Supporting Information

for

Helicobacter pylori α1–3/4-fucosyltransferase (Hp3/4FT)-catalyzed one-pot multienzyme (OPME) synthesis of Lewis antigens and human milk fucosides

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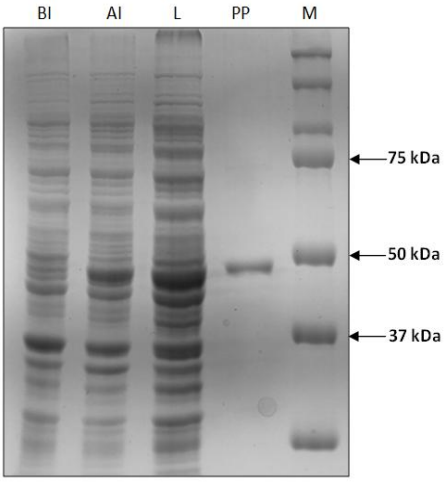

Figure S1. SDS-PAGE analysis of Hp3/4FT. It indicates an apparent molecular weight of 49 kDa for the purified enzyme which is close to the calculated molecular weight (51.5 kDa). Lanes: BI, whole cell extract before induction; AI, whole cell extract after induction; L, lysate after induction; P, Ni^{2+}-NTA column purified protein; M, protein markers (Bio-Rad precision Plus Protein Standards, 10–250 kDa).

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Hp3/4FucT : MFQPLLLAFIDSTHLDET---IHKPPLNVALANWWPLKNSDKKGFRDFIIHFILKQRYKIILLSNPNEPSDLVFGNPLEQA :  78
Hp4FucT   : MFQPLLLAYIDSTRLDET---DYKPPLKIAVANWWGG----VEIFKKSTLYFILSQRYTITLHRNPDKPADIVFGNPLGSA :  74
Hp3FucT_1 : MFQPLLLAYVESASIEKMASKSP-PPLKIAVANWWGDE--EIKEFKNSVLYFILSQRYTITLHQNPNEFSDLVFGNPLGSA :  78
Hp3FucT_2 : MFQPLLLAFIESASIEKMASKSPEPPLKIAVANWWGDE--EIKEFKKSVLYFILSQRYAITLHQNPNEFSDLVFSNPLGAA :  79

Hp3/4FucT : RKILSYQNTKRVFYTGENEVPNFNLFDYAIGFDELDFNDRYLRMPLYYAYLHYKAMLVNDTTSPYKLK--ALYTLKKPSHK : 157
Hp4FucT   : RKILSYQNAKRVFYTGENEVPNFNLFDYAIGFDELDFNDRYLRMPLYYAHLHYEAELVNDTTSPYKIKDNSLYALKKPSHH : 155
Hp3FucT_1 : RKILSYQNAKRVFYTGENESPNFNLFDYAIGFDELDFNDRYLRMPLYYDRLHHKAESVNDTTAPYKLKDNSLYALKKPSHC : 159
Hp3FucT_2 : RKILSYQNTKRVFYTGENESPNFNLFDYAIGFDELDFNDRYLRMPLYYAHLHYKAELVNDTTAPYKLKDNSLYALKKPSHH : 160

Hp3/4FucT : FKENHPNLCALIHNESDPWKRGFASFVASNPNAPIRNAFYDALNAIEPVASGGSVKNTLGYKVKNKNEFLSQYKFNLCFEN : 238
Hp4FucT   : FKENHPNLCAVVNNESDPLKRGFASFVASNPNAPKRNAFYDALNSIEPVTGGGSVKNTLGYNVKNKNEFLSQYKFNLCFEN : 236
Hp3FucT_1 : FKEKHPNLCAVVNDESDPLKRGFASFVASNPNAPIRNAFYDALNSIEPVTGGGSVRNTLGYNVKNKNEFLSQYKFNLCFEN : 240
Hp3FucT_2 : FKENHPNLCAVVNDESDLLKRGFASFVASNANAPMRNAFYDALNSIEPVTGGGSVRNTLGYKVGNKSEFLSQYKFNLCFEN : 241

Hp3/4FucT : SQGYGYVTEKILDAYFSHTIPIYWGSPSVAKDFNPKSFVNVHDFNNFDEAIDYIRYLHAHQNAYLDMLYENPLNTIDGKAG : 319
Hp4FucT   : SQGYGYVTEKILDAYFSHTIPIYWGSPSVAKDFNPKSFVNVHDFNNFDEAIDHVRYLHTHPNAYLDMLYENPLNTLDGKAY : 317
Hp3FucT_1 : TQGYGYVTEKIIDAYFSHTIPIYWGSPSVAKDFNPKSFVNVHDFKNFDEAIDYIKYLHTHKNAYLDMLYENPLNTLDGKAY : 321
Hp3FucT_2 : SQGYGYVTEKILDAYFSHTIPIYWGSPSVAKDFNPKSFVNVHDFNNFDEAIDYIKYLHTHPNAYLDMLYENPLNTLDGKAY : 322

Hp3/4FucT : FYQDLSFEKILDFFKNILENDTIYHCNDAHYSALHRDLNEPLVSVDDLRRDHDDLRVNYDDLRVNYDDLRVNYDDLRVNYD : 400
Hp4FucT   : FYQNLSFKKILDFFKTILENDTIYHCDAHNYSALHRDLNEPLVSIDDLRINYDDLRIN---------------------- : 375
Hp3FucT_1 : FYQNLSFKKILAFFKTILENDTIYHDNP--F-IFCRDLNEPLVTIDDLRVNYDDLRVNYDDLRINYDDLRVNYDDLRINYD : 399
Hp3FucT_2 : FYQDLSFKKILDFFKTILENDTIYHKFSTSF-MWEYDLHKPLVSIDDLR------------------------------- : 370

Hp3/4FucT : DLRVN----------YDDLRRDHDDLRRDHERLLSKAT----------------------------------- : 428
Hp4FucT   : ----------YDDLRINYDDLRINYERLLQNASPLLELSQNTSFKIYRKAYQKSLPLLRAIRRWVKK--- : 432
Hp3FucT_1 : DLRVNYDDLRVNYDDLRINYDDLRVNYDDLRVNYERLLSKATPLLELSQNTTSKIYRKAYQKSLPLLRAIRRWVKKLGL : 478
Hp3FucT_2 : ----------VNYDDLRVNYDRLLQNASPLLELSQNTTFKIYRKAYQKSLPLLRAVRKLVKKLGL : 425
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Figure S2. Protein sequence alignment of Hp3/4FT and other fucosyltransferases from *H. pylori*. GenBank accession number: Hp4FT (GenBank accession number AAR88243.1, 82% identity); Hp3FT_1, (GenBank accession number O30511.1, 80% identity); Hp3FT_2 (GenBank accession number WP_000487428.1, 79% identity).

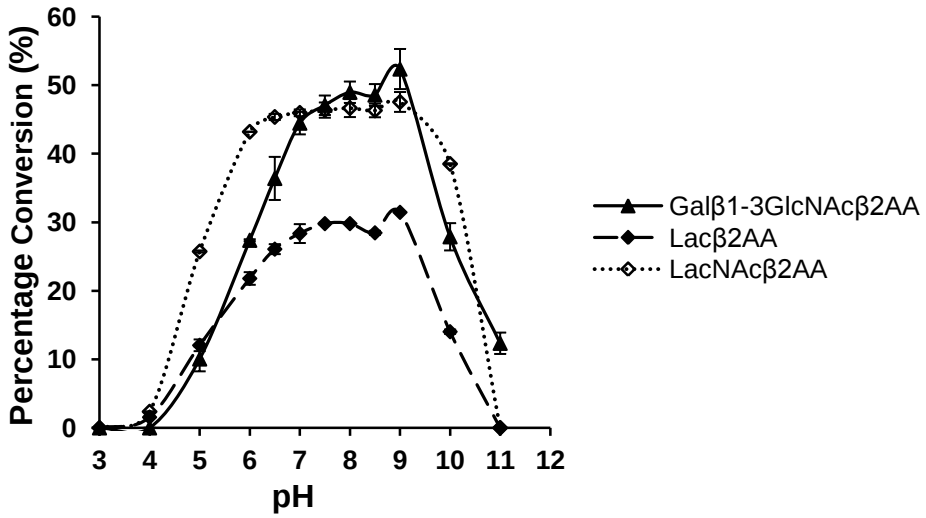

Figure S3. pH profiles of Hp3/4FT using Galβ1–3GlcNAcβPro2AA (filled triangle with solid line), LacβPro2AA (filled diamond with dashed line), or LacNAcβPro2AA (open diamond with dotted line) as the acceptor. Buffers used were: citric acid-Na_2HPO_4, pH 3.0–4.0; MES, pH 5.0–6.5; Tris-HCl, pH 7.0–9.0; CAPS, pH 10.0–11.0. Amount of the enzyme used: for LacβPro2AA, 1.9 μg; for LacNAcβPro2AA and Galβ1–3GlcNAcβPro2AA, 0.24 μg. Reactions were allowed to proceed for 10 min at 37 °C. Error bars represent the differences of the experimental data and the averages of the experimental data.

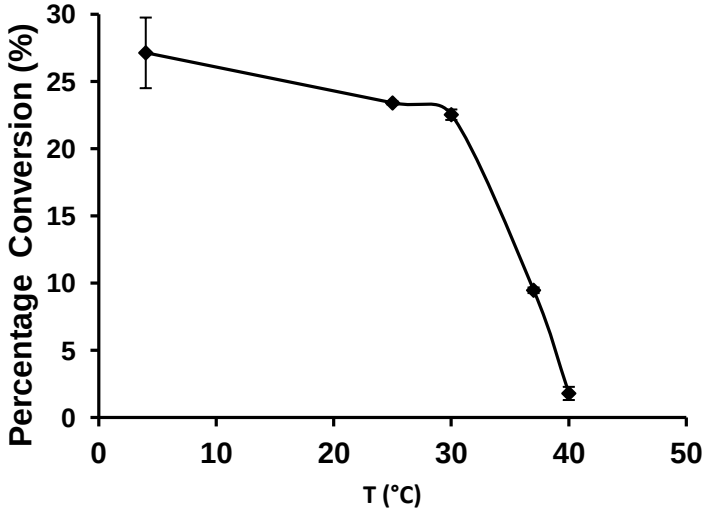

Figure S4. Thermostability assays for the fucosyltransferase activity of Hp3/4FT using LacNAcβPro2AA as the acceptor. Error bars represent the differences of the experimental data and the averages of the experimental data.

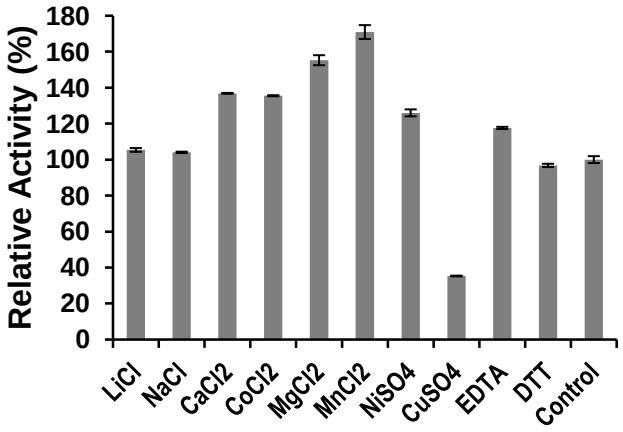

Figure S5. Effects of metal ions, EDTA, and DTT on the fucosyltransferase activity of Hp3/4FT. Error bars represent the differences of the experimental data and the averages of the experimental data.

