Torsion Angles of Epothilones

Derivation of Dihedral Angles from CH–CH Dipolar–Dipolar Cross-Correlated Relaxation Rates: A C–C Torsion Involving a Quaternary Carbon Atom in Epothilone A Bound to Tubulin**

Teresa Carlomagno, Víctor M. Sánchez, Marcel J. J. Blommers, and Christian Griesinger**

Cross-correlated relaxation (CCR) rates constitute a valid alternative to coupling constants for the determination of dihedral angles.[1] For high-molecular-weight systems the measurement of scalar coupling constants poses serious problems caused by large linewidths and by differential relaxation effects.[2] Conversely, CCR measurements are feasible also for molecules of large size and have been successfully employed in the past years to determine dihedral angles in biomolecules.[3–6] We have recently reported the usage of cross-correlated relaxation rates to derive angular

[*] Dr. T. Carlomagno, Prof. Dr. C. Griesinger, Dr. V. M. Sánchez
Max-Planck-Institut für Biophysikalische Chemie
Am Fassberg 11, 37077 Göttingen (Germany)
Fax: (+49) 551-201-2201
E-mail: taco@nmr.mpibpc.mpg.de
cigr@nmr.mpibpc.mpg.de
Dr. M. J. J. Blommers
Novartis Pharma AG
P.O. Box, 4002 Basel (Switzerland)

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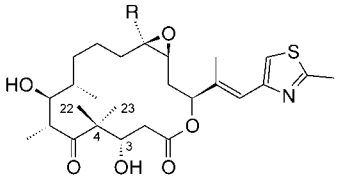
structural information for a small ligand weakly bound to a macromolecule.[7,8] Similarly to "transferred-NOEs",[9,10] "transferred-CCR rates" are informative of the bound conformation of the ligand when the complex is in the fast-exchange regime on the NMR timescale. Information on the bound conformation of the ligand cannot be obtained from coupling constants, because their value is independent of the molecular correlation time.[7]

Herein we introduce a pulse sequence that measures CH–CH cross-correlated relaxation in a H^aC^a–$C^b(CH_3)^c$ moiety, in which the second C atom is quaternary. This rate allows the calculation of the dihedral angle about the C^a—C^b bond.

We have applied the pulse sequence to a 100:1 mixture of ^{13}C-labeled epothilone A (**1**) and tubulin to determine the dihedral angle about the C3—C4 bond of epothilone in its bound conformation. Epothilones are very potent antitumor drugs that exert cytotoxic activity by binding to the 100-kDa tubulin α/β heterodimers.[11a] Their conformation in the bound state is not known, whereas the unbound conformation was previously determined by X-ray crystallography.[11b] The equilibrium and kinetic constants of the epothilone A–tubulin complex ($K_D = 10$–100 μM) render it suitable for "transferred" CCR experiments. Furthermore, it could be shown by competition experiments with the strong specific binder epothilone B that there is only one specific binding site for epothilone A. A sample of ^{13}C-labeled epothilone A was obtained by growing the myxobacterium *Sorangium cellulosum* on ^{13}C-rich medium.



1a R = H (epothilone A)
1b R = CH_3 (epothilone B)

The dihedral angle about the C3—C4 bond of epothilone is accessible by measuring the dipolar–dipolar CCR rates between the C3–H vector and the C22–H or C23–H vectors. The direction of the three C—H bonds in each methyl group is averaged, as a consequence of the fast rotation around the C4—C22 and C4—C23 bonds. Therefore, the C22–H and C23–H vectors assume an average orientation that is collinear with the C4—C22 and C4—C23 bonds, respectively.

The pulse sequence used for the measurement of the $\Gamma_{CH,CH}$ rate between the C3–H vector and the $(C22/23–H)_{av.}$ vectors is shown in Figure 1. Two experiments are recorded, yielding a cross and a reference spectrum (quantitative Γ approach).[12,13] The CCR rate is extracted from the ratio of peak intensities in the cross and reference experiments. The pulse sequence is optimized for maximum sensitivity of the cross experiment: the $8H_z^{22/23}C_y^{22/23}C_z^{4}C_y^{3}$ coherence present at point c is transformed by $\Gamma_{C23–H,C3–H}$ into $8C_x^{22/23}C_z^{4}C_x^{3}H_z^{3}$ with an efficiency equal to $3\sinh(\Gamma_{C22/23–H,C3–H}T_{rel})\cosh^2(\Gamma_{C22/23–H,C3–H}T_{rel})$. The $8C_x^{22/23}C_z^{4}C_x^{3}H_z^{3}$ term is then transformed into H_x^3 for detection. The delays are optimized for optimal refocusing and defocusing of the couplings. Selective π pulses on the carbon atoms prevent loss of magnetization to other passively coupled carbons. The details of the coherence transfers are provided in Figure 1 and its legend.

