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Synthesis of Fluorous Tags for Incorporation of Reducing Sugars into a Quantitative Microarray Platform

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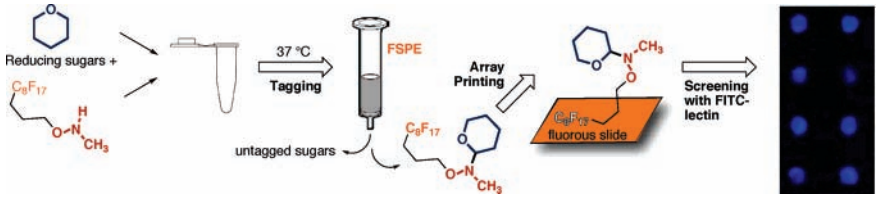
ABSTRACT



Carbohydrate microarrays can map out key interactions of carbohydrates and proteins in a high-throughput manner, but require the inclusion of a range of sugars for their optimal use. Here we present the synthesis and use of a new hydroxylamine-modified fluorous tag that allows the facile incorporation of reducing sugars into a noncovalent fluorous-based microarray after simple purification by fluorous solid-phase extraction (FSPE). The microarray supports quantitative screening against carbohydrate-binding proteins.

Carbohydrate−protein interactions are critical in many important biological events, including cell−cell communication, pathogen invasion, and inflammatory responses.[1] Plotting these carbohydrate−protein interactions using a minimal amount of material has been facilitated to a large extent by the development of carbohydrate microarrays. Multiple sugars can be screened in parallel in these chip-based approaches. The successful fabrication of carbohydrate microarrays depends on efficient and facile immobilization methods for carbohydrate probes on a proper surface. The orientation and glycosidic stereochemistry of the incorporated sugars must be consistent for reproducible results. A range of methods to immobilize sugars for preparing carbohydrate microarrays have been described to date.[2] Unfortunately, these methods require optimization of the reaction chemistry for attachment of sugars to the solid support and are not readily amenable to the quick fabrication of small arrays to ask targeted questions of a small range of isolated carbohydrates by nonexperts. Recently we demonstrated a simple method to synthesize oligosaccharides using fluorocarbon tags that also allows the direct formation of microarrays by noncovalent fluorous−fluorous interactions.[3] More recently, this microarray system has been applied by other groups to the fabrication of small molecule arrays for the discovery of histone deacetylase inhibitors[4] and protein−ligand binding interactions.[5] Our carbohydrate microarray method does not require unique reactive functional handles and multiple

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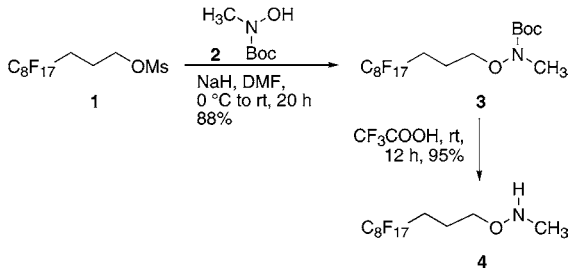
chemical steps required by covalent immobilization methods. The introduction of fluorous tags also can substantially simplify the purification of synthetic carbohydrate intermediates. Although isolation of homogeneous sugars from natural sources is challenging, some reducing sugars are available and ideally also could be interfaced easily with this carbohydrate array platform. Herein we report the design and synthesis of a fluorous tag for reaction with unprotected reducing sugars and incorporation of the tagged sugars in a fluorous microarray platform as the first step in producing a carbohydrate microarray kit for incorporation of natural sugars. We also demonstrate that the fluorous array platform with this new tag can support quantitative binding measurements.

A fluorous tag for incorporation of reducing sugars into our microarray platform would require addition of a functional group that can easily react with unmodified sugars at the reducing end and produce primarily the chain-closed version of the sugar. The conjugation method must be under mild reaction conditions and be amenable to parallel synthesis. Reactions of aminooxy or hydrazide groups with free carbohydrates are known to be chemoselective and have been widely used for the synthesis of various glycoconjugates.[6] Because formation of the hydrazide resulted in a cyclized product (see the Supporting Information), we next tried to build a fluorous tag with an aminooxy reactive group. Previously, a peptide bearing an aminooxy function was reacted with the anomeric center of a free aldose in a chemoselective way without using protecting groups[7] and neoglycolipid probes have been made by oxime ligations for microarrays.[8] After we began our studies, the functional group has also been reacted with a range of reducing sugars.[9] Although reaction of a primary-O−NH_2 group produces mostly open-chain sugar oximes, the cyclic form of the sugar is restored when a peptide with a secondary-hydroxylamino group (R−O−NH−R′) is used.[7] Preparation of the desired aminooxy-functionalized fluorous tag commenced with protection of commercially available *N*-methylhydroxylamine hydrochloride to form the known *N*-*tert*-butylcarbamate **2**[10] followed by reaction of this protected hydroxylamine with 3-(perfluorooctyl)propanyl methyl sulfonate[3b] to provide **3** (Scheme 1). Removal of the Boc protecting group with trifluoroacetic acid gave the desired fluorous tag **4**.

