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Conformational Constraints: Nature Does It Best with Sialyl Lewis^x

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Selectins play a key role in the inflammatory cascade. The interaction with their physiological ligands containing the tetrasaccharide sialyl Lewis^x (sLe^x) leads to the recruitment of leukocytes from the vascular system to the site of injury. To facilitate the interaction under the shear stress conditions present in the blood vessel, the conformation of sLe^x is stabilized via lipophilic inter-residual contacts. sLe^x and two analogs were synthesized and evaluated for selectin binding, average conformation, and conformational dynamics. We could show that the methyl group in L-fucose is optimally suited to stabilize the sLe^x core through an interaction with the β-face of D-galactose and thus enables binding to the selectins under shear stress conditions.

Introduction

The recruitment of leukocytes to inflamed tissue mediated by the cell adhesion molecules E-, P-, and L-selectin is essential for immune defense. The interaction of selectins leads to the characteristic tethering and rolling of leukocytes on the vascular endothelium, followed by firm adhesion and migration to the site of injury.[1] However, excessive extravasation of leukocytes can cause acute or chronic reactions, as observed in reperfusion injury, stroke, or rheumatoid arthritis.[2] Therefore, the antagonism of the selectins is generally considered to be a potential therapeutic approach for the treatment of inflammatory diseases.[3]

The known physiological ligands of E-, P-, and L-selectin contain the carbohydrate epitope sialyl Lewis^x [sLe^x (**1a**), Figure 1].[4] Although the affinity of sLe^x to E-selectin is in the micro- to millimolar range – K_d values between 107 and 1800 μM are reported[5] – the sLe^x motif serves as a lead structure in the search for high-affinity selectin antagonists.[6]

For dynamic non-equilibrium processes, such as E-selectin-mediated rolling of leukocytes on activated endothelial cells, conformational entropy is expected to play an important role. Under the influence of shear stress present in blood vessels, preferably the fraction of the oligosaccharide ligand that is preorganized in the bioactive conformation binds to the receptor. Because information regarding the bound conformation of sLe^x is available from trNOE NMR experiments[7] and X-ray crystallography,[8] a possible strategy to high-affinity mimetics is their preorganization in the bioactive conformation. In an initial attempt, the Glc*N*Ac moiety was replaced with cyclic 1,2-*trans*-diols.[9,10] A significant reduction in the affinity associated with the replacement of the Glc*N*Ac moiety by conformationally flexible diols impressively demonstrated the effect of preorganization. However, an improved affinity was achieved when the Lewis^x (Le^x) core was stabilized by steric constraints, that is, with an equatorial methyl substituent adjacent to the linking position of the L-fucose residue.[10] Because this additional methyl substituent is not in direct contact with the protein surface (deduced from the sLe^x/E-selectin X-ray structure[8] and STD-NMR investigations[10b]), the increased affinity was assigned to a higher degree of preorganization of the core in its bioactive conformation.

Results and Discussion

In this communication, we investigated a second constraint contributing to the stabilization of the Le^x core. Numerous reports reviewed the replacement of the L-Fuc moiety in sLe^x antagonists.[6,11–13] Thus, when in glycopeptide **2** L-fucose (rIC_{50} = 1) was replaced by D-arabinose (**2b**, rIC_{50} = 2.8), a substantial loss of affinity resulted, whereas with L-galactose (**2c**, rIC_{50} = 1.25) affinity remained nearly unchanged (Figure 1).[13b]

According to Lemieux, monosaccharides can establish – besides polar interactions – lipophilic contacts via their ring methine protons. Some monosaccharides even display ex-

