Glycopeptides

Synthesis of Asparagine Derivatives Harboring a Lewis X Type DC-SIGN Ligand and Evaluation of their Impact on Immunomodulation in Multiple Sclerosis

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Abstract: The protein myelin oligodendrocyte glycoprotein (MOG) is a key component of myelin and an autoantigen in the disease multiple sclerosis (MS). Post-translational *N*-glycosylation of Asn_{31} of MOG seems to play a key role in modulating the immune response towards myelin. This is mediated by the interaction of Lewis-type glycan structures in the *N*-glycan of MOG with the DC-SIGN receptor on dendritic cells (DCs). Here, we report the synthesis of an unnatural Lewis X (Le^X)-containing Fmoc-SPPS-compatible asparagine building block (SPPS = solid-phase peptide synthesis), as well as asparagine building blocks containing two Le^X-derived oligosaccharides: LacNAc and Fucα1-3GlcNAc. These building blocks were used for the glycosylation of the immunodominant portion of MOG ($MOG_{31\text{-}55}$) and analyzed with respect to their ability to bind to DC-SIGN in different biological setups, as well as their ability to inhibit the citrullination-induced aggregation of $MOG_{31\text{-}55}$. Finally, a cytokine secretion assay was carried out on human monocyte-derived DCs, which showed the ability of the neoglycopeptide decorated with a single Le^X to alter the balance of pro- and anti-inflammatory cytokines, inducing a tolerogenic response.

Introduction

Multiple sclerosis (MS) is a group of autoimmune neurodegenerative diseases characterized by the formation of lesions in the patient's brain that lead to the loss of functions.[1] The pathology of MS is not fully understood, but degradation of the myelin sheath seems to be a critical step in the process.[2] Myelin sheaths are comprised of myelin, an insulating substance consisting of lipids, proteins, and other molecules, and are responsible for fast information transfer through axons.[3] Indeed, some proteinogenic components of myelin sheaths have been shown to become antigenic upon their degradation.[4] For example, myelin oligodendrocyte glycoprotein (MOG), an exclusively central nervous system (CNS)-resident protein found on the surfaces of oligodendrocytes and myelin sheaths, acts as an autoantigen in the MS-like animal model, experimental autoimmune encephalomyelitis (EAE).[5]

MOG is a glycoprotein, decorated with an *N*-glycan[6] on Asn_{31}, with a molecular mass of 26–28 kDa.[7,8] It comprises 245 amino acids (AAs) and belongs to the immunoglobulin (Ig) superfamily. Over the last few decades, it has been shown that antibodies against MOG are circulating in the bloodstream of patients suffering from various demyelinating diseases such as MS and *N*-methyl-D-aspartate receptor encephalitis,[9] and that the peptide fragment comprising AAs 35–55, $MOG_{35\text{-}55}$, is a key T-cell epitope in EAE.[10,11]

We have recently discovered a potential reason for the pathogenicity of this $MOG_{35\text{-}55}$ peptide in EAE: After post-translational citrullination (deimination of the guanidine in arginine), the peptide can form amyloid-like aggregates intracellularly, where they appear to be cytotoxic.[12,13] Citrullination of myelin proteins is considered to be critical in MS. For example, another antigenic myelin protein, myelin basic protein (MBP), has been shown to exhibit increased citrullination in myelin samples from MS patients.[14] Together, these advances led to the hypothesis that post-translational citrullination of MOG could in part be responsible for the shift of the disease pathogenesis in EAE towards neurodegeneration rather than autoimmunity.

In light of the above findings, we wished to explore whether the native *N*-glycan at position 31 has an effect on the aggregation behavior of the citrullinated peptide, as the *O*-glycosyla-

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tion of serine or threonine residues has previously been shown to have inhibitory effects on the aggregation of a tau-derived peptide, a highly aggregation-prone protein family involved in Alzheimer's disease.[15] The introduction of *N*-glycans and mimics thereof onto peptides derived from prion protein[16] and the full-length prion protein[17] itself has also been shown to decrease or even abrogate aggregation.

Furthermore, previous studies on the glycosylation of MOG suggested that the nature of the carbohydrate structures of *N*-glycan plays an important role in the modulation of immunological tolerance through glycan interactions with the dendritic cell-specific intercellular adhesion molecule-3-grabbing nonintegrin (DC-SIGN) receptor.[18] This receptor has been shown to recognize the fucose-containing Lewis-type glycans,[19] especially the trisaccharide Galβ1-4(Fucα1-3)GlcNAc, better known as Lewis X (Le^X), which has been shown to be highly abundant on natively glycosylated MOG.[20] Hence, studies using synthetic neoglycopeptides bearing DC-SIGN-binding *N*-glycan mimics may shed light on the role of the putative interaction between DC-SIGN and MOG in MS. We have synthesized MOG_{31-55} peptides decorated with Le^X and Le^X-derived oligosaccharides (LacNAc and Fucα1-3GlcNAc) on the N-terminal asparagine (Asn_{31}) and assessed the effect of these modifications on the tendency of these proteins to aggregation. It was our aim to link the glycans to the peptides through amide linkages, to minimize artefacts stemming from various non-native linkers.[21–23] To achieve this, we extended our recently published method for the synthesis of glycosylated asparagine derivatives using larger oligosaccharides.[24] By using these asparagine building blocks together with our previously established model peptide, MOG_{31-55},[12] we were able to evaluate the effect of glycosylation on the citrullination-dependent aggregation of MOG. Subsequently, the binding of Le^X-decorated neoglycopeptides to DC-SIGN was confirmed by solid-phase immunoassays. Finally, a cytokine secretion assay in monocyte-derived dendritic cells (moDCs) from human donors was used to analyze the degree of modulation of interleukin 10 (IL-10, anti-inflammatory) and IL-12p70 (pro-inflammatory) production by Le^X-decorated peptides.

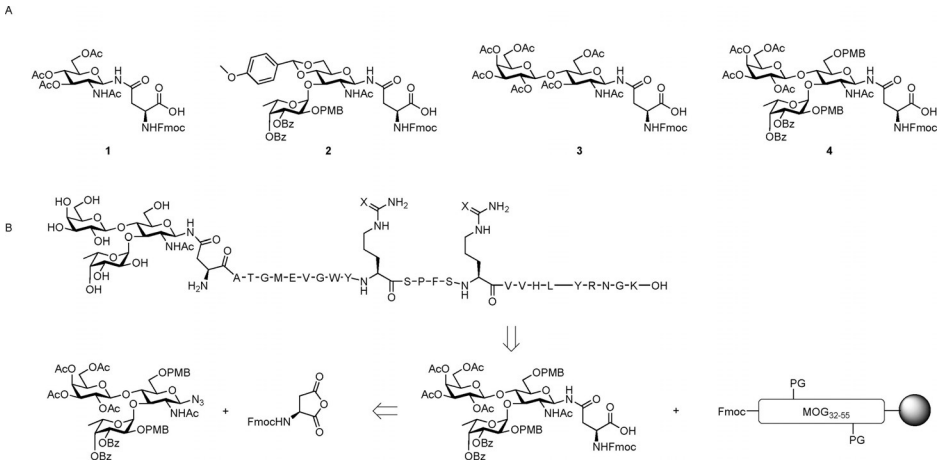
The outcomes of these biochemical and immunological studies suggest that 1) the aggregation behavior of citrullinated MOG_{31-55} can be halted or abrogated depending on the glycan, 2) our amide-linked Le^X ligand indeed binds to DC-SIGN in an ELISA (enzyme-linked immunosorbent assay), and 3) the Le^X-decorated neoglycopeptide has an in vitro tolerogenic effect (cytokine secretion assay), thus potentially preventing inflammation.

We report here the first synthesis of MOG_{31-55} derivatives that are site-specifically decorated with DC-SIGN ligands and analyze the immunological consequences of exposure of moDCs to the Le^X-decorated peptide.

Results and Discussion

While *N*-glycosylation of asparagine is of prime importance for a variety of protein functions, such as signaling and folding,[25] the typical size and complexity of the *N*-glycan poses a considerable synthetic challenge. *N*-Glycosylated peptides have been generated by using semisynthetic methods involving the synthesis and/or isolation of carbohydrate segments that can be linked covalently using endohexosaminidases,[26,27] or extended through specific glycosyltransferases, as recently demonstrated by Boons and co-workers.[28,29] The synthesis of an entire peptide bearing a natural *N*-glycan has also been reported.[30]

Previous work from our group and others[31–35] demonstrated that fucosylated glycans interact with DC-SIGN without the need for an *N*-glycan core structure. This inspired us to synthesize a Le^X *N*-glycan derivative similar to the one developed by von dem Bruch and Kunz.[36] We have designed and synthesized three glycosyl amide derivatives of asparagine as Fmoc-SPPS (solid-phase peptide synthesis)-compatible building blocks, containing Le^X and two Le^X derivatives, LacNAc and Fucα1-3GlcNAc, attached to the asparagine side chain through the reducing ends of the respective sugars (Scheme 1 A, **1**–**4**). The LacNAc construct (**3**) served as a negative control for DC-SIGN binding, as the interaction of Le^X with the receptor has been shown to be fucose-dependent.[32]



Scheme 1. A) Structures of glycosylated asparagine derivatives **1**–**4**. B) Retrosynthetic analysis of the synthesis of Le^X-decorated MOG_{31-55} peptides. X = NH (Arg) or X = O (Cit).

We chose to base our synthesis on acid-labile *p*-methoxybenzyl (PMB) and *p*-methoxybenzylidene groups, which would be removed during global peptide deprotection in standard Fmoc-based SPPS, and on esters, which can be selectively removed using hydrazine in methanol after the acidic global deprotection of the peptide. By including these protecting groups from the start of the oligosaccharide synthesis, late-stage protecting group manipulation could mostly be avoided.

