Chemoenzymatic Synthesis of N-glycan Positional Isomers and Evidence for Branch Selective Binding by Monoclonal Antibodies and Human C-type Lectin Receptors

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Chemoenzymatic Synthesis of N-glycan Positional Isomers and Evidence for Branch Selective Binding by Monoclonal Antibodies and Human C-type Lectin Receptors

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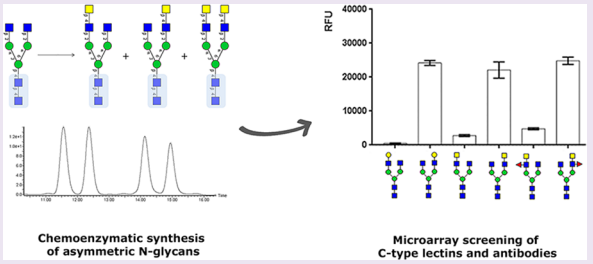
Supporting Information

ABSTRACT: Here, we describe a strategy for the rapid preparation of pure positional isomers of complex N-glycans to complement an existing array comprising a larger number of N-glycans and smaller glycan structures. The expanded array was then employed to study context-dependent binding of structural glycan fragments by monoclonal antibodies and C-type lectins. A partial enzymatic elongation of semiprotected core structures was combined with the protecting-group-aided separation of positional isomers by preparative HPLC. This methodology, which avoids the laborious chemical differentiation of antennae, was employed for the preparation of eight biantennary N-glycans with Galβ1,4GlcNAc (LN), GalNAcβ1,4GlcNAc (LDN), and GalNAcβ1,4-[Fucα1,3]GlcNAc (LDNF) motifs presented on either one or both antennae. Screening of the binding specificities of three anti-Le^X monoclonal IgM antibodies raised against *S. mansoni* glycans and three C-type lectin receptors of the innate immune system, namely DC-SIGN, DC-SIGNR, and LSECtin, revealed a surprising context-dependent fine specificity for the recognition of the glycan motifs. Moreover, we observed a striking selection of one individual positional isomer over the other by the C-type lectins tested, underscoring the biological relevance of the structural context of glycan elements in molecular recognition.



N-glycosylation is a very common modification of the majority of eukaryotic proteins. N-glycans have important intracellular functions in protein folding, targeting, and degradation and play extracellular roles in protein stability, antigenicity, and molecular binding and signaling in cellular communication.[1−3] The heterogeneity generally observed in protein glycosylation is a consequence of the nontemplate nature of glycan biosynthesis that is controlled by expression levels of glycosyltransferases and transporter proteins, nucleotide sugar donor concentrations, and protein−protein and protein−carbohydrate interactions around the glycosylation sites. Hence, glycan profiles are not only species-, tissue-, cell-, or protein-specific but often altered under pathological or changing physiological conditions, underscoring their potential as disease markers.[4]

The structural heterogeneity of complex N-glycans is largely due to the variable enzymatic elongation of terminal *N*-acetylglucosamine (GlcNAc) moieties with galactose, sialic acid, and fucose residues on bi-, tri-, and tetra-antennary glycans. The majority of N-glycan structures found in nature have nonidentical antennae, giving rise to isomeric and structural variations in the terminal motifs that form specific ligands for glycan-binding proteins.[5−7]

Molecular recognition processes based on carbohydrate−protein interactions have been found to mediate a plethora of biological processes including cell adhesion, cell development, protein trafficking, or the differentiation of self-and nonself by the immune system.[4,8] The usually weak monovalent interactions between carbohydrates and proteins are reinforced by a multivalent presentation of glycans on cell surfaces and through multimerization of receptors and glycan binding domains. Therewith, affinities can be increased up to 6 orders of magnitude, leading to binding strengths comparable to those of protein−protein interactions.[9,10] On the molecular level, carbohydrate−protein interactions are stabilized by hydrogen and water mediated hydrogen bonding, calcium coordination, hydrophobic interactions between aliphatic amino acid side chains with CH groups, and CH−π interactions between

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aromatic amino acid side chains and sugar residues.[11−13] Ultimately, glycan motif presentation, *e.g.*, by glycan clustering, as repeating units in polysaccharides or on the antennae glycans of secreted glycoproteins, can influence the strength and specificity of glycan protein interactions.[8,10]

In relation to pathogen recognition and the discrimination between self and nonself on the basis of glycan determinants, two important classes of carbohydrate binding proteins are antiglycan antibodies and lectins of the innate immune system. Immune responses to viral, bacterial, and parasitic infections are often orchestrated by glycan-induced molecular mechanisms.

In various parasitic worm infections, including the major human disease schistosomiasis, the antibody response is dominated by antibodies directed against antigenic glycans expressed on the parasite surface or on secreted glycoprotein antigens.[14] Knowledge of the exact binding specificities of these (monoclonal) antibodies is highly relevant for the development of diagnostic assays, in imaging, and in the development of glycan-targeting therapeutics.

One important class of mammalian glycan-binding receptors of the innate immune system is the C-type lectin receptors (CLRs). CLRs are pattern recognition receptors (PRRs) located on immune cells and involved in the recognition and uptake of both self and nonself glycoconjugates. Beyond their important functions in pathogen and/or antigen recognition and uptake, some CLRs are multifunctional receptors mediating cell adhesion, glycoprotein clearance, or further signaling events.[10,15] With few exceptions, CLRs with sugar binding affinity share a common fold for their carbohydrate binding domain and a well-defined primary sugar-binding site that involves the coordination of calcium ions.[15] On the basis of the specificity of their carbohydrate binding domain (CRD), CLRs are roughly grouped into mannose/fucose and galactose/GlcNAc binding receptors, but the fine specificity and additional affinity is gained through binding of oligosaccharides to an extended binding domain or *via* secondary binding sites.[15,16]

In this study, we have explored the influence of the structural context of N-glycan antenna ligands for binding of three anti-Lewis X antibodies directed against *S. mansoni* glycans and three C-type lectins, DC-SIGN, DC-SIGNR, and LSECtin.[17,18] DC-SIGN and DC-SIGNR are closely related CLRs that bind Ebola, hepatitis C, and human immune deficiency viruses *via* high mannose and complex type N-glycans of the viral surface proteins. While DC-SIGN induces specific immune responses upon interaction with numerous pathogens, DC-SIGNR in contrast seems to be only an adhesion receptor.[17,19] Both receptors have also been reported to enhance trans-infection of T-cells even at low viral loading by capture and efficient surface presentation of HIV to CD4 presenting cells.[20,21] L-SECtin recognizes Ebola virus via complex-type N-glycans[22] of the viral envelope protein, as well as West Nile filovirus and SARS coronavirus,[23] but not HIV or hepatitis C virus.[24] It also interacts with activated T-cells.[25]

