# Measurement of C',C Coupling Constants in $^{13}$C-Labeled Proteins: A New Method for the Stereospecific Assignment of γ-Methyl Groups in Valine Residues†

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The precision of structure determination of peptides and proteins is increased if the stereochemical assignment of diastereotopic groups or atoms is available. A general method for the stereochemical assignment of the δ- and γ-methyl groups in leucines and valines has been published relying on stereoselective biochemical synthesis.[1] Recently, Bax and co-workers published a method for measuring long-range carbon–carbon couplings from the integral ratio of cross peak to diagonal peak in long-range C,C correlation experiments, leading to stereochemical assignments of methyl groups in leucines and valines.[2] In this communication, we present an approach to measure $^3J(C',C_\gamma)$ couplings in proteins that are uniformly $^{13}$C labeled for the stereospecific assignments of valine methyl groups. The procedure proposed here relies on the E. COSY[3,4] method to measure small coupling constants, which has successfully been applied in homo- and heteronuclear spin systems.[5-14] Because of the long relaxation times of the C', we do not expect to introduce any systematic errors due to differential relaxation.[15]

The new method introduced here relies on the rather large $^1J(C',C_\alpha)$ coupling of about 55 Hz between the C' and $C_\alpha$ carbons in amino acids. Correlating the $C_\alpha$ with $C_\gamma$ without touching the carbonyl carbon allows one to measure $^3J(C',C_\gamma)$. $C_\alpha$ and $C_\gamma$ are correlated via $C_\beta$ using a relay segment. For sensitivity reasons, the magnetization on $C_\gamma$ is transferred to $H_\gamma$ for detection. Selective pulses that are broadband in the aliphatic region and do not affect the carbonyls have to be used for polarization transfer. The pulse sequence employed, HCCC-COSY, is an INEPT-C-RELAYED-CT-C,C-COSY-C,H-COSY. The experiment is necessarily a 3D experiment, where the $C',C_\gamma$ coupling constant is measured in the indirectly sampled $\omega_2$ dimension. Evolution of the chemical shift of the $C_\alpha$ takes place in a constant time segment in order to gain sensitivity and to reduce multiplet structure and thus possible overlap. The constant time delay $2\tau$ is set to $(2(J(C_\alpha,C_\beta))^{-1}$ to employ the first maximum in the transfer function. Therefore, linear prediction methods have to be applied to resolve the $J(C_\alpha,C')$ coupling in $\omega_1$.

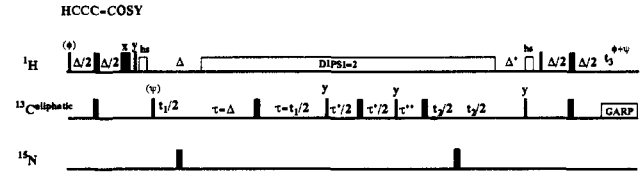


**Figure 1.** Pulse sequence of the $C_{aliphatic}$-selective HCCC-COSY for molecules uniformly $^{13}$C labeled. Two scans per $t_1$ (64 complex points, 4483 Hz) and $t_2$ (129 complex points, 1000 Hz) were recorded, with 512 complex points in $t_3$. The delays were set as follows: $\Delta = 3.4$ ms, $\tau = 7.1428$ ms, $\tau' = 4.2857$ ms, $\tau'' = 1.4284$ ms, $\Delta' = 1.3794$ ms, to yield spectra without phase correction in $\omega_1$ and $\omega_2$ and to achieve optimal polarization transfer. Homospoil pulses (2 and 1.5 ms) and a purge pulse with full rf-power of 2 ms were applied. After Fourier transformation in $\omega_3$ and in $\omega_2$, linear prediction in $\omega_1$ from 127 to 383 complex points was used after mirror imaging[16] the complex data. Apodization was done over 383 points with a squared cosine bell function. The final matrix contained 256*512*256 real points. The pulse phases that are not explicitely given were set to 0, except for $\psi = x, -x$. Ruben–States–Haberkorn in $t_1$[17] and States–TPPI in $t_2$[18] were used for frequency sign discrimination. Carbon pulses are aliphatic selective (alternating G4 and time reversed *G4* for 90° and G3 for 180° pulses[13,19,20]). Total measuring time was 27 h.

