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Strategies for chemoenzymatic synthesis of carbohydrates

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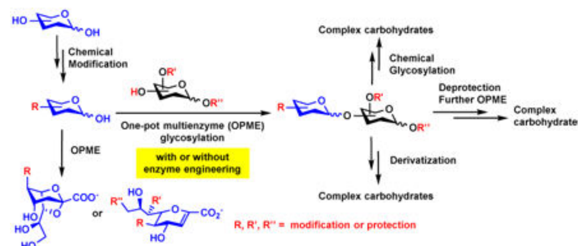
Abstract

Carbohydrates are structurally complex but functionally important biomolecules. Therefore, they have been challenging but attractive synthetic targets. While substantial progress has been made on advancing chemical glycosylation methods, incorporating enzymes into carbohydrate synthetic schemes has become increasingly practical as more carbohydrate biosynthetic and metabolic enzymes as well as their mutants with synthetic application are identified and expressed for preparative and large-scale synthesis. Chemoenzymatic strategies that integrate the flexibility of chemical derivatization with enzyme-catalyzed reactions have been extremely powerful. Briefly summarized here are our experiences on developing one-pot multienzyme (OPME) systems and representative chemoenzymatic strategies from others using glycosyltransferase-catalyzed reactions for synthesizing diverse structures of oligosaccharides, polysaccharides, and glycoconjugates. These strategies allow the synthesis of complex carbohydrates including those containing naturally occurring carbohydrate postglycosylational modifications (PGMs) and non-natural functional groups. By combining these strategies with facile purification schemes, synthetic access to the diverse space of carbohydrate structures can be automated and will not be limited to specialists.

Graphical Abstract



Keywords

carbohydrate synthesis; chemoenzymatic synthesis; glycolipid; glycosyltransferase; regioselective; enzyme engineering

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1. Introduction

Carbohydrates are the most abundant and structurally diverse naturally occurring organic compounds. They are presented on the surfaces of all types of cells from different organisms [1]. The cell surface carbohydrate coating (or glycocalyx) can be readily observed under microscopes [2]. Carbohydrates on mammalian cells are involved in numerous biological and pathological processes including homeostasis, cell-cell interaction, cell migration, development, bacterial and viral infection, inflammation, immunology, cancer metastasis, etc. [3, 4]. The variety of these properties are the results of the structural diversity of carbohydrates. Unlike proteins and nucleic acids, carbohydrates are not the products of template-driven biosynthesis but are directly dependent on the expression and substrate specificity of glycosyltransferases as well as the availability of corresponding sugar nucleotides [5]. Diverse monosaccharide building blocks and various stereo- and regiochemistry in glycosidic linkages contribute to the complexity of the linear and branched structures of carbohydrates. Additional modifications, such as epimerization, *O*-acetylation, *O*-methylation, *O*-phosphorylation, and *N*- or *O*-sulfation etc., can take place on the carbohydrates. [6, 7]. Most of these modifications occur after the formation of glycosidic linkages and are named as carbohydrate post-glycosylation modifications (PGMs) [6] in analogy to protein post-translational modifications. Therefore, PGMs add another layer of complexity in naturally occurring carbohydrates. To unravel the important functions "coded" by carbohydrates [8, 9], obtaining pure compounds in sufficient amount is necessary. While substantial progress has been made on advancing chemical glycosylation methods, incorporating enzymes into carbohydrate synthetic schemes has become increasingly practical with the increasing availability of carbohydrate-biosynthetic and metabolic enzymes as well as their mutants. Chemoenzymatic strategies that integrate the flexibility of chemical derivatization with enzyme-catalyzed reactions have been extremely powerful. The development of synthetic strategies using exoglycosidases, endoglycosidases, and their mutants in the production of oligosaccharides and glycoconjugates can be found in several recent reviews [10-12]. The current review focuses on one-pot multienzyme (OPME) systems and other representative chemoenzymatic strategies using glycosyltransferase-catalyzed reactions for synthesizing diverse structures of oligosaccharides, polysaccharides, and glycoconjugates.

2. One-pot multienzyme (OPME) chemoenzymatic glycosylation systems

Most carbohydrates in nature are synthesized by Leloir-type glycosyltransferase-catalyzed reactions. These glycosyltransferases require the use of sugar nucleotides as activated sugar donor substrates. Although expensive, the sugar nucleotides of some common human monosaccharide building blocks are commercially available. These compounds can be used directly for enzymatic and chemoenzymatic synthesis of carbohydrates if only small amounts of the desired products are needed. To decrease the synthetic cost and reduce potential inhibition of glycosyltransferases by nucleotide formed, methods for in situ recycling sugar nucleotide have been developed and were reviewed previously [13].

For carbohydrates containing naturally occurring PGMs [6] or non-natural occurring modifications, an efficient strategy is to use one-pot multienzyme (OPME) chemoenzymatic

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systems (Figure 1) [14]. This strategy relies on in situ production of a sugar nucleotide from a simple monosaccharide or its derivative, which is used as the donor for a glycosyltransferase in the same pot for the synthesis of desired carbohydrates without the isolation of intermediates. This strategy helps to decrease the cost for the synthesis of target carbohydrates in large amounts. More importantly, it allows the introduction of modified monosaccharides as potential precursors for sugar nucleotides for the synthesis of carbohydrates with modifications at specific sites. Multiple OPME systems can be carried out in sequential for producing longer chain oligosaccharides and glycoconjugates.

2.1. OPME sialylation systems for the chemoenzymatic synthesis of sialosides

An example of OPME glycosylation reactions is shown in Figure 2 for sialylation. Sialyltransferases (SiaTs) are the key enzymes for installing sialic acids. The most common form of sialic acid is *N*-acetylneuraminic acid (Neu5Ac) [15]. Sialyltransferases use cytidine 5'-monophosphate (CMP)-linked sialic acids such as CMP-*N*-acetylneuraminic acid (CMP-Neu5Ac) as an activated donor substrate. CMP-Neu5Ac can be prepared by a sugar activation (SA) system SA1 (Figure 2) from its 6-carbon precursor *N*-acetylmannosamine (ManNAc) involving a sialic acid aldolase-catalyzed aldol-addition reaction from ManNAc and pyruvate to produce Neu5Ac followed by a CMP-sialic acid synthetase (CSS)-catalyzed reaction using cytidine 5'-triphosphate (CTP). SA1 can be combined with a suitable sialyltransferase in one pot to produce sialosides with a desired sialyl linkage from simple ManNAc, pyruvate, CTP, and the acceptor without the isolation of intermediates. Products with different sialyl linkages (α2–3/6/8/9) can be produced by the specificity of different sialyltransferases used.

By introducing structural modification at the monosaccharides such as ManNAc or Neu5Ac by chemical synthesis, OPME sialylation systems can be used for direct synthesis of sialosides containing desired structurally modified sialic acid forms, diverse internal glycans, and different sialyl linkages. For synthesizing sialosides containing sialic acid with C5, C7, C8, and/or C9-modifications, the derivatives of sialic acid six-carbon precursors can be chemically synthesized and used as the starting material for one-pot three-enzyme (OP3E) sialylation systems. For sialosides containing C4-modifications at sialic acids, sialic acid derivatives should be used in one-pot two-enzyme (OP2E) systems for the synthesis (Figure 3). Sialic acid analogs with C3-modification can also be introduced by using 3-substituted pyruvate derivative in a sialic acid aldolase-catalyzed reaction. After purification, sialosides containing C3-modified sialic acid residue can be readily formed using this OP2E system [16]. The key for the success of these chemoenzymatic strategies is to access enzymes that have promiscuous substrate specificity to tolerate desired modification on the substrates. Ideally, the enzymes can also be expressed in *Escherichia coli* in large amounts to allow low cost production of sufficient quantity of catalysts for synthesis.

Quite remarkably, many wild-type enzymes from bacterial sources have desired properties of high activity, good expression in *Escherichia coli*, and substrate promiscuity. Using this OPME chemoenzymatic sialylation strategy, libraries of α2–3/6-linked sialosides [17-19] containing naturally occurring sialic acid forms (Figure 4A) and non-natural sialic acid modifications (Figure 4B) have been produced using bacterial sialic acid aldolases (e.g. from

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Escherichia coli [20] or *Pasteurella multocida* [21]), CMP-sialic acid synthetases (from *Neisseria meningitidis* [20] or *Pasteurella multocida* [22]), and sialyltransferases including *Pasteurella multocida* α2–3-sialyltransferase 1 (PmSTl) [17], *Photobacterium damselae* α2–6-sialyltransferase (Pd2,6ST) [18], *Photobacterium* sp. JT-ISH-224 a2–6-sialyltransferase (Psp2,6ST) [23]. These include sialoside containing modification at C3 [16], C4 [24], C5 [17-19, 25], C7 [19, 26, 27], C8 [19, 28], and/or C9 [17-19, 25, 27, 29, 30] of sialic acid. Similarly, α2–3/6-linked sialosides can be further extended by α2–8-linked sialic acids using a *Campylobacter jejuni* α2–3/8-sialyltransferase (CjCstII)-containing OPME α2–8-sialylation system (Figure 5) [31]. Typical yields for these OPME sialylation systems were over 50%, many were over 90%, and some reached quantitative yields.

