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Enzymatic Synthesis of Human Milk Fucosides α1,2-Fucosyl *para*-Lacto-*N*-Hexaose and its Isomeric Derivatives

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Abstract. Enzymatic synthesis of *para*-lacto-*N*-hexaose and its isomeric structures as well as those α1,2-fucosylated variants naturally occurring in human milk oligosaccharide (HMOs) was achieved using a sequential one-pot enzymatic system. Three glycosylation routes comprising bacterial glycosyltransferases and corresponding sugar-nucleotide–generating enzymes were developed to facilitate efficient production of extended type-1 and type-2 *N*-acetyllactosamine (LacNAc) backbones and hybrid chains. Further fucosylation efficiency of two α1,2-fucosyltransferases on both type-1 and type-2 chains of the hexasaccharide was investigated to achieve practical synthesis of the fucosylated glycans. The availability of structurally defined HMOs offers a practical approach for investigating future biological applications.

Keywords: Human milk oligosaccharides; Chemoenzymatic synthesis; Glycosyltransferases; Fucosylation; Human microbiota

Human milk, which contains a variety of bioactive compounds such as proteins, fat globules, and oligosaccharides, is an essential source of nutrition for infants.[1-2] Human milk oligosaccharides (HMOs) are a family of structurally diverse unconjugated glycans and are the third most abundant solid component of human milk following lactose and lipids. Instead of being digested by infants, HMOs serve as prebiotics for beneficial bacteria, thus shaping the intestinal microbiota.[3-5] Because of their structural similarity to some intestinal mucosal cell surface glycans, HMOs also act as decoys and disrupt the binding of microbial or viral lectins to host cell receptors, thereby preventing infections of the host by these organisms.[6-7] Increasing evidence has demonstrated that HMOs modulate epithelial and immune responses and reduce excessive mucosal leukocyte infiltration and activation, decreasing the risk of necrotizing enterocolitis.[8-10]

HMOs comprise five monosaccharides, namely glucose (Glc), galactose (Gal), *N*-acetyl glucosamine (GlcNAc), fucose (Fuc), and sialic acid. The core structure of HMOs is a lactose moiety at the reducing end, which can be extended by disaccharide units and rarely found monosaccharide, for example, GlcNAc serves as the terminal unit at the non-reducing end of the lacto-*N*-triose. The *N*-acetyllactosamine (Galβ1,4GlcNAc, type-2 LacNAc) can serve as both internal and terminal disaccharide units, while the lacto-*N*-biose (Galβ1,3GlcNAc, type-1 LacNAc) can only serve as the non-reducing end terminal disaccharide. By assembling these building blocks into linear and/or branched structures, HMOs with large structural complexity can be produced (>200 discovered in nature).[11-12] Although most of the functions of HMOs have not yet been elucidated,[11] HMOs with high molecular weight are expected to be biologically important. The fucosylated portion of HMOs containing the H blood group epitope (Fucα1,2Gal) has antiadhesive antimicrobial functions, including antibacterial, antiyeast, and antiviral activities.[13-15] The α1,2-linked fucosyl HMOs are therefore considered attractive targets for drug development or infant food additives. For instance, the European Food Safety Authority has reported that 2'-*O*-fucosyllactose (2'-FL) is safe to be used in human food additives.[16]

Linear HMO hexasaccharides with type-2 disaccharide repeat units (*para*-lacto-*N*-neohexaose, Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glc) and an extended type-1–type-2 chain (*para*-lacto-*N*-hexaose, Galβ1-3GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glc) were first identified in human milk in 1977.[17] In addition to human milk, these hexasaccharides with fucosyl extensions are found acting as glycan antigens in erythrocyte membranes and are considered to have essential biological functions.[18-19] In addition to the extended type-2 and type-1–type-2 hybrid chain structures, the isomeric form of hexasaccharides with extended type-1 disaccharide units (Galβ1-3GlcNAcβ1-3Galβ1-3GlcNAcβ1-3Galβ1-4Glc) and its fucosyl derivatives have been observed in colonic and gastric cancer cell lines.[20-21] Identification of the linkages with internal LacNAc (β1-3- or β1-4-linked) through mass spectrometry is challenging, and the glycan

standard is usually required to obtain unambiguous results.[22]

