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Synthesis of Oligomeric Mannosides and Their Structure-Binding Relationship with Concanavalin A

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Abstract: Small glycodendrimers with α-mannosyl ligands were synthesized by using copper-catalyzed azide–alkyne coupling chemistry and some of these molecules were used as multivalent ligands to study the induction of concanavalin A (Con A) precipitation. The results showed that the monovalent mannose ligand could induce the precipitation of Con A. This unexpected finding initiated a series of studies to characterize the molecular basis of the ligand–lectin interaction. The atypical precipitation is found to be specific to the mannose, fluorescein moiety (FITC), and Con A. Apparently the mannose ligand binds to Con A through hydrogen-bonding interactions, whereas the binding of FITC is mediated by hydrophobic forces.

Keywords: carbohydrates · dendrimers · glycopeptides · multivalent effect · precipitation

Introduction

Carbohydrate–protein interactions are prevalent in biological systems and most of the interactions are closely associated with physiological processes, such as modulation of immunological responses,[1] fertilization,[2] tumor metastasis,[3] and bacteria and viral invasion.[4] Although monovalent carbohydrate–protein interaction is weak,[5,6] nature overcomes the weak association by 1) displaying the carbohydrate ligands in multiple copies and 2) presenting the protein receptors in oligomeric complexes. Multivalent presentation of ligands and receptors strengthens the carbohydrate–protein interactions. These natural architectures have inspired chemists to design multivalent ligand mimics. Representative examples include glyconanoparticles,[7] glycopolymers,[8] glycodendrimers,[9] glycoclusters,[10] and glycoliposomes.[11] Of these synthetic ligands, glycodendrimers are particularly attractive because of their well-defined structure and controllable ligand density. In addition, dendritic molecules can be functionalized by specific chemical groups.

In a project to develop a lectin-sensing method, we explored sugar-binding-induced precipitation as a lectin-recognition mechanism.[12] This precipitation process was first studied by Brewer et al., who found that lectins underwent precipitation when binding to specific glycopeptides.[13] Such a binding-induced precipitation event is attributed to the crosslinking of lectin molecules by carbohydrate ligands in glycopeptides.[14] The requirement for the crosslinking of lectin is the multivalent architecture of the binding ligand.

Initially, monovalent mannoside **1** and a series of oligomeric mannosides, including glycodendritic molecules **2a**–**2c**, **3a**–**3c**, **4a**, and **4b**, were prepared. The bodies of the glycodendritic molecules were constructed from galactosyl (Gal) branching units, which provide attachment sites for the placement of the mannosyl ligands (Figure 1). For the assembly of the glycodendritic molecules, the classic convergent approach was applied, which allowed purification of the intermediate in each reaction step.[15] Of the synthetic mannosyl ligands, monovalent mannosyl ligand **1**, trivalent dendritic mannosyl ligand **2c**, and hexavalent dendritic mannosyl ligand **3c** were conjugated to a fluorescein label (FITC), which enabled naked-eye detection of the binding-induced precipitation.

In the lectin precipitation experiments, monovalent mannosyl ligands **1**, trivalent dendritic mannosyl **2c**, and **3c** were used to bind concanavalin A (Con A).[16] Con A lectin is widely used for the study of the multivalent carbohydrate-protein interactions.[17] We assumed that if crosslinking between the mannosyl ligand and Con A lectin occurred, the precipitation of lectin would be induced. Interestingly, FITC-labeled monovalent mannosyl ligand **1** induced Con A precipitation. This result is unexpected because there is only a single copy of the mannosyl ligand in **1**. To understand the

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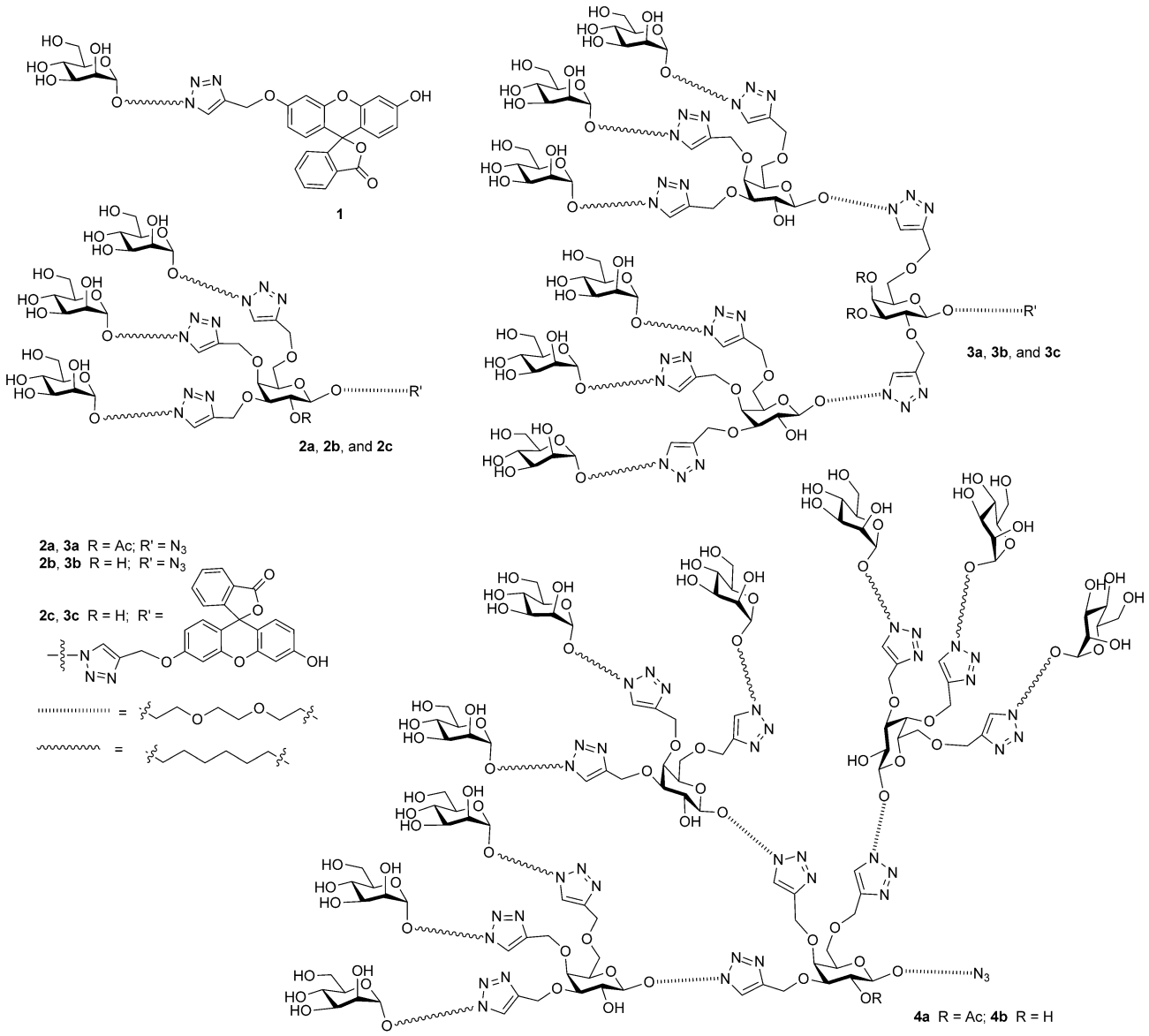


Figure 1. The structure of monovalent mannosyl ligand **1** and multivalent glycodendritic mannosyl ligands **2a**–**2c**, **3a**–**3c**, **4a**, and **4b**.

molecular basis of the precipitation, additional experiments were performed, including ligand competition, structural truncation study, measurement of dissociation constants (K_d), and dynamic light scattering (DLS) analysis. Taken together, these results provide an explanation for this unexpected precipitation that is useful for future studies of carbohydrate–protein interactions. A full account of our investigations is described herein.

