NMR Spectroscopic Determination of Angles α and ζ in RNA from CH-Dipolar Coupling, P-CSA Cross-Correlated Relaxation

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Abstract: A new method is introduced to measure the backbone torsion angles α and ζ in ^{13}C-labeled oligonucleotides. The experiments relies on the quantification of the cross-correlated relaxation of C,P double and zero quantum coherence caused by the C,H dipolar coupling and the P chemical shift anisotropy. Two-dimensional surfaces that reveal the angular dependence of the cross-correlated relaxation rates depend on the backbone angles α and β as well as ϵ and ζ and are interpreted using torsion angle information for the angles β and ϵ from experiments measuring ^{3}J(H,P) and ^{3}J(C,P) coupling constants. The experiments have been carried out on the 10mer RNA 5′-CGC**UUUU**GCG-3′ that forms a hairpin and in which the four uridine residues are ^{13}C-labeled in the ribofuranoside moiety.

Over the last years, NMR spectroscopy of isotope labeled RNA[1,2] has become a very powerful tool to determine RNA structures in solution.[3] The local conformational preferences of nucleotides such as the sugar pucker mode and backbone angles can be obtained from measurement of homo- and heteronuclear coupling constants[4,5,6] and H,C-dipole, dipole cross-correlated relaxation rates.[7,8] However, no methods have been developed for a direct determination of the backbone angles α and ζ in oligonucleotides. Conformational analysis has relied so far on a qualitative interpretation of ^{31}P chemical shifts[9] indicating noncanonical conformations around α and ζ not differentiating between either an unusual α or ζ angle.

Here, a new method for the direct determination of the phosphodiester backbone angles α and ζ is introduced. The proposed experiment requires oligonucleotides that are ^{13}C-labeled in the sugar moiety. The method relies on the quantification and structural interpretation of cross-correlated relaxation[10,11] of ^{1}H,^{13}C-dipolar coupling and ^{31}P-chemical shift anisotropy. The cross-correlated relaxation rates can be obtained from the modulation of the two submultiplets of an ^{1}H-coupled constant time spectrum (Figure 1) of ^{13}C,^{31}P double- and zero-quantum coherence (DQC and ZQC, respectively) shown in Figure 2.

Cross-correlated relaxation[10,11] has a different effect on DQC and ZQC. From the differences in the intensities of the two doublet components (see Figure 2b), the cross-correlated relaxation $\Gamma^{DD,CSA}$ of H,C-dipolar coupling and the ^{13}C-chemical shift anisotropy (CSA) as well as the ^{31}P-CSA can be extracted:

$$\Gamma_{CH,P}^{DD,CSA} = \frac{1}{4T}\ln\frac{I_{DQC}^{\alpha} I_{ZQC}^{\beta}}{I_{ZQC}^{\alpha} I_{DQC}^{\beta}} = -\frac{2}{15}\gamma_P B_0 \tau_c \hbar \frac{u_0}{4\pi}\frac{\gamma_H \gamma_C}{(r_{CH})^3} S_{CSA,DD}^2 \cdot \begin{Bmatrix} (\sigma_{22}^P - \sigma_{11}^P) & (3\cos^2\theta_{CH,\sigma_{22}} - 1) \\ +(\sigma_{33}^P - \sigma_{11}^P) & (3\cos^2\theta_{CH,\sigma_{33}} - 1) \end{Bmatrix} \quad (1)$$

