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Enzymatic transglycosylation for glycoconjugate synthesis

Lai-Xi Wang[*] and **Wei Huang**
Institute of Human Virology and Department of Biochemistry & Molecular Biology, University of Maryland School of Medicine, Baltimore, MD 21201, USA

Summary

Remarkable advances have been made in recent years in exploiting the transglycosylation activity of glycosidases and glycosynthase mutants for oligosaccharide and glycoconjugate synthesis. New glycosynthases were generated from retaining glycosidases, inverting glycosidases, and those that proceed in a mechanism of substrate-assisted catalysis. Directed evolution coupled with elegant screening methods has led to the discovery of an expanding number of glycosynthase mutants that show improved catalytic activity and/or altered substrate specificity. In particular, enzymatic transglycosylation strategy has been recently extended to the synthesis of complex glycoconjugates, including glycosphingolipids, N-glycoproteins, and other glycosylated natural products.

Introduction

Glycosylation is an effective strategy that nature takes to expand the biological information of biomolecules by adding a new level of structural diversity. Glycans in glycoconjugates modulate the physicochemical properties of the conjugates and directly participate in many important biological recognition processes such as cell adhesion, cancer progression, host-pathogen interactions, and immune responses [1-4]. Understanding the roles of glycans in the context of glycoconjugates is thus fundamentally important. However, progress in functional glycomics studies is often hampered by the lack of homogeneous glycoconjugates, which are difficult to obtain in pure forms from natural sources. To meet with the urgent need for structurally defined oligosaccharides and glycoconjugates, a number of elegant chemical and enzymatic synthesis methods have been developed [5-8]. In contrast to conventional chemical synthesis that usually requires tedious protection/de-protection manipulations in order to achieve regio- and stereo-selectivity, enzymatic glycosylation usually provides perfect control of the anomeric configuration and high regio-selectivity without the need of any protecting groups. Both glycosyltransferases and glycosidases have been vigorously studied for synthetic purposes [9,10]. In comparison with glycosyltransferase-catalyzed reaction that uses complex sugar nucleotide as the donor and usually has very stringent substrate specificity, glycosidase-catalyzed transglycosylation has several advantages, including the use of readily available donor substrates, the relaxed substrate specificity for acceptors, the easy access to the enzymes, and the potential for a single-step block oligosaccharide transfer (as in the case of endoglycosidases). Nevertheless, the use of glycosidases in synthesis is subject to two major limitations: the low transglycosylation yield and the product hydrolysis.

*Corresponding author: Tel: 410-706-4982 LWang3@ihv.umaryland.edu.

Significant progresses have been made in recent years to overcome these problems. A major breakthrough in the field is the invention of glycosynthases, a class of novel glycosidase mutants that can promote glycosidic bond formation when a suitable activated glycosyl donor is provided, but do not hydrolyze the newly formed glycosidic linkage. Moreover, protein engineering, including directed evolution coupled with elegant screening methodology has led to the discovery of an expanding number of novel glycosynthases with enhanced transglycosylation activity and/or altered substrate specificity. In this article, we highlight recent advances in this area, with a focus on new transglycosylation reactions and novel glycosynthases that are particularly valuable for the synthesis of complex glycoconjugates.

Enzymatic transglycosylation for glycosidic bond formation

For a typical retaining β-glycosidase, its catalysis usually proceeds in a two-step, double displacement mechanism involving two key carboxylate residues that are spatially proximal (usually within ca. 6.0 Å). In this mechanism, the nucleophilic residue attacks the anomeric center to form an enzyme-substrate complex, while the the general acid/base residue protonates the glycosidic oxygen and subsequently activates water (in hydrolysis) or a sugar acceptor (in transglycosylation) (Figure 1a). For the reactions catalyzed by family GH18 chitinases, GH20 hexosaminidases, or GH85 endo-β-N-acetylglucosaminidases (ENGases), a mechanism of substrate-assisted catalysis was proposed (Figure 1b). In this mechanism, the 2-acetamido group in the substrate acts as the nucleophile to promote the breakdown of the glycosidic bond, resulting in the formation of an oxazolinium ion intermediate that is then reacted with water or a sugar acceptor. Transglycosylation efficiency would thus depend on how the enzyme could favorably accommodate a sugar acceptor to compete with water. In addition, product hydrolysis is another pressing issue in using glycosidases for synthesis. Early attempts to address these problems include the incorporation of organic solvents in the reaction media to decrease the activity of water, and the use of activated species (e.g., glycosyl fluoride and synthetic sugar oxazoline) as donors to achieve a more favorable kinetic control [9,11,12]. For example, a series of oligosaccharides and artificial polysaccharides have been prepared by the transglycosylation of disaccharide glycosyl fluoride under the catalysis of endoglycosidases (cellulose, α-amylase, 1,3-1,4-β-glucanase, and xylanase) [11]. Artificial chitin and other glycosaminoglycan derivatives have been synthesized by polymerization of modified disaccharide oxazolines using family GH18 chitinase and GH56 hyaluronidase as the catalyst [13-16]. Moreover, the use of synthetic sugar oxazoline in the ENGase-catalyzed transglycosylation has significantly expanded the synthetic repertoire for the construction of homogeneous N-glycopeptides and N-glycoproteins [12].

