Recent advances in the enzymatic synthesis of sugar-nucleotides

Sanaz Ahmadipour, Laura Beswick and Gavin J. Miller*

Lennard-Jones Laboratory, School of Chemical and Physical Sciences, Keele University, Keele, Staffordshire, ST5 5BG, U.K.
**Corresponding author. Email: g.j.miller@keele.ac.uk*

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1. Introduction

Sugar-nucleotides (sugar-nucleoside diphosphates) are imperative to carbohydrate metabolism and glycoconjugate biosynthesis. They are composed of an activated sugar donor that is glycosylated onto a diverse range of acceptors, typified by glycosyltransferase catalysed processes for the assembly of glycosides[1] and oligo- or polysaccharides.[2] They are of considerable interest as carbohydrate-based tools for the study of glycoconjugate biosynthesis and for their potential as enzyme inhibitors in new therapeutic strategies.[3] In addition, they are important for unambiguous biochemical assay development and for the provision of structurally defined homogenous analytical standards. We recently reviewed the current start of the art (2009 onwards) regarding chemical approaches to synthesise sugar-nucleotides and present here a complimentary appraisal of the enzymatic methods that have emerged during the same period.

1.1. Enzymatic approaches towards sugar-nucleotides

Sugar-nucleotides are structurally diverse, consisting of a sugar linked to a nucleoside diphosphate (sugar nucleoside monophosphates, such as CMP-sialic acid, are not covered here). Figure 1a illustrates a generic sugar-nucleotide, alongside the common enzymatic strategies that have been used for their synthesis, starting from the hemi-acetal. The advent of one-pot multi-enzyme (OPME) approaches to complex carbohydrates[4] has meant that access to sugar-nucleotides and the glycosyltransferases (GTs) that utilise them is much simplified compared to classic synthetic chemistry methods. From the perspective of the promiscuity of the enzymes involved, there is still development required to access diverse/non-native targets and, generally speaking, bacterial enzymes have shown greater promise in this regard.

In animal cells the most commonly occurring sugar-nucleotides utilise a uridine or guanidine-containing nucleoside diphosphate (UDP or GDP) along with a sugar; this includes aldopentose (UDP-Xyl), aldohexose (UDP-Glc, UDP-Gal, GDP-Man), aldohexosamine (UDP-GlcNAc, UDP-GalNAc) and uronic acid (UDP-GlcA) components.[5] These common mammalian examples are illustrated in Figure 1b), alongside some examples found in bacteria, such as GDP-ManA, UDP-Gal*f* and dTDP-Glc.

We have divided this review into sections, first covering enzymes that are available for the provision of glycosyl 1-phosphates, followed by the use of uridylyltransferases for nucleoside diphosphate formation. Finally, we consider emergent new strategies such as the reversal of GT activity to produce sugar-nucleotides.

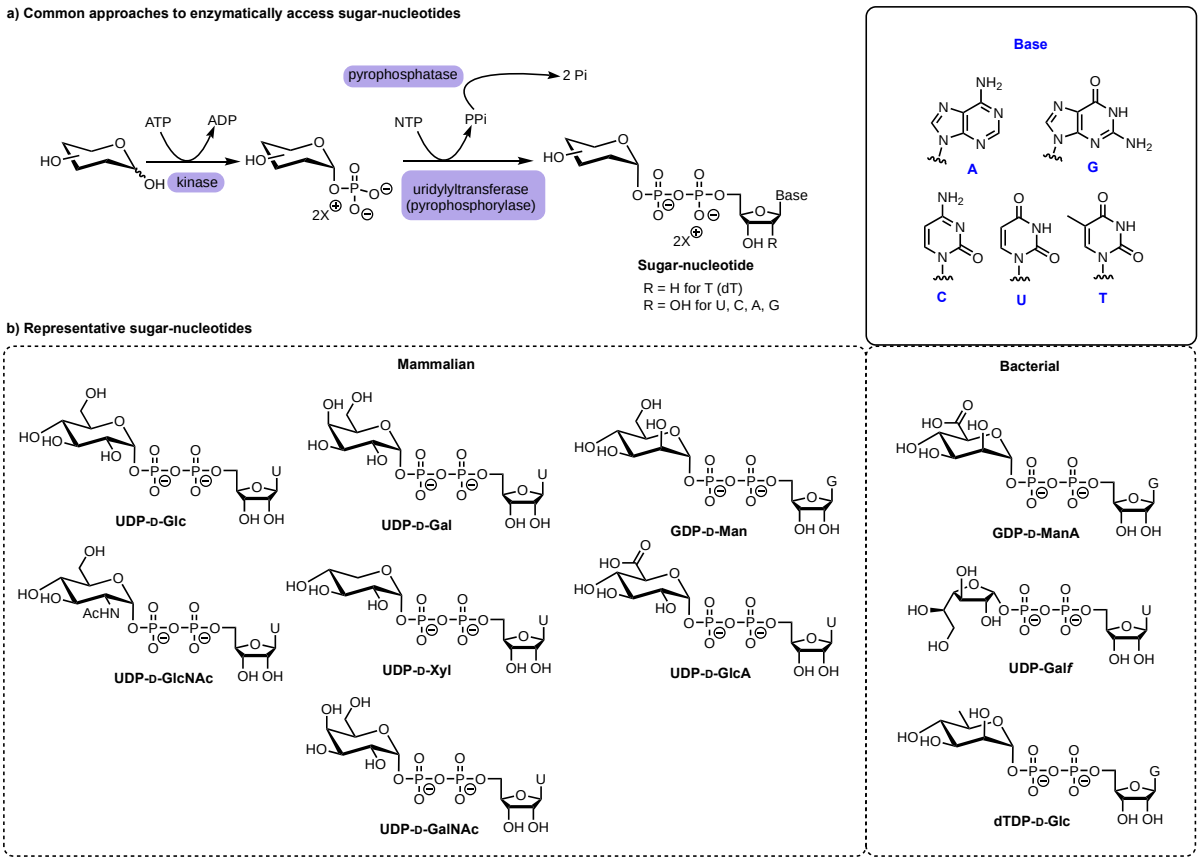
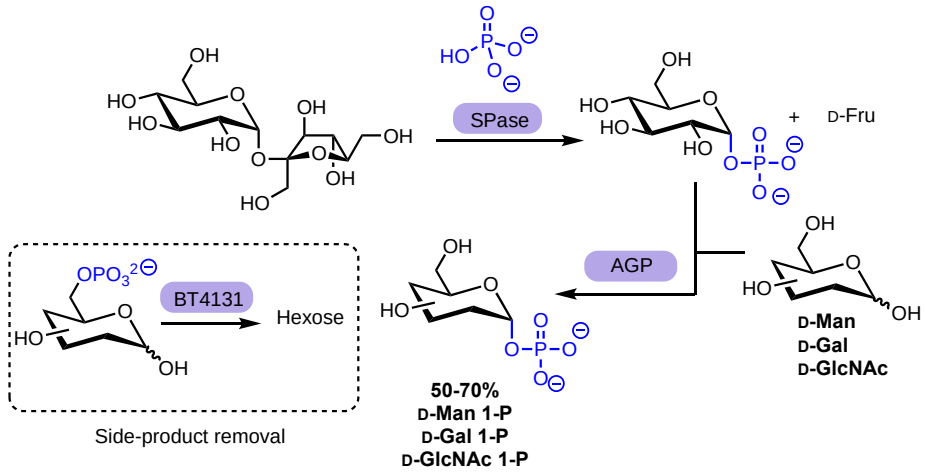


