Evidence for two populated conformations for the dimeric Le^{X} and $\text{Le}^{\text{A}}\text{Le}^{\text{X}}$ tumor associated carbohydrate antigens.

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ABSTRACT

The conformational behavior of tumor associated carbohydrate antigens (TACAs) dimLe^{X} and $\text{Le}^{\text{A}}\text{Le}^{\text{X}}$ was studied using a combination of NMR experiments and molecular dynamic simulations. It is shown that within the hexasaccharides, the Le^{X} and Le^{A} branched trisaccharides fragments adopt the rigid “stacked” conformation known for the isolated trisaccharide antigens. In contrast, the $\beta\text{-D-GlcNAc-(1}\rightarrow\text{3)-D-Gal}$ glycosidic bond that connects the two Le^{X} trisaccharides in dimLe^{X} , and the Le^{A} trisaccharide to the Le^{X} trisaccharide in $\text{Le}^{\text{A}}\text{Le}^{\text{X}}$ was found to be very flexible in both hexasaccharides. Our results show that two distinct conformations differing by the value of the Ψ angle for this glycosidic bond, are populated in solution. While

the relative proportions of the two conformations in solution could not be determined accurately, experimental measurements indicate that both conformations are populated in significant amounts.

INTRODUCTION

Since the discovery half a century ago that glycolipids accumulated in adenocarcinomas,¹ the quest for carbohydrate-based anti-cancer immunotherapies has been relentless.² Amongst the many Tumor Associated Carbohydrate Antigens (TACAs) that have been identified,³ hexasaccharide dimeric Lewis X (dimLe^x, **1**) was found to accumulate in colonic and liver adenocarcinoma⁴ while hexasaccharide Le^aLe^x (**2**) has been found to be over expressed on the surface of squamous lung carcinoma (SLC) cells.⁵ While the hexasaccharides are clearly associated to

tumor cells, the non-reducing end trisaccharide fragments Le^x and Le^a are widely expressed at the surface of normal cells and tissues (Le^x ref.⁶, Le^a ref.⁷). Therefore, when considering the development of a therapeutic anti-cancer vaccine based on the TACAs dimLe^x or Le^aLe^x, an important factor to consider is the expected autoimmune responses to the native Le^x and Le^a antigens. Interestingly, two monoclonal antibodies (mAbs, FH4, SH2) isolated when immunizations were carried out with the dimLe^x glycosphingolipid, were shown to recognize the

hexasaccharide antigen selectively, while only weakly binding to the Le^x antigen.⁸ Similarly, immunization of mice with SLC cells led to the cloning of mAb 43-9F which was shown to specifically recognize Le^aLe^x while it only weakly bound to the Le^a trisaccharide.^{7b,9} Thus it is clear that these mAbs recognize internal epitopes displayed by dimLe^x and Le^aLe^x. Most importantly, immunostaining experiments with FH4 showed that these internal epitopes were selectively displayed on stomach, colon and breast cancer tissues^{8b} while the epitope recognized by 43-9F was associated with lung cancer and the tumorigenicity of SLC cells.^{7b,9-10}

It has been well established that the Le^x (ref¹¹) and Le^a (ref¹²) branched trisaccharides are rather rigid and adopt mostly well-defined “stacked” conformations. Therefore, the conformational behavior of the β -D-GlcNAc-(1 $\rightarrow$ 3)-D-Gal glycosidic bond that connects the two Le^x trisaccharides in dimLe^x and the Le^a trisaccharide to Le^x in Le^aLe^x appears to be the main factor determining the shape of the hexasaccharide antigens and thus the internal epitopes that are presented to the immune system. We describe here the results of our study of the conformational behavior of hexasaccharide antigens dimLe^x and Le^aLe^x combining molecular dynamics experiments with NMR experiments on hexasaccharide **2** and **4**. We have also studied the conformation of tetrasaccharide **5**, a common fragment to both antigens which lacks the fucosyl and galactosyl residues at the non-reducing end.

RESULTS AND DISCUSSION

The orientations adopted around the glycosidic linkages are described below by the dihedral angles: $\Phi = \text{O5-C1-O1-Cx}$ and $\Psi = \text{C1-O1-Cx-Cx+1}$ and the signs of the torsions are in agreement with the recommendations of the IUPAC-IUB Commission of Biochemical Nomenclature.¹³

NMR experiments. NMR data for the hexasaccharides **2** (7 mM) and **4** (4.5 mM) were acquired at 300 K for D₂O solutions prepared in Shigemi tubes. To achieve better resolution an 800 MHz spectrometer was used for the dimLe^x analogue, while better sensitivity was achieved using a 600 MHz spectrometer equipped with a cryoprobe for the Le^aLe^x analogue. NMR data for tetrasaccharide **5** (21 mM) were also acquired at 300 K using the cryoprobe-equipped 600 MHz spectrometer. The ¹H chemical shifts for oligosaccharides **2**, **4**, **5** were assigned using COSY, HSQC, HMBC experiments as well as 1D selective TOCSY and ROESY experiments, these assignments are reported in the supporting information and for dimLe^x are in excellent agreement with previously reported assignments.¹⁴ The vicinal coupling constants measured for the three sugar units support an average ⁴C₁ conformation for the galactose, glucose and *N*-acetylglucosamine rings and a ¹C₄ conformation of the fucose rings.

