JOURNAL OF MAGNETIC RESONANCE 88, 627–635 (1990)

A Three-Dimensional Heteronuclear Multiple-Quantum Coherence Homonuclear Hartmann–Hahn NOESY Experiment

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Received February 13, 1990

The main advantage of 3D over 2D NMR experiments lies in their potential to remove overlap of resonances, as has been demonstrated in a number of published homonuclear (*1–5*) and heteronuclear (*6–10*) 3D NMR experiments. Heteronuclei such as ^{15}N and ^{13}C have a larger chemical-shift dispersion than ^{1}H. Consequently, hetero 3D NMR experiments, involving ^{15}N and ^{13}C, have the additional advantage over 3D homonuclear ^{1}H NMR experiments of improved spectral resolution. Recently, we have demonstrated the feasibility of a 3D heteronuclear multiple-quantum HOHAHA experiment (HMQC-HOHAHA) (*11*). The experiment was performed on a mixture of uniformly ^{13}C-enriched α- and β-D-glucose. We discussed the usefulness of such experiments for the analysis of polysaccharides as well as nucleic acids. Because of the presence of relay peaks in a HOHAHA experiment, 3D HMQC-HOHAHA is ideally suited for assignment purposes when strong overlap of resonances is present. However, for a full analysis one also needs spatial information, i.e., NOE information, which can be obtained, for example, from a 3D NOESY-HMQC experiment. Such 3D NOESY-HMQC experiments have been carried out successfully on ^{15}N-enriched proteins (*8–10*). For a complete analysis, therefore, two heteronuclear 3D experiments are needed, such as a 3D HMQC-HOHAHA and a 3D HMQC-NOESY. The first provides the *J*-coupling information and the second the spatial information.

In this communication we present a new heteronuclear 3D NMR experiment in which both *J*-coupling information (via spin locking) and NOE information are present (*12*). In this 3D NMR experiment the 3D HMQC-HOHAHA pulse sequence (*11*) is combined with a 2D NOESY experiment. Instead of placing the second evolution time (t_2) between the HMQC part of the pulse sequence and the spin-lock mixing period, as is done in the 3D HMQC-HOHAHA experiment (*11*), in this experiment the second evolution time (t_2) is placed directly after the spin-lock mixing period. The evolution time (t_2), in turn, is followed by a NOESY mixing time, while, finally, during t_3 acquisition is done. We call this experiment a 3D HMQC(HOHAHA)-NOESY, where the parentheses indicate the absence of an evolution period between the HMQC and HOHAHA. To test this pulse sequence, a mixture of α- and β-D-glucose was used, with both α-D-glucose and β-D-glucose being ^{13}C-enriched only in the C1 position. In this Communication we analyze this 3D experiment and present

0022-2364/90 $3.00

in detail the results of the application of this pulse sequence to this sample. Finally, we discuss the potential usefulness of this and similar experiments for resonance assignment and structure determination of oligosaccharides, nucleic acids, and proteins in comparison with other related 3D experiments.

The complete pulse sequence of the 3D HMQC(HOHAHA)-NOESY is shown in Fig. 1A. As can be seen from this figure, the heteronucleus is decoupled during all of the pulse sequence, except during the HMQC part, to enhance sensitivity. We did not use extensive phase cycling; only the signals stemming from protons bonded to the ^{12}C were suppressed (*11*). It is our experience that additional phase cycling of the proton pulses (e.g., Cyclops) leads to poorer suppression of these signals. TPPI was applied to achieve quadrature detection in both t_1 and t_2 directions. In the 3D NMR spectrum, absorptive lineshapes in all three directions can thus be obtained after 3D Fourier transformation.

