Recent progress in chemoenzymatic synthesis of human glycans (Postprint)

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Abstract

Glycan is an essential cell component that usually exists in either a free form or a glycoconjugated form. Glycosylation affects the regulatory function of glycoconjugates in health and disease development, indicating the key role of glycan in organisms. Because of the complexity and diversity of glycan structures, it is challenging to prepare structurally well-defined glycans, which hinders the investigation of biological functions at the molecular level. Chemoenzymatic synthesis is an attractive approach for preparing complex glycans, because it avoids tedious protecting group manipulations in chemical synthesis and ensures high regio- and stereo-selectivity of glucosides during glycan assembly. Herein, enzymes, such as glycosyltransferases (GTs) and glycosidases (GHs), and sugar donors involved in the chemoenzymatic synthesis of human glycans are initially discussed. Many state-of-the-art chemoenzymatic methodologies are subsequently displayed and summarized to illustrate the development of synthetic human glycans, for example, N- and O-linked glycans, human milk oligosaccharides, and glycosaminoglycans. Thus, we provide an overview of recent chemoenzymatic synthetic designs and applications for synthesizing complex human glycans, along with insights into the limitations and perspectives of the current methods.This work reviewed the recent progress in the chemoenzymatic synthesis of human glycans and provides insights into the limitations and perspectives of the current methods.

Full Text

Preamble

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Recent progress in chemoenzymatic synthesis of human glycans

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Glycans are essential cellular components that exist in either free form or as glycoconjugates. Glycosylation profoundly influences the regulatory functions of glycoconjugates in health and disease, underscoring the critical biological roles of glycans. However, the structural complexity and diversity of glycans pose significant challenges for preparing well-defined structures, which has hindered mechanistic investigations at the molecular level. Chemoenzymatic synthesis offers an attractive alternative for accessing complex glycans, as it circumvents tedious protecting-group manipulations inherent in chemical synthesis while ensuring high regio- and stereoselectivity during glycan assembly. This review first discusses key enzymes—including glycosyltransferases (GTs) and glycosidases (GHs)—and sugar donors employed in the chemoenzymatic synthesis of human glycans. We then survey and summarize state-of-the-art chemoenzymatic methodologies that have advanced the synthesis of human glycans, including N- and O-linked glycans, human milk oligosaccharides, and glycosaminoglycans. By providing this overview of recent synthetic designs and applications, we offer insights into current limitations and future perspectives for chemoenzymatic glycan synthesis.

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Introduction

Glycans represent some of the most structurally complex and diverse molecules in nature, existing either as free oligosaccharides or covalently attached to proteins, lipids, or RNA to form glycoconjugates such as glycoproteins, proteoglycans, glycolipids, and glycoRNAs.1,2 Glycosylation regulates numerous biological processes, including signal transduction, cell migration, inflammation, immune evasion, cancer metastasis, and microbial infection.3–6 Despite their importance, the molecular mechanisms through which glycans mediate these events remain poorly understood, largely due to the limited availability of structurally well-defined glycans. Unlike nucleic acid and protein synthesis, glycan biosynthesis is a non-template-driven process. In vivo glycan formation depends on the expression levels of glycosyltransferases (GTs) and glycosidases (GHs),7 as well as the availability of sugar nucleotide donors.8–10 Two primary approaches exist for obtaining glycans: extraction from natural sources11 and in vitro artificial synthesis.

The low abundance and microheterogeneity of endogenous glycans make it difficult to isolate structurally homogeneous materials from nature, even with advanced analytical tools. Artificial synthesis therefore offers a more attractive alternative. While chemical synthesis can produce diverse natural and non-natural glycans and their derivatives,12–14 preparing complex glycans at scale remains challenging due to laborious protecting-group manipulations and demanding stereoselective glycosylations.

Enzymatic glycosylation provides another synthetic route.15,16 This approach offers exceptional regio- and stereoselectivity without requiring protecting groups. However, a single enzyme—such as one GT—typically catalyzes only one specific glycosidic bond formation, limiting its scope. To overcome these limitations, chemoenzymatic strategies that integrate chemical synthesis with enzymatic glycosylation have been developed and widely adopted over the past few decades for complex glycan synthesis.17,18 This hybrid approach eliminates tedious protecting-group manipulations while maintaining high regio- and stereoselectivity, offering tremendous potential for efficient synthesis of highly branched glycans (Fig. 1 [Figure 1: see original paper]). This mini-review surveys recent advances in chemoenzymatic methods for synthesizing complex human glycans.

Enzymes and Sugar Donors

GTs and GHs constitute the two major enzyme classes used in (chemo)enzymatic glycan synthesis. GTs employ nucleotide sugars as activated donors to catalyze glycan and glycoconjugate biosynthesis natively,19 making them the most widely used enzymes in glycan synthesis. The Carbohydrate-Active Enzymes (CAZy) database (https://www.cazy.org) classifies over 1,000,000 GTs into 135 families (as of 30th May 2024), with an additional 60,000 GT sequences remaining unclassified. Although thousands of GTs have been discovered, the hundreds successfully applied to human glycan chemoenzymatic synthesis are primarily glucosyltransferases (GlcTs), galactosyltransferases (GalTs), N-acetylglucosaminyltransferases (GlcNAcTs), glucuronyltransferases (GlcATs), N-acetylgalactosaminyltransferases (GalNAcTs), mannosyltransferases (ManTs), sialyltransferases (SiaTs), and fucosyltransferases (FucTs).

Ideal GTs for chemoenzymatic synthesis should exhibit high expression levels, stability, catalytic activity, regio- and stereoselectivity, minimal side activities, and promiscuity toward modified substrate analogs.20 However, wild-type GTs rarely possess all these properties. To address this, prokaryotic and eukaryotic expression systems21,22 have been engineered, often combined with protein engineering, to generate optimized GTs and mutants.

GHs, or glycoside hydrolases, cleave glycosidic linkages in glycans and glycoconjugates.23 They are classified as exo-glycosidases (removing terminal non-reducing sugars) or endo-glycosidases (cleaving internal bonds).24 This hydrolytic activity has been exploited as a complementary tool in chemoenzymatic synthesis. For example, treatment of a designed N-glycan precursor with various GHs and chemical deprotection yielded a high-mannose N-glycan library.25 Critically, GH hydrolysis is reversible, enabling glycoside synthesis.

Many oligosaccharides have been synthesized using GHs with intrinsic transglycosylation activity.26–28 Engineered GH mutants show enhanced transglycosylation efficiency for glycans,29,30 glycoproteins,31 and antibody glycoengineering.32 Mutant GHs have also enabled cell-surface transglycosylation for site-

specific glycoprotein analysis.33–35 Glycosynthases—GH mutants that synthesize glycosidic bonds without hydrolysis—have been reviewed extensively.36–39 This review focuses on biomimetic synthesis using activated donors and GTs.

Monosaccharides must be converted to activated donors for GT recognition and transfer.40 Donors include sugar nucleotides, dolichol phosphate sugars, and monosaccharide analogs. Sugar nucleotides are most common, recognized by most GTs,41 though some use dolichol phosphate sugars in early endoplasmic reticulum glycosynthesis.42 Phosphorylated and fluorinated sugars serve as glycosynthase substrates.43,44

Humans possess nine common sugar nucleotides: UDP-Gal, UDP-GalNAc, UDP-Xyl, GDP-Man, GDP-Fuc, and CMP-Neu5Ac (Fig. 2a [Figure 2: see original paper]). Their in vivo formation involves complex multi-enzyme pathways,45,46 making biomimetic in vitro synthesis challenging. One-pot multi-enzyme strategies47,48 and in situ regeneration systems49,50 have been developed for large-scale preparation. Wen and coworkers reported an efficient cofactor-driven cascade for rare sugar nucleotides—important precursors for uncommon sugar-containing glycans.10,51,52

Unnatural sugar nucleotide analogs have been designed for glycan synthesis. Recognized by GTs, they incorporate unnatural residues for precise manipulation. UDP-N-trifluoroacetylglucosamine (UDP-GlcNHTFA) serves as a widely used UDP-GlcNAc analog.53–55 UDP-6-N3-GlcNAc, UDP-4-N3-GlcNAc, UDP-4-SH-GlcNAc, and UDP-4-N3-GalNAc have also been prepared and applied (Fig. 2b).48,56,57 These analogs enable in situ cell-surface glycan synthesis for visualization and labeling.58–60

N-Linked Glycans

Human N-glycans are categorized as complex, hybrid, or high-mannose types, all sharing a common Man3GlcNAc2 core pentasaccharide attached to the Asn-X-Ser/Thr sequon.5,61–64 Access to this core is prerequisite for N-glycan chemoenzymatic synthesis. Current methods involve core preparation followed by enzymatic extension,65–68 enabling diverse symmetric and asymmetric N-glycan synthesis.

Wong and co-workers pioneered GT-mediated N-glycan elongation, developing modular assembly.69,71,72 This approach chemically synthesizes acceptor and donor modules, couples them to form the N-glycan core, then performs enzymatic extension (Fig. 3a [Figure 3: see original paper]). Despite challenging chemistry, this method accesses many complex N-glycans.

