Supporting Information

A Bacterial β1–3-Galactosyltransferase Enables Multigram-Scale Synthesis of Human Milk Lacto-*N*-tetraose (LNT) and Its Fucosides

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†These authors contributed similarly to this work.

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A)

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Yiβ3GalT  MGLIESINKMNNKNRNPLVSVILPVYNSEKFLTEALLSIIEQLYSNIEIIIINDGSTDNS  60
EcWbgO    MI-ID--EAESAESTHPVVSVILPVNKKNPFLDEAINSILSQTFSSFEIIIVANCCTDDF  57
Seβ3GalT  -M-LT--EVRAVSTTKPLVSVILPVNKFNPYLDRAIHSILSQSYPSIELIIIANNCTNDF  56
Yiβ3GalT  ------------MINNPLVSVVIPVNKHNPFLKEAIESIQNQSYSNIEIILIANNCSDCF  48
Cvβ3GalT  --------MDTIMIKRPLVSVILPVNKNNPHLEEAIQSIKNQTYKELELIIIANNCEDNF  52
                      .*:***::** : : .* .*: ** .* : .:*:*:: : . :

Yiβ3GalT  LEIIKYFQKRDHRIKIISRENRGLVSSLNEGISNAEGDFIARMDADDISAPDRIYKQLNY  120
EcWbgO    YNELKHKV--NDKIKLIRTNIAYLPYSLNKAIDLSNGEFIARMDSDDISHPDRFTKQVDF  115
Seβ3GalT  FDALKKRE--CETIKVLRTNIAYLPYCLNKGLDLCNGDFVARMDSDDISHPERIDRQVDF  114
Yiβ3GalT  FNSLQEFS--NEKTKLIRTDLSFLPFSLNLGIHIANGEFIARMDSDDIADVDRIAKQVVY  106
Cvβ3GalT  YSLLLKYQ--DQKTKIIRTSIKYLPFSLNLGVHLSQGEYIARMDSDDISVLDRIEKQVKR  110
           . :       .  *::  .   *  .** .:  .:*:::****:***:  :*: :*:

Yiβ3GalT  ILANPSIDILGSNIEYINDSSEKIGESKYPLSNLQIRKTLPFWCCLAHPTILAKKSVFVA  180
EcWbgO    LKNNPYVDVVGTNAIFIDDKGREINKTKLPEENLDIVKNLPYKCCIVHPSVMFRKKVIAS  175
Seβ3GalT  LINNPDIDVVGTNAVYIDEDDVELEKSNLPENNNAIKKMLPYKCCLVHPSVMFRKNVVIS  174
Yiβ3GalT  MRAHSDVAVVGSNVKFIDERGIITGMSDYPISHRNIKRRMRYNCCVAHPSVMMRRDVIVK  166
Cvβ3GalT  FLNTPELSILGSNVEYINEASESIGYSNYPLDHSSIVNSFPFRCNLAHPTIMVKKEVITT  170
          :     : ::*:*  :*:: .     :. * .:  * . : : * :.**::: ::.*.

Yiβ3GalT  HEQYKSKYRSEDYELWLRLRRNKKIQFGNMRESLLKYRLHGSQMTDVKNLKLIVKSNLSL  240
EcWbgO    IGGYMFSNYSEDYELWNRLSLAK-IKFQNLPEYLFYYRLHEGQSTAKKNLYMVMVNDLVI  234
Seβ3GalT  SGGYMFANYSEDYELWNRLAVEG-RTFYNLSEYLLYYRLHNNQSTSKNNLFMVMVNDVAI  233
Yiβ3GalT  AGGYMYGSLSEDYDLWLRLLQDKNVVFHNINEPLLQYRIHANQATGKNNLYKIFIYDLCL  226
Cvβ3GalT  LGGYMYGSLSEDYDLWIRASRHGNFKFSNIDEPLLKYRIHKGQATNKSNAYNIFAFDSSL  230
             *     ****:** *        * *: * *: **:* .* *  .*   :.  :  :

Yiβ3GalT  KMRELVLTRDIVFLLGMLRDIISLVYSKVKTFL   273
EcWbgO    KMKCFFLTGNINYLFGGIRTIASFIYCKYIK--   265
Seβ3GalT  KVKYFLLTKKVSYLLGIIRTVFSVFYCKYIK--   264
Yiβ3GalT  KLRFFLLYPNVFYFFGCIRGFLSYIYCRYIKK-   258
Cvβ3GalT  KIREFLLNGNVQYLLGAARGFFAFLYVRFIKK-   262
          *:: :.*  .: :::*  * . : .* :  .
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B)

	EcWbgO	**Cvβ3GalT**	**Ppβ3GalT**	**Seβ3GalT**	**Yiβ3GalT**
EcWbgO	-	45.38	49.22	63.26	41.13
Cvβ3GalT	45.38	-	53.49	44.23	42.75
Ppβ3GalT	49.22	53.49	-	46.48	39.15
Seβ3GalT	63.26	44.23	46.48	-	37.12
Yiβ3GalT	41.13	42.75	39.15	37.12	-

Figure S1. Multiple sequence alignment of EcWbgO and four of its analogs (**A**) and the corresponding sequence identity matrix (**B**).

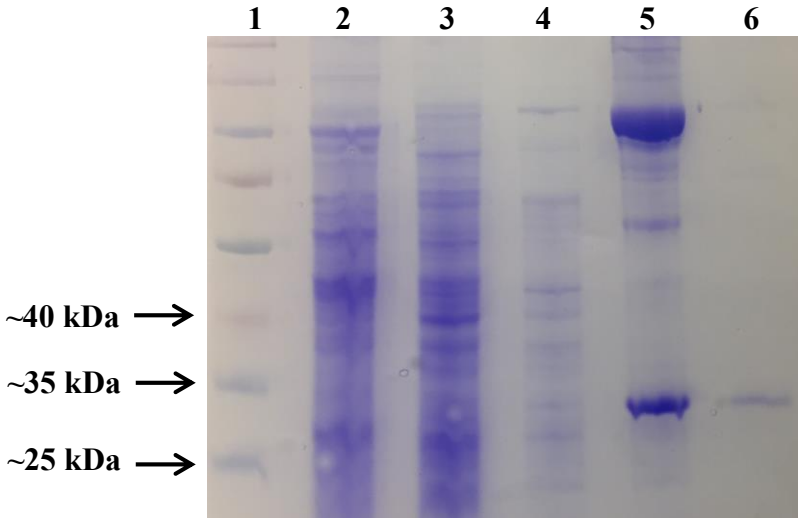

Figure S2. SDS-PAGE analysis of Cvβ3GalT (theoretical MW 30 kDa). Lanes: 1, protein markers (Thermo Scientific Spectra multicolor broad range protein ladder); 2, whole cell extraction before induction; 3, whole cell extraction after induction; 4, cell lysate; 5, Ni^{2+}-column-purified Cvβ3GalT; and 6, purified Cvβ3GalT after Ni^{2+}-column and size exclusion column chromatography.

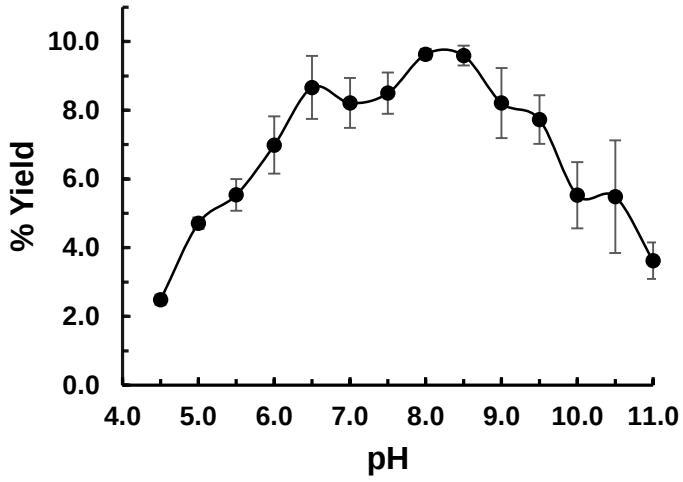

Figure S3. pH profile of Cvβ3GalT activity using LNT II-βProNHFmoc as the acceptor. Buffers used were: MES, pH 4.5–6.5; Tris-HCl, pH 7.0–9.0; CAPS, pH 9.5–11.0.

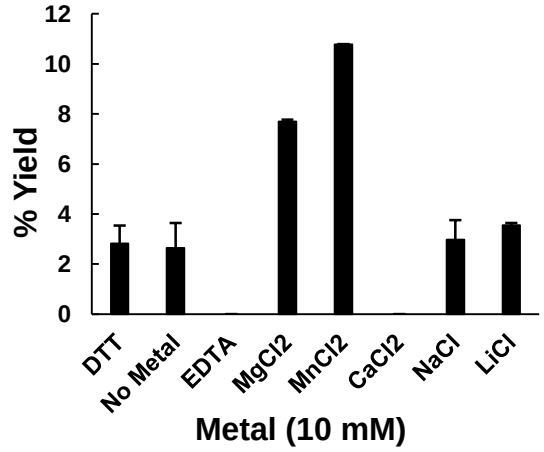

Figure S4. Effects of metal ions, EDTA, and DTT on the activity of Cvβ3GalT.

Experimental section

Bacterial strains, plasmids, and materials

Chemically competent *E. coli* DH5α and BL21 (DE3) cells were purchased from Invitrogen (Carlsbad, CA, USA). Vector pET22b(+) was purchased from EMD Millipore (Billerica, MA, USA). Nickel-nitrilotriacetic acid agarose (Ni^{2+}-NTA agarose) was from Qiagen (Valencia, CA, USA).

Cloning

The gene of Cvβ3GalT optimized for *E. coli* expression was synthesized by Invitrogen and cloned into pET-22b(+) at the NdeI and SalI restriction sites using the following primers: 5'-AGATAACATATGGACACCATCATGATTAAACGTCCGCTGGTTAGCGTTATTCTGCCGG-3' (Cvβ3GalT_F) and 5'-ATGAAGGTCGACTTTTTTGATGAAGCGCACATACAGAAATGCAAAAAAACCACGTGCTGC ACCCAGC-3' (Cvβ3GalT_R). Polymer chain reactions (PCRs) were performed using a two-step procedure with Phusion DNA Polymerase. Cycling conditions were 30 second initial denaturation at 98 °C, thirty-five cycles of 10 seconds at 98 °C, and 45 seconds at 72 °C. The reaction was ended with a 10-minute final extension at 72 °C. Double digested PCR product and vector were ligated with T4 DNA ligase and transformed into chemically competent DH5α cells. The plasmid was isolated by miniprep (Qiagen, CA) and transformed into chemically competent BL21(DE3) cells.