Targeting antigen to densely expressed dendritic cell receptors such as DC-SIGN is an attractive strategy for vaccine efficacy improvement and immunotherapy[26] and has been achieved both with antilectin antibodies and with glycans recognized by the targeted lectin receptor.[27−30] A lack of specificity due to an overlap in glycan binding specificities of lectin receptors can be problematic for dendritic cell targeting approaches, especially if various receptors with opposing functions are responsive. The identification of high affinity and selective ligands that specifically target a single receptor on dendritic cells is an active area of research not only for immunotherapy and vaccine development but also for understanding lectin receptor function in antigen recognition, uptake, and processing. Previous glycan array profiling experiments have shown that DC-SIGN and DC-SIGNR both recognize Manα1,2Man residues, preferentially in high mannose type glycans, but that only DC-SIGN shows affinity to blood group antigens by binding to Fucα1,3GlcNAc and Fucα1,4GlcNAc residues.[17,31,31] Recently, Guo et al. have shown differentiated binding patterns of tetravalent DC-SIGN R and DC-SIGN as a function of the mannose residue density on glycan functionalized quantum dots. While DC-SIGN bound efficiently to quantum dots displaying mannose residues at high density, the spatially differently oriented DC-SIGNR CRDs could not engage in a sufficient number of contacts for strong binding.[32] Other screening efforts have also shown strong binding of DC-SIGN to biantennary GlcNAc terminating glycans, which is however completely lost in the presence of a core α1,2 xylose residue.[33]

All binding studies in this work were performed on an expanded version of our existing synthetic glycan array. It includes a large number of N-glycans reflecting a significant part of the structural heterogeneity found for antennae type and number, core modifications, and terminal sugar residues and an additional collection of smaller glycan fragments and blood group antigens.[33,34] For this study, we broadened our glycan array with a series of complex biantennary N-glycans presenting Galβ1,4GlcNAc (LacNAc), GalNAcβ1,4GlcNAc (LDN), and GalNAcβ1,4[Fucα1,3]GlcNAc (LDNF) motifs on either one or both antennae (Figures 2 and 3). In 2012, Benevides *et al.* observed the unusual specificity of a lectin from the legume *Platypodium elegans* for asymmetrically branched glycans.[35] A year later, the Boons group presented a strategy for the synthesis of N-glycans with differentiated antennae and found significant differences in the binding affinities of some antennae isomers to certain plant lectins and to hemagglutinins involved in influenza virus binding. This work suggested a role for positional isomers in molecular recognition processes.[36] Since then, other reports of differential binding patterns of asymmetrically branched glycans and strategies for their synthesis have appeared.[37−39] The chemo-enzymatic synthesis of asymmetrically extended complex multiantennary glycan isomers was first reported by the Boons group, based on an orthogonally protected central pentasaccharide scaffold and on a set of partially protected advanced glycosyl donors.[36] Subsequent enzymatic glycosylations elongated only fully deprotected antennae residues while acetate protected terminal sugars remained unchanged. However, this elegant approach suffered from incomplete enzymatic reactions, leading to compound mixtures that were arduous to separate.[40] Moreover, an elaborate chemical synthesis has to be accomplished for every underlying scaffold structure. This prompted us to seek an alternative approach for the preparation of N-glycan positional isomers.

Recently, we have developed PADS, the Protecting-group-Aided Detection and Separation of glycans, as a very robust methodology to quickly prepare libraries of all enzymatically accessible positional isomers from just a handful of multiantennary basic glycan scaffolds.[38] LDN and LDNF constitute CLR ligands that are very selectively expressed on mammalian N-glycoproteins[41] but that are highly abundant for instance in helminth parasites, including *S. mansoni*, one of the most significant worm parasites of humans. We studied the interaction of positional N-glycan isomers with several antiglycan

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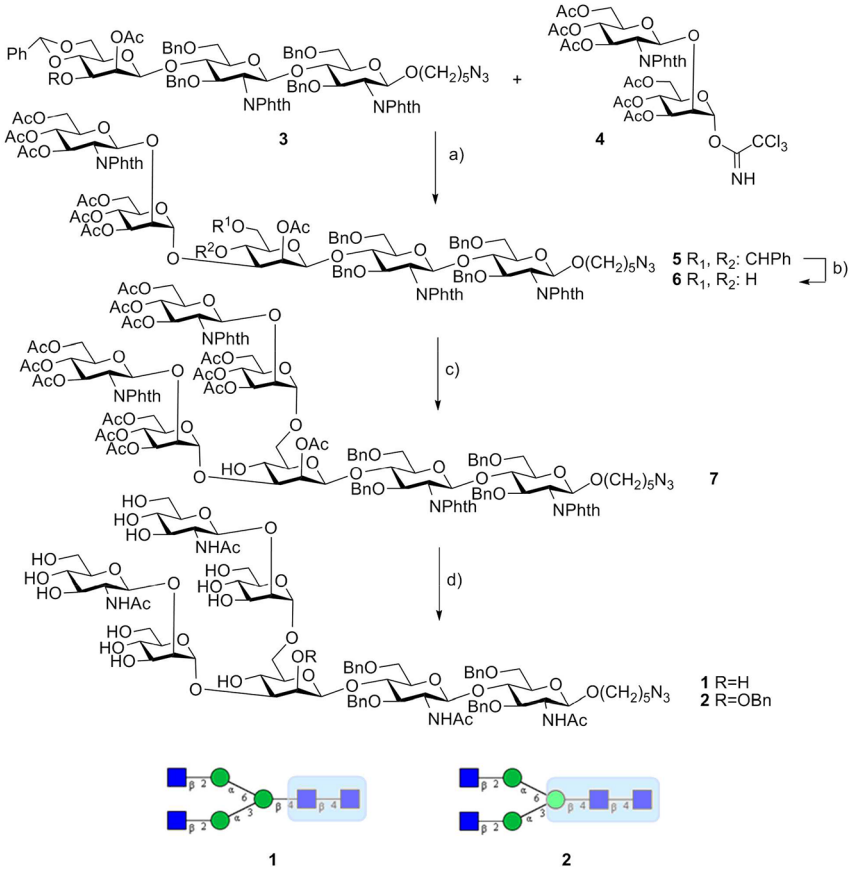
Scheme 1. Chemical Synthesis of Biantennary N-Glycan 1: (a) 10% TMSOTf, CH_2Cl_2, m.s., −20°C, 76%; (b) BF_3·OEt_2, EtSH, CH_2Cl_2, 79%; (c) 4, 10% TMSOTf, CH_2Cl_2, m.s., −45°C, 52%; (d, i) $NH_2(CH_2)_2NH_2$, *n*BuOH; (ii) Ac_2O, pyridine; (iii) NaOMe, MeOH; Below: Pictogram Presentation of Semiprotected Glycans 1 and 2 with Benzyl Protected Monosaccharide Residues Boxed in Light Blue



