Biological Evaluation of Rationally Modified Analogs of the H-Type II Blood Group Trisaccharide. A Correlation between Solution Conformation and Binding Affinity

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Abstract: The role of local steric influences on the solution conformation and the biological activity of the H-type II blood group determinant **1** has been evaluated using structurally modified trisaccharides **2–4** and their corresponding C1-substituted *C*-glycosides **5–8** as conformational models. The preference of the *C*-glycosidic bond to adopt the gauche "*exo*-anomeric" conformation and the removal of destabilizing 1,3-diaxial-like interactions on the *C*-aglyconic bond have been used to create predictable conformational characteristics in *C*-trisaccharides **5–8**. Vicinal coupling constants from ^{1}H NMR spectroscopy and 2D NOESY spectroscopy demonstrate that structural modifications in the *C*-trisaccharides result in large changes in their conformational preferences. To test the impact of solution conformation on receptor–ligand recognition, the affinities of compounds **1–8** toward the lectin I of *Ulex europaeus* (UEA-I) have been investigated using a quantitative binding assay. The binding affinities of the H-type II trisaccharide **1** and the corresponding carbon analog **5** are virtually identical. The activities of the structurally modified *C*-trisaccharides **6–8** decrease sharply relative to the unmodified *C*-trisaccharide **5**, correlating conformation to binding affinity. A parallel gradient in binding affinity is observed for the *O*-trisaccharides **1–4**. The selectivity of UEA-I for epitopes **1–8** validates the assumption that its receptor site largely defines a bound conformation for the substrates, and establishes that the conformational behavior of *O*-glycosides such as **1–4** is similar to that of *C*-glycosides such as **5–8**.

Introduction

The growing body of evidence that oligosaccharides play vital roles in intercellular communication and cell-mediated processes has been accompanied by a commensurate surge of interest in carbohydrates.[1] Oligosaccharide solution conformation and macromolecular carbohydrate recognition have both become important topics of research. Stoichiometric binding of carbohydrate ligands by antibodies and lectins has been characterized thus far by (1) polar interactions and key hydrogen bonds,[2] (2) complementary hydrophilic/hydrophobic domains on the ligand and receptor site,[3] and (3) thermodynamic parameters optimizing hydrogen bond networks among the carbohydrate, the protein, and interstitial water molecules.[4] Less well understood is the role of ligand molecular shape in protein–sugar interactions. Manipulation of an oligosaccharide's conformational bias through the modification of steric influences could in principle result in a more tightly binding ligand.[5] Some attempts have been made to explore this issue in terms of conformational

flexibility,[6] but a rational approach toward this challenge has not been suggested, until now.

In this paper we establish a clear relationship between the solution conformation of an oligosaccharide and its binding affinity toward a protein receptor, using the H-type II human blood group determinant as our model system. The trisaccharide **1** is known to possess the basic structural requirements for high specificity and affinity to several leguminous lectins, including lectin I of *Ulex europaeus* (UEA-I).[7,8] A variety of opportunistic microorganisms are also known to recognize human blood group antigens, a specific example being the bacteria *Helicobacter pylori*, which has been implicated in the formation of stomach ulcers and gastric lymphoma.[9] Pathogens such as this one may be attractive targets for carbohydrate-based therapeutics.[10]

We predict that strategic modifications in structure **1**, as represented by trisaccharides **2–4** (Figure 1), would result in dramatic changes of molecular shape and subsequent biological activity. Our prediction is based on the well-established

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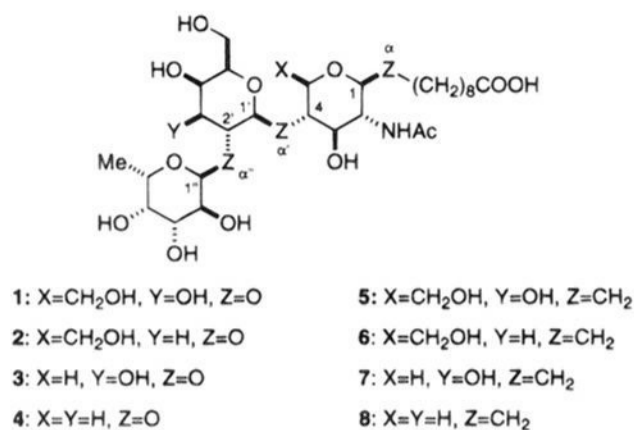
Analogs of the H-Type II Blood Group Trisaccharide

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Figure 1.