To address the issue of product hydrolysis by glycosidases, a conceptual breakthrough was fulfilled in 1998 by the invention of novel glycosidase mutants termed “glycosynthase” that are able to catalyze transglycosylation but are devoid of product hydrolysis activity [10,17, 18]. Withers and co-workers reported the first glycosynthase by engineering the β-glycosidase from *Agrobacterium sp.* by mutating the nucleophile residue E358 to a non-nucleophilic residue [19] (Figure 1c); Planas and co-workers reported the first endo-acting glycosynthase by site-directed mutation of the nucleophile E134 of the retaining 1,3-1,4-β-glucanase from *Bacillus licheniformis* [20]; and Moracci and co-workers described yet another interesting glycosynthase approach, which used an activated glycosyl species as substrate but rescued the transglycosylation activity of the non-hydrolyzing glycosidase mutant with sodium formate as an external nucleophile [21]. Following these pioneering studies, various new glycosynthases have been discovered from glycosidases belonging to more than a dozen of glycoside hydrolase families. Notable recent examples include: the generation of novel thioglycosynthases [22-24]; the expansion of the glycosynthase strategy to inverting glycosidases such as the exo-β-oligoxylanase [25,26•] and the 1,2-α-L-fucosidase[27]; the creation of glycosynthases from the *Thermotoga maritime* β-glucuronidase [23]; and the discovery of first-generation

glycosynthases from the endoglycosidases that proceed in a mechanism of substrate-assisted catalysis [28••,29•]. Recently, amylosucrase, a transglucosidase, was successfully engineered to glycosylate nonnatural acceptors for the synthesis of cell-surface oligosaccharide antigens [30]. On the other hand, directed evolution coupled with an efficient high-throughput screening strategy has become a powerful tool to improve the catalytic efficiency of existing glycosynthases and/or to discover new glycosynthases with altered substrate specificity [31-34]. A general, high-throughput assay for screening those glycosynthase mutants that use glycosyl fluoride as donors was recently described [33•]. In combination with chemical synthesis, enzymatic transglycosylation has emerged as an important tool for the synthesis of complex oligosaccharides and glycoconjugates.

Synthesis of glycosphingolipids by endoglycoceramidase-catalyzed transglycosylation

Glycosphingolipids play essential roles in a number of biological processes and have demonstrated a great potential as therapeutic agents for the treatment of human diseases. However, structurally defined glycosphingolipids has been difficult to obtain from nature because of their structural heterogeneity. Early work has shown that some endoglycoceramidases possess transglycosylation activity and are useful for neoglycolipid synthesis [35,36]. But the yield is only moderate and the product formed is subject to hydrolysis by the enzyme. Through engineering the endoglycoceramidase II (EGC II) from *Rhodococcus sp*, a glycosphingolipid-hydrolyzing enzyme, Withers and coworkers generated the first glycosynthase for glycosphingolipid synthesis [37]. They found that mutation of the putative nucleophile E351 resulted in the loss of EGC II-catalyzed hydrolysis activity, but the mutants were able to use synthetic 3'-O-sialyl-α-D-lactosyl fluoride as the donor to glycosylate D-erythro-sphingosine. The E351S mutant was identified as the most efficient glycosynthase that could promote the formation of the corresponding lyso-GM3 ganglioside product in over 90% yield (Figure 2). Despite the observed substrate promiscuity, however, the first-generation catalyst has shown a very low activity to some lipid acceptors including the phytosphingosine. Kinetic studies indicated that the activity of E351S for phytosphingosine is about 10,000-fold lower than that for sphingosine, as estimated by the k_{cat}/K_m value [38]. To overcome this limitation, Withers and co-workers have recently made a significant progress in expanding the synthetic scope of the EGCII-based glycosynthase through directed evolution of the E351S glycosynthase [39]. An elegant ELISA-based assay was developed to screen the EGC mutant libraries, and novel glycosynthases with improved catalytic efficiency were discovered [39••]. A special mutant, D314Y/E351S, was identified to have almost the same k_{cat}/K_m value for phytoshingosine as that for sphingosine, indicating a few thousand-fold enhancement of the catalytic activity (Figure 2). This study showcases the power of directed evolution in improving on glycosynthase activity.

Synthesis of homogeneous N-glycoproteins through enzymatic transglycosylation

Synthesis of homogeneous glycoproteins carrying well-defined oligosaccharides poses a significant challenge to synthetic chemists [7,8]. Among other methods, the endoglycosidase catalyzed transglycosylation has attracted much attention in recent years [8,12,40]. For sugar chain extension, the family GH85 endo-beta-*N*-acetylglucosaminidases (ENGases), including the Endo-A from *Arthrobacter protophormiae* and the Endo-M from *Mucor hiemalis*, have been particularly useful, as they are able to transfer an intact N-glycan *en bloc* to a GlcNAc-peptide acceptor to form a new natural N-glycopeptide, in a regio- and stereo-specific manner [12,41]. Two major advances have been achieved recently aiming to overcome the limitations

associated with the ENGase-catalyzed synthesis such as substrate limitation, low transglycosylation efficiency and product hydrolysis.