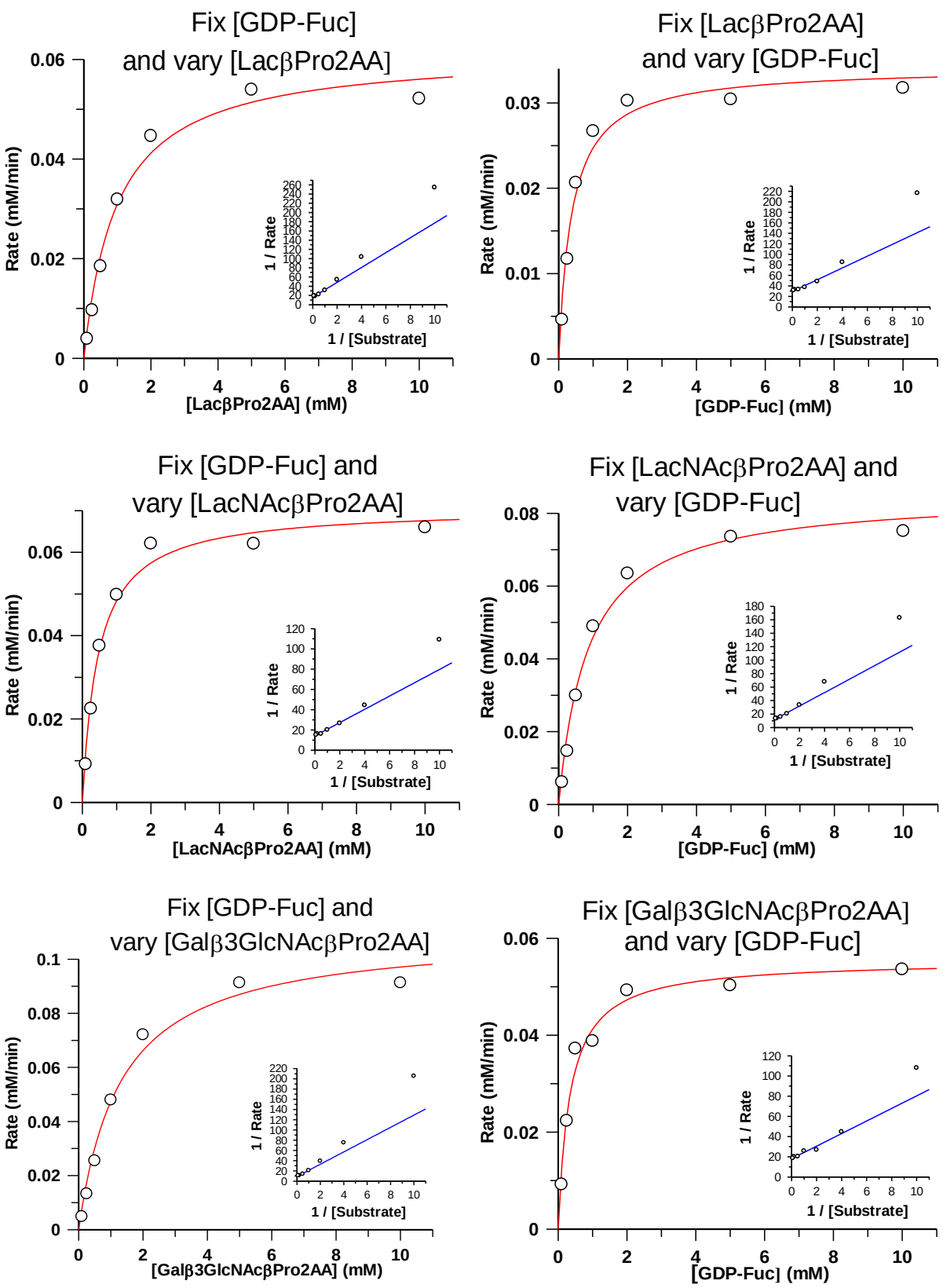

Figure S6. Kinetics plots for the fucosyltransferase activity of Hp3/4FT using different acceptor substrates.

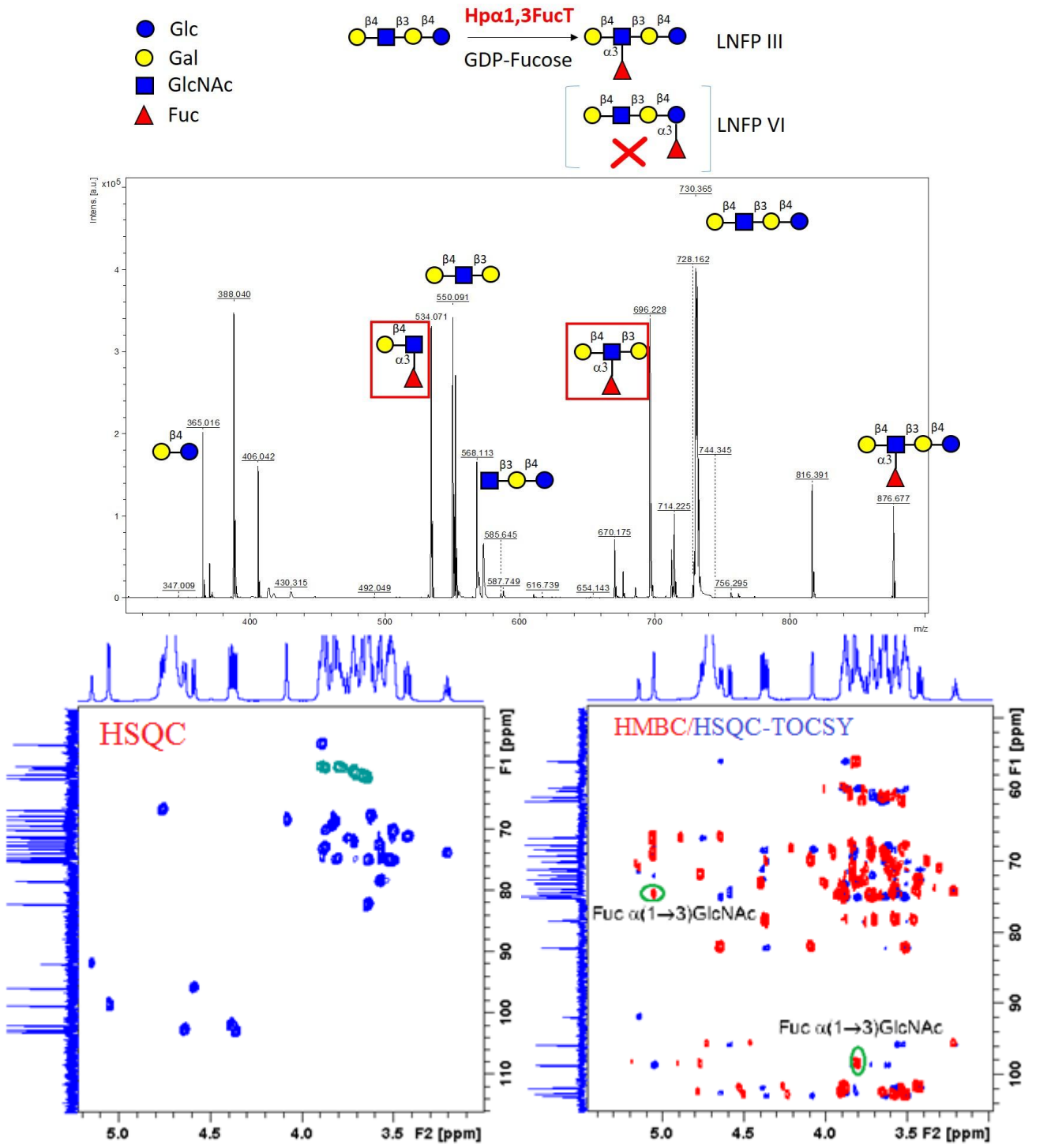

Figure S7. LNFP III (**18**) characterization by MALDI-TOF tandem mass spectrometry and 2D NMR. In MALDI-TOF tandem mass spectrometry, only peak (m/z: [M+Na]=534.071) for Galβ4(Fucα3)GlcNAc, rather than peak (m/z: [M+Na]=511.122) for Galβ4(Fucα3)Glc was found, indicating that LNFP III, rather than LNFP VI, was synthesized by Hp3/4FT-catalyzed OP3E fucosylation of LNnT (**8**).

Experimental sections

Bacterial strains, plasmids, and materials

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

Cloning

Gene sequence (GenBank accession number AF194963.2) of the C-terminal 34 amino acids truncated α1–3/4-fucosyltransferase from *Helicobacter pylori* UA948 was codon optimized (host: *E. coli*) and synthesized with N-terminal NdeI and C-terminal XhoI restriction Enzyme Sites from GenScript (Piscataway, NJ). The gene was then cleaved by NdeI and XhoI, and ligated with vector pET22b(+) treated with same restriction enzymes, yielding target plasmid containing C-terminal His_6-tagged Hp3/4FT. The plasmid was confirmed by DNA sequencing.

Full gene sequence of codon optimized and synthesized Hp3/4FT gene sequence:
CATATG(NdeI)TTCCAACCGCTGCTGGACGCTTTTATTGACTCTACCCATCTGGACGAAACG
ACGCACAAACCGCCGCTGAATGTGGCACTGGCAAACTGGTGGCCGCTGAAAAATTCAGA
GAAAAAAGGCTTTCGCGATTTCATTCTGCACTTCATCCTGAAACAGCGTTACAAAATCATC
CTGCATAGCAACCCGAATGAACCGTCTGATCTGGTTTTTGGTAATCCGCTGGAACAGGCG
CGCAAAATTCTGTCGTACCAAAATACCAAACGTGTCTTCTATACGGGCGAAAACGAAGTG
CCGAACTTTAACCTGTTTGATTACGCCATCGGTTTTGATGAACTGGACTTCAATGATCGTT
ATCTGCGCATGCCGCTGTATTACGCATATCTGCACTACAAAGCTATGCTGGTGAACGATAC
CACGAGCCCGTATAAACTGAAAGCACTGTACACCCTGAAAAAACCGTCTCACAAATTCAA
AGAAAACCATCCGAATCTGTGCGCTCTGATTCATAATGAAAGCGATCCGTGGAAACGCGG
CTTTGCATCATTCGTTGCTTCGAACCCGAATGCGCCGATTCGTAACGCCTTTTATGATGCG
CTGAATGCCATCGAACCGGTTGCAAGTGGCGGTTCCGTCAAAAACACGCTGGGTTACAAA
GTGAAAAACAAAAACGAATTTCTGAGTCAGTACAAATTCAACCTGTGTTTCGAAAATTCC
CAAGGCTATGGTTACGTTACCGAAAAAATTCTGGATGCGTACTTCAGTCACACGATTCCG
ATCTATTGGGGCAGCCCGTCTGTCGCCAAAGATTTTAACCCGAAATCCTTCGTGAATGTTC
ATGACTTCAACAACTTCGACGAAGCAATCGATTACATCCGCTACCTGCATGCGCACCAGA
ACGCCTATCTGGATATGCTGTACGAAAACCCGCTGAATACCATTGACGGCAAAGCTGGTT
TCTACCAGGATCTGAGCTTTGAAAAAATCCTGGACTTTTTCAAAAACATTCTGGAAAACG
ACACGATCTATCATTGCAATGATGCACACTACTCAGCTCTGCATCGTGATCTGAACGAACC
GCTGGTCTCGGTGGATGACCTGCGTCGCGATCATGATGACCTGCGTGTGAACTATGATGA
CCTGCGCGTTAATTACGATGACCTGCGTGTCAATTACGATGACCTGCGCGTGAACTACGA
CGACCTGCGTGTTAACTATGATGACCTGCGTCGCGACCACGACGACCTGCGTCGCGACCA
TGAACGCCTGCTGAGCAAAGCCACCCTCGAG(XhoI)

Transformation, expression, and purification of Hp3/4FT

The plasmid containing the target gene was transformed into *E. coli* BL21 (DE3) chemical competent cells as the expression host. *E. coli* strains were cultured in LB rich medium (10 g L^{-1} tryptone , 5 g L^{-1} yeast extract, and 10 g L^{-1} NaCl) supplemented with ampicillin (100 μg mL^{-1}). Over-expression of Hp3/4FT was achieved by inducing the *E. coli* BL21 (DE3) cell culture with 0.1 mM of isopropyl-1-thio-D-galactopyranoside (IPTG) when the $OD_{600\ nm}$ of the culture reached 0.8–1.0 followed by incubation at 20 °C for 20 h. Bacterial cells were harvested by centrifugation at 4 °C in a Sorvall LYNX 6000 centrifuge with a fixed rotor at 7000 × rpm for 20 min. Harvested cells were resuspended in lysis buffer (Tris-HCl buffer, 100 mM, pH 8.0 containing 0.1% v/v Triton X-100) (20 mL for cells

collected from one liter cell culture). The cell suspension was subjected to sonication (amplitude at 68% for big tip, 2 s pulse on and 3 s pulse off for 120 cycles) and the mixture was centrifuged at 4 °C, 12,000 rpm for 15 min. Cell lysate (supernatant) was obtained by centrifugation at 12000 × rpm for 15 min. Purification was carried out by loading the supernatant onto a Ni^{2+}-NTA column pre-equilibrated with 10 column volumes of binding buffer (pH 7.5) containing 5 mM imidazole, 0.5 M NaCl, and 50 mM Tris-HCl. The column was washed with 10 column volumes of binding buffer and 10 column volumes of pH7.5 washing buffer containing 36 mM imidazole, 0.5 M NaCl, 50 mM Tris-HCl. The target protein was eluted with pH7.5 elute buffer containing 50 mM Tris-HCl,200 mM imidazole and 0.5 M NaCl . The fractions containing the purified enzymes were collected, and stored at 4 °C.

