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An Alternative Method for Pucker Determination in Carbohydrates from Residual Dipolar Couplings: A Solution NMR Study of the Fructofuranosyl Ring of Sucrose

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Abstract: A simple solution NMR method is presented for pucker determination of five-membered rings using only residual dipolar couplings obtained in a single liquid crystalline medium, DMPC/DHPC bicelles (DMPC = dimyristoylphosphatidylcholine; DHPC = dihexanoylphosphatidylcholine). The method was applied to determine the pucker of the fructofuranosyl ring of sucrose. The results indicate a fructofuranosyl pucker phase in the 20°−70° range. The pucker phases are in agreement with those from previous NMR and optical spectroscopic studies and, importantly, do not rely on empirically parametrized Karplus curves. Furthermore, the analysis implies more than one stable pucker phase and rapid ring interconversion in this range. The present results suggest that using residual dipolar couplings alone can reveal multiple conformations present in solution. Hence, when a sufficient number of residual dipolar coupling constants is measured, the outcome is a robust, reliable, and independent route for determining carbohydrate and nucleic acid structure by NMR spectroscopy.

Introduction

Three-dimensional carbohydrate structure is poorly understood, despite the fact that carbohydrates make up one of the largest classes of biological molecules. Carbohydrates serve as recognition molecules in many biological systems, for example, in blood groups. They also make up bacterial cell walls and, consequently, are used in polysaccharide vaccines. Therefore, carbohydrate structure determination is crucial for a detailed understanding of features responsible for binding and subsequent immunity.

In principle, structural details can be obtained at atomic resolution using nuclear magnetic resonance (NMR) spectroscopy through the nuclear overhauser effect (NOE).[1] In practice, solution structure determination of carbohydrates by NMR is difficult because few NOEs between sequence-remote residues are observed. ^{1}H-^{1}H dipolar couplings can be observed indirectly through the NOE, which depends on r^{-6}. Unfortunately, NOEs can only be observed if the interatomic distance is less than 5 Å, thus complicating three-dimensional structural studies.[1]

Another method for obtaining structural information is through residual dipolar couplings (RDCs), which are simply scaled dipolar couplings. The dipolar coupling between a carbon and hydrogen atom is given by

$$\mathscr{D} = (\hbar\gamma_H\gamma_C)(r^3)^{-1}(3\cos^2\theta - 1)$$

where θ is the angle between the interatomic vector and the static magnetic field, $\hbar$ is Planck's constant divided by 2π, and γ_H and γ_C are the magnetogyric ratios of hydrogen and carbon in c.g.s. units, respectively. Dipolar coupling cannot be directly observed for an isotropically tumbling molecule because the average $\langle 3\cos^2\theta - 1\rangle = 0$. In contrast, bicelles[2−4] and other water-soluble weakly aligning liquid crystalline media[5−10] restrict isotropic molecular reorientation and $\langle 3\cos^2\theta - 1\rangle \neq 0$. Therefore RDCs can be measured directly from high-resolution NMR spectra. These RDCs yield angular relations between interatomic vectors separated by large distances because these vectors can be related to the same coordinate system. RDCs have been used to refine protein structures[11−14] and to find the relative orientation of protein domains with respect to one another.[8−10] It is important to note that scalar coupling constants and RDCs have different origins. Nevertheless, they

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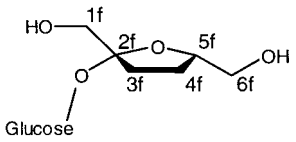

Figure 1. A schematic representation of the fructofuranosyl ring in sucrose with hydrogen atoms and ring OH groups omitted for clarity. The atoms are numbered to correspond to column 2 in Table 1.

are observed simultaneously in spectra of oriented molecules. RDCs can be extracted using the relation RDC $= J' - J_{iso}$, where J' is the observed coupling under oriented conditions, and J_{iso} is the scalar coupling (observed under isotropic conditions). The alignment tensor requires at least five RDCs to fit a given structure to the equation $\mathrm{RDC}(\psi,\phi) = (\hbar\gamma_H\gamma_C)(r^3)^{-1}(D_a(3\cos^2\psi - 1) + {}^3/_2 D_r(\sin^2\psi\cos 2\phi))$. Here, D_a is the axial component of the alignment tensor, D_r is its rhombicity parameter, and ψ and ϕ are the polar angles that define the internuclear vector in the molecular alignment frame.[10]

RDCs also reflect internal motion within a molecule. A formalism that accounts for motional averaging of RDCs in complex carbohydrates and proteins was recently proposed.[15,16] To use this approach, the structures of the smallest rigid molecular units had to be defined[16] before they were combined to define the macromolecule. These RDCs, in turn, were used to relate the rigid subunits and provide a new framework for solution structure determination in molecules with few long-range NOEs.

