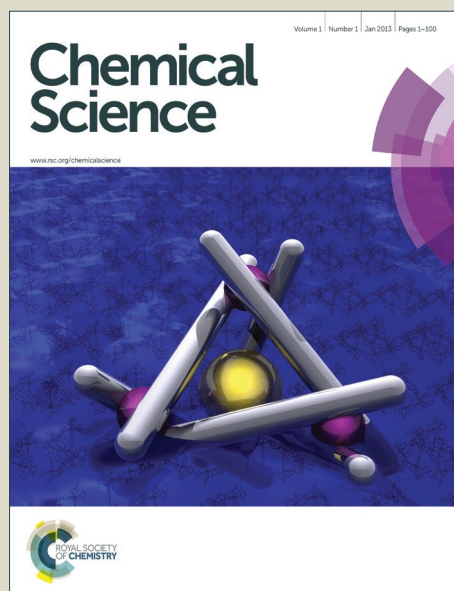


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Efficient Chemoenzymatic Synthesis of an N-glycan Isomer Library

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Quantification, characterization and biofunctional studies of N-glycans on proteins remain challenging tasks due to complexity, diversity and low abundance of these glycans. The availability of structurally defined N-glycans (especially isomers) libraries is essential to help on solving these tasks. We reported herein an efficient chemoenzymatic strategy, namely Core Synthesis/Enzymatic Extension (CSEE), for rapid production of diverse N-glycans. Starting with 5 chemically prepared building blocks, 8 N-glycan core structures containing one or two terminal N-acetyl-D-glucosamine (GlcNAc) residue(s) were chemically synthesized via consistent use of oligosaccharyl thioethers as glycosylation donors in the convergent fragment coupling strategy. Each of these core structures was then extended to 5 to 15 N-glycan sequences by enzymatic reactions catalyzed by 4 robust glycosyltransferases. Success in synthesizing N-glycans with Neu5Gc and core-fucosylation further expanded the ability of enzymatic extension. High performance liquid chromatography with an amide column enabled rapid and efficient purification (>98% purity) of N-glycans in milligram scales. A total of 73 N-glycans (63 isomers) were successfully prepared and characterized by MS² and NMR. The CSEE strategy provides a practical approach for "mass production" of structurally defined N-glycans, which are important standards and probes for Glycoscience.

Introduction

Glycans are ubiquitous and play diverse roles in a wide range of biological processes, such as protein folding and degradation, glycoproteostasis, cell adhesion and trafficking, cell signaling, fertilization and embryogenesis, as well as pathogen recognition and immune responses.¹ Abnormal cell surface glycoforms and/or glycan-profiles are usually related to diseases such as cancer and atherosclerosis. Accordingly, glycan/glycoprotein biomarkers have been developed.² Thus, elucidating the structures and functions of glycans is essential for understanding carbohydrate related biological and pathological processes, and for developing diagnostics and therapeutics for human diseases.

N-glycans found in nature possess an inherited complexity and diversity. These are mainly due to the variable and multiple connectivity of glycan building blocks (monosaccharides) and the process that they are assembled in biosystems. In mammalian glycomes, numerous glycan structures can be formed including branched-, regio- and stereo-isomers from only 10 common monosaccharide building blocks.³ Unlike precise template directed transcription/translation of nucleic acids/proteins, glycan structures are determined by the activities of glycosyltransferases (GTs),

glycosidases, and other glycan biosynthetic enzymes, as well as the availability of donor substrates. For example, more than 30 GTs and glycosidases in the Golgi of human cells are involved in processing N-glycans.⁴ The expression, activity, substrate specificity, and localization of each enzyme have the potential to influence the assembly of N-glycans. It is thus understandable that N-glycans are extremely micro-heterogeneous even in one particular N-glycosylation site. For example, 58 different complex N-glycan structures were identified at one N-glycan site in mouse zona pellucida glycoprotein 3.⁴ As a result, despite decades of efforts in developing novel approaches for glycan analysis,⁵ absolute quantification and characterization of complex mixtures of N-glycans remain challenging tasks. At present, the main approach for characterizing N-glycan isomers is ion-trap mass spectrometry (MS), analysis of permethylated glycans, which requires large quantities of samples, therefore not suitable for low abundance glycans and rare biological samples. The availability of libraries of structurally defined N-glycans (especially isomers) provides essential standards and probes for MS-based N-glycan analysis and glycan microarray studies of carbohydrate binding proteins.

Given the difficulties in separating structurally defined glycans from natural resources, chemical or chemoenzymatic approaches have been developed for the synthesis of mostly symmetric N-glycans in the last two decades.⁶ Among chemically synthesized N-glycans, only few contains terminal sialic acid (Sia) due to difficulties in sialic acid chemistry,⁷ which was later overcome by enzymatic glycosylation using sialyltransferases.⁸ Most recently, Boons developed a strategy for chemoenzymatic synthesis of asymmetrical N-glycans, and 14 tri-antennary complex N-glycans were obtained.⁹ Nevertheless, only a few N-glycan structures were prepared in each report, mainly due to their complexity and diversity. A simple and robust strategy for efficient production of large numbers of N-glycan structures is still highly desirable.

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†Electronic Supplementary Information (ESI) available: Materials, detailed experimental protocols, Supporting schemes and figures, synthetic methods, analytical data including HPLC profiles, mass and NMR spectrums of synthesized N-glycans are available. See DOI: 10.1039/x0xx00000x

