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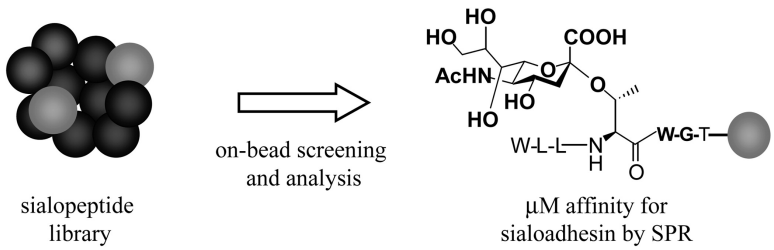

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Glycopeptides as Oligosaccharide Mimics: High Affinity Sialopeptide Ligands for Sialoadhesin from Combinatorial Libraries

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Two different sialic acid containing glycopeptide (sialopeptide) libraries were synthesized using the portion mixing method and ladder synthesis. The libraries were attached via an IMP spacer and a photolabile linker to $PEGA_{1900}$ resin in order to facilitate rapid and unambiguous structural analysis of hits by MALDI-TOFMS. One library contained a lactamized sialic acid moiety at the N terminus of a pentapeptide, while a second library displayed a sialic acid residue at the center of a heptapeptide. The sialopeptide libraries were screened against the recombinant binding domain (SnD1) of a sialic acid binding Ig-like protein, sialoadhesin (Siglec-1). No ligands were identified from the lactamized sialic acid library, underscoring the importance of the carboxylic acid moiety for binding. Screening of the second gave few distinct hits (∼0.03% of library) with a high consensus. The high-affinity ligands contained, in most cases, a WG motif following the sialylated Thr. The strength of binding of selected ligands was determined by surface plasmon resonance. The best sialopeptide ligand, WLLT(Sa)WGT, exhibited micromolar affinity of SnD1; >10 times the affinity of SnD1 to 3′-sialyl lactose.

Introduction

Sialic acids, a group of related acidic monosaccharides usually found as the terminal sugar of complex oligosaccharides of glycoproteins and glycolipids, are instrumental in regulating biological processes either by inhibiting particular interactions through masking or by mediating the binding of carbohydrate binding proteins.[1] Sialic acid binding proteins are ubiquitous in nature and are of animal, microbial, or plant origin.[1] In mammals, much focus has been placed on the selectins because of their implication in the inflammatory response[2] and their possible involvement in cancer metastases.[3] In the last 10 years, a novel set of membrane-bound, I-type lectins that recognize sialylated glycans on cell surfaces have been discovered[4,5] and recently reviewed.[6] These proteins, designated "Siglecs",[7] comprise among others, sialoadhesin (Siglec-1),[4] CD22 (Siglec-2),[5] CD33 (Siglec-3),[8] and the myelin-associated glycoprotein (Siglec-4).[9] Each Siglec exhibits a characteristic preference for both the type of sialic acid recognized and its linkage to the penultimate sugar. Furthermore, they are expressed in a highly tissue-specific manner, suggesting that they perform distinct, nonoverlapping functions in both the hemopoietic (Siglecs 1−3 and 5−10), and nervous (Siglec 4) systems.

Sialoadhesin is the largest cell surface member of the Siglec family and consists of a membrane-distal V-set immunoglobulin-like domain followed by 16 C2-set domains.[10] It is expressed uniquely by macrophage subsets in various tissues and by macrophages recruited to sites of chronic inflammation and recognizes the oligosaccharide sequence Neu5Acα(2 → 3/6)Galβ(1 → 3/4)-GlcNAc/GalNAc in either sialoglycoproteins or gangliosides.[6,9] It has been shown that the N-terminal V-set immunoglobulin-like domain of sialoadhesin is necessary and sufficient for binding of the ligand.[11−13]

In the quest for successfully designing carbohydrate-based therapeutics, there has been a mandate to develop simpler, smaller molecules that can mimic the activity of complex oligosaccharides that are notoriously challenging to synthesize or isolate in sufficient quantities. Although there have been various approaches to identifying different classes of compounds that mimic oligosaccharides,[14−16] it has been unequivocally demonstrated that complex oligosaccharides can be mimicked by O-linked glycopeptides.[17−19] In principle, the carbohydrate portion of the glycopeptide should provide the specificity of the interaction, while the relatively flexible peptide scaffold may actively participate in the ligand−receptor interaction, thus enhancing the overall affinity of the ligand to the receptor. These types of synthetic neoglycopeptides mimetics can be rapidly generated and can give important information about the nature and topology of the ligand−receptor interaction. Furthermore, they may give rise to lead compounds for the development of drugs. Combinatorial library synthesis has emerged into powerful methodology for the rapid identification of lead compounds for drug discovery.[20] Although there are several methods for the

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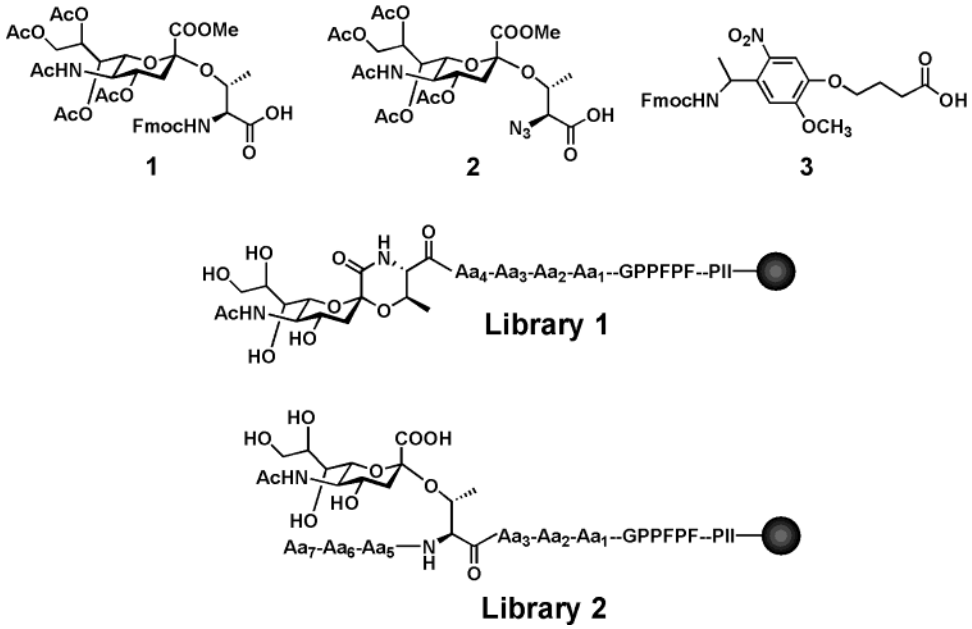

Figure 1. Schematic representation of resin-bound sialopeptide libraries 1 and 2. Sialylated threonine building blocks **1** and **2** and photolabile linker **3** (Pll) were used. Amino acids (Aa) were introduced as mixtures (9:1 ratio) of N^{α}-Fmoc- and Boc-protected amino acids to achieve ladder synthesis of the libraries.

generation of libraries, the portion-mixing or split-and-mix method[21,22] has been most frequently implemented for the generation of "one-bead-one-compound" libraries.[23] The unambiguous identification of the minute amounts (~80 pmoles) of compound attached to a single bead has been a liability of this method. Modification of the ladder synthesis strategy,[24,25] a method in which a small portion of the growing oligomer is capped in each synthetic step,[26] has been used to solve this problem for glycopeptide libraries. For this method to be successful, the chemical reactivity of the capping agent and building block must be equal. Consequently, in an Fmoc-based synthesis of a glycopeptide, the capping agent was the Boc-protected counterpart of the Fmoc amino acid building block.

