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A Novel Composite 90° Pulse Sequence Which Provides Distortionless NMR Spectra and Suppresses without Destroying the Water Magnetization[1]

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A novel 90° composite pulse sequence which allows one to record 1D and 2D NMR spectra without disturbing the water magnetization is described. A home-written program was used to optimize the pulse angles for which the pulse sequence response fitted best the desired excitation profile, producing a neat and distortionless spectrum with a broad null excitation at the carrier frequency. The resulting pulse sequence was first evaluated using the simulation program "PENCIL" and then tested on two protein samples. A 3.5° phase shift of the last pulse was required to cancel correctly the water signal. The pulse scheme was appended to a NOESY pulse sequence. Inspection of the water cross section revealed interactions between water and some protons of drosomycine, a small insect antifungal protein. © 1998 Academic Press

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In protein ^{1}H NMR studies, it is often necessary to observe exchangeable protons and bound water molecules which give a wealth of information on structure and dynamics of these molecules. Several pulse sequences have been proposed to record ^{1}H NMR spectra without presaturation of the water signal. Among them, the binomial pulse sequences which give no net excitation at the frequency of the solvent resonance are well known (*1*). However, most such composite pulses suffer from a nonuniform excitation profile and a consequent variation in signal phase across the excitation bandwidth. Recently pulse sequences using inhomogeneity of either the radiofrequency field or the static magnetic field to scramble the H_2O magnetization during the NMR pulse sequence have been described (*2, 3*). Unfortunately, due to the very long T_1 relaxation time of H_2O protons compared to protein protons, water remains in a semisaturated state since the delay time between scans is usually much shorter than the H_2O T_1 relaxation time. In this note we describe a composite 90° pulse sequence which does not excite the water magnetization and which provides a nearly uniform excitation profile across the spectral region of interest with minimal phase gradient.

For the design of the composite 90° pulse, a program named "PULSE" was written to search for the pulse angles giving the best sequence output, in agreement with predefined requirements: (1) the pulse sequence should be symmetrical and should contain six pulses, (2) all pulses should be applied along only one axis, and (3) the pulse sequence must provide a clean excitation profile along the regions of interest with minimum phase distortion and a broad null excitation at the offset resonance. Since the effects of pulses and of the evolution during any time interval upon chemical shift on magnetization are rotation around axis, the influence of the pulse sequence on *Mx*, *My*, and *Mz* magnetizations is calculated via rotation matrices. At any time of the sequence, the system is represented by a matrix $P[i][j]$ which provides the evolution of the *Mx*, *My*, and *Mz* magnetizations,

$$P[i][j] = \begin{bmatrix} a_{11ij} & a_{12ij} & a_{13ij} \\ a_{21ij} & a_{22ij} & a_{23ij} \\ a_{31ij} & a_{32ij} & a_{33ij} \end{bmatrix},$$

where j indicates the position of the pulse in the sequence. Eleven pulses are considered: six real pulses applied on the y axis corresponding to odd j numbers, and five rotations around the z axis corresponding to even j numbers; these are frequency dependent and represent the evolution of the frequencies during the interpulse delay τ. Therefore each matrix element is calculated for several frequencies ν_i where $\nu_i = 2\pi\Delta\nu\tau i$, $\Delta\nu$ being the frequency increment and τ the interpulse delay. For odd j numbers the matrix $P[i][j]$ is

$$P[i][2k+1] = \begin{bmatrix} \cos(\theta_k) & 0 & \sin(\theta_k) \\ 0 & 1 & 0 \\ -\sin(\theta_k) & 0 & \cos(\theta_k) \end{bmatrix},$$

where $j = 2k + 1$ and θ_k represents the flip angle of the kth pulse. Since the offset frequency magnetization is submitted to

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an overall pulse equal to the sum of all θ_k, a nonexcitation of that frequency requires the constraint $\Sigma_k\ \theta_k = 0$. As in binomial pulse sequences, we use a symmetrical solution. Therefore, three additional constraints are added which in turn save calculation time since only three pulse angles are incremented and searched for, $\theta_1 = -\theta_6$, $\theta_2 = -\theta_5$, and $\theta_3 = -\theta_4$.

