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Authors

Na, Lan
Li, Riyao
Chen, Xi

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Recent progress in synthesis of carbohydrates with sugar nucleotide-dependent glycosyltransferases

Lan Na, **Riyao Li**, **Xi Chen**

Department of Chemistry, University of California Davis, Davis, California, USA.

Abstract

Sugar nucleotide-dependent glycosyltransferases are key enzymes that catalyze the formation of glycosidic bonds in nature. They have been increasingly applied in the synthesis of complex carbohydrates and glycoconjugates with or without *in situ* generation of sugar nucleotides. Human glycosyltransferases are becoming more accessible and new bacterial glycosyltransferases have been identified and characterized. An increasing number of crystal structures elucidated for glycosyltransferases from mammalian and bacterial sources facilitate structure-based design of mutants as improved catalysts for synthesis. Automated platforms have also been developed for chemoenzymatic synthesis of carbohydrates. Recent progress in applying sugar nucleotide-dependent glycosyltransferases in enzymatic and chemoenzymatic synthesis of mammalian glycans and glycoconjugates, bacterial surface glycans, and glycosylated natural products from bacteria and plants are reviewed.

Keywords

carbohydrate; biocatalyst; chemoenzymatic synthesis; enzymatic synthesis; glycosyltransferase; sugar nucleotide

1. Introduction

Carbohydrates have been found in all kinds of organisms and are the most abundant organic compounds on earth. They not only are structural components and energy sources of diverse organisms but also play important roles in their molecular recognition and interaction, bioprocess regulation, protection, and many other events. Unlike the biosynthetic processes of nucleic acids or proteins which are template-driven, carbohydrates are biosynthesized by the orchestrated actions of carbohydrate-active enzymes without any templates. Different types of enzymes for the formation of glycosidic bonds have been discovered [1]. Leloir-type glycosyltransferases (GTs) use sugar nucleotides as activated donor substrates and are

xiichen@ucdavis.edu.

Declaration of competing interest

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the key enzymes for the formation of glycosidic bonds in nature. Either a nucleoside diphosphate (NDP)- or a nucleoside monophosphate (NMP)-activated monosaccharide is used by Leloir-type GTs (Figure 1a) to transfer the monosaccharide to the acceptor with either inverting or retaining of the stereochemistry of the anomeric carbon of the donor monosaccharide. An NDP-activated monosaccharide can also be used by Leloir-type glycosyl-1-phosphotransferases (GPTs) (Figure 1b) to transfer the monosaccharide-1-phosphate to the acceptor. These enzymes and their recent applications in synthesizing mammalian glycans and glycoconjugates, bacterial surface glycans, and glycosylated natural products are the subject of discussion here. Strategies for regioselective glycosylation of mammalian glycans are highlighted with several examples. Oligosaccharyltransferases (OSTs) that use an oligosaccharide-diphosphate-lipid as the donor substrate [2–4] and glycosidic linkage formations catalyzed by glycoside phosphorylases (GPs) [5,6] or transglycosylases (TGs) [7–9] will not be discussed.

2. Enzymatic and chemoenzymatic synthesis of mammalian glycans and glycoconjugates

Both mammalian and bacterial GTs have been applied in the synthesis of mammalian glycans and glycoconjugates. The establishment of general mammalian and insect cell expression platforms as well as the efforts of expressing all known human GTs [10] greatly facilitate the application of mammalian GTs in the synthesis of mammalian glycans. The efforts also led to the access to the crystal structures of an increasing number of mammalian GTs [11]**[10–12], which helps to better understand the substrate specificities of these enzymes and guide their applications in enzymatic and chemoenzymatic synthesis. On the other hand, an increasing number of bacterial GTs have been identified, characterized, and applied to the synthesis of mammalian glycans and glycoconjugates with or without *in situ* generation of sugar nucleotide donors in one-pot multienzyme (OPME) [13] or enzyme module (EM) [14,15] systems. In these systems, NDP-monosaccharide (e.g. UDP-D-GlcNAc, UDP-D-GalNAc, UDP-D-Glc, UDP-D-Gal, UDP-D-GlcA, GDP-L-Fuc, or GDP-D-Man) is obtained from a free monosaccharide, adenosine 5’-triphosphate (ATP), and a nucleoside triphosphate (NTP) by multienzymes including a sugar-1-phosphate kinase, an NDP-monosaccharide synthetase, with or without an inorganic pyrophosphatase (PpA) to break down the pyrophosphate generated to drive the reaction towards the formation of NDP-monosaccharide. In comparison, NMP-monosaccharide (e.g. CMP-sialic acid, CMP-5,7-di-*N*-acetyllegionanimic acid) is usually synthesized from monosaccharide, or its precursor and pyruvate, and NTP by a NMP-monosaccharide synthetase with or without an aldolase [16,17]. Examples of recent success on GT-dependent synthesis of human milk oligosaccharides, carbohydrate antigens, glycoprotein *N*-glycans and *O*-glycans, and glycosphingolipids are presented below. Strategies for controlling glycosylation regiospecificity are highlighted with examples. The applications of GTs in modifying mammalian cell surface glycans [18–20] are not discussed. Efforts on GT-dependent synthesis of glycosaminoglycans [21–24] are the subjects of several recent reviews [25,26] and are not covered here.

2.1. Human milk oligosaccharides (HMOSs)

Numerous bacterial GTs have been identified and used for synthesizing human milk oligosaccharides (HMOSs), a family of more than 100 structurally distinguished oligosaccharides that have been shown to contribute to the health benefits of human milk to breast-feeding infants [27,28]. Most HMOSs are extended from lactose with GlcNAcβ1–3/6Gal and Galβ1–3/4GlcNAc with or without additional Fucα1–2Gal, Fucα1–3/4GlcNAc, Fucα1–3Glc, Neu5Acα2–3/6Gal, and/or Neu5Acα2–6GlcNAc linkages [29]. Type 1 glycan containing the Galβ1–3GlcNAc linkage is an important structural component of HMOSs. Several bacterial β1–3-galactosyltransferases (β3GalTs) that can be used for the synthesis of the linkage have been identified but usually have low expression levels in *E. coli*. Recently, a β3GalT from *Chromobacterium violaceum* (Cvβ3GalT) was expressed in a relatively large amount in *E. coli* and used together with *Streptococcus pneumoniae* TIGR4 galactokinase (SpGalK), *Bifidobacterium longum* UDP-sugar pyrophosphorylase (BLUSP), and *Pasteurella multocida* inorganic pyrophosphatase (PmPpA) in an OPME galactosylation reaction for multigram-scale (>10 g) synthesis of lacto-*N*-tetraose (LNT) in high yields with *in situ* generation of uridine 5'-diphosphate galactose (UDP-Gal) from galactose (Gal), ATP, and uridine 5'-triphosphate (UTP). It also allowed the synthesis of various fucosylated and/or sialylated LNT-containing HMOSs including a difucosyl LNT that has a fucose α1–2-linked to the Gal on the non-reducing end and another fucose α1–3-linked to the Glc at the reducing end of the LNT, a target that would be impossible to obtain enzymatically without a suitable β3GalT such as Cvβ3GalT [30]. Numerous fucosylated HMOSs were also synthesized using sequential OPME glycosylation reactions including OPME fucosylation reactions containing an α1–3/4-fucosyltransferase cloned from *Helicobacter pylori* DSM 6709 (HpFucTIII) [31] or an α1–3-fucosyltransferase cloned from *Bacteroides fragilis* (Bf3FT) [32]. A list of α1–2-fucosylated HMOSs were also obtained [33].

