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A ^{13}C double-filtered NOESY with strongly reduced artefacts and improved sensitivity

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Summary

A ^{1}H NOESY experiment with two ^{13}C half-filters is described which has, compared to previously reported versions, an enhanced overall sensitivity and strongly reduced intramolecular cross peaks in any part of the spectrum edited for intermolecular NOEs. By adding a shaped ^{13}C pulse to the half-filter which selectively inverts the aromatic resonances, the filter can be tuned separately and simultaneously for the aliphatic and aromatic regions. Contrary to recently proposed schemes, no magnetization is destroyed, so that full sensitivity is retained for symmetric systems such as homodimers. Furthermore, by replacing the rectangular 180° ^{13}C pulses by high-power hyperbolic secant pulses for inversion of the complete ^{13}C spectral range, offset effects (which are another source of signal loss and artefacts) are eliminated. The spectra edited for intermolecular NOEs clearly demonstrate that residual artefacts are considerably smaller than in the original version of the experiment.

During the last few years, isotope-edited NMR experiments have been shown to be very useful for specific selection of intra- and intersubunit NOEs in macromolecular complexes in which one of the components has been uniformly labelled with a stable isotope (^{13}C, ^{15}N) (Folkers et al., 1993; Lee et al., 1994a; Zhang et al., 1994). A well-known experiment is the double X half-filtered 2D ^{1}H NOESY (Otting and Wüthrich, 1989). By choosing the appropriate linear combination of subspectra, for instance NOEs between an unlabelled protein and a labelled ligand can be selected, as was first demonstrated for the complex of cyclosporin A bound to cyclophilin (Fesik et al., 1991; Weber et al., 1991). More recently, this method has also been applied to observe intersubunit NOEs in homodimeric proteins (Burgering et al., 1993a; Folkers et al., 1993).

Like many other editing techniques, X half-filters rely on the efficiency of subtraction of unwanted signals. In this respect, an intrinsic difficulty is posed by the non-uniform distribution of $^{1}J_{^{1}H^{13}C}$ couplings, both in proteins and nucleic acids. The conventional ^{13}C half-filter is tuned to an average J-value, and problems arise from ^{1}H-^{13}C pairs with a J-coupling deviating from this mean value. As was pointed out earlier (Otting and Wüthrich, 1990; Gemmecker et al., 1992), this results in imperfect suppression of signals from ^{13}C-bound protons when selecting the ^{12}C-bound proton resonances. This 'leaking' ^{13}CH magnetization gives rise to unwanted cross peaks in each of the subspectra containing a ^{13}C-filtered dimension.

Several methods have been proposed addressing this problem. For instance, Ikura and Bax (1992) described an $[F_1,F_2]$ double ^{13}C-filtered 2D NOESY experiment using purge pulses (vide infra), in which the first filter (F_1) is optimized for the aliphatic region of the spectrum, while the second is matched for the aromatic region. Gemmecker and co-workers (Gemmecker et al., 1992), on the other hand, introduced a double filter that can be tuned to two different one-bond coupling constants prior to each of the two evolution times, using spin-lock pulses to eliminate the ^{13}C-bound proton magnetization. Contrary

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to the aforementioned approach, which optimizes the experiment only for one region of the 2D spectrum (namely, F_1/F_2 = aliphatic/aromatic), this method allows the same efficiency of filtering in each region (aromatic/aromatic, aliphatic/aliphatic, aromatic/aliphatic and aliphatic/aromatic) of the spectrum. This, however, occurs at the expense of two times two extra delays of length $(2J)^{-1}$ (a total of about 14 ms), which may be unacceptable for larger systems due to relaxation.

A fundamentally different approach for studying macromolecular complexes in which one of the components has been uniformly labelled has been proposed by Ikura and Bax (1992). This method involves the application of filter sequences using purge pulses, specifically optimized for full suppression of ^{13}C-bound magnetization. The desired spectrum is obtained by suppression of the unwanted magnetization in each of the filters, rather than by choosing a linear combination of subspectra (Otting and Wüthrich, 1989). Thus, for instance NOE contacts within an unlabelled subunit can be obtained in an $[F_1,F_2]$ double ^{13}C-filtered experiment. Recently, Bax et al. (1994) reported an improved sequence for this particular experiment, using heteronuclear Hartmann–Hahn dephasing to eliminate the signals from the enriched compound. Futhermore, Ikura and Bax (1992) state that, knowing the chemical shifts of the unlabelled subunit, the interaction between an unlabelled peptide and a ^{13}C-enriched protein can usually be studied in a regular 3D ^{13}C-edited NOESY, using the ^{13}C shifts of the protein to separate these interactions.

