Multivalency Effects in Protein−Carbohydrate Interaction: The Binding of the Shiga-like Toxin 1 Binding Subunit to Multivalent *C*-Linked Glycopeptides

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A series of monovalent and bivalent glycopeptides displaying a *C*-linked analogue of the Pk trisaccharide, the in vivo ligand for the pentavalent Shiga-like toxin binding subunit (SLT-1B), were prepared and evaluated as ligands for SLT-1B by isothermal titration microcalorimetry and competitive enzyme-linked immunosorbent assay (ELISA). Although none of the monovalent ligands showed any enhancement in affinity compared to *O*-methyl glycoside, two bivalent ligands show significant enhancements in affinity in assays. This observation represents the first calorimetric observation of an enhancement in affinity for this system. In contrast, only one of the two ligands shows an enhancement in the competitive ELISA. Together, these data signal a difference in the means by which the two ligands achieve affinity, apparently triggered by a change in the nature of the linker domain. These results provide a rationalization for apparently contradictory reports from the recent literature and again emphasize the importance of investigating complex binding phenomena by multiple techniques.

Introduction

Protein−carbohydrate interaction plays a central role in myriad functions in normal human biology as well as in an array of pathological recognition events.[1] From this basis, the development of high-affinity soluble ligands designed to inhibit pathogen−host adhesion has become a priority in the carbohydrate research community.[2−5] To date, most enhancements in affinity, typically measured in aggregation assays, have come through the use of multivalent ligands.[6] Understanding the molecular basis of these enhancements in activity is not straightforward; indeed, in the general case the structure of the final complex is not known. At least two models of multivalent complexation are conceivable. In the first, a bivalent ligand spans two binding sites on a single receptor. The thermodynamic parameters describing this complexation relative to the corresponding monovalent interaction are described by the expression

$$\Delta J_{\mathrm{bi}} = 2\Delta J_{\mathrm{mono}} + \Delta J_{\mathrm{i}}$$

where ΔJ_{bi} represents any thermodynamic parameter for bivalent complexation, ΔJ_{mono} represents the corresponding term for monovalent association, and ΔJ_{i} represents an interaction term, the energetic consequence of physical linkage.[7,8] Various effects contribute to interaction free energies; these effects contribute incrementally to the overall enthalpy (e.g., alteration of ligand fit within the binding site and interactions between linker and protein) and entropy (e.g., translational and conformational "savings" and conformational "penalties") of binding. Alternatively, a bivalent ligand could span two binding sites on different proteins, generating aggregates. Such aggregates might be stabilized by protein−protein interactions; alternatively, a diminished solubility of the aggregate would lead to an apparently enhanced affinity through a coupled equilibrium.

We have recently reported studies designed to discern the mechanism of binding by multivalent ligands to the plant lectin concanavalin A.[9,10] The paradigm for our studies is based on the notion that different assays of protein−carbohydrate interaction differentially weigh the various intermolecular events that typify such interactions; differences in the results of these studies thus shed light on the mechanism of binding. Our results with concanavalin A strongly suggest that enhancements in affinity result from aggregation and/or precipitation. Here, we consider the binding of bivalent ligands to a bacterial lectin, the binding subunit of the Shiga-like toxin binding subunit (SLT-1B). This pentavalent lectin, which recognizes the αGal(1→4)βGlc(1→4)βGlc carbohydrate domain of the glycolipid Gb_3, or CD77, is ideally suited for the evaluation of enhancements in affinity

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available from intramolecular bivalent binding.[11,12] The protein is exceptionally small, with a subunit molecular weight of only 7.7 kDa, minimizing distances between binding sites. Second, the lectin directs all five binding sites along a single vector, minimizing the propensity of the lectin to aggregate multivalent saccharides.[13]

The precise location of binding sites on the SLT pentamer is unclear, and homologous or heterologous sites could be spanned by bivalent ligands with linkers ranging from 10 to 38 Å.[13−16] Recently, two high-affinity multivalent ligands were reported for the SLT-B subunit, although the two apparently achieve high affinity by different molecular mechanisms. The first reported high-affinity species, a decavalent ligand disclosed by Bundle and co-workers, involves the formation of at least dimers.[17] Unfortunately, in the absence of calorimetric data the energetic consequence of dimer formation is impossible to discern. In a second case, a pentavalent ligand reportedly binds without the formation of any higher order species, signaling an entirely different mechanism of binding.[18]

Here, we report the binding properties of bivalent peptide-linked ligands. We use peptide linkers for two reasons. First, the peptide linkages of α-amino acids provide a balance of the flexibility required to maximize favorable interactions of the carbohydrate recognition domains within the binding site while minimizing the unfavorable conformational entropy penalty that develops as the linker region is restricted. Second, peptides offer the possibility of combinatorially searching for favorable enthalpic contributions to the interaction free energy from adventitious contacts between the linker domain and the protein, either at the periphery of the binding site or with the surface of the protein between binding domains.