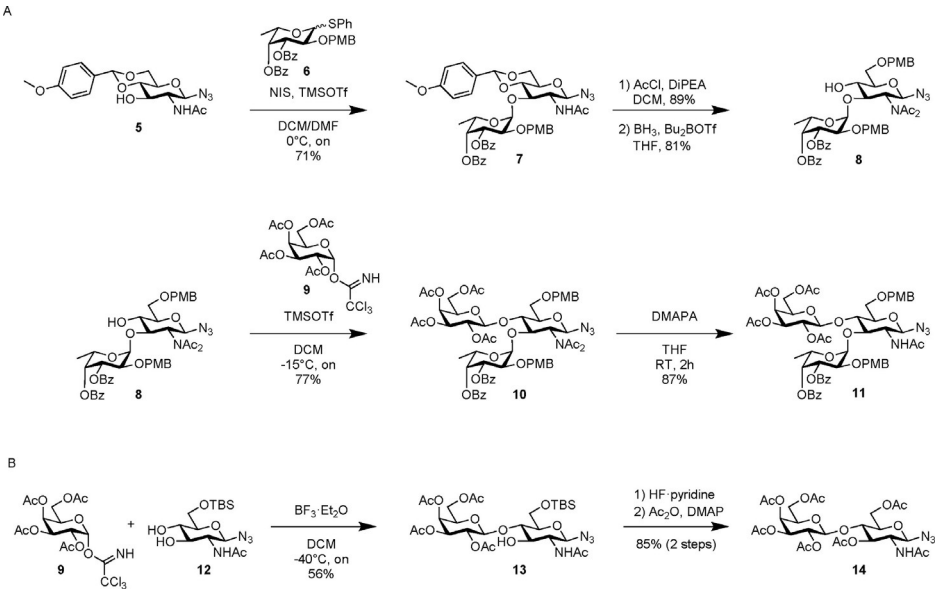
The linkages between the oligosaccharides and the asparagine side chain were installed by using our recently developed two-step one-pot approach for the synthesis of glycosylated asparagine derivatives.[24] Here, we combined a Staudinger reduction to transform a glycosyl azide into a glycosylamine, as reported by many others,[37–39] followed by aspartic anhydride ring-opening to generate a protected glycosyl asparagine derivative (Scheme 1 B). The synthesis of the protected Le^X glycosyl azide **11** (Scheme 2) was initiated from the *p*-methoxybenzylidene-protected glycosyl azide **5** (see the Supporting Information) by means of an *N*-iodosuccinimide (NIS)/TMSOTf-promoted fucosylation reaction with the thioglycoside **6** (see the Supporting Information) to afford disaccharide **7** in a yield of 71%. The presence of the acetamido group was detrimental to the results of the following glycosylation, an often encountered problem with *N*-acetylglucosamine-derived acceptors.[40] Accordingly, disaccharide **7** was treated with an excess of acetyl chloride and diisopropylethylamine (DiPEA) to convert the amide functionality into the less interfering imide in a yield of 89%.[41] Reductive opening of the *p*-methoxybenzylidene with BH_3/Bu_2BOTf was performed as described previously[42] to afford compound **8** in a yield of 81%. Finally, galactosylation with the trichloroacetimidate donor **9** (see the Supporting Information) yielded the desired protected trisaccharide **10** in a yield of 77%. Chemoselective deacetylation of **10** using *N*,*N*-dimethylaminopropylamine (DMAPA)[43] afforded **11** in a yield of 87%.

The protected lactosaminyl azide **14** was prepared using a literature protocol for the regioselective glycosylation of 1,6-protected GlcNAc derivatives.[44,45] Silyl ether protected glycosyl azide **12** was subjected to BF_3·Et_2O-promoted galactosylation with trichloroacetimidate donor **9** to afford the partially protected disaccharide **13** in a yield of 56% (Scheme 2). This compound was treated with HF·pyridine to remove the *tert*-butyldimethylsilyl (TBS) group followed by acetylation to afford the desired peracetylated glycosyl azide **14** in a yield of 85% over two steps.

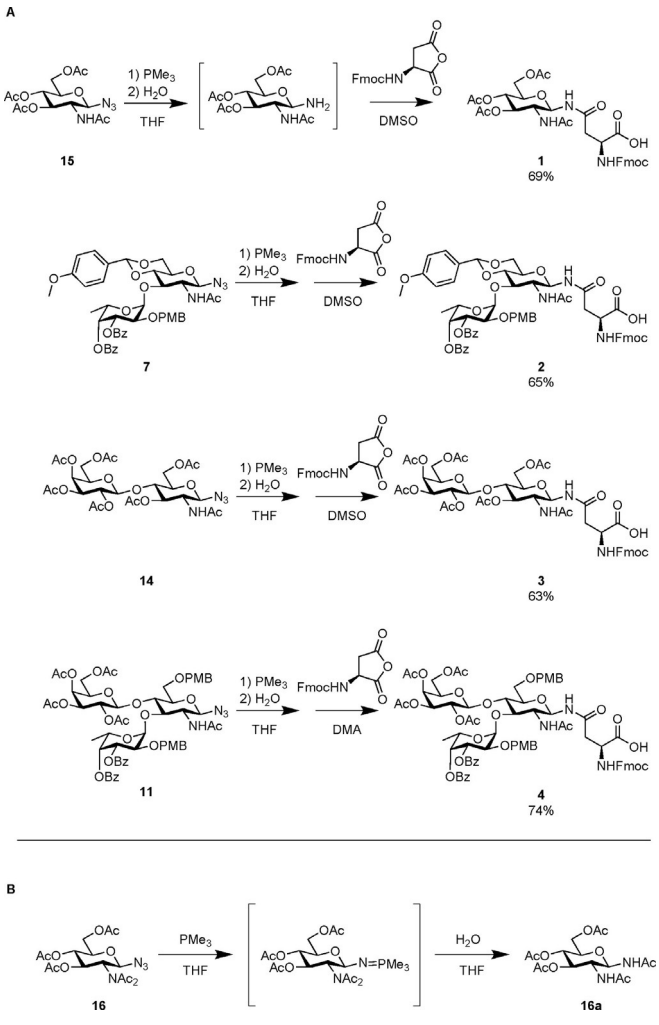
Asparagine derivatives **1**–**4** were prepared following a general synthetic strategy involving Staudinger reduction of the corresponding glycosyl azide, followed by direct ligation of the resulting glycosylamine with Fmoc-aspartic anhydride, a sequence reported recently by us (Scheme 3 A).[24] Accordingly, Fmoc-Asn(GlcNAc)-OH (**1**) was synthesized from the readily obtained glycosyl azide **15**[46] in three steps by PMe_3-mediated azide reduction, followed by the addition of H_2O to the crude iminophosphorane to obtain the intermediate glycosylamine. The desired asparagine derivative was formed by redissolving the crude glycosylamine in DMSO followed by the addition of Fmoc-aspartic anhydride. Precipitation directly afforded the desired SPPS building block **1** in a yield of 69%.

The above sequence proved similarly useful for the preparation of the other desired glycosylated asparagine building blocks **2**–**4** (Scheme 3 A). However, precipitation or extraction was found to be less efficient for small-scale purification of the more complex carbohydrates, and therefore we purified these compounds by silica gel chromatography. By using this approach, the fucosylated glycosyl azide **7** was converted into its corresponding SPPS building block **2** in a yield of 65%, and lactosyl compound **14** was similarly converted into compound **3** in a yield of 63% (Scheme 3 A).

For the trisaccharide glycosyl azide, transformation of the NAc_2 functionality into the acetamide was required, as Stau-



Scheme 2. Synthesis of (A) Lewis X azide **11** and (B) LacNAc azide **14**.



Scheme 3. A) Synthesis of glycosylated Fmoc-asparagine derivatives **1**–**4** by the two-step Staudinger reduction/aspartic anhydride coupling approach. B) Reaction observed when performing the Staudinger reduction of NAc_2-protected glycosyl azide **16**.

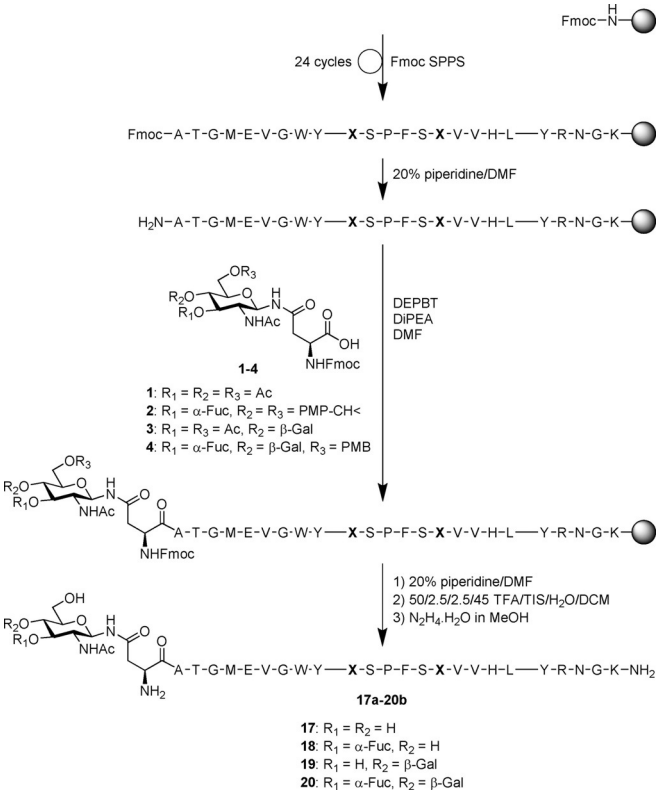
dinger reduction of **10** afforded conversion into an unknown side product. Acetyl migration is a likely explanation, as Staudinger reduction of the simpler NAc_2-protected glycosyl azide **16** afforded clean conversion into the more readily assignable glycosylacetamide **16a** (Scheme 3B). Glycosyl azide **11** was coupled to Fmoc-aspartic anhydride to yield the desired Le^X SPPS building block **4** as an inseparable 10:1 mixture with its corresponding isoasparagine isomeric product. It has been shown that dimethylacetamide (DMA) shows similar regioselectivity to DMSO when used as a solvent for aspartic anhydride ring-opening reactions.[47] However, the lower melting point of this solvent allows for aspartic anhydride ring-opening at 0 °C, potentially increasing the regioselectivity. Indeed, this solvent and temperature change resulted in the desired Le^X asparagine **4** being formed in a yield of 74% with complete regioselectivity.

The syntheses of the desired glycopeptides were initiated by automated SPPS of the $MOG_{32\text{-}55}$ peptide on Tentagel® S-RAM resin using HCTU (2-(6-chloro-1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethylaminium hexafluorophosphate) as the coupling reagent. These peptides where then manually elongated at the N terminus with the glycosylated asparagines **1**–**4** by using DEPBT (3-(diethoxyphosphoryloxy)-1,2,3-benzotriazin-4(3*H*)-one) as the coupling reagent to prevent aspartimide formation, as described by Yamamoto et al.[48] The general synthetic strategy used for the synthesis of the glycopeptides is outlined in Scheme 4.

The peptides were cleaved from the solid support under acidic conditions (95:2.5:2.5 TFA/TES/H_2O for 2 h; TFA = trifluoroacetic acid; TES = triethylsilane) for the non-fucosylated peptides, and under more dilute acidic conditions (50:2.5:2.5:45 TFA/TES/H_2O/DCM for 4 h) for the fucose-containing ones, to prevent hydrolysis of the acid-labile α-fucosyl bond.[49] The reaction time under these less acidic conditions had to be extended to ensure complete removal of the 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl (Pbf)-protecting groups, which are more acid-stable than the usual side-chain protecting groups (Boc/*t*Bu) in Fmoc-SPPS.[50]

To remove the remaining ester protecting groups on the carbohydrates, the crude peptides were treated with 10% hydrazine monohydrate in methanol. The crude glycopeptides were purified by preparative reversed-phase (RP) HPLC. All four different glycosylated asparagine building blocks exhibited good coupling efficiencies under the conditions used here (Scheme 4) and the neoglycopeptides **17a**--**20a** were isolated in moderate-to-good yields after RP-HPLC (Table 1).

To test whether glycosylation has an impact on the aggregation behavior of citrullinated MOG-derived peptides, we pre-



Scheme 4. Synthetic strategy employed for the synthesis of $MOG_{31\text{-}55}$ glycopeptides. X = Arg (**17a**–**20a**) or X = Cit (**17b**–**20b**).

