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A Robust Method for Determining the Magnitude of the Fully Asymmetric Alignment Tensor of Oriented Macromolecules in the Absence of Structural Information

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It has recently been shown that the degree of alignment of macromolecules in an aqueous dilute liquid crystalline medium of bicelles is sufficient to permit accurate values of residual ^{15}N–^{1}H, ^{13}C–^{1}H, and $^{13}C^{\alpha}$–C′ dipolar couplings to be obtained on a routine basis, thereby providing potentially unique long-range structural information. To make use of this information in macromolecular structure determination, the magnitude of the axial and rhombic components of the molecular alignment tensor must be determined. This can be achieved by taking advantage of the fact that different, fixed-distance internuclear vector types are differently distributed relative to the alignment tensor. A histogram of the ensemble of normalized residual dipolar couplings for several such vector types approximates a powder pattern from which the magnitude of the axial and rhombic components are readily extracted in the absence of any prior structural information. The applicability of this method is demonstrated using synthetic data derived from four proteins representative of different sizes, topologies, and secondary structures, and experimental data measured on the small protein ubiquitin.

Key Words: **dipolar couplings; molecular alignment; liquid crystal; rhombicity; solution NMR structure determination; long-range restraints; powder pattern.**

Residual dipolar couplings in high-resolution NMR spectra, arising from small degrees of alignment of molecules in the magnetic field, provide unique long-range structural restraints that can significantly improve the quality of NMR structures (*1*). Alignment can be induced in a number of ways, including the magnetic field itself (*2–6*), an electric field (*7–9*), or the use of a liquid crystalline medium (*10–13*). Alignment arising from anisotropy of the molecular magnetic susceptibility tensor scales with the square of the magnetic field, but is generally very small even at the highest magnetic field strengths available (*1, 14–16*). Consequently, degrees of alignment sufficient to obtain accurate dipolar couplings for macromolecular structure determination are only attained for a limited number of systems (e.g., paramagnetic proteins, nucleic acids, and proteins complexed to nucleic acids).

Recently, it has been shown that moderate degrees of alignment, while retaining the resolution, sensitivity, and simplicity obtained in the isotropic phase, can be obtained by dissolving macromolecules in a very dilute liquid crystalline phase (*17, 18*) of so-called bicelles (*19*), comprising a ~5% wt/vol, 3:1 molar ratio of DMPC:DHPC in water. This permits accurate measurement of residual dipolar couplings for a variety of different fixed-distance internuclear vector types, including one-bond ^{15}N–^{1}H, $^{13}C^{\alpha}$–$^{1}H^{\alpha}$, $^{13}C^{\alpha}$–$^{13}C'$ vectors (*18*). In order to make use of residual dipolar couplings for macromolecular structure refinement, the magnitude of the axial and rhombic components of the alignment tensor must be known. Knowledge of the orientation of the alignment tensor, however, is not required since it is treated as a freely floating variable during the structure calculation (*1, 20*).

In a recent paper, we showed that it was possible, providing a reliable estimate of the minimum value of the residual dipolar couplings could be obtained, to simultaneously refine the structures and ascertain the values of the axial and rhombic components by a grid search procedure in which a series of structures were calculated for different rhombicities (*20*). Such an approach, however, can only be employed in the final stages of a structure determination and is only suitable when a sufficient number of NOE restraints is available to generate a well-defined polypeptide fold (*20*). In this paper, we outline a simple and robust procedure for reliably estimating the values of the axial and rhombic components of the alignment tensor in the absence of any prior structural information by examining the distribution of dipolar couplings.

In principle, the problem of finding the magnitude of the generally asymmetric alignment tensor is the same as that encountered in determining the Saupe ordering matrix for small molecules in a liquid crystalline environment (*10–12*). For globular proteins, however, one may safely assume a shape which is not distorted by transient interactions with the liquid crystalline medium and a negligible effect of internal motions on the alignment tensor, so that finding the Saupe ordering matrix or alignment tensor is far simpler.