Because of the biological significance and clinical importance of HMOs, several elegant approaches have been explored for the synthesis of HMOs with hexasaccharide cores, including chemical synthesis,[23-25] enzymatic synthesis,[22][26] and whole-cell fermentation.[27] Despite the numerous synthetic approaches reported to date, the collective synthesis of high-molecular-weight HMOs including linear hexasaccharides with fucosylation through straightforward mammalian glycosyltransferase (GT)-based multienzyme synthesis has only recently been achieved.[26] As an alternative, bacterial GTs with high-expression yields and broad substrate specificity are known as a versatile tool for complex oligosaccharide preparation.[28-30] Herein, we report an effective enzyme-catalyzed system using bacterial GTs for the synthesis of nonfucosylated HMO-type gylcan backbones from disaccharide to hexasaccharide (*para*-lacto-*N*-hexaose) and their isomeric structures. Subsequently, backbone fucosylation was performed to yield α1,2-fucosylated HMO-type glycan products, as shown in Figure 1.

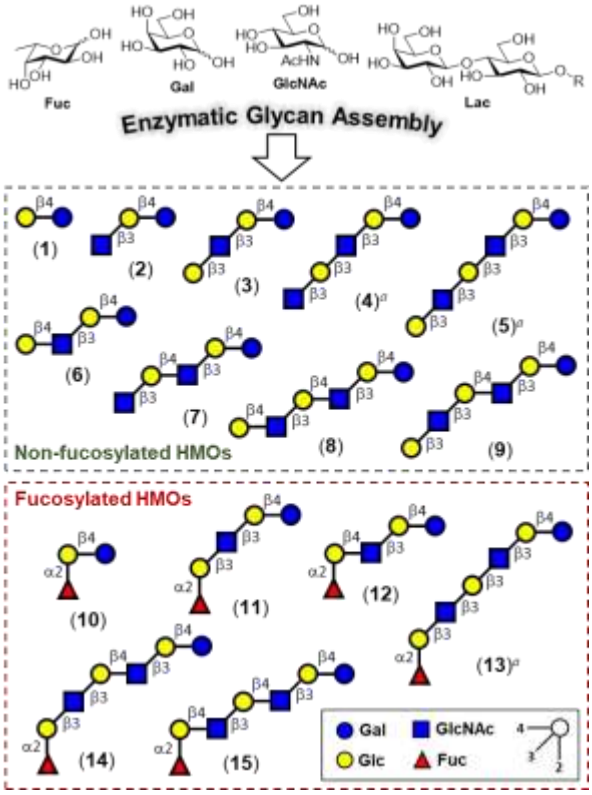
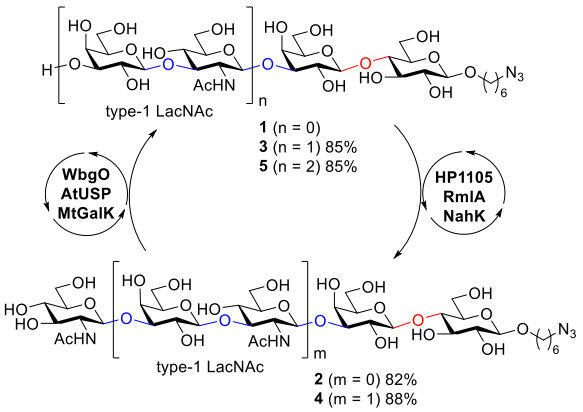


Figure 1. Fifteen human milk oligosaccharide (HMO)-type gly-cans and related isomeric glycans synthesized in this study. [a]Not reported in human milk, but found as the backbones of the human tumor-associated antigen.

