[8] Homonuclear Hartmann–Hahn Experiments

By AD BAX

Introduction

The principles and applications of homonuclear correlation experiments have been discussed by Markley.[1] In this chapter one of these techniques, known as HOHAHA or TOCSY, will be discussed in more detail, with particular emphasis on the experimental details required for recording optimal spectra on biological samples. Total correlation spectroscopy (TOCSY) was first proposed by Braunschweiler and Ernst.[2] This experiment has particular potential for protein NMR studies since it permits correlation of all protons within a scalar coupling network. Hence, a complete subspectrum can be obtained for every amino acid, making resonance assignments considerably easier. However, the TOCSY experiment never gained much popularity, mainly because of the limited band width that could be covered with the mixing schemes used in the original pulse sequence. We accidentally rediscovered this type of magnetization transfer when analyzing artifacts[3] occurring in rotating frame NOE (ROESY[4,5]) spectra. It was shown that these artifacts are caused by a homonuclear Hartmann–Hahn (HOHAHA) effect that could be described quantitatively by mathematical expressions derived for the heteronuclear case by Müller and Ernst[6] and Chingas *et al.*[7] Magnetization transfer via the Hartmann–Hahn effect occurs only when the difference in the absolute magnitudes of the effective rf fields experienced by two coupled spins is smaller than the scalar interaction between the two spins. The effective rf field is the vector sum of the resonance offset, δ, and the applied rf field. Its magnitude is therefore strongly dependent on the resonance offset.

By considering the spurious magnetization transfer in the ROESY

[1] J. L. Markley, this volume [2].
[2] L. Braunschweiler and R. R. Ernst, *J. Magn. Reson.* **53,** 521 (1983).
[3] A. Bax and D. G. Davis, *J. Magn. Reson.* **63,** 207 (1985).
[4] A. A. Bothner-By, R. L. Stephens, J. T. Lee, C. D. Warren, and R. W. Jeanloz, *J. Am. Chem. Soc.* **106,** 811 (1984).
[5] L. R. Brown and B. T. Farmer II, this volume [11].
[6] L. Müller and R. R. Ernst, *Mol. Phys.* **38,** 963 (1979).
[7] G. C. Chingas, A. N. Garroway, R. D. Bertrand, and W. B. Moniz, *J. Chem. Phys.* **74,** 127 (1981).

experiment as a case of homonuclear Hartmann–Hahn cross-polarization, it was relatively straightforward to develop methods that would overcome the effects of rf offset. A series of different mixing schemes is now available and their advantages and disadvantages will be discussed. As will be shown, the HOHAHA experiments are very efficient at establishing direct (COSY[8]) and indirect (RELAY[9]) types of spin–spin connectivities. Of particular importance for the study of proteins is the fact that the efficiency of HOHAHA magnetization transfer is less sensitive to short transverse relaxation times than the alternative COSY and RELAY type experiments. As will be demonstrated here, methods for recording the HOHAHA spectra in H_2O solution are especially useful for connecting the side-chain protons with the usually better resolved amide resonances. At presence, most commercial spectrometers require some special hardware for producing the rf fields that have to be generated during the mixing period. Therefore, a brief discussion of the hardware requirements of the HOHAHA experiment is also included.

Principles of Homonuclear Hartmann–Hahn Spectroscopy

A detailed theoretical analysis of Hartmann–Hahn cross-polarization is beyond the scope of this chapter. For background information on heteronuclear Hartmann–Hahn cross-polarization in liquids the reader is referred to the papers by Müller and Ernst[6] and Chingas *et al.*[7] For analogous descriptions of the homonuclear case, the reader could consult a variety of papers.[2,3,10–14] Here, the principles will be briefly reiterated to provide some insight into selecting the optimal parameters for a particular experiment.

As pointed out by Braunschweiler and Ernst, the magnetization of a spin, I, under isotropic mixing conditions is periodically converted into magnetization of a second spin, S, provided that I and S are scalar cou-

[8] K. Nagayama, A. Kumar, K. Wuethrich, and R. R. Ernst, *J. Magn. Reson.* **40,** 321 (1980); A. Bax and R. Freeman, *ibid.* **44,** 542 (1981).

[9] G. Eich, G. Bodenhausen, and R. R. Ernst, *J. Am. Chem. Soc.* **104,** 3732 (1982).

[10] A. Bax and D. G. Davis, *in* "Advanced Magnetic Resonance Techniques in Systems of High Molecular Complexity" (N. Niccolai and G. Valensin, eds.), pp. 21–48. Birkhaeuser, Basel, 1986.

[11] J. S. Waugh, *J. Magn. Reson.* **68,** 189 (1986).

[12] R. Bazzo and J. Boyd, *J. Magn. Reson.* **75,** 452 (1987).

[13] R. R. Ernst, G. Bodenhausen, and A. Wokaun, "Principles of Nuclear Magnetic Resonance in One and Two Dimensions," p. 444. Oxford Univ. Press (Clarendon), London and New York, 1987.

[14] A. Bax, *J. Magn. Reson.* **77,** 134 (1988).

pled. Mathematically, this is described by the expression

$$I_x \xrightarrow{J\tau IS} I_x[1 + \cos(2\pi J\tau)]/2 + S_x[1 - \cos(2\pi J\tau)]/2 + (I_yS_z - I_zS_y)\sin(2\pi J\tau) \quad (1)$$

where τ is the duration of the mixing period. I_x and S_x denote the x components of transverse I and S spin magnetization, and similarly the indices y and z refer to the y and z components of magnetization in the regular rotating frame. This expression shows how the x component of I spin magnetization is converted periodically (with period $1/J$) into the x component of S spin magnetization. Similarly, during isotropic mixing, I_y is periodically interconverted with S_y and likewise I_z with S_z. If the magnetization transfer takes place during the mixing period of a two-dimensional (2D) experiment (Fig. 1), this generates off-diagonal cross-peaks in the 2D spectrum. Equation (1) shows that for two coupled spins all I spin magnetization can be transferred to S (in the absence of relaxation) if the duration of the mixing period, τ, is set to $1/(2J)$. The diagonal peaks in this case would disappear from the 2D spectrum and *all* intensity would be present in the cross-peaks. For systems consisting of more than two spins, an analytical expression analogous to Eq. (1) for the transfer of magnetization becomes much more complicated,[15,16] and complete transfer of magnetization from the diagonal to the cross-peaks is generally not possible. Moreover, in protein studies short relaxation times prohibit the use of long mixing periods needed for transferring the maximal amount of magnetization to the cross peaks.