Cvβ3GalT Synthetic DNA sequence

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ATGGACACCATCATGATTAAACGTCCGCTGGTTAGCGTTATTCTGCCGGTGAATAAAAACAATCCGCA
TCTGGAAGAAGCAATCCAGAGCATTAAAAACCAGACCTATAAAGAGCTGGAACTGATCATTATTGCCA
ACAACTGCGAGGATAACTTTTATAGCCTGCTGCTGAAATATCAGGACCAGAAAACCAAAATTATCCGC
ACCAGCATCAAATATCTGCCGTTTAGCCTGAATCTGGGTGTTCATCTGAGCCAGGGTGAATATATTGC
ACGTATGGATTCAGATGATATCAGCGTTCTGGATCGCATTGAAAAACAGGTTAAACGCTTTCTGAATA
CACCGGAACTGAGCATTCTGGGTAGCAATGTTGAATATATCAATGAAGCCAGCGAAAGCATTGGCTAT
AGCAACTATCCGCTGGATCATAGCAGCATTGTTAATAGCTTTCCGTTTCGTTGTAATCTGGCACATCC
GACCATTATGGTTAAAAAAGAAGTGATTACCACGCTTGGTGGCTATATGTATGGTAGCCTGAGCGAAG
ATTATGATCTGTGGATTCGTGCAAGCCGTCATGGCAATTTCAAATTTAGCAATATTGATGAACCGCTG
CTGAAGTACCGTATTCATAAAGGTCAGGCAACCAATAAAAGCAACGCCTATAACATCTTTGCCTTTGA
TAGCAGCCTGAAAATCCGTGAATTTCTGCTGAATGGTAATGTGCAGTATCTGCTGGGTGCAGCACGTG
GTTTTTTTGCATTTCTGTATGTGCGCTTCATCAAAAAA
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Cvβ3GalT Protein sequence

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MDTIMIKRPLVSVILPVNKNNPHLEEAIQSIKNQTYKELELIIIANNCEDNFYSLLLKYQDQKTKIIR
TSIKYLPFSLNLGVHLSQGEYIARMDSDDISVLDRIEKQVKRFLNTPELSILGSNVEYINEASESIGY
SNYPLDHSSIVNSFPFRCNLAHPTIMVKKEVITTLGGYMYGSLSEDYDLWIRASRHGNFKFSNIDEPL
LKYRIHKGQATNKSNAYNIFAFDSSLKIREFLLNGNVQYLLGAARGFFAFLYVRFIKK
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Overexpression and purification

Flasks (4 L) containing 1 L of autoclaved LB media supplemented with ampicillin (0.1 mg mL^{-1}) were inoculated with 1 mL of overnight cultured *E. coli* BL21(DE3) cells harboring the plasmid. The 1 L cultures were grown at 37 °C until $OD_{600\ nm}$ reached 0.6 to 1.0, then expression was induced with isopropyl β-D-1-thiogalactoside (IPTG) with a final concentration of 1 mM and the cells were shaken at 20 °C and 250 rpm for overnight (20 h). Cells were harvested by centrifugation at 5000 × g for 20 minutes, resuspended in 20 mL of Tris-HCl (pH 7.5, 100 mM) and lysed by sonication with the following method: amplitude at 65%, 10 s pulse on and 30 s pulse off for 18 cycles. The lysate was collected after centrifugation at 8000 pm for 30 minutes and then loaded onto a Ni^{2+}-NTA affinity column at 4 °C that

was pre-equilibrated with 6 column volumes of binding buffer (50 mM Tris-HCl buffer, pH 7.5, 10 mM imidazole, 0.5 M NaCl). The column was washed with 10 column volumes of binding buffer and 10 column volumes of washing buffer (50 mM of Tris-HCl buffer, pH 7.5, 50 mM of imidazole, 0.5 M of NaCl) sequentially to wash away the nonspecific binding protein. The target protein was eluted using Tris-HCl buffer (50 mM, pH 7.5) containing 200 mM imidazole and 0.5 M NaCl. Fractions containing the purified protein were combined. For storage at -20 °C, 50% glycerol was added to a final concentration of 10% glycerol.

For further purification, Ni^{2+}-column-purified protein was subjected to a size exclusion column chromatography using a NGC Quest 10 Chromatography System (Bio-Rad) with an ENrich SEC 650 10 × 300 column (Bio-Rad). Tris-HCl buffer (50 mM, pH 7.5) supplemented with NaCl (200 mM) was used as the eluent at a constant flow rate of 1 mL min^{-1}.

Bioreactor overexpression

The bioreactor (Eppendorf BioFlo 120 with a 5 L Fermentation Vessel) contained 5 L of autoclaved LB media supplemented with ampicillin (0.1 mg mL^{-1}) and Antifoam 204 (Sigma, 200 μL). It was inoculated with 100 mL of overnight culture of *E. coli* BL21(DE3) cells harboring the plasmid. The cells were grown at 37 °C and 500 rpm agitation with air flow at 5 SPLM and its pH maintained between 6.9 and 7.1 until $OD_{600\ nm}$ reached 1.0. The expression was induced with IPTG to a final concentration of 1 mM and the cells were further cultured at 20 °C for overnight (20 h). Purification was performed in the same manner as described for the shake flask expression.

pH Profiles

Reactions were performed in duplicate at 37 °C for 30 minutes in a buffer (100 mM) selected from (MES for pH 4.5–6.5; Tris-HCl for pH 7.0–9.0; or CAPS for pH 9.5–11.0) containing $MnCl_2$ (10 mM), LNT II-βProNHFmoc (0.5 mM), and UDP-Gal (2 mM). Reactions were stopped by heat denaturation at 70 °C for 10 minutes. The mixtures were incubated at 4 °C and centrifuged at 13,000 rpm for 5 minutes. Supernatants were analyzed using an Infinity 1290-II HPLC equipped with a ZORBAX Eclipse Plus C18 Rapid Resolution HD column (1.8 μm particle, 2.1 × 50 mm, Agilent Technologies, CA) and a UV-Vis detector (Agilent Technologies, CA) monitored at $A_{315\ nm}$. An isocratic flow of 0.5 mL min^{-1} for a 30% acetonitrile and 70% aqueous solution containing 0.1% TFA was used for elution.

Metal ion effect assays

Reactions were performed in duplicate at 37 °C for 1 h in Tris-HCl buffer (100 mM, pH 7.0) containing GlcNAcβ*p*NP (5 mM), and UDP-Gal (15 mM) in the presence or the absence of dithiothreitol (DTT, 10 mM), ethylenediaminetetraacetic acid (EDTA, 10 mM), or a metal salt (10 mM) selected from $MgCl_2$, $MnCl_2$, $CaCl_2$, NaCl, or LiCl. Reactions were stopped by heat denaturation at 70 °C for 10 minutes. The mixtures were incubated at 4 °C and centrifuged at 13,000 rpm for 5 minutes. Supernatants were analyzed similarly as described above for pH profile studies except for a different eluant (an isocratic flow of 0.5 mL min^{-1} for a 20% acetonitrile and 80% aqueous solution containing 0.1% TFA) was used.

Kinetics

Reactions were performed in duplicate at 37 °C for 30 minutes in Tris-HCl buffer (100 mM, pH 7.5) containing $MnCl_2$ (10 mM), Cvβ3GalT (9.4 nM for variable LNT II-βProNHFmoc, 94 nM for variable UDP-Gal), a fixed concentration of UDP-Gal (5 mM) or LNT II-βProNHFmoc (2 mM) with varying concentrations (0.2, 0.5, 1.0, 2.0, and 5.0 mM) of LNT II-βProNHFmoc or UDP-Gal, respectively. Reactions were stopped by heat denaturation at 70 °C for 10 minutes. The mixtures were incubated at 4 °C and centrifuged at 13,000 rpm for 5 minutes. The reactions were analyzed similarly as described above for pH profile studies. The apparent kinetic parameters were obtained by fitting the experimental

data into the Michaelis-Menten equation using Grafit 5.0.

General methods for synthesis

Chemicals were purchased and used without further purification. ^{1}H NMR (800 MHz) and ^{13}C NMR (200 MHz) spectra were recorded on a Bruker Avance-800 NMR spectrometer. High resolution electrospray ionization (ESI) mass spectra were obtained using Thermo Electron LTQ-Orbitrap Hybrid MS at the Mass Spectrometry Facility in the University of California, Davis. Silica gel 60 Å (230–400 mesh, Sorbent Technologies) was used for flash column chromatography. Thin-layer chromatography (TLC, Sorbent Technologies) was performed on silica gel plates using anisaldehyde sugar stain for detection. Gel filtration chromatography was performed with a column (100 cm × 2.5 cm) packed with Bio-Gel P-2 Fine resins (Bio-Rad, Hercules, California, USA). D-Galactose (Gal) and D-lactose (Lac) were purchased from Fisher Scientific (Pittsburgh, Pennsylvania, USA. D-GalNAc was from Carbosynth US. L-Fucose (Fuc) was from V-LABS (Covington, USA). Neu5Ac were bought from NingBo Hongxiang Bio-Chem (Ningbo, China). ATP, UTP, CTP, and GTP were bought from Hangzhou Meiya Pharmacy (Hangzhou, China). Recombinant enzymes Recombinant enzymes *Bifidobacterium longum* strain ATCC55813 *N*-acetylhexosamine-1-kinase (BLNahK),[1] *Pasteurella multocida N*-acetylglucosamine uridylyltransferase (PmGlmU),[2] *Pasteurella multocida* inorganic pyrophosphatase (PmPpA),[3] *Neisseria meningitidis* β1–3-*N*-acetylglucosaminyltransferase (NmLgtA),[4] *Streptococcus pneumoniae TIGR4* galactokinase (SpGalK),[5] *Bifidobacterium longum* UDP-sugar pyrophosphorylase (BLUSP),[6] *Neisseria meningitidis* CMP-sialic acid synthetase (NmCSS),[7] *Pasteurella multocida* α2–3-sialyltransferase 1 M144D mutant (PmST1 M144D),[8] *Bacteroides fragilis* strain NCTC9343 bifunctional L-fucokinase/GDP-fucose pyrophosphorylase (BfFKP),[9] *Helicobacter pylori* α1–3/4-fucosyltransferase (Hp3/4FT),[10] *Thermosynechococcus elongates* α1–2-fucosyltransferase (Te2FT),[11] and *Escherichia coli* O126 α1–2-fucosyltransferase (EcWbgL)[12] were expressed and purified as described previously.

