C-13-detected IPAP-INADEQUATE for simultaneous measurement of one-bond and long-range scalar or residual dipolar coupling constants

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Rapid Communication

^{13}C-detected IPAP-INADEQUATE for simultaneous measurement of one-bond and long-range scalar or residual dipolar coupling constants

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The sensitivity of cryoprobes, which are rapidly becoming available, means that the measurement of coupling constants involving ^{13}C, ^{13}C pairs at the natural abundance of ^{13}C can now, in principle, be done by using tens rather then hundreds of milligrams of compounds. However, a robust method that would yield reliable values of small long-range carbon--carbon coupling constants is still missing. In this Communication, we describe a novel ^{13}C–detected incredible natural-abundance double-quantum transfer experiment (INADEQUATE) experiment for simultaneous correlation of one-bond and long-range ^{13}C–^{13}C pairs and the measurement of both types of coupling constants in ^{13}C natural abundance samples. This method yields accurate values of one-bond and long-range coupling constants by manipulation of pure phase in-phase (IP) and antiphase (AP) doublets, and is referred to as ^{13}C-detected IPAP-INADEQUATE. It is illustrated by the measurement of interglycosidic $^{3}J_{CCOC}$ coupling constants in a disaccharide molecule providing important information about the conformation of the glycosidic linkage. Owing to the simplicity of INADEQUATE spectra the carbon–carbon coupling constants are particularly suitable for studies of partially oriented molecules through the measurement of carbon–carbon residual dipolar couplings (RDCs). An example of this approach is presented. We expect the method to find a variety of applications in the conformational analysis of small molecules, determination of diastereoisomers and enantiomers, and studies of molecules in aligned media. Copyright © 2007 John Wiley & Sons, Ltd.

KEYWORDS: NMR; ^{13}C; INADEQUATE; IPAP; carbon--carbon coupling constants; RDC; aligned media; conformation; carbohydrates

INTRODUCTION

The ultimate NMR experiment for the structure determination of small and medium size molecules is based on the tracing of the carbon–carbon connectivities via $^{1}J_{CC}$ coupling constants. In addition, the size of long-range carbon–carbon coupling constants reports on the stereochemistry and conformation of molecules. In 1980, incredible natural-abundance double-quantum transfer experiment (INADEQUATE) has been proposed[1,2] making it possible to obtain carbon–carbon chemical shift correlation on samples with natural abundance of ^{13}C. To this day, numerous implementations of DQ filtration – the essence of INADEQUATE – have appeared focusing on direct[3–8] or remote carbon–carbon pairs.[5,7,9–12] Most of the more recent versions of INADEQUATE employ ^{1}H detection,[1–3,3–13] an approach which is only partially successful in increasing the sensitivity of this experiment, as discussed previously.[4,13,14]

At the natural abundance of ^{13}C, INADEQUATE is indeed a rather insensitive experiment, as only one in 8734 molecules contains a ^{13}C,^{13}C pair. Nevertheless, with the increasing availability of cryoprobes, INADEQUATE is about to realize its potential to become a mainstream NMR experiment. In order to achieve this, an ideal INADEQUATE experiment needs to offset its inherently lower sensitivity by maximizing its information content. Such an experiment should provide (i) simultaneous one-bond and long-range correlation, and (ii) accurate values of both $^{1}J_{CC}$ and $^{n}J_{CC}$ coupling constants, all within a single measurement.

We present here a ^{13}C-detected INADEQUATE experiment that fulfils these criteria and illustrate its use on the accurate measurement of scalar and dipolar carbon–carbon coupling constants of carbohydrates.