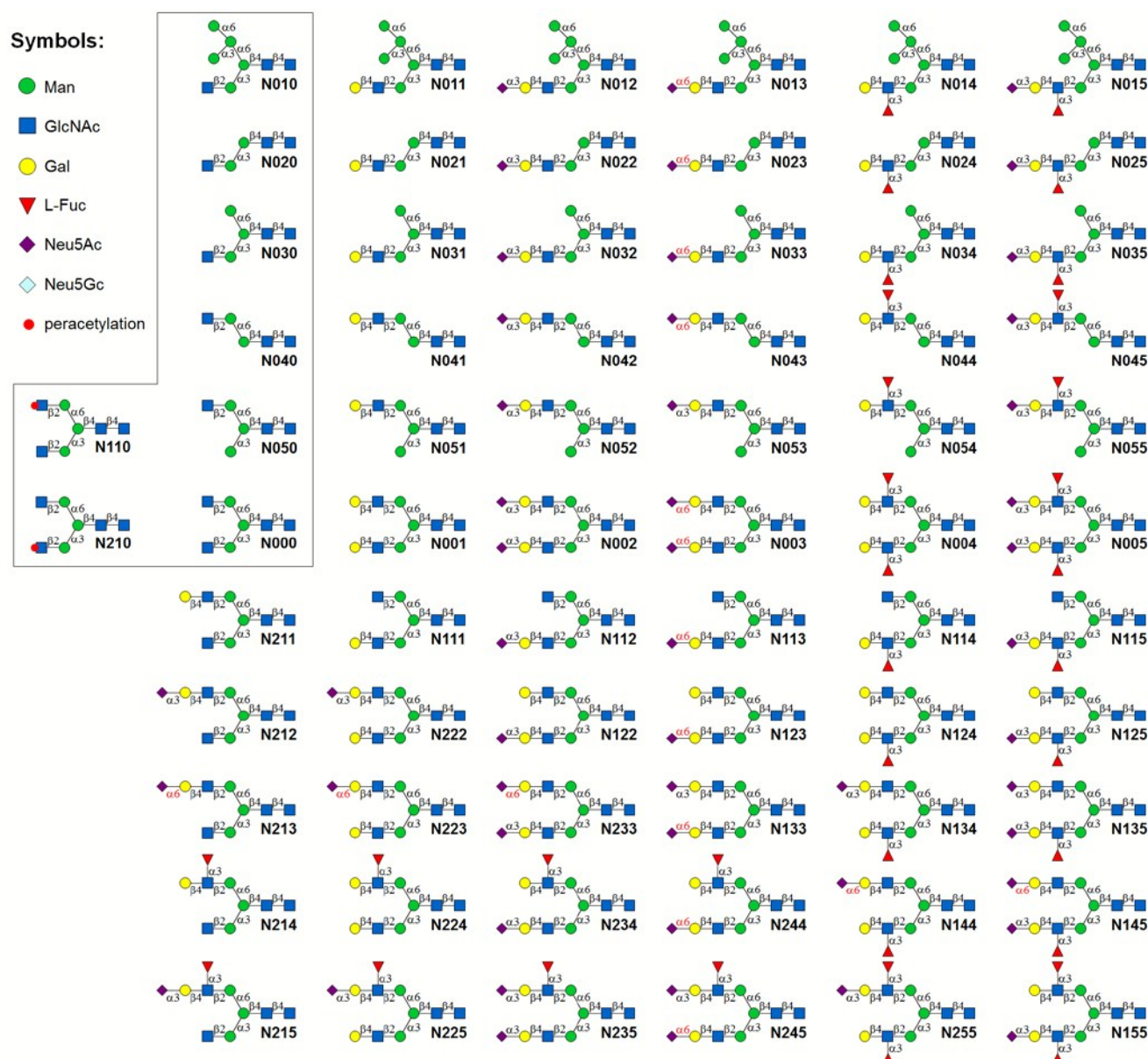


Figure 1. N-glycans synthesized by the Core Synthesis/Enzymatic Extension (CSEE) strategy. N-glycan core structures (boxed) were chemical synthesized. Glycans N0xx were enzymatically synthesized from corresponding cores N0xx0, whereas N1xx and N2xx were enzymatically synthesized from N110 or N210.

Another roadblock in the rapid access of glycans in high purity is the purification strategy, which now largely relies on gel filtration chromatography (usually Sephadex G-25 or Bio-gel P2).^{8,9} However, even though gel filtration has been applied for decades in purifying glycans, it is time-consuming, less efficient, and may waste significant portion of products in preparing small quantities of precious N-glycans. Therefore, a more reliable and rapid N-glycan purification approach is yet to be developed.

Lewis^x [Le^x, Galβ1,4-(Fucα1,3-)GlcNAc] and sialyl Lewis^x [SLe^x, Siaα2,3-Galβ1,4-(Fucα1,3-)GlcNAc] are among the most biologically significant glycan epitopes. For example, also known as CD15 antigen or SSEA-1 trisaccharide, Le^x plays a role in the development of central nervous systems in vertebrates,¹⁰ and interferes with pathogen transfer in breastfed infants.¹¹ SLe^x is a specific ligand on human leukocytes for E-, L-, and P-selectins, and was shown to mediate leukocyte recruitment.¹² SLe^x (on both N- and O-glycans of glycoprotein zona pellucida) was also shown to

mediate human sperm binding during fertilization.¹³ In addition, Le^x and SLe^x are usually overexpressed on the surface of cancer cells¹⁴. Granted all the significances, N-glycans containing these epitopes were not synthesized until recently.⁹ In this study, we described an efficient Core Synthesis/Enzymatic Extension (CSEE) strategy and a HPLC based purification approach for rapid preparation of N-glycans with/without (S)Le^x epitopes. In this strategy, 8 N-glycan core structures with GlcNAc residue(s) at the non-reducing terminal were firstly synthesized by convergent assembly of 5 building blocks. A set of robust GTs were then used to elongate these cores to yield a library of 73 N-glycans (Fig. 1 & 3). The development of an HPLC based approach using an amide column enabled rapid purification of milligrams of the chemoenzymatically synthesized N-glycans to minimum 98% purity. In addition, MS² analysis of selected N-glycans yielded unique fragmentation patterns that may be used for distinguishing certain isomers.

Results

Convergent Core Synthesis. A good amount of work had been reported in chemically synthesizing N-glycan structures. For example, by Danishefsky^{7a, 15} constructed a core pentasaccharide by glycosylating a trisaccharide with a monosaccharide thioether donor; Unverzagt¹⁶ synthesized multi-antennary complex type N-glycans with acetated Schmidt's trichloroacetimidate donor; Wong^{8a} used oligosaccharyl fluorides as donors to produce bi-, tri- and tetra-antennary complex type N-glycans; Boons⁹ developed a strategy by sequential removal of the protecting groups, and then by chemical glycosylation using a diverse set of trifluoroacetimidate donors. In these cases, to prepare various glycosyl donors, a temporary anomeric protecting group was used and then transformed into trichloroacetimidates, fluorides or trifluoroacetimidates, depending on the choice of glycosylation reaction. In this study, we developed an efficient convergent strategy that utilized oligosaccharyl thioether as a versatile donor for glycosylation, facilitating antennae assembly in just one or two glycosylation step(s) with excellent yield and good stereoselectivity.

