Synthetic peptides as tools in chemical immunology

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Synthesis of asparagine derivatives harboring a Lewis^X-type DC-SIGN ligand and evaluation of their immunomodulation

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Introduction

Multiple Sclerosis (MS) is a group of auto-immune neurodegenerative diseases characterized by the formation of lesions in the patient's brain that lead to loss of function.[1] The pathology of MS is not fully understood, but degradation of myelin sheath seems to be a critical step in the disease pathogenesis.[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] Several proteinogenic components of myelin sheath have been shown to become antigenic upon their degradation.[4] For example, myelin oligodendrocyte glycoprotein (MOG), an exclusively CNS-resident protein found on the surface of

oligodendrocytes and myelin sheaths, acts as an autoantigen in an MS-like animal model, the so-called experimental autoimmune encephalomyelitis (EAE).[5]

MOG is a membrane bound glycoprotein, decorated with an *N*-glycan[6] on Asn_{31}, with an approximate molecular mass of 26 kDa.[7,8] It comprises 245 amino acids (AA) and belongs to the immunoglobulin superfamily (Ig). 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] It has also been shown that a peptide fragment comprising AAs 35-55, MOG_{35-55}, is an immunodominant peptide in EAE.[10,11]

Recently, a potential mechanism behind the pathogenicity of this MOG_{35-55}-peptide in EAE was discovered: after post-translational citrullination (deimination of the guanidine moiety of arginine), the peptide was shown to 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 have increased citrullination in myelin samples from MS patients.[14] Together, these advances led to the hypothesis that post-translational citrullination of MOG, via cytotoxic peptide aggregates, could be in part responsible for the neurodegeneration observed in MS and EAE.

In light of the above findings, the effect of the native *N*-glycan at position 31 on the aggregation behavior of the citrullinated peptide was questioned. Inhibition of aggregation by glycosylation could be expected based on the work on *O*-glycosylation of serine or threonine residues, which has previously been shown to inhibit 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 their mimics) on peptides derived from prion protein[16] and the full-length prion protein[17] has also been shown to decrease or even abrogate aggregation. Therefore, a study on the effects of glycosylation on the previously described aggregation of citrullinated MOG peptides was called for.

Furthermore, the N-glycan present on MOG may play a second role in its pathological mechanisms: previous studies on the glycosylation of MOG suggest that specific *N*-glycan structures can modulate the immunological tolerance through 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]

Studies using synthetic neoglycopeptides bearing DC-SIGN binding *N*-glycan mimics may shed light on the role of a putative interaction between DC-SIGN and MOG in MS. MOG_{31-55}-peptides decorated with Le^X and Le^X derived oligosaccharides (LacNAc and Fucα1-3GlcNAc) on the N-terminal asparagine (Asn_{31}) were therefore synthesized and the effect of these

modifications on the aggregation-proneness of the peptides was assessed, and the results of these studies are described in this Chapter. To minimize artefacts stemming from various non-native linkers[21–23], the oligosaccharides are attached via the native anomeric amide linkage that normally occurs in N-glycans. To achieve this, the recently published method for the synthesis of glycosylated asparagine derivatives was extended to larger oligosaccharides.[24] By using the asparagine building blocks obtained in this manner with the previously established model peptide, MOG_{31-55}[12], the effect of glycosylation on citrullination-dependent aggregation of MOG could be evaluated. Subsequently, the binding of Le^X-decorated neoglycopeptides to DC-SIGN was confirmed by solid-phase immunoassays using recombinant DC-SIGN-Fc fusion protein.[25] Finally, a cytokine secretion assay in monocyte derived dendritic cells (moDCs) from human donors was utilized to analyze the degree of modulation for IL-10 (anti-inflammatory) and IL-12p70 (pro-inflammatory) production by Le^X-decorated peptides.

Results and Discussion

N-glycosylation of asparagine is of prime importance for a variety of protein functions such as signaling and folding.[26] Studying these functions however, is challenging, as the typical size and complexity of an *N*-glycan poses a considerable synthetic challenge. *N*-glycosylated peptides have been generated using semisynthetic methods involving synthesis and/or isolation of carbohydrate segments which can be linked covalently using endohexosaminidases[27,28], or extended via specific glycosyltransferases as recently demonstrated by Boons and colleagues.[29,30] Synthetic preparation of an entire peptide bearing a natural *N*-glycan has also been reported.[31–33]

Previous work has shown that fucosylated glycans interact with DC-SIGN without the need for an *N*-glycan core structure.[34–38] This formed the inspiration to synthesize a Le^X *N*-glycan derivative similar to the one developed by von dem Bruch and Kunz.[39] This glycosylated asparagine could then be incorporated as residue 31 on the MOG_{31-55} peptide, enabling the facile synthesis of MOG_{31-55} derived neoglycopeptides. These would enable the study of the DC-SIGN binding properties of glycosylated MOG_{31-55}, without needing to produce a full Lewis^X containing N-glycan (Figure 1). To study the effect glycosylation has on the citrulline driven aggregation of MOG_{31-55}, citrullinated forms of MOG_{31-55}, also bearing different N-glycan derivatives, could be synthesized. Since MOG_{31-55} has three arginine residues, seven different permutation of citrullinated derivatives could be considered. Replacing Arg_{41} and Arg_{46} with citrulline seemed to most interesting, as it had previously been shown that this citrullination pattern has some of the most pronounced aggregation behaviour.[12] These arginine residue are also within the reported MHC-I restricted epitope (for non-human primates) MOG_{40-48}.[40] Furthermore, the citrullination of either of these positions has previously been extensively studied in a rodent EAE model.[41]

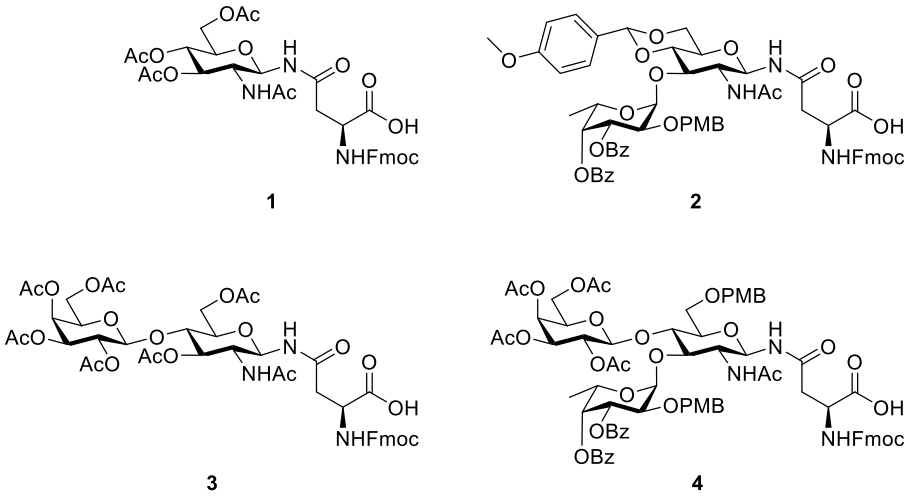


Figure 1. Desired glycosylated Fmoc-asparagine building blocks **1**-**4**.

Three Fmoc-SPPS (solid phase peptide synthesis) compatible glycosyl amide derivatives of asparagine were designed and synthesized (Figure **1**, **1-4**), one (compound **4**) containing a Le^X structure and two (compounds **2** and **3**) featuring a Fucα1-3GlcNAc and LacNAc, respectively, attached to the asparagine side chain via the reducing ends of respective sugars. The LacNAc construct (**3**) was included as a negative control for DC-SIGN binding, as the interaction of Le^X with the receptor has been shown to be fucose dependent.[35]

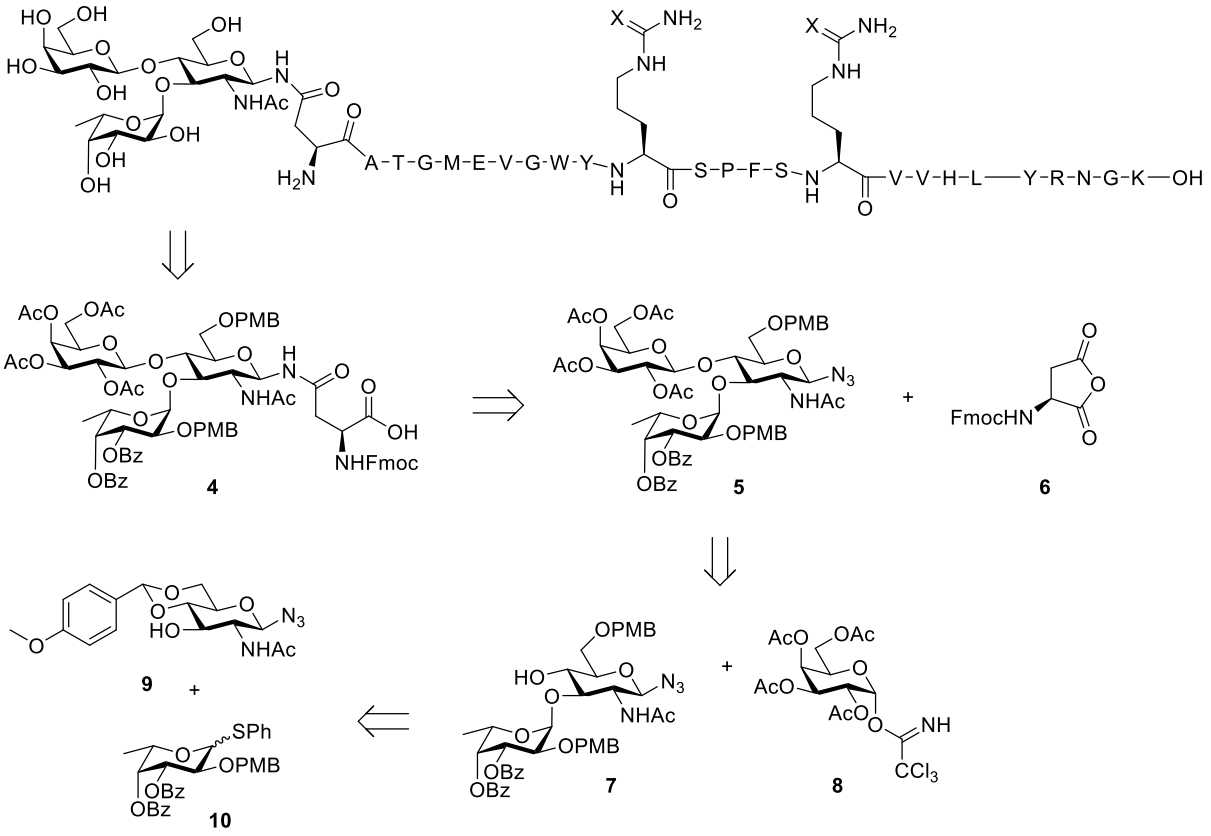


Figure 2. Retrosynthetic analysis of the synthesis of Le^X decorated MOG$_{31-55}$ peptides using building block **4**. A similar approach can be used to produce building blocks **1-3** and incorporate these into peptides. X = NH (Arg) or X = O (Cit).

As exemplified by the structure of Le^X containing protected building block **4** (Figure 1), the synthetic strategy was based on the utilization of acid labile *para*-methoxybenzyl (PMB) and *para*-methoxybenzylidene groups, which would be removed during the TFA mediated global peptide deprotection in standard Fmoc-based solid phase peptide synthesis, and on ester protective groups, which can be removed using hydrazine in methanol after the acidic global deprotection of the peptide. A synthetic strategy was designed so that these protecting groups would be introduced on the monosaccharide building blocks and used throughout the synthesis, avoiding late-stage protecting group manipulation.

The condensation between the glycosyl amine and the sidechain of asparagine for the synthesis of the Fmoc-asparagine building blocks was accomplished using the recently developed two-step one-pot approach for the synthesis of glycosylated asparagine derivatives.[24] This method uses Fmoc-aspartic anhydride (**6**) as an activated Fmoc-aspartic acid derivative, while avoiding protecting group manipulation on the C$^\alpha$-carbonyl. In this method, a Staudinger reduction is used to transform a glycosyl azide into a glycosyl amine.[42–44] The crude glycosyl amine is then redissolved in DMSO, and reacted with Fmoc-aspartic anhydride. The glycosyl amine regioselectively opens the anhydride ring on the C$^\gamma$ side, generating a protected glycosyl asparagine amino acid (Figure 2).[45] The polarity of the solvent is crucial for this regioselectivity, with DMSO generally giving the best results.[46] The synthesis

of the Le^X azide derivative **5** itself will be carried out by first introducing the fucose moiety on the 3-OH of GlcNAc, using donor **10** and acceptor **9**, followed by reductive opening of the *para*-methoxybenzylidine function to generate **7**. This disaccharide acceptor can then be glycosylated with galactoside donor **8**, producing Le^X azide **5**.

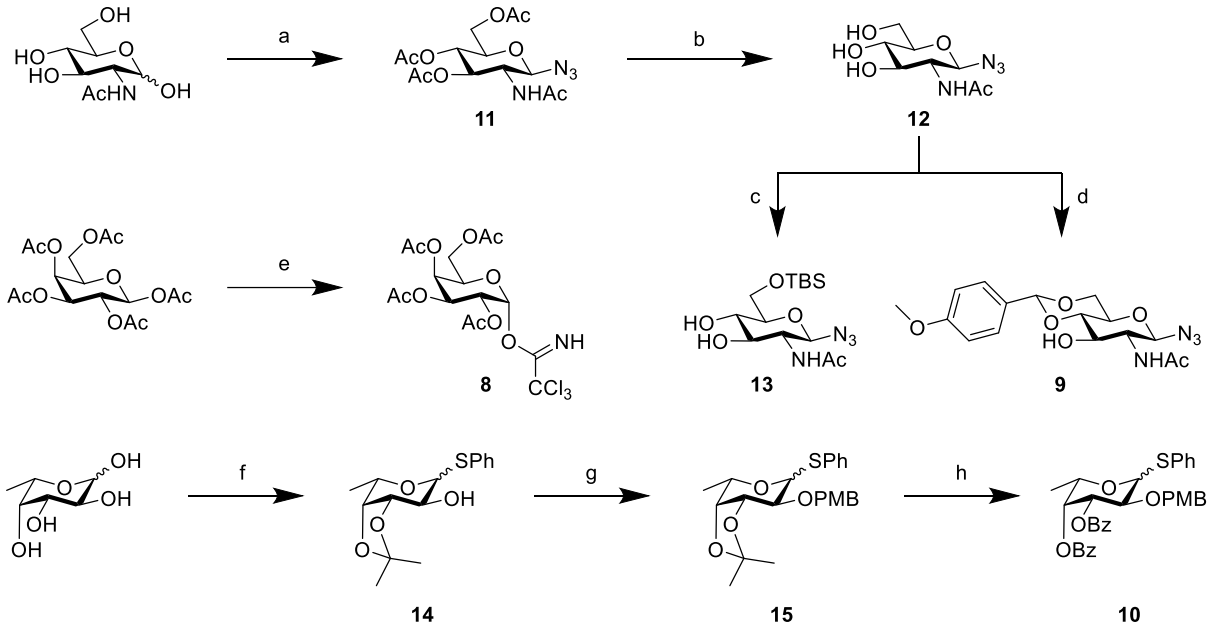


Figure 3. Synthesis of acceptor glycosides **9** and **13**, donor galactoside **8** and donor fucoside **10**. Reagents and conditions: a) i) AcCl, RT ii) NaN_3, DMF, RT, 2h, 42% b) Na, MeOH, RT, 1h c) TBSCl, pyridine, RT, 2h, 84% d) anisaldehyde dimethyl acetal, CSA, ACN, 50 °C, 300 mbar, 3h, 65% e) i) N,N-dimethylaminopropylamine, THF, RT 1h ii) CCl_3CN, DBU, DCM, 1 h RT, 60% over two steps f) i) Ac_2O, pyridine ii) $BF_3{\cdot}Et_2O$, PhSH, toluene iii) Na, MeOH iv) 2,2-dimethoxypropane, CSA, acetone, 80% over four steps g) NaH, PMB-Cl, DMF, 0°C → RT, 2h, 84% h) i) AcOH, H_2O, 80°C, 1h ii) BzCl, pyridine, RT, 2h, 64% over two steps

In the first step towards the glycosyl azides, the monosaccharide building blocks were constructed (Figure 3). N-acetyl glucosaminyl azide **11** was synthesized from N-acetyl glucosamine by acetylation and simultaneous introduction of the anomeric chloride,[47] followed by substitution of the chloride with sodium azide in DMF. This gave **11** in 42% yield over two steps. The acetyl groups were removed under Zemplén conditions and the resulting compound **12** was reacted either with anisaldehyde dimethyl acetal in the presence of CSA to fashion *para*-methoxybenzylidene protected acceptor **9** in 65% yield, or with TBS-Cl in pyridine to produce acceptor **13** in 84% yield. The synthesis of galactosyl imidate donor **8** was accomplished by selective hydrolysis of the anomeric acetate in galactose pentaacetate using N,N-dimethylaminopropylamine[48], followed by formation of the anomeric imidate using trichloroacetonitrile in the presence of catalytic DBU. Lastly, fucosyl donor **10** was constructed starting from L-fucose. Sequential acetylation, introduction of the thiophenol, deacetylation and formation of an isopropylidene gave **14** in 80% yield over four steps. The free 2-OH was then alkylated with *para*-methoxybenzyl chloride, mediated by NaH, to produce compound **15**. Hydrolysis of the isopropylidene group in **15** using 50% aqueous acetic acid at 80°C resulted in liberation of the diol without removing the acid labile PMB group. This was

followed by benzoylation with benzoyl chloride in pyridine, producing donor **10** in 64% over two steps.