The 3D NMR experiment was carried out on a Bruker AM 400 spectrometer equipped with a 5 mm QNP probe. Decoupling of the ^{13}C nucleus was accomplished with an external WALTZ decoupler. The use of selectively enriched glucose (vide supra) in the 3D HMQC(HOHAHA)-NOESY experiment mimics a similar 3D experiment in which, by selective excitation, only the $^{13}C1$ resonances of α- and β-D-glucose are excited. Because in the f_1 (^{13}C) direction only the spectral region of $^{13}C1$ resonances is excited, the spectral width in f_1 can be kept relatively small. A value of 1000 Hz was used to cover the spectral range of the $^{13}C1\alpha$ and $^{13}C1\beta$ resonances. The number of t_1 increments can consequently also be kept quite small (16 steps were taken), thereby reducing the measuring time considerably, while one still obtains sufficient resolution in f_1. For f_2 and f_3 the spectral widths were set to 1000 Hz to cover the spectral range of all sugar protons H1 to H6. For each of the 16 t_1 values, FIDs were measured for 108 different t_2 values. Each FID of 512 data points (t_3) was the accumulation of 48 scans with each scan being preceded by 4 dummy scans to establish steady state. To produce the multiple coherences in the HMQC sequence, the waiting period Δ was taken to be 3.0 ms, which is approximately equal to $1/2J$, with J being the direct ^1H–^{13}C coupling constant. The spin-lock mixing period length was set to a relatively large value of 100 ms in order to achieve coherence relay from H1 throughout the J-coupled spin system. Since glucose is a small molecule with $\omega\tau_c \ll 1$, NOE effects are negative and relatively small. To obtain sufficient mixing the NOE mixing time was set to 1 s. The 3D spectrum was obtained from the 3D data set by Fourier transformation in all three directions on an ASPECT 3000 computer. In the t_1 and t_2 directions, the FIDs were zero-filled to 128 and 256 data points, respectively. In t_3, only the first 256 data points of the 512 measured data points were used. In all three directions the same squared cosine window function was applied. Finally, after phase correction to pure absorption, only the real points were retained, giving a 3D NMR spectrum of $64 \times 128 \times 128$ data points.

To analyze 3D NMR experiments we feel a scheme as presented in Fig. 2 is most useful. This method basically shows how to follow in a concise manner the coherence and/or magnetization transfer during the 3D pulse sequence. We apply it here to the 3D HMQC(HOHAHA)-NOESY experiment. In Fig. 2 we show, schematically, the cross peaks that may appear in a plane perpendicular to the f_1 axis (the ^{13}C axis) for the sugar moiety incorporated in this figure. The plane is assumed to cross the ^{13}C

