CHEMICAL REVIEWS

REVIEW

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Enzymes in the Synthesis of Glycoconjugates

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CONTENTS

1. INTRODUCTION

1.1. Aims

This review is intended to update our previous discussion on the chemoenzymatic synthesis of carbohydrates and glycoconjugates,[1] with new emphasis placed on enzymatic and whole-cell engineering techniques for the production of homogeneous glycoproteins. Glycans, the carbohydrate portion of glycoconjugates, have diverse structures and far-reaching biological functions that remain to be elucidated. The need to understand glycans and glycoconjugates has motivated an extraordinary amount of work toward developing synthetic and

Special Issue: 2011 Enzymes in Synthesis

Received: April 8, 2011
Published: July 13, 2011

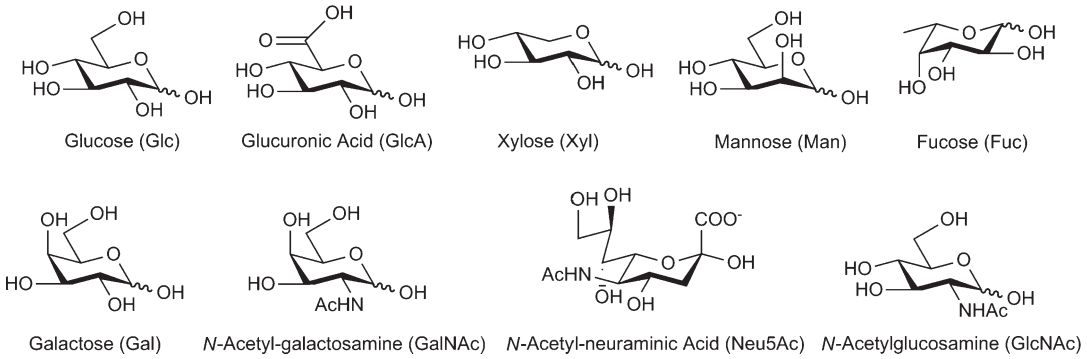


Figure 1. Biologically relevant monosaccharide subunits.

chemoenzymatic methods to produce them in a pure form over the past ten years. While many of these routes have been the subject of focused reviews,[2−25] this account provides a thorough resource of the chemoenzymatic and whole-cell tools that are available for producing glycans and glycoconjugates with defined structures. Because the covered topics are broad, succinct discussions of key concepts are provided in the text along with a few representative examples, while more in-depth consideration of subjects can be gained by consulting the extensive sources to the primary literature provided throughout.

1.2. Glycoconjugate Diversity and the Need for Homogeneous Glycoproteins

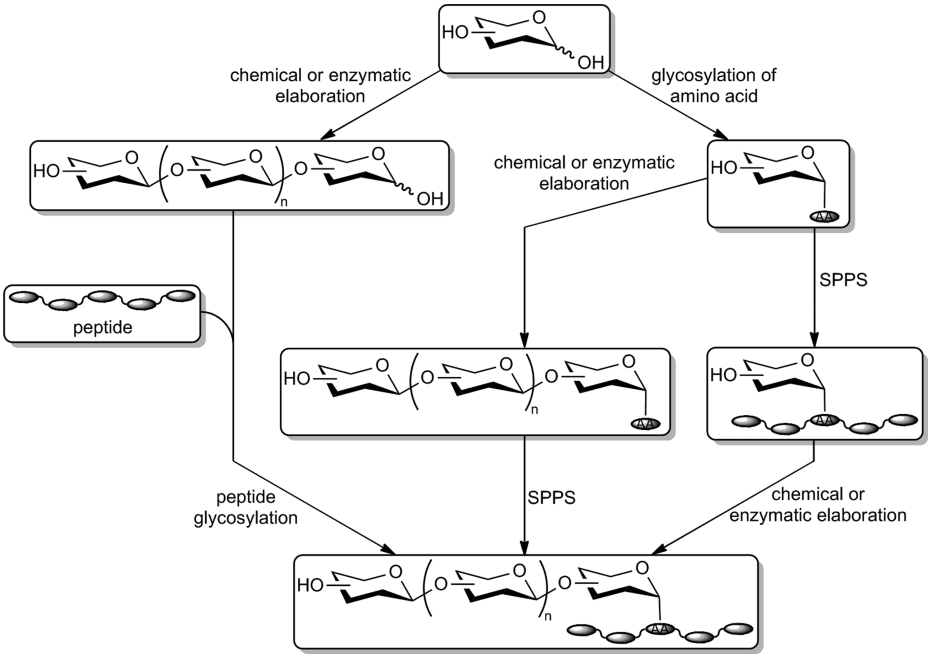
Carbohydrates are the most abundant and structurally diverse class of biomolecules. They are essential to all forms of life and play key roles in varied cellular processes, from modulating membrane structure, hydration, and stability, to governing intercellular events of recognition, adhesion, and communication.[26−34] Carbohydrates abound on all cell surfaces and in extracellular matrices, as free polysaccharides and as constituents of glycoconjugates (i.e., small molecules, lipids, and proteins appended with glycan structures). The functional significance of cell-surface glycans is underscored by dramatic shifts in their composition associated with cellular transformations, like differentiation, infection, and oncogenesis.[35,36] Glycans are co- or post-translationally appended to the majority of human proteins, a process known as glycosylation, which greatly augments the structure and function of the proteome.[6] Conserved glycan structures are actively involved in the early phases of protein biosynthesis, where they aid in folding and cellular trafficking.[37,38] Mature glycans have diverse structures and functions that remain challenging to characterize and classify. These challenges are compounded because functional glycoprotein populations are composed of glycoforms, or an invariant protein sequence bearing many different glycans of significant structural variability, also termed glycan heterogeneity. Still, it has become increasingly clear that the carbohydrate moieties can tune activities and govern specific tasks, both intrinsically, by modulating the properties of the protein to which they are attached, and extrinsically, by modulating interactions with other biomolecules; they are able to do so with remarkable control by virtue of their structural diversity.[39] Glycan diversity may explain human complexity in light of a paradoxically small human genome;[40] and the structural divergence of glycans may be key to organismal evolution and speciation.[40−43]

The chemical and biological factors that govern glycan diversity pose significant challenges to the isolation, structural characterization, and chemical synthesis of carbohydrate-containing molecules. From a chemical standpoint, the structural diversity of glycans arises from the vast theoretical potential presented by the various regio- and stereochemical linkage combinations between the nine common monosaccharide building blocks in mammalian cells (Figure 1).[44] These chemical factors cannot be easily controlled, and they greatly complicate synthesis by the automated and iterative strategies that are easily accessible to linear nucleic acids and peptides. From a biological standpoint, the dynamic and diverse glycan structures needed for cellular function arise because carbohydrate structures are not genetically encoded or synthesized in a templated process.[45] Indeed, an array of over 250 enzymes in the human secretory pathway support glycan synthesis and processing, including a suite of glycosyltransferases (GTases), which add sugars using activated sugar donors, and glycosides, which cleave them.[41,46] In this network, competing or overlapping substrate/donor specificities, substrate availability, and varying levels of enzyme expression, activation, and localization along the secretory pathway all contribute to functionally significant glycan heterogeneity.[45] Glycoconjugate glycosylation can also be established through multiple kinds of linkages (most commonly, N- and O-linked); this process is also nontemplated, and occupancy of glycosylation sites cannot always be predicted or controlled.[47] Thus, glycoconjugates obtained from cellular sources are composed of significant heterogeneity from which the isolation of homogeneous forms is often impractical, if not impossible. For example, one glycoprotein can have hundreds of glycoforms, characterized not only by different glycan structures but also by different glycosylation sites, different glycan-protein linkages, and varying degrees of glycosylation site occupancy.[48] Such heterogeneous mixtures are inadequate for many purposes, including the important task of establishing how the molecular architectures of glycans convey specific biological properties (i.e., establishing glycan-related structure−activity relationships, gSARs).

Access to homogeneous glycoproteins is necessary to first determine the molecular details of a glycan's function and then to produce those glycoforms conveying desired properties. Which glycoforms are needed and how they can be accessed will depend on the stage of research and/or application. In initial glycoprotein studies, simplified glycans are often required to overcome the heterogeneity that disrupts the establishment of gSARs and the determination of X-ray crystal structures.[49,50] Bench-scale quantities of simplified glycoforms can be accessed using enzymatic trimming of recombinantly

dx.doi.org/10.1021/cr200113w |*Chem. Rev.* 2011, 111, 4259–4307

Scheme 1. Strategies for Glycoprotein Synthesis



expressed or commercially available glycoproteins; however, more complex homogeneous glycoforms may require more sophisticated chemoenzymatic manipulation of glycoproteins and/or de novo synthesis of them, as covered herein. The establishment of gSARs using these bench-scale techniques has been, and will continue to be, critical to the identification of glycosylation patterns and/or glycoforms with distinct properties, such as enhanced glycoprotein stability,[51−53] altered binding or immunogenic properties,[32,54] and increased therapeutic efficacy,[55−58] to name a few. The identification of desired glycoforms will spur large-scale production needs. This is especially pertinent for biologic glycoforms (e.g., glycoproteins with increased α2-3 sialylation of *N*-glycans have longer serum half-lives,[59,60] and monoclonal antibodies lacking core α1,6-fucosylation on an *N*-glycan in a conserved Fc region have more therapeutic antibody-dependent cellular cytotoxicity, or ADCC).[61,62] Unfortunately, recombinant expression in mammalian cells is the only practical method for the large-scale production of glycoproteins, and this process results in heterogeneity that does not maximize glycoform efficacy.[63] Viable routes to targeted human glycoforms by recombinant means will require advances in the glycoengineering of cellular glycosylation pathways in whole cells, a field that is quickly emerging. Thus, synthetic, chemoenzymatic, and whole-cell routes to glycans/glycoconjugates/glycoproteins are key to the ongoing process of understanding glycan function and producing active glycoforms.

1.3. Overview of the Synthesis of Glycopeptides and Glycoproteins

Because access to homogeneous glycoproteins is essential, a tremendous amount of work has emerged for preparing them using de novo synthesis, chemoenzymatic remodeling, and whole-cell glycoengineering. Each route has strengths and weaknesses that must be considered for meeting a particular need for a homogeneous glycoform. De novo glycoprotein synthesis is practical for bench-scale research and has the advantage of precise chemical control of many aspects of glycoprotein structures, including the site-specific incorporation of the glycan into the peptide component (section 2); the synthesis of the peptide backbone, including methods to ligate together small, chemically accessible fragments into large protein fragments (section 3); and, finally, the synthesis of the glycan, which poses a large challenge due to the precise regio- and stereochemical control needed to create glycan structures (section 4). Fortunately, enzymatic steps can be combined with de novo chemical approaches at various points to facilitate the process, especially with regard to glycan synthesis. Enzymes can also be employed to create homogeneous glycans by remodeling heterogeneous glycoprotein populations from natural sources, as showcased with RNase (section 1.4) and discussed in more detail in section 4. Importantly, enzymes are amenable to industrial-scale needs for glycan synthesis.[64] Finally, although cellular production of glycoconjugates is the source of troublesome glycoforms, recombinant expression represents the most efficient and cost-effective route for their production. In section 5, glycoengineering efforts are presented that seek to harness cellular machinery and orchestrate controlled synthesis of glycan structures.

De novo glycoprotein synthesis can be broken down into the synthesis of glycopeptide fragments that can be tackled by several synthetic strategies. To obtain a glycopeptide, there are various ways in which a glycan can be incorporated into the peptide, the peptide can be elongated, and the glycan can be elongated, as shown in Scheme 1. Factors such as the number, position, and type of appended glycans within the glycopeptide will dictate which approach should be selected for a particular target glycoprotein. The most prevalent regioselective strategy is to incorporate a glycosyl amino acid at a specific point in a solid-phase peptide synthesis (SPPS).[65−68] This strategy is effective for the synthesis of O-linked and N-linked glycopeptides; however, glycan size and stability must be carefully considered.

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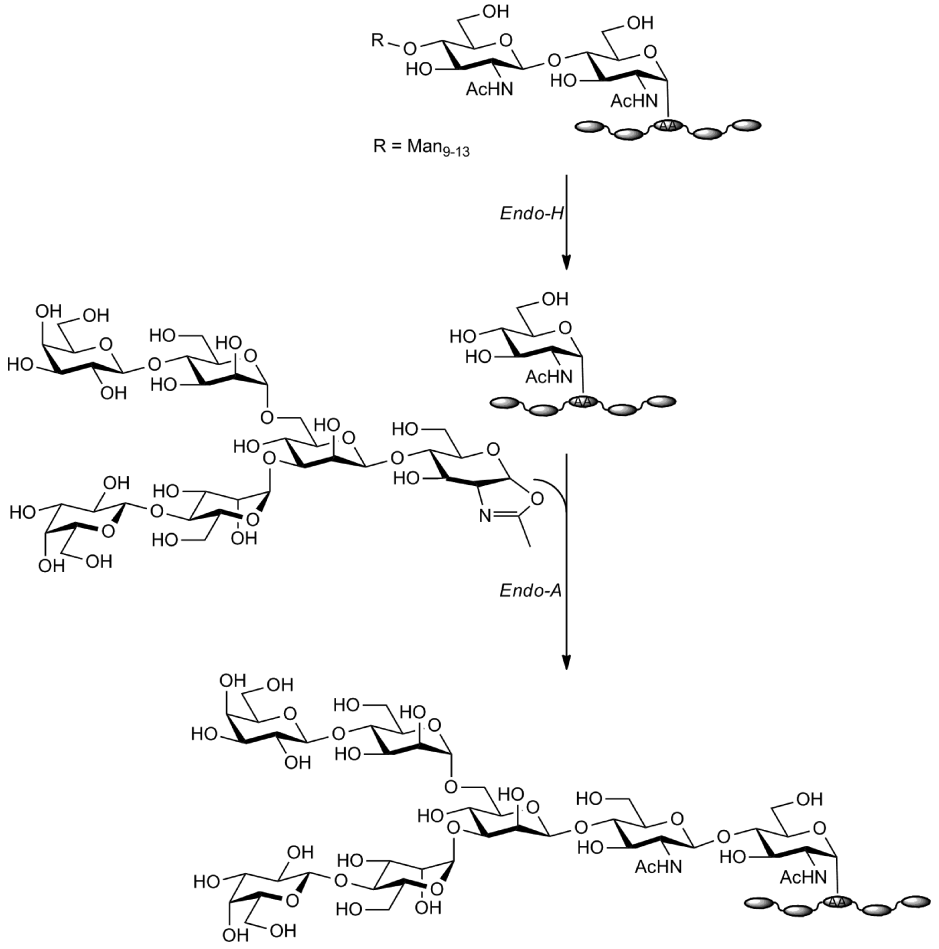
Scheme 2. ENGase-Catalyzed Glycan Remodeling



An impressive example of this technique is the synthesis of WW domain of human Pin 1 variants bearing an N-linked GlcNAc residue in order to study the effects of glycosylation on protein folding energetics.[52,53] A convergent method for site-specific chemical N-glycosylation is the Lansbury aspartylation, which couples a free glycosylamine and an activated aspartyl amino acid in the peptide (Scheme 1).[69] A full sized *N*-glycan structure can be introduced into a peptide at a late stage using this powerful method; however, coupling efficiency and regioselectivity must be carefully considered.[70,71] The synthesis of prostate specific antigen, envelope glycoprotein gp120 (gp120), and β-human follicle stimulating hormone demonstrate the utility of this aspartylation.[72−77] Emerging technologies for glycopeptide synthesis and their ligation into larger proteins have increased the number of synthetically accessible glycoproteins, although the majority still remain synthetically elusive. The challenge of glycoprotein synthesis is highlighted by the fact that the first de novo synthesis of a native N-linked glycoprotein was only realized in 2008, when Yamamoto and co-workers combined SPPS[78] and native chemical ligation (NCL)[79] to synthesize monocyte chemotactic protein-3, which is composed of 76 amino acids and one N-glycosylation site.[80]

For the controlled synthesis of glycans, enzymatic, chemoenzymatic, and whole-cell glycoengineering methods provide attractive options. Enzymes have exquisite regio- and stereoselectivity and can cleave specific linkages (i.e., glycosidases) and synthesize specific bonds (i.e., GTases and glycosynthases) under mild conditions in vivo and in vitro, without the complicated protecting groups required in purely synthetic endeavors. Enzymes can be used to elaborate synthetic peptide products at various stages, including on glycosyl amino acid building blocks and larger glycopeptides (Scheme 1). In the latter case, incorporation of simple glycosyl amino acids in SPPS combines early-stage regioselectivity with the convenience of introducing larger glycan structures at a later stage when they will not interfere with, or be lost during, synthesis. As an example, Glucagon-like peptide 1 was chemically synthesized and chemoenzymatically elaborated with GTases to extend serum half-life, without negatively affecting the peptide activity.[81] A powerful alternative to de novo synthesis is to use enzymes to remodel glycoproteins from natural sources. Remodeling schemes can enzymatically homogenize diverse protein glycoforms through trimming; the trimmed population can also be enzymatically elaborated to a single glycoform of complex structure (see the RNase example in section 1.4). The Endos (see section 4.2.2) provide a mild, selective way to accomplish the remodeling of *N*-glycans and have even been applied to the challenging synthesis of homogeneous glycans in the Fc-domain of therapeutic antibodies (Scheme 2).[82] Glycomodifying enzymes are most powerful in the biosynthetic pathways of their native cellular environments. As such, glycoengineering efforts seek to intervene, manipulate, and/or augment glycosylation pathways in

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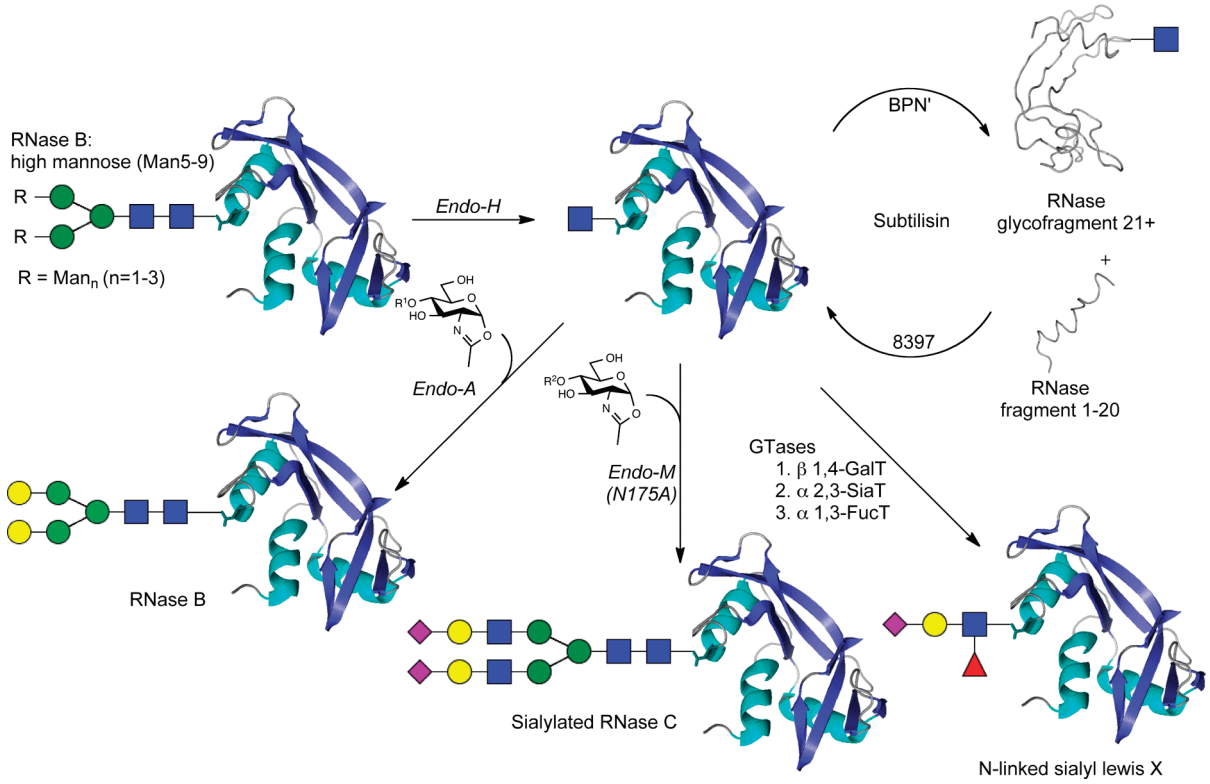
Scheme 3. Enzyme Versatility in RNase Syntheses



whole cells in an effort to customize the synthesis of glycan structures. Promisingly, the introduction of human-type enzymes in the N-glycosylation pathways has been successful in many different eukaryotic systems commonly used for recombinant protein expression, as demonstrated by the production of human therapeutics of controlled glycoform in glycoengineered yeast,[83−86] insect,[87−89] and plant[90−92] systems. Exciting prospects for the future of glycoengineering, including the production of glycosylated proteins in *Escherichia coli*, are presented by diverse glycomodifying enzymes that are now being characterized from very recently discovered protein glycosylation pathways in all kingdoms of life.

1.4. RNase: A Demonstration of Enzyme Potential in Glycoprotein Synthesis

Ribonuclease (RNase) has several naturally occurring forms, including the nonglycosylated form of RNase A, and the two glycosylated forms of RNase B and RNase C, which have a single N-glycosylation site displaying high-mannose and complex populations, respectively. These easily accessed glycoform populations have made RNase an excellent model system to study the purpose of N-linked glycans on glycoproteins. Indeed, RNase has been used in pioneering evaluations of folding, structure, and function, and it was the first model system to definitively show that the glycan components can alter enzyme activity.[93] As the glycoprotein prototype, RNase has also been used to showcase many synthetic and chemoenzymatic remodeling approaches toward homogeneous glycoproteins.[94−97] Scheme 3 shows representative enzymatic applications for the synthesis of homogeneous glycoproteins using RNase. Monoglycosylated RNase, containing a protein proximal GlcNAc residue, can be obtained by Endo-catalyzed trimming of an isolated mixture of RNase B glycoforms (in this case, *endo*-glycosidase H, Endo-H).[94] The homogeneous GlcNAc bearing RNase is a useful intermediate for several re-elaboration approaches. For example, RNase protein fragments generated using Subtilisin-BPN′ digestions can be rejoined, or can be coupled with unnatural or modified protein sequences using Subtilisin 8397 catalyzed ligation.[95] Most frequently, the GlcNAc is elaborated with GTases and glycosidases to realize homogeneous RNase bearing mammalian-type glycoforms. In the first chemoenzymatic synthesis of a novel RNase glycoform, a derivative containing an N-linked sialyl Lewis X (sLe^X) was prepared using iterative GTases.[95] Another useful technique is the enzymatic re-elaboration using Endos in the synthetic direction by supplying synthetically derived oxazoline substrates for transfer. In two powerful examples of homogeneous RNase synthesis, Endo-A was applied to catalyze the transfer of synthetic Gal_2Man_3 oxazoline derivates to afford homogeneous RNase analogue,[94] while an optimized synthetic mutant of Endo-M (Endo-M with N175A) successfully transferred a complex-type glycan to yield a sialylated RNase C derivative.[96,97]

2. REGIOSELECTIVE INTRODUCTION OF CARBOHYDRATES TO SYNTHETIC PEPTIDES

Many biosynthetic products, such as proteins and lipids, are either co- or posttranslationally glycosylated. Enzymatic glycosylation is critical to the proper function of these conjugates, and the structural diversity of the glycan appendages has been well documented in all kingdoms of life.[98,99] The types of carbohydrate−conjugate linkages differ dramatically among cell types.[47] At least eight different amino acids are known to be glycosylated (i.e., arginine [Arg], asparagine [Asn], hydroxylysine [Hyl],

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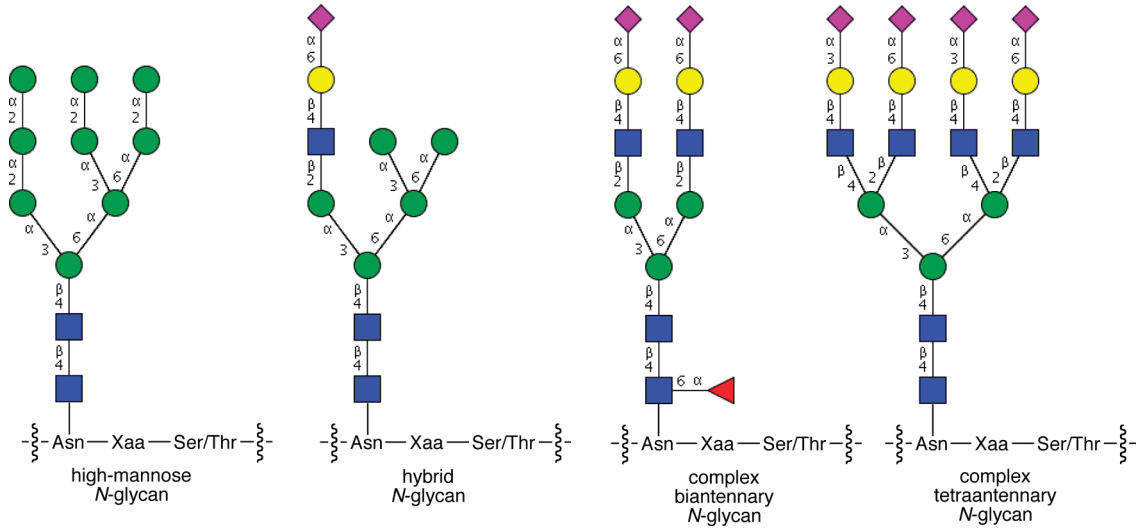


Figure 2. Common *N*-glycan motifs in mammals.

hydroxyproline [Hyp], serine [Ser], threonine [Thr], tryptophan [Trp], and tyrosine [Tyr]) by 13 distinct proximal monosaccharide units (i.e., arabinose [Ara], diactrideoxyhexose, fucose [Fuc], *N*-acetylfucosamine [FucNAc], galactose [Gal], *N*-acetylgalactosamine [GalNAc], glucose [Glc], *N*-acetylglucosamine [GlcNAc], mannose [Man], pseudaminic acid [PSE], rhamnose [Rha], xylose [Xyl], and EthN-6-P-Man), and these conjugate proximal sugars are often further elaborated. Glycoconjugate classes are defined according to their basal chemical linkage, with N-linked and O-linked glycans to amino acids constituting the majority, whereas other notable classes include C- and P-linked glycans.