This paper reports a rapid method for geometry determination of five-membered rings using only RDCs in DMPC/DHPC bicelles, and its application to pucker determination in the fructofuranosyl ring of sucrose (Figure 1). It involves evaluating the fits of experimentally determined RDCs to 20 possible structures of sucrose's fructofuranosyl ring, which differ only in their pucker phase. Application of this method would simplify structure determination of di-,[17] oligo-, and polysaccharides, where motion is known to complicate structural elucidation. Structure determination of the fructofuranosyl ring of sucrose is expected to aid in structure determination of sucrose using the generalized degree of order approach.[15,16] This methodology could also be extended to study the conformation of five-membered rings in nucleic acids. The method presented here, an alternative to others,[18] is more accessible than those which rely on complex relaxation analysis,[19,20] does not rely on empirically parametrized Karplus curves,[21,22] and can be applied to systems in which few ^{1}H-^{1}H scalar coupling constants are available.

Experimental Section

Data were collected for two samples at 315 K: (a) a 75 mM sucrose sample in 20 mM phosphate buffer in D_2O at pD* = 7.1; and (b), identical to (a) with added 22% DMPC/DHPC bicelles. DMPC and DHPC, purchased from Avanti Polar Lipids (Alabaster, AL), were used without further purification. Bicelles ($q = 3$) were prepared as previously reported[23,24] in a 20 mM phosphate buffer in D_2O (pD* = 7.1). The samples were placed in Shigemi (Alison Park, PA) microcells, degassed under reduced pressure, and the tops were tightly wrapped with Teflon tape and then Parafilm.

Two-dimensional HSQC and long-range quantitative J spectra were taken at a ^{1}H frequency of 500.13 MHz and a ^{13}C frequency of 125.76 MHz on a Bruker Avance 500. Spectral widths were set to 8 and 20 ppm in the ^{1}H and ^{13}C dimensions, respectively, and the carriers were placed on HOD for ^{1}H and 58 ppm for ^{13}C. All two-dimensional ^{1}H-^{13}C correlation spectra were acquired using gradients for coherence selection.[25] One-bond coupling constants for both samples were measured from frequency differences in a ^{1}H-^{13}C constant time HSQC spectra acquired without decoupling ^{1}H's during ^{13}C evolution, with 1536 complex points in the ^{1}H dimension, 200 complex points in the ^{13}C dimension in natural abundance, and a recycle delay of 1.8 s. Long-range ^{1}H-^{13}C coupling constants were measured in natural abundance from quantitative J spectra in a manner similar to Grzesiek and co-workers, except that the reference peaks used were the auto-correlation peaks in the long-range quantitative J spectra.[26,27] These spectra were acquired with 404 complex points in the ^{1}H dimension and 200 complex points in the ^{13}C dimension and a recycle delay of 3.05 s. Coupling constants were determined according to

$$J = (2\pi\Delta)^{-1} \times \left(\sin^{-1}\left([\sin(2\pi\Delta^1 J)]\sqrt{[V_{lr}(T_2^{13C}/T_2^{12C})/V_{auto}(1-\exp(-\tau/T_1))]}\right)\right)$$

where ^1J is the ^{1}H-^{13}C one-bond coupling constant measured from HSQC spectra, Δ is the INEPT delay, V_{lr} and V_{auto} are the peak volumes for the correlation peaks and auto-correlation peaks in the long-range spectra, respectively, T_2^{13C} is the T_2 for ^{1}H's attached to ^{13}C, T_2^{12C} is the T_2 for ^{1}H's attached to ^{12}C, τ is the recycle delay, and T_1 is the ^{1}H T_1 for a given resonance.[28] T_2^{13C} and T_2^{12C} were obtained from a program that calculates ^{1}H relaxation times from the spectral densities using 90 ps as an overall correlation for sucrose.[29]