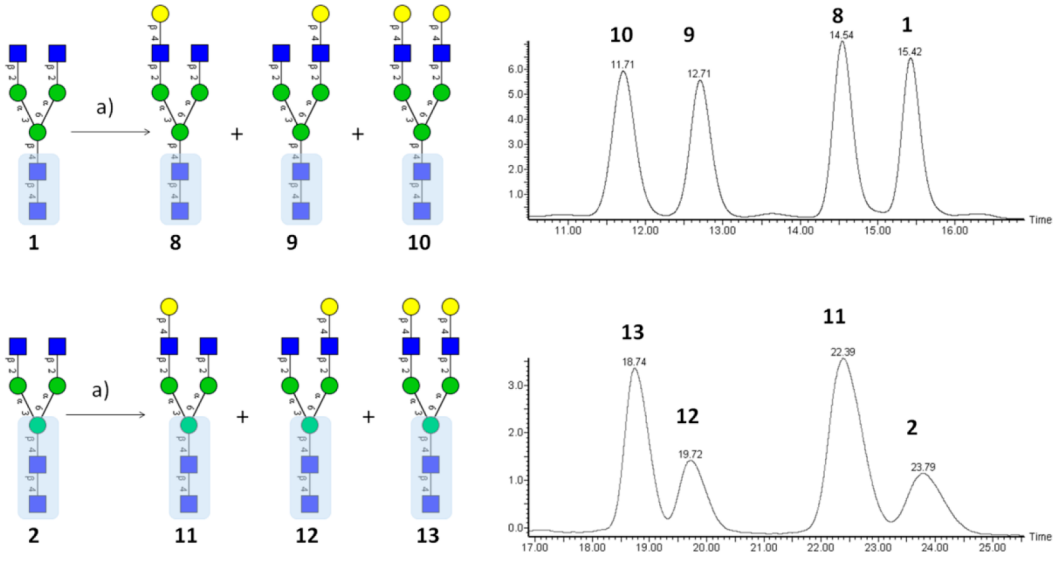


Figure 1. Galactosylation reaction of **1** and **2** and the corresponding purification chromatograms. (a) GalT, UDP-Gal, HEPES 50 mM at pH 8.5, $MnCl_2$ 10 mM, 37 °C. Galactosylation reaction of **1**, product distribution: **8**, 26.5%; **9**, 22.3%; **10**, 25.4%; **1**, 25.8%. Galactosylation reaction of **2**, product distribution: **11**, 44.1%; **12**, 12.4%, **13**, 29.2%, **2**, 14.3%.

monoclonal antibodies and human C-type lectins involved in pathogen and self recognition. These new binding data support the idea of isomer-specific roles in carbohydrate mediated molecular recognition.

RESULTS AND DISCUSSION

Synthesis. Enzymatic reactions toward the preparation of biantennary N-glycans presenting LDN and LDNF motifs were performed on the two partially benzylated glycan scaffolds **1** and

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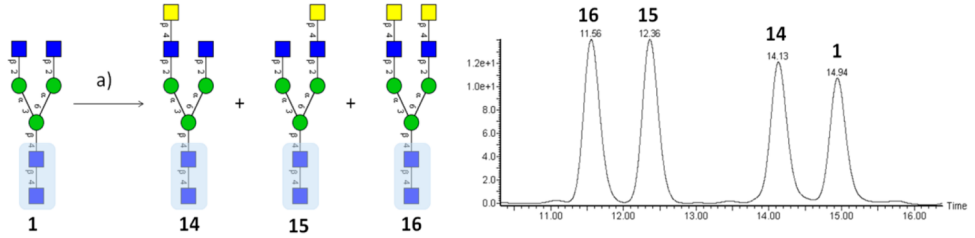

Figure 2. Enzymatic N-acetyl-galactosamination of **1**. (a) GalT mutant, UDP-GalNAc, HEPES 50 mM pH 8.5, $MnCl_2$ 10 mM, 37 °C. Product distribution: **14**, 24.4%; **15**, 27.5%, **16**, 27.6%, **1**, 20.5%.

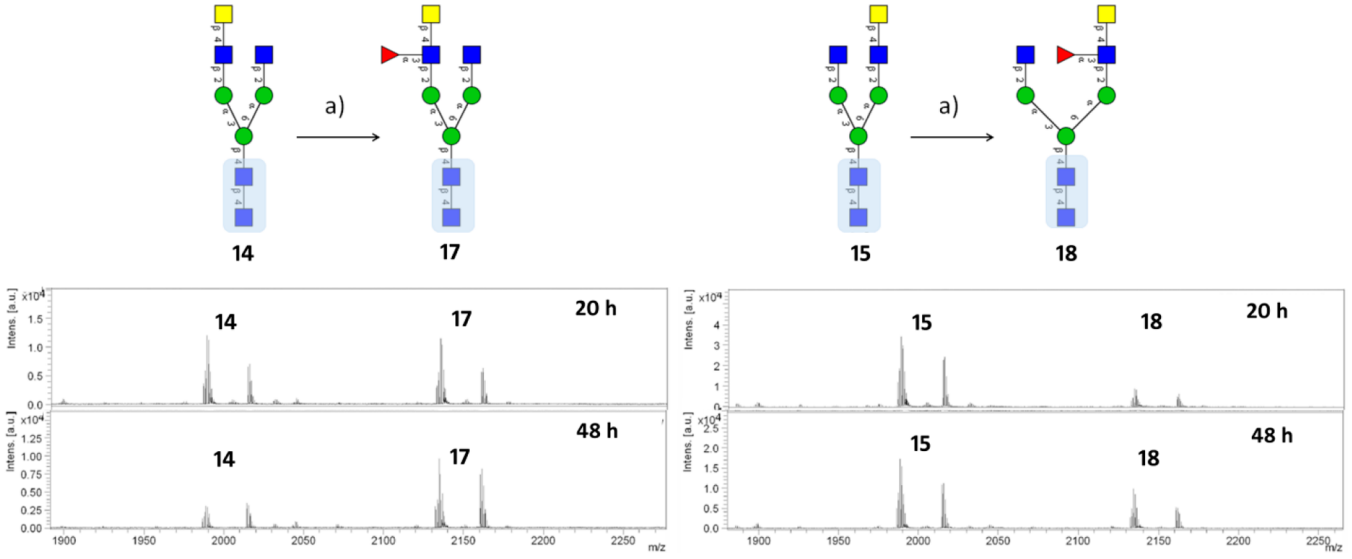

Figure 3. MALDI-TOF MS spectra of the fucosylation of **14** (left) and **15** (right) after 20 h and 48 h, respectively. (a) GDP-Fuc, CeFUT6, MES 40 mM, $MnCl_2$ 10 mM.

2, with compound **2** presenting an additional benzyl group at C2 of the central mannose residue. The synthesis of both scaffolds **1** and **2**[42] follows a [3 + 2 + 2] building block approach and is described in detail in the Methods section (see also Scheme 1).

