Published in final edited form as:

Adv Carbohydr Chem Biochem. 2016 ; 73: 73–116. doi:10.1016/bs.accb.2016.07.001.

Endoglycosidases for the Synthesis of Polysaccharides and Glycoconjugates

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1. INTRODUCTION

1.1 Biological Functions of Glycans and Glycoconjugates

Glycans are the most abundant biological molecules on earth and are widely present in all living organisms including bacteria, fungi, plants, animals, and humans. They can exist either in free forms like some common polysaccharides, or in conjugation with a wide variety of other biological molecules to form glycoconjugates, such as glycoproteins, glycolipids, proteoglycans, and glycosylated natural products. Glycans are one of the four fundamental molecular building blocks of life, along with nucleic acids, proteins, and lipids. The last three decades have witnessed an explosion of knowledge on the functions of glycans in biological systems.^{1–3} For example, the oligosaccharide components of glycoproteins can have profound effects on a protein's intrinsic properties such as folding, stability, serum half-life, and intracellular trafficking^{4,5}; cell-surface glycans can directly participate in a wide variety of biological recognition processes including cell adhesion, signaling, development, and immune response.^{1,2,6–9} Alteration of cell-surface glycans is often associated with the progression of cancer, implicating a role for glycans in cancer development and metastasis,^{10–12} as glycans are key mediators in host–pathogen interactions such as bacterial attachment and viral infections.^{13–17} Thus, a detailed understanding of the essential roles of glycans in these biological and disease processes not only provides new knowledge but also offers great promise for developing novel diagnostic and therapeutic methods for improving human health.^{18–23} Despite the implications of the diverse roles of glycans, we have only begun to decode the glycome of living organisms. Advances in glycoscience research are still lagging behind the studies on other biological molecules such as proteins and nucleic acids. In particular, the enormous structural complexity (branching structures, stereoisomers, and large molecular mass) and heterogeneity of complex oligosaccharides, glycoproteins, and proteoglycans have made them formidable targets for structural analysis, chemical/enzymatic synthesis, and functional studies. In 2011–12, the National Academy of Sciences (NAS) called for a Committee on Glycosciences to assess the importance and impact of glycoscience, to explore the landscape of current research, and to identify the challenges that will need to be addressed to enable the field to move forward. The NAS report (http://www.nap.edu/openbook.php?record_id=13446), which provides a roadmap for the future of glycoscience, concludes that glycoscience is a central field with links to many disciplines, and that a better understanding of glycoscience is critical for advancement of human health and for sustainable development. Among others, a major roadblock preventing a detailed understanding of the biological functions of glycans remains

the limited availability of diverse, structurally well-defined, homogeneous glycans and glycoconjugates, as well as robust and facile tools for their synthesis.

1.2 Synthetic Glycans and Glycoconjugates for Deciphering Functions

Synthesis has played an essential role in elucidating the functions of glycans and glycoconjugates and has significantly accelerated their applications as functional biomaterials and therapeutic agents (drugs and vaccines).^{19,24,25} The past decade has witnessed tremendous progress in the development of efficient chemical and chemoenzymatic methods for the synthesis of structurally well-defined complex carbohydrates, which have been used for addressing critical biological problems that were otherwise difficult to tackle by genetic approaches.^{21,23,25–32} For example, synthetic oligosaccharides are essential for identifying glycan-binding proteins and for diagnosis using glycan microarrays.^{22,33–37} Novel multivalent carbohydrate ligands have been designed and used for characterizing cell-surface carbohydrate–protein interactions involved in signaling and B-cell activation.^{38,39} Novel enzyme inhibitors have been designed for probing cellular functions with potential for development of novel therapeutics,^{40,41} and sophisticatedly designed glyconanoparticles and glycodendrimers have been used for specific targeting of lectins for drug and vaccine delivery.^{42–44} In particular, a very active area of research in carbohydrate chemistry and glycobiology is design and synthesis of carbohydrate-based vaccines against cancer, bacteria, and viruses.^{45–49} Another impressive example is the development of a fully synthetic heparin oligosaccharide analog, Arixtra® (fondaparinux sodium), as an anticoagulant agent for the treatment of deep vein thrombosis.^{50–52} More recently, highly efficient chemoenzymatic synthesis of low-molecular-weight (LMW) heparin was achieved via in vitro enzymatic conversion of a nonsulfated *N*-acetylheparonan polysaccharide produced by *Escherichia coli* strain K5 to ATIII-binding heparin oligosaccharides, using a series of enzymes cloned from the heparin biosynthetic pathway.⁵³ These examples provide a testament to the fact that synthetic glycans and glycoconjugates are indeed indispensable for both basic research and various applications.

1.3 Enzymes as a Tool for the Synthesis of Glycans and Glycoconjugates

Despite the tremendous progresses in synthetic carbohydrate chemistry, new synthetic methods and strategies are still in demand to meet with the challenges in constructing complex carbohydrates. In contrast to chemical glycosylation that usually requires tedious protection/deprotection manipulations in order to achieve regio- and stereoselectivity, enzymatic glycosylation usually provides perfect control of the anomeric configuration and high regioselectivity without the need of protecting groups.²⁵ Thus, incorporation of enzymatic transformation in a complex carbohydrate synthesis could significantly simplify the synthetic schemes and enhance the overall efficiency of the synthesis. Indeed, chemoenzymatic synthesis, the combination of chemical synthesis with enzymatic transformation, has emerged as one of the most promising approaches in recent years for constructing complex oligosaccharides and glycoconjugates.^{25,32,54}

Both glycosyltransferases and glycosidases have been vigorously explored for synthetic purposes.^{25,54–56} While glycosyltransferases are the natural enzymes used for glycosidic bond formation in carbohydrate biosynthesis, a subset of glycosidases have also been found

to be capable of promoting glycosidic bond formation via transglycosylation reactions. In comparison with common glycosyltransferase-catalyzed reactions that require sugar nucleotides or sugar phospholipids as donor substrates and transfer monosaccharides one at a time, glycosidase-catalyzed synthesis has several advantages, including the use of relatively simple and cheap donor substrates, a relatively easy access to the enzymes, and in the case of an endoglycosidase-catalyzed reaction, an en bloc oligosaccharide transfer in a single step. Moreover, the invention of glycosynthases, a class of novel glycosidase mutants that are devoid of hydrolytic activity but are capable of using a suitable activated glycosyl donor for transglycosylation, provides a beautiful solution to the two fundamental problems associated with glycosidase-catalyzed synthesis: the low transglycosylation efficiency and product hydrolysis.^{32,57–64} On the other hand, recent advances have shown that both glycosyltransferases and glycosidases can in fact have relaxed substrate specificity, allowing the synthesis of both natural and selectively modified complex carbohydrates.^{25,56} In this chapter, we highlight recent advances in the use of endoglycosidases for the synthesis of biologically interesting glycans and glycoconjugates, including natural and artificial polysaccharides, glycoproteins, and glycolipids.

2. ENDOGLYCOSIDASES IN THE SYNTHESIS OF NATURAL AND ARTIFICIAL POLYSACCHARIDES

Enzymatic and chemoenzymatic synthesis of natural and artificial polysaccharides together form an important area of interest because of the diverse applications of synthetic polysaccharides. Several excellent reviews have been published that cover different aspects of enzymatic synthesis and applications of polysaccharides using endoglycosidases as the key biocatalyst.^{62,65–70} Here we focus the discussions on recent progress in using three types of endoglycosidases (cellulase, chitinase, and hyaluronidase) for enzymatic polymerization of activated monomers for the synthesis of natural and selectively modified cellulose, chitin, and glycosaminoglycan derivatives.

