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Synthesis of a BSA-Le^x glycoconjugate and recognition of Le^x analogues by the anti-Le^x monoclonal antibody SH1: The identification of a non-cross reactive analogue

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ABSTRACT

A Le^x trisaccharide functionalized with a cysteamine arm was prepared and this synthesis provided additional information on the reactivity of *N*-acetylglucosamine O-4 acceptors when they are glycosylated with trichloroacetimidate donors activated with excess $BF_3{\cdot}OEt_2$. In turn, this trisaccharide was conjugated to BSA lysine side chains through a squarate–mediated coupling. This BSA-Le^x glycoconjugate displayed 35 Le^x haptens per BSA molecule. The relative affinity of the anti-Le^x monoclonal antibody SH1 for the Le^x antigen and analogues of Le^x in which the D-glucosamine, L-fucose or D-galactose residues were replaced with D-glucose, L-rhamnose and D-glucose, respectively, was measured by competitive ELISA experiments. While all analogues were weaker inhibitors than the Le^x antigen, only the analogue of Le^x in which the galactose residue was replaced by a glucose unit showed no binding to the SH1 mAb. To confirm that the reduced or loss of recognition of the Le^x analogues by the anti-Le^x mAb SH1 did not result from different conformations adopted by the analogues when compared to the native Le^x antigen, we assessed the conformational behavior of all trisaccharides by a combination of stochastic searches and NMR experiments. Our results showed that, indeed, the analogues adopted the same stacked conformation as that identified for the Le^x antigen. The identification of a trisaccharide analogue that does not cross-react with Le^x but still retains the same conformation as Le^x constitutes the first step to the design of a safe anti-cancer vaccine based on the dimeric Le^x tumor associated carbohydrate antigen.

1. Introduction

Aberrant glycosylation in human cancer was first indirectly suggested in the mid-sixties when several papers reported the accumulation of fucose-containing glycolipids in adenocarcinomas.[1] One such glycoplipid displaying the dimeric Le^x hexasaccharide (dimLe^x, Chart 1) was reported to accumulate in colonic and liver adenocarcinoma and it was suggested that additional fucosylation of internal GlcNAc residues in polylactosamine chains was associated with the progression of colorectal cancer.[2–4] However since it was first characterized,[5] the Le^x antigenic determinant or X determinant (β-D-Gal*p*(1→4)[α-L-Fuc*p*(1→3)]-D-GlcNA*cp*) displayed at the non-reducing end of dimLe^x has also been found on numerous normal cells and tissues such as kidney tubules, gastrointestinal epithelial cells, and cells of the spleen and brain.[6] Thus, when attempting to use the Tumor Associated Carbohydrate Antigen (TACA) dimLe^x as a target for anti-cancer vaccine development, an important factor to consider is the possibility that such vaccine would also elicit an immune response against the X determinant that would eventually lead to the destruction of healthy cells. In this context, our research program aims at discovering analogues of the dimLe^x hexasaccharide that would retain internal epitopes displayed on the surface of cancer cells by dimLe^x but no longer possess epitopes associated with the Le^x trisaccharide **A′(B′)C′**. Indeed, the occurrence of such internal epitopes has been repeatedly[2,4] established when monoclonal antibodies against dimLe^x (FH4, SH2) were shown to recognize selectively the dimLe^x and trimLe^x antigens but only weakly bind to the Le^x antigen.[2,4] In contrast anti-Le^x monoclonal antibodies (e.g. FH3, SH1) were clearly shown to also recognize the X determinant when it was displayed at the non-reducing end of dimLe^x.[2,4] In turn, these antibodies have been extensively used to differentiate dimLe^x expressed by colorectal tumor cells from the Le^x antigen expressed on healthy tissues.[2,4] Thus, our goal is to discover dimLe^x analogues that retain the internal epitopes recognized by anti-dimLe^x SH2-like antibodies but no longer possess epitopes recognized by anti-Le^x SH1-like antibodies. We are investigating if hexasaccharides **2**–**4** (Chart 1) in

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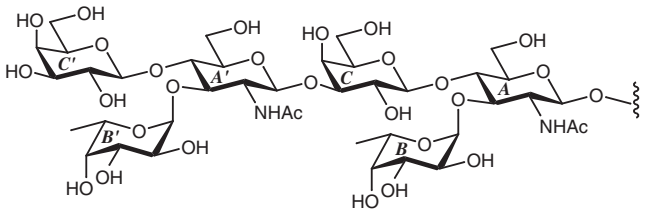
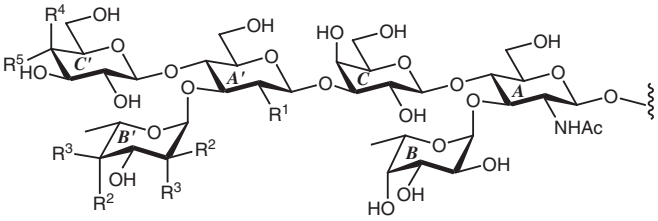


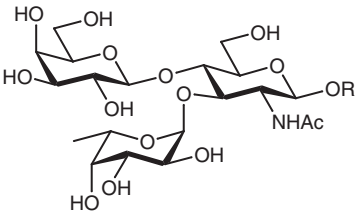
1 DimLe^x



2 $R^1 = R^2 = R^4 = OH$, $R^3 = R^5 = H$
3 $R^1 = NHAc$, $R^2 = R^5 = H$, $R^3 = R^4 = OH$
4 $R^1 = NHAc$, $R^2 = R^5 = OH$, $R^3 = R^4 = H$

Chart 1.

which one of the sugar units **A**′, **B**′ or **C**′ defining the X determinant in dimLe^x (**1**) is replaced by another sugar residue, that is, *N*-acetylglucosamine by glucose (**2**), fucose by rhamnose (**3**), and galactose by glucose (**4**), would meet these requirements. Since our first goal with these analogues is to abolish their cross-reactivity with the Le^x antigen we have first embarked on studying the cross-reactivity of the Le^x-OMe trisaccharide **5** with the Le^x analogue trisaccharides **6**–**9** shown in Chart 2. In analogue **6** *N*-acetylglucosamine is replaced by glucose; in analogue **7** fucose is replaced by rhamnose; in analogue **8** galactose is replaced by glucose and in analogue **9** *N*-acetylglucosamine and fucose are replaced with glucose and rhamnose, respectively. To complete our study of the cross-reactivity of these analogues with the natural Le^x antigen towards binding with the monoclonal antibody SH1,[4] we report here the preparation of a Le^x-BSA glycoconjugate. In turn, the affinity of anti-Le^x mAb SH1 for analogues **5**–**9** was studied by ELISA and a non-cross reactive analogue was identified. Finally, the conformations adopted by the Le^x trisaccharide and the analogues were studied by a combination of NMR and stochastic searches.



10 $R = (CH_2)_3S(CH_2)_2NH_2$
11 R = All

Chart 3.

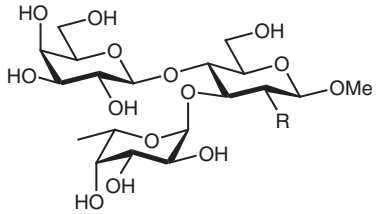
2. Results and discussion

2.1. Synthesis of Le^x-BSA conjugate 23

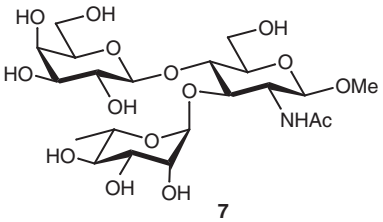
The Le^x-BSA glycoconjugate **23** was obtained through the squarate–mediated[7–10] coupling of trisaccharide **10** to the BSA lysine residues (Chart 3). We describe here the preparation of trisaccharide **10** from the allyl Le^x trisaccharide **11** prepared in our laboratory by Asnani[11] as well as through an alternate synthetic scheme from the individual monosaccharide building blocks.

2.1.1. Synthesis of trisaccharide 10: additional results on the glycosylation at O-4 of *N*-acetylglucosamine glycosyl acceptors

There are numerous reports in the literature that deal with the chemical[12–17] or chemoenzymatic[18] preparation of Le^x analogues as well as that of Le^x intermediate building blocks to be further converted into the Sialyl Le^x tetrasaccharide. It has been established early on by Jacquinet and Sinaÿ[12] as well as by Hindsgaul et al.[13] that the glycosylation at O-4 of *N*-acetylglucosamine in such syntheses was often difficult. Indeed, the majority of these syntheses employed acceptors that carried an *N*-Phth, *N*-TCP, *N*-Troc, azido or oxazolidinone at C-2, and only two reports[16,17] in addition to these early syntheses[12,13] use an *N*-acetylglucosamine acceptor. The first one is that of Roy and co-workers[16] that describes the regioselective glycosylation of an acceptor free at O-3 and O-4, and the second one is our recent synthesis[17] of Le^x in which we have applied conditions (2 equiv $BF_3{\cdot}OEt_2$, 5 equiv donor, rt or 40 °C) that we had found[19–21] to efficiently promote glucosylation at O-4 of *N*-acetylglucosamine. Indeed over the past few years our studies have shown that the success of such glycosylation depended on the nature of the substituent already present



5 R = NHAc (Le^x)
6 R = OH



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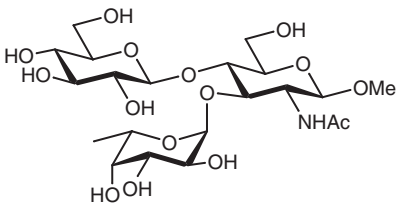

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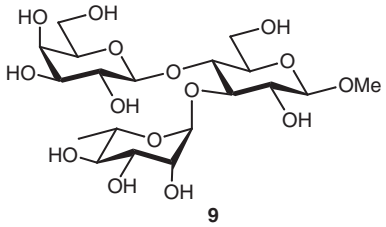


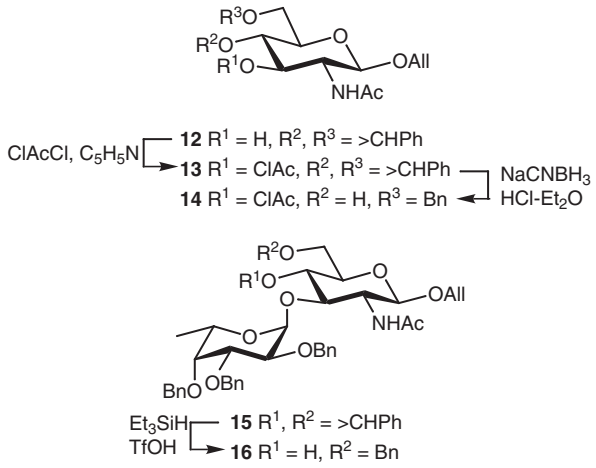
9

Chart 2.

at O-3 in the acceptor[20] but also and more surprisingly[17] on the structure of the aglycone carried by the acceptor. Since our research required the preparation of additional cysteamine adduct **10** we investigated the O-4 galactosylation of *N*-glucosamine glycosyl acceptors carrying an allyl aglycone at C-1. Thus, we report here our results when galactosylating monosaccharide acceptor **14** and disaccharide acceptor **16** which were prepared from the known[22] intermediates **12** and **15**, respectively (Scheme 1). Alcohol **12** was chloroacetylated at O-3 and the benzylidene ring in the resulting intermediate **13** was reductively opened to give acceptor **14** while disaccharide acceptor **16** was obtained through the reductive opening of the benzylidene ring in disaccharide **15**.