Figure 1. a) *Enzymatic approach to sugar-nucleotides, using a kinase to access a glycosyl 1-phosphate for subsequent reaction with a uridylyltransferase (pyrophosphorylase). The use of pyrophosphorylases and a NTP is often combined with a pyrophosphatase to degrade the diphosphate by-product, providing a thermodynamic sink to the process* ***b)*** *representative examples of common mammalian and bacterial sugar-nucleotides. PPi = pyrophosphate, Pi = phosphate.*

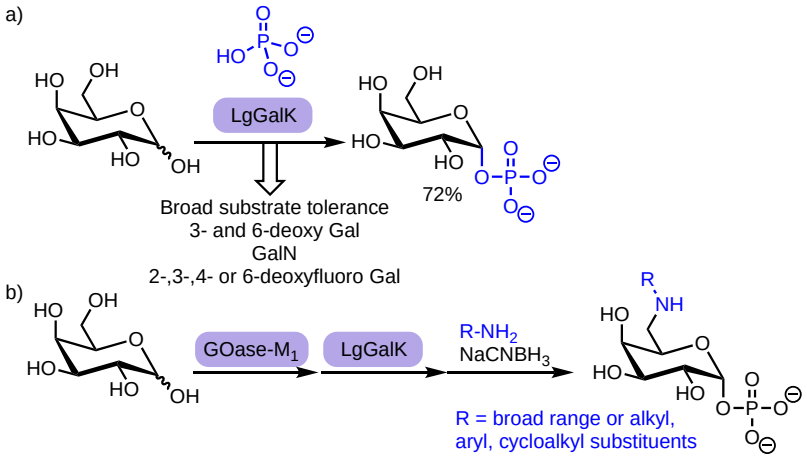
2. Enzymes for the preparation of glycosyl 1-phosphates

In an effort provide access to useful glycosyl 1-phosphates and an economic benefit in not having to utilise expensive NTP donors for such processes, Nidetzky and colleagues described a two-step diastereoselective phosphorylation of pyranoses (*Scheme 1*).[6] Their studies demonstrated an *endo*-anomeric centre phosphorylation of sucrose by inorganic phosphate, catalysed by sucrose phosphorylase (SPase), followed by a selective transphosphorylation of the product Glc 1-phosphate with a sugar acceptor and glucose 1-phosphatase (AGP). This enzyme combination delivered α-configured glycosyl 1-phosphates of D-Man, D-Gal and D-GlcNAc in yields between 50 and 70%. The authors also developed a useful enzymatic purification procedure whereby the phosphatase (BT4131) was able to effectively distinguish between desired anomeric and unwanted 6-position phosphates, thereby hydrolysing any side reaction that produced 6-phosphates. This method produced up to 40 mg from a single small-scale reaction and demonstrated that AGP is capable of transphosphorylation to a broad range of acceptors, using Glc 1-phosphate as a substrate.



Scheme 1. *Synthesis of glycosyl phosphates using SPase from Leuconostoc mesenteroides and AGP from E. coli in a combination catalyst system. BT4131 from Bacteroides thetaiotaomicron.*

In 2018 the Flitsch group described the production and utilisation of a new bacterial galactokinase, LgGalK, which produced Gal 1-phosphate with a conversion rate of 72% (*Scheme 2a*).[7] Crucially, the enzyme also exhibited a broad substrate tolerance, as evidenced by the synthesis of a range of Gal 1-phosphate analogues, including deoxy and deoxy-fluoro substituted systems.



Scheme 2. a) *Synthesis of Gal 1- phosphates by using LgGalK from Leminorella grimontii* ***b)*** *Use of LgGalK for a one-pot, two-step access to 6-position modified Gal 1-phosphates.*

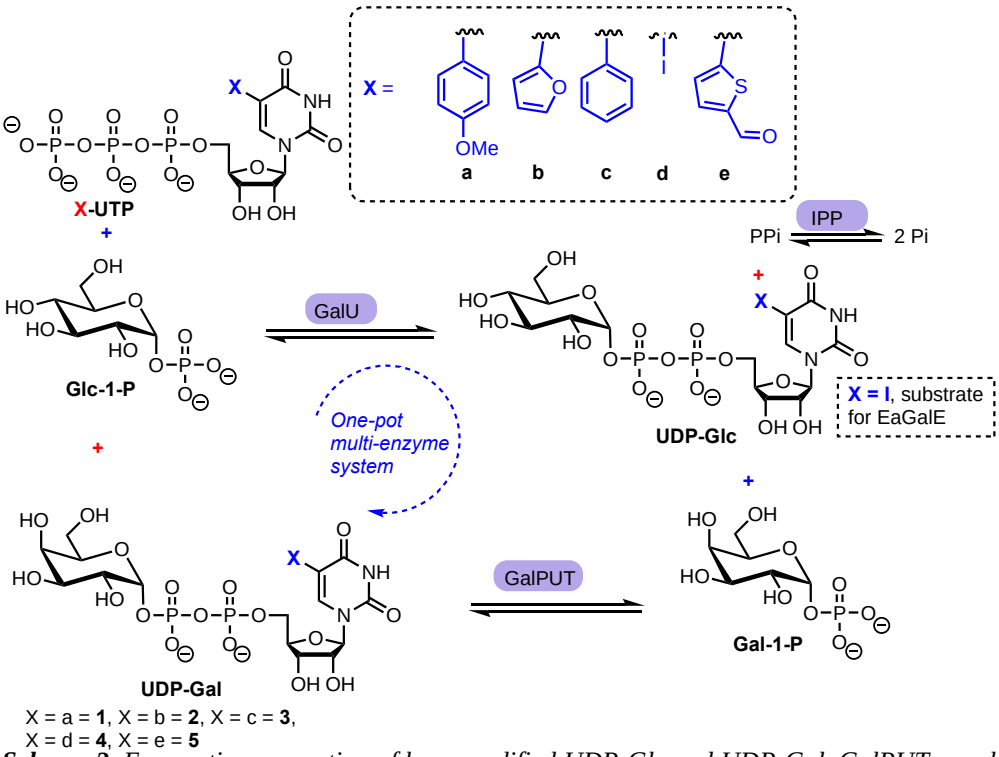
LgGalK was also used for a one-pot, two-step strategy towards a series of more complex 6-position modified galactose 1-phopshates. Using their galactose oxidase, GOase-M_1, the C6 position was first oxidised to the corresponding aldehyde, followed by treatment with LgGal to the 1-phosphate and *in situ* reductive amination to produce a matrix of thirty 6-position amino Gal-1-phosphate derivatives, confirmed by HRMS analysis (*Scheme 2b*). This modification strategy could also be used with 2-deoxy-2-fluoro Gal as the starting hexose, further extending the matrix of products and providing a powerful approach to deliver modified glycosyl 1-phosphate chemical tools.

Since 2009 several other kinases have been utilised to access a range of glycosyl 1-phosphates, however, as these were generally combined within multi-enzyme approaches towards sugar-nucleotides, we have discussed these inclusively in the following sections.

