HHS Public Access

Author manuscript

*J Am Chem Soc*. Author manuscript; available in PMC 2020 March 06.

Published in final edited form as:

*J Am Chem Soc*. 2019 March 06; 141(9): 3735–3754. doi:10.1021/jacs.8b11005.

# Oligosaccharide Synthesis and Translational Innovation

**Larissa Krasnova**[†] and **Chi-Huey Wong**[†,‡]

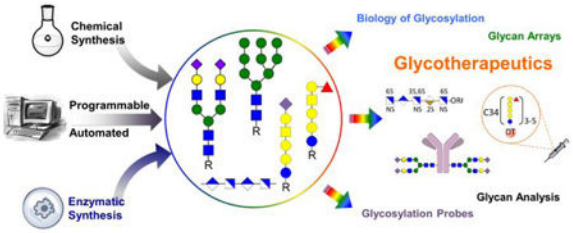
[†]Department of Chemistry, The Scripps Research Institute, 10550 N. Torrey Pines Road, San Diego, CA 92037.

[‡]Genomics Research Center, Academia Sinica, Taipei 115, Taiwan.

## Abstract

The translation of biological glycosylation in humans to the clinical applications involves systematic studies using homogeneous samples of oligosaccharides and glycoconjugates, which could be accessed by chemical, enzymatic or other biological methods. However, the structural complexity and wide-range variations of glycans and their conjugates represent a major challenge in the synthesis of this class of biomolecules. To help navigate within many methods of oligosaccharide synthesis, this Perspective offers a critical assessment of the most promising synthetic strategies with an eye on the therapeutically relevant targets.

## Graphical Abstract




## INTRODUCTION

Oligosaccharides (or glycans) are the ubiquitous molecules of life. While nucleic acids (DNA and RNA) are the information molecules and templates for making proteins, glycans are used by Nature to modulate the properties of biomolecules and for the communications between proteins and cells. However, the role of glycosylation in life has not been well understood. Glycosylation is a complex process, which varies among different cells and tissues, and could be affected by the environment. In the past two decades, a great progress has been made in understanding the biology of glycosylation.[1] These advances can be attributed in part to the recent developments in oligosaccharide synthesis, predominantly the synthesis of human-type sequences to assist biomedical research.[2]

**Corresponding Author** wong@scripps.edu, chwong@gate.sinica.edu.tw.

The authors declare no competing financial interest.

In human cells, glycans are often conjugated to lipids and proteins. As part of glycolipids, oligosaccharides participate in cell-cell recognition and signaling events. The discovery of tumor-associated carbohydrate antigens (TACAs) has been explored for the development of anticancer vaccines.[2c-f] When expressed on glycoproteins, *N*-glycans can affect protein structure, function and stability, thus making glycosylation a critical parameter in the optimization of therapeutic glycoproteins,[2g] whereas *O*-glycosylation can also influence protein folding and post-translational phosphorylation. In mucins, *O*-glycans provide lubrication and protection of epithelial surfaces against acid-exposure and pathogenic infection, and serve as an inspiration for the design of new biomaterials. The extracellular glycosaminoglycan (GAG)-containing proteins (or proteoglycans) modify the physicochemical properties of the environment and participate in many recognition processes. The physiological roles of GAGs have been translated into a plethora of pharmaceutical applications ranging from thrombosis and inflammation to design of new biomaterials for drug delivery, and tissue engineering.[2h-j] The glycosylphosphatidylinositol (GPI) oligosaccharides, which connect proteins to membrane lipids, were shown to affect protein conformations and organization of lipid rafts. Although fully-assembled GPI-anchored proteins are still difficult to study, the GPI oligosaccharides have been pursued in the context of antiparasitic vaccines, as well as for the development of diagnostic glycan arrays.[2k,l] As such, the term “oligosaccharides” covers many types of glycans with compositions and linkages specific to each class (Figure 1). In addition, within each type, there is a high degree of heterogeneity as glycan biosynthesis depends not only on genetic parameters, but also environmental factors, which could influence the expression of enzymes involved in glycan biosynthesis, and affect the availability of glycosylation substrates. For this reason, isolation of homogeneous glycoforms is almost impossible. Instead, only chemical and *in vitro* enzymatic methods are suitable for the preparation of homogeneous samples except for glycoproteins with multiple glycosylation sites. The chemoenzymatic synthesis of these biomolecules is yet to become practical, therefore stimulating development of new methods. As the result, synthesis of complex oligosaccharides requires a multidisciplinary approach encompassing practical synthetic methods and effective tools of cellular and molecular biology, biochemistry and glycoproteomics. Remarkably, the complementary use of both chemical and biochemical methods for oligosaccharide synthesis could be traced back to the work of Emil Fischer and his systematic fermentation experiments of simple oligosaccharides.[3]

Historically, chemical synthesis has always been a method of exploratory research as this mature discipline can provide access to almost any structure. To meet the synthetic challenges associated with the preparation of homogeneous glycans, biochemical methods have been developed to complement chemical and cell-based methods. In particular, if the demand of a glycoconjugate is confirmed, then process optimization is often performed using an enzymatic or chemoenzymatic method. This cycle could be illustrated with the development of carbohydrate-based anticancer vaccines,[2f,4] and heparin-based anticoagulants.[5] In the case of therapeutic antibodies, protein glycoengineering by a combination of cell-based and chemoenzymatic methods has the potential to optimize both efficacy and safety of monoclonal antibodies (mAbs), reduce batch-to-batch variability and manufacturing cost by switching to non-mammalian expression systems.[6]

In this Perspective, we review the key innovative methodologies of chemical and enzymatic synthesis, and highlight the application of these methods towards the development of glycotherapeutics, without going through a detailed process of development. To provide a better oulook of how the progress in oligosaccharide synthesis impacts the development of glycotherapeutics, we summarized some of the pharmaceutically relevant targets and products in Table S1 (SI). From commercialized drugs to candidates at early and late stages, one can note the increasing complexity of biomolecules that are being accessed by the evolving methods of oligosaccharide synthesis.

## CHEMICAL VS. ENZYMATIC SYNTHESIS

Until the early 1980s, chemical synthesis was the only method that could deliver homogeneous samples of oligosaccharides. Although the retrosynthetic analysis is relatively more obvious for oligosaccharides than other natural products, the chemical synthesis of oligosaccharides has several challenges, including: (*i*) control of stereoselectivity in the glycosidic bond formation; (*ii*) glycosylation of substrates with high-density of functional groups; (*iii*) differentiation of multiple functional groups with orthogonal protection to control the regioselectivity of glycosylation and downstream modifications (*e.g.*, sulfation); (*iv*) numerous protections and deprotections, which decrease overall efficiency of synthesis. These highlighted issues, which have to be resolved for at least 10 human-type monosaccharides, explain the absence of one general method for the assembly of glycans. Nevertheless, solutions to many of these problems have been reported (Table 1),[7] and in its current state, given enough time and resources, chemical methods can deliver most glyco-products of any complexity, as illustrated by the synthesis of mycobacterial 92-mer arabinogalactan,[8] and certain glycoforms of EPO.[9]

Control of stereoselectivity at the anomeric center is the central topic of the chemical method. Depending on the mechanism,[22] glycosylation can proceed with either *trans*- or *cis*-selectivity between substituents at C-1 and C-2. Among many parameters (*e.g.*, solvent, temperature, nucleophilicity of acceptor), the structure and reactivity of the donor affected by protecting groups (PGs) have the strongest impact on the stereochemistry of glycosylation. For example, acyl PG at C-2 can direct *trans*-configuration by forming the 1,2-*cis*-fused five-membered cyclic intermediate permitting nucleophilic attack from the *trans*-face.[10] On the other hand, the influence of non-assisting groups (*e.g.*, $N_3$, OBn), which could favor 1,2-*cis*-configuration due to the anomeric effect, is relatively weak leading to a mixture of isomers. Thus, historically, the synthesis of 1,2-*cis*-glycosides,[13e,15b] as well as glycosides lacking the hydroxyl group next to the anomeric position (*i.e.*, 2-deoxyglycosides,[23] and sialosides with sterically hindered quaternary anomeric center), represented the hardest problem in chemical synthesis (Table 1, entry *i*).

Using the thioether auxiliaries, Boons and others have extended the neighboring group participation strategy to 1,2-*cis*-glycosylation through a decalintype intermediate and formation of a *trans*-configured sulfonium ion.[11] Although the directing acyl groups at remote positions (C-3, C-4, and C-6) do not provide the same degree of stereoselectivity as those at C-2,[12] the properly designed donors with remote acetyl groups can give high stereoselectivity in α-gluco- and α-galactosylations. This strategy is particularly suited for

the automated synthesis of oligosaccharides as common acyl groups do not require extra steps for installation and deprotection.[21c] Unlike acyl groups, which facilitate *anti*-addition of a nucleophile, the picoloyl (Pico) substituents at remote positions favor *syn*-additions. The hydrogen bond-mediated aglycon delivery (HAD) strategy developed by the Demchenko group has been applied to the synthesis of various glycosides with high anomeric selectivity.[13j] The Mong group also reported the synthesis of nonsymmetrical α −1,1'-disaccharides using a Pico-protected trimethylsilyl acceptor and an α- or β-directing donor to achieve desired stereoselectivity.[13k]

In the intramolecular aglycon delivery (IAD) method, acceptor and donor are tethered together to increase the efficiency of coupling and to improve the facial selectivity of the acceptor. Initially developed for the synthesis of β-mannosides,[14a-f] the IAD strategy has been applied to a variety of targets[14f-h]. However, the most straightforward procedure for β-mannosylation is based on the conformation-restraining 4,6-*O*-benzylidene acetal PG.[15a] A detailed discussion on the origin of β-selectivity in this transformation could be found in a recent review.[15b] Other examples of the conformation-restraining PGs include 4,6-*O*-di-tert-butylsilylene for α-galactosylation,[15c] 2,6-lactone for β-mannosylation,[15d] 3,4-*O*-tetra-iso-propyldisiloxane for α-glycoaslation from glycals,[23c] 2,3-oxazolidinone for α-glycosylation of GlcNAc,[15e] and equatorially selective glycosylation of pseudaminic acid donor with controlled side-chain conformation.[15f] To improve the reactivity and α-selectivity of the sialoside donor,[15a,g] the Wong group introduced the *N*-acetyloxazolidinone-protected sialoside with a dibutylphosphate leaving group (LG).[15h,i]

Among many LGs developed to date,[24] 1-thioglycosides[24a,b] and *O*-glycosyl imidates[24c] are the most popular donors for glycosylation. Typically, these donors provide the desired window of reactivity for the construction of common linkages; however, the use of acidic promoters could be detrimental in the case of acidsensitive substrates or stagnant glycosylations, which require long reaction times leading to decomposition of glycosyl intermediates. A general solution to this problem is the development of mild and neutral glycosylation conditions (Table 1, entry *ii*) by optimizing LGs,[16a] promoters[16–17,25] and catalysts.[16b,25e,f] One prominent example is the gold-catalyzed glycosylation with alkynyl donors developed by Yu *et al.* that has been applied to the synthesis of a number of natural products.[16b] Examples of mild promotors include photochemical activation of thioglycoside donors[25a,b] and synthesis of 2-deoxyglycosides from glycals,[25c] electrochemical generation of glycosyl triflate intermediates from thioglycosides,[25d] and use of organocatalytic systems (*e.g.*, chiral phosphoric acid) for glycosylation reactions.[25e,f] The Jacobsen group reported the use of macrocyclic bis-thioureas as catalysts for the stereoselective glycosylation with chloride donors.[26] The reaction was shown to proceed through the inverted and cooperative mechanism in which both the electrophile and the nucleophile were activated to effect the stereoselectivity.

In certain cases, generation of highly reactive glycosyl donors *in situ* could improve the yield of stagnant glycosylations, as demonstrated in the coupling of ceramide acceptors with trimethylsilyl iodide donors generated *in situ*[25g] and α-selective glycosylation with 2-deoxysugar donors via *in situ* generated bromide donors using cyclopropenone- (and cyclopropene-1-thione-)-derived promoters.[17]

Methods for the selective protection of monosaccharide building blocks are based on the well-established differences in the intrinsic reactivities of the hydroxyl groups.[27] While orthogonally protected sugars are still at the core of chemical synthesis,[27b] various methodologies for glycosylation of unprotected donors and acceptors have emerged. Many of these methods are either organocatalytic[25f] or organoboron-mediated glycosylations.[18a,25e,28] The more general boron-assisted methods rely on the propensity of boronic/borinic reagent to reversibly form a complex with 1,2- or 1,3-diol.[18–19,29] Thus, cyclic boronates could serve as transient masking groups to differentiate the hydroxyls on the acceptor,[29a] or conceal the hydroxyl(s) of a donor as in the case of Bu2BOTf reagent, which provided 1,2-*trans*-selectivity even in the presence of non-assisting groups at C-2, including free OH.[29b] However, the most valuable application of organoboron reagents is based on the ability of the tetracoordinated organoboron complex to activate the equatorial oxygen of 1,2-*cis*-diol towards reaction with electrophiles (Table 1, entry *iii*). Using this strategy, Taylor and colleagues developed a Ph2BOH-catalyzed monofunctionalization of diols and triols. When applied to the Koenigs-Knorr-type glycosylation of acceptors protected at C-1/C-6, the borinic acid catalyst afforded a high regio- and 1,2-*trans*-selectivity.[18a] Later, the same group reported an oxaboraanthracene-derived catalyst effective at low temperatures.[18b,c]

Taking the utility of boron reagents even further, Takahashi and Toshima developed a conceptually new method, where a boron reagent/catalyst not only differentiates the hydroxyls of acceptor but also acts as a Lewis acid activating the 1,2-anhydrosugar donor towards the 1,2-*cis*-selective attack by acceptor.[19] While diarylborinic acids are suitable for the glycosylation of mono-deprotected acceptors, the aryl boronic acids can be used to differentiate the 1,3-diol at C-6 and C-4 offering excellent 1,4-regioselectivity for most of the substrates, with the exception of galactosyl acceptor, which favors 1,6-regioselectivity. Recently, a glycosylation of fully deprotected acceptors in the presence of water and $^{p\text{-}NO2}$PhB(OH)2 as a catalyst has been reported.[19b]

Perhaps the most useful improvement for the preparative synthesis of oligosaccharides was the development of one-pot glycosylation procedures (Kahne, Fraser-Reid, Ley)[30] to improve the overall efficiency of synthesis by minimizing the number of steps and eliminating the work-up and purification routines. Since the various strategies for the assembly of oligosaccharides and the expedite one-pot procedures for the protection of monosaccharide building blocks[31] have been recently reviewed,[21f,32] herein, we briefly discuss some popular methods and their limitations. Essentially, the one-pot procedures exploit the differences in reactivities of the acceptor hydroxyls and/or the LGs of the donors. However, many practical strategies operate on the assembly from non-reducing end to reducing end, thus relying on the properties of donors. For example, the orthogonal one-pot protocol is based on the sequential chemoselective activation of different LGs under the orthogonal conditions.[33] Despite the simplicity of the idea, the design of orthogonal one-pot synthesis requires a certain level of familiarity with oligosaccharide synthesis. To transform the highly specialized carbohydrate synthesis into a routine operation, a programmable one-pot method has been developed by the Wong group (Table 1, entry iv).[20a] Inspired by the seminal work of Ley[32c], the Wong group performed systematic measurements of the relative reactivity values (RRVs) of thioglycoside donors in the competition experiments against a peracetylated thiomannoside standard (RRV=1) and developed the OptiMer software to

search database for the optimal combination of coupling partners. [20a,b] The next-generation software Auto-CHO based on the artificial intelligence can now identify suitable building blocks for the complex glycan synthesis from 150 monosaccharides and fragments with validated RRV and 50,000 virtual building blocks with predicted RRV.[20c] Over the years, the programmable one-pot method has been successfully used to synthesize various oligosaccharides, including heparin pentasaccharides, LacNAc oligomers, the cancer antigens $Le^y$, $sLe^x$, fucosyl $GM_1$, Globo H and SSEA-4, and the embryonic stem cell surface carbohydrates $Lc_4$, and $IV^2Fuc\text{-}Lc_4$. [20,32] Using the thioglycoside donors and promoters (*i.e.*, NIS, TMSOTf), the programmable one-pot method is probably the most general method applicable to the synthesis of linear and branched sequences commonly found in human. However, if the availability of thioglycoside building blocks is an issue, then a preactivation-based iterative onepot strategy could be considered,[32,34] in which, a thioglycoside donor is activated with a promoter prior to the addition of acceptor. This method is independent of donor reactivity but requires a complete consumption of the initial reagents before adding the next set. As the result, glycosylation with low-reactivity substrates could be problematic and may require process optimization. Nevertheless, the preactivation one-pot method is particularly suited for the construction of repetitive oligosaccharide sequences.[8]

Another strategy for expediting the preparation of oligosaccharides is represented by the solid-phase synthesis, which eliminates the need for the purification of reaction intermediates and could be transformed into an automated process. The most advanced technology is the automated glycan assembly (AGA) developed by the Seeberger group.[21a,b] Following years of optimization of synthetic protocols and instrumentation, a fully automated glycan synthesizer, Glyconeer 2.1™, is now commercially available. In the AGA, the oligosaccharide sequence is constructed from the reducing end (*i.e.*, resin-bound acceptor) by iteration of glycosylation-capping-deprotection cycles with monitoring of the coupling efficiency by UV-trace of dibenzofulvene, which is formed upon deprotection of the Fmoc-group.[21d,e] Since the solid-phase synthesis requires the use of donors in excess and often encounters low-yields for the traditionally difficult glycosylations (*e.g.*, β-mannosylation, α-sialylation),[21e] AGA gives best results for optimized sequences using “approved” building blocks. Nonetheless, the list of AGA-compatible substrates is expanding, and the versatility of method has been demonstrated with the synthesis of different classes of glycans, covering blood group determinants, oligosaccharides of glycolipids, GAGs, parasitic GPIs, glycopeptides, microbial and plant polysaccharides.[21a-e] Other notable examples of automated methods (*e.g.*, one-pot solution-phase oligosaccharide synthesis by Quest-210 (Takahashi), the fluorous-tag-assisted synthesis based on the automated liquid handler and fluorous solid-phase extraction (FSPE) technique (Pohl), the HPLC-assisted oligosaccharide synthesis (Demchenko, Stine), and the automated electrochemical synthesis (Nokami)) have been covered in a recent review.[21f]

A more desirable technology, however, could combine the advantages of solidphase synthesis, enzymatic catalysis, and automation.[20b,35] While the first prototype of automated glycosynthesizer employing immobilized glycosyltransferases and polymer-bound substrates was reported in the early 2000s by Nishimura,[20b,35a,b] the application of this technology has been stalled by the limited availability of compatible enzymes with the desired selectivities.

Recent examples of the preparative automated enzymatic synthesis of glycans include a process based on the FSPE technique (Chen),[35c] and a microwave peptide synthesizer (Wang)[35d,e] with oligosaccharides bound to the water soluble thermoresponsive PNPAM-based polymer.[36]

Unlike the chemical synthesis of oligosaccharides, which had been practiced for more than 100 years, the enzymatic synthesis has gained its momentum only in the 1980s, following the development of recombinant DNA methodology and polymerase chain reaction (PCR) that permitted engineering and overexpression of enzymes with optimized stability, activities, and specificities.[37] The *in vitro* synthesis of oligosaccharides employs glycosyltransferases (GTs),[38] glycosidases (or glycosyl hydrolases, (GH))[38a,39] and phosphorylases.[40] Some of the first procedures of the preparative enzymatic synthesis utilized GTs that require sugar nucleotides as donors. To eliminate the use of expensive sugar nucleotides and prevent the feedback inhibition caused by the nucleoside phosphate byproduct, a sugar nucleotide recycling system was developed in 1982 (Figure 2A).[41] At present, the multienzymatic protocols for the regeneration of all nine nucleotide donors have been etsablished[4a,42] and applied to the synthesis of sialyl Lewis X,[42b] hyaluronic acid,[42c] heparin oligosaccharides,[42d] disialyllacto-N-tetraose,[42e] glycolipids[4a] and other complex glycoconjugates, including the first example of homogeneous glycoprotein synthesis.[42f] In recent years, many efficient protocols have been further developed by the groups of Chen and Wang, who coined the term OPME (One-Pot Multi Enzyme) synthesis.[42g]

Glycosidases and phosphorylases cleave glycosidic bonds releasing free sugar or glycoside-1-phosphate respectively. The reversibility of these transformations makes glycosidases and phosphorylases applicable to the synthesis of glycans. However, the product yield usually does not exceed 20–30% due to competitive hydrolysis. In 1998, the Withers group reported the first glycosynthase, which was generated from glycosidase by mutating the nucleophilic residue at the active site to abolish the hydrolytic activity.[43] Selective site mutations and directed evolution techniques have been used to generate glycosynthases with relaxed substrate specificities.[43d] Glycosidases of *endo*-type, such as *endo*-β-*N*-acetylglucosaminidase (ENGase), became essential for the preparation of homogeneous glycoproteins (Figure 2C),[39a,44] particularly therapeutic mAbs with well-defined glycan structure for optimal efficacy.[45]

Although the enzymatic synthesis of oligosaccharides provides a high control of stereoselectivity in glycosylation without the use of PGs, optimization of reaction conditions and purification protocols are often required to obtain practical yields. One potential limitation of the enzymatic method is the availability of enzymes with specificities for the desired linkages. Nevertheless, the number of carbohydrate-modifying enzymes is expanding. As of September 2013, the CAZy (Carbohydrate-Active enZyme) database had listings of 1936 characterized GTs (139 with structure) and 9221 glycosidases (817 with structure).[46] In addition, Moremen and Jarvis have reported an expression vector library encoding all known human glycoenzymes for the production in mammalian (HEK293) and baculovirus-infected insect cells.[47] Overall, the drawbacks of both chemical and enzymatic methods could be compensated by a combined chemoenzymatic approach.

