Supporting Information

for

The one-pot multienzyme (OPME) synthesis of human blood group H antigens and a human milk oligosaccharide (HMOS) with highly active *Thermosynechococcus elongatus* α1–2-fucosyltransferase

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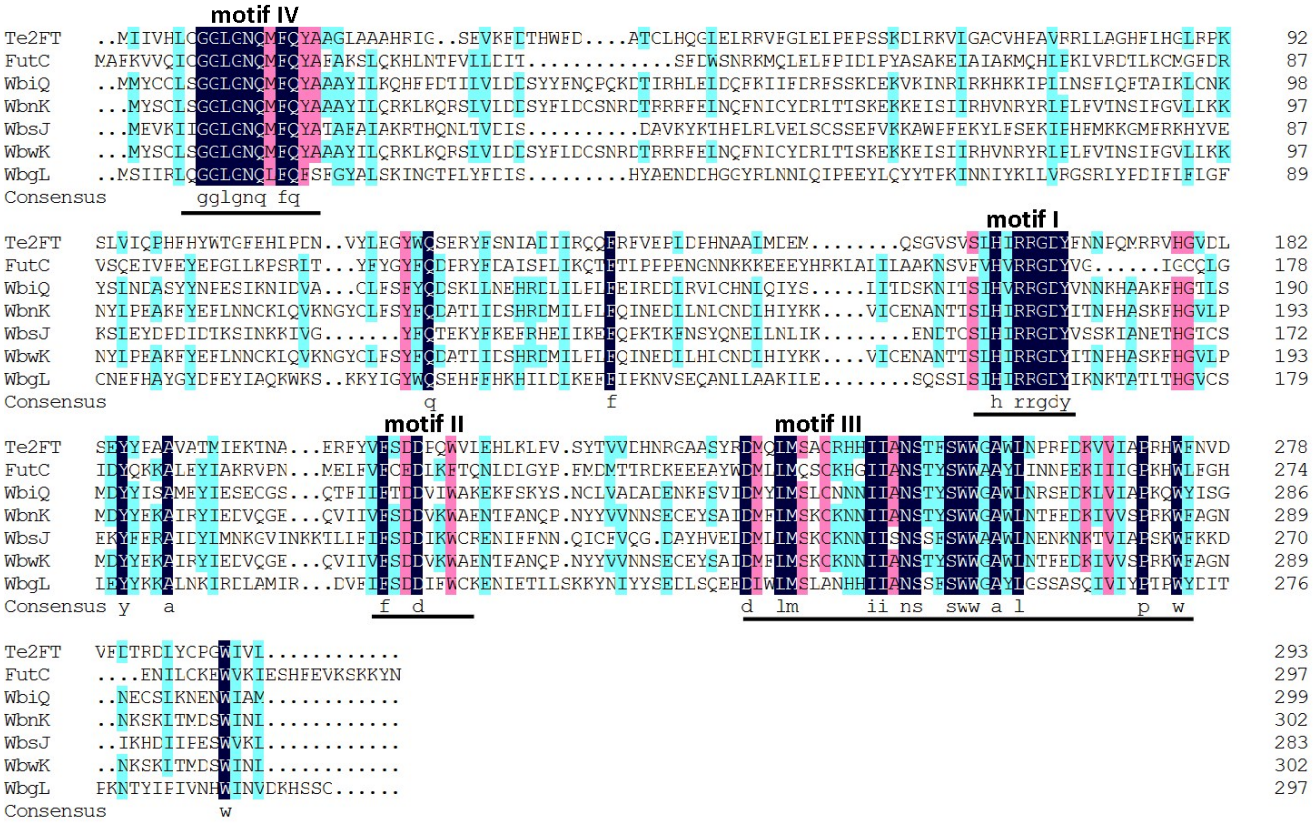


Fig. S1. Alignment of Te2FT (UniProtKB:Q8DK72, GenBank: BAC08546.1), *H. pylori* FutC (UniProtKB:A4L7J1), *E. coli* O127:K63 WbiQ (UniProtKB:Q5J7C6), *E. coli* O86:K62:H2 WbnK (UniProtKB:Q58YV9), *E. coli* O128:B12 WbsJ (UniProtKB:Q6XQ53), *E. coli* O86:B7 WbwK (GenBank:AAO37719.1), and *E. coli* O126 WbgL (UniProtKB: A6M9C2). The sequence alignment of the investigated genes indicates four common motifs (I–IV). The highly conserved motif I ($H^{162}xR^{164}R^{165}xD^{167}$) suggests a potential binding site for GDP-fucose. Residues R^{164} and D^{167} were indicated to play critical roles in donor binding and enzyme activity.[1]

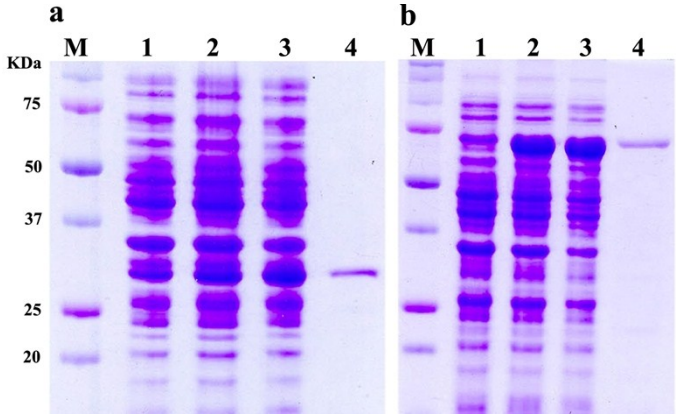


Fig. S2. SDS-PAGE analysis of His_6-Te2FT (**a**) and MBP-Te2FT-His_6 (**b**) expression and purification.

Lanes: M, protein standards; 1, whole cell extraction before induction; 2, whole cell extraction after induction; 3, cell lysate after induction; 4, Ni^{2+}-column purified protein.

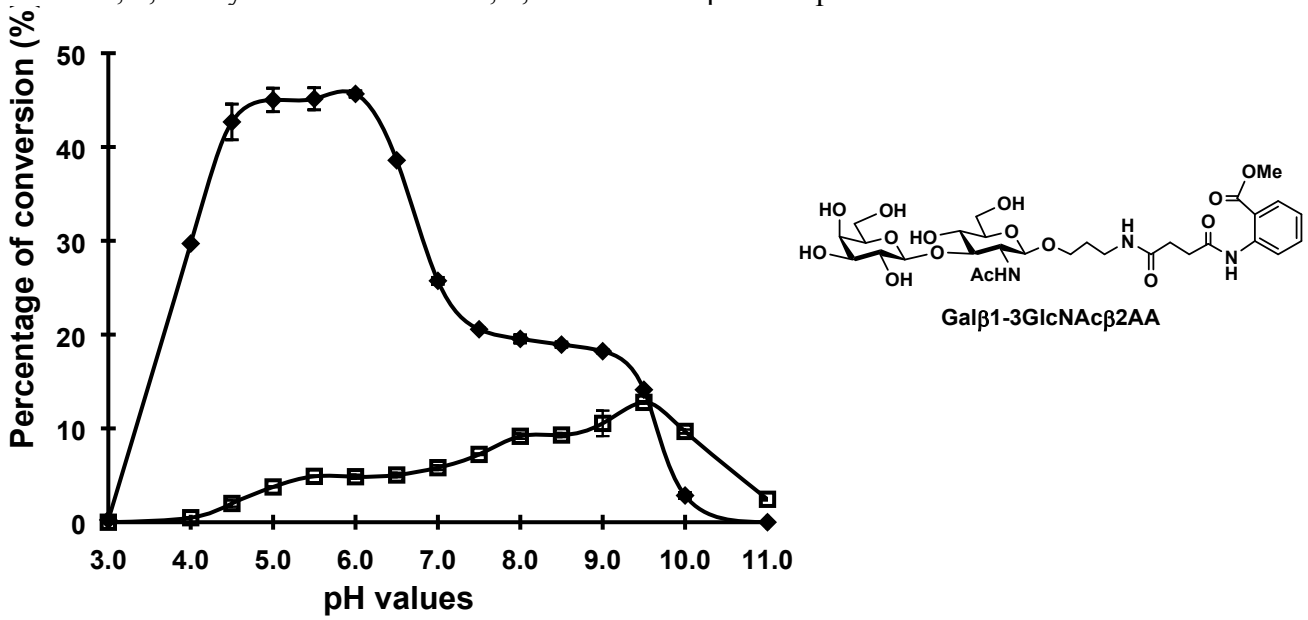


Fig. S3. The pH profiles of the α1–2-fucosyltransferase activity (filled diamond, Galβ1–3GlcNAcβ2AA was used as an acceptor substrate) and the GDP-fucose hydrolysis activity (open square) of His_6-Te2FT. Please notice that 15-fold more enzyme and 3-fold longer incubation time were used in the GDP-fucose hydrolysis activity assays than that in the α1–2-fucosyltransferase activity assays. Buffers used: Citric, pH 3.0–4.0; NaOAc/HOAc, pH 4.5; MES, pH 5.0–6.0; Tris-HCl, pH 7.0–9.0; *N*-cyclohexyl-3-aminopropanesulfonic acid (CAPS), pH 10.0–11.0.