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RESULTS AND DISCUSSION

We take, as the basis for our development, the original nonrefocused INADEQUATE experiment,[2] with a modified phase cycling,[15] and optimize its preparation period, τ for long-range correlations (Fig. 1(a)). Unlike a similar experiment proposed in the past,[16] we record two experiments by setting $\tau = 0.5/^{n}J_{CC}$ or $\tau = 0.5/^{n}J_{CC} + 0.5/^{1}J_{CC}$. Such variation of the evolution interval only marginally affects the intensity of long-range cross peaks, but ensures that the one-bond cross peaks appear at least in one of the two spectra. We use, wherever possible, broad-band excitation and inversion pulses[17,18] – an approach proposed previously for inversion pulses.[19]

The determination of $^{1}J_{CC}$ values from the nonrefocused INADEQUATE spectra is trivial because of large separation of the two components of the one-bond antiphase doublets. Prior to peak picking, it maybe beneficial to add or subtract the two spectra, providing both contain one-bond cross peaks with sufficient intensity. When analyzing the long-range cross peaks, it is always beneficial to add the two spectra. The determination of $^{n}J_{CC}$ coupling constants is however non trivial, as partial cancellation of closely spaced antiphase lines produces an apparent splitting that is larger than the true coupling constant.[11,20,21] The use of inverse FT methods[22,23] in the analysis of antiphase doublets is also problematic. These methods rely on significant presence of the signal after the zero crossing, which is difficult to achieve in noise INADEQUATE cross peaks containing small coupling constants.

It has been illustrated by numerous NMR experiments that editing of the corresponding antiphase and in-phase multiplets is a possible route towards accurate determination of coupling constants from unresolved multiplets.[20,21,24–26] This approach has since been successfully applied to the measurement of scalar and dipolar coupling constants of biomolecules under the acronym IPAP.[27] We therefore record two additional refocused INADEQUATE[28] spectra by inserting a refocusing interval, followed by an efficient purging of the residual antiphase component, which suppresses both DQ and ZQ coherences[29] (Fig. 1(b)). As the coupling constants are obtained by editing of the in-phase and antiphase doublets, we refer to the proposed methods as ^{13}C-detected IPAP-INADEQUATE.

However, for poorly resolved multiplets, editing of in-phase and antiphase multiplets works reliably only when the relationship between the intensities of the two multiplets is known.[30] We therefore calculate the scaling factor for the antiphase multiplets by using the relevant transfer functions and effective ^{13}C spin–spin relaxation times determined in separate experiments. The details of this procedure are provided in the Experimental section. We note that the accuracy of the scaling factors may be affected by the low signal-to-noise ratio of INADEQUATE spectra. We therefore perform simulations assessing the effects of inaccurate scaling factors on coupling constant determination by IPAP-INADEQUATE. Simulated editing of poorly resolved in-phase and antiphase Lorentian doublets (Fig. 2) showed that (i) larger coupling constants are less susceptible to errors arising from the use of improper scaling factors (ii) underestimating the scaling factors leads to larger errors than overestimating them and (iii) deviations of 50% from the correct value of the scaling factors result in <20% error in the determination of small (2–4 Hz), unresolved coupling constants. We can therefore conclude that any inaccuracies in the determination of the scaling factors are, to a large extend, absorbed by the method.

We used 70 mg of Me-β-D-xylopyranoside (**1**) dissolved in 350 µl of D_2O for the initial tests of our method. The experiment was repeated using an aligned[31] sample of **1**. The analysis of one-bond and long-range cross peaks from both the experiments is shown in Figs 3 and 4, respectively. The determined coupling constants are given in Table 1. The experimental D_{CC} coupling constants agree very well with

