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Spin-Echo Water Suppression for the Generation of Pure-Phase Two-Dimensional NMR Spectra

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A new approach is described for water suppression in one- and two-dimensional NMR, generating absorption-mode spectra that are free of baseline distortions. This method involves the use of a 1–1 hard pulse as a read pulse, followed by a 1–1 refocusing pulse which is phase cycled to obtain the greatest possible water suppression. Examples of this approach for water suppression are demonstrated for a 10 mg sample of the decapeptide LH-RH in 90% H_2O by recording 2D NOE, 2D HOHAHA, and heteronuclear ^1H–^{15}N shift correlation spectra. © 1987 Academic Press, Inc.

A number of different approaches are available for suppression of the intense H_2O resonance in the NMR spectra of water soluble compounds. Most commonly one employs presaturation of the H_2O resonance using low-power irradiation via the decoupler channel prior to the observation pulse (*1*, *2*). Recently an interesting modification of this approach has been proposed that increases the effectiveness of this method (*3*). A disadvantage of presaturation is that resonances of exchangeable NH protons and signals near the H_2O frequency are also saturated. Selective soft pulse excitation with a null at the water resonance avoids this problem (*4*, *5*). Excitation with the specifically designed composite "hard pulse" sequences achieves the same purpose while being less sensitive to RF inhomogeneity and more convenient for routine use. A large variety of different hard pulse sequences has been proposed in recent years (*6*–*16*); however, all but the simplest 90°_x–τ–90°_{-x} (1–1) sequence yield spectra that require a large linear phase correction. The baseline roll that results from such a phase correction can lead to intense artifacts in phase-sensitive 2D NMR spectra. We describe a new approach that uses a hard pulse 1–1 excitation sequence (*7*) immediately followed by a 1–1 refocusing pulse. This provides excellent water suppression and as will be derived below, the resulting spectra do not require any first- or higher-order phase correction.

PHASE AND INTENSITY PROFILE

A refocusing pulse ideally is a 180° rotation about an axis in the transverse plane. Imperfections in such a pulse can result in severe amplitude and phase distortions in

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the resulting spectrum. In 1977, Bodenhausen *et al.* (*17*) demonstrated that the spurious effects of this pulse imperfection can be eliminated by the use of a four-step phase cycle which they named Exorcycle. In this phase-cycling procedure the phase of the refocusing pulse is incremented in 90° steps in consecutive scans and the corresponding data are alternately added to and subtracted from memory. It was shown by Bodenhausen *et al.* that upon completion of the four-step Exorcycle all resulting magnetization had undergone a 180° rotation about an axis in the transverse plane. To calculate the phase profile as a function of resonance offset it must be determined about which axis in the transverse plane the magnetization is effectively rotated.

The effect of an arbitrary pulse can be described by a Cartesian rotation matrix **R**, given by

$$\mathbf{R}=\begin{bmatrix} a & b & c \\ d & e & f \\ g & h & i \end{bmatrix}. \qquad [1]$$

If the phase of the pulse (or of all its components) is incremented by an amount ϕ, the rotation operator $\mathbf{R}_\phi$ is given by

$$\mathbf{R}_\phi = R_z(-\phi)\mathbf{R}R_z(\phi). \qquad [2]$$

The overall effect of the pulse after completion of a four-step Exorcycle (ϕ = 0, $\pi/2$, π, $3\pi/2$; Acq. = +, −, +, −) is described by a matrix $\mathbf{R}^\mathrm{E} = \mathbf{R}_0 - \mathbf{R}_{\pi/2} + \mathbf{R}_\pi - \mathbf{R}_{3\pi/2}$:

$$\mathbf{R}^\mathrm{E} = 1/2\begin{bmatrix} a-e & b+d & 0 \\ b+d & e-a & 0 \\ 0 & 0 & -1 \end{bmatrix}. \qquad [3]$$

This matrix represents rotation of transverse magnetization by 180° about the y axis combined with subsequent rotation around the z axis by an angle Φ:

$$\Phi = \arctan[(b+d)/(e-a)]. \qquad [4]$$

In addition, the transverse magnetization is modulated in amplitude by a factor k:

$$k = [(a-e)^2 + (b+d)^2]^{1/2}/2. \qquad [5]$$

A pulse or a set of pulses does not affect the first- or higher-order phase corrections if the rotational operator $\mathbf{R}^\mathrm{E}$ can be described by a matrix for which Φ is a constant independent of RF offset.