1. Results and Discussion

1.1. Syntheses of Monovalent and Multivalent Dendritic Mannosyl Ligands

The synthesis of the target glycodendritic molecules required mannosyl building block **5**, Gal branching units **6** and **7**, and FITC propargyl ether derivative **8** (Figure 2, Scheme 1). Preparation of **5**, **6**, and **7** followed literature procedures (see the Supporting Information).[18] FITC prop-

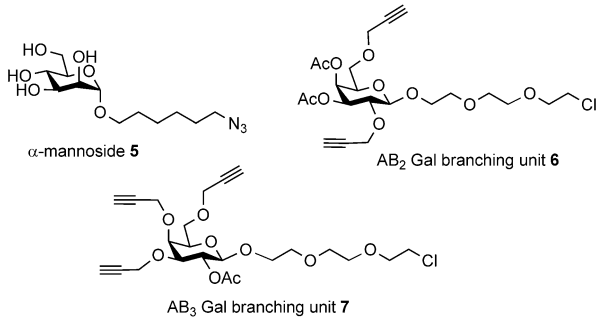
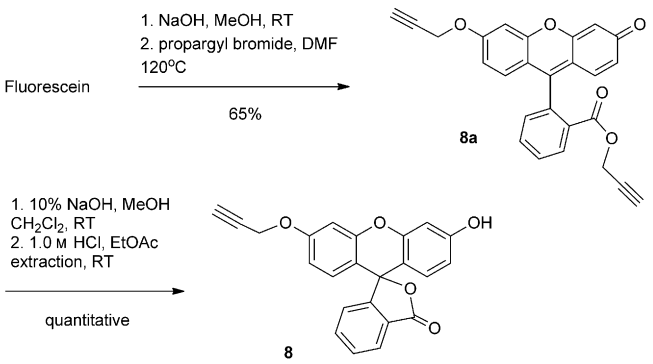


Figure 2. Mannosyl building block **5** and Gal branching units **6** and **7**.



Scheme 1. Preparation of propargyl FITC ether derivative **8** from fluorescein.

argyl ether derivative **8** was synthesized from unmodified FITC through three steps, including 1) alkylation of FITC with propargyl bromide to ester **8a**, 2) hydrolysis of ester **8a** to an acid derivative, and 3) lactonization of the acid derivative to the desired product **8** (Scheme 1).[19] Galactosyl derivatives **6** and **7** functioned as the AB_2 and AB_3 branching units, respectively, which provided the terminal alkyne groups for coupling with mannosyl building block **5** or the glycodendritic intermediate (**2b**, **3b**, and **4b**). The coupling of **5** (or **2b**, **3b**, and **4b**) with the Gal branching units (**6** or **7**) relies on copper(I)-catalyzed azide–alkyne coupling (CuAAC) chemistry.[20] Note that building blocks **2b**, **3b**, **4b**, and **5** were unprotected so as to reduce the steric hindrance in the coupling reactions.

The synthetic routes of mannosyl ligand **1**, **2a**–**2c**, **3a**–**3c**, **4a**, and **4b** are depicted in Scheme 2a–d. For the synthesis of FITC-labeled monovalent mannosyl ligand **1**, mannosyl building block **5** was reacted with FITC propargyl ether derivative **8** at 55 °C under CuAAC conditions to give **1** in 64 % isolated yield (Scheme 2a). Purification of **1** was achieved by standard normal-phase chromatography.

Concerning the synthesis of glycodendritic molecules **2a**–**2c**, AB_3 branching unit **7** was employed to couple with mannosyl building block **5** at 50 °C by using the CuAAC method (Scheme 2b).

The CuAAC method produced a trivalent glycodendritic molecule with a chloride terminus, which upon $Cl \rightarrow N_3$ substitution was converted to glycodendritic molecule **2a** with a terminal azido function. The C2 acetyl group at the Gal unit of **2a** was deprotected to give deacetylated glycodendritic molecule **2b**. Glycodendritic molecule **2b** could be used for 1) preparation of the FITC-labeled trivalent glycodendritic mannosyl ligand **2c** through CuAAC reaction with FITC propargyl ether derivative **8** or 2) iterative growth of the second-generation glycodendritic molecule through CuAAC reaction with one of the branching units (either **6** or **7**). Due to the high polarity of the glycodendritic molecules, size-exclusion chromatography was applied for purification of intermediates **2b** and **2c**. To remove the residual copper ions, the glycodendritic molecules were subjected to dialysis in a solution containing ethylenediaminetetraacetate (EDTA).

A hexavalent or nonavalent glycodendritic mannosyl ligand could be prepared from glycodendritic molecule **2b**, depending on which of the branching units (**6** or **7**) was used (Scheme 2c and d). Hexavalent glycodendritic molecule **3a** was obtained by coupling of glycodendritic molecule **2b** with AB_2 branching unit **6**, followed by $Cl \rightarrow N_3$ substitution. Subsequent deprotection of the C2 acetyl function of **3a** afforded hexavalent glycodendritic molecule **3b**, which was treated with FITC ether derivative **8** to give FITC-labeled hexavalent glycodendritic mannosyl ligand **3c** (Scheme 2c). Nonavalent glycodendritic molecules **4a** and **4b** were simply prepared by using the same synthetic route as for **3**, but AB_3 branching unit **7** and glycodendritic molecule **2b** was used in the coupling reaction (Scheme 2d).

Other than the hexavalent and nonavalent glycodendritic molecules, the aforementioned strategy is applicable for preparation of a dodecavalent glycodendritic mannosyl ligand from the coupling of hexavelant glycodendritic molecule **3b** with branching unit **6** (see the Supporting Information for reference). However, the present strategy is ineffective for the synthesis of a glycodendritic molecule with a higher ligand density (i.e., >12 ligands) probably due to steric factors.

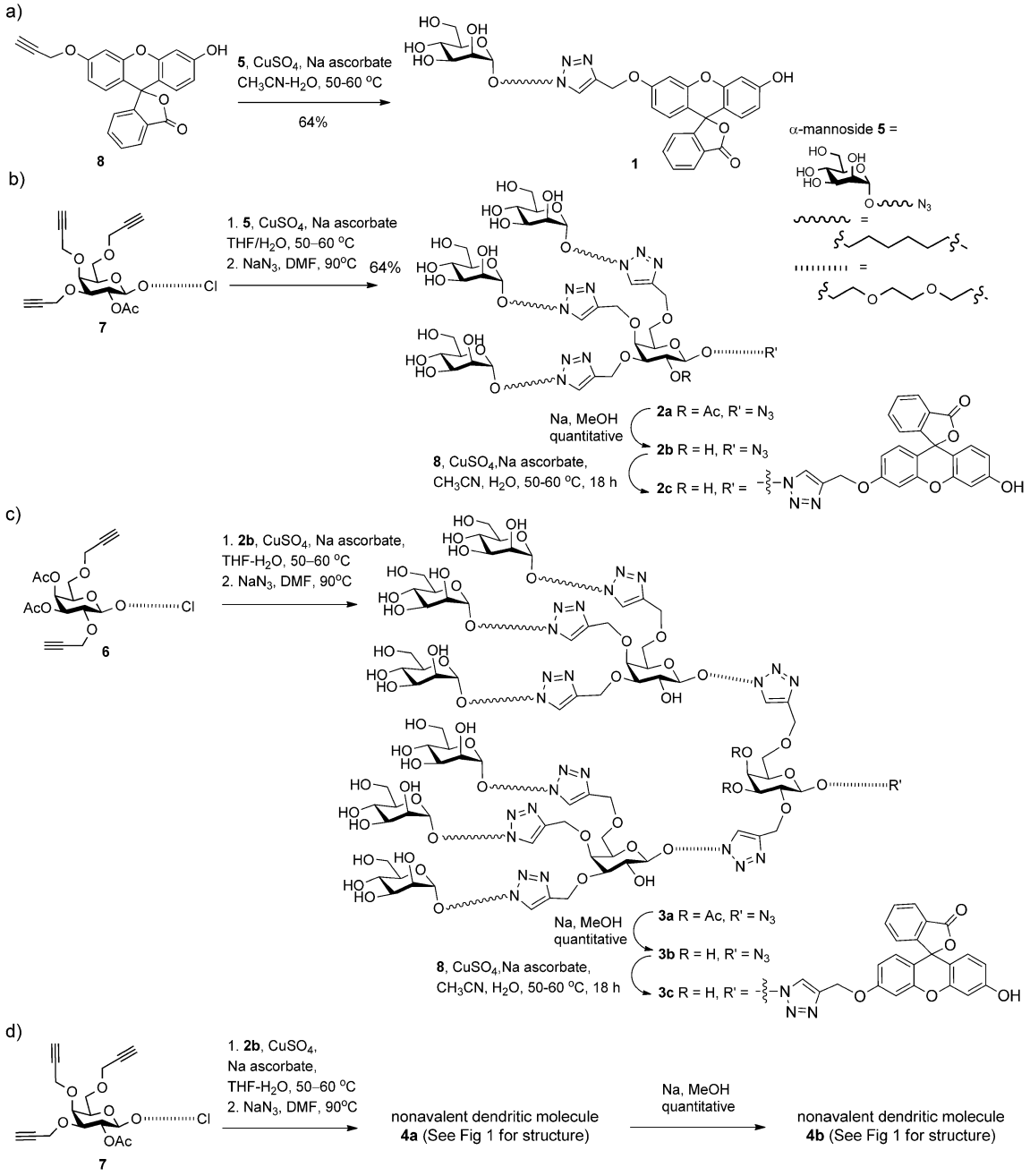
1.2. Characterization of Glycodendritic Compounds