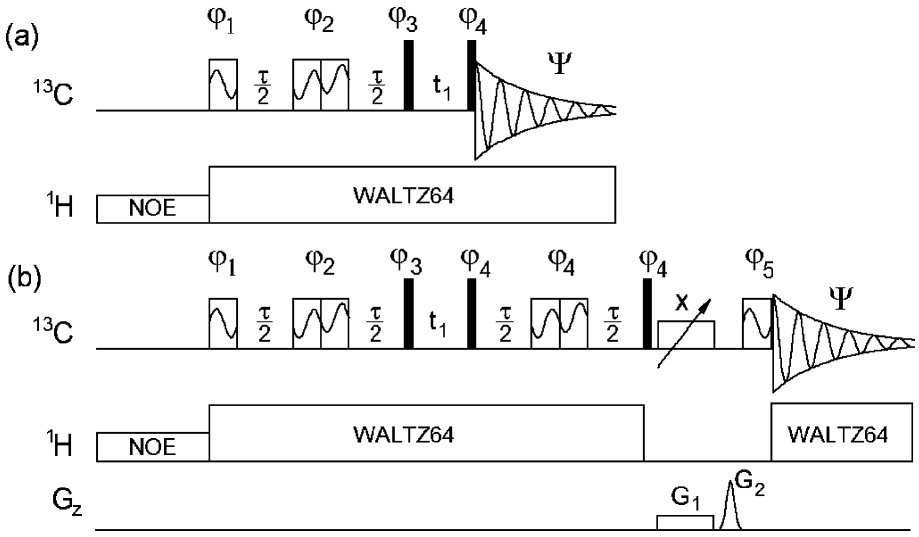

Figure 1. ^{13}C-detected, phase cycled IPAP INADEQUATE. (a) Nonrefocused (AP), and (b) refocused (IP) INADEQUATE: $\tau = 0.5/^{n}J_{CC}$ or $0.5/^{n}J_{CC} + 0.5/^{1}J_{CC}$; the 90° rectangular pulses are shown as filled rectangles; 90° BEBOP and 180° BIBOP pulses[17,18] are indicated as narrow and wide rectangles, respectively; the adiabatic inversion pulse is designated by an inclined arrow; the phase cycling[15] is given in the Experimental section.

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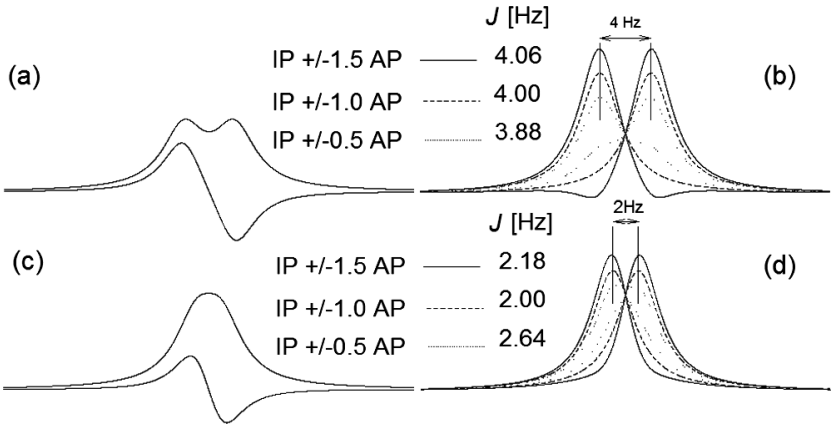

Figure 2. Simulated editing of in-phase and antiphase Lorentian lines. (a) and (c) show in-phase and antiphase 4 and 2 Hz doublets, respectively. (b) and (d) show corresponding edited multiplets obtained using the scaling factor given in the inset. The coupling constants determined by peak picking of the edited doublets are given. The effective relaxation time of 80 ms was used for the simulations. The scaling factor was varied by ±50% from its correct value.

