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Solid-State Dipolar INADEQUATE NMR Spectroscopy with a Large Double-Quantum Spectral Width

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A technique for obtaining dipolar-mediated INADEQUATE NMR spectra with a large spectral window in the double-quantum dimension is presented. Using the dipolar recoupling sequence C7 to excite the double-quantum coherence under magic-angle spinning, the technique involves incrementing the evolution period in synchrony with the phase of the radiofrequency pulses in the C7 sequence. The technique is demonstrated on a uniformly ^{13}C-labeled amino acid and an extensively ^{13}C-labeled protein to identify ^{13}C connectivity patterns for spectral assignment. © 1999 Academic Press

Key Words: **solid-state NMR; INADEQUATE; double-quantum; C7; dipolar recoupling.**

Recently, solid-state homonuclear double-quantum NMR spectroscopy has been increasingly employed to obtain spectral assignment, torsion angles, and distances in biological solids (*1–7*), synthetic polymers (*8*), inorganic glasses (*9, 10*), and zeolites (*11, 12*). The utilization of double-quantum (DQ) coherence suppresses signals from isolated spins so that the spectrum is simplified to contain only signals from spin pairs (*13*). The DQ coherence can be exploited in various ways in the experimental design (*14, 15*). In two-dimensional INADEQUATE spectroscopy (*16*), homonuclear DQ coherence is excited before the evolution period (t_1) and is then reconverted to observable, single-quantum, coherence for detection (t_2). This gives rise to 2D spectra in which the indirect dimension (ω_1) exhibits the sum chemical shift of the coupled spins that survive the double-quantum filter and is correlated with the isotropic chemical shifts of the individual spins in the direct dimension (ω_2). Compared to single-quantum correlation spectroscopy, which gives rise to spectra with both diagonal and off-diagonal peaks, the double-quantum spectra have the distinct advantage that coupled spins with small chemical shift differences can be observed clearly without interference from diagonal peaks.

The double-quantum coherence can be excited by either the dipolar coupling or the scalar coupling between the two spins. The scalar-coupling-mediated INADEQUATE experiment was demonstrated originally in solutions (*16*) and more recently also in solids (*1, 17*). Since the dipolar coupling permits spatial proximity to be probed, a dipolar-mediated INADEQUATE experiment is potentially useful for structure determination. Furthermore, it can be used in place of the scalar-coupling-mediated version for resonance assignments in solids. Due to its strong distance dependence, the dipolar coupling between directly bonded ^{13}C spins is more than five times stronger than the two-bond couplings and other long-range couplings. Therefore, at short mixing times the dipolar INADEQUATE experiment is as valid as the scalar version for resonance assignment. In addition, the dipolar interaction allows faster excitation of the DQ coherence, thereby reducing T_2-induced signal losses. Such a dipolar-mediated solid-state assignment approach has been shown recently in a ^{15}N–^{13}C heteronuclear correlation experiment (*18*). When applying dipolar INADEQUATE spectroscopy to unoriented solids, chemical site resolution must be achieved by magic-angle spinning (MAS). However, since MAS also averages out the dipolar interaction, which drives the DQ excitation and reconversion, special radiofrequency (RF) pulse sequences must be applied to reintroduce the dipolar coupling. Many such homonuclear dipolar recoupling sequences are now available (*19–24*).

One feature of the INADEQUATE experiment is that the ω_1 dimension intrinsically has a large spectral range since it reflects the sum chemical shifts of pairs of coupled spins. For polypeptides, the ^{13}C DQ spectral range is at least 250 ppm, considering that DQ coherence between aromatic carbons, which resonate at about 110 ppm downfield from the center of the aliphatic region, can occur easily (while carbonyl–carbonyl couplings are weak enough to be ignored). Although the ω_1 spectral width may be reduced by a factor of two using delayed acquisition or foldover correction (*14, 25, 26*), folding crowds the spectrum and complicates the interpretation of the connectivity patterns for complex biological macromolecules. To obtain an INADEQUATE spectrum with a large, unfolded ω_1 width on a static solid or a solution sample, one can simply make the t_1 dwell time small. However, to carry out a MAS-INADEQUATE experiment involving dipolar recoupling sequences, an additional requirement often arises: the recoupling pulses must be synchronized with the sample rotation. This can severely restrict the choice of the t_1 dwell times. For example,