2.2. Carbohydrate antigens

Cancer-related carbohydrate antigens continue to be attractive synthetic targets. GT-dependent chemoenzymatic synthesis of carbohydrate antigens has been facilitated by streamlined chemical synthesis of suitable GT acceptors. A protecting group-free chemical glycosylation strategy via an *N*′-glycosyl *p*-toluenesulfonohydrazide intermediate was used for facile synthesis of GlcNAcβProN_3 from free GlcNAc. The resulting monosaccharide glycoside was readily converted to disaccharide Galβ1–3GlcNAcβProN_3 using *Bifidobacterium infantis* D-galactosyl-β1–3-*N*-acetyl-D-hexosamine phosphorylase (BiGalHexNAcP) together with *Streptococcus pneumoniae* TIGR4 galactokinase (SpGalK). Gram-scale synthesis of Lewis a (Le^a) antigen was then successfully achieved by *Helicobacter pylori* α1–3/4-fucosyltransferase (Hp3/4FT)-containing OPME fucosylation reaction with *in situ* generation of guanosine 5'-diphosphate-L-fucose (GDP-Fuc) from L-fucose, ATP, and guanosine 5'-triphosphate (GTP) by a bifunctional *Bacteroides fragilis* enzyme with both L-fucokinase and GDP-Fuc pyrophosphorylase activities (BfFKP) and PmPpA. OPME sialylation reaction containing *Pasteurella multocida* sialyltransferase 1 (PmST1) M144D mutant was shown to be highly efficient in α2–3-sialylating Le^a to produce sialyl Lewis a antigens containing different forms of sialic acids and derivatives [34]. The combination of BiGalHexNAcP-based OPME β1–3-galactosylation followed by

PmST1-based OPME α2–3-sialylation reactions was also used for synthesizing sialyl T-antigen and its fluorine-derivatives [35].

Disialylglobo-heptasaccharide (DSGb5) was produced from chemically synthesized globo-pentasaccharide Gb5 with human ST3Gal-I-catalyzed α2–3-sialylation of the terminal Gal followed by human ST6GalNAc-VI-catalyzed α2–6-sialylation of the penultimate residue, GalNAc, in Gb5 [36]. The unique acceptor substrate specificities of the two human sialyltransferases made the enzymatic sialylation a straight forward process.

The reverse acylation activity of a *Cyclobacterium marinum N*-acetylglucosamine deacetylase (CmCDA) was discovered and its substrate promiscuity towards different carboxylic acids allowed its application in an OPME system together with a sialyltransferase, a CMP-sialic acid synthetase, a sialic acid aldolase, and a GlcNAc C2-epimerase for high-yield synthesis of α2–6-linked sialosides containing a sialic acid derivative with different *N*-acyl chains (acetyl, propyl, or glycolyl) from glucosamine, carboxylic acid, pyruvate, CTP, and a galactoside [37].

2.3. Glycoprotein N- and O-glycans

Efficient chemoenzymatic synthetic processes have also been developed for an increasing number of glycoprotein *N*- and *O*-glycans including more challenging branched and asymmetric structures. Advances on protein/peptide site-specific glycosylation have also been made. One noticeable development is the **Glyco**sylation **S**equence **C**haracterization and **O**ptimization by **R**apid **E**xpression and **S**creening (GlycoSCORES) platform. It combines *E. coli*-based cell-free protein synthesis (CFPS) with self-assembled monolayers for matrix-assisted desorption/ionization (SAMDI) enzyme activity assay method. Recently, it was used to screen a large number (3,480) of unique peptides to identify substrates and glycosylation sites of human polypeptide *N*-acetylgalactosaminyltransferases ppGalNAcT1 and ppGalNAcT2 as well as human *O*-GlcNAc transferase (hOGT) in addition to polypeptide *N*-glycosyltransferases (NGTs) from *Actinobacillus pleuropneumoniae* (ApNGT), *Mannheimia haemolytica* (MhNGT), and *Haemophilus ducreyi* (HdNGT). The optimal peptide sequences (so called “GlycTags”) identified can be inserted to the proteins of interest to introduce *N*-glycosylation at desired sites [38]**. The strategy was further applied to screen 41 putative bacterial NGTs using 1254 peptides. *N*-Glycosylation of target proteins at multiple sites with different glycan forms can be achieved by inserting orthogonal peptide substrates of different NGTs [39].

To obtain asymmetric multi-antennary *N*-glycans, chemical synthesis of core structures followed by differentiating enzymatic extension has been a common strategy. The chemical synthetic process, however, can be long. To simplify the process of accessing the core, biantennary GlcNAc-terminated *N*-glycan structure was produced in gram-scales by pronase and glycosidase treatment of a glycopeptide isolated from egg yolk powder. Uridine 5’-diphosphate-*N*-trifluoroacetylglucosamine (UDP-GlcNTFA) was used as an alternative sugar nucleotide donor for mammalian *N*-acetylglucosaminyltransferases MGAT4 and MGAT5 to introduce additional GlcNTFA-terminated branches to the biantennary *N*-glycan core. The *N*-TFA group can be easily removed under a basic condition to form an amino group which can be further protected by conversion to an azido group. Unlike GlcNAc, the

resulting $GlcNH_2$ and $GlcN_3$ in the oligosaccharide acceptors are not suitable sites for enzymatic galactosylation, thus allowing regio-selective galactosylation of GlcNAc to take place. Both $GlcNH_2$ and $GlcN_3$ were readily converted back to GlcNAc to form suitable galactosylation sites to produce desired asymmetric glycan structures [40]**. An example of applying the strategy in synthesizing a complex asymmetric tetra-antennary *N*-glycan is shown in Figure 2a.

For glycoprotein *O*-glycans, in addition to *O*-GalNAc glycan synthesis [41]** which is discussed in more details below, protein *O*-mannosylated glycans have attracted much attention for chemoenzymatic synthesis. The combination of chemical synthesis of core structures (Cores M1–M3) with glycan extension by GT-based enzymatic extension with or without *in situ* generation of sugar nucleotide donors in OPME reactions has been found to be an efficient approach [14,42]. More than ninety *O*-mannosylated glycans with or without different forms of α2–3- or α2–6-sialic acids, α1–3-linked fucose [14,42], and/or glucuronic acid (with or without 3-*O*-sulfation) β1–3-linked to LacNAc (e.g. in glycans containing human natural killer-1 epitope) [43]** have been synthesized and used for binding studies of carbohydrate-binding proteins in glycan microarray studies.

2.4. Glycosphingolipids

Gangliosides are a class of important sialic acid-containing glycosphingolipids in human. A highly efficient chemoenzymatic strategy for total synthesis of gangliosides has been developed [44]. The strategy started by chemical synthesis of water-soluble lactosyl phingosine (LacβSph) followed by extension of the lactoside using GT-dependent sequentially OPME systems. Chemical acylation as the last step converted the glycosylsphingosines readily to the desired glycosphingolipids [45]. The tolerance of the lipid-tagged glycan, LacβSph, as a suitable acceptor by *Pasteurella multocida* α2–3-sialyltransferase 3 (PmST3) is critical for the gram-scale high-yield production of GM3 sphingosine, a core structure for the synthesis of other ganglioside sphingosines (glycosylsphingosines of GM2, GM1, fucosyl GM1, GD3, and GD2). The sphingosine in the glycosylsphingosines serves as a natural hydrophobic tag to facilitate the facile product purification of the OPME glycosylation reactions using a C18 cartridge [45].