Results and Discussion

Prior to initiating a combinatorial search of backbone structures, we set out to prepare a series of ligands with which to conduct preliminary binding studies; such studies would confirm the utility of *C*-glycopeptides as ligands for the SLT-1B subunit. Two monovalent hydrophobic peptides, **1** and **2**, were synthesized to evaluate the effect of carboxy- versus amino-terminal positioning of the carbohydrate (Figure 1). On the basis of previous studies indicating the importance of the hydrophobic character for monovalent ligands of SLT1, hydrophilic

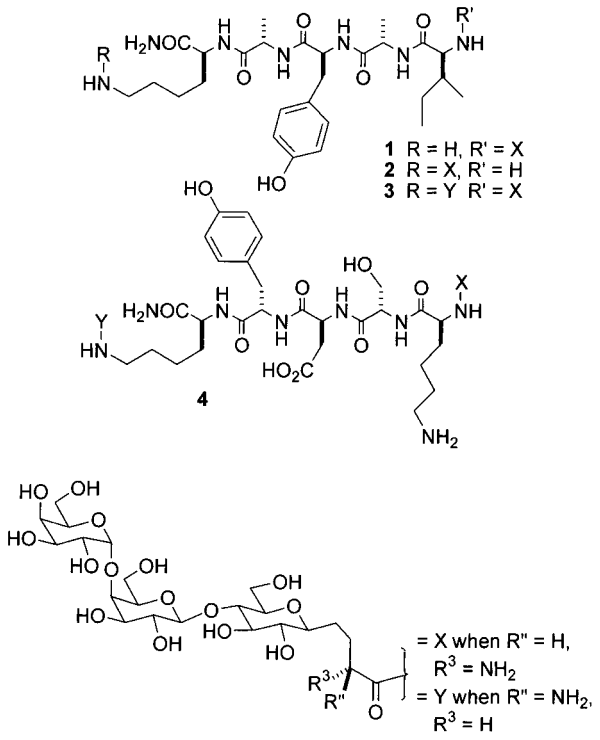

Figure 1. Design of ligands synthesized for use in this study.

monovalent ligands were not utilized.[19−21] Two different bivalent ligands, **3** and **4**, were synthesized to determine the effect of increased valency compared to the monovalent ligands. Two different linkers, one containing predominantly hydrophobic and one containing predominantly hydrophilic side chains, were utilized to investigate the possible contributions of adventitious contacts between the linker region and the lectin. Finally, to avoid β-elimination during peptide synthesis, we utilized carbon-linked glycosyl serines as replacements for the more labile *O*-glycosides.[22−26]

The glycopeptides were prepared on Rink amide MBHA resin using standard Fmoc solid-phase peptide synthesis. Preparation of the required *C*-glycosyl serines has been described elsewhere.[22] Commercially available amino acids activated as the pentafluorophenol esters (Pfp) were utilized where possible; in all other cases, HBTU/NEM activation was utilized. During addition of the glycosylated amino acids, 1.2−1.5 equiv of amino acid were used. All coupling reactions were monitored for completion by ninhydrin assay (Kaiser test).

Two assays, competitive ELISA and isothermal titration microcalorimetry, were used to evaluate ligand binding to the SLT-B subunit; details of both assays have been reported elsewhere.[17,27] Inhibition curves from the ELISA experiment for compounds **1**−**3** are shown in Figure 2; reference peptides and compound **4** showed no

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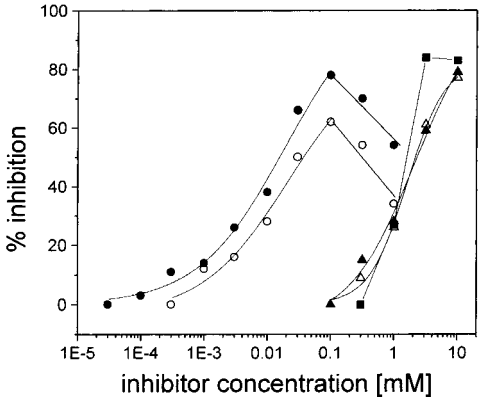

Figure 2. Inhibition of SLT-1B binding as determined by enzyme-linked immunosorbent assay (ELISA). The solid line denotes the best-fit curve for the following ligands: ■, monovalent ligand **1**; ▲ and △, monovalent ligand **2**; ● and ○, bivalent ligand **3**.

Table 1. Thermodynamic Properties of SLT-1B Binding to Glycopeptides As Determined by ITC

compound	K (M^{-1})	n	ΔG [a]	$T\Delta S$ [a]	ΔH [a]
Pk trisaccharide	500−1000	1	−3.6	−8.4	−12
monovalent **1**	700	1.4	−3.9	−2.5	−1.4
monovalent **2**	900	0.97	−4.0	−4.9	+0.90
bivalent **3**	11000	1.08	−5.6	−1.1	−4.5
bivalent **4**	6800	2.0	−5.2	−4.6	−0.67

[a] In kcal mol^{-1}.

inhibitory activity below 10 mM. Curve fitting provided IC_{50} values of of 1.9 and 1.1 mM for compounds **1** and **2**, respectively, whereas the bivalent ligand **3** demonstrated a marked increase of inhibitory activity with an IC_{50} of 20 μM.