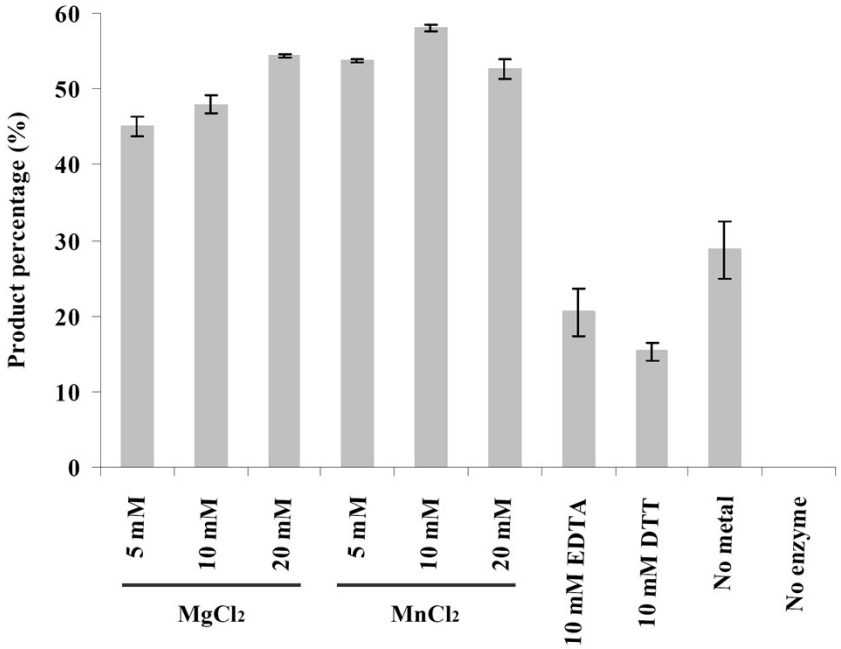


Fig. S4. Effects of metal ions ($Mg^{2+}$and Mn^{2+}), EDTA, and DTT (in the presence of 20 mM Mg^{2+}) on

the fucosyltransferase activity of His_6-Te2FT when Galβ1–3GlcNAcβ2AA was used as an acceptor substrate.

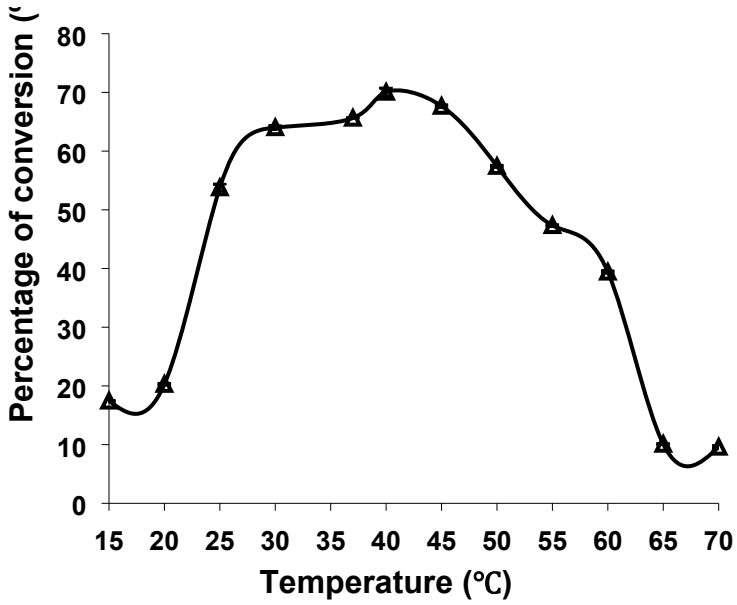


Fig. S5. Temperature profile of the fucosyltransferase activity of His_6-Te2FT when Galβ1–3GlcNAcβ2AA was used as an acceptor substrate.

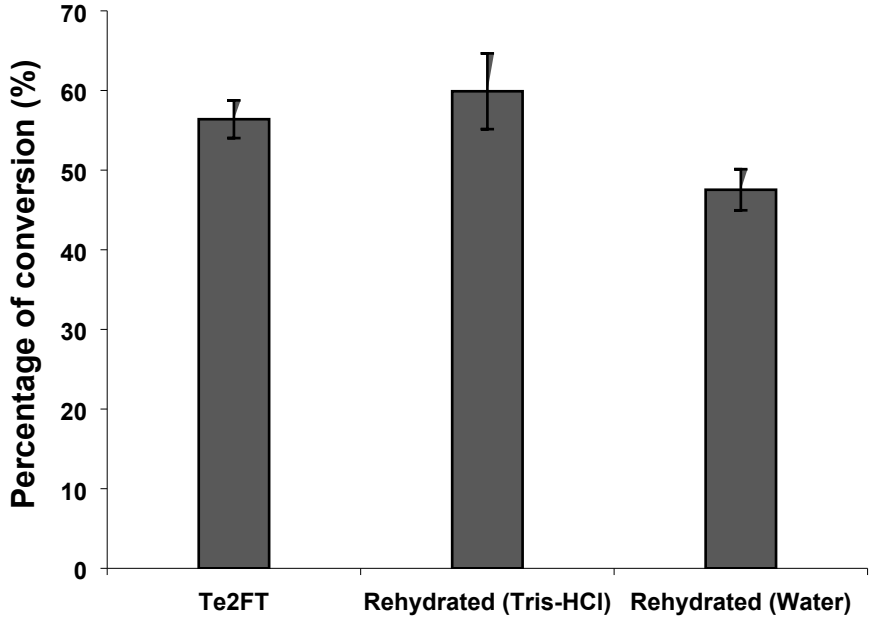


Fig. S6. Activities of His_6-Te2FT with or without lyophilization and rehydration (Galβ1–

3GlcNAcβ2AA was used as an acceptor substrate).

Bacterial strains, plasmids, and materials

Escherichia coli electrocompetent cells DH5α and chemically competent cells BL21 (DE3) were from Invitrogen (Carlsbad, CA, USA). Vector plasmids pET15b and pMAL-c4X were purchased from Novagen (EMD Biosciences Inc., Madison, WI, USA). QIAprep spin miniprep kit and QIAquick gel extraction kit were from Qiagen (Valencia, CA, USA). Herculase-enhanced DNA polymerase was from Stratagene (La Jolla, CA, USA). T4 DNA ligase and 1 kb DNA ladder were from Promega (Madison, WI, USA). *Nde*I, *BamH*I, *EcoR*I, and *Sal*I restriction enzymes, *Taq* 2× Master Mix, and amylose resin were from New England Biolabs (Beverly, MA, USA). Nickel-nitrilotriacetic acid agarose (Ni^{2+}-NTA agarose) was from 5 PRIME (Gaithersburg, MD, USA).

