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# Measurement of Long-Range $^1$H–$^{13}$C Coupling Constants from Quantitative 2D Heteronuclear Multiple-Quantum Correlation Spectra

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Heteronuclear three-bond $J$ values for $^1$H–$^{13}$C, $^3J_{CH}$, are related to the intervening dihedral angle via well-parametrized Karplus-type relations (*1, 2*) and they therefore contain important information regarding molecular structure. However, measurement of these couplings from the $^{13}$C multiplet structure is frequently very difficult because the multitude of protons coupled to a given $^{13}$C frequently give rise to a very complex $^{13}$C multiplet. One solution to this problem, selective $^1$H-flip spectroscopy, ensures that the $^{13}$C resonance is split in the $F_1$ dimension of a 2D spectrum only by the coupling to a selected $^1$H of interest (*3*). Because in this experiment the low-$\gamma$ $^{13}$C nucleus is detected, sensitivity is low. Moreover, couplings to only a single proton are obtained in any given 2D experiment. More recently, alternative approaches have been proposed that rely on E.COSY-based techniques (*4–8*) or on the measurement of the $^1$H {–$^{13}$C} multiplet splittings in $^1$H-detected $^1$H–$^{13}$C correlation experiments (*9–12*). This latter approach requires the $^1$H {–$^{13}$C} multiplet to be resolved, at least partially, and the multiplet structure then is compared with the multiplet structure of the protons not coupled to $^{13}$C.

Here we present a different approach for measurement of the $^1$H–$^{13}$C long-range coupling constants. The values of $J$ for $^1$H–$^{13}$C can be calculated from the resonance intensities in a $^1$H-detected $^1$H–$^{13}$C multiple-quantum correlation by comparing them with the intensities in a 2D "reference spectrum." The method is functionally analogous to experiments described recently for the measurement of long-range $J$ values for $^{13}$C–$^{13}$C in $^{13}$C-enriched proteins (*13*) and for measurement of values of $J$ for $^1$H–$^{113}$Cd and $^1$H–$^{199}$Hg in metalloproteins (*14*).

The pulse sequence used in the present experiment is

$$
\begin{array}{llll}
{}^1\mathrm{H}: & 90^\circ_{\phi 1}—\Delta— & —t_1/2—180^\circ_x—t_1/2— & —\Delta \\
{}^{13}\mathrm{C}: & 90^\circ_{\phi 2} & & 90^\circ_{\phi 3} \\
 & & & —\text{Acquire } (\psi) \\
 & & & —\text{Decouple} \qquad [1]
\end{array}
$$

with phase cycling $\phi_1 = x, y, -x, -y$; $\phi_2 = 4(x), 4(-x)$; $\phi_3 = 8(x), 8(-x)$; $\psi = x, -y, -x, y, -x, y, x, -y, -x, y, x, -y, x, -y, -x, y$. The spectrum is recorded in the phase-sensitive mode, and quadrature in the $t_1$ dimension is obtained in the States–TPPI manner by repeating the entire experiment with $\phi_2$ incremented by 90° and by inverting $\phi_2$ each time $t_1$ is incremented. This scheme for correlating long-range-coupled $^1$H and $^{13}$C nuclei is equivalent to the well-known HMQC one-bond correlation pulse scheme, with the dephasing and rephasing intervals $\Delta$ adjusted to a suitably long value (30–60 ms) (*15, 16*). The experiment is somewhat less sensitive than the HMBC experiment (*17, 18*), since in the present case no data acquisition takes place during the second interval, $\Delta$, and no further $^1$H{–$^{13}$C} rephasing occurs during data acquisition because of the applied $^{13}$C decoupling. The reference spectrum is obtained with the same $^1$H pulse scheme, but with the 90° $^{13}$C pulses replaced by short delays equal to the 90° $^{13}$C pulse width (12 $\mu$s), and with use of only the first four steps of the phase cycle. Also, the second spectrum for each $t_1$ value, needed to obtain quadrature in the $t_1$ dimension, is not recorded, and zeros are inserted for these data prior to the $t_1$ Fourier transformation. This results in a "reference spectrum" that is symmetric about the carrier in the $F_1$ dimension of the 2D spectrum after complex Fourier transformation. As will be discussed below, the peak shapes in the $^1$H–$^{13}$C correlation spectrum are identical to those in the reference spectrum. Their relative intensities are related in a straightforward manner to the size of the long-range $^1$H–$^{13}$C coupling.