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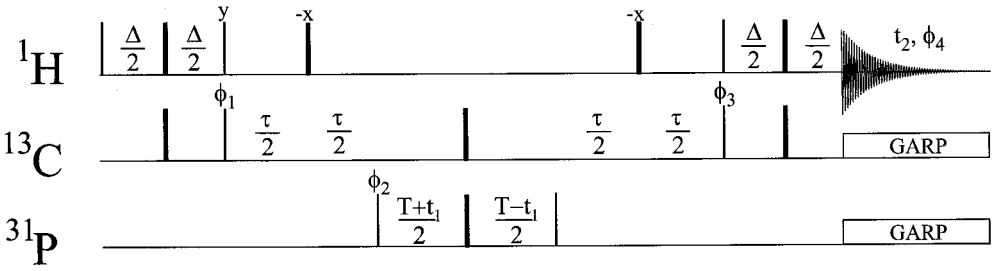
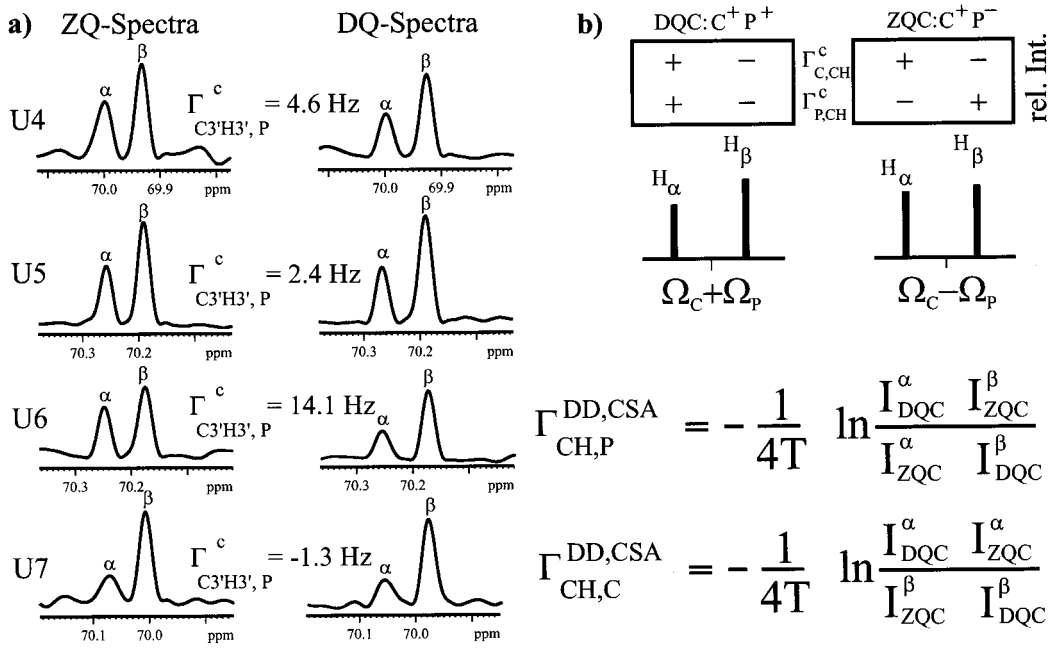


Figure 1. Pulse sequence for the 2D *constant time* Γ-DQ/ZQ-HCP. Pulse phases are along *x* if not stated otherwise. Δ = 3.2 ms, $T + 2\tau = 2/^1J(C,C) = 50$ ms, $T = 10$ ms. ^{13}C- and ^{31}P-decoupling during acquisition was applied with a field strength of 2.3 and 1.7 kHz, respectively. The relaxation delay was 1.5 s. The experiments were carried out on a Bruker DRX600 with a $^1H,^{13}C,^{31}P,^{19}F$ QXI-probe and actively shielded *z* gradients. Separation of DQC and ZQC was achieved by recording four spectra for each t_1-increment with $\phi_1 = \phi_2 = x,y,-x,-y$ and addition (ZQC: FID1 + FID2 + FID3 + FID4) or alternating addition and subtraction (DQC: FID1 − FID2 + FID3 − FID4) to create four independently stored FIDs. Sign discrimination according to States-TPPI was achieved by an independent phase cycle on ϕ_1.[14] 224 experiments per t_1 point (90 complex points, spectral width: 5882 Hz) were recorded with 4096 complex points in t_2 (spectral width: 4000 Hz). $\phi_1 = x,-x$; $\phi_2 = x,x,-x,-x$; $\phi_3 = x,x,x,x,-x,-x,-x,-x$; $\phi_4 = x,-x,-x,x,-x,x,x,-x$. Total measurement time was 34 h on a 0.9 mM sample of 5′-CGC**UUUU**GCG-3′.