3. Enzymes for the pyrophosphorylation of sugar 1-phosphates

3.1. Towards UDP and dTDP sugar-nucleotides

In 2015, Field *et al*. reported a one-pot multienzyme approach to base modified UDP-Glc and UDP-Gal (*Scheme 3*).[8] This provided enzymatic access to a new class of 5-position modified sugar-nucleotides, which had previously demonstrated promise as GT inhibitors[9] and as fluorescent tools for GT assay development.[10] Their strategy employed a small panel of 5-position modified UTPs and Glc 1-phosphate, incubated with UDP-glucose pyrophosphorylase (GalU) to generate, *in situ*, a modified UDP-Glc in catalytic quantity, to be continuously recycled by GalU. Subsequently, Gal 1-phosphate uridylyltransferase (GalPUT) catalysed the reaction of base-modified UDP-Glc into Glc 1-phosphate and the corresponding UDP-Gal.



Scheme 3. *Enzymatic preparation of base-modified UDP-Glc and UDP-Gal. GalPUT = galactose-1-phosphate uridylyltransferase from E. coli. GalU = UDP-glucose pyrophosphorylase from E. coli. EaGalE from Erwinia amylovora. IPP = pyrophosphatase.*

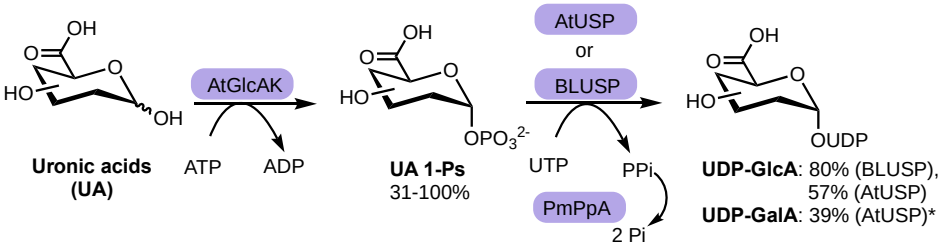
Reaction of these enzymes with 5-substituted UTPs was slower in comparison to the parent UTP, but furnished 5-substituted UDP-Gal derivatives **1** (5%), **2** (23%) and **3** (<5%), albeit in lower yields than the wild-type. For 5-iodo- and 5-(5-formyl-2-thienyl)-UTP no product (**4** or **5**) was observed. A series of reverse reactions and inhibition experiments led to the conclusion that GalPUT was the limiting factor in this one-pot system. Furthermore, there was an observed degree of relaxed specificity towards the nucleotide triphosphate component using GalU, which was able to independently convert all the UTP analogues to UDP-Glc derivatives. Finally, a 4''-epimerase was used to independently convert 5-iodo UDP-Glc into **4**, achieving a ca 7.5:2.5 equilibrium ratio of Glc/Gal after 30 mins.

This strategy was also used by the group for an enzymatic synthesis of 6-deoxy 6-fluoro-UDP-Glc.[11] Starting from Gal, GalK, GalPUT and GalE were used to deliver the 6-deoxy 6-fluoro sugar-nucleotide and, coupled with an expedient purification method, demonstrated a capability to provide ^{19}F radio-labelled sugar-nucleotide derivatives. Lowary and co-workers also utilised a three-enzyme system, containing GalPUT, GalU and a

phosphatase for their enzymatic synthesis of native and modified UDP-Gal*f* derivatives.[12] Whilst deoxy modified 1-phosphates (3-, 5- and 6-positions) were substrates for the system, OMe capping of hydroxy groups (at positions 2, 5 and 6) was not tolerated. The group also recently showed that GalPUT was unable to produce UDP-GalfNAc, UDP-GalfN_3 or UDP-GalfNH_2, confirming the sensitivity of this nucleotidyltransferase to the nature of the C2 group.[13]

The Chen group have made several important contributions to access sugar-nucleotides using enzymatic approaches[14] and most recently the group developed a OPME for the synthesis of UDP-uronic acids (*Scheme 4*).[15] Notably, this avoided the more expensive use of an NAD^+ dependant oxidative uronyl dehydrogenase to access UDP-GlcA.[16] The workers evaluated a bacterial glucuronokinase (AtGlcAK) and demonstrated its capability to accept GlcA, GalA and ManA substrates with good conversion to the desired uronate 1-phosphates in quantitative, 31% and 95% yields respectively. A galactokinase (BiGalK) also showed activity here for just GalA, however evaluations for L-IdoA showed it was not a substrate for either kinase.

Accordingly, their sugar 1-phosphates were evaluated as substrates for two UDP-sugar pyrophosphorylases, BLUSP and AtUSP. BLUSP delivered UDP-GlcA in 80% yield, , but did not produce UDP-GalA. Comparatively, AtUSP delivered UDP-GlcA in a lower yield of 57%, but was shown to work in accessing UDP-GalA (39% yield) and neither enzyme was able to produce GDP-ManA. This OPME approach was subsequently harnessed within a sequential process for the synthesis of heparosan oligosaccharides.

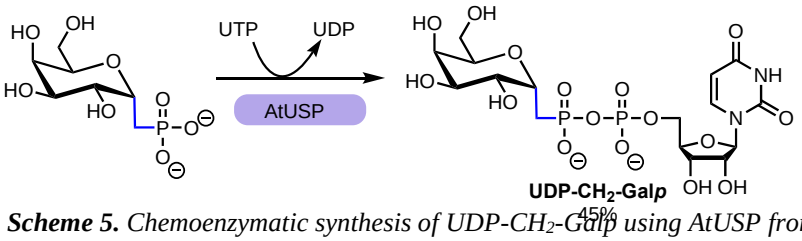


Scheme 4. *One-pot three-enzyme synthetic approach to UDP-GlcA and UDP-GalA. AtGlcAK from Arabidopsis thaliana, BLUSP from Bifidobacterium longum, AtUSP (from Arabidopsis thaliana). *not from BLUSP*

AtUSP has also been used by Wong *et al.* to produce UDP-Gal in their large-scale synthesis of cancer associated antigens Globo H and SSEA4.[17] The group focused on salvage pathway enzymes to successfully regenerate the required sugar-nucleotides. Alongside AtUSP, GlmU (for regeneration of UDP-GalNAc) and FKP, a bifunctional enzyme found in *Bacteroides* with L-fucose-1-phosphate guanyltransferase and L -fucose kinase activities, all showed capability for a powerful *in situ* nucleoside diphosphate regeneration strategy.