Protein sequence alignment

EMBOSS Needle alignment tool (http://www.ebi.ac.uk/bwK5Tools/psa/emboss_needle/) was used for protein sequence alignment. As shown in Figure S1, the hypothetical α1–2-fucosyltransferase encoded in cyanobacterium *T. elongatus* BP-1 genome (Te2FT) shares 32.3% identity and 48.3% similarity to *E. coli* O86:B7 WbwK belonging to Carbohydrate Active Enzyme (CAZy) glycosyltransferase family 11 (GT 11) that is involved in the biosynthesis of lipopolysaccharide (O-antigen) of the bacterium.[2] It also shares 32% identity to *E. coli* O128:B12 WbgL that has been used for the synthesis of 2′-fucosyllactose.[3] Sequence alignment indicates that Te2FT has four conserved motifs shared by GT11 family fucosyltransferases including motif I which is likely involved in GDP-fucose binding.[1]

Cloning, expression, and purification of His_6-Te2FT and MBP-Te2FT-His_6

Full-length synthetic gene for Te2FT with codons optimized for *E. coli* expression was customer synthesized by Biomatik (Wilmington, DE, USA) and provided in a pBSK(+) vector. The primers used for cloning His_6-Te2FT in pET15b vector were: forward primer 5′-GATCCATATGATTATCGTTCACCTGTGCG-3′ (*Nde*I restriction site is underlined) and reverse primer 5′-AAGGGATCCTTACAGAACAATCCAACCC-3′ (*BamH*I restriction site is underlined). The primers used for cloning the MBP-Te2FT-His_6 in pMAL-c4X vector were: forward primer 5′-GATCCATATGGTGAAAGTACTGACTGTATT-3′ (*EcoR*I restriction site is underlined) and reverse primer 5′-ACGCGTCGACTTAGTGGTGGTGGTGGTGGTGCAGAACAATCCAACCC-3′ (*Sal*I restriction site is underlined). Polymerase chain reactions (PCRs) for amplifying the target gene were performed in a 50 μL reaction mixture containing plasmid DNA (10 ng), forward and reverse primers (0.2 μM each), *Taq* 2× Master Mix. The PCR procedure included an initial cycle of 30 s at 95 °C, followed by 30 cycles of 30 s at 95 °C, 30 s at 56 °C, and 1 min at 68 °C. For the final extension, the reaction was held at 68 °C for 10 min. The resulting PCR product was purified and double digested with *Nde*I and *BamH*I or *EcoR*I and *Sal*I restriction enzymes. The purified and digested PCR products were ligated with the predigested pET15b vector or pMAL-c4X and transformed into electrocompetent *E. coli* DH5α cells. Positive plasmids were selected and transformed into *E. coli* BL21 (DE3) chemical competent cells. The plasmid-bearing *E. coli* strains were cultured in LB 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}). Overexpression of the target protein was achieved by inducing the *E. coli* culture with 0.1 mM of isopropyl-1-thio-β-D–galactopyranoside (IPTG) when $OD_{600\ nm}$ reached 0.6–0.8 followed by incubating at 16 °C for 20 h with shaking at 160 rpm in a C25KC incubator shaker (New Brunswick Scientific, Edison, NJ).

His_6-tagged target proteins were purified from cell lysate. To obtain the cell lysate, cell pellet harvested by centrifugation at 4000 rpm for 1 h was resuspended (30 mL L^{-1} cell culture) in lysis buffer (pH 8.0, 100 mM Tris-HCl containing 0.1% Triton X-100). Sonication protocol was 2s (sonication)/3s (rest) for a total of 7 min on ice. Cell lysate was obtained by centrifugation at 11,000 rpm for 25 min as the supernatant. Purification of His_6-tagged proteins from the lysate was achieved using a Ni^{2+}-resin column. The column was pre-equilibrated with 10 column volumes of binding buffer (5 mM imidazole, 0.5 M NaCl, 50 mM Tris-HCl, pH 7.5) before the lysate was loaded. After washing with 8 column volumes of binding buffer and 10 column volumes of washing buffer (50 mM imidazole, 0.5 M NaCl, 50 mM Tris-HCl, pH 7.5), the protein was eluted with an elute buffer (200 mM imidazole, 0.5 M NaCl, 50 mM Tris-HCl, pH 7.5).

The purified proteins were quantified in a 96-well plate using a Bicinchoninic acid (BCA) Protein Assay Kit (Pierce Biotechnology, Rockford, IL) with bovine serum albumin as a protein standard. The absorbance of samples was measured at 562 nm by a BioTek Synergy™ HT Multi-Mode Microplate Reader.

Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of His_6-Te2FT and MBP-Te2FT-His_6 (Fig. S2)

SDS-PAGE was performed in a 12% Tris-glycine gel using a Bio-Rad Mini-protein III cell gel electrophoresis unit (Bio-Rad) at DC = 125 V. Bio-Rad Precision Plus Protein Standards (10–250 kDa) were used as molecular weight standards. Gels were stained with Coomassie Blue.

pH profile of α1–2-fucosyltransferase activity of His_6-Te2FT by high-performance liquid chromatography (HPLC) (Fig. S3)

Assays were carried out in a total volume of 10 μL in a buffer (200 mM) with pH varying from 3.0 to 11.0. The buffers used were: Citric, pH 3.0–4.0, NaOAc/HOAc, pH 4.5, MES, pH 5.0–6.5; Tris-HCl, pH 7.0–9.0; and CAPS, pH 10.0–11.0. Reaction system was: GDP–fucose (1 mM), fluorophore 2-anthranilic acid (2AA)-labeled oligosaccharide (Galβ1–3GlcNAcβ2AA, 1 mM), $MgCl_2$ (20 mM), and the recombinant His_6-Te2FT (0.25 μg). All reactions were allowed to proceed for 10 min at 37 °C. The reaction mixture was stopped by boiling for 2 min and then adding equal volume water (10 μL) to make ten-fold dilution for Galβ1–3GlcNAcβ2AA. The samples were analyzed by a Shimadzu LC-2010A system equipped with a membrane online degasser, a temperature control unit and a fluorescence detector. A reverse-phase Premier C18 column (250 × 4.6 mm i.d., 5 μm particle size, Shimadzu) protected with a C18 guard column cartridge was used. The mobile phase was 20% acetonitrile. The ratio of the absorbance for fluorescent-labeled product at 315–400 nm was determined. All assays were carried out in duplicate.

pH profile of GDP–fucose hydrolysis of His_6-Te2FT by capillary electrophoresis assays (Fig. S3)

Assays were performed in a total volume of 10 μL in a buffer (200 mM) with pH varying from 3.0 to 11.0 (see above for the buffers used) containing GDP-fucose (1 mM), $MgCl_2$ (20 mM) and the recombinant enzyme (3.75 μg). Reactions were allowed to proceed for 30 min at 37 °C before being stopped by boiling for 2 min followed by adding an equal volume of water (10 μL) to make a two-fold dilution. The samples were kept on ice until aliquots of 6 μL were withdrawn and analyzed by a Beckman P/ACE MDQ capillary electrophoresis system (60 cm × 75 μm i.d.) with a PDA detector. The ratio of the absorbance for GDP–fucose and GDP at 254 nm was determined at different concentrations (2.5, 5, and 10 mM). All assays were carried out in duplicate.

Effects of metal ions, dithiothreitol (DTT) and ethylenediaminetetraacetic acid (EDTA) (Fig. S4)
Different concentrations (5 mM, 10 mM, 20 mM) of $MgCl_2$ or $MnCl_2$, EDTA (10 mM) and DTT (10 mM) were used in a Tris-HCl buffer (pH 7.5, 200 mM) to analyze their effects on the α1–2-fucosyltransferase activity of His_6-Te2FT for acceptor Galβ1–3GlcNAcβ2AA. Reaction without metal ions, EDTA and DTT was used as a control. The effects of metal ions Mg^{2+} and Mn^{2+} as well as DTT and the chelating agent EDTA on the α1–2-fucosyltransferase activity of His_6-Te2FT toward and Galβ1–3GlcNAcβ2AA were examined at pH 7.5.

Optimal temperature for the α1–2-fucosyltransferase activity of His_6-Te2FT (Fig. S5)
Assays were carried out in a total volume of 10 μL in a Tris-HCl buffer (pH 6.0, 200 mM) at temperatures ranging from 15 °C to 70 °C. Reaction system was: GDP–fucose (1 mM), $MgCl_2$ (20 mM), Galβ1–3GlcNAcβ2AA (1 mM), and the recombinant His_6-Te2FT (1.5 μg). All reactions were allowed to proceed for 10 min at different temperatures. The reaction mixture was stopped by boiling for 2 min, and diluted 60-fold for detection.

