Selective Detection of the Carbohydrate-Bound State of Concanvalin A at the Single Molecule Level

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Abstract: The labeling of molecules with charge-transfer dyes, such as 5-(dimethylamino)-1-napthalenesulfonyl (dansyl) chloride, is a powerful tool for examining the solvent shell of attached substances. This investigation describes the synthesis and application of a new charge transfer label, based on *trans*-1-[*p*-(*N*,*N*-dimethylamino)phenyl]-*trans*-4-(*p*-nitrophenyl)-1,3-butadiene (NND). Unlike many commonly used fluorophores, the quantum yield of NND decreases over 4 orders of magnitude upon changing from nonpolar to polar environments. In addition, several derivatives of NND undergo little photodecomposition and can be detected at the picomolar level in a confocal fluorescence correlation spectrometer. In conjunction with recent detection of single molecules in solution, this paper describes a method to discriminate between single free and carbohydrate-bound aggregates of the Jack Bean lectin, concanavalin A (Con A). To this end, two derivatives of NND were constructed possessing an additional functional handle. One derivative, alkenyne **4**, was efficiently attached to the β-anomeric position of glucopyranosides. Transients from single aggregates of this fluorophore were detected in solutions which contained both Con A and maltoside **1**, and not the corresponding glucoside **2**. This result is in agreement with the known affinity of Con A for α-glucopyranosides and not β-glucopyranosides. A full description of the synthesis of these dyes, their solvochromatic properties, and the method used for single aggregate detection is provided herein.

Introduction

Within the last decade, the development of improved electron tunneling probes and optical methods has made it possible to detect single molecules in vacuum, solution, and the solid state and on surfaces.[1] In contrast to classical spectroscopy which monitors an average molecular ensemble, single molecule detection (SMD) examines unique events within a population. This ability enables one to examine minor species whose function would normally be lost by inclusion within an average. In solution, single molecules have been detected by monitoring their laser-induced fluorescence (LIF).[2,3] While the detection limit of LIF is far superior to that of traditional fluorescence spectroscopy, the method cannot directly operate at the single molecule level (i.e., 10^{-24} M). Amplification to the molecular level can be accomplished by reducing the probed volume to or below a femtoliter. Modern developments in optical imaging now provide confocally-imaged pinholes which when placed in the path of a diffraction-limited laser beam provide an illuminated cylinder with a diameter of 400 nm, a length of 2 μm, and a volume of approximately 0.2 fL (1 fL = 10^{-15} L). In this cavity, the concentration of a single molecule now corresponds to 8.3 nM, a value within the capacity of LIF. Using this and other methods for generation of small volumes, single fluorescent molecules have been detected in flowing and static solutions.

Since its discovery in 1974,[4] fluorescence correlation spectroscopy (FCS) has provided new insight into a wide variety of investigations, including diffusion, aggregation, chemical reactions, and conformational analysis.[5] The principle of this method is based on monitoring the fluctuation in fluorescence intensity as molecules diffuse through a specified illuminated

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cavity. When a molecule or group of molecules pass into a cavity tuned near their absorption maximum, they undergo cycles of excitation and relaxation by emission of a second photon. When these events are recorded in a time-dependent matter, the quanta which belong to these molecules can be determined through autocorrelation.[6] In 1994, Rigler and Eigen described a method for detecting single rhodamine-labeled DNA molecules, based on application of confocal microscopy to FCS.[7] Concurrently, Zare and colleagues devised a similar method for detecting single molecules of YOYO intercalated DNA in real time, without the need for autocorrelation.[8] Since these discoveries, Webb and Gratton have expanded the spectral window to include the UV region through use of multiple photon excitation.[9] When used in conjunction with techniques for concentrating or separating particles, such as electrical focusing or optical tweezers, the detection limit of this method becomes infinite.[7,8] To date, FCS has been applied to a number of biophysical investigations, including the study of protein folding, neuroreceptor-binding, the motion of actin filaments, and membrane dynamics.[10] This investigation describes the first application of single molecule FCS to monitor the interaction between carbohydrates and proteins.

Recently, increased attention has been devoted to gaining a better understanding of the biological significance of carbohydrate−protein recognition, due to the participation of these events in a wide variety of disease-related processes including cellular growth−development, fertilization, metastasis, and inflammatory response, as well as bacterial and viral recognition.[11] Low affinity, often with an association constant (K_a) as low as 10^{-4} M^{-1}, has been a major problem facing these investigations. One solution to this problem has appeared through enhancement of the binding by modification of one partner. Several groups have reported dramatically increased affinities of molecules possessing multiple (polyvalent) carbohydrates.[12] The affinity of these ligands and their monomeric counterparts can be determined with techniques such as fluorescence anisotropy or microcalorimetry.[13,14] The advantage of the latter is that it provides a complete energetic description, including entropic and enthalpic terms. These methods have been used to determine the affinity of sialosides for influenza hemagglutinin, various oligosaccharides for E-selectin, Salmonella trisaccharide epitope for a monoclonal antibody Se 155-4, and numerous *C*- and *O*-glycosides for lectins. Although accurate, both methods rely on a comparison between the free and bound states. To date, neither method is capable of specifically detecting molecules in one state nor can either method operate at the single molecule level. One question that becomes important to the understanding of carbohydrate−protein binding events is the role and mechanistic aspects of aggregation before and after binding. Early on, it was recognized that several of the carbohydrate binding proteins exist in aggregated (dimeric, tetrameric, or polymeric) forms. Multivalent ligands, which are already polymeric, are biased toward aggregated forms and therefore do not easily allow one to examine this aggregation. Therefore, discovery of a method which selectively detects only the carbohydrate-bound or free state provides an ideal tool for this type of investigation. On the basis of low affinities of these events, the design must incorporate a means to detect at very low concentration, ideally at the single molecule level.

One limitation to the development of confocal FCS and further laser-based fluorometric techniques is the photophysical and spectroscopic properties of the fluorescent molecule or tag. In fluorescence-based single molecule detection, a laser beam tuned near the absorption maximum of the fluorophore is used to initially provide high-lying rotational and vibrational states which then undergo picosecond nonradiative decay to a low-lying singlet state (S^1). In doing so, molecules which contain degrees of rotational freedom can adopt more than one singlet state, such as twisted intramolecular charge transfer (TICT) states.[15] These states originate from internal rotation to conformers where the orbitals of one portion of the molecule are oriented orthogonally with the other. Observation of these states was first seen in the fluorescence spectrum of *p*-(*N*,*N*-dimethylamino)benzonitrile and soon thereafter attributed internal twisting about the dimethylamino group through analogy to several locked and rotationally restricted derivatives, such as those shown in Figure 1. Emission from these states is typically sensitive to solvent polarity, low in energy and intensity, and of short lifetime. In addition to formation of TICT states, several fluorophores readily undergo spin-forbidden relaxation from the S^1 state to a long-lived triplet state (T^1), additionally decreasing in their fluorescence. For single molecule detection, the efficiency of a chromophore is measured with its absorption cross-section (σ), its fluorescence quantum yield (Φ_f), and its photodecomposition.

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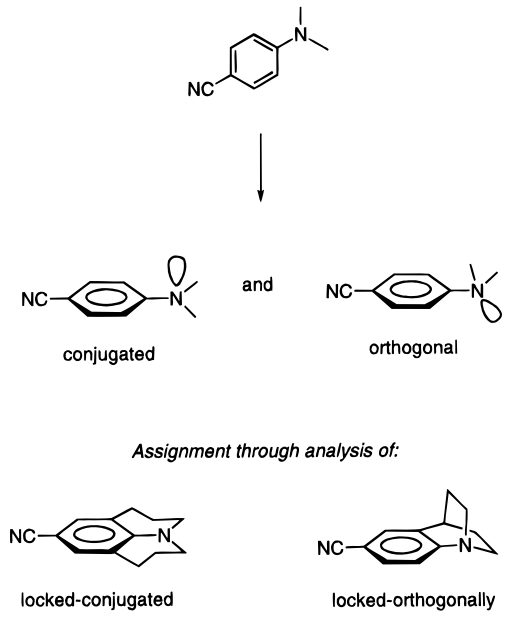

Figure 1.

