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Chemo-Enzymatic Synthesis of Isomeric I-branched Polylactosamines Using Traceless Blocking Groups

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Poly-*N*-acetyl lactosamines (polyLacNAc) are common structural motifs of *N*- and *O*-linked glycan, glycosphingolipids and human milk oligosaccharides. They can be branched by the addition of β1,6-linked *N*-acetyl-glucosamine (GlcNAc) moieties to internal galactoside (Gal) residues by the I-branching enzyme beta-1,6-*N*-acetylglucosaminyltransferase 2 (GCNT2). I-branching has been implicated in many biological processes and is also associated with various diseases such as cancer progression. Currently, there is a lack of methods that can install, in a regioselective manner, I-branches and allows the preparation of isomeric poly-LacNAc derivatives. Here, we described a chemo-enzymatic strategy that addresses this deficiency and is based on the enzymatic assembly of an oligo-LacNAc chain that at specific positions is modified by a GlcNTFA moiety. Replacement of the trifluoroacetyl (TFA) moiety by *tert*-butyloxycarbonyl (Boc) gives compounds in which the galactoside at the proximal site is blocked from modification by GCNT2. After elaboration of the antennae, the Boc group can be removed, and the resulting amine acetylated to give natural I-branched structures. It is also shown that fucosides can function as a traceless blocking group that can provide complementary I-branched structures from a single precursor. The methodology made it possible to synthesize a library of polyLacNAc chains having various topologies.

Introduction

Poly-*N*-acetyl lactosamines (polyLacNAc) are glycan substructures that are composed of repeating units of type 2 LacNAc disaccharides [Galβ1,4GlcNAcβ1−]$_n$. These substructures form backbones of many complex carbohydrates and can be presented as linear and branched architectures. The topology of polyLacNAc chains also forms the basis of the Ii blood group system classification.[1] The i antigen is a linear β1,3-linked polyLacNAc chain that enzymatically can be converted into an I antigen by the I-branching enzyme beta-1,6-*N*-acetylglucosaminyltransferase 2 (GCNT2), which introduces a β-linked GlcNAc moiety at the 6-position of internal galactosides of i antigens (Figure 1A). This branching point can be extended by glycosyltransferases in a similar manner as the main glycan chain to form I-branched multi-antennary glycans.[2] I-branched structures are found on *N*- and *O*-linked glycoproteins, glycolipids and unconjugated human milk oligosaccharides (HMOs) (Figure 1B, C).[2–3]

Developing fetuses and neonates solely express i antigens until several months postpartum, after which I antigen expression is initiated.[4] Almost all adults express I antigens, whilst the i_{adult} phenotype, characterized by the absence of I antigen on red blood cells, is extremely rare (1 in 4400).[5] Interestingly, the delay in biosynthesis of I antigens in neonates correlates with a similar delay of blood group A expression. This delay in biosynthesis has been linked to the low incidence of hemolytic disease of the fetus and newborn (HDFN).[4–6] Destruction of fetal red blood cells by HDFN is caused by maternal alloimmunization through antibodies crossing the placental membrane during pregnancy. Infant red blood cells only express i antigen which have lower antigenicity compared to I-branched carrying red blood cells in adults that allows multivalent presentation of ABO antigens.[7]

In adults, rare mutations/deletions in GCNT2 that prevent the biosynthesis of the I antigen are associated with development of congenital cataracts.[8] Moreover, alterations in GCNT2 expression and thus I-branch biosynthesis have been linked to cancer progression and metastasis.[3d,9] I-branched *N*-glycans have also been implicated in regulation of B cell activation through modulation of galectin 9 binding.[10]

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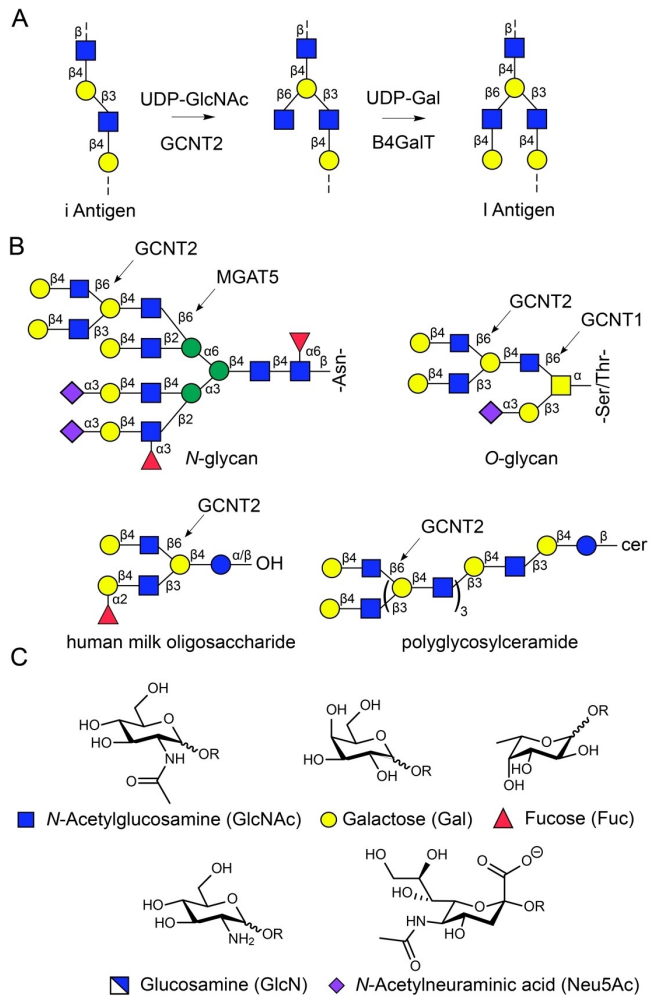


Figure 1. A) Biosynthetic pathway of the Ii blood group system; B) Overview of glycan classes carrying I-antigens. β1,6-GlcNAc modifications are annotated with the appropriate enzyme responsible for the transfer. Oligosaccharides represent only one of the possible structures; C) Chemical structures of monosaccharides and their associated symbols.