Using the reactions conditions that we have previously established[17,19–21] (2 equiv of $BF_3{\cdot}OEt_2$, 40 °C) to glycosylate O-4 of *N*-acetylglucosamine, we then attempted the coupling of monosaccharide **14** with 5 equiv of the known[23] trichloroacetimidate donor **17**. Under these conditions, the reaction proceeded slowly and unreacted acceptor was still present in the reaction mixture after the usual reaction time of 1 h.[17,20] After an extended reaction time, the desired disaccharide **18** was isolated in a disappointing 43% yield. This reaction was difficult to follow by normal phase TLC and our attempts to use reverse phase HPLC to either follow the glycosylation or purify the product were also unsuccessful.

In contrast galactosylation of disaccharide acceptor **16** with donor **17** could easily be followed by RP HPLC and to limit the expected[20] degradation of the product at 40 °C, the reaction was carried out at room temperature. Two HPLC traces at 50 and 100 min of reaction are shown on Figure 1a and b, respectively. As can be seen on Figure 1a most of the acceptor **16** had been converted to the trisaccharide **19** after 50 min of reaction and the presence of two additional products **20** and **21** indicated that degradation was already taking place. Indeed, at 100 min of reaction most of the trisaccharide product **19** had disappeared and two new products were now clearly present in the reaction mixture. After additional 40 min at 40 °C, the reaction was quenched and three products were separated by flash chromatography (Scheme 2). The two compounds seen by RP HPLC were identified as the known[24] fucose hemiacetal **21** (15% yield) and the endo/exo mixture of orthoester **20** (60% yield). The third product which eluted with the solvent front in the RP HPLC was identified as disaccharide **22** (25% yield). The major isomer present in compound **20** (75%) was assumed to be the less sterically hindered exo orthoester and presented in the ^{1}H NMR spectrum a characteristic signal at 1.74 ppm corresponding to the methyl orthoacetate.[25] In addition, the galactosyl H-2 signal was found at



Scheme 1.

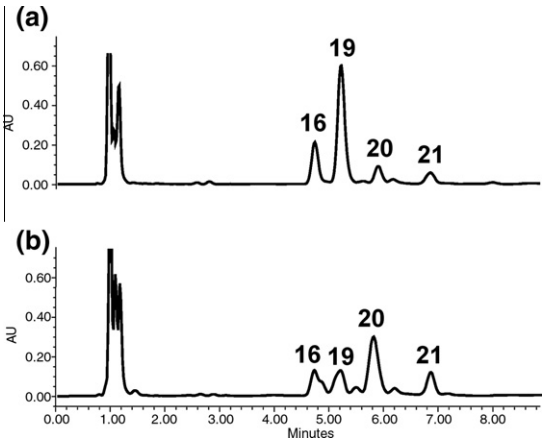


Figure 1. HPLC trace after (a) 50 min and (b) 100 min of reaction for the glycosylation of disaccharide acceptor **16** with donor **17** (5 equiv) activated with 2 equiv of $BF_3{\cdot}OEt_2$ at rt.

4.05 ppm which was, as expected, considerably up field from the signal that is typically found for this hydrogen if the galactosyl residue is acetylated at C-2 (see for instance H-2′ in disaccharide **22** found at ~5.2 ppm). When the reaction was quenched after 40 min the desired trisaccharide **19** was obtained in 64% yield.

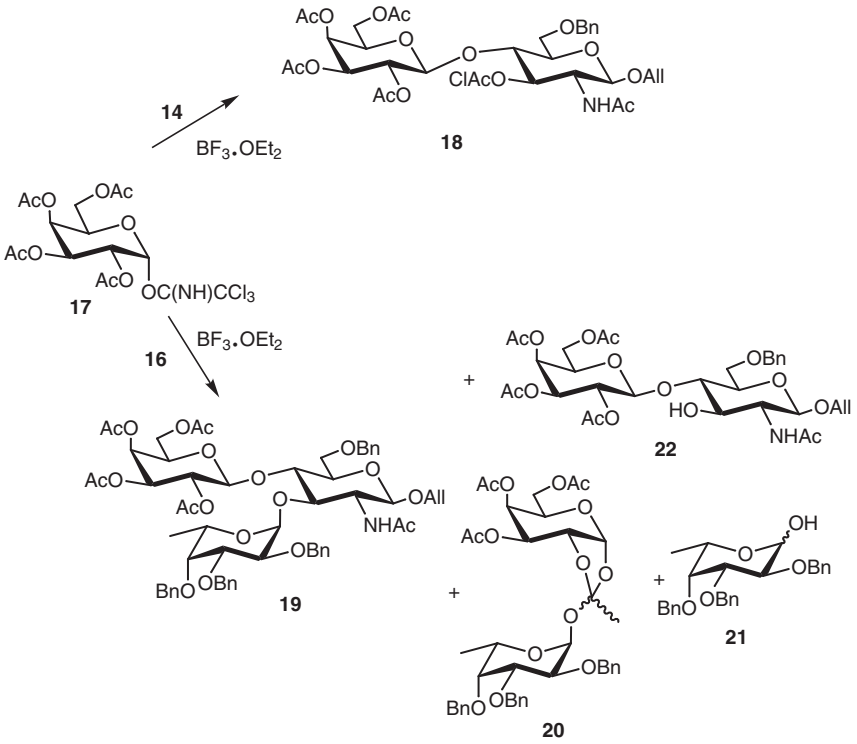
The protected trisaccharide **19** was then converted to the cysteamine derivative **10** in two steps: the UV-promoted addition of 2-aminoethanethiol hydrochloride to allyl glycoside **19** followed by the removal of all protecting groups under dissolving metal conditions [$Na/NH_3(l)$] (Scheme 3).[17] The resulting trisaccharide **10** was purified by gel permeation chromatography and was obtained pure as the acetate salt in 73% yield after lyophilization from water. The cysteamine adduct **10** was also prepared via the UV-promoted addition of 2-aminoethanethiol hydrochloride[8] to allyl glycoside **11**.[11]

2.1.2. Squarate mediated coupling to BSA

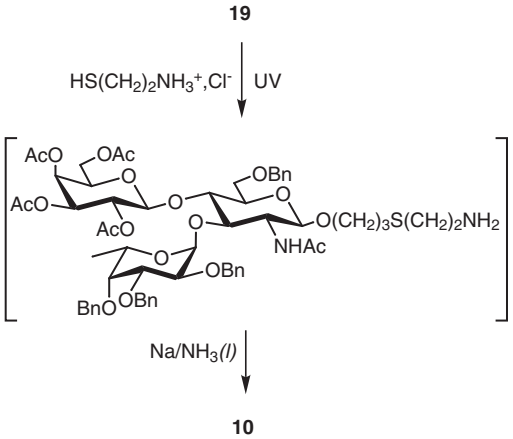
Following the well established squarate mediated conjugation method[7–10] glycoconjugate **23** was prepared in two steps from the cysteamine adduct **10** (Scheme 4). Trisaccharide **10** was dissolved in methanol, allowed to react with diethylsquarate at room temperature and when TLC showed its complete conversion to the less polar squarate derivative the solvent was evaporated. The squarate adduct (51 equiv) was allowed to react at a 30 mM hapten concentration with the lysine side chains of BSA (Sigma Cat. No. A4378) in a pH 10 (0.1 M) carbonate buffer for 7 days at room temperature. The resulting glycoconjugate was dialyzed against water and the average level of Le^x hapten incorporation per BSA molecule was determined by the Dubois[26] colorimetric assay to be of 35 ± 3. This loading corresponds to a 70% coupling yield (based on the hapten) and a 59% occupancy level of the 59 lysine residues present in the protein.[27] This result is in excellent agreement with results obtained by Kováč and co-workers[10] in their recent thorough study of this coupling method.

2.2. Competitive binding experiments with the Le^x analogues 5–9

The relative affinity of the anti-Le^x monoclonal antibody SH1[4] for the Le^x antigen **5**[15] and analogues (**6–9**)[15,19] was measured by competitive ELISA experiments using glycoconjugate **23** as the immobilized ligand.[28] Figure 2 shows the inhibition curves of mAb SH1 for each of the soluble ligands **5–9** that were fitted to a four parameters logistic sigmoidal equation.[29] The concentration of each inhibitor required for 50% inhibition (IC_{50}) and the



Scheme 2.

