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Conformation and dynamics at a flexible glycosidic linkage revealed by NMR spectroscopy and molecular dynamics simulations: Analysis of β -L-Fucp-(1 $\rightarrow$ 6)- α -D-Glcp-OMe in water solution[‡]

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ABSTRACT

The intrinsic flexibility of carbohydrates facilitates different three-dimensional structures in response to altered environments. At glycosidic (1 $\rightarrow$ 6)-linkages three torsion angles are variable and herein the conformation and dynamics of β -L-Fucp-(1 $\rightarrow$ 6)- α -D-Glcp-OMe are investigated using a combination of NMR spectroscopy and molecular dynamics (MD) simulations. The disaccharide shows evidence of conformational averaging for the ψ and ω torsion angles, best explained by a four-state conformational distribution. Notably, there is a significant population of conformations having $\psi = 85^\circ$ (*clinal*) in addition to those having $\psi = 180^\circ$ (*antiperiplanar*). Moderate differences in ^{13}C R_1 relaxation rates are found to be best explained by axially symmetric tumbling in combination with minor differences in librational motion for the two residues, whereas the isomerization motions are occurring too slowly to be contributing significantly to the observed relaxation rates. The MD simulation was found to give a reasonably good agreement with experiment, especially with respect to diffusive properties among which the rotational anisotropy, $D_{\parallel}/D_{\perp}$, is found to be 2.35. The force field employed showed too narrow ω torsion angles in the *gauche-trans* and *gauche-gauche* states as well as overestimating the population of the *gauche-trans* conformer. This information can subsequently be used in directing parameter developments and emphasizes the need for refinement of force fields for (1 $\rightarrow$ 6)-linked carbohydrates.

INTRODUCTION

Carbohydrates in the form of glycans are involved in a multitude of biochemically important phenomena such as protein folding, protein regulation, cells distinguishing between self and non-self, as well as cellular organization.¹ Compared to the other biopolymers the field of structural biology of glycans is less developed, largely due the poor understanding of their three-dimensional (3D) structure.² However, recent developments in computational methods have made it possible to address the conformational dynamics of mono- and oligosaccharides on longer time scales, well into the μ s time regime.^{3,4}

For the monosaccharides the 3D structure is given by their stereochemistry at the different carbon atoms and the hydroxyl groups are generally either axially or equatorially oriented for six-membered sugar rings (pyranoses) or pseudo-axially or pseudo-equatorially positioned, typically observed for five-membered sugar rings (furanoses). In addition, conformational equilibria and intra-residue dynamics may be present for some of the sugars. In forming oligosaccharides, the substitution may take place at the hydroxyl group of a secondary carbon atom, e.g. at C2, C3 or C4 of glucopyranose, or at the hydroxyl group of a primary carbon atom such as C6 of glucopyranose. In the former case the conformational flexibility of a disaccharide is governed by the two glycosidic torsion angles ϕ and ψ ,⁵ but in the latter case also the ω torsion angle (O5-C5-C6-O6) is involved as a major degree of freedom leading to a more complicated situation when the conformational preferences at the glycosidic linkage should be described. The conformations available to the exocyclic hydroxymethyl group of different sugars have been investigated⁶⁻⁹ and commonly two out of three staggered or nearly so conformations are highly preferred whereas the last one is only populated to a small extent, based on preferences due to the *gauche* effect^{10,11} and the 1,3-diaxial steric/repulsive interactions.¹²

Previous studies of (1 $\rightarrow$ 6)-linked carbohydrates with *gluco*-configuration (hydroxyl group at C4 equatorial for D-Glcp in the 4C_1 conformation) in aqueous solution using NMR spectroscopy combined with computational methods have found that ϕ prefers the conformation predicted by the exo-anomeric effect, while ψ mainly adopts an extended anti-conformation around 180° with either a broad distribution or limited excursions to conformations having values of ψ around either +90° or -90°. The ω torsion angle adopts conformations similar to that of glucose as a monomer, i.e., essentially an equal distribution between the in the *gauche-trans* (*gt*) and *gauche-gauche* (*gg*) conformations.¹³⁻¹⁸ The conformational and dynamical properties of other (1 $\rightarrow$ 6)-linked disaccharides^{17,19-21} and larger oligosaccharides having this linkage^{15,22} have been investigated.

The combination of experimental NMR techniques and molecular simulations offers detailed analysis of molecular structures, conformational equilibria and dynamics.²³ The disaccharide β -L-Fucp-(1 $\rightarrow$ 6)- α -D-Glcp-OMe, shown schematically in Figure 1,²⁴ (F6G) which was previously investigated only with respect to ^1H and ^{13}C glycosylation shifts, i.e., the magnitudes and signs of chemical shift changes due to glycosylation, is herein studied by ^1H , ^1H NOEs, homo- and heteronuclear spin-spin coupling constants, NMR spin simulation, ^{13}C spin-lattice relaxation and translational diffusion measurements in conjunction with molecular dynamics (MD) simulations of the disaccharide with explicit water molecules as solvent. By this approach we are able to present a detailed picture of the conformation, flexibility and dynamics as well as motional aspects of F6G, to highlight strengths and weaknesses in the force field used for the MD simulations as well as giving an indication of how the force field changes should be carried out in future improvements and which conformational preferences that should be targeted when a force field is evaluated for carbohydrates.

THEORY

^1H , ^1H -NOESY. Cross-relaxation rates determined from NOESY experiments are dependent on both the internuclear distance and the motions affecting the connecting vector. Under the isolated spin-pair approximation, differences stemming from the motional part are negligible and the cross-relaxation rates, σ , are determined completely by the distances between the interacting spins, allowing the following relationship to be established:²⁵

$$r_{ij} = r_{\text{ref}} \left(\sigma_{\text{ref}} / \sigma_{ij} \right)^{1/6} \quad (1)$$

where σ_{ref} and σ_{ij} are the cross-relaxation rates for the reference interaction and for the interaction between spins i and j . Using the known distance for the reference interaction, r_{ref} , the distance r_{ij} can be determined.

The areas of peaks of interest, I_j , determined in a 1D NOESY experiment can be normalized against the area of the inverted peak, I_i , allowing the fitting to either a first- or second-order equation of the form:²⁶

$$\frac{-I_j(t_{\text{mix}})}{I_i(t_{\text{mix}})} \approx \sigma_{ij} t_{\text{mix}} - \frac{1}{2} \sigma_{ij} (R_j - R_i) t_{\text{mix}}^2 \quad (2)$$

where t_{mix} is the cross-relaxation delay and R_i and R_j are the auto-relaxation rate constants for the respective spins.