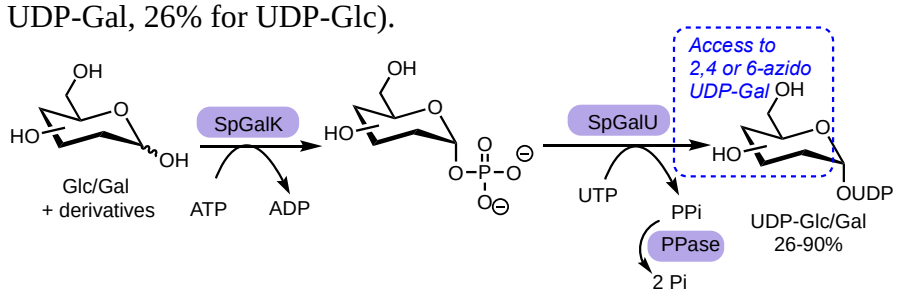
UDP-galactopyranose mutase (UGM) plays a key role in the cell wall biosynthesis of many pathogenic microorganisms, including *Mycobacterium tuberculosis*, catalysing a reversible isomerization of UDP-Gal*p* to UDP-Gal*f*. The absence of Gal*f* in humans makes UGM a potential target for developing novel antibacterial agents and, as such, there is a need for sugar-nucleotide tools to study the associated chemical and structural biology. The phosphonate analogue of UDP-Gal*p*, UDP-phosphono-galactopyranose (UDP-CH_2-Gal*p*) had previously been proposed and synthesised as a probe for deciphering the UGM mechanism.[18] Seeking to improve upon prior chemical syntheses of UDP-CH_2-Gal*p* where side reactions and decomposition following the key chemical pyrophosphorylation were observed, Sanders and co-workers described a novel chemoenzymatic synthesis of UDP-CH_2-Gal*p*.[19] Starting from the phosphonate analogue of Gal 1-phosphate and catalysed by recombinant AtUSP (*Scheme 5*), their strategy proved successful, delivering 19 mg of the target sugar-nucleotide with an isolated, purified yield of 45% (from a recovery of 25% starting Gal 1-phosphonate). The

authors noted that recombinant AtUSP was approximately 50-fold less active towards the glycosyl 1-phosphonate than native sugar 1-phosphates.



Scheme 5. *Chemoenzymatic synthesis of UDP-CH_2-Galp using AtUSP from Arabidopsis thaliana.*

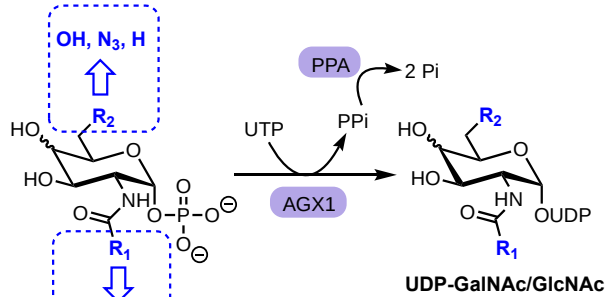
In 2013, a one-pot three-enzyme synthesis of UDP-Glc/Gal and their derivatives was reported by Chen and co-workers.[20] Using their catalytic system, UDP-Glc and six unnatural UDP-Gal derivatives (including UDP-2-deoxy-Gal and UDP-GalN_3 were efficiently synthesised in a one pot procedure (*Scheme 6*). Initially, anomeric phosphorylation of Glc/Gal by a galactokinase (SpGalK, previously characterised by the group[21]) afforded Gal/Glc 1-phosphates which were subsequently pyrophosphorylated by glucose pyrophosphorylase, SpGalU. A commercial inorganic pyrophosphatase (PPase) was added to degrade PPi, preventing its inhibition of SpGalU. The method was scalable (7 mg of UDP-Glc could be produced) and offered access to natural sugar-nucleotides (Gal/Glc/L-Man) and mimetic (seven Gal analogues with modifications at C2, C4 and C6) species with variable yields (90% for UDP-Gal, 26% for UDP-Glc).



Scheme 6*. One-pot three-enzyme synthesis of UDP-Glc/Gal and derivatives, SpGalK and SpGalU from S. pneumoniae.*

Wang and colleagues described the synthesis of eight UDP-GlcNAc analogues and three UDP-GalNAc analogues using a recombinant *N*-acetylglucosamine 1-phosphate uridylyltransferase (GlmU) with moderate yields (10-65%) and on relatively large scales (35-75 mg).[22] During this work the authors noted a narrow substrate specificity for GlmU in accepting GalNAc 1-phosphate analogues and duly explored a recombinant human UDP-GalNAc pyrophosphorylase (AGX1) to enable mimetic sugar-nucleotide synthesis.[23]A total of nine GalNAc 1-phosphate and five GlcNAc 1-phosphate derivatives were prepared chemoenzymatically using *N*-acetylhexosamine 1-kinase (NahK), as previously described by the group,[24] and then tested using AGX1, which exhibited a good tolerance of NAc and 4- and 6-position modifications of Gal and Glc (*Scheme 7*).

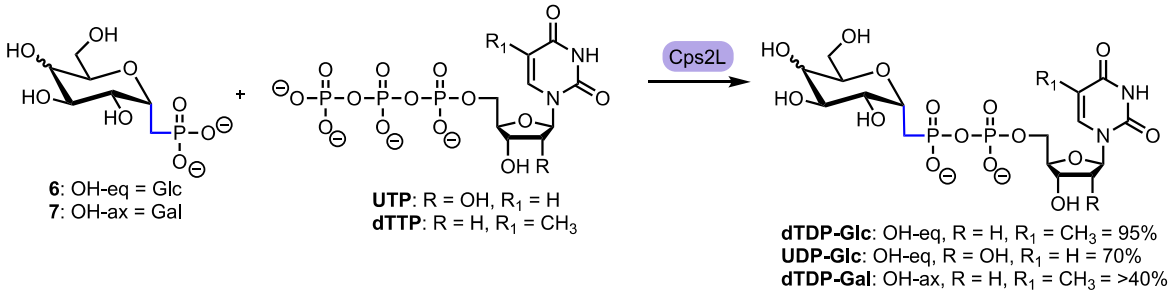
Comparatively, AGX1 was observed to exhibit a broader specificity than GlmU towards modified UDP-Glc/Gal targets, although neither enzyme was capable of accepting 4-position substituents larger than OH. GlmU differentiated Gal/GlcNAc-1-phosphate analogues with bigger *N*-acyl modifications whereas AGX1 was only slightly affected by the bulkiness of *N*-acyl groups in both Gal/GlcNAc systems. Moreover, AGX1 also showed good tolerance to C-6 modified 1-phosphate analogues 6-deoxy GalNAc-1-P and 6-azido-GalNAc-1-P.



Scheme 7. *AGX1 uridylyltransferase to access to UDP-GalNAc/GlcNAc analogues.*

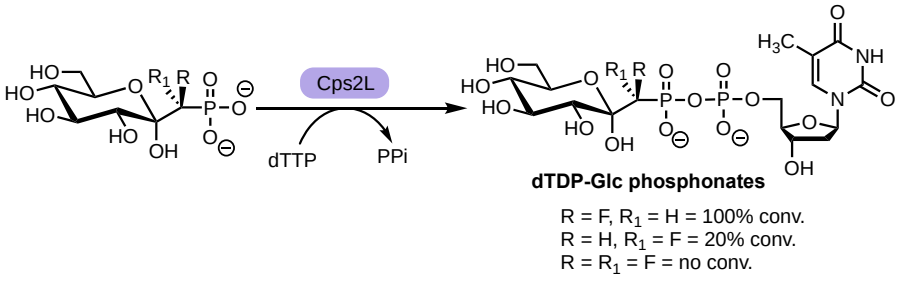
Anomeric phosphonates, a mimetic of native glycosyl 1-phosphates, have significant potential as metabolically stable glycosyltransferase probes. In 2009, Jakeman *et al.* reported the use of Cps2L in a nucleotidylyltransferase catalysed coupling of dTTP and UTP nucleotides with phosphonate analogues of Glc and Gal 1-phosphate (*Scheme 8*).[25] Previous chemical synthesis approaches to such analogues had been encumbered by slow reaction times and diminished yields.[26]

They probed the interaction of phosphonate analogues **6** and **7** with Cps2L to afford the desired sugar-nucleotide analogues. Incubation of **6** with dTTP and UTP proceeded with conversions of 95% and 70% respectively, but conversion of **7** was significantly lower (≥40%, as adjudged by HPLC analysis). Though the geometric and ionisation state changes between phosphate and phosphonate were found to partially account for a decrease in turnover efficiency, compared to the physiological substrate, the change in stereochemistry at C4 was deemed to have a greater significance on conversion.