For even j values the matrix represents the evolution of the *Mx*, *My*, and *Mz* magnetization related to chemical shifts,

$$P[i][2k] = \begin{bmatrix} \cos(\beta_i) & -\sin(\beta_i) & 0 \\ \sin(\beta_i) & \cos(\beta_i) & 0 \\ 0 & 0 & 1 \end{bmatrix},$$

where $j = 2k$, $\beta_i = 2\pi\Delta\nu\tau i$, $0 \le i \le N_{\max}$, $\Delta\nu$ being the frequency increment, τ the interpulse delay, $N_{\max} = \nu_{\max}/\Delta\nu$ and $\nu_{\max}$ the highest sweep width frequency. Owing to the symmetry of the sequence response, only frequencies between the offset resonance and $\nu_{\max}$ are considered for calculations.

The output of the pulse sequence is reduced to the matrix multiplication

$$S[i] = \prod_{j<12} P[i][j] = \begin{bmatrix} s_{11i} & s_{12i} & s_{13i} \\ s_{21i} & s_{22i} & s_{23i} \\ s_{31i} & s_{32i} & s_{33i} \end{bmatrix},$$

where $P[i][0]$ is the identity matrix.

Since we are only interested in the in-plane magnetizations *Mx* and *My* arising from the evolution of the *Mz* magnetization after the pulse sequence, only the two matrix elements s_{13i} and s_{23i} which refer to the *Mx* and *My* magnetizations, respectively, evolving at the frequency ν_i, are considered.

The desired response of the pulse sequence is then introduced as a two-dimensional column matrix $D[i]$ where elements are zero or one:

$$D[i] = \begin{bmatrix} d_{1i} \\ d_{2i} \end{bmatrix}.$$

$d_{1i} = 0.0$ for $0 \le i \le N$ represents the frequency interval from the offset where no *Mx* magnetization excitation is expected and $d_{1i} = 1.0$ for $N \le i \le N_{\max}$ represents the frequency sweep where a uniform excitation of the *Mx* magnetization is required. Since no dispersive componant of the magnetization, *My*, is wanted, we impose $d_{2i} = 0.0$ for $0 \le i \le N_{\max}$.

The program increments step by step the first three pulse angles, the three last ones being deduced by a sign inversion and calculates for each increment the theoritical response $S[i]$ for all frequenies ν_i in the frequency range 2100 Hz from the offset. Then it estimates the deviation between the output of the pulse sequence and the desired excitation profile,

$$\mathrm{rms}_x = \sqrt{\left(\sum_i (s_{13i} - d_{1i})^2/N\right)},$$

for the *Mx* magnetization and

$$\mathrm{rms}_y = \sqrt{\left(\sum_i (s_{23i} - d_{2i})^2/N\right)}$$

for the dispersive component.

In this calculation, the null window from the offset frequency is set to 80 Hz for the absorptive component to ensure that the water signal is not excited. The initial solution calculated by PULSE is in agreement with the predefined requirements. The only discrepancies are two large symmetrical dispersive bulges in a window of 80 Hz around the offset resonance. The intensity of these unwanted dispersive componants is strongly reduced by oversampling the frequency axis in this critical frequency window. Eighty points are chosen to describe the first 130 Hz from the offset and 30 points for the rest of the frequency sweep width, i.e., 1950 Hz. In this case, rms_x and rms_y values are dominated by the response of the pulse sequence in the null window region.

The solution showing the lowest rmsds corresponds to the following three pulse angles: $\theta_1 = 35°$, $\theta_2 = 56°$, and $\theta_3 = 132°$ (see Fig. 1). These values are close to those found in the 3–9–19 sequence (20.8°, 62.3°, 131.5°) defining a selective 180° pulse in the WATERGATE sequence (*4*). The main difference between the two sequences is a sign inversion of the first and last pulse phases.