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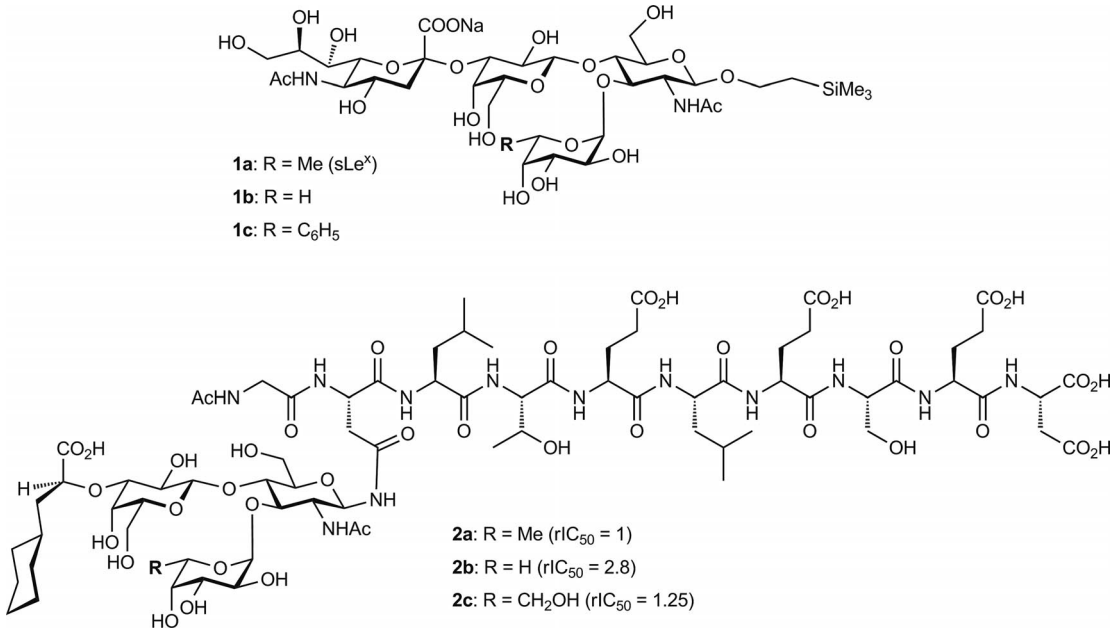


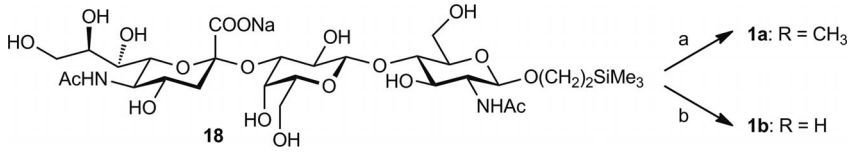
Figure 1. The carbohydrate epitope recognized by the selectin family. In sLe^x (**1a**) and sLe^x derivatives **1b** and **1c** (containing a modified fucose residue) the influence of an intramolecular lipophilic stabilization was studied. Glycopeptide derivatives **2a**–**c** with modified fucose were reported by Kunz et al. to improve their biological half-life.[13b] Relative IC_{50} values (rIC_{50}) were calculated by dividing the IC_{50} of the substance of interest by the IC_{50} of the reference compound, here **2a**.

tended lipophilic patches, also called their lipophilic face.[14] Because of the unusually high conformational stability of the Le^x core,[15] we hypothesized that in sLe^x (**1a**) the methyl group of the L-fucose moiety establishes a lipophilic contact with the β-face of D-galactose and thereby contributes to the stability of the core. Because the 5-methyl group of L-fucose is not in direct contact with the protein,[8,16] no contribution to the enthalpy is expected. In contrast, in arabinose derivative **1b** where this stabilizing effect is no longer present, affinity should be markedly reduced.