It can be derived that for a single refocusing pulse (of arbitrary duration) upon completion of the Exorcycle, the angle Φ (Eq. [4]) is offset-independent. For example, for a soft pulse corresponding to an α_y rotation on-resonance, $\Phi = 0$ and the amplitude is modulated by a factor $k = [1 - \cos\{\alpha(\beta^2 + 1)^{1/2}\}]/2(\beta^2 + 1)$, with $\beta = \omega/\omega_1$, where ω is the resonance offset and ω_1 is the RF field strength.

When a composite water suppression pulse is applied for refocusing, the situation becomes more complicated because in general the term $(b + d)$ in the matrix of Eq. [3] will be offset-dependent. Therefore, the extent of z rotation (Φ) for such a pulse will also be offset-dependent. However, if a 90°_x-τ-90°_{-x} ("jump and return") sequence is used, the rotation matrix is antisymmetric, resulting in $b + d = 0$:

$$\mathbf{R}_{1\text{-}1} = R_{-x}(\pi/2)R_z(\omega\tau)R_x(\pi/2) = R_y(\omega\tau) = \begin{bmatrix} \cos\omega\tau & 0 & -\sin\omega\tau \\ 0 & 1 & 0 \\ \sin\omega\tau & 0 & \cos\omega\tau \end{bmatrix}. \qquad [6]$$

Upon completion of the Exorcycle this results in an effective rotation described by the matrix

$$\mathbf{R}^{\mathrm{E}}_{1\text{-}1} = 1/2\begin{bmatrix} \cos\omega\tau - 1 & 0 & 0 \\ 0 & 1-\cos\omega\tau & 0 \\ 0 & 0 & -1 \end{bmatrix} \qquad [7]$$

which represents a 180° rotation about the y axis independent of resonance offset with the amplitude modulated by a factor $(1 - \cos\omega\tau)/2$.

WATER SUPPRESSION USING THE 1-1 ECHO SEQUENCE

When the 90°_ϕ–τ_2–$90^\circ_{-\phi}$ refocusing pulse is combined with a 90°_x–τ_1–90°_{-x} excitation pulse (Fig. 1a), there results a sequence that provides excellent water suppression and does not introduce any linear or higher-order phase errors. The sequence of Fig. 1a has an intensity profile that depends on $\sin\omega\tau_1(1 - \cos\omega\tau_2)$. Most commonly one will choose $\tau_2 = 2\tau_1$, resulting in an excitation profile proportional to $\sin^3\omega\tau_1$. Figure 2 displays the experimental offset dependence of the excitation, showing the absorption character of the resonance, independent of offset, and the $\sin^3\omega\tau$ intensity dependence. Of course, the water suppression in an individual scan is not very high, being on the same order as that obtained with the regular 1–1 (90°_x–τ–90°_{-x}) sequence, which typically provides an attenuation of the water signal by about a factor of 50. Very high suppression (5000–10,000) of the water is obtained only after combining the results of four scans in the Exorcycle manner. A factor of 50 suppression per individual scan is sufficient to overcome the dynamic range problems. Subsequent phase cycling then removes the residual H_2O signal from the spectrum.

When necessary, water suppression in a single scan by a factor much larger than 50 can be obtained if two short homogeneity spoiling (homospoil) pulses of identical length are used during the echo delays, as indicated in Fig. 1a. These homospoil pulses destroy residual transverse water magnetization components which are generated mainly by imperfect phase shifts, transient effects, and H_0 inhomogeneity. Signals of interest which lose phase coherence during the first homospoil pulse interval are recovered by the 1–1 refocusing pulse. For this application the duration of the homospoil pulses can be kept relatively short. On our Nicolet NT-500 spectrometer 2 ms homospoil pulses followed by 2 ms recovery times suppress H_2O by a factor of about 300 for a single scan. However, when the 1–1 echo sequence is incorporated in 2D NMR experiments it is important to keep the time between the 1–1 excitation pulse and the 1–1 refocusing pulse very short, because otherwise coherent transfer between J-coupled spins is generated by the 1–1 refocusing pulse. In many cases this type of transfer is undesirable (for example, in the 2D NOE experiment) and the time needed for homospoil pulse and subsequent recovery prohibits application of the version of the 1–1 echo sequence that uses these homospoil pulses.