$^1$H chemical shifts during pulse scheme [1] may safely be neglected as the 180° ($^1$H) pulse refocuses the effect of resonance offset. First we will consider the simple case where homonuclear $^1$H–$^1$H $J$ modulation is absent and only heteronuclear coupling is present. Note that because of the low isotopic abundance of $^{13}$C, the case where a proton is coupled to more than one $^{13}$C may safely be ignored. If the effective



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flip angle of the $^{13}C$ pulses is $\alpha$ ($\alpha \sim 90°$), the signal $S_{CH}(t_1, t_2)$ of a $^1H-^{13}C$ correlation is given by

$$S_{CH}(t_1, t_2) = CNA \sin^2(\pi J_{CH}\Delta)\sin^2(\alpha)\cos(\Omega_C t_1)\exp(i\Omega_H t_2), \quad [2a]$$

where $\Omega_C$ and $\Omega_H$ are the angular $^{13}C$ and $^1H$ offset frequencies, respectively, $C$ is a constant, $N$ is the number of scans, and $A$ is the isotopic abundance of $^{13}C$. In the reference spectrum, the signal is given by

$$S_{ref}(t_1, t_2) = CN_{ref}\exp(i\Omega_H t_2), \quad [2b]$$

where $N_{ref}$ is the number of scans per increment for the reference spectrum. Both the reference and the correlation spectra are affected in exactly the same way by the presence of homonuclear $^1H-^1H$ couplings, and the shape of a multiplet in the reference spectrum (centered at $F_1 = 0$) is identical to that of the corresponding cross peak in the $^1H-^{13}C$ correlation spectrum. As is clear from Eqs. [2a] and [2b], for any given proton, the $^1H-^{13}C$ correlation/reference intensity ratio is given by

$$S_{CH}(t_1, t_2)/S_{ref}(t_1, t_2) = A(N/N_{ref})\sin^2(\pi J_{CH}\Delta)\sin^2(\alpha). \quad [3]$$

The natural abundance of $^{13}C$ is known with good precision ($A = \sim 1.108\%$). The factor $\sin^2(\alpha)$ is a constant determined by the RF inhomogeneity of the probehead, provided the average effective flip angle $\langle\alpha\rangle$ is carefully adjusted to 90° and the effect of $^{13}C$ resonance offset is insignificant. Application of the experiment to $\beta$-acetonaphtalene, a compound for which long-range coupling constants were previously reported with high precision (*19*), indicated that $\sin^2(\alpha) = 0.88 \pm 0.01$ for our probehead. Provided that $\sin(\pi J_{CH}\Delta) \ll 1$, measurement of the intensity ratio then allows the accurate determination of $J_{CH}$ from Eq. [3].

In the above discussion, the effect of relaxation has been ignored because $^{13}C$ nuclei two or more bonds removed from a given $^1H$ do not significantly affect the $T_1$ or $T_2$ relaxation of this proton, and both the reference and the $^1H-^{13}C$ correlation spectra are affected identically by other relaxation mechanisms. Relaxation of the $^{13}C-^1H$ multiple-quantum coherence during the evolution period $t_1$ may differ slightly from the relaxation of $^1H$ coherence during $t_2$, but this difference can be ignored because the acquisition time in this dimension is typically much shorter than the applicable transverse relaxation times. Relative intensity differences between the correlation and the reference peaks can arise, however, during the dephasing and rephasing delays since $^1H\{-^{13}C\}$ antiphase coherence relaxes slightly faster than in-phase $^1H$ coherence (*20*). For the case where the proton I and carbon S have a negligible dipolar interaction, the relaxation rate of the antiphase magnetization, $I_xS_z$, equals to a good approximation the sum of the $^1H$ transverse relaxation rate, $1/T_{2I}$, and the $^{13}C$ longitudinal relaxation rate, $1/T_{1S}$. For the present case, where $T_{1S}$ is nearly an order of magnitude longer than the $\Delta$ delays, this difference in relaxation affects the intensity ratio by less than $\sim 10\%$, and the derived couplings are therefore no more than $\sim 5\%$ smaller than their true value (*21*).