We envisaged that trisaccharide **1** (Fig. 2) containing a crucial β -mannoside would be a versatile precursor for the synthesis of core structures, where the C4, C6-hydroxyl groups (OH) of the β -Man are protected with benzylidene and the C3-OH is unprotected to allow further chemical glycosylation. Installation of β -mannoside, the most challenging task in N-glycan synthesis, was accomplished using Crich-Kahne conditions with satisfying yield and β -selectivity.¹⁷ The benzylidene acetal ring can be selectively opened at either C6-OH⁹ or C4-OH¹⁸ of the β -mannoside for further chemical assembly. In order to prepare target N-glycans, 8 GlcNAc terminated core structures (Fig. 2) were designed. Among these, **N110** and **N210** were partially protected by peracetylation of GlcNAc residue on either α 1,6Man or α 1,3Man branch for the synthesis of asymmetric bi-antennary N-glycans. After enzymatic extension of the unprotected branch, the acetyl groups can be removed easily for further elongation.

The versatile precursor trisaccharide **1**^{7b} and donor fragments **2**,¹⁹ **3**,²⁰ **4** and **5**²¹ were prepared as previously reported (ESI[†]). Using these building blocks, the synthesis of the 8 core structures were performed in a convergent strategy (Scheme 1). For example, to synthesize core structure **N010**, thioether donor **4** was first stereoselectively installed onto C3-hydroxyl of trisaccharide **1** in the presence of N-iodosuccinimide (NIS)/AgOTf with a yield of 93%. Pentasaccharide **7** was then obtained in an excellent yield (96%) by selective opening of the benzylidene ring at C6 using Et₃SiH/PhBCl₂. The octasaccharide **8** was assembled by stereoselective installation of **5** onto C6-hydroxyl of β -Man of acceptor **7** with a yield of 85%. The two phthalimides of **8** were then converted into acetamides, followed by the global deprotection of Bn by catalytic hydrogenolysis with Pd(OH)₂/H₂ in MeOH/H₂O (10:1).

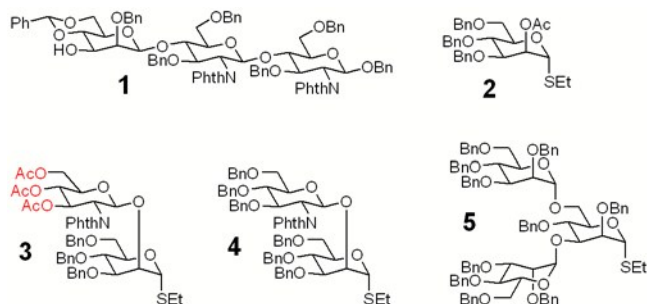
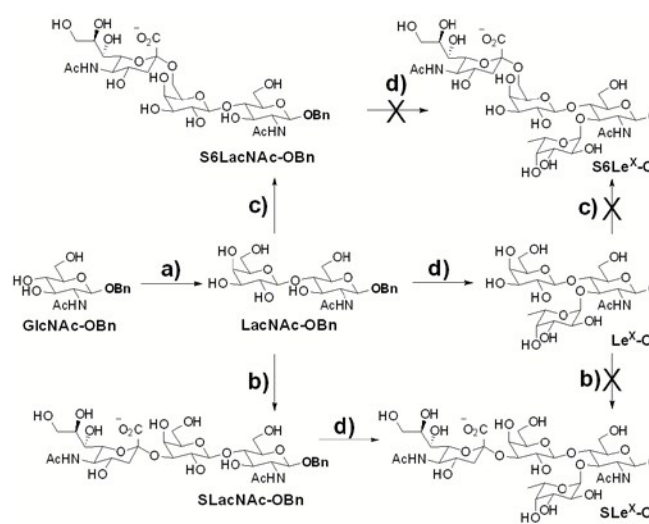


Figure 2. The versatile trisaccharide precursor **1** and four donor fragments (**2**, **3**, **4**, **5**) for the assembly of 8 core structures.

The core structure **N010** was produced in a total yield of 63% over the three steps.

Similarly, cores **N000**, **N020**, **N030**, **N050**, **N110**, and **N210** were synthesized by first installing **2**, **3** or **4** onto C3-hydroxyl of β -man of **1**, then followed by installation of corresponding building blocks onto the α 1,6Man branch. For the synthesis of **N040**, simple 3-O-benzoylation and controlled reductive cleavage of the benzylidene acetal of **1** was performed to afford acceptor **15**, which was then glycosylated with **4** to yield pentasaccharide **16** in 91% yield and a satisfactory stereoselectivity ($\alpha/\beta = 3.5:1$). Compound **16** was further deprotected to yield **N040** as previously described. The structures and stereochemistry of all glycosidic linkages was confirmed by NMR (ESI[†]).

Enzymatic Extension of N-glycans. We intend to develop an enzymatic extension strategy that can efficiently generate 6 glycans (including Le^x and SLe^x) starting with any terminal GlcNAc residue. To this end, several robust GTs were chosen for the proof-of-concept experiment: β 1,4-galactosyltransferase from bovine milk (B4GALT1); α 2,3-sialyltransferase 1 mutant E271F/R313Y from *Pasteurella multocida* (PmST1m) with reduced α 2,3-sialidase activity;²² α 2,6-sialyltransferase from *Photobacterium damsela* (Pd2,6ST);²³ and C-terminal 66 amino acids truncated α 1,3-fucosyltransferase from *Helicobacter pylori* (Hp α 1,3FT).²⁴ Except for the commercially available B4GALT1, all GTs were from bacteria and have high expression levels in *Escherichia coli*, high activity, and relatively relaxed substrate specificities. Using GlcNAc-OBn as a starting material, we validated the activities of these GTs (Scheme 2). Same as previously reported, the enzymes could efficiently catalyze the formation of LacNAc (Gal β 1,4-GlcNAc)-, SLacNAc (Sia α 2,3-Gal β 1,4-GlcNAc)-, S6LacNAc (Sia α 2,3-Gal β 1,4-GlcNAc)-, Le^x- and SLe^x-OBn. For reactions catalyzed by B4GALT1, Pd2,6ST and Hp α 1,3FT, no product hydrolysis was observed (analyzed by MS) even with excessive