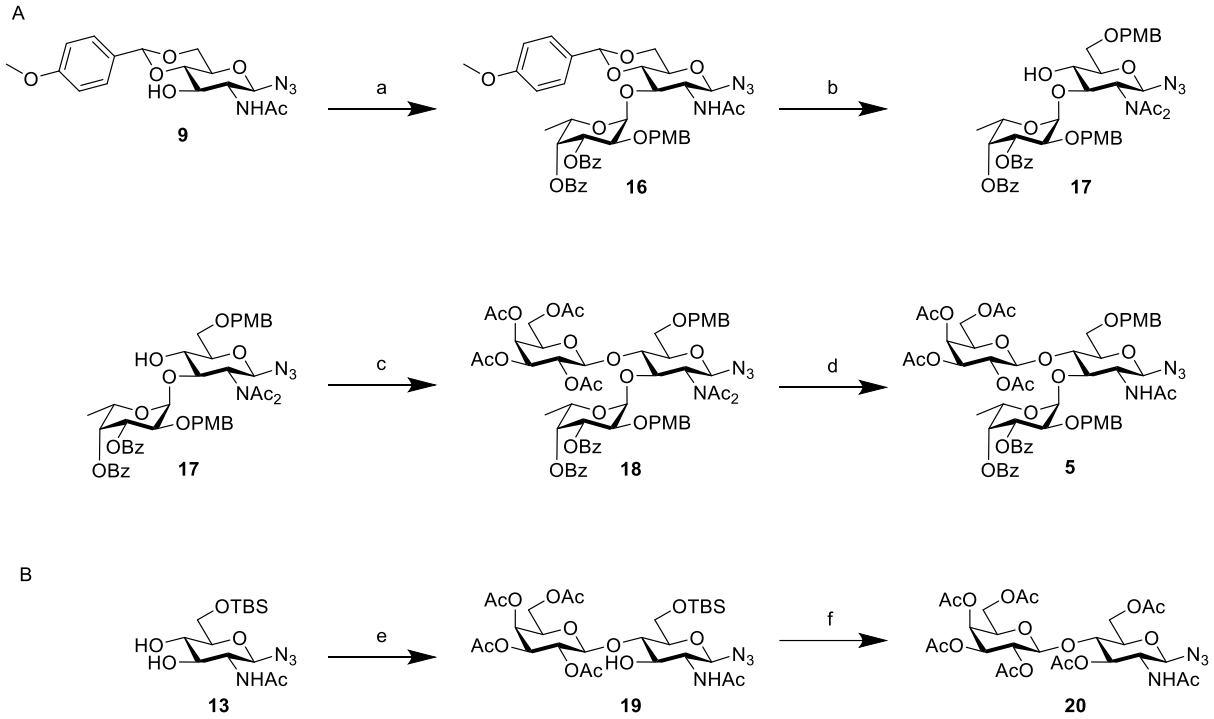


Figure 4. Synthesis of Lewis X azide (A) **15** and LacNAc azide **17** (B). Reagents and conditions: a) **11**, NIS, TMSOTf, 4 Å molecular sieves, DCM, DMF, 0°C, 71% b) i) AcCl, DiPEA, DCM, 89% ii) BH_3, Bu_2BOTf, THF, -50 °C, 81% c) **8**, TMSOTf, DCM, 4 Å molecular sieves, DCM, -15°C, 77% d) N,N-dimethylaminopropylamine, THF, 87% e) **8**, $BF_3{\cdot}Et_2O$, DCM, -40°C, 56% f) i) HF·pyridine ii) Ac_2O, DMAP, DCM, 85% over two steps

The synthesis of protected Le^X glycosyl azide **5** (Figure 4A) started from *para*-methoxybenzylidene-protected glycosyl azide **9** by NIS/TMSOTf-promoted fucosylation with thioglycoside **10** to afford disaccharide **16** in 71% yield. Next, reductive opening of the *para*-methoxybenzylidene group in **16** was carried out to produce **7** (Figure 2). However, the presence of the acetamido group in this glycosyl acceptor hindered the glycosylation, an often encountered problem with *N*-acetyl-glucosamine derived acceptors.[49] Accordingly, disaccharide **16** was first treated with an excess of acetyl chloride and diisopropylethylamine (DiPEA) to convert the amide into the less interfering imide in 89% yield.[50] Reductive opening of the *para*-methoxybenzylidene with BH_3/Bu_2BOTf was performed as described,[51] affording compound **17** in 81% yield. Then, galactosylation with trichloroacetimidate donor **8** yielded the desired protected trisaccharide **18** in 77% yield. Chemoselective deacetylation of **18** using N,N-dimethylaminopropylamine[48] afforded **5** in 87% yield.

The protected lactosaminyl azide **17** was prepared using a literature protocol for regioselective glycosylation of 1,6-protected GlcNAc derivatives.[52,53] Silyl-protected glycosyl azide **13** was subjected to $BF_3{\cdot}Et_2O$ promoted galactosylation with trichloroacetimidate donor **8**, affording the partially protected disaccharide **19** in a 56% yield (Figure 4B). This compound

was treated with HF·pyridine for removal of the *tert*-butyldimethylsilyl (TBS) group, followed by acetylation to afford the desired peracetylated glycosyl azide **20** in 85% yield over 2 steps.

Asparagine derivatives **1-4** were prepared following a general synthetic strategy involving the Staudinger reduction of a glycosyl azide followed by direct reaction of the resulting glycosyl amine with Fmoc aspartic anhydride to perform a nucleophilic ring opening (Figure 5A).[24] Accordingly, Fmoc-Asn(Ac_4GlcNAc)-OH (**1**) was synthesized from glycosyl azide **11**[54] in three steps by PMe_3-mediated azide reduction, followed by addition of H_2O to the crude iminophosphorane to obtain the intermediate glycosyl amine. The desired asparagine derivative was formed by redissolving the crude glycosyl amine in DMSO followed by addition of Fmoc aspartic anhydride. Precipitation directly afforded the desired SPPS building block **1** in 69% yield.

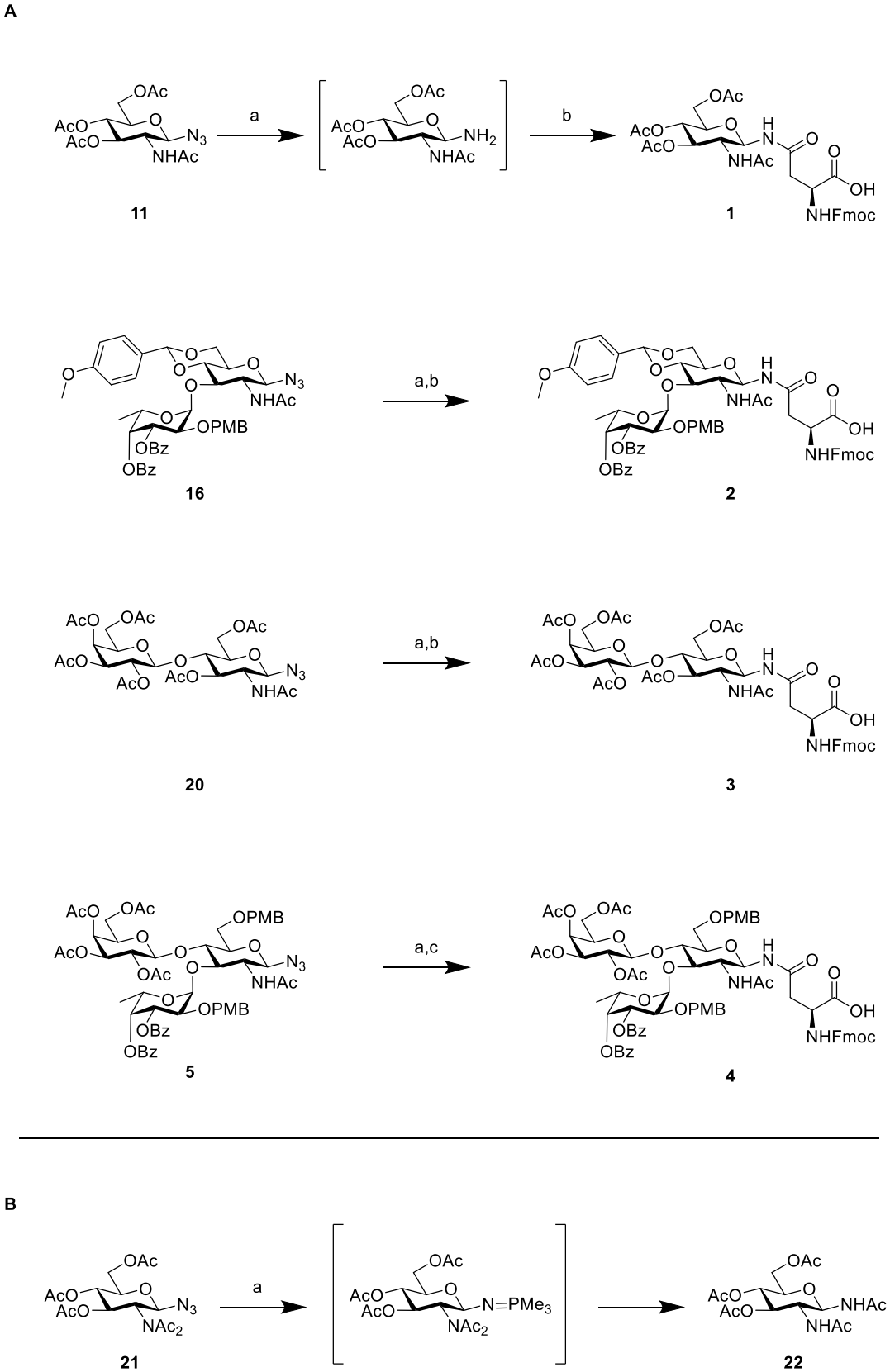


Figure 5. A) Synthesis of glycosylated Fmoc-asparagine derivatives **1**-**4** via the two step Staudinger reduction/aspartic anhydride coupling approach. B) observed reaction when performing Staudinger reduction of diacetylimide **21**. Reagents and conditions: a) i) PMe_3, THF ii) H_2O b) Fmoc-aspartic anhydride, DMSO, 69% (**1**), 65% (**2**), 63% (**3**) c) Fmoc-aspartic anhydride, DMA, 74%

The above sequence proved similarly useful for the preparation of the other desired glycosylated asparagine building blocks (**2**-**4**, Figure 5A). However, precipitation or extraction were found to be less efficient for small scale purification of the more complex carbohydrates, and therefore these compounds were subjected to silica gel chromatography for purification. Using this approach, the fucosylated glycosyl azide **16** was converted to its corresponding SPPS building block **2** in 65% yield, while lactosyl compound **20** was similarly converted to compound **3** in 63% yield. (Figure 5). For the trisaccharide glycosyl azide, conversion of the NAc_2 functionality back to the acetamide was required, as Staudinger reduction of **18** afforded conversion to an unknown side product. Acetyl migration is a likely explanation, as Staudinger reduction of the model NAc_2 protected glycosyl azide **21** afforded clean conversion to the more readily assignable glycosyl acetamide **22** (Figure 5B). Glycosyl azide **5** was coupled to Fmoc aspartic anhydride yielding the desired Le^X SPPS building block **4** as an inseparable 10:1 mixture with its corresponding iso-asparagine isomeric product. It has been shown that dimethylacetamide (DMA) gives similar regioselectivity as DMSO when used as solvent for aspartic anhydride ring opening reactions.[46] However, the lower melting point of this solvent allows for aspartic anhydride ring-opening at 0°C, potentially increasing regioselectivity. Indeed, this solvent and temperature change resulted in the desired Le^X asparagine **4** being formed in 74% yield with complete regioselectivity.

The syntheses of the desired glycopeptides started with the automated SPPS of the immobilized MOG_{32-55} peptide **23** on Tentagel®S-RAM resin, using HCTU as the coupling reagent. This peptide was then manually elongated at the N-terminus with either of the glycosylated asparagine derivatives **1**-**4** using DEPBT as the coupling reagent to prevent aspartimide formation, as described by Yamamoto *et al*.[55] The general synthetic strategy used for the synthesis of the glycopeptides is outlined in Figure 6.

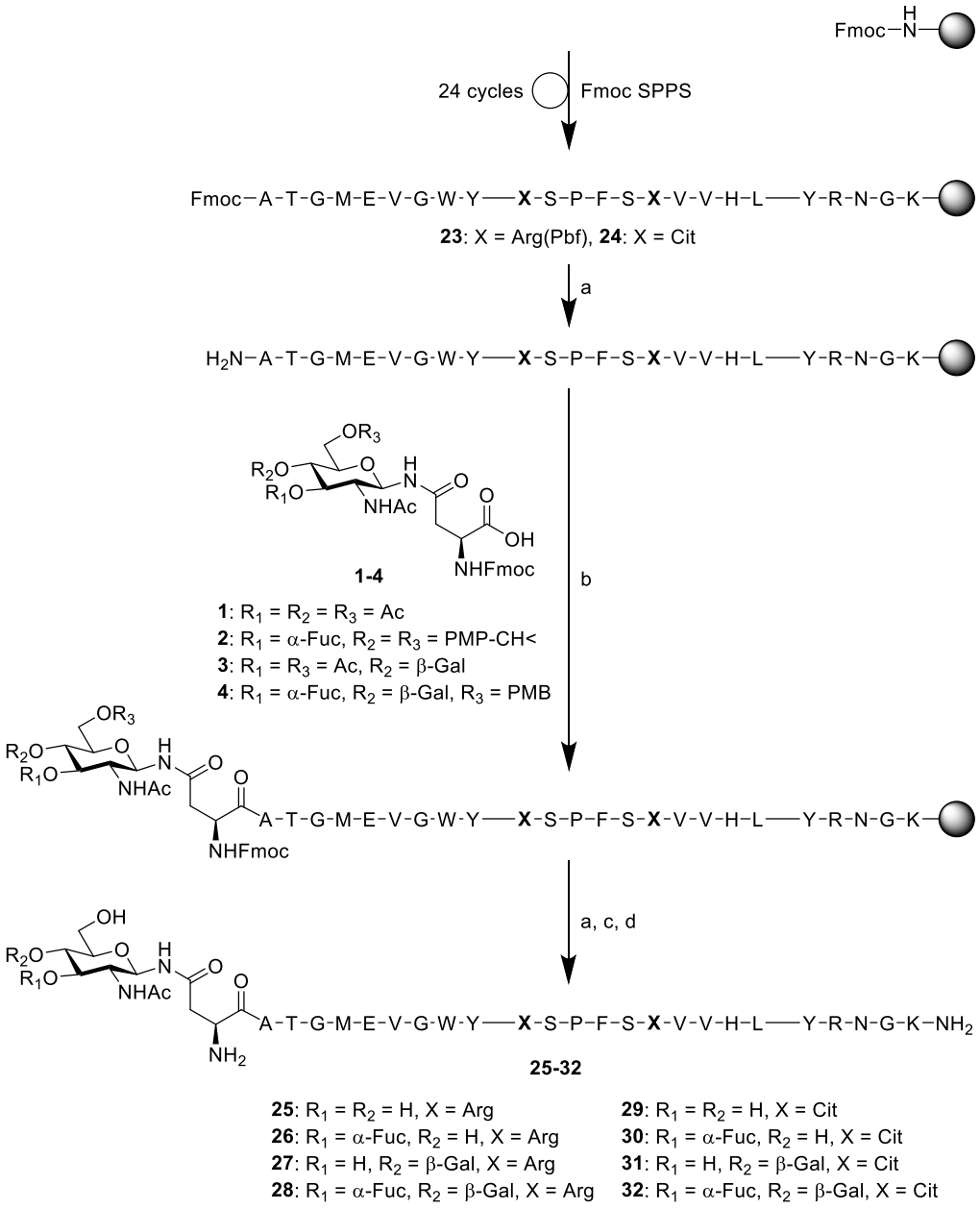


Figure 6. Synthetic strategy employed for the synthesis of MOG_{31-55} glycopeptides **25-32** starting from fully protected immobilized peptides **23** and **24**. X = Arg (**25-28**) or Cit (**29-32**). Reagents and conditions: a) 20% piperidine in DMF b) **1-4**, DEPBT. DiPEA, DMF c) TFA, TIS, H_2O, DCM d) $H_2H_4 \cdot H_2O$, MeOH.

Immobilized peptides **25** and **27** were cleaved from the solid support under standard cleavage conditions (95:2.5:2.5 TFA/TIS/H_2O (v/v) mixture for 2 hours). To prevent potential hydrolysis of the acid labile α-fucosyl bonds[56] in peptides **26** and **28**, more dilute acidic conditions (50:2.5:2.5:45 TFA/TIS/H_2O/DCM (v/v) mixture for 4 hours) were applied. 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 other side chain protecting groups (Boc/tBu/Trt) used in the synthesis.[57]

To remove the remaining ester protecting groups on the carbohydrate moieties, the crude peptides were treated with 10% hydrazine monohydrate in methanol. The resulting fully

deprotected glycopeptides were then purified by preparative reverse-phase (RP) HPLC and the target neoglycopeptides **25-28** were isolated in moderate to good yields after RP-HPLC (Table 1).

Table 1. Yields of glycopeptides **25-32** obtained using the synthetic strategy outlined in Figure 6 after preparative HPLC. The number for each compound is given together with the HPLC yield based on crude mass.

Amino acid	X = Arg	X = Cit
GlcNAc (**1**)	**25** (4.0 %)	**29** (8.6 %)
Fucα1-3GlcNAc (**2**)	**26** (5.6 %)	**30** (2.1 %, 5.7 %[a])
LacNAc (**3**)	**27** (5.8 %)	**31** (5.6 %)
Lewis X (**4**)	**28** (4.1 %)	**32** (6.1 %, 4.8 %[a])

[a] The product containing methionine oxidation was isolated separately.

Next, peptides carrying both post-translational modifications under investigation, glycosylation and citrullination, were synthesized. These peptide were designated peptides **29-32** and prepared using the same procedures as the non-citrullinated glycopeptides, starting from peptide **24**, synthesized using Fmoc-citrulline as the 41st and 46th amino acid. Similar levels of glycosyl amino acid incorporation and similar RP-HPLC yields were achieved during the synthesis of these glycopeptides (Table 1).

The influence of glycosylation on the structure and behavior of MOG_{31-55} was assessed using a variety of biophysical and biochemical experiments. First, solution circular dichroism (CD) spectra of peptides **26-28** and **30-32** were taken to see if these would indicate any difference in biophysical behavior. All peptides showed a pre-dominantly random-coiled structure. The effect of addition of the α-helix stabilizer TFE (50% v/v in PBS) or SDS at non-micellar concentrations (4 mM) was also evaluated (Figure 7). These results indicate the peptides are not prone to β-sheet formation.