Asymmetric N-glycans, bearing unique saccharide appendages at each branch point,5,73 have been targeted through several strategies. In 2013, Boons and co-workers reported a general chemoenzymatic route using a core pentasaccharide protected orthogonally with levulinoyl (Lev), fluorenylmethyloxycarbonate (Fmoc), allyloxycarbonate (Alloc), and 2-naphthylmethyl (Nap) groups at po-

tential branch sites.65 This common precursor enabled selective chemical glycosylation and subsequent GT-mediated branch elongation to generate asymmetric N-glycan libraries (Fig. 3b). Ito et al. simultaneously reported a top-down chemoenzymatic approach to high-mannose N-glycan libraries.25 Related methods have produced various N-glycans.76–79

Trimming sialoglycopeptide (SGP) from egg sources with GHs provides another core access route.80–82 Boons and co-workers developed a "stop and go" strategy from SGP to synthesize asymmetric multi-antennary N-glycans within $ $10 steps.55 Using UDP-GlcNHTFA as a donor, human MGAT4 and MGAT5 transferred GlcNHTFA to the bi-antennary heptasaccharide, creating controllable arms. GlcNHTFA could be converted to GlcNH2 and then to GlcN3—neither recognized by human β-1,4-galactosyltransferase (B4GalT1)—enabling regioselective GlcNAc galactosylation. Subsequent conversion back to GlcNAc provided B4GalT1 substrates (Fig. 3c). MGAT1 and MGAT2 also utilize UDP-GlcNHTFA, enabling synthesis of 32 asymmetric bi-antennary N-glycans from airway tissues.83

Large-scale core pentasaccharide production remains challenging via chemical synthesis or isolation. Though human mannosyltransferases Alg1 and Alg2 have been characterized,84–86 large-scale core preparation proved elusive. Wen and coworkers recently addressed this with a concise chemoenzymatic platform.70 Starting from inexpensive commercial GlcNAc$ $1,4GlcNAc, they chemically coupled an 11-carbon lipid tail (undecanol) to create a starter recognized by recombinant Alg1 and Alg2, which installed α-1,3- and α-1,6-mannoses. This enabled efficient large-scale core synthesis in few steps, yielding >60 N-glycans (Fig. 3d).

N-glycans on antibodies significantly affect effector functions.87–89 Chemoenzymatic methods have prepared IgG N-glycan panels. In 2020, Unverzagt and co-workers galactosylated bisecting GlcNAc in rare IgG N-glycans using β-1,4-galactosyltransferase, finding activity only on bi-antennary bisected structures.90 Li and co-workers constructed a 64-member IgG N-glycan library from SGP.91 Antibody N-glycan remodeling using endo-β-N-acetylglucosaminidase mutants has been reviewed elsewhere.31,92,93

O-Linked Glycans

O-GalNAc and O-mannose represent the major human O-glycan types,94,95 attracting extensive chemoenzymatic development.96,97

A regioselective one-pot multienzyme (OPME) strategy systematically synthesized sialyl Core 2 glycans using Pasteurella multocida α-2,3-sialyltransferase 3 and rationally designed acceptors (Fig. 4a [Figure 4: see original paper]).98 Site-specific modifications like sulfation further complicate synthesis. Li and coworkers prepared >30 defined Core 2 glycans enzymatically from chemically synthesized sulfated and non-sulfated Core 2 trisaccharide starters (Fig. 4b).99

A robust modular assembly strategy accessed O-GalNAc Cores 1–4 and 6 using three chemical building blocks.100 Enzymatic decoration yielded 83 O-GalNAc glycans covering diverse epitopes, which were arrayed to probe lectins, anti-glycan antibodies, and colorectal cancer serum samples. Recently, Li's group prepared three α-linked rare cores (Core 5, 7, 8) and their sialylated forms.103 Photobacterium damselae α-2,6-sialyltransferase (Pd2,6ST) showed restricted specificity, installing α-2,6 sialic acid at the 6-OH of internal GalNAc in Cores 5 and 8, and terminal GalNAc in Core 7.

O-mannosyl glycans, typically bi-antennary with β-1,2- and β-1,6-linked GlcNAc residues,95 present regioselective synthesis challenges. Cao and co-workers assembled 58 complex O-mannosyl glycans from five synthetic cores, with 55 being first-time syntheses (Fig. 4c).101 Li and coworkers used a scaffold synthesis/enzymatic extension strategy to prepare 45 human O-mannosyl glycans.104 Peng and coworkers synthesized O-mannosyl glycopeptides via microwave-assisted SPPS.107

The HNK-1 epitope (3-S-GlcA$ 1, 3*Gal* $1,4GlcNAc) is a unique O-mannosyl component in brain tissue. Cao and co-workers synthesized HNK-1-bearing O-mannosyl glycans via chemoenzymatic assembly of chemically prepared trisaccharide lactone donors, selective lactone cleavage, sulfation, and bacterial GT elaboration (Fig. 4d).102

Poly-LacNAc Glycans

Poly-N-acetyllactosamine (poly LacNAc) glycans, composed of type II LacNAc repeats, are common motifs in N- and O-glycans and human milk oligosaccharides (HMOs). Fucosylation and sialylation generate biologically important epitopes like Lewis X and sialyl Lewis X, driving interest in regioselective poly LacNAc synthesis.

Elling and coworkers chemoenzymatically synthesized branched LacNAc oligomers bearing LacNAc and/or LacdiNAc epitopes using sequential enzymatic/chemical steps, galactose oxidase, and reductive amination (Fig. 5a [Figure 5: see original paper]).108 Galactose oxidase oxidation generated 6 -aldehyde LacNAc, which served as a $ $3GlcNAcT acceptor for chain elongation while the aldehyde enabled reductive amination coupling.

Cao's group reprogrammed the enzymatic assembly line for site-specific fucosylation by using α-2,6-linked sialic acid as a protecting group on LacNAc repeats (Fig. 5b).109 After controlled fucosylation, sialidase removed the sialic acid. They also reported a redox-controlled site-specific sialylation strategy using galactose oxidase to oxidize the Gal C-6 hydroxyl, solving regioselectivity challenges.110 This approach was later extended to site-specific fucosylation of N- and O-glycans.111

Boons and coworkers developed a protecting group-based strategy using a hexasaccharide precursor containing GlcNH2, GlcNHTFA, and GlcNHBoc (Fig.

5c).112 Recombinant fucosyltransferases Hp39-FT and FUT5 recognized LacNAc and LacNHTFA for α-1,3 fucosylation, but not LacNH2/LacNHBoc. Modulating these moieties before/after enzymatic steps yielded diversified poly LacNAc libraries.

Boons et al. found that LacNHBoc blocks GCNT2-mediated branching at proximal Gal residues, enabling controlled synthesis.113 LacNHBoc is galactosylated by bacterial HpGalT and CvGalT but not fucosylated. Lin and coworkers leveraged bacterial GT promiscuity and acceptor-mediated glycosylation for regioselective fucosylation (Fig. 5d).114

Human Milk Oligosaccharides (HMOs)

HMOs serve as prebiotic substrates for neonatal microbiome establishment118,119 and as soluble decoys against viral and parasitic infection.120,121 Their structural complexity impedes access to defined HMOs for biological study and scale-up. Synthesis begins with lactose, elongated by LacNAc repeats that are fucosylated or sialylated.119,122 Chemoenzymatic methods have enabled diverse HMO synthesis.123,124

Cao and coworkers chemoenzymatically synthesized lacto-N-tetrasaccharide and sialylated variants via OPME in 2015.125 They first prepared lacto-N-tetrasaccharide-proN3 acceptor, then installed sialic acid at the non-reducing end. Wang and coworkers used a core synthesis/enzymatic extension strategy with thioether and bromide donors to prepare three cores convergently, which GTs extended to a 31-HMO library (Fig. 6a [Figure 6: see original paper]).115

To reduce chemical steps, Chen's group enzymatically assembled lacto-N-tetrasaccharide-proN3 using a highly efficient C. violaceum β-1,3-galactosyltransferase.126 Yu and coworkers applied H. pylori DSM 6709 α-1,3/4-fucosyltransferase (FucTIII) in a sequential one-pot strategy to synthesize fucosylated HMOs containing Lewis X, Lewis A, and sialyl Lewis X.127 FucTIII preferred type II LacNAc acceptors, with efficiency enhanced by terminal GlcNAc.

Yu and coworkers demonstrated that a sulfo-fluorous tag (SF17) at the reducing end of lactose enables site-selective enzymatic fucosylation (Fig. 6b).116 SF17-tagged acceptors allowed: (1) site-selective fucosylation by wild-type Bacteroides fragilis α-1,3/4-fucosyltransferase (Bf13FT) and H. pylori α-1,3/4-FucT; (2) selective α-2,6-sialylation of terminal Gal by Pd2,6ST (avoiding multi-sialylation seen with untagged acceptors); and (3) enhanced catalytic efficiency of $ $1,3-GalT, $ $1,3-GlcNAcT, and Bf13FT. These strategies highlight bacterial GTs as powerful HMO synthesis tools.