1: X=CH₂OH, Y=OH, Z=O   5: X=CH₂OH, Y=OH, Z=CH₂

2: X=CH₂OH, Y=H, Z=O   6: X=CH₂OH, Y=H, Z=CH₂

3: X=H, Y=OH, Z=O   7: X=H, Y=OH, Z=CH₂

4: X=Y=H, Z=O   8: X=Y=H, Z=CH₂

Figure 2. Preferred conformation of *C*-trisaccharide **8**, as represented in a diamond-lattice diagram. The conformational analysis of **8** is based on ¹H NMR coupling constants across the interglycosidic bonds.

preference of glycosidic bonds to adopt the "*exo*-anomeric" conformation,[11] in which the C1—C2 ring bond is antiperiplanar to the *exo*-glycosidic bond, and steric conformational analysis. In order to obtain unambiguous evidence that oligosaccharide conformation is influenced by specific substituents, we synthesized the structurally analogous *C*-trisaccharides **5**−**8**, in which the three bridging glycosidic oxygen atoms are each replaced with a methylene (CH₂) group. These additional methylene protons provide a spectroscopic "handle" in the form of vicinal coupling constants from ¹H NMR spectroscopy, which define to the first degree of approximation the torsional relationship between each pyranose ring. The variance in conformation between *O*- and *C*-glycosides is expected to be small, because discrepancies in the C—C and C—O sp³ bond lengths (1.54 Å vs 1.43 Å on average) are compensated by differences in the interglycosidic C—C—C and C—O—C bond angles (109° vs 116° from crystallographic data for β-cellobiose[12]). Furthermore, comparable values in the NOE and T_1 data of the *O*- and *C*-disaccharides suggest their conformational similarities.[13]

Results and Discussion

Solution Conformation of *C*-Trisaccharides 5−8. We have demonstrated in previous studies that the overall backbone conformation of *C*-disaccharides and *C*-trisaccharides can be predicted solely on the basis of steric considerations.[14] Vicinal coupling constants from ¹H NMR spectroscopy verified experimentally that (1) the *C*-glycosidic bond preferentially adopts the staggered *exo*-anomeric conformation like its parent *O*-glycosidic bond does and (2) the adjacent *C*-aglyconic bond adopts a well-defined staggered conformation in the absence of 1,3-diaxial-like interactions.

