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REVIEW ARTICLE

Synthesis of selected unnatural sugar nucleotides for biotechnological applications

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ABSTRACT

Sugar nucleotides are the principal building blocks for the synthesis of most complex carbohydrates and are crucial intermediates in carbohydrate metabolism. Uridine diphosphate (UDP) monosaccharides are among the most common sugar nucleotide donors and are transferred to glycosyl acceptors by glycosyltransferases or synthases in glycan biosynthetic pathways. These natural nucleotide donors have great biological importance, however, the synthesis and application of unnatural sugar nucleotides that are not available from *in vivo* biosynthesis are not well explored. In this review, we summarize the progress in the preparation of unnatural sugar nucleotides, in particular, the widely studied UDP-GlcNAc/GalNAc analogs. We focus on the "two-block" synthetic pathway that is initiated from monosaccharides, in which the first block is the synthesis of sugar-1-phosphate and the second block is the diphosphate bond formation. The biotechnological applications of these unnatural sugar nucleotides showing their physiological and pharmacological potential are discussed.

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Introduction

Despite the structural complexity of carbohydrates and glycoconjugates in nature, there are surprisingly only nine common building blocks for human glycoproteins and glycolipids: glucose (Glc), galactose (Gal), *N*-acetylglucosamine (GlcNAc), *N*-acetylgalactosamine (GalNAc), glucuronic acid (GlcA), xylose (Xyl), mannose (Man), fucose (Fuc) and *N*-acetylneuraminic acid (Neu5Ac; Figure 1) [1]. These building blocks must be activated into sugar nucleotide donors, which are the primary building blocks to prepare complex carbohydrates to participate in the construction of different sugar chains [2,3]. Sugar nucleotides can be structurally divided into two categories, nucleoside diphosphate (NDP) sugars, and nucleoside monophosphate (NMP) sugars, and NDP-sugars are the most naturally occurring [4]. Uridine diphosphate (UDP) monosaccharides are the most common NDP-sugar donors and are transferred to glycosyl acceptors by glycosyltransferases or synthases in glycan biosynthesis. Both natural and unnatural UDP-sugars can be chemically, enzymatically, or chemoenzymatically synthesized [4–8]. Although the synthesis of natural UDP-sugars is well established in the literature, the synthesis and application of unnatural UDP-sugars that are not available from *in vivo* biosynthesis, are not well explored. Such unnatural donors have great potential as enzymatic substrates in carbohydrate synthesis, as enzyme inhibitors in biochemical studies, as tools for assay development, and as reagents for the study of glycoconjugate biosynthesis [4,9]. Therefore, developing efficient methods to obtain unnatural sugar nucleotides has attracted increasing attention during the past decade. Wagner and coworkers described the progress of chemical synthesis of NDP-sugars, NMP-sugars, and their derivatives from 1999 to 2009 [4]. Thorson and coworkers summarized in detail the enzymic synthesis pathway for sugar nucleotides as well as their substrates and enzyme-catalyzed conversions, especially those involving anomeric kinases and pyrophosphorylases, in an article about glycosylation published in 2011 [10]. In this review, we summarize the progress in

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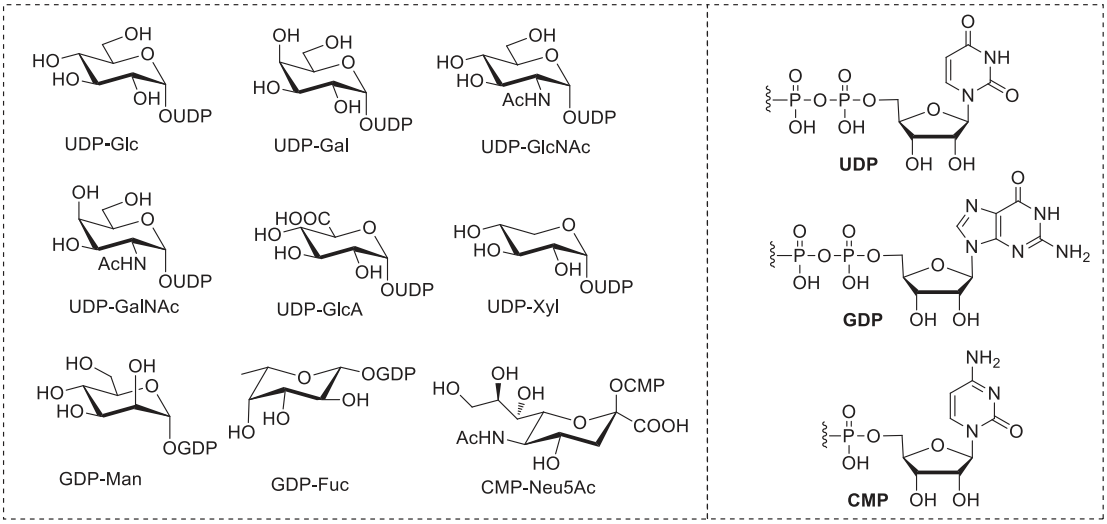

Figure 1. Nine common sugar nucleotides involved in the biosynthesis of human glycoproteins and glycolipids. UDP, uridine diphosphate; GDP, guanosine diphosphate; CMP, cytidine monophosphate.

the chemical, enzymatic and chemoenzymatic preparation of unnatural sugar nucleotides, in particular, the widely studied UDP-GlcNAc/GalNAc analogs, and their biotechnological applications that show physiological and pharmacological potential. Since it is very difficult to derivatize natural sugar nucleotides to obtain unnatural sugar nucleotides [4], a "two-block" synthetic strategy that is initiated from monosaccharides was widely utilized. The first block is the synthesis of sugar-1-phosphate (sugar-1-P) where structure derivatization can be manipulated at this stage, and the second block is the diphosphate bond formation. This review fills the gap of new applications in sugar nucleotides reviewed over the past decade, with the synthesis of size-controlled polysaccharides, stable isotope labeling technology, enzymatic and metabolic bioorthogonal reporter strategies, and the in-depth structure-activity relationship (SAR) study of sugar nucleotides.

Synthesis of sugar nucleotides

Most of these common sugar nucleotides share a similar biosynthetic pathway that is catalyzed by a kinase and a nucleotidyltransferase, using glucose-6-phosphate and fructose-6-phosphate as key intermediates. GlcNAc and GalNAc are fundamental components of many important polysaccharides and glycoconjugates, such as glycosaminoglycans (GAGs), which are linear polysaccharides interacting with many biologically important proteins and dominating the chemical and biological properties of proteoglycans [11,12]. Thus, the corresponding nucleotide donors, UDP-GlcNAc and UDP-GalNAc, involved in GAG synthesis have been widely studied. These donors are obtained from enzymatic pyrophosphorylation of GlcNAc-1-phosphate (GlcNAc-1-P), which is biosynthesized from either glucose (*de novo* pathway) or GlcNAc/GalNAc (salvage pathways) through several enzymatic steps (Figure 2) [13]. The biosynthesis of UDP-GlcNAc/GalNAc can be simplified as proceeding through (1) sugar to sugar-6-P by a 6-kinase; (2) sugar-6-P is converted to sugar-1-P by a mutase; and (3) then sugar-1-P is pyrophosphorylated with uridine triphosphate (UTP) to give UDP-sugar (Figure 2) [7].