The second-order term becomes significant in the case when relaxation is much faster for the detected spin than for the inverted spin, and at large values of t_{mix} . In practice, the choice between first- or second-order fittings can be made by applying a statistical F -test to the first- and second-order fit.²⁷

From the ratio of cross-relaxation rates determined in NOESY experiments and those determined in T-ROESY experiments, effective correlation times, τ_{eff} , can be determined:^{17,28}

$$\frac{\sigma_{\text{NOE}}}{\sigma_{\text{T-ROE}}} = \frac{10 + 2\omega_{\text{H}}^2\tau_{\text{eff}}^2 - 8\omega_{\text{H}}^4\tau_{\text{eff}}^4}{10 + 23\omega_{\text{H}}^2\tau_{\text{eff}}^2 + 4\omega_{\text{H}}^4\tau_{\text{eff}}^4} \quad (3)$$

^{13}C T_1 relaxation. For ^{13}C nuclei undergoing dipolar relaxation with directly bound ^1H nuclei, the longitudinal relaxation rate R_1 can be expressed in terms of the dipolar coupling constant and the reduced spectral density, $J(\omega)$, at three frequencies:

$$R_1 = T_1^{-1} = \frac{d_{\text{CH}}^2}{4} [J(\omega_{\text{H}} - \omega_{\text{C}}) + 3J(\omega_{\text{C}}) + 6J(\omega_{\text{H}} + \omega_{\text{C}})] \quad (4)$$

in which $d_{\text{CH}} = -(\mu_0 / 4\pi)\hbar\gamma_{\text{C}}\gamma_{\text{H}}r_{\text{CH}}^{-3}$ is the dipolar coupling constant, μ_0 is the permeability of vacuum, $\hbar$ is Planck's constant divided by 2π , γ_{C} and γ_{H} are the magnetogyric ratios of carbon and proton respectively, and the proton-carbon distance, r_{CH} , is equal to 1.117 Å.²⁹ The reduced spectral density function of the particular Larmor frequencies, ω , for an isotropically tumbling molecule with limited and rapid internal flexibility can be approximated by:³⁰

$$J(\omega) = \frac{2}{5} \left(\frac{S^2\tau_{\text{c}}}{1 + \omega^2\tau_{\text{c}}^2} \right) \quad (5)$$

in which S^2 and τ_{c} are the generalized order parameter and the molecular correlation time, respectively.

However, if the molecule is undergoing axially symmetric tumbling, a more complicated spectral density function must be used:^{31,32}

$$J(\omega) = \frac{2}{5} \left(S^2 \sum_{k=1,2,3} A_k \frac{\tau_k}{1 + \omega^2 \tau_k^2} \right) \quad (6)$$

being the Fourier transform of the following correlation function:

$$C(t) = S^2 \sum_{k=1,2,3} A_k \exp(-t/\tau_k) \quad (7)$$

with the coefficients $A_1 = (3\cos^2\theta - 1)^2/4$, $A_2 = 3\sin^2\theta\cos^2\theta$, and $A_3 = 3\sin^4\theta/4$ and the correlation times $\tau_1 = (6D_\perp)^{-1}$, $\tau_2 = (5D_\perp + D_\parallel)^{-1}$, and $\tau_3 = (2D_\perp + 4D_\parallel)^{-1}$. Here, θ is the angle between the vector connecting the interacting nuclei (i.e. for ^{13}C relaxation, the CH vector) and the unique axis of the diffusion tensor. $D_\parallel$ and $D_\perp$ are the parallel and perpendicular rotational diffusion constants, respectively.

From an MD simulation, the correlation function in equation 7 is accessible by calculation of the P_2 correlation function in the laboratory frame

$$C_2(t) = \langle P_2(\vec{\mu}_0 \cdot \vec{\mu}_t) \rangle \quad (8)$$

where $\vec{\mu}_t$ is the orientation for the chosen vector at time t , and P_2 is the second order Legendre polynomial, $P_2(x) = (3x^2 - 1)/2$, and the angular brackets indicate averaging over the trajectory. Together with a representative geometry, the rotational diffusion constants along with the orientation of the diffusion tensor can be determined. After removing overall rotation, the P_2 correlation function reaches a plateau which indicates the extent of internal motion, i.e., the generalized order parameter S^2 .

Separation of time scales. For the molecule under study, dynamical processes on at least three different time scales are occurring. In addition to tumbling, there is librational motion as well as conformational transitions. If the former is sufficiently much faster than tumbling, the effect is simply a fast initial drop in the value of the correlation function, resulting in an amplitude factor, S^2 , in the spectral density function. On the other hand, if the conformational transitions are sufficiently much slower than tumbling, the correlation function will be insensitive to this motion. The observed NMR relaxation rates will simply be the population-weighted average of the rates in the individual conformations and do not contain information on the time-scale of conformational transitions.

Population fitting. In order to perform a meaningful fitting of populations using different types of experimental data, viz., different scalar coupling constants and cross-relaxation rates, the estimated uncertainty can be included in the error function, as defined by the χ^2 statistic:²⁷

$$\chi^2 = \sum_i \left(\frac{\langle O_{i,\text{pred}} \rangle - O_{i,\text{expt}}}{\sigma_i} \right)^2 \quad (9)$$

in which i refers to all experimental parameters, O_i is the value of the parameter, either as determined from experiment or from the population-weighted average for a set of conformations. The estimated uncertainty in the i^{th} parameter, σ_i , should reflect both errors in the experimentally determined values as well as the uncertainty in the predicted values. Assuming librational fluctuations in the torsion angles within each conformation, values of the parameters can be calculated according to:

$$O_{i,\text{pred}} = \int p(\phi, \psi, \omega) O_i(\phi, \psi, \omega) d\phi d\psi d\omega \quad (10)$$

where $O_i(\phi, \psi, \omega)$ is the value of the parameter as a function of the three torsion angles. Assuming that librations are independent, a wrapped three-dimensional probability density, $p(\phi, \psi, \omega)$, for a conformational state can be obtained using the von Mises's distribution:³³

$$p(\phi, \psi, \omega) = \frac{1}{Z} \exp \left[\sum_{\theta=\phi, \psi, \omega} \kappa_\theta \cos(\theta - \theta_0) \right] \quad (11)$$

where Z normalizes the total probability to unity, θ_0 is the mean angle for the distribution, and $1/\kappa_\theta$ is equivalent to the variance in the normal distribution.

For a given set of conformations, parameters can be predicted using equations 10 and 11, and subsequently the populations optimized by minimizing the value of χ^2 in equation 9. The inclusion of an additional conformational state is justified if the prediction of parameters is significantly improved, as determined from the F value:²⁷

$$F = \frac{\chi^2(m) - \chi^2(m+1)}{\chi^2(m+1)/(N - (m+1))} \quad (12)$$

where N is the number of independent NMR parameters, m is the number parameters used in the fitting (equal to the number of conformational states minus one).

MATERIALS AND METHODS

General Considerations. The disaccharide β -L-Fucp-(1 $\rightarrow$ 6)- α -D-Glcp-OMe (F6G) was previously synthesized and its ^1H and ^{13}C NMR chemical shifts assigned by Baumann et al.²⁴ Atoms in the terminal fucosyl residue are denoted by an *f* while atoms in the *O*-methyl glucoside are labeled with the letter *g*. The glycosidic torsion angles are defined as follows: $\phi = \text{H1}f\text{-C1}f\text{-O6}g\text{-C6}g$, $\psi = \text{C1}f\text{-O6}g\text{-C6}g\text{-C5}g$, and $\omega = \text{O6}g\text{-C6}g\text{-C5}g\text{-O5}g$.

