Exploration of the conformational flexibility of the Le^X related oligosaccharide GalNAcα(1→3)Galβ(1→4)[Fucα1→3)]Glc by ^1H NMR relaxation measurements and molecular dynamics simulations

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^1H NMR relaxation data, measured at two magnetic fields, and molecular dynamics simulations indicate that a tetrasaccharide related to the Le^X antigen is conformationally flexible.

Oligosaccharides are involved in a variety of recognition events such as cell adhesion, metastasis, and embryonic development, among others.[1] To play a role in these functions, the three dimensional structure of the carbohydrate is of primary importance.[2] The extent and nature of the motion around the glycosidic linkages of oligosaccharides remains an open question,[3] and even detailed analyses of results have give either constrained conformations[4] or conformational averaging[5] for different—or even the same—carbohydrate structures. At the present moment, it is evident that complete rigidity can be discarded. However, the ways of looking at the concept of flexibility are fairly subjective and may range[2–8] from the consideration of small torsional oscillations around a given conformer to the recognition of the simultaneous presence of two or more significantly different geometries. To the best of our knowledge, unambiguous indication of internal motion around glycosidic linkages has only been directly obtained in small sugars such as sucrose,[6] and other disaccharides,[3,7,8] but not in branched oligosaccharides. The existence of rigid or flexible structures is of prime importance in recognition phenomena, since any bimolecular binding process is entropically unfavourable.[9]

The conformation of the Le^X trisaccharide and its analogues has been the subject of intensive research during the past few years due to their implication in inflammatory processes.[10] The reported results indicate that, in all the studied compounds, the branched Le^X trisaccharide moiety, Galβ-(1→4)[Fucα(1→3)]GlcNAc, is rather rigid.[10] It has been recently reported that a Le^X-related compound, namely, GalNAcα(1→3)Galβ(1→4)[Fucα(1→3)]GlcβOMe **1**, shows inhibitory activity against the proliferation of astrocytes and transformed neural cell lines.[11a] In addition, a variety of analogues were synthesised that showed different inhibitory potencies.[11,12]

We now report on the application of NMR relaxation measurements, measured at different temperatures and magnetic fields, to unequivocally characterise that, in contrast to the common belief, a Le^X tetrasaccharide analogue **1** is conformationally flexible in solution.

Intra- and inter-residue NOEs were obtained through 2D-NOESY (five mixing times between 100 and 800 ms and 2D-ROESY (four mixing times between 100 and 400 ms) experiments at five different temperatures (between 299 and 323 K) and two different magnetic fields (300 and 500 MHz). Cross relaxation rates (σ_{ROE}, σ_{NOE}) were obtained from these measurements.[6] A first attempt to characterize the presence of internal motion was performed by obtaining $\sigma_{ROE}/\sigma_{NOE}$ ratios at two magnetic fields[13], since they allow the estimation of specific correlation times. Direct experimental evidence of differential flexibility for the GalNAc and Fuc residues of **1** was obtained by evaluation of these $\sigma_{ROE}/\sigma_{NOE}$ ratios at different temperatures. We will comment on the results at 300 MHz. Indeed, although in the NOESY spectra at 299 K, NOE effects for all the residues were negative, NOESY cross peaks for the fucose residue at 302 K were positive. At this temperature, the corresponding intraresidue cross peaks for the Glc, Gal, and GalNAc are negative or barely detectable. In addition, and at 313 K, cross peaks for both Fuc and GalNAc were clearly positive, while those for Gal and Glc were approximately zero. Therefore, according to the experimental results and unexpectedly, the glycosidic linkage for the Fuc residue at the branching Glc moiety is as flexible or even more flexible than that for the terminal GalNAc ring. The observed results at 500 MHz are similar but the temperature required for observing differential signs is higher, *ca.* 318 K. In addition, when the ratios of non-selective longitudinal relaxation times (T_1) at different magnetic fields were calculated, it could be observed that, at all the temperatures studied, the T_1 ratios[14] for the internal Gal and Glc residues were significantly larger than the T_1 ratios of the external GalNAc and Fuc residues (Table 1). Therefore, both the T_1 ratios and the existence of opposite signs for cross peaks belonging to different residues, within the same NOESY spectrum, unequivocally indicate that, even in a branched tetrasaccharide, there is a substantial amount of conformational averaging, as well as distinct flexibility for the different glycosidic linkages.

Information on the accesible conformational space was obtained through MD simulations of **1** in presence of 545 explicit water molecules, using the CVFF force field (Biosym Technologies, USA). It can be observed that, although the glycosidic torsion angles do not flip between widely differing-values, they cover a substantial part of the complete Φ/Ψ map. In order to characterise the time scale and relative restriction of the internal motion around the glycosidic linkages, correlation functions were calculated for the different intra- and inter-residue proton–proton vectors of each of the residues of **1**, using a molecule-fixed coordinate frame, whereby global reorienta-

Table 1 Longitudinal non-selective relaxation times T_1 at two magnetic fields ($T_1^{500\ MHz}/T_1^{300\ MHz}$) for different protons of the branched tetrasaccharide **1**[a]

	Temperature/k				
Proton	299	302	313	320	333
Fuc H-1	2.35	2.03	1.92	1.86	1.74
GalNAc H-1	2.32	2.10	1.94	1.94	1.93
Gal H-1	2.53	**2.27**	**2.24**	overlap H_2O	**2.48**
Glc H-1	**2.65**	2.17	2.15	**2.12**	overlapH_2O
Fuc H-5	1.80	1.87	1.86	1.82	1.79
Fuc CH_3-6	1.62	1.72	1.85	1.57	1.64
GalNAc H-2	2.24	2.04	1.88	1.88	1.87

[a] The higher the ratio (in bold), the higher the restriction to internal motion (ref. 14). Several independent measurements were performed.