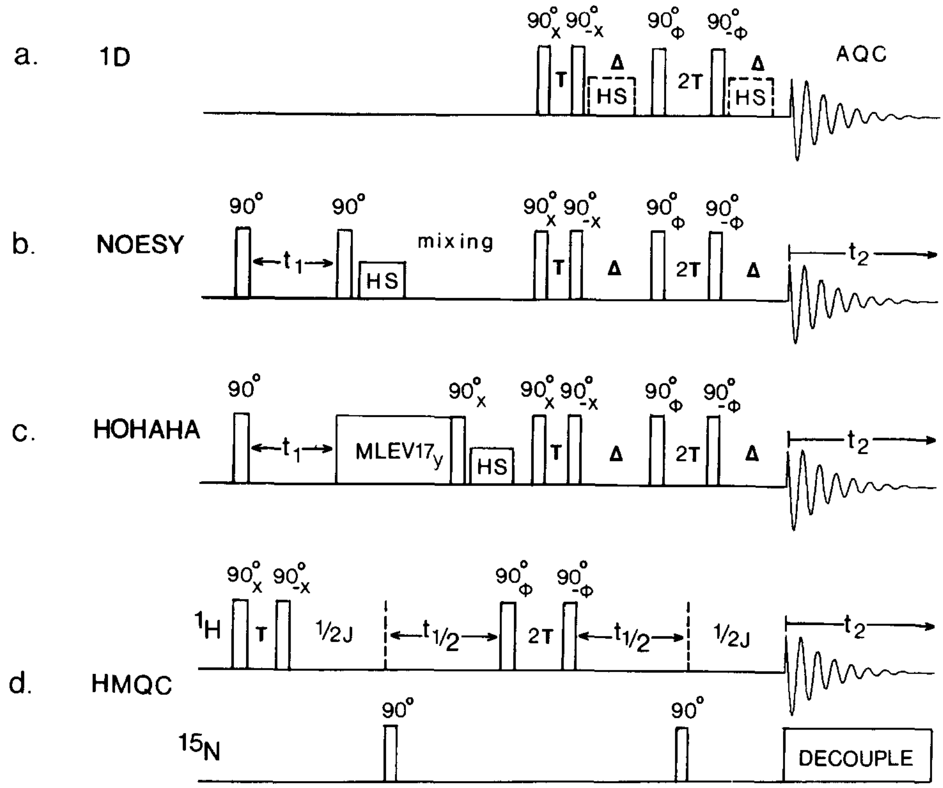


FIG. 1. Schemes for water suppression using the 1-1 echo sequence. In all schemes the phase ϕ is cycled along the x, y, $-x$, and $-y$ axis in the Exorcycle manner. Phases of all pulses not indicated in the figure are cycled as in the regular experiment without water suppression. (a) The 1-1 echo scheme for generating a one-dimensional spectrum. To increase the suppression per individual scan, short homospoil pulses (HS) can be applied during the delays, Δ. (b) The modified NOESY scheme; an intense homospoil pulse is applied during the mixing period. (c) The modified HOHAHA sequence. (d) The modified HMQC experiment for correlating ^{1}H and ^{15}N chemical shifts.

INCORPORATION IN TWO-DIMENSIONAL EXPERIMENTS

The 1-1 echo water suppression sequence of Fig. 1a is easily incorporated in a variety of two-dimensional NMR experiments. The sequence must be used as the final read pulse; for maximum water suppression per individual scan it is required that no transverse water magnetization be present just before this final read pulse unit. Most experiments require an additional pulse in combination with a homospoil pulse to create such a situation. Below, incorporation of the 1-1 echo sequence will be discussed for some of the 2D NMR experiments where water suppression often is a problem.

The NOE experiment. Incorporation in the 2D NOE experiment is most easily accomplished by replacing the last pulse of the NOESY sequence by the 1-1 echo scheme of Fig. 1a. It is important to minimize the presence of transverse H_2O magnetization before this echo scheme, otherwise the suppression in a single scan will be