NMR Spectroscopy. The disaccharide was prepared for NMR spectroscopy by dissolution in D_2O (0.5 mL, 50 mM) followed by treatment with CHELEX 100 resin to remove any paramagnetic ions present, degassing with three freeze-pump-thaw cycles and finally flame-sealed. NMR experiments were performed at 14.1 T on a Bruker Avance III 600 MHz spectrometer equipped with a TCI Z-gradient probe and on a Varian INOVA 600 MHz spectrometer equipped with a 5 mm PFG triple resonance probe, except for ^{13}C relaxation measurements, which were carried out on a Bruker Avance 400 MHz spectrometer equipped with a BBO probe. Pulsed field gradient strengths are reported as fractions of the maximum strength, $55.7 \text{ G}\cdot\text{cm}^{-1}$, and all gradient pulses were of 1 ms length unless otherwise stated. All experiments were performed at 303 K, unless otherwise stated. Temperatures were calibrated using methanol-*d*₄.³⁴

Proton-proton cross-relaxation rates from $\text{H1}f$, $\text{H6}_{\text{pro-}R}$ and $\text{H6}_{\text{pro-}S}$ were measured using 1D ^1H , ^1H -NOESY³⁵ and 1D ^1H , ^1H -T-ROESY³⁶ experiments. In addition, the cross-relaxation rate from $\text{H5}g$ to $\text{H1}f$ was measured using 1D ^1H , ^1H -STEP-NOESY³⁷ experiments. In all cases, selective excitation was achieved by single or double PFGSEs utilizing 40-100 ms r-SNOB or i-SNOB-2³⁸ shaped pulses for the NOESY and 80 ms r-SNOB shaped pulses for the T-ROESY and STEP-NOESY experiments. The strengths of the first and second gradient pairs were 2.7–17% and 4.5–40%, respectively, for the NOESY experiments, and 5.7% and 12.5% for the T-ROESY experiments. For the STEP-NOESY, the strengths of the gradients were set to 10% and 6.5% for the first and 45% and 15% for the second excitation, respectively. Due to the spectral overlap of the $\text{H5}f$ resonance with that of $\text{H5}g$, the resonance from $\text{H2}g$ was selectively excited and magnetization transferred to $\text{H5}g$ using a 70 ms, 9.3 kHz DIPSI-2 spin lock prior to selective excitation of $\text{H5}g$.

Zero-quantum coherences were suppressed using the scheme devised by Thrippleton et al.³⁹ A 10-20 ms adiabatic Chirp pulse with a bandwidth of 20–60 kHz was applied together with a gradient pulse with 3.5–8.5% of the maximum power, except in the STEP-NOESY experiment where a 50 ms adiabatic Chirp pulse with a bandwidth of 20 kHz was used in combination with a gradient pulse at 3% of the maximum power. The NOESY experiments on the Varian spectrometer were performed without zero-quantum suppression. One to two inversion pulses were placed during the

NOE mixing time³⁵ consisting of 1 ms hyperbolic secant adiabatic pulses flanked by gradient echo pairs of 1% and 1.3% of the maximum strength for the first and second pulse, respectively.

In the NOESY experiments, 10–11 series of five to nine cross-relaxation delays between 50–500 ms were collected for each of the excited spins. For T-ROESY, six cross-relaxation delays between 60 and 250 ms were used, and the STEP-NOESY employed five cross-relaxation delays in the range 100–500 ms. During the cross-relaxation delay in the T-ROESY experiment, a 3.5 kHz T-ROESY spin-lock was applied. A spectral width of 5 or 10 ppm was sampled using 16k or 32k data points and for each spectrum, 256–1024 transients were averaged. The repetition time was 10–15 s, i.e., in all cases longer than $5 \times T_1$.

Prior to Fourier transformation, the FIDs of the 1D experiments were zero-filled to 256k points and multiplied by an exponential line-broadening function of 2 Hz. Base-line correction was performed prior to integration which used the same integration limits for all experiments within a series. The areas of relevant peaks were divided by the area of the inverted peak⁴⁰ and least-square fitted to a first or second order function yielding the cross-relaxation rate constant as the first order parameter. The choice between first and second order fitting was made by an *F*-test, discarding the second order fit if $\text{Pr}(> F) = 0.01$ or higher.²⁷

Proton-proton homonuclear coupling constants were extracted by fitting a spin-simulated NMR spectrum to a 1D proton spectrum in the program PERCH.⁴¹ Heteronuclear proton-carbon coupling constants were measured using the constant-time ¹H,¹³C-J-HMBC experiment with a third order low-pass *J*-filter ($\tau_1 = 3.57$, $\tau_2 = 3.13$ – 3.26 and $\tau_3 = 2.78$ ms)⁴² and with adiabatic pulses replacing hard 180° pulses on carbon. The adiabatic pulse for inversion was a 60 kHz smoothed Chirp of 500 μ s duration and for refocusing a 2 ms long 60 kHz composite Chirp pulse was used. Pulsed field gradients were set to 18%, –10%, –5% and –3% for the low-pass filter and 80%:–48% and –48%:80% for echo/anti-echo selection. Spectral widths of 100 ppm (15 091 Hz) to 110 ppm (16 600 Hz) for ¹³C and 5.99 ppm (3 597 Hz) for ¹H were sampled using 512×8k data points in the *F*₁ and *F*₂ dimensions, respectively, averaging 16–64 transients for each increment. Data matrices were reshaped to 8k×4k data and multiplied by squared cosine-bell window functions prior to Fourier transformation. Coupling constants were determined from absolute value mode projections.

The ¹H,¹³C-HSQC-HECADE experiment⁴³ was acquired using an isotropic mixing period of 110 ms and a *J*-scaling factor (t_1^*/t_1) of 0.7 in the indirect dimension. A data matrix of 512×24k data points was filled, sampling 65 ppm and 5 ppm in the indirect and direct dimensions, respectively. Each increment was averaged over 32 transients. Zero-filling to 2k×32k and multiplication with a

squared 90° shifted sine-bell and a 2 Hz exponential line-broadening function were applied in the F_1 and F_2 dimensions, respectively, prior to Fourier transformation. Heteronuclear coupling constants were determined using F_2 slices for the different ^{13}C spin states.

Translational diffusion was measured at 298 K using the bipolar pulse longitudinal eddy current delay (BPPLIED) pulse sequence⁴⁴ having gradient pulses with a duration ($\delta/2$) of 2 ms and a constant diffusion delay Δ of 100 ms. The eddy current delay was set to 5 ms. A spectral window of 7 ppm was sampled with 16k data points. Eight such experiments were performed measuring intensity as a function of 32 linearly spaced gradient strengths between 0.01% and 34% of the maximum. Integration was performed between 4.13 ppm and 3.20 ppm, corresponding to the non-anomeric protons of the molecule, and the resulting integrals fitted to the Stejskal-Tanner equation.⁴⁵ The diffusion measurements were calibrated using a sample of 1% H_2O in D_2O doped with $1\text{ mg}\cdot\text{L}^{-1}$ GdCl_3 with a known diffusion constant of $1.90\times 10^{-9}\text{ m}^2\cdot\text{s}^{-1}$. The effect of the nonlinear gradient profile was compensated for, following the protocol by Damberg et al.⁴⁶

^{13}C T_1 relaxation was measured using the inversion recovery experiment with a WALTZ-65 decoupling element⁴⁷ applied to the proton channel during the entire experiment. Nine experiments were performed, each determining the intensity at 10 different relaxation delays between 10 ms and 2 s. A spectral width of 149.7 ppm was sampled during 1 s and averaged over 1k transients for each delay. Between transients, a 1.5 s delay was used ($\sim 2\times T_1$). Zero-filling to 256k and multiplication by a 1 Hz line-broadening function was performed before Fourier transformation. Intensities were fitted to a three-parameter exponential function in the T_1/T_2 -Relaxation module of the TopSpin software (Bruker).