To efficiently and quickly produce hexasaccharide backbones with different linkages, we used three recombinant bacterial GTs, including β1,3-*N*-acetyl-glucosaminyltransferase from *Helicobacter pylori* strain 26695 (HP1105),[31] β1,4-galactosyltransferase from *H. pylori* strain NCTC11637 (HP0826),[32] and β1,3-galactosyltransferase from *Escherichia coli* O55:H7 (WbgO),[33] overexpressed in *E. coli* (Figure S1 and Table S1). The sugar donors required for the GT-catalyzed reaction, uridine diphosphate galactose (UDP-Gal) and uridine diphosphate *N*-acetyl glucosamine (UDP-GlcNAc), were generated through sequential one-pot enzymatic reaction with slight modification.[34] UDP-GlcNAc was prepared by the action of *N*-acetyl hexosamine kinase from *Bifidobacterium longum* (NahK)[34] and glucose-1-phosphate thymidylyltransferase from *Aneurinibacillus thermoaerophilus* (RmlA);[35] in addition, UDP-Gal was generated using galactokinase from *Meiothermus taiwanensis* (MtGalK)[36] and UDP-sugar pyrophosphorylase from *Arabidopsis thaliana* (AtUSP).[37] By combining HP1105 and WbgO with the corresponding UDP-sugar-generating enzymes in the sequential one-pot system, lactose **1** was smoothly converted into defined lengths of HMO backbone containing lacto-*N*-biose (type-1 LacNAc) repeats (**2-5**), as shown in Scheme 1.



Scheme 1. Enzymatic synthesis of human milk oligosaccharides with type-1 LacNAc backbones. The circle containing HP1105, RmlA, and NahK represents sequential one-pot β1,3-*N*-acetyl glucosaminylation; the circle containing WbgO, AtUSP, and MtGalK represents β1,3-galactosylation.

First, 6-azidohexyl lactoside acceptor (Lac, **1**) was prepared, and the azide group should facilitate compound immobilization onto glass slides for further glycan array development. Trisaccharide lacto-*N*-triose (Lc3) **2** was prepared by treatment of **1** with HP1105 in the presence of UDP-GlcNAc through sequential one-pot synthesis (see Supporting Information). By combining the use of an SPE tC18 cartridge and size-exclusion chromatography, the desired trisaccharide (**2**) was obtained in 82% yield (165 mg).

The lacto-*N*-tetraose (LNT, **3**) was synthesized through WbgO-catalyzed sequential one-pot galactosylation with *in situ* UDP-Gal preparation (Scheme 1); the desired LNT **3** was obtained in 85% yield (106 mg). As previously reported, MtGalK

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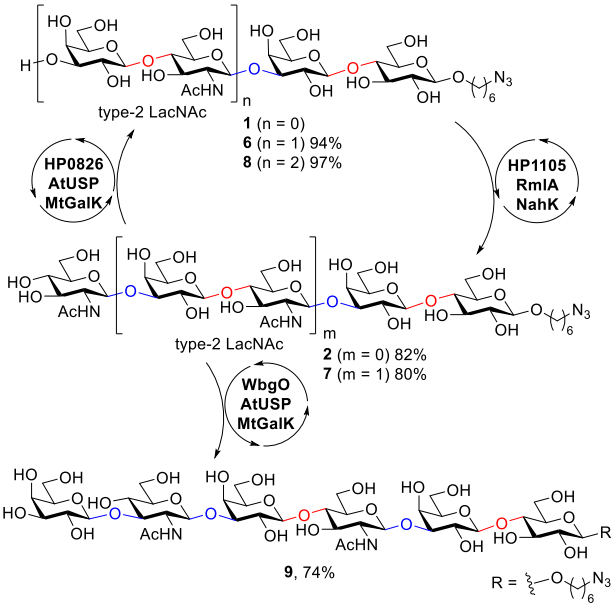
exhibited excellent activity at a steady working temperature ranging from 40 to 65 °C.[36] Therefore, in the *in situ* one-pot UDP-Gal preparation, the reaction temperature was set at 42 °C to avoid the additional step of temperature elevation. In addition, the UDP-Gal could be simply purified from the reaction mixture through recrystallization using ethanol and water in gram-scale amounts (see Supporting Information). Following the sequential one-pot procedure, type-1 pentasaccharide (**4**, 88%, 44 mg) and type-1 hexasaccharide (**5**, 85%, 22 mg) were readily prepared through the subsequent treatment of **3** with HP1105 in the presence of UDP-GlcNAc, followed by WbgO and UDP-Gal (Scheme 1). It is worth mentioned that the glycan structures of type-1 pentasaccharide **4** and type-1 hexasaccharide **5** have not been reported in human milk, but found as the backbones of the human tumor-associated antigen.[20]



Scheme 2. Enzymatic synthesis of human milk oligosaccharides containing type-2 LacNAc backbones. The circle containing HP1105, RmlA, and NahK represents sequential one-pot β1,3-N-acetyl glucosaminylation; the circle containing WbgO, AtUSP, and MtGalK represents β1,3-galactosylation; the circle containing HP0826, AtUSP, and MtGalK represents β1,4-galactosylation.