## LIPID-LINKED OLIGOSACCHARIDES

Mammalian glycolipids, or glycosphingolipids (GSLs), are oligosaccharides β-linked to ceramide, a sphingoid amine (*i.e.*, sphingosine, sphinganine, phytosphingosine) acetylated with fatty acids ($C_{14}$-$C_{32}$).[48] The heterogeneity of GSLs arises from the structures of lipid and oligosaccharide. Depending on the glycan composition, GSLs could be classified into cerebrosides (*i.e.*, GlcCer and GalCer), sulfated, neutral and sialylated GSLs (or gangliosides). Generally, GSLs are derived from the simple Glc/GalCer, which are extended with Gal, GlcNAc, GalNAc and modified by GlcA, sulfation, phosphorylation, fucosylation and sialylation (Figure 1D), as in the Lewis histo-type antigens. Another level of heterogeneity stems from sialylation, which could be present in α2,3-, α2,6- or α2,8-linkages covering modifications with Neu5Ac, Neu5Gc, ketodeoxynonulosonic acid (KDN) and their acetylated, sulfated and methylated derivatives.

As in the synthesis of other glycoconjugates, the synthetic task could be broken down into syntheses of aglycon, oligosaccharide, and efficient conjugation of glycan to the lipid. Due to the relative structural simplicity and the strong link between abridged GSLs and tumorigenesis, the GLS glycans became some of the first targets of synthesis[48b] allowing investigation of these oligosaccharides as cancer biomarkers and antigens for the design of therapeutic vaccines.[2e,f] The most advanced vaccine candidate Globo H-KLH/QS21 (Phase III for metastatic breast cancer),[2f] has been further improved by the new generation Globo H-DT vaccine which is combined with an analog of α- galactosylceramide as adjuvant to enhance the antibody response to Globo H and the class switch from IgM to IgG.[4b,c] The induced antibodies showed specificity against Globo H, SSEA-3 and SSEA-4, which are overexpressed in breast cancer and its stem cells, as well as 15 other types of cancer cells. In another study, a Globo H vaccine candidate with 6N3-Fuc modification induced a stronger IgG response than the parent unmodified Globo H-DT conjugate.[4d] To assist the development of Globo H vaccine, an effective enzymatic process was developed by Wong for the gram-scale synthesis of Globo H and SSEA-4 oligosaccharides (Figure 2A).[4a]

The enzymatic synthesis could provide access to many GSL sequences,[48a,49] including ganglio-, lacto-, neolacto-, globo-, isoglobo-,[49a] and muco-series,[49b] as well as histo-blood group antigens;[49c,d] whereas chemical and chemoenzymatic approaches are useful for the synthesis of oligosaccharides with unusual modifications and linkages, such as *O*-sulfated $sLe^x$,[49e] or echinoderm-type gangliosides with potent neuritogeneic activity.[48b,c,49f] A list of enzymes suitable for the synthesis of GSLs, including fucosylsynthase (BbAfcBD703S) for the α1,4-fucosylation of GlcNAc[49g] and the α2,3/8-sialyltransferase (CjCstII),[49h] could be found in a recent review.[48a] Sialyltransferases for the synthesis of human-type α2,3/6/8- and bacterial α2,9-sialosides have been surveyed elsewhere.[49i]

The functional studies of glycolipids require the full-length oligosaccharide attached to a lipid,[48a] and the most straightforward synthetic strategy is based on the adaptation of biosynthetic pathways to *in vitro* synthesis. Although, this appoach was successfully applied to synthesize a bacterial Lipid II,50 it is not suitable for the human-type GSLs yet. The chemical synthesis of GSLs involves conjugation of a monosaccharide to the sphingosine base, followed by acetylation and glycan extension. In a chemoenzymatic approach, an

oligosaccharide (prepared by enzymatic method) is protected by acetylation and transformed into an imidate donor, which is then coupled to a sphingosine azide.[49j] After azide reduction and total deprotection, the oligosaccharide substrate could be further elaborated with GTs.[49k] To avoid solubility problems, installation of the fatty acid is carried out during the last step.

As an alternative approach, Withers and colleagues developed a glycosynthase-mediated synthesis of GSLs from unprotected glycosyl fluorides and sphingosine (Figure 2B) using the glycosynthase of *endo*-glycosylceramidase II (EGC II) from *Rhodococcus* strain M-777.[43b,c] The most active mutant EGC II$^{E351S}$ had relaxed substrate specificities for the glycan donor (*i.e.*, *lyso*-GM3, -GM1, -Gb3 and -Lac) accepting sphingosine and sphinganine substrates. Further mutagenesis experiments have led to the synthases with improved activities towards phytosphingosine.[43d] As in the case of chemoenzymatic approach, the late-stage acetylation with fatty acid gave fully elaborated GSLs.[43c]

Overall, the synthesis of GSL oligosaccharides with enzymes is well established and could be adjusted to other glycan sequences. The robustness of enzymatic protocols has been demonstrated with their adaptation to the automated synthesis.[35c,d] Chemical methods, however, are still used to create unusual linkages and modifications (*e.g.*, site-specific labeling of GSLs probes with reporting tags).[48a]

## PROTEIN-LINKED GLYCANS AND RELATED OLIGOSACCHARIDES

### Synthesis of *N*-glycans.

Oligosaccharides are often linked to proteins either at the side-chain nitrogen of Asn (*N*-glycans) or the hydroxyl group (*O*-glycans) of Ser, Thr, Tyr, *etc.* This section predominantly discusses proteins modified with *N*-glycans and mucin-type *O*-glycans with therapeutic significance. The biosynthesis of *N*-linked oligosaccahrides starts in ER51 and involves a step-wise synthesis of dolichol-linked $Glc_3Man_9GlcNAc_2$, which is transferred by oligosaccharyltransferase (OST) to the N-X-S/T (X ≠ P) sequon of a growing polypeptide (Figure 1A). After going through the CLX/CRT cycle(s), a correctly folded protein is relocated to the Golgi apparatus, where a high-mannose structure is converted into hybrid-or complex-type glycans. The enzymatic diversification of *N*-glycans in Golgi is affected by many factors, including the interplay of glycosyltransferases,[52] leading to heterogeneous mixtures of glycoforms representative of cell type and glycoprotein structure.

To address the great diversity of *N*-glycans, two main strategies for the generation of *N*-glycan libraries have been explored: (1) enzymatic diversification of a common precursor (Figure 3A);[53] and (2) modular glycan assembly (Figure 3B).[54] In 2013, Takeda and Ito introduced a top-down chemoenzymatic synthesis of the high-mannose-type glycans to study the substrate specificities of the glycan-modifying enzymes in ER. Starting from the synthetic $Man_9GlcNAc_2$-derived oligosaccharide with different non-native glycosides at terminal ends, the glycan library was generated by selective deprotection of each arm followed by enzymatic trimming of the Man-α1,2-Man linkages.[53a,b] Boons and colleagues reported several strategies towards complex-type *N*-glycans including the $Man_3GlcNAc_2$ precursor orthogonally protected at the O-2/4 and O-2/6 positions of terminal mannoses;

[53c-g] GlcNX4Man3GlcNAc2 precursor with 'masking' terminal GlcN3 and GlcNH2;[53e] and a $GalGlcNAc_4Man_3GlcNAc_2$ oligosaccharide modified with non-native Gal-α1,4-GlcNAc and Man-β1,4-GlcNAc. When used as a substrate for the carefully devised enzymatic sequences, a structurally diverse library of *N*-glycans was generated.[53f] Wang and colleagues designed a Core Synthesis/Enzymatic Extension (CSEE) approach, which utilized bacterial GTs and simple glycan cores to generate *N*-glycan and *O*-mannosyl glycan libraries.[53h-k] Using a similar logic, DeLisa and colleagues reported the enzymatic elaboration of *N*-glycan cores obtained from microbial glycoproteins and glycolipids.[53l]

In the modular method developed by Wong, three types of glycan modules are prepared by chemoenzymatic synthesis and converted to the glycosyl fluoride for coupling to the $Man_3GlcNAc_{2/1}$ core, generating a large number of multiantennary *N*-glycans.[54a-c] In addition to the fluoride approach, the Fukase and Unverzagt groups demonstrated glycosylation with trifluoroimidate[54d,e] and trichloroimidate[54f] donors respectively. A general chemical sequence for the synthesis of multi-branched *N*-glycan cores (Unverzagt) included a successive installation of the α1,3-arm, α1,6-arm, and the final insertion of the bisecting β1,4-GlcNAc.[54f] The optimized procedure for the chemical fucosylation of the α1,6-core has been also developed. As a general strategy, the modular method is applicable to all types of *N*-glycans, including unusual hybrid-type glycans, which have been identified in an array format as preferred epitopes of broadly neutralizing antibodies (bNAbs) against HIV-1,[54c] as well as non-native *N*-glycan cores modified with both bisecting GlcNAc and core fucose.[54f] This method is suitable for the synthesis of *N*-glycan substrates for the glycan remodeling of glycoproteins.[45f,g,55]

A major bottleneck in the syntheses of *N*-glycans is the availability of core oligosaccharides. The recent breakthroughs in the expression and purification of Alg1 and Alg2 allowed replication of the lipid-linked $Man_3GlcNAc_2$ biosynthesis *in vitro* (Figure 1A).[56] However, the Alg2-catalyzed reaction was found to be effective only for the $C_{20}$-$C_{25}$-long isoprenyl lipids[56b] making this process unsuitable for the synthesis of the core. Currently, apart from the chemical synthesis, the core oligosaccharides can be obtained from natural sources (*e.g.*, sialylglycopeptide (SGP) from egg yolk[53i] or recombinant bovine fetuin[53j]) albeit the purification process is laborious.

### Synthesis of mucin-type *O*-glycans and human milk oligosaccharides.

The biosynthesis of mucin-type *O*-glycans involves installation of GalNAc sites by a cohort of polypeptide *N*-α-acetylgalactosaminyltransferases (ppGalNAcTs),[57] and further modifications of the GalNAc residue by GTs (Figure 1B).[57b] Overall, the enzymatic synthesis of mucin-type oligosaccharides is not a significant challenge, as many GTs of the pathway have been reported,[38a,39b,47] whereas the chemical synthesis of mucin-type *O*-glycans (*e.g.*, Tn, sTn, T and sT antigens) has also been used for the exploration of therapeutic vaccines against cancer.[2e,58]

The human milk oligosaccharides (HMOs) have been investigated for their beneficial properties to the health of breastfed infants,[59] and shown to regulate intestinal microbiome, modulate epithelial and immune cell responses, and provide nutrients (*i.e.*, sialic acid) to the brain. As in the case of *O*-glycans, the "haphazard" biosynthesis leads to a high degree of

heterogeneity among HMOs, where a lactose core could be extended by type 2 structures (linear i-or β3/6-branched I-antigens), and terminated with type 1, Lewis-or blood-type antigens (Figure 1C). Over the years, methods for the chemical, enzymatic and chemoenzymatic syntheses of relatively short HMOs have been reported.[59a] In 2017, an enzymatic synthesis of the asymmetric multi-antennary HMOs was established by Boons (Figure 3C).[59d] The preparative enzymatic synthesis of HMOs could be performed with inexpensive bacterial enzymes coupled with sugar-nucleotide regeneration.[59a-c]

## HOMOGENEOUS GLYCOPROTEINS

### The glycosylation effect.

Although the effect of glycosylation has to be considered in the context of a specific protein, the production of therapeutic glycoproteins in nonhuman cells should avoid immunogenic epitopes, such as non-human α-Gal (*i.e.*, terminal Gal-α1,3-Gal), Neu5Gc, insect-type α1,3-fucosylation of the core, or yeast-type mannans. In addition, receptor-specific glycans could affect the distribution of the protein. For example, the ability of mannose-6-phosphate receptors (M6PRs) to transfer Man-6P-conjugated proteins to lysosome could help optimize the enzyme replacement therapies for the treatment of lysosomal storage disorders.[2g] Overall, the extended *N*- and *O*-glycans protect the underlying peptide sequence from proteolysis. Sialylation prevents protein aggregation and prolongs half-life by shielding the Gal residue, a ligand of the hepatic asialoglycoprotein receptor, which removes Gal-associated proteins from the bloodstream. Moreover, the pharmacokinetic properties of therapeutic proteins could be improved by protein modification with polysialic acid (PSA).[60] The effect of glycosylation has been also investigated in the context of glycoprotein stabilization.[2g] For example, the complex-type glycoforms of Fc region were found to be thermodynamically more stable, than the hybrid type, high-mannose or mono-GlcNAc glycoforms.[61]

Numerous experiments have been designed to understand the intrinsic effect of *O*-[62] and *N*-glycosylation[62f,g,63] on the stabilization and folding kinetics of glycoproteins. Several reports showed that the first sugar has the most influence on the conformational preferences of the glycopeptide. For example, the *O*-β-GlcNAc on an RNA polymerase II model was shown to promote type II β-turn structure and facilitate protein folding.[62a] The effect of α-GalNAc glycans on the local peptide conformation of MUC1 has been investigated in the context of the anticancer vaccine design.[62d] Overall, the *O*-glycosylation prompts “stiffening” and favors the extended conformation of the peptide backbone of MUC1. On the other hand, peptides modified with the β-GalNAc linkage exhibit the conformational behavior similar to the naked peptide, which is dynamic and relatively unrestrained. To explain why anti-MUC1 antibodies recognize Tn-Thr and have low affinities to the Tn-Ser moiety, the conformational differences between these antigens have been examined. Compared to GalNAc-α-Ser, the glycosidic linkage of GalNAc-α-Thr is rather rigid in solution occupying the eclipsed conformation caused by the interactions between the endocyclic oxygen and the methyl group of Thr. The recent study by Corzana *et al* demonstrated that the eclipsed conformation of Tn-Thr offers the space for a water pocket (Figure 4A), which was observed in solution, as well as in a protein-bound state.[62e]

Although *O*-glycosylation does not have a designated glycosylation sequon, studies aimed at identification of the preferred peptide acceptors of ppGalNAcTs have been performed. For example, using the GlycoSCORE technique, which combines *in vitro* screening of GTs and a library of synthetic peptides on self-assembled monolayers for matrix-assisted desorption/ionization mass spectrometry analysis, the preferred peptide sequences of *N*-acetylglucosamine transferase (OGT) have been determined.[64]

In general, the *N*-glycosylation of folded proteins (in contrast to glycopeptides) does not affect the average backbone fold; but provides a long-range stabilization of the tertiary or quaternary fold of glycoprotein.[63h-k] For example, the presence of GlcNAc-β1,4-GlcNAc-β at different sites of bacterial immunity protein Im7 was shown to have the most prominent stabilizing effect in the compact turn motifs between segments of ordered structure, where glycosylation promotes folding and enhances the overall stability of the native protein.[63k] This result may explain why glycosylation is commonly present at the transition between different types of secondary structures, where it may shield the disordered sequences from proteolysis.[63i]

Similar to *O*-glycosylation, the first couple of monosaccharides of *N*-glycan have been shown to affect protein stability most significantly.[63h] Using the cell adhesion and signaling molecule hCD2ad, Kelly and Wong determined that a total of ~3 kcal/mol of stabilization could be attributed to the $ManGlcNAc_2$ core trisaccharide, where the first GlcNAc (from the reducing end) contributes 2/3 and the core Man-β1,4-GlcNAc disaccharide provides 1/3 of the energy. The peripheral sugars have an insignificant effect on stabilization (Figure 4B).[63a] Further analysis revealed that aromatic residues, Aro (*i.e.*, Phe, Tyr, His, or Trp), at the [i-2] position to GlcNAc-Asn [i] offer an additional stabilization.[63b-d] The stabilizing enhanced-aromatic sequon (EAS)/reverse-turn pairings include the Phe-Asn-Xxx-Thr sequon for simple type I β-turns, Phe-Yyy-Asn-Xxx-Thr sequon for type I β-bulge turns, and the Phe-Yyy-Zzz-Asn-Xxx-Thr sequon for type II six-residue loop.[63c] To better understand the nature of interactions between the first sugar and the aromatic residue in EAS, the systematic studies of Pin WW domain with variable Aro[63d] and monosaccharides[63e] have been performed.

The apparent stabilizing interactions of EAS coincide with an increased probability of aromatic residues at positions [i-1] and [i-2].[63i] Later, it was shown that the EAS increased the glycosylation efficiency of OST and decreased the *N*-glycan heterogeneity by suppressing glycan processing in the Golgi complex.[63f] To further investigate the impact of peptide sequence on the efficiency of *N*-glycosylation, a systematic screening of the amino acids of the five-residue EAS of hCD2ad has been carried out (Figure 4B).[63g] In summary, the preferred sequon for *N*-glycosylation included aromatic (especially Thr) and sulfur-containing residues at the [i-2] position; aliphatic, hydroxyl and thiol-containing (particularly Cys) residues at [i-1]; and small residues at [i+1]. Incorporation of the EAS sequons into therapeutic glycoproteins is expected to lower the equilibrium concentration of the misfolded or unfolded proteins and improve the *in vivo* glycosylation of proteins.

### Glycan remodeling with ENGases and synthesis of homogeneous mAbs.

In the past decade, ENGase catalysis has emerged as a powerful tool for the synthesis of homogeneous glycoproteins, including therapeutic mAbs. Since the synthetic applications of ENGases have been recently reviewed,[39,44c] we briefly outline the basic principles of transglycosylation for the site-specific glycan modification focusing on the most recent examples. As a subclass of *endo*-hexosaminidases, ENGases cleave the GlcNAc-β1,4-GlcNAc linkage in *N*-glycans releasing the GlcNAc-β-protein. The active site of ENGases contains two catalytic residues: a general acid/base residue E and an assisting residue D (GH18 family), or N (GH85 family) (Figure 2C). Hydrolysis takes place with retention of configuration at the anomeric position through a substrate-assisted mechanism, where residues D/N do not directly trap the glycosyl intermediate but aid oxazolinium ion formation in the first step and its reaction with a water molecule in the second step. The investigation of the synthetic utility of ENGases began with the realization of the Man-β1,4-GlcNAc-oxazoline donor in 2001 by Shoda and colleagues,[44e] and development of the first glycosynthase mutant of Endo $M^{N175A}$ by Wang and Yamamoto in 2008 (Figure 2C).[44a] In general, glycosynthases are generated by mutating residues D/N, hence minimizing oxazoline towards hydrolysis. ENGases from many species with varied specificities to cover a wide spectrum of substrates have been identified.[39a] The list of acceptors includes GlcNAc-β-protein/peptide, GlcNAc-O*p*NP, as well as Glu and Man primers, and the scope of *N*-glycan substrates comprises of truncated, high mannose, hybrid, and mult-antennary complex-type *N*-glycans with sialylation and core-fucosylation.

The overall process of glycosylation remodeling consists of two steps: (a) trimming of heterogeneous glycans to a single GlcNAc-glycoform; and (b) *en bloc* transfer of a synthetic glycan onto the GlcNAc-site using either glycosidase-deffecient ENGase with a reactive oxazoline donor or wild-type ENGase with a stable *N*-glycan donor (Figure 5A). Until recently, the direct glycosylation of peptides was possible only with Glc by *N*-glucosyltransferase (NGT). However, in 2017, Wang, Cheng, and co-workers reported an engineered NGT ($ApNGT^{Q469A}$ from *Actinobacillus pleuropneumoniae*) with flexible acceptor and donor specificity, capable of transferring GlcN from UDP-GlcN onto N-X-S/T. Subsequent acetylation of GlcN with a glucosamine *N*-acetyltransferase from *Clostridium acetobutylicum* (GlmA) produced GlcNAc-modified peptides suitable for further transglycosylation with ENGases (Figure 5A).[65] The preferred glycosylation sequences for NGT have been identified using the GlycoSCORES technique.[64]

Despite apparent utility of the approach, the chemoenzymatic glycan remodeling has several limitations: (1) the use of glycosidase-deficient ENGases requires a large excess of oxazoline donor, which could lead to non-specific modifications of nucleophilic amino acids;[45i] (2) the high affinity of ENGase to glycoprotein may complicate the purification step leaving behind traces of enzyme, which could hydrolyze the glycoprotein sample with time; (3) the relatively high cost of chemoenzymatic method; (4) the specificity of ENGases for glycan structures at different glycosites can be affected by protein substrate. To overcome these drawbacks, alternative donors and new ENGases have been pursued. Among recent examples are the discovery of Endo E with low affinity for IgG substrate;[45g] Endo S double mutants (D233Q/E350Q and D233Q/D405A) with improved transglycosylation

efficiency;[45j] cost-efficient yeast expression and glycan rxemodeling with a stable SGP donor;[45g,j] transglycosylation with immobilized enzymes to simplify the prurification step[44d,45k] and site-selective glycoengineering of a therapeutic antibody using the differences in substrate specificity of Endo-S, Endo-S2 and Endo-F3.[45d] The major limitation of the method is the inability to selectively modify one GlcNAc-primed site in the presence of another. Although a recent report from the Wang group demonstrated that Endo F3$^{D165A}$ can modify two out of three glycosylation sites in EPO,[44f] the difference in reactivity was attributed to the steric hindrance at N24, and may not be generalized to other substrates. Overall, currently, the ENGase catalysis is limited mainly to glycoproteins with single glycosylation site, such as therapeutic antibodies.