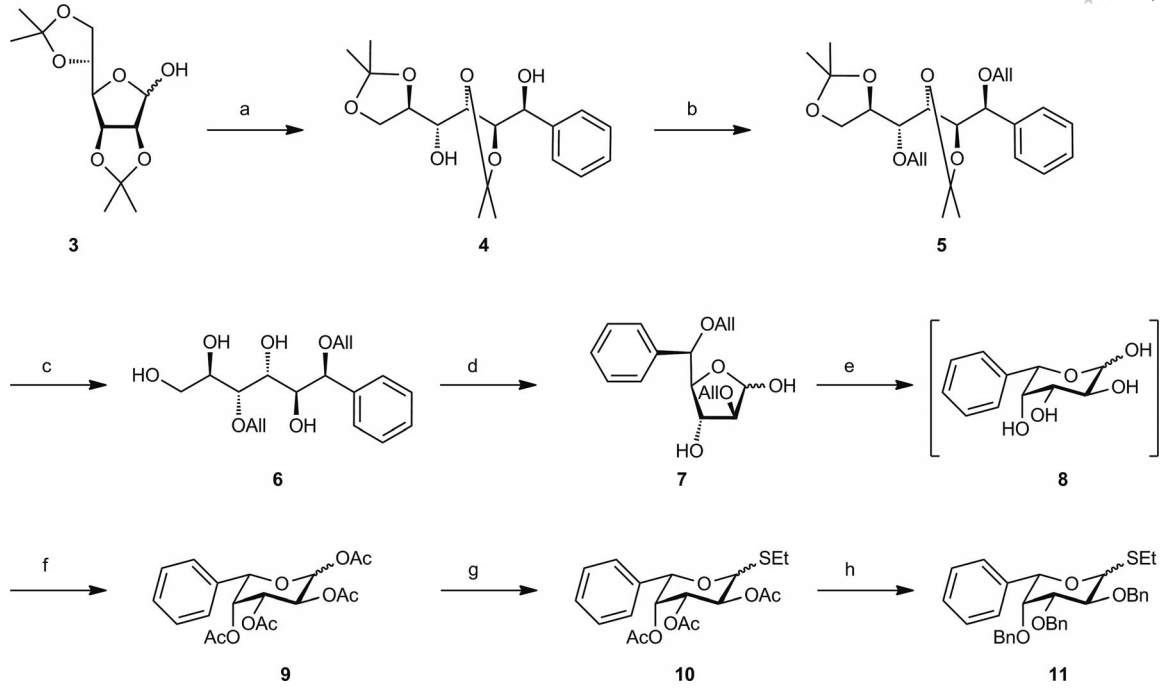
Hydrophobic contacts of carbohydrates with their binding partners have been broadly investigated.[17] Thus, in galectins[18] or carbohydrate-specific antibodies[19] aromatic side chains of amino acids mediate hydrophobic contacts with a carbohydrate ligand. In another example, the alignment of the β-face of galactosides with toluene was reported,[20] which occurs in a manner similar to that of the hydroxyphenyl side chain of tyrosine in an *Artocarpus hirsute* lectin.[21] Furthermore, by NMR and molecular modeling, Jiménez-Barbero and Bartik could show the modes of stacking interaction of a variety of monosaccharides with either phenol or aromatic amino acid side chains.[22] Finally, intramolecular conformational stabilization as a result of such stacking interactions between carbohydrates and aromatic groups in glycoconjugates was reported by Terraneo et al.[23]

For our study on the stabilization of the core of sLe^x by an intramolecular hydrophobic contact, we synthesized derivatives **1a**–**c** (Figure 1). When the L-fucose moiety in **1a** is replaced by D-arabinose (→**1b**), a loss of intramolecular stabilization should result. In contrast, a phenyl group (→**1c**) might restore this effect. For the synthesis of **1a** and **1b**, trisaccharide **18**[24] was glycosylated with fucosyltransferase FucT-III by using the glycosyl donors GDP-Fuc (→**1a**) or GDP-Ara (→**1b**), respectively (Scheme 1, for experimental details see Supporting Information).[25]