2.2. One-pot multienzyme (OPME) systems for the chemoenzymatic synthesis of sialidase inhibitors

The OPME α2–3-sialylation system can be combined with a sialidase for synthesizing sialidase inhibitors or inhibitor precursors. For example, *Streptococcus pneumoniae* strains collectively have three sialidases [32] SpNanA, SpNanB, and SpNanC. While SpNanA catalyzes the hydrolysis of α2–3/6/8-linked sialosides to produce free monosaccharide Neu5Ac, SpNanB and SpNanC specifically recognize α2–3-linked sialosides as substrates. Furthermore, the product of SpNanB is 2,7-anhydro-*N*-acetylneuraminic acid (2,7-anhydro-Neu5Ac), a potential prebiotic that can be used as the sole carbon source of a common human gut commensal anaerobic bacterium *Ruminoccocus gnavus* [33] and the product of SpNanC is 2,3-dehydro-2-deoxy-*N*-acetylneuraminic acid (Neu5Ac2en or DANA), the general transition state analog inhibitor against common hydrolytic sialidases [32]. By including SpNanB [34] or SpNanC [35] into the OPME scheme, one-pot multienzyme systems for synthesizing sialidase inhibitors or precursors were formed (Figure 6). All enzymes in the systems have some level of tolerance towards substrate modifications. A single mutant of PmST1, PmST1 M144D, was used to replace wild-type PmST1 to decrease its sialoside hydrolysis activity to provide high efficiency in the system. In addition, separating sialoside generation and Sia2en production by SpNanC into two steps was shown to improve the yields for some compounds. The 2,7-anhydro-Neu5Ac and Neu5Ac2en analogs obtained can be further chemically derivatized to generate inhibitors with improved inhibitory activity or improved selectivity [34, 35].

2.3. OPME activation and glycosylation systems for monosaccharides other than sialic acid

OPME glycosylation reactions using bacterial enzymes have also been developed for activating and transferring common monosaccharides in human glycome other than sialic acid (Figure 7 including L-fucose (Fuc), D-galactose (Gal), *N*-acetylglucosamine (GlcNAc), *N* acetylgalactosamine (GalNAc), and glucuronic acid (GlcA) [14]. For these systems, the simplest routes are the combination of a glycokinase (GlyK), a nucleotidyltransferase (NucT), and a glycosyltransferase (GlyT). An inorganic pyrophosphatase (PpA) can also be added to breal down the pyrophosphate formed in the nucleotidyltransferase-catalyzed reaction to shift the reaction process towards sugar nucleotide formation. The glycokinase is responsible for the formation of a monosaccharide-1-phosphate from a simple monosaccharide and adenosine 5’ triphosphate (ATP). The nucleotidyltransferase then

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catalyzes the formation of a suga nucleotide (NDP) such as uridine 5'-diphosphate (UDP)-activated sugars (for Gal, GlcNAc GalNAc, and GlcA) or guanosine 5'-diphosphate (GDP)-activated sugar (for Fuc) Glycosyltransferases are used for synthesizing products with different linkage specificities Similar OPME systems have been developed for synthesizing sugar nucleotides for D-mannose (Man) and *N*-acetylmannosamine (ManNAc) [14] but have not been coupled with the corresponding glycosyltransferases.

3. Enzymatically synthesized oligosaccharides as building blocks for chemical glycosylation

Just as chemical synthesis can provide building blocks for enzymatic assembly, OPME reactions can provide building blocks for subsequent chemical glycosylations. For example, glycosyl thiotoluenes are common glycosyl donors for chemical glycosylation. Oligosaccharyl thiotoluene building blocks can be synthesized by enzymatic glycosylation of simpler monosaccharyl thiotoluenes and used for further chemical glycosylation. Such a strategy has been successfully used for chemoenzymatic synthesis of sialyl Lewis × tetrasaccharide (sLe^{x}βProN$_3$) [36] and lacto-*N*-tetraose derivative (LNTβProN$_3$) [37]. For synthesizing sLe^{x}βProN$_3$ without a desired β1–4-galactosyltransferase and α1–3-fucosyltransferase, sialylated disaccharyl thiotoluene (Neu5Acα2–3GalβSTol) was chemoenzymatically synthesized from galactosyl thiotolune (GalβSTol), ManNAc, pyruvate, and CTP using a OPME α2–3-sialylation reaction containing *Pasteurella multocida* sialic acid aldolase (PmAldolase), *Neisseria meningitidis* CMP-sialic acid synthetase (NmCSS) and PmST1. It was protected and used as a building block for chemical glycosylation processes for the production of the desired tetrasaccharide (Figure 8A) [36]. Similarly, for the synthesis of LNTβProN$_3$ (Figure 8B), disaccharyl thiotolune of lacto-*N*-biose (Galβ1–3GlcNAcβSTol) was enzymatically synthesized from *N*-acetylglucosaminyl thiotolune (GlcNAcβSTol), galactose (Gal), and ATP using a one-pot two-enzyme reaction containing *Escherichia coli* galactokinase (EcGalK) and *Bifidobacterium infantis* D-galactosyl-β1–3-*N*-acetyl-D-hexosamine phosphorylase (BiGalHexNAcP). Chemical protection, followed by chemical glycosylation with selectively protected disaccharide acceptor led to the formation of protected tetrasaccharide. After chemical deprotection, acetylation, and global deprotection, the target tetrasaccharide LNTβProN$_3$ was successfully obtained [37].

OPME enzymatic glycosylation followed by chemical protection and activation, chemical glycosylation, and deprotection has also been used for synthesizing glycosphingolipids (GSLs), including globotrihexosylceramide (Gb3) and isoglobotrihexosylceramide (iGb3) (Figure 8C) [38], as well as GM3, blood group antigens H and A GSLs (Figure 8D) [39]. In these systems, isoglobotriaose was synthesized from lactose, glucose-1-phophate (Glc-1-P), and uridine 5'-triphosphate (UTP) by a one-pot three-enzyme system containing UDP-glucose pyrophosphorylase (GalU), UDP-Gal 4-epimerase (GalE), and an α1–3-galactosyltransferase (α1–3GalT). GM3 oligosaccharide was synthesized from lactose, Neu5Ac, and CTP using a one-pot two-enzyme system containing NmCSS and PmSTl M144D. Blood group antigen H oligosaccharide was synthesized from lactose, fucose (Fuc), ATP, and guanosine 5'-triphosphate (GTP) using a one-pot three-enzyme system containing a bifunctional L-fucokinase/GDP-fucose pyrophosphorylase (FKP), PmPpA, and

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Helicobacter mustelae α1–-fucosyltranferase (Hmα1,2FT). Blood group antigen A oligosaccharide was obtained from blood group antigen H oligosaccharide, *N*-acetylgalactosamine (GalNAc), ATP, and UTP using a one-pot four-enzyme reaction containing *Bifidobacterium infantis N*-acetylhexamine kinase (BiNahK), *Homo sapiens* UDP-GalNAc pyrophosphorylase (hAGX1), PmPpA, and *H. mustelae* α1–3-GalNAc transferase (HmBgtA). The free oligosaccharides, including globotriaose, were protected by pivoyl or benzoyl groups and activated to form trichloroacetimidate or *N*-phenyl trifluoroacetimidate glycosyl donors for chemical glycosylation. Reduction, acylation, followed by deprotection led to the formation of desired GSLs.

4. Regioselective enzymatic glycosylation of acceptors containing multiple glycosylation sites

Although OPME systems allow facile synthesis of many oligosaccharides without protection strategies, glycan structures may contain multiple potential glycosylation sites for glycosyltransferases. Regioselectivity in some cases can be achieved by the acceptor substrate specificity of glycosyltransferases. Some mammalian glycosyltransferases may possess the desired selectivity. However, desired regioselectivity may not always be achieved, especially when bacterial glycosyltransferases with higher substrate promiscuous are used. In these cases, several advanced strategies have been developed to generate products with desired regioselectivity. These include chemical and/or enzymatic protection of selected glycosylation sites in acceptor substrates, altering sequences of multiple glycosylation processes, and protein engineering of glycosyltransferases.

