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Noncarbohydrate Glycomimetics and Glycoprotein Surrogates as DC-SIGN Antagonists and Agonists

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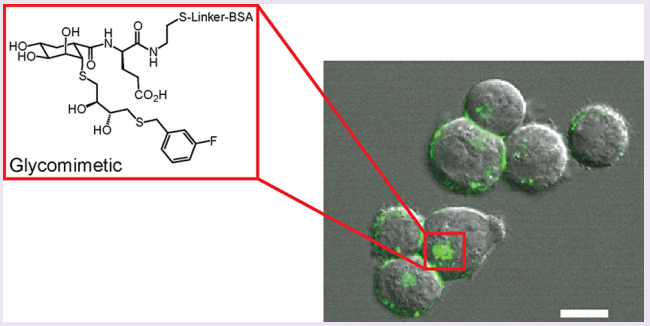
Supporting Information

ABSTRACT: An understanding of the biological roles of lectins will be advanced by ligands that can inhibit or even recruit lectin function. To this end, glycomimetics, noncarbohydrate ligands that function analogously to endogenous carbohydrates, are being sought. The advantage of having such ligands is illustrated by the many roles of the protein DC-SIGN. DC-SIGN is a C-type lectin displayed on dendritic cells, where it binds to mannosides and fucosides to mediate interactions with other host cells or bacterial or viral pathogens. DC-SIGN engagement can modulate host immune responses (*e.g.*, suppress autoimmunity) or benefit pathogens (*e.g.*, promote HIV dissemination). DC-SIGN can bind to glycoconjugates, internalize glycosylated cargo for antigen processing, and transduce signals. DC-SIGN ligands can serve as inhibitors as well as probes of the lectin's function, so they are especially valuable for elucidating and controlling DC-SIGN's roles in immunity. We previously reported a small molecule that embodies key features of the carbohydrates that bind DC-SIGN. Here, we demonstrate that this noncarbohydrate ligand acts as a true glycomimetic. Using NMR HSQC experiments, we found that the compound mimics saccharide ligands: It occupies the same carbohydrate-binding site and interacts with the same amino acid residues on DC-SIGN. The glycomimetic also is functional. It had been shown previously to antagonize DC-SIGN function, but here we use it to generate DC-SIGN agonists. Specifically, appending this glycomimetic to a protein scaffold affords a conjugate that elicits key cellular signaling responses. Thus, the glycomimetic can give rise to functional glycoprotein surrogates that elicit lectin-mediated signaling.



Carbohydrate−lectin interactions are crucial for many biological processes, including cellular adhesion, migration, signaling, and infection.[1] Because carbohydrates are displayed on the exterior of all cells, lectins have critical roles in immunity and tolerance. One large family of lectins that can function in this capacity is the C-type lectin class, whose members are named for their dependence on calcium ions to facilitate carbohydrate binding by chelation to carbohydrate hydroxyl groups.[2] Several members of this class are found on dendritic cells (DCs), the major antigen-presenting cells of the immune system,[3] where they can function as antigen receptors and control DC migration and interactions with other immune cells.[4,5] These multiple functions all contribute to mounting appropriate immune responses. One DC receptor, dendritic cell-specific ICAM-3-grabbing nonintegrin (DC-SIGN), is an intriguing lectin with varied functions.[6,7] Through its interactions with high mannose glycans or fucose-containing Lewis-type antigens on self-glycoproteins ICAM-3 and ICAM-2, DC-SIGN can mediate T cell interactions and trans-endothelial migration, respectively.[8,9] It also has been implicated in antigen processing because it promotes uptake of anti-DC-SIGN antibodies for processing and presentation to T cells.[10] Although these data emphasize the roles of DC-SIGN in giving rise to immune responses, the lectin can interact with a variety of glycosylated pathogens to facilitate infection. For example, DC-SIGN binds to the mannosylated surface glycoprotein gp120 on HIV to mediate *trans*-infection of T cells.[11,12] The infectious agent *Mycobacterium tuberculosis* exploits DC-SIGN interactions for a different end. The bacteria, which display a mannosylated surface component, are internalized and processed *via* interactions with DC-SIGN. The outcome is a dampening of pro-inflammatory signaling and inhibition of DC maturation, leading to immunosuppression.[13]