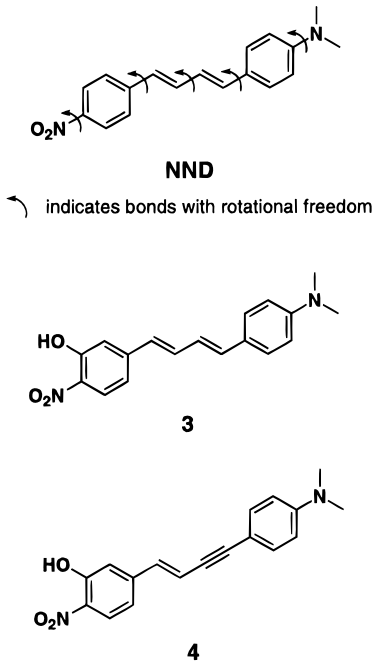

Figure 2.

In addition to photophysical considerations, fluorometric detection of binding requires a substance which undergoes significant modification of its absorption or emission maxima, emission quantum yield, and/or excited state lifetime upon binding. Unlike many commonly used fluorophores,[16] the quantum yield of *trans*-1-[*p*-(*N*,*N*-dimethylamino)phenyl]-*trans*-4-(*p*-nitrophenyl)-1,3-butadiene (NND) decreases upon changing from nonpolar to polar solvent systems as seen in the comparison of heptane ($\Phi_f = 0.097$) to methanol ($\Phi_f = 4 \times 10^{-6}$).[17] In addition, the absorption and fluorescence maxima of these materials are red shifted by a respective 130 and 109 nm over the same interval. Even more remarkably, the quantum yield of this material increased to 0.14, a 44% improvement over that seen in the most nonpolar solvent, when embedded in a phospholipid vesicle.[17] This fact clearly shows that restriction of the space for internal rotation results in a dramatic gain of fluorescence. Similar findings have been seen in the insertion of *trans*-stilbene into vesicle membranes.[18] In this case, the increased fluorescence quantum yield was attributed to inhibiting photoisomerization, to *cis*-stilbene, which is known to compete for the singlet excited state with fluorescence. Comparable isomerizations have been seen in *trans*-1-[*p*-(*N*,*N*-dimethylamino)phenyl]-2-(*p*-nitrophenyl)ethylene (NNS).[19] The isomerization yield of this process ($\Phi_{t-c} = 0.034$ in toluene) as well as the amount of crossing to the triplet state decreased with solvent polarity, suggesting that nonradiative relaxation was the major path back to the ground state in polar solvents. When extensively photolyzed, NND in cyclohexane or toluene reached a photostationary state containing a mixture of the initial *trans*,*trans*-isomer (67%) and the corresponding *cis*,*trans*-isomers.[17] Inhibition of this isomerization may be one factor contributing to the increased fluorescence seen in vesicles. Alternatively, this fluorescence gain can be explained by restricting the formation of TICT states. Although there is no direct evidence for the presence of TICT states in NND, due to the complicated nature associated with several positions for rotation, the decreased fluorescence and red-shifted absorption upon increasing solvent polarity are comparable with those of substances known to have TICT states. Here, the goal is to apply this fluorescence response to distinguish between the environment surrounding a carbohydrate as it passes from saline solution to the surface of a protein. Since many carbohydrate−protein complexes exist in aggregated forms, the appended fluorophore will respond not only to the surface interaction with the protein but also the spatial considerations imparted by inclusion in an aggregate. In addition, this aggregation increases the number of fluorescent units per molecular entity, as each aggregate can contain up to one ligand per protein. This investigation chose to examine the lectin concanavalin A (Con A) since its carbohydrate affinity has been determined,[20] it is known to exist as a tetramer at pH 7.2,[21] and its binding pocket has been reported to be more hydrophobic than predicted.[22] The first steps toward this experimentation required derivatization of NND so that it could be attached to carbohydrates.

Results and Discussion

The investigation began with the synthesis of two analogs of NND, compounds **3** and **4** (Figure 2), which contain a phenolic handle for linkage to the anomeric center of a carbohydrate. Prior to this work, Aykiyama reported that NND can be converted to **3** by exposing its DMF solution to potassium *tert*-butoxide in air.[23] This procedure provided a 13% yield of **3** along with several alkynic products from oxidation of the internal alkenes. The synthetic approach employed herein uses

(16) These quantum yields can be compared to other commonly used fluorophores such as fluorescein (0.91 in 0.1 M NaOH) or rhodamine B (0.70 in ethanol). Chen, R. F. *Anal. Biochem.* **1967**, *20*, 339.

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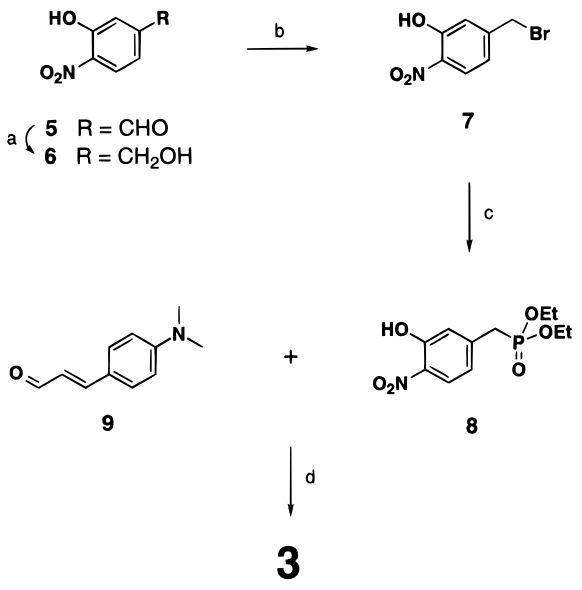
Scheme 1[a]



[a] Conditions: (a) $NaBH_4$, MeOH, THF, Et_2O (5:5:1), 0 °C to rt, 97%. (b) CBr_4, PPh_3, CH_2Cl_2, 0 °C to rt, 86%. (c) $P(OEt)_3$, DMF, 155 °C, 1.5 h, 99%. (d) (i) Add NaHMDS (2.2 equiv) in THF to crude **8** in DMF, 0 °C to rt, 1 h; (ii) add **9** in THF, −20 °C to rt, 6 h, 55%.

a direct construction, providing **3** and **4** in significantly higher overall yield and without the need for tedious chromatographic separation. This was accomplished through coupling of phosponate **8** with the corresponding aldehydes **9** and **13** by means of a Wadsworth−Horner−Emmons modified Wittig reaction.[24] This disconnection was chosen on the basis the high *trans*-stereoselectivity of this method and the fact that aldehyde **9** is commercially-available and the other materials were obtained in three steps. Phosphonate **8** was prepared through functional manipulation of aldehyde **5**. This sequence began by reducing the carbonyl group in **5** with $NaBH_4$ as described in Scheme 1. The resulting alcohol **6** was then converted to bromide **7** using the phosphonium salt method of Appel.[25] In turn, this bromide was displaced with triethyl phosphite at 150 °C in DMF, providing phosphonate **8**. This sequence was readily conducted on a 10 g scale with an 83% overall yield. In accordance with the Wadsworth−Horner−Emmons modified Wittig procedure, the ylide of **8** was generated by the addition of 2.2 equiv of NaHMDS in THF to the crude displacement mixture at 0 °C. The production of a deep purple color upon surpassing addition of the first equivalent of base provided a convenient internal standard. Condensation of this dianion with *p*-(*N*,*N*-dimethylamino)cinnamaldehyde (**9**) provided a 54% yield of NND-OH (**3**).