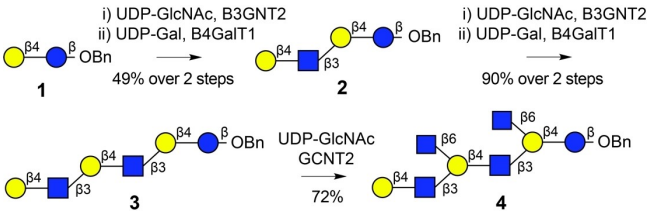
In I^+ adults, both linear and I-branched glycan structures are ubiquitously expressed.[11] Despite the prevalence of such structural motifs, it has been difficult to investigate how I-branching influences recognition by glycan binding proteins. Moreover, the distinction between I-branched and elongated linear polyLacNAc chains is difficult to resolve by conventional glycomic analysis due to identical glycan compositions. It is to be expected that well-defined I-branched glycan standards will be valuable tools to develop mass spectrometry-based methodologies that can distinguish linear from I-branched structures. Furthermore, such compounds will also be valuable to develop glycan microarrays to examine selectivities of glycan binding proteins.

I-branches have been installed using recombinant GCNT2,[10a,12] however, this enzyme lacks regioselectivity and cannot selectively modify a specific galactoside moiety of a poly-LacNAc chain. Chemical synthesis can in principle address this deficiency, however, these approaches are very time consuming and has only been employed to prepare human milk oligosaccharides with a branching GlcNAc moiety at the lactose moiety[13] or at single galactose that at the C-6 position is extended by an oligo-LacNAc moiety.[14] In an alternative approach, linear polyLacNAc structures were synthesized having an unnatural galactoside with a C-6 aldehyde that was employed to generate I-branching glycomimetics.[15] Thus, the regioselective modification of polyLacNAc chains with natural I-branches has not been reported yet and hence such compounds with different topologies are not available. To address this deficiency, we explored the regioselective introduction of I-branches using the enzyme GCNT2 in combination with polyLacNAc chains that are modified by traceless blocking groups that exerts regioselective control over GCNT2 activity. It was found that a glucosamine residue modified by *t*-butyloxycarbamate (Boc) or 1,3-fucoside can function as temporary blocking group for I-branching allowing the preparation of isomeric structures. The methodology made it possible, for the first time, to prepare a library of well-defined I-branched glycans with different topologies.

Results and Discussion

Examining the regioselectivity of GCNT2

Hexasaccharide **3**, which contains two internal galactosides, was prepared to investigate possible regioselectivity of the I-branching enzymes, GCNT2 (Scheme 1). This glycan structure is based on a lactose core that is present on HMOs and polyglycosylceramides, which are glycan classes that are often modified by I-branches.[1,16] Benzyl lactoside **1** was used as the starting material because its anomeric center is locked in one anomeric configuration and furthermore the benzyl moiety increases sensitivity for MS and UV absorption based detection thereby facilitating identification and purification. Benzyl glycoside **1**, which was generated by a four-step procedure involving Koenigs–Knorr glycosylation of lactose with benzyl alcohol, was enzymatically extended to lacto-neo-*N*-tetraose **2** and then to lacto-neo-*N*-hexaose **3** by the alternating action of beta-1,3-*N*-acetylglucosaminyltransferase 2 (B3GNT2) and beta-1,4-galactosyltransferase 1 (B4GalT1). Treatment of **3** with the I-branching enzyme GCNT2 generated biantennary octasaccharide **4** when two or more equivalents of UDP-GlcNAc was employed. The addition of only one equivalent of UDP-GlcNAc led to a mixture of bi-antennary octasaccharide, mono-antennary heptasaccharide and starting material, which indicates that GCNT2 does not



Scheme 1. I-branching of **3** reveals no inherent selectivity of GCNT2 on oligosaccharides containing multiple I-branch acceptor sites.

possess inherent regioselectivity for this type of substrate. Control over I-branch activity of GCNT2 is necessary for the enzymatic synthesis of well-defined I-branched polyLacNAc chains. To address this challenge, we developed methodologies that could give access to a library of polyLacNAc oligosaccharides that carry I-branches of various length on the proximal, distal or both non-terminal galactosides of a hexasaccharide HMO scaffold.