The current work applies a combinatorial strategy toward the identification of sialic acid-containing glycopeptides (sialopeptides), which are mimics of the natural ligands of sialoadhesin. The high affinity ligands thus obtained can be used to further our understanding of the ligand−receptor interactions of Siglecs and may also find application as targeting devices for tissues that express this lectin and related proteins. Previously, we reported the synthesis of the two sialic acid containing amino acid building blocks, **1** and **2** (Figure 1).[27] Currently, we describe the utilization of these building blocks in the synthesis of two sialopeptide libraries (Figure 1) and present herein the results of screening the libraries with the carbohydrate binding domain of sialoadhesin (SnD1)[28] as well as a quantitative measurement of the affinity of the library hits for the receptor using surface plasmon resonance.

Results and Discussion

Synthesis of Libraries 1 and 2. *General Construct.* A general library construct depicted in Figure 1 was synthesized in order to allow direct, on-bead assays and to facilitate expedient cleavage of active compounds from the solid support for rapid analysis. The acid- and base-stable photolabile linker (**3**)[29] allowed a one-step evaluation of the ladder of oligomer fragments by MALDI-TOFMS, thus providing the sequence of the sialopeptide. Phenylalanine was incorporated into the peptide mass spacer, GPPFPF, to improve sensitivity in MALDI-TOFMS analysis[30] and to ensure a reasonably uniform response from the various fragments. Arginine, previously reserved for this purpose,[25] was not used in the present study because the prolonged acidic conditions required for removal of the Pmc side chain protecting group induced cleavage of the acid-sensitive *O*-sialic acid linkage.

Library 1. For matching the reactivity of capping agent and building, Boc-protected amino acids were used as capping agents in a Fmoc-based glycopeptide synthesis protocol.[24] In the library, all natural amino acids except for Arg, Asp, Cys, Glu, and Lys were included. Four amino acids, Tyr, His, Trp, and Phe, were doubly represented, and the unnatural amino acid cyclohexylalanine (Cha) was included as an additional lipophilic moiety. A mixture of N^{α}-Fmoc- and N^{α}-Boc-protected amino acids (9:1 ratio; 2 equiv total) was coupled to the peptide mass spacer, GPPFPF, using TBTU/NEM methodology (the last amino acid of the IMP spacer, Gly, had been coupled as a mixture of Fmoc and Boc amino acids in order to generate the "base peak" in the MALDI-TOF spectra of resin-bound sialopeptides). After the introduction of randomized positions, building block **1** was introduced after TBTU/NEM activation. Removal of the Fmoc-protecting group using piperidine resulted in an immediate attack of the α-amino group on the sialic acid methyl ester to form the lactam.[27] Results from MALDI-TOFMS analysis of some library members indicated that the ladder synthesis was very efficient. The truncated oligomer fragments were clearly detected, and amino acid mass differences could be distinguished with 0.2 mass unit accuracy (Figure 2).

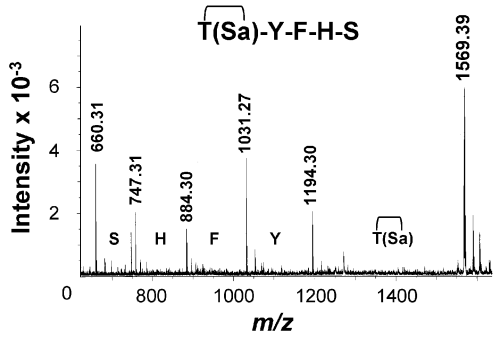

Figure 2. Example of MALDI-TOF mass spectrum of a bead from library 1 recorded in positive mode using CHC matrix. Each peak in the spectrum represents the mass of a capped oligomer fragment. The mass difference between peaks 1569.39 and 1194.30 corresponds to the lactamized moiety formed by reaction between the amino terminus of Thr and the sialic acid methyl ester.

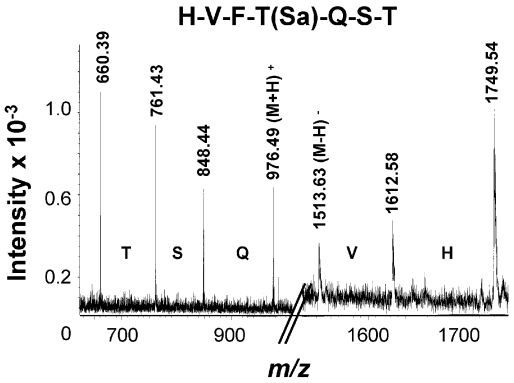

Figure 3. Example of MALDI-TOF mass spectrum of a bead from library 2. The left part of the figure shows the spectrum recorded in the positive ion mode, while the right part represents the spectrum recorded in the negative ion mode. The mass difference between the last peak of the spectrum recorded in the positive ion mode (M = 975.49) and the first peak of the spectrum recorded in the negative ion mode (M = 1514.63) is equal to the mass of the sialic acid-linked threonine building block (392.36) plus the following amino acid, 147.14, corresponding to Phe.

Library 2. The synthesis of library 2 (Figure 1) up to the first three randomized positions was carried out as described for the synthesis of library 1. At that point, building block **2** was introduced after TBTU/NEM activation. The methyl ester and acetyl groups from the carbohydrate residue were removed by treatment with LiOH/$CaCl_2$ in *i*-propyl alcohol−water solution, as previously described.[27] After reduction of the azido group using DTT and DBU in DMF,[31] three more randomized positions were introduced. It should be noted that no acid-labile amino acid side chain protecting groups other than Boc were utilized during the synthesis of both libraries because of the acid lability of the sialic acid linkage. The Boc group was removed by treatment with 10% TFA in CH_2Cl_2 for 30 min, conditions which left the glycosidic linkage intact. The hydroxyl function of Ser, Thr, and Tyr were unprotected during the library synthesis. Analysis of the completed library by MALDI-TOFMS presented a veritable conundrum. The truncated oligomer fragments *without* the sialic acid building block were detectable only in the *positive* ion mode, whereas fragments *containing* the sialic acid building block were seen only in the *negative* ion-mode. Furthermore, despite extensive washing of the beads before analysis, each fragment peak displayed several sodium and potassium adduct peaks, thus complicating the readout of the laddered sequence. The problem was solved by combining spectra acquired in both the positive and negative ion modes using the basic matrix, 6-aza-2-thiothymine (Figure 3). In the spectrum shown in Figure 3, the mass difference between the last peak of the spectrum recorded in the positive ion-mode (M = 975.49) and the first peak of the spectrum recorded in the negative ion-mode (M = 1514.63) is equal to the mass of the sialic acid linked threonine building block (M = 392) plus the following amino acid. In this spectrum, the mass of that amino acid is 147.14, corresponding to phenylalanine. The number of multiple ion peaks was reduced by addition of cation-exchange resin (∼5 beads/library bead) to the extracted sialopeptide after photolytic release.

