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Simultaneous CT-^{13}C and VT-^{15}N chemical shift labelling: Application to 3D NOESY-CH_3NH and 3D ^{13}C,^{15}N HSQC-NOESY-CH_3NH

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Abstract

Based on the HSQC scheme, we have designed a 2D heterocorrelated experiment which combines constant time (CT) ^{13}C and variable time (VT) ^{15}N chemical shift labelling. Although applicable to all carbons, this mode is particularly suitable for simultaneous recording of methyl-carbon and nitrogen chemical shifts at high digital resolution. The methyl carbon magnetisation is in the transverse plane during the whole CT period ($1/J_{CC}$ = 28.6 ms). The magnetisation originating from NH protons is initially stored in the $2H_zN_z$ state, then prior to the VT chemical shift labelling period is converted into $2H_zN_y$ coherence. The VT -^{15}N mode eliminates the effect of $^1J_{N,CO}$ and $^{1,2}J_{N,CA}$ coupling constants without the need for band-selective carbon pulses. An optional editing procedure is incorporated which eliminates signals from CH_2 groups, thus removing any potential overlap with the CH_3 signals. The CT-$^{13}CH_3$,VT-^{15}N HSQC building block is used to construct two 3D experiments: 3D NOESY-CH_3NH and 3D ^{13}C,^{15}N HSQC-NOESY-CH_3NH. Combined use of these experiments yields proton and heteronuclear chemical shifts for moieties experiencing NOEs with CH_3 and NH protons. These NOE interactions are resolved as a consequence of the high digital resolution in the carbon and nitrogen chemical shifts of CH_3 and NH groups, respectively. The techniques are illustrated using a double labelled sample of the CH domain from calponin.

Introduction

Evaluation of interproton distances is the essential step in determination of protein structures by NMR spectroscopy. For ^{13}C and ^{15}N double-labelled proteins, 4D NOESY experiments with two proton and two heteronuclear chemical shift axes provide, in principle, all the data required for identification of protons located close in space (Kay et al., 1990; Clore et al., 1991; Zuiderweg et al., 1991). However, the limited digital resolution and sensitivity inherent to 4D experiments mean that, in practice, several 3D NOESY experiments usually have to be recorded instead (Bax and Grzesiek, 1993). Nonetheless, the fundamental limitations in the digital resolution of the ^{13}C dimension in 3D ^{13}C-edited NOESY spectra together with degeneracy of chemical shifts make the assignment of 3D NOESY spectra of proteins one of the most challenging problems in structure determination by NMR.

Several 3D experiments have shown that for methyl groups, whose carbon spin–spin relaxation times are generally longer than those of the rest of the amino acid side chains, the digital resolution in the ^{13}C dimension can be increased substantially without dramatic decrease of the cross peak intensities (Hu and Zuiderweg, 1996; Diercks et al., 1998; Zwahlen et al., 1998a, b; Uhrín et al., 2000). High digital resolution NOE data for methyl protons increase the accuracy of NMR structures as methyl-containing amino acids occur frequently in hydrophobic cores of proteins