Numerous methods were examined for attaching **3** to the anomeric center of carbohydrates.[26] Unfortunately, NND-OH (**3**) decomposed under the conditions generated during activation of phenylthio, phenylselenyl, fluoro, and pentenyl glycosides. Classical Konigs−Knorr coupling with α-D-glucopyranosyl bromide tetraacetate resulted in either recovery or slow decomposition of **3**. Recently, Roy developed a phase transfer method which primarily operates through an S_N2 displacement of a glycosyl bromide by a phenolate.[27] This method provided a high degree of stereoinversion and was amenable to several carbohydrate and phenolic units. Application of this method to NND-OH (**3**) was complicated by the low solubility of **3** in applicable solvents. At saturation in methylene chloride, glycosylation of **3** was only 20% complete after 2 weeks. In addition, purification of this material was complicated by the presence of several unwanted side products which arose from *in situ* acetate hydrolysis and further reaction with α-D-glucopyranosyl bromide tetraacetate. In an attempt to improve the efficiency of this process, attention was turned to construction alkyne **4**, with the hope that it would be more soluble.

Application of the previously described Wittig protocol for **4** required construction of aldehyde **13**. This material was prepared in three steps from *N*,*N*-dimethylaniline (**10**) (Scheme 2). The sequence began by regioselectively introducing an iodine atom onto the *para*-position of **10** through reaction with iodine in an aqueous sodium bicarbonate buffer. Upon one recrystallization from an ether−hexane mixture, iodide **11** could be coupled to propargyl alcohol using a procedure described by Takalo and Haenninen.[28] The resulting alkynol **12** was then oxidized with a slurry of MnO_2 to the desired aldehyde **13**. Condensation of this aldehyde with the previously described ylide of **8** provided alkenyne **4**, as evidenced by the presence of a single 16 Hz vinylic coupling constant in its ^{1}H-NMR spectrum. Unlike NND-OH (**3**), alkenyne **4** was soluble in methylene chloride up to 0.2 M and readily reacted with 2,3,4,6-tetraacetoxy-α-D-glucopyranosyl bromide under the conditions of Roy. Since partial hydrolysis of the *C*(6)-acetate often occurred under these conditions, the crude material was directly subjected to methanolysis, providing a single compound, **2**. Alternatively, buffering of the methanolysis reaction with benzoic acid provided the readily recrystallized benzoate salt of **2**. Encouraged by this success, efforts were directed at preparing a derivative which contained an α-glucopyranosidic linkage for binding to Con A. This was accomplished by appending maltose, as it already contained an α-glucopyranoside and the association constant for the binding of maltose to Con A was determined to microcalorimetrically to be 1.6×10^3 M^{-1}.[14g] Under conditions previously described for construction of **2**, the benzoate of β-maltoside **1** was obtained in 73% yield from **4** and the corresponding peracetylated α-pyranosyl bromide of maltose. The free bases **1** and **2** were obtained by warming solutions of their benzoate salts in 1,4-dioxane containing powdered 4 Å molecular sieves.

The absorption and fluorescence spectra of **1** and **2** were examined in solvents ranging from THF to methanol (see Table 1). These materials absorbed in two regions, one centered at about 390−435 nm and the other between 280 and 320 nm. The position and intensity of this absorption were nearly identical for both **1** and **2**. With the exception of methylene chloride and chloroform, the position of the lower energy band deviated only 3% from ∼400 nm and was on average 20−30 nm lower than that of **4**. The fluorescence maxima were nearly identical for **1** and **2**, and their quantum yields in methanol were 36% and 39% less than those in THF, respectively. In addition, fluorescence from maltoside **1** and glucoside **2** could be detected up to 720 nm in THF, while no fluorescence was detected above 580 nm in methanol. The closest line of an argon laser to the

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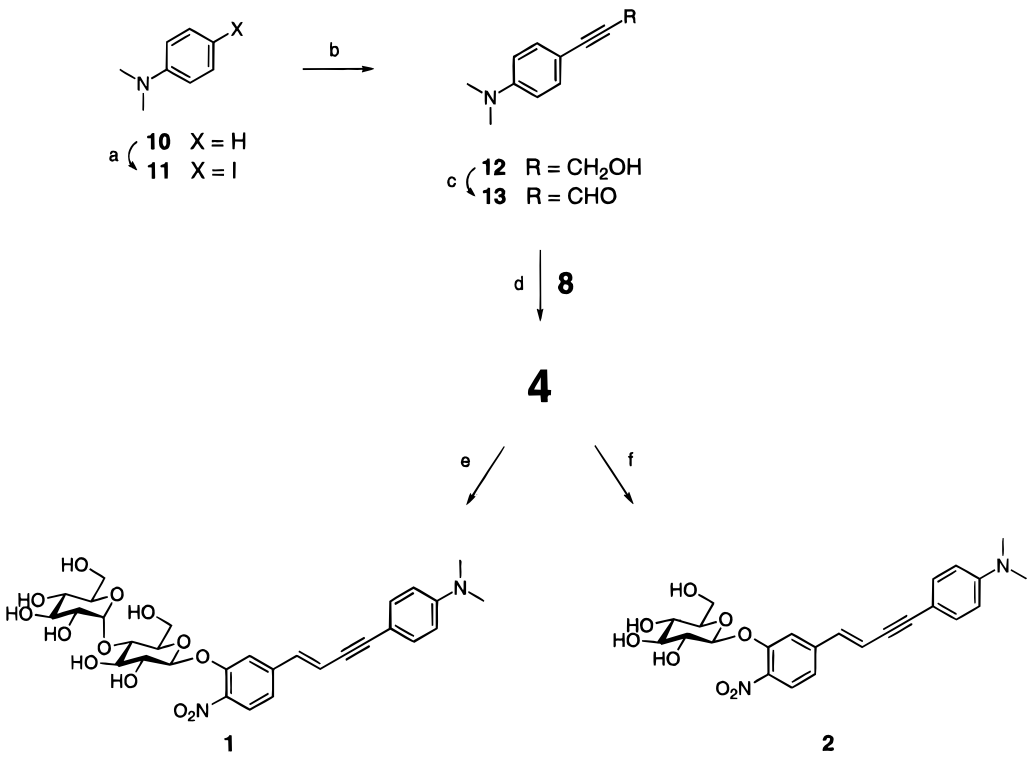
Scheme 2[a]



[a] Conditions: (a) (i) iodine, $NaHCO_3$, H_2O, 12−15 °C, 10 min; (ii) then rt, 1 h, 86%. (b) Propargyl alcohol, $Cl_2Pd(PPh_3)_2$ (1.4 mol %), CuI (1.4 mol %), Et_3N, rt, 18 h, 89%. (b) MnO_2, CH_2Cl_2, rt, 4 h, 89%. (d) (i) Add NaHMDS (2.2 equiv) in THF to crude **8** in DMF, 0 °C to rt, 1 h; (ii) add **13** in THF, −20 °C to rt, 6 h, 56%. (e) (i) α-D-Maltopyranosyl bromide heptaacetate, *N*-benzyltriethylammonium chloride, 1 M NaOH, CH_2Cl_2, rt, 12 h; ii. $NaOCH_3$, CH_3OH, rt, 1 h; (iii) $PhCO_2H$; (iv) Powdered 4 Å molecular sieves, 1,4-dioxane, 70 °C, 2 h; 69%. (f) (i) α-D-Glucopyranosyl bromide tetraacetate, *N*-benzyltriethylammonium chloride, 1 M NaOH, CH_2Cl_2, rt, 12 h; (ii) $NaOCH_3$, CH_3OH, Ph, rt, 1 h; (iii) $PhCO_2H$; (iv) Powdered 4 Å molecular sieves, 1,4-dioxane, 70 °C, 2.5 h; 71%.