Having prepared the glycodendritic molecules, we characterized these compounds by using ^{1}H NMR spectroscopy (see the Supporting Information), MALDI-TOF spectrometry, and gel permeation chromatography (GPC) methods, which adequately confirm the chemical identity and homogeneity. However, due to the highly unsymmetrical structure and aggregation behavior of the glycodendritic molecules, a full assignment of the ^{1}H NMR spectra is difficult unless multidimensional NMR spectroscopy experiments are applied.[21] Nevertheless, the structure of glycodendritic molecules **2**–**4** can reasonably be inferred by studying the ratio of the characteristic proton signals. For illustration, columns 2 and 3 of Table 1 depict the theoretical and experimental ratios of the

Table 1. Characterization of glycodendritic molecules **2a**, **3a**, and **4a**.

	Triazole-*H*/CH_3C=O ratio		MALDI-TOF MS	
	calcd	exptl	$[M+H]^+$ or $[M+Na]^+$	found (calcd)
2a	1.0	1.0	$C_{57}H_{98}N_{12}NaO_{26}$	1431.6 (1431.7)
3a	2.7	2.5	$C_{136}H_{227}N_{27}NaO_{62}$	3253.4 (3253.5)
4a	4.0	4.2	$C_{194}H_{327}N_{39}O_{87}$	4594.1 (4594.2)

triazole protons (triazole-*H*) (at $\delta = 7.8$–8.1 ppm) to the acetyl protons (CH_3C=O) of the Gal branching unit (at $\delta =$ 1.9–2.0 ppm). Further support of the chemical identity is provided by analysis of the MALDI-TOF mass spectra. The molecular ions (m/z) of glycodendritic molecules **2a**, **3a**, and **4a** were detected (Table 1, columns 4 and 5). To confirm the homogeneity of the glycodendritic molecules, compounds **2a**, **3a**, and **4a** were analyzed by using gel permeation chromatography (GPC). As shown in Figure 3, these



Scheme 2. Preparation of FITC-labeled monovalent mannosyl ligand **1** and multivalent dendritic mannosyl ligands **2a**–**2c**, **3a**–**3c**, **4a**, and **4b**.

compounds are monodisperse, thus they are free from structurally defective products.

1.3. Mannose-Binding-Induced Precipitation of Con A

After characterization of the glycodendritic mannosyl ligands, we performed sugar-binding-induced precipitation experiments. The experimental method was established on the base of a known precipitation procedure.[13b] Thus, a solution of Con A in Tris buffer (pH 7.4) at concentrations of 10, 20, or 40 μM was mixed with the FITC-labeled mannosyl ligand **1**, **2c**, or **3c** (Figure 4). After mixing and standing at RT for 30 min, the mixture was centrifuged and if lectin precipitation had occurred then a precipitate pellet was found at the bottom of the centrifuge tube. The supernatant was decanted and the precipitate was washed with clean buffer solution, then the precipitate pellet was examined under UV illumination. Pellets with fluorescent emission imply that ligand-bound lectin precipitation has occurred.

Trivalent and hexavalent dendritic mannosyl ligands **2c** and **3c** were able to induce precipitation of Con A at lectin concentrations of 10, 20, or 40 μM, which can be explained

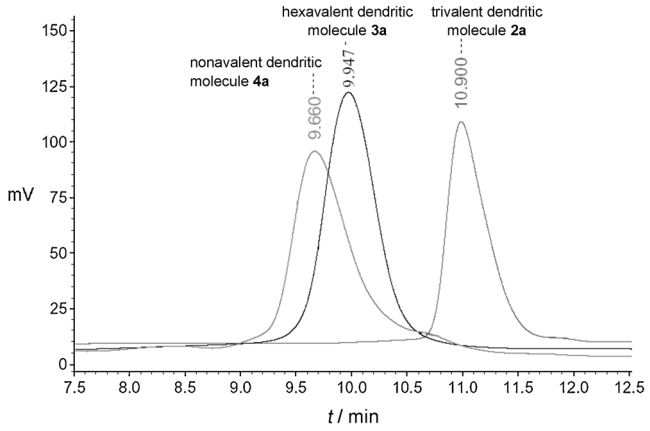


Figure 3. Overlay of the GPC chromatograms of multivalent glycodendritic molecules **2a**, **3a**, and **4a**.

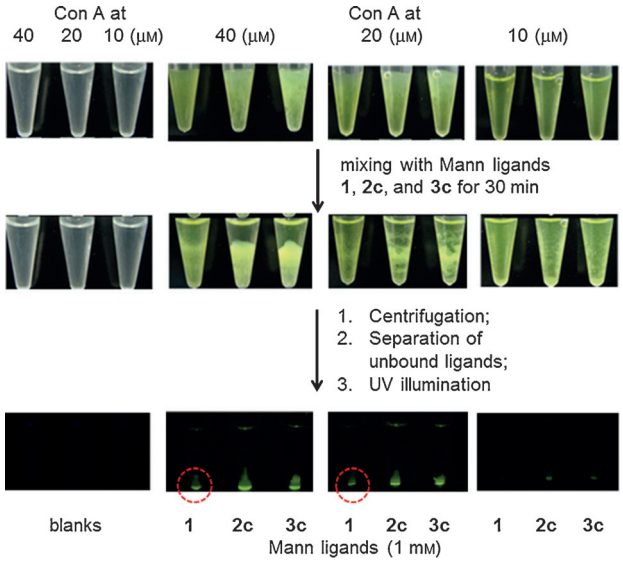


Figure 4. Photographs of the results of the induced precipitation experiment obtained by using synthetic ligands **2c**, **3c**, and **1** (1.0 mM, 75 μL) to interact with different concentrations of Con A (10, 20, and 40 μM; 50 μL). Panel 1: Photographs taken immediately after mixing; Panel 2: photographs taken after the samples stood at RT for 30 min; Panel 3: photographs of the lectin precipitate under UV illumination after a washing cycle (experimental details are given in the Supporting Information).

by the multivalent presentation of ligands in these molecules (Figure 4, panels 1 and 2). What appears to be unusual is the Con A precipitation induced by **1** (Figure 4, columns 2 and 3). Is the precipitation resulting from a nonspecific interaction? If this was the case, this precipitation should occur for other proteins. Thus, proteins of different sizes, including myoglobin (Myo), α-casein (α-Ca), β-casein (β-Ca), carbonic anhydrase (CAH), Con A, bovine serum albumin (BSA),[22] and lectins with different sugar binding sites, including peanut agglutinin (PNA)[23] and *Ricinus communis* agglutinin (RCA_{120}),[24] were treated with monovalent mannosyl ligand **1**. The resulting mixture was put through the same precipitation procedure (Figure 5a). With the exception of Con A, all proteins were stable in solution and no induced precipitation occurred. Apparently, the precipitation of Con A by monovalent ligand **1** is specific to lectin.