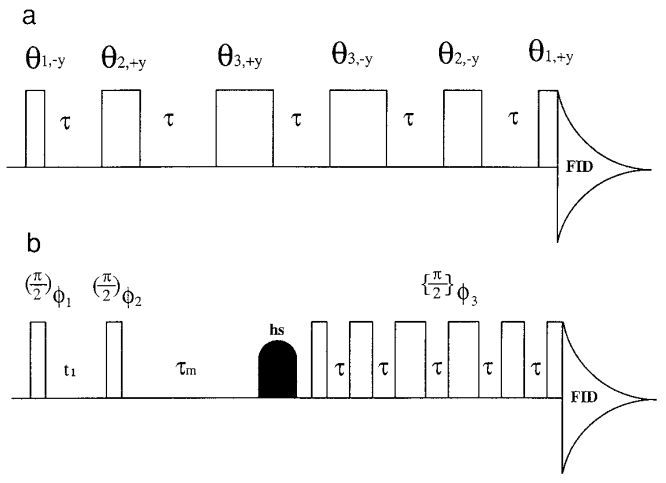
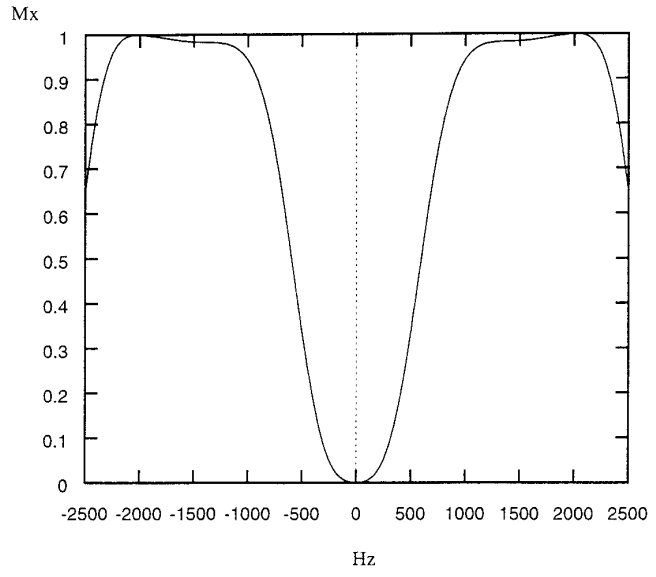


FIG. 1. (a) Composite pulse sequence used to record 1D spectra. The pulse angles are 35°, 56°, and 132° for θ_1, θ_2, and θ_3, respectively. The interpulse delay τ was set to 290 μs in all NMR experiments. To overcome the detection of miror image signals a 4-step CYCLOPS cycle can be used. (b) NOESY pulse sequence used to record spectra of wheat ns-LTP and drosomycine. The phase cycling is a classical 16-step cycling: $\phi_1(4x, 4(-x))$, $\phi_2(8x, 8(-x))$, $\phi_3(x, y, -x, -y)$, $\phi_{\mathrm{rec}} \cdot (y, -x, -y, x, 2(-y, x, y, -x), y, -x, -y, x)$. The phase ϕ_3 refers to the phase of the first pulse of the composite 90° pulse sequence. The phase ϕ_2 was shifted by an angle of 45° to allow a rapid return of the water magnetization onto the $+z$ axis during the mixing time via radiation damping (*8*). A 1-ms homospoil pulse is applied at the end of the mixing time to ensure that all magnetizations are aligned with the z axis before applying the composite 90° pulse for signal detection.