2.1 Synthesis of Artificial Cellulose and Derivatives via Enzymatic Polymerization of Glycosyl Fluorides Catalyzed by Cellulase and Other Related Endoglycosidases

Cellulose is a linear polysaccharide composed of β -1,4-linked glucose units. It is a primary structural component of the plant cell wall and an important bioresource for many applications including biofuels.⁷¹ In 1991, Kobayashi and coworkers reported the first example of in vitro enzymatic polymerization for making artificial cellulose, using wild-type retaining cellulase from *Trichoderma viride* as the enzyme and β -cellobiosyl fluoride as the activated donor substrate (Scheme 1).^{72–74} It was found that the degree of polymerization and the morphology of the resulting artificial cellulose were dependent on the reaction conditions and the source and purity of the cellulases used. Artificial cellulose with a dp of ca. 22 glucose units could be obtained in over 50% yield by the enzymatic polymerization. As a way to improve the polymerization efficiency, Kimura and coworkers engineered a cellulase from *T. viride* by deleting the cellulose-binding domain.⁷⁵ In 1996, Moreau and Driguez reported the first enzymatic synthesis of thiocellooligosaccharides with alternative *O*- and *S*-glycosidic linkages by enzymatic polymerization of 4-thio- β -cellobiosyl fluoride, using the cellulase Cel7B from *Humicola insolens* as the enzyme (Scheme 1b).⁷⁶ It was

shown that cellulases could tolerate certain modifications on the β -cellobiosyl fluoride substrate at the 2, 3, and/or 6 positions, allowing the synthesis of selectively modified cellulose, including the cellulose–chitin hybrid (Scheme 1c).^{77–79}

To address the problem of product hydrolysis when using wild-type cellulases, which usually results in relatively low yields, Driguez and coworkers generated a glycosynthase from a retaining cellulase derived from *H. insolens* (Cel7B) by site-directed mutation at the critical nucleophilic residue in the enzyme.⁸⁰ The mutant Cel7B E197A showed excellent transglycosylation activity with α -cellobiosyl fluoride to various mono- and disaccharide acceptors in excellent yields. In addition, the mutant Cel7B E197A could tolerate certain modifications such as amino, bromo, and sulfur substitutions at the C-6' position of the α -cellobiosyl fluoride donors, making it possible to synthesize selectively modified cello-oligosaccharides and polysaccharides (Scheme 1d–f). More recently, Planas and coworkers reported a glycosynthase-catalyzed polymerization using the *H. insolens* cellulase Cel7B E197A mutant for the synthesis of modified cellulose derivatives with perfect control of the substitution pattern of the subunits in the polysaccharides.⁸¹ The 6'-azido group introduced in the cellobiose subunits in the artificial cellulose could be subject to site-selective reactions to provide a variety of functionalized cellulose (Scheme 1g). As for the glycosynthase concept, the reversed anomeric configuration of the activated substrate (α -glycosyl fluoride for β -endoglycosidase) mimics the glycosyl–enzyme intermediate in the double inversion mechanism. Thus the glycosidase mutant lacking the nucleophilic residue would not be able to hydrolyze the ground-state substrate, but could accommodate glycosyl fluoride and catalyze the transglycosylation with a suitable acceptor to form a new glycosidic linkage.

In addition to cellulases, other endoglycosidases have also been explored for the synthesis of modified cellulose and related derivatives. Brumer and coworkers reported that three retaining glycosynthases derived from different glycoside hydrolase families (GH7, 12, and 16) were capable of synthesizing various large xyloglucan polymers, a hemicellulose occurring on plant cell wall, with the molecule weight up to 60,000 Da, which represents an impressive high-molecular-weight polysaccharide synthesized by a glycosynthase-catalyzed polymerization.^{82,83} The glycosynthase of previously reported Cel7B (new mutant E197S) can form a nongalactosylated, XXXG-based homoxyloglucan, while the xyloglucanase mutant from *Tropaeolum majus* (TmNXG1 E94A) can make XLLG-based xyloglucan oligosaccharides. However, the xyloglucanase mutant from *Bacillus licheniformis* (BIXG12 E155A) showed poor activity in the condensation of xyloglucosyl fluorides. Recently, a xyloglucanase from *Paenibacillus pabuli* was successfully converted into a glycosynthase and was applied to the synthesis of structurally complex plant polysaccharides (Scheme 2).⁸⁴ The PpXG5 E323G mutant showed broad-spectrum activities on various oligosaccharyl fluorides as the donor substrates, including XLLGaF and XXXGaF, and could efficiently catalyze their polymerization to synthesize homogeneously branched xyloglucans with high molecular weight. Interestingly, a fucosyltransferase from *Arabidopsis thaliana* was designed to combine with this glycosynthase to achieve the synthesis of fucogalactoxyloglucans, which are found in the primary cell walls of dicotyledons. Considering the difficulty to obtain these polysaccharides from natural sources in homogeneous forms, this glycosynthase–glycosyltransferase system provides a promising tool for the synthesis of well-defined xyloglucans. In addition, following the first report of

endoglycosidase-based glycosynthase,⁵⁸ the glycosynthase mutants based on the 1,3–1,4- β -glucanase from *Bacillus* were generated and used for the in vitro synthesis of crystalline (1,3–1,4)- β -D-glucans.⁸⁵ In studying the transglycosylation of endo- β -N-acetylglucosaminidases using sugar oxazolines as substrates, Wang and coworkers observed 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, and the Endo-A enzyme could promote a tandem transglycosylation of a disaccharide oxazoline, Glc β –1,4-GlcNAc oxazoline, leading to the synthesis of novel cellulose–chitin hybrid oligosaccharides and glycopeptides carrying repeating Glc-GlcNAc structure.⁸⁶ Similar to the cellulase-catalyzed polymerization of β -cellobiosyl fluoride, an α -amylase-catalyzed polymerization of α -maltosyl fluoride was fulfilled to give synthetic maltooligosaccharide in an aqueous organic medium.⁸⁷

2.2 Chitinase-Catalyzed Synthesis of Artificial Chitin and Derivatives Using Sugar Oxazolines as Activated Substrates

Chitin is a polysaccharide consisting of β –1,4-linked *N*-acetylglucosamine (GlcNAc) monosaccharide units.⁸⁸ It is a major component of the cell walls of insects and the exoskeletons of shell fish. Lipochitooligosaccharides are a class of signal molecules that can modulate plant host immunity and enable the establishment of nitrogen-fixation symbiosis between bacteria and legumes.⁸⁹ Chitin and chitosan oligosaccharides also possess anticancer and antiinflammatory activities.⁹⁰ Chitinases, a class of glycosidases that hydrolyze chitin, belong to two distinct glycoside hydrolase families (GH18 and GH19), which showed different catalytic mechanisms including retaining and inverting processes. Both types of chitinases have been explored for in vitro synthesis of chitin and chitosan-related oligo- and polysaccharides. The GH18 chitinases hydrolyze chitin via a substrate-assisted mechanism in which the 2-acetamido group of the GlcNAc at the –1 sub-site acts as the nucleophile to form an oxazolinium intermediate.⁹¹ In 1996, Kobayashi and coworkers demonstrated the first chitinase-catalyzed self-condensation of synthetic *N*-acetylchitobiose oxazoline to make artificial chitin.⁹² The authors used chitinase from *Bacillus* sp. as the catalyst and elegantly chose basic reaction conditions (pH 10.6) under which the chitinase-catalyzed hydrolysis of product was suppressed, but the enzyme still kept sufficient activity on the activated sugar oxazoline for polymerization. The condensation led to the formation of insoluble artificial chitin with a molecular weight of >4000 Da. Similar to the cellulase-catalyzed polymerization, the chitinase-catalyzed condensation of chitobiose oxazolines also can tolerate certain modifications on the oxazoline subunits, permitting the synthesis of a range of selectively modified chitooligosaccharides and functionalized chitin derivatives. These include chitoheptaose derivatives,⁹³ 2-, 3-, and 6-substituted chitins,^{94–96} chitosan-, cellulose-, galactose-, or xylose-chitin hybrids,^{79,97–99} β –1,4- β –1,6-linked derivatives,¹⁰⁰ fluorinated chitin,¹⁰¹ *N*-sulfonated chitin,¹⁰² *N*-phthaloyl chitin,¹⁰³ and *N*-L-cysteinyl chitin¹⁰⁴ (Scheme 3). In spite of the many successful examples of the chitinase-catalyzed condensation of chitobiose oxazoline derivatives, the degree of polymerization was often relatively low due to the precipitation of chitooligosaccharides. To improve the water solubility of oligomers so that the sugar can be further elongated, Kobayashi and coworkers elegantly designed an *N*-sulfonate derivative by introducing an *N*-sulfonate group into the disaccharide oxazoline unit. Polymerization of this *N*-sulfonated sugar oxazoline under the