2.5. Strategies for regioselective glycosylation

Due to the multifunctionality of individual monosaccharides, glycans and glycoconjugates are consist of monosaccharides linked at different sites. Branch structures are also common. While regioselectivity for the formation of some glycosidic linkages can be achieved by choosing GTs or mutants with desired specificity, such enzymes may not be available for accessing many glycosyl linkages. Several strategies that have been developed to overcome the challenges include varying the ratio of donor and acceptor substrates, designing altering chemoenzymatic synthetic routes, and introducing protecting groups by chemical or enzymatic approaches in addition to chemically synthesizing asymmetric acceptors that is discussed above [14,42].

Mammalian GTs are well known for their high acceptor substrate specificities [40]** which are supported by the specific acceptor binding site modules in solved GT crystal structures

[11]**. Different acceptor substrate preferences have also been reported for bacterial GTs. The feature has been used for synthesizing complex targets in a regioselective manner as demonstrated in a recent report for sequential chemoenzymatic synthesis of sialyl core 2 glycans [41]**. Three sialyltransferases including *Pasteurella multocida* α2–3-sialyltransferase 1 M144D mutant (PmST1 M144D), PmST3, and *Photobacterium* species α2–6-sialyltransferase A366G mutant (Psp2,6ST A366G) were used in OPME sialylation systems to introduce α2–3- or α2–6-linked sialic acid at different sites. As shown in Figure 2b, starting from a chemically synthesized branched trisaccharide **F2b_1**, OPME β1–4-galactosylation reaction using *Neisseria meningitidis* β1–4-galactosyltransferase (NmLgtB) with *in situ* generation of UDP-Gal produced Core 2 (**F2b_2**) which can readily form disialyl Core 2 **F2b_3** using acceptor promiscuous PmST1 M144D or form monosialylated Core 2 **F2b_4** using PmST3 which preferred to sialylate the β1–4-linked galactoside branch. On the other hand, sialylating the Gal in chemically synthesized trisaccharide **F2b_1** using OPME sialylation system containing PmST1 M144D, monosialylated Core 2 glycan **F2b_5** was obtained. OPME β1–4-galactosylation reaction using NmLgtB with *in situ* generation of UDP-Gal produced **F2b_6** which was further sialylated with OPME reaction containing Psp2,6ST A366G to produce disialyl Core 2 **F2b_7** containing an α2–3- and an α2–6-linked sialic acid, respectively, on two branches. A good understanding of the acceptor substrate preference (preferring LacNAc to Lac) of *Helicobacter pylori* α1–3-fucosyltransferase (Hp3FT) also allowed the regio-selective control of the sites and the number of fucosylation by varying the ratio of the donor and the acceptor of the GT [46].

On the other hand, selective α1–3-fucosylation of internal GlcNAc residues in poly-LacNAc oligosaccharides was also achieved by alternating the order of OPME reactions by carrying out OPME α1–3-fucosylation reaction before the OPME β1–4-galactosylation reaction without [46] or with [47] an additional OPME sialylation reaction to obtain a large number of fucose-containing oligosaccharides [46,47].

Regioselective glycosylation has also been achieved by chemically synthesize GT acceptors with selectively protected sites. Such a method has been applied for regioselective enzymatic fucosylation of oligo-LacNAc structures using NH_2 or NHBoc as a substitute of the NHAc in GlcNAc to protect selective sites from fucosylation [48].

Enzymes have also been used to selectively protect specific glycosylation sites of the acceptors during enzymatic chain elongation processes to afford regioselectivity. One example of applying the strategy is discussed above for the synthesis of asymmetric *N*-glycans as shown in Figure 2a [40]**. Another is showcased in site-selective α1–3-fucosylation of the internal GlcNAc residues in poly-LacNAc structures using enzymatic α2–6-sialylation of the neighboring Gal at the non-reducing end as a protecting strategy. The α2–6-sialic acid introduced can be removed in the last step by sialidase-catalyzed reaction for the formation of target fucosides with desired fucosylation patterns. An impressive list of eighty-three structurally distinct glycans were successfully obtained [49].

To overcome the lack of regioselectivity in α2–6-sialylation using *Photobacterium damselae* α2–6-sialyltransferase (Pd2,6ST), an enzyme that has been used broadly for synthesizing α2–6-linked sialosides but does not differentiate internal and terminal β-linked galactose

(Gal) and/or *N*-acetylgalactosamine (GalNAc) residues for sialylation, an oxidation and reduction process was developed. Commercially available galactose oxidase (GOase) and a peroxidase were used to oxidize the C-6 of terminal Gal or GalNAc in oligosaccharide acceptor substrates to form an aldehyde or its hydrated germinal diol form, serving as a small-size protecting group to prevent C-6 from α2–6-sialylation. Upon further chain elongation for the synthesis of poly-LacNAc structures with desired protection pattern, Pd2,6ST-containing OPME sialylation reaction was performed to sialylated non-protected Gal and/or GalNAc. The aldehyde can be reduced using sodium borohydride ($NaBH_4$) in aqueous solution to form the desired unprotected Gal. Sixty-six structurally distinguished glycans were successfully obtained [50]**. An example of synthesizing sialosides containing different sialic acid forms with various sialyl linkages in a regio-specific manner with the GOase-oxidation and downstream reduction strategy is shown in Figure 2c.

Protein engineering is also an efficient strategy to improve the regioselectivity of GTs. For example, bulkier groups were introduced to the amino acid residues in the acceptor binding pocket of Pd2,6ST by mutagenesis and the resulting Pd2,6ST A200Y/S232Y double mutant allowed selective sialylation of the terminal Gal in the presence of internal Gal or GalNAc residue in the acceptor substrate while retaining its promiscuity towards donor substrates [51].

3. Chemoenzymatic synthesis of bacterial surface glycans

Various advances have been made in chemoenzymatic synthesis of numerous bacterial surface glycans (Figure 3). Bacterial surface polysaccharides [52] are biosynthesized mainly via three (Wzx/Wzy-dependent, ATP-binding cassette (ABC) transporter-dependent, and synthase-dependent) major biosynthesis and export processes. While the former two processes start by transfer a monosaccharide-1-phosphate to a lipid phosphate, the synthase-dependent process usually does not need the lipid phosphate in the substrate [53]. New GPTs and GTs have been characterized. A chemoenzymatic synthon strategy was developed to extend the scope of products of a given set of enzymes. OPME systems have been used to lower the synthetic cost. Better understanding of polysaccharide synthases has been achieved and their application in synthesizing structurally defined oligosaccharides are facilitated by the use of hydrophobic UV-detectable tags. Reverse glycosylation activity has been identified for a polysaccharide synthase and mutagenesis has been found to be effective in overcoming its drawback in synthesizing longer chain oligosaccharides. High-throughput screening platforms have been developed for directed evolution of polysialyltransferase [54] and *Pasteurella multocida* hyaluronan synthase [55].

3.1. Bacterial glycosyl-1-phosphotransferases (GPTs) or phosphoglycosyltransferases

Glycosyl-1-phosphotransferases (GPTs) or phosphoglycosyltransferases catalyze the transfer of glycosyl-1-phosphate from the corresponding nucleoside-diphosphate sugar donor to a suitable acceptor (Figure 1b). The acceptor can be a lipid phosphate, such as a polyprenyl monophosphate, or a glycoside.

The tolerance of alternative UDP-sugars by GPTs such as *Salmonella enterica* WbaP (SeWbaP) and *Aeromona hydrophilia* WecP (AhWecP) that are involved in initiating the

biosynthesis of polyprenyl diphosphate oligosaccharide repeating units for the Wzx/Wzy-dependent process was recently demonstrated using fluorescent tagged polyprenyl monophosphate derivatives [56].