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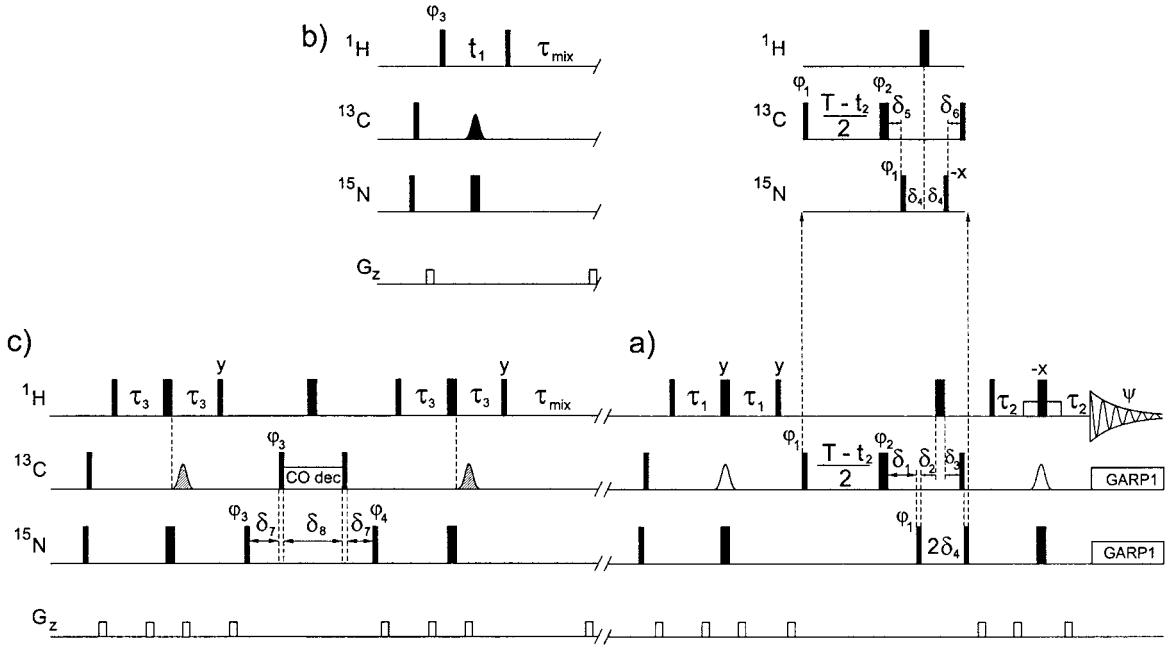

Figure 1. (a) Pulse sequence of CT-$^{13}CH_3$, VT-^{15}N HSQC. Thin and thick bars represent 90° and 180° non-selective pulses and are applied along the x-axis unless otherwise stated. The delays are: $\tau_1 = 2.25$ ms, $\tau_2 + pw_{sel} = \tau_1$, where pw_{sel} is a 90° ^1H selective pulse, T = 28.6 ms, $\delta_1 = (T - t_2)/2 - pwn + (4/\pi)pwn + pwc$, $\delta_4 = t_2/2 - (2/\pi)$ pwn, $\delta_2 = \delta_4 - pwh$, $\delta_3 = \delta_4 - pwh - pwc$, $\delta_5 = (T - t_2)/2 - pwn + (4/\pi)$ pwn $- \tau_a$, $\delta_6 = \tau_a - pwn$, where $\tau_a = 4.0$ ms and pwn, pwc and pwh are 90° ^{15}N, ^{13}C and ^1H pulses, respectively. The following phases were applied: φ_1 = x,−x, φ_2 = x and ψ = y,−y,−y,y; every two scans the central part of the sequence was replaced by the one shown in the inset. The ^{13}C and ^{15}N carriers were set to 20 and 118 ppm, respectively. The ^{13}C inversion pulse was a 692 μs I-SNOB-5 pulse (Kupče, personal communication). The sign discrimination in F_1 was achieved by the States-TPPI method with a 90° incrementation of the phases φ_1 and φ_2. GARP1 decoupling covering the CH_3 and NH regions ($\gamma B_2/2\pi = 2000$ Hz) was used. (b+a) 3D NOESY-CH_3NH, φ_3 = 4x,4(−x), ψ = y,−y,−y,y,−y,y,y,−y; the carbon decoupling during t_1 was achieved by a 126 μs broadband inversion pulse (Smith and Shaka, 2000). The sign discrimination in F_1 was achieved by the States-TPPI method incrementing the phase φ_3 by 90°. (c+a) 3D ^{13}C,^{15}N HSQC-NOESY-CH_3NH, $\tau_3 = 2.6$ ms, $\delta_7 = t_1/2 - pwc - (2/\pi)$ (pwn − pwc), $\delta_8 = t_1 - (4/\pi)$ pwc. The adiabatic inversion WURST-20 pulse applied after the 180° ^1H pulse was optimised to cover only the aliphatic region. The field was swept through 27 kHz during 1.098 ms from higher to lower field with the carrier positioned at 30 ppm (Kupče and Freeman, 1997). Phases φ_3 and ψ were identical to those used in (b), phase φ_4 = x was changed to −x after eight scans and the data were stored into a separate memory location.