Enzymatic Synthesis. The enzymatic extension of **1** and **2** synthesis to glycans presenting terminal LN (*N*-acetyl lactosamine), LDN, and LDNF moieties was carried out with a recombinant bovine milk galactosyltransferase (GalT), a mutant of bovine milk galactosyltransferase with GalNAc-transferase (GalNAcT) activity,[43] and a Lewis X type fucosyltransferase (FucT) from *C. elegans*. The presence of benzyl groups distant to the acceptor site had little or no influence on the outcome of the enzymatic reactions. To ensure a largely equimolar product distribution, we monitored the enzymatic reaction by UPLC-MS and carefully adjusted the substrate concentration, the incubation time, and the amounts of both added enzyme and nucleotide donor UDP-Galactose. Tuning of reaction conditions including time, enzyme, and donor concentrations afforded both regioisomers **8** and **9**, alongside the bis-elongated product **10** and the starting glycan **1** in nearly equimolar amounts, in line with the known indiscriminate action of the enzyme on the 3- and 6-arms of biantennary N-glycans[44] (Figure 1).

For the galactosylation of **2**, however, with an additional 2-*O*-benzyl group in the central mannose residue (Scheme 1), we observed the preferred formation of one regio-isomer over the other (Figure 1). Separation of the reaction components by preparative HPLC and structural assignment of both isomers by NMR revealed the 3-arm as the preferred branch for galactosylation (**11**, 44.1%). The added steric bulk conveyed by the benzyl group apparently gave rise to an unfavorable interaction with the enzyme for the elongation of the GlcNAc residue on the 6-arm. Consequently, only 12.4% of the 6-isomer **12** was formed. This protecting group induced selectivity was surprising, and a more systematic investigation of the steric effects of residual protecting groups as a means of modulating the selectivity of glycosyltransferases and glycosidases is currently underway in our lab.

For the rapid preparation of near equimolar amounts of all regio-isomers, however, the 2-*O*-benzyl-protected glycan **2** seemed less suited and was therefore abandoned as a scaffold for further enzymatic reactions. For the preparation of terminal GalNAc glycans, we incubated glycan **1** with UDP-GalNAc and GalNAcT and carefully adjusted the amount of enzyme, of nucleotide donor and the reaction time to produce similar amounts of both regioisomers **14** and **15**, starting material **1**, and bis-glycosylated product **16**, which were easily separated by preparative HPLC (Figure 2). Benzyl protected aliquots of **14**, **15**, and **16** were subjected to further enzymatic derivatization with a Lewis type fucosyltransferase to produce the LDNF immunogenic element on one or both arms of the biantennary N-glycan. Compounds **14** and **15** were incubated with CeFUT6 and GDP-fucose and the enzymatic conversion monitored for each reaction by MALDI-TOF mass spectrometry (Figure 3). After 20 h of incubation, around 50% of isomer **14** had reacted, and after 48 h, the fucosylation had reached completion. The

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isomer **15** with the GalNAc residue on the 6-arm reacted substantially slower, showing only 10% conversion to the fucosylated product **18** after 20 h (Figure 3). The reaction was left for 48 h until no further conversion to **18** was observed by MALDI-TOF MS, and the unreacted starting material was removed by preparative HPLC.

Treatment of the glycan **16** with CeFUT6, an α-1,3 fucosyltransferase that catalyzes the addition of a fucose residue at the distal GlcNAc of the chiotobiose core in *C. elegans* N-glycans[45] and which also shows activity toward terminal LN and LDN residues,[46] provided a mixture of regioiomers **19** and **20** and bis-glycosylated glycan **21**. Although the enzyme adds fucose on GalNAc residues of both antennae, the chromatographic profile (Figure 4) suggests preferential glycosylation of the 3-arm prior to addition on the 6-arm, a selectivity which we believe has not been described before. Even after 5 days of incubation with CeFUT6 and the GDP-fucose donor and after most starting glycan had been consumed, only trace amounts of the 6-isomer **20** (1.7%) were formed. To gain access to useful amounts of region-isomer **20**, we treated the monofucosylated glycan **18** with GalNAcT and UDP-GalNAc.

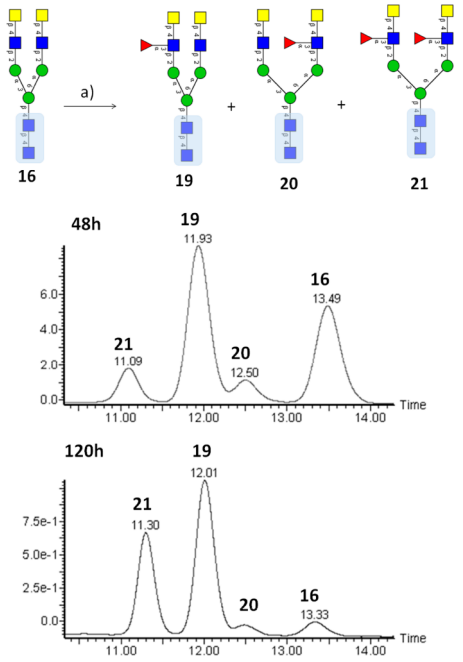

Figure 4. α-1,3-Fucosylation of compound **16**. (a) GDP-Fuc, CeFUT6, MES 40 mM, $MnCl_2$ at 10 mM. Chromatogram of the reaction after 48 and 120 h, respectively. Product distribution after 48 h: **19**, 55.6%; **20**, 4.6%; **21**, 11.8%; **16**, 28.0%. After 120 h: **19**, 55.8%; **20**, 1.7%; **21**, 35.7%; **16**, 6.8%.

After purification by semipreparative HPLC, compounds **8**−**10**, **14**−**15**, and **17**−**21** groups were deprotected by Pd/C catalyzed continuous flow hydrogenation (for details, refer to the SI) providing eight new positional isomers **22**−**29** with LN, LDN, and LDNF structural elements (Figure 5), which were included in our N-glycan microarray alongside our existing collection of 126 synthetic glycans onto NHS-activated glass microarray slides (see SI for all structures included on the glycan array).[33]

Carbohydrate Fine Specificity of Anti-LeX Antibodies Derived from *S. mansoni* Infected Mice. Printed arrays were first used to study the glycan binding specificities of monoclonal antibodies produced by hybridomas generated from the spleen cells of *S. mansoni* infected mice.[47,48] Schistosomiasis is an infectious disease caused by blood flukes of the *Schistosoma* genus that affects more than 200 million people worldwide.[49] *S. mansoni* parasites are highly glycosylated during all developmental stages and present a structurally rich variety of glycans including fucosylated LN and LDN motifs on their cell surface and secreted glycoproteins. Both innate and adaptive immune responses toward schistosome glycans are generated upon infection in humans, including the formation of antiglycan antibodies.[48,50] Several years ago, Remoortere *et al.* studied the glycan binding specificities of a library of 188 mAbs derived from *Schistosoma*-infected or immunized mice employing a number of small synthetic glycan conjugates.[49,51,52] Antibodies were grouped by binding specificity for Le^x, LDN, and LDNF epitopes, found in different developmental stages of *S. mansoni* and in addition classified for distinguishing monomeric from polymeric epitope presentation. The antibodies were then employed to visualize the spatial distribution of glycan epitopes in various stages of the parasite by immunofluorescence assays.[49,53] Here, we have screened a selection of mAbs known to bind Le^x epitopes to assess the effect of epitope presentation within the context of a larger N-glycan by glycan array methodology. Printed glycan arrays were incubated with diluted antibody solutions, and binding was detected by incubation with a fluorescently labeled secondary antimouse immunoglobulin antibody. Figure 6 shows the specificities of three Le^X binding monoclonal IgM antibodies for glycans presenting the Le^X epitope. The mAb 128−4F9 recognizes Le^x only as the free nonextended trisaccharide epitope, but not within the context of a larger N-glycan as neither **GL32**−**GL36**, **GL38**, **GL82**, nor **GL92** presenting the same epitope on the 3- and/or the 6-arm are bound. In contrast, the mAb 99−1G3 recognizes Le^X