The details of the synthesis and conformational analysis of *C*-trisaccharides **5**−**8** have recently been reported.[14] In summary, it was demonstrated that these compounds each possess a unique conformational preference. Although all four compounds exhibit a strong tendency to adopt the *exo*-anomeric conformation about the C1′−Cα′ and C1″−Cα″ glycosidic bonds, they vary widely with respect to their aglyconic conformations. Removing the C3′-hydroxyl, i.e., **5** → **6**, induces a large change in orientation about the C2′−Cα″ bond to a well-defined staggered conformation. Removing the C5-hydroxymethyl, i.e., **5** → **7**, creates a similarly large change in conformational preference about the C4−Cα′ bond. It is therefore not surprising that the bisdefunctionalized trisaccharide **8** preferentially adopts only 1 out of its 81 possible staggered
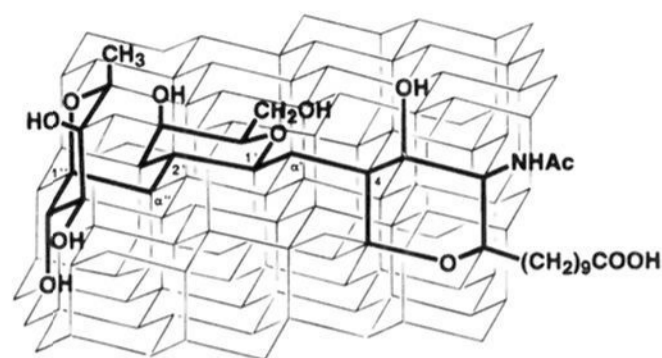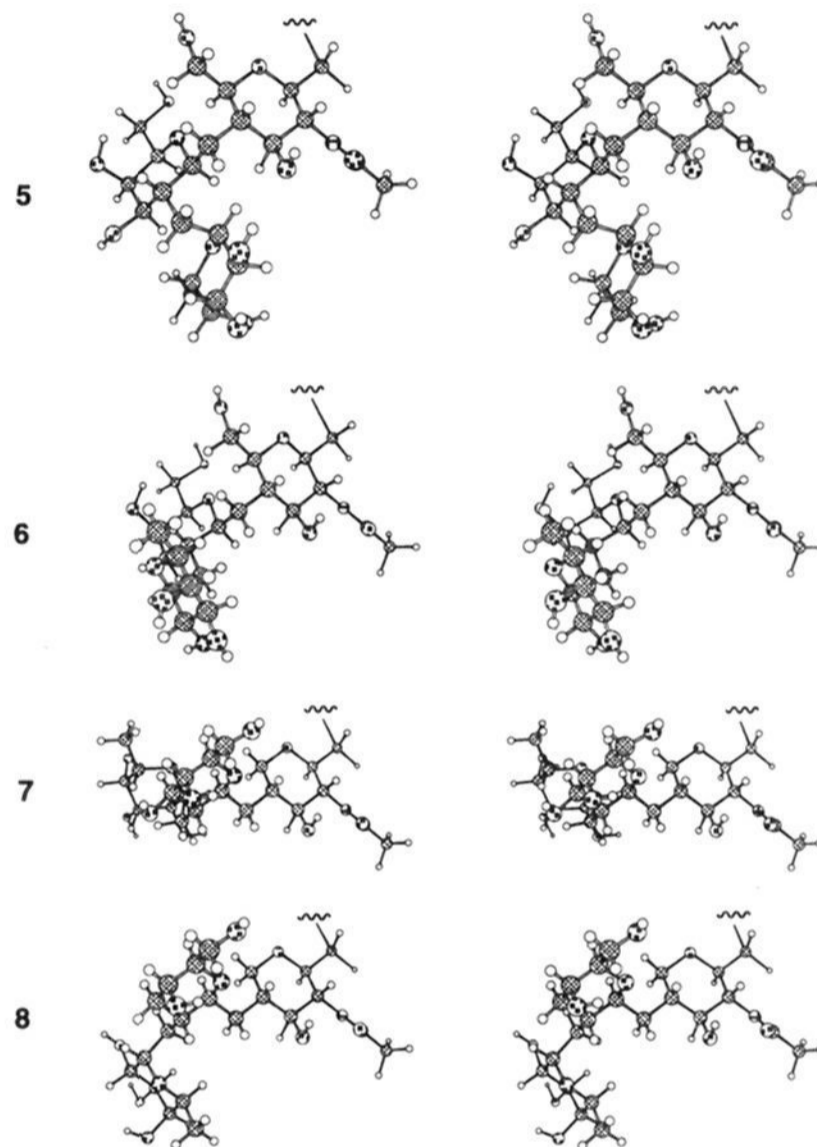

Figure 3. Stereoview images of *C*-trisaccharide **5**−**8**. In each compound, the C1 hydrocarbon side chain has been truncated, and the relative orientation of the N-acetylglucosamine ring is fixed.

interglycosidic conformations (Figure 2). It is notable that variations in the coupling constants due to changes in solvent are small, and the conformations about the interglycosidic bonds are mutually independent, indicating that hydrogen bonding and solvent effects have insignificant roles in defining the overall shapes of the *C*-trisaccharides.

The differences in secondary structure for these *C*-trisaccharides are striking (Figure 3). Time-averaged conformations were generated for **5**−**8** using the data provided by the vicinal coupling constants (Table 1), complemented by information obtained from 2D NOESY NMR spectroscopy. An example of local steric influence on shape can be illustrated by comparing interglycosidic nuclear Overhauser effect (NOE) enhancements in C3′-deoxy-*C*-trisaccharide **6** and C5-deshydroxymethyl derivative **7** (Figure 4). A strong NOE interaction is observed across the galactose and N-acetylglucosamine rings between the protons at C1′ and C3 in **6**, but is replaced with an interaction between the C1′ proton and the C5 equatorial proton in **7**. Similarly, NOEs across the fucose and galactose rings are

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Table 1. Selected ^{1}H NMR (500 MHz) Coupling Constants (Hz) for Compounds **5−8** at Room Temperature[14,a]

compd	J(1',α')	J(4,α')		J(1'',α'')	J(2',α'')