Wang and co-workers explored synthetic sugar oxazolines as donor substrates for ENGase-catalyzed N-glycopeptide synthesis [42-44]. This was based on the assumption that the ENGase-catalyzed reaction proceeds via a mechanism of the substrate-assisted catalysis involving an oxazolinium ion intermediate. It was found that the oligosaccharide oxazoline corresponding to the *N*-glycan core $Man_3GlcNAc$, and its truncated and selectively modified forms, were able to serve as donors for transglycosylation to form corresponding N-glycopeptides in good to excellent yields [42-44]. Interestingly, the resulting glycopeptides were poor substrates for hydrolysis because of the structural modifications on the N-glycans. This chemoenzymatic approach was successfully extended to the glycosylation remodeling of glycoproteins [45,46•]. An expeditious chemoenzymatic synthesis of various homogeneous glycoforms of ribonuclease B carrying azido tags and oligosaccharide ligands was reported [46•] (Figure 3). Glycosylation engineering of human IgG1-Fc by the chemoenzymatic approach was also achieved [47•]. The approach includes the expression of human IgG1-Fc in yeast *Pichia pastoris*, enzymatic de-glycosylation, and Endo-A catalyzed transglycosylation with sugar oxazolines to the exposed GlcNAc to provide homogeneous glycoforms of IgG1-Fc. A remarkable observation in this study is that the Endo-A could efficiently glycosylate the native GlcNAc-containing IgG1-Fc homodimer. Independently, Fairbanks and co-workers have synthesized series of sugar oxazolines and evaluated their transglycosylation activities with Endo-A and Endo-M [48-50]. A very interesting finding is that the core β-linked mannose residue in the N-glycan oxazoline could be replaced with a β-glucose residue without loss of its transglycosylation activity, showing again a great potential of the chemoenzymatic method for constructing novel neoglycopeptides. A tandem transglycosylation by Endo-A with a disaccharide oxazoline, Glcβ-1,4-GlcNAc oxazoline, was also reported, leading to the synthesis of novel oligosaccharides and glycopeptides carrying repeating disaccharide structure [51].

Despite the remarkable efficiency of the transglycosylation using modified N-glycan oxazolines as the substrates, product hydrolysis remains a serious problem when glycoproteins carrying full-size natural N-glycans are the synthetic targets. To diminish the hydrolytic activity, the conventional approach to generating glycosynthase by “knocking out” the nucleophilic residue in the enzyme [10,17] could not be applied as the ENGase-catalyzed reaction proceeds via a substrate-assisted mechanism, in which the nucleophile is the 2-acetamido group in the substrate. Yamamoto, Wang, and co-workers reported the first-generation of ENGase-based glycosynthases by screening a series of Endo-M mutants generated by site-directed mutagenesis [28••]. Their transglycosylation activity was examined using a synthetic sugar oxazoline derived from the full-size natural N-glycan Man9GlcNAc2. Several interesting mutants were identified that showed improved activity. A special mutant, N175A, was identified as a glycosynthase that promotes the transglycosylation with Man9GlcNAc-oxazoline, but does not hydrolyze the resulting natural glycopeptide. A plausible mechanism was proposed, in which the N175 residue plays a role in orientating the N-acetamido group to promote oxazoline formation upon protonation of the glycosidic oxygen by the general acid/base residue E177. Mutation of the N175 to an Ala residue renders the enzyme incompetent of oxazoline formation and product hydrolysis, but the resulting N175A mutant was still able to take pre-synthesized sugar oxazoline for transglycosylation (Figure 4a). An analogous Endo-A mutant, EndoA-N171A, was also created, which could transglycosylate Man9GlcNAc oxazoline effectively [52•]. Extension of the chemoenzymatic method to the synthesis of homogeneous glycoforms of ribonuclease B carrying full-size natural N-glycans was achieved [52•]. In another study, Fairbanks and co-workers reported a different Endo-A glycosynthase by site-directed mutation at the general acid/base residue E173 [29•]. It was found that the Endo-A mutants E173H and E173Q were able to use the

Man3GlcNAc-oxazoline for transglycosylation but almost lacked hydrolytic activity (Figure 4b).

Recently, important progresses were made in the structural studies of the family GH85 endo-β-N-acetylglucosaminidases. Liu and co-workers reported the crystal structures of Endo-A and its complex with Man3GlcNAc-thiazoline, an oxazoline analog [53•]. Taylor and co-workers solved the structure of an Endo-A mutant, E173Q [54]. These structural studies have indentified several essential catalytic residues, including E173, N171, and Y205, which are within hydrogen bonding distance of the substrate. The observations are consistent with previous mutational studies that the E173 play a role as a general acid/base, while the N171 is essential for orientating the 2-acetamido group and promoting oxazoline formation. Meanwhile, the crystal structure of another family GH85 ENGase, Endo-D from *Streptococcus pneumoniae* was solved by Boraston and co-workers [55]. Endo-D and Endo-A were shown to have almost identical topology in folding of the catalytic domain and share a general mechanism of substrate-assisted catalysis. The structural information provides a foundation for rational engineering of ENGases for improving their transglycosylation activity or for discovering new glycosynthase mutants with altered substrate specificity.