Enzymatic synthesis of proximal I-branched polyLacNAc chains

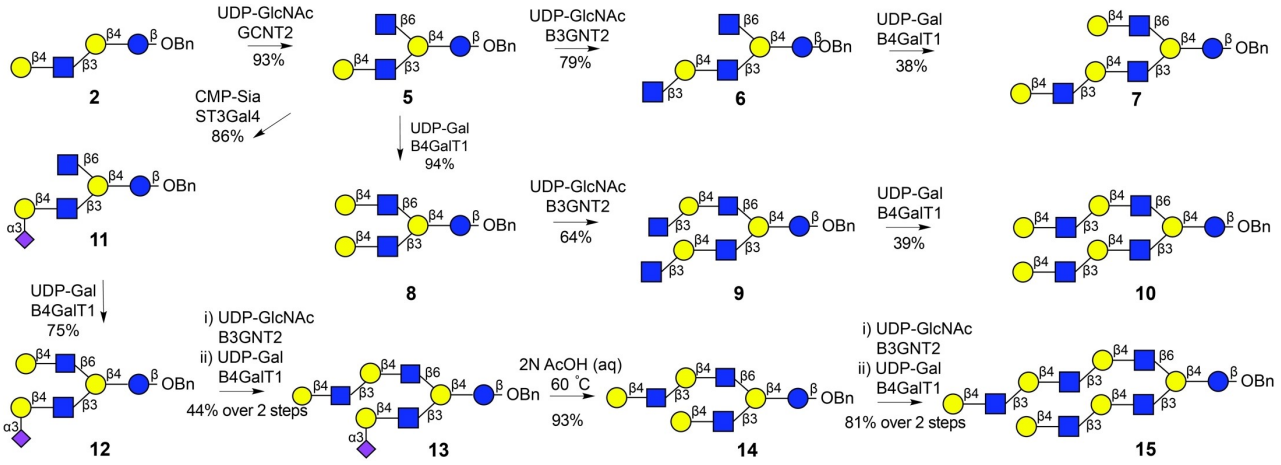
PolyLacNAc chains that have an I-branch on the proximal galactoside were prepared by exploiting the inherent selectivity of GCNT2 for internal galactosides.[10a] Thus, a compound such as **2** has only one acceptor site for this enzyme and therefore could readily be converted into common precursor **5**. The latter derivative has an asymmetrical architecture in which the main chain terminates as galactoside and the I-branch as a GlcNAc residue (Scheme 2). We exploited this asymmetry to selectively extend the terminal galactoside of main by a β1,3-linked GlcNAc moiety by treatment with B3GNT2 in the presence of UDP-GlcNAc to give compound **6**. Asymmetrical derivative **7** was readily obtained by further extension of both arms of **6** with a β1,4-galactoside by employing B4GalT1 and UDP-Gal. Alternatively, treatment of compound **5** with B4GalT1 in the presence of UDP-Gal gave symmetrical derivative **8** which could readily be converted into **10** that has two LacNAc moieties at the I-branch.

To allow further selective extension of the I-branch, it was necessary to temporarily deactivate the main chain from LacNAc extension. Sialic acid was selected as an appropriate blocking group because it occupies the acceptor hydroxyl and can be installed with high regioselectivity and be removed by selective acid hydrolysis. Sialylation of **5** was achieved by using alpha-2,3-sialyltransferase 4 (ST3Gal4) and CMP-Neu5Ac to give in compound **11**. The I branch of **11** could selectively be extended by a LacNAc moiety to give **13** by subsequent treatment with B3GNT2 and B4GalT1. Next, the sialoside of the main chain of **13** was removed by treatment with aqueous acetic acid to generate **14**, which is an isomer of polyLacNAc **7**. Both arms of **14** could be further extended by a LacNAc moiety to give derivative **15**.

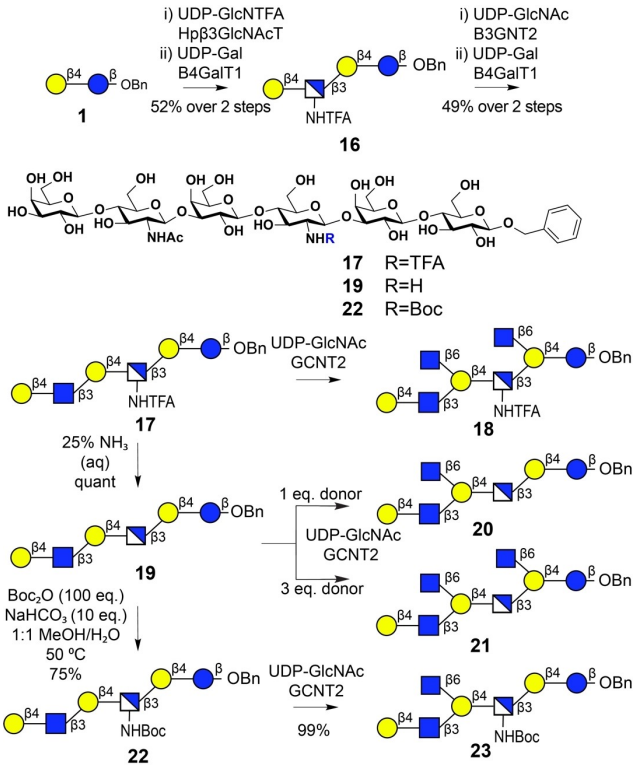
Selective I-branch activity imposed by N-modified glucosamine derivatives

The approach described above makes it possible to prepare oligo-LacNAc chains having an I-branch of different length on the proximal lactose moiety. To prepare compounds having I-branching at one of the LacNAc moieties, we pursued a strategy in which a linear oligo-LacNAc chain such as **22** was prepared having a *t*-butyl-oxycarbamate (Boc) modified glucosamine moiety at the central LacNAc position (Scheme 3). It was found that the Boc moiety blocks I-branching by GCNT2 at the proximal galactoside moiety to give selective formation of compound **23**. Key precursor **22** could readily be prepared by exploiting the finding that various GlcNAc transferases can employ UDP-GlcNTFA as unnatural sugar nucleotide donor to give oligosaccharides having a GlcNTFA moiety.[17] At an appropriate stage of synthesis, the TFA moiety can selectively be removed under mild basic conditions to give $GlcNH_2$ that can be further functionalized by for example a Boc masking group. Previously, we demonstrated that $GlcNH_2$ and GlcNBoc are resistant to α1,3-fucosylation to give access to polyLacNAc chains having various patterns of Lewis^x epitopes.[18] Therefore, we were compelled to investigate how these modifications influence the regioselective activity of GCNT2. For this purpose, we prepared model hexasaccharides **17**, **19** and **22** containing various modifications on the internal LacNAc moiety (Scheme 3).