Quantification of purified protein

The concentration of purified enzyme was determined in a 96-well plate using a BCA Protein Assay Kit (Pierce Biotechnology, Rockford, IL) with bovine serum albumin as a protein standard. Ample absorbance was measured at 562 nm using a BioTek SynergyTM HT Multi-Mode Microplate Reader.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE)

Protein samples were loaded to a 10% (w/v) Tris-glycine gel and proteins were separated using Bio-Rad Mini-protean III cell gel electrophoresis unit (Bio-Rad, Hercules, CA) at DC = 150 V. Bio-Rad Precision Plus Protein Standards (10–250 kDa) were used as molecular weight standards. Gels were stained with Coomassie Blue R-250.

pH Profile of Hp3/4FT

Standard enzymatic assays were carried out in duplicate in a final volume of 20 μL reaction system containing a buffer (200 mM) with a pH range from 3.0–11.0, a 2-anthranilic acid (2AA)-labeled acceptor substrate (1.0 mM), GDP-Fuc (1.0 mM), $MgCl_2$ (10 mM), and Hp3/4FT. The acceptor substrates tested were Lacβ2AA (1.9 μg Hp3/4FT), LacNAcβPro2AA or Galβ1–3GlcNAcβPro2AA (0.24 μg Hp3/4FT). Buffers used here were: citric acid-Na_2HPO_4, pH 3.0–4.0; MES, pH 5.0–6.5; Tris-HCl, pH 7.0–9.0; and CAPS, pH 9.5–11. Reactions were incubated at 37 °C for 10 min and quenched by adding 20 μL of pre-chilled ethanol. Samples were centrifuged at 13,000 rpm for 5 min, the supernatants were analyzed by a Agilent Ultra-Performance Liquid Chromatography (UPLC) system equipped with a UV detector. A reverse phase EclipasePlus C18 RRHD column (1.8 μm, 2.1 × 50 mm, Agilent) protected with a 1290 Infinity In-Line Filter was used. The mobile phase was 18% acetonitrile in H_2O (v/v).

Thermostability assays of Hp3/4FT

Thermostability assays were carried out by incubating Hp3/4FT at different temperatures for 60 min. The fucosyltransferase activity assays were then carried out in duplicate in Tris-HCl buffer (pH 8.0, 200 mM) in 20 μL reaction mixtures containing Hp3/4FT (0.12 μg) and LacNAcβPro2AA (1.0 mM). Other reaction conditions and analysis method were the same as described above for the pH profile studies on the fucosyltransferase activity of Hp3/4FT. The enzyme without pre-incubation was used as a positive control.

EDTA, dithiothreitol (DTT), and metal effects of Hp3/4FT

EDTA, DTT, or different metal salts (10 mM) were used in Tris-HCl buffer (pH 8.0, 200 mM) to analyze their effects on the fucosyltransferase activity using Lacβ2AA (10 mM) as the acceptor substrate . The metal ions used were: LiCl, NaCl, $CaCl_2$, $MgCl_2$, $CoCl_2$, $MnCl_2$, $CuSO_4$, $NiSO_4$. The reaction without EDTA, DTT and metal ions was used as a control. Other reaction conditions were same as described above for the pH profile assays.

Kinetic study of Hp3/4FT

Enzymatic assays were carried out in duplicate in a total volume of 20 μL in Tris-HCl buffer (pH 8.0, 200 mM). Reactions were allowed to proceed for 10 min at 37 °C. Apparent kinetic parameters were obtained by varying the donor GDP-Fuc concentration from 0.1–10.0 mM (0.1, 0.25, 0.5, 1, 2, 5, and 10 mM) and a fixed concentration of acceptor (LacβPro2AA or LacNAcβPro2AA or Galβ1–3GlcNAcβPro2AA, 1 mM), or a fixed concentration of donor GDP-Fuc (1 mM) and varied concentrations of acceptor (0.1, 0.25, 0.5, 1, 2, 5, and 10 mM). Apparent kinetic parameters were obtained by fitting the data to the Michaelis–Menten equation using Grafit 5.0.

Fucosidase activity studies

Assays were carried out in a total volume of 10 μL in MES buffer (200 mM, pH 6.0) containing a fucoside substrate (10 mM) and Hp3/4FT (12 μg). The substrates used were $Le^x\beta ProN_3$, $Le^x\beta$*p*NP, and $Le^a\beta ProN_3$. Reactions were incubated at 37 °C for 20 min and analyzed by high-resolution mass spectrometry (HRMS) and thin-layer chromatography (TLC) assays (developing solvent used was EtOAc:MeOH:H_2O = 5:2:1 by volume).

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 and ^{1}H NMR (600 MHz) and ^{13}C NMR (150 MHz) spectra were recorded on a Bruker Avance-III HD 600 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-Lactose was from Fisher Scientific (Pittsburgh, Pennsylvania, USA). L-Fucose was from V-LABS (Covington, Louisiana, USA). Lacto-*N*-tetraose (LNT, **7**) was from Elicityl (Crolles, France). Guanidine 5'-triphosphate (GTP) and Adenosine 5'-triphosphate (ATP) were from Hangzhou Meiya Pharmacy (Hangzhou, China). Recombinant enzymes *Bacteroides fragilis* strain NCTC9343 bifunctional L-fucokinase/GDP-fucose pyrophosphorylase (BfFKP)[1] and *Pasteurella multocida* inorganic pyrophosphatase (PmPpA)[2] were expressed and purified as described previously. Compounds Galβ1–4GlcβProN_3 (**2**),[3] Galβ1–4GlcNAcβProN_3 (**3**),[2] Galβ1–4GlcNAc6SβProN_3 (**4**),[4] Galβ1–3GlcNAcβProN_3 (**5**),[5] Galβ1–3GlcNAcαProN_3 (**6**),[5] Galβ1–4GlcNAcβ1–3Galβ1–4Glc (**8**),[6] Galβ1–4GlcNAcβ1–3Galβ1–4GlcβProN_3 (**9**),[7] Neu5Acα2–3Galβ1–4GlcNAcβProN_3 (**10**),[8] and Fucα1–2Galβ1–3GlcNAcβProN_3 (**21**)[9] were synthesized as described previously.

General procedure of one-pot three-enzyme preparative-scale synthesis of α1–3/4-linked monofucosylated glycans

Galactosides (**1**–**6** and **10**, 30–100 mg, 20 mM), L-fucose (1.3 equiv.), ATP (1.3 equiv.), and GTP (1.3 equiv.) were dissolved in Tris-HCl buffer (8 mL, 100 mM, pH 7.5) containing $MgCl_2$ (20 mM) and recombinant L-fucokinase/GDP-fucose pyrophosphorylase (BfFKP, 1.5–2.0 mg),[1] *Pasteurella multocida* inorganic pyrophosphatase (PmPpA) (1.0–2.0 mg), and Hp3/4FT (1.5–2.0 mg). All reactions were incubated in an incubator shaker at 37 °C for around 1–2 days with agitation at 100 rpm. The product formation was monitored by mass spectrometry. When an optimal yield was achieved, the reaction was stopped by adding the same volume of cold ethanol (EtOH) and kept at 4 °C for 30 min. The mixture was centrifuged at 7000 rpm for 30 minutes and the precipitates were removed. The

supernatant was concentrated, passed through a BioGel P-2 gel filtration column, and eluted with water to obtain partially purified product. A silica gel column was then used for further purification using EtOAc:MeOH:H_2O = 5:2:1 (by volume) as the mobile phase. The final pure fucosylated products were obtained by passing through a BioGel P-2 gel filtration column again for removing any silica gel dissolved.

LNnT (**8**, 10 mg, 10 mM), L-fucose (1.2 equiv.), ATP (1.2 equiv.), and GTP (1.2 equiv.) were dissolved in Tris-HCl buffer (1.5 mL, 100 mM, pH 8.0) containing $MgCl_2$ (10 mM) and recombinant BfFKP (1 mg), PmPpA (0.5 mg), and Hp3/4FT (0.5 mg). All reactions were incubated in an incubator shaker at 37 °C for around two days with agitation at 100 rpm. The product formation was monitored by mass spectrometry. When an optimal yield was achieved, the reaction was stopped by boiling the solution for 5 min. The mixture was centrifuged at 12000 rpm for 20 minutes and the precipitates were removed. The supernatant was concentrated by rotovap and purified by HPLC using a XBridge BEH Amide Column (130Å, 5 μm, 4.6 mm × 250 mm). Mobile phase A: 100 mM ammonium formate, pH 3.46; Mobile phase B: acetonitrile; Gradient: 80% to 60% B (by volume) over 35 minutes, 60% to 0% B (by volume) over 1 minute, 0% B for 2 minutes, 0% to 80% B (by volume) over 2 minutes, 80% B (by volume) for 5 minutes. HPLC eluent was monitored by absorption at 210 nm, and glycan-containing fractions were analyzed by MS.

General procedure of one-pot three-enzyme preparative-scale synthesis of α1–3/4-linked difucosylated glycans.

Tetrasaccharide (**7** or **9**, 31–40 mg, 20 mM), L-fucose (3.0 equiv.), ATP (3.0 equiv.), and GTP (3.0 equiv.) were dissolved in Tris-HCl buffer (8 mL, 100 mM, pH 7.5) containing $MgCl_2$ (20 mM) and recombinant BfFKP (3.0 mg), PmPpA (2.0 mg), and Hp3/4FT (3.0 mg). All reactions were incubated in an incubator shaker at 37 °C for around 4–5 days with agitation at 100 rpm. The product formation was monitored by mass spectrometry. When an optimal yield was achieved, the reaction was quenched by adding the same volume of ice-cold ethanol and incubation at 4 °C for 30 min. The mixture was centrifuged and the precipitates were removed. The supernatant was concentrated, passed through a BioGel P-2 gel filtration column and eluted with water. The fractions containing the product were collected, concentrated, and further purified by HPLC over a XBridge BEH Amide Column (130Å, 5 μm, 4.6 mm × 250 mm). Mobile phase A: 100 mM ammonium formate, pH 3.46; Mobile phase B: acetonitrile; Gradient: 65% to 50% B (by volume) over 25 minutes, 50% to 0% B (by volume) over 1 minute, 0% B for 2 minutes, 0% to 65% B (by volume) over 2 minutes, 65% B (by volume) for 5 minutes. HPLC eluent was monitored by absorption at 210 nm, and glycan-containing fractions were analyzed by TLC and MS. The fractions containing the pure product were collected and concentrated to obtain the final pure fucosylated glycans.

Galβ3(Fucα4)GlcNAcβProN$_3$ (11). 51 mg, 95%; ^{1}H NMR (800 MHz, D_2O) δ 5.00 (d, *J* = 4.0 Hz, 1H), 4.85 (m, 1H), 4.50 (d, *J* = 8.0 Hz, 1H), 4.47 (d, *J* = 8.0 Hz, 1H), 4.05–3.32 (m, 19 H), 2.03 (s, 3 H), 1.82 (m, 2H), 1.15 (d, *J* = 6.4 Hz, 3H); ^{13}C NMR (200 MHz, D_2O) δ 174.23, 102.76, 100.91, 97.95, 75.97, 75.26, 74.68, 72.29, 72.17, 71.83, 70.35, 69.00, 68.24, 67.66, 67.08, 66.74, 61.54, 59.55, 55.63, 47.66, 28.00, 22.17, 15.26. HRMS (ESI) m/z calculated for $C_{23}H_{40}N_4NaO_{15}$ (M+Na) 635.2388, found 635.2391.

Galβ3(Fucα4)GlcNAcαProN$_3$ (12). 50 mg, 96%; ^{1}H NMR (800 MHz, D_2O) δ 5.02 (d, *J* = 4.0 Hz, 1H), 4.90 (m, 1H), 4.51 (d, *J* = 8.0 Hz, 1H), 4.19–4.15 (m, 2 H), 3.89–3.43 (m, 18 H), 2.03 (s, 3 H), 1.89 (m, 2H), 1.17 (d, *J* = 6.4 Hz, 3H); ^{13}C NMR (200 MHz, D_2O) δ 174.11, 102.73, 97.94, 96.94, 74.58, 74.20, 72.31, 72.14, 71.86, 71.33, 70.33, 69.00, 68.30, 67.68, 66.73, 64.79, 61.60, 59.45, 53.60,

48.01, 27.90, 21.89, 15.27. HRMS (ESI) m/z calculated for $C_{23}H_{40}N_4NaO_{15}$ (M+Na) 635.2388, found 635.2392.

