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Large-scale chemoenzymatic synthesis of blood group and tumor-associated poly-*N*-acetyllactosamine antigens

Daniela Vasiliu, Nahid Razi, Yingning Zhang, Nathan Jacobsen, Kirk Allin, Xiaofei Liu, Julia Hoffmann, Ognian Bohorov and Ola Blixt*

Glycan Array Synthesis Core D, Consortium for Functional Glycomics. The Scripps Research Institute, Department of Molecular Biology, CB 248A 10550 N. Torrey Pines Road, La Jolla, CA 92037, USA

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Abstract—Poly-*N*-acetyllactosamines (pLNs) are common terminal sugars of many N- and O-linked glycan structures present in glycoproteins and glycolipids. Utilizing various glycosyltransferases, we developed new and efficient chemoenzymatic methods for the synthesis of pLNs in gram-scale. Specifically, the use of sialyltransferases and fucosyltransferases enabled us to synthesize and purify 24 blood group and tumor-associated pLN derivatives with α-(2→3)- and α-(2→6)-linked sialic acid, as well as with α-(1→2)- and α-(1→3)-linked fucose. All synthesized derivatives were linked to a short 2-azidoethyl spacer for further modification.

Keywords: Poly-*N*-acetyllactosamines; Oligosaccharides; Chemoenzymatic synthesis; Glycosyltransferases; Blood group; Tumor antigens

1. Introduction

A large number of diverse carbohydrate motifs are structural elements of glycoproteins and glycolipids. More than 50% of all secreted and cell-surface proteins are glycosylated. There has been a growing interest in the N- and O-linked glycans due to their newly discovered roles in many specific biological recognition events, such as cell–cell interactions, cancer invasion and metastasis, viral and bacterial cell attachment, and inflammation and immunity.[1–3]

Poly-*N*-acetyllactosamines (pLNs), unique carbohydrate structures composed of *N*-acetyllactosamine (LN) repeats, are known to represent the backbone of many glycan structures that participate in different cell functions.[4,5] Sialylated and fucosylated derivatives of pLNs have been characterized as specific ligands for different lectins such as selectins and galectins as well as being tumor-associated antigens.[6–9] Most of the carbohydrate-based antigens are, as a practical matter, not available by isolation. Therefore, the preparation of lactosamine derivatives is crucial for studying their structures and precise interactions with other biomolecules.

Chemical syntheses of pLNs have been developed using a variety of advanced synthetic strategies, yet the methods always involve tedious multiple protection and deprotection steps.[10–14] A limited number of structures have been synthesized in small amounts using glycosyltransferases as an alternative approach to the chemical synthesis.[15–18]

In this work, we developed an efficient enzymatic strategy to produce 24 pLN derivatives in gram-scale amounts to supply our glycan compound library (www.functionalglycomics.org). We have taken advantage of the relaxed substrate specificity of the recombinant bacterial enzymes β-(1→4)-galactosyltransferase (β4GalT; EC 2.4.1.38) and β-(1→3)-*N*-acetylglucosaminyltransferase (β3GlcNAcT; EC 2.4.1.149), which, by concerted action, generate repeating LN units.[19,20] These structures were further modified with different recombinant fucosyltransferases (FUTs) and sialyltransferases (STs) to obtain a diverse collection of pLN derivatives. All compounds are synthesized with

* Corresponding author. Tel.: +1 858 784 7538; fax: +1 858 784 7542; e-mail: olablixt@scripps.edu

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the 2-azidoethyl aglycone, which can be converted to a primary amino moiety to allow further modifications or to be incorporated onto our glycan microarray.[21,22]

The 2-azidoethyl glycosides based on the LN core that we describe here are the following oligosaccharides: LNβsp (**2**, β-D-Gal*p*-(1→4)-β-D-Glc*p*NAcOCH$_2$CH$_2$N$_3$), DiLNβsp (**3**, β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-(1→4)-β-D-Glc*p*NAcOCH$_2$CH$_2$N$_3$), TriLNβsp (**4**, β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-(1→4)-β-D-Glc*p*NAcOCH$_2$CH$_2$N$_3$), TetraLNβsp (**5**, β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-(1→4)-β-D-Glc*p*NAcOCH$_2$CH$_2$N$_3$), Le^{X}βsp (**6**, β-D-Gal*p*-(1→4)-[α-L-Fuc*p*-(1→3)]-β-D-Glc*p*NAcOCH$_2$CH$_2$N$_3$), Le^X–Le^{X}βsp (**7**, β-D-Gal*p*-(1→4)-[α-L-Fuc*p*-(1→3)]-β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-(1→4)-[α-L-Fuc*p*-(1→3)]-β-D-Glc*p*NAcOCH$_2$CH$_2$N$_3$), Le^X–Le^X–Le^{X}βsp (**8**, β-D-Gal*p*-(1→4)-[α-L-Fuc*p*-(1→3)]-β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-(1→4)-[α-L-Fuc*p*-(1→3)]-β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-(1→4)-[α-L-Fuc*p*-(1→3)]-β-D-Glc*p*NAcOCH$_2$CH$_2$N$_3$), H-type2βsp (**9**, α-L-Fuc*p*-(1→2)-β-D-Gal*p*-(1→4)-β-D-Glc*p*NAcOCH$_2$CH$_2$N$_3$), H-type2-LNβsp (**10**, α-L-Fuc*p*-(1→2)-β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-(1→4)-β-D-Glc*p*NAcOCH$_2$CH$_2$N$_3$), H-type2-LN–LNβsp (**11**, α-L-Fuc*p*-(1→2)-β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-(1→4)-β-D-Glc*p*NAcOCH$_2$CH$_2$N$_3$), Le^{Y}βsp (**12**, α-L-Fuc*p*-(1→2)-β-D-Gal*p*-(1→4)-[α-L-Fuc*p*-(1→3)]-β-D-Glc*p*NAcOCH$_2$CH$_2$N$_3$), Le^Y–Le^{X}βsp (**13**, α-L-Fuc*p*-(1→2)-β-D-Gal*p*-(1→4)-[α-L-Fuc*p*-(1→3)]-β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-(1→4)-[α-L-Fuc*p*-(1→3)]-β-D-Glc*p*NAc-OCH$_2$CH$_2$N$_3$), Le^Y–Le^X–Le^{X}βsp (**14**, α-L-Fuc*p*-(1→2)-β-D-Gal*p*-(1→4)-[α-L-Fuc*p*-(1→3)]-β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-(1→4)-[α-L-Fuc*p*-(1→3)]-β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-(1→4)-[α-L-Fuc*p*-(1→3)]-β-D-Glc*p*NAcOCH$_2$CH$_2$N$_3$), Neu*p*5Ac2, 6LNβsp (**15**, α-Neu*p*5Ac-(2→6)-β-D-Gal*p*-(1→4)-β-D-Glc*p*NAcOCH$_2$CH$_2$N$_3$), Neu*p*5Ac2,6LN–LNβsp (**16**, α-Neu*p*5Ac-(2→6)-β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-(1→4)-β-D-Glc*p*NAcOCH$_2$CH$_2$N$_3$), Neu*p*5Ac2,6LN–LN–LNβsp (**17**, α-Neu*p*5Ac-(2→6)-β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-(1→4)-β-D-Glc*p*NAcOCH$_2$CH$_2$N$_3$), Neu*p*5Ac2,3LNβsp (**18**, α-Neu*p*5Ac-(2→3)-β-D-Gal*p*-(1→4)-β-D-Glc*p*NAcOCH$_2$CH$_2$N$_3$), Neu*p*5Ac2,3LN–LNβsp (**19**, α-Neu*p*5Ac-(2→3)-β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→3)-Gal*p*-(1→4)-β-D-Glc*p*NAcOCH$_2$CH$_2$N$_3$), Neu*p*5Ac2,3LN–LN–LNβsp (**20**, α-Neu*p*5Ac-(2→3)-β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→3)-Gal*p*-(1→4)-β-D-Glc*p*NAcOCH$_2$CH$_2$N$_3$), Neu*p*5Ac2,3Le^{X}βsp (**21**, α-Neu*p*5Ac-(2→3)-β-D-Gal*p*-(1→4)-[α-L-Fuc*p*-(1→3)]-β-D-Glc*p*NAcOCH$_2$CH$_2$N$_3$), Neu*p*5Ac2,3Le^X–Le^{X}βsp (**22**, α-Neu*p*5Ac-(2→3)-β-D-Gal*p*-(1→4)-[α-L-Fuc*p*-(1→3)]-β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-(1→4)-[α-L-Fuc*p*-(1→3)]-β-D-Glc*p*NAcOCH$_2$CH$_2$N$_3$), Neu*p*5Ac2,3Le^X–Le^X–Le^{X}βsp (**23**, α-Neu*p*5Ac-(2→3)-β-D-Gal*p*-(1→4)-[α-L-Fuc*p*-(1→3)]-β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-(1→4)-[α-L-Fuc*p*-(1→3)]-β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-(1→4)-[α-L-Fuc*p*-(1→3)]-β-D-Glc*p*NAcOCH$_2$CH$_2$N$_3$), Neu*p*5Ac2,6LN-Le^X–Le^{X}βsp (**24**, α-Neu*p*5Ac-(2→6)-β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-(1→4)-[α-L-Fuc*p*-(1→3)]-β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-(1→4)-[α-L-Fuc*p*-(1→3)]-β-D-Glc*p*NAcOCH$_2$CH$_2$N$_3$), LN-Le^X–Le^{X}βsp (**25**, β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-(1→4)-[α-L-Fuc*p*-(1→3)]-β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-(1→4)-[α-L-Fuc*p*-(1→3)]-β-D-Glc*p*NAcOCH$_2$CH$_2$N$_3$).