where they have an intrinsic structural role involving many inter-residue interactions. Methyl groups are also represented on protein surfaces where they can be involved in intermolecular interactions (Gardner et al., 1997). NOESY experiments focusing on methyl groups (Diercks et al., 1998; Zwahlen et al., 1998a, b) can also be designed to incorporate detection of the NOE connectivity of NH protons (Zwahlen et al., 1998b). Such a combination is a part of a continued effort to reduce the number of necessary 3D NOESY experiments by maximising the information content of each experiment (Sørensen, 1990; Pascal et al., 1994; Vis et al., 1994; Jarela and Rule, 1995; Zwahlen et al., 1998b).

We discuss here two 3D ^{13}C,^{15}N NOESY experiments designed to yield interproton NOEs between the following pairs of groups; CH_3 and CH_n, NH and CH_n, and NH and NH (n = 1,2,3). The spectra are acquired with high digital resolution in the combined $^{13}CH_3$ and ^{15}N F_2 dimension. The directly detected dimension contains CH_3 and NH protons only, while the F_1 dimension is used for labelling of either ^1H or ^{13}C and ^{15}N chemical shifts. The experiments are illustrated using a double labelled sample (12.5 kDa, 1.2 m M, pH 7.0, 18 °C) of the CH domain from calponin (**I**).

Results and discussion

Combined CT-$^{13}CH_3$, VT-^{15}N HSQC

At the heart of the proposed NOESY experiments is a combination of a constant (CT) and variable (VT)

