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# A comparison of biological and calorimetric analyses of multivalent glycodendrimer ligands for concanavalin A

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**Abstract**

The cluster glycoside effect — the observation that multivalent glycosides bind to their polyvalent protein receptors with apparent affinities greater than those that can be rationalized solely on the basis of valency — is by now a well established phenomenon. As part of a continuing effort to provide a molecular basis for the cluster glycoside effect, we report here the synthesis of two series of mannosylated dendritic ligands and their performance in a range of competitive and non-competitive binding assays, including hemeagglutination inhibition (HIA), enzyme-linked lectin assays (ELLA) and isothermal titration microcalorimetry (ITC). The first series of ligands contained a semi-rigid glycylglycine spacer and showed no significant performance enhancement in any binding studies. The second series of ligands contained a flexible tetraethylene glycol spacer; these ligands showed marked enhancements at tetravalent and hexavalent levels in both HIA ($IC_{50}$=3 and<0.8 μM, respectively) and ITC ($K_A=6.2\times10^4$ and $1.5\times10^6$ $M^{-1}$, respectively) studies. In all cases, the thermodynamic parameters of association are consistent with non-specific aggregation rather than enhanced lectin–ligand affinity. This conclusion is reinforced by the lack of enhancements in ligand activity observed in ELLA studies. 

## 1. Introduction

The role of protein–carbohydrate interactions in biological communication is by now widely appreciated. Myriad processes in normal mammalian biology are mediated by such interactions including neutrophil recruitment, fertilization, and immunological surveillance.[1–7] Alternatively, carbohydrate-based recognition facilitates the initiation of a variety of infectious diseases that commence with pathogen recognition of and adhesion to a host through a protein–carbohydrate interaction.[8] This common motif has raised interest in the development of non-cytotoxic carbohydrate-based pharmaceuticals that would prevent infection by inhibiting pathogen–host recognition.

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The intellectual appeal of such a strategy notwithstanding, the development of carbohydrate-based therapeutics has been thwarted by the exceptionally weak affinity of native saccharide ligands for their protein receptors; individual interactions between lectins and their ligands typically proceed with dissociation constants in the millimolar to micromolar range.[9] Nature has overcome this weak affinity, and cell to cell contacts mediated by protein–carbohydrate binding are frequently irreversible on a biologically relevant timescale. Lectins are typically found as oligomeric structures; frequently many copies of each oligomers are displayed on the surface of the cell. Together, these observations suggest that nature has compensated for weak individual protein–carbohydrate affinities through multivalency.

Following this observation, several groups have sought to develop high affinity lectin ligands through multivalency.[3,8,10–23] Many such compounds exhibit superior performance in a range of competitive binding assays relative to the corresponding monovalent ligand, with potencies greater than those expected solely on the basis of their valency. This phenomenon has been termed the 'cluster glycoside effect'.[24,25] Despite many observations of the phenomenon, the physical origin of the effect is still unclear. Although most often interpreted in terms of an entropically assisted, or chelate-type, binding, extrapolation of values of translational and rotational entropies for bimolecular complexation and conformational entropy losses during the restriction of rigid rotors make this explanation unattractive.[26] Here we continue work designed to provide an understanding of the molecular processes that underlie the cluster glycoside effect through the construction and evaluation of the binding behavior of two series of dendritic ligands.

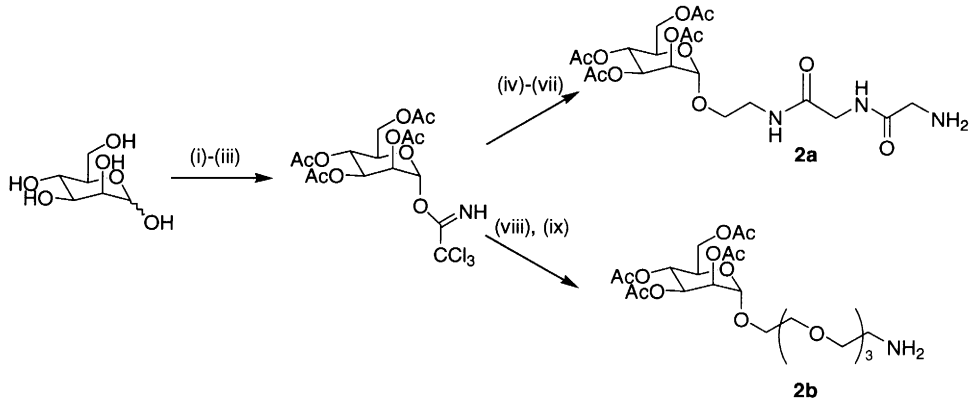
## 2. Results and discussion

We have previously reported the synthesis of a series of mannosylated dendrimers and their performance as ligands for the plant lectin concanavalin A.[27] Our initial series of ligands consisted of mannosyl units tethered to an aromatic core via propyl linkers. The binding of these ligands to concanavalin A was evaluated by isothermal titration calorimetry (ITC), hemeagglutination inhibition assay (HIA), and enzyme-linked lectin assay (ELLA). The study suggested that the apparent affinity of a ligand is highly dependent on the type of assay used, and that observed enhancements are not due to alterations in the thermodynamics of protein–ligand interaction. A body of evidence suggests that the behavior of multivalent ligands in binding assays is highly dependent on the nature of the linker domain.[28–31] Here we extend our earlier studies through the preparation of ligands incorporating distinctly different linker regions: a polypeptide spacer and a spacer based on tetraethylene glycol. The linkers were chosen to extend the size of our previous ligands and evaluate the effect of linker flexibility on affinities.

### *2.1. Synthesis of ligands*

Peptidic dendrimers were constructed on an azidoethyl glycylglycine linker. 2,3,4,6-Tetra-*O*-acetyl-mannosyl trichloroacetimidate was coupled to 2-azidoethanol under TMSOTf promotion to form the α-azidoethyl mannopyranoside **1**. Reduction to the free amine preceded DCC/HOBT coupling to *N*-Cbz glycylglycine which, following hydrogenolytic removal of the benzyl carbamate, provided the semi-rigid mannosyl-spacer domain **2a**. Alternatively, coupling of the trichloroacetimidate to 11-azido-3,6,9-trioxaundecanol followed by hydrogenolysis yielded the flexible TEG-linked mannoside **2b** (Scheme 1).

With glycosylated linkers in hand, dendrimer assembly proceeded according to our previously published protocols. Coupling of the amino-terminated mannopyranosides to benzoyl chloride, 5-azidomethyl-




Scheme 1. Synthesis of precursors for glycodendrimer assembly. Reagents: (i) $Ac_2O$, pyridine; (ii) hydrazine acetate, 50°C; (iii) $CCl_3CN$, $K_2CO_3$; (iv) 2-azidoethanol, TMSOTf; (v) $H_2$ (1 atm), Pd/C; (vi) Cbz-GlyGly, DCC, HOBT; (vii) $H_2$ (1 atm), Pd/C; (viii) TMSOTf, 11-azido-3,6,9-trioxaundecanol; (ix) $H_2$ (1 atm), Pd/C

1,5-benzenedicarbonyl dichloride, and 1,3,5-benzenetricarbonyl trichloride yielded mono-, bi-, and trivalent ligands **3a**–**5a** and **3b**–**5b** in the glycylglycine and TEG-linked series, respectively. Reduction of bivalent dendrons **4a** and **4b** and coupling of the resulting amines to the *N*-hydroxysuccinimide derivatives of 5-azidomethyl-1,5-benzenedicarboxylate **6** and 1,3,5-benzenetricarboxylate **7** yielded tetravalent **8a**, **8b** and hexavalent members **9a**, **9b** of both ligand series. Zemplen deprotection provided the fully deprotected species **10a**–**14a** and **10b**–**14b** required for binding studies (Scheme 2).

