

Fluorescence Anisotropy Assays Reveal Affinities of C- and O-Glycosides for Concanavalin A¹

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Received August 2, 1995²

The free energies of binding of various C- and O-glycosides to the lectin concanavalin A were measured using fluorescence anisotropy. Fluorescein derivatives of mannose and glucose were synthesized and were shown to bind to concanavalin A with free energies of -5.1 and -4.3 kcal mol⁻¹, respectively. Competition experiments were performed to determine the binding energies of different nonfluorescent carbohydrates, and the results were in excellent agreement with the binding energies determined by microcalorimetry. The minimum carbohydrate epitope that fills the lectin carbohydrate binding site, methyl 3,6-di-O-(α -mannopyranosyl)- α -mannopyranoside, competes directly for the site with the fluorescent ligands, indicating that the fluorescent ligands bind specifically. The binding affinities of C-glycosides to concanavalin A were compared with those of O-glycosides. The free energies of binding for corresponding C- and O-glycosides differed by less than 0.5 kcal mol⁻¹, indicating that recognition properties of C- and O-glycosides are very similar. It was found that for some ligands the use of a carbon linkage rather than an oxygen linkage caused the specificity of binding to decrease slightly.

Introduction

Protein-carbohydrate interactions mediate critical biological processes. These recognition events facilitate many events, such as cell signaling in growth and differentiation, fertilization, and the inflammatory response. For example, protein binding to cell surface carbohydrates promotes leukocyte rolling along the inflamed endothelium in a variety of inflammatory conditions.² With the identification of new roles for carbohydrates, interest in modulating such interactions is growing.

One approach to interfering with carbohydrate function is to generate saccharide analogs that possess non-natural, enzymatically stable linkages. Recent advances in the synthesis of one class of these compounds, the C-glycosides, have resulted in the generation of a variety of saccharide analogs.³ Despite previous studies that have shown that C-glycosides behave similarly to O-glycosides in their ability to prevent viral or bacterial attachment to cells in adhesion assays,⁴ little is known about the energetics and specificity of the interaction of C-glycosides with lectins.

The low affinity of saccharides for lectins complicates

the measurement of these interactions. The measurement of inhibition constants in cellular adhesion assays does not reflect thermodynamic dissociation constants because of the polyvalent nature of cell surface interactions. One method that has proven useful in obtaining accurate dissociation constants is fluorescence anisotropy, which has been applied in two cases to monitor carbohydrate-protein association. This technique has been employed to measure the binding of sialosides to influenza hemagglutinin⁵ and the binding of E-selectin to various carbohydrate ligands.⁶

This report details the design and use of an assay based on fluorescence anisotropy to determine the interaction of various monovalent C- and O-glycosides with concanavalin A. It represents the first determination of the binding energies of an important class of carbohydrate analogs, the C-glycosides. We compare these results with those obtained by titration calorimetry^{7,8} and discuss the strengths and weaknesses of methods for analysis of fluorescence anisotropy data for these low-affinity interactions.

Results and Discussion

The most widely studied lectin, concanavalin A, binds specifically to glucose and mannose residues that possess free hydroxyl groups at positions 3, 4, and 6 of the α -pyranose ring. The strength of these interactions is typical of monovalent saccharide-protein interactions ($K_a \approx 10^4$ M⁻¹), which tend to be of low affinity. The lectin discriminates between these ligands by displaying

¹ Abstract published in *Advance ACS Abstracts*, December 15, 1995.
 (1) Abbreviations used: α -MeMan, methyl α -D-mannopyranoside; α -MeGlc, methyl α -D-glucopyranoside; C-allyl man, mannose derivative **1**; C-allyl glc, glucose derivative **2**; Fl-man, fluorescein mannose derivative **3**; Fl-glc, fluorescein glucose derivative **4**; ox-(man)₂, oxanorbornene mannose derivative **5**; ox-(glc)₂, oxanorbornene bis(glucose) derivative **6**; α -Man(1 \rightarrow 6)- α -ManOMe, 6-O-(α -mannopyranosyl)- α -mannopyranoside; α -Man(1 \rightarrow 3)- α -ManOMe, 3-O-(α -mannopyranosyl)- α -mannopyranoside; α -Man(1 \rightarrow 6)(α -Man(1 \rightarrow 3))- α -ManOMe, methyl 3,6-di-O-(α -mannopyranosyl)- α -mannopyranoside. All unspecified sugar residues are of the D-configuration. Anisotropy values are represented by the variable *r*.

