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Regioselective One-Pot Multienzyme (OPME) Chemoenzymatic Strategies for Systematic Synthesis of Sialyl Core 2 Glycans

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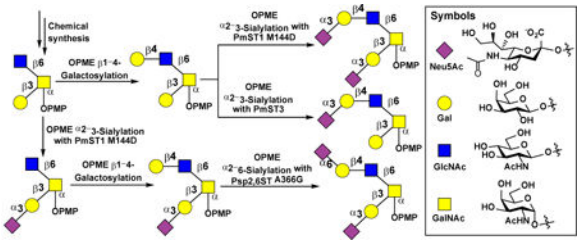
Abstract

O-GalNAc glycans or mucin-type glycans are common protein post-translational modifications in eukaryotes. Core 2 O-GalNAc glycans are branched structures that are broadly distributed in glycoproteins and mucins of all types of cells. To better understand their biological roles, it is important to obtain structurally defined Core 2 O-GalNAc glycans. We present here regioselective one-pot multienzyme (OPME) chemoenzymatic strategies to systematically access a diverse array of sialyl Core 2 glycans. Regioselectivity can be achieved by using OPME systems containing a glycosyltransferase with restricted acceptor specificity or by differentiating the branches using altered glycosylation sequences. This work provides a general regioselective strategy to access diverse Core 2 O-GalNAc glycans which can be extended for the synthesis of other complex branched glycans.

Graphical Abstract



Keywords

biocatalysis; enzymatic synthesis; glycosyltransferase; O-GalNAc glycan; sialyltransferase

O-GalNAc glycosylation at serine (Ser) or threonine (Thr) residues of proteins is a common protein post-translational modification (PTM) in eukaryotes. O-GalNAc glycans are also named as mucin-type O-glycans as they were initially found and are the most abundant in

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ASSOCIATED CONTENT

Supporting Information.

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Detailed synthetic procedures, nuclear magnetic resonance (NMR) spectroscopy and high-resolution mass spectrometry (HRMS) data, and NMR spectra of products (PDF)

mucins. All O-GalNAc glycans are extended from a monosaccharide *N*-acetylgalactosamine (GalNAc) that is α-linked to a serine or threonine residue (GalNAcαSer/Thr) on glycoproteins. Most O-GalNAc glycans are derived from four major core structures namely Cores 1–4, including linear structures Galβ1–3GalNAcαOR (Core 1) and GlcNAcβ1–3GalNAcαOR (Core 3) and branched structures Galβ1–3(GlcNAcβ1–6)GalNAcαOR (Core 2) (**1** in Chart 1) and GlcNAcβ1–3(GlcNAcβ1–6)GalNAcαOR (Core 4).[1] The biosynthesis of these complex glycans is not template driven and the structures are more diverse and less predictable than their N-glycan counterparts in eukaryotes.[2]

Core 2 glycans are extended from Core 2 and are broadly distributed in glycoproteins and mucins of all types of cells.[1] Core 2 glycans have been found to be the major O-GalNAc glycans on glycoproteins of mouse zona pellucida (mZP) which are major oocyte surface glycoproteins and the targets of sperm binding for initiating fertilization.[3] They have also been shown to influence the adhesion of Enteropathogenic *E. coli* (EPEC) and Enterohemorrhagic *E. coli* (EHEC) O157:H7 to human epithelial cell surface.[4] Several common Core 2 glycans are shown in Chart 1. Among these, extended Core 2 tetrasaccharide Galβ1–3(Galβ1–4GlcNAcβ1–6)GalNAcαOR (**2**), disialyl Core 2 hexasaccharide Neu5Acα2–3Galβ1–3(Neu5Acα2–3Galβ1–4GlcNAcβ1–6)GalNAcαOR (**3**), monosialyl Core 2 pentasaccharides Galβ1–3(Neu5Acα2–3Galβ1–4GlcNAcβ1–6)GalNAcαOR (**4**) and Neu5Acα2–3Galβ1–3(Galβ1–4GlcNAcβ1–6)GalNAcαOR (**6**) and their fucosylated derivatives have been found in several breast cancer cell lines.[5] Disialyl Core 2 hexasaccharide Neu5Acα2–3Galβ1–3(Neu5Acα2–3Galβ1–4GlcNAcβ1–6)GalNAcαOR (**3**) was also found as the major O-GalNAc glycan on glycocalicin released from human platelet membrane.[6–7] While the major Core 2 glycan of human synovial lubricin and erythrocyte membrane glycoproteins of normal mice was found to be monosialyl Core 2 pentasaccharide Neu5Acα2–3Galβ1–3(Galβ1–4GlcNAcβ1–6)GalNAcαOR (**6**),[8–9] monosialyl Core 2 tetrasaccharide Neu5Acα2–3Galβ1–3(GlcNAcβ1–6)GalNAcαOR (**5**) was the major Core 2 glycan in β1–4-galactosyltransferase I (GalT-I)-knock out mice.[8] Disialyl Core 2 hexasaccharide with an α2–6-linked sialic acid residue on the GlcNAc branch of the Core 2 structure and an α2–3-linked sialic acid residue on the Gal branch Neu5Acα2–3Galβ1–3(Neu5Acα2–6Galβ1–4GlcNAcβ1–6)GalNAcαOR (**7**) was identified together with Core 2 glycans **2–4** and **6** in a glycan pool of advanced breast cancer serum MUC1.[10]

