Carbohydrate Microarrays

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Synthesis and Quantitative Evaluation of *Glycero*-D-*manno*-heptose Binding to Concanavalin A by Fluorous-Tag Assistance**

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Herein we report the first use of a quantitative fluorous microarray strategy to show that the mannose-binding lectin concanavalin A (conA), contrary to prevailing belief, actually can accept modifications of the mannose at the C-6 position in the form of *glycero-manno*-heptoses found on pathogenic bacteria (Figure 1). The well-known mannose–conA interaction was reported earlier in the first demonstration of a microarray fabrication strategy based on noncovalent fluorous interactions.[1] Mannose and other monosaccharides tagged by single C_8F_{17} chains were noncovalently immobilized to fluorocarbon-coated glass slide surfaces and shown to tolerate conditions necessary for identifying binding partners such as conA. More recently, this fluorous-based microarray approach has proven valuable for the probing of other classes of small molecules.[2] In the case of histone deacetylase inhibitors with dissocation constants of less than 0.1 s^{-1}, the hits found by fluorous microarrays were comparable to those found by techniques such as surface plasmon resonance (SPR) and solution-based biochemical assays.[2a] Ideally, of course, the relative quantification of these binding interactions could also be carried out within the same fluorous microarray screening format.

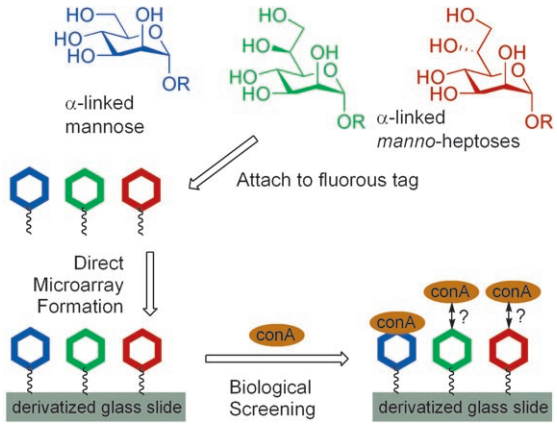

Figure 1. Structure of mannose and heptomannose, and the strategy for their incorporation into a microarray by noncovalent fluorous interactions for screening with concanavalin A.

ConA is a plant lectin that is widely used like antibodies as research tools and diagnostics to identify the presence of specific sugars, such as mannose, on cells;[3] however, in reality the sugar specificities of lectins have not been tested broadly, especially against less readily available carbohydrates. ConA is the most-studied lectin and is usually considered to bind terminal alpha-linked mannose, glucose, and N-acetylglucosamine. Earlier inhibition data suggest that modifications at the C-3, C-4, and C-6 positions of the D-mannopyranose deter binding to conA.[4] In particular, the loss of the hydroxy group in the C-6 position as in 6-deoxy-D-mannose and 1,6-anhydro-β-D-*manno*-pyranose result in complete loss of activity. To date, no studies have been realized on the addition of another hydroxymethyl group at this same position. In fact, surprisingly little is known about how seven-carbon sugars might mimic six-carbon sugars or interact with proteins.[5]

The seven-carbon sugars L-*glycero*-D-*manno*- and D-*glycero*-D-*manno*-heptose (Figure 1) are common constituents in pathogenic bacteria of lipopolysaccharides (LPS)[6] that mediate numerous responses to bacterial infections.[7] The increasing resistance of many bacterial strains against conventional antibiotics is prompting the need for a detailed understanding of the immunological responses against these outer carbohydrate coats to inform the development of new therapeutic agents such as bacterial cell wall biosynthesis inhibitors and vaccines.[6] Although humans lack *manno*-heptoses, the success of a vaccine against this bacterial sugar would presume the lack of cross-reactivity of antibodies generated against this distinct heptose antigen with the many mannose residues found in humans. As lectins are often used as antibody replacements in cellular carbohydrate-binding experiments, we reasoned that a study of the feasibility of using *manno*-heptose motifs in vaccine design should begin with assessing the ability of a well-characterized mannose-binding protein, conA, to cross-react with heptopyranoside motifs.