Human GTs also enable HMO synthesis. Boons and coworkers used a limited set of human GTs to synthesize asymmetric multi-antennary HMOs.117 Lactose bearing a hydrophobic coumarin tag was converted to LNnT tetrasaccharide using human β-1,3-N-acetylglucosaminyltransferase 2 and B4GalT1. GCNT2 then

introduced a branching GlcNAc, and subsequent modification by human GTs (α-1,2-fucosyltransferase 1, α-1,3/4-fucosyltransferase 3, α-2,6-sialyltransferase 1) generated a 60-member asymmetric multi-antennary HMO library for glycan microarray analysis (Fig. 6c). Recently, Fang and coworkers expressed GCNT2 in *Pichia pastoris* for an enzymatic modular synthesis of branched HMOs.128

Glycosaminoglycans (GAGs)

GAGs are linear anionic polysaccharides with specific sulfation patterns, composed of alternating hexosamine and uronic acid/galactose residues.129–131 Based on disaccharide repeats, they are classified as: hyaluronic acid (HA, (-$ 4*GlcA*− 3*GlcNAc*1−)*n*); *heparin/heparansulfate*(*HP*/*HS*, (− 4*IdoA*− 4*GlcNAc*−)*nwithvariablesulfatio* with 6-O sulfation).131,132

GAGs have substantial medical and cosmetic value—hyaluronan for arthritis/cosmetics,133 heparin as an anticoagulant,134 and HP/HS as potential SARS-CoV-2 inhibitors.135 However, limited access to homogeneous GAGs hampers scaled production, making chemoenzymatic synthesis promising.

Pasteurella multocida hyaluronan synthase (PmHAS) polymerizes GlcA and GlcNAc into HA. Its two GT domains recognize UDP-GlcNAc and UDP-GlcA,136,137 enabling HA elongation with a single enzyme.138,139 OPME systems using inexpensive monosaccharides (e.g., sucrose, GlcNAc) instead of costly nucleotides were developed for economic HMw-HA synthesis with in situ UDP-sugar regeneration (Fig. 7a).140,141

HP/HS synthesis employs bifunctional *P. multocida* PmHS1/PmHS2142 or *E. coli* K5 KfiA/KfiC.143 Sulfotransferases (6-OST, 3-OST, NST) introduce structural diversity. Liu and coworkers tailored two homogeneous ultra-low molecular weight (ULMW) heparins from a disaccharide using PmHS2/KfiA and UDP-GlcNHTFA, which KfiA incorporated and could be converted to GlcNH2 for N-sulfation. C5-epimerases and OSTs then produced the final heparins (Fig. 7b).53 Other efforts achieved cost-effective, controlled, gram-scale HP/HS synthesis.144–147

CS/DS synthesis uses *E. coli* K4 GT KfoC, which transfers both GalNAc and GlcA. Liu and coworkers synthesized 15 CS glycans (trisaccharide to nonasaccharide) with diverse sulfation patterns.154 They also reported seven homogeneous HS/CS chimeras coupling a CS heptasaccharide domain to an HS pentasaccharide.155 Huang and coworkers recently developed the first solid-phase chemoenzymatic CSPG synthesis using Sepharose beads with a cleavable linker.150 Sequential GT reactions (xylosyltransferase-1, β-1,4-galactosyltransferases 7/6, β-1,3-glucuronic acid transferase 3) after xylose 2-O phosphorylation yielded CSPG glycopeptides (Fig. 7c).

DS synthesis from chondroitin requires DS-epimerase and D4ST1 sulfotransferase.156 KS synthesis remains underdeveloped despite its biological importance. One approach uses mutant keratanase II transglycosylation with oxazo-

line donors.30,157 Boons and coworkers recently employed human CHST2 and CHST1 sulfotransferases: CHST2 sulfates only terminal GlcNAc, while CHST1 sulfates internal Gal in LacNAc chains.158 α-1,3-fucosylated LacNAc is not a CHST1 substrate, and Gal-6-O sulfation blocks α-1,3-fucosylation, enabling programmed synthesis of diverse KS glycans (Fig. 7d).151

Summary and Outlook

Decades of research have revealed critical physiological and pathological glycan functions, creating urgent demand for well-defined glycans to probe their biology. Chemoenzymatic synthesis is a powerful tool for preparing complex biologically relevant glycans and glycoconjugates (glycopeptides, glycoproteins,31 glycolipids159,160). Combining enzymatic glycosylation with chemical handles avoids tedious protecting-group manipulations while enhancing regio- and stereoselectivity.

Two main factors limit this methodology. First, GT availability and understanding remain limited. Expression systems (e.g., *E. coli* for bacterial GTs, insect cells for mammalian GTs) and enzyme engineering address this, while structural and mutagenesis studies deepen mechanistic insight. Second, multi-step syntheses require numerous purification steps (e.g., a decasaccharide needs $ $9 isolations). Automated enzymatic synthesis platforms161,162 and advanced analytical tools (UPLC,163 HRMS,164 nanopore sequencing,165–167 machine learning168) are streamlining production and structural analysis. Future protocols may enable glycan synthesis as routine as PCR amplification.

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References

1. R. A. Flynn, K. Pedram, S. A. Malaker, P. J. Batista, B. A. H. Smith, A. G. Johnson, B. M. George, K. Majzoub, P. W. Villalta, J. E. Carette and C. R. Bertozzi, Small RNAs are modified with N-glycans and displayed on the surface of living cells, *Cell*, 2021, 184(12), 3109–3124.
2. S. S. Shivatare, V. S. Shivatare and C. H. Wong, Glycoconjugates: Synthesis, Functional Studies, and Therapeutic Developments, *Chem. Rev.*, 2022, 122(20), 159–202.
3. A. Varki, Biological Roles of Oligosaccharides - All of the Theories Are Correct, *Glycobiology*, 1993, 3(2), 97–130.

4. K. Ohtsubo and J. D. Marth, Glycosylation in cellular mechanisms of health and disease, *Cell*, 2006, 126(5), 855–867.

5. K. W. Moremen, M. Tiemeyer and A. V. Nairn, Vertebrate protein glycosylation: diversity, synthesis and function, *Nat. Rev. Mol. Cell Biol.*, 2012, 13(7), 448–462.

6. H. J. Joshi and H. Clausen, Global view of human protein glycosylation pathways and functions, *Nat. Rev. Mol. Cell Biol.*, 2020, 21(12), 729–749.

7. S. M. Hancock, M. D. Vaughan and S. G. Withers, Engineering of glycosidases and glycosyltransferases, *Curr. Opin. Chem. Biol.*, 2006, 10(5), 509–519.

8. G. K. Wagner, T. Pesnot and R. A. Field, A survey of chemical methods for sugar-nucleotide synthesis, *Nat. Prod. Rep.*, 2009, 26(9), 1172–1194.

9. S. Sanz, G. Bandini, D. Ospina, M. Bernabeu, K. Marino, C. Fernandez-Becerra and L. Izquierdo, Biosynthesis of GDP-fucose and other sugar nucleotides in the blood stages of *Plasmodium falciparum*, *J. Biol. Chem.*, 2013, 288(23), 16506–16517.

10. S. Wang, J. Zhang, F. Wei, W. Li and L. Wen, Facile Synthesis of Sugar Nucleotides from Common Sugars by the Cascade Conversion Strategy, *J. Am. Chem. Soc.*, 2022, 144(22), 9980–9989.

11. X. Song, H. Ju, Y. Lasanajak, M. R. Kudelka, D. F. Smith and R. D. Cummings, Oxidative release of natural glycans for functional glycomics, *Nat. Methods*, 2016, 13(6), 528–534.

12. A. Pardo-Vargas, M. Delbianco and P. H. Seeberger, Automated glycan assembly as an enabling technology, *Curr. Opin. Chem. Biol.*, 2018, 46, 48–55.

13. Q. Zhu, Z. Shen, F. Chiodo, S. Nicolardi, A. Molinaro, A. Silipo and B. Yu, Chemical synthesis of glycans up to a 128-mer relevant to the O-antigen of *Bacteroides vulgatus*, *Nat. Commun.*, 2020, 11(1), 4142.

14. W. Yao, D.-C. Xiong, Y. Yang, C. Geng, Z. Cong, F. Li, B.-H. Li, X. Qin, L.-N. Wang, W.-Y. Xue, N. Yu, H. Zhang, X. Wu, M. Liu and X.-S. Ye, Automated solution-phase multiplicative synthesis of complex glycans up to a 1,080-mer, *Nat. Synth.*, 2022, 1(11), 854–863.

15. T. Rexer, D. Laaf, J. Gottschalk, H. Frohnmeyer, E. Rapp and L. Elling, Enzymatic Synthesis of Glycans and Glycoconjugates, *Adv. Biochem. Eng./Biotechnol.*, 2021, 175, 1–30.

16. C. C. Liu, J. Ye and H. Cao, Chemical Evolution of Enzyme-Catalyzed Glycosylation, *Acc. Chem. Res.*, 2024, 57(3), 456–467.