2. Results and discussion

2.1. Synthesis of core pLN

All glycans were enzymatically generated starting from a monosaccharide precursor. The chemical synthesis of this building block, 2′-azidoethyl 2-acetamido-2-deoxy-β-D-glucopyranoside (GlcNAcβsp, **1**), was described previously.[23]

The synthesis of pLNs was accomplished by successive additions of Gal*p* and Glc*p*NAc to **1** using the bacterial glycosyltransferases β4GalT-GalE and β3GlcNAcT, respectively. The β4GalT-GalE enzyme is a fusion protein that consists of a β-(1→4)-galactosyltransferase and an UDP-galactose-4′ epimerase (GalE; EC 5.1.3.2) that converts inexpensive UDP-glucose (UDP-Glc) to UDP-galactose (UDP-Gal).[20] The fusion protein transfers galactose from the in situ generated UDP-Gal donor to acceptor **1** to give a monomeric LN (**2**) (Fig. 1).

The relaxed acceptor substrate specificity of β3GlcNAcT (lgtA)[19] enabled further elongation by the addition of Glc*p*NAc β-(1→3) to galactose using UDP-GlcNAc as the donor substrate. Despite the <10% relative activity toward terminal LN acceptors compared to lactose, with the use of increased amounts of enzyme (2–10 U/mmol acceptor), the gram-scale reactions were quantitative. Subsequent additions of another galactose residue β-(1→4) to the Glc*p*NAc-terminated LN generated compounds **3** (4.0 g, 62%), **4** (3.2 g, 45%), and **5** (0.1 g, 20%) in excellent yields (Fig. 1).

2.2. Fucosylation of pLN

The synthesized pLN structures were further used as precursors in a systematic synthesis of fucosylated pLN derivatives using recombinant fucosyltransferases (Fig. 2). Compounds **2**, **3**, and **4** were fully fucosylated by the α-(1→3)-fucosyltransferase VI (FUT-VI; EC 2.4.1.152)[24,25] on the Glc*p*NAc residues using GDP-Fucose (GDP-Fuc) as a donor substrate. With our reaction conditions, FUT-VI transferred fucose well to terminal, internal, and penultimate Glc*p*NAc moieties on a LN

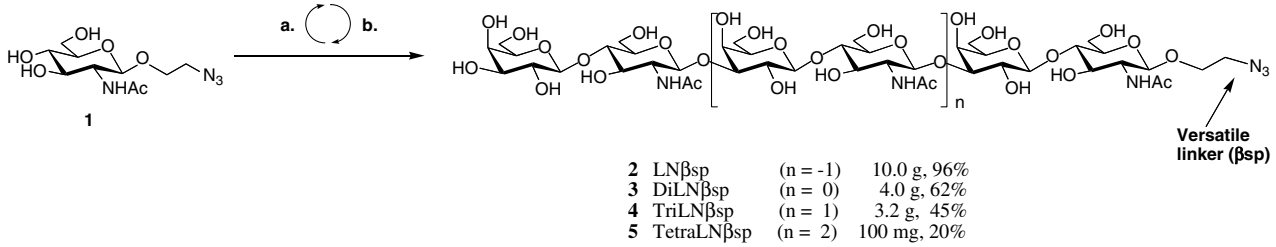

Figure 1. Chemoenzymatic synthesis of poly-*N*-acetyllactosamine structures by concerted action of two recombinant glycosyltransferases starting from **1**. Buffers: sodium cacodylate (200 mM), pH 7.5, $MnCl_2$ (40 mM) and enzyme reactions: (a) β4GalT-GalE (10 U/mmol), UDP-Glc (1.3 equiv); (b) β3GlcNAc-T (20 U/mmol), UDP-GlcNAc (1.3 equiv).

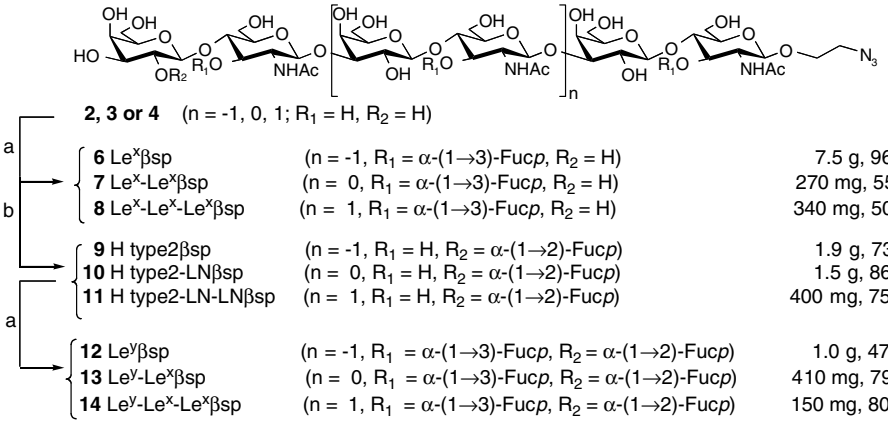

Figure 2. Fucosylation of poly-*N*-acetyllactosamine structures using two recombinant fucosyltransferases FUT-II and FUT-VI. Buffers: sodium cacodylate (200 mM), pH 7.5, $MnCl_2$ (40 mM) and enzyme reactions: (a) FUT-VI (10 U/mmol), GDP-Fuc (1.3–6 equiv); (b) FUT-II (2–4 U/mmol), GDP-Fuc (1.3–6 equiv).

acceptor substrates to generate Lewis^X (Le^X), Le^X–Le^X, and Le^X–Le^X–Le^X structures **6** (7.5 g, 96%), **7** (270 mg, 55%), and **8** (340 mg, 50%), respectively. To accomplish complete fucosylation and obtain **7** and **8**, an excess of GDP-Fuc was used and, after 3 days of incubation when the reaction would not go further, gel filtration was used to remove the inhibitory nucleotide-donor by-products. The resulting oligosaccharide mixture produced consisted of the fully fucosylated and monofucosylated derivatives. Fucosylation was pushed to completion by the addition of more GDP-Fuc and fresh enzyme to give Le^X–Le^X and Le^X–Le^X–Le^X. Thus, in contrast to traditional chemical synthesis, our enzymatic approach allowed the synthesis of these rarely available tumor-associated polymeric Le^X structures in gram-scale in 2–3 weeks.

Other structures related to malignancy are the α-(1→2)-fucose-extended Le^X structures, such as Lewis^Y–Lewis^X (Le^Y–Le^X). Years of elegant chemical work generated about 13 mg of Le^YLe^X heptasaccharide.[26] A recent communication describing an automated solid-phase approach significantly shortened the total synthesis time (excluding the time of building block synthesis), to days instead of years, although yields (<7%) and amounts were still very limited. By contrast, the enzymatic approach allows these compounds to be produced faster and in gram-scale as follows. First, the LNs **2**, **3**, and **4** were terminally fucosylated using recombinant α-(1→2)-fucosyltransferase-II (FUT-II; EC 2.4.1.69). This enzyme transfers fucose from a GDP-Fuc to the terminal galactose of LacNAc in an α-(1→2)-linkage, generating the blood group H-type structures **9** (1.9 g, 73%), **10** (1.5 g, 86%), and **11** (400 mg, 75%). Further α-(1→3) fucosylation of these α-(1→2)-monofucosylated LNs was again possible with FUT-VI, to obtain Le^Y structures **12** (1.0 g, 47%), **13** (410 mg, 79%), and **14** (150 mg, 80%) in good yields (Fig. 2).[24]

2.3. Sialylation of pLN

Enzymatic transfer of sialic acid residues to pLN using two sialyltransferases with different regioselectivities allowed structural diversification to produce known sialylated glycoconjugates (Fig. 3).[27] The recombinant human α-(2→6)-sialyltransferase (hST6Gal-I; EC 2.4.99.1)[20] was used to quantitatively transfer sialic acid in an α-(2→6)-linkage to the terminal galactose of oligosaccharides **2**, **3**, and **4**. The expensive cytidine-5′-monophospho-*N*-acetylneuraminic acid (CMP-Neu5Ac) donor was