Identification of the roles DC-SIGN can play in pathogenesis has prompted efforts to identify chemical inhibitors. DC-SIGN binds weakly to monosaccharides such as *N*-acetyl mannosamine (ManNAc, K_d = 8.7 mM) and L-fucose (K_d = 6.7 mM).[14] Oligosaccharides bind with slightly higher affinities (*e.g.*, $Man_9GlcNAc$ has a K_d of 0.21 mM). These observations, in conjunction with the finding that DC-SIGN is tetrameric, have prompted the exploration of multivalent presentation as a strategy to generate potent inhibitors.[14−16] Several multivalent glycan inhibitors have been developed;[17−20] however, a complication of studying DC-SIGN in natural settings is the presence of additional C-type lectins that have similar

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specificities.[4] Oligomannose and oligofucose ligands may therefore interact with multiple lectins, complicating the dissection of DC-SIGN function *in vivo*. Thus, there is a need for alternative compounds with more specificity as well as higher affinity for DC-SIGN.

We have synthesized small molecules that serve as ligands for DC-SIGN.[21−23] In our pursuit, we sought to devise new types of glycomimetics. The term glycomimetic has been applied widely and often is used to refer to lectin-binding compounds in which some or even most glycosidic linkages are preserved. Carbohydrate derivatives of this type that bind DC-SIGN have been identified and can be more potent inhibitors than canonical carbohydrate ligands.[24−28] Here, we define "glycomimetic" to mean a compound that is lacking standard glycosidic linkages but resembles a carbohydrate and can mimic or inhibit its function. We therefore set out to identify noncarbohydrate building blocks that would possess the critical features of the carbohydrates that bind DC-SIGN. Specifically, we have used (−)-shikimic acid as a scaffold to generate compounds designed to function as mannoside or fucoside surrogates.[29,30] The natural product (−)-shikimic acid was subjected to amide formation followed by conjugate addition of a thiolate to afford a compound with hydroxyl groups in the requisite stereochemical arrangement to mimic D-mannosides (Figure 1). This strategy yields compounds wherein several positions can be modified to take advantage of secondary-site interactions to increase binding affinity and specificity. We previously used the approach to generate DC-SIGN inhibitors.[23] One lead compound, triol **1**, was about 4-fold more active than ManNAc. A polymer bearing the compound exhibited an increase in potency of approximately 1,000-fold. These studies validate the utility of multivalency for designing noncarbohydrate mimics of natural carbohydrates and glycoproteins.

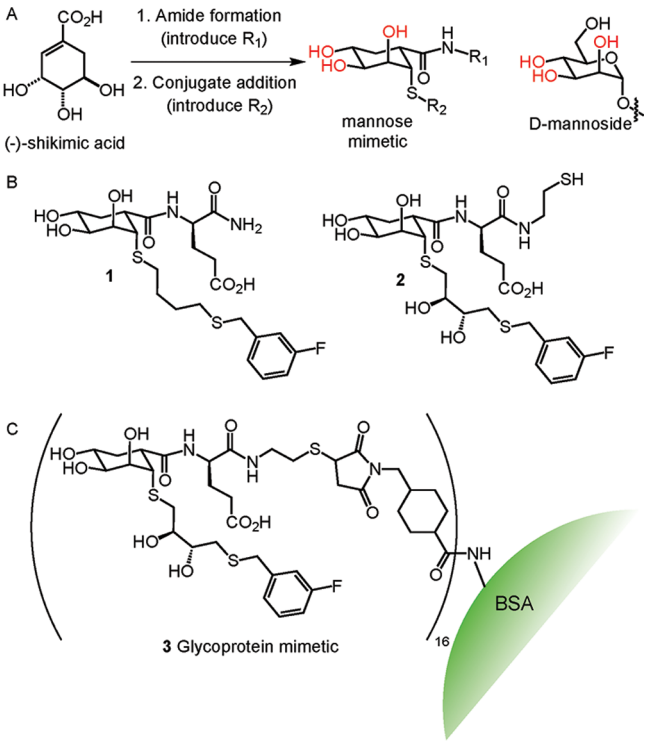

Figure 1. Strategy for glycomimetic design. (A) Three key hydroxyl groups (red) on mannosides contribute to C-type lectin binding.[32] Compounds can be synthesized from (−)-shikimic acid with hydroxyl groups in the relevant orientations that mirror D-mannosides. (B) Lead compound **1** and hydroxylated analogue **2** bearing a cysteamine moiety are inhibitors of DC-SIGN.[23] (C) Compound **2** was appended to BSA and the conjugate was converted into fluorescent glycoprotein surrogate **3**.