Selective 1D ROESY experiments irradiating anomeric signals H-1*B*, H-1*C*, H-1*B*' and H-1*C*' (256 scans) in dimLe^x (**2**) gave the inter-residue NOE contacts expected for the Le^x stacked conformation^{11d-f} both for the reducing and non-reducing end trisaccharides (Figure 1a). Thus, correlations were observed between: the fucosyls H-1*B* and H-1*B*' with the GlcNAc H-3*A* and H-3*A*', respectively; the galactosyls H-1*C* and H-1*C*' with the GlcNAc H-4*A* and H-4*A*', respectively, as well as with the GlcNAc methylenes H-6*aA*, H-6*bA* and H-6*aA*', H-6*bA*', respectively. The expected correlations between the fucosyls H-5*B* and H-5*B*' with galactosyls H-2*C* and H-2*C*', respectively, could not be assigned with certainty using selective 1D ROESY as both H-5*B* and H-5*B*' gave overlapping signals. However, a 2D NOESY experiment clearly showed the presence of these close contacts (Figure 2) which are most characteristic of the Le^x “stacked” conformation.^{11d-f} With respect to the glycosidic linkage β-D-GlcNAc-(1→3)-D-Gal that connects the two Le^x trisaccharides in hexasaccharide **2**, irradiation of H-1*A*' combined with

the overlapping HDO signal gave, as expected,¹⁵ cross-relaxation signals to H-3C and H-4C. Cross-relaxation buildup curves were obtained over 10 mixing times ranging from 20 ms to 350 ms with 5 mixing times chosen between 20 and 100 ms. Normalized buildup curves for the resolved inter-residue correlations (Figure 1b) were fitted to double exponential equations and used to evaluate inter-proton distances using the isolated spin pair approximation (reference buildup curves are given in the supporting information).¹⁶ As we have previously observed, the apparent slower buildup of signals H-5A' (supporting information), H-3C and H-4C (Figure 1b) upon irradiation of H-1A', resulted from the normalization against the overlapping signals H-1A' and HDO.¹⁷

Figure 1. NMR data for hexasaccharide dimLe^x (**2**) at 800 MHz and 300 K: a) ¹H NMR spectrum and ¹H, ¹H ROESY spectra upon selective excitation of protons H-1*B*, H-1*C*, H-1*A*' combined with HDO, H-1*B*' and H-1*C*' (200 ms). b) Normalized cross-relaxation buildup curves for resolved signals.

Figure 2. 800 MHz NOESY spectrum recorded for hexasaccharide **2** at 300 K (300 ms) showing clear correlations between H-5*B* and H-2*C* as well as between H-5*B*' and H-2*C*'.

The measured inter-proton distances (Table 1) for the Le^x trisaccharide fragments in dimLe^x are consistent with the distances that we have reported^{11f} for the Le^xOMe trisaccharide and support the “stacked” conformations for both Le^x trisaccharide fragments within the hexasaccharide. As reported^{12e,15,18} for β-D-GlcNAc-(1→3)-D-Gal glycosidic bonds in smaller oligosaccharides and as discussed below, the distance (3.3 Å) measured between H-1*A*' and H-4*C* indicates flexibility around this glycosidic linkage.

Selective 1D ROESY experiments (512 scans) acquired for the Le^aLe^x hexasaccharide **4** (Figure 3a) gave the inter-residue NOE contacts expected for the Le^x and Le^a stacked conformations.^{11d-f,12g-k} Thus, correlations were observed between: H-1*B* and H-3*A*, H-1*C* and H-4*A*, H-1*C* and H-6*aA*, H-6*bA* for the reducing end Le^x trisaccharide, while for the non-reducing end Le^a trisaccharide we observed correlations between: H-1*C*' and H-3*A*', H-1*B*' and H-4*A*', H-1*B*' and H-6*aA*', H-6*bA*'. Here again, selective irradiation of H-1*A*' gave, as expected, cross-

relaxation signals to H-3C and H-4C. As described above for dimLe^x, normalized cross relaxation buildup curves (Figure 3b, 5 mixing times, 40–120 ms) were fitted to double exponential equations and used to evaluate inter-proton distances using the isolated spin pair approximation (reference buildup curves are given in the supplementary information).¹⁶ As for dimLe^x, the inter-proton distances measured (Table 2) for hexasaccharide Le^aLe^x (**4**) were in agreement with the distances known^{11f,12k} for the “stacked” conformation of both the Le^x and the Le^a trisaccharides. Here again as seen in smaller oligosaccharides^{12e,15,18} and as discussed below, the distance (3.3 Å) measured between H-1A' and H-4C indicates flexibility around the β-D-GlcNAc-(1→3)-D-Gal glycosidic bond.

Table 1. Calculated and measured distances (Å) for dimLe^x [**1**(R = Me) and **2**], tetrasaccharide **5**, and measured distances for Le^xOMe (ref^{11f}).