17. S. Muthana, H. Cao and X. Chen, Recent progress in chemical and chemoenzymatic synthesis of carbohydrates, *Curr. Opin. Chem. Biol.*,

2009, 13(5–6), 573–581.

18. R. M. Schmaltz, S. R. Hanson and C. H. Wong, Enzymes in the synthesis of glycoconjugates, *Chem. Rev.*, 2011, 111(7), 4259–4307.

19. M. Kumar, C. K. Bandi and S. P. Chundawat, High-throughput screening of glycosynthases using azido sugars for oligosaccharides synthesis, in *Methods Enzymol*, Elsevier, 2023, vol. 682, pp. 211–245.

20. J. B. McArthur and X. Chen, Glycosyltransferase engineering for carbohydrate synthesis, *Biochem. Soc. Trans.*, 2016, 44(1), 129–142.

21. R. Chen, Bacterial expression systems for recombinant protein production: *E. coli* and beyond, *Biotechnol. Adv.*, 2012, 30(5), 1102–1107.

22. K. W. Moremen, A. Ramiah, M. Stuart, J. Steel, L. Meng, F. Forouhar, H. A. Moniz, G. Gahlay, Z. Gao, D. Chapla, S. Wang, R. J. Wood, J. Y. Yang, P. K. Prabhakar, M. D. Rosa, C. Geisler, A. V. Nairn, J. Seetharaman, S. C. Wu, L. Tong, H. J. Gilbert, J. LaBaer and D. L. Jarvis, Expression system for structural and functional studies of human glycosylation enzymes, *Nat. Chem. Biol.*, 2018, 14(2), 156–162.

23. Y. Bourne and B. Henrissat, Glycoside hydrolases and glycosyltransferases: families and functional modules, *Curr. Opin. Struct. Biol.*, 2001, 11(5), 593–600.

24. A. Kobata, Exo- and endoglycosidases revisited, *Proc. Jpn. Acad., Ser. B*, 2013, 89(3), 97–117.

25. A. Koizumi, I. Matsuo, M. Takatani, A. Seko, M. Hachisu, Y. Takeda and Y. Ito, Top-down chemoenzymatic approach to a high-mannose-type glycan library: synthesis of a common precursor and its enzymatic trimming, *Angew. Chem., Int. Ed.*, 2013, 52(29), 7426–7431.

26. L. Guo, X. Chen, L. Xu, M. Xiao, L. Lu and H. Atomi, Enzymatic Synthesis of 6 -Sialyllactose, a Dominant Sialylated Human Milk Oligosaccharide, by a Novel exo-α-Sialidase from *Bacteroides fragilis* NCTC9343, *Appl. Environ. Microbiol.*, 2018, 84(13), e00071–e00018.

27. H. T. T. Pham, G. A. T. Kate, L. Dijkhuizen and S. S. van Leeuwen, Synthesis and Characterization of Sialylated Lactose- and Lactulose-Derived Oligosaccharides by Trans-sialidase, *J. Agric. Food Chem.*, 2019, 67(12), 3469–3478.

28. B. Zeuner and A. S. Meyer, Enzymatic transfucosylation for synthesis of human milk oligosaccharides, *Carbohydr. Res.*, 2020, 493, 108029.

29. B. Zeuner, M. Vuillemin, J. Holck, J. Muschiol and A. S. Meyer, Loop engineering of an alpha-1,3/4 l-fucosidase for improved synthesis of human milk oligosaccharides, *Enzyme Microb. Technol.*, 2018, 115, 37–44.

30. S. Yuge, A. Tateishi, K. Numata and M. Ohmae, Sulfo-Synthesis of Potent Siglec-8 Ligands Catalyzed by Chemoenzymatic Transglycosylation of Keratanase, *Biomacromolecules*, 2022, 23(1), 316–325.

31. C. Li and L. X. Wang, Chemoenzymatic Methods for the Synthesis of Glycoproteins, *Chem. Rev.*, 2018, 118(17), 8359–8413.

32. L. X. Wang, X. Tong, C. Li, J. P. Giddens and T. Li, Glycoengineering of Antibodies for Modulating Functions, *Annu. Rev. Biochem.*, 2019, 88, 433–459.

33. Y. Tian, Y. Wang, H. Yin, Y. Luo, F. Wei, H. Zhou and L. Wen, A Sensitive and Reversible Labeling Strategy Enables Global Mapping of the Core–Fucosylated Glycoproteome on Cell Surfaces, *Angew. Chem., Int. Ed.*, 2022, 134(49), e202206802.

34. Y. Luo, Y. Wang, Y. Tian, H. Zhou and L. Wen, "Two Birds One Stone" Strategy for the Site-Specific Analysis of Core Fucosylation and O-GlcNAcylation, *J. Am. Chem. Soc.*, 2023, 145(29), 15879–15887.

35. Y. Wang, R. Yuan, B. Liang, J. Zhang, Q. Wen, H. Chen, Y. Tian, L. Wen and H. Zhou, A "One-Step" Strategy for Global Characterization of Core-Fucosylated Glycoproteome, *JACS Au*, 2024, 4(5), 2005–2018.

36. S. J. Williams and S. G. Withers, Glycosynthases: Mutant glycosidases for glycoside synthesis, *Aust. J. Chem.*, 2002, 55(1–2), 3–12.

37. M. R. Hayes and J. Pietruszka, Synthesis of Glycosides by Glycosynthases, *Molecules*, 2017, 22(9), 1434.

38. T. M. Gloster, Exploitation of carbohydrate processing enzymes in biocatalysis, *Curr. Opin. Chem. Biol.*, 2020, 55, 1–9.

39. C. Moulis, D. Guieysse, S. Morel, E. Séverac and M. Remaud-Siméon, Natural and engineered transglycosylases: Green tools for the enzyme-based synthesis of glycoproducts, *Curr. Opin. Chem. Biol.*, 2021, 61, 96–105.

40. J. P. Dolan, S. C. Cosgrove and G. J. Miller, Biocatalytic Approaches for Enzymatic and Chemical Glycan Synthesis, *JACS Au*, 2023, 3(1), 47–61.

41. L. Mestrom, M. Przypis, D. Kowalczykiewicz, A. Pollender, A. Kumpf, S. R. Marsden, I. Bento, A. B. Jarzebski, K. Szymanska, A. Chrusciel, D. Tischler, R. Schoevaart, U. Hanefeld and P. L. Hagedoorn, Leloir Glycosyltransferases in Applied Biocatalysis: A Multidisciplinary Approach, *Int. J. Mol. Sci.*, 2019, 20(21), 5263.

42. Y. Maeda and T. Kinoshita, Dolichol-phosphate mannose synthase: structure, function and regulation, *Biochim. Biophys. Acta*, 2008, 1780(6), 861–868.

43. H. Yu, V. Thon, K. Lau, L. Cai, Y. Chen, S. Mu, Y. Li, P. G. Wang and X. Chen, Highly efficient chemoenzymatic synthesis of beta1–3-linked galactosides, *Chem. Commun.*, 2010, 46(40), 7507–7509.

44. C. Li, S. Zhu, C. Ma and L. X. Wang, Designer alpha1,6-Fucosidase Mutants Enable Direct Core Fucosylation of Intact N-Glycopeptides and N-Glycoproteins, *J. Am. Chem. Soc.*, 2017, 139(42), 15074–15087.

45. M. Bar-Peled and M. A. O'Neill, Plant nucleotide sugar formation, interconversion, and salvage by sugar recycling, *Annu. Rev. Plant Biol.*, 2011, 62, 127–155.

46. L. Cai, Recent Progress in Enzymatic Synthesis of Sugar Nucleotides, *J. Carbohydr. Chem.*, 2012, 31(7), 535–552.

47. H. Yu, H. Yu, R. Karpel and X. Chen, Chemoenzymatic synthesis of CMP-sialic acid derivatives by a one-pot two-enzyme system: comparison of substrate flexibility of three microbial CMP-sialic acid synthetases, *Bioorg. Med. Chem.*, 2004, 12(24), 6427–6435.

48. Y. Chen, V. Thon, Y. Li, H. Yu, L. Ding, K. Lau, J. Qu, L. Hie and X. Chen, One-pot three-enzyme synthesis of UDP-GlcNAc derivatives, *Chem. Commun.*, 2011, 47(38), 10817–10819.

49. T. I. Tsai, H. Y. Lee, S. H. Chang, C. H. Wang, Y. C. Tu, Y. C. Lin, D. R. Hwang, C. Y. Wu and C. H. Wong, Effective sugar nucleotide regeneration for the large-scale enzymatic synthesis of Globo H and SSEA4, *J. Am. Chem. Soc.*, 2013, 135(39), 14831–14839.

50. M. T. Anwar, S. K. Kawade, Y. R. Huo, A. K. Adak, D. Sridharan, Y. T. Kuo, C. Y. Fan, H. R. Wu, Y. S. Lee, T. Angata and C. C. Lin, Sugar nucleotide regeneration system for the synthesis of Bi- and triantennary-glycans and exploring their activities against siglecs, *Eur. J. Med. Chem.*, 2022, 232, 114146.