Thus, lactoside **1** was extended by a GlcNTFA moiety by treatment with UDP-GlcNTFA in the presence of HpB3GlcNAcT.[12a,19] The GlcNTFA moiety of the resulting product was tolerated as an acceptor substrate for B4GalT1 resulting in the formation of LacNTFA containing tetrasaccharide **16**.



Scheme 2. Enzymatic synthesis of compounds **7**, **10** and **15**.



Scheme 3. Chemo-enzymatic synthesis of **23**. Only one of the I-branching sites is blocked by GlcNBoc.

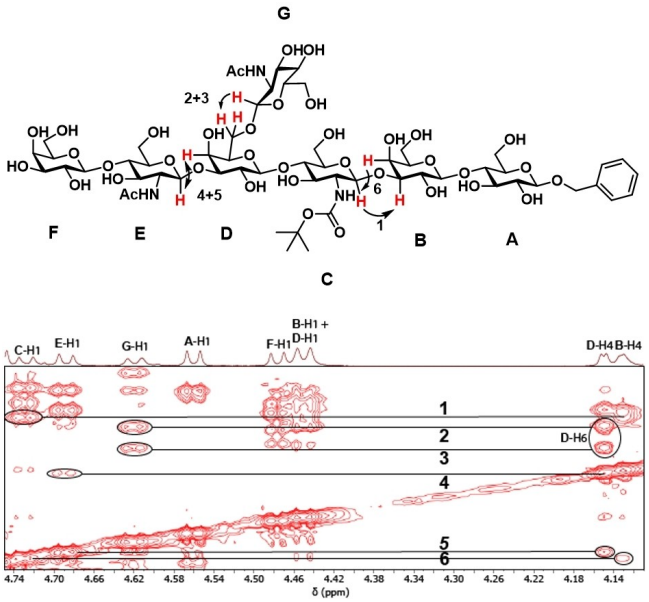


Figure 2. NOESY NMR spectrum of compound **23**; NOESY transfer signals indicative for the structural characterization of **23** are noted by numbers 1–6 and are between the red annotated protons.

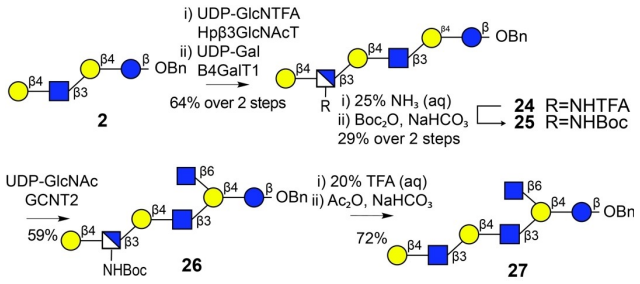
Consecutive LacNAc extension of **17** using B3GNT2 and B4GalT1 gave hexasaccharide **17**. Aminolysis of the TFA protecting group of **17** was accomplished by treatment with aqueous ammonia to give amine **19** that was readily converted into **22** by reaction with Boc_2O in the presence of sodium bicarbonate.

Treatment of **17** with I-branching enzyme GCNT2 resulted in the modification of the two internal galactosides to give biantennary **18** demonstrating the TFA moiety does not exert control over the regioselectivity. Similar treatment of **19** led to full conversion to heptasaccharide **20**, however, extended reaction times still resulted in the slow formation of the undesired octasaccharide **21**. This residual activity suggests that GCNT2 still has some activity for the second I-branching site which may become problematic when blocking multiple sites on longer polyLacNAc chains are present. Gratifyingly, treatment of hexasaccharide **22** with GCNT2 led to the selective formation of I-branched heptasaccharide **23** and in this case no formation of octasaccharide was observed. Even a large excess of enzyme and sugar nucleotide did not result in progression of the reaction supporting that *N*-Boc fully deactivates one of the I-branching sites.

The position of I-branch regioselectivity imposed by *N*-modified glucosamine was confirmed by a combination of ^{1}H, COSY, NOESY, TOCSY and HSQC NMR experiments on compound **23** (Figure 2). GlcNBoc **C** was identified by a large up field shift of the H2 signal relative to the other glucosamines (COSY). NOE correlations of GlcNBoc **C** and correlations of GlcNAc **E** with neighboring galactosides revealed the H4 signals of galactoside **B** and **D**, respectively. The I-branching modification resulted in a substantial increase in NOE transfer between galactoside **D** H4 and H6/H6′ which is absent in galactoside without modification on the C6 position. NOE correlations between GlcNAc **G**-H1to H6/H6′ of **D** were detected indicating that GlcNAc **G** is connected to galactose **D** through the expected β1,6 linkage. The assignment of galactoside **D** as the I-branched point was confirmed by a downshift of H6 in the HSQC spectrum. Thus, it is revealed that the GlcNBoc modification blocks GCNT2 activity on the neighboring reducing end galactoside, while not interfering with I-branch activity on the neighboring non-reducing end galactoside.