A fully ^{13}C labeled sucrose sample (Isotec, Miamisburg, OH) provided ^{13}C-^{13}C coupling constants, which were measured from one-dimensional ^{13}C data acquired with homonuclear decoupling at 125.76 MHz. Decoupling was accomplished using an RF bridge to simultaneously deliver two independent RFs, and the pulse program was modified so that irradiation took place while the receiver was gated off between data points. ^{1}H-^{1}H coupling constants were measured from COSY spectra taken at 800.13 MHz using ACME.[30]

The geometry of the fructose ring was evaluated with only RDCs, utilizing singular value decomposition.[31] The initial structure of the fructofuranosyl ring that was used to generate different pucker geometries was taken from the neutron diffraction sucrose structure.[32] The fructose ring pucker phase[33] was set to 0° and then varied in 18° increments to generate a total of 20 structures using a pucker amplitude of 40°.[33] The geometry of each pucker form was optimized (CHARMM) with ring torsion restraints using an adopted basis Newton−Raphson

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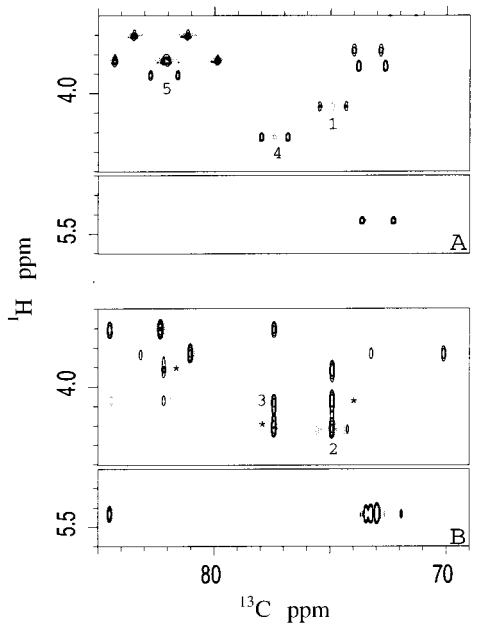

Figure 2. Partial spectra from typical NMR experiments used to determine RDCs. The spectra were taken at 42 °C under isotropic conditions in D_2O. Peaks in the spectrum are labeled with a number which corresponds to a peak number listed in Table 1. Peaks with asterisks (*) are auto-correlation peaks in the long-range quantitative *J* spectra. (a) A constant-time ^{1}H-^{13}C HSQC without decoupling, using gradients for coherence selection. The doublets are well resolved, and their frequency difference yields reliable measurement of $^1J_{CH}$ values. (b) A long-range constant-time ^{1}H-^{13}C HSQC using gradients for coherence selection. Coupling constants were determined from ratios of the cross-peak to the auto-correlation peak volume in the spectrum, except for C5f−H4f (see footnote in Table 1).

minimization and previously reported force-field parameters.[34,35] Fits to the data were based on χ^2, which is defined as $(D_{calc} - D_{exp})^2/(D_{err})^2$.

Results and Discussion

Overall, values for the one-bond RDCs measured from ^{1}H-^{13}C two-dimensional (HSQC) spectra (Figure 2a) are of reasonable magnitude,[36] with the largest observed for H5f−C5f (Table 1). Three additional long-range ^{1}H-^{13}C RDCs were obtained from long-range quantitative *J* spectra (Figure 2b), taken of sucrose at natural abundance. Peaks in the spectra were well-resolved and easy to interpret (Figure 2b).[27] Furthermore, the quantitative *J* method employed here for measurement of heteronuclear coupling constants was not limited by digital resolution and provided reproducible and reliable *J* values (Table 1).[27] Table 1 also lists two ^{1}H-^{1}H RDCs measured from ^{1}H-^{1}H COSY spectra at 800 MHz[30] and five ^{13}C-^{13}C RDCs measured from one-dimensional decoupling experiments. Thus, four independent data types were measured, which minimizes the systematic error in the final fits of RDCs to structure.