With a similar procedure to the sequential one-pot synthesis, the type-2 HMOs with *N*-acetyl lactosamine (LacNAc) repeats (**6**, **7**, and **8**) were synthesized in good to excellent yields (80%–97%). HP0826 was the enzyme that efficiently catalyzed the formation of β1,4-galactosidic linkages during the type-2 HMO synthesis. As illustrated in Scheme 2, Lc3 **2** was galactosylated by HP0826 in the presence of UDP-Gal, with incubation for 30 min at 37 °C, using the sequential one-pot enzymatic synthesis procedure to produce lacto-*N*-neotetraose (LNnT, **6**) in 94% yield (58 mg). The LNnT **6** was used as the substrate for HP1105 and reacted with UDP-GlcNAc for 63 h at 25 °C to obtain type-2 pentasaccharide **7** in 80% yield (23 mg). The resulting product was treated with UDP-Gal under catalysis with HP0826 for 30 min at 37 °C to obtain *para*-lacto-*N*-neohexaose (*p*-LNnH) **8** in 97% yield (59 mg). In addation, hundreds of mg scale syntheses of both LNnT **6** (517 mg) and *p*-LNnH **8** (137 mg) could be achieved from **1** and **6**, respectively, after two cycles of enzymatic glycosylation reactions (see Supporting Information). To prepare *para*-lacto-*N*-hexaose (*p*-LNH, **9**), **7** was used in the WbgO-catalyzed reaction and incubated with UDP-Gal at 37 °C for 14 h to produce **9** in 74% yield (45 mg), as illustrated in Scheme 2. These results indicate that HP1105 exhibited excellent activity on both type-1 and type-2 acceptors (**3** and **6**), regardless of their penultimate glycan sequence. The results are similar to those of another β1,3GlcNAcTs from *H. pylori* strain J99 (JHP1032), which had a very similar amino acid sequence to HP1105.[38]

To determine the suitable α1,2-fucosyltransferase (α1,2-FucT) for the preparation of fucosylated HMOs, two bacterial α1,2-FucTs, FutC from *H. pylori* strain 26695 and WbsJ from *E. coli* O128:B12 were recombinant-overexpressed in *E. coli* for testing (Figure S1). FutC[39] was employed in whole-cell fermentation to produce 2'-FL and lacto-*N*-neofucopentaose-I (LNnFP-I).[40] Although the substrate specificity of FutC from *H. pylori* strain NCTC 11639 was higher for type-1 than type-2 acceptors,[41] the substrate tolerance of FutC from strain 26695 for *in vitro* glycan synthesis, especially in HMO production, was slightly unclear. WbsJ was reported to show broad acceptor specificity with β1,3-linked acceptors (Galβ1,3GalNAc) and β1,4 linked acceptors (lactose);[42] however, synthetic application to HMO synthesis was not available. We employed a simple thin-layer chromatography assay using guanosine diphosphate (GDP)-fucose as a donor to access the ability of these two α1,2-FucTs (FutC and WbsJ) to transfer fucose to 9 HMO backbone types for the production of potential H-antigen-containing HMOs. The bifunctional L-fucokinase/GDP-fucose pyrophosphorylase from *Bacteroides fragilis* (FKP)[43] was responsible for the preparation of GDP-fucose from L-fucose in the presence of ATP, guanosine triphosphate, and $MnCl_2$ in Tris–HCl buffer (100 mM, pH 7.5).

The percentage conversion of a series of HMOs containing di-, tri-, tetra-, penta-, and hexasaccharides upon enzymatic fucosylation is illustrated in Figure 2. The degree of α1,2-fucosylation of **1** to form 2'-FL **10** was 71% and 17% for FutC and WbsJ, respectively. The formation of LNFP-I **11** from **3** was conducted by FutC and WbsJ in excellent conversion rates (92%–96%); the reaction rate of FutC was slightly higher than that of WbsJ. Notably, no significant activity in the difucosylation of LNT **3** was detected by WbsJ and FutC in the presence of more GDP-fucose (2.5 eq.); however, increased monofucosyl transfer yields were observed (97%–