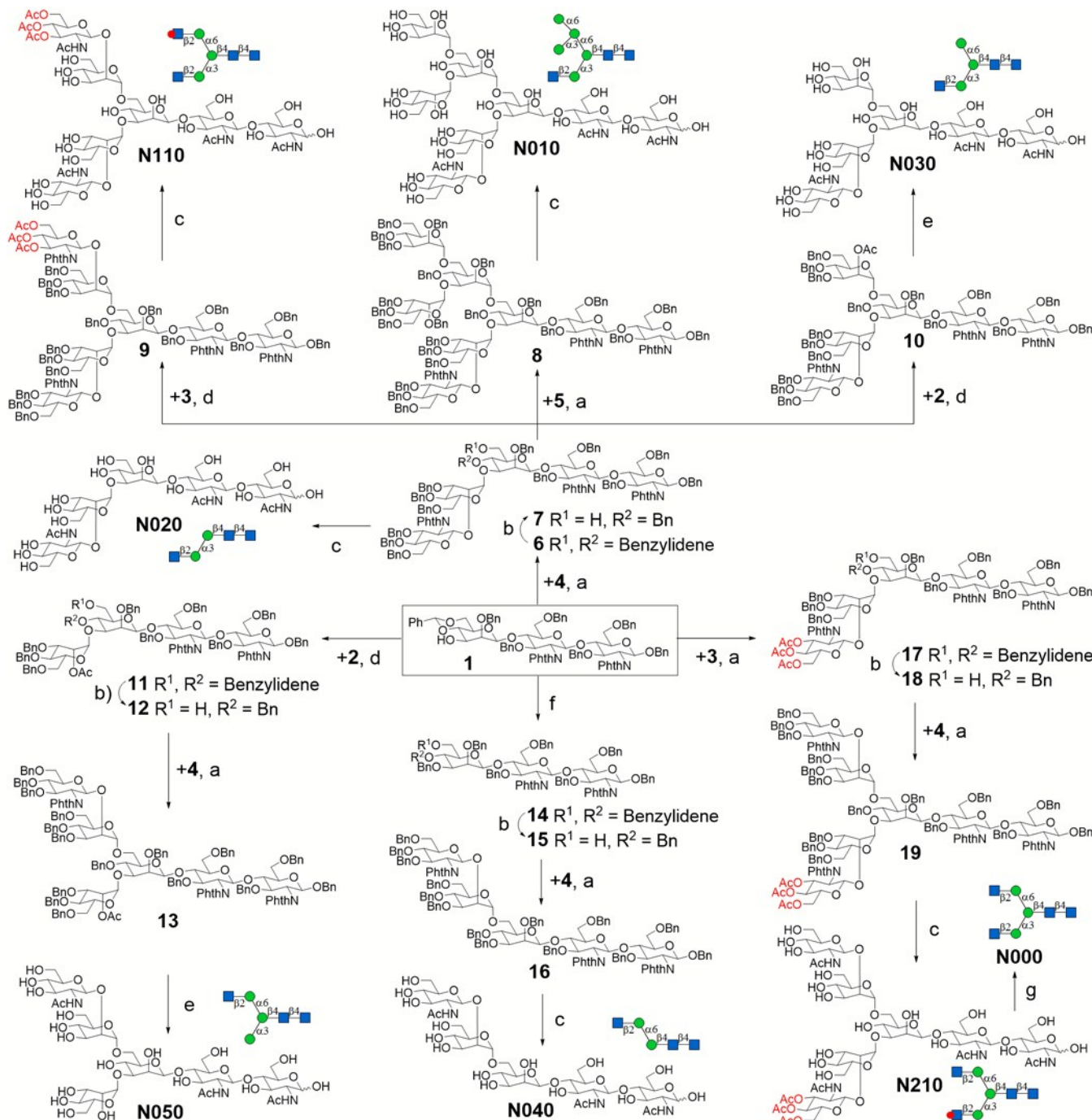


Scheme 2^a Proof-of-concept experiment for the proposed enzymatic extension strategy. ^aReagents and conditions: a) UDP-Gal, Mn²⁺ and β 1,4-galactosyltransferase from bovine milk (B4GALT1); b) CMP-Sia and double mutant E271F/R313Y of α 2,3-sialyltransferase 1 from *Pasteurella multocida* (PmST1m); c) CMP-Sia and α 2,6-sialyltransferase from *Photobacterium damsela* (Pd2,6ST); d) GDP-Fuc, Mn²⁺ and C-terminal 66 amino acids truncated α 1,3-fucosyltransferase from *Helicobacter pylori* (Hp α 1,3FT).

amounts of enzymes and up to 24 h of incubation. However, product hydrolysis was observed for PmST1m with an excess amount of enzyme and an extended reaction time (>1 h), even the α 2,3-sialidase activity has been reduced for 6333-fold by mutation of two amino acid residues.²² Therefore, controlling the amount of PmST1m and the reaction time is still important for the synthesis of α 2,3-sialosides. The attempt to synthesize unnatural tetrasaccharide S6Le^x [Sia α 2,6-Gal β 1,4-(Fuc α 1,3-)GlcNAc] failed utilizing either

Hpa1,3FT or Pd2,6ST, with the formation of only MS detectable product after 24 h of Pd2,6ST incubation (Fig. S3). In addition, PmST1m can hardly catalyze the sialylation of Le^x to form SLe^x. Such information is important for designing sequential enzymatic N-glycan synthesis schemes.

Utilizing the enzymatic extension strategy, N011 - N015 were prepared starting with chemically prepared cores N010 (Fig. 3A).



Firstly, in a 1.5 mL reaction system, 9 mg of **N010** (4 mM) was incubated with UDP-Gal (8 mM), MnCl_2 (5 mM), and B4GALT1 (20 $\mu\text{U}/\mu\text{mole}$ acceptor). One microliter of the reaction mixture was aliquoted every hour for analysis. MS analysis showed a peak at $m/z = 719.7645$, corresponding to **N011** $[\text{M}+2\text{H}]^{2+}$. Meanwhile, on the HPLC-ELSD (Evaporative Light Scattering Detector) profile, a new peak ($T_R = 16.79$ min) was observed, the area of which grew while the peak corresponding to **N010** ($T_R = 14.86$ min) became smaller. After 6 h of incubation, the reaction was freeze-quenched at -80°C for 30 min, and condensed into 300 μL for HPLC purification using a water/acetonitrile gradient elution, yielding 9.4 mg of **N011** (94 % yield). The purified **N011** (99% pure) was then utilized for the synthesis of **N012**, **N013**, and **N014** (Fig 3A) catalyzed by PmST1m, Pd2,6ST, and Hp α 1,3FT, respectively (see ESI[†] for details). It is worth noting that the reaction for the synthesis of **N012** was only allowed to proceed for 30 min due to the sialidase activity of PmST1m. **N015** was then synthesized from **N012** using Hp α 1,3FT.