In order to elucidate their biological functions, it is necessary to obtain these structurally defined Core 2 O-GalNAc glycans in pure forms. Nevertheless, synthetic efforts in accessing these compounds have been limited. Chemical synthesis of sialylated Core 2 glycans[11] suffered from low overall yield because of the presence of one or more sialic acids which are challenging to introduce by chemical sialylation.[12–14] Chemoenzymatic synthesis of various sialylated core 2 glycans was also reported using rat recombinant α2–3-sialyltransferase and CMP-Neu5Ac.[15–16] Recently, glycopeptides containing some of the Core 2 glycan structures were synthesized in small amounts for a competition enzyme-linked immunosorbent assay (ELISA)[17] and mass spectrometry analysis.[18] We report herein highly efficient regioselective one-pot multienzyme (OPME) chemoenzymatic strategies to

systematically access these important Core 2 glycans (**1–7**, Chart 1) using bacterial enzymes.

To facilitate product purification and reaction monitoring, a *p*-methoxyphenyl (PMP) group was installed at the reducing end of target Core 2 glycans. To allow regioselective enzymatic glycosylation of the Galβ1–3- and GlcNAcβ1–6-branches of Core 2, PMP-tagged Core 2 trisaccharide Galβ1–3(GlcNAcβ1–6)GalNAcαOR (**1**) was chemically synthesized from properly protected monosaccharide intermediates **8**,[19] **9**,[20] and **10**[21] by regio- and stereoselective glycosylation reactions. Briefly, iodonium ion-promoted stereoselective glycosylation of glycosyl acceptor **8** with thiotolyl glycoside derivative **9** using *N*-iodosuccinimide (NIS)-trimethylsilyl trifluoromethanesulfonate (TMSOTf) produced disaccharide derivative **11** in 84% yield (Scheme 1). It was converted to disaccharide diol derivative **12** in 92% yield using acetic acid-catalyzed selective deprotection of the benzylidene acetal group.[22] Reactivity-based regio- and stereoselective glycosylation of glycosyl acceptor **12** with thiotolyl donor **10** in the presence of NIS-TMSOTf at −55 °C furnished trisaccharide derivative **13** in 78% yield. Compound **13** was subjected to a series of deprotection reactions including (a) transformation of *N*-phthaloyl group to acetamido group by the treatment with ethylene diamine followed by acetylation[23] using acetic anhydride and pyridine; (b) thioacetic acid-catalyzed one-pot conversion of azido to acetamido group;[24] and (c) saponification using sodium methoxide followed by purification using C18-column chromatography to afford the target Core 2 trisaccharide Galβ1–3(GlcNAcβ1–6)GalNAcαOPMP (**1**) in three steps with an overall yield of 79%. The overall yield for synthesizing compound **1** from properly protected monosaccharide building blocks was 48% in six steps. This represents an improved synthetic route compared to the previous report of synthesizing a *p*-nitrophenyl glycoside of Core 2 trisaccharide (**1**) using a 4,6-di-*O*-benzoyl-2,3-*N*,*O*-oxazolidinone-protected donor with a step-by-step glycosylation process in an overall yields of 19%.[25] The current synthetic process is also more efficient than the reported chemoenzymatic synthesis of an octyl glycoside of Core 2 trisaccharide (**1**) in a 5 mg-scale which involved the chemical synthesis of Core 2 tetrasaccharide (**2**) using a [2+2] block glycosylation strategy with an overall 22% yield from properly protected monosaccharides followed by jack bean β-galactosidase-catalyzed reaction.[26]