Synthesis of LNT II GlcNAcβ3Galβ4Glc

A reaction mixture (70 mL) in Tris-HCl buffer (100 mM, pH 8.0) containing lactose (1.0 g, 2.92 mmol), GlcNAc (0.775 g, 3.51 mmol), ATP (2.09 g, 3.80 mmol), UTP (2.01 g, 3.80 mmol), $MgCl_2$ (20 mM), BLNahK (9.0 mg), PmGlmU (8.0 mg), PmPpA (4.0 mg), and NmLgtA (5.0 mg) was incubated in an incubator shaker at 30 °C for 72 h with shaking (100 rpm). The reaction progress was monitored by thin-layer chromatography (TLC) (*i*-PrOH:H_2O:$NH_3{\cdot}H_2O$ = 7:2:1, by volume, detected with *p*-anisaldehyde sugar stain) and mass spectrometry (MS). Upon completion, pre-cooled ethanol (70 mL) was added and the mixture was incubated at 4 °C for 1 h. After centrifugation (4300 × g), the supernatant was concentrated and passed through a Bio-Gel P-2 gel filtration column (2.5 cm × 80 cm, water was used as an eluant). The fractions containing the product were collected, concentrated, and further purified by the Bio-Gel P-2 column again to obtain trisaccharide LNT II (1.54 g, 97%). ^{1}H NMR (800 MHz, D_2O) δ 5.16 (d, *J* = 4.0 Hz, 0.4H), 4.62 (d, *J* = 8.0 Hz, 0.4H), 4.61 (d, *J* = 8.0 Hz, 0.6H), 4.60 (d, *J* = 8.0 Hz, 0.6H), 4.38 (d, *J* = 8.0 Hz, 1H), 4.09 (d, *J* = 3.2 Hz, 1H), 3.91–3.19 (m, 17H), 1.98 (s, 3H). ^{13}C NMR (200 MHz, D_2O) δ 174.80, 102.75, 102.72, 95.57, 91.64, 81.78, 81.76, 78.12, 78.02, 75.48, 74.72, 74.63, 74.17, 73.60, 73.37, 71.23, 70.94, 69.95, 69.86, 69.83, 69.48, 68.21, 68.18, 62.08, 60.81, 60.28, 59.87, 59.74, 55.47, 21.98. HRMS (ESI) m/z calculated for $C_{20}H_{35}NNaO_{16}$ $[M+Na]^+$ 568.1854, found 568.1864.

Synthesis of LNT (1) Galβ3GlcNAcβ3Galβ4Glc

A reaction mixture (8 mL) in Tris-HCl buffer (100 mM, pH 8.0) containing LNT II (100 mg, 0.18 mmol), galactose (43 mg, 0.24 mmol), ATP (132 mg, 0.24 mmol), UTP (0.127 g, 0.24 mmol), $MgCl_2$ (20 mM), SpGalK (2 mg), BLUSP (2 mg), PmPpA (2 mg), and Cvβ3GalT (1.5 mg) was incubated in an incubator

shaker at 37 °C for 30 h with shaking (100 rpm). The product formation was monitored by TLC (*n*-PrOH:H_2O:$NH_3{\cdot}H_2O$ = 5:2:1, by volume, detected with *p*-anisaldehyde sugar stain) and mass spectrometry (MS). When an optimal yield was achieved, pre-cooled ethanol (80 mL) was added and the mixture was incubated at 4 °C for 30 min. The precipitates were removed by centrifugation and the supernatant was concentrated (4300 × g) and purified by a Bio-Gel P-2 gel column (2.5 cm × 80 cm, water was the eluent) to obtain LNT (0.13 g, 99%). ^{1}H NMR (800 MHz, D_2O) δ 5.22 (d, *J* = 4.0 Hz, 0.3H), 4.74 (d, *J* = 8.0 Hz, 0.3H), 4.73 (d, *J* = 8.0 Hz, 0.7H), 4.67 (d, *J* = 8.0 Hz, 0.7H), 4.44 (d, *J* = 8.0 Hz, 2H), 4.16 (d, *J* = 3.2 Hz, 1H), 3.97–3.27 (m, 23H), 2.03 (s, 3H). ^{13}C NMR (200 MHz, D_2O) δ 174.87, 103.39, 102.83, 102.79, 102.46, 95.64, 91.72, 81.97, 81.89, 81.86, 78.30, 78.20, 75.18, 75.09, 74.80, 74.70, 74.26, 73.69, 72.37, 71.31, 71.04, 70.58, 70.03, 69.94, 69.91, 68.44, 68.36, 68.25, 68.23, 60.94, 60.89, 60.87, 60.41, 59.99, 59.86, 54.61, 22.14. HRMS (ESI) m/z calculated for $C_{26}H_{45}NNaO_{21}$ $[M+Na]^+$ 730.2382, found 730.2397.

Sequential OPME 10-gram-scale synthesis of LNT (1) Galβ3GlcNAcβ3Galβ4Glc

A reaction mixture (500 mL) in Tris-HCl buffer (100 mM, pH 8.0) containing lactose monohydrate (10.0 g, 27.7 mmol, 55.5 mM), GlcNAc (7.4 g, 33.4 mmol), ATP (20.7 g, 37.5 mmol), UTP (19.8 g, 37.5 mmol), $MgCl_2$ (20 mM), BINahK (90.0 mg), PmGlmU (75 mg), NmLgtA (70 mg), and PmPpA (60 mg) was incubated in an incubator shaker at 30 °C with shaking (100 rpm). Additional NmLgtA (30 mg) was added in day 3. The reaction progress was monitored by TLC and MS until the reaction was complete (~7 days). The reaction mixture was then concentrated and used directly for the next step of OPME reaction without purification.

The volume of the above reaction was decreased to 200 mL by blowing air to the container. Galactose (6.5 g, 36.1 mmol), ATP (20.7 g, 37.5 mmol), UTP (19.8 g, 37.5 mmol), SpGalK (80 mg), BLUSP (90 mg), PmPpA (47 mg), Cvβ3GalT (77 mg) were added and the final volume of the mixture was adjusted to 500 mL and the pH of the solution was adjusted to 8.0 using 4 N of NaOH. The reaction mixture was incubated in an incubator shaker at 30 °C for 6 days with shaking (100 rpm). The product formation was monitored by mass spectrometry. When the LNT II was completely consumed, the reaction mixture was incubated in a boiling water bath for 15 minutes. The precipitates were removed by centrifugation at 4300 × g for 20 min and the supernatant was concentrated to 200 mL by blowing air to the container. A solution of 16 mL (1/12.5 of the total sample volume) was loaded to Dowex 1 × 8-200 (formate form) anion exchange column (40 cm × 2.5 cm). The product was eluted with H_2O (250 mL) and monitored by TLC (*i*-PrOH:H_2O:$NH_3{\cdot}H_2O$ = 5:2:1, by volume, detected with *p*-anisaldehyde sugar stain). Product-containing fractions were combined and evaporated under vacuum to produce the crude LNT which was dissolved in H_2O and purified further by a Bio-Gel P-2 fine column (90 cm × 2.5 cm, water as the eluent) to produce 1.56 g of pure LNT (**1**). The remaining of the reaction mixture was purified similarly. The total yield of synthesizing LNT (**1**) from Lac using combined OPME glycosylation reactions was 99.3%.

Synthesis of LNFP II (2) Galβ3(Fucα4)GlcNAcβ3Galβ4Glc

A reaction mixture (30 mL) in Tris-HCl buffer (100 mM, pH 7.5) containing LNT (**1**) (500 mg, 0.71 mmol), L-fucose (128 mg, 0.78 mmol), ATP (467 mg, 0.85 mmol), GTP (481 mg, 0.85 mmol), $MgCl_2$ (20 mM), BfFKP (10 mg), PmPpA (4.5 mg), and Hp3/4FT (5 mg) was incubated at 30 °C for 2 days with shaking (100 rpm). Upon the completion of the reaction, pre-cooled ethanol (30 mL) was added and the mixture was incubated at 4 °C for 1 h. After centrifugation, the supernatant was concentrated and passed through a Bio-Gel P-2 gel filtration column (water was used as an eluant) to give the LNFP II (**2**) (488 mg, 81%). ^{1}H NMR (800 MHz, D_2O) δ 5.20 (d, *J* = 4.0 Hz, 0.4 H), 5.11 (d, *J* = 4.0 Hz, 0.4 H), 5.01 (d, *J* = 4.0 Hz, 0.6 H), 4.88–4.85 (m, 0.6 H), 4.84–4.82 (m, 0.4 H), 4.70–4.68 (m, 1 H), 4.64 (d, *J* = 8.0 Hz, 0.6 H), 4.49 (d, *J* = 8.0 Hz, 0.6 H), 4.44 (d, *J* = 8.0 Hz, 0.4 H), 4.42 (d, *J* = 8.0 Hz, 1 H), 4.14 (d, *J* = 3.2 Hz, 1H), 4.08–3.25 (m, 26 H), 2.02–2.02 (m, 3 H), 1.17–1.16 (m, 3H); ^{13}C NMR (200 MHz,

D_2O) δ 174.65, 174.58, 102.81, 102.78, 102.74, 102.52, 101.65, 98.49, 97.90, 95.61, 91.69, 81.95, 81.93, 78.24, 78.22, 78.14, 78.12, 75.78, 75.09, 74.99, 74.80, 74.76, 74.75, 74.69, 74.68, 74.63, 74.23, 73.66, 72.93, 72.34, 72.19, 72.00, 71.83, 71.79, 71.28, 71.00, 70.93, 70.37, 70.00, 69.87, 69.84, 69.07, 69.01, 68.23, 68.22, 68.19, 67.67, 67.58, 66.73, 66.57, 61.54, 61.39, 60.86, 60.85, 59.95, 59.82, 59.51, 59.47, 59.24, 55.75, 22.17, 22.13, 15.26, 15.19. HRMS (ESI) m/z calculated for $C_{32}H_{55}NNaO_{25}$ $[M+Na]^+$ 876.2961, found 876.2955.