^{a} The spectra were recorded in the following solvents: (a) 95:5 pyridine-d_5/methanol-d_4 (**5** methyl ester); (b) methanol-d_4; (c) 75:25 methanol-d_4/DMSO-d_6; (d) D$_2$O.
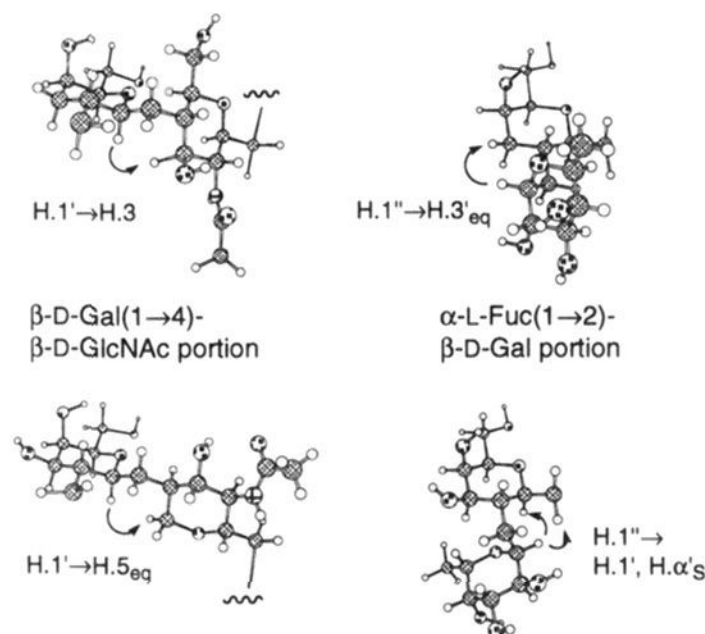

Figure 4. NOE interactions across the β-D-Gal(1→4)-β-D-GlcNAc linkages and the α-L-Fuc(1→2)-β-D-Gal linkages of C3'-deoxy-*C*-trisaccharide **6** (upper) and C5-deshydroxymethyl-*C*-trisaccharide **7** (lower).

evident between the C1'' proton and the C1' and *pro*-(*S*)-Cα' protons in **7**, whereas **6** gives rise instead to a strong interaction between the C1'' proton and the C3' equatorial proton.

Biological Evaluation of H-Type II Trisaccharide Analogs 1−8. To establish the role of oligosaccharide solution conformation in biological activity, we have quantitatively measured the binding affinities of *O*-trisaccharides **1−4** and *C*-trisaccharides **5−8** to the lectin UEA-I. Our objectives are 3-fold. First, we wish to confirm the conformational similarities of the H-type II determinant **1** and its carbon analog **5** by demonstrating their comparable binding affinities. There is no *a priori* reason to believe that the electronic character of the bridging glycosidic oxygen is important for protein−carbohydrate recognition.[15] Second, we wish to correlate the widely different solution

(15) A recent example of a protein−sugar[14] complex solved by X-ray crystallography suggests a hydrogen-bonding role for one of the bridging glycosidic oxygens: Merritt, E. A.; Sarfaty, S.; Akker, F. v. d.; L'Hoir, C.; Martial, J. A.; Hol, W. G. J. *Protein Sci.* **1994**, *3*, 166.

structures of *C*-trisaccharides **6−8** to a loss in binding affinity with respect to the unmodified *C*-trisaccharide **5**. We rationalize on the basis of torsional strain energy that the preferred staggered geometries adopted by the C2'−Cα'' or the C4−Cα' aglyconic bond of **6** or **7** result in larger free energy differences between the free and bound states of these ligands (*vide infra*). Thus, bisdefunctionalized derivative **8**, the *C*-trisaccharide with the lowest conformational energy, is expected to have the weakest binding affinity of all. Third, we wish to integrate the first two by correlating conformation with biological activity in *O*-glycosides. We expect the *O*-trisaccharides **1−4** to exhibit a gradient in binding activity in a manner parallel to that of the corresponding *C*-trisaccharides **5−8**, thus demonstrating the importance of local conformational influences in protein−carbohydrate recognition. It should be emphasized that a general method for unambiguously determining the conformations of *O*-glycosides has yet to be established. The unique advantages of using *C*-glycosides as models of carbohydrate solution structure are the reliable assessment of their conformational behavior and the accurate appraisal of the elements which influence them.