Solid-Phase Screening of Libraries 1 and 2 with Sialoadhesin SnD1. A portion of library 1 (200 mg, ∼82 000 beads) was incubated with the fluorescent labeled SnD1 (the monomeric form of the membrane-distal V-set immunoglobulin-like domain of sialoadhesin (MW 13 298 Da)) overnight. After washing, careful inspection of the library showed no beads with any degree of fluorescence, that is, no hits. This result is in keeping with literature data that show that for most sialic acid binding proteins, a negatively charged carboxylate group at the C-1 position is critical for recognition.[1] Through X-ray crystallography,[13] site-directed mutagenesis[12] and NMR studies,[32] the molecular basis for carbohydrate recognition by sialoadhesin has been elucidated. It was established that when in the α configuration, the carboxyl group of sialic acid forms a key stabilizing salt bridge with Arg97.

Incubation of a similar quantity of library 2 (200 mg, ∼82 000 beads) with fluorescent-labeled SnD1 resulted in very few distinctly fluorescent beads (∼23). Intensely fluorescent beads were visually distinguished from less fluorescent ones and transferred to the MALDI-TOFMS target where spectra were recorded in both the positive and negative ion-modes. The recording of spectra in the negative mode was generally less sensitive as compared to the positive mode. Of the 23 library hits, the complete sequence of 19 sialopeptides was obtained, and these are listed in Table 1. The remaining four beads yielded only partial sequences from the spectra obtained in the positive-ion mode.

The hits from the screening of library 2 displayed a high consensus for a WG dipeptide motif on the C-terminal side of the sialic acid building block (13 out of 19). A higher sequence consensus was observed for intensely fluorescent beads than for the less fluorescent beads, suggesting that the dipeptide motif may be essential for tight binding. In these hits, the WG dipeptide motif was followed by either Thr or His. The three amino acids on the amino side of the sialylated threonine did not display any consensus but could be loosely classified as nonpolar. The larger aromatic amino acids (W, F, Y, H, and Cha) that were doubly represented during the library synthesis were clearly not preferred at the amino terminus of lead sialopeptides (only 14 out of 57 possible positions in the 19 lead compounds were occupied by these amino acids). Of the less intensely fluorescent library hits, the WG dipeptide consensus was observed in 40% of the

Table 1. Library Hits: Mass of Sialopeptides Recognized by SnD1 during Solid-Phase Library Screening[a]

intensely fluorescent beads				fluorescent beads			
peptide[b]	sequence[c]	mass expected	mass observed	peptide[b]	sequence[c]	mass expected	mass observed
4	WLLT(Sa)**WG**T	1806.73	1805.81	**13**	GSAT(Sa)**WG**G	1565.56	1565.20
5	AGLT(Sa)**WG**T	1635.63	1636.79	**14**	LMPT(Sa)**WG**Q	1762.68	1760.78
6	FAFT(Sa)**WG**T	1759.67	1760.49	**15**	GAYT(Sa)**WG**Cha	1737.56	1737.95
7	AHGT(Sa)**WG**H	1695.62	1694.49	**16**	ChaMVT(Sa)**WG**N	1790.59	1791.11
8	LFGT(Sa)**WG**H	1747.68	1746.91	**17**	MTTT(Sa)**YW**M	1863.66	1863.32
9	GGPT(Sa)**WG**H	1641.59	1641.49	**18**	HFTT(Sa)**LY**T	1812.71	1813.68
10	MTMT(Sa)**WG**G	1713.59	1713.69	**19**	ChaTVT(Sa)**YV**M	1796.63	1797.62
11	AQMT(Sa)**WG**L	1737.64	1738.16	**20**	GFLT(Sa)**HH**A	1712.67	1713.60
12	QNTT(Sa)**WG**V	1735.66	1736.61	**21**	G**GW**T(Sa)P**SY**	1697.61	1698.33
				22	QNWT(Sa)APV	1746.66	1747.28

[a] All MALDI-TOF mass spectra were obtained using 6-aza-2-thiothymine as matrix. [b] All structures are attached to the solid phase via the IMP spacer (mass 659.79). [c] T(Sa) = Thr(α-Neu5Ac), Cha = cyclohexylalanine.

hits. However, five of the remaining six sequences contained at least one amino acid with an aromatic/planar side chain (His, Trp, or Tyr). As for the intensely fluorescent hits, the less fluorescent hits contained primarily small or hydrophobic amino acids on the N-terminal side of the sialylated threonine. The finding of ligands with an aromatic or hydrophobic moiety in close proximity to the glycan is in agreement with previous work in which glycopeptides identified as ligands for the *Lathyrus odoratus* lectin contained a high proportion of aromatic residues (particularly phenylalanine) adjacent to the mannosylated amino acid.[25] This preponderance of hydrophobic residues may be attributed to specific interactions between the hydrophobic amino acids and the receptor binding site. It has been established that lectins generally contain a number of aromatic amino acids in the binding site, and these help augment binding of a carbohydrate through interactions with the β-face or C−H groups of the saccharide.[33]

Inhibition of Binding of Sialoadhesin to Fetuin. To establish that the glycopeptides obtained from the library screen are indeed mimics of one of the natural ligand of sialoadhesin (oligosaccharides containing Neu5Acα2 → 3Galβ1 → 3GalNAc/GlcNAc) and to get a measure of their solution affinity for SnD1, a competition assay was carried out using surface plasmon resonance. The binding of the SnD1 to fetuin type IV in the presence of various sialopeptides, sialic acid derivatives and fetuin type IV at different concentrations in solution was measured. To determine the K_i values of the sialic acid compounds, the K_a value of SnD1 for fetuin, and F_{im}, the concentration of binding sites available were first determined. Bovine fetuin type IV that contains both 2 → 3 and 2 → 6 sialylated complex type *N*-glycans was immobilized in the active flow cell at 6900 RU. A suitable reference surface was created by immobilizing ribonuclease B, a high-mannose-type glycoprotein, at 3579 RU.

