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A constant-time ^{13}C-^{1}H HSQC with uniform excitation over the complete ^{13}C chemical shift range

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Summary

An improved version of the constant-time HSQC experiment is presented that gives uniform sensitivity over the complete ^{13}C bandwidth in ^{13}C-^{1}H correlation experiments without creating artifacts in the methyl and aromatic regions of the spectra. The improvement is achieved by replacing the refocussing ^{13}C 180° pulse in the evolution time by a combination of a full-power (22 kHz) hyperbolic secant 180° pulse that inverts and refocusses the entire ^{13}C window, immediately followed by a selective 180° pulse on the CO region. Further improvement in signal-to-noise in the aromatic and methyl regions, although less spectacular, is obtained by replacing the other two 180° ^{13}C pulses in the INEPT parts of the pulse sequence by full-power hyperbolic secant pulses. Results of simulations and experimental data are presented that demonstrate the excellent performance of the hyperbolic secant pulse for broadband inversion and show that refocussing of transverse magnetization occurs over the same bandwidth, albeit with a ^{13}C signal phase that depends quadratically on offset. A further modification, in which one of the selective pulses on the CO region is omitted, is also presented. Implications for other 2D and 3D experiments performed at high fields, where uniform ^{13}C inversion and refocussing is desirable, are discussed.

Introduction

The advent of triple-resonance experiments and their application to proteins, uniformly and doubly ^{13}C and ^{15}N labelled, has led to a drastic increase in the molecular weight limit of the protein assignment problem. Further advances in 3D and 4D NMR, like ^{13}C and ^{15}N editing, filtering and/or labelling of ^{1}H NOEs, have allowed protein structure determination by NMR to follow this upward trend. However, the introduction of close to 100% ^{13}C and ^{15}N isotopes also has some disadvantages, such as enhanced spin–spin relaxation and the appearance of complicated multiplet patterns in the ^{13}C dimension of nD experiments. This latter problem has been addressed by Vuister and Bax (1992) in a constant-time version of the ^{1}H-^{13}C HSQC experiment (ct-HSQC), where a significant improvement in ^{13}C resolution was obtained by adjusting the constant evolution time to 26.6 or 53 ms for refocussing of the one-bond ^{13}C-^{13}C couplings in the non-

aromatic side chains. In analogy to the constant-time ^{1}H COSY experiment described earlier (Bax and Freeman, 1981), the chemical shift evolution in F1 is obtained by moving a central 180° ^{13}C pulse across half of the constant-time window, as indicated in Fig. 1.

Modified constant-time HSQC or HS-HSQC pulse sequence

A 180° ^{1}H and a 180° selective ^{13}C pulse on the carbonyls are used in combination with the central 180° ^{13}C pulse to refocus the ^{1}J$_{CH}$ and ^{1}J$_{C\alpha CO}$ couplings (53 Hz). This experiment, which was clearly not intended to observe ^{13}C-^{1}H correlations in aromatic side chains, can be modified to observe all one-bond correlations simultaneously, with uniform sensitivity over the entire ^{13}C spectral window. Due to the requirement in the ct-HSQC experiment that the central 180° ^{13}C pulse acts as a 360° off-resonance pulse at the position of the carbonyls, the aromatic carbons will be incompletely inverted and re-

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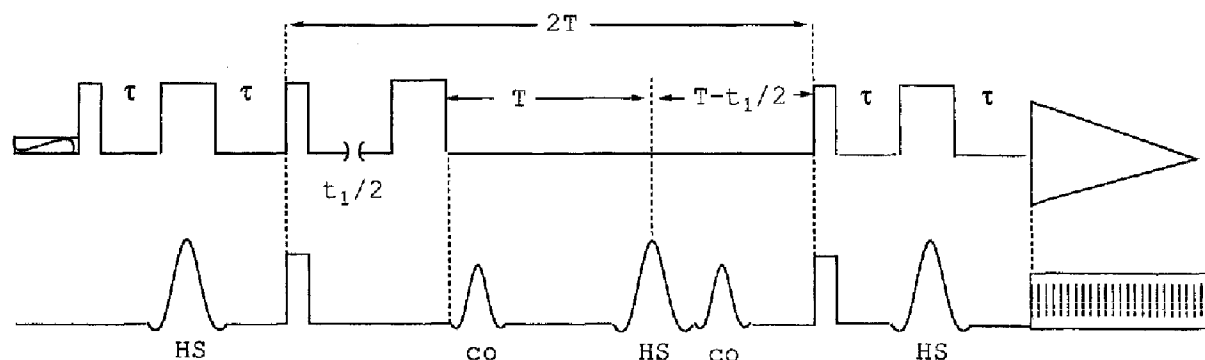**Present address: SCMB, Institut Pasteur de Lille, 1 Rue du Prof. Calmette, B.P. 245, F-59019 Lille, France.