	2	Le ^x OMe	5	1	5
	NMR ^a	NMR ^b	NMR ^a	MD ^c	MD ^c
H-1B/H-3A	2.51	^d	^d	2.58	2.59
H-5B/H-2C	^d	2.66	2.53	2.45	2.45
H-1C/H-4A	2.40	2.44	2.48	2.48	2.48
H-1C/H-6aA	2.77	2.69	2.75	3.48	2.78
H-1C/H-6bA	2.51	2.49	2.52	2.35	2.42
H-1A'/H-3C	2.25	^e	2.29	2.35	2.32
H-1A'/H-4C	3.32	^e	3.26	3.16	3.20
H-1B'/H-3A'	2.52	^e	^e	2.58	^e
H-5B'/H-2C'	^d	^e	^e	2.50	^e
H-1C'/H-4A'	^d	^e	^e	2.51	^e

H-1C'/H-6aA'	^d	^e	^e	2.88	^e
H-1C'/H-6bA'	2.49	^e	^e	2.39	^e

^a $r_{ij} = r_{\text{ref}} (S_{\text{ref}}/S_{ij})^{1/6}$. ^b Reported^{11f}. ^c $1/r = \langle r^{-6} \rangle^{1/6}$. ^d Signal overlap precluded measurement. ^e Not applicable.

Figure 3. NMR data for hexasaccharide Le^aLe^x (**4**) at 600 MHz (cryoprobe) and 300 K: a) ¹H NMR spectrum and ¹H, ¹H ROESY spectra upon selective excitation of protons H-1B, H-5B, H-

1C, H-1A', H-1B', H-5B' and H-1C' (mixing time 120 ms for all signals except for H-1A' shown at 200 ms). b) Normalized cross-relaxation buildup curves for resolved signals.

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Table 2. Calculated and measured distances (Å) for Le^aLe^x [**3** (R = Me) and **4**], and measured distances for Le^xOMe and Le^aOMe (refs ^{11f,12k}).

	4	Le^xOMe	Le^aOMe	3
	NMR ^a	NMR ^b	NMR ^c	MD ^d
H-1B/H-3A	2.45	^e —	^f —	2.58
H-5B/H-2C	2.47	2.66	^f —	2.43
H-1C/H-4A	2.43	2.44	^f —	2.48
H-1C/H-6Aa	2.77	2.69	^f —	2.86
H-1C/H-6Ab	2.22	2.49	^f —	2.40
H-1A'/H-3C	2.25	^f —	^f —	2.39
H-1A'/H-4C	3.27	^f —	^f —	3.32
H-1B'/H-4A'	2.65	^f —	2.39	2.53
H-1B'/H-6A'a	2.65	^f —	2.60	2.50
H-1B'/H-6A'b	2.32	^f —	2.39	2.24
H-5B'/H-2C'	2.77	^f —	2.65	2.50
H-1C'/H-3A'	2.44	^f —	2.57	2.47

^a $r_{ij} = r_{\text{ref}} (S_{\text{ref}}/S_{ij})^{1/6}$. ^b Reported^{11f}. ^c Reported^{12k}. ^d $1/r = \langle r^{-6} \rangle^{1/6}$. ^e Signal overlap precluded measurement. ^f Not applicable.

Selective 1D ROESY experiments were also acquired for tetrasaccharide **5** (128 scans), the experiments and normalized buildup curves are shown in the supporting information. As described above for hexasaccharides **2** and **4**, the curves were used to evaluate inter-proton distances (Table 1). As previously seen for dimLe^x and Le^aLe^x , the measured inter-proton distances were also in agreement with those reported^{11f} for the “stacked” conformation of the Le^x

trisaccharide and the distance (3.3 Å) measured between H-1A' and H-4C also indicated flexibility around the β -D-GlcNAc-(1 $\rightarrow$ 3)-D-Gal glycosidic bond.^{12e,15,18}

Molecular dynamic simulations. To further investigate the flexibility of the hexasaccharide antigens dimLe^x (**1**, R = Me), Le^aLe^x (**3**, R = Me) and tetrasaccharide fragment **5** we ran 20 ns molecular dynamic simulations in explicit water at 300 K using AMBER10¹⁹ with the inclusion of the Glycam06 parameters for carbohydrates.²⁰ Initial structures were built using the global minimum torsions expected for the Le^x and Le^a trisaccharides¹¹⁻¹² and the known^{11a} global minimum torsions for disaccharide β -D-GlcNAc(A')-(1 $\rightarrow$ 3)-Gal(C) and were solvated with 10 shells of TIPS3P water molecules.²¹ We define the Φ/Ψ torsions as follow: Φ_B/Ψ_B = Fuc(B)-(1 $\rightarrow$ 3)-GlcNAc(A) in dimLe^x (**1**), Le^aLe^x(**3**) and fragment **5**; Φ_C/Ψ_C = Gal(C)-(1 $\rightarrow$ 4)-GlcNAc(A) in dimLe^x (**1**), Le^aLe^x(**3**) and fragment **5**; $\Phi_{A'}/\Psi_{A'}$ = GlNAc(A')-(1 $\rightarrow$ 3)-Gal(C) in dimLe^x (**1**), Le^aLe^x(**3**) and fragment **5**; $\Phi_{B'}/\Psi_{B'}$ = Fuc(B')-(1 $\rightarrow$ 3)-GlcNAc(A') in dimLe^x (**1**) and Fuc(B')-(1 $\rightarrow$ 4)-GlcNAc(A') in Le^aLe^x (**3**); $\Phi_{C'}/\Psi_{C'}$ = Gal(C')-(1 $\rightarrow$ 4)-GlcNAc(A') in dimLe^x (**1**) and Gal(C')-(1 $\rightarrow$ 3)-GlcNAc(A') in Le^aLe^x (**3**). The Φ/Ψ trajectories for the fucosidic (Φ_B/Ψ_B , $\Phi_{B'}/\Psi_{B'}$) and galactosidic (Φ_C/Ψ_C , $\Phi_{C'}/\Psi_{C'}$) bonds in the two Le^x trisaccharide fragments of dim Le^x (**1**) and in the Le^a (left; $\Phi_{B'}/\Psi_{B'}$, $\Phi_{C'}/\Psi_{C'}$) and Le^x (right ; Φ_B/Ψ_B , Φ_C/Ψ_C) trisaccharide fragments of Le^aLe^x (**3**) were superimposed over the MM3 grid search energy maps obtained for the corresponding disaccharide linkages^{11a} (Figures 4 and 5). Similar trajectories for Le^x in tetrasaccharide **5** are shown in the supporting information. The values of the Φ and Ψ dihedral angles averaged during the 20 ns simulations for each glycosidic linkages in the dimLe^x (**1**, R = Me), Le^aLe^x (**3**, R = Me) and fragment **5** as well as the associated calculated standard deviations are given in Table 3. Calculated inter-proton distances averaged over r^{-6} for comparison with experimental distances are given in Table 1 for analogues **1** and **5** and Table 2 for analogue **3**.