Pucker of the Fructofuranosyl Ring of Sucrose. A total of 13 RDCs, including ^{1}H-^{13}C, ^{1}H-^{1}H, and ^{13}C-^{13}C RDCs, were fit to each of the 20 structures generated as described in the Experimental Section, and the goodness of fit results are plotted for each pucker in Figure 3. The best fit structures are located in the NE quadrant of the pseudorotational wheel (defined as 0°−90°),[33] with puckers of 18°, 36°, 54°, and 72°; indeed, values

Table 1. Scalar and Residual Dipolar Couplings for the Fructofuranosyl Ring of Sucrose

peak number	atom pair	J_{iso}	J'	RDC
1	C3f−H3f[a]	143.1 ± 0.1	129.9 ± 0.1	−13.2 ± 0.2
2	C3f−H4f[b]	−2.15 ± 0.03	−1.65 ± 0.02	0.5 ± 0.05
3	C4f−H3f[b]	−3.59 ± 0.05	−2.22 ± 0.05	1.4 ± 0.1
4	C4f−H4f[a]	138.4 ± 0.1	130.0 ± 0.1	−8.4 ± 0.2
	C5f−H4f[b]	−1.2 ± 0.2	−0.6 ± 0.2	0.6 ± 0.4
5	C5f−H5f[a]	147.1 ± 0.1	128.9 ± 0.1	−18.2 ± 0.2
	H3f−H4f[c]	8.63 ± 0.1	8.2 ± 0.1	−0.43 ± 0.2
	H4f−H5f[c]	8.4 ± 0.1	5.8 ± 0.1	−2.6 ± 0.2
	C1f−C2f[d]	51.6 ± 0.15	52.1 ± 0.15	0.5 ± 0.3
	C2f−C3f[d]	43.6 ± 0.15	47.0 ± 0.15	3.4 ± 0.3
	C3f−C4f[d]	38.8 ± 0.15	39.9 ± 0.15	1.1 ± 0.3
	C4f−C5f[d]	39.9 ± 0.15	40.3 ± 0.15	0.4 ± 0.3
	C5f−C6f[d]	41.5 ± 0.15	41.0 ± 0.15	−0.5 ± 0.3

[a] Obtained from data 2-D ^{1}H-^{13}C (constant time HSQC) spectra collected using gradient coherence selection[25] with the ^{1}H decoupling pulse omitted during ^{13}C evolution. RDCs for H1f−C1f and H6f−C6f are not reported because their hydrogen atoms have overlapping ^{1}H chemical shifts at 500 MHz. [b] Obtained from long-range quantitative *J* spectra by using gradient coherence selection[25] with INEPT delays of 25 ms (isotropic) and 19.5 ms (oriented). Values for C5f−H4f were measured with an INEPT delay of 41.05 ms (isotropic and oriented). Spectra for the 41.05 ms INEPT delay are not shown. Peak volume ratios were used to quantify coupling constants in the fructofuranosyl ring and obtain reliable *J*'s and RDCs. [c] Measured from ^{1}H-^{1}H COSY experiments at 800 MHz and fitted with ACME.[30] [d] Measured from frequency differences in ^{13}C spectra acquired with ^{13}C decoupling. The sample was uniformly ^{13}C labeled.

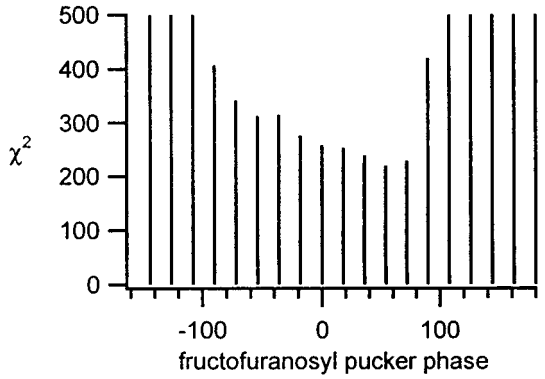

Figure 3. Plot of χ^2 vs fructofuranosyl ring pucker showing the goodness of fit for different fructose puckers. The plot is magnified to show the minimum χ^2 values occur at puckers of 18°, 36°, 54°, and 72°.

of D_a for these four puckers are nearly identical. Poor fits were obtained for puckers in the NW, SW, and SE quadrants of the pseudorotational wheel. Thus, Figure 3 shows that RDCs are sensitive to molecular geometry and, hence, ring pucker. This analysis cannot rule out the presence of multiple fructofuranosyl conformations.