2.1. N-Linked Glycans

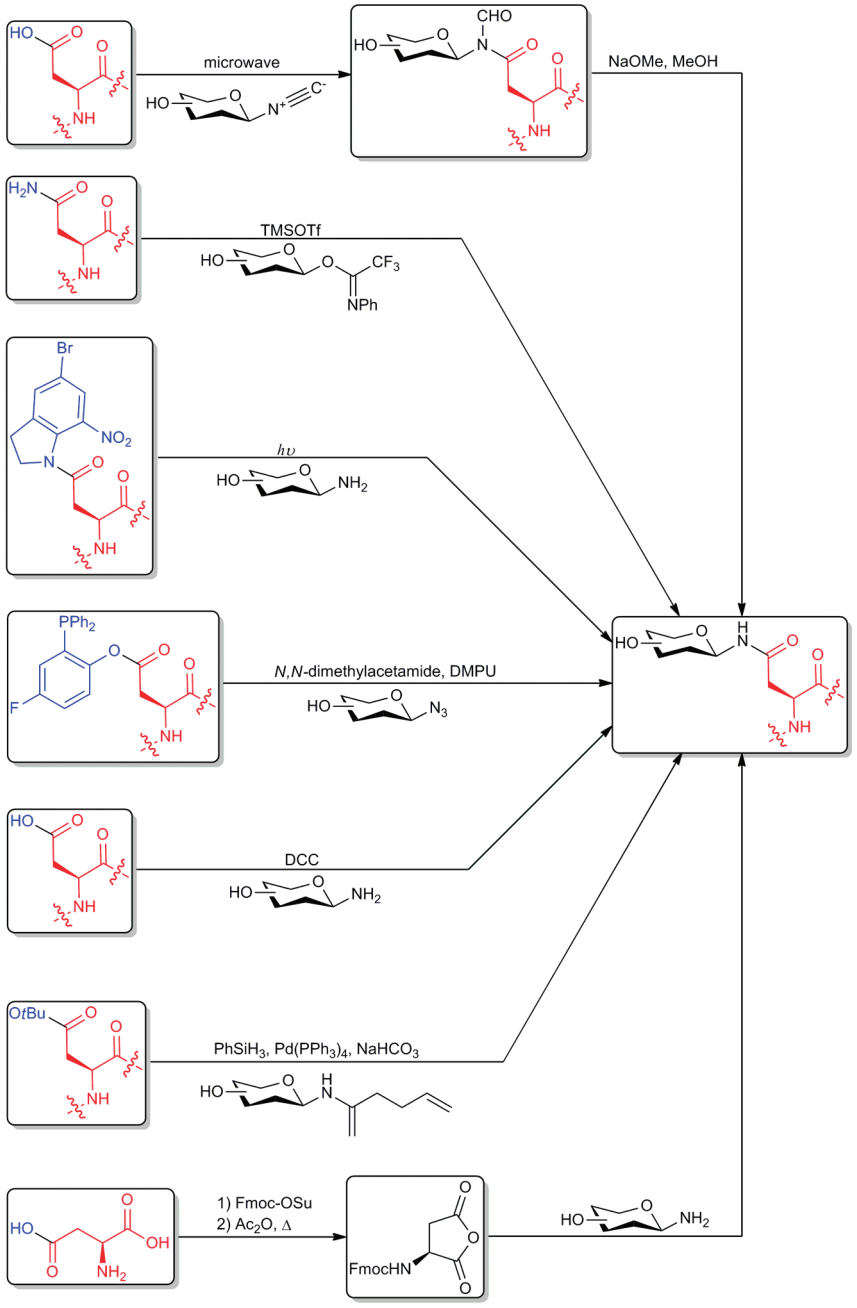
2.1.1. Biosynthesis. In eukaryotes, N-linked glycans are nearly always connected to the side-chain amide nitrogen of Asn residues found within the consensus peptide sequence: Asn-Xaa-Ser/Thr; where Xaa is any amino acid except proline.[33] *N*-Glycans share a common pentasaccharide core, ($Man_3GlcNAc_2$), and variable distal composition that is categorized into the three subtypes of high mannose, hybrid, and complex types (Figure 2). Complex structures can be further categorized as bi-, tri-, and tetra-antennary, which refers to the number of carbohydrate branches originating from the trimannosyl core. Each *N*-glycan derives from a lipid-linked oligosaccharide (LLO) precursor composed of triglucosylated polymannosyl core linked to a dolichol carrier through a diphosphate bond (LLO = $Glc_3Man_9GlcNAc_2$-PP-Dol). The LLO is synthesized in the endoplasmic reticulum (ER) membrane by a family of dedicated GTases, in processes that begin on the cytosolic face and are completed in the ER lumen. In the ER, the LLO is transferred to a nascent polypeptides en bloc by the oligosaccharyltransferase (OST).[33] Initial enzymatic processing involves the iterative removal of three glucose residues by glucosidases I and II, and then recycling of the first glucose by α-glucosyltransferase and glucosidase I, along with trimming of mannose residues; a process that, although not entirely understood, is critical to determining if a protein is properly folded and should be secreted or if it should be targeted for degradation.[33] Properly folded N-linked proteins are exported from the ER and subject to trimming and glycan elaboration by a suite of glycoprocessing enzymes in the Golgi.

Recently, bacterial and archaeal organisms have been identified to harbor protein N-glycosylation pathways.[100−102] These pathways show striking similarities to the eukaryotic system in that they are composed of an OTase that can glycosylate sequons on protein by catalyzing the en bloc transfer of an LLO, which is biosynthetically generated in a separate cellular compartment.[100−102] Although most of these systems remain to be functionally characterized, the bacterial system from *C. jejuni* has been studied in-depth.[103] The bacterial N-glycosylation pathway is greatly simplified, with the entire system encoded by a *pgl* gene cluster that is functionally transportable.[104,105] Key differences in the bacterial system include: (1) the identity of the LLO, which is $GalNAc_2(Glc)GalNAc_3Bac$ (where Bac is bacilosamine); (2) the consensus sequence, which is extended to Asp/Glu-Xaa-Asn-Xaa[2]-Ser/Thr, where Xaa is not proline; (3) the cellular location for glycosylation, which is in the periplasm (LLO is built up in the cytosol); and (4) the nature of the protein substrate, which is a sequon in an unstructured region of folded protein. The discovery that key N-glycosylation steps are conserved throughout all kingdoms of life has further emphasized the biological importance of glycosylation. Furthermore, the discovery of these pathways also has important applications for whole-cell engineering, especially with respect to developing human glycosylation in bacterial cell systems (see section 5).

2.1.2. Chemical Synthesis. Formation of the correct sugar−amino acid linkage in a regio- and stereocontrolled fashion is a critical step in homogeneous glycoprotein synthesis. Regioselective installation of *N*-glycans in peptide synthesis is often accomplished in two ways, both of which are compatible with the introduction of complete glycan structures or the introduction of monosaccharide handles that set the stage for elaboration. One approach establishes the key *N*-glycan linkage by incorporating the requisite glycosyl amino during SPPS; the other establishes the linkage by coupling the glycan amine to an Asp residue on a short peptide sequence. Synthesis of the N-linked glycosyl amino acids is accomplished through amidation of an activated aspartic acid residue,[106,107] although the technique has been expanded to include primary amide glycosylation,[108] phototransamidation,[109] isonitrile-based coupling,[110] and amidation of aspartic anhydrides[111] (Scheme 4). The most

dx.doi.org/10.1021/cr200113w |*Chem. Rev.* 2011, 111, 4259–4307

Scheme 4. *N*-Glycan Installation



common strategy for creating an N-linkage is by simply coupling a glycosyl amine and an activated Asp amino acid.[77,112] This method can be used for an "on-resin" aspartylation, which takes advantage of a 2-phenylisopropyl protecting group on the Asp residue that first suppresses undesired aspartimide formation during SPPS and then can be selectively removed after the peptide is completed.[113] A less reactive functionality, such as a glycosyl azide, can also be used on the glycone and coupled to an Asp residue via Staudinger reaction to minimize product degradation and undesired side reactions.[114,115] This is quite useful for incorporating unprotected sugar substrates into glycoprotein synthesis with a minimal number of protection and deprotection steps.[116,117]

2.2. O-Linked Glycans

2.2.1. Biosynthesis. In mammals, O-linked glycans are added to folded proteins, wherein the addition of a single sugar is catalyzed by specialized protein glycosylating enzymes, and following which glycan structures can be iteratively elongated by GTases. O-Linked glycans are extremely diverse in chemical composition and they have also been found anchored to nearly all hydroxyl-containing amino acids, although they are most typically linked to Ser and Thr residues.[47] The most abundant O-linking sugar is the *O*-GalNAc of mucins, whereas others include the *O*-Fuc found in epidermal growth factor, the *O*-Xyl that anchors glycosaminoglycans, and the cytosolic *O*-GlcNAc modification of proteins.[47] No specific consensus sequence has

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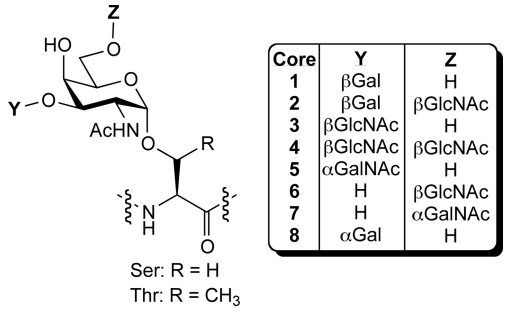
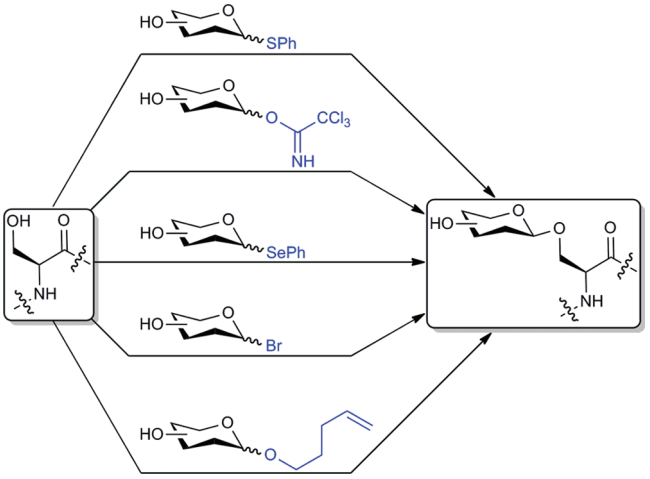


Figure 3. Core *O*-glycan structures.

Scheme 5. *O*-Glycan Installation



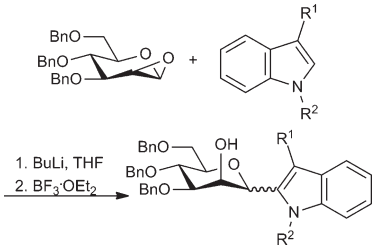
been identified for *O*-glycan glycosylation. In the case of mucins, biosynthesis is controlled by a family of ppGalNAcTs, which catalyze the addition of UDP-GalNAc to either Ser/Thr residue within a characteristic mucin peptide region, often with a proline at the −1 or +3 position.[47] There are eight core mucin structures that are built by dedicated GTases (Figure 3). Notably, there are also sets of GTases that can elaborate both mucin core structures and *N*-glycan structures with common glycan motifs, such as poly-LacNAc extensions and blood group and Lewis antigens.

2.2.2. Chemical Synthesis. Introducing an O-linked monosaccharide at an early stage in de novo O-linked glycoprotein synthesis is imperative, owing to the prevalence of free hydroxyl groups in proteins. This is typically accomplished through the chemical synthesis of small glycosyl amino acids that can be introduced during SPPS. The O-glycosidic bond between a synthetic glycan and a Ser or Thr residue is often achieved through standard carbohydrate couplings between hydroxyl acceptor on the amino acids and glycosyl donors, commonly halogens or trichloroacetimidates (Scheme 5).[118−120] A stable glycosyl donor can greatly increase synthetic adaptability and robust glycosyl donors employed in mucin-type O-linked glycopeptide include *n*-pentenyl-,[121] seleno-,[122] and thioglycosides.[123,124] In addition to being more stable, thioglycosides have also provided outstanding α-stereoselectivity, as demonstrated in the synthesis of T_n and T_f antigen glycosyl amino acids.[123]

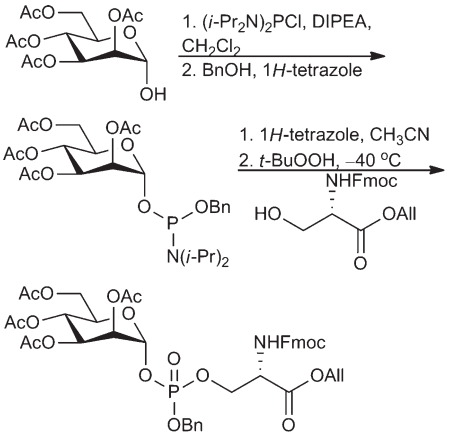
2.3. Rare and Unnatural Glycan Linkages

A number of other linkages have been identified in glycoconjugates in addition to the common O- and N-linked glycans.

Scheme 6. Synthesis of C-Linked Glycosyl-Trp Amino Acids



Scheme 7. Synthesis of Phosphoglycosides

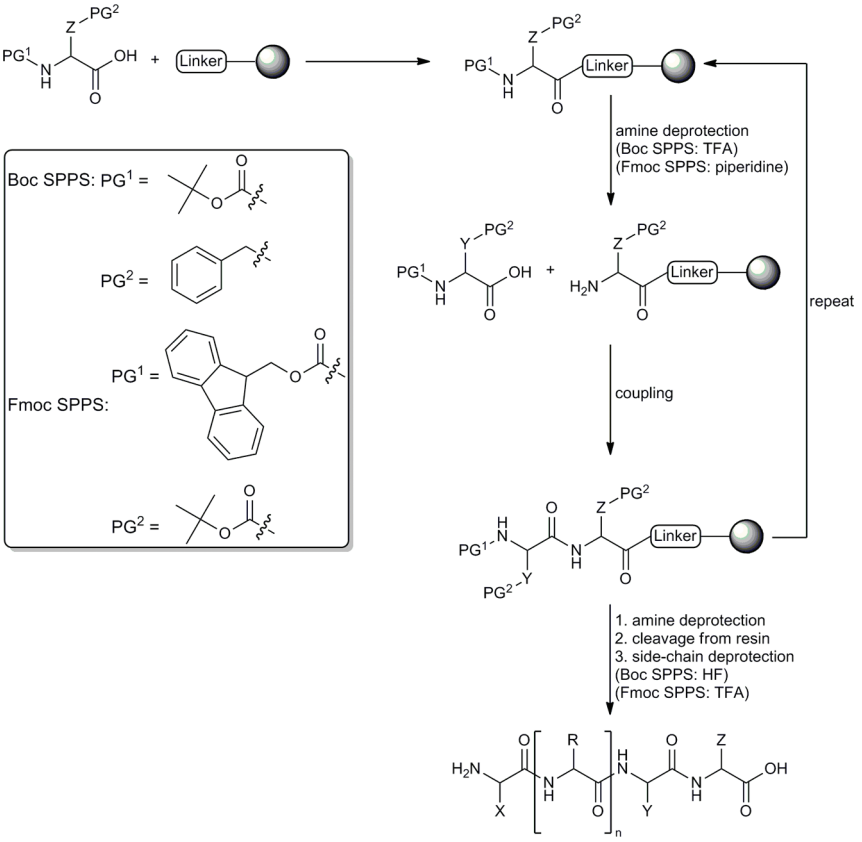


C-mannosylation can occur at the indole C-2 carbon of the first tryptophan (Trp) residue in the amino acid sequence Trp-Xaa-Xaa-Trp.[125,126] This linkage has been found in human proteins such as thrombospondins, RNase2, interleukin-12, mucins, and properdin and is formed by an unknown mechanism in the secretory pathway.[126−129] Although C-linked glycosyl-Trp amino acids have been difficult to access, Ito and co-workers have described the synthesis of *C*-mannopyranosyl glycosyl amino acids via ring-opening of benzyl-protected 1,2-anhydro-Man with a lithiated indole derivative (Scheme 6).[130]

Phosphoglycans represent a rare *O*-glycan linkage, characterized by a phosphodiester bond between the glycan and the hydroxyl group of Ser/Thr residues.[131,132] This linkage has been observed in the Golgi-resident proteins in lower eukaryotic organisms such as *Dictyostelium*, *Leishmania*, and *Trypanosoma*.[132−135] Phosphoglycans are believed to arise from enzymes in the phosphotransferase class.[136] Chemical routes are available to access Man and GlcNAc phosphoglycan derivatives (Scheme 7),[137,138] wherein the desired tetra-acetate protected sugar is converted to an anomeric phosporamidite, which can be activated with 1*H*-tetrazole and coupled to a suitably protected amino acid with a free hydroxyl group.[138]

Though uncommon in nature, S-linked glycans have emerged as a useful class of unnatural glycan linkages that can emulate regioselective glycoprotein modifications.[17,139] For example, introducing S-linked glycans regioselectively in place of O-linked glycans can help to overcome the otherwise insurmountable challenge of adding a late-stage O-linked glycan, owing to the ubiquitous free hydroxyl functionality characteristic of full-length proteins.[139,140] In addition to providing a means for late-stage

Scheme 8. Boc and Fmoc SPPS Strategies



glycosylation, S-linked glycans may also endow traits that are characteristic of a native linkage including increased in vivo stability and enzymatic resistance.[141] Chemical methods for the synthesis of S-linked glycans has been the subject of a detailed review.[17]

3. PROTEIN BACKBONE SYNTHESIS

3.1. Linear Peptide Synthesis

Although classic solution-phase peptide synthesis can be used to access short, biologically active peptides,[142−144] SPPS is the method of choice for de novo peptide construction because it provides access to longer peptides based on iterative solid-phase methodology performed with superior yields and simplified purification.[78] SPPS employs two different protecting group strategies: the *tert*-butoxycarbonyl (Boc) protection strategy and the 9-fluorenylmethoxycarbonyl (Fmoc) protection strategy (Scheme 8).[145,146] Traditional Boc SPPS protects the α-amino group of the free amino acid with Boc groups during peptide couplings to the growing chain, and employs benzyl ethers as permanent side-chain protecting groups. To release the N-terminal amino group for another round of coupling, Boc groups are removed by treatment with trifluoroacetic acid (TFA), while HF treatment is enacted at peptide completion to remove benzyl ether protecting groups and to cleave from the resin. Unfortunately, this strongly acidic environment precludes the use of Boc SPPS for *O*-glycopeptide synthesis because most O-glycosidic linkages are acid-labile. On the other hand, Fmoc SPPS protects the α-amino groups as Fmoc derivatives and amino acid side-chains with Boc groups. The mildly basic conditions required to cleave Fmoc pose little threat to the integrity of the glycopeptide. Likewise, the TFA treatment necessary to liberate Boc-protected side-chains and cleave the peptide from the resin will leave most glycosidic linkages intact. Fmoc-based SPPS of glycopeptides is ideal for the incorporation of small glycosyl amino acids for lengths up to ∼50 amino acids.

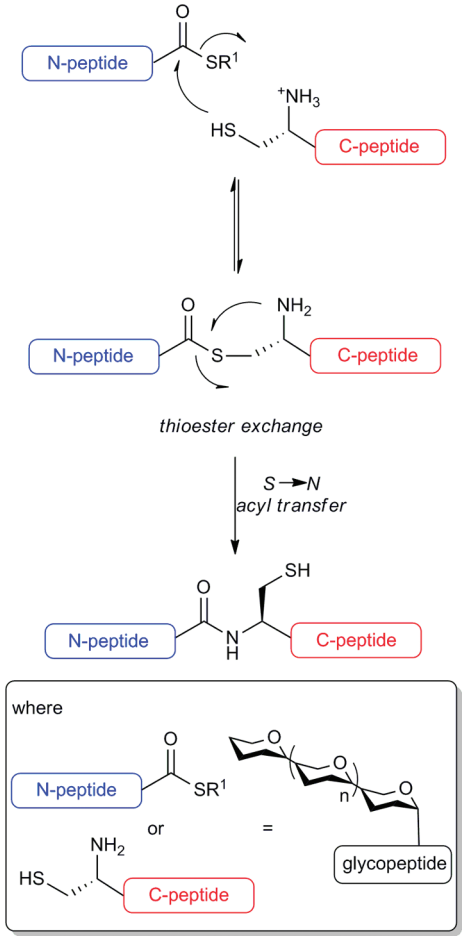
3.2. Convergent Peptide Synthesis

Convergent synthetic methods are required to synthesize larger glycoproteins, especially those containing more complex glycan structures. Ligation strategies seek to divide large glycoprotein targets into synthetically accessible fragments, which can be joined by ligations in convergent fashion at a late stage. To do so, regioselective couplings in the presence of all protein functionality must be enacted, because ligations will necessarily entail the use of deprotected peptide fragments in aqueous media (the only environment in which they will be soluble). Several such ligation techniques have been developed, including NCL, auxiliary assisted ligation (AAL), expressed protein ligation (EPL), and protease-catalyzed ligation (PCL). These chemical ligation strategies have provided tools to synthesize large peptides and proteins, as detailed in a recent review.[18] Here, we will highlight several key chemical ligation strategies for glycopeptides and glycoproteins, with emphasis on enzymatic methods.

3.2.1. Native Chemical Ligation. NCL is the most commonly employed method of peptide ligation. It is a powerful

dx.doi.org/10.1021/cr200113w |*Chem. Rev.* 2011, 111, 4259–4307

Scheme 9. Mechanistic Pathway for NCL



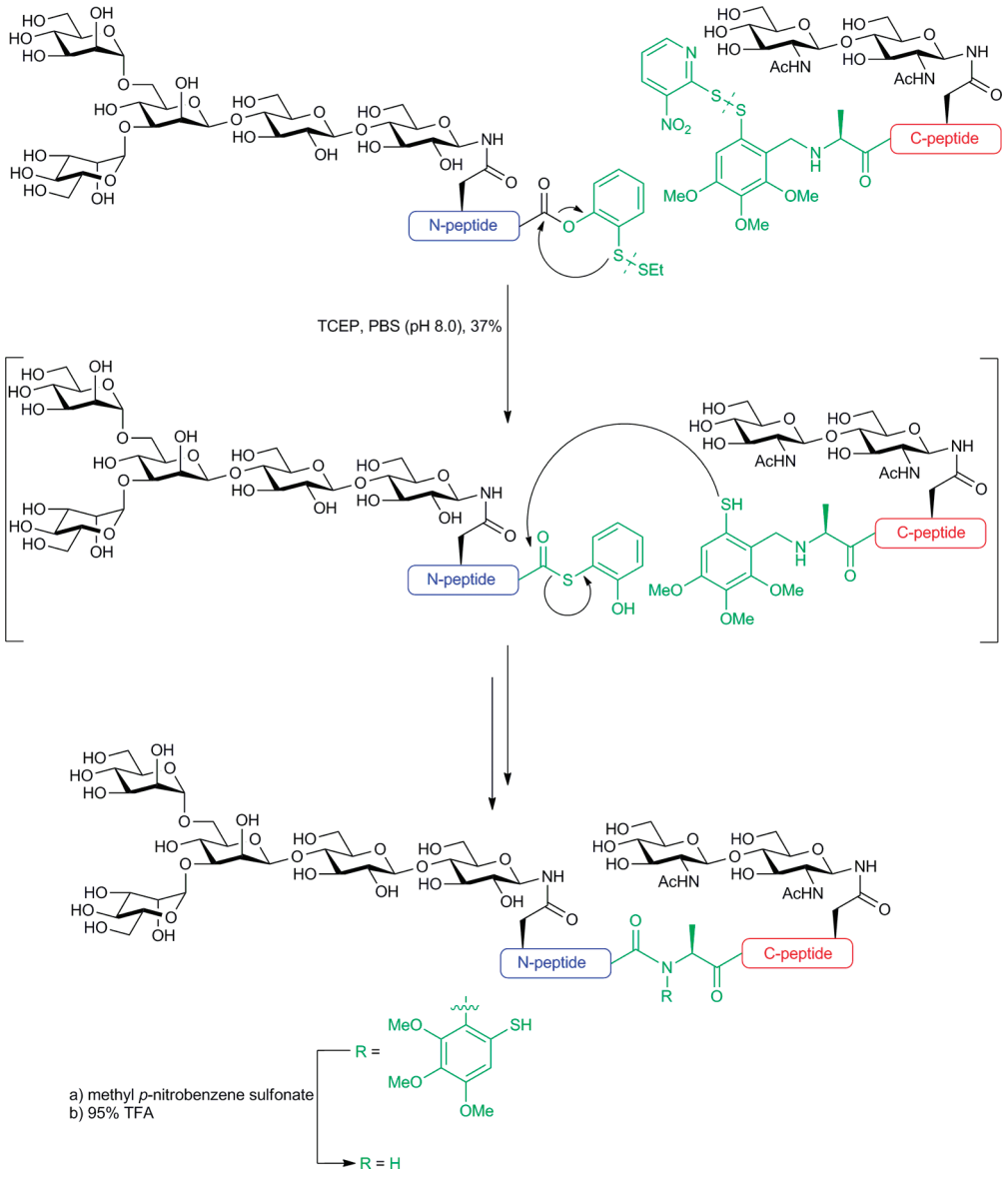
ligation strategy because it creates the native peptide bond that is imperative for accurate synthesis of protein structures. NCL proceeds through a reversible transthioesterification of a C-terminal thioester with a N-terminal cysteine residue, followed by irreversible $N \rightarrow C$ acyl migration (Scheme 9).[147] This intermediate can subsequently undergo a spontaneous and irreversible $S \rightarrow N$ acyl shift to afford a new peptide bond in nearly quantitative yields. Since the transthioesterification is completely reversible, a neighboring free amine must be present for the ligation, which originally limited this technique to N-terminal cysteines.[11] Notably, the traditional thioester–resin linkages used to build thioesters for standard NCL with Boc SPPS are not stable under the Fmoc SPPS conditions required for glycopeptide synthesis. As such, a number of elegant solutions, including the employment of a "safety catch"[148,149] or "double"[150] linker or with a side-chain anchoring strategy,[151] have been designed to create glycopeptide thioesters compatible with NCL.

3.2.2. Auxiliary Assisted Ligation. NCL-based synthetic strategies have broad applications; however, the cysteine requirement presents a major drawback. Cysteines severely limit the choice of natural ligation sites as these residues occur rarely, at a rate of only 1.7 per hundred residues. To circumvent this limitation, a variety of methods have been developed that employ thiol auxiliaries that can be removed after they have facilitated NCL.[152−165] The first such demonstrations were actually with cysteines used for NCL and subsequently desulfurized to afford the more commonly occurring amino acid alanine.[163−166] Since this first example of desulfurization, several thio-amino acids have been developed for incorporation into NCL-based strategies[18,23] including thiovaline,[163,167,168] thiophenylalanine,[165] and thiolysine.[169−171] Nonamino acid-based auxiliaries are also utilized. For example, Danishefsky and co-workers developed a thiol-based (4,5,6-trimethoxy-2-mercaptobenzyl) auxiliary-assisted ligation strategy in an impressive synthesis of a hindered bifunctional glycopeptide (Scheme 10).[157] The thioester was formed through an $O \rightarrow S$ acyl migration under reducing ligation conditions, which was then coupled to the auxiliary-modified glycopeptide, and a subsequent $S \rightarrow N$ acyl shift yielded the full-length glycopeptide. Methylation of the aromatic sulfur residue blocked the competing $N \rightarrow S$ acyl transfer, and the addition of TFA promoted acid-catalyzed cleavage of the auxiliary. In the case that auxiliary removal conditions are incompatible with labile glycosidic bonds,[172] in situ-generated phenolic esters can be used, which mediate a direct amide coupling without a covalent auxiliary linkage to the peptide.[172] Alternatively, ligations can be affected using a cysteine-free peptide ligation strategy, which implements a key mixed solvent buffer system to drive direct aminolysis of thioesters by the free N-terminal amine on the ligation partner.[173] The direct aminolysis reaction avoids the use of auxiliaries and overcomes problems with epimerization of the C-terminal residues that plague the activating agent-dependent strategies (i.e., thioester-based and phenolic ester directed couplings); however, Lys residues may need to be protected, which can affect peptide solubility.