The reaction took 20 h to achieve complete conversion (Fig. S4). Similarly, starting with other chemically synthesized cores (**N000**, **N020**, **N030**, **N040** and **N050**), N-glycans **N001** - **N005**, **N021** - **N025**, **N031** - **N035**, **N041** - **N045** and **N051** - **N055** were prepared in a manner analogous to that described above. All prepared N-glycans were analyzed by HPLC-ELSD, ESI/MALDI-MS, and NMR to confirm purity and structures (ESI[†]).

The synthesis of asymmetric bi-antennary N-glycans **N1xx** and **N2xx** (Fig. 1) was carried out by enzymatic extension of the unprotected antenna first and then the other. The synthesis of **N1xx** was illustrated in Figure 3B. Firstly, Gal was added by B4GALT1 to the GlcNAc residue in the α 1,3Man branch of **N110** to form **N110a**, galactosylation on the α 1,6Man branch was avoided by peracetylation of corresponding GlcNAc residue. It should be noted that partial de-acetylation was observed when the reaction was incubated for over 12 h. After HPLC purification, **N110a** was de-acetylated using 30% of ammonium hydroxide: H_2O (1: 10) to

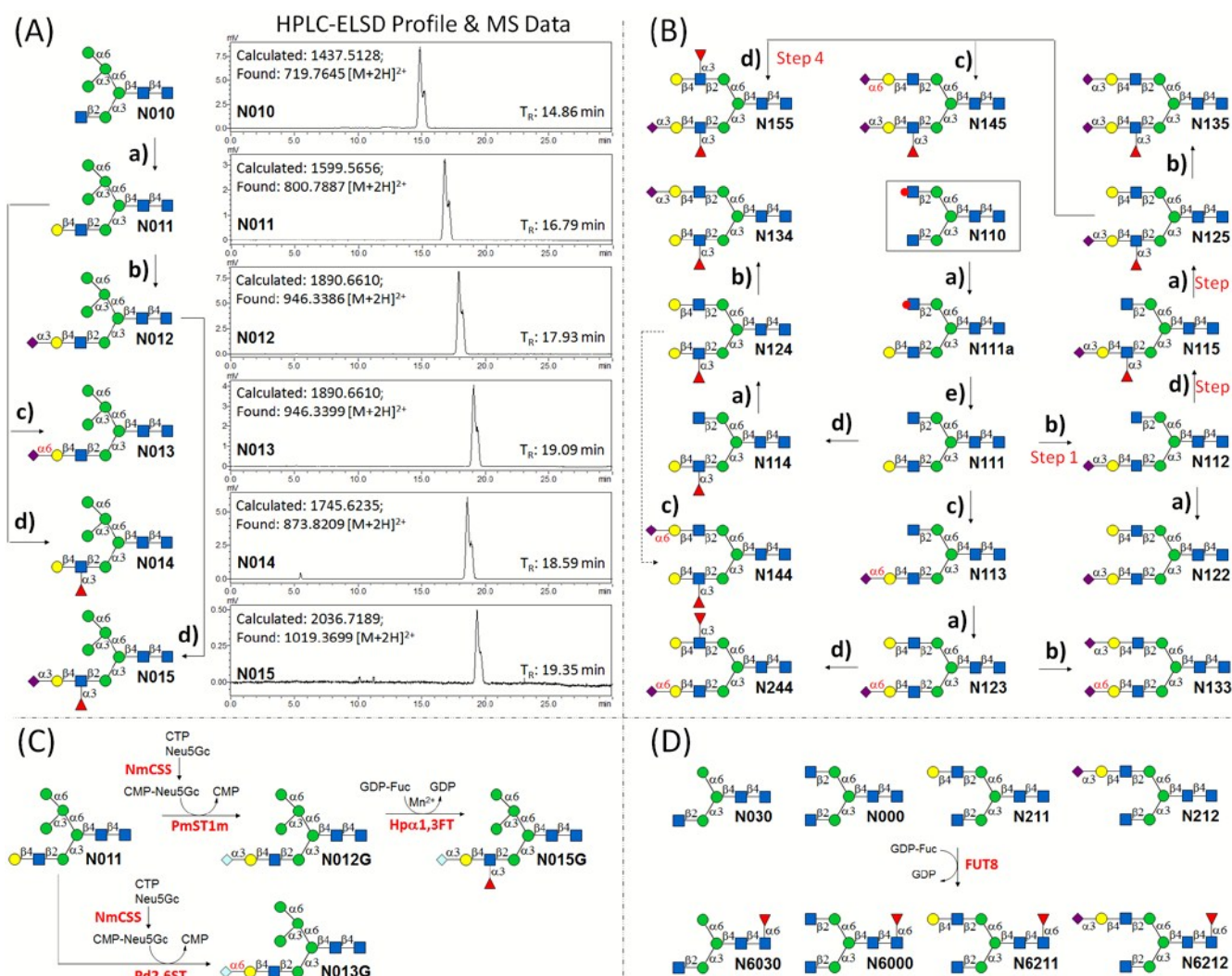


Figure 3. Enzymatic Extension of N-glycans. a) B4GALT1, UDP-Gal, Mn^{2+} ; b) PmST1m, CMP-Neu5Ac; c) Pd2,6ST, CMP-Neu5Ac; d) Hp α 1,3FT, GDP-Fuc, Mn^{2+} ; e) 30% ammonium hydroxide: H_2O (1:10), 6 h. PmST1m, *Pasteurella multocida* α 2,3-sialyltransferase 1 mutant E271F/R313Y; Pd2,6ST, *Photobacterium damsela* α 2,6-sialyltransferase; NmCSS, *Neisseria meningitidis* CMP-sialic acid synthetase; Hp α 1,3FT, c-terminal 66 amino acids truncated *Helicobacter pylori* α 1,3-fucosyltransferase; FUT8, human α 1,6-fucosyltransferase. Each N-glycan was purified into minimum 98% pure by HPLC with a semi-preparative amide column (10 \times 250 mm). HPLC analysis of purified N-glycans were performed using an analytical amide column (4.6 \times 250 mm) under a gradient condition (solution A: 100 mM ammonium formate, pH 3.4; solution B: acetonitrile; flow rate: 1 mL/min; B%: 65 - 50% within 25 min) monitored by ELSD. The adjacent two peaks on HPLC profiles compound corresponding to α and β anomers of N-glycans. MS data for purified N-glycans were obtained by ESI-MS.

afford **N111**, which was then used as a substrate for synthesizing other **N1xx** glycans in a strictly controlled sequential manner. For example, to obtain **N155**, the α 1,3Man branch was firstly extended by PmST1m (Step 1) and Hp α 1,3FT (Step 2) to yield **N115**, the α 1,6Man branch was then extended by B4GALT1 (Step 3) and Hp α 1,3FT (Step 4) (Fig. 3B). Such synthetic routes were designed according to the substrate specificities of GTs to avoid undesirable glycosylation. Particularly, **N144** was not designed to be synthesized from **N124** to avoid potential sialylation on the α 1,3Man branch by Pd2,6ST. Instead, N-glycan **N244** was synthesized from **N123** catalyzed by Hp α 1,3FT (Fig. 3B). Similarly, N-glycans **N2xx** and **N144** were synthesized from **N210** (Fig. S5).