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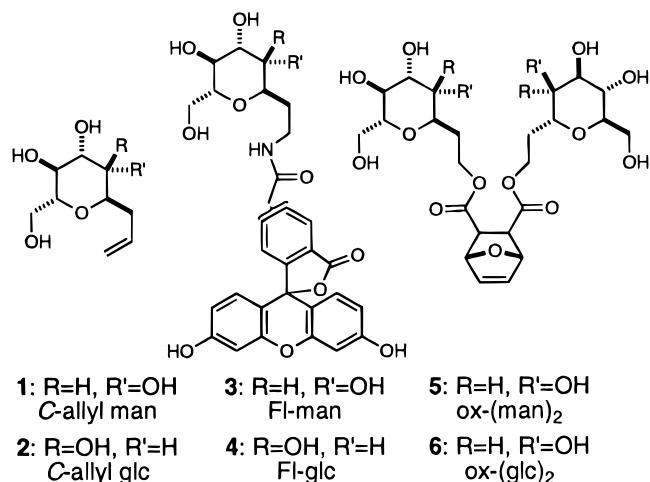


Figure 1. Structures and abbreviations of synthetic *C*-glycosides used in this study.

an approximate 4-fold preference for the *manno* over *gluco* configuration at C2.⁷ In addition to binding low molecular weight saccharide derivatives, tetrameric concanavalin A is able to agglutinate cells through multivalent interactions with cell surface carbohydrates. The agglutination can be inhibited by the addition of either glucose- or mannose-containing saccharides,⁹ with natural¹⁰ and non-natural polyvalent saccharide ligands being more effective inhibitors than monosaccharides. For example, we have shown that neoglycopolymers prepared by aqueous ring-opening metathesis polymerization are at least 2000 times more effective at inhibiting this interaction.¹¹ Because of its weak binding to saccharide ligands and its ability to engage in multivalent interactions, concanavalin A is an excellent tool for model studies aimed at studying carbohydrate-facilitated cell-cell interactions.

Although the binding of carbohydrates to concanavalin A has been characterized by X-ray crystallographic studies¹² and by microcalorimetry,^{7,8} neither technique alone allows the determination of binding constants and confirmation that the ligands are competing directly for the same saccharide binding site. Fluorescence anisotropy can accomplish both these tasks. The basis of the method is the detection of changes in the anisotropy of fluorescence of a fluorophore attached to a carbohydrate ligand. When a protein binds to the labeled ligand, there is an increase in the rotational correlation time of the fluorophore. This increase is due to the large size of the complex relative to the unbound carbohydrate ligand. This method is sensitive and can detect the weak binding characteristic of carbohydrate-protein interactions.

To determine the ligand affinities for concanavalin A, we synthesized fluorescein-labeled derivatives of mannose and glucose (compounds 3 and 4, Figure 1). Fluorescent ligands 3 and 4 were generated by coupling carboxyfluorescein to a free amine of the corresponding glucose or mannose derivative, which was obtained from

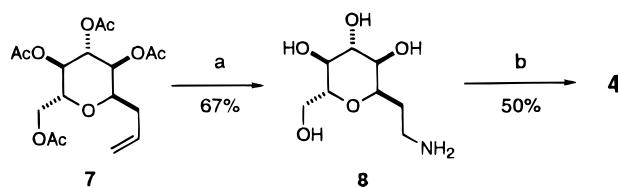
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Scheme 1^a



^a Synthesis of fluorescein-labeled glucose derivative. The mannose derivative was made by the same method with similar yield. Reagents: (a) (i) O₃, MeOH/CH₂Cl₂, –78 °C, (ii) Ph₃P polymer, MeOH/CH₂Cl₂, (iii) BnNH₂, HOAc, pyr-BH₃, MeOH/CH₂Cl₂, reflux, (iv) NaOMe, MeOH/CH₂Cl₂, (v) Pd(OH)₂/C, H₂ (50 atm), MeOH/H₂O; (b) *N*-hydroxysuccinimide-activated carboxyfluorescein, DMF/H₂O.