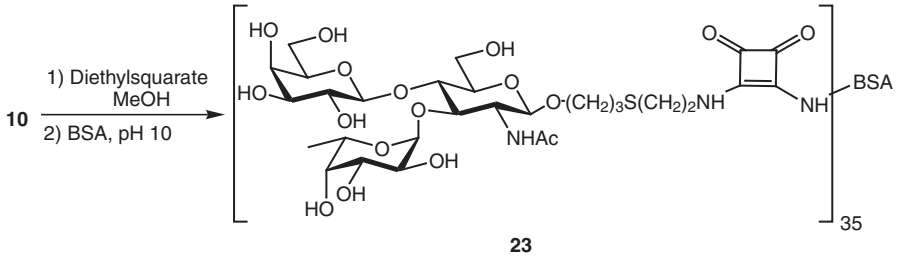


Scheme 3.

corresponding changes in free energy $\Delta(\Delta G)$ of binding referenced to the Le^x soluble antigen **5** are given in Table 1. As can be seen from these results all analogues **6**–**9** were weaker inhibitors than the natural Le^x antigen. Replacing the glucosamine unit by a glucose residue (entry 2) resulted in only a small decrease in free energy of binding to SH1 while replacing the fucose residue by a rhamnose unit (entry 3) led to a change in free energy of binding consistent with the loss of a key polar interaction to the SH1 combining site.[30] A cumulative effect was observed when both the *N*-acetylglucosamine and fucose sugars were substituted by glucose and rhamnose, respectively (entry 4) but cross-reactivity with the native antigen was nonetheless still observed. In sharp contrast, analogue **8** in which the galactose residue is replaced by a glucose unit, showed no binding to the SH1 mAb even at high concentration (entry 5). This result suggests that the non-reducing end galactose residue is essential to conserve cross-reactivity with the natural Le^x antigen and that modification of this residue and particularly at O-4 may totally abolish this cross-reactivity. To confirm that the loss of recognition of analogue **8** by the anti-Le^x mAb SH1 did not result from different conformations adopted by analogue **8** when compared to the native Le^x antigen we embarked on the conformational analysis of analogue **8** as well as that of compounds **5**–**7** and **9**.

2.3. Conformational analysis on trisaccharide analogues 5–9

In a previous paper,[31] we have described the results of biased stochastic conformational searches on reducing analogues of trisaccharides **5**–**8** using an early version of the Molecular Operating



Scheme 4.

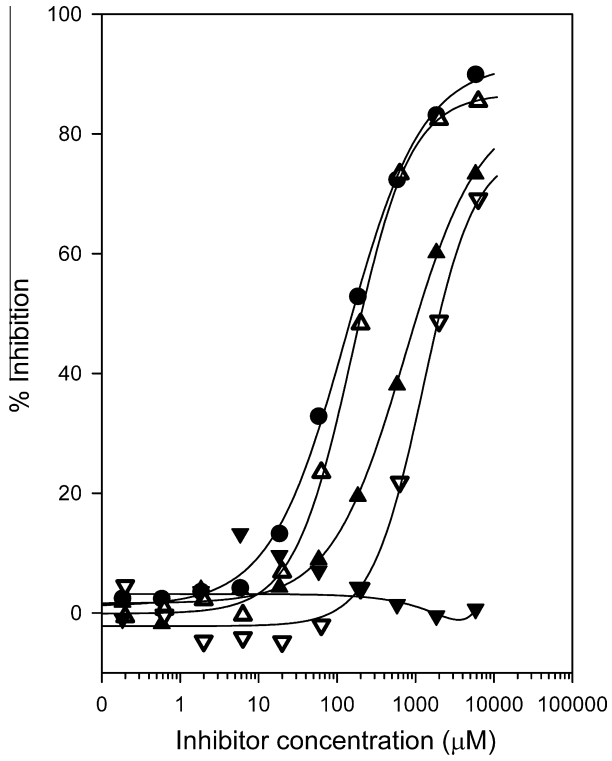


Figure 2. Inhibition curves for mAb SH1 using glycoconjugate **23** as immobilized antigen and analogues **5**–**9** as soluble competitive inhibitors: **5** (●), **6** (△), **7** (▲), **8** (▼), **9** (▽).

Table 1
Inhibition data for MAb SH1 with analogues **5**–**9**

Entry	Inhibitor	IC50[a] (μM)	$\Delta(\Delta G)$[b] (kcal mol^{-1})
1	**5**	156	0
2	**6**	204	0.2 ± 2%
3	**7**	1068	1.1 ± 4%
4	**9**	2037	1.5 ± 3%
5	**8**	≫5800	—[c]

[a] Concentration of inhibitor required for 50% inhibition using solid phase glycoconjugate **23** as immobilized antigen.

[b] Values determined from the expression $\Delta(\Delta G) = rt\ \ln([I_1]/[I_2]$ where $[I_2]$ is the IC_{50} measured for the reference inhibitor analogue **5** and $[I_1]$ the IC_{50} measured for each analogue **6**–**9** with $R = 1.98$ cal K^{-1}and $T = 296$ K.

[c] No inhibition observed at 5800 μM.

Environment program suite (MOE2001)[32] which offered a limited choice of force fields. In that study the MMFF94 force field[33] and the Generalized Born/Surface Area continuum solvation model[34] (GB/SA) were used to identify preferred conformations adopted by the Le^x trisaccharide. The global minima that were identified for the reducing trisaccharide Le^x and for the analogues in which the glucosamine or galactosyl unit were replaced by glucose were in agreement with the preferred conformation reported in the literature for the Le^x antigen.[35–38] In contrast, these preliminary results suggested that the Le^x analogue in which the fucose residue was replaced by a rhamnose unit was adopting a minimum energy conformation drastically different from that adopted by the Le^x trisaccharide.[31] Having now synthesized analogues **5**–**9**[15,19] and received a version of MOE (MOE2005) that implements the AMBER94 force field[39] known to be efficient for carbohydrate modeling,[40] we performed new stochastic searches on analogues **5**–**9** and supported these results by NMR experiments. Since the different orientations around the glycosidic linkages have the most substantial impact on the overall molecular shape of oligosaccharides,[41] we focused our attention on the conformational features associated with these linkages in the trisaccharides **5**–**9**. The orientations adopted around the glycosidic linkages are described below by the dihedral angles: Φ = O5–C1–O1–Cx and Ψ = 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.[42]

2.3.1. Stochastic searches: conformational families of trisaccharides 5–9

In these stochastic searches, minima in the potential energy surface are randomly sampled through the rotation of all bonds (including ring bonds) to random dihedral angles as well as through a random 0.4 Å Cartesian perturbation of atom positions. For each analogues **5**–**9**, two searches of 150,000 iterations were carried out to generate new conformers that were each minimized using the AMBER94 force field[39] with implicit solvation by the GB/SA continuum model.[34] In the first search a RMS tolerance of 0.01 Å on heavy atoms was used to determine whether generated conformations were considered as new. In the second search a RMS tolerance of 0.1 Å was applied on all atoms including all hydrogens atoms to account for possible hydrogen bonding. For each compound, the databases containing the conformations selected by each search were combined and only those conformations found within 10 kcal mol^{-1} from the global minima were retained. In turn we focused our attention on those conformations found within 3 kcal mol^{-1} from the global minima since these conformations may be populated in solution. As shown in Table 2, the searches yielded between 836 and 1865 conformations within 10 kcal mol^{-1} from the global minima but the numbers of conformations found within 3 kcal mol^{-1} of the global minima were considerably smaller from 36 conformations for analogue **7** to 100 for analogue **6**.

These conformations clustered around well-defined values of the glycosidic torsions that fell into 4 distinct families which are represented by the overlaid lowest energy conformations found for each family shown on Figure 3a (more detailed results of the stochastic searches are given in the Supplementary data). As can be seen on Figure 3a and in Table 3 (entry 1), the same global minimum (**A**) was found for all analogues. This conformation corresponds to the global minimum identified by numerous modeling studies of the Le^x trisaccharide[35–38] and which was experimentally supported by NMR[36,37] and X-ray crystallography.[38] For all analogues (**5**–**9**) this stacked conformation was in fact the most represented conformation in the final databases (Fig. 3b). Indeed, no other conformation was found for analogue **9** while 98% of the conformations found for analogue **6** clustered around these glycosidic torsions (Fig. 3b). The first and second local minima (Fig. 3a, **B** and **C**) found for the Le^x analogue **5** were also represented in the databases obtained for analogues **7** and **8** but were not found for analogues **6** or **9**. Finally, a third local minimum (Fig. 3a, **D**) was found for analogues **6** and **8** which was not represented in the databases of analogues **5**, **7**, and **9**. The energy as well as glycosidic torsions measured for the lowest energy representatives of each family (**B**–**D**) and for each analogue (**5**–**9**) are given in the Supplementary data. Shown Table 3 (entries 2–6) are the Boltzman-averaged

Table 2
Number of conformations found within 10 and 3 kcal mol^{-1} from the global minima

Analogue	10 kcal mol^{-1}	3 kcal mol^{-1}
5	1318	56
6	1646	100
7	1524	36
8	836	61
9	1865	52

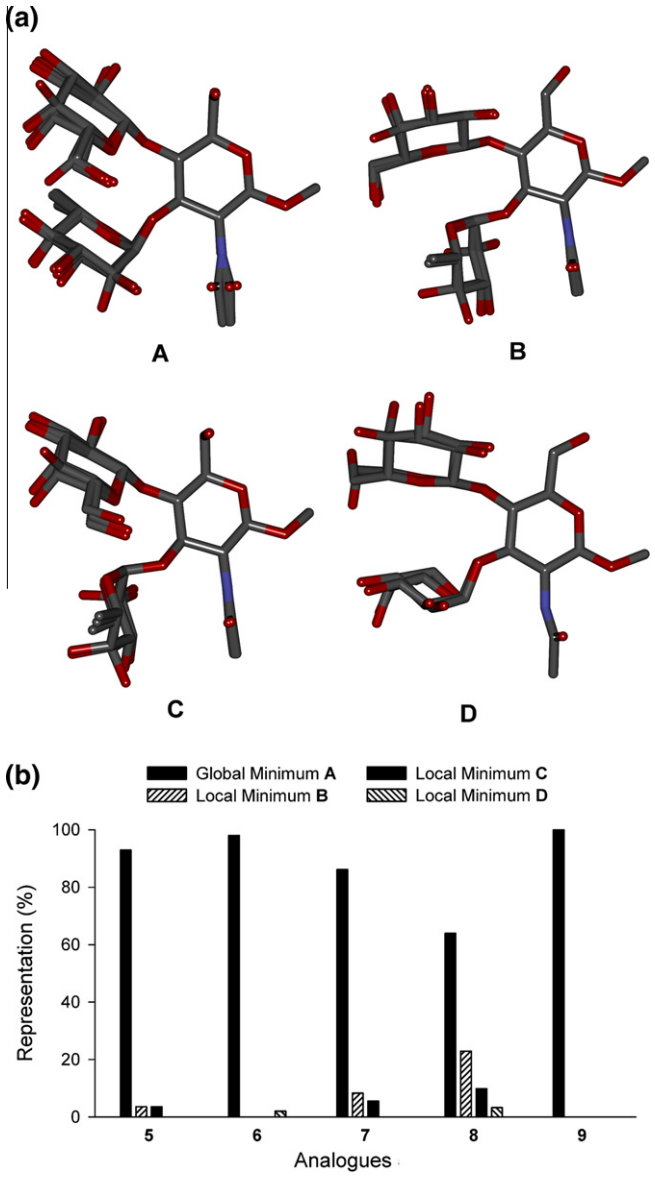


Figure 3. (a) Overlaid global (**A**) and local (**B**–**C**) minima identified in the stochastic searches for compounds **5**–**9**. (b) Representation (%) of each conformation **A**–**D** in the databases (3 kcal mol^{-1}) calculated for analogue **5**–**9**.

