

Design and Evaluation of a Tightly Binding Fluorescent Ligand for Influenza A Hemagglutinin

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Abstract: Attachment of influenza virus to susceptible cells is mediated by the viral protein hemagglutinin, which recognizes cell-membrane-bound glycoconjugates that terminate in α -sialosides. We have synthesized a fluorescent α -sialoside that has the highest affinity of any reported monovalent ligand for hemagglutinin, and it is not a substrate for the viral neuraminidase. This α -sialoside provides a convenient fluorescence competition assay for the binding of other ligands. Since each of the currently used binding assays has significant disadvantages, such a simple assay is of great importance for the study of potential inhibitors of viral attachment.

Introduction

Influenza is a common and widespread disease of humans, against which no satisfactory drug has yet been developed. This lack is surprising, considering the extensive scrutiny to which the influenza virus has been subjected, that has led to the X-ray crystal structure determination of both the viral surface proteins hemagglutinin (HA)^{1,2} and neuraminidase (NA)³ and to a profound understanding of the virus life cycle.⁴ One step in this cycle which is interesting from the standpoint of inhibitor design is the initial adsorption of virus particles to the host cell membrane. This attachment is achieved by specific interactions between viral HA and cell-surface glycoproteins and glycolipids that terminate in α -sialosides.⁵ Molecules with high affinity for the sialoside-binding site of HA could interfere with this initial attachment process and thus prevent or limit influenza infection.⁶ Compared to the natural immune defense system, this approach might have an important advantage. Since antibodies recognize epitopes that are large compared to the receptor binding site of HA, the virus can easily prevent binding of an antibody by point mutations at sites somewhat removed from the sialoside-binding locus. However, it should be more difficult for the virus to prevent binding of a small molecule that binds only to a functional region, such as the sialoside-binding site, because point mutations within this site will necessarily also affect the binding of the natural ligand.

One prerequisite for evaluating and developing competitive inhibitors is, of course, the availability of an assay to measure the strength of the binding interaction. Unfortunately, all of the commonly used binding assays have serious disadvantages. These assays either measure the inhibition of the association of virus particles and polyvalent ligands⁷ (e.g., erythrocytes in the hemagglutination inhibition assay) or follow ligand binding directly by NMR spectroscopy.⁸ Because of the multivalent character of the interaction between virus particles and erythrocytes, a complex kinetic competition between the inhibitor and the polyvalent ligand, rather than a thermodynamic dissociation constant, is measured. In the NMR assay, although the binding of ligands is measured directly and true equilibrium constants are obtained, relatively large quantities of protein and extensive use of a high-field NMR spectrometer are required.

Fluorescence spectroscopy is a powerful method for studying the interactions between proteins and their ligands.⁹ Binding can be followed by changes in fluorescence intensity or fluorescence polarization or, if the affinity is very high, by fluorescence energy transfer. Studies of the fluorescence of the tryptophan residues of HA in the presence and absence of ligand did not, however, show significant differences in fluorescence intensity. It was therefore necessary to introduce a fluorescent reporter group into the ligand molecule. Since only small quantities of protein can be used, the binding of a fluorescent ligand to HA should be tight, in order to maximize any observable fluorescence change. Un-

fortunately, the binding affinity of paradigmatic monovalent sialosides such as α (2,6)-sialyllactose is very weak ($K_D = 2.1$ mM).⁸ However, Toogood et al.¹⁰ have reported recently that the binding affinity of α -sialosides can be increased by introducing an aromatic group into the aglycon. We have exploited this observation in the design of the tight-binding fluorescent ligand **1a**, which contains the (6-((naphthylmethyl)amino)carbonyl)hexyl aglycon. On the basis of the X-ray crystal structure of HA complexed with α (2,6)-sialyllactose, we decided to attach the fluorophore at the 4-position of the α -sialoside. In this complex, the 4-hydroxy group projects out of the binding pocket into solution, and we reasoned that substitution on this hydroxyl group would not interfere with the binding to HA. In contrast to HA, the other surface protein of the influenza virus, NA, which catalyzes the hydrolysis of the glycosidic bond in α -sialosides, binds sialic acid with the 4-hydroxy group buried in the protein. Although this crystal structure was solved with NA from influenza B and this protein shows only ~30% sequence similarity to NA from influenza A, we expected that substitution at the 4-position would still prevent binding to NA and result in species resistance to NA cleavage. We chose the dansyl group as the fluorophore for three reasons: Its excitation maximum differs sufficiently from the tryptophan absorption of proteins, its fluorescence intensity depends on the polarity of the environment, and its fluorescence

(1) The following abbreviations are used: HA, hemagglutinin; NA, neuraminidase; BHA, bromelain-released HA; BOP, (benzotriazol-1-yloxy)tris-(dimethylamino)phosphonium hexafluorophosphate.

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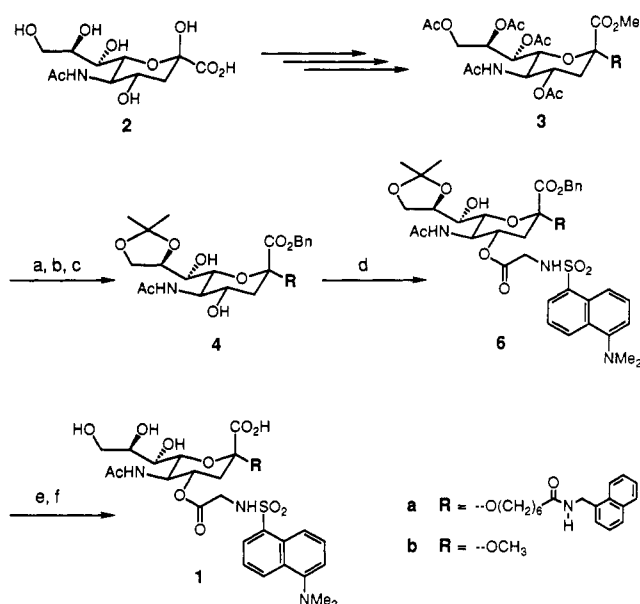
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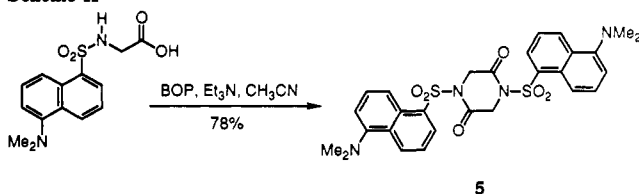
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Scheme I. Synthesis of Fluorescent α -Sialosides^a

Scheme II



lifetime makes it suitable for fluorescence polarization studies. To ensure a stable linkage to the 4-hydroxy group under assay conditions, as well as to provide some conformational flexibility for the large dansyl group, a glycine linker was introduced between the dansyl group and the α -sialoside.