catalysis of the chitinases from *Bacillus* sp. and *Serratia marcescens* did enhance the degree of polymerization due to the increased solubility of the oligomers, giving a synthetic chitin derivative with a higher molecular weight (up to 5000 Da). Recently, an endo chitinase from *Serratia proteamaculans* was reported to possess hyper transglycosylation activity to synthesize long-chain chitooligosaccharides.¹⁰⁵

Following the glycosynthase concept proposed for endo- β -*N*-acetylglucosaminidases that use a substrate-assisted mechanism via the formation of a sugar oxazolinium ion intermediate,^{59,60,106} glycosynthase mutants were also generated from chitinases by site-directed mutagenesis of the key residue that promotes sugar oxazolinium ion formation during hydrolysis. Cottaz and coworkers reported that mutation of the D202 residue in the chitinase A1 from *Bacillus circulans* gave a mutant (D202A) that could significantly diminish the hydrolytic activity and increase the yield of sugar oxazoline polymerization.¹⁰⁷ Fukamizo and coworkers reported two mutants, D313A and D313N, of GH18 chitinase A from *Vibrio harveyi* that were capable of synthesizing chitooligosaccharide without obvious hydrolysis of products.¹⁰⁸ While most of the chitinases employed in the synthesis of artificial chitins are retaining enzymes from the GH18 family, an inverting GH19 endo-chitinase from moss *Bryum coronatum* was also successfully engineered as a glycosynthase.¹⁰⁹ The mutant could use α -chitobiosyl fluoride as the substrate for polymerization to form chitooligosaccharides. While the catalytic mechanism involving the action on the α -chitobiosyl fluoride for condensation is not well understood, it is likely that the catalysis proceeds by the Hehre resynthesis–hydrolysis mechanism.

2.3 Hyaluronidase-Catalyzed Construction of Glycosaminoglycans Using Sugar Oxazoline as the Activated Substrates

Glycosaminoglycans (GAGs) are an important class of mammalian polysaccharides. They exist either as free polysaccharides such as hyaluronans (HAs) or as large (and tremendously heterogeneous) glycans conjugated with proteins (ie, proteoglycans) such as heparan sulfate (HS), dermatan sulfate (DS), and chondroitin sulfate (ChS). Enzymatic and chemoenzymatic synthesis of glycosaminoglycans with well-defined compositions and structures has attracted great interest in recent years, because of their diverse biological functions and also because of the difficulties in isolating structurally homogeneous materials from natural sources for functional studies. While several types of endoglycosidases have been explored for in vitro glycosaminoglycan synthesis, a major advance in this field was the discovery of hyaluronidase as a class of enzymes that can catalyze the condensation of diverse sugar oxazolines to form a wide variety of GAG structures with remarkably relaxed substrate specificity.¹¹⁰

Hyaluronidase (HAase) belongs to the glycoside hydrolase family GH56 that hydrolyzes the β -glycosidic bond of the *N*-acetylglucosamine or *N*-acetylgalactosamine moiety in hyaluronic acids and related glycosaminoglycans. It proceeds via a retaining, substrate-assisted mechanism.^{111,112} The transglycosylation activity of HAase was first reported as early as 1955 by Weissman and coworkers and was subsequently demonstrated by several other groups.^{113–116} Although its synthetic ability had been proven a long time ago, the

efficiency of hyaluronidase-catalyzed transglycosylation reactions using ground-state substrates was relatively low due to the natural hydrolytic activity of the enzyme.^{117–120}

This situation changed when Kobayahsi and coworkers explored the use of the corresponding sugar oxazoline (the transition-state analog) as an activated substrate for hyaluronidase-catalyzed transglycosylation, which is kinetically favorable to form the polymerization product over its hydrolysis. In 2001, Kobayashi and coworkers reported the first in vitro enzymatic synthesis of artificial HA using a disaccharide oxazoline as the monomer, yielding an artificial HA with a molecular weight (M_n) up to 1.74×10^4 Da with perfect control of the structure¹²¹ (Scheme 4). HA is a class of GAGs consisting of a repeating β -1,3-linked disaccharide unit of D-glucuronic acid and *N*-acetyl-D-glucosamine connected with a β -1,4-glycosidic linkage. HA plays a role in tumor progression and other biological processes.^{122,123} The bovine testicular hyaluronidase and ovine testicular hyaluronidase that hydrolyze β -glycosidic bond in the GlcNAc- β -(1,4)-GlcA linkage in hyaluronan, chondroitin, chondroitin sulfate, and dermatan sulfate are the most commonly used endoglycosidase for the synthesis of HA and related glycosaminoglycans.¹¹² In contrast to the chitinase- and cellulase-catalyzed polymerizations where each enzyme catalyzes a single type of condensation reaction, it was found that the hyaluronidase could catalyze multiple enzymatic polymerizations with a wide variety of disaccharide oxazolines and their derivatives to form various GAGs with perfect control of the regio- and stereoselectivity.¹¹⁰ By an elegant design of the disaccharide oxazoline monomers (with or without modifications), the synthesis of hyaluronan,¹²¹ chondroitin,¹²⁴ and chondroitin sulfate,¹²⁵ and their selectively modified derivatives^{124,126,127} were achieved using a single hyaluronidase (Scheme 4).

In addition to condensation of single monomers, hybrid GAGs were prepared via copolymerization of two oxazoline monomers by hyaluronidase.¹²⁸ For example, copolymerization of two disaccharide oxazolines, one derived from the *N*-acetylhyalobiuronate and the other from *N*-acetylchondrosine, could proceed in a highly regio- and stereoselective manner without homopolymerization of the individual oxazolines, to give the hybrid HA-Ch polysaccharide with high molecular weight ($M_n = 1.4 \times 10^4$) (Scheme 5).¹²⁸ The copolymer composition could be easily controlled by adjusting monomer ratio. More recently, Endo and coworkers reported a facile enzymatic synthesis of glycosaminoglycan oligosaccharides using immobilized hyaluronidase as the catalyst to facilitate the product purification.¹²⁹ It is expected that the hyaluronidase-catalyzed self-condensation and copolymerization of sugar oxazolines will find wide applications for constructing various natural and modified GAGs for functional studies and potential biomedical applications.