Fig. 1. Pulse sequence for the constant-time HSQC experiment or HS-HSQC with hyperbolic secant pulses replacing the hard 180° ^{13}C pulses. The narrow and wide rectangular pulses are hard 90° and 180° pulses, respectively. The intense shaped pulses marked HS are full-power hyperbolic secant pulses centered in the ^{13}C chemical shift range. The low-intensity shaped pulses marked CO are selective inversion pulses for the carbonyl region. The constant time 2T contains the central HS pulse at time $t_1/2 + T$, immediately followed by the second CO pulse. The delays τ are equal to $1/4J_{CH}$. A GARP ^{13}C decoupling sequence is applied during detection.

focussed. In fact, such a 180° pulse centered at 43 ppm gives a 240°–270° rotation at the aromatic carbons, bringing the latter somewhere between the x,y plane and the +z axis. Equally adverse effects occur for refocussing of transverse magnetization, leading to a severe loss of sensitivity in this region. Off-resonance effects also affect carbons at the right edge of the carbon chemical shift range, giving rise to the presence of spurious triplets in the methyl region (Vuister and Bax, 1992). Even a hard 180° pulse of 2*14 μs or 2*11 μs (maximum power on our instrument), while leading to almost complete inversion of the aliphatic region, still gives poor inversion of the aromatic carbons, leading to artifacts and severe loss of sensitivity in this region of the spectrum. In addition, a constant-time evolution period of 2*13.3 ms is not optimal for the larger ^{13}C-^{13}C couplings of the aromatic rings that vary between 55 and 75 Hz, as determined from a high-resolution HMQC experiment on a 98% enriched protein. As a result, one obtains ^{1}H-^{13}C correlation spectra with very well resolved correlations in the alpha-aliphatic region, but with an aromatic region that is almost completely invisible when using the conditions described in the ct-HSQC experiment. This loss of information in 2D correlation spectra is much more dramatic in ^{13}C-edited NOESY spectra, where it was recognized that the off-resonance effects in the aromatic region prevent the observation of intra- and/or intermolecular NOEs with aromatic ring protons (Billeter et al., 1993).

We present here a modification of the ct-HSQC experiment, called HS-HSQC, that leads to a resolution improvement over the regular HSQC experiment similar to that obtained by Vuister and Bax, but with simultaneous and uniform detection of the complete carbon range of interest (10–140 ppm), while maintaining good refocussing of the carbonyl couplings to the α-carbons. As explained above, the main problem stems from the central 180° pulse; one would like this to obey the following criteria:

(1) Complete inversion of the whole alpha and ali-

phatic carbon region (10–70 ppm) and of the aromatic carbons (110–140 ppm);

(2) Zero excitation of the carbonyl carbons.

These criteria are not easily fulfilled simultaneously on present-day spectrometers. However, we propose to make use of the final carbonyl pulse in the ct-HSQC experiment (applied for compensation of the Bloch–Siegert effects of the first carbonyl pulse) to reach this goal. We will use a full-power hyperbolic secant carbon pulse, immediately followed by a selective pulse on the carbonyl carbons to fulfill both requirements: the hard pulse will invert/refocus the whole spectrum, including the carbonyl carbons, while the selective pulse on the carbonyl carbons (while maintaining its Bloch–Siegert compensation effect) immediately brings them back to their original position, allowing the carbonyl ^{13}CO-^{13}C$^\alpha$ coupling to refocus. This procedure is similar to the one described by Brüschweiler et al. (1988) for the elimination of specific couplings in ^{1}H spectra. The shaped pulse on the carbonyl carbons is quite easy to produce, as it should only invert a small spectral window that is quite isolated from the rest of the carbon spectrum. It is important to realize that the first selective CO pulse must be given *after* the 180° proton pulse at the end of the first half of the evolution time, see Fig. 1, in order to establish exact refocussing of the ^{13}CO-^{13}C$^\alpha$ couplings.

The hyperbolic secant pulse

Broadband population inversion has been obtained by the use of phase-shifted composite pulses with constant amplitude (Levitt, 1982; Tycko, 1983). A general description of phase-modulated as well as phase- *and* amplitude-modulated broadband inversion pulses (including the hyperbolic secant) and their relation to adiabatic inversion in NMR and to self-induced transparency in optical two-level systems has been given by Baum et al. (1985). The hyperbolic secant or sech pulse (an exact solution of the Bloch equations) has been demonstrated to give excel-

lent inversion over a limited bandwidth, provided that the B_1 field has a minimum value determined by the width of the inversion window (Silver et al., 1984a,b). More recently, broadband excitation has been demonstrated with adiabatic half-passage using a rapid phase continuous frequency sweep (Kentgens, 1991,1993). The general form of the sech pulse (Silver et al., 1984a,b) is given by:

$$\Omega(t) = \Omega_o \left[\text{sech} \{\beta(t - t_o)\} \right]^{1 + i\mu}$$

where Ω is the B_1 field amplitude, μ a real constant determining the oscillatory behaviour of the pulse and β its time scaling factor. From a series expansion for $|\beta(t - t_o)| \ll 1$ and $|\beta(t - t_o)| \gg 1$ it can easily be shown that application of the hyperbolic secant pulse is equivalent to a frequency sweep through resonance, with maximum speed $\mu(\beta)^2$ and amplitude Ω_o around $t = t_o$, while at the beginning and the end of the pulse ($\beta(t - t_o) \gg 1$; $\beta(t - t_o) \ll -1$) the frequency is constant with offset $+\beta$ and $-\beta$ respectively, while the amplitude becomes vanishingly small (see also Silver et al., 1984a).