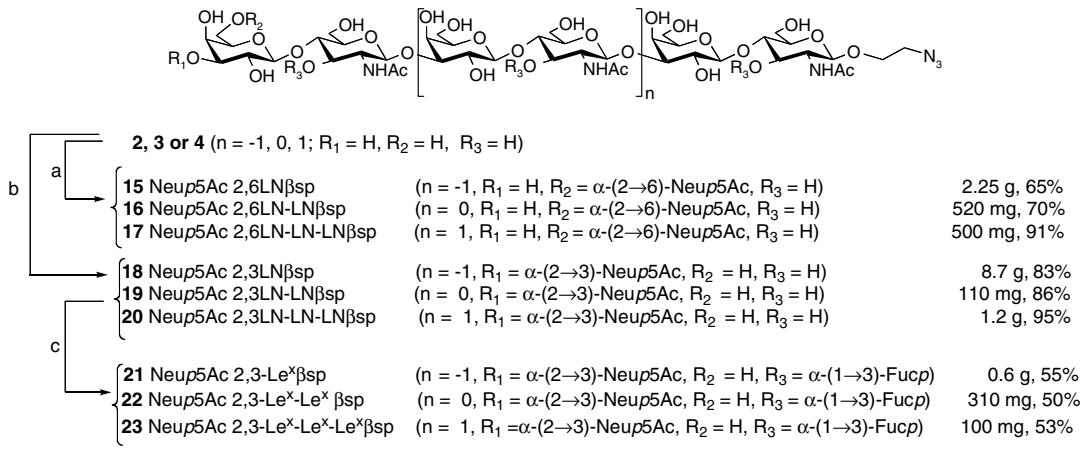

Figure 3. Sialylated poly-*N*-acetyllactosamine derivatives using two recombinant sialyltransferases hST6Gal-I and ST3-CMP-Neu5Ac fusion protein. Buffers: sodium cacodylate (200 mM), pH 7.0–7.5, $MnCl_2$ (40 mM) and enzyme reactions: (a) hST6Gal-I (1–2 U/mmol), CMP-Neu5Ac (1.5 equiv); (b) ST3-CMP-Neu5Ac fusion protein (2–5 U/mmol), CMP-Neu5Ac (2 equiv); (c) FUT-VI (10 U/mmol), GDP-Fuc (1.3–6 equiv).

enzymatically generated from Neu5Ac and cytidine 5′-triphosphate (CTP) using a recombinant bacterial CMP-Neu5Ac synthetase (ST3-CMP-Neu5Ac; EC 2.4.99.4).[28] Compounds **15** (2.25 g, 65%), **16** (520 mg, 70%), and **17** (500 mg, 91%) were obtained and isolated in high yields. A similar approach was taken for the α-(2→3)-sialylation of **2**, **3**, and **4** using the ST3-CMP-Neu5Ac-fusion protein.[20] The resulting α-(2→3)-sialosides, **18** (8.7 g, 83%), **19** (110 mg, 86%), and **20** (1.2 g, 95%), were further fucosylated with FUT-VI using GDP-Fuc as a sugar donor to give sialylated Le^X derivatives **21** (0.6 g, 55%), **22** (310 mg, 50%), and **23** (100 mg, 53%).

Neutrophils often express a series of long unbranched pLNs having α-(2→3)-sialylation on terminal Gal*p* residues and α-(1→3)-fucosylation on internal Glc*p*NAc residues, which were identified as the epitope of *E*-selectin.[29] The internal α-(1→3)-fucosylation is catalyzed by FUT-IV in vivo; however, as a recombinant enzyme, it gives a complex mixture of branched fucosylated products.[30] To avoid fucosylation of the terminal LacNAc, we capped the terminal galactose with an α-(2→6)-Neu*p*5Ac (Fig. 4). As a consequence, the FUT-VI does not add a fucose to the Glc*p*NAc residue on the terminal LacNAc but only to the internal and penultimate Glc*p*NAc residues. Thus, Neu*p*5Ac-α-(2→6)-tri-LN (**17**) was partially fucosylated to produce compound **24** (0.44 g, 70%). When the terminal α-(2→6)-Neu5Ac residue was removed with neuraminidase (EC 3.2.1.18), compound **25** was obtained as a difucosylated tri-LN structure. The same strategy was applied toward production of LN–Le^Xβsp via the sialyl-α-(2→6)-VIM2 antigen (Siaα2-6LNLe^X, data not shown).

In summary, based on the defined specificities of the glycosyltransferases, synthetic strategies were designed to obtain site-specific mono-, di-, and tri-fucosylated pLNs. Sialyltransferases were exploited to add sialic acid in α-(2→3)- and α-(2→6)-linkages to the terminal Gal*p* residue of mono-, di-, and tri-LNs. The creative use of different glycosyltransferases allowed the synthesis of a variety of LNs in large amounts in a fairly short time period, compared to traditional oligosaccharide synthesis.

3. Experimental

3.1. General methods

All enzymatic reactions were performed in aqueous buffered system with the appropriate pH for each enzyme. The reactions were monitored by thin layer chromatography (TLC) performed on Silica Gel 60F pre-coated TLC plates (EMD Chemicals Inc., Gibbstown, NJ, USA). After development with appropriate eluants, the spots were visualized by UV light for nucleotides and/or dipping in 5% sulfuric acid in ethanol, followed by charring to detect sugars. Nuclear magnetic resonance (NMR) spectra were recorded on Bruker DRX-500 and DRX-600 MHz instruments at 25 °C and were referenced to acetone δ 2.225 (^1H in D_2O) and δ 29.9 (^{13}C in D_2O). Mass spectrometry (MS) profiles were recorded with an LC MSD TOF (Agilent Technologies, Foster City, California, USA) using dihydroxybenzoic acid as the matrix. Water was purified by NanoPure Infinity Ultrapure water system (Barnstead/Thermolyne, Dubuque, Iowa, USA) and degassed by vacuum treatment before use. Large scale enzyme concentration was performed in a UF/MF Tangential Flow Filtration (Pall Filtron, Bioseparation Company). The purity of the synthesized compounds is estimated to be 90–95% by TLC and NMR (see ^1H NMR spectra in Supplementary data).

3.2. Materials

Uridine 5′-diphospho-*N*-acetylglucosamine (UDP-GlcNAc) and guanidine-5-diphospho-fucose (GDP-Fuc) were a gift from Tokyo Research Laboratories, Kyowa Hakko Kogyo Co. Ltd. [^{14}C]-GDP-Fuc (200–370 mCi/

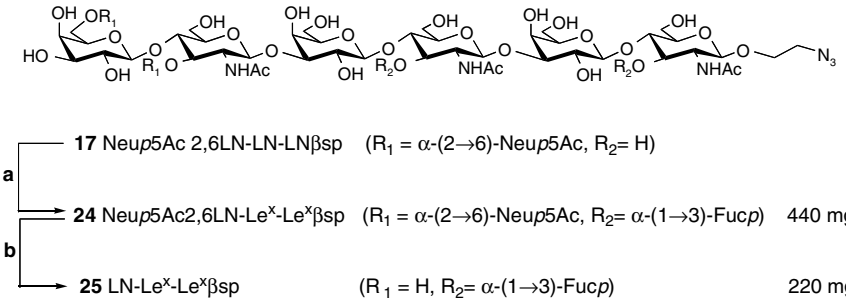

Figure 4. Partial fucosylation of poly-*N*-acetyllactosamine using FUT-VI. The α-(2→6)-sialylated pLN (**17**) was partially fucosylated with FUT-VI (10 U/mmol), GDP-Fuc (3 equiv) in sodium cacodylate (200 mM), pH 7.0–7.5, $MnCl_2$ (40 mM). Removal of Neu5Ac with neuraminidase (6 U/mmol) in sodium phosphate buffer (50 mM), pH 6.0, gave **25** in 53% yield.

mmol) was from GE Healthcare (Little Chalfont, UK). The radioactive nucleotide sugars were diluted with unlabeled UDP-sugars to obtain the desired specific radioactivity. All other chemicals were of highest purity and purchased from Sigma Chemical Co. (St. Louis, MO, USA). Neuraminidase (Sialidase from *Arthrobacter ureafaciens*; 1 U/100 μL) was purchased from Roche Applied Science (Indianapolis, IN, USA).