2.4 Endo- β -Xylosidase-Catalyzed Transglycosylation in the Synthesis of Proteoglycans

Proteoglycans play essential roles in many important molecular recognition processes such as cell adhesion and development.¹³⁰ Several studies have implicated that the endo- β -xylosidase, the enzyme that cleaves GAG from proteoglycans by hydrolyzing the Xyl-Ser linkage, possesses transglycosylation activity capable of transferring a GAG back to a given polypeptide to reconstitute a new proteoglycan.⁶⁸ In natural proteoglycans, the GAG is

linked to the protein backbone through a tetrasaccharide linkage, GlcA- β -1,3-Gal- β -1,3-Gal- β -1,4-Xyl- β -, which is attached to a serine residue in the protein.¹³¹ In nature, the biosynthesis of GAGs is initiated with site-specific transglycosylation from UDP-xylose to proteins catalyzed by two xylosyltransferase isoenzymes I and II with recognition of a G-S-G or G-S-X-G site on the amino acid sequence of proteins.^{132–134} It was reported that the endo-xylosidases from the *Molluscus patinopecten* can hydrolyze a donor GAG such as the peptidochondroitin sulfate (30 kDa) and transfer the releasing GAG to a tetrapeptide acceptor in a transglycosylation manner to form a new conjugate.^{135–137} In addition, dermatan sulfate and heparan sulfate were also active donor substrates for transglycosylation (Scheme 6). This endo-xylosidase-catalyzed synthesis of neoproteoglycans appears as a very promising technology to make neoproteoglycans. However, the major problem of this endo-xylosidase-catalyzed transglycosylation is the very low yield (usually less than 10%), and so far the transglycosylation of a GAG to an intact protein has been elusive. It is expected that exploration of new active donor substrates and enzyme engineering of the endo- β -xylosidase to improve the transglycosylation efficiency could lead to a more efficient approach for proteoglycan synthesis.⁶⁸ Interestingly, a cellulase from *Aspergillus niger* was found to be able to cleave the β -xyloside-Ser linkage in proteoglycans to release the GAGs.¹³⁸ This cellulase might be further explored for transglycosylation for proteoglycan synthesis.

3. ENDOGLYCOSIDASES IN THE SYNTHESIS OF N-GLYCOPEPTIDES AND N-GLYCOPROTEINS

Synthesis of homogeneous glycoproteins carrying well-defined oligosaccharides poses a significant challenge to synthetic chemists.^{32,139} While chemical synthesis of complex oligosaccharides involves a series of hurdles in terms of the control of regio- and stereoselectivity, the conjugation of free glycan to a free (nonprotected) polypeptide or protein in a site-specific manner to form a natural glycan-peptide linkage adds a new level of difficulty because of the presence of multiple functional groups on both the sugar and polypeptide portions. Among other methods, the chemoenzymatic method involving the enzymatic transglycosylation with endo- β -*N*-acetylglucosaminidases (ENGases) is emerging as a very promising method for glycopeptide synthesis and glycosylation remodeling.³² ENGases are a class of endoglycosidases that cleave *N*-glycans from glycoproteins by hydrolyzing the β -1,4-glycosidic bond in the *N,N'*-diacetylchitobiose core. These enzymes are widely distributed in microorganisms, plants, animals, and humans.^{140–142} In addition to their hydrolytic activity, some ENGases such as Endo-A from *Arthrobacter protophormiae*^{143–145} and Endo-M from *Mucor hiemalis*¹⁴⁶ possess transglycosylation activity, ie, the ability to transfer the released oligosaccharide to a suitable acceptor to form a new glycosidic linkage. Several reviews have been published that provide a nice coverage of early studies in the ENGase-catalyzed transglycosylation for oligosaccharide and glycopeptide synthesis.^{147–152} Significant progress in the methodology has been made in recent years, including the use of sugar oxazolines as activated donor substrates for transglycosylation, the discovery of ENGase-based glycosynthase mutants that lack hydrolysis activity but can take a sugar oxazoline as an activated donor substrate for transglycosylation, and the discovery of new endoglycosidases and related glycosynthase

mutants, which has significantly expanded the scope of the chemoenzymatic method for various applications. This section will highlight the major recent advances in this field.

3.1 Exploration of Glycan Oxazolines as Donor Substrates for ENGase-Catalyzed Synthesis of Complex Glycopeptides and Glycoproteins

The original protocol of the ENGase-catalyzed transglycosylation used natural *N*-glycans or *N*-glycopeptides as donor substrates.¹⁴⁷ However, a broad application of the endoglycosidase-catalyzed transglycosylation has been hampered by several limitations, including the relatively low transglycosylation yield, product hydrolysis, and the restriction to the use of only natural *N*-glycans as donor substrates that are difficult to obtain. A major progress in the field was made by the use of sugar oxazoline as a kinetically favorable substrate for ENGase-catalyzed transglycosylation. This was based on the assumption that the ENGase-catalyzed reaction proceeded by a substrate-assisted mechanism involving a sugar oxazolinium ion intermediate, similar to that of some GH family-18 chitinases^{153–155} and some GH family-20 *N*-acetyl- β -hexosaminidases.^{156,157} It was previously shown that chitobiose oxazolines as transition-state mimics could serve as substrates for chitinase-catalyzed polymerizations.¹⁵⁸ Shoda and coworkers first reported that a simple disaccharide oxazoline derived from Man β 1,4GlcNAc could serve as a substrate of Endo-A and Endo-M for transglycosylation to form a *p*-nitrophenyl trisaccharide derivative, which was designed as a substrate for detecting transglycosylation activity in a coupled assay.¹⁵⁹ The yield was about 50% when a twofold excess of the sugar oxazoline was used. Wang and coworkers first explored synthetic sugar oxazolines as donor substrates for ENGase-catalyzed *N*-glycopeptide synthesis (Scheme 7).^{160–164} Independently, Fairbanks and coworkers synthesized a series of sugar oxazolines and evaluated their transglycosylation activities with Endo-A and Endo-M.^{165–167} It was found that the oligosaccharide oxazoline corresponding to the *N*-glycan core Man3GlcNAc, and some of its truncated and selectively modified forms, was able to serve as donors for transglycosylation to form corresponding *N*-glycopeptides in good to excellent yields. Interestingly, the resulting glycopeptides that carry truncated or modified *N*-glycans were poor substrates for hydrolysis because of the structural modifications. Thus the high transglycosylation activity of the activated sugar oxazoline, coupled with the low hydrolytic activity of the resulting truncated or modified *N*-glycans in the product, ensures formation of high yields and accumulation of the *N*-glycopeptide product. This chemoenzymatic approach was successfully extended to the glycosylation remodeling of glycoproteins and resulted in an expeditious chemoenzymatic synthesis of an array of homogeneous glycoforms of ribonuclease B (Scheme 8).^{163,164} Notably, the enzyme Endo-A could tolerate modifications of the outer mannose residues in the Man3GlcNAc-oxazoline core, thus permitting site-specific introduction of small-molecule tags (eg, an azide group) as well as large oligosaccharides (eg, lectin ligands) into a protein while preserving the natural *N*-pentasaccharide core in the resulting glycoproteins.¹⁶⁴ Recently, Fairbanks and coworkers reported that Endo-A was also able to transfer a phosphorylated *N*-glycan from the corresponding glycan oxazoline to deglycosylated ribonuclease B to form a phosphoglycoprotein (Scheme 8).¹⁶⁸ This approach may find applications for enhancing cellular uptake of therapeutic enzymes via the M6P receptor-mediated delivery in enzyme-replacement therapy.