With the *N*,*O*-substituted hydroxylamine modified fluorous tag **4** in hand, we next experimented with the glycosylation

Scheme 1. Synthesis of Fluorous Tag



reaction conditions to produce a small library of sugars including polysaccharides and *N*-acetylamino sugars commonly found as ligands for plant lectins (Figure 1). Of

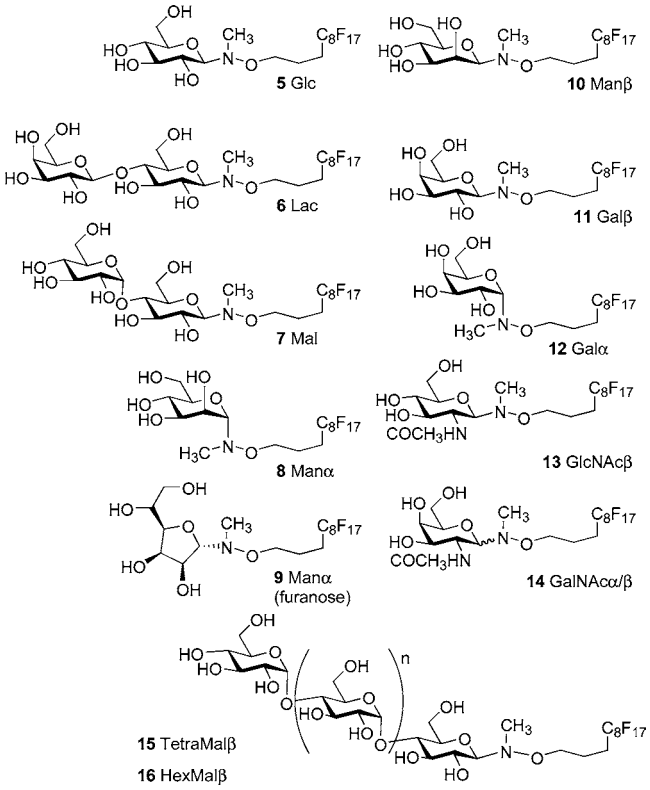


Figure 1. Fluorous-tagged sugars synthesized as targets for carbohydrate microarray production.

particular interest was testing the amount of cyclized versus open-chain form of monosaccharides formed after tagging as only the closed-chain pyranose forms will bind to a range of lectins. We also wished to find conditions to separate anomers if mixtures were formed in order to test pure compounds in our arrays and delineate if this separation was actually always necessary. The reducing sugars glucose, maltose, lactose, galactose, and mannose were mixed with the fluorous tag **4** in a polar organic solvent mixture (DMF/AcOH, v:v = 1:1). As expected, compounds **5**−**7** were obtained exclusively in the pyranose form with a typical $^4C_1$

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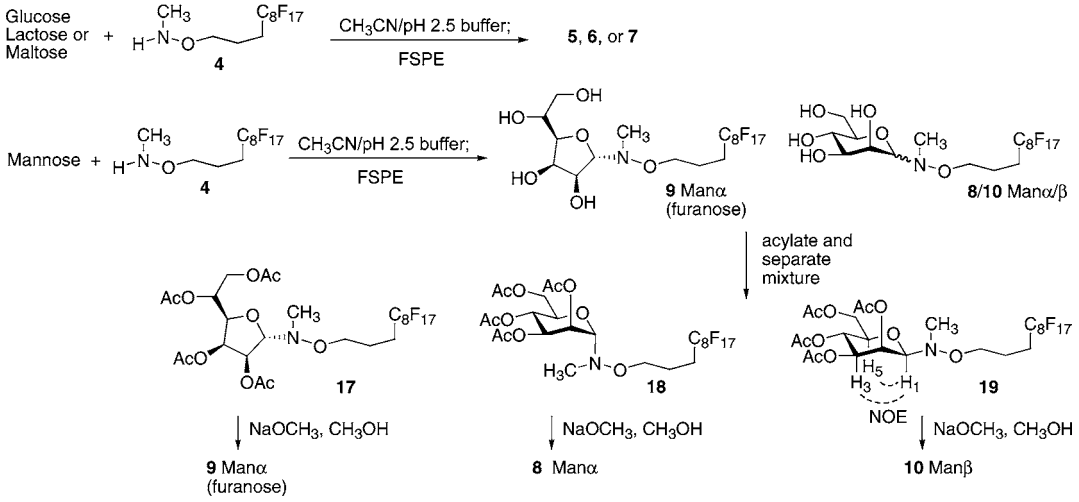
Scheme 2. Addition of Fluorous Tag to Reducing Sugars with Fluorous Solid-Phase Extraction (FSPE) and Separation of Mixtures