Figure 4. Trajectories (20 ns dynamics simulations, 300 K) of the fucosidic (Φ_B/Ψ_B and $\Phi_{B'}/\Psi_{B'}$) and galactosidic (Φ_C/Ψ_C and $\Phi_{C'}/\Psi_{C'}$) linkages in the Le^x trisaccharides portions of dimLe^x (**1**) superimposed on the MM3 grid search energy maps obtained for the disaccharide linkages (contours).^{11a}

Figure 5. Trajectories (20 ns dynamics simulations, 300 K) of the fucosidic (Φ_B/Ψ_B and $\Phi_{B'}/\Psi_{B'}$) and galactosidic (Φ_C/Ψ_C and $\Phi_{C'}/\Psi_{C'}$) linkages in the Le^a (left) and Le^x (right) trisaccharide portions of Le^aLe^x (**3**) superimposed on the MM3 grid search energy maps obtained for the disaccharide linkages (contours).^{11a}

Table 3. Calculated average Φ/Ψ angles for analogues **1**, **3** and **5**

Entry		1 (R = Me)	3 (R = Me)	5
Fuc(B)		(1→3)-GlcNAc	(1→3)-GlcNAc	(1→3)-GlcNAc
1	Φ_B	-68 ± 9	-69 ± 9	-68 ± 10
2	Ψ_B	142 ± 8	141 ± 8	142 ± 7
Gal(C)		(1→4)-GlcNAc	(1→4)-GlcNAc	(1→4)-GlcNAc
3	Φ_C	-68 ± 8	-67 ± 8	-67 ± 9
4	Ψ_C	-108 ± 7	-108 ± 7	-108 ± 8
GlcNAc(A')		(1→3)-Gal	(1→3)-Gal	(1→3)-Gal
5	$\Phi_{A'}$	-81 ± 16	-80 ± 14	-83 ± 14
6	$\Psi_{A'}$	112 ± 36	122 ± 32	113 ± 32
Fuc(B')		(1→3)-GlcNAc	(1→4)-GlcNAc	
7	$\Phi_{B'}$	-69 ± 9	-68 ± 9	
8	$\Psi_{B'}$	142 ± 8	-101 ± 7	
Gal(C')		(1→4)-GlcNAc	(1→3)-GlcNAc	
9	$\Phi_{C'}$	-68 ± 8	-70 ± 8	
10	$\Psi_{C'}$	-108 ± 7	134 ± 8	

As can be seen from Table 3 (entries 1–4 and 7–10) and Figures 4, 5, our simulations supported very limited flexibility around either fucosidic and galactosidic bonds in the Le^x and Le^a branched trisaccharide sections of the dimLe^x and Le^aLe^x hexasaccharide antigens (a similar observation is made for the Le^x fragment of tetrasaccharide **5**). Furthermore, the calculated

distances (Tables 1 and 2) are in good agreement with the expected “stacked” conformations for the Le^x and Le^a trisaccharide fragments.^{11f,12k} Thus, these results concur with previous dynamic simulations on the Le^x (ref.^{11d-f}) and Le^a (ref.^{12d,e,12i,12k}) antigens which also described these trisaccharides as rather rigid entities with limited flexibility around the glycosidic bonds. Figure 6 (left) shows the $\Phi_{A'}/\Psi_{A'}$ trajectories of the β -D-GlcNAc-(1→3)-D-Gal linkage in each of hexasaccharides **1** and **3** (Figures 6a and 6b, respectively) and tetrasaccharide **5** (Figure 6c), superimposed over the MM3 grid search energy maps obtained for the corresponding disaccharide linkage.^{11a}

Figure 6. Trajectories (20 ns dynamics simulations, 300 K) for a) dimLe^x (**1**); b) Le^aLe^x (**3**) and c) tetrasaccharide **5**. Left: glycosidic torsion angles $\Phi_{A'}/\Psi_{A'}$ for the β -GlcNAc-(1→3)-Gal linkages superimposed on the MM3 grid search energy maps obtained for the disaccharide linkages (contours).^{11a} Right: individual trajectories for $\Phi_{A'}$ (i), $\Psi_{A'}$ (ii) and distances H-1A' to H-4C (iii).