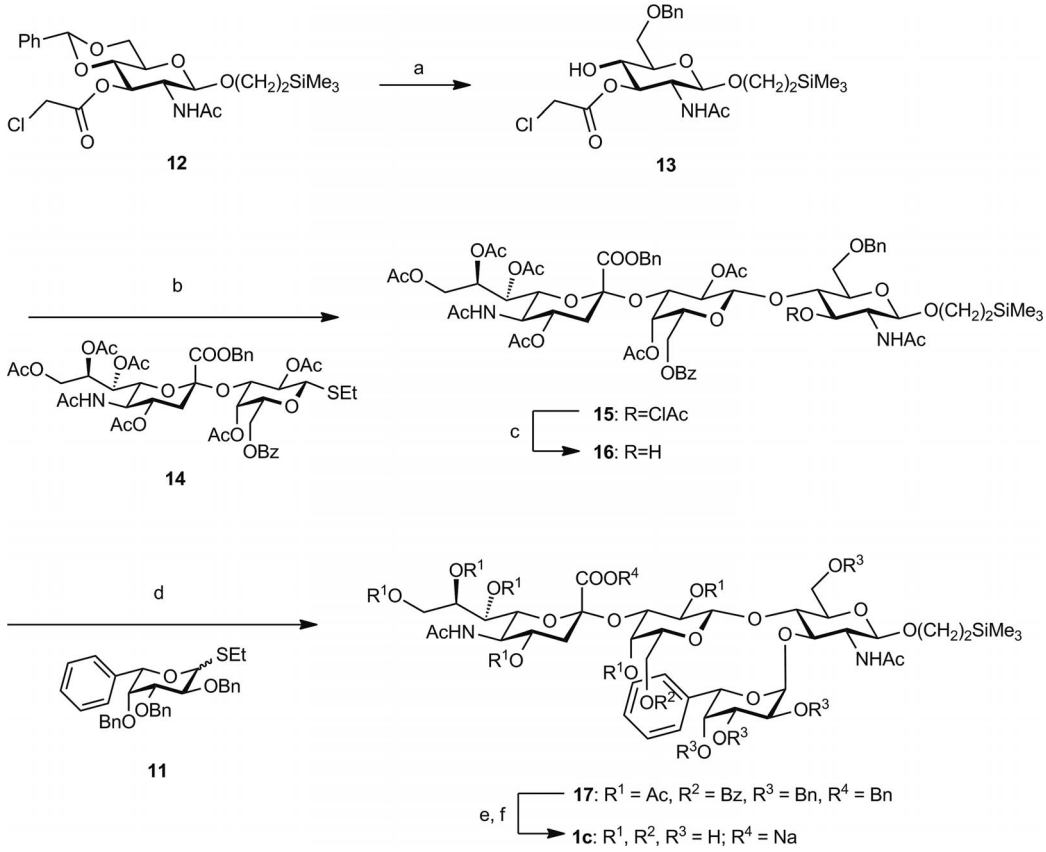
For the synthesis of derivative **1c**, phenyllithium was added diastereoselectively to D-mannofuranose diacetonide **3**, following a strategy reported by Mekki et al. (Scheme 2).[26] The free hydroxy groups in **4** were transformed into allyl ethers (i.e., **5**). Cleavage of the acetonides under acidic conditions (→**6**) followed by iodate-mediated oxidative cleavage of the terminal diol yielded bis-allylated 5-C-phenylarabinose derivative **7** in high yield. After cleavage of the allyl ethers, **8** was acetylated (→**9**) and treated with ethyl mercaptan under Lewis acid catalysis. 5-C-Phenylarabinose donor **11** was finally obtained by replacing the acetate protection in **10** by benzyl ethers.



Scheme 1. Reagents and conditions: (a) FucT-III, GDP-Fuc (89%), (b) FucT-III, GDP-Ara (81%).



Scheme 2. Reagents and conditions: (a) PhLi, Et_2O, –78 to –20 °C, 20 h, 76%, 4:1 *dr*; (b) AllBr, NaH, DMF, 0 °C to r.t., 1 h, 93%; (c) HOAc/H_2O (4:1), r.t., 10 h, quant.; (d) $NaIO_4$, CH_2Cl_2, aq. $NaHCO_3$, 0 °C to r.t., 1 d, 77%; (e) Pd/C, CSA, dioxane/H_2O, 95 °C, 2 d; (f) Ac_2O, DMAP, pyridine, r.t., 45 min, 69% (over 2 steps, α/β = 1:2); (g) EtSH, CH_2Cl_2, TMSOTf, 0 °C to r.t., 3 h, 65% (α/β = 1:2); (h) 1. NaOMe, MeOH, r.t., 3 h; 2. NaH, BnBr, DMF, 0 °C to r.t., 1 h, 88% (over 2 steps).



Scheme 3. Reagents and conditions: (a) $BH_3{\cdot}NMe_3$, $AlCl_3$, H_2O, THF, r.t., 2 h, 92%; (b) DMTST, 4 Å MS, CH_2Cl_2, 0 °C, 2.5 d, 68%; (c) $H_2NC(S)NH_2$, 2,6-di-*tert*-butylpyridine, DMF, 70 °C, 22 h, 63%; (d) 1. **11**, Br_2, CH_2Cl_2, 0 °C, 30 min; 2. **16**, Et_4NBr, 4 Å MS, DMF/CH_2Cl_2, r.t., 3 d, 37%; (e) H_2 (1 atm), Pd/C, dioxane/H_2O, r.t., 14 h; (f) NaOMe, MeOH, r.t., 12 h, 59%.

For the assembly of tetrasaccharide **1c** (Scheme 3), the benzylidene in the chloroacetylated Glc*N*Ac derivative **12**[24] was selectively reduced[27] to yield acceptor **13**, which was glycosylated with donor **14**[28] to give trisaccharide **15**. The chloroacetyl group was then selectively removed by using thiourea, and resulting acceptor **16** was glycosylated with 5-*C*-phenylarabinose donor **11**. Tetrasaccharide **17** was then deprotected by treatment with base and careful hydrogenolysis to selectively yield **1c** without affecting the sterically inaccessible benzylic position of the 5-C-phenylarabinose.