The results of isothermal titration microcalorimetry (ITC) experiments are reported in Table 1. Data were reduced utilizing a binding model that assumes identical noninteracting sites. Stoichiometries are reported in all cases on a *per mole of ligand basis using B-subunit monomer concentrations*, regardless of the valence of the ligand. Both bivalent ligands show significant enhancements in affinity relative to the monovalent ligands (see Figure 3 for representative titration). Significantly, however, the two ligands bind with distinctly different stoichiometries. Specifically, hydrophobic ligand **3** binds such that each bivalent ligand apparently completes the binding requirements of one monomer, whereas for the hydrophilic ligand each bivalent ligand occupies only a single subunit. *Together, the stoichiometry and binding constants suggest a variable model of binding that is highly dependent on the nature of the linker region*. The observations leading to this conclusion are described below.

First, *neither of the hydrophobic monovalent ligands binds with significantly higher affinity than does the reference monomeric O-methyl trisaccharide*, although both bind with significantly reduced enthalpies of binding compared to monovalent *O*-methyl glycoside. Assuming that the monovalent peptide ligand binds in the same binding site and in the same orientation as does the reference *O*-methyl glycoside, this observation requires that a large endothermic interaction of the peptide with the B-subunit diminishes the overall enthalpy of binding. This interaction, however, apparently proceeds with little effect on the free energy of binding. Alternatively, addition of the peptide tether might alter the orientation of the bound carbohydrate, either directing it to another site or shifting the carbohydrate within the binding pocket. Neither peptide in the absence of trisaccharide shows measurable binding, minimizing the likelihood of this eventuality (data not shown).

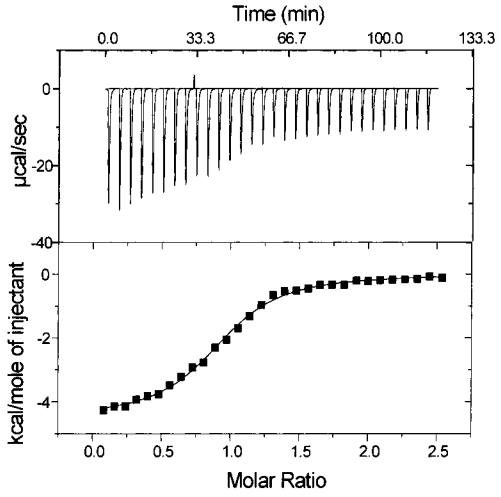

Figure 3. Example of calorimetric titration of SLT-1B with ligand **3** at 25 °C: (top) raw data for 30 7-μL injections and (bottom) the integrated curve showing experimental points (■) and the least-squares fit to the integrated data as a line. The buffer was 10 mM MOPS, 138 mM NaCl, and mM KCl at pH 7.4.

In contrast to the monovalent affinities, both bivalent ligands show significantly greater affinity for the B-subunit than any previously reported monovalent ligand. The hydrophilic ligand **4** binds with an affinity roughly 10 times that of the reference *O*-methyl glycoside, whereas the binding of the hydrophobic bivalent ligand is enhanced by nearly 20-fold. Indeed, bivalent ligands **3** and **4** are the highest-affinity ligands for SLT-IB evaluated calorimetrically to date.

The bivalent ligands bind with markedly different stoichiometries. The hydrophobic bivalent ligand binds with a stoichiometry of 1:1, that is, the binding of one bivalent ligand or *two carbohydrate recognition domains* per B-subunit monomer. On the other hand, the hydrophilic bivalent ligand binds with a stoichiometry of 2:1; that is, two bivalent ligands or *four saccharide recognition domains* are bound per B-subunit monomer. Both of the hydrophobic monovalent ligands bind with a stoichiometry of 1:1, or *one carbohydrate recognition domain* per protein monomer. We have previously reported that the reference *O*-methyl glycoside binds with a stoichiometry of 1:1, although a second class of binding sites was weakly occupied.[27]

Competitive ELISA both differentiates the two bivalent ligands and offers an unexpected concentration dependence of the inhibition. Figure 2 shows fractional inhibition as a function of inhibitor concentration for the two hydrophobic monovalent inhibitors and for the bivalent hydrophobic ligand; all other ligands tested were inactive at any concentration. Both hydrophobic monovalent ligands show IC_{50} values of roughly 1 mM, and the bivalent ligand provides 50% inhibition at 20 μM; both values are in good agreement with calorimetrically derived binding constants.