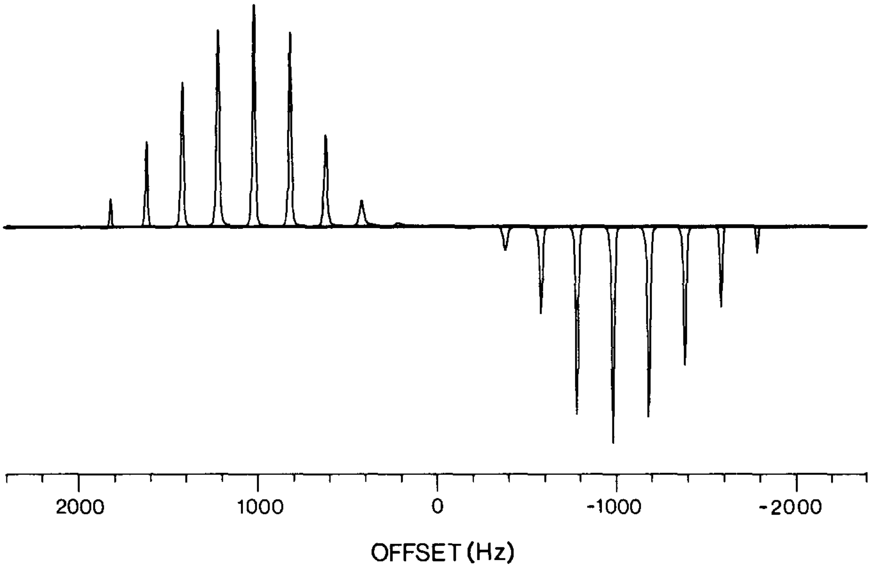


FIG. 2. Experimental offset dependence of the 1–1 echo excitation after a four-step Exorcycle. A constant frequency-independent phase correction was used for adjusting all resonances to the absorption mode.

much poorer and the dynamic range of the spectrometer will become a problem. To minimize transverse H_2O magnetization, a homospoil pulse is used in the mixing period. To improve the response time of this pulse, a minor modification was made to the Oxford Instruments shim coil power supply (details available on request). Use of the homospoil pulse has the additional advantage of removing coherent transfers through single-quantum and higher orders of coherence. This reduces the need for phase cycling commonly used to eliminate this type of coherent transfer. The minimum number of scans required per t_1 value therefore remains 64 (32 for TPPI): 4 steps for separating positive and negative modulation frequencies and suppression of axial peaks (*18, 19*) (2 for TPPI), 4 steps for the Exorcycle of the 1–1 refocusing pulse, and 4 steps for CYCLOPS cycling to remove quadrature artifacts (*20*). On our spectrometer an 8 ms homospoil pulse is sufficient to eliminate the water almost completely. An equal length delay following this homospoil pulse is needed for recovery of the lock and for decay of eddy current effects. It should be noted, however, that the required duration of the homospoil pulse depends strongly on the design of the receiver coil. The time Δ between the 90°_x–τ–90°_{-x} excitation and the 90°_x–2τ–90°_{-x} refocusing pulse should be kept very short ($\ll 1$ ms). For Δ durations longer than 1 ms, significant amounts of coherent transfer can be introduced by the 90°_x–2τ–90°_{-x} pulse. For the same reason, the delay between the 90°_x and 90°_{-x} pulses should be short, prohibiting the use of this sequence in cases where exchangeable protons resonate near the H_2O resonance. Therefore, the sequence is best suited for high-field spectrometers where the frequency difference between amide protons and the water resonance is typically several kilohertz.

The method is demonstrated for a solution of 10 mg of the hormone LH–RH (*p*Glu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2) in 0.4 ml 90% H_2O/10% D_2O, containing 100 m*M* NaCl, pH 5.8, 1 °C. Experiments are carried out at 500 MHz on a modified NT-500 spectrometer. Figure 3 shows part of the NOESY spectrum recorded with the scheme of Fig. 1b, using a mixing time of 200 ms including the homospoil pulse of 30 ms and Δ periods of 100 μs. The τ delay was set to 160 μs and the 90° pulse width was 20 μs, optimizing excitation at about ±1.4 kHz offset from the carrier. For correct quantitation of the NOE cross peaks, one has to correct for the $\sin^3 \pi\omega/2\omega_m$ dependence of the cross-peak intensities in the F_2 dimension of the spectrum, where ω is the resonance offset from the carrier (H_2O) frequency and ω_m is the frequency at which excitation is maximal. The relative intensities of resonances on individual slices taken parallel to the F_1 axis are not affected by the read pulse.

Apart from NOE cross peaks, the spectrum also shows intense peaks indicating exchange between the amide protons and the water resonance. As demonstrated by Dobson *et al.* (*21*) the intensities of these peaks can be used for a quantitative study of the exchange rates. The most intense exchange cross peaks show small tails pointing in the $F_1 = F_2$ direction and are due to inhomogeneity of the static magnetic field.