4.1 Chemical protection of selected glycosylation sites in acceptor substrates

Partial chemical modification or protection of the acceptor substrates of glycosyltransferases can be used to control the regioselectivity of enzyme-catalyzed reactions. Such a strategy named as “substrate engineering” by Withers et al. has been used to manipulate the substrate regioselectivity of *Neisseria meningitidis* α1–4-galactosyltransferase NmLgtC and bovine α1–3-galacosyltransferase (α3GalT) by installing a hydrophobic protection group at different locations of monosaccharide acceptors [40]. On the other hand, while some conformationally constrained oligosaccharides may improve catalytic efficiency of certain glycosyltransferases as shown by Boons et al. [41, 42], conformational constraining of acceptor substrates can also be used to block the recognition of glycosyltransferases. For example, Cao et al. used an easy-to-install lactone to protect one of the glycosylation sites of *Photobacterium damselae* α2–6-sialyltransferase (Pd2,6ST) in the acceptor substrate to allow regioselective sialylation. The lactone can be easily removed after the formation of the desired glycosidic linkage for the synthesis of target ganglioside disialyl tetrasaccharide (Figure 9) [43]. In comparison, without lactone protection, Pd2,6ST-catalyzed sialylation led to the formation of mixtures of disialylated tetrasaccharides and trisialylated pentasaccharide.

Selective *O*-acetylation or per-*O*-acetylation of acceptor glycosylation sites has also been used as a strategy to target glycosylations to a single desired site. This has been demonstrated for chemoenzymatic synthesis of polysaccharide analogs containing sialyl

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galactoside disaccharide repeats (Figure 10A) [44] as well as asymmetric N-glycans (Figure 10B and 10C) [45, 46]. While selected *O*-acetylation or per-*O*-acetylation in the glycosyltransferase acceptor substrates for the synthesis of N-glycans [45, 46] was introduced during chemical construction of the compounds, in the synthesis of sialyl galactoside polysaccharide analogs, per-*O*-acetylated galactose was attached to an azido-modified CMP-sialic acid by Cu(I)-catalyzed azide-alkyne cyclization reaction under mild coupling conditions. The resulting CMP-activated, triazole-containing disaccharide analog was tolerated as a donor substrate for Pd2,6ST-catalyzed formation of longer oligosaccharide. De-acetylation of the resulting oligosaccharide provided an elongated acceptor for another round of chain extension by sialylation [44].

Chemically synthesized acceptors with selective sites protected by monosaccharide caps have also been used as a strategy to control the downstream enzymatic glycosylation at the desired sites. The monosaccharide caps can then be selectively cleaved off by reactions catalyzed by the corresponding glycosidases. For example, β–4-linked mannose, α1–4-linked galactose, and β–4/6-linked GlcNAc were successfully used as the terminal monosaccharide caps on a precursor of complex N-glycans. They were readily removed by the corresponding α-galactosidase, β-mannosidase, and β-GlcNAcidase, respectively, before or after glycosyltransferase-catalyzed reactions taking place at other sites in the acceptor. This strategy allowed multi-dimensional enzymatic derivatization for the generate of a library of N-glycans from a common chemically synthesized precursor [47].

4.2 Enzymatic protection of selected glycosylation sites in acceptor substrates

Pairs of a glycosyltransferase and a glycosidase can also be used as a protection and deprotection strategy for regioselective glycosylation by glycosyltransferase-catalyzed reactions. This has been demonstrated for chemoenzymatic synthesis of an asymmetric human milk oligosaccharide derivative [48]. As shown in Figure 11, terminal LacNAc moiety of the GlcNAcβ1–6-branch of a complex human milk oligosaccharide structure was protected by an α2–6-linked sialic acid residue introduced by an α2–6-sialyltransferase (ST6GAL-I)-catalyzed reaction, preventing the terminal LacNAc from fucosylation by FUT3. Therefore, FUT3 catalyzed the addition of an α1–3-linked fucose selectively to the internal LacNAc residue. The sialic acid residue was removed by *Arthrobacter ureafaciens* sialidase for the formation of the desired difucosylated oligosaccharide.

4.3 Altering the order of multiple glycosylation processes

Carefully choosing reaction sequences provides another solution to selective glycosylation of substrates containing multiple acceptor moieties. For example, lacto-*N*-neotetraose (LNnT) (Figure 12) can be synthesized from lactose, an inexpensive disaccharide, using GlcNAc activation and transfer OPME system (OPME1) followed by Gal activation and transfer OPME system (OPME2). Direct α2–6-sialylation of the resulting LNnT with a OPME α2–6-sialylation system containing Pd2,6ST (OPME3) led to the addition of sialic acid residues to both Gal moieties [49, 50]. Alternating the sequence of OPME2 and OPME3 reactions led to the formation of monosialylated product with only one sialic acid α2–6-linked to the internal Gal of LNnT moiety. It went through a OPME α2–3-sialylation reaction (OPME4) for the formation of a disialylated analog DS'LNT containing a sialic

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acid α2-6-linked to the internal Gal and a sialic acid α2–3-linked to the terminal Gal. A similar strategy was used for the synthesis of a monofucosylated and internally α2–6-monosialylated hexasaccharide [51].

5. Chemoenzymatic synthons

When enzymes involved in the one-pot multienzyme (OPME) glycosylation reactions cannot tolerate the desired substrate modifications, "chemoenzymatic synthons" method can be used. In this strategy, substrates modified with an alternative group are used for OPME enzymatic synthesis of oligosaccharide derivatives which can then be enzymatically or chemically converted to the desired products. Such a strategy has been used for chemoenzymatic synthesis of heparan sulfate oligosaccharides [52]. *N*-Sulfated analogs of heparan sulfate oligosaccharides [53], *N*-acyl derivatives of sialidase transition state analog inhibitors [34, 35], and 5,7-di-*N*-acetyl-legionaminic acid (Leg5,7diNAc)-containing oligosaccharides [27].

Trifluoroacetamido (-NTFA) has been found to be an excellent analog of acetamido (-NAc) group in *N*-acetylhexosamine-containing substrates for carbohydrate biosynthetic enzymes [54]. For example, *N*-trifluoroacetylglucosamine (GlcNTFA) can be converted to UDP-GlcNTFA by the combined function of *N*-acetylhexosamine 1-kinase (NahK) and *N*-acetylglucosamine-1-phosphate uridylyltransferase (GlmU) [55]. The resulting UDP-GlcNTFA was readily used as a donor substrate of *Escherichia coli* α1–4-GlcNAc transferase (EcKfiA) [52] or *Pasteurella multocida* heparosan synthase 2 (PmHS2) [53] for the synthesis of *N*-TFA-analog of heparan sulfate oligosaccharides or analogs. The *N*-TFA group can then be easily removed selectively under mild basic conditions to provide an amino group to allow enzymatic [52] or chemical *N*-sulfation [53]. Derivatization with different acyl groups can also be carried out to form a library of analogs [34, 55].

Another excellent *N*-sulfate or *N*-acyl precursor moiety for chemoenzymatic reactions is azido (-N_3) group. Substitution of a hydroxyl group by an azido group in the substrate can be tolerated by many carbohydrate biosynthetic enzymes. For example, 6-azido-6-deoxy-*N*-acetylglucosamine (GlcNAc6N_3) can be tolerated by NahK for the synthesis of GlcNAc6N_3-1-phosphate. It was converted by *Pasteurella multocida N*-acetylglucosamine-1-phosphate uridylyltransferase (PmGlmU) to form UDP-GlcNAc6N_3, which was used as a donor substrate of PmHS2 for the synthesis of GlcNAc6N_3-containing heparosan oligosaccharide analog. The azido group can be chemically reduced to an amino group followed by *N*-sulfation for the formation of *N*-sulfated analogs of heparan sulfate oligosaccharides [53]. A similar strategy has been used for the synthesis of *N*-acyl derivatives of sialidase transition state analog inhibitors where 6-azido-6-deoxyl-*N*-acetylmannosamine (ManNAc6N_3) was used as a starting material in a one-pot multienzyme (OPME) system for the synthesis of 9-azido-2,3-dehydro-2,9-dideoxy-*N*-acetylneuraminic acid (Neu5Ac9$N_3$2en). The azido group was then readily reduced and acylated to form desired derivatives [35]. The strategy was recently used for the synthesis of oligosaccharides containing di-*N*-acetyllegionaminic acid (Leg5,7Ac_2) (Figure 13), a sialic acid analog in lipopolysaccharides of several Gram-negative pathogenic bacteria [27]. In this case, Leg5,7Ac_2 itself was not a suitable substrate of *Neisseria meningitidis* CMP-sialic acid

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synthetase (NmCSS) for the synthesis of CMP-Leg5,7Ac_2. In contrast, its diazido-analog Leg5,7diN_3 was readily obtained from chemically synthesized 6-carbon precursor, 2,4-diazido-2,4,6-trideoxymannose (6deoxyMan2,4diN_3), by a sialic acid aldolase-catalyzed reaction and was tolerated by NmCSS for the synthesis of CMP-Leg5,7diN_3 which was further accepted by bacterial α2–3- and α2–6-sialyltransferases for the synthesis of Leg5,7 diN_3-containing oligosaccharides. These compounds can be easily converted to the desired Leg5,7Ac*2*-containing oligosaccharides [27].