Scatter plots of experimental versus calculated RDCs for pucker values of 18°, 36°, 54°, and 72° show that RDCs are well reproduced for these four puckers; in contrast, the remaining puckers show lower correlations between experimental and calculated RDCs (Supporting Information). On close examination, the scatter plots of experimental versus calculated RDCs for the 18°−72° range reveal that most calculated RDCs agree with experimentally determined RDCs within experimental error. Calculated RDCs for C4f−H3f, H3f−H4f, and H4f−H5f do not reproduce experimental RDCs as well. This implies that the calculated errors for these pairs of atoms may be underestimated. Alternatively, the errors may arise from strong coupling artifacts.[37]

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To evaluate potentially erroneous fits of the data, RDCs were removed one at a time from the input matrix. For each RDC removed, χ^2 versus pucker profiles are similar to those obtained from fitting all the RDCs. The evaluation indicates that fitting the complete set of dipolar couplings to different puckers of fructose is a sound way to analyze the conformation of the fructose ring of sucrose. Interestingly, exclusion of the ^{13}C-^{13}C RDCs, which left only eight RDCs, also yielded a best fit in the NE quadrant of the pseudorotational wheel. The best puckers were found to be 18°, 36°, 54°, and 72°, in agreement with fits which used 13 RDCs. Potentially erroneous fits were evaluated in the same manner as above by removing one RDC at a time and repeating the fit. In all but one case, the fits obtained yield similar χ^2 versus pucker profiles for the fructose ring. In the one exception, when H5f−C5f (RDC = −18.2) is removed, the best fits were found in the NW quadrant of the pseudorotational wheel. Such behavior is expected since the RDC for H5f−C5f is large and thus strongly influences fitting. This result shows that even when the number of RDCs is reduced to eight, the results agree with those obtained from 13 RDCs. Nevertheless, removal of one RDC at a time from the data set with only eight RDCs suggests that it is desirable to include as many RDCs as possible. Thus, the ^{13}C-^{13}C RDCs were included in the fits presented here. Inclusion of the ^{13}C-^{13}C RDCs is extremely relevant because examples of partially and fully ^{13}C labeled polysaccharides and ^{13}C, ^{15}N RNA have been reported.[38,39] Hence, the method presented here has wide applicability because it can be extended to study other classes of biomolecules.

As stated earlier, this work reports a means of structure determination on the basis of RDCs alone and is an alternative to other methods.[18,20,21,40,41] An argument can be made for using well-parametrized Karplus curves to determine the fructose ring pucker from scalar coupling constants. However, Duker and Serianni previously noted that there are only two measurable ^{1}H-^{1}H scalar couplings measured for the fructofuranosyl ring in sucrose.[21] These authors combined ^{1}H-^{1}H couplings with heteronuclear coupling constants measured from partially labeled sucrose samples and fitted them to Karplus curves. Still, this approach implied that a pucker localized to the NE quadrant of the pseudorotational wheel was most consistent with the data, but only a small number of scalar couplings were actually measured.[21] Thus, RDCs and the well-parametrized empirical Karplus relation independently yield the same fructose pucker, demonstrating that the latter is not necessary to obtain this pucker. A combination of RDCs and well-parametrized Karplus curves does not significantly change the above conclusion. Thus, using ^{1}H-^{13}C in addition to ^{1}H-^{1}H and ^{13}C-^{13}C RDCs to obtain molecular structure offers an alternative to COSY and NOESY techniques.

Another possibility is that two or more conformers may be interconverting to yield one set of averaged RDCs or, similarly, *J* couplings. If these conformers differed in pucker phase, the large $J_{H3f-H4f}$ and $J_{H4f-H5f}$ values would be reduced. That they are still large implies that any interconversion is limited to the NE quadrant. Additionally, fitting two puckers to one set of experimental RDCs would require at least 10 experimentally determined RDCs, five for each pucker. Because thirteen RDCs are measured for the fructofuranosyl ring, the significance of the fit would be in question since only three degrees of freedom would be left. Furthermore, fitting two puckers would require knowledge of the populations of each. Since these are not available and their estimation is difficult, the simplest approach is preferred.