Inspired by their biosynthetic pathways, the key step in the synthesis of sugar nucleotides is the introduction of the pyrophosphate function between the 5′-OH position of a nucleoside and the anomeric hydroxyl of a saccharide unit (Figure 3) [4]. A variety of procedures have been reported that can be generally classified into two main categories (Figure 3). Path A comprises the direct glycosylation of a protected glycosyl donor such as an anomeric halide with nucleoside 5′-diphosphate (Figure 3, path A). This approach is often hampered by the limited availability of suitable sugar donors and with some exceptions a low anomeric diastereoselectivity (the α-linkage is often desired). In Path B, the pyrophosphate moiety can be introduced by coupling a monosaccharide-1-phosphate with an activated nucleoside 5′-monophosphate (Figure 3, path B), such as nucleoside phosphoromorpholidates, an extensively explored method developed by Khorana and Moffat [14,15]. The anomeric configuration of the final product can be controlled by the stereo-selective synthesis of

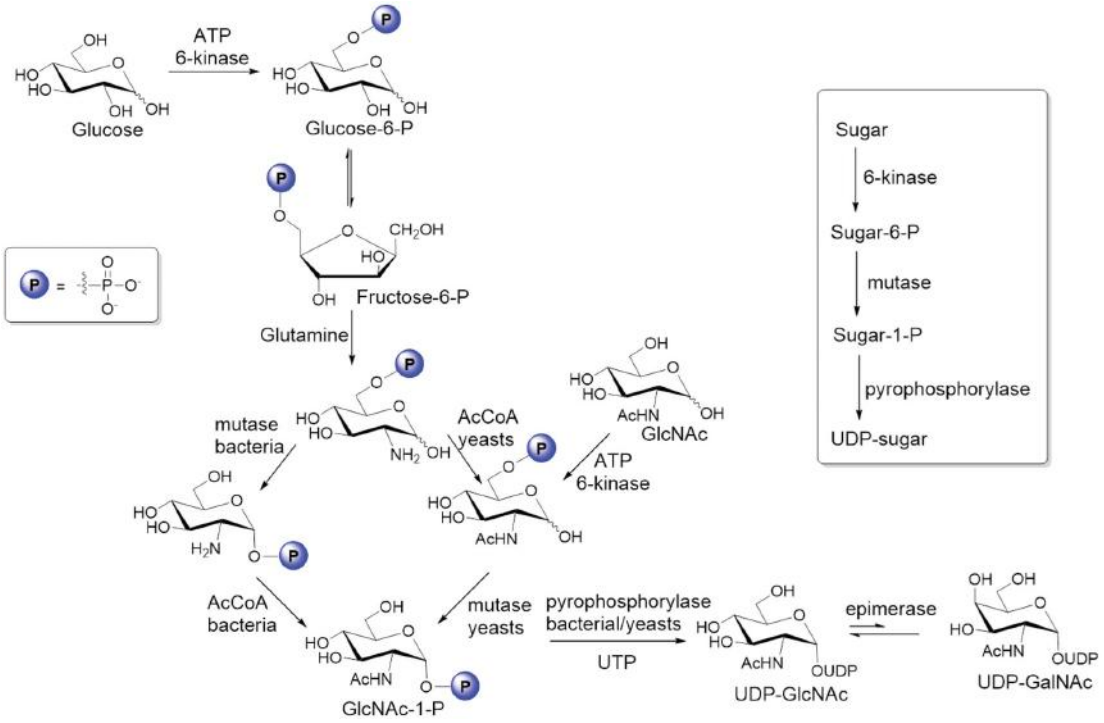

Figure 2. Pathways for the biosynthesis of UDP-GlcNAc/GalNAc. The general biosynthetic scheme is sugar→sugar-6-phosphate→sugar-1-phosphate→UDP-sugar. ATP, adenosine triphosphate; UTP, uridine triphosphate; AcCoA, acetyl-coenzyme A.

the sugar-1-phosphate. Since the second strategy is commonly favored, in this mini-review we discuss recent advances of both chemical and chemoenzymatic approaches toward UDP-sugars synthesis by introducing the synthesis of sugar-1-P first followed by the discussion of diphosphate bond formation.

Synthesis of sugar-1-P

α-Linked sugar-1-P

Sugar-1-phosphates with either anomeric α- or β-configuration can be obtained from the corresponding monosaccharide precursor through different synthetic manipulations. For making UDP-sugars, the most prominent of these nine sugar nucleotides, the relatively more prevalent α-configuration can be controlled through the stereo-selective synthesis of the sugar-1-P prior to coupling with an activated nucleoside 5′-monophosphate. Approaches to access α-linked sugar-1-P have been extensively investigated.

Macdonald reaction. The MacDonald reaction of a sugar bearing an anomeric acetyl group with phosphoric acid under vacuum is a classic method and is still widely used in the phosphorylation of sugars to afford α-linked configured sugar-1-phosphate [16]. Based on this approach, Linhardt and coworkers (Figure 4) prepared a small library of unnatural GlcNAc-1-P and GalNAc-1-P derivatives in 30% to 50% yields [17]. This study also demonstrated that azido and alkyne motifs were generally stable under such acidic conditions, thus, carrying additional moieties associated with click chemistry that are potentially applicable in conjugation chemistry. On account of the stability of trifluoroacetamide groups under such conditions, MacDonald reaction can be applied in the preparation of UDP-*N*-trifluoroacetylglucosamine (GlcNTFA), an important unnatural glycosyl donor currently used in the chemoenzymatic synthesis of heparan sulfate and heparin [18]. This method can also be applied into the synthesis of other sugar nucleotides, such as α-D-arabinofuranose-1-phosphate (Ara-1-P) [19] and 3-deoxy-α-D-arabinohexose-1-phosphate [20], and 2-deoxy-α-D-glucose-1-phosphate [20]. However, the typically harsh conditions associated with the MacDonald reaction have resulted in relatively low yields and restrict its wide application especially in synthesis of the chemically and thermally less stable sugars that are exclusively found in lower organisms [4].

Phosphorylation with hemiacetals. Condensation between sugar hemiacetal and various phosphorylation

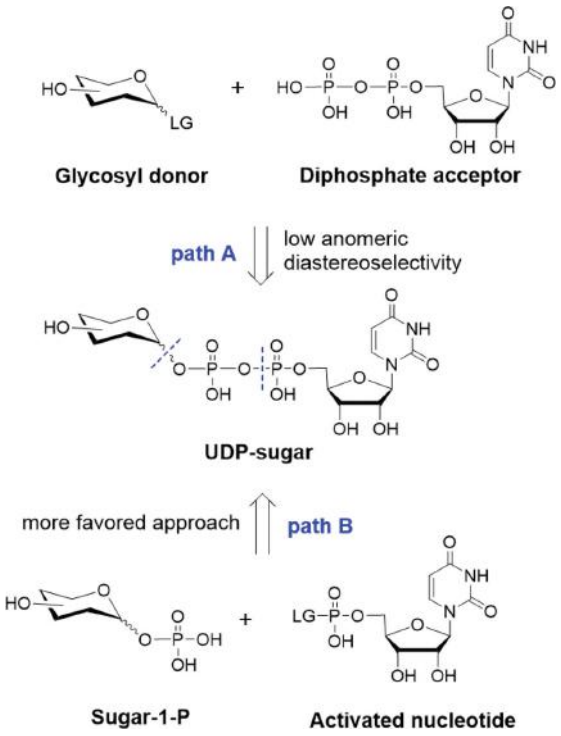

Figure 3. General strategies for UDP-sugar synthesis are shown where LG is leaving group. Path A shows the direct glycosylation of glycosyl donor with diphosphate acceptor, and Path B shows the coupling between sugar-1-phosphate and the activated nucleoside 5′-monophosphate.