Results and Discussion

Synthesis of Fluorescent Ligands. The synthesis of the fluorescent ligand **1a** and of its α -methyl derivative **1b**, which serves as a control compound, is outlined in Scheme I. Isolation of sialic acid (**2**) from birds' nest substance,¹¹ esterification, and protection of the hydroxy groups¹² are steps well described in the literature. The (6-(((naphthylmethyl)amino)carbonyl)hexyl) fragment (the aglycone of **1a**) was introduced according to Toogood et al.¹⁰ to give the α -sialoside **3a**. Subsequent removal of the acetyl protecting groups was accomplished with sodium methoxide in methanol. The methyl ester was converted into the benzyl ester, and the 8- and 9-hydroxy groups were protected as the acetonide to yield **4a**. Selective modification of the 4-hydroxy group of 8,9-protected sialosides in the presence of the unprotected 7-hydroxy group is described in the literature,¹³ and reaction of **4a** with 1 equiv of the piperazinedione derivative **5** (Scheme II) and triethylamine gave the product **6a**. Simply stirring a solution of dansylglycine, the BOP reagent, and triethylamine in acetonitrile

Table I. Comparison of Binding and Inhibition Constants

	K_i by hemagglutination inhibition (μM)	K_D by fluorescence polarization titration (μM)	K_D by NMR titration (μM)
1a	2.5–5.0	3.7 ± 0.6	
1b	300		
7	45 ^a	47 ± 24	
8	2500 ^b	2900 ± 1200	2800 ± 300^c
10	350 ^a	262 ± 75	$600\text{--}1400^d$
11	175 ^a	131 ± 49	
12	90 ^a	83 ± 47	180 ^e

^a From Toogood et al.¹⁰ ^b From Glick et al.²⁴ ^c From Sauter et al.⁸

^d Nicholas Sauter, personal communication. ^e Peter Toogood and Michael Eisen, personal communication.

produced the anhydride **5** as a precipitate that can be directly recrystallized from the reaction mixture (Scheme II). This activated form of dansylglycine reacts readily with alcohols under mildly basic conditions and is a useful reagent to label alcohols with the fluorescent dansyl group. The position of the dansylglycyl group in compound **6a** is readily confirmed by the large downfield shift of the ¹H NMR signal for H-4 as compared to the H-4 signal in the starting material **4a**.¹⁴ Hydrolysis of the acetonide protecting group and removal of the benzyl ester group by catalytic hydrogenation provided the target sialoside **1a**. The α -methyl derivative **1b** was synthesized analogously.

Binding of the Fluorescent Ligands to HA and NA. Binding of the fluorescent ligand **1a** to HA was measured by direct titration with bromelain-released hemagglutinin (BHA), which is a soluble form of HA that lacks the C-terminal anchoring peptide.¹⁵ Since only a small change in fluorescence intensity (less than 20%) was observed, the binding was followed by fluorescence polarization. If a fluorophore is excited with linear polarized light and the fluorescence lifetime is large compared to the rotational relaxation time of the fluorophore in solution, the emitted light will be almost completely depolarized. If the fluorophore binds to a macromolecule, however, the longer relaxation time results in a higher fluorescence polarization. From the increase in fluorescence polarization with increasing protein concentration, a dissociation constant of $3.7 \mu\text{M}$ for **1a** was obtained, which is in good agreement with that derived from the hemagglutination inhibition assay (Table I). This α -sialoside has the highest affinity for HA of any reported monovalent ligand. However, it is not known how tightly an effective inhibitor must bind in order to inhibit viral attachment in vivo. In contrast to the titration of the fluorescent α -sialoside **1a**, addition of BHA to the fluorescent α -methyl derivative **1b** results in only a very small increase in fluorescence polarization. This observation corresponds to the much lower affinity of **1b** to HA as measured by the hemagglutination inhibition. Binding specificity of the fluorescent ligand **1a** for the receptor binding site in HA is established by the fact that a range of other α -sialosides clearly competes for the sialoside-binding site of HA (see below).¹⁶

The presence of the dansylglycyl group in compounds **1a** and **1b** leads to a 10-fold increase in binding affinity compared to those of the α -sialosides **7** and **8** (Figure 1), each of which lacks the dansylglycyl fluorophore. Two phenomena may contribute to this observation. On the one hand, binding affinity could simply be a consequence of the more hydrophobic character of **1a** and **1b**, as has been observed with other carbohydrates binding to lectins.¹⁸ On the other hand, the dansylglycyl group might adopt a con-

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(16) Although recent crystallographic studies¹⁷ have located a second binding site in HA of unknown biological function, the 4-hydroxy group of the ligand bound in this second site forms a complex network of polar contacts with the protein, and it is very unlikely that **1a** can bind there.

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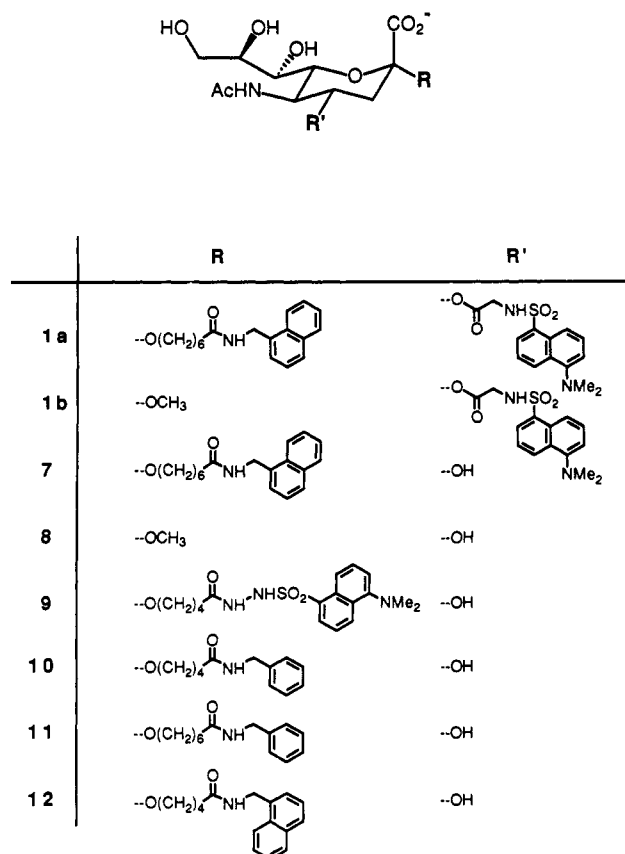


Figure 1. Structures of monovalent α -sialosides.

formation in which it makes specific contacts with the protein. The nature of these interactions may be evident from protein crystallographic studies, and work to obtain structural information of this protein-ligand complex is in progress.

Viral NA does not cleave the glycosidic bond of the 4-substituted α -sialosides **1a** and **1b**. No cleavage products could be detected even after 24 h of incubation of either sialoside with virus-bound NA. Under the same conditions, the α -sialoside **9**, which has the dansyl group in the aglycon and a free hydroxyl group at the 4-position, is completely hydrolyzed after 2 h. Cleavage of compound **9** is fully inhibited by the presence of the NA inhibitor, 2-deoxy-2,3-didehydro-*N*-acetylneuraminic acid.¹⁹ α -Sialosides that are acetylated in the 4-position are almost completely resistant to cleavage by influenza NA,²⁰ and it appears reasonable that the larger dansylglycyl group leads to complete resistance to hydrolysis of the α -sialosides **1a** and **1b**. Substitution at the 4-position is evidently a simple way of protecting potential inhibitors from NA-mediated cleavage.