conformation as ascertained by ^{1}H NMR in deuterated methanol. It was noteworthy that, based on analysis of the J_{H1-H2} coupling constant values, the reactions proceed with complete diastereoselectivity; only the β-pyranose forms were observed for glucose, maltose, and lactose, which all contain D-glucose at the reducing end.

To make this technique more amenable for a simple microarray kit, we wanted to eliminate the need for dimethylformamide, a solvent that is known to decompose upon prolonged storage, as well as simplify the procedure for use only with equipment found in most molecular biology laboratories. As expected, aminoxy-functionalized fluorous tag **4** is not very water soluble and requires an organic cosolvent. Acetonitrile was chosen based on its ease of removal, miscibility with water, and stability in room temperature storage. Our glycan derivatives then were prepared in milligram scale using an aqueous/organic mixture (Scheme 2) and identified by ^{1}H NMR and ESI mass spectrometry. Briefly, representative disaccharide maltose and the fluorous tag **4** (molar ratio 1.5:1) were each separately dissolved in aqueous buffer at pH 2.5 in NaH_2PO_4/H_3PO_4 (0.7 mL) and acetonitrile (0.2 mL) (pH 3.0 after addition of CH_3CN). The two solutions were then mixed in a centrifuge tube and shaken at 37 °C. After 24 h, the reaction mixture was directly loaded on a cartridge packed with silica gel containing a perfluorooctylethylsilyl ($Si(CH_2)_2C_8F_{17}$) bonded phase for fluorous solid-phase extraction (FSPE).[11] Fluorous-tagged sugars were eluted with methanol whereas other components, including the unreacted sugar and buffer salts, were eluted first with a fluorophobic phase. Two other buffers were also assayed: pH 4 buffer (NaOAc/HOAc, 0.2 M) and pH 1.5 buffer (HCl/KCl, 0.2 M). After mixing with acetonitrile, the pH values were found to be 4.1 and 1.9. The NaH_2PO_4/H_3PO_4 buffer provided the most reliable results.

This aqueous/organic method was successfully applied to tag a range of sugars (Figure 1) and could be easily run on a larger micromolar scale. These reaction conditions worked well to quantitatively tag *N*-acetylglucosamine (Glc*N*Ac) and *N*-acetylgalactosamine (Gal*N*Ac) in 24 h. Only the β-pyranose form of the glycosylation product of Glc*N*Ac (**13**) was detected. In contrast, a 1:1 ratio of Gal*N*Ac derivative anomers (**14**) was evident from ^{1}H NMR data. For maltotetraose and maltohexaose, a molar ratio of sugar to fluorous tag of 2:1 was used to affect quantitative conversion of the fluorous tag after 36 h. In these cases also, only the β-pyranose forms **15** and **16** were detected. Unlike the glucose and glucosamine conjugations, the reactions with mannose and galactose were more complex. The ^{1}H NMR spectrum of the D-galactose derivative revealed the presence of a mixture of pyranose forms (α to β: 2 to 3, as calculated from the integration ratio of the methyl group on the hydroxylamine linker). Three forms of D-mannose derivatives were evident in the ^{1}H NMR data (ratio of 5:2:3 from less polar to more polar) and could not be readily separated by silica gel chromatography. However, acylation of the mannose and galactose reaction mixtures with acetic anhydride in pyridine resulted in easily separable acylated derivatives (Scheme 2 and Supporting Information). Because the anomeric position of mannose cannot be characterized simply by J_{H1-H2} coupling constants,[12] NOESY NMR experiments were performed for compounds **17**−**19** to establish the anomeric stereochemistry. In the NOE spectrum of **19**, cross-peaks between H_1 and H_3 as well as H_1 and H_5 were seen, but no comparable cross-peaks were found in compounds **17** and **18**. Proton identities were assigned by COSY NMR. Standard Zemplén deacylation conditions[13] readily converted the acylated compounds to their final forms for incorporation into microarrays.