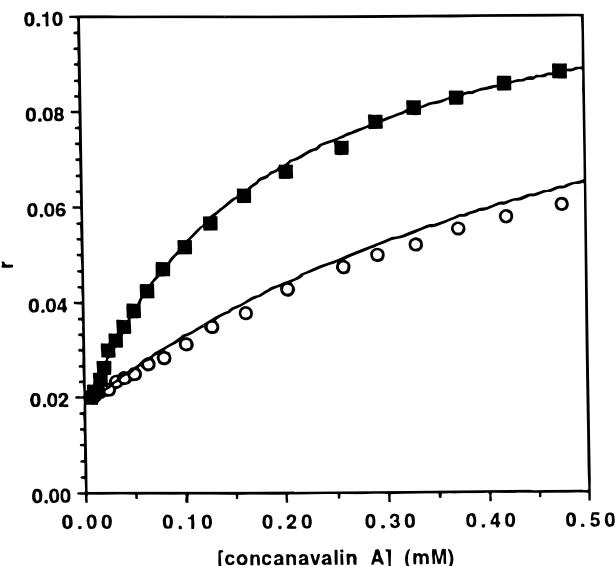


Figure 2. Fluorescence anisotropy titration of labeled ligands with fitted curves superimposed. Fluorescence anisotropy measurements were executed with either Fl-glc 4 (○) or Fl-man 3 (■) at various concentrations of native concanavalin A.

the protected *C*-allyl glycoside¹³ (Scheme 1). The labeled molecules were synthesized such that the linker bearing the fluorophore was attached in the α -configuration, the preferred configuration for concanavalin A binding.⁹ The orientation of the substituent attached to the C1 position was critical for obtaining accurate binding data (*vide infra*). These ligands were then assayed for binding by measurement of the anisotropy of fluorescence as a function of concanavalin A concentration. The fluorescein derivatives of both mannose and glucose were bound by the lectin as shown in the titration curve in Figure 2. Unlike previous studies,⁶ our work shows that detectable changes in fluorescence anisotropy can be observed with a flexible linker between the carbohydrate and the fluorophore. Analysis of the titration curves¹⁴ gave free energies of binding of –4.3 kcal mol^{–1} for Fl-glc 3 and –5.1 kcal mol^{–1} for Fl-man 4 (Table 1).

These binding energies were determined with a program that uses a numerical rather than an analytical method to fit the data.¹⁴ This procedure is superior to standard approaches using double reciprocal plots to calculate the dissociation constants,⁶ because it does not require an estimate of the anisotropy value for free ligand in solution nor is it as sensitive to small errors at low

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Table 1. Binding Free Energies of Various Carbohydrates to Concanavalin A

carbohydrate ligand	ΔG by fluorescence anisotropy ^a	ΔG by microcalorimetry ^a
Interaction with Tetrameric Native Concanavalin A		
Fl-Man 3	-5.1 ± 0.1	NA ^b
Fl-Glc 4	-4.3 ± 0.1	NA
α-MeMan	-5.1 ± 0.1	-5.3 ± 0.2 ⁶
α-MeGlc	-4.4 ± 0.1	-4.6 ± 0.3 ⁶
α-Man(1→3)-α-ManOMe	-5.7 ± 0.4	-5.7 ¹²
α-Man(1→6)-α-ManOMe	-5.0 ± 0.1	-5.3 ¹²
α-Man(1→6)-α-Man(1→3)-α-ManOMe	-7.1 ± 0.4	-7.2 ¹²
α-C-allyl Man 1	-4.9 ± 0.1	NA
α-C-allyl Glc 2	-4.7 ± 0.1	NA
ox-(man) ₂ 5	caused precipn	NA
ox-(glc) ₂ 6	caused precipn	NA
Interaction with Dimeric Succinyl Concanavalin A		
Fl-Man 3	-5.3 ± 0.1	NA
α-MeMan	-5.2 ± 0.1	-5.4 ¹²
α-MeGlc	-4.6 ± 0.1	NA
ox-(man) ₂ 5	-5.3 ± 0.1	NA
ox-(glc) ₂ 6	-4.9 ± 0.1	NA

^a All values are expressed in kcal mol⁻¹. ^b Data is not available.

protein concentrations. This feature is particularly significant for the measurement of weak association constants. In anisotropy assays with a labeled ligand, the concentration of protein must range from below to above the dissociation constant. This criterion is particularly difficult to meet for weak interactions, especially when relatively small supplies of protein are available or when the protein is not soluble at high concentrations. In our assays with concanavalin A, the maximum accessible protein concentrations were 3-fold higher than the determined dissociation constant. Even with this limitation, when the dissociation constants obtained using the BIOEQS program¹⁴ were used to analyze data from competition experiments with known ligands, the results were in excellent agreement with those obtained by other methods (*vide infra*).