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when using the C7 sequence (*23*) to excite and reconvert the DQ coherence, the reconversion pulses normally must start at integer multiples of the rotor cycle after the end of the excitation period to maintain correspondence with the rotor phase (*27*). This would require a minimum t_1 dwell time of one rotor cycle. At currently feasible RF field strengths and spinning speeds, one rotor cycle is usually much larger than the dwell times required for a sufficiently wide ^{13}C double-quantum spectrum, as we discuss in more detail below. In a previous demonstration of the dipolar INADEQUATE experiment incorporating C7 (*15*), this problem was circumvented fortuitously because ^{31}P signals with a small chemical shift dispersion were detected.

In this article, we show a phase-permuted version of the original C7 sequence which permits the measurement of ^{13}C dipolar INADEQUATE spectra with a large DQ spectral width in the ω_1 dimension. We chose C7 as our dipolar recoupling sequence since it offers one of the highest DQ excitation efficiencies available so far. Our discussion below uses much of the original formalism of the C7 sequence (*23*). Briefly, C7 is a train of continuous sevenfold phase-switched RF pulse cycles whose field strength fulfills the condition $\omega_1 = 7\omega_r$ to achieve dipolar recoupling. Due to the weak orientation dependence of its average Hamiltonian, C7 and its variants (*28, 29*) have high recoupling efficiencies and are thus ideal for DQ excitation and reconversion in the INADEQUATE experiment. In a DQ experiment, the C7 reconversion block not only changes its overall phase relative to that of the excitation block in order to select the DQ coherence, but also continues the sevenfold phase switching from the end of the excitation period. The latter is a manifestation of the central requirement of the C7 sequence, which is that *the sevenfold RF phase shifts must remain synchronous with the sample rotation.* This requirement is reflected in the average Hamiltonian of a basic C7 unit, expressed in the interaction frame of the RF field,

$$\bar{H}_p^{(0)} = \sum_Q \sum_{\lambda\mu lm} \bar{\omega}^Q_{\lambda\mu lm}(\Omega_{PR}, t^0) \times \exp(i\phi_p(2m-\mu))T^Q_{\lambda\mu}. \quad [1]$$

The symbols have the same meanings as defined in Ref. (*23*). For the present discussion, the relevant parameters are the pulse phase of the pth C7 unit, $\phi_p = 2\pi p/7$, and the time-averaged anisotropic frequency $\bar{\omega}^Q_{\lambda\mu lm}(\Omega_{PR}, t^0)$,

$$\bar{\omega}^Q_{\lambda\mu lm}(\Omega_{PR}, t^0) = \frac{1}{\tau_c}\int_0^{\tau_c} \tilde{\omega}^Q_{\lambda\mu lm}(\Omega_{PR}, t^0, \tau)d\tau$$

$$= A(\Omega_{PR}) \int_0^{\tau_c} d^\lambda_{\mu 0}(-\beta_{RF}(\tau)) \times \exp(im\omega_r(\tau + t^0))d\tau. \quad [2]$$