Table 1. Absorption Maxima, Extinction Coefficients (ϵ), Fluorescence Maxima, and Quantum Yields (Φ_f) for Compounds **1** and **2**

		β-glucoside **1**		β-maltoside **2**		β-glucoside **1**		β-maltoside **2**	
solvent	E_t	λ_A (max), nm	ϵ, 10^4 cm^{-1} M^{-1}	λ_A (max), nm	ϵ, 10^4 cm^{-1} M^{-1}	λ_f (max), nm	Φ_f	λ_f (max), nm	Φ_f
THF	37.4	358−427	3.23	381−419	3.97	610	0.000 068	602	0.000 073
		298	4.04	298	4.13	478	0.000 094	482	0.000 097
$CHCl_3$	39.1	428	3.41			519	0.000 088		
		314	3.54						
CH_2Cl_2	41.4	419	3.52			531	0.000 082		
acetone	42.2	359−431	4.85	375−421	4.89	492	0.000 071	488	0.000 061
DMF	43.8	402	3.41	406	3.32	495	0.000 075	480	0.000 073
		301	3.23	302	3.23			434	0.000 082
DMSO	45.0	406	4.52	410	4.64	472	0.000 11	476	0.000 094
		304	3.54	306	3.97				
acetonitrile	46.0	366−421	4.52	401	4.63	499	0.000 063	495	0.000039
		299	4.06	300	4.07				
isobutanol	49.0	397	4.02	405	4.13	551	0.000 099	548	0.000096
		301	4.21	300	4.25				
l-butanol		361−452	3.89	376−425	4.05	556	0.000 1	542	0.000 11
		282	4.21	297	4.32				
ethanol	51.9	356−419	3.01	401	3.12	550	0.000 053	537	0.000 055
		298	2.82	299	2.96				
methanol	55.5	367−422	3.07	398	3.31	541	0.000 027	499	0.000 027
		296	3.75		3.76				

absorption maxima of **1** and **2** was at 457 nm. At this excitation wavelength, the emission from **1** and **2** was ∼25% of that at 400 nm in THF. Fluorescence from **1** and **2** in media established for monosaccharide binding to the tetrameric form of Con A (i.e., 0.05 M in PIPES (pH 7.2), 10 mM in $MnCl_2$, 10 mM in $CaCl_2$, and 1 M in NaCl) could not be detected in a SLM-Aminco fluorimeter, even at saturation or at its absorption maximum.

Confocal fluorescence correlation spectrometry was measured using the spectrometer described by Eigen and Rigler.[7] Samples of **1**−**4** were examined in several organic solvents by placing a droplet of the appropriate solution in a ∼20 μL conical gold well and bringing the droplet in contact with a thin microscope slide which hung from a water immersible microscope objective by a drop of water.[29] Excitation was provided by passing the 457 nm line of an argon ion laser through the objective at 0.5

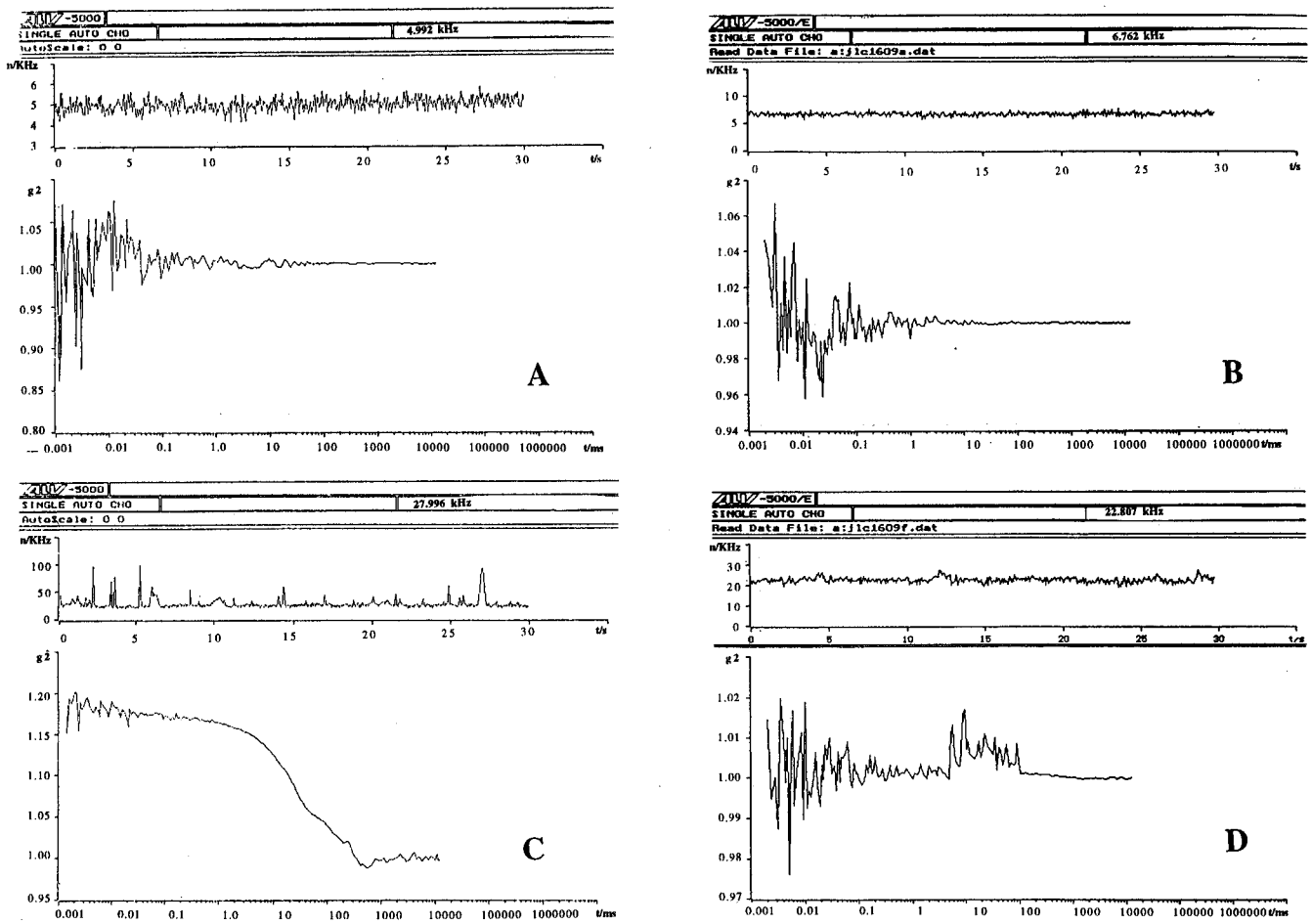


Figure 3. Real time and autocorrelated traces demonstrating carbohydrate binding to Con A. Samples were in a buffer which was 10 mM in $MnCl_2$, 10 mM in $CaCl_2$, 0.05 M in PIPES (pH 7.2), and 1 M in NaCl, irradiated with a laser tuned to 457 nm (0.5 mW) and collected through an Omega Optics 545 cutoff filter. For each sample, the upper trace indicates the frequency of emitted photons (kHz) and shows transients from aggregates in real time. The lower trace provides the autocorrelation function, where the number of particles inside the volume element is given by $1/g2$ as $t \rightarrow 0$. The midpoint of the autocorrelated curve provides an approximation of the diffusion time. (A) 51 μM **1**, (B) 12 μM **2**, (C) 51 μM **1** with 170 μM Con A, (D) 12 μM **2** with 170 μM Con A.