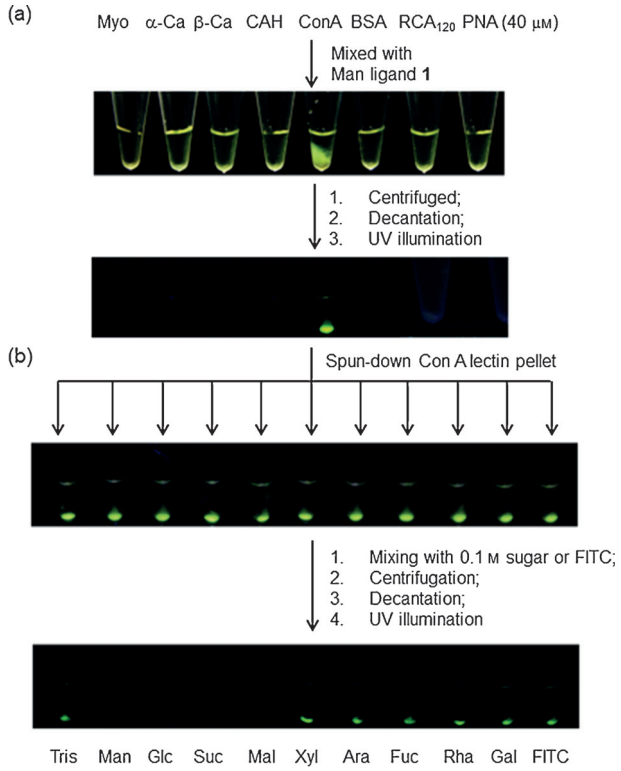


Figure 5. a) Induced precipitation experiment for mixing monovalent ligand **1** with other proteins and b) treatment of the precipitate pellet of Con A with 0.10 M of sugar or FITC solution.

Another question that attracts our attention is the specificity of the precipitation with respect to the sugar ligand. Typical ligand-binding-induced precipitation is attributed to the crosslinking interaction between carbohydrate ligands and lectin molecules. Because such hydrogen-bonding-mediated interactions are weak and, therefore, bound sugar ligand **1** can be displaced by excessive amounts of free ligand molecules, that is, mannose or glucose in the present case.[14] To confirm if the binding-induced precipitation by **1** is associated with hydrogen-bonding (H-bonding) interactions, ligand competition experiments were performed. In these experiments, the Con A precipitate pellet obtained from the above experiment was mixed in 0.10 M free-sugar solution. The mixture was centrifuged and then the supernatant was decanted. At this point, the centrifugation tubes were re-examined for pellet recovery (Figure 5b).

When the Con A pellet was suspended in a solution of 0.1 M mannose (Man), glucose (Glc), sucrose (Suc), or maltose (Mal), the precipitate was completely dissolved and no trace of the precipitation pellet were recovered (Figure 5). In contrast, when the pellet was suspended in a solution of 0.1 M xylose (Xyl), arabinose (Ara), fucose (Fuc), rhamnose (Rha), galactose (Gal), or fluorescein (FITC), the precipitation pellet was recovered. These results indicate that the present precipitation involves a H-bonding interaction between the mannose in **1** and the binding domains in Con A.

1.4. Structural Truncation Study

After verifying the specificity of the Con A precipitation, we performed a structural truncation study to elucidate the molecular basis of the precipitation above. Thus, unmodified fluorescein (FITC), FITC-labeled monovalent mannosyl ligand **1**, unlabeled monovalent mannosyl ligand **5**, unlabeled trivalent mannosyl ligand **2b**, and FITC-labeled trivalent mannosyl ligand **2c** were also tested in the precipitation experiment described above. In addition, FITC-labeled monovalent galactosyl ligand **9**[25] was included in the study (Figure 6). The truncated analogues that either lack the FITC moiety, such as **2b** and **5**, or lack the mannose residue, such as unmodified FITC and **9**, did not induce the precipitation of Con A, which implies that the presence of the mannose and FITC moieties in ligands **1** and **2c** is crucial for induced precipitation.

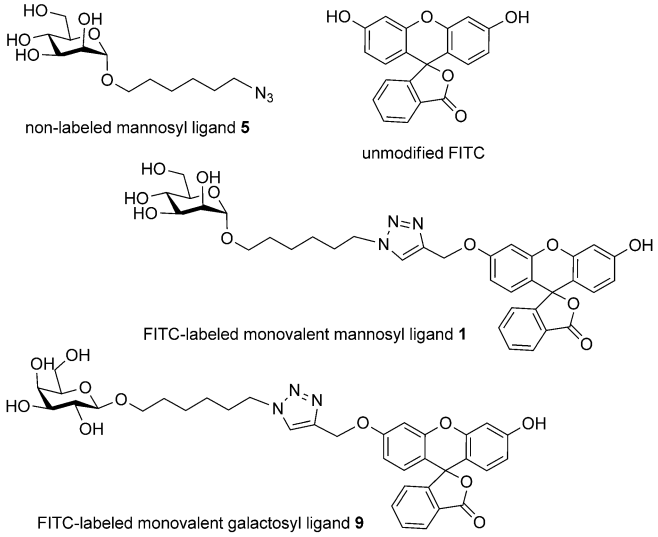


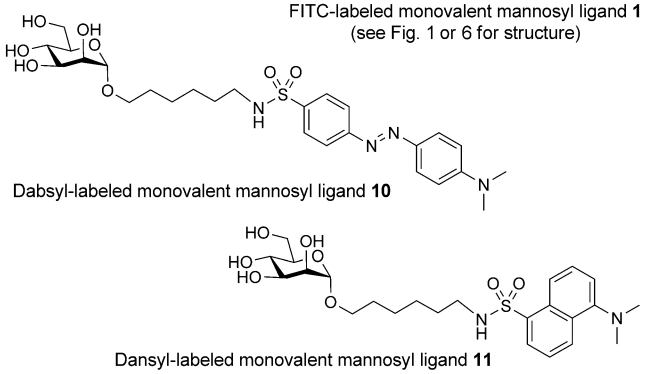
Figure 6. Structures of FITC-labeled monovalent mannosyl ligand **1**, unlabeled monovalent mannosyl ligand **5**, and FITC-labeled monovalent galactosyl ligand **9**.

In light of the structural truncation study, we initially thought that the induced precipitation by ligand **1** was triggered by a binding interaction from the mannose and FITC moieties.[26] In the literature, a body of reports reveal the binding of nonpolar molecules to lectins through hydrophobic interactions,[27,28] and the incorporation of a hydrophobic linker to a synthetic ligand has been shown to enhance its binding affinity.[29,30,17b,26,31–33] However, based on the experimental results of Figure 5b, FITC alone is unlikely to be a ligand to Con A. At this point, we decided to determine the contribution of FITC in ligands **1**, **2c**, and **3c** to Con A binding. To this end, the dissociation constants (K_d) of the ligands and corresponding structural analogues were measured. From intuition, the obtained K_d values may correlate with the results of the lectin precipitation in Figures 4 and 5.