3.2 Generation of ENGase-Based Glycosynthases for Transglycosylation

Despite the remarkable efficiency of the chemoenzymatic method for the synthesis of glycopeptides and glycoproteins carrying truncated and/or unnaturally modified *N*-glycans, product hydrolysis remains a serious problem when the target is a glycoprotein carrying full-size natural *N*-glycans that are good substrates for the respective enzymes. A rational solution for the problem is to create novel glycosynthase mutants that are able to promote transglycosylation using activated donor substrates that are devoid of hydrolytic activity.^{57,61,169,170} However, the conventional approach to generating a glycosynthase by “knocking out” the nucleophilic residue in a retaining glycosidase could not be applied directly to ENGases, as the ENGase-catalyzed reaction proceeds via a substrate-assisted mechanism in which the nucleophile is the 2-acetamido group in the substrate. In 2008, Wang, Yamamoto, and coworkers reported the first example of ENGase-based glycosynthases by using a glycan oxazoline corresponding to the natural Man9GlcNAc *N*-glycan to screen a series of Endo-M mutants generated by site-directed mutagenesis on residues in or around the putative catalytic region of wild-type Endo-M.⁵⁹ Several interesting mutants were identified. The Y217F mutant was found to possess a much enhanced transglycosylation activity and relatively low hydrolytic activity. Kinetic analysis suggests that the increased transglycosylation activity can be attributed to a reduction of the K_m value of the acceptor substrate (ie, an increased affinity to the acceptor). Another mutant, N175A, was identified as a glycosynthase that promotes the transglycosylation of the highly activated sugar oxazoline Man9GlcNAc-oxazoline, but does not hydrolyze the resulting glycopeptide. A plausible mechanism was proposed for the Endo-M-catalyzed reaction.⁵⁹ The N175 residue was proposed to play a role in orientating and promoting the oxazoline formation upon protonation of the glycosidic oxygen by the general acid/base residue E177. Then the E177 facilitates the attack of the oxazoline by water (leading to hydrolysis) or an acceptor (leading to transglycosylation). Mutation of the N175 to an alanine residue renders the enzyme incompetent for hydrolysis, as it could not promote oxazoline formation; however, the mutant could still catalyze the transglycosylation of the supplied synthetic sugar oxazoline to form the transglycosylation product, which would not be hydrolyzed by the mutant because of the mutation. An analogous Endo-A mutant, Endo-A N171A, was also created, which could transglycosylate Man9GlcNAc oxazoline effectively.⁶⁰ Kinetic studies revealed that the dramatically enhanced synthetic efficiency of the Endo-A N171A mutant over the wild-type Endo-A was due to its significantly reduced hydrolytic activity toward both the Man9GlcNAc oxazoline and the product, as well as to its enhanced activity for transglycosylation. Extension of the chemoenzymatic method to the synthesis of homogeneous glycoforms of ribonuclease B carrying full-size natural *N*-glycans was achieved (Scheme 9).^{60,171} The Endo-M N175A mutant could take both high-mannose-type and complex-type *N*-glycan oxazolines as substrate for transglycosylation, while the Endo-A N171A mutant is specific only for transferring high-mannose-type *N*-glycans. Further systematic site-directed mutagenesis at the N175 of Endo-M revealed that the Endo-M N175Q mutant showed remarkable enhanced transglycosylation efficiency over other mutants.¹⁰⁶ These mutants derived from Endo-M and Endo-A represent the first examples of ENGase-based glycosynthases. Later on, glycosynthases (eg, the N322A mutant) derived from Endo-D were generated that were specific for glycans of the *N*-glycan core.¹⁷² In a separate study, Fairbanks and coworkers reported a different Endo-A glycosynthase by site-

directed mutation at the general acid/base residue E173.¹⁷³ 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. However, a similar study showed that a copper-binding mutant at general acid/base residue E177 of the Endo-M enzyme was unable to convert it into glycosynthase.⁶⁰ In 2012, Wang and coworkers described the first example of glycosynthases from the GH18 family ENGases. They generated an array of mutants (Endo-S D233A and D233Q) by site-directed mutagenesis of an endoglycosidase from *Streptococcus pyogenes*, Endo-S, which were found to be capable of efficiently transferring predefined *N*-glycans from corresponding glycan oxazolines to the Fc-deglycosylated intact IgGs without product hydrolysis.¹⁷⁴ More recently the same research group described a glycosynthase from Endo-F3, another GH18 ENGase, which could transfer tri-antennary *N*-glycans to peptides and intact antibodies.¹⁷⁵ In parallel to the site-directed mutagenesis to generate glycosynthases, important progress was also made in the structural studies of the family GH85 and GH18 ENGases. In 2009, Liu and coworkers reported the crystal structures of Endo-A and its complex with Man3GlcNAc-thiazoline, an oxazoline analog.¹⁷⁶ Taylor and coworkers solved the structure of an Endo-A mutant, E173Q.¹⁷⁷ These structural studies identified 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 establish that the E173 plays a role as a general acid/base, while the N171 is essential for promoting oxazoline formation by orientating the 2-acetamido group. Meanwhile, Boraston and coworkers solved the structure of Endo-D from *Streptococcus pneumoniae*, another family GH85 ENGase.¹⁷⁸ Endo-D and Endo-A had almost identical topology in folding of the catalytic domain and share a general mechanism of substrate-assisted catalysis. In 2014, Sundberg and coworkers reported the crystal structure of Endo-S that showed an unusual V-shaped structure.¹⁷⁹ The structural information will be highly valuable for the rational engineering of ENGases aiming to improve their transglycosylation activity or for discovering new glycosynthase mutants with altered substrate specificities.

3.3 ENGase-Catalyzed Synthesis of Selected Biologically Interesting Glycopeptides and Glycoproteins

The discovery of ENGase-based glycosynthases has opened a new avenue for the synthesis of complex *N*-glycopeptides and *N*-glycoproteins that have been hitherto difficult to construct by other methods. Here we highlight several recent examples to showcase the advance. One example is the chemoenzymatic synthesis of different glycoforms of the CD52 glycopeptide antigens.^{175,180} CD52 is a glycosylphosphatidylinositol-anchored glycopeptide antigen found on sperm cells and human lymphocytes. Recent structural studies indicate that sperm-associated CD52 antigen carries both a complex-type *N*-glycan and an *O*-glycan on the polypeptide backbone. To facilitate functional and immunological studies of distinct CD52 glycoforms, Wang and coworkers reported the first chemoenzymatic synthesis of homogeneous CD52 glycoforms carrying both *N*- and *O*-glycans¹⁸⁰ (Scheme 10). The synthetic strategy consists of two key steps: (1) monosaccharide primers GlcNAc and GalNAc were first installed at the predetermined *N*- and *O*-glycosylation sites by a facile solid-phase peptide synthesis, and (2) the *N*- and *O*-glycans were extended in very good yields by enzymatic glycosylations employing Endo-M N175A mutant and T-synthase,

respectively. This chemoenzymatic approach is highly convergent and permits easy construction of various homogeneous CD52 glycoforms from a common polypeptide precursor.