Contrary to the analysis of protein–ligand complexes, in symmetric systems such as homodimers and tetramers it is not possible to assign NOEs as being intra- or intersubunit solely on the basis of the chemical shifts. Instead, intersubunit NOEs can be obtained from an $[F_1,F_2]$ ^{13}C-selected ^{13}C-filtered 2D NOESY employing purge filters, similar to the scheme recently published by Lee et al. (1994b). However, in the case of a homodimer this would result in a twofold decrease in signal-to-noise ratio for intermolecular cross peaks in comparison with a nonfiltered analogue; in a regular NOESY, each intermolecular cross peak (ω_A,ω_B) has a 1:1 contribution from the $^{13}CH_A \rightarrow {}^{12}CH_B$ and $^{12}CH_A \rightarrow {}^{13}CH_B$ NOE pathways, but an $[F_1,F_2]$ ^{13}C-selected ^{13}C-filtered experiment would only allow the detection of the first of these contributions.

In this communication, we describe a double half-filtered NOESY experiment, in which each of the two half-filters can be tuned separately and simultaneously for the aromatic and aliphatic region of the spectrum, without destroying any ^{13}C-bound proton magnetization. Hence, full sensitivity for intersubunit NOEs in symmetric systems is retained. The approach will be demonstrated for a mutant ($Phe^{36} \rightarrow$ His) of the homodimeric single-stranded DNA-binding protein (ssDBP) of bacteriophage Pf3, which uses *Pseudomonas aeruginosa* as a host (Folmer et al., 1994). Pf3 ssDBP is a β-sheet protein (78 residues; the molecular mass of the dimer is 18 kDa), similar in function to the gene V protein encoded by filamentous phage M13 (Folkers et al., 1994).

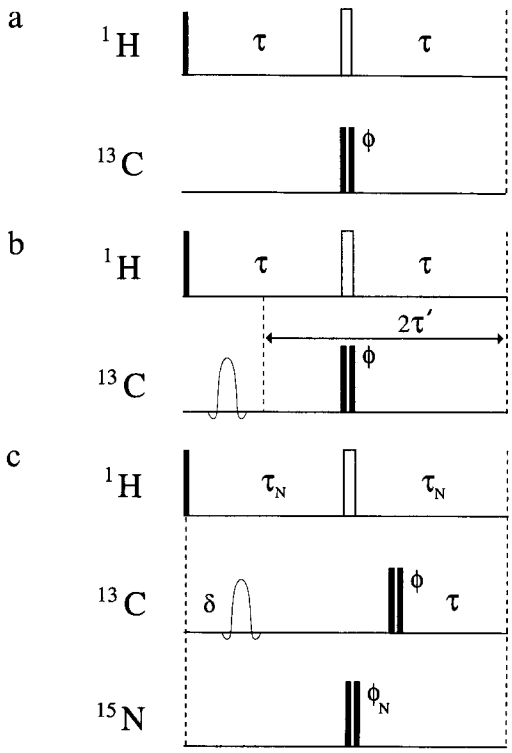

Fig. 1. Comparison of half-filter building blocks. Solid and open bars denote 90° and 180° pulses, respectively. All pulses are applied along the x-axis, except where phases ϕ and ϕ_N are indicated. These editing phases are alternated between x and −x, resulting in either an effective 180° pulse or an effectively omitted pulse. (a) Pulse scheme of the conventional half-filter. The value of τ is $(2\ {}^1J_{H,C})^{-1}$. (b) Scheme of the double-tuned half-filter. The selective pulse is applied at the aromatic region, only when ϕ = −x (indicated with dashes). The delay τ is optimized for the aliphatic region, while τ′ is tuned to the aromatic J-couplings. (c) Scheme of the ^{13}C double-tuned, $[^{13}C,^{15}N]$ time-shared half-filter. The delay τ_N is $(2\ {}^1J_{H,N})^{-1}$, and δ is (τ − τ′). Delays τ and τ′ are identical to those in (b).