3.3. Enzyme production

Bacterial β-(1→4)-galactosyltransferase/UDP-Galactose 4′-epimerase fusion construct (β4GalT-GalE) and β-(1→3)-*N*-acetylglucosaminyltransferase (β3GlcNAcT) were expressed in AD202 bacterial cells and prepared in large scale as described previously.[19,20,31] The ST3-CMP-Neu5Ac-fusion protein was produced as described by Gilbert et al.[28] The preparation and activity assays for human α-(2→6)-sialyltransferase (hST6Gal-I) were described in our previous work.[20]

The baculoviral constructs expressing α-(1→2)-fucosyltransferase II (FUT-II) and α-(1→3)-fucosyltransferase VI (FUT-VI) were kindly donated by NEOSE Technologies Inc. (Horsham, PA, USA). The viruses were purified by conventional plaque-forming assay to determine the viral titers. Viral amplification was performed in S*f*9 cells by infecting 1×10^6 cells with multiplicity of infection, MOI = 0.5–1. The viral production was initiated in T-flasks (20 mL) and further adapted to 500 mL shaker flask (150 mL culture), incubating at 27 °C for 48 h. The enzyme expression was also performed in S*f*9 cells in 350 mL culture in 1 L shaker flasks with serum-free media in the presence of 10% conditioned media to maintain the optimal cell growth and protein expression. S*f*9 cells were infected ($1.5–2 \times 10^6$ cells/mL) with the desired purified viral stock with the MOI = 5. Incubation continued with vigorous shaking (120 rpm) at 27 °C. The enzyme expression was monitored for up to 7 days, by fucosyltransferase assay. The culture medium was collected by centrifugation for 30 min at 5000 rpm when the cell viability decreased to about 50%, and the assay showed a decrease in the activity.

3.4. Fucosyltransferase assays

Both FUT-II and FUT-VI were assayed in a final volume of 100 μL of Tris buffer (50 mM), pH 7.5, MnCl (10 mM), using [^{14}C]-GDP-Fuc sugar nucleotide (specific activity of 8000, 8 nmol) as donor substrate and lactose or LN (10 mM) as acceptor substrate. The enzyme activity was up to 10 and 25 U/L of culture media for FUT-II and FUT-VI, respectively, using the above assay conditions. The collected medium was 20-fold concentrated by a Tangential Flow Filtration system to be used as a crude mixture in our synthesis. The enzymes are stable in 50% glycerol in −20 °C for at least 1 year.

3.5. 2′-Azidoethyl 2-acetamido-2-deoxy-β-D-glucopyranoside (1)

This compound was synthesized as previously described.[23] Selected ^{1}H NMR (500 MHz, D_2O): δ 4.58 (d, 1H, $J = 8$ Hz, H-1), 4.05 (m, 2H, $OCH_2CH_2N_3$), 3.93 (dd, 1H, H-2), 3.53 (dd, 1H, H-3), 2.04 (s, 3H, $NHCOCH_3$); Selected ^{13}C NMR (500 MHz, D_2O): δ 174.35, 100.70, 75.57, 73.52, 69.54, 68.42, 60.35, 55.15, 50.01, 21.87; ESIMS *m*/*z* calcd for $[M+Na]^+$: 313.1124. Found: 313.1110.

3.6. General procedure for the synthesis of compounds 2, 3, 4, and 5

Compound **1** (6.67 g, 23 mmol), UDP-Glc (29 mmol, 1.3 equiv), and β4GalT-GalE (10 U/mmol) were added and the reaction mixture (pH 7.5) was gently stirred for 24 h at rt. The reaction was monitored by TLC (6:3:3:2, EtOAc/HOAc/CH_3OH/H_2O). The mixture was centrifuged and the supernatant was purified on a Sephadex (5 × 170 cm) G15 column eluted with 5% BuOH in H_2O. Pure product fractions were collected and lyophilized to produce **2** (10.0 g, 22 mmol, 96%). To each of the Gal-terminated LN structures **2**–**4** (0.3–7.6 mmol), β3GlcNAcT (20 U/mmol substrate), and UDP-GlcNAc (0.9–19 mmol, 2.5–3 equiv) were dissolved in cacodylate buffer (200 mM) containing $MnCl_2$

(40 mM), the pH adjusted to 7.5 and the reaction mixture was stirred at rt overnight. The products were purified, further galactosylated as described above and the corresponding fractions were collected and lyophilized to give **3**, **4**, or **5** (0.1–4.0 g, 0.06–5.0 mmol, 20–62%).

3.6.1. 2′-Azidoethyl β-D-galactopyranosyl-(1→4)-2-acetamido-2-deoxy-β-D-glucopyranoside (2). Selected ^{1}H NMR (500 MHz, D_2O): δ 4.60 (d, 1H, $J = 8$ Hz, Glc*p*NAc, H-1), 4.47 (d, 1H, $J = 8$ Hz, Gal*p*, H-1), 3.92 (d, 1H, Gal*p*, H-4), 4.06–4.03 (2m, 2H, $OCH_2CH_2N_3$), 3.99 (dd, 1H, Glc*p*NAc, H-2), 3.83 (dd, 1H, Glc*p*NAc, H-3), 3.72 (dd, 1H, Glc*p*NAc, H-4), 3.67 (dd, 1H, Glc*p*NAc, H-3), 3.55 (dd, 1H, Gal*p*, H-2), 3.40–3.50 (2m, 2H, $OCH_2CH_2N_3$), 2.038 (s, 3H, $NHCOCH_3$); Selected ^{13}C NMR (500 MHz, D_2O): δ 174.26, 102.53, 100.60, 78.11, 75.00, 74.46, 72.16, 72.13, 70.61, 68.39, 68.20, 60.67, 59.70, 54.67, 50.01, 21.92; ESIMS *m*/*z* calcd for $[M+Na]^+$: 475.1653. Found: 475.1643.

3.6.2. 2′-Azidoethyl β-D-galactopyranosyl-(1→4)-2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→3)-β-D-galactopyranosyl-(1→4)-2-acetamido-2-deoxy-β-D-glucopyranoside (3). Selected ^{1}H NMR (500 MHz, D_2O): δ 4.70 (d, 1H, $J = 8$ Hz, GlcNAc2, H-1), 4.60 (d, 1H, $J = 8$ Hz, GlcNAc1, H-1), 4.48–4.46 (m, 3H, Gal1, Gal2, H-1), 2.039 (s, 3H, $NHCOCH_3$), 2.034 (s, 3H, $NHCOCH_3$); Selected ^{13}C NMR (500 MHz, D_2O): δ 174.54, 174.28, 102.57, 102.52, 102.44, 100.63, 81.72, 78.07, 77.77, 75.01, 74.54, 74.46, 74.21, 72.14, 71.84, 70.62, 69.61, 68.43, 68.20, 67.98, 60.70, 60.62, 59.67, 59.49, 54.84, 54.63, 50.02, 21.92, 21.84; ESIMS *m*/*z* calcd for $[M+H]^+$: 818.3155. Found: 818.3139.

3.6.3. 2′-Azidoethyl β-D-galactopyranosyl-(1→4)-2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→3)-β-D-galactopyranosyl-(1→4)-2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→3)-β-D-galactopyranosyl-(1→4)-2-acetamido-2-deoxy-β-D-glucopyranoside (4). Selected ^{1}H NMR (500 MHz, D_2O): δ 4.70 (d, 2H, $J = 8$ Hz, GlcNAc2, GlcNAc3, H-1), 4.60 (d, 1H, $J = 8$ Hz, GlcNAc1, H-1), 4.48–4.46 (m, 3H, Gal1, Gal2, Gal3, H-1), 2.04 (s, 3H, $NHCOCH_3$), 2.033 (s, 6H, $NHCOCH_3$); Selected ^{13}C NMR (500 MHz, D_2O): δ 174.55, 174.30, 102.52, 102.43, 100.62, 81.71, 78.05, 77.77, 75.01, 74.53, 74.46, 74.20, 72.13, 71.82, 70.62, 69.61, 68.43, 68.20, 67.98, 60.69, 60.62, 59.66, 59.49, 54.83, 54.80, 54.62, 50.01, 21.918, 21.84; ESIMS *m*/*z* calcd for $[M+H]^+$: 1183.4476. Found: 1183.4462.

3.7. General fucosylation conditions for reactions catalyzed by FUT-VI

The sugar acceptor substrates **2**, **3**, **4**, **9**, **10**, **11**, **17**, **18**, **19**, or **20** (0.1–12.6 mmol) and the GDP-Fuc donor (1.3–6.0 equiv) were dissolved in cacodylate buffer (200 mM, pH 7.5) containing $MnCl_2$ (40 mM). FUT-VI (10 U/mmol) was added and the reaction mixture was gently stirred at rt for 2–4 days. When the reaction looked complete by TLC, the reaction mixture was centrifuged and purified through a size exclusion column Sephadex G15 (5 × 140 cm), equilibrated and eluted with 5% butanol in H_2O (v/v). Fractions containing the product were lyophilized and further purified on a size exclusion column of Sephadex G25 (5 × 140 cm), equilibrated and eluted with 5% butanol in H_2O (v/v). Fractions containing the product were lyophilized to give pure α-(1→3)-fucosylated products **6**, **7**, **8**, **12**, **13**, **14**, **21**, **22**, **23**, and **24** (0.1–7.5 g, 50–96%).