The method is demonstrated for the cyclic decapeptide gramicidin S, *c*(Pro–Val–Orn–Leu–Phe)$_2$, 18 m*M*, dissolved in DMSO-$d_6$. Experiments were carried out on a Bruker AMX-600 spectrometer equipped with an inverse probehead. Using a dwell time in the $t_1$ dimension of 80 μs and 256 $t_1$ increments in both experiments, the 2D reference spectrum was recorded with 4 scans per $t_1$ increment and the $^1H-^{13}C$ correlation spectrum with 64 scans for the $x$ and 64 scans for the $y$ component of each complex $t_1$ increment. Total measuring times were 19 min for the reference spectrum and 10 h for the $^1H-^{13}C$ correlation spectrum. After zeros were inserted for the imaginary component in the $t_1$ domain of the reference spectrum, both spectra were processed identically, using cosine-bell apodization in both the $t_1$ and the $t_2$ dimensions and zero filling to yield a $1024(F_1) \times 4096(F_2)$ matrix for the absorptive part of the final spectrum. The digital resolution was 12 Hz ($F_1$) and 1.75 Hz ($F_2$). To test the reproducibility of the $J$ values measured with the new method, the experiment was performed twice, once with a $\Delta$ of 30 ms and once with a 60 ms $\Delta$ value.

Figure 1 compares a small region of the reference spectrum (Fig. 1A) with a corresponding region of the $^1H-^{13}C$ correlation spectrum (Fig. 1B). In the reference spectrum, all peaks are centered around zero frequency in the $F_1$ dimension, as the $F_1$ frequency is determined only by the (unresolved) $^1H-^1H$ couplings. In the $^1H-^{13}C$ correlation spectrum, the $F_1$ frequency is determined by the $^{13}C$ chemical shift, with the unresolved $^1H-^1H$ couplings superimposed. The lineshape in the reference spectrum is therefore identical to that in the correlation spectrum, as can be seen by comparing the peak shapes observed in Figs. 1A and 1B. Their relative intensities can be calculated either by peak picking or, more sensitively, by calculating a scaling factor that gives the "best fit" between the scaled reference peak and the correlation of interest. The latter procedure was used in our study of gramicidin S for all protons with nonoverlapping resonances in the reference spectrum. For protons with partial overlap, a scaling factor was used which visually gave the best fit.

Figure 2 shows the $H^\alpha$ region of a cross section taken through the reference spectrum at $F_1 = 0$, together with the corresponding cross sections for $^{13}C$ nuclei coupled to these $H^\alpha$ protons. In all cross sections, lineshapes are affected in