Figure 1a depicts the pulse scheme of the conventional half-filter (Otting et al., 1986). The delay τ is tuned to $(2\ {}^1J_{H,C})^{-1}$ for optimal rephasing of proton in-phase magnetization at the end of the two delays. Because in proteins the heteronuclear ^{13}C-^1H one-bond couplings range from ~125 Hz for methyl groups to ~170 Hz for aromatic spin pairs (and even up to 220 Hz in histidine residues), the filter delay has to be matched to a mean value. For protons attached to ^{12}C the length of the delay is obviously irrelevant, as only their chemical shifts, which are refocussed by the 180° proton pulse, will evolve during the filter period. Refocussing of the ^{13}C-bound protons is also independent of the delay when the 180° (^{13}C) editing pulse is effectively not applied, because the ^{13}C-^1H couplings are refocussed by the 180° proton pulse as well. In the presence of the 180° carbon pulse, however, imperfect refocussing occurs if the one-bond coupling deviates from the

value for which the delay has been matched. Ignoring multiple-bond couplings, the product operator description (Sørensen et al., 1983; Van de Ven and Hilbers, 1983) of ^{13}C-bound proton magnetization at the end of the half-filter is

$$I_y \cos(2\pi\tau J) + 2I_xS_z \sin(2\pi\tau J)$$

in which J is the one-bond coupling in a given ^{13}C^{1}H spin pair. Therefore, as soon as this coupling constant deviates from the mean value, subtraction of the two recordings with and without the editing pulse no longer leads to perfect cancellation of the ^{13}C-bound protons. In practice, this may result in the observation of ^{13}CH-^{13}CH NOEs in subspectra in which, for instance, only ^{12}CH-^{13}CH NOEs should appear. To quantify this effect, we choose as an example a filter delay τ of 3.45 ms. This corresponds to a J-coupling of 145 Hz, which is an appropriate mean value if one would be interested in both the methyl and aromatic resonances. The in-phase component of a ^{13}C-bound methyl proton with J = 125 Hz at the end of the half-filter is then only $\cos(2\pi\tau J) = 0.91$, which is equivalent to a 9% difference in intensity between the two recordings with and without the editing pulse. This may seem a relatively small amount, but a 9% leakage of a very strong NOE cross peak is likely to be significant. For this reason, a 20 Hz deviation for the J-coupling with respect to the filter delay should be considered to be unacceptable. In contrast, if one focusses solely on the aliphatic region of the spectrum, the average J-coupling is about 135 Hz. The maximum difference between the aliphatic couplings and this mean value is then about 10 Hz, giving rise to a maximum leakage of only 2.7%. Now, the aromatic coupling constants are clearly deviating too much and, as a consequence, only the aliphatic part of the X-filtered NOESY can be reliably interpreted (Qian et al., 1993).

However, by applying a selective 180° pulse on the aromatic carbons it is possible to decouple the aromatic protons for the period corresponding to the difference in aliphatic and aromatic J-couplings, which is shown in Fig. 1b. Here, the delays τ and τ' are matched to the average values of the aliphatic (135 Hz, 3.7 ms) and aromatic (165 Hz, 3.0 ms) couplings, respectively. The selective 180° pulse decouples the aromatic protons for the period $2(\tau - \tau')$, which hence will nicely refocus as in-phase proton magnetization after the period $2\tau'$. There is approximately 1.4 ms time to produce this selective pulse, which is sufficient as the spectral region of the aromatic carbons is quite isolated from the aliphatic signals. Of course, the pulse is only applied in the recordings where $\phi = -x$. Since the delays for the aromatic and aliphatic J-couplings can now be tuned separately, the maximum offset for any J-coupling with respect to the delay is reduced to about 10 Hz (excluding the histidines). In this way ^{13}C-H signal leakage is at most 2–3% per half-filter, which is quite reasonable. Figure 1c shows that this double-tuned ^{13}C half-filter is also easily combined with a ^{15}N filter in a time-shared [^{15}N,^{13}C] half-filter (Burgering et al., 1993b).