Molecular dynamics simulations. MD simulations were performed using CHARMM c35b4⁴⁸ with the PARM22/SU01 force field.⁴⁹ The disaccharide was placed in a pre-equilibrated water box consisting of 900 TIP3P molecules in a cube with a side length of 29.972 Å. Water molecules less than 2.6 Å from the solute were removed, resulting in 877 water molecules. After energy minimization, using 500 steps of steepest descent followed by 3000 steps of adopted basis Newton-Raphson, heating from 103 K to 303 K during 50 ps and equilibration for 100 ps followed. Electrostatics were handled using the particle mesh Ewald method⁵⁰ while other non-bonded interactions were forced to zero at 12 Å using a switching function acting from 10 Å. Leap-frog integration was performed using a time step of 2 fs until 500 ns had been sampled with constant temperature and pressure, saving coordinates every 2 ps. Bonds to hydrogen atoms were kept rigid using SHAKE.⁵¹

The translational self-diffusion constant was determined from the derivative of the mean-squared displacement function at displacement times where the derivative was at a plateau, i.e., the displacement was linear. Calculations were performed for 60 blocks of 4 ns length, and the derivative at the plateau was averaged using the inverse of the variances among the 60 blocks at each displacement time as weights. The calculated diffusion constant was corrected for the finite size of the box,⁵² using the viscosity, $\eta = 0.299$ cP, reported for TIP3P at 303 K using PME for electrostatic interactions.⁵³

For the two major conformations, the 1000 longest continuous states were merged and used to calculate P_2 correlation functions for CH bond vectors as well as the proton-proton interaction vectors for which cross-relaxation had been measured. S^2 values were calculated from the plateau of the P_2 correlation functions after removing overall rotation by fitting to the first set of coordinates using non-hydrogen atoms, averaging the coordinates and subsequently fitting to the average coordinates.

The parameters of the diffusion tensor, $D_{\parallel}$, $D_{\perp}$, and the direction of the unique axis, were obtained by fitting equation 7 to P_2 correlation functions obtained as described above but without removing overall rotation, together with representative coordinates for the two major conformations. In practice, the average structure was calculated for each major conformation and subsequently the coordinates having the lowest heavy-atom RMSD from these two structures in the trajectory were used. Spatial distribution functions were generated at 0.2 Å resolution in CHARMM and smoothened using a Gaussian function ($\sigma = \sqrt{2}/5$ Å) before interpolating to 0.1 Å resolution using the ‘spline’ option in Octave, version 3 (<http://www.gnu.org/>).⁵⁴ Hydrogen bonds and water bridges were analyzed in CHARMM using < 2.5 Å and $> 135^\circ$ as the criteria for hydrogen-oxygen distances and H–O $\cdots$ H angles, respectively.

Parameter prediction. Scalar coupling constants were calculated using the pertinent Karplus equations for $^3J_{\text{H5g,H6gR/S}}$, $^2J_{\text{H6gR,H6gS}}$, and $^3J_{\text{C4g,H6gR/S}}$ published by Thibaudau et al.⁵⁵ and for $^3J_{\text{C1f,H6gR/S}}$ and $^3J_{\text{C6g,H1f}}$ published by Swn et al.⁵⁶ Effective distances were calculated from the MD simulation using $r_{\text{eff}} = \langle r^{-6} \rangle^{-1/6}$. In order to include in equation 10 areas of the conformational space not sampled by the MD simulation, r^{-6} was determined from a potential-energy relaxed three-dimensional map calculated at 10° intervals with a dielectric constant of 3 and subsequently interpolated to a resolution of 5° . Probability densities were calculated for each conformational state using equation 11 with $1/\sqrt{\kappa} = 15^\circ$ for all torsion angles; these were then used together with equation 10 to predict parameters for each state.

Population fitting. For the vicinal homo- and heteronuclear J -coupling constants, the error was estimated to be 0.6 Hz and 0.8 Hz, respectively, whereas for ${}^2J_{\text{H}6gR,\text{H}6gS}$ the error estimate was set to 2 Hz since the pertinent Karplus-type relationship was developed using compounds bearing a hydrogen atom at O6⁵⁵ and has, to the best of our knowledge, not been validated for compounds where O6 is glycosylated. For effective distances, errors were set to 6% and 12% of the experimental value in data from NOESY and T-ROESY experiments, respectively. For the determination if the addition of an additional conformational state was justified, the F value was calculated according to equation 12 and the additional parameter considered statistically significant if $\text{Pr}(>F, 1, N-(m+1))$ was less than 0.01.²⁷

RESULTS AND DISCUSSION

Conformation from NMR experiments. By a series of NMR experiments, from which a selection of spectra are shown in Figure 2, NMR parameters related to conformationally defining the torsion angles were determined. The values for scalar coupling constants and proton-proton distances are shown in Tables 1 and 2, respectively, together with the torsion angles which they report on.

The measured ${}^3J_{\text{C}6g,\text{H}1f}$ coupling constant is consistent with a single conformational state for ϕ with $\phi_{\text{H}} = -40^\circ$, in agreement with the exo-anomeric effect. The deviation from a perfectly staggered torsion angle can be explained by the greater repulsive interactions with the endocyclic oxygen than with the anomeric hydrogen. For the ψ torsion angle, a single-state analysis gives a global minimum deviation from the experimental values at $\psi = 0^\circ$, with a somewhat higher local error minimum at $\psi = 180^\circ$. The former state is unlikely from a sterical point of view, while the latter conformation alone would give coupling constant values being of too small magnitude, 1.7 Hz for both ${}^3J_{\text{C}1f,\text{H}6g}$ interactions (cf. Table 1). A single broad distribution, as has been suggested for methyl α -gentiobioside,¹⁶ could account for the observed coupling constants. However, unhindered large scale libration within such a broad conformation would very likely have a profound effect on ${}^{13}\text{C}$ T_1 relaxation times. Based on the absence of such effects (vide infra), we conclude that at least two conformational states are contributing to the experimentally determined coupling constant values. Interpreting the homonuclear three-bond coupling constant values 4.89 Hz and 2.15 Hz for the $\text{H}5g,\text{H}6g_{\text{pro-}R}$ and $\text{H}5g,\text{H}6g_{\text{pro-}S}$ interactions, respectively, related to the ω torsion angle as a three-state equilibrium gives the populations 0.42:0.50:0.08 for the gt ($+65^\circ$), gg (-65°), and tg (180°) states, respectively.⁷

${}^1\text{H}, {}^1\text{H}$ -NOESY and ${}^1\text{H}, {}^1\text{H}$ -T-ROESY experiments were used to determine effective distances from $\text{H}1f$ and from the $\text{H}6g$ protons. Cross-relaxation rates were analyzed using the PANIC approach as

demonstrated in Figure 3. From the NOESY experiments where H1f was inverted, a small enhancement at H5g could be observed. The distance between H1f and H5g is expected to be longer than 4 Å in all conformations having ψ around 180°. However, the overlap with H5f prevented quantification of the cross-relaxation rate. This limitation was circumvented by using the STEP-NOESY experiment, in which magnetization is transferred from a well-resolved resonance to the resonance of interest by the use of a TOCSY spin-lock (Figure 4). As can be seen from Figure 5, this allowed the clean excitation of H5f to be performed and subsequently the cross-relaxation rate to H1f could be determined. The effective distance is indeed shorter than 4 Å (cf. Table 2), indicating that there is some population of ψ in other conformations than the one defined by $\psi = 180^\circ$. However, because this distance is influenced by all three torsion angles, a quantitative interpretation cannot be made without relatively accurate molecular models.