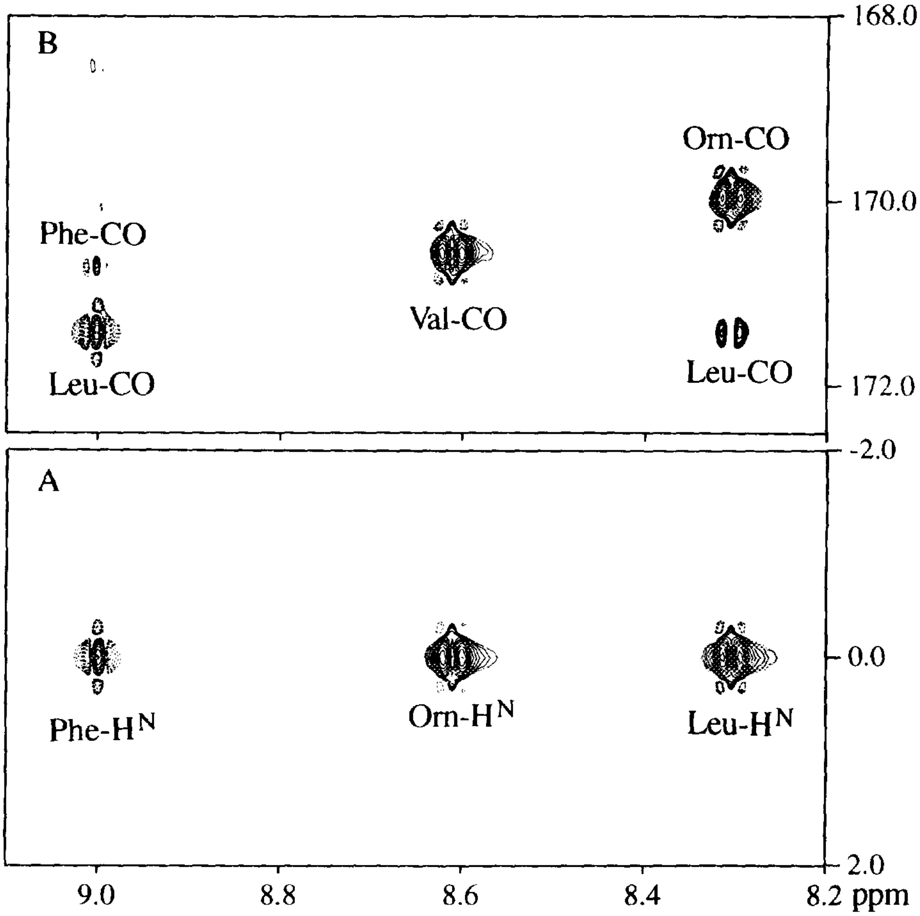


FIG. 1. Small sections of the amide region of (A) the reference spectrum and (B) the $^1$H–$^{13}$C correlation spectrum of gramicidin S. For the reference spectrum, only the region around $F_1 = 0$ is displayed. For the $^1$H–$^{13}$C correlation spectrum, the region that contains the correlations to the carbonyl resonances is shown. Both the reference and the $^1$H–$^{13}$C correlation spectra have been recorded with $\Delta = 60$ ms.

the same way by the homonuclear $^1$H–$^1$H couplings, which are different for the different H$^\alpha$ protons and give rise to different phases for the various multiplet components. Hence, the multiplet shapes in the $^1$H–$^{13}$C correlation spectrum are identical to those of the corresponding resonances in the reference spectrum. Only the correlation to Phe–C$\gamma$ is of opposite sign compared to the reference spectrum, because this correlation is aliased once in the $^{13}$C dimension, and the experiment was set up to ensure a 180° linear phase correction in the $F_1$ dimension (*22*).

A precise $J$ value can be derived only if the corresponding correlation is observable in the $^1$H–$^{13}$C correlation spectrum. In practice, for our study of gramicidin S, this requires a $J$ value of at least $\sim$1–2 Hz, depending on the intensity of the $^1$H multiplet in the reference spectrum. However, if no cross peak is observed for a given $^1$H–$^{13}$C correlation, this nevertheless provides information on the upper limit for this coupling; any coupling larger than this upper limit would have given an observable $^1$H–$^{13}$C correlation. The absence of a $J$ correlation therefore also provides useful information on the size of J.

Using the approach outlined above, 65 values for two- and three-bond $J_{CH}$ values in gramicidin S were measured (Table 1). Also listed in Table 1 are the upper limits for 12 three-bond $J_{CH}$ values for which no correlation could be observed. For correlations that were observable in both the spectra recorded with $\Delta = 30$ and 60 ms, the values listed in Table 1 are the average of these two measurements. The rms difference between the two sets of measurements was only 0.3 Hz, indicating that the measurements are highly reproducible. The measured $J$ values provide an indication of the range of values in a conformationally constrained cyclic peptide and illustrate the large number of structural parameters that may be derived from such a simple correlation experiment.