1.5 Thermodynamic Binding Affinity and Dynamic Laser Light Scattering Study

The dissociation constants (K_d) of the FITC-labeled mannosyl ligands (**1**, **2c**, and **3c**), unlabeled mannosyl ligands (**5**, **2b** and **3b**), structure analogue **9** (different in the carbohydrate ligand), dabsylate (Dab)-labeled mannosyl ligand **10**, and dansyl (Dan)-labeled mannosyl ligand **11** (different in the terminal hydrophobic group) were measured by using a known fluorescent polarization method.[34] Mannosyl ligands **10** and **11** contain an aromatic group to mimic the FITC moiety of mannosyl ligand **1** (Table 2) and the hydrophobicity of the ligands are inferred from the retention time of the HPLC analysis.[35]

Table 2. Dissociation constants (K_d) for Con A binding.



Entry	Ligand	Hydrophobic group	Ligand	K_d [μM][a]
1	**1** (monovalent)	FITC	Man	11.2 ± 1.0
2	**5** (monovalent)	–	Man	940 ± 20
3	**9** (monovalent)	FITC	Gal	1570 ± 72
4	**10** (monovalent)	Dabsyl	Man	N.D.
5	**11** (monovalent)	Dansyl	Man	38.8 ± 1.0
6	–	FITC	–	N.D.
7	**2c** (trivalent)	FITC	Man	7.20 ± 1.5
8	**2b** (trivalent)	–	Man	366 ± 10
9	**3c** (hexavalent)	FITC	Man	15.8 ± 2.8
10	**3b** (hexavalent)	–	Man	48.3 ± 3.0

[a] N.D. indicates not detectable at a concentration of 1.0 mM.

Remarkably, the K_d value for FITC-labeled monovalent mannosyl ligand **1** (11.2 μM) is about 50 and 150 times smaller than that for unlabeled monovalent mannosyl ligand **5** (940 μM) and FITC-labeled monovalent galactosyl ligand **9** (1570 μM), respectively (Table 2, entries 1–3). These results correlate well with the ability of **1** to induce precipitation of Con A. At this point, we investigated the effect of substituting the FITC moiety in **1** with other aromatic groups to see if the resulting analogues would afford similar binding affinity. By comparing the K_d values for Dab-labeled mannosyl ligand **10** (not detectable at [**10**] ≥ 1 mM) and Dan-labeled mannosyl ligand **11** (38.6 μM) with that of the FITC-labeled mannosyl ligand **1** (11.2 μM), it can be seen that the substitution of the FITC derivative with other aromatic structure does modify the binding affinity (Table 2, entries 1, 4, and 5). Apparently, the greater the hydrophobicity (shorter re-

tention time) of the ligand (i.e., **1** and **11**), the higher the binding affinity (smaller K_d value). Although the binding affinity of ligand **1** is likely contributed from the mannose and FITC moieties, FITC alone does not compete with **1** for Con A binding (Figure 5b). Taken together, these data imply that a chemical connection between the mannose and FITC is essential for lectin precipitation and high binding affinity. To confirm this, we measured the K_d value of unmodified FITC and no significant binding could be detected (not detectable at [FITC] $\geq$ 1 mM; Table 2, entry 6).

In addition to the K_d value for **1**, the K_d value for FITC-labeled and unlabeled multivalent ligands **2b–2c** and **3b–3c** were obtained. Based on these data (K_d = 366 μM for **2b**; K_d = 7.2 μM for **2c**), the binding affinity of FITC-labeled trivalent ligand **2c** is higher than that of unlabeled counterpart **2b** (Table 2, entries 7 and 8). This set of data reinforces the fact that FITC is responsible for the higher binding affinity. A similar trend is also observed for hexavalent ligands **3c** and **3b** (K_d = 15.8 and 48 μM, respectively; Table 2, entries 9 and 10). Furthermore, FITC-labeled hexavalent mannosyl ligand **3c** has similar binding affinity compared with FITC-labeled monovalent ligand **1** and FITC-labeled trivalent ligand **2c** (Table 2, entries 1, 7, and 9, respectively). Apparently, the mannose itself plays a primary role in the Con A binding, whereas the role of FITC (aromatic group) is secondary and yet necessary for enhancement of the binding affinity.

It is clear from the K_d values of the FITC-labeled mannosyl ligands (11.2 μM for monovalent ligand **1**, 7.2 μM for trivalent ligand **2c**, and 15.8 μM for hexavalent ligand **3c**) that no multivalent effect is observed by simply increasing the number of mannose ligands (Table 2, entries 1, 7, and 9, respectively). Interestingly, when unlabeled monovalent ligand **5** (K_d = 940 μM) was used as a reference to compare the K_d values of unlabeled multivalent ligands **2b** (K_d = 366 μM) and **3b** (K_d = 48 μM), it seems that the binding affinity gradually increases as the number of the mannosyl ligands is increased (Table 2, entries 2, 8, and 10). However, such a mild increase trend still deviates from the general conception of a multivalent effect in which the binding affinity increases exponentially on increasing the number of the ligands. A possible explanation is insufficient spatial separation and/or orientation of the mannose ligands in **2b** and **3b** (also in **2c** and **3c**) to affect the overall binding efficiency gained by multivalency. However, if the K_d value of **5** is used as a reference to compare with the K_d value of **2c** and **3c**, an impression of multivalent effect is obvious.

In the literature, a number of multivalent ligands have been synthesized for studies of carbohydrate–protein interactions. Because such ligands contain nonpolar supporting structures and/or reporters, the possible contribution of the aglycone components to the protein binding must be considered with a great caution before reaching a conclusion.[36]

1.6 CMC Measurement and DLS Studies

Although the measurement of K_d values above confirms the contribution of FITC to Con A binding, it does not answer the question about the mechanism of Con A precipitation as induced by monovalent ligand **1**. In the literature, glycolipids are known to form micelle aggregates at high concentrations, which is expected to display the glyco ligands in a multivalent manner.[37] It is thus necessary to examine whether there is any micelle formation in our experimental context and, if so, at what concentration. In this vein, the dynamic light scattering method was used to measure the critical micelle concentration (CMC) of **1** (Figure 7 and Figure S1 in Supporting Information).[38] At concentrations of

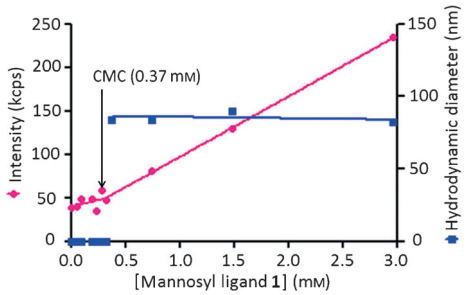


Figure 7. Measurement of CMC and detection of micelle formation for monovalent ligand **1**.

0.37 mM or above, ligand **1** forms aggregates with an approximate hydrodynamic diameter (d_h) of (96.4 ± 27.6) nm. A consistent result was also observed in the study of Con A precipitation in which the concentration of **1** was varied from 0.05 to 3.0 mM. The Con A lectin clearly appeared to precipitate at [**1**] $\geq$ 0.37 mM and the extent of precipitation increases in proportion to the ligand concentration (Figure 8).

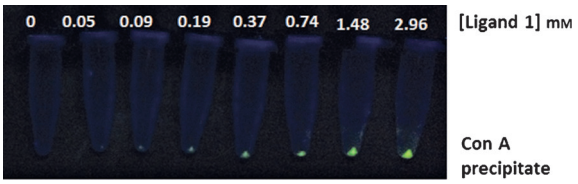


Figure 8. Concentration dependence of the induced precipitation of Con A by mannosyl ligand **1**.

Collectively, the above data suggest a possible scenario in which ligand **1** at 0.6 mM forms micelle aggregates that constitute a multivalent platform to crosslink the lectin molecules and lead to precipitation. At this stage, in spite of the observed aggregate-based multivalency, we are still not certain if other sugar ligands (such as **2c** and **3c**) also behave similarly in solution and thus cannot exclude the effect of possible hydrophobic interaction between FITC and Con A.