Thus, introducing this selective pulse allows editing of the aliphatic and aromatic regions of the spectrum within the same experiment, without an unacceptable mismatch of the delay and the J-couplings. Nevertheless, especially at high fields (>500 MHz) the large spectral window of ^{13}C becomes another source of filter artefacts, because of off-resonance effects of the 180° editing pulse. On present-day instruments with ^{13}C B_1 fields around 20 kHz these effects are significant, even when the transmitter is positioned in-between the aromatic and aliphatic signals (~78 ppm). Consequently, ^{13}C spins resonating near the edges of the spectrum will not be completely inverted by the 180° editing pulse, resulting again in nonperfect refocussing of proton magnetization at the end of the half-filter. To minimize and even remove these effects, we propose to use a full-power hyperbolic secant carbon pulse to replace the $90^\circ_x 90^\circ_x$ pulse pair. The hyperbolic secant (sech) pulse was introduced in spectroscopy already in 1932, when it was applied in the double Stern–

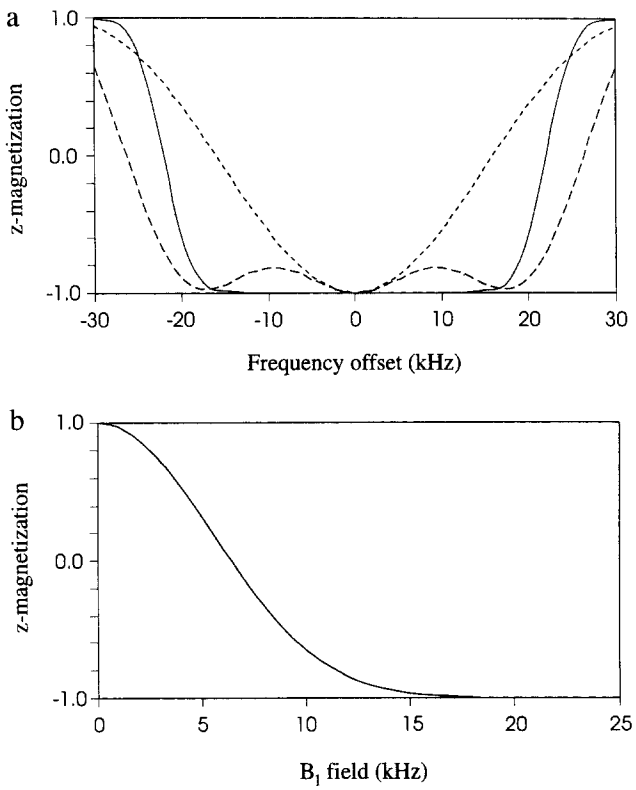

Fig. 2. (a) Simulation of the action of a 180° square pulse (------), a $90^\circ_x 180^\circ_y 90^\circ_x$ composite pulse (----) and a 180° hyperbolic secant pulse (——), applied on +z-magnetization of unit length, as a function of the transmitter offset frequency. (b) Simulation of the z-magnetization (at zero offset) for a 360 µs hyperbolic secant as a function of the B_1 field strength. The graphs were calculated with the 'pulsetool' package implemented in the Varian VNMR software.