Table 1. Yields of glycopeptides obtained by using the synthetic strategy outlined in Scheme 4 after preparative HPLC.[a]

	X = Arg	X = Cit
GlcNAc (**1**)	**17a** (4.0%)	**17b** (8.6%)
Fucα1-3GlcNAc (**2**)	**18a** (5.6%)	**18b** (2.1%, 5.7%[b])
LacNAc (**3**)	**19a** (5.8%)	**19b** (5.6%)
Lewis X (**4**)	**20a** (4.1%)	**20b** (6.1%, 4.8%[b])

[a] The number of each product is given together with the HPLC yield based on the crude product mass. [b] The product containing methionine oxidation was isolated separately.

pared MOG_{31-55} peptides carrying both post-translational modifications, namely citrullination and glycosylation. For the citrullination pattern, we chose to replace both Arg_{41} and Arg_{46} with citrullines, as we have previously shown that this citrullination pattern enhances aggregation behavior.[12] Furthermore, the location of the modifications in the putative MHC-I restricted non-human primate epitope MOG_{40-48}[51] was interesting, as citrullination of one of these positions has been demonstrated to exacerbate ongoing EAE.[52]

The citrullinated peptides **17b**–**20b** were synthesized by following the same methodology as used for their non-citrullinated counterparts, using Fmoc-citrulline as the 41st and 46th amino acids. Similar levels of glycosyl-amino acid incorporation and similar RP-HPLC yields were achieved in the synthesis of these glycopeptides (Table 1).

To assess the influence of glycosylation on immune-relevant MOG_{31-55}, we opted for different biophysical and biochemical experiments. First, we determined the secondary structure in solution by circular dichroism (CD). All the peptides showed a predominantly random-coil structure. The effect of addition of the α-helix stabilizer trifluoroethanol (TFE) (50% v/v in phosphate buffered saline (PBS)) or sodium dodecyl sulfate (SDS) at non-micellar concentrations (4 mM) was also evaluated (see Figure S1 in the Supporting Information). The results indicated that the peptides are not prone to β-sheet formation.

Next, inspired by the recently reported aggregation behavior of citrullinated MOG_{31-55} peptides, we evaluated the susceptibility of all the glycopeptides to amyloid-like aggregation by using the previously described Thioflavin T (ThT) fluorescence assay.[12] In this assay, a fluorogenic substrate, Thioflavin T, with a selectivity towards cross-β-sheet structures as found in amyloid-like aggregates, is used to detect whether such aggregation occurs: The non-citrillunated peptides did not show aggregation at physiologically relevant concentrations (10 μM, Figure 1 A). For the citrullinated peptides, the effect of glycosylation seemed to be structure-dependent.

Although all forms of glycosylation had an inhibitory effect on aggregation (Figure 1 B), the inclusion of a single GlcNAc modification (**17b**) was sufficient to completely abrogate the aggregation, displaying the powerful effect glycosylation can have on peptide aggregation. The DC-SIGN ligand Le^X (**20b**) showed a similar inhibition of aggregation to that of GlcNAc, which suggests potential in controlling the immune household and not the neurodegenerative mechanism in MS. However,

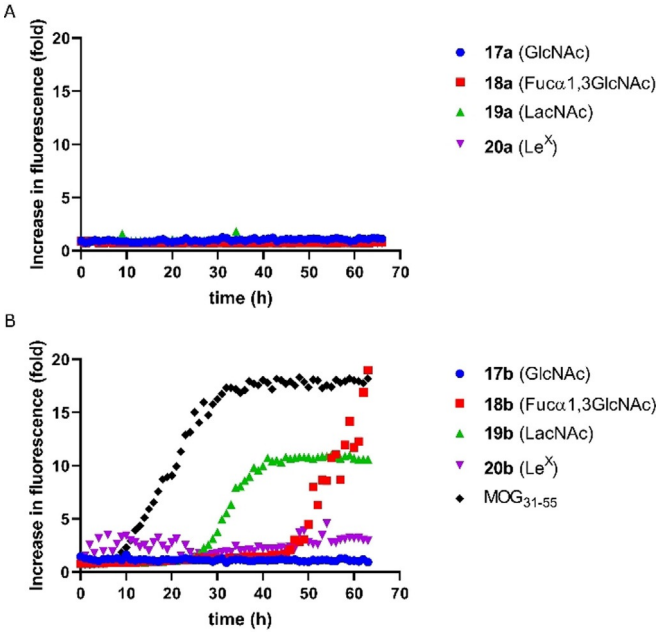


Figure 1. ThT aggregation assay of (A) non-citrullinated and (B) citrullinated glycosylated MOG_{31-55} peptides **17a**–**20b**. The peptides were tested at a concentration of 10 μM. The positive control (black diamonds) is non-glycosylated MOG_{31-55} citrullinated at positions 41 and 46. All the data were recorded at an excitation wavelength of 444 ± 9 nm and an emission wavelength of 485 ± 9 nm. All samples were used at pH 5.0 and the aggregation assays were performed at least three times and with experimental triplicates.

the other glycosylation patterns tested, Fucα1-3GlcNAc (**18b**) and LacNAc (**19b**), did not fully inhibit aggregation, delaying only its onset (Figure 1 B). In a previous study,[12] we showed that citrullinated MOG_{35-55} peptides are cytotoxic to murine bone marrow derived dendritic cells (BMDCs). Citrullinated MOG_{31-55}, however, has not yet been tested. To analyze whether native, glycosylated, or citrullinated MOG_{31-55} variants show similar cytotoxicity to those of citrullinated MOG_{35-55}, we conducted cell viability assays using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT assay), as described previously (see Figure S1 in the Supporting Information).[12] The BMDCs were treated with the citrullinated peptides **18b**–**20b**, as well as their non-glycosylated counterpart (cit MOG_{31-55}) at four different concentrations (40, 20, 10, and 5 μM). None of the tested peptides showed a significant decrease in viability of the BMDCs at any concentration tested. Furthermore, no significant drop in the viability of the BMDCs was observed with the glycosylated MOG_{31-55} derivatives or the native variant.

Overall, it can be concluded that the glycosylation of MOG_{31-55} does not alter its biophysical properties, as measured by CD, whereas GlcNAc and Le^X modifications abrogate the amyloid-like behavior of MOG_{31-55}. Moreover, no major cytotoxic effects were observed for the citrullinated and glycosylated MOG_{31-55} derivatives in the BMDCs, which renders them useful for subsequent studies to explore the impact of DC-SIGN binding on moDCs.

Next, we investigated the physiological relevance of our simplified *N*-glycan structures. To assess the ability of the model

N-glycans to bind DC-SIGN, a DC-SIGN binding ELISA was carried out.[53,54] Briefly, the peptides were coated onto the bottom of high-binding plates. DC-SIGN binding was then assessed by incubation with a recombinant DC-SIGN-Fc construct (the N-terminally truncated extracellular domain (K_{62}-A_{404}) of human DC-SIGN expressed with the fused Fc region (fragment crystallizable region) of human IgG1 at the N terminus), followed by an horseradish peroxidase (HRP)-conjugated secondary antibody for a qualitative readout of the binding. The results of this assay are displayed in Figure 2.

As expected,[19] Le^X peptides **20a** (Figure 2A) and **20b** (Figure S3 in the Supporting Information) were readily recognized by DC-SIGN-Fc, whereas the other glycopeptides were recognized to a lesser extent (**18a**,**b**, see Figure S3) or not at all (**19a**,**b**, Figure 2A and Figure S3). Note that we observed an increase in the binding affinity of the GlcNAcylated peptide **17a** to DC-SIGN (see Figure S3). This can be explained by GlcNAc being a weak binder to DC-SIGN with an IC_{50} of 5 mM in vitro.[55] Citrullination of this peptide, **17b**, inhibited the increase in binding affinity (see Figure S3).

Finally, we investigated the downstream effects of the stimulation of human moDCs with Le^X-decorated peptide **20a**. Because DC-SIGN is absent on murine DCs,[56] human dendritic cells, derived from donor blood, are a useful alternative. Furthermore, the pathophysiology of MS is not completely mimicked by murine EAE,[57] further necessitating the use of human model systems when feasible. We used a well-established assay[58] to measure the release of the anti- and pro-inflammatory cytokines IL-10 and IL-12p70, respectively. It has been shown that the stimulation of DC-SIGN with fucosylated glycoconjugates (in the presence of TLR4 ligands) induces an upregulation of IL-10 and a downregulation of IL-12p70, switching the immune response towards tolerance instead of inflammation. In this assay, moDCs, derived from peripheral blood monocytes (PBMCs) of three donors, were stimulated with peptide **20a** or non-glycosylated MOG_{31-55} at distinct concentrations (14, 7, and 3.5 μM) in the presence or absence of the TLR4 ligand lipopolysaccharide (LPS) (from *E. coli* at 10 ng mL^{-1}), and their cytokine secretion levels were measured.[59] As expected, no cytokine production was observed upon stimulation of the moDCs with peptide in the absence of LPS (see Figure S4 in the Supporting Information). However, upon co-stimulation with LPS, we observed an Le^X-dependent effect for MOG_{31-55} on IL-12p70 secretion at all concentrations tested for peptide **20a**. The ratio of IL-10/IL-12p70 secretion is plotted for a single donor in Figure 2B (representative of three independent experiments, $N=3$), which shows a higher ratio for the Le^X-decorated neoglycopeptide **20a** compared with the non-glycosylated control at all concentrations tested. This increase in IL10/IL12p70 ratio shows that stimulation with peptide **20a** leads to a more tolerogenic response compared with non-glycosylated MOG_{31-55}. Figure 2C shows plots of the ratio of cytokine secretion for the stimulation of moDCs with **20a** and non-glycosylated MOG_{31-55} for all donors ($N=3$) at three different concentrations. A reduction in secretion of pro-inflammatory cytokine IL-12p70 is observed, whereas the secretion of anti-inflammatory IL-10 remains unchanged. Because the DC-SIGN-Fc binding ELISA shows a binding interaction between the Le^X-decorated peptide and not the non-glycosylated peptide, a DC-SIGN-driven process is strongly suggested.

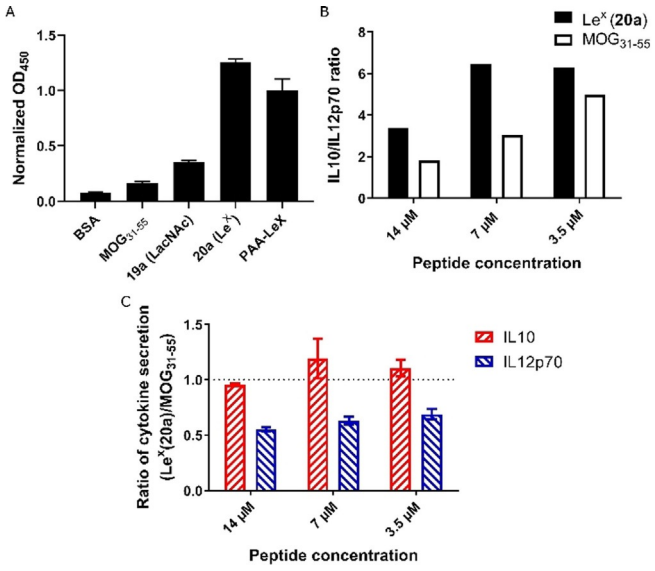


Figure 2. In vitro DC-SIGN binding assay and moDC cytokine profiling upon exposure to **20a**. (A) In vitro DC-SIGN-FC ELISA binding assay. Lewis X decorated polymer (PAA-LeX) was used as the positive control, whereas for the negative control, no peptide was added, meaning they are fully blocked with bovine serum albumin (BSA). The DC-SIGN ELISA assay was performed three times showing similar results. The graph shows the data from one representative experiment out of three independent experiments performed in duplicate. Error bars represent standard deviation. (B) Ratio of IL10/IL12p70 secretion measured upon moDC stimulation with either **20a** or the non-glycosylated control in the presence of 10 ng mL^{-1} of LPS. This graph is a representative plot from one donor ($N=3$). (C) Normalized ratios for IL-10 and IL-12p70 secretion for peptide **20a** harboring Le^X and non-glycosylated peptide MOG_{31-55} incubated with moDCs at different concentrations in the presence 10 ng mL^{-1} LPS. Here, a ratio of 1 means cytokine production is the same for both peptides, whereas a ratio of 0.5 means cytokine production is halved for **20a** compared with the non-glycosylated peptide. The results are the average of three experiments performed using cells from three separate donors, each measured in duplicate.