The optimum choice for the duration of the delay $\Delta$ de-

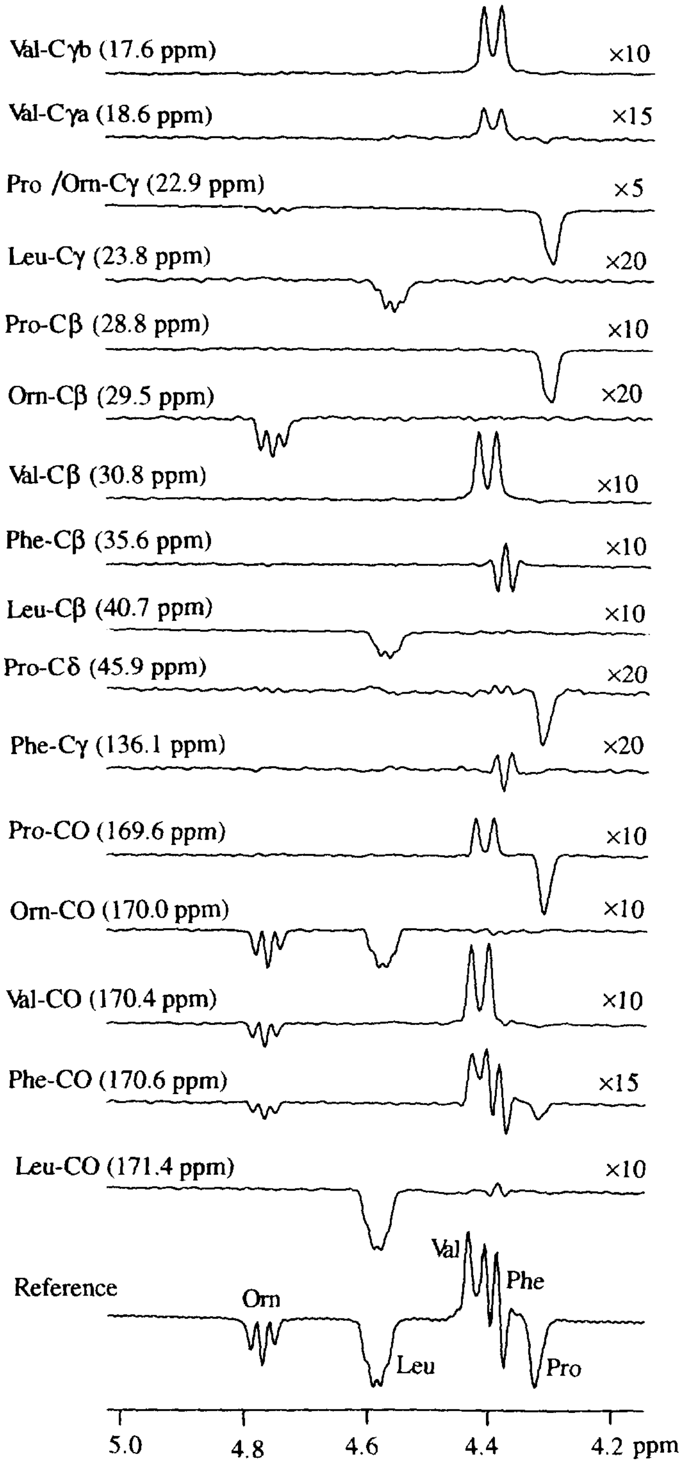


FIG. 2. $H^{\alpha}$ region of 1D $F_2$ cross sections through the reference spectrum (bottom trace) and the $^{1}H-^{13}C$ correlation spectrum (other traces) taken at the marked $F_1(^{13}C)$ frequencies. The reference spectrum was recorded with 4 scans per $t_1$ increment and the $^{1}H-^{13}C$ correlation spectrum with 64 scans. The vertical scale of the cross sections shown is expanded by the factor shown in the right margin. This scaling is in addition to the factor 16, resulting from the difference in the number of scans. Spectra shown have been recorded with $\Delta$ = 60 ms.