More recently, Wang and coworkers reported the synthesis of core-fucosylated bi- and tri-antennary complex glycoforms of CD52, which was enabled a glycosynthase mutant (D165A) derived from Endo-F3.¹⁷⁵ By screening of various ENGases through the use of appropriate synthetic donor and acceptor substrates, the research group found that the *Flavobacterium meningosepticum* endo- β -*N*-acetyl-glucosaminidases (GH family 18), including Endo-F2 and Endo-F3, were able to glycosylate α -1,6-fucosylated GlcNAc derivative to provide natural, core-fucosylated complex-type *N*-glycopeptides.¹⁸¹ In contrast, the commonly used endoglycosidases such as Endo-A and Endo-M, which belong to GH family 85, were unable to take α -1,6-fucosyl-GlcNAc derivative as acceptors for transglycosylation. The novel activity of Endo-F2 and Endo-F3 was successfully applied for a highly convergent chemoenzymatic synthesis of a full-sized CD52 glycopeptide antigen carrying both terminal sialic acid and core fucose.¹⁸¹ This is the first report on endoglycosidases that shows the ability to glycosylate α -1,6-fucosylated GlcNAc derivatives to form natural core-fucosylated glycopeptides. Very recently, the same group described the discovery of glycosynthase mutants derived from Endo-F3 of the GH18 family, which are devoid of the inherent hydrolytic activity but are able to take glycan oxazolines for transglycosylation. Notably, the Endo-F3 D165A and D165Q mutants demonstrated high acceptor substrate specificity toward α -1,6-fucosyl-GlcNAc-polypeptide in transglycosylation, enabling a highly convergent synthesis of core-fucosylated, complex CD52 glycopeptide antigen (Scheme 10).¹⁷⁵ The Endo-F3 mutants were able to use both bi- and tri-antennary glycan oxazolines as substrates for transglycosylation, in contrast to previously reported endoglycosidases derived from Endo-S, Endo-M, Endo-D, and Endo-A mutants that could not recognize tri-antennary *N*-glycans. The Endo-F3 mutants represent the first endoglycosidase-based glycosynthases capable of transferring tri-antennary complex *N*-glycans.

As another notable example, Wang and coworkers reported the design, synthesis, and antigenic evaluation of new cyclic V1V2 glycopeptides carrying defined *N*-linked glycans at the conserved glycosylation sites (Asn160 and Asn156 or Asn173) derived from gp120 of two HIV-1 strains (CAP45 and ZM109).¹⁸² Using the endoglycosynthase-catalyzed approach, the group synthesized more than 25 homogeneous glycopeptides to probe the nature of the neutralizing epitopes.¹⁸² For the synthesis of the V1V2 glycopeptides carrying one *N*-glycans, a precursor GlcNAc peptide was prepared by automated solid-phase peptide synthesis (SPPS) by installing a GlcNAc moiety at the predetermined site. Then various *N*-glycans were ligated to the glycosylation sites by the glycosynthase-catalyzed transglycosylation using respective glycan oxazolines, leading to a quick assembly of a glycopeptide library (Scheme 11).

To synthesize the V1V2 glycopeptides carrying two different *N*-glycans, the GlcNAc primers were first installed at the N160 and N173 sites by SPPS, and then transglycosylation was controlled to give two monoglycosylated intermediates, which were separated by HPLC. Subsequent enzymatic glycosylation of the free GlcNAc at the second site of the

intermediates with a different glycan gave the glycopeptides carrying two distinct *N*-glycans (Scheme 12). While the method enabled the synthesis of doubly glycosylated glycopeptides, the HPLC separation of the monoglycosylated intermediates was still tedious. A new synthetic method capable of distinguishing among different glycosylation sites in enzymatic transglycosylation is thus required. SPR and ELISA binding analysis indicate that both the glycan structure and the glycosylation site in the right context of the polypeptide are important for antibody recognition. These studies confirmed the importance of a Man₅GlcNAc₂ glycan at N160 for recognition by PG9 and PG16 and further revealed a critical role of a sialylated *N*-glycan at the N156 or N173 site for high-affinity binding, which was not revealed by the original PG9 structural study.¹⁸³ In addition to defining the glycan specificities of the broadly neutralizing antibodies (PG9 and PG16), the identified synthetic glycopeptides provide a valuable template for HIV-1 vaccine design.

In addition to glycosylation remodeling of glycoproteins such as the synthesis of ribonuclease B glycoforms, the glycosynthase approach is also particularly useful for total glycoprotein synthesis when coupled with native chemical ligation. Nakahara and coworkers recently reported the total chemoenzymatic synthesis of saposin C, a hydrophobic glycoprotein.¹⁸⁴ This impressive work included the solid-phase peptide synthesis coupled with native chemical ligation to make the GlcNAc-containing saposin C protein backbone and its folding, the chemical synthesis of a complex *N*-glycan and its conversion into the corresponding glycan oxazoline, and finally the enzymatic transfer of the glycan to the GlcNAc-saposin C by the glycosynthase mutant (Endo-M N175Q) to give the full-size glycosylated saposin C (Scheme 13). This chemoenzymatic approach should be equally applicable for the construction of other biologically interesting *N*-glycoproteins. For glycoprotein synthesis via the ENGase-catalyzed transglycosylation, a key precursor is the GlcNAc-containing protein. While GlcNAc protein can be prepared by total chemical synthesis incorporating native chemical ligation as described above for saposin C synthesis, it would be highly desirable if an *E. coli* system, biotechnology's working horse for protein expression, could be explored for making GlcNAc-proteins. However, *E. coli* usually cannot produce glycoproteins as it lacks the protein *N*-glycosylation machinery. In 2010, Wang, Aebi, and coworkers reported a method for producing homogeneous glycoproteins with humanized glycosylation by a combination of novel *E. coli* glycoprotein expression and in vitro glycosylation remodeling.¹⁸⁵ The method involves glycosylation pathway engineering and functional transfer of the *Campylobacter jejuni* glycosylation machinery¹⁸⁶ in *E. coli* to express a glycoprotein, in which the Asn-linked monosaccharide bacillosamine in the Asn-linked bacterial *N*-glycan was changed to the eukaryotic GlcNAc moiety. This was achieved by deleting the genes involved for the biosynthesis (*pglD*, *pglE*, and *pglF*) and transfer (*pglC*) of bacillosamine and by taking advantage of the *E. coli* existing biosynthetic pathway for making the GlcNAc-diphosphate-lipid precursor. Fortunately, the *C. jejuni* oligosaccharyl transferase *pglB* was able to use the resulting GlcNAc-containing glycolipid as substrate to glycosylate the target protein. Then the bacterial *N*-glycans were trimmed down to the key GlcNAc protein. Finally eukaryotic *N*-glycans of high-mannose type or complex type were introduced by the enzymatic transglycosylation to fulfill the homogeneous eukaryotic glycoproteins (Scheme 14). This method was applied for making glycosylated human IgG-Fc CH2 domain and a single-chain antibody. This method

combines the convenience of protein expression in *E. coli* with the flexibility of in vitro chemoenzymatic glycan remodeling and therefore provides a potentially general platform for producing eukaryotic *N*-glycoproteins. Future studies should be directed to enhancing the efficiency of glycosylation of biomedically important glycoproteins through optimizing this engineered *E. coli* expression system.