Solution viscosity has been reported to affect anisotropy measurements.⁶ A comparison of the anisotropy of the fluorescent ligands in protein-free buffer ($r = 0.020$) with the anisotropy of fluorescent ligands in concentrated concanavalin A solutions that have been completely displaced by competitor ($r = 0.023$) revealed that viscosity effects on the anisotropy of fluorescence were minimal in our system. This insensitivity of the assay to viscosity is probably due to the relatively small size of the ligand compared to ligands used in other systems.

After demonstrating that the changes in fluorescence anisotropy were indicative of the saccharide-concanavalin A interaction, we performed competition experiments using other concanavalin A ligands. In these assays, the fluorescence anisotropy of the fluorescein-labeled derivatives **3** and **4** at a high concanavalin A concentration was monitored in the presence of increasing concentrations of inhibitor. The accuracy of the assay was determined by comparing the binding energies determined by fluorescence anisotropy with those obtained by titration calorimetry^{7,8} (Table 1). For example, competition experiments using the trisaccharide methyl 3,6-di-O-(α-mannopyranosyl)-α-mannopyranoside, the minimum oligosaccharide needed to fill the lectin binding site,¹⁵ gave binding energies similar to those obtained in the micro-

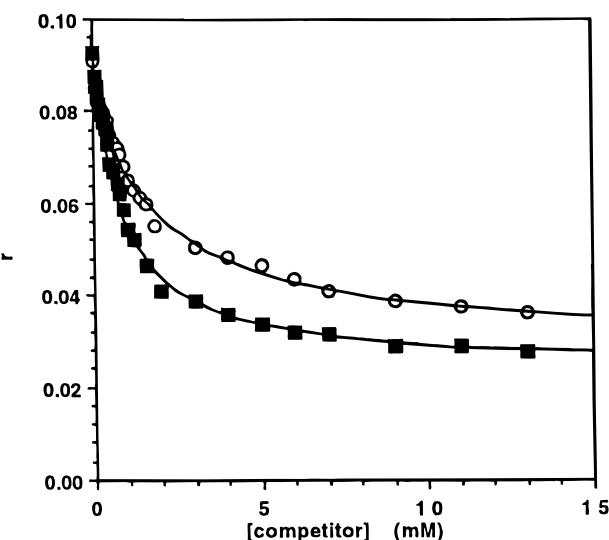


Figure 3. Typical fluorescence anisotropy competition experiment with fitted curves superimposed. To a solution containing Fl-man and native concanavalin A, increasing amounts of nonfluorescent ligands were added. (Shown here are methyl α-D-glucopyranoside (○) and methyl α-D-mannopyranoside (■)).

calorimetry experiments (Table 1). Likewise, the disaccharides methyl 3-O-(α-mannopyranosyl)-α-mannopyranoside and 6-O-(α-mannopyranosyl)-α-mannopyranoside also gave consistent results.⁷ The fluorescence anisotropy assay was also able to discriminate between the binding affinities of methyl α-D-glucopyranoside and methyl α-D-mannopyranoside (Figure 3). Combined, these data strongly suggest that the fluorescent ligands are binding specifically in the carbohydrate binding site.

Demonstration that the fluorescent carbohydrate derivative is not interacting at a site distinct from that in which unlabeled saccharides bind is critical when studying lectins; some lectins possess binding sites for aromatic residues. Concanavalin A possesses a binding site for aromatic groups that differs from that used for saccharide recognition.¹⁶ Competitive binding at this site was inferred from anisotropy data derived using the commercially available fluorescein mono-β-D-glucopyranoside. This glucose derivative bearing the fluorescent reporter group in the β-configuration did not produce results in agreement with those determined by microcalorimetry. Although the ligand gave rise to large anisotropy changes when mixed with increasing concentrations of concanavalin A, competition experiments afforded free energies of binding that consistently differed from those calorimetrically determined by values up to 0.5 kcal mol⁻¹. Consequently, the mode and stereochemistry of attachment of the fluorescent reporter moiety is a critical parameter in the design of anisotropy assays of this type.