Antibodies consist of the Fragment antibody binding (Fab) and Fragment crystallizable (Fc) domains responsible for antigen targeting and effector functions respectively. The Fc glycosylation site N297 is located in between heavy chains and near the antibody hinge, where it can influence antibody-receptor interactions by affecting the structure of the hinge or directly binding to the receptor. As such, glycosylation could influence IgG interactions with classical Fc receptors (*i.e.*, FcγRI, FcγRIIa-c, FcγRIIIa-b), complement proteins (C1q and mannose-binding lectin) and C-type lectin receptors (DC-SIGN). Different combinations of antibody-receptor interactions correspond to distinct immune cell responses, or antibody effector functions, such as antibody-dependent cellular phagocytosis (ADCP), antibody-dependent cellular cytotoxicity (ADCC), complementdependent cytotoxicity (CDC), and the anti-inflammatory activity.[6,66] Thus, optimization of N297 glycan provides means to tune the effector functions of mAb for the desired therapeutic application.[6] For example, it has been established that agalactosylation accompanies chronic inflammation, whereas α2,6-sialylation is associated with anti-inflammatory activity. Presence of core fucosylation is linked to the reduced ADCC, and galactosylated glycoform improves CDC. Despite the observed correlations between antibody glycosylation and immune response, the exact mechanisms of immune activation are difficult to decipher due to the heterogeneity of antibody glycoforms produced by the current cell-culture and fermentation methods. To address this challenge, a substantial effort has been directed towards the preparation of homogeneous antibodies for structural and functional studies[67]. Along this effort, a universal glycan was identified to optimize the anti-cancer, anti-infective and anti-inflammatory activities of IgG.[45f]

Figure 5B illustrates some of the notable achievements in the synthesis of IgG glycoforms, including the discovery of the Fc-specific Endo S,[45a,b] and Endo S2,[45c,d] as well as development of new enzymes (*e.g.*, Endo S mutants D233A, D233Q,[45e] and D233N;[45f] an efficient fucosidase (BfFucH) for the removal of core fucose;[55a] and Endo F3$^{D165A}$ for glycosylation with tri-antennary substrate[68]). Taking advantage of the relaxed substrate specificity of Endo S2, the Wong group generated a series of Endo S2 mutants, which were tested for the transglycosylation activity using a diverse array of oxazoline donors.[54a] The study identified three Endo S2 mutants (D226Q, D182Q and T138Q) with substrate specificities ranging from high-mannose to hybrid, and sialylated complex bi- and tri-antennary structures (Figure 5C).[55b] Other improvements to the ENGase method include the discovery of Endo E and A7283, which can accept tri-and tetra-antennary *N*-glycans;[45g] development of Endo S2$^{D184M}$ with enhanced transglycosylation activity;[45m] one-pot

enzymatic remodeling with Endo S based on the reduced affinity of enzyme towards high-mannose and hybrid-type *N*-glycans;[45l] the one-pot system comprised of Endo $M^{N175Q}$ (to generate reactive intermediate from SGP/SG-Asn donors) and Endo $S^{D233Q}$ (to modify GlcNAc-primed IgG);[45j] and the discovery of α1,6-fucosyntase $AlfC^{E274A}$ for the core fucosylation of intact *N*-glycoproteins with α-fucosyl fluoride donor.[45n]

## Synthesis of homogeneous glycoproteins and glycopeptides with multiple glycosylation sites.

Currently, the only way to obtain a homogeneous protein with variable glycans at different sites is to chemoselectively couple glycopeptide fragments.[69] Among many methods developed for the ligation of peptides, the native chemical ligation (NCL) is the most general technique.[69f] The scope and limitations of NCL are defined by the methodological developments in the area of peptide synthesis,[70] which is beyond the scope of this review.[69a-c] Herein, we focus on the applications of NCL and expressed protein ligation (EPL)[69e] to synthesize homogeneous glycoproteins.[69c,d,71]

In the NCL, peptides with N-to-D substitution are prepared by the solid phase peptide synthesis (SPPS), followed by installation of the desired glycan via Lansbury aspartylation[70a] or aminolysis of the activated thioester (Figure 6A).[70b] The installation of the pseudoproline motif by protecting Ser/Thr at [n+2] was found to facilitate glycosylation and prevent intramolecular aspartimide formation.[70c,d] The so-called convergent approach is optimal for the installation of large oligosaccharides, such as *N*-glycans. In the alternative "cassette" strategy, GlcNAc-Asn or Ser/Thr modified with short oligosaccharides are introduced directly into SPPS sequence. Next, deprotected peptides with GlcNAc/GalNAc-sites could be extended by enzymatic synthesis.

The key step of the NCL is a coupling of the activated thioester at the C-terminal with Cys (or thiol-modified amino acid) at the N-terminal, followed by S->N acyl transfer and subsequent desulfurization, if required (Figure 6B). The use of thiol auxiliaries at the N-terminal could improve the efficiency of ligation and eliminate the need for desulfurization.[70e] In the sugar-assisted ligation (SAL), the thiol auxiliary is placed at the amide/ester of GlcNAc/GalNAc, which is distant from the junction site.[70f-l] In general, the auxiliary-mediated ligations give the best results for the junctions containing unhindered Gly or Ala. The introduction of mild and aqueous-phase compatible desulfurization conditions[70m] by Danishefsky stimulated the development of NCL strategies using thiol-modified amino acids.[69a]

As an extension of NCL, the EPL permits incorporation of activated thioesters and cysteines into the expressed protein fragments by engineering protein constructs fused with intein or protease-specific peptide tag.[69e,72] The C-terminal thioester component is usually obtained from intein extrusion,[72a] whereas the N-terminal cysteine peptide fragment could be released by factor Xa,[72b] tobacco etch virus (TEV) protease,[72c,d] SUMO protease[72e] or DnaB-intein extrusion (Figure 6B).[72e] To simplify the isolation and purification of the expressed fragments, the affinity-specific tags (*e.g.*, $His_6$, or chitin-binding domain) are usually introduced next to the cleavable fragments of protein constructs. Some of the glycoprotein targets of pharmaceutical interest[9,71,72e,73] synthesized by NCL and EPL

include EPO[9], α-hGPH,[71b] hFSH,[71c] hLH,[71d] hCG,[71d] hTSH,[71e] GM-CSF,[71f] G-CSF3,[71g] hINF-β−1a,[73b] monoglycosylated GM2-activator protein,[73a] IL-6,[72e] and IL-8.[73c] In the case of the GPI-anchored proteins,[74] the ligation of the protein C-terminal to the N-terminal linked to the GPI-anchor has been carried out using NCL,[74a,b] EPL[74c] and Sortase A-mediated ligation. The last method, however, requires a non-native LPXTG peptide tag.[74d,e] Despite the advances, synthesis of full-length and functional glycoproteins with well-defined glycans at multiple sites remains a major challenge.

While indispensable for the study of glycosylation, the chemoenzymatic method cannot compete with the cell-based method yet, which involves the use of glycosylation pathway engineering to produce certain enriched human-like glycoforms. In addition, the emergence of technologies for the precise gene editing is opening up new possibilities for producing homogeneous glycoproteins.[75] Some recent examples include the GlycoDelete technology to give recombinant glycoproteins with simplified glycosylation;[75f] genetic manipulation of *N*-glycosylation in CHO cells using ZFN;[75c] and development of the SimpleCell technology to yield glycoproteins with simplified *O*-GalNAc-type[75d,e] and *O*-Mantype[75b] glycosylations; as well as application of the CRISPR/Cas9 technology to optimize *N*-glycosylation in insect cells[75g,h] and to edit the human glycosyltransferase genome using gRNA library.[75i] With the emergence of new tools of genetic engineering, we could expect more studies aimed at designing the glycosylation pathways tailored to the glycoform of interest.

## SYNTHESIS OF GLYCOSAMINOGLYCANS

The term GAGs covers several classes of linear polysaccharides (*i.e.*, HA, CS, DS, HS, and HP), which could be conjugated to the membrane-bound and GPI-anchored proteins, or secreted into the extracellular matrix, where GAGs interact with a variety of plasma proteins. Depending on the nature of proteoglycans and the expression conditions, proteoglycans can be modified with one or more GAG chains.[76] Due to the complexity of GAGs and the presence of negatively charged groups, the synthesis of proteoglycans and glycopeptides is less established as compared to the synthesis of *N*-glycosylated proteins. Instead, the major effort is focused on deciphering the sulfation code of GAGs with the help of analytical methods and homogeneous samples of GAG oligosaccharides.[77]

The least complex HA with GlcA-β1,3-GlcNAc-β1,4- repeating disaccharide is the only modification-free, unbound GAG (Figure 1E). KSs consist of LacNAc repeats with possible sulfation at *O*-6 and α1,3-fucosylation of the internal GlcNAc. The LacNAc part could extend from the protein-bound *N*-glycans, mucin-type and mannosyl *O*-glycans. The remaining GAGs are expressed on proteoglycans attached through a Xyl-β-linkage to Ser. Although poorly understood, the S-G-X-G (X ≠ P) sequon for xylosylation has been proposed.[78] Both CS and DS contain the GlcA-β1,3-GalNAc-β1,4- repeats with possible GlcA2S/3S and GalNAc4S/6S modifications; however in DS, a small portion of GlcA-β1,3- is epimerized into IdoA-α1,3-. HP and HS contain GlcA-β1,4-GlcNAc-α1,4- and IdoA-α1,4-GlcNAc- α1,4- units with deacetylated GlcN, sulfated GlcNS, and possible sulfation of GlcNS3S/6S and GlcA2S/IdoA2S. Compared to HS, HP contains a higher number of IdoA, sulfate groups and a smaller amount of unmodified GlcNAc. As such, the heterogeneity of

GAGs arises from the composition of glycan chain, sulfation pattern and the degree of polymerization. The polyanionic character of GAGs promotes interactions with positively charged proteins (*e.g.*, cytokines, growth hormone receptors, protease inhibitors, proteases, chemokines, and morphogens), and the associated therapeutic applications, many of which are pending the availability of homogeneous samples.[2h-j] Although chemical synthesis is currently the only method that could provide samples with desired sulfation pattern and backbone composition, the development of enzymatic synthesis of GAGs has been in a steady progress.[79] Since the chemical methods have been recently reviewed,[79a,b] herein, we highlight examples of the chemoenzymatic synthesis of well-defined GAG sequences and low-dispersity polysaccharides.[79c,d]

In general, the chemoenzymatic synthesis could be performed either by a stepwise elongation of GAG chain using GTs or polymerization of disaccharide oxazoline donor by hyaluronidase (Figure 7A). The latter approach developed by Ohmae, Kobayashi, and co-workers, has been applied for the polymerization of HA, chondroitin, CS-A, and oligomerization of KS.[79c,80] However, the GT-mediated synthesis is the most flexible method, which could be used to make polymers and oligosaccharides of defined structure.[5,42c,81] For example, a highly efficient HA synthase from *Pasteurella multocida* (PmHAS) containing two GT domains for UDP-GlcA and UDP-GlcNAc was shown to produce HA polymers with dispersity index ~1–1.3.[81a] A stepwise polymerization of HA using monofunctional mutants of PmHAS provided a better control of the degree of polymerization.[81b] In addition, the GT-mediated synthesis of HA is compatible with sugar-nucleotide regeneration.[42c] The oligosaccharides of CS have been obtained by the step-wise synthesis using bifunctional GT from *Escherichia coli* K4 (KfoC), followed by sulfation (Figure 7B).[81c]

Among the GAGs, HP is the most complex and the most studied polysaccharide due to its potent anticoagulant activity and the corresponding clinical application (Table 1).[2h-j] Using chemical synthesis, a pentasaccharide sequence required for the binding of antithrombin has been established leading to the development of Fondaparinux for the outpatient treatment of thrombotic disorders. Although the commercial drug is still produced by the chemical synthesis, the original process entails >50 steps with an overall yield of 0.1%. On the contrary, the chemoenzymatic synthesis of the active pentasaccharide developed by the Liu group involved only 12 steps with 37% overall yield (Figure 7C),[5a] The process started with *N*-deacetylation and *N*-sulfation of GlcNAc, followed by epimerization of the nearby GlcA to IdoA and sequential sulfations by 2-*O*-sulfotransferase (2-OST), 6-OST and 3-OST.[79d] Since only the NST domain of bifunctional *N*-deacetylase/*N*-sulfotransferase could be efficiently expressed in *E. coli*, the *N*-deacetylation was performed chemically by introducing the trifluroacetyl (TFA) protection. Thus, the bacterial heparan synthases KfiA (with UDP-GlcNTFA donor) and pmHS2 (with UDP-GlcA donor) were used to assemble heparosan backbone. Removal of the TFA group and *N*-sulfation with NST installed a recognition motif (*i.e.*, GlcNS at the preceding [−1] position) for $C_5$-epi. To ensure the irreversibility of $C_5$-epi-catalysis, the *N*-acetylated GlcNTFA at the succeeding [+3] position was introduced. Fixation of IdoA is also achieved by sulfation at O-2. A similar strategy has been applied to the synthesis of 8–12mer LMWHs with *p*NP at the reducing end.[5b] In the mouse model, the 10–12mer oligosaccharides were metabolized in the liver, suggesting that

these LMWHs are safe for the treatment of renal-impaired patients. In addition, protamine antidote was shown to neutralize the oligosaccharides larger than decasaccharide. Particularly, the 12mer LMWH with an extra GlcNS3S6S had the highest sensitivity to neutralization by protamine. Later, the gram-scale synthesis of the 12mer HP oligosaccharide was achieved by optimizing the enzyme and co-factor production.[5c] Linhardt *et al* have extended this strategy to produce a library of HS and HP oligosaccharides, which included the structures containing rare GlcA2S and IdoA.[77a] Other improvements to the enzymatic synthesis of HP include OPME synthesis,[81d] and processes based on the enzyme and substrate engineering to improve reaction efficiencies and selectivities.[81e-g] For example, a structural study of 6-OST has helped identify an optimized substrate for the efficient sulfation of all *O*-6 positions and revealed the potential amino acid mutations that could switch the substrate specificity of the enzyme.[81e] Characterization of 3-OST-3 demonstrated that unlike the 3-OST-1 isoform, 3-OST-3 preferentially sulfates substrates that do not carry 6-*O*-sulfation.[81f] In another study, protection of GlcA from epimerization by $C_5$-epi has been achieved by masking GlcNS at the succeeding [+1] position with methyl ether at O-6.[81g]

The recent progress in the enzymatic synthesis of GAGs, and particularly HP, is about to bring new GAG-derived therapeutics with improved potencies, pharmacokinetic and safety parameters. However, it is important to understand that the global demand of GAGs could be only fulfilled by the recombinant expression method. In order to move away from the large-scale extraction of HPs from the porcine trachea, new methods for the microbial production of HA, heparosan, and chondroitin, as well as enzymatic methods for the subsequent processing of these polysaccharides are being developed.[82] In any event, further development of effective methods for the synthesis of heparin saccharides with regiodefined sulfation patterns to enable the study of their role in receptor binding and functions is needed. In addition to the identification of specific sulfotransferases for regioselective sulfation, the development of chemical methods for access to regiodefined sulfate derivatives could be an alternative approach[20e].

## CONCLUSION AND FUTURE PROSPECTS

In this review we deliberately focused on the evolution of methods for oligosaccharide synthesis with the goal to understand the roles of glycans in biology and to translate this knowledge into biomedical applications. However, the availability of homogeneous samples of glycans in sufficient quantities is vital for the study of chemical glycobiology[2b,83] and development of essential tools (*e.g.*, glycan arrays,[83a] glycosylation probes,[2b] and new techniques for studying glycosylation at the single-molecule level[83b]), as well as methods for glycan analysis (*e.g.*, glycoproteomics and glycomics).[83c] The structural complexity of oligosaccharides and the technical challenges associated with their synthesis resulted in a unique situation, where both chemical and enzymatic methods are now commonly used to prepare homogeneous glycans, glycoproteins and other glycoconjugates, including the preparation of homogeneous samples which contain a probe, a drug or a prodrug through glycoengineering.[84] We believe that in the next decade, many therapeutically relevant human-type sequences could be assembled by the methods described in this Perspective. The synthesis of oligosaccharides will continue to play a major role in exploratory research

for the understanding of biological glycosylation, illuminating the path for glycoscience and glycomedicines towards a new frontier.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## ACKNOWLEDGMENT

This work was supported by the National Institutes of Health (AI072155), the National Science Foundation (CHE-1664283), Academia Sinica and the Kwang Hua Foundation.

## REFERENCES

(1). Varki A “Biological roles of glycans” Glycobiology 2017, 27, 3–49. [PubMed: 27558841]

(2). aDalziel M; Crispin M; Scanlan CN; Zitzmann N; Dwek RA “Emerging principles for the therapeutic exploitation of glycosylation” Science 2014, 343, 1235681; [PubMed: 24385630] bHudak JE; Bertozzi CR “Glycotherapy: new advances inspire a reemergence of glycans in medicine” Chem. Biol 2014, 21, 16–37; [PubMed: 24269151] cRodriguez E; Schetters STT; van Kooyk Y “The tumour glyco-code as a novel immune checkpoint for immunotherapy” Nat Rev Immunol 2018, 18, 204–211; [PubMed: 29398707] dWei MM; Wang YS; Ye XR “Carbohydrate-based vaccines for oncotherapy” Med Res Rev 2018, 38, 1003–1026; [PubMed: 29512174] eWilson RM; Danishefsky SJ “A vision for vaccines built from fully synthetic tumor-associated antigens: from the laboratory to the clinic” J. Am. Chem. Soc 2013, 135, 14462–14472; [PubMed: 23944352] fDanishefsky SJ; Shue Y-K; Chang MN; Wong C-H “Development of Globo-H cancer vaccine” Acc. Chem. Res 2015, 48, 643–652; [PubMed: 25665650] gSola RJ; Griebenow K “Glycosylation of therapeutic proteins: an effective strategy to optimize efficacy” BioDrugs 2010, 24, 9–21; [PubMed: 20055529] hLima M; Rudd T; Yates E “New applications of heparin and other glycosaminoglycans” Molecules 2017, 22, 749;iGulati K; Poluri KM “Mechanistic and therapeutic overview of glycosaminoglycans: the unsung heroes of biomolecular signaling” Glycoconj. J 2016, 33, 1–17; [PubMed: 26635091] jKowitsch A; Zhou G; Groth T “Medical application of glycosaminoglycans: a review” J. Tissue Eng. Regen. Med 2018, 12, e23–e41; [PubMed: 28079984] kYu S; Guo Z; Johnson C; Gu G; Wu Q “Recent progress in synthetic and biological studies of GPI anchors and GPI-anchored proteins” Curr. Opin. Chem. Biol 2013, 17, 1006–1013; [PubMed: 24128440] lTsai Y-H; Liu X; Seeberger PH “Chemical biology of glycosylphosphatidylinositol anchors” Angew. Chem. Int. Ed. Engl 2012, 51, 11438–11456. [PubMed: 23086912]

(3). Lichtenthaler FW “100 Years “Schlüssel-Schloss-Prinzip”: What made Emil Fischer use this analogy?” Angew. Chem. Int. Ed. Engl 1995, 33, 2364–2374.