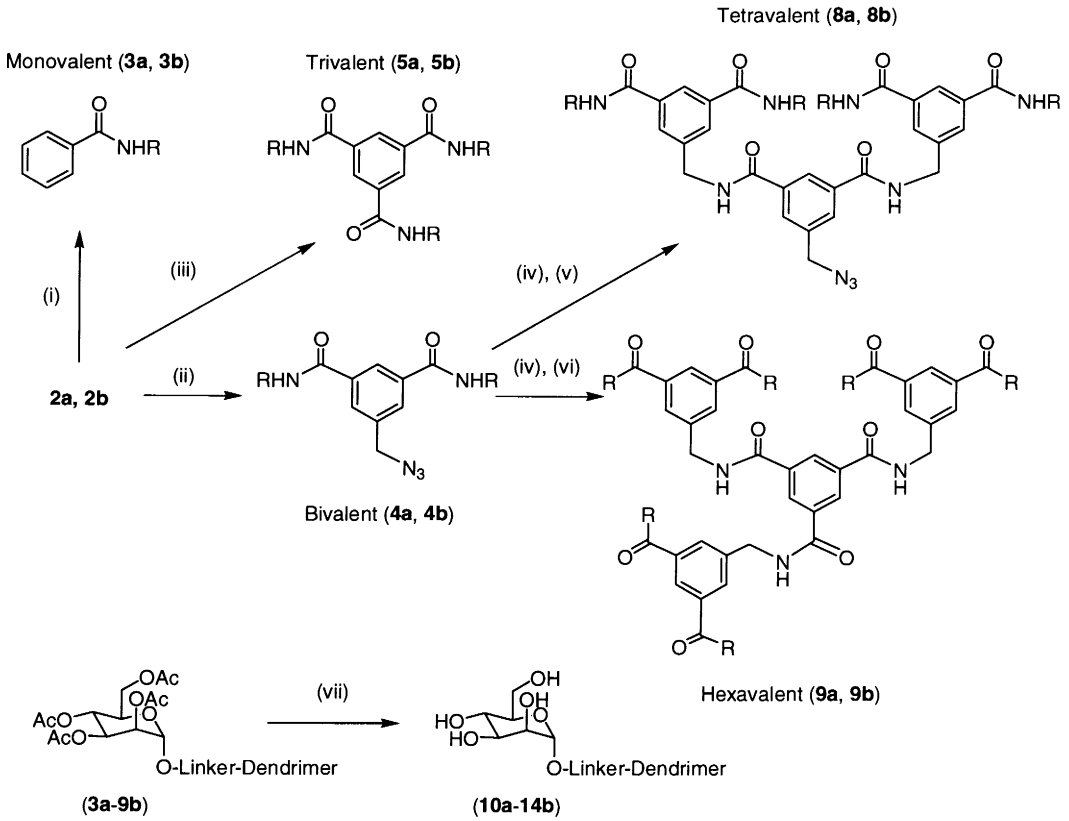
### *2.2. Hemeagglutination inhibition assays (HIA)*

The inhibition of hemeagglutination has been used extensively for the analysis of protein–carbohydrate interactions, primarily due to the simplicity of the technique and the relatively low material requirements.[32] Previous assays have typically utilized chicken erythrocytes; because of their ready availability, porcine erythrocytes were used here. To exclude effects based on variability of erythrocyte surface composition, we first compared $IC_{50}$ values for a series of previously determined carbohydrates with chicken and porcine erythrocytes.[33] Table 1 demonstrates that although agglutination of porcine erythrocytes is inhibited at slightly lower levels of ligand than are chicken erythrocytes, the two cells provide comparable $IC_{50}$s, within experimental error.

Minimum inhibitory concentrations for both series of dendrimeric ligands were analyzed using porcine erythrocytes. In accordance with previous observations, no significant benefit of multivalency was observed at low ligand valency (Table 2). Rather, low-valency ligands bind in a similar fashion to α-methyl mannose, a trend that continues into the second generation for the peptide-linked ligands. Alternatively, the TEG-linked series differs markedly from the peptide-linked series. With this group of ligands significant enhancements in inhibition are observed with second generation ligands: the tetra- and hexavalent TEG dendrimers show valence-corrected inhibition enhancements of 50- and greater than 112-fold over the monovalent reference ligand.

### *2.3. Enzyme linked lectin assay*

The enzyme linked lectin assay (ELLA) is a variation of the commonly used ELISA.[34,35] ELLA evaluates the ability of a soluble ligand to inhibit the adhesion of a multivalent lectin to a reference ligand non-covalently immobilized on the surface of a microtitre plate. Readout is accomplished through




Scheme 2. Synthesis of multivalent glycodendrimers. (i) BzCl, $Et_3N$; (ii) 5-azidomethyl benzene-1,3-dicarbonyl dichloride, DMAP, pyridine; (iii) benzene-1,3,5-tricarbonyl trichloride, DMAP, pyridine; (iv) $H_2$ (1 atm), Pd/C; (v) 5-azidomethylbenzene-1,3-dicarboxylate di-*N*-hydroxysuccinimide ester, $Et_3N$; (vi) 1,3,5-benzenetricarboxylate tri-*N*-hydroxysuccinimide ester, $Et_3N$; (vii) 0.1 M NaOMe, MeOH

Table 1
Comparison of hemeagglutination assay results for erythrocytes from chickens and pigs. Reported $IC_{50}$ values are from Gupta et al.[33]

| Compound | Measured $IC_{50}$ (mM) | Reported $IC_{50}$ (mM) |
|---|---|---|
| Mannose | 10 | 15 |
| α-methyl mannose | 1.3 | 3.1 |
| α-methyl glucose | 10 | 13 |
| Galactose | Not measurable | Not measurable |

the use of lectin directly labelled with peroxidase, eliminating washing steps that potentially lead to the dissociation of weakly interacting systems. The most significant advantage of ELLA over the hemeagglutination assay lies in the spectrometric analysis of binding: the assay thus provides a more precise determination of $IC_{50}$ values than does HIA.

Table 2 shows the results of ELLA experiments with the two series of ligands. Again, peptide-linked ligands provide no significant enhancement in $IC_{50}$ relative to the monovalent ligand. For this series, increases in inhibitory power are solely a function of increased saccharide concentration. In contrast to

Table 2
Biological assay results for peptide- and TEG-linked glycodendrimers. Parenthetical values are corrected for carbohydrate concentration

| | HIA | | ELLA | |
|---|---|---|---|---|
| compound | $IC_{50}$ (mM) | Potency | $IC_{50}$ (μM) | Potency |
| α-methyl mannose | 0.56 | 1.0 | 530 | 1 |
| peptide-linked | | | | |
| monovalent | 0.55 | 1.2 (1.2) | 466 | 1.4 (1.4) |
| bivalent | 0.25 | 2.6 (1.3) | 152 | 4.3 (2.1) |
| trivalent | 0.22 | 3.0 (1.0) | 188 | 3.4 (1.1) |
| tetravalent | 0.18 | 3.1 (0.8) | 188 | 2.8 (0.7) |
| hexavalent | 0.16 | 3.5 (0.6) | 62 | 8.5 (1.4) |
| TEG-linked | | | | |
| monovalent | 0.81 | 0.81 (0.81) | 156 | 3.4 (3.4) |
| bivalent | 0.18 | 3.6 (1.8) | 60 | 4.7 (2.3) |
| trivalent | 0.08 | 8.6 (2.9) | 98 | 5.4 (1.8) |
| tetravalent | 0.003 | 186 (47) | 45 | 12 (3.0) |
| hexavalent | < 0.0008 | >672 (>112) | 35 | 15 (2.5) |

the results of HIA, the TEG-linked series of ligands demonstrate no enhancement in performance with increased valency; rather all five ligands behave essentially identically.

### 2.4. *Isothermal titration microcalorimetry*

A third technique that has found extensive utility in ligand binding studies during the last decade is isothermal titration microcalorimetry.[36–43] In this experiment, soluble ligand is titrated into a solution containing the binding protein, and the heat evolved or absorbed during binding is measured as a function of ligand concentration. Deconvolution of these data yields a binding constant, an enthalpy of binding, and the stoichiometry of the interaction (Fig. 1). The binding constant is simply related to the free energy of association, and the entropy of the association is thus available by subtraction. Because the assay is soluble and homogeneous, ITC reports protein–carbohydrate interactions solely in terms of reversible binding processes. Effects contributing to $IC_{50}$s arising from, for example, surface display and phase change are disregarded.

The results of ITC evaluation of the binding of both ligand series are presented in Table 3. The reduction of ITC data requires an assumed model of ligand binding. The nature of this model is extremely important; values of binding constants and enthalpies vary roughly linearly as ligand concentrations, in turn dependent on the model of binding. Here we have used the simplest possible model of binding, that of individual saccharides binding to individual, discrete binding sites in a non-cooperative fashion. From this model the ITC data are fitted by Eq. (1), where $V_o$ is the cell volume, $1/r=[P]_{tot}K$, $[L]_r=[P]_{tot}/[L]_{tot}$, and requires the use of the total saccharide concentration for $[L]_{tot}$ and the total binding site concentration for $[P]_{tot}$.

