One-Dimensional ROESY Experiments with Full Sensitivity and Reliable Cross-Peak Integration When Applied to Natural Products

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The three-dimensional structure determination of a medium-size natural product often requires recording time-consuming 2D ROESY spectra. When the amount of sample available is limited, a series of suitable 1D selective ROESY experiments provide the same information using much less experimental time. The setup and the choice of 1D selective ROESY experiments are critical to obtain reliable results. Often, organic and natural products chemists overlook these aspects and do not make the most effective possible use of existing ROESY experiments. We propose a 1D selective ROESY experiment tailor-made for investigating the structure of natural products. The experiment has been tested on a sample of cyclosporine, and analyses of the spectra demonstrate that the experiment provides clean spectra, allowing reliable cross-peak integration while maintaining high sensitivity. In addition, the experiment does not need a sample-specific setup and can be used as it is starting from standard parameters set by inexperienced users as well as under automated conditions.

High-resolution NMR is a powerful tool for the analysis of natural products at an atomic level.[1,2] Three-dimensional structure elucidation of natural products depends mainly on the use of several NMR parameters. Information about dihedral angles from ^3J scalar couplings extracted from simple 1D spectra is in general not sufficient and must be combined with the information on distances obtained from NOE enhancements. Cross-relaxation rates measured with NOESY experiments[3] have become one of the most important tools among the NMR experiments arsenal and remains an essential part of the structure determination process by NMR.[4,5] However, NOESY experiments do not work well for molecules with molecular weights of approximately 1000−2000 g/mol, where the NOEs are very close to zero. The buildup rate of the NOE depends on the correlation time τ_c of the compound and the observation frequency ω, and no NOE can be observed at $\omega\tau_c \approx 1.12$. During the past decade, access to high-field NMR spectrometers has been greatly facilitated, with the consequence that compounds with molecular weights of only about 500 g/mol meet the above criterion where the NOEs are very close to zero. This problem is solved by using rotating-frame Overhauser effect spectroscopy (ROESY), which yields positive Overhauser enhancements irrespective of the molecular weight.[6,7] The number of natural products having molecular weights between 500 and 2000 g/mol is undeniably important, which makes ROESY experiments a tool that cannot be ignored by natural product chemists.

However, ROESY experiments are not as easy to handle as NOESY experiments, and several experimental problems must be avoided during the setup and the interpretation must be done carefully. Running ROESY experiments implies not only choices between different pulse sequences, but also choices of acquisition and processing parameters. In our view, organic chemists and natural product chemists are generally not making the most effective possible use of existing ROESY experiments. This is in fact hardly surprising since most organic chemists have limited theoretical and practical training in NMR spectroscopy and the overall impact of breakthrough articles upon nonexpert NMR users is limited since they are mostly published in NMR specialized journals. In this report, we propose a selective 1D ROESY experiment tailor-made for natural products that benefit from the latest advances in order to obtain artifact-free ROESY spectra that can be reliably and easily integrated while maintaining a high degree of sensitivity. The amount of natural products available for NMR studies may not always be suitable for recording the recommended full battery of NMR experiments, and especially 2D ROESY experiments.[1] Therefore, we felt that it was worthwhile to propose a selective 1D ROESY experiment rather than a 2D NMR method, as sensitivity is an essential aspect of natural products. Of course, the proposed 1D ROESY experiment can be easily transformed into a 2D version if needed. This specific experiment eliminates the weaknesses of standard ROESY experiments, and the overall performance of the pulse sequence is improved significantly. In addition, we paid particular attention to the experimental setup, with the intention that the proposed ROESY experiment can be used as it is starting from standard parameters set by inexperienced users as well as under automated conditions.

Among this basic set of recommended NMR experiments for structure elucidation of natural products,[1] the ROESY experiment is probably the most demanding in terms of sensitivity and experimental time, as ROE responses can be extremely weak when medium- to long-range interactions are searched. While 2D or 3D NOESY-type experiments are the methods of choice for measuring NOE enhancements in large molecules, they have not been as attractive for smaller molecules where the NOE or ROE enhancements are weaker and for which a complete set of enhancements is not usually needed. It is well established that 1D NMR experiments using selective shaped pulses are advantageous compared to the corresponding 2D and 3D techniques because of the much faster acquisition, higher digital resolution, and greatly simplified analysis.[8,9] Such 1D selective experiments become unavoidable when the amount of sample available is limited and for the measurement of medium to long-range distances, for which the NOE or ROE enhancements are inevitably much weaker.