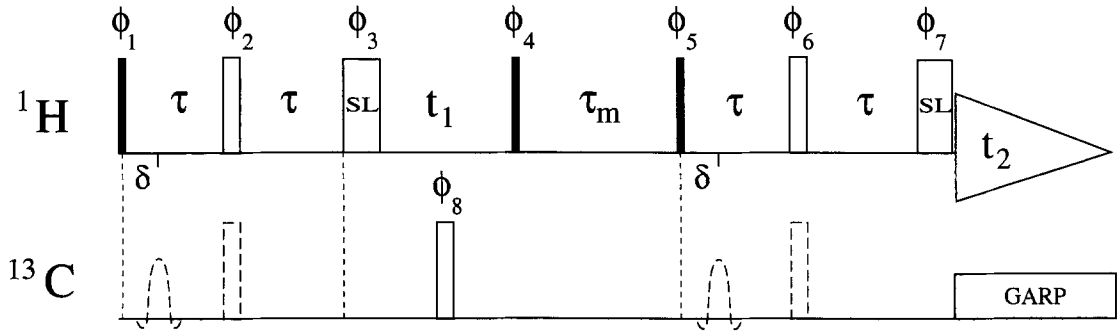

Fig. 3. Pulse sequence of the improved double half-filtered NOESY. In cases of symmetric dimers, two data sets are recorded, one in which the dashed pulses are applied, and one where they are effectively omitted (see text). The delay τ is chosen as 3.7 ms, corresponding to a one-bond coupling of 135 Hz. Delay $\delta = 0.7$ ms. The spin-lock pulses have durations of 1.3 and 1.7 ms. The 180° ^{13}C pulses are applied as 360 µs sech pulses at 20.0 kHz field strength with the transmitter at 78 ppm. An off-resonance I-BURP-2 pulse (Geen and Freeman, 1991) of 1.0 ms (10.0 kHz field) was used to selectively decouple the aromatic resonances during the period 2δ. During the recovery delay and the NOE mixing time, low-power H_2O irradiation was applied. The phase cycling used is as follows: ϕ_1 = 4(x,–x), 4(y,–y); ϕ_2 = 4(x,–x), 4(y,–y), 4(–x,x), 4(–y,y); ϕ_3 = 4(y,–y), 4(x,–x), 4(–y,y), 4(–x,x); $\phi_4 = \phi_8$ = 8(x), 8(y), 8(–x), 8(–y); ϕ_5 = 2(x), 2(y), 2(–x), 2(–y), 2(y), 2(–x), 2(–y), 2(x); ϕ_6 = 2(x), 2(y), 2(–x), 2(–y), 2(y), 2(–x), 2(–y), 2(x), 2(–x), 2(–y), 2(x), 2(y), 2(–y), 2(x), 2(y), 2(–x); ϕ_7 = 2(y), 2(–x), 2(–y), 2(x), 2(–x), 2(–y), 2(x), 2(y), 2(–y), 2(x), 2(y), 2(–x), 2(x), 2(y), 2(–x), 2(–y); Rec. = x,–x,y,–y,–x,x,–y,y,y,–y,–x,x,–y,y,x,–x,–x,x,–y,y,x,–x,y,–y,–y,y,x,–x,y,–y,x,–x. Quadrature detection in the indirect dimension was achieved by the States-TPPI method (Marion et al., 1989), incrementing ϕ_1 and ϕ_3.

Gerlach experiment (Rosen and Zener, 1932). To our knowledge, Silver et al. (1984a,b) were the first to introduce the hyperbolic secant in NMR as a pulse that allows selective inversion with good phase behaviour. Only quite recently it has been recognized that a hyperbolic secant, when applied with maximum power, can also be used to both invert and refocus very large spectral regions without creating artefacts (Hallenga and Lippens, 1995). To illustrate the behaviour of the pulse acting on magnetization aligned along the z-axis, we simulated its excitation profile with the 'pulsetool' package integrated in the Varian VNMR software. In this routine, use is made of the classical model of nuclear spin evolution described by the Bloch equations. Figure 2a compares the simulated z-magnetization as a function of the transmitter offset after the action of a rectangular 180° pulse, a $90^\circ_x 180^\circ_y 90^\circ_x$ composite pulse and a 360 µs hyperbolic secant pulse, all at 20 kHz power. The figure shows that only for the latter the z-magnetization is perfectly inverted over a band width of no less than 25 kHz. This profile is significantly better than that obtained by a rectangular pulse ($90^\circ_x 90^\circ_\phi$), which is normally used in half-filter experiments. This means that even on 750 MHz spectrometers, both the aromatic and aliphatic regions of the ^{13}C spectrum can be perfectly inverted when using the sech pulse.

An additional advantage of the hyperbolic secant is demonstrated in Fig. 2b. Here, the z-magnetization (at zero offset) is simulated for a 360 µs pulse as a function of the B_1 field strength. Since the sech pulse is equivalent to an adiabatic rapid passage (Baum et al., 1983; Warren and Silver, 1988), it behaves rather differently from other shaped pulses because the requirement for adiabaticity leads to the situation that only a minimum power limit is necessary to obtain full inversion; increasing the power above this threshold value does not affect the band width, nor the rotation angle. This characteristic makes the hyperbolic secant insensitive to B_1 inhomogeneities once the power used is larger than is necessary for adiabatic inversion. Then, spins near the edges of the NMR tube, which usually experience lower field strengths, may be completely inverted as well. Thus, the sech pulse has two remarkable properties, which we expect to make it a very useful pulse for a variety of high-field NMR experiments, i.e., the broad excitation which is of course particularly