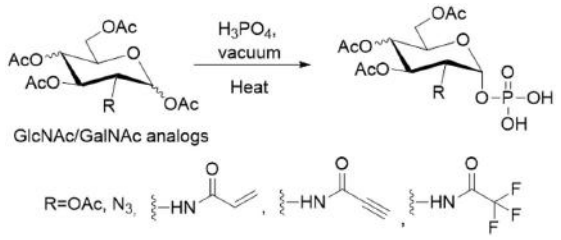

Figure 4. Synthesis of GlcNAc/GalNAc-1-phosphate analogs is shown using the MacDonald reaction, where acetyl, azido and amide groups are tolerated.

reagents represents another method for preparing α-linked anomeric phosphates. Subsequent hydrogenation is often employed to remove the protecting group from phosphate to typically afford the phosphate monoester product in quantitative yield. Sabesan and Neira developed a simple coupling method involving sugar hemiacetal and diphenylphosphoryl chloride coupling catalyzed by DMAP (4-dimethylaminopyridine) [21]. Williams and coworkers prepared ^{13}C-labeled peracetylated glucose-1-phosphate in 83% yield using the same method (Figure 5, panel I) [22]. Linhardt and coworkers reported the phosphorylation with tetrabenzyl pyrophosphate in the presence of lithium diisopropylamide (LDA), affording the phosphorylated product having only the α-configuration (Figure 5, panel II) [23,24]. The fluoride, azido and benzoate groups tolerated these conditions well. Nishimura and coworkers (Figure 5, panel III) reported a two-step phosphorylation reaction from hemiacetals treated with dibenzyl diethylphosphoramidite (DDP) in the presence of triazole, yielded the corresponding dibenzyl phosphites as anomeric mixture (α:β = 1:1) [25]. The two isomers could then be treated with *tert*-butylhydroperoxide (TBHP) in tetrahydrofuran to be rapidly isomerized into the desired α-1-phosphate. It is noteworthy that this oxidation was unsuccessful when performed with hydrogen peroxide because of the labile O–P bond. Similarly, Kosma and coworkers successfully prepared GDP-D-glycero-α-D-manno-heptopyranose using bis(benzyloxy)-*N*, *N*-diisopropylamino-phosphine/1H-tetrazole followed by TBHP-mediated oxidation to obtain the key intermediate α-1-phosphate [26]. This method is relatively versatile and can be applied to the synthesis of a series of sugar-1-phosphates, such as D-mannuronic acid (ManA)-1-P [27], *N*-acetylmuramic acid (MurNAc)-l-P [28], 2-deoxy-α-D-glucose-1-P, 3-deoxy-α-D-arabino-hexose-1-P, α-D-lyxose-1-P and 4-deoxy-α-D-lyxo-hexose-1-P [20].

Enzymatic approach. The chemical synthesis of sugar nucleotides is complicated due to the poor solubility of sugar nucleotides in organic solvents and the instability of glycosidic and pyrophosphate bonds to hydrolytic cleavage. This usually needs multi-step synthetic manipulations and purifications that result in a tedious processes and low overall yields. Thus, an enzymatic route may represent a promising alternative [7].

NahK, a *N*-acetylhexosamine kinase, is the first wild type gluco-type 1-kinase reported and can be used to quickly and efficiently catalyze the synthesis of GlcNAc-1-P from a GlcNAc acceptor and a adenosine 5′-triphosphate (ATP) phosphate donor [29]. Wang and coworkers successfully utilized NahK to transform the unnatural GlcNAc/GalNAc analogues to their 1-phosphorylated products, regardless of the stereo-configuration at the C-4 position of the sugar ring (Figure 6) [30,31]. More significantly, the relaxed tolerance to the *N*-acyl chain modifications can facilitate the incorporation bio-orthogonal functionalities that carry additional tags or labels into carbohydrate containing biomolecules. In addition, NahK can be combined with the other two enzymes, PmGlmU and PmPpA in a one-pot synthesis of UDP-sugar derivatives [32].

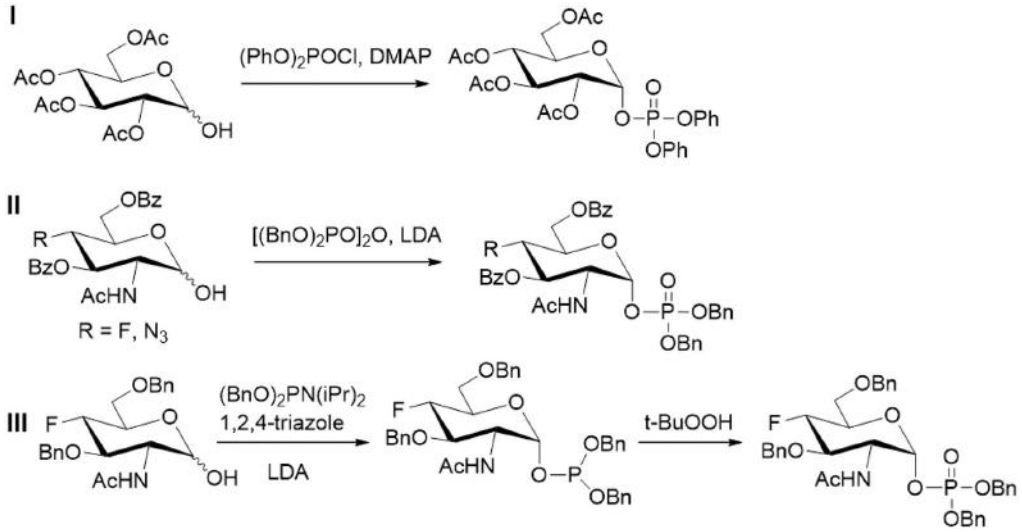

Figure 5. Condensation between sugar hemiacetal and phosphorylation reagents is shown in the synthesis of a α-linked anomeric phosphate. Varieties of phosphate donors such as diphenylphosphoryl chloride (panel I), tetrabenzyl pyrophosphate (panel II) and dibenzyl diethylphosphoramidite (panel III) can be used.

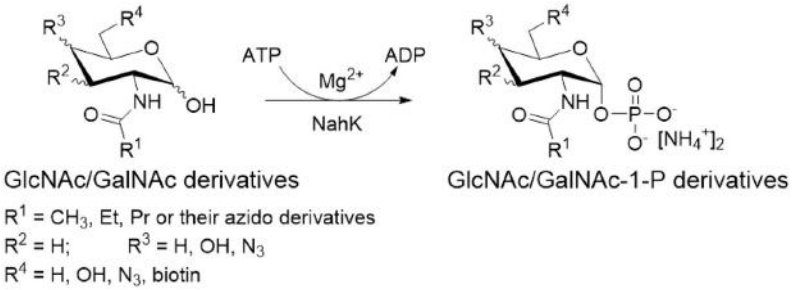

Figure 6. Enzymatic synthesis of GlcNAc/GalNAc-1-P derivatives catalyzed by NahK is shown where a number of suitable structural modifications of the monosaccharide substrates are accepted. Et, ethyl; Pr, propyl; ADP, adenosine diphosphate.

β-*Linked sugar-1-P*

Hexopyranose phosphates in the β-configuration are not stabilized by the anomeric effect and are more challenging synthetic targets than the corresponding α-anomers. Furthermore, these β-anomeric phosphates are usually not accessible using the methods previously described and there are few reports of the synthesis of β-linked sugar-1-P. Kahne and coworkers prepared β-configured 2-deoxy sugar phosphates from α-configured donor glycosyl halides and tetrabutylammonium salts through an S_N2 displacement reaction (Figure 7, panel I) [33]. These researchers suggest that more stable anomeric leaving groups are required to shift the substitution of glycosyl halides from the S_N1 to the S_N2 pathway. In addition, neighboring group participation is helpful in the stereocontrol at the phosphorylation step. Timmons and Jakeman have successfully prepared α-L-arabinose-1-phosphate involving the coupling of acetyl- or benzoyl-protected glycosyl bromides with dibenzylphosphate (Figure 7, panel II) [34]. Prihar and coworkers used *o*-phenylene phosphorochloridate to phosphorylate the hemiacetal hydroxyl group of mannopyranose, and obtained β-D-mannopyranosyl-1-P [35]. Moreover, the same group also used the MacDonald reaction to obtain a 12:5 anomeric mixture of β- and α-L-fucofuranosyl-1-phosphate [36]. In addition, enzymatic pathways can also achieve the synthesis of the β-configured sugar-1-P. For example, Stiller and coworkers accomplished the synthesis of β-L-fucose-1-P using fucose kinase [37].