51. Y. Zheng, J. Zhang, J. Meisner, W. Li, Y. Luo, F. Wei and L. Wen, Cofactor-Driven Cascade Reactions Enable the Efficient Preparation of Sugar Nucleotides, *Angew. Chem., Int. Ed.*, 2022, 61(20), e202115696.

52. F. Wei, R. Yuan, Q. Wen and L. Wen, Systematic Enzymatic Synthesis of dTDP–Activated Sugar Nucleotides, *Angew. Chem.*, 2023, 135(20), e202217894.

53. Y. Xu, S. Masuko, M. Takieddin, H. Xu, R. Liu, J. Jing, S. A. Mousa, R. J. Linhardt and J. Liu, Chemoenzymatic synthesis of homogeneous ultralow molecular weight heparins, *Science*, 2011, 334(6055), 498–501.

54. Y. Xu, C. Cai, K. Chandarajoti, P. H. Hsieh, L. Li, T. Q. Pham, E. M. Sparkenbaugh, J. Sheng, N. S. Key, R. Pawlinski, E. N. Harris, R. J. Linhardt and J. Liu, Homogeneous low-molecular-weight heparins with reversible anticoagulant activity, *Nat. Chem. Biol.*, 2014, 10(4), 248–250.

55. L. Liu, A. R. Prudden, C. J. Capicciotti, G. P. Bosman, J. Y. Yang, D. G. Chapla, K. W. Moremen and G. J. Boons, Streamlining the chemoenzymatic synthesis of complex N-glycans by a stop and go strategy, *Nat. Chem.*, 2019, 11(2), 161–169.

56. X. Zhang, D. E. Green, V. L. Schultz, L. Lin, X. Han, R. Wang, A. Yaksic, S. Y. Kim, P. L. DeAngelis and R. J. Linhardt, Synthesis of 4-azido-N-acetylhexosamine uridine diphosphate donors: clickable glycosaminoglycans, *J. Org. Chem.*, 2017, 82(18), 9910–9915.

57. P. He, X. Zhang, K. Xia, D. E. Green, S. Baytas, Y. M. Xu, T. Pham, J. Liu, F. M. Zhang, A. Almond, R. J. Linhardt and P. L. DeAngelis, Chemoenzymatic synthesis of sulfur-linked sugar polymers as heparanase inhibitors, *Nat. Commun.*, 2022, 13(1), 7438.

58. L. Wen, D. Liu, Y. Zheng, K. Huang, X. Cao, J. Song and P. G. Wang, A One-Step Chemoenzymatic Labeling Strategy for Probing Sialylated Thomsen–Friedenreich Antigen, *ACS Cent. Sci.*, 2018, 4(4), 451–457.

59. L. Q. Wen, M. R. Gadi, Y. Zheng, C. Gibbons, S. M. Kondengaden, J. B. Zhang and P. G. Wang, Chemoenzymatic Synthesis of Unnatural Nucleotide Sugars for Enzymatic Bioorthogonal Labeling, *ACS Catal.*, 2018, 8(8), 7659–7666.

60. Y. P. Tian, S. Z. Ma and L. Q. Wen, Towards chemoenzymatic labeling strategies for profiling protein glycosylation, *Curr. Opin. Chem. Biol.*, 2024, 80, 102460.

61. A. Helenius and M. Aebi, Intracellular functions of N-linked glycans, *Science*, 2001, 291(5512), 2364–2369.

62. L. Ellgaard and A. Helenius, Quality control in the endoplasmic reticulum, *Nat. Rev. Mol. Cell Biol.*, 2003, 4(3), 181–191.

63. E. Weerapana and B. Imperiali, Asparagine-linked protein glycosylation: from eukaryotic to prokaryotic systems, *Glycobiology*, 2006, 16(6), 91R–101R.

64. F. Schwarz and M. Aebi, Mechanisms and principles of N-linked protein glycosylation, *Curr. Opin. Struct. Biol.*, 2011, 21(5), 576–582.

65. Z. Wang, Z. S. Chinoy, S. G. Ambre, W. Peng, R. McBride, R. P. de Vries, J. Glushka, J. C. Paulson and G. J. Boons, A general strategy for the chemoenzymatic synthesis of asymmetrically branched N-glycans, *Science*, 2013, 341(6144), 379–383.

66. P. Wang, J. Zhu, Y. Yuan and S. J. Danishefsky, Total synthesis of the 2,6-sialylated immunoglobulin G glycopeptide fragment in homogeneous form, *J. Am. Chem. Soc.*, 2009, 131(46), 16669–16671.

67. C. Gao, M. S. Hanes, L. A. Byrd-Leotis, M. Wei, N. Jia, R. J. Kardish, T. R. McKitrick, D. A. Steinhauer and R. D. Cummings, Unique Binding

Specificities of Proteins toward Isomeric Asparagine-Linked Glycans, *Cell Chem. Biol.*, 2019, 26(4), 535–547.

68. M. S. Bunyatov, I. A. Gagarinov, S. Delgado, N. G. A. Abrescia, A. Arda and G. J. Boons, Chemoenzymatic Synthesis of Complex N-Glycans of the Parasite *S. mansoni* to Examine the Importance of Epitope Presentation on DC-SIGN recognition, *Angew. Chem., Int. Ed.*, 2021, 60(35), 19287–19291.

69. S. S. Shivatare, S. H. Chang, T. I. Tsai, S. Y. Tseng, V. S. Shivatare, Y. S. Lin, Y. Y. Cheng, C. T. Ren, C. C. Lee, S. Pawar, C. S. Tsai, H. W. Shih, Y. F. Zeng, C. H. Liang, P. D. Kwong, D. R. Burton, C. Y. Wu and C. H. Wong, Modular synthesis of N-glycans and arrays for the heteroligand binding analysis of HIV antibodies, *Nat. Chem.*, 2016, 8(4), 338–346.

70. F. Wei, L. Zang, P. Zhang, J. Zhang and L. Wen, Concise chemoenzymatic synthesis of N-glycans, *Chem*, 2024, DOI: 10.1016/j.chempr.2024.05.006.

71. S. S. Shivatare, S. H. Chang, T. I. Tsai, C. T. Ren, H. Y. Chuang, L. Hsu, C. W. Lin, S. T. Li, C. Y. Wu and C. H. Wong, Efficient convergent synthesis of bi-, tri-, and tetra-antennary complex type N-glycans and their HIV-1 antigenicity, *J. Am. Chem. Soc.*, 2013, 135(41), 15382–15389.

72. S. Pawar, L. Hsu, T. N. Reddy, M. Ravinder, C. T. Ren, Y. W. Lin, Y. Y. Cheng, T. W. Lin, T. L. Hsu, S. K. Wang, C. H. Wong and C. Y. Wu, Synthesis of Asymmetric Glycans as Common Core Substrates for Structural Diversification through Selective Enzymatic Glycosylation, *ACS Chem. Biol.*, 2020, 15(9), 2382–2394.

73. S. J. North, P. G. Hitchen, S. M. Haslam and A. Dell, Mass spectrometry in the analysis of N-linked and O-linked glycans, *Curr. Opin. Struct. Biol.*, 2009, 19(5), 498–506.

74. I. A. Gagarinov, T. Li, S. Torano, T. Caval, A. D. Srivastava, J. A. Kruijtzer, A. J. Heck and G. J. Boons, Chemoenzymatic Approach for the Preparation of Asymmetric Bi-, Tri-, and Tetra-Antennary N-Glycans from a Common Precursor, *J. Am. Chem. Soc.*, 2017, 139(2), 595–602.

75. Z. S. Chinoy, F. Friscourt, C. J. Capicciotti, P. Chiu and G. J. Boons, Chemoenzymatic Synthesis of Asymmetrical Multi-Antennary N-Glycans to Dissect Glycan-Mediated Interactions between Human Sperm and Oocytes, *Chemistry*, 2018, 24(31), 7970–7975.

76. K. Fujikawa, A. Koizumi, M. Hachisu, A. Seko, Y. Takeda and Y. Ito, Construction of a high-mannose-type glycan library by a renewed top-down chemo-enzymatic approach, *Chemistry*, 2015, 21(8), 3224–3233.

77. L. Li, Y. Liu, C. Ma, J. Qu, A. D. Calderon, B. Wu, N. Wei, X. Wang, Y. Guo, Z. Xiao, J. Song, G. Sugiarto, Y. Li, H. Yu, X. Chen and P. G. Wang, Efficient Chemoenzymatic Synthesis of an N-glycan Isomer Library, *Chem. Sci.*, 2015, 6(10), 5652–5661.

78. Z. Wu, Y. Liu, C. Ma, L. Li, J. Bai, L. Byrd-Leotis, Y. Lasanajak, Y. Guo, L. Wen, H. Zhu, J. Song, Y. Li, D. A. Steinhauer, D. F. Smith, B. Zhao, X. Chen, W. Guan and P. G. Wang, Identification of the binding roles of terminal and internal glycan epitopes using enzymatically synthesized N-glycans containing tandem epitopes, *Org. Biomol. Chem.*, 2016, 14(47), 11106–11118.