3.7.1. 2′-Azidoethyl β-D-galactopyranosyl-(1→4)-[α-L-fucopyranosyl-(1→3)]-2-acetamido-2-deoxy-β-D-glucopyranoside (6). Selected ^{1}H NMR (500 MHz, D_2O): δ 5.11 (d, 1H, $J = 4$ Hz, Fuc, H-1), 4.61 (d, 1H, $J = 8$ Hz, GlcNAc, H-1), 4.45 (d, 1H, $J = 8$ Hz, Gal, H-1), 2.04 (s, 3H, $NHCOCH_3$), 1.17 (d, 3H, Fuc, H-6); Selected ^{13}C NMR (500 MHz, D_2O): δ 174.07, 101.50, 100.48, 98.35, 75.05, 74.60, 74.56, 73.02, 72.10, 71.55, 70.68, 68.86, 68.37, 67.99, 67.33, 66.38, 61.15, 59.38, 55.34, 50.03, 21.98, 14.97; ESIMS *m*/*z* calcd for $[M+Na]^+$: 621.2232. Found: 621.2220.

3.7.2. 2′-Azidoethyl β-D-galactopyranosyl-(1→4)-[α-L-fucopyranosyl-(1→3)]-2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→3)-β-D-galactopyranosyl-(1→4)-[α-L-fucopyranosyl-(1→3)]-2-acetamido-2-deoxy-β-D-glucopyranoside (7). Selected ^{1}H NMR (500 MHz, D_2O): δ 5.14 (d, 1H, $J = 4$ Hz, Fuc2, H-1), 5.10 (d, $J = 4$ Hz, Fuc1, H-1), 4.71 (d, 1H, $J = 8$ Hz, GlcNAc2, H-1), 4.61 (d, 1H, $J = 8$ Hz, GlcNAc1, H-1), 4.47 (d, 2H, Gal2, H-1), 4.44 (d, 2H, Gal1, H-1), 2.04 (3H, s, $NHCOCH_3$), 2.02 (3H, s, $NHCOCH_3$), 1.18–1.14 (dd, 6H, 2Fuc, H-6); Selected ^{13}C NMR (500 MHz, D_2O): δ 174.298, 174.083, 102.14, 101.45, 101.40, 100.46, 98.40, 98.22, 81.26, 75.04, 74.73, 74.57, 74.42, 74.10, 72.76, 72.70, 72.13, 71.57, 71.51, 71.39, 70.71, 70.17, 69.24, 68.85, 68.35, 68.01, 67.88, 67.36, 67.30, 66.36, 61.15, 61.10, 59.41, 59.28, 55.61, 55.37, 50.19, 50.04, 48.27, 21.98, 21.91, 14.97, 14.92; ESIMS *m*/*z* calcd for $[M+Na]^+$: 1132.4133. Found: 1132.4123.

3.7.3. 2′-Azidoethyl β-D-galactopyranosyl-(1→4)-[α-L-fucopyranosyl-(1→3)]-2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→3)-β-D-galactopyranosyl-(1→4)-[α-L-fucopyranosyl-(1→3)]-2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→3)-β-D-galactopyranosyl-(1→4)-[α-L-fucopyranosyl-(1→3)]-2-acetamido-2-deoxy-β-D-glucopyranoside (8). Selected ^{1}H NMR (500 MHz, D_2O): δ 5.13 (2d, 2H, $J = 4$ Hz, Fuc2, Fuc3, H-1), 5.10 (d, 1H, $J = 4$ Hz, Fuc1, H-1), 4.70 (d, 2H, $J = 8$ Hz, GlcNAc2, GlcNAc3, H-1), 4.60 (d, 1H, $J = 8$ Hz, GlcNAc1, H-1), 4.45 (dd, 3H, Gal1,

Gal2, Gal3, H-1), 2.03 (3H, s, $NHCOCH_3$), 2.015 (6H, s, $2NHCOCH_3$), 1.17 (d, 3H, Fuc, H-6), 1.14 (d, 6H, 2Fuc, H-6); Selected ^{13}C NMR (500 MHz, D_2O): δ 174.06, 173.85, 101.91, 101.21, 101.15, 100.23, 98.18, 98.12, 98.01, 81.01, 74.78, 74.46, 74.33, 74.16, 73.85, 72.48, 72.40, 72.13, 71.87, 71.31, 71.26, 70.45, 69.92, 68.59, 68.13, 67.76, 67.65, 67.10, 67.03, 60.93, 60.88, 59.13, 59.02, 55.36, 55.10, 49.79, 21.72, 21.64, 14.73, 14.69. ESIMS m/z calcd for $[M+Na]^+$: 1643.6034. Found: 1643.6026.

3.8. General fucosylation conditions for reactions catalyzed by FUT-II

The sugar acceptor substrates **2**, **3**, or **4** (0.4–2.2 mmol) and the GDP-Fuc donor (0.8–4.4 mmol, 2 equiv) were dissolved in cacodylate buffer (200 mM, pH 7.5) containing $MnCl_2$ (40 mM). FUT-II (2–4 U/mmol substrate) was added and the reaction mixture gently stirred for 2 days at rt. The reaction was observed on TLC (6:3:3:2, EtOAc/HOAc/CH_3OH/H_2O). When complete, the reaction mix was centrifuged and purified through a size exclusion column Sephadex G15 (5×140 cm), equilibrated and eluted with 5% butanol in H_2O (v/v). Only the fractions containing the product were collected, lyophilized and further purified using Sephadex G25 (5×170 cm) to give pure α-(1→2)-fucosylated products **9**, **10**, and **11** (0.4–1.9 g, 73–86%).

3.8.1. 2′-Azidoethyl α-L-fucopyranosyl-(1→2)-β-D-galactopyranosyl-(1→4)-2-acetamido-2-deoxy-β-D-glucopyranoside (9). Selected ^1H NMR (600 MHz, D_2O): δ 5.31 (d, 1H, $J = 3$ Hz, Fucα1–2, H-1), 4.58 (d, 1H, $J = 8$ Hz, GlcNAc1, H-1), 4.56 (d, 1H, $J = 8$ Hz, Gal, H-1), 2.05 (3H, s, $NHCOCH_3$), 1.234 (d, 3H, Fuc, H-6); Selected ^{13}C NMR (600 MHz, D_2O): δ 174.30, 100.72, 99.90, 99.07, 76.06, 75.73, 75.00, 74.92, 73.19, 72.05, 71.33, 69.27, 68.78, 68.51, 67.84, 66.57, 59.81, 60.80, 54.85, 50.01, 21.95, 14.97; ESIMS m/z calcd for $[M+Na]^+$: 621.2232. Found: 621.2211.

3.8.2. 2′-Azidoethyl α-L-fucopyranosyl-(1→2)-β-D-galactopyranosyl-(1→4)-2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→3)-β-D-galactopyranosyl-(1→4)-2-acetamido-2-deoxy-β-D-glucopyranoside (10). Selected ^1H NMR (600 MHz, D_2O): δ 5.30 (d, 1H, $J = 3$ Hz, Fucα1–2, H-1), 4.70 (d, 2H, $J = 8$ Hz, GlcNAc2, H-1), 4.60 (d, 1H, $J = 8$ Hz, GlcNAc1, H-1), 4.55 (d, 1H, $J = 8$ Hz, Gal2, H-1), 4.46 (d, 1H, $J = 8$ Hz, Gal1, H-1), 2.04 (s, 6H, $2NHCOCH_3$), 1.23 (d, 3H, Fuc, H-6); Selected ^{13}C NMR (600 MHz, D_2O): δ 174.56, 174.30, 102.55, 102.45, 100.62, 99.90, 99.07, 81.65, 78.067, 76.09, 75.49, 74.91, 74.75, 74.52, 74.46, 73.19, 72.13, 71.73, 71.32, 69.64, 69.27, 68.43, 67.97, 67.85, 66.61, 60.79, 60.58, 59.66, 55.03, 54.63, 50.01, 21.92, 21.86, 14.98. ESIMS m/z calcd for $[M+Na]^+$: 986.3554. Found: 986.3541.