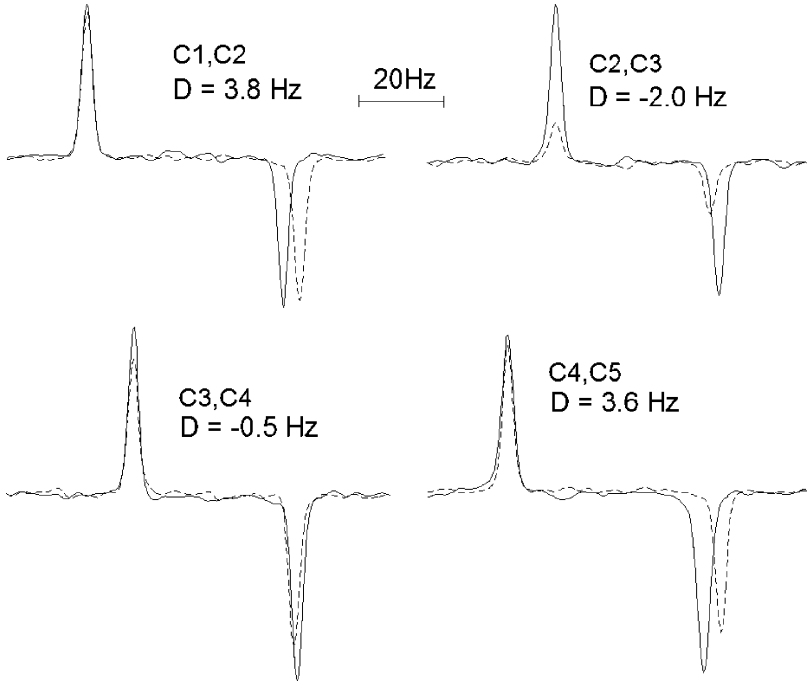

Figure 3. One-bond cross peaks extracted from AP INADEQUATE spectra of **1**. Solid and dashed lines show antiphase doublets acquired in isotropic and aligned media, respectively. The signals are overlaid on the left part of the doublet in order to visualize the differences between the splitting in the two media.

the predicted residual dipolar couplings (RDCs) calculated using the solution structure[32] of **1**.

The use of carbon–carbon RDCs for the structural investigation of small organic molecules in aligned media is a very attractive proposition.[33] Many media proposed thus far for the organic solvents tend to align molecules rather strongly. The consequent appearance of higher order effects prevents accurate measurement of D_{HH} and $^1D_{CH}$ coupling constants. Since only two ^{13}C spins are selected in each molecule by the INADEQUATE pulse sequence, the extraction of numerous carbon–carbon RDCs is no different from the measurement of J_{CC} coupling constants (Figs 3 and 4). The ^1H-detected INADEQUATE is not particularly suited for the measurement of carbon--carbon RDCs because of the broadening of ^1H multiplets by numerous ^1H–^1H RDCs in the aligned media.

Our second example focuses on the measurement of $^3J_{CC}$ coupling constants across the glycosidic linkage of carbohydrates. These coupling constants are extremely valuable for the conformational analysis of carbohydrates as they complement $^nJ_{CH}$ coupling constants,[34] which by themselves do not fully characterize the conformation of this flexible part of the carbohydrate molecules.[35] For this experiment we have chosen a more realistic amount of sample – 25 mg of a disaccharide, Me-β-D-lactoside (**2**, M_W = 356 g mol^{-1}) – to record the IPAP-INADEQUATE spectra in 42 h. Figure 5 shows traces extracted at DQ frequencies correlating carbons across the glycosidic linkage.

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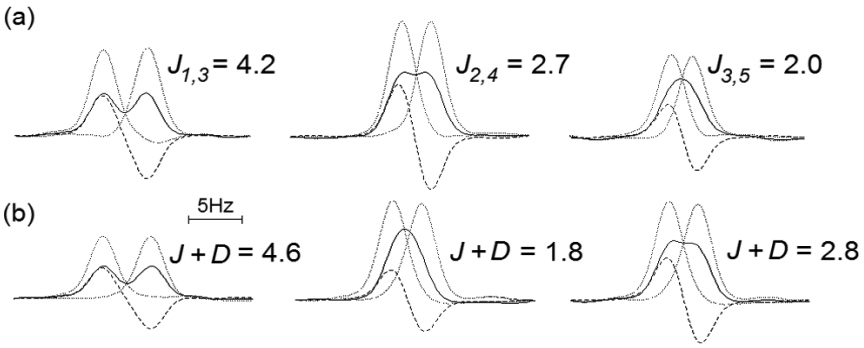

Figure 4. Analysis of long-range cross peaks: (a) isotropic and (b) aligned sample of **1**. Dashed and solids lines show antiphase and in-phase multiplets. Dotted singlets were obtained by the addition and subtraction of the in-phase and antiphase multiplets using appropriate scaling factors.