Survive of freeze-dry cycle of His_6-Te2FT (Fig. S6)
The purified His_6-Te2FT samples were dialyzed against 20 mM Tris-HCl without any glycerol and lyophilized. The Te2FT powders were stored at –70 °C for 20 days. After that, the dried powder was dissolved in water and 20 mM Tris-HCl buffer, respectively. The activities were compared with the same amount and concentration of freshly prepared Te2FT samples. Reaction setup was: GDP–fucose (1 mM), Galβ1–3GlcNAcβ2AA, (1 mM), $MgCl_2$ (20 mM), and His_6-Te2FT (0.75 μg). All reactions were allowed to proceed for 10 min at 37 °C. All assays were carried out in duplicate.

Kinetics of α1–2-fucosyltransferase activity by HPLC assays
Typical enzymatic assays were carried out in a total volume of 10 μL in Tris-HCl buffer (pH 6.0, 200 mM) containing $MgCl_2$ (20 mM), GDP-fucose, Galβ1–3GlcNAcβ2AA, and the recombinant His_6-Te2FT (0.75 μg). All reactions were allowed to proceed for 10 min at 37 °C. Apparent kinetic parameters were obtained by varying the GDP-fucose concentration from 0.1 to 10.0 mM (0.1, 0.2, 0.4, 1.0, 5.0, and 10.0 mM) and a fixed concentration of Galβ1–3GlcNAcβ2AA (1 mM); or a fixed concentration of GDP-fucose (1 mM) and varied concentrations of Galβ1–3GlcNAcβ2AA from 0.1 to 10.0 mM (0.1, 0.2, 0.4, 5.0, and 10.0 mM). The reaction mixture was quenched by boiling for 2 min, and diluted 60-fold for detection. All assays were carried out in duplicate. Apparent kinetic parameters were obtained by fitting the data into the Michaelis–Menten equation using Grafit 5.0.

Kinetics of GDP–fucose hydrolysis by capillary electrophoresis assays
The enzymatic assays were carried out in a total volume of 10 μL in Tris–HCl buffer (200 mM, pH 9.0) containing $MgCl_2$ (20 mM), GDP–fucose and the recombinant protein (4.5 μg). Reactions were allowed to proceed for 30 min at 37 °C. Apparent kinetic parameters were obtained by varying the final GDP-fucose concentration from 0.4 to 2.0 mM (0.4, 0.5, 0.6, 0.8, 1.0 and 2.0 mM). Apparent kinetic parameters were obtained by fitting the data (the average values of duplicate assay results) 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 or 5% sulfuric acid in ethanol 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, D-galactose, and *N*-acetyl-D-glucosamine (D-GlcNAc) were from Fisher Scientific (Pittsburgh, Pennsylvania, USA). L-Fucose was from V-LABS (Covington, Louisiana, USA). Lacto-*N*-tetraose (LNT) was from Elicityl (Crolles, France). Guanidine 5'-triphosphate (GTP) was from Hangzhou Meiya Pharmacy (Hangzhou, China). Adenosine 5'-triphosphate (ATP) was from Beta Pharma Scientific, Inc. (Branford, Connectic, USA) Recombinant enzymes *Bacteroides fragilis* strain NCTC9343 bifunctional L-fucokinase/GDP-fucose pyrophosphorylase (FKP)[4] and *Pasteurella multocida* inorganic pyrophosphatase (PmPpA)[5] were expressed and purified as described previously. Compounds Galβ1–3GalNAcαProN$_3$, Galβ1–3GalNAcβProN$_3$, Galβ1–3GlcNAcαProN$_3$, Galβ1–3GlcNAcβProN$_3$ were synthesized as described previously.[6]

General procedures for the synthesis of 2AA-labeled saccharides Galβ1–3GlcNAcβ2AA, Galβ1–4GlcNAcβ2AA, and Galβ1–4Glcβ2AA

Galβ1–3GlcNAcβProN$_3$, Galβ1–4GlcNAcβProN$_3$, or Galβ1–4GlcβProN$_3$ (50–60 mg) was dissolved in 5 mL water and 100 mg Pd/C was added. The mixture was shaken under H_2 (4 Bar) for 1 h and filtered. The filtrate was evaporated to dryness to afford the corresponding amine product which was used for the next step without purification. To the solution of glycan-amine in 5 mL anhydrous DMF was added triethylamine (60 μL) under argon. Two equivalents of *N*-hydroxysuccinimidyl 2AA were then added at 0 °C. The resulted solution was stirred at room temperature for overnight. The reaction mixture was concentrated and the residue was purified by flash column chromatography (EA:MeOH:H_2O = 8:2:1, by volume) to produce the corresponding 2AA-labeled oligosaccharides.

Galβ1–3GlcNAcβ2AA. 51 mg, 92%. ^{1}H NMR (800 MHz, D_2O): δ 7.91 (d, *J* = 8.0 Hz, 1H), 7.84 (d, *J* = 8.0 Hz, 1H), 7.59 (t, *J* = 8.0 Hz, 1H), 7.26 (t, *J* = 8.0 Hz, 1H), 4.43 (d, *J* = 8.8 Hz, 1H), 4.37 (d, *J* = 8.0 Hz, 1H), 3.88 (d, *J* = 4.0 Hz, 1H), 3.87 (s, 3H), 3.86–3.84 (m, 2H), 3.79–3.59 (m, 6H), 3.61 (dd, *J* = 9.6 and 3.2 Hz, 1H), 3.53 (m, 1H), 3.49 (t, *J* = 8.0 Hz, 1H), 3.48 (t, *J* = 8.8 Hz, 1H), 3.37 (m, 1H), 3.24 (m, 1H), 3.14 (m, 1H), 2.71 (t, *J* = 7.2 Hz, 2H), 2.58 (t, *J* = 7.2 Hz, 2H), 1.98 (s, 3H), 1.71 (m, 2H); ^{13}C NMR (200 MHz, D_2O): δ 174.48, 174.24, 173.09, 168.90, 137.18, 133.93, 130.77, 124.95, 122.86, 120.11, 103.40, 100.77, 82.39, 75.19, 75.15, 72.35, 70.53, 68.55, 68.38, 67.48, 60.87, 60.56, 54.38, 52.64, 35.98, 32.29, 30.75, 28.20, 22.07. HRMS (ESI) m/z calcd for $C_{29}H_{43}N_3NaO_{15}$ (M+Na) 696.2592, found 696.2585.

Galβ1–4GlcNAcβ2AA. 68 mg, 90%. ^{1}H NMR (800 MHz, D_2O): δ 7.96 (d, *J* = 8.0 Hz, 1H), 7.81 (d, *J* = 8.0 Hz, 1H), 7.62 (t, *J* = 8.0 Hz, 1H), 7.31 (t, *J* = 8.0 Hz, 1H), 4.42 (d, *J* = 8.0 Hz, 1H), 4.40 (d, *J* = 8.8 Hz, 1H), 3.98–3.51 (m, 16H), 3.46 (m, 1H), 3.25 (m, 1H), 3.14 (m, 1H), 2.74 (t, *J* = 8.0 Hz, 2H), 2.59 (t, *J* = 8.0 Hz, 2H), 1.98 (s, 3H), 1.71 (m, 2H); ^{13}C NMR (200 MHz, D_2O): δ 174.37, 174.31, 173.31, 168.97, 136.87, 133.85, 130.76, 125.20, 123.32, 120.87, 102.75, 100.97, 78.30, 75.21, 74.57, 72.40, 72.30, 70.82, 68.41, 67.47, 60.88, 59.89, 54.90, 52.65, 35.95, 32.17, 30.76, 28.18, 21.99. HRMS

(ESI) m/z calcd for $C_{29}H_{43}N_3NaO_{15}$ (M+Na) 696.2592, found 696.2589.

Galβ1–4Glcβ2AA. 55 mg, 91%. ^{1}H NMR (800 MHz, D_2O): δ 7.95 (d, *J* = 8.0 Hz, 1H), 7.84 (d, *J* = 8.8 Hz, 1H), 7.61 (t, *J* = 8.0 Hz, 1H), 7.30 (t, *J* = 8.0 Hz, 1H), 4.41 (d, *J* = 8.0 Hz, 1H), 4.33 (d, *J* = 8.0 Hz, 1H), 3.92–3.52 (m, 13H), 3.45 (m, 1H), 3.31–3.23 (m, 5H), 2.74 (t, *J* = 7.2 Hz, 2H), 2.59 (t, *J* = 7.2 Hz, 2H), 1.77 (m, 2H); ^{13}C NMR (200 MHz, D_2O): δ 174.34, 173.23, 168.95, 137.03, 133.90, 130.78, 125.10, 123.12, 120.52, 102.81, 101.93, 78.24, 75.22, 74.58, 74.21, 72.65, 72.41, 70.82, 68.41, 67.50, 60.89, 59.91, 52.65, 36.04, 32.27, 30.85, 28.24. HRMS (ESI) m/z calcd for $C_{27}H_{40}N_2NaO_{15}$ (M+Na) 655.2326, found 655.2326.