6. Enzyme engineering for altered glycosylation sequence or improved regioselectivity

Many carbohydrate biosynthetic wild-type enzymes from bacterial sources display remarkable promiscuity toward modified substrates, allowing the production of carbohydrates with post-glycosylation modifications using the OPME strategies discussed above. Nevertheless, when efforts to identify an enzyme with suitable activity toward a desired substrate fail, enzyme engineering can provide an alternative [56]. Such effort is greatly facilitated by the availability of protein crystal structures in the presence of substrates or analogs. For example, PmST1 M144D, designed based on crystal structures of PmST1 [57, 58], allows the tolerance of a fucosylated glycan, Lewis^x [Le^x, Galβ1-4(Fucα1-3)GlcNAcβOR] as an acceptor substrate for efficient synthesis of sialyl Lewis^x [sLe^x, Siaα2-3Galβ1-4(Fucα1-3)GlcNAcβOR] structures, which are well-known tumor-associated carbohydrate antigens and candidates for developing cancer vaccine [59]. In addition, Psp2,6ST A366G has an increased expression level and is more efficient in synthesizing Neu5Acα2-6GalNAcαOSer/Thr STn antigens [60].

Recently, the identification of engineered α2–6-sialyltransferases with selective sialylation activity towards terminal galactose or GalNAc residue in oligosaccharides has been described. Two strategies have been reported to achieve this by protein engineering of CAZy GT80 bacterial sialyltransferases. One strategy was by protein crystal structure-based site-specific saturated mutagenesis to switch PmST1, an α2–3-sialyltransferase, to α2–6-sialyltransferase mutants with desired regioselectivity. PmST1 P34H/M144L was identified as the best candidate which not only has the desired regioselectivity, but also has the highest reported catalytic efficiency for CAZy GT80 α2–6-sialyltransferases. The mutant displayed minor activity toward internal acceptor moieties and required careful reaction monitoring to prevent accumulation of multi-sialylated products. It was successfully used for preparative-scale synthesis of desired monosialylated pentasaccharide Neu5Acα2–6LNnTβProN_3 in 90% yield. Disialylated hexasaccharide was also obtained in 5% yield [61]. Another strategy was to engineer the acceptor binding pocket of Pd2,6ST. A double mutant A200Y/S232Y with bulkier amino acid residues was found to successfully block its binding to internal Gal or GalNAc residues while retain its α2–6-sialylation activity towards terminal Gal or GalNAc sites. A number of monosialylated products were successfully obtained in high yields without the formation of multi-sialylated compounds [62].

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7. Chemoenzymatic synthesis of glycolipids with facile purification

Considerable efforts have been made recently toward simplifying the purification of oligosaccharide products from chemoenzymatic synthetic reactions [63]. Inspired by using hydrocarbon-conjugated glycans for enzyme activity assays of glycosyltransferases [64], light perfluoroaklyl tags have been used to facilitate the purification of the oligosaccharide products by fluorous solid-phase extraction (FSPE), allowing non-tagged reaction components, such as nucleotides, sugar nucleotides, and monosaccharides to be washed away with water before eluting the tagged product and any remaining glycosyltransferase acceptor with methanol. A triethylene glycol (TEG) or a hexa-ethylene glycol (HEG) linker in C_8F_{17}- or the C_6F_{13}-tagged glycosyltransferase acceptor was found to improve their compatibility with enzymatic glycosylation reactions [65]. More recently, a sulfo-fluorous tag with a photo-cleavable linker was found to improve the water solubility of fluorous-tagged glycosyltransferase acceptors to achieve high-yields in enzymatic glycosylation reactions while facilitate the product purification by fluorous solid-phase extraction (FSPE) [66].

The hydrophobic tail is an intrinsic component of naturally existing glycolipids. It can be used to facilitate the product purification from chemoenzymatic synthetic reactions without the need to remove it afterwards. Therefore, glycolipids can be readily synthesized by enzyme-catalyzed reactions with facile product purification by C18-cartridges. Such strategy has been successfully applied for the synthesis and purification of GSLs, a class of biologically important glycolipids found in the cell membranes of various organisms. To do this, lactosyl sphingosine (LacβSph) was chemically synthesized from inexpensive starting materials including lactose and phytosphingosine. It was then used readily in one-pot multienzyme (OPME) glycosylation reactions that can be carried out in sequential for building up a large library of glycosyl sphingosines which upon one-step acylation, leading to the formation of desired GSLs. In contrast to glycosylceramide with short glycan chains which have low water solubility, the glycosyl sphingosines are soluble in water and are compatible with enzymatic reactions. The sphingosine component facilitated the facile C18-cartridge-based purification processes (Figure 14). Ganglio- and neolacto-series glycosphingolipids have been successfully obtained using the strategy [67, 68]. Compared to *en bloc* transfer of oligosaccharides from chemoenzymatically synthesized oligosaccharyl fluorides for the synthesis of glycosphingolipids and glycosphingosines containing a sphingoid base or its derivative using endoglycoceramidase mutants (endoglycoceramidase glycosynthase) [69, 70], the OPME glycosylation of LacβSph strategy is more flexible in accessing a more diverse array of products and also provides an easier C18 cartridge-purification process.

8. Solid phase-assisted chemoenzymatic synthesis and purification strategies

Carbohydrate biosynthetic enzymes have been immobilized on solid supports to improve their stability and allow their reuse in the synthesis [71-75]. To permit facile product purification which is a critical factor to consider for automated synthesis of carbohydrates

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and glycoconjugates, substrate/product immobilization is a more attractive approach [63]. Substrate/product immobilization on a solid phase support can be done before or after enzymatic reactions. The latter allows enzymatic reactions to be carried out in a solution phase with facile solid-phase purification. Other than attaching a hydrophobic tail similar to the strategies discussed above for the synthesis of fluorous/hydrocarbon tagged-oligosaccharides and glycosphingolipids, the use of thermo-responsive water soluble polymer support [76] and cationic aglycon-containing substrates [77] are attractive approaches. Aspects for consideration towards automated enzymatic synthesis of carbohydrates were detailed recently in an excellent review with discussion on the availability and the choice of enzymes, glycosyltransferase donors, solid-phase supports, linkers and spacers, and instruments [63]. Much progresses have been made and it is expected that chemoenzymatic synthesis of carbohydrates can be automated to allow the access of targets by even non-specialists.

9. Conclusions and future perspectives

Innovations in chemoenzymatic approaches for glycan synthesis are granting access to carbohydrates with increasing structural complexity. The use of OPME systems with chemically modified monosaccharides, strategically designed synthetic routes, protective groups introduced onto acceptor substrates, synthons of glycosyltransferase donor precursors for targets containing exotic sugars, tags to facilitate purifications, and other strategies described here are important for the synthesis of structurally diverse carbohydrates including those containing PGMs, asymmetric branches, and/or repeating motifs. The introduction of selective glycoside hydrolases into OPME systems opens access to the production of defined compounds with demonstrated value as therapeutic sialidase inhibitors and potential prebiotics. Wild-type enzymes can be cloned and characterized from different sources to identify suitable candidates for the synthesis of desired target compounds. Crystal structure-guided mutagenesis and screening is an effective approach to obtain mutants of desired properties if wild-type enzymes with desired functions are not readily available. Chemoenzymatic synthesis of oligosaccharide building blocks can be used for additional chemical glycosylation for the formation of more complex structures. When glycosyltransferases and other carbohydrate biosynthetic enzymes with desired substrate promiscuity are not available, suitable chemically synthesized synthons can be used as the substrates for enzyme-catalyzed version of intermediates which can be converted to the desired targets chemically. Progresses made on strategies to facilitate product purifications can be combined with advances on chemoenzymatic synthesis to access the enormous chemical space of naturally occurring and non-natural derivatives of carbohydrates and glycoconjugates not only by experts in the carbohydrate field but also by non-specialists. Lack of sufficient amounts of glycosyltransferases for the formation of desired glycosidic bonds and sugar nucleotide biosynthetic enzymes for producing nucleotide-activated uncommon sugars remains to be the key challenge for chemoenzymatic synthesis. Methods to improve the expression level and increase the stability of highly active enzymes are in high demand. Continuous efforts are needed for crystal structure studies of synthetic useful enzymes which will help to design synthetic useful mutants with improved properties. A clearer landscape of glycomes is also needed to define the chemoenzymatic synthetic

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targets. Therefore, combined efforts of glycan analysis, enzyme discovery and characterization, protein crystallography and mutagenesis, synthetic method development, and functional application of carbohydrate products will greatly advance the carbohydrate chemoenzymatic synthesis and also the overall glycoscience field.