The HOHAHA experiment. As shown previously, homonuclear Hartmann–Hahn-type cross polarization can be very effective for obtaining direct and relayed scalar connectivity (*22–26*). A composite pulse mixing scheme (*26*), based on the MLEV-16 decoupling sequence (*27*), can cover a large chemical-shift range and minimizes the magnetization loss due to relaxation precesses during the mixing period. Unless very high RF power is used, mixing with the original MLEV-16 sequence leads to

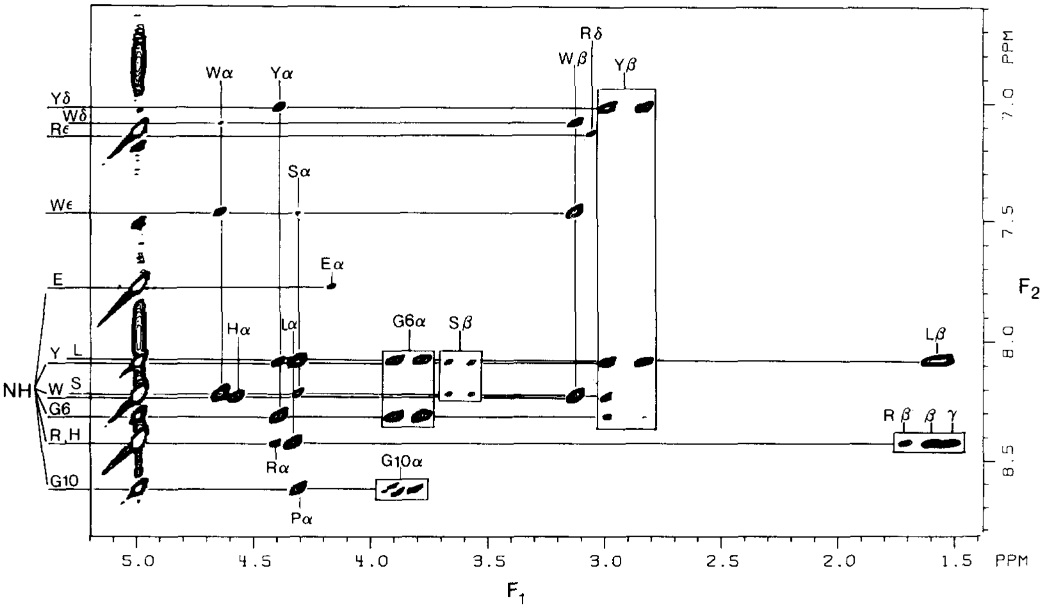


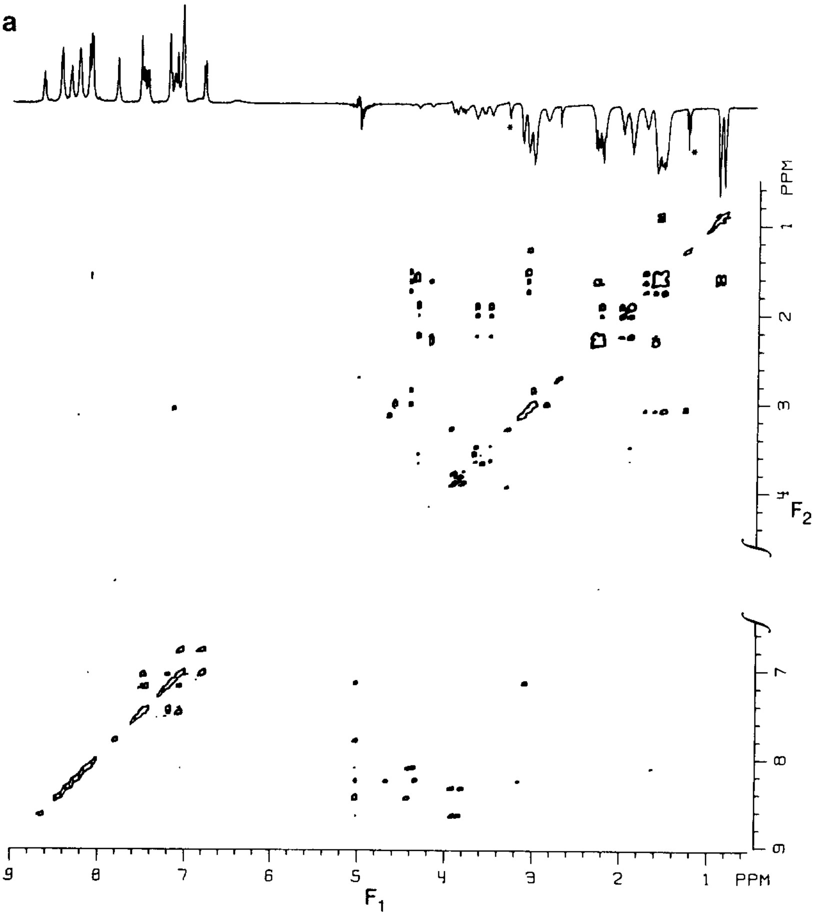
FIG. 3. Absorption-mode 2D NOE spectrum of the decapeptide LH-RH, recorded with the scheme of Fig. 1b. Expansion of the "fingerprint region." The spectrum results from a 2 × 350 × 1024 data matrix, corresponding to 70 and 102 ms acquisition times in the t_1 and t_2 dimensions, respectively. The mixing time was 200 ms, including a 10 ms homospoil pulse. A total of 64 transients were recorded per t_1 value and the total measuring time was 9 h.