With the chemically synthesized Core 2 trisaccharide **1** in hand, neutral extended Core 2 tetrasaccharide Galβ1–3(Galβ1–4GlcNAcβ1–6)GalNAcαOPMP (**2**) containing both β1–4- and β1–3-linked galactoside branches was readily synthesized in 89% yield (Scheme 2) using an improved one-pot multienzyme (OPME) galactose (Gal)-activation and transfer system[27] containing *Streptococcus pneumoniae* TIGR4 galactokinase (SpGalK),[28] *Bifidobacterium longum* UDP-sugar pyrophosphorylase (BLUSP),[27] *Pasteurella multocida* pyrophosphatase (PmPpA),[29] and *Neisseria meningitidis* β1–4-galactosyltransferase (NmLgtB)[29–30] in Tris-HCl buffer (100 mM, pH 8.0) at 37 °C for 14 hours. SpGalK, BLUSP, and PmPpA allowed in situ formation of the donor substrate of NmLgtB, uridine 5’-diphosphate-galactose (UDP-Gal), from uridine 5’-triphosphate (UTP) and galactose-1-phosphate (Gal-1-P) formed from monosaccharide galactose (Gal) and adenosine 5’-triphosphate (ATP).

The purified extended Core 2 tetrasaccharide (**2**) was used as the acceptor substrate for sialylation using OPME α2–3-sialylation system containing *Neisseria meningitidis* CMP-sialic acid synthetase (NmCSS)[31] and an α2–3-sialyltransferase. The most common sialic acid form, *N*-acetylneuraminic acid (Neu5Ac), was introduced. NmCSS was responsible for the formation of cytidine 5'-monophosphate *N*-acetylneuraminic acid (CMP-Neu5Ac), the donor substrate of sialyltransferases, from Neu5Ac and cytidine 5'-triphosphate (CTP). Two different α2–3-sialyltransferases were used. The first one was *Pasteurella multocida* α2–3-sialyltransferase 1 M144D (PmST1 M144D),[32] a mutant engineered from wild-type multifunctional PmST1[33] to decrease its CMP-sialic acid (donor) hydrolysis activity (20-fold reduction) and α2–3-sialidase activity (5588-fold decreased). Due to its high efficiency in sialylating both β1–4- and β1–3-linked galactosides in the extended Core 2 tetrasaccharide Galβ1–3(Galβ1–4GlcNAcβ1–6)GalNAcαOR (**2**), disialyl Core 2 hexasaccharide Neu5Acα2–3Galβ1–3(Neu5Acα2–3Galβ1–4GlcNAcβ1–6)GalNAcαOR (**3**) was readily produced in 73% yield (Scheme 2). The α2–3-sialylation of both Gal residues in **3** was confirmed by comparing the chemical shifts of ^{13}C nuclear magnetic resonance (NMR) data of compounds **2** and **3**. As shown in Table S1, signals for two newly added Neu5Ac residues were observed in compound **3**. In addition, the chemical shifts of C-3 (72.11 ppm and 72.00 ppm) of both β1–4- and β1–3-linked Gal residues in compound **2** were moved downfield to 75.11 ppm and 74.95 ppm, respectively, in compound **3**. These represented the most significant chemical shift changes and indicated the sites of sialylation.