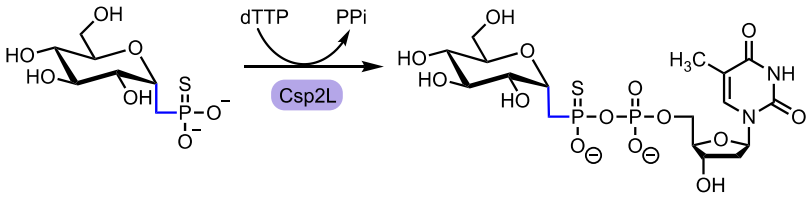
Scheme 8. *Synthesis of dTDP and UDP Glc and Gal phosphonate analogues using Cps2L.*

In order to evaluate the effect of α-monofluorination in probing Cps2L, Jakeman also reported the synthesis of a series of ketose phosphonates (*Scheme 9*).[27] They observed that an *S*-configured α-monofluorinated phosphonate was a substrate for Cps2L, showing 100% conversion, whilst the diastereomeric *R*-phosphonate was only converted in 20%. An α-*gem*-difluoro analogue was not turned over by the enzyme. Their method provided access to 5-10 mg quantities of the phosphonate sugar-nucleotides in 10-55% yields and the broader results of their study provided important insight into the effect of α-monofluorination upon glycosyltransferase activity, correlating factors of enzyme turnover, ketose mutarotation and phosphonate acidity for this class of mimetic sugar-nucleotide.



Scheme 9. *dTDP-ketose phosphonate sugar-nucleotide synthesis catalysed by Cps2L.*

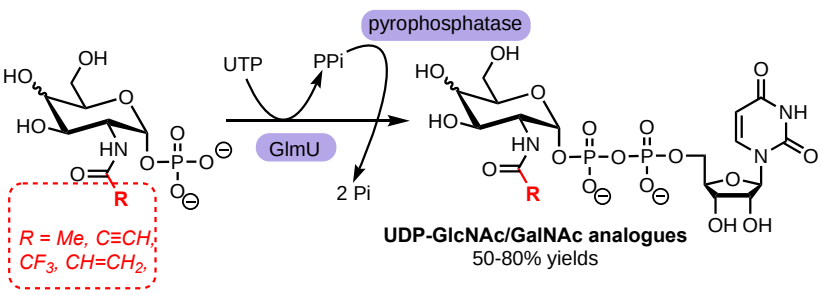
The group also reported the first synthesis of a dTDP-Glc-1-thiophosphonate again using Cps2L.[28] A non-scissile glycosyl thiophosphonate analogue (*Scheme 10*) was evaluated as a Cps2L substrate, forming the desired dTDP-glycosyl thiophosphonate with a 15% conversion rate (ESI-MS confirmed that an *O*-pyrophosphate had formed). Only one peak was observed by HPLC for the thiophosphonate product, which inferred that Cps2L may distinguish between *pro*-R and *pro*-S thiophosphonate oxygen atoms; a similar observation having been made for pro-*R* and pro-*S* α-monofluorinated phosphonate (*Scheme 9*).



Scheme 10. *Csp2L catalysed synthesis of the first dTDP-Glc-1-thiophosphonate analogue of Glc-1-phosphate.*

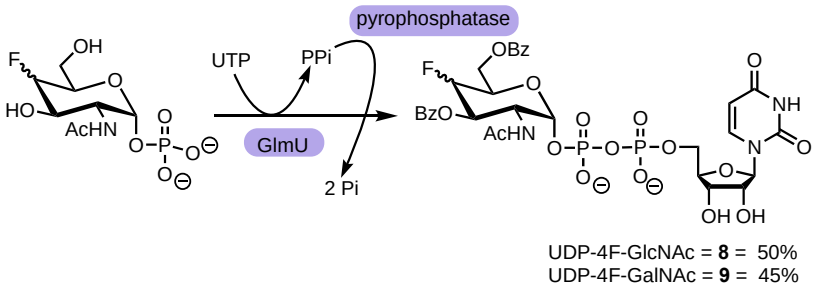
Most recently the group have extended their investigation of Cps2L promiscuity (and a GDP-ManPP) against a panel of eight fluorinated isosteric Glc 1-phosphates, broadly reaffirming a defined utility for Cps2L in the advancement of enzymatic synthesis of mimetic sugar nucleotides and indicating that the acidity of the phosphate nucleophile and the hexose configuration each contribute to substrate specificity.[29] The Lowary group also used Cps2L for an enzymatic synthesis of dTDP-Gal*f*, obtaining the sugar-nucleotide in 50% yield from Gal*f* 1-phosphate using Cps2L immobilised on a Ni-NTA agarose resin.[12a=12a]

In 2012, Linhardt *et al.* reported a chemical synthesis of eight different GlcNAc 1-phosphate and GalNAc 1-phosphate analogues and tested their capability with GlmU (*Scheme 11*).[30] Substrates containing an amide linkage at C2 were transferred by GlmU to afford their corresponding UDP sugar-nucleotides in good yields (50-80%). The studies demonstrated that whilst the presence of an amide at C2 was essential (no conversion was observed with $GlcN_3$ or $GlcNH_2$), the C4 OH did not appear to play a critical role in enzyme substrate recognition as UDP-GalNAc was obtained in 70% yield. The group were also able to demonstrate immobilisation of GlmU onto Ni Sepharose beads and subsequent sugar-nucleotide formation, providing a useful platform to develop a technology for GlmU recycling and its use in continuous processes.



Scheme 11. *Enzymatic synthesis of modified UDP-GlcNAc and UDP-GalNAc using GlmU from E. coli*

Following on from this the group also reported a first chemoenzymatic synthesis of 4''-fluorinated UDP-GlcNAc and UDP-GalNAc using GlmU (*Scheme 12*).[31] Synthetically prepared 4-fluoro-hexosamine 1-phosphates were accepted by GlmU, providing the corresponding UDP-sugar nucleotides in moderate yields (45-50%) and further concluding that the configuration of the pyranose 4-OH appeared not to play a critical role for turnover by the enzyme.