Binding Assay by Competitive Fluorescence Polarization Titration. Nonfluorescent ligands can compete with the fluorescent ligand **1a** for the sialoside-binding site of HA, and this competition of two ligands for one site can be used to determine the binding constant of one ligand if the binding constant for the other ligand is known. In the present case, binding of the fluorescent ligand **1a** can be followed by fluorescence polarization spectroscopy. Typical displacement titration curves are shown in Figure 2. The fluorescence polarization obtained upon binding of the fluorescent ligand **1a** to HA falls as the concentration of the nonfluorescent ligand increases. This depolarization results from the displacement of **1a** from the receptor binding site of HA. To obtain the dis-

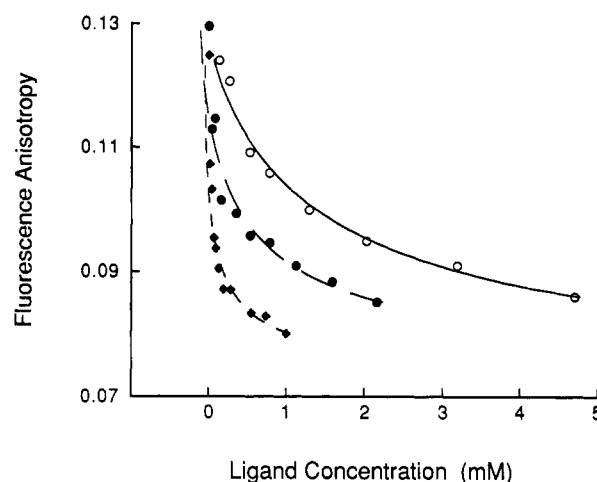


Figure 2. Competitive fluorescence polarization titration. To a solution of the fluorescent α -sialoside **1a** and BHA was added increasing amounts of nonfluorescent ligands: $\circ$, **10**; $\bullet$, **11**; $\blacklozenge$, **7**.

sociation constant K_D of the nonfluorescent ligand, the total concentration of the nonfluorescent ligand, $[L]_T$, and the observed fluorescence anisotropy, r , are fitted to eq 1.²¹ (K_I is the dis-

$$[L]_T = \frac{K_D}{K_I} \left([BHA]_T \frac{(r_B - r)}{(r - r_F)} - [I]_T \frac{(r_B - r)}{(r_B - r_F)} \right) - K_D \quad (1)$$

sociation constant of **1a**, $[BHA]_T$ and $[I]_T$ are the total concentrations of BHA and **1a**, respectively, and r_B and r_F are the values of the fluorescence anisotropy when all of the fluorescent ligand is either bound to the protein (r_B) or free in solution (r_F). In Table I the affinities of a variety of α -sialosides obtained by different assays are compared. It is clear that the dissociation constant K_D measured by fluorescence polarization accords well with the inhibition constants K_i obtained by inhibition of hemagglutination and is comparable with the dissociation constants determined by NMR titration. In a control titration, β -2-*O*-methyl-*N*-acetylneuraminic acid, the epimer of the α -methyl-sialoside **8**, was added to the assay mixture and no detectable fluorescence depolarization was observed. This result is in accord with a reported binding constant of greater than 200 mM⁸ and shows the insensitivity of the fluorescence assay toward effects other than direct competition for the receptor binding site. In another control experiment, the nonfluorescent ligand **10** was added to a mixture of the fluorescent α -methyl derivative **1b** and BHA at concentrations used in the fluorescence assay. During this titration, no significant change in the fluorescence anisotropy was observed, and it may be noted that the value of the fluorescence anisotropy corresponds to the extrapolated anisotropy r_F , when all of fluorescent ligand **1a** is free in solution. This agreement shows that the fluorescent α -methyl derivative **1b** does not bind tightly to HA and that all of the fluorescent ligand **1a** can be replaced in a competitive titration.

Conclusions

This work illustrates the design and synthesis of a tight-binding fluorescent α -sialoside for influenza virus HA. One potential problem of α -sialoside-based inhibitors can arise from inactivation by influenza virus NA. By attaching a large substituent at the 4-position of the α -sialoside, however, resistance toward NA cleavage can be incorporated into the inhibitor. This approach eliminates one possible path by which a potential α -sialoside-based

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(21) In a displacement titration, the equilibria for L and I must be considered. These equilibria are coupled by the condition that the total concentration of binding sites is conserved. Under the conditions used, it is valid to use the approximation $[L] = [L]_T$ to measure binding constants as low as 10 μ M. $[PI]$ and $[I]$ can be determined directly from the average anisotropy r , because binding of the fluorescent ligand results only in a small change in fluorescence intensity so that r becomes the sum of the anisotropies of the bound fluorophore r_B and the free fluorophore r_F , multiplied by their mole fractions. For the derivation of eq 1, see the supplementary material.

inhibitor can be inactivated in vivo. In addition, the fluorescent ligand provides a simple assay to measure binding constants for the binding of other (nonfluorescent) ligands to HA. For this assay, less than 0.5 mg of BHA is required, which is about one-fifth the amount used in the NMR assay. Each fluorescence polarization titration can be performed in less than 1 h, which is much faster than the NMR assay. Because of the nature of a displacement assay, only binding to the receptor site is measured, and meaningful structure-function relationships can thus be inferred.

Experimental Section

Materials. The α -sialosides **3a**, **7**, **9**, **10**, **11**, and **12** have been described earlier.¹⁰ The synthesis of α -sialoside **3b** has also been reported.²² α -2-O-methyl-N-acetylneuraminic acid (**8**)²³ and β -2-O-methyl-N-acetylneuraminic acid¹² were prepared according to literature methods. 2-Deoxy-2,3-didehydro-N-acetylneuraminic acid was obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN). Dichloromethane, acetonitrile, and triethylamine were distilled from CaH₂ under N₂. Anhydrous methanol was obtained from Mallinckrodt Inc. (Paris, KY). Dansylglycine and Dowex 50W (H⁺ form; 8% cross-linked) were purchased from Sigma Chemical Co. (St. Louis, MO). All other reagents were purchased from Aldrich Chemical Co., Inc. (Milwaukee, WI). Influenza virus X-31²⁵ (H3N2) and isolated BHA¹⁵ were gifts from Dr. J. J. Skehel. NA impurities in the BHA preparation were removed by chromatography on an anti-neuraminidase antibody column.⁸ Chicken erythrocytes were from SPAFAS Inc. (Norwich, CT).