(4). aTsai T-I; Lee H-Y; Chang S-H; Wang C-H; Tu Y-C; Lin Y-C; Hwang D-R; Wu C-Y; Wong C-H “Effective sugar nucleotide regeneration for the large-scale enzymatic synthesis of Globo H and SSEA4” J. Am. Chem. Soc 2013, 135, 14831–14839; [PubMed: 24044869] bHuang Y-L; Hung J-T; Cheung S-K; Lee H-Y; Chu K-C; Li S-T; Lin Y-C; Ren C-T; Cheng T-J; Hsu T-L; Yu A-L; Wu C-Y; Wong C-H “Carbohydrate-based vaccines with a glycolipid adjuvant for breast cancer” Proc. Natl. Acad. Sci. U. S. A 2013, 110, 2517–2522; [PubMed: 23355685] cHung T-C; Lin C-W; Hsu T-L; Wu C-Y; Wong C-H “Investigation of SSEA-4 binding protein in breast cancer cells” J. Am. Chem. Soc 2013, 135, 5934–5937; [PubMed: 23574147] dLee H-Y; Chen C-Y; Tsai T-I; Li S-T; Lin K-H; Cheng Y-Y; Ren C-T; Cheng T-J; Wu C-Y; Wong C-H “Immunogenicity study of Globo H analogues with modification at the reducing or nonreducing end of the tumor antigen” J. Am. Chem. Soc 2014, 136, 16844–16853. [PubMed: 25371992]

(5). aXu Y; Masuko S; Takieddin M; Xu H; Liu R; Jing J; Mousa SA; Linhardt RJ; Liu J “Chemoenzymatic synthesis of homogeneous ultralow molecular weight heparins” Science 2011, 334, 498–501; [PubMed: 22034431] bXu Y; Cai C; Chandarajoti K; Hsieh PH; Li L; Pham TQ; Sparkenbaugh EM; Sheng J; Key NS; Pawlinski R; Harris EN; Linhardt RJ; Liu J “Homogeneous low-molecular-weight heparins with reversible anticoagulant activity” Nat. Chem. Biol 2014, 10,

248–250; [PubMed: 24561662] cXu Y; Chandarajoti K; Zhang X; Pagadala V; Dou W; Hoppensteadt DM; Sparkenbaugh EM; Cooley B; Daily S; Key NS; Severynse-Stevens D; Fareed J; Linhardt RJ; Pawlinski R; Liu J "Synthetic oligosaccharides can replace animal-sourced low-molecular weight heparins" Sci. Transl. Med 2017, 9, eaan5954. [PubMed: 28878012]

(6). Mastrangeli R; Palinsky W; Bierau H "Glycoengineered antibodies: towards the next-generation of immunotherapeutics" Glycobiology 2018.

(7). aHung SC; Zulueta MML Glycochemical Synthesis: Strategies and Applications; First ed.; Wiley & Sons: Hoboken, New Jersey, 2016;bBennett CS Selective Glycosylations: Synthetic Methods and Catalysts; Wiley-VCH Verlag GmbH & Co. KGaA: Weinheim, Germany, 2017.

(8). Wu Y; Xiong D-C; Chen S-C; Wang Y-S; Ye X-S "Total synthesis of mycobacterial arabinogalactan containing 92 monosaccharide units" Nat. Commun 2017, 8, 14851. [PubMed: 28300074]

(9). aWang P; Dong S; Shieh J-H; Peguero E; Hendrickson R; Moore M; Danishefsky S "Erythropoietin derived by chemical synthesis" Science 2013, 342, 1357–1360; [PubMed: 24337294] bMurakami M; Kiuchi T; Nishihara M; Tezuka K; Okamoto R; Izumi M; Kajihara Y "Chemical synthesis of erythropoietin glycoforms for insights into the relationship between glycosylation pattern and bioactivity" Sci. Adv 2016, 2, e1500678. [PubMed: 26824070]

(10). Goodman L Neighboring-Group Participation in Sugars; In Advances in Carbohydrate Chemistry; Wolfrom ML, Tipson RS, Eds.; Academic Press: 1967; Vol. 22, p 109–175.

(11). aKim JH; Yang H; Park J; Boons GJ "A general strategy for stereoselective glycosylations" J. Am. Chem. Soc 2005, 127, 12090–12097; [PubMed: 16117550] bFascione MA; Adshead SJ; Stalford SA; Kilner CA; Leach AG; Turnbull WB "Stereoselective glycosylation using oxathiane glycosyl donors" Chem. Commun 2009, 5841–5843;cFang T; Mo KF; Boons GJ "Stereoselective assembly of complex oligosaccharides using anomeric sulfonium ions as glycosyl donors" J. Am. Chem. Soc 2012, 134, 7545–7552; [PubMed: 22475263] dSingh GP; Watson AJ; Fairbanks AJ "Achiral 2-hydroxy protecting group for the stereocontrolled synthesis of 1,2-cis-alpha-glycosides by six-ring neighboring group participation" Org. Lett 2015, 17, 4376–4379; [PubMed: 26308903] eFang T; Gu Y; Huang W; Boons G-J "Mechanism of glycosylation of anomeric sulfonium ions" J. Am. Chem. Soc 2016, 138, 3002–3011; [PubMed: 26878147] fMensink RA; Boltje TJ "Advances in stereoselective 1,2-cis glycosylation using C-2 auxiliaries" Chem. Eur. J 2017, 23, 17637–17653; [PubMed: 28741787] gElferink H; Mensink RA; White PB; Boltje TJ "Stereoselective β-mannosylation by neighboring-group participation" Angew. Chem. Int. Ed. Engl 2016, 55, 11217–11220. [PubMed: 27405102]

(12). Komarova BS; Tsvetkov YE; Nifantiev NE "Design of alpha-Selective Glycopyranosyl Donors Relying on Remote Anchimeric Assistance" Chem. Rec 2016, 16, 488–506. [PubMed: 26785933]

(13). aYasomanee JP; Demchenko AV "Effect of remote picolinyl and picoloyl substituents on the stereoselectivity of chemical glycosylation" J. Am. Chem. Soc 2012, 134, 20097–20102; [PubMed: 23167454] bPistorio SG; Yasomanee JP; Demchenko AV "Hydrogen-bond-mediated aglycone delivery: focus on beta-mannosylation" Org. Lett 2014, 16, 716–719; [PubMed: 24471826] cYasomanee JP; Demchenko AV "Hydrogen bond mediated aglycone delivery: synthesis of linear and branched alpha-glucans" Angew. Chem. Int. Ed. Engl 2014, 53, 10453–10456; [PubMed: 25115776] dYasomanee JP; Demchenko AV "Hydrogen-bond-mediated aglycone delivery (HAD): a highly stereoselective synthesis of 1,2-cis alpha-D-glucosides from common glycosyl donors in the presence of bromine" Chem. Eur. J 2015, 21, 6572–6581; [PubMed: 25765479] eNigudkar SS; Demchenko AV "Stereocontrolled 1,2-glycosylation as the driving force of progress in synthetic carbohydrate chemistry" Chem. Sci 2015, 6, 2687–2704; [PubMed: 26078847] fLiu QW; Bin HC; Yang JS "beta-Arabinofuranosylation using 5-O-(2-quinolinecarbonyl) substituted ethyl thioglycoside donors" Org. Lett 2013, 15, 3974–3977; [PubMed: 23879464] gRuei JH; Venukumar P; Ingle AB; Mong KK "C6 picoloyl protection: a remote stereodirecting group for 2-deoxy-beta-glycoside formation" Chem. Commun 2015, 51, 5394–5397;hEscopy S; Geringer SA; De Meo C "Combined effect of the picoloyl protecting group and triflic acid in sialylation" Org. Lett 2017, 19, 2638–2641; [PubMed: 28453277] iWu YF; Tsai YF "Assistance of the C-7,8-picoloyl moiety for directing the glycosyl acceptors into the alpha-orientation for the glycosylation of sialyl donors" Org. Lett 2017, 19, 4171–4174;

[PubMed: 28753308] jHuang W; Zhou YY; Pan XL; Zhou XY; Lei JC; Liu DM; Chu Y; Yang JS "Stereodirecting effect of C5-carboxylate substituents on the Glycosylation stereochemistry of 3-deoxy-d-manno-oct-2-ulosonic acid (Kdo) thioglycoside donors: stereoselective synthesis of alpha-and beta-Kdo glycosides" J. Am. Chem. Soc 2018, 140, 3574–3582; [PubMed: 29481074] kLu YL; Ghosh B; Mong KT "Unusually stable picoloyl-protected trimethylsilyl glycosides for nonsymmetrical 1,1'-glycosylation and synthesis of 1,1'-disaccharides with diverse configurations" Chem. Eur. J 2017, 23, 6905–6918. [PubMed: 28272765]

(14). aBarresi F; Hindsgaul O "Synthesis of beta-mannopyranosides by intramolecular aglycon delivery" J. Am. Chem. Soc 1991, 113, 9376–9377;bStork G; Kim G "Stereocontrolled synthesis of disaccharides via the temporary silicon connection" J. Am. Chem. Soc 1992, 114, 1087–1088;cYukishige I; Tomoya O "A novel approach to the stereoselective synthesis of β-mannosides" Angew. Chem. Int. Ed. Engl 1994, 33, 1765–1767;dAbdel-Rahman AA; El Ashry el SH; Schmidt RR "Efficient intramolecular beta-mannoside formation using m-xylylene and isophthaloyl derivatives as rigid spacers" Carbohydr. Res 2002, 337, 195–206; [PubMed: 11844489] eIshiwata A; Munemura Y; Ito Y "NAP ether mediated intramolecular aglycon delivery: a unified strategy for 1,2-cis-glycosylation" Eur. J. Org. Chem 2008, 2008, 4250–4263;fFairbanks AJ "Intramolecular aglycon delivery (IAD): the solution to 1,2-cis stereocontrol for oligosaccharide synthesis?" Synlett 2003, 2003, 1945–1958;gJia XG; Demchenko AV "Intramolecular glycosylation" Beilstein J. Org. Chem 2017, 13, 2028–2048; [PubMed: 29062425] hFujikawa K; Seko A; Takeda Y; Ito Y "Approaches toward high-mannose-type glycan libraries" Chem. Rec 2016, 16, 35–46. [PubMed: 26493153]

(15). aCrich D "Methodology development and physical organic chemistry: a powerful combination for the advancement of glycochemistry" J. Org. Chem 2011, 76, 9193–9209; [PubMed: 21919522] bSasaki K; Tohda K "Recent topics in β-stereoselective mannosylation" Tetrahedron Lett 2018, 59, 496–503;cImamura A; Ando H; Korogi S; Tanabe G; Muraoka O; Ishida H; Kiso M "Di-tert-butylsilylene (DTBS) group-directed α-selective galactosylation unaffected by C-2 participating functionalities" Tetrahedron. Lett 2003, 44, 6725–6728;eHashimoto Y; Tanikawa S; Saito R; Sasaki K "beta-Stereoselective mannosylation using 2,6-lactones" J. Am. Chem. Soc 2016, 138, 14840–14843; [PubMed: 27782395] fManabe S; Ishii K; Ito Y "N-benzyl-2,3-oxazolidinone as a glycosyl donor for selective alpha-glycosylation and one-pot oligosaccharide synthesis involving 1,2-cis-glycosylation" J. Am. Chem. Soc 2006, 128, 10666–10667; [PubMed: 16910646] gDhakal B; Crich D "Synthesis and stereocontrolled equatorially selective glycosylation reactions of a pseudaminic acid donor: importance of the side-chain conformation and regioselective reduction of azide protecting groups" J. Am. Chem. Soc 2018, 140, 15008–15015; [PubMed: 30351022] hTanaka H; Nishiura Y; Takahashi T "Stereoselective synthesis of oligo-α-(2,8)-sialic acids" J. Am. Chem. Soc 2006, 128, 7124–7125; [PubMed: 16734441] iHsu C-H; Chu K-C; Lin Y-S; Han J-L; Peng Y-S; Ren C-T; Wu C-Y; Wong C-H "Highly alpha-selective sialyl phosphate donors for efficient preparation of natural sialosides" Chem. Eur. J 2010, 16, 1754–1760; [PubMed: 20066711] jChu K-C; Ren C-T; Lu C-P; Hsu C-H; Sun T-H; Han J-L; Pal B; Chao T-A; Lin Y-F; Wu S-H; Wong C-H; Wu C-Y "Efficient and stereoselective synthesis of alpha-2,9 oligosialic acids: from monomers to dodecamers" Angew. Chem. Int. Ed. Engl 2011, 50, 9391–9395. [PubMed: 21948436]

(16). Yu B "Gold(I)-catalyzed glycosylation with glycosyl o-alkynylbenzoates as donors" Acc. Chem. Res, 2018, 51, 507–516; [PubMed: 29297680] Nielsen MM; Pedersen CM "Catalytic glycosylations in oligosaccharide synthesis" Chem. Rev 2018, 118, 8285–8358. [PubMed: 29969248]

(17). Nogueira JM; Bylsma M; Bright DK; Bennett CS "Reagent-controlled alpha-selective dehydrative glycosylation of 2,6-dideoxy-and 2,3,6-trideoxy sugars" Angew. Chem. Int. Ed. Engl 2016, 55, 10088–10092. [PubMed: 27431663]

(18). aTaylor MS "Catalysis based on reversible covalent interactions of organoboron compounds" Acc. Chem. Res 2015, 48, 295–305; [PubMed: 25493641] bD'Angelo KA; Taylor MS "Borinic acid catalyzed stereo-and regioselective couplings of glycosyl methanesulfonates" J. Am. Chem. Soc 2016, 138, 11058–11066; [PubMed: 27533523] cMancini RS; McClary CA; Anthonipillai S; Taylor MS "Organoboron-promoted regioselective glycosylations in the synthesis of a saponin-derived pentasaccharide from spergularia ramosa" J. Org. Chem 2015, 80, 8501–8510. [PubMed: 26292956]

(19). aTakahashi D; Tanaka M; Nishi N; Toshima K “Novel 1,2-cis-stereoselective glycosylations utilizing organoboron reagents and their application to natural products and complex oligosaccharide synthesis” Carbohydr. Res 2017, 452, 64–77; [PubMed: 29080430] bTanaka M; Nakagawa A; Nishi N; Iijima K; Sawa R; Takahashi D; Toshima K “Boronic-acid-catalyzed regioselective and 1,2-cis-stereoselective glycosylation of unprotected sugar acceptors via SNi-type mechanism” J. Am. Chem. Soc 2018, 140, 3644–3651. [PubMed: 29457892]

(20). aZhang Z; Ollmann IR; Ye X-S; Wischnat R; Baasov T; Wong C-H “Programmable One-Pot Oligosaccharide Synthesis” J. Am. Chem. Soc 1999, 121, 734–753;bHsu C-H; Hung S-C; Wu C-Y; Wong C-H “Toward automated oligosaccharide synthesis” Angew. Chem. nt. Ed. Engl 2011, 50, 11872–11923;cCheng C-W; Zhou Y; Pan W-H; Dey S; Wu C-Y; Hsu W-L; Wong C-H “Hierarchical and programmable one-pot automated synthesis of oligosaccharides.” Nat. Commun 2018;dTing C-Y; Lin Y-W; Wu C-Y; Wong C-H “Design of disaccharide modules for a programmable one-pot synthesis of building blocks with LacNAc repeating units for asymmetric N-glycans” Asian J. Org. Chem 2017, 6, 1800–1807;eDey S; Wong C-H “Programmable one-pot synthesis of heparin pentasaccharides enabling access to regiodefined sulfate derivatives” Chem. Sci 2018, 9, 6685–6691. [PubMed: 30310602]

(21). aPlante OJ; Palmacci ER; Seeberger PH “Automated solid-phase synthesis of oligosaccharides” Science 2001, 291, 1523–1527; [PubMed: 11222853] bSeeberger PH “The logic of automated glycan assembly” Acc. Chem. Res 2015, 48, 1450–1463; [PubMed: 25871824] cHahm HS; Hurevich M; Seeberger PH “Automated assembly of oligosaccharides containing multiple cis-glycosidic linkages” Nat. Commun 2016, 7, 12482; [PubMed: 27580973] dHahm HS; Schlegel MK; Hurevich M; Eller S; Schuhmacher F; Hofmann J; Pagel K; Seeberger PH “Automated glycan assembly using the Glyconeer 2.1 synthesizer” Proc. Natl. Acad. Sci. U. S. A 2017, 114, E3385–E3389; [PubMed: 28396442] ePardo-Vargas A; Delbianco M; Seeberger PH “Automated glycan assembly as an enabling technology” Curr. Opin. Chem. Biol 2018, 46, 48–55; [PubMed: 29715619] fPanza M; Pistorio SG; Stine KJ; Demchenko AV “Automated chemical oligosaccharide synthesis: novel approach to traditional challenges” Chem. Rev 2018, 118, 8105–8150. [PubMed: 29953217]

(22). aAdero PO; Amarasekara H; Wen P; Bohé L; Crich D “The experimental evidence in support of glycosylation mechanisms at the SN1–SN2 interface” Chem. Rev 2018, 118, 8242–8284; [PubMed: 29846062] bFrihed TG; Bols M; Pedersen CM “Mechanisms of glycosylation reactions studied by low-temperature nuclear magnetic resonance” Chem. Rev 2015, 115, 4963–5013. [PubMed: 25923428]

(23). aBennett CS; Galan MC “Methods for 2-deoxyglycoside synthesis” Chem. Rev 2018, 118, 7931–7985; [PubMed: 29953219] bZeng J; Xu Y; Wang H; Meng L; Wan Q “Recent progress on the synthesis of 2-deoxy glycosides” Sci. China Chem 2017, 60, 1162–1179;cBalmond EI; Benito-Alifonso D; Coe DM; Alder RW; McGarrigle EM; Galan MC “A 3,4-trans-fused cyclic protecting group facilitates alpha-selective catalytic synthesis of 2-deoxyglycosides” Angew. Chem. Int. Ed. Engl 2014, 53, 8190–8194. [PubMed: 24953049]

(24). aLian G; Zhang X; Yu B “Thioglycosides in carbohydrate research” Carbohydr. Res 2015, 403, 13–22; [PubMed: 25015586] bCodee JDC; Litjens REJN; van den Bos LJ; Overkleeft HS; van der Marel GA “Thioglycosides in sequential glycosylation strategies” Chem. Soc. Rev 2005, 34, 769–782; [PubMed: 16100617] cZhu X; Schmidt RR “New principles for glycoside-bond formation” Angew. Chem. Int. Ed. Engl 2009, 48, 1900–1934. [PubMed: 19173361]

(25). aSpell ML; Deveaux K; Bresnahan CG; Bernard BL; Sheffield W; Kumar R; Ragains JR “A visible-light-promoted O-glycosylation with a thioglycoside donor” Angew. Chem. Int. Ed. Engl 2016, 55, 6515–6519; [PubMed: 27086646] bMao R-Z; Xiong D-C; Guo F; Li Q; Duan J; Ye X-S “Light-driven highly efficient glycosylation reactions” Org. Chem. Front 2016, 3, 737–743;cZhao G; Wang T “Stereoselective synthesis of 2-deoxyglycosides from glycals by visible-light-induced photoacid catalysis” Angew. Chem. Int. Ed. Engl 2018, 57, 6120–6124; [PubMed: 29569307] dNokami T; Saito K; Yoshida J “Synthetic carbohydrate research based on organic electrochemistry” Carbohydr. Res 2012, 363, 1–6; [PubMed: 23089173] eWilliams R; Galan MC “Recent advances in organocatalytic glycosylations” Eur. J. Org. Chem 2017, 2017, 6247–6264;fWang HY; Blaszczyk SA; Xiao G; Tang W “Chiral reagents in glycosylation and modification of carbohydrates” Chem. Soc. Rev 2018, 47, 681–701; [PubMed: 29206256]

gGervay-Hague J "Taming the reactivity of glycosyl iodides to achieve stereoselective glycosidation" Acc. Chem. Res 2016, 49, 35–47. [PubMed: 26524481]

(26). Park Y; Harper KC; Kuhl N; Kwan EE; Liu RY; Jacobsen EN "Macrocyclic bisthioureas catalyze stereoselective glycosylation reactions" Science 2017, 355, 162–166. [PubMed: 28082586]

(27). aLawandi J; Rocheleau S; Moitessier N "Regioselective acylation, alkylation, silylation and glycosylation of monosaccharides" Tetrahedron 2016, 72, 6283–6319;bÁgoston K; Streicher H; Fügedi P "Orthogonal protecting group strategies in carbohydrate chemistry" Tetrahedron: Asymmetry 2016, 27, 707–728.

(28). Peng P; Schmidt RR "Acid–base catalysis in glycosidations: A nature derived alternative to the generally employed methodology" Acc. Chem. Res 2017, 50, 1171–1183. [PubMed: 28440624]

(29). aKaji E; Yamamoto D; Shirai Y; Ishige K; Arai Y; Shirahata T; Makino K; Nishino T "Thermodynamically controlled regioselective glycosylation of fully unprotected sugars through bis(boronate) intermediates" Eur. J.. Org. Chem 2014, 2014, 3536–3539;bHoang KLM; He J.-x.; Báti G; Chan-Park MB; Liu X-W "A minimalist approach to stereoselective glycosylation with unprotected donors" Nat. Commun 2017, 8, 1146. [PubMed: 29079775]

(30). aMootoo DR; Konradsson P; Udodong U; Fraser-Reid B "Armed and disarmed n-pentenyl glycosides in saccharide couplings leading to oligosaccharides" J. Am. Chem. Soc 1988, 110, 5583–5584;bRaghavan S; Kahne D "A one step synthesis of the ciclamycin trisaccharide" J. Am. Chem. Soc 1993, 115, 1580–1581;cDouglas NL; Ley SV; Lucking U; Warriner SL "Tuning glycoside reactivity: New tool for efficient oligosaccharide synthesis" J. Chem. Soc., Perkin Trans 1 1998, 51–66.