phase distortions (*28*) that are partly of a fundamental nature and in part caused by pulse imperfections. It was shown (*26*) that if at the end of every MLEV-16 cycle a 180° pulse is applied along the same axis as the 180° pulses of the MLEV-16 cycle, this provides an effective spin-lock axis and removes phase distortion from the spectrum. A recent experimental and computational analysis (*29*) shows that one can reduce the flip angle of this final pulse from 180° to about 60° without introducing serious phase distortions. This reduction in flip angle widens by about 40% the spectral width that can be covered by a given amount of RF power. Previously, so-called trim pulses (*26*) were applied to further eliminate the signals from H_2O magnetization that builds up during the evolution period and that is perpendicular to the spin-lock axis. In the present scheme these trim pulses are not required because the 1–1 echo sequence eliminates the water signal. The pulse scheme is shown in Fig. 1c. The MLEV-17_y scheme consists of an integer number of repeats of the sequence ABBA BBAA BAAB AABB 60_y, where A = $90^\circ_x 180^\circ_y 90^\circ_x$ and B = $90^\circ_{-x} 180^\circ_{-y} 90^\circ_{-x}$. This scheme spin locks the magnetization along the y axis and the subsequent 90°_x pulse, applied at the end of the mixing period, turns the spin-locked magnetization towards the z axis. After that a strong homospoil pulse (8 ms followed by a recovery delay of 8 ms) is used to eliminate any remaining transverse magnetization. The following 1–1 echo sequence then reads the z magnetization and suppresses the H_2O signal. Note that NOE effects will build up in the delay between the pulse that stores magnetization along the z axis and the final 1–1 echo read sequence. Because the delay is relatively short (16 ms in our case) the NOE effect will generally be dwarfed by the much stronger HOHAHA effect. The same approach as described here for the HOHAHA experiment can be used for incorporation of the 1–1 echo read pulse sequence into COSY and spin-locked NOE spectroscopy (*30*).

As an example, Fig. 4a shows the HOHAHA spectrum of LH–RH, obtained with a 35 ms mixing period. Near the F_2 frequency of the water (5.0 ppm) all intensities are very low and this part of the spectrum is not shown. An expansion of the region displaying connectivities between the amide protons and the α and β protons is shown in Fig. 4b. The assignments indicated in this figure are based on the combined information of the NOE and the HOHAHA spectrum and complete earlier assignments made by Chary *et al.* (*31*). In the HOHAHA experiment cross peaks between the amide protons and the H_2O resonance are also observed. These are due to hydrogen exchange during the MLEV-17 scheme and during the subsequent homospoil and recovery delays.

Heteronuclear multiple-quantum correlation. For correlating the chemical shifts of protons and ^{15}N nuclei in peptides, it often is necessary to work in H_2O solution. Previously, weak 90° and 180° ^1H pulses were used in this experiment; the length of these pulses was optimized for minimal excitation of the water resonance (*32*). The spectral width of amide protons that can be covered with this soft pulse approach is very narrow. As first proposed by Griffey *et al.* (*33*) one can also use 1–1 type excitation and refocusing pulses for suppression of the H_2O resonance in this heteronuclear multiple-quantum-type shift correlation. As shown above, pure-phase spectra can be obtained by including Exorcycle phase cycling of the 90°_x–2τ–90°_{-x} refocusing pulse. Due to synthesizer noise (*34*) and very small random variations in the pulse width, the suppression of signals not coupled to ^{15}N is lower than with the sequence that