N-glycolylneuraminic acid (Neu5Gc), often found on mammalian glycans, is another common Sialic acid molecule besides N-acetylneuraminic acid (Neu5Ac).²⁵ Even though human cells cannot produce Neu5Gc because of the inactivation of gene encoding CMP-Neu5Ac hydroxylase,²⁶ it was frequently detected on glycans of cancer cells, probably by metabolic incorporation from Neu5Gc-containing structures in the diet.²⁵ Previously, the two sialyltransferases (PmST1, Pd2,6ST) used in the enzymatic extension were shown to be extremely promiscuous towards sugar donors, and were applied in efficient synthesis of numbers of sialosides and derivatives.^{23, 27} To further expand current library, N-glycans with Neu5Gc residue (**N012G** and **N013G**) were synthesized via a one-pot two-enzyme system (Fig. 3C). In detail, for the synthesis of **N012G**, 3 mM of **N011** was incubated with 5 mM of Neu5Gc and cytidine 5'-triphosphate (CTP), 5 μ g/mL of PmST1m, and excessive amounts of CMP-Sia synthetase (NmCSS). After 30 min of incubation at 37 °C (94% conversion as detected by HPLC), the reaction mixture was concentrated and subjected to HPLC purification. The synthesis of **N013G** was achieved by simply replace PmST1m to Pd2,6ST. Surprisingly, it was found that the incorporation of a Neu5Gc residue resulted in a longer retention time shift (>1 min) on the amide column compared that to the Neu5Ac-counterpart (**N012**, T_R = 17.93 min; **N012G**, T_R = 19.13 min; **N013**, T_R = 19.09 min; **N013G**, T_R = 20.16 min) (ESI[†]). Fucosylation of **N012G** (to generate **N015G**) was shown to be as efficient as that of **N012**, indicating that Hp α 1,3FT can tolerate substrates with Neu5Gc. Theoretically, another set of 57 N-glycans can be easily synthesized by simply replacing Neu5Ac of the glycans in Figure 1 with Neu5Gc.

Core-fucosylated N-glycans were widely found in mammalian glycoproteins and are particularly abundant in brain tissues.²⁸ The alteration of core-fucosylation was proven to be associated with human cancers, chronic hepatitis, etc.²⁹ Thus, the ability of preparing homogenous core-fucosylated N-glycans was believed to be important. N-glycans prepared above are perfect substrates for specificity study of α 1,6-fucosyltransferase (FUT8), the sole enzyme responsible for core-fucosylation of N-glycans, and for preparing core-fucosylated N-glycan library. Specifically, 4 N-glycans with an identical α 1,3Man branch but a different α 1,6Man branch were selected for FUT8-catalyzed core-fucosylation (Fig. 3D). The results showed that FUT8 was highly active in using all 4 N-glycans as acceptors. Corresponding core-fucosylated N-glycans (**N6030**, **N6000**, **N6211**, **N6212**) (0.5 - 1 mg each) were synthesized accordingly. Further substrate specificity study showed that FUT8 may have a stricter requirement for structures on the α 1,3Man branch than that of α 1,6Man branch (detailed study is undergoing).

A HPLC based approach for rapid access of pure N-glycans. Gel filtration (Bio-gel P2, 1 \times 110 cm) was first applied in the purification of synthesized N-glycans. Taking the separation of **N001** (synthesized from **N000** by B4GALT1 catalyzed reaction) as an example, MS of the P2 purified product showed a major peak at m/z = 821.2992 corresponding to **N001** [M+2H]²⁺, and a minor peak

at m/z = 740.2735 corresponding to **N001** minus a Gal residue [M+2H]²⁺, possibly comes from incomplete galactosylation of **N000**. This result again indicated that gel filtration is inefficient for purifying N-glycans into high purities, especially when incomplete glycosylation occurred. As a consequence, previously reported N-glycan synthesis usually employed excessive enzymes and long incubation times to push GT-catalyzed reactions towards completion.⁹

Hydrophilic interaction liquid chromatography (HILIC) provides a rapid and effective strategy for separating small polar compounds, and has been used extensively in glycan analysis.³⁰ In these cases, N-glycans from biological samples were usually fluorescent labeled via reductive amination and then detected in picomole scales by UPLC-HILIC. However, HILIC has not been applied in milligram scale N-glycan purification. Using an analytical HILIC column (XBridge BEH amide column, 5 μ m, 4.6 mm \times 250 mm, Waters) under a gradient running condition (solvent A: 100 mM ammonium formate, pH 3.4; solvent B: acetonitrile; flow rate: 1 mL/min; B%: 70 - 50% within 50 min), the abovementioned Bio-gel P2-purified products were analyzed. Four peaks were observed in the HPLC profile using an evaporative light scattering detector (Fig. S1). Peaks 1 (T_R = 21.68 min) and 2 (T_R = 22.16 min) were next to each other and partially overlapped. The same observation was found for peaks 3 (T_R = 24.51 min) and 4 (T_R = 25.04 min). These peaks were collected in a parallel run monitored at $A_{210\text{ nm}}$ and subjected to MS analysis. Same m/z values were observed for peaks 3 (821.2997) and 4 (821.2991) (Fig. S1), implying that both peaks represented N-glycan **N001** [M+2H]²⁺, possibly for α and β anomers, which is common for free glycans due to the process of mutarotation in water. This was confirmed by ¹H NMR analysis that showed chemical shifts of both α and β anomer protons (ESI[†]). Similarly, peaks 1 and 2 represented to α and β anomers of **N001** minus a Gal residue [M+2H]²⁺.