The average values of the $\Phi_{A'}/\Psi_{A'}$ dihedral angles as well as the associated calculated standard deviations are given in Table 3 (entries 5 and 6). As can be seen from Table 3 and Figure 6, the

β -D-GlcNAc-(1 $\rightarrow$ 3)-D-Gal linkage in tetrasaccharide **5** as well as more surprisingly in hexasaccharides **1** and **3**, appeared to be quite flexible as all three trajectories explored a large conformational space around the global minimum ($\Phi_{A'}/\Psi_{A'} = -75^\circ/+60^\circ$) calculated^{11a,18} for this glycosidic bond. Though trajectories showed flexibility around both Φ and Ψ angles, flexibility around $\Psi_{A'}$ was much greater than around $\Phi_{A'}$ [Figure 6, compare (i) and (ii)] with deviations averaging for the three analogues $\pm 15^\circ$ and $\pm 33^\circ$ for $\Phi_{A'}$ and $\Psi_{A'}$, respectively (Table 3, entries 5 and 6). The flexibility around $\Psi_{A'}$ translated directly into large fluctuations of the distance between H-1 GlcNAc(A') and H-4 Gal(C) [Figure 6 a–c (iii)] for which our simulations gave r^{-6} averaged values for all analogues of ~ 3.2 Å (see Tables 1 and 2). Careful comparisons of the $\Psi_{A'}$ trajectories for analogues **1**, **3** and **5** [Figure 6 a–c (ii)] indicates that the orientation around $\Psi_{A'}$ in tetrasaccharide **5** varied through the conformational space at a higher frequency than the change in orientations around $\Psi_{A'}$ in hexasaccharides **1** and **3**. Probability analyses (based on a 5° binning) of each database of $\Phi_{A'}$ and $\Psi_{A'}$ angles (Figure 7) show that while the flexibility of $\Phi_{A'}$ is centered around one favored orientation ($\Phi_{A'} \sim -80^\circ$), the $\Psi_{A'}$ torsion adopts two favored orientations at $\Psi_{A'} \sim 150^\circ$ and $\Psi_{A'} \sim 80^\circ$ in all three databases.

Figure 7. Probability curves (5° binning) for torsion angle $\Phi_{A'}$ (left) and $\Psi_{A'}$ (right) in dimLe^x (×), Le^aLe^x (♦) and tetrasaccharide **5** (▼).

We define conformation **I** in which $\Psi_{A'}$ adopts values ranging from 50° to 115° and conformation **II** in which $\Psi_{A'}$ adopts values ranging from 116° to 175° . The relative populations, average $\Phi_{A'}/\Psi_{A'}$ angles, and corresponding standard deviations for conformations **I** and **II** in the database of each compound **1**, **3** and **5** are given in Table 4, together with the calculated experimental distance between GlcNAc(*A'*) H-1 and Gal(*C*) H-4 for each conformational family. For all analogues, conformation **I** adopts $\Phi_{A'}/\Psi_{A'}$ torsions of $-83^\circ/86^\circ$ leading to a distance between GlcNAc(*A'*) H-1 and Gal(*C*) H-4 of ~ 2.9 Å and conformation **II** adopts $\Phi_{A'}/\Psi_{A'}$ torsions of $-80^\circ/143^\circ$ leading to a distance between GlcNAc(*A'*) H-1 and Gal(*C*) H-4 of ~ 4.3 Å (Table 4). While both conformations are almost equally populated in the dimLe^x (**1**) and tetrasaccharide **5** databases, the dynamic simulation on Le^aLe^x (**3**) seems to favor conformation **II** (Table 4). Even though the distance between GlcNAc(*A'*) H-1 and Gal(*C*) H-4 in conformation **I** is much shorter than that observed for conformation **II**, calculation of theoretical (r^6) averaged distances to be compared with NMR data led to very similar distances for all three analogues (Table 2).

Comparison of the NMR and molecular dynamic simulations results. As can be seen in Tables 1 and 2, the distances measured by NMR are in very good agreement with those calculated in the molecular dynamic simulations. Both NMR and MD simulations support that the Le^x and Le^a branched trisaccharides in all compounds adopt preferentially the so-called “stacked” conformations and that these conformations are fairly rigid. NMR also supports flexibility of the β -D-GlcNAc-(1 $\rightarrow$ 3)-D-Gal glycosidic linkage in agreement with the molecular dynamic simulations. The molecular dynamics experiments clearly indicate that the oligosaccharides are likely adopting two distinct conformations (**I** and **II**, Table 4). These conformations differ mostly by the value of the $\Psi_{A'}$ angle (86° and 143° in conformations **I** and **II**, respectively) of the β -D-GlcNAc-(1 $\rightarrow$ 3)-D-Gal glycosidic linkage.

Table 4. Conformations **I** and **II**: calculated populations, average angles and corresponding calculated H-1A'/H-4C distance (Å) for dimLe^x (**1**), Le^aLe^x (**3**) and fragment GlcNAcLe^x (**5**).