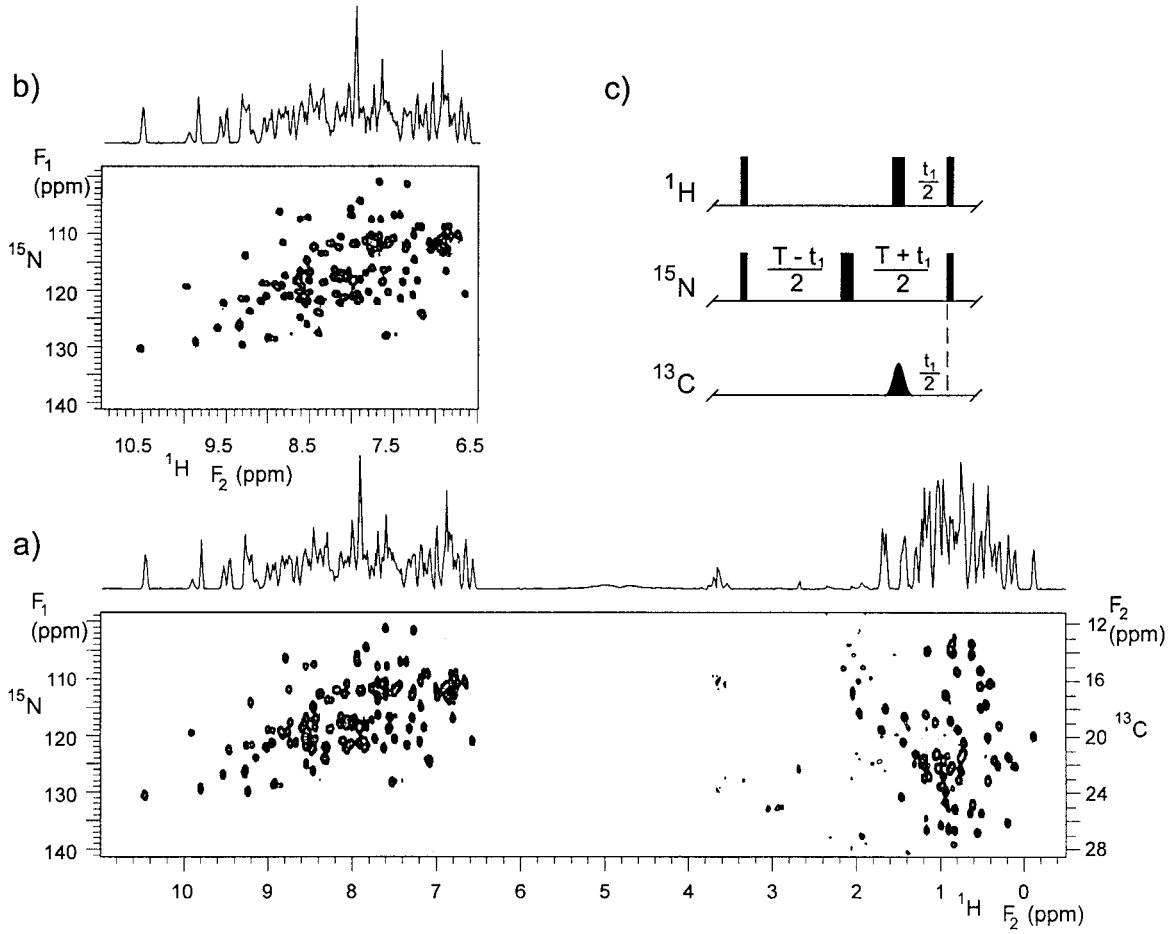

Figure 2. (a) CT-$^{13}CH_3$, VT-^{15}N HSQC spectrum of **I** acquired using the pulse sequence of Figure 1a. The sweep width in F_1 was 2600 Hz, 4 scans were accumulated into each of 54 complex points. The constant time interval, T, was set to 28.6 ms. Acquisition times were 20.8 and 64 ms in t_1 and t_2, respectively and the total acquisition time was 10 min. (b) CT-^{15}N HSQC spectrum acquired using identical parameters to those in (a) using the constant-time chemical shift labelling scheme as shown in (c). The nitrogen–carbon interactions were decoupled by a 126 μs ^{13}C broadband inversion pulse (Smith and Shaka, 2000).

time $^{13}CH_3$ and ^{15}N-HSQC (Figure 1a). This combination of labelling periods has, to our knowledge, not been described previously. The magnetisation of the NH protons is initially stored in the form of two-spin order ($2H_zN_z$) and converted into nitrogen antiphase magnetisation ($2H_zN_y$) only during the later part of the CT interval. The length of the CT CH_3 period ($1/^1J_{CC}$) is dictated by the need to refocus the one-bond carbon–carbon coupling constants. The amount of time for which the two-spin order exists is gradually reduced as the length of the VT-^{15}N chemical shift labelling period increases. This period is centred around the ^1H 180° pulse which serves to refocus the $^1J_{NH}$ (and also $^1J_{CH3}$) coupling constants. Elimination of the evolution of $^1J_{N,CO}$ and $^{1,2}J_{N,CA}$ is not required, since these are not resolved in F_1 during the typical acquisition time of around 20 ms.

For medium size proteins the relaxation of the two-spin order, $2H_zN_z$, is similar to that of the ^{15}N transverse magnetisation (Dayie and Wagner, 1994). The ^{15}N interferogram of the VT-^{15}N t_1 period obtained in the CT-$^{13}CH_3$, VT-^{15}N HSQC therefore resembles that of a CT experiment. The NH part of the spectrum of **I** acquired using the pulse sequence of Figure 1a, and the spectrum acquired using the CT-^{15}N t_1 period of Figure 2c are shown for comparison in Figures 2a and b, respectively. Horizontal projections of these spectra are almost identical; the slight broadening of the resonances in F_1 seen in the former spectrum is due to unresolved nitrogen–carbon coupling constants.