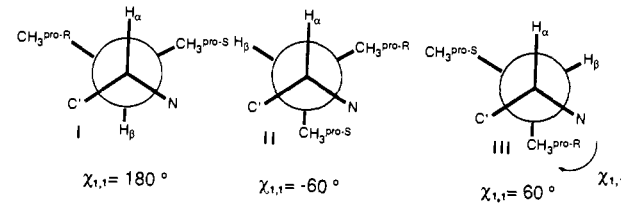


**Figure 2.** Staggered conformations around the $C_\alpha$–$C_\beta$ bond in valine.

**Table I.** Couplings Determined for the Valines in Ribonuclease $T_1$[a]

| | $H_\gamma$ [ppm] | $C_{\gamma d}$ [ppm] | $^3J(C',C_{\gamma d})$ [Hz] | $H_\gamma$ [ppm] | $C_{\gamma u}$ [ppm] | $^3J(C',C_{\gamma u})$ [Hz] |
|---|---|---|---|---|---|---|
| Val 16 | 0.68 | 24.38 | 4.3 ± 0.12[c] | 0.73 | 23.05 | 1.1 ± 0.14 |
| Val 33 | 1.03 | 23.45 | 2.3 ± 0.12 | 1.12 | 21.62 | 1.2 ± 0.15 |
| Val 52 | 0.86 | 22.92 | 3.7 ± 0.11 | 0.69 | 19.43 | 1.4 ± 0.15 |
| Val 67 | 1.20 | 23.44 | 1.5 ± 0.46 | 1.02 | 23.10 | 3.8 ± 0.09 |
| Val 78 | *b* | *b* | *b* | *b* | *b* | *b* |
| Val 79 | 0.34 | 23.68 | 1.3 ± 0.31 | 0.00 | 21.41 | 4.5 ± 0.36 |
| Val 89 | 0.01 | 22.94 | 4.4 ± 0.12 | 0.68 | 21.81 | 0.3 ± 0.12 |
| Val 101 | 1.03 | 23.25 | 4.8 ± 0.60 | 0.89 | 20.42 | 1.1 ± 0.07 |

[a] The indices u and d refer to the upfield and downfield carbons, respectively. [b] γ protons and γ carbons are both degenerate. [c] For detection of error, see Figure 4.

We demonstrate the method on a uniformly $^{13}$C,$^{15}$N-labeled 1.5 mM sample of ribonuclease $T_1$ in $D_2O$. The cross peaks between $C_\alpha$ and $C_\gamma$ for valine 16 are shown in Figure 3. The following coupling constants for valine 16 can be extracted: $^3J(C',C_{\gamma d}^{pro\text{-}S}) = 4.3 \pm 0.1$ Hz (indicative of antiperiplanar orientation) and $^3J(C',C_{\gamma u}^{pro\text{-}S}) = 1.1 \pm 0.1$ Hz. (The indices u and d refer to upfield and downfield carbon resonance.) Together with the $^3J(H_\alpha,H_\beta) = 12.4$ Hz and $^3J(C',H_\beta) = 0.5$ Hz,[13] this leads to the unambiguous assignment of rotamer II. $C_{\gamma d}$ is $C_\gamma^{pro\text{-}S}$, and $C_{\gamma u}$ is $C_\gamma^{pro\text{-}R}$.

Table I lists the couplings determined for the valines in ribonuclease $T_1$.

In conclusion, we have shown a new and versatile method for the stereochemical assignments of methyl groups in valines. A detailed conformational analysis of all eight valines in ribonuclease $T_1$ is under way.