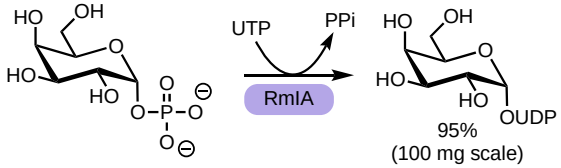


Scheme 12. *Chemoenzymatic synthesis of **8** and **9** using GlmU.*

Liu and co-workers have also utilised the promiscuity of GlmU for the provision of UDP-GlcNTFA, from GlcNTFA 1-phosphate.[32] This has proven to be a powerful sugar-nucleotide tool for the chemoenzymatic assembly of heparan sulfate glycosaminoglycans, offering a unique nitrogen protecting group to effect site specific *N*-sulfation within the final oligosaccharide targets.[33]

GlmU from *Pasteurella multocida* (PmGlmU) has also been used as part of a successful cascade process for the development of tools to highlight epigenetic modifications. PmGlmU successfully transferred 6-deoxy-6-azido-Glc to UTP which was then used by a glucosyltransferase to glycosylate DNA 5-hydroxymethylcytosine residues, affording the required '6-azide for isolation or fluoro-tagging using click chemistry.[34] PmGlmU was also used successfully by Chen as part of a OPME system for synthesising a series of UDP-GlcNAc derivatives, including modifications to the *N*- and 6-poistions of Glc, including the incorporation of *O*-sulfate.[35]

In 2012, Lin and co-workers reported the synthesis of UDP-Gal using RmIA, a glucose 1-phosphate thimidylyltransferase used to synthesise TDP-Glc in nature.[36] Direct synthesis of UDP-Gal was achieved in the presence of wild-type RmIA, with this method proving scalable, delivering 100 mg of UDP-Gal in 90% yield (*Scheme 13*). The group also utilised RmIA for a sequential synthesis of a series of sialylated oligo-LacNAcs.[37] RmLA was harnessed in a cost saving method to synthesise UDP-Gal and UDP-GlcNAc from the corresponding sugar 1-phosphates in one pot. These sugar-nucleotides were in turn derived from their free sugars using the kinases MtGalK (for Gal 1-phosphate) and NahK (for GlcNAc 1-phosphate). UDP-Gal and UDP-GlcNAc were then used with two bacterial glycosyltransferases to produce defined lengths of oligo-LacNAc. Their approach showed a powerful application of a thermophilic thymidylyltransferase to effect multiple sugar-nucleotide syntheses in one pot.



Scheme 13. *Scaled synthesis of UDP-Gal using RmIA from A. thermoaerophilus.*

In Table 1 we summarise the enzymes available for the synthesis of UDP and dTDP sugar-nucleotides. There are several plant, bacterial, human and archaeal options, offering access to a range of targets and a growing resource of substrate-enzyme SAR for both functional group modifications and hexose substrate promiscuity. New enzymes are also emerging, typified by a recent report from Routier *et al.* who described the characterisation of a new bacterial UDP-sugar pyrophosphorylase from *Leishmania*.[38] The enzyme displayed a close relation to plant UDP-sugar pyrophosphorylases, capable of providing UDP-Gal without the Leloir pathway dependence of UDP-Glc and was able to process Gal- and Glc 1-phosphates along with other hexose and pentose 1-phosphates, but notably not hexosamine 1-phosphates.

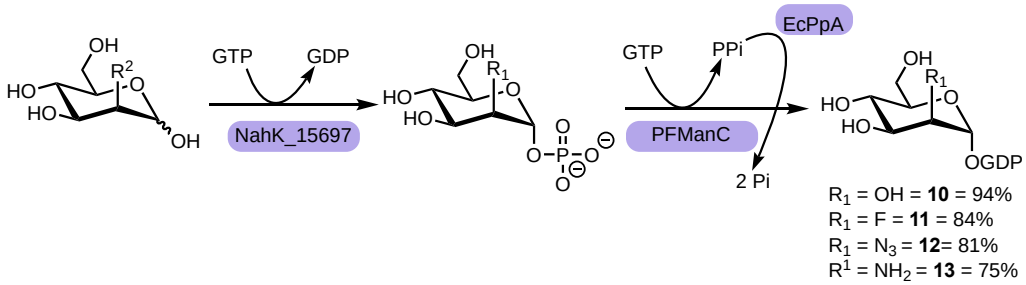
UDP and dTDP Sugar Nucleotides			
Enzyme	Source	Target sugar-nucleotide(s)	Ref(s).
GalU	*E. coli*	UDP-Glc 5-position base modified UDP-Glc (5-I, 5-furanyl)	8
BLUSP	*B. longum*	UDP-GlcA	15
AtUSP	*A. thaliana*	UDP-GlcA UDP-GalA UDP-Gal UDP-CH_2-Gal*p*	15 15 17 19
SpGalU	*S. pneumoniae*	UDP-Glc UDP-Gal UDP- L-Man 2, 4 and 6 modified UDP-Gal	20
AGX1	*Human*	UDP-GlcNAc UDP-GalNAc NAc and 6-position modified UDP-Gal and UDP-Glc	23
GalPUT*	*E. coli*	UDP-Gal 5-position base modified UDP-Gal (5-I, 5-furanyl) 6-deoxy-6-fluoro UDP-Glc	8 11
GalPUT		UDP-Gal*f* and 2,5,6-OMe modifications	12
Cps2L	*S. pneumoniae*	dTDP-gluco-ketose phosphonates dTDP-Glc phosphonate UDP-Glc phosphonate dTDP-Gal phosphonate dTDP-Glc thiophosphonate dTDP-Gal*f*	27 25 25 25 28 12a
GlmU	*E. coli*	UDP-4-F-GlcNAc	31

	P. multocida	UDP-4-F-GalNAc UDP-GlcNTFA UDP-Glc/GalNAc *N*-modifications UDP-GalNAc UDP-6-deoxy-6-azido-Glc UDP-GlcNAc *N*- and 6-position modifications	31 39 30 17 34 35
RmIA	*A. thermoaerophilus*	UDP-Gal UDP-GlcNAc	36 37

Table 1. *Summary of enzymes recently used to synthesise UDP and dTDP sugar-nucleotide targets. *with GalE and Galk.*

3.2. Towards GDP sugar-nucleotides

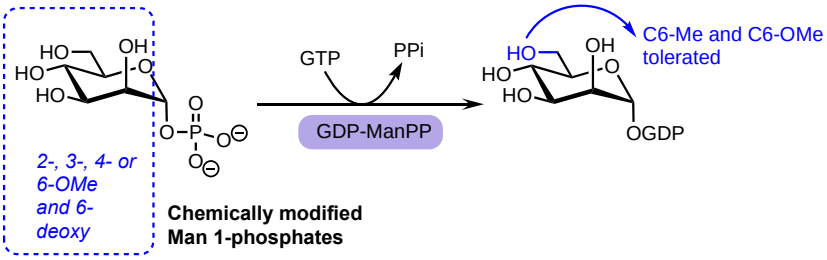
Wang and co-workers reported an efficient enzymatic synthesis of GDP sugar-nucleotides and their derivatives using a one-pot, three enzyme approach, starting from monosaccharide hemi-acetals (*Scheme 14*).[39] Glycoside 1-phosphate formation was catalysed by *N*-acetylhexosamine 1-kinase (NahK_15697, previously established by the group as having mannose 1-kinase activity[40]), followed by a GDP-mannose pyrophosphorylase (PFManC), forming the required GDP-sugar. Pleasingly, the authors noted that whilst PFManC was known to work with ATP (to make ADP-sugars), they were able to use GTP as the sole phosphate donor for the combined process. Finally, an inorganic pyrophosphatase (EcPpA) catalysed hydrolysis of pyrophosphate. Their approach was demonstrated for a variety of GDP-Man 2-position analogues, **10**-**13** **(***Scheme 13*), in high yields and also 4-azido GDP-Man. Furthermore, they were able to access a GDP-Glc series along with GDP-Tal, but also discovered that NahK_15697 was incompatible with 6-position modified mannose derivatives (ManA and 6-deoxy-Tal). Bulkier groups at C2 (NAc) were not accepted by PFManC for either Man or Glc 1-phosphates.