Equation (1) indicates that in-phase I spin magnetization (I_x) can be transferred to in-phase S spin magnetization. This is very similar to, for example, the NOESY experiment in that one can record the spectrum in the absorption mode, with all cross-peaks having the same sign as diagonal peaks. However, more careful inspection of Eq. (1) shows that I_x magnetization can also be transferred into I_yS_z and I_zS_y terms. These latter terms denote antiphase magnetization components; for example, I_zS_y refers to S spin transverse magnetization along the $\pm y$ axis, i.e., the magnetization vector that corresponds to the S spin doublet component with spin I in the α spin state points along the y axis and the second doublet component points along the $-y$ axis. Because the line widths in protein spectra often are large relative to the J coupling, the overlapping antiphase multiplet components largely cancel one another and the spectrum still appears to be absorptive. Glycine residues can be an exception to this rule because their doublet components often are partially resolved

[15] N. Chandrakumar, *J. Magn. Reson.* **71,** 322 (1987).
[16] N. Chandrakumar and S. Subramanian, *J. Magn. Reson.* **62,** 346 (1985).

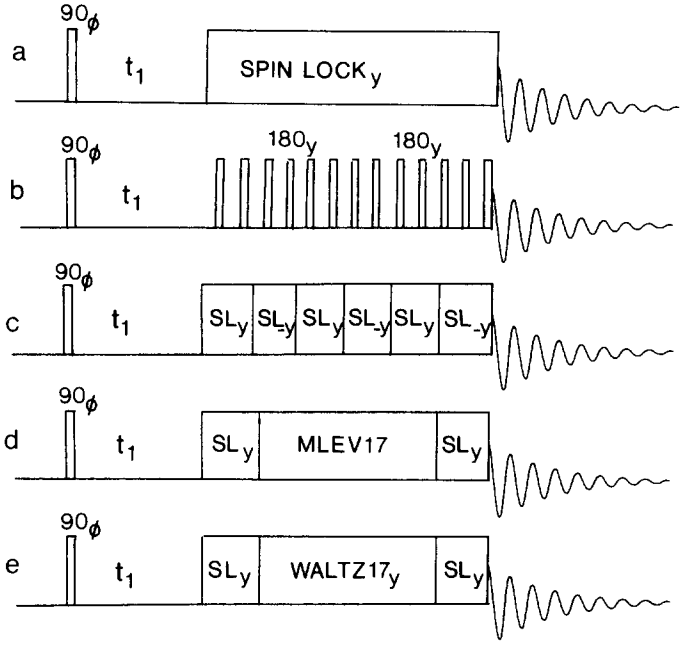

FIG. 1. Pulse schemes for 2D homonuclear Hartmann–Hahn and TOCSY experiments. (a) Using a spin-lock field of constant phase, requiring excessively strong rf fields for cross-polarization over a wide band width, but possibly useful for restricting magnetization transfer to spins that are close in chemical shift. (b) The original TOCSY experiment,[2] using a series of closely spaced 180° pulses to accomplish magnetization transfer. The shortest possible 180° pulse width (or composite 180° pulses[13]) should be used and pulse spacing should be less than 1/(2SW), where SW is the spectral width in hertz. (c) Magnetization transfer using a phase-alternated spin-lock scheme. (d) Mixing using an MLEV17 cycle, preceded and followed by trim pulses (typically 1–2 msec each). The $MLEV17_y$ mixing consists of an integral number of repetitions of the following sequence: *ABBA BBAA BAAB AABB* 60_y, where $A = 90_x180_y90_x$ and $B = 90_{-x}180_{-y}90_{-x}$. (e) Mixing using the $WALTZ17_y$ cycle, consisting of an integral number of repetitions of *ABBA* $-\ \alpha_y$, where $A = 270_{-x}360_x180_{-x}270_x90_{-x}180_x360_{-x}180_x270_{-x}$ and $B = 270_x360_{-x}180_x270_{-x}90_x180_{-x}360_x180_{-x}270_x$; the flip angle α is adjusted between 0 and 90°. The phase cycling used is $\phi = x, -x$ and Acq $= x, -x$ for TPPI type experiments and $\phi = x, y, -x, -y$ and Acq $= x, x, -x, -x$ for hypercomplex data acquisition. In addition, CYCLOPS may be added by incrementing all phases (ϕ, trim pulses, spin lock or MLEV or WALTZ and Acq) by 90° after completion of the short phase cycle.

(large coupling with no passive spins if the NH proton has been exchanged for ^2H) and the phase distortion of the cross-peak between the geminal protons then can be observed.

Equation (1) is only valid if all chemical shift and rf terms of the Hamiltonian have identical values for each of the two spins. For example,

if the J-coupled spins I and S have identical chemical shifts, periodic oscillation of magnetization between spins I and S occurs without any rf field being present (infinitely strong coupling case). In general, spins I and S have different chemical shifts and the purpose of the rf irradiation is to eliminate the difference in the chemical shifts between the two protons. This then would create a situation where the spins would become infinitely strongly coupled, resulting in periodic transfer of magnetization between the two coupled spins. One simple way to accomplish this is the application of a strong rf field (Fig. 1a). We will consider the effective rf fields experienced by the two coupled spins. Each effective rf field corresponds to the vector sum of the applied rf field (along the y axis) and the resonance offset vector (along the z axis) for that particular spin (Fig. 2). The magnitude of the effective rf field, ν_{eff}, for a particular spin with resonance offset δ from the carrier is then given by

$$\nu_{\text{eff}} = (\delta^2 + \nu^2)^{1/2} \tag{2}$$

where ν is the nominal rf field strength (in frequency units). For cases where the rf field strength is much larger than the offset ($\nu \gg \delta$) the difference, Δ, in effective field strengths for two spins I and S is

$$\Delta = \nu_{\text{effI}} - \nu_{\text{effS}} \approx (\delta_I^2 - \delta_S^2)/2\nu \tag{3}$$