A new GPT, mannosyl-1-phosphotransferase (CdManPT) that is responsible for the formation of *C. difficile* polysaccharide II (PSII), was identified and characterized using a chemoenzymatically synthesized phenylated disaccharyl diphosphate lipid to produce a trisaccharyl diphosphate lipid (**F3a_1**). It requires a glycosyl diphosphate lipid acceptor and guanidine 5'-diphosphate (GDP) as the nucleotide component in the sugar nucleotide donors. It can, however, tolerate various sugars and derivatives in the sugar component of the sugar nucleotides as donors. GDP-mannose (GDP-Man) and its derivatives containing C2-azido, C2-amino-, C2-fluoro, or C4-azido-modified mannose were suitable donor substrates. GDP-talose as well as GDP-glucose and its derivatives containing C2-azido or C2-amino-modified glucose were also tolerated [57]**. These studies paved the way for future chemoenzymatic synthesis of desired oligosaccharides and analogs which have been underexplored previously.

3.2. Bacterial glycosyltransferases (GTs) for the synthesis of bacterial surface glycans

Recent identification and characterization of major GTs and *O*-acetyltransferases involved in the biosynthesis of Enterobacteriaceae colanic acid containing an *O*-acetylated hexasaccharide repeat [58] have a great potential for future chemoenzymatic synthesis of related targets.

A chemoenzymatic synthon strategy was developed recently to synthesize glycosides containing rare sugars, such as 5,7-diacetamido-3,5,7,9-tetradeoxyd-glycero-D-galacto-non-2-ulosonic acid (Leg5,7Ac_2), a bacterial nonulosonic acid (NulO) analogue of sialic acid. A diazido-analog 2,4-diazido-2,4,6-trideoxy mannose (6deoxyMan2,4diN_3) was designed as the six-carbon precursor of Leg5,7Ac_2. It was chemically synthesized as used as a chemoenzymatic synthon in OPME reactions to obtain Leg5,7diN_3-containing glycosides which were readily converted to the desired di-NAc products. The OPME reactions each contained a GT (PmST1 M144D and Psp2,6ST) and other enzymes involved in the synthesis of the corresponding sugar nucleotide donor. The success of the strategy demonstrated the substrate promiscuity of the enzymes involved in the OPME synthesis. The OPME systems with *in situ* generation of activated sugar nucleotides made the activated unnatural sugars available and avoided their challenging purification processes and potential instability issues during storage. The chemoenzymatic synthon strategy allowed the expansion of the product scope of a limit set of available enzymes [59]. More recently, with the access to a recombinant *Legionella pneumophila* CMP-5,7-di-*N*-acetyllegionaminic acid synthetase (LpCLS), efficient OPME synthesis of α2–3-linked Leg5,7Ac_2-containing glycosides including a trisaccharide component (**F3a_2**) of the tetrasaccharide repeating unit of *Enterobacter cloacae* C6285 capsular polysaccharide (CPS) was achieved directly from Leg5,7Ac_2 or its six-carbon precursor 2,4-diacetamido-2,4,6-trideoxy mannose (6deoxyMan2,4diNAc) [17].

3.3. Bacterial polysaccharide synthases

Several polysaccharide synthases that catalyze the transfer of either sugar-1-phosphate and/or monosaccharide from the corresponding sugar nucleotide donors have been characterized an applied recently for the chemoenzymatic synthesis of polysaccharides and structurally defined oligosaccharides with repeating units containing one, two, or three sugar residues with or without a phosphate (**F3a_3–9**).

The Nm serogroup X CPS synthase (or *N*-acetylglucosamine-1-phosphotransferase) (NmCsxA) is the key enzyme for the synthesis of NmX CPS, a homopolymer containing α1–4-linked GlcNAc-1-P. NmX CPS oligosaccharides with an average degree of polymerization of 15 (avDP15) (**F3a_4**) were produced by immobilized a truncated NmCsxA with a controlled donor to acceptor ratio of 20:1. A similar strategy was used to synthesize NmA CPS oligosaccharides (**F3a_3**), a homopolymer containing α1–6-linked ManNAc-1-P, by immobilizing a C-His_6-tagged fusion protein of NmA CPS synthase (NmCsaB) and NmA UDP-GlcNAc 2-epimerase (NmCsaA) for in-situ generation of UDP-ManNAc from UDP-GlcNAc [60]**. On another report, a list of UDP-GlcNAc derivatives were shown to be suitable substrates of NmCsxA for producing NmX CPS polysaccharides or oligosaccharides which can be potential inhibitors against NmCsxA [61].

Noticeably, a new family of polymerases with both GT and hexose/polyol-phosphotransferase activities for producing polymers with oligosaccharide phosphate or glycosylpolyol phosphate repeating units were identified by searching homologs of *N. meningitidis* serogroup L CPS synthase NmCslB. The activities of CPS synthases from *H. influenzae* serotype c (HiCcs2) and various serotypes of *A. pleuropneumoniae* including ApCps1B, ApCps12B, ApCps3D, and ApCps7D were confirmed by NMR characterization of the polymers produced (**F3a_5–9**) [62]**. The effort enriched the catalyst tool box for enzymatic and chemoenzymatic synthesis of group 2 CPS and oligosaccharides.

Most recently, total synthesis of structurally defined oligosaccharides of NmW CPS consisting of −6Galα1–4Neu5Acα2- disaccharide repeating unit was successfully demonstrated using a sequential OPME platform [63]**. From a chemically synthesized chromophore-tagged monosaccharide (**F3b_1a**) (Figure 3b), NmW CPS polysaccharide synthase NmSiaDw, a bifunctional GT with both α1–4-galactosyltransferase and α2–6-sialyltransferase activities, was used in alternate galactose activation and transfer system (**OPME1**) and sialic acid activation and transfer system (**OPME2**) to form structurally defined galactosides (**F3b_2a–e**) and sialosides (**F3b_1b–e**) in up to gram-scale (0.22–1.26 g) with high yields (83–96%) [63]**. The removable benzyloxycarbonyl (Cbz)-tag in the substrate facilitated reaction monitoring, product purification by C18 cartridges, and biochemical characterization of the NmW CPS synthase NmSiaDw.

A dual-functional polysaccharide synthase, *Pasteurella multocida* heparosan synthase 2 (PmHS2) having both α1–4GlcNAcT and β1–4GlcAT GT activities, has been widely utilized for the synthesis of heparan sulfate and heparin derivatives. *N*-Terminal 80-amino-acid truncation of PmHS2 was found to improve its solubility, expression level, and thermostability. The resulting Δ80PmHS2 was successfully applied in sequential OPME reactions (**OPME3** for GlcNAc activation and transfer and **OPME4** for GlcA activation and

transfer) for the synthesis of heparosan oligosaccharides (**F3c_1** and **F3c_2**) up to a hexasaccharide from a chemically synthesized fluorescent-tagged monosaccharide (**F3c_1a**) (Figure 3c). Reverse glycosylation of Δ80PmHS2 was observed which led to low yields in OPME synthesis of a heptasaccharide. Δ80PmHS2 mutants with single function GT activities (Δ80PmHS2_D569N as a single function GlcAT and Δ80PmHS2_D291N as a single function GlcNAcT) were designed and generated. They were found to be more efficient catalysts in sequential OPME synthesis of structurally defined heparosan oligosaccharide of larger sizes (heptasaccharide-decasaccharide) [64]**.