	Conformation I	Conformation II
	50°<Ψ _{A'} <115°	116°<Ψ _{A'} <175°
DimLe ^x (1)	50%	49%
Φ _{A'}	-85 ± 19	-78 ± 12
Ψ _{A'}	86 ± 15	142 ± 12
<i>r</i> _{H-1A'/H-4C} (Å) ^a	2.89	4.26
Le ^a Le ^x (3)	35%	63%
Φ _{A'}	-82 ± 17	-79 ± 12
Ψ _{A'}	85 ± 16	144 ± 12
<i>r</i> _{H-1A'/H-4C} (Å) ^a	2.91	4.28
GlcNAcLe ^x (5)	50%	49%
Φ _{A'}	-84 ± 15	-81 ± 13
Ψ _{A'}	86 ± 15	143 ± 12
<i>r</i> _{H-1A'/H-4C} (Å) ^a	2.92	4.23

$$^a \frac{1}{r} = \langle r^{-6} \rangle^{1/6}$$

The simulations also suggest that the relative ratios of conformers **I** and **II** populated in solution varies with the structure considered. As expected, the smaller pentasaccharide undergoes rapid exchange between the two conformations while the hexasaccharides seem to reside for a longer time around one conformation before adopting the second conformation. The exchange between the two conformations is, however, fast on the NMR time scale and NMR only show a conformational average. The NMR experimental distance measured between

GlcNAc(*A'*) H-1 and Gal(*C*) H-4 (~ 3.3 Å) for all three analogues provides clear evidence that both conformations **I** and **II** are populated in solutions in all cases. These measured distances are in good agreement with the calculated distances found in the molecular dynamic experiments. Unfortunately, while it is clear from all data that not one single conformation (**I** or **II**) is predominant for any of dimLe^x (**1**), Le^aLe^x (**3**) or tetrasaccharide **5**, the current data does not allow determining the relative ratio of the two conformational populations in any of the analogues.

¹H chemical shifts vs temperature. It has been suggested that conformational flexibility around glycosidic bonds resulted in linear variation of the corresponding glycosidic hydrogen chemical shift with temperature.^{12e} To support our findings that tumor associated hexasaccharides **2** and **4** underwent conformational exchange around the β-D-GlcNAc-(1→3)-D-Gal glycosidic linkage in solution, we measured the chemical shift of the glycosidic hydrogen H-1*A'* at temperatures varying from 280 to 330 K. As a measure of comparison we also recorded the variation in chemical shifts observed for H-1*B'*, and H-1*C'* of the non-reducing end trisaccharide. In addition, we recorded selective 1D TOCSY on H-1*C* and selective 1D ROESY on H-1*A'* to determine the chemical shifts of H-3*C* and H-4*C* at various temperatures for the two hexasaccharides. Figure 8 shows the chemical shift variations Δ(δ) for these signals in hexasaccharide **2** (black) and **4** (blue) as a function of the temperature increasing from 280 to 330 K. As can be seen the impact of temperature increase was large on the glycosidic hydrogens H-1*A'* in both analogues with linear increases Δ(δ)/Δ(T) of 1.3 and 1.7 ppb.K⁻¹ for hexasaccharides **2** and **4**, respectively. In contrast the chemical shifts measured for the glycosidic hydrogens H-1*B'* and H-1*C'* hardly changed with the increasing temperatures and led only to linear decreases Δ(δ)/Δ(T) comprised between 0 and -0.3 ppb.K⁻¹.

Figure 8. ^1H Chemical shift displacements for signals H-1A' (▲), H-1B' (●), H-1C' (■), H-3C (◆) and H-4C (▼) as a function of the temperature increasing from 280 to 330 K. Signal shifts measured for dimLe^x (2) and Le^aLe^x (4) are shown in black and blue, respectively.

Interestingly, the chemical shifts measured for H-3C and H-4C remained relatively inert to temperature variations with linear decreases $\Delta(\delta)/\Delta(T)$ comprised between -0.2 and -0.3 ppb.K⁻¹. Thus the local electronic environment around these hydrogens is not affected by the conformational flexibility around the β -D-GlcNAc-(1→3)-D-Gal glycosidic linkage. Although, additional MD simulations at various temperatures could shed light on the temperature dependence of the H-1A' chemical shift resulting from conformational exchange, such calculations are beyond the scope of this paper. However, the observation that H-1A' in both hexasaccharides underwent significant linear downfield shift with increasing temperatures supports conformational flexibility and exchange around the β -D-GlcNAc-(1→3)-D-Gal glycosidic linkage.

CONCLUSION

We have clearly established that the tumor associated carbohydrate antigens dimLe^x and Le^aLe^x adopt two distinct conformations (I and II) in solution shown on Figure 9. These conformations differ by the value of the torsion angle Ψ_A for the β -D-GlcNAc-(1→3)-D-Gal

glycosidic bond that links the two rigid branched trisaccharides: Le^x to Le^x and Le^a to Le^x in dimLe^x and Le^aLe^x , respectively.

Figure 9. a) and b) Conformations **I** and **II** for dimLe^x , respectively. c) and d) Conformations **I** and **II** for Le^aLe^x , respectively [GlcNAc (A') and Gal (C) ring hydrogens shown in purple defining non-polar surfaces].