mW. Aqueous solutions were more easily sampled by directly hanging the droplet (approximately 20 μL) from the microscope slide. The resulting fluorescence were collected through the same objective by harvesting the emitted photons through a dichroic mirror, followed by filtering with an Omega Optics 545 nm cutoff filter, and counting with a SPAD detector. Autocorrelation was provided online with an ALV-card attached to a PC. The fluorescence fluctuation from compounds **1**−**4** deviated within 5% in most solvents, with the exception of chloroform and methanol (Table 2). The enhanced decomposition in these solvents can be attributed to slow cleavage of the anomeric center by methanolysis or trace acidity often found in chloroform. In the confocal FCS spectrometer, autocorrelation from samples of **3** and **4** was readily detected at the picomolar level in heptane and decreased to nearly the micromolar level with increased polarity, as reflected by the loss of quantum efficiency. Autocorrelation from **1** and **2** could be detected at a 10-fold lower concentration in THF than in methanol. Samples of **1** and **2** in the buffer commonly used for binding of carbohydrates to Con A did not autocorrelate at any concentration, nor were transients detected as shown in Figure 3 (traces A and B). Samples of 51 μM **1**, 12 μM **2**, and a blank fluoresced with a fluctuation of about 5.0, 6.8, and 4.3 kHz, respectively. As shown in the upper portion of trace C, transients from the diffusion of single molecules containing the fluorophore into the volume element were observed in a solution which contained 170 μM Con A and 51 μM **1** at pH 7.2. The number of molecules in the cavity is $N = 0.83$, as given by $N = 1/g2$, and therefore the concentration of the bound material in this cavity (volume 2×10^{-15} L) is 0.69 nM.[30] On the basis of the reported affinity of Con A for α-glucopyranosides and lack of β-glucopyranosides, the transients seen originate from

Table 2: Behavior of Compounds **1**−**4** in the Eigen−Rigler Confocal Fluorescence Correlation Spectrometer[a]

	detection limit, nM				photostability, % deviation			
solvent	1	2	3	4	1	2	3	4
heptane			0.002	0.011			1	1
THF	70	62	14	12	5	1	1	2
$CHCl_3$		79	0.28	0.85		8	13	9
acetone	74	58	14	20	5	4	4	3
DMF	350	165	49	61	4	1	2	2
acetonitrile	240	230	220	240	2	2	3	4
ethanol	170	120	62	93	3	2	2	2
methanol	510	290	160	380	7	9	2	2

[a] Excitation was provided at 455 nm (0.5 mW), and the fluorescence was collected through a 545 nm cutoff filter.

(29) The absorption cross-sectional area for alkenyne **4** at 457 nm is maximally 3×10^{-16} cm^2. A laser beam with a single line power of 0.5 mW provides a photon density of 10^{24} photons/(cm^2 s). Within this beam, the singlet excitation rate of this fluorophore would be 3×10^{-8} s^{-1}, as given by the product of its cross-sectional area and photon density.

the complex of **1** with Con A. This was verified by the fact that transients due to aggregates were not detected in samples of **1** without Con A (trace A) or **2** with and without the addition of Con A (traces B and D, respectively).[31] Upon complexation, the weight about the fluorescent label dramatically increases from a formula weight of 632 to approximately 100 000. This increase would therefore be translated into a slower diffusion time upon binding, as the diffusion is dependent on the size of a material. As given by autocorrelation, the midpoint of descent in the autocorrelated curve is an approximation of the size about the fluorescent moiety. The diffusion times of **1** and **2** ranged between 0.04 and 0.06 ms in various solvents, while that attributed to complexes of **2** with Con A was approximately 19 ms, as seen in Figure 3. Similar increases in diffusion time have been seen in the complexation of a BODIPY-labeled DNA primer to M13-DNA.[7]

Conclusion

This investigation describes a new scheme for monitoring interactions between carbohydrates and proteins. Although the study so far is limited to the interaction between Con A and a β-maltopyranoside, the method provides selective detection of carbohydrate-bound lectin. Combined with the fact that this fluorescent tag responds not only to solvent polarity but also to confinement, the method should be applicable to a wide variety of events where the label encounters restriction upon binding. Investigations into the reason for this fluorescence enhancement and application of this method to study aggregation of these complexes are currently underway.

Experimental Section

Chemical Synthesis. All reactions were conducted under an argon atmosphere in rigorously dried glassware and were magnetically-stirred with a Teflon-coated stir bar, unless otherwise indicated. Reagents were added to reaction vessels *via* a cannula or dry syringe. Anhydrous tetrahydrofuran (THF) was distilled from sodium−benzophenone ketyl. Methylene chloride, methanol, 1,4-dioxane, and *N*,*N*-dimethylformamide (DMF) were purchased dry from Aldrich. Materials reacted under anhydrous conditions were dried extensively with toluene azeotrope prior to use. Thin layer chromatography (TLC) on Merck silica gel DC 60 plates was routinely used to monitor all reactions. TLC plates were developed by staining with iodine absorbed on silica gel. All R_f values were collected from runs in 33% ethyl acetate/hexane. Melting points were measured on a Büchi 520 and are uncorrected. Infrared spectra (IR) were collected on a Perkin-Elmer Paragon 1000 PC FT-IR spectrometer. Samples were prepared on sodium chloride (NaCl) plates, neat or in a chloroform smear. UV−vis and fluorescence spectra were measured on a Perkin-Elmer Lambda 17 UV−vis spectrometer, Perkin-Elmer LS-5B luminescence spectrometer, and SLM-Aminco SPF-500C. ^{1}H-NMR and ^{13}C-NMR spectra were obtained at 300 and 75 MHz, respectively, on a Bruker MSL300. Chemical shifts (δ) are given in parts per million and coupling constants (J) in hertz. Microanalyses were obtained from Beller Microanalytisches Labor (Göttingen, Germany). Standard flash chromatography was performed on Merck 9395 silica gel using a gradient from hexane to the solvent listed. Samples of **1** and **2** were recrystallized three times from spectral grade methanol at −20 °C to ensure purity.

***m*-Hydroxy-*p*-nitrobenzyl Alcohol (6).** The synthesis of phosphonate **8** was accomplished in three operations from commercial aldehyde **5**. This functional conversion began by reducing aldehyde **5** (3.74 g, 22.4 mmol) with $NaBH_4$ (3.46 g, 91.5 mmol) in a 5:5:1 mixture of methanol, ether, and THF (77 mL). This was accomplished by adding $NaBH_4$ to the solution of **5** at 0 °C over 30 min and then warming over 2 h to room temperature (rt). The reaction was quenched with 10% aqueous HCl (until the pH was approximately 6), poured onto 80 mL of brine (80 mL), extracted with 200 mL of CH_2Cl_2 (3×), dried with Na_2SO_4, and concentrated. The crude product was used directly for the next step. Pure material could be obtained through flash chromatography (SiO_2, 50% ethyl acetate/hexanes), yielding 3.67 g (97%) of **6**: mp 79.7−81.3 °C; $R_f = 0.37$; ^{1}H NMR ($CDCl_3$) δ 10.60 (s, 1H), 8.06 (d, $J = 8.8$ Hz, 1H), 7.14 (s, 1H), 6.94 (d, $J = 8.8$ Hz, 1H), 4.73 (d, $J = 5.5$ Hz, 2H), 1.97 (t, $J = 5.5$ Hz, 1H); ^{13}C NMR ($CDCl_3$) δ 63.7, 116.9, 117.9, 125.2, 151.7, 155.3; IR ($CHCl_3$) 3459, 2356, 1620, 1579, 1520, 1475 cm^{-1}. Anal. Calcd for $C_7H_7NO_4$: C 49.71, H 4.17, N 8.28. Found: C 50.13, H 4.30, N 8.20.

