

Accurate Measurements of Multiple-Bond ^{13}C - ^1H Coupling Constants from Phase-Sensitive 2D INEPT Spectra

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Measurements of multiple-bond ^{13}C - ^1H coupling constants are of great interest for the assignment of nonprotonated ^{13}C resonances and the elucidation of molecular conformation in solution. Usually, the heteronuclear multiple-bond coupling constants were measured either by the J_{CH} splittings mostly in selective 2D spectra or in 3D spectra, which are time consuming, or by the cross peak intensity analysis in 2D quantitative heteronuclear J correlation spectra (1994, G. Zhu, A. Renwick, and A. Bax, *J. Magn. Reson. A* **110, 257; 1994, A. Bax, G. W. Vuister, S. Grzesiek, F. Delaglio, A. C. Wang, R. Tschudin, and G. Zhu, *Methods Enzymol.* **239**, 79.), which suffer from the accuracy problem caused by the signal-to-noise ratio and the nonpure absorptive peak patterns. Concerted incrementation of the duration for developing proton antiphase magnetization with respect to carbon-13 and the evolution time for proton chemical shift in different steps in a modified INEPT pulse sequence provides a new method for accurate measurements of heteronuclear multiple-bond coupling constants in a single 2D experiment.** © 1999 Academic Press

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A variety of 2D and 3D experiments have been developed for the measurements of ^1H - ^{13}C multiple-bond coupling constants (1–20). In most 2D experiments, selective techniques were used (3–11), and to complete the measurement, a series of these selective spectra should be obtained, which is equivalent to measure a 3D spectrum. Since the multiple-bond J_{CH} and J_{HH} are in the range 0–20 Hz and the spectral width of proton chemical shifts is about 4 kHz in a 9.4 T magnetic field as an example, the digital resolution normally set in the ω_1 dimension in a 2D ^1H - ^{13}C correlation spectrum is not enough to resolve the antiphase doublets of J_{CH} couplings, so that a third frequency dimension should be introduced for observing the coupling splittings. It is well known that 3D experiment is extremely time-consuming. Accordion spectroscopy (21) is a useful tool to observe two interactions at different time scales in a single frequency dimension, which can be used to reduce the dimensions of some 3D or 4D experiments. Using the principle of accordion spectroscopy, a new method can be

proposed for accurate measurements of heteronuclear multiple-bond coupling constants.

As shown in Fig. 1A the pulse sequence is modified from refocused INEPT (22, 23) sequence by setting the interpulse delay between the first 90° pulse (^1H) and the refocusing 180° pulses (^1H , ^{13}C) to $(n + 1)t_1/2$ and the interpulse delay between the refocusing 180° pulses (^1H , ^{13}C) and the second 90° pulses (^1H , ^{13}C) to $(n - 1)t_1/2$. This is equivalent to setting the evolution time for proton chemical shifts to t_1 and the evolution time for heteronuclear and homonuclear couplings to nt_1 . The constant interpulse delay Δ is optimized for multiple-bond J_{CH} couplings the same as in the COLOC (24) experiment. For a given parameter n , t_1 is incremented, in a series of experiments, by the step of Δt_1 determined by the spectral width of proton chemical shifts, which results in a 2D spectrum with the cross peaks split by both heteronuclear and homonuclear couplings. In this spectrum, the splittings of heteronuclear and homonuclear couplings are increased by n times. The pulse sequence in Fig. 1B is obtained by replacing the first 90° pulse (^1H) in 1A by a BIRD sequence (25) to suppress one-bond J_{CH} correlations.

The parameter n is set so that the multiplets of J_{CH} couplings can be well resolved at the set digital resolution in ω_1 dimension and there is no severe sensitivity loss due to too much noise acquired at longer nt_1 as well as an n -time-increase in the apparent linewidths along the ω_1 dimension. The measured J_{CH} splittings divided by n gives the true values of J_{CH} coupling constants. In practice, that n is about 10 can be suggested. In this case, the sensitivity of the methods approaches that of the COLOC experiment. Since the heteronuclear coupling splitting gives an antiphase doublet (25) and the homonuclear ones give in-phase multiplets, the splittings of J_{CH} couplings can be easily distinguished from the splittings of homonuclear ones in a phase-sensitive 2D spectrum. The phase cycles of the pulse sequences in Fig. 1 are described in detail in the figure caption, where the TPPI procedure (26, 27) is employed to record 2D phase-sensitive spectra.