These two orientations of the $\Psi_{A'}$ angle are consistent with the orientations Ψ^+ and Ψ^- identified for the same glycosidic bond in the smaller milk oligosaccharides and other structures.^{12e,15,22} However, while the β -D-GlcNAc-(1 $\rightarrow$ 3)-D-Gal glycosidic bond is expected to be flexible in smaller oligosaccharides,^{12e,15,22} the identification of two conformations clearly populated in solution for these large branched hexasaccharides was unexpected. The hydrophobic patch of the β -galactosyl α face defined by H-1C, H-3C, H-4C and H-5C (shown in purple on Figure 9),²³ is known to constitute an important recognition element due to its interaction with aromatic amino acid residues present in anti-carbohydrate antibodies and lectins binding sites.^{17,24} It is interesting to notice that in conformation **I** of both hexasaccharides (see a and c, Figure 9) this patch is extended by the hydrophobic surface defined by H-1, H-3 and H-5 (also shown in purple on Figure 9) of the GlcNAc (A') residue. In contrast, rotation around the β -D-GlcNAc-(1 $\rightarrow$ 3)-D-Gal glycosidic bond in conformation **II** (see b and d, Figure 9) places the GlcNAc (A') hydrophobic face perpendicular to the Gal (C) hydrophobic patch. Thus we

conclude that the two conformations are presenting different internal epitopes to the immune system and will thus possibly trigger two distinct families of antibodies. However, the implications on rationale vaccine design of the conformational flexibility and occurrence of two defined conformations for these tumor associated carbohydrate antigens has yet to be established. Indeed, while the importance of conformational epitopes and epitope presentation is well recognized when attempting to develop carbohydrate-based vaccines against bacterial²⁵ and fungal diseases²⁶ or HIV,²⁷ research has, so far, failed to yield efficient vaccines based on well-defined epitopes. Studies in our laboratory will aim at establishing if any, of these two conformations is immuno-dominant and leads to SH2- and 43-9F-like antibodies that recognize selectively the internal epitopes displayed by dimLe^x and Le^aLe^x on cancer tissues.^{7b,8b,9-10}

EXPERIMENTAL SECTION

Molecular dynamics simulations. The molecular dynamics simulations were carried out using the SANDER module of AMBER10¹⁹ with the inclusion of Glycam06 parameters²⁰ for carbohydrates. The initial structures were built using the XLEAP module of AMBER using the global minimum torsions expected for the Le^x and Le^a trisaccharides¹¹⁻¹² and the known^{11a} global minimum torsions for the disaccharide linkage β -D-GlcNAc(*A'*)-(1 $\rightarrow$ 3)-D-Gal(*C*) disaccharide. The molecules were solvated with 10 shells of TIP3P water molecules²¹ giving a total of 1070 solvent molecules in a 30 Å cubic box. Each simulation was carried out in five stages. First the water molecules were minimized at constant volume (NVT) whereas the oligosaccharides were held fixed. A 1000 cycle limit was employed with 50 cycles of steepest descent followed by conjugate gradient minimization. A 1×10^{-3} kcal.mol⁻¹.Å gradient convergence criteria was used. A 10 Å cut-off was set, and non-bonded updates were made every 10 steps. The dielectric constant was set to 1. Then, the entire systems were minimized without any restraints. A 2500

cycle limit was used with 500 cycles of steepest descent followed by conjugate gradient minimization. Afterward, the systems were heated for 20 ps from 0 to 300 K with the saccharides weakly restrained. Time steps of 2 fs were used and translational momentum was removed every 1000 steps. Bonds involving hydrogens were constrained using the SHAKE algorithm with 0.00001 Å tolerance. Coordinates were output every 250 steps. The systems were then equilibrated at a constant pressure (NPT) of 1 atm for 100 ps with no restraints. Finally, dynamic trajectories production runs of 20 ns were carried out at 300 K. The PTRAJ module of AMBER was used to analyze the results.

Nuclear magnetic resonance spectroscopy. Sample preparation Pure samples of hexasaccharide **2** and **4** were obtained from the Consortium for Functional Glycomics and the synthesis of tetrasaccharide **7** has been reported.²⁸ The purity of all compounds was estimated by ¹H and ¹³C NMR to be greater than 95%. Hexasaccharides **2** (2.4 mg) and **4** (1 mg) were lyophilized three times from D₂O (99.9%) and dissolved in 0.3 mL and 0.2 mL of D₂O (99.96%), respectively. The solutions were then transferred to Shigemi NMR tubes (BMS 005B), the tubes were flushed with nitrogen and carefully sealed with parafilm. Tetrasaccharide **5** (11 mg) was lyophilized three times from D₂O (99.9%) and dissolved in 0.7 mL of D₂O (99.96%). The solution was transferred to a 5 mm NMR tube and the tube was flushed with nitrogen and carefully sealed with parafilm.