Table 2 Experimental 300 MHz longitudinal cross relaxation rates, σ_{NOE} at 299 and 302 K for different inter- and intra-residue proton pairs of the branched tetrasaccharide **1**. Positive cross peaks are given in bold.[a] The calculated (from MD simulations) generalized order parameters (S^2) and the comparison between the experimental, r_{exp}, (from $\sigma_{NOE}/\sigma_{ROE}$ and $\sigma_{NOE500}/\sigma_{NOE300}$ ratios[13]) and calculated distances (from $<r^{-6}>^{-1/6}$ and $<r^{-3}>^{-1/3}$ averaging) are also presented

Proton pair	σ_{NOE}^{299}	σ_{NOE}^{302}	r_{exp} (Å)	$<r^{-6}>^{-1/6}$	$<r^{-3}>^{-1/3}$	$S^2_{H_2O}$	S^2_{vacuo}
Gal H-1/Glc H-4	−0.140	−0.065	2.2–2.3	2.49	2.50	0.92	0.78
Gal H-1/H-3	−0.062	−0.043	2.6–2.7	2.53	2.53	0.96	0.85
GalNAc H-1/H-2	−0.090	−0.042	2.3–2.5	2.41	2.41	0.91	0.65
GalNAc H-1/Gal H-3	−0.021	−0.005	2.6–2.7	2.51	2.58	0.71	0.38
GalNAc H-1/Gal H-4	−0.035	−0.006	2.4–2.5	2.42	2.46	0.70	0.39
Gal H-1/Glc H-6a	−0.010	−0.004	2.8–3.0	2.45	2.49	0.80	0.50
Gal H-1/Glc H-6b	overlap	overlap	overlap	4.01	4.02	0.89	0.50
Glc H-1/H-3	−0.075	−0.034	2.5–2.7	2.56	2.56	0.89	0.81
Fuc H-1/H-2[b]	−0.032	**0.042**	2.2–2.3	2.51	2.51	0.85	0.52
Fuc H-1/Glc H-3[b]	−0.032	**0.042**	2.2–2.3	2.54	2.56	0.77	0.58
Fuc H-1/Glc H-2	−0.004	**0.006**	3.0–3.2	3.59	3.60	0.85	0.58
Fuc H-5/Gal H-2	−0.040	**0.003**	2.6–2.7	2.56	2.61	0.70	0.40
Fuc H-5/H-4	−0.040	**0.007**	2.4–2.6	2.52	2.52	0.83	0.47
Fuc CH_3-6/Gal H-2	**0.005**	**0.009**	3.0–3.3	3.25	3.36	n.d.[c]	n.d.[c]
Fuc CH_3-6/H-5	**0.009**	**0.028**	2.4–2.6	2.20	2.31	n.d.[c]	n.d.[c]

[a] It is noteworthy to mention that positive and negative cross peaks are observed simultaneously for both NOESY spectra. At 299 K, cross peaks involving the Fuc CH_3 group are positive, while at 302 K, all the intra- and inter-residue cross peaks for the Fuc residue are positive. The other cross peaks for the Gal, Glc and GalNAc moieties are negative. [b] Overlapping signals. [c] N.d. = not determined.

tion was removed.[15] The correlation functions were used to derive generalized order parameters and internal motion correlation times, using the Lipari and Szabo formalism.[16] The calculated results from the solvated simulation (Table 2) also indicated a higher degree of restriction for the Glc and Gal residues (larger S^2), while the amplitude of motion is higher for GalNAc and Fuc moieties (smaller S^2), in agreement with the experimental data. In all cases, the internal correlation times around the glycosidic linkages were calculated to be around a few tens of picoseconds. An *in vacuo* MD simulation was also performed, which indicated an even higher degree of conformational freedom. Finally, average expected interproton distances were compared to those obtained from the experimental cross-relaxation rates. It can be observed that there is satisfactory agreement between both quantities. A superimposition of different conformers found in the solvated MD simulation is shown in the graphical abstract.

In conclusion, ^{1}H NMR data have shown that the glycosidic bonds of **1** in solution are fairly flexible, in contrast with previously reported results for different analogues which present an acetamido group on the reducing Glc residue.[10] From a general point of view, it is shown that the recording of NOESY spectra at different temperatures provide a direct and fast means to detect the presence of differential motions in oligosaccharides. The existence of flexibility for **1**, and thus possibly for the closely related Le^X analogues, indicates that care should be taken when designing new substrates with potential therapeutic use, since the biologically active conformation may not be the major one existing in solution.

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