$$\Gamma^{DD,CSA}_{CH,P} = -\frac{1}{4T}\ln\frac{I^{\alpha}_{DQC}\, I^{\beta}_{ZQC}}{I^{\alpha}_{ZQC}\, I^{\beta}_{DQC}}$$

$$\Gamma^{DD,CSA}_{CH,C} = -\frac{1}{4T}\ln\frac{I^{\alpha}_{DQC}\, I^{\alpha}_{ZQC}}{I^{\beta}_{ZQC}\, I^{\beta}_{DQC}}$$

Figure 2. (a) ZQ and DQ spectra taken at the positions Ω$_2$=H3′ for $^{13}C-U$ labeled[2a] nucleotides U4, U5, U6, and U7 of the RNA oligonucleotide 5′-CGC**UUUU**GCG-3′. (b) Schematics of intensity modulation of the doublet in DQ and ZQ spectra due to $\Gamma^{DD,CSA}_{CH,P}$ and $\Gamma^{DD,CSA}_{CH,C}$ cross-correlated relaxation effects.

The observed cross-correlated relaxation rate $\Gamma^{DD,CSA}_{CH,P}$ depends on the projection angles $\theta_{CH,\sigma_{22}}$ and $\theta_{CH,\sigma_{33}}$ of the CH-dipole tensor parallel to the CH bond vector and the $(\sigma_{22}-\sigma_{11})$ and $(\sigma_{33}-\sigma_{11})$ components of the ^{31}P-CSA-tensors. γ_C, γ_H, γ_P are the gyromagnetic ratios, r_{CH} the CH-bond distance, μ_0 the susceptibility of vacuum, $h/2\pi$ the Planck constant, B_0 the field strength, and *T* the DQC,ZQC evolution time. For the hairpin under study, τ_c has been determined to be 2.5 ± 0.2ns from ^{13}C-T_1-times and $^1H,^{13}C$ heteronuclear NOE. The components of the ^{31}P-CSA-tensor have to be calibrated from model compounds. The closest model system reported in the literature for a phosphodiester in oligonucleotides is the barium salt of diethyl phosphate. For this molecule, the ^{31}P-CSA tensor is found to be asymmetric[12] with σ_{11}, σ_{22}, and σ_{33} being −76 ppm, −16 ppm, and 103 ppm as shown in Figure 3. These values are to within 5% identical to the ^{31}P-CSA values found in polynucleotides and phospholipids[13] and therefore considered to be applicable to RNA. A small variation of the CSA in oligonucleotides is corroborated by the small range of the isotropic ^{31}P chemical shifts in RNA. The propagation of CSA

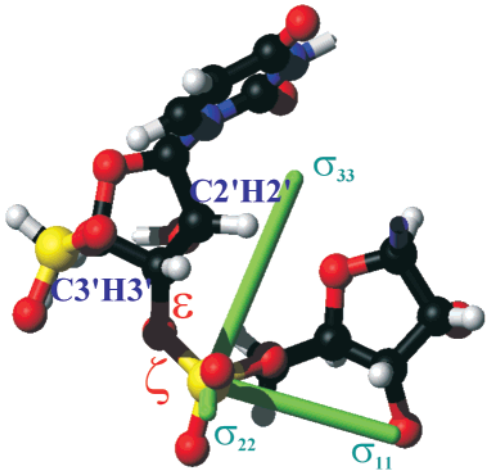


Figure 3. The orientation of the ^{31}P-CSA-tensor as determined in diethyl phosphate in the dinucleotide molecular frame.