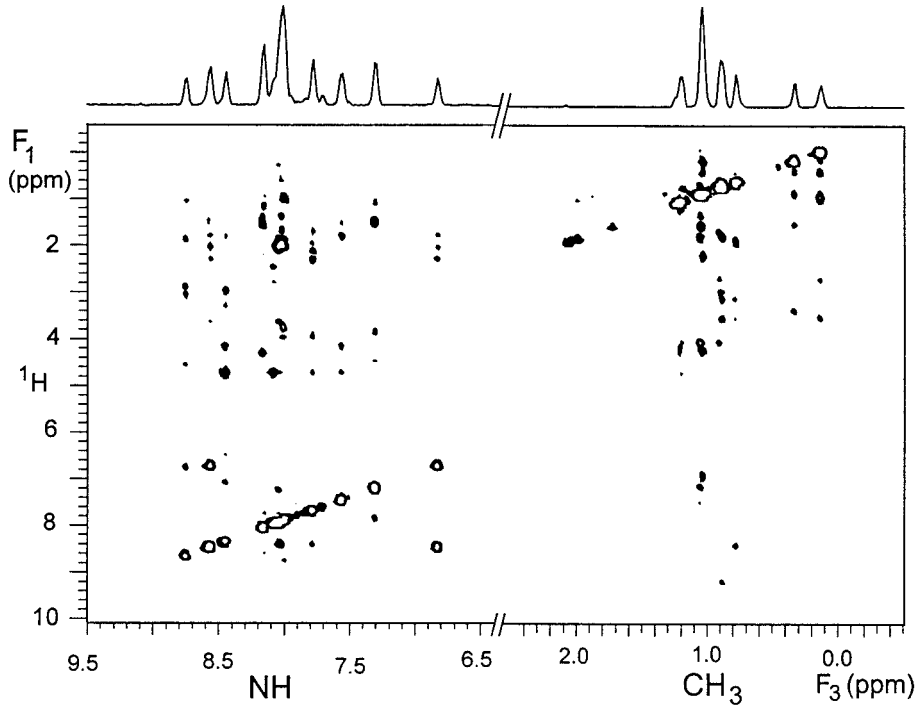

Figure 3. The F_1F_3 plane from a 3D NOESY-CH_3NH spectrum of **I** acquired using the pulse sequence of Figure 1(b+a). The NOE mixing time was 100 ms; the acquisition times t_1, t_2 and t_3 were 15, 19.2 and 64 ms respectively, corresponding sweep widths were 6400, 2500 and 8000 Hz and 96, 48 and 512 complex points were accumulated during 63 h using eight scans per increment.

A broad-band inversion ^{13}C pulse, which was used in the CT-^{15}N experiment (Figure 2c), is not applicable in experiments with combined CT-$^{13}CH_3$,^{15}N periods (Zwahlen et al., 1998b). Selective 180° C′, Cα and CH_3 pulses must be used with a Bloch–Siegert shift compensation for at least the Cα pulse. This means that a total of four selective ^{13}C pulses are required in an experiment with a shared CT-^{15}N,^{13}C period (Zwahlen et al., 1998b), while only one non-selective 180° ^{13}C pulse is needed in the proposed combination of the CT-^{13}C and VT-^{15}N chemical shift labelling.