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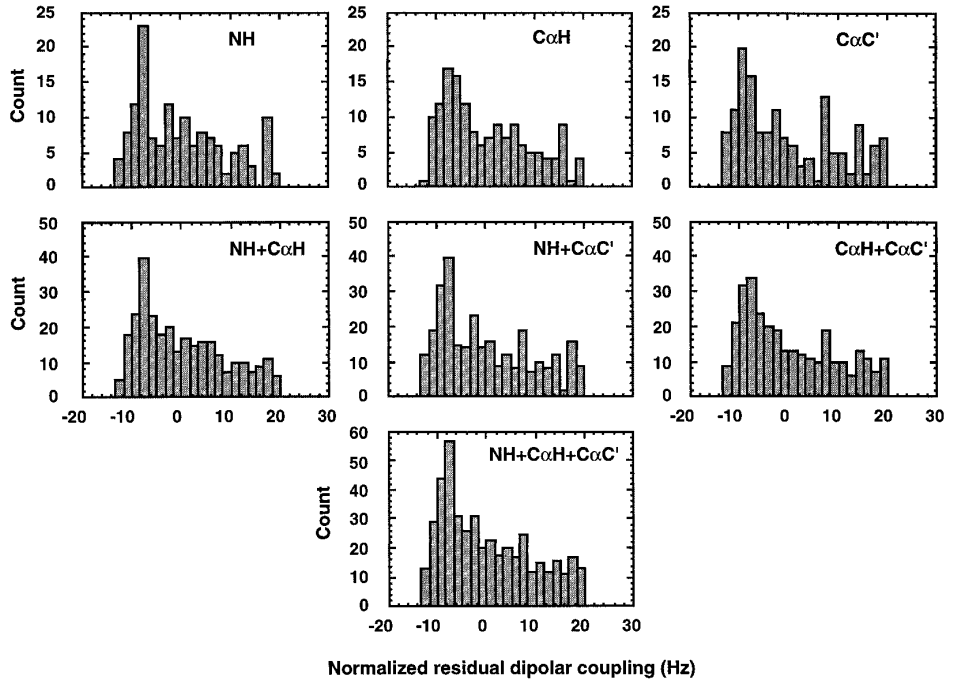

FIG. 1. Histograms of normalized residual dipolar couplings calculated for $D_a^{NH} = 10.0$ and $R = 0.2$ for interleukin-1β. Plots are shown for the individual normalized ${}^1D^{NH}$, ${}^1D^{C\alpha H\alpha}$(NH), and ${}^1D^{C\alpha C'}$(NH) dipolar couplings, for the pairwise ensemble of the normalized dipolar couplings (${}^1D^{NH}$ and ${}^1D^{C\alpha H\alpha}$(NH), ${}^1D^{NH}$ and ${}^1D^{C\alpha C'}$(NH), and ${}^1D^{C\alpha H\alpha}$(NH) and ${}^1D^{C\alpha C'}$(NH), and for the ensemble of all three normalized dipolar couplings (${}^1D^{NH}$, ${}^1D^{C\alpha H\alpha}$(NH) and ${}^1D^{C\alpha C'}$(NH)). The residual dipolar couplings, normalized relative to the one-bond NH dipolar coupling, are calculated from the structures using the coordinate frame to represent the alignment tensor. The coordinates employed for interleukin-1β are taken from Ref. (*28*).

Moreover, assignment of dipolar interactions to specific pairs of atoms is trivial in the macromolecular case of very weak ordering, where they are simply obtained from the change in the corresponding J splitting relative to the isotropic case.

The general expression for the residual dipolar coupling $D^{AB}(\theta, \phi)$ between two directly coupled nuclei A and B can be simplified to the form (*20*)

$$D^{AB}(\theta, \phi) = D_a^{AB}\left\{(3\cos^2\theta - 1) + \frac{3}{2}R(\sin^2\theta \cos 2\phi)\right\}, \quad [1]$$