TABLE 1
Multibond $^{1}H-^{13}C$ $J$ Couplings in Gramicidin S

| Pro | $CO^{Phe}$ | CO | $C^{\alpha}$ | $C^{\beta}$ | $C^{\gamma}$ | $C^{\delta}$ |
|---|---|---|---|---|---|---|
| $H^{\alpha}$ | 1.6 | 4.3 | | 4.8 | 8.4 | 3.2 |
| $H^{\beta a}$ | | 3.7 | <2.6 | | <2.6 | 4.6 |
| $H^{\delta a}$ | <1.1 | | <1.1 | 4.2 | 3.1 | |
| $H^{\delta b}$ | <1.8 | | <1.8 | <1.8 | 5.0 | |
| **Leu** | $CO^{Orn}$ | CO | $C^{\alpha}$ | $C^{\beta}$ | $C^{\gamma}$ | $C^{\delta}$ |
| $H^{N}$ | 4.5 | 1.6 | 2.2 | <0.7 | | |
| $H^{\alpha}$ | 3.2 | 4.5 | | 2.8 | 2.2 | |
| $H^{\beta a}$ | | 2.6 | 3.3 | | 4.5 | 3.3* |
| $H^{\beta b}$ | | 4.7 | 5.2 | | | 3.5* |
| $H^{\gamma}$ | | | 2.7 | 3.1 | | 3.0 |
| **Orn** | $CO^{Val}$ | CO | $C^{\alpha}$ | $C^{\beta}$ | $C^{\gamma}$ | $C^{\delta}$ |
| $H^{N}$ | 4.3 | <0.8 | 1.5 | <0.8 | | |
| $H^{\alpha}$ | 3.0 | 4.1 | | 3.7 | 2.8 | |
| $H^{\beta a}$ | | 3.4 | 4.4 | | 4.3 | 4.3 |
| **Phe** | $CO^{Leu}$ | CO | $C^{\alpha}$ | $C^{\beta}$ | $C^{\gamma}$ | $C^{\delta}$ |
| $H^{N}$ | 4.4 | 1.6 | 2.5 | 2.4 | | |
| $H^{\alpha}$ | 1.4 | 2.6 | | 2.9 | 1.7 | |
| $H^{\beta a}$ | | 6.5 | 5.4 | | 5.4 | 4.8* |
| $H^{\beta b}$ | | 3.0 | 5.5 | | 5.3 | 4.6* |
| **Val** | $CO^{Pro}$ | CO | $C^{\alpha}$ | $C^{\beta}$ | $C^{\gamma a}$ | $C^{\gamma b}$ |
| $H^{N}$ | 4.4 | <1.0 | | <1.0 | | |
| $H^{\alpha}$ | 2.8 | 4.7 | | 4.3 | 2.2 | 4.3 |
| $H^{\beta}$ | | 1.7 | 3.7 | | 3.9 | 3.6 |
| $H^{\gamma b}$ | | | 4.2 | 4.0 | | 4.7 |

*Note.* Superscripts a and b refer to the downfield and upfield resonating nucleus, respectively.

* Because the $^{13}C$ resonance represents the superposition of two $^{13}C$ nuclei, the intensity of the $^{1}H-^{13}C$ correlation is halved before $J_{CH}$ is calculated. For leucine, this approximation requires rapid rotameric averaging about the $C^{\beta}-C^{\gamma}$ bond.

pends on the size of the coupling one wants to measure most accurately. A value for $\Delta$ close to $1/(2J_{CH})$ yields an inaccurate measurement for $J_{CH}$ because the factor $\sin^2(\pi J_{CH}\Delta)$ in Eq. [3] depends only weakly on $J_{CH}$. A $\Delta$ duration shorter than $\sim 1/(10J_{CH})$ gives rise to a very low intensity $^{1}H-^{13}C$ correlation, which may be difficult to quantitate. For the measurement of $^{1}H-^{13}C$ couplings in the 2–8 Hz range, a $\Delta$ duration of $\sim$40 ms appears to be a good compromise value. Alternatively, as in the present study, two data sets may be recorded with different $\Delta$ durations.

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