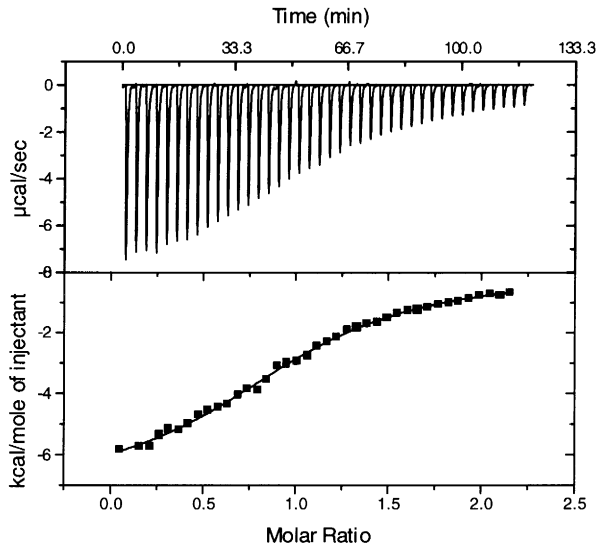



Fig. 1. Representative calorimetric data for the titration of peptide-linked trivalent **5a** (6 mM) with concanavalin A (0.57 mM) at 25°C. (A) Raw data for forty 2.2 µL injections and (B) the integrated curve showing experimental points (●) and the least squares fit (−) to the integrated data. The buffer was 50 mM dimethyl glutaric acid, 250 mM NaCl, 1 mM $CaCl_2$, and 1 mM $MnCl_2$, at pH 5.2. The fit gives the following values: n=1.02, K=7900 $M^{-1}$, $\Delta H=-7.8$ kcal $mol^{-1}$

$$1/V_0(dq/d[L]_{tot}) = \Delta H\left[\frac{1}{2} + \frac{1-(1+r)/2-[L]_r/2}{\sqrt{[L]_r^2 - 2[L]_r(1-r)+(1+r)^2}}\right] \quad (1)$$

Table 3
Isothermal titration microcalorimetry results for peptide- and TEG-linked glycodendrimers. All values listed above are corrected for the valency of the ligand in question

| compound | K ($M^{-1}$) | ΔG (kcal $mol^{-1}$) | ΔH (kcal $mol^{-1}$) | TΔS (kcal $mol^{-1}$) |
|---|---|---|---|---|
| α-methyl mannose | 7600 | -5.3 | -6.6 | -1.3 |
| peptide-linked | | | | |
| monovalent | 9200 | -5.4 | -7.4 | -2.0 |
| bivalent | 8000 | -5.3 | -7.5 | -2.2 |
| trivalent | 7900 | -5.3 | -7.8 | -2.5 |
| tetravalent | 7500 | -5.3 | -4.2 | +1.1 |
| hexavalent | ND | | | |
| TEG-linked | | | | |
| monovalent | 8000 | -5.3 | -6.4 | -1.1 |
| bivalent | 8600 | -5.3 | -7.7 | -2.3 |
| trivalent | 4700 | -5.0 | -7.1 | -2.1 |
| tetravalent | 62000 | -6.6 | -2.3 | +4.3 |
| hexavalent | 1500000 | -8.5 | -1.3 | +7.2 |

In accordance with the previous results, multivalent peptide-linked ligands behave similarly to the monovalent parent compound. Our model of binding is validated by the excellent fit of the data by Eq.

(1). Only slight changes in thermodynamic parameters occur through the second generation, notably a diminution in the enthalpy of binding accompanied by a compensating increase in the entropic term, a trend we have observed previously.[27] The effect is more pronounced for higher valent ligands. Unfortunately, the insolubility of the hexavalent ligand precluded calorimetric evaluation of binding. The TEG-linked ligands follow similar trends in the first generation of ligands, with thermodynamic parameters of binding essentially equivalent to those of the monovalent ligand. As was previously observed for the propyl-linked dendritic ligands, the trivalent ligand shows a weaker interaction than would be expected solely on the basis of saccharide concentration.[27] Crystallographic studies in that instance demonstrated the apparent loss in affinity was in fact due to the binding of only two of the three saccharide ligands available, because of constraints applied by the lattice structure of the cross-linked macroscopic structure. Thus, the apparent binding constant of the trivalent ligand is reduced because of an overestimate of the available ligand, rather than from any alteration in the nature of the protein–carbohydrate couple. Significant enhancements in affinity are observed during binding of the second-generation TEG-linked ligands, although the enhancement is less than that observed in hemeagglutination assays. Additionally, the decrease in enthalpy of association is greatly exaggerated; at the hexavalent ligand fully 85% of the apparent free energy of association arises from a favorable entropy of binding.

The thermodynamic pattern of association observed here is most consistent with an initial protein–carbohydrate interaction, which presumably proceeds with thermodynamic parameters equivalent to those of a simple monovalent saccharide, followed by a reversible aggregation process. While exceptions certainly exist, specific associations in aqueous solution are typified by an enthalpy of association greater than the free energy and an unfavorable entropy of binding.[9,39,44,45] In contrast, non-specific aggregation processes typically proceed as entropically driven process, characterized by a small negative or positive enthalpy. For example, the micellization of pure octyl-β-D-glucopyranoside proceeds spontaneously with an enthalpy of +1.5 kcal $mol^{-1}$.[46,47] Several other studies of aqueous micelle formation reinforce these trends. Thus, micelle formation of octyl-linked tetra- or pentaethylene glycol ($\Delta H_{mic}$ +3.6 kcal $mol^{-1}$ in both cases), sodium decyl sulfate ($\Delta H_{mic}$ +5.5 kcal $mol^{-1}$), and TX-100 ($\Delta H_{mic}$ +3.1 kcal $mol^{-1}$) are all endothermic processes.[48–50] We suggest that the steadily diminishing enthalpy of binding is the thermodynamic signature of an endothermic aggregation process superimposed on an exothermic ligand binding event.

This model of association is consistent with the lack of enhancement in ligand performance by ELLA. The origin of the discrepancy between ligand performance in ELLA and HIA can be readily explained by the nature of the lectin involved. The lectin utilized in ELLA is directly labelled with horseradish peroxidase, a 40 kDa protein. Our crystallographic study of the aggregates formed during concanavalin A binding of dendritic ligands demonstrates the need for tight protein–protein packing.[27] The attachment of a large protein label presumably prevents the formation of large ordered aggregates during ligand binding. Although very long linkers might overcome this effect, these observations suggest that ELLA might more faithfully report protein–carbohydrate affinities in the traditional sense of the term than other assays.

The thermodynamic contribution of aggregation to apparent ligand affinities is readily deducible by assuming initial protein–carbohydrate interactions proceed with parameters equivalent to those of the parent, monovalent compound. This assumption is justified on the basis of our previously reported crystallographic studies of ligand cross-linking, work that demonstrates that the orientation of individual saccharide recognition domains of a multivalent ligand within the concanavalin A binding site is identical to that of methyl α-D-mannopyranoside. Overall thermodynamic parameters can then be separated as:

$\Delta J = \Delta J_i + \Delta J_a$

Where $\Delta J_i$ refers to the intrinsic contribution, or contribution of protein–carbohydrate interaction, and $\Delta J_a$ refers to the contribution arising from aggregation effects. Table 4 shows that the enthalpy of aggregation is significantly endothermic in the second-generation dendrimers for both series. In the case of the TEG-linked series, the increase in the entropic component is significantly larger than the enthalpic penalty, leading to an overall increase in the binding constant.

Table 4
Thermodynamic effects of aggregation. All values are corrected for ligand valency

| Compound | $\Delta H_{aggregation}$ (kcal mol$^{-1}$) | $T\Delta S_{aggregation}$ (kcal mol$^{-1}$) |
|---|---|---|
| Peptide-linked trivalent | -0.4 | -0.5 |
| Peptide-linked tetravalent | +2.4 | +2.3 |
| TEG-linked trivalent | -0.7 | -1.0 |
| TEG-linked tetravalent | +4.1 | +5.4 |
| TEG-linked hexavalent | +5.1 | +8.3 |

## 3. Conclusions

The performance of multivalent saccharide ligands is strongly a function of the assay used to evaluate them. It is inappropriate to draw conclusions regarding ligand binding based on either agglutination or microtitre plate inhibition assays. Observed enhancements in apparent affinity appear to be the result of entropically driven protein aggregation phenomena that are highly dependent on the precise molecular details of the ligand, including valency and linker composition, and protein concentration. In some circumstances the contribution of aggregation events to overall thermodynamic parameters rivals that of protein–carbohydrate interaction. We are continuing our studies of the role of multivalency in protein–carbohydrate interaction and will report our results in due course.