The pulse program is first evaluated using the simulation program ''PENCIL'' (5). The expected excitation profile of the composite 90° pulse sequence is provided in Fig. 2. The pulse sequence nicely fulfills the desired requirements since a clean excitation of the *Mx* magnetization is achieved over most of the spectral sweep width. The *My* magnetization being the source of the phase distortions along the spectral width is almost equal to zero except for two small symmetrical bulges located around the offset resonance. Both magnetizations show a sufficiently broad null at the carrier frequency to abolish the detection of any signal arising from the solvent magnetization. This is illustrated by the low phase values observed over the spectral width except for a region around the offset resonance where some phase distortions are expected.



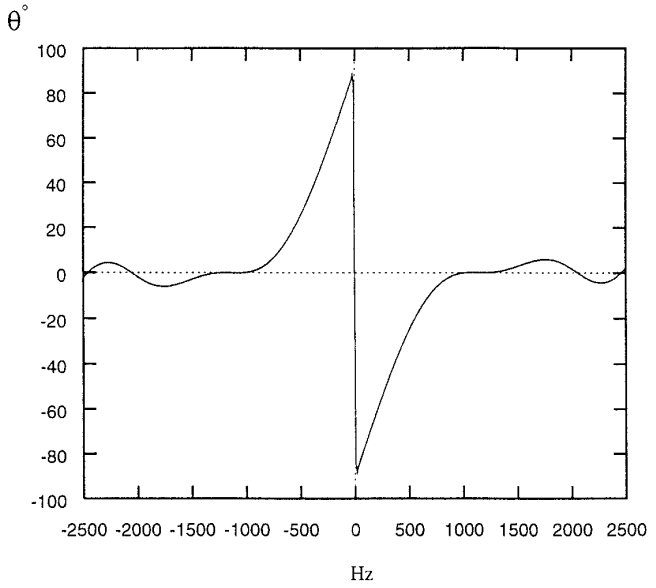


FIG. 2. Excitation profile of the composite 90° pulse sequence calculated by the simulation program ''PENCIL.'' The interpulse delay was set to 300 μs and the RF power to 25 kHz. The initial magnetization was *Mz*. All pulses were applied on the *y* axis and the composite sequence generates a clean excitation of the *Mx* component of the magnetization. The phase distortions calculated from the *Mx* and *My* magnetizations are also reported.