Table 3
Calculated torsion angles[a]

Entry		Φ^1	ψ^1	Φ^2	ψ^2
Global minimum[b]					
1	**A**	−72 ± 4[c]	148 ± 3[c]	−72 ± 5[c]	−107 ± 3[c]
Boltzman average[d]					
2	**5** (Le^x)	−71	148	−69	−111
3	**6**	−70	150	−70	−110
4	**7**	−75	145	−74	−108
5	**8**	−75	145	−77	−110
6	**9**	−63	155	−72	−109

[a] Φ^1, ψ^1 = α-L-Fuc/Rha-(1→3)-GlcNAc/Glc; Φ^2, ψ^2 = β-D-Gal/Glc-(1→4)-GlcNAc/Glc.
[b] Torsions calculated for global minimum **A** averaged on all analogues.
[c] Standard deviation amongst the analogues.
[d] Boltzman-averaged torsions on the 3 kcal mol^{-1} databases for each analogue.

values for the Φ^1, ψ^1 and Φ^2, ψ^2 angles measured respectively for the α-L-Fuc/Rha-(1→3)-GlcNAc/Glc and β-D-Gal/Glc-(1→4)-GlcNAc/Glc glycosidic bonds for the five analogues. In addition, Table 4 gives for each analogue (entries 2–6) the Boltzman-averaged inter-proton distances: H-1′ to H-3, H-1″ to H-4, H-1″ to H-6a and H-6b, H-5′ to H-2″, that are typically short in the stacked minimum energy conformation[35–38] (entry 1) and have been supported experimentally for the Le^x antigen.[36–38] As can be seen, these values are essentially identical to the average torsions and distances measured in the global minimum (Tables 3 and 4, entry 1) common to all analogues. Thus, it is clear that the stacked conformation (**A**) previously identified as the rigid global minimum adopted by the Le^x antigen is also the preferred conformation adopted by all our analogues **6**–**9**. Indeed, although other conformations were fairly well represented in the database obtained for analogue **8** (Fig. 3b) the Boltzman-averaged values of the glycosidic torsions and distances given in Tables 3 and 4 (entry 5) show that the energetically most favored conformation is the global minimum. Thus, the results obtained in these stochastic searches confirm, as we had previously found,[31] that replacing the *N*-acetyl-D-glucosamine or D-galactose unit by a D-glucose residue in the Le^x trisaccharide does not lead to a change in the conformation. In contrast our observation here that replacing the L-fucose residue with an L-rhamnose unit did not either lead to a different conformation than the Le^x antigen contradicts our original findings using the MMFF94 force field.[31] This difference is likely resulting from the different parameterizations of the force fields involved. To confirm that indeed the analogues **6**–**9** adopt the same conformation than the Le^x antigen **5** the conformational behavior of compounds **5**–**9** was assessed by NMR experiments.

2.3.2. NMR measurements

The ^{1}H chemical shift assignments for trisaccharides **5–9** measured at 300 K in D_2O are reported in the Supplementary data. The vicinal coupling constants measured for the three sugar units supported an average $^4C_1$ conformation for the galactose and *N*-acetylglucosamine rings and a $^1C_4$ conformation of the fucose and rhamnose rings and have been reported.[15,19] Inter-residue NOE interactions were evaluated using 1D ROESY experiments at 300 K selectively irradiating H-1′, H-5′, and H-1″ for each analogues. Since we are particularly interested in the conformational behavior of analogue **8** that is not recognized by the mAb SH1, we show here in Figure 4a the ^{1}H NMR spectrum and the ^{1}H, ^{1}H ROESY spectra (mixing time 200 ms) upon selective irradiation of protons H-1′, H-5′, and H-1″ for this analogue. The ^{1}H NMR and ^{1}H, ^{1}H ROESY spectra obtained for analogue **5**–**7** and **9** are given in the Supplementary data. For all analogues, the expected intra-residue cross-relaxation signals were evident: cross-relaxation signals were observed for the glycosidic linkages between H-1′ and H-3 as well as between H-1″ and H-4; cross-relaxation signals were observed from H-1″ to H-6a and H-6b; and cross-relaxation signals between H-5′ and H-2″ were also seen in all spectra. Although for the rhamnosylated analogues **7** and **9**, H-1″ and H-5′ gave overlapping signals, the intensity of the cross-relaxation signal to H-2″ when both H-1″ and H-5′ were irradiated together was clearly indicative of a close proximity of H-5′ and H-2″. Cross-relaxation buildup curves were obtained for those isolated signals over 9–10 mixing times ranging from 20 to 400 ms. The normalized buildup curves obtained for analogue **8** are given here Figure 4b, while those obtained for analogue **5**–**7** and **9** are given in the Supplementary data. The normalized cross relaxation buildup curves (Fig. 3) were fitted to a double exponential equation and the slopes at initial buildup were calculated and used to evaluate the inter-proton distances using the isolated spin pair approximation.[43] In all cases the R^2 values for the exponential fits were at least 0.99. The measured distances are given in Table 4, entries 7–11.

As can be seen these experimental distances are in very good agreement with the results obtained for all analogues in the stochastic searches. It is therefore clear that all analogues adopt

Table 4
Calculated and measured inter-proton distances

Entry		H-1′/H-3 (Å)	H-1″/H-4 (Å)	H-1″/H-6a (Å)	H-1″/H-6b (Å)	H-5′/H-2″ (Å)
Lowest energy confomers[a]						
1	**A**	2.5	2.4	2.4	3.8	2.7
Boltzman average[b]						
2	**5**	2.6	2.4	2.9	2.9	2.6
3	**6**	2.6	2.4	3.1	3.1	2.6
4	**7**	2.5	2.3	2.6	3.3	2.9
5	**8**	2.6	2.4	2.8	3.5	3.0
6	**9**	2.8	2.3	3.0	3.1	2.8
NMR measurements						
7	**5**	ND[c]	2.4	2.5	2.7	2.7
8	**6**	2.6[d]	ND[c]	ND[c]	2.6	2.7
9	**7**	ND	2.3	2.7[d]	2.7	2.8[d]
10	**8**	ND	2.4	2.4	2.7	2.7
11	**9**	2.5	ND[c]	ND[c]	2.7	2.8[d]

[a] Distances calculated for global minimum **A** averaged on all analogues.
[b] Boltzman-averaged distances on the 3 kcal mol^{-1} databases for each analogue.
[c] Signal overlap did not allow distance calculation.
[d] Signals were corrected as they overlapped with cross relaxation signals generated from known short inter-proton distances.

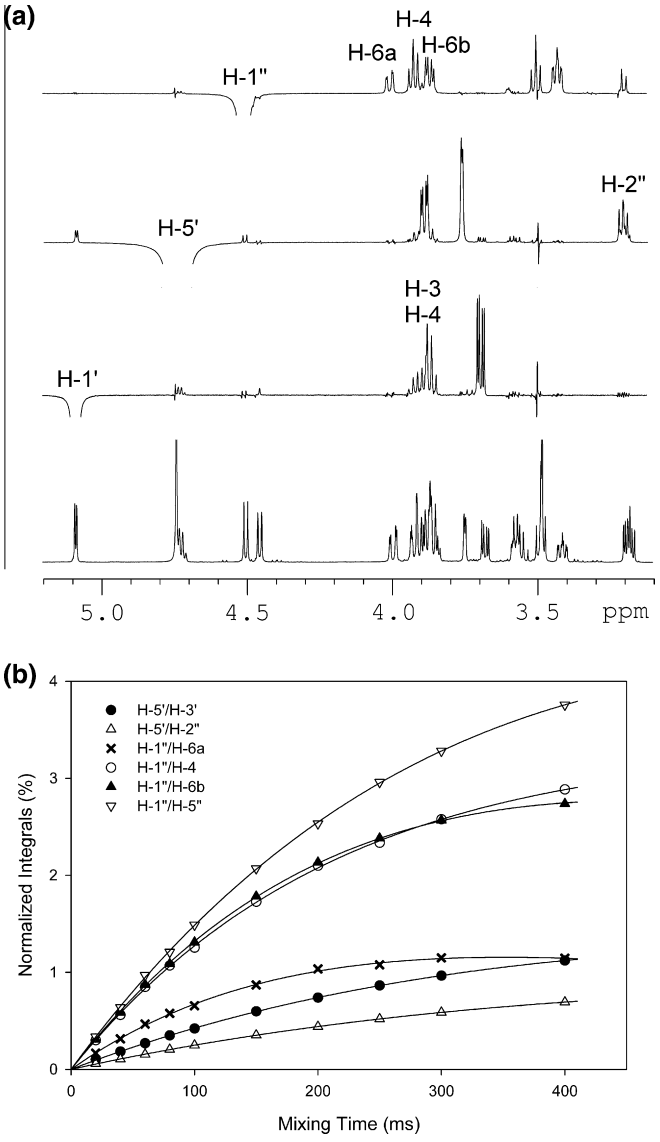


Figure 4. (a) ^{1}H NMR spectrum (600 MHz, 300 K) of trisaccharide **8** and ^{1}H, ^{1}H ROESY spectra (mixing time 200 ms) upon selective excitation of proton H-1, H-5′, and H-1″. (b) ^{1}H, ^{1}H ROESY cross-relaxation buildup curves for trisaccharide **8**.

a similar conformation in solution which corresponds to the stacked conformation already known for the Le^x antigen.[35–38]

3. Conclusions

From a synthetic standpoint, the results presented here for the preparation of trisaccharide **10** support once again that the outcome of galactosylations at O-4 of *N*-acetylglucosamine acceptors depend on the structure of the acceptor. Together with our published results[17,20,21] this study indicates that glycosylations of monosaccharide acceptors using trichloroacetimidate donors activated with 2 equiv of $BF_3{\cdot}OEt_2$ are successful (~70%) when the acceptor carries a methyl or 6-chlorohexyl aglycone. In contrast glycosylation of monosaccharides carrying an allyl and 6-azidohexyl aglycone are less successful giving yields of 30–40%. In addition, while such activation conditions have proven useful to achieve glycosylation at O-4 of *N*-acetylglucosamine in good yields, it is important to recognize that they may lead to the degradation of the desired product when the acceptor carries an α-linked sugar unit at O-3.[20] Indeed, the results presented here show that the galactosylation of the allyl glycoside disaccharide acceptor **16** was faster than the glucosylation of the analogous methyl glycoside disaccharide acceptor[20] but that, concurrently, the degradation of the acceptor and/or trisaccharide also occurred at a faster rate.