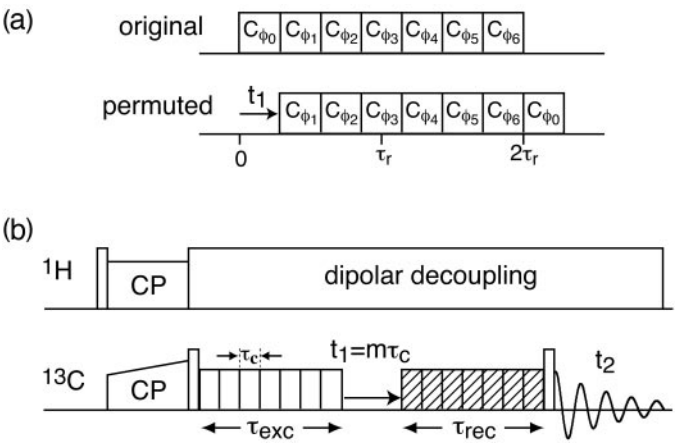

FIG. 1. (a) The original C7 sequence and its equivalent, phase-permuted version used to acquire a dipolar INADEQUATE spectrum with a large double-quantum width. (b) Pulse sequence for the dipolar INADEQUATE experiment incorporating C7 for homonuclear dipolar recoupling under MAS. After cross-polarization, double-quantum ^{13}C–^{13}C coherence is excited by a train of C7 pulses. It evolves under the sum chemical shifts before being reconverted into observable single-quantum magnetization for detection. The excitation and reconversion periods consist of an integer multiple of seven basic C7 units, $\tau_{exc} = \tau_{rec} = n\tau_c$ ($n = 7, 14, \cdots$). The evolution time t_1 is chosen to be $m\tau_c$ ($m = 0, 1, \cdots$); thus the t_1 dwell time is equal to the duration of one C7 unit, τ_c. The phases of the reconversion pulses are shifted as described in the text to maintain synchrony with the rotor phase.

The dependence of the averaged frequency on the rotor orientation is reflected in the time t^0 when the C7 sequence is initiated. To emphasize this time dependence, we include all time-independent terms in $A(\Omega_{PR})$, where Ω_{PR} is the set of Euler angles describing the relative orientation of the principal-axis frame of the interaction tensor and a rotor-fixed frame.

A large ω_1 width in the dipolar INADEQUATE experiment is derived by inserting an evolution period that matches the duration of one (or integer multiples of one) C7 unit, $t_1 = \tau_c = 2\tau_r/7$, between the double-quantum excitation and reconversion periods, which are chosen to comprise an integer multiple of seven basic C7 units, C_{ϕ_p}. If, instead of continuing the RF phase from the end of the excitation period to the beginning of the reconversion period, we shift the phase of the latter by $\Delta\phi = 2\pi/7$ (or integer multiples of this value) (Fig. 1a), then the average Hamiltonian for the reconversion block is identical to that of the excitation block. This can be proved as follows. It is obvious from Fig. 1a that the first six C7 units in the phase-permuted and time-shifted C7 sequence have the same average Hamiltonians $\bar{H}_p^{(0)}$ as before, since neither the timing of the pulses (t^0) relative to the rotor period nor the phases (ϕ_p) of the pulses have changed. For the last C7 unit, the pulse phase has returned to the initial value $\phi_0 = 0$ of the original sequence, while the pulse cycle occurs exactly one rotor period after the first C7 unit C_{ϕ_0} of the original sequence, due to $t^0 = t_1 + 6\tau_c = 7\tau_c = \tau_r$. Thus the amplitude of the average Hamiltonian during this last C7 unit is identical to that of C_{ϕ_0} in the original sequence,