(31). aWang C-C; Lee J-C; Luo S-Y; Kulkarni SS; Huang Y-W; Lee C-C; Chang K-L; Hung S-C "Regioselective one-pot protection of carbohydrates" Nature 2007, 446, 896–899; [PubMed: 17443183] bKo YC; Tsai CF; Wang CC; Dhurandhare VM; Hu PL; Su TY; Lico LS; Zulueta MM; Hung SC "Microwave-assisted one-pot synthesis of 1,6-anhydrosugars and orthogonally protected thioglycosides" J. Am. Chem. Soc 2014, 136, 14425–14431; [PubMed: 25291402] cDhurandhare VM; Wen Y-S; Gawande SD; Liao P-H; Wang C-C "Synthesis of d-galactosamine and d-allosamine derivatives via a microwave-assisted preparation of 1,6-anhydroglucosamine" J. Org. Chem 2016, 81, 11521–11528. [PubMed: 27775348]

(32). Kulkarni SS; Wang CC; Sabbavarapu NM; Podilapu AR; Liao PH; Hung SC ""One-Pot" protection, glycosylation, and protection-glycosylation strategies of carbohydrates" Chem. Rev 2018, 118, 8025–8104. [PubMed: 29870239]

(33). Yamada H; Harada T; Miyazaki H; Takahashi T "One-pot sequential glycosylation: A new method for the synthesis of oligosaccharides" Tetrahedron Lett 1994, 35, 3979–3982.

(34). Huang X; Huang L; Wang H; Ye XS "Iterative one-pot synthesis of oligosaccharides" Angew. Chem. Int. Ed. Engl 2004, 43, 5221–5224. [PubMed: 15455432]

(35). aNishimura S-I Solution and Polymer-Supported Synthesis of Carbohydrates; In Carbohydrate-Based Drug Discovery; Wong C-H, Ed.; Wiley-VCH Verlag GmbH & Co. KGaA: 2003, p 129–136;bMatsushita T; Nagashima I; Fumoto M; Ohta T; Yamada K; Shimizu H; Hinou H; Naruchi K; Ito T; Kondo H; Nishimura S "Artificial Golgi apparatus: globular protein-like dendrimer facilitates fully automated enzymatic glycan synthesis" J. Am. Chem. Soc 2010, 132, 16651–16656; [PubMed: 21033706] cChen X; Yu H; Hwang J "Automated carbohydrate synthesis" patent WO/2014/201462 12 18, 2014;dWen L; Edmunds G; Gibbons C; Zhang J; Gadi MR; Zhu H; Fang J; Liu X; Kong Y; Wang PG "Toward automated enzymatic synthesis of oligosaccharides" Chem Rev 2018, 118, 8151–8187; [PubMed: 30011195] eZhang J; Chen C; Gadi MR; Gibbons C; Guo Y; Cao X; Edmunds G; Wang S; Liu D; Yu J; Wen L; Wang PG "Machine-Driven Enzymatic Oligosaccharide Synthesis by Using a Peptide Synthesizer" Angew. Chem. Int. Ed. Engl 2018, 57, 16638–16642. [PubMed: 30375138]

(36). Huang X; Witte Krista L.; Bergbreiter David E.; Wong C-H "Homogenous enzymatic synthesis using a thermo-responsive water-soluble polymer support" Adv. Synth. Catal 2001, 343, 675–681.

(37). Reetz MT "Biocatalysis in organic chemistry and biotechnology: past, present, and future" J. Am. Chem. Soc 2013, 135, 12480–12496. [PubMed: 23930719]

(38). aSchmaltz RM; Hanson SR; Wong C-H "Enzymes in the synthesis of glycoconjugates" Chem. Rev 2011, 111, 4259–4307; [PubMed: 21749134] bNidetzky B; Gutmann A; Zhong C "Leloir

glycosyltransferases as biocatalysts for chemical production" ACS Catal 2018, 6283–6300;cChang A; Singh S; Phillips GN Jr.; Thorson JS "Glycosyltransferase structural biology and its role in the design of catalysts for glycosylation" Curr. Opin. Biotechnol 2011, 22, 800–808; [PubMed: 21592771] dLairson LL; Henrissat B; Davies GJ; Withers SG "Glycosyltransferases: structures, functions, and mechanisms" Annu. Rev. Biochem 2008, 77, 521–555. [PubMed: 18518825]

(39). aFairbanks AJ "The ENGases: versatile biocatalysts for the production of homogeneous N-linked glycopeptides and glycoproteins" Chem. Soc. Rev 2017, 46, 5128–5146; [PubMed: 28681051] bLi C; Wang L-X "Chemoenzymatic methods for the synthesis of glycoproteins" Chem. Rev 2018, 118, 8359–8413. [PubMed: 30141327]

(40). aO'Neill EC; Field RA "Enzymatic synthesis using glycoside phosphorylases" Carbohydr. Res 2015, 403, 23–37; [PubMed: 25060838] bNakai H; Kitaoka M; Svensson B; Ohtsubo K "Recent development of phosphorylases possessing large potential for oligosaccharide synthesis" Curr. Opin. Chem. Biol 2013, 17, 301–309. [PubMed: 23403067]

(41). Wong C-H; Haynie S; Whitesides G "Enzyme-catalyzed synthesis of N-acetyllactosamine with in situ regeneration of uridine 5'-diphosphate glucose and uridine 5'-diphosphate galactose" J. Org. Chem 1982, 47, 5416–5418.

(42). aCai L "Recent progress in enzymatic synthesis of sugar nucleotides" J. Carbohydr. Chem 2012, 31, 535–552;bIchikawa Y; Lin YC; Dumas DP; Shen GJ; Garcia-Junceda E; Williams MA; Bayer R; Ketcham C; Walker LE; Paulson JC; Wong C-H "Chemical-enzymatic synthesis and conformational analysis of sialyl Lewis X and derivatives" J. Am. Chem. Soc 1992, 114, 9283–9298;cDe Luca C; Lansing M; Martini M; Crescenzi F; Shen G-J; O'Regan M; Wong C-H "Enzymatic synthesis of hyaluronic acid with regeneration of sugar nucleotides" J. Am. Chem. Soc 1995, 117, 5869–5870;dZhou X; Chandarajoti K; Pham TQ; Liu R; Liu J "Expression of heparan sulfate sulfotransferases in Kluyveromyces lactis and preparation of 3'-phosphoadenosine-5'-phosphosulfate" Glycobiology 2011, 21, 771–780; [PubMed: 21224284] eYu H; Lau K; Thon V; Autran CA; Jantscher-Krenn E; Xue M; Li Y; Sugiarto G; Qu J; Mu S; Ding L; Bode L; Chen X "Synthetic disialyl hexasaccharides protect neonatal rats from necrotizing enterocolitis" Angew. Chem. Int. Ed. Engl 2014, 53, 6687–6691; [PubMed: 24848971] fWitte K; Sears P; Martin R; Wong C-H "Enzymatic glycoprotein synthesis: preparation of ribonuclease glycoforms via enzymatic glycopeptide condensation and glycosylation" J. Am. Chem. Soc 1997, 119, 2114–2118;gYu H; Chen X "One-pot multienzyme (OPME) systems for chemoenzymatic synthesis of carbohydrates" Org. Biomol. Chem 2016, 14, 2809–2818. [PubMed: 26881499]

(43). aMackenzie LF; Wang Q; Warren RAJ; Withers SG "Glycosynthases: mutant glycosidases for oligosaccharide synthesis" J. Am. Chem. Soc 1998, 120, 5583–5584;bVaughan MD; Johnson K; DeFrees S; Tang X; Warren RA; Withers SG "Glycosynthase-mediated synthesis of glycosphingolipids" J. Am. Chem. Soc 2006, 128, 6300–6301; [PubMed: 16683778] cRich JR; Cunningham A-M; Gilbert M; Withers SG "Glycosphingolipid synthesis employing a combination of recombinant glycosyltransferases and an endoglycoceramidase glycosynthase" Chem. Commun 2011, 47, 10806–10808;dHancock SM; Rich JR; Caines ME; Strynadka NC; Withers SG "Designer enzymes for glycosphingolipid synthesis by directed evolution" Nat. Chem. Biol 2009, 5, 508–514. [PubMed: 19525967]

(44). aUmekawa M; Huang W; Li B; Fujita K; Ashida H; Wang LX; Yamamoto K "Mutants of Mucor hiemalis endo-beta-N-acetylglucosaminidase show enhanced transglycosylation and glycosynthase-like activities" J. Biol. Chem 2008, 283, 4469–4479; [PubMed: 18096701] bHuang W; Li C; Li B; Umekawa M; Yamamoto K; Zhang X; Wang LX "Glycosynthases enable a highly efficient chemoenzymatic synthesis of N-glycoproteins carrying intact natural N-glycans" J. Am. Chem. Soc 2009, 131, 2214–2223; [PubMed: 19199609] cLi C; Wang LX "Endoglycosidases for the synthesis of polysaccharides and glycoconjugates" Adv. Carbohydr. Chem. Biochem 2016, 73, 73–116; [PubMed: 27816108] dHaneda K; Oishi T; Kimura H; Inazu T "Development of a microbioreactor for glycoconjugate synthesis" Bioorg. Med. Chem 2018, 26, 2092–2098; [PubMed: 29555418] eFujita M; Shoda S; Haneda K; Inazu T; Takegawa K; Yamamoto K "A novel disaccharide substrate having 1,2-oxazoline moiety for detection of transglycosylating activity of endoglycosidases" Biochim. Biophys. Acta 2001, 1528, 9–14; [PubMed: 11514092] fYang Q; An Y; Zhu S; Zhang R; Loke CM; Cipollo JF; Wang LX "Glycan

remodeling of human erythropoietin (EPO) through combined mammalian cell engineering and chemoenzymatic transglycosylation." ACS Chem Biol 2017, 12, 1665–1673. [PubMed: 28452462]

(45). aGoodfellow JJ; Baruah K; Yamamoto K; Bonomelli C; Krishna B; Harvey DJ; Crispin M; Scanlan CN; Davis BG "An endoglycosidase with alternative glycan specificity allows broadened glycoprotein remodelling" J. Am. Chem. Soc 2012, 134, 8030–8033; [PubMed: 22551167] bTrastoy B; Klontz E; Orwenyo J; Marina A; Wang LX; Sundberg EJ; Guerin ME "Structural basis for the recognition of complex-type N-glycans by Endoglycosidase S" Nat. Commun 2018, 9, 1874; [PubMed: 29760474] cSjogren J; Cosgrave EF; Allhorn M; Nordgren M; Bjork S; Olsson F; Fredriksson S; Collin M "EndoS and EndoS2 hydrolyze Fc-glycans on therapeutic antibodies with different glycoform selectivity and can be used for rapid quantification of high-mannose glycans" Glycobiology 2015, 25, 1053–1063; [PubMed: 26156869] dGiddens JP; Lomino JV; DiLillo DJ; Ravetch JV; Wang LX "Site-selective chemoenzymatic glycoengineering of Fab and Fc glycans of a therapeutic antibody." Proc. Natl. Acad. Sci. U. S. A 2018, 115, 12023–12027; [PubMed: 30397147] eHuang W; Giddens J; Fan S-Q; Toonstra C; Wang L-X "Chemoenzymatic glycoengineering of intact IgG antibodies for gain of functions" J. Am. Chem. Soc 2012, 134, 12308–12318; [PubMed: 22747414] fLin C-W; Tsai M-H; Li S-T; Tsai T-I; Chu K-C; Liu Y-C; Lai M-Y; Wu C-Y; Tseng Y-C; Shivatare S-S; Wang C-H; Chao P; Wang S-Y; Shih H-W; Zeng Y-F; You T-H; Liao J-Y; Tu Y-C; Lin Y-S; Chuang H-Y; Chen C-L; Tsai C-S; Huang C-C; Lin N-H; Ma C; Wu C-Y; Wong C-H "A common glycan structure on immunoglobulin G for enhancement of effector functions." Proc. Natl. Acad. Sci. U. S. A 2015, 112, 10611–10616; [PubMed: 26253764] gLiu C-P; Tsai T-I; Cheng T; Shivatare VS; Wu C-Y; Wu C-Y; Wong C-H "Glycoengineering of antibody (Herceptin) through yeast expression and in vitro enzymatic glycosylation." Proc. Natl. Acad. Sci. U. S. A 2018, 115, 720–725; [PubMed: 29311294] hKurogochi M; Mori M; Osumi K; Tojino M; Sugawara S; Takashima S; Hirose Y; Tsukimura W; Mizuno M; Amano J; Matsuda A; Tomita M; Takayanagi A; Shoda S; Shirai T "Glycoengineered monoclonal antibodies with homogeneous glycan (M3, G0, G2, and A2) using a chemoenzymatic approach have different affinities for FcgammaRIIIa and variable antibody-dependent cellular cytotoxicity activities." PLoS One 2015, 10, e0132848; [PubMed: 26200113] iParsons TB; Struwe WB; Gault J; Yamamoto K; Taylor TA; Raj R; Wals K; Mohammed S; Robinson CV; Benesch JLP; Davis BG "Optimal synthetic glycosylation of a therapeutic antibody" Angew. Chem. Int. Ed. Engl 2016, 55, 2361–2367; [PubMed: 26756880] jIwamoto M; Sekiguchi Y; Nakamura K; Kawaguchi Y; Honda T; Hasegawa J "Generation of efficient mutants of endoglycosidase from Streptococcus pyogenes and their application in a novel one-pot transglycosylation reaction for antibody modification" PLoS One 2018, 13, e0193534; [PubMed: 29474426] kLi T; Li C; Quan DN; Bentley WE; Wang LX "Site-specific immobilization of endoglycosidases for streamlined chemoenzymatic glycan remodeling of antibodies" Carbohydr. Res 2018, 458–459, 77–84;lTong X; Li T; Orwenyo J; Toonstra C; Wang LX "One-pot enzymatic glycan remodeling of a therapeutic monoclonal antibody by endoglycosidase S (Endo-S) from Streptococcus pyogenes" Bioorg. Med. Chem 2018, 26, 1347–1355; [PubMed: 28789910] mLi T; Tong X; Yang Q; Giddens JP; Wang LX "Glycosynthase mutants of endoglycosidase S2 show potent transglycosylation activity and remarkably relaxed substrate specificity for antibody glycosylation remodeling." J. Biol. Chem 2016, 291, 16508–16518; [PubMed: 27288408] nLi C; Zhu S; Ma C; Wang LX "Designer alpha1,6-Fucosidase Mutants Enable Direct Core Fucosylation of Intact N-Glycopeptides and N-Glycoproteins" J Am Chem Soc 2017, 139, 15074–15087. [PubMed: 28990779]

(46). Lombard V; Golaconda Ramulu H.; Drula E; Coutinho PM; Henrissat B "The carbohydrate-active enzymes database (CAZy) in 2013" Nucleic Acids Res 2014, 42, D490–495. [PubMed: 24270786]

(47). Moremen KW; Ramiah A; Stuart M; Steel J; Meng L; Forouhar F; Moniz HA; Gahlay G; Gao Z; Chapla D; Wang S; Yang JY; Prabhakar PK; Johnson R; Rosa MD; Geisler C; Nairn AV; Seetharaman J; Wu SC; Tong L; Gilbert HJ; LaBaer J; Jarvis DL "Expression system for structural and functional studies of human glycosylation enzymes" Nat. Chem. Biol 2018, 14, 156–162. [PubMed: 29251719]

(48). aHunter CD; Guo T; Daskhan G; Richards MR; Cairo CW "Synthetic strategies for modified glycosphingolipids and their design as probes" Chem. Rev 2018, 118, 8188–8241; [PubMed: 29979587] bKulkarni SS SYNTHESIS OF GLYCOSPHINGOLIPIDS; In Glycochemical

Synthesis; First Edition ed.; Hung S-C, Zulueta M, Eds.; Wiley & Sons, Inc.: 2016;cAndo H; Komura N; Imamura A; Kiso M; Ishida H "A synthetic challenge to the diversity of gangliosides for unveiling their biological significance" J. Synth. Org. Chem., Jpn 2017, 75, 1162–1170.

(49). aYu H; Li Y; Zeng J; Thon V; Nguyen DM; Ly T; Kuang HY; Ngo A; Chen X "Sequential one-pot multienzyme chemoenzymatic synthesis of glycosphingolipid glycans" J. Org. Chem 2016, 81, 10809–10824; [PubMed: 27736072] bMeng X; Yao W; Cheng J; Zhang X; Jin L; Yu H; Chen X; Wang F; Cao H "Regioselective chemoenzymatic synthesis of ganglioside disialyl tetrasaccharide epitopes" J. Am. Chem. Soc 2014, 136, 5205–5208; [PubMed: 24649890] cYe J; Liu X.-w.; Peng P; Yi W; Chen X; Wang F; Cao H "Diversity-oriented enzymatic modular assembly of ABO histo-blood group antigens" ACS Catal 2016, 6, 8140–8144;dTsering D; Chen C; Ye J; Han Z; Jing BQ; Liu XW; Chen X; Wang F; Ling P; Cao H "Enzymatic synthesis of human blood group P1 pentasaccharide antigen" Carbohydr. Res 2017, 438, 39–43; [PubMed: 27960098] eSantra A; Yu H; Tasnima N; Muthana MM; Li Y; Zeng J; Kenyond NJ; Louie AY; Chen X "Systematic chemoenzymatic synthesis of O-sulfated sialyl Lewis x antigens" Chem. Sci 2016, 7, 2827–2831; [PubMed: 28138383] fRich JR; Withers SG "A chemoenzymatic total synthesis of the neurogenic starfish ganglioside LLG-3 using an engineered and evolved synthase" Angew. Chem. Int. Ed. Engl 2012, 51, 8640–8643; [PubMed: 22821741] gSakurama H; Fushinobu S; Hidaka M; Yoshida E; Honda Y; Ashida H; Kitaoka M; Kumagai H; Yamamoto K; Katayama T "1,3–1,4-alpha-L-fucosynthase that specifically introduces Lewis a/x antigens into type-1/2 chains" J. Biol. Chem 2012, 287, 16709–16719; [PubMed: 22451675] hTasnima N; Yu H; Li Y; Santra A; Chen X "Chemoenzymatic synthesis of para-nitrophenol (pNP)-tagged alpha2–8-sialosides and high-throughput substrate specificity studies of alpha2–8-sialidases" Org. Biomol. Chem 2016, 15, 160–167; [PubMed: 27924345] iYu C-C; Withers SG "Recent developments in enzymatic synthesis of modified sialic acid derivatives" Adv. Synth. Catal 2015, 357, 1633–1654;jLiu Y; Wen L; Li L; Gadi MR; Guan W; Huang K; Xiao Z; Wei M; Ma C; Zhang Q; Yu H; Chen X; Wang PG; Fang J "A general chemoenzymatic strategy for the synthesis of glycosphingolipids" Eur. J. Org. Chem 2016, 2016, 4315–4320;kSantra A; Li Y; Yu H; Slack TJ; Wang PG; Chen X "Highly efficient chemoenzymatic synthesis and facile purification of alpha-Gal pentasaccharyl ceramide Gal-alpha3n-Lc4-beta-Cer" Chem. Commun 2017, 53, 8280–8283.

(50). Huang L-Y; Huang S-H; Chang Y-C; Cheng W-C; Cheng T-J; Wong C-H "Enzymatic synthesis of lipid II and analogues" Angew. Chem. Int. Ed. Engl 2014, 53, 8060–8065. [PubMed: 24990652]

(51). aHelenius A; Aebi M "Intracellular functions of N-linked glycans." Science 2001, 291, 2364–2369; [PubMed: 11269317] bMoremen KW; Tiemeyer M; Nairn AV "Vertebrate protein glycosylation: diversity, synthesis and function" Nat. Rev. Mol. Cell. Biol 2012, 13, 448–462. [PubMed: 22722607]

(52). aTseng T-H; Lin T-W; Chen C-Y; Chen C-H; Lin J-L; Hsu T-L; Wong C-H "Substrate preference and interplay of fucosyltransferase 8 and N-acetylglucosaminyltransferases" J. Am. Chem. Soc 2017, 139, 9431–9434; [PubMed: 28678517] bChuang P-K; Hsiao M; Hsu T-L; Chang C-F; Wu C-Y; Chen B-R; Huang H-W; Liao K-S; Chen C-C; Chen C-L; Yang S-M; Kuo C-W; Chen P; Chiu P-T; Chen I-J; Lai J-S; Yu C-DT; Wong C-H "Signaling pathway of globo-series glycosphingolipids and b1,3-galactosyltransferase V (b3GalT5) in breast cancer." Proc. Natl. Acad. Sci. U. S. A 2018, in press.