The second sialyltransferase used was *Pasteurella multocida* α2–3-sialyltransferase 3 (PmST3),[34] a monofunctional sialyltransferase that can use oligosaccharides, glycolipids, and glycopeptides[35] as acceptor substrates. Because of its preference in using β1–4-linked galactosides but not β1–3-linked galactosides as acceptor substrates,[34] OPME sialylation system containing PmST3 led to selective mono-sialylation of the β1–4-linked galactoside branch of the tetrasaccharide **2** to form monosialylated Core 2 pentasaccharide Galβ1–3(Neu5Acα2–3Galβ1–4GlcNAcβ1–6)GalNAcαOR (**4**) in 83% yield (Scheme 3). The selective α2–3-sialylation of compound **2** on the β1–4-galactoside branch to form compound **4** was confirmed by comparing the ^{13}C NMR data of compounds **2** and **4**. As shown in Table S1, the most significant changes on chemical shifts were the appearance of the signals for the newly added single Neu5Ac residue and downfield shift of the signal of the C-3 of β1–4-linked Gal residue (72.11 ppm in **2** to 74.70 ppm in **4**).

The strategy for regioselective sialylation of the β1–3-galactoside branch of the Core 2 was to use trisaccharide Galβ1–3(GlcNAcβ1–6)GalNAcαOR (**1**) as the acceptor substrate in the OPME system containing NmCSS[31] and PmST1 M144D[33] which can sialylate terminal galactose residue with either β1–3- or β1–4-linkage but not terminal *N*-acetylglucosamine (GlcNAc) residue. The Galβ1- branch, not the GlcNAcβ1-branch, in Core 2 trisaccharide (**1**) was selectively sialylated to form monosialyl Core 2 tetrasaccharide Neu5Acα2–3Galβ1–3(GlcNAcβ1–6)GalNAcαOR (**5**) in 94% yield (Scheme 3). Galactosylation of compound **5** using the same OPME β1–4-galactosylation system described above for the synthesis of **2** from **1** led to the formation of monosialyl Core 2 pentasaccharide Neu5Acα2–3Galβ1–3(Galβ1–4GlcNAcβ1–6)GalNAcαOR (**6**) with the sialylation on the β1–3-linked galactoside branch of the Core 2 glycan in 91% yield. Final sialylation using an OPME α2–

6-sialylation system containing NmCSS and *Photobacterium species* α2–6-sialyltransferase[36] A366G mutant (Psp2,6ST A366G)[36] with improve expression level and slightly increased activity successfully synthesized disialyl Core 2 hexasaccharide Neu5Acα2–3Galβ1–3(Neu5Acα2–6Galβ1–4GlcNAcβ1–6)GalNAcαOR (**7**) containing both α2–3- and α2–6-linked sialic acid residues at desired sites in 84% yield. The α2–3-sialylation of β1–3-linked galactoside branch in compound **6** was confirmed by comparing the ^{13}C NMR data of compounds **2** and **6** (Table S1) by the presence of the signals of a single Neu5Ac residue in compound **6** and the downfield shift of the signal for the C-3 of β1–3-linked Gal residue from 72.00 ppm in compound **2** to 75.16 ppm in compound **6**. Similarly, disialylation in compound **7** was obvious as the signals for two Neu5Ac residues was observed. The significant downfield shifts of C-3 of β1–3-linked Gal (72.00 ppm in **2** and 75.15 ppm in **7**) and C-6 of β1–4-linked Gal (60.51 ppm in **2** and 62.77 ppm in **7**) were clear by comparing the ^{13}C NMR data of compounds **2** and **7**, which showed the sites of the sialylation.

In conclusion, regioselective one-pot multienzyme (OPME) chemoenzymatic strategies have been developed for systematic accessing a library of Core 2 O-GalNAc glycans. Regioselective sialylation was successfully achieved by using sialyltransferases with restricted acceptor specificity such as PmST3 and by differentiating the branches of chemically synthesized Core 2 trisaccharide **1** for sialylation using altered OPME glycosylation sequences. The two general regioselective strategies demonstrated here can be extended for the chemoenzymatic synthesis of other complex branched glycans.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENT