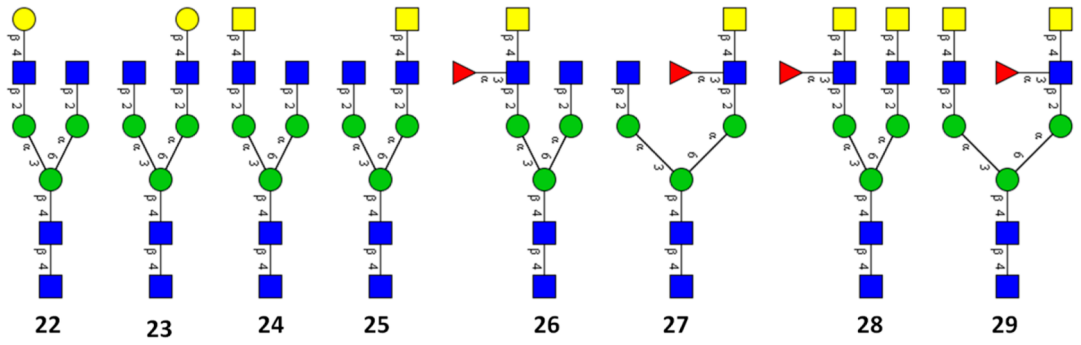

Figure 5. Pictogram representation of the eight newly synthesized positional LN, LDN, and LDNF isomers.

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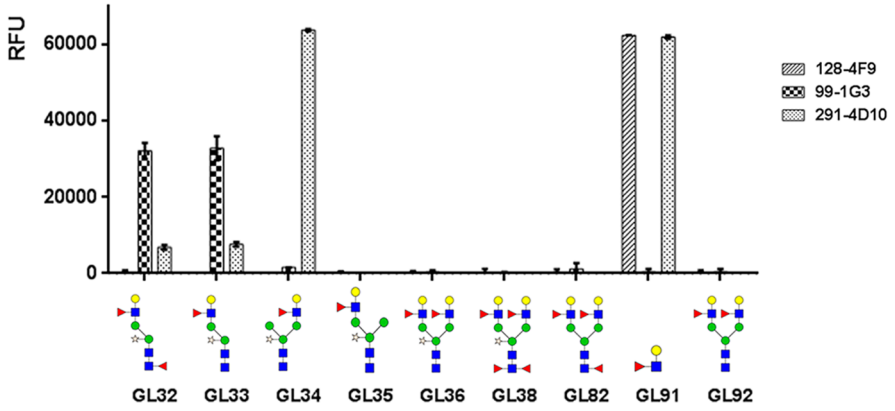

Figure 6. Glycan binding profile of *S. mansoni*-related monoclonal IgM antibodies 128−4F9, 99−1G3, and 291−4D10 toward Le^x containing structures. RFU values were analyzed after incubation with Alexa Fluor-555 antimouse IgM. Each histogram represents the mean RFU values for four spots with the SD of the mean.