Synthesis of S-LNF II (3) Neu5Acα3Galβ3(Fucα4)GlcNAcβ3Galβ4Glc

A reaction mixture (30 mL) in Tris-HCl buffer (100 mM, pH 8.5) containing LNFP II (**2**) (19 mg, 0.022 mmol, 5 mM), *N*-acetylneuraminic acid (Neu5Ac, 10 mg, 0.033 mmol), CTP (18 mg, 0.033 mmol), $MgCl_2$ (20 mM), NmCSS (0.5 mg), and PmST1 M144D (1 mg) was incubated at 30 °C for 2 days with shaking (100 rpm). The progress was monitored by TLC and MS. Upon the completion of the reaction, pre-cooled ethanol (30 mL) was added and the mixture was incubated at 4 °C for 1 h. After centrifugation, the supernatant was concentrated and passed through a Bio-Gel P-2 gel filtration column (2.5 cm × 80 cm, water was used as an eluant). The fractions containing the product were collected, concentrated, and further purified by HPLC using a C18 column to produce pure S-LNF II (**3**) (21 mg, yield 81%) as a white powder. ^{1}H NMR (800 MHz, D_2O) δ 5.21 (d, *J* = 4.0 Hz, 0.4H), 5.17 (d, *J* = 3.2 Hz, 0.4H), 5.11 (d, *J* = 4.0 Hz, 0.6H), 4.81–4.80 (m, 1H), 4.68 (d, *J* = 8.0 Hz, 0.6H), 4.65 (d, *J* = 8.0 Hz, 0.6H), 4.64 (d, *J* = 8.0 Hz, 0.4H), 4.54 (d, *J* = 8.0 Hz, 0.4H), 4.51 (d, *J* = 8.0 Hz, 0.6H), 4.42 (d, *J* = 8.0 Hz, 0.6H), 4.40 (d, *J* = 8.0 Hz, 0.4H), 4.15–4.06 (m, 2H), 3.97–3.26 (m, 33H), 2.77–2.73 (m, 1H), 2.03 (s, 6H), 1.80–1.76 (m, 1H), 1.17 (d, *J* = 6.4 Hz, 3H). ^{13}C NMR (200 MHz, D_2O) δ 175.00, 174.98, 174.84, 174.66, 173.84, 102.90, 102.50, 101.70, 101.54, 100.68, 99.75, 99.63, 98.55, 98.40, 95.81, 95.70, 82.07, 82.05, 78.34, 78.23, 77.99, 76.97, 75.63, 75.52, 75.45, 75.34, 75.14, 75.00, 74.96, 74.89, 74.86, 74.77, 74.62, 74.49, 74.32, 73.75, 73.01, 72.88, 72.86, 72.69, 72.65, 72.35, 72.23, 72.06, 71.88, 71.83, 71.74, 71.37, 71.09, 70.94, 70.92, 70.09, 69.96, 69.94, 69.36, 69.24, 69.18, 69.15, 68.32, 68.28, 68.23, 68.07, 68.05, 67.77, 67.68, 67.44, 67.28, 66.63, 66.47, 62.59, 62.56, 62.54, 61.47, 61.01, 60.96, 60.04, 59.91, 59.49, 55.13, 51.67, 51.65, 39.76, 39.60, 22.24, 22.22, 22.13, 22.01, 15.36, 15.25. HRMS (ESI) m/z calculated for $C_{43}H_{71}N_2O_{33}$ $[M-H]^-$ 1143.3945, found 1143.3927.

Synthesis of LNDFH I (4) Fucα2Galβ3(Fucα4)GlcNAcβ3Galβ4Glc

LNFP I (**8**) was synthesized as described previously.[11] For the synthesis of LNDFH I (**4**), a reaction mixture (8 mL) in Tris-HCl buffer (100 mM, pH 7.5) containing LNFP I (**8**) (100 mg, 0.12 mmol), L-fucose (38 mg, 0.23 mmol), ATP (161 mg, 0.30 mmol), GTP (166 mg, 0.30 mmol), $MgCl_2$ (20 mM), BfFKP (2 mg), PmPpA (1.5 mg), and Hp3/4FT (2 mg) was incubated at 30 °C for 2 days with shaking (100 rpm). Upon the completion of the reaction as monitored by TLC and MS, pre-cooled ethanol (8 mL) was added and the mixture was incubated at 4 °C for 1 h. After centrifugation (4300 × g), the supernatant was concentrated and passed through a Bio-Gel P-2 gel filtration column (2.5 cm × 80 cm, water was used as an eluant) to produce pure LNDFH I (**4**) (107 mg, 92%). ^{1}H NMR (800 MHz, D_2O) δ 5.21 (d, *J* = 4.0 Hz, 0.4H), 5.14 (d, *J* = 4.0 Hz, 1H), 5.01 (d, *J* = 4.0 Hz, 1H), 4.88–4.84 (m, 2H), 4.65 (d, *J* = 8.0 Hz, 1.6H), 4.60 (d, *J* = 8.0 Hz, 1H), 4.41 (d, *J* = 8.0 Hz, 1 H), 4.33 (dd, *J* = 6.4 and 13.6 Hz, 1H), 4.14–4.11 (m, 2H), 3.94–3.25 (m, 27H), 2.05 (s, 3H), 1.26 (d, *J* = 7.2 Hz, 3H), 1.25 (d, *J* = 6.4 Hz, 3H); ^{13}C NMR (200 MHz, D_2O) δ 174.09, 103.12, 102.86, 102.83, 100.52, 100.49, 99.46, 99.43, 97.66, 95.63, 95.60, 91.71, 91.68, 81.43, 78.13, 78.02, 76.38, 75.04, 74.70, 74.61, 74.35, 74.21, 73.67, 73.51, 71.91, 71.86, 71.66, 71.01, 70.06, 69.32, 69.00, 68.65, 68.60, 68.52, 68.17, 67.71, 66.93, 66.90, 66.14, 66.11, 61.48, 61.34, 60.87, 59.23, 59.21, 59.19, 55.65, 22.09, 15.26, 15.21. HRMS (ESI) m/z calculated for $C_{38}H_{65}NNaO_{29}$ $[M+Na]^+$ 1022.3540, found 1022.3577.

Synthesis of Fuc-LNT II (10) GlcNAcβ3Galβ4(Fucα3)Glc

A reaction mixture (20 mL) in Tris-HCl buffer (100 mM, pH 7.5) containing trisaccharide LNT II (300 mg, 0.55 mmol), L-fucose (135 mg, 0.82 mmol), ATP (485 mg, 0.88 mmol), and GTP (499 mg, 0.88 mmol) were dissolved $MgCl_2$ (20 mM), FKP (6.0 mg), Hp3/4FT (5.5 mg), and PmPpA (4.0 mg) was incubated in an incubator shaker at 30 °C for 48 h with shaking (100 rpm). The product formation was monitored by TLC (*n*-PrOH:H_2O:$NH_3{\cdot}H_2O$ = 4:2:1, by volume, detected with *p*-anisaldehyde sugar stain) and mass spectrometry (MS). Upon the completion of the reaction, pre-cooled ethanol (10 mL) was added and the mixture was incubated at 4 °C for 1 h. The precipitates were removed by centrifugation (4300 × g) and the supernatant was concentrated and purified by a Bio-Gel P-2 gel filtration column (2.5 cm × 80 cm, water was used as an eluant) to produce Fuc-LNT II (**10**) (373 mg, 98%). ^{1}H NMR (800 MHz, D_2O) δ 5.42 (d, *J* = 4.0 Hz, 0.6 H), 5.36 (d, *J* = 4.0 Hz, 0.4 H), 5.17 (d, *J* = 4.0 Hz, 0.4 H), 4.82–4.78 (m, 1 H), 4.68 (d, *J* = 8.8 Hz, 0.4 H), 4.67 (d, *J* = 8.8 Hz, 0.6 H), 4.64 (d, *J* = 8.0 Hz, 0.6 H), 4.41 (d, *J* = 8.0 Hz, 1 H), 4.08 (dd, *J* = 4.0 Hz, 1H), 3.96–3.67 (m, 14 H), 3.59–3.42 (m, 6 H), 2.02 (s, 3 H), 1.16–1.14 (m, 3H); ^{13}C NMR (200 MHz, D_2O) δ 174.80, 102.68, 101.61, 98.44, 98.33, 95.72, 92.01, 81.35, 81.27, 76.89, 75.51, 75.42, 75.26, 74.58, 74.39, 74.38, 73.38, 72.56, 72.26, 72.16, 71.82, 71.80, 70.83, 70.61, 70.56, 69.59, 69.58, 69.16, 69.10, 68.19, 68.15, 67.92, 67.89, 66.37, 66.33, 61.38, 61.35, 60.35, 59.69, 59.61, 55.51, 22.03, 15.11, 15.10. HRMS (ESI) m/z calculated for $C_{26}H_{45}NNaO_{20}$ $[M+Na]^+$ 714.2433, found 714.2430.

Synthesis of LNFP V* (5) *Galβ3GlcNAcβ3Galβ4(Fucα3)Glc

A reaction mixture (15 mL) in Tris-HCl buffer (100 mM, pH 8.0) containing tetrasaccharide Fuc-LNT II (**10**) (200 mg, 0.29 mmol), Gal (78 mg, 0.43 mmol), ATP (287 mg, 0.52 mmol), UTP (0.274 g, 0.52 mmol), $MgCl_2$ (20 mM), SpGalK (3.5 mg), BLUSP (3.0 mg), PmPpA (2.5 mg), and Cvβ3GalT (3.0 mg) was incubated in an incubator shaker at 30 °C for 48 h with shaking (100 rpm). The product formation was monitored by TLC (*n*-PrOH:H_2O:$NH_3{\cdot}H_2O$ = 4:2:1, by volume, detected with *p*-anisaldehyde sugar stain) and MS. Upon completion of the reaction, pre-cooled ethanol (70 mL) was added and the mixture was incubated at 4 °C for 1 h. After centrifugation (4300 × g), the supernatant was concentrated and passed through a Bio-Gel P-2 gel filtration column (2.5 cm × 80 cm, water was used as an eluant) to produce pentasaccharide LNFP V (**5**) (242 mg, 98%). ^{1}H NMR (800 MHz, D_2O) δ 5.49 (d, *J* = 4.0 Hz, 0.6 H), 5.43 (d, *J* = 4.0 Hz, 0.4 H), 5.24 (d, *J* = 4.0 Hz, 0.4 H), 4.88–4.83 (m, 1 H), 4.77 (d, *J* = 8.0 Hz, 1 H), 4.70 (d, *J* = 8.0 Hz, 0.6 H), 4.50 (d, *J* = 8.0 Hz, 1 H), 4.48 (d, *J* = 8.0 Hz, 1 H), 4.15 (d, *J* = 4.0 Hz, 1H), 4.03–3.75 (m, 19 H), 3.70 (dd, *J* = 4.0 and 10.4 Hz, 1H), 3.66–3.51 (m, 6 H), 2.07 (s, 3 H), 1.23–1.20 (m, 3H); ^{13}C NMR (200 MHz, D_2O) δ 174.88, 103.41, 102.43, 101.68, 98.52, 98.40, 95.81, 92.08, 82.01, 81.48, 81.41, 77.01, 75.52, 75.35, 75.24, 75.14, 74.70, 74.47, 74.46, 72.66, 72.44, 72.40, 72.31, 71.91, 71.89, 70.94, 70.69, 70.65, 69.26, 69.20, 68.50, 68.44, 68.43, 68.22, 68.19, 68.02, 67.99, 66.44, 66.40, 61.43, 61.40, 60.98, 60.49, 59.80, 59.72, 54.66, 22.19, 15.18, 15.17. HRMS (ESI) m/z calculated for $C_{32}H_{55}NNaO_{25}$ $[M+Na]^+$ 876.2961, found 876.2970.