The cross and reference spectra are shown in Figure 2. Two peaks are observed in the reference spectrum at the

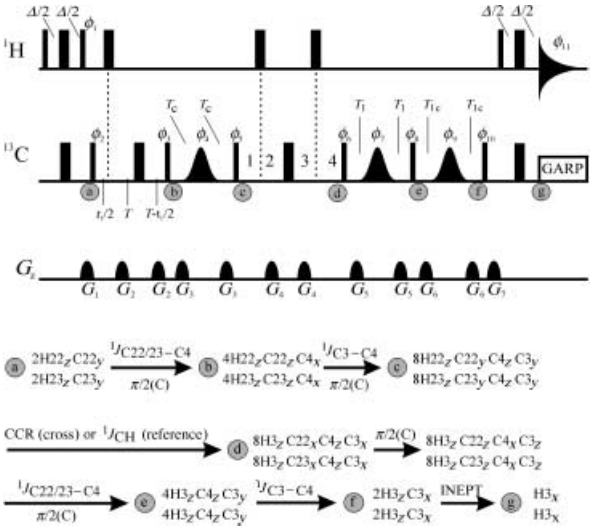


Figure 1. Pulse sequence for the measurement of dipolar–dipolar cross-correlated relaxation between the $(C22/23–H)_{av.}$ vector and the C3–H vector in epothilone. $\Delta = 1/(2^1J_{C,H}) = 7$ ms; $T = 1/(4^1J_{C,C}) = 7$ ms; $T_c = 1/(4^1J_{C,C}) = 7$ ms; $T_1 = 1/(8^1J_{C,C}) = 3.5$ ms; $T_{1c} = T_c$. Delays 1–4 are equal to $T_{rel}/4$ ($T_{rel} = 1/^1J_{C,C} = 28$ ms) in the cross-experiment; in the reference experiment, delay $1 = T_{rel}/4+\Delta/8 =$ delay 3 and delay $2 = T_{rel}/4-\Delta/8 =$ delay 4. $\phi_1 = y$; $\phi_2 = x, -x$; $\phi_3 = 2(y), 2(-y)$; $\phi_5 = 4(x), 4(-x)$; $\phi_6 = 8(y), 8(-y)$; $\phi_8 = x$; $\phi_{10} = y$; $\phi_{11} = 2(x,-x), 4(-x,x), 2(x,-x)$; $\phi_4 = \phi_7 = \phi_9 = x$. The pulses with phase ϕ_4 and ϕ_9 are Q3 pulses of 768 ms duration, centered at $\delta = 63$ ppm, to invert C3 and C4 selectively (C3 resonates at 70.9 ppm and C4 at 53.0 ppm); the pulse with phase ϕ_7 is a Q3 pulse with a duration of 512 ms centered at $\delta = 37$ ppm, to invert the C22/23 and C4 selectively (C22 resonates at $\delta = 19.1$ ppm and C23 at $\delta = 21.7$ ppm). The proton carrier was at $\delta = 4.78$ ppm and the carbon carrier at $\delta = 37$ ppm; spectral widths were 4807.69 Hz for ^{1}H and 8333.33 Hz for ^{13}C. Carbon decoupling in acquisition consisted of a GARP modulated pulse train at 2.38 kHz field strength.

chemical shifts of C22 and C23 in ω_1 and the chemical shift of H3 in ω_2. In the cross-spectrum, the C23(ω1)–H3(ω2) peak is missing, indicating that the dipolar–dipolar CCR between the $(C23–H)_{av.}$ vector and the C3–H vector is close to zero. The quantitative value for the two CCR rates can be extracted according to [Eq. (1)] (I_{cross} and I_{ref} are the intensities of the peaks in the cross and reference experiment, respectively and Γ indicates the cross-correlated relaxation rate).

$$I_{cross}/I_{ref} = \tanh(\Gamma T_{rel})/[\sin^2(\pi J_{C,H}\Delta/2)\cos^2(\pi J_{C,H}\Delta/2)] \quad (1)$$

The measured CCR rates between the C3–H vector and the $(C23–H)_{av.}$ and $(C22–H)_{av.}$ vectors are 0.1 ± 0.1 and -1.4 ± 0.4, respectively. The error was calculated applying error propagation theory, assuming that the noise represents the experimental uncertainty of the peak intensities.

The dependence of the two CCR rates on the C2-C3-C4-C5 dihedral angle θ is given in [Eq. (2)] and [Eq. (3)].

$$\Gamma_{C23–H,C3–H} = -0.3296\, k\, S^2 \tau_c P_2[\cos^2 109.7^o \sin^2 109.7^o \cos(\theta)] \quad (2)$$

$$\Gamma_{C22–H,C3–H} = -0.3296\, k\, S^2 \tau_c P_2[\cos^2 109.7^o \sin^2 109.7^o \cos(\theta - 120^o)] \quad (3)$$