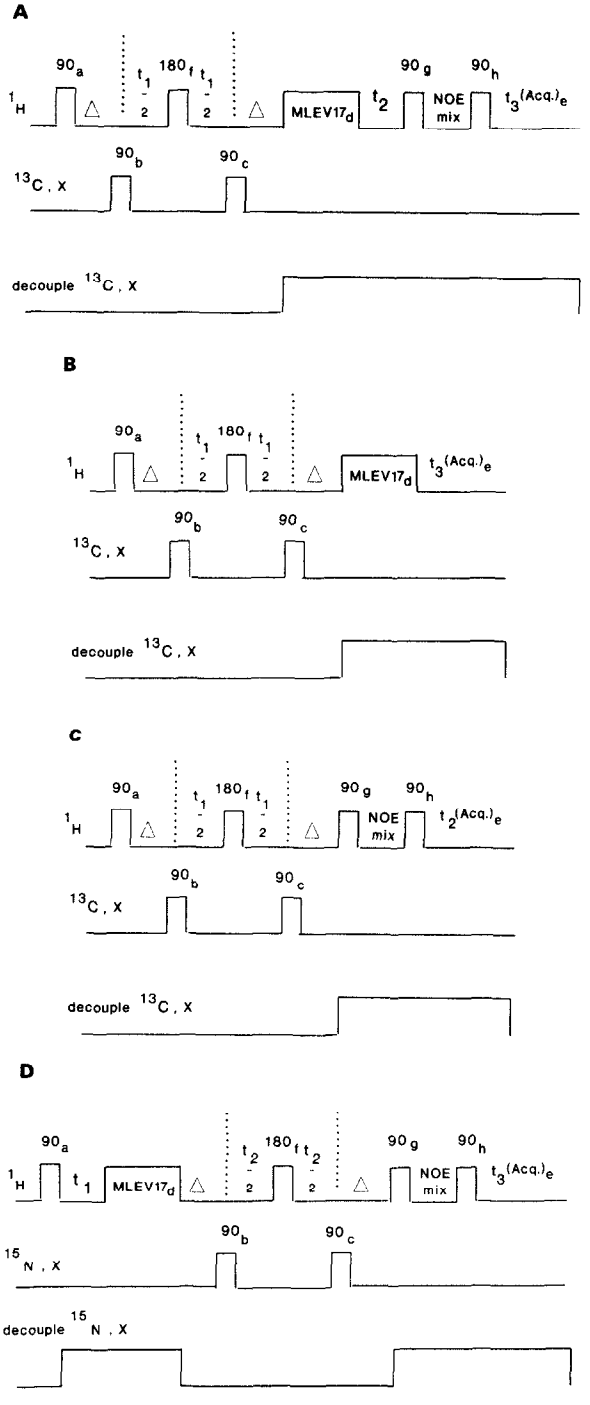

FIG. 1. Pulse sequence for 3D HMQC(HOHAHA)-NOESY (A), 2D HMQC(HOHAHA) (B), 2D HMQC(NOESY) (C), and 3D HOHAHA-HMQC(NOESY) (D). The phase cycling used is as follows: (A) a = x(+TPPI(t_2)); b = (x, $-x$, x, $-x$)(+TPPI(t_1)); f = x(+TPPI (t_2)); c = x, x, $-x$, $-x$; d = y(+TPPI (t_2)); g = x; h = x; acquisition e = x, $-x$, $-x$, x. (B) a = x; b = (x, $-x$, x, $-x$)(+TPPI(t_1)); f = x; c = x, x, $-x$, $-x$; d = y; acquisition e = x, $-x$, $-x$, x. (C) a = x; b = (x, $-x$, x, $-x$)(+TPPI(t_1)); f = x; c = x, x, $-x$, $-x$; g = x; h = x; acquisition e = x, $-x$, $-x$, x. (D) a = x(+TPPI(t_1)); d = y; b = (x, $-x$, x, $-x$)(TPPI(t_2)); f = x; c = x, x, $-x$, $-x$; g = x; h = x; acquisition e = x, $-x$, $-x$, x. TPPI(t_1) stands for TPPI in the t_1 direction.