After titration (Figure 4A) of the active surface with various concentrations of SnD1 (15.4−0.48 μM), the strength of the association was calculated by nonlinear fitting of the rectangular hyperbolic plot of R_{eq} versus concentration of SnD1 to the equation[34]

$$R_{eq} = (K_a R_{max}[\text{SnD1}])/(1 + K_a[\text{SnD1}]) \quad (1)$$

where R_{max} represents the biosensor response when all the

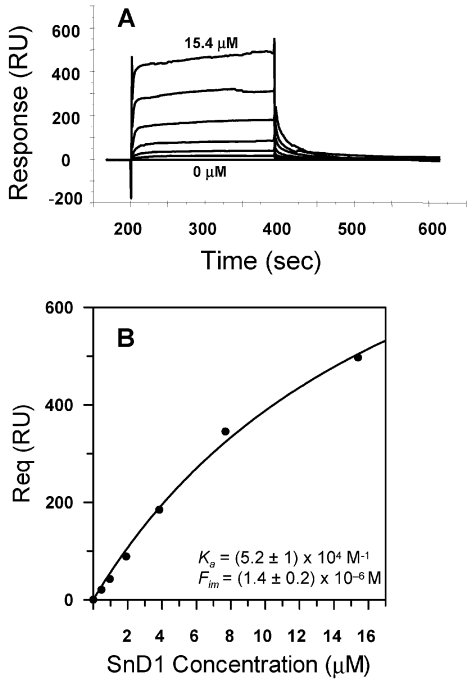

Figure 4. Characterization of the binding interaction between SnD1 and immobilized fetuin on the basis of equilibrium surface plasmon resonance responses. (A) Concentration dependence of binding as monitored by SPR. Solutions of SnD1 (15.4−0.48 μM) were injected over the surface for 3 min at 30 μL/min. (B) Nonlinear regression analysis of the data from A in terms of the rectangular hyperbolic equation yielded values of K_a and F_{im}, the active concentration of immobilized fetuin.

matrix binding sites are occupied (Figure 4B),

$$R_{max} = (k'_p M_{SnD1}) F_{im} \quad (2)$$

where k'_p is the proportionality constant of 60 000 response units per unit concentration (g/L) of protein.[34] The value of R_{max} from the nonlinear fit was then used to calculate the active concentration of immobilized binding sites. Values of (5.2 ± 1) × 10^4 M^{-1} and (1.4 ± 0.2) × 10^{-6} M were obtained for K_a and F_{im}, respectively. The affinity of SnD1 for the immobilized glycoprotein fetuin is an order of magnitude less than the binding of a sialic acid binding lectin from *Maackia amurensis* to immobilized fetuin, determined using surface plasmon resonance (K_a = 5.42 × 10^5).[35] This difference could be attributed to the multimeric nature of

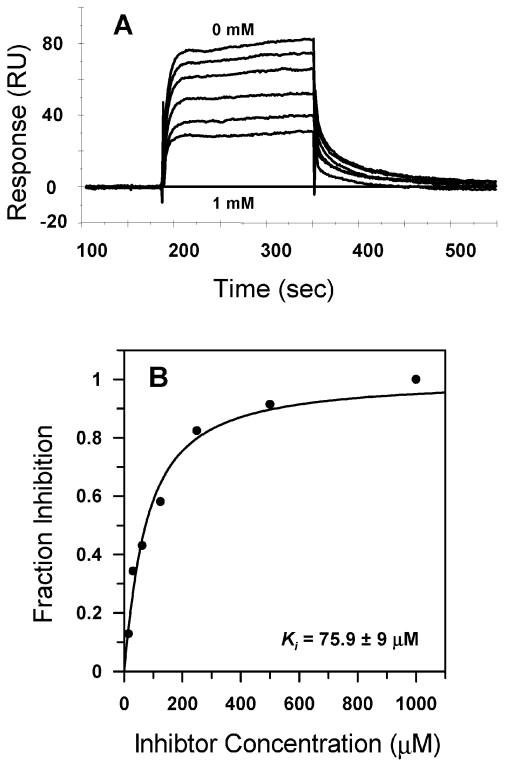

Figure 5. Inhibition of SnD1 binding to fetuin by sialopeptide, WLLT(Sa)WGT (**4**). (A) Binding curves for 7.7 μM SnD1 and 0, 0.03, 0.06, 0.13, 0.25, 0.5, and 1.0 mM. (B) Fractional inhibition curve for inhibition of SnD1 binding.

M. amurensis and the fact that it displays an unusual carbohydrate binding site that accommodates all three residues of 3′-sialyllactose in an extensive hydrogen-bond network.[36] SnD1, on the other hand, binds extensively only to the sialic acid residue of 3′-sialyllactose.[13,32]

In competition assays, immobilized fetuin and soluble sialic acid derivatives (inhibitors) competed for binding to 7.7 μM SnD1. A typical binding response in the presence of increasing concentrations of inhibitor is shown in Figure 5A. Fractional inhibition values were calculated using the equilibrium response in the presence and absence of inhibitor. Inhibition constants (K_i) were determined from nonlinear fits of the data in plots (Figure 5B) of fractional inhibition (f) versus concentration of inhibitor to the equation[37]

$$f = [I]/([I] + K_i(1 + F_{im}/K_d)) \quad (3)$$

The inhibition constants for the various ligands are shown in Table 2. All of the sialopeptides were better ligands for SnD1 than both sialic acid and 3′-sialyllactose. All ligands were at least 10-fold less active than the glycoprotein fetuin, which had a K_i value of 7.6 μM. This value is slightly different from the dissociation constant obtained for the binding of SnD1 to immobilized fetuin ($K_d = 19.2$ μM) and may be attributed to the fact that some of the fetuin binding glycans may be inaccessible because of the immobilization process. The K_i value of 3′-sialyl lactose was 0.83 mM, which is comparable to the dissociation constant (K_d) from NMR experiments (1.4 mM looking at protein chemical shifts or 0.8 mM looking at sugar *N*-acetyl chemical shifts).[32] Sialic acid bound much more weakly (8×; $K_i = 6$ mM) than 3′-sialyllactose and α-methyl sialoside, which bound to SnD1 with an affinity of 3 mM on the basis of NMR experiments.[32] This difference in binding is due to the fact that the sialic acid used was a mixture of α and β- anomers (∼1:9)[38] and to the absence of added interactions between the methyl group of α-methyl sialoside and the protein. Indeed, a study of the interactions of various sialic acid binding lectins with derivatives of sialic acid revealed that there was a 2−10-fold increase in binding for the α-methyl glycoside as compared to sialic acid.[39]

Table 2. Inhibition of SnD1 Binding to Fetuin by Selected Sialopeptides and Sialic Acid Derivatives

compd	structure	K_i (μM)
	fetuin type IV[a]	7.6 ± 0.4
4	WLLT(Sa)WGT	75.9 ± 9
7	AGHT(Sa)WGH	206.7 ± 52
21	GGWT(Sa)PSY	111.6 ± 14
23	AcT(Sa)WGT	154.7 ± 13
24	WLLTWGT	no DDI[b]
25	ManNGGSGG	no inhib.
26	Neu5Ac	5992 ± 456
27	Neu5Ac(2 → 3)Gal(1 → 4)Glc	826 ± 50

[a] 68 000 glycoprotein containing both 2 → 3 and 2 → 6 linked sialic acid residues. [b] No dose-dependent inhibition (DDI) detected.