98%). In addition, the use of excess GDP-fucose (10 eq.) resulted in only terminal monofucosylated products, indicating the infeasibility of internal α1,2-fucosylation on HMOs by these two bacterial enzymes (Figure S2 and S3). The conversion of the FutC-catalyzed α1,2-fucosylation reaction on LNnT **6** to form LNnFP-I **12** was moderate (54%), while complete conversion was achieved with longer incubation (95% for 24 h). No significant improvement in the conversion was observed on LNnT **6** when more GDP-fucose was employed. The lower conversion for FutC on type-2 glycans showed a similar tendency to the reported FutC from strain 11639.[41][44] In addition, WbsJ was almost inactive on LNnT **6**. The α1,2-fucosylation conversion on hexasaccharides **5** and **9** by FutC were excellent (86% and 88%). In comparison, WbsJ-catalyzed α1,2-fucosylation resulted in a moderate conversion on **5** (68%) and **9** (80%). Interestingly, increasing the amount of GDP-fucose to 2.5 eq. resulted in excellent conversion for both the WbsJ and FutC reactions (80%–99%). The formation of **15** (α1,2-fucosyl *para*-lacto-*N*-neohexaose) from **8** catalyzed by WbsJ in the presence of GDP-fucose (1.2 and 2.5 eq.) was not observed. Notably, FutC could α1,2-fucosylate the type-2 glycan *p*LNnH **8** with moderate conversion (58%–61%). These results indicate the preference of FutC for the type-1 HMOs, with WbsJ only active on type-1 HMOs. In addition, the elongation of the type-1 chain and the presence of internal type-2 linkage did not considerably affect WbsJ- or FutC- catalyzed α1,2-fucosyl transfer. Wang and co-workers recently reported the use of α1,2-FucT from *Helicobacter mustelae* (Hmα1,2-FT) that could fucosylate the branched HMOs with terminal type-2 LacNAcs in excellent yields.[45] Sequence alignment of the these α1,2-FucTs reveals the differences in the conserved motif II and III, both are expected as the acceptor binding domains (Figure S4).[46]

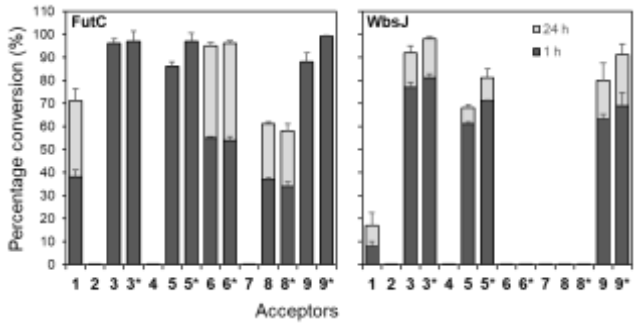


Figure 2. Enzymatic fucosylation of human milk oligosaccharides using α1,2-fucosyltransferases FutC and WbsJ. Percentage conversion was determined through thin-layer chromatography and using the ImageJ software after staining and is illustrated in a vertical bar chart. Data were averaged from three independent experiments. *Reaction was conducted in the presence of 2.5-eq. of GDP-fucose.

Table 1. Fucosylation of type-1 and type-2 glycans by fucosyltransferases FutC or WbsJ.

			Yield (%)	
Entry	Acceptor	Product	FutC[a]	WbsJ[b]
1	**1**	**10**	31	n.r.[c]
2	**3**	**11**	71	76
3	**5**	**13**	98	82
4	**6**	**12**	39[d]	n.r.
5	**8**	**15**	45	n.r.
6	**9**	**14**	80	55

[a] Conditions: HEPES 50 mM, pH 5.0, $MnCl_2$ 5 mM, acceptor 10 mM, GDP-fucose 25 mM, FutC 0.35 mg/mL, alkaline phosphatase (AP) 5 U/mL, 25 °C. [b] Conditions: Tris-HCl 50 mM, pH 7.4, $MnCl_2$ 5 mM, acceptor 10 mM, GDP-fucose 25 mM, WbsJ 0.35 mg/mL, AP 5 U/mL, 25 °C. [c] n.r. = no reaction. [d] Yield could be improved to 98% (123 mg) in the presence of high concentration of FutC (1 mg/mL).