a



PPM
1
2
3
4
F2
7
8
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9
8
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6
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4
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2
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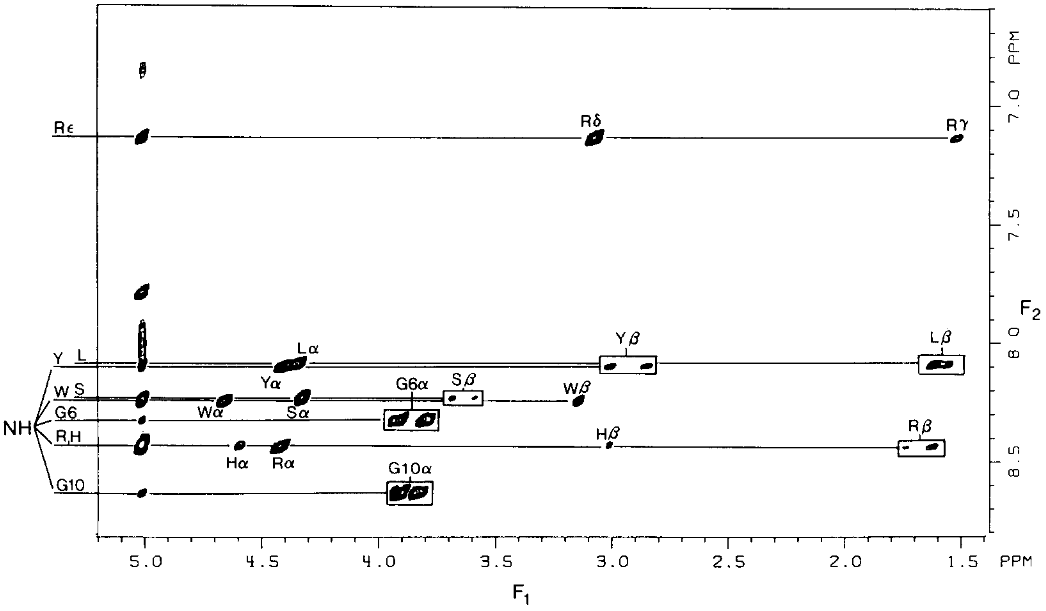
PPM
F1

b



PPM
7.0
7.5
8.0
8.5
F2
Rε
Rδ
Rγ
Y L
Lα
Yβ
Lβ
W S
Yα
G6α
Sβ
Wβ
G6
Wα
Sα
NH
R,H
Hβ
Rβ
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F1

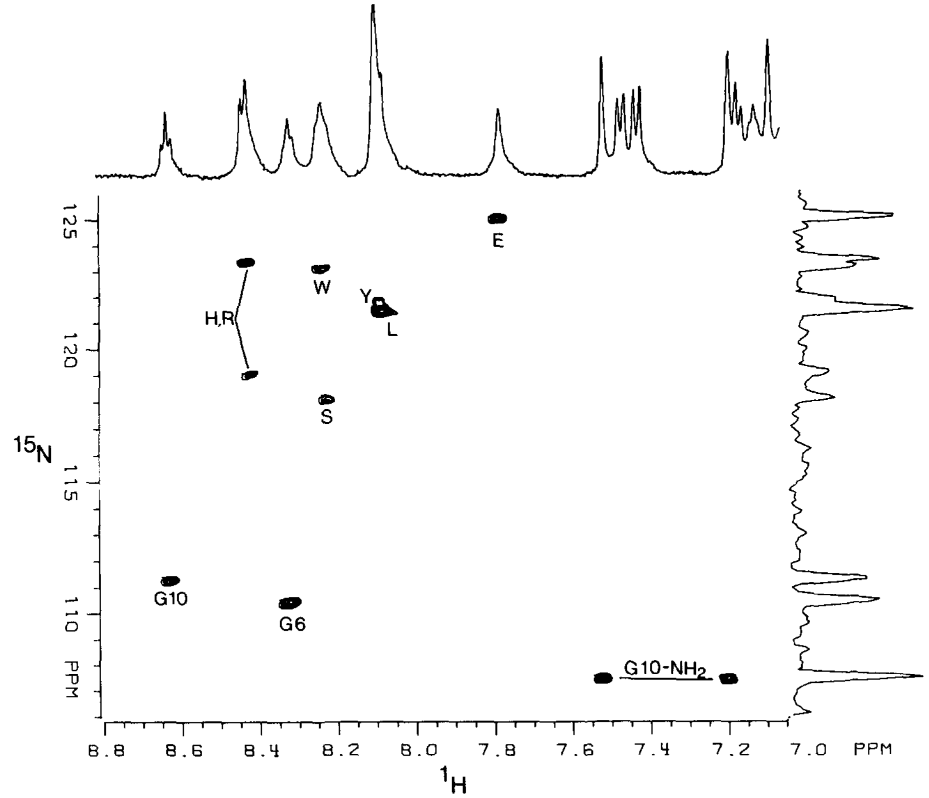

FIG. 5. Absorption-mode natural abundance 2D ^{1}H–^{15}N correlation of LH-RH, measured with the scheme of Fig. 1d. The spectrum results from a 2 × 128 × 1024 data matrix, corresponding to 26 and 102 ms acquisition times in the t_1 and t_2 dimensions, respectively. A total of 512 transients were recorded per t_1 value and the total measuring time was 17 h.