Conclusions

We have developed a synthetic route to three novel SPPS-compatible glycosylated Fmoc-asparagine building blocks, including an asparagine derivative of the important DC-SIGN ligand Le^X. These building blocks were synthesized from the corresponding glycosyl azides by using our Staudinger reduction/aspartic anhydride ring-opening approach. By careful choice of protecting groups during the oligosaccharide assembly, the number of protecting group manipulations could be kept to a minimum and glycopeptide deprotection was accomplished in a straightforward manner. To demonstrate this, we synthesized glycosylated derivatives of the peptide MOG_{31-55} in good yields and purity, as well as derivatives that are both glycosylated and citrullinated.

Using these synthetic neoglycopeptides, we have demonstrated that glycosylation has a powerful effect on the citrullination-driven aggregation of this model peptide. Interestingly,

the effect glycosylation has on citrullination-driven aggregation also seems to be dependent on oligosaccharide structure. Furthermore, we have shown by ELISA that Le^X, when linked to asparagine directly through an amide bond, is capable of binding to DC-SIGN. Finally, we have shown that a peptide decorated with Le^X on asparagine is able to elicit a tolerogenic response (reduced IL12p70 secretion compared with the non-glycosylated counterpart) when used to stimulate moDCs.

Experimental Section

Disclaimer for the use of human moDCs: Peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats obtained by Sanquin Blood bank, Amsterdam, The Netherlands, from healthy adult volunteers (blood donors) following written informed consent in accordance with the Declaration of Helsinki. The Molecular Cell Biology and Immunology department at VU Medisch Centrum has a written agreement with the mentioned Sanquin Blood bank, Amsterdam (NVT0203.01) approved and in agreement with the local ethical committee.

General methods for SPPS: An automated synthesizer (PTI Tribute UV-IR synthesizer, Gyros Protein Technologies) was used for SPPS. Unless stated otherwise, the peptides were synthesized on Tentagel® S-RAM resin (Rapp Polymere, Germany) on a 100 μmol scale using 5.0 equiv of each amino acid with respect to the resin loading. Fmoc-protected amino acids were purchased from either Novabiochem or Sigma–Aldrich. For the amino acids that requires side-chain protection, the following protecting groups were used: *t*Bu for Ser, Thr, and Tyr; O*t*Bu for Asp and Glu; Trt for Asn, Gln, and His; Boc for Lys and Trp; Pbf for Arg. An equimolar quantity of HCTU was used as activator. Coupling cycles of 1 h were used, and unreacted amines were capped after each cycle by using a solution of acetic anhydride (500 μL), DiPEA (250 μL), and DMF (4.25 mL) for 5 min at room temperature twice. Fmoc deprotection was accomplished with 20% piperidine in DMF (3×5 min). Cleavage of non-glycosylated peptides was accomplished by using a 95:2.5:2.5 mixture of TFA/TES/H_2O for 3 h, followed by precipitation from cold diethyl ether and recovery of the precipitate by centrifugation. The peptides were characterized by electrospray ionization mass spectrometry (ESI-MS) on a Thermo Finnigan LCQ Advantage Max LC-MS instrument with a Surveyor PDA plus UV detector on an analytical C18 column (Phenomenex, 3 μm, 110 Å, 50 mm×4.6 mm) in combination with buffers A (H_2O), B (MeCN), and C (1% aq. TFA). The quality of the crude peptides was evaluated by using a linear gradient of 10–50% B with a constant 10% C over 10 min, and the final peptide quality was evaluated by using a linear gradient of 5–65% B with a constant 10% C over 30 min.

Incorporation of glycosylated amino acids: The glycopeptides was synthesized on the 25 μmol scale. The Fmoc group was removed from the resin-bound peptide by using 20% piperidine in DMF (2×2 mL, 3+7 min). After Fmoc deprotection, the resin was washed five times with DMF (5×5 mL). Fully protected glycosylated asparagine (2 equiv, 50 μmol) was dissolved in a 0.3 M solution of DEPBT in DMF (500 μL) with the addition of DiPEA (8.7 μL, 2 equiv, 50 μmol). The mixture was agitated for at least 5 min or until all the amino acid had been dissolved. The solution containing the activated amino acid was added to the resin, and the resin was incubated overnight under mild agitation. After overnight coupling, the resin was washed with DMF (5×5 mL) and a small portion was deprotected to confirm incorporation of the glycosylated amino acid. Fmoc deprotection was carried out as normal by using a freshly prepared piperidine solution. Full cleavage of the peptide was achieved by using 2 mL of a 95:2.5:2.5 mixture of TFA/TES/H_2O for 2 h or a 50:2.5:2.5:45 mixture of TFA/TES/H_2O/DCM for 4 h for fucose-containing peptides. The deprotected peptide was precipitated in cold diethyl ether (10 mL) and the resin was washed with DCM (1 mL), added to the ether phase. After centrifuging, the pellet was washed with a small amount of diethyl ether (3–5 mL) and centrifuged again. To facilitate the removal of the ester-protecting groups, the peptide was suspended in methanol (2.25 mL) in a round-bottomed flask and placed under N_2 atmosphere, followed by the addition of hydrazine monohydrate (0.25 mL). After stirring overnight, the reaction progress was checked by LC-MS. When complete deprotection was verified, the volatiles were removed in vacuo to yield the crude glycopeptide. Preparative RP-HPLC on a Waters AutoPurification system (eluent A: H_2O + 0.2% TFA; eluent B: CH_3CN) with a preparative Gemini C18 column (5 μm, 150×21.2 mm) yielded the final products.

***N*$^{\gamma}$-[3,4,6-Tri-*O*-acetyl-2-deoxy-2-acetamido-β-D-glucopyranosyl]-*N*$^{\alpha}$-fluorenylmethoxycarbonyl-L-asparagine (1)**: Glycosyl azide **15** (200 mg, 0.54 mmol) was dissolved in THF (0.76 mL) and the solution cooled in an icebath. A 1 M solution of trimethylphosphine (1.0 equiv, 0.54 mmol, 0.54 mL) in THF was added dropwise over 2 min, during which gas evolution was observed. The icebath was removed and the reaction was stirred for 5 min before H_2O (10 equiv, 97 μL, 5.4 mmol) was added. The reaction mixture was stirred at room temperature for 1.5 h, after which it was concentrated. The residue containing the crude glycosylamine was redissolved in DMSO (1.8 mL) and Fmoc-aspartic anhydride[60] (1.0 equiv, 181 mg, 0.54 mmol) was added. The reaction mixture was stirred for 2 h at room temperature. The DMSO solution was then added dropwise to a centrifuge tube containing a 2:1 mixture of diethyl ether and ethyl acetate (30 mL) and a precipitate started to form. The compound was left to fully precipitate for 16 h at room temperature, after which it was collected by centrifugation. The supernatant was discarded and the pellet was washed with a small amount of the diethyl ether/ethyl acetate (2:1) mixture. After removing the volatiles under reduced pressure, the title compound was obtained as a white amorphous solid (255 mg, 0.37 mmol, 69%). ^{1}H NMR (500 MHz, [D$_6$]DMSO): δ = 8.60 (d, J = 9.8 Hz, 1H; γN-H), 7.99–7.78 (m, 3H; N*H*C(O)CH$_3$), 7.71 (d, J = 7.5 Hz, 2H; Fmoc-Ar), 7.51 (d, J = 8.5 Hz, 1H; αN-H), 7.41 (t, J = 7.5 Hz, 2H; Fmoc-Ar), 7.32 (t, J = 7.4 Hz, 2H; Fmoc-Ar), 5.18 (t, J = 9.8 Hz, 1H; 1-H), 5.10 (t, J = 9.8 Hz, 1H; 3-H), 4.82 (t, J = 9.8 Hz, 1H; 4-H), 4.38 (q, J = 7.5 Hz, 1H; Asn-CH), 4.33–4.13 (m, 4H; Fmoc-CH$_2$, Fmoc-CH, 6a-H), 3.94 (d, J = 11.3 Hz, 1H; 6b-H), 3.88 (q, J = 9.8 Hz, 1H; 2-H), 3.84–3.78 (m, 1H; 5-H), 2.66 (dd, J = 16.3, 5.4 Hz, 1H, Asn-C*H*H), 1.99 (s, 3H; OC(O)CH$_3$), 1.96 (s, 3H; OC(O)CH$_3$), 1.90 (s, 3H; OC(O)CH$_3$), 1.72 ppm (s, 3H; NHC(O)C*H*$_3$); ^{13}C NMR (126 MHz, [D$_6$]DMSO): δ = 173.0 (C=O), 170.1 (C=O), 169.9 (C=O), 169.6 (C=O), 169.6 (C=O), 169.4 (C=O), 155.9 (C=O), 143.8 (Fmoc-Ar), 143.8 (Fmoc-Ar), 140.7 (Fmoc-Ar), 127.7 (Fmoc-Ar), 127.1 (Fmoc-Ar), 125.3 (Fmoc-Ar), 120.2 (Fmoc-Ar), 78.1 (C-1), 73.4 (C-3), 72.3 (C-5), 68.4 (C-4), 65.8 (Fmoc-CH$_2$), 61.9 (C-6), 52.2 (C-2), 50.0 (Asn-CH), 46.6 (Fmoc-CH), 36.9 (Asn-CH$_2$), 22.6 (NHC(O)*C*H$_3$), 20.6 (OC(O)*C*H$_3$), 20.4 (OC(O)*C*H$_3$), 20.4 ppm (OC(O)*C*H$_3$); HRMS (ESI): *m/z*: calcd for $C_{33}H_{37}N_3O_{13}H$: 684.23991; found: 684.23920 $[M+H]^+$.