The sialopeptide with the highest affinity for SnD1 was WLLT(Sa)WGT (**4**), which bound 10 times more tightly than 3′-sialyllactose. Glycopeptides AGHT(Sa)WGH (**7**) and GGWT(Sa)PSY (**21**) also bound to SnD1 more tightly than 3′-sialyllactose, but to a lesser degree than **4**. It is noteworthy that sialopeptide **21**, which was not an intensely fluorescent bead during the library screening, was a very good inhibitor in the solution-phase assay. The reasons for this finding are not immediately clear but can be attributed to several factors. First, the fluorescence intensity of the beads was not quantified but classified as bright and intensely bright on the basis of visual inspection. The bead containing sialopeptide **21** was one of the more intensely bright "less fluorescent" ones. Furthermore, **21** does contain the WG motif, but on the N-terminal side of the T(Sa) instead of on the C-terminal side, as the other sialopeptides. During screening, SnD1 probably interacts with the resin-bound sialopeptide **21** such that that the Trp3 occupies the site of another hydrophobic pocket present close to R48 and Y41 (the cleft occupied by His3 of peptide **7**) (vide infra). However, in solution phase, **21** possibly binds to the receptor in the reverse manner, with the **WG** motif in the optimal position for interacting with Y44, V109, and L107, as in the case of the other sialopeptides. Shortening the peptide (**23**) by omission of the WLL tripeptide and acetylation of the amino terminus resulted in only a 2-fold loss of binding. To examine the specificity of the inhibition and the importance of the sialic acid moiety in the binding interaction, the peptide WLLTWGT (**24**) was synthesized, and its ability to inhibit binding of SnD1 to fetuin was examined. Peptide **24** reduced the binding of SnD1 to fetuin, albeit in a non-dose-dependent manner. The percent inhibition was 40−50% over the entire concentration range of 0.03−1 mM. Given the hydrophobic (greasy) nature of the peptide, it is likely that it simply interacts nonspecifically with either SnD1 or

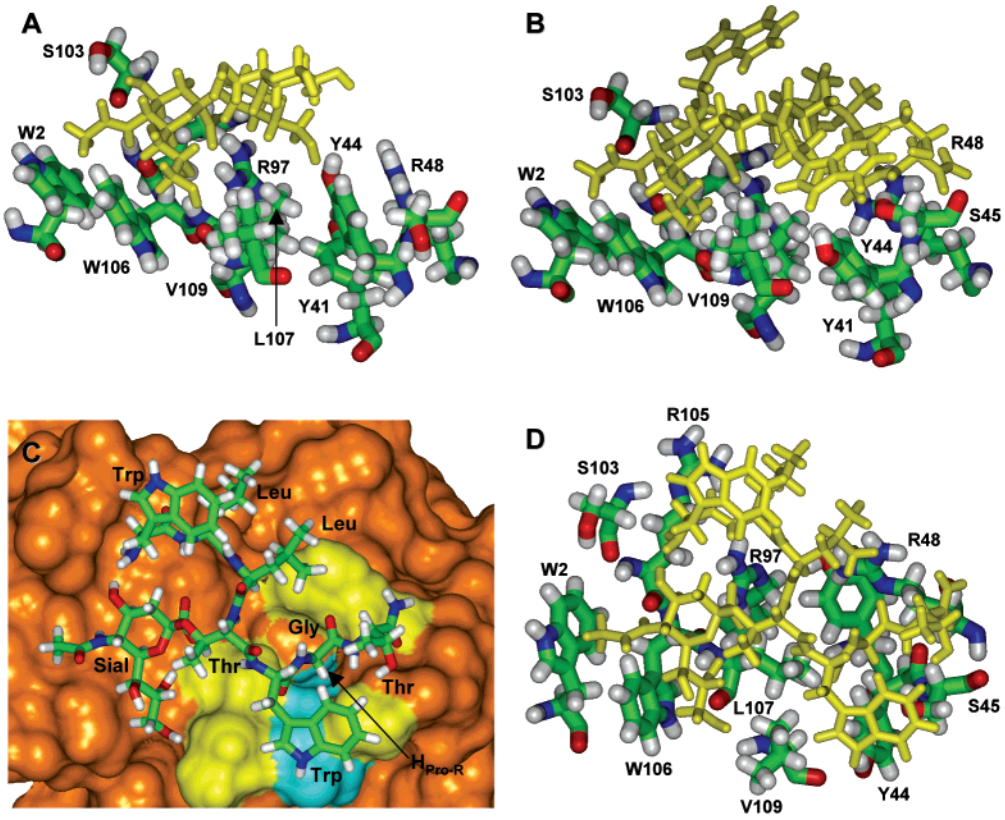

Figure 6. Models of the complexes formed between sialic acid compounds and SnD1 as determined from molecular dynamics calculations. (A) Complex with 3′-sialyllactose. The sialic acid moiety forms numerous hydrogen bonds and van der Waals interactions, particularly to W2, W106, and S103 and a salt bridge to R97, while mainly Gal 6-OH of the lactose moiety hydrogen-bonds to Y44. (B and D) Side and top view of the interaction of sialopeptide **4** (WLLT(Sa)WGT) with SnD1 in the same orientation as 3′-sialyllactose. The peptide portion forms hydrophobic interactions with Y44 (stacking), L107, and V109. (C) Top view of the complex in (B) Requirement for a Gly in the sialopeptide is probably due to steric interactions between L-amino acid side chains larger than an H and SnD1. The pro-S proton of Gly points towards the protein.

immobilized fetuin, thus blocking access to the binding sites. In addition, a mannose-containing peptide, (ManN)GGSGG, failed to inhibit the binding of SnD1 to fetuin at the highest concentration used in the study, that is, 1 mM.