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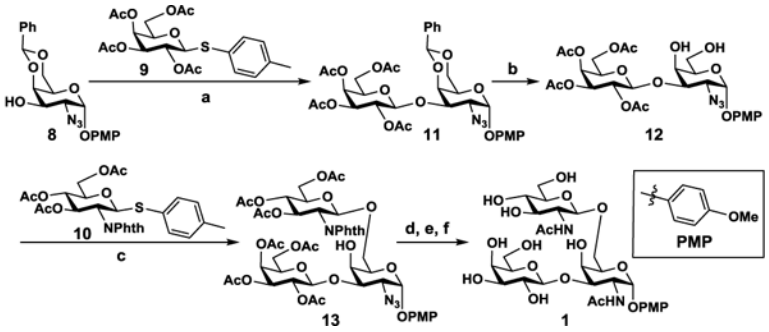
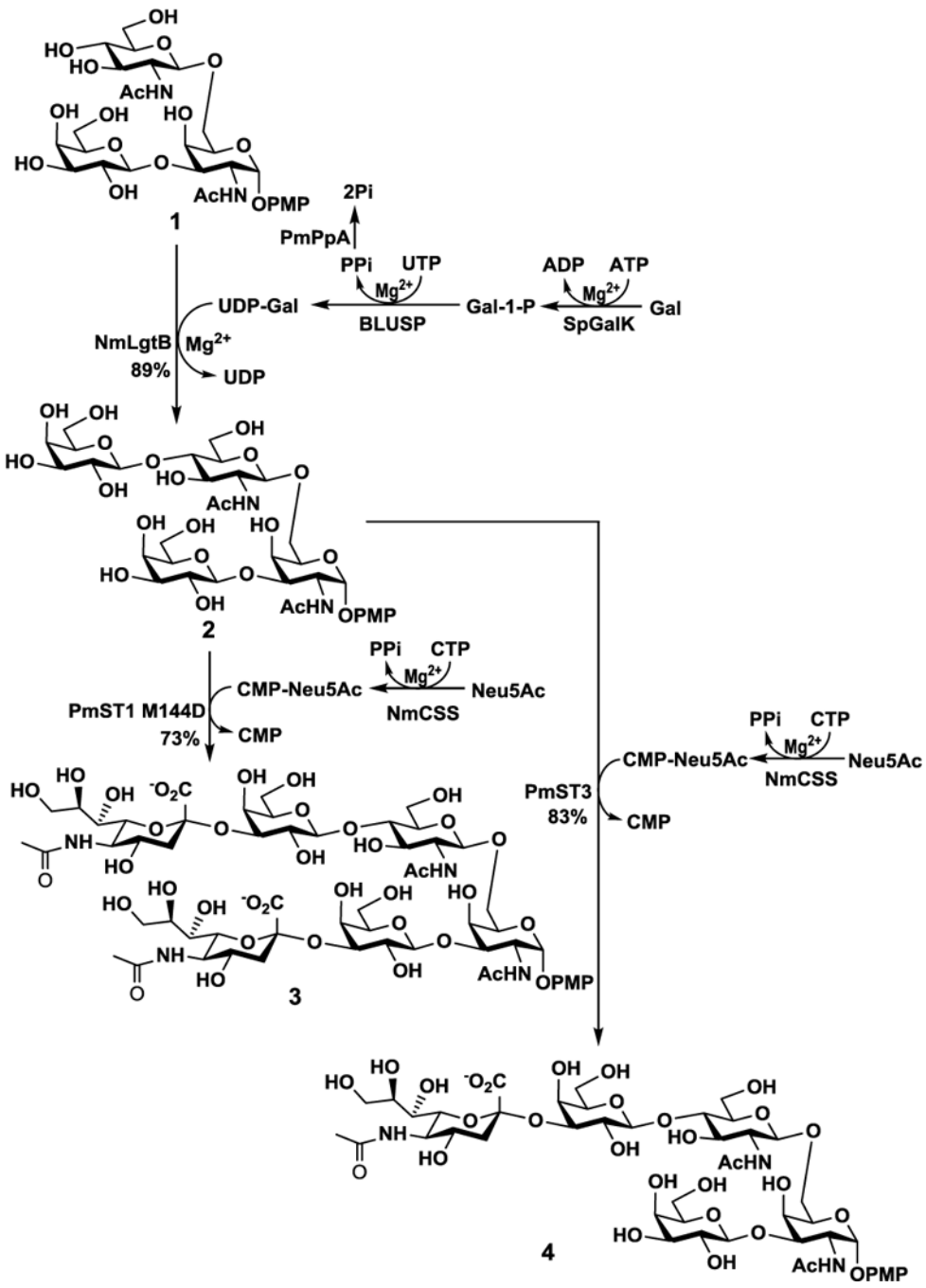