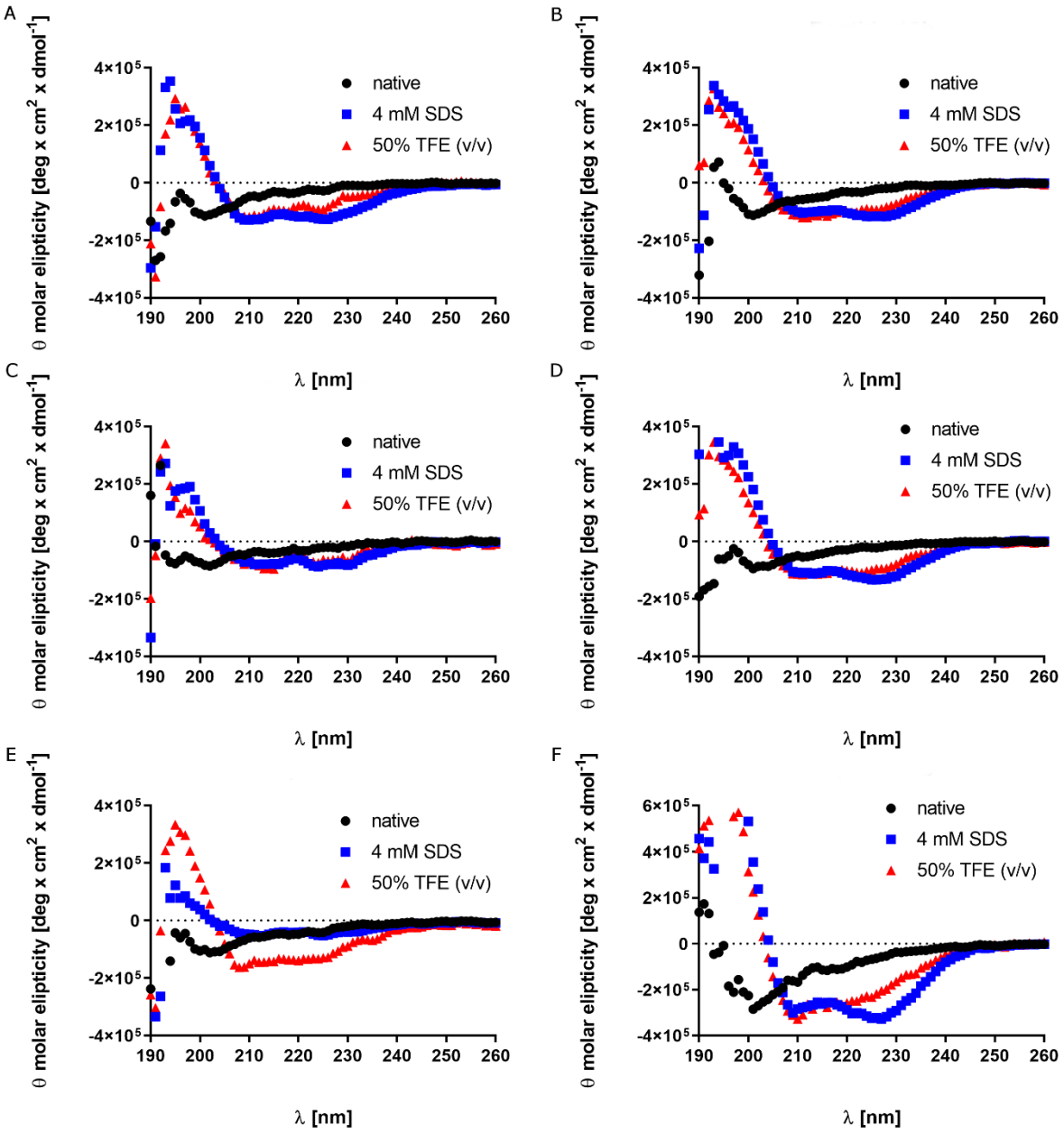


Figure 7. Circular Dichroism spectra of the glycopeptides. A) peptide **26** B) peptide **27** C) peptide **28** D) peptide **30** E) peptide **31** F) peptide **32**

Next, the susceptibility of all glycopeptides to form amyloid-like aggregates was evaluated using the previously described Thioflavin T (ThT) fluorescence assay.[12] In this assay, a fluorogenic substrate, Thioflavin T, was used to detect whether such aggregation occurs. This dye, upon binding to the typical cross β-sheet structures found in amyloid-like aggregates, undergoes an increase in fluorescence quantum yield and a shift of absorption/emission maxima, resulting in an increase in observed fluorescence.[58] The non-citrullinated peptides did not show aggregation at 10 µM (Figure 8A). For the citrullinated peptides, the differently glycosylated peptides displayed distinct aggregation behavior.

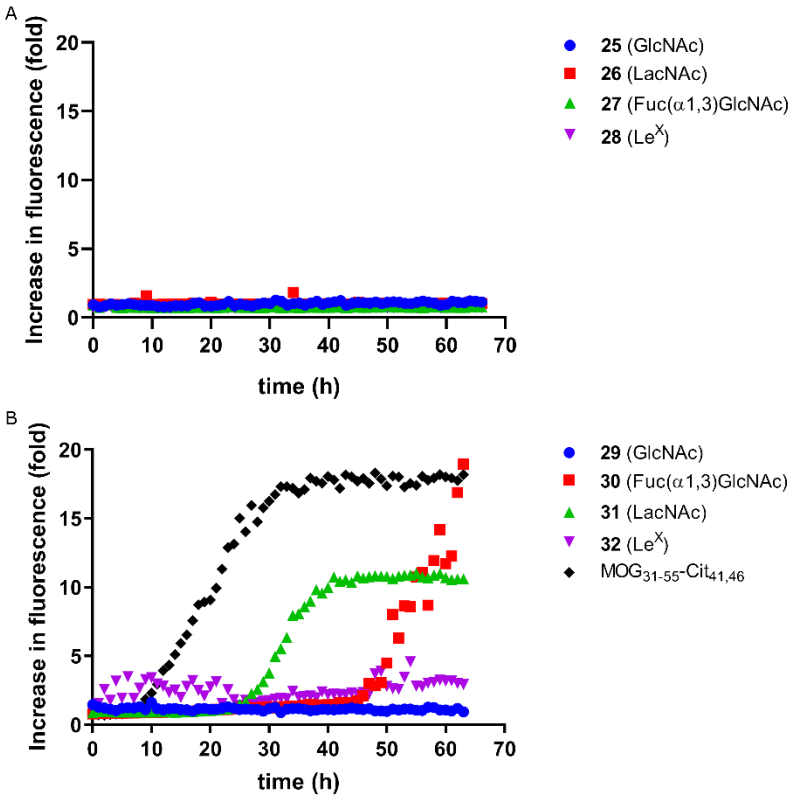


Figure 8. ThT aggregation assay of non-citrullinated (A) and citrullinated (B) glycosylated MOG31-55 peptides 17a-20b. Peptides were tested at a concentration of 10 µM. Positive control (black diamonds) is nonglycosylated MOG31-55 citrullinated at positions 41 and 46. All data were recorded at an excitation wavelength of 444 ± 9 nm and an emission wavelength of 485 ± 9 nm. All samples were used at a pH of 5.0 and aggregation assays were performed at least three times and with experimental triplicates.

While all glycosylated peptides showed reduced aggregation propensity compared to the non-glycosylated control, large differences between the differently glycosylated structures were found (Figure 8B). The peptide containing a single GlcNAc (**29**) did not show any aggregation over the entire duration of the assay. This exemplifies the powerful effect glycosylation can have on peptide aggregation. The peptide containing the DC-SIGN ligand Le^X (**32**) showed a similar inhibition of aggregation to that of GlcNAc, suggesting the potential in controlling immune household and not the neurodegenerative mechanism in MS. However, the other glycosylated peptides tested, that is Fucα1-3GlcNAc containing peptide **30** and LacNAc containing peptide **31**, did still aggregate after longer incubation times, indicating that glycan structure plays a role in this process (Figure 8B).

In previous studies[12] it was shown that citrullinated MOG_{35-55} peptides are cytotoxic to murine bone marrow derived dendritic cells (BMDCs). Citrullinated MOG_{31-55} however was not yet tested. To analyze whether native, glycosylated or citrullinated MOG_{31-55} variants show similar cytotoxicity to those of citrullinated MOG_{35-55}, cell viability assays were conducted using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) as described previously (Figure 9).[12] In this assay, the mitochondrial activity of the cells under investigation is determined by their ability to enzymatically convert MTT into formazan. Formation of this

compound is determined by detection of absorption at 540 nm. By comparing the amount of signal at 540 nm to that produced to the same amount of non-treated cells, the percentage of living cells can be determined.

BMDCs were treated with citrullinated peptides **29-32** as well as their non-glycosylated counterpart (MOG_{31-55}-$Cit_{41,46}$) at four different concentrations (40, 20, 10 and 5 µM). None of the tested peptides showed significant decrease in viability of BMDCs at any concentration tested. As expected, the remaining glycosylated MOG_{31-55} derivatives **25-28** as well as the native variant did also not exhibit any significant drop in cell viability in BMDCs.

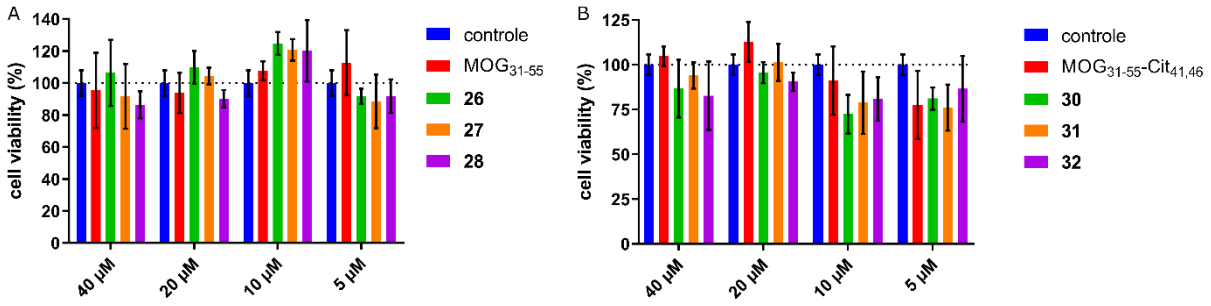


Figure 9. Cell viability as determined by MTT of BMDCs incubated for 3-4 hours with varying concentrations of A) non-citrullinated MOG peptides **21a**-**23a** (N=3) or B) citrullinated MOG peptides **21b**-**23b** (N=6)

From this data, it may be concluded that the glycosylated MOG_{31-55} peptides do not display altered biophysical properties as measured by CD. Furthermore, glycosylated peptide **29** and **32** showed a complete absence of aggregation propensity. No major cytotoxic effects were observed for citrullinated and glycosylated MOG_{31-55} derivatives **29-32** in BMDCs, which renders them useful for subsequent studies to explore the impact of DC-SIGN binding on moDCs.

Next, the ability of the *N*-glycosylated peptides to bind DC-SIGN was assessed by ELISA.[59] For this, the peptides were adsorbed on high-binding 96-well plates and incubated with a recombinant DC-SIGN-Fc construct consisting of the N-terminally truncated extracellular domain (K_{62}-A_{404}) of human DC-SIGN fused to the Fc region of human IgG1 at the N-terminus.[25] This was followed by incubation with a HRP-conjugated anti-IgG1 and 3,3',5,5'-tetramethylbenzidine (TMB) as a substrate for the conjugated HRP. The amount of DC-SIGN-Fc bound to the glycopeptides was determined readout of absorbence at 450 nm (Figure 10). Le^X peptides **28** and **32** were recognized by DC-SIGN-Fc, while the other fucosylated glycopeptides were recognized to a lesser extent (**26, 30**). LacNAc decorated peptides **27** and **31** showed no binding, as expected. A detectable binding of the GlcNAcylated peptide **25** was also observed, in line with previous reports that GlcNAc itself is a ligand for DC-SIGN; albeit a weak one with an IC_{50} of 5 mM *in vitro*.[60] This DC-SIGN binding was not seen for the other GlcNAc containing peptide, **29**.

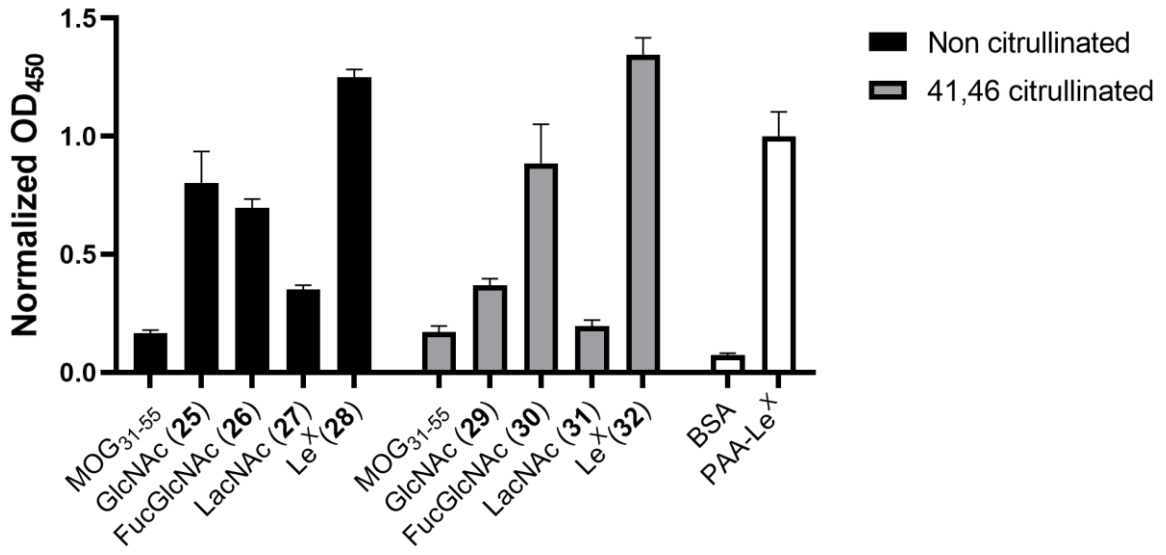


Figure 10. DC-SIGN-Fc ELISA. Lewis X decorated polymer (PAA-LeX) was used as the positive control, while for the negative control no peptide was added, meaning they are fully blocked with BSA. The DC-SIGN ELISA has been performed three times showing similar results. The graph shows data of one representative experiment out of three independent experiments performed in duplicate. Error bars represent standard deviation.

Finally, the downstream effects of stimulation of human monocytes-derived dendritic cells (moDCs) with Le^X decorated peptide **28** was investigated, as this peptide showed good binding to DC-SIGN in the ELISA. Since DC-SIGN is absent on murine DCs,[61] human dendritic cells, derived from donor blood, were used for this experiment. A well-established assay[62] was utilized, where the release of anti- and pro-inflammatory cytokines, IL-10 and IL-12p70 respectively, is measured. When stimulated with TLR4 ligands, DCs become pro-inflammatory, inducing the secretion of IL12p70. Simultaneous stimulation of DC-SIGN with fucosylated glycans induces an upregulation of IL-10 secretion and a down-regulation of IL-12p70 secretion, switching the immune response towards tolerance instead of inflammation. MoDCs from three donors were stimulated with peptide **28** or non-glycosylated MOG_{31-55} at multiple concentrations (14, 7 and 3.5 µM). This was done in presence or absence of the TLR4 ligand LPS (from *E. coli* at 10 ng/mL). After 16 hours, the concentrations of secreted cytokines were measured.[63] No cytokine production was observed upon stimulation of moDCs with peptide in the absence of LPS (Supporting Figure S1). However, upon co-stimulation with LPS, a glycan-dependent effect on IL-12p70 secretion at all concentration tested was observed. In Figure 11A the ratio of IL-10/IL-12p70 secretion is plotted for a single donor (representative for three independent experiments, N=3). An increase of the IL10/IL12P70 ratio was found for the Le^X decorated neoglycopeptide **28** over the non-glycosylated control at all concentrations tested. This increase in IL10/IL12p70 ratio shows that stimulation with peptide **28** leads to a more tolerogenic response compared to non-glycosylated MOG_{31-55}.

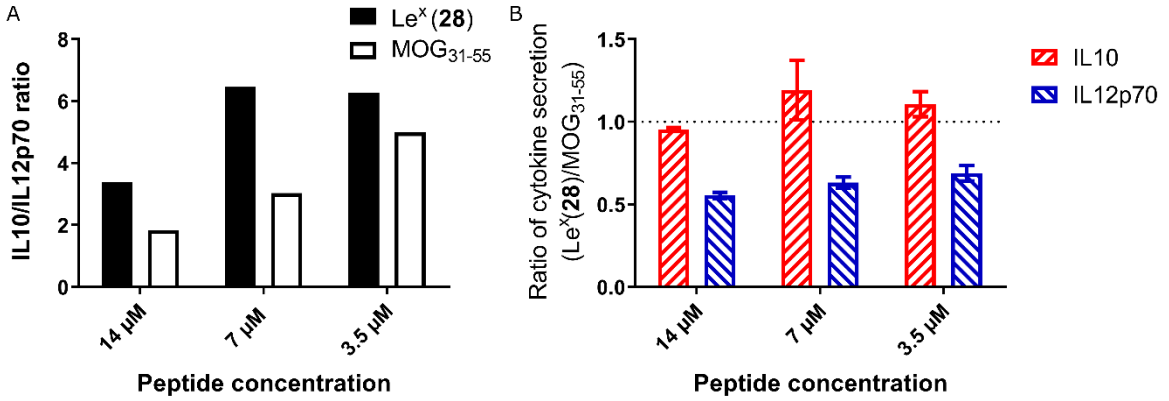


Figure 11. In vitro moDC cytokine profiling upon exposure to **28** A) Ratio of IL10/IL12p70 secretion measured upon moDC stimulation with either **28** or non-glycosylated control in the presence of 10 ng/mL of LPS. This graph is a representative plot from one donor (N=3). B) Normalized ratios for IL-10 and IL-12p70 secretion between non-glycosylated peptide MOG$_{31-55}$ and peptide **28** harboring Le^X incubated with moDCs at different concentrations in the presence 10 ng/mL LPS. Here a ratio of 1 means cytokine production is the same for both peptides, while a ratio of 0.5 means cytokine production is halved for **28** compared to non-glycosylated peptide. The results are the average of three experiments performed using cells from three separate donors, each measured in duplicate.

Figure 11B shows the ratio of cytokine secretion between stimulation of moDCs with **28** and non-glycosylated MOG$_{31-55}$ for all donors (N=3). A reduction in secretion of pro-inflammatory cytokine IL-12p70 is observed, while secretion of anti-inflammatory IL-10 remains unchanged. This indicates the increase in IL10/IL12p70 ratio is mostly driven by a decrease in IL12p70 secretion. Since 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.