We have also prepared a BSA-Le^x glycoconjugate and used it as the immobilized ligand to measure the relative affinity of the anti-Le^x mAb SH1[8] for the Le^x antigen **5**[15] and analogues (**6–9**)[15,19] by competitive ELISA. Our results showed that all analogues were weaker inhibitors of the anti-Le^x mAb SH1 than the natural antigen. To evaluate whether conformational differences between the Le^x analogues and Le^x could induce these drops in affinity to the SH1 mAb, the conformational behavior of analogues **6–9** was compared to that of the natural Le^x trisaccharide **5** by a combination of stochastic searches and NMR experiments. Our results indicate that all analogues adopt as a preferred conformation the stacked conformation that has been identified for the Le^x trisaccharide.[35–38] Therefore, it is clear that the lower binding of analogues **6–9** to mAb SH1 when compared to the natural antigen Le^x does not result from changes in conformational behavior of the analogues. While replacing the glucosamine unit by a glucose residue or the fucose residue by a rhamnose unit led to analogues having lower affinity for the SH1 combining site, some cross-reactivity with the native antigen Le^x was nonetheless still retained.

Therefore, it is likely that vaccine candidates **2** or **3** that display analogues of Le^x at their reducing end in which the glucosamine and fucose residues are replaced by a glucose and a rhamnose unit, respectively, may still induce the production of antibodies cross-reacting with the Le^x antigen.

In sharp contrast, analogue **8** in which the galactose residue is replaced by a glucose unit, showed no binding to the SH1 mAb even at high concentration. The reason for this observed loss of binding is unknown at this time. Indeed, it has been well-recognized in the past two decades that binding affinities between proteins and carbohydrates resulted from a combination of factors.[30,44,45] These include the formation of direct or water-mediated hydrogen bonds between the ligand and amino acid residues in the combining site, the favorable interactions between non-polar amino acid residues and hydrophobic patches displayed by the ligand, as well as the favorable displacement of high energy water molecules from the combining site. Thus binding affinities result from a combination of enthalpic, entropic and solvation effects and are often the result of favorable enthalpic contributions that are offset by unfavorable entropic contributions.[30,44,45] It is interesting to point out that replacing the galactose residue by a glucose residue in analogue **8** brought a polar hydroxyl group within the α-face of what used to be the exposed hydrophobic patch of galactose[44] (see conformation A, Fig. 3a). Thus we may speculate that the presence of this hydroxyl group disturbs favorable hydrophobic interactions between the ligand and the combining site of mAb SH1 and leads to this loss of binding. However, one may also speculate that either a difference in solvation of analogue **8** versus Le^x, or the loss of a strong H bond involving the axial 4″-OH group in Le^x contribute to the lack of recognition of analogue **8** by mAb SH1. Thus, only additional studies with 4″-deoxy, 4″-fluoro, or 4″-methyloxy Le^x analogue inhibitors will provide a better understanding of these phenomena[30] and these analogues are currently being synthesized in our group. Nevertheless based on the loss of recognition of analogue **8** by mAb SH1, we can postulate that a vaccine candidate displaying an analogue of dimeric Lewis X such as hexasaccharide **4** in which the non-reducing end galactosyl residue is replaced by a glucose unit would no longer trigger the production of antibodies that cross-react with Le^x. Most importantly since the galactose residue and particularly the 4″-OH are not likely involved in the internal epitopes displayed by the TACA dimLe^x, we may expect that this vaccine candidate will trigger the production of antibodies that are cross-reactive with the natural TACA dimLe^x. We are currently preparing a glycoconjugate that displays the hexasaccharide **4** conjugated to an immunostimulant protein. This glycoconjugate will be used to immunize mice and the polyclonal serum will be tested for its cross-reactivity with the dimLe^x TACA. Since it is known that polymeric Le^x antigens can trigger the production of antibodies against internal epitopes,[2,4,46] we expect that our vaccine candidate displaying hexasaccharide **4** will trigger the production of antibodies that recognize the TACA dimLe^x while they do not bind to the Le^x trisaccharide. The results described in this paper place us one step closer to the design of a safe anti-cancer vaccine based on the TACA dimLe^x though a considerable amount of research remains to be carried out.

4. Experimental procedures

4.1. Synthesis of Le^x-BSA conjugate

4.1.1. Materials and methods

^1H NMR (300.13, 400.13 and 600.13 MHz) and ^{13}C NMR (75.5, 100.6 and 150.9 MHz) spectra were recorded in $CDCl_3$ (internal standard, for ^1H residual $CHCl_3$ δ 7.24; for ^{13}C: $CDCl_3$ δ 77.0), CD_3OD (internal standard, for ^1H residual CH_3OD δ 3.30; for ^{13}C: CD_3OD δ 49.0) or D_2O (external standard 3-(trimethylsilyl)-propionic acid-d_4, sodium salt (TSP) for ^1H δ 0.00, for ^{13}C δ 0.00). ^1H NMR and ^{13}C NMR chemical shifts are reported in parts per million (ppm). Coupling constants (J) are reported in hertz (Hz). Chemical shifts and coupling constants were obtained from a first-order analysis of one-dimensional spectra. Assignments of proton and carbon resonances were based on two dimensional ^1H–^1H COSY and ^{13}C–^1H HSQC correlation experiments. Multiplicities are abbreviated as follows: singlet (s), doublet (d), triplet (t), quartet (q) and multiplet (m). Analytical thin-layer chromatography (TLC) was performed using silica gel 60 F_{254} precoated plates (250 μm), were visualized under UV and/or charring with 10% H_2SO_4 in EtOH. Flash chromatography was performed using silica gel 60 (230–400 mesh) from EM science. All reactions were carried out under nitrogen atmosphere with anhyd freshly distilled solvents unless otherwise noted. Solvents were distilled and dried according to standard procedures,[47] and organic solutions were dried over Na_2SO_4 and concentrated under reduced pressure. Molecular sieves were activated by flame drying under reduced pressure. All reagents were purchased from commercial suppliers and used without further purification unless otherwise noted. Reverse-phase HPLC purification were carried out on Prep Nova Pak® HR C18, 6 μm 60 Å (8 × 100 mm) column using mixtures of acetonitrile and water as eluent. Purifications by gel permeation chromatography were carried out on a Biogel P2 column (95 cm × 1.5 cm) eluted with water unless otherwise stated. UV irradiation was accomplished with an Ace Glass Incorporated Hanovia photochemical (medium pressure) quartz mercury-vapor lamp (approx. 40–48% of the total energy radiated is in the UV portion of the spectrum) unless otherwise noted. One time crystallized BSA (fatty acid-free, ⩾97% by agarose gel electrophoresis) was purchased as lyophilized powder from Sigma; catalogue number: A4378. For Dubois tests, absorbance values were read at 490 nm employing a Biomate 5 Thermo Electron Corporation plate reader. Optical rotations were measured on a Rudolph Research Autopol III automatic polarimeter at 298 K and are given in deg mL dm^{-1} g^{-1}. High-resolution electrospray ionization mass spectra (HRESIMS) were recorded by the analytical services of the McMaster Regional Center for Mass Spectrometry, Hamilton, Ontario.

4.1.2. 3-(2-Aminoethylthio)propyl 2-acetamido-2-deoxy-3-*O*-(α-L-fucopyranosyl)-4-*O*-(β-D-galactopyranosyl)-β-D-glucopyranoside (10)

4.1.2.1. From protected trisaccharide 19. MeOH (900 μL) and 2-aminoethanethiol hydrochloride (110 mg, 0.97 mmol, 49 equiv) were added to a solution of the allyl glycoside **19** (22 mg, 0.020 mmol) dissolved in a minimum amount of CH_2Cl_2 (250 μL) and placed into a quartz tube. The mixture was stirred until full dissolution and irradiated by UV light (Rayonet photochemical reactor) for 50 min. The solution was then diluted with CH_2Cl_2 (50 mL) and washed with 1 M NaOH (5 × 50 mL). The aqueous layers were re-extracted with CH_2Cl_2 (50 mL) and the organic layers were combined, dried and concentrated to give the crude cysteamine Le^x adduct. A solution of this adduct in a mixture of THF (1.5 mL) and EtOH (1.5 mL) was added at −78 °C to liquid NH_3 (~15 mL), and Na (70 mg, 3.0 mmol, 152 equiv) was added to the resulting mixture. After 50 min at −78 °C, the reaction was quenched with MeOH (2 mL), the ammonia allowed to evaporate at rt and the remaining solvent evaporated. The resulting solid was dissolved in water (5 mL) and neutralized with acetic acid (180 μL, 3.0 mmol, 1 equiv to Na). After freeze-drying the crude trisaccharide was passed twice through a Biogel P2 column eluted with 0.05 M ammonium acetate. The fractions containing trisaccharide **10** were combined, freeze-dried, concentrated from MeOH (4 × 10 mL), and freeze dried again from distilled water

(2 × 10 mL) to remove the ammonium acetate. The pure acetate salt of cysteamine Le^x **10** (9.4 mg, 73% over two steps) was obtained as a white amorphous solid.

4.1.2.2. From Le^{x}OAll trisaccharide 11. An aq solution of cysteamine hydrochloride (7.5 M, 75 μL, 57 equiv) was added to a solution of allyl glycoside **11** (6.1 mg, 0.011 mmol)[11] in water (125 μL) and placed in a quartz tube. The mixture was irradiated under UV light for 90 min, diluted with aq ammonium acetate (0.25 M, 250 μL) and submitted to gel permeation chromatography on a Biogel P2 column eluted with 0.25 M ammonium acetate. The fractions containing trisaccharide **10** were combined and after freeze-drying the crude adduct was dissolved in distilled water and de-ionized with Dowex HO$^-$ resin. After filtration and freeze-drying, compound **10** (4 mg, 58%) was obtained pure as a white amorphous powder.