To test the ability of conA to bind to L-*glycero*-D-*manno*- and D-*glycero*-D-*manno*-heptoses, we first needed a route to readily access both diastereomers. Several methods have been reported.[8] To start from a less expensive six-carbon sugar, we required an approach to differentiate the alcohol at C-2 from

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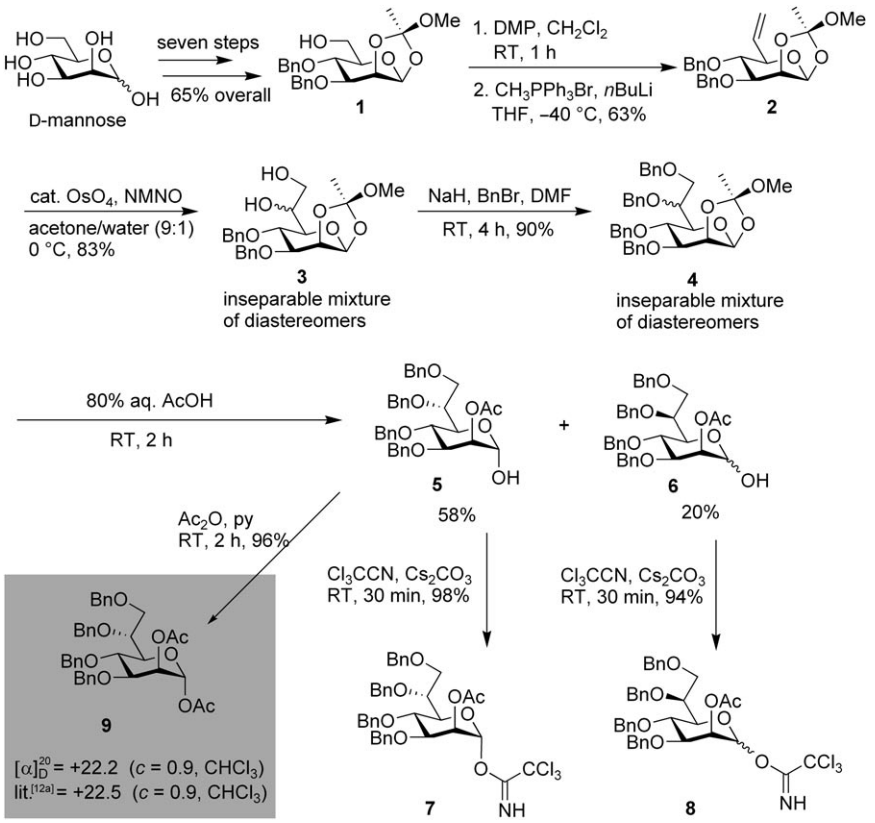
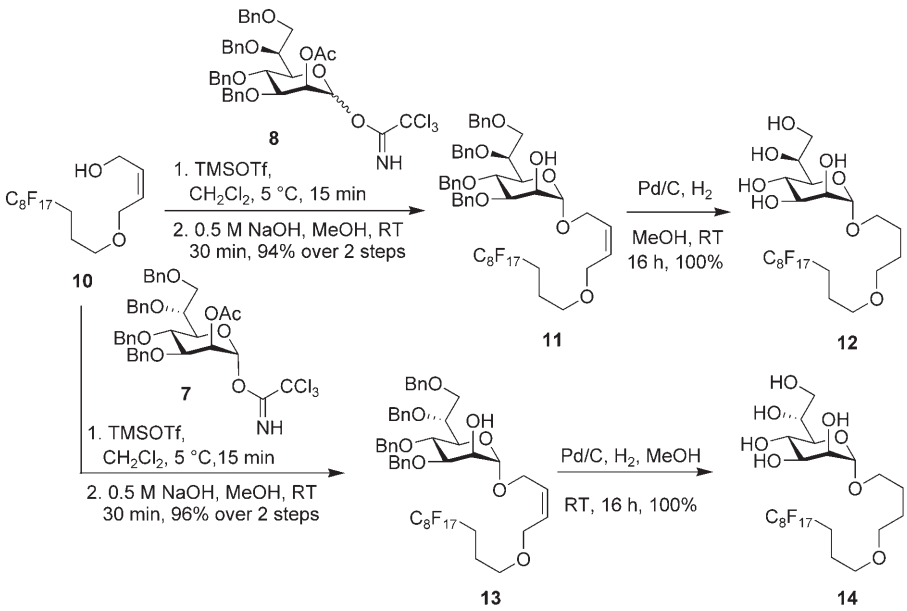
those at C-3, C-4, and C-6 to ensure alpha-selectivity in the glycosylation steps. This selectivity was accomplished by temporarily protecting C-2 as an orthoester. The next step was to protect the other positions without decomposition of the orthoester, which will be converted into a 2-acetyl group. We selected the method published by Crich and Banerjee that gives a 3-5:1 ratio of D-*glycero*-D-*manno*-heptose and L-*glycero*-D-*manno*-heptose.[8d] The requisite orthoester **1** was synthesized from D-mannose as previously reported[9] in seven steps and 65% overall yield (Scheme 1). Dess–Martin oxidation[10] provided the best yield of the desired aldehyde, which was used directly for a Wittig reaction[11] to give alkene **2** in 63% yield over two steps. Osmylation[8d] of **2** provided a 3:1 mixture of diastereomers that was predicted to favor the *R* configuration (Scheme 1) at the newly generated stereocenter, in accordance with Kishi's empirical rule.[12] To verify the stereochemical assignment of the major diastereomer, lactol **5** was converted into the known compound **9** {$[\alpha]_D^{20} = +22.2\ \mathrm{deg\,cm^3\,g^{-1}\,dm^{-1}}$ ($c = 0.9\ \mathrm{g\,cm^{-3}}$, $CHCl_3$); reported:[8a] $[\alpha]_D^{20} = +22.5\ \mathrm{deg\,cm^3\,g^{-1}\,dm^{-1}}$ ($c = 0.9\ \mathrm{g\,cm^{-3}}$, $CHCl_3$)}.