Conclusion

In this chapter, the development of a synthetic route for three novel SPPS compatible glycosylated Fmoc-asparagine building blocks, including an asparagine derivative of the important DC-SIGN ligand Le^X is described. These building blocks have been synthesized from the corresponding glycosyl-azides using a Staudinger-reduction/aspartic anhydride ring-opening approach. By careful choice of protecting groups during the oligosaccharide assembly, the amount of protecting group manipulations could be kept to a minimum, and final glycopeptide deprotection was accomplished in a straightforward manner. This was demonstrated by the synthesis of 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, it was demonstrated that glycosylation has a powerful effect on the citrullination driven aggregation of this model peptide. All evaluated peptides carrying both glycosylation and citrullination had slowed induction of aggregation compared to the non-glycosylated control, with the GlcNAc (**29**) and Le^X (**32**) decorated peptides showing no glycosylation at all. Furthermore, it was shown that Le^X, while linked to

asparagine directly via an amide bond, is capable of binding to DC-SIGN, *via* ELISA. In a final experiment it was shown that peptide **28**, decorated with Le^X on asparagine, is able to elicit a tolerogenic response (reduced IL12p70 secretion compared to non-glycosylated counterpart), when used to stimulate moDCs. This indicates that peptides bearing the simplified Le^X N-glycan described in this chapter could be useful tools to study the role of DC-SIGN stimulation on immunotolerance. Given the straightforward synthesis of building block **4** and efficient incorporation of this structure into peptides using SPPS, this offers a new tool to perform experiments on lectin driven immunomodulation.

Experimental Section

General methods for synthesis and characterization of compounds

Solvents were purchased from Honeywell, VWR or Alfa Aesar. Anhydrous solvents were prepared by drying over 4Å molecular sieves. Reagents purchased from chemical suppliers were used without further purification, unless stated otherwise. All reactions were performed under nitrogen atmosphere and/or under exclusion of H_2O, unless stated otherwise. Reactions were followed by thin layer chromatography which was performed using TLC silica gel 60 F254 on aluminium sheets, supplied by Merck. Compounds were visualized using UV absorption (254 nm) and/or a spray reagent, either permanganate (5 g/L $KMnO_4$, 25 g/L K_2CO_3) or sulfuric acid (10% v/v in EtOH). ^1H and ^{13}C NMR spectra were recorded using a Brüker AV400 (400 /101 MHz) and COSY and HSQC 2D experiments were used to assign peaks. Recorded data was interpreted and analyzed using MestReNova 12 software. Chemical shifts are reported in ppm (δ) in reference to an internal standard (TMS) or the residual solvent peak. High resolution mass spectra were recorded by direct injection (2 µL of a 2 µM solution in H_2O/MeCN 1:1 and 0.1% formic acid) on a mass spectrometer (Thermo Fisher Exactive HF Orbitrap) equipped with an electrospray ion source in positive mode. The high resolution mass spectrometer was calibrated prior to measurements with a calibration mixture (Thermo Finnigan). The optical rotation of chirally pure compounds was measured on an Anton Paar MCP110 polarimeter at 25°C. IR spectra were recorded using a Shimadzu IRSpirit fourier transform infrared spectrometer.

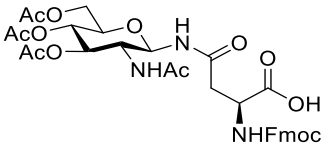
General methods for SPPS

An automated synthesizer (PTI Tribute UV-IR synthesizer, Gyros Protein Technologies) was utilized. If not stated otherwise, peptides were synthesized on Tentagel S RAM resin (Rapp Polymere GmbH, Germany) on a 100 µmol scale using 5.0 equiv of each amino acid (AA) with respect to the resin loading. Fmoc protected amino acids were purchased from either Novabiochem or Sigma-Aldrich. For the amino acids that require sidechain protection, the following protecting groups were used: tBu for Ser, Thr and Tyr; OtBu for Asp and Glu; Trt for Asn, Gln and His; Boc for Lys and Trp; Pbf for Arg. An equimolar quantity of 2-(6-chloro-1-H-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium hexafluorophosphate (HCTU) was used as activator. Coupling cycles of 1 h were utilized, and unreacted amines were capped after each cycle using a solution of 500 µL of acetic anhydride, 250 µL of DIPEA, and 4.25 mL of DMF for 5 min at room temperature twice. Fmoc deprotection was accomplished with 20% piperidine in DMF (3 x 5 min). Cleavage of non-glycosylated peptides was accomplished using a 95:2.5:2.5 mixture of TFA/TES/H_2O for 3 hours, followed by precipitation from cold diethyl ether and recovery of the precipitate by centrifugation. Peptides were characterized using 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). Quality of crude peptides was evaluated with a linear gradient of 10-50% B with a constant 10% C over 10 minutes, while final peptide quality was evaluated using a linear gradient of 5-65% B with a constant 10% C over 30 minutes.

Incorporation of glycosylated amino acids

Synthesis of glycopeptides was carried out at 25 µmol scale. Fmoc group was removed from the resin bound peptide using 2 x 2 mL of 20% (v/v) piperidine in DMF (3 + 7 min). After Fmoc deprotection, the resin was washed five times with DMF (5 x 5 mL). Fully protected glycosylated asparagine (2 eq, 50 µmol) was dissolved in 500 µL of a 0.3 M solution of 3-(diethoxyphosphoryloxy)-1,2,3-benzotriazin-4(3H)-one (DEPBT) in DMF by the addition of DIPEA (8.7 µL, 2 eq, 50 µmol). The mixture was agitated for at least 5 minutes or until all 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 x 5 mL) and a small portion was deprotected to confirm incorporation of the glycosylated amino acid. Fmoc deprotection was carried out as normal using a freshly prepared piperidine solution. Full cleavage of the peptide was achieved using 2 mL of 95:2.5:2.5 (v/v) mixture of TFA/TES/H_2O for 2 hours or 50:2.5:2.5:45 (v/v) mixture of TFA/TES/H_2O/DCM for 4 hours for fucose containing peptides. The deprotected peptide was precipitated in cold diethyl ether (10 mL) and the resin was washed with DCM (1 mL) which was added to the ether phase. After centrifugation, the pellet was washed with a small amount of diethyl ether (3-5 mL) and centrifugated again. To facilitate the removal of the ester protection groups, the peptide was suspended in methanol (2.25 mL) in a roundbottom 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 confirmed the volatiles were removed *in vacuo* to yield the crude glycopeptide. Preparative reverse phase HPLC on a Waters AutoPurification system (eluent A: H_2O + 0.2% TFA; eluent B: ACN) with a preparative Gemini C18 column (5 µm, 150 x 21.2 mm) yielded the final products.

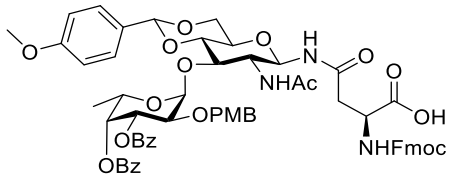
N^{γ}-[3,4,6-tri-O-acetyl-2-deoxy-2-acetamido-β-D-glucopyranosyl]-N^{α}-fluorenylmethoxycarbonyl-L-asparagine (1)



Glycosyl azide **11** (200 mg, 0.54 mmol) was dissolved in THF (0.76 mL) and the solution was cooled in an icebath. 0.54 mL of a 1 M solution of trimethylphosphine (1.0 eq, 0.54 mmol) in THF was added dropwise over 2 minutes, during which gas evolution was observed. The icebath was removed, and the reaction was stirred for 5 minutes before H2O (10 eq, 97 µL, 5.4 mmol) was added. The reaction was stirred at room temperature for 1.5 hours, after which it was concentrated. The residue containing the crude glycosyl amine was redissolved in DMSO (1.8 mL) and Fmoc-aspartic anhydride[45] (1.0 eq, 181 mg, 0.54 mmol) was added. The reaction was stirred for 2 hours at room temperature. The DMSO solution was added dropwise to a centrifuge tube containing 30 mL of a 2:1 mixture of diethyl ether and ethyl acetate and a precipitate started to form. The compound was left to fully precipitate for 16 hours 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 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, DMSO-d6) δ 8.60 (d, J = 9.8 Hz, 1H, $N^{\gamma}H$), 7.99 – 7.78 (m, 3H, N<u>H</u>C(O)CH_3, Fmoc-Ar), 7.71 (d, J = 7.5 Hz, 2H, Fmoc-Ar), 7.51 (d, J = 8.5 Hz, 1H, $N^{\alpha}H$), 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, H1), 5.10 (t, J = 9.8 Hz, 1H, H3), 4.82 (t, J = 9.8 Hz, 1H, H4), 4.38 (q, J = 7.5 Hz, 1H, Asn-CH), 4.33 – 4.13 (m, 4H, Fmoc-CH_2, Fmoc-CH, H6a), 3.94 (d, J = 11.3 Hz, 1H, H6b), 3.88 (q, J = 9.8 Hz, 1H, H2), 3.84

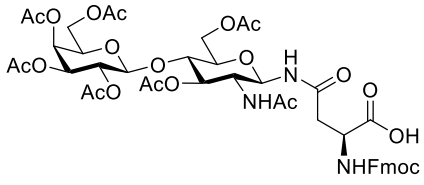
– 3.78 (m, 1H, H5), 2.66 (dd, J = 16.3, 5.4 Hz, 1H, Asn-CHH), 1.99 (s, 3H, OC(O)CH_3), 1.96 (s, 3H, OC(O)CH_3), 1.90 (s, 3H, OC(O)CH_3), 1.72 (s, 3H, NHC(O)CH_3). **^{13}C NMR** (126 MHz, DMSO-d6) δ = 173.0 (C=O), 170.1 (C=O), 169.9 (C=O), 169.6 (C=O), 169.6 (C=O), 169.4 (C=O), 155.9 (C=O), 143.8 (Fmoc-Ar), 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 (C1), 73.4 (C3), 72.3 (C5), 68.4 (C4), 65.8 (Fmoc-CH_2), 61.9 (C6), 52.2 (C2), 50.0 (Asn-CH), 46.6 (Fmoc-CH), 36.9 (Asn-CH_2), 22.6 (NHC(O)CH_3), 20.6 (OC(O)CH_3), 20.4 (OC(O)CH_3), 20.4 (OC(O)CH_3). **HRMS** (ESI) m/z: [M + H^+] calcd for $C_{33}H_{37}N_3O_{13}H$ 684.23991, found 684.23920.

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 **16** (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 eq, 220 µL, 0.22 mmol). The reaction was stirred for 10 minutes at room temperature and H_2O (50 eq, 180 µL, 10 mmol) was added. After stirring for 1 hour at room temperature, the reaction was concentrated and the residue was dissolved in DMSO (2 mL). Fmoc-aspartic anhydride[45] (1.0 eq, 67 mg, 0.2 mmol) was added and the reaction mixture was stirred for 1 hour at room temperature. The solvent was removed *in vacuo* and the crude was subjected to silica gel column chromatography (0 → 8% MeOH in DCM, Δ = 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, DMSO-d6) δ 8.46 (d, J = 9.4 Hz, 1H, N$^\gamma$H), 8.16 (d, J = 9.0 Hz, 1H, NHC(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, N$^\alpha$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, H1', H3'), 5.23 (d, J = 3.5 Hz, 1H, H4'), 5.15 (t, J = 9.5 Hz, 1H, H1), 4.55 – 4.43 (m, 2H, H5', PMB-CHH), 4.39 – 4.30 (m, 2H, PMB-CHH, Asn-CH), 4.30 – 4.17 (m, 4H, Fmoc-CH_2, H5, Fmoc-CH), 4.13 (t, J = 9.5 Hz, 1H, H3), 3.99 (dd, J = 10.7, 3.5 Hz, 1H, H2'), 3.95 – 3.84 (m, 1H, H2), 3.76 – 3.60 (m, 9H, H6, H4, OCH_3, OCH_3), 2.66 (dd, J = 16.1, 5.6 Hz, 1H, Asn-CHH), 1.82 (s, 3H, NHC(O)CH_3), 0.46 (d, J = 6.4 Hz, 3H, H6'). **^{13}C NMR** (101 MHz, DMSO-d6) δ = 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 (C1'), 79.4 (C1, C4), 75.3 (C3), 72.3 (C4'), 71.5 (C2'), 70.0 (PMB-CH_2), 69.6 (C3'), 68.0 (C5), 67.8 (C6), 65.8 (Fmoc-CH_2), 63.9 (C5'), 55.1 (OCH_3, C2), 55.0 (OCH_3), 50.4 (Asn-CH), 46.6 (Fmoc-CH), 37.3 (Asn-CH_2), 23.1 (NHC(O)CH_3), 15.2, (C6'). **HRMS** (ESI) m/z: [M + H^+] calcd for $C_{63}H_{63}N_3O_{18}H$ 1150.41794, found 1150.41741.

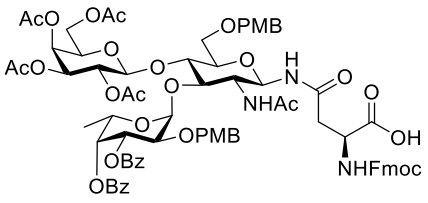
N$^\gamma$-[2,3,4,6-tetra-O-acetyl-β-D-galactopyranoside-(1→4)-6,3-di-O-acetyl-2-deoxy-2-acetamido-β-D-glucopyranosyl]-N$^\alpha$-fluorenylmethoxycarbonyl-L-asparagine (3)



Glycosyl azide **20** (0.74 mmol, 488 mg) was dissolved in THF (7.4 mL) and a 1 M solution of trimethylphosphine in THF (1.5 eq, 1.1 mL, 1.1 mmol) was added and the reaction was stirred at room temperature. H_2O (50 eq, 0.67 mL, 37 mmol) was added and the

reaction was further stirred for 60 minutes. The volatiles were removed *in vacuo* and the crude glycosyl amine was redissolved in DMSO (7.4 mL). Fmoc-aspartic anhydride (1 eq, 0.74 mmol, 249 mg) was added and the reaction was stirred for 75 minutes. The solvent was removed *in vacuo* and the crude was subjected to silica gel column chromatography (0 → 8% MeOH in DCM, Δ = 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, DMSO-d6) δ 8.58 (d, J = 9.1 Hz, 1H, N$^{\gamma}$H), 7.89 (d, *J* = 7.7 Hz, 2H, Fmoc-Ar), 7.86 (d, J = 9.5 Hz, 1H, NHC(O)CH$_3$), 7.71 (d, J = 7.5 Hz, 2H, Fmoc-Ar), 7.42 (t, J = 7.3 Hz, 3H, Fmoc-Ar, N$^{\alpha}$H), 7.33 (t, J = 7.4 Hz, 2H, Fmoc-Ar), 5.23 (d, J = 3.7 Hz, 1H, H4'), 5.16 (dd, J = 10.3, 3.6 Hz, 1H, H3'), 5.10 (t, J = 9.5 Hz, 1H, H1), 4.97 (t, J = 9.5 Hz, 1H, H3), 4.84 (dd, J = 10.3, 8.0 Hz, 1H, H2'), 4.70 (d, J = 8.0 Hz, 1H, H1'), 4.36 – 4.15 (m, 6H, Asn-CH, Fmoc-CH$_2$, H6a, H5', Fmoc-CH), 4.09 – 3.95 (m, 3H, H6b, H6'), 3.81 (q, J = 9.5 Hz, 1H, H2), 3.73 – 3.55 (m, 2H, H4, H5), 2.63 (dd, J = 16.3, 5.3 Hz, 1H, Asn-CH$_2$), 2.11 (s, 3H, C(O)CH$_3$), 2.07 (s, 3H, C(O)CH$_3$), 2.01 (s, 3H, C(O)CH$_3$), 2.01 (s, 3H, C(O)CH$_3$), 1.94 (s, 3H, C(O)CH$_3$), 1.90 (s, 3H, C(O)CH$_3$), 1.71 (s, 3H, NH(CO)CH$_3$). **^{13}C NMR** (101 MHz, DMSO-d6) δ = 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 (C1'), 77.9 (C1), 76.2 (C4), 73.8 (C3), 73.5 (C5), 70.4 (C3'), 69.7 (C5'), 68.9 (C2'), 67.1 (C4'), 65.7 (Fmoc-CH$_2$), 62.5 (C6), 60.9 (C6'), 52.3 (C2), 50.3 (Asn-CH), 46.6 (Fmoc-CH), 37.1 (Asn-CH$_2$), 22.7 (NHC(O)CH$_3$), 20.7 (C(O)CH$_3$), 20.6 (C(O)CH$_3$), 20.5 (C(O)CH$_3$), 20.4 (C(O)CH$_3$), 20.4 (C(O)CH$_3$) **HRMS** (ESI) m/z: [M + H$^+$] calcd for $C_{45}H_{53}N_3O_{21}H$ 972.32443, found 972.32357

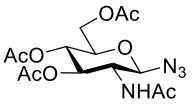
N$^{\gamma}$-{2,3,4,6-tetra-O-acetyl-β-D-galactopyranoside-(1→4)-[3,4-di-O-benzoyl-2-O-(4-methoxybenzyl)-α-L-fucopyranoside-(1→3)]-6-O-(4-methoxybenzyl)-2-deoxy-2-acetamido-β-D-glucopyranosyl}-N$^{\alpha}$-fluorenylmethoxycarbonyl-L-asparagine (4)