A condition for Hartmann–Hahn transfer is that $\Delta \ll J$. As can be seen from Eq. (3), one way to obtain this is to use a very strong rf field. Alternatively, the carrier can be put exactly halfway between the two

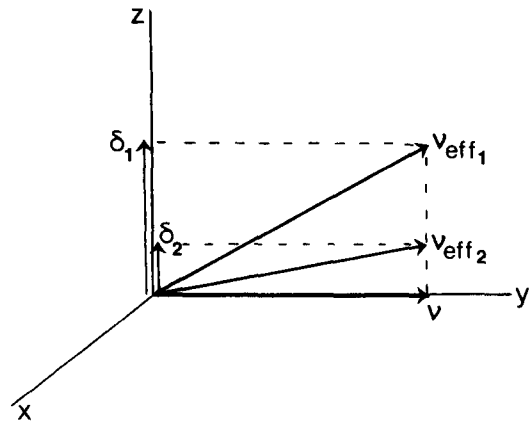

FIG. 2. Vector diagram of the orientations of the effective rf fields in the rotating frame, experienced by two spins with offsets δ_1 and δ_2, when an rf field of strength ν is applied along the y axis.

resonances, such that $\delta_I = -\delta_S$. A second point worth noting is that the effective J coupling during spin-lock conditions is reduced because the two effective fields point in different directions. This slightly slows down the rate of magnetization transfer between coupled spins.[11,12,14] In practice, the method of Fig. 1a is not suitable for wide-band cross-polarization: an rf field of several megahertz would be needed to cover a 10-ppm band width at 500-MHz ^{1}H frequency! In addition, transverse NOE would also give rise to intense cross-peaks which might cancel the Hartmann–Hahn transfers which are of opposite sign. The same is true for the mixing scheme used in the original TOCSY experiment (Fig. 1b). Although the power required for wide-band homonuclear cross-polarization is significantly reduced with this method relative to continuous irradiation, transverse NOE's are also present and the band width that can be covered effectively is still quite narrow.

Mixing Based on Composite Pulse Decoupling Schemes

Effective methods for wide-band cross-polarization in proteins must satisfy three criteria: (1) the effects of transverse NOE's must be minimized; (2) minimal rf power must be used during the cross-polarization to avoid sample heating; and (3) they must be easy to use in a routine fashion. Below, two sequences that satisfy these criteria will be discussed briefly.

Arguments presented by Waugh on the theory of spin decoupling[17] suggested to us that sequences effective at broad-band heteronuclear decoupling might be useful for obtaining wide-band homonuclear cross-polarization at low rf power. The first improvement we made on the scheme of Fig. 1a was to modulate the phase of the spin-lock field (Fig. 1c), the analog of square-wave heteronuclear decoupling.[18] Much more effective decoupling schemes have been developed by Freeman, Levitt, and co-workers.[19,20] In a first attempt, we tried to use their MLEV16 scheme[21] for wide-band homonuclear cross-polarization. The MLEV16 composite pulse sequence consists of a large number of 90 and 180° pulses that are phase shifted relative to one another, as described in the caption of Fig. 1. The first problem encountered was that this scheme preserves both the x and the y component of transverse magnetization present at the end of the evolution period. However, the relaxation rates of these two

[17] J. S. Waugh, *J. Magn. Reson.* **50,** 30 (1982).
[18] J. B. Grutzner and A. E. Santini, *J. Magn. Reson.* **19,** 178 (1975).
[19] T. A. Frenkiel, M. H. Levitt, and R. Freeman, *Adv. Magn. Reson.* **11,** 47 (1983).
[20] A. J. Shaka and J. Keeler, *Prog. NMR Spectrosc.* **19,** 47 (1987).
[21] M. H. Levitt, R. Freeman, and T. A. Frenkiel, *J. Magn. Reson.* **47,** 328 (1982).

components differ from one another during the MLEV16 irradiation (*vide infra*), resulting in quadrature artifacts and phase distortions. As discussed later, trim pulses before and after the mixing period can be used to eliminate either the x or the y component. The MLEV16 scheme is supposed to accomplish isotropic mixing of the protons over a wide band width, which means that the magnetization vector of any isolated spin in the rotating frame ends up in its starting orientation after a single MLEV16 pulse cycle has been completed. As calculated by Waugh,[11] even in theory the MLEV 16 sequence is not quite perfect at doing this. In practice, small errors in phase shifting and pulse width aggravate this imperfection. So it can happen that y magnetization present at the end of the evolution period finishes along the z axis at the end of the mixing period (after many MLEV16 cycles), and remains undetected during the t_2 period of the 2D experiment. This then results in phase and amplitude distortions in the 2D spectrum. Our solution to this problem was to add a seventeenth pulse ($60^\circ_{\bar{y}}$) which prevents the y spin magnetization from rotating away from the y axis during subsequent MLEV16 cycles. Note that the addition of the seventeenth pulse severely reduces the band width over which this MLEV17 sequence[22] provides efficient homonuclear cross-polarization (or heteronuclear decoupling). However, its beneficial effects are to ensure that pure-phase spectra free of anomalous amplitude distortions can be recorded. It also can be shown that the addition of this seventeenth pulse makes the mixing nonisotropic, which eliminates magnetization (sensitivity) loss that otherwise would be caused by the generation of multiple-quantum coherence.[11,23]

As mentioned earlier, in principle both the x and the y components of transverse magnetization are preserved during the mixing period. The undesirable x component can be eliminated by the application of so-called trim pulses before and after the mixing period. Because of the severe inhomogeneity of the rf field, any magnetization not parallel to the effective field vector (the y axis in Fig. 1d) is rapidly defocused. The effect of any nondefocused x magnetization that can give rise to a weak antidiagonal in the 2D spectrum can be eliminated by coadding the results of two 2D experiments recorded under identical conditions except that the second experiment uses trim pulses that are 90° longer than the first experiment.[23]

Use of the MLEV17 sequence for protein structure determination has been pioneered by Clore, Gronenborn, and co-workers,[24] and it is now

[22] A. Bax and D. G. Davis, *J. Magn. Reson.* **65,** 355 (1985).
[23] A. Bax, *Isr. J. Chem.* **28,** in press (1989).
[24] G. M. Clore, S. R. Martin, and A. M. Gronenborn, *J. Mol. Biol.* **191,** 553 (1986).

used routinely in a number of laboratories. The MLEV17 sequence is quite forgiving; the sequence is not particularly sensitive to the calibration of hardware rf phase shifts or balancing of the amplitudes of the phase-shifted pulses. Exact calibration of the pulse widths also is not very critical. For effective cross-polarization over a 10-ppm band width at 500 MHz (assuming the worst case of an NH proton removed by 5 ppm from the carrier and $C_\alpha H$ on resonance, with a J coupling of 4 Hz), an rf field strength of about 10 kHz is needed. Typically this requires about 3- to 5-W rf power. Even if this power is applied for relatively short mixing period durations of 30–60 msec, this can result in significant sample heating effects. The inhomogeneous sample heating may cause deterioration of the lock signal and an increase in t_1 noise.