Direct enzymatic transglycosylation of small-molecule natural products

Many natural products such as secondary metabolites are glycosylated. Engineering the sugar moiety in natural products expands the structural diversity and will facilitate the understanding of the roles of glycosylation. Thorson and co-workers have discovered a novel reverse glycosylation activity of a subset of glycosyltransferases involved in the biosynthesis of calicheamicin and vancomycin [56]. They have found that the glycosyltransferases could release sugar from calicheamicin and vancomycin to nucleotide TDP and then transfer the sugar from the resulting TDP-sugar (as activated donor) to another aglycon to form a new glycosylated natural product. These glycosyltransferases, acting as a typical transglycosidase, allow sugar and aglycons to be exchanged with ease. A library of 70 differentially glycosylated calicheamicin and vancomycin was established by this approach.

Davis and co-workers reported a novel property of a glycosynthase mutant E197S of the *Humicola insolens* cellulase Cel7B, showing that this Cel7B-E197S mutant was able to directly glycosylate flavonoids (non-sugar substrates) with high efficiency [57••]. Cel7B is an endoglucanase that hydrolyzes the α-1,4-linked glucosidic bonds in cellulose. Using a high-throughput MS-based method, they have screened 80 different acceptors and more than 20 glycosyl donors for substrate activity, leading to the identification of a subclass of flavonoids as the acceptors of Cel7B-E197S catalyzed transglycosylation (Figure 5). Kinetic studies indicated that the rates of Cel7B-E197S catalyzed sugar transfer to flavonoids were comparable to those of natural glycosyltransferases. The ability of the Cel7B-E197S mutant to glycosylate the non-sugar flavonoids in a highly regio-selective manner is very impressive. In another study, Wang and co-workers reported an efficient chemoenzymatic method for introducing N-glycans to various lipophilic natural products by Endo-A catalyzed transglycosylation [58•]. It was found that Endo-A was able to transfer N-glycans to a GlcNAc and/or Glc moiety in the context of aglycon species other than a polypeptide, such as bile acid and flavonoids (Figure 6), indicating a very broad substrate specificity of Endo-A. Since N-glycans of glycoprotein origin can serve as ligands for various lectins and cell-surface receptors, site-specific introduction of a defined N-glycan may bestow novel properties onto the natural products.

Conclusion

Chemoenzymatic synthesis involving enzymatic transglycosylation has emerged as a powerful method for making complex oligosaccharides and glycoconjugates that are highly valuable for

functional studies and for biomedical applications as well. The invention of glycosynthases that overcome the limitations associated with a traditional glycosidase-catalyzed transglycosylation represents a conceptual breakthrough in this field. It is almost certain that continuous studies in this field will lead to the discovery of an expanding number of glycosynthases that cover new types of transglycosylation, possess improved catalytic activity, and/or gain broadened substrate specificity. The potential of enzymatic transglycosylation for complex glycoconjugate synthesis is just begun to unfold.