Scheme 1. Synthesis of *manno*-heptose building blocks. DMP = Dess–Martin periodinate, NMNO = *N*-methylmorpholine-*N*-oxide.



Scheme 2. Synthesis of fluorous-tagged sugars. TMSOTf = trimethylsilyl trifluoromethanesulfonate.

Finally, benzylation of the epimeric alcohols **3**, with subsequent opening of the orthoester, resulted in a separable mixture of lactols **5** and **6**. These alcohols were converted into the corresponding trichloroacetimidate donors **7** and **8**, respectively.[13] For integration into our fluorous microarray platforms,[1,14] we next needed to glycosylate the trichloroacetimidate building blocks with fluorous alcohol **10**.[14] Both the donors were individually coupled and purified on flourous solid phase extraction (FSPE) column.[15] Addition of sodium methoxide directly to the methanolic eluent produced the deacetylated fluorous-tagged heptomannose compounds (**11** and **13**) in 94–96% yields (Scheme 2). Finally, hydrogenolysis to remove the benzyl groups with concomitant reduction of the alkene in the fluorous tag to the alkane resulted in the formation of fully deprotected fluorous-tagged heptomannose compounds **12** and **14**.

With the desired compounds in hand, we could test whether fluorous-coated glass slides[1] could support the

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seven-carbon sugars for screening against conA, and for quantitative binding experiments. To this end, our fluorous-tagged heptomannose monosaccharides, dissolved in a solvent mixture, were arrayed by using a standard DNA microarray spotting robot. The spotted glass slides were incubated with a solution of fluorescein isothiocyanate-labeled concanavalin A (FITC-ConA) in phosphate-buffered saline (PBS) and scanned with a laser at 488 nm to visualize the carbohydrate–conA binding (Figure 2). As expected, the

Figure 2. Left: Fluorescence images of arrayed carbohydrates probed with FITC-labeled conA. Columns of five spots each of 125 μM carbohydrates were incubated for 2 h with FITC-ConA with 1% bovine serum albumin. Man = fluorous-tag-linked mannose. Right: Graph of average fluorescence intensities.

fluorous-tagged β-D-mannose[14] exhibits a robust fluorescent response indicating protein binding, in contrast to the negative control fluorous-tagged β-D-galactose (not shown),[14] which shows no binding. Surprisingly, both fluorous-tagged heptomannose monosaccharides show binding to conA. On the basis of fluorescence intensity, the *R* diastereomer binds comparably to the *S* diastereomer to conA. For quantification, the fluorinated sugars were spotted (2×12 spots) on the fluorinated microarray slide at a concentration of 125 μM and let dry for 30 minutes in a dark humidified chamber. The slide was then incubated for 2 hours at eight different concentrations of conA (0.1–2 μM) and washed (five to seven times) with deionized water. The relative intensities of the spots were determined with ImaGene software to calculate the K_d value for each sugar.[16] The fluorous-tagged β-D-mannose (Figure 3) bound with a value of $K_d = 1.9 \pm$