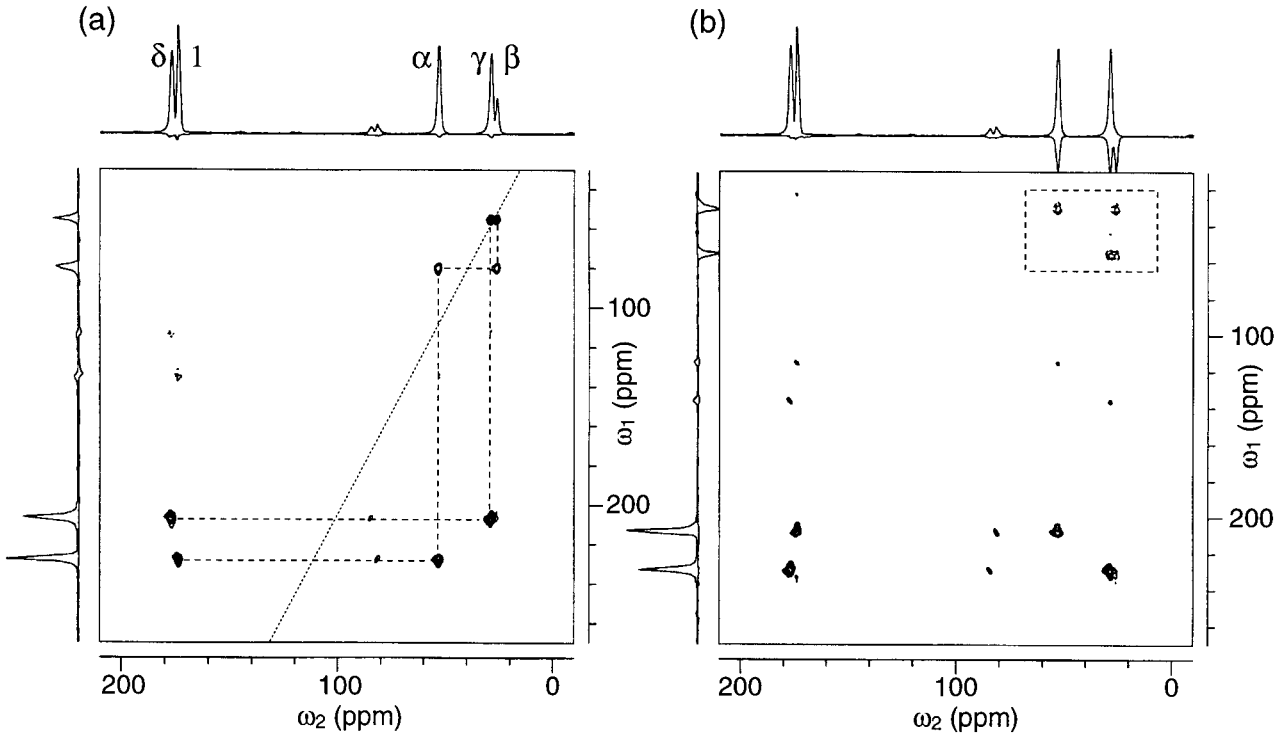

FIG. 2. (a) Two-dimensional ^{13}C dipolar INADEQUATE spectra of uniformly ^{13}C-labeled glutamine. Dashed lines indicate the connectivities, and Greek letters represent the spectral assignment. The expected tilted diagonal across the center of each pair of double-quantum signals is shown as a dotted line. The projections of positive and negative signals along each dimension are also shown. The spectrum was acquired with a double-quantum excitation time of 285.6 μs ($=2\tau_r$) and a t_1 dwell time of 40.8 μs. A total of 160 complex t_1 points were acquired. The total acquisition time, which was limited by the length of the phase cycle (32 steps), was 8.5 h. (b) ^{13}C INADEQUATE spectrum of the same sample acquired without the synchronous phase switching of the C7 pulses in the DQ reconversion block. Otherwise, the conditions were the same as those in (a). The dashed rectangle in the upper right corner of the spectrum indicates the negative peaks observed. Note the inequality between the DQ frequencies in the ω_1 dimension and the sum of the single-quantum frequencies in the ω_2 dimension. The chemical shift scale along the ω_1 axis was made arbitrarily to represent the same (Cβ, Cγ) sum chemical shift as that in the correct spectrum of (a).