The Molecular Basis for Binding to SnD1. To explore the molecular basis for the specificity observed in the library hits, sialopeptides WLLT(Sa)WGT (**4**), AGHT(Sa)WGH (**7**), and GGWT(Sa)PSY (**21**) were docked into the crystal structure of sialoadhesin SnD1. Molecular dynamics calculations were performed using annealing (Insight/Discover program, MSI) during the docking of the glycopeptide ligands from a 15-Å distance. The crystal structure of the protein was maintained except for those residues in contact with the ligand, which were allowed to move to optimize the induced fit. The proper alignment of the sialic acid in the binding site was ensured by imposing weak constraints between atom pairs in close proximity in the crystal structure. In this way, a bound conformation of the peptide such that the amino acid side chains fitted into the shallow pockets next to the sialic acid reducing end was obtained. The rough binding model was established relatively early in the calculations and did not change considerably with time. The validity of the model was confirmed for sialopeptide **4** when the use of several peptide start conformations during docking resulted in the same binding mode. The predicted binding of sialopeptide **4** to SnD1 was compared to the binding of 3′-sialyllactose (Figure 6). As previously described, the 3′-sialyllactose is bound almost exclusively through the sialic acid, except for a single hydrogen bond between Gal C-6 hydroxyl and the phenolic hydroxyl group of Y44, and a van der Waals contact between Gal C-5 and L107 (Figure 6A).[13] Sialopeptide **4**, on the other hand, interacts more tightly with the protein, covering a greater surface area and bonding with a number of aromatic, hydrophobic, and polar residues (Figure 6B,D). Since the binding mode of the sialic acid moiety was fixed to resemble the conformation in 3′-sialyllactose, it remained only to examine the interactions of the amino acids. As observed from the library hits (Table 1), there was a requirement for a Trp residue at the C-terminal end of the glycopeptide adjacent to the sialylated Thr. From the modeling, it appears that the presence of a Trp in that position is ideal because of possible stacking or edge−face interactions with Y44, hydrophobic bonding to V109 or L107, and a hydrogen bond to S45 of the protein. Preliminary X-ray crystallographic data of the complex between SnD1 and **7** support this position of Trp 5 close to Y44.[40] In some calculations, it seemed that the interaction of Trp5 with Y44 was optimal only when the sialopeptide had a conformation such that the side chain of the subsequent amino acid pointed toward the protein (Figure 6C). This requirement is best satisfied with the smallest possible amino acid side chain, that is, hydrogen; hence, the necessity of the **WG** dipeptide motif in all of the intensely fluorescent library hits. The C-terminal Thr hydrogen bonds to R48 and

G46. At the N-terminus of the glycopeptide, the terminal Trp1 appears not to interact significantly with the protein and instead folds back against the two hydrophobic Leu residues. Leu2 and Leu3 have a few backbone contacts to the D102 and R105, whereas the side chains have little or no contact. Overall, there is minimal contact between the N-terminal tripeptide moiety of the glycopeptide and the protein, and this is reflected in the lack of consensus for this portion of the identified ligands (Table 1) and in the modest (2-fold) reduction in binding when sialopeptide **4** was truncated to **23** (Table 2). The modeling also suggests that Leu3 could be advantageously substituted with an appropriate D-amino acid in order to fill a pocket in that part of the receptor. The interaction of sialopeptide **21** with SnD1 according to our modeling is essentially similar to that of **4**, although one cannot rule out the possibility that the peptide binds in the inverse conformation. However, since the **WG** dipeptide is not at the C-terminal end of the glycopeptide, and the Pro5 residue creates a kink in the chain, another aromatic residue, Tyr7, interacts with Y44 and S45. Trp3 on the N-terminus of the glycopeptide occupies a small cleft near R48 with a face−guanidino group interaction with R48 and an edge−face interaction with Y41. That cleft may be occupied by Leu3 of sialopeptide **4** or His3 of sialopeptide **7**.

Conclusions. Urged by the conceptual appeal of a carbohydrate-based drug or easily attainable glyco-research tools, researchers have designed and developed oligosaccharide mimetics using myriad approaches ranging from simplified "monomer" ligands presenting the essential functional groups of the natural ligand to a variety of multivalent structures. These research efforts have been complicated by the complexity of carbohydrate−protein interactions and a poor understanding of the nature of the forces at play during the in vivo binding event. We and others have focused on the use of simplified compounds, and we have demonstrated that glycopeptides can be efficient mimics of complex oligosaccharides. In the present work, we have used a solid-phase combinatorial library approach and have allowed the receptor of interest, sialoadhesin, to select the best sialopeptides (oligosaccharide mimic) from thousands of possible structures. The library was efficiently synthesized using the building block approach. The use of a PEG-based resin designed for library work and a photolabile linker facilitated rapid on-bead screening and identification of hits. The sialopeptides identified from the library showed high consensus and proved to bind much more tightly to the receptor than the trisaccharide analogue of a natural binding oligosaccharide epitope. Through molecular modeling, we gained an insight into the contacts important for the binding of the sialopeptide to the lectin binding site as well as an understanding of the basis for the high consensus in the library hits. In addition, the preponderance of hydrophobic and aromatic residues present in the identified sialopeptides point to a key requirement of high-affinity ligands for carbohydrate receptors; namely, that in keeping with the results of structural studies of lectin−ligand complexes, hydrophobic interactions between the ligand and the protein contribute significantly to the overall binding affinity of a ligand. This information can be utilized for designing even tighter binding ligands specific for sialoadhesin.

Experimental Section

Materials and General Methods. All solvents were of HPLC grade and were used without further purification unless otherwise indicated. VLC was performed on Merck silica gel 60 H and chromatography under dry conditions was performed on dried silica gel 60 (120 °C; 24 h) eluting with dry solvents. Solid-phase peptide coupling reactions were monitored using the Kaiser test,[41] and solution-phase reactions were monitored by thin-layer chromatography (TLC) performed on Merck Silica Gel 60 F_{254} aluminum-backed sheets with detection by charring with sulfuric acid or by UV light when applicable. $PEGA_{1900}$ resin (0.2 mmol/g, 300−500 μm) was obtained from Polymer Laboratories (Amherst, MA). Suitably protected N^{α}-Fmoc and N^{α}-Boc amino acids were purchased from Bachem (Bubendorf, Switzerland) and NovaBiochem (Läufelfingen, Switzerland). Preparative HPLC of glycopeptides was performed over a 25 × 200 mm semipreparative RP-18 column (Millipore Delta Pak 15 μ). Eluent A (1% TFA in H_2O) and B (10% of A with 1% TFA in acetonitrile) were mixed using a linear gradient starting from 85% A to 15% B with a slope of 0.5% per min and a flow rate of 10 mL min^{-1}. Sonication was carried out using a Branson 3210 sonicator. ES-MS spectra were recorded in the positive mode on a Fisons VG Quattro Instrument. NMR spectra were recorded in $CDCl_3$ on a Bruker AMX-250 spectrometer, and compounds were assigned by ^1H and $^1H-^1H$ COSY experiments. Chemical shifts are given in ppm and referenced to $CDCl_3$ (δ_H 7.29 and δ_C 77.0). Water was obtained at 18.4 Ω. SPR measurements were carried out on a BIAcore 3000 using 10 mM HEPES buffer, pH 7.14, augmented with 150 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 0.005% Tween 20, and data collected were deconvoluted using BIAeval 3.1 software. All buffers were filtered and degassed before use. Building blocks **1** and **2** were prepared as previously described.[27] The photolabile linker **3** was synthesized as previously described.[29]