4. Glycosylated natural products from plants, bacteria, and fungi

Carbohydrates are an integral components of many natural products [65] and glycosylation often affects the therapeutic activities and pharmacokinetics of natural products [66]. Significant advances have also been made in synthesizing glycosylated natural products with *O*-, *C*-, *N*-, and *S*-glycosidic bonds. These have been achieved by discovering new GTs including those with donor and acceptor substrate promiscuities from both plant and bacterial sources. Protein engineering has also been explored to obtaining GTs with new functions.

Among plant natural product GTs, those in the carbohydrate-active enzyme database (http://www.cazy.org) [67] GT1 family are the most well studied. Although they all use UDP-activated monosaccharides as donor substrates, the sugar component in the UDP-sugar donors and acceptor substrate can differ. Terpenoids [68], flavonoids, and coumarins are common acceptors substrates. Flavonoid glucosyltransferases have recently identified from maize (UFGT2) [69] and tomato (Twi1) [70]. On the other hand, a tomato UDP-glucosyltransferase (UGTSL2) was used to synthesize rebaudioside D (a diterpene glycoside) with recycling of UDP-Glc catalyzed by a potato sucrose synthase (StSUS1) [71]. A glucosyltransferase from indigo plant *Polygonum tinctorium* (PtUGT1) was identified and used in an environmental friendly biosynthetic process to generate indican as a soluble and stable glycoside for storage. Upon hydrolysis catalyzed by a β-glucosidase, the indoxyl released is spontaneously oxidized to form indigo dye [72].

A new benzophenone *C*-GT from mango plant *Mangifera indica* (MiCGTb) was shown to be able to catalyze the formation of bis-*C*-glycosylated product. Mutants of MiCGTb and a previously identified mono-C-GT MiCGT with improved donor and acceptor substrate promiscuities along with enhanced bis-C-glycosylation activity were obtained [73]**. A quadruple mutant MiCGTb-GAGM (S60G/V100A/T104G/I152M) was shown to have the desired improved *C*-glucosylation activity towards a diverse array of coumarins [74]. Both *C*- and *O*-glucoside products were observed and UDP-Gal was a suitable but poorer donor substrate. In addition to enzyme-catalyzed synthesis, MiCGTb-GAGM-containing whole cells were successfully used for synthesizing *C*-glucosyl coumarin products with comparable yields for most compounds [74].

Mutagenesis strategies including random mutagenesis, alanine scanning and sequence consensus analysis were used to improve the activity of a triterpene O-GT from plant *Siraitia*

grosvenorii SgUGT74AC1 [75]. A mutant with 10^4-fold improved activity and mutants with improved activity to non-native donors and acceptors were identified.

More recently, remarkable donor and acceptor substrate promiscuities of two plant GTs [76]** [77]** have been discovered. The crystal structures of both belong to GT-B fold. *Trollius chinensis* C-GT (TcCGT1) [76]** was shown to be able to add a glucose to the C-8 of a large number of flavones and flavonoids (**F4a_1**, Figure 4a). Mono-*C*-glucosylation of other compounds (**F4a_2** and **F4a_3**), di-*C*-glucosylation (**F4a_4** and **F4a_5**), *O*-glucosylation (**F4a_6** and **F4a_7**), as well as *N*- and *S*-glucosylation (**F4a_8**) were observed. In addition to UDP-Glc, TcCGT1 was able to use UDP-Xyl, UDP-Gal, and UDP-Ara as donor substrates although the latter two were poorer substrates. *Glycyrrhiza glabra* di-*C*-GT GgCGT [77]** was shown to be able to use numerous phenolic compounds as acceptors for *C*-glucosylation to form di-*C*-glucosylated or mono-C-glycosylated products with flopropione as the minimal structure component for di-*C*-glucosylation. GgCGT was also shown to be able to catalyze the formation of *N*- and *S*-glucosylated products (**F4a_8**). In addition to UDP-Glc, GcCGT was able to use UDP-Xyl, UDP-Gal, and UDP-Ara as donor substrates with decreased efficiencies.

Two bacterial GTs (Bs-GT from *Bacillus subtilis* 168 and Bc-GTA from *Bacillus cereus* WQ9–2, respectively) that can glycosylate a diterpenoid crocetin to produce plant glycosides monoglucosylated crocin-5 and diglucosylated crocin-3 have also been discovered [78].

Three bacterial CAZy GT1 family UDP-GTs (BLYjiC, BLYdhE, and BLYojK) have been identified from *Bacillus licheniformis* [79]. BLYjiC was used to glycosylate the glycan on avermectin B_{1a} with Glc, Gal, L-Fuc, or 2-deoxy-D-glucose. Glycosylation was shown to improve the water solubility and the antinematodal activity of avermectin B_{1a} [80]. The enzyme was also shown to be able to add a glucose to several tetracyclines [81]. On the other hand, both BLYdhE and BLYojK [79] have high promiscuity toward sugar nucleotide donors and acceptors. Their transglycosylation activities have been demonstrated. In addition to *O*-glycosylation, they could also catalyze the transfer of Glc to suitable acceptors to form *N*- and *S*-glycosides, respectively.

Glycosylated natural products containing uncommon sugars are challenging targets. Some of the uncommon sugars are formed at the sugar nucleotide level. For example, nogalamycin is an anticancer anthracycline glycoside containing two uncommon L-sugars. From TDP-Glc (**F4b_1,** Figure 4b), TDP-L-rhodosamine (**F4b_2**) was synthesized using seven enzymes in one pot (**OPME5**). It was then used as the donor substrate of rhodosaminyltransferase SnogD to *O*-glycosylate **F4b_3** to form a product which was converted to nogalamycine R (**F4b_4**) by oxygenase SnoK in another OPME reaction (**OPME6**) [82].

In addition to plant and bacterial GTs that have been identified for glycosylating natural products, a bifunctional fungal methylglucosyltransferase was identified from *Beauveria bassiana* (Bb). The promiscuous enzyme can use macrolides, anthraquinones, flavonoids, and naphthalene as suitable acceptor substrates for *O*- and *N*-glucosylation. Further 4-*O*-methylation of the newly introduced glucose residue was also achieved [83].

5. Automated chemoenzymatic synthesis

Recently, automated carbohydrate chemoenzymatic synthetic platforms have been developed. One [84]** uses a commercially available peptide synthesizer and takes advantage of a thermosensitive polymer that can be conjugated to an acceptor to allow enzymatic synthesis to be carried out in aqueous solution at room temperature or 37 °C and easy product purification by polymer aggregates at higher temperature followed by filtration. By switching to an amine-functionalized silica gel resin as a solid support that is suitable for both peptide synthesis in organic solvents and enzymatic extension of glycans, the peptide synthesizer platform was successfully used for producing glycopeptides [85]**. The other [86]** uses an commercial automation workstation and anionic sulfonate-tagged GT acceptors for enzymatic glycosylation to allow easy product purification by ion exchange resins. With the development of more efficient enzymes, protocols, reaction monitoring, product purification and characterization processes, these platforms will see increasing applications.