## 4. Experimental

### *4.1. General*

Concanavalin A was purchased from Sigma. D-Mannose and glycylglycine were purchased from Aldrich and used without further purification. Dichloromethane was distilled from calcium hydride prior to use. BioGel P2 and SX-1 resins were obtained from BioRad. Trichloroacetonitrile was fractionally distilled prior to use. Porcine erythrocytes were collected fresh and stored in Alsever's solution. All other chemicals were reagent grade and used without further purification. Column chromatography was carried out using flash grade silica gel. Thin layer chromatography slides were silica gel 60 $F_{254}$ (Merck) and detected by using Hanessian's dip and UV detection. All $^1$H NMR spectra were obtained on GE QE300, Varian Mercury, or Varian Unity spectrometers operating at 300.015, 300.07 and 399.956 MHz, respectively. All $^{13}$C NMR spectra were taken on a GE QE300 spectrometer, operating at 75.48 MHz or a Varian Mercury NMR spectrometer operating at 75.452 MHz. All spectra were obtained in $CDCl_3$ or $D_2O$ and referenced to TMS, an internal solvent peak, or sodium acetate. FAB-MS was carried out on a

JEOL SX-102 mass spectrometer. Protein concentrations were determined by the method of Edelhoch,[51] and carbohydrate concentrations were determined by phenol–sulfuric acid chars according to DuBois.[52]

### 4.2. Syntheses

#### 4.2.1. 1-O-(2-Azidoethyl)-2,3,4,6-tetra-O-acetyl-α-D-mannopyranoside **1**

2,3,4,6-Tetra-*O*-acetyl-α-D-mannosyl trichloroacetimidate (1.4 g, 2.4 mmol) was dissolved in dichloromethane (10 mL) with 2-azidoethanol (1.1 mL, 12 mmol) and 4 Å molecular sieves (1 g) under a nitrogen atmosphere. The mixture was cooled to −30°C, TMSOTf (150 μL, 0.96 mmol) was added, and the reaction was stirred for 5 h at 25°C. The mixture was filtered over Celite, concentrated in vacuo, and purified by silica gel chromatography (petroleum ether:ethyl acetate 2:1) to yield **1** (570 mg, 53% yield) as a colorless oil. $^1$H NMR (300 MHz, $CDCl_3$): $\delta$ 2.00 (s, 3H), 2.06 (s, 3H), 2.12 (s, 3H), 2.17 (s, 3H), 3.45–3.51 (m, 2H), 3.65–3.71 (m, 1H), 3.84–3.91 (m, 1H), 4.04–4.15 (m, 2H), 4.3 (dd, 1H, *J*=5.28, 12.30 Hz), 4.88 (d, *J*=1.63 Hz), 5.27–5.39 (m, 3H) ppm. $^{13}$C NMR (75 MHz, $CDCl_3$): $\delta$ 20.59, 20.64, 20.67, 20.80, 50.24, 62.33, 65.84, 66.96, 68.73, 69.26, 97.60, 169.73, 169.93, 170.54 ppm. IR (neat): $\nu$ 2954, 2955, 2893, 2106, 1744, 1368, 1228, 1136, 1048, 979 cm$^{-1}$.

#### 4.2.2. 1-O-[2-N-(N-Benzyloxycarbonyl glycylglycyl)ethyl]-2,3,4,6-tetra-O-acetyl-α-D-mannopyranoside **2a**

Azidosugar **1** (150 mg, 0.31 mmol) was dissolved in ethanol (10 mL). Degussa Pd/C (30 mg) was added, and $H_2$ was bubbled through the solution for 3 h. The mixture was filtered over Celite and concentrated in vacuo. The residue was dissolved in anhydrous DMF (10 mL) and Cbz-glycylglycine (90 mg, 0.3 mmol), DCC (80 mg, 0.3 mmol), and HOBT (45 mg, 0.33 mmol) were added. The solution was stirred under a nitrogen atmosphere at 25°C for 12 h, filtered over Celite, concentrated in vacuo, and purified by silica gel chromatography (petroleum ether:ethyl acetate:ethanol 4:3:1) to yield **2a** (120 mg, 63% yield) as a colorless oil. $^1$H NMR (300 MHz, $CDCl_3$): $\delta$ 1.97 (s, 3H), 2.04 (s, 3H), 2.09 (s, 3H), 2.12 (s, 3H), 3.42–3.47 (m, 2H), 3.55–3.63 (m, 1H), 3.89–3.97 (m, 4H), 3.98–4.02 (m, 1H), 4.10–4.15 (m, 1H), 4.22–4.28 (dd, 1H, *J*=5.50, 12.23 Hz), 4.81–4.82 (d, 1H, *J*=1.13 Hz), 5.11 (s, 2H), 5.22–5.32 (m, 3H), 6.0 (broad t, 1H), 7.1 (broad t, 1H), 7.2 (broad t, 1H), 7.07–7.34 (m, 5H) ppm. $^{13}$C NMR (75 MHz, $CDCl_3$): $\delta$ 20.65, 20.69, 20.82, 20.80, 38.85, 43.08, 62.42, 66.00, 66.78, 67.10, 68.53, 69.08, 69.31, 97.50, 127.98, 128.17, 128.46, 136.04, 156.23, 169.08, 169.68, 170.04, 170.21, 170.34, 170.79 ppm. HR FAB-MS (pos) calcd for $C_{28}H_{37}N_3O_{14}$: 639.2276, obsd $MH^+$: 640.2342.

#### 4.2.3. 1-O-(11-Azido-3,6,9-trioxaundecyl)-2,3,4,6-tetra-O-acetyl-α-D-mannopyranoside **2b**

2,3,4,6-Tetra-*O*-acetyl-α-D-mannosyl trichloroacetimidate (500 mg, 1.0 mmol) was dissolved in dichloromethane (5 mL) with 11-azido-3,6,9-trioxaundecanol (1.0 mL, 5.01 mmol) and 4 Å molecular sieves (0.2 g) under a nitrogen atmosphere. TMSOTf (80 μL, 0.4 mmol) was added, and the reaction was stirred at 25°C for 6 h. Solid sodium bicarbonate (200 mg) was added. The mixture was filtered over Celite, concentrated in vacuo, and purified by silica gel chromatography (petroleum ether:ethyl acetate 2:1) to yield **2b** (570 mg, 53% yield) as a colorless oil. $^1$H NMR (300 MHz, $CDCl_3$): $\delta$ 2.00 (s, 3H), 2.05 (s, 3H), 2.11 (s, 3H), 2.16 (s, 3H), 3.40 (t, 2H, *J*=5.0 Hz), 3.62–3.84 (m, 14H), 4.07–4.12 (m, 2H), 4.30 (dd, 1H, *J*=5.0, 12.3 Hz), 4.88 (d, 1H, *J*=1.6 Hz), 5.26–5.35 (m, 3H) ppm. $^{13}$C NMR (75 MHz, $CDCl_3$): $\delta$ 20.67, 20.74, 20.88, 50.61, 62.33, 66.06, 67.33, 68.31, 69.03, 69.50, 69.92, 70.60, 70.64, 70.68, 97.65, 169.69, 169.86, 170.01, 170.67 ppm. IR (neat) $\nu$ 2922, 2105, 1746, 1369, 1224, 1135, 1086, 1047, 978 cm$^{-1}$. HR FAB-MS (pos) calcd for $C_{22}H_{35}N_3O_{13}$: 549.2171, obsd $(M-N_2+3H)^+$: 524.2361.

*4.2.4. 1-*O*-[2-(*N*-Benzylamidoglycylglycyl)ethyl]-2,3,4,6-tetra-*O*-acetyl-α-*D*-mannopyranoside **3a***

Protected glycopeptide **2a** (135 mg, 0.21 mmol) was dissolved in absolute ethanol (25 mL). Degussa Pd/C (50 mg) was added, and $H_2$ was bubbled through the mixture for 2.5 h. The solution was filtered over Celite and concentrated in vacuo. The residue was dissolved in dichloromethane (20 mL) and benzoyl chloride (70 μL, 0.6 mmol) and triethylamine (20 μL) were added. The reaction was stirred under a nitrogen atmosphere at 25°C for 24 h then washed with water (1×20 mL), 1 M HCl (1×20 mL), saturated sodium bicarbonate (1×20 mL), and brine (1×20 mL). The organic layer was dried ($MgSO_4$), filtered, and concentrated in vacuo. Silica gel chromatography (ethyl acetate:ethanol 2:1) yielded **3a** (86 mg, 70% yield) as a colorless oil. $^1$H NMR (300 MHz, $CDCl_3$): $\delta$ 1.96 (s, 3H), 2.02 (s, 3H), 2.09 (s, 3H), 2.11 (s, 3H), 3.4 (m, 2H), 3.6 (m, 1H), 3.95–3.97 (m, 2H), 4.08–4.14 (m, 3H), 4.11–4.16 (m, 1H), 4.22–4.28 (dd, 1H, *J*=5.3, 12.0 Hz), 4.82–4.83 (d, 1H, *J*=1.24 Hz), 5.25–5.29 (m, 3H), 7.2 (broad t, 1H), 7.28–7.50 (m, 4H), 7.7 (br t, 1H), 7.76–7.85 (m, 2H) ppm. $^{13}$C NMR (75 MHz, $CDCl_3$): $\delta$ 20.61, 20.66, 20.79, 25.05, 25.52, 38.92, 53.68, 62.34, 65.89, 66.68, 68.44, 69.2, 69.30, 97.54, 130.37, 133.69, 136.73, 166.60, 169.60, 169.73, 170.17, 170.27, 170.60, 170.73 ppm. HR FAB-MS (pos) calcd for $C_{27}H_{35}N_3O_{13}$: 609.2171, obsd $MH^+$: 610.2227.