Motion in the Fructofuranosyl Ring of Sucrose. That the RDCs yield acceptable fits for more than one structure may be interpreted as a poorly defined fit. Alternatively, a good fit to more than one pucker of the fructofuranosyl ring in sucrose could reflect more than one possible pucker. The good fit of the RDCs to multiple fructofuranosyl puckers is interpreted here as suggesting the presence of multiple puckers in solution. Similar conclusions were reached in a recent study of furanose dynamics in Dickerson's dodecamer.[42] Meints et al. show that fitting solid-state ^{2}H NMR line shapes and ^{2}H relaxation data is more consistent with a continuous diffusive process, rather than an activated exchange between two conformers. They further show that the diffusive process takes place on the nanosecond time scale (9.9×10^8 Hz). Taken together, the low barrier predicted from furanose rings in DNA[42] and the good fits of multiple fructose conformations in solution from RDCs are interpreted as suggesting the presence of multiple puckers in solution.

The above results contrast with recent analyses of sucrose, also known as saccharose, and raffinose, which contains sucrose as a substructure.[18] In this study, RDCs, well-parametrized Karplus curves, and NOEs were combined to determine the structure of the above sugars. Interestingly, only one pucker phase of the fructofuranosyl ring was found in each carbohydrate. This was taken to mean that the fructofuranosyl ring in each of these molecules is present in only one conformation. A solution NMR study based on ^{13}C relaxation finds support for a rigid fructofuranosyl ring.[43] However, ^{13}C relaxation could not detect interconversion between multiple pucker phases if the motion in this ring was in the 10 ns to 100 μs regime in solution (a little slower than in the solid state, perhaps due to interactions with water) because overall molecular tumbling, not fructose ring pucker motion, would be the major source of relaxation. Thus, the molecule and the fructofuranosyl ring would appear rigid, even though motion is taking place.

Duker and Serianni reported increasing values for $J_{H3f-H4f}$ and $J_{H4f-H5f}$ upon going from free fructose to fructose as a part of sucrose, which implies that the five-membered ring is nonrigid and can easily change conformation.[21] Furthermore, it is well established that five-membered rings are flexible and that multiple puckers may be present due to the low pseudorotation barrier.[44−46] Nevertheless, conformational flexibility of the fructofuranosyl ring in sucrose is limited.[21,22] Using solely RDCs, the fits indicate that the ring pucker is localized to the

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NE quadrant of the pseudorotational wheel, most likely in the 20°−70° range. The results obtained here are in excellent agreement with results obtained in previous reports.[21,22] Optical rotation studies show that the best fits are obtained when the pucker is 18°−54°,[22] while the neutron structure shows a pucker near 0°.[32] That all three methods find puckers in the NE quadrant demonstrates the strength of using RDCs to determine the overall geometry of the fructofuranosyl ring of sucrose. Additionally, the range of good fits implies that four pucker states may be present in the five-membered ring.

Conclusions

The fitting results obtained from RDCs rule out many of the pucker possibilities for the fructofuranosyl ring of sucrose and suggest that puckers in the 20°−70° range are virtually indistinguishable. This is in accord with molecular dynamics simulations,[47] which show a flat potential in the minimum energy region.[48] It is also noteworthy that the barrier to pseudorotation was calculated to be ∼2 kcal/mol,[45] allowing facile interconversion from one pucker phase to the next. Evidence for this type of motion was found in a recent solid-state ^{2}H NMR study of Dickerson's dodecamer.[42] Fits of the fructofuranosyl ring in sucrose in the present study indicate potential rapid interconversion in the 20°−70° range.

The results presented in this report rely solely on RDCs and define a pucker range for the fructofuranosyl ring of sucrose without relying on the Karplus relation. They also are in agreement with more than one pucker phase. The puckers are accessible due to the low potential energy barrier in five-membered rings. It can also be concluded that RDCs may be used to detect motion not observable by ^{13}C relaxation measurements. This study also stresses the great sensitivity of RDCs to molecular structure and requires only a single liquid crystalline medium. Consequently, this robust methodology can be applied to oligo- and polysaccharides containing five-membered rings and to sugar rings in nucleic acids.

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Supporting Information Available: Twenty scatter plots of experimental versus calculated residual dipolar couplings (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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