These projections are based on the premise that the receptor binding site is relatively fixed, rather than participating in an "induced fit" with the substrate.[16] The exclusive selectivity of the lectin UEA-I for the H-type II trisaccharide L-α-Fuc(1→2)-D-β-Gal(1→4)-D-β-GlcNAc over the H-type I trisaccharide L-α-Fuc(1→2)-D-β-Gal(1→3)-D-β-GlcNAc is a typical example of specificity observed in oligosaccharide-binding proteins.[7] We have thus made the assumption that UEA-I largely defines a specific bound conformation for the trisaccharide epitopes **1−8**, for which we have qualitatively predicted relative differences in binding energy.

The binding affinities of the trisaccharide epitopes **1−8** have been determined using a competitive enzyme-linked biotin−avidin assay, with the biotin hydrazide conjugate of **1** as the wild-type reference.[17] IC$_{50}$ values in the micromolar range have been observed for all compounds tested (Table 2). This dispels any concerns that the glycosidic ether oxygens have an important role in binding, at least for this particular system. The relative differences in IC$_{50}$ values for *O*-trisaccharide **1** and *C*-trisaccharide **5** are almost negligible, demonstrating our first objective. Considering the deviations in bond lengths and angles due to the substitution of three oxygen atoms by three methylene groups, the retention of binding observed for the *C*-trisaccharide **5** is gratifyingly consistent with our assumption of conformational equivalency relative to the corresponding *O*-trisaccharide **1**.

Comparison of the IC$_{50}$ values of the *C*-trisaccharide epitopes reveals a substantial decrease in binding affinities for the structurally modified compounds **6−8**. As predicted, the absence of either the C3'-hydroxyl or the C5-hydroxymethyl substituent results in a loss of biological activity for **6** and **7**, respectively. In the case of C3'-deoxy-C5-deshydroxymethyl derivative **8**, binding is weakened to an even greater extent, demonstrating that the effect of removing both substituents is cumulative. The binding profile for compounds **5−8** correlates directly with the changes in conformational preferences determined for these ligands.

A gradient in binding affinity was also observed for the *O*-trisaccharides **1−4**. The losses in biological activity due to the absence of the C3'-hydroxyl and the C5-hydroxymethyl

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(17) We have observed that the methyl esters of ligands **1−8** are readily saponified to their corresponding carboxylic acids in phosphate-buffered saline solution (pH 7.4). All binding assays were therefore conducted on the free carboxylates.

Table 2. Binding Assay Results for O-Trisaccharides **1−4** and C-Trisaccharides **5−8** Against Wild-Type Reference **1**−Biotin Conjugate

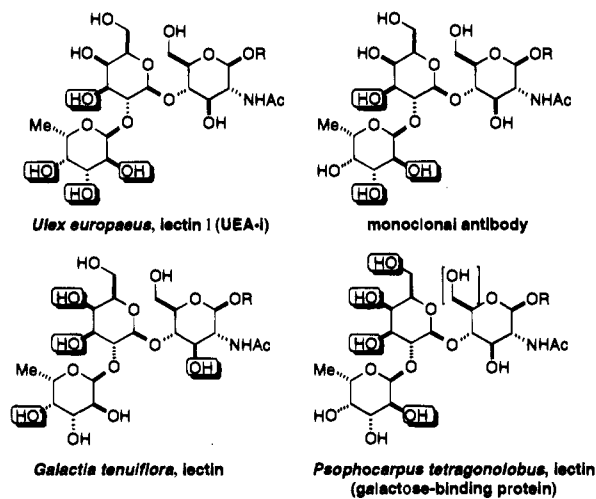| O-trisaccharide	IC$_{50}$ (μM)	$\Delta\Delta G°$ (kcal/mol)	C-trisaccharide	IC$_{50}$ (μM)	$\Delta\Delta G°$ (kcal/mol)
1 (X = CH$_2$OH, Y = OH)	1.94 ± 0.28	+0.0	**5** (X = CH$_2$OH, Y = OH)	2.65 ± 0.62	+0.2
2 (X = CH$_2$OH, Y = H)	59.0 ± 18.4	+2.0	**6** (X = CH$_2$OH, Y = H)	55.5 ± 1.4	+2.0
3 (X = H, Y = OH)	14.1 ± 2.4	+1.2	**7** (X = H, Y = OH)	13.7 ± 2.1	+1.2
4 (X = Y = H)	107 ± 2	+2.4	**8** (X = Y = H)	97 ± 29	+2.4

Figure 5. Epitope map of the H-type II trisaccharide (R = (CH$_2$)$_8$-CO$_2$Me) for four different receptors.[8] Removal of certain substituents (shaded boxes) resulted in a large drop in binding affinity ($\Delta\Delta G°$ ≥ 1.8 kcal/mol). Removal of the C6-hydroxyl alone did not affect binding negatively, but for the one case in which the C5-hydroxymethyl group was absent (brackets), a loss of affinity was noted ($\Delta\Delta G°$ ≈ 1.0 kcal/mol).