The magnetisations of CH_2 and CH_3 differ in sign at the end of the constant time interval ($1/^1J_{CC}$) due to the different number of interacting carbon nuclei. In the case of overlap, the destructive interference between these two types of signals can decrease the quality of NOESY spectra. The CH_2 signals can be removed from the CT-$^{13}CH_3$, VT-^{15}N HSQC spectra in a way similar to that used in the 3D HCCH_3-TOCSY experiment (Uhrín et al., 2000): the 180° ^{1}H pulse is shifted by 4 ms ($= 1/2^1J_{CH3}$) towards the beginning of the pulse sequence during half of the scans accumulated for each increment. This causes a change in the sign of the CH and CH_3 resonances, but does not affect the CH_2 signals, which are then eliminated, by a suitable phase cycling of the receiver. A part of the CH_2 signal which was not completely eliminated due to the differences in $^1J_{CH}$ between the methyl and methylene groups, is substantially reduced by the fast spin–spin relaxation of CH_2 carbons. The CH signals from Cα carbons are not excited at all due to the methyl selective inversion used during the de- and refocusing INEPT periods. The only signals, except for those from CH_3 carbons, which pass this filter, albeit much attenuated due to faster spin-spin relaxation, are those from CH groups resonating in the methyl region. Since their phase is identical to that of the CH_3 signals, they do not cause signal cancellation. By employing this procedure for selection of methyl resonances rather than coherence selection by pulsed field gradients, the sensitivity of the proposed method is higher than the sensitivity of the CT-QQF-HSQC experiment (Diercks et al., 1998).

3D NOESY-CH_3NH

A 3D NOESY-CH_3NH experiment is obtained by placing the proton chemical shift labelling followed by the NOE mixing time before the CT-$^{13}CH_3$,VT-