Mixing based on the WALTZ16[25] decoupling cycle can be slightly more efficient than the MLEV17 scheme, providing a reduction in rf power by up to about 40%. However, our computer simulations and experimental results suggest that this type of mixing is more critical with respect to exact balancing of the amplitudes of the two phase-shifted rf transmit channels. On some of the newer types of NMR instruments (which often use digital rf phase shifting) this presents no particular problem and the WALTZ-based mixing scheme (Fig. 1e) is then preferable over the MLEV17 scheme. For similar reasons as for the MLEV17 scheme, a seventeenth pulse may be added at the end of each WALTZ16 cycle. In practice, on our Bruker AM600 spectrometer, a length of 30–60° for this seventeenth pulse is sufficient. To determine what duration is needed for the seventeenth pulse, the following simple experiment should be conducted. Use a compound with well-resolved resonances spread over at least 8 ppm. Record the spectrum that corresponds to the first increment of the 2D experiment. First use a short mixing time (one WALTZ17 cycle) followed by a 3-msec trim pulse and determine what phase parameters are needed for phasing the 1D spectrum to the absorption mode. Next, using a 75-msec mixing period, remove the final trim pulse, and reduce the width of the seventeenth pulse down from 180° until phase distortions become apparent in the spectrum (when phased with the parameters for the short mixing period with trim pulse). The seventeenth pulse should be set to the minimal width for which the spectrum still has an absorption mode (±15°) appearance. All spectra shown in this chapter have been recorded with the scheme of Fig. 1e, using a 60° flip angle for the seventeenth pulse.

Reducing Relaxation during Mixing

In the scheme of Fig. 1a, the spin-locked magnetization decays with a time constant $T_{1\rho}$, which for proteins is identical to T_2. In the scheme of

Fig. 1d, the y magnetization present at the start of the MLEV17 sequence goes through a trajectory where it spends one-half its time along the z axis (relaxing with T_1) and one-half its time in the transverse plane where it relaxes with T_2. Because for macromolecules, ^1H T_1 values typically are much larger than T_2 values, the magnetization decay is reduced by almost a factor of two relative to Schemes a–c in Fig. 1.[22]

Similarly, during the WALTZ mixing scheme of Fig. 1e, magnetization perpendicular to the axis along which the WALTZ pulses are applied (the x axis in Fig. 1e) relaxes by almost a factor of two more slowly relative to magnetization parallel to that axis. Note that the advantage in relaxation only applies to in-phase spin-locked magnetization; the antiphase I_yS_z and I_zS_y terms, essential in the HOHAHA process, are not reduced in relaxation rate.

Interference from NOE Effects

As mentioned earlier, transverse NOE effects can interfere with the Hartmann–Hahn effects. Since, under spin-locked conditions, the NOE effect is always positive, NOE cross-peaks are of opposite sign to the diagonal. During the MLEV and WALTZ sequences, however, the "spin-locked" magnetization spends half its time along the z axis, where the NOE is negative for macromolecules. Although the spin-locked NOE effect is larger than the regular NOE effect, the fact that they are of opposite sign strongly reduces the net NOE effect. This minimizes reduction of HOHAHA cross-peak intensities in cases where both HOHAHA and NOE effects are present. Note that for the WALTZ sequence the NOE effects would persist if the phase of the WALTZ pulses were changed by 90° with respect to the trim pulses.

Applications of HOHAHA to Peptides and Proteins

The power of the HOHAHA experiment to solve spectral assignment problems (cf. Basus [7], Vol. 177, this series) is probably most clearly demonstrated for peptides and proteins by correlating amide protons with C_α and side-chain protons. As an example, Fig. 3 shows the complete 2D correlation spectrum for the antimicrobial peptide, magainin 2 (23 amino acids).[26] The spectrum was recorded in a mixture of trifluoroethanol (TFE) and H_2O (25/75, v/v) using Scheme e of Fig. 1, with presaturation of the H_2O resonance prior to the evolution period. Because of the acidic sample conditions used (pH 4 prior to addition of TFE) the amide proton exchange is sufficiently slow that presaturation does not obliterate any of

[25] A. J. Shaka, J. Keeler, T. A. Frenkiel, and R. Freeman, *J. Magn. Reson.* **52,** 335 (1983).