Generally, those J_{CH} splittings being larger than the apparent linewidth can be accurately measured if there is no FID truncation in the t_1 dimension and the digital resolution is enough

in the ω_1 dimension, and for J_{CH} splittings being less than the apparent linewidth there are errors due to displacement of peak maxima (28), even if there is no FID truncation in the t_1 dimension and the digital resolution is enough in the ω_1 dimension. Therefore, the apparent linewidth can serve as the accuracy-limit of J_{CH} measurements. In normal 2D experiments, the data points acquired during t_1 period are limited, which causes t_1 -FID truncation and poor digital resolution in ω_1 dimension. Because of t_1 -FID truncation and poor digital resolution in ω_1 dimension, the accurately measurable splittings cannot be accurately measured due to displacement of peak maxima (28) or even become unresolved. The introduction of accordion factor n overcomes the problem of FID truncation in t_1 dimension and the poor digital resolution in ω_1 dimension. Although both J_{CH} splitting and the apparent linewidth are increased by n times, their ratio remains unchanged. For J_{CH} splittings being larger than the apparent linewidth, accurate measurements can be achieved using the proposed method. For J_{CH} splittings being less than the apparent linewidth, it may be suggested to combine data fitting procedures (29, 30) to obtain accurate results.

To demonstrate this method, an example of a 2D spectrum recorded using the pulse sequence in Fig. 1B is shown in Fig. 2. The sample for recording the spectrum is the sodium salt of cefuroxime in D_2O . Cefuroxime is an antibacterial medicine.

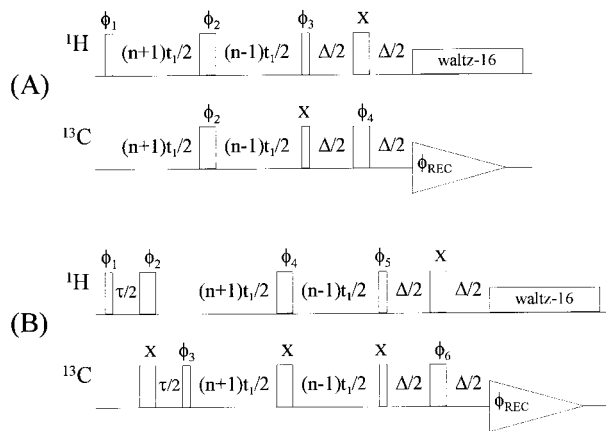


FIG. 1. (A) The pulse sequence for accurate measurements of heteronuclear multiple-bond coupling constants without suppression of one-bond correlation. The pulse phases are cycled as follows: $\phi_1 = x, -x$; $\phi_2 = 4(x), 4(y), 4(-x), 4(-y)$; $\phi_3 = y, y, -y, -y$; $\phi_4 = 16(x), 16(y), 16(-x), 16(-y)$; $\phi_{\text{REC}} = 2(x, -x, -x, x, -x, x, x, -x), 2(-x, x, x, -x, x, -x, -x, x)$, and ϕ_1 is incremented by 90° in concert with t_1 increment to ensure pure absorptive peak pattern. (B) The pulse sequence for accurate measurements of heteronuclear multiple-bond coupling constants with suppression of one-bond correlation by BIRD. The pulse phases are cycled as follows: $\phi_1 = x$; $\phi_2 = 2(x), 2(y), 2(-x), 2(-y)$; $\phi_3 = x, -x$; $\phi_4 = 4(x), 4(y), 4(-x), 4(-y)$; $\phi_5 = y$; $\phi_6 = 8(x), 8(y), 8(-x), 8(-y)$; $\phi_{\text{REC}} = x, x, -x, -x, -x, x, x, -x, -x, -x, x, x, x, -x, -x$, and ϕ_1 is incremented by 90° in concert with t_1 increment to ensure pure absorptive peak pattern. The interpulse delays $\tau, \Delta = 1/2J_{\text{CH}}$, where J_{CH} 's are one-bond and multiple-bond coupling constants, respectively.