(53). aKoizumi A; Matsuo I; Takatani M; Seko A; Hachisu M; Takeda Y; Ito Y "Topdown chemoenzymatic approach to a high-mannose-type glycan library: synthesis of a common precursor and its enzymatic trimming" Angew. Chem. Int. Ed. Engl 2013, 52, 7426–7431; [PubMed: 23740650] bFujikawa K; Koizumi A; Hachisu M; Seko A; Takeda Y; Ito Y "Construction of a high-mannose-type glycan library by a renewed top-down chemo-enzymatic approach" Chem. Eur. J 2015, 21, 3224–3233; [PubMed: 25586968] cWang Z; Chinoy ZS; Ambre SG; Peng W; McBride R; de Vries RP; Glushka J; Paulson JC; Boons GJ "A general strategy for the chemoenzymatic synthesis of asymmetrically branched N-glycans" Science 2013, 341, 379–383; [PubMed: 23888036] dLi T; Huang M; Liu L; Wang S; Moremen KW; Boons G-J "Divergent chemoenzymatic synthesis of asymmetrical-core-fucosylated and core-unmodified N-glycans" Chem. Eur. J 2016, 22, 18742–18746; [PubMed: 27798819] eLiu L; Prudden AR; Capicciotti CJ; Bosman GP; Yang J-Y; Chapla DG; Moremen KW; Boons G-J "Streamlining the

chemoenzymatic synthesis of complex N-glycans by a stop and go strategy" Nat. Chem 2019, 11, 161–169; [PubMed: 30532014] fGagarinov IA; Li T; Torano JS; Caval T; Srivastava AD; Kruijtzer JA; Heck AJ; Boons GJ "Chemoenzymatic approach for the preparation of asymmetric bi-, tri-, and tetra-antennary N-glycans from a common precursor" J. Am. Chem. Soc 2017, 139, 1011–1018; [PubMed: 28002670] gChinoy ZS; Friscourt F; Capicciotti CJ; Chiu P; Boons GJ "Chemoenzymatic synthesis of asymmetrical multi-antennary N-glycans to dissect glycan-mediated interactions between human sperm and oocytes" Chem. Eur. J 2018, 24, 7970–7975; [PubMed: 29603480] hLi L; Liu Y; Ma C; Qu J; Calderon AD; Wu B; Wei N; Wang X; Guo Y; Xiao Z; Song J; Sugiarto G; Li Y; Yu H; Chen X; Wang PG "Efficient chemoenzymatic synthesis of an N-glycan isomer library" Chem. Sci 2015, 6, 5652–5661; [PubMed: 26417422] iWu Z; Liu Y; Ma C; Li L; Bai J; Byrd-Leotis L; Lasanajak Y; Guo Y; Wen L; Zhu H; Song J; Li Y; Steinhauer DA; Smith DF; Zhao B; Chen X; Guan W; Wang PG "Identification of the binding roles of terminal and internal glycan epitopes using enzymatically synthesized N-glycans containing tandem epitopes" Org. Biomol. Chem 2016, 14, 11106–11116; [PubMed: 27752690] jCalderon AD; Zhou J; Guan W; Wu Z; Guo Y; Bai J; Li Q; Wang PG; Fang J; Li L "An enzymatic strategy to asymmetrically branched N-glycans" Org Biomol Chem 2017, 15, 7258–7262; [PubMed: 28832049] kWang S; Zhang Q; Chen C; Guo Y; Gadi MR; Yu J; Westerlind U; Liu Y; Cao X; Wang PG; Li L "Facile chemoenzymatic synthesis of O-mannosyl glycans" Angew. Chem. Int. Ed 2018, 57, 9268–9273;lHamilton BS; Wilson JD; Shumakovich MA; Fisher AC; Brooks JC; Pontes A; Naran R; Heiss C; Gao C; Kardish R; Heimburg-Molinaro J; Azadi P; Cummings RD; Merritt JH; DeLisa MP "A library of chemically defined human N-glycans synthesized from microbial oligosaccharide precursors" Sci. Rep 2017, 7, 15907. [PubMed: 29162910]

(54). aShivatare SS; Chang S-H; Tsai T-I; Tseng S-Y; Shivatare V-S; Lin Y-S; Cheng Y-Y; Ren C-T; Lee C-C; Pawar S; Tsai C-S; Shih H-W; Zeng Y-F; Liang C-H; Kwong P-D; Burton D-R; Wu C-Y; Wong C-H "Modular synthesis of N-glycans and arrays for the hetero-ligand binding analysis of HIV antibodies" Nat. Chem 2016, 8, 338–346; [PubMed: 27001729] bShivatare SS; Chang S-H; Tsai T-I; Ren C-T; Chuang H-Y; Hsu L; Lin C-W; Li S-T; Wu C-Y; Wong C-H "Efficient convergent synthesis of bi-, tri-, and tetra-antennary complex type N-glycans and their HIV-1 antigenicity" J. Am. Chem. Soc 2013, 135, 15382–15391; [PubMed: 24032650] cShivatare V-S; Shivatare S-S; Lee C-D; Liang C-H; Liao K-S; Cheng Y-Y; Saidachary G; Wu C-Y; Lin N-H; Kwong P-D; Burton D-R; Wu C-Y; Wong C-H "Unprecedented role of hybrid N-glycans as ligands for HIV-1 broadly neutralizing antibodies" J. Am. Chem. Soc 2018, 140, 5202–5210; [PubMed: 29578688] dNagasaki M; Manabe Y; Minamoto N; Tanaka K; Silipo A; Molinaro A; Fukase K "Chemical synthesis of a complex-type N-glycan containing a core fucose" J. Org. Chem 2016, 81, 10600–10616; [PubMed: 27775350] eManabe Y; Shomura H; Minamoto N; Nagasaki M; Takakura Y; Tanaka K; Silipo A; Molinaro A; Fukase K "Convergent synthesis of a bisecting N-acetylglucosamine (GlcNAc)-containing N-glycan" Chem. Asian J 2018, 13, 1544–1551; [PubMed: 29665315] fUnverzagt C; Luber T; Niemietz M; Karagiannis T; Mönnich M; Ott D; Perkams L; Walcher J; Berger L; Pischl M; Weishaupt M; Eller S; Siebenhaar J "A single route to mammalian N-glycans substituted with core fucose and bisecting GlcNAc" Angew. Chem. Int. Ed. Engl 2018, 57, 14543–14549. [PubMed: 30144245]

(55). aTsai T-I; Li S-T; Liu C-P; Chen K-Y; Shivatare SS; Lin C-W; Liao S-F; Lin C-W; Hsu T-L; Wu Y-T; Tsai M-H; Lai M-Y; Lin N-H; Wu C-Y; Wong C-H "An effective bacterial fucosidase for glycoprotein remodeling" ACS Chem. Biol 2016, 12, 63–72; [PubMed: 28103685] bShivatare SS; Huang L-Y; Zeng Y-F; Liao J-Y; You T-H; Wang S-Y; Cheng T; Chiu C-W; Chao P; Chen L-T; Tsai T-I; Huang C-C; Wu C-Y; Lin N-H; Wong C-H "Development of glycosynthases with broad glycan specificity for the efficient glyco-remodeling of antibodies." Chem. Commun 2018, 54, 6161–6164.

(56). aLi ST; Wang N; Xu S; Yin J; Nakanishi H; Dean N; Gao XD "Quantitative study of yeast Alg1 beta-1, 4 mannosyltransferase activity, a key enzyme involved in protein N-glycosylation" Biochim. Biophys. Acta 2017, 1861, 2934–2941;bRamirez AS; Boilevin J; Lin CW; Ha Gan B; Janser D; Aebi M; Darbre T; Reymond JL; Locher KP "Chemo-enzymatic synthesis of lipid-linked GlcNAc2Man5 oligosaccharides using recombinant Alg1, Alg2 and Alg11 proteins" Glycobiology 2017, 1–8.

(57). aBennett EP; Mandel U; Clausen H; Gerken TA; Fritz TA; Tabak LA "Control of mucin-type O-glycosylation: a classification of the polypeptide GalNAc-transferase gene family" Glycobiology

2012, 22, 736–756; [PubMed: 22183981] bJu T; Otto VI; Cummings RD “The Tn antigen-structural simplicity and biological complexity” Angew. Chem. Int. Ed. Engl 2011, 50, 1770–1791. [PubMed: 21259410]

(58). Seitz O; Wong C-H “Chemoenzymatic solution-and solid-phase synthesis of O-glycopeptides of the mucin domain of MAdCAM-1. A general route to O-LacNAc, O-Sialyl-LacNAc, and O-Sialyl-Lewis-X peptides” J. Am. Chem. Soc 1997, 119, 8766–8776.

(59). aChen X “Human milk oligosaccharides (HMOS): Structure, function, and enzyme-catalyzed synthesis” Adv. Carbohydr. Chem. Biochem 2015, 72, 113–190; [PubMed: 26613816] bChen C; Zhang Y; Xue M; Liu XW; Li Y; Chen X; Wang PG; Wang F; Cao H “Sequential one-pot multienzyme (OPME) synthesis of lacto-N-neotetraose and its sialyl and fucosyl derivatives” Chem. Commun 2015, 51, 7689–7692;cXiao Z; Guo Y; Liu Y; Li L; Zhang Q; Wen L; Wang X; Kondengaden SM; Wu Z; Zhou J; Cao X; Li X; Ma C; Wang PG “Chemoenzymatic synthesis of a library of human milk oligosaccharides” J. Org. Chem 2016, 81, 5851–5865; [PubMed: 27305319] dPrudden AR; Liu L; Capicciotti CJ; Wolfert MA; Wang S; Gao Z; Meng L; Moremen KW; Boons G-J “Synthesis of asymmetrical multiantennary human milk oligosaccharides” Proc. Natl. Acad. Sci. U. S. A 2017, 114, 6954–6959. [PubMed: 28630345]

(60). Lindhout T; Iqbal U; Willis LM; Reid AN; Li J; Liu X; Moreno M; Wakarchuk WW “Site-specific enzymatic polysialylation of therapeutic proteins using bacterial enzymes.” Proc. Natl. Acad. Sci. U. S. A 2011, 108, 7397–7402. [PubMed: 21502532]

(61). Bowden TA; Baruah K; Coles CH; Harvey DJ; Yu X; Song BD; Stuart DI; Aricescu AR; Scanlan CN; Jones EY; Crispin M “Chemical and structural analysis of an antibody folding intermediate trapped during glycan biosynthesis” J. Am. Chem. Soc 2012, 134, 17554–17563. [PubMed: 23025485]

(62). aSimanek E; Huang D-H; Pasternack L; Machajewski T; Seitz O; Millar D; Dyson H; Wong C-H “Glycosylation of threonine of the repeating unit of RNA polymerase II with beta-linked N-acetylglucosamine leads to a turn like structure” J. Am. Chem. Soc 1998, 120, 11567–11575;bChu C-S; Lo P-W; Yeh Y-H; Hsu P-H; Peng S-H; Teng Y-C; Kang M-L; Wong C-H; Juan L-J “O-GlcNAcylation regulates EZH2 protein stability and function” Proc. Natl. Acad. Sci. U. S. A 2014, 111, 1355–1360; [PubMed: 24474760] cLo P-W; Shie J-J; Chen C-H; Wu C-Y; Hsu T-L; Wong C-H “O-GlcNAcylation regulates the stability and enzymatic activity of the histone methyltransferase EZH2” Proc. Natl. Acad. Sci. U. S. A 2018, 115, 7302–7307 [PubMed: 29941599] dMartinez-Saez N; Peregrina JM; Corzana F “Principles of mucin structure: implications for the rational design of cancer vaccines derived from MUC1-glycopeptides” Chem. Soc. Rev 2017, 46, 7154–7175; [PubMed: 29022615] eBermejo IA; Usabiaga I; Companon I; Castro-Lopez J; Insausti A; Fernandez JA; Avenoza A; Busto JH; Jimenez-Barbero J; Asensio JL; Peregrina JM; Jimenez-Oses G; Hurtado-Guerrero R; Cocinero EJ; Corzana F “Water sculpts the distinctive shapes and dynamics of the tumor-associated carbohydrate Tn antigens: Implications for their molecular recognition” J. Am. Chem. Soc 2018, 140, 9952–9960; [PubMed: 30004703] fO’Conner SE; Imperiali B “A molecular basis for glycosylation-induced conformational switching” Chem. Biol 1998, 5, 427–437; [PubMed: 9710565] gChaffey PK; Guan X; Li Y; Tan Z “Using chemical synthesis to study and apply protein glycosylation” Biochemistry 2018, 57, 413–428. [PubMed: 29309128]

(63). aHanson SR; Culyba EK; Hsu TL; Wong C-H; Kelly JW; Powers ET “The core trisaccharide of an N-linked glycoprotein intrinsically accelerates folding and enhances stability” Proc. Natl. Acad. Sci. U. S. A 2009, 106, 3131–3136; [PubMed: 19204290] bCulyba EK; Price JL; Hanson SR; Dhar A; Wong C-H; Gruebele M; Powers ET; Kelly JW “Protein native state stabilization by placing aromatic side chains in N-glycosylated reverse turns” Science 2011, 331, 571–575; [PubMed: 21292975] cPrice JL; Culyba EK; Chen W; Murray AN; Hanson SR; Wong C-H; Powers ET; Kelly JW “N-glycosylation of enhanced aromatic sequon to increase glycoprotein stability” Biopolymers 2012, 98, 195–211; [PubMed: 22782562] dChen W; Enck S; Price JL; Powers DL; Powers ET; Wong C-H; Dyson HJ; Kelly JW “Structural and energetic basis of carbohydrate-aromatic packing interactions in proteins” J. Am. Chem. Soc 2013, 135, 9877–9884; [PubMed: 23742246] eHsu CH; Park S; Mortenson DE; Foley BL; Wang X; Woods RJ; Case DA; Powers ET; Wong C-H; Dyson HJ; Kelly JW “The dependence of carbohydrate-aromatic interaction strengths on the structure of the carbohydrate” J. Am. Chem. Soc 2016, 138, 7636–7648; [PubMed: 27249581] fMurray AN; Chen W; Antonopoulos A; Hanson SR;

Wiseman RL; Dell A; Haslam SM; Powers DL; Powers ET; Kelly JW “Enhanced aromatic sequons increase oligosaccharyltransferase glycosylation efficiency and glycan homogeneity” Chem. Biol 2015, 22, 1052–1062; [PubMed: 26190824] gHuang Y-W; Yang H-I; Wu Y-T; Hsu T-L; Lin T-W; Kelly JW; Wong C-H “Residues comprising the enhanced aromatic sequon influence protein N-glycosylation efficiency” J. Am. Chem. Soc 2017, 139, 12947–12955; [PubMed: 28820257] hWormald MR; Dwek RA “Glycoproteins: glycan presentation and protein-fold stability” Structure 1999, 7, R155–160; [PubMed: 10425673] iPetrescu AJ; Milac AL; Petrescu SM; Dwek RA; Wormald MR “Statistical analysis of the protein environment of N-glycosylation sites: implications for occupancy, structure, and folding” Glycobiology 2004, 14, 103–114; [PubMed: 14514716] iImperiali B; O’Connor S “Effect of N-linked glycosylation on glycopeptide and glycoprotein structure” Curr. Opin. Chem. Biol 1999, 3, 643–649; [PubMed: 10600722] jChen MM; Bartlett AI; Nerenberg PS; Friel CT; Hackenberger CPR; Stultz CM; Radford SE; Imperiali B “Perturbing the folding energy landscape of the bacterial immunity protein Im7 by site-specific N-linked glycosylation” Proc. Nat. Acad. of Sci. U. S. A 2010, 107, 22528–22533.

(64). Kightlinger W; Lin L; Rosztoczy M; Li W; DeLisa MP; Mrksich M; Jewett MC “Design of glycosylation sites by rapid synthesis and analysis of glycosyltransferases” Nat. Chem. Biol 2018, 14, 627–635. [PubMed: 29736039]

(65). Xu Y; Wu Z; Zhang P; Zhu H; Zhu H; Song Q; Wang L; Wang F; Wang PG; Cheng J “A novel enzymatic method for synthesis of glycopeptides carrying natural eukaryotic N-glycans” Chem. Commun 2017, 53, 9075–9077.

(66). Jennewein MF; Alter G “The immunoregulatory roles of antibody glycosylation” Trends. Immunol 2017, 38, 358–372. [PubMed: 28385520]

(67). Chen C-L; Hsu J-C; Lin C-W; Wang C-H; Tsai M-H; Wu C-Y; Wong C-H; Ma C “Crystal structure of a homogeneous IgG-Fc glycoform with the N-glycan designed to maximize the antibody dependent cellular cytotoxicity.” ACS Chem. Biol 2017, 12, 1335–1345. [PubMed: 28318221]

(68). Giddens JP; Lomino JV; Amin MN; Wang LX “Endo-F3 glycosynthase mutants enable chemoenzymatic synthesis of core fucosylated tri-antennary complex-type glycopeptides and glycoproteins” J. Biol. Chem 2016, 291, 9356–9370. [PubMed: 26966183]

(69). aBurke HM; McSweeney L; Scanlan EM “Exploring chemoselective S-to-N acyl transfer reactions in synthesis and chemical biology” Nat. Commun 2017, 8, 15655; [PubMed: 28537277] bBondalapati S; Jbara M; Brik A “Expanding the chemical toolbox for the synthesis of large and uniquely modified proteins” Nat. Chem 2016, 8, 407–418; [PubMed: 27102674] cUnverzagt C; Kajihara Y “Chemical assembly of N-glycoproteins: a refined toolbox to address a ubiquitous posttranslational modification” Chem. Soc. Rev 2013, 42, 4408–4420; [PubMed: 23403448] dKajihara Y; Murakami M; Unverzagt C Chemical Glycoprotein Synthesis; In Glycochemical Synthesis; Hung S-C, Zulueta M, Eds.; John Wiley & Sons, Inc.: 2016, p 263–292;eMuir TW “Semisynthesis of proteins by expressed protein ligation” Annu. Rev. Biochem 2003, 72, 249–289; [PubMed: 12626339] fDawson PE; Muir TW; Clark-Lewis I; Kent SB “Synthesis of proteins by native chemical ligation” Science 1994, 266, 776–779. [PubMed: 7973629]

(70). aWilson R; Dong S; Wang P; Danishefsky S “The winding pathway to erythropoietin along the chemistry–biology frontier: A success at last” Angew. Chem. Int. Ed. Engl 2013, 52, 7646–7665; [PubMed: 23775885] bDu J-J; Gao X-F; Xin L-M; Lei Z; Liu Z; Guo J “Convergent synthesis of N-linked glycopeptides via aminolysis of ω-Asp p-nitrophenyl thioesters in solution” Org. Lett 2016, 18, 4828–4831; [PubMed: 27619788] cUllmann V; Radisch M; Boos I; Freund J; Pohner C; Schwarzinger S; Unverzagt C “Convergent solid-phase synthesis of N-glycopeptides facilitated by pseudoprolines at consensus-sequence Ser/Thr residues” Angew. Chem. Int. Ed. Engl 2012, 51, 11566–11570; [PubMed: 22945377] dWang P; Aussedat B; Vohra Y; Danishefsky SJ “An advance in the chemical synthesis of homogeneous N-linked glycopolypeptides by convergent aspartylation” Angew. Chem. Int. Ed. Engl 2012, 51, 11571–11575; [PubMed: 23011954] eWu B; Chen J; Warren JD; Chen G; Hua Z; Danishefsky SJ “Building complex glycopeptides: development of a cysteine-free native chemical ligation protocol” Angew. Chem. Int. Ed. Engl 2006, 45, 4116–4125; [PubMed: 16710874] fBrik A; Yang YY; Ficht S; Wong C-H “Sugar-assisted glycopeptide ligation” J. Am. Chem. Soc 2006, 128, 5626–5627; [PubMed:

16637616] gBrik A; Ficht S; Yang Y-Y; Bennett CS; Wong C-H “Sugar-assisted ligation of N-linked glycopeptides with broad sequence tolerance at the ligation junction” J. Am. Chem. Soc 2006, 128, 15026–15033; [PubMed: 17105315] hBrik A; Wong C-H “Sugar-assisted ligation for the synthesis of glycopeptides” Chem. Eur. J 2007, 13, 5670–5675; [PubMed: 17508364] iFicht S; Payne RJ; Brik A; Wong C-H “Second-generation sugar-assisted ligation: a method for the synthesis of cysteine-containing glycopeptides” Angew. Chem. Int. Ed. Engl 2007, 46, 5975–5979; [PubMed: 17607677] jYang Y-Y; Ficht S; Brik A; Wong C-H “Sugar-assisted ligation in glycoprotein synthesis” J. Am. Chem. Soc 2007, 129, 7690–7701; [PubMed: 17523644] kPayne RJ; Ficht S; Tang S; Brik A; Yang YY; Case DA; Wong C-H “Extended sugar-assisted glycopeptide ligations: development, scope, and applications” J. Am. Chem. Soc 2007, 129, 13527–13536; [PubMed: 17935327] lBennett CS; Dean SM; Payne RJ; Ficht S; Brik A; Wong C-H “Sugar-assisted glycopeptide ligation with complex oligosaccharides: scope and limitations” J. Am. Chem. Soc 2008, 130, 11945–11952; [PubMed: 18698778] mWan Q; Danishefsky SJ “Free-radical-based, specific desulfurization of cysteine: a powerful advance in the synthesis of polypeptides and glycopolypeptides” Angew. Chem. Int. Ed. Engl 2007, 46, 9248–9252; [PubMed: 18046687] nMitchell NJ; Malins LR; Liu X; Thompson RE; Chan B; Radom L; Payne RJ “Rapid additive-free selenocystine-selenoester peptide ligation” J. Am. Chem. Soc 2015, 137, 14011–14014. [PubMed: 26487084]