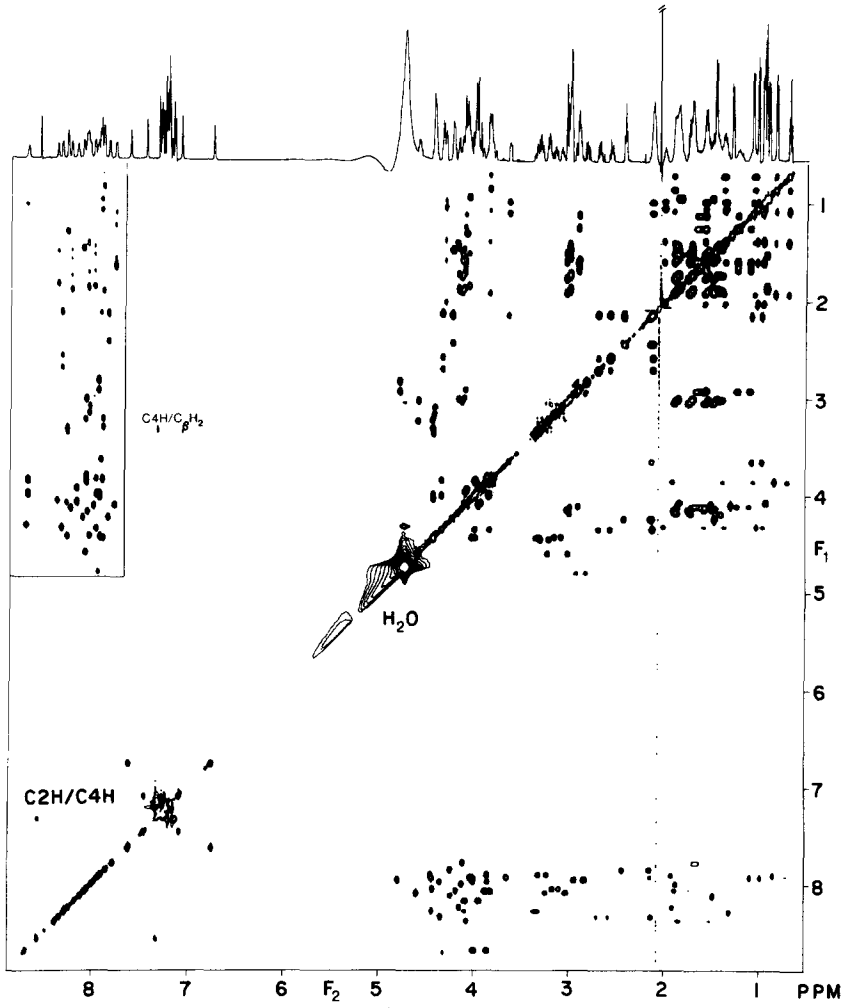

FIG. 3. Two-dimensional HOHAHA spectrum of the peptide magainin 2, 15 mg in 0.375 ml H_2O/0.125 ml trifluoroethanol, 27°. The spectrum was recorded at 600 MHz, using a WALTZ17 mixing sequence with $\alpha = 60°$. The 90° pulse width was 35 μsec, duration of the WALTZ irradiation was 75 msec, and the trim pulse duration was 2 msec. Presaturation of the H_2O resonance was used during the recycling delay. Data were acquired in the TPPI mode and the size of the acquired data matrix was 700 × 2048, corresponding to acquisition times of 42 and 120 msec in the t_1 and t_2 dimension, respectively. Eight scans, preceded by two dummy scans, were recorded per t_1 value, and the total measuring time was 3.5 hr. Zero filling was used to yield a digital resolution of 4 Hz in both dimensions.

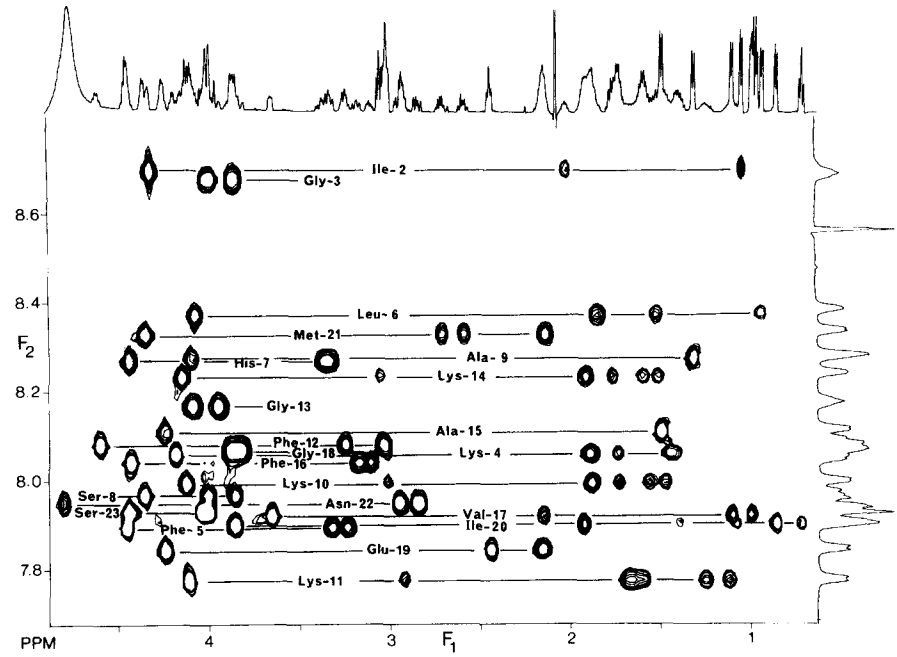

FIG. 4. Expansion of the boxed region of Fig. 3, showing the amide to side-chain connectivities. With the exceptions of Gly-1 and Ile-2, for each amide proton nearly all side-chain resonances connected directly or indirectly via scalar couplings to the C_α–H resonance are observed, despite relatively small NH–C_αH J couplings (α helix).

the exchangeable peptide protons. Under these conditions the peptide adopts an α-helical conformation,[27] and consequently the NH–C_αH J couplings are relatively small (4–5 Hz). Nevertheless, for the mixing time used in this experiment (79 msec) a large proportion of the amide magnetization has been transferred to the aliphatic protons. An expansion of the boxed region of Fig. 3 is shown in Fig. 4, showing the complete coupled spin systems of the side chains. For example, all four lysine side chains can be clearly identified, correlating the NH proton directly with the terminal $C_\varepsilon H_2$ protons. Similarly, the complete spin system of Ile-20 can be recognized immediately. Analogous connectivities for Ile-2 are considerably weaker because of the broadening of the NH resonance. No connectivities between the aromatic ring protons of the phenylalanine residues and the aliphatic protons are seen, although small couplings between C_δ and C_β protons have been reported for such residues. In contrast, the isolated C2H and C4H protons of the histidine residue show intense cor-

[26] M. Zasloff, *Proc. Natl. Acad. Sci. U.S.A.* **84**, 5449 (1987).

relations to each other and C4H shows a correlation to the C_β protons. In our experience, this latter connectivity is usually not observable in molecules significantly larger than the magainin peptide. Note also that the cross-peak for transfer from $C_\beta H_2$ (F_1) to C4H (F_2) is much stronger than the cross-peak corresponding to transfer in the opposite direction. This is caused by too short a delay time between scans: by waiting insufficiently long the slowly relaxing C4H protons have less magnetization available for transfer to the $C_\beta H_2$ protons than vice versa. This type of asymmetry artifact caused by too-short delay times can be seen in many types of 2D experiments.