A major issue was whether compound **1** could serve as a functional mimic of the saccharides that bind DC-SIGN. NMR experiments with recombinant DC-SIGN reveal the addition of compound **1** results in the types of chemical shift perturbations caused by mannose and fucose. We leveraged the similarity of the binding modes of carbohydrates and the glycomimetic to generate a glycoprotein surrogate that acts as a DC-SIGN agonist. The resulting conjugate is internalized by DC-SIGN-expressing cells and initiates cellular signaling. These findings highlight the potential of synthetic ligands for exploring the diverse roles of DC-SIGN.

RESULTS AND DISCUSSION

Chemical Shift Perturbation Analysis of Glycomimetic Binding. While compound **1** serves as an effective inhibitor, its mechanism of inhibition was not known. The scaffold was designed to interact with the carbohydrate recognition domain of DC-SIGN, but as with most putative glycomimetics, structural information regarding the mode of binding of compound **1** to DC-SIGN was lacking. Such information is important if glycomimetics and glycoprotein surrogates are to be used to probe and control lectin function. For example, antibodies that interact with DC-SIGN at sites other than its carbohydrate recognition domain fail to trigger DC-SIGN internalization.[10] Thus, for synthetic ligands to be effective cellular probes of DC-SIGN function they should mimic glycan binding. We therefore sought a method to determine whether compound **1** engages DC-SIGN in the same manner as carbohydrate ligands.

Our strategy was to employ NMR spectroscopy to monitor binding of different ligands to the DC-SIGN carbohydrate recognition domain (CRD) (Figure 2). We reasoned that $^{1}H-^{15}N$ HSQC experiments would be valuable for this purpose as they reveal chemical shift perturbations of backbone amide NMR resonances upon ligand addition.[31] Because chemical shifts are so sensitive to environment, perturbations can be valuable indicators of ligand binding. This method is especially illuminating when complexation occurs with minimal protein conformational change. We postulated that the C-type lectins would be excellent substrates for analysis using this method because the data from X-ray crystallographic analysis indicates that the bound and unbound structures of these proteins are similar.[32] Consequently, we anticipated that HSQC shift perturbations would be localized to residues in the ligand-binding site. To this end, spectra were collected of unbound (apo) DC-SIGN and of the lectin in the presence of *N*-acetyl mannosamine (ManNAc), fucose (Fuc), or compound **1**.

When ManNAc or fucose was added, a subset of peaks shifted compared to the apo spectrum, indicating that the corresponding residues were affected by ligand binding (Figure 2). That the addition of each type of carbohydrate afforded similar chemical shift perturbations is consistent with structural data indicating that the modes by which these saccharides engage DC-SIGN are analogous (for a list of relevant chemical shifts, see Figure S1). The structures determined from crystallographic analysis also indicate that nearly identical lectin side chains participate in binding to either *N*-

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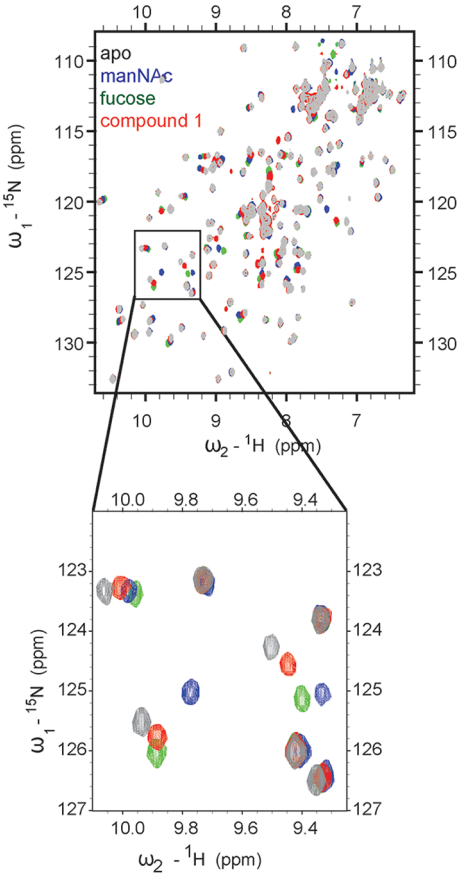

Figure 2. Glycomimetic interacts with DC-SIGN in the sugar binding pocket. Superimposed two-dimensional HSQC spectra are shown of DC-SIGN CRD apo (gray) or in the presence of 20 mM *N*-acetylmannosamine (ManNAc) (blue), fucose (green), or compound **1** (red). The inset highlights a region of the spectrum containing three signals that undergo significant shifts upon ligand binding.