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Highlights

- Recent glycosyltransferase-dependent chemoenzymatic strategies are summarized
- One-pot multienzyme (OPME) glycosylation systems are highly efficient
- Enzymatically synthesized building blocks can be used for chemical glycosylation
- Identifying suitable enzymes and mutants is the key to success
- Strategies for regioselective glycosylation and facile purification are discussed

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Figure 1.
Schematic illustration of one-pot multienzyme (OPME) chemoenzymatic reactions for the synthesis of carbohydrates with structural modifications.

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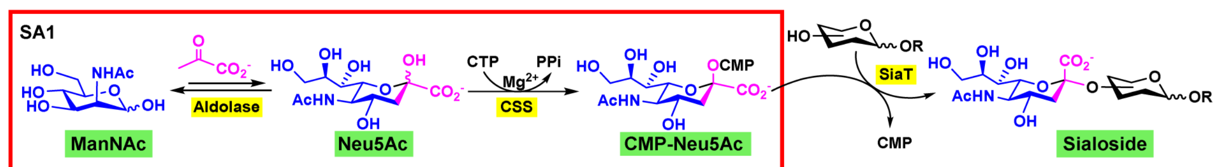

Figure 2.

OPME sialylation systems for synthesis of sialosides. (Adapted from H. Yu, X. Chen, Org Biomol Chem, 14 (2016) 2809-2818.)

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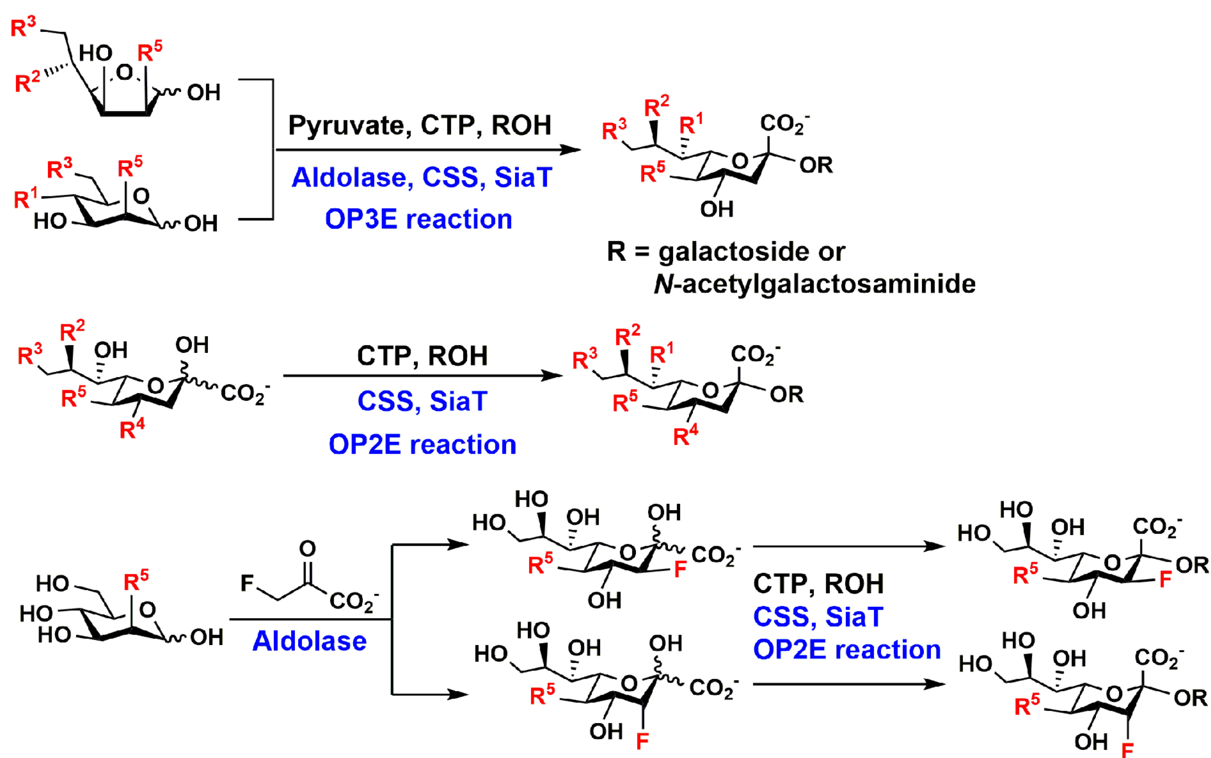

Figure 3.
OPME chemoenzymatic sialylation systems for synthesizing sialosides with modified sialic acid residues.

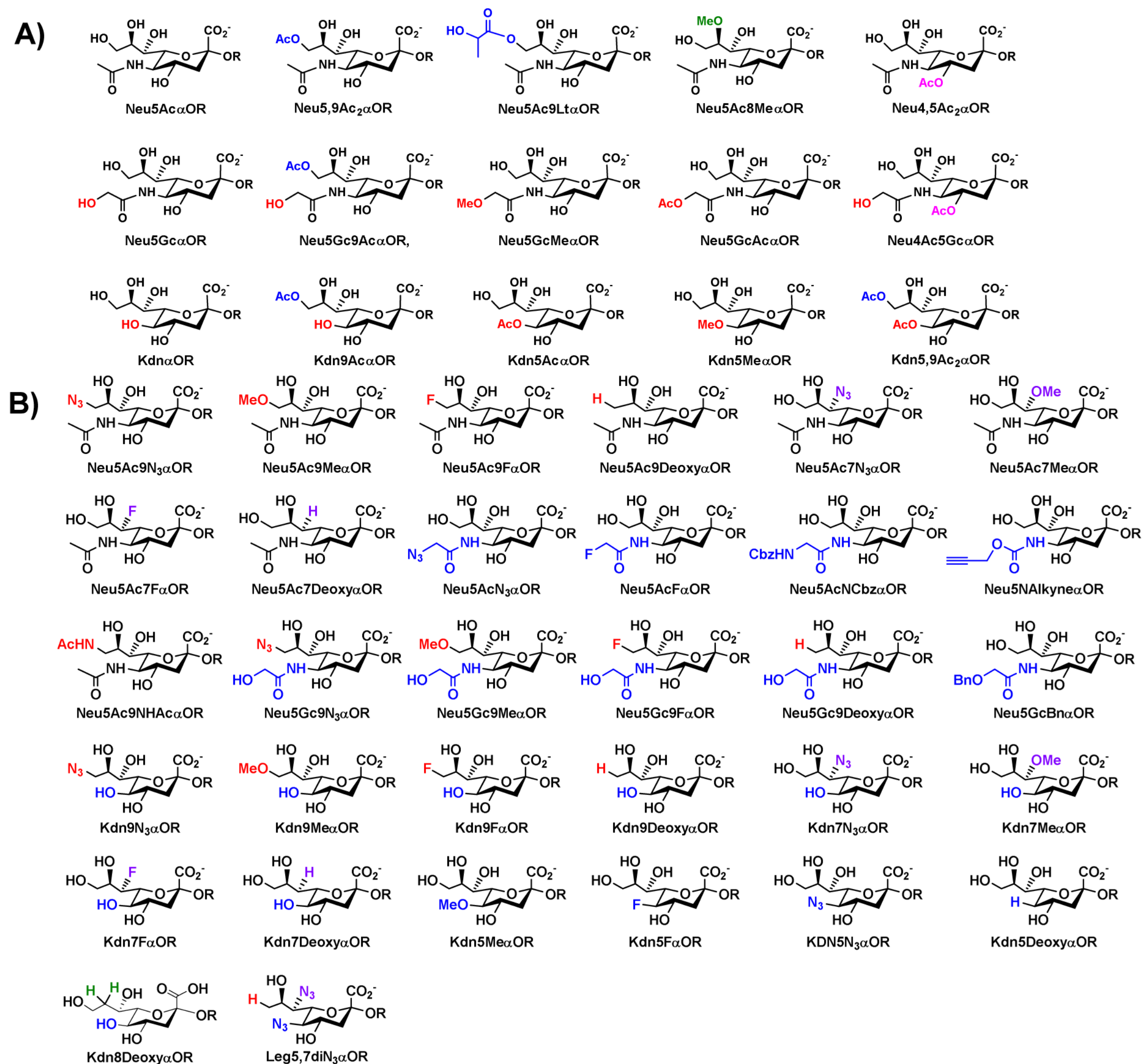

Figure 4.

Sialosides containing naturally occurring sialic acid forms (**A**) or non-natural modifications (**B**) that have been synthesized by OPME α2–3/6-sialylation systems.