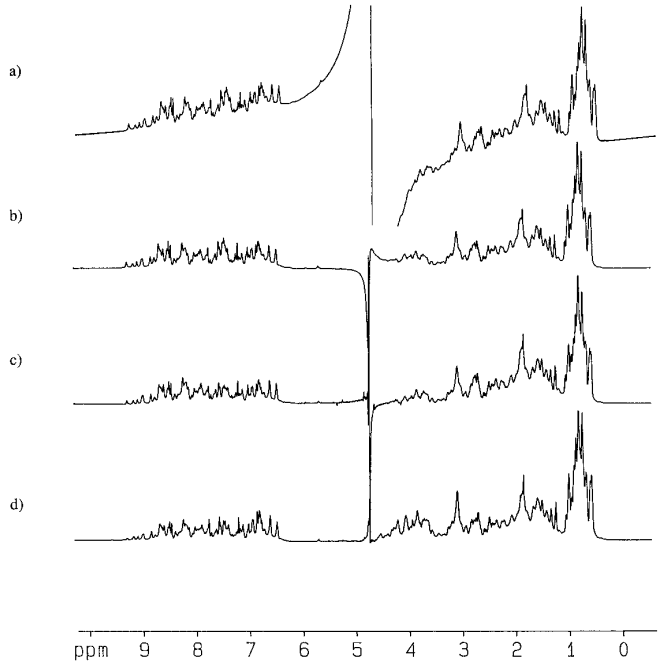


FIG. 3. All spectra of wheat ns-LTP sample presented here were recorded under the same experimental conditions. The temperature was set to 20°C, the protein concentration was 6 mM, and the solution pH was set to 5.5. The spectrometer used was a Bruker AMX500 operating at a frequency of 500 MHz for proton nucleus and equipped with three axis gradient coils. The interpulse delay τ was set to 290 μs. The RF field power corresponds to a 90° of 13.8 μs. The recycling delay was 1.4 s in all cases. For all spectra, no postacquisitional water suppression and baseline correction were applied. Only zero order phase correction was used. (a) 1D spectrum of a 6 mM wheat ns-LTP sample solubilized in nondeuterated solvent recorded with the pulse scheme of Fig. 1a. Although strongly attenuated, a strong water signal was detected. (b) Same conditions as in (a) except for a 3.5° phase shift of the last pulse phase. The spectrum is almost water free. (c) Fourier transform of the first FID of a NOESY spectrum recorded on the ns-LTP sample. The mixing time was set to 200 ms and the interpulse delay of the composite sequence to 290 μs. The phase of the last pulse was shifted by 3.5°. The number of scans was 64. (d) Spectrum of wheat ns-LTP recorded with a presaturation scheme. The presaturation delay was 1.2 s with a RF power of 60 Hz.

Several frequencies are not excited by this kind of composite pulse. The first null occuring at the offset frequency, theoritically the other unexcited frequencies experience a multiple of 2π rotation during the interpulse delay, $2\pi\nu\tau = k2\pi$, where k is an integer.

Theoretically the second null must be set outside the frequency sweep width. Therefore, for a given sweep width SW, the interpulse delay τ is chosen in such a way that the highest

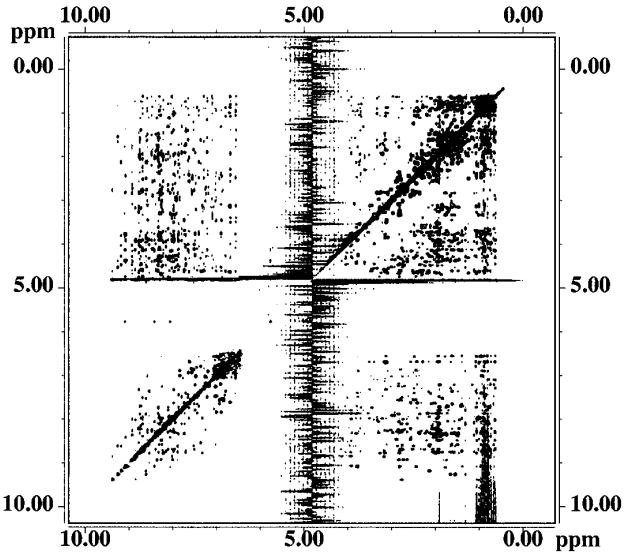


FIG. 4. NOESY spectrum of wheat ns-LTP recorded with the pulse scheme of Fig. 1b. The mixing time was 200 ms. The experimental conditions were 512 increments, 64 scans per FID. The quadrature detection along ω_1 was achieved by the use of the States method (*10*). The overall relaxation delay between two scans was 1.4 s. The interpulse delay of the composite 90° pulse sequence was set to 290 μs. The RF power corresponds to 90° pulse of 13.8 μs. A simple zero-order phase correction was used along the ω_2 axis.

spectral frequency, SW/2, experiences during τ a rotation lower than 2π, $2\pi\mathrm{SW}/2\tau < 2\pi$, and therefore $\tau < 2/\mathrm{SW}$.