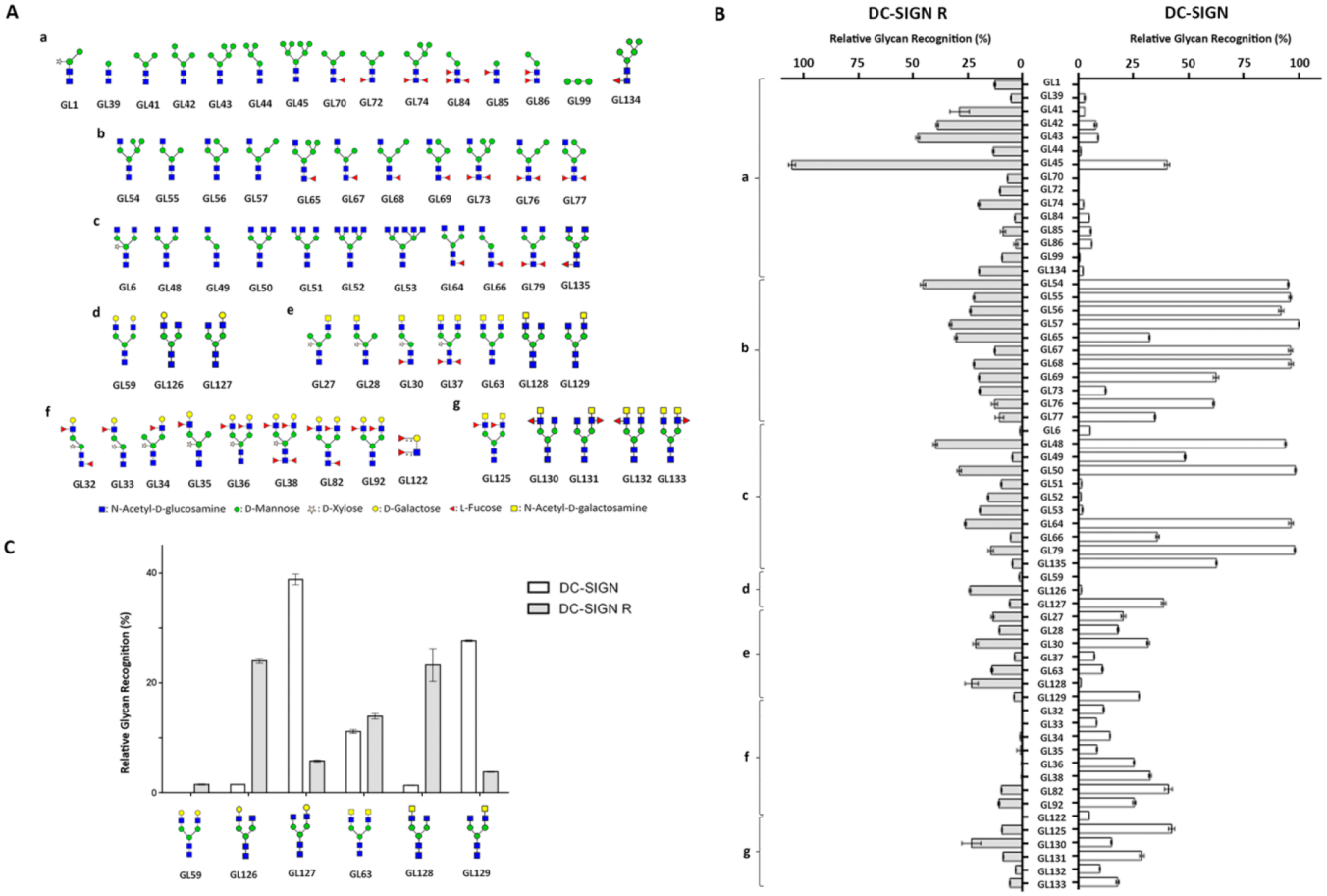

Figure 7. Glycan binding profile of DC-SIGN ECD and DC-SIGNR ECD. (A) Schematic representation of glycan structures binding to DC-SIGN and/or DC-SIGNR grouped by structure: (a) high mannose related structures, (b) hybrid N-glycans, (c) complex GlcNAc terminating N-glycans, (d) galactose containing N-glycans. (e) LDN containing N-glycans, (f) Le^x containing N-glycans and Le^y, (g) LDNF containing N-glycans. (B) Histograms representing the % RFU values for DC-SIGN and DC-SIGNR binding. (C) Comparison of %RFU values of monogalactosylated and N-glycans with terminal single GalNAc residues in DC-SIGN and DC-SIGNR binding.

exclusively on the 3-arm of the two core xylosylated glycans (**GL32**, **GL33**), while other compounds presenting the epitope in a spatially different manner, including the free epitope, are not bound. The third Le^x binding mAb 291−4D10 displays yet another specific binding profile as it recognizes both the free epitope and a structure presenting the epitope on the 6-arm of a core-xylosylated N-glycan (**GL34**). Compounds presenting the epitope on the 3-arm (**GL35**) or bis-functionalized compounds, **GL36** and **GL38**, were however not bound at all by 291−4D10. A rationale for this highly specific context-dependent binding is difficult to provide without additional structural information regarding the carbohydrate antibody complex. However, a possible explanation for the observed fine-specificity of these mAbs would be that their binding site extends beyond the Le^x

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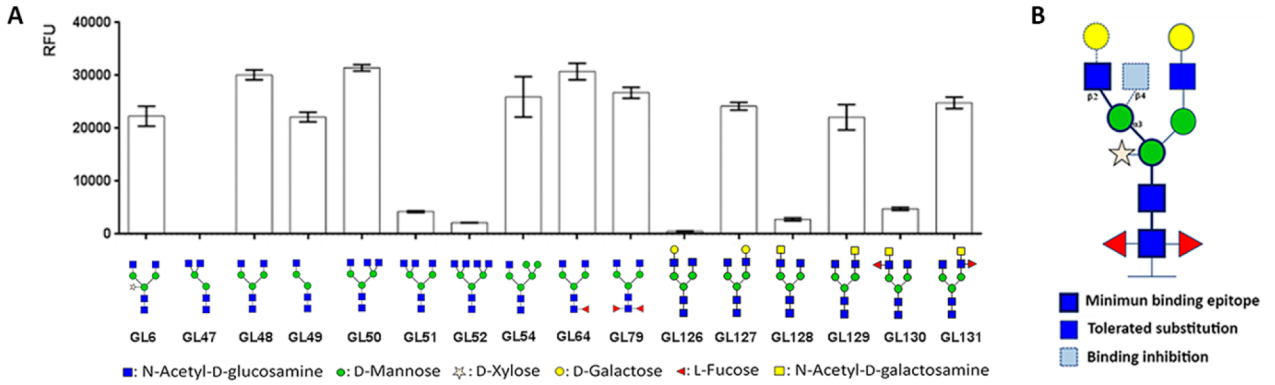

Figure 8. (A) Cy3-labeled L-SECtin CRD binding profile toward selected N-glycan structures. Each histogram represents the mean RFU values for four spots with the SD of the mean. (B) Preferred epitope found in complex and hybrid N-glycans for binding toward L-SECtin CRD.

trisaccharide itself. The mAbs 99−1G3 and 291−4D10 appear to be selective for the α3- and α6-arms of core xylosylated glycans, respectively, indicating that the mannose residue and the context of the trimannosyl core may take part in the mAb−glycan interactions. The observed specificity might also be the result of compound exclusion due to sterical clashes of antennae residues with the lectin rather than binding of a larger epitope *via* an extended binding groove. In contrast, 128−4F9 may prefer a more freely accessible Le^x motif such as in the context of the synthetic trisaccharide-linker conjugate. Crystallographic studies[54] indicated that glycan-binding pockets of Fab fragments can indeed accommodate oligosaccharides larger than a trisaccharide, including a linear trimer of Le^x. Interestingly, an at that time unspecified difference in the binding of Le^X-containing N-glycans between 99 and 1G3 and 291−4D10 was observed earlier using a glycan microarray of naturally occurring glycans isolated from *S. mansoni*.[55] N-glycans containing a single Le^X antenna were bound by 291−4D10, but not by 99−1G3, suggesting that the so far unspecified location of the Le^X motif in these cercarial glycans was on the α6-arm rather than the α3-arm. Therefore, unraveling the fine specificity of antiglycan mAbs by defined synthetic glycan arrays is helpful in defining isomeric and asymmetric glycan structures from natural sources.

The exquisite antiglycan specificity of some monoclonal antibodies found in this study highlights the importance of epitope selection and presentation and could have important consequences, *e.g.*, in immunoassays employing natural or synthetic glycan antigens such as those that detect allergen-directed IgEs.[56]

Screening of the Carbohydrate Binding Specificity of C-Type Lectin Receptors. Here, printed arrays including the novel structures **22**−**29** were used to screen the C-type lectins DC-SIGN, DC-SIGNR, and LSECtin. Both DC-SIGN and DC-SIGNR extracellular domains (ECDs) were incubated on our array seeking ligands with selectivity for one of these two closely related lectins. Figure 7 shows strong binding of DC-SIGN to hybrid (**GL54**−**GL57**) and complex type glycans (**GL48**−**GL50**) and N-glycans presenting Le^x epitopes (**GL82** and **GL92**). One of the most strongly bound glycans in our collection was, as previously shown, the complex type biantennary glycan **GL48**.[33] We also observed a significant binding to the biantennary glycan monogalactosylated on the 6-arm (**GL127**) but not to the positional isomer galactosylated on the 3-arm (**GL126**). A similar behavior was observed for N-glycans with a single terminal *N*-acetylgalactosamine (GalNAc) residue (**GL128** and **GL129**). Incubation of DC-SIGNR on the same arrays gave a slightly different picture than DC-SIGN (Figure 7). The strongest binding glycan was the high mannose glycan **GL45** with an atypical non-natural branching pattern presenting the Manα1−3(Manα1−6Man) trisaccharide on both 3 and 6 arms. **GL45** was less strongly bound by DC-SIGN. Even more interestingly, we observed opposite binding behavior of DC-SIGNR and DC-SIGN toward the monogalactose and mono-GalNAc positional isomers. Both galactosylated and GalNAc-terminating glycans **GL126** and **GL128** were specifically recognized by DC-SIGNR, while the corresponding positional isomers **GL127** and **GL129** were bound with far lower affinity.