Having established the accuracy of the assay, we used it to probe the effects of using C-glycosides on the binding affinity and specificity of concanavalin A. C-Glycosides have been used as carbohydrate analogs to increase stability, and it has been suggested that they might act as conformationally restricted carbohydrate mimics.³ C-Linked saccharide derivatives have been shown to inhibit the adhesion of *Escherichia coli* to yeast as well as the adhesion of influenza virus to erythrocytes,⁴ but neither of these investigations addressed the effect of using a carbon linkage on the binding specificity. In this

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study, three different sets of *C*-linked derivatives of mannose and glucose were used to address the specificity question. While all the carbohydrates bound in the binding site, each set gave slightly different results (Table 1). Concanavalin A bound to the fluorescein-labeled derivatives with affinities and specificity similar to those of the *O*-methyl glycosides. This result indicates that the fluorescent reporter group does not greatly alter the affinity of the carbohydrate determinant when it is attached at the reducing end of the saccharide group.

In contrast, the specificity of concanavalin A binding to the *C*-allyl glycosides **1** and **2** decreased. Consequently, the difference in binding energies between methyl α -D-glucopyranoside and methyl α -D-mannopyranoside ($0.7 \text{ kcal mol}^{-1}$) is greater than that between α -*C*-allyl glucoside and α -*C*-allyl mannoside ($0.2 \text{ kcal mol}^{-1}$). This result is surprising because the allyl substituent for both manno and gluco isomers **1** and **2** would be expected to occupy the same site in both complexes with concanavalin A. The origin of the decreased specificity was an increase in the binding free energy of concanavalin A and *C*-allyl glucoside relative to methyl α -D-glucopyranoside. A similar decrease in the binding specificity was also observed for the interaction of the oxanorbornene derivatives **5** and **6** with a dimeric form of the lectin, succinylconcanavalin A.

This change in specificity is not due to loss of direct concanavalin A–carbohydrate contacts upon switching from an oxygen linker to a carbon linker. An analysis of the crystal structure of concanavalin A bound to methyl α -D-mannopyranoside¹² does not show interactions between the protein and any atoms at the C1 position. The change in specificity is most likely due to subtle changes in the solvation of the mannose- and glucose-derived ligands upon switching from an oxygen to a methylene group. Lemieux has proposed that the driving force for carbohydrate–protein complexation may be due to changes in the interactions between the water molecules hydrating the complexing species.¹⁷ The energetic differences we observed are small and difficult to predict. For example, the C1-substituted fluorescein-substituted glucose and mannose derivatives **3** and **4** bind with the same specificity as the corresponding methyl *O*-glycosides. In this case the energetic consequences of employing *C*-glycoside-linked derivatives are not large; however, the erosion of recognition specificity may be more significant for other analogs.

Bivalent ligands were also tested in competition experiments to determine whether there was an effect of increased valency on the binding of concanavalin A. The bisglycosylated oxanorbornene derivatives of glucose and mannose, compounds **5** and **6**, are the immediate precursors to the neoglycopolymers that have been shown to inhibit concanavalin A-induced hemagglutination of erythrocytes.¹¹ When they were used in competition experiments with native concanavalin A at pH 7.2, they caused the aggregation and precipitation of the protein. This precipitation was not seen, however, when binding was assayed to succinylconcanavalin A, which exists as a dimer rather than tetramer. Although succinylconcanavalin A bound to Fl-man **3** with the same affinity ($\Delta G = -5.3 \text{ kcal mol}^{-1}$), competition studies with the oxanorbornene derivatives afforded binding energies similar to those reported for the *C*-allyl glycosides binding

to native concanavalin A (Table 1). Similar binding affinities were also found in competition studies with native concanavalin A at pH 5.2, where it also exists predominately as a dimer (data not shown). These observations indicate that the bivalent ligands interact differently with the tetrameric form of the protein than with the dimeric form. Future investigations will address the origins of the observed differences.