Of particular interest for the synthesis of glycopeptides is sugar-assisted ligation (SAL), which places the thiol auxiliary on the sugar moiety. The thiosugar auxiliary is prepared as a glycosyl amino acid building block and is installed during SPPS in an amino acid near the ligation site, where they can mediate amide bond formation with the neighboring peptide ester (Scheme 11).[158] Once the ligation has occurred, the thiosugar can then be reduced to the natural sugar or removed through treatment with PNGase. The remaining sugar can be elaborated by treatment with β-1,4-galactosyltransferase if the thiol was desired,[158] or the thiol handle can be removed using desulfurization conditions to afford the unmodified O-linked GlcNAc residue for subsequent testing or elaboration.[159] SAL is amenable to the majority of both O- and N-linked glycoproteins using SAL auxiliaries based on β-O- and β-N-linked glycans[158,159] as well as α-O-linked sugars[162] of both thio-GlcNAc and thio-GalNAc derivatives. The proximal sugars can be further elaborated to afford a desired glycan motif. Extended SAL demonstrated that additional N-terminal amino acids were tolerated following the thiosugar auxiliary residue, broadening the applicability of the method to more ligation junctions, including a large number of O-glycosylation sites that are not accessible by other ligation methods.[161]

3.2.3. Expressed Protein Ligation. EPL is a semisynthetic ligation method that can be used to generate large glycoproteins that are not accessible using NCL and SPPS alone. In EPL, large peptide/protein fragments are obtained by recombinant expression methods in cells, whereas shorter synthetic glycopeptide fragments are fabricated using SPPS; which segment is the thioester will depend on the site of glycosylation. EPL is a particularly attractive method for targeting glycoproteins with a glycosylation site near a terminus (~50 amino acids) because SPPS is very effective for generating short peptides with specific

Scheme 10. Removable Auxiliary-Assisted Ligation



glycan structures.[174] EPL strategies to modify both the N- and C-terminus of glycoproteins have been developed wherein chemoenzymatic steps, along with SPPS and recombinant expression, play important roles in generating the appropriate fragments, thioesters, and ligation junctions.[175]

EPL using N-terminal engineering is advantageous for synthetically controlling glycan structures that are found on target glycoproteins bearing N-terminal glycosylation sites. For N-terminal engineering the requirements are (1) a synthetic N-terminal glycopeptide thioester fragment and (2) a recombinant C-terminal peptide bearing a N-terminal ligation auxiliary to engage NCL with the thioester (Scheme 12a).[176] The glycopeptide thioester can be made using SPPS (see section 3.1). Bacterial expression systems can be used to make the C-terminal protein fragment bearing a N-terminal Cys. This fragment can be designed using a N-terminal fusion tag that will first aid in purification and then will unveil the Cys ligation junction by selective removal. For example, Wong and co-workers masked Cys ligation junctions using a protease-cleavable N-terminal fusion composed of a hexahistidine affinity purification tag, followed by the tobacco etch virus protease (TEV) recognition sequence ENLYFQ. EPL between the protease cleaved fragments and glycopeptide thioesters proceeded with 95% ligation efficiency and led to the synthesis of homogeneous glycoforms of both an HIV entry inhibitor (C37-H6) and human interleukin-2.[177,178] Unverzagt and co-workers elegantly displayed the capacity N-terminal engineering in the synthesis of full-length RNase C (Scheme 13).[179,180] Overall, this EPL strategy required three fragments and two ligations. The largest C-terminal fragment (RNase 40-124) was expressed as a N-terminal fusion composed of a chitin-binding domain (CBD) for purification and an intein domain for excision and unveiling of the Cys ligation junction. This expressed protein fragment required treatment

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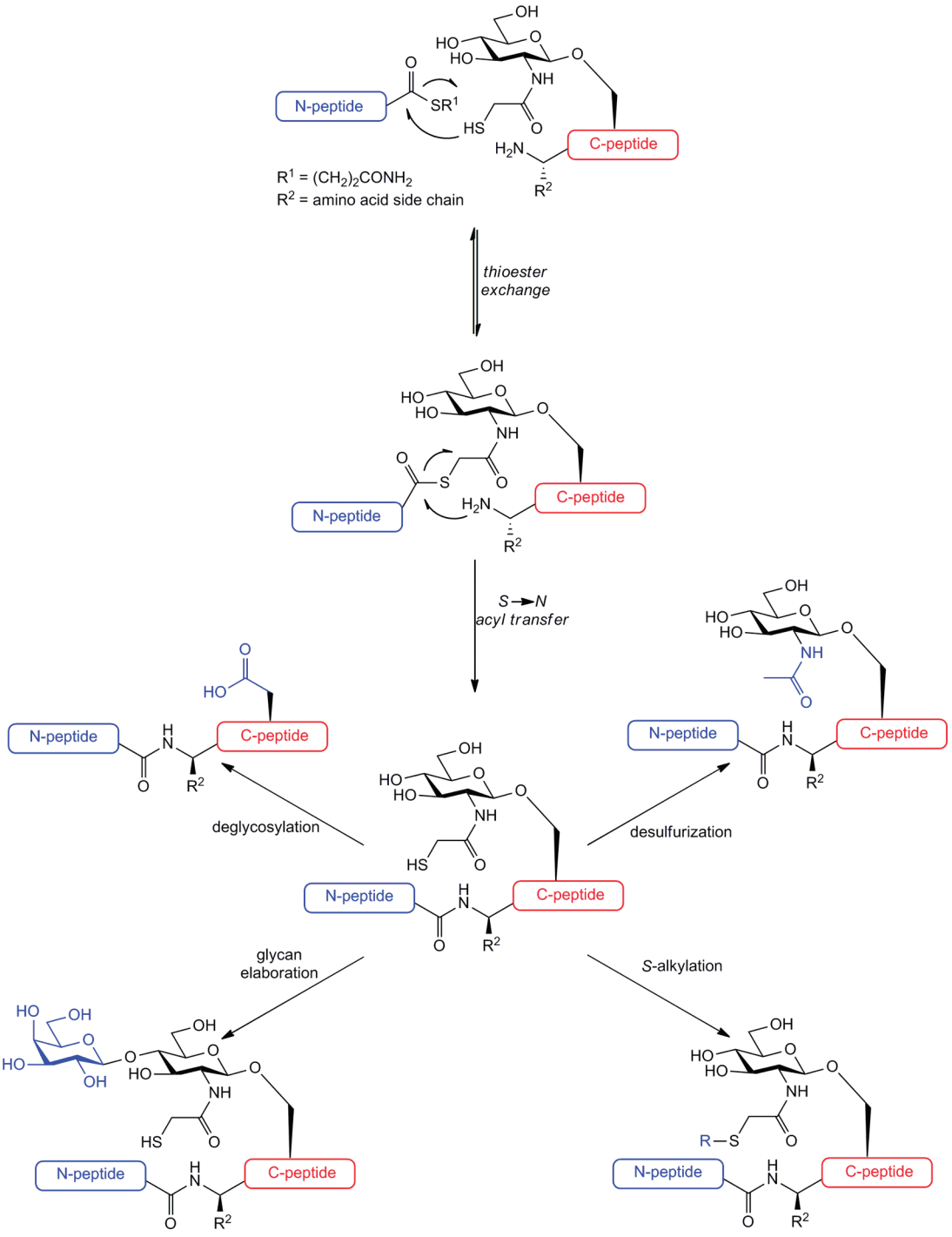
Scheme 11. Sugar-Assisted Ligation



with carboxyethyl methanethiosulfonate (CEMTS) to form the requisite mixed disulfides for solubility and intein excision.[181] The expressed peptide was then ligated to the glycopeptide thioester fragment (RNase 26-39), where the N-terminal Cys26 was protected as a thiazolidine, which is synthetically remarkable for the incorporation a glycosyl amino acid bearing a full complex *N*-glycan structure during SPPS. The full-length RNase was afforded by a final ligation with the N-terminal thioester fragment (RNase 1-25), demonstrating the first synthesis of a full-length complex N-linked glycoprotein over 100 amino acids in length.

Proteins bearing a glycan structure near the C-terminus require C-terminal engineering. The EPL requirements in this case are (1) a recombinantly expressed N-terminal fragment bearing a C-terminal thioester and (2) a C-terminal SPPS peptide bearing a N-terminal ligation auxiliary (Scheme 12b). C-terminal engineering is slightly more difficult as it relies on modified intein technology to generate the expressed C-terminal thioester. Intact

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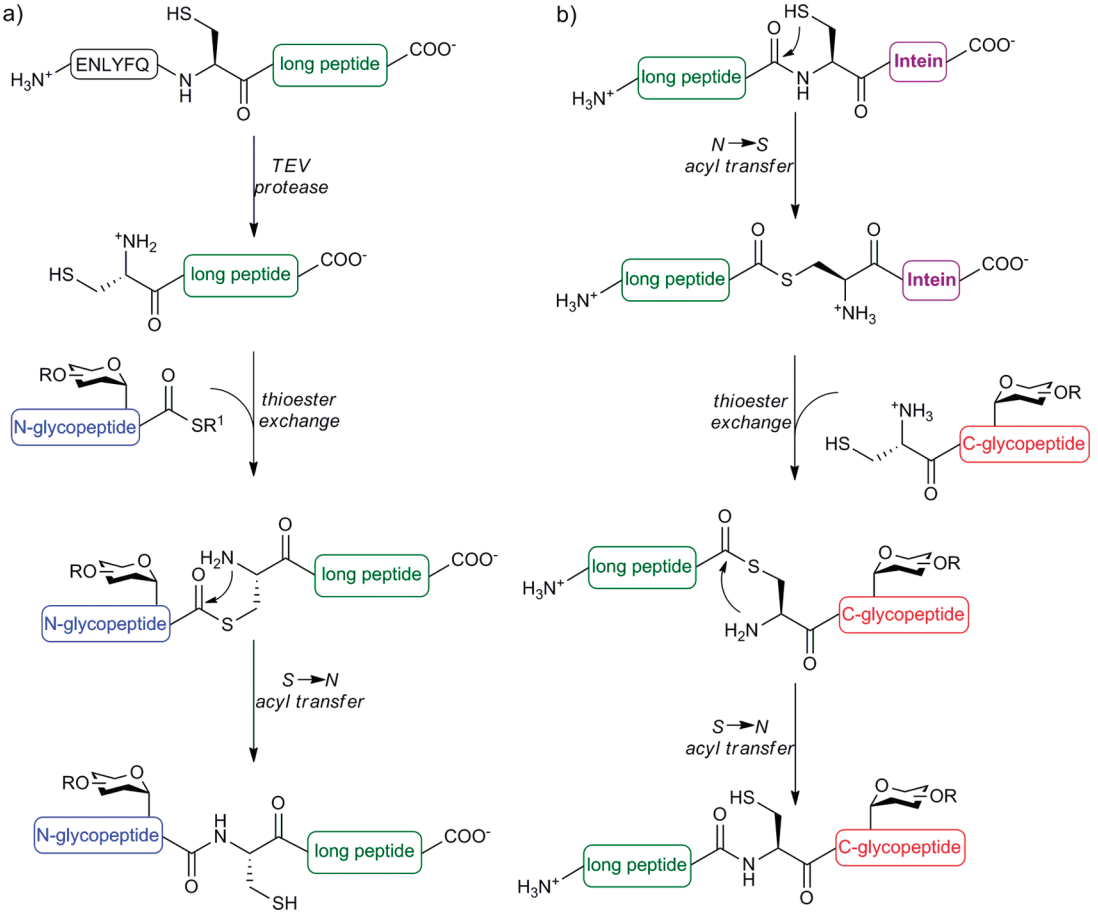
Scheme 12. Expressed Protein Ligation: (a) N-Terminal Engineering and (b) C-Terminal Engineering



inteins catalyze their own excision by facilitating an ordinarily unfavorable $N \rightarrow S$ acyl transfer (Scheme 12b);[182−184] however, modified inteins contain a mutation that inhibits excision after the formation of the thioester, which makes it possible to generate a C-terminal thioester intermediate for EPL.[185,186] To further facilitate thioester purification, inteins are resin-bound and modified with an affinity tag for purification. After ligation, the resin-bound intein can be filtered to afford purified semisynthetic protein. The application of this technique to glycoprotein synthesis has recently been showcased in a number of laboratories,[184,187−192] and the versatility of the method was highlighted in a homogeneous synthesis of glycoprotein variants of a 392 amino acid maltose-binding protein, which contained examples of C-terminal carbohydrate labeling, fluorescence labeling, metal chelator addition, and nucleotide incorporation.[184]

ECL approaches can also combine N- and C-terminal engineering, which is important because most glycoproteins have multiple glycosylation sites. The work of Macmillan and Bertozzi demonstrates the power of applying both N- and C-terminal engineering to gain access to the leukocyte homing glycoprotein GlyCAM-1 (Scheme 14).[188] In this approach the C-terminal fragment GlyCAM-1 (78-132) and the N-terminal glycopeptide thioester fragment GlycCAM-1 (1-40) were synthesized by SPPS, while the central fragment GlyCAM-1 (41-77) was expressed as a recombinant fusion construct containing an N-terminal FXa protease site to mask Cys41 and a C-terminus containing a modified intein for purification and thioester formation at S77.[191] The intein-mediated thioester was generated first and ligated to the C-terminal GlyCAM-1 fragment via NCL to form ligated fragment GlyCAM-1 (41-132). Then, Cys41 was unmasked from the ligated fragment using Factor Xa and coupled with the N-terminal glycopeptide thioester to afford full-length GlyCAM-1.

3.2.4. Protease-Catalyzed Ligation. Proteases provide another chemoenzymatic approach to selectively ligate peptide fragments. Proteases can catalyze "reverse hydrolysis" ligation reactions, or PCLs, under carefully controlled conditions, without racemization, and have been employed in many synthetic efforts for site-specific modification.[193] PCL couples an activated peptide ester to a N-terminal amine, typically in the presence of an organic cosolvent. The organic solution minimizes undesired hydrolysis of the peptide ester but often decreases enzyme activity and stability. Engineered mutants of Subtilisin BPN′, a Ser protease that has been well established as a nonspecific catalyst of peptide ligation, are available with improved PCL activity.[53,143−148] Thiosubtilisin contains a catalytic Cys in place of the wild-type Ser residue, which optimizes aqueous enzyme activity by engendering a thioester intermediate that favors attack by an amine group (over wild-type hydrolysis). The mutant 8397 has enhanced stability in organic conditions and has a half-life in dimethylformamide (DMF) of 14 days, an improvement over the wild-type half-life

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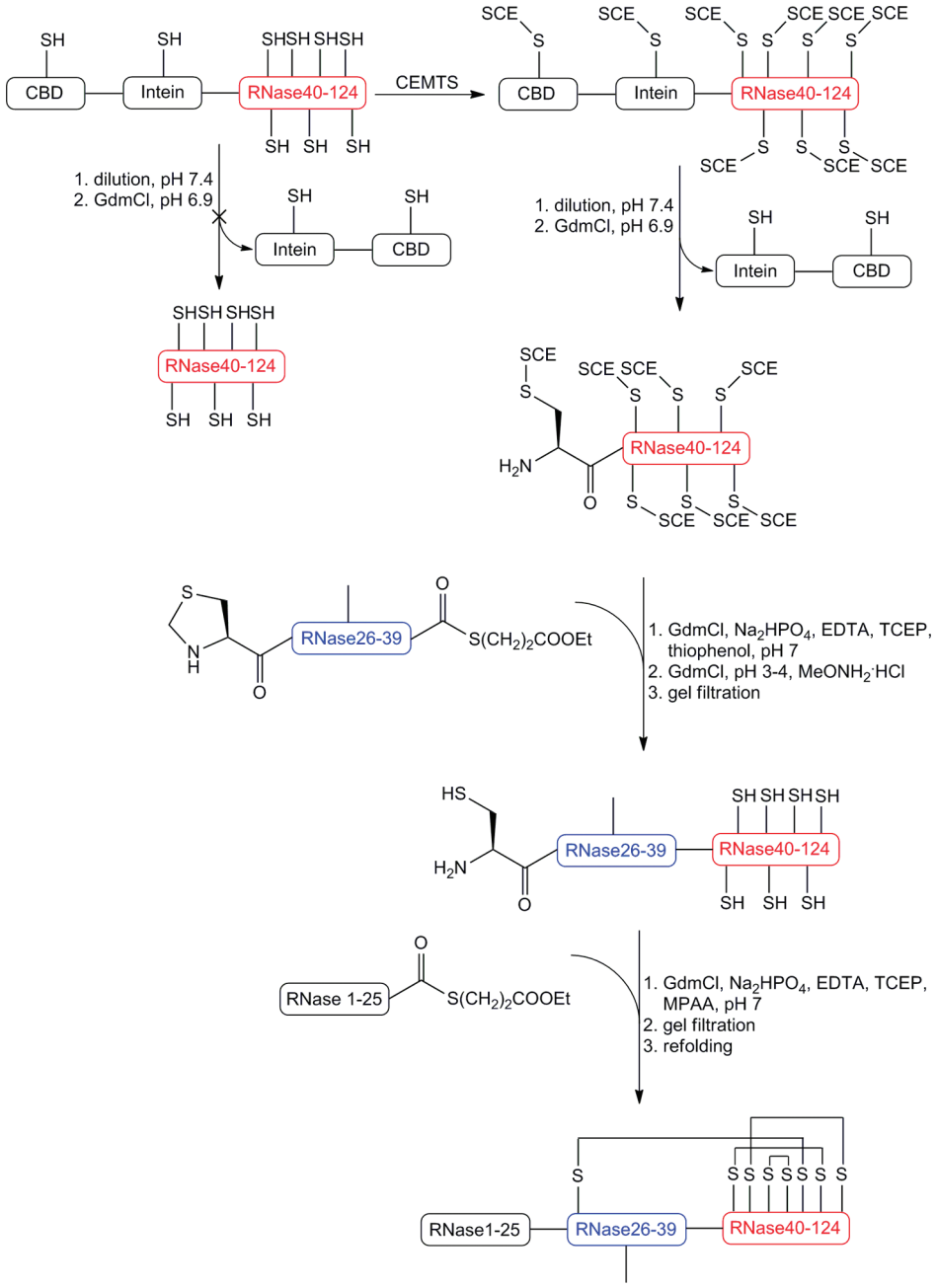
Scheme 13. Synthesis of RNase C



of 30 min. Subtilisin is suitable for peptide ligation of fragments containing both O- and N-linked monosaccharides near, but not in, the active site and is sensitive to glycosylation at a few key positions.[194] Importantly, the Davis group has shown that substrate specificity can be significantly broadened through mutagenesis.[195]

4. CHEMOENZYMATIC SYNTHESIS, ELABORATION, AND REMODELING OF CARBOHYDRATES

Carbohydrate synthesis poses a unique synthetic challenge to carefully control the regio- and stereochemical selectivity of glycosyl reactions. Unfortunately, no universal method has emerged for the controlled synthesis of carbohydrate bonds, although several chemical methods can be used to synthesize glycans.[25] These methods primarily rely on the use of protecting groups to direct regioselectivity and carefully controlled reaction conditions to favor a desired anomeric outcome, although absolute stereoselectivity is rarely observed. Although it is hoped that enzyme-catalyzed protecting group schemes may facilitate solution-phase synthesis,[196,197] many synthetic routes to carbohydrates remain elusive, as the inherent differences between neighboring hydroxyl groups are often inconsequential and employment of protecting group protocols can be prohibitively tedious.[6,25] The most traditional method for carbohydrate synthesis is solution-phase synthesis,[198] while solid-phase carbohydrate synthesis has also emerged in an effort to develop an automated synthetic strategy.[199] Programmable one-pot synthesis is another strategy that exploits

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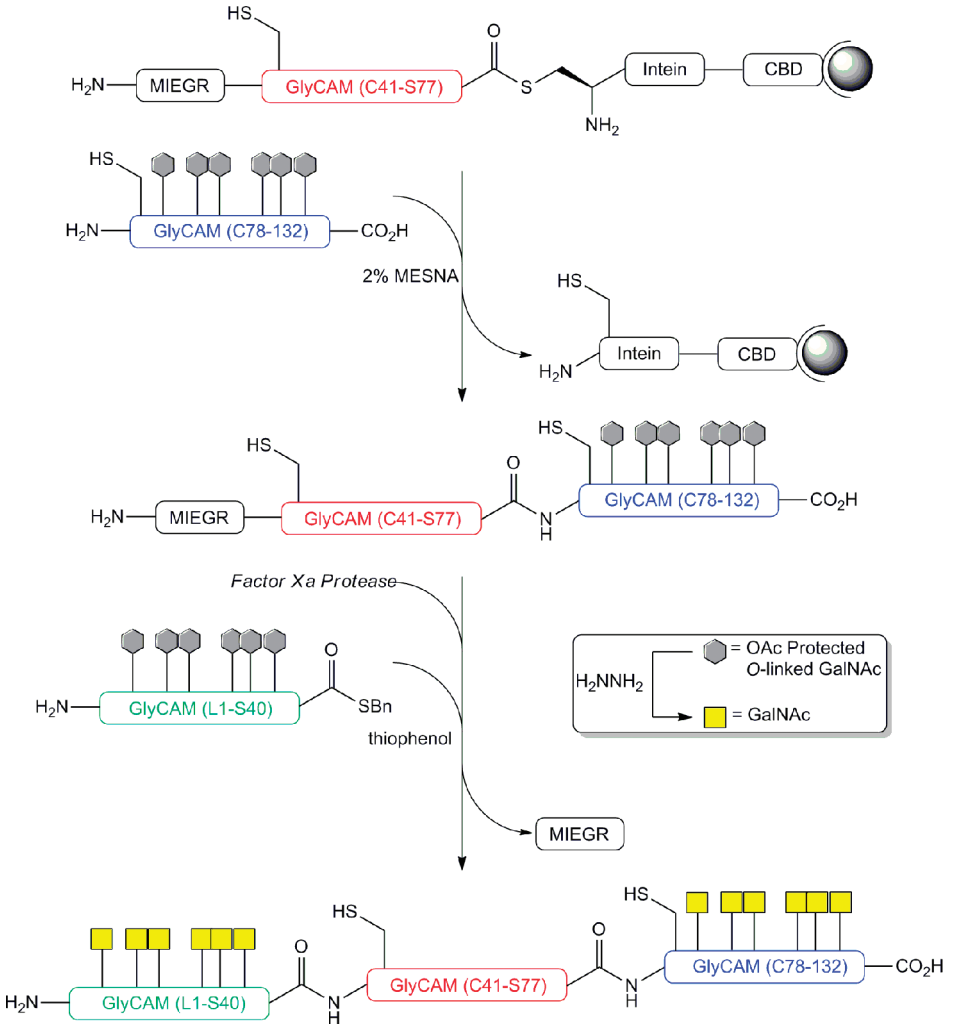
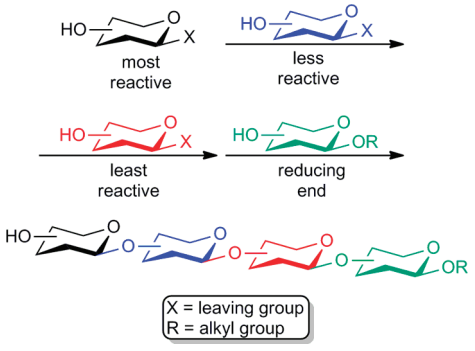
Scheme 14. EPL−Double Ligation



Scheme 15. Programmable One-Pot Oligosaccharide Synthesis



differences in glycosyl donor reactivity to control the elongation of a carbohydrate chain in a one-pot, stepwise procedure (Scheme 15).[200] This approach still relies on the synthesis of carefully designed and suitably protected sugar subunits, but has proven to be invaluable in synthesizing several interesting oligosaccharides.[201−205] Chemical synthesis has been the subject of extensive review and will not be discussed in further detail.[6,198,199,206−210]

Alternatively, enzyme-catalyzed carbohydrate synthesis offers strategies for glycosidic bond formation between both mono- and oligosaccharide building blocks with high regio- and stereoselectivity, and also permits the use of unprotected donors and acceptors.[19] Three classes of enzymes are employed for carbohydrate synthesis: GTases, *exo*-glycosidases, and *endo*-glycosidases. Additionally, these classes of enzymes have been enhanced over the past decade through genetic engineering to form mutant enzyme classes, such as glycosynthases and thioglycoligases, which are designed or selected for increased yields, enzyme stability, and improved donor and acceptor specificity.[15,211,212] Enzymes can also be exploited in the glycoengineering of cells (see section 5).

4.1. Glycosyltransferases

The GTases (EC 2.4) comprise a class of naturally occurring enzymes that establish glycosidic bonds with exquisite regio- and stereoselectivity by catalyzing the transfer of an activated saccharide donor to an acceptor, which may be a specific carbohydrate hydroxyl group or a specific protein or lipid nucleophile.[213−216] The vast majority of mammalian GTases

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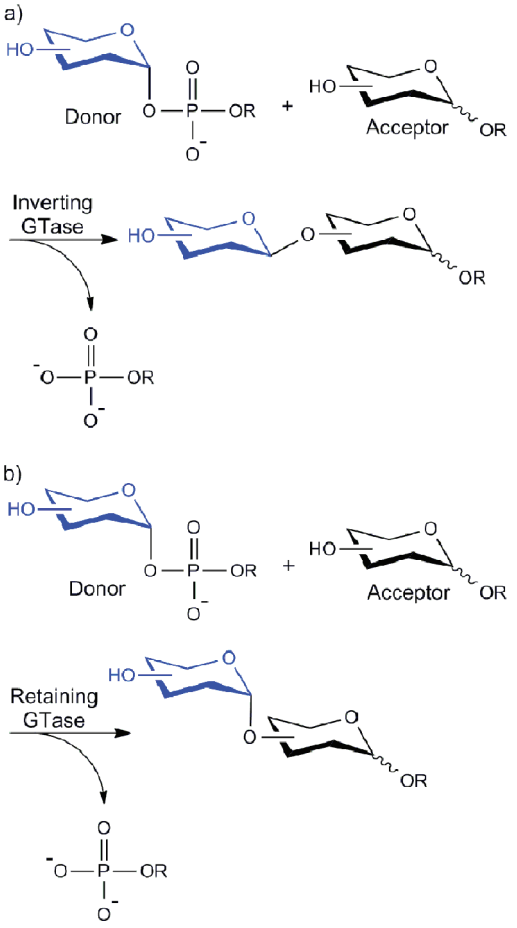
Scheme 16. GTase Types: (a) Inversion of Anomeric Configuration and (b) Retention of Anomeric Configuration



are of the Leloir type that build glycan structures by transferring sugars from a nucleotide donor, including UDP-Glc, UDP-Gal, UDP-GlcNAc, UDP-GalNAc, UDP-Xyl, UDP-GlcA, GDP-Man, GDP-Fuc, and CMP-sialic acid.[217] The nucleotide component of these Leloir donors does much more than simply activate the sugar for transfer; it is critical for GTase folding, recognition, and function, and thus there is little tolerance for nucleotide modifications, although substrate engineering is a topic of interest.[19,212,218] Leloir GTases are found in all kingdoms of life and fall into the two structural superfamilies GT-A and GT-B, which reflects conservation of their nucleotide-binding Rossman fold and also suggests evolution from a small number of progenitor sequences, perhaps primitive archaeal enzymes.[213] In addition to the C-terminal catalytic domain, eukaryotic GTases typically have a type II transmembrane domain for embedding into secretory membranes. Although the mechanistic details of many GTases are unknown, they are generally categorized as retaining and inverting to indicate the relative anomeric configuration between donor and product (Scheme 16).[213] Non-Leloir GTases use sugar phosphates or polyprenol sugar phosphates as their glycosyl donors. These enzymes are uncommon in mammals and many await characterization and chemoenzymatic application, although we note that newly discovered OTases have promising whole-cell applications (see section 5.3.3).