6. Conclusion and perspectives

With an increased access to GTs and GPTs from various sources and a better understanding of these important carbohydrate biosynthetic power tools, complex oligosaccharides, polysaccharides, glycolipids, glycopeptides, and glycosylated natural products are becoming more readily available. It is now not uncommon to see the production of a large library of complex glycans with the combination of a limited sets of GTs and GPTs in a single report. The discovery of the reserve glycosylation, glycosidase, and donor hydrolysis side activities of GTs make the biochemical characterization and reaction condition optimization essential for their synthetic applications. Enzymes involved in the formation of sugar nucleotides are becoming more important, not only to decrease the cost for the production of target compounds with common sugar building blocks, but also to allow an easier access to glycans with uncommon sugar building blocks. Crystal structure and mutagenesis studies will further facilitate the identification and improvement of synthetically useful and efficient enzymes. Compared to chemical synthesis of carbohydrates, there is a lower technical barrier for non-specialists to adopt enzymatic and chemoenzymatic synthetic strategies in their own laboratories. Automation of the carbohydrate enzymatic and chemoenzymatic synthetic processes will further decrease the barrier. Discovery of new enzymes (e.g. by genome mining, high-throughput screening, sequence similarity network and machine learning [87]), biochemical and structural characterization, protein engineering, improving enzyme formulation [88], making carbohydrate biosynthetic enzymes and necessary building blocks (e.g. acceptors, uncommon sugars) commercially available will attract and allow the broader community to study the important functions of carbohydrate and develop carbohydrate-based diagnostics and therapeutics.

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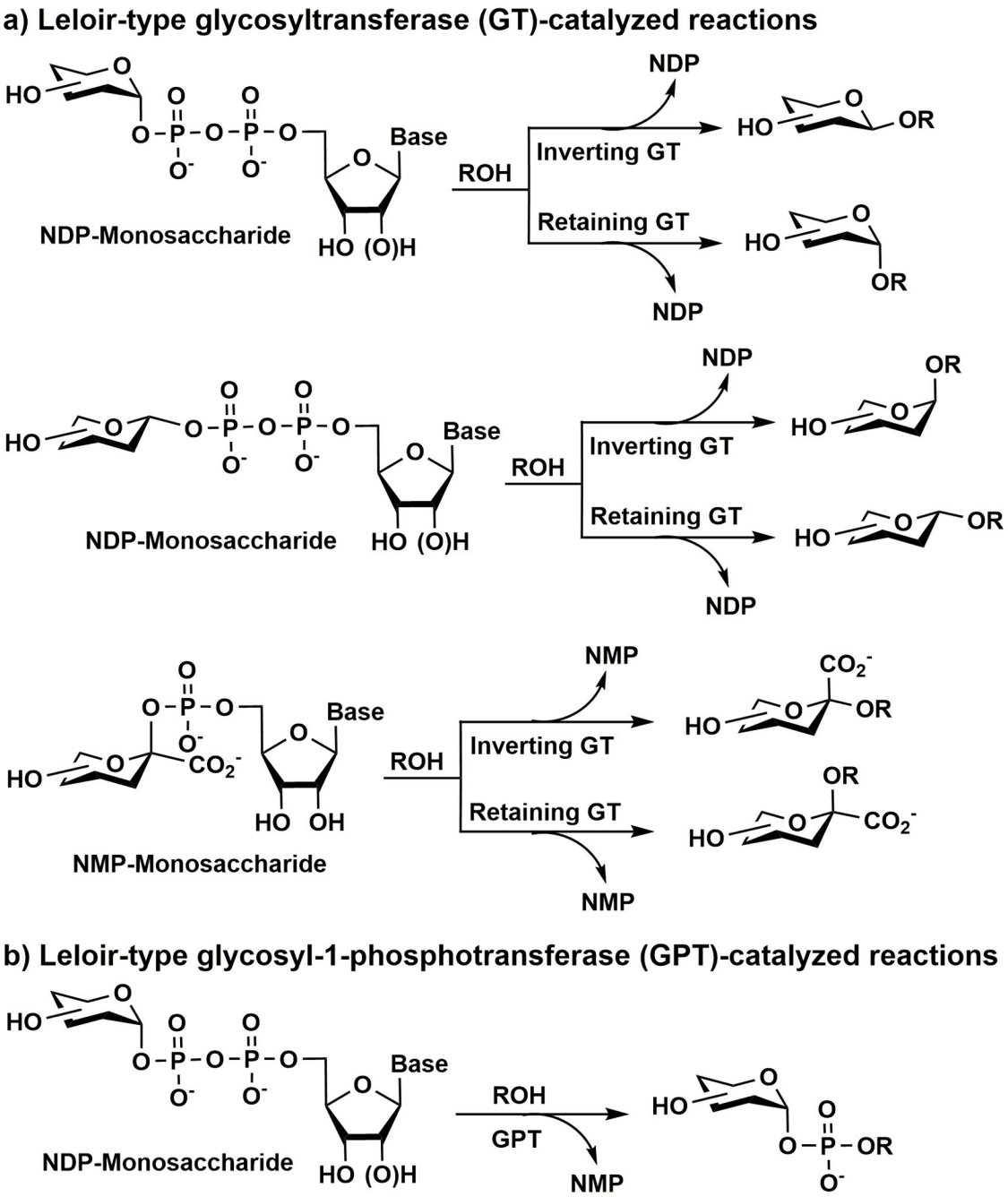


Figure 1.

Schematic illustration of reactions for the formation of glycosidic linkages by (**a**) Leloir-type glycosyltransferases (GTs) and (**b**) Leloir-type glycosyl-1-phosphotransferases (GPTs). While either a nucleoside diphosphate (NDP)- or a nucleoside monophosphate (NMP)-activated sugar could be a suitable sugar nucleotide donor substrate for GTs for transferring a monosaccharide to acceptors with inverting or retaining of the stereochemistry of the anomeric carbon in the sugar of the donor, GPTs usually use NDP-monosaccharides as donor substrates for transferring monosaccharide-1-phosphate to acceptors.