In toto, these observations suggest a model of binding that invokes an *intramolecular* bivalent interaction for

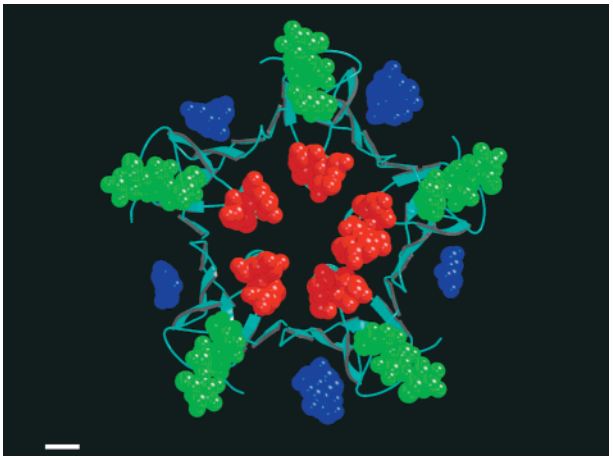

Figure 4. Crystal structure of SLT-1B as determined by Read and co-workers.[13] Trisaccharides are displayed as spacing-filling molecules and are color-coded as follows: blue represents sites denoted as site 1, green denotes site 2, and red denotes site 3, with all sites numbered as in the reference. The white bar is approximately 5 Å in length. The schematic was generated by MOLSCRIPT and rendered in Raster3D (see: Kraulis, P. J. *J. Appl. Crystallogr.* **1991**, *24*, 946−950. Merritt, E. A.; Bacon, D. J. *Methods Enzymol.* **1997**, *277*, 505−524.).

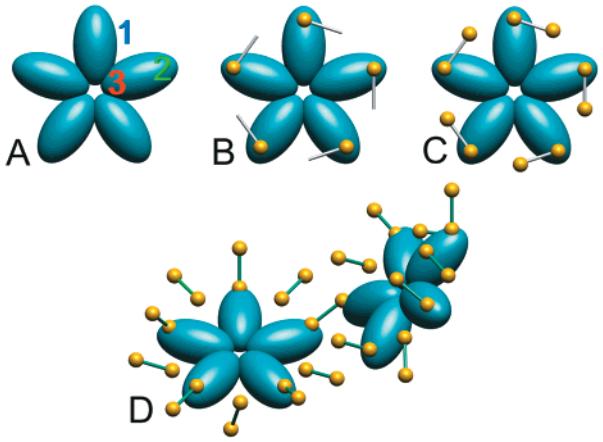

Figure 5. Models for the different modes of binding: (A) labeling convention of the different binding sites in the crystal structure; (B) monovalent binding of ligands **1** and **2** proceeds with tail directed toward the adjacent binding site; (C) bivalent **3** binding occurs with one ligand per protein monomer, with the ligand spanning sites between two adjacent protein monomers; (D) bivalent **4** binding proceeds with two ligands binding per protein monomer, with intermolecular cross-linking between pentamers occurring periodically.

the bivalent hydrophobic ligand and an *intermolecular* interaction for the hydrophilic spacer. The stoichiometry of binding of both bivalent ligands requires that two saccharide binding sites per protein monomer are occupied. This observation is consistent with previous efforts to identify the location of the saccharide binding site that together show the presence of at least two binding sites, although the two likely have different affinities.[15,16] The hydrophobic bivalent ligand apparently binds in an intramolecular sense, spanning two heterologous binding sites. The most likely sites are those designated as sites one and two by Read; the distance between these two sites on a single monomer is roughly 20 Å, whereas that between sites one and two on adjacent monomers is 18.5 Å (Figure 4).[13] We cannot distinguish here the precise mode of binding because the two are identical with respect to the reported stoichiometry.

An alternative model of binding involves primary interaction of the peptide linker with the protein, in a site remote from the carbohydrate binding domains. Such a binding would proceed with the correct stoichiometry. Although we cannot rule out the possibility that the hydrophobic ligand as a whole binds in a discrete and unique site, peptide alone shows no interaction with the protein. On this basis, we have discounted the possibility of a primarily peptide-driven association.

On the other hand, binding of the hydrophilic bivalent ligand proceeds through the interaction of two bivalent ligands with each monomer. The change in binding orientation presumably derives from the change in the nature of the linker. In this model, the hydrophobic linker makes favorable contacts with the surface of the protein that directs binding of the second recognition domain toward the second, presumably lower-affinity, binding site. Alternatively, the hydrophilic ligand, through either the absence of favorable contacts or the presence of unfavorable contacts, binds only one of two available saccharides. The observation that monovalent hydrophobic ligands bind with a stoichiometry of 1:1, that is, only one saccharide recognition domain per monomer, suggests that contacts between the linker and protein preclude binding at the second, lower-affinity site (Figure 5).