NMR Experiments. NMR experiments were recorded at 300 K on a 600 MHz spectrometer equipped with a cryoprobe or using a 800 MHz spectrometer (see below for specific compounds). One-dimensional ROESY spectra were acquired with selective excitations of protons using a 5 s relaxation delay between scans. The optimum irradiation range for each signal was used to determine the best length of the soft pulse applied. This value was

automatically calculated by Bruker software (BUTSEL-NMR). 1D ROESY spectra were recorded using a minimum of five mixing times (up to 10) from 20 ms to 350 ms. Before Fourier transformation, the FIDs were zero-filled once and multiplied with a 1 Hz line broadening factor. Spectra were phase and baseline corrected and integrated. The integrals measured for the irradiated signals were plotted against mixing time and the obtained curves were fitted to a double exponential decaying function:

$$f(\tau_m) = -A[\exp(B\tau_m) + \exp(C\tau_m)]$$

where τ_m is the mixing time and A , B and C are adjustable parameters. The values of these integrals were extrapolated to 0 ms mixing time, and the integrals from cross-relaxation peaks were normalized through division by these extrapolated values. The % normalized cross-relaxation integrals were plotted against the mixing times and the build-up curves were fitted to a double exponential equation of the form:

$$f(\tau_m) = A[\exp(B\tau_m) - \exp(C\tau_m)]$$

and the initial slopes at 0 ms mixing times were determined from the calculated first derivatives:

$$f'(0) = A(B-C)^{\text{ref. 16}}$$

Interproton distances were calculated based on the isolated spin pair approximation (ISPA):

$$r_{ij} = r_{\text{ref}} (S_{\text{ref}}/S_{ij})^{1/6}$$

where S is the initial slope at $\tau_m = 0$, and r is the proton-proton distance.

Hexasaccharide **2**: Selective 1D ROESY were acquired at 800 MHz using 256 scans/experiment and 10 mixing times 20–350 ms for protons H-1B', H-1B, H-1A' overlapping with HDO, H-1C', and H-1C. Evidence of cross-relaxation transfers from H-5B to H-2C and H-5B' to H-2C was obtained from a 2D NOESY experiment using a 300 ms mixing time (see

Figure 2). The intra-residue cross-peaks used as reference for distance determinations in the selective 1D ROESY experiments were: H-1*B*'/H-2*B*' and H-1*B*/H-2*B* (2.42 Å); H-1*A*'/H-5*A*' (2.36 Å); H-1*C*/H-5*C* and H-1*C*'/H-5*C*' (2.42 Å). The known distances H-1*B*'/H-3*B*' and H-1*B*/H-3*B* (3.76 Å) were used to correct the slopes when measuring H-1*B*'/H-3*A*' and H-1*B*/H-3*A*. The R^2 values for the exponential fits were greater than 0.9 for all signals.

Hexasaccharide **4**: Selective 1D ROESY were acquired at 600 MHz (cryoprobe) using 512 scans/experiment and 5 mixing times (40–120 ms) for protons H-1*B*', H-1*B*, H-5*B*', H-5*B*, H-1*C*', H-1*C* and 7 mixing times (40–200 ms) for proton H-1*A*'. The intra-residue cross-peaks used as reference for distance determinations in the selective 1D ROESY experiments were: H-1*B*/H-2*B* and H-1*B*'/H-2*B*' (2.42 Å); H-5*B*'/H-4*B*' and H-5*B*/H-4*B* (2.43 Å); H-1*A*'/H-5*A*' (2.36 Å); H-1*C*'/H-5*C*' and H-1*C*/H-5*C* (2.42 Å). The known distance H-1*A*'/H-3*A*' (2.62 Å) was used to correct the slope when measuring H-1*A*'/H-4*C*. The R^2 values for the exponential fits were all greater than 0.9.

Tetrasaccharide **5**: Selective 1D ROESY were acquired at 600 MHz (cryoprobe) using 128 scans/experiment and 6 mixing times (20–120 ms) for protons H-5*B*, H-1*A*', H-1*C*. The intra-residue cross-peaks used as reference for distance determinations in the selective 1D-ROESY experiments were: H-5*B*/H-4*B* (2.43 Å); H-1*A*'/H-3*A*' (2.62 Å); H-1*C*/H-5*C* (2.42 Å). The R^2 values for the exponential fits were all greater than 0.99.

Variable temperature NMR experiments. ^1H , 1D selective ROESY (irradiation of H-1*A*') and 1D selective TOCSY (irradiation of H-1*C*) experiments were carried out at 600 MHz on samples of hexasaccharide **2** (1.9 mg in 0.6 mL D_2O) and **4** (0.9 mg in 0.25 mL in a Shigemi tube (BMS 005B) at temperatures varying from 280 K and 330 K. The chemical shifts were

referenced to external DSS at each temperature and the temperature was allowed to equilibrate for 30 min between each series of experiments.

ASSOCIATED CONTENT

Supporting Information. Proton chemical shifts for compounds **2**, **4** and **5** in D₂O at 300 K; normalized cross-relaxation buildup curves for signals used as references in distance calculation for dimLe^x (**2**), Le^aLe^x (**4**); NMR data for tetrasaccharide **5**; trajectories of the fucosidic (Φ_B/Ψ_B) and galactosidic (Φ_C/Ψ_C) linkages in the Le^x trisaccharides portions of tetrasaccharide **5** are given in the supporting information. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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ABBREVIATIONS USED

TACA, tumor associated carbohydrate antigen; Le^x, Lewis X; Le^a, Lewis A, dimLe^x, dimeric Lewis X, Le^aLe^x, Lewis A-LewisX; SLC, squamous lung carcinoma; mAbs, monoclonal antibodies.

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