Glycosyl azide **5** (53 mg, 45 µmol) was dissolved in dry THF (0.45 mL) and cooled to 0°C in an icebath. 75 µL of a 1 M trimethylphosphine solution in THF was added dropwise. The reaction was stirred for 5 minutes at 0°C and for 5 minutes at room temperature. H_2O (50 eq, 40 µL, 2.25 mmol) was added and the reaction was stirred for 2 hours at room temperature. The volatiles were removed *in vacuo* and the crude glycosyl amine was redissolved in DMA (450 µL). The reaction mixture was again cooled in an icebath and aspartic anhydride[45] (1 eq, 15 mg, 45µmol) was added. The reaction was stirred and allowed to warm to room temperature overnight. The solvent was removed by evaporation and the crude glycoaminoacid 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, 73%). Traces of acetic acid were removed by sequential co-evaporation with dioxane (3 x 2 mL), toluene (3 x 2 mL) and $CHCl_3$ (3 x 2 mL). $[\alpha]_D^{25}$ = -94.2 (c 1.00 in $CHCl_3$). **^{1}H NMR** (400 MHz, $CDCl_3$) δ 7.98 – 7.89 (m, 2H, CH_{arom}), 7.78 – 7.64 (m, 5H, N$^{\gamma}$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, NHC(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, N$^{\alpha}$H), 5.63 – 5.54 (m, 2H, H4', H3'), 5.47 (d, J = 3.4 Hz, 1H, H1'), 5.33 (d, J = 3.7 Hz, 1H, H4'), 5.09 (dd, J = 10.4, 8.0 Hz, 1H, H2''), 4.99 (t, J = 7.8 Hz, 1H, H1), 4.85 (dd, J = 10.4, 3.6 Hz, 1H, H3''), 4.81 – 4.70 (m, 1H, H5'), 4.69 – 4.43 (m, 5H, PMB-CH$_2$, Asn-CH, Fmoc-CH, H1''), 4.39 – 4.22 (m, 5H, Fmoc-CH$_2$, PMB-CHH, H6''), 4.22 – 4.03 (m, 4H, PMB-CHH, H2, H2', H4), 3.95 (t, J = 8.4 Hz, 1H, H3), 3.82 – 3.63 (m, 8H, OCH$_3$, H6, OCH$_3$), 3.57 – 3.44 (m, 2H, H5, H5''), 2.90 – 2.72 (m, 2H, Asn-

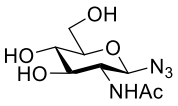
CH_2), 2.17 (s, 3H, C(O)CH_3), 2.07 – 1.91 (m, 12H, 4 x C(O)CH_3), 1.24 (d, J = 6.5 Hz, 3H, H6'). **^{13}C NMR** (101 MHz, $CDCl_3$) δ = 173.5 (C=O), 173.2 (C=O), 171.8 (C=O), 170.5 (C=O), 170.4 (C=O), 170.0 (C=O), 169.8 (C=O), 165.9 (C=O), 165.3 (C=O), 159.6 (C_q), 159.5 (C_q), 156.4 (C=O), 143.9 (Fmoc-Ar), 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 (C1''), 97.4 (C1'), 79.7 (C1), 76.0 (C5, C3), 73.3 (C4), 73.3 (C2'), 73.3 (PMB-CH_2), 72.7 (PMB-CH_2), 72.5 (C4'), 71.0 (C5''), 70.8 (C3''), 70.1 (C3'), 69.3 (C2''), 67.8 (C6), 67.2 (Fmoc-CH_2), 66.9 (C4''), 65.8 (C5'), 61.0 (C6''), 55.3 (OCH_3), 55.2 (OCH_3), 53.6 (C2), 50.5 (Asn-CH), 47.1 (Fmoc-CH), 37.9 (Asn-CH_2), 22.8 (NHC(O)$\underline{C}H_3$), 20.8 (C(O)$\underline{C}H_3$), 20.8 (C(O)$\underline{C}H_3$), 20.7 (C(O)$\underline{C}H_3$), 20.6 (C(O)$\underline{C}H_3$), 16.1 (C6'). **HRMS** (ESI) m/z: [M + Na^+] calcd for $C_{77}H_{83}N_3O_{27}Na$ 1504.51061, found 1504.51004

Azido 3,4,6-tri-O-acetyl-2-deoxy-2-acetamido-β-D-glucopyranoside (11)



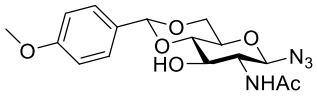
N-acetyl glucosamine was converted into the 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-α-D-glucopyranosyl chloride as described by Horton[47]. Briefly, N-acetyl glucosamine (11.0 g, 50 mmol) was carefully added to acetyl chloride (25.0 ml, 350 mmol). The resulting suspension was heated to 30°C for 30 minutes, followed by overnight stirring at room temperature. The reaction mixture was diluted with chloroform (100 mL) and washed with ice water (100 g ice and 25 mL water). The organic layer was washed with ice cold saturated $NaHCO_{3\ (aq)}$ (100 mL) and dried over $MgSO_4$. The solution was concentrated to 20% of the original volume and Et_2O (100 mL) was added. The product was allowed to crystalized overnight to yield 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-α-D-glucopyranosyl chloride in a 3.6:1 mixture with 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-α-D-glucopyranosyl acetate, which was collected by filtration and dried *in vacuo*. This mixture was used without further purification in the next step. The crude glycosyl chloride was dissolved in DMF (125 mL) and NaN_3 (3.25 g, 50 mmol) was added. The reaction was stirred for 2 hours at room temperature until TLC (100% EtOAc) confirmed complete consumption of the glycosyl chloride. The reaction mixture was diluted with saturated aqueous $NaHCO_3$ and the product was extracted with DCM. The organic layer was washed with a second portion of saturated aqueous $NaHCO_3$ and dried over $MgSO_4$, filtered and concentrated. The mixture containing the β-glycosyl azide and α-glycosyl acetate was dissolved in THF (125 mL) and N,N-dimethylaminopropylamine (70 mmol, 8.8 mL) was added. The reaction mixture was stired at room temperature two hours, resulting in the selective hydrolysis of the anomeric acetate as described by Andersen et al.[48] The reaction mixture was diluted with DCM and washed with 1 M aqueous HCl. The organic layer was dried over $MgSO_4$, filtered and concentrated. Silica gel column chromatography (80% EtOAc in DCM) gave the title compound (7.82 g, 21 mmol, 42%). $\mathbf{[\alpha]_D^{25}}$ = -41.6° (c 1.00 in $CHCl_3$) $\mathbf{\nu_{max}/cm^{-1}}$ 2117.80 (N_3), 1747.18 (CO), 1664.19 (CO) **^{1}H NMR** (400 MHz, $CDCl_3$) δ 5.66 (d, J = 8.9 Hz, 1H, NH), 5.25 (dd, J = 10.6, 9.3 Hz, 1H, H3), 5.11 (t, J = 9.7 Hz, 1H, H4), 4.76 (d, J = 9.3 Hz, 1H, H1), 4.28 (dd, *J* = 12.5, 4.8 Hz, 1H, H6a), 4.17 (dd, J = 12.4, 2.3 Hz, 1H, H6b), 3.92 (dt, J = 10.7, 9.1 Hz, 1H, H2), 3.79 (ddd, J = 10.0, 4.8, 2.3 Hz, 1H, H5), 2.11 (s, 3H, C(O)CH_3), 2.05 (s, 3H, C(O)CH_3), 2.04 (s, 3H, C(O)CH_3), 1.99 (s, 3H, C(O)CH_3) **^{13}C NMR** (101 MHz, $CDCl_3$) δ = 171.0 (C=O), 170.8 (C=O), 170.7 (C=O), 169.4 (C=O), 88.5 (C1), 74.0 (C5), 72.2 (C3), 68.3 (C4), 62.0 (C6), 54.1 (C2), 23.3 (NHC(O)$\underline{C}H_3$), 20.8 (C(O)$\underline{C}H_3$), 20.7 (C(O)$\underline{C}H_3$), 20.7 (C(O)$\underline{C}H_3$) **HRMS** (ESI) m/z: [M + H^+] calcd for $C_{14}H_{20}N_4O_8H$ 373.13539, found 373.13521.

Azido 2-deoxy-2-acetamido-β-D-glycopyranoside (12)



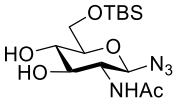
Acetylated glycosyl azide **11** (4.3g, 11.6 mmol) was dissolved in methanol (115 mL) and put under inert atmosphere. Elemental sodium was added until the pH reached 11 (as indicated by wet pH paper) and the reaction was stirred at room temperature for 2 hours, after which TLC (100% EtOAc) indicated full consumption of starting material. The reaction was neutralized with amberlite H^+ resin and the resin was filtered off. The volatiles were removed *in vacuo* to yield the title compound in quantitative yield. This compound was used without further purification. **ν_{max}/cm^{-1}** 2117.80 (N_3) 1644.16 (CO) **^{1}H NMR** (400 MHz, MeOD) δ 4.51 (d, J = 9.2 Hz, 1H, H1), 3.90 (dd, J = 12.1, 1.8 Hz, 1H, H6a), 3.74 – 3.63 (m, 2H, H6b, H2), 3.47 (dd, J = 9.6, 8.5 Hz, 1H, H3), 3.41 – 3.36 (m, 2H, H4, H5), 2.00 (s, 3H, NHC(O)C$\underline{H}_3$) **^{13}C NMR** (101 MHz, MeOD) δ 173.8 (C=O), 90.1 (C1), 80.3 (C4), 75.7 (C3), 71.6 (C5), 62.6 (C6), 56.7 (C2), 22.9 (NHC(O)$\underline{C}H_3$) **HRMS** (ESI) m/z: [M + H+] calcd for C8H14N4O5H 247.10370, found 247.10360

Azido 4,6-O-(4-methoxybenzylidene)-2-deoxy-2-acetamido-β-D-glucopyranoside (9)



Deacetylated glycosyl azide **12** (492 mg, 2.0 mmol) was suspended in dry acetonitrile (8 mL) and anisaldehyde dimethyl acetal (2 eq., 660 µL, 4.0 mmol) and CSA (0,1 eq., 46 mg, 0,2 mmol) were added. The reaction was kept at 50 °C and 300 mbar on a rotary evaporator for 3h. The reaction was quenched by the addition of trimethylamine (100 µL) and the volatiles were removed under reduced pressure. The crude was recrystallized from methanol to yield the title compound (475 mg, 1,3 mmol, 65%) **$[\alpha]_D^{25}$** = -70.0° (c 0.10 in $CHCl_3$) **ν_{max}/cm^{-1}** 2114.94 (N_3), 1651.31 (CO) **^{1}H NMR** (400 MHz, DMSO-d_6) δ 7.99 (d, J = 8.4 Hz, 1H, NH), 7.37 (d, J = 8.8 Hz, 2H, CH_{arom}), 6.93 (d, J = 8.8 Hz, 2H, CH_{arom}), 5.57 (s, 1H, PMP-CH_{acetal}), 5.45 (d, J = 5.2 Hz, 1H, 3-OH), 4.60 (d, J = 8.8 Hz, 1H, H1), 4.21 (dd, J = 10.1, 4.3 Hz, 1H, H6), 3.75 (s, 3H, OCH_3), 3.74 – 3.44 (m, 5H, H6, H2, H3, H4, H5), 1.86 (s, 3H, NHC(O)C$\underline{H}_3$) **^{13}C NMR** (101 MHz, DMSO-d_6) δ 169.62 (NH$\underline{C}$(O)CH_3), 159.63, 129.98 (C_q), 127.74, 113.38 (CH_{arom}), 100.73 (PMP-$\underline{C}$H), 88.83 (C1), 80.79 (C4), 70.26 (C3), 68.19 (C5), 67.49 (C6), 55.42 (C2), 55.16 (OCH_3), 22.97 (NHC(O)$\underline{C}H_3$) **HRMS** (ESI) m/z: [M + H^+] calcd for $C_{16}H_{20}N_4O_6H$ 365.14556, found 365.14532

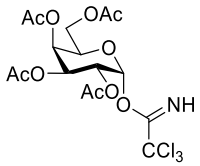
Azido 6-(*tert*-butyldimethylsilyl)-2-deoxy-2-acetamido-β-D-glucopyranoside (13)



Deacetylated glycosyl azide **12** (1.23 g, 5 mmol) was co-evaporated three times with toluene and dissolved in 50 mL of dry pyridine. TBS-Cl (1.5 eq, 2.7 mL, 7.5 mmol) was added and the reaction was stirred at room temperature. After 2 hours, TLC (10% MeOH in EtOAc) indicated complete consumption of the starting material. The reaction mixture was poured into H_2O (100 mL) and transferred to a separatory funnel. DCM (200 mL) and 1 M HCl (aq) (100 mL) were added and the organic layer was collected. The organic layer was dried over $MgSO_4$, filtered and concentrated. Traces of pyridine were removed with toluene co-evaporation. Silica gel column chromatography (0% → 1% → 2% → 5% → 10% MeOH in EtOAc) yielded the title compound (1.51 g, 4.19 mmol, 84%). **$[\alpha]_D^{20}$** = -57,4 (c 1.00 in $CHCl_3$) **ν_{max}/cm^{-1}** 2114.94 (N_3), 1648.45 (CO) **^{1}H NMR** (400 MHz, $CDCl_3$) δ 6.90 (d, J = 7.9 Hz, 1H, NH), 5.08 (d, J = 4.2 Hz, 1H, 3-OH), 4.63 (d, J = 8.7 Hz, 1H, H1), 4.30 (d, J = 3.2 Hz, 1H, 4-OH), 3.91 (ddd, J = 19.5, 11.2, 4.1 Hz, 2H, H6), 3.73 – 3.56 (m, 2H, H2, H3), 3.52 (td, J = 8.8, 8.4, 3.2 Hz, 1H, H4), 3.42 (dt, J = 8.8, 4.5 Hz, 1H, H5), 2.04 (s, 3H, NHC(O)C$\underline{H}_3$), 0.91 (s, 9H, tBu), 0.11 (d, J = 1.3 Hz, 6H, Si-CH_3) **^{13}C NMR** (101 MHz, $CDCl_3$) δ = 172.7 (C=O), 88.5 (C1), 77.7 (C5), 74.5 (C3),

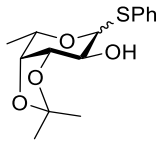
71.6 (C4), 63.6 (C6), 55.4 (C2), 25.9 (tBu), 23.3 (NHC(O)$\underline{C}H_3$), 18.4 (Si-C), -5.2 (Si-CH_3) **HRMS** (ESI) m/z: [M + Na^+] calcd for $C_{14}H_{28}N_4O_5SiNa$ 383.1721, found 383.1729

2,3,4,6-tetra-O-acetyl-α-D-galactopyranosyl trichloroacetimidate (8)



1,2,3,4,6-tetra-O-acetyl-β-D-galactopyranose (7.8 g, 20 mmol) was dissolved in dry THF (100 mL) and N,N-dimethylaminopropylamine (5 eq, 12,8 mL) was added according to the procedure of Andersen *et al.*[48] The reaction was stirred at room temperature for 1.5 hours, after which TLC (1/1 EtOAc/pentane) showed full conversion. The reaction was diluted with DCM and washed with 1 M aqueous HCl. The organic layer was collected, dried over $MgSO_4$, filtered and concentrated. The crude was co-evaporated once with toluene and dissolved in dry DCM (100 mL). Trichloroacetonitrile (5 eq, 10 mL, 100 mmol) and DBU (0.1 eq, 0.3 mL, 2 mmol) were added. The reaction was stirred at room temperature for 1 h, after which TLC (1/1 EtOAc/pentane) showed full conversion. Celite was added to the reaction and the volatiles were removed *in vacuo*. Silica gel column chromatography on neutralized silica (30% → 40% → 50% Et_2O in pentane) yielded the title compound (5.87 g, 11.6 mmol, 58%, corrected for residual diethyl ether), which was immediately stored at -20°C under an N_2 atmosphere. **^{1}H NMR** (400 MHz, $CDCl_3$) δ 8.69 (s, 1H, NH), 6.60 (d, J = 3.5 Hz, 1H, H1), 5.57 (dd, J = 3.2, 1.3 Hz, 1H, H4), 5.43 (dd, J = 10.8, 3.2 Hz, 1H, H3), 5.36 (dd, J = 10.8, 3.5 Hz, 1H, H2), 4.49 – 4.41 (m, 1H, H5), 4.17 (dd, J = 11.3, 6.6 Hz, 1H, H6a), 4.09 (dd, J = 11.3, 6.7 Hz, 1H, H6b), 2.18 (s, 3H, C(O)CH_3), 2.04 (s, 3H, C(O)CH_3), 2.03 (s, 3H, C(O)CH_3), 2.02 (s, 3H, C(O)CH_3) **^{13}C NMR** (101 MHz, $CDCl_3$) δ 170.4 (C=O), 170.2 (C=O), 170.0 (C=O), 161.0 (C=NH), 93.6 (C1), 69.0 (C5), 67.6 (C3), 67.4 (C4), 66.9 (C2), 61.3 (C6), 20.7 (C(O)$\underline{C}H_3$), 20.7 (C(O)$\underline{C}H_3$), 20.7 (C(O)$\underline{C}H_3$), 20.6 (C(O)$\underline{C}H_3$)

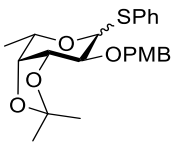
Phenyl 3,4-O-isopropylidene-1-thio-L-fucopyranoside (14)