Galβ4(Fucα3)GlcNAcβProN$_3$ (13). 60 mg, 98%; ^{1}H NMR (800 MHz, D_2O) δ 5.18 (d, *J* = 4.0 Hz, 1H), 4.89 (m, 1H), 4.62 (d, *J* = 8.0 Hz, 1H), 4.52 (dd, *J* = 8.0 Hz, 1H), 4.07–3.66 (m, 16 H), 3.55 (d, *J* = 9.6 Hz, 1H), 3.43 (m, 2H), 2.11 (s, 3 H), 1.90 (m, 2H), 1.23 (d, *J* = 6.4 Hz, 3H); ^{13}C NMR (200 MHz, D_2O) δ 174.13, 101.70, 100.83, 98.51, 75.20, 74.78, 73.23, 72.32, 71.79, 70.90, 69.08, 68.23, 68.20, 67.56, 67.08, 66.60, 66.57, 61.38, 59.63, 55.69, 47.63, 27.99, 22.12, 15.19. HRMS (ESI) m/z calculated for $C_{23}H_{40}N_4NaO_{15}$ (M+Na) 635.2388, found 635.2395.

Galβ4(Fucα3)GlcNAc6SβProN$_3$ (14). 94 mg, 93%; ^{1}H NMR (800 MHz, D_2O). ^{1}H NMR (800 MHz, D_2O) δ 5.10 (d, *J* = 4.0 Hz, 1H), 4.81 (m, 1H), 4.54 (d, *J* = 8.0 Hz, 1H), 4.51 (d, *J* = 8.0 Hz, 1H), 4.35 (m, 2H), 4.00–3.57 (m, 14H), 3.48 (t, *J* = 8.0 Hz, 1H), 3.45 (m, 2H), 2.02 (s, 3H), 1.82 (m, 2H), 1.16 (d, *J* = 6.4 Hz, 3H); ^{13}C NMR (200 MHz, D_2O) δ 173.74, 101.01, 100.44, 98.10, 74.46, 74.28, 72.45, 72.25, 71.91, 71.40, 70.48, 68.69, 67.93, 67.26, 66.77, 66.22, 65.45, 60.95, 55.19, 47.27, 27.61, 21.72, 14.81. HRMS (ESI) m/z calculated for $C_{23}H_{39}N_4O_{18}S$ (M-H) 691.1980, found 691.1978.

Galβ4(Fucα3)GlcβProN$_3$ (15). 74 mg, 92%; ^{1}H NMR (800 MHz, D_2O) ^{1}H NMR (800 MHz, D_2O) δ 5.41 (d, *J* = 4.0 Hz, 1H), 4.80 (m, 1 H), 4.45 (d, *J* = 8.0 Hz, 1H), 4.40 (dd, *J* = 8.0 Hz, 1H), 3.98–3.42 (m, 19 H), 1.88 (m, 2 H), 1.15 (d, *J* = 6.4 Hz, 3H); ^{13}C NMR (200 MHz, D_2O) δ 102.08, 101.64, 98.25, 76.81, 75.18, 74.80, 74.38, 72.57, 72.29, 71.83, 71.01, 69.09, 68.19, 67.91, 67.27, 66.37, 61.36, 59.61, 47.71, 28.08, 15.12. HRMS (ESI) m/z calculated for $C_{21}H_{37}N_3NaO_{15}$ (M+Na) 594.2122, found 594.2132.

Galβ4(Fucα3)Glc (16). 121 mg, 90%; ^{1}H NMR (800 MHz, D_2O) δ 5.41 (d, *J* = 4.0 Hz, 0.5 H), 5.35 (d, *J* = 4.0 Hz, 0.5 H), 5.15 (d, *J* = 4.0 Hz, 0.5 H), 4.81 (m, 1 H), 4.62 (d, *J* = 8.0 Hz, 0.5 H), 4.40 (dd, *J* = 8.0 Hz, 1H), 3.94–3.43 (m, 15 H), 1.16–1.15 (m, 3H); ^{13}C NMR (200 MHz, D_2O) δ 101.67, 101.64, 98.38, 98.24, 95.69, 91.96, 76.88, 75.40, 75.24, 74.82, 74.58, 72.56, 72.54, 72.48, 72.29, 72.27, 71.85, 71.83, 71.03, 71.01, 70.78, 69.16, 69.10, 68.22, 68.20, 67.95, 67.91, 66.37, 66.33, 61.42, 61.38, 59.65, 59.56, 59.16, 15.13, 15.11. HRMS (ESI) m/z calculated for $C_{18}H_{32}NaO_{15}$ (M+Na) 511.1639, found 511.1631.

Galβ3(Fucα4)GlcNAcβ3Galβ4(Fucα3)Glc (17). 56 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), 5.02 (d, *J* = 4.0 Hz, 1.0 H), 4.87 (m, 2H), 4.68 (d, *J* = 8.8 Hz, 1H), 4.63 (d, *J* = 8.0 Hz, 0.6 H), 4.50 (d, *J* = 7.2 Hz, 1H), 4.40 (d, *J* = 8.0 Hz, 1H), 4.09–3.44 (m, 30 H), 2.02 (s, 3 H), 1.22–1.15(m, 6H); ^{13}C NMR (200 MHz, D_2O) δ 174.62, 102.73, 102.47, 101.62, 98.43, 98.32, 97.88, 95.72, 92.01, 81.47, 81.40, 76.87, 75.80, 75.42, 75.25, 75.06, 74.69, 74.56, 74.39, 74.38, 72.56, 72.25, 72.18, 72.16, 71.99, 71.83, 71.81, 71.79, 70.83, 70.55, 70.50, 70.37, 69.15, 69.10, 69.01, 68.23, 68.15, 68.12, 67.91, 67.88, 67.66, 66.73, 66.37, 66.33, 62.61, 61.54, 61.38, 61.36, 59.67, 59.60, 59.47, 55.75, 22.15, 15.25, 15.10, 15.09. HRMS (ESI) m/z calculated for $C_{38}H_{65}NNaO_{29}$ (M+Na) 1022.3540, found 1022.3551.

Galβ4(Fucα3)GlcNAcβ3Galβ4Glc (18). 10.5 mg, 88%; ^{1}H NMR (600 MHz, D_2O) δ 5.14 (d, *J* = 3.6 Hz, 0.4H), 5.05 (d, *J* = 4.2 Hz, 1H), 4.76 (d, *J* = 6.6 Hz, 1H), 4.63 (d, *J* = 8.4 Hz, 1H), 4.58 (d, *J* = 8.4 Hz, 0.6H), 4.38 (d, *J* = 10.8 Hz, 1 H), 4.36 (d, *J* = 7.8 Hz, 1H), 4.08 (d, *J* = 3.0 Hz, 1H), 3.90–3.48 (m, 26.4H), 3.43–3.40 (m, 1H), 3.21–3.18 (m, 0.6H), 1.94 (s, 3H), 1.09 (d, *J* = 6.6 Hz, 3H); ^{13}C NMR (150 MHz, D_2O) δ 174.71, 102.93, 102.90, 102.54, 101.78, 98.59, 95.74, 91.81, 82.08, 78.27, 75.12, 74.92, 74.87, 74.80, 74.74, 74.36, 73.80, 73.06, 72.48, 71.92, 71.40, 71.14, 71.05, 70.12, 69.98, 69.20, 68.35,

67.70, 66.69, 61.51, 60.96, 60.08, 59.95, 59.63, 55.96, 22.26, 15.31. HRMS (ESI) m/z calculated for $C_{32}H_{55}N_1NaO_{25}$ (M+Na) 876.2955, found 876.2911.

Galβ4(Fucα3)GlcNAcβ3Galβ4(Fucα3)GlcβProN$_3$ (19). 42 mg, 99%; ^{1}H NMR (800 MHz, D_2O) δ 5.42 (d, *J* = 4.0 Hz, 1H), 5.12 (d, *J* = 4.0 Hz, 1H), 4.83 (m, 2H), 4.69 (d, *J* = 8.8 Hz, 1H), 4.46 (d, *J* = 7.2 Hz, 1H), 4.69 (d, *J* = 7.2 Hz, 1H), 4.40 (d, *J* = 8.0 Hz, 1H), 4.08 (d, *J* = 4.0 Hz, 1H), 4.00–3.43 (m, 33 H), 2.01 (s, 3 H), 1.90 (m, 2 H), 1.17 (d, *J* = 6.4 Hz, 3H), 1.15 (d, *J* = 6.4 Hz, 3H); ^{13}C NMR (200 MHz, D_2O) δ 174.54, 102.39, 102.11, 101.63, 101.60, 98.47, 98.33, 81.45, 76.80, 75.19, 74.95, 74.79, 74.63, 74.40, 74.35, 72.90, 72.35, 72.27, 71.79 (2C), 70.93, 70.52, 69.08, 69.07, 68.23, 68.11, 67.87, 67.58, 67.30, 66.57, 66.37, 61.39, 61.34, 59.65, 59.51, 55.83, 47.73, 28.09, 22.12, 15.19, 15.08. HRMS (ESI) m/z calculated for $C_{41}H_{70}N_4NaO_{29}$ (M+Na) 1105.4023, found 1105.4020.

Neu5Acα3Galβ4(Fucα3)GlcNAcβProN$_3$ (20). 26 mg, 84%; ^{1}H NMR (600 MHz, D_2O) δ 5.08 (d, *J* = 3.6 Hz, 1 H), 4.80 (m, 1 H), 4.51 (d, *J* = 8.4 Hz, 1 H), 4.50 (d, *J* = 7.8 Hz, 1 H), 4.06 (dd, *J* = 9.6 and 3.0 Hz, 1 H), 4.00-3.53 (m, 22 H), 3.50 (t, *J* = 8.4 Hz, 1 H), 3.35 (m, 2 H), 2.74 (dd, *J* = 12.0 and 4.2 Hz, 1 H), 2.01 (s, 6 H), 1.81 (m, 2 H), 1.77 (t, *J* = 12.0 Hz, 1 H), 1.14 (d, *J* = 6.6 Hz, 3 H); ^{13}C NMR (150 MHz, D_2O) δ 175.18, 174.39, 174.03, 101.78, 101.14, 99.83, 98.76, 75.82, 75.42, 75.07, 74.98, 73.52, 73.08, 72.07, 72.02, 69.43, 69.35, 68.47, 68.28, 67.87, 67.47, 67.36, 66.85, 62.77, 61.64, 59.82, 55.98, 51.86, 47.94, 39.95, 28.28, 22.40, 22.21, 15.44. HRMS (ESI) m/z calcd for $C_{34}H_{56}N_5O_{23}$ $(M-1)^-$ 902.3372, found 902.3344.

Fucα2Galβ3(Fucα4)GlcNAcβProN$_3$ (22). 36 mg, 98%; ^{1}H NMR (800 MHz, D_2O) δ 5.14 (d, *J* = 4.0 Hz, 1H), 5.01 (d, *J* = 4.0 Hz, 1H), 4.82 (m, 2H), 4.64 (d, *J* = 8.0 Hz, 1H), 4.39 (d, *J* = 8.0 Hz, 1H), 4.33 (dd, *J* = 6.4 and 13.6 Hz, 1H), 4.11 (t, *J* = 9.6 Hz, 1H), 3.97–3.30 (m, 20 H), 2.06 (s, 3 H), 1.82 (m, 2H), 1.25 (d, *J* = 6.4 Hz, 3H), 1.24 (d, *J* = 6.4 Hz, 3H); ^{13}C NMR (200 MHz, D_2O) δ 173.46, 101.75, 100.53, 99.44, 97.71, 76.35, 75.24, 74.65, 74.55, 73.50, 72.00, 71.86, 71.77, 69.31, 68.97, 68.62, 68.14, 67.67, 66.99, 66.93, 66.14, 61.49, 59.42, 55.51, 47.62, 28.06, 22.10, 15.24, 15.14. HRMS (ESI) m/z calculated for $C_{29}H_{50}N_4NaO_{19}$ (M+Na) 781.2967, found 781.2965.