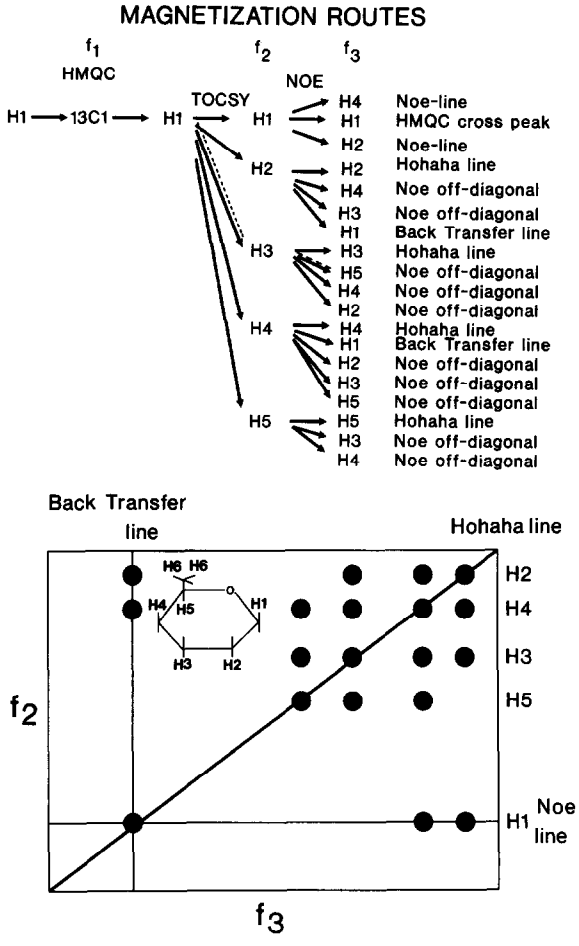

FIG. 2. Magnetization transfer scheme of the 3D HMQC(HOHAHA)-NOESY experiment for the sugar moiety shown in the lower part of the figure. The resulting cross peaks, as they may appear in the plane perpendicular to the f_1 axis (hetero axis), are also shown. The plane is assumed to dissect the f_1 axis at the ^{13}C1 resonance frequency. The H6 cross peaks are not shown. The terms TOCSY and HOHAHA are used interchangeably.

frequency axis at the C1 resonance position. As follows from the transfer scheme in the upper part of Fig. 2, ^{1}H coherence is first transferred from H1 to ^{13}C1; then frequency labeling occurs in the f_1 direction after which coherence is transferred back to H1, which has then become labeled with the ^{13}C frequency. Subsequently, coherence is transferred during the spin-lock period to other J-coupled protons labeling them with the ^{13}C frequency as well. Then the protons are labeled with their own Larmor frequencies during t_2. In the NOE mixing time, magnetization may be transferred further through NOE contacts, which adds, during the acquisition period, the Larmor frequency of the last proton in the pathway to the resultant magnetization. Using this scheme it can easily be shown that, in the planes perpendicular to f_1, intensity will fall on the diagonal, either if no transfer of magnetization takes place at all

(this intensity is designated the HMQC cross peak; in Fig. 2 this is the cross peak situated at the point where the HOHAHA line, the NOE line, and the back-transfer line cross; vide infra) or if transfer occurs only during the spin-lock mixing period. Because of the last condition the diagonal may be called the HOHAHA line.

Off-diagonal cross peaks evolve due to magnetization transfer during the NOE mixing time. This may be gleaned from the scheme in Fig. 2 as well. In the special case that transfer occurs only during the NOE mixing time the magnetization ends up on a line parallel to f_3, which may thus be called the NOE line. The line parallel to f_2 through the HMQC cross peak stems from the magnetization that is first transferred from H1, during the spin-lock period, to some other proton, and then during the NOE mixing time is transferred back to H1. This line is thus designated the back-transfer line. The other off-diagonal cross peaks are due to magnetization that is first transferred during the spin-lock period and is then relayed further in the NOE mixing period. For example, the NOE cross peak between H3 and H5 stems from the following route: the H1 magnetization is transferred to C1, so that during f_1 it is labeled with the C1 frequency; it is then transferred back to H1α and during the spin-lock period transferred to H3. During t_2 it is then frequency labeled as H3 and subsequently is transferred to H5 in the NOE mixing period, so that during t_3 this magnetization obtains the H5 frequency label. In Fig. 2 this particular pathway is indicated by the dashed lines. Each cross peak can be identified quickly using the shorthand notation given in Fig. 2.

Finally, we note that in case NOE cross peaks have a diagonal neighbor, these peaks refer to NOEs between *J*-coupled protons. If, however, the NOE is to a proton that does not belong to the set of *J*-coupled protons, a cross peak occurs that has no counterpart on the diagonal. Consider, for instance, a sequence of three sugar rings connected via the glycosidic C(1, 4) bond as depicted in Fig. 3. An NOE contact is expected between the H1 of one ring and the H4 resonance of the next ring across the glycosidic C(1, 4) bond. As long as the C1 resonances and the ^{1}H1 of the different rings do not coincide, one can readily identify the ^{1}H resonance positions of the protons of a particular ring from a plane perpendicular to f_1 through the corresponding ^{13}C1 resonance. As indicated in Fig. 3, a nonmatching NOE from H4 identifies H1 of the previous sugar ring. From the NOEs between the *J*-coupled spins within the ring, one can characterize the ring structure.

