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NMR investigation of oligosaccharide conformation using dipolar couplings in an aqueous dilute liquid crystalline medium

Torgny Rundlöf,[1] Clas Landersjö,[1] Kristina Lycknert,[1] Arnold Maliniak[2] and Göran Widmalm[1]*

[1] Department of Organic Chemistry, Arrhenius Laboratory, Stockholm University, S-106 91 Stockholm, Sweden
[2] Division of Physical Chemistry, Arrhenius Laboratory, Stockholm University, S-106 91 Stockholm, Sweden

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ABSTRACT: The tetrasaccharide lacto-*N*-neotetraose β-D-Gal*p*-(1 → 4)-β-D-Glc*p*NAc-(1 → 3)-β-D-Gal*p*-(1 → 4)-D-Glc*p* was dissolved in a dilute mixture of dimyristoyl- and dihexanoylphosphatidylcholine in deuterium oxide and ^1J(C,H) spin–spin coupling constants were measured in an isotropic solution at 25 °C. On raising the temperature to 38 °C, an ordering of the system occurs which permits determination of dipolar couplings. An energy minimized molecular mechanics model was generated showing that the molecule is anisotropic and can be treated as a cylinder. A fit of the expression for the carbon–proton dipolar couplings in a uniaxial molecule in a uniaxial liquid crystal revealed good agreement with experimental data, emphasizing the future potential of the method in studies of carbohydrate conformation. © 1998 John Wiley & Sons, Ltd.

KEYWORDS: NMR; ^{13}C NMR; dipolar coupling; liquid crystal; oligosaccharide; conformation

INTRODUCTION

Determination of the three-dimensional structure is a prerequisite for further investigations of molecular properties and/or function of molecules. For organic molecules in general and biomolecules such as proteins, nucleic acids and carbohydrates in particular, the use of nuclear Overhauser effects and nuclear spin–spin coupling constants are most important for structure determination in solution. In addition, ^{13}C and ^{15}N spin relaxation measurements have been essential in the description of flexibility and dynamics in biomolecules.[1] In the field of carbohydrates the overall structure and flexibility of an oligosaccharide are determined by the torsional angles at the glycosidic linkage. NOEs[2] and *trans*-glycosidic ^3J(C,H) coupling constants[3] give information on conformation, but this is usually not sufficient for an extensive description and additional experimental techniques are needed. These include measurements of *trans*-glycosidic ^3J(C,C) coupling constants,[4] optical rotation[5] and Raman optical activity.[6] For peptides in the solid state[7] and proteins in solution,[8] multiple quantum coherence NMR techniques have been developed which allow direct measurements of torsional angles without relying on a Karplus-type relationship. Studies of through-space dipole–dipole interactions constitute an extremely powerful tool for molecular structure determination. Unfortunately, this information is essentially lost in isotropic solutions and can only be partially recovered by measuring cross-relaxation rates.[9] In anisotropic systems, on the other hand, the dipole–dipole couplings can be observed but the resulting proton spectrum, even for a medium-sized molecule, becomes very complicated. Several techniques, including multiple quantum NMR[10] and deuterium labelling,[11] have been applied in order to simplify the spectra and yet retain the information about the structure and dynamics. At high magnetic fields it has been possible to measure dipolar couplings in solution. These couplings originate from a partial alignment in the static field of the NMR spectrometer due to the magnetic susceptibility of molecules, which facilitates refinement of molecular structure.[12,13] Another method to align molecules is to use liquid crystalline solvents. Since the magnitude of dipolar couplings is proportional to the molecular order, the solvent can be used as a selection tool, i.e. in a low-order liquid crystal only strong couplings will be observed. The formation of bilayer-like assemblies (bicelles) upon mixing of dimyristoylphosphatidylcholine (DMPC) and dihexanoylphosphatidylcholine (DHPC) in water was described some time ago,[14] and has been further studied.[15] This mixture was successfully used in the investigation of molecular orientation in model membranes[16] and of the structure of a non-spherically symmetric protein.[17,18]

RESULTS AND DISCUSSION

In the present study we investigate the tetrasaccharide lacto-*N*-neotetraose (LNnT) β-D-Gal*p*-(1 → 4)-β-D-Glc*p*NAc-(1 → 3)-β-D-Gal*p*-(1 → 4)-D-Glc*p* (Fig. 1),