Finally, we looked at the carbohydrate binding profile of the C-type lectin L-SECtin CRD to our glycan array. L-SECtin is widely expressed in lymph node and liver endothelial tissue, peripheral blood, and on macrophages.[18] Its carbohydrate binding specificities have not been studied in much detail yet. A report from Dominguez-Soto *et al.* mentions binding to GlcNAc functionalized sepharose but not to mannan or GalNAc analogues.[18] A later published binding profile to an array of 300 glycans showed high affinity of the CRD to GlcNAcβ-1,2-Man containing glycan fragments, while fucosylated and mannosylated glycans were only identified as low affinity binders at very high receptor concentrations.[22] Our results indicate an even higher selectivity of L-SECtin CRD for GlcNAc1,2-Man residues on the 3-arm of the complex and hybrid N-glycans. An additional GlcNAc residue in position C4 inhibited binding (structures **GL47**, Figure 8). The same GlcNAc1,2-Man disaccharide presented on the 6-arm of di-, tri-, or tetra-antennary complex N-glycans was bound to a far lesser extent (structures **GL48**, **GL51**, **GL52**). An additional capping with galactose on the 3-arm (**GL126**) completely abolishes the low binding of GlcNAc1,2-Man residues on the 6-arm. An *N*-acetylgalactosamine cap on the same arm is not as efficient in inhibiting this interaction (structures **GL128** and **GL129**). The striking observation comes again from the differential interaction of LSECtin toward two positional isomers **GL126** and **GL127** where a galactose is capping the 3- and 6-arm, respectively. Similar behavior, albeit to a lesser extent, can be observed with two other couples of positional isomers with an LDN (**GL128**, **GL129**) motif or LDNF motif (**GL130**, **GL131**) present on the 3- and 6-arms, respectively. It is yet another example for the context specific binding of a well-defined structural element. This highlights the biochemical relevance of truncated or incompletely extended structures here for the

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molecular recognition of glycans and glycoproteins by C-type lectin immune receptors.

The binding data of L-SECtin can be resumed in the preferred ligand GlcNAcβ1,2Manα1,3Man. Substitution of this structural element with core xylose is tolerated, while capping with galactose or additional branching with β1−4GlcNAc completely inhibits binding. Capping with GalNAc on the 3-arm or branching with β1−6GlcNAc on the 6-arm does not affect the moderate binding observed for structures presenting a GlcNAcβ1,2Man residue on the 6-arm (structures **GL48**, **GL51**, **GL52**).

CONCLUSIONS

We and others have reported a growing number of examples where previously defined minimal ligand structures are differentially recognized by antibodies, lectins, and glycosyltransferases when presented in different structural contexts. This common feature in glycan recognition can be explained by the existence of more extended binding domains that can accommodate larger structural elements than previously thought. Another possible explanation could be the existence of different binding modes between an oligosaccharide and protein binding domain, with at times exquisite selectivity as suggested by some of our positional isomer selective binding data. The ever more extended structural annotation of glycan structures with simple pictograms that neglect glycosidic binding information might nurture the misconception of symmetrically equivalent antennae branches in N-glycans similar to a dendrimer. Detailed structural analysis, e.g., by NMR has shown large differences in the accessibility and the three-dimensional orientation for the different arms of the same N-glycan backbone in line with the different connectivity of monosaccharide residues. The structural asymmetry between antennae can now be visualized by NMR by attaching a paramagnetic lanthanide tag to the anomeric position of a multiantennary glycan. The induced pseudo-contact shifts decrease with the distance to the paramagnetic metal and provide spectral separation of peaks for the same residues present on different antennae.[57] The identification of a pair of positional N-glycan isomers that selectively bind to DC-SIGN and DC-SIGNR was an unexpected but welcome finding, which opens the door to further improvement on the selectivity and affinity of the identified ligands by medicinal chemistry approaches. Likewise, the discovery of the monogalactosylated N-glycan **GL127** as a very strong binder of L-SECtin while the isomeric glycan does not show any binding requires further structural analysis, *e.g.*, by NMR. Additional screening of other human lectins will show if this lead structure could eventually become a selective probe for targeting this important lectin involved in pathogen recognition and T-cell activation. Finally, the differences we observed for mAbs binding the Le^x epitope indicates that not only lectins but also the adaptive immune system (antibodies) can differentiate glycan motifs presented in different contexts.

METHODS

Chemical Synthesis of Semiprotected N-Glycan Precursors 1 and 2. Briefly, **1** was prepared by the sequential glycosylation of trisaccharide **3** with the disaccharide donor **4** on the central mannose residue. Glycosylation at position C3 under TMSOTf promotion provided pentasaccharide **5** in 76% yield. The Bn-acetal was subsequently removed by transacetalization with thioethanol and $BF_3{\cdot}OEt_2$ to produce **6** in 79% yield. Glycosylation on OH-6 was achieved with complete regioselectivity at low temperature and dilute reactant concentration again employing imidate **4** as a glycosyl donor and TMSOTf promotion to furnish the protected heptasaccharide **7** in 52% yield (Scheme 1). Phthalimide groups were removed by microwave assisted aminolysis with diaminoethane and the intermediate amine treated subsequently with acetic anhydride and sodium methanolate to arrive at glycan **1** with four remaining benzyl groups in the core chitobiose moiety as our key scaffold for enzymatic diversification. The synthesis of compound **2** followed an analogous route which has been described earlier.[42]

C-Type Lectin Expression Cloning. Standard pUC57 plasmids containing optimized synthetic human genes encoding human DC-SIGN R ECD (amino acids 80−399) and LSECtin CRD (amino acids 162−292) designed for efficient production in *E. coli* were manufactured by GeneCust Europe. PCR amplification using suitable primers and restriction enzyme digestion were used to subclone DC-SIGN R ECD and N-terminal 6-His tagged LSECtin CRD into the pET30-b (Novagen) between the NdeI and *Hin*dIII restriction sites. The sequencing of each construction was done by Genewiz.