1D selective NMR experiments are recorded exclusively using pulsed field gradients (PFGs), either for selecting coherence pathways[10] or for coherence rejection.[11,12] Coherence rejection methods rely on phase cycling for the final desired coherence, and the intensity of cancellation artifacts is greatly reduced since the unwanted coherences are attenuated significantly by the action of the PFGs. For example, the DPFGSE sequence, which was originally developed for water suppression, is the basis of 1D coherence rejection selective methods.[11,13,14] However, molecules that yield sharp, intense signals or signals with very different

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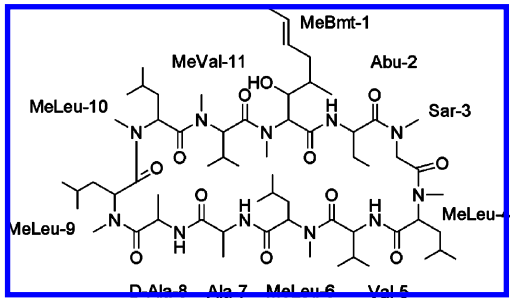

Figure 1. Cyclosporine A. The amino-acid residues were abbreviated following the notation given by Kessler.[25]

intensities can potentially leave severe cancellation artifacts, even after the action of PFGs. While the occurrence of these artifacts can be tolerated to a certain extent, their presence in the ROESY spectra of natural products is not desirable. Indeed, ^{1}H NMR spectra of natural products are often very crowded, and the smallest residual subtraction artifact can hamper key ROE enhancements for the elucidation of the structure or the discrimination between potential isomers. Efficient purging methods exist for 1D selective NOESY experiments, which have been extensively described in the report of Stott et al.[12] However, these methods cannot be implemented into 1D ROESY experiments, because the mixing time of ROESY experiments cannot be modified as readily as the mixing time of NOESY experiments.

On the other hand, spectra in which coherence pathways are selected by PFGs do not suffer from cancellation artifacts since signals that would give rise to them are not digitized at all. On the negative side, however, such spectra contain only half of the signal, which is further attenuated by incomplete gradient rephasing due to molecular diffusion.[12] The loss of sensitivity due to PFGs can be particularly critical in 1D selective ROE experiments (GROESY).[15] The required mixing time when ROE experiments are applied to natural products is usually of the order of several hundred milliseconds or even longer if very small enhancements between distant protons are investigated. This long mixing time causes important signal attenuation and, under unfavorable conditions, its complete extinction. For that reason, experiments based on a coherence rejection strategy are preferred to experiments based on coherence selection, in spite of the possible occurrence of subtraction artifacts.[9,12] For natural products, however, experiments based on coherence selection are preferable, because the problem of cancellation artifacts is inherently significantly reduced. The experiment we propose is therefore based on a standard GROESY experiment[15] in which both the suppression of cancellation artifacts and the sensitivity have been optimized. The timing diagram of the proposed 1D selective GROESY experiment is depicted in Figure 2a along with a standard SPFGSE-ROESY sequence (Figure 2b), which is the standard ROESY experiment based on coherence rejection strategy.[9] In the subsequent parts of this article, these experiments will be denoted as Tr-GROESY (Figure 2a) and Tr-ROESY (Figure 2b), respectively. In the Tr-ROESY experiment, the magnetization of one or more proton resonances is selected by an SPFGSE sequence,[10] whereas the magnetization of the other spins is dephased. During the subsequent mixing time τ_m, the magnetization of the target resonance can exchange magnetization through cross-correlation. As unwanted magnetization from the other spins due to self-relaxation may contribute to the final spectrum, a basic two-scan phase cycle is required to complete the selection process. This is achieved using the familiar EXORCYCLE phase cycle[16] for the selective 180° pulse while alternating the phase of the receiver.