Synthesis. A. Methods. Proton nuclear magnetic resonance (¹H NMR) spectra were recorded on Bruker AM 400- or 500-MHz spectrometers. All NMR spectra were measured at ambient probe temperature using CHCl₃ (δ = 7.24), H₂O (δ = 4.80), or CD₂OD (δ = 3.35) as internal reference. Mass spectra (MS) and high-resolution mass spectra (HRMS) were measured by fast atom bombardment (FAB) at the Harvard University Chemistry Department Mass Spectrometry Facility. For thin-layer chromatography, Kieselgel 60 F₂₅₄ plates from Merck (Darmstadt, Germany) were used. Silica gel 60 for column chromatography was obtained from EM Science (Gibbstown, NJ). High-performance liquid chromatography (HPLC) was performed with two Waters Associates (Milford, MA) Model 510 pumps, a Model 680 gradient controller, and a Model 494 absorbance detector. For reverse-phase HPLC, a Waters μ Bondapak column (10 μ m, 19 $\times$ 300 mm) was used.

B. Methyl [(6'-(((Naphthylmethyl)amino)carbonyl)hexyl) 5-Acetamido-3,5-dideoxy-D-glycero- α -D-galacto-nonulopyranosid]onate. The fully protected α -sialoside **3a**¹⁰ (669 mg, 886 μ mol) was dissolved in anhydrous MeOH, and sodium methoxide (95 mg, 1.75 mmol) was added. The solution was stirred under Ar at room temperature for 30 min. The reaction mixture was neutralized with Dowex 50W (H⁺ form). After filtration of the reaction mixture the solvent was removed under reduced pressure to give the product (520 mg, 99%) as a clear glass that was used without further purification (*R*_f 0.53, 20% MeOH in CH₂Cl₂): ¹H NMR (400 MHz, CD₃OD) δ 8.09 (dd, 1 H, *J* = 1.6, 7.5 Hz, naphthyl-H), 7.93 (dd, 1 H, *J* = 2.5, 6.9 Hz, naphthyl-H), 7.86 (dd, 1 H, *J* = 2.0, 7.3 Hz, naphthyl-H), 7.60–7.52 (m, 2 H, naphthyl-H), 7.52–7.45 (m, 2 H, naphthyl-H), 4.87 (s, 2 H, CH₂naphthyl), 3.87 (s, 3 H, CO₂Me), 3.91–3.83 (m, 2 H), 3.82–3.75 (m, 2 H), 3.71–3.63 (m, 2 H), 3.58 (dd, 1 H, *J* = 1.6, 10.4 Hz), 3.54 (dd, 1 H, *J* = 1.6, 8.6 Hz), 3.39–3.32 (m, 1 H), 2.71 (dd, 1 H, *J* = 4.6, 12.6 Hz, 3-H_{ax}), 2.27 (t, 2 H, *J* = 7.4 Hz, 6'-H), 2.04 (s, 3 H, NAc), 1.76 (t, 1 H, *J* = 12.6 Hz, 3-H_{ax}), 1.70–1.62 (m, 2 H), 1.55–1.47 (m, 2 H), 1.40–1.29 (m, 4 H). HRMS (FAB, 3-nitrobenzyl alcohol) calculated for [M + H]⁺, 591.2917; found (*m/z*), 591.2923.

C. Benzyl [(6'-(((Naphthylmethyl)amino)carbonyl)hexyl) 5-Acetamido-3,5-dideoxy-D-glycero- α -D-galacto-nonulopyranosid]onate. Methyl [(6'-(((naphthylmethyl)amino)carbonyl)hexyl) 5-acetamido-3,5-dideoxy-D-glycero- α -D-galacto-nonulopyranosid]onate (520 mg, 880 μ mol) was dissolved in anhydrous benzyl alcohol (25 mL), and potassium *tert*-butoxide (33 mg, 290 μ mol) was added. The solution was stirred under Ar at room temperature for 6 h. The reaction mixture was neutralized with acetic acid and chromatographed on silica gel (eluting with

50% ether in hexane followed by 12% MeOH in CH₂Cl₂) to give the product (467 mg, 79%) as a clear glass (*R*_f 0.17, 10% MeOH in CH₂Cl₂): ¹H NMR (500 MHz, CD₃OD) δ 8.09 (d, 1 H, *J* = 8.2 Hz, naphthyl-H), 7.93 (d, 1 H, *J* = 7.6 Hz, naphthyl-H), 7.85 (d, 1 H, *J* = 7.7 Hz, naphthyl-H), 7.59–7.34 (m, 9 H, aryl-H), 5.22 (s, 2 H, CH₂phenyl), 4.88 (s, 2 H, CH₂naphthyl), 3.91–3.83 (m, 2 H), 3.78 (q, 1 H, *J* = 10.3 Hz), 3.74–3.61 (m, 4 H), 3.55 (d, 1 H, *J* = 8.6 Hz), 3.22 (q, 1 H, *J* = 7.0 Hz), 2.74 (dd, 1 H, *J* = 4.5, 12.5 Hz, 3-H_{ax}), 2.25 (t, 2 H, *J* = 7.4 Hz, 6'-H), 2.04 (s, 3 H, NAc), 1.77 (t, 1 H, *J* = 12.5 Hz, 3-H_{ax}), 1.66–1.58 (m, 2 H), 1.47–1.38 (m, 2 H), 1.32–1.20 (m, 4 H). HRMS (FAB, glycerol) calculated for [M + Na]⁺, 689.3050; found (*m/z*), 689.3088.

D. Benzyl [(6'-(((Naphthylmethyl)amino)carbonyl)hexyl) 5-Acetamido-3,5-dideoxy-8,9-O-isopropylidene-D-glycero- α -galacto-nonulopyranosid]onate (4a). Benzyl [(6'-(((naphthylmethyl)amino)carbonyl)hexyl) 5-acetamido-3,5-dideoxy-D-glycero- α -D-galacto-nonulopyranosid]onate (450 mg, 675 μ mol) was dissolved in dry CH₃CN (27 mL), and 2,2-dimethoxypropane (1.14 mL, 9.3 mmol) and Dowex 50W (H⁺ form) were added. The reaction mixture was stirred under Ar at room temperature for 30 min. Dowex was removed by filtration, and the solvent was evaporated under reduced pressure to yield compound **4a** (463 mg, 97%) as a white foam that was used without further purification (*R*_f 0.37, 10% MeOH in CH₂Cl₂): ¹H NMR (500 MHz, CDCl₃) δ 7.98 (dd, 1 H, *J* = 0.9, 8.3 Hz, naphthyl-H), 7.85 (dd, 1 H, *J* = 1.6, 7.8 Hz, naphthyl-H), 7.78 (dd, 1 H, *J* = 3.3, 6.2 Hz, naphthyl-H), 7.54–7.46 (m, 2 H, naphthyl-H), 7.43–7.38 (m, 2 H, naphthyl-H), 7.36–7.26 (m, 5 H, phenyl-H), 5.91 (br t, 1 H, *J* = 5.2 Hz, NH), 5.76 (d, 1 H, *J* = 8.1 Hz, NH), 5.17 and 5.15 (AB spectrum, 2 H, *J* = 12.1 Hz, CH₂phenyl), 4.85 (d, 2 H, *J* = 5.2 Hz, CH₂naphthyl), 4.16 (ddd = q, 1 H, *J* = 6.1 Hz, 8-H), 4.03 and 4.00 (AB part of ABM spectrum, 2 H, *J*_{AB} = 8.4 Hz, *J*_{AM} = 6.2 Hz, *J*_{BM} = 6.6 Hz, 9-H and 9'-H), 3.80 (ddd = dt, 1 H, *J* = 8.1, 10.2 Hz, 5-H), 3.66 (dt, 1 H, *J* = 9.1, 6.5 Hz, 1'-H), 3.63–3.55 (m, 2 H, 4-H and 7-H), 3.46 (dd, 1 H, *J* = 1.2, 10.2 Hz, 6-H), 3.25 (dt, 1 H, *J* = 9.1, 6.4 Hz, 1'-H), 2.66 (dd, 1 H, *J* = 4.8, 13.0 Hz, 3-H_{ax}), 2.12 (t, 2 H, *J* = 7.7 Hz, 6'-H), 1.96 (s, 3 H, NAc), 1.78 (dd, 1 H, *J* = 10.8, 13.0 Hz, 3-H_{ax}), 1.63–1.54 (m, 2 H), 1.45–1.38 (m, 2 H), 1.33 (s, 3 H, isopropylidene-Me), 1.28–1.18 (m, 7 H). FAB MS (3-nitrobenzyl alcohol, NaI added) (*m/z*): 729 (100%) [M + Na]⁺.