N-Modified glucosamines only block the proximal galactoside

To act as a selective blocking group that can direct I-branching on longer polyLacNAc chains, it is necessary that GlcNBoc does not influence other I-branching sites such as the reducing lactose moiety. To investigate this property, a hexasaccharide was prepared that places the GlcNBoc moiety on the terminal position (Scheme 4). The latter compound was prepared by treatment of **2** with UDP-GlcNTFA in the presence of Hpβ3GlcNAcT followed by galactosylation of the resulting product with B4GalT1 to give hexasaccharide **24** that was subjected to exchange of TFA by Boc by standard manipulations. Treatment of **25** with UDP-GlcNAc in the presence of GCNT2 resulted only in modification of the galactoside of the lactose moiety to give the expected heptasaccharide **26**. Hydrolysis of the Boc masking group followed by acetylation of the resulting free amine gave target compound **27**. The successful preparation of this derivative supports that GlcNBoc



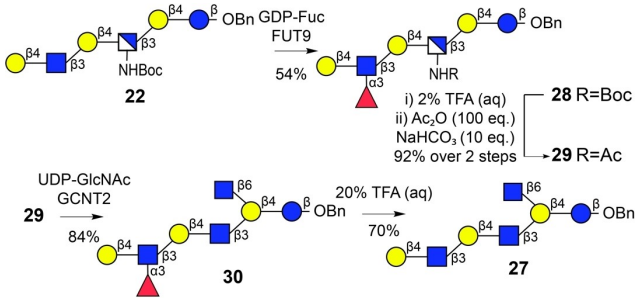
Scheme 4. Chemo-enzymatic synthesis of **27** shows that Boc only blocks the reducing-end neighboring galactose.

only affects the neighboring reducing end galactoside and does not influence other I-branching sites. Because only one acceptor site is blocked by GlcNBoc, it is expected that this synthetic methodology is suitable to control I-branching on larger polyLacNAc chains.

Control of I-branch selectivity by a fucosyl blocking group

Next, we explored whether α1,3-fucosides can be exploited as a potential I-branch directing group. This approach was investigated for several reasons. First, an α1,3-fucoside is bulky and it was expected that like *N*-Boc, it may direct the action of the I-branching enzyme GCNT2. Furthermore, GlcNBoc is known to block fucosylation, and can direct α1,3-fucosyltransferase activity to an unmodified LacNAc residue (e.g. **22**→**28**, Scheme 5). Thus, subsequent removal of a Boc masking group and acetylation of the resulting amine was expected to give a precursor (e.g. **29**) that allows regioselective installation of an I-branch at an alternative site.[18]

To investigate the proposed strategy, hexasaccharide **22** was selectively fucosylated by treatment with alpha-1,3-fucosyltransferase 9 (FUT9) and GDP-fucose to form compound **28**. The Boc protecting group prevented FUT9 activity on the inner LacNBoc, whilst activity on lactose was not observed when GDP-fucose was limited to 1.1 equivalents. The latter regioselectivity is explained by a lower activity of FUT9 for lactose aided by a preference of this enzyme for distal LacNAc moieties.[20] After fucosylation, it was necessary to reactivate the



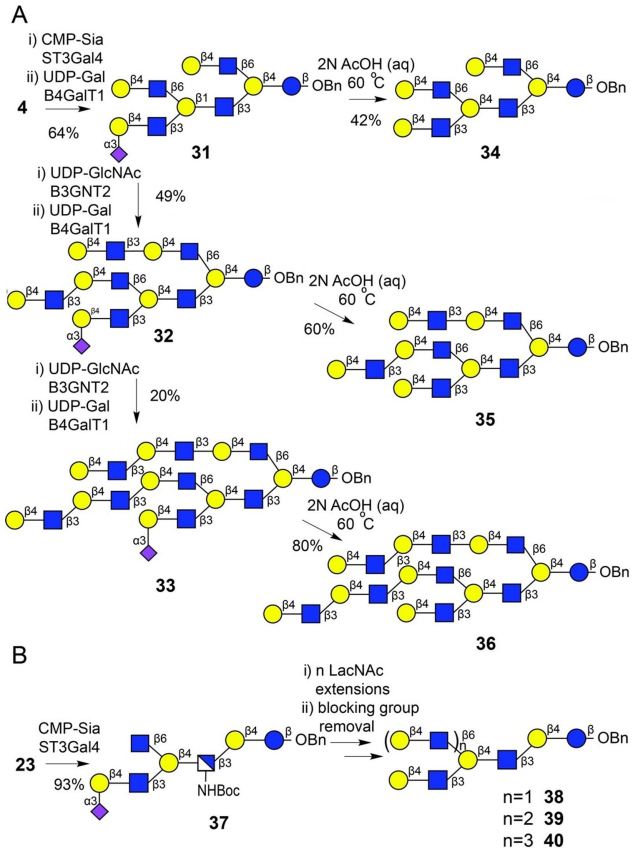
Scheme 5. Chemo-enzymatic synthesis of **27** using fucose as a traceless blocking group.