The Tr-GROESY differs from the Tr-ROESY experiment in that the G_1 gradients are now set such that the magnetization of the target spin is phase-encoded using the same SPFGSE block, whereas

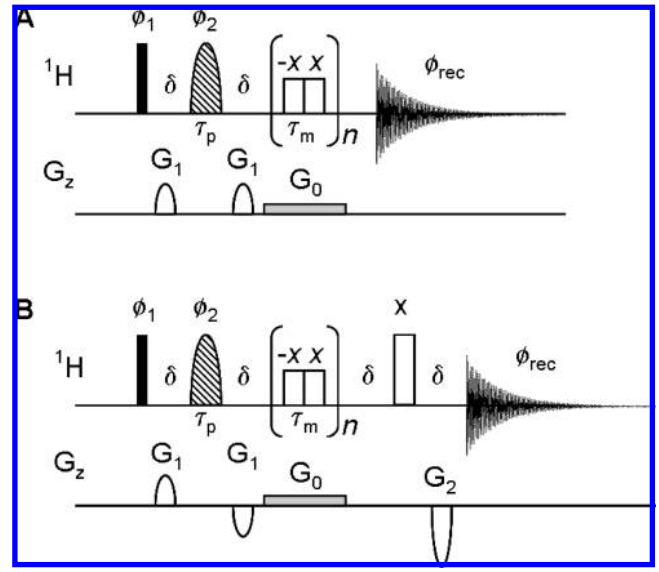

Figure 2. Timing diagrams of the 1D Tr-ROESY (a) and the proposed 1D Tr-GROESY experiment (b). For both experiments, thin black bars represent 90° hard pulses, thick bars 180° hard pulses, and shaded half-ellipsis selective 180° pulses. Half-height thick bars represent the phase-alternating 180° pulses of the Tr scheme. Gradient pulses are represented by unfilled half-ellipses denoted by G_1 and G_2. For the Tr-GROESY experiment, they were applied in the ratio 1:−1:−2. For diminishing the signal loss due to diffusion, the value of G_1 should be around 5 G cm^{-1}. The gradient for eliminating ZQ coherences is represented by a gray bar and denoted G_0. δ is the duration of the gradients and the gradient recovery delay. τ_p is the length of the selective 180° pulse, τ_m is the mixing time, and n is the number of phase-alternating 180° pulses. The following phase cycle was applied: $\phi_1 = x, -x$; $\phi_2 = x, x, y, y, -x, -x, -y, -y$; $\phi_{rec} = x, -x, -x, x$. This phase cycling leads to substantially better artifact suppression.

the magnetization of all other spins is rephased before the ROESY mixing time. At the end of the mixing time, a final gradient G_2 dephases all magnetizations except that of the target proton resonance. The final observed magnetization is that of the target spin or magnetization arising from cross-relaxation with the target spin. Hence, ultraclean 1D spectra can be obtained without phase cycling. However, as in all gradient-selected methods, half of the signal is lost compared to a gradient rejection experiment, because either N- or P-type data are selected as a function of the sign of the refocusing gradient.[9] In addition, the signal is further attenuated by incomplete gradient rephasing due to molecular diffusion.[12]

A prerequisite for reliable analysis of ROESY data is to obtain spectra with good signal-to-noise ratios, free from artifacts, and which allow observing ROE enhancements originating exclusively from cross-relaxation. If a ROESY experiment is recorded without precaution,[7,17] it is well known that the cross-peaks have the embarrassing propensity to originate from different magnetization transfer processes, which greatly complicates the analysis. Among existing methods, we have chosen the Tr-ROESY scheme[18] with addition of a weak continuous magnetic field gradient during the mixing time.[19] This scheme has emerged as one of the simplest and most efficient to ensure the observation of cross-peaks originating exclusively from cross-relaxation. For this gradient, the strength should not be too strong (around 0.5 G/cm), because applying a gradient creates a resonance-offset effect, which, in turn, can lessen the spin-lock efficiency, reducing the available magnetization.[19]

In Figure 3, spectra recorded on a sample of cyclosporine using a standard Tr-GROESY and a Tr-GROESY with application of a weak continuous magnetic field gradient during the mixing time are shown, together with a standard ^{1}H NMR spectrum. For the