Acknowledgments

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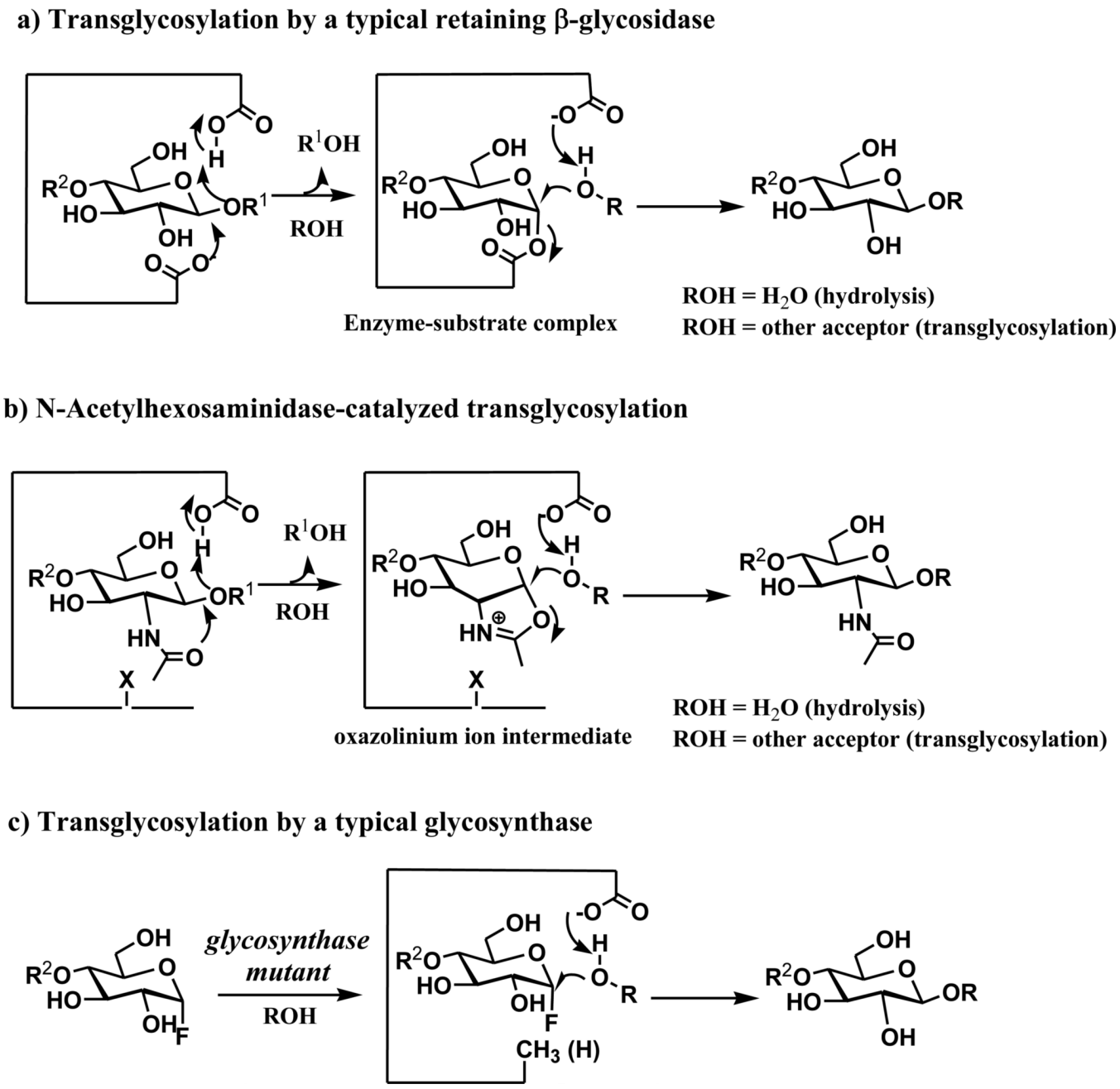