E. Benzyl [(6'-(((Naphthylmethyl)amino)carbonyl)hexyl) 5-Acetamido-3,5-dideoxy-4-O-(N-((5-(dimethylamino)naphth-1-yl)sulfonyl)glycyl)-8,9-O-isopropylidene-D-glycero- α -D-galacto-nonulopyranosid]onate (6a). The acetamide **4a** (141 mg, 200 μ mol) was dissolved in dry CH₂Cl₂ (12 mL), and the labeling reagent **5** (58 mg, 100 μ mol), 4-(dimethylamino)pyridine (4.9 mg, 40 μ mol), and triethylamine (35 μ L, 250 μ mol) were added. The solution was stirred in the dark under Ar at room temperature for 40 h. The reaction mixture was concentrated under reduced pressure and chromatographed on silica gel (eluting with 2% MeOH in CH₂Cl₂ followed by 3% MeOH in CH₂Cl₂) to give compound **6a** (94 mg, 48%) as a green fluorescent solid (*R*_f 0.52, 10% MeOH in CH₂Cl₂): ¹H NMR (500 MHz, CDCl₃) δ 8.55 (d, 1 H, *J* = 8.5 Hz, dansyl-H), 8.25 (d, 1 H, *J* = 8.7 Hz, dansyl-H), 8.19 (dd, 1 H, *J* = 1.1, 7.3 Hz, dansyl-H), 7.98 (dd, 1 H, *J* = 0.9, 8.2 Hz, naphthyl-H), 7.84 (dd, 1 H, *J* = 1.6, 7.7 Hz, naphthyl-H), 7.78 (dd, 1 H, *J* = 2.8, 6.6 Hz, naphthyl-H), 7.56 (dd, 1 H, *J* = 7.6, 8.5 Hz, dansyl-H), 7.53–7.46 (m, 3 H), 7.40–7.37 (m, 2 H, naphthyl-H), 7.32–7.26 (m, 5 H, phenyl-H), 7.17 (d, 1 H, *J* = 7.6 Hz, dansyl-H), 5.91 (d, 1 H, *J* = 8.2 Hz, NH), 5.80 (br t, 1 H, *J* = 5.2 Hz, NH), 5.58 (br t, 1 H, *J* = 5.9 Hz, NH), 5.19 and 5.12 (AB spectrum, 2 H, *J* = 12.1 Hz, CH₂phenyl), 5.00 (ddd = dt, 1 H, *J* = 5.0, 11.0 Hz, 4-H), 4.86 (d, 2 H, *J* = 5.2 Hz, CH₂naphthyl), 4.17 (d, 1 H, *J* = 5.1 Hz, 7-OH), 4.12 (ddd = q, 1 H, *J* = 5.9 Hz, 8-H), 4.02 and 3.96 (AB part of ABM spectrum, 2 H, *J*_{AB} = 8.4 Hz, *J*_{AM} = 6.1 Hz, *J*_{BM} = 6.6 Hz, 9-H and 9'-H), 3.94 (ddd = dt, 1 H, *J* = 8.2, 10.4 Hz, 5-H), 3.71 and 3.61 (AB part of ABM spectrum, 2 H, *J*_{AB} = 18.0 Hz, *J*_{AM} = 6.7 Hz, *J*_{BM} = 5.1 Hz, Gly-H and Gly-H'), 3.62 (m, 1 H, 1'-H), 3.55 (ddd = br t, 1 H, *J* = 5.1 Hz, 7-H), 3.41 (dd, 1 H, *J* = 1.1, 10.4 Hz, 6-H), 3.25 (dt, 1 H, *J* = 9.0, 6.5 Hz, 1'-H), 2.86 (s, 6 H, NMe₂), 2.53 (dd, 1 H, *J* = 5.0, 12.8 Hz, 3-H_{ax}), 2.11 (t, 2 H, *J* = 7.6 Hz, 6'-H), 1.89 (s, 3 H, NAc), 1.75 (dd, 1 H, *J* = 11.0, 12.8 Hz, 3-H_{ax}), 1.61–1.54 (m, 2 H), 1.42–1.34 (m, 2 H), 1.31 (s, 3 H, isopropylidene-Me), 1.28–1.15 (m, 7 H). HRMS (FAB, 3-nitrobenzyl alcohol, NaI added) calculated for [M + Na]⁺, 1019.4088; found (*m/z*), 1019.4127.

F. Benzyl [(6'-(((Naphthylmethyl)amino)carbonyl)hexyl) 5-Acetamido-3,5-dideoxy-4-O-(N-((5-(dimethylamino)naphth-1-yl)sulfonyl)glycyl)-D-glycero- α -D-galacto-nonulopyranosid]onate. Compound **6a** (82 mg, 85 μ mol) was dissolved in aqueous acetic acid (10 mL, 80% (v/v) glacial acetic acid in H₂O) and stirred in the dark at room temperature for 40 h. The solvent was removed under reduced pressure, and the residue was chromatographed on silica gel (eluting with 5% MeOH in CH₂Cl₂ followed by 7.5% MeOH in CH₂Cl₂) to afford the product (70 mg, 89%) as a green fluorescent solid (*R*_f 0.30, 10% MeOH in CH₂Cl₂): ¹H NMR (500 MHz, CDCl₃) δ 8.54 (d, 1 H, *J* = 8.5 Hz, dansyl-H),