acetylmannosamine or fucose, and our NMR data are consistent. These results highlight the value of $^{1}H-^{15}N$ HSQC NMR spectroscopy for studying ligand binding to a C-type lectin, and we posit that this approach will be useful for future studies of proteins in this class

When we tested compound **1**, its addition resulted in chemical shift perturbations similar to those obtained for ManNAc and fucose. These findings indicate that compound **1** has a binding mode analogous to that of saccharide ligands. In addition, several peaks appeared upon glycomimetic addition that were not present in any of the other spectra (Figure 2). The appearance of these peaks is consistent with the conformational restriction of residues that were flexible in the apo form. Thus, some conformational heterogeneity exists in the apo protein, and the glycomimetic appears to be unique among ligands tested in its ability to decrease this heterogeneity. These data suggest that there are glycomimetic-specific interactions with DC-SIGN, which presumably arise from binding to adjacent secondary sites. They also augur well for optimization of the glycomimetic by enhancing its secondary site interactions. Overall, these data demonstrate that the molecular basis of the interaction of compound **1** with DC-SIGN parallels that of *N*-acetylmannosamine and fucose. Compound **1** is therefore deserving of the term "glycomimetic."

A Glycoprotein Surrogate Undergoes DC-SIGN-Mediated Uptake. Given that compound **1** mimics the interactions of carbohydrates with DC-SIGN, we next tested whether it would promote the cellular functions of DC-SIGN. While other synthetic ligands have been shown to inhibit DC-SIGN, none have been shown to act as functional agonists. There are two critical functions that DC-SIGN agonists can promote: antigen internalization and activation of signaling pathways. Both types of processes should be facilitated by clustering of DC-SIGN. Thus, we generated multivalent versions of our glycomimetic by appending compound **2**, a dihydroxylated analogue of compound **1**, to bovine serum albumin (BSA). Derivative **2** binds to DC-SIGN with affinity similar to that of compound **1** but is more water-soluble.[23] Compound **2** was appended to BSA (~16 copies, see the Supporting Information), and the resulting conjugate was coupled to an AlexaFluor 488 dye equipped with an *N*-hydroxysuccinimidyl ester to yield a fluorophore-labeled glycoprotein mimic. The fluorophore was similarly attached to mannose-substituted BSA and fucose-substituted BSA. The resulting glycoprotein conjugates and the glycoprotein surrogate were assessed in several assays.

We first tested the ability of the glycoconjugates and glycoconjugate mimic to undergo DC-SIGN-mediated endocytosis. The cytoplasmic tail of DC-SIGN contains conserved internalization motifs, and anti-DC-SIGN antibodies are taken up by dendritic cells, processed, and presented to T cells.[10] If synthetic glycomimetic conjugates such as **3** are endocytosed, they could be used as novel vaccines to deliver antigens to dendritic cells. The observation that HIV is not processed following engagement of DC-SIGN,[11,12] however, suggests that cargo that interacts with DC-SIGN can have other fates. The ability to generate tailored DC-SIGN ligands therefore provides the opportunity to examine what factors influence DC-SIGN-mediated internalization. To assess the feasibility of this approach, confocal microscopy was used to monitor DC-SIGN-specific internalization of fluorescent conjugates: mannose-BSA, fucose-BSA, and glycomimetic-BSA (compound **3**, Figure 3). Each glycoconjugate or the glycoprotein mimetic was exposed to Raji cells, a human B cell line derived from Burkitt's lymphoma, or Raji cells stably transfected with the gene encoding DC-SIGN (Raji/DC-SIGN).[33] Mannosylated and fucosylated BSA conjugates were internalized by the Raji/DC-SIGN but not the Raji cells (Figure 3). These results indicate that uptake of the carbohydrate-decorated proteins depends on the presence of DC-SIGN. The glycoprotein surrogate also was internalized only by the DC-SIGN-expressing cells. Thus, the glycoprotein mimic undergoes DC-SIGN-mediated uptake, thereby acting in accord with glycoconjugates decorated with carbohydrates. The data indicate that a specific lectin can be targeted to deliver cargo to a cell's interior using a glycomimetic or glycoprotein surrogate.