Synthesis of DiFuc-LNT (6) Fucα2Galβ3GlcNAcβ3Galβ4(Fucα3)Glc

A reaction mixture (5 mL) in Tris-HCl buffer (100 mM, pH 7.5) containing LNFP V (**5**) (100 mg, 0.12 mmol), L-fucose (29 mg, 0.17 mmol), ATP (103 mg, 0.19 mmol), GTP (106 mg, 0.19 mmol), $MgCl_2$ (20 mM), BfFKP (1.0 mg), PmPpA (0.8 mg), and EcWbgL (0.3 mg) was incubated in an incubator shaker at 30 °C with shaking (100 rpm) until the reaction was completed as detected by TLC and MS. Pre-cooled ethanol (5 mL) was added and the mixture was incubated at 4 °C for 1 h. After centrifugation 4300 × g), the supernatant was concentrated and passed through a Bio-Gel P-2 gel filtration column (2.5 cm × 80 cm, water was used as an eluant) to produce hexasaccharide DiFuc-LNT (**6**) (116 mg, 99%). ^{1}H NMR (800 MHz, D_2O) δ 5.40 (d, *J* = 4.0 Hz, 0.6 H), 5.35 (d, *J* = 4.0 Hz, 0.4 H), 5.17 (d, *J* = 4.0 Hz, 1 H), 5.16 (d, *J* = 4.0 Hz, 0.4 H), 4.83–4.79 (m, 2 H), 4.63 (d, *J* = 8.0 Hz, 1.6 H), 4.61 (d, *J* = 8.8 Hz, 0.5 H), 4.60 (d, *J* = 8.8 Hz, 0.5 H), 4.39 (d, *J* = 8.8 Hz, 1 H), 4.29–4.26 (m, 1H), 4.06 (d, *J* = 4.0 Hz, 1 H), 3.97 (t, *J*

= 9.6 Hz, 1 H), 3.95–3.43 (m, 27 H), 2.03 (s, 3 H), 1.22 (d, *J* = 6.4 Hz, 1 H), 1.15–1.13 (m, 3H); ^{13}C NMR (200 MHz, D_2O) δ 174.11, 103.10, 101.58, 100.12, 99.40, 98.50, 98.40, 95.74, 92.02, 81.06, 80.99, 77.02, 76.99, 76.55, 75.39, 75.27, 75.06, 74.93, 74.67, 74.30, 74.29, 73.35, 72.52, 72.05, 71.95, 71.82, 71.80, 71.76, 70.87, 70.85, 70.80, 69.28, 69.15, 69.10, 69.00, 68.43, 68.40, 68.35, 67.88, 67.85, 67.83, 66.36, 66.35, 66.32, 61.39, 61.36, 61.03, 60.25, 59.69, 59.62, 54.92, 22.01, 15.18, 15.16, 15.15. HRMS (ESI) m/z calculated for $C_{38}H_{65}NNaO_{29}$ $[M+Na]^+$ 1022.3540, found 1022.3556.

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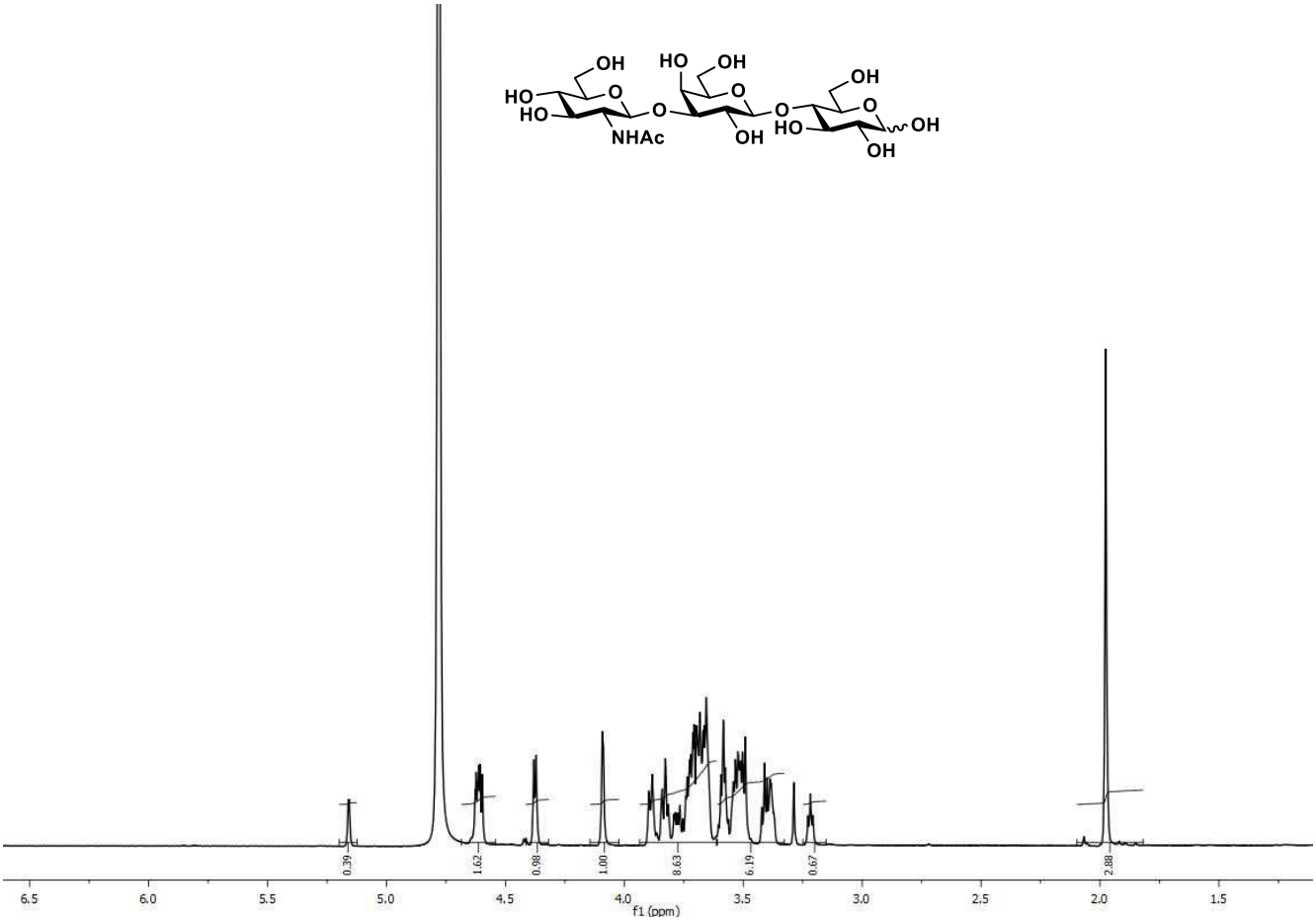
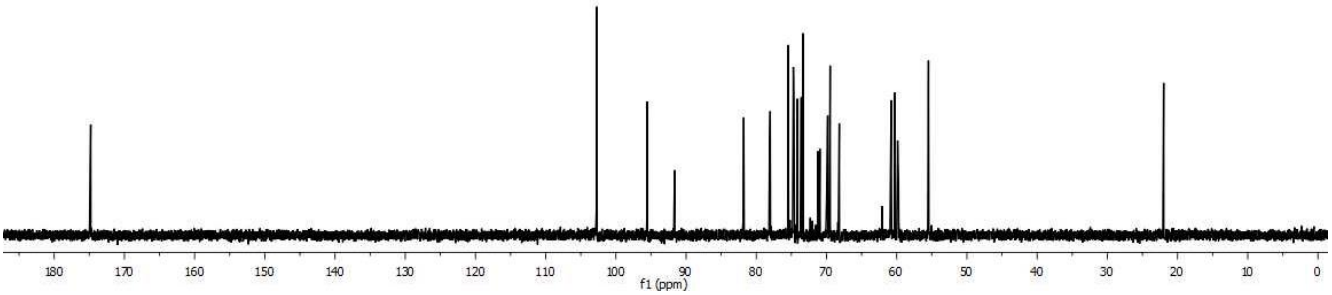
^{1}H and ^{13}C NMR spectra of LNT II (GlcNAcβ3Galβ4Glc)





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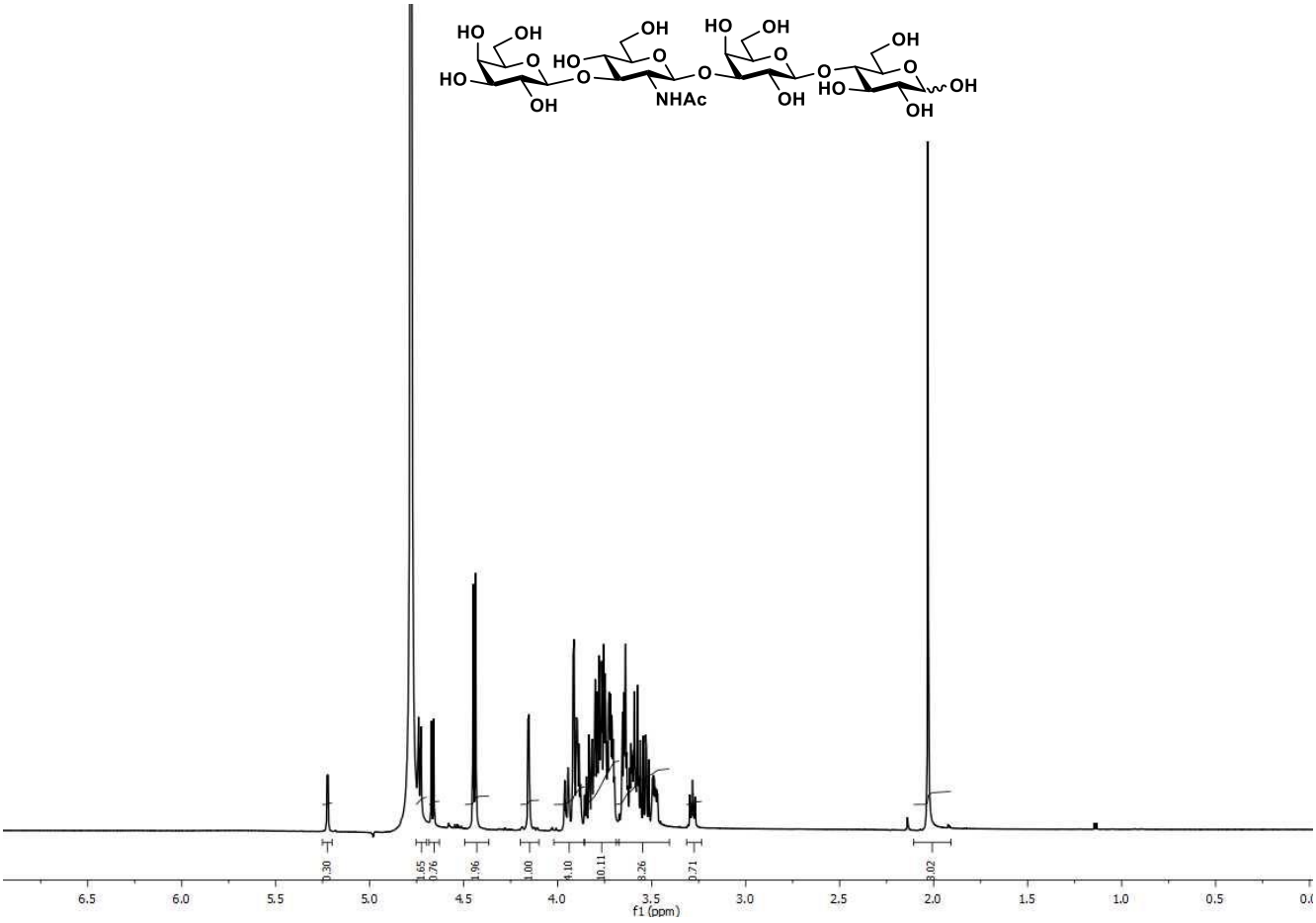