where D_a^{AB} and D_r^{AB} in units of hertz are the axial and rhombic components of the traceless second rank diagonal tensor **D** given by $\frac{1}{3}[D_{zz}^{AB} - (D_{xx}^{AB} + D_{yy}^{AB})/2]$ and $\frac{1}{3}[D_{xx}^{AB} - D_{yy}^{AB}]$, respectively, with $|D_{zz}^{AB}| > |D_{yy}^{AB}| \geq |D_{xx}^{AB}|$; R is the rhombicity defined by D_r^{AB}/D_a^{AB} and is always positive; θ is the angle between the A–B interatomic vector and the z axis of the tensor; and ϕ is the angle which describes the position of the projection of the A–B interatomic vector on the x–y plane, relative to the x axis. D_a^{AB} subsumes various constants, including the gyromagnetic ratios of the two nuclei γ_A and γ_B, the inverse cube of the distance between the two nuclei $\langle r_{AB}^{-3}\rangle$, where the $\langle\ \rangle$ brackets indicate vibrational averaging, and the generalized order parameter S for fast angular fluctuations of the internuclear vector (*21*) which provides a first-order correction for the effect of rapid internal motion on D^{AB} (*1*, *16*, *22*). In the liquid crystal bicelle medium $D_a^{AB} = -(\mu_o h/16\pi^3)S\gamma_A\gamma_B\langle r_{AB}^{-3}\rangle A_a$, where A_a is the unitless axial component of the molecular alignment tensor **A** (*12*). Note that for the bicelle medium, where the liquid crystal director is orthogonal to the magnetic field, **A** differs from the Saupe ordering matrix by a factor of $-\frac{1}{2}$. In the case of alignment induced by magnetic susceptibility anisotropy $D_a^{AB} = -(B_o^2 h/60kT\pi^2)S\gamma_A\gamma_B\langle r_{AB}^{-3}\rangle\chi_a$, where B_o is the magnetic field strength and χ_a the axial component of the magnetic susceptibility tensor χ in units of m^3/molecule (*6*). Since S^2 values obtained from heteronuclear relaxation measurements typically range from 0.7 to 0.9 for the structured regions in proteins, S falls in the 0.85 to 0.95 range so that the assumption of a uniform S value introduces a negligible error of at most a few percent in the calculated dipolar couplings (*1*).

For a given type of fixed distance (r_{AB}) A–B interaction, the extreme D^{AB} values measured correspond to orientations of A–B vectors closest to the z ($\theta = 0°$) and y ($\theta = 90°$; $\phi = 90°$) principal axes of the alignment tensor. If the A–B vectors are distributed uniformly and isotropically, a histogram describing the probability of finding values of D^{AB} between these two extremes will have the same shape as a chemical shift anisotropy (CSA) powder pattern (*23*). The highest probability di-