One-pot three-enzyme preparative-scale synthesis of α1–2-linked fucosides

Galactoside (30–60 mg each, 1 equiv.), 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 (FKP, 1.5 mg),[4] *Pasteurella multocida* inorganic pyrophosphatase (PmPpA) (1.0 mg), and His_6-Te2FT (1.5–2.0 mg). All other reactions were carried out at pH 7.5. 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 was obtained by passing through a BioGel P-2 gel filtration column again for removing any silica gel dissolved.

Gram-scale synthesis of Fucα1–2LNT (LNFP I, Fucα1–2Galβ1–3GlcNAcβ1–3Galβ1–4Glc)

Lacto-*N*-tetraose (LNT, 1 g), L-fucose (1.5 equiv.), ATP (1.5 equiv.), and GTP (1.5 equiv.) were dissolved in Tris-HCl buffer (140 mL, 100 mM, pH 7.0) containing $MgCl_2$ (20 mM) and appropriate amounts of a recombinant L-fucokinase/GDP-fucose pyrophosphorylase (FKP, 30 mg), *Pasteurella multocida* inorganic pyrophosphatase (PmPpA, 30 mg), and His_6-Te2FT (25–30 mg). The reactions were incubated in an incubator shaker at 37 °C for 1–2 days with agitation at 100 rpm. The product formation was monitored by mass spectrometry and thin layer chromatography (TLC). 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 then 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 used for further purification of LNFP-I using EtOAc:MeOH:H_2O = 5:3:2 (by volume) as the mobile phase. The obtained LNFP I sample was further purified used activated charcoal. To a 50 mL centrifuge tube, 2 g of charcoal were added. Ethanol (absolute ethanol or 90%+ ethanol) (30 mL) was added to the tube and it was mixed thoroughly by inverting the tube. The tube was centrifuged at 12,000 rpm for 30 min and the supernatant was decanted. The process was repeated once. Water (30 mL) was added to tube and it was mixed thoroughly by inverting the tube followed by centrifugation for 30 min and supernatant was decanted. The tube was left at room temperature for 30 min in a fume hood to evaporate any residual of ethanol. Crude LNFP I (100 mg) was added to tube with 30 mL of water and the tube was mixed thoroughly. The tube was then put into a shaker with agitation (100 rpm) for 1–2 h at 37 °C. The tube was centrifuged at 12,000 rpm for 30 min to spin down the charcoal and

the supernatant was discarded by decanting. Finally, 50% methanol (30 mL) was added to tube and it was mixed thoroughly by inverting the tube followed by centrifugation at 12,000 rpm for 30 min. The supernatant was collected and passed through a paper filter to remove any charcoal particles. The process was repeated. The filtered solution was combined and lyophilized to produce pure LNFP I as a white solid with an excellent yield of 95%. NMR and HRMS analyses were carried out to confirm the identify and the purity of the product.

Fucα1–2Galβ1–3GalNAcαProN$_3$ (1). 35.7 mg, 95%, ^{1}H NMR (800 MHz, D_2O): δ 5.17 (d, *J* = 4.0 Hz, 1H), 4.62 (d, *J* = 8.0 Hz, 1H), 4.21 (dd, *J* = 13.6 and 6.4 Hz, 1H), 4.12 (t, *J* = 9.6 Hz, 1H), 3.84-3.42 (m, 19 H), 2.04 (s, 3 H), 1.87 (m, 2 H), 1.19 (d, *J* = 7.2 Hz, 3H); ^{13}C NMR (200 MHz, D_2O): δ 173.52, 101.91, 99.14, 96.63, 76.09, 74.89, 73.90, 73.39, 71.71, 70.43, 69.46, 68.95, 67.94, 66.68, 64.64, 62.55, 61.08, 60.82, 49.41, 48.01, 27.91, 21.78, 15.26. HRMS (ESI) m/z calculated for $C_{23}H_{40}N_4NaO_{15}$ (M+Na) 635.2388, found 635.2383.

Fucα1–2Galβ1–3GalNAcβProN$_3$ (2). 43.8 mg, 98%, ^{1}H NMR (800 MHz, D_2O): δ 5.21 (d, *J* = 4.8 Hz, 1H), 4.59 (d, *J* = 8.0 Hz, 1H), 4.31 (d, *J* = 8.0 Hz, 1H), 4.21 (dd, *J* = 13.6 and 7.2 Hz, 1H), 4.08 (d, *J* = 2.4 Hz, 1H), 3.97-3.58 (m, 16H), 3.34 (m, 2 H), 2.04 (s, 3 H), 1.81 (m, 2 H), 1.19 (d, *J* = 6.4 Hz, 3H); ^{13}C NMR (200 MHz, D_2O): δ 173.58, 102.54, 101.95, 99.08, 76.48, 75.88, 74.94, 74.67, 73.43, 71.66, 69.39, 68.99, 68.38, 67.90, 66.86, 66.68, 60.86, 60.82, 51.29, 47.61, 28.07, 22.14, 15.14. HRMS (ESI) m/z calculated for $C_{23}H_{40}N_4NaO_{15}$ (M+Na) 635.2388, found 635.2395.

Fucα1–2Galβ1–3GlcNAcαProN$_3$ (3). 51.3 mg, 95%, ^{1}H NMR (800 MHz, D_2O): δ 5.21 (d, *J* = 3.2 Hz, 1H), 4.85 (d, *J* = 3.2 Hz, 1H), 4.62 (d, *J* = 8.0 Hz, 1H), 4.21 (dd, *J* = 13.6 and 6.4 Hz, 1H), 4.17 -3.41 (m, 19H), 2.03 (s, 3 H), 1.87 (m, 2H), 1.18 (d, *J* = 6.4 Hz, 3H); ^{13}C NMR (200 MHz, D_2O): δ 173.54, 100.12, 99.36, 96.82, 76.48, 75.27, 74.89, 73.38, 71.71, 71.48, 69.38, 69.03, 68.54, 67.91, 66.37, 64.77, 60.96, 60.31, 53.25, 48.03, 27.91, 21.72, 15.19. HRMS (ESI) m/z calculated for $C_{23}H_{40}N_4NaO_{15}$ (M+Na) 635.2388, found 635.2391.

Fucα1–2Galβ1–3GlcNAcβProN$_3$ (4). 50.5 mg, 96%, ^{1}H NMR (800 MHz, D_2O): δ 5.16 (d, *J* = 3.2 Hz, 1H), 4.62 (d, *J* = 7.2 Hz, 1H), 4.40 (d, *J* = 7.2 Hz, 1H), 4.27-3.32 (m, 20H), 2.05 (s, 3H), 1.81 (m, 2H), 1.20 (d, *J* = 6.4 Hz, 3H); ^{13}C NMR (200 MHz, D_2O): δ 173.55, 101.66, 100.07, 99.37, 77.11, 76.51, 75.29, 74.93, 73.32, 71.66, 69.29, 69.00, 68.61, 67.91, 66.97, 66.37, 61.03, 60.55, 54.73, 47.61, 28.04, 22.06, 15.07. HRMS (ESI) m/z calculated for $C_{23}H_{40}N_4NaO_{15}$ (M+Na) 635.2388, found 635.2393.