L-fucose (16,4 g, 100 mmol) was suspended in ethyl acetate (250 mL) and pyridine (7.5 eq., 60 mL, 750 mmol) was added. The mixture was cooled in an icebath and acetic anhydride (7.5, eq., 70 mL, 750 mmol) and DMAP (0.1 eq., 1,2 g, 10 mmol) were added. The reaction was stirred and allowed to warm to room temperature. After 4 hours, TLC indicated starting material consumption and all compound was dissolved. The reaction was again cooled with an icebath and methanol was added to quench the reaction. The organic phase was washed with 1 M HCl, saturated $NaHCO_3$ and brine, dried over $MgSO_4$, filtered, and concentrated. This compound was used in the next reaction without purification. **^{1}H NMR** (400 MHz, $CDCl_3$) δ 6.34 (d, J = 3.0 Hz, 1H, H1), 5.39 – 5.28 (m, 3H, H2, H3, H4), 4.28 (q, J = 6.6 Hz, 1H, H5), 2.19 (s, 3H, C(O)CH_3), 2.16 (s, 3H, C(O)CH_3), 2.02 (s, 3H, C(O)CH_3), 2.01 (s, 3H, C(O)CH_3), 1.16 (d, J = 6.6 Hz, 3H, H6) **^{13}C NMR** (101 MHz, $CDCl_3$) δ 170.5 (C=O), 170.2 (C=O), 170.0 (C=O), 169.2 (C=O), 90.0 (C1), 70.6, 67.8, 67.3, 66.5 (C2, C3, C4, C5), 21.0 (C(O)$\underline{C}H_3$), 20.7 (C(O)$\underline{C}H_3$), 20.6 (C(O)$\underline{C}H_3$), 20.6 (C(O)$\underline{C}H_3$), 16.0 (C6). The intermediate was coevaporated once with toluene and then dissolved in toluene (100 mL). Thiophenol (1.1 eq., 11,3 mL, 110 mmol) was then added and the mixture was cooled in an icebath. $BF_3{\cdot}Et_2O$ (3.0 eq., 37 mL, 300 mmol) was added and after 15 minutes the icebath was removed. After one hour TLC indicated complete consumption of the starting material. The reaction was diluted with toluene and washed five

times with saturated aqueous $NaHCO_3$. The organic phase was dried over $MgSO_4$, filtered, and concentrated to yield the thioglycoside in a 1:4 α:β ratio. This compound was used in the next reaction without purification. **^{1}H NMR** (300 MHz, $CDCl_3$) δ 7.55 – 7.24 (m, 6H, CH_{arom}), 5.94 (d, J = 4.9 Hz, 1H, H1α), 5.40 – 5.29 (m, 3H, H2α, H3α, H4α), 5.27 (dd, J = 3.3, 1.2 Hz, 1H, H4β), 5.21 (t, J = 9.9 Hz, 1H, H2β), 5.05 (dd, J = 9.9, 3.3 Hz, 1H, H3β), 4.71 (d, J = 9.9 Hz, 1H, H1β), 4.61 (q, J = 6.5 Hz, 1H, H5α), 3.84 (q, J = 6.4 Hz, 1H, H5β), 2.17 (s, 3H, C(O)CH_3 (α)), 2.15 (s, 3H, C(O)CH_3 (β)), 2.10 (s, 3H, C(O)CH_3 (α)), 2.09 (s, 3H, C(O)CH_3 (β)), 2.02 (s, 3H, C(O)CH_3 (α)), 1.98 (s, 3H, C(O)CH_3 (β)), 1.24 (d, J = 6.4 Hz, 3H, C6β), 1.13 (d, J = 6.5 Hz, 3H, C6α) **^{13}C NMR** (75 MHz, $CDCl_3$) δ 170.7 (C=O), 170.2 (C=O), 169.6 (C=O), 132.4 $C(C_q)$, 131.8 (CH_{arom}), 129.2 (CH_{arom}), 129.0 (CH_{arom}), 128.0 (CH_{arom}), 127.6 (CH_{arom}), 86.6 (C1β), 85.6 (C1α), 73.2 (C5β), 72.5 (C3β), 71.0 (C4α), 70.4 (C4β), 68.7, 68.2 (C2α, C3α), 67.4 (C2β), 65.6 (C5α), 21.0 (C(O)$\underline{C}H_3$), 20.7 (C(O)$\underline{C}H_3$), 16.6 (C6β), 16.0 (C6α). The acetylated thioglycoside was suspended in methanol (500 mL). Metallic sodium was added until the pH was 12 and the reaction was left to stir overnight. The reaction was neutralized with amberlite H^+ resin, filtered and concentrated to give the deacetylated intermediate. This compound was used in the next reaction without purification. **^{1}H NMR** (400 MHz, MeOD) δ 7.56 – 7.45 (m, 2H, CH_{arom}), 7.32 – 7.18 (m, 4H, CH_{arom}), 5.60 (d, J = 5.6 Hz, 1H, H1α), 4.58 (d, J = 9.6 Hz, 1H, H1β), 4.40 (q, J = 6.6 Hz, 1H, H5α), 4.17 (dd, J = 10.1, 5.6 Hz, 1H, H2α), 3.75 – 3.70 (m, 2H, H3α, H4α), 3.70 – 3.57 (m, 3H, H4β, H2β, H5β), 3.53 (dd, J = 9.2, 3.3 Hz, 1H, H3β), 1.25 (d, J = 6.5 Hz, 3H, H6β), 1.19 (d, J = 6.6 Hz, 3H, H6α). **^{13}C NMR** (101 MHz, MeOD) δ 132.8 (C_q), 131.9 (CH_{arom}), 129.8 (CH_{arom}), 129.7 (CH_{arom}), 127.9 (CH_{arom}), 91.0 (C1α), 89.7 (C1β), 76.2 (C3β), 75.7 (C5β), 73.2 (C3α), 72.9 (C4β), 72.3 (C4α), 70.6 (C2β), 69.5 (C2α), 68.5 (C5α), 17.0 (C6β), 16.5 (C6α). The crude triol was dissolved in acetone (500 mL). 2,2-Dimethoxypropane (4.0 eq., 49 mL, 400 mmol) and CSA (0,1 eq., 2,3 g, 10 mmol) were added. The reaction was stirred at room temperature for 1 hour, after which TLC analysis indicated consumption of stating material. The reaction was quenched with Et_3N (2 mL) and concentrated. Silica gel column chromatography (50% Et_2O/pentane) yielded the title compound as an inseparable mixture of anomers (1:4, α:β) (23,7 g, 80 mmol, 80%). **^{1}H NMR** (400 MHz, $CDCl_3$) δ 7.62 – 7.46 (m, 2H, CH_{arom}), 7.36 – 7.25 (m, 4H, CH_{arom}), 5.56 (d, *J* = 4.9 Hz, 1H, H1α), 4.57 (qd, *J* = 6.6, 2.3 Hz, 1H, H5α), 4.43 (d, *J* = 10.2 Hz, 1H, H1β), 4.20 – 4.08 (m, 3H, H2α, H3α, H4α), 4.08 – 4.02 (m, 2H, H3β, H4β), 3.88 (qd, *J* = 6.6, 2.0 Hz, 1H, H5β), 3.55 (ddd, *J* = 10.2, 6.4, 2.5 Hz, 1H, H2β), 2.72 (d, *J* = 6.0 Hz, 1H, 2-OHα), 2.65 (d, *J* = 2.5 Hz, 1H, 2-OHβ), 1.52 (s, 3H, C(CH_3)$C\underline{H}_3$ α), 1.46 – 1.41 (m, 6H, H6β, C(CH_3)$C\underline{H}_3$ β), 1.38 – 1.35 (m, 6H, H6α, C($C\underline{H}_3$)CH_3 α), 1.35 (s, 3H, C($C\underline{H}_3$)CH_3 β). **^{13}C NMR** (101 MHz, $CDCl_3$) δ 132.7 (CH_{arom}), 132.3 (C_q), 131.4 (CH_{arom}), 129.2 (CH_{arom}), 129.0 (CH_{arom}), 128.1 (CH_{arom}), 127.4 (CH_{arom}), 110.0 ($C_{q,acetal}$β), 109.5 ($C_{q,acetal}$α), 88.4 (C1α), 87.9 (C1β), 79.2 (C3β), 76.4 (C4β), 75.9 (C3α, C4α), 72.9 (C5β), 71.4 (C2β), 70.1 (C2α), 65.5 (C5α), 28.2 (C(CH_3)$\underline{C}H_3$ β), 28.0 (C(CH_3)$\underline{C}H_3$ α), 26.5 (C($\underline{C}H_3$)CH_3 β), 26.1 (C($\underline{C}H_3$)CH_3 α), 17.1 (C6β), 16.4 (C6α) **HRMS** (ESI) m/z: [M + Na^+] calcd for $C_{15}H_{20}O_4SNa$ 319.09745, found 319.09718

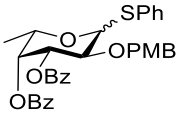
Phenyl 3,4-O-isopropylidene-2-O-(4-methoxybenzyl)-1-thio-L-fucopyranoside (15)



Thioglycoside **14** (1.48 g, 5.0 mmol) was dissolved in dry DMF (25 mL). The reaction was cooled in an icebath and a 60% w/w NaH dispersion in mineral oil (1,2 eq., 240 mg, 6.0 mmol) was added. The reaction was stirred for 15 minutes before PMB-Cl (2 eq., 1,56 g, 10 mmol) was added. The reaction was cooled for an additional 15 minutes before the icebath was removed. The reaction was then stirred for an additional 2 hours at room temperature. The reaction mixture was diluted with chloroform, and washed twice with water

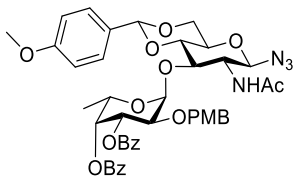
and once with brine. The organics were dried over $MgSO_4$, filtered and concentrated. Silicagel column chromatography (10% → 15% → 20% Et_2O in pentane) gave the title compound (1.75 g, 4.2 mmol, 84%) as a mixture of anomers (1:9, α:β). **^{1}H NMR** (400 MHz, $CDCl_3$) δ 7.52 (d, J = 7.4 Hz, 2H, CH_{arom}β), 7.47 (d, J = 7.7 Hz, 2H, CH_{arom}α), 7.33 (d, J = 8.2 Hz, 2H, CH_{arom}), 7.30 – 7.12 (m, 4H, CH_{arom}), 6.85 (d, J = 8.2 Hz, 2H, CH_{arom}), 5.58 (d, J = 5.2 Hz, 1H, H1α), 4.74 (d, J = 10.9 Hz, 1H, PMB-CH_2β), 4.71 – 4.61 (m, 2H, PMB-CH_2α), 4.61 – 4.45 (m, 3H, PMB-CH_2β, H1β, H5α), 4.27 (t, J = 6.3 Hz, 1H, H3α), 4.18 (t, J = 5.9 Hz, 1H, H3β), 4.05 (d, J = 5.3 Hz, 1H, H4α), 3.97 (d, *J* = 5.9 Hz, 1H, H4β), 3.86 (t, J = 6.0 Hz, 1H, H2α), 3.81 – 3.65 (m, 4H, OCH_3, H5β), 3.47 (dd, J = 9.7, 5.9 Hz, 1H, H2β), 1.40 (s, 3H, C(CH_3)C$\underline{H}_3$), 1.36 (d, J = 6.7 Hz, 3H, H6β), 1.34 (s, 3H, C(C$\underline{H}_3$)CH_3), 1.27 (d, J = 6.7 Hz, 3H, H6α) **^{13}C NMR** (101 MHz, $CDCl_3$) δ 159.2 (C_q), 133.7 (C_q), 131.8 (CH_{arom}), 130.6 (CH_{arom}), 130.0 (CH_{arom}), 129.8 (CH_{arom}), 129.5 (CH_{arom}), 128.8 (CH_{arom}), 128.7 (CH_{arom}), 127.2 (CH_{arom}), 126.6 (CH_{arom}), 113.7 (CH_{arom}), 113.6 (CH_{arom}), 109.5 ($C_{q,acetal}$β), 109.1 ($C_{q,acetal}$α), 85.9 (C1β), 85.7 (C1α), 79.7 (C3β), 77.6 (C2β), 76.3 (C4β), 75.7 (C4α), 75.4 (C2α), 74.5 (C3α), 72.9 (PMB-CH_2), 72.2 (C5β), 64.9 (C5α), 55.1 (OCH_3), 27.8 (C(CH_3)C$\underline{H}_3$β), 27.6 (C(CH_3)C$\underline{H}_3$α), 26.3 (C(C$\underline{H}_3$)CH_3β), 25.9 (C(C$\underline{H}_3$)CH_3α), 16.8 (C6β), 16.2 (C6α) **HRMS** (ESI) m/z: [M + H^+] calcd for $C_{23}H_{28}O_5SH$ 417.17302, found 417.17284

Phenyl 3,4-di-O-benzoyl-2-O-(4-methoxybenzyl)-1-thio-L-fucopyranoside (10)



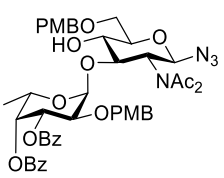
Thioglycoside **15** (7.98g, 19.2 mmol) was suspended in water (50 mL) and acetic acid (50 mL) and heated to 80°C for one hour. The volatiles were removed under reduced pressure and were further removed with toluene co-evaporation. The crude intermediate was then dissolved in pyridine (125 mL) and cooled in an icebath. Benzoyl chloride (3.0 eq., 6.8 mL, 57.6 mmol) was added and the reaction was stirred at room temperature for 2 hours. The reaction was quenched with water, diluted with ethyl acetate and washed successively with 1 M HCl and saturated $NaHCO_3$. The organic layer was dried over $MgSO_4$, filtered and concentrated. Silica gel column chromatography (10% → 20% → 30% Et_2O in pentane) gave the title compound (7.19 g, 12.3 mmol, 64%) as a mixture of anomers (1:4, α:β). **^{1}H NMR** (400 MHz, $CDCl_3$) δ 8.00 – 7.90 (m, 3H, CH_{arom}), 7.85 – 7.67 (m, 5H, CH_{arom}), 7.67 – 7.56 (m, 2H, CH_{arom}), 7.56 – 7.42 (m, 5H, CH_{arom}), 7.42 – 7.34 (m, 3H, CH_{arom}), 7.34 – 7.16 (m, 4H, CH_{arom}), 7.11 – 7.04 (m, 2H, CH_{arom}), 6.74 (d, J = 8.7 Hz, 2H, CH_{arom}), 6.70 – 6.61 (m, 2H CH_{arom}), 5.80 (d, J = 5.4 Hz, 1H, C1α), 5.69 (dd, J = 3.3, 1.1 Hz, 1H, H4α), 5.63 (dd, J = 3.3, 0.9 Hz, 1H, H4β), 5.42 (dd, J = 9.6, 3.3 Hz, 1H, H3β), 4.80 (d, J = 9.6 Hz, 1H, H1β), 4.73 (d, J = 10.3 Hz, 1H, PMB-C$\underline{H}$Hβ), 4.67 (d, J = 12.0 Hz, 1H, PMB-C$\underline{H}$Hα), 4.57 (d, J = 12.0 Hz, 1H, PMB-CH$\underline{H}$α), 4.52 (d, J = 10.3 Hz, 1H, PMB-CH$\underline{H}$β), 4.39 (dd, J = 10.5, 5.4 Hz, 1H, H2α), 4.02 (qd, *J* = 6.6, 0.9 Hz, 1H, H5β), 3.95 (t, J = 9.6 Hz, 1H, H2β), 3.74 (s, 3H, OCH_3α), 3.68 (s, 3H, OCH_3β), 1.32 (d, J = 6.6 Hz, 3H, H6β), 1.19 (d, J = 6.5 Hz, 3H, H6α) **^{13}C NMR** (101 MHz, $CDCl_3$) δ 165.9 (C=O), 165.5 (C=O), 159.3 (C_q), 133.5 (CH_{arom}), 133.4 (CH_{arom}), 133.2 (CH_{arom}), 133.1 (C_q), 132.8 (CH_{arom}), 131.7 (CH_{arom}), 130.0 (CH_{arom}), 129.9 (CH_{arom}), 129.8 (CH_{arom}), 129.8 (CH_{arom}), 129.7 (CH_{arom}), 129.6 (C_q), 129.6 (C_q), 129.1 (CH_{arom}), 128.9 (CH_{arom}), 128.6 (CH_{arom}) , 128.6 (CH_{arom}), 128.4 (CH_{arom}), 128.3 (CH_{arom}), 127.9 (CH_{arom}), 127.3 (CH_{arom}), 113.8 (CH_{arom}), 113.7 (CH_{arom}), 87.2 (C1α), 87.1 (C1β), 75.3 (C3β), 75.0 (PMB-CH_2β), 74.5 (C2β), 73.5 (C5α), 72.5 (C2α), 72.2 (C3α), 72.0 (PMB-CH_2α), 71.8 (C4β), 71.1 (C3α), 66.0 (C5α), 55.2 (OCH_3), 16.9 (C6β), 16.2 (C6α) **HRMS** (ESI) m/z: [M + NH_4^+] calcd for $C_{34}H_{32}O_7SNH_4$ 602.22070, found 602.22042

Azido 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-glucopyranoside (16)



Donor **10** (1.5 eq., 1.76 mg, 3.0 mmol) and acceptor **9** (728 mg, 2.0 mmol) were co-evaporated 3 times with toluene, backfilling the flask with N_2 after every co-evaporation round, and placed under a N_2 atmosphere. The sugars were dissolved in dry DCM (36 mL) with dry DMF (4 mL). Activated 4Å molecular sieves (1 g) were added and the solution was stirred for 90 minutes. The reaction mixture was then cooled in an icebath and NIS (2.0 eq., 900 mg, 4.0 mmol) and TMSOTf (0.1 eq., 37 µL) were added. The reaction was stirred and allowed to warm to room temperature overnight. The reaction 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 $CHCl_3$) **νmax/cm^{-1}** 2117.80 (N_3), 1724.29 (CO) **^{1}H NMR** (400 MHz, $CDCl_3$) δ 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, H3'), 5.53 (s, 1H, PMP-CH_{acetal}), 5.50 (dd, J = 3.4, 1.4 Hz, 1H, H4'), 5.28 (d, J = 9.3 Hz, 1H, H1), 5.15 (d, J = 3.5 Hz, 1H, H1'), 4.61 (d, J = 11.4 Hz, 1H, PMB-C<u>H</u>H), 4.54 (d, J = 11.4 Hz, 1H, PMB-CH<u>H</u>), 4.51 – 4.42 (m, 2H, H3, H5'), 4.38 (dd, J = 10.5, 4.2 Hz, 1H, H5), 4.13 (dd, J = 10.5, 3.4 Hz, 1H, H2'), 3.84 – 3.70 (m, 7H, 2 x OCH_3, H6a), 3.70 – 3.57 (m, 2H, H6b, H4), 3.25 (td, J = 9.3, 6.9 Hz, 1H, H2), 1.81 (s, 3H, NHC(O)C<u>H</u>$_3$), 0.73 (d, J = 6.5 Hz, 3H, H6'). 13C NMR (101 MHz, $CDCl_3$) δ 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-CH), 98.6 (C1'), 87.9 (C1), 80.7 (C4), 75.5 (C3), 74.2 (C2'), 73.4 (PMB-CH_2), 72.6 (C4'), 70.9 (C3'), 68.6 (C6), 68.5 (C5), 65.6 (C5'), 58.4 (C2), 55.4 (OCH_3), 55.3 (OCH_3), 23.4 (NHC(O)<u>C</u>H$_3$), 15.6 (C6') **HRMS** (ESI) m/z: [M + H$^+$] calcd for $C_{44}H_{46}N_4O_{13}H$ 839.31341, found 839.31311

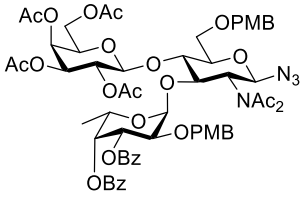
Azido 3,4-di-O-benzoyl-2-O-(4-methoxybenzyl)-α-L-fucopyranoside-(1→3)-6-O-(4-methoxybenzyl)-2-deoxy-2-(N-acetylacetamido)-β-D-glucopyranoside (17)