In order to test the pulse sequence, a one dimensional spectrum of a 6 mM sample of wheat nonspecific lipid transfer protein (ns-LTP), a 90 amino-acid protein, was recorded in H_2O on a Bruker AMX 500-MHz spectrometer (Fig. 3a). Clearly, the suppression of the water magnetization does not appear to be neat, as expected. Although considerably attenuated, a strong residual water signal is still present in the spectrum, providing large baseline distortions. This discrepancy between experimental and theoretical results may result from the radiation damping of the water magnetization which occurs during the interpulse delays. Such phenomenon has been extensively described and may be very critical for NOESY experiments where no presaturation and gradient pulses are applied. Indeed the first pulses create transverse magnetizations of the solvent signal. This magnetization generates an oscillating current in the coil of the NMR probe, which in turn generates a weak RF field. This soft pulse has a tendency to rotate the solvent magnetization back to the $+z$ axis (*6*). Although a 290-μs interpulse delay is used, the influence of the radiation damping hampers the efficiency of the composite sequence as already described in the jump and return pulse sequence (*7*). The water magnetization position when applying the last pulse is not accurately defined. This problem can be alleviated by slightly moving the phase of the last pulse. With our Bruker AMX500 spectrometer, operating at 500 MHz for ^{1}H, a decrease of the last pulse phase of 3.5° suppresses almost completely the water signal as displayed in Fig. 3b. In this case, the receiver gain setting is mainly limited by the signals arising from the protein. Although the WATERGATE pulse sequence is more robust and less sensitive to parameter missettings, it requires two necessary extra delays during which gradient pulses are applied. The composite 90° pulse sequence described herein reduces the delay between the protein signal excitation, and this time saving can be crucial for the study of proteins, the protons of which are relaxing fast. For comparison we have added a one-dimensional spectrum recorded with a simple presaturation scheme in Fig. 3d. It clearly appears that the presaturation induces an overall intensity decrease of the signals arising from the amide protons of the protein via an exchange process with the presaturated bulk water. The main drawback of the composite sequence use is an attenuation of resonances lying close to the offset frequency. A comparison of Figs. 3b and 3d shows that resonances of CαH protons of the protein are strongly reduced in intensity. However, the most interesting region in this spectrum recorded in H_2O is the amide region. Indeed this spectral region, which is uniformly and properly excited and not attenuated, contains crucial informations on the secondary structure elements of the protein.

The composite 90° pulse sequence can easily be appended to a NOESY pulse sequence to record NOESY spectra of proteins

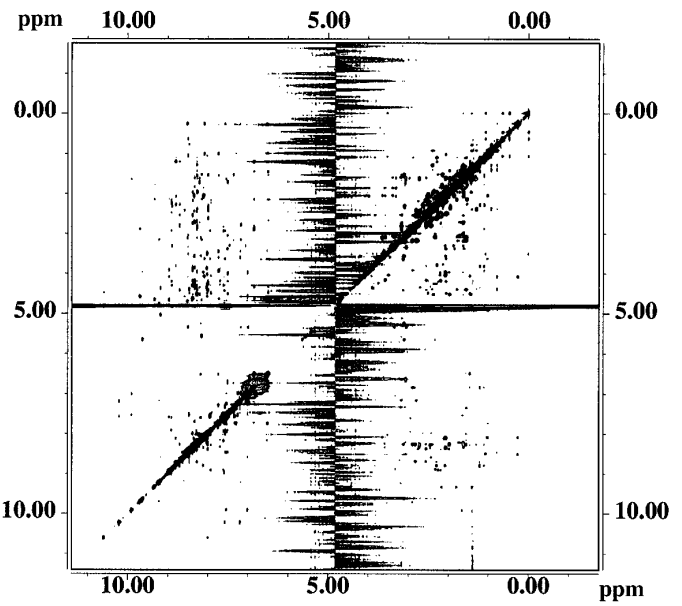


FIG. 5. NOESY spectrum of drosomycine recorded at 20°C with the pulse scheme of Fig. 1b. The mixing time was 250 ms, and 80 scans per FID were coadded. The total recycling delay was 1.4 s, and 512 FIDs were recorded and the States method was used to record phase sensitive spectrum. The RF power corresponds to a 90° pulse of 9.4 μs. The protein concentration was 1.5 mM. The sample volume being 300 μl, a Shigemi nmr tube was used. No baseline correction or digitalized filter was used to reduce the water signal in the 2D spectrum. Only zero-order phase correction was applied along the ω_2 axis.