$$\bar{\omega}^{Q}_{\lambda\mu lm}(\Omega_{PR}, t^0 = \tau_r) = A(\Omega_{PR}) \int_0^{\tau_c} d^{\lambda}_{\mu 0}(-\beta_{RF}(\tau)) \times \exp(im\omega_r(\tau + \tau_r))d\tau = \bar{\omega}^{Q}_{\lambda\mu lm}(\Omega_{PR}, t^0 = 0). \quad [3]$$

Combined, the zeroth-order average Hamiltonian of the permuted C7 sequence is identical to that of the original sequence

$$\bar{H}^{(0)}_0(0) + \bar{H}^{(0)}_1(\tau_c) + \cdots + \bar{H}^{(0)}_6(6\tau_c) = \bar{H}^{(0)}_1(t_1 = \tau_c) + \cdots \bar{H}^{(0)}_6(6\tau_c) + \bar{H}^{(0)}_0(\tau_r). \quad [4]$$

Therefore, to carry out a 2D INADEQUATE experiment, we simply need to increment t_1 with a step of $\tau_c = 2\tau_r/7$, and shift the overall phase of the reconversion pulses by $\Delta\phi = 2\pi/7$ for successive t_1 values. The durations of the excitation and reconversion periods must be integer multiples of the rotor period. In this way, we maintain the phase correspondence between the DQ excitation and reconversion blocks while still having a t_1 dwell time small enough to ensure a large ω_1 spectral width. The pulse sequence of this dipolar INADEQUATE experiment is shown in Fig. 1b.

This dipolar INADEQUATE experiment is demonstrated on a uniformly ^{13}C- and ^{15}N-labeled sample of glutamine (Cambridge Isotope Laboratories, Andover, MA). The experiment was carried out on a Bruker DSX-300 spectrometer using a triple-resonance MAS probe equipped with a 4-mm spinning module. Phase-sensitive chemical shift spectra in the ω_1 dimension were acquired in the hypercomplex manner (*30*). This involves changing the phases of the DQ reconversion pulses by 45° between the cosine and the sine data sets (*31*). The sample was spun at 7 kHz, requiring a C7 RF field strength of 49 kHz. According to the above discussions, these conditions constrained the t_1 dwell time to $\tau_c = 40.8$ μs. This corresponds to a DQ spectral width of 325 ppm. Figure 2a displays the properly acquired ^{13}C dipolar INADEQUATE spectrum of glutamine. Only 90% of the ω_1 window is shown. Four pairs of DQ cross peaks in the ω_1 dimension are observed for the six-carbon molecule. The tilted "diagonal" with slope 2, characteristic of INADEQUATE spectra, is found by connecting

