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Flexible enzymatic and chemo-enzymatic approaches to a broad range of uridine-diphospho-sugars†