Table 1. Carbon–carbon coupling constants of **1**, in Hz, determined from in- and antiphase spectra of the isotropic and aligned sample

	C_1C_2	C_2C_3	C_3C_4	C_4C_5	C_1C_3	C_2C_4	C_3C_5
J	46.8	38.7	39.0	39.8	4.2	2.7	2.0
$J + D$	50.6	36.7	38.5	43.5	4.6	1.8	2.8
D_{exp}	3.8	−2.0	−0.5	3.6	0.4	−0.9	0.8
D_{cal}	3.8	−2.0	−0.4	3.6	0.3	−0.8	0.6

Parameters of the alignment tensor: $S_{x'x'} = (2.43 \pm 0.02) \times 10^{-4}$, $S_{y'y'} = (9.09 \pm 0.13) \times 10^{-4}$, $S_{z'z'} = (-1.15 \pm 0.01) \times 10^{-3}$. The Euler angles in the molecular coordinate system of **1**: $\alpha = 260.0 \pm 0.3$, $\beta = 128.3 \pm 0.2$ and $\gamma = 85.9 \pm 1.1$ degrees. Six $^1D_{CH}$ coupling constants (from −1.4 to −10.8 Hz) were also used during this calculation.

The average S/N ratio of 10 : 1 (no window function in F_2) was obtained for long-range cross peaks, which is adequate for accurate coupling constant determination. Coupling constants $^3J_{C1,C5'} = 2.0$ Hz and $^3J_{C2,C4'} = 3.1$ Hz were obtained, while no cross peaks were observed for the third pair of carbons (C1, C3′) across the glycosidic linkage as the corresponding coupling constant is close to zero.[35] These coupling constants allowed characterizing the dihedral angles across the glycosidic linkage of **2**.[35]

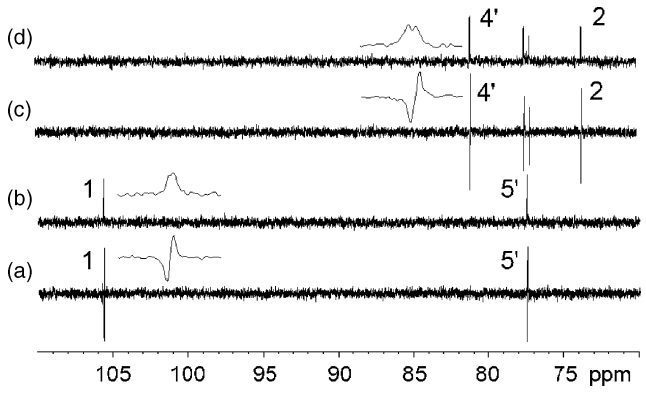

Figure 5. F_2-traces from the AP (a), (c) and IP (b), (d) INADEQUATE spectra of **2** at DQ frequencies of C_1C_5' (a), (b) and C_1C_4' (c), (d). The insets show the expansions of individual doublets. All the spectra are plotted using identical vertical scale.

Using the same sample, spectrometer, and identical experimental time, a ^{1}H-detected J-modulated IPAP-INADEQUATE experiment combining the existing J-modulated INADEQUATE experiments[10,11] yielded S/N ratio of 8 : 1 (no window function in F_1, data not shown). General reasons for such a good performance of the ^{13}C-detected INADEQUATE were discussed previously.[4,13,14] Specific to our experiment is the use of ^{1}H decoupling during long evolution intervals that prolongs the effective relaxation of carbon coherences and eliminates the leakage of magnetization for protonated carbon pairs.[13] The use of phase cycling, rather than pulsed field gradients for coherence selection, not only increases the S/N ratio by a factor of 1.41, but also eliminates diffusion related losses that could be severe for small molecules and long evolution intervals. Our data were acquired using inverse triple-resonance probes with a cold ^{13}C preamplifier. A further factor of two in S/N could be gained by acquiring spectra on a direct ^{13}C cryoprobe.