Conclusion

In summary, the convergent syntheses of small glycodendritic molecules are reported. Selected glycodendritic molecules were functionalized with a fluorescein label and used for the study of the binding-induced precipitation. The FITC-labeled monovalent mannosyl ligand was unexpectedly found able to induce precipitation of Con A. This finding triggered a series of studies, including 1) lectin precipitation experiments; 2) structural truncation studies; 3) thermodynamic binding analysis; and 4) dynamic light scattering studies, which were aimed at understanding the molecular basis of the unexpected precipitation. These results demonstrate that although it is known that multivalent carbohydrate–protein interaction can contribute to the binding affinity, the linker and the labeling group (if present) also participate in the binding event. Particularly worthy of note is the aggregation behavior of particular monovalent ligands, which when aggregated may display the carbohydrate ligands in a multivalent architecture. Such subtle events may easily have been overlooked in previous studies, leading to inappropriate conclusions. The present study systematically deciphers the roles of the ligand, the linker, and the hydrophobic labeling group in the carbohydrate–protein binding event. We hope that the information herein will be useful for future studies of carbohydrate–protein interactions.

Experimental Section

*Synthesis of trivalent glycodendritic molecule **2a***

A mixture of AB_3 Gal branching unit **7** (1.45 g, 2.98 mmol) and $CuSO_4{\cdot}5H_2O$ (75 mg, 0.30 mmol) in 1:1 THF/H_2O (20 mL) was stirred for 15 min at RT, followed by addition of a freshly prepared solution of sodium ascorbate (590 mg, 2.98 mmol) in H_2O (2.0 mL) and mannoside **5** (3.18 g, 10.43 mmol). The resulting mixture was stirred at 50 °C for 12 h and the solvent was removed by rotary evaporator. The residue was dissolved in H_2O (10 mL) and filtered through a syringe filter over 0.45 μm pore size PVDF membrane. The concentrated filtrate was purified by using size-exclusion chromatography (elution over 75×2.5 cm Superdex 30 prep-grade gel purchased from GE; deionized water was used as the eluent) to remove remaining mannoside **5**. The resulting product was further purified by using dialysis (0.25 wt% EDTA (2×2 L) and deionized water (2×2 L)). Upon removal of water by lyophilization, the trivalent dendritic molecule was obtained as yellowish amorphous powder (yield: 3.05 g, 75%). For the trivalent dendritic molecule, R_f=0.40 (IPA/EtOH/H_2O/AcOH 5:1:1:1); ^{1}H NMR (300 MHz, CD_3OD): δ=8.03 (br s, 3H; triazole-H×3), 5.09 (t, J=9.0 Hz, 1H; Gal H2), 4.74 (s, 3H; Man H1×3), 4.50 (d, J=9.0 Hz, 1H; Gal H1), 2.02 (s, 3H; CH_3C=O), 1.93 (br s, 6H; CH_2×3), 1.59 (br s, 6H; CH_2×3), 1.42–1.36 ppm (m, 12H; CH_2×6). The trivalent dendritic chloride derivative (1.31 g, 0.94 mmol) was dissolved in DMF (10 mL), followed by addition of sodium azide (NaN_3; 0.31 g, 4.70 mmol) and the mixture was stirred at 90 °C under N_2 for ≈10 h to achieve Cl→N_3 substitution. The complete conversion of Cl→N_3 was confirmed by using ^{13}C NMR spectroscopy. Upon completion of the reaction, DMF was removed by distillation under reduced pressure and the residue was dissolved in H_2O (5 mL) for purification with size-exclusion chromatography (Superdex 30 prep-grade gel from GE Healthcare). Upon removal of water by lyophilization, trivalent glycodendritic molecule **2a** was obtained as white amorphous powder (yield: 1.23 g, 93%). For **2a**: R_f=0.4 (IPA/EtOH/H_2O/AcOH 5:1:1:1); GPC retention time 10.900 min, PDI=1.022 (Jordi Gel DVB 500A column (×2), DMF elution at 0.8 mL min^{-1}); ^{1}H NMR (300 MHz, D_2O): δ=7.95–7.87 (m, 3H; triazole-*H*), 4.80 (t, J=9.0 Hz, 1H; Gal H2, partly overlapped with residual proton signals in D_2O), 4.77–4.63 (m, 7H), 4.43–4.23 (m, 7H; -CH_2CH_2C-triazole×3 and Gal H1×1), 3.97 (br s, 1H), 3.82–3.31 (m, 42H), 2.10 (s, 3H; acetyl-CH_3×1), 1.74 (br s, 6H; C_6-linker-CH_2×3), 1.40 (br s, 6H; C_6-linker-CH_2×3), 1.20–1.11 ppm (br s, 12H; C_6-linker-CH_2×6); MALDI-TOF: m/z calcd for $C_{59}H_{100}N_{12}NaO_{27}$: 1431.67; found: 1431.62 $[M+Na]^+$.

*Synthesis of trivalent glycodendritic molecule **2b***

A solution of trivalent glycodendritic molecule **2a** (1.42 g, 1.01 mmol) in MeOH (10 mL) was treated with freshly cut sodium (≈20 mg) and stirred at RT for ≈6 h. Progress of the reaction was monitored by ^{1}H NMR spectroscopy of the crude reaction mixture. Upon completion of deacetylation, the mixture was neutralized with resin IR-120 H^+, filtered, concentrated, and then dialyzed in deionized water for 5 h (2×2 L). The resulting residue was lyophilized to give trivalent glycodendritic molecule **2b** as a yellowish amorphous powder (yield: 1.21 g, 88%). For **2b**: R_f=0.4 (IPA/EtOH/H_2O/AcOH 5:1:1:1); ^{1}H NMR (300 MHz, CD_3OD): δ=8.08 (s, 1H; triazole-*H*), 8.02 (s, 1H; triazole-*H*), 7.98 (s, 1H; triazole-*H*), 4.90–4.87 (s, 6H; -OCH_2C-triazole, partly overlapped with residual H_2O signal in CD_3OD), 4.74 (s, 3H; Man H1×3), 4.70–4.60 (m, 4H), 4.43–4.37 (m, 6H; -CH_2CH_2C-triazole ×3), 4.32 (d, J=9.0 Hz, 1H; Gal H-1), 4.00–3.31 (m, 41H), 1.96–1.89 (m, 6H; C_6-linker-CH_2×3), 1.60–1.56 (m, 6H; C_6-linker-CH_2×3), 1.44–1.35 ppm (m, 12H; C_6-linker-CH_2×6); MALDI-TOF: m/z calcd for $C_{57}H_{98}N_{12}NaO_{26}$: 1389.6; found: 1390.0 $[M+Na]^+$.

*Synthesis of FITC-labeled trivalent glycodendritic ligand **2c***

A mixture of glycodendritic molecule **2b** (100 mg, 0.073 mmol) and $CuSO_4{\cdot}5H_2O$ (2 mg, 0.007 mmol) in CH_3CN/H_2O (4 mL, 4:1 v/v) was stirred for 15 min at RT, followed by addition of freshly prepared sodium ascorbate (5 wt%, 6.0 mg, 0.029 mmol) and propargyl FITC derivative **8** (32 mg, 0.088 mmol). The resulting mixture was stirred at 50 °C for 12 h and then the reaction temperature was brought to RT. The upper CH_3CN layer of the reaction mixture was removed and the lower aqueous phase was concentrated by rotary evaporator for size-exclusion chromatography (elution over 70×1.5 cm TOSPEAR HW 40-F column with deionized water as eluting buffer at 0.5 mL min^{-1}). The resulting product was further purified by dialysis with 0.25 wt% EDTA (2×2 L), followed by deionized water (2×2 L) to remove copper ions. Upon lyophilization, FITC-labeled trivalent glycodendritic mannosyl ligand **2c** was obtained as a yellow amorphous powder (yield: 92 mg, 72%). For **1c**: R_f=0.40 (IPA/EtOH/H_2O/AcOH 5:1:1:1); ^{1}H NMR (500 MHz, D_2O): δ=8.12 (s, 1H; triazole-*H*), 7.91–7.68 (m, 5H; triazole-*H*×3 and Ar-*H*×2), 7.58 (s, 1H; Ar-*H*), 7.50 (s, 1H; Ar-*H*), 7.11 (s, 3H; Ar-*H*), 7.03 (s, 1H; Ar-*H*), 6.85 (s, 1H; Ar-*H*), 6.54 (s, 1H; Ar-*H*) 6.41 (s, 1H; Ar-*H*), 5.22 (br s, 2H; -OCH_2C- triazole), 4.62–4.15 (m, 17H), 3.81–3.26 (m, 41H), 1.67–1.62 (m, 6H; C_6-linker-CH_2×3), 1.36–1.34 (m, 6H; C_6-linker-CH_2×3), 1.15–1.14 (m, 6H; C_6-linker-CH_2×3), 1.05 ppm (s, 6H; C_6-linker-CH_2×3); MALDI-TOF: m/z calcd for $C_{80}H_{112}N_{12}NaO_{31}$: 1759.75; found: 1759.71 $[M+Na]^+$.