General Methods for Solid-Phase Peptide Synthesis. All resins were washed with CH_2Cl_2 (6×) then dried under vacuum (lyophilizer) for at least 24 h before use. All manipulations (synthesis and screening) of peptides linked to the resin via the photolabile linker were carried out in subdued light (protected from UV radiation). Linkers were attached to the amino groups of the resin via an acid (3 equiv) activated with TBTU (2.9 equiv)−NEM (4.5 equiv) for 6 min. Fmoc/Boc−Aa-OH were coupled using the TBTU/NEM methodology and reaction completeness monitored using the Kaiser test. Ser, Thr, and Tyr were coupled with unprotected hydroxyl side chains. Fmoc-Aa-OPfp esters (3 equiv) were activated for coupling with Dhbt-OH (1 equiv) which also served as a measure of reaction completeness. After each acylation, the resin was washed with DMF (8×), and then dried by suction for 1 h using a water aspirator or by lyophilization overnight. The Fmoc-protecting groups were removed by treatment with 20% piperidine in DMF (2 + 18 min). The resin was then washed with DMF (10×).

For removal of the Boc group, the resin was first washed with CH_2Cl_2 (10×), dried, then treated with a 10% TFA/CH_2Cl_2 solution for 30 min. The resin was then washed with CH_2Cl_2 (3×); 5% DIPEA in DMF (2×); DMF (4×); and finally, MeOH (5×) before drying by suction for 1 h or by lyophilization overnight. Carbohydrate acetyl protecting groups were removed by hydrolysis with hydrazine hydrate (55 μL) in methanol (1 mL) for 6 h followed by washing with MeOH (3×), H_2O (3×), MeOH (3×), toluene (3×), then acetonitrile (3×). Simultaneous hydrolysis of the sialic acid methyl ester and acetate groups was effected by treatment with a solution of 1 M LiOH (50 μL) in 0.8 M $CaCl_2$ dissolved in 70% *i*-PrOH/H_2O (1.45 mL).[42] The mixture was sonicated for 4 h at 0 °C, the reagents were removed by suction, then the resin was washed with H_2O (8×) and DMF (6×).

Ladder Synthesis of Library 1. To $PEGA_{1900}$ resin (600 mg, ~250 000 beads, 300−500 μm, 0.22 mmol/g loading) was coupled linker **3** (3 equiv) under TBTU activation. The IMP-spacer, GPPFPF, was then synthesized in a syringe using standard Fmoc-OPfp methodology for the first five amino acids. Gly was coupled as a mixture (9:1) of Boc and Fmoc amino acids (2 equiv) under TBTU activation. The four randomized positions of the library were generated using the split-and-mix approach in a 20-well custom-made (2.0 mL capacity) multiple column library generator.[43] For the coupling of nonglycosylated amino acids, a mixture of the Fmoc- and Boc-protected amino acid (90% Fmoc and 10% Boc, 4 equiv) from stock solutions was activated with TBTU/NEM for 6 min and then added to the wells. Coupling times ranged from 4 to 12 h, and reaction completion was checked by the Kaiser test. After each coupling, the resin was pooled, mixed, and divided prior to Fmoc removal. The usual washing protocol followed each coupling and deprotection step. Building block **1** (2 equiv) was activated with TBTU/NEM for 5 min and then added to all wells overnight. The Fmoc group was removed by treatment with piperidine, and the resulting product was immediately cyclized to form the lactamized analogue, as evidenced by a negative Kaiser test. The Boc and acetyl protecting groups were removed as described in the deprotection protocol, and the dried library was stored at 4 °C.

Ladder Synthesis of Library 2. Synthesis of Library 2 proceeded essentially in the same manner as described for library 1 using $PEGA_{1900}$ resin (600 mg, ~250 000 beads, 300−500 μm, 0.22 mmol/g loading). After the first three randomized positions of the library were generated, the library was transferred to a syringe, the Fmoc group was removed, and building block **2** (1.5 equiv) was coupled using the TBTU/NEM methodology. After 12 h and a positive Kaiser test, the library was washed with DMF (8×) and a 70% *i*-PrOH/H_2O solution (3×). The acetates and methyl ester of sialic acid were removed using a mixture of LiOH/$CaCl_2$ with sonication as described above. The resin was then washed with H_2O (8×) and DMF (6×), and the azido group was reduced by treatment with 0.1 M DTT in DMF containing DBU (1 equiv) for 10 h. After washing with DMF (6×), a positive Kaiser test confirmed the presence of an amine. The library was placed once more in the 20-well library generator, and three more randomized amino acid couplings were carried out according to the ladder principle. After the final coupling, the library was deprotected as described above, washed, and stored at 4 °C.

Synthesis of Soluble Peptides. Soluble peptides **4**, **7**, **21**, **23**, and **24** were synthesized on $PEGA_{800}$ resin (150−300 μm beads, 0.26 mmol/g loading). The resin was first derivatized with a Rink amide linker. The synthesis was carried out on a 90-μmol scale essentially as described for the resin-bound lead glycopeptides using the Fmoc-OPfp ester methodology for the nonglycosylated amino acids (4.0 equiv) and the TBTU/NEM methodology for incorporation of building block **2** (2.0 equiv). After reduction of the azide, treatment with 20% Ac_2O in DMF for 30 min at room temperature yielded **23**. After completion of the synthesis, the peptides were deprotected with concomitant release from the resin by treatment with 95% aqueous TFA containing 2% TIS (2 × 30 min). The cleaved glycopeptide solutions were lyophilized, purified by preparative HPLC, and analyzed by ES-MS or MALDI-TOFMS.

MALDI-TOF Mass Spectrometry. *General Methods.* Beads were irradiated on stainless steel targets with a UV lamp for 30 min. The analyte was extracted on the target from the beads using 0.2 μL of 70% acetonitrile and then dried at room temperature.

Procedure for Library 1. CHC matrix (10 mg/mL in 70% acetonitrile) was added (0.2 μL), and the sample was dried at 40 °C. Spectra were recorded in the positive ion mode using 5−200 pulses at the lowest power required to facilitate desorption and ionization. Bradykinin (1060.2 mu), renin (1759.0 mu), and mellitin (2846.5 mu) were used as standards for internal calibration of the mass spectrometer.

Procedure for Library 2. After extraction of the analyte on the target, 0.2 μL of H_2O and ~5 cation exchange beads (Bio-Rad, AG 50W-X2 resin, 100−200 mesh, hydrogen form) were added, and the sample was dried at RT. The matrix 6-aza-2-thiothymine (10 mg/mL in 70% acetonitrile) was added (0.2 μL), and the sample was dried at room temperature. The spectra were recorded in both the positive and negative ion mode as described for library 1.