Figure 1.
Mechanisms for glycosidase-catalyzed transglycosylation

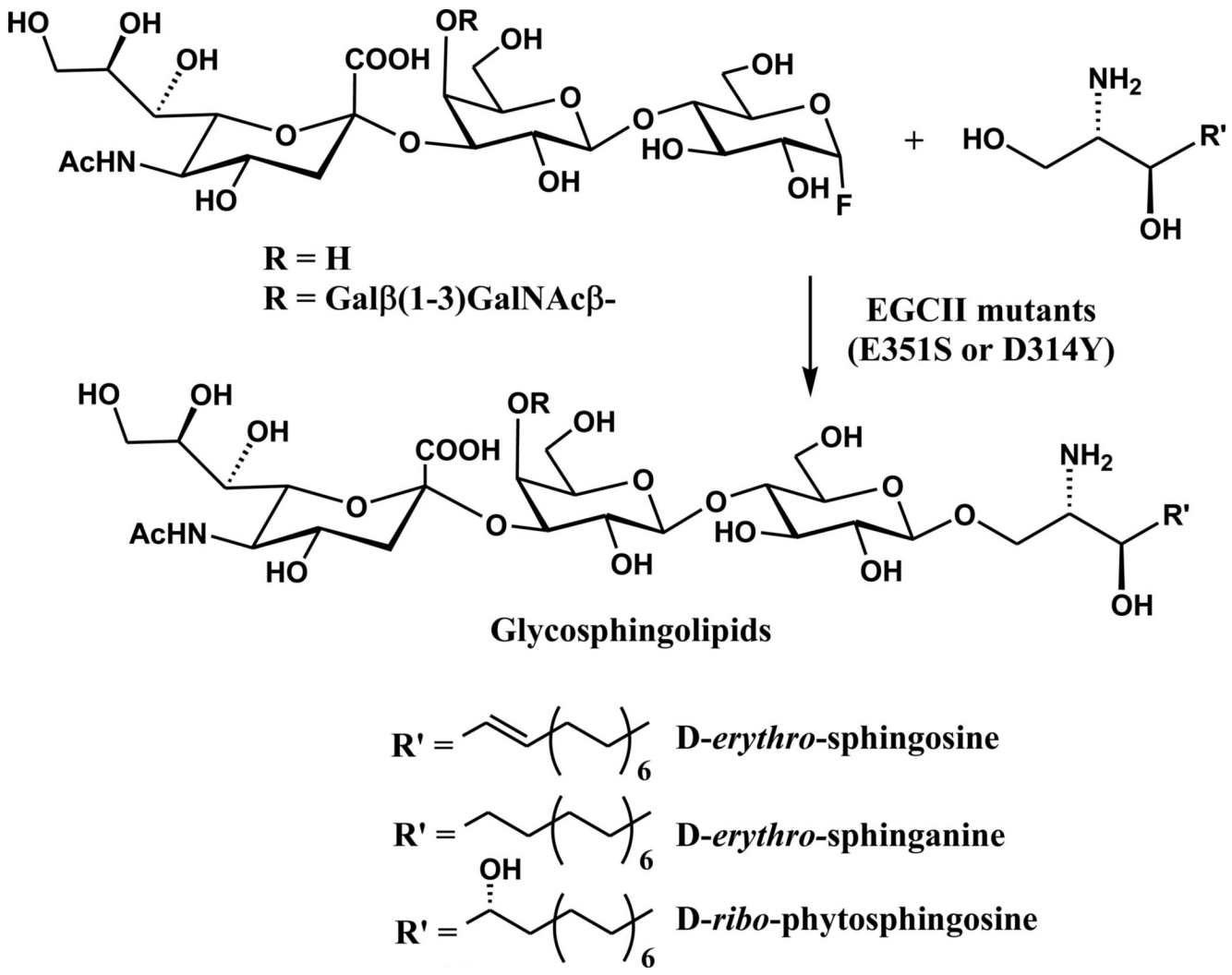

Figure 2.
Synthesis of glycosphingolipids by EGC-based glycosynthase

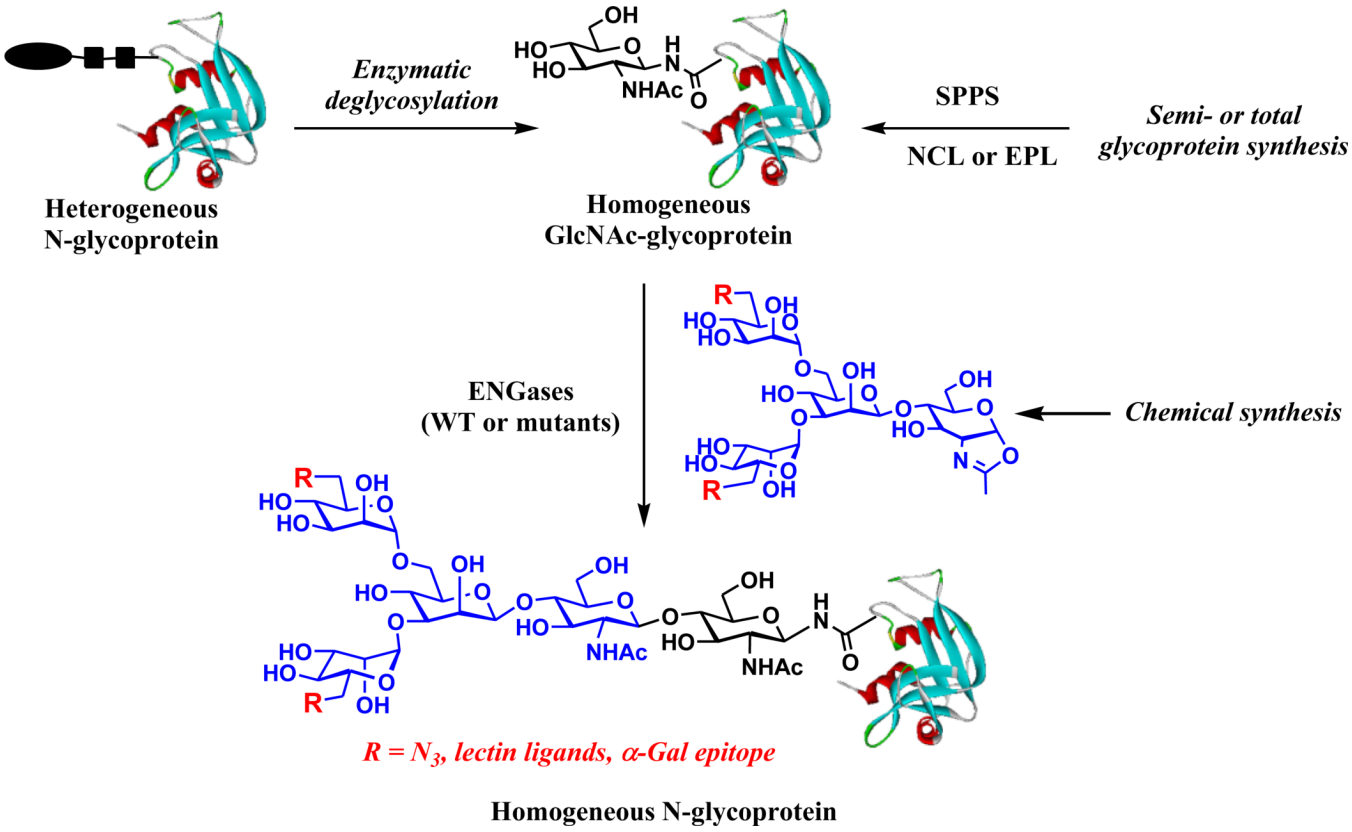