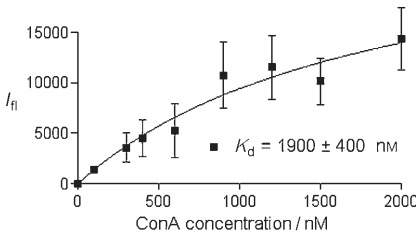

Figure 3. Binding curve of fluorous-tag-linked mannose.

0.4 μM, which is comparable to the seven-carbon sugars (*R* and *S* diastereomer: $K_d = 1.1 \pm 0.3$ μM). As observed previously,[16] the one-point threshold-based qualitative analysis (Figure 2) does not necessarily accurately reflect the relative binding strengths of even closely related substrates.

These experiments are the first demonstration that microarrays based on noncovalent fluorous interactions can be used not only for qualitative but also for the relative quantitative assessment of carbohydrate/protein interactions in a way that traditional single concentration assays with microarrays cannot. Interestingly, the values reported for mannose binding to conA with various surface-based techniques differ widely; comparisons should only be made to rank order compounds measured with the same protocol. Kiessling, Corn, and co-workers used an SPR technique with thiol-modified mannose linked to a gold surface to measure a K_d value of 200 ± 50 μM with conA.[17] The authors specifically note that the inverse of the association value (179 nM for mannose-conA) could not be used to determine the dissociation directly and that the method should be used to compare compounds only with the same technique. More recently, a microarray based on reaction of carbohydrates with an epoxy-coated glass surface and blocked with a proprietary protein solution showed an unusually strong dissociation constant of 80 nM for bound mannose and conA.[16] Clearly, the nature of the linker and surface matters and quantitative comparisons between ligands binding to a protein should be limited to systems in which only the ligands themselves are a variable.

These new results also show, for the first, time that concanavalin A can recognize seven-carbon mannose analogues as ligands. In 1965 Goldstein et al.[4] suggested that the modifications of any of the hydroxy groups at C-3, C-4, and C-6 positions of the D-mannopyranose ring resulted in a complete loss of affinity with conA. However, our study demonstrates that the substitution of one of the hydrogen atoms in the C-6 position by a hydroxymethyl group does not abrogate carbohydrate-protein binding. The presence of the second hydroxy group could perhaps even stabilize the monosaccharide in the binding site of the protein. In an X-ray crystallographic study of conA binding to α-(1→2) mannobiose, Naismith and co-workers noted the presence of a water molecule located near the C-6 hydroxy group of the nonreducing mannose that helps mediate the interaction of the sugar with the lectin.[18] This "structural" water molecule, first described with conA in 1996 by Naismith and Field,[19] links and stabilizes the nonreducing sugar to the protein (Asp16). In our case, the extra hydroxymethyl group in the heptomannose could possibly play the role of this water molecule and even replace it as a stabilizing link between sugar and protein. The positioning of this C-7 hydroxy group may also account for the differences in binding affinity seen between the heptamannose diastereomers.

Fluorous-based carbohydrate microarrays clearly show that both diastereomers of *glycero*-D-*manno*-heptoses found in bacteria bind to conA and that this well-studied lectin can accept modifications at the C-6 position of its usual mannose ligand. These results illustrate the imperative to incorporate an ever greater range of carbohydrates into screening arrays and to very cautiously interpret lectin-binding data as proof of the presence of distinct structures. In addition, attempts to use *glycero*-D-*manno*-heptoses in the construction of antibacterial vaccines should proceed with caution as proteins that bind to this motif can clearly cross-react with mannose residues that are found in a range of human proteins. Even the most-studied lectin conA has revelations to offer in its binding affinities. Microarrays promise to uncover more, and more surprising carbohydrate-binding protein/sugar complexes.[20] The fluorous tag described herein not only facilitates the

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construction of carbohydrate microarrays, but also accelerates the purification of intermediates in automated glycosylation–deprotection cycles required to build up larger structures. In addition, these fluorous-based microarrays can support quantitative assessments of protein–ligand binding.

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