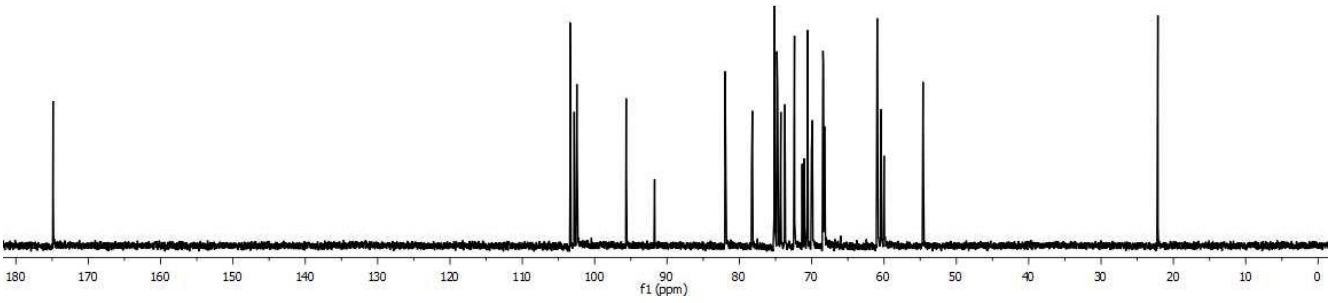

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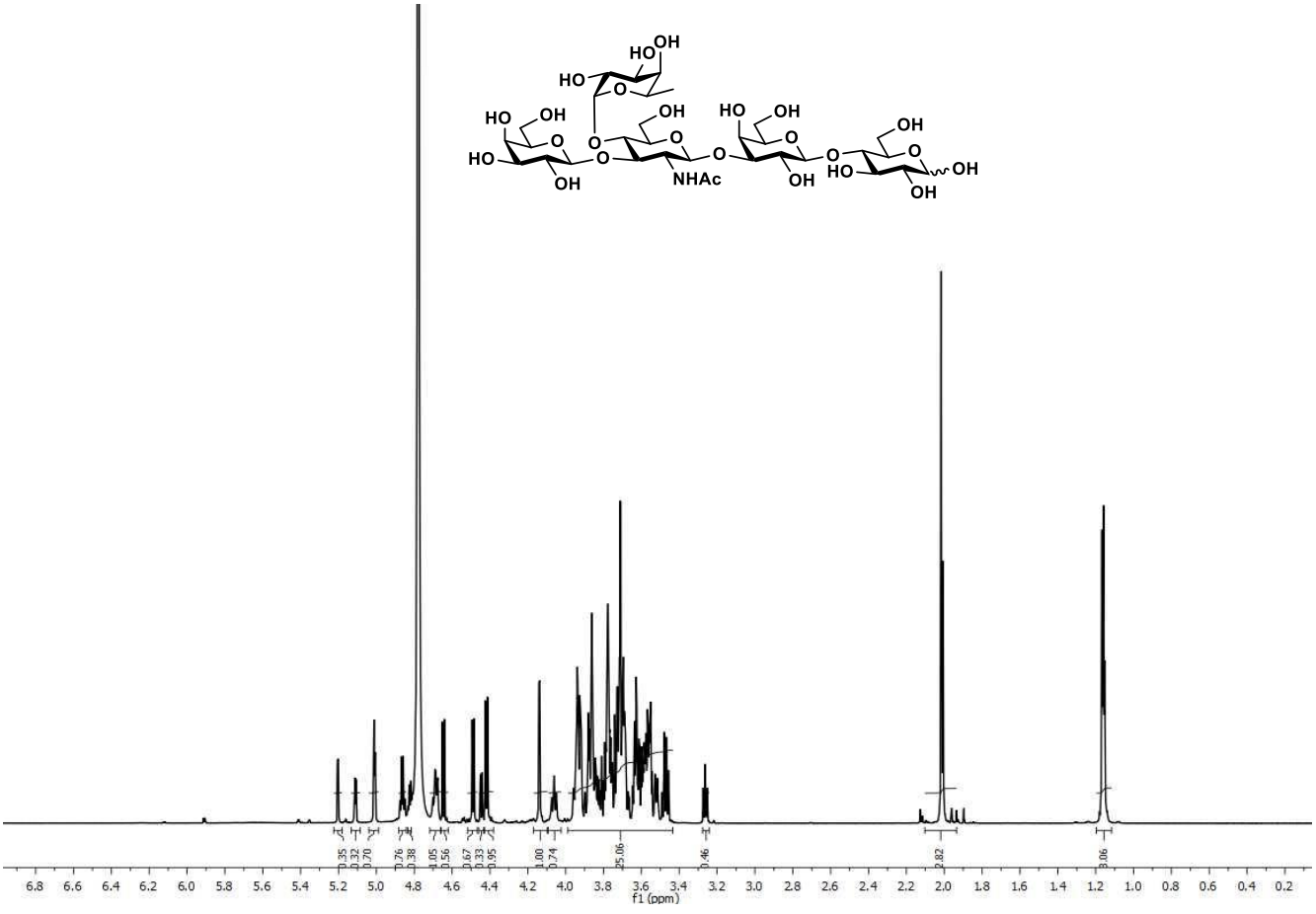