Figure 6. Oligosaccharide inhibitors and antigens from other structure-binding affinity studies. Substituents which impose a steric influence on conformation have been identified by the analysis of 1,3-diaxial-like interactions[23] (shaded boxes). For all cases tested, the removal of these groups resulted in a loss of biological activity.[24]

groups for these epitopes are parallel to that observed for the C-trisaccharides **5−8**. From this, we can make the transitive assumption that the decreased binding affinities of the defunctionalized O-trisaccharides **2−4** are due to changes in conformational preference. In addition, these relative affinities validate our assumption that the UEA-I binding site largely defines a specific bound conformation for the O- and C-trisaccharide epitopes **1−8**.

Other Binding Studies Concerning Protein−Carbohydrate Recognition. An exhaustive survey of epitopes based on the H-type II human blood group determinant was undertaken by Lemieux and co-workers, and is of particular relevance to our own work.[8] They observed that the removal of certain hydroxyl groups from trisaccharide **1** methyl ester resulted in a sharp decrease in its ability to bind to various protein receptors, including lectin UEA-I (Figure 5). It is noteworthy that the responses of the receptors to the epitopes of **1** methyl ester are different for each case, but they all have in common a sensitivity to the loss of the C3′-hydroxyl. Furthermore, the absence of the C6-hydroxyl from **1** methyl ester did not cause a decrease in binding for the receptors studied, but in fact resulted in a slight increase. We cannot rigorously exclude the possibility that the C3′-hydroxyl participates in a hydrogen bond with the protein in any of the four receptor−ligand systems, but its relevance in defining conformation correlates strongly with its invariable importance in binding. It is clear that the C6-hydroxyl is not an important polar substituent, so a loss in binding due to the removal of the C6 carbon is even more convincingly related to a change in shape. Although Lemieux and co-workers do not cite steric influences in the H-type II trisaccharide epitopes as a relevant element in protein−
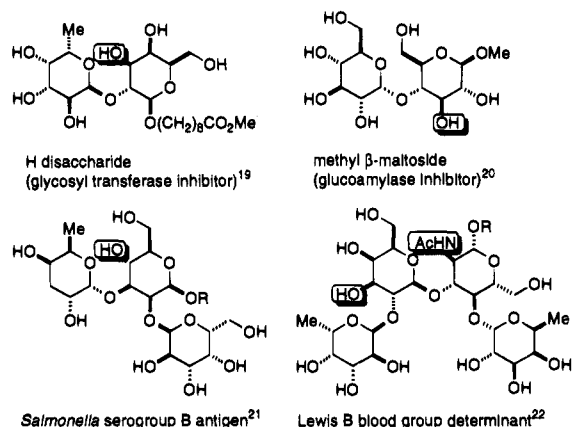
carbohydrate recognition,[18] their data provide additional supportive evidence that the ground-state conformation of carbohydrates is an important factor in binding strength.

The importance of oligosaccharide conformational preference in biomolecular recognition can be extended to other receptor−ligand systems. Epitopes of various oligosaccharides have been prepared and tested[19−22] in the same fashion as the H-type II trisaccharide studies by Lemieux and co-workers. We have identified the substituents which impose a steric influence on these substrates' conformations (Figure 6), and cross-examined them with the results of the epitope studies. For every case in which such a substituent was removed, a loss in binding affinity was observed, thus supporting the generality of this relationship.

To the best of our knowledge, this is the first study to rationally demonstrate the relationship between oligosaccharide solution conformation and protein−carbohydrate recognition. The replacement of three bridging glycosidic oxygens with three methylene groups has a minimal impact on the biological activity of the H-type II blood group trisaccharide, whereas the removal of the conformationally influential C3′-hydroxyl and C5-hydroxymethyl substituents results in a substantial decrease in binding affinity. The nearly identical gradients in biological activity for the O-trisaccharides **1−4** and C-trisaccharides **5−8** validate the utility of C-glycosides as conformational models,

(18) Interestingly, Lemieux and others have previously cited nonbonded interactions in oligosaccharide solution conformation in isolated cases: Lemieux, R. U.; Bock, K.; Delbaere, L .T. J.; Koto, S.; Rao, V. S. *Can. J. Chem.* **1980**, *58*, 631. Yan, Z.-Y.; Rao, B. N. N.; Bush, C. A. *J. Am. Chem. Soc.* **1987**, *109*, 7663.