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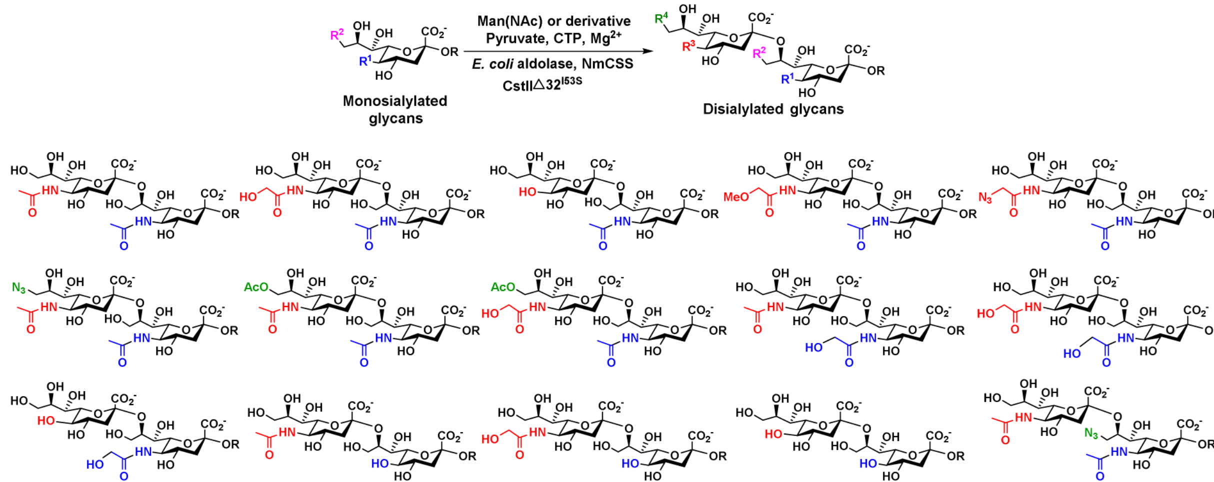

Figure 5.

Sialosides containing naturally occurring sialic acid forms or non-natural modifications that have been synthesized by OPME α2–3/6- and α2–8-sialylation systems.

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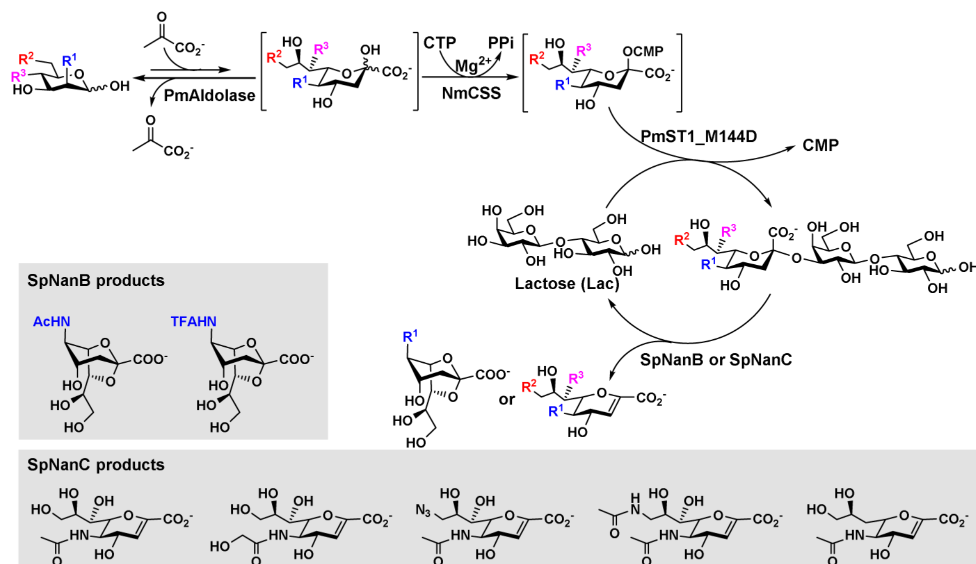

Figure 6.

OPME systems for the synthesis of sialidase inhibitors or sialidase inhibitor precursors 2,3-dehydro-2-deoxy-sialic acids (Sia2ens) and 2,7-anhydro-sialic acids (2,7-anhydro-Sias).

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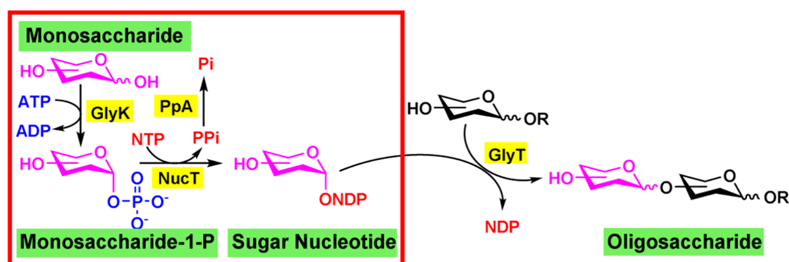

Figure 7.

OPME systems for synthesizing oligosaccharides using glycosyltransferases that require nucleoside diphosphate-sugars as donor substrates. Enzyme abbreviations: GlyK – glycokinase; NucT – nucleotidyltransferase; PpA – inorganic pyrophosphatase; GlyT – glycosyltransferase. (Adapted from H. Yu, X. Chen, Org Biomol Chem, 14 (2016) 2809-2818.)[14]

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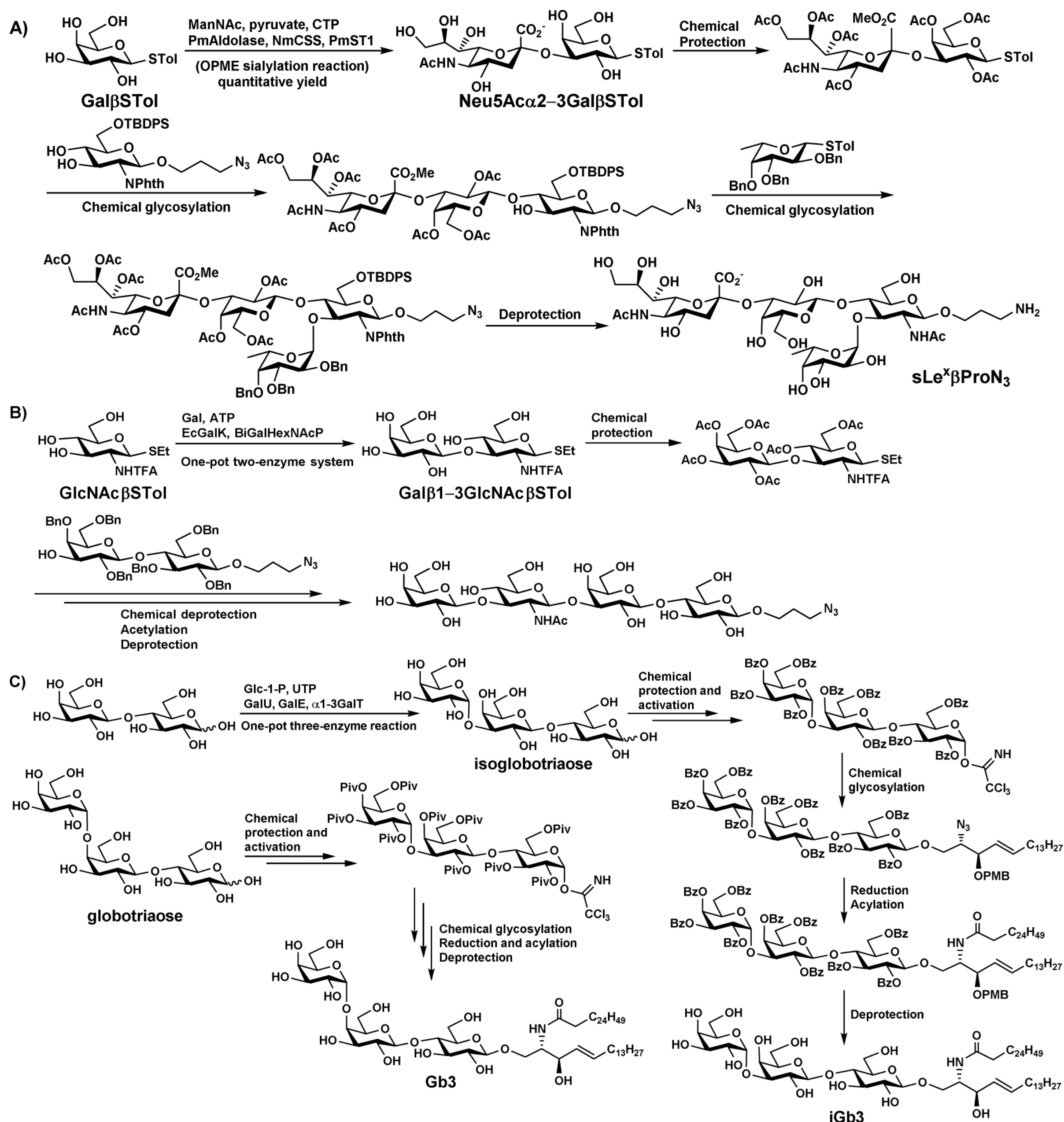