Dissacharide **16** (436 mg, 0.52 mmol) was dissolved in anhydrous DCM and DiPEA (10 eq., 870 µL, 5 mmol) and acetyl chloride (50 eq., 1.8 mL, 25 mmol) were added. The reaction was stirred for 2 hours 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 $CHCl_3$). **ν_{max}/cm^{-1}** 2119.23 (N_3), 1727.15 (CO) **^{1}H NMR** (400 MHz, $CDCl_3$) δ 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, H1, H3'), 5.51 (s, 1H, PMP-CH_{acetal}), 5.42 (dd, J = 3.3, 1.4 Hz, 1H, H4'), 4.79 (dd, J = 9.6, 8.6 Hz, 1H, H3), 4.74 (d, J = 3.5 Hz, 1H, H1'), 4.52 – 4.37 (m, 4H, PMB-CH_2, H5', H5), 4.06 (dd, J = 10.6, 3.5 Hz, 1H, H2'), 3.85 – 3.70 (m, 8H, 2 x OCH_3, H6), 3.70 – 3.61 (m, 2H, H4, H2), 2.50 (s,

3H, N(C(O)CH_3)C(O)C$\underline{H}_3$), 2.30 (s, 3H, N(C(O)C$\underline{H}_3$)C(O)CH_3), 0.52 (d, J = 6.4 Hz, 3H, H6'). **^{13}C NMR** (101 MHz, $CDCl_3$) δ 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$\underline{H}$), 98.8 (C1'), 87.5 (C1), 80.9 (C4), 73.6 (C3), 73.4 (PMB-CH_2), 72.6 (C4'), 71.8 (C2'), 71.3 (C3'), 68.6 (C6), 68.0 (C5), 65.4 (C5'), 64.1 (C2), 55.4 (OCH_3), 55.2 (OCH_3), 28.6 (N(C(O)CH_3)C(O)$\underline{C}H_3$), 25.6 (N(C(O)$\underline{C}H_3$)C(O)CH_3), 15.2 (C6'). **HRMS** (ESI) m/z: [M + Na^+] calcd for $C_{46}H_{48}N4O_{14}Na$ 903.30592, found 903.30478. 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 eq, 2.6 mmol, 2.6 mL) and the reaction was stirred for 15 minutes at this temperature. Then Bn_2BOTf was added as a 1.0 M solution in DCM (2 eq, 1 mmol, 1 mL) and the reaction was stirred for an additional 15 minutes at -70°C. The reaction was then heated to -50°C and stirred overnight. The reaction was quenched by careful addition of 0.5 mL of Et_3N followed by 15 mL MeOH and was stirred at room temperature for 30 minutes. The reaction mixture was concentrated *in vacuo* and subjected to silica gel column chromatography (40% → 50% →60 % Et_2O in pentane). This yielded the title compound (370 mg, 0.42 mmol, 81%). **$[\alpha]_D^{25}$** = -93.2 (c 1.00 in $CHCl_3$) **ν_{max}/cm^{-1}** 2117.80 (N_3), 1724.29 (CO) **^{1}H NMR** (400 MHz, $CDCl_3$) δ 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, H1, H3', H4'), 4.93 (d, *J* = 3.6 Hz, 1H, H1'), 4.66 – 4.39 (m, 6H, PMB-CH_2, PMB-CH_2, H3, H5'), 4.10 (dd, *J* = 10.3, 3.6 Hz, 1H, H2'), 4.01 (s, 1H, 4-OH), 3.85 – 3.79 (m, 4H, H6a, OCH_3), 3.78 – 3.72 (m, 4H, H6b, OCH_3), 3.71 – 3.62 (m, 3H, H2, H4, H5), 2.41 (s, 3H, N(C(O)CH_3)C(O)C$\underline{H}_3$), 2.37 (s, 3H, N(C(O)C$\underline{H}_3$)C(O)CH_3)), 1.21 (d, *J* = 6.5 Hz, 3H, H6') **^{13}C NMR** (101 MHz, $CDCl_3$) δ 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 (C1'), 86.8 (C1), 82.6 (C3), 76.7 (C4), 73.4 (PMB-CH_2), 72.7 (PMB-CH_2), 72.1 (C4'), 71.4 (C5), 71.3 (C2'), 70.3 (C3'), 68.7 (C6), 66.7 (C5'), 62.3 (C2), 55.4 (OCH_3), 55.3 (OCH3), 28.4 (N(C(O)$\underline{C}H_3$)C(O)CH_3), 25.6 (N(C(O)CH_3)C(O)$\underline{C}H_3$), 16.2 (C6') **HRMS** (ESI) m/z: [M + NH_4^+] calcd for $C_{46}H_{50}N_4O_{14}NH_4$ 900.36618, found 900.36581

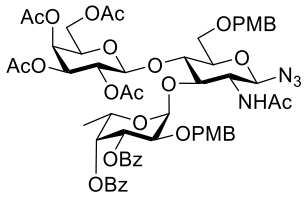
Azido 2,3,4,6-tetra-O-acetyl-β-D-galactopyranoside(1→4)-[3,4-di-O-benzoyl-2-O-(4-methoxybenzyl)-α-L-fucopyranoside-(1→3)]-6-O-(4-methoxybenzyl)-2-deoxy-2-(N-acetylacetamido)-β-D-glucopyranoside (18)



Donor **8** (5 eq., 737 mg, 1.5 mmol) and acceptor **17** (266 mg, 0,3 mmol) were co-evaporated 3 times with toluene, backfilling the flask with N_2 after every co-evaporation round, and placed under a N_2 atmosphere. The sugars were dissolved in dry DCM (3 mL) and activated 4Å molecular sieves (300 mg) were added. The mixture was stirred 30 minutes at room temperature and subsequently cooled to -10°C. TMS triflate (0.1 eq, 5.6 µl, 0.03 mmol) was added and the reaction was stirred over night at -10°C. The reaction was quenched by addition of Et_3N (0.1 mL) and allowed to warm to room temperature. The reaction mixture was 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, $CDCl_3$) δ 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, H4'), 5.64 – 5.54 (m, 2H, H3', H1), 5.38 (dd, J = 3.6, 1.0 Hz, 1H, H4''), 5.14 (q, J = 6.5 Hz, 1H, H5'), 5.04 (dd, J = 10.3, 8.3 Hz, 1H, H2''), 4.86 – 4.67 (m, 5H, H3'', PMB-C<u>H</u>H, H1', H1'', H3), 4.60 (dd, J = 11.5, 6.1 Hz, 1H, H6''a), 4.50 (s, 2H, PMB-CH_2), 4.46 – 4.38 (m, 2H, PMB-CH<u>H</u>, H6''b), 4.11 (dd, J = 10.6, 3.7 Hz, 1H, H2'), 4.05 (dd, J = 10.0, 8.9 Hz, 1H, H4), 3.88 – 3.68 (m, 8H, OCH_3, H6, OCH_3), 3.59 (t, J = 9.4 Hz, 1H, H2), 3.56 – 3.49 (m, 2H, H5, H5''), 2.51 (s, 3H, N(C(O)CH_3)C(O)C<u>H</u>$_3$), 2.25 (s, 3H, N(C(O)C<u>H</u>$_3$)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 (d, J = 6.6 Hz, 3H, H6') **^{13}C NMR** (101 MHz, $CDCl_3$) δ = 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 (C1''), 97.8 (C1'), 86.9 (C1), 76.6 (C5''), 74.3 (C4), 73.7 (PMB-CH_2), 73.5 (PMB-CH_2), 72.9 (C4'), 71.8 (C3', C3, C2'), 71.3 (C3''), 71.1 (C5), 69.2 (C2''), 67.0 (C4''), 66.9 (C6), 64.9 (C5'), 64.3 (C2), 61.1 (C6''), 55.4 (OCH_3), 55.3 (OCH_3), 28.8 (N(C(O)CH_3)C(O)C<u>H</u>$_3$), 25.8 (N(C(O)C<u>H</u>$_3$)C(O)CH_3), 21.0 (C(O)C<u>H</u>$_3$), 20.9 (C(O)C<u>H</u>$_3$), 20.8 (C(O)C<u>H</u>$_3$), 20.7 (C(O)C<u>H</u>$_3$), 16.0 (C6'). **HRMS** (ESI) m/z: [M + Na^+] calcd for $C_{60}H_{68}N_4O_{23}Na$ 1235.41666, found 1235.41654

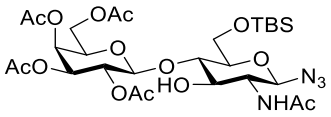
Azido 2,3,4,6-tetra-O-acetyl-β-D-galactopyranoside-(1→4)-[3,4-di-O-benzoyl-2-O-(4-methoxybenzyl)-α-L-fucopyranoside-(1→3)]-6-O-(4-methoxybenzyl)-2-deoxy-2-acetamido-β-D-glucopyranoside (5)



Protected trisaccharide **18** (61 mg, 50 µmol) was dissolved in dry THF (1 mL) and N,N-dimethylaminopropylamine (10 eq, 63 µL, 0.5 mmol) was added. The reaction was stirred for 30 minutes at room temperature and another portion of N,N-dimethylaminopropylamine (10 eq, 63 µL, 0.5 mmol) was added. After further stirring for 1 hour, 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, $CDCl_3$) δ 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, H4', H3'), 5.38 (dd, J = 3.6, 1.1 Hz, 1H, H4''), 5.26 (d, J = 8.2 Hz, 1H, H1), 5.21 (d, J = 3.6 Hz, 1H, H1'), 5.08 (dd, J = 10.4, 8.1 Hz, 1H, H2''), 4.99 – 4.86 (m, 2H, H5', H3''), 4.73 – 4.67 (m, 2H, PMB-C<u>H</u>H, H1''), 4.64 (d, J = 11.6 Hz, 1H, PMB-C<u>H</u>H), 4.57 (d, J = 11.7 Hz, 1H, PMB-CH<u>H</u>), 4.46 – 4.31 (m, 4H, PMB-CH<u>H</u>, H6'', H3), 4.18 (dd, J = 9.7, 3.5 Hz, 1H, H1'), 4.06 (t, J = 8.3 Hz, 1H, H4), 3.85 – 3.78 (m, 5H, OCH_3, H6), 3.75 (s, 3H, OCH_3), 3.64 – 3.55 (m, 2H, H5'', H5), 3.33 (q, J = 8.1 Hz, 1H, H2), 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<u>H</u>$_3$), 1.25 (d, J = 6.6 Hz, 3H, H6') **^{13}C NMR** (101 MHz, $CDCl_3$) δ = 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 (C1''), 97.3 (C1'), 87.2 (C1), 76.7 (C5), 73.6 (C2', C4), 73.5 (C3), 73.4 (PMB-CH_2), 73.1 (PMB-CH_2), 72.8 (C4'), 71.1 (C5''), 71.0 (C3''), 71.0 (C3'), 69.2

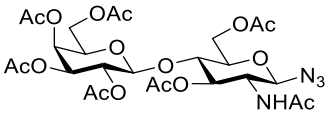
(C2''), 67.4 (C6), 67.0 (C4''), 65.2 (C5'), 61.1 (C6''), 57.0 (C2), 55.4 (OCH_3), 55.3 (OCH_3), 23.5 ($NHC(O)\underline{C}H_3$), 20.9 ($C(O)\underline{C}H_3$), 20.9 ($C(O)\underline{C}H_3$), 20.7 ($C(O)\underline{C}H_3$), 16.1 (C6'). **HRMS** (ESI) m/z: [M + Na^+] calcd for $C_{58}H_{66}N_4O_{22}Na$ 1193.40609, found 1193.40573

Azido 2,3,4,6-tetra-O-acetyl-β-D-galactopyranoside-(1→4)-6-(*tert*-butyldimethylsilyl)-2-deoxy-2-acetamido-β-D-glucopyranoside (19)



Donor **8** (1.5 eq, 368 mg, 0.75 mmol) and acceptor **13** (180 mg, 0.5 mmol) were co-evaporated 3 times with toluene and put 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 hours at room temperature. The reaction was cooled to -40°C and $BF_3{\cdot}Et_2O$ (1.6 eq, 100 µL, 0.8 mmol) was added. The reaction was stirred at -40°C overnight and formation of 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%). $\mathbf{[\alpha]_D^{20}}$ = +5,8 (c 1.00 in $CHCl_3$) $\mathbf{\nu_{max}/cm^{-1}}$ 2115.65 (N_3), 1752.19 (CO) **^{1}H NMR** (400 MHz, $CDCl_3$) δ 6.17 (d, J = 8.5 Hz, 1H, NH), 5.40 (dd, J = 3.4, 1.0 Hz, 1H, H4'), 5.22 (dd, J = 10.5, 8.0 Hz, 1H, H2'), 4.99 (dd, J = 10.5, 3.4 Hz, 1H, H3'), 4.69 – 4.61 (m, 2H, H1, H1'), 4.15 (d, J = 6.5 Hz, 2H, H6'), 4.06 (bs, 1H, 3-OH), 4.01 (t, J = 6.5 Hz, 1H, H5'), 3.90 – 3.72 (m, 3H, H6, H3), 3.69 – 3.57 (m, 2H, H4, H2), 3.43 (ddd, J = 9.6, 3.4, 1.5 Hz, 1H, H5), 2.17 (s, 3H, $C(O)CH_3$), 2.08 (s, 3H, $C(O)CH_3$), 2.07 (s, 3H, $C(O)CH_3$), 2.03 (s, 3H, $NHC(O)C\underline{H}_3$), 1.99 (s, 3H, $C(O)CH_3$), 0.92 (s, 9H, tBu), 0.11 (s, 3H, Si-CH_3), 0.10 (s, 3H, Si-CH_3) **^{13}C NMR** (101 MHz, $CDCl_3$) δ = 171.0 (C=O), 170.6 (C=O), 170.2 (C=O), 170.1 (C=O), 169.4 (C=O), 101.6 (C1'), 87.9 (C1), 80.5 (C4), 76.7 (C5), 71.9 (C3), 71.4 (C5'), 70.9 (C3'), 68.7 (C2'), 66.8 (C4'), 61.4 (C6'), 61.2 (C6), 55.6 (C2), 25.9 (tBu), 23.4 ($NHC(O)\underline{C}H_3$), 20.7 ($C(O)\underline{C}H_3$), 20.6 ($C(O)\underline{C}H_3$), 20.6 ($C(O)\underline{C}H_3$), 20.6 ($C(O)\underline{C}H_3$), 18.3 (Si-C_q), -5.0 (Si-CH_3), -5.2 (Si-CH_3) **HRMS** (ESI) m/z: [M + Na^+] calcd for $C_{28}H_{46}N_4O_{14}SiNa$ 713.2672, found 713.2695

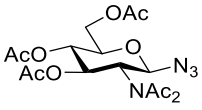
Azido 2,3,4,6-tetra-O-acetyl-β-D-galactopyranoside-(1→4)-6,3-di-O-acetyl-2-deoxy-2-acetamido-β-D-glucopyranoside (20)



Silyl protected disaccharide **19** (517 mg, 0.75 mmol) was dissolved in dry THF (7.5 mL) in a plastic tube. HF·pyridine complex (16 eq, 310 µL, 12 mmol) was added and the reaction was stirred overnight. Completion of the reaction was assessed by TLC (100% EtOAc) and the reaction mixture was diluted with DCM. The organic layer was washed with aqueous saturated $NaHCO_3$ (3:1 ratio of DCM:H_2O) and the aqueous layer was back extracted with DCM. The combined organic layers were dried over $MgSO_4$, filtered and concentrated, yielding 380 mg (0.66 mmol) of crude intermediate. The crude desilylated disaccharide was dissolved in dry pyridine (6.6 mL) and cooled to 0°C in an ice bath. Acetic anhydride (10 eq, 620 µL, 6.6 mmol) and DMAP (0.1 eq, 9 mg, 0.07 mmol) were added. The reaction was stirred overnight at room temperature and reaction completion was confirmed by TLC (100% EtOAc). The reaction was quenched with methanol and concentrated. Pyridine traces were removed with toluene co-evaporation. Silica gel column chromatography (70% → 80% → 90% EtOAc in pentane) yielded the title compound (421 mg, 0.64 mmol, 85%). $\mathbf{[\alpha]_D^{20}}$ = -26,4 (c 1.00 in $CHCl_3$) $\mathbf{\nu_{max}/cm^{-1}}$ 2116.37 (N_3), 1744.32 (CO) **^{1}H NMR** (400 MHz, $CDCl_3$) δ 6.53 (d, J = 9.6 Hz, 1H, NH), 5.37 (dd, J = 3.4, 1.2 Hz, 1H, H4'), 5.19 – 5.03 (m, 2H, H3, H2'), 4.99 (dd, J = 10.5, 3.4 Hz, 1H, H3'), 4.64 – 4.50 (m, 3H, H1, H1', H6a), 4.21

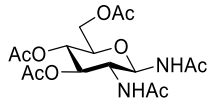
– 4.01 (m, 4H, H6', H6b, H2), 3.93 (t, J = 7.1 Hz, 1H, H5'), 3.84 (t, J = 9.1 Hz, 1H, H4), 3.73 (ddd, J = 9.1, 5.0, 2.2 Hz, 1H, H5), 2.17 (s, 3H, C(O)CH_3), 2.14 (s, 3H, C(O)CH_3), 2.11 (s, 3H, C(O)CH_3), 2.07 (s, 3H, C(O)CH_3), 2.07 (s, 3H, C(O)CH_3), 1.99 (s, 3H, NHC(O)C$\underline{H}_3$), 1.97 (s, 3H, C(O)CH_3) **^{13}C NMR** (101 MHz, $CDCl_3$) δ = 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 (C1'), 88.3 (C1), 76.1 (C4), 74.5 (C5), 73.1 (C3), 70.8 (C3'), 70.7 (C5'), 69.0 (C2'), 66.6 (C4'), 61.9 (C6), 60.6 (C6'), 53.0 (C2), 23.0 (NHC(O)$\underline{C}H_3$), 20.9 (C(O)$\underline{C}H_3$), 20.8 (C(O)$\underline{C}H_3$), 20.6 (C(O)$\underline{C}H_3$), 20.6 (C(O)$\underline{C}H_3$), 20.5 (C(O)$\underline{C}H_3$), 20.5 (C(O)$\underline{C}H_3$) **HRMS** (ESI) m/z: [M + Na^+] calcd for $C_{26}H_{36}N_4O_{16}Na$ 683.2019, found 683.2029