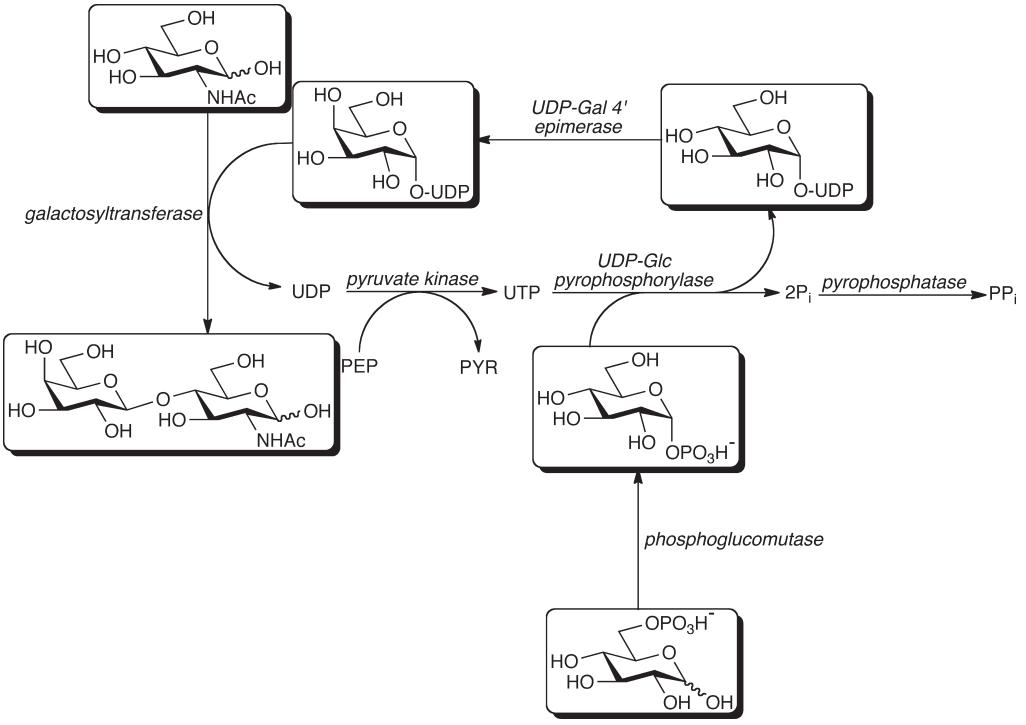
Table 1. Commercially Available GTases

GTase	suppliers
β1,4-GalT	Sigma-Aldrich/Calbiochem-Merck
α1,3-GalT	Sigma-Aldrich
α1,3-FucT	Sigma-Aldrich
α1,2-ManT	Calbiochem-Merck
α2,3-(N)-SiaT	Calbiochem-Merck
α2,3-(O)-SiaT	Calbiochem-Merck
α2,6-SiaT	Calbiochem-Merck

The application of Leloir GTases in the synthesis of glycan structures is of keen interest because of their ability to form the bonds of mammalian glycoconjugate structures with high regio- and stereospecificity in excellent yield. Furthermore, GTases remain one of the only efficient ways to enact challenging bonds, such as sialylation. However, their widespread application in synthesis can be restricted by challenges, including GTase availability, reaction expense, and feedback inhibition, each of which must be considered when applying GTases to chemoenzymatic endeavors. To begin, only a few GTases are commercially available (Table 1), necessitating that most be isolated or recombinantly expressed, which limits their accessibility to synthetic laboratories, especially in the case of eukaryotic enzymes requiring eukaryotic cell culture. Furthermore, eukaryotic GTases with transmembrane domains may preclude soluble applications; and although the list of soluble variants continues to grow, enzymes capable of catalyzing the complete spectrum of glycan linkages still await discovery. The next consideration is the cost of GTase systems, which can be high due to the expense of sugar nucleotide donors, although advances in sugar-nucleotide synthesis continue to alleviate this problem.[219] Finally, it must be recognized that GTase systems suffer from feedback inhibition, as the GTase is inactivated by nucleoside diphosphate byproducts generated during the course of the glycosylation reaction. Fortunately, the latter two problems related to sugar nucleotide expense and byproduct inhibition can be overcome with multienzyme systems that can break down detrimental byproducts or regenerate sugar donors in situ from inexpensive starting materials (Scheme 17).[220,221] The importance of GTases for the synthesis of carbohydrates is underscored by the number of multienzyme systems that have been developed to optimize sugar-nucleotide regeneration.[64,222−228] Notably, the large-scale synthesis of sLe^X was only possible using GTases.[64] To date, over 65 GTases have been identified and characterized for chemoenzymatic applications of glycans, as summarized in Table 2. In the following sections, chemoenzymatic enzymes and their applications are summarized under the category of GTases and donor substrates, and we remind the reader to consult our previous review for many useful chemoenzymatic applications developed before 2000.[1]

4.1.1. Glucosyltransferases/Glucouronyltransferases (GlcTs/GlcATs). GlcTs/GlcATs can be used to transfer glucose from UDP-Glc/UDP-GlcA to a number of acceptors (Table 2). Protein *O*-glucosylation is a rare posttranslational modification observed on several serum proteins including notch, factor VII, factor IX, protein Z, and thrombospondin. The most thoroughly studied glucose residues on glycoproteins are the three terminal glucose residues found on the highly conserved N-linked glycan transferred to all eukaryotic glycoproteins.[318,319] These Glc residues are rarely found on the mature *N*-glycan structures

Scheme 17. Sugar-Nucleotide Regeneration



but play an important role in folding and secretion or targeting proteins for degradation if improperly folded.[33] GlcA is a saccharide variant of Glc that is oxidized to a carboxylate at C-6. GlcA is found in many glycan structures, such as the human natural killer-1 (HNK-1) carbohydrate, and it is a key component of the repeating disaccharide units of glycosaminoglycans (GAGs), such as hyaluronan (HA), chondroitin (CH), and heparin sulfate (HS),[292] making GlcATs useful for chemoenzymatic synthesis of such structures. For example, the synthesis of HNK has been examined using GlcAT-P and GlcAT-S.[291] The synthesis of HA, which is composed of a repeating (-GlcA-β1,3-GlcNAc-β1,4-)$_n$ polymer, has been demonstrated using the bifunctional GTase hyaluronan synthase 1 (HAS1), which is responsible for both polymeric additions from the corresponding nucleotide sugars—UDP-GlcA and UDP-GlcNAc.[320] The synthesis of CH, which is composed of a repeating (-GlcA-β1,3-GalNAc-β1,4-)$_n$ polymer, has been achieved using a similar bifunctional enzyme K4CP from *E. coli*.[321] Furthermore, K4CPs, which have been mutated to contain only one of the GTase activities (i.e., D241K has only GlcAT activity), can be bound to resins for iterative synthesis of CH derivatives of controlled length that can be easily purified (Scheme 18).[294]

4.1.2. Mannosyltransferases (ManTs). ManTs can be used to transfer Man residues to a number of acceptors using GDP-Man (Table 2). Man is a component of N-linked glycans, which all contain the strictly conserved core of $Man_3GlcNAc_2$. This pentasaccharide is structurally composed of a bridging Man that is β-linked to chitobiose and that displays two α-linked Man residues at the 3- and 6-positions, which are poised to bear the remaining features of the glycan structure, save for a possible core fucose at the protein proximal GlcNAc (Figure 2). Because of the biological importance of *N*-glycans, synthesis of the $Man_3GlcNAc_2$ core is desired; however, it contains one of the most chemically challenging β-Man bonds.[322] Fortunately, the trisaccharide Manβ1 → 4GlcNAcβ1 → 4GlcNAc can be accessed chemoenzymatically by using a method with a recombinant β1,4-ManT, as developed by Flitsch and coworkers.[300,301] Notably, this β1,4-ManT is commercially available (Table 1). Another β1,4-ManT cloned from *Salmonella* and expressed in *E. coli* was chemoenzymatically employed to synthesize the bacterial O-antigen group E_1 by Zhao and Thorson.[302] A unique ManT from *T. maritima* adds an α1,2-linked Man onto another Man residue that is a constituent of di-*myo*-inositol phosphate, and it may have chemoenzymatic applications for deriving P-linked poly-Man derivatives.[299] Shibata and Okawa synthesized a series of mannoprotein mimics with α1,2-ManT from *C. parapsilosis* and α1,6-ManTs from *C. albicans*.[296] These ManTs were found to be useful for fluorescent protein labeling via addition of azido-modified Man, from GDP-azidodeoxymannoses, which can be chemoselectively labeled via Staudinger ligation.[323]

4.1.3. Sialyltransferases (SiaTs). SiaTs can be used to transfer Neu5Ac groups to a number of acceptors using CMP-Neu5Acs (Table 2). Neu5Acs are extensively integrated into the glycoconjugates of higher animal species, where they are often terminally displayed and recognized by a diverse set of Neu5Ac-binding lectins.[324] In doing so, Neu5Acs modulate many critical biological processes, such as inflammation, immune response, cancer metastasis, and microbial pathogenesis.[239,325,326] Being able to study these processes by synthesizing sialylated glycans will lead to a greater understanding of their biology and will lead to new therapeutic targets. Notably, the chemical synthesis of sialic acid-containing oligosaccharides is particularly challenging because of their hindered and charged tertiary anomeric center, the lack of a participating group to aid in stereochemical control.[327] As a result, chemoenzymatic methods of sialylation

dx.doi.org/10.1021/cr200113w |*Chem. Rev.* 2011, 111, 4259–4307

Table 2. Synthetically Relevant Glycosyltransferases

enzyme (donor)	entry	glycosyl acceptor(s)	known acceptor specificity	enzyme name (source)	linkage(s) synthesized	ref
FucT (GDP-Fuc)	1[a]	Gal, Xyl, Fuc, Mal, IsoMal, Melibiose, Cel, Lac, L-Xyl	Multiple	FTF (*B. subtilis*)	α-(1→2)-	*(1)*
	2	Gal	Lac/Lactulose/Galβ1→4Man/ T antigen	WbsJ (*E. coli*)	α-(1→2)-	*(2)*−*(6)*
	3	Gal	preferentially fucosylates Galβ1→ 3GlcNAc over Galβ1→4GlcNAc	α1,2-FucT (*H. pylori*)	α-(1→2)-	*(7)*
	4	GlcNAc	Lewis antigens/LacNAc	α(1,3/4)-FucT (*H. pylori*)	α-(1→2)-/ α-(1→3)-	*(8)*
	5	GlcNAc	M3/M3F[6]/LDNT/LN-*p*NP/LNnT	CEFT(1−4) (*C. elegans*)	α-(1→3)-	*(9)*, *(10)*
	6[b]2	GlcNAc	LacNAc	FucT-III (*H. sapiens*)	α-(1→3)-	*(11)*
	7	GlcNAc	Galβ1→4GlcNAc	FucT-V (*H. Sapiens*)	α-(1→3)-	*(12)*−*(14)*
	8	GlcNAc	NeuAcα2→3Galβ1→4GlcNAc	FucT-VI (*H. sapiens*)	α-(1→3)-	*(15)*, *(16)*
	9	GlcNAc	distal GlcNAc in polylactosamine chain	FucT-IX (*H. sapiens*)	α-(1→3)-	*(17)*
	10	GlcNAc	Lewis antigens/LacNAc	α1,4-FucT (*H. pylori*)	α-(1→4)-	*(18)*
	11	GlcNAc	LacNAc/*N,N*′-diacetylchitobiose	α1,6-FucT (*Rhizobium* sp.)	α-(1→6)-	*(19)*
	12	GlcNAc	Galβ1→4GlcNAcβProN$_3$/ NeuAcα2→3Galβ1→ 4GlcNAcβProN$_3$	HhFT1 (*H. hepaticus*)	α-(1→3)-	*(20)*
GalT (UDP-Gal)	13	Man/GlcNAc/Glc	multiple	NmLgtB (*N. meningitidis*)	β-(1→4)-	*(21)*−*(23)*
	14	GlcNAc	multiple	Hp1−4GalT (*H. pylori*)	β-(1→4)-	*(22)*
	15	Gal	lactose/LacNAc/Lac-β-N$_3$	α1,3-GalT (bovine)	α-(1→3)-	*(3)*, *(23)*, *(24)*
	16	Gal	lactose/Lactosyl ceramide	LgtC (*N. meningitidis*)	α-(1→4)-	*(6)*, *(25)*−*(27)*
	17	Gal	lactose	α1,4-GalT (*N. gonorrheae*)	α-(1→4)-	*(28)*
	18	GalNAc	GalNac/II[3]-α-Neu5Ac-Gg3Cer	CgtB (*C. jejuni*), C1GalT (*H. sapiens*)	β-(1→3)-	*(3)*, *(29)*, *(30)*
	19	Gal	β-Gal-*O*-NM/Galβ1→4Xylβ-*O*-Bn	β1,3-GalT6 (*H.* sapiens)	β-(1→3)-	*(31)*
	20	GlcNAc	GlcNAcβ1−3Galβ1−4Glcβ1-Cer	β1,3-GalT (*H. sapiens*)	β-(1→3)-	*(32)*
		GlcNAc/GalNAc	multiple	β1,3-GalT (murine)	β-(1→3)-	*(33)*
	21	GlcNAc	LacNAc[33]/Multiple [23]	β1,4-GalT (*N. meningitidis*[33], *H. sapiens*[23])	β-(1→4)-	*(10)*, *(12)*, *(13)*, *(30)*, *(33)*−*(36)*
	22	Xyl	β-Xyl-MU	β1,4-GalT7 (*H. sapiens*)	β-(1→4)-	*(37)*
αGalNAcT (UDP-GalNAc)	23	Gal	Fucα1→2Gal	GTA (*H. sapiens*)	α-(1→3)-	*(38)*, *(39)*
	24	Gal	sialyllactose	CgtA (*C. jejuni*)	β-(1→4)-	*(29)*
	25	Gal	globotriose	CgtE (*C. jejuni*)	β-(1→3)-	*(40)*
	26	Gal	globotriose	LgtD (*H. influenzae*)	β-(1→3)-	*(41)*
	27	GalNAc	GalNAc-α-PP-alkyl	WbnH (*E. coli*)	α-(1→3)-	*(3)*, *(42)*
		GalNAc	globotetrose	Pm1138 (*P. multocida*)	α-(1→3)-	*(44)*
	28	GalNAc	multiple	β1,4-GalNAcT (*T. ni*)	β-(1→4)-	*(43)*
	29	GlcNAc	GlcNAc	β1,4-GalNAcT (CHO)	β-(1→4)-	*(43)*, *(44)*
	30	GlcA	GlcAβ1→3Galβ-R	β1,4-GalNAcT (*H. sapiens*)	β-(1→4)-	*(45)*, *(46)*
	31	GlcNAc	*N,N*′-diacetylchitobiose	β1,4-GalNAcT (*C. elegans*)	β-(1→4)-	*(16)*
GlcNAcT (UDP-GlcNAc)	32	α-heptose(II)/ α-Man	multiple	LgtK (*N. gonorrheae*)	α-(1→2)-	*(47)*
	33	Gal	LacNAc	β3GnTI (*H. sapiens*)	β-(1→3)-	*(10)*, *(35)*
		Gal	LacNAc	β3GnTII (*H. sapiens*)	β-(1→3)-	*(37)*
	34	Gal	Lac/LacNAc/Others	LgtA (*N. meningitidis*)	β-(1→3)-	*(48)*
	35	GalNAc	Galβ1→3GalNAcα-Bn/Galβ1→ 3GalNAcα-*p*NP/Galβ1→ 3GalNAcα-octyl	C2GnT (mouse)	β-(1→6)-	*(30)*, *(49)*
	36	Gal	central Gal residue in polyLacNAc	CIGnT6 (*H. sapiens*)	β-(1→6)-	*(50)*
	37	Man	multiple	GnT-V (*H. sapiens*)	β-(1→6)-	*(51)*−*(53)*

dx.doi.org/10.1021/cr200113w |*Chem. Rev.* 2011, 111, 4259–4307

Table 2. Continued

enzyme (donor)	entry	glycosyl acceptor(s)	known acceptor specificity	enzyme name (source)	linkage(s) synthesized	ref
	38	Man	O-linked and N-linked glycans	GnT-Vb (*H. sapiens*)	β-(1→6)-	(52)
	39	GlcNAc	multiple	β1,4-GlcNAcT (*L. stagnalis, A. caulinodans*)	β-(1→4)-	(10), (33), (54)
	40	GlcA	multiple	α1,4-GlcNAcT (*H. sapiens*)	α-(1→4)-	(46), (55), (56)
	41	GlcA	hyaluronan	PmHAS (*P. multocida*)	β-(1→4)-	(57)
	42	Man	$Man_5GlcNAc_2$	GlcNAcT (rabbit)	β-(1→2)-	(58)
Glc(A)T	43	Fuc	α-Fuc-R	β1,3-GlcT (CHO)	β-(1→3)-	(59)
(UDP-Glc(A))	44	Glc	$Glc_2Man_9GlcNAc_2$	α1,2-GlcT (*H. sapiens*)	α-(1→2)-	(60)
	45	Gal	Galβ1→3Galβ1→4Xyl-R	GlcAT-I (*H. sapiens*)	β-(1→3)-	(61)−(64)
	46	Gal	multiple	GlcAT-P, GlcAT-S (rat)	β-(1→3)-	(65), (66)
	47	Gal	Galβ1→3Galβ1→4Xyl	DmGlcAT-I (*D. melanogaster*)	β-(1→3)-	(67)
	48	Gal	multiple	DmGlcAT-BS (*D. melanogaster*)	β-(1→3)-	(67)
	49	GalNAc	chondroitin	K4CP (*E. coli*)	β-(1→3)-	(68)
	50	GlcNAc	hyaluronan	PmHAS (*P. multocida*)	β-(1→3)-	(57)
ManT	51	Man	polymannose	α1,2-ManT (*C. albicans*)	α-(1→2)-	(69), (70)
(GDP-Man)	52	Man	α-1,3-mannobiose	α1,3-ManT (*C. neoformans*)	α-(1→3)-	(71)
	53	Man	multiple	α1,6-ManT (*M. smegmatis*)	α-(1→6)-	(72)
	54	Man	di-*myo*-inositol-1,3′-phosphate	β1,2-ManT (*T. maritima*)	β-(1→2)-	(73)
	55	GlcNAc	*N*,*N*′-diacetylchitobiose	β1,4-ManT (*S. cerevisiae*)	β-(1→4)-	(74), (75)
	55	Rham	Rhamα1→3Gal	β1,4-ManT (*Salmonella* sp.)	β-(1→4)-	(76)
SiaT	56	Gal	LacNAc	α2,3-SiaT (*S. frugiperda*)	α-(2→3)-	(36), (77)
(CMP-Neu5Ac)	58	Gal	LacNAc	α2,6-SiaT (*S. frugiperda*)	α-(2→6)-	(36), (77)−(79)
	59	Gal	fucosylated acceptors (Lewis a and Lewis x)	v-ST3Gal I (myxoma virus)	α-(2→3)-	(80)
	60	Sia	multiple	α2,8/2,9-SiaT (*E. coli*)	α-(2→8)-	(81)
	61	Gal	Lac/LacNAc/α and β galactosides/multiple[72]	α2,3-SiaT (*N. meningitidis*)	α-(2→3)-	(29), (82)−(86)
	62	Gal	multiple	α2,3-SiaT (*N. gonorrheae*)	α-(2→3)-	(87)
	63	Gal	multiple	tPm0188Ph (*P. multocida*)	α-(2→3)-	(79)
	64	Gal	Fuc and Sia trisaccharides	α2,6-SiaT (*P. damsela*)	α-(2→6)-	(88)
	65	Gal	multiple	α2,6-SiaT (*P. multocida*)	α-(2→6)-	(78), (79)
XylT (UDP-Xyl)	66	Glc	β-Glc-R	α1,3-XylT (*H. sapiens*)	α-(1→3)-	(89), (90)
	67	Xyl	Xylα1→3Glc	α1,3-XylT (*H. sapiens*)	α-(1→3)-	(90), (91)

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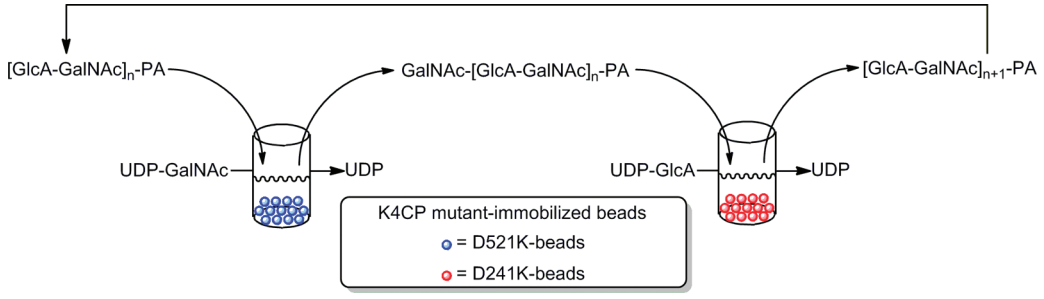
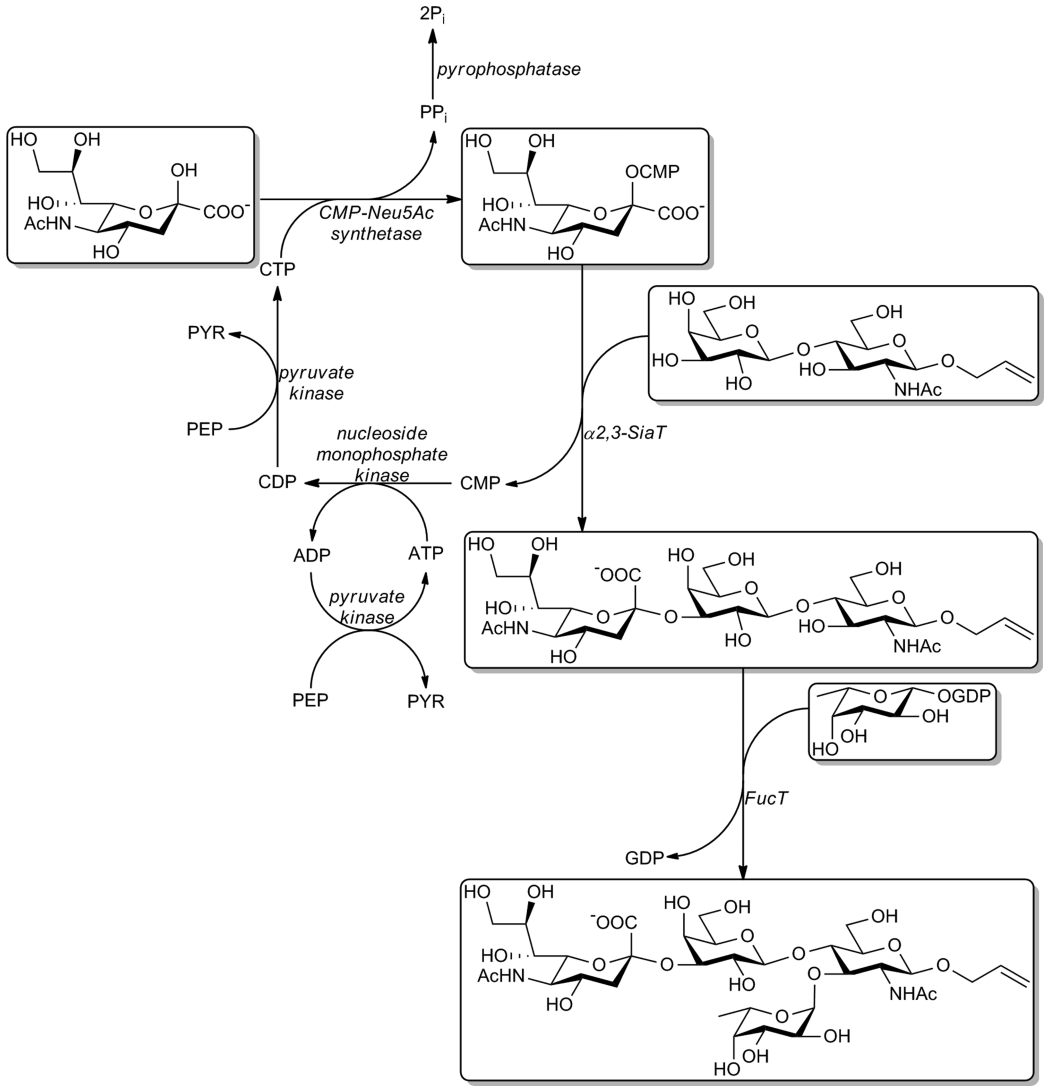
Scheme 18. Synthesis of Chondroitin by Resin-Bound GTase Mutants

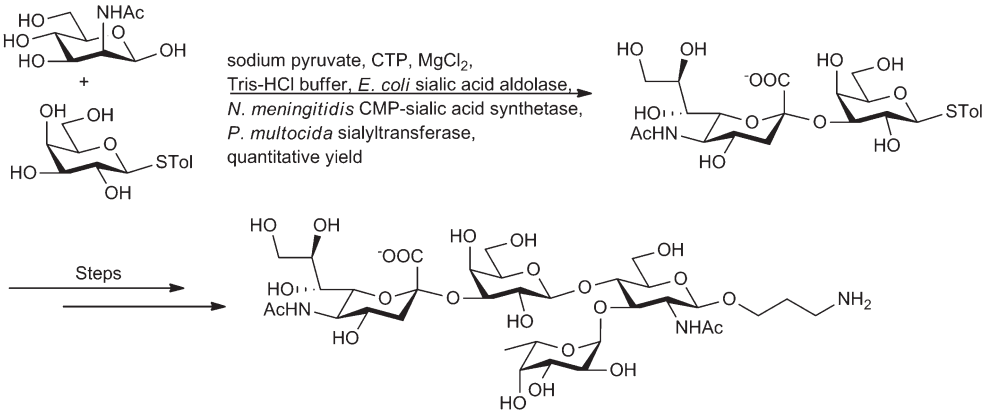


are particularly important and have been incorporated into numerous carbohydrate and glycoprotein syntheses. For example, P- and E-selections are two important and well-studied lectins that bind sLe^X and recruit leukocytes to regions of normal or damaged tissue, a process that is elevated in inflammatory diseases and cancer. An attractive model to inhibit overactive inflammation is the sLe^X bearing P-Selectin glycoprotein ligand-1 (PSGL-1), which is a known ligand for both selectins, and whose N-terminus was synthesized chemoenzymatically.[328] Paulson, Wong, and co-workers accomplished a landmark enzymatic synthesis of sLe^X in 1992 by incorporating a SiaT into a multienzyme approach (Scheme 19).[329] This enzyme system has many applications for syntheses of bivalent[330] and dendritic[331] sLe^X analogues. In an elegant display of SiaT utility, Lin and co-workers employed an α2,3-SiaT in their multienzyme, one-pot synthesis of a sLe^X containing PSGL-1 glycopeptide.[238] Cao and co-workers enhanced the chemoenzymatic synthesis of sLe^X by creating a scheme for in situ generation of CMP-sialic acid from ManNAc using biosynthetic enzymes from bacterial sources (Scheme 20).[332] In a more

Scheme 19. Multienzyme Synthesis of sLe^X



Scheme 20. Chemoenzymatic Synthesis of sLe^X Via Conversion of ManNAc to Neu5Ac



recent example, α2,3- and α2,6-SiaTs were used to synthesize sialoglycopolypeptides as glycomimetics in the inhibition of influenza infection.[305] In another large-scale example of chemoenzymatic synthesis, multigram quantities of 24 homogeneous

dx.doi.org/10.1021/cr200113w |*Chem. Rev.* 2011, 111, 4259–4307

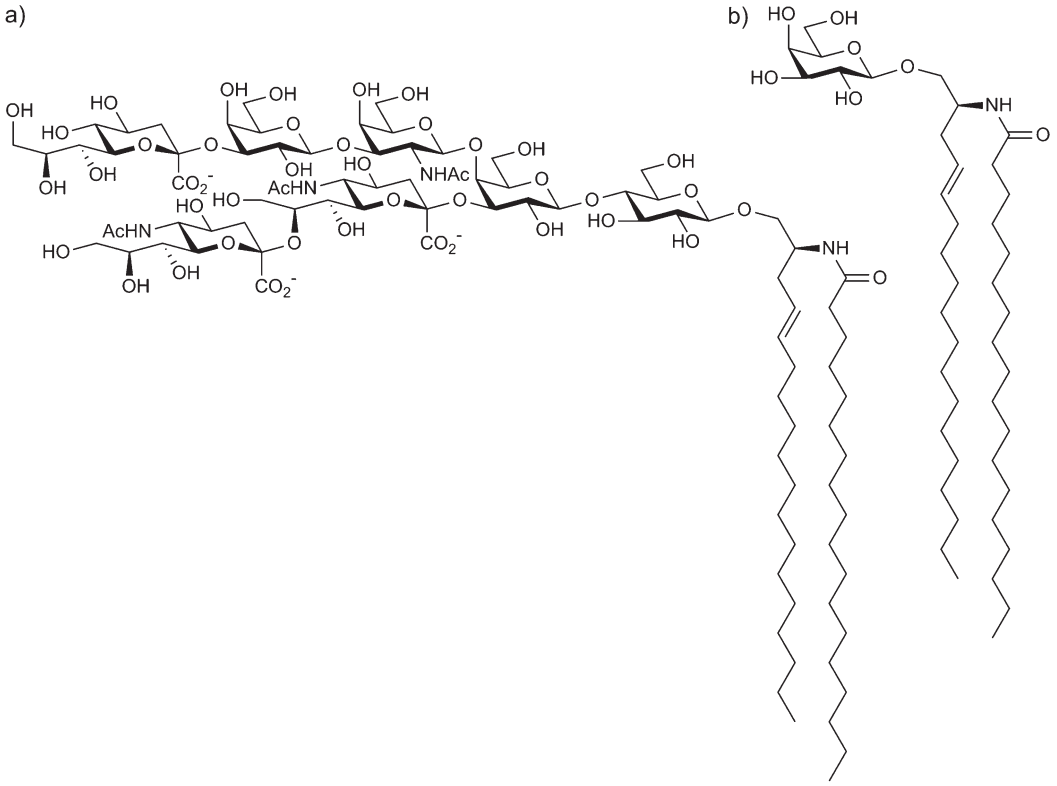


Figure 4. Glycosphingolipids: (a) ganglioside (GT1b) and (b) galactosylceramide.