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8.22 (d, 1 H, $J = 8.6$ Hz, dansyl-H), 8.17 (dd, 1 H, $J = 1.1, 7.3$ Hz, dansyl-H), 7.98 (dd, 1 H, $J = 0.8, 8.1$ Hz, naphthyl-H), 7.84 (dd, 1 H, $J = 1.5, 7.8$ Hz, naphthyl-H), 7.78 (dd, 1 H, $J = 2.4, 7.0$ Hz, naphthyl-H), 7.56 (dd, 1 H, $J = 7.6, 8.6$ Hz, dansyl-H), 7.53–7.45 (m, 3 H), 7.42–7.37 (m, 2 H, naphthyl-H), 7.35–7.28 (m, 5 H, phenyl-H), 7.17 (d, 1 H, $J = 7.6$ Hz, dansyl-H), 6.28 (d, 1 H, $J = 8.0$ Hz, NH), 5.88 (br t, 1 H, $J = 5.2$ Hz, NH), 5.57 (br s, 1 H), 5.26 and 5.20 (AB spectrum, 2 H, $J = 12.0$ Hz, CH_2phenyl), 4.95 (ddd, 1 H, $J = 4.9, 10.6, 11.9$ Hz, 4-H), 4.86 and 4.85 (AB part of ABM spectrum, 2 H, $J_{AB} = 14.5$ Hz, $J_{AM} = J_{BM} = 5.2$ Hz, $\text{CH}_2\text{naphthyl}$), 4.72 (d, 1 H, $J = 3.9$ Hz, OH), 3.96–3.80 (m, 3 H), 3.71–3.57 (m, 5 H), 3.50–3.43 (m, 2 H), 3.09 (dt, 1 H, $J = 8.9, 6.5$ Hz), 2.85 (s, 6 H, NMe_2), 2.65 (dd, 1 H, $J = 4.9, 12.6$ Hz, 3- H_{ax}), 2.53 (br s, 1 H, OH), 2.12 (t, 2 H, $J = 7.6$ Hz, 6'-H), 1.94 (s, 3 H, NAc), 1.79 (t, 1 H, $J = 12.6$ Hz, 3- H_{ax}), 1.62–1.54 (m, 2 H), 1.43–1.33 (m, 2 H), 1.25–1.12 (m, 4 H). HRMS (FAB, glycerol) calculated for $[\text{M} + \text{H}]^+$, 957.3955; found (m/z), 957.3936.

G. (6'-((Naphthylmethyl)amino)carbonyl)hexyl 5-Acetamido-3,5-dideoxy-4-*O*-(*N*-((5-(dimethylamino)naphth-1-yl)sulfonyl)glycyl)-*D*-glycero- α -*D*-galacto-nonulopyranosidonic Acid (**1a**). Benzyl [(6'-((naphthylmethyl)amino)carbonyl)hexyl] 5-acetamido-3,5-dideoxy-4-*O*-(*N*-((5-(dimethylamino)naphth-1-yl)sulfonyl)glycyl)-*D*-glycero- α -*D*-galacto-nonulopyranosidionate (58 mg, 63 μmol) was dissolved in MeOH (10 mL), and 10% Pd/C (23 mg) was added. The reaction mixture was stirred under 1 atm of H_2 in the dark at room temperature for 2.5 h and then filtered through Celite. The solvent was removed under reduced pressure to give a green fluorescent solid, which was purified by reverse-phase HPLC chromatography (eluting with 40% CH_3CN in aqueous trifluoroacetic acid (0.01%) at a flow rate of 10 mL min^{-1}). The solvent was removed under reduced pressure, and the resulting solid material was dissolved in MeOH (7.5 mL). H_2O (75 mL) was added and the solution lyophilized overnight to afford the fluorescent α -sialoside **1a** (39 mg, 50%): ^1H NMR (500 MHz, CD_3OD) δ 8.60 (d, 1 H, $J = 8.6$ Hz, dansyl-H), 8.37 (d, 1 H, $J = 8.6$ Hz, dansyl-H), 8.22 (dd, 1 H, $J = 1.1, 7.3$ Hz, dansyl-H), 8.08 (d, 1 H, $J = 8.3$ Hz, naphthyl-H), 7.91 (dd, 1 H, $J = 1.5, 7.9$ Hz, naphthyl-H), 7.84 (d, 1 H, $J = 7.8$ Hz, naphthyl-H), 7.65–7.44 (m, 6 H), 7.31 (d, 1 H, $J = 7.5$ Hz, dansyl-H), 5.00–4.93 (m, 1 H, 4-H), 4.87 (s, 2 H, $\text{CH}_2\text{naphthyl}$), 3.93–3.81 (m, 3 H), 3.79–3.69 (m, 4 H), 3.67–3.61 (m, 1 H), 3.52 (br d, 1 H, $J = 8.9$ Hz), 3.47–3.40 (m, 1 H), 2.92 (s, 6 H, NMe_2), 2.58 (dd, 1 H, $J = 4.6, 12.2$ Hz, 3- H_{ax}), 2.28 (t, 2 H, $J = 7.4$ Hz, 6'-H), 1.93 (s, 3 H, NAc), 1.72–1.62 (m, 2 H), 1.56–1.48 (m, 2 H), 1.47 (t, 1 H, $J = 12.2$ Hz, 3- H_{ax}), 1.42–1.29 (m, 4 H). HRMS (FAB, 3-nitrobenzyl alcohol, NaI added) calculated for $[\text{M} + \text{Na}]^+$, 889.3306; found (m/z), 889.3321. Calculated for $[\text{M} + 2\text{Na} - \text{H}]^+$, 911.3125; found (m/z), 911.3138.

H. Benzyl [Methyl 5-Acetamido-3,5-dideoxy-4-*O*-(*N*-((5-(dimethylamino)naphth-1-yl)sulfonyl)glycyl)-8-*O*-isopropylidene-*D*-glycero- α -*D*-galacto-nonulopyranosidionate (**6b**). The α -methyl acetamide **4b**²² (130 mg, 287 μmol) was dissolved in dry CH_2Cl_2 (15 mL), and the labeling reagent **5** (83 mg, 140 μmol), 4-(dimethylamino)pyridine (7.0 mg, 57 μmol), and triethylamine (40 μL , 287 μmol) were added. The solution was stirred in the dark under Ar at room temperature for 40 h. The reaction mixture was concentrated under reduced pressure and chromatographed on silica gel (eluting with 2% MeOH in CH_2Cl_2) followed by 3% MeOH in CH_2Cl_2) to give compound **6b** (153 mg, 71%) as a green fluorescent solid (R_f 0.48, 10% MeOH in CH_2Cl_2): ^1H NMR (500 MHz, CDCl_3) δ 8.57 (d, 1 H, $J = 8.5$ Hz, dansyl-H), 8.27 (d, 1 H, $J = 8.6$ Hz, dansyl-H), 8.22 (dd, 1 H, $J = 1.2, 7.3$ Hz, dansyl-H), 7.59 (dd, 1 H, $J = 7.6, 8.6$ Hz, dansyl-H), 7.52 (dd, 1 H, $J = 7.3, 8.5$ Hz, dansyl-H), 7.37–7.32 (m, 5 H, phenyl-H), 7.19 (d, 1 H, $J = 7.6$ Hz, dansyl-H), 5.93 (d, 1 H, $J = 8.2$ Hz, NH), 5.60 (dd, 1 H, $J = 5.2, 6.6$ Hz, NH), 5.26 and 5.17 (AB spectrum, 2 H, $J = 12.1$ Hz, CH_2phenyl), 5.06 (ddd, 1 H, $J = 5.1, 10.6, 11.8$ Hz, 4-H), 4.18 (ddd = q, 1 H, $J = 6.1$ Hz, 8-H), 4.17 (d, 1 H, $J = 5.5$ Hz, 7-OH), 4.07 and 4.03 (AB part of ABM spectrum, 2 H, $J_{AB} = 8.4$ Hz, $J_{AM} = 5.9$ Hz, $J_{BM} = 6.6$ Hz, 9-H and 9-H'), 3.98 (ddd = dt, 1 H, $J = 8.2, 10.6$ Hz, 5-H), 3.76 and 3.66 (AB part of ABM spectrum, 2 H, $J_{AB} = 18.1$ Hz, $J_{AM} = 6.6$ Hz, $J_{BM} = 5.2$ Hz, Gly-H and Gly-H'), 3.55 (ddd = dt, 1 H, $J = 1.1, 5.5$ Hz, 7-H), 3.46 (dd, 1 H, $J = 1.1, 10.6$ Hz, 6-H), 3.31 (s, 3 H, OMe), 2.88 (s, 6 H, NMe_2), 2.52 (dd, 1 H, $J = 5.1, 12.6$ Hz, 3- H_{ax}), 1.94 (s, 3 H, NAc), 1.76 (dd, 1 H, $J = 11.8, 12.6$ Hz, 3- H_{ax}), 1.36 (s, 3 H, isopropylidene-Me), 1.28 (s, 3 H, isopropylidene-Me). HRMS (FAB, 3-nitrobenzyl alcohol, NaI added) calculated for $[\text{M} + \text{Na}]^+$, 766.2621; found (m/z), 766.2643.