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errors into angular information is weak. A variation of the principal values of the CSA tensor, σ_{11}, σ_{22}, and σ_{33} by 10% leads to changes of ζ of U4 by only 5° (Table S1 and Figures S1 and S2 in the Supporting Information).

As described in ref 12, the principal axes of the ^{31}P tensor $\vec{e}_{11}$, $\vec{e}_{22}$, $\vec{e}_{33}$ and the phosphate reference frame described by the three vectors $\vec{e}_x$ (the unit vector along the intersection of the O3−P−O4 bond angle), $\vec{e}_z$ (the unit vector orthogonal to $\vec{e}_x$ in the O3−P−O4 plane), and $\vec{e}_y$ (the unit vector orthogonal to $\vec{e}_z$ and $\vec{e}_x$) by the axes of the unit cell $\vec{a}$, $\vec{b}$, and $\vec{c}$ is defined by the set of direction cosines (eq 2):

$$\begin{bmatrix} e_{11} \\ e_{22} \\ e_{33} \end{bmatrix} = A_\sigma \cdot \begin{pmatrix} a \\ b \\ c \end{pmatrix} = \begin{pmatrix} 0.7330 & -0.5281 & -0.5915 \\ 0.0443 & -0.5919 & 0.8048 \\ -0.6787 & -0.6090 & -0.4105 \end{pmatrix} \cdot \begin{pmatrix} a \\ b \\ c \end{pmatrix} \quad (2a)$$

Consequently, the orientations of the CSA tensor in the

$$\begin{bmatrix} e_x \\ e_y \\ e_z \end{bmatrix} = A \cdot \begin{pmatrix} a \\ b \\ c \end{pmatrix} = \begin{pmatrix} 0.6345 & -0.4976 & -0.5915 \\ 0.1577 & -0.6658 & 0.7293 \\ -0.7567 & -0.5560 & -0.3440 \end{pmatrix} \cdot \begin{pmatrix} a \\ b \\ c \end{pmatrix} \quad (2b)$$

phosphate frame can be expressed as:

$$\begin{bmatrix} e_{11} \\ e_{22} \\ e_{33} \end{bmatrix} = A_\sigma \cdot A^{-1} \begin{pmatrix} e_x \\ e_y \\ e_z \end{pmatrix} \quad (2c)$$

The projection cosines can then be calculated using: $\cos\theta_{CH,\sigma_{22}} = \overrightarrow{CH}\vec{e}_{22}/|\overrightarrow{CH}|$ and $\cos\theta_{CH,\sigma_{33}} = \overrightarrow{CH}\vec{e}_{33}/|\overrightarrow{CH}|$. The dependence of the cross-correlated relaxation rate $\Gamma^{DD,CSA}_{(C2'_i,H2'_i),(P_{i+1})}$ on the backbone angles ϵ and ζ is shown in Figure 4a, the dependence of $\Gamma^{DD,CSA}_{(C3'_i,H3'_i),(P_{i+1})}$ on ϵ and ζ (for the ribofuranoside ring in C3′-endo conformation) is shown in Figure 4b. With $\tau_c = 2.5 \pm 0.2$ ns, the cross-correlated relaxation rates around the angles ϵ and ζ vary from −10 to 20 Hz. The blue and the green areas are allowed for U4, according to the measured cross correlated relaxation rates of 4.6 and 9.5 Hz, respectively. Both plots have been derived from eq 1. The relevant $\overrightarrow{CH}$, $\vec{e}_{22}$, and $\vec{e}_{33}$ vectors were obtained from dinucleotide models built in InsightII with variation of the two torsion angles ϵ and ζ or β and α, respectively, in steps of 10° and assuming either C2′-endo or C3′-endo conformation for the ribose.