Fucα1–2LNT or LNFP I (5). 68.1 mg, 94% and 1.146 g, 95%, ^{1}H NMR (800 MHz, D_2O): δ 5.19 (d, *J* = 4.0 Hz, 0.4H), 5.17 (d, *J* = 4.0 Hz, 1H), 4.64 (d, *J* = 8.0 Hz, 0.6H), 4.62 (d, *J* = 8.0 Hz, 1H), 4.60 (dd, *J* = 8.0 nad 2.4 Hz, 1H), 4.40 (d, *J* = 8.0 Hz, 1H), 4.26 (dd, *J* = 13.6 and 6.4 Hz, 1H), 4.15 (d, *J* = 3.2 Hz, 1H), 3.98-3.24 (m, 26H), 2.03 (s, 3H), 1.21 (d, *J* = 6.4 Hz, 3H); ^{13}C NMR (200 MHz, D_2O): δ 174.13, 103.11, 102.82, 102.78, 100.12, 99.39, 95.60, 91.68, 81.43, 81.40, 78.06, 77.98, 77.00, 76.54, 75.11, 74.93, 74.69, 74.19, 73.65, 73.35, 71.72, 71.24, 70.99, 70.10, 70.07, 70.02, 69.29, 68.99, 68.49, 68.47, 68.33, 67.91, 66.36, 61.02, 60.83, 60.25, 59.92, 59.79, 54.84, 22.00, 15.13. HRMS (ESI) m/z calculated for $C_{32}H_{55}NnaO_{25}$ (M+Na) 876.2961, found 876.2959.

Growth of *B. infantis* 15697 and *B. animalis* 27536 on medium supplemented with glucose (Glc), human milk oligosaccharides (HMOS), or LNFP I.

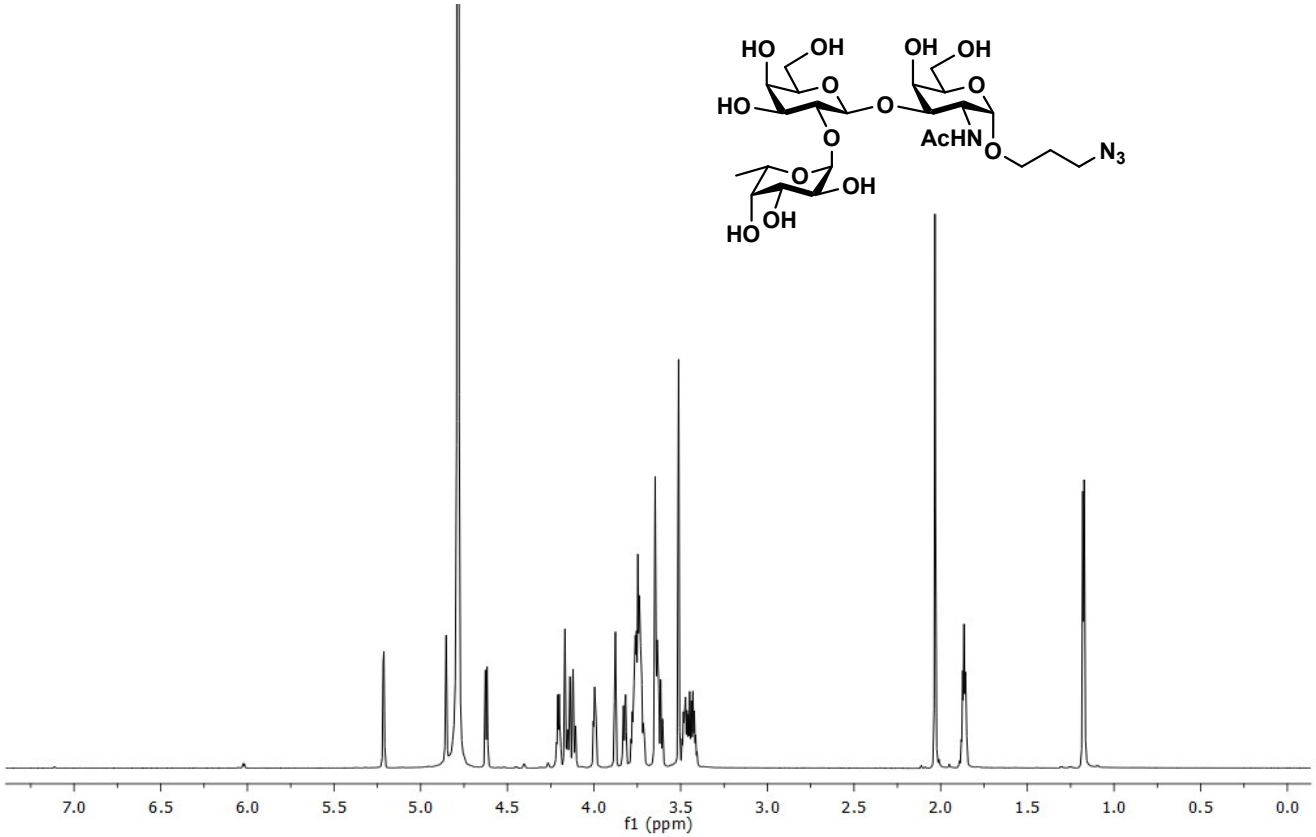
B. infantis 15697 and *B. animalis* 27536 were tested for growth in the presence of glucose, HMO, and

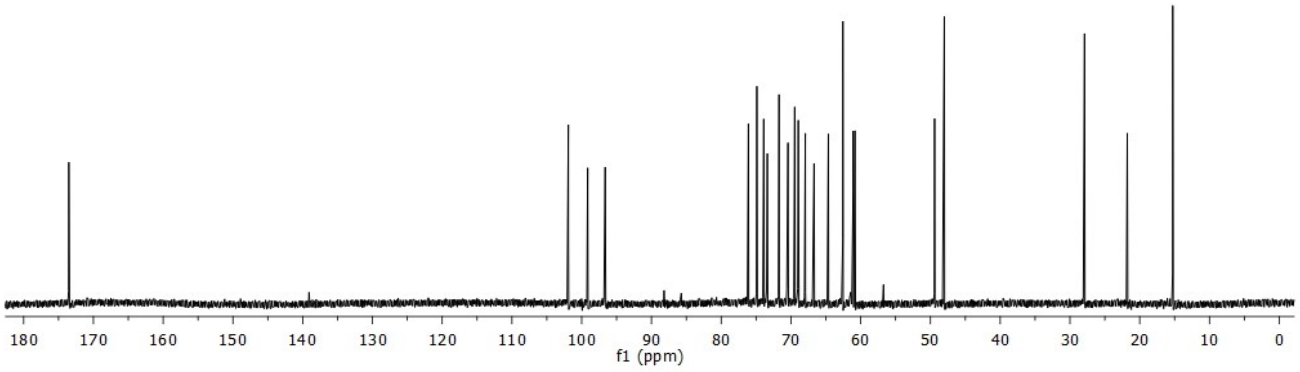
LNFP I. Growth assays were performed as previously described.[7] Briefly, two microliters of each resulting overnight culture was used to inoculate 150 μL of modified MRS medium (mMRS), devoid of glucose and supplemented with 2% (wt/vol) of each sterile-filtered substrate as the sole carbohydrate source. The medium was supplemented with 0.05% (wt/vol) l-cysteine, and in all the cases the cultures in the wells of the microtiter plates were covered with 30 μL of sterile mineral oil to avoid evaporation. The incubations were carried out at 37 °C in an anaerobic chamber (Coy Laboratory Products). Cell growth was monitored in real time by assessing optical density at 600 nm (OD600) using a BioTek PowerWave 340 plate reader (BioTek, Winoosky, VT) every 30 min, preceded by 30 s of shaking at variable speed. Two biological replicates and three technical replicates each were performed for every studied strain. Fermentations were carried out in triplicate.