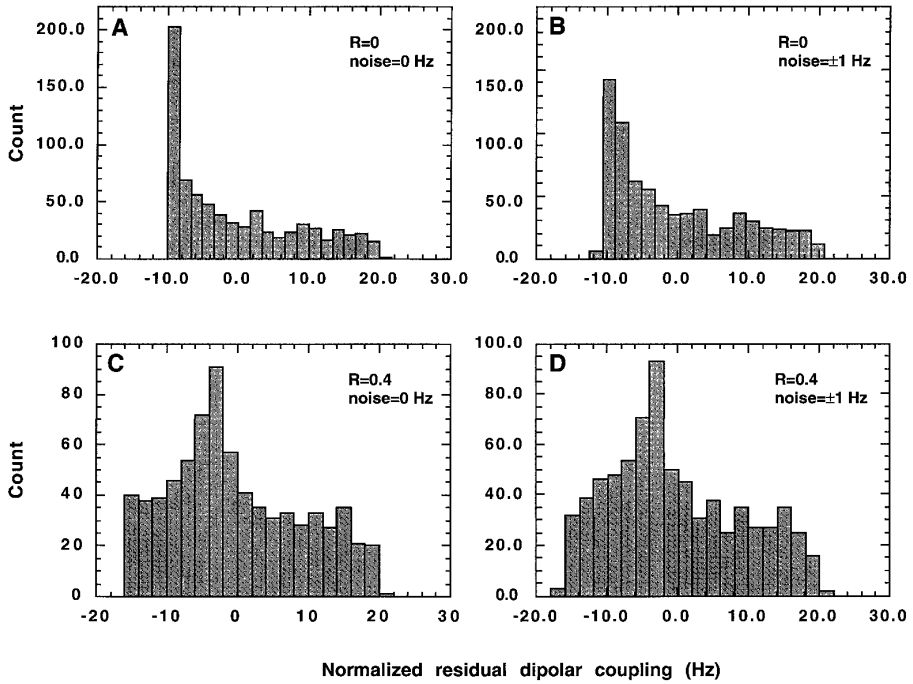

FIG. 2. Histograms of the ensemble of the normalized ${}^1D^{\mathrm{NH}}$, ${}^1D^{\mathrm{C}\alpha\mathrm{H}\alpha}$(NH), and ${}^1D^{\mathrm{C}\alpha\mathrm{C}'}$(NH) dipolar couplings for enzyme I calculated for $D_{\mathrm{a}}^{\mathrm{NH}} = 10.0$. (A) and (C) are calculated for $R = 0$ and $R = 0.4$, respectively, with no random added error. (B) and (D) are calculated for $R = 0$ and $R = 0.4$, respectively, with a random added rms error of ± 1 Hz, to simulate experimental uncertainty. The residual dipolar couplings, normalized relative to the one-bond NH dipolar coupling, are calculated from the structure using the coordinate frame to represent the alignment tensor. The coordinates employed for enzyme I (which consists of 259 residues) are taken from Ref. (*29*).

polar coupling value, therefore, coincides with the magnitude of a bond vector aligned along the x axis of the alignment tensor ($\theta = 90°$, $\phi = 0°$). Since $D_{xx}^{\mathrm{AB}} + D_{yy}^{\mathrm{AB}} + D_{22}^{\mathrm{AB}} = 0$, it follows from Eq. [1] that

$$\begin{aligned} D_{zz}^{\mathrm{AB}} &= 2D_{\mathrm{a}}^{\mathrm{AB}} \\ D_{yy}^{\mathrm{AB}} &= -D_{\mathrm{a}}^{\mathrm{AB}}(1 + 1.5R) \\ D_{xx}^{\mathrm{AB}} &= -D_{\mathrm{a}}^{\mathrm{AB}}(1 - 1.5R). \end{aligned} \qquad [2]$$

In practice, the number of dipolar couplings measured will be limited and the assumption of a uniform isotropic distribution of orientations need not necessarily apply. Thus, for example, N–H vectors in a helix are roughly parallel to the helix axis so that in largely helical proteins clustering of N–H vector orientations may occur. In the case of residual dipolar couplings there are at least three backbone one-bond interactions per residue that can be accurately measured: ${}^1D^{\mathrm{NH}}$, ${}^1D^{\mathrm{C}\alpha\mathrm{H}\alpha}$, and ${}^1D^{\mathrm{C}\alpha\mathrm{C}'}$. In addition, ${}^1D^{\mathrm{C}'\mathrm{N}}$ can also be measured, albeit with slightly less relative accuracy than the other three dipolar couplings. As the orientation of these vectors with regard to the alignment tensor will be different for any given residue, it follows that the distribution for the ensemble of the different vector types will be more uniform than for any of the individual vector types alone (*24*). The distribution is further enhanced by the fact that the angles between the N–H and C′–N bond vectors and between the C^α–H^α and C^α–C′ bond vectors are 120° and 109°, respectively. Such an ensemble is obtained by normalizing the different residual dipolar couplings. Thus, the residual dipolar coupling, D^{AB}(NH), between atoms A and B, normalized relative to ${}^1D^{\mathrm{NH}}$, is given by

$$D^{\mathrm{AB}}(\mathrm{NH}) = D^{\mathrm{AB}}(\gamma_{\mathrm{N}}\gamma_{\mathrm{H}}\langle r_{\mathrm{NH}}^{-3}\rangle/(\gamma_{\mathrm{A}}\gamma_{\mathrm{B}}\langle r_{\mathrm{AB}}^{-3}\rangle). \qquad [3]$$

(The distances we employ are 1.02, 1.08, 1.341, and 1.525 Å for the N–H, C^α–H^α, N–C′, and C^α–C′ bonds, respectively (*25*).)

Experimentally, the normalized values of D_{zz} and D_{yy} are obtained by taking the average of the high and low extreme values of the normalized residual dipolar couplings, respectively, such that the standard deviations in the estimated values of D_{zz} and D_{yy} are equal to the measurement error. The value of D_{xx} corresponds to the most populated value in the histogram of the observed normalized residual dipolar couplings. With two unknowns and three observables (D_{xx}, D_{yy}, and D_{zz}), the values of D_{a} and R are then calculated by nonlinear least-squares optimization of Eq. [2].