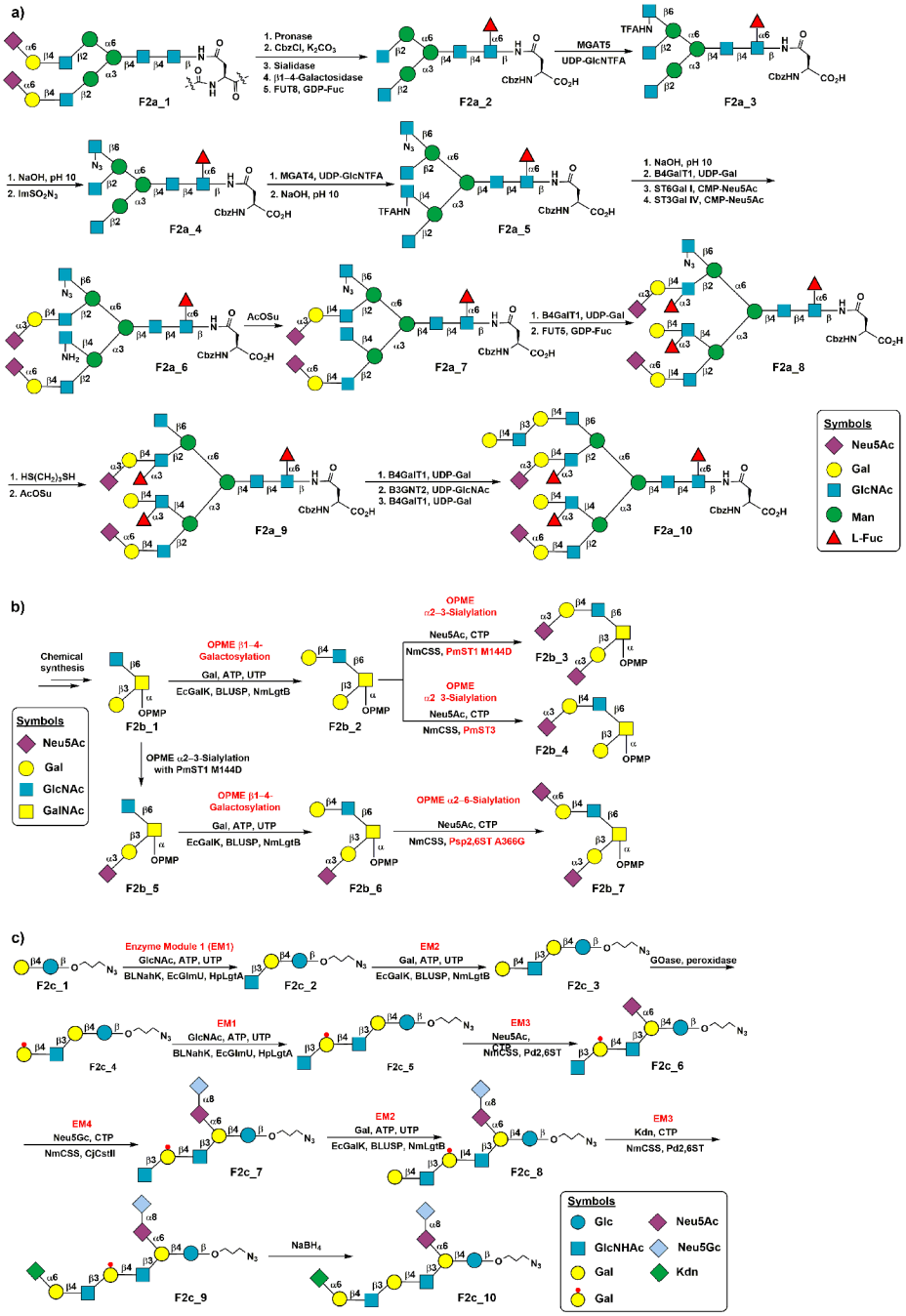
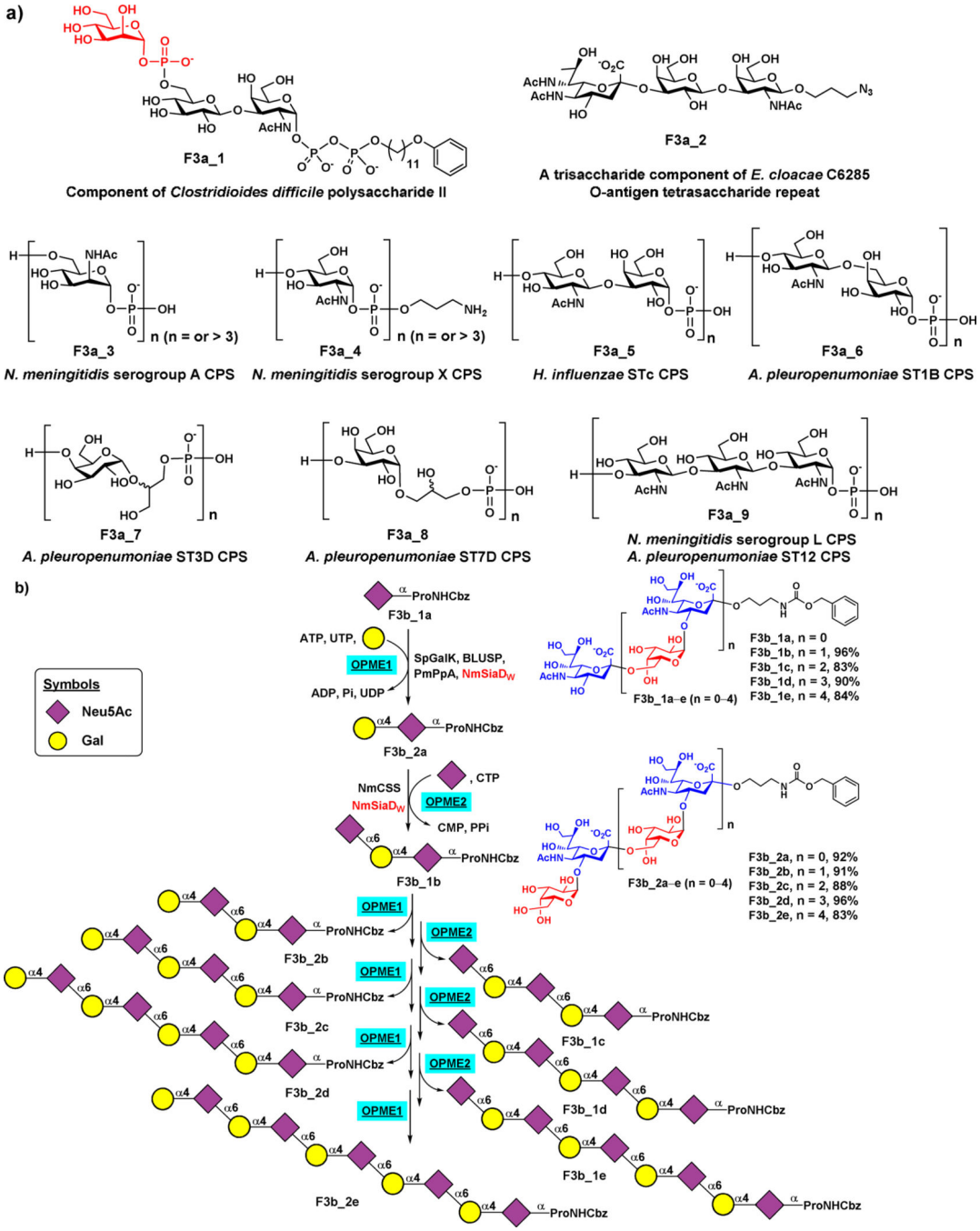


Figure 2.

Examples of representative regio-selective glycosylation strategies for the synthesis of asymmetric *N*-glycans [40]** **(a)**, sialyl *O*-glycans [41]** **(b)**, and sialyl poly-LacNAc structures [50]** **(c)** by GT-catalyzed reactions.