Some distortion of the 2D spectrum of Fig. 3 in the region of the residual water is seen. This residual water always tends to be a much larger problem in HOHAHA experiments in comparison with, for example, NOESY or COSY methods. The reason lies in the relatively long trim pulses used. Water magnetization that is relatively far removed from the receiver coil and is present in a very poor homogeneous region of the magnetic field is excited by these long pulses, giving a significant response in the 2D spectrum. As discussed in the next section, an alternative method that does not use ^1H presaturation will automatically avoid excitation of this spurious H_2O signal.

Tracing Connectivities along the Aliphatic Side Chains

For proteins larger than 60 or 70 amino acids, the T_2 (T_{1_ρ}) values of the amide protons often become extremely short (<20 msec) and the long mixing periods needed for connecting the amide resonances with all side-chain resonances necessarily lead to very low signal to noise in the final spectrum. However, connectivities to the C_α and C_β protons often are visible. In this case it can be very useful to record a second HOHAHA spectrum in D_2O solution. Typically, magnetization propagates very rapidly along the aliphatic side chains, and a mixing time of 35–50 msec is often sufficient to observe all possible connectivities. As a result, the spectrum becomes very crowded; and to distinguish direct from indirect connectivities, it may be necessary to record HOHAHA spectra for at least two mixing times or to compare the spectrum with an absorption-mode COSY spectrum.

The T_2 values of the aliphatic resonances often are substantially longer than those of the amide protons and excellent spectra can be obtained for quite large proteins. Figure 5 shows the aromatic and aliphatic regions of staphylococcal nuclease, complexed with pdTp and calcium (18 kDa).

[27] D. Marion, M. Zasloff, and A. Bax, *FEBS. Lett.* **227**, 21 (1988).

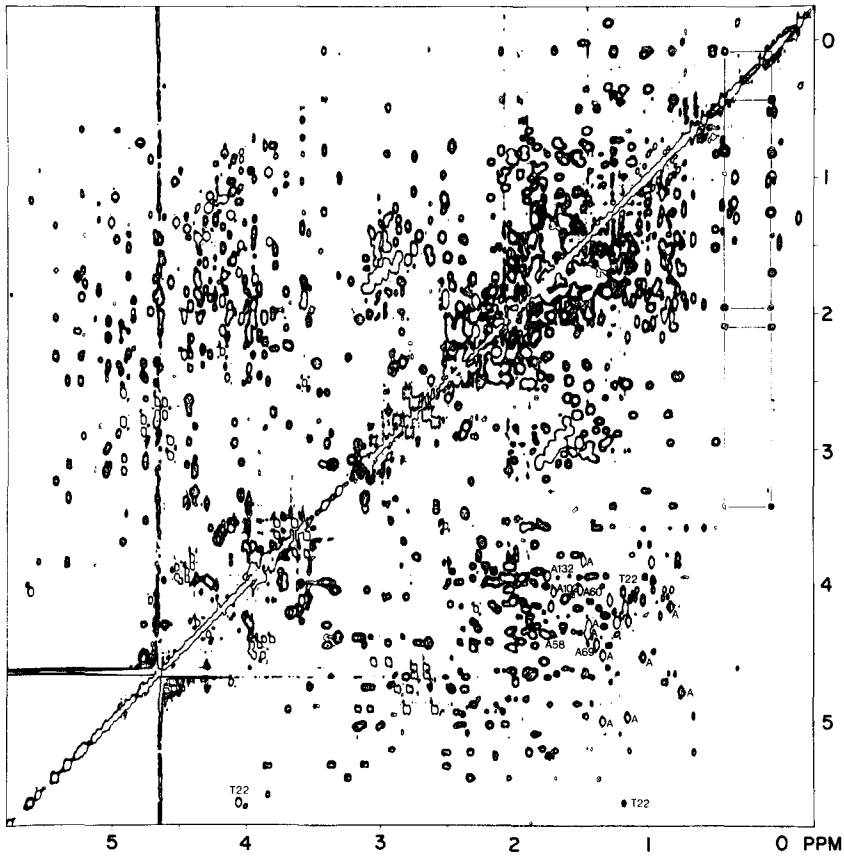

FIG. 5. Two-dimensional HOHAHA spectrum of the aliphatic region of a staphylococcal nuclease/pdTp/Ca^{2+} complex (18 kDa), 1.5 m*M* in D_2O, 100 m*M* NaCl, pD 7.4. The spectrum was recorded with a mixing time of 35 msec and the measuring time was 6 hr. Duration of the trim pulses was 1.5 msec and the 90° pulse width was 28 μsec. Labeled cross-peaks follow the assignments of Torchia *et al.*[28]

Some of the assignments, based on isotopic labeling studies,[28] are indicated in the figure. The phase distortion observed for the intense resonance of the residual HDO resonance is largely due to the finite duration of the trim pulses. As mentioned earlier, coadding data recorded with two different durations of the trim pulses can remove this type of artifact.

The relative intensities of resonances from nonmobile methylene resonances can become very low if the strongest resolution enhancement is

[28] D. A. Torchia, S. W. Sparks, and A. Bax, *Biochemistry* **27**, 5135 (1988).

used. For this reason it may be useful to process the data twice, using different line-broadening functions. The same, of course, is also true for processing the data from NOESY and COSY spectra.

Recording HOHAHA Spectra without Presaturation

As mentioned above, presaturation of H_2O when recording HOHAHA spectra can lead to serious problems caused by water from outside the receiver coil. Moreover, presaturation obliterates a band of C_α protons resonating close to the H_2O resonance. In addition, when working at or near physiological pH, the amide exchange rates for many of the residues are such that H_2O presaturation also saturates the amide resonances, which in turn may partially saturate other protons that have a strong NOE interaction with these amide protons. For these reasons, it may be better to record the HOHAHA spectrum without presaturation, by using one of the nonexcitation water suppression schemes. One such scheme[29] has been demonstrated to function well for the HOHAHA method. On our Bruker AM600 spectrometer we prefer to use a slight variation of this method which has a more favorable excitation profile. Its pulse sequence is sketched in Fig. 6. Essentially, this is the same sequence as the one of Fig. 1e, with the following minor modifications: After the mixing sequence, the spin-locked magnetization is stored along the z axis by means of the 90_ψ flip-back pulse. Subsequently, the z magnetization is "read" by a conventional 1–1 "jump-and-return"[30] sequence. One has to prevent the water resonance from getting inverted, which would lead to severe radiation damping problems. Therefore, the first excitation pulse is cycled only in a two-step (not the regular four-step) fashion. Below, the procedure typically followed during the setup of this experiment is described briefly. First, the 90° pulse is determined, simply by taking one-half the pulse width of a 180° pulse or one-fourth the width of a 360° pulse. Next, the scheme of Fig. 6 is executed with the pulse widths of the final 1–1 sequence set to zero. The width of the flip-back pulse is then finely adjusted to minimize the amount of H_2O signal. The 1–1 sequence is then reinstated and the receiver gain is adjusted to avoid overload.