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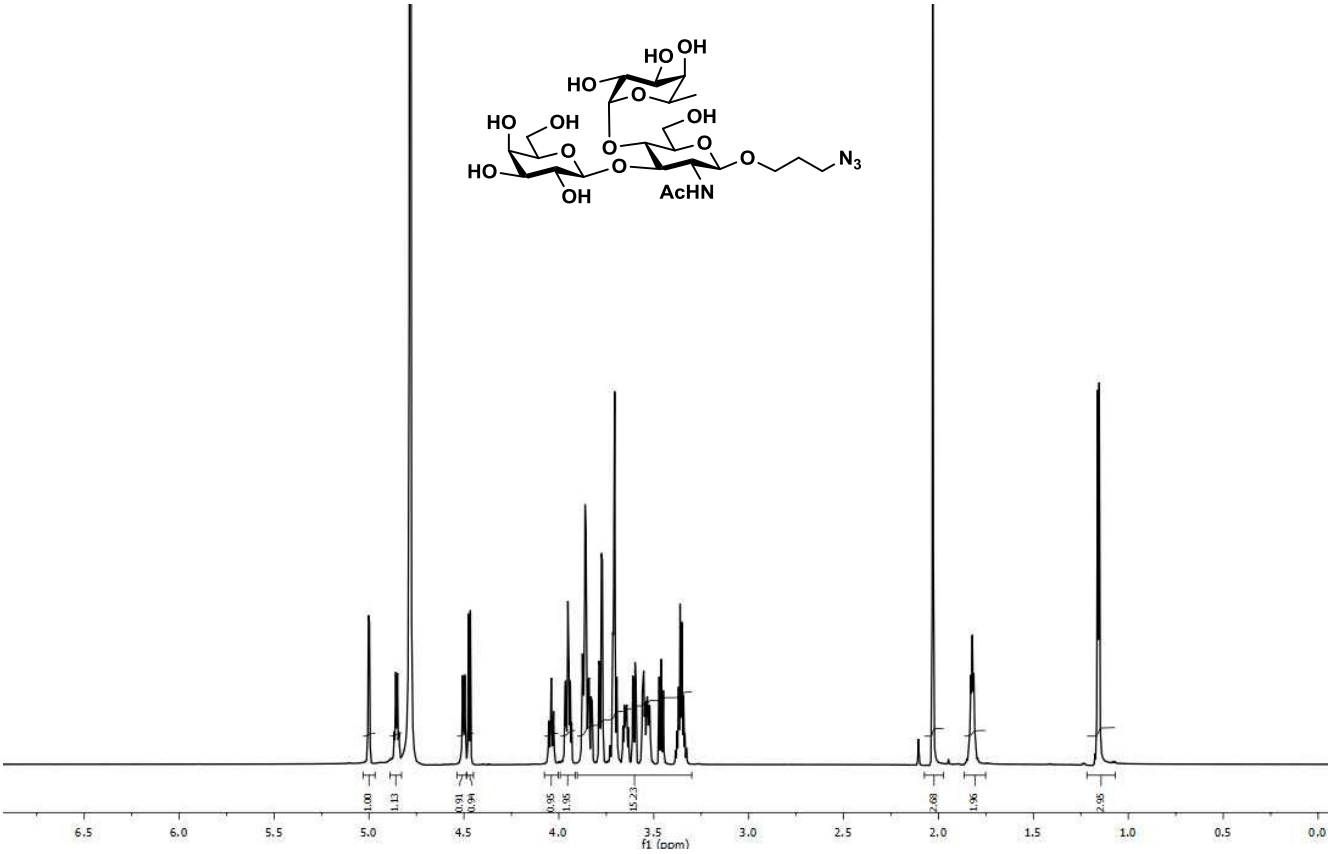
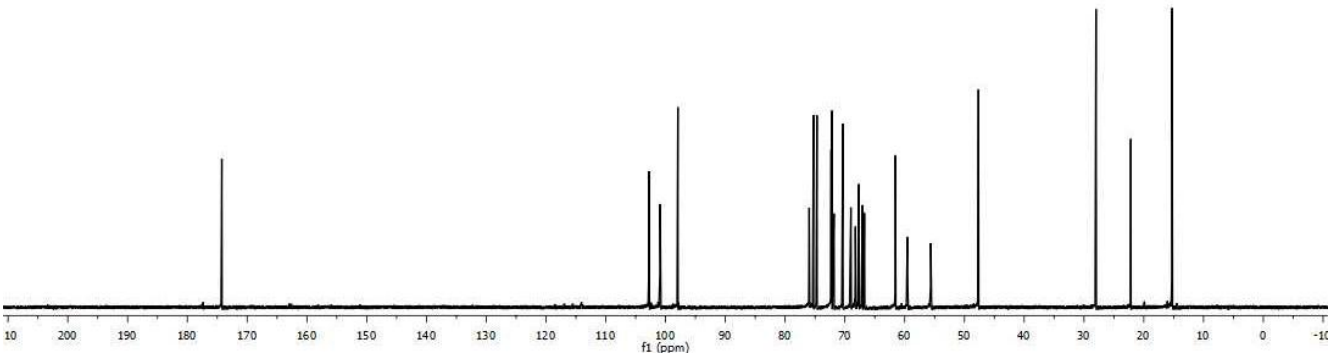
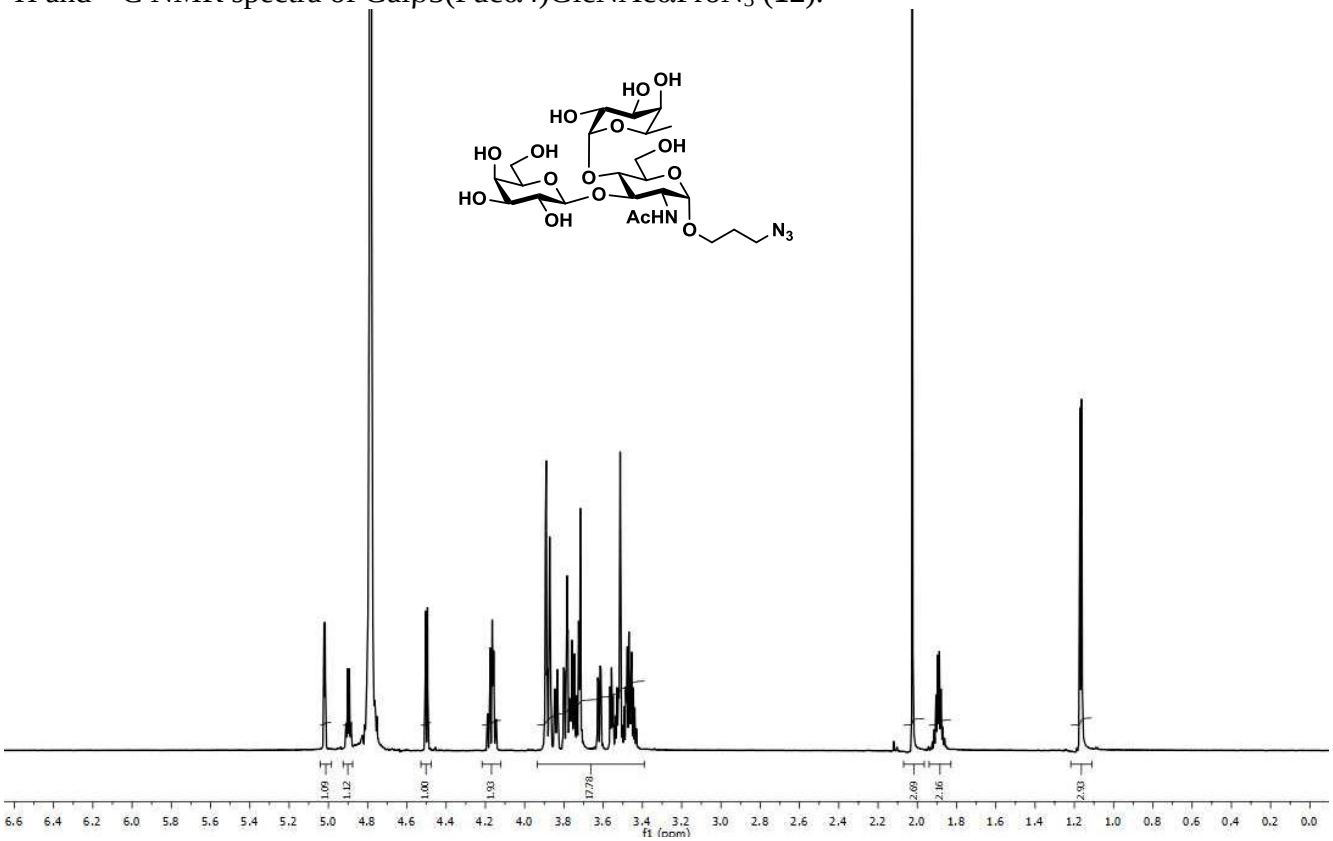
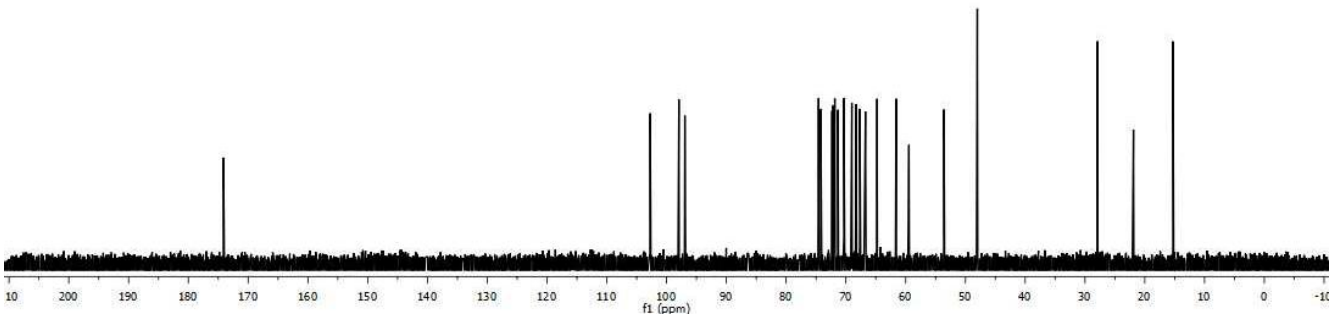
^{1}H and ^{13}C NMR spectra of Galβ3(Fucα4)GlcNAcβProN_3 (**11**).