I. Benzyl [Methyl 5-Acetamido-3,5-dideoxy-4-*O*-(*N*-((5-(dimethylamino)naphth-1-yl)sulfonyl)glycyl)-*D*-glycero- α -*D*-galacto-nonulopyranosidionate. Compound **6b** (125 mg, 168 μmol) was dissolved in aqueous acetic acid (15 mL, 80% (v/v) glacial acetic acid in H_2O) and stirred in the dark at room temperature for 40 h. The solvent was removed under reduced pressure, and the residue was chromatographed on silica gel (eluting with 5.5% MeOH in CH_2Cl_2) followed by 8% MeOH in CH_2Cl_2) to afford the product (108 mg, 91%) as a green fluorescent

glass (R_f 0.27, 10% MeOH in CH_2Cl_2): ^1H NMR (500 MHz, acetone- d_6) δ 8.56 (dd, 1 H, $J = 0.9, 8.5$ Hz, dansyl-H), 8.39 (d, 1 H, $J = 8.6$ Hz, dansyl-H), 8.22 (dd, 1 H, $J = 1.2, 7.3$ Hz, dansyl-H), 7.61–7.57 (m, 2 H, dansyl-H), 7.46–7.34 (m, 5 H, phenyl-H), 7.27 (d, 1 H, $J = 7.2$ Hz, dansyl-H), 7.20 (br s, 1 H), 7.15 (d, 1 H, $J = 8.5$ Hz), 5.31 and 5.27 (AB spectrum, 2 H, $J = 12.0$ Hz, CH_2phenyl), 4.93 (ddd, 1 H, $J = 5.0, 10.4, 11.8$ Hz, 4-H), 4.58 (d, 1 H, $J = 4.9$ Hz), 3.88–3.78 (m, 4 H, 5-H, Gly- CH_2 , 8-H), 3.78–3.73 (m, 1 H, 9-H), 3.62 (dd, 1 H, $J = 1.6, 10.6$ Hz, 6-H), 3.60–3.54 (m, 1 H, 9-H'), 3.48 (ddd, 1 H, $J = 1.7, 4.8, 8.8$ Hz, 7-H), 3.26 (s, 3 H, OMe), 2.87 (s, 6 H, NMe_2), 2.48 (dd, 1 H, $J = 5.0, 12.7$ Hz, 3- H_{ax}), 1.91 (s, 3 H, NAc), 1.40 (dd, 1 H, $J = 11.8, 12.7$ Hz, 3- H_{ax}). HRMS (FAB, 3-nitrobenzyl alcohol, NaI added) calculated for $[\text{M} + \text{Na}]^+$, 726.2309; found (m/z), 726.2310.

J. Methyl 5-Acetamido-3,5-dideoxy-4-*O*-(*N*-((5-(dimethylamino)naphth-1-yl)sulfonyl)glycyl)-*D*-glycero- α -*D*-galacto-nonulopyranosidonic Acid (**1b**). Benzyl [methyl 5-acetamido-3,5-dideoxy-4-*O*-(*N*-((5-(dimethylamino)naphth-1-yl)sulfonyl)glycyl)-*D*-glycero- α -*D*-galacto-nonulopyranosidionate (94 mg, 130 μmol) was dissolved in MeOH (20 mL), and 10% Pd/C (19 mg) was added. The reaction mixture was stirred under 1 atm of H_2 in the dark at room temperature for 2 h and then filtered through Celite. The solvent was removed under reduced pressure to give a green fluorescent solid, which was purified by reverse-phase HPLC chromatography (eluting with 18% CH_3CN in aqueous trifluoroacetic acid (0.01%) at a flow rate of 10 mL min^{-1}). The solvent was removed under reduced pressure, and the resulting solid material was dissolved in H_2O . The solution was lyophilized overnight to afford the fluorescent α -sialoside **1b** (54.8 mg, 67%): ^1H NMR (500 MHz, D_2O) δ 8.82 (d, 1 H, $J = 8.8$ Hz, dansyl-H), 8.50 (d, 1 H, $J = 8.8$ Hz, dansyl-H), 8.42 (dd, 1 H, $J = 0.8, 7.5$ Hz, dansyl-H), 8.16 (d, 1 H, $J = 7.9$ Hz, dansyl-H), 7.97 (dd, 1 H, $J = 7.9, 8.8$ Hz, dansyl-H), 7.94 (dd, 1 H, $J = 7.5, 8.8$ Hz, dansyl-H), 4.63 (ddd, 1 H, $J = 5.0, 10.4, 12.2$ Hz, 4-H), 4.02 and 3.97 (AB spectrum, 2 H, $J_{AB} = 18.7$ Hz, Gly-H and Gly-H'), 3.86 (dd, 1 H, $J = 2.5, 12.3$ Hz, 9-H), 3.84 (m, 1 H, 8-H), 3.70 (dd, 1 H, $J = 1.7, 10.4$ Hz, 6-H), 3.63 (dd, 1 H, $J = 6.4, 12.3$ Hz, 9-H'), 3.57 (s, 6 H, NMe_2), 3.52 (dd, 1 H, $J = 1.7, 9.2$ Hz, 7-H), 3.48 (t, 1 H, $J = 10.4$ Hz, 5-H), 3.31 (s, 3 H, OMe), 2.01 (dd, 1 H, $J = 5.0, 12.2$ Hz, 3- H_{ax}), 1.92 (s, 3 H, NAc), 0.68 (t, 1 H, $J = 12.2$ Hz, 3- H_{ax}). HRMS (FAB, 3-nitrobenzyl alcohol, NaI added) calculated for $[\text{M} + \text{Na}]^+$, 636.1839; found (m/z), 636.1837.