Generally, it is considered that the advantage of 3D experiments lies in the removal of overlap. If overlap is still present in the 3D experiment, relay peaks due to the HOHAHA part of the sequence may further help to distinguish between different spin systems. Herein lies the relative advantage of a HOHAHA over a COSY experiment. Because it is difficult to give general guidelines as to when overlap problems may be resolved by using certain 3D experiments, we have considered in Fig. 3 the special situation of an oligosaccharide in which only the H1 and ^{13}C1 resonances do not overlap, while all other resonances do; i.e., all H2 to H5 as well as all ^{13}C2 to ^{13}C5 resonances of the three-ring system mutually overlap. In such a case, as can be seen from Fig. 3, the HMQC(HOHAHA)-NOESY experiment can still provide all the assignments and the structural information for the individual rings. No combination of 2D experiments can provide all the assignment and structural information. Interestingly enough, two separate 3D experiments, a 3D HMQC-HOHAHA for the as-

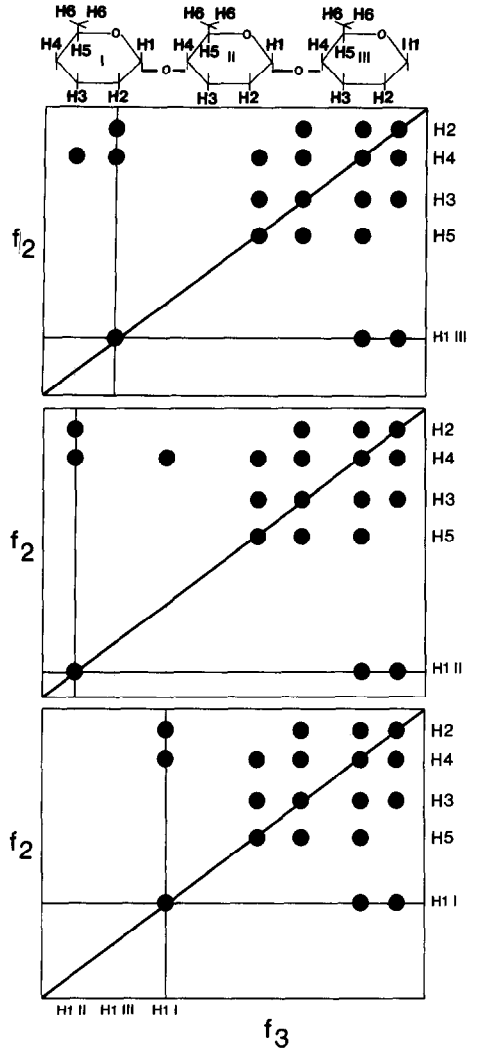

FIG. 3. The expected cross-peak patterns in a 3D HMQC(HOHAHA)-NOESY spectrum of the trisaccharide shown. From top to bottom three planes are shown, which were taken perpendicular to the f_1 axis (hetero axis). They are assumed to dissect the f_1 axis at the $^{13}C1$ resonance frequency of the sugar moieties I, II, and III, respectively.

signment and a 3D HMQC-NOESY for the structural information, do not make it possible to identify the different ring systems. Only through the combined use of the 3D HMQC(HOHAHA)-NOESY can such an identification be done.

To test the feasibility of the proposed experiment and to get a feeling for the required length of the spin-lock time and the NOE mixing time we first performed, on the test sample, two separate 2D experiments, namely a HMQC(HOHAHA) and a HMQC(NOESY) experiment, which in fact compose the 3D HMQC(HOHAHA)-NOESY experiment. The pulse sequences of these experiments are given in Figs. 1B and 1C, respectively. Here only the results of the HMQC(HOHAHA) experiment