Figure 3.
ENGase-catalyzed transglycosylation for N-glycoprotein synthesis

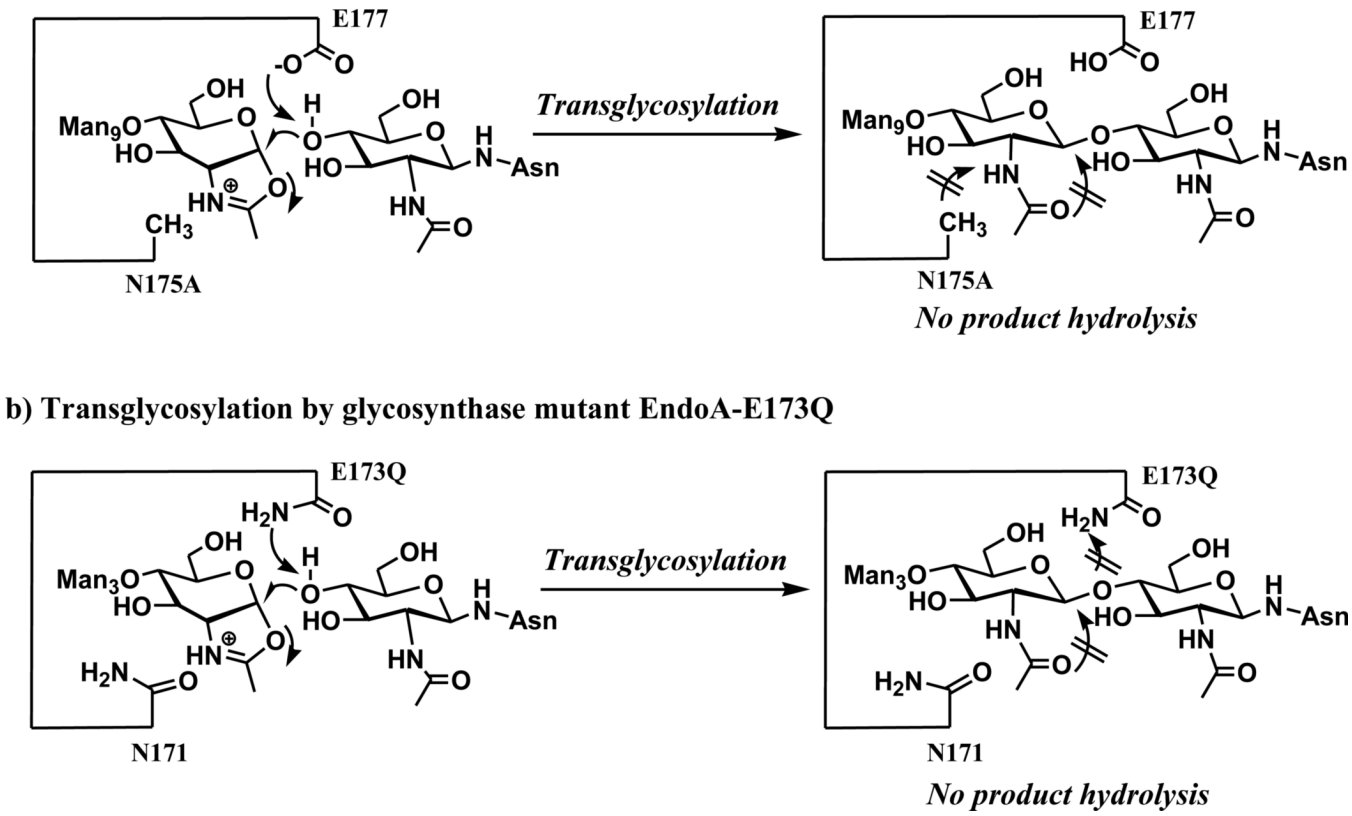

Figure 4.
Plausible catalytic mechanisms of ENGase-derived glycosynthases