Although this model satisfies the observations of ligand stoichiometry, it fails to rationalize the apparently enhanced affinity of the bivalent hydrophilic ligand in the calorimetric assay but not the competitive ELISA. The most reasonable explanation for this effect derives from the difference in protein concentrations used in the two assays, a difference of roughly 10^5. The failure of a ligand with an apparent affinity of 10^4 M^{-1} to show any inhibitory effect in the competitive ELISA signals a dependence of the binding constant on the protein concentration; this binding motif must involve the formation of species with more than one SLT-B subunit. Such species might or might not have a defined stoichiometry but involve more than one pentamer and are ultimately either stabilized by protein−protein interactions or show diminished solubilities. Indeed, solutions of the SLT-B subunit that had been titrated with bivalent ligands showed considerable cloudiness; a diminished solubility provides a coupled equilibrium with a corresponding increase in the apparent "binding" free energy. The observed 2:1 stoichiometry suggests that the number of cross-links, or intermolecular bivalent binding events, is small.

Finally, competitive ELISA displays a "tailing" effect in assays of the bivalent hydrophobic ligand as a function of ligand concentration. Two events might reasonably give rise to this behavior. First, the product of ligand binding might change over the ligand concentration range investigated. In this eventuality, ligand binding at low ligand concentration would give rise to a species in which a single ligand spanned binding sites on more than one B-subunit pentamer, providing a high-affinity complex. Increasing ligand concentration would then increase the population of a lower-affinity complex, one in which one ligand is bound to each monomer in a monovalent sense, similar to the interaction of the hydrophilic bivalent ligand. Such an effect would lead to

a lower apparent IC_{50}. We discount this explanation on the basis of the good agreement between association constants from ITC and IC_{50} values over a 10^5-fold range of protein concentrations. Alternatively, high ligand concentrations might lead to ligand−ligand interactions. This eventuality seems reasonable given the hydrophobic nature of the peptide tether. To the extent that ligand−ligand interactions are lost during binding to the B-subunit, the effect would reduce the apparent affinity of monovalent ligand for its protein receptor. We note parenthetically that a stabilization of less than 1.5 kcal mol^{-1}, or an essentially unmeasurable association constant of 10 M^{-1}, would lead to a 10-fold reduction in IC_{50} values.

In summary, a series of *C*-linked glycopeptide ligands of varying composition and valency were synthesized. Multivalency produces a profound effect on affinity, although through multiple molecular mechanisms that are apparently strongly functions of the linker region. Evaluation of the ligand binding to the SLT-B subunit by isothermal titration microcalorimetry demonstrates the importance of adventitious contacts between the linkage region of a bivalent ligand and lectin. Because the structures of the linkers used here were essentially chosen at random, the maximum extent of the increase in affinity from linker−protein contacts has yet to be determined. Additionally, the exact nature of the binding of the ligands to the pentameric toxin has not been determined structurally. We continue our studies in this regard and will report our results in due course.

Experimental Section

General. The glycopeptides were prepared on Rink amide MBHA resin (Novabiochem) using standard Fmoc solid-phase peptide synthesis techniques.[28] *C*-Glycosyl serine **5***R* and **5***S* have been previously synthesized.[22] DMF (Amresco) was anhydrous and was used without further purification. A ninhydrin assay (Kaiser test) was used to monitor the reaction for completion. When available, the amino acids were activated as the pentafluorophenol esters (Pfp). If they were unavailable, HBTU/NEM activation was utilized. Buffer salts were purchased from Aldrich Chemical Co. (Milwaukee, WI) and used without further purification. Water was purified with a Millipore purification system consisting of charcoal, reverse osmosis, and ion exchange filters; the resistance of the water was greater than 10 MΩ. SLT-1B was isolated essentially as previously published.[29] Protein and ligand concentrations were determined by the method of Edelhoch.[30]