*Synthesis of hexavalent glycodendritic molecule **3a***

A mixture of AB_2 Gal branching unit **6** (120 mg, 0.24 mmol) and $CuSO_4{\cdot}5H_2O$ (15 mg, 0.06 mmol) in THF/H_2O (20 mL, 1:1) was stirred for 15 min at RT, followed by addition of freshly prepared sodium ascorbate (5 wt%, 115 mg, 0.58 mmol) and glycodendritic molecule **2b** (800 mg, 0.58 mmol). The resulting mixture was stirred at 60 °C for 12 h, the solvent was removed by rotary evaporator, and the residue was dried in vacuo for 3 h without further purification. The crude residue was dissolved in DMF (10 mL), followed by addition of NaN_3 (78 mg, 1.20 mmol), and the mixture was stirred at 90 °C under N_2 for ≈14 h to effect Cl→N_3 substitution. The complete conversion of Cl→N_3 was confirmed by using ^{13}C NMR spectroscopy. Upon completion of the reaction, DMF was removed by distillation under reduced pressure and the residue was absorbed in H_2O (10 mL) for purification with size-exclusion chromatography gel (elution over 70 cm TOSPEAR HW 40-F, deionized

water was used as the eluent). Hexavalent glycodendritic molecule **3a** was obtained as a yellowish amorphous powder after lyophilization (yield: 650 mg, 84%). For **3a**: R_f=0.3 (IPA/EtOH/H_2O/AcOH 5:1:1:1); GPC retention time 9.947 min; PDI 1.112 (Jordi Gel DVB 500A column (×2), DMF elution at 0.8 mL min^{-1}); ^{1}H NMR (300 MHz, D_2O): δ=8.07–8.03 (br m, 8H; triazole-*H*), 5.24 (s, 1H; Gal H-4), 4.79–3.35 (m, 138H), 2.13, (s, 3H; CH_3C=O), 1.92–1.86 (br s, 15H; acetyl-CH_3×1 and C_6-linker-CH_2×6), 1.51 (br s, 12H; C_6-linker-CH_2×6), 1.29–1.24 ppm (br s, 24H; C_6-linker-CH_2×12); MALDI-TOF: *m/z* calcd for $C_{136}H_{227}N_{27}NaO_{62}$: 3253.5; found: 3253.4 [*M*+Na]$^+$.

*Synthesis of hexavalent glycodendritic molecule **3b***

A solution of hexavalent glycodendritic molecule **3a** (520 mg, 0.16 mmol) in MeOH (5 mL) was treated with freshly cut Na (≈10 mg) and stirred at RT. Upon completion of deacetylation (≈8 h), as determined by using ^{1}H NMR spectroscopy, the reaction mixture was neutralized with resin IR-120 H$^+$, filtered, concentrated, and then dialyzed in deionized water for 5 h (2×2 L). Upon lyophilization, hexavalent glycodendritic molecule **3b** was obtained as a yellow amorphous powder (yield: 440 mg, 86%). For **3b**: R_f (IPA/EtOH/H_2O/AcOH 5:1:1:1) 0.30. Because **3b** was used as an intermediate for the subsequent CuAAC reaction, and the NMR spectroscopy was skipped. MALDI-TOF: *m/z* calcd for $C_{132}H_{223}N_{27}NaO_{60}$: 3169.5; found: 3169.7 [*M*+Na]$^+$.

*Synthesis of FITC-labeled hexavalent glycodendritic ligand **3c***

A mixture of hexavalent glycodendritic molecule **3b** (95 mg, 0.030 mmol) and $CuSO_4$·5H_2O (3.8 mg, 0.015 mmol) in CH_3CN/H_2O (3 mL, 4:1 v/v) was stirred for 15 min at RT, followed by addition of freshly prepared Na ascorbate (5 wt%, 12 mg, 0.060 mmol) and propargyl fluorescein derivative **8** (17 mg, 0.045 mmol). The resulting mixture was stirred at 55 °C for 18 h and then the reaction temperature was brought to RT. The upper CH_3CN layer of reaction mixture was removed and the lower aqueous phase was concentrated by rotary evaporator for size-exclusion chromatography (elution over 70×1.5 cm TOSPEAR HW 40-F column with deionized water as the eluting buffer at 0.3 mL min^{-1}). Upon removal of water by lyophilization, hexavalent glycodendritic mannosyl ligand **3c** was obtained as a yellow amorphous powder (yield: 92 mg, 72%). For **3c**: R_f=0.25 (IPA/EtOH/H_2O/AcOH 4:1.5:1.5:0.6); ^{1}H NMR (300 MHz, CD_3OD): δ=8.13 (br s, 1H; triazole-*H*), 7.94–7.81 (m, 10H; triazole-*H*× 8 and Ar-*H*×2), 7.61 (m, 1H; Ar-*H*), 7.21–7.11 (br s, 4H; Ar-*H*), 6.93 (br s, 1H; Ar-*H*), 6.60 (br m, 1H; Ar-*H*), 6.51 (br s, 1H; Ar-*H*), 5.21 (br s, 2H; -OCH_2C-triazole), 4.67–3.37 (br m, 86H; signals are partly overlapped with residual H_2O and broaden; thus the observed H-integral is less than theoretical 139H), 1.72 (br s, 12H; linker-CH_2×6), 1.38 (br s, 12H; linker-CH_2×6), 1.16 ppm (br s, 24H; linker-CH_2×12); MALDI-TOF: *m/z* calcd for $C_{155}H_{237}N_{27}NaO_{65}$: 3539.6; found: 3539.7 [*M*+Na]$^+$.