utilizes soft pulses (*32*). However, on our spectrometer system sufficient suppression, by a factor of about 3000, can be obtained provided the 90° pulse width is at least on the order of 25 μs. If the phase ϕ of the 1–1 refocusing pulse is incremented by 90° this creates a new steady-state magnetization at the beginning of the next scan, which leads to imperfect cancellation of signals from protons not coupled to ^{15}N. To minimize this systematic imperfect cancellation, the phase ϕ is incremented only four times per t_1 value; i.e., if N scans are performed per t_1 value, ϕ is incremented after $N/4$ scans.

Figure 5 shows the ^{1}H-^{15}N correlation obtained with the sequence of Fig. 1d. A total of 512 scans were recorded per t_1 value and the total measuring time was 17 h.

FIG. 4. (a) Phase-sensitive 2D HOHAHA spectrum of LH-RH, recorded with the scheme of Fig. 1c, using a 35 ms mixing time. The delay between the MLEV17 sequence and the 1–1 echo sequence was 16 ms, including a 8 ms homospoil pulse. The acquisition parameters were identical to those of the NOE spectrum of Fig. 3. The spectrum shown along the F_1 axis has been recorded with the scheme of Fig. 1a. (b) Expansion of the 2D HOHAHA spectrum showing connectivities between amide protons and the α and β protons.

Along the ^1H axis the regular ^1H spectrum is shown; along the ^{15}N axis a projection of the 2D spectrum onto this axis is presented. Spectral assignments are based on the sequential assignment of the backbone protons using the spectra of Figs. 3 and 4.

The higher resolution obtained with this phase-sensitive pulse scheme, compared with the simpler absolute-value mode version of the HMQC experiment (*35*), may be more important than the higher degree of suppression that can be obtained with the simpler version of the experiment. If spectrometer stability is sufficient to permit use of the absorption version (Fig. 1d) this generally will be preferable. The relatively narrow $\sin^3$ profile decreases the efficiency of excitation to about 50% at the borders of the amide region. The sequence utilizing soft 90° and 180° pulses (when applied using identical RF power for both pulses) (*32*) provides a window even more narrow in the F_2 dimension but gives somewhat better suppression (by about 40% on our spectrometer) of protons not coupled to ^{15}N than the scheme of Fig. 1d.

DISCUSSION

A large number of different approaches for eliminating the water signal from a ^1H spectrum have been proposed in the past. Apart from presaturation, which is not considered here, these include the selective nonexcitation procedures (for example, the composite hard pulse sequences and the Redfield 2-1-4 sequence), tricks to minimize the water signal that enters the ADC (in its simplest form a high-pass filter with the RF carrier positioned on the H_2O resonance), and manipulations performed on the data after the data have been digitized (for example, double-quantum filtering). Since the dynamic range of the preamplifier and of the RF mixers is limited, every effective procedure for water suppression must include some form of selective nonexcitation of the H_2O resonance. In the 1-1 echo sequence proposed here the excitation of the water is minimized by using both 1-1 excitation and refocusing pulses. Although the water suppression obtained in one scan with this echo sequence is relatively poor, of the same order of magnitude as obtained with a single 1-1 jump and return excitation, the residual H_2O signal does not present serious problems of dynamic range for the preamplifier, mixers, or ADC. Exorcycle-type phase cycling then provides a very effective means for further suppression of the H_2O signal. The combined excitation profile (upon completion of the four-step Exorcycle) has a $\sin^3$ shape, with zero excitation at the H_2O resonance. This profile has a less desirable shape than other schemes proposed recently but it has the strong advantage of providing pure-phase spectra that have a flat baseline. Upon completion of the Exorcycle, the 1-1 echo sequence provides water suppression by a factor of over 5000. As demonstrated here, the 1-1 echo sequence is easily incorporated in most 2D experiments where H_2O suppression can be a problem.

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