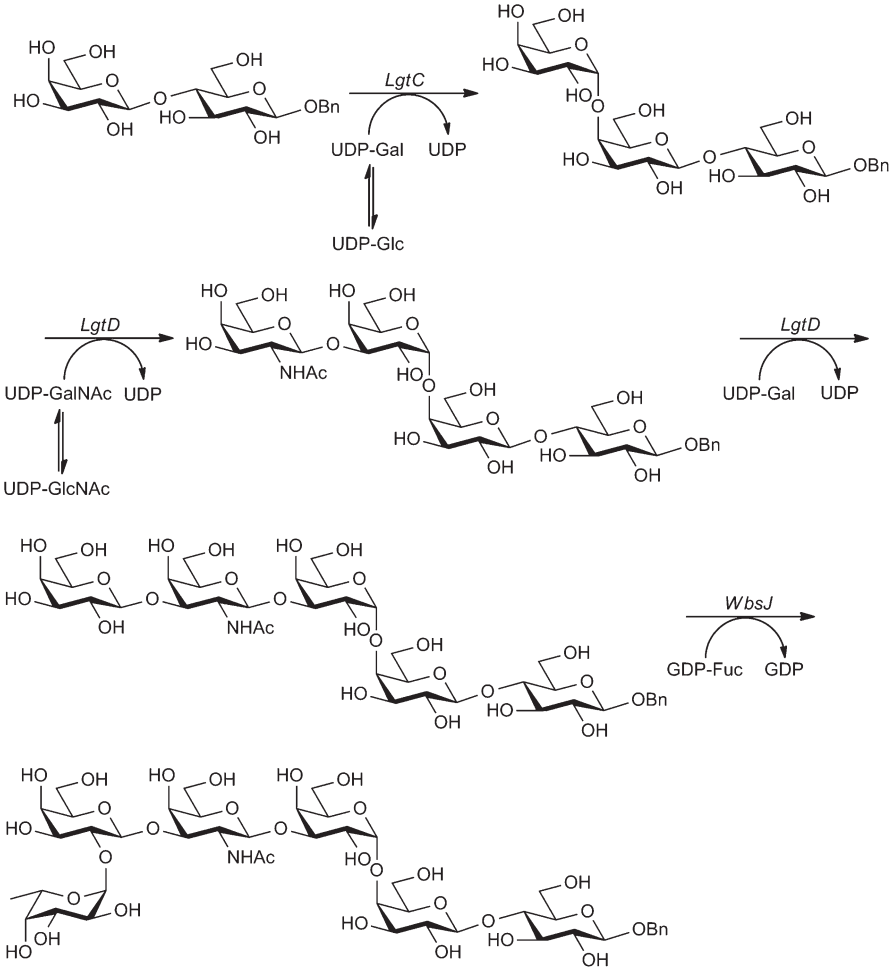
blood group and tumor-associated poly-*N*-acetyllactosamine antigens were synthesized by incorporating a variety of GTases including an α2,3- and an α2,6-SiaT.[333]

4.1.4. Galactosyltransferases (GalTs). GalTs can be used to transfer Gal residues to a number of acceptors using UDP-Gal (Table 2). These GTases are well characterized and extensively applied to chemoenzymatic syntheses of Lac and LacNAc-based motifs common to N- and O-linked glycoproteins as well as glycolipids.[247,256,257,263,334−339] Glycosphingolipids that have been linked to cancer, malaria, and many autoimmune disorders have Gal-rich sequences that can be targeted for chemoenzymatic synthesis using GalTs (Figure 4).[340] Indeed, a number of glycosphingolipid family members have been synthesized using GalTs cloned from their biosynthetic pathways.[341,342] In pioneering examples, α1,4-GalTs were used to enzymatically transfer Gal onto lactose acceptors to create globotriose,[252,253,343−345] including a metabolic engineering approach in bacteria by Ozaki and co-workers (see section 5.4).[253] A β-1,4-GalT was employed, along with several other GTases, toward the synthesis of sialylated IgG1 Fc with enhanced activity.[346] Chemoenzymatic methods with GalTs were improved by attaching the glycan to a solid support, a measure which did not affect GTase activity but drastically simplified product purification.[345] Importantly, bacterial α1,4-GalTs can be recombinantly expressed in *E. coli*, purified, and used for large-scale synthesis of globotriose;[252,343] they can also be employed for in vivo glycoengineering,[344] which will be discussed in section 5.

4.1.5. Fucosyltransferases (FucTs). FucTs can be used to transfer Fuc residues to a number of acceptors using GDP-Fuc (Table 2). Fuc is a C-6 deoxy L-sugar that endows unique binding properties to glycan structures, similarly to Neu5Ac. Fuc is commonly attached to N-linked glycan structures at the core and branch termini, and it is an integral member of important biological groups, such as the ABO and Lewis blood groups, which are found as terminal epitopes on many glycoconjugates. Along with SiaTs, FucTs have been integral to chemoenzymatic syntheses of selectin ligands and other cell-surface glycans.[238,329,332,347] FucTs have a high tolerance to variations with respect to donor and acceptor specificity that is unique among GTases, and which extends their utility and flexibility in carbohydrate synthesis.[230,231,235,237,240,242,244,348−354] Exemplifying donor versatility, a recombinant α1,3-FucT from *H. pylori* was implemented in the synthesis of sLe^X and five conformationally constrained sLe^X variants via promiscuous transfer of synthetically garnered GDP-fucose analogues.[347] A study with α1,2-FucT from *H. pylori* by Lin and co-workers exploited acceptor flexibility to gain chemoenzymatic access to a variety of tumor-associated Lewis antigens.[235] Surprisingly, even human FucT-VI has a relatively high acceptor tolerance, with an affinity for the unusual GalNAcβ1 → 4GlcNAc expressed by the human parasite *S. mansoni*.[241] Globo-H is a fucosylated member of the Globo family of gangliosides that is only expressed on tumor cells, making its structure of interest as a cancer biomarker and also as a potential cancer-vaccine antigen.[355−359] While Globo-H is accessible through chemical methods,[360−366] a facile and high-yielding chemoenzymatic route developed in 2008 employs three glycosyltransferases (LgtC, LgtD, and WbsJ) and two epimerases (GalE and WbgU), which elongate the Lac-based starting material to Globo-H in six steps with a remarkable overall yield of 57% (Scheme 21).[233,234]

4.1.6. *N*-Acetylhexosaminyltransferases (GlcNAcT and GalNAcT). GlcNAcTs and GalNAcTs can be used to transfer *N*-acetylhexosamine (HexNAc) residues to a number of acceptors using UDP-HexNAcs (Table 2). HexNAcs are common protein proximal glycan anchors (i.e., *N*-GlcNAc and *O*-GalNAc)

Scheme 21. Chemoenzymatic Synthesis of Globo-H-OBn



and connective residents within all types of glycan structures. As such, HexNAcTs are a diverse group of GTases for chemoenzymatic applications. For example, HexNAcTs can be chemoenzymatically employed in the building of GAGs, the repeating disaccharide units of which all contain HexNAcs. Betaglycan contains two GAG glycosylation sites, where Ser[535] may display either HS and CS, and Ser[546] only displays CS. Through chemical synthesis of tetraosyl peptide acceptor mimetics, Tamura and co-workers found that the neighboring amino acid residues near the GAG attachment modulate the relative activities of the competing GTases, β4GalNAcT-I (CS) and α4GlcNAcT-I (HS).[272] DeAngelis and co-workers identified a bifunctional GTase (PmHAS) from *P. multocida* that catalyzes the polymeric transfer of GlcNAc and GlcA subunits and can be used in the synthesis of HA.[283] In another example, Gilbert and co-workers employed the GalNAcTs, CgtE, and Pm1138 to synthesize the Forssman antigen (Scheme 22).[367] Although humans may not produce the Forssman antigen natively,[368,369] Pm1138 synthesizes a rare α1,3-GalNAc linkage that affords another tool for the enzymatic synthesis of uncommon carbohydrate linkages.

4.2. Glycosidases and Glycosynthases

Glycan trimming and breakdown is enacted by glycoside hydrolases (EC 3.2), a class of glycosidases that hydrolytically cleave glycosidic bonds. There are two broad subclasses of these glycosidases differing in the site of glycosidic bond cleavage and in the number of sugars that are released from the nonreducing end of an oligosaccharide: *exo*-glycosidases hydrolyze the nonreducing glycosidic bond to cleave a single terminal sugar (this includes cleavage of disaccharides), whereas *endo*-glycosidases hydrolyze an internal bond to cleave more than one sugar. Glycosidases comprise over 100 different families encompassing a diverse range of structural folds that have converged upon similar active site features for hydrolysis.[212] In general, glycosidase active sites contain two proximal carboxyl side-chains that mediate acid/base-catalyzed cleavage with water by three common mechanisms (Scheme 23): (1) a direct displacement mechanism leading to anomeric inversion, which entails glycosidic bond cleavage directly by an activated water; (2) a double inversion mechanism leading to net anomeric retention, which entails glycosidic cleavage via attack by an enzyme nucleophile to form a glycosylated-enzyme intermediate that is subsequently released by activated water attack; and (3) a substrate-assisted mechanism leading to anomeric retention, which entails glycosidic cleavage via an enzyme-activated intramolecular 1,2-trans attack by the C2-acetamido group on a nonreducing HexNAc, thereby cleaving it from its reducing end and forming an oxazolinium intermediate that is subsequently opened by an activated water molecule. Over 2500 glycosidases are known with capabilities for cleaving nearly every glycan bond, and many are

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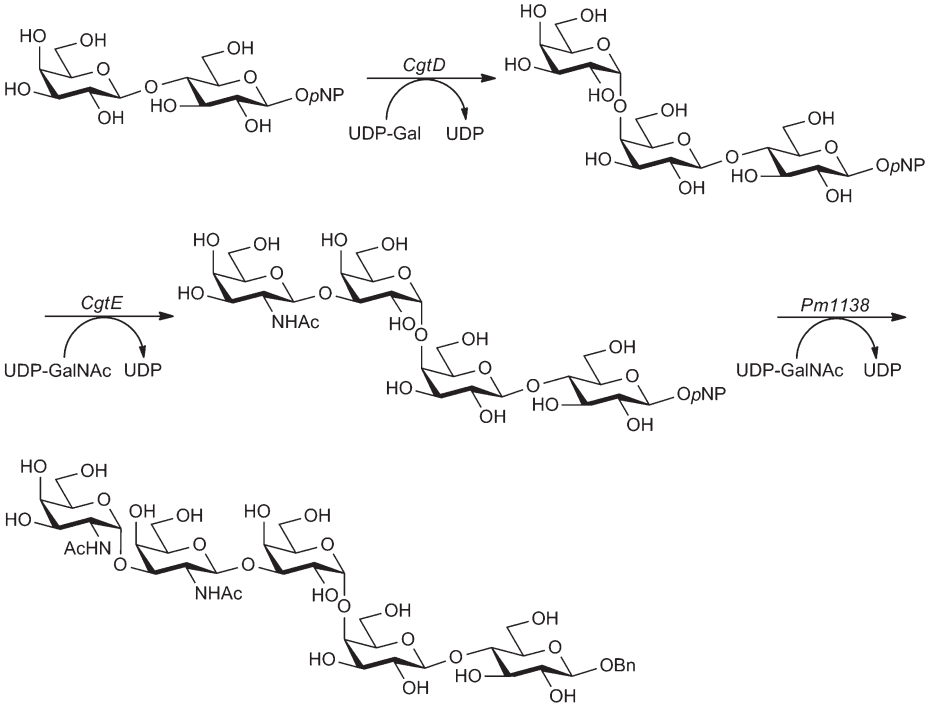
Scheme 22. Chemoenzymatic Synthesis of Forssman Antigen with *Pm1138*



commercially available or can be easily expressed and purified using *E. coli*. Glycosidases are long-standing tools in many fields of glycobiology, where they can be used to obtain information about glycosidic bonds and/or simplify glycan structures for glycan sequencing, glycoproteomics,[24,370] crystallography,[371,372] and gSARs.[54,373,24] Key enzymes for trimming and remodeling mammalian glycoprotein structures are shown in Figure 5.

Implementing and/or engineering glycosidases for chemoenzymatic glycan synthesis is of interest because, like GTases, glycosidases can create glycosidic bonds; however, glycosidases are more tractable, because they provide access to diverse glycan bonds, using inexpensive and synthetically accessible starting materials.[374−376] For chemoenzymatic purposes, glycosidases can be compelled toward synthesis by making the carbohydrate hydroxyl group a superior nucleophile over water (Scheme 24).[20,377−380] For example, thermodynamic conditions for reverse hydrolysis have been successful, although kinetic conditions for transglycosylation using an activated glycosyl donor, organic cosolvent, or excess glycosyl donor produce superior yields.[22] Transglycosylation has been successfully applied to a few glycan syntheses for *exo*-[381−384] and *endo*-glycosidases.[381−384] However, in reversed- and transglycosylation reactions, attention must be paid to undesired side-reactions of self-condensation and regiocondensation and, particularly, to product hydrolysis.[5,212,385] A combination of substrate engineering and mutational engineering of glycosidases can be used to overcome such limitations.[386] In particular, product cleavage can be averted by creating a rational mechanism-based glycosynthase, a glycosidase where a key catalytic residue has been removed to prevent hydrolysis, while the catalytic residue driving transglycosylation remains intact.[13,386,387] Beginning from the first rationally designed glycosynthase by Withers et al. nearly 20 years ago from a retaining *exo*-glycosidase,[388] there are now many examples of glycosynthase engineering that exploit all three hydrolytic glycosidase mechanisms (Scheme 23).[13,212,386,389−393] The utility of glycosidases and glycosynthases in glycan and glycoprotein synthesis is increasing, and several applications are listed in Table 3. Highlights include the synthesis of such challenging carbohydrate-containing molecules as homogeneous IgG,[82] glycosphingolipids,[394] glycan-containing flavonoids,[395] and cyclic glycans.[396]

4.2.1. *exo*-Glycosidases and *exo*-Glycosynthases. *exo*-Glycosidases can be turned into useful synthetic tools if their native activity of cleaving the nonreducing glycosidic residue off an oligosaccharide chain is reversed into the synthetic direction. Creating such glycosynthases is possible by both rational engineering[212] and directed evolution,[386] which, respectively, seek to eliminate hydrolytic activity and then enhance enzyme stability and specificity toward desired donors and acceptors. To date, most *exo*-glycosynthases have been designed from a parental double-displacement mechanism (Scheme 25). In this process, the enzymatic nucleophile, typically Asp, is mutated to a non-nucleophilic derivative, typically Ala, which disables cleavage of the glycosidic bond and formation of the glycosyl-enzyme intermediate (Scheme 25). However, this intermediate can be mimicked with a synthetic derivative, which, in place of the transient enzyme bond, bears an activated anomeric leaving group, such as fluoride. When the enzyme binds this synthetic donor, it will activate a carbohydrate acceptor for nucleophilic attack using the intact catalytic base, thereby forming a new glycosidic bond.[386] Detailed accounts of evolving and applying glycosynthases have been provided by Withers and coworkers.[16,212,389,392,397] Notably, this mechanism-based engineering technique has been expanded to create novel thioglycosynthases.[398] Glycosynthases have also been more recently generated from the inverting glycosidases, including *exo*-β-oligoxylanase[15,393] and 1,2-α-fucosidase,[13,399] expanding

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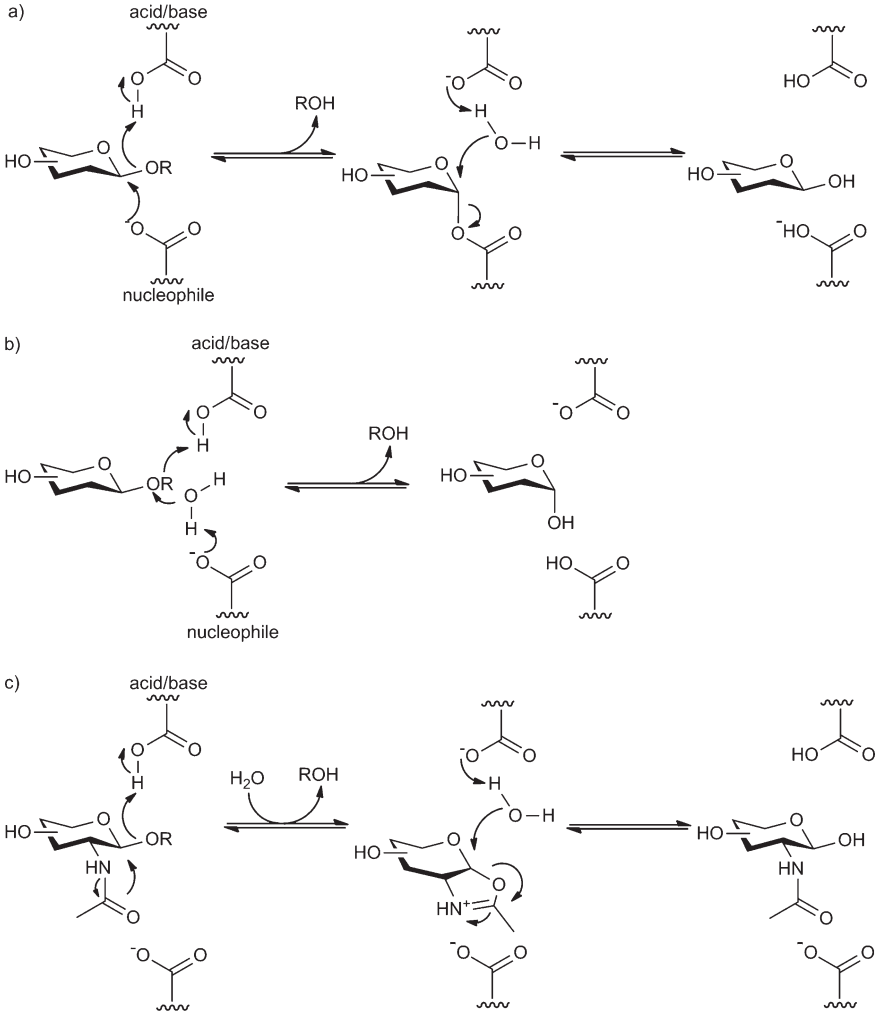
Scheme 23. Glycosidase Mechanisms for (a) Retaining, (b) Inverting, (c) (*R*)-*N*-Acetylhexosaminidases



the types of glycosidases that can be engineered for synthetic ends. Undoubtedly, engineering new glycosynthases will continue to create useful enzymes for glycan synthesis, and the current success of the technique is underscored by the variety of enzymes that are available to enact various stereo- and regiochemical linkages, as summarized in Table 3.[212,400−404]

4.2.2. *endo*-Glycosidases and *endo*-Glycosynthases. *endo*-Glycosidases are capable of cleaving internal glycosidic linkages in glycan structures, which provides another benefit to remodeling and synthetic endeavors.[12] For example, *endo*-glycosidases can be employed for facile homogenization of heterogeneous glycan populations, when they cleave at predictable sites within conserved glycan regions. After cleavage, a similar *endo*-glycosidase enzyme can be used in the synthetic direction to reelaborate the truncated glycan back into controlled homogeneous structure. One of the most significant classes of *endo*-glycosidases in this respect are the *endo*-β-*N*-acetylglucosaminidases (EC 3.2.1.96, commonly known as Endos and ENGases;), which act on *N*-glycan structures. ENGases hydrolyze the β-(1→4)-glycosidic bond comprising the *N*,*N*′-diacetylchitobiose core of *N*-glycans, leaving a single protein proximal N-linked GlcNAc residue. They are found widely distributed through nature with common chemoenzymatic variants including Endo-D, which is specific for pauci mannose; Endo-A and Endo-H, which are specific for high mannose; Endo-F subtypes, which range from high mannose to biantennary complex; and Endo-M, which can cleave most *N*-glycan structures (high mannose/complex-type/hybrid-type), as defined in Figure 5 (notably, Endos D, H, and F are commercially available). These ENGases show specificity toward the distal *N*-glycan structure and not the protein displaying it, making them useful for cleaving most N-linked glycans from glycoproteins under native conditions. For example, Endo-H has been used to truncate glycoproteins for crystallographic studies;[371,372] for glycoproteomic N-glycosylation site mapping;[24,370] and for establishing *N*-glycan-driven SARs, such as affects on protein folding and immune response,[54,430] as well as to create a basis for subsequent synthetic elaboration to different glycan structures, as illustrated by RNase (Scheme 3).[94,95] Some ENGases have more defined specificity, as exemplified by Endo-S, which is specific for cleaving only one conserved *N*-glycan in the FC domain of human IgGs.[431−435]

The transglycosylation activity of ENGases holds particular promise in the synthesis of homogeneous N-linked glycans, since they can elaborate a single N-linked GlcNAc that is accessible through methods of synthesis and enzymatic remodeling.[12] Transglycosylation activity has been characterized with

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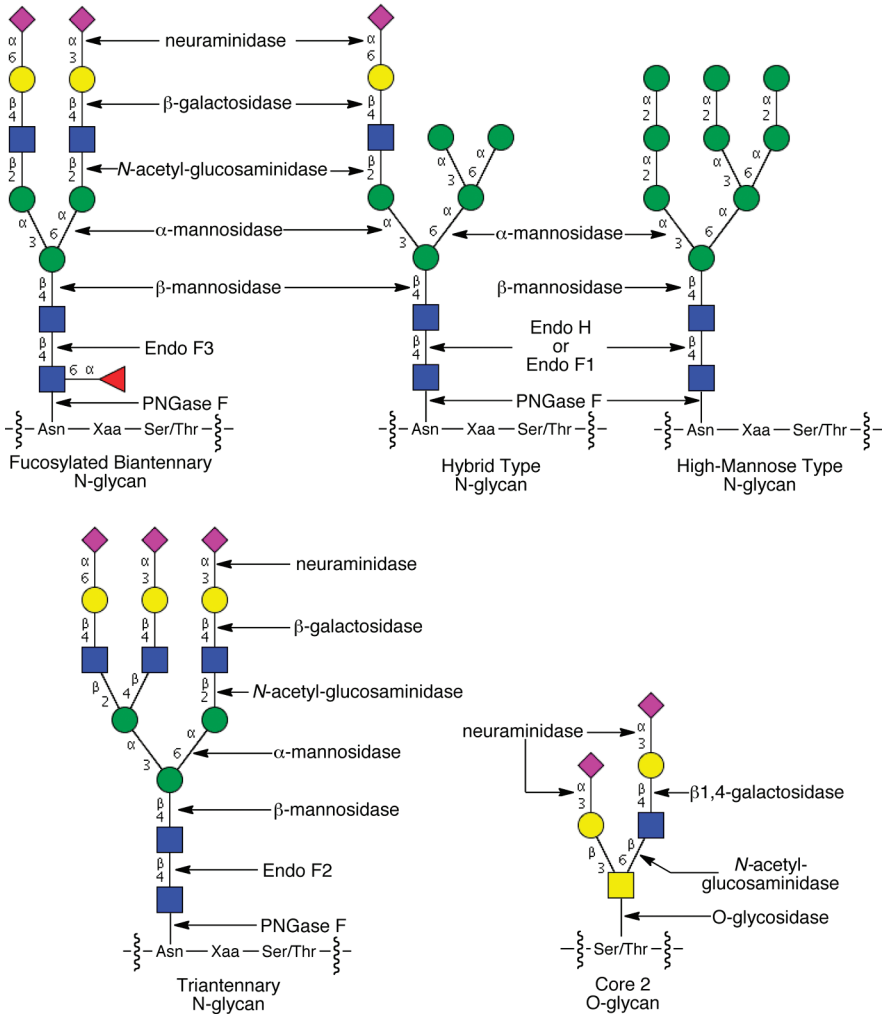
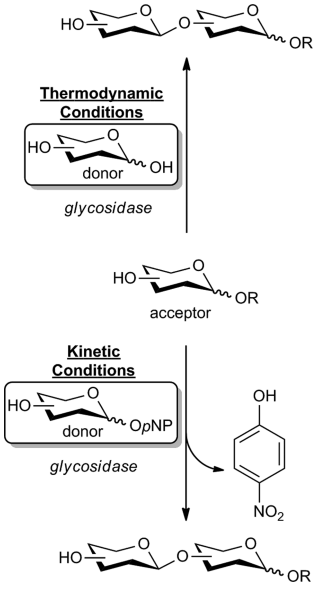


Figure 5. Glycosidase cleavage sites for N- and O-linked glycans.

Scheme 24. General Methods for Glycosidase-Catalyzed Carbohydrate Synthesis



Endo-A,[436,437] Endo-BH,[438] Endo-CE,[439] and Endo-M,[440] and of these, Endos -A and -M[441] have been the most thoroughly investigated for synthetic applications.[403,404,408−411,94,441−447] In line with its cleavage preference, Endo-A most efficiently transglycosylates high mannose structures, whereas Endo-M, which is rather flexible with respect to *N*-glycan cleavage, prefers complex structures for transglycosylation.[12] The difference in the transglycosylation specificities of these ENGases can be exploited to divergently elaborate a truncated GlcNAc-bearing intermediate, as demonstrated by the synthesis of homogeneous RNase B and C by respective Endo-A and -M mediated elaboration of GlcNAcylated RNase (Scheme 3).[94,96,97] These ENGases can also be used to introduce different *N*-glycan structures at unique glycosylation sites, as demonstrated by the ENGase-based chemoenzymatic synthesis at two glycosylation site on eel calcitonin by Haneda and co-workers (Scheme 26).[448] A more comprehensive understanding of the ENGase substrate specificities, and in particular acceptor requirements,[449−451] may lead to important regioselective ENGase applications.