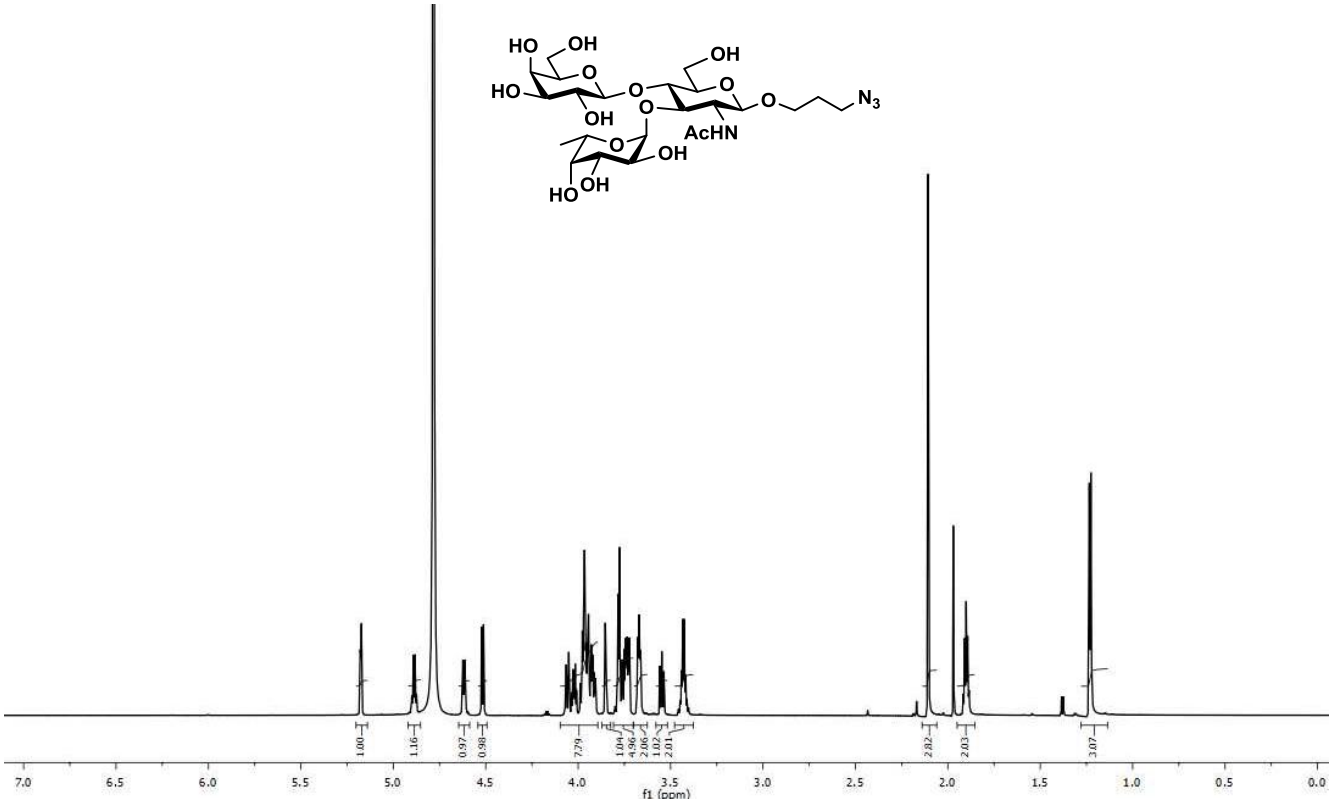


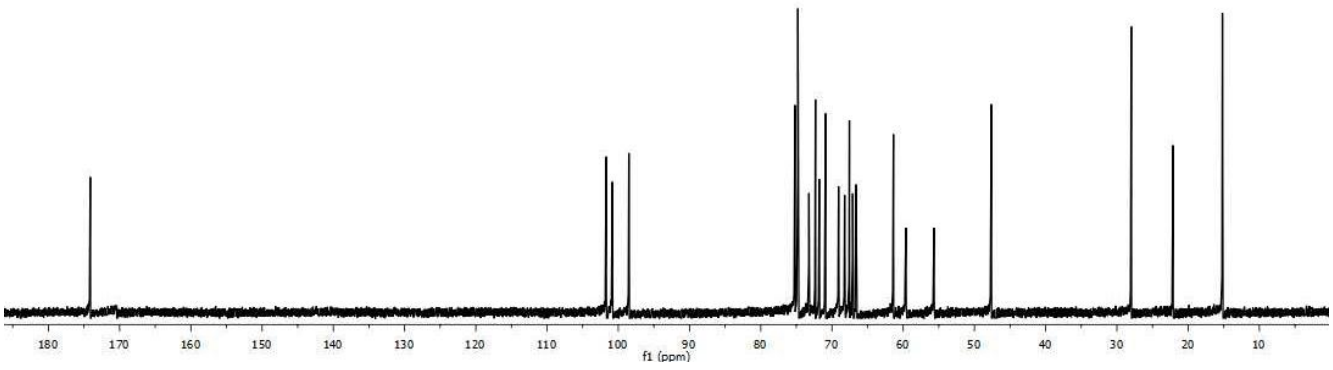
^{1}H and ^{13}C NMR spectra of Galβ3(Fucα4)GlcNAcαProN_3 (**12**).



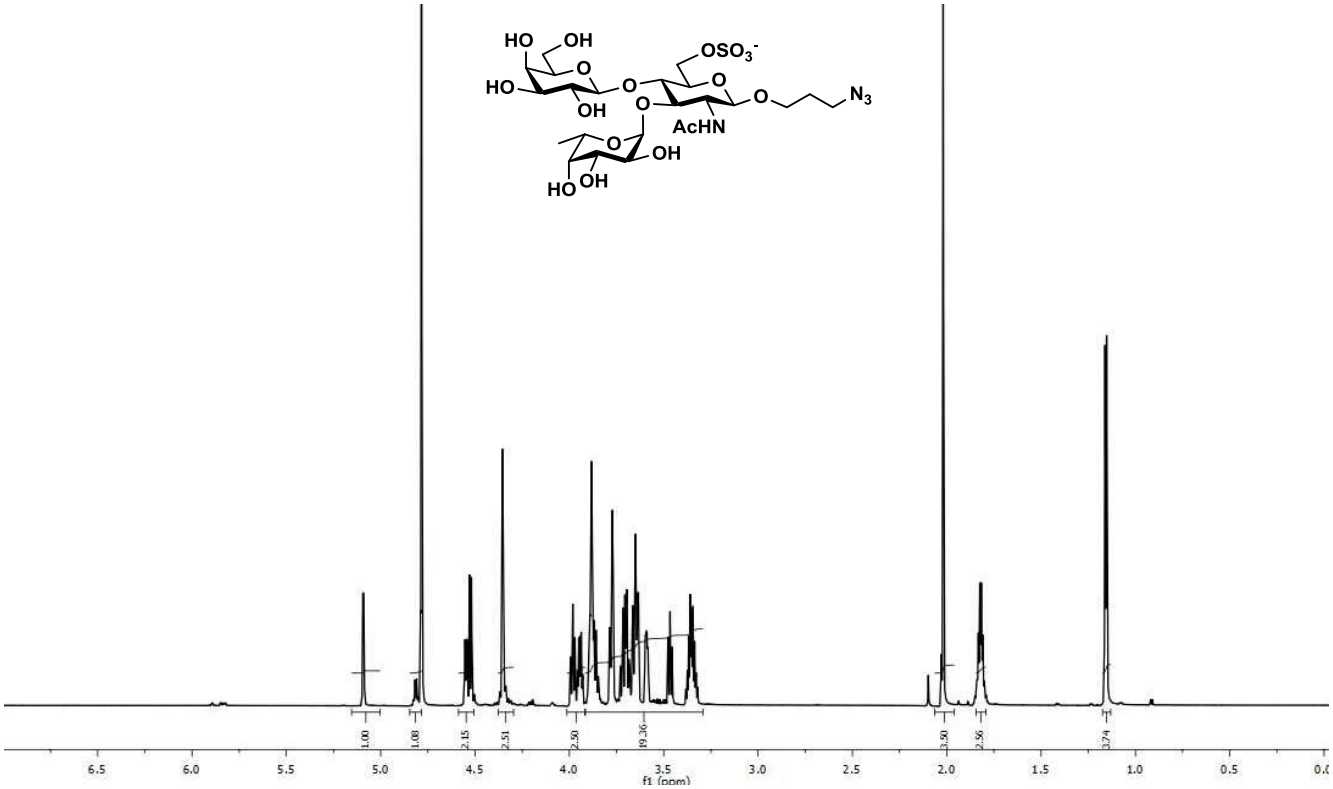


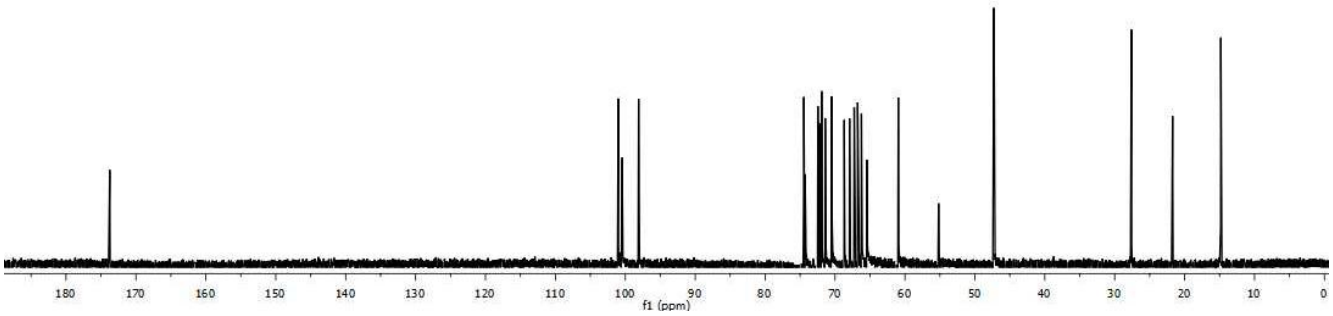
^{1}H and ^{13}C NMR spectra of Galβ4(Fucα3)GlcNAcβProN_3 (**13**).



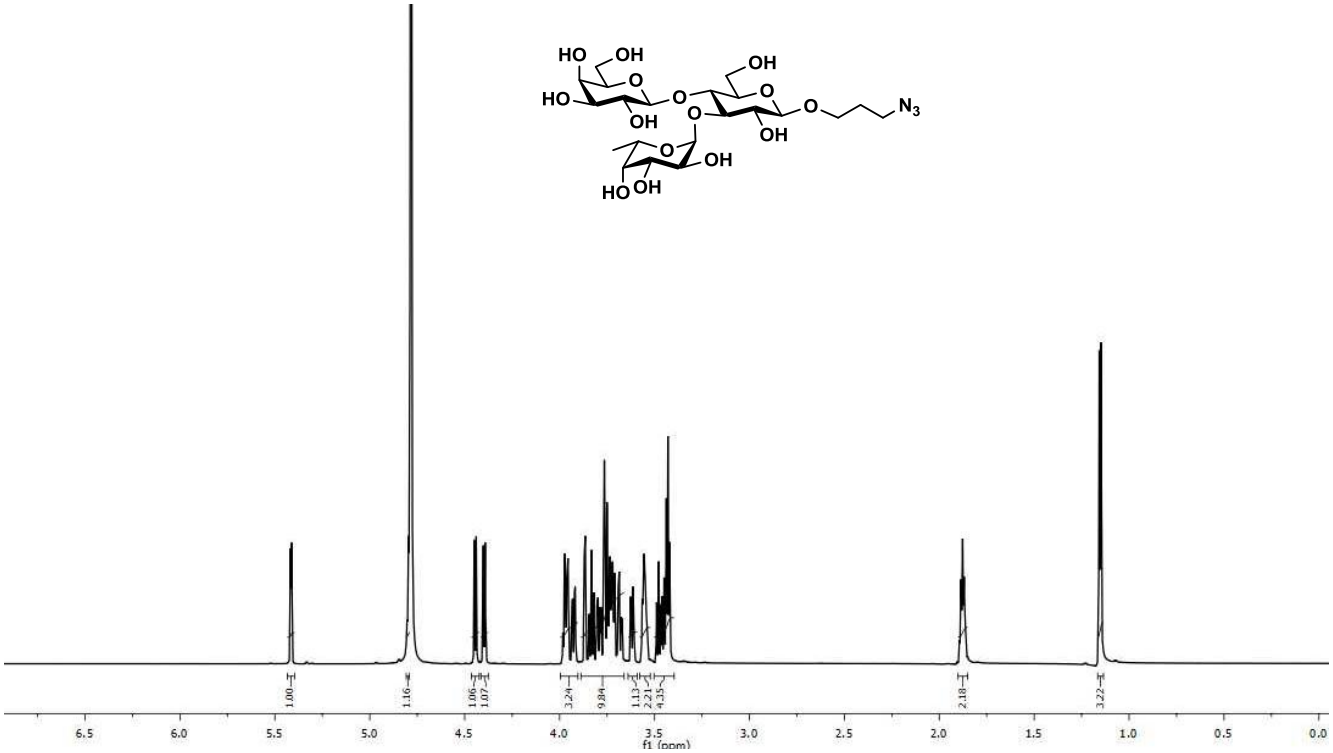


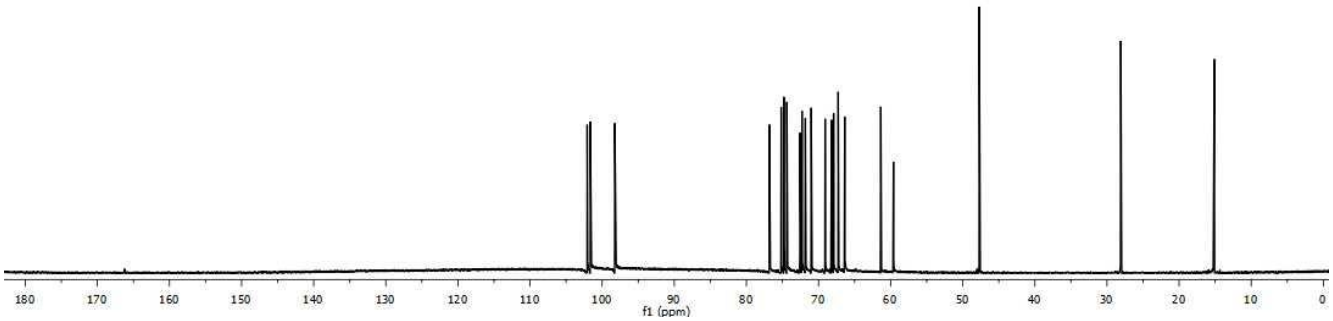
^{1}H and ^{13}C NMR spectra of Galβ4(Fucα3)GlcNAc6SβProN_3 (**14**).