The intersections of the blue and green areas of the two plots define the allowed pairs of ϵ and ζ for U4 (yellow circles in Figure 4c). Due to the independently measured angle $\epsilon = -146° \pm 10°$ [6] (red error bars) we find ζ close to −100°.

Figure 5 shows a similar graph for the backbone torsion angles α and β. For the angle α the sum of $\Gamma^{DD,CSA}_{(C5'_i,H5'_i),(P_i)} + \Gamma^{DD,CSA}_{(C5'_i,H5''_i),(P_i)}$ representing the arithmetic average of the two dipole tensors can be measured. This allows the restraint of the angle α to four, or in favorable cases two torsion angles as summarized in Table 1.

The analysis is based on the assumption that the phosphodiester backbone conformation is rigid. Fast internal dynamics have been modeled by assuming local motion with an amplitude of ±10° shown in Figure 6. Comparison of Figure 6 with Figure 4a shows that the cross-correlated relaxation rates are scaled but that the shape of the torsion angle dependence is not affected.

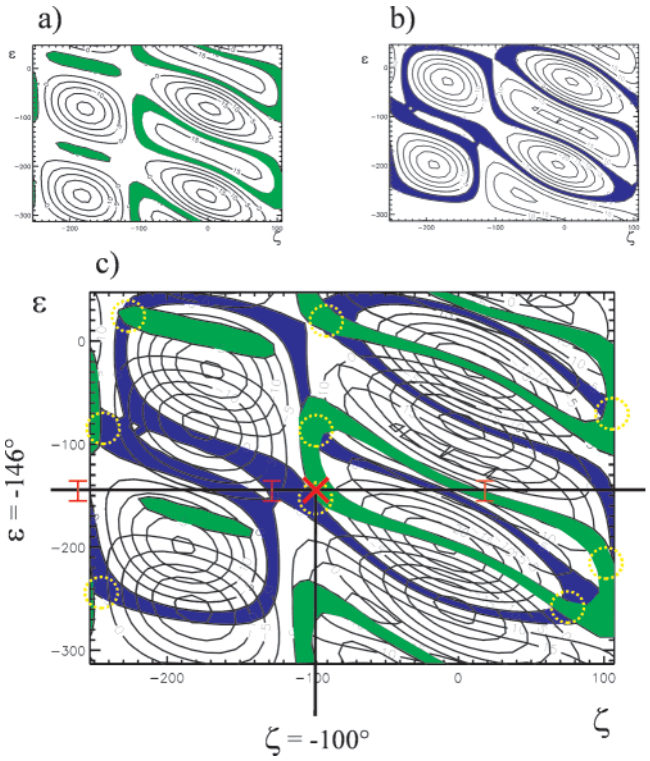

Figure 4. (a) Dependence of $\Gamma^{DD,CSA}_{(C2'_i,H2'_i),(P_{i+1})}$ and b) $\Gamma^{DD,CSA}_{(C3'_i,H3'_i),(P_{i+1})}$ on the angle ϵ and ζ for the nucleotide in the C3′-endo conformation. The experimenal rates for U4 define the green and blue areas. (c) Superposition of the two plots. U4 adopts the C3′-endo conformation ($P = 44°$, $\nu^{max} = 44°$).[7] The yellow circles indicate the conformational regions which fulfill the experimental cross-correlated relaxation rates. The red bars reflect the error of ϵ derived from the $^3J(C2'_i,P_{i+1})$, $^3J(H3'_i,P_{i+1})$ and $^3J(C4'_i,P_{i+1})$ coupling constants yielding $\epsilon = -146°$.[6] The combination of the coupling constants and the cross-correlated relaxation rates yields $\zeta = -100°$.