*4.2.5. 1-*O*-(11-Benzylamido-3,6,9-trioxaundecyl)-2,3,4,6-tetra-*O*-acetyl-α-*D*-mannopyranoside **3b***

Glycoconjugate **2b** (65 mg, 0.12 mmol) was dissolved in absolute ethanol (10 mL). Degussa Pd/C (20 mg) was added, and $H_2$ was bubbled through the mixture for 1.5 h. The solution was filtered over Celite and concentrated in vacuo. The residue was dissolved in dichloromethane (10 mL) and benzoyl chloride (28 μL, 0.25 mmol), triethylamine (165 μL, 10 equiv.), and DMAP (20 mg) were added. The reaction was stirred under a nitrogen atmosphere at 25°C for 18 h then washed with 1 M HCl (1×10 mL), saturated sodium bicarbonate (1×10 mL), and brine (1×10 mL). The organic layer was dried ($MgSO_4$), filtered, and concentrated in vacuo. Silica gel chromatography (ethyl acetate:petroleum ether:methanol 4:3:1) yielded **3b** (53 mg, 71%) as a colorless oil. $^1$H NMR (300 MHz, $CDCl_3$): $\delta$ 1.98 (s, 3H), 2.03 (s, 3H), 2.10 (s, 3H), 2.15 (s, 3H), 3.63–3.79 (m, 16 H), 4.03–4.11 (m, 2H), 4.30 (dd, 1 H, *J*=5.0, 12.3 Hz), 4.86 (d, 1H, *J*=1.6 Hz) 5.26–5.35 (m, 3H), 6.86 (br s, 1 H), 7.40–7.53 (m, 3H), 7.81 (d, 2H, *J*=7.1 Hz) ppm. $^{13}$C NMR (75 MHz, $CDCl_3$): $\delta$ 20.67, 20.74, 39.71, 62.34, 67.33, 69.02, 69.56, 69.77, 69.85, 69.92, 70.21, 70.49, 70.68, 70.81, 97.66, 127.02, 128.44, 131.34, 134.56, 167.43, 169.92, 170.71 ppm. IR (neat) $\nu$ 3408, 2090, 1647, 1371, 1227, 1048 $cm^{-1}$. FAB-MS (pos) calcd for $C_{29}H_{41}NO_{14}$: 627.25, obsd $MNa^+$: 650.25.

*4.2.6. 5-Azidomethyl-*N,N′*-bis[2-*O*-(2,3,4,6-tetra-*O*-acetyl-α-*D*-mannopyranosyl)-*N*-ethylglycylglycyl]-benzene-1,3-dicarboxamide **4a***

Protected glycopeptide **2a** (810 mg, 1.21 mmol) was dissolved in absolute ethanol (25 mL). Degussa Pd/C (50 mg) was added, and $H_2$ was bubbled through the mixture for 5 h. The solution was filtered over Celite and concentrated in vacuo. The residue was dissolved in dichloromethane (35 mL) and 5-azidomethylbenzene-1,3-dicarbonyl dichloride (110 mg, 0.4 mmol), HOBT (190 mg, 1.4 mmol), and DCC (285 mg, 1.4 mmol) were added. The mixture was stirred under a nitrogen atmosphere at 25°C for 48 h then filtered over Celite and washed with water (1×40 mL), 1 M HCl (1×40 mL), saturated sodium bicarbonate (1×40 mL), and brine (1×40 mL). The organic layer was dried ($MgSO_4$), filtered, and concentrated in vacuo. Silica gel chromatography (ethyl acetate:ethanol 2:1) yielded **4a** (314 mg, 66% yield) as a colorless oil. $^1$H NMR (400 MHz, $CDCl_3$): $\delta$ 1.90 (s, 6H), 1.96 (s, 6H), 2.02 (s, 6H), 2.06 (s, 6H), 3.3 (m, 4H), 3.5 (m, 2H), 3.7 (m, 2H), 3.8 (m, 4H), 3.95–3.97 (m, 4H), 4.01–4.07 (m, 2H), 4.17–4.21 (dd, 1H, *J*=5.1, 12.0 Hz), 4.30 (s, 2H), 4.77 (br s, 2H), 5.17–5.24 (m, 3H), 7.4 (broad t, 2H), 8.0 (broad t, 2H), 8.4 (broad t, 2H), 7.83 (s, 2H), 8.20 (s, 1H) ppm. $^{13}$C NMR (75 MHz, $CDCl_3$): $\delta$ 19.69, 19.73,

19.88, 24.13, 24.58, 33.02, 37.99, 42.04, 52.78, 61.44, 65.00, 65.76, 67.56, 68.28, 68.37, 96.62, 129.44, 132.80, 135.84, 165.92, 168.71, 168.84, 169.28, 169.39, 169.70, 169.84 ppm. IR (neat): diagnostic $\nu$ 2102 $cm^{-1}$ azide. HR FAB-MS (pos) calcd for $C_{45}H_{65}O_{26}N_9$: 1195.4042, obsd $MH^+$: 1196.4136.

*4.2.7. 5-Azidomethyl*-N,N′-*bis[11*-O-*(2,3,4,6-tetra*-O-*acetyl*-α-D-*mannopyranosyl)-3,6,9-trioxaundecyl]benzene-1,3-dicarboxamide* ***4b***

Glycoconjugate **2b** (80 mg, 0.14 mmol) was dissolved in absolute ethanol (10 mL). Degussa Pd/C (20 mg) was added, and $H_2$ was bubbled through the mixture for 1.5 h. The solution was filtered over Celite and concentrated in vacuo. The residue was dissolved in tetrahydrofuran (10 mL). Pyridine (100 μL), DMAP (20 mg), and 5-azidomethylbenzene-1,3-dicarbonyl dichloride (12 mg, 0.054 mmol) were added, and the solution was stirred under a nitrogen atmosphere at 25°C for 16 h. The solvent was removed in vacuo, and the crude reaction was dissolved in ethyl acetate (15 mL) and washed with 1 M HCl (1×10 mL), saturated sodium bicarbonate (1×10 mL), and brine (1×10 mL). The organic layer was dried ($MgSO_4$), filtered, and concentrated in vacuo. Silica gel chromatography (ethyl acetate:petroleum ether 2:1) yielded **4b** (43 mg, 63%) as a colorless oil. $^1$H NMR (300 MHz, $CDCl_3$): $\delta$ 1.98 (s, 6H), 2.04 (s, 6H), 2.10 (s, 6H), 2.16 (s, 6H), 3.57–3.78 (m, 32H), 4.05–4.14 (m, 4H), 4.23–4.27 (dd, 2H, *J*=4.6, 12.1 Hz), 4.44 (s, 2H), 4.84 (d, 2H, *J*=1.4 Hz), 5.22–5.37 (m, 6H), 7.65 (s, 2H), 8.01 (s, 1H) ppm. $^{13}$C NMR (75 MHz, $CDCl_3$): $\delta$ 19.77, 19.84, 21.44, 21.51, 67.26, 69.94, 68.41, 68.59, 69.20, 69.57, 69.85, 69.86, 70.22, 70.42, 70.45, 70.49, 72.26, 96.43, 128.59, 135.34, 166.12, 169.90, 169.92 ppm. HR FAB-MS (pos) calcd for $C_{53}H_{77}N_5O_{28}$: 1231.4755, obsd $MH^+$: 1232.4891.

*4.2.8.* N,N′,N′′-*Tris[2*-O-*(2,3,4,6-tetra*-O-*acetyl*-α-D-*mannopyranosyl)*-N-*ethylglycyglycyl]benzene-1,3,5-tricarboxamide* ***5a***

Protected glycopeptide **2a** (135 mg, 0.21 mmol) was dissolved in absolute ethanol (25 mL). Degussa Pd/C (50 mg) was added, and $H_2$ was bubbled through the mixture for 2.5 h. The solution was filtered over Celite and concentrated in vacuo to yield deprotected glycopeptide. The deprotected glycopeptide (100 mg, 0.2 mmol) was dissolved in dichloromethane (10 mL). 1,3,5-Benzenetricarbonyl trichloride (12 mg, 0.05 mmol) and triethylamine (25 μL) were added, and the reaction was stirred under a nitrogen atmosphere at 25°C for 24 h. The solution was washed with water (1×20 mL), 1 M HCl (1×20 mL), saturated sodium bicarbonate (1×20 mL), and brine (1×20 mL). The organic layer was dried ($MgSO_4$), filtered, and concentrated in vacuo. Silica gel chromatography (ethyl acetate:ethanol 2:1) yielded **5a** (58 mg, 69%) as a colorless oil. $^1$H NMR (400 MHz, $CDCl_3$): $\delta$ 1.99 (s, 9H), 2.06 (s, 9H), 2.11 (s, 9H), 2.15 (s, 9H), 3.37 (br m, 3H), 3.48–3.57 (br m, 6H), 3.70–3.77 (m, 6H), 4.01–4.15 (m, 18H), 4.26–4.30 (dd, 3H, *J*=5.3, 12.4 Hz), 4.82 (br s, 3H), 5.25–5.35 (m, 9H), 7.1 (br s, 3H), 8.4 (s, 3H), 8.6 (br s, 3H), 9.22 (br s, 3H) ppm. $^{13}$C NMR (75 MHz, $CDCl_3$): $\delta$ 20.66, 20.75, 20.84, 20.92, 29.62, 39.12, 43.05, 44.45, 44.47, 62.36, 65.93, 66.99, 68.62, 69.04, 69.25, 97.77, 129.09, 133.41, 165.88, 169.67, 170.05, 170.11, 170.40, 170.46, 170.51, 170.86 ppm. HR FAB-MS (pos) calcd for $C_{69}H_{93}N_9O_{39}$: 1671.5572, obsd $MH^+$: 1672.5642.