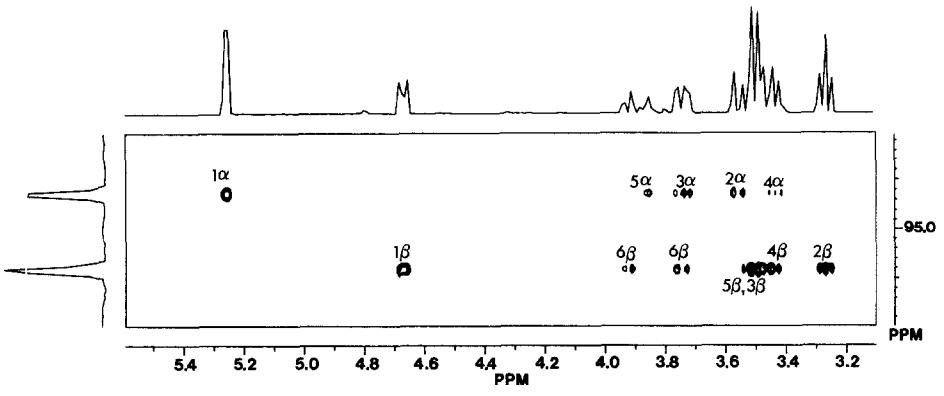

FIG. 4. 2D HMQC(HOHAHA) spectrum of ^{13}C1-enriched α- and β-D-glucose. Spin-lock mixing time was 100 ms. The numbers attached to the cross peaks refer to the sugar protons as sketched in Fig. 5.

are given. It can be seen in Fig. 4 that, for a mixing time of 100 ms and starting from the H1α and H1β cross peaks, sufficient coherence transfer takes place through the sugar spin system to generate cross peaks even at the H5 position of α-D-glucose and at the H6 position of β-D-glucose, respectively. In a similar way the NOE mixing time was tested.

In the 3D spectrum, obtained by application of the HMQC(HOHAHA)-NOESY sequence to the test sample, the cross-peak intensity accumulates in two planes perpendicular to the ^{13}C frequency axis (f_1). One set of cross peaks is located at the C1α resonance frequency, and the other set at the C1β resonance frequency. Detailed plots of two planes are given in Figs. 5A and 5B, representing the cross sections through the 13Cα and 13Cβ resonance position, respectively. Each plane has a diagonal on which the spin-lock peaks are located, while off the diagonal one finds cross peaks due to NOE transfer (vide supra).

For α- and β-D-glucose, it can be seen in Figs. 5A and 5B that via the NOE contacts the sugar system is easily identified and characterized. For example, H3 and H5 have an NOE contact as expected from the structure (Fig. 5A). Furthermore, H1α has an intense NOE contact with H2α, while this is not the case for H1β and H2β, because H1α is in an equatorial and H1β is in an axial position. Consistent with the structure of β-D-glucose, H1β shows a strong cross peak to the overlapping resonance positions of H3β and H5β. Also, other NOE contacts conform to the structure; e.g., in both α- and β-D-glucose H2 shows NOE cross peaks to H3 and to H4 as expected. Starting from H1α and H1β, it is seen that sufficient coherence transfer takes place through the sugar spin system during the spin-lock mixing time to observe on the diagonal cross peaks at the H5α resonance position and the H6β resonance position, respectively (see Fig. 4). This difference between α-D-glucose and β-D-glucose is not very surprising if one realizes that the coupling constant between H1β and H2β is much larger than that between H1α and H2α. As is thus clearly shown this type of 3D experiment is feasible and provides both coupling (assignment) information and spatial information, which for this class of sugars can be used for assignment purposes as well.