Encouraged by these results, preparative synthesis of fucosylated HMOs (**10**–**15**) was performed with the same enzyme concentration for comparison (Table 1). FutC-catalyzed α1,2-fucosylations on β1,3-linked galactosides (**3**, **5**, and **9**; each 10 mM) were completed within 3.5 h at 25 °C in HEPES buffer (50 mM, pH 5.0) in the presence of GDP-fucose (25 mM), $MnCl_2$ (5 mM), and alkaline phosphatase (AP) to produce **11**, **13** (α1,2-fucosyl type-1 hexasaccharide), and **14** (α1,2-fucosyl *para*-lacto-*N*-hexaose) with isolation yields of 71%, 98% and 80%, respectively. Longer incubation (24 h) was required to α1,2-fucosylate the β1,4-linked acceptors (**1**, **6**, and **8**) to produce **10** (31% yield), **12** (39% yield), and **15** (45% yield). Notably, although the β1,4-linked glycan was not the best substrate for FutC, the complete α1,2-fucosylation on type-2 glycan **6** to form **12** could still be achieved in the presence of high enzyme concentration. The WbsJ-catalyzed reactions were only active on terminal β1,3-linked galactosides (**3**, **5**, and **9**), producing α1,2-fucosylatd HMOs **11** (76% yield), **13** (82% yield), and **14** (55% yield) in Tris–HCl (50 mM, pH 7.4) in the presence of GDP fucose (25 mM), $MgCl_2$ (5 mM), 1,4-dithiothreitol (1 mM), and AP and incubated at 37 °C for 24 h. The products were analyzed in terms of their purity and structure through electrospray ionization mass spectroscopy and nuclear magnetic resonance (NMR) spectroscopy. The structures of the compounds were confirmed through 1D and 2D NMR analysis with full assignments (Table S2 and S3).

The structural analysis of these compounds using heteronuclear multiple bond correction (HMBC) 2D NMR spectroscopy is exemplified for compound **13** in Figure 3. A cross-signal for the anomeric proton of residue F and the C3 carbon atom of residue E was observed (blue double arrow in Figure 3); similarly, a cross-signal was observed for the H3 proton of residue E and anomeric carbon atom of residue F (red

double arrow in Figure 3). These cross-peaks suggest that the glycosidic linkage was a 1→3 link. Using a similar approach, we confirmed the 1→3 glycosidic linkage with the backbone structure. In addition, the terminal fucosylation was determined by the cross-peak signal between the F and G residues. The anomeric proton–proton coupling constants ($^3J_{H,H}$) of residues B, C, D, E, F, and G were determined through 1D NMR analysis to be 8.1, 8.5, 8.1, 8.3, 7.5, and 3.9 Hz, respectively. The measured $^3J_{H,H}$ values suggest that the glycosidic linkages of the backbone in compound **13** were in the β-form, whereas the terminal fucose unit was in the α-form.

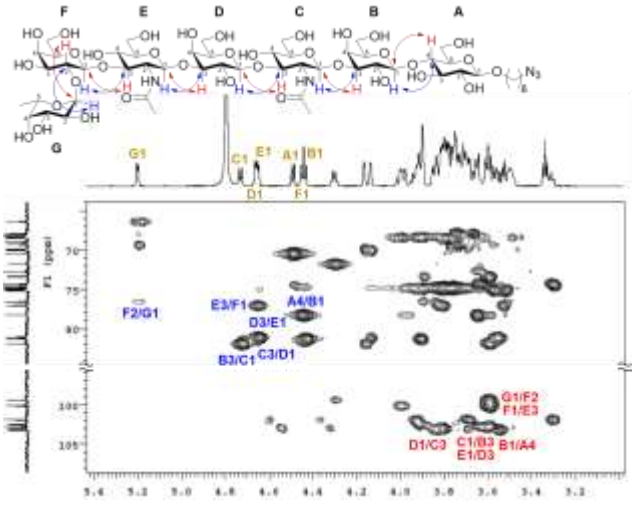


Figure 3. The structure of fucosylated type-1 hexasaccharide **13** and its HMBC spectrum. Anticipated cross-peaks are indicated by either blue or red double arrows. The results of HMBC analysis confirmed that the linkages of the HMO backbone were β1→3 links and the α1,2-fucosylation on terminal galactose. No resonances were detected from 85 to 95 ppm, and thus, this region was truncated. Residues represented are: A, Glc; B, Gal; C, GlcNAc; D, Gal; E, GlcNAc; F, Gal; G, Fuc.