Compound 1. Rink amide MBHA resin (1.0 g, 0.59 mmol) was swollen in DMF (25 mL) for 1 h. The resin was rinsed with DMF (3 × 10 mL). FmocLys(Dde)OH (2.0 equiv, 586 mg) preactivated for 6 min with HBTU (2.0 equiv, 730 mg) and NEM (6.0 equiv, 0.42 mL) was added to a suspension of the resin in DMF (10 mL). After 20 h, the resin was rinsed with DMF (3 × 25 mL). The Fmoc group was removed upon treatment of the resin with piperidine (20% v/v in DMF) (2 × 15 min × 25 mL). Attachment of FmocAlaOPfp, FmocTyr(*t*-Bu)Opfp, FmocAlaOPfp, and FmocIleOPfp was accomplished through the use of DIC/HOBt activation using 3.0 equiv of each amino acid and activating reagent. Deprotection of the Fmoc groups was carried out by treatment with piperidine (20% v/v in DMF) (2 × 15 min × 25 mL) and a rinse with DMF (3 × 10 mL). After the amino acid sequence K(Dde)AYAI is installed, (2*R*)-1-((2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranosyl)-(1→4)-(2,3,6-tri-*O*-acetyl-*β*-D-galactopyranosyl)-(1→4)-2,3,6-tri-*O*-acetyl-*β*-D-glucopyranosyl)-2-ene-2-(*N*-*t*-butyloxycarbonyl)butanoic acid (**5***R*) (1.2 equiv, 313 mg) was attached to the amino terminus. The Dde group was removed from lysine upon treatment of the resin with hydrazine hydrate (2% v/v in DMF) (3 × 3 min × 15 mL). The resin was rinsed with DMF (3 × 15 mL) and MeOH (3 × 15 mL) and incubated with 100 mM NaOMe (2 × 15 mL × 15 min). The resin was rinsed with MeOH (3 × 15 mL) and CH_2Cl_2 (3 × 10 mL). The resin was cleaved by treatment with TFA/water/TES (9:1:1) (10 mL) for 1 h, followed by washing of the resin with fresh TFA (3 × 10 mL) and concentrating in vacuo. Any remaining TFA was neutralized by the addition of 50 mM NH_4HCO_3 (10 mL). Once at pH 7, the reaction was concentrated to provide **1** (93.6 mg, 82%) as a clear film. ^{1}H NMR (500 MHz, D_2O): δ 6.99−6.96 (m, 2H), 6.68−6.67 (d, J = 8.5 Hz, 2H), 4.80−4.79 (d, J = 4.0 Hz, 1H), 4.37−4.22 (m, 2H), 4.20−4.17 (t, J = 7.0 Hz, 1H), 4.16−4.11 (m, 2H), 4.09−4.06 (m, 1H), 3.98−3.96 (d, J = 8.0 Hz, 1H), 3.91−3.89 (m, 2H), 3.84−3.74 (m, 3H), 3.71−3.68 (dd, J = 3.5, 10.8 Hz, 2H), 3.64−3.60 (m, 4H), 3.58−3.55 (m, 3H), 3.46−3.41 (m, 3H), 3.35−3.34 (m, 1H), 3.18−3.15 (m, 1H), 3.10−3.06 (m, 1H), 2.92−2.76 (m, 9H), 2.70 (s, 1H), 1.89 (s, 1H), 1.88−1.51 (m, 9H), 1.34−1.22 (m, 4H), 1.22−1.19 (m, 5H), 1.17−1.12 (dd, J = 7.0, 19.0 Hz, 2H), 1.05−0.98 (m, 2H), 0.71−0.68 (t, J = 7.0 Hz, 3H), 0.66−0.65 (d, J = 6.5 Hz, 3H). ^{13}C NMR (125 MHz, D_2O): δ 178.9, 177.0, 176.96, 176.9, 176.6, 176.1, 175.6, 175.3, 175.2, 167.4, 157.0, 133.0, 130.4, 118.0, 105.7, 102.8, 81.7, 80.9, 80.8, 79.9, 78.4, 77.9, 75.5, 74.7, 73.4, 73.3, 71.6, 71.4, 71.1, 63.0, 62.9, 61.2, 60.8, 57.5, 56.1, 55.8, 52.0, 51.9, 41.7, 38.6, 38.5, 32.8, 29.0, 28.7, 19.0, 18.8, 18.7, 17.2, 17.1.

Compound 2. Rink amide MBHA resin (200 mg, 0.12 mmol) was swollen in DMF (20 mL) for 1 h prior to use. FmocLys(Dde)OH, Ala, Tyr, and Ala were installed sequentially. BocIleOSu (5 equiv, 197 mg) was added after the second alanine residue. The Dde group was removed upon treatment with hydrazine hydrate (2% v/v in DMF) (3 × 3 min × 33 mL). *C*-Glycosyl serine **5***R* (1.5 equiv, 200 mg) was coupled to the newly deprotected lysine side chain. The resin was then rinsed with DMF (4 × 20 mL) and MeOH (4 × 20 mL) and treated with 50 mM NaOMe (5 mL × 5 min × 3). The resin was rinsed with MeOH (4 × 20 mL) and CH_2Cl_2 (4 × 20 mL) and dried on the high vacuum for 15 h. The peptide was cleaved from the resin upon treatment with TFA/TES/water (95:5:5) for 1 h. The resin was filtered and washed with fresh TFA. The filtrate was combined and concentrated to provide **2** as a clear film (74.8 mg, 55%). Selected NMR data follow. ^{13}C NMR (125 MHz, D_2O): δ 179.1, 176.8, 176.4, 175.1, 171.9, 171.2, 164.1, 157.0, 133.0, 130.4, 118.0, 117.7, 105.8, 102.9, 81.7, 80.9, 80.7, 79.9, 78.4, 78.0, 75.3, 74.7, 73.5, 73.4, 71.7, 71.5, 71.1, 63.1, 62.9, 60.0, 57.6, 56.0, 55.9, 51.9, 51.8, 46.9, 41.8, 41.6, 38.8, 32.9, 31.2, 30.3, 29.6, 28.5, 26.6, 25.0, 19.2, 16.4, 12.9.