In a competitive binding assay,[29] the affinity of sLe^x (**1a**) and its derivatives **1b** and **1c** for E-selectin was determined. sLe^x (**1a**) showed an affinity of 1 mM (= rIC_{50} of 1).[30] Arabinoside **1b** was inactive up to 10 mM ($rIC_{50} > 10$), whereas 5-C-phenylarabinoside **1c** could partially restore binding to E-selectin ($rIC_{50} = 2.8$).

To rationalize the biological affinities, the preferred conformation of sLe^x and its two analogs in aqueous solution was studied by jump-symmetrized ROESY[31] NMR experiments (Figure 2). The average distances between the axial H5 of L-fucose and its analogs (→**1a**–**c**) and the axial H2 of the D-galactose moiety were determined by analysis of the build-up curves of the rotating frame nuclear Overhauser effect. In sLe^x (**1a**) and arabinoside **1b**, the average distance between those two nuclei is nearly identical (**1a**: 2.54 Å, **1b**: 2.49 Å), whereas in 5-C-phenylarabinoside **1c** (2.75 Å) it is increased by 10%.

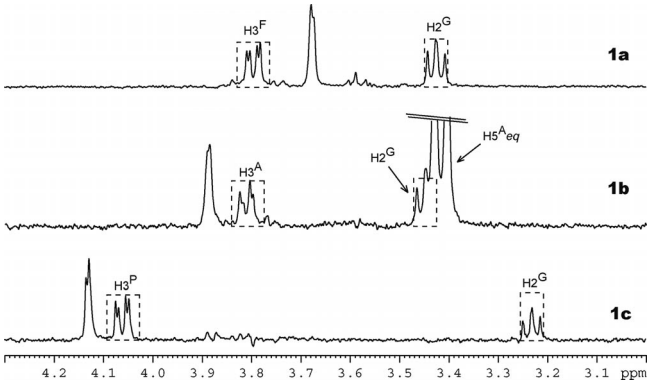


Figure 2. Selective ROESY experiments measuring the transfer of magnetization from the axial H5 of L-fucose or its analogs in selectin antagonists **1a**, **1b**, and **1c** to $H2^G$ at equal mixing times. The distance measurement was calibrated on the ROE intensities of the axial H5 – axial H3 ROEs in the $^1C_4$ chair conformation of the fucose/fucose derivatives (equal intensities are indicated by boxes of equal height on $H3^F$, $H3^A$, and $H3^P$ for **1a**, **1b**, and **1c**, respectively). Inter-residue distances between the axial H5 of L-Fuc, D-Ara, or 5-C-phenyl-D-Ara and axial $H2^G$ can be calculated from the analysis of the build-up curves of the ROE transfer. The distance differences among the selectin ligands are illustrated by the height of the boxes fixed at the value for **1a**. Superscript A is used for arabinose, F for fucose, G for galactose, and P for 5-C-phenylarabinose.

To gain deeper insight into the dynamic range of the distances between the fucose-derived residues and the D-galactose moiety, molecular dynamics (MD) simulations were performed. MD simulations (Figure 3) in an aqueous environment clearly demonstrate the flexibility of the arabinose residue in **1b** vs. the conformational stability in derivatives **1a** and **1c**.