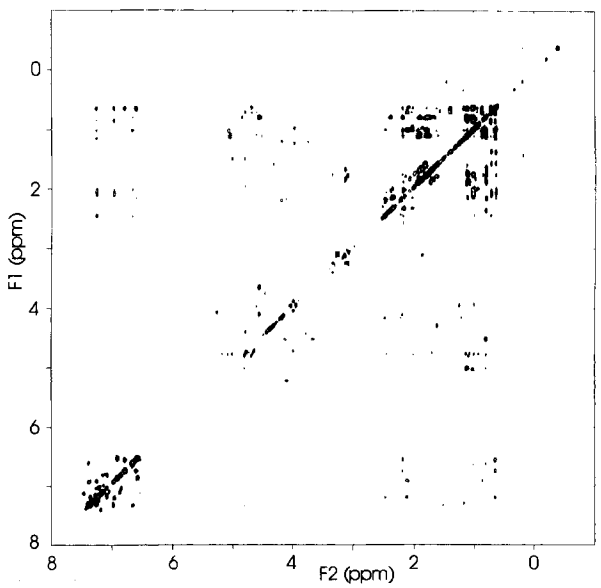

Fig. 4. Complete subspectrum of the double half-filtered 2D NOESY experiment, obtained after subtraction of the two data sets (see text). The spectrum was recorded at 500 MHz on a Varian Unity+ spectrometer equipped with a (^{13}C,^{15}N) triple-probe. The effective heterodimer concentration was 0.7 mM. The experiment was recorded at 27 °C, with a mixing time of 120 ms. Both data sets consisted of 200 complex t_1 increments, for which 256 transients were accumulated.

important for carbon, and the remarkable dependence of the B_1 field strength. Both features increase the overall sensitivity of this and other experiments where one or more ^{13}C broadband inversion pulses are required.

Figure 3 depicts the pulse sequence we used to record the double X half-filtered NOESY. All 180° carbon pulses are implemented by full-power hyperbolic secant pulses of 360 μs length. Now, X-filter editing can no longer be achieved by alternating the phase of the second pulse in the $90°_x 90°_\phi$ pair. Instead, we switch between applying the pulse centered in the ^{13}C spectrum, and at 60 kHz offset. The latter case is then again equivalent to omitting the pulse. The selective pulses are treated in a similar fashion. Furthermore, two spin-lock pulses are added to destroy the antiphase components of magnetization that is not completely refocused at the end of the half-filter.

The proposed sequence has been tested on a mutant ($Phe^{36} \rightarrow His$) of the single-stranded DNA-binding protein of bacteriophage Pf3, which forms a dimer in solution (Folmer et al., 1994). To obtain a suitable NMR sample, a 1:1 mixture of uniformly (99%) ^{13}C,^{15}N-labelled and unlabelled protein was prepared at 0.04 mM concentration. We established that at this concentration the monomers exchange sufficiently rapid to allow the formation of heterodimers with one monomer labelled and the other unlabelled. This volume was concentrated on a monoS cation-exchange FPLC column, lyophilized and dissolved in D_2O to a 1.4 mM protein solution, containing the desired heterodimers and the two types of homodimers in a 2:1:1 ratio.

Because in the case of a dimer molecule one is basically only interested in the intermonomer NOEs, it suffices to record two data sets: one in which both 180° editing pulses are applied, and one in which they are both omitted (Folkers et al., 1993). Figure 4 shows the 500 MHz spectrum that was obtained with this new sequence, after subtraction of the two recordings. Due to the 1.1% occurrence of ^{13}C at natural abundance in the unlabelled monomers, as well as the ^{12}C fraction in the 99% ^{13}C-labelled monomers, the intramonomer NOEs will always be attenuated by about 2% in the recording where both editing pulses are applied (assuming a random distribution of the minor isotope in both fractions). Therefore, it is stressed that this experiment will only work properly for isotopically highly pure subunits. To compensate for this effect, the recording with the editing pulses was multiplied by 1.02 before subtracting it from the other one. The resulting spectrum displays a large number of intermonomer NOEs, but more importantly, only very few off-diagonal artefacts. Unwanted intramonomer contacts are almost exclusively found between aromatic protons. These NOEs are usually so intense that apparently even the presence of a few percent leakage still gives rise to fairly strong cross peaks. Figure 5 compares the methyl regions of the 'regular' NOESY spectrum, obtained by addition of the two subspectra, and of the filtered spectrum, obtained by subtracting the subspectra. The efficiency of the sequence is best illustrated by the NOEs between the δ-methyl groups of Leu^{68}, indicated in Fig. 5b. This spectrum clearly shows that these NOEs have an intermonomer contribution, indicating that the side chains of the two Leu^{68} residues are in close contact. The strong intensity of the diagonal obscures these NOEs in the left-hand spectrum, but the nearly perfect subtraction of intramonomeric NOEs and diagonal peaks allows them to be easily detected in the filtered spectrum. Recently, we determined the three-dimensional structure of the protein, which confirmed that Leu^{68} is very close to the dyad axis