without presaturation or any kind of water suppression. The pulse sequence with the phase cycling is reported in Fig. 1b. The efficiency of the composite 90° detection pulse sequence relies on the position of the water magnetization. For optimum efficiency, the water magnetization must be aligned along the $+z$ axis before applying the composite pulse. Owing to the phase cycling in the NOESY sequence, the water magnetization is aligned along the $-z$ axis half of the time. This two-step phase cycling is necessary to remove axial peaks in the resulting two-dimensional spectrum. In probes with high quality Q factor, the mixing time should be long enough to allow the return of the solvent magnetization onto the $+z$ axis before applying the detection pulse (*6*). With our probe, a mixing time of 200 ms is not long enough and still a small but noticeable residual transverse water magnetization is present. In order to overcome this problem, we took advantage of the radiation damping by shifting the phase of the second 90° pulse by 45°. Therefore, regardless of the phase cycling, the water magnetization is never completely aligned along the $-z$ axis and a strong residual water transverse magnetization is always present at the beginning of the mixing time, speeding up the return to the $+z$ axis via radiation damping (*8*). To ensure that no transverse magnetization arising from the solvent and the solute is still present at the end of the mixing time a 1-ms homospoil pulse is applied. Under these conditions a neat and almost water-free spectrum is recorded. The result is shown in Fig. 3c where the Fourier transform of the first increment is plotted. No postacquisitional water suppression is applied. Furthermore only a zero-order phase correction is used, avoiding strong baseline rolling which is often obtained when composite 90° pulse sequences are used. The double Fourier transform of the NOESY spectrum is shown in Fig. 4. Again no postacquisitional procedures to reduce the water signal, i.e., digital filtering or baseline correction and zero-order phase correction along ω_2 frequency axis, are applied. This high quality demonstrates the efficiency of the pulse scheme.

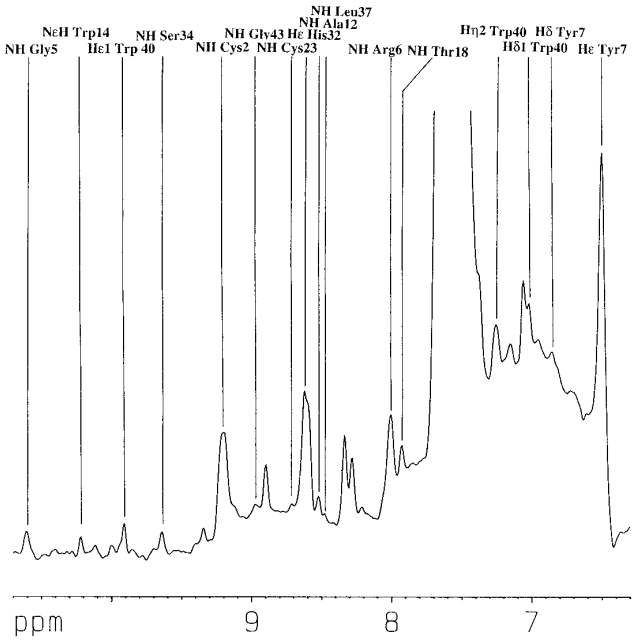


FIG. 6. Cross section of the NOESY spectrum of drosomycin corresponding to the water resonance along the ω_1 axis. All peaks which are identified correspond to the protein protons which interact with water via a NOE or an exchange process. Some cross peaks are not assigned corresponding to NOEs between CαH proton resonating at the water frequency and amide protons, in agreement with the solution structure of the protein.