4.1.2.3. Analytical data for 10. $[\alpha]_D$ = +31.2 (*c* 0.5, MeOH). ^{1}H NMR (400 MHz, CD_3OD, 295 K) δ 5.03 (d, 1H, *J* = 3.9 Hz, C-1′); 4.86–4.79 (m, 1H, H-5′); 4.47–4.40 (m, 2H, H-1″, H-1); 4.01–3.82 (m, 7H, H-2, H-4, H-6, H-3′, H-4″, OC*H*H); 3.82–3.67 (m, 4H, H-4′, H-3″, H-6″); 3.66–3.55 (m, 2H, H-2′, OCH*H*); 3.53–3.39 (m, 4H, H-3, H-5, H-2″, H-5″); 3.06 (m, 2H, SCH_2CH$_2$N); 2.76 (m, 2H, SCH$_2$CH_2N); 2.63 (m, 2H, OCH$_2$CH$_2$CH_2S); 1.97 (s, 3H, *N*-acetate); 1.83 (m, 2H, OCH$_2$CH_2CH$_2$S); 1.18 (d, 3H, *J* = 6.5 Hz, H-6′). ^{13}C NMR (100 MHz, CD_3OD, 295 K): 173.7 (C=O); 103.8 (C-1″); 102.5 (C-1); 100.2 (C-1′); 77.4 (C-5); 76.7 (C-5″); 76.5 (C-4′); 75.0 (C-4); 74.9 (C-3); 73.7 (C-4′); 72.7 (C-2″); 71.2 (C-3′); 69.9 (C-2″); 69.9 (C-3″); 68.6 (OCH$_2$); 67.7 (C-5′); 62.8 (C-6″); 61.3 (C-6); 57.4 (C-2) 39.7 (SCH$_2$CH$_2$N); 30.4 (OCH$_2$CH$_2$CH$_2$S); 29.7 (SCH$_2$CH$_2$N); 28.8 (OCH$_2$CH$_2$CH$_2$N); 23.2 (CH$_3$ acetate); 16.7 (C-6′). HRESIMS calcd for $C_{25}H_{46}N_2O_{15}S$ [M+H]$^+$: 647.2673, found: 647.2697.

4.1.3. Allyl 2-acetamido-4,6-*O*-benzylidene-3-*O*-chloroacetyl-2-deoxy-β-D-glucopyranoside (13)

A solution of the known[22] benzylidene alcohol **12** (400 mg, 1.15 mmol) in anhyd CH_2Cl_2 (20 mL) and pyridine (1.6 mL) was cooled to 0 °C under N_2 and chloroacetyl chloride (194 μL, 2.1 equiv) was added drop wise over 10 min. The reaction mixture was allowed to slowly warm up to rt and stirred overnight. Solvents were evaporated and residual pyridine was removed by co-concentration with toluene (3 × 20 mL). The resulting amorphous solid was washed with ethanol (2 × 20 mL) and the pure chloroacetate **13** (411 mg, 84%) was obtained as an amorphous white powder after drying under high vacuum. $[\alpha]_D$ = −4.1 (*c* 1.0, DMF). ^{1}H NMR (400 MHz, $CDCl_3$, 295 K) δ 7.39–7.26 (m, 5H, Ar); 5.84–5.73 (m, 1H, –C*H*=CH$_2$); 5.46 (s, 1H, C*H*Ph); 5.42 (d, 1H, *J* = 9.1 Hz, NH); 5.31 (t, 1H, *J* = 9.9 Hz, H-3); 5.26–5.13 (m, 2H, –CH=CH_2); 4.61 (d, 1H, *J* = 8.4 Hz, H-1); 4.33–4.26 (m, 2H, H-6a, OC*H*H allyl); 4.06–3.95 (m, 4H, H-2, OCH*H* allyl, *O*-chloroacetate); 3.76 (t, 1H, *J* = 10.3 Hz, H-6b); 3.67 (t, 1H, *J* = 9.4 Hz, H-4); 3.48 (m, 1H, H-5); 1.95 (s, 3H, *N*-acetate). ^{13}C NMR (100 MHz, $CDCl_3$, 295 K) δ 133.3 (–CH=CH$_2$); 131.9, 129.1, 128.2, 126.1 (Ar); 117.9 (–CH=CH$_2$); 101.8 (C*H*Ph); 100.4 (C-1); 78.4 (C-4); 73.4 (C-3); 70.1 (OCH$_2$ allyl); 68.6 (C-6); 66.3 (C-5); 54.9 (C-2); 40.7 (*O*-chloroacetate); 23.4 (*N*-acetate). HRESI calcd for $C_{20}H_{24}ClNO_7$ [M+H]$^+$: 426.1320, found: 426.1325.

4.1.4. Allyl 2-acetamido-6-*O*-benzyl-3-*O*-chloroacetyl-2-deoxy-β-D-glucopyranoside (14)

A solution of benzylidene chloroacetate **13** (270 mg, 0.63 mmol) and $NaCNBH_3$ (420 mg, 6.9 mmol, 10.1 equiv) in anhyd THF (12 mL) containing activated 3 Å MS (770 mg) and methyl orange (5 mg) was cooled to 0 °C and stirred under N_2 for 30 min. A 1 M solution of HCl in Et_2O (6.3 mL, 0.63 mmol, 1 equiv) was slowly added over 10 min to the reaction mixture, the solution turned dark pink and H_2 (g) was generated. The reaction mixture was stirred for 1 h at rt, filtered through Celite® and the solids were rinsed with MeOH (100 mL). The combined filtrate and washings were concentrated and flash chromatography (15:1, $CHCl_3$–MeOH) gave the pure chloroacetate acceptor **14** (151 mg, 56%) as an amorphous solid. $[\alpha]_D$ = −12.3 (*c* 0.3, $CHCl_3$). ^{1}H NMR (400 MHz, $CDCl_3$, 295 K) δ 7.37–7.21 (m, 5H, Ar); 5.88–5.76 (m, 2H, –C*H*=CH$_2$, NH); 5.28–5.12 (m, 2H, –CH=CH_2); 4.62–4.51 (m, 3H, H-1, CH_2Ph); 4.32–4.26 (m, 1H, OC*H*H allyl); 4.13–4.01 (m, 3H, OCH*H* allyl, *O*-chloroacetate); 3.95–3.86 (m, 1H, H-2); 3.83–3.71 (m, 3H, H-4, H-6a, H-6b); 3.58–3.51 (m, 1H, H-5); 3.24 (s, 1H, OH); 1.95 (s, 3H, *N*-acetate). ^{13}C NMR (100 MHz, $CDCl_3$, 295 K) δ 133.5 (–CH=CH$_2$); 128.5, 128.0, 127.8 (Ar); 117.7 (–CH=CH$_2$); 99.8 (C-1); 76.6 (C-3); 73.8 (OCH$_2$Ph); 73.6 (C-5); 70.6 (C-4); 70.2 (C-6); 69.9 (OCH$_2$ allyl); 54.1 (C-2); 40.9 (CH$_3$ chloroacetate); 23.4 (CH$_3$ acetate). HRESIMS calcd for $C_{20}H_{26}ClNO_7$ [M+H]$^+$: 428.1476, found: 428.1481.

4.1.5. Allyl 2-acetamido-3-*O*-(2,3,4-tri-*O*-benzyl-α-L-fucopyranosyl)-6-*O*-benzyl-2-deoxy-β-D-glucopyranoside (16)

A solution of the known[22] benzylidene disaccharide **15** (128 mg, 0.17 mmol) in anhyd CH_2Cl_2 (2.5 mL) containing powdered activated 4 Å MS (625 mg) was stirred under N_2 for 1 h then cooled to −78 °C. Et_3SiH (80 μL, 0.50 mmol, 3.0 equiv) and TfOH (38 μL, 0.57 mmol, 3.4 equiv) were added to the reaction mixture and the solution was stirred for 1 h. The reaction was quenched with Et_3N (333 μL, 2.4 mmol, 14 equiv) and MeOH (333 μL) and the reaction mixture diluted with CH_2Cl_2 (50 mL) was filtered through Celite®. The filtrate was washed with satd aq $NaHCO_3$ (50 mL), dried, concentrated, and flash chromatography (1:1, EtOAc–hexanes) of the residue gave the pure *N*-acetate acceptor **16** (99 mg, 77%) as a colorless glass. $[\alpha]_D$ = −31.6 (*c* 1.0, $CHCl_3$). ^{1}H NMR (400 MHz, $CDCl_3$, 298 K) δ 7.35–7.14 (m, 20H, Ar); 5.86–5.74 (m, 1H, –C*H*=CH$_2$); 5.41 (d, 1H, *J* = 6.7 Hz, NH); 5.22–5.06 (m, 2H, –CH=CH_2); 4.92–4.85 (m, 2H, H-1′, C*H*HPh); 4.84–4.66 (m, 4H, H-1, C*H*HPh, CH_2Ph); 4.62–4.49 (m, 4H, 2 × CH*H*Ph, CH_2Ph); 4.27 (dd, 1H, *J* = 13.0, 5.2 Hz, OC*H*H allyl); 4.09–3.97 (m, 4H, H-2′, H-5′, OH, OCH*H* allyl); 3.90–3.72 (m, 3H, H-3, H-3′, H-6a); 3.66–3.58 (m, 2H, H-4′, H-6b); 3.46–3.24 (m, 3H, H-2, H-4, H-5); 1.52 (s, 3H, *N*-acetate); 1.08 (d, 3H, *J* = 6.4 Hz, H-6′). ^{13}C NMR (100 MHz, $CDCl_3$, 298 K): 169.2 (C=O); 138.2 (Ar quaternary); 134.2 (–C*H*=CH$_2$); 128.4, 128.2, 128.1, 127.9, 127.7, 127.6 (Ar); 117.3 (–CH=CH_2); 99.2 (C-1, C-1′); 83.9 (C-3); 79.2 (C-3′); 77.2 (C-4′); 76.0 (C-2′); 75.0 (OCH_2Ph); 74.9 (C-5); 74.1, 73.5, 72.8 (OCH_2Ph); 70.4 (C-4); 69.8 (OCH$_2$ allyl); 69.6 (C-6); 68.1 (C-5′); 56.2 (C-2); 22.1 (CH$_3$ acetate); 16.6 (C-6′). HRESIMS calcd for $C_{45}H_{53}NO_{10}$ [M+H]$^+$: 768.3748, found: 768.3777.