Diphosphate bond formation

Chemical approach

Improvements for the conversion of sugar-1-P to sugar nucleotide, such as UDP-sugar, in terms of reaction time and yield, have been reported by utilizing a catalyst or through the use of a variety of active nucleoside

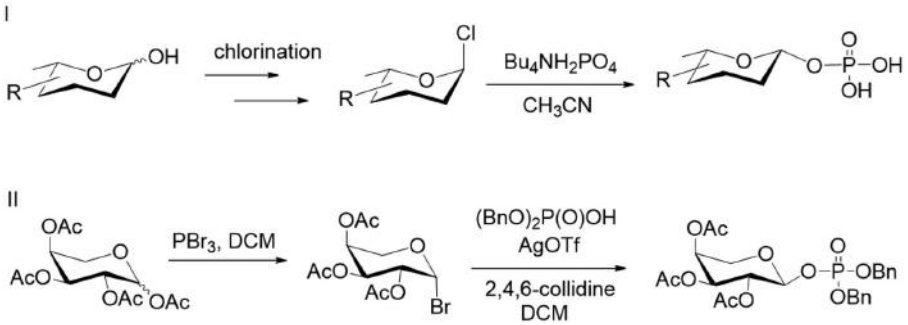

Figure 7. Chemical synthesis of β-linked sugar-1-P. Panel I shows the synthesis of 2-deoxy β-L-sugar phosphates involving the coupling between α-configured glycosyl chlorides with tetrabutylammonium salts, where R is protecting group. Panel II shows the synthesis of α-L-arabinose-1-phosphate using the coupling of acetyl-protected glycosyl bromides with dibenzylphosphate as a key step. DCM, dichloromethane.

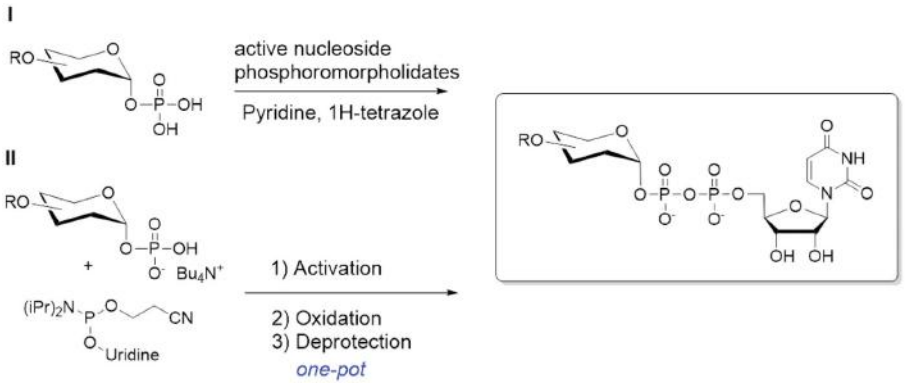

Figure 8. Diphosphate formation using a chemical approach. Panel I shows the direct coupling with active nucleoside phosphoromorpholidates. Panel II shows one-pot procedure based on the coupling of a sugar phosphate and a nucleoside phosphoramidite followed by oxidation.

phosphoromorpholidates (Figure 8, panel I). For example, the coupling of phosphoric acid with uridine-monophosphoromorpholidate in pyridine is typically quite sluggish [26], but the introduction of 1H-tetrazole catalyst can significantly accelerate the reaction rate [22]. Uridine 5′-monophosphomorpholidate 4-morpholine-*N,N*-dicyclohexylcarboxamidine salt has been widely utilized as active monophosphomorpholidate to produce parent UDP-sugars [23]. The van der Marel group developed a one-pot procedure based on the coupling of a sugar phosphate and a nucleoside phosphoramidite for the rapid construction of UDP-*N*-acetylglucosamine derivatives in good yields (63–76%) [38]. The phosphatephosphite intermediate formed *in situ* was subsequently oxidized with *t*-BuOOH to form the desired products (Figure 8, panel II). In addition to UDP-GlcNAc/GalNAc, chemical pathways can also be used in the synthesis of other sugar nucleotides, such as UDP-4′′-deoxy-α-D-xylohexopyranose (UDP-D-Glc) [39], UDP-*N*-acetylmuramic acid [28], UDP-D-fucose [40], as well as GDP-D-ManA [27].

Enzymatic and chemoenzymatic approaches

Pyrophosphorylase is typically utilized for the connection between a glycosyl phosphate and a nucleoside monophosphate to form UDP-sugars. For example, unnatural UDP-GlcNAc/UDP-GalNAc sugar donors could be prepared by GlmU, a GlcNAc-1-P uridyltransferase (pyrophosphorylase), from the sugar-1-P and UTP [41]. In addition, yeast inorganic pyrophosphatase was added to drive the GlmU reaction forward by degrading the pyrophosphate (PPi) byproduct (Figure 9, panel I) [42]. Wang and coworkers have made systematic efforts in the synthesis of unnatural UDP-GlcNAc/UDP-GalNAc analogs relying on a platform that combines chemical synthesis and enzymatic synthesis with GlmU [42]. This has resulted in the facile preparation of a library of unnatural sugar nucleotides modified with diverse bioorthogonal reactive groups, especially involving the functionalization of C-2 nitrogen group (Figure 9, panel I) [42]. Wang and coworkers also investigated the substrate specificity of GlmU and found that GlmU showed relaxed tolerance for modifications at *N*-acyl, C-3, C-4,

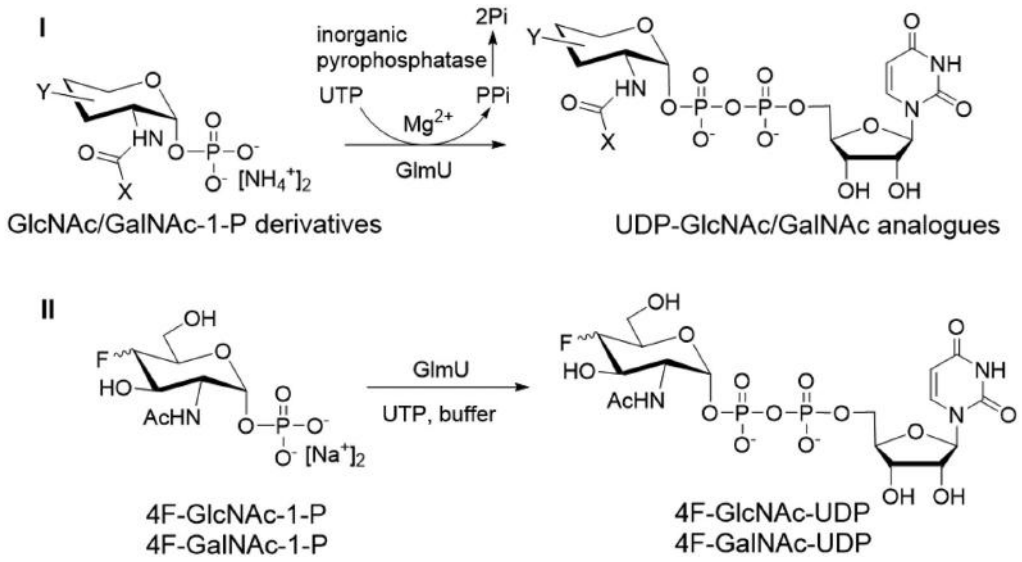

Figure 9. Diphosphate bond formation catalyzed by enzyme. Panel I shows the mechanism of pyrophosphorylation catalyzed by GlmU. Group X is methyl, ethyl or their azido derivative and group Y is hydrogen or hydroxyl. Panel II shows the synthesis of unnatural 4F-UDP-GlcNAc/GalNAc catalyzed by GlmU.

and C-6 positions, with a preference for small substituents. It is noteworthy that only substrates that have an amide linkage to the C-2 nitrogen could be transferred by GlmU [17].