***m*-Hydroxy-*p*-nitrobenzyl Bromide (7).** Carbon tetrabromide (2.86 g, 8.63 mmol) was added over 30 min to a solution of **6** (∼1.22 g, ∼7.22 mol) and PPh_3 (2.45 g, 9.35 mmol) in 25 mL of dry CH_2Cl_2 at 0 °C. The reaction was warmed to rt over 1.5 h and allowed to stand for an additional 1 h, at which point the mixture was poured onto water, extracted with 100 mL of CH_2Cl_2 (2×), dried with Na_2SO_4, and concentrated. Pure material was obtained by flash chromatography (SiO_2, 33% ethyl acetate/hexanes) yielding 1.42 g (86%) of **7**: mp 68.9−70.2 °C; $R_f = 0.58$; ^{1}H NMR ($CDCl_3$) δ 10.58 (s, 1H), 8.07 (d, $J = 8.6$ Hz, 1H), 7.16 (d, $J = 1.9$ Hz, 1H), 6.99 (dd, $J = 1.9$, 8.8 Hz, 1H), 4.39 (s, 2H); ^{13}C NMR ($CDCl_3$) δ 30.6, 120.1, 120.8, 125.7, 147.8, 155.2; IR ($CHCl_3$) 2354, 1622, 1584, 1479, 1328, 1258, 1159, 968, 885, 844, 652 cm^{-1}. Anal. Calcd for $C_7H_6NO_3Br$: C 36.23, H 2.61, N 6.04. Found: C 36.10, H 2.69, N 5.92.

Diethyl (*m*-Hydroxy-*p*-nitrobenzyl)phosphonate (8). A mixture of **7** (0.91 g, 3.98 mmol) and triethyl phosphite (0.87 mL, 5.09 mmol) was refluxed (bath temperature 155 °C) in 2.5 mL of dry DMF for 1.5 h. Pure material was obtained through flash chromatography (SiO_2, 25% ethyl acetate/hexanes), yielding 1.14 g (99%) of **8**: mp 59.3−62.1 °C, $R_f = 0.09$; ^{1}H NMR ($CDCl_3$) δ 10.56 (s, 1H), 8.01 (d, $J = 8.7$ Hz, 1H), 7.04 (dd, $J = 2.2$ Hz, $J_{H-P} = 2.2$ Hz, 1H), 6.91 (dd, $J = 2.2$, 8.7 Hz, $J_{H-P} = 2.2$ Hz, 1H), 4.04 (qd $J = 7.1$ Hz, $J_{H-P} = 8.1$ Hz, 2H), 3.04 (d, $J_{H-P} = 22.5$ Hz, 2H), 1.25 (q, $J = 7.1$ Hz, 3H); ^{13}C NMR ($CDCl_3$) δ 16.2 (d, $J_{C-P} = 5.3$ Hz), 34.2 (d, $J_{C-P} = 136.7$ Hz), 62.4 (d, $J_{C-P} = 6.5$ Hz), 120.7 (d, $J_{C-P} = 8.3$ Hz), 121.8 (d, $J_{C-P} = 6.0$ Hz), 125.0 (d, $J_{C-P} = 2.4$ Hz), 143.2 (d, $J_{C-P} = 8.8$ Hz), 154.9 (d, $J_{C-P} = 3.8$ Hz); IR ($CHCl_3$) 3850, 3444, 2985, 1623, 1587, 1520, 1480, 1443 cm^{-1}. Anal. Calcd for $C_{11}H_{16}NO_6P$ C 45.68, H 5.58, N 4.84. Found: C 45.60, H 5.74, N 4.83.

***trans*-1-[*p*-(*N*,*N*-Dimethylamino)phenyl]-*trans*-4-(m-hydroxy-*p*-nitrophenyl)-1,3-butadiene (3).** Sodium bis(trimethylsilyl)amide (0.99 mL, 1.0 M in THF) was added to the crude solution of phosphonate **8** (143.6 mg, 0.453 mmol in 0.8 mL of DMF) from the above step at 0 °C. Thirty minutes later, the solution was warmed to room temperature and kept there for 20 min, at which point, the contents were recooled to −20 °C and reacted with a solution of (*N*,*N*-dimethylamino)-cinnamaldehyde (74.2 mg, 0.430 mmol) in 4 mL of THF. After 8 h at ambient temperature, 15 mL of ice cold brine was added. The pH was adjusted to 7 with dilute HCl, and the crude product was obtained by repetitive extraction with 10% THF in CH_2Cl_2, drying with Na_2SO_4, and concentration. Flash chromatography (33% $CHCl_3$/hexane) and recrystallization from 10:1 heptane/THF provided 302.7 mg (55%) of **3**: mp 216.3−217.2 °C; $R_f = 0.43$; ^{1}H NMR ($CDCl_3$) δ 10.74 (s, 1H), 7.99 (d, $J = 8.7$ Hz, 1H), 7.24 (d, $J = 8.7$ Hz, 2H), 7.08 (dd, $J = 7.8$, 15.5 Hz, 1H), 7.03 (dd, $J = 7.8$, 16.9 Hz, 1H), 7.02 (d, $J = 16.9$ Hz, 1H), 6.75 (d, $J = 7.8$ Hz, 2H), 6.74 (s, 1H), 6.65 (d, $J = 8.7$ Hz, 2H), 6.47 (d, $J = 15.4$ Hz, 1H), 2.98 (s, 6H); ^{13}C NMR (DMSO-d_6): δ 30.6, 112.3, 115.9, 117.0, 124.1, 124.6, 125.8, 127.4, 128.0, 133.9, 135.2, 136.7, 145.6, 150.6, 153.6; IR (trace $CHCl_3$) 3850, 3741, 2357, 2169, 1574, 1470, 1219, 962, 944, 772, 674 cm^{-1}. Anal. Calcd for $C_{18}H_{18}N_2O_3$: C 69.66, H 5.85, N 9.03. Found: C 69.72, H 5.97, N 9.06.

***N*,*N*-Dimethyl-*p*-iodoaniline (11).** Resublimed iodine (4.28 g, 16.8 mmol) was added in small portions over 45 min to a mixture of *N*,*N*-dimethylaniline (**10**) (2.37 mL, 18.7 mmol) and $NaHCO_3$ (2.35 g, 27.9 mmol) in 16 mL of water between 12 and 15 °C. Ten minutes after

(30) The concentration provided is a description of the Con A−maltoside **1** complex which undergoes fluorescence. The total concentration of the Con A−maltoside **1** complex is much larger and requires knowledge of its quantum yield. Since there is no direct means to determine this yield, the affinity of this interaction cannot be calculated with this method.