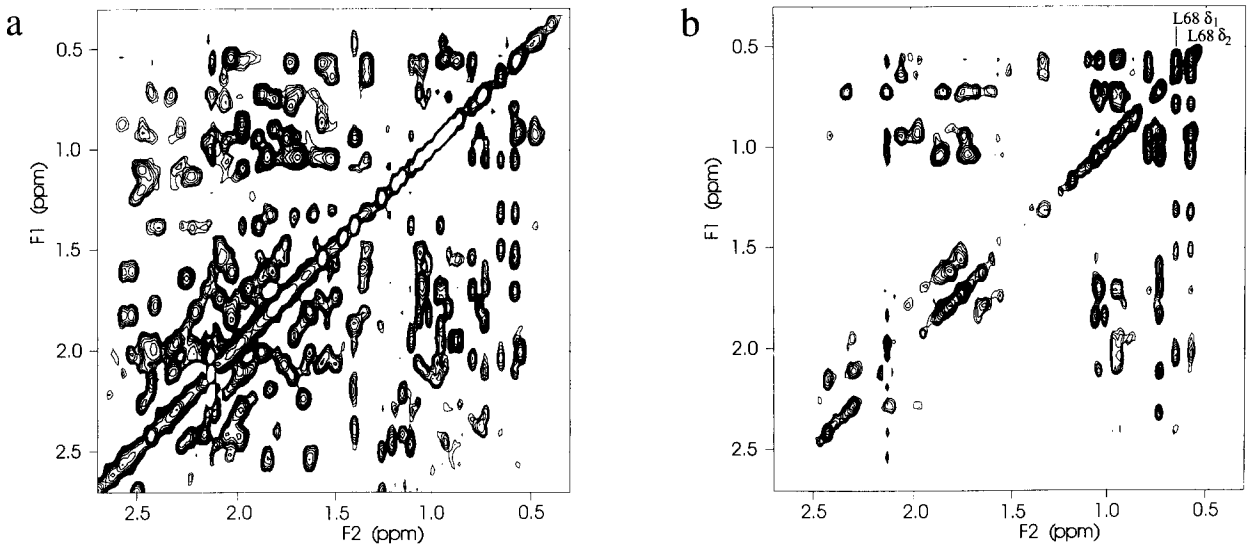

Fig. 5. Comparison of the methyl regions from the double half-filtered NOESY spectrum recorded with the sequence of Fig. 3. (a) Subspectrum obtained after addition of the two data sets, which is equivalent to a regular 2D NOESY. (b) Subspectrum obtained after subtraction of the two data sets, displaying the intermonomer NOE contacts. As an example, the intersubunit NOEs between the methyl groups of Leu^{68} are indicated.

of the molecule (unpublished results). The interpretation of this spectrum resulted in the nearly complete assignment of the intermonomer NOEs, which is generally of great importance for structure calculations of dimeric proteins. In this respect, it should be noted that the spectrum of the recording in which the two editing pulses are applied can be quite useful too. Provided that the mixture of hetero- and homodimers is exactly 2:1:1, precisely half of the intermonomeric NOEs occur as ^{12}CH-^{13}CH NOEs, whereas the other half are contacts inside the homodimers. As these have opposite signs in the recording with the editing pulses, they will totally cancel and, consequently, the intermonomer NOEs are absent from this particular subspectrum. In this way, one obtains a double-check for the identification of intermonomeric NOEs in symmetric dimers.

In conclusion, it has been demonstrated that it is possible to apply ^{13}C half-filter editing simultaneously to aromatic and aliphatic proton–carbon spin pairs. Although it was shown here for a protein, it is equally applicable to nucleic acids, in which the $^{1}J_{H^{13}C}$ couplings in the sugar and the base are also quite different. Applied to the Pf3 ssDBP, the improved double ^{13}C-filtered NOESY allowed the unambiguous identification of many intermonomer NOEs, particularly between methyl groups, which were essential for the calculation of the structure of the protein.

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