Scheme 14. *One-pot three-enzyme synthetic approach to GDP-Man and 2-position derivatives* ***10-13****. NahK_15697 from Bifidobacterium infantis, PFManC from Pyrococcus furiosus, EcPpA from E. coli.*

In 2012, Lowary reported a chemoenzymatic synthesis of GDP-Man analogues starting from a series of chemically modified deoxy and methoxy mannose-1-phosphates (*Scheme 15*).[41] The group utilised a pyrophosphorylase (GDP-ManPP) and demonstrated that whilst the enzyme was able to turnover their modified substrates, it was largely intolerant of methoxy substituents at the C2, C3 or C4 positions of mannose, exhibiting 6-, 14- and 17-fold decreases in activity respectively. However, 6-methoxy and 6-deoxy-mannose 1-phosphates showed relatively good activity (>75%), concluding that the 6-position hydroxyl group may not be inherently involved in substrate binding, the inverse of the requirements at C2, C3 and C4.

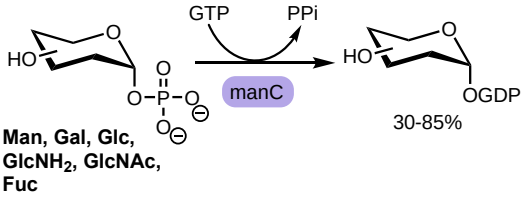
These findings correlated to previous work surrounding the substrate promiscuity of GDP-ManPP.[42]



Scheme 15. *Chemoenzymatic synthesis of GDP-Man analogues to probe the promiscuity of a GDP-ManPP from Salmonella enterica.*

In 2009, Pohl *et al.* reported a first chemoenzymatic synthesis of a range of sugar-nucleotides using a thermostable bifunctional phosphomannose isomerase/GDP-Man pyrophosphorylase (manC).[43] Previous work indicated that such archaeal enzymes had broad substrate specificity, providing potential for the synthesis of natural and non-natural sugar-nucleotides, otherwise difficult to access chemically.[44] ManC was first investigated for NTP tolerance, with results showing that the enzyme was highly permissive, accepting all five naturally occurring NTPs in the presence of mannose-1-phosphate; the corresponding NDP-mannoses were afforded in yields exceeding 80%, with the exception of dTDP-mannose (43%, *Scheme 16*).

The enzyme also showed relatively high activity against Gal-, Glc-, $GlcNH_2$-, GlcNAc- and Fuc-1-phosphates when reacted with GTP, affording the corresponding sugar-nucleotides in yields between 30-85% on multi-milligram scales. Given the unusual substrate promiscuity observed for manC, a truncated mutation was created to investigate the role of the C-terminal domain in overall enzyme activity and substrate specificity. The mutant accepted its natural substrates Man 1-phosphate and GTP, but exhibited 100-fold lower GDP-mannose pyrophosphorylase activity and no longer accepted other NTPs or sugar-1-phosphates, suggesting the C-terminal domain was crucial for the observed substrate promiscuity.

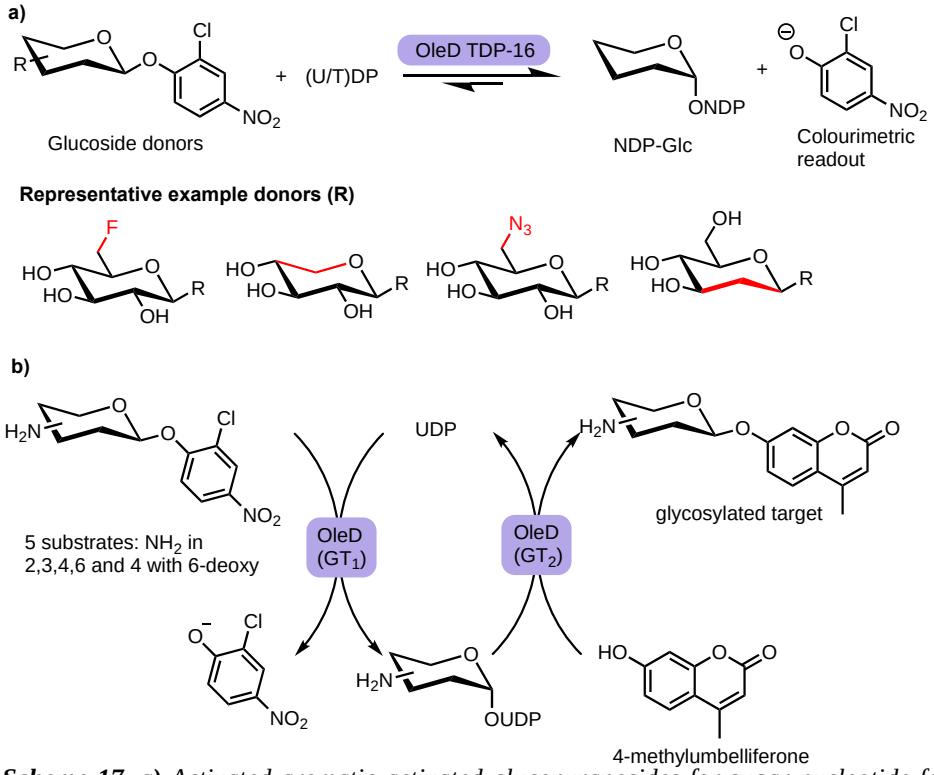


Scheme 16. *Sugar-nucleotide synthesis catalysed by manC from Pyrococcus furiosus.*

3.3. Emergent enzymes for sugar-nucleotide synthesis

GT catalysed reactions are known to be readily reversible and highly substrate dependent. The majority of GTs are Leloir (sugar-nucleotide dependent) enzymes and consume NDP-sugars as donors for glycoside bond formation to an aglycone partner. This renders the reverse reaction, to form a sugar-nucleotide, thermodynamically unfavourable. In 2011, Thorson and co-workers reported a landmark discovery for the use of activated sugar donors that could dramatically and favourably shift the equilibrium of a GT-catalysed reaction towards sugar-nucleotide formation.[45] In order to overcome the thermodynamic barrier, activated sugar donors, aromatic *O*-glycosides, were used to alter the equilibrium position of an inverting macrolide-inactivating glycosyltransferase mutant, OleD, variant TDP-16 (*Scheme 17a*). 2-chloro-4-nitrophenyl β-D-glucopyranoside was optimised as the donor from a screen of 32 putative glycosyl donors.

The OleD mutant was shown to catalyse the formation of UDP-Glc and TDP-Glc in good yield (55% and 61% respectively) using a 1:1 molar ratio of UDP or TDP to glycoside donor and on a representative scale (6-7mg). $\Delta G°_{pH8.5}$ for the reaction was calculated at -2.78 kcal mol^{-1}, notably exothermic and corresponding to a dramatic shift in K_{eq}, favouring sugar-nucleotide formation. An important observation was that donor hydrolysis was not a detrimental competing reaction. A wider evaluation of the Glc-donor component revealed OleD TDP-16 to tolerate deoxygenation at C2, C3, C4 or C6, C3 epimerization and an array of substitution at C6 (SH, N_3, Hal), providing an impressive access to 22 natural and non-natural T/UDP-sugars. The group proceeded to use their sugar-nucleotide forming platform for single and dual GT coupled reactions, alongside a colourimetric screen for NDP-sugar formation (provided by the phenolate by-product of the GT reaction), opening access to glycoconjugates derived from sugar-nucleotide donors.