Typically at 500 or 600 MHz, a 90° pulse width of 30 or 25 μsec will be optimal for coverage of a 10-ppm spectral width. More power will result in too much sample heating, causing lock problems and t_1 noise. Also, to minimize the effect of unavoidable rf heating, the sample should be

[29] A. Bax, V. Sklenář, A. M. Gronenborn, and G. M. Clore, *J. Am. Chem. Soc.* **109**, 6511 (1987).

[30] P. Plateau and M. Guéron, *J. Am. Chem. Soc.* **104**, 7310 (1982).

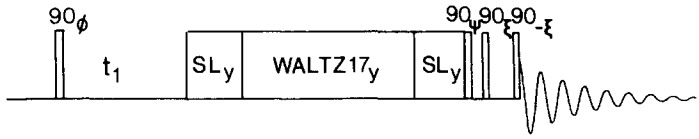

FIG. 6. Pulse scheme of the HOHAHA experiment for recording spectra in H_2O solution without the use of presaturation. This scheme has additional pulses at the end of the mixing period (compare to Fig. 1e). The 90_ψ pulse flips the spin-locked magnetization back to the z axis. An additional saturation pulse (not shown) may be applied at the end of data acquisition.[29] Phase cycling used on our Bruker AM-600 spectrometer is as follows: $\phi = -45°, 135°$; $\psi = -x, x; \xi = x, x, y, y, -x, -x, -y, -y$; Acq $= x, x, y, y, -x, -x, -y, -y$. On successive t_1 increments the phase ϕ is incremented (from odd to even numbered spectra) or decremented by 90° (from even to odd). This procedure prevents the water resonance from getting inverted and still permits the suppression of axial peaks and the recording of 2D quadrature spectra. Similar procedures can be used for recording the data in the hypercomplex format.[29]

brought to a steady state by starting the 2D experiment for about 5 min, immediately followed by a restart of the real experiment.

The procedure described above is demonstrated for a sample of 4.5 m*M* hen egg white lysozyme (14 kDa), pH 4.2, 36°, 80 m*M* NaCl. The fingerprint region of the HOHAHA spectrum, obtained with a 36-msec mixing time is shown in Fig. 7. Complete assignments for this protein, based on very careful double-quantum-filtered COSY and RELAY experiments, were given recently by Redfield and Dobson.[31] Comparison of their double-quantum-filtered COSY spectrum with the spectrum of Fig. 7 indicates that the resolutions in the fingerprint regions of the two spectra are quite similar, despite the 20% higher field strength used for the HOHAHA experiment. However, the lowest contour level in the HOHAHA spectrum is far above the thermal noise level, so much stronger resolution enhancement and higher digital resolution probably could be used to further enhance spectral resolution. Baseline correction of the final 2D spectrum was not used but would enhance the appearance of the spectrum significantly, particularly in the lower right-hand corner of the spectrum closest to the H_2O resonance.

Because of the relatively short mixing period used (36 msec), the amount of relay observed in the spectrum of Fig. 7 is relatively limited. However, NH cross-peaks to both the $C_\alpha H$ and the $C_\beta H$ resonances are present for most residues. For Val-2, the entire spin system is clearly visible. The amount of relay is significantly larger than observed for other Val residues, perhaps because this amino acid at the N-terminus may have increased mobility of the side chain. NH–$C_\beta H_3$ connectivities for

[31] C. Redfield and C. M. Dobson, *Biochemistry* **27**, 122 (1988).