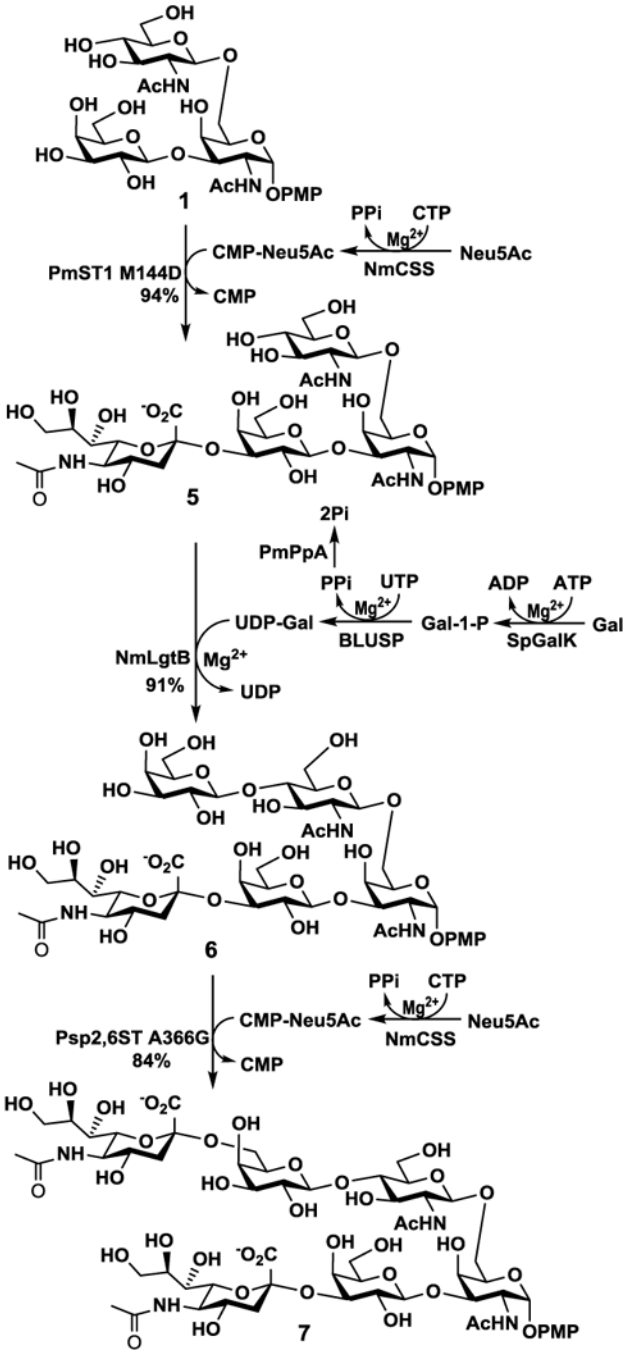
Scheme 1.

Chemical synthesis of Core 2 trisaccharide (1). Reagents and conditions: (a) *N*-iodosuccinimide (NIS), TMSOTf, MS 4 Å, CH_2Cl_2, −25 °C, 1 h, 84%; (b) 80% acetic acid in water, 80 °C, 3 h, 92%; (c) *N*-iodosuccinimide (NIS), TMSOTf, MS 4 Å, CH_2Cl_2, −55 °C, 1 h, 78%; (d) (i) $H_2N(CH_2)_2NH_2$, *n*-BuOH, 90 °C, 8 h; (ii) pyridine, Ac_2O, r.t., 14 h; (e) pyridine, thioacetic acid, r.t., 18 h; (f) 0.1 M NaOMe, MeOH, r.t., 14 h; 79% in three steps.