I-branch position blocked by GlcNBoc by removal of Boc followed by *N*-acetylation of the resulting amine using Ac_2O and $NaHCO_3$ to give compound **29**. It was observed that the use of 20% aqueous TFA resulted in the hydrolysis of Boc as well as the fucoside, however, selective Boc removal could be accomplished by lowering the TFA concentration to 2%. Exposure of **29** to the I-branching enzyme GCNT2 in the presence of UDP-GlcNAc resulted in the selective modification of the proximal lactose moiety to give compound **30** demonstrating that the α1,3-fucoside of **29** directs the regioselectivity of I-branching which was confirmed by detailed NMR experiments (for the details of the analysis, see the Supporting Information). Unsubstituted polyLacNAc **27** could be generated by hydrolysis of the fucoside of **30** by exposure to 20% aqueous TFA for 8 h to give clean formation of heptasaccharides **27**. Both *N*-Boc and α1,3-fucosylation can thus be used as traceless directing group for the I-branching enzyme GCNT2. Regio-isomer **23** could readily be prepared by exposure of **22** to GCNT2 in the presence of UDP-GlcNAc highlighting that the combined use of GlcNBoc and fucoside can provide isomeric I-branched structures from a single structure.

Selective enzymatic extensions of I-branches

To selectively extend the I-branches of compound **4** with several LacNAc moieties, it was necessary to block the terminal LacNAc moiety of the main chain from enzymatic modification. This was achieved by α2,3-sialylation because it occupies the C3 position on the galactoside blocking further extension (Scheme 6A). Furthermore, sialosides can readily be removed under mild acid conditions without affecting other moieties.[21] As expected, ST3Gal4 could install an α2,3-sialoside on the terminus of the main chain of glycan **4**, and subsequent treatment with B4GalT1 in the presence of UDP-Gal resulted in extension of the I-branches to give compound **31**. The terminal LacNAc moieties of **31** could be further extended by alternating action of B3GNT2 and B4GalT1 resulted in the formation of compound **32**. Repeating the enzymatic module gave compound **33** having the I-branches extended by three LacNAc repeating units. Treatment of compounds **31**–**33** with 12% AcOH(aq) at 60 °C for 18 h resulted in the hydrolysis of the sialosides providing unsubstituted polyLacNAc derivatives **34**–**36**.

Selective extension of an I-branch could also be performed on compound **23** which has a GlcNBoc moiety to give access to derivatives **38**–**40** (Scheme 6B). Thus, treatment of **23** with ST3Gal4 in the presence of CMP-Neu5Ac resulted in the formation of sialoside **37**. The terminal GlcNAc moiety of the latter compound could be converted into LacNAc by treatment with B4GalT1, which was followed by additional enzymatic treatment with B3GNT2 and B4GalT1 to install additional LacNAc moieties. The Boc protecting groups could readily be cleaved using 20% aqueous TFA at room temperature for 1 h to give amines that were acetylated using Ac_2O and $NaHCO_3$. Interestingly, the sialosides were stable under these conditions, however, could readily be removed by heating in aqueous



Scheme 6. Sialic acid is used as a temporary protecting group on the main chain of I-branched oligosaccharides to allow enzymatic extension of I-branches on oligosaccharides. A) Symmetric I-branched polyLacNAc pathway to compounds **34**, **35** and **36**; B) Pathway to compounds **38**, **39** and **40**.

acetic acid to provide target compounds **38**–**40**. Collectively, the results show that I-branches can be selectively modified without affecting the main chain structure making it possible to prepare multiple glycans from a single precursor.

Conclusions

Poly-*N*-acetyl lactosamines (polyLacNAc) are common structural motifs of *N*- and *O*-linked glycan, glycosphingolipids and human milk oligosaccharides that can be branched by the addition of β1,6-linked N-acetyl-glucosamine (GlcNAc) moieties to internal galactoside (Gal) residues by the I-branching enzyme GCNT2. Currently, there are no methods for installing I-branches in regioselective manner hindering the preparation of isomeric poly-LacNAc derivatives. Such compounds are need as analytical standards, to develop glycan microarrays and as substrates to examine biosynthetic pathways. Here, a chemo-enzymatic methodology is described that can install, in controlled manner, I-branches on polyLacNAcs scaffolds using traceless blocking groups. It is based on the assembly of a poly-LacNAc chain using UDP-GlcNAc and UDP-GlcNTFA as donors to give poly-LacNAc chains that at specific positions. Selective chemical manipulation of the GlcNTFA group on the unprotected oligosaccharide allowed the installation of *N*-blocking groups. Using this methodology, modified hexasaccharides with GlcNTFA, $GlcNH_2$ and GlcNBoc were prepared. It was found that the *N*Boc selectively blocks I-branching activity on the neighboring reducing end galactosides. Naturally occurring, highly complex, I-branched polyLacNAcs could be obtained after removal of the *N*Boc group and re-acetylation. Fucose as a I-branch directing group was also explored by exploiting the previously reported fucosyltransferase directing capabilities of *N*Boc. This method allowed the synthesis of complementary I-branched architectures from a single precursor.

It is anticipated that I-branched polyLacNAcs can be derivatized with glycan epitopes such as blood group antigens to study the effects of multivalent glycan presentation. We also expect that this methodology can be adapted to the synthesis of complex glycans, such as I-branched *N*-glycans, *O*-glycans and glycolipids. Such glycans will provide reference compounds for the development of analytical methodologies that can resolve polyLacNAc topologies. In this respect, mass spectrometric approaches can in general only provide glycan compositions but not exact structures. Although tandem mass spectrometry that induces glycosidic linkage or cross-ring fragmentation can provide snippets of structural information, it usually does not reveal exact glycan structures.[22] It is the expectation that well-defined glycan standards in combination with orthogonal analytical methods such as tandem mass spectrometry, ion mobility spectrometry and gas-phase IR action spectroscopy can provide exact structures of complex glycans. It has also been difficult to investigate how I-branching influences recognition by glycan binding proteins. A library of well-defined I-branched oligosaccharides will provide opportunities to investigate in a systematic manner how branching influences binding of glycan binding proteins such as galectins. The latter can be accomplished by printing synthetic glycans as a microarray for rapid structure-binding studies.[23]