The hyperbolic secant (HS) pulse has been used for quite some time to obtain a selective inversion with a good phase behaviour: using $\mu = 5$, low power levels and a reasonably long duration, one can indeed obtain a selective inversion of a spectral region as narrow as a few hertz. In our application, however, there are two main differences from this classical approach. The first one is that we want an inversion over as broad a frequency range as possible. The second difference is that this pulse has traditionally been used to invert z-magnetization, whereas in our experiment it also serves to refocus transverse magnetization. We experimented with composite rectangular and HS pulses to obtain a better excitation profile than the one obtained with a rectangular hard pulse. It was found that the HS pulse can be used to invert *and* refocus a significantly larger spectral region than the rectangular pulse, without creating artifacts, when applied with the same (maximum) power.

In order to satisfy the two criteria mentioned above, we used a hyperbolic secant pulse of 310 μs length, with a maximum B_1 field of 22 kHz. The excitation behaviour of the pulse acting on magnetization aligned along the z- or x-axis is simulated with the 'pulsetool' package integrated in the Varian VNMR software. Starting from a magnetization vector aligned along the z- or x-axis, we apply the pulse around the y-axis and examine the resulting x-, y- and z-magnetization. In Fig. 2 (trace a), we represent the fraction of magnetization that is created perpendicular to the initial magnetization vector. This implies that z-magnetization is inverted for 95% or more over a band width of at least 25 kHz, while transverse magnetization also remains transverse for at least 95% upon refocussing. This is sufficient to invert/refocus the entire carbon spectral region, if we shift the transmitter to

about 90 ppm. This pulse, immediately followed by the selective carbonyl pulse, resulted in an inversion and refocussing profile that satisfies the two requirements mentioned above.

The refocussing simulations by the hyperbolic secant revealed an unusual phase behaviour, as shown in Fig. 2 (traces b and c): depending on frequency offset, a certain amount of y-magnetization is created when x-magnetization is inverted (Fig. 2b). The phase of the refocussed transverse magnetisation shows a nonlinear dependence on frequency offset, with a 30° deviation on-resonance and perfect refocussing for an offset of ±8 kHz. In fact, the phase variation seems to be purely quadratic over the complete observation window, as shown in Fig. 2c. Both in simulated and experimental data, we have observed that the quadratic nature of the phase correction is obeyed independently of the initial position of the magnetization in the xy-plane prior to the pulse. Since there is no need for a linear phase correction when the evolution delay is properly corrected, one can thus write a purely quadratic phase correction routine and obtain a properly phased spectrum.

Refocussing of CO-C$^\alpha$ and C-C couplings

In the ct-HSQC experiment, the first selective 180° pulse inverting the carbonyl carbons is used to allow a refocussing of the CO-C$^\alpha$ coupling. If this pulse is not applied, an effective evolution of the coupling would occur in t_1, leading to a term $\cos(\pi J t_1)$, which would transform to doublet peaks in the t_1 direction.