CONCLUSION

In conclusion, we have presented a robust method for a simultaneous determination of one-bond and long-range carbon–carbon correlations and the measurement of corresponding carbon–carbon coupling constants. The ^{13}C-detected IPAP-INADEQUATE experiment is expected to find a variety of applications in the conformational analysis of small molecules, determination of diastereoisomers and enantiomers, and studies of molecules in aligned media.

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EXPERIMENTAL

The following phase cycling was used for IPAP-INADEQUATE experiment.

$$\varphi_1 = (8)\,2\,2\,6\,6\,2\,2\,6\,6\,4\,4\,0\,0\,4\,4\,0\,0$$
$$6\,6\,2\,2\,6\,6\,2\,2\,0\,0\,4\,4\,0\,0\,4\,4$$
$$\varphi_2 = (8)\,4\,0\,0\,4\,4\,0\,0\,4\,6\,2\,2\,6\,6\,2\,2\,6$$
$$0\,4\,4\,0\,0\,4\,4\,0\,2\,6\,6\,2\,2\,6\,6\,2$$
$$\varphi_3 = (8)\,0\,0\,4\,4\,0\,0\,4\,4\,2\,2\,6\,6\,2\,2\,6\,6$$
$$4\,4\,0\,0\,4\,4\,0\,0\,6\,6\,2\,2\,6\,6\,2\,2$$
$$\varphi_4 = \quad 0\,1\,2\,3\,1\,0\,3\,2\,2\,3\,0\,1\,3\,2\,1\,0$$
$$\varphi_5 = \quad 0\,1\,2\,3\,1\,0\,3\,2\,2\,3\,0\,1\,3\,2\,1\,0$$
$$\psi = \quad 0\,3\,2\,1\,3\,0\,1\,2$$

The phase increment is equal to 90°, except for when symbol (8) is used, where the phase increment is equal to 45°.

The IPAP-INADEQUATE spectra of **1** were acquired using a 600 MHz triple-resonance cryoprobe with a cold ^{13}C preamplifier and 70 mg of compound, dissolved in 350 μL of D_2O (Shigemi tube). The t_1 and t_2 acquisition times were 23.2 and 452 ms, respectively. Using the relaxation time of 1s, 32 scans were acquired in each of 182 complex points of four interleaved IPAP-INADEQUATE experiments. The overall experimental time was 22 h. The experiment was repeated on the aligned sample of **1** using identical conditions. The aligned sample was prepared using a mixture of $C_{12}E_5$ and hexanol. 128 μl of $C_{12}E_5$, 38.8 μl of hexanol and 300 μl of D_2O were used to dissolve 70 mg of **1**. The mass fraction of the medium was 22% with the molar ratio r = 0.99 of $C_{12}E_5$/hexanol. The splitting of the deuterium signal at 25 °C was 138.8 Hz.

An 800 MHz triple-resonance cryoprobe with a cold ^{13}C preamplifier was employed to acquire IPAP-INADEQUATE spectra using 25 mg of a disaccharide, **2**. The t_1 and t_2 acquisition times were 9.3 and 511 ms, respectively. Using relaxation time of 0.5 s, 256 scans were acquired in each of 112 complex points of two interleaved experiments. The overall experimental time was 42 h. The variation of the evolution intervals was not activated, as the focus of the experiment were the long-range coupling constants.

The evolution interval, τ, was optimized for $^nJ_{CC} = 3.0$ Hz in all the experiments. The 90° BEBOP and 180° BIBOP pulses[17,18] were 700 and 1400 μs long and applied at $\gamma B_1/2\pi = 12.5$ KHz. A 20 ms adiabatic pulse[36] was used in pulse sequence of Fig. 1(b) simultaneously with a 2.1 Gcm^{-1} pulsed field gradient, G_1, followed by a purging gradient, $G_2 = 23.8\ G\ cm^{-1}$. The 2D spectra were zero filled to yield the digital resolution of 0.25 Hz/point in F_2. No window function was used in F_2.