3.8.3. 2′-Azidoethyl α-L-fucopyranosyl-(1→2)-β-D-galactopyranosyl-(1→4)-2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→3)-β-D-galactopyranosyl-(1→4)-2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→3)-β-D-galactopyranosyl-(1→4)-2-acetamido-2-deoxy-β-D-glucopyranoside (11). Selected ^1H NMR (600 MHz, D_2O): δ 5.31 (d, 1H, $J = 3$ Hz, Fuc α1–2, H-1), 4.70 (d, 2H, $J = 8$ Hz, GlcNAc2, GlcNAc3, H-1), 4.60 (d, 1H, $J = 8$ Hz, GlcNAc1, H-1), 4.55 (d, 1H, Gal3, H-1), 4.47–4.45 (dd, 2H, Gal2, Gal1, H-1), 2.039 (s, 6H, $NHCOCH_3$), 2.032 (s, 3H, $NHCOCH_3$), 1.225 (d, 3H, Fuc, H-6); Selected ^{13}C NMR (600 MHz, D_2O): δ 174.56, 174.53, 174.30, 102.57, 102.53, 102.42, 102.38, 100.62, 99.90, 99.07, 81.71, 78.07, 77.79, 76.10, 75.51, 74.91, 74.75, 74.53, 74.46, 74.20, 73.18, 72.13, 71.82, 71.72, 71.32, 69.64, 69.61, 69.27, 68.77, 68.42, 67.97, 67.86, 66.60, 60.78, 60.61, 59.66, 55.03, 54.80, 54.62, 50.02, 21.92, 21.86, 21.84, 14.9. ESIMS m/z calcd for $[M+Na]^+$: 1351.4876. Found: 1351.4848.

3.8.4. 2-Azidoethyl α-L-fucopyranosyl-(1→2)-β-D-galactopyranosyl-(1→4)-[α-L-fucopyranosyl-(1→3)]-2-acetamido-2-deoxy-β-D-glucopyranoside (12). Selected ^1H NMR (600 MHz, D_2O): δ 5.28 (d, 1H, $J = 3$ Hz, Fuc α1–2, H-1), 5.10 (d, 1H, $J = 4$ Hz, Fucα1–3, H-1), 4.59 (d, 1H, $J = 8$ Hz, GlcNAc, H-1), 4.50 (d, 1H, $J = 8$ Hz, Gal, H-1), 2.04 (s, 3H, $NHCOCH_3$), 1.27 (d, 3H, Fuc, H-6), 1.24 (d, 3H, Fuc, H-6); Selected ^{13}C NMR (600 MHz, D_2O): δ 174.11, 100.05, 99.90, 99.07, 98.31, 75.98, 75.29, 74.58, 74.51, 73.22, 72.99, 71.59, 71.36, 69.38, 68.84, 68.45, 68.40, 67.91, 67.34, 66.55, 66.49, 61.12, 59.56, 55.50, 50.03, 22.00, 15.10. ESIMS m/z calcd for $[M+Na]^+$: 767.2811. Found, 767.2801.

3.8.5. 2′-Azidoethyl α-L-fucopyranosyl-(1→2)-β-D-galactopyranosyl-(1→4)-[α-L-fucopyranosyl-(1→3)]-2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→3)-β-D-galactopyranosyl-(1→4)-[α-L-fucopyranosyl-(1→3)]-2-acetamido-2-deoxy-β-D-glucopyranoside (13). Selected ^1H NMR (600 MHz, D_2O): δ 5.28 (d, 1H, $J = 3$ Hz, Fucα1–2, H-1), 5.12 (d, 1H, $J = 4$ Hz, Fucα1–3, H-1), 5.10 (d, 1H, $J = 4$ Hz, Fucα1–3, H-1), 4.70 (d, 2H, $J = 8$ Hz, GlcNAc2, H-1), 4.61 (d, 1H, $J = 8$ Hz, GlcNAc1, H-1), 4.51 (d, 1H, $J = 8$ Hz, Gal2, H-1), 4.44 (d, 1H, $J = 8$ Hz, Gal1, H-1), 2.03 (s, 3H, $2NHCOCH_3$), 2.02 (s, 3H, $2NHCOCH_3$), 1.27 (d, 3H, Fuc, H-6), 1.24 (d, 3H, Fuc, H-6), 1.15 (d, 3H, Fuc, H-6); Selected ^{13}C NMR (600 MHz, D_2O): δ 174.32, 174.11, 101.46, 100.48, 99.85, 99.08, 98.44, 98.27, 81.16, 76.01, 75.04, 74.97, 74.60, 74.52, 74.47, 74.08, 73.22, 72.76, 72.69, 71.60, 71.51, 71.36, 70.22, 69.39, 68.84, 68.41, 68.38, 67.93, 67.89, 67.35, 67.29, 66.58, 66.46, 66.38, 61.15, 61.08, 59.40, 55.71, 55.36, 50.04, 21.97, 21.92, 15.12,

15.10, 14.93. ESIMS *m/z* calcd for $[M+H]^+$: 1256.4892. Found: 1256.4889.

3.8.6. 2′-Azidoethyl α-L-fucopyranosyl-(1→2)-β-D-galactopyranosyl-(1→4)-[α-L-fucopyranosyl-(1→3)]-2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→3)-β-D-galactopyranosyl-(1→4)-[α-L-fucopyranosyl-(1→3)]-2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→3)-β-D-galactopyranosyl-(1→4)-[α-L-fucopyranosyl-(1→3)]-2-acetamido-2-deoxy-β-D-glucopyranoside (14). Selected ^{1}H NMR (500 MHz, D_2O): δ 5.27 (d, 1H, $J = 3$ Hz, Fucα1–2, H-1), 5.12 (d, 2H, $J = 4$ Hz, Fuc2, Fuc3, H-1), 5.10 (d, 1H, $J = 4$ Hz, Fuc1, H-1), 4.70 (d, 2H, $J = 8$ Hz, GlcNAc2, GlcNAc3, H-1), 4.61 (d, 1H, $J = 8$ Hz, GlcNAc1, H-1), 4.51 (d, 1H, Gal3, H-1), 4.45 (dd, 2H, Gal1, Gal2, H-1), 2.03 (3H, s, $NHCOCH_3$), 2.018 (6H, s, $2NHCOCH_3$), 1.26 (d, 3H, Fuc, H-6), 1.24 (d, 3H, Fuc, H-6), 1.15 (dd, 6H, Fuc, H-6); Selected ^{13}C NMR (600 MHz, D_2O): δ 215.06, 193.06, 174.32, 158.02, 157.80, 156.88, 154.86, 145.76, 124.09, 108.75, 102.14, 102.09, 101.47, 101.37, 100.48, 99.86, 99.09, 98.42, 98.25, 89.56, 81.83, 81.25, 81.18, 76.03, 74.99, 74.52, 74.48, 74.42, 74.08, 73.22, 72.76, 72.71, 72.56, 72.42, 71.60, 71.52, 71.36, 70.24, 70.17, 69.39, 69.12, 68.84, 68.42, 68.37, 67.94, 67.89, 67.30, 66.58, 66.45, 66.34, 65.48, 61.09, 59.41, 55.62, 55.35, 55.19, 50.05, 47.42, 21.90, 15.10, 14.92. ESIMS *m/z* calcd for $[M+Na]^+$: 1789.6607. Found, 1789.6620.

3.9. General sialylation conditions for reactions catalyzed by hST6Gal-I

The sugar acceptor substrates **2**, **3**, **4** (0.4–4.4 mmol) and the CMP-Neu*p*5Ac donor (0.5–6.6, 1.5 equiv) were dissolved in cacodylate buffer (200 mM, pH 7.5) containing $MnCl_2$ (40 mM). hST6Gal-I (1–2 U/mmol) was added and the pH was adjusted to 7.5. The reaction mixture was stirred at rt for 24 h. The reaction mixture was centrifuged (8000 rpm, 15 min) and the supernatant was loaded on Sephadex (5 × 170 cm) G25 column. Product fractions were collected and lyophilized to give pure α-(2→6)-sialylated oligosaccharides **15**, **16**, and **17** (0.5–2.3 g, 65–91%).

3.9.1. 2-Azidoethyl 5-*N*-acetyl-α-neuraminyl-(2→6)-β-D-galactopyranosyl-(1→4)-2-acetamido-2-deoxy-β-D-glucopyranoside (15). Selected ^{1}H NMR (500 MHz, D_2O): δ 4.63 (d, 1H, $J = 8$ Hz, GlcNAc, H-1), 4.45 (d, 1H, $J = 8$ Hz, Gal, H-1), 2.67 (dd, 1H, $J = 4$ Hz, NeuAc, H-3eq), 2.066 (s, 3H, $NHCOCH_3$ of NeuAc), 2.026 (s, 3H, $NHCOCH_3$ of GlcNAc), 1.68 (t, 1H, $J = 12$ Hz, NeuAc, H-3ax); Selected ^{13}C NMR (500 MHz, D_2O): δ 103.16, 100.48, 80.41, 74.18, 73.34, 72.23, 72.20, 72.08, 71.36, 70.38, 68.42, 68.06, 68.02, 62.99, 59.99, 54.43, 51.56, 50.02, 39.76, 22.06, 21.70. ESIMS *m/z* calcd for $[M+Na]^+$: 788.2426. Found, 788.2420.