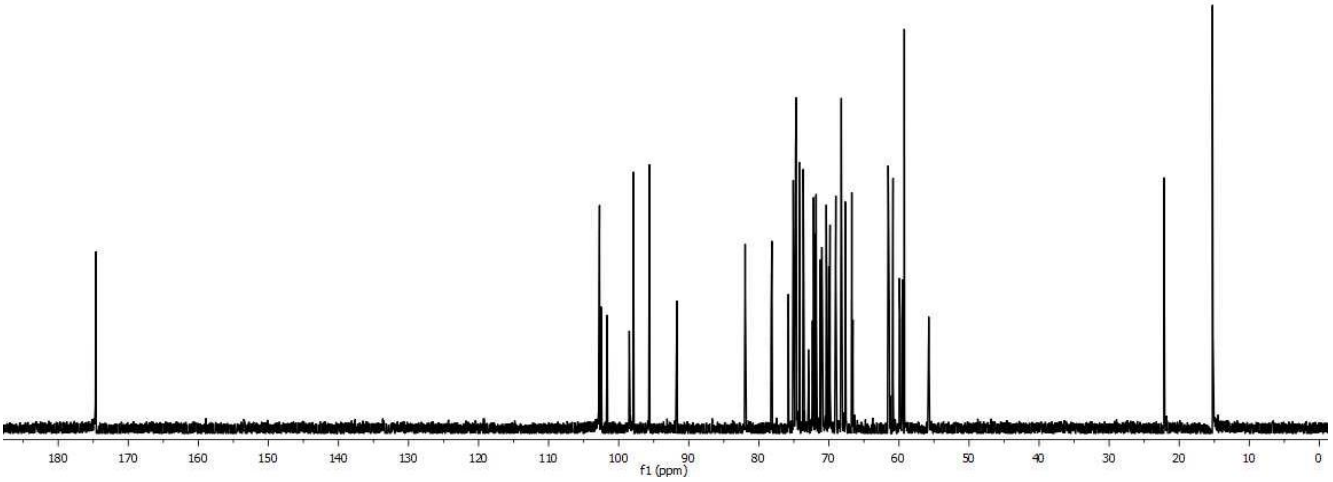

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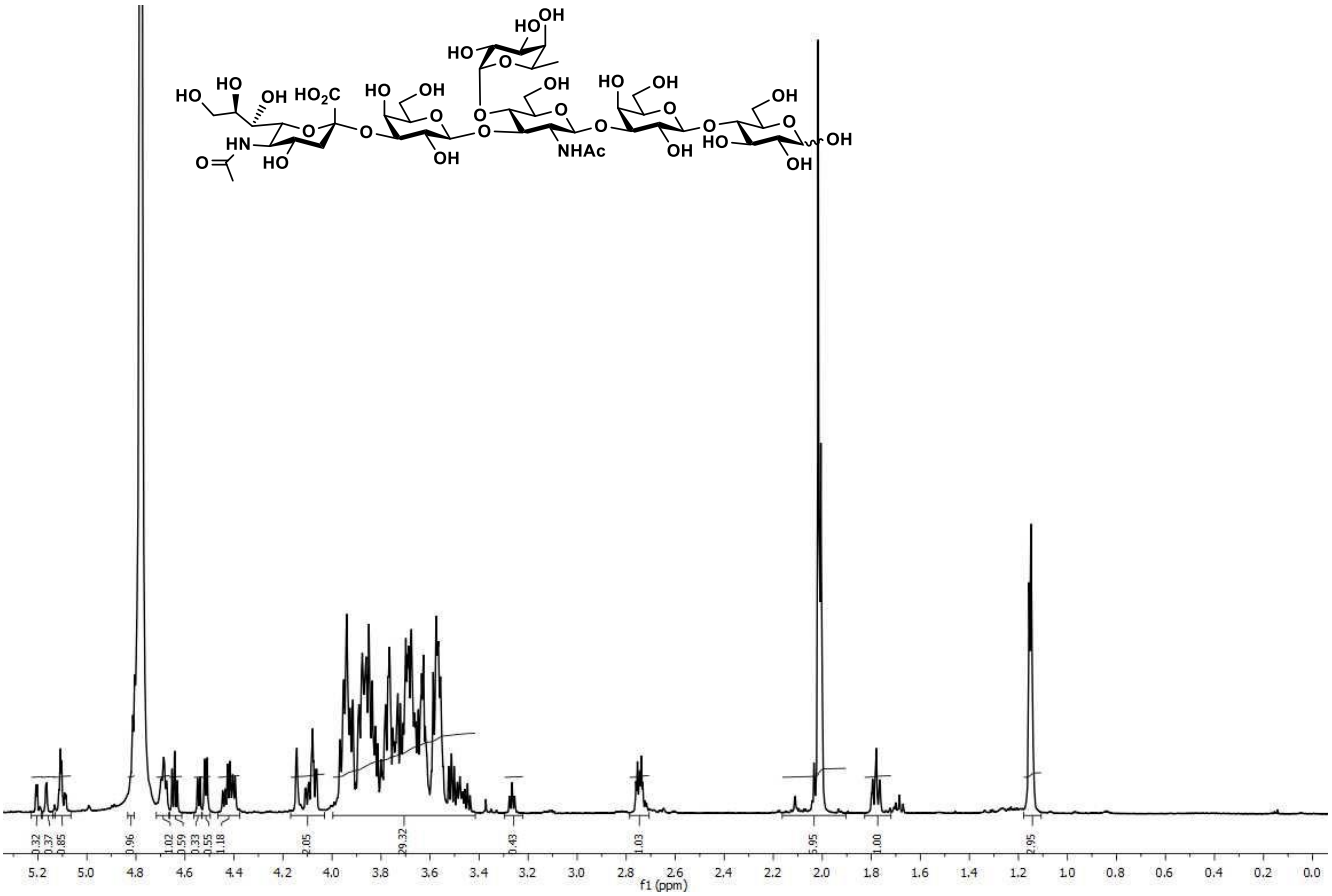
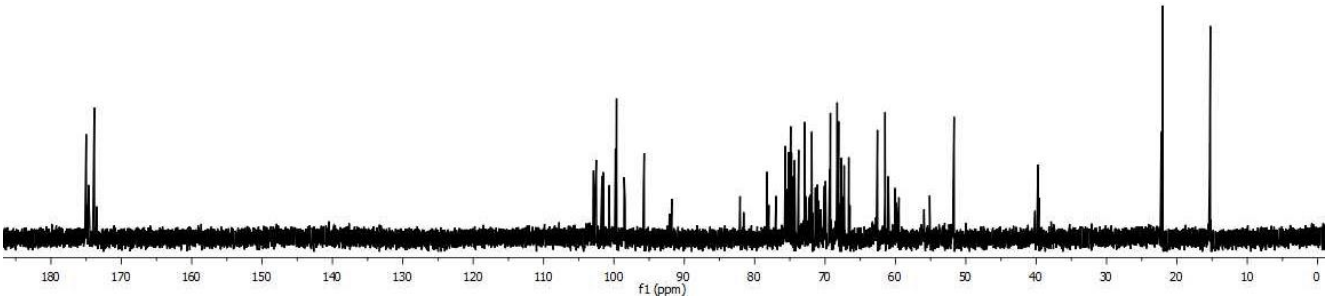
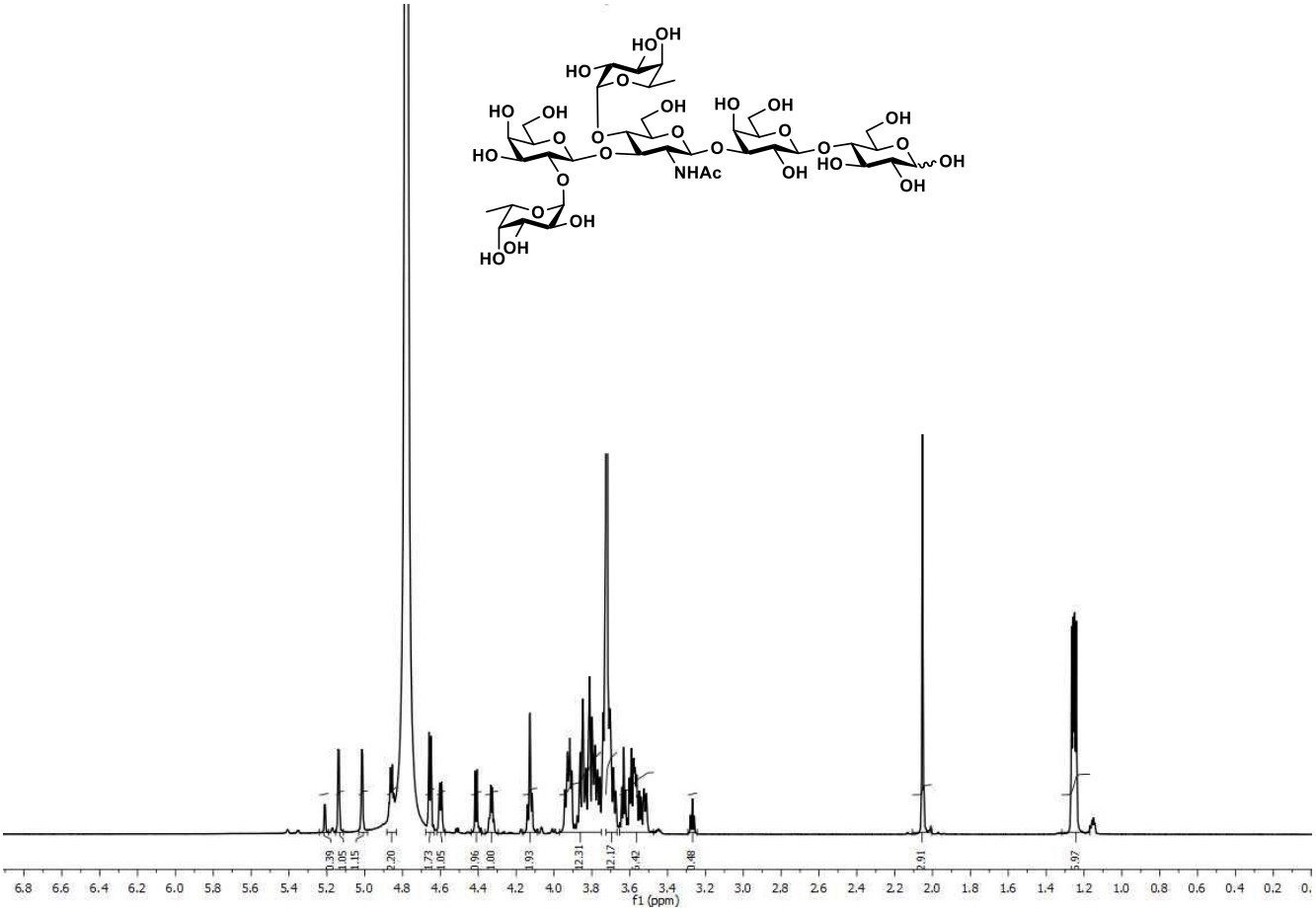
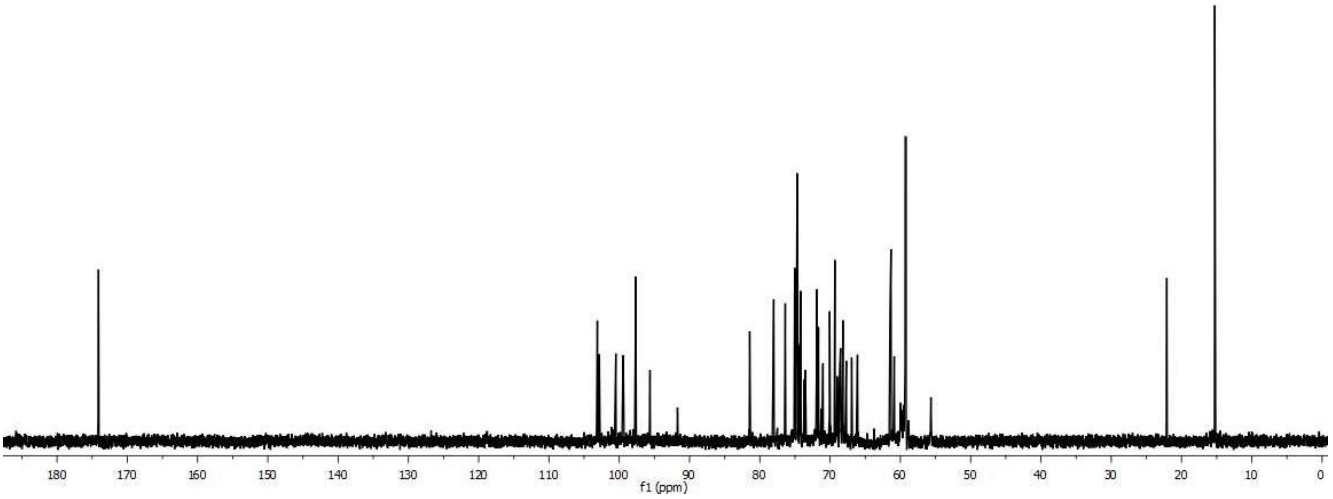