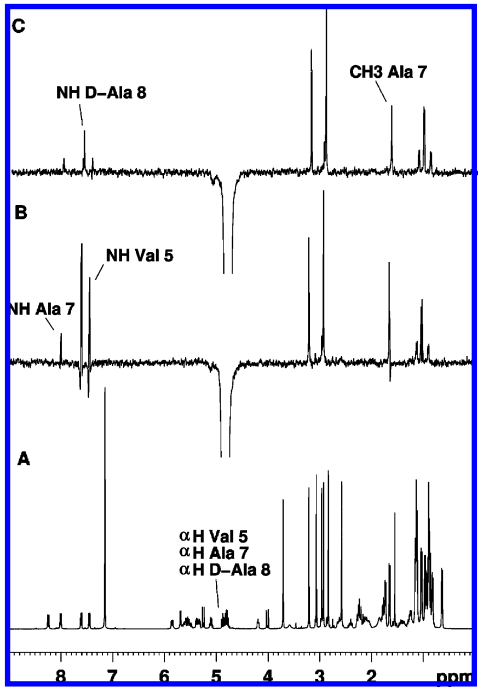

Figure 3. 1D (a) and 1D Tr-GROESY spectra recorded on cyclosporine without (b) and with (c) a weak continuous magnetic field gradient during the mixing time. Both spectra were recorded using the parameters given in the experimental part. The multiplet between 4.80 and 4.90 ppm, including the αH protons of Val 5, Ala 7, and D-Ala 8, has been selectively inverted using a 50 ms Gaussian pulse. The assignments are from ref 25.

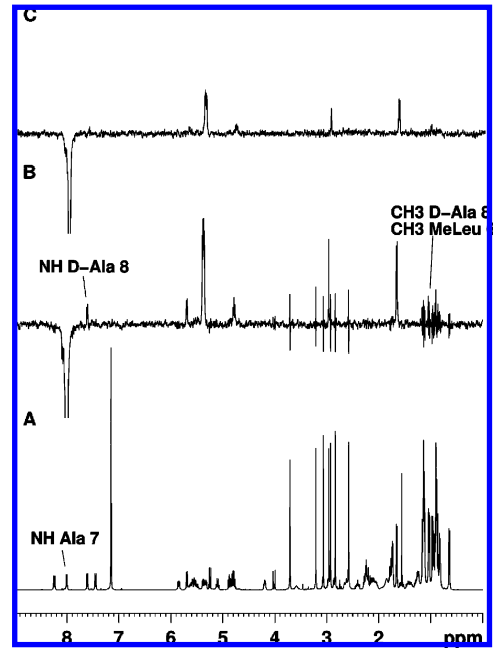

Figure 4. 1D (a), 1D Tr-ROESY (b), and 1D Tr-GROESY (c) spectra recorded on cyclosporine. The spectra have been recorded using the parameters given in the Experimental Section. The NH resonance of Ala 7 at 8.00 ppm was selectively inverted using a 50 ms Gaussian pulse. The assignments are from ref 25.

1D ROESY spectra, the multiplet between 4.80 and 4.90 ppm, which contains the αH protons of Val 5, Ala 7, and D-Ala 8, has been selectively inverted. The effect of a weak gradient for dephasing the unwanted magnetization is readily shown. In Figure 3b, ZQ coherence that was not dephased produced COSY-type peaks, in particular for the NH Val 5, NH D-Ala 8, and CH_3 Ala 7 resonances. In Figure 3c, by adding a small gradient (0.5 G/cm) during the entire mixing time, the COSY-type peaks were removed, revealing clean ROE enhancements. As mentioned before, other methods exist for significantly reducing those artifacts,[7,17] but they require a judicious setting of the carrier frequency and the use of a weak radiofrequency field for the spinlock. The Tr-ROESY scheme with an additional small gradient during the mixing time is efficient as well, but does not require further adjustments and can be used as it is starting from a predefined parameters set, under automation conditions or by inexperienced users.

In 1D selective experiments, the selection step represents another source of signal attenuation due to relaxation that is active during the selective pulse. Therefore, the length of this selection step should be kept to a minimum. When applied to natural products, the single pulsed-field-gradient echo (SPFGSE) block[10] would be more suitable than the standard DPFGSE sequence for three main reasons: First, the loss of sensitivity is strongly reduced. When using long selective pulses on samples where T_1 or T_2 is of the same order of magnitude as the pulse length, one cannot ignore relaxation during the pulse and the sensitivity losses can be dramatic.[20,21] The DPFGSE scheme, which consists of two selective 180 pulses, each one flanked by two gradients, is twice longer than the SPFGSE scheme and therefore much more subjected to sensitivity losses. Second, the SPFGSE technique offers high-quality selective excitation, identical to the DPFGSE sequence, when Gaussian-shaped inversion pulses are used.[9] Third, for the 1D-GROESY experiment, additional losses of magnetization due to self-diffusion must be taken into account. This can be a serious problem using the DPFGSE scheme, as it contains four dephasing gradients[12] instead of two as for the SPFGSE block.