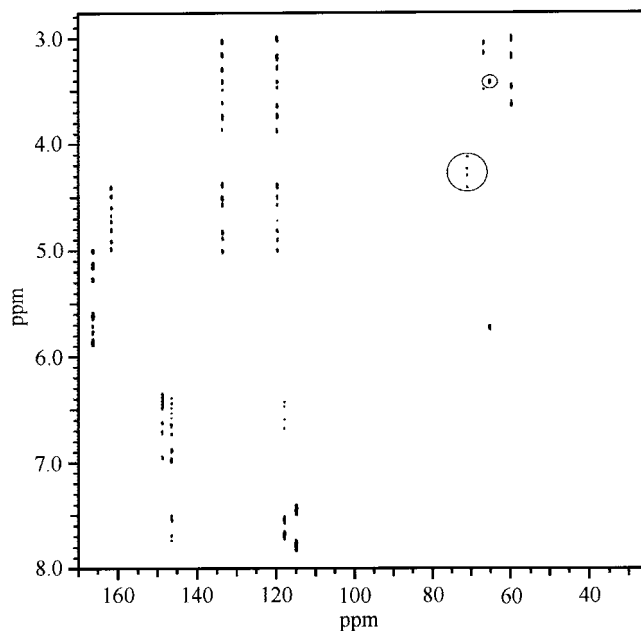
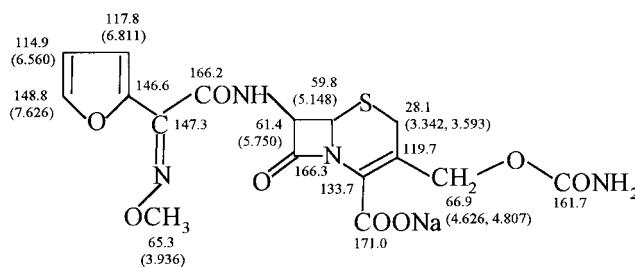


FIG. 2. A 2D spectrum of cefuroxime (its sodium salt) in D_2O was recorded by using the pulse sequence in Fig. 1B with $n = 10$, $\tau = 2.976$ ms, and $\Delta = 33.334$ ms. The time domain data set $\text{TD}_1 \times \text{TD}_2 = 512 \times 1024$. The frequency domain data set $\text{SI}_1 \times \text{SI}_2 = 2048 \times 2048$ and the spectral widths $\text{sw}_1 \times \text{sw}_2 = 2250 \text{ Hz} \times 16025.64 \text{ Hz}$. The 90° -shifted sinbell window function was used in the 2D Fourier transformation. The encircled multiplets are cross peaks of impurities, and the other peak encircled is folded from one of the doublet of the methyl group.

Its molecular structure is shown below with ^1H (in parentheses) and ^{13}C resonance assignments on it.



From this 2D spectrum, 23 multiple-bond $^{13}\text{C}-^1\text{H}$ coupling constants, as listed in Table 1, and 5 vicinal $^1\text{H}-^1\text{H}$ coupling constants, as listed in Table 2, can be extracted.

A practical aspect of this method is that the overlapping of two antiphase doublets leads to the cancellation of two peaks respectively from these two doublets. As an example, a region at the neighborhood of cross peaks (6.56–146.6 ppm) and (6.811–148.8 ppm) in Fig. 2 is expanded and shown in Fig. 3A. The cross peaks of ^1H at 6.56 ppm are split into inphase quadruplets by homonuclear couplings, each of which split further into antiphase doublets by heteronuclear coupling. Similarly, the cross peaks of ^1H at 6.811 ppm are split into in-phase

TABLE 1
The Multiple-Bond Carbon–Proton Coupling Constants in Hz Are Extracted from the 2D Spectrum Shown in Fig. 2

$(^1\text{H}, ^{13}\text{C})$ pair (ppm)	J_{CH} (Hz)	$(^1\text{H}, ^{13}\text{C})$ pair (ppm)	J_{CH} (Hz)
3.34, 59.8	6.5	4.81, 161.7	2.9
3.34, 66.9	3.9	5.15, 166.3	6.1
3.34, 119.7	6.9	5.75, 166.2	6.0
3.34, 133.7	4.6	6.56, 117.8	4.8
3.59, 119.7	5.7	6.56, 146.6	7.3
3.59, 133.7	4.6	6.56, 148.8	11.3
4.63, 28.1	4.9 ^a	6.81, 146.6	10.0
4.63, 119.7	4.1	6.81, 147.3	1.5
4.63, 133.7	5.2	6.81, 148.8	7.4
4.63, 161.7	3.3	7.63, 114.9	13.4
4.81, 28.1	5.4 ^a	7.63, 117.8	5.8
4.81, 119.7	4.2	7.63, 146.6	7.8
4.81, 133.7	4.9		