3.4 ENGase-Catalyzed Transglycosylation for Glycosylation Remodeling of Therapeutic Monoclonal Antibodies

Monoclonal antibodies (mAbs) of the IgG type are an important class of therapeutic proteins used for the treatment of cancer, autoimmune, and infectious diseases. Antibody glycosylation, in particular, the fine structures of Fc *N*-glycans, can modulate the effector functions of IgG antibodies.^{7,8} However, controlling the glycosylation patterns during recombinant production is still a challenging task. In 2008, Wang and coworkers reported the first example of glycosylation remodeling of a recombinant human Fc domain by the endoglycosidase-catalyzed transglycosylation.¹⁸⁷ The group first expressed the human IgG1-Fc in yeast *Pichia pastoris*. The large heterogeneous high-mannose-type *N*-glycans were then removed by Endo-H to leave only the innermost GlcNAc residue at the Asn-297 glycosylation site, giving the GlcNAc-Fc homodimer. Finally a preassembled glycan oxazoline was transferred by Endo-A to the GlcNAc-Fc to produce the desired homogeneous glycoform of IgG-Fc. An important finding was that the Endo-A-catalyzed transglycosylation could proceed smoothly on the GlcNAc residue at the C_H2 domain of the Fc homodimer without the need of denaturing the IgG-Fc protein.¹⁸⁷ Thus the structural integrity of the IgG-Fc was preserved. Based on this proof-of-concept preliminary study, they have further shown that various truncated and modified *N*-glycans could be attached to the Fc domain to probe the effects of *N*-glycan core structures on the binding of Fc to FcγIIIa receptor, which correspond to the antibody's ADCC activity.^{172,188} In 2012, the same research group reported the first Fc glycosylation remodeling of an intact therapeutic mAb, rituximab.¹⁷⁴ This was enabled by the discovery of two Fc-specific glycosynthase mutants (Endo-S D233A and D233Q) by site-directed mutagenesis of an endoglycosidase from *S. pyogenes*. It was found that wild-type Endo-S could efficiently deglycosylate rituximab under physiological conditions, and the Endo-S glycosynthase mutants were capable of efficiently transferring predefined *N*-glycans from corresponding glycan oxazolines to the Fc-deglycosylated intact IgGs under mild conditions without product hydrolysis (Scheme 15). By this approach, a variety of homogeneous glycoforms, including the sialylated complex type, nonfucosylated complex type, and azide-tagged glycoforms, could be synthesized efficiently. Several research groups have applied this method to synthesize specific glycoforms of antibodies for functional studies.^{189–192} More recently, Wang and coworkers reported glycosynthase mutants from Endo-F3, another GH18 family endoglycosidase.¹⁷⁵ They demonstrated that the D165A mutant of Endo-F3 was highly specific for core-fucosylated *N*-glycan and was able to transfer tri-antennary *N*-glycan to the Fc domain of the intact antibody (Scheme 15). This was the first glycosynthase capable of transferring highly branched *N*-glycans. It is expected that the field will continue to develop with discoveries of new and more potent glycosynthase mutants. The endoglycosidase-catalyzed glycosylation remodeling system is emerging as a general platform for glycoengineering of antibodies for both functional studies and biomedical applications.

4. ENDOGLYCOSIDASES FOR THE SYNTHESIS OF NEOGLYCOLIPIDS AND GLYCOSPHINGOLIPIDS

4.1 Ceramide Glycanase-Catalyzed Transglycosylation for Glycolipid Synthesis

Glycosphingolipids play essential roles in a number of biological processes and have demonstrated great potential as therapeutic agents for the treatment of human diseases. But because of the heterogeneity from the sugar and lipid components, it has been hitherto difficult to obtain pure glycosphingolipids from natural sources or by chemical synthesis. In 1991, Li and coworkers reported that ceramide glycanase (CGase), a class of endoglycosidases capable of cleaving the glycosidic linkage between oligosaccharides and ceramides in various glycosphingolipids, possessed transglycosylation activities and was capable of transferring the released oligosaccharide to various simple alcohols and ceramide derivatives to form neoglycolipids.¹⁹³ It was shown that the CGase isolated from the leech *Macrobdella decora* was able to transfer the intact II3NeuAcGgOse4 from GM1 to 4-phenyl-1-butanol, 1,8-octanediol, and various 1-alkanols having a chain length of six or more carbons (Scheme 16). In 2001, Ito and coworkers reported that the endoglycoceramidase isolated from jellyfish *Cyanea nozakii* could catalyze both transglycosylation and reverse hydrolysis reactions.¹⁹⁴ It was found that this enzyme transferred oligosaccharides from various glycosphingolipids to simple alcohols as well as to the NBD ceramide, a fluorescent ceramide, to yield simple glycolipids or NBD-labeled glycosphingolipids. Moreover, the enzyme was also shown to catalyze the reverse hydrolysis reaction, as demonstrated by the enzymatic condensation of lactose and ceramide to give β -lactosylceramide, indicating the potential of this type of endoglycosidase for synthesizing various glycolipids.

4.2 Endoglycoceramidase-Based Glycosynthase for the Synthesis of Glycosphingolipids

Despite the discovery of the novel transglycosylation properties of ceramide glycanase or endoglycoceramidase, the efficiency of the enzymatic synthesis using the wild-type enzyme is moderate, and the glycolipid product is also subject to hydrolysis by the enzyme. Withers and coworkers reported the first glycosynthase of endoglycoceramidase by initial engineering and later directed the evolution of the endoglycoceramidase II (EGC II) from *Rhodococcus* sp. II.^{195,196} First, the residue E351 was identified as the potential catalytic nucleophile in EGCII based on sequence alignments of EGCII with other Family 5 glycosyl hydrolases to which the EGCII was assigned. Mutation of E351 with other residues such as Ser, Ala, Gly, and Asp resulted in the loss of EGC II-catalyzed hydrolysis activity, supporting the postulated role of E351 in the catalysis mechanism. These mutants were then tested for glycosynthase activity using synthetic 3'-O-sialyl- α -D-lactosyl fluoride as the donor and D-erythro-sphingosine as the acceptor. The E351S mutant was found to be an excellent glycosynthase, promoting the formation of the corresponding lyso-GM3 ganglioside product in over 90% yield (Scheme 17). It was further demonstrated that the glycosynthase mutants are promiscuous to accept a range of substrates varied on the sugar (donor substrate) and lipid (acceptor substrate) portions for transglycosylation, thus providing a potentially powerful tool for the synthesis of various glycosphingolipids. Despite the substrate promiscuity, however, the first-generation catalyst has shown a very

low activity with 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. A single-crystal structure of EGCII revealed a narrow, hydrophobic pocket for binding to the ceramide lipid, thus explaining the narrow lipid specificity of the wild-type enzyme.¹⁹⁷ To overcome this limitation, Withers and coworkers in 2007 made significant progress in expanding the synthetic scope of the EGCII-based glycosynthase through directed evolution.¹⁹⁶ An elegant ELISA-based assay was developed to screen the EGC mutant libraries, and novel glycosynthases with dramatically enhanced phytosphingosine activity and improved catalytic efficiency were discovered.¹⁹⁶ EGCII D314Y, a single-point mutant, showed almost the same k_{cat}/K_m value for phytosphingosine as that for sphingosine, suggesting a dramatic enhanced activity of the enzyme for this special lipid acceptor (Scheme 17). Structural analysis implicates that the D314Y mutation might result in the perturbation of the lipid-recognizing hydrophobic tunnel, expanding its substrate specificity. Moreover, a number of other mutants, including some double and triple mutants, were identified that showed better activity with the phytosphingosine and natural sphingosine lipid acceptor than the original mutant E351S. This study showcases the power of directed evolution in improving on glycosynthase activity. More recently, Withers and coworkers have shown that combination of glycosyltransferase-catalyzed manipulations of the oligosaccharide structures and the glycosynthase-catalyzed oligosaccharide transfer would be able to further expand the scope of the endoglycosidase-catalyzed synthesis of a large variety of glycosphingolipids.^{198,199} The new EGC-based glycosynthases are expected to find wide applications for the synthesis and functional studies of various glycosphingolipids.