The scaling factor between the long-range in-phase and antiphase multiplets was determined as follows. A resolved, long-range (or one-bond) in-phase doublet was used as a reference peak, for which the carbon–carbon coupling constant was determined by peak picking. All in-phase long-range doublets were integrated and the ratios of the integrals with regard to the reference signal were used to determine an initial value of the coupling constant, J_{ini}. Generally, these integral ratios need to be corrected by taking into account differences in ^{13}C T_1 relaxation times. This was not necessary for carbohydrates **1** and **2** due to uniform relaxation of their CH carbons. The integrals were either determined by using the integration routine or signal deconvolution. In these calculations, the effective relaxation times of individual carbons, determined in separate experiments, were used. The initial value of the coupling constant was calculated as:

$$J_{\mathrm{ini}} = \frac{1}{\pi\tau}\arcsin\left(\left(\frac{I}{I_{\mathrm{ref}}}\right)^{1/2}\frac{\sin(\pi J_{\mathrm{ref}}\tau)\exp\left(-\tau/T_{2\mathrm{eff}}^{\mathrm{ref}}\right)}{\exp\left(-\tau/T_{2\mathrm{eff}}\right)}\right) \quad (1)$$

where I_{ref} and I are the integrals of the in-phase cross peaks of the reference doublet and that of the investigated doublet; J_{ref} and J and T_{2eff}^{ref} and T_{2eff} are corresponding coupling constants and effective carbon spin–spin relaxation times. The scaling factor for the antiphase doublet prior to its addition/subtraction with the in-phase double is given by:

$$S = A(\upsilon)\sin(\pi J_{\mathrm{ini}}\tau)\exp(-\tau/T_{2\mathrm{eff}}) \quad (2)$$

where $A(\nu)$ is a constant, which accounts for the fact that the refocused pulse sequence contains more pulses and therefore the intensity of in-phase multiplets will be affected more by the pulse imperfections and off resonance effects than those of the in-phase multiplets. This correction factor is frequency dependent, following the excitation profile of the 90 rectangular pulse used to flip the magnetization into the z-axis prior to the purging of ZQ/DQ coherences. All the other pulses are not offset dependent. For carbohydrates, with a narrow spread of ^{13}C chemical shifts, this frequency dependence can, however, be safely ignored. A uniform correction factor, A = 0.9 was used in this work, as determined through the intensity changes of one-bond correlation cross speaks of $^{13}C1$ labeled glucose measured in AP and IP INADEQUATE spectra. When a short 90° broad-band universal rotator with negligible off resonance effects becomes available, it is advisable to use it in place of the remaining rectangular 90° pulses. This will remove the frequency dependence of A.

The relaxation during the de- and refocusing τ intervals of the IPAP-INADEQUATE pulse sequence involves coherences of coupled carbons i and j in the form of $C_{i,xy}$ and $2C_{i,xy}\,C_{j,z}$. Assuming that both coherences exist for one halve of τ, the effective relaxation rate of C_i is given[9] as: $[T_{2\mathrm{eff}}]^{-1} = [T_2\ (C_i)]^{-1} + 0.5[T_1\ (C_j)]^{-1}$. The T_2 and T_1 relaxation times of **1** and **2** were determined, respectively, by acquiring a series of ^{13}C spin-echoes with proton decoupling (effectively 90-τ/2-180-τ/2 of the INADEQUATE pulse sequence) and the inversion recovery spectra, and also using ^1H decoupling during the recovery delay. The peak intensities were fitted as single exponential decays. Given the concentrations required for the INADEQUATE, these additional experiments required only minutes to complete.

In case when none of the long-range cross peaks is resolved, one-bond cross peaks could be used as reference signals. In principle, this requires estimating the additional

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contribution to the effective carbon spin–spin relaxation times caused by $^{13}C-^{13}C$ dipolar relaxation of directly bonded carbon pairs. In ^{13}C1 labeled glucose, the relaxation times of C1 and C2 atoms were, respectively, 10 and 20% shorter than those of the corresponding carbons in the unlabeled glucose. According to our simulations (see the main text), the uncertainty of the scaling factor of this magnitude has a negligible effect on the obtained values of coupling constants. This makes the one-bond cross peaks an attractive alternative to the long-range cross peaks to act as reference signals.

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