* Correspondence to: G. Widmalm, Department of Organic Chemistry, Arrhenius Laboratory, Stockholm University, S-106 91 Stockholm, Sweden.
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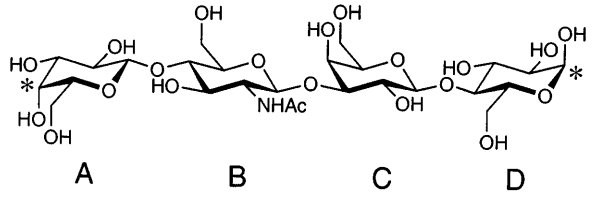

Figure 1. Schematic representation of the tetrasaccharide lacto-*N*-neotetraose β-D-Gal*p*-(1 → 4)-β-D-Glc*p*NAc-(1 → 3)-β-D-Gal*p*-(1 → 4)-D-Glc*p*. The molecule is drawn with the α-anomeric configuration at its reducing end, which was used in the analysis. Sugar residues are labelled A–D. The C-4 atom in A and the C-1 atom in D are highlighted with asterisks.

which is found in human milk.[19] LNnT was chosen as a model system since it has non-parallel C—H vectors in three out of the four sugar residues and a limited number of resonances, which simplifies the spectral analysis. Furthermore, we have previously investigated its motional properties using ^{13}C nuclear spin relaxation measurements.[20] LNnT was dissolved in a dilute mixture of DMPC and DHPC in deuterium oxide and ^{1}J(C,H) spin–spin coupling constants were measured at 25 °C from a $^{1}H,^{13}C$ gradient selected HSQC spectrum.[21,22] At this temperature and lipid concentration the mixture forms an isotropic solution. On raising the temperature to 38 °C an ordering of the system occurs in which the bicelles orient with their normal perpendicular to the external magnetic field.[14] The solute molecule, i.e. in our case LNnT, will exhibit a small degree of alignment, which is proportional to the concentration of bicelles. However, it is assumed that the molecular structure is essentially unaffected.[17] This minute ordering facilitates contributions from dipolar couplings to be measured, which may be either positive or negative. This is shown in Fig. 2, in which the apparent $^{1}H,^{13}C$ splitting is changed in the ordered phase compared with the $^{1}H,^{13}C$ splitting due to ^{1}J(C,H) spin–spin coupling constants in the isotropic phase.

An energy-minimized molecular mechanics model was generated using the HSEA force field[23] taking into account that the predominant conformation at the glycosidic linkage is a *'syn'* conformer.[2] The torsional angles are denoted by ϕ (H-1—C-1—O-X—C-X) and ψ(C-1—O-X—C-X—H-X), where X is the linkage atom. The glycosidic linkages are denoted by a subscript referring to the pertinent sugar residue and the conformation of LNnT as its α-anomeric configuration of residue D is described by $\phi_A = 52°$, $\psi_A = 3°$, $\phi_B = 53°$, $\psi_B = -9°$, $\phi_C = 53°$, $\psi_C = 4°$. The molecular shape of LNnT is clearly anisotropic, as indicated by Fig. 1, which is also confirmed by the fact that the ratio between principal elements of the moment of inertia tensor, $I_{zz}:I_{yy}:I_{xx}$, is 1.0:6.3:6.5. We consider, therefore, the molecule as a cylinder with the symmetry axis essentially coinciding with the vector from the C-4 atom in residue A to the C-1 atom in residue D. For a uniaxial molecule in a uniaxial liquid crystal the expression for the carbon–proton dipolar couplings is given by[24]