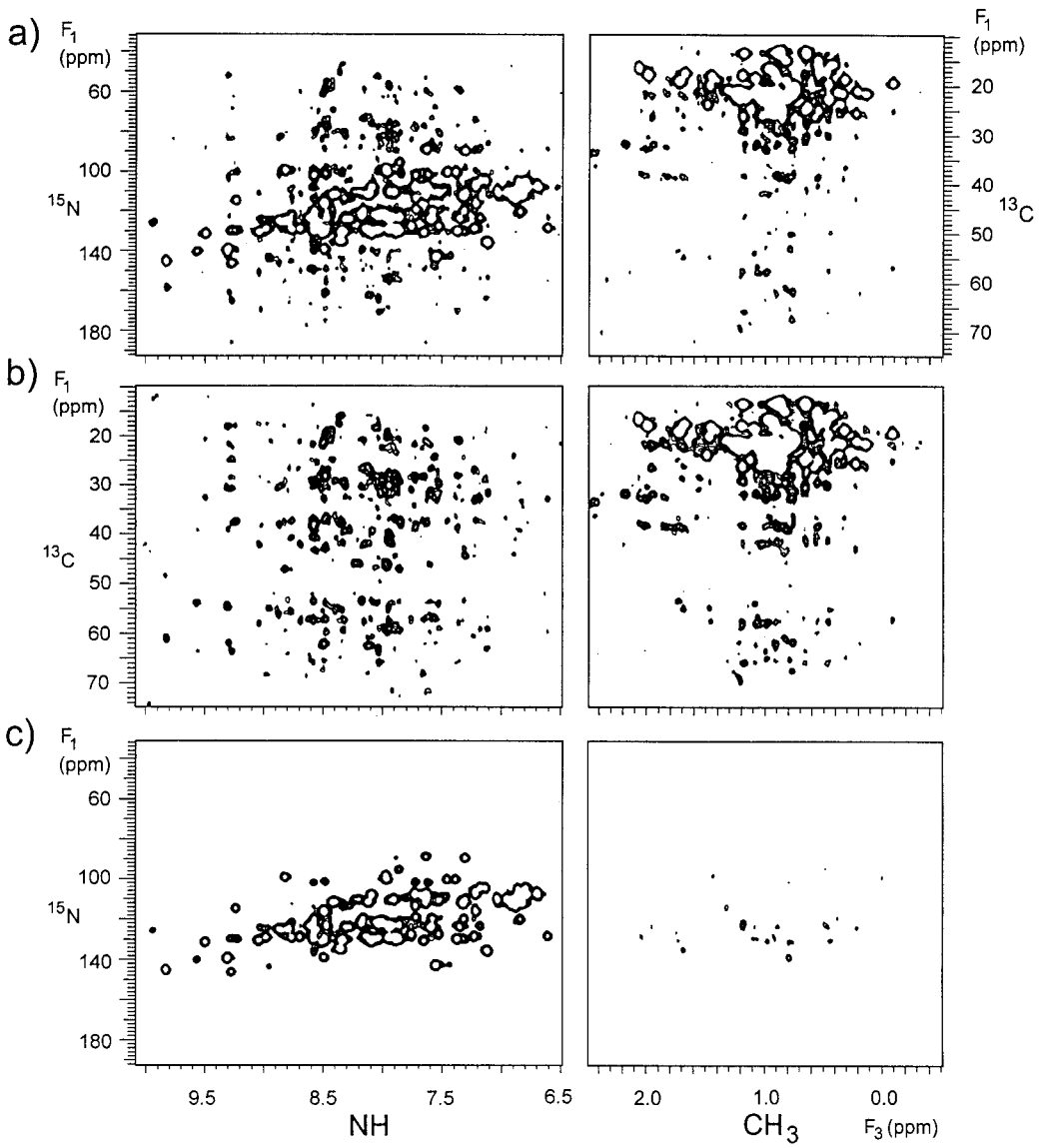

Figure 4. Parts of the first F_1F_3 plane from a 3D ^{13}C,^{15}N HSQC-NOESY-CH_3NH spectrum of **I** containing NH and CH_3 correlations acquired using the pulse sequence of Figure 1(c+a). (a) Contains both CH and NH cross peaks, while the spectra in (b) and (c) were obtained by adding or subtracting, respectively, the data sets acquired by changing the phase φ_4 and stored in separate memory blocks. In this manner the CH and NH cross peaks were separated. Only one half of the data were used to give the spectrum in (a). The NOE mixing time was 100 ms. The ^{13}C carrier was set to 30, 45 and 20 ppm for adiabatic inversion and ^{13}C chemical shift labelling during t_1 and t_2, respectively. Only the NOEs from aliphatic carbons were recorded using a ^{13}C selective inversion pulse described in the legend of Figure 1c. The acquisition times t_1 and t_3 were 6.4 and 64 ms respectively, corresponding sweep widths were 9955 and 8000 Hz; 64 and 512 complex points were acquired; 16 scans were accumulated per increment using $\varphi_4 = x$ and another 16 scans using $\varphi_4 = -x$. The total acquisition time was 1.5 h.

^{15}N HSQC block (Figure 1(b+a)). A 2D plane from the 3D NOESY-CH_3NH spectrum of **I** is shown in Figure 3. Four different types of cross peaks are visible here: CH_3-CH_n, CH_3-NH, NH-NH and NH-CH_n ($n = 1,2,3$), where CH_n includes both aliphatic and aromatic protons. Superior digital resolution in the ^{15}N,^{13}C-shared F_2 dimension is a consequence of a ca. 20 ms acquisition time, which is considerably longer than the 5–8 ms typically used for ^{13}C-edited spectra. The same acquisition time applies to the ^{15}N chemical shift labelling. This is comparable to acquisition times used in the 3D ^{15}N-edited NOESY exper-

iments, but much longer than the acquisition achievable in the shared ^{13}C,^{15}N-edited NOESY experiment with simultaneous carbon and nitrogen pulses (Pascal et al., 1994). Unfortunately, there is an initial loss of magnetisation caused by the relaxation of the $2N_zH_z$ two-spin order during the 28.6 ms period when compared with the standard 3D ^{15}N-edited NOESY, causing some signal attenuation.