These results encouraged us to purify **N001** using a semi-reparative HILIC column (10 \times 250 mm). Under a similar gradient running condition (solvent A: 100 mM ammonium formate; solvent B: acetonitrile; flow rate: 4 mL/min; B%: 70 - 50% within 50 min; monitored at $A_{210\text{ nm}}$), 10.5 mg of **N001** were separated by 3 injections (Fig. S2) with a purity of higher than 98% as analyzed by HPLC-ELSD (ESI[†]). Different solvent combinations were later tested for N-glycan purification (Fig. S6). Results showed that 100 mM ammonium formate/acetonitrile gradient elution gave the best separation of all N-glycans tested. In addition, N-glycans without Sia residues were able to be separated to similar level using water/acetonitrile gradient elution, where sialylated N-glycans were eluted rapidly (T_R < 3 min). Furthermore, it was found that a shorter running time with a narrower B% gradient (65 - 50% in 25 min) was able to achieve a similarly good separation level. Such running conditions were applied to separate enzymatically synthesized N-glycan into 98% (ESI[†]).

Under a standard running condition (solvent A: 100 mM ammonium formate; solvent B: acetonitrile; flow rate: 1 mL/min; B%: 65 - 50% within 25 min), all purified N-glycans were analyzed by HPLC-ELSD (ESI[†]). It was found that when different sugar residues were added to N-glycans, the retention time shifts of peaks on HPLC chromatograms generally decrease in the following order: Neu5Gc α 2,3 with Fuc α 1,3 > Neu5Gc α 2,6 > Neu5Ac α 2,3 with Fuc α 1,3 > Neu5Gc α 2,3 > Neu5Ac α 2,6 > Gal β 1,4 > Fuc α 1,3 > Neu5Ac α 2,3 > Fuc α 1,6. For example, the retention time of **N015G**, **N013G**, **N015**, **N012G**, **N013**, **N014**, **N012** are 20.66, 20.16, 19.39, 19.13, 19.09, 18.59, 17.93 min respectively. Such regularity may be found useful in HILIC-based profiling and identification of N-glycans.

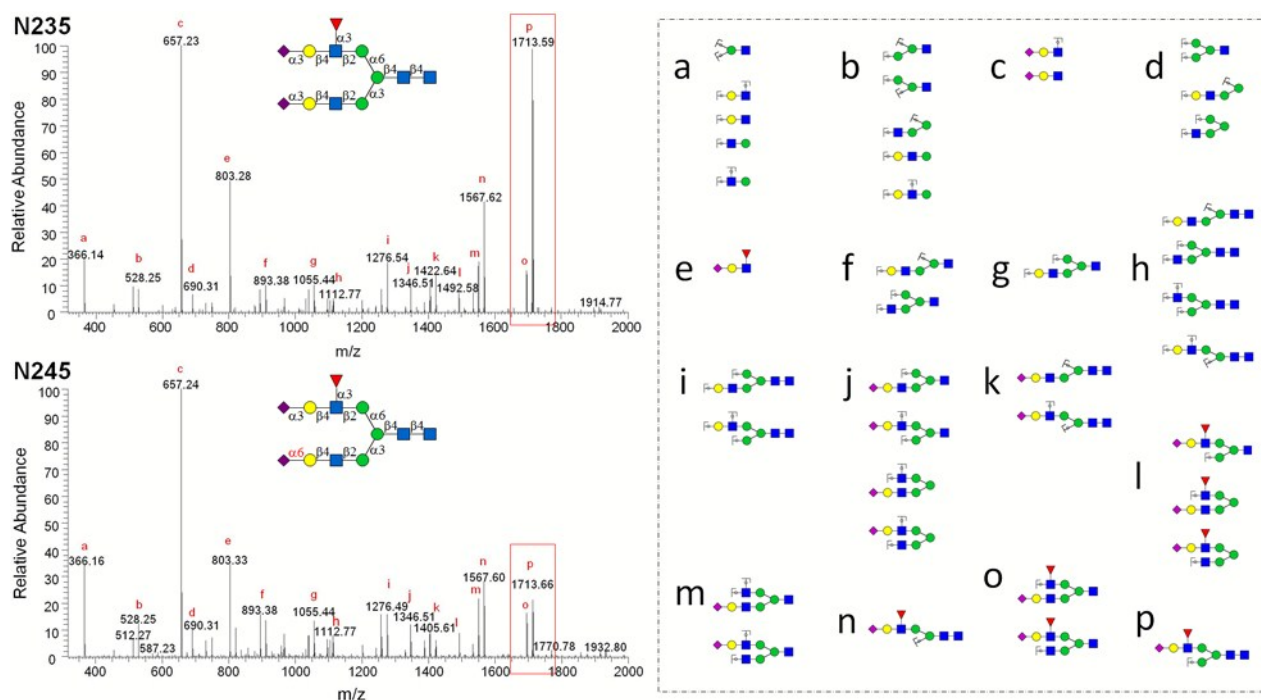


Figure 4. MS² analysis of N-glycan isomers N235 and N245.

MS² analysis of selected N-glycan isomers. The structurally well-defined N-glycans were subjected to MS² analysis, forming a specific spectra library (see **Supporting Data Set**). The library may be used readily to differentiate some N-glycan isomers in biological samples. For example, glycan structural isomers of α 2,3 and α 2,6Neu5Ac terminated N-glycans can be distinguished from their MS² spectra. N235 and N245 are a pair of isomers with the only difference on the sialyl linkage of the terminal Neu5Ac residue on the α 1,3Man branch. In the MS² spectrum of N235, the base peak at $m/z = 1713.59$ is gained from losing Neu5Ac, Gal, and GlcNAc (break between GlcNAc and Man residues of α 1,3Man branch), whereas the same ion in MS² spectrum of N245 is significantly weaker (**Fig. 4**). It is thus presumed that the glycosidic bond between GlcNAc and Man in N235 is easier to break than that in N245. Same phenomena were also observed in other Neu5Ac α 2,3, Neu5Ac α 2,6 isomer pairs, such as N042 and N043, N212 and N213, N222 and N223. Therefore, signal intensity of fragmentation ions can be reliable evidence in distinguishing linkage patterns between Gal and Neu5Ac residues. Interestingly, this phenomenon was only observed for the structures on the α 1,3Man branch, for example, while changing linkage on the α 1,3Man branch (isomers N002 and N133, or N003 and N233) resulted in similar changes in spectra as described above, no significant changes were observed between N002 and N233 or N003 and N133, which differ only on the α 1,6Man branch (**Fig. S8**). This phenomenon is interesting yet hard to comprehend. Several other unique MS² fragmentation patterns were also found and may be used in distinguishing isomers in the further.