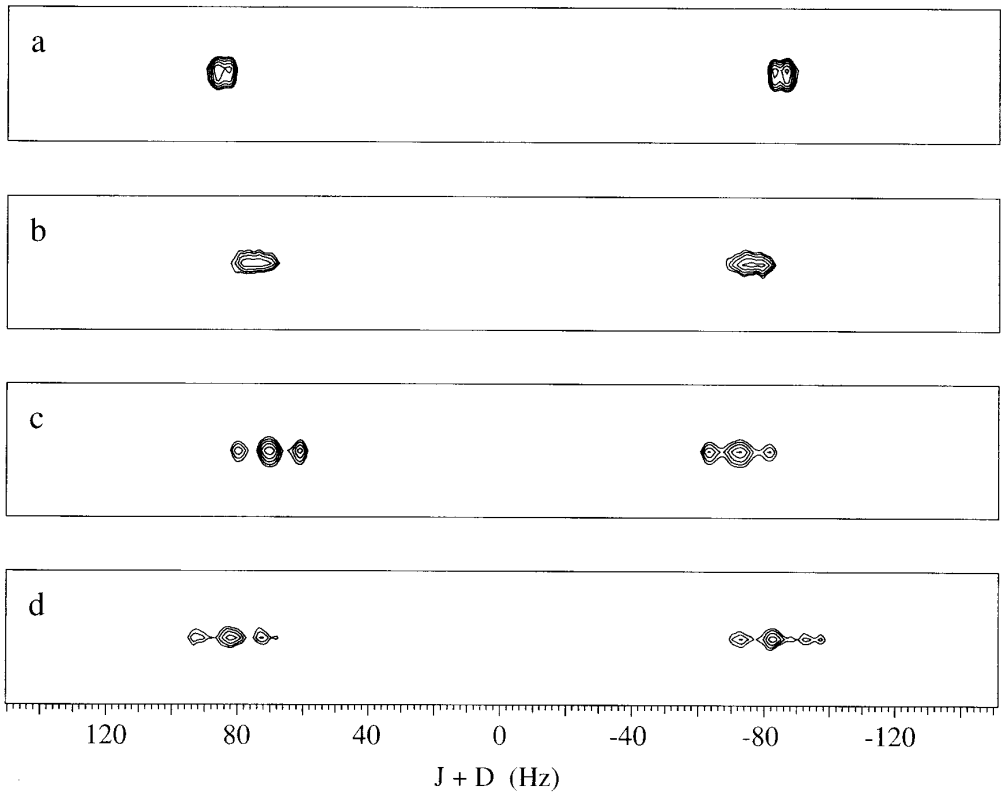

Figure 2. Part of the ^{1}H, ^{13}C gradient selected HSQC spectrum of LNnT (lower order sample), with splittings from spin–spin (J) and dipole–dipole (D) couplings. Cross peak of C-1 in residue D (α-form) at (a) 25 °C (isotropic) and (b) 38 °C. Cross peak of C-2 in residue B at (c) 25 °C and (d) 38 °C.

Table 1. Dipolar couplings in LNnT (α-anomeric form) at 600 MHz

Sugar residue	Atom pair	Dipolar coupling (Hz) 7.5% lipid	10% lipid
A	H-4–C-4	−16.0	−26.9
A	H-5–C-5	15.5	25.5
B	H-2–C-2	20.8	30.1
B	H-3–C-3	22.8	30.0
C	H-2–C-2	18.6	27.4
C	H-4–C-4	−8.2	−11.1
D	H-1–C-1	−17.7	−28.3
D	H-5–C-5	15.7	26.8

$$D_{CH} = -\frac{\mu_0}{4\pi}\frac{\gamma_C\gamma_H\hbar}{2\pi r_{CH}^3}[S \cdot S_{zz}\tfrac{1}{2}(3\cos^2\theta - 1)] \quad (1)$$

where r_{CH} is the spin–spin distance, θ is the angle between the spin–spin vector and the molecular symmetry axis, S_{zz} is the second-rank orientational order parameter related to the symmetry axis and S is an order parameter which reflects the internal motion of the spin–spin vector. All other symbols have their usual meanings. The order parameter, S_{zz}, increases with increase in bicelle concentration, resulting in an increased magnitude of the dipolar couplings. We determined dipolar couplings at two bicelle concentrations (Table 1). In the more ordered sample the values range between −28 and 30 Hz, while at the lower concentration the corresponding values are −18 and 23 Hz. Contributions from homonuclear proton–proton dipolar couplings were also observed in the oriented phase, which excluded some signals from the analysis since reliable values of heteronuclear dipolar couplings could not be extracted. The internal motion can be related to the generalized order parameter S, which can, in turn, be obtained from nuclear spin relaxation measurements.[25] To a first approximation, a common value of the order parameter, S, for all dipolar vectors can be assumed.