With the fluorous-tagged sugars in hand, we probed the ability of the new fluorous tag to bind to a fluorous-coated

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glass slide surface and support plant lectin binding (Figure 2). Differences in binding between the pure α-/β-isomers

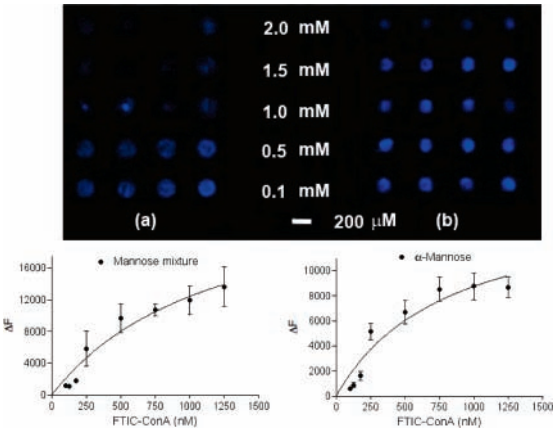

Figure 2. Two scans at 488 nm and the binding curve of glass slides arrayed with a fluorous-tagged carbohydrate mixture (a) and pure mannose **11** (b) after incubation with FITC-labeled ConA. The curve was obtained with use of FITC-ConA. The $K_{D,surf}$ values were obtained by fitting the curve to Langmuir isotherms.

and their mixture (containing the three forms of fluorous tagged mannose with the same ratio from reaction) were also of interest. The fluorous-tagged carbohydrates were dissolved in methanol/DMSO/water (2:2:1) and were spotted onto commercially available fluoroalkylsilane-derivatized glass slides[6a] employing a standard robot used for DNA array formation. The resulting slides were then incubated with FITC (fluorescein isothiocyanate)-labeled concanavalin A (jack bean, FITC-ConA). Clearly the new tag could support qualitative microarray experiments.

We next wished to test the feasibility of getting quantitative binding data of both pure α-mannose **9** and the mixture of mannose isomers **8**, **9**, and **10** to ConA. To determine a suitable sugar concentration for these quantitative measurements, control experiments were first carried out for different sugar concentrations (Figure 2). The lower sugar concentrations (0.1 M), especially for the mixture of mannose isomers, proved optimal. Higher concentrations of sugars that cannot bind to ConA (namely **9** and **10** in the mixture) then would only crowd the surface and provide a suboptimal binding surface for the protein.

The arrays of compound **8** and mannose mixture at 250 μM were then probed with eight concentrations of protein-FITC labeled ConA, ranging from 1.25 μM to 100 nM. ConA concentrations were plotted against mean fluorescence intensities of replicate spots to give a curve (Figure 2). The curves were analyzed as Langmuir isotherms, assuming that the system reached equilibrium during incubation (see the Supporting Information). $K_{D,surf}$ is found to be 1.2 ± 0.2 μM for the mannose mixture and 0.8 ± 0.1 μM for **9**. We also calculated the binding constants for the sugar mixture and **9** at different concentrations, $K_{D,surf}$ = 1.2 ± 0.2 μM for the mixture at 6.25 μM, and 0.8 ± 0.1 μM for **9** at 100 μM. These results show that the binding constants measured are not affected by sugar concentrations in a certain range. As expected, the $K_{D,surf}$ value of **9** is smaller than that of the mixture, which contains only 50% of **9**. The nonbinding components of the mixture did not significantly affect the binding constant under these conditions and therefore even mixtures of sugars obtained after conjugation can be used directly in a microarray screen after analysis of the component ratio. Compared to other mannose-ConA surface binding results with multivalent displays, our value is in excellent agreement with surface plasmon resonance (SPR) data (K_{ADS} = 5.6 ± 1.7 × 10^6 M).[14] Interestingly, a recently reported study on a carbohydrate microarray capped with a proprietary protein prior to ConA binding found K_D = 80.4 nM.[15] Clearly, because the linkers to mannose and the surfaces differ substantially between techniques, quantitative data should be compared only with those of comparable systems.

In conclusion, the new fluorous glycosylation method can directly link reducing saccharides from natural sources to a fluorous tag with retention of a cyclic structure and without the requirement of activation of the anomeric center. The tag can be synthesized in three linear steps from commercial material in 84% overall yield prior to reaction with reducing sugars and arraying on commercially available fluorous-coated glass slides. Because of the ease of the overall procedure, we envision this new tag as the basis of a simple kit aimed at glycobiologists for the fabrication of small targeted carbohydrate microarrays.

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Supporting Information Available: Experimental procedures and copies of ^{1}H, ^{13}C, and ^{1}H−^{1}H COSY NMR spectra for all new compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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