Initiation of Cell Signaling by the Glycoprotein Surrogate. Ligand binding to DC-SIGN can promote cellular signaling.[34] A critical functional test of our glycoprotein surrogate is whether it effects signaling. The ability of a synthetic ligand to elicit cellular signals opens new avenues of investigation. Some pathogens are known to modulate DC-SIGN-mediated signaling, but the mechanisms by which they do so have not been fully elucidated.[13,35−37] If chemically defined DC-SIGN agonists can initiate signaling events, they can be crafted to reveal how ligand structure modulates signal output. Such information could guide the design of compounds that control DC-SIGN-mediated signaling to enhance immunity and thereby combat infection or to suppress unwanted immune responses. A clinically important role for

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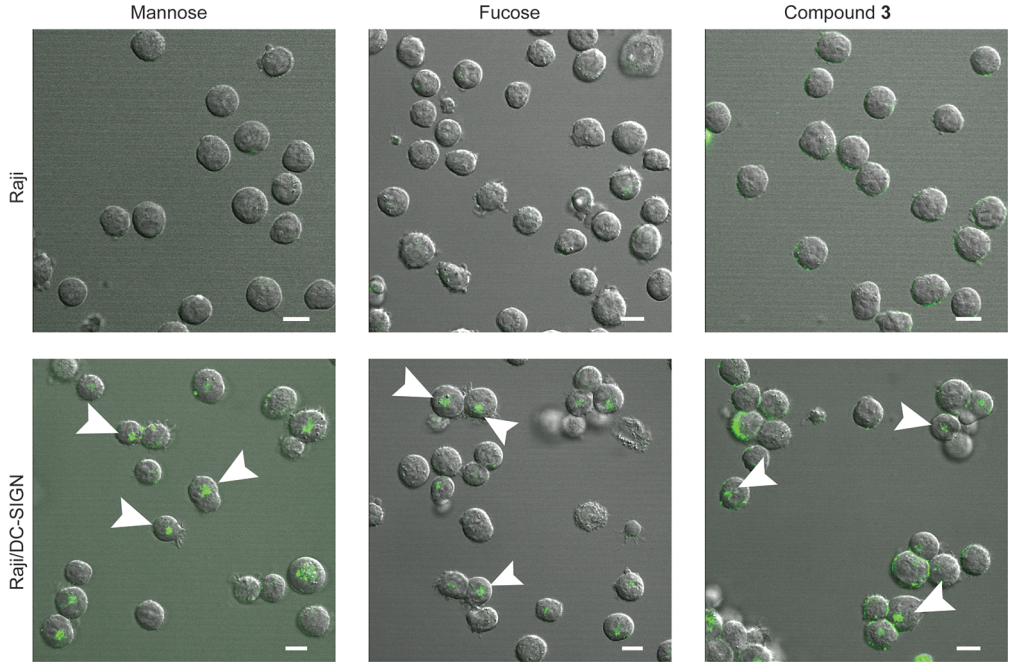

Figure 3. Glycocoprotein surrogate is internalized by DC-SIGN-expressing cells. Raji cells (top panels) and Raji/DC-SIGN cells (bottom panels) were treated with glycoconjugates mannose-BSA-AF488 (left), fucose-BSA-AF488 (center), or compound **3** (right). After exposure to glycoconjugate or glycomimetic conjugate, live cells were imaged by confocal microscopy. Examples of punctate intracellular staining are indicated by white arrows. Scale bars indicate 10 μm.

DC-SIGN in regulating immune responses has emerged from investigations of the mechanism of action of intravenous immunoglobulin (IVIG).[38] This blood product, composed of pooled IgG antibodies, is used for a number of different purposes, one of which is to suppress deleterious inflammation. DC-SIGN interacts with endogenous sialylated Fc to upregulate IL-33, a cytokine that can suppress serum-induced arthritis.[39] As such, ligands that trigger DC-SIGN-mediated signaling may act as novel agents to combat inflammatory and immune diseases. Accordingly, we tested whether our glycoconjugate surrogate initiates signaling *via* DC-SIGN.