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(23) Wang, Y.; Goekjian, P. G.; Ryckman, D. R.; Kishi, Y. *J. Org. Chem.* **1988**, *53*, 4151. Wang, Y.; Goekjian, P. G.; Ryckman, D. M.; Miller, W. H.; Babirad, S. A.; Kishi, Y. *J. Org. Chem.* **1992**, *57*, 482.

(24) In the case of the Lewis B blood group determinant, removal of the sterically influential −NHAc group was not experimentally addressed.[22]

Scheme 1[a]
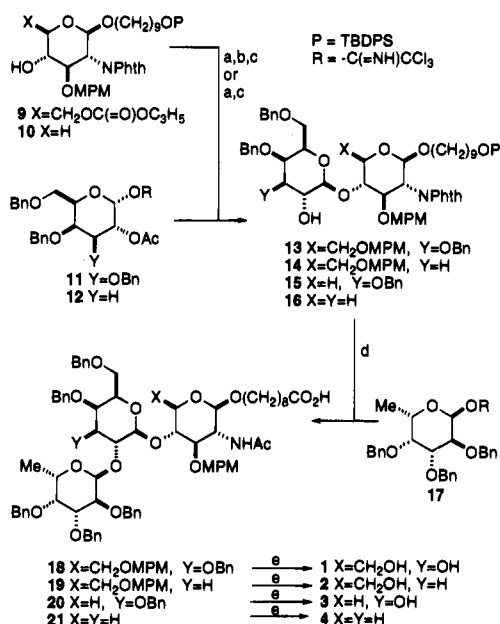
a Conditions: (a) 4 Å molecular sieves, 4:1 hexanes/CH₂Cl₂, TMSOTf, −30 °C, yields: **9 + 11**, 56%; **9 + 12**, 62%; **10 + 11**, 79%; **10 + 12**, 74%; (b) Pd(PPh₃)₄, PPh₃, HCO₂H, *n*-BuNH₂, THF; *p*-methoxybenzyl 2,2,2-trichloroacetimidate, TfOH, Et₂O, CH₂Cl₂; (c) for Y = OBn: K₂CO₃, MeOH, CH₂Cl₂, and then phthalic anhydride, Et₃N, 3 Å molecular sieves, MeOH, reflux; for Y = H: DBU, MeOH, CH₂Cl₂; overall postcoupling yields: **13**, 54%, **14**; 56%; **15**, 50%; **16**, 89%; (d) alcohol, Et₂O, TMSOTf, 25 °C, add **17**, yields: **13 + 17**, 90%; **14 + 17**, 83%; **15 + 17**, 70%; **16 + 17**, 96%; H₂NNH₂·H₂O, MeOH 80 °C; Ac₂O, pyridine, DMAP; TBAF, THF; oxalyl chloride, DMSO, Et₃N, CH₂Cl₂; NaClO₂, NaH₂PO₄·H₂O, 2-methyl-2-butene, *tert*-butyl alcohol, H₂O; overall postcoupling yields: **18**, 52%; **19**, 38%; **20**, 28%; **21**, 56%; (e) Pd(OH)₂ on C, H₂, MeOH, followed by C₁₈ reversed-phase chromatography.

and establish the importance of local conformational behavior in defining shape at the molecular level. The trends in binding affinity also validate the assumption that the UEA-I receptor site largely defines a specific bound conformation. The relevance of nonbonded interactions in oligosaccharide conformation opens up the possibility of designing carbohydrate ligands with novel conformations, resulting in increased biological activity.

Experimental Section

Materials. The synthesis of the *C*-trisaccharides **5−8** has been carried out and recently published.[14] The synthesis of the *O*-trisaccharides **1−4** has been accomplished as shown in Scheme 1. Experimental procedures and spectroscopic data for the intermediates **9−16** and **18−21** and final compounds **1−4** are included in the supporting information.