Conformation from MD simulation. The average values and standard deviations of the glycosidic torsion angles from the 500 ns MD simulation of the disaccharide were $\langle\phi\rangle = -48 (17)^\circ$, $\langle\psi\rangle = 177 (25)^\circ$, $\langle\omega\rangle = 26 (48)^\circ$. The time scale of torsional processes can be observed qualitatively from Figure 6 which shows a 20 ns excerpt from the torsion angle trajectories. The main conformational state for the ϕ and ψ torsion angles, centered at -48° and 179° , respectively, is populated to approximately 95%, as presented in Figure 7. The remaining parts of the trajectory are essentially in a secondary state having $\psi = 85^\circ$. These two conformations of ψ will be referred to as the *antiperiplanar* and *clinal* states, respectively. For the ω torsion angle, significant flexibility is observed, being in equilibrium between *gt/gg/tg* conformations in a ratio of 72:27:1, in qualitative agreement with the population distribution obtained from the aforementioned three-state analysis of vicinal homonuclear proton-proton coupling constants. The average value of the ω torsion angle is $+53^\circ$ and -47° in the *gt* and *gg* conformations, respectively, with corresponding average values for ϕ/ψ being $-48^\circ/177^\circ$ and $-47^\circ/180^\circ$, respectively. From the two-dimensional potential of mean force plot for the ψ and ω torsion angles (Figure 8), there appears to be some correlation between the two; the *gt* conformation, with the ω torsion angle decreased by approximately 10° , is slightly more favored in the *clinal* state.

As expected, inter-residual hydrogen bonding is scarce owing to the greater distance between the residues in a (1→6)-linkage compared to a linkage at a position carrying a secondary carbon atom. The most frequent inter-residual hydrogen bond is found between O5g and HO2f which populates only 0.5% of the trajectory. This hydrogen bond is exclusively found when ψ is in the *clinal* state; the presence is 8% and 11% in the *gt/clinal* state (Figure 9a) and in the *gg/clinal* state, respectively.

Solvent interactions from MD simulation: In order to investigate the water structure around the disaccharide, spatial distribution functions⁵⁷ were calculated for the two major conformational states (Figure 10). In both states, regions with high water density are found between the two residues, clustering between O5g and O6g, and close to O5f and HO2f. In the *gt* conformation of ω the high density region associated with O5f is partially overlapping with the O5g/O6g cluster, whereas in the *gg* conformation these regions are clearly separated and instead the HO2f-associated density is close to the O5g/O6g density.

The analysis of water bridges found in the different conformations is consistent with this picture. In both states water bridges to O5g and O6g are present in 9% of the trajectory. Whereas bridges in the *gt* state are formed between O5g and O4f (5%) (Figure 9b) and O5f (4%) (Figure 9c), and between O6g and O4f (5%) (Figure 9b), they are in the *gg* conformation absent between O5g and the oxygen atoms in the fucose residue, while the population of bridges between O6g and O4f is diminished (2%). Instead, bridges between O5g and HO2f are present to 22% (Figure 9d), compared to 1% in the *gt* conformation.

The hydroxyl torsion angles are found to be confined to certain torsion angles which are populated to more than 70% throughout the simulation. The probability of finding all hydroxyl torsions in the fucose residue in a clockwise arrangement is 59%, and a counterclockwise orientation in the glucose residue is present in 63% of the trajectory.

No significant differences in the number of hydrogen bonds to the solvent is found between the different hydroxyl groups; the average numbers are between 1.3 and 1.5 while the acetal oxygens on average form 0.5 hydrogen bonds to water. Residence times were not found to be considerably longer for water molecules interacting with O5f, O5g or O6g (10 ps) compared to those interacting with hydroxyl groups (8 ps). The observation of similar residence times for bridging water molecules at the glycosidic linkage compared to water molecules engaged with hydroxyl groups has also been observed in sucrose,⁵⁸ and indicate that the regions of the spatial distribution function with high density are due to a larger degree of ordering of water molecules rather than a stronger binding.

Comparison of NMR and MD data: Conformation. The coupling constants related to the ω torsion angle were calculated using the torsion angles found in the MD simulation, which yielded values of the $^3J_{C4g,H6gR}$ and $^3J_{H5g,H6gS}$ coupling constants that were significantly larger than the experimentally observed values, being 1.3 Hz and 2.2 Hz, respectively (Table 1), in all of the three MD conformations (viz. *gt*, *gg* and *tg*). Consequently it was concluded that the torsion angle of ω in

the *gt* and *gg* conformations needed adjustment. In X-ray crystallographic studies typical magnitudes of these conformations are closer to $\pm 65^\circ$ ^{59,60} than the smaller values observed for the ω torsion in the present MD simulation. Hence, NMR parameters related to the ω torsion angle were calculated using the larger values of the torsions.

Although the effective distances related to only ψ and ϕ determined directly from the MD simulation are in excellent agreement with the experimental values, the coupling constants indicate that the conformational equilibrium is somewhat different from the simulated one. As previously mentioned, there was no reasonable single (and narrow) conformation able to rationalize the observed coupling constants related to the ψ torsion angle. Consequently, we investigated the possibility of a larger population of the secondary *clinal* state.

From Tables 1 and 2 it is clear that only two NMR parameters report solely on ψ , viz., the heteronuclear coupling constants between C1f and the two methylene protons bound to C6g. Fitting these two values to a two-state equilibrium gives the populations 61:39 for the *antiperiplanar* and *clinal* states, respectively. There are, however, several NMR parameters which are sensitive to ψ together with either ϕ or ω as well as the H1f-H5g distance, which is sensitive to all three torsion angles (Tables 1 and 2). Hence, NMR parameters were calculated for all combinations of $\phi = -40^\circ$, $\psi = 85^\circ/180^\circ$ and $\omega = 65^\circ/-65^\circ/180^\circ$; in total six conformations. The populations resulting from the fit were 45:48:7 for the *gt/gg/tg* conformations of ω and 68:32 for the *antiperiplanar/clinal* conformations of ψ when the conformations were assumed to be uncorrelated by keeping the ratios *gt/gg/tg* to the same values in both conformations of ψ . Allowing correlated populations reduced the error slightly, leading to a ratio of the ω conformations as 50:40:10 in the *antiperiplanar* conformation and 36:64:0 in the *clinal* conformation, without changing the *antiperiplanar/clinal* ratio. These fitted populations gave reasonable agreement between predicted and experimental parameters, as shown in Table 1.