Discussion

Given the diversity and micro-heterogeneity nature of complex glycans, as well as significant roles they played in biological processes, libraries of structurally well-defined glycans are in urgent demand for studies in Glycobiology and Glycomedicine. Decades of efforts has been paid for developing chemical methodologies and enzymatic approaches to synthesize N-glycans, however, none was

able to (cost-)efficiently generate large numbers of N-glycans. This is mainly due to: (1) Chemical methodologies developed so far are not cost-effective and rapid enough to prepare numerous complex N-glycans. Generally, each methodology was developed specifically for certain N-glycan structures, thus not suitable for efficient synthesis of other complex N-glycans. (2) GTs applied in the enzymatic or chemoenzymatic synthesis approaches were mostly from eukaryotes,^{8a, 9, 31} which are typically hard to access and exhibit narrow substrate specificity towards glycan acceptors. Most recently, several N- and O-glycans with multisialylated poly-N-acetylglactosamine extensions were successfully synthesized using a single bacterial α 2,6-sialyltransferase (Pd2,6ST).^{8b} This revealed the power of bacterial GTs in diversifying glycans. (3) A highly efficient and rapid N-glycan purification approach is lacking. So far the only reported approach is gel-filtration, which takes many hours to separate each target, and is not able to separate complex glycans with only one monosaccharide difference.

This work found answers to the above three obstacles. Firstly, a highly efficient strategy was developed based on the consistent use of oligosaccharyl thioether for the convergent installation of branched GlcNAc-terminated antennae to achieve high stereoselectivity with excellent yields. This approach minimized synthetic steps and maximized yield, which proceeded very efficiently with less glycosyl donor (1.3 equivalents) and mild conditions (at 0 °C). Notably, when the (Ac₃)GlcNAc β 1,2-Man disaccharide thioether **3** and Bn-GlcNAc β 1,2-Man disaccharide thioether **4** were used as donors, installations on 3-OH of tor **1** were achieved with excellent yield and high stereoselectivity. We were also able to install the Man₃ thioether donor **5** on the 6-OH of the β -Man in good yield and high stereoselectivity, as seen before in our previous report.^{18, 32} Using this strategy, 8 N-glycan core structures with 5 - 8 monosaccharide residues were convergently synthesized. We expect this strategy would allow us to prepare more N-glycans with various glycoforms for enzymatic extension.

Secondly, a general enzymatic extension strategy is developed that can extend any GlcNAc terminated glycans to 5 more glycans (including Le^x and SL^x) using B4GALT1 and three robust bacterial GTs. Such a strategy enabled generation of 5 - 15 more N-glycans

from each chemically synthesized core structure. During the synthesis of these N-glycans, each of the GTs was tested towards 10 to 21 N-glycan acceptors. For example, PmST1m showed comparable high activities towards **N001**, **N011**, **N021**, **N031**, **N041**, **N051**, **N111**, **N123**, **N124**, **N125**, **N211**, **N223**, **N224** and **N225** (which share a common Gal β 1,4-GlcNAc motif), and efficiently catalyzed the formation of corresponding α 2,3Sialylated N-glycans. In addition, the successful synthesis of Neu5Gc terminated N-glycan **N012G** indicated that PmST1m is also promiscuous towards sugar donors. Furthermore, substrate specificity study revealed that Hp α 1,3FT can well accept various N-glycans terminated with LacNAc or Siaa2,3LacNAc (ESI[†], Table S1). Similar relaxed substrate specificities were also found for B4GALT1 and Pd2,6ST towards various N-glycan acceptors. These results clearly indicated that: a) the 4 robust GTs only recognize the most adjacent one or two monosaccharide residues in glycosylation reactions, thus has a great potential to extend various N-glycans; b) the promiscuity of the bacterial GTs towards sugar donors dose not affected by acceptors, no matter simple oligosaccharides^{23, 27, 33} or complex N-glycans were used, thus has a great potential to synthesize N-glycan derivatives.

Thirdly, instead of generally used gel-filtration, each N-glycan was purified to >98% by HPLC utilizing a HILIC column in milligram scales (up to 4 mg per run). This HPLC-based approach could well separate complex N-glycans with only one monosaccharide difference, and takes only 30 min per injection.

Among the synthesized structures, only a few (e.g. **N011**, **N001**, **N002**, **N003**, **N6000**) were previous synthesized via chemical^{7b, 15c, 34} or chemoenzymatic approaches.^{8b, 31, 35} This library covers a number of low molecular weight N-glycans which have or have not been identified,³⁶ including most common hybrid and bi-antennary complex types. More importantly, this work represents the first report in preparing high pure N-glycan isomers. This N-glycan library contains 21 groups of isomers (Fig. S7) (2 to 6 distinct structures in each group), e.g. glycans **N125**, **N134**, **N144**, **N225**, **N234** and **N244** are isomers with the same molecular weight of 2077.7455. These groups of isomers are valuable standards that may be applied in absolute quantification and structural identification of N-glycans by MS.

Conclusions

We have successfully developed a Core Synthesis/Enzymatic Extension (CSEE) strategy for efficient synthesis of structurally defined N-glycans and a HPLC-based approach for rapid purification of these compounds. The combination of CSEE and HPLC purification allowed rapid access of 0.5 - 2 mg of 73 homogenous N-glycans in high purities (>98%), including 63 isomers (21 groups). These N-glycans are valuable materials for glycan analysis and bioactivity evaluation. In this work, oligosaccharyl thioether was used as a chemical glycosylation donor for the convergent installation of branched GlcNAc-terminated antennae, this general and efficient approach produced 8 N-glycan core structures with high stereoselectivity and excellent overall yields. This work also demonstrated that any GlcNAc terminated glycans can be enzymatically extended to 5 or more longer glycans (including Le^x and SLe^x) using several robust glycosyltransferases. Moreover, this work showed that complex N-glycans are best purified using HPLC utilizing a HILIC column. In summary, the CSEE strategy described here provides a practical approach for rapid production of structurally defined N-glycans, and has the potential to become a general approach to solve the complexity and diversity of glycomes. This may mark a beginning of "mass production" of glycomes.

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Author Contribution

L.L., Y.L., C.M. and P.G.W. designed experiments, analyzed data and wrote the manuscript. L.L. performed enzymatic synthesis, purification and HPLC identification of glycans; Y.L., B.W., and Z.X. performed chemical synthesis and NMR analysis; C.M., J. Q. and N.W. performed MS analysis. All other authors expressed and purified enzymes.

Competing financial interests

The authors declare no competing financial interests.

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