References

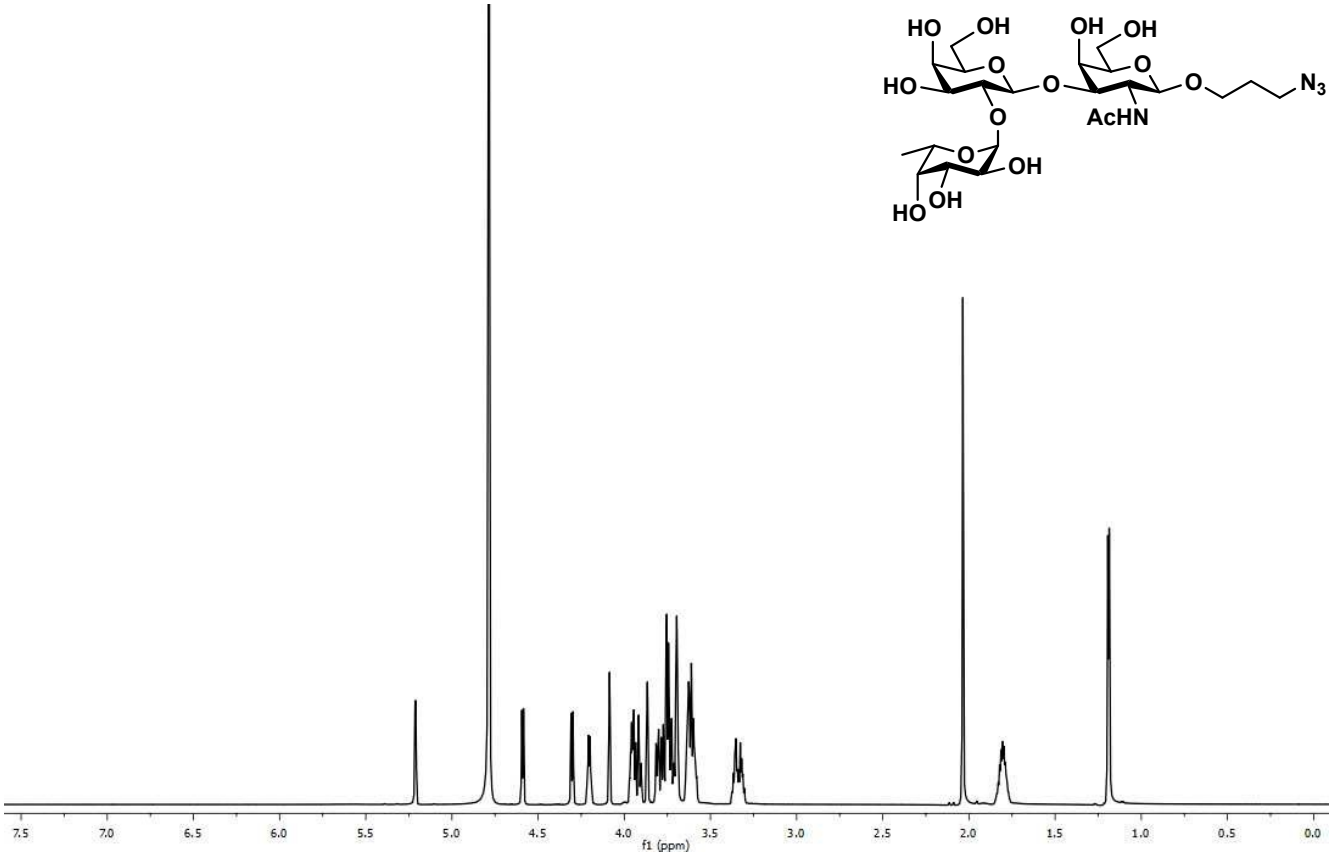
1. M. Li, J. Shen, X. Liu, J. Shao, W. Yi, C. S. Chow and P. G. Wang, *Biochemistry*, 2008, **47**, 11590–11597.
2. J. Shao, M. Li, Q. Jia, Y. Lu and P. G. Wang, *FEBS Lett.*, 2003, **553**, 99–103.
3. L. Engels and L. Elling, *Glycobiology*, 2014, **24**, 170–178.
4. W. Yi, X. Liu, Y. Li, J. Li, C. Xia, G. Zhou, W. Zhang, W. Zhao, X. Chen and P. G. Wang, *Proc. Natl. Acad. Sci. U. S. A.*, 2009, **106**, 4207–4212.
5. K. Lau, V. Thon, H. Yu, L. Ding, Y. Chen, M. M. Muthana, D. Wong, R. Huang and X. Chen, *Chem. Commun.*, 2010, **46**, 6066–6068.
6. H. Yu, V. Thon, K. Lau, L. Cai, Y. Chen, S. Mu, Y. Li, P. G. Wang and X. Chen, *Chem. Commun.*, 2010, **46**, 7507–7509.
7. S. Ruiz-Moyano, S. M. Totten, D. A. Garrido, J. T. Smilowitz, J. B. German, C. B. Lebrilla and D. A. Mills, *Appl. Environ. Microbiol.*, 2013, **79**, 6040–6049.

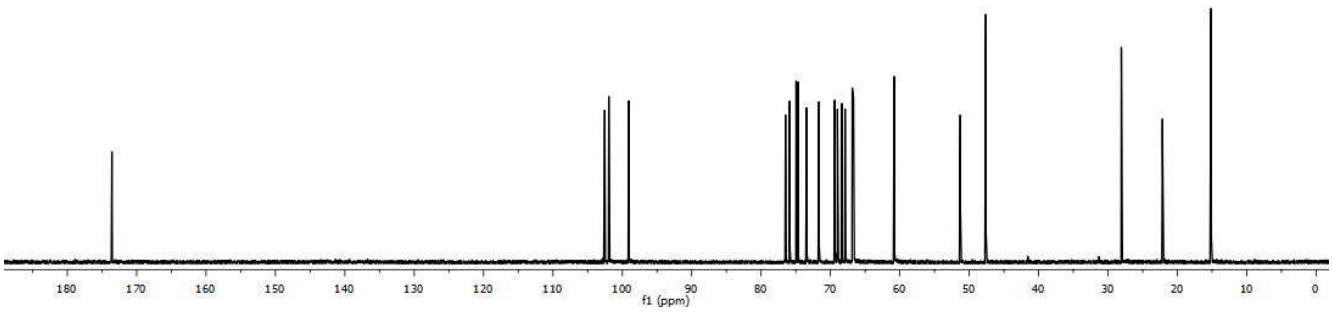
^{1}H and ^{13}C NMR spectra of Fucα1–2Galβ1–3GalNAcαProN_3 (**1**)