Although inclusion of the *tg* conformation reduced the error slightly, the reduction in χ^2 was not significant. Using a smaller ω torsion angle in the ψ -*clinal* state the ω -*gt* state as observed in MD simulations (Figure 8) was not found to improve the quality of the fit. Additionally, the possibility of a ψ -*clinal* conformation having $\psi = -85^\circ$ rather than $+85^\circ$ was excluded as the corresponding six-state fitting allowing correlated populations yielded a large error ($\chi^2 = 22.1$).

The large deviation of the geminal coupling constant, which was given a large estimated error (2 Hz), indicates that the Karplus relationship needs to be revised to account for the effects of glycosylation at O6.

Dynamics from NMR experiments. From pulsed field gradient NMR experiments, the translational self-diffusion constant, D_t , was determined to be $4.61 \times 10^{-10} \text{ m}^2 \cdot \text{s}^{-1}$ (scaled from the measured value, $4.00 \times 10^{-10} \text{ m}^2 \cdot \text{s}^{-1}$ at 298 K, to 303 K) for the disaccharide under study. Under the assumptions of being a rigid sphere this corresponds to a rotational correlation time, τ_c , of 116.4 ps. Effective correlation times were also determined from the ratio between the cross-relaxation rates from NOESY and T-ROESY experiments¹⁷ which are in very good agreement with that calculated from translational diffusion (Table 3). To further investigate the dynamics ^{13}C T_1 relaxation rates were measured (Figure 11a, Table 4). The overall slower relaxation for carbons in the fucose residue than for carbons in the glucose residue indicates that the latter residue is more rigid. Interestingly, C4f relaxes significantly faster than the other carbons in the same residue. Since it is unlikely that this position experiences less internal motion, the probable explanation is that the molecule is tumbling anisotropically. The remaining CH vectors in the fucose residue are all axial (assuming a $^1\text{C}_4$ conformation of the pyranose ring) and thus parallel to each other, whereas the equatorial C4f-H4f vector has a roughly orthogonal orientation, as seen from Figure 12. A similar phenomenon has been observed for the C4 carbons in the galactose residue in both lactose and methyl β -lactoside.^{61,62} In order to determine if there is in fact any difference in flexibility between the two residues, the effect of the anisotropic diffusion must be quantified.³²

Dynamics from MD simulation. Generalized order parameters, S^2 , obtained from internal P_2 correlation functions for the CH vectors for the *gt* and *gg* states are shown in Figure 13a,b and Table 5. These show that flexibility is higher for the fucose residue, (f235) having S^2 values around 0.82, compared to the higher S^2 values in the glucose ring being around 0.87, a difference that is more pronounced in the *gg* state than in the *gt* state. For the proton-proton interaction vectors investigated with respect to NOEs, the calculated S^2 values are in the range 0.86–0.97 and hence are expected to have a negligible impact on the determined effective distances.

Laboratory-frame P_2 correlation functions were calculated for the *gt* and *gg* states having ϕ in the exo-anomeric and ψ in the *anti*-conformation, shown for the *gt* state in Figure 14a. From these, the diffusion tensor could be determined using equation 7, yielding the following rotational diffusion constants; $D_{\parallel}^{\text{D}_2\text{O}} = 2.81 \times 10^9 \text{ s}^{-1}$ and $2.93 \times 10^9 \text{ s}^{-1}$ in the *gt* and *gg* states, respectively, and $D_{\perp}^{\text{D}_2\text{O}} = 1.21 \times 10^9 \text{ s}^{-1}$ and $1.20 \times 10^9 \text{ s}^{-1}$ in *gt* and *gg*, respectively, after adjusting for the low viscosity of the water model. Equivalently, $\tau_{\text{iso}} = 95.5 \text{ ps}$ and $D_{\parallel}/D_{\perp} = 2.32$ in the *gt* state and $\tau_{\text{iso}} = 93.4 \text{ ps}$ and $D_{\parallel}/D_{\perp} = 2.43$ in the *gg* state. Assuming that the diffusion tensor is not significantly affected by the conformation, the population-weighted average was calculated, giving $\tau_{\text{iso}} = 95.0$ and $D_{\parallel}/D_{\perp} = 2.35$.

The diffusion tensor was found to be aligned with the moment of inertia tensor, deviating less than 8° (Figure 12), allowing us to use the main axis of the moment of inertia tensor in lieu of the diffusion tensor.

From the mean-squared displacement function, the translational self-diffusion constant was determined to be $4.95 \times 10^{-10} \text{ m}^2 \cdot \text{s}^{-1}$ after correcting for the viscosity ratio between the water model⁵³ and D₂O⁶³, and for the finite size of the simulated system,⁵² overestimating the experimental value by only 5%. The isotropic correlation time calculated from the translational self-diffusion constant is 94.6 ps, i.e., very close to the correlation times found from fitting the diffusion tensor.

The average angles between the largest principal component of the inertia tensor and the CH vectors are shown in Figure 11b, supporting the rationalization for the deviating T_1 relaxation of C4f. In addition, the faster relaxation for carbons 2 – 5 in the glucose residue compared to C1g and C6g can, at least partially, be explained by anisotropy, mainly due to their smaller angles to the unique axis of the moment of inertia tensor in the *gg* state. The fact that the C1g-H1g vector has an angle of 83°, i.e., at least as large as the CH vectors in the fucose residue suggests that the faster relaxation of the glucose residue also must have another source, such as less extensive internal motion.

The interconversions between the respective states for the ω and ψ torsions were investigated by their number correlation functions⁶⁴ (Figure 14b), which upon fitting to an exponential decay gave $\tau_N^\omega = 88 \text{ ps}$ and $\tau_N^\psi = 47 \text{ ps}$. Assuming that these processes have a proportional response to viscosity, these correlation times correspond to $\tau_N^\omega = 289 \text{ ps}$ and $\tau_N^\psi = 155 \text{ ps}$ in D₂O, i.e., slightly slower than overall rotation. As a comparison, the transition rate for the ω torsion in gentiobiose has been suggested to be on the order of 10^9 to 10^{10} s^{-1} , with reorientational motion being faster than the transition rate at ambient temperature.⁶⁵

Comparison of NMR and MD data: Dynamics. Together with the above discussion on CH vector angles to the main axis of symmetry, the observed ^{13}C T_1 relaxation times (Figure 11a) are most likely the result of a combination axially symmetric reorientation and higher flexibility in the fucose residue than for the glucose residue. ^{13}C T_1 relaxation times were calculated for each coordinate set in the MD simulation using the population-weighted average rotational diffusion constants (vide supra) to calculate the relaxation times for each conformation. ^{13}C T_1 relaxation times using the conformational distribution derived from other NMR parameters are shown in Figure 11c. The inclusion of separate S^2 values for the two rings increased the quality of the prediction, whereas using the S^2 values determined from each CH vector did not bring any further improvement.

Importantly, the good agreement suggests that the torsional motions are indeed sufficiently slow, allowing an averaging of relaxation rates. That is, the decay of the correlation functions due to reorientation is complete before any significant effect of torsional motion can occur.