(71). aFernandez-Tejada A; Brailsford J; Zhang Q; Shieh JH; Moore MA; Danishefsky SJ “Total synthesis of glycosylated proteins” Top. Curr. Chem 2015, 362, 1–26; [PubMed: 25805144] bAussedat B; Fasching B; Johnston E; Sane N; Nagorny P; Danishefsky SJ “Total synthesis of the alpha-subunit of human glycoprotein hormones: toward fully synthetic homogeneous human follicle-stimulating hormone” J. Am. Chem. Soc 2012, 134, 3532–3541; [PubMed: 22280541] cNagorny P; Sane N; Fasching B; Aussedat B; Danishefsky SJ “Probing the frontiers of glycoprotein synthesis: the fully elaborated beta-subunit of the human follicle-stimulating hormone” Angew. Chem. Int. Ed. Engl 2012, 51, 975–979; [PubMed: 22162182] dFernandez-Tejada A; Vadola PA; Danishefsky SJ “Chemical synthesis of the beta-subunit of human luteinizing (hLH) and chorionic gonadotropin (hCG) glycoprotein hormones” J. Am. Chem. Soc 2014, 136, 8450–8458; [PubMed: 24806200] eBrailsford JA; Stockdill JL; Axelrod AJ; Peterson MT; Vadola PA; Johnston EV; Danishefsky SJ “Total chemical synthesis of human thyroid-stimulating hormone (hTSH) β-subunit: Application of arginine-tagged acetamidomethyl (AcmR) protecting groups” Tetrahedron 2018, 74, 1951–1956; [PubMed: 30853725] fZhang Q; Johnston EV; Shieh JH; Moore MA; Danishefsky SJ “Synthesis of granulocyte-macrophage colony-stimulating factor as homogeneous glycoforms and early comparisons with yeast cell-derived material” Proc. Natl. Acad. Sci. U. S. A 2014, 111, 2885–2890; [PubMed: 24516138] gRoberts AG; Johnston EV; Shieh JH; Sondey JP; Hendrickson RC; Moore MA; Danishefsky SJ “Fully synthetic granulocyte colony-stimulating factor enabled by isonitrile-mediated coupling of large, side-chain-unprotected peptides” J. Am. Chem. Soc 2015, 137, 13167–13175. [PubMed: 26401918]

(72). aTolbert TJ; Wong C-H “Intein-mediated synthesis of proteins containing carbohydrates and other molecular probes” J. Am. Chem. Soc 2000, 122, 5421–5428;bMacmillan D; Bertozzi CR “Modular assembly of glycoproteins: Towards the synthesis of GlyCAM-1 by using expressed protein ligation” Angew. Chem. Int. Ed. Engl 2004, 116, 1379–1383;cTolbert TJ; Wong C-H “New methods for proteomic research: preparation of proteins with N-terminal cysteines for labeling and conjugation” Angew. Chem. Int. Ed. Engl 2002, 41, 2171–2174; [PubMed: 19746634] dTolbert TJ; Wong C-H “Conjugation of glycopeptide thioesters to expressed protein fragments: semisynthesis of glycosylated interleukin-2” Methods Mol. Biol 2004, 283, 255–266; [PubMed: 15197317] eReif A; Siebenhaar S; Troster A; Schmalzlein M; Lechner C; Velisetty P; Gottwald K; Pohner C; Boos I; Schubert V; Rose-John S; Unverzagt C “Semisynthesis of biologically active glycoforms of the human cytokine interleukin 6” Angew. Chem. Int. Ed. Engl 2014, 53, 12125–12131. [PubMed: 25243720]

(73). Sato K; Shigenaga A; Kitakaze K; Sakamoto K; Tsuji D; Itoh K; Otaka A “Chemical synthesis of biologically active monoglycosylated GM2-activator protein analogue using N-sulfanylethylanilide peptide” Angew. Chem. Int. Ed. Engl 2013, 52, 7855–7859; [PubMed: 23765733] aSakamoto I; Tezuka K; Fukae K; Ishii K; Taduru K; Maeda M; Ouchi M; Yoshida K; Nambu Y; Igarashi J; Hayashi N; Tsuji T; Kajihara Y “Chemical synthesis of homogeneous human glycosyl-interferon-beta that exhibits potent antitumor activity in vivo” J. Am. Chem. Soc

2012, 134, 5428–5431; [PubMed: 22404596] bIzumi M; Makimura Y; Dedola S; Seko A; Kanamori A; Sakono M; Ito Y; Kajihara Y “Chemical synthesis of intentionally misfolded homogeneous glycoprotein: A unique approach for the study of glycoprotein quality control” J. Am. Chem. Soc 2012, 134, 7238–7241. [PubMed: 22497239]

(74). aPaulick MG; Wise AR; Forstner MB; Groves JT; Bertozzi CR “Synthetic analogues of glycosylphosphatidylinositol-anchored proteins and their behavior in supported lipid bilayers” J. Am. Chem. Soc 2007, 129, 11543–11550; [PubMed: 17715922] bBecker CFW; Liu X; Olschewski D; Castelli R; Seidel R; Seeberger PH “Semisynthesis of a glycosylphosphatidylinositol-anchored prion protein” Angew. Chem. Int. Ed. Engl 2008, 47, 8215–8219; [PubMed: 18814158] cSchumacher MC; Resenberger U; Seidel RP; Becker CF; Winklhofer KF; Oesterhelt D; Tatzelt J; Engelhard M “Synthesis of a GPI anchor module suitable for protein post-translational modification” Biopolymers 2010, 94, 457–464; [PubMed: 20593476] dGuo X; Wang Q; Swarts BM; Guo Z “Sortase-catalyzed peptide-glycosylphosphatidylinositol analogue ligation” J. Am. Chem. Soc 2009, 131, 9878–9879; [PubMed: 19583255] eWu Z; Guo X; Wang Q; Swarts BM; Guo Z “Sortase A-catalyzed transpeptidation of glycosylphosphatidylinositol derivatives for chemoenzymatic synthesis of GPI-anchored proteins” J. Am. Chem. Soc 2010, 132, 1567–1571. [PubMed: 20078124]

(75). aSteentoft C; Bennett EP; Schjoldager KT; Vakhrushev SY; Wandall HH; Clausen H “Precision genome editing: a small revolution for glycobiology” Glycobiology 2014, 24, 663–680; [PubMed: 24861053] bVester-Christensen MB; Halim A; Joshi HJ; Steentoft C; Bennett EP; Levery SB; Vakhrushev SY; Clausen H “Mining the O-mannose glycoproteome reveals cadherins as major O-mannosylated glycoproteins” Proc. Natl. Acad. Sci. U. S. A 2013, 110, 21018–21023; [PubMed: 24101494] cYang Z; Wang S; Halim A; Schulz MA; Frodin M; Rahman SH; Vester-Christensen MB; Behrens C; Kristensen C; Vakhrushev SY; Bennett EP; Wandall HH; Clausen H “Engineered CHO cells for production of diverse, homogeneous glycoproteins” Nat. Biotechnol 2015, 33, 842–844; [PubMed: 26192319] dSteentoft C; Vakhrushev SY; Vester-Christensen MB; Schjoldager KT; Kong Y; Bennett EP; Mandel U; Wandall H; Levery SB; Clausen H “Mining the O-glycoproteome using zinc-finger nuclease-glycoengineered SimpleCell lines” Nat. Methods 2011, 8, 977–982; [PubMed: 21983924] eYang Z; Halim A; Narimatsu Y; Jitendra Joshi H.; Steentoft C; Schjoldager KT; Alder Schulz M.; Sealover NR; Kayser KJ; Paul Bennett E.; Levery SB; Vakhrushev SY; Clausen H “The GalNAc-type O-Glycoproteome of CHO cells characterized by the SimpleCell strategy” Mol. Cell Proteomics 2014, 13, 3224–3235; [PubMed: 25092905] fMeuris L; Santens F; Elson G; Festjens N; Boone M; Dos Santos A; Devos S; Rousseau F; Plets E; Houthuys E; Malinge P; Magistrelli G; Cons L; Chatel L; Devreese B; Callewaert N “GlycoDelete engineering of mammalian cells simplifies N-glycosylation of recombinant proteins” Nat. Biotechnol 2014, 32, 485–489; [PubMed: 24752077] gMabashi-Asazuma H; Jarvis DL “CRISPR-Cas9 vectors for genome editing and host engineering in the baculovirus-insect cell system” Proc. Natl. Acad. Sci. U. S. A 2017, 114, 9068–9073; [PubMed: 28784806] hMabashi-Asazuma H; Kuo CW; Khoo KH; Jarvis DL “Modifying an Insect Cell N-Glycan Processing Pathway Using CRISPR-Cas Technology” ACS Chem. Biol 2015, 10, 2199–2208; [PubMed: 26241388] iNarimatsu Y; Joshi HJ; Yang Z; Gomes C; Chen YH; Lorenzetti FC; Furukawa S; Schjoldager KT; Hansen L; Clausen H; Bennett EP; Wandall HH “A validated gRNA library for CRISPR/Cas9 targeting of the human glycosyltransferase genome” Glycobiology 2018, 28, 295–305. [PubMed: 29315387]

(76). Karamanos NK; Piperigkou Z; Theocharis AD; Watanabe H; Franchi M; Baud S; Brézillon S; Götte M; Passi A; Vigetti D; Ricard-Blum S; Sanderson RD; Neill T; Iozzo RV “Proteoglycan chemical diversity drives multifunctional cell regulation and therapeutics” Chem. Rev 2018, 118, 9152–9232. [PubMed: 30204432]

(77). aZhang X; Pagadala V; Jester Hannah M.; Lim AM; Pham TQ; Goulas AMP; Liu J; Linhardt RJ “Chemoenzymatic synthesis of heparan sulfate and heparin oligosaccharides and NMR analysis: paving the way to a diverse library for glycobiologists” Chem. Sci 2017, 8, 7932–7940; [PubMed: 29568440] bZong C; Huang R; Condac E; Chiu Y; Xiao W; Li X; Lu W; Ishihara M; Wang S; Ramiah A; Stickney M; Azadi P; Amster IJ; Moremen KW; Wang L; Sharp JS; Boons G-J “Integrated approach to identify heparan sulfate ligand requirements of Robo1” J. Am. Chem. Soc 2016, 138, 13059–13067. [PubMed: 27611601]

(78). Yu Y; Linhardt RJ “Xylosyltransferase 1 and the GAG attachment site” Structure 2018, 26, 797–799. [PubMed: 29874596]

(79). aMende M; Bednarek C; Wawryszyn M; Sauter P; Biskup MB; Schepers U; Bräse S “Chemical synthesis of glycosaminoglycans” Chem. Rev 2016, 116, 8193–8255; [PubMed: 27410264] Tsai CT; Zulueta MML; Hung SC “Synthetic heparin and heparan sulfate: probes in defining biological functions” Curr. Opin. Chem. Biol 2017, 40, 152–159; [PubMed: 29032302] bDeAngelis PL; Liu J; Linhardt RJ “Chemoenzymatic synthesis of glycosaminoglycans: re-creating, re-modeling and re-designing nature’s longest or most complex carbohydrate chains” Glycobiology 2013, 23, 764–777; [PubMed: 23481097] cLiu J; Linhardt J “Chemoenzymatic synthesis of heparan sulfate and heparin” Nat. Prod. Rep 2014, 31, 1676–1685. [PubMed: 25197032]

(80). aFujikawa S; Ohmae M; Kobayashi S “Enzymatic synthesis of chondroitin 4-sulfate with well-defined structure” Biomacromol 2005, 6, 2935–2942;bOhmae M; Sakaguchi K; Kaneto T; Fujikawa S; Kobayashi S “Keratanase II-catalyzed synthesis of keratan sulfate oligomers by using sugar oxazolines as transition-state analogue substrate monomers: a novel insight into the enzymatic catalysis mechanism” Chembiochem 2007, 8, 1710–1720. [PubMed: 17705309]

(81). aFu X; Shang W; Wang S; Liu Y; Qu J; Chen X; Wang PG; Fang J “A general strategy for the synthesis of homogeneous hyaluronan conjugates and their biological applications” Chem. Commun 2017, 53, 3555–3558;bDeAngelis PL; Oatman LC; Gay DF “Rapid chemoenzymatic synthesis of monodisperse hyaluronan oligosaccharides with immobilized enzyme reactors” J. Biol. Chem 2003, 278, 35199–35203; [PubMed: 12840012] cLi J; Su G; Liu J “Enzymatic synthesis of homogeneous chondroitin sulfate oligosaccharides” Angew. Chem. Int. Ed. Engl 2017, 56, 11784–11787; [PubMed: 28731518] dChen Y; Li Y; Yu H; Sugiarto G; Thon V; Hwang J; Ding L; Hie L; Chen X “Tailored design and synthesis of heparan sulfate oligosaccharide analogues using sequential one-pot multienzyme systems” Angew. Chem. Int. Ed. Engl 2013, 52, 11852–11856; [PubMed: 24038939] eXu Y; Moon AF; Xu S; Krahn JM; Liu J; Pedersen LC “Structure based substrate specificity analysis of heparan sulfate 6-O-sulfotransferases” ACS Chem. Biol 2017, 12, 73–82; [PubMed: 28103688] fWang Z; Hsieh PH; Xu Y; Thieker D; Chai EJ; Xie S; Cooley B; Woods RJ; Chi L; Liu J “Synthesis of 3-O-sulfated oligosaccharides to understand the relationship between structures and functions of heparan sulfate” J. Am. Chem. Soc 2017, 139, 5249–5256; [PubMed: 28340300] gLu W; Zong C; Chopra P; Pepi LE; Xu Y; Amster IJ; Liu J; Boons GJ “Controlled chemoenzymatic synthesis of heparan sulfate oligosaccharides” Angew. Chem. Int. Ed. Engl 2018, 57, 5340–5344. [PubMed: 29512241]

(82). aBadri A; Williams A; Linhardt RJ; Koffas MAG “The road to animal-free glycosaminoglycan production: current efforts and bottlenecks” Curr. Opin. Biotechnol 2018, 53, 85–92; [PubMed: 29291494] bVaidyanathan D; Williams A; Dordick JS; Koffas MAG; Linhardt RJ “Engineered heparins as new anticoagulant drugs” Bioeng Transl Med 2017, 2, 17–30. [PubMed: 28516163]

(83). aRillahan CD; Paulson JC “Glycan microarrays for decoding the glycome” Annu. Rev. Biochem 2011, 80, 797–823; [PubMed: 21469953] bLakshminarayanan A; Richard M; Davis BG “Studying glycobiology at the single-molecule level” Nat. Rev. Chem 2018, 2, 148–159;cRuhaak LR; Xu G; Li Q; Goonatilleke E; Lebrilla CB “Mass spectrometry approaches to glycomic and glycoproteomic analyses” Chem. Rev 2018, 118, 7886–7930. [PubMed: 29553244]

(84). avan Geel R; Wijdeven MA; Heesbeen R; Verkade JM; Wasiel AA; van Berkel S; van Delft FL “Chemoenzymatic conjugation of toxic payloads to the globally conserved N-glycan of native mAbs provides homogeneous and highly efficacious antibody-drug conjugates.” Bioconj. Chem 2015, 26, 2233–2242;bZhou Q; Stefano JE; Manning C; Kyazike J; Chen B; Gianolio DA; Park A; Busch M; Bird J; Zheng X; Simonds-Mannes H; Kim J; Gregory RC; Miller RJ; Brondyk WH; Dhal PK; Pan CQ “Site-specific antibody–drug conjugation through glycoengineering.” Bioconj. Chem 2014, 25, 510–520;cLi X; Fang T; Boons GJ “Preparation of well-defined antibody-drug conjugates through glycan remodeling and strain-promoted azide-alkyne cycloadditions.” Angew. Chem. Int. Ed. Engl 2014, 53, 7179–7182; [PubMed: 24862406] dTang F; Wang LX; Huang W “Chemoenzymatic synthesis of glycoengineered IgG antibodies and glycosite-specific antibody-drug conjugates.” Nat. Protoc 2017, 12, 1702–1721; [PubMed: 28749929] ede Graaf M; Boven E; Scheeren HW; Haisma HJ; Pinedo HM “Beta-glucuronidase-mediated drug release” Curr. Pharm. Des 2002, 8, 1391–1403. [PubMed: 12052215]

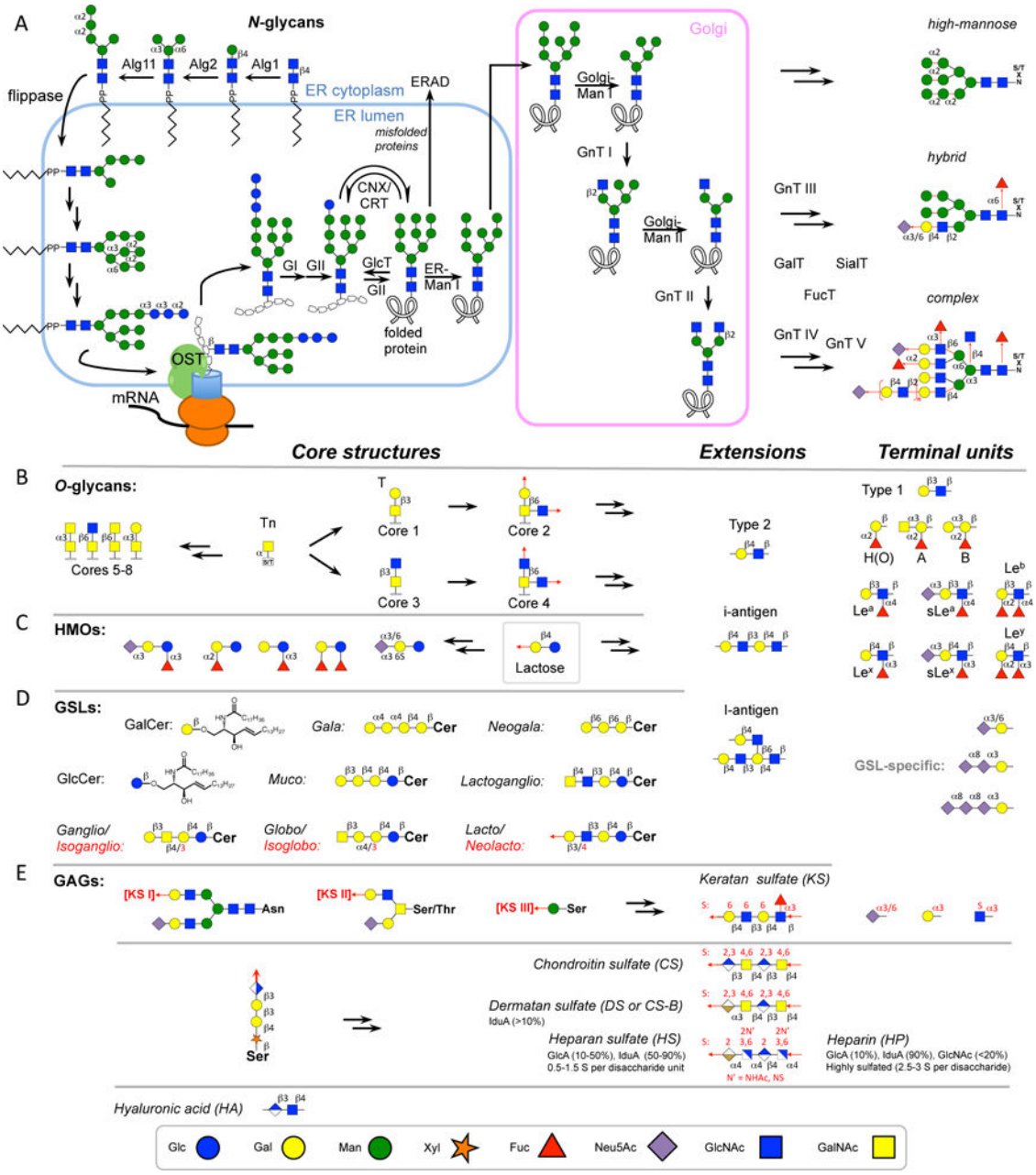



**Figure 1.**

(A) Biosynthesis of *N*-glycans in ER and further diversification in Golgi; (B) Mucin-type *O*-glycans; (C) Human milk oligosaccharides (HMOs); (D) Glycosphingolipids (GSLs); (E) Glycosaminoglycans (GAGs). Abbreviations: Calreticulin (CRT), Calnexin (CLX), endoplasmic reticulum (ER), Glucosidase I and II (GI and II), glycosyl transferases (GlcT, GlcNAcT, GalT and SialT), Mannosidase I and II (ManI and ManII), oligosaccharyltransferase (OST).