(31) Transients were also not detected in samples with concentrations of **2** ranging from 0.012 μM up to the point where the background was to large for the detector (∼200 μM) with and without Con A.

complete addition, the mixture was warmed to rt. This mixture was diluted with 500 mL of ether and the organic phase extracted consecutively with 50 mL of water, 100 mL of sodium thiosulfate, and 100 mL of water (2×). Afterward, the crude product was dried with Na_2SO_4, concentrated, and recrystallized from 10:1 hexane/ether to yield 3.98 g (86 %) of **11**: mp 63.5−66.1 °C; $R_f = 0.66$; ^{1}H NMR ($CDCl_3$) δ 7.45 (d, $J = 8.9$ Hz, 2H), 6.46 (d, $J = 8.9$ Hz, 2H), 2.90 (s, 6H); ^{13}C NMR ($CDCl_3$) δ 40.2, 114.8, 137.3, 149.9.

3-[*p*-(*N*,*N*-dimethylamino)phenyl]prop-2-ynol (12). A mixture of iodide **11** (309.7 mg, 1.25 mmol), $Cl_2Pd(PPh_3)_2$ (6.4 mg, 0.0091 mmol), and CuI (9.5 mg, 0.0499 mmol), in 1.2 mL of triethylamine was degassed by a rapid bubbling of argon. After 30 min, 2-propyn-1-ol (0.087 ml, 1.37 mmol) was added via microliter syringe. Three hours later, a second batch of $Cl_2Pd(PPh_3)_2$ (6.4 mg) and CuI (9.5 mg) was added, and the reaction went to completion within 18 h. A strict maintenance of an argon atmosphere was crucial to the yield of this manipulation. The crude solution was filtered through 20 g of silica gel with ethyl acetate and concentrated. Pure material was obtained through flash chromatography (SiO_2, 25% ethyl acetate/hexanes), yielding 195.2 mg (89%) of **12**: mp 51.2−53.7 °C; $R_f = 0.31$; ^{1}H NMR ($CDCl_3$) δ 7.30 (d, $J = 8.9$ Hz, 2H), 6.60 (d, $J = 8.9$ Hz, 2H), 4.45 (d, $J = 4.1$ Hz, 2H), 2.95 (s, 6H); ^{13}C NMR ($CDCl_3$) δ 40.1, 51.8, 85.1, 88.7, 109.5, 111.8, 132.8, 150.3; IR (trace $CHCl_3$) 3355, 2860, 1608, 1520, 1445, 1360, 1225, 1190, 1024, 955, 818 cm^{-1}. Anal. Calcd for $C_{11}H_{13}NO$: C 75.40, H 7.48, N 7.99. Found: C 75.33, H 7.42, N 7.96.

[3-*p*-(*N*,*N*-dimethylamino)phenyl]prop-2-ynal (13). Activated MnO_2 (1.148 g, 13.2 mmol) was added to a solution of **12** (421.5 mg, 2.41 mol) in 10 mL of CH_2Cl_2 at rt. After 4 h, the reaction was purified directly by flash chromatography (SiO_2, 25% ethyl acetate/hexanes), yielding 371.8 mg (89%) of **13**: mp 81.4−82.3 °C; $R_f = 0.49$; ^{1}H NMR ($CDCl_3$) δ 9.33 (s, 1H), 7.44 (d, $J = 9.0$ Hz, 2H), 6.60 (d, $J = 9.0$ Hz, 2H), 3.01 (s, 6H); ^{13}C NMR ($CDCl_3$) δ 39.8, 96.0, 99.9, 104.9, 111.5, 135.2, 152.1, 176.2; IR (trace $CHCl_3$) 2149, 1643, 1595, 1380, 1190, 981 cm^{-1}. Anal. Calcd for $C_{11}H_{11}NO$: C 76.28, H 6.40, N 8.09. Found: C 76.42, H 6.41, N 8.07. Alternatively, large-scale preparations can be purified by recrystallization from 20:1 heptane/THF.

1-[*p*-(*N*,*N*-dimethylamino)phenyl]-*trans*-4-(*p*-nitrophenyl)-1-buten-3-yne (4). Sodium bis(trimethylsilyl)amide (6.27 mL, 1.0 M in THF, 6.27 mmol) was added to the crude solution of the phosphonate **8** (∼852.0 mg, ∼2.95 mmol) in 2.0 mL of DMF at 0 °C. A dramatic color change (light yellow to deep magenta) occurred upon exceeding the first equivalent of base. This internal standardization was routinely used to ensure proper addition of base. The solution was warmed to rt after 30 min at 0 °C and kept there for 20 min, at which point it was cooled to −20 °C and aldehyde **6** (310.0 mg, 1.79 mmol) in 5 mL of THF was added *via* cannula. After 8 h at rt, 5 mL of water was added, the pH was adjusted to 7 with dilute HCl, and then the solution was further diluted with 10 mL of brine. Crude product was obtained by extraction with 40 mL of 10% THF in CH_2Cl_2 (3×), dried with Na_2SO_4, and concentrated. Flash chromatography (SiO_2, 33% $CHCl_3$/hexanes) and recrystallization from heptane/THF (10:1) yielded 308.2 mg (56%) of **4**: mp 185.6−186.9 °C; $R_f = 0.28$; ^{1}H NMR ($CDCl_3$) δ 10.65 (s, 1H), 8.02 (d, $J = 8.8$ Hz, 1H), 7.34 (d, $J = 8.9$ Hz, 2H), 7.07 (d, $J = 1.6$ Hz, 1H), 6.99 (dd $J = 1.6$, 8.9 Hz, 1H), 6.84 (d, $J = 16.0$ Hz, 1H), 6.62 (d, $J = 8.9$ Hz, 2H), 6.55 (d, $J = 16.0$ Hz, 2H), 2.98 (s, 6H); ^{13}C NMR (DMSO-d_6): δ 39.6, 87.4, 96.6, 108.9, 111.2, 113.8, 116.6, 117.0, 125.7, 132.6, 135.2, 136.8, 143.7, 150.5, 153.1; IR (trace $CHCl_3$) 3850, 3741, 2357, 2169, 1574, 1470, 1219, 962, 944, 772, 674 cm^{-1}. Anal. Calcd for $C_{18}H_{16}N_2O_3$: C 70.12, H 5.23, N 9.09. Found: C 70.22, H 5.49, N 9.07.

Glucopyranoside 2. *N*-Benzyltriethylammonium chloride (254.8 mg, 1.12 mmol) was added to a suspension of **4** (345.1 mg, 1.12 mmol) and 2,3,4,6-tetraacetoxy-α-D-glucopyranosyl bromide (922.0 mg, 2.24 mmol) in 4 mL of CH_2Cl_2 and 10 mL of 1 M NaOH. After 12 h, the reaction mixture was diluted with 100 mL of ethyl acetate and 20 mL of water and extracted. The aqueous phase was further extracted with 50 mL of ethyl acetate (2×), and the combined organic layers were washed with 10 mL of water (2×). Crude material was obtained by drying with Na_2SO_4 and concentrating. This material was dissolved in 10 mL of methanol and 6 mL of benzene and treated with 0.25 mL of 1 M $NaOCH_3$ in methanol. After 1 h, 330 mg of benzoic acid was added followed by 1.2 g of $NaHCO_3$, 15 min later. The residual $NaHCO_3$ was filtered off, and the excess methanol was removed by rotary evaporation. Flash chromatography (SiO_2, 10:1:1 ethyl acetate/methanol/toluene) and recrystallization from methanol afforded 510.2 mg (86%) of a 1:1 mixture of **2** and its benzoate salt. The pure amine was obtained by heating this material for 2 h in dry 1,4-dioxane containing 1.3 g of 4 Å molecular sieves. The crude material was obtained by filtration aided by extensive washing with methanol and concentration. Flash chromatography (SiO_2, 10:1:1 ethyl acetate/methanol/toluene) and recrystallization from methanol (3×) yielded 375.2 mg (71%) of pure **2**: mp = 177.9−182.5 °C; $R_f = 0.28$ (10:1:1 ethyl acetate/methanol/toluene); ^{1}H NMR ($CDCl_3$) δ 7.72 (d, $J = 8.4$ Hz, 1H), 7.44 (d, $J = 1.4$ Hz, 2H), 7.20 (d, $J = 8.7$ Hz, 1H), 7.17 (dd $J = 1.4$, 8.4 Hz, 1H), 6.85 (d, $J = 16.2$ Hz, 1H), 6.62 (d, $J = 8.7$ Hz, 2H), 6.60 (d, $J = 16.0$ Hz, 2H), 5.03 (d, $J = 7.3$ Hz, 2H), 3.86 (dd, $J = 2.1$, 12.0 Hz, 2H), 3.67−3.57 (m, 2H), 3.50−3.37 (m, 3H), 3.28−3.22 (m, 1H), 2.90 (s, 6H); ^{13}C (CD_3OD) δ 40.6, 63.1, 71.8, 75.2, 78.4, 79.0, 88.0, 97.4, 103.2, 111.4, 113.4, 115.1, 116.5, 121.1, 127.0, 134.0, 139.1, 144.7, 152.4; HRMS (FAB) ($C_{24}H_{26}N_2O_8$) found 470.1689, found 470.1699.