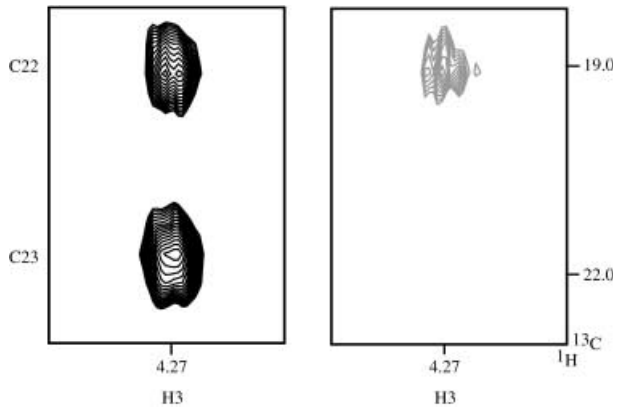


Figure 2. Reference (left) and cross-spectra (right) acquired with the sequence of Figure 1 on a Bruker DRX 600 MHz spectrometer. The peaks visible in the reference spectrum are at the chemical shift of C22 ($\delta = 19.1$ ppm) and C23 ($\delta = 21.7$ ppm) in the ^{13}C dimension and at the chemical shift of H3 ($\delta = 4.27$ ppm) in the ^{1}H dimension. The cross-experiment was four times longer than the reference experiment. During the experiment, 1024 and 64 complex points were acquired in t_2 and t_1, respectively. The final matrices were 2048×128 points. The gray lines represent negative contours. The sample contained 0.5 mM epothilone A and 5 μM tubulin dissolved in D_2O.

The $(C22–H)_{av.}$ and $(C23–H)_{av.}$ vectors lie along the direction defined by the C4–C22 and C4–C23 bonds, respectively. The CCR rates $\Gamma_{C22–H,C3–H}$ and $\Gamma_{C23–H,C3–H}$ are scaled by $P_2[\cos 109.7°] = {}^1/_2[3\cos^2(109.7°)-1] = -0.3296$ as a result of the averaging of the C–H directions in each methyl group, ($k = (\gamma_C\gamma_H h\mu_0/8\pi^2)^2$, τ_c is the effective correlation time of the molecule, θ is the torsion angle C^2-C^3-C^4-C^5 and S^2 is the order parameter accounting for internal motions.)

A graphical representation of the dependence of the two CCR rates on the torsion angle θ is shown in Figure 3 for a τ_c of 0.74 ns, which is the effective τ_c of epothilone in the presence of 5 μM tubulin, as estimated from other CCR measurements, and an order parameter $S^2 = 0.8$ to account for

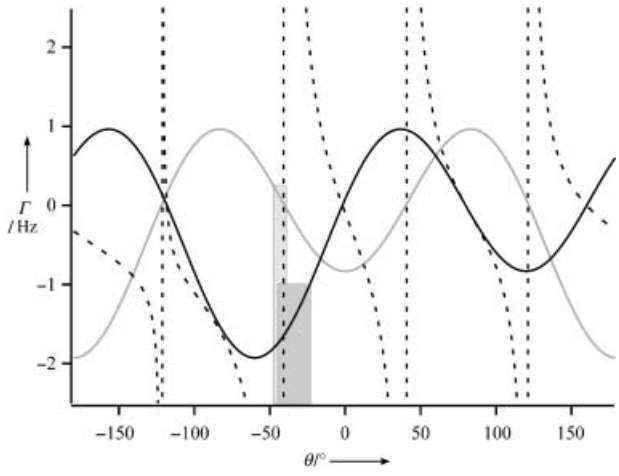


Figure 3. Dependence of the two cross-correlated relaxation rates $\Gamma_{C22–H,C3–H}$ (in black) and $\Gamma_{C23–H,C3–H}$ (in gray) on the dihedral angle θ(C^2-C^3-C^4-C^5). The two gray shadowed regions represent the value of θ that satisfy the measured CCR rates. The dashed curve represents the ratio $\Gamma_{C23–H,C3–H}/\Gamma_{C23–H,C3–H}$.

internal motions. The stereospecific assignment of the methyl groups (C22 in *pro-S* and C23 in *pro-R*) was obtained by the combination of NOE interactions and $^3J_{H,C}$ and $^3J_{C,C}$ coupling-constant data for epothilone B in the absence of tubulin and is opposite to that reported in the literature.[11b,14]

The dihedral angle θ that complies with the ratio between the two CCR rates is $-45 \pm 5°$. We used the ratio of the two CCR rates, instead of the rates themselves, to determine the dihedral angle because this approach is independent of both the correlation time and the internal motion order parameter S^2, if equal internal reorientation of all the C–H vectors is assumed.

The change in the θ dihedral angle from the gauche+ conformation in the free form to the gauche− conformation in the bound form is supported by transferred-NOE data. The structure of the bound form of epothilone determined by transferred-NOE and transferred-CCR was reported in the previous communication.[14]

The new experiment allows the determination of X-C^a-C^b-Y dihedral angles by CCR rates when X is a proton and Y is a methyl group. The method is broadly applicable to any HC-CCH_3 moiety, and therefore to a number of amino acid side chains in proteins.

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