5. CONCLUDING REMARKS

The endoglycosidase-catalyzed transglycosylation has emerged as a powerful method for making complex oligosaccharides, polysaccharides, and glycoconjugates. A unique feature of this approach is the block transfer of an entire oligosaccharide moiety in a given step. The invention of glycosynthases that are devoid of product hydrolysis activity but are capable of taking an appropriate glycosyl fluoride or sugar oxazoline as the activated substrate for transglycosylation represents a truly conceptual breakthrough in this field. Moreover, many endoglycosidases demonstrate remarkably relaxed substrate specificity, making it possible to construct not only naturally existing carbohydrates but also selectively modified and/or specially functionalized polysaccharides and glycoconjugates. It is expected that continuing studies in this field will lead to the discovery of an expanding repertoire of endoglycosidases and related glycosynthases that enable new transglycosylation reactions and possess remarkably enhanced catalytic efficiencies. The potential of the endoglycosidase-catalyzed transglycosylation for the synthesis of complex oligosaccharides, polysaccharides, and glycoconjugates such as glycoproteins has just begun to unfold. These structurally well-defined, homogeneous glycans and glycoconjugates are highly valuable for functional glycomics studies and for biomedical applications as well.

ACKNOWLEDGMENT

This work was supported by the National Institutes of Health (NIH Grant No. R01 GM080374).

ABBREVIATIONS

ADCC	antibody-dependent cell-mediated cytotoxicity
Ch	chondroitin
ChS	chondroitin sulfate
DP	degree of polymerization
DS	dermatan sulfate
ENGase	endo- β - <i>N</i> -acetylglucosaminidase
Fab	antigen-binding region
Fc	fragment crystallizable region
Fmoc	fluorenylmethyloxycarbonyl
G0F	asialo-, agalacto-, core-fucosylated bi-antennary complex-type <i>N</i> -glycan
GAGs	glycosaminoglycans
Gal	galactose
GH	glycoside hydrolase
Glc	glucose
GlcA	glucuronic acid
GlcNAc	<i>N</i> -acetylglucosamine
Glycan-ox	<i>N</i> -glycan oxazoline donor
HA	hyaluronan
HAase	hyaluronidase
HS	eparan sulfate
IgG	immunoglobulin G
Man	mannose
Man9	high-mannose-type glycan
RNase B	ribonuclease B
S2G2F	fully sialylated core-fucosylated bi-antennary complex-type <i>N</i> -glycan
SCT	sialylated complex-type glycan
SGP	sialylglycopeptide

SPPS	solid-phase peptide synthesis
UDP	uridine diphosphate
Xyl	xylose

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Scheme 1.

Cellulase-catalyzed synthesis of artificial cellulose.

Scheme 2.

Xyloglucan glycosynthase-promoted synthesis of artificial cellulose derivatives.

a	R ¹ = H	R ² = OH	R ³ = NHCOCH ₃	R ⁴ = H	R ⁵ = OH
b	R ¹ = H	R ² = OH	R ³ = OH	R ⁴ = H	R ⁵ = OH
c	R ¹ = CH ₃	R ² = OH	R ³ = NHCOCH ₃	R ⁴ = H	R ⁵ = OH
d	R ¹ = H	R ² = OH	R ³ = NHCOCH ₃	R ⁴ = CH ₃	R ⁵ = OH
e	R ¹ = CH ₃	R ² = OH	R ³ = NHCOCH ₃	R ⁴ = CH ₃	R ⁵ = OH
f	R ¹ = H	R ² = OH	R ³ = NHCOCH ₃	R ⁴ = H	R ⁵ = OCH ₂ COONa
g	R ¹ = H	R ² = OH	R ³ = NH ₂	R ⁴ = H	R ⁵ = OH
h	R ¹ = H	R ² = F	R ³ = NHCOCH ₃	R ⁴ = H	R ⁵ = OH
i	R ¹ = H	R ² = OH	R ³ = NHCOCH ₃	R ⁴ = H	R ⁵ = F
j	R ¹ = H	R ² = F	R ³ = NHCOCH ₃	R ⁴ = H	R ⁵ = F
k	R ¹ = H	R ² = OH	R ³ = NHCOCH ₃	R ⁴ = H	R ⁵ = H
l	R ¹ = H	R ² = OH	R ³ = NHSO ₃ Na	R ⁴ = H	R ⁵ = OH
m	R ¹ = H	R ² = OH	R ³ = <i>N</i> -phthaloyl	R ⁴ = H	R ⁵ = OH

Scheme 3.

Chitinase-catalyzed construction of chitins and their derivatives.

a	$R^1 = \text{CH}_3$	$R^2 = \text{H}$	$R^3 = \text{OH}$	h	$R^1 = \text{CH}_3$	$R^2 = \text{OH}$	$R^3 = \text{H}$
b	$R^1 = \text{CH}_2\text{CH}_3$	$R^2 = \text{H}$	$R^3 = \text{OH}$	i	$R^1 = \text{CH}_2\text{CH}_3$	$R^2 = \text{OH}$	$R^3 = \text{H}$
c	$R^1 = (\text{CH}_2)_2\text{CH}_3$	$R^2 = \text{H}$	$R^3 = \text{OH}$	j	$R^1 = (\text{CH}_2)_2\text{CH}_3$	$R^2 = \text{OH}$	$R^3 = \text{H}$
d	$R^1 = \text{CH}(\text{CH}_3)_2$	$R^2 = \text{H}$	$R^3 = \text{OH}$	k	$R^1 = \text{CH}(\text{CH}_3)_2$	$R^2 = \text{OH}$	$R^3 = \text{H}$
e	$R^1 = \text{Ph}$	$R^2 = \text{H}$	$R^3 = \text{OH}$	l	$R^1 = \text{Ph}$	$R^2 = \text{OH}$	$R^3 = \text{H}$
f	$R^1 = \text{CH}=\text{CH}_2$	$R^2 = \text{H}$	$R^3 = \text{OH}$	m	$R^1 = \text{CH}=\text{CH}_2$	$R^2 = \text{OH}$	$R^3 = \text{H}$
g	$R^1 = \text{C}(\text{CH}_3)=\text{CH}_2$	$R^2 = \text{H}$	$R^3 = \text{OH}$	n	$R^1 = \text{CH}_3$	$R^2 = \text{OSO}_3^-$	$R^3 = \text{H}$

Scheme 4.

Hyaluronidase-catalyzed construction of hyaluronan, chondroitin, and their derivatives.

Scheme 5.
Hyaluronidase-catalyzed copolymerizations.

Scheme 6.

Endo- β -xylosidase-catalyzed construction of a peptidoglycan.

Scheme 7.

Sugar oxazolines for ENGase-catalyzed synthesis of N-glycopeptides.

Scheme 8.

Chemoenzymatic synthesis of homogeneous glycoproteins using ENGases and sugar oxazolines.

Scheme 9.

Chemoenzymatic synthesis of homogenous glycoforms ribonuclease B by ENGase-derived glycosynthases.

Scheme 10.
Chemoenzymatic synthesis of CD52 glycopeptide antigens.

Scheme 11.

Chemoenzymatic synthesis of ZM109 V1V2 glycopeptides carrying defined *N*-glycans.

Scheme 12.
Chemoenzymatic synthesis of HIV-1 glycopeptides carrying two different *N*-glycans.

Scheme 13.
Chemoenzymatic synthesis of hydrophobic glycoprotein saposin C.

Scheme 14.
Protein expression and glycosylation-engineered system.

Scheme 15.
Glycosylation remodeling of therapeutic monoclonal antibodies.

Scheme 16.
Ceramide glycanase-catalyzed synthesis of neoglycolipids.

Scheme 17.

Glycosynthase-catalyzed synthesis of glycosphingolipids.