*4.2.9.* N,N′,*N′′-Tris[11*-O-*(2,3,4,6-tetra*-O-*acetyl*-α-D-*mannopyranosyl)-3,6,9-trioxaundecyl]benzene-1,3,5-tricarboxamide* ***5b***

Glycoconjugate **2b** (161 mg, 0.29 mmol) was dissolved in absolute ethanol (10 mL). Degussa Pd/C (30 mg) was added, and $H_2$ was bubbled through the mixture for 1.5 h. The solution was filtered over Celite and concentrated in vacuo. The residue was dissolved in tetrahydrofuran (10 mL), and pyridine (100 μL), DMAP (20 mg), and 1,3,5-benzenetricarbonyl trichloride (25 mg, 0.097 mmol) were added. The reaction mixture was stirred under a nitrogen atmosphere at 25°C for 16 h. The solution was concentrated

in vacuo, and the residue was dissolved in ethyl acetate (15 mL). The solution was washed with 1 M HCl (1×10 mL), saturated sodium bicarbonate (1×10 mL), and brine (1×10 mL). The organic layer was dried ($MgSO_4$), filtered, and concentrated in vacuo. Purification by silica gel chromatography (ethyl acetate:petroleum ether 5:1) yielded **5b** (43 mg, 63%) as a colorless oil. $^1$H NMR (300 MHz, $CDCl_3$): $\delta$ 1.96 (s, 9H), 2.02 (s, 9H), 2.07 (s, 9H), 2.13 (s, 9H), 3.54–3.78 (m, 48H), 4.00–4.13 (m, 6H) 4.24–4.29 (dd, 3H, *J*=5.1, 12.3 Hz), 4.82 (d, 3H, *J*=1.4 Hz), 5.22–5.33 (m, 9H), 7.19 (br s, 3H), 8.39 (s, 3H) ppm. FAB-MS (pos) calcd for $C_{75}H_{111}N_3O_{42}$: 1725.7, obsd $MH^+$: 1726.7.

*4.2.10. 5-Azidomethylbenzene-1,3-dicarboxylate di-*N*-hydroxysuccinimide ester **6***

5-Azidomethylbenzene-1,3-dicarboxylic acid (200 mg, 0.9 mmol), *N*-hydroxysuccinimide (230 mg, 2.22 equiv.), and DCC (413 mg, 2 mmol) were dissolved in tetrahydrofuran (25 mL). The reaction was stirred under a nitrogen atmosphere at 25°C for 18 h. The mixture was filtered, and dichloromethane (25 mL) was added. The solution was concentrated in vacuo, and the residue was dissolved in 1:1 ethanol:dichloromethane. Upon cooling, a white precipitate formed which was separated by gravity filtration. The precipitate **6** (330 mg, 88% yield) was dried in vacuo and used without further purification. $^1$H NMR (300 MHz, $CDCl_3$): $\delta$ 2.94 (s, 8H), 4.58 (s, 2H), 8.38 (s, 2H), 8.84 (s, 1H) ppm. $^{13}$C NMR (75 MHz, $CDCl_3$): $\delta$ 24.65, 130.91, 134.27, 159.34, 167.67 ppm. HR FAB-MS (pos) calcd for $C_{17}H_{13}N_5O_8$: 415.0764, obsd $MH^+$: 416.0824.

*4.2.11. 1,3,5-Benzenetricarboxylate tri-*N*-hydroxysuccinimide ester **7***

Benzene 1,3,5-tricarboxylate (300 mg, 1.4 mmol), *N*-hydroxysuccinimide (540 mg, 4.7 mmol), and DCC (1.2 g, 6 mmol) were dissolved in tetrahydrofuran (25 mL). The reaction was stirred under a nitrogen atmosphere at 25°C for 18 h. The mixture was filtered, and dichloromethane (25 mL) was added. The solution was concentrated in vacuo, and the residue was dissolved in 1:1 ethyl acetate:dichloromethane. Upon cooling, a white precipitate formed which was separated by gravity filtration. The precipitate **7** (420 mg, 59.8% yield) was concentrated in vacuo and used without further purification. $^1$H NMR (300 MHz, $CDCl_3$): $\delta$ 2.94 (s, 12H), 9.14 (s, 3H) ppm. $^{13}$C NMR (75 MHz, $CDCl_3$): $\delta$ 25.76, 137.32, 168.26 ppm. FAB-MS (pos) calcd for $C_{21}H_{15}N_3O_{12}$: 501.0656, obsd $MH^+$: 502.0720.

*4.2.12. 5-Azidomethyl-*N,N′*-bis[*N,N′*-bis[2-*O*-(2,3,4,6-tetra-*O*-acetyl-α-*D*-mannopyranosyl)-*N*-ethyl-glycylglycyl]-1-phenylmethyl-3,5-dicarboxamide]benzene-1,3-dicarboxamide **8a***

Protected bivalent **4a** (100 mg, 0.08 mmol) was dissolved in methanol (15 mL). Degussa Pd/C (30 mg) was added, and $H_2$ was bubbled through the mixture for 4.5 h. The solution was filtered over Celite, washed with hot methanol, and concentrated in vacuo. The residue was dissolved in tetrahydrofuran (10 mL) and triethylamine (50 μL). Compound **6** (8 mg, 0.023 mmol) was added, and the reaction was stirred under a nitrogen atmosphere at 25°C for 1 week. The reaction was concentrated in vacuo and purified by size-exclusion chromatography (BioGel SX-1 resin in tetrahydrofuran) to yield **8a** (27 mg, 47% yield) as a colorless oil. $^1$H NMR (300 MHz, $CDCl_3$): uninterpretable. $^{13}$C NMR: unable to obtain. FAB-MS (pos) calcd for $C_{107}H_{137}N_{17}O_{54}$: 2524, obsd $MH^+$: 2525.

*4.2.13. 5-Azidomethyl-*N,N′*-bis[*N,N′*-bis[11-*O*-(2,3,4,6-tetra-*O*-acetyl-α-*D*-mannopyranosyl)-3,6,9-trioxaundecyl]-1-phenylmethyl-3,5-dicarboxamide]benzene-1,3-dicarboxamide **8b***

Protected bivalent **4b** (43 mg, 0.035 mmol) was dissolved in absolute ethanol (10 mL). Degussa Pd/C (60 mg) was added, and $H_2$ was bubbled through the mixture for 2 h. The solution was filtered over Celite, washed with hot ethanol (40 mL), and concentrated in vacuo. The residue was dissolved in tetrahydrofuran (10 mL) and triethylamine (50 μL). Compound **6** (5.7 mg, 0.014 mmol) was added,

and the reaction was stirred under a nitrogen atmosphere at 25°C for 48 h. The reaction was concentrated in vacuo and purified by size-exclusion chromatography (BioGel SX-1 resin in tetrahydrofuran) to yield **8b** (23 mg, 63%) as a colorless oil. $^1$H NMR (300 MHz, $CDCl_3$): $\delta$ 1.98 (s, 12H), 2.03 (s, 12H), 2.09 (s, 12H), 2.14 (s, 12H), 2.27 (s, 2H), 3.59–3.74 (m, 56H), 4.02–4.10 (m, 14H), 4.13–4.30 (dd, 4H, *J*=4.9, 12.3 Hz), 4.41 (s, 2H), 4.60 (s, 4H), 4.84 (d, 4H, *J*=1.5 Hz), 4.99 (s, 2H), 5.24–5.30 (m, 18H), 6.98 (s, 2H), 7.56 (s, 2H), 7.80 (s, 1H), 7.87 (s, 2H), 7.91 (s, 1H), 8.40 (s, 1H) ppm. HR FAB-MS (pos) calcd for $C_{115}H_{161}N_9O_{58}$: 2595.9926, obsd $MNa^+$: 2618.9912.