The occurrence of cancellation artifacts by the use of PFGs as a means of coherence rejection is illustrated by comparison of 1D Tr-ROESY and 1D Tr-GROESY spectra recorded on cyclosporine. Both experiments have been recorded with standard parameters, described in the Experimental Section. For these spectra, the NH resonance of Ala 7 at 8.00 ppm was selectively inverted using a 50 ms Gaussian pulse. Cyclosporine contains numerous methyl groups, which yield sharp, intense singlets, as shown in Figure 4a. As can be seen from the Tr-ROESY spectrum (Figure 4b), the intensity of subtraction artifacts that originate from these methyl groups is comparable to or higher than the ROE enhancements and interfere with them. For instance, the response of the CH_3 β-protons of D-Ala 8 at 1.06 ppm is hampered because of the presence of a strong subtraction artifact originating from the $CH_3(\delta_2)$ protons of MeLeu 6 at 1.05 ppm. Likewise, the ROE response of the CH_3N protons of MeLeu 9 at 2.93 ppm is overlapped by a subtraction artifact, which skews the integration and would lead to an erroneous internuclear distance in the case of a quantitative measurement.

Subtraction artifacts are due to spectrometer instability, and, as a matter of course, instabilities cannot be controlled. We have recorded many different Tr-ROESY spectra in which the number of scans, the relaxation time, the gradient strength, and the gradient length have been varied, without being able to significantly reduce the intensity of these subtraction artifacts. We must point out that an almost perfect spectrum can be occasionally obtained, but in an unpredictable way and without any relation with a parameter change. By increasing significantly the number of transients, because of an averaging process, the intensity of the ROE signals may gradually exceed that of the artifacts. However, this procedure increases the experimental time, which, in turn, increases the risk of subtraction artifacts' occurrence. Any way, increasing the number of transients is not a satisfactory solution for diluted samples, for which the measurement time would become prohibitive.

Concentrating on subtraction artifacts first, the spectrum recorded using the Tr-GROESY scheme (Figure 4c) is clearly superior to its Tr-ROESY variant. The cancellation artifacts are completely

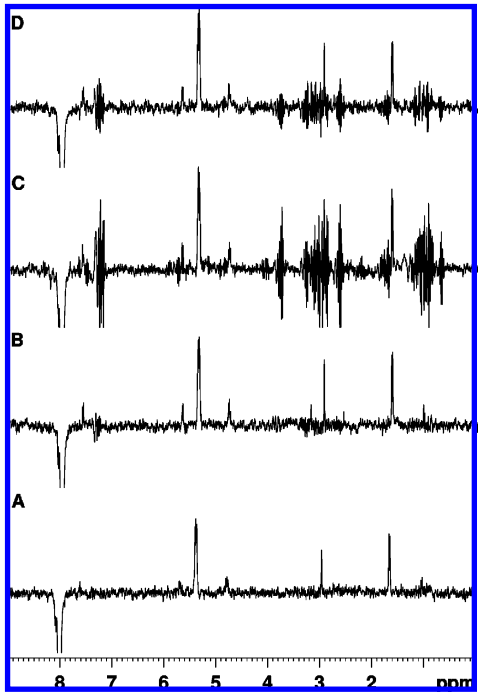

Figure 5. 1D Tr-GROESY spectra recorded on cyclosporine using different gradient parameters: (a) length 1.5 ms, $G_1 = 8$ G/cm, $G_2 = -16$ G/cm; (b) length 1 ms, $G_1 = 8$ G/cm, $G_2 = -16$ G/cm; (c) length 0.5 ms, $G_1 = 8$ G/cm, $G_2 = -16$ G/cm; (d) length 1 ms, $G_1 = 5$ G/cm, $G_2 = -10$ G/cm. The spectra have been otherwise recorded using the parameters given in the Experimental Section. The NH resonance of Ala 7 at 8.00 ppm was selectively inverted using a 50 ms Gaussian pulse. The assignments are from ref 25.