Conclusion

We have employed fluorescence anisotropy to measure the binding of various *C*- and *O*-linked carbohydrates to the lectin, concanavalin A. We have demonstrated that this technique can be used to accurately quantitate small energetic differences in the binding of low-affinity ligands to the lectin. Fluorescein derivatives of mannose and glucose were synthesized and were shown to bind specifically to concanavalin A. Competition experiments were performed to determine the binding energies of different nonfluorescent carbohydrates, and the results were in excellent agreement with the binding energies determined by microcalorimetry.

A comparison of the binding affinities of *C*-linked glycosides to concanavalin A with those of *O*-linked glycosides revealed that both these sets of molecules bind with similar affinities. Still, for some ligands the use of a carbon linkage rather than an oxygen linkage caused the specificity of binding to decrease. Our studies indicate that changes in parts of the carbohydrate that do not interact with the protein, such as switching from an *O*-glycoside linkage to a *C* linkage, can affect the binding specificity in unpredictable ways. Despite these small effects, our investigations provide further validation of the use of *C*-glycosides as carbohydrate analogs.

Experimental Procedures

General Methods. Native and succinyl concanavalin A, methyl α -D-mannopyranoside, methyl α -D-glucopyranoside, α -Man(1→3)- α -ManOMe, α -Man(1→6)- α -ManOMe, and α -Man(1→6)(α -Man(1→3))- α -ManOMe were purchased from commercial sources and used without further purification. Both native and succinyl concanavalin A were dissolved in 0.1 M HEPES buffer, pH 7.2, containing 0.9 M NaCl, 1 mM CaCl₂, and 1 mM MnCl₂. The concentration of native concanavalin A was determined spectrophotometrically at 280 nm using $A^{1\%, 1\text{cm}} = 13.7$ at pH 7.2 and expressed in terms of the monomer ($M_r = 26\,500$).¹⁸ The concentration of succinyl concanavalin A was determined spectrophotometrically at 280 nm using $A^{1\%, 1\text{cm}} = 13.7$ at pH 7.2 and expressed in terms of the monomer ($M_r = 28\,000$).¹⁹ C-allyl glc, C-allyl man, ox-(glc)₂, and ox-(man)₂ (Figure 1) were synthesized in a manner previously described.¹¹

Reaction solvents were freshly distilled from calcium hydride (dichloromethane and dimethylformamide) or magnesium metal (methanol). Chromatography solvents were ACS grade. Analytical thin layer chromatography was performed on 0.25 mm Merck precoated silica gel plates (60F-254) and flash chromatography on E. M. Science silica gel 60 (230–400 mesh).

Fluorescence Anisotropy Measurements. After each addition of the sample, a 5 s delay was included before each reading to allow the system to reach equilibrium. Fluorescence anisotropy measurements were made of native concanavalin A binding to both Fl-glc **4** and Fl-man **3** and succinylcon-

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canavalin A binding to Fl-man **3** in 0.1 M HEPES buffer, pH 7.2, containing 0.9 M NaCl, 1 mM CaCl₂, 1 mM MnCl₂, and 200 nM ligand by varying the protein concentration from 1 to 480 μM. The binding energies were then determined using the BIOEQUIS curve fitting program.¹⁴

Competition titration experiments of nonfluorescent ligands were performed using 480 μM concanavalin A in 0.1 M HEPES buffer, pH 7.2, containing 0.9 M NaCl, 1 mM CaCl₂, 1 mM MnCl₂, and 200 nM of either Fl-glc **4** or Fl-man **3**. The unlabeled ligand concentration was then gradually increased until further addition of ligand failed to significantly affect the anisotropy measurement. The binding energies of nonfluorescent ligands were obtained from dissociation constants calculated by using the curve fitting program Sigma Plot to fit the competition curves to the following equation:⁵

$$[L]_T = \frac{K_D}{K_F} \left([P]_T \frac{(r_B - r)}{(r_B - r_F)} - [F]_T \frac{(r_B - r)}{(r - r_F)} \right) - K_D$$

where [L]_T is the nonfluorescent ligand concentration, [F]_T is the fluorescent ligand concentration, *K*_D is the dissociation constant of the nonfluorescent ligand, *K*_F is the dissociation constant of the fluorescent ligand, [P]_T is the total concanavalin A concentration, *r* is the observed anisotropy, and *r*_B and *r*_F are the anisotropy values when the fluorescent ligand is bound by protein or free in solution, respectively.