However, if one leaves out the selective pulse on the carbonyls and replaces the central 180° pulse by the hard hyperbolic secant pulse, inverting simultaneously the alpha, aliphatic and aromatic transverse magnetization and the carbonyl longitudinal magnetization components, one obtains unperturbed evolution of the CO-C$^\alpha$ coupling over the constant-time period, corresponding to a term $\cos(\pi J 2T)$, leading to a scaling factor of the cross peaks but not to a doublet structure. Ideally, one would like to choose the constant time T in such a manner that this term becomes 1, but there is also the requirement that this time allows the refocussing of the aliphatic ^{13}C-^{13}C couplings. These couplings are typically on the order of 32–40 Hz, whereas the carbonyl CO-C$^\alpha$ coupling is typically 53 Hz. Moreover, as one of the major aims of the proposed method concerns the observation of the aromatic correlation peaks, we also require that the aromatic ^{13}C-^{13}C coupling, with coupling constants between 55 and 75 Hz, is well refocussed. Figure 3a (solid traces) shows the $\cos(2\pi J T)$ behaviour for the three different classes of coupling constants (CO-C$^\alpha$, aliphatic ^{13}C-^{13}C, and aromatic ^{13}C-^{13}C) and Fig. 3b (dashed curve) contains the sum of the squares of the three curves in Fig. 3a. From this figure it is clear that the best compromise is reached for values of T equal to 9 or 16 ms. The first value is
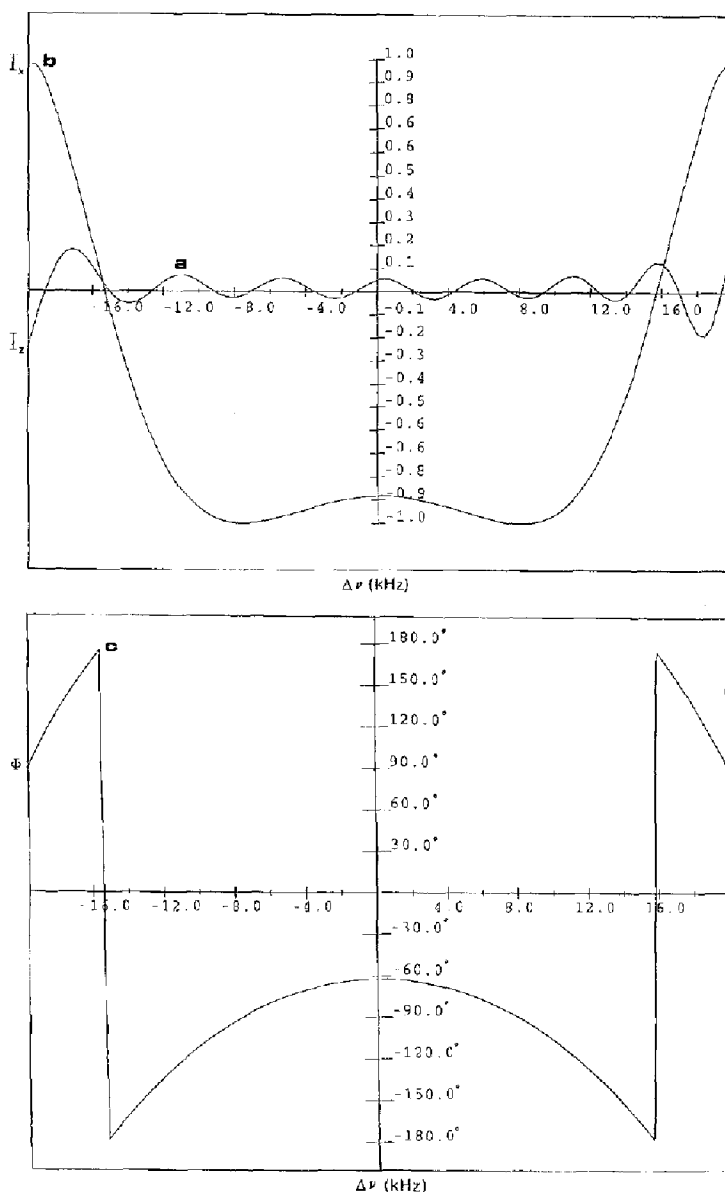
Fig. 2. Simulation of the action of a 180° hyperbolic secant pulse, applied along the y-axis, on x-magnetization of unit length. In traces a and b the amount of z-, respectively -x-magnetization generated is shown. In trace c the phase of the transverse magnetization with respect to the x-axis is displayed.

short and will limit the maximum number of increments possible to 96 (taking a spectral width of 6000 Hz), and so we loose one of the major advantages, i.e., omitting the carbonyl pulses. Indeed, as this pulse has to be selective on the carbonyl carbons, its length is typically on the order of 4 ms when a hyperbolic secant is used, which reduces the maximum number of increments considerably (by 25% if T = 16 ms). However, for large proteins with fast transverse relaxation, the 9 ms optimum for T might be the only value to retain sufficient signal intensity.

Materials and Methods

The performances of the different pulse sequences are demonstrated for a 1.5 mM solution of a small protein,

tick anticoagulating peptide (M_r = 6700), uniformly enriched (>98%) in ^{13}C and dissolved in D_2O at pH 2.5. The experiments were recorded at 25 °C and 33 °C, using only two channels of our Varian Unity 600 spectrometer equipped with waveform generators.

All experiments were carried out with the ^{13}C transmitter positioned at 50 ppm, using a 90° pulse width of 11 µs, except in the first tests where a 90° pulse of 14 µs was chosen. The ^{13}C spectral width was set to 40 ppm, causing the methyl resonances around 10 ppm to be folded once, whereas the aromatic carbons (115-135 ppm) undergo double aliasing. This folding pattern allows a good resolution in the ^{13}C dimension, without the necessity of an excessive number of increments (Marion et al., 1989).
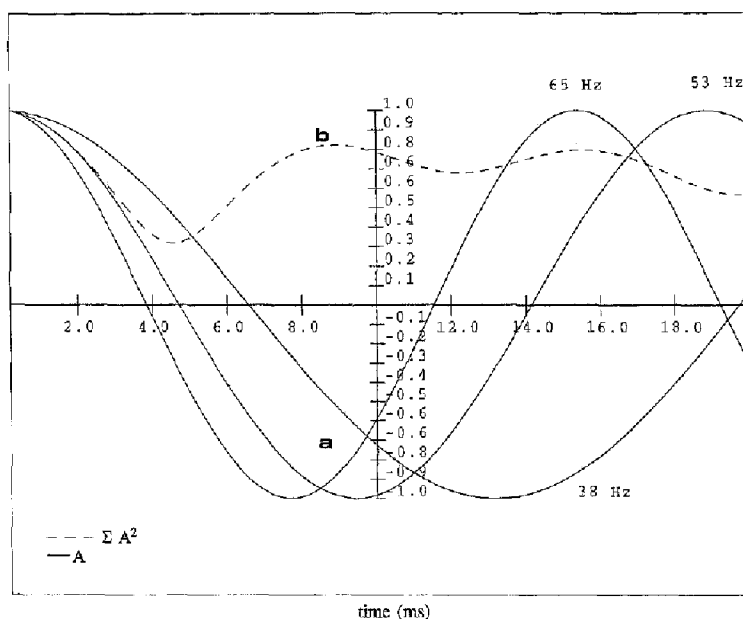
Fig. 3. Solid lines (a): refocussing of the ^{13}C magnetization as a function of the length of the constant evolution time T (in ms) for coupling constants of 38, 53 and 65 Hz representing the various one-bond C-C couplings that occur in a protein. Dashed line (b): the sum of squares of the three solid curves shown in a.