Compound 3. Resin (0.118 mmol) containing the sequence K(Dde)AYAI(S-Trisacc), synthesized as described above, was treated with hydrazine hydrate (2% v/v in DMF) (3 × 3 min × 15 mL) and washed with DMF (3 × 15 mL). A solution of (2*S*)-1-((2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranosyl)-(1→4)-(2,3,6-tri-*O*-acetyl-*β*-D-galactopyranosyl)-(1→4)-2,3,6-tri-*O*-acetyl-*β*-D-glucopyranosyl)-2-ene-2-(*N*-*t*-butyloxycarbonyl)butanoic acid (**5***S*) (1.2 equiv, 313 mg) preactivated with HBTU/NEM for 7 min was added to a suspension of the resin in DMF (10 mL). Additional **5***S* (100 mg) was added after 20 h. Once addition was complete, the resin was washed with DMF (3 × 15 mL) and MeOH (3 × 15 mL). The resin was treated with 100 mM NaOMe (2 × 20 mL × 15 min). The resin was rinsed with MeOH (3 × 15 mL) and CH_2Cl_2 (3 × 15 mL) and dried for 15 min. The resin was treated with TFA cocktail (10 mL) for 1 h. The beads were filtered, washed with fresh TFA (3 × 10 mL), and concentrated. NH_4HCO_3 (50 mM, 30 mL) was added until pH 7 was reached. The mixture was concentrated to provide **3** (114 mg, 57%) as a clear film. ^{1}H NMR (500 MHz, D_2O): δ 6.98−6.97 (d, J = 6.5 Hz, 2H), 6.68−6.66 (d, J = 8.5 Hz, 2H), 4.80−4.79 (d, J = 3.5 Hz, 1H), 4.37−4.31 (m, 5H), 4.21−4.13 (m, 6H), 4.05−4.02 (m, 2H), 4.00−3.98 (d, J = 7.5 Hz, 2H), 3.94−3.91 (m, 2H), 3.88−3.87 (d, J = 3.0 Hz, 2H), 3.84−3.74

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(m, 4H), 3.70−3.67 (m, 5H), 3.63−3.52 (m, 7H), 3.45−3.41 (m, 6H), 3.36−3.34 (m, 3H), 3.19−3.15 (m, 2H), 3.10−3.04 (m, 2H), 3.03−3.00 (m, 2H), 2.90−2.86 (m, 2H), 2.81−2.78 (m, 2H), 2.75 (bs, 2H), 1.96−1.94 (d, J = 11.0 Hz, 2H), 1.89 (s, 1H), 1.83−1.80 (m, 3H), 1.66 (m, 3H), 1.58−1.57 (m, 3H), 1.43−1.35 (m, 4H), 1.31−1.26 (m, 5H), 1.21−1.16 (m, 4H), 1.14−1.12 (d, J = 7.5 Hz, 2H), 1.04−0.98 (m, 2H), 0.71−0.68 (t, J = 14.0 Hz, 4H), 0.66−0.65 (d, J = 6.5 Hz, 4H). ^{13}C NMR (125 MHz, D_2O): δ 179.1, 177.0, 176.8, 176.1, 175.3, 175.1, 173.1, 172.5, 157.0, 133.0, 130.3, 118.0, 105.8, 102.8, 81.7, 80.9, 80.7, 79.9, 78.4, 77.9, 75.5, 75.4, 74.7, 73.4, 73.3, 71.6, 71.5, 71.1, 63.0, 62.9, 61.2, 60.8, 57.6, 56.0, 55.8, 55.5, 52.1, 51.9, 46.8, 41.6, 41.5, 38.6, 32.9, 31.2, 30.3, 29.9, 28.9, 28.6, 27.1, 25.0, 24.1, 19.0, 18.9, 18.8, 17.2, 12.8, 12.5.