Access to the C4 position of GlcNAc/GalNAc can be complicated by its low relative reactivity compared to neighboring sites [43]. In a complementary approach, Linhardt and coworkers have investigated unnatural modifications at the C4 position (Figure 9, panel II) [23,24]. The configuration of the 4-OH group appeared to play a lesser role in GlmU recognition, for example, 4F-GlcNAc-1-phosphate and 4F-GalNAc-1-phosphate analogues have been successfully used as GlmU substrates. The yields have been greatly improved compared to the previous studies that use UDP-GlcNAc pyrophosphorylase produced in *Escherichia coli* JM109 [25], and are comparable to chemical approaches while offering significantly simplified purification processes. However, the authors did find that GlmU would not accept $4N_3$ *N*-actylhexosamine-1-phosphate, possibly the result of the considerably larger size of the azido group.

In some instances, it might be more efficient to transform complicated multiple-enzyme-catalyzed reaction sequences into one-pot reactions, where UDP-sugars or product oligosaccharides are prepared without required isolation or purification of intermediates. Chen et al. reported an efficient one-pot multi-enzyme (OPME) system, containing NahK_ATCC55813, PmGlmU and PmHS2, to prepare unnatural UDP-donor followed by glycosylation (Figure 10, panel I) [32]. Fang and coworkers developed similar alternative sequential OPME synthetic strategy, by the *in situ* combination of two sugar nucleotides generated with PmHAS-catalyzed polymerization to prepare homogeneous hyaluronan (HA; Figure 10, panel II) [44]. This approach can convert inexpensive starting monosaccharides to HA polymers in a bio-safe manner without consumption of expensive sugar nucleotide donors.

Similarly, other sugar nucleotides can be prepared using this strategy as well. For instance, Wang and coworkers reported the synthesis of GDP-Man from Man-1-P using a recombinant GDP-mannose pyrophosphorylase that was from *Salmonella enterica* [45]. The same group subsequently prepared GDP-fucose, UDP-xylose and UDP-arabinose with corresponding pyrophosphorylases [30,46]. While different from UDP-sugars, CMP-Neu5Ac and its analogs can be obtained directly from Neu5Ac rather than sugar-1-phosphate, which is catalyzed by *N*-acylneuraminate cytidylyltransferase [47].

Applications of sugar nucleotides

Controlled preparation of polysaccharides/ oligosaccharides

UDP-sugars can be transferred to the non-reducing end of carbohydrate chains by glycosylation reactions with glycosyltransferases. By chemically modifying the sugar moieties of these UDP-sugars, the fine structure of the

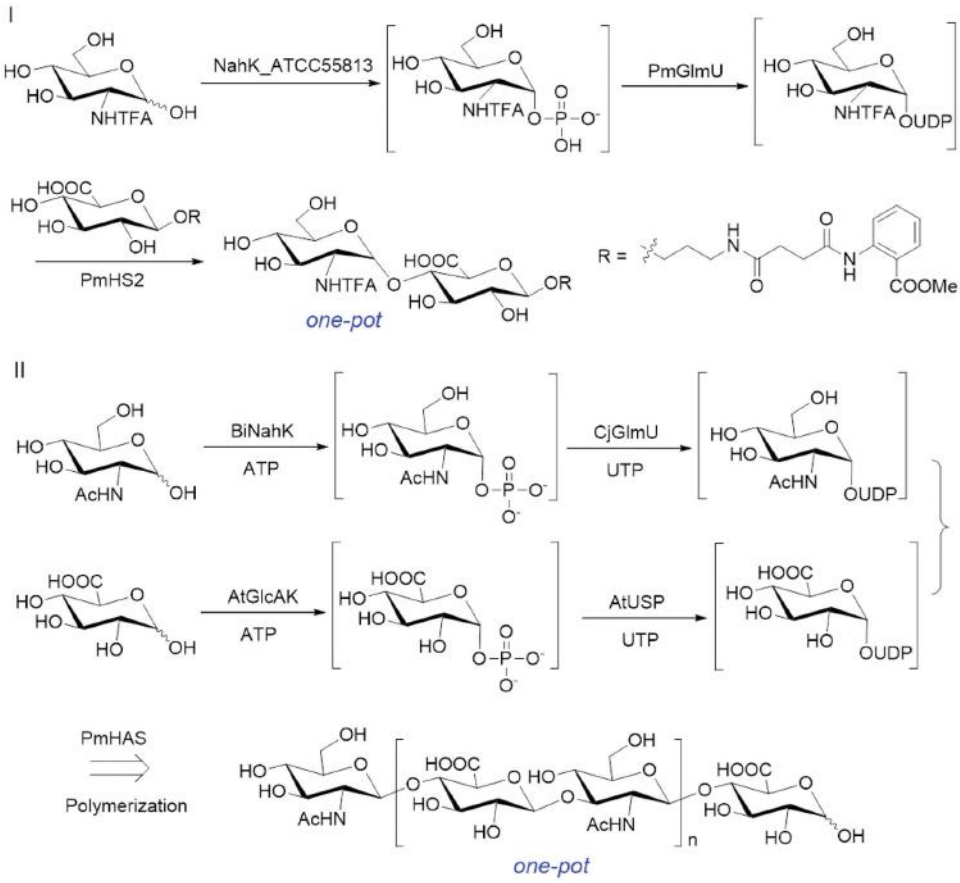

Figure 10. One-pot multienzyme reactions. Panel I shows the sequential enzymatic synthesis of heparin sulfate oligosaccharide precursor catalyzed by NahK_ATCC55813, PmGlmU and PmHS2. Panel II shows the synthesis of HA polymers through the *in situ* combination of two sugar nucleotides followed by PmHAS-catalyzed polymerization.

target carbohydrate can be manipulated offering an additional level of structural control.

Currently commercially available GAGs (with the exception of HA, for which there are microbial sources) are isolated from animal sources. GAG biosynthesis *in vivo* results in a variety of chain lengths and modification patterns, making a commercial GAG a complex, heterogeneous mixture of molecules [11]. Linhardt and coworkers have reported the synthesis of 4F-UDP-GlcNAc and found that this unnatural donor could be accepted by glycosyltransferase PmHS1 [24] and subsequently served as a chain terminator [48], offering an approach to synthesize HS/heparin oligosaccharides with well defined lengths (Figure 11, panel I). In addition, fluorinated carbohydrates have a wide range of applications in the glycosciences [49]. For example, fluorine is a useful probe for nuclear magnetic resonance (NMR) analysis and imparts a mass defect useful in mass spectrometry (MS), so the ability to enzymatically incorporate fluorinated sugars can facilitate polysaccharide structural characterization [50].