Maltopyranoside 1. A saturated solution of HBr in HOAc (1.5 mL) was added to an 18:1 mixture of β/α peracetylated maltose in 0.4 mL of HOAc at 0 °C. The mixture was warmed to 15 °C over 30 min and kept there for an additional 30 min, at which point it was concentrated to dryness by warming to 40 °C at 10 mmHg (generated by a water aspirator pump). *N*-Benzyltriethylammonium chloride (106.0 mg, 0.32 mmol) was added to a suspension of **4** (0.0987 mg, 0.32 mmol) and the above maltopyranosyl bromide (891.0 mg, 1.27 mmol) in 8 mL of CH_2Cl_2 and 8 mL of 1 M NaOH. After 12 h, the reaction mixture was diluted with 4 mL of cyclohexane and filtered. The filter paper was dried, washed extensively with methanol (∼100 mL), and concentrated to 80 mL. This solution was treated with 1.2 mL of a 1 M methanolic solution of $NaOCH_3$ for 1 h. The reaction was buffered by addition of 380 mg of benzoic acid. The crude product was obtained by filtering, washing extensively with methanol, and concentrating. Flash chromatography (SiO_2, 10:1:1 ethyl acetate/methanol/toluene to methanol) and recrystallization from methanol yielded the benzoate salt of **1**. The pure amine was obtained by heating the above material for 2 h in 5 mL of dry 1,4-dioxane containing 0.8 g of 4 Å molecular sieves. The crude material was obtained by filtration aided by extensive washing with methanol and concentration. Recrystallization from methanol (3×) yielded 139.7 mg (69%) of pure **1**: mp = 229.2−232.7 °C; $R_f = 0.54$ (CH_3OH); ^{1}H NMR ($CDCl_3$) δ 7.72 (d, $J = 8.6$ Hz, 1H), 7.42 (d, $J = 1.2$ Hz, 2H), 7.20 (d, $J = 8.7$ Hz, 1H), 7.17 (dd $J = 1.2$, 8.6 Hz, 1H), 6.66 (d, $J = 16.2$ Hz, 1H), 6.62 (d, $J = 8.7$ Hz, 2H), 6.60 (d, $J = 16.2$ Hz, 2H), 5.13 (d, $J = 3.7$ Hz, 2H), 5.07 (d, $J = 7.6$ Hz, 2H), 3.88 (dd, $J = 12.2$ Hz, 2H), 3.79−3.41 (m, 8H), 3.37 (dd, $J = 3.7$, 9.7 Hz, 1H), 3.35−3.21 (m, 2H), 2.90 (s, 6H); ^{13}C (DMSO-d_6) δ 39.6, 61.5, 62.4, 67.1, 67.7, 71.2, 73.5, 73.6, 74.0, 74.4, 76.6, 77.0, 80.5, 87.3, 101.8, 102.1, 112.3, 113.9, 115.1, 120.2, 125.5, 125.8, 128.9, 129.9, 132.9, 133.1, 137.1, 143.2. Anal. Calcd for $C_{30}H_{36}N_2O_{13}$: C 56.96, H 5.74, N 4.43. Found: C 56.89, H 5.62, N 4.41.

Absorption and Fluorescence Measurements. Samples of **1** and **2** were prepared by dissolving between 0.2 and 3.8 mg in a 5 mL volumetric flask using the appropriate solvent. Absorption spectra were run in quartz cuvettes with a width of 0.5 cm. Fluorescence intensities were compared at a standard concentration of 10 μM, and the quantum yields were standardized against 0.70 for rhodamine B in ethanol.[16] A list of the absorption maxima, extinction coefficients, fluorescence maxima, and quantum yields provided is an average of three repetitions of the above; the data deviated within 4%. Several samples were diluted 10-fold (to 1 μM) and their fluorescence spectra retaken to ensure that the data were not enhanced by aggregation.

Concanavalin A Binding Studies. Concanavalin A, type VI, was purchased from Sigma (Lot 105H9567) and used as is. Water used for FCS studies was distilled twice following deionization. $MnCl_2$ and $CaCl_2$ were purchased from Sigma, molecular biology grade. The buffer was prepared with 0.05 M PIPES (pH 7.2), 10 mM $MnCl_2$, 10 mM $CaCl_2$, and 1 M NaCl. Stock solutions of glucosides **1** and **2** were prepared at 0.24 and 0.12 mM in the above buffer by first dissolving in 100 μL of ethanol and then diluting with buffer to 10 mL. These

samples were warmed to 45 °C prior to usage to ensure complete solvation. A 0.5 mM stock solution of Con A was prepared in each buffer and verified by comparison to the reported $\epsilon = 1.14$ cm^2/mg at 280 nm.[32] Samples were filtered through a Cameo 25 GAS syringe filter immediately prior to use to remove any interfering particles. The stability of Con A to the conditions was verified by electrophoresis upon completion of the measurements.

FCS studies were conducted by excitation with the 457 nm line of an argon laser (Lexel Argon Ion, Waldbroon, Germany) at 0.5 mW which was focused through a water-immersion microscope objective (Zeiss Plan Neofluar 40 × 0.9), providing a volume element of approximately 2×10^{-16} L. Fluorescent molecules were excited for 0.1−50 ms as they passed through this volume element, as given by diffusion coefficients. The fluorescence was collected by the same objective, and light scattering was blocked with a dichroic mirror and passed through a 545 cutoff filter (Omega Optics) and a pinhole in image space. Fluorescence was detected by a SPAD (EG & G-Chemie, Steinheim, Germany), and signal autocorrelation was carried out by a PC with a digital autocorrelator card (ALV-5000 Fa. Peters, Langen, Germany).

(32) Wang, J. L.; Becker, J. W.; Reeke, J. N.; Edelman, G. M. *Proc. Natl. Acad. Sci. U.S.A.* **1971**, *68*, 1130.

Samples containing 51 μM **1** and 12 μM **2** were prepared with a gradient of Con A from 0 μM, to 0.5 μM, 5.0 μM to 50 mM to 170 μM. These samples were stored at room temperature for 12 h prior to measurement. A single droplet of these solutions (∼20 μL) was hung from fresh microscope slides and its fluorescence observed over 30 s. Repetition with three different preparations provided intensities of fluctuation within 3% of the original run. Signals due to aggregates were detected in a solution with 50 μM **1** and 170 μM Con A. Signals were not detected in samples of **2** with 170 μM Con A nor in the presence of 1 mM Con A.

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