^a Values obtained from a 2D spectrum recorded using pulse sequence in Fig. 1A.

doublets by homonuclear coupling, each of which split further into antiphase doublets by heteronuclear coupling. Since the overlapping between the cross peaks of ^1H at 5.56 ppm and ^1H at 6.811 ppm, some peaks are missing. However, both the heteronuclear and the homonuclear coupling constants can still be extracted without ambiguity, by using the isotropic chemical shift of the proton and the remaining multiplets. Although mutual cancellation from adjacent multiplets does not affect the extraction of coupling constants, it might be helpful to run the experiment with a different value of n . As a comparison, an ideal case is shown in Fig. 3B, where the extraction of coupling constants is straightforward. In addition, it may be the case where only the sum of homonuclear and heteronuclear couplings can be obtained if they are comparable. In this situation, comparison of the splittings in the 2D spectrum with the relevant 1D ^1H spectrum is necessary, or E-COSY type methods (14, 16) can be employed.

All the measurements were performed on a Bruker DRX-400 spectrometer equipped with a 5 mm QNP ($^1\text{H}/^{13}\text{C}/^{15}\text{N}/^{31}\text{P}$) probe tuned at 400.13 MHz for ^1H and 100.613 MHz for ^{13}C . The high power 90° pulse widths are 10.3 and 10 μs , respectively, in ^1H and ^{13}C channels. The low power 90° pulse width

TABLE 2
The Vicinal Proton–Proton Coupling Constants in Hz Are Extracted from the 2D Spectrum Shown in Fig. 2

$(^1\text{H}, ^1\text{H})$ pair (ppm)	J_{HH} (Hz)
3.34, 3.59	17.9
4.63, 4.81	12.6
5.15, 5.75	4.7
6.56, 6.81	3.6
6.56, 7.63	1.9

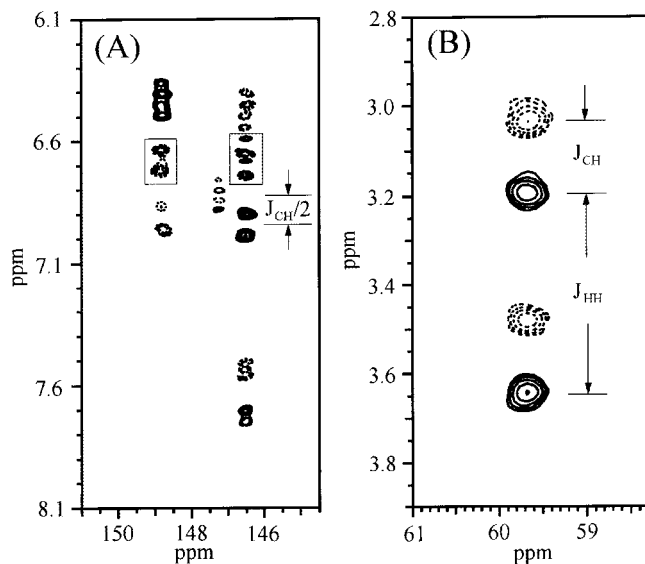


FIG. 3. Two expanded regions in Fig. 2 are shown. (A) The cross peaks of (6.56–146.6 ppm), (6.56–148.8 ppm), (6.811–146.6 ppm), and (6.811–148.8 ppm). Mutual cancellation from adjacent multiplets is present in the regions denoted by boxes and the coupling constants can still be extracted through comparing the remaining multiplet peaks with ^1H chemical shift position. (B) The cross peak of (3.34–59.8 ppm), where the extraction of coupling constants is straightforward. The positive peaks are shown by solid lines, while the negative peaks are shown by dashed lines.

is 100 μs in the composite pulse decoupling channel. The experimental temperature was 24°C .

In conclusion, a new 2D INEPT experiment based on the accordion scheme is proposed for the measurements of heteronuclear multiple-bond coupling constants. Since the detection is on carbon-13, the sensitivity of this experiment is 4 times less than the inverse-detected methods (11–16), which makes its application restricted in small molecules (at natural abundance) or larger molecules with isotope enrichment. Compared to COLOC (24), the resultant spectra show cross peaks containing information of n -time-amplified J splittings from both homonuclear and heteronuclear couplings, which allows easy measurement of these coupling constants. In addition, this method supplies an example of using the accordion scheme to study two kinds of interactions at different time scales in a single frequency dimension, which may be applicable to reducing the dimension of other 3D or 4D experiments.

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