UDP-GlcNTFA is an unnatural UDP–sugar and also an excellent substrate for PmHS2 to elongate the heparan sulfate backbone with high yield [18,51]. The resulting GlcNTFA residue can then be easily converted to GlcNS by removing the trifluoroacetyl group under mildly alkaline conditions followed by the introduction of *N*-sulfo groups (Figure 11, panel II) [52,53]. Liu, Linhardt and coworkers have utilized this strategy for the regioselective synthetic incorporation of GlcNS residues, and, thus, successfully prepared a variety of low-molecular-weight heparins (LMWHs) and ultra-low-molecular-weight heparins (ULMWHs) [51,54]. Using a similar strategy, well defined heparan sulfate polysaccharides could be prepared as well, and further applied to structure-activity studies on fibroblast growth factor (FGF)1 and FGF2 signaling [55]. Furthermore, sulfated UDP-sugar derivatives, such as UDP-6-SO_3-GlcNAc and UDP-

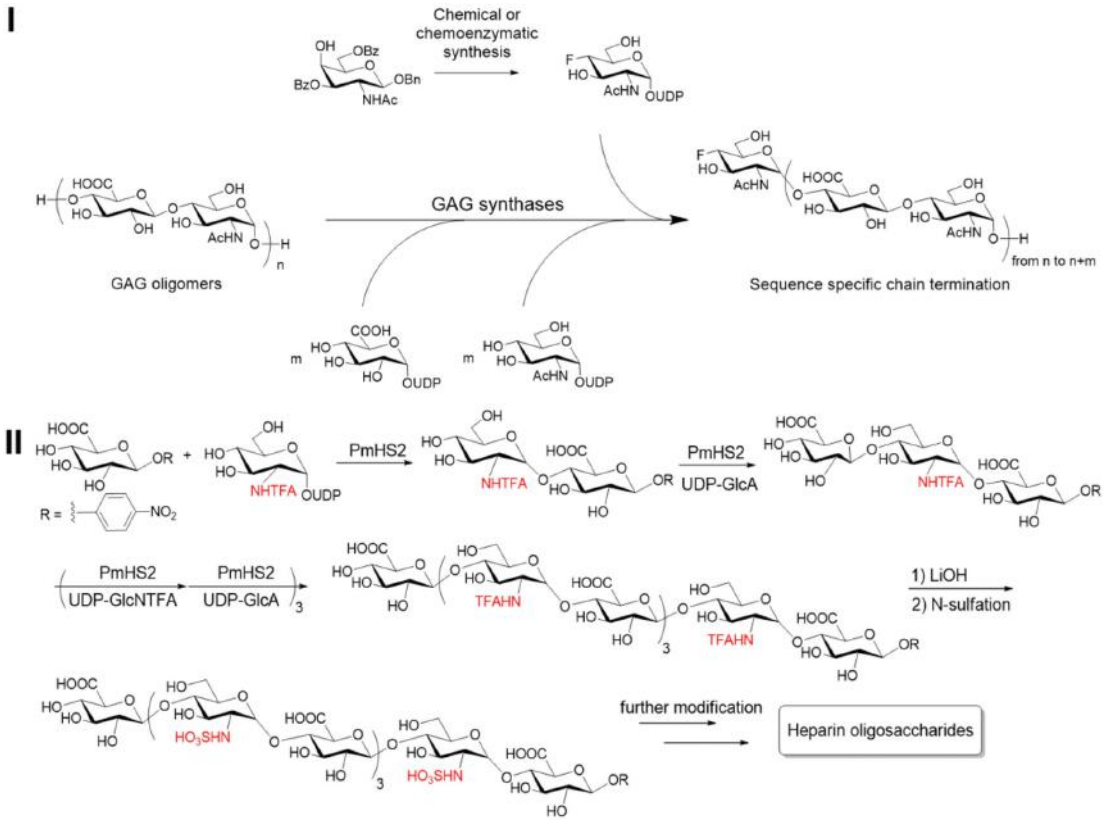

Figure 11. Unnatural UDP-sugars applied in the controlled preparation of polysaccharides/oligosaccharides. Panel I shows incorporation of unnatural UDP-sugars into GAGs polysaccharide synthesis. Panel II shows the application of UDP-GlcNTFA in the chemoenzymatic synthesis of heparin oligosaccharides.

sulfoquinovose, developed by Chen and DeAngelis, respectively, can be potentially utilized to directly introduce sulfate groups into sugar chains [56,57]. Unlike the typical enzymatic glycosylation that transferring monosaccharide residue to saccharide acceptor, Li and coworkers discovered a novel mannosyl-1-phosphotransferase that could transfer mannose-α1-phosphate from GDP-man to disaccharide acceptor, forming a unique mannose-α1-phosphate-6-glucose linkage [58].

Inhibitors for cancer therapy

It was reported that cell-surface glycans are dysregulated in many different states of cancer progression such as proliferation, invasion, angiogenesis, and metastasis [59]. It is also well documented that the expression of highly branched *N*-glycans is distinctly enhanced during the proliferation of different cancer cells and metastasis [60]. Therefore, inhibitors and modifiers of the biosynthesis of such highly branched glycans may become novel candidates as cancer therapeutics. Increasing the intracellular UDP-GlcNAc concentration leads to the increased branching of *N*-glycans of such glycoprotein receptors [61] and enhances the affinity with the cell-surface galectin-3, a key lattice-forming lectin [62,63].

Nishimura et al. developed GlcNAc analogues bearing a fluorine atom at the C4 position [64]. These derivatives could be exogenously added and penetrate into cells across the plasma membrane and are converted into UDP-4F-GlcNAc with in human prostate cancer PC-3 cells. This group demonstrated that the unnatural UDP donors depressed the expression levels of such hyperbranched *N*-glycans in PC-3 cells by perturbing the hexosamine biosynthetic pathway of native UDP-GlcNAc. This may result in promising leads in the discovery of a new class of anti-prostate-cancer drugs.

Stable isotope labeling technology

Stable isotope labels (SILs) are one of the most promising ways to investigate the roles of biomacromolecules in metabolic pathways. SILs allow tracking the stable isotope-enriched analogs of biomolecules that are

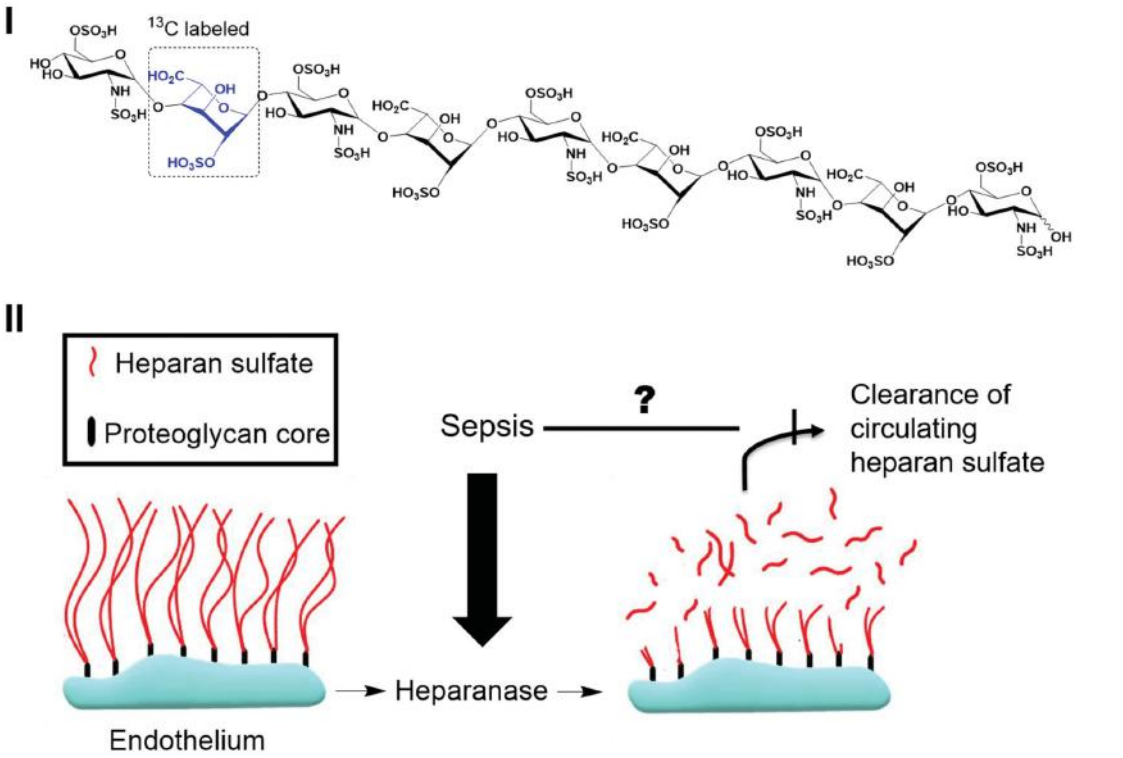

Figure 12. Unnatural UDP-sugars applied in stable isotope labeling technology. Panel I shows the structure of heparin nonasaccharide probe in which one of the iduronic acid residues is ^{13}C-labeled. Panel II shows the application of this ^{13}C-labeled heparin oligosaccharides for sepsis study. Reproduced from ref. [66].