***N*$^{\gamma}$-[3,4-Di-*O*-benzoyl-2-*O*-(4-methoxybenzyl)-α-l-fucopyranoside-(1→3)-4,6-*O*-(4-methoxybenzylidene)-2-deoxy-2-acetamido-β-D-glucopyranosyl]-*N*$^{\alpha}$-fluorenylmethoxycarbonyl-L-asparagine (2)**: Glycosyl azide **7** (168 mg, 0.2 mmol) was dissolved in dry THF (2 mL) and trimethylphosphine was added as a 1 M solution in THF (1.1 equiv, 220 μL, 0.22 mmol). The reaction mixture was stirred for 10 min at room temperature and H_2O (50 equiv, 180 μL, 10 mmol) was then added. After stirring for 1 h at room temperature, the re-

action mixture was concentrated and the residue dissolved in DMSO (2 mL). Fmoc-aspartic anhydride[60] (1.0 equiv, 67 mg, 0.2 mmol) was added and the reaction mixture stirred for 1 h at room temperature. The solvent was removed in vacuo and the crude was subjected to silica gel column chromatography (0→8% MeOH in DCM, $\Delta = 1\%$). This yielded the title compound (150 mg, 0.13 mmol, 65%). $[\alpha]_D^{25} = -73.3$ ($c = 1.00$ in $CHCl_3$); ^{1}H NMR (400 MHz, [D$_6$]DMSO): $\delta = 8.46$ (d, $J = 9.4$ Hz, 1H; γN-H), 8.16 (d, $J = 9.0$ Hz, 1H; N*H*C(O)CH$_3$), 7.87 (t, $J = 8.0$ Hz, 3H; CH$_{arom}$), 7.81–7.64 (m, 5H; CH$_{arom}$), 7.64–7.47 (m, 5H; CH$_{arom}$), 7.47–7.26 (m, 8H; αN-H, CH$_{arom}$), 7.18–7.04 (m, 2H; CH$_{arom}$), 6.97–6.89 (m, 2H; CH$_{arom}$), 6.73–6.62 (m, 2H; CH$_{arom}$), 5.71 (s, 1H; PMP-CH$_{acetal}$), 5.42–5.33 (m, 2H; 1′-H, 3′-H), 5.23 (d, $J = 3.5$ Hz, 1H; 4′-H), 5.15 (t, $J = 9.5$ Hz, 1H; 1-H), 4.55–4.43 (m, 2H; 5′-H, PMB-C*H*H), 4.39–4.30 (m, 2H; PMB-CH*H*, Asn-CH), 4.30–4.17 (m, 4H; Fmoc-CH$_2$, 5-H, Fmoc-CH), 4.13 (t, $J = 9.5$ Hz, 1H; 3-H), 3.99 (dd, $J = 10.7$, 3.5 Hz, 1H; 2′-H), 3.95–3.84 (m, 1H; 2-H), 3.76–3.60 (m, 9H; 6-H, 4-H, OCH$_3$, OCH$_3$), 2.66 (dd, $J = 16.1$, 5.6 Hz, 1H; Asn-C*H*H), 1.82 (s, 3H; NHC(O)C*H*$_3$), 0.46 ppm (d, $J = 6.4$ Hz, 3H; 6′-H); ^{13}C NMR (101 MHz, [D$_6$]DMSO): $\delta = 170.2$ (C=O), 169.7 (C=O), 165.6 (C=O), 164.8 (C=O), 159.7 (C$_q$), 158.8 (C$_q$), 155.9 (C=O), 143.9 (Fmoc-Ar), 143.8 (Fmoc-Ar), 140.7 (Fmoc-Ar), 133.7 (CH$_{arom}$), 133.5 (CH$_{arom}$), 130.0 (C$_q$), 129.2 (CH$_{arom}$), 129.1 (C$_q$), 129.0 (CH$_{arom}$), 128.8 (CH$_{arom}$), 128.5 (CH$_{arom}$), 127.8 (CH$_{arom}$), 127.8 (CH$_{arom}$), 127.7 (CH$_{arom}$), 127.1 (CH$_{arom}$), 125.3 (CH$_{arom}$), 120.1 (CH$_{arom}$), 113.4 (CH$_{arom}$), 100.9 (PMP-CH), 96.2 (C-1′), 79.4 (C-1, C-4), 75.3 (C-3), 72.3 (C-4′), 71.5 (C-2′), 70.0 (PMB-CH$_2$), 69.6 (C-3′), 68.0 (C-5), 67.8 (C-6), 65.8 (Fmoc-CH$_2$), 63.9 (C-5′), 55.1 (OCH$_3$, C-2), 55.0 (OCH$_3$), 50.4 (Asn-CH), 46.6 (Fmoc-CH), 37.3 (Asn-CH$_2$), 23.1 (NHC(O)C*H*$_3$), 15.2 ppm (C-6′); HRMS (ESI): *m/z*: calcd for $C_{63}H_{63}N_3O_{18}H$: 1150.41794; found: 1150.41741 $[M+H]^+$.

N^{γ}-[2,3,4,6-Tetra-*O*-acetyl-β-D-galactopyranoside-(1→4)-3,6-di-*O*-acetyl-2-deoxy-2-acetamido-β-D-glucopyranosyl]-N^{α}-fluorenylmethoxycarbonyl-L-asparagine (3): Azido sugar **14** (0.74 mmol, 488 mg) was dissolved in THF (7.4 mL), a 1 M solution of trimethylphosphine in THF (1.5 equiv, 1.1 mL, 1.1 mmol) was added, and the reaction mixture was stirred at room temperature. H_2O (50 equiv, 0.67 mL, 37 mmol) was added and the reaction mixture further stirred for 60 min. The volatiles were removed in vacuo and the crude glycosylamine was redissolved in DMSO (7.4 mL). Fmoc-aspartic anhydride (1 equiv, 0.74 mmol, 249 mg) was then added and the reaction mixture was stirred for 75 min. The solvent was removed in vacuo and the crude was subjected to silica gel column chromatography (0→8% MeOH in DCM, $\Delta = 1\%$) to yield the title product (455 mg, 0.47 mmol, 63%). $[\alpha]_D^{20} = +0{,}2$ ($c = 1.00$ in MeOH); ^{1}H NMR (400 MHz, [D$_6$]DMSO): $\delta = 8.58$ (d, $J = 9.1$ Hz, 1H; γN-H), 7.89 (d, $J = 7.7$ Hz, 2H; Fmoc-Ar), 7.86 (d, $J = 9.5$ Hz, 1H; N*H*C(O)CH$_3$), 7.71 (d, $J = 7.5$ Hz, 2H; Fmoc-Ar), 7.42 (t, $J = 7.3$ Hz, 3H; Fmoc-Ar, αN-H), 7.33 (t, $J = 7.4$ Hz, 2H; Fmoc-Ar), 5.23 (d, $J = 3.7$ Hz, 1H; 4′-H), 5.16 (dd, $J = 10.3$, 3.6 Hz, 1H; 3′-H), 5.10 (t, $J = 9.5$ Hz, 1H; 1-H), 4.97 (t, $J = 9.5$ Hz, 1H; 3-H), 4.84 (dd, $J = 10.3$, 8.0 Hz, 1H; 2′-H), 4.70 (d, $J = 8.0$ Hz, 1H; 1′-H), 4.36–4.15 (m, 6H; Asn-CH, Fmoc-CH$_2$, 6a-H, 5′-H, Fmoc-CH), 4.09–3.95 (m, 3H; 6b-H, 6′-H), 3.81 (q, $J = 9.5$ Hz, 1H; 2-H), 3.73–3.55 (m, 2H; 4-H, 5-H), 2.63 (dd, $J = 16.3$, 5.3 Hz, 1H; Asn-CH$_2$), 2.11 (s, 3H; C(O)C*H*$_3$), 2.07 (s, 3H; C(O)C*H*$_3$), 2.01 (s, 3H; C(O)C*H*$_3$), 2.01 (s, 3H; C(O)C*H*$_3$), 1.94 (s, 3H; C(O)C*H*$_3$), 1.90 (s, 3H; C(O)C*H*$_3$), 1.71 ppm (s, 3H; NH(CO)C*H*$_3$); ^{13}C NMR (101 MHz, [D$_6$]DMSO): $\delta = 173.1$ (C=O), 170.4 (C=O), 170.0 (C=O), 169.9 (C=O), 169.6 (C=O), 169.5 (C=O), 169.3 (C=O), 169.2 (C=O), 155.8 (C=O), 143.8 (Fmoc-Ar), 140.7 (Fmoc-Ar), 127.7 (Fmoc-Ar), 127.1 (Fmoc-Ar), 125.3 (Fmoc-Ar), 120.2 (Fmoc-Ar), 99.9 (C-1′), 77.9 (C-1), 76.2 (C-4), 73.8 (C-3), 73.5 (C-5), 70.4 (C-3′), 69.7 (C-5′), 68.9 (C-2′), 67.1 (C-4′), 65.7 (Fmoc-CH$_2$), 62.5 (C-6), 60.9 (C-6′), 52.3 (C-2), 50.3 (Asn-CH), 46.6 (Fmoc-CH), 37.1 (Asn-CH$_2$), 22.7 (NHC(O)C*H*$_3$), 20.7 (C(O)C*H*$_3$), 20.6 (C(O)C*H*$_3$), 20.5 (C(O)C*H*$_3$), 20.4 (C(O)C*H*$_3$), 20.4 ppm (C(O)C*H*$_3$); HRMS (ESI): *m/z*: calcd for $C_{45}H_{53}N_3O_{21}H$: 972.32443; found: 972.32357$[M+H]^+$.