For the central 180° pulse in the ct-HSQC experiment we used a 50 µs pulse, yielding zero excitation at the carbonyls. In our modified experiment, the excitation frequency of the high-power sech 180° pulse used as the central inversion pulse was shifted to 92 ppm in order to allow a good inversion of the whole ^{13}C spectral range, extending from the carbonyl resonances (180 ppm) to the methyl groups (5 ppm). Shifts of the central frequency were generated according to the 'shifted laminar pulse' technique (Patt, 1991), which is also integrated in the Varian software. The constant time was set to a value of 2T = 32 ms in all experiments. In order to eliminate the linear F1 phase correction that causes baseline distortion, all delays were corrected for the finite width of the first 90° ^{13}C pulse and for 'hidden' instrumental delays (such as the delays required to implement power level switching). These corrections allowed us to reproduce the ct-HSQC experiment with a zero linear phase correction in the F1 direction, without sign inversion of folded peaks. In the 1D experiments 128 transients of 1K complex points were collected. For all 2D spectra, 140 complex (t_1) × 1024 complex (t_2) data points were acquired. Quadrature detection in F1 and suppression of axial peaks were achieved employing the TPPI-States method (Marion et al., 1989). For each complex t_1 increment, 64 scans were recorded in both the ct-HSQC and the HS-HSQC experiment with a recycling delay of 1 s, resulting in 4.8 h of measuring time.

For all data sets, we applied a shifted Gaussian filter in the t_2 direction, and after phasing and Fourier transformation, the interferograms were zero-filled to 512
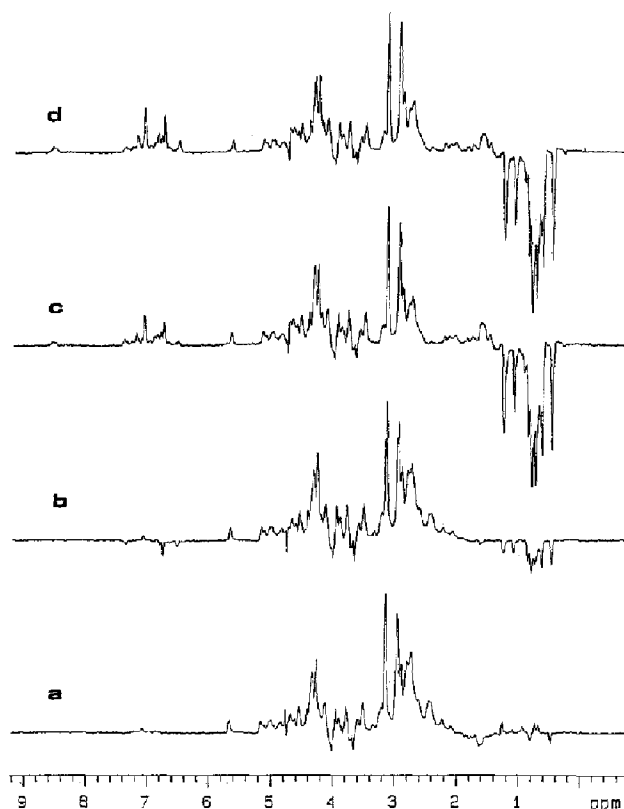