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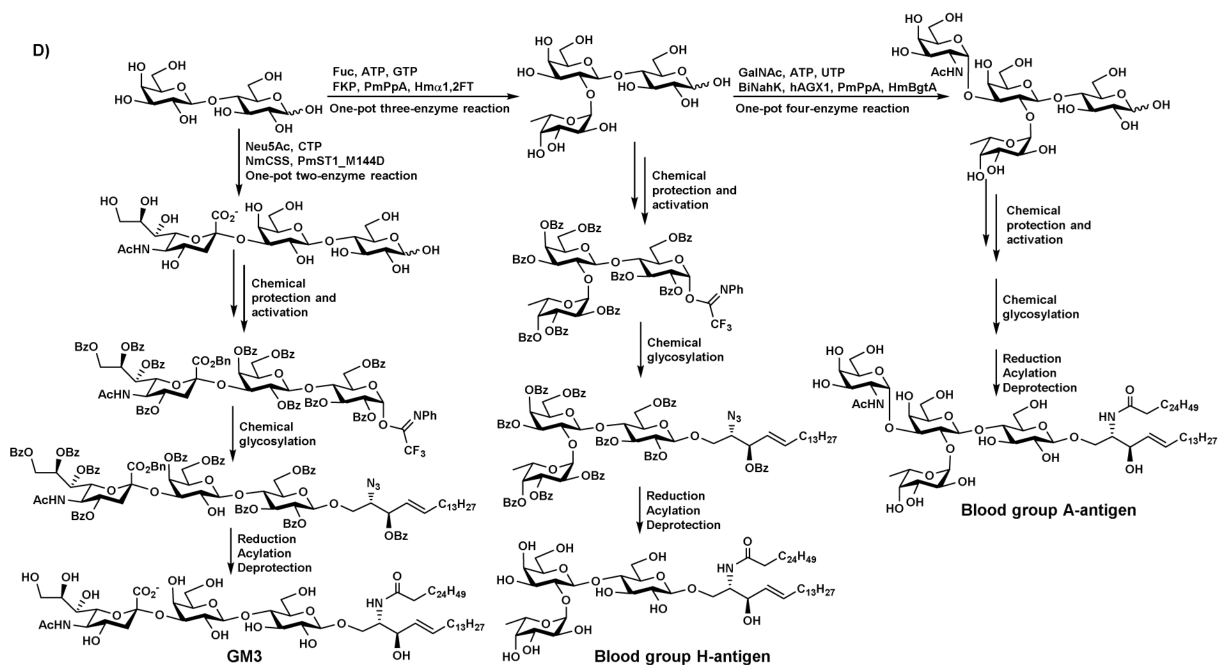

Figure 8.

Chemoenzymatic synthesis of sLe^{x}βProN$_3$ (**A**), LNTβProN$_3$ (**B**), iGb3 and Gb3 (**C**), as well as GM3, blood group H- and A-antigens (**D**) by chemical glycosylation using building blocks synthesized by OPME reactions.

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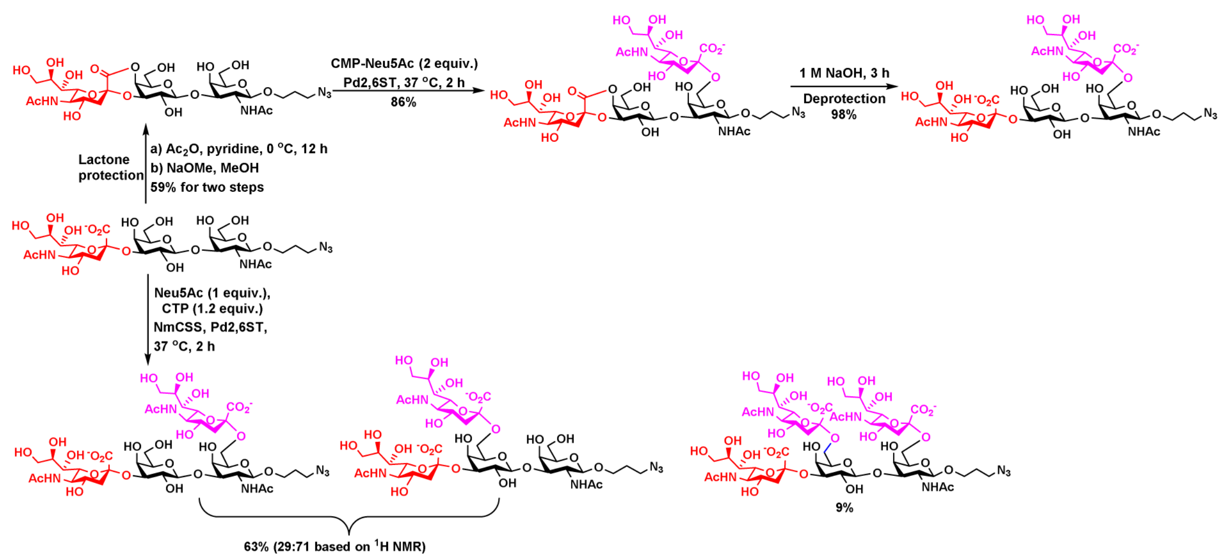

Figure 9.

Use of lactone protection as a strategy to provide regioselectivity for chemoenzymatic synthesis of ganglioside disialyl tetrasaccharide. In the absence of lactone protection, mixture of disialyl tetrasaccharides and trisialyl pentasaccharide were formed in *Photobacterium damselae* α2–6-sialyltransferase (Pd2,6ST)-catalyzed reaction.

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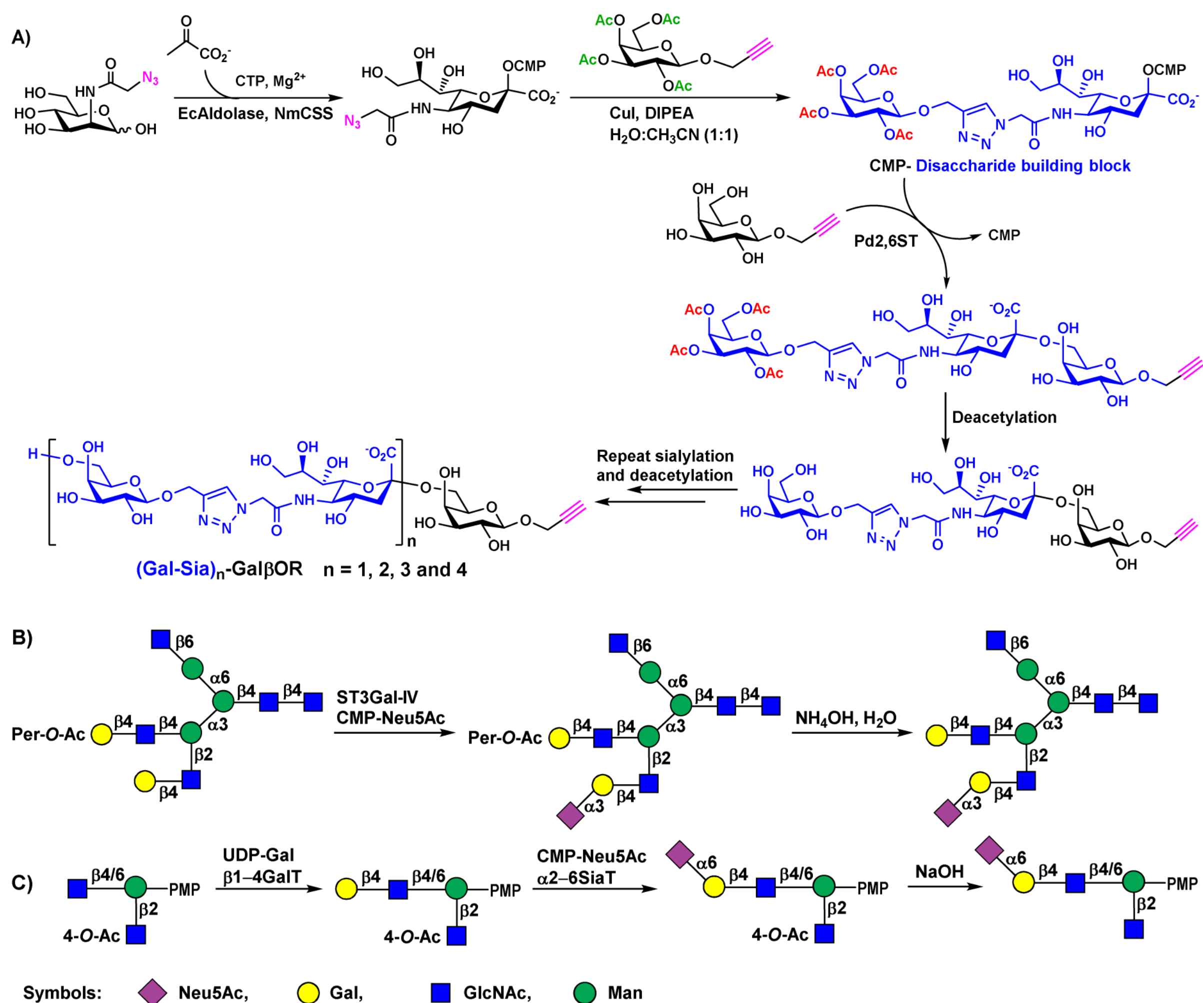

Figure 10.

