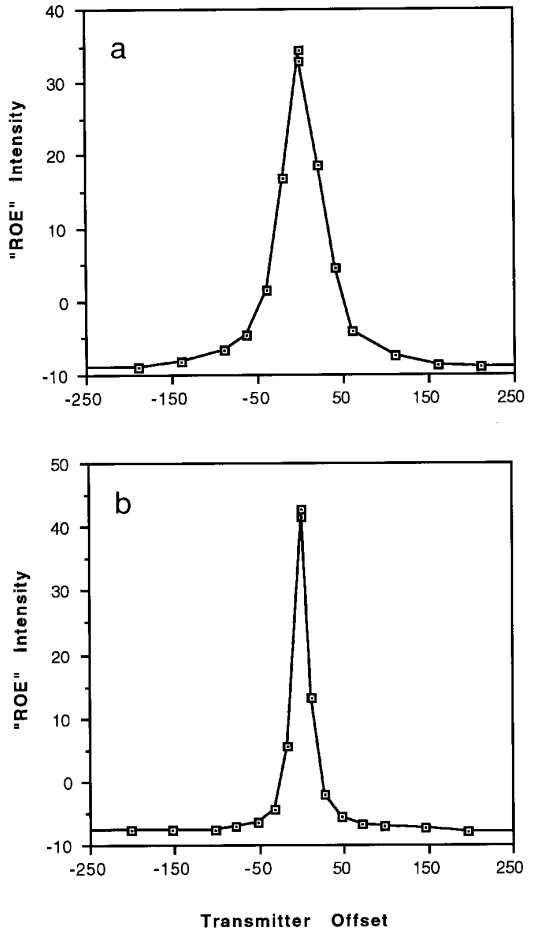

FIG. 3. Apparent ROE intensities as a function of transmitter offset for (a) *t* Boc–Phe–Ala–OBn (MW 412) and (b) thiostrepton (MW 1665).

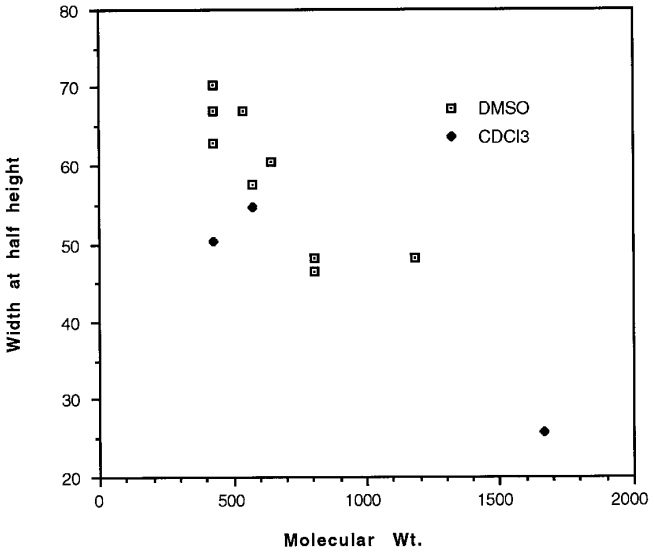

FIG. 4. Bandwidth of HOHAHA onset as a function of molecular weight. Compounds used were (1) Val–Phe–Ala–NHBn, 410 Da; (2) *t*-Boc–Phe–Ala–OBn, 412 Da; (3) Asn–Val–Phe–Ala–OBn, 525 Da; (4) *t*Boc–N(CH_3)–Ala–Val–Phe–Ala–OBn, 570 Da; (5) Tyr–(D)Thr–Gly–Phe–Leu–Thr, 629 Da; (6) Tyr–(D)Ala–Phe–Gly–Tyr–Pro–Ser–NH_2, 760 Da; (7) (oxo)Pro–His–Trp–Ser–Tyr–(D)Ala–Leu–Arg–Pro–Gly–NHEt, 1223 Da; (8) thiostrepton, 1665 Da.

zero-quantum coherence in the rotating frame and is the one responsible for the HOHAHA transfer. This coherence is subject to spin relaxation. When spin relaxation is dominated by dipole–dipole terms, the following expression pertains

$$R_{on} = d^2[J(0) + 3J(\omega_0) + 6J(2\omega_0)], \qquad [1]$$

where d is the dipolar interaction constant and J is the spectral density function. It is noteworthy that the relaxation rate has a rather small dependence on $J(0)$ in comparison to rates for single-quantum coherence and longitudinal cross relaxation.

Even with a slight mismatch, the above modes provide a reasonable description of the primary mechanism of HOHAHA transfer. However, transmitter offset causes additional mixing of both transverse and longitudinal modes. Under these conditions, relaxation rates become averages over time spent in these various modes. Briand and Ernst (*12*) treat a similar averaging caused by toggling frame shifts in multipulse sequences used for isotropic mixing, and they have supplied relaxation-rate formulas which account for the sampling of longitudinal modes. Using their formula with a sampling fraction of $\frac{1}{2}$ (for purposes of illustration), the following expression for relaxation of the zero-quantum mode results:

$$R_{off} = (d^2/2)[5J(0) + 9J(\omega_0) + 6J(2\omega_0)]. \qquad [2]$$

From the above two expressions, it is clear that large and small molecules will behave differently. In the large molecule limit ($\omega\tau_c \gg 1$), $R_{on} = d^2J(0)$ and $R_{off} = 5d^2J(0)/2$. In the small-molecule limit, $R_{on} = R_{off} = 10d^2J(0)$. Thus, off match, a large molecule experiences an enhanced relaxation that a small molecule does not. The actual functional form of the offset dependence of HOHAHA transfer inherently depends on the strength, homogeneity, and duration of the RF spin-lock field. However, the above analysis suggests that this basic functional dependence on offset can be modified by relaxation of the primary mode responsible for HOHAHA transfer. For small molecules, at long mixing times, all parts of the function are scaled equally. For large molecules, at long mixing times, those parts of the function far from match are additionally scaled by relaxation making the apparent bandwidth smaller. We are in the process of developing code to simulate these effects for a more complete verification.

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