*4.2.14.* N,N′,N′′-*Tris-[*N,N′-*bis[2*-O-*(2,3,4,6-tetra*-O-*acetyl-*α-D-*mannopyranosyl)*-N-*ethylglycyl-glycyl]-1-phenylmethyl-3,5-dicarboxamide]benzene-1,3,5-tricarboxamide* ***9a***

Protected bivalent **4a** (80 mg, 0.06 mmol) was dissolved in methanol (15 mL). Degussa Pd/C (30 mg) was added, and $H_2$ was bubbled through the mixture for 4 h. The solution was filtered over Celite, washed with hot methanol, and concentrated in vacuo. The residue was dissolved in tetrahydrofuran (10 mL) and triethylamine (50 μL). Compound **7** (4 mg, 0.008 mmol) was added, and the reaction was stirred under a nitrogen atmosphere at 25°C for 1 week. The reaction was concentrated in vacuo and purified by size-exclusion chromatography (BioGel SX-1 resin in tetrahydrofuran) to yield **9a** (18 mg, 61%) as a colorless oil. Both $^1$H and $^{13}$C NMR spectra showed only broad featureless resonances. FAB-MS (pos) calcd for $C_{156}H_{201}O_{81}N_{21}$: 3664, obsd $MNa^+$: 3687.

*4.2.15.* N,N′,N′′-*Tris-[*N,N′-*bis[11*-O-*(2,3,4,6-tetra*-O-*acetyl-*α-D-*mannopyranosyl)-3,6,9-trioxa-undecyl]-1-phenylmethyl-3,5-dicarboxamide]benzene-1,3,5-tricarboxamide* ***9b***

Protected bivalent **4b** (95 mg, 0.08 mmol) was dissolved in absolute ethanol (10 mL). Degussa Pd/C (50 mg) was added, and $H_2$ was bubbled through the mixture for 2.5 h. The solution was filtered over Celite, washed with hot ethanol (30 mL), and concentrated in vacuo. The reduced bivalent compound was dissolved in tetrahydrofuran (10 mL) and triethylamine (50 μL). Compound **7** (10 mg, 0.02 mmol) was added, and the reaction was stirred under a nitrogen atmosphere at 25°C for 72 h. The reaction was concentrated in vacuo and purified by size-exclusion chromatography (BioGel SX-1 resin in tetrahydrofuran) to yield **9b** (36 mg, 48%) as a colorless oil. $^1$H NMR (300 MHz, $CDCl_3$): $\delta$ 1.98 (s, 18H), 2.03 (s, 18H), 2.09 (s, 18H), 2.14 (s, 18H), 3.60–3.65 (m, 84H), 3.75 (s, 6H), 4.05–4.16 (m, 21H), 4.25–4.31 (dd, 6H, *J*=4.9, 12.3 Hz), 4.84 (d, 6H, *J*=1.5 Hz), 5.24–5.34 (m, 21H), 7.90 (br m, 12H) ppm. FAB-MS (pos) calcd for $C_{163}H_{227}N_9O_{87}$: 3774, obsd $MNa^+$: 3797.

*4.2.16. 1*-O-*[2-(*N-*Benzylamidoglycylglycyl)ethyl]-*α-D-*mannopyranoside* ***10a***

Protected monovalent **3a** (50 mg, 0.08 mmol) was dissolved in 0.1 M sodium methoxide in methanol (15 mL), and the reaction stirred at 25°C for 18 h. The solution was neutralized with Dowex $H^+$ resin, filtered, and concentrated in vacuo. The residue was dissolved in deionized water and purified by size-exclusion chromatography (BioRad P2 gel in water) to yield **10a** (quant.) as a white powder. $^1$H NMR (400 MHz, $D_2O$): $\delta$ 3.41–3.55 (m, 2H), 3.58–3.65 (m, 3H), 3.70–3.82 (m, 3H), 3.84–3.87 (m, 1H), 3.91–3.93 (dd, 1H, *J*=1.7, 3.4 Hz), 3.96 (s, 2H), 4.16 (s, 2H), 4.84 (d, 1H, *J*=1.71 Hz), 7.46–7.68 (m, 3H), 7.88–7.84 (m, 2H) ppm. $^{13}$C NMR (75 MHz, $D_2O$): $\delta$ 38.88, 42.42, 43.12, 60.80, 65.58, 66.66, 69.90, 70.38, 72.68, 99.54, 127.20, 128.20, 128.67, 128.75, 131.13, 132.50, 171.31, 171.41, 172.29 ppm. FAB-MS (pos) calcd for $C_{19}H_{27}O_9N_3$: 441.11, obsd $MNa^+$: 464.1.

*4.2.17. 1*-O-*(11-Benzylamido-3,6,9-trioxaundecyl)-*α-D-*mannopyranoside* ***10b***

Protected monovalent **3b** (30 mg, 0.033 mmol) was dissolved 0.1 M sodium methoxide in methanol (5 mL), and the reaction stirred at 25°C for 18 h. The solution was neutralized with Dowex $H^+$ resin, filtered,

and concentrated in vacuo. The residue was dissolved in deionized water and purified by size-exclusion chromatography (BioRad P2 gel in water) to yield **10b** (quant.) as a colorless oil. $^1$H NMR (400 MHz, $D_2O$): $\delta$ 3.39–3.73 (m, 21H), 3.77 (dd, 1H, *J*=1.7, 3.3 Hz), 4.68 (d, 1H, *J*=1.5, H-1, Hz), 7.38 (t, 2H, *J*=7.5 Hz), 7.47 (t, 1H, *J*=7.5 Hz), 7.62 (d, 2H, *J*=7.0 Hz) ppm. FAB-MS (pos) calcd for $C_{21}H_{33}NO_{10}$: 459.21, obsd $MNa^+$: 482.20.

*4.2.18. 5-Azidomethyl*-N,N′-*bis[2*-O-*(*α-D-*mannopyranosyl)*-N-*ethylglycylglycyl]benzene-1,3-dicarboxamide* ***11a***

Protected bivalent **4a** (75 mg, 0.06 mmol) was dissolved in 0.1 M sodium methoxide in methanol (10 mL), and the reaction stirred at 25°C for 18 h. The solution was neutralized with Dowex $H^+$ resin, filtered, and concentrated in vacuo. The residue was dissolved in deionized water and purified by size-exclusion chromatography (BioRad P2 gel in water) to yield **11a** (quant.) as a white solid. $^1$H NMR (400 MHz, $D_2O$): $\delta$ 3.41–3.55 (m, 4H), 3.58–3.65 (m, 6H), 3.70–3.81 (m, 6H), 3.83–3.87 (m, 2H), 3.91–3.92 (dd, 2H, *J*=1.7, 3.5 Hz), 3.97 (s, 4H), 4.20 (s, 4H), 4.60 (s, 2H), 4.85 (d, 2H, *J*=1.71 Hz), 8.04 (d, 2H, *J*=1.68 Hz), 8.23 (t, 1H, *J*=1.68 Hz) ppm. $^{13}$C NMR (75 MHz, $D_2O$): $\delta$ 24.42, 24.47, 38.86, 42.42, 43.10, 51.57, 60.78, 65.56, 66.64, 67.43, 69.87, 70.35, 72.65, 72.74, 99.51, 125.94, 130.53, 133.79, 137.43, 169.66, 171.35, 172.02 ppm. FAB-MS (pos) calcd for $C_{33}H_{49}N_9O_{18}$: 859.32, obsd $MNa^+$: 882.3.

*4.2.19. 5-Azidomethyl*-N,N′-*bis[11*-O-*(*α-D-*mannopyranosyl)-3,6,9-trioxaundecyl]benzene-1,3-dicarboxamide* ***11b***

Protected bivalent **4b** (56 mg, 0.05 mmol) was dissolved in 0.1 M sodium methoxide in methanol (5 mL), and the reaction stirred at 25°C for 18 h. The solution was neutralized with Dowex $H^+$ resin, filtered, and concentrated in vacuo. The residue was dissolved in deionized water and purified by size-exclusion chromatography (BioRad P2 gel in water) to yield **11b** (quant.) as a white solid. $^1$H NMR (400 MHz, $D_2O$): $\delta$ 3.60–3.97 (m, 28H), 4.59 (s, 2H), 4.82 (d, 2H, *J*=1.6 Hz), 7.96 (s, 2H), 8.13 (s, 1H) ppm. FAB-MS (pos) for $C_{37}H_{61}N_5O_{20}$: 895.39, obsd $MNa^+$: 918.38.