Fig. 4. 1D spectra taken with 128 transients, representing the first increments of the ct-HSQC (a,b) and HS-HSQC (c,d) experiments. The ct-HSQC experiments were recorded using a 50 µs central 180° ^{13}C pulse. The 90° and 180° pulses in the INEPT transfer were 14 and 28 µs, respectively in trace a and 11 and 22 µs, respectively in trace b. In the HS-HSQC experiments sech 180° pulses of 310 µs were used for the central 180° ^{13}C pulse. In trace c the 90° and 180° pulses in the INEPT transfer were 11 and 22 µs, while in trace d the INEPT 180° pulses were also replaced by sech 180° pulses of 310 µs.
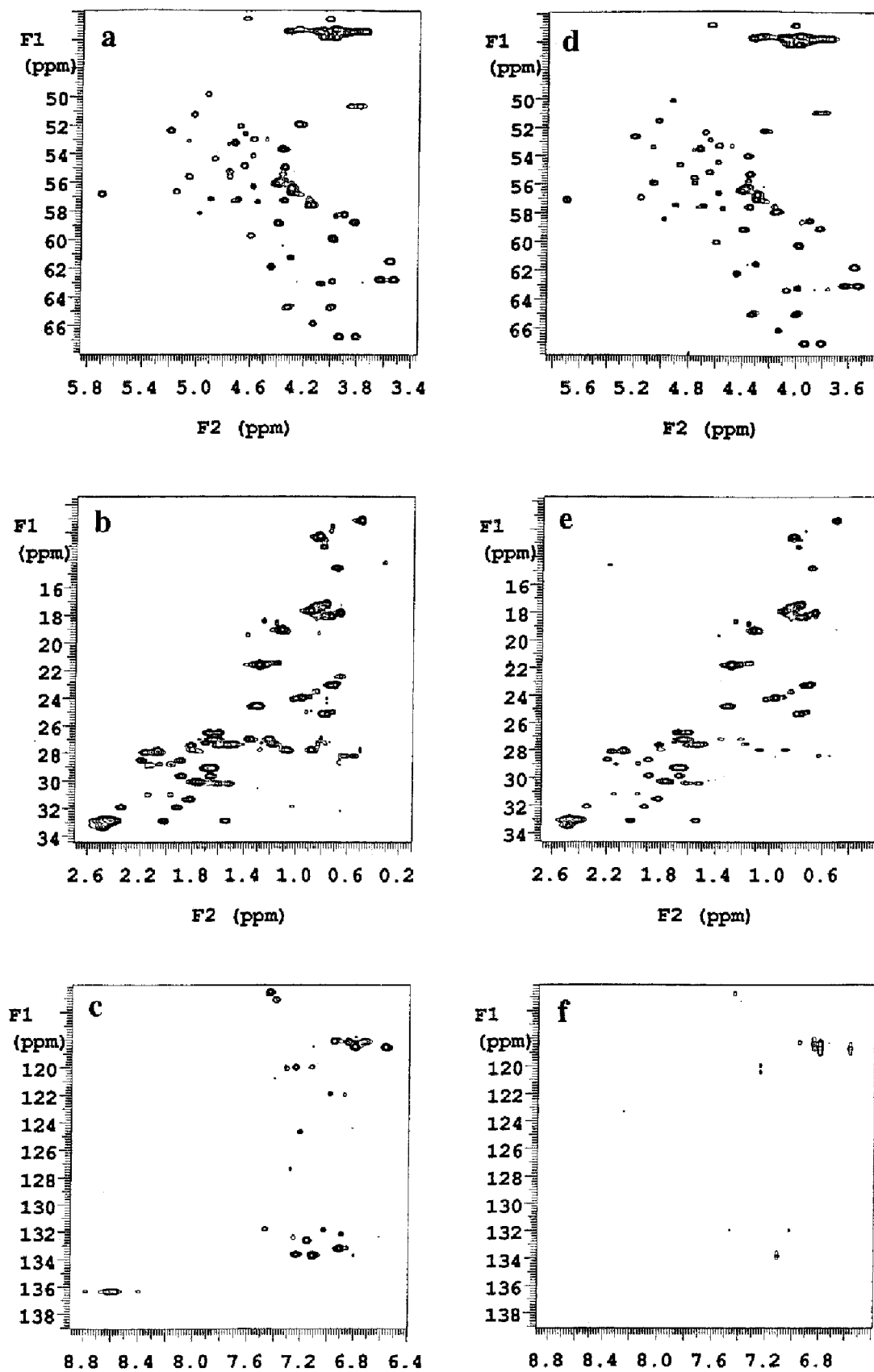
Fig. 5. Three representative regions of the HS-HSQC and ct-HSQC experiments are shown on the left and the right (a, b, c and d, e, f, respectively). The proton and carbon dimensions are F2 and F1, respectively. a and d compare the α-regions, b and e the aliphatic regions and c and f the aromatic regions of the spectra.

complex points. After applying a shifted sine bell function in t_1 and Fourier transformation, a real data set of 1024 × 512 points was obtained, with digital resolutions of 6.0 Hz (F2) and 11.9 Hz (F1).