C-1-(2'-Aminoethyl) α-Glucoside 8. A stirred solution of C-1-allyl O-2,3,4,6-tetraacetyl-α-glucoside (**7**)¹³ (138 mg, 0.371 mmol) in 1:1 methanol/dichloromethane (5 mL) was treated at -78 °C with ozone for approximately 5 min or until a persistent blue color was observed. The mixture was then purged with nitrogen for 5 min. Polymer-supported triphenylphosphine (247 mg, 0.742 mmol) was then added, and the resulting stirred solution was allowed to warm to room temperature. After stirring for an additional 3 h, the solution was filtered through cellulose and concentrated to yield a clear oil.

The oil was dissolved in 1:1 methanol/dichloromethane solution (5 mL), and in succession, benzylamine (0.200 mL, 1.85 mmol), glacial acetic acid (0.100 mL, 1.85 mmol), and pyridine–borane (0.190 mL, 1.85 mmol) were added. The stirred solution was then heated at reflux for 3 h. Dichloromethane (20 mL) was added, and the resulting organic layer was washed twice with water (20 mL). The organic fraction was concentrated to a clear oil, which was dissolved in 1:1 methanol/dichloromethane (5 mL), and 25% (w/v) sodium methoxide in methanol was added until the pH of the solution was 11. The resulting mixture was stirred for 12 h. The reaction was quenched by the addition of Amberlyst 15 strongly acidic cation exchange resin until the pH of the solution was 7. The solution was then filtered, and dichloromethane (10 mL) was added. The organic layer was washed with water (3 × 15 mL), and the aqueous fraction was concentrated and partially purified by silica gel chromatography (5:4:1 CHCl₃/MeOH/water) to yield a white solid.

To a solution of the white solid in a 1:1 methanol/water solution (3 mL) was added 20% (w/w) Pd(OH)₂ on carbon (100 mg), and the solution was shaken in a Paar apparatus under 50 atm of hydrogen for 14 h. The solution was then filtered through cellulose, concentrated, and purified using Sephadex SP C-25 cation exchange resin with 0.5 M triethylammonium bicarbonate buffer as the eluent. Concentration afforded a clear syrup (52 mg, 0.25 mmol, 67% yield over four steps): ¹H NMR (300 MHz) δ 1.55–1.85 (m, 2H), 2.55–2.80 (m, 1H), 2.85–3.05 (m, 1H), 3.16 (app t, *J* = 9.3 Hz, 1H), 3.34–3.57 (m, 4H), 3.68 (d, *J* = 8.3 Hz, 1H), 3.94–3.87 (m, 1H); ¹³C NMR (75 MHz) δ 73.9, 73.1, 72.7, 70.8, 70.2, 61.0, 37.1, 24.8; HRMS (EI) calcd for C₈H₁₇NO₅ 207.1107, obsd 207.1124.

C-1-(2'-aminoethyl)-α-mannoside Using the Procedure for 8. C-1-Allyl O-2,3,4,6-tetrabenzoyl-α-mannoside (0.38 g, 0.61 mmol) in 1:1 methanol/dichloromethane (5 mL) was treated with ozone, followed by addition of polymer-supported triphenylphosphine (406 mg, 1.22 mmol). The reductive amination was then performed with benzylamine (0.30 mL, 3.0 mmol), glacial acetic acid (0.15 mL, 3.0 mmol), and pyridine–borane (0.30 mL, 3.0 mmol) in 1:1 methanol/dichlo-

romethane (5 mL) followed by deacylation with 25% (w/v) sodium methoxide in methanol. Hydrogenation performed with 20% (w/w) Pd(OH)₂ on carbon (75 mg) afforded the title compound (64 mg, 0.31 mmol, 51% yield over four steps): ¹H NMR (300 MHz) δ 1.50–1.62 (m, 1H), 1.85–1.95 (m, 1H), 2.76 (app sextet, *J* = 5.6 Hz, 1H), 2.82–3.00 (m, 1H), 3.32–3.72 (m, 6H), 3.83 (d, *J* = 9.2, 1H); ¹³C NMR (75 MHz) δ 75.8, 73.9, 71.1, 70.6, 67.3, 61.0, 37.2, 27.9; HRMS (EI) calcd for C₈H₁₇NO₅ 207.1107, obsd 207.1121.