The observation that the glucose residue displays more rigidity than the fucose residue could have several explanations. The presence of a methyl group instead of a hydroxymethyl group on the fucose residue means that this residue is less solvated than the glucose residue, while the attachment of the *O*-methyl group at the anomeric position of the glucose residue shifts the center of mass closer to the glucose residue. In addition, the two rings could be differently sensitive to librational motion in the glycosidic torsion angles. Based on only this molecule, it is not possible to decipher which, if any, of these effects are the cause of the observed difference in rigidity. Available ^{13}C T_1 relaxation data for gentiobiose ($\beta\text{-D-Glcp-(1}\rightarrow\text{6)-D-Glc}$)⁶⁶ show slightly faster relaxation for the terminal residue than for the reducing end residue, whereas the opposite pattern is found for isomaltose ($\alpha\text{-D-Glcp-(1}\rightarrow\text{6)-D-Glc}$).⁶⁷ However, as in the present case, effects of anisotropic diffusion must be separated from contributions due to internal motion before conclusions about the latter can be drawn.

The observed anisotropy could possibly complicate the use of the isolated spin-pair approximation for the determination of experimental proton-proton distances, since it requires that differences in the spectral density functions, related to the motions, are negligible compared to the dipolar coupling constants, which is related to the distances of the interacting spin pairs. However, for the determined rotational diffusion constants, the maximum errors in cross-relaxation rates due to neglecting anisotropy are 6.0% and 28% for NOESY and T-ROESY, respectively, corresponding to errors in effective distances less than 1.0% for the former and 4.2% for the latter. Hence, the effect of anisotropy on the proton-proton effective distances can be neglected in this case.

As evident from the good agreement between the translational diffusion constant as well as the ^{13}C T_1 relaxation times from NMR spectroscopy and MD simulation, the force field performs well in this regard, after compensating for the well-known low viscosity of the TIP3P water model.

CONCLUSIONS

A dynamic model for the disaccharide $\beta\text{-L-Fucp-(1}\rightarrow\text{6)-}\alpha\text{-D-Glcp-OMe}$ in the study has been devised. NMR spectroscopic data allowed us to refine the model from MD simulations, correcting the slightly deviating populations for the ω torsion angle conformations as well as the torsion angles in these conformations. Evidence is presented supporting a significant population of conformations having ψ around 85° in addition to the intuitively preferred extended conformation having $\psi = 180^\circ$.

The access to secondary conformational states at the ψ torsion angle may affect interactions with other molecules, as for β -D-GlcpNAc-(1 $\rightarrow$ 6)- α -D-Manp-OMe which binds to Wheat germ agglutinin in a conformation having $\psi = -90^\circ$.¹⁴

Together these deviations highlight the slight weaknesses of the force field used in the study which preclude a direct prediction of conformation, but still allow a model to be devised in conjunction with experimental parameters, as was done herein.

The motion of the disaccharide was found to have a rather large anisotropy in MD simulation, an effect that was clearly observed in ^{13}C T_1 relaxation data. The fact that relaxation data is accurately predicted without including torsional isomerization indicates that these motions occur on a slower timescale than reorientation for the studied system.

The present study, as well as a different one using another more recent force field for a trisaccharide containing a (1 $\rightarrow$ 6)-linkage,⁶⁸ underscores the need for improved force fields properly representing the glycosidic (1 $\rightarrow$ 6)-linkage. Furthermore, determination of the experimental parameters related to the conformational distributions in other compounds containing this type of linkage is essential in order to decipher possible influences on the distribution of populated states. Developments along these lines are presently underway and will be reported in due course.

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Table 1. Scalar coupling constants as determined by NMR spectroscopy, calculated from MD simulation and from population fitting.

$^{2/3}J$	NMR ^a /Hz	MD /Hz	Fitted /Hz		Pertinent torison angle(s)
			6 states ^b	4 states ^c	
H5g,H6g _{pro-R}	4.89	7.55	5.19	5.23	ω
H5g,H6g _{pro-S}	2.15	3.35	2.19	1.82	ω
H6g _{pro-R} ,H6g _{pro-S}	-11.7	-8.95	-10.4	-10.3	ψ, ω
C4g,H6g _{pro-R}	1.26, 1.0	3.36	1.88	1.66	ω
C4g,H6g _{pro-S}	3.20, 3.1	4.93	3.81	3.84	ω
C1f,H6g _{pro-R}	3.23	2.18	3.20	3.07	ψ
C1f,H6g _{pro-S}	3.03	1.83	2.59	2.52	ψ
C6g,H1f	4.39	3.27	—	—	ϕ
χ^2	—	41.1	2.2	2.5	

^aFor heteronuclear coupling constants, the first value is determined by J-HMBC and the second using HECADE experiments.

^bFitted using the combinations of three states in ω (*gt*, *gg*, and *tg*) and two in ψ (*antiperiplanar* and *clinal*).

^cFitted using the combinations of the *gt* and *gg* states in ω and two in ψ (*antiperiplanar* and *clinal*).

Table 2. Effective distances as determined by NMR spectroscopy, calculated from MD simulation and from population fitting.

Proton pair	NMR ^a /Å	MD /Å	Fitted /Å		Pertinent torsion angle(s)
			6 states ^b	4 states ^c	
H1 <i>f</i> -H6g _{pro-R}	2.77, 2.78	2.80	2.89	2.88	φ, ψ
H1 <i>f</i> -H6g _{pro-S}	2.41, 2.45	2.40	2.43	2.43	φ, ψ
H1 <i>f</i> -H5g	3.18, n.d. ^d	3.72	3.17	3.12	φ, ψ, ω
H4g-H6g _{pro-R}	2.72, 2.76	2.53	2.74	2.72	ω
H4g-H6g _{pro-S}	3.02, 3.03	3.17	3.11	3.17	ω
χ ²	—	10.9	1.1	1.5	

^aThe first value is distance determined from NOESY and second from T-ROESY, in all cases using H1*f*-H3*f* (2.59 Å) as the reference interaction.

^bFitted using the combinations of three states in ω (*gt*, *gg*, and *tg*) and two in ψ (*antiperiplanar* and *clinal*).

^cFitted using the combinations of the *gt* and *gg* states in ω and two in ψ (*antiperiplanar* and *clinal*).

^dNot determined.

Table 3. ^1H , ^1H cross-relaxation rates and effective correlation times.

		$\sigma_{ij} / 10^{-2} \text{ s}^{-1}$		$\sigma_{\text{NOE}} / \sigma_{\text{T-ROE}}$	$\tau_{\text{eff}} / \text{ps}$
Proton pair		NOE	T-ROE		
H1 <i>f</i>	H3 <i>f</i>	4.41	6.62	0.67	123
H1 <i>f</i>	H5 <i>g</i>	1.31	n.d. ^a	n.d.	n.d.
H1 <i>f</i>	H6 _{pro-<i>R</i>}	2.98	4.48	0.66	123
H1 <i>f</i>	H6 _{pro-<i>S</i>}	6.76	9.79	0.69	117
H4 <i>g</i>	H6 _{pro-<i>R</i>}	3.20	4.75	0.67	121
H4 <i>g</i>	H6 _{pro-<i>S</i>}	1.80	2.71	0.66	123

^aNot determined.

Table 4. ^{13}C T_1 relaxation times at 9.4 T with standard deviation in the last decimal given in parenthesis.