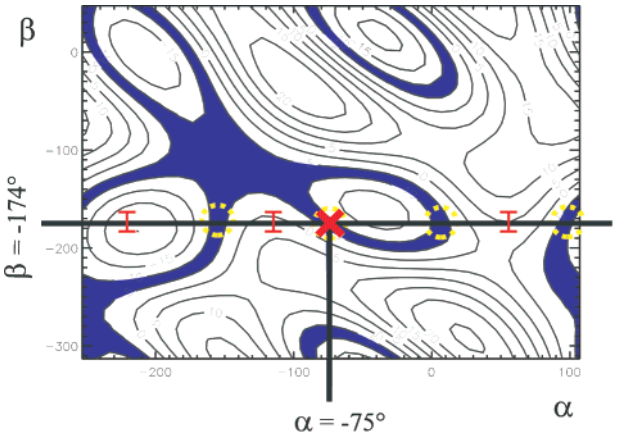

Figure 5. Dependence of $\Gamma^{DD,CSA}_{(C5'_i,H5'_i),(P_i)} + \Gamma^{DD,CSA}_{(C5'_i,H5''_i),(P_i)}$ on the angles β and α for the U4 nucleotide. The yellow circles indicate the conformational regions which fulfill the experimental cross-correlated relaxation rates. The red bars reflect the errors derived from the $^3J(H5'_i,P_i)$, $^3J(H5''_i,P_i)$ and $^3J(C4'_i,P_i)$ coupling constants yielding $\beta = -174°$.[6] The combination of the coupling constants and the cross-correlated relaxation rates yields $\alpha = -160°, -75°, 0°, 180°$. $\alpha = -75°$ is the canonical value.

Table 1 summarizes the experimental cross-correlated relaxation rates and the angles derived from interpretation of scalar coupling constants measured previously and the relaxation rates. It shows that a near complete definition of all torsion angles can be obtained for this block labeled oligonucleotide.

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Table 1. CH-dipolar Coupling−^{31}P-CSA Cross-correlated Relaxation Rates in Hz measured for the RNA Oligonucleotide 5′-CGC**UUUU**GCG-3′ [a]

	U4	U5	U6	U7
$\Gamma^{DD,CSA}_{(C2'_i,H2'_i),(P_{i+1})}$	9.5 ± 0.3	4.0 ± 0.4	3.8 ± 0.6	1.2 ± 0.1
$\Gamma^{DD,CSA}_{(C3'_i,H3'_i),(P_{i+1})}$	4.6	2.4 ± 0.6	14.1 ± 0.2	−1.3
$\Sigma\Gamma^{DD,CSA}_{(C5'_i,H5'_i+C5'_i,H5''_i),(P_i)}$	7.0 ± 1.5	2.2	n.d.	2.7 ± 0.9
$\Gamma^{DD,CSA}_{(C2',H2'),(C2')}$	5.9 (5.5)	6.5 (7.1)	6.6 (5.7)	6.9 (6.8)
$\Gamma^{DD,CSA}_{(C3',H3'),(C3')}$	n.d.	6.1 (5.3)	6.4 (6.1)	n.d.
$E\Gamma^{DD,CSA}_{(C5',H5'+C5',H5'')(C5')}$	7.0 (4.9)	(5.2)	n.d.	4.8 (6.1)
α	−160°, −75° 0°, 180°	−120°, 50°	n.d.	−120°, 50°
β	−174 ± 10°	176 ± 10°	n.d.	−165 ± 10°
ϵ	−146 ± 10°	129 ± 10°	126 ± 10°	123 ± 10°
ζ	−100°	−125°	−105°	180°

[a] The experimental error is of the order of 2 Hz. n.d.= nondetermined. The ^{13}CH-dipole,dipole, ^{13}C-CSA cross-correlated relaxation were determined independently in a non-decoupled ^{1}H,^{13}C-HSQC given in parentheses, Σ = the sum of two rates. Errors in the rates and angles were estimated from the rms between experimental and predicted coupling constants as reported in ref 6 and cross-correlated relaxation rates as reported in the text.