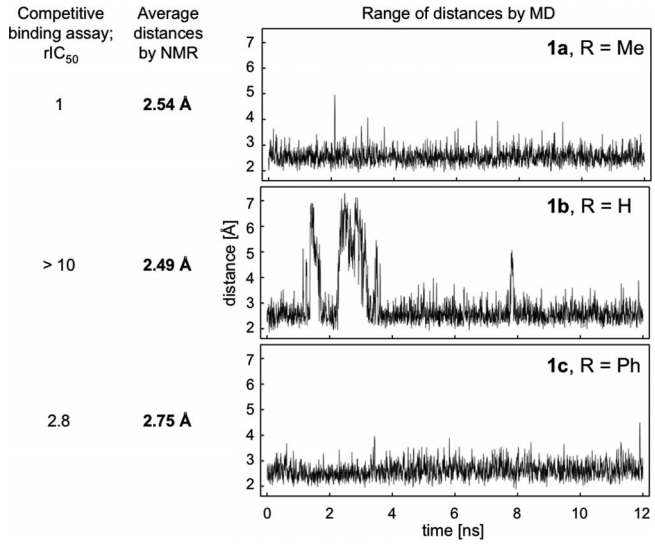


Figure 3. Affinities of selectin ligands **1a**–**c** determined in a competitive binding assay[29] Whereas **1a** and **1c** show comparable affinities, arabino derivative **1b** did not show binding to E-selectin. The average inter-residue distances were measured by ROESY NMR experiments between the axial $H2^G$ and the axial H5 of L-Fuc, D-Ara, or 5-C-phenyl-D-Ara. The dynamic range of distances between these two nuclei in **1a**, **1b**, and **1c** was assessed in a 12-ns molecular dynamics simulation and are plotted against time.

It should be noted that the population of **1b** at internuclear distances of 5–7 Å results from torsion of the glycosidic linkage of the arabinose moiety and not from a ring flip as a consequence of a reduced number of equatorial substituents compared to **1a** and **1c** (for the conformational stability of fucose and its derivatives, see the Supporting Information).

Conclusions

Sialyl Lewis^x (**1a**) and analogs **1b** and **1c** were synthesized and analyzed for E-selectin binding. Although the average conformation of the core in sLe^x (**1a**) and arabinoside **1b** as determined by NMR spectroscopy are similar, their bioactivities differ dramatically. Because the methyl group of L-fucose does not participate in protein binding, the loss of activity of **1b** is related to an increased flexibility of the core, as observed from molecular dynamics simulations. When the methyl group of L-fucose was replaced by the lipophilic phenyl group (**1a** → **1c**), biological activity was regained as a result of the increased conformational stability originating from the inter-residual contact between L-fucose and 5-C-phenyl-D-Ara with the galactose moiety.

Nature has chosen sLe^x (**1a**) as a binding epitope for the selectins for a number of reasons. First, specificity is gained through the large number of hydrogen bonds involved in the interaction.[8,32] In blood vessels, leukocytes bind to the

selectins under shear stress, conditions where preorganization of the pharmacophoric groups of sLe^x (**1a**) in their bioactive conformation[7] is essential for success. Our data are a further example of lipophilic saccharide interactions[14] and document for the first time a lipophilic interresidual stabilization of an oligosaccharide structure.

Experimental Section

Conformational Analysis by NMR: The samples for the ROESY analysis consisted of ca. 5 mg of either **1a**, **1b**, or **1c**, solvated in 99.8% D_2O (Armar Chemicals) at pH ≈ 7.0 (uncorrected for D_2O) and were measured without the addition of a buffer. Shigemi NMR tubes were used to reduce the sample volume (200 μL) needed for measurement. Measurements were performed at 25 °C using a Bruker DMX 500 NMR spectrometer. Chemical shifts were referenced with respect to earlier work,[10a] which assigned a chemical shift of 4.60 ppm to the H5^F resonance of **1a**.

The doubly-selective homonuclear Hartmann–Hahn scheme[33] was used to selectively transfer magnetization from H6^F to H5^F. This scheme allowed a highly selective transfer of magnetization from H6^F to H5^F through their scalar coupling. The selective excitation of H5^F allowed an accurate quantification of this resonance by avoiding the excitation of residual H_2O, which has a similar chemical shift. To remove any remaining magnetization from H6^F, a selective gradient echo at the frequency of H5^F was applied. A 200 ms REBURP[34] 180° refocusing pulse was applied to the H5^F resonance. The REBURP pulse was sandwiched by a pair of Gaussian shaped gradients of 1 ms each and an amplitude of 20 G/cm. This additional spectral filter ensured that the observed ROESY[35] peaks were due to magnetization that originated from the H5^F resonance.[36]

The jump-symmetrized CW-ROESY variation of the ROESY sequence was used in all experiments to minimize TOCSY artifacts.[31] This sub-element of the pulse sequence was inserted following the selective gradient echo. During the ROESY period, the transmitter frequency was shifted up or downfield during the first or second half of the mixing-time, respectively. The highfield spin lock was applied at 4.9 ppm and the lowfield at 0.9 ppm. The spin lock was a rectangular pulse of 2 kHz amplitude. For each compound, 10 experiments were run to record a build-up curve of the ROE transfer. The 10 experiments were sampled with increasing durations of the spin lock, beginning after 50 ms, and repeated after each 50 ms increment, resulting in a 500 ms spin lock duration for the final experiment.