N^{γ}-{2,3,4,6-Tetra-*O*-acetyl-β-D-galactopyranoside-(1→4)-[3,4-di-*O*-benzoyl-2-*O*-(4-methoxybenzyl)-α-L-fucopyranoside-(1→3)]-6-*O*-(4-methoxybenzyl)-2-deoxy-2-acetamido-β-D-glucopyranosyl}-N^{α}-fluorenylmethoxycarbonyl-L-asparagine (4): Glycosyl azide 11 (53 mg, 45 μmol) was dissolved in dry THF (0.45 mL) and cooled to 0 °C in an icebath. A 1 M trimethylphosphine solution in THF (75 μL) was added dropwise. The reaction mixture was stirred for 5 min at 0 °C and for 5 min at room temperature. H_2O (50 equiv, 40 μL, 2.25 mmol) was added and the reaction mixture stirred for 2 h at room temperature. The volatiles were removed in vacuo and the crude glycosylamine was redissolved in DMA (450 μL). The reaction mixture was again cooled in an icebath and aspartic anhydride[60] (1 equiv, 15 mg, 45 μmol) was added. The reaction mixture was stirred and then allowed to warm to room temperature overnight. The solvent was removed by evaporation and the crude glyco-amino acid was subjected to silica gel column chromatography (0→25% acetone in DCM + 0.5% acetic acid, $\Delta_{acetone} = 5\%$) to yield the title compound (49 mg, 33 μmol, 74%). Traces of acetic acid were removed by sequential co-evaporation with dioxane (3× 2 mL), toluene (3×2 mL), and $CHCl_3$ (3×2 mL). $[\alpha]_D^{25} = -94.2$ ($c = 1.00$ in $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 7.98$–7.89 (m, 2H; CH$_{arom}$), 7.78–7.64 (m, 5H; γN-H, CH$_{arom}$), 7.64–7.51 (m, 3H; CH$_{arom}$), 7.51–7.40 (m, 3H; CH$_{arom}$), 7.40–7.19 (m, 9H; N*H*C(O)CH$_3$, CH$_{arom}$), 7.07 (d, $J = 8.6$ Hz, 2H; CH$_{arom}$), 6.91 (d, $J = 8.6$ Hz, 2H; CH$_{arom}$), 6.66 (d, $J = 8.3$ Hz, 2H; CH$_{arom}$), 6.41 (d, $J = 8.4$ Hz, 1H; αN-H), 5.63–5.54 (m, 2H; 4′-H, 3′-H), 5.47 (d, $J = 3.4$ Hz, 1H; 1′-H), 5.33 (d, $J = 3.7$ Hz, 1H; 4′-H), 5.09 (dd, $J = 10.4$, 8.0 Hz, 1H; 2″-H), 4.99 (t, $J = 7.8$ Hz, 1H; 1-H), 4.85 (dd, $J = 10.4$, 3.6 Hz, 1H; 3″-H), 4.81–4.70 (m, 1H; 5′-H), 4.69–4.43 (m, 5H; PMB-CH$_2$, Asn-CH, Fmoc-CH, 1″-H), 4.39–4.22 (m, 5H; Fmoc-CH$_2$, PMB-C*H*H, 6″-H), 4.22–4.03 (m, 4H; PMB-CH*H*, 2-H, 2′-H, 4-H), 3.95 (t, $J = 8.4$ Hz, 1H; 3-H), 3.82–3.63 (m, 8H; OCH$_3$, 6-H, OCH$_3$), 3.57–3.44 (m, 2H; 5-H, 5″-H), 2.90–2.72 (m, 2H; Asn-CH$_2$), 2.17 (s, 3H; C(O)CH$_3$), 2.07–1.91 (m, 12H; 4×C(O)CH$_3$), 1.24 ppm (d, $J = 6.5$ Hz, 3H; 6′-H); ^{13}C NMR (101 MHz, $CDCl_3$): $\delta =$ 173.5 (C=O), 173.2 (C=O), 171.8 (C=O), 170.5 (C=O), 170.4 (C=O), 170.0 (C=O), 169.8 (C=O), 165.9 (C=O), 165.3 (C=O), 159.6 (C$_q$), 159.5 (C$_q$), 156.4 (C=O), 143.9 (Fmoc-Ar), 143.7 (Fmoc-Ar), 141.2 (Fmoc-Ar), 141.2 (Fmoc-Ar), 133.3 (CH$_{arom}$), 133.1 (CH$_{arom}$), 130.3 (CH$_{arom}$), 129.8 (CH$_{arom}$), 129.7 (CH$_{arom}$), 129.6 (CH$_{arom}$), 129.6 (C$_q$), 129.5 (C$_q$), 128.9 (C$_q$), 128.5 (CH$_{arom}$), 128.3 (CH$_{arom}$), 127.7 (CH$_{arom}$), 127.1 (CH$_{arom}$), 125.3 (CH$_{arom}$), 125.2 (CH$_{arom}$), 119.9 (CH$_{arom}$), 114.1 (CH$_{arom}$), 114.0 (CH$_{arom}$), 99.4 (C-1″), 97.4 (C-1′), 79.7 (C-1), 76.0 (C-5, C-3), 73.3 (C-4), 73.3 (C-2′), 73.3 (PMB-CH$_2$), 72.7 (PMB-CH$_2$), 72.5 (C-4′), 71.0 (C-5″), 70.8 (C-3″), 70.1 (C-3′), 69.3 (C-2″), 67.8 (C-6), 67.2 (Fmoc-CH$_2$), 66.9 (C-4″), 65.8 (C-5′), 61.0 (C-6″), 55.3 (OCH$_3$), 55.2 (OCH$_3$), 53.6 (C-2), 50.5 (Asn-CH), 47.1 (Fmoc-CH), 37.9 (Asn-CH$_2$), 22.8 (NHC(O)C*H*$_3$), 20.8 (C(O)C*H*$_3$), 20.8 (C(O)C*H*$_3$), 20.7 (C(O)C*H*$_3$), 20.6 (C(O)C*H*$_3$), 16.1 ppm (C-6′); HRMS (ESI): *m/z*: calcd for $C_{77}H_{83}N_3O_{27}Na$: 1504.51061; found: 1504.51004 $[M+Na]^+$.

3,4-di-*O*-benzoyl-2-*O*-(4-methoxybenzyl)-α-L-fucopyranoside-(1→3)-4,6-*O*-(4-methoxybenzylidene)-2-deoxy-2-acetamido-β-D-glucopyranosyl azide (7): Donor **6** (1.5 equiv, 1.76 mg, 3.0 mmol) and acceptor **5** (728 mg, 2.0 mmol) were co-evaporated three times with toluene, backfilling the flask with N_2 after every co-evaporation, and placed under a N_2 atmosphere. The sugars were dissolved in a mixture of dry DCM (36 mL) and dry DMF (4 mL). Activated 4 Å molecular sieves (1 g) were added and the solution was stirred for 90 min. The reaction mixture was then cooled in an icebath and NIS (2.0 equiv, 900 mg, 4.0 mmol) and TMSOTf (0.1 equiv, 37 μL) were added. The reaction mixture was stirred and allowed to warm

to room temperature overnight. The reaction mixture was filtered, diluted with DCM, and washed with a 1:1 mixture of 10% $Na_2S_2O_3$ (aq) and saturated $NaHCO_3$ (aq). The organic layer was dried over $MgSO_4$, filtered, and concentrated. Silica gel column chromatography (30%→40%→50%→60% EtOAc in pentane) yielded the title compound (1.19 g, 1.42 mmol, 71%). $[\alpha]_D^{25} = -144.0$ ($c = 1.00$ in $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 8.00–7.89$ (m, 2H; CH_{arom}), 7.82–7.75 (m, 2H; CH_{arom}), 7.64–7.56 (m, 1H; CH_{arom}), 7.54–7.40 (m, 5H; CH_{arom}), 7.33–7.27 (m, 2H; CH_{arom}), 7.15–7.08 (m, 2H; CH_{arom}), 6.88 (d, $J = 8.8$ Hz, 2H; CH_{arom}), 6.74 (d, $J = 8.6$ Hz, 2H; CH_{arom}), 6.05 (d, $J = 6.9$ Hz, 1H; NH), 5.73 (dd, $J = 10.5$, 3.3 Hz, 1H; 3′-H), 5.53 (s, 1H; PMP-CH_{acetal}), 5.50 (dd, $J = 3.4$, 1.4 Hz, 1H; 4′-H), 5.28 (d, $J = 9.3$ Hz, 1H; 1-H), 5.15 (d, $J = 3.5$ Hz, 1H; 1′-H), 4.61 (d, $J = 11.4$ Hz, 1H; PMB-C*H*H), 4.54 (d, $J = 11.4$ Hz, 1H; PMB-CH*H*), 4.51–4.42 (m, 2H; 3-H, 5′-H), 4.38 (dd, $J = 10.5$, 4.2 Hz, 1H; 5-H), 4.13 (dd, $J = 10.5$, 3.4 Hz, 1H; 2′-H), 3.84–3.70 (m, 7H; PMB-OCH_3,PMP-OCH_3, 6a-H), 3.70–3.57 (m, 2H; 6b-H, 4-H), 3.25 (td, $J = 9.3$, 6.9 Hz, 1H; 2-H), 1.81 (s, 3H; NHC(O)CH_3), 0.73 ppm (d, $J = 6.5$ Hz, 3H; 6′-H); ^{13}C NMR (101 MHz, $CDCl_3$): $\delta = 171.4$ (C=O), 166.0 (C=O), 165.7 (C=O), 160.4 (C_q), 159.6 (C_q), 133.4 (CH_{arom}), 133.2 (CH_{arom}), 129.8 (CH_{arom}), 129.7 (CH_{arom}), 129.7 (CH_{arom}), 129.6 (C_q), 129.6 (C_q), 128.6 (CH_{arom}), 128.4 (CH_{arom}), 127.8 (CH_{arom}), 114.1 (CH_{arom}), 113.7 (CH_{arom}), 102.1 (PMP-C*H*), 98.6 (C-1′), 87.9 (C-1), 80.7 (C-4), 75.5 (C-3), 74.2 (C-2′), 73.4 (PMB-CH_2), 72.6 (C-4′), 70.9 (C-3′), 68.6 (C-6), 68.5 (C-5), 65.6 (C-5′), 58.4 (C-2), 55.4 (OCH_3), 55.3 (OCH_3), 23.4 (NHC(O)CH_3), 15.6 ppm (C-6′); IR (KBr): $\tilde{\nu}_{max} = 2117.80$ (N_3), 1724.29 (CO) cm^{-1}; HRMS (ESI): *m/z*: calcd for $C_{44}H_{46}N_4O_{13}H$: 839.31341; found: 839.31311 $[M+H]^+$.

3,4-di-*O*-benzoyl-2-*O*-(4-methoxybenzyl)-α-L-fucopyranoside-(1→3)-6-*O*-(4-methoxybenzyl)-2-deoxy-2-(*N*-acetylacetamido)-β-D-glucopyranosyl Azide (8): Disaccharide **7** (436 mg, 0.52 mmol) was dissolved in anhydrous DCM and DiPEA (10 equiv, 870 μL, 5 mmol) and acetyl chloride (50 equiv, 1.8 mL, 25 mmol) were added. The reaction mixture was stirred for 2 h at room temperature, after which TLC (10% EtOAc in DCM) indicated full conversion. The reaction mixture was diluted with DCM and the organic layer was washed with saturated aqueous $NaHCO_3$. The organic layer was dried over $MgSO_4$, filtered, and concentrated. Silica gel column chromatography (30%→40%→50% Et_2O in pentane) yielded the diacetylated intermediate (406 mg, 0.46 mmol, 89%). $[\alpha]_D^{25} = -105.2$ ($c = 0.50$ in $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 7.94–7.87$ (m, 2H; CH_{arom}), 7.78–7.71 (m, 2H; CH_{arom}), 7.63–7.55 (m, 1H; CH_{arom}), 7.52–7.39 (m, 5H; CH_{arom}), 7.32–7.26 (m, 2H; CH_{arom}), 7.12–7.04 (m, 2H; CH_{arom}), 6.91–6.83 (m, 2H; CH_{arom}), 6.68 (d, $J = 8.7$ Hz, 2H; CH_{arom}), 5.75–5.66 (m, 2H; 1-H, 3′-H), 5.51 (s, 1H; PMP-CH_{acetal}), 5.42 (dd, $J = 3.3$, 1.4 Hz, 1H; 4′-H), 4.79 (dd, $J = 9.6$, 8.6 Hz, 1H; 3-H), 4.74 (d, $J = 3.5$ Hz, 1H; 1′-H), 4.52–4.37 (m, 4H; PMB-CH_2, 5′-H, 5-H), 4.06 (dd, $J = 10.6$, 3.5 Hz, 1H; 2′-H), 3.85–3.70 (m, 8H; PMB-OCH_3, PMP-OCH_3, 6-H), 3.70–3.61 (m, 2H; 4-H, 2-H), 2.50 (s, 3H; N(C(O)CH_3)C(O)CH_3), 2.30 (s, 3H; N(C(O)CH_3)C(O)CH_3), 0.52 ppm (d, $J = 6.4$ Hz, 3H; 6′-H); ^{13}C NMR (101 MHz, $CDCl_3$): $\delta = 175.2$ (C=O), 174.6 (C=O), 165.9 (C=O), 165.8 (C=O), 160.5 (C_q), 159.5 (C_q), 133.3 (CH_{arom}), 133.2 (CH_{arom}), 130.5 (CH_{arom}), 129.8 (CH_{arom}), 129.7 (CH_{arom}), 129.4 (C_q), 129.2 (C_q), 128.5 (CH_{arom}), 128.4 (CH_{arom}), 128.0 (CH_{arom}), 113.8 (CH_{arom}), 113.7 (CH_{arom}), 102.5 (PMP-C*H*), 98.8 (C-1′), 87.5 (C-1), 80.9 (C-4), 73.6 (C-3), 73.4 (PMB-CH_2), 72.6 (C-4′), 71.8 (C-2′), 71.3 (C-3′), 68.6 (C-6), 68.0 (C-5), 65.4 (C-5′), 64.1 (C-2), 55.4 (OCH_3), 55.2 (OCH_3), 28.6 (N(C(O)CH_3)C(O)CH_3), 25.6 (N(C(O)CH_3)C(O)CH_3), 15.2 ppm (C-6′); IR (KBr): $\tilde{\nu}_{max} = 2119.23$ (N_3), 1727.15 (CO) cm^{-1}; HRMS (ESI): *m/z*: calcd for $C_{46}H_{48}N_4O_{14}Na$: 903.30592; found: 903.30478 $[M+Na]^+$.