To test how well this approach performs in practice, we have calculated normalized residual dipolar couplings (using the coordinate frame to represent the alignment tensor) for the N–H, C^α–H, and C^α–C′ vectors of four proteins covering different sizes, topologies, and secondary structures: namely, the GB1 domain of protein G (56 residues, mixed α-helix and

TABLE 1

Estimated Values of D_a^{NH} and R Obtained by Analysis of Histograms of Normalized Residual Dipolar Couplings for a Target Value of $D_a^{NH} = 10$ Hz, Rhombicities of 0.1 to 0.6, and a Random Added rms Error of ±1 Hz

		Estimated values of D_a^{NH}(Hz) and R							
		[$^1D^{NH}$, $^1D^{C\alpha H\alpha}$(NH) and $^1D^{C\alpha C'}$(NH)]				[$^1D^{NH}$ and $^1D^{C\alpha H\alpha}$(NH)]			
		100% data		50% data		100% data		50% data	
Protein	Target R	D_a (Hz)	R	D_a (Hz)	R	D_a (Hz)	R	D_a (Hz)	R
Enzyme I	0.1	10.0	0.11	9.9	0.11	9.9	0.11	9.9	0.09
IL-1β	0.1	9.9	0.10	9.9	0.09	10.0	0.09	9.8	0.06
IL-4	0.1	10.0	0.11	10.1	0.07	9.8	0.11	9.7	0.11
GB1	0.1	9.9	0.12	10.0	0.09	10.1[a]	0.10[a]	9.9[a]	0[a]
Enzyme I	0.2	10.1	0.19	10.0	0.19	9.9	0.17	9.4	0.20
IL-1β	0.2	9.9	0.19	9.7	0.19	9.6	0.16	9.6	0.12
IL-4	0.2	9.9	0.18	9.8	0.19	9.9	0.19	9.7	0.23
GB1	0.2	9.9	0.17	9.4	0.23	10.0[a]	0.11[a]	10.0[a]	0.11[a]
Enzyme I	0.4	10.2	0.37	9.8	0.42	9.9[a]	0.38[a]	9.5[a]	0.34[a]
IL-1β	0.4	9.9	0.35	9.7	0.36	9.6	0.36	9.5	0.37
IL-4	0.4	9.9	0.37	9.9	0.39	10.0	0.40	9.7	0.36
GB1	0.4	9.6	0.44	9.5	0.44	10.0[a]	0.34[a]	10.0[a]	0.23[a]
Enzyme I	0.6	9.7	0.66	9.5	0.66	9.6[a]	0.57[a]	9.6[a]	0.50[a]
IL-1β	0.6	9.6	0.64	9.6	0.60	9.5	0.58	9.3	0.54
IL-4	0.6	9.7	0.63	9.6	0.60	9.6	0.63	9.6	0.63
GB1	0.6	9.4	0.63	9.4	0.63	9.7	0.62	9.9[a]	0.38[a]

Note. Estimated values of D_a^{NH} and R are obtained by nonlinear least-squares optimization of Eq. [2] using the values of D_{xx}, D_{yy}, and D_{zz} measured from the histograms of normalized residual dipolar couplings. Results are given for the ensemble of the normalized $^1D^{NH}$, $^1D^{C\alpha H\alpha}$(NH), and $^1D^{C\alpha C'}$(NH) couplings, as well as for the ensemble of only the $^1D^{NH}$ and $^1D^{C\alpha H\alpha}$(NH) couplings. The residual dipolar couplings, normalized relative to the one-bond NH coupling, are calculated from the structures using the coordinate frame to represent the alignment tensor. The coordinates for the B1 domain of protein G (GB1), interleukin-4 (IL-4), interleukin-1β (IL-1b), and enzyme I are taken from Refs. (*26–29*), respectively.

[a] D_{xx} was ill-defined from the histogram, so D_a^{NH} and R were calculated from D_{zz} and D_{yy} only.

β-sheet (*26*)), interleukin-4 (123 residues, four α-helix bundle (*27*)), interleukin-1β (153 residues, β-sheet (*28*)), and enzyme I (259 residues, mixed α-helix and β-sheet (*29*)). The normalized residual dipolar couplings were calculated for a value of $D_a^{NH} = 10.0$, which corresponds to values typically obtained using dilute bicelle liquid crystals (*17, 18*), and different values of the rhombicity R.