Following the application of the spin lock, the transmitter was returned to the center of the spectrum, at 2.9 ppm, and the FID measured using 4096 complex points to sample a bandwidth of 7 ppm. To achieve a high signal-to-noise ratio, 1024 scans were measured for each mixing time. Using a prescan delay of 3 s, on average the experiments lasted approximately 1.2 h each. The NMR spectroscopic data were analyzed using XWINNMR version 3.0 operating on a Silicon Graphics O2. The spectra were apodized with an exponential decay function with 2 Hz line broadening. An additional advantage of the selective experiments was the lack of signal overlap, which allowed to integrate the signals without interference from other resonances.

To determine the internuclear distances, the rotating-frame cross-relaxation rates were calculated from the build up curves. Traditionally the cross-relaxation rate is determined from fitting the spectra to a biexponential function that depends upon both the cross- and auto-relaxation rates.[37] The extent to which accurate cross-relaxation rates can be determined by this manner depends upon how well the auto-relaxation rate can be defined. Alternatively, it is possible to remove the dependence on the auto-relaxation by dividing the target-peak by the source-peak for each value of the mixing time.[38] The resulting function is a hyperbolic tangent, the argument of which is the product of the cross-relaxation rate and the mixing time. For the longest mixing times performed and highest rate of cross-relaxation expected for the compounds studied herein, the hyperbolic tangent function is indistinguishable from a linear function, hence offering the potential to apply linear regression to extract the cross-relaxation rate. The above procedure resulted in values that were well described by linear functions. Removal of autorelaxation through the conversion of biexponential into hyperbolic tangent functions has as well been recently applied to determine accurate relaxation rates in cross-correlation measurements.[39]

Molecular Modeling: The 3D structures of all compounds were generated using MacroModel.[40] Next, a conformational search was performed to identify the global minimum conformation by sampling a total of 10 000 structures (MacroModel, mixed torsional/low-mode sampling method, extended torsional sampling, OPLS 2005 force-field,[41] implicit water solvent model). A periodic boundary system was created by placing the global minimum in a box of preorganized TIP3P water molecules. The system charge was neutralized and sodium and chloride ions were added to reach a physiological electrolyte concentration of 0.15 M. Special attention was paid to building a proper solvation shell around the solute: First, the solvent environment was minimized by using a gradient criterion of 0.1 kcal/mol followed by a 24 ps molecular dynamics (MD) simulation, so that the water molecules could reorganize around the solute (the geometry of the solute was kept fixed). The whole system was then completely minimized by using a gradient criterion of 0.05 kcal/mol. A 12-ns MD simulation was performed by using NPT ensemble and standard conditions (T = 300 K, p = 101.325 kPa) with frames sampled every 1.2 ps. All MD simulations were done using Desmond.[42] For the statistical analysis, structural data (dihedral angle and interatomic distance values) were determined from the 9′950 frames collected during the MD run (first 50 frames were skipped due to equilibration of the system).

Supporting Information (see footnote on the first page of this article): Synthetic and analytical details of the described structures. Traces of the molecular dynamics simulation of ring dihedral angles of the fucose residue in **1a**, the arabinose residue in **1b**, and phenylarabinose residue in **1c**. Copies of the ^{1}H NMR and ^{13}C NMR spectra.

Acknowledgments

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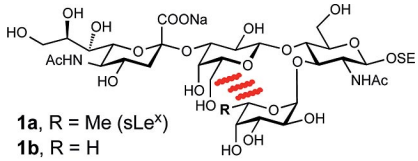
Conformation Analysis

To facilitate the interaction under the shear stress conditions in the blood vessel, the conformation of sLe^x is stabilized via lipophilic inter-residual contacts. The methyl group in fucose is optimally suited to stabilize the sLe^x core through an interaction with the galactose moiety.



1a, R = Me (sLe^x)
1b, R = H
1c, R = C_6H_5

A. Titz, A. Marra, B. Cutting, M. Smieško, G. Papandreou, A. Dondoni, B. Ernst* 1–7

Conformational Constraints: Nature Does It Best with Sialyl Lewis^x

Keywords: Glycoproteins / Conformational analysis / Carbohydrates / Stacking interactions