4.1.6. Allyl 2-acetamido-4-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-6-*O*-benzyl-3-*O*-chloroacetyl-2-deoxy-β-D-glucopyranoside (18)

The chloroacetate acceptor **14** (27 mg, 0.063 mmol) and the known[23] trichloroacetimidate **17** (164 mg, 0.33 mmol, 5.3 equiv) were dissolved in anhyd CH_2Cl_2 (2 mL) and the solution was warmed up to 40 °C under N_2. $BF_3{\cdot}OEt_2$ (17 μL, 0.14 mmol, 2.1 equiv) was added and the mixture was stirred for 1 h 40 min at 40 °C. The reaction was quenched with Et_3N (21 μL, 0.15 mmol, 2.4 equiv), the mixture diluted in CH_2Cl_2 (30 mL) and washed with satd aq $NaHCO_3$ (30 mL). The aq phase was re-extracted with CH_2Cl_2 (20 mL) and the combined organic layers were dried and concentrated. After two successive flash chromatography columns (6:4 EtOAc–hexanes followed by 50:1 $CHCl_3$–MeOH) the pure disaccharide **18** (20.9 mg, 43%) was isolated as an amorphous glass. $[\alpha]_D$ = −5.5 (*c* 1.0, $CHCl_3$). ^{1}H NMR (400 MHz, $CDCl_3$, 295 K) δ 7.40–7.29 (m, 5H, Ar); 5.89–5.77 (m, 1H, –C*H*=CH$_2$); 5.57 (d, 1H, *J* = 9.2 Hz, NH); 5.27–5.08 (m, 4H, H-3, H-4′, –CH=CH_2); 4.97 (dd, 1H, *J* = 10.4, 8.0 Hz, H-2′); 4.81 (dd, 1H, *J* = 10.3, 3.5 Hz, H-3′); 4.73 (d, 1H, *J* = 12.1 Hz, C*H*HPh); 4.51 (d, 1H, *J* = 7.8 Hz, H-1); 4.45

(d, 1H, J = 12.1 Hz, CH*H*Ph); 4.39 (d, 1H, J = 8.0 Hz, H-1′); 4.31 (m, 1H, OC*H*H allyl); 4.09–3.99 (m, 6H, H-2, H-6a′, H6b′, OCH*H* allyl, *O*-chloroacetate); 3.94 (t, 1H, J = 8.1 Hz, H-4); 3.70 (m, 2H, H-6a, H-6b); 3.63 (m, 1H, H-5′); 3.45 (m, 1H, H-5); 2.13–1.92 (5 s, 5 × 3H, *O*-acetates and *N*-acetate). ^{13}C NMR (75 MHz, $CDCl_3$, 300 K): 171.0, 170.9, 170.7, 169.7, 168.0 (C=O); 138.4 (Ar quaternary); 133.6 (–*C*H=CH$_2$); 128.6, 128.1, 128.0 (Ar); 117.6 (–CH=*C*H$_2$); 100.2 (C-1′); 99.8 (C-1); 74.5 (C-5); 74.4 (C-3); 74.2 (C-4); 73.7 (OCH$_2$Ph); 70.8 (C-3′); 70.7 (C-5′); 69.6 (OCH$_2$ allyl); 69.2 (C-2′); 67.4 (C-6); 66.8 (C-4′);61.1 (C-6′); 53.4 (C-2); 40.8 (CH$_2$Cl); 23.3, 20.7, 20.5 (CH$_3$ acetates). HRESIMS calcd for $C_{34}H_{44}ClNO_{16}$ $[M+H]^+$: 758.2427, found: 758.2455.

4.1.7. Allyl 2-acetamido-4-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-3-*O*-(2,3,4-tri-*O*-benzyl-α-L-fucopyranosyl)-6-*O*-benzyl-2-deoxy-β-D-glucopyranoside (19)

The disaccharide acceptor **16** (22 mg, 0.029 mmol) and the trichloroacetimidate **17** (70 mg, 0.14 mmol, 4.9 equiv) were dissolved in anhyd CH_2Cl_2 (2 mL) under N_2. $BF_3{\cdot}OEt_2$ (7.2 μL, 0.057 mmol, 2.0 equiv) was added and the mixture was stirred for 40 min at rt. The reaction was quenched with Et_3N (10 μL, 0.072 mmol, 2.5 equiv) and the mixture diluted with CH_2Cl_2 (30 mL) was washed with satd aq $NaHCO_3$ (30 mL). The aq phase was re-extracted with CH_2Cl_2 (20 mL) and the combined organic layers were dried and concentrated. Flash chromatography (1:1–6:4 EtOAc–hexanes) followed by RP HPLC (65:35–90:10 CH_3CN–H_2O) gave the pure trisaccharide **19** (19.9 mg, 64%) as a white amorphous solid. $[\alpha]_D$ = −27.2 (*c* 1.0, $CHCl_3$). ^{1}H NMR (600 MHz, $CDCl_3$, 310 K) δ 7.38–7.21 (m, 20H, Ar); 5.91 (d, 1H, J = 7.9 Hz, NH); 5.84–5.76 (m, 1H, –C*H*=CH$_2$); 5.29–5.26 (d, 1H, J = 3.2 Hz, H-4″); 5.25–5.20 (dd, 1H, J = 1.4, 17.2 Hz, –CH=C*H*H); 5.11–5.07 (m, 2H, H-1′, –CH=CH*H*); 5.04 (dd, 1H, J = 8.2, 10.4 Hz, H-2″); 4.93 (d, 1H, J = 11.8 Hz, C*H*HPh); 4.87–4.70 (m, 6H, H-1, H-3″, 2 × C*H*$_2$Ph); 4.66 (d, 1H, J = 11.8 Hz, CH*H*Ph); 4.58 (d, 1H, J = 12.0 Hz, C*H*HPh); 4.53 (s, 1H, J = 8.1 Hz, H-1″); 4.41 (d, 1H, J = 12.0 Hz, CH*H*Ph); 4.25 (m, 1H, H-5′); 4.19 (dd, 1H, J = 5.1, 13.0 Hz, OC*H*H allyl); 4.14–4.07 (m, 3H, H-3, H-2′, H-6a″); 4.02–3.96 (m, 2H, H-6b″, OCH*H* allyl); 3.91 (t, 1H, J = 6.5 Hz, H-4); 3,87 (dd, 1H, J = 2.5, 10.1 Hz, H-3′); 3.86–3.73 (m, 2H, H-6a, H-6b); 3.64–3.58 (m, 3H, H-2, H-4′, H-5″); 3.56 (m, 1H, H-5); 2.0–1.77 (5 s, 5 × 3H, *O*-acetates and *N*-acetate); 1.13 (d, 3H, J = 6.5 Hz, H-6′). ^{13}C NMR (150 MHz, $CDCl_3$, 310 K): 170.0, 169.9 (C=O); 138.9, 138.9, 138.7, 138.0 (Ar quaternary); 134.1 (–*C*H=CH$_2$); 128.5, 128.4, 128.3, 128.3, 128.2, 127.9, 127.8, 127.7, 127.6, 127.5, 127.3, 127.2 (Ar); 117.0 (–CH=*C*H$_2$); 99.6 (C-1″); 98.7 (C-1); 97.3 (C-1′); 79.8 (C-3′); 77.2 (C-5″); 76.5 (C-2′); 74.5 (O*C*H$_2$Ph); 74.2 (C-4); 74.1 (C-5); 73.4, 73.4 (O*C*H$_2$Ph); 73.3 (C-3); 72.7 (O*C*H$_2$Ph); 70.6 (C-5′, C-3″); 69.5 (OCH$_2$ allyl); 68.9 (C-2″); 68.9 (C-6); 66.8 (C-4″); 66.6 (C-5′); 60.5 (C-6″); 23.2, 20.7, 20.5, 20.5 (CH$_3$ acetates); 16.7 (C-6′). HRESIMS calcd for $C_{59}H_{71}NO_{19}$ $[M+H]^+$: 1098.4699, found: 1098.4678.

4.1.8. Allyl 2-acetamido-4-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-6-*O*-benzyl-2-deoxy-β-D-glucopyranoside (22); 2,3,4-tri-*O*-benzyl-α,β-L-fucose (21) and 3,4,6-tri-*O*-acetyl-1,2-*O*-(2,3,4-tri-*O*-benzyl-α-L-fucos-1′-yl orthoacetyl)-α-D-galactopyranose (20)

The disaccharide **16** (22 mg, 0.029 mmol) and the trichloroacetimidate **17** donor (70 mg, 0.14 mmol, 4.9 equiv) were dissolved in anhyd CH_2Cl_2 (2 mL) under N_2. $BF_3{\cdot}OEt_2$ (7.2 μL, 0.057 mmol, 2.0 equiv) was added and the mixture was stirred for 2 h 20 min at rt. The reaction was quenched with Et_3N (10 μL, 0.072 mmol, 2.5 equiv), the reaction mixture diluted with CH_2Cl_2 (30 mL) and washed with satd aq $NaHCO_3$ (30 mL). The aq phase was re-extracted with CH_2Cl_2 (20 mL) and the combined organic layers were dried and concentrated. Flash chromatography (6:4 EtOAc–hexanes to pure EtOAc) yielded the known[24] fucose hemiacetal **21** (2 mg, 15%), the pure disaccharide **22** (5 mg, 25%) and the impure orthoester **20**. The isomeric mixture of endo and exo orthoesters (**20**) was obtained free of other contaminants (13 mg, 60%) upon RP HPLC (65:35–90:10 CH_3CN–H_2O). ^{1}H NMR showed that one isomer assumed to be the less sterically hindered exo orthoester was predominant (75%).