The interest in driving ENGases in the synthetic direction has led to efficient routes that can minimize product hydrolysis.[12] As with all glycosidases, optimized conditions involving excess

dx.doi.org/10.1021/cr200113w |*Chem. Rev.* 2011, 111, 4259–4307

Table 3. Synthetically Relevant Glycosynthases

entry	glycosyl donor	glycosyl acceptor(s)	source (abbreviation)	mutation	linkage(s) synthesized	ref
			Cellobiose			
1	α-Cel-F	β-Cel-*p*NP	Cel5A (*C. cellulolyticum*)	E307G	β-(1→3)-/β-(1→4)-	*(1)*
			Fucose			
2	β-Fuc-F	Lac	AfcA (*B. bifidum*)	D766G/N423G/N421G	α-(1→2)	*(2)*
3	β-Fuc-N_3	β-Xyl-*p*NP/β-GlcNAc-*p*NP/β-Gal-*p*NP	Ss (*S. solfataricus*)	D242S	α-(1→2)-/α-(1→3)-/α-(1→4)-/α-(1→6)-	*(3)*
4	β-Fuc-N_3	β-Xyl-*p*NP	Ss (*S. solfataricus*)	D224G	α-(1→3)-/α-(1→4)-	*(3)*
			Galactose			
5	α-Gal-F	β-Glc-*p*NP/β-Xyl-*p*NP/β-Glc-2-Cl-2,4-DNP/β-Man-*p*NP/β-Cel-*p*NP/β-Cel-OPh-4-OMe	Abg (*Agrobacterium* sp.)	E358A	β-(1→3)-/β-(1→4)-	*(4), (5)*
6	α-Gal-F	β-Glc-Ph	Tt (*T. thermophilus*)	E338A	β-(1→3)-	*(6)*
7	α-Gal-F	β-Glc-Ph/β-Gal-Ph	Tt (*T. thermophilus*)	E338S	β-(1→3)-	*(6)*
8	α-Gal-F	β-Glc-Ph/β-Glc-*p*NP/β-Glc-Bn/β-Glc-4-CN-Ph/β-Glc-1-S-Ph	Tt (*T. thermophilus*)	E338G	β-(1→3)-/β-(1→6)-	*(6), (7)*
9	α-Gal-F	6-*O*-Bn-D-glucopyranose/6-*O*-Bz-D-glucopyranose/6-*O*-(4-nitrobenzyl)-D-glucopyranose	Abg (*Agrobacterium* sp.)	E358S	β-(1→2)-/β-(1→3)-	*(8)*
10	α-Gal-F	β-Glc-*p*NP/Man-*p*NP/β-Cel-*p*NP/GlcNAc-*p*NP	Abg (*Agrobacterium* sp.)	E358S	β-(1→3)-/β-(1→4)-	*(9)*
11	α-Gal-F	Glc-2-AP/Glc-3-AP/Glc-4-AP (Resin Bound)	Abg (*Agrobacterium* sp.)	E358G	β-(1→3)-/β-(1→4)-	*(10)*
12	α-Gal-F	β-Glc-*p*NP/β-Gal-*p*NP/β-Xyl-*p*NP/β-Man-*p*NP	*Streptomyces* sp.	E383A	β-(1→3)-/β-(1→4)-	*(11)*
13	α-Gal-F	β-Glc-*p*NP/β-Cel-*p*NP/β-Glc-Ph	LacZ (*E. coli*)	E537S	β-(1→6)-	*(12)*
13	α-Gal-*p*NP	melibiose	Aga2 (*B. breve* 203)	recombinant	α-(1→4)-	*(13)*
14	β-Gal-DNP	β-Glc-4S-*p*NP	Abg (*Agrobacterium* sp.)	E171G	β-(1→4)-	*(14)*
15	β-Gal-2-NP	α-Xyl-4P, β-Xyl-4P	Ss (*S. solfataricus*)	E387G	β-(1→3)-/β-(1→4)-	*(15)*
16	β-Gal-2-NP	β-Xyl-4P	Ta (*T. aggregans*)	E386G	β-(1→3)-/β-(1→4)-	*(15)*
17	α-GalUA-F	β-Glc-*p*NP/β-Xyl-*p*NP/β-Cel-*p*NP	Tm (*T. maritima*)	E476A	β-(1→3)-	*(16)*
18	β-GalUA-*p*NP	β-Glc-4S-*p*NP/β-GlcNAc-4S-*p*NP/β-Glc-3S-*p*NP	Tm (*T. maritima*)	E383A	β-(1→4)-	*(16)*
			Glucose			
19	α-Glc-F	β-Glc-Ph/β-Glc-*p*NP/β-Glc-2-NP/β-Glc-2-F-2,4-DNP/β-Glc-MU/β-Glc-OPh-4-OMe/β-Xyl-*p*NP/β-Man-*p*NP/β-Cel-*p*NP	Abg (*Agrobacterium* sp.)	E358A	β-(1→3)-/β-(1→4)-	*(4)*
20	α-Glc-F	β-Glc-*p*NP/β-Man-*p*NP/β-Cel-*p*NP	Abg (*Agrobacterium* sp.)	E358S	β-(1→3)-/β-(1→4)-	*(9)*
21	α-Glc-F	β-Glc-2-NP	Ss (*S. solfataricus*)	E387G	β-(1→3)-/β-(1→4)-/β-(1→6)-	*(17)*
22	α-Glc-F	β-Glc-Ph/β-Gal-Ph	Tt (*T. thermophilus*)	E338A	β-(1→3)-	*(6)*
23	α-Glc-F	β-Glc-Ph/β-Cel-*p*NP/β-Glc-Bn/β-Glc-4-CN-Ph	Tt (*T. thermophilus*)	E338G	β-(1→3)-	*(6), (7)*
24	α-Glc-F	β-Glc-Ph/β-Gal-Ph/β-Fuc-*p*NP/β-Cel-*p*NP/β-Glc-Me	Tt (*T. thermophilus*)	E338S	β-(1→3)-	*(6)*
25	α-Glc-F	β-Glc-*p*NP/β-Gal-*p*NP/β-Xyl-*p*NP/β-Man-*p*NP/β-Fuc-*p*NP/β-Lam-*p*NP/β-Cel-*p*NP	(*Streptomyces* sp.)	E383A	β-(1→3)-/β-(1→4)-	*(11)*
26	α-Glc-F	β-Glc-4S-*p*NP/β-Glc-4S-MU	Abg (*Agrobacterium* sp.)	E171A and E358G	β-(1→4)-	*(18)*
27	β-Glc-F	α-Glc-*p*NP/α-Xyl-*p*NP/β-Glc-*p*NP/α-Man-*p*NP	Sp (*S. pombe*)	D481G	α-(1→4)-/α-(1→6)-	*(19)*
28	β-Glc-DNP	β-Glc-4S-*p*NP/β-Xyl-4S-*p*NP/1,4-dithio-D-glucopyranoside	Abg (*Agrobacterium* sp.)	E171A	β-(1→4)-	*(20), (21)*
29	β-Glc-DNP	β-Lac-4S-*p*NP	Abg (*Agrobacterium* sp.)	E171G	β-(1→4)-	*(14)*

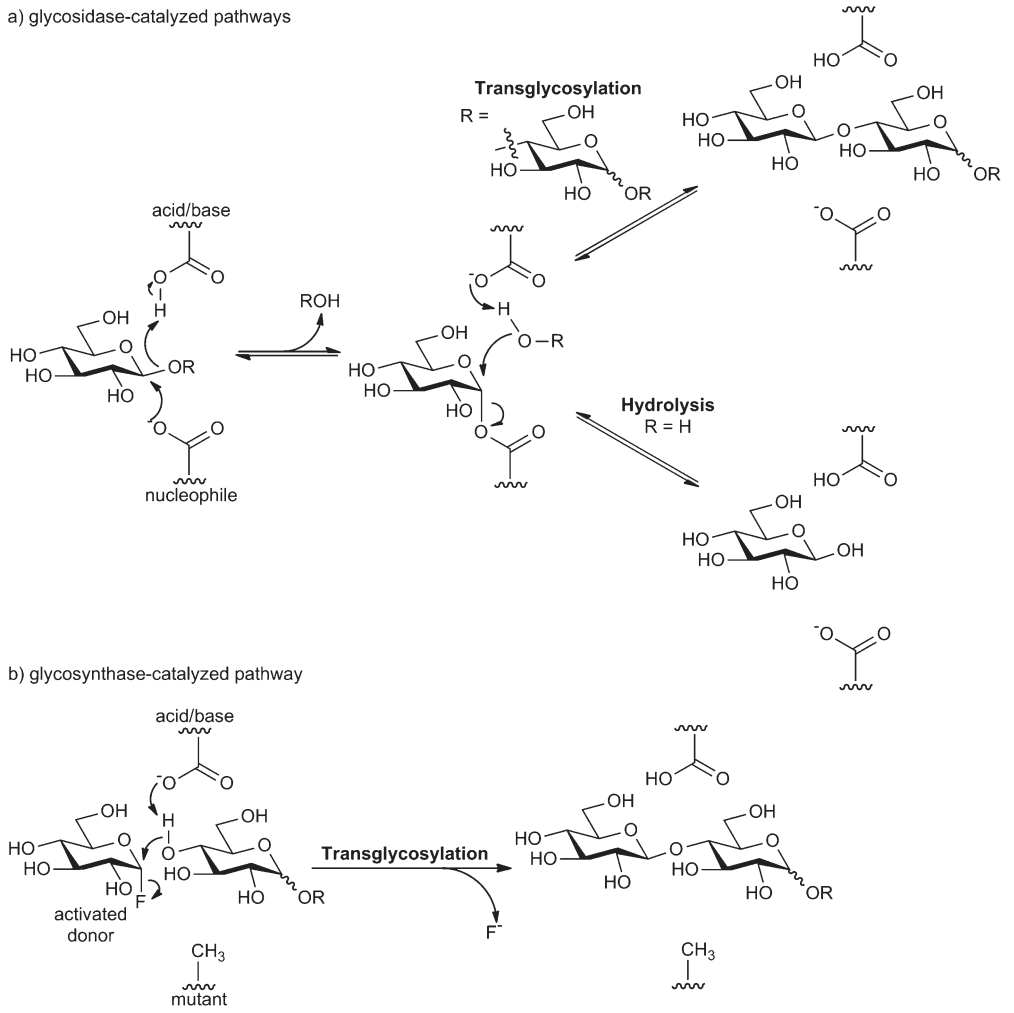
dx.doi.org/10.1021/cr200113w |*Chem. Rev.* 2011, 111, 4259–4307

Table 3. Continued

entry	glycosyl donor	glycosyl acceptor(s)	source (abbreviation)	mutation	linkage(s) synthesized	ref
30	β-Glc-DNP	β-GlcNAc-4S-*p*NP	Abg (*Agrobacterium* sp.)	E171Q	β-(1→4)-	(14)
31	β-Glc-2-NP	α-Glc-*p*NP/β-Glc-*p*NP/β-Glc-MU/ α-Glc-OMe/β-Glc-OMe/α-Gal-*p*NP/ α-Man-pNP/α-GlcNAc-2-NP/ β-Lam-2-NP/α-Mal-*p*NP	Ss (*S. solfataricus*)	E387G	β-(1→3)-/β-(1→4)-/ β-(1→6)-	(22)
32	β-Glc-2-NP	β-Glc-MU/ β-Lam-MU	Ta (*T. aggregans*)	E386G	β-(1→3)-/β-(1→4)-	(15)
33	α-GlcUA-F	β-Glc-*p*NP/β-Xyl-*p*NP/β-Cel-*p*NP/ β-GlcUA-*p*NP	Tm (*T. maritima*)	E476A	β-(1→3)-	(16)
34	β-GlcUA-*p*NP	β-Glc-4S-*p*NP/β-GlcNAc-4S-*p*NP/ β-Glc-3S-*p*NP	Tm (*T. maritima*)	E383Q	β-(1→4)-	(16)
			Lactose			
35	α-Lac-F	β-Cel-*p*NP	Cel5A (*C. cellulolyticum*)	E307G	β-(1→3)-	(1)
36	α-Lac-F	N^{I}-acetyl-2^{II}-azido-chitobiose	Cel7B (*H. insolens*)	E197A/H209A/ A211T	β-(1→3)-	(23)
37	α-Lac-F	Glc-OBn/Xyl-OBn/Man-S-*p*NP/ β-Cel-OMe/(C-2^{I}-Br) β-Cel-OMe/ $GlcNH_2$-β-(1−4)-glcNAc/ β-Lam-OBn	Cel7B (*H. insolens*)	E197A	β-(1→4)-	(24), (25)
			Laminaribiose			
38	α-Lam-F	β-Glc-MU/β-Lam-MU/β-Cel-MU	Blg (*B. licheniformis*)	E134A	β-(1→4)-	(25), (26)
39	α-Lam-F	α-Lam-F/Xyl-*p*NP/β-Gal-*p*NP/ β-Cel-*p*NP/Gent-*p*NP	Hv (*H. vulgare*)	E231G	β-(1→3)-	(27), (28)
	α-Lam-F					
			Mannose			
40	α-Man-F	β-Man-*p*NP/2-F-Man-2,4-DNP/ β-Glc-*p*NP/β-Xyl-*p*NP/ β-Cel-*p*NP/β-Gent-*p*NP	Man2A (*C. fimi*)	E519S	β-(1→3)-/β-(1→4)-	(29), (30)
41	α-Man-F	β-Glc-*p*NP	Abg (*Agrobacterium* sp.)	2F6	β-(1→3)-/β-(1→4)-	(31)
42	β-Man-DNP	4-S-Glc-*p*NP/4-S-Xyl-*p*NP	Man2A (*C. fimi*)	E429A	β-(1→4)-	(20)
43	α-Mannobiosyl-F	β-Glc-*p*NP/β-Man-*p*NP/β-Xyl-*p*NP/ β-Cel-*p*NP	Man26A (*C. japonicus*)	E320G	β-(1→4)-	(32)
			Xylose			
44	α-Xyl-F	β-Glc-*p*NP/β-Xyl-*p*NP/Xyl2-*p*NP	Abg (*Agrobacterium* sp.)	2F6	β-(1→3)-/β-(1→4)-	(31), (33)
45	α-Xyl-F	β-Glc-*p*NP/β-Xyl-*p*NP/β-Man-*p*NP/ α-XylF	Gs (*G. stearothermophilus*)	E335G	β-(1→3)-/β-(1→4)-	(34)
46	α-Xyl-2-F	β-Xyl-*p*NP/Xyl-2-SBn	Cf (*C. fimi*)	E235G	β-(1→4)-	(35)

(1) Tao, H. Y.; Peralta-Yahya, P.; Decatur, J.; Cornish, V. W. *ChemBioChem* **2008**, *9*, 681. (2) Wada, J.; Honda, Y.; Nagae, M.; Kato, R.; Wakatsuki, S. et al. *FEBS Lett.* **2008**, *582*, 3739. (3) Wang, L.-X. *Chem. Biol.* **2009**, *16*, 1026. (4) Mackenzie, L. F.; Wang, Q. P.; Warren, R. A. J.; Withers, S. G. *J. Am. Chem. Soc.* **1998**, *120*, 5583. (5) Shaikh, F. A.; Withers, S. G. *Biochem. Cell. Biol.* **2008**, *86*, 169. (6) Drone, J.; Feng, H. Y.; Tellier, C.; Hoffmann, L.; Tran, V. et al. *Eur. J. Org. Chem.* **2005**, 1977. (7) Marton, Z.; Tran, V.; Tellier, C.; Dion, M.; Drone, J. et al. *Carbohydr. Res.* **2008**, *343*, 2939. (8) Stick, R. V.; Stubbs, K. A.; Watts, A. G. *Aust. J. Chem.* **2004**, *57*, 779. (9) Mayer, C.; Zechel, D. L.; Reid, S. P.; Warren, R. A.; Withers, S. G. *FEBS Lett.* **2000**, *466*, 40. (10) Tolborg, J. F.; Petersen, L.; Jensen, K. J.; Mayer, C.; Jakeman, D. L. et al. *J. Org. Chem.* **2002**, *67*, 4143. (11) Faijes, M.; Saura-Valls, M.; Perez, X.; Conti, M.; Planas, A. *Carbohydr. Res.* **2006**, *341*, 2055. (12) Jakeman, D. L.; Withers, S. G. *Can. J. Chem.* **2002**, *80*, 866. (13) Zhao, H.; Lu, L.; Xiao, M.; Wang, Q.; Lu, Y. et al. *FEMS Microbiol. Lett.* **2008**, *285*, 278. (14) Mullegger, J.; Jahn, M.; Chen, H. M.; Warren, R. A.; Withers, S. G. *Protein Eng., Des. Sel.* **2005**, *18*, 33. (15) Trincone, A.; Giordano, A.; Perugino, G.; Rossi, M.; Moracci, M. *Bioorg. Med. Chem. Lett.* **2003**, *13*, 4039. (16) Mullegger, J.; Chen, H. M.; Chan, W. Y.; Reid, S. P.; Jahn, M. et al. *ChemBioChem* **2006**, *7*, 1028. (17) Trincone, A.; Perugino, G.; Rossi, M.; Moracci, M. *Bioorg. Med. Chem. Lett.* **2000**, *10*, 365. (18) Jahn, M.; Chen, H.; Mullegger, J.; Marles, J.; Warren, R. A. et al. *Chem. Commun.* **2004**, 274. (19) Okuyama, M.; Mori, H.; Watanabe, K.; Kimura, A.; Chiba, S. *Biosci. Biotechnol. Biochem.* **2002**, *66*, 928. (20) Jahn, M.; Marles, J.; Warren, R. A.; Withers, S. G. *Angew. Chem., Int. Ed.* **2003**, *42*, 352. (21) Stick, R. V.; Stubbs, K. A. *Tetrahedron: Asymmetry* **2005**, *16*, 321. (22) Trincone, A.; Giordano, A.; Perugino, G.; Rossi, M.; Moracci, M. *ChemBioChem* **2005**, *6*, 1431. (23) Blanchard, S.; Armand, S.; Couthino, P.; Patkar, S.; Vind, J. et al. *Carbohydr. Res.* **2007**, *342*, 710. (24) Fort, S.; Boyer, V.; Greffe, L.; Davies, G.; Moroz, O. et al. *J. Am. Chem. Soc.* **2000**, *122*, 5429. (25) Faijes, M.; Fairweather, J. K.; Driguez, H.; Planas, A. *Chemistry* **2001**, *7*, 4651. (26) Malet, C.; Planas, A. *FEBS Lett.* **1998**, *440*, 208. (27) Fairweather, J. K.; Hrmova, M.; Rutten, S. J.; Fincher, G. B.; Driguez, H. *Chemistry* **2003**, *9*, 2603. (28) Hrmova, M.; Imai, T.; Rutten, S. J.; Fairweather, J. K.; Pelosi, L. et al. *J. Biol. Chem.* **2002**, *277*, 30102. (29) Mayer, C.; Jakeman, D. L.; Mah, M.; Karjala, G.; Gal, L. et al. *Chem. Biol.* **2001**, *8*, 437. (30) Nashiru, O.; Zechel, D. L.; Stoll, D.; Mohammadzadeh, T.; Warren, R. A. et al. *Angew. Chem., Int. Ed.* **2001**, *40*, 417. (31) Kim, Y. W.; Lee, S. S.; Warren, R. A.; Withers, S. G. *J. Biol. Chem.* **2004**, *279*, 42787. (32) Jahn, M.; Stoll, D.; Warren, R. A.; Szabo, L.; Singh, P. et al. *Chem. Commun.* **2003**, 1327. (33) Kim, Y. W.; Chen, H.; Withers, S. G. *Carbohydr. Res.* **2005**, *340*, 2735. (34) Ben-David, A.; Bravman, T.; Balazs, Y. S.; Czjzek, M.; Schomburg, D. et al. *ChemBioChem* **2007**, *8*, 2145. (35) Kim, Y. W.; Fox, D. T.; Hekmat, O.; Kantner, T.; McIntosh, L. P. et al. *Org. Biomol. Chem.* **2006**, *4*, 2025.

Scheme 25. Nucleophilic Mutation in Glycosynthases Inhibits Product Hydrolysis



donor, optimized pH, and mixed-solvent systems can be used to boost synthetic yields with ENGases.[452−455] For example, the addition of 10−30% acetone can improve the yields of Endo-A (e.g., in study, 30% acetone (v/v) improved yields from 32% to 89%) and Endo-M, a property that Fairbanks and co-workers have attributed to a decreased product hydrolysis.[456] A powerful method for improving rates of transglycosylation is to engineer the sugar donor to be more reactive. This is accomplished by using sugar donors preactivated as oxazolines, presumably because the oxazole mimics the substrate-assisted enzyme transition state, thereby driving higher rates of conversion using less overall donor (Scheme 27).[82,94,449,457−462] In a pioneering study using oxazoline starting material, Wang et al. used Endo-A, a high-mannose oxazole, and a GlcNAc-bearing peptide (prepared by Fmoc SPPS) to synthesize C34, a truncated HIV-1 transmembrane protein gp41 peptide sequence containing a highly conserved N-linked high mannose that is essential for HIV fusion with the host cell membrane (Scheme 28).[463] Competing product hydrolysis in oxazole reactions can be suppressed by producing *N*-glycan products that are incompatible with the hydrolytic binding pocket, although this often means that non-native *N*-glycan structures are produced.[449,457,464] Transglycosylation with oxazoles can be driven further when combined with glycosynthases engineering, as demonstrated by Yamamoto and co-workers with Endo-M[465] and Fairbanks and co-workers with Endo-A.[466] Both of these endeavors found ENGases devoid of hydrolytic activity, including Endo-M-N175A and Endo-A-E173Q, both of which carry a key Asn mutation that is believed to render the enzyme unable to activate the GlcNAc 2-acetamido group for formation of the oxazole intermediate and cleavage of the glycosidic bond; however, these Asn mutants can still perform transglycosylation when provided the preformed oxazole donor. In addition to generating glycosynthases by blocking anchimeric assistance, hydrolysis can be reduced by removing a key tyrosine residue that activates water for attack on oxazole intermediates.[465] Optimizing ENGase transglycosylations has led to the practical synthesis of a large number of carbohydrates and glycopeptides, with some yields approaching 90%.[12,82,94,97,449,457−460,462,465−469] Notable examples include the synthesis of CD52,[470] HIV−I V3,[458] HIV-1 C34 glycopeptide,[465] RNase glycoprotein derivatives,[94,468] and human IgG1-Fc.[82]

In addition to the ENGases, other glycosidases have been engineered into useful *endo*-glycosynthases.[12,97,394,395,400,427,465,471−480] Most of these *endo*-glycosynthases are like the *exo*-glycosynthases in that they use the double inversion mechanism and require mutation of the enzymatic nucleophile that cleaves the glycosidic bond and forms the enzyme intermediate (see Scheme 25 for

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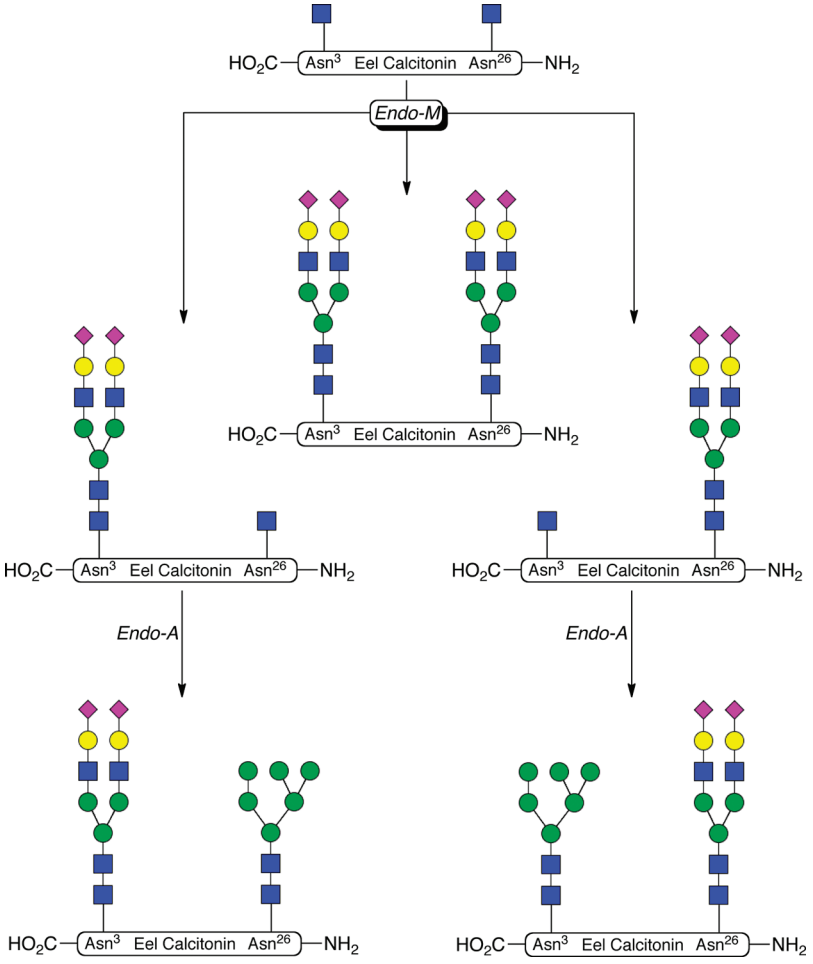
Scheme 26. Synthesis of Three Distinct Eel Calcitonin Glycoforms

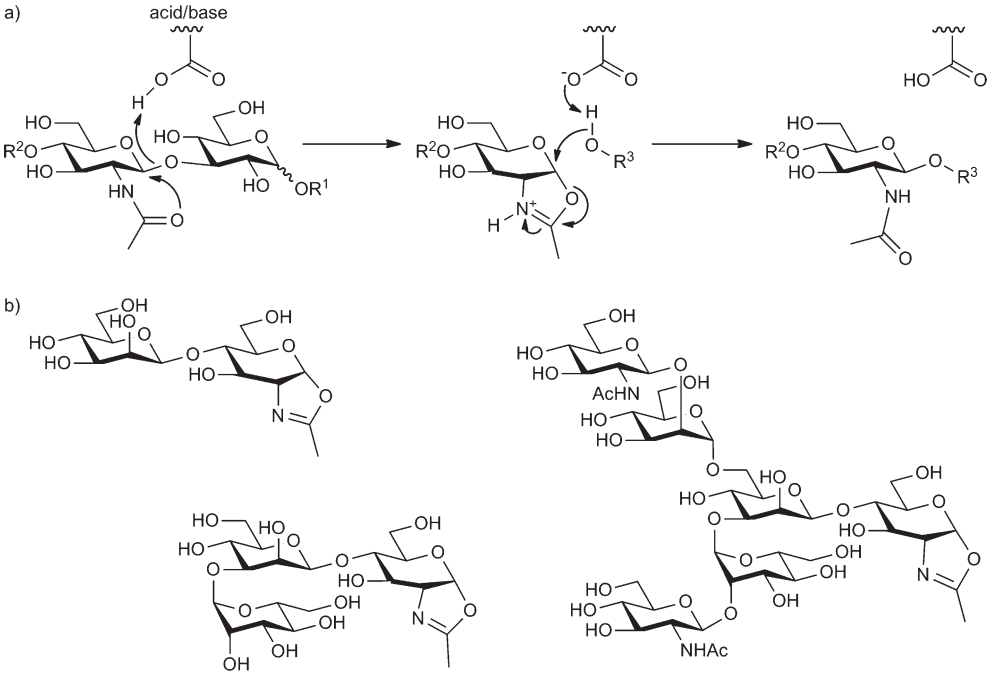


exodouble displacement mechanism) for glycosynthase engineering. Cel7B from *H. insolens* was mutated into several variants, including the single E197A variant,[400,404] the double E197A:H209A and E197A:H209G, and the triple E197A:H209A:A211T variant.[404] Interestingly, when subjected to a lactosyl fluoride donor and an *O*-allyl N^{I}-acetyl-2^{II}-azido-β-chitobioside acceptor, the double mutants preferentially catalyzed the β-(1→4) linkage, whereas the single and triple mutants yielded predominately β-(1→3) products, showing for the first time modulation of regioselectivity through site-directed mutagenesis by an *endo*-glycosynthase.[404] In an effort to provide additional strategies to form the often problematic β-mannosidic linkages, a β-Mannanase (Man26A) engineered to Glu320Gly was applied to catalyze the transfer of an α-mannobiosyl fluoride to Glc-*p*NP, Man-*p*NP, Xyl-*p*NP and Cel-*p*NP, forming exclusively β-(1→4) linkages.[427] *endo*-Glycosynthases can also be applied to transfer full-length oligosaccharides to acceptors other than sugars, as demonstrated by engineered glycosylation of glycosphingolipids[340,394] and flavonoids.[395]

5. GLYCOENGINEERING IN CELLS

Because they are covered with glycan structures, all cells have endogenous machinery for the synthesis of carbohydrates and glycoconjugates. The glycomodifying infrastructure within cells includes glycosidases, GTases, mechanisms for activated-sugar synthesis and transport, and supporting functions, which are under coordinated control to orchestrate the formation of precise glycan/glycoconjugate structures. The vast majority of these structures pose significant challenge to synthetic endeavors ex vivo and can only be made efficiently in vivo. As such, the goal of glycoengineering approaches is to manipulate the biosynthetic power of cells to transform them into synthetic tools for the production of specific glycan products. Over the past 10 years, glycoengineering approaches have surged. They have provided new routes to producing human glycoproteins of controlled glycoform, by manipulating mammalian glycosylation pathways (section 5.1), by creating glycoprocessing enzymes with novel properties (section 5.2), and by modifying nonmammalian systems (section 5.3), including humanizing the N-glycosylation pathways of yeast, insect, and plant cell systems and introducing protein glycosylation pathways into *E. coli*. Glycoengineering efforts have also transformed bacteria into factories for complex carbohydrate production and into tools to study and thwart glycan-related pathogenic mechanisms (section 5.4). Key to these strategies is the manipulation and augmentation of the endogenous cellular glycoprocessing infrastructure with novel enzyme activity. As an example, glycan sialylation pathways have been introduced into yeast,[83,85] insect,[481,482] plant,[483] and bacterial systems,[484,485] to produce biologically important sialylated glycans. This involves

Scheme 27. Oxazolines in ENGase-Catalyzed Glycoprotein Elaboration: (a) Proposed ENGase Reaction Mechanism and (b) Examples of Oxazolines Used in ENGase-Catalyzed Carbohydrate Transfer



the engineering of several genes for (i) biosynthesis, (ii) activation, (iii) transport, and (iv) transfer of Neu5Ac (Scheme 29a). Other examples discussed in this section demonstrate how harnessing and augmenting the synthetic power cells through glycoengineering can lead to powerful synthetic tools for glycans and glycoconjugates.