The reference compound used in the binding assays (*vide infra*) was synthesized by coupling the H-type II trisaccharide **1** and biotin hydrazide with *N*-ethyl-*N'*-[3,3-(dimethylamino)propyl]carbodiimide in pyridine, and purified by reversed-phase HPLC (*μ*Bondapak C18; Waters). *U. europaeus* lectin I (UEA-I; Vector Laboratories) and bovine serum albumin (BSA; Sigma) were obtained as dry powders. ExtrAvidin−horseradish peroxidase (HRP) conjugate (Sigma) was obtained as a concentrated solution. Enzyme-linked biotin−avidin assays were conducted in polystyrene 96-well microtiter plates (NuncImmuno Maxisorp, flat-bottomed wells) using the biotin conjugate

of **1** as the wild-type reference, and trisaccharides **1−8** as competitive inhibitors. Concentration-dependent absorbance values were determined by the rate of enzymatic oxidation of *o*-phenylenediamine (OPD), and recorded on a microplate reader (Bio-Rad 3550-UV) at 450 nm.

Quantitative Binding Assays. Plates were incubated overnight at 5 °C with 100 *μ*L/well of a 5 *μ*g/mL solution of UEA-I in 10 mM phosphate buffered saline (PBS), pH 7.4, with 0.08% NaN₃ and 0.1 mM CaCl₂. The wells were rinsed out three times in rapid succession with PBS using a multichannel microtiter plate washer (Nunc Immuno-Wash 8). Plates were then incubated for 2 h at room temperature with 150 *μ*L/well of a 1% BSA solution in PBS with 0.08% NaN₃ and 0.1 mM CaCl₂ and then rinsed out as before. Wells were incubated for 4 h at room temperature with 37.5 *μ*L/well of a 1.25 *μ*g/mL solution of **1**−biotin conjugate and 37.5 *μ*L/well of a solution of inhibitor in PBS containing 0.05% poly(oxyethylene)sorbitan monolaurate (Tween 20). Each series of inhibitors was tested in triplicate, and included one set of wells containing wild-type reference (37.5 *μ*L of a 1.25 *μ*g/mL solution of **1**−biotin conjugate) and excess inhibitor (37.5 *μ*L of a 250 *μ*g/mL solution of **1** or **5**) as a measure of nonspecific binding. The following ranges of inhibitor concentrations were used prior to dilution with reference solution:

1: 0, 0.04, 0.11, 0.34, 1.03, 3.09, 9.26, 27.8, 83.3 *μ*g/mL

5: 0, 0.98, 1.95, 3.91, 7.81, 15.6, 31.3, 62.5, 125 *μ*g/mL

2, 3, 6, 7: 0, 3.91, 7.81, 15.6, 31.3, 62.5, 125, 250, 500 *μ*g/mL

4, 8: 0, 7.81, 15.6, 31.3, 62.5, 125, 250, 500, 1000 *μ*g/mL

Wells were rinsed three times with 0.05% Tween 20 in PBS and then incubated for 1.5 h at room temperature with 100 *μ*L/well of a 1 *μ*g/mL solution of ExtrAvidin−HRP conjugate and 1% BSA solution in PBS. Wells were rinsed again, followed by addition of 150 *μ*L/well of an OPD/urea−hydrogen peroxide solution in freshly prepared 25 mM citrate−phosphate buffer (OPD-Fast; Sigma). After 6−10 min with occasional agitation, absorbance readings were recorded at 450 nm. Specific binding was determined by subtracting out the signal from wells containing excess inhibitor (nonspecific binding). A regressive line-fitting program was used to calculate the IC₅₀ values from the linear portion of the inhibition curve.

NMR Analysis. The conformational analyses of *C*-trisaccharides **5−8** using vicinal coupling constant data have recently been published.[14] 2D NOESY ¹H NMR spectra were recorded on a 500 MHz spectrometer (Bruker Magnetics) at 263 K. A series of 512 experiments of 16 scans each were performed with a variable mixing time of 250 ± 20 ms, and a relaxation delay of 1.67 s between pulses.

Supporting Information Available: Experimental procedures and spectroscopic data for intermediates leading to and including *O*-trisaccharides **1−4** and 2D NOESY NMR spectra for *C*-trisaccharides **6** and **7** (14 pages). This material is contained in many libraries on microfiche, immediately follows this article in the microfilm version of the journal, can be ordered from the ACS, and can be downloaded from the Internet; see any current masthead page for ordering information and Internet access instructions.

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