79. A. D. Calderon, J. Zhou, W. Guan, Z. Wu, Y. Guo, J. Bai, Q. Li, P. G. Wang, J. Fang and L. Li, An enzymatic strategy to asymmetrically branched N-glycans, *Org. Biomol. Chem.*, 2017, 15(35), 7258–7262.

80. A. Seko, M. Koketsu, M. Nishizono, Y. Enoki, H. R. Ibrahim, L. R. Juneja, M. Kim and T. Yamamoto, Occurrence of a sialylglycopeptide and free sialylglycans in hen's egg yolk, *Biochim. Biophys. Acta, Gen. Subj.*, 1997, 1335(1–2), 23–32.

81. B. Sun, W. Bao, X. Tian, M. Li, H. Liu, J. Dong and W. Huang, A simplified procedure for gram-scale production of sialylglycopeptide (SGP) from egg yolks and subsequent semi-synthesis of Man3GlcNAc oxazoline, *Carbohydr. Res.*, 2014, 396, 62–69.

82. L. Liu, A. R. Prudden, G. P. Bosman and G. J. Boons, Improved isolation and characterization procedure of sialylglycopeptide from egg yolk powder, *Carbohydr. Res.*, 2017, 452, 122–128.

83. S. Ma, L. Liu, D. Eggink, S. Herfst, R. A. M. Fouchier, R. P. de Vries and G.-J. Boons, Asymmetrical Biantennary Glycans Prepared by a Stop-and-Go Strategy Reveal Receptor Binding Evolution of Human Influenza A Viruses, *JACS Au*, 2024, 4(2), 607–618.

84. J. D. Valderrama-Rincon, A. C. Fisher, J. H. Merritt, Y. Y. Fan, C. A. Reading, K. Chhiba, C. Heiss, P. Azadi, M. Aebi and M. P. DeLisa, An engineered eukaryotic protein glycosylation pathway in *Escherichia coli*, *Nat. Chem. Biol.*, 2012, 8(5), 434–436.

85. A. S. Ramirez, J. Boilevin, C. W. Lin, B. H. Gan, D. Janser, M. Aebi, T. Darbre, J. L. Reymond and K. P. Locher, Chemo-enzymatic synthesis of lipid-linked GlcNAc2Man5 oligosaccharides using recombinant Alg1, Alg2 and Alg11 proteins, *Glycobiology*, 2017, 27(8), 726–733.

86. M. H. Xiang, X. X. Xu, C. D. Wang, S. Chen, S. Xu, X. Y. Xu, N. Dean, N. Wang and X. D. Gao, Topological human Alg2 mannosyltransferase reveals its role in lipid-linked oligosaccharide biosynthetic pathway, *Commun. Biol.*, 2022, 5(1), 117.

87. N. I. Majewska, M. L. Tejada, M. J. Betenbaugh and N. Agarwal, N-Glycosylation of IgG and IgG-Like Recombinant Therapeutic Proteins: Why Is It Important and How Can We Control It?, *Annu. Rev. Chem. Biomol. Eng.*, 2020, 11, 311–338.

88. S. Malik, I. Grunert, M. F. von Roman, H. Walch, T. Dams, M. Thomann and R. Falkenstein, Implementation of in vitro glycoengineering of monoclonal antibodies into industrial production, downstream processing, *Glycobiology*, 2022, 32(2), 123–135.

89. N. Cohen Saban, A. Yalin, T. Landsberger, R. Salomon, A. Alva, T. Feferman, I. Amit and R. J. S. i. Dahan, Fc glycoengineering of a PD-L1 antibody harnesses Fcγ receptors for increased antitumor efficacy, *Sci. Immunol.*, 2023, 8(81), eadd8005.

90. M. Weiss, D. Ott, T. Karagiannis, M. Weishaupt, M. Niemietz, S. Eller, M. Lott, M. Martinez-Orts, A. Canales, N. Razi, J. C. Paulson and C. Unverzagt, Efficient Chemoenzymatic Synthesis of N-Glycans with a beta1,4-Galactosylated Bisecting GlcNAc Motif, *ChemBioChem*, 2020, 21(22), 3212–3215.

91. W. Ma, Z. Xu, Y. Jiang, J. Liu, D. Xu, W. Huang and T. Li, Divergent Enzymatic Assembly of a Comprehensive 64-Membered IgG N-Glycan Library for Functional Glycomics, *Adv. Sci.*, 2023, 10(30), e2303832.

92. F. Tang, Y. Yang, Y. Tang, S. Tang, L. Yang, B. Sun, B. Jiang, J. Dong, H. Liu, M. Huang, M. Y. Geng and W. Huang, One-pot N-glycosylation remodeling of IgG with non-natural sialylglycopeptides enables glycosite-specific and dual-payload antibody-drug conjugates, *Org. Biomol. Chem.*, 2016, 14(40), 9501–9518.

93. A. J. Fairbanks, The ENGases: versatile biocatalysts for the production of homogeneous N-linked glycopeptides and glycoproteins, *Chem. Soc. Rev.*, 2017, 46(16), 5128–5146.

94. D. J. Gill, H. Clausen and F. Bard, Location, location, location: new insights into O-GalNAc protein glycosylation, *Trends Cell Biol.*, 2011, 21(3), 149–158.

95. S. H. Stalnaker, R. Stuart and L. Wells, Mammalian O-mannosylation: unsolved questions of structure/function, *Curr. Opin. Struct. Biol.*, 2011, 21(5), 603–609.

96. L. Na, R. Li and X. Chen, Recent progress in synthesis of carbohydrates with sugar nucleotide-dependent glycosyltransferases, *Curr. Opin. Chem. Biol.*, 2021, 61, 81–95.

97. Z. F. Hu, K. Zhong and H. Cao, Recent advances in enzymatic and chemoenzymatic synthesis of N- and O-glycans, *Curr. Opin. Chem. Biol.*, 2024, 78, 102417.

98. A. Santra, Y. Li, T. Ghosh, R. Li, H. Yu and X. Chen, One-Pot Multienzyme (OPME) Chemoenzymatic Strategies for Systematic Synthesis of Sialyl Core 2 Glycans, *ACS Catal.*, 2019, 9(1), 211–215.

99. Z. Xu, Y. Deng, Z. Zhang, W. Ma, W. Li, L. Wen and T. Li, Diversity-Oriented Chemoenzymatic Synthesis of Sulfated and Nonsulfated Core 2 O-GalNAc Glycans, *J. Org. Chem.*, 2021, 86(15), 10819–10828.

100. S. Wang, C. Chen, M. R. Gadi, V. Saikam, D. Liu, H. Zhu, R. Bollag, K. Liu, X. Chen, F. Wang, P. G. Wang, P. Ling, W. Guan and L. Li, Chemoenzymatic modular assembly of O-GalNAc glycans for functional glycomics, *Nat. Commun.*, 2021, 12(1), 3573.

101. C. Meng, A. Sasmal, Y. Zhang, T. Gao, C. C. Liu, N. Khan, A. Varki, F. Wang and H. Cao, Chemoenzymatic Assembly of Mammalian O-Mannose Glycans, *Angew. Chem., Int. Ed.*, 2018, 57(29), 9003–9007.

102. T. Gao, J. Y. Yan, C. C. Liu, A. S. Palma, Z. M. Guo, M. Xiao, X. Chen, X. M. Liang, W. G. Chai and H. Z. Cao, Chemoenzymatic Synthesis of O-Mannose Glycans Containing Sulfated or Nonsulfated HNK-1 Epitope, *J. Am. Chem. Soc.*, 2019, 141(49), 19351–19359.

103. M. R. Gadi, C. C. Chen, S. M. Bao, S. S. Wang, Y. X. Guo, J. H. Han, W. D. Xiao and L. Li, Convergent chemoenzymatic synthesis of GalNAc rare cores 5, 7, 8 and their sialylated forms, *Chem. Sci.*, 2023, 14(7), 1837–1843.

104. S. Wang, Q. Zhang, C. Chen, Y. Guo, M. R. Gadi, J. Yu, U. Westerlind, Y. Liu, X. Cao, P. G. Wang and L. Li, Facile Chemoenzymatic Synthesis of O-Mannosyl Glycans, *Angew. Chem., Int. Ed.*, 2018, 57(30), 9268–9273.

105. M. O. Sheikh, S. M. Halmo and L. Wells, Recent advancements in understanding mammalian O-mannosylation, *Glycobiology*, 2017, 27(9), 806–819.

106. M. Van Scherpenzeel, E. Willems and D. J. Lefeber, Clinical diagnostics and therapy monitoring in the congenital disorders of glycosylation, *Glycoconjugate J.*, 2016, 33(3), 345–358.

107. T. Li, Y. Zhang, T. Li, H. Zhuang, F. Wang, N. Wang, R. R. Schmidt and P. Peng, Divergent Synthesis of Core m1, Core m2 and Core m3 O–Mannosyl Glycopeptides via a Chemoenzymatic Approach, *Chin. J. Chem.*, 2022, 40(13), 1329–1338.