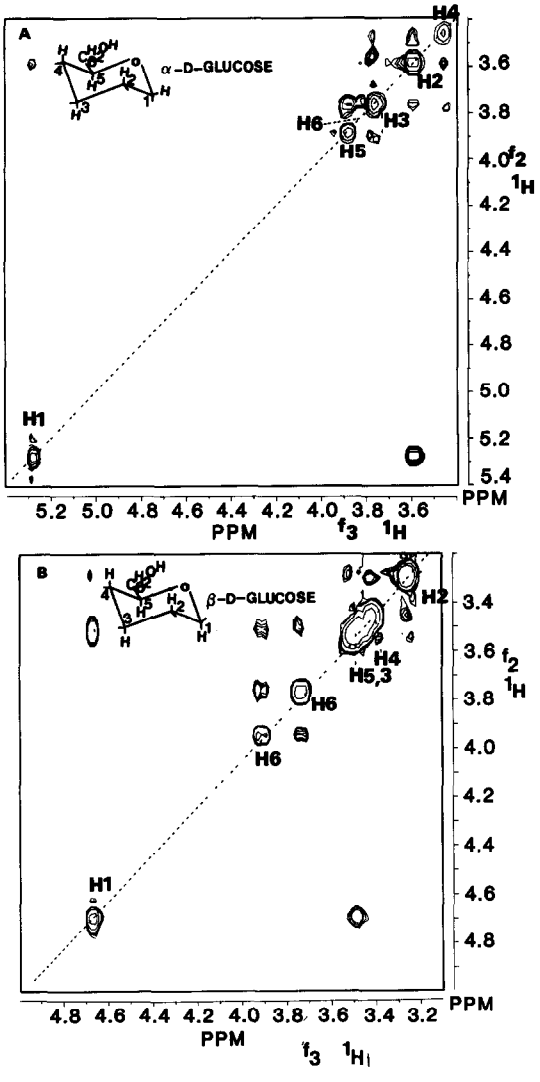

FIG. 5. Cross sections perpendicular to the f_1 axis (^{13}C axis) of the 3D HMQC(HOHAHA)-NOESY spectrum of $^{13}C1$-enriched α- and β-D-glucose. (A) Cross section through $^{13}C1\alpha$ resonance frequency. (B) Cross section through $^{13}C1\beta$ resonance frequency. Capital H's refer to the protons in the sugar ring. In A the resonance position of the H6 is indicated but the HOHAHA cross peak cannot be observed (see also Fig. 4 and text).

We expect that the present experiment may also be quite useful for the study of nucleic acids, where severe overlap often occurs in the region of H4′, H5″, and H5′ protons. For the study of ^{15}N-enriched proteins the combination of a HMQC pulse sequence with a NOESY pulse sequence has already proven to lead to a powerful 3D experiment (*7–10*). As has been pointed out, one needs for the resonance assignment apart from the NOE information *J*-coupling information as well. The latter can be obtained from a separate 3D HMQC-HOHAHA (or HOHAHA-HMQC) experi-

ment. Combination of HMQC-HOHAHA and HMQC-NOESY, as we suggest (Fig. 1A), will provide both the *J*-coupling information and the spatial information in one heteronuclear 3D experiment. To permit the observation of NOEs to $C_\alpha H$ resonances close to the H_2O resonance, it might be better (^{15}N-enriched proteins dissolved in H_2O) to utilize a slightly different pulse scheme in which the HOHAHA pulse sequence is placed in front of the HMQC sequence and the NOE mixing time after the HMQC sequence, resulting in the pulse sequence of Fig. 1D. In that case the f_2 axis becomes the hetero axis. Analysis of the magnetization transfer routes, with a scheme similar to the one used in Fig. 2, shows that in the planes perpendicular to the f_2 axis, one finds the spin-lock pattern of the individual amino acids parallel to the f_1 axis (the HOHAHA line). Parallel to the f_3 axis, through the HMQC cross peak, lies the NOE line, while the diagonal is the back-transfer line. It is readily shown using the magnetization transfer route scheme that the amino acid spin-lock pattern repeats itself for each NOE contact of the NH proton. In fact, this is the same type of cross-peak pattern found in a plane perpendicular to f_2 in a 3D homonuclear ^1H TOCSY-NOESY experiment (*13*).

ACKNOWLEDGMENTS

This work was supported by the Dutch Foundation for Chemical Research (SON). Spectra were recorded at Dutch National HF-NMR Facility (Nijmegen, The Netherlands) supported by SON. We thank Ing. J. van Os and Ing. J. J. Joordens for technical assistance.

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