*Synthesis of nonavalent glycodendritic molecule **4a***

Gal branching unit **7** (210 mg, 0.43 mmol) and $CuSO_4$·5H_2O (10 mg, 0.04 mmol) in THF/H_2O (10 mL, 1:1) was stirred at RT for 15 min, followed by addition of freshly prepared 5 wt% sodium ascorbate (85 mg, 0.43 mmol) and glycodendritic molecule **2b** (2.08 g, 1.52 mmol). The reaction was stirred at 60 °C for 40 h and monitored by using TLC. Upon completion of click coupling, the solvent was removed by rotary evaporator and filtered through a syringe filter. The resulting residue was dissolved in H_2O (10 mL) for purification with size-exclusion chromatography (70×1.5 cm TOSPEAR HW 40-F column with deionized water as an eluent). Removal of the residual copper ions was achieved by dialysis (0.25 wt% EDTA (2×2 L) and deionized water (2×2 L)) and followed by lyophilization. The nonavalent glycodendritic molecule was obtained as a yellowish amorphous powder (yield: 1.43 g, 62%). R_f=0.35 (IPA/EtOH/H_2O/AcOH 4:1:2:1); ^{1}H NMR (300 MHz, D_2O): δ=7.97–7.91 (br m, 12H; triazole-*H*×12), 4.48–3.26 (br m, 199H), 1.87 (s, 3H; CH_3C=O×1), 1.73 (br s, 18H; C_6-linker-CH_2×9), 1.40 (br s, 18H; C_6-linker-CH_2×9), 1.18–1.11 ppm (m, 36H; C_6-linker-CH_2×18). The aforementioned nonavalent glycodendritic molecule (1.43 g, 0.31 mmol) was dissolved in DMF (5.0 mL), and followed by the addition of NaN_3 (101 mg, 1.56 mmol). The mixture was stirred at 90 °C under N_2 for ≈18 h. The complete conversion of Cl→N_3 was confirmed by using ^{13}C NMR spectroscopy. The DMF solvent was removed by distillation under reduced pressure and the reaction residue was dissolved in H_2O (5 mL) and purified by using size-exclusion chromatography (70×1.5 cm TOSPEAR HW 40-F column with deionized water as an eluent). Upon lyophilization, desired nonavalent glycodendritic molecule **4a** was obtained as white amorphous powder (yield: 1.24 g, 87%). For **4a**: R_f=0.35 (IPA/EtOH/H_2O/AcOH 4:1:2:1); GPC retention time 9.660 min; PDI 1.029 (Jordi Gel DVB 500A column (×2), DMF elution at 0.8 mL min^{-1}); ^{1}H NMR (300 MHz, D_2O): δ=7.97–7.91 (br m, 12H; triazole-*H*×12), 4.48–3.26 (br m, 199H), 1.87 (s, 3H; CH_3C=O×1), 1.73 (br s, 18H; C_6-linker-CH_2×9), 1.40 (br s, 18H; C_6-linker-CH_2×9), 1.18 (br s, 18H; C_6-linker-CH_2×9), 1.11 ppm (br s, 18H; C_6-linker-CH_2×9); MALDI-TOF: *m/z* calcd for $C_{194}H_{326}N_{39}O_{87}$: 4594.2; found: 4594.1 [*M*+H]$^+$.

*Synthesis of nonavalent glycodendritic molecule **4b***

A solution of nonavalent glycodendritic molecule **4a** (1.02 g, 0.22 mmol) in MeOH (10 mL) was treated with freshly cut sodium (≈20 mg) and stirred at RT. Upon completion of deacetylation (≈8 h), as judged by using ^{1}H NMR spectroscopy, the reaction mixture was neutralized with resin IR-120 H$^+$, filtered, concentrated, and then dialyzed with deionized water for 5 h (2×2 L) to obtain nonavalent glycodendritic molecule **4b** as a yellowish amorphous powder (yield: 710 mg, 70%). For **4b**: R_f=0.35 (IPA/EtOH/H_2O/AcOH 4:1:2:1); ^{1}H NMR (300 MHz, CD_3OD): δ=8.13–7.97 (br m, 12H; triazole-*H*×12), 4.74 (s, 9H; Man H-1×9), 4.69–4.55 (m, 20H), 4.44–4.36 (m, 18H), 4.31 (d, *J*=9.0 Hz, 4H; Gal H-1×4), 4.00–3.55 (m, 136H), 3.50–3.36 (m, 12H), 1.92–1.90 (m, 18H; C_6-linker-CH_2×9), 1.59–1.57 (m, 18H; C_6-linker-CH_2×9), 1.42–1.31 ppm (m, 36H; C_6-linker-CH_2×18); MALDI-TOF: *m/z* calcd for $C_{192}H_{322}N_{39}NaO_{86}$: 4574.2; found: 4574.6 [*M*+Na]$^+$.

Procedure of ligand-binding-induced Con A precipitation (Figure 4 and 5a)

The examined ligands (**1**, **2c**, and **3c**; 1 mM) in Tris (pH 7.4, 75 μL) were mixed individually with solutions of Con A (50 μL; 10, 20, or 40 μM) in Tris (12.5 mM, pH 7.4) and allowed to stand at RT. Photographs of these mixtures under UV illumination (λ_{max}=365 nm) were taken at time points of 0, 5, and 30 min (Figure 4). In the control experiments, Con A (50 μL; 10, 20, or 40 μM) was mixed with a blank Tris buffer (75 μL, 12.5 mM, pH 7.4). After standing for 30 min at RT, the samples were centrifuged at 2000 rpm for 10 min, then the supernatant was decanted. The resulting ligand-bound precipitate pellets were washed with fresh Tris buffer (125 μL, 12.5 mM, pH 7.4), followed by centrifugation at 8000 rpm. After decanting the supernatant, the washed precipitate pellets (if any) were examined under UV illumination (λ_{max}=365 nm).

Procedure of competitive ligand binding experiment (Figure 5b)

A series of (ligand **1**)-bound Con A precipitate pellets were obtained from mixing a solution of Con A (0.1 mM, 10 μL) in Tris (12.5 mM, pH 7.4) with monovalent mannose ligand **1** (1.0 mM, 15 μL) in Tris (12.5 mM, pH 7.4) for 1 h, followed by centrifugation at 4000 rpm (4 °C) for 10 min. After decanting the supernatants, the ligand-bound precipitate pellets were then mixed with Tris buffer, a solution of saccharide (0.1 M; mannose (Man), Glucose (Glc), sucrose (Suc), maltose (Mal), xylose (Xyl), arabinose (Ara), fucose (Fuc), rhamnose (Rha), or galactose (Gal)) or unmodified fluorescein (25 μL) for 30 min, followed by centrifugation at 4000 rpm (4 °C) for 10 min. After decanting the supernatants, the remaining precipitate pellets were examined under UV illumination (λ_{max}=365 nm).

Fluorescence polarization binding assay

The measurement of fluorescence polarization assays is based on the rotation speed of a fluorophore. When a fluorophore-containing compound is bound to the protein counterpart (e.g., Con A herein), the fluorophore rotates at a slower rate than when it is unbound and the resulting fluorescence polarization is higher. Herein, we carried out all measurements according to a reported procedure.[27] A fluorescent compound was added to the final sample volume (70 μL) in each assay to achieve a final con-

centration of 20 μM. All the measurements were carried out in Tris buffer (12.5 mM, pH 7.4) at 4 °C. For the direct binding assay of **1** (the fluorescent probe or reference compound), **2c, 3c**, **9**, **10**, **11** and FITC, the data (anisotropy (A) vs. Con A concentration [Con A]) were fitted to the formula $A = A_0 + A_{max} \times [\text{Con A}]/(K_d + [\text{Con A}])$ in which A_0 is the value measured in the absence of Con A and A_{max} is the maximum value approached with increasing Con A concentration. For the inhibitor competition assay (**2b**, **3b** and **5**), K_d values for the Con A–inhibitor interactions were calculated from the following equations:

$$[\text{PG}] = [(A - A_0)/(A_{max} - A_0)] \times [\text{P}]_{tot}$$

$$[\text{P}] = [\text{P}]_{tot} - [\text{PG}]$$

$$[\text{G}] = K_d \text{ (fluorescent compound)} \times [\text{PG}]/[\text{P}]$$

$$[\text{IG}] = [\text{G}]_{tot} - [\text{PG}] - [\text{G}]$$

$$[\text{I}] = [\text{I}]_{tot} - [\text{IG}]$$

$$K_d \text{ (inhibitor)} = [\text{I}][\text{G}]/[\text{IG}].$$

[I], [P], and [G] are the concentrations of free inhibitor, probe (**1**), and Con A, respectively, and [IG] and [PG] are the concentrations of inhibitor–Con A complex and probe–Con A complex, respectively. To solve the seven unknowns in these equations, five more independent equations were needed. The known total concentrations of each component govern the following three equations:

$$[\text{I}]_{tot} = [\text{I}] + [\text{IG}],$$

$$[\text{P}]_{tot} = [\text{P}] + [\text{PG}],$$

$$[\text{G}]_{tot} = [\text{G}] + [\text{IG}] + [\text{PG}].$$

The measured anisotropy value (A) was used to calculate the amount of bound probe: $[\text{PG}] = (A - A_0)/(A_{max} - A_0) \times [\text{P}]_{tot}$.

A_{max} was obtained as the maximum value approached in a separate direct binding experiment as described above; A_0 is the value for the probe without Con A and inhibitor.

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