Experimental Section

Material and methods: ^{1}H NMR are recorded on a 600 MHz Avance Neo or 400 MHz Agilent NMR. Chemical shifts are recorded in parts per million (ppm) relative to TMS or the residual solvent peak. NMR signals are represented as: chemical shift, multiplicity (s = singlet, d = doublet, app.t. = apparent triplet, t = triplet, dd = doublet of doublets, m = multiplet), J coupling and integration. Assignment of ^{1}H NMR signals was conducted based on ^{1}H NMR, 2D COSY (COSYGPSW), 2D TOCSY (MLEVPHSW), 2D NOESY (NOESYPHSW) and 2D HSQC (HSQCEDETGPSISP). ^{13}C NMR signal assignment was extracted from 2D HSQC spectra. Mass spectra are recorded by ESI on a Bruker micrOTOF-QII system as m/z. Reaction progress for unprotected carbohydrates was determined with a SeQuant Zic HILIC guard column (20×2.1 mm) coupled to a Bruker micrOTOF-QII MS system running a gradient of 90% AcCN:H_2O to 50% AcCN:H_2O over 5 min followed by isocratic 50% AcCN:H_2O until product was detected. MilliQ water was purified by a Millipore Synergy water purification system. Size exclusion was conducted using Bio-Gel P2, P4 or P6 gel polyacrylamide beads from BioRad, eluding with MilliQ water or 20 mM Ammonium Bicarbonate for compounds containing a primary amine. Column chromatography was performed on silica gel G60. Thin Layer Chromatography (TLC) was conducted on

Silica gel 60F254 (EMD Chemicals Inc.) on prefabricated glass slides with visualization by UV and, spraying with 10% H_2SO_4 in ethanol, p-Anisaldehyde stain or Hennesian's stain and subsequent charring of the TLC plate. UDP-Galactose, UDP-*N*-Acetylglucosamine and CMP-Neu5Ac were bought from Roche Diagnostics. Calf intestine alkaline phosphatase (CIAP) was obtained from Invitrogen. GDP-Fucose was prepared using L-fucokinase/GDP-fucose pyrophosphorylase.[24] Glycosyltransferases were expressed and purified according to published protocols.[10a,12a] Reagents purchased from commercial sources were used without further purification. UDP-GlcNTFA was prepared according to published protocol.[25]

General method for transfer of β1,3-*N*-acetylglucosamine using B3GNT2: To a solution of 10 mM acceptor oligosaccharide in HEPES buffer (HEPES 50 mM, KCl 25 mM, $MgCl_2$ 2 mM, DTT 1 mM, pH 7.3) was added UDP-*N*-acetylglucosamine (1.5 equiv.), CIAP (1 U/μL) and β3GNT2 (10 μg/μmol). The reaction was incubated at 37 °C for 16 h with gentle shaking. Reaction progress was monitored by LC-ESI-Q-TOF-MS. The reaction mixture was lyophilized if no more starting material was observed otherwise 5 μg/μmol β3GNT2 was added. The residue was re-dissolved in minimal MilliQ water and loaded on a Biogel size exclusion column loaded with a resin of the appropriate pore size. Carbohydrate containing fractions were identified by LC-ESI-Q-TOF-MS, pooled and lyophilized.

General method for transfer of β1,3-*N*-trifluoroacetylglucosamine using HpB3GlcNAcT: To a solution of 10 mM acceptor oligosaccharide in TRIS buffer (TRIS 100 mM, $MnCl_2$ 10 mM, pH 8) was added UDP-*N*-trifluoroacetylglucosamine (1.5 equiv.), CIAP (1 U/μL) and HpB3GlcNAcT (10 μg/μmol). The reaction was incubated at 37 °C for 16 h with gentle shaking. Reaction progress was monitored by LC-ESI-Q-TOF-MS. The reaction mixture was lyophilized if no more starting material was observed otherwise 5 μg/μmol HpB3GlcNAcT was added. The residue was re-dissolved in minimal MilliQ water and loaded on a Biogel size exclusion column loaded with a resin of the appropriate pore size. Carbohydrate containing fractions were identified by LC-ESI-Q-TOF-MS, pooled and lyophilized.

General method for transfer of β1,6-*N*-acetylglucosamine using GCNT2: To a solution of 10 mM acceptor oligosaccharide in TRIS buffer (TRIS 100 mM, $MnCl_2$ 10 mM, pH 8) was added UDP-*N*-acetylglucosamine (1.5 equiv.), CIAP (1 U/μL) and GCNT2 (10 μg/μmol). The reaction was incubated at 37 °C for 16 h with gentle shaking. Reaction progress was monitored by LC-ESI-Q-TOF-MS. The reaction mixture was lyophilized if no more starting material was observed otherwise 5 μg/μmol GCNT2 was added. The residue was re-dissolved in minimal MilliQ water and loaded on a Biogel size exclusion column loaded with a resin of the appropriate pore size. Carbohydrate containing fractions were identified by LC-ESI-Q-TOF-MS, pooled and lyophilized.