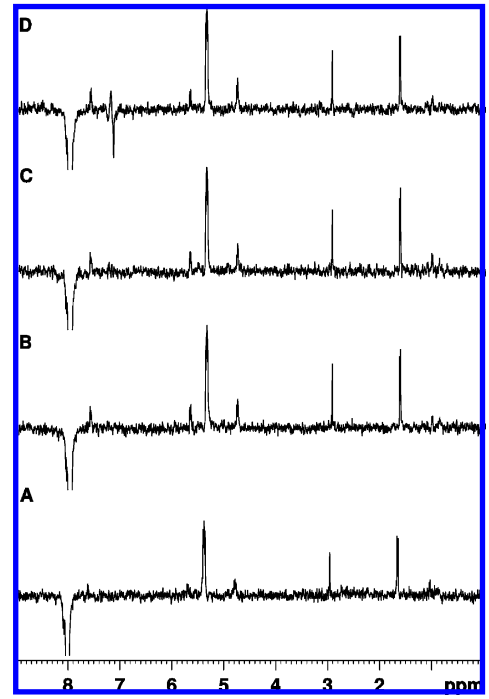

Figure 6. 1D Tr-GROESY spectra recorded on cyclosporine using the same gradient parameters as those for Figure 5, but with introduction of a difference step in the Tr-GROESY pulse sequence.

suppressed and the spectrum is ultraclean. However, the intensity of the signals has considerably decreased, because of incomplete gradient rephasing due to molecular diffusion. For example, the ROE response of the amide proton of D-Ala 8 at 7.63 ppm can hardly be detected.

Pulsed field gradient NMR spectroscopy can be used to measure translational diffusion of molecules. In 1965, Stejskal et al. proposed the gradient spin echo sequence to this end.[22] More generally, molecular diffusion will cause a loss of signal intensity in any experiment in which magnetization is dephased by one gradient and then subsequently rephased by a second. In 1D GROESY experiments, molecules are spatially labeled depending on their position in the sample tube by use of gradients during the SPGSE selection block. As molecules move after these encoding gradients during the subsequent mixing time τ_m, their new position can be only partly decoded by the third gradient. The NMR signal intensity is attenuated depending on the mixing time and the gradient parameters (length and strength). In many laboratories and under automation conditions, standard experiments, like COSY or HSQC, are run using standard gradient values. For GROESY experiments, however, these gradient parameters are not adapted. For instance, for a molecule having a self-diffusion constant of 5×10^{-10} m^2 s^{-1}, a typical value for medium-sized molecules,[23] less than 12% of the magnetization is refocused at the end of a spin echo experiment run with standard gradient parameters (20 G/cm and 1.5 ms) and a diffusion time of 0.3 s, a typical ROESY mixing time. Thus, decreasing significantly the length and the strength of the gradients results in noticeable effects on the sensitivity.[22,23]

In Figure 5, Tr-GROESY spectra in which the NH resonance of Ala 7 at 8.00 ppm was selectively inverted are shown. The experiment has been recorded as previously described,[15] without applying any phase cycling. It can be readily seen that the length and the strength of the field gradient pulses can be reduced only to a certain extent. The results in Figure 5a have been obtained using a gradient length of 1.5 ms and strength of 8 G/cm (G_1) and -16 G/cm (G_2). These values are the lowest we could use for obtaining an artifacts-free spectrum. The spectra in Figures 5b and 5c have been obtained using gradient strengths of 8 G/cm (G_1) and -16 G/cm (G_2) and lengths of 1 and 0.5 ms, respectively, while the spectra in Figure 5d have been obtained using gradient strengths of 5 G/cm (G_1) and -10 G/cm (G_2) and lengths of 1 ms. Evidently, these three spectra contain subtraction artifacts, particularly intense in spectra 5c and 5d. With our spectrometer, a gradient strength inferior to 8 G/cm and a gradient length inferior to 1 ms cannot efficiently suppress unwanted responses. Using these gradient parameters, the intensity losses due to transversal diffusion remain significant and problematic for diluted samples, especially when long mixing times are required and when nonviscous solvents are used.

Figure 6 shows the results when the same experiments were performed with introduction of a basic phase cycling in the Tr-GROESY pulse sequence.[24] This phase cycling uses the familiar EXORCYCLE phase cycle[16] for the selective 180° pulse while alternating the phase of the receiver. It can be readily seen that the spectra remain ultraclean, even when the length and the strength of the field gradients pulses are reduced to minimal values. The only artifact, arising from the residual solvent peak (C_6D_5H) at 7.15 ppm, emerges with gradient strengths of 1 G/cm (G_1) and -2 G/cm (G_2) and lengths of 1 ms.