Results and Discussion

Spectra obtained with the ct-HSQC and HS-HSQC experiments are compared below. Figure 4 shows a number of 1D spectra. In Fig. 4a the ct-HSQC is reproduced with 90° and 180° ^{13}C pulses of 14 and 28 μs, respectively. Increasing the transmitter power to its maximum value, giving values of 11 and 22 μs for the ^{13}C pulses while maintaining the central 180° pulse at 50 μs, results in some improvement for the methyl region but hardly any for the aromatics, as shown in Fig. 4b. A spectacular improvement is shown in Figs. 4c and d. In Fig. 4c only the central 180° pulse is replaced by a hyperbolic secant, while in Fig. 4d also the hard rectangular 180° pulses of 22 μs in the INEPT part of the sequence were replaced by full-power sech pulses. While the central spectral region, comprising the α-carbons and most of the β-carbons, is of similar intensity in all four spectra, the methyl groups clearly have a much higher intensity in the HS-HSQC experiment. More dramatic, however, is the improvement for the aromatic resonances. Whereas these are mostly invisible in the first increments of the ct-HSQC experiments (Figs. 4a and b) and thus display greatly reduced intensity in the 2D plot, we observe them with full intensity in the
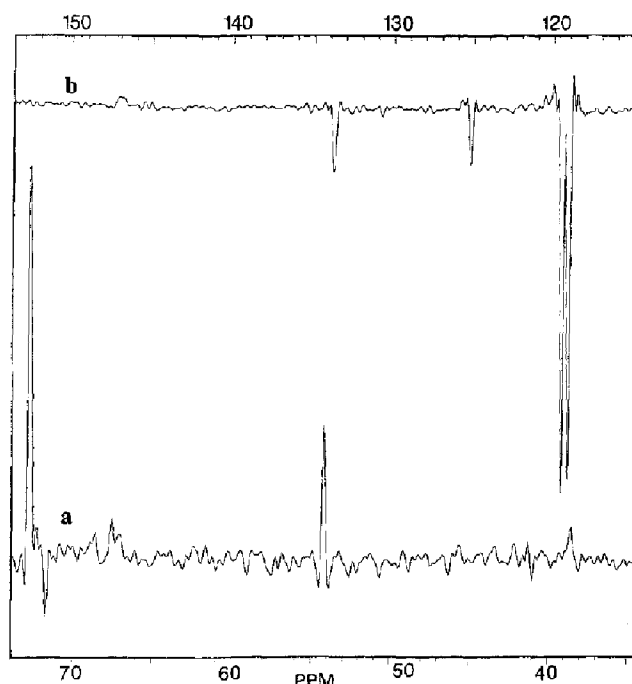
Fig. 7. The same cross-sections as in Fig. 6, using a zero-order plus a second-order phase correction with identical values in both traces.

HS-HSQC experiments. In addition, we note the presence of a small peak at 8.6 ppm representing a histidine C4 signal with a carbon frequency of 136.5 ppm, which is still visible in the HS-HSQC (Figs. 4c and d) but totally absent in the ct-HSQC experiment (Figs. 4a and b).

Replacing the 50 μs 180° pulse by a full-power pulse of 22 ms, as suggested by Vuister and Bax (1992), does indeed improve the signal-to-noise ratio of the edge of the aliphatic region and eliminates artifacts. However, this leads to the appearance of distorted CO-C^α coupling patterns in the C^α correlation peaks, because now the CO magnetization is affected adversely by the full-power 180° pulse. Therefore, this increase in power does not appear to be a good alternative.

For the 2D experiments we will compare only the ct-HSQC and HS-HSQC experiments with the full-power 180° and the three sech 180° pulses (Figs. 4b and d). In Fig. 5 we show the different spectral regions of the 2D plots obtained with both methods. The trends already observed in the 1D plots are fully confirmed: next to a similar quality of the central aliphatic region (Fig. 5a versus 5d), we get a better intensity for the methyl resonances (Fig. 5b versus 5e) and a dramatic improvement at the level of the aromatic resonances (Fig. 5c versus 5f), where the few correlations observed in the ct-HSQC experiment (Fig. 5f) clearly show triplet structures in the F1 dimension.

One remaining problem for the observation of phenyl rings, as mentioned by Vuister and Bax (1992) is the occurrence of strong couplings between ^{13}C nuclei. This can lead to serious distortions and loss of signal and
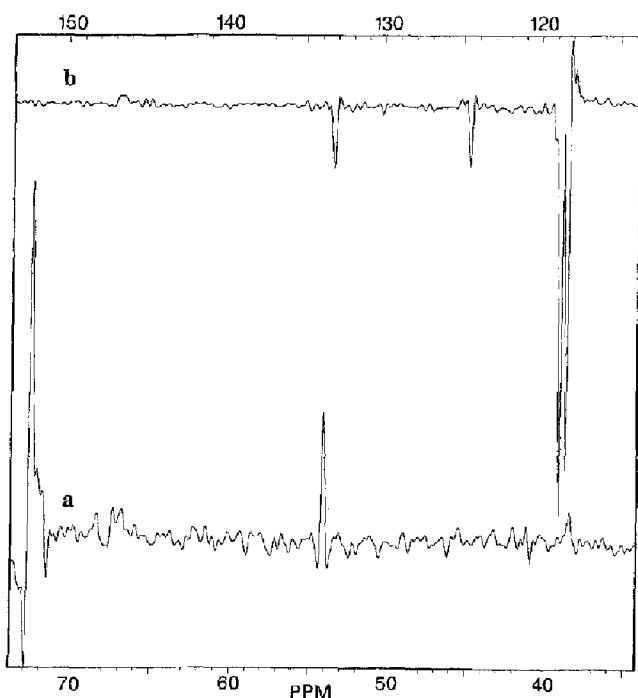

Fig. 6. Cross-sections along F1 through the aliphatic and aromatic regions of the HS-HSQC spectrum, with only a zero-order phase correction in traces a and b, respectively.

might also occur for aliphatic side chains (leucine C^γ-C^δ). We have found some indications for such effects in the spectra obtained with the hyperbolic secant pulses. It is clear, however, that the imperfect inversion of the aromatic resonances by a rectangular pulse forms a major contribution to these distortions.