K. *N,N'*-bis((5-(Dimethylamino)naphth-1-yl)sulfonyl)-2,5-piperazinedione (Dansylglycine Anhydride) (**5**). Dansylglycine (308 mg, 1 mmol) was dissolved in dry CH_3CN , BOP (443 mg, 1 mmol) and triethylamine (278 μL , 2 mmol) were added, and the solution was stirred under Ar at room temperature for 8 h. The resulting precipitate was directly recrystallized from the reaction mixture to yield the labeling reagent **5** as green fluorescent needles (226 mg, 78%): ^1H NMR (300 MHz, CDCl_3) δ 8.60 (d, 1 H, $J = 8.4$ Hz, arom-H), 8.49 (dd, 1 H, $J = 1.2, 7.6$ Hz, arom-H), 7.65 (d, 1 H, $J = 8.6$ Hz, arom-H), 7.58 (dd, 1 H, $J = 7.6, 8.4$ Hz, arom-H), 7.30 (dd, 1 H, $J = 7.7, 8.6$ Hz, arom-H), 7.06 (d, 1 H, $J = 7.7$ Hz, arom-H), 4.63 (s, 2 H, Gly-H), 2.88 (s, 6 H, NMe_2). HRMS (FAB, 3-nitrobenzyl alcohol) calculated for $[\text{M}]^+$, 580.1450; found (m/z), 580.1460.

Assays A. Competitive Fluorescence Polarization Titration. To a freshly prepared solution (300 μL) of **1a** (15 μM) and BHA¹⁵ (15 μN in receptor sites) in phosphate-buffered saline were added increasing amounts of a mixture (total 100 μL) of nonfluorescent ligand (7, 4 mM; 8, 80 mM; 10, 20 mM; 11, 10 mM; 12, 4 mM), **1a** (15 μM) and BHA (15 μN) at 25 $^\circ\text{C}$. Fluorescence polarization measurements were performed on a Perkin Elmer LS 50 luminescence spectrometer equipped with polarizers using a dual path length quartz cell (10 $\times$ 2 mm) from NSG Precision Cells Inc. (Farmingdale, NY). The fluorophore was excited with vertical polarized light at 340 nm (excitation slit width 5 nm), and the intensity of the emitted light was observed through vertical (V_V) and horizontal (V_H) polarizers at 546 nm (emission slit width 10 nm). The correction factor G was determined by excitation (340 nm) with horizontal polarized light and observation (546 nm) of the emitted light through vertical (H_V) and horizontal (H_H) polarizers. The fluorescence anisotropy r was calculated from:

$$r = \frac{V_V - GV_H}{V_V + 2GV_H} \quad \text{with} \quad G = \frac{H_V}{H_H}$$

The BHA concentration was determined by UV absorption at 280 nm using an extinction coefficient of $\epsilon^{280} = 1.5 \text{ cm}^{-1} (\text{mg/mL})^{-1}$ and a molecular weight per monomer of 69 474 g mol^{-1} .⁸ The concentration of the fluorescent ligand **1a** was obtained using an extinction coefficient of $\epsilon^{248} = 13 400 \text{ cm}^{-1} \text{ M}^{-1}$. Ligand-free BHA can be recovered (90–95%) by repeated (5 $\times$) addition of buffer and concentration by ultrafiltration (Centricon 10, 3000 $\times$ g, 90 min).

B. Hemagglutination Inhibition Assay. The hemagglutination inhibition assay was performed as described previously.²⁴

C. Neuraminidase Assay. Fluorescent α -sialosides (1 mM) in phosphate-buffered saline were incubated with influenza virus X-31²⁵ (0.1 mg mL⁻¹) at 37 °C. Portions were removed after 0.25, 0.5, 1, 2, 12, and 24 h and analyzed by thin-layer chromatography on silica gel using a mixture of 2-propanol, water, and glacial acetic acid (30:8:1 by volume) as eluant.²⁶ Fluorescent compounds were visualized in the UV and had the following R_f values: **1a**, 0.71; **1b**, 0.59; **9**, 0.55; the fluorescent cleavage product of **9**, 0.79. A control experiment contained, in addition to the

α -sialosides and virus, the neuraminidase inhibitor 2-deoxy-2,3-dehydro-*N*-acetylneuraminic acid (1 mM).

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Supplementary Material Available: Derivation of eq 1 (3 pages). Ordering information is given on any current masthead page.

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Trimesoyltris(3,5-dibromosalicylate): Specificity of Reactions of a Trifunctional Acylating Agent with Hemoglobin¹

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Abstract: Trimesoyltris(3,5-dibromosalicylate)(TTDS) was prepared and evaluated as a trifunctional site-directed protein cross-linking reagent. It is synthesized by reaction of trimesoyl chloride with *tert*-butyl 3,5-dibromosalicylate, followed by deprotection with trifluoroacetic acid. TTDS reacts with the deoxy form of human hemoglobin A and with carbonmonoxyhemoglobin to produce amides from the ϵ -amino groups of Lys-82 of each of the β chains of hemoglobin. The third 3,5-dibromosalicylate ester group from TTDS reacts much more slowly, principally undergoing hydrolysis. Minor products include materials with α -chain modification and triply linked trimesoyl (β 82Lys, β 1Val, β '82Lys)hemoglobin. Comparison of these results with results from other reagents indicates that the nature of the leaving group and the structure of the acylating core control the observed specificity.

The chemical cross-linking of proteins can produce stabilized materials for use in a wide variety of applications.^{2,3} Products of the method can be complementary to those obtained with site-directed mutagenesis.⁴⁻⁷ A limitation of chemical cross-linking is its tendency to produce highly heterogeneous materials. Functional group specificity (chemoselectivity) is insufficient to give homogeneity since proteins contain many instances of the same functional group. However, reagents can be developed which have added specificity for small regions of a protein.^{8,9} Such site-directed group-specific cross-linking reagents can improve the chances of reducing heterogeneity.

Hemoglobin is an important target protein for modification, being the basis for the production of a red-cell substitute to be used in transfusions.^{4,7,10} The protein is a tetramer consisting of $\alpha\beta$ dimers. Amino groups are good targets for directing cross-linking reagents, but in the case of hemoglobin, each $\alpha\beta$ dimer has 24 primary amino groups which can react, giving diverse products unless the reagent has further specificity.¹¹⁻¹⁴ It has been observed that anionic acylating reagents show selectivity for certain amino groups within hemoglobin, particularly those located in the site which binds the polyanionic regulator 2,3-diphosphoglycerate.^{8,9,13-22} The charge of such a reagent directs it to cationic sites on the protein, consistent with the known importance of electrostatic control of association in protein binding sites.²³

In addition to electrostatic effects, selectivity and efficiency can be enhanced by taking advantage of steric effects and the presence of additional functional groups. Steric effects play an important role in determining the regioselectivity of organic reagents,²⁴ and it can be expected that such effects can increase the selectivity

of a protein reagent. The combination of charge direction, steric bulk, and multifunctionality in a single reagent should lead to

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