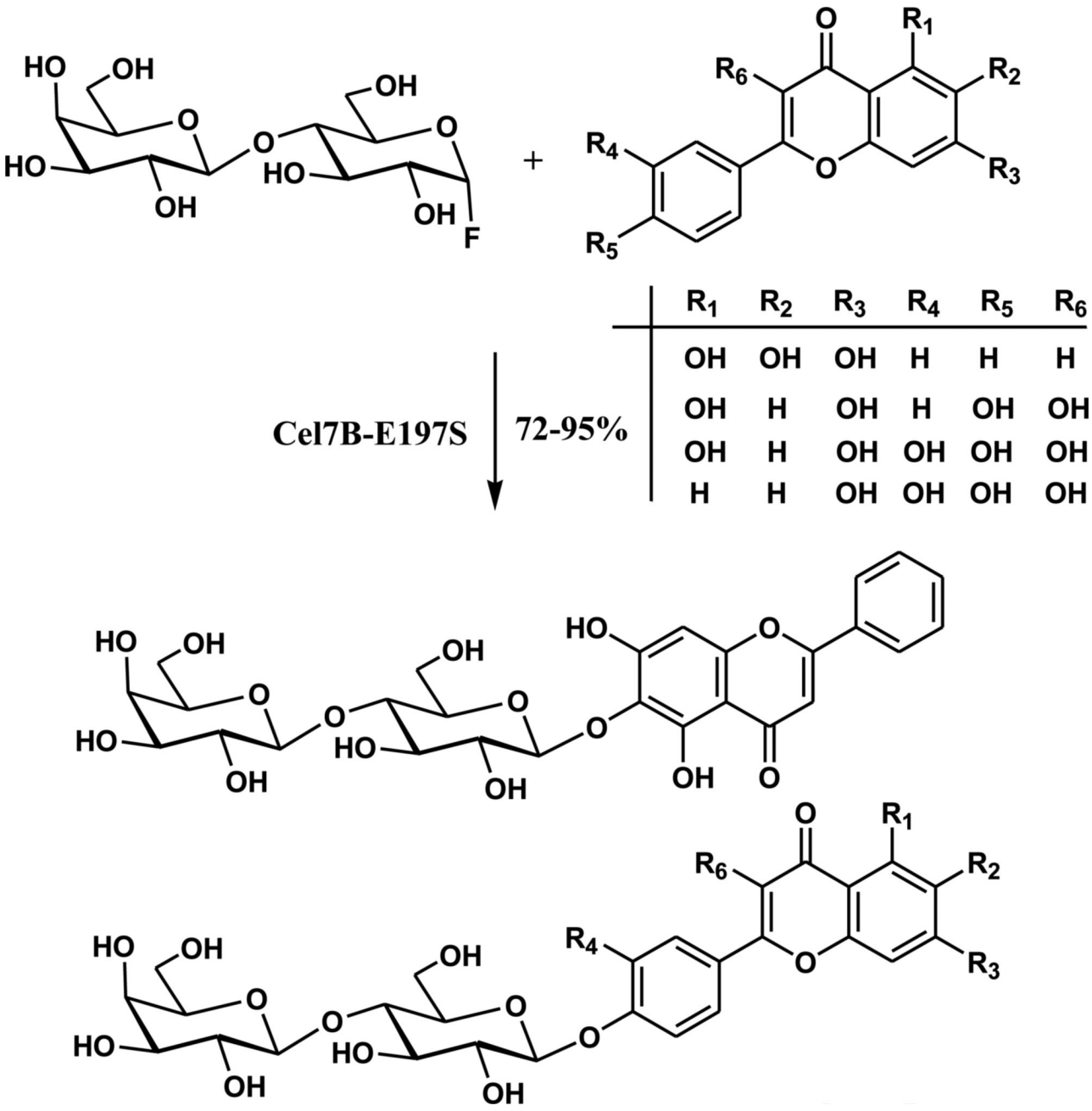

Figure 5.
Glycosylation of flavonoids by Cel7B-E197S mutant

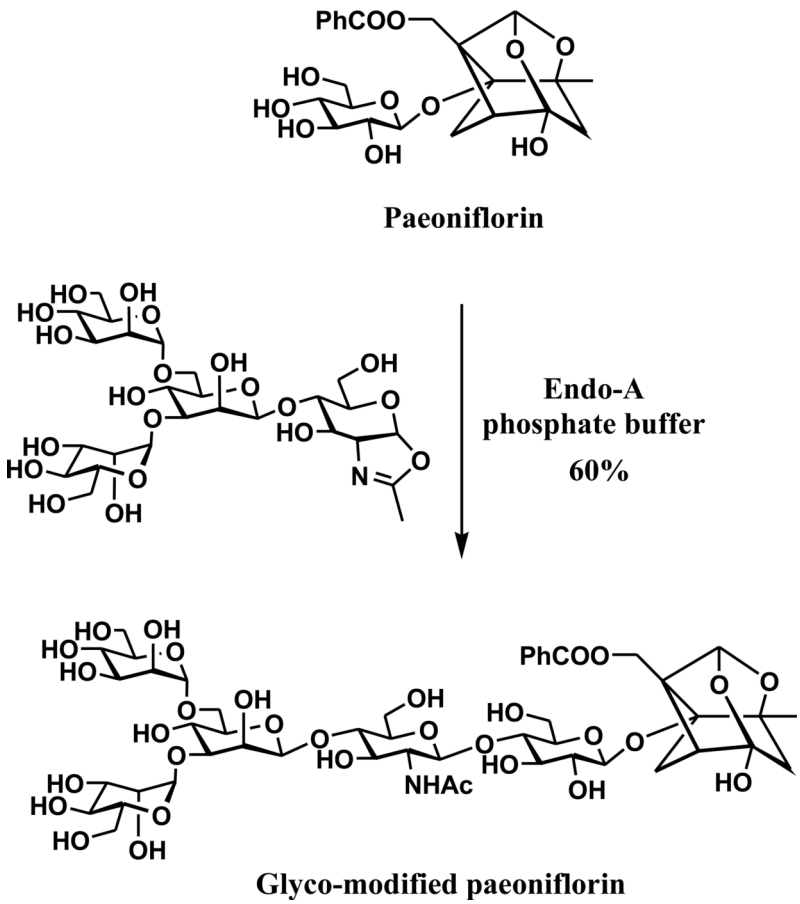

Figure 6.
Endo-A catalyzed introduction of N-glycans into natural products