4.1.8.1. Analytical data for orthoester 20. ^{1}H NMR (400 MHz, $CDCl_3$, 298 K, for the major isomer) δ 7.42–7.25 (m, 5H, Ar); 6.29 (d, 1H, J = 3.6 Hz, H-1′); 5.49 (d, 1H, J = 5.0 Hz, H-4′); 5.36 (dd, 1H, J = 10.5, 3.1 Hz, H-3′); 5.06 (d, 1H, J = 3.6 Hz, H-1); 4.97 (d, 1H, J = 11.6 Hz, C*H*HPh); 4.82–4.62 (m, 5H, CH*H*Ph, 2 × C*H*$_2$Ph); 4.29 (t, 1H, J = 6.2 Hz, H-5); 4.15 (dd, 1H, J = 10.6, 3.7 Hz, H-2′); 4.09 (d, 2H, J = 5.5 Hz, H-6); 4.05 (dd, 1H, J = 10.2, 4.8 Hz, H-2); 3.85 (m, 1H, H-5′); 3.78 (dd, 1H, J = 10.1, 2.5 Hz, H-3); 3.65 (s, 1H, H-4); 2.19–2.02 (3 s, 3 × 3H, *O*-acetates); 1.74 (s, 3H, orthoester CH_3); 1.13 (d, 3H, J = 8.5 Hz, H-6′). ^{13}C NMR (100 MHz, $CDCl_3$, 298 K, for the major isomer): 170.4, 170.1, 169.4 (C=O); 138.7, 138.6, 138.5 (Ar quaternary); 128.5, 128.4, 128.3, 128.2, 128.0, 127.7, 127.5, 127.5, 127.3 (Ar); 99.7 (C-1); 91.4 (C-1′); 78.7 (C-3); 78.6 (C-4); 75.8(C-2); 74.8,72.8, 72.7 (O*C*H$_2$Ph); 71.5 (C-2′); 69.4 (C-3′); 68.4 (C-3′); 67.7 (C-5); 67.4 (C-5′); 61.3 (C-6); 21.0, 20.7, 20.5 (CH_3 acetates and orthoacetate); 16.5 (C-6′). HRESIMS calcd for $C_{47}H_{53}NO_{11}$ $[M+NH_4]^+$: 782.3388, found: 782.3370.

4.1.8.2. Analytical data for 22. $[\alpha]_D$ = −6.6 (*c* 0.5, $CHCl_3$). ^{1}H NMR (400 MHz, $CDCl_3$, 294 K) δ 7.39–7.21 (m, 5H, Ar); 5.92–5.81 (m, 1H, –C*H*=CH$_2$); 5.57 (d, 1H, J = 7.7 Hz, NH); 5.33 (s, 1H, J = 3.1 Hz, H-4′); 5.29–5.11 (m, 3H, H-2′, –CH=C*H*$_2$); 4.90 (dd, 1H, J = 10.5, 3.4 Hz, H-3′); 4.78 (d, 1H, J = 8.2 Hz, H-1); 4.70 (d, 1H, J = 12.1 Hz, C*H*HPh); 4.50–4.43 (2 d, 2H, J = 12.0, 7.9 Hz, H-1, CH*H*Ph); 4.31 (dd, 1H, J = 12.9, 5.0 Hz, OC*H*H allyl); 4.13–3.98 (m, 4H, H-3, H-6a′, H-6b′, OCH*H* allyl); 3.99 (s, 1H, OH); 3.86 (t, 1H, J = 6.2 Hz, H-5′); 3.69–3.59 (m, 3H, H-4, H-6a, H-6b); 3.52–3.37 (m, 2H, H-2, H-5); 2.15–1.94 (5 s, 5 × 3H, *O*-acetates and *N*-acetate). ^{13}C NMR (100 MHz, $CDCl_3$, 295 K): 170.5, 170.1, 170.0, 169.2 (C=O); 138.0 (Ar quaternary); 133.8 (–*C*H=CH$_2$); 128.5, 128.4, 128.3, 127.9, 127.8, 127.4 (Ar); 117.7 (–CH=*C*H$_2$); 101.2 (C-1); 99.1 (C-1′); 80.9 (C-4); 73.9 (C-5); 73.6 (O*C*H$_2$Ph); 71.3 (C-3); 71.1 (C-5′); 70.7 (C-3′); 69.9 (OCH$_2$ allyl); 68.7 (C-2′); 68.0 (C-6); 66.8 (C-4′); 61.3 (C-6′); 57.2 (C-2); 23.7, 20.7, 20.6, 20.5 (CH_3 acetates). HRESIMS calcd for $C_{32}H_{43}NO_{15}$ $[M+H]^+$: 682.2711, found: 682.2709.

4.1.9. Preparation of the glycoconjugate Le^x-BSA (23)

A solution (10 μL/mL, 90 μL, 1 equiv) of 3,4-diethyoxy-3-cyclobutene-1,2-dione (diethyl squarate) in freshly distilled MeOH was added to a solution of cysteamine Le^x **10** (4 mg, 6.1 μmol) in freshly distilled MeOH (600 μL). The reaction mixture was left at rt for 3 h and TLC (6:3:1 EtOAC–MeOH–H_2O) showed that the starting amine **10** was quantitatively converted to the desired squarate compound. After evaporation of the methanol, a solution of BSA (8.1 mg, 0.120 μmol) in a pH 10 carbonate buffer (0.1 M, 100 μL) was added to the squarate adduct obtained above (4.3 mg, 5.9 μmol, 50 equiv). The vial that contained the BSA solution was washed with more buffer (100 μL) which was added to the reaction mixture and the reaction was left to proceed for 7 days at rt. The glycoconjugate was filtered against distilled H_2O (6 × 8 mL) using an Amicon ultrafiltration cell equipped with a Diaflo membrane (25 mm, 30 kDa cut-off). The residue was then taken up in water and lyophilized to give the pure Le^x-BSA conjugate **23** (9.0 mg). The level of incorporation was measured by the Dubois test[26] that gave an average hapten loading (n) of 35 hapten molecules per BSA carrier protein.

4.2. Competitive binding experiments

A NUNC 96-well ELISA microtiter plate was coated with a dilution of the Le^x-BSA glycoconjugate **23** (100 μL per well, 3 μg/mL) in a 0.05 M carbonate-bicarbonate buffer at pH 9.6. The plate was covered with Saran wrap and incubated at 4 °C for 16 h. The antigen solution was discarded and the plate was washed (3 × 5 min incubation period with buffer) with a PBS buffer at pH 7.4 containing 0.05% Tween 20. The plate was then rinsed with PBS and blocked with skim milk (DIFCO, 5% in PBS-0.05% Tween, 400 μL per well). After a 1 h incubation at 37 °C, the plate was rinsed (3×) with PBS. Each well received a solution containing 50 μL of competitor (concentration range from 0.2 μg/mL to 20 mg/mL in 2% skim milk in PBS) and 50 μL of a solution of SH1 antibodies (1:125 dilution in 2% skim milk in PBS). The plate was incubated for 4 h at 23 °C. The plate was subsequently washed (3 × 5 min incubation period with buffer) with PBS-0.05% Tween and rinsed with PBS. A dilution of commercially available alkaline phosphatase-labeled goat anti-mouse antibody (1:1500 in 2% skim milk in PBS, 100 μL per well) was added to each well. After 1 h of incubation at 37 °C, the plate was washed (3 × 5 min incubation period with buffer) with PBS-0.05% Tween, and rinsed with PBS. A solution of the chromogenic substrate *p*-nitrophenyl phosphate (100 μL per well) at 1 mg/mL in 0.05 M sodium carbonate buffer pH 9.8 containing 10 mM $MgCl_2$ was added. After a 14 h incubation period at 23 °C, the absorbance values were read at 405 nm employing a PowerWave XS plate reader. All samples were prepared in triplicate. The absorbance values were plotted as the percentage inhibition against an increasing concentration of competitor, calculated using wells containing no competitor as the reference point. The values were fitted to a 4 parameter logistics sigmoidal equation:[29] $y = y_0 + a/[1 + (x/x_0)^b]$ using Sigma Plot 10.0.

4.3. Conformational analysis

4.3.1. Stochastic searches

The trisaccharide was subjected to all atom and heavy atom stochastic conformational searches using the AMBER94 force field[39] in the Molecular Operating Environment (MOE2005)[32] program suite. In this implementation of AMBER94, any parameters missing for a class of compound are approximated by MOE based on parameter values for similar molecular fragments. The starting structure was built using the carbohydrate builder in MOE2005 and submitted to the stochastic search. This search is similar to the random incremental pulse search (RIPS) method[48] in which the coordinates of each atom are randomly perturbed, after which the entire molecule is minimized to attempt to generate a new conformer. In these stochastic searches, new conformations are generated by the rotation of all bonds (including ring bonds) to random dihedral angles. In our searches a bias of 30° was used. This means that dihedral angles were selected with a sum-of-Gaussians distribution with peaks at multiples of 30°. In addition to bond rotations, atom positions were randomly perturbed by 0.4 Å. These random perturbations were repeated 150,000 times per conformational search and each generated conformer was subjected to 500 steps of minimization with full degrees of freedom with implicit solvation by the Generalized Born/Surface Area (GB/SA) continuum solvation model[34] using a dielectric constant of 78. Two separate searches were performed and the databases were combined for analysis. Initially each new conformation was checked against the previously found conformations using a root-mean-square (RMS) tolerance of 0.1 Å on all atoms, including the hydrogen atoms to account for possible hydrogen bonding. The second search was carried out using a more stringent RMS value (0.01 Å) applied to the heavy atoms only. In both searches the calculations terminated at the end of the 150,000 iterations as the number of failures criteria to find new conformations (10,000 iterations in a row) was never met. Structures that did not have the $^4C_1$ conformation for D-glucosamine or D-galactose or those that did not maintain a $^4C_1$ conformation for L-fucose were eliminated. The results of the two searches were combined for analysis and conformations with energies over 10 kcal mol^{-1} above the global minima were rejected.

4.3.2. NMR experiments

NMR experiments were recorded in 5 mm NMR tubes at 400 MHz (HSQC) and 600 MHz (^1H, ^{13}C, COSY, HMBC and NOESY). All spectra were recorded at 300 K in D_2O. One-dimensional ROESY spectra were acquired with selective excitations of protons: H-1′, H-1″ and H-5′ 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 at nine or ten mixing times from 20 ms to 300 or 400 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 buildup 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,

$$F'(0) = A(B - C).^{\text{Ref.43}}$$

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

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

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

The intra-residue cross-peaks used as reference for distance determinations were: H-5′/H-3′ (2.50 Å) and H-1″/H-3″ (2.64 Å) for compounds **5** and **6**; H-1″/H-3″ (2.64 Å) for compound **7**; H-5′/H-3′ (2.50 Å) and H-1″/H-5″ (2.39 Å) for compound **8**; H-1′/H-2′ (2.51 Å), H-5′/H-3′ (2.59 Å) and H-1″/H-3″ (2.64 Å) for compound **9**.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2010.08.040.

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