Figure 7 compares ROE buildup curves of the $CH_3(\delta_2)$ of MeLeu 4 recorded using the Tr-ROESY and Tr-GROESY experiments after selective inversion of the multiplet spanning from 4.80 to 4.90 ppm. The ROE enhancements have been quantified by careful integration of the enhanced multiplet. It is obvious that the presence of subtraction artifacts in the Tr-ROESY spectra leads to considerable scatter on the plots, particularly because artifacts occur in an unpredictable way. With the use of the Tr-GROESY pulse sequence, the scatter is eliminated. If an actual distance is needed, one may use the well-known approach in which the ROE is inversely proportional to the distance to the sixth power.[5] Given a known distance between two protons and its ROE integral, a distance can be calculated from another ROE integral.

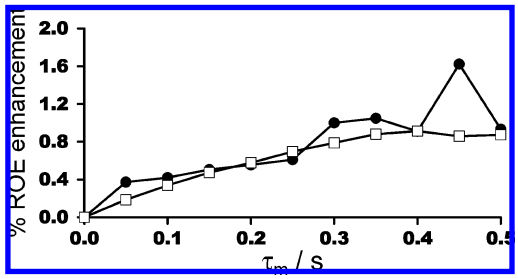

Figure 7. Plots of the measured ROE buildup of the $CH_3(\gamma_1)$ resonance of MeVal 11 at 0.97 ppm in the 1D Tr-ROESY spectrum (●) and 1D Tr-GROESY (□) after selective excitation of the CH_3 resonance of MeBmt 1 at 3.73 ppm. During the spin-lock period, the transmitter offset was placed at the frequency of the MeBmt 1 CH_3 resonance. The presence of subtraction artifacts in the spectra recorded using the Tr-ROESY spectra leads to scatter on the data, but this is eliminated using the Tr-GROESY sequence.

For medium-size natural products, standard 1D ROESY spectra are likely to be hindered by the occurrence of subtraction artifacts. We have shown that the choice of the experimental scheme and the proper setting of experimental parameters result in a substantial improvement in the quality of ROE spectra, thus making both qualitative and quantitative measurements easy and more reliable. Antiphase contributions, which are frequently troublesome in ROE experiments, can be easily suppressed by a simple addition of a gradient during the entire mixing time. It has also been shown that the use of the SPFGSE rather than the DPFGSE sequence significantly reduces intensity losses due to molecular diffusion. In addition, the setup of the proposed GROESY experiment is straightforward and the experiment can be used as it is starting from a standard set of parameters. Thus, the GROESY experiment described in this contribution is robust and reliable and can be used by inexperienced users as well as under automated conditions.

Experimental Section

General Experimental Procedures. For all experiments, a sample of 50 mmol of cyclosporine (Figure 1) dissolved in 0.6 mL of benzene-d_6 was used. All NMR experiments were acquired at 25 °C on a Bruker Avance II 400 spectrometer operating at a nominal proton resonance frequency of 400.13 MHz, equipped with a 5 mm broadband direct (BBFOplus) probehead with an additional *z*-gradient accessory. For all experiments, the ^{1}H 90° pulse length was 9.3 μs and the radio frequency strength for the Tr-ROESY sequence was 6 kHz. The selective inversion was obtained using a 50 ms Gaussian pulse. The relaxation delay was 3 s for all experiments. The spectra were acquired in 16 scans with a spectral width of 12 ppm, using 8192 complex points, leading to an acquisition time of 1.74 s. Before processing, data were zero filled to 16 384 points and apodized with exponential windows using a 2 Hz line-broadening. NMR spectra were processed using the Bruker Topspin 2.0 package (Bruker-Biospin, Fällanden, Switzerland). Cyclosporine dissolved in C_6D_6 was purchased from Bruker-Biospin.

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Supporting Information Available: The 1D-GROESY pulse sequence (Bruker Avance), spectra, a table providing relationships between the duration of a Gaussian pulse and the width of the excitation window, and theoretical data are available. This material is provided free of charge via the Internet at http://pubs.acs.org.

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