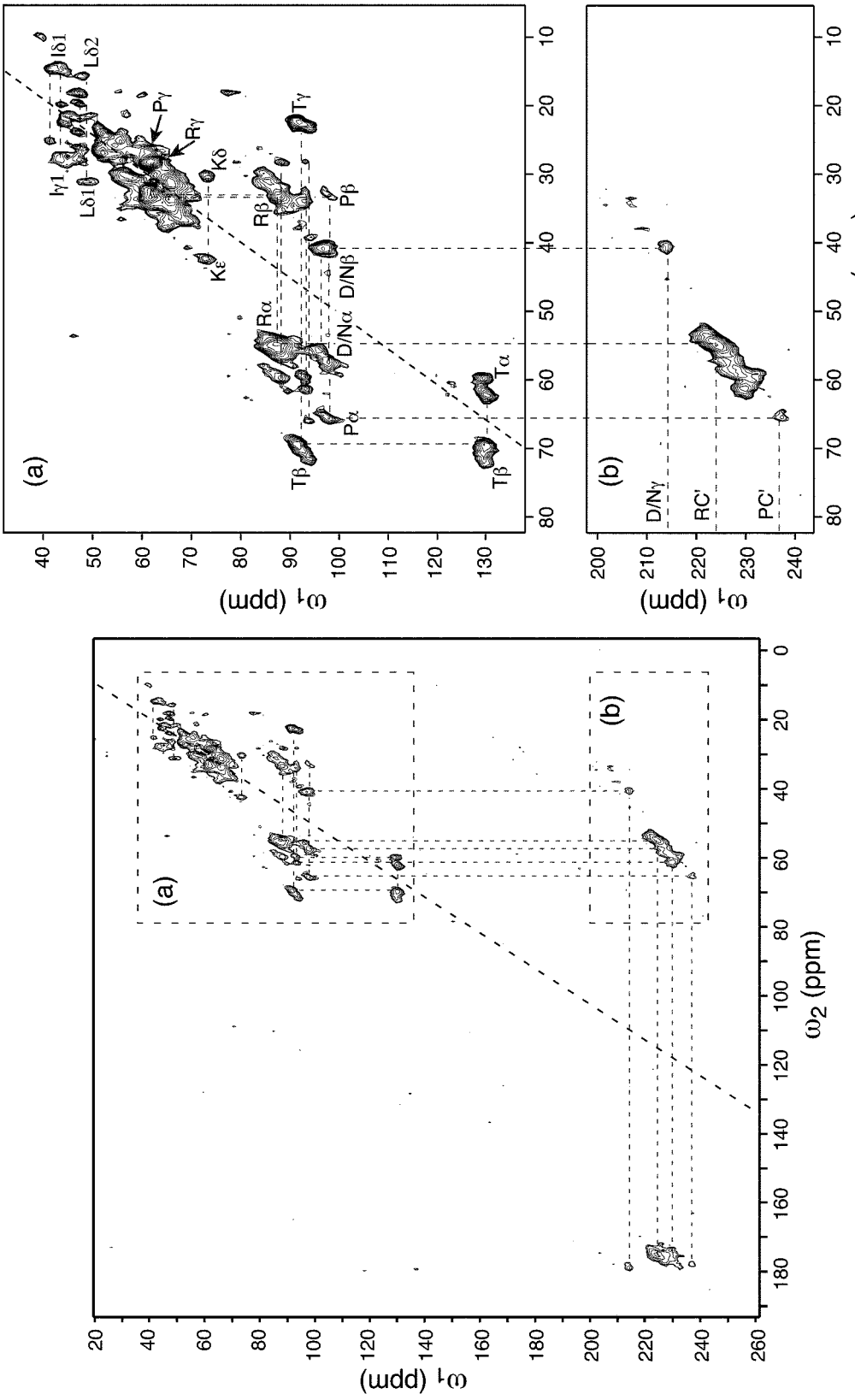

FIG. 3. ^{13}C dipolar INADEQUATE spectrum of extensively ^{13}C-labeled ubiquitin (M_r = 8565). (a) Aliphatic region. (b) Cross-peak region between the aliphatic and the carbonyl carbons. The double-quantum dimension (ω_1) has a total spectral width of 325 ppm. To reduce the offset dependence of the recoupling sequence, CMR7 (29), a variant of the C7 sequence, was used for DQ excitation and reconversion. The DQ excitation time was 571 μs. The spinning speed was 7 kHz. Total acquisition time was 21 h.

the midpoint of the two signals in each cross section. The two carbonyl resonances are assigned based on their connectivities with the aliphatic carbons. The upfield carbonyl is coupled to the well-resolved Cα signal (which is assigned based on the chemical shift alone); thus it results from C1. This makes the downfield carbonyl carbon the sidechain Cδ, whose aliphatic coupling partner must then be assigned to Cγ. Cγ is in turn coupled to Cβ, the most upfield resonance of all, which exhibits a coupling to Cα. In the spectrum, couplings between nondirectly bonded carbons are suppressed due to the short mixing time ($\tau_{exc} = \tau_{rec} = 285.6$ μs) used to excite the DQ coherence. In addition to the isotropic peaks, the spectrum also exhibits weak spinning sidebands in both dimensions.