A) Chemoenzymatic synthesis of disaccharide building block and controlled chemoenzymatic synthesis of size-defined polysaccharides by Pd2,6ST-catalyzed block transfer of disaccharide building blocks. **B**) Chemoenzymatic synthetic route of asymmetrically multi-antennary glycans using selected *O*-acetylated acceptor. **C**) Chemoenzymatic synthesis of modules with selected *O*-acetylation for N-glycan assembly.

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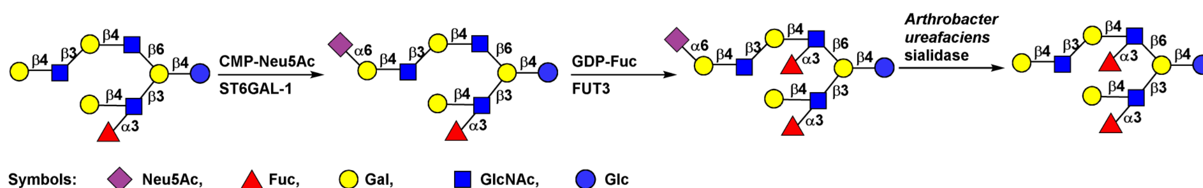

Figure 11.
An example by the Boons group of using a glycosyltransferase (ST6Gal-I) and a glycosidase (*Arthrobacter ureafaciens* sialidase) pair as a protection and deprotection strategy for regioselective glycosylation (fucosylation) by glycosyltransferase (FUT3)-catalyzed reaction.

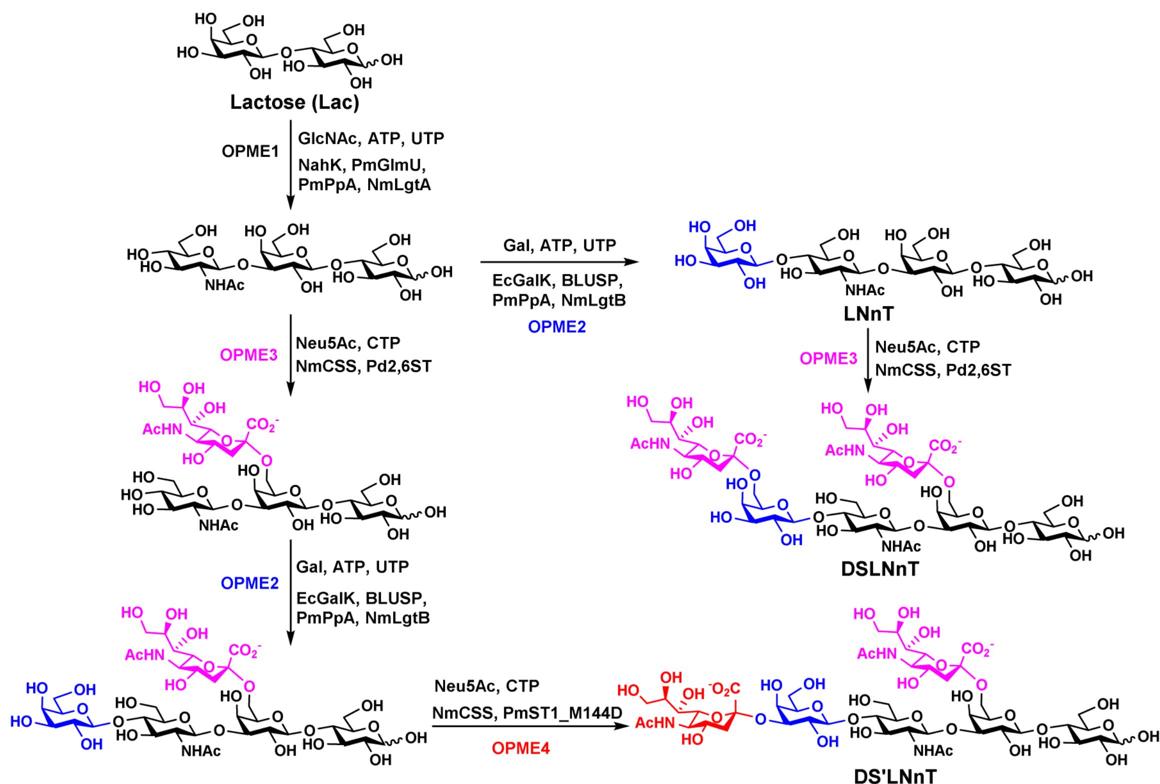

Figure 12.

Synthesis of disialylated hexasaccharides DSLNnT and DS'LNnT by sequential OPME reactions with altered sequence of OPME2 and OPME3.

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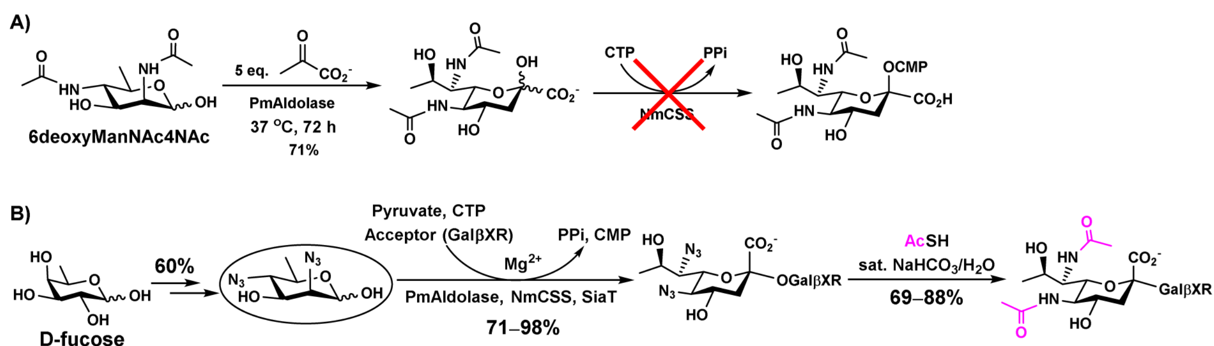

Figure 13.

Application of a chemoenzymatic synthon strategy in the synthesis of Leg5,7Ac$_2$-containing oligosaccharides. A) Leg2,7Ac$_2$ produced from 2,4-diacetamido-2,4,6-trideoxy-D-mannose (6deoxyManNAc4NAc) by PmAldolase-catalyzed reaction cannot be used by NmCSS as the substrate. B) Production of Leg2,7Ac$_2$-containing oligosaccharides by OPME synthesis of Leg2,7diN$_3$-containing glycosides followed by chemical derivatization.

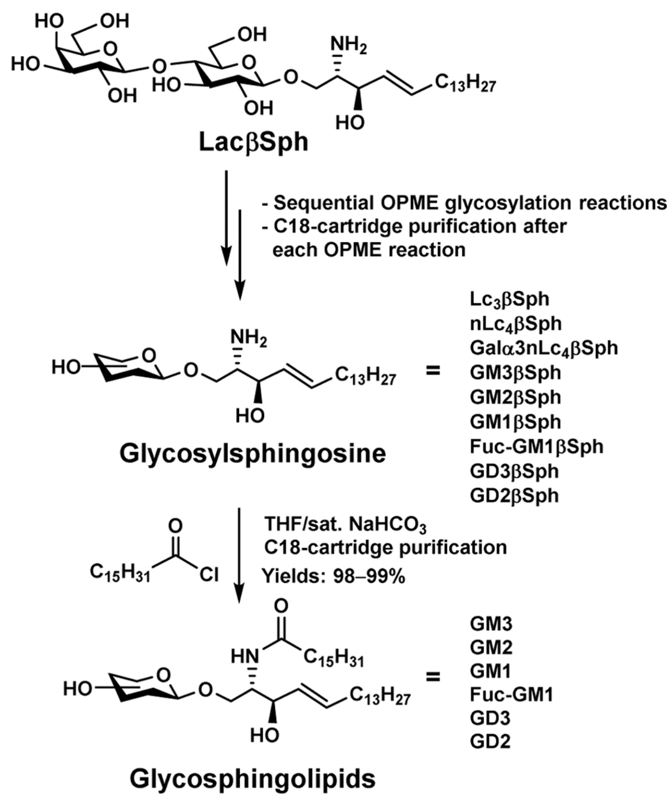

Figure 14.

Efficient chemoenzymatic strategies for synthesizing complex glycosphingolipids by enzymatic extension of lactosyl sphingosine using OPME reactions followed by acylation reaction.

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