3D ^{13}C,^{15}N HSQC-NOESY-CH_3NH

Even when there is no overlap between NOE cross peaks in 3D ^{13}C NOESY-HSQC spectra, their assignment is not unambiguous. This is mostly due to our lack of knowledge of the chemical shift of the heteronucleus to which one of the pair of interacting protons is attached. This information can be provided by searching for symmetry related cross peaks, a procedure which is time consuming and not always successful. The missing heteronuclear chemical shift information can alternatively be obtained in a complementary 3D experiment, which employs the initial chemical shift labelling of ^{13}C or ^{15}N rather than that of protons (Frenkiel, 1990; Vis et al., 1994; Jarela and Rule, 1995). There, by working simultaneously with two 3D NOESY spectra differing only by the nucleus labelled in the F_1 dimension (^1H or X), and comparing the chemical shifts of cross peaks with the resonance assignment table, it is usually possible to identify unambiguously the interacting proton pair. An additional advantage of ^{13}C-edited NOESY spectra with two ^{13}C axes comes from the larger dispersion of ^{13}C chemical shifts which may uncover more NOE contacts than had been assumed previously based on the analysis of a standard ^{13}C-edited NOESY spectrum (Diercks et al., 1999).

It is also possible for ^{15}N and ^{13}C to share the chemical shift labelling period prior to the NOE mixing time. In this case, however, the problem of overlap of ^{15}N and ^{13}C resonances in F_1 must be addressed. The first solution, which involves elimination of the diagonal peaks by a suitable phase cycling, also results in the elimination of all NH-NH and CH-CH cross peaks (Jarela and Rule, 1995). The second, and the preferred solution, relies on the independent phase cycling of the 90° ^{15}N and ^{13}C pulses of the first INEPT transfer (Sørensen, 1990). The latter solution was adopted for the proposed 3D ^{13}C,^{15}N HSQC-NOESY-CH_3NH experiment (Figure 1(c+a)). In this way, the separation of CH and NH cross peaks in F_1 is achieved by spectral editing, as exemplified in Figure 4 using the first plane of the 3D ^{13}C,^{15}N HSQC-NOESY-CH_3NH spectrum of **I**. The VT-^{15}N,^{13}C labelling was adopted during t_1, which limits the acquisition time in the ^{13}C dimension to ca. 5–8 ms due to evolution of carbon–carbon coupling constants. The t_1 acquisition time for ^{15}N was twice that for ^{13}C resonances, which was made possible by storing the carbon magnetisation in the form of two-spin order ($2C_zH_z$) during parts of the ^{15}N labelling (Boelens et al., 1994; Vis et al., 1994).

In principle, chemical shifts of all ^{13}C resonances, including those of aromatic carbons, can be labelled during the t_1 period of the 3D ^{13}C,^{15}N HSQC-NOESY-CH_3NH experiment. This would, however, lead to some very long acquisition times since spectral folding is difficult to optimise in this indirectly detected dimension. We have therefore focused on the aliphatic carbons by using an adiabatic band-selective WURST inversion (Kupce and Freeman, 1995). The ^{13}C inversion pulse starts after the 180° ^1H pulse of the INEPT spin echo and the RF field is swept from aliphatic to aromatic resonances. The length of this pulse, the frequency of the ^{13}C carrier, the frequency sweep range and the length of the surrounding delays were calculated (Kupče and Freeman, 1997) (see legend of Figure 1) by taking into account the difference in $^1J_{CH}$ in different chemical shift regions (Zwahlen et al., 1997). The calculated length of the INEPT interval (5.2 ms) is, at the same time, optimised for the transfer of magnetisation from NH protons to nitrogen. This part of the sequence can be easily modified should only aromatic or methyl resonances be of interest.

Conclusions

We have designed a 2D CT-$^{13}CH_3$, VT-^{15}N HSQC experiment which combines constant time ^{13}C and variable time ^{15}N chemical shift labelling, and illustrated its usefulness for simultaneous recording of methyl-carbon and nitrogen chemical shifts during the indirectly detected periods of 3D NOESY spectra. The resulting 3D NOESY-CH_3NH and 3D ^{13}C,^{15}N HSQC-NOESY-CH_3NH experiments provide the ^1H chemical shifts and the ^{13}C or ^{15}N chemical shift, respectively of the directly attached heteronucleus, for protons showing NOE interaction with CH_3 and NH protons. The NOE cross peaks are resolved by the methyl-carbon, or the nitrogen chemical shifts which are acquired with a high digital resolution during a time-shared CT-$^{13}CH_3$, VT-^{15}N interval.

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