Figure 1 illustrates a series of histograms obtained from the individual normalized $^1D^{NH}$, $^1D^{C\alpha H\alpha}$(NH), and $^1D^{C\alpha C'}$(NH) couplings, from the pairwise ensembles of the three types of couplings, and for the ensemble of all three couplings calculated for interleukin-1β, using $D_a^{NH} = 10.0$ and $R = 0.2$. It is readily apparent that the resemblance of the histograms to a CSA powder pattern improves as more internuclear vector types are employed, thereby permitting more accurate values of D_{xx}, D_{yy}, and D_{zz} to be obtained. The effects of introducing a random added rms error of ± 1 Hz, which corresponds to the typical error obtained experimentally for a protein in the 20- to 40-kDa range, are illustrated in Fig. 2 for enzyme I, both for the case of axial symmetry ($R = 0$) and a rhombicity, R, of 0.4. In the absence of added random errors (Fig. 2A,C), the histograms look like almost perfect powder patterns, since the protein is large enough (259 residues) to ensure that the N–H, C^α–H, and C^α–C' vectors are approximately uniformly and isotropically distributed in space. Analogous to the effect of line broadening on the CSA powder pattern, the principal values of the dipolar coupling tensor become less well determined in the presence of added random error (Fig. 2B,D). However, provided that the measurement uncertainty is much smaller than $|D_{zz}|$, the extracted values remain fully adequate for the required purpose.

Table 1 summarizes the results of a series of calculations for the four proteins in which the values of D_a^{NH} and R have been calculated by nonlinear least-squares optimization of Eq. [2], using the values of D_{xx}, D_{yy}, and D_{zz} obtained from the histograms of normalized residual dipolar couplings [$^1D^{NH}$, $^1D^{C\alpha H\alpha}$(NH) and $^1D^{C\alpha C'}$(NH); and $^1D^{NH}$ and $^1D^{C\alpha H\alpha}$(NH)] calculated with a random added rms error of ± 1 Hz, a target value of $D_a^{NH} = 10$ Hz, and target values of R ranging from 0.1 to 0.6. In general, values of D_a^{NH} and R are obtained within an error of $\leq$5% and $\leq \pm 0.1$, respectively, even when only 50% of the residual dipolar couplings are employed. These uncertainties are sufficiently small to have little or no effect on the results of simulated annealing refinement (*1*).

To further test the robustness of the approach we have analyzed the distribution of the 54 $^1D^{NH}$, 42 $^1D^{C\alpha H\alpha}$, and 54 $^1D^{C\alpha C'}$ dipolar couplings measured for the 76-residue protein ubiquitin by taking the difference in the one-bond J splittings obtained in the 5% wt/vol bicelle (2.9:1 DMPC:DHPC) liquid crystalline medium and in an isotropic medium (*18*). A nonlinear least-squares procedure to determine the magnitude and orientation of the alignment tensor (five adjustable parameters) on the basis of the measured $^1D^{NH}$ couplings and the N–H vector orientations obtained from the 1.8 Å resolution crystal structure (*29*), yielded values of D_a^{NH} and R of -9.6 Hz and 0.17, respectively (*18*). This compares to values of -10.0 Hz and 0.14, respectively, obtained from an analysis of the histogram of the combined ensemble of normalized $^1D^{NH}$, $^1D^{C\alpha H\alpha}$(NH), and $^1D^{C\alpha C'}$(NH) couplings, and -9.6 Hz and 0.10, respectively, from an analysis of the histogram of the ensemble of normalized $^1D^{NH}$ and $^1D^{C\alpha H\alpha}$(NH) couplings. Even if the histogram is generated with only the $^1D^{NH}$ couplings, reasonable estimates of D_a^{NH} (-9.3 Hz) and R (0.10) are still obtained.

In conclusion, we have shown that the magnitude of the generally asymmetric alignment tensor can be obtained in a reliable manner from the distribution of normalized residual dipolar couplings in the absence of any structural information. In the final stages of structure refinement, the present approach may be combined with that described in Ref. (*20*), that is, searching over a small range of D_a and R values to minimize the difference between the measured dipolar couplings and those predicted from the structures calculated with the dipolar couplings included. Thus, we anticipate that residual dipolar couplings can be employed on a routine basis for macromolecular structure determination, thereby providing long-range structural restraints that may significantly increase the accuracy of NMR structures.

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