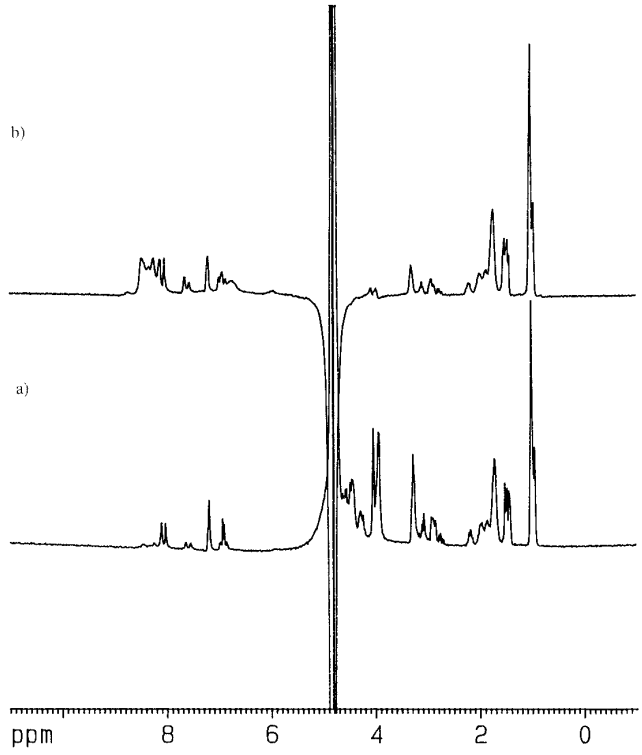


FIG. 7. Spectra of a 5 mM peptide derived from the defensin A protein recorded at 27°C and at pH 6.5 on a Bruker AM300 operating at a 300 MHz frequency for ^{1}H nucleus. In (a) the spectrum was recorded with a simple presaturation scheme, and in (b) the spectrum was recorded under the same experimental conditions as in (a) but with the composite pulse sequence. Eight scans for both spectra were coadded. The RF power was set in order to have a 90° pulse length of 10 μs in both cases. The presaturation delay was set to 1.5 s and the relaxation delay in (b) to 1.5 s. The interpulse delay τ was 400 μs, and the phase shift of the last pulse was 16°.

Another NOESY spectrum is recorded with a diluted sample of drosomycin in H_2O, a 44-residue protein whose solution structure was solved in our laboratory (*9*) and which is involved in the antifungal defense of insects. In order to increase the protein concentration, the protein was dissolved in 300 μl of a mixture 90/10 of H_2O/D_2O in a Shigemi NMR tube. The resulting NOESY spectrum (Fig. 5) is obtained without applying baseline corrections, and postacquisitional water suppression, and without first-order phase correction. This clearly demonstrates that even for diluted sample, the use of the composite 90° pulse sequence allows one to record a sensitive

NOESY spectrum without destruction of the water magnetization.

Since there is no attempt to suppress the water magnetization at any time of the pulse sequence, the cross section of the water signal can be used to detect protein protons which interact with water. However, the effective mixing time for water/protein proton NOEs is less than the nominal NOESY mixing time since radiation damping brings the water magnetization back onto the $+z$ axis during this delay. Figure 6 displays the cross section of the NOESY spectrum corresponding to the water frequency along the ω_1 frequency axis. All amide protons depicted in the figure giving a cross peak with water were previously found to be fast exchanging with deuterons. The only exceptions are concerning the amide protons of Cys23, Ser34, and Leu37. However, these residues are located very close to Ser residues in the protein solution structures, and the interactions seen in the cross-section for these three amino acids may result from NOEs with the hydroxyl protons of Ser29 for Cys23 and Ser36 for Ser34 and Leu37.

In conclusion, the results provided in this paper clearly show that the novel composite 90° pulse sequence we propose can be used to record very sensitive multidimensional spectra of compounds dissolved in H_2O. Since this sequence does not require gradient pulses it can be implemented on any spectrometer. Several spectra have been acquired with this simple sequence on a Bruker AM300. In that case the phase shift of the last pulse was $+16°$ and nearly water-free spectra were recorded. For instance, two spectra of a 28-residue peptide corresponding to the CS$\alpha\beta$ motif of defensin A (*11*) where Cys residues were substituted by serines are reported in Fig. 7. This clearly emphasizes the improvement obtained in the amide proton region when the composite pulse is used. All examples provided in this work show that the composite pulse sequence can be confidently used in peptide and protein studies. However, some phase distortions and signal attenuations may occur in high frequency regions due to off-resonance effects and may be troublesome in DNA and RNA imino proton studies.

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