† Dedicated to Prof. R. R. Ernst on the occasion of his 60th birthday.

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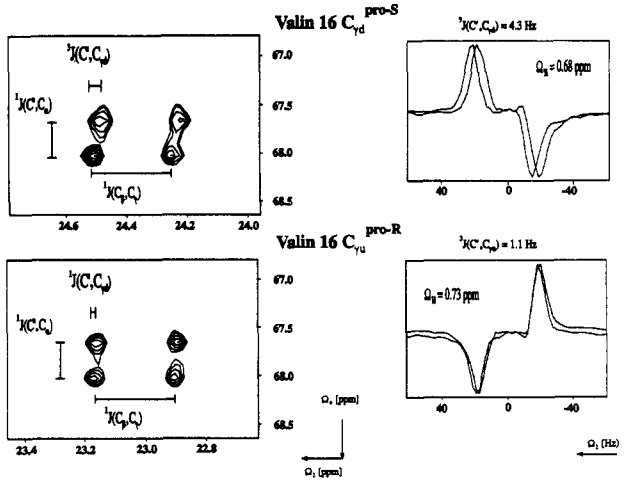


**Figure 3.** 1D columns and 2D slices through the spectrum of ribonuclease $T_1$ showing the $C_\alpha$,$C_\gamma$ cross peaks of valine 16 with the displacement vectors due to C′. The rows were taken from the 3D spectrum, inverse Fourier transformed after addition over appropriate slices in $\omega_1$ and $\omega_3$ to increase S/N, zerofilled to 4*K* complex points, and Fourier transformed to increase digitization.

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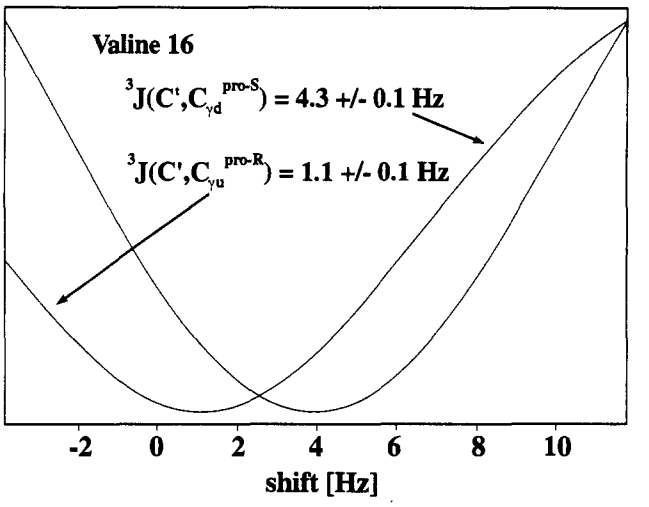


**Figure 4.** Power integral over the difference spectra of the $\omega_2$ rows displaced in $\omega_1$ by $^1J(C_\alpha,C')$ for two valine 16 resonances as a function of the shift. In order to determine the coupling precisely, the two $\omega_2$ rows displaced in $\omega_1$ by $^1J(C_\alpha,C')$ for both valine 16 resonances were shifted with respect to each other by an incremented frequency shift from 0 to 8 Hz, with a step size 0.224 Hz, and subsequently subtracted from each other to yield a difference spectrum. The power integral over this difference spectrum (error) as a function of the shift in Hz is shown here. The rms of the $^3J(C',C_\gamma)$ value was determined in the following way. The integration region of the power difference spectrum was varied, which translates the noise of the spectrum into the noise of the error integral. The rms of this noise normalized to the square root of the integral region was added to the minimum of the error integral shown here, and the shift values ($J_{lower}$, $J_{upper}$) corresponding to this value of the error integral were read off by parabolic extrapolation of the curve in Figure 4. The error given in Table I was set to $(J_{lower} - J_{upper})/2$.