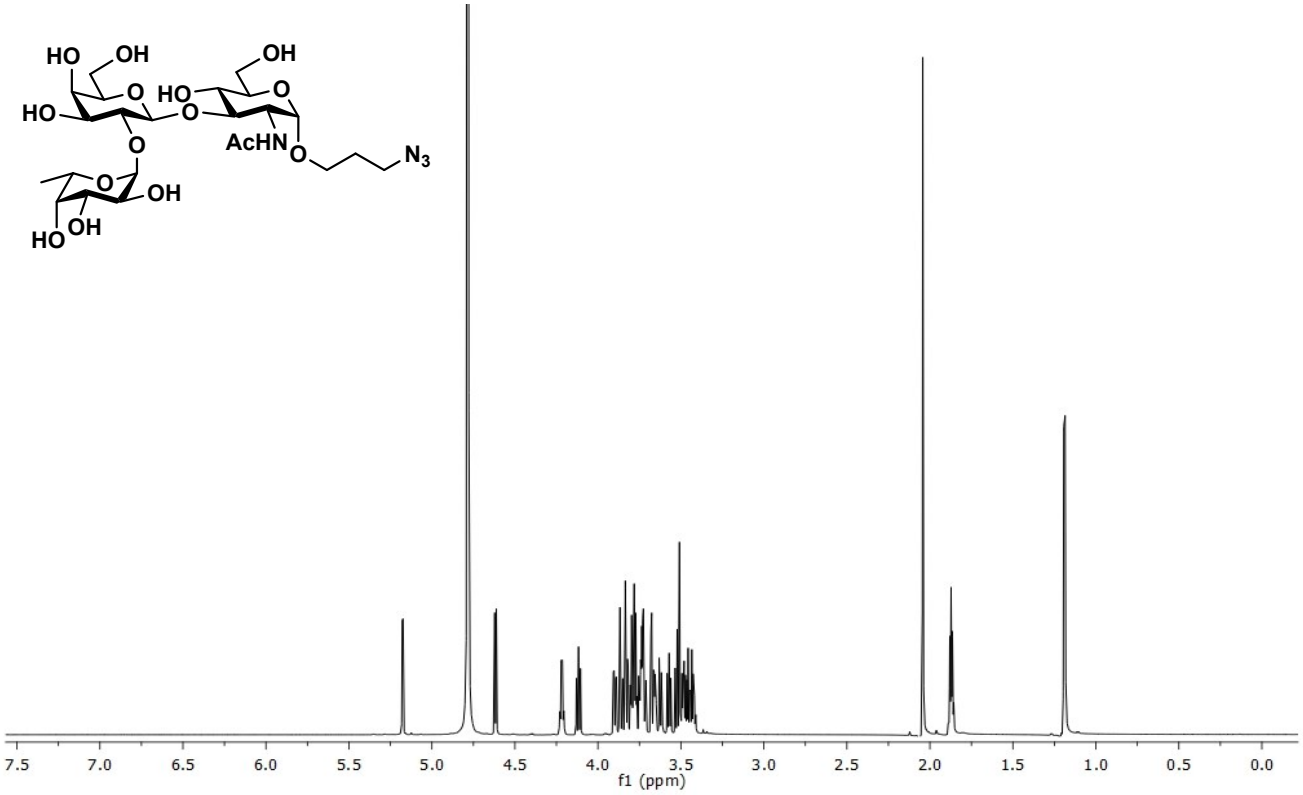


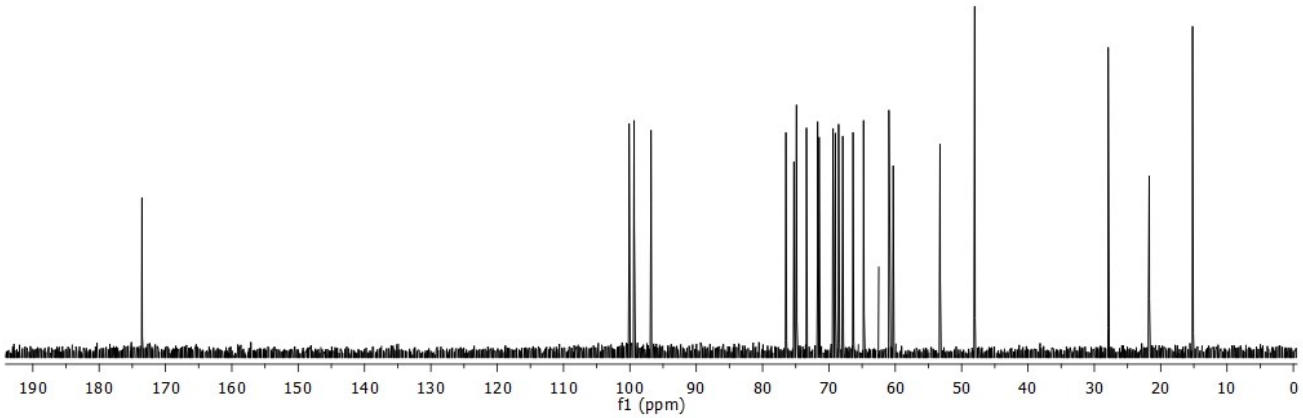
^{1}H and ^{13}C NMR spectra of Fucα1–2Galβ1–3GalNAcβProN_3 (**2**)



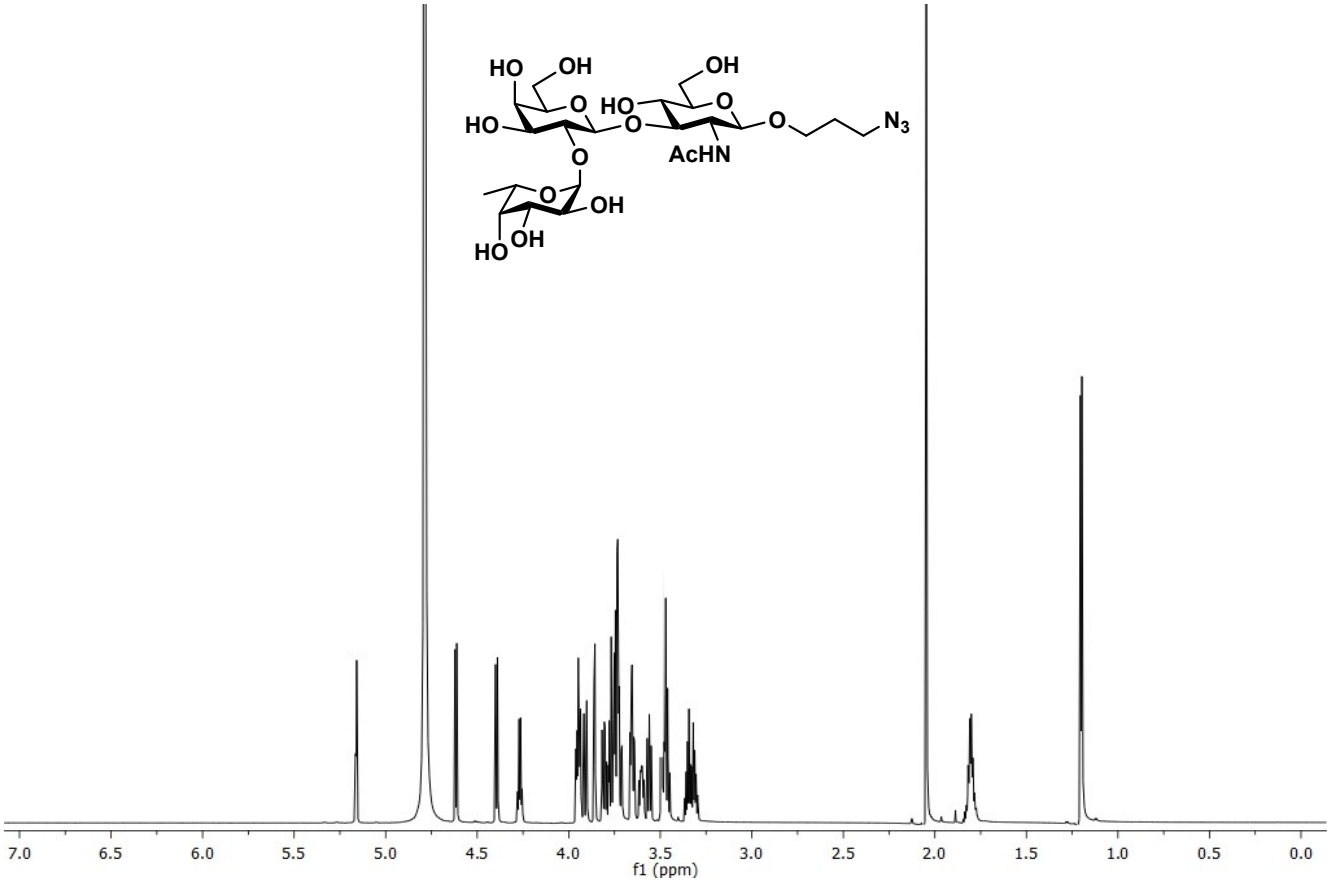


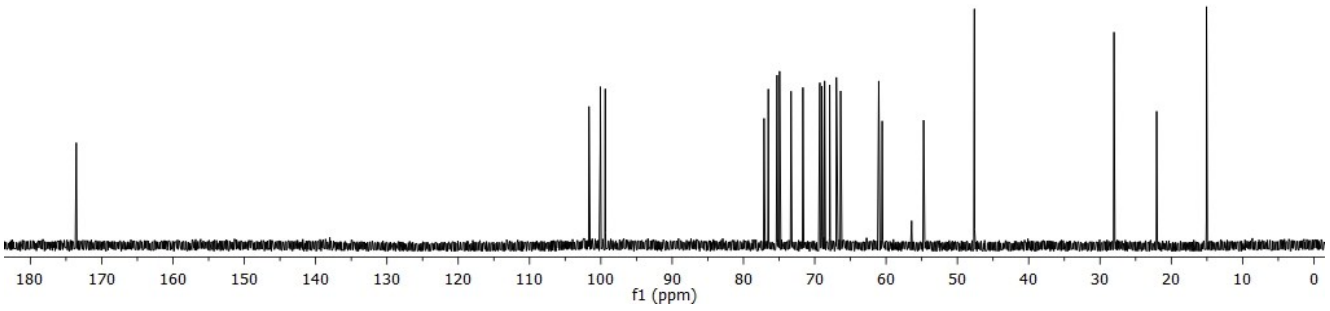
^{1}H and ^{13}C NMR spectra of Fucα1–2Galβ1–3GlcNAcαProN_3 (**3**)





^{1}H and ^{13}C NMR spectra of Fucα1–2Galβ1–3GlcNAcβProN_3 (**4**)





^{1}H and ^{13}C NMR spectra of Fucα1–2LNT or LNFP I (**5**)