*4.2.20.* N,N′,N′′-*Tris[2*-O-*(*α-D-*mannopyranosyl)*-N-*ethylglycylglycyl]benzene-1,3,5-tricarboxamide* ***12a***

Protected trivalent **5a** (20 mg, 0.02 mmol) was dissolved in 0.1 M sodium methoxide in methanol (10 mL), and the reaction stirred at 25°C for 18 h. The solution was neutralized with Dowex $H^+$ resin, filtered, and concentrated in vacuo. The residue was dissolved in deionized water and purified by size-exclusion chromatography (BioRad P2 gel in water) to yield **12a** (quant.) as a white solid. $^1$H NMR (400 MHz, $D_2O$): $\delta$ 3.41–3.53 (m, 6H), 3.58–3.65 (m, 9H), 3.70–3.81 (m, 9H), 3.84–3.87 (m, 3H), 3.91–3.93 (dd, 3H, *J*=1.7, 3.5 Hz), 3.98 (s, 6H), 4.23 (s, 6H), 4.85 (d, 3H, *J*=1.71 Hz), 8.47 (s, 1H) ppm. $^{13}$C NMR (75 MHz, $D_2O$): $\delta$ 27.29, 32.42, 39.32, 42.91, 43.60, 61.19, 65.97, 67.04, 70.28, 73.03, 99.85, 129.92, 134.23, 169.16, 171.54, 172.16 ppm. FAB-MS (pos) calcd for $C_{45}H_{69}N_9O_{27}$: 1167, obsd $MNa^+$: 1190.

*4.2.21.* N,N′,N′′-*Tris[11*-O-*(*α-D-*mannopyranosyl)-3,6,9-trioxaundecyl]benzene-1,3,5-tricarboxamide* ***12b***

Protected trivalent **5b** (58 mg, 0.05 mmol) was dissolved in 0.1 M sodium methoxide in methanol (5 mL), and the reaction stirred at 25°C for 18 h. The solution was neutralized with Dowex $H^+$ resin, filtered, and concentrated in vacuo. The residue was dissolved in deionized water and purified by size-exclusion chromatography (BioRad P2 gel in water) to yield **12b** (quant.) as a white solid. $^1$H NMR (400 MHz, $D_2O$): $\delta$ 3.60–3.97 (m, 54H), 4.82 (d, 3H, *J*=1.6 Hz), 8.37 (s, 3H) ppm. FAB-MS (pos) calcd for $C_{51}H_{87}N_3O_{30}$: 1222, obsd $MNa^+$: 1245.

*4.2.22. 5-Azidomethyl*-N,N′-*bis[*N,N′-*bis[2*-O-*(α*-D-*mannopyranosyl)*-N-*ethylglycylglycyl]-1-phenylmethyl-3,5-dicarboxamide]benzene-1,3-dicarboxamide* ***13a***

Protected tetravalent **8a** (20 mg, 0.008 mmol) was dissolved in 0.1 M sodium methoxide in methanol (10 mL), and the reaction stirred at 25°C for 18 h. The solution was neutralized with Dowex $H^+$ resin, filtered, and concentrated in vacuo. The residue was dissolved in deionized water and purified by size-exclusion chromatography (BioRad P2 gel in water) to yield **13a** (quant.) as a white solid. $^1$H NMR (400 MHz, $D_2O$, 60°C): $\delta$ 3.40–3.48 (m), 3.55–3.65 (m), 3.69–3.81 (m), 3.82–3.84 (m), 3.87–3.89 (m), 3.96 (s), 4.17 (s, 2H), 4.40 (s), 4.55 (br s), 8.02 (m), 8.17 (m) ppm.

*4.2.23. 5-Azidomethyl*-N,N′-*bis[*N,N′-*bis[11*-O-*(α*-D-*mannopyranosyl)-3,6,9-trioxaundecyl]-1-phenylmethyl-3,5-dicarboxamide]benzene-1,3-dicarboxamide* ***13b***

Protected tetravalent **8b** (23 mg, 0.008 mmol) was dissolved in 0.1 M sodium methoxide in methanol (10 mL), and the reaction stirred at 25°C for 24 h. The solution was neutralized with Dowex $H^+$ resin, filtered, and concentrated in vacuo. The residue was dissolved in deionized water and purified by size-exclusion chromatography (BioRad P2 gel in water) to yield **13b** (quant.) as a white solid. $^1$H NMR (400 MHz, $D_2O$): $\delta$ 3.60–3.97 (m, 56H), 4.60 (s, 2H), 4.82 (d, 4H, $J$=1.6 Hz), 7.97 (br m, 6H), 8.13 (br m, 3H) ppm. FAB-MS (pos) calcd for $C_{83}H_{129}N_9O_{42}$: 1924, obsd $MNa^+$: 1947.

*4.2.24.* N,N′,N′′-*Tris[*N,N′-*bis[2*-O-*(α*-D-*mannopyranosyl)*-N-*ethylglycylglycyl]-1-phenylmethyl-3,5-dicarboxamide]benzene-1,3,5-tricarboxamide* ***14a***

Protected hexavalent **9a** (18 mg, 0.005 mmol) was dissolved in 0.1 M sodium methoxide in methanol (10 mL), and the reaction stirred at 25°C for 18 h. The solution was neutralized with Dowex $H^+$ resin, filtered, and concentrated in vacuo. The residue was dissolved in deionized water and purified by size-exclusion chromatography (BioRad P2 gel in water) to yield **14a** (quant.) as a colorless oil. $^1$H NMR (400 MHz, $D_2O$): $\delta$ 3.41–3.51 (m), 3.59–3.68 (m), 3.72–3.82 (m), 3.84–3.87 (m), 3.92–3.94 (m), 3.98 (s), 4.20 (s, 2H), 7.43–7.44 (m), 7.70–7.72 (m), 7.82–7.84 (m) ppm.

*4.2.25.* N,N′,N′′-*Tris[*N,N′-*bis[11*-O-*(α*-D-*mannopyranosyl)-3,6,9-trioxaundecyl]-1-phenylmethyl-3,5-dicarboxamide]benzene-1,3,5-tricarboxamide* ***14b***

Protected hexavalent **9b** (18 mg, 0.005 mmol) was dissolved in 0.1 M sodium methoxide in methanol (10 mL), and the reaction stirred at 25°C for 18 h. The solution was neutralized with Dowex $H^+$ resin, filtered, and concentrated in vacuo. The residue was dissolved in deionized water and purified by size-exclusion chromatography (BioRad P2 gel in water) to yield **14b** (quant.) as a colorless oil. Both $^1$H and $^{13}$C NMR spectra showed only broad featureless resonances. FAB-MS (pos) calcd for $C_{115}H_{179}N_9O_{63}$: 2694, obsd $MNa^+$: 2717.

*4.2.26. Hemeagglutination inhibition assay*

Prior to each assay, the minimum lectin concentration for agglutination of a 2% porcine erythrocyte solution was determined, and a sixfold excess of lectin in phosphate-buffered saline pH 7.3 (PBS) was utilized during inhibition assays. Serial twofold dilutions of ligand were incubated with concanavalin A for 2 h at 37°C. A 2% solution of porcine erythrocytes in phosphate was added to the lectin solution before subsequent incubation at 37°C. The minimum concentration of ligand required to inhibit agglutination was determined and reported relative to α-D-methyl mannopyranoside.

*4.2.27. ELLA analysis*

ELLA assays were carried out as previously described. All incubations were carried out at 37°C.[34,35] Briefly, Falcon microtiter plates were coated overnight with yeast mannan (100 μL of a 10 μg/mL solution in PBS). Excess mannan was removed by three washes with PBS containing 0.05% v/v Tween-20 (PBST). Coated wells were blocked with 1% w/v bovine serum albumin in PBS (150 μL) for 1 h. During this period, serial twofold dilutions of ligand were also incubated with peroxidase labeled concanavalin A (a dilution of a 0.1 mg/mL solution which had been determined to give $A_{410}$ of 1.0–0.8 AU). The lectin solutions were then transferred to the blocked plate for an additional 1 h incubation. These plates were subsequently washed twice with PBST and once in PBS prior to development with ABTS (50 μL of a 250 μg/mL solution in citrate–phosphate buffer pH 4.0 with 0.02% v/v hydrogen peroxide). The absorbance at 410 nm was monitored after a 20 min period. Fractional inhibitions are based on $A_{410}$ without inhibitor present and referenced to the inhibitory potential of an α-D-methyl mannose standard determined for each plate. All assays were carried out in duplicate.

*4.2.28. Titration microcalorimetry*

Calorimetric experiments were carried out on a MicroCal Omega isothermal titration microcalorimeter; details on the instrument and mathematical analyses are described elsewhere.[37] The reaction cell (volume=1.3678 mL) contained a solution of concanavalin A (0.60 mM monomer) in buffer (50 mM dimethyl glutaric acid, 250 mM sodium chloride, 1 mM calcium chloride, 1 mM manganese chloride, at pH 5.2). A series of forty injections of dendrimer solution (4–28 mM mannoside) were made. The volume of each injection was 2.2 μL, the duration of injection was 4.4 s, and the time between injections was 3 min. The data from the resulting injections were integrated to generate a titration curve. A non-linear 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.

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