The orientation of the dipolar vectors in the molecular frame, i.e. the angles θ, were determined using the molecular structure generated in the molecular mechanics procedure described above. Experimental dipolar couplings were analyzed in a subsequent fitting procedure where the product, $S \cdot S_{zz}$, was used as a single adjustable parameter. In the lower order sample $SS_{zz} = 1.6 \times 10^{-3}$ was obtained while the corresponding value in the higher order system was $S \cdot S_{zz} = 2.5 \times 10^{-3}$. Experimental dipolar couplings determined in the lower order sample are shown in Fig. 3, together with the result of the numerical fitting. We note that the measured couplings correspond to essentially two orientations: (i) $\theta = 35°$ and (ii) vectors orthogonal to the molecular symmetry axis. In fact, these are the only two orientations of sugar ring C—H vectors present in the molecule. In order to account quantitatively for the effect of internal motion, different S values should be used for vectors corresponding to different sugar residues. Further refinements of models for the interpretation of experimental data should be possible using a distribution function or via conformational averaging as performed by Monte Carlo or molecular dynamics simulations, routes that are currently under investigation. In the present study we have shown that dipolar couplings in aqueous dilute liquid crystalline media are in good agreement with a model generated by molecular mechanics and we consider that their use will be of paramount importance for the determination of carbohydrate structure and conformation.

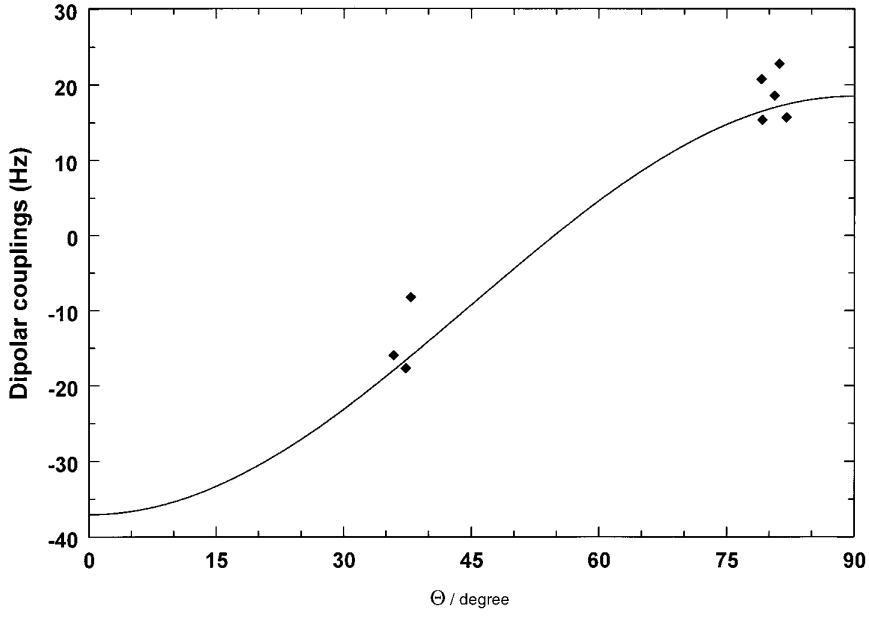

Figure 3. Experimental dipolar couplings of LNnT in the lower order sample as a function of the orientation of the C—H vectors relative to the molecular symmetry axis (determined using the moment of inertia tensor). The best fit curve using Eqn (1) is included.

EXPERIMENTAL

LNnT (4.0 mg) was dissolved in 600 μl of D_2O containing 10% (w/v) of DMPC–DHPC in a molar ratio of 2.9:1 determined by integration of the peaks in the ^{31}P NMR spectrum (the ^2H quadrupolar splitting at 38 °C was 19.8 Hz), resulting in a 9.4 mM solution. Subsequent dilution of the sample with 200 μl of D_2O gave a sample with a concentration of 7.5% with respect to the lipids (resulting in a ^2H quadrupolar splitting of 14.5 Hz at 38 °C). Spectra were acquired using the inverse proton detected ^1H, ^{13}C-gHSQC technique on a Varian Inova 600 MHz NMR spectrometer with an experimental time of 16 h for each concentration and temperature. The digital resolution in the F_2 dimension was 2.2 Hz, and spectra were subsequently zero-filled twice prior to the spectral analysis. The GEGOP program, version 2.7, was used for the generation of the molecular mechanics model and subsequent energy minimization.[26]

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