5.1. Engineering Glycosylation for the Production of Homogeneous Glycoproteins in Mammalian Cells

Mammalian cells harbor extensive glycosylation machinery that is capable of producing a wide array of glycan structures in accord with the functional needs of the cell. While this complexity makes mammalian cells a poor host for the production of homogeneous glycoproteins, the capability of these cells to perform the proper post-translational modification, folding, and function of many recombinant glycoproteins necessitates their use.[58,60,371,372] In fact, the manufacture of glycoprotein biologics is predominantly performed in mammalian systems, and mainly in Chinese hamster ovary (CHO) cell lines, wherein glycoform heterogeneity is monitored as a "glycan profile" that must be reliably reproduced using carefully controlled bioprocessing conditions.[63,486] Glycan profiles effectively address standards for safety (i.e., a particular biologic with a particular glycan profile will not lead to disadvantageous effects, such as an immune reaction); however, they often do not maximize the advantageous effects of the most therapeutically active glycoform.[487] Mammalian cells present other notable challenges, with regard to: (1) the expense of maintenance, (2) the time for recombinant protein production, and (3) the possibility of human pathogen contamination, all in addition to glycan heterogeneity. Glycoengineering approaches can be used to simplify, control, and/or enhance glycosylation in mammalian cell lines when their use is necessary. A prominent limitation to glycoengineering mammalian cells is their intractability toward homologous recombination, which complicates directly simplifying glycosylation pathways through targeted genetic knockdown.[488] Mutations in glycosylation pathways can be engineered using toxic lectin-resistance (Lec^R) selections, and fortunately, a wide variety of such cell lines are available (section 5.1.1). Alternatives to control glycan structures in mammalian cells are to temporarily block glycosylation pathways leading to undesired structures using RNA interference and small-molecule inhibitors (section 5.1.1). Glycoprocessing activity can also be transiently coexpressed to block, introduce, and/or enhance specific glycan structures in mammalian cells (section 5.1.2).

5.1.1. Simplified Glycosylation in Mammalian Cell Lines Through Mutation, Knockout, and Inhibition. Mutation, knockout, and inhibition present possibilities for controlling or simplifying N- and/or O-linked glycosylation profiles in mammalian cell lines. Within this category, cell lines with genetic mutations in glycosylation pathways have been a valuable resource for studying glycobiology and continue to represent powerful tools for producing glycoconjugates with more tailored glycans.[489] Toxic lectin selection schemes in CHO cells have yielded diverse Lec^R cell lines, which yield altered glycan structures through loss-of-function (i.e., *Lec*) or gain-of-function (i.e., *LEC*) gene mutations.[490] Figure 6 shows well-characterized Lec^R mutants and associated changes to *N*- and *O*-glycan structures in terms of sugars lost (−) or added (+).[490] These cell lines can be used as a starting point for producing glycoproteins with a dominant N- or O-linked glycosylation profile (although care must be taken to properly characterize glycan profiles[491]). Notable CHO cell lines for producing simplified glycoforms include *Lec13* cells that reduce core fucosylation of *N*-glycans;[492] *Lec2* cells that lack sialylaiton;[493] and *Lec1* cells that produce predominantly high-mannose *N*-glycans.[494] Engineering in human embryonic kidney (HEK) cells has now afforded the

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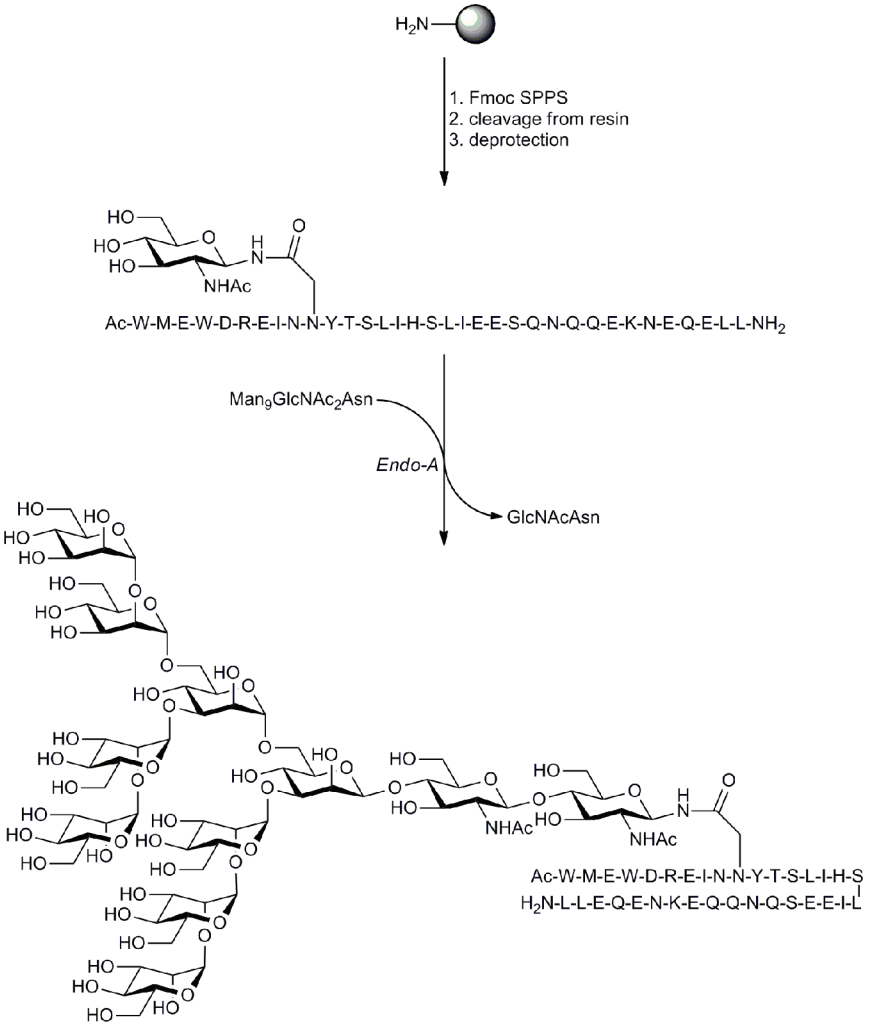
Scheme 28. Chemoenzymatic Synthesis of HIV-1 gp41

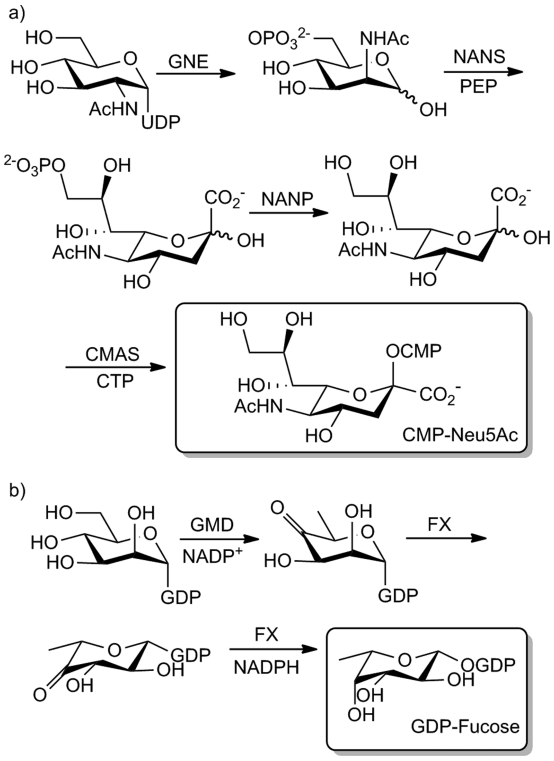


capability of producing a representative mammalian *N*-glycan panel of high-mannose (*Lec1*),[495] hybrid (*Lec36*),[496] and sialylated complex *N*-glycans (wild-type HEK 293 cells).[371] These cell lines can be exploited for glycoprotein production and studies. For example, the simplified glycan structures on recombinant glycoproteins produced in *Lec1* cells have witnessed broad application, from enhancing lysosomal targeting via mannose receptors for enzyme-replacement therapies[497,498] to improving crystallization for glycoprotein structural studies,[499] as well as facilitating enzymatic remodeling to homogeneous glycoforms for crystallography[499] and gSARs.[50] Notably, insect and engineered yeast cells can also be in place of *Lec1* cell lines to produce high-mannose glycoforms for similar objectives.[49,51,500]

Lec^R cell lines vary in their degree of altered function, and their phenotypes can arise from the mutation of several different genes,[490] which can lead to the production of recombinant glycoproteins with more complicated glycosylation profiles than expected.[491] To gain more complete control of glycosylation, further glycoengineering may be necessary in the form of additional toxic Lectin selections or gene knockout. Engineering out core α1,6-fucosylation in the N-glycosylation pathway serves as an excellent example of such efforts. While *Lec13* CHO cells were important for establishing fucose-related effects on antibody function, these cells harbor a mutation in the GDP-fucose biosynthetic pathway gene encoding GDP-fucose 4,6-dehydratase (GMD, Schemes 29b and 30), which only reduces core fucosylation.[492] Several attempts were put forth to create a more robust fucosylation-deficient CHO strain using toxic *Lens culinaris* Agglutinin (LCA) selection,[501] targeted gene disruption of the α1,6-FucT (Fut8) alleles,[502] and Fut8 knockout coupled with glycosylation inhibitors (see below).[503] To ablate fucosylation, a double knockout of the genes encoding Fut8 and GMD was necessary.[504] This double knockout protocol has been streamlined for general application to primary antibody-producing CHO cells.[505] These knockouts of core fucosylation are a notable success of homologous recombination in mammalian cells, which is often time-consuming and difficult to achieve.[488]

Although not technically glycoengineering, RNA interference[488] and small-molecule inhibitors of glycosylation[506] can also be used to knock down the activity of glycoprocessing enzymes and produce simplified glycan structures. Inhibitors are particularly useful for quickly probing glycosylation patterns on a small scale when glycoengineered mutants are not readily

Scheme 29. Biosynthetic Pathways for (a) Neu5Ac-CMP and (b) GDP-Fucose



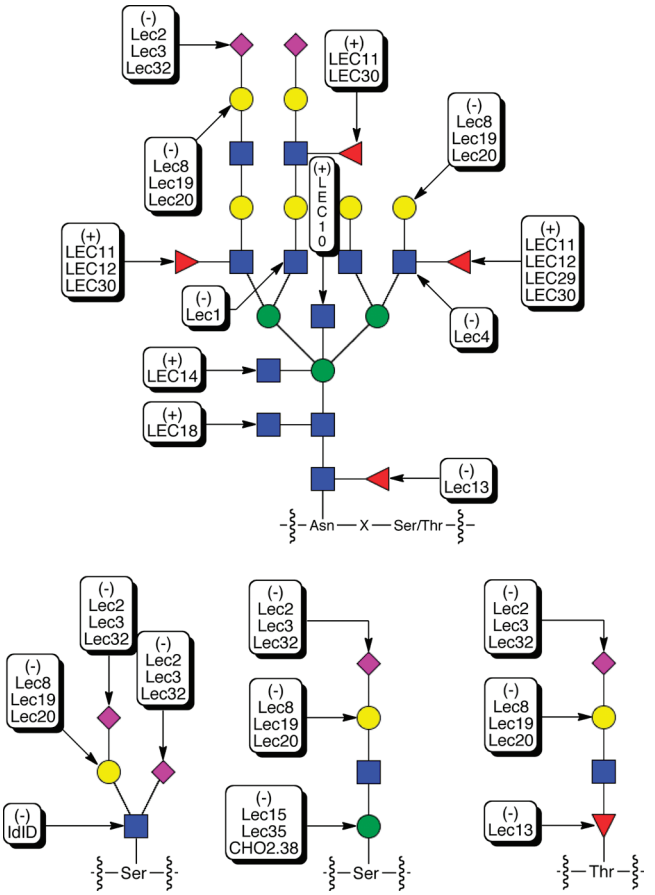


Figure 6. Well-characterized Lec^R mutants.

available. Several useful inhibitors of early N-glycosylation steps in the ER and Golgi (Figure 7 parts a and b) are commercially available, including (1) Tunicamycin, which blocks N-glycosylation by inhibiting formation of the LLO at the first step catalyzed by GnP-T; (2) Deoxynojirimycin, which produces Glc_1Man_9-glycoproteins by inhibiting α-glucosidase trimming by Glcase I; (3) Kifunensin, which produces Man_9-glycoproteins by inhibiting Mns-I; and (4) Swainsonine, which produces high-mannose or hybrid-type *N*-glycans by inhibiting Mns-II (Figure 8). These inhibitors are essential tools for modulating and studying glycosylation pathways[506−508] and can be applied to produce simplified glycoproteins for crystallographic studies[49,496,509,510] as well as for gSARs.[50,503,511]

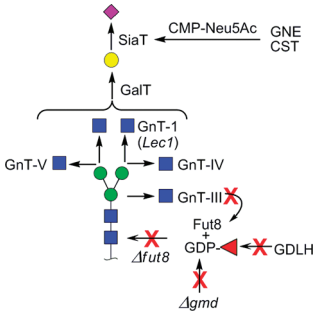
5.1.2. Controlling Glycosylation in Mammalian Cells by Expressing Glycoprocessing Enzymes. Glycan structures can be manipulated quickly and effectively by transient expression of recombinant glycoprocessing enzymes. For example, the less sophisticated O-glycosylation machinery of CHO cells can be transiently augmented with human enzymes, to "humanize" their glycosylation profile.[512] In this fashion, many human-type mucin structures of interest, including sLe^X, HNK, and 6-sulfo-*N*-acetyl lactosamine, have been built upon the basic core1 mucin structures in CHO cells.[512−515] Early experiments of this type demonstrated that specific mucin structures had important biological functions. For example, by displaying sLe^X on core1 structures through the simple overexpression of human FucT-VII, it was established that sLe^X is the glycan ligand for Selectins.[516−518] The importance of sulfated sLe^X was similarly established by expressing the requisite human sulfotransferases.[513] Because CHO cells lack or have Lec^R backgrounds deficient in human sulfating infrastructure, isolated expression of sulfotransferases in these backgrounds continues to be a tool for probing how differentially sulfated structures in the GAG family modulate important biological interactions, such as embryogenesis, blood clotting, and viral pathogenesis, to name a few.[519]

As the pharmacological properties endowed by *N*-glycans to glycoproteins have come to be appreciated, particularly with respect to the beneficial effects of α2,3-sialylation on biologic half-life and detrimental effects of core α1,6-fucosylation on antibody effector functions,[60,62] increased focus has been placed on tailoring desired *N*-glycan structures on biologics. As summarized in Scheme 30, many glycoprocessing enzymes can be coexpressed to ascertain *N*-glycan structures with increased sialylation and decreased fucosylation. Methods to increase sialylation include (1) increasing the number of *N*-glycan antenna by overexpressing branching GlcNAc transferases (GnT-IV and GnT-V),[520] or by overexpressing GnT-1 into a *Lec1* CHO cell background (i.e., △*GnT1*);[521] (2) boosting the installation of Neu5Ac or the Neu5Ac-Gal complex glycan cap onto branches by expression of α2,3-SiaTs or by coexpression of α2,3-SiaTs and β1,4-GalT;[522−525] and (3) increasing the pool of CMP-Neu5Ac by overexpressing enzymes involved in Neu5Ac biosynthesis and transport, including a highly productive GNE mutant and cytidine monophosphate-sialic acid synthatase (CMAS).[526−528] These techniques can be used alone, or in conjunction, and screening N-glycomodifying enzymes and infrastructure can help to find the best conditions to improve sialylation in a cell line.[524525] Another tactic to increase sialylation

dx.doi.org/10.1021/cr200113w |*Chem. Rev.* 2011, 111, 4259–4307

has been to engineer in additional N-glycosylation sites in the protein sequence of therapeutic proteins.[57,529−534] Notably, the introduction of novel N-glycosylation sites should be greatly facilitated by new engineering principles that also reliably guide structural stability.[53] Although core fucosylation can be knocked out in CHO cells,[504,505] enzyme overexpression offers faster alternatives. For example, Fut8 activity can be blocked when a bisecting β1,4-linked GlcNAc residue is present on the central β-linked mannose of the conserved Man3 *N*-glycan core.[535,536] This non-native activity can be introduced into CHO cells by expression of the GnT-III enzyme[537−540] and can be enhanced using a domain engineered GnT-III in conjunction with Mns-II (leading to antibodies with improved ADCC bearing nonfucosylated bisected complex glycans).[541,542] In an alternative approach, the pool of cytosolic GDP-fucose can be decreased using

Scheme 30. Knockout and Overexpression in CHO Cells



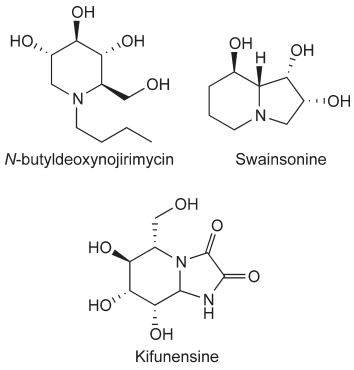


Figure 8. Glycosylation inhibitors.

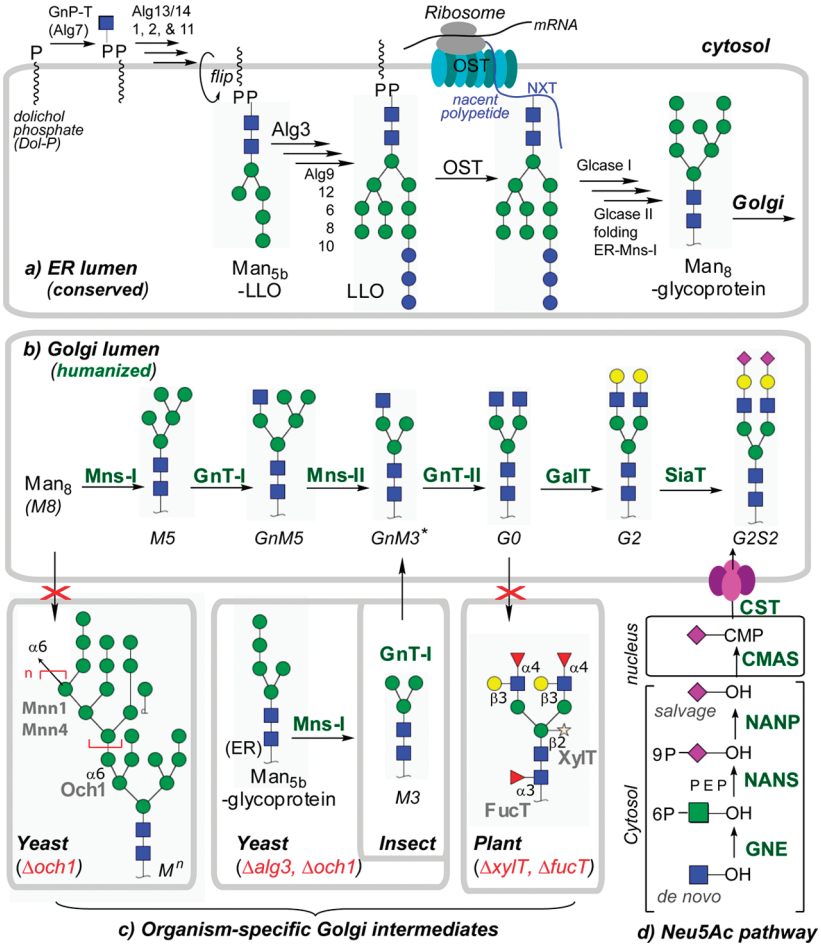


Figure 7. Glycoengineering in the N-glycosylation pathway of eukaryotes: (a) conserved steps in the ER that lead to glycoproteins bearing Man8 *N*-glycans (*M8*), which are trafficked to the Golgi; (b) processing and diversification steps in the Golgi that lead to human-type sialylated complex *N*-glycans (*G2S2*), with the necessary humanizing enzymes shown over arrows in green; (c) organism-specific glycoprotein intermediates diverted to feed into the humanized pathway; (d) glycoprocessing enzymes leading to the production of CMP-Neu5Ac for SialylT. (Abbreviations for the glycoprotein's *N*-glycan are indicated below structures, where Man [*M*], GlcNAc [*Gn*], Gal [*G*], Neu5Ac [*S*]).

dx.doi.org/10.1021/cr200113w |*Chem. Rev.* 2011, 111, 4259–4307

a bacterial GDP-6-deoxy-D-lyxo-4-hexulose (GDLH) that effectively depletes intermediates from the de novo synthesis pathway (Schemes 29b and 30).[543] A final enzyme that has been used to modulate antibody glycan structures for therapeutic application is the IgG-specific *endo*-glycosidase, Endo S, isolated from *Streptococcus pyogenes*.[432−435,544,545] Interestingly, when expressed in vivo, this enzyme only truncates the conserved Fc *N*-glycan down to the bridgehead GlcNAc, thereby eliminating its effector functions; this is promising toward autoimmunity treatment.[434]

5.2. Domain Engineering of Glycoprocessing Enzymes

Domain engineering is an important technique in the advancement of eukaryotic glycoengineering. Domain engineering approaches focus on controlling the location of glycoprocessing infrastructure along the secretory pathway.[546] Secretory placement colocalizes enzymes and glycan substrates, making it a key factor in determining which glycan structures will be presented on glycoconjugates. Glycoprocessing enzymes in the secretory pathway are primarily type-II membrane-bound proteins, which are composed of two distinct and interchangeable modular domain architectures: the first unit contains a cytoplasmic tail, transmembrane spanning region, and stem region (CTS), and the second unit contains the enzymatic domain.[547] Whereas the glycoprocessing activity is a function of the catalytic domain alone, localization in the secretory pathway is controlled by CTS, although the precise governing factors are not fully understood.[548,549] In domain engineering approaches, CTS and enzymatic modules are swapped to create chimeric enzymes with new properties to alter the fate of glycan structures. Domain swapping is possible in many secretory pathways, and its power to provide more precise control over glycosylation pathways in mammalian cells and to augment nonmammalian pathways with new functionality should continue to grow as more is understood about CTS localization signals.

In mammalian systems, domain engineering has been used to alter glycosylation profiles and to precisely control the activity of engineered glycoprocessing enzymes. Engineering chimeric enzymes with earlier accesses to native glycan substrates can deplete a glycan substrate or block the action of a native GTase, thereby diverting the formation of an undesired glycoform. In murine cell lines, the formation of the immunogenic α-Gal epitopes by endogenous GalT activity was diverted by engineering an earlier-acting SiaT.[550] In CHO cells, undesired *N*-glycan core fucosylation on IgG was effectively obstructed by using a chimeric GnTIII to add a bisecting GlcNAc.[541] In a further measure of domain engineering, enzymatic localization and enzymatic activity have been placed under a chemical control switch. In this approach, the CTS and catalytic domains are expressed as separate fusion proteins that are each equipped with a unit of a chemically induced heterodimerization domain (CID, such as the rapimycin-dependent human rapamycin-associated protein (KFBP) and rapamycin-binding domain of mTOR (FRB) heterodimerizing domains).[546] In CHO cells, several CID schemes for controlled reconstitution of FucT and sulfotransferase activity have been demonstrated.[551−554]

Nonmammalian systems demonstrate the power of domain engineering to provide novel catalytic activity in the secretory pathway. In yeast and plant systems, domain engineering has been critical to introducing the necessary mammalian-type glycoprocessing activity to generate mammalian N- and O-linked glycans, a process called "mammalianizing" or "humanizing" (see section 5.3).[555−557] In these approaches, non-native catalytic domains representative of mammalian glycoprocessing enzymes were paired with appropriate ER-directing tags or CTS-domains for proper secretory localization. For example, ER localization can be achieved by combining a catalytic domain with a four amino acid C-terminal HDEL-tag (which will redirect activity to the ER-based HDEL receptor),[558−561] or with an ER-based CTS domain (which embeds in the ER membrane).[562] The majority of domain-engineered mammalian-type glycoprocessing enzymes have been implemented in humanizing the N-glycosylation pathway in the yeast strain *P. Pastoris* and have been localized to the yeast Golgi.[555,563] Successful chimeric combinations were both hand-selected[563] and library-generated.[555] Moreover, the yeast approaches paved the way for domain-engineered glycoprocessing enzymes in plants.[556] The fact that multiple chimeric enzymes have been created to reenact mammalian steps of N- and O-linked glycoprocessing in nonmammalian secretory pathways (see section 5.3) suggests a fair amount of tolerance and flexibility for engineering non-native glycoprocessing infrastructure using domain engineering.