Compound 4. Resin (0.118 mmol) containing the sequence K(Dde)YDSK, prepared using similar procedures as described above, was treated with piperidine (20% v/v in DMF) (2 × 25 mL × 15 min). The resin was rinsed with DMF (3 × 15 mL). *C*-glycosyl serine **5***R* (1.2 equiv, 157 mg) preactivated with HBTU/NEM for 7 min was added to a suspension of the resin in DMF (10 mL). After 16 h, additional **5***R* (0.5 equiv) was preactivated with HBTU/NEM and added. The reaction was complete by ninhydrin assay after 16 h. The resin was rinsed with DMF (3 × 15 mL) and treated with hydrazine hydrate (2% v/v in DMF) (3 × 3 min × 15 mL). The resin was washed with DMF (3 × 15 mL). A solution of **5***S* (1.2 equiv, 313 mg) preactivated with HBTU/NEM for 7 min was added to a suspension of the resin in DMF (10 mL). Additional **5***S* (100 mg) was added after 20 h. Ninhydrin was negative after 20 h. The resin was washed with DMF (3 × 15 mL) and MeOH (3 × 15 mL). The resin was treated with 100 mM NaOMe (2 × 20 mL × 15 min). The resin was rinsed with MeOH (3 × 15 mL) and CH_2Cl_2 (3 × 15 mL) and dried for 15 min. The resin was treated with TFA cocktail (10 mL) for 1 h. The beads were filtered, washed with fresh TFA (3 × 10 mL), and concentrated. NH_4HCO_3 (50 mM, 30 mL) was added until pH 7 was reached. The mixture was concentrated to provide **4** (312.7 mg, 100%) as a clear film. ^{1}H NMR (500 MHz, D_2O): δ 6.97−6.96 (d, J = 7.5 Hz, 2H), 6.69−6.67 (d, J = 8.0 Hz, 2H), 4.78−4.77 (d, J = 3.5 Hz, 1H), 4.43−4.41 (m, 2H), 4.37−4.24 (m, 8H), 4.19−4.17 (t, J = 6.5 Hz, 2H), 4.14−4.12 (m, 3H), 4.00−3.97 (m, 3H), 3.86 (bs, 2H), 3.82−3.77 (m, 3H), 3.75−3.72 (m, 3H), 3.70−3.66 (m, 5H), 3.64−3.57 (m, 8H), 3.55−3.50 (m, 5H), 3.43−3.41 (m, 4H), 3.34 (bs, 2H), 3.18−3.14 (m, 3H), 3.08−3.07 (m, 3H), 3.00−2.96 (m, 3H), 2.86−2.80 (m, 6H), 2.75 (bs, 2H), 2.44−2.41 (m, 2H), 1.90−1.80 (m, 4H), 1.62−1.49 (m, 8H), 1.35−1.28 (m, 7H), 1.10 (bs, 4H). ^{13}C NMR (125 MHz, D_2O): δ 178.8, 177.0, 176.6, 175.4, 174.1, 172.9, 171.8, 157.1, 133.1, 133.0, 130.4, 118.2, 118.1, 105.8, 103.0, 102.9, 81.8, 81.0, 80.8, 80.1, 80.0, 78.4, 78.0, 77.9, 75.6, 75.5, 74.8, 74.7, 73.6, 73.4, 73.3, 71.7, 71.6, 71.2, 71.1, 63.5, 63.4, 63.3, 63.2, 63.1, 62.9, 62.8, 58.3, 58.0, 57.9, 57.8, 57.0, 56.5, 56.4, 56.2, 56.0, 55.9, 55.8, 55.5, 55.4, 54.0, 53.8, 46.9, 41.7, 41.6, 40.3, 38.4, 32.9, 31.2, 30.2, 29.8, 28.8, 28.5, 25.0, 24.8, 24.6.

Isothermal Titration Calorimetry. Calorimetric experiments were carried out on a MicroCal Omega isothermal titration microcalorimeter; details on the instrument and mathematical analyses are detailed elsewhere.[31] The reaction cell had a volume of 1.3678 mL and contained a solution of SLT-1B (ranging in concentration from 1 to 2.5 mM monomer) in buffer (10 mM MOPS, 138 mM NaCl, and mM KCl at pH 7.4). A series of 30 7-μL injections, with 7 s per injection every 3 min, of ligand solution (13−18 mM ligand) were made. The data from the resulting injections were integrated to generate a titration curve. A nonlinear least-squares fit of the data was used to determine the binding constant, K, the enthalpy of binding, ΔH, and the stoichiometry of binding, n.

Solid-Phase Binding Assays. A synthetic Pk trisaccharide attached to a C16 aglycon terminated by an ω-thiol and oxidized to the corresponding disulfide[32] was dissolved in PBS (10 μg/mL), and 96-well ELISA plates were coated (100 μL, 18 h at 4 °C). The plate was washed with PBST (×five times) and blocked for 1 h at room temperature by incubation with 2% BSA in PBS (100 μL). The plate was washed with PBST (×three times), and SLT solution (0.05 μg/mL in PBS) with or without inhibitor (tested over a final inhibitor concentration range from 10.0 mmol to 10 nmol) in a total volume of 100 μL was added to the plate. The coated plate and SLT solution with or without inhibitor were incubated for 18 h at room temperature. The plate was washed with PBST (×five times), and rabbit anti-SLT-I or SLT-II solution (100 μL) diluted (1:1000) in PBS was incubated for 1 h at room temperature. After the plate was washed with PBST (×five times), commercial horseradish-peroxidase-labeled goat anti-rabbit antibody solution (100 μL) diluted (1:2000) in PBS was incubated for 1 h at room temperature. The plate was washed with PBST (×five times), and streptavidin horseradish peroxidase conjugate (100 μL) was added and incubated for 1 h at room temperature. The plate was washed with PBST (×five times), 3,3′,5,5′-tetramethylbenzidine (TMB, 100 μL) was added, and after 2 min the color reaction was stopped by the addition of 1 M phosphoric acid (100 μL). Absorbance was read at 450 nm, and percent inhibition was calculated using wells containing no inhibitor as the reference point.

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Supporting Information Available: ^{1}H NMR and ^{13}C NMR spectra of compounds **1**−**4**. This material is available free of charge via the Internet at http://pubs.acs.org.

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