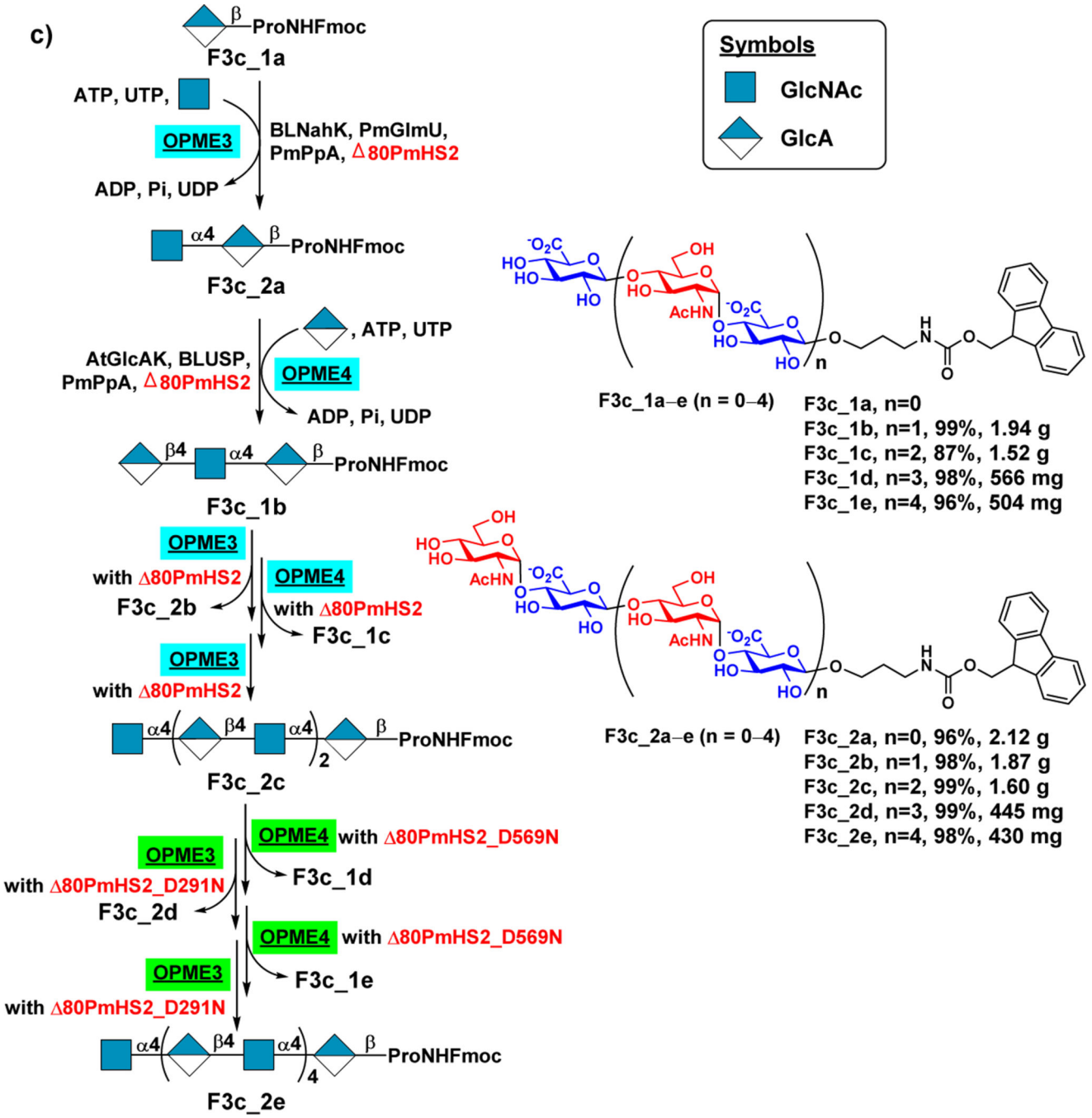


Figure 3.

Structures of some bacterial surface glycans [57]**[17] [60]**[62]**synthesized recently by enzymatic or chemoenzymatic methods **(a)**; an example of sequential one-pot multienzyme (OPME) chemoenzymatic synthesis of NmW CPS oligosaccharides [63]** **(b)**; and an example of sequential OPME synthesis of bacterial heparosan oligosaccharides using engineered polysaccharide synthases Δ80PmHS2 and its mutants [64]** **(c)**.

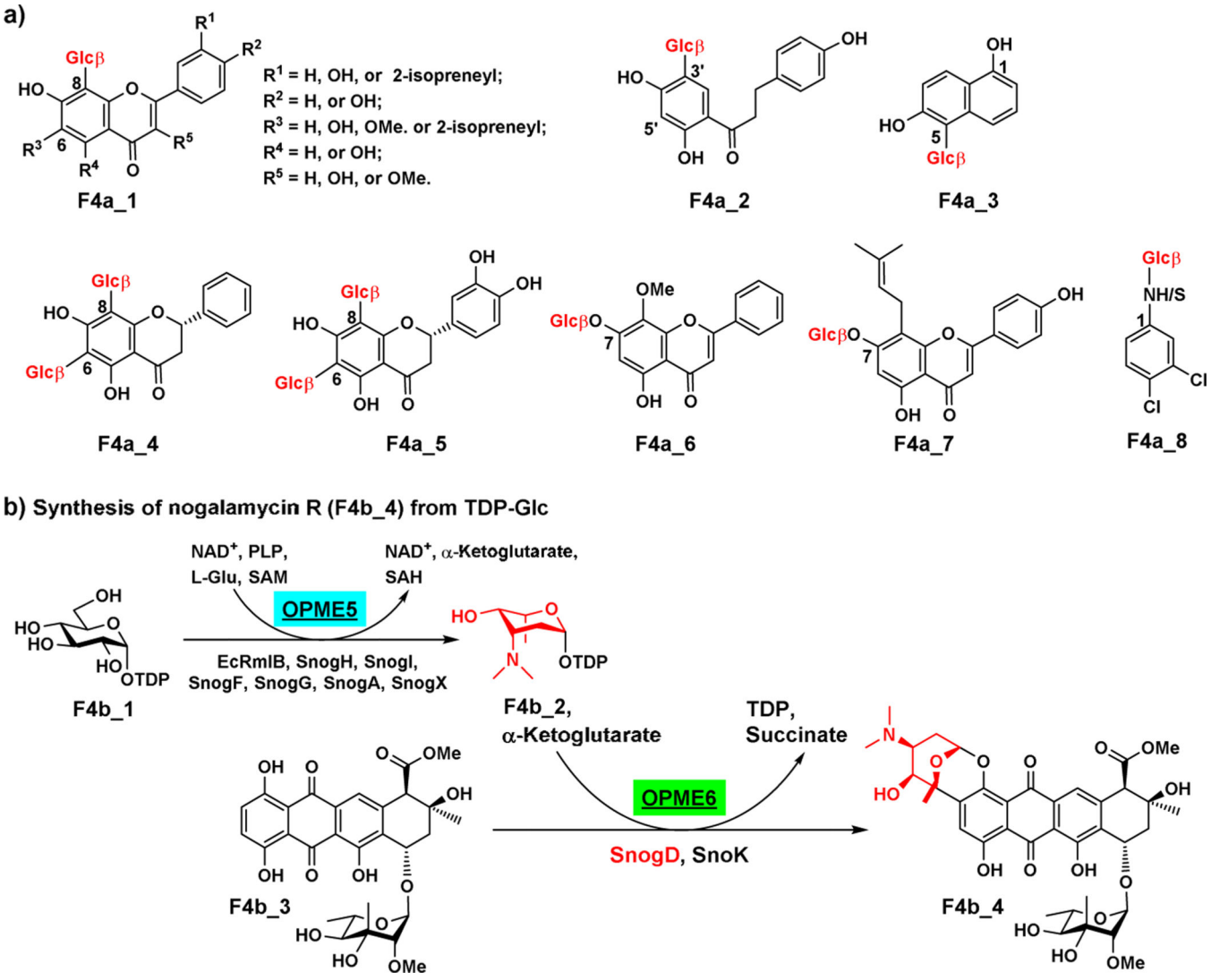


Figure 4.

Structures of selected glucosylated products [76]** [77]** catalyzed by *Trollius chinensis* C-GT (TcCGT1) **(a)**, and a simplified schematic illustration for the enzymatic synthesis of nogalamycine R [82] from TDP-glucose **(b)**. Enzyme abbreviations: EcRmlB, *E. coli* 4,6-dehydratase; SnogH, *S. nogalater* 2,3-dehydratase; SnogI, *S. nogalater* aminotransferase; SnogF, *S. nogalater* 5-epimerase; SnogG, *S. nogalater* 4-ketoreductase; SnogA and Snog X, *S. nogalater* methyltransferase; SnogD, *S. nogalater* rhodosaminyltransferase; SnoK, *S. nogalater* α-ketoglutarate dependent oxygenase.