5.3. Engineering Glycosylation Pathways in Non-Mammalian Organisms

The expense, time, and complexities of glycoprotein production in mammalian cells has motivated glycoengineering geared toward "humanizing" or "mammalianizing" glycosylation in nonmammalian systems that are utilized for recombinant protein expression. Plant, insect, and yeast systems provide many attractive features for glycoengineering: first, these systems have well-established protocols for the expression of recombinant glycoproteins that are cost-effective, high-yielding, and contamination-free; second, these systems have genetic manipulation techniques for engineering that are well-established and much more tractable than mammalian cells; and, most importantly, these systems harbor secretory pathways with greatly simplified but homologous glycosylation infrastructures that can serve as the basis for introducing humanized glycoprocessing using domain engineering (section 5.3). Bacteria, although the most genetically tractable and robust of recombinant protein production systems, present additional challenges to humanizing glycosylation because they do not have a secretory pathway, and if they have protein glycosylation machinery (most do not), it is substantially different from that of eukaryotes. The basic approach to humanizing glycosylation pathways in nonmammalian systems is the same regardless of similarity: organism-specific enzymes responsible for generating any interfering or immunogenic glycan structures must be removed, whereas enzymes and supporting infrastructure needed to build human glycans must be introduced. The glycoengineering of N-glycosylation pathways demonstrates the variety of ways that this can be achieved in different, or even the same, organism (section 5.3.1). Examples of engineering humanized O-linked glycosylation are fewer, although efforts in yeast have paved the way for the development of humanized mucin-type glycosylation (section 5.3.2). New OTase glycosylation pathways characterized in lower eukaryotes, bacteria, and archaea have functional similarity to eukaryotic N-glycosylation, providing new avenues for glycoengineering microbes. Although OTase-driven glycoengineering is only beginning to be explored, it shows great potential toward the production of diverse N- and O-linked glycoproteins in *E. coli* (section 5.3.3).

5.3.1. Engineering Mammalian *N*-Glycosylation Pathways in Eukaryotic Secretory Pathways. In a true demonstration of the power and versatility of glycoengineering, humanized *N*-glycosylation culminating in sialylated complex structures has been engineered into yeast,[555,564] insect,[500,565] and

plant[556,566] systems in the past 10 years (Figure 7). Because these notable glycoengineering achievements have been the subject of individual review,[548,557,500,556,565,566] our purpose here is to summarize these accomplishments and highlight recent developments. Glycoengineering of mammalian N-glycosylation into nonmammalian eukaryotic secretory pathways has been particularly successful both because of the highly conserved steps and the key glycan intermediates in N-glycosylation pathways and because of the pathway's glycoprocessing enzymes, which are tractable to domain engineering (see section 5.2). As shown in Figure 7A, strictly conserved steps of the N-glycosylation pathway in the ER entail (1) the synthesis of the LLO ($Glc_3Man_9GlcNAc_2$-PP-Dol by the Alg family of GTs), (2) cotranslational protein glycosylation by the OST complex, (3) *N*-glycan-associated protein folding, quality control, and degradation (processes linked to Glu and Man trimming on the *N*-glycan), and (4) trafficking of folded Man_8 glycoproteins from the ER into the Golgi.[37,38,567−569] In the Golgi, cell-dependent maturing processes act on the Man_8 glycan structure (*M8*), transforming them into organism-specific forms. Representative organism-specific *N*-glycans include sialylated complex in humans (*G2S2*, Figure 7B); hypermannosyl in yeast (M^n, Figure 7C); paucimannosyl (*M3*), with possible core α1,6- and α1,3-fucosylation, in insects (Figure 7C); and, in plants, biantennary complex structures of *G0* bearing bisecting β1,2-Xyl, core α1,3-fucosylation, and often terminal Le^a motifs (via α1,4-fucosylation and β1,3-galactosylation of the GlcNAc antennae) (Figure 7C). To glycoengineer humanized glycans in these nonmammalian secretory pathways, appropriate humanizing glycoprocessing infrastructure is introduced (Figure 7B) to exploit endogenously produced *N*-glycan intermediates (Figure 7C).

The nonmammalian features of *N*-glycan structures are very immunogenic, making engineering out such features as important as adding in the appropriate mammalian features. Figure 7C shows representative nonmammalian glycans and indicates where intermediates can be intercepted by engineering to provide glycan intermediates to feed into a mammalian pathway (Figure 7B). In insect and yeasts, mannosyl intermediates can be accessed for this purpose. The *M3* structures, predominantly processed in insect cells, can feed directly into the mammalian pathway as substrates for the GlcNAcTs, GnT-1, and GnT-II (although the potential for α1,3-fucosylation in insect cells may be a residual problem).[500] In yeast, two different mannosyl glycoprotein intermediates can be engineered for mammalianization (Figure 7C). *M8* intermediates are accessible by averting the yeast-specific hypermannosylation pathway, characterized by the α-1,6-linked polymeric mannan branch (Figure 7C, indicated by red brackets in M^n).[559−562,570,571] In many yeast strains, including *P. Pastoris*, it is sufficient to knock out the *och1* gene encoding the enzyme that initiates the mannan arm by adding the first α-1,6-mannose residue,[378,379] although other strains may require additional mannan gene knockouts (including *och1*, *mnn1*, and *mnn4* in *S. cerevisiae*) and mutational optimization.[571−574] Glycoprotein intermediates bearing Man_{5b} can be generated by deleting the *alg3* gene in conjunction with *och1*.[575−578] Without Alg3, LLO biosynthesis will be arrested at the Man_{5b}-LLO intermediate, forcing OST to transfer the suboptimal substrate, which will then be trimmed to an *M3* intermediate for entry into the human pathway.[571,579] In plants, the N-glycosylation machinery leads to more advanced *G0* structures that can be diverted into the mammalian pathway by knocking out the genes encoding XylT and FucT, which are responsible for the immunogenic additions of the β1,2-Xyl and α1,3-Fuc.[566] Double knockouts of this type are viable in a number of plant systems.[556] Once key intermediates are engineered, they can be subjected to the humanized pathways, such as that shown in Figure 7B.

To date, the most sophisticated "humanized" strains can generate sialylated biantennary complex *N*-glycan structures. This *G2S2* structure can be recapitulated from *M8* intermediates using the set of glycoprocessing infrastructure shown in Figure 7 parts B and D (where green type indicates necessary glycosidases, GTases, and elements for sialic acid biosynthesis and transport that are necessary), although fewer components may be required depending on the organism-specific intermediate that is feeding into the pathway (Figure 7 part C). Domain engineering is essential to generating the humanized glycoprocessing enzymes, as described in section 5.2. Glycan substrate flexibility, including in the trimming of high-mannose residues by Mns-1and Mns-II and in the transfer of GlcNAc to the α1,3 Man branch by GnT-1, allows for several mannosyl intermediates from yeast and insect cell (*M8-M3*) to be rendered into *GnM3*, the first committed structure to complex glycan synthesis.[563,575,580,581] After the biantennary *G0* base is afforded by transfer of GlcNAc to the α1,6-Man branch by GnT-II, subsequent addition of β1,4-linked Gal residues by GalT leads to mammalianized asialo complex glycans (*G2*). Finally, *N*-glycan sialylation can be supported by introducing the appropriate SiaT and by generating Neu5Ac-CMP, which requires the introduction of up to five genes in the CMP-Neu5Ac pathway (Figure 7D). In yeast, all five genes must be engineered.[85] Plants have an endogenous UDP-*N*-acetylglucosamine 2-epimerase/*N*-acetylmannosamine kinase (NANP), and therefore only need GNE, *N*-acetylneuraminic acid phosphate synthase (NANS), CMAS, and CST for *N*-glycan sialylation.[483] Insect cells only require the addition of NANS and CSS along with media supplementation with ManNAc to bolster an endogenous salvage pathway.[481,582,583]

5.3.2. Engineering Mammalian O-Glycosylation Pathways in Eukaryotic Secretory Pathways. The feasibility of introducing mammalian O-glycosylation into secretory pathways where it is absent has been demonstrated in glycoengineered yeast. In *S. cerevisiae* two different kinds of O-linked glycan structures have been engineered, including the most common mucin core1 structure (Gal-β3-GalNAc-O-Thr/Ser) and the rarer α-O-linked Fuc. The mucin core1 disaccharide was recreated by introducing (1) the genes for GalNAc synthesis and transport, including UDP-Gal/GalNAc 4-epimerase (GalE, to produce UDP-GalNAc from GlcNAc) and UDP-Gal/GalNAc transporter (UGT2, to bring the nucleotide sugar from the cytosol into the secretory lumen); and (2) the requisite domain-engineered GTs, containing the catalytic domains of human ppGalNAcT (for the transfer of the proximal α-O-GalNAc to Ser/Thr) and a β-1-3-GalT (to finish the core1 disaccharide), each fused to the mannosyltransferase9 (MMN9) CTS from yeast for Golgi localization.[557] Another *S. cerevisiae* strain was engineered to produce α-O-linked Fuc residues through introduction of three enzymes, GMD, FX, and *O*-FucT-1. GMD and FX comprise the GDP-Fuc de novo pathway responsible for converting endogenous GDP-Man to GDP-Fuc (Scheme 29b) (these genes were taken from *Arabidopsis thaliana*),[584] whereas *O*-FucT-1 catalyzes the transfer of GDP-Fuc to Ser/Thr residues.[585] Surprisingly, this study found that yeast have an endogenous Fuc transport mechanism even though yeast are not known to generate fucosylated glycans naturally, and that the introduction of the human Golgi transporter for Fuc was detrimental to the fucosylation of epidermal growth factor (EGF).[585] Notably, endogenous yeast *O*-mannosylation can be attenuated with rhodanine-3-acetic acid derivatives during recombinant protein

expression, which is important because such modification is highly immunogenic to humans.[586]

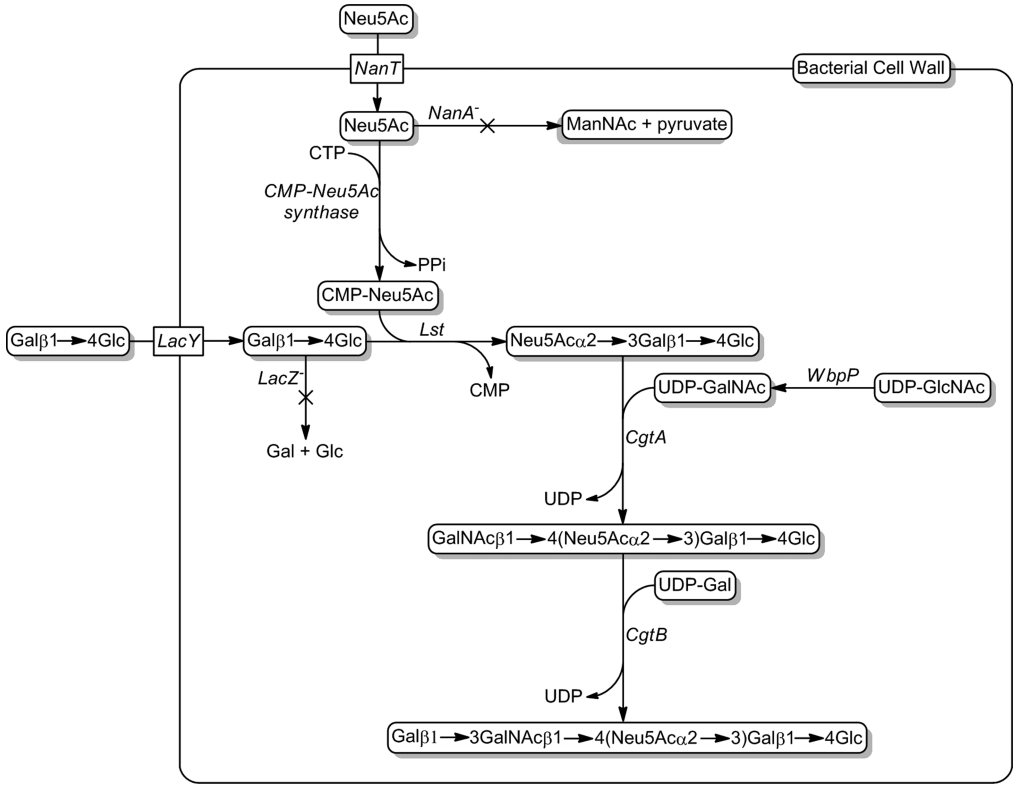
5.3.3. Engineering Glycosylation in Microorganisms Using Novel OTases. OTases are non-Leloir GTases that use LLOs as donors in glycosylation reactions. For a long time, the eukaryotic OST was the singular example in this class capable of glycosylating proteins. This picture has now changed, as all domains of life have been found to harbor OTases and supporting LLO biosynthetic pathways to *N*-glycosylate (N-OTases) and *O*-glycosylate (O-OTases) proteins.[100,101] The catalytic subunit of OST, Stt3, is in fact a member of an N-OTase family, composed of eukaryotic-Stt3, bacterial-PglB, and archaeal-AglB members.[587] Features of the Stt3/PglB/AglB family include en bloc glycosylation from an LLO to an Asn sequon containing at least N-X-S/T (where X is not Pro). LLO donors are built up by a dedicated suite of GTases, primarily in a segregated cellular compartment, before being translocated for copresentation with the protein substrate to the N-OTase. Variable features include LLO composition, glycosylation sequon identify/preference, and subcellular locations for LLO biosynthesis and transfer. For example, the eukaryotic OST transfers $Glc_3Man_9GlcNAc_2$-PP-Dol, biosynthetically initiated on the cytoplasmic side of the secretory membrane, onto Asn residues in N-X-T sequons in unfolded polypeptides cotranslationally translocated in the ER lumen (Figure 7a), whereas the *C. jejuni* PglB transfers $GalNAc_2$(Glu) $GalNAc_3$Bac-PP-Und, biosynthetically initiated on the cytosolic face of the cytosolic membrane, to Asn residues in D/E-Z-N-X-S/T sequons within flexible regions of folded proteins in the periplasm.[103] Several bacterial pathogens *O*-glycosylate proteins using LLO substrates, which are biosynthesized by pathways that are related to those of the N-OTase LLOs, but with distinct O-OTases, which are related instead to O-antigen ligases.[588,589] Although OTase protein glycosylation is only in the early stages of characterization, archaeal glycosylation systems appear to have the most diversity.[102] For example, alignment of the Stt3/PglB/AglB family indicate three types of catalytic center motifs in archaeal AglBs (DM, MI, and DK), whereas eukaryotic Stt3s only have DK, and bacterial PglBs only have MI.[587] Glycoengineering with OTases already shows promise as many can be functionally transported into heterologous systems and LLO substrates can be engineered, as discussed in more detail in this section. OTase pathways in all kingdoms of life may indeed have the unique balance of functional conservation and genetic diversity needed to greatly expand the tools of glycoengineering.

The eukaryotic OST is a complicated heterooctomeric complex, the basic workings of which are still not fully understood.[590] Although long precluded by complicated and essential function, glycoengineering around OST in yeast is now possible by replacing the OST complex with a single subunit N-OTase of the Stt3/AlgB/PglB N family. The single subunit Stt3s of several protists, including *Toxoplasma gondii*, *Trypanosoma cruzi*, *Trypanosoma brucei*, and *Leishmania major*, were capable of reconstituting N-glycosylation activity in yeast with otherwise lethal OST complex knock outs.[591−595] The Stt3 paralogs appear to function independently, and their unique specificities can be conveyed. For example, *T. brucei* has two Stt3 paralogs, TbStt3a and TbStt3b, which are responsible for parallel and selective routes to glycoproteins bearing oligomannose and complex glycans. The route to oligomannose is controlled by TbSTT3b, which will transfer LLOs bearing Man9 to most unoccupied sequons, whereas the route to complex glycans is controlled by TbSTT3a, which will only transfer $Man5_b$-LLOs to sequons flanked by acidic amino acids.[596] These preferences can be manifested in yeast through glycoengineering, as demonstrated by the high glycosylation of acidic sequons with $Man5_b$ upon coexpression of TbSTT3a in a yeast strain deficient of OST and Alg3 activity (notably, $\triangle alg3$ produce $Man5_b$-type LLOs for transfer, see Figure 7A).[593] Because primitive eukaryotes harbor a unique array of single subunit Stt3 N-OTases and LLO pathways for the synthesis of truncated human *N*-glycan structures at specific sequons,[597] they provide much greater latitude to glycoengineering than that provided by the conserved pathway (Figure 7A). These possibilities would be extended even further if the bacterial PglB and archaeal AlgB N-OTases can be functionally transferred into yeast.

Glycoengineering of protein N-glycosylation in *E. coli* is possible because of the Stt3/AlgB/PglB N-OTase family. Although much remains to be done to reconcile the differences in bacterial and mammalian N-OTase processes,[103] progress using the *C. jejuni* bacterial system has promising glycoengineering features. Importantly, the entire *C. jejuni* N-OTase pathway encoded by the *pgl* locus (CjPgl), as well as just the N-OTase activity encoded by *pglB*, can be functionally transported into *E. coli* through heterologous gene expression[598] (remarkably, the CjPgl system can even be reconstituted in vitro using the purified enzyme members).[599] The fundamental difference of the bacterial and mammalian LLO donors presents one of the biggest hurdles toward mammalianizing the CjPgl system. Fortunately, PglB is promiscuous with regard to LLO specificity, accepting vastly different structures from GlcNAc-PP-Und, in vitro, to $GalNAc_5$GlcNAc-PP-Und and O-antigen-PP-Und, as engineered in vivo substrates.[600−602] The generation of the $GalNAc_5$GlcNAc-LLO demonstrates that the Pgl LLO pathway is amenable to engineering and is a key step toward generating mammalian LLO structures and mammalian glycoproteins. Importantly, α-galactosaminidase trimming of $GalNAc_5$GlcNAc-bearing glycoproteins can unveil the native N-linked GlcNAc of mammalian glycoproteins. Such GlcNAcylated proteins can be enzymatically elaborated to full mammalian structure by using synthetic ENGases and sugar oxazolines,[82,94] although other methods discussed in section 4.2.2 could also be used for elaboration. The combined latitude in heterologous pathway transfer, LLO substrate tolerance, and LLO engineering bode well for ultimately constructing mammalian-type LLOs for PglB transfer in *E. coli*, although the task of expressing human or finding human-type GTs in bacteria remains a challenge.[603] Further characterization and transfer of Stt3/AlgB/PglB *N*-OTase pathways into *E. coli* will likely provide more tools toward humanizing efforts and will present opportunities to make glycoproteins bearing a broad range of novel glycans for SARs expanding to all kingdoms of life.

Bacterial O-OTases represent a new set of glycoengineering tools for introducing O-glycosylation into *E. coli*. Very recently characterized systems in *Neisseria gonorrheae*,[604] *Neisseria meningitides*,[605] and *Bacteroides fragilis*[606] represent O-OTase systems that can generally *O*-glycosylate proteins. The O-OTases are related to bacterial O-antigen ligases, which are responsible for generating LPS by glycosylating lipid A with an O-antigen-PP-Und donor.[588] The identity of the LLO donors vary between organisms. Some O-OTases use O-antigen-PP-Und from LPS pathways, as is the case of for PilO, from *P. aeruginosa*.[607] Other O-OTases have a dedicated biosynthetic cluster to build LLOs (which closely resembles the *pgl* locus in *C. jejuni*),[100,101] as is the case for the *Neisseria* O-OTase orthologs PglL and PglO, from *N. meningitides* and *N. gonorrheae*, respectively.[604,608] Transfer by O-OTases may in fact be modulated by the type of LLOs an organism can produce. Such behavior is consistent with the engineering of

Scheme 31. Production of Gangliosides with an Engineered Bacterial System



O-glycosylation in *E. coli* using O-OTases and LLOs. For example, PilO and PglL expressed heterologously in *E. coli* were able to *O*-glycosylate proteins with the *C. jejuni* LLO, when coexpressed with the *pgl* LLO biosynthetic genes; however, when coexpressed with the biosynthetic genes for O7-antigen-PP-Und LLOs, proteins were O-glycosylated with O7-antigens.[609] In *E. coli*, active PglL was capable of transferring an entire library of natural UndPP-bound glycans engineered for biosynthesis in vivo, in addition to a synthetic library of lipid-modified donor substrates in vitro, which indicates that this O-OTase has extreme substrate tolerance with respect to glycan and lipid components.[609,610] Although human-type O-glycosylation remains to be established, O-OTases present a promising combination of relaxed substrate specificity and en bloc transfer that may provide a novel solution to homogeneous O-mucin glycosylation, if the requisite LLOs can be assembled.

5.4. Engineering Bacteria for the Production and Display of Complex Carbohydrates

Metabolic glycoengineering of *E. coli* has been a successful method for producing human complex carbohydrate structures. These schemes focus on generating the glycan component in the bacterial cytosol by employing microbial glycoprocessing enzymes. Key steps include (1) engineering an appropriate carbohydrate acceptor scaffold that cannot be metabolically diverted and (2) engineering appropriate glycoprocessing infrastructure to build upon acceptor scaffolds, including GTs and supporting nucleotide sugar synthesis and transport. In the past 10 years, two primary applications of metabolically glycoengineered *E. coli* have been demonstrated. In one application, *E. coli* are transformed into factories for the production of high-value glycans.[611] In another application, glycan structures are display on chimeric lipooligosaccharides (LOS) at the bacterial cell surface, which can be used to study bacterial glycomimicry,[612] develop probiotics,[613] and screen GTases.[614] Both approaches demonstrate that biologically relevant glycan epitopes can be efficiently generated using very different engineered acceptor molecules, suggesting further applications for metabolic glycoengineering can be developed through the engineering of appropriate acceptor molecules, perhaps even glycoproteins. More detailed examples of these systems are provided below.

Metabolically glycoengineered *E. coli* are compatible with large-scale fermentation and can provide access to significant quantities of complex carbohydrates, making them of interest for both research and clinical applications. Successful glycoengineering examples include such high-value glycan structures as gangliosides (G_{M3}, G_{M2}, and G_{M1});[254,615,616] P blood group (Gb_3 and Gb_4) and Forssman (Gb_5) antigens;[367,615,617] H-antigen;[618] and Lewis X motifs;[262] along with polysaccharides, such as HA, alginate, and *exo*-polysaccharides.[611] An example of an engineered system for the large-scale synthesis of gangliosides in *E. coli* is shown in Scheme 31. The ganglioside system requires the internalization of two non-native sugar moieties—lactose, which will serve as the acceptor, and Neu5Ac, which will be used for elaboration—and the introduction of the appropriate series of GTs and enzymes for Neu5Ac-CMP synthesis. The non-native sugars must be exogenously fed to the cells in the growth media, imported into the cytosol, and diverted from metabolic degradation, while enzyme activity is generated using recombinant expression. As shown in Scheme 31, lactose and Neu5Ac are internalized via the native β-galactoside permease (LacY) and sialic acid transporter (NanT),[619] and their metabolic processing is diverted by knockout of β-galactosidase ($\triangle lacZ$) and sialic acid aldolase ($\triangle nanA$), respectively. To elaborate lactose into the ganglioside series, the following GTases are coexpressed: (1) α2,3-SiaT (Lst), which sialylates the Gal terminus of lactose to produce G_{M3}; (2) β1,4-GalNAcT

dx.doi.org/10.1021/cr200113w |*Chem. Rev.* 2011, 111, 4259–4307

(CgtA), which adds GlcNAc the G_{M3}-Gal to form G_{M2}; and (3) β1,3-GalT (CgtB), which adds Gal to the G_{M2}-GalNAc to yield G_{M1}.[254] Notably, activated sugars for the GTases are endogenously produced, except in the case of CMP-Neu5Ac, which is generated from internalized Neu5Ac by an engineered CMP-Neu5Ac synthetase (SiaB), as shown in Scheme 31. De novo CMP-sialic acid from GlcNAc has also engineered by expressing the *C. jejuni* biosynthetic genes *neuABC*, encoding *N*-acetylglucosamine-6-phosphate-epimerase (NeuC), sialic acid synthase (NeuB), and CMP-Neu5Ac synthetase (NeuA).[484,485] This should make the production of sialylated carbohydrates more economical and versatile by coupling to various engineered SiaTs, including α2,3-SiaTs[254,484] and α2,6-SiaTs.[620]

Metabolically glycoengineered *E. coli*, which display complex carbohydrates on chimeric LOS at their cell surface, are providing new methods to study and thwart glycan-related disease mechanisms in pathogenic bacteria. The chimeric LOS strategy uses standard metabolic engineering techniques to build complex carbohydrate structures (i.e., engineering of GTases and nucleotide sugar transport), with the key substitution of the acceptor molecule to a terminal glucose on an engineered Lipid A intermediate. This truncated Lipid A is diverted from the endogenous LPS pathway by the knockout of one or both of the requisite elaborating GTases, WaaO and WaaB.[612,621] Coexpressed GTases can build the target carbohydrate molecules upon the Lipid A scaffold, and the resulting chimeric LOS is displayed at the bacterial cell surface, in place of native LPS. Using this approach, ganglioside-LOS have displayed on *E. coli*. For example, a G_{M3}-LOS chimera was produced using an appropriately knocked out *E. coli* K12 derivative (Δ*nanA*, Δ*waaO*, Δ*waaB*) to overexpress Neu5Ac synthetase (SiaB) the α2,3 SiaT (Lst),[612] very similarly to Scheme 31. The G_{M3}-LOS was used to probe the connection between pathogenic ganglioside and autoimmune disorders. In another approach, G_{M1}-LOS was engineered into *E. coli* CWG308 (i.e., *E. coli* R1 with Δ*waaO*) using coexpression of a β1,4-GalT (LgtE), along with the *C. jejuni* CMP-sialic acid biosynthetic genes *neuABC*.[485] The G_{M1}-LOS expressing cells were capable of binding cholera toxin. The binding of other bacterial toxins has been affected using other engineered chimeric LOS, including Gb_3- and Gb_4-LOS, which can bind Shigella toxins,[622] and lacto-*N*-neotetraose- and G_{M2}-LOS, which can bind heat-labile entrotoxin.[623] Display of toxin-binding chimeric LOS on the surface of nonpathogenic bacteria may transform them into probiotics that can sequester and neutralize the harmful toxins leading to disease.[613]

6. CONCLUDING REMARKS

Nature uses glycan structures to tune and expand the functional repertoire of glycoconjugates, especially in the case of glycoproteins. Although our ability to understand glycan-driven properties has been shrouded by biological heterogeneity and formidable synthetic challenges, new chemoenzymatic and whole-cell engineering techniques are now providing much-needed routes to homogeneous glycans. We have presented a comprehensive overview of these methods herein, with the goal of providing a valuable resource to researchers who desire access to glycoconjugates of homogeneous glycoform for diverse research objectives. Importantly, the areas of chemoenzymatic synthesis and whole-cell engineering will continue to grow and become more practical, allowing for the efficient production of glycoconjugates and the assignment of glycan-related structure−activity relationships, in the near term, and, hopefully, the extrapolation of universal principles for glycoengineering and therapeutic efficacy, in the future.

ASSOCIATED CONTENT

Supporting Information

List of abbreviations used in this manuscript. This material is available free of charge via the Internet at http://pubs.acs.org.

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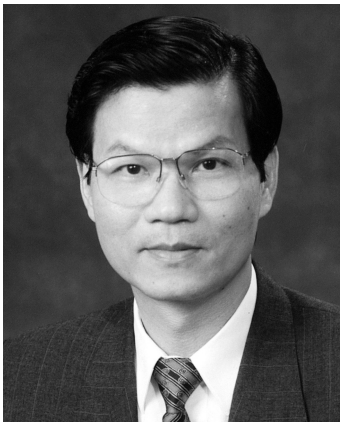

Professor Chi-Huey Wong received his B.S. (1970) and M.S. (1977) degrees from National Taiwan University, and Ph.D. (1982) in Chemistry from Massachusetts Institute of Technology. He then worked at Harvard University as a postdoctoral fellow for another year. He started his independent career as Assistant Professor of Chemistry at Texas A&M University in 1983 and became Associate Professor in 1986 and Professor in 1987. He was Professor and Ernest W. Hahn Chair in Chemistry at the Scripps Research Institute (1989−2006) and Director of the Genomics Research Center at Academia Sinica, Taipei (2003−2006). Since October 2006, he has been President of Academia Sinica and Professor of Chemistry at the Scripps Research Institute and National Taiwan University. He is the recipient of numerous awards and is a member of the National Academy of Sciences. He has over 700 publications, 90 patents, and 4 books. His research interests are in the areas of bioorganic and synthetic chemistry and biocatalysis, including synthesis of complex carbohydrates, glycoproteins, and small-molecule probes for the study of carbohydrate-mediated biological recognition, glycosylation, and drug discovery.

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