The 4-methoxybenzylidene-protected disaccharide (461 mg, 0.52 mmol) was dissolved in dry THF and cooled to −70 °C. BH_3·THF was added as a 1.0 M solution in THF (5 equiv, 2.6 mmol, 2.6 mL) and the reaction mixture was stirred for 15 min at this temperature. Then, Bu_2BOTf was added as a 1.0 M solution in DCM (2 equiv, 1 mmol, 1 mL) and the reaction mixture stirred for an additional 15 min at −70 °C. The reaction mixture was then heated to −50 °C and stirred overnight. The reaction was quenched by careful addition of Et_3N (0.5 mL) followed by MeOH (15 mL) and the mixture was stirred at room temperature for 30 min. It was then concentrated in vacuo and subjected to silica gel column chromatography (40%→50%→60% Et_2O in pentane) to yield the title compound (370 mg, 0.42 mmol, 81%). $[\alpha]_D^{25} = -93.2$ ($c = 1.00$ in $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 7.95–7.88$ (m, 2H; CH_{arom}), 7.80–7.73 (m, 2H; CH_{arom}), 7.67–7.59 (m, 1H; CH_{arom}), 7.54–7.42 (m, 3H; CH_{arom}), 7.35–7.26 (m, 4H; CH_{arom}), 7.11–7.04 (m, 2H; CH_{arom}), 6.90 (d, $J = 8.6$ Hz, 2H; CH_{arom}), 6.74 (d, $J = 8.6$ Hz, 2H; CH_{arom}), 5.67–5.59 (m, 3H; 1-H, 3′-H, 4′-H), 4.93 (d, $J = 3.6$ Hz, 1H; 1′-H), 4.66–4.39 (m, 6H; PMB-CH_2, PMB-CH_2, 3-H, 5′-H), 4.10 (dd, $J = 10.3$, 3.6 Hz, 1H; 2′-H), 4.01 (s, 1H; 4-OH), 3.85–3.79 (m, 4H; 6a-H, OCH_3), 3.78–3.72 (m, 4H; 6b-H, OCH_3), 3.71–3.62 (m, 3H; 2-H, 4-H, 5-H), 2.41 (s, 3H; N(C(O)CH_3)C(O)CH_3), 2.37 (s, 3H; N(C(O)CH_3)C(O)CH_3), 1.21 ppm (d, $J = 6.5$ Hz, 3H; 6′-H); ^{13}C NMR (101 MHz, $CDCl_3$): $\delta = 175.4$ (C=O), 174.3 (C=O), 165.8 (C=O), 165.4 (C=O), 159.5 (C_q), 159.4 (C_q), 133.5 (CH_{arom}), 133.3 (CH_{arom}), 130.0 (CH_{arom}), 129.9 (CH_{arom}), 129.7 (CH_{arom}), 129.5 (CH_{arom}), 129.2 (C_q), 128.6 (CH_{arom}), 128.4 (CH_{arom}), 113.9 (CH_{arom}), 99.7 (C-1′), 86.8 (C-1), 82.6 (C-3), 76.7 (C-4), 73.4 (PMB-CH_2), 72.7 (PMB-CH_2), 72.1 (C-4′), 71.4 (C-5), 71.3 (C-2′), 70.3 (C-3′), 68.7 (C-6), 66.7 (C-5′), 62.3 (C-2), 55.4 (OCH_3), 55.3 (OCH_3), 28.4 (N(C(O)CH_3)C(O)CH_3), 25.6 (N(C(O)CH_3)C(O)CH_3), 16.2 ppm (C-6′); IR (KBr): $\tilde{\nu}_{max} = 2117.80$ (N_3), 1724.29 (CO) cm^{-1}; HRMS (ESI): *m/z*: calcd for $C_{46}H_{50}N_4O_{14}NH_4$: 900.36618; found: 900.36581 $[M+NH_4]^+$.

2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranoside(1→4)-[3,4-di-*O*-benzoyl-2-*O*-(4-methoxybenzyl)-α-L-fucopyranoside-(1→3)]-6-*O*-(4-methoxybenzyl)-2-deoxy-2-(*N*-acetylacetamido)-β-D-glucopyranosyl Azide (10): Donor **9** (5 equiv, 737 mg, 1.5 mmol) and acceptor **8** (266 mg, 0,3 mmol) were co-evaporated three times with toluene, backfilling the flask with N_2 after every co-evaporation, and placed under a N_2 atmosphere. The sugars were dissolved in dry DCM and activated 4 Å molecular sieves (300 mg) were added. The mixture was stirred for 30 min at room temperature and subsequently cooled to −10 °C. TMS triflate (0.1 equiv, 5.6 μL, 0.03 mmol) was added and the reaction mixture stirred overnight at −10 °C. The reaction was quenched by the addition of triethylamine (TEA) (0.1 mL) and allowed to warm to room temperature. The reaction mixture was then diluted with DCM, filtered, further diluted with toluene, and concentrated in vacuo. Silica gel column chromatography (40%→70% Et_2O in pentane, Δ = 5%) yielded the title compound (283 mg, 0.23 mmol, 77%). $[\alpha]_D^{25} = -104.4$ ($c = 1.00$ in $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 7.99–7.92$ (m, 2H; CH_{arom}), 7.78–7.71 (m, 2H; CH_{arom}), 7.65–7.58 (m, 1H; CH_{arom}), 7.50–7.42 (m, 3H; CH_{arom}), 7.36–7.30 (m, 2H; CH_{arom}), 7.26 (dd, $J = 8.3$, 7.4 Hz, 3H; CH_{arom}), 7.13 (d, $J = 8.6$ Hz, 2H; CH_{arom}), 6.97 (d, $J = 8.7$ Hz, 2H; CH_{arom}), 6.70 (d, $J = 8.6$ Hz, 2H; CH_{arom}), 5.66 (dd, $J = 3.3$, 1.4 Hz, 1H; 4′-H), 5.64–5.54 (m, 2H; 3′-H, 1-H), 5.38 (dd, $J = 3.6$, 1.0 Hz, 1H; 4″-H), 5.14 (q, $J = 6.5$ Hz, 1H; 5′-H), 5.04 (dd, $J = 10.3$, 8.3 Hz, 1H; 2″-H), 4.86–4.67 (m, 5H; 3″-H, PMB-C*H*H, 1′-H, 1″-H, 3-H), 4.60 (dd, $J = 11.5$, 6.1 Hz, 1H; 6″a-H), 4.50 (s, 2H; PMB-CH_2), 4.46–4.38 (m, 2H; PMB-CH*H*, 6″b-H), 4.11 (dd, $J = 10.6$, 3.7 Hz, 1H; 2′-H), 4.05 (dd, $J = 10.0$, 8.9 Hz, 1H; 4-H), 3.88–3.68 (m, 8H; OCH_3, 6-H, OCH_3), 3.59 (t, $J = 9.4$ Hz, 1H; 2-H), 3.56–3.49 (m, 2H; 5-H, 5″-H), 2.51 (s, 3H; N(C(O)CH_3)C(O)CH_3), 2.25 (s, 3H; N(C(O)CH_3)C(O)CH_3), 2.24 (s, 3H; C(O)CH_3), 2.10 (s, 3H; C(O)CH_3), 2.00 (s, 3H; C(O)CH_3), 1.98 (s, 3H; C(O)CH_3), 1.24 ppm (d, $J = 6.6$ Hz, 3H; 6′-H); ^{13}C NMR (101 MHz, $CDCl_3$): $\delta = 175.5$ (C=O), 174.8 (C=O), 170.9 (C=O), 170.5 (C=O), 170.2 (C=O), 168.9 (C=O), 166.1 (C=O), 165.4 (C=O), 159.8 (C_q), 159.5 (C_q), 133.3 (CH_{arom}), 133.0 (CH_{arom}), 130.8 (CH_{arom}), 130.0 (C_q),

130.0 (CH_{arom}), 129.9 (CH_{arom}), 129.8 (C_q), 129.6 (CH_{arom}), 129.4 (C_q), 128.5 (CH_{arom}), 128.3 (CH_{arom}), 114.3 (CH_{arom}), 113.7 (CH_{arom}), 99.7 (C-1″), 97.8 (C-1′), 86.9 (C-1), 76.6 (C-5″), 74.3 (C-4), 73.7 (PMB-CH_2), 73.5 (PMB-CH_2), 72.9 (C-4′), 71.8 (C-3′, C-3, C-2′), 71.3 (C-3″), 71.1 (C-5), 69.2 (C-2″), 67.0 (C-4″), 66.9 (C-6), 64.9 (C-5′), 64.3 (C-2), 61.1 (C-6″), 55.4 (OCH_3), 55.3 (OCH_3), 28.8 (N(C(O)CH_3)C(O)CH_3), 25.8 (N(C(O)CH_3)C(O)CH_3), 21.0 (C(O)CH_3), 20.9 (C(O)CH_3), 20.8 (C(O)CH_3), 20.7 (C(O)CH_3), 16.0 ppm (C-6′); HRMS (ESI): *m/z*: calcd for $C_{60}H_{68}N_4O_{23}Na$: 1235.41666; found: 1235.41654 $[M+Na]^+$.