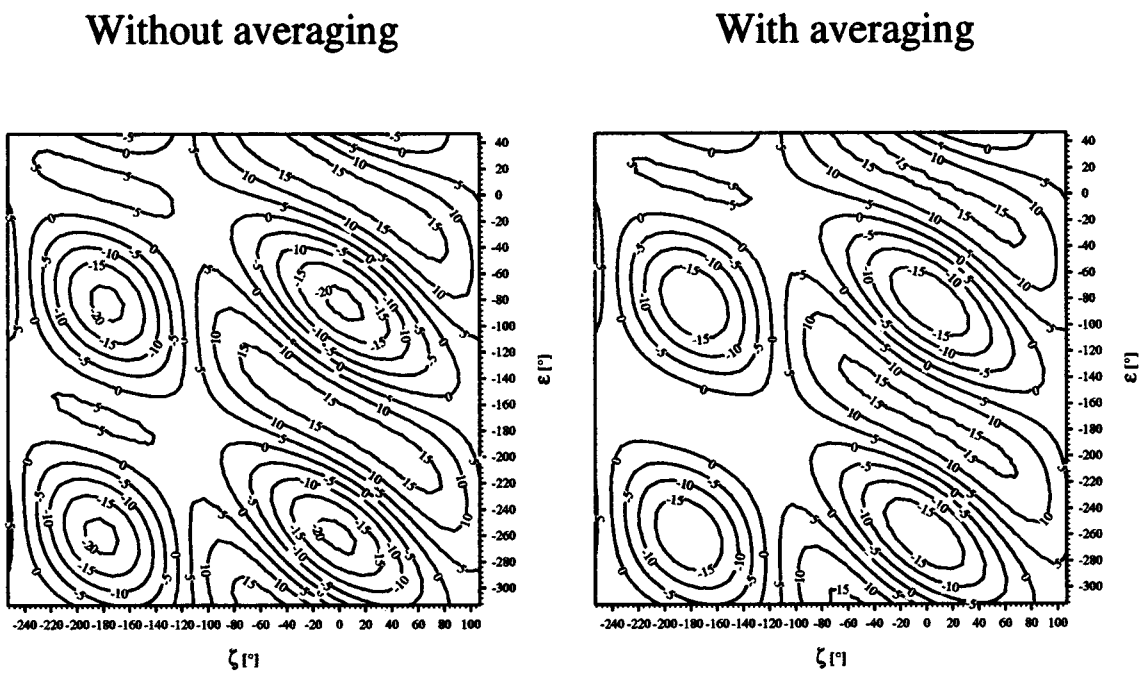

Figure 6. Graphical representation of the variation of the ϵ,ζ dependence of the cross-correlated relaxation rates assuming fast linear conformational averaging by ±10° around the preferred backbone conformation.

The experimental uncertainty in the proposed new experiment has been tested by measuring the C,H-dipolar, ^{13}C-CSA cross-correlated relaxation rate $\Gamma^{DD,CSA}_{(C,H),(C)}$ in an ω_1-coupled HSQC and comparing it to the same rate measured form the 2D *constant time* Γ-DQ/ZQ-HCP experiment. This comparison of measuring the same rate in two different experiments yields an experimental uncertainty of 1.5 Hz or less for the relaxation rates of interest here (see Table 1).

In summary, the interpretation of cross-correlated relaxation of ^{1}H,^{13}C-dipole, dipole and ^{31}P-chemical shift anisotropy completes the analysis of phosphodiester backbone conformation based on interpretation of coupling constants and makes accessible the backbone angles α and *ζ*. Cross-correlated relaxation does not require any Karplus parametrization. The assumption that the ^{31}P-CSA tensor does not change with changing conformation is supported by the fact that the variation in the ^{31}P isotropic chemical shift is only few ppm for oligonucleotides. Work is under way to provide more accurate information on the ^{31}P-CSA tensor and to develop experiments for larger oligonucleotides.

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Supporting Information Available: Experimental details (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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