easily distinguished from their endogenously biosynthesized counterparts when analyzed by MS [65]. Linhardt and coworkers successfully synthesized a heparin nonasaccharide containing a ^{13}C-labeled internal 2-sulfoiduronic unit through chemoenzymatic approach using a ^{13}C-labeled UDP-GlcA (Figure 12, panel I) [66]. The resulting ^{13}C-labeled compound was subsequently intravenously administered to septic and non-septic mice, and the results of this study suggested that circulating heparan sulfates are rapidly cleared from the plasma during sepsis and selectively penetrate the hippocampus, where they may have functional consequences (Figure 12, panel II) [67].

Bioorthogonal reporter strategies

Enzymatic bioorthogonal labeling strategies

In recent years, the development of a bioorthogonal reporter strategy has emerged as a powerful tool for glycan analysis [68]. Bioorthogonal functional groups, such as azido and alkyne that can be carried by unnatural sugar nucleotides enzymatically incorporated into glycans, can allow for further covalent conjugation by bioorthogonal chemical reactions. These strategies allow the detection of specific glycoconjugate types on cells, in living organisms or the selective capture of glycoproteins from cell or tissue lysates [23]. For example, the azido moiety is inert to natural processing or reactivity within biological systems but can be readily covalently tagged with imaging probes or other epitopes using an azide-specific reaction, such as Staudinger ligation with phosphines [69,70] or [3 + 2] cycloadditions with alkynes [71,72]. Linhardt and coworkers synthesized 4N_3-UDP-GlcNAc, and this unnatural donor was enzymatically added to the non-reducing end of the sugar chain in hyaluronan and heparosan [23]. The resulting 4N_3-GlcNAc-terminated polysaccharides were then successfully conjugated with Alexa Fluor 488 DIBO alkyne, achieving the regioselective labeling and detection of GAGs.

Wang and coworkers recently reported a large library of UDP-GlcNAc and UDP-GalNAc derivatives modified with diverse groups which could be subjected to well-established bioorthogonal reactions [73], such as click chemistry [71] (azido and alkyne groups), Staudinger–Bertozzi ligation [74] (ketone group), Diels–Alder reaction [75] (alkene group), one-step labeling strategy [76] (biotin group), Raman reporter

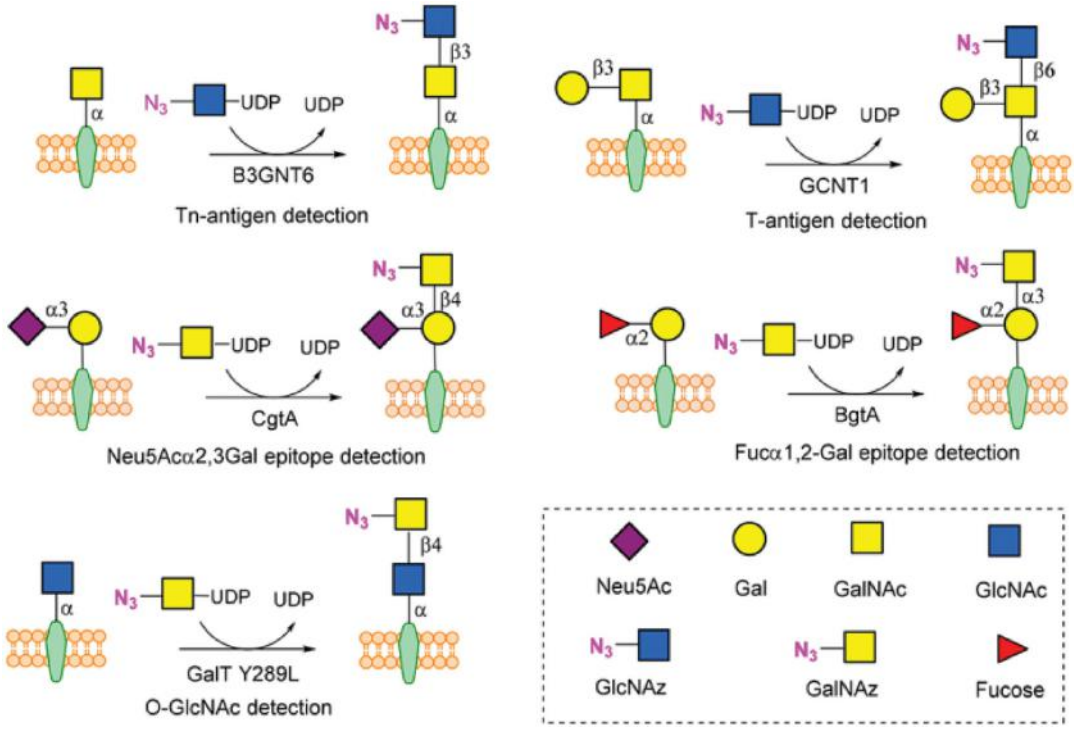

Figure 13. Enzymatic bioorthogonal labeling of Tn antigen, T antigen, Neu5Acα2,3-Gal, Fucα1,2-Gal, and *O*-GlcNAc with UDP-GlcNAz and UDP-GalNAz. Reproduced from ref. [73]. Copyright 2018 American Chemical Society.

strategy [77] (nitrile group) and photoactivatable cross-linking [78] (diazirine groups; Figure 13). Some of these UDP-sugar derivatives even have better activity than their natural substrates in the donor specificity studies with glycosyltransferases. The authors also evaluated the application of enzymatic bioorthogonal labeling reactions with several known glycan epitopes, such as the well-known mucin-type *O*-glycans Tn antigen and T antigen, which have no expression on normal cells but are highly expressed on many cancer cells (Figure 13). These UDP-sugar analogues can be used in the versatile labeling of glycan epitopes and show great potential for application in immunotherapy and clinical diagnosis.

Metabolic bioorthogonal labeling strategies

In addition, bioorthogonal labeling using unnatural sugar nucleotides catalyzed by glycosyltransferases afford unnatural monosaccharide analogues carrying these functional groups and can be metabolically incorporated *in vivo* into glycans for imaging and functional studies [79–81]. However, this strategy has only been investigated in animal systems. Chen and coworkers reported the development of this strategy for metabolic labeling and imaging of protein *N*-glycans in a plant, *Arabidopsis thaliana*, probing the biosynthesis and biological functions of plant glycans (Figure 14) [82]. The synthesized Ac4GlcNAz was metabolically incorporated and the bioorthogonal conjugation of the incorporated fluorescent probes allowed direct visualization of newly synthesized *N*-glycans in the root tissue. More importantly, this strategy provided a means to visualize the protein *N*-linked glycosylation in *Arabidopsis* seedlings with spatial and temporal resolution. Although these metabolic chemical reporters (MCRs) are powerful reagents for the unbiased identification of glycoproteins, they do have certain limitations. For example, they are incorporated substoichiometrically into glycans, and most MCRs are not selective for one class of glycoprotein [83]. Using a similar strategy, this group was able to fluorescent image cell-surface sialoglycans and perform proteomic profiling of sialoglycoproteins based on the metabolic incorporation of unnatural 9-azido analogues of sialic acids [84]. Baskin and coworkers successfully visualized glycosylation in embryos and larvae during zebrafish development using 6-alkynyl UDP-Gal followed by click chemistry tagging [85].