C-1-(2'-N-(5"- and 6"-carboxyfluoresceinylaminoethyl) α-Glucoside (4). A stirred solution of a 1:1 isomeric mixture of 5'- and 6'-carboxyfluorescein (30 mg, 0.080 mmol) in dimethylformamide (0.74 mL) was cooled to 0 °C, and then *N*-hydroxysuccinimide (10 mg, 0.087 mmol) and dicyclohexylcarbodiimide (18 mg, 0.088 mmol) were added. The resulting solution was allowed to stir in the dark at 0 °C for 14 h. The reaction was filtered through a 5 μm syringe filter into a solution of 2'-aminoethyl α-glucoside (13 mg, 0.062 mmol) in water (0.310 mL). The solution was allowed to stir in the dark at room temperature for 5 h. The solution was concentrated under vacuum, and the residue was purified by silica gel chromatography (5:4:1 CHCl₃/MeOH/water) followed by anion exchange chromatography using DEAE–Sephadex A-25 anion exchange resin with a gradient of triethylammonium bicarbonate as the eluent. Fractions containing the purified product were then pooled and concentrated to afford a red solid (12 mg, 0.021 mmol, 35% yield). Anisotropy assays were performed with a mixture of both isomers: ¹H NMR (300 MHz) δ 1.65–2.35 (m, 2H), 3.10–3.70 (m, 8H), 3.85–4.05 (m, 1H), 6.38–6.48 (m, 4H), 6.92 (dd *J* = 9.2, 4.2 Hz, 2H), 7.04–7.33 (m, 1H), 7.69–8.03 (m, 2H); ¹³C NMR (125 MHz) δ 169.1, 168.7, 157.4, 134.5, 131.5, 130.1, 128.5, 121.1, 114.3, 114.4, 103.3, 73.9, 72.7, 70.9, 70.2, 61.0, 36.9, 23.3; IR (KBr) 3685, 2999, 1790, 1637, 1502, 1464, 1171, 819, 769, 717 cm⁻¹; MS (LSIMS, MH⁺) 566.2.

C-1-(2'-N-(5"- and 6"-carboxyfluoresceinylaminoethyl) α-Mannoside (3). Using the procedure for **4**, a 1:1 isomeric mixture of 5'- and 6'-carboxyfluorescein (108 mg, 0.32 mmol) in dimethylformamide (1.4 mL), *N*-hydroxysuccinimide (36 mg, 0.32 mmol), dicyclohexylcarbodiimide (71 mg, 0.34 mmol), and **8** (50 mg, 0.025 mmol) in water (1.2 mL) afforded the title compound as a mixture of regioisomers (65 mg, 0.11 mmol, 47% yield): ¹H NMR (300 MHz) δ 1.75–2.02 (m, 1H), 2.87–2.95 (m, 1H), 3.12–3.67 (m, 8H), 3.88–3.95 (m, 1H), 6.34–6.46 (m, 4H), 6.87–7.10 (m, 3H), 7.61–8.08 (m, 2H); ¹³C NMR (125 MHz) δ 178.4, 178.3, 158.3, 131.5, 131.4, 131.0, 130.3, 130.0, 129.6, 128.6, 122.2, 113.3, 113.1, 103.4, 73.6, 73.0, 72.9, 70.5, 70.0, 60.9, 36.8, 22.2; IR (KBr) 3710, 2999, 1790, 1637, 1502, 1464, 1171, 819, 769, 717 cm⁻¹; MS (LSIMS, MH⁺) 566.1.

Acknowledgment. We thank Prof. C. Royer (UW-Madison) for assistance with data analysis, Prof. R. Raines (UW-Madison) for use of the fluorimeter, Prof. E. Toone (Duke) for helpful discussions, and K. H. Mortell for providing some of the carbohydrate samples. This work was supported by a grant from the National Institutes of Health (GM49975). R.V.W. thanks the NIH for a Predoctoral Biotechnology Training Fellowship (T32GM08349). L.L.K. is a Beckman Young Investigator, a Shaw Scientist, and an NSF National Young Investigator.

Supporting Information Available: ¹H NMR spectra of compounds **3**, **4**, **8**, and C-1-(2'-aminoethyl)-α-mannoside (**4** pages). This material is contained in libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.