It is clear from the above discussion that if the C7 reconversion pulses always start with the same phase at the end of t_1, then the average Hamiltonian $\Sigma_p \bar{H}_p^{(0)}(t^0 = t_1 + p\tau_c)$ of the reconversion period differs from that of the excitation period $\Sigma_p \bar{H}_p^{(0)}(t^0 = p\tau_c)$. To show that the pulse-synchronized t_1 incrementation and the rotor-synchronized phase switching are indeed both necessary for the success of this dipolar INADEQUATE experiment, we performed an alternative experiment without these features for comparison. It was acquired with the same t_1 dwell time but without the synchronous phase-switching scheme in the reconversion block. The resulting spectrum (Fig. 2b) exhibits several remarkable features. First, the basic coupling pattern is correctly manifested. There are no dispersive intensities or spurious sidebands, which might be expected for such a time-sensitive pulse sequence. However, the aliphatic DQ signals exhibit *negative* intensities while the carbonyl-aliphatic cross peaks remain *positive*. In addition, the double-quantum frequency is no longer the sum of the single-quantum chemical shifts, i.e., $(\omega_1, \omega_2) \neq (\Omega_i + \Omega_j, \Omega_{i,j})$. This means no tilted diagonal exists for the spectrum. In fact, the chemical shift scale of the ω_1 dimension seems to have no meaning, and it is not possible to derive the altered spectrum by folding the correct double-quantum spectrum. These peculiar artifacts may be qualitatively understood by considering that the insertion of the evolution period without subsequent compensation by the phase of the DQ reconversion pulses is equivalent to imparting an additional modulation factor to the indirectly detected signals.

To further demonstrate the utility of this large-spectral-width dipolar INADEQUATE experiment, we applied it to a 76-residue protein, ubiquitin ($M_r = 8565$). The sample was extensively enriched with ^{13}C (*32, 33*). Over 50 peaks are completely or partially resolved in the spectrum. About half of them can be assigned to the amino acid types based on the connectivity patterns, the ^{13}C chemical shifts, and the labeling pattern resulting from the biosynthetic expression. The spectral resolution is quite high for a protein of this molecular weight: the peak widths (full width at half maximum) in the ω_2 dimension are <1 ppm for methyl and carbonyl carbons and $\sim$1.5 ppm for methylene carbons. More complete assignment may be obtained by combining other techniques such as 2D ^{15}N–^{13}C correlation.

This large-spectral-width dipolar INADEQUATE experiment is practically limited to spinning speeds between about 4000 and 8000 Hz. According to the C7 resonance condition and the phase permutation scheme discussed here, these spinning speeds would yield t_1 dwell times between 71.4 and 35.7 μs, which correspond to DQ spectral widths from 14 to 28 kHz. The upper limit is sufficient for ^{13}C spectroscopy on spectrometers up to 100 MHz ^{13}C Larmor frequency, while the lower limit is insufficient for a DQ spectrum except for ^{13}C Larmor frequencies below 50 MHz. Higher spinning speeds ($>$8 kHz) would require correspondingly higher RF fields ($>$56 kHz) on the ^{13}C channel, which would induce signal loss due to CP leakage to protons under the typical ^{1}H decoupling fields accessible today ($\sim$100 kHz).

In conclusion, we have shown that high-resolution ^{13}C dipolar INADEQUATE spectroscopy can be used to assign the ^{13}C spectra of unoriented ^{13}C-labeled solids. The dipolar-based polarization transfer can be achieved with the recoupling sequence C7 or its variants. The INADEQUATE spectrum has a large unfolded DQ spectral width while maintaining rotor synchronization of the C7 sequence. This is achieved by making the t_1 dwell time equal to the duration of one basic C7 unit and shifting the phase of the reconversion pulses correspondingly forward. This large-double-quantum spectral-width dipolar INADEQUATE experiment, with the principal of rotor-synchronized t_1- and RF-phase incrementation, can be equally performed using other DQ dipolar recoupling sequences such as MELODRAMA (*22*).

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