We developed an effective method for synthesizing 15 fucosylated and nonfucosylated HMO-type glycans and the isomeric glycans by using a few bacterial glycosyltransferases and sugar-nucleotide–generating enzymes. The fucosylation on HMOs from lactose to *para*-lacto-*N*-hexaose and its isomeric structures were examined using two α1,2-fucosyltransferases, FutC and WbsJ. FutC exhibited favorable catalytic properties over WbsJ in terms of catalytic efficiency and substrate tolerance. Additionally, both enzymes performed the α1,2-fucosyl transfer only on the terminal Gal but not on the internal ones. This approach is highly divergent and can be used to prepare several oligosaccharide linkage isomers in the scale of tens to hundreds of milligrams, enabling complete structural characterization through NMR spectroscopy and supplemental biological applications.

Experimental Section

Fucosyl type 1 hexasaccharide-C_6N_3 (13). To a buffered (50 mM HEPES, pH 5.0) solution (835 μL) of **5** (10 mg, 8.3 μmol, 10 mM), GDP-fucose (13.2 mg, 21 μmol, 25 mM), manganese(II) chloride (5 mM) and alkaline phosphatase (5 U/mL) was incubated at 25 °C in the presence of FutC (0.35 mg/mL) with agitation at 200 rpm for 24 h. The product formation was monitored by TLC (*n*-butanol/ water/ acetic acid = 2:1:1 (v/v/v), 2 runs, R_f = 0.41). The reaction solution was quenched by heating at 100 °C for 10 min as TLC analysis indicated the completion of the reaction. The resulting mixture was centrifuged (4 °C, 10,000 x *g*, 10 min) to remove the proteins and insoluble precipitates. The supernatant was concentrated, purified by Sep-Pak® Vac tC18 cartridges (Waters) followed by passing through a TOYOPEAL® HW-40F gel (packed in BioRad column, 1.5 cm x 120 cm) with water (10 mL/h) to obtain the desired product with an isolation yield of 98% (11 mg) as the white powder. $[\alpha]^{28}_D$ = -15.4° (*c* 1.0, H_2O); ^{1}H NMR (700 MHz, D_2O) δ 5.20 (d, *J* = 3.9 Hz, 1H), 4.73 (d, *J* = 8.5 Hz, 1H), 4.65 (d, *J* = 7.5 Hz, 1H), 4.64 (d, *J* = 8.3 Hz, 1H), 4.49 (d, *J* = 8.0 Hz, 1H), 4.44 (d, *J* = 8.1 Hz, 1H), 4.43 (d, *J* = 8.1 Hz, 1H), 4.30 (q, *J* = 6.5 Hz, 1H), 4.16 (d, *J* = 2.5 Hz, 1H), 4.13 (d, *J* = 2.6 Hz, 1H), 4.02-3.87 (m, 6H), 3.86-3.62 (m, 24H), 3.62-3.46 (m, 8H), 3.36-3.27 (m, 3H), 2.06 (s, 3H), 2.04 (s, 3H), 1.69-158 (m, 4H), 1.45-1.36 (m, 4H), 1.24 (d, *J* = 6.6 Hz, 3H); ^{13}C NMR (175 MHz, D_2O) δ 174.93, 174.17, 103.22, 102.99, 102.83, 102.51, 101.90, 100.13, 99.41, 81.92, 81.30, 81.10, 78.27, 77.05, 76.55, 75.15, 75.05, 74.96, 74.79, 74.65, 74.60, 74.34, 73.38, 72.72, 71.75, 70.44, 70.03, 69.90, 69.31, 69.02, 68.49, 68.41, 68.32, 68.20, 67.93, 66.38, 61.05, 60.85(x2), 60.39, 60.32, 60.00, 54.90, 54.67, 51.03 28.47, 27.77, 25.55, 24.49, 22.16, 22.04, 15.16; HRMS (ESI-TOF) *m/z* calcd for $C_{52}H_{89}N_5O_{35}$ $[M+Na]^+$: 1366.5236; found 1366.5189.

Acknowledgements

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