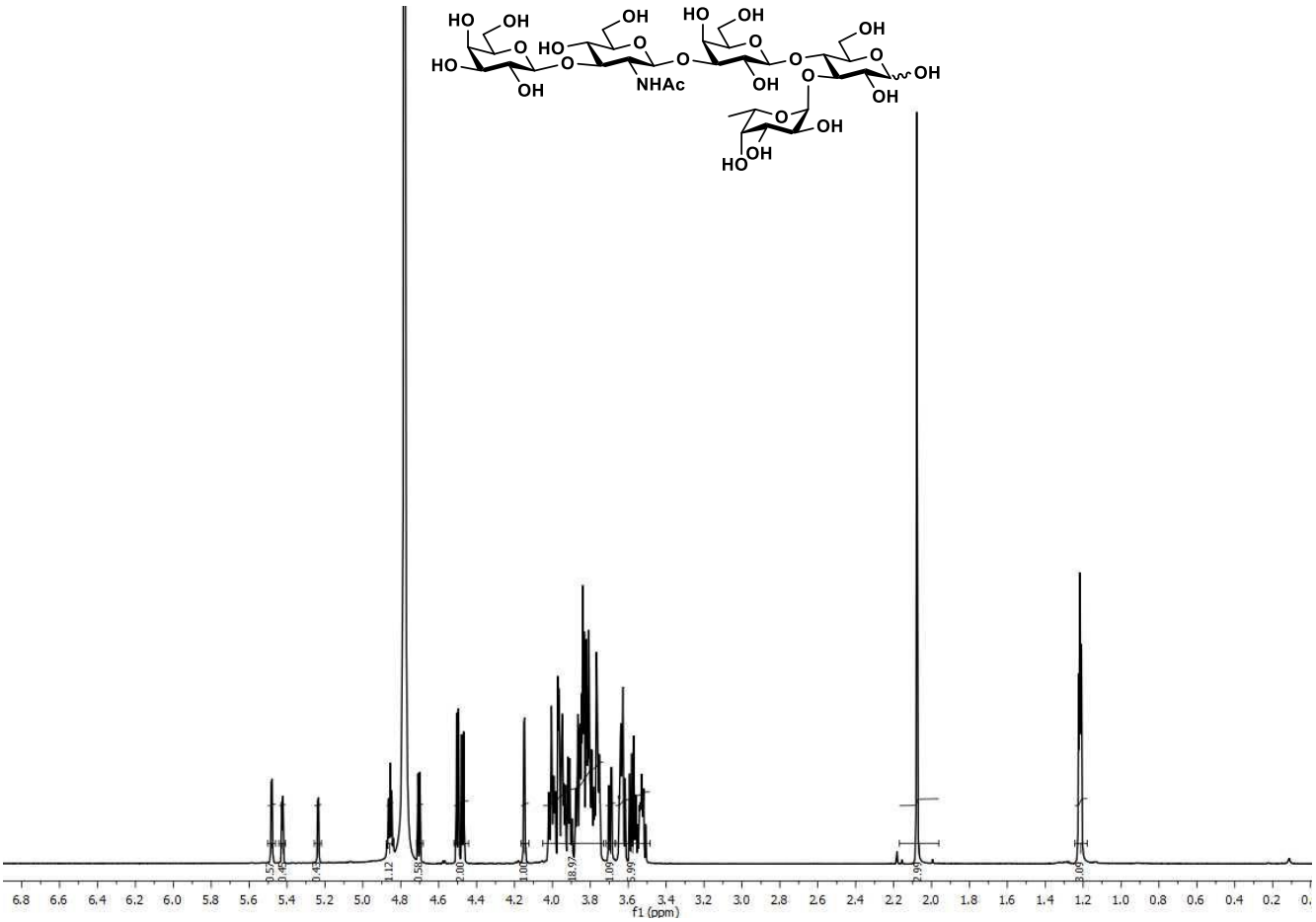


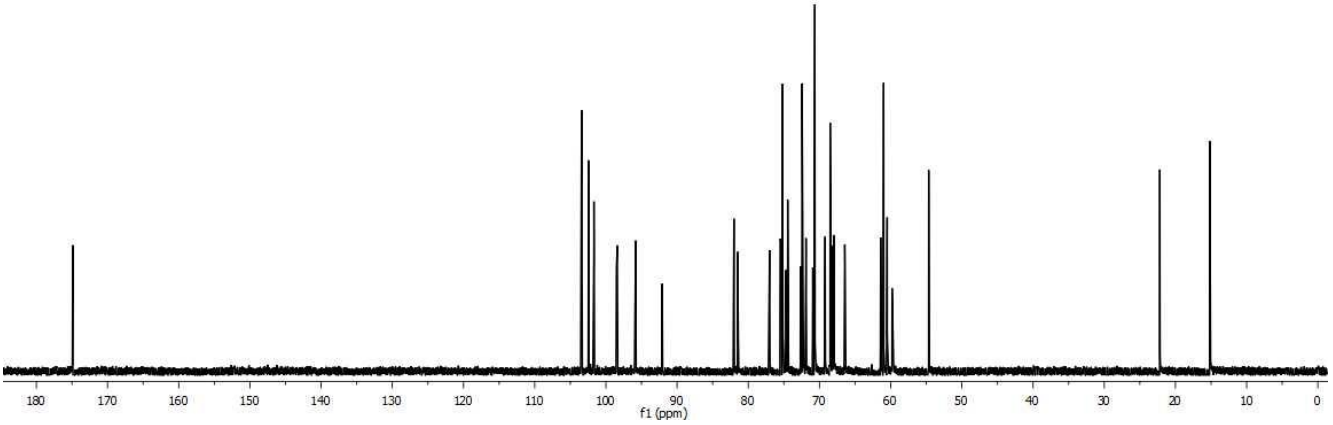
^{1}H and ^{13}C NMR spectra of LNDFH I (**4**) Fucα2Galβ3(Fucα4)GlcNAcβ3Galβ4Glc



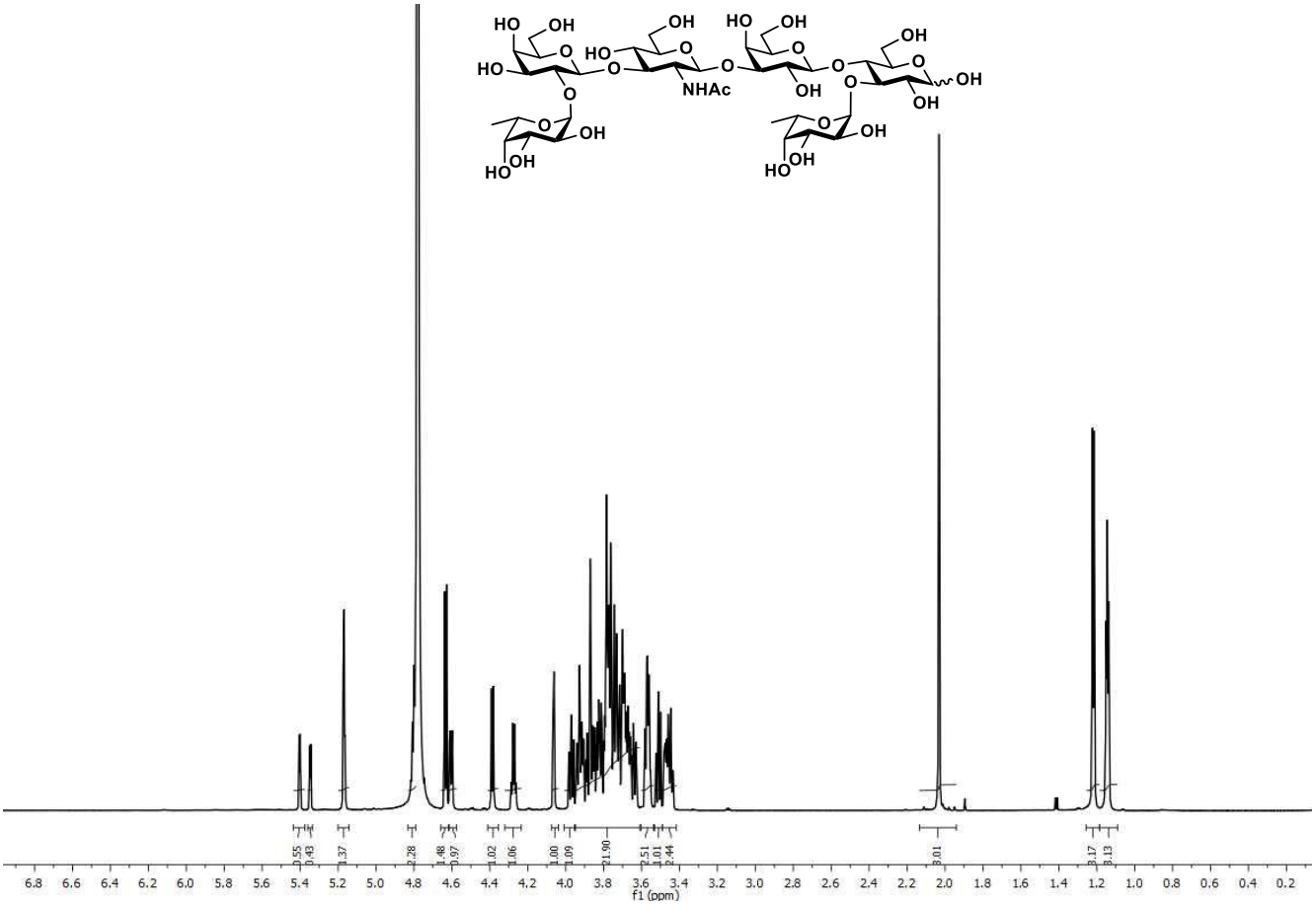


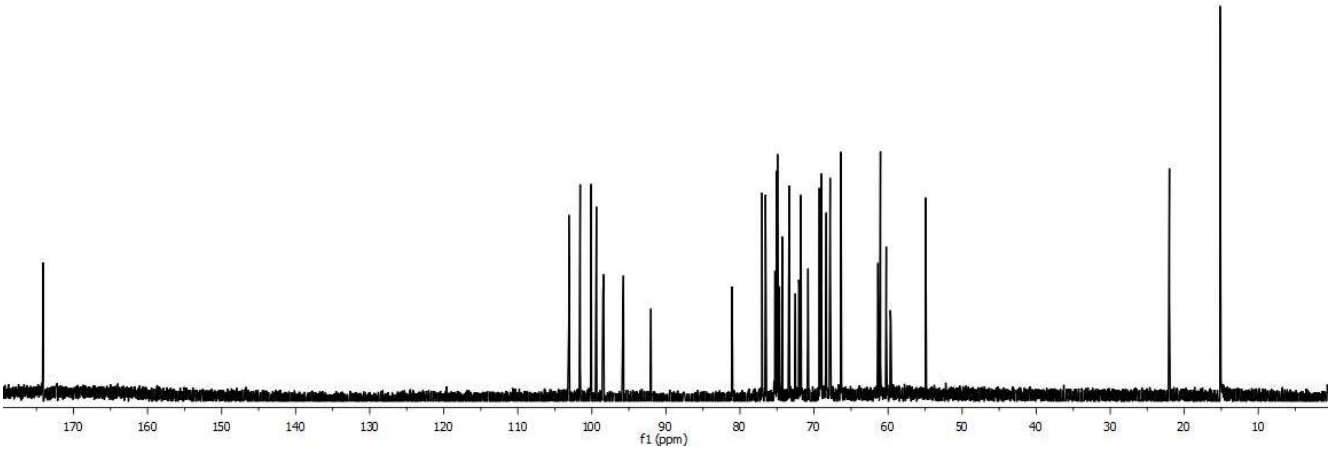
^{1}H and ^{13}C NMR spectra of LNFP V (**5**) Galβ3GlcNAcβ3Galβ4(Fucα3)Glc





^{1}H and ^{13}C NMR spectra of DiFuc-LNT (**6**) Fucα2Galβ3GlcNAcβ3Galβ4(Fucα3)Glc





^{1}H and ^{13}C NMR spectra of Fuc-LNT II (**10**) GlcNAcβ3Galβ4(Fucα3)Glc