2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranoside-(1→4)-[3,4-di-*O*-benzoyl-2-*O*-(4-methoxybenzyl)-α-L-fucopyranoside-(1→3)]-6-*O*-(4-methoxybenzyl)-2-deoxy-2-acetamido-β-D-glucopyranosyl Azide (11): Protected trisaccharide **10** (61 mg, 50 μmol) was dissolved in dry THF (1 mL) and DMAPA (10 equiv, 63 μL, 0.5 mmol) was added. The reaction mixture was stirred for 30 min at room temperature and another portion of DMAPA (10 equiv, 63 μL, 0.5 mmol) was added. After further stirring for 1 h, TLC (15% EtOAc in DCM) indicated full conversion. The reaction mixture was diluted with DCM and washed with 1 M HCl (aq). The organic layer was dried over $MgSO_4$, filtered, and concentrated in vacuo. Silica gel column chromatography (0%→10%→15%→20% EtOAc in DCM) yielded the title compound (51 mg, 42 μmol, 87%). $[\alpha]_D^{25} = -76.0$ ($c = 1.00$ in $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 8.02–7.94$ (m, 2H; CH_{arom}), 7.79–7.73 (m, 2H; CH_{arom}), 7.65–7.58 (m, 1H; CH_{arom}), 7.51–7.44 (m, 3H; CH_{arom}), 7.33–7.28 (m, 3H; CH_{arom}), 7.17 (d, $J = 8.6$ Hz, 2H; CH_{arom}), 6.95 (d, $J = 8.6$ Hz, 2H; CH_{arom}), 6.76 (d, $J = 8.7$ Hz, 2H; CH_{arom}), 6.03 (d, $J = 7.5$ Hz, 1H; NH), 5.68–5.61 (m, 2H; 4′-H, 3′-H), 5.38 (dd, $J = 3.6$, 1.1 Hz, 1H; 4″-H), 5.26 (d, $J = 8.2$ Hz, 1H; 1-H), 5.21 (d, $J = 3.6$ Hz, 1H; 1′-H), 5.08 (dd, $J = 10.4$, 8.1 Hz, 1H; 2″-H), 4.99–4.86 (m, 2H; 5′-H, 3″-H), 4.73–4.67 (m, 2H; PMB-C*H*H, 1″-H), 4.64 (d, $J = 11.6$ Hz, 1H; PMB-C*H*H), 4.57 (d, $J = 11.7$ Hz, 1H; PMB-CH*H*), 4.46–4.31 (m, 4H; PMB-CH*H*, 6″-H, 3-H), 4.18 (dd, $J = 9.7$, 3.5 Hz, 1H; 1′-H), 4.06 (t, $J = 8.3$ Hz, 1H; 4-H), 3.85–3.78 (m, 5H; OCH_3, 6-H), 3.75 (s, 3H; OCH_3), 3.64–3.55 (m, 2H; 5″-H, 5-H), 3.33 (q, $J = 8.1$ Hz, 1H; 2-H), 2.21 (s, 3H; C(O)CH_3), 2.07 (s, 3H; C(O)CH_3), 2.02 (s, 3H; C(O)CH_3), 1.98 (s, 3H; C(O)CH_3), 1.89 (s, 3H; NHC(O)C*H*$_3$), 1.25 ppm (d, $J = 6.6$ Hz, 3H; 6′-H); ^{13}C NMR (101 MHz, $CDCl_3$): $\delta = 171.0$ (C=O), 170.6 (C=O), 170.5 (C=O), 170.2 (C=O), 169.4 (C=O), 166.1 (C=O), 165.3 (C=O), 159.6 (C_q), 159.6 (C_q), 133.3 (CH_{arom}), 133.0 (CH_{arom}), 129.9 (CH_{arom}), 129.8 (C_q), 129.8 (CH_{arom}), 129.7 (CH_{arom}), 129.7 (CH_{arom}), 128.6 (CH_{arom}), 128.3 (CH_{arom}), 114.1 (CH_{arom}), 114.0 (CH_{arom}), 99.6 (C-1″), 97.3 (C-1′), 87.2 (C-1), 76.7 (C-5), 73.6 (C-2′, C-4), 73.5 (C-3), 73.4 (PMB-CH_2), 73.1 (PMB-CH_2), 72.8 (C-4′), 71.1 (C-5″), 71.0 (C-3″), 71.0 (C-3′), 69.2 (C-2″), 67.4 (C-6), 67.0 (C-4″), 65.2 (C-5′), 61.1 (C-6″), 57.0 (C-2), 55.4 (OCH_3), 55.3 (OCH_3), 23.5 (NHC(O)C*H*$_3$), 20.9 (C(O)CH_3), 20.9 (C(O)CH_3), 20.7 (C(O)CH_3), 16.1 ppm (C-6′); HRMS (ESI): *m/z*: calcd for $C_{58}H_{66}N_4O_{22}Na$: 1193.40609; found: 1193.40573 $[M+Na]^+$.

2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranoside-(1→4)-6-(*tert*-butyldimethylsilyl)-2-deoxy-2-acetamido-β-D-glucopyranosyl Azide (13): Donor **9** (1.5 equiv, 368 mg, 0.75 mmol) and acceptor **12** (180 mg, 0.5 mmol) were co-evaporated three times with toluene and placed under N_2. The sugars were dissolved in dry DCM (5 mL) and stirred with activated 4 Å molecular sieves (0.5 g) for 2 h at room temperature. The reaction mixture was cooled to −40 °C and BF_3·Et_2O (1.6 equiv, 100 μL, 0.8 mmol) was added. The reaction mixture was stirred at −40 °C overnight and the formation of the disaccharide product was confirmed by TLC (70% EtOAc in pentane). The reaction was quenched with Et_3N (0.5 mL), diluted with DCM, filtered, diluted with toluene, and concentrated. Silica gel column chromatography (60%→70%→80% EtOAc in pentane) yielded the title compound (193 mg, 0.28 mmol, 56%). $[\alpha]_D^{20} = +5{,}8$ ($c = 1.00$ in $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 6.17$ (d, $J = 8.5$ Hz, 1H; NH), 5.40 (dd, $J = 3.4$, 1.0 Hz, 1H; 4′-H), 5.22 (dd, $J = 10.5$, 8.0 Hz, 1H; 2′-H), 4.99 (dd, $J = 10.5$, 3.4 Hz, 1H; 3′-H), 4.69–4.61 (m, 2H; 1-H, 1′-H), 4.15 (d, $J = 6.5$ Hz, 2H; 6′-H), 4.06 (br s, 1H; 3-OH), 4.01 (t, $J = 6.5$ Hz, 1H; 5′-H), 3.90–3.72 (m, 3H; 6-H, 3-H), 3.69–3.57 (m, 2H; 4-H, 2-H), 3.43 (ddd, $J = 9.6$, 3.4, 1.5 Hz, 1H; 5-H), 2.17 (s, 3H; C(O)C*H*$_3$), 2.08 (s, 3H; C(O)C*H*$_3$), 2.07 (s, 3H; C(O)C*H*$_3$), 2.03 (s, 3H; NHC(O)C*H*$_3$), 1.99 (s, 3H; C(O)C*H*$_3$), 0.92 (s, 9H; *t*Bu), 0.11 (s, 3H; $SiCH_3$), 0.10 ppm (s, 3H; $SiCH_3$); ^{13}C NMR (101 MHz, $CDCl_3$): $\delta = 171.0$ (C=O), 170.6 (C=O), 170.2 (C=O), 170.1 (C=O), 169.4 (C=O), 101.6 (C-1′), 87.9 (C-1), 80.5 (C-4), 76.7 (C-5), 71.9 (C-3), 71.4 (C-5′), 70.9 (C-3′), 68.7 (C-2′), 66.8 (C-4′), 61.4 (C-6′), 61.2 (C-6), 55.6 (C-2), 25.9 (*t*Bu), 23.4 (NHC(O)CH_3), 20.7 (C(O)CH_3), 20.6 (C(O)CH_3), 20.6 (C(O)CH_3), 20.6 (C(O)CH_3), 18.3 (Si-*C*), −5.0 (Si-CH_3), −5.2 ppm (Si-CH_3); IR (KBr): $\tilde{\nu}_{max} = 2115.65$ (N_3), 1752.19 (CO) cm^{-1}; HRMS (ESI): *m/z*: calcd for $C_{28}H_{46}N_4O_{14}SiNa$: 713.2672; found: 713.2695 $[M+Na]^+$.

2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranoside-(1→4)-3,6-di-*O*-acetyl-2-deoxy-2-acetamido-β-D-glucopyranosyl Azide (14): Silyl-protected disaccharide **13** (517 mg, 0.75 mmol) was dissolved in dry THF (7.5 mL) in a plastic tube. HF·pyridine complex (16 equiv, 310 μL, 12 mmol) was added and the reaction mixture stirred overnight. Completion of the reaction was assessed by TLC (100% EtOAc) and the reaction mixture was then diluted with DCM. The organic layer was washed with aqueous saturated $NaHCO_3$ (3:1 DCM/H_2O) and the aqueous layer was back-extracted with DCM. The combined organic layers were dried over $MgSO_4$, filtered, and concentrated to yield 380 mg (0.66 mmol) of the crude intermediate. The crude desilylated disaccharide was dissolved in dry pyridine (6.6 mL) and the mixture cooled to 0 °C in an ice bath. Acetic anhydride (10 equiv, 620 μL, 6.6 mmol) and 4-dimethylaminopyridine (DMAP; 0.1 equiv, 9 mg, 0.07 mmol) were then added. The reaction mixture was stirred overnight at room temperature and reaction completion was confirmed by TLC (100% EtOAc). The reaction was quenched with methanol and the mixture concentrated. Pyridine traces were removed by toluene co-evaporation. Silica gel column chromatography (70%→80%→90% EtOAc in pentane) yielded the title compound (421 mg, 0.64 mmol, 85%). $[\alpha]_D^{20} = -26{,}4$ ($c = 1.00$ in $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 6.53$ (d, $J = 9.6$ Hz, 1H; NH), 5.37 (dd, $J = 3.4$, 1.2 Hz, 1H; 4′-H), 5.19–5.03 (m, 2H; 3-H, 2′-H), 4.99 (dd, $J = 10.5$, 3.4 Hz, 1H; 3′-H), 4.64–4.50 (m, 3H; H1, 1′-H, 6a-H), 4.21–4.01 (m, 4H; 6′-H, 6b-H, 2-H), 3.93 (t, $J = 7.1$ Hz, 1H; 5′-H), 3.84 (t, $J = 9.1$ Hz, 1H; 4-H), 3.73 (ddd, $J = 9.1$, 5.0, 2.2 Hz, 1H; 5-H), 2.17 (s, 3H; C(O)C*H*$_3$), 2.14 (s, 3H; C(O)C*H*$_3$), 2.11 (s, 3H; C(O)C*H*$_3$), 2.07 (s, 3H; C(O)C*H*$_3$), 2.07 (s, 3H; C(O)C*H*$_3$), 1.99 (s, 3H; NHC(O)C*H*$_3$), 1.97 ppm (s, 3H; C(O)C*H*$_3$); ^{13}C NMR (101 MHz, $CDCl_3$): $\delta = 171.0$ (C=O), 170.5 (C=O), 170.4 (C=O), 170.3 (C=O), 170.1 (C=O), 170.0 (C=O), 169.3 (C=O), 101.3 (C-1′), 88.3 (C-1), 76.1 (C-4), 74.5 (C-5), 73.1 (C-3), 70.8 (C-3′), 70.7 (C-5′), 69.0 (C-2′), 66.6 (C-4′), 61.9 (C-6), 60.6 (C-6′), 53.0 (C-2), 23.0 (NHC(O)CH_3), 20.9 (C(O)CH_3), 20.8 (C(O)CH_3), 20.6 (C(O)CH_3), 20.6 (C(O)CH_3), 20.5 (C(O)CH_3), 20.5 ppm (C(O)CH_3); IR (KBr): $\tilde{\nu}_{max} = 2116.37$ (N_3), 1744.32 (CO) cm^{-1}; HRMS (ESI): *m/z*: calcd for $C_{26}H_{36}N_4O_{16}Na$: 683.2019; found: 683.2029 $[M+Na]^+$.

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Conflict of interest

The authors declare no conflict of interest.

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