3,4,6-tri-O-acetyl-2-deoxy-2-(acetylacetamido)-β-D-glucopyranosyl azide (21)



Glycosyl azide **11** (186 mg, 0.5 mmol) was dissolved in anhydrous DCM (5 mL). DiPEA (2 eq, 175 µL, 1.0 mmol) and acetyl chloride (10 eq, 350 µL, 5.0 mmol) were added and the reaction was stirred at room temperature. After 2 hours TLC (20% pentane in EtOAc) inducated complete consumption of the starting material. The reaction mixture was diluted with DCM and washed with saturated aqueous $NaHCO_3$. The organic layer was dried over $MgSO_4$ and concentrated. Silica gel column chromatography (1/1 Et_2O/pentane) yielded the title compound (112 mg, 0.27 mmol, 54%). **^{1}H NMR** (400 MHz, $CDCl_3$) δ 5.86 (d, J = 8.7 Hz, 1H, H1), 5.82 (dd, J = 10.3, 8.9 Hz, 1H, H3), 5.10 (dd, J = 10.3, 8.9 Hz, 1H, H4), 4.37 (dd, J = 12.5, 4.6 Hz, 1H, H6a), 4.14 (dd, J = 12.5, 2.2 Hz, 1H, H6b), 3.92 (ddd, J = 10.3, 4.6, 2.2 Hz, 1H, H5), 3.62 (dd, J = 10.3, 8.7 Hz, 1H, H2), 2.38 (s, 3H, NHC(O)CH_3), 2.37 (s, 3H, NHC(O)C$\underline{H}_3$), 2.11 (s, 3H, C(O)CH_3), 2.04 (s, 3H, C(O)CH_3), 2.00 (s, 3H, C(O)CH_3) **^{13}C NMR** (101 MHz, $CDCl_3$) δ = 174.7 (C=O), 173.4 (C=O), 170.6 (C=O), 169.6 (C=O), 87.1 (C1), 73.6 (C5), 70.2 (C3), 68.8 (C4), 61.7 (C2), 61.7 (C6), 27.8 (NHC(O)C$\underline{H}_3$), 25.1 (NHC(O)$\underline{C}H_3$), 20.7 (C(O)$\underline{C}H_3$), 20.6 (C(O)$\underline{C}H_3$), 20.4 (C(O)$\underline{C}H_3$)

3,4,6-tri-O-acetyl-2-deoxy-2-(acetamido)-β-D-glucopyranosyl acetamide (22)



Acetylimide protected sugar **21** (40 mg, 0.1 mmol) was dissolved in THF (2 mL) and a 1 M solution of trimethylphosphine (1.5 eq, 150 µL, 0.15 mmol) was added. The reaction was stirred for 5 minutes before H_2O (50 eq, 90 µL 5 mmol) was added. The reaction was stirred for 1 hour and the volatiles were removed *in vacuo*. NMR analysis (DMSO-d6) indicated full conversion to the anomeric acetamide. **^{1}H NMR** (400 MHz, DMSO-d_6) δ 8.56 (d, J = 9.3 Hz, 1H, C1-N$\underline{H}$), 7.99 (d, J = 9.2 Hz, 1H, C2-N$\underline{H}$), 5.15 (t, J = 9.6 Hz, 1H, H1), 5.09 (dd, J = 10.3, 9.5 Hz, 1H, H3), 4.80 (t, J = 9.8 Hz, 1H, H4), 4.16 (dd, J = 12.4, 4.3 Hz, 1H, H6a), 3.94 (dd, J = 12.4, 2.2 Hz, 1H, H6b), 3.90 – 3.76 (m, 2H, H2, H5), 1.99 (s, 3H, C(O)CH_3), 1.96 (s, 3H, C(O)CH_3), 1.90 (s, 3H, C(O)CH_3), 1.82 (s, 3H, C(O)CH_3), 1.74 (s, 3H, C(O)CH_3)

Asn(GlcNAc)-Ala-Thr-Gly-Met-Asp-Val-Gly-Trp-Tyr-Arg-Ser-Pro-Phe-Ser-Arg-Val-Val-His-Leu-Tyr-Arg-Asn-Gly-Lys-NH_2 (25)

Using the general method of glycopeptide synthesis, asparagine derivative **1** was coupled to immobilized peptide **23**, producing compound **25** in 4.0% (3.1 mg) yield after RP-HPLC. **LC-MS** RT = 14.0 min (C18, 5-65% B over 30 minutes) **LRMS** calcd $[M+3H]^{3+}$ = 1043.18; observed M/z = 1043.42

Asn(FucGlcNAc)-Ala-Thr-Gly-Met-Asp-Val-Gly-Trp-Tyr-Arg-Ser-Pro-Phe-Ser-Arg-Val-Val-His-Leu-Tyr-Arg-Asn-Gly-Lys-NH_2 (26)

Using the general method of glycopeptide synthesis, asparagine derivative **2** was coupled to immobilized peptide **23**, producing compound **26** in 5.6% (2.4 mg) yield after RP-HPLC. **LC-MS** RT = 14.0 min (C18, 5-65% B over 30 minutes) **LRMS** calcd $[M+3H]^{3+}$ = 1091.87, $[M+2H]^{2+}$ = 1637.30; observed M/z = 1092.50, 1637.83

Asn(LacNAc)-Ala-Thr-Gly-Met-Asp-Val-Gly-Trp-Tyr-Arg-Ser-Pro-Phe-Ser-Arg-Val-Val-His-Leu-Tyr-Arg-Asn-Gly-Lys-NH_2 (27)

Using the general method of glycopeptide synthesis, asparagine derivative **3** was coupled to immobilized peptide **23**, producing compound **27** in 5.8% (2.3 mg) yield after RP-HPLC. **LC-MS** RT = 13.9 min (C18, 5-65% B over 30 minutes) **LRMS** calcd $[M+3H]^{3+}$ = 1097.20, $[M+2H]^{2+}$ = 1645.30; observed M/z = 1097.92, 1645.92

Asn(Le^X)-Ala-Thr-Gly-Met-Asp-Val-Gly-Trp-Tyr-Arg-Ser-Pro-Phe-Ser-Arg-Val-Val-His-Leu-Tyr-Arg-Asn-Gly-Lys-NH_2 (28)

Using the general method of glycopeptide synthesis, asparagine derivative **4** was coupled to immobilized peptide **23**, producing compound **28** in 4.1% (1.7 mg) yield after RP-HPLC. **LC-MS** RT = 13.9 min (C18, 5-65% B over 30 minutes) **LRMS** calcd $[M+3H]^{3+}$ = 1145.89, $[M+2H]^{2+}$ = 1718.34; observed M/z = 1146.50, 1718.83

Asn(GlcNAc)-Ala-Thr-Gly-Met-Asp-Val-Gly-Trp-Tyr-Cit-Ser-Pro-Phe-Ser-Cit-Val-Val-His-Leu-Tyr-Arg-Asn-Gly-Lys-NH_2 (29)

Using the general method of glycopeptide synthesis, asparagine derivative **1** was coupled to immobilized peptide **24**, producing compound **29** in 8.6% (6.7 mg) yield after RP-HPLC. **LC-MS** RT = 15.1 min (C18, 5-65% B over 30 minutes) **LRMS** $[M+3H]^{3+}$ = 1043.84, $[M+2H]^{2+}$ = 1565.26; observed M/z = 1044.08, 1565.50

Asn(FucGlcNAc)-Ala-Thr-Gly-Met-Asp-Val-Gly-Trp-Tyr-Cit-Ser-Pro-Phe-Ser-Cit-Val-Val-His-Leu-Tyr-Arg-Asn-Gly-Lys-NH_2 (30)

Using the general method of glycopeptide synthesis, asparagine derivative **2** was coupled to immobilized peptide **24**, producing compound **30** in 2.1% (2.2 mg) yield as well as the product containing a single methionine oxidation (5.7 %, 6.1 mg) after RP-HPLC. **LC-MS** RT = 15.1 min (C18, 5-65% B over 30 minutes) **LRMS** calcd $[M+3H]^{3+}$ = 1092.50, $[M+2H]^{2+}$ = 1638.29; observed M/z = 1092.58, 1638.17

Asn(LacNAc)-Ala-Thr-Gly-Met-Asp-Val-Gly-Trp-Tyr-Cit-Ser-Pro-Phe-Ser-Cit-Val-Val-His-Leu-Tyr-Arg-Asn-Gly-Lys-NH_2 (31)

Using the general method of glycopeptide synthesis, asparagine derivative **3** was coupled to immobilized peptide **24**, producing compound **31** in 5.6% (3.9 mg) yield after RP-HPLC. **LC-MS** RT = 14.9 min (C18, 5-65% B over 30 minutes) **LRMS** calcd $[M+3H]^{3+}$ = 1097.86, $[M+2H]^{2+}$ = 1646.29; observed M/z = 1098.17, 1646.33

Asn(Le^X)-Ala-Thr-Gly-Met-Asp-Val-Gly-Trp-Tyr-Cit-Ser-Pro-Phe-Ser-Cit-Val-Val-His-Leu-Tyr-Arg-Asn-Gly-Lys-NH_2 (32)

Using the general method of glycopeptide synthesis, asparagine derivative **4** was coupled to immobilized peptide **24**, producing compound **32** in 5.3% (1.9 mg) yield as well as the product containing a single methionine oxidation (3.9 %, 1.4 mg) after RP-HPLC. **LC-MS** RT = 15.0 min (C18, 5-65% B over 30 minutes) **LRMS** calcd $[M+3H]^{3+}$ = 1146,54, $[M+2H]^{2+}$ = 1719.31; observed M/z =1146.75, 1719.42

Biophysical and biochemical methods

ThT Fluorescence Aggregation Assays

Aggregation assays were modified according to Araman et al.[12] Briefly, a mixture of 199 µL of peptide (10 µM) and 1 µL of ThT pipetted to 96-well plates and the fluorescence in each well was measured with a kinetic interval of 10 min peptides at 37 °C using the CLARIOstar® *Plus* plate reader. The measurements were performed with an excitation wavelength of 444 nm and an emission wavelength of 485 nm with a bandwidth of 10 nm.

Circular Dichroism (CD) Spectroscopy

Circular dichroism spectroscopy was performed at room temperature using a Jasco J-815 CD spectrometer with a 1 mm path-length cell and a bandwidth of 2.0 nm. The peptides were prepared in 20 mM NaOAc buffer (pH 5.0) with a final peptide concentration of 0.1−0.2 mg/mL.. Spectra were recorded from 260 to 190 nm at an interval of 1 nm. Each spectrum was the average of five scans and blank subtraction. Further analysis was performed via addition of 4 mM sodium dodecyl sulfate (SDS) (β-sheet enhancer) or 50% trifluoroethanol (TFE) (α-helix enhancer).

Cell Viability Assay with 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide (MTT)

The cell viability of BMDCs was tested via the MTT assay. In a typical experiments, BMDCs were seeded in a 96-well plate at a density of 2.5×10^4 cells/well and treated with varying amounts of peptides **26-28** as well as **29-32** in IMDM (40, 20, 10, and 5 µM) incubated overnight at 37 °C and 5% CO_2. Medium with (control) and medium without 2.5×10^4 cells (blank) served used as controls. Upon overnight incubation, cells were centrifuged (300*g* for 5 min at 4 °C), the supernatant was discarded, and 100 µL of 0.5 mg/mL MTT in PBS was added to each well. Upon incubation for 3–4 h at 37 °C and formation of intracellular formazan crystals, the supernatant was removed and the crystals were dissolved in DMSO (75-100 µL). The plate was incubated for a further 30 min at 37 °C, and the absorbance was measured at 540 nm (A_{540}) as well as 570 nm (CLARIOstar® *Plus*). The following equation was used to assess cell viability:

$$\%\ \text{cell viability} = \frac{A_{540}(\text{sample}) - A_{540}(\text{blank})}{A_{540}(\text{control}) - A_{540}(\text{blank})}$$

DC-SIGN ELISA

DC-SIGN ELISA was performed according to the method published elsewhere.[59] Briefly, 2 mM peptide stock solutions in DMSO were diluted to 14 µM with PBS before incubating 50 µL/well in high-binding 96-well plates (Nunc Maxisorp) at room temperature for 2 hours for coating. The wells were then washed twice with 150 µL of a calcium and magnesium-containing buffer [TSM: 20 mM tris(hydroxymethyl)aminomethane (Tris)-HCl, pH 8.0; 150 mM NaCl; 1 mM $CaCl_2$; 2 mM $MgCl_2$] and blocked using 100 uL TSM buffer containing 1% BSA for 45 min. at room temperature. The blocking solution was removed and wells were then incubated 45 min. at room temperature with 1µg/mL of DC-SIGN-Fc (extracellular portion of DC-SIGN, residues 64 to 404, fused at the C-terminus to a human IgG1/Fc fragment into the Sig-pIgG1-Fc vector) in TSM buffer containing 0.5% BSA. After two washes with TSM buffer, HRP-linked goat anti-human IgG-Fc, 400 µg/mL diluted 1:250 in TSM containing 0.5% BSA was added and incubated for 1 hour at room temperature. After 2 washes with TSM buffer, TMB substrate in citric acid buffer containing a catalytic amount of H_2O_2 was added, and the plate was read on a spectrophotometer at 450 nm. Polyacrylamide polymers (PAA), functionalized with LeX was purchased from Lectinity, MW approx. 20 KDa, carbohydrate content around 20% mol., and used as positive control (20 µg/mL to coat the ELISA wells).

Generation and stimulation of moDC

Peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats of healthy volunteers (Sanquin, Amsterdam, The Netherlands) by centrifugation on a Ficoll gradient as previously described.[64] Briefly, blood was mixed with PBS 1% citrate and layered on the Ficoll. After 30 min centrifugation, the interphase containing monocytes and lymphocytes was collected, washed with PBS/Citrate and the pellet was resuspended in complete RPMI medium. PBMCs were then loaded on a Percoll layer (GE Healthcare, Chicago, U.S.) and after centrifugation, the interphase was collected, washed and resuspended in complete RPMI. moDC were generated by culturing monocytes for 5-7 days at a concentration of 1.25×10^6/mL in complete RPMI (Lonza, Basel, Switzerland) containing 500 U/mL IL-4 (ImmunoTools, Friesoythe, Germany) and 800 U/mL Granulocyte Macrophage Colony stimulating Factor (GM-CSF) (ImmunoTools). On day four moDC ($0.5\text{-}1 \times 10^5$) were stimulated with different concentration of the reported glycosylated peptides (14, 7 and 3.5 µM) in RPMI-1640, supplemented with 10% FCS, L-Glutamine (2 mM), and penicillin/streptomycin (100 U/ml) at 37°C, 5% CO_2 for 16 hours. Lipopolysaccharide (LPS, *E. coli* 0111:B4, Sigma-Aldrich, cat#L4391, 10 ng/mL) was used as control.

Cytokine ELISA

Human IL-10 and IL-12p70 ELISA Kit Duo Set R&D systems were used following the manufacturer instructions. Briefly, anti-human IL-10 or IL-12p70 was coated overnight in 50 mM Na2CO3, pH 9.7, in Nunc Maxisorp plates. After washing with PBS 0.05% Tween, and blocked for 30 min with 1% BSA in PBS, moDC supernatants were added together with the corresponding detection antibody for 2 h at RT. Cytokines were detected with Streptavidin-PO (Biosource Finnigan, Waltham, U.S.) adding substrate buffer [(110 mM citric acid (Merck), 110 mM sodium acetate (Fisher scientific, Waltham, U.S.), pH 4] and 100 µg/mL TMB solution (Sigma) with a catalytic amount of H2O2, and stopping the enzymatic reaction with 0.8M H2SO4. UV absorbance was measured at 450 nm on an ELISA reader (Bio-Rad Benchmark, Hercules, U.S.).

Supporting Figures

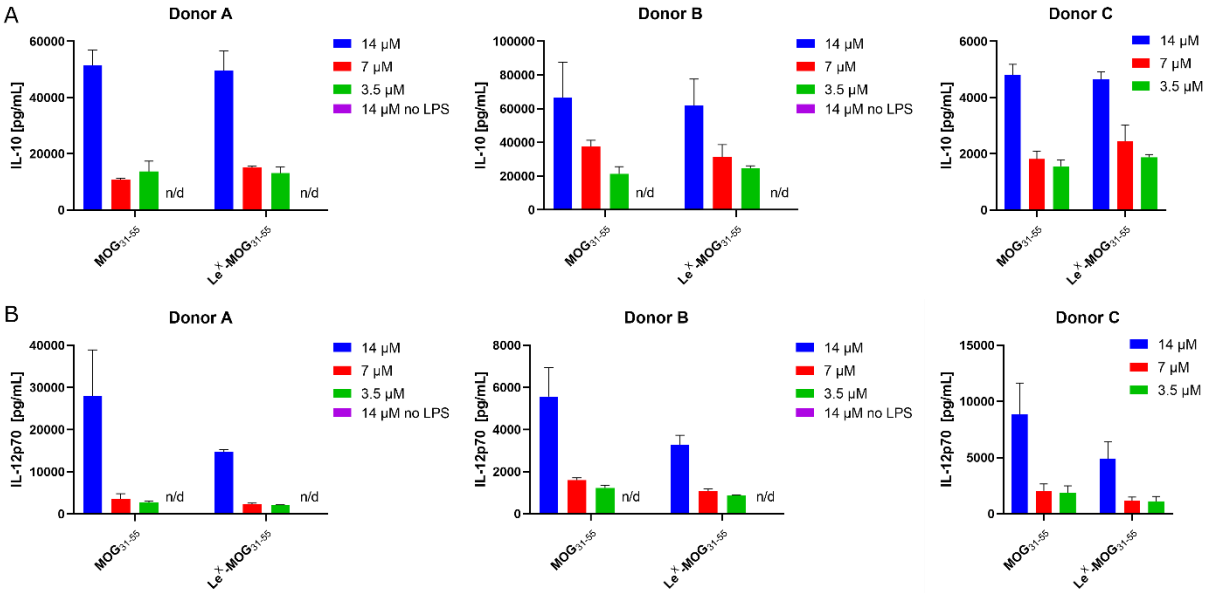


Figure S1. Concentrations of secreted of IL10 (A) and IL12p70 (B) measured for Donors A, B and C upon stimulation with MOG_{31-55} or the glycosylated peptide Le^X-MOG_{31-55} (**28**). Cells were stimulated with 10 ng/mL of LPS unless stated otherwise ("no LPS"). n/d = not detected

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