108. D. Laaf, H. Steffens, H. Pelantová, P. Bojarová, V. Křen and L. Elling, Chemo–Enzymatic Synthesis of Branched N–Acetyllactosamine Glycan Oligomers for Galectin–3 Inhibition, *Adv. Synth. Catal.*, 2017, 359(22), 4015–4024.

109. J. F. Ye, H. Xia, N. Sun, C. C. Liu, A. R. Sheng, L. L. Chi, X. W. Liu, G. F. Gu, S. Q. Wang, J. Zhao, P. Wang, M. Xiao, F. S. Wang and H. Z. Cao, Reprogramming the enzymatic assembly line for site-specific fucosylation, *Nat. Catal.*, 2019, 2(6), 514–522.

110. N. Lu, J. Ye, J. Cheng, A. Sasmal, C. C. Liu, W. Yao, J. Yan, N. Khan, W. Yi, A. Varki and H. Cao, Redox-Controlled Site-Specific alpha2–6-Sialylation, *J. Am. Chem. Soc.*, 2019, 141(11), 4547–4552.

111. N. Lu, Y. Li, H. Xia, K. Zhong, C. Jia, J. Ye, X. Liu, C. C. Liu and H. Cao, A Redox–Controlled Substrate Engineering Strategy for Site–Specific Enzymatic Fucosylation, *Angew. Chem.*, 2022, 134(50), e202211032.

112. I. A. Gagarinov, T. Li, N. Wei, J. S. Torano, R. P. de Vries, M. A. Wolfert and G. J. Boons, Protecting-Group-Controlled Enzymatic Glycosylation of N-Acetyllactosamine Derivatives, *Angew. Chem., Int. Ed.*, 2019, 58(31), 10547–10552.

113. G. M. Vos, Y. Wu, R. van der Woude, R. P. de Vries and G. J. Boons, Chemo-Enzymatic Synthesis of I-branched Polylactosamines Using Traceless Blocking Groups, *Chemistry*, 2024, 30(5), e202302877.

114. H. K. Tseng, H. K. Wang, C. Y. Wu, C. K. Ni and C. C. Lin, Exploring Regioselective Fucosylation Catalyzed by Bacterial Glycosyltransferases through Substrate Promiscuity and Acceptor-Mediated Glycosylation, *ACS Catal.*, 2023, 13(16), 10661–10671.

115. Z. Xiao, Y. Guo, Y. Liu, L. Li, Q. Zhang, L. Wen, X. Wang, S. M. Kondengaden, Z. Wu, J. Zhou, X. Cao, X. Li, C. Ma and P. G. Wang, Chemoenzymatic Synthesis of a Library of Human Milk Oligosaccharides, *J. Org. Chem.*, 2016, 81(14), 5851–5865.

116. Y. T. Huang, Y. C. Su, H. R. Wu, H. H. Huang, E. C. Lin, T. W. Tsai, H. W. Tseng, J. L. Fang and C. C. Yu, Sulfo-Fluorous Tagging Strategy for Site-Selective Enzymatic Glycosylation of Human Milk Oligosaccharides, *ACS Catal.*, 2021, 11(5), 2631–2643.

117. A. R. Prudden, L. Liu, C. J. Capicciotti, M. A. Wolfert, S. Wang, Z. Gao, L. Meng, K. W. Moremen and G. J. Boons, Synthesis of asymmetrical multiantennary human milk oligosaccharides, *Proc. Natl. Acad. Sci. U. S. A.*, 2017, 114(27), 6954–6959.

118. M. R. Charbonneau, L. V. Blanton, D. B. DiGiulio, D. A. Relman, C. B. Lebrilla, D. A. Mills and J. I. Gordon, A microbial perspective of human developmental biology, *Nature*, 2016, 535(7610), 48–55.

119. R. E. Moore, L. Y. L. Xu and S. D. Townsend, Prospecting Human Milk Oligosaccharides as a Defense Against Viral Infections, *ACS Infect. Dis.*, 2021, 7(2), 254–263.

120. C. Kunz and S. Rudloff, Potential anti-inflammatory and anti-infectious effects of human milk oligosaccharides, *Adv. Exp. Med. Biol.*, 2008, 606, 455–465.

121. S. Etzold and L. Bode, Glycan-dependent viral infection in infants and the role of human milk oligosaccharides, *Curr. Opin. Virol.*, 2014, 7,

101–107.

122. M. Lu, I. Mosleh and A. Abbaspourrad, Engineered Microbial Routes for Human Milk Oligosaccharides Synthesis, *ACS Synth. Biol.*, 2021, 10(5), 923–938.

123. X. Chen, Human Milk Oligosaccharides (HMOS): Structure, Function, and Enzyme-Catalyzed Synthesis, *Adv. Carbohydr. Chem. Biochem.*, 2015, 72, 113–190.

124. J. Zheng, H. Xu, J. Fang and X. Zhang, Enzymatic and chemoenzymatic synthesis of human milk oligosaccharides and derivatives, *Carbohydr. Polym.*, 2022, 291, 119564.

125. W. Yao, J. Yan, X. Chen, F. Wang and H. Cao, Chemoenzymatic synthesis of lacto-N-tetrasaccharide and sialyl lacto-N-tetrasaccharides, *Carbohydr. Res.*, 2015, 401, 1–7.

126. J. B. McArthur, H. Yu and X. Chen, A Bacterial beta1-3-Galactosyltransferase Enables Multigram-Scale Synthesis of Human Milk Lacto-N-tetraose (LNT) and Its Fucosides, *ACS Catal.*, 2019, 9(12), 10721–10726.

127. T.-W. Tsai, J.-L. Fang, C.-Y. Liang, C.-J. Wang, Y.-T. Huang, Y.-J. Wang, J.-Y. Li and C.-C. Yu, Exploring the Synthetic Application of Helicobacter pylori $ $1,3/4-Fucosyltransferase FucTIII toward the Syntheses of Fucosylated Human Milk Glycans and Lewis Antigens, *ACS Catal.*, 2019, 9(12), 10712–10720.

128. Y. Li, Y. Li, Y. Guo, C. Chen, L. Yang, Q. Jiang, P. Ling, S. Wang, L. Li and J. Fang, Enzymatic modular synthesis of asymmetrically branched human milk oligosaccharides, *Carbohydr. Polym.*, 2024, 333, 121908.

129. U. Lindahl and M. Hook, Glycosaminoglycans and Their Binding to Biological Macromolecules, *Annu. Rev. Biochem.*, 1978, 47(1), 385–417.

130. N. S. Gandhi and R. L. Mancera, The Structure of Glycosaminoglycans and their Interactions with Proteins, *Chem. Biol. Drug Des.*, 2008, 72(6), 455–482.

131. X. Zhang, L. Lin, H. Huang and R. J. Linhardt, Chemoenzymatic Synthesis of Glycosaminoglycans, *Acc. Chem. Res.*, 2020, 53(2), 335–346.

132. J. Gottschalk and L. Elling, Current state on the enzymatic synthesis of glycosaminoglycans, *Curr. Opin. Chem. Biol.*, 2021, 61, 71–80.

133. G. Kogan, L. Soltes, R. Stern and P. Gemeiner, Hyaluronic acid: a natural biopolymer with a broad range of biomedical and industrial applications, *Biotechnol. Lett.*, 2007, 29(1), 17–25.

134. L. A. Linkins, A. L. Dans, L. K. Moores, R. Bona, B. L. Davidson, S. Schulman and M. Crowther, Treatment and prevention of heparin-induced

thrombocytopenia: Antithrombotic Therapy and Prevention of Thrombosis, 9th ed: American College of Chest Physicians Evidence-Based Clinical Practice Guidelines, *Chest*, 2012, 141(2 Suppl), e495S–e530S.

135. L. Liu, P. Chopra, X. Li, K. M. Bouwman, S. M. Tompkins, M. A. Wolfert, R. P. de Vries and G. J. Boons, Heparan Sulfate Proteoglycans as Attachment Factor for SARS-CoV-2, *ACS Cent. Sci.*, 2021, 7(6), 1009–1018.

136. P. H. Weigel and P. L. DeAngelis, Hyaluronan synthases: a decade-plus of novel glycosyltransferases, *J. Biol. Chem.*, 2007, 282(51), 36777–36781.

137. J. Mandawe, B. Infanzon, A. Eisele, H. Zaun, J. Kuballa, M. D. Davari, F. Jakob, L. Elling and U. Schwaneberg, Directed Evolution of Hyaluronic Acid Synthase from *Pasteurella multocida* towards High-Molecular-Weight Hyaluronic Acid, *ChemBioChem*, 2018, 19(13), 1414–1423.

138. W. Jing and P. L. DeAngelis, Synchronized chemoenzymatic synthesis of monodisperse hyaluronan polymers, *J. Biol. Chem.*, 2004, 279(40), 42345–42349.

139. X. Fu, W. J. Shang, S. S. Wang, Y. P. Liu, J. Y. Qu, X. Chen, P. G. Wang and J. Q. Fang, A general strategy for the synthesis of homogeneous hyaluronan conjugates and their biological applications, *Chem. Commun.*, 2017, 53(25), 3495–3498.