Scheme 2.

Sequential one-pot multienzyme (OPME) synthesis of extended Core 2 tetrasaccharide (2) by OPME β1–4-galactosylation, disialyl Core 2 hexasaccharide (3) using PmST1 M144D in OPME sialylation reaction, and monosialyl Core 2 pentasaccharide (4) using PmST3 in OPME sialylation reaction.



Scheme 3.

Sequential one-pot multienzyme (OPME) synthesis of monosialyl Core 2 tetrasaccharide (5) using PmST1 M144D in OPME sialylation reaction, monosialyl Core 2 pentasaccharide (6) by OPME β1–4-galactosylation, and disialyl Core 2 hexasaccharide (7) using Psp2,6ST A366G in OPME sialylation reaction.

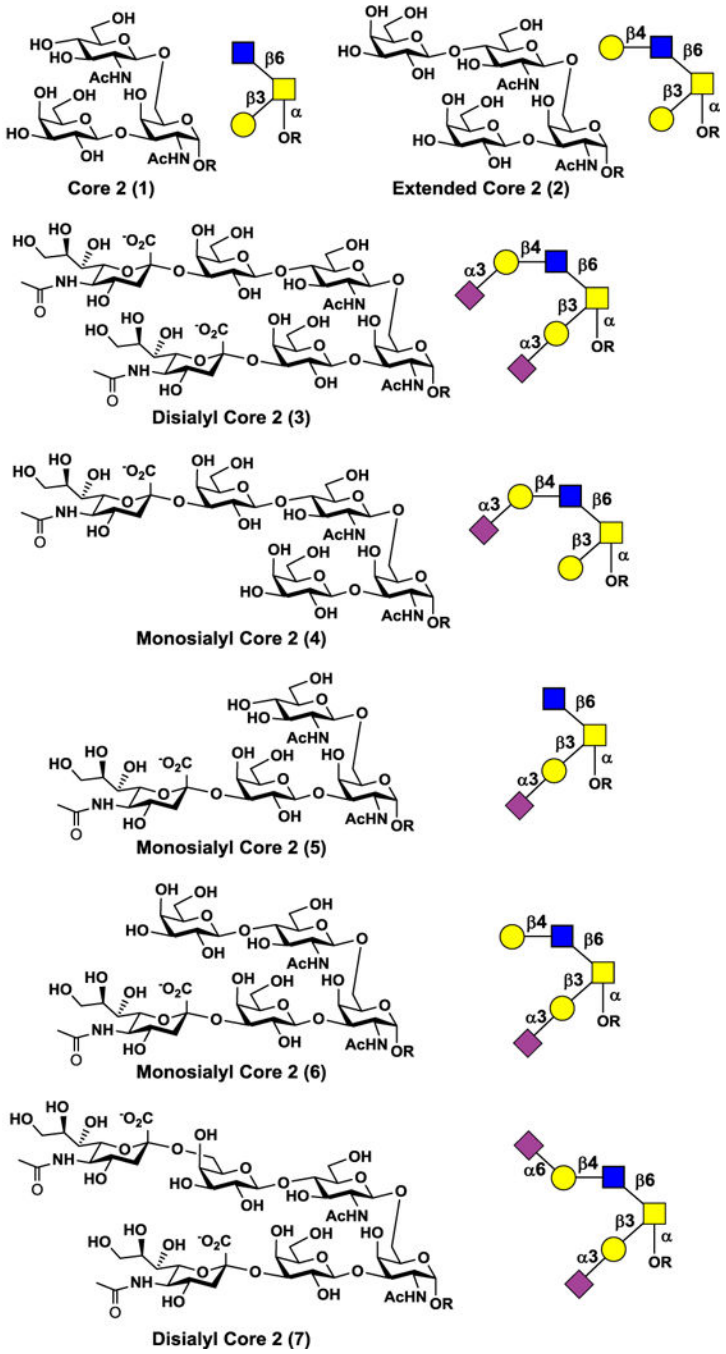


Chart 1.
Structures of O-GalNAc Core 2 glycans (1–7) that are synthesized in the current study.