Atom	$N \times T_1$ /s
C1 f	0.74 (2)
C2 f	0.74 (2)
C3 f	0.74 (3)
C4 f	0.58 (1)
C5 f	0.74 (2)
C6 f	2.61 (11)
C1 g	0.71 (2)
C2 g	0.65 (1)
C3 g	0.65 (1)
C4 g	0.63 (1)
C5 g	0.61 (2)
C6 g	0.69 (2)

Table 5. Generalized order parameters (S^2) and average angles (θ) to the unique axis of the moment of inertia tensor for the bond vectors between carbon and hydrogen atoms in the *gt* and *gg* conformations.

Atom pair	S^2		$\theta / ^\circ$	
	gt	gg	gt	gg
C1 <i>f</i> -H1 <i>f</i>	0.86	0.84	83	81
C2 <i>f</i> -H2 <i>f</i>	0.82	0.81	79	76
C3 <i>f</i> -H3 <i>f</i>	0.81	0.80	78	76
C4 <i>f</i> -H4 <i>f</i>	0.85	0.86	27	20
C5 <i>f</i> -H5 <i>f</i>	0.82	0.81	81	79
C1 <i>g</i> -H1 <i>g</i>	0.86	0.87	83	83
C2 <i>g</i> -H2 <i>g</i>	0.84	0.86	78	52
C3 <i>g</i> -H3 <i>g</i>	0.85	0.87	79	53
C4 <i>g</i> -H4 <i>g</i>	0.85	0.87	79	52
C5 <i>g</i> -H5 <i>g</i>	0.87	0.90	81	55
C6 <i>g</i> -H6 <i>g</i> _{pro-R}	0.87	0.89	82	73
C6 <i>g</i> -H6 <i>g</i> _{pro-S}	0.87	0.89	84	84

FIGURES AND LEGENDS

Figure 1. Schematic of disaccharide β -L-Fucp-(1 $\rightarrow$ 6)- α -D-Glcp-OMe (F6G) with pertinent glycosidic torsion angles.

Figure 2. (a) Selected spectral region of a 1D ^1H , ^1H -DPFGSE-NOESY NMR spectrum of F6G recorded at 303 K on a 600 MHz spectrometer with selective excitation of H1f and a mixing time of 500 ms; (b) selected spectral region of a ^1H , ^{13}C -J-HMBC spectrum where the apparent splitting of the doublet components in the F_1 dimension, given by $\kappa \times ^3J_{\text{CH}}$, was achieved with a scaling factor $\kappa = 18.4$; (c) selected region from the ^1H , ^{13}C -HSQC-HECADE spectrum showing the tilting of the cross-peak and the $^3J_{\text{C4g,H6gR}}$ coupling constant of 1.0 Hz measured in the F_2 dimension.

Figure 3. (a) ^1H , ^1H -NOE build-up curves for F6G obtained at a 600 MHz spectrometer frequency employing the PANIC approach. Values for $-I_j^{t_{\text{mix}}}/I_i^{t_{\text{mix}}}$ are shown as error bars corresponding to the standard deviations from 10–11 experiments at each t_{mix} . Lines show the fits obtained using equation 2. Selective excitation was carried out for H1f and cross-relaxation rates were determined to H3f (red), H6g_{pro-R} (blue) and H6g_{pro-S} (black); (b) ^1H , ^1H -T-ROE build-up curves from a single experiment at each t_{mix} and analyzed in the same way.

Figure 4. Illustrative overview of the STEP-NOESY experiment for measuring the cross-relaxation rate between H5g and H1f; (a) selective excitation of H2g, followed by isotropic mixing transfers magnetization to H5g (b), which then can be selectively inverted (c), for subsequent cross-relaxation to H1f (d).

Figure 5. Selected spectral region of (a) 1D ^1H NMR spectrum, (b) 1D ^1H , ^1H -TOCSY collected with excitation of H2g and an isotropic mixing time of 70 ms, and (c) 1D ^1H , ^1H -STEP-NOESY collected with excitation of H2g followed by isotropic mixing, 70 ms, and subsequent excitation of H5g and a cross-relaxation delay of 400 ms. (d) Double difference spectrum³⁷ obtained by subtracting a spectrum with shortest possible cross-relaxation delay (60.5 ms) from the spectrum shown in (c); note the recovery of the correct multiplicity for the H6g protons.

Figure 6. Selected time dependence over 20 ns of the 500 ns MD simulation for the glycosidic torsion angles in F6G: ϕ (top), ψ (middle) and ω (bottom).

Figure 7. Probability distributions of the glycosidic torsion angles in F6G from the MD simulation: ϕ (top), ψ (middle) and ω (bottom).

Figure 8. Potential of mean force as a function of the ψ and ω torsion angles, calculated from $W(\psi, \omega) = -\ln p(\psi, \omega)$, with contour levels at $1 k_B T$ steps up to $7 k_B T$.

Figure 9. Molecular models with hydrogen bonding between O5g and HO2f (a), and with water bridges between O5g, O6g and O4f (b), between O5g and O5f (c), and between HO2f and O5g (d).

Figure 10. Spatial distribution functions calculated for the *gt/antiperiplanar* (a) and *gg/antiperiplanar* (b) conformations shown as surfaces enclosing regions with a density 5.2 and 2.7 times the bulk density for water oxygen (red) and hydrogen (white), respectively.

Figure 11. (a) ^{13}C NMR T_1 relaxation times for carbon atoms in F6G obtained at 303 K and 9.4 T (N equals the number of hydrogen atoms directly attached to the carbon atom being analyzed); (b) CH vector orientations (θ) to the principle axis of the moment of inertia tensor averaged for the gt conformation at ω (black bars) and for the gg conformation at ω (white bars). (c) Relaxation times calculated using rotational diffusion constants, residue-averaged S^2 values, geometries (adjusted to $\phi = -40^\circ$ and $\omega = -65^\circ/65^\circ$) from MD simulation together with the experimentally determined four-state conformational distribution.

Figure 12. Molecular model of F6G based on the structure with the lowest heavy-atom RMSD to the average structure with glycosidic torsion angles $\phi = -47^\circ$, $\psi = -178^\circ$ and $\omega = 48^\circ$ in the *gt* conformation (a); the structure with the lowest RMSD to the average structure with glycosidic torsion angles $\phi = -57^\circ$, $\psi = 180^\circ$ and $\omega = -46^\circ$ in the *gg* conformation (b). Rods indicate the main axes of the fitted diffusion tensor (light blue) and of the moment of inertia tensor (black).

Figure 13. Reorientational P_2 time correlation functions calculated after removing overall motion for the CH vectors in the *gt* conformation (a) and the *gg* conformation (b); for the ^1H - ^1H vectors in the *gt* conformation (c) and in the *gg* conformation (d). Pertinent atom pair interactions are color coded and these are given in the panels.

Figure 14. (a) Selected reorientational P_2 time correlation functions in the laboratory frame (crosses) for the gt conformation used for fitting of the diffusion tensor: unique axis of the moment of inertia tensor (black, angle: 7°), C5g–C2g vector (blue, 42°) and C5g–H5g vector (red, 85°). Lines are calculated using the determined diffusion tensor. (b) Number correlation functions $C_N^\omega(t)$ (solid line) and $C_N^\psi(t)$ (dashed line) for the isomerization process in F6G at the ω and ψ torsion angles, respectively.

TOC.