140. S. Li, S. Wang, X. Fu, X. W. Liu, P. G. Wang and J. Fang, Sequential one-pot multienzyme synthesis of hyaluronan and its derivative, *Carbohydr. Polym.*, 2017, 178, 221–227.

141. A. Eisele, H. Zaun, J. Kuballa and L. Elling, In Vitro One–Pot Enzymatic Synthesis of Hyaluronic Acid from Sucrose and N–Acetylglucosamine: Optimization of the Enzyme Module System and Nucleotide Sugar Regeneration, *ChemCatChem*, 2018, 10(14), 2969–2981.

142. A. E. Sismey-Ragatz, D. E. Green, N. J. Otto, M. Rejzek, R. A. Field and P. L. DeAngelis, Chemoenzymatic synthesis with distinct *Pasteurella* heparosan synthases: monodisperse polymers and unnatural structures, *J. Biol. Chem.*, 2007, 282(39), 28321–28327.

143. N. Hodson, G. Griffiths, N. Cook, M. Pourhossein, E. Gottfridson, T. Lind, K. Lidholt and I. S. Roberts, Identification That KfiA, a Protein Essential for the Biosynthesis of the *Escherichia coli* K5 Capsular Polysaccharide, Is an α-UDP-GlcNAc Glycosyltransferase, *J. Biol. Chem.*, 2000, 275(35), 27311–27315.

144. X. Zhang, V. Pagadala, H. M. Jester, A. M. Lim, T. Q. Pham, A. M. P. Goulas, J. Liu and R. J. Linhardt, Chemoenzymatic synthesis of heparan sulfate and heparin oligosaccharides and NMR analysis: paving the way to a diverse library for glycobiologists, *Chem. Sci.*, 2017, 8(12), 7932–7940.

145. Y. Yu, L. Fu, P. He, K. Xia, S. Varghese, H. Wang, F. Zhang, J. Dordick and R. J. Linhardt, Chemobiocatalytic Synthesis of a Low-Molecular-Weight Heparin, *ACS Chem. Biol.*, 2022, 17(3), 637–646.

146. G. Zhang, K. Yang, L. Wang, Y. Cheng and C. Liu, Facile chemoenzymatic synthesis of unmodified anticoagulant ultra-low molecular weight heparin, *Org. Biomol. Chem.*, 2022, 20(42), 8323–8330.

147. S. Zhao, T. Zhang, Y. Kan, H. Li and J. P. Li, Overview of the current procedures in synthesis of heparin saccharides, *Carbohydr. Polym.*, 2024, 339, 122220.

148. W. Lu, C. Zong, P. Chopra, L. E. Pepi, Y. Xu, I. J. Amster, J. Liu and G. J. Boons, Controlled Chemoenzymatic Synthesis of Heparan Sulfate Oligosaccharides, *Angew. Chem., Int. Ed.*, 2018, 57(19), 5340–5344.

149. L. F. Sun, P. Chopra and G. J. Boons, Chemoenzymatic Synthesis of Heparan Sulfate Oligosaccharides having a Domain Structure, *Angew. Chem., Int. Ed.*, 2022, 61(47), e202211112.

150. P.-H. Lin, Y. Xu, S. K. Bali, J. Kim, A. Gimeno, E. T. Roberts, D. James, N. M. S. Almeida, N. Loganathan, F. Fan, A. K. Wilson, I. J. Amster, K. W. Moremen, J. Liu, J. Jiménez-Barbero and X. Huang, Solid–Phase–Supported Chemoenzymatic Synthesis and Analysis of Chondroitin Sulfate Proteoglycan Glycopeptides, *Angew. Chem.*, 2024, e202405671.

151. Y. Wu, G. P. Bosman, D. Chapla, C. Huang, K. W. Moremen, R. P. de Vries and G.-J. Boons, A Biomimetic Synthetic Strategy Can Provide Keratan Sulfate II Oligosaccharides with Diverse Fucosylation and Sulfation Patterns, *J. Am. Chem. Soc.*, 2024, 146(13), 9230–9240.

152. S. G. Chen, C. H. Xue, L. A. Yin, Q. J. Tang, G. L. Yu and W. G. Chai, Comparison of structures and anticoagulant activities of different fucosylated chondroitin sulfates from sea cucumbers, *Carbohydr. Polym.*, 2011, 83(2), 688–696.

153. E. Tykesson, M. Maccarana, H. Thorsson, J. Liu, A. Malmström, U. Ellervik and G. Westergren-Thorsson, Recombinant dermatan sulfate is a potent activator of heparin cofactor II-dependent inhibition of thrombin, *Glycobiology*, 2019, 29(6), 446–451.

154. J. Li, G. Su and J. Liu, Enzymatic Synthesis of Homogeneous Chondroitin Sulfate Oligosaccharides, *Angew. Chem., Int. Ed.*, 2017, 56(39), 11784–11787.

155. E. Stancanelli, W. Liu, R. Wander, J. Li, Z. Wang, K. Arnold, G. Su, A. Kanack, T. Q. Pham, V. Pagadala, A. Padmanabhan, Y. Xu and J. Liu, Chemoenzymatic Synthesis of Homogeneous Heparan Sulfate Chondroitin Sulfate Chimeras, *ACS Chem. Biol.*, 2022, 17(5), 1207–1214.

156. E. Tykesson, A. Hassinen, K. Zielinska, M. A. Thelin, G. Westergren-Thorsson, A. Malmström, S. Kellokumpu and M. Maccarana, Dermatan sulfate epimerase 1 and dermatan 4–sulfotransferase 1 form complexes that generate long epimerized 4–sulfated blocks, *J. Biol. Chem.*, 2018, 293(35), 13725–13734.

157. Y. Yamazaki, S. Kimura and M. Ohmae, Reaction specificity of keratanase II in the transglycosylation using the sugar oxazolines having keratan sulfate repeating units, *Carbohydr. Res.*, 2018, 456, 61–68.

158. Y. Wu, G. M. Vos, C. Huang, D. Chapla, A. L. M. Kimpel, K. W. Moremen, R. P. de Vries and G.-J. Boons, Exploiting Substrate Specificities of Keratan Sulfate 6-O-Sulfotransferases to Enzymatically Synthesize Oligosaccharides, *JACS Au*, 2023, 3(11), 3155–3164.

159. R. C. R. Jala, S. Vudhgiri and C. G. Kumar, A comprehensive review on natural occurrence, synthesis and biological activities of glycolipids, *Carbohydr. Res.*, 2022, 516, 108547.

160. H. Ando and N. Komura, Recent progress in the synthesis of glycosphingolipids, *Curr. Opin. Chem. Biol.*, 2024, 78, 102418.

161. J. Zhang, C. Chen, M. R. Gadi, C. Gibbons, Y. Guo, X. Cao, G. Edmunds, S. Wang, D. Liu, J. Yu, L. Wen and P. G. Wang, Machine–Driven Enzymatic Oligosaccharide Synthesis by Using a Peptide Synthesizer, *Angew. Chem.*, 2018, 130(51), 16880–16884.

162. T. Li, L. Liu, N. Wei, J. Y. Yang, D. G. Chapla, K. W. Moremen and G. J. Boons, An automated platform for the enzyme-mediated assembly of complex oligosaccharides, *Nat. Chem.*, 2019, 11(3), 229–236.

163. R. Basharat, V. Kotra, L. Y. Loong, A. Mathews, M. M. Kanakal, C. H. B. P. Dev, S. Nyamathulla, R. Varala, L. C. Ming, K. R. S. S. Rao, B. H. Babu and M. M. Alam, A Mini-review on Ultra Performance Liquid Chromatography, *Orient. J. Chem.*, 2021, 37(4), 847–857.

164. J. Q. Wang, J. Zhao, S. P. Nie, M. Y. Xie and S. P. Li, Mass spectrometry for structural elucidation and sequencing of carbohydrates, *TrAC, Trends Anal. Chem.*, 2021, 144, 116426.

165. B. Xia, J. Fang, S. Ma, M. Ma, G. Yao, T. Li, X. Cheng, L. Wen and Z. Gao, Mapping the Acetylamino and Carboxyl Groups on Glycans by Engineered α-Hemolysin Nanopores, *J. Am. Chem. Soc.*, 2023, 145(34), 18623–18630.

166. G. Yao, Y. Tian, W. Ke, J. Fang, S. Ma, T. Li, X. Cheng, B. Xia, L. Wen and Z. Gao, Direct Identification of Complex Glycans via a Highly Sensitive Engineered Nanopore, *J. Am. Chem. Soc.*, 2024, 146(19), 13356–13366.

167. G. Yao, W. Ke, B. Xia and Z. Gao, Nanopore-based glycan sequencing: state of the art and future prospects, *Chem. Sci.*, 2024, 15(17), 6229–6243.

168. J. L. Abrahams, G. Taherzadeh, G. Jarvas, A. Guttman, Y. Zhou and M. P. Campbell, Recent advances in glycoinformatic platforms for glycomics and glycoproteomics, *Curr. Opin. Struct. Biol.*, 2020, 62, 56–69.

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