We focused our analysis on the JNK pathway, which has been implicated in DC-SIGN-mediated immune signaling.[40,41] JNK is a mitogen-activated protein kinase that is linked to dendritic cell maturation and the response to "danger" signals.[42] The net result is changes in gene expression *via* downstream effects on a variety of transcription factors, including c-Jun. When Raji or Raji/DC-SIGN cells were treated with mannose-substituted BSA, the level of phospho-JNK was increased. Modest enhancement was detected after 5 min, while larger increases were observed at later time points (10 and 15 min) (Figure 4). The increases in phospho-JNK depend on the presence of DC-SIGN. Intriguingly, cellular responses to glycoprotein surrogate **3** paralleled those obtained with the mannose-substituted glycoconjugate. Our data indicate that the glycoprotein surrogate effectively initiates signaling events. Thus, our strategy provides the means to probe how the structures of different glycoconjugates influence DC-SIGN-mediated cellular responses.

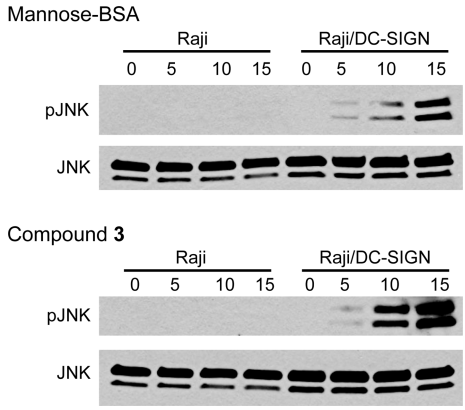

Figure 4. Glycoprotein surrogate stimulates DC-SIGN-mediated JNK signaling. Raji or Raji/DC-SIGN cells were treated with mannose-BSA (top) or compound **3** (bottom) and incubated at 37 °C for the indicated time (min). Samples were subject to lysis, products were separated by SDS-PAGE, and the gels were analyzed by immunoblotting for phospho-JNK (pJNK). Blots were then stripped and reprobed for JNK to assess the total protein loaded in each lane.

Conclusions. In summary, we synthesized a glycomimetic that is functionally equivalent to glycans that bind DC-SIGN. The utility of DC-SIGN ligands relies on their ease of synthesis and the opportunities for their chemical manipulation to explore and/or trigger specific cellular responses. To interpret functional data from such probes, however, the probes must mimic glycan ligands in binding to their target lectin. The shikimic acid-based glycomimetic we have devised does just that. Accordingly, it merits the descriptor "glycomimetic". Our results also highlight the utility of NMR shift perturbations for identifying and comparing glycan binding sites within lectins. Finally, we show that our glycomimetic can be used to generate a glycoprotein surrogate that acts as a full DC-SIGN agonist: it is capable of initiating DC-SIGN-mediated internalization and signaling. Thus, we have identified a probe of DC-SIGN that goes beyond inhibition. We therefore anticipate that compounds **1** and **3** can be used to interrogate DC-SIGN function.

These data also serve as validation that shikimic acid can serve as a useful scaffold to generate noncarbohydrate

glycomimetics. Compound **1** is a starting point to optimize probes for specific attributes, such as broad activity for subsets of C-type lectins, increased affinity, or enhanced DC-SIGN binding specificity. Our group has also recently described a related approach to generate fucoside mimics based on (−)-4-epi-shikimic acid,[30] and we anticipate that compounds of this class can be converted into agonists for fucose-binding lectins. We envision a general glycomimetic approach based on different shikimic acid scaffolds that will allow for generation of a wide range of compounds useful for specifically inhibiting undesirable lectin interactions with pathogens, as well as for elucidating the biological functions of immune lectins. We expect that the strategy that we have described here can serve as a blueprint to devise synthetic ligands that can promote lectin signaling.

METHODS

Cells and Constructs. Raji and Raji/DC-SIGN cells were obtained from the NIH AIDS Reference and Reagent Program.[33] Cells were maintained in RPMI (Gibco) supplemented with 10% fetal bovine serum and penicillin/streptomycin at 37 °C with 5% carbon dioxide. A plasmid for expressing DC-SIGN carbohydrate recognition domain (residues 250−404) was obtained from Kurt Drickamer[14] and transformed into *E. coli* strain BL21/DE3.

Chemical Methods. Full synthesis and characterization of compound **2** and glycoconjugate **3** is provided in the Supporting Information. Mannose and fucose glycoconjugate probes were generated by coupling an AF488 succinimidyl ester (Invitrogen) to mannose-BSA and fucose-BSA (Dextra) following the manufacturer's instructions. The resulting surrogates were purified using a PD-10 column (GE Healthcare) and dialysis into PBS.

NMR Spectroscopy, Confocal Microscopy, and Western Blotting. Described in detail in the Supporting Information.

ASSOCIATED CONTENT

Supporting Information

This material is available free of charge *via* the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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