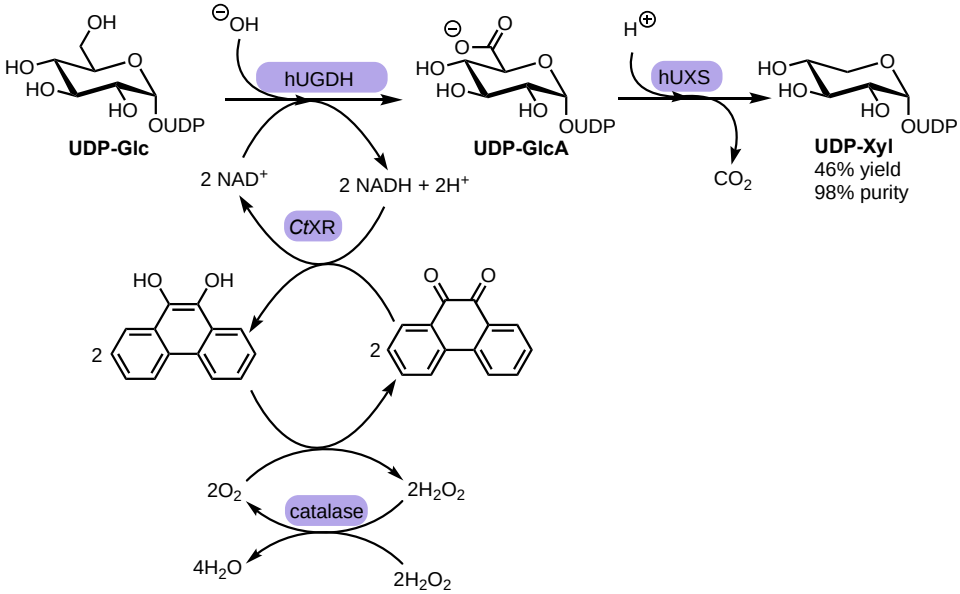


Scheme 17. a) *Activated aromatic activated glucopyranosides for sugar-nucleotide formation with GT mutant OleD, from Streptomyces antibioticus* ***b)*** *OleD Loki catalysed transglycosylation reactions of 2-chloro-4-nitrophenyl glucosamino/xylosamino donors to afford 4-methylumbelliferone glucosamino-/xylosaminosides.*

The workers have recently expanded the capability of their GT mutant, evolving the OleD sequence to an OleD Loki variant, capable of recognising six uniquely functionalised sugar donors *and* five structurally different NDP acceptors, broadening the scope of the process further to 30 distinct sugar-nucleotides.[46] OleD Loki has also been used to access aminosugar-nucleotides, which serve as important structural building blocks for a range of glycoconjugates.[47] The mutant enzyme converted 12 of 14 aminosugar glycosyl donors to the target T/UDP-aminosugars and, despite showing overall preference for glucosides, also accepted non-native donors such as Glc-6-NAc (*Scheme 17b*). The OleD Loki system was also able to mediate glycosylation of a model acceptor, fluorescent 4-methylumbelliferone, permitting coupled transglycosylation processes and a series of model 4-methylumbelliferone glucosamino-/xylosaminosides were produced (*Scheme 17b*).

UDP-Xyl acts as the donor substrate for xylosylation of different acceptor molecules, typified by proteoglycan biosynthesis where it transferred to a protein acceptor by a xylosyltransferase (XylT). Access to this sugar-nucleotide is vital for the study of XylTs from the perspective of inhibitor strategies and the functional cell biology of the enzyme. In 2014, Nidetzky reported an *in vitro* one-pot synthesis of UDP-Xyl using UPD-Glc as a donor substrate *via* an oxidative decarboxylation process (*Scheme 18*).[48] A two-step conversion of UDP-Glc to UDP-Xyl *via* UDP-GlcUA was catalysed by UDP-glucose dehydrogenase (hUGDH) and UDP-xylose synthase (hUXS). hUGDH was coupled to a NAD^+ coenzyme regeneration cascade using a xylose reductase (*Ct*XR) and bovine liver catalase.

NAD^+ was regenerated by reduction of 9,10-phenanthrenequinone (PQ) with *Ct*XR, affording 9,10-phenantheren hydroquinone (PQH_2), which was spontaneously re-oxidised by molecular oxygen, supplied *in situ* by periodic feeding with H_2O_2. Moreover, the reduction of molecular oxygen provided a strong thermodynamic driving force for the cycle. The workers optimised an efficient one pot, two-step system as they initially observed that a one pot, one-step process produced UDP-Xyl which inhibited hUGDH, retarding the efficiency of the system. Henceforth, they first converted UDP-Glc to UDP-GlcA, optimising the reaction parameters to 37 °C and pH 7.5, which were amenable to the second hUXS step. Their approach enabled production of 19.5 mM (10.5 g L^{-1}) of UDP-Xyl which, after a two-step chromatographic purification, was obtained in high purity (>98%) and good overall yield (5.3 mg, 46%).



Scheme 19. *Synthesis of UDP-Xyl via chemoenzymatic redox cascade through the combined action of human hUGDH, hUXS, CtXR from Candidia tenuis and bovine catalase.*

The results provide a strong case for application of multi-step redox cascades in the synthesis of sugar-nucleotides as both the enzymes utilised exhibit no observable back reaction, which presents a clear advantage in having eliminated the thermodynamic restrictions of nucleotidyltransferase catalysed conversions or the need for the thermodynamic pull of a pyrophosphatase.

Recently, an in-microbe methodology to produce normal and labelled NDP-sugars has been developed by Bar-Peled and colleagues,[49] who demonstrated the use of *E. coli*, metabolically engineered, to contain genes that encode proteins to convert monosaccharides into their respective monosaccharide-1-phosphates and subsequently into the corresponding sugar-nucleotide shown to yield 5 to 12.5 microg/ml cell culture. This presents an alternative method from classical *in vitro* chemoenzymatic synthesis and OPME processes.

5. Summary and Outlook

Since 2009 there have been considerable developments in enzymatic methods to access sugar-nucleotides. Broadly speaking, pyrophosphorylases dominate for the formation of UDP, GDP and dTDP systems, however, the advent of the OleD GT system has now extended this classification. Additionally, the volume of structure-activity data that now exists for these enzymes regarding their capability to accept and process non-native substrates is impressive and ever expanding. This is vital to the relevance of these sugar-nucleotide tools to interrogate biological glycosylation processes. Coupled to this are ever improving analytical capabilities for multi-enzyme cascade reactions, evidenced most recently by Elling's multiplexed capillary electrophoresis (MP-CE), a fast analytical tool for the optimisation of sugar-nucleotide synthesis using multi-enzyme cascade reactions.[50]

Combined with these enzymatic capabilities is the contributions of chemical synthesis for modification of both the glycosyl 1-phosphate and nucleotide components and the symbiosis of these two approaches will ensure that that the field of sugar-nucleotide synthesis continues to prosper.

Acknowledgements

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