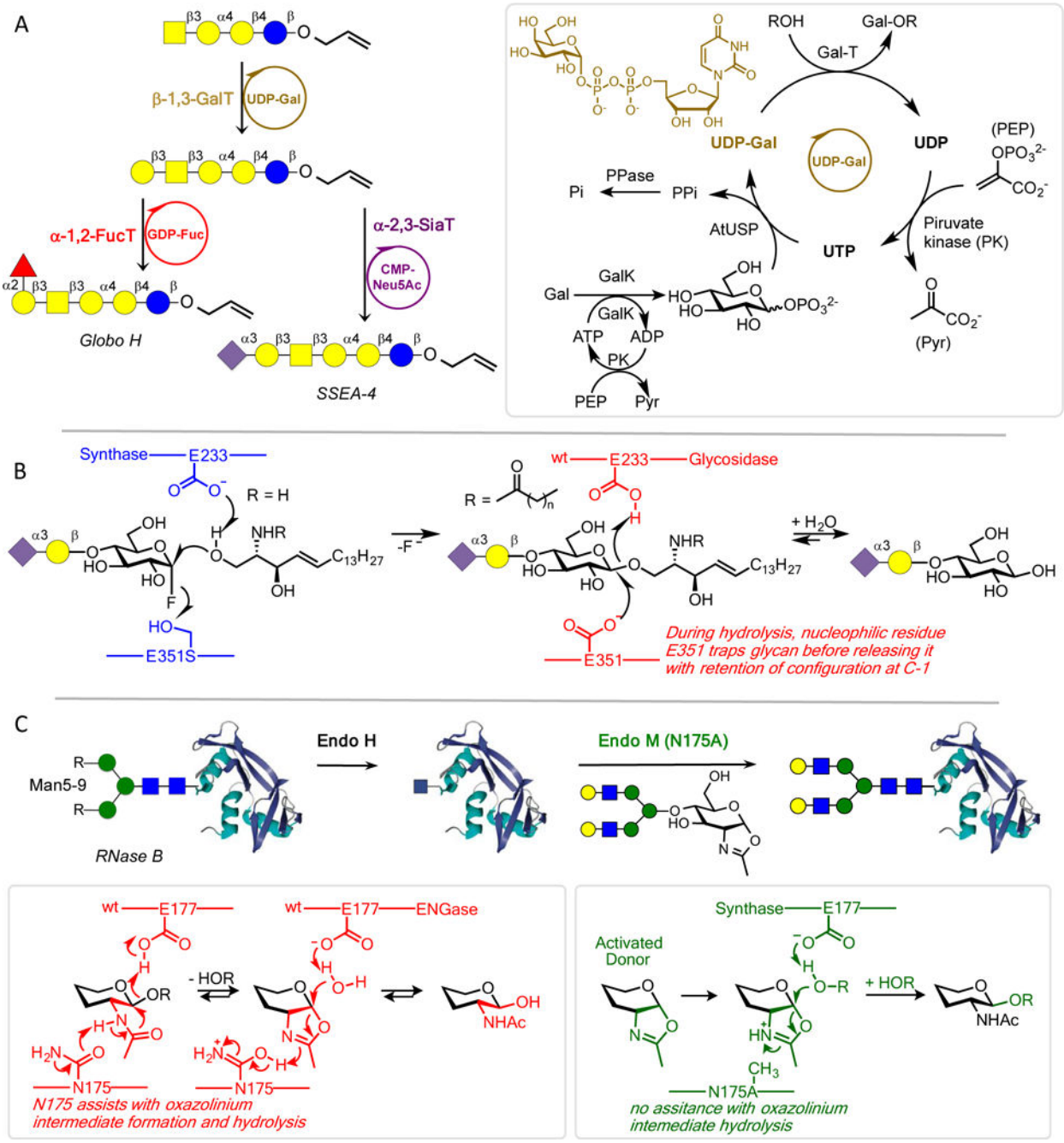



**Figure 2.**

(A) Synthesis of Globo H and stage-specific embryonic antigen 4 (SSEA-4) with GTs and an improved *in situ* UDP-Gal recycling system.[4a] (B) Synthesis of *lyso*-$GM_3$ with activated fluoride donor and hydrolytically inactive *endo*-glycoceramidase II mutant (E351S),[43b,c] and depiction of the mechanism of a synthase mutant (in blue) and a wt. glycosidase (in red). (C) Glycosylation remodeling of ribonuclease B (RNase B) with ENGases and activated oxazoline donor.[41f, 44b] Depiction of hydrolysis with wt. Endo M (in red), and transglycosylation with Endo $M^{N175A}$ synthase (in green).

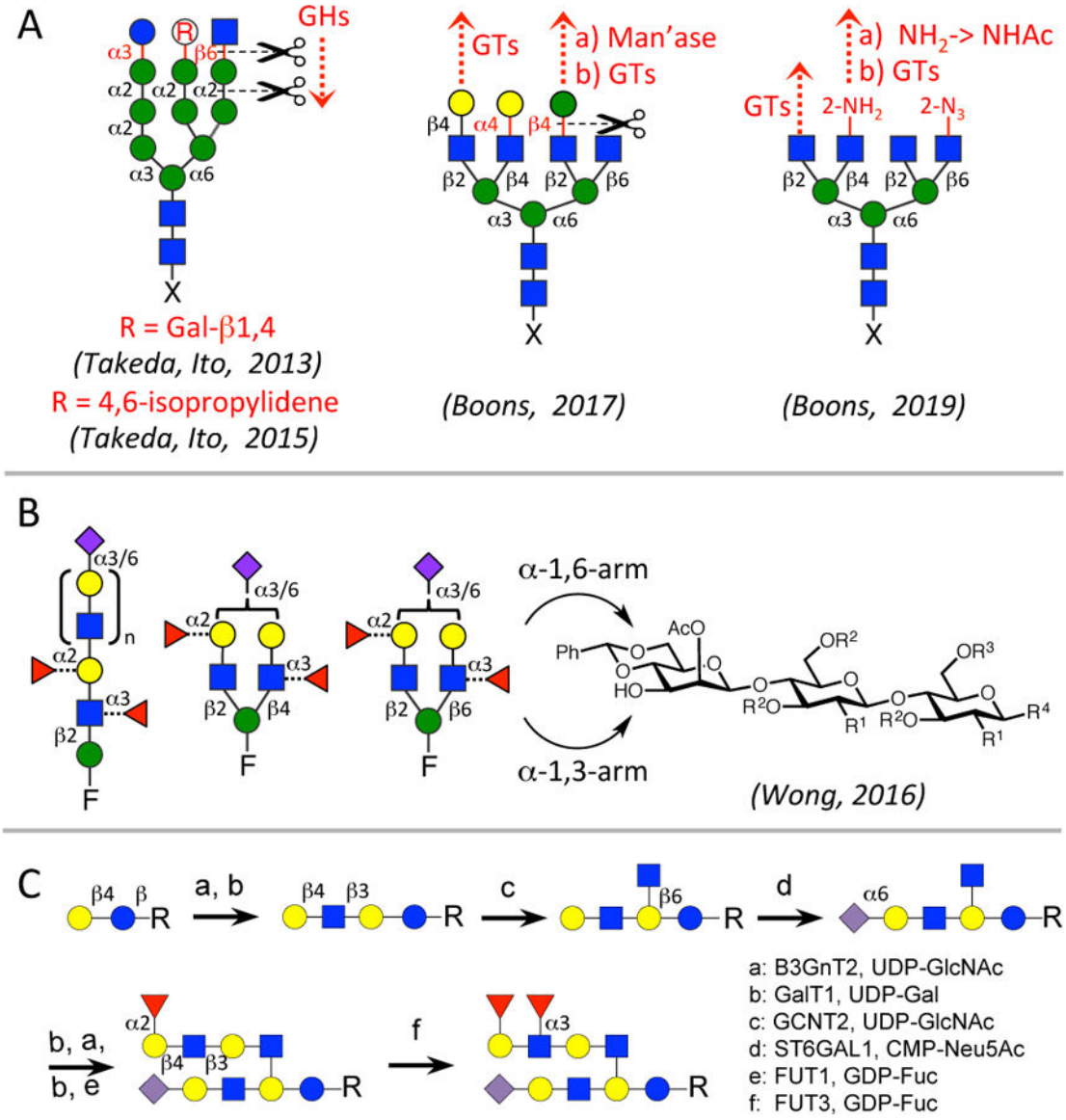



**Figure 3.**

(A) Generation of *N*-glycans libraries from a common precursor.[53a,b,e,f] (B) Modular glycan assembly by Wong.[54a-c] (C) Enzymatic synthesis of the branched HMOs.[59d]

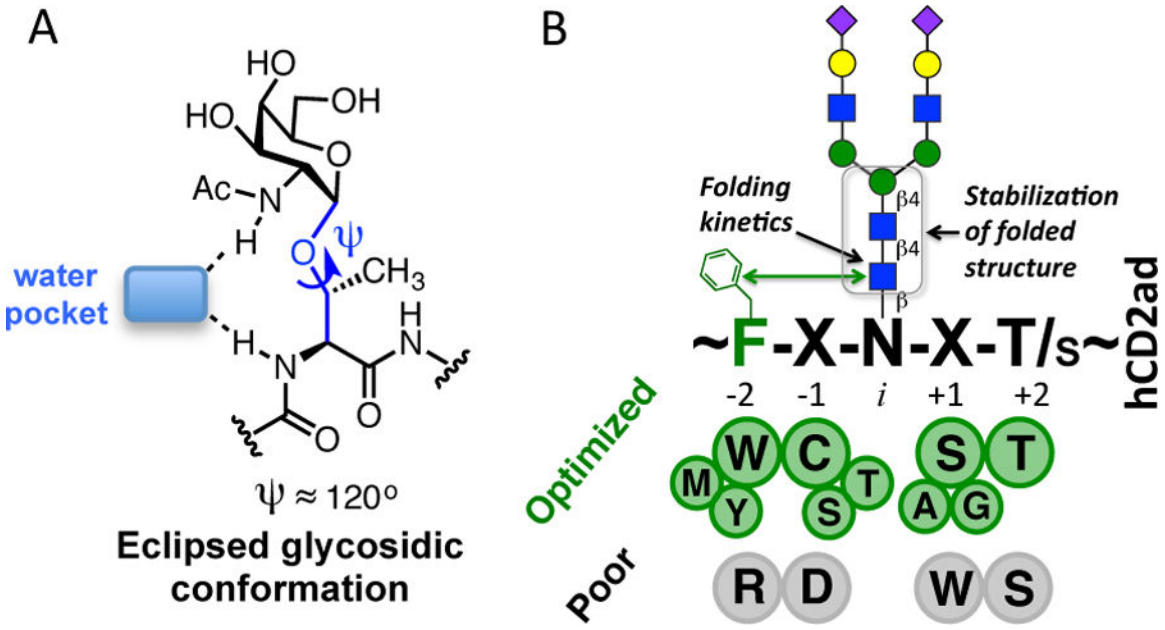



**Figure 4.**

(A) The Tn-Thr antigen in the eclipsed conformation.[62e] (B) Stabilizing effect of the $ManGlcNAc_2$ core on the protein backbone, and the preffered amino acids of the EAS.[63a-g]

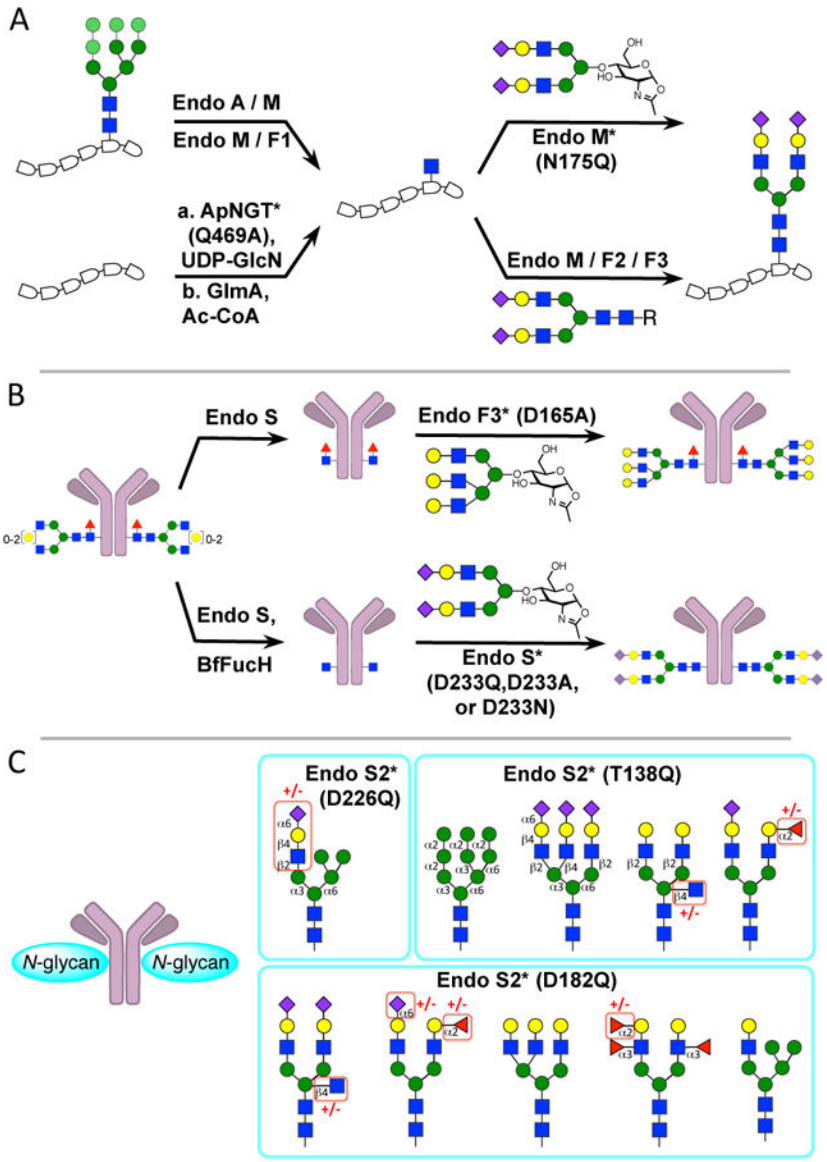



**Figure 5.**

(A) Summary of the *in vitro* enzymatic glycan remodeling of proteins and peptides. (B) Optimized ENGases for the synthesis of homogenous mAbs. (C) Substrate specificities of Endo S2 mutants.[55b]

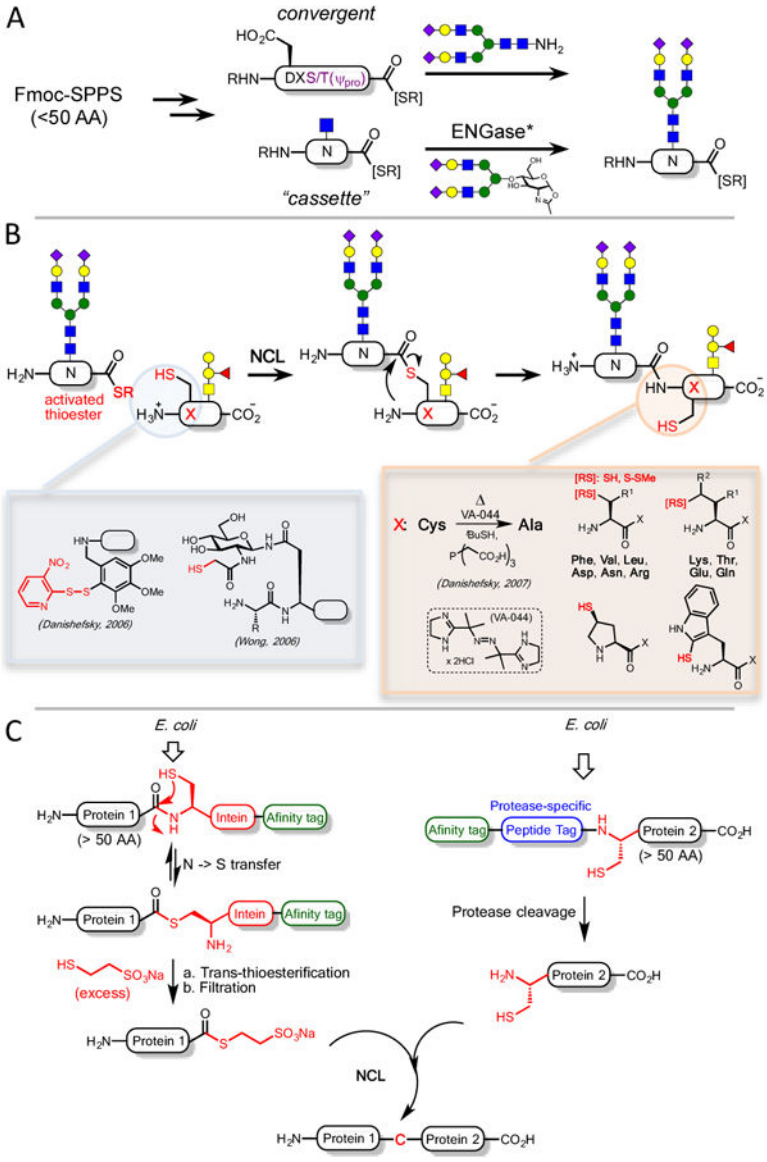



**Figure 6.**

(A) Convergent and "cassette"-based syntheses of glycopeptide fragments. (B) Illustration of NCL and selected examples of enabling methodologies. An up-to-date list of axillaries, thiol-modified amino acids and desulfurization methods could be found in a recent review.[69a] (C) Semi-synthesis of proteins by EPL.

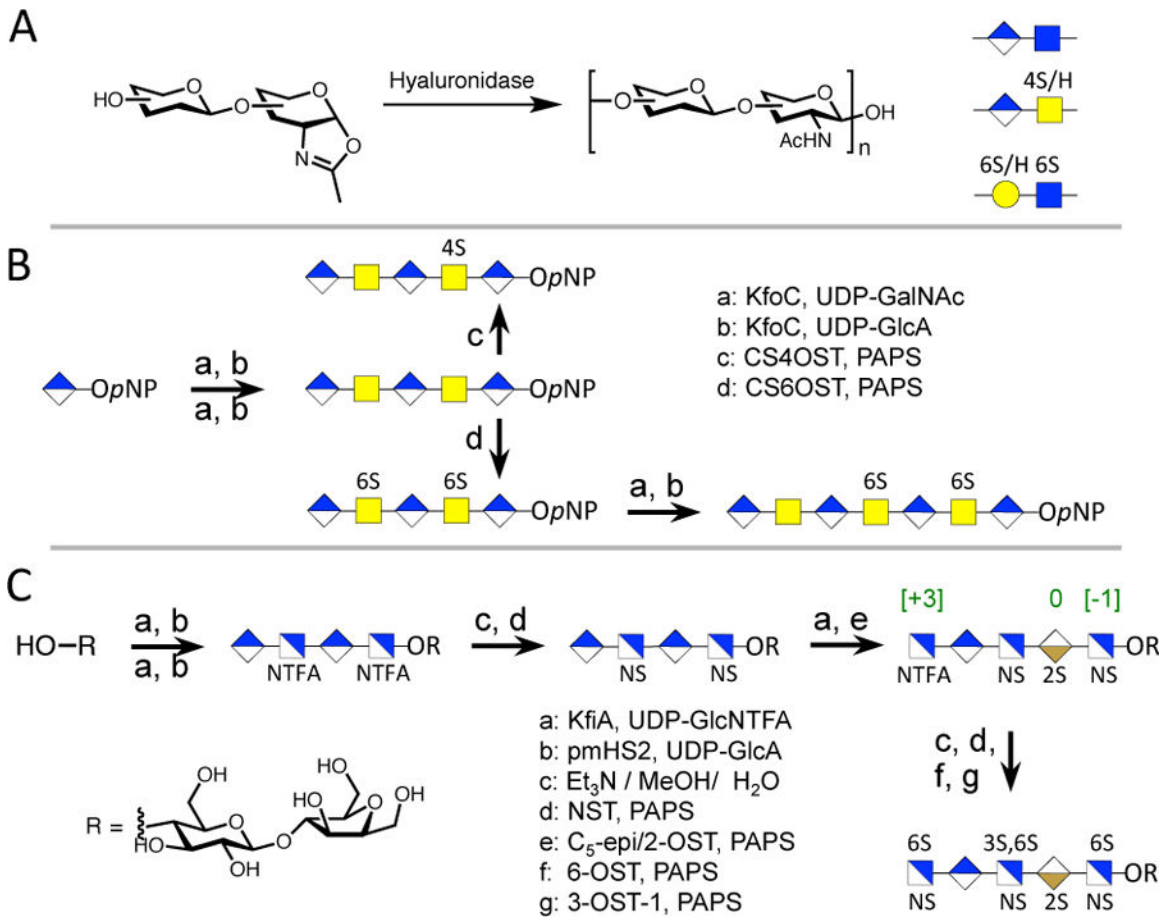



**Figure 7.**

(A) Polymerization of oxazoline donors.[80] (B) Enzymatic synthesis of CS with GTs.[81c] (C) Chemoenzymatic synthesis of ULMWH containing antithrombin-III binding site.[5a]

**Table 1.**

Selected methodologies for the synthesis of oligosaccharides.