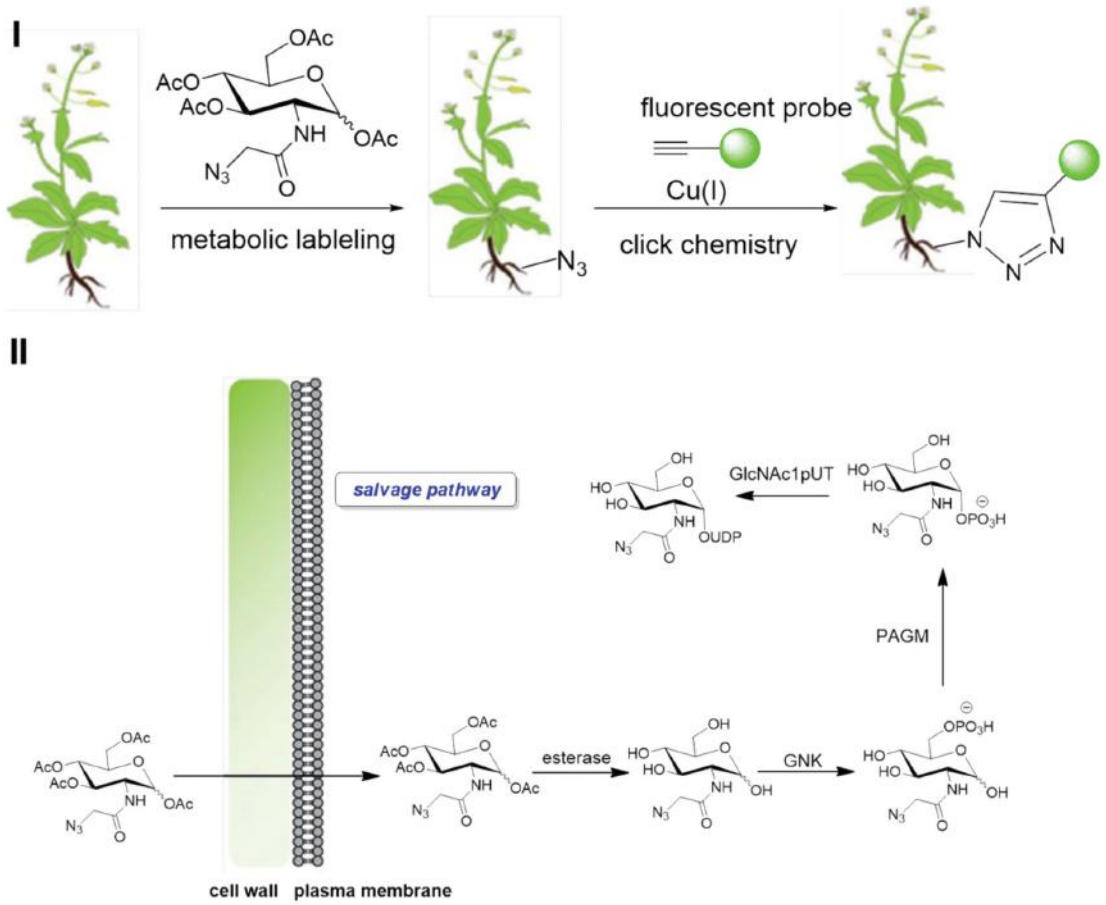

Figure 14. Metabolic labeling of *Arabidopsis N*-linked glycans with Ac4GlcNAz. Panel I shows Ac4GlcNAz is fed to *Arabidopsis* seedlings grown in liquid medium and incorporated into *N*-linked glycans through the GlcNAc salvage pathway. Panel II shows the mechanism of exogenous Ac4GlcNAz conversion into UDP-GlcNAz. Reproduced from ref [82]. Copyright 2016 John Wiley & Sons, Inc. GNK, GlcNAc kinase; PAGM, phosphoacetylglucosamine mutase.

Glycosylation of natural products

Glycosylation with sugar nucleotides is a common modification reaction in the biosynthesis of natural compounds, enhancing the solubility and stability relative to its aglycones. Moreover, glycosylation of some antibiotics is required for their biological activities because sugars usually participate in the molecular recognition of their cellular targets [86]. Thus, glycosylation is of great importance in developing the next generation of novel therapeutics.

The natural flavonoids, especially their glycosides, are the most abundant polyphenols in foods and play key roles in the prevention and management of chronic diseases such as cancer, diabetes, and cardiovascular conditions [87]. Dai and coworkers efficiently produced structurally diverse flavone bis-*C*-glycosides with two identical or different sugar moieties using UDP-α-D-glucose, UDP-Glc, UDP-α-D-galactose, or UDP-β-L-arabinose (Figure 15) [88]. However, the extensive investigation of *C*-glycosyltransferase is needed to broaden the scope of both donors and acceptors to efficiently produce structurally diverse bis-*C*-glycosides with two identical or different sugar moieties in drug discovery.

Conclusion and future perspectives

Sugar nucleotides are essential intermediates in carbohydrate metabolism and glycoconjugate biosynthesis. These natural and unnatural sugar nucleotides could participate in various biological processes, showing considerable interest as carbohydrate-based tools for the study of glycoconjugate biosynthesis and for their potential as enzyme inhibitors in therapeutic intervention strategies. Replacement of GlcNAc/GalNAc residues by GlcNAc/GalNAc analogs in a polysaccharide or a

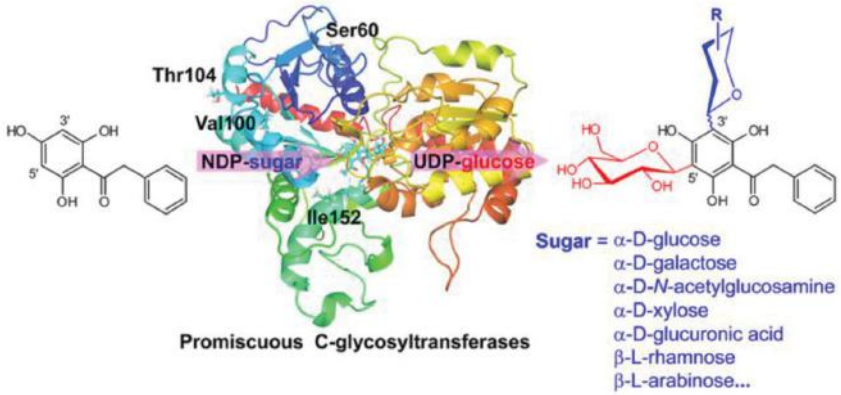

Figure 15. *C*-glycosylation of natural and unnatural flavones with a series sugar nucleotides catalyzed by *C*-glycosyltransferases. Reproduced from ref [88]. Copyright 2018 American Chemical Society.

glycoconjugate is a good approach to understand the mechanism of GlcNAc/GalNAc-related pathways. Changes in glycosylation are correlated to disease and associated with differentiation processes. In addition, by chemically modifying these UDP moieties, the fine structure of the target carbohydrate can be manipulated or functionalized. Therefore, methods for the efficient preparation can greatly benefit synthetic, biological and medicinal chemistry.

Chemical approaches are available to synthesize many sugar nucleotides of interest. However, the scale-up of chemical synthesis proves to be uneconomical and impractical due to serious drawbacks such as long coupling reaction times and generally low overall yields. In contrast to chemical synthesis, enzymes have exquisite stereo-selectivity and regio-selectivity for sugar nucleotide synthesis. However, purely enzymatic synthesis has its own limitations such as limited availability of key enzymes or limited substrate tolerance of enzymes. Therefore, chemoenzymatic synthesis, integrating the flexibility of chemical derivatization with the specificity of enzyme-catalyzed reactions, mimicking the biosynthetic pathway of sugar nucleotides, represents a promising strategy to address many of these problems.

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