General method for transfer of β1,4-galactose using B4GalT1: To a solution of 10 mM acceptor oligosaccharide in TRIS buffer (TRIS 50 mM, $MnCl_2$ 10 mM, BSA 0.1 wt%, pH 7.3) was added UDP-galactose (1.5 equiv.), CIAP (1 U/μL) and B4GalT1 (10 μg/μmol). The reaction was incubated at 37 °C for 16 h with gentle shaking. Reaction progress was monitored by LC-ESI-Q-TOF-MS. The reaction mixture was lyophilized if no more starting material was observed otherwise 5 μg/μmol B4GalT1 was added. The residue was re-dissolved in minimal MilliQ water and loaded on a Biogel size exclusion column loaded with a resin of the appropriate pore size. Carbohydrate containing fractions were identified by LC-ESI-Q-TOF-MS, pooled and lyophilized.

General method for transfer of α2,3-*N*-acetylneuraminic acid using ST3Gal4: To a solution of 10 mM acceptor oligosaccharide in TRIS buffer (TRIS 50 mM, BSA 0.1 wt%, pH 7.3) was added CMP-Neu5Ac (1.5 equiv.), CIAP (1 U/μL) and ST3Gal4 (10 μg/μmol). The reaction was incubated at 37 °C for 16 h with gentle shaking. Reaction progress was monitored by LC-ESI-Q-TOF-MS. The reaction mixture was lyophilized if no more starting material was observed otherwise 5 μg/μmol ST3Gal4 was added. The residue was re-dissolved in minimal MilliQ water and loaded on a Biogel size exclusion column loaded with a resin of the appropriate pore size. Carbohydrate containing fractions were identified by LC-ESI-Q-TOF-MS, pooled and lyophilized.

General for *N*-acetylneuraminic acid hydrolysis: Sialoside was dissolved in 2 N AcOH (aq) (200 μL/mg carbohydrate) and the solution was incubated for 16 h at 60 °C. Reaction progress was monitored by LC-ESI-Q-TOF-MS. The reaction mixture was lyophilized when no more starting material was observed. The residue was re-dissolved in minimal MilliQ water and loaded on a Biogel size exclusion column loaded with a resin of the appropriate pore size. Carbohydrate containing fractions were identified by LC-ESI-Q-TOF-MS, pooled and lyophilized.

General procedure for removal of *N*-trifluoroacetamide group: Oligosaccharide was dissolved in a 25 wt% aqueous NH_3 solution and the solution was incubated for 3 h at 37 °C. Reaction progress was monitored by LC-ESI-Q-TOF-MS. NH_3 was removed under N_2 stream and the reaction mixture was lyophilized when no more starting material was observed. The residue was re-dissolved in minimal MilliQ water and loaded on a P2 Biogel size exclusion column and eluded with 10 mM NH_4HCO_3(aq). Carbohydrate containing fractions were identified by LC-ESI-Q-TOF-MS, pooled and lyophilized.

General procedure for removal of *N*-tert-butyloxycarbonyl group: Oligosaccharide was dissolved in a 2 v/v% aqueous TFA solution and the solution was incubated for 4 h at 37 °C. Reaction progress was monitored by LC-ESI-Q-TOF-MS. The reaction mixture was diluted with MiliQ water and lyophilized when no more starting material was observed. If no fucose was present on the starting material 20 v/v% aqueous TFA was used and the reaction was completed in 1 h. The residue was re-dissolved in minimal MilliQ water and loaded on a P2 Biogel size exclusion column MilliQ water, which eluded with 10 mM NH_4HCO_3(aq). Carbohydrate containing fractions were identified by LC-ESI-Q-TOF-MS, pooled and lyophilized.

General procedure for fucoside hydrolysis: Fucoside was dissolved in a 20 v/v% aqueous TFA solution and the solution was incubated for 8 h at 37 °C. Reaction progress was monitored by LC-ESI-Q-TOF-MS. The reaction mixture was diluted with MiliQ water and lyophilized when no more starting material was observed. The residue was re-dissolved in minimal MilliQ water and loaded on a P2 Biogel size exclusion column MiliQ water, which was eluded with 10 mM NH_4HCO_3(aq). Carbohydrate containing fractions were identified by LC-ESI-Q-TOF-MS, pooled and lyophilized.

General procedure for installation of *N*-tert-butyloxycarbonyl: Oligosaccharide was dissolved in 1:1 MeOH:H_2O (100 μL/mg carbohydrate) and $NaHCO_3$ (10 equiv.) and Boc anhydride (100 equiv.) were added. The solution was sonicated for 1 h at 50 °C, reaction progress was monitored by LC-ESI-Q-TOF-MS. If starting material was observed another 10 equiv. of $NaHCO_3$ and Boc anhydride were added. The reaction mixture was lyophilized when no more starting material was observed. The residue was re-dissolved in minimal MilliQ water and loaded on a P2 Biogel size exclusion column, which was eluded with 10 mM NH_4HCO_3(aq). Carbohydrate containing fractions were identified by LC-ESI-Q-TOF-MS, pooled and lyophilized.

General procedure for selective installation of *N*-acetamide: Oligosaccharide was dissolved in 1:1 MeOH:H_2O mixture (40 μL/

mg carbohydrate) and acetic anhydride (20 μL/mg carbohydrate) was added. The solution was sonicated for 1 h at 37 °C, and reaction progress was monitored by LC-ESI-Q-TOF-MS. If starting material was observed another 20 μL/mg carbohydrate of H_2O and acetic anhydride were added. The reaction mixture was lyophilized when no more starting material was observed. The residue was re-dissolved in minimal MilliQ water and loaded on a P2 Biogel size exclusion column, which was eluded with 10 mM NH_4HCO_3(aq). Carbohydrate containing fractions were identified by LC-ESI-Q-TOF-MS, pooled and lyophilized.

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Conflict of Interests

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available in the supplementary material of this article.

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