3.9.2. 2-Azidoethyl 5-*N*-acetyl-α-neuraminyl-(2→6)-β-D-galactopyranosyl-(1→4)-2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→3)-β-D-galactopyranosyl-(1→4)-2-acetamido-2-deoxy-β-D-glucopyranoside (16). Selected ^{1}H NMR (600 MHz, D_2O): δ 4.73 (d, 1H, $J = 8$ Hz, GlcNAc2, H-1), 4.60 (d, 1H, $J = 8$ Hz, GlcNAc1, H-1), 4.47 (d, 1H, Gal2, H-1), 4.45 (d, 1H, Gal1, H-1), 2.67 (dd, 1H, $J = 4$ Hz, NeuAc, H-3ax), 2.055 (s, 3H, NeuAc $NHCOCH_3$), 2.040 (s, 3H, $NHCOCH_3$ of GlcNAc2), 2.029 (s, 3H, $NHCOCH_3$ of GlcNAc1), 1.718 (t, 1H, $J = 12$ Hz, NeuAc, H-3ax); Selected ^{13}C NMR (600 MHz, D_2O): δ 174.57, 174.30, 173.18, 103.12, 102.57, 102.24, 100.63, 99.81, 81.72, 81.67, 80.13, 78.13, 74.55, 74.47, 73.93, 72.20, 72.14, 71.90, 71.38, 70.40, 69.64, 68.40, 68.07, 67.97, 67.87, 62.33, 60.63, 59.81, 59.68, 54.62, 51.55, 50.03, 39.75, 21.95, 21.70. ESIMS *m/z* calcd for $[M+Na]^+$: 1131.3929. Found: 1131.3961.

3.9.3. 2-Azidoethyl 5-*N*-acetyl-α-neuraminyl-(2→6)-β-D-galactopyranosyl-(1→4)-2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→3)-β-D-galactopyranosyl-(1→4)-2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→3)-β-D-galactopyranosyl-(1→4)-2-acetamido-2-deoxy-β-D-glucopyranoside (17). Selected ^{1}H NMR (600 MHz, D_2O): δ 4.73 (d, 1H, $J = 8$ Hz, Glu3, H-1), 4.70 (d, 1H, $J = 8$ Hz, GlcNAc2, H-1), 4.60 (d, 1H, $J = 8$ Hz, GlcNAc1, H-1), 4.48–4.44 (m, 3H, Gal1, Gal2, Gal3, H-1), 2.67 (dd, 1H, $J = 4$ Hz, NeuAc, H-3eq), 2.054 (s, 3H, NeuAc $NHCOCH_3$), 2.040 (s, 3H, $NHCOCH_3$ of GlcNAc3), 2.033 (s, 3H, $NHCOCH_3$ of GlcNAc2), 2.029 (s, 3H, $NHCOCH_3$ of GlcNAc1), 1.718 (t, 1H, $J = 12$ Hz, NeuAc, H-3ax). ESIMS *m/z* calcd for $[M+Na]^+$: 1519.5064. Found, 1519.5063.

3.10. General sialylation conditions for reactions catalyzed by ST3Gal-CMP-Neu*p*5Ac fusion protein

The sugar acceptor substrates **2**, **3**, or **4** (0.1–13.3 mmol) and the CMP-Neu*p*5Ac (0.2–26.6 mmol, 2 equiv) were dissolved in cacodylate buffer (200 mM, pH 7.0) containing $MnCl_2$ (40 mM). ST3Gal-CMP-Neu5Ac fusion protein (2–5 U/mmol) was added and the pH was adjusted to 7.0. After 18 h at rt, the reaction was stopped by centrifugation. The mixture and the supernatant were loaded on a Sephadex G25 column (2.5 × 170 cm). The collected fractions were freeze-dried. The synthesized structures were further purified on Sephadex G25 to give pure α-(2→3)-sialylated products **18**, **19**, and **20** (0.1–8.7 g, 83–96%), respectively.

3.10.1. 2-Azidoethyl 5-*N*-acetyl-α-neuraminyl-(2→3)-β-D-galactopyranosyl-(1→4)-2-acetamido-2-deoxy-β-D-glucopyranoside (18). Selected ^{1}H NMR (500 MHz, D_2O): δ 4.60 (d, 1H, $J = 8$ Hz, GlcNAc, H-1), 4.55 (d, 1H, $J = 8$ Hz, Gal, H-1), 2.75 (dd, 1H, $J = 4$ Hz, NeuAc,

H-3eq), 2.04 (s, 3H, $NHCOCH_3$), 2.03 (s, 3H, $NHCOCH_3$), 1.80 (t, 1H, $J = 12$ Hz, NeuAc, H-3ax); Selected ^{13}C NMR (500 MHz, D_2O): δ 174.50, 173.54, 102.22, 100.66, 99.46, 77.90, 75.12, 74.83, 74.46, 72.53, 72.10, 71.41, 69.03, 68.41, 67.99, 67.74, 67.13, 62.23, 60.69, 59.66, 58.98, 54.65, 51.34, 50.01, 39.27, 21.92, 21.70. ESIMS m/z calcd for $[M+Na]^+$: 788.2426. Found: 788.2452.

3.10.2. 2-Azidoethyl 5-*N*-acetyl-α-neuraminyl-(2→3)-β-D-galactopyranosyl-(1→4)-2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→3)-β-D-galactopyranosyl-(1→4)-2-acetamido-2-deoxy-β-D-glucopyranoside (19). Selected ^1H NMR (600 MHz, D_2O): δ 4.70 (d, 1H, $J = 8$ Hz, GlcNAc2, H-1), 4.60 (d, 1H, $J = 8$ Hz, GlcNAc1, H-1), 4.555 (d, 1H, Gal2, H-1), 4.46 (d, 1H, Gal1, H-1), 2.76 (dd, 1H, $J = 4$ Hz, NeuAc, H-3eq), 2.03 (s, 9H, $NHCOCH_3$), 1.80 (t, 1H, $J = 12$ Hz, NeuAc, H-3ax); Selected ^{13}C NMR (600 MHz, D_2O): δ 114.20, 102.56, 102.48, 98.31, 96.61, 93.29, 81.73, 78.07, 77.60, 75.13, 74.83, 74.56, 74.20, 72.55, 72.13, 71.80, 71.43, 69.62, 69.25, 68.42, 68.01, 67.73, 62.88, 62.23, 60.72, 60.63, 59.66, 58.10, 54.83, 54.62, 51.34, 50.02, 41.53, 41.30, 39.30, 31.63, 30.16, 21.92, 21.84, 21.70. ESIMS m/z calcd for $[M+Na]^+$: 1131.3929. Found: 1131.3912.

3.10.3. 2-Azidoethyl 5-*N*-acetyl-α-neuraminyl-(2→3)-β-D-galactopyranosyl-(1→4)-2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→3)-β-D-galactopyranosyl-(1→4)-2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→3)-β-D-galactopyranosyl-(1→4)-2-acetamido-2-deoxy-β-D-glucopyranoside (20). Selected ^1H NMR (500 MHz, D_2O): δ 4.70 (d, 2H, $J = 8$ Hz, GlcNAc2, GlcNAc3, H-1), 4.60 (d, 1H, $J = 8$ Hz, GlcNAc1, H-1), 4.555 (d, 1H, Gal3, H-1), 4.47–4.46 (m, 2H, Gal1, Gal2, H-1), 2.76 (dd, 1H, $J = 4$ Hz, NeuAc, H-3eq), 2.03 (s, 12H, $NHCOCH_3$), 1.80 (t, 1H, $J = 12$ Hz, NeuAc, H-3ax); Selected ^{13}C NMR (500 MHz, D_2O): δ 174.66, 102.55, 102.47, 102.42, 102.19, 100.61, 81.71, 78.07, 77.80, 75.14, 74.83, 74.53, 74.45, 74.20, 72.54, 72.12, 71.80, 71.40, 69.61, 69.25, 69.03, 68.42, 67.97, 67.73, 67.13, 62.23, 60.70, 60.62, 59.49, 54.83, 54.79, 54.62, 51.33, 50.01, 39.27, 21.92, 21.83, 21.70. ESIMS m/z calcd for $[M+H]^+$: 1496.5250. Found, 1496.5208.

3.10.4. 2-Azidoethyl 5-*N*-acetyl-α-neuraminyl-(2→3)-β-D-galactopyranosyl-(1→4)-[α-L-fucopyranosyl-(1→3)]-2-acetamido-2-deoxy-β-D-glucopyranoside (21). Selected ^1H NMR (600 MHz, D_2O): δ 5.11 (d, 1H, $J = 4$ Hz, Fucα1–3, H-1), 4.61 (d, 1H, $J = 8$ Hz, GlcNAc, H-1), 4.53 (d, 1H, $J = 8$ Hz, Gal, H-1), 2.76 (dd, 1H, $J = 4$ Hz, NeuAc, H-3eq), 2.034 (s, 3H, $NHCOCH_3$), 1.79 (t, 1H, $J = 12$ Hz, NeuAc, H-3ax), 1.17 (d, 3H, Fuc, H-6); Selected ^{13}C NMR (600 MHz, D_2O): δ 174.69, 174.09, 116.97, 115.04, 101.30, 100.53, 99.35, 98.32, 75.30, 74.97, 74.57, 74.51, 73.01, 72.57, 71.55, 68.92, 68.86, 68.38, 67.97, 67.77, 67.35, 66.97, 66.36, 62.26, 61.15, 59.27, 55.36, 51.37, 50.04, 39.44, 21.98, 21.70, 14.94. ESIMS m/z calcd for $[M+Na]^+$: 890.3366. Found: 890.3352.