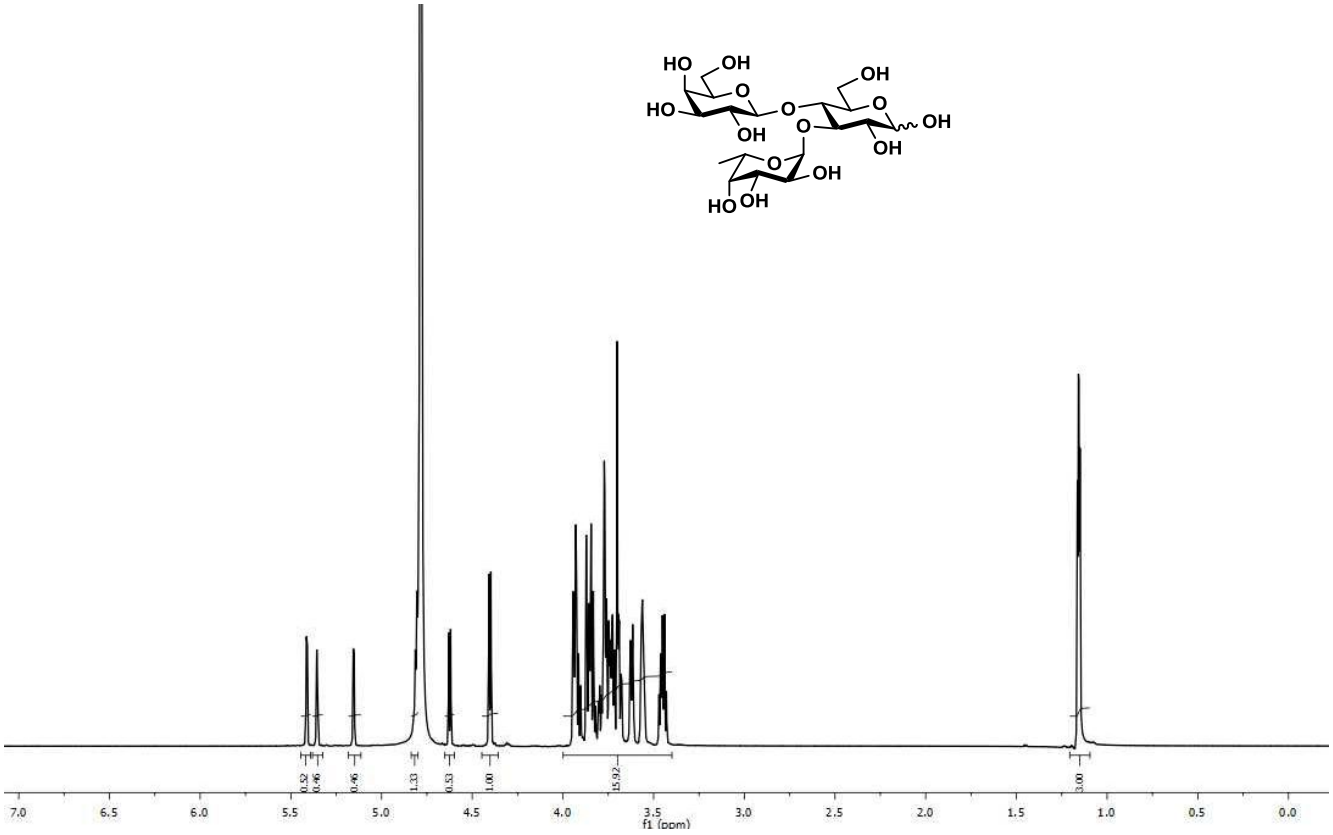


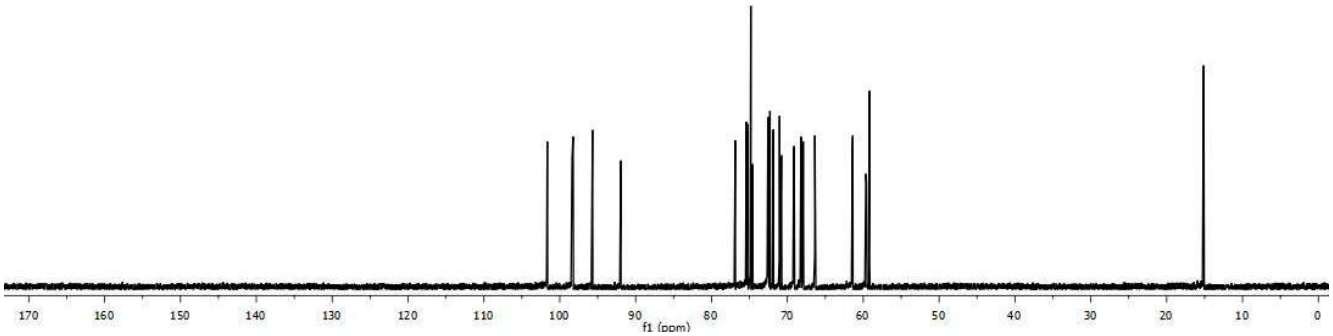
^{1}H and ^{13}C NMR spectra of Galβ4(Fucα3)GlcβProN_3 (**15**).



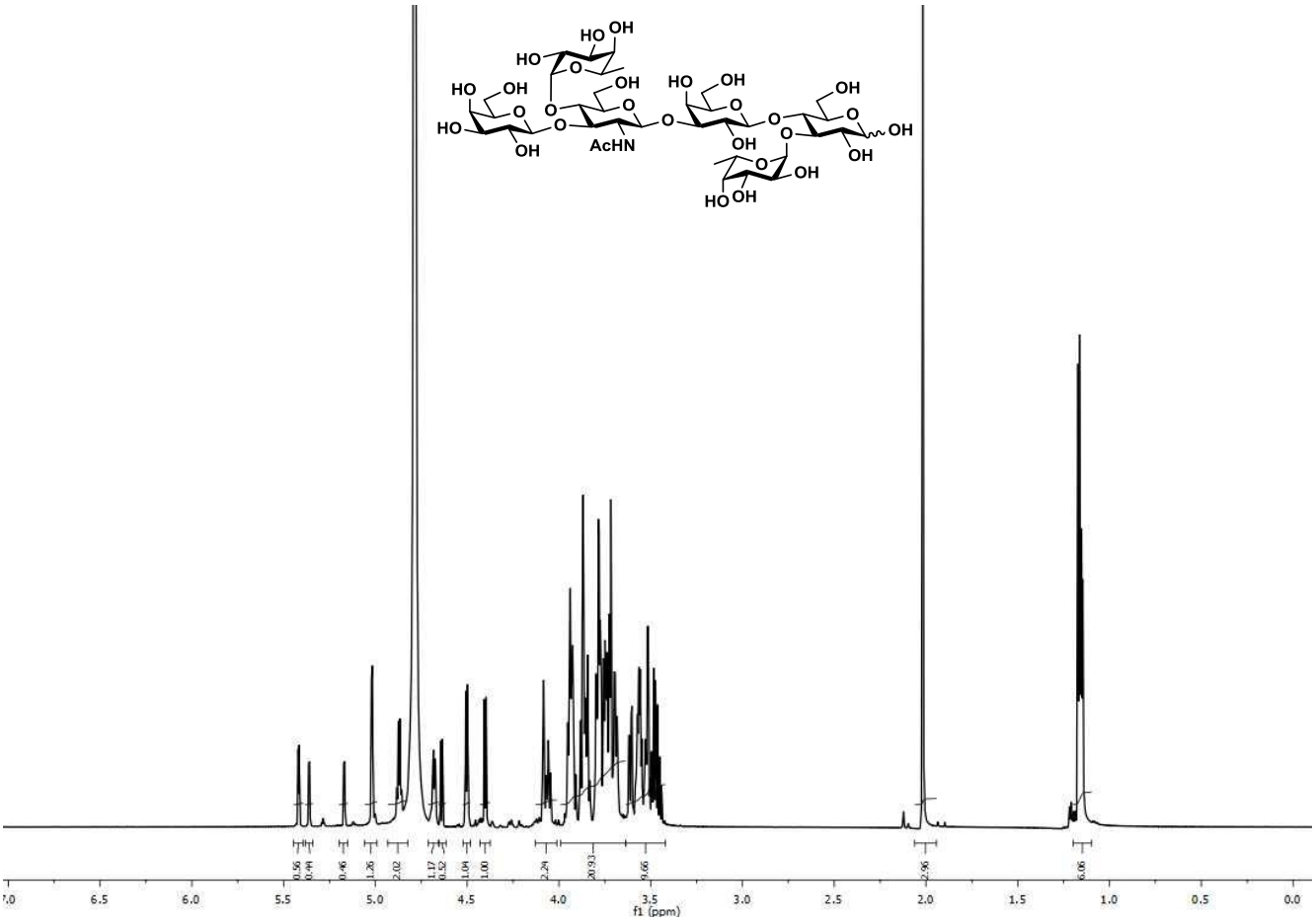


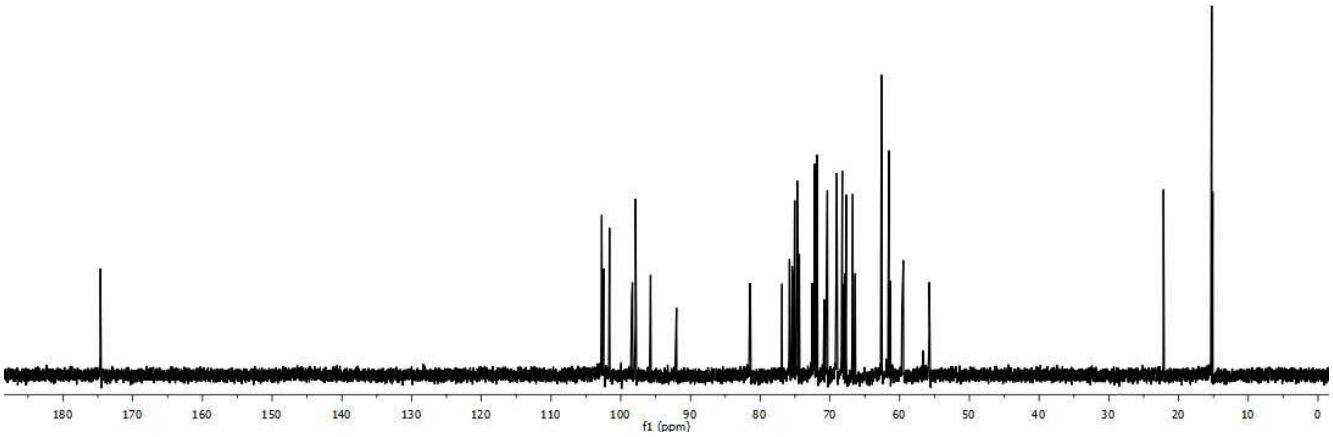
^{1}H and ^{13}C NMR spectra of Galβ4(Fucα3)Glc (**16**).



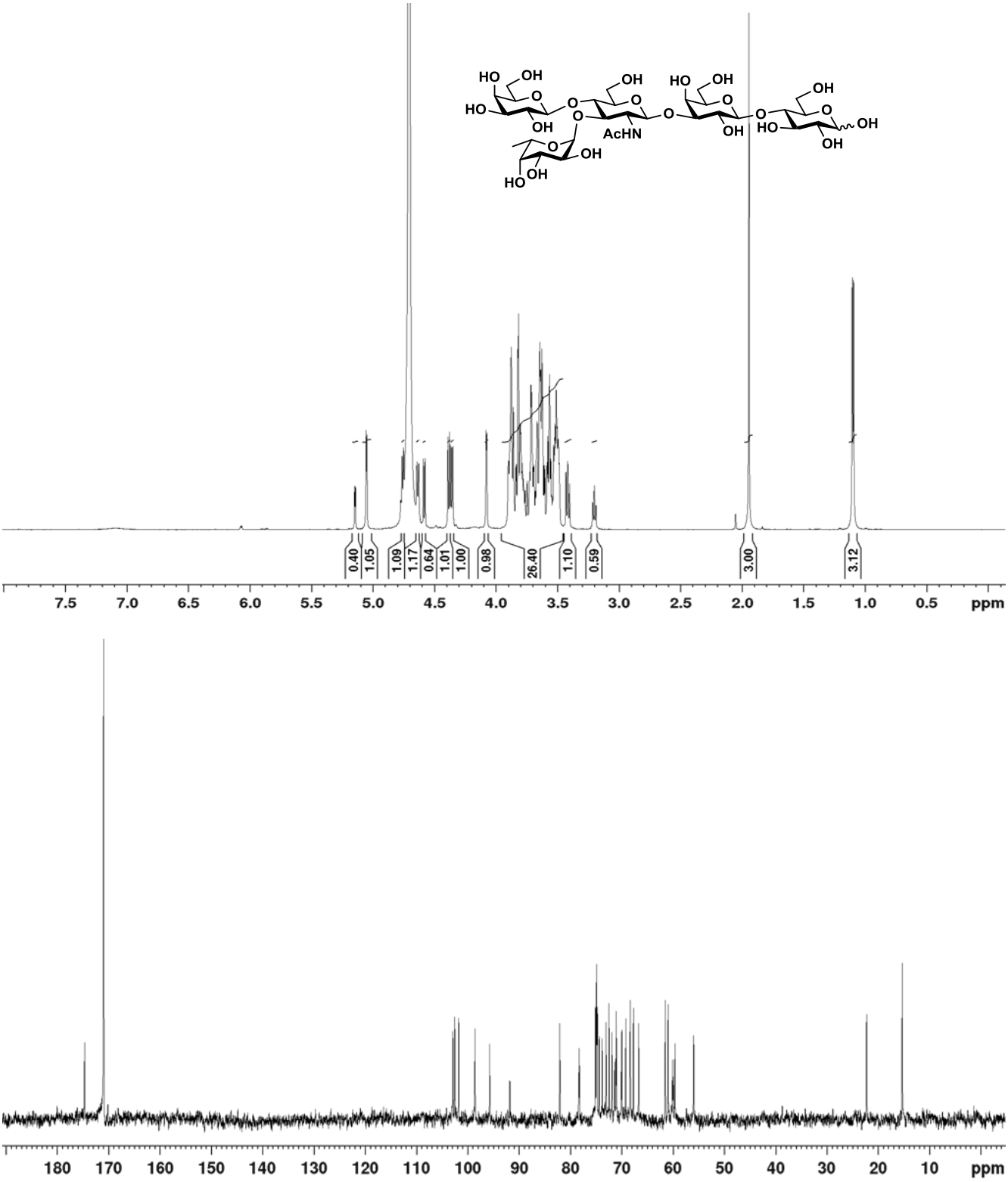


^{1}H and ^{13}C NMR spectra of Galβ3(Fucα4)GalNAcβ3Galβ4(Fucα3)Glc (**17**).