Protein Expression and Purification. The DC-SIGN extracellular domain (DC-SIGN ECD) was produced and purified as previously described.[58] DC-SIGNR ECD and L-SECtin CRD were expressed in *E. coli* BL21(DE3) in 1 L of LB culture supplemented with 50 μg/mL kanamycin at 37 °C. Expression was induced by the addition of 1 mM isopropyl 1-thio-D-galactopyranoside (IPTG) when the culture had reached an A_{600nm} of 0.8 and maintained for 3 h. The protein was expressed in the cytoplasm as inclusion bodies. Cells were harvested by a 20 min centrifugation cycle at 5000*g* at 4 °C. The pellet was resuspended in 30 mL of a solution containing 150 mM NaCl, 25 mM Tris-HCl at pH 8, and one antiprotease mixture tablet (Complete EDTA free, Roche). Cells were disrupted by sonication and cell debris eliminated by ultracentrifugation at 100 000*g* for 45 min at 4 °C with a Beckman 45Ti rotor. The pellet was solubilized in 30 mL of 6 M guanidine-HCl containing 25 mM Tris-HCl at pH 8, 150 mM NaCl, and 0.01% (v/v) β-mercaptoethanol. The mixture was centrifuged at 100 000*g* for 45 min at 4 °C, and the supernatant was diluted 5-fold with 1.25 M NaCl, 25 mM $CaCl_2$, and 25 mM Tris-HCl or 200 mM Tris-HCl at pH 8 (for Dc-SIGNR or L-SECtin respectively) by slow addition with stirring. The diluted mixture was dialyzed against 10 volumes of 25 mM Tris-HCl at pH 8, 150 mM NaCl, and 4 mM $CaCl_2$ (buffer A) with three buffer changes. After dialysis, insoluble precipitate was removed by centrifugation at 100 000*g* for 1 h at 4 °C.

The supernatant containing DC-SIGNR ECD was loaded onto a mannan agarose column (Sigma) equilibrated with buffer A for purification by affinity chromatography. After loading, DC-SIGNR ECD was tightly bound to the column and eluted in the same buffer without $CaCl_2$ but supplemented with 1 mM EDTA (buffer B). This step was followed by Size Exclusion Chromatography (SEC) using a Superose 6 column equilibrated with buffer A. Fractions were analyzed by SDS-PAGE (12%), and DC-SIGN R ECD containing fractions were pooled and concentrated by ultrafiltration (YM10 Amicon membrane from Merck-Millipore).

The supernatant containing the His tagged LSECtin CRD was loaded onto a HisTrap HP column (GE Heathcare) at 4 °C. Unbound proteins were washed away with buffer A before LSECtin CRD was eluted with buffer C (150 mM NaCl, 25 mM Tris-HCl at pH 8, 4 mM $CaCl_2$, 0.5 M imidazole). Eluted fractions were analyzed by SDS-PAGE (15%), and the LSECtin CRD containing fractions were pooled and concentrated by ultrafiltration (YM10 Amicom membrane from Merck-Millipore). Each construct was checked by N-terminal amino acid sequencing and mass spectrometry.

Cy3 Labeling of DC-SIGN ECD, DC-SIGNR ECD, and LSECtin CRD. A total of 150 μL of 10 mg mL^{-1} solutions of DC-SIGN ECD, 250 μL of 3.77 mg mL^{-1} solutions of DC-SIGNR ECD, and 1 mL of 0.5 mg mL^{-1} solution of LSECtin CRD in 25 mM HEPES at pH 7.25 and 4 mM $CaCl_2$ were prepared. Corresponding volumes (4.5, 3, and 2 μL, respectively) of 10 mg mL^{-1} Cy3-NHS ester (GeneCopoeia) were added to the DC-SIGN ECD, DC-SIGNR ECD, and LSECtin CRD solutions individually, and the reactions were gently shaken at RT for 2 h and then at 4 °C for 4 h. Excess dye was removed by two dialyses (3.5k

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Z-lyser from Thermo Scientific) of 3 h against 25 mM Tris at pH 8, 150 mM NaCl, and 4 mM $CaCl_2$. The amount of attached Cy3 was estimated spectrophotometrically based on the dye (ε_{550} 150 000 cm^{-1} M^{-1}) and protein molar absorption coefficients (ε_{280} DC-SIGN ECD 70 400 cm^{-1} M^{-1}, ε_{280} DC-SIGNR ECD 60 890 cm^{-1} M^{-1}, ε_{280} LSECtin CRD 48 845 cm^{-1} M^{-1}). The obtained degree of labeling (DOL) was 0.95 for both DC-SIGN ECD and DC-SIGNR ECD and 0.2 for LSECtin CRD.

Microarray Preparation. Glycan microarrays were prepared as previously described.[33] Briefly, ligand solutions (50 μM, 1.25 nL, five drops, drop volume: 250 pL) in sodium phosphate buffer (300 mM, pH 8.4, 0.005% Tween-20) were spatially arrayed employing a robotic noncontact piezoelectric spotter (SciFLEXARRAYER S11, Scienion) onto *N*-hydroxysuccinimide (NHS) activated glass slides (Nexterion H, Schott AG). After printing, the slides were placed in a 75% humidity chamber at 25 °C for 18 h. The remaining NHS groups were quenched with 50 mM solution of ethanolamine in 50 mM sodium borate buffer, at pH 9.0, for 1 h. The slides were washed with PBST (PBS solution containing 0.05% Tween 20), PBS, and water. The slides were dried in a slide spinner and stored at −20 °C until use.

Incubation with Monoclonal Antibodies. Monoclonal antibodies (mAbs) were produced as previously described.[49] Culture supernatants containing IgM mAbs against Le^x epitopes (128−4F9, 99−1G3, and 291−4D10) were diluted 1:200 in PBS containing 1% (w/v) bovine serum albumin (BSA) and 0.01% (v/v) Tween-20. MAbs solutions (200 μL per array) were used to incubate individual wells on a glycan array slide at RT for 1 h. The slides were washed with PBST (PBS containing 0.05% Tween-20) followed by PBS. Slides were then incubated with antimouse IgM-555 (1:1000) in PBS containing 1% BSA and 0.01% Tween-20 for 1 h in the dark. Arrays were washed from unbound secondary antibodies with PBST, PBS, and water. Microarrays were dried in a slide spinner, and fluorescence measurements were performed on a microarray scanner (Agilent G2565BA, Agilent Technologies) at 10 μm resolution. Quantification of fluorescence was performed by ProScanArray Express software (PerkinElmer) employing an adaptive circle quantification method from 50 μm (minimum spot diameter) to 300 μm (maximum spot diameter). Average RFU values with local background subtraction of four spots and standard deviation of the mean were reported using Microsoft Excel and GraphPad Prism software.

Incubation with C-type Lectins. Cy3 labeled C-type lectins were diluted in incubation buffer (25 mM Tris·HCl, 150 mM NaCl, 4 mM $CaCl_2$, pH 7.5 containing 0.5% bovine serum albumin (BSA), and 0.005% Tween-20). C-type lectin solutions (200 μL per array) were used to incubate individual wells on a glycan array slide at 4 °C for 18 h. Arrays were washed with incubation buffer without BSA and with water and dried in a slide spinner. Fluorescence measurements and analysis were performed as described above for monoclonal antibodies.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acschembio.8b00431.

Protocols for the chemo-enzymatic synthesis of asymmetric N-glycan structures, list of glycans printed on microarrays, and full histograms and images of microarray experiments (DOCX)

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Notes

The authors declare no competing financial interest.

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