3.10.5. 2-Azidoethyl 5-*N*-acetyl-α-neuraminyl-(2→3)-β-D-galactopyranosyl-(1→4)-[α-L-fucopyranosyl-(1→3)]-2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→3)-β-D-galactopyranosyl-(1→4)-[α-L-fucopyranosyl-(1→3)]-2-acetamido-2-deoxy-β-D-glucopyranoside (22). Selected ^1H NMR (600 MHz, D_2O): δ 5.12 (d, 1H, $J = 4$ Hz, Fuc2, H-1), 5.10 (d, $J = 4$ Hz, Fuc1, H-1), 4.70 (d, 1H, $J = 8$ Hz, GlcNAc2, H-1), 4.61 (d, 1H, $J = 8$ Hz, GlcNAc1, H-1), 4.53 (d, 1H, Gal2, H-1), 4.43 (d, 1H, Gal1, H-1), 2.76 (dd, 1H, 4Hz, NeuAc, H-3eq) 2.033 (3H, s, $NHCOCH_3$), 2.016 (3H, s, $NHCOCH_3$), 1.85 (t, 1H, $J = 12$ Hz, NeuAc, H-3ax), 1.17–1.14 (dd, 6H, 2Fuc, H-6); Selected ^{13}C NMR (600 MHz, D_2O): δ 102.20, 101.46, 101.20, 100.47, 98.43, 98.22, 94.98, 92.28, 84.86, 81.30, 76.87, 75.32, 75.04, 74.64, 74.59, 74.31, 74.12, 72.73, 72.67, 72.57, 71.52, 70.16, 69.25, 68.92, 68.83, 68.37, 67.96, 67.89, 67.76, 67.37, 67.29, 66.97, 66.73, 66.38, 66.32, 62.25, 61.16, 59.39, 56.81, 55.61, 55.36, 51.35, 50.04, 39.45, 35.20, 34.49, 28.63, 24.60, 21.97, 21.90, 21.70, 14.93. ESIMS m/z calcd for $[M+Na]^+$: 1423.5086. Found, 1423.5070.

3.10.6. 2-Azidoethyl 5-*N*-acetyl-α-neuraminyl-(2→3)-β-D-galactopyranosyl-(1→4)-[α-L-fucopyranosyl-(1→3)]-2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→3)-β-D-galactopyranosyl-(1→4)-[α-L-fucopyranosyl-(1→3)]-2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→3)-β-D-galactopyranosyl-(1→4)-[α-L-fucopyranosyl-(1→3)]-2-acetamido-2-deoxy-β-D-glucopyranoside (23). Selected ^1H NMR (600 MHz, D_2O): δ 5.12 (dd, 2H, $J = 4$ Hz, Fuc3 H-1, Fuc2, H-1), 5.10 (d, $J = 4$ Hz, Fuc1, H-1), 4.70 (d, 2H, $J = 8$ Hz, GlcNAc2, GlcNAc3, H-1), 4.60 (d, 1H, $J = 8$ Hz, GlcNAc1, H-1), 4.52 (d, 1H, Gal3, H-1), 4.44 (dd, 2H, Gal1, Gal2, H-1), 2.76 (dd, 1H, $J = 4$ Hz, NeuAc, H-3eq), 2.03 (3H, s, $NHCOCH_3$), 2.015 (9H, s, $3NHCOCH_3$), 1.85 (t, 1H, $J = 12$ Hz, NeuAc, H-3ax), 1.15 (m, 9H, 3Fuc, H-6). ESIMS m/z calcd for $[M+Na]^+$: 1934.6987. Found: 1934.6972.

3.10.7. 2-Azidoethyl 5-*N*-acetyl-α-neuraminyl-(2→6)-β-D-galactopyranosyl-(1→4)-2-acetamido-2-deoxy-b-D-glucopyranosyl-(1→3)-β-D-galactopyranosyl-(1→4)-[α-L-fucopyranosyl-(1→3)]-2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→3)-β-D-galactopyranosyl-(1→4)-[α-L-fucopyranosyl-(1→3)]-2-acetamido-2-deoxy-β-D-glucopyranoside (24). Selected ^1H NMR (600 MHz, D_2O): δ 5.12 (d, 1H, $J = 4$ Hz, Fucα1–3, H-1), 5.10 (d, 1H, $J = 4$ Hz, Fucα1–3, H-1), 4.70 (dd, 2H, $J = 8$ Hz, GlcNAc2, GlcNAc3, H-1), 4.61 (d, 1H, $J = 8$ Hz, GlcNAc1, H-1), 4.46–4.43 (m, 3H, Gal1, Gal2, Gal3, H-1), 2.67 (dd, 1H, $J = 4$ Hz, NeuAc, H-3eq), 2.047–2.016 (12H, m,

$NHCOCH_3$), 1.72 (t, 1H, $J = 12$ Hz, NeuAc, H-3ax), 1.17–1.16 (m, 6H, 2Fuc, H-6); Selected ^{13}C NMR (600 MHz, D_2O): δ 161.99, 159.42, 157.94, 156.28, 135.52, 112.04, 111.78, 108.20, 102.20, 101.47, 101.37, 98.44, 98.39, 98.36, 81.26, 82.08, 75.13, 75.04, 74.91, 74.43, 74.11, 73.89, 73.37, 72.73, 72.40, 72.20, 72.08, 71.88, 71.51, 71.38, 70.39, 70.17, 69.25, 69.12, 68.85, 68.38, 68.02, 67.90, 67.30, 66.38, 63.02, 62.69, 62.32, 61.14, 60.94, 60.02, 59.79, 59.39, 59.26, 55.20, 54.55, 51.54, 50.04, 34.42, 34.31, 21.90, 21.69, 15.74, 15.69, 15.66, 15.60, 15.58, 14.95, 13.31. ESI-MS m/z calcd for $[M - H]^-$: 1765. Found: 1765.

3.10.8. 2-Azidoethyl β-D-galactopyranosyl-(1→4)-2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→3)-β-D-galactopyranosyl-(1→4)-[α-L-fucopyranosyl-(1→3)]-2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→3)-β-D-galactopyranosyl-(1→4)-[α-L-fucopyranosyl-(1→3)]-2-acetamido-2-deoxy-β-D-glucopyranoside (25). Compound **24** (305 mg, 0.17 mmol) was dissolved in sodium phosphate buffer (50 mM, 5 mL, pH 6.0). Roche neuraminidase (1 U) was added, and the reaction was stirred at 37 °C overnight. Complete de-sialylation was observed by TLC (6:3:3:2, EtOAc/HOAc/CH_3OH/H_2O). The reaction was centrifuged and the supernatant was loaded on a Sephadex G25 column (1.6 × 170 cm). The collected fractions were freeze-dried to give **25** (0.22 g, 0.13 mmol, 75%). Selected ^1H NMR (600 MHz, D_2O): δ 5.12 (d, 1H, $J = 4$ Hz, Fucα1–3, H-1), 5.10 (d, 1H, $J = 4$ Hz, Fucα1–3, H-1), 4.70 (d, 2H, $J = 8$ Hz, GlcNAc2, GlcNAc3, H-1), 4.61 (d, 1H, $J = 8$ Hz, GlcNAc1, H-1), 4.48 (d, 1H, Gal3, H-1), 4.45–4.42 (m, 2H, Gal1, Gal2, H-1), 2.033–2.016 (9H, m, $NHCOCH_3$), 1.17–1.14 (m, 6H, Fuc, H-6); Selected ^{13}C NMR (600 MHz, D_2O): δ 102.53, 102.34, 101.47, 101.37, 100.48, 98.42, 98.34, 82.58, 81.28, 79.17, 77.86, 75.12, 75.08, 74.72, 74.18, 74.10, 72.44, 72.17, 71.79, 71.51, 70.63, 70.16, 69.25, 68.84, 68.35, 68.21, 67.91, 67.30, 66.34, 61.11, 60.68, 60.62, 59.41, 59.30, 58.08, 55.62, 55.36, 54.83, 50.04, 48.35, 21.98, 21.90, 21.83, 15.74, 15.69, 14.93. ESIMS m/z calcd for $[M+Na]^+$: 1497.5455. Found: 1497.5430.

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Supplementary data

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