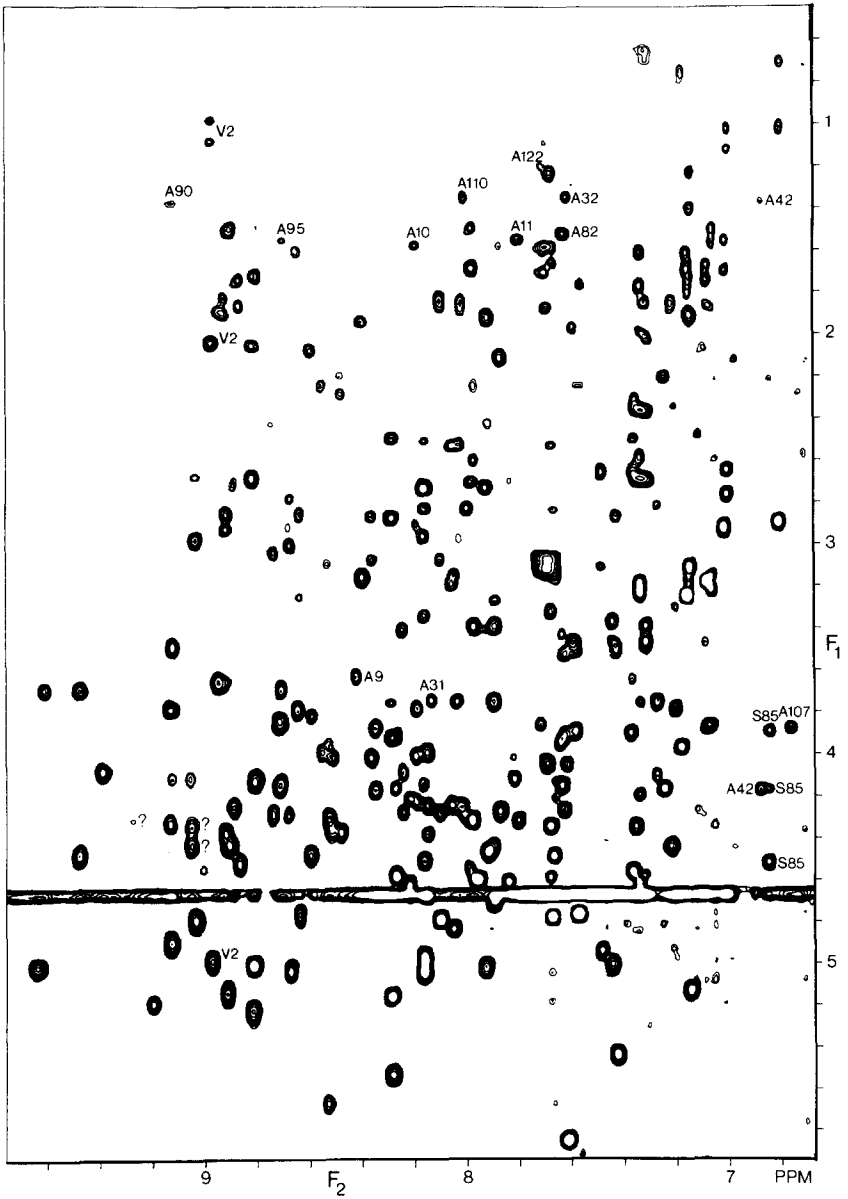

FIG. 7. Two-dimensional HOHAHA spectrum of hen egg white lysozyme, 4.5 m*M*, pH 4.2, 75 m*M* NaCl, 35°. The spectrum was recorded with the scheme of Fig. 6, using a mixing time of 35 msec, 24-μsec 90° pulse width, 800 × 2048 data matrix, acquisition times of 40 and 102 msec in t_1 and t_2 dimension, respectively. Sixteen scans were recorded per t_1 value and the total measuring time was 7 hr. Assignments are taken from Redfield and Dobson.[31] No baseline correction procedure of the frequency domain spectra was used and the lowest

the alanine residues are labeled in Fig. 1. At the contour level shown, these relay connectivities are absent for Ala-9, Ala-31, and Ala-107. At lower contour levels these connectivities are also observed, but it is not clear why the relay intensities would be weaker for these residues, considering that the coupling between C_α and C_β protons is always 7 Hz. Three unidentified cross-peaks, not present in the COSY spectrum of Ref. 30 (near 9.1/4.4 ppm), are marked "?."

Discussion

Appropriate hardware is required for the recording of high-quality HOHAHA spectra. First, an rf amplifier at ^1H frequencies is needed that can produce the power needed for a 25- to 35-μsec 90° pulse (typically 2–5 W) and that can produce this amount of rf power without any droop for at least 50 msec. Attenuating the high "observe pulse power" is usually not an adequate solution and may result in burned attenuators or in a collapse of the power supply of the amplifier. A linear or near-linear amplifier as is often used for ^1H decoupling is better suited for the HOHAHA irradiation. To keep the complexity of the 2D experiment to a minimum, we use the same power for pulses, trim pulses, and mixing sequences. A slight loss in sensitivity and a small linear phase error in the F_1 dimension resulting from rf offset effects at these medium levels of rf power cause no major problems in practice. If more effective mixing sequences are developed in the future, it may become necessary to switch between high-power pulses and low-power mixing for their application, as has been proposed for the ROESY experiment.[32] At present, there appears no need for such power switching.

If one wants to use the ^1H decoupler amplifier for generating rf pulses, the decoupler rf must be phase coherent with the receiver. Although in principle this latter requirement can be avoided by using an interesting z-filtered version of the experiment,[33] in practice this approach causes extra t_1 noise and with it we have been unable to generate spectra of a quality similar to that obtained with the sequence described in this chapter.

[32] C. Griesinger and R. R. Ernst, *J. Magn. Reson.* **75,** 261 (1987).
[33] M. Rance, *J. Magn. Reson.* **74,** 557 (1987).

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contour level is far above the thermal noise, but close to the baseline distortions caused by incomplete water suppression and by frequency-dependent phase correction used in the F_1 dimension. Three of the 12 NH–$C_\beta H_3$ relay connectivities for Ala residues (A31, A9, A107) are not observed at this contour level, despite intense NH–C_αH cross-peaks. Resonances marked "?" were not observed in the COSY spectrum.

Of the various mixing schemes discussed here, the MLEV17 and WALTZ17 schemes appear to be most suitable for protein studies. All of the earlier schemes (Fig. 1a–c) give strong additional NOE cross-peaks which can be very confusing and which also can decrease the sensitivity of the experiment significantly in cases where NOE and HOHAHA effects compete. Comparison of the MLEV17 and WALTZ17 schemes reveals a few differences. From a practical point of view, the MLEV17 mixing scheme appears to be less sensitive to imperfections in the phase-shifting hardware. However, the band width that can be covered with MLEV17 for a given amount of rf power is about 25% smaller. WALTZ17 mixing has the intrinsic disadvantage that the effective size of the coupling during mixing (and thus the transfer rate) is reduced more than it is for the MLEV sequence. For offsets used in practice (smaller than $\nu_{rf}/2$), this effect is small. The maximal band width covered by the WALTZ17 sequence is about $\pm 0.4\nu_{rf}$ and for MLEV17, about $\pm 0.33\nu_{rf}$. For most diamagnetic proteins this allows effective cross-polarization over the entire spectral range with only a few watts of rf power. Overall, if spectrometer hardware is sufficiently good, the WALTZ sequence appears preferable over the MLEV mixing scheme.

Two-dimensional HOHAHA experiments are particularly useful for delineating individual spin systems in proteins. The method provides high-sensitivity, high-resolution spectra that contain a wealth of relayed connectivity information. There are two reasons why HOHAHA spectroscopy may be more sensitive than conventional COSY-type experiments. First, because of the in-phase nature of the transfer of magnetization, cancellation of absorptive components within a cross-multiplet does not occur. Second, no magnetization is lost to the generation of spurious multiple-quantum coherence, a major source of sensitivity loss in conventional RELAY experiments. Resolution in the fingerprint region of double-quantum-filtered COSY and HOHAHA spectra is quite similar; although in our experience, the sensitivity of the HOHAHA spectra is typically much higher. For studying spectral regions close to the diagonal, the HOHAHA spectrum usually offers the best appearance because of the relatively low intensity of the diagonal.

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