Preparation of Sialoadhesin SnD1. The N-terminal V-set immunoglobulin-like domain of sialoadhesin (SnD1) was prepared and purified as previously described.[28] Before experiments, the SnD1 monomer was separated from traces of the contaminating dimer (~3%) by FPLC (Äkta, Pharmacia Biotech) using a HiLoad 16/60 superdex 75 prep grade gel filtration column.[28] For the library screening, the protein was labeled with Alexa 488 fluorescent dye (Molecular Probes) according to the manufacturer's protocol, with the exception that excess dye was removed by filtration using centricon YM-3 with a 3000 mwco (Amicon) accompanied by washing with PBS buffer (6×).

Library Screening. Library screening was carried out at room temperature (25 °C) in 5-mL syringes that had been cut to a height of 1.5 cm and fitted with a Teflon filter (70 μm) and a stopper. Library beads were washed (3×) with 10 mM PBS buffer pH 6.8 augmented with $CaCl_2$ (1 mM), and then the resin was blocked with 1% BSA in PBS buffer solution (750 μL) for 30 min. The beads were then incubated

with the fluorescent-labeled monomeric form of the set immunoglobulin-like domain of sialoadhesin (SnD1) (0.0625 mg/mL) in PBS buffer containing 1% BSA (750 μL) and then incubated overnight. The SnD1 solution was removed by suction, and the resin was washed with PBS buffer (3×) and water (3×), transferred to a glass plate in small portions (~5000 beads at a time), swelled in water, and visualized using a fluorescent microscope. Very bright and bright beads were isolated with a closed, roughened glass capillary tip and transferred directly onto the MALDI-TOF stainless steel target for MS analysis.

Surface Plasmon Resonance. *Preparation of Surfaces*. All reagents for surface preparation were injected at a flow rate of 5 μL/min. The carboxylated dextran layer in flow cell 4 of a CM5 chip was activated by injection of a 1:1 mixture (35 μL) of 0.2 M EDC and 0.05 M NHS. Type IV fetuin (22.5 μL, 200 μg/mL in NaOAc buffer, pH 4.5) was then injected, and untreated NHS esters were capped by injection of 1 M ethanolamine, pH 8.5 for 7 min. A reference surface was created in flow cell 3 by injection of ribonuclease B (40 μL, 200 μg/mL in NaOAc buffer, pH 4.5) over a similarly activated surface.

Binding and Inhibition of SnD1 to Fetuin. Experiments were carried out in duplicate, and the average of the two results is reported. Varying concentrations of SnD1 in HEPES buffer were injected (flow rate 30 μL/min) into flow cells 3 and 4 for 3 min, allowing 3 min for dissociation. The concentration range covered was 15.4−0.48 μM by 2-fold dilution. The surfaces were regenerated by a single injection of a 1:1 mixture of 5 mM NaOH containing 200 mM NaCl, pH 11.2, and HEPES running buffer for 20 s. The data were double-reference-subtracted and K_a and F_{im} (the effective concentration of immobilized fetuin) were determined from nonlinear fitting of the rectangular hyperbolic plot of the equilibrium response (R_{eq}) 170 s after injection versus the concentration of SnD1.[34]

Competition experiments with fetuin, sialopeptides, and sialic acid derivatives were carried out as follows: SnD1 (7.7 μM) was mixed with inhibitor, and the mixture was injected (flow rate 20 μL/min) over flow cells 3 and 4 for 3 min, allowing 3 min for dissociation. Inhibitor concentration ranged from 1 to 0.031 mM by 2-fold dilution. Inhibitors were dissolved in buffer to make 10 or 20 mM stock solutions, except for **29,** which was dissolved in DMF and buffer added to make a 10% DMF stock solution. The response to bound SnD1 was measured in the equilibrium part of the curve, that is, at $t = 345.5$ s. K_i values were obtained by fitting the data to the fractional inhibition equation: $f = [1]/([I] + K_i\,(1 + F_{im}/K_d))$.[37]

Modeling. Molecular Dynamics calculations were carried out on a Silicon Graphics Octane workstation using the InsightII/Discover program. The coordinates from the crystal structure of sialoadhesin complexed with 3′-sialyl lactose determined at 1.85 Å resolution[13] were used as input for the calculations after removal of 3′-sialyllactose. The calculations were performed with three different sialopeptides, WLLT-(Sa)WGT (**4**), AGHT(Sa)WGH (**7**), and GGWT(Sa)PSY (**21**), in order to compare their binding interactions with the receptor and to explain the high selectivity for the WG dipeptide motif in virtually all the hits. During all calculations, most of the amide backbone and side chains of sialoadhesin SnD1 were fixed, while a few residues likely to have important contacts with the ligand were allowed to move. Each calculation was carried out as annealing at decreasing temperatures, 650 and then 500 and 300 K. At 500 and 300 K, the SnD1 residues in contact with the ligand were allowed to move freely in sequence ranges of three successive amino acids or less. The ligands were initially energy-minimized in extended conformations and added at a distance of 15 Å from the receptor binding site with several distance constraints between the sialic acid moiety and SnD1, as determined from the crystal structure. These constraints were maintained throughout all calculations. For compound **4**, several random starting conformations resulted in roughly the same structure of the complex. The calculations were initiated with 10 minimization steps followed by 100 000 to 200 000 steps calculated at each temperature at 1-fs intervals. The development of each calculation was monitored, and when obstructions to the progress (e.g., the formation of a cis amide bond in the ligand at high temperature) of the calculation was observed, these were manually remedied, and the calculations continued.

Abbreviations

AIBN, α,α-azoisobutyronitrile; Boc, *tert*-butyloxycarbonyl; BSA, bovine serum albumin; CHC, α-cyano-4-hydroxycinnamic acid; DBU, 1,8-diazabicyclo[5.4.0]undec-7-ene; Dhbt-OH, 3,4-dihydro-3-hydroxy-4-oxobenzotriazine; DTT, dithiothreitol; EDC, *N*-ethyl-*N*′-(dimethylaminopropyl)carbodiimide; FITC, fluorescein isothiocyanate; Fmoc, 9-fluorenylmethoxycarbonyl; IMP, ionization-mass-peptide; MALDI-TOFMS, matrix-assisted laser desorption ionization mass spectrometry; NEM, *N*-ethyl morpholine; NHS, *N*-hydroxysuccimimide; PBS, phosphate-buffered saline; PEGA, poly(ethylene glycol) dimethylacrylamide copolymer; Pfp, pentafluorophenol; Pmc, 2,2,5,7,8-pentamethylchroman-6-sulfonyl; TIS, trisisopropylsilane; TBTU, *O*- (1*H*-benzotriazoyl-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate; TFA, trifluoroacetic acid; VLC, vacuum liquid chromatography.

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