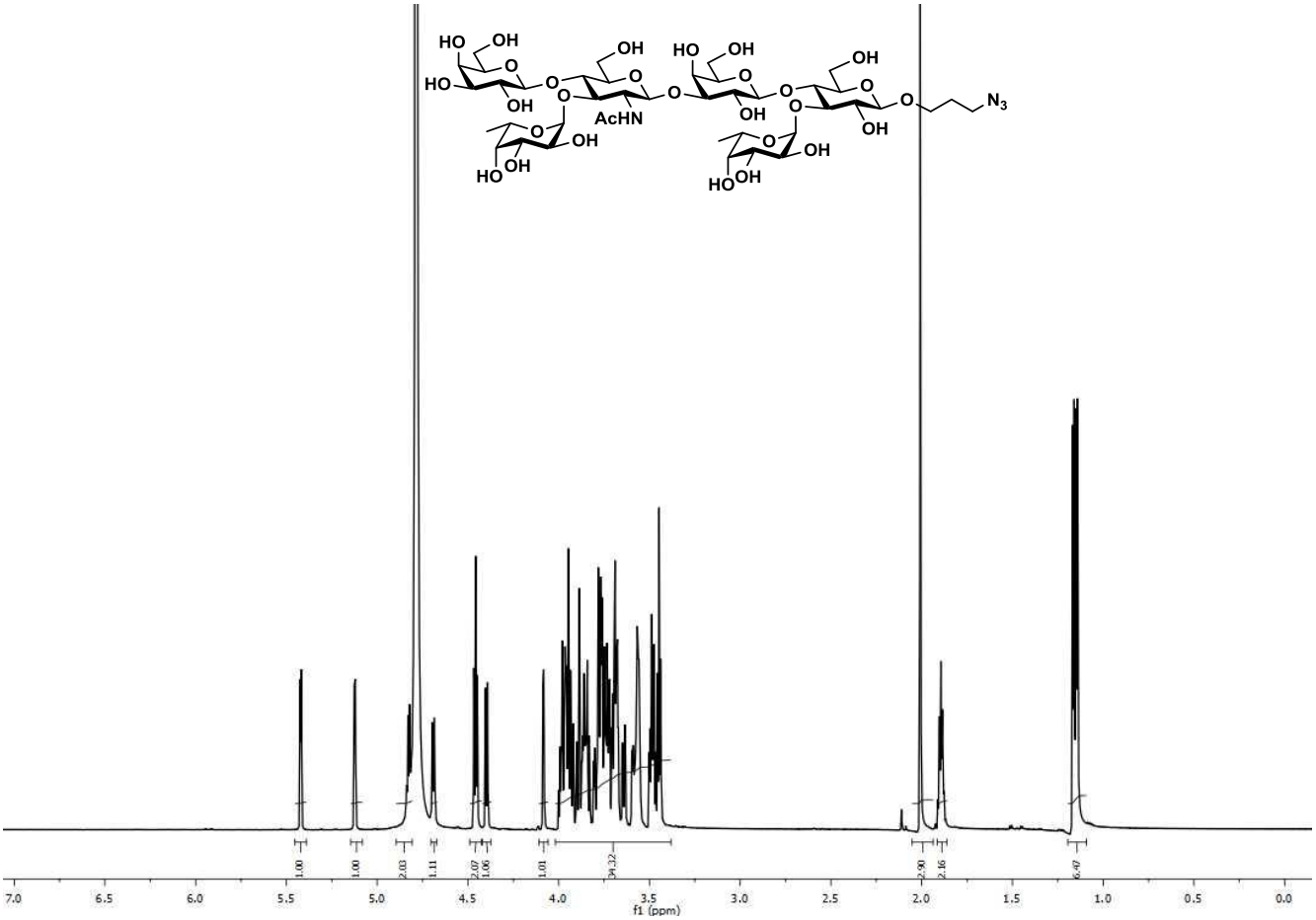


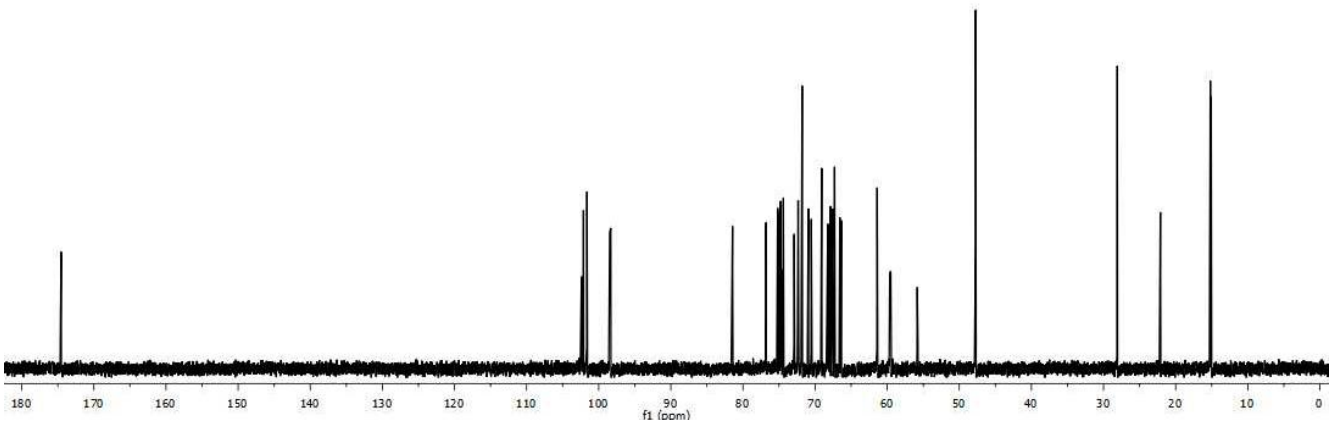


^{1}H and ^{13}C NMR spectra of Galβ4(Fucα3)GlcNAcβ3Galβ4Glc (**18**)

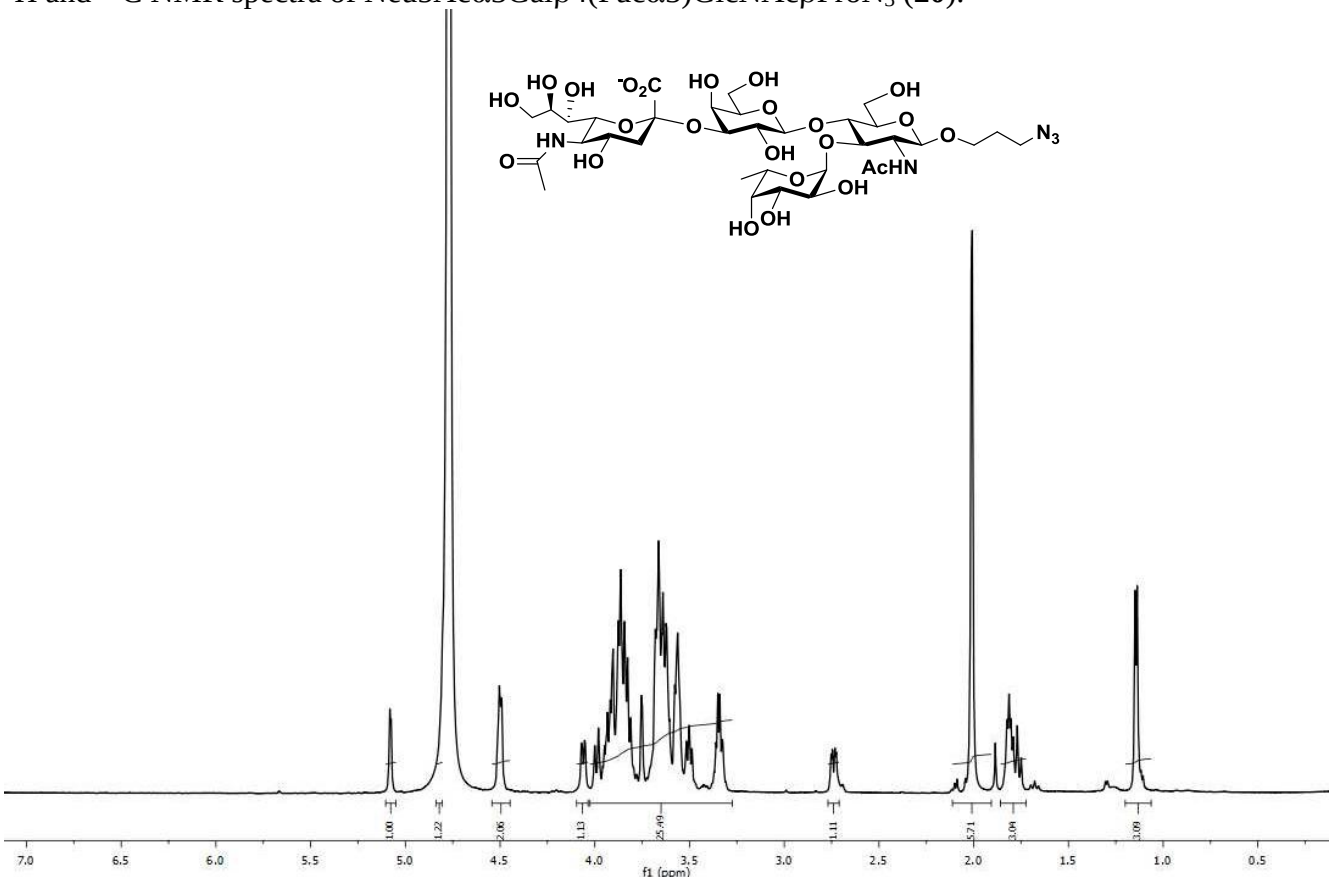


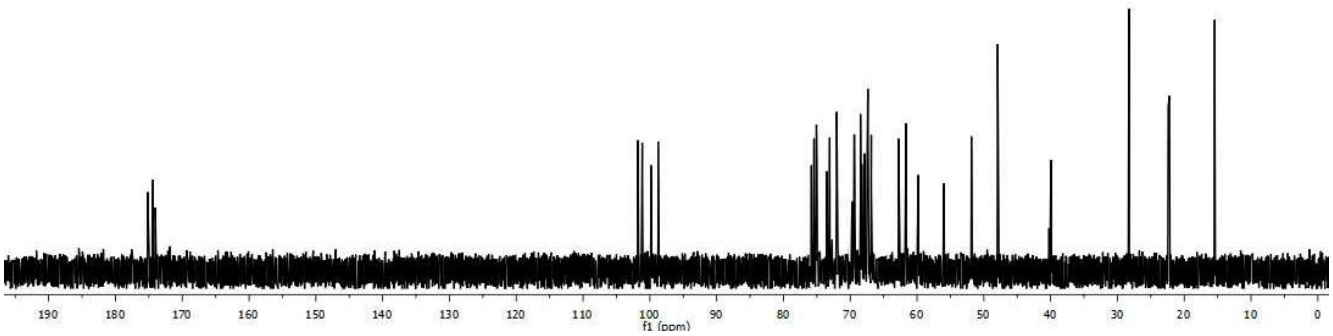
^{1}H and ^{13}C NMR spectra of Galβ4(Fucα3)GalNAcβ3Galβ4(Fucα3)GlcβProN_3 (**19**).





^{1}H and ^{13}C NMR spectra of Neu5Acα3Galβ4(Fucα3)GlcNAcβProN$_3$ (**20**).





^{1}H and ^{13}C NMR spectra of Fucα2Galβ3(Fucα4)GlcNAcβProN_3 (**22**).