As mentioned above, the different delays were adjusted so as to obtain a zero linear phase correction in the F1 direction of the ct-HSQC experiment. However, if one applies only the zero-order phase correction to a trace of a HS-HSQC experiment, spectra as shown in Fig. 6 result. The trace in Fig. 6a is taken through the α,β region. The central peak is correctly phased, but the peak at the edge is not. Taking into account that the peak is folded once, the quadratic phase correction, as required by the use of a sech 180° pulse as an inversion pulse for transversal magnetization, results in the spectrum of Fig. 7a, where all peaks are correctly phased. A similar effect is seen on a trace through the aromatic resonances (Figs. 6b and 7b). However, because we shifted the center of the sech 180° pulse from 50 to 92 ppm, the quadratic phase correction will be less pronounced, even though the aromatic resonances at 130 ppm are folded twice. The phase-corrected trace, using the same zero-order and second-order phasing parameters as in Fig. 7a, is displayed in Fig. 7b, and shows all peaks perfectly in phase. Although it is not possible to obtain spectra that are properly phased in the carbon dimension with only a zero-order phase correction, no serious baseline distortion has been observed in the HS-HSQC experiment. The data were processed without any baseline correction.

As discussed above, the CO-C^α couplings can also be eliminated by omitting both soft carbonyl pulses and using the hard sech 180° pulse as the central pulse of the constant-time period. This modification leads to a scaling factor of $\cos(2\pi JT)$ for those carbon resonances that are coupled to a carbonyl carbon, but should leave the other resonances unaffected. A very similar experiment, where the position of the first carbonyl pulse is switched between two positions and the difference spectrum is considered, has in fact been developed for measuring the coupling constants between the backbone carbonyls and methyl carbons in isotopically enriched proteins (Grzesiek et al., 1993). For a constant-time value 2T of 2 × 16 ms, the scaling factor should be 0.58. We have compared the spectra with and without the carbonyl pulses, and we observe a ratio of 0.62 ± 0.07 for the α-carbons, whereas the effect on other resonances, due to the much smaller coupling constants and the short constant time used, is much less pronounced. Therefore, in this version of the experiment the hard sech 180° pulse can advantageously replace the combination of the soft rectangular pulse with zero excitation at the carbonyls and the selective pulses on the carbonyls.

Conclusions

We have shown that it is possible to obtain a uniform sensitivity for a given constant-time value in the ct-HSQC experiment over the complete ^{13}C chemical shift range of a protein enriched in ^{13}C. The novelty of the experiment presented here is in the use of a shaped sech 180° pulse as the central inversion pulse. Because this pulse inverts a larger spectral width than the traditional hard rectangular 180° pulse, with substantial increase in sensitivity at the edges of the ^{13}C chemical shift range, we believe that it can be of general use in many of the existing 3D and 4D pulse sequences that are used to assign larger proteins. This includes ^{13}C editing, as well as ^{13}C filtering experiments currently under development in our laboratory. However, care should be exercised when this pulse is used as a refocussing pulse for magnetization in the xy-plane rather than as a pulse that inverts z-magnetization, because it leads to a quadratic phase correction. We have also shown that this pulse, in combination with a soft 'back-turning' pulse on the carbonyl carbons, can be used to generate a 'wide-band' selective pulse, inverting selectively the alpha, aliphatic and aromatic carbons while leaving the backbone carbonyls essentially unaffected.

Acknowledgements

References

Baum, J., Tycko, R. and Pines, A. (1985) *Phys. Rev. A*, **32**, 3435–3448.

Bax, A. and Freeman, R. (1981) *J. Magn. Reson.*, **44**, 542–548.

Billeter, M., Qian, Y.Q., Otting, G., Müller, M., Gehring, W. and Wüthrich, K. (1993) *J. Mol. Biol.*, **234**, 1084–1097.

Brüschweiler, R., Griesinger, C., Sørensen, O.W. and Ernst, R.R. (1988) *J. Magn. Reson.*, **78**, 178–185.

Grzesiek, S., Vuister, G.W. and Bax, A. (1993) *J. Biomol. NMR*, **3**, 487–493.

Ikura, M., Kay, L.E., Tschudin, R. and Bax, A. (1990) *J. Magn. Reson.*, **86**, 204–209.

Kentgens, A.P.M. (1991) *J. Magn. Reson.*, **95**, 619–624.

Kentgens, A.P.M. (1993) *J. Magn. Reson.*, **104**, 302–307.

Levitt, M.H. (1982) *J. Magn. Reson.*, **48**, 234–264.

Marion, D., Ikura, M., Tschudin, R. and Bax, A. (1989) *J. Magn. Reson.*, **85**, 393–399.

Patt, S.L. (1991) *J. Magn. Reson.*, **95**, 94–106.

Silver, M.S., Joseph, R.I. and Hoult, D.I. (1984a) *Phys. Rev. A*, **31**, 2753–2755.

Silver, M.S., Joseph, R.I. and Hoult, D.I. (1984b) *J. Magn. Reson.*, **59**, 347–353.

Tycko, R. (1983) *Phys. Rev. Lett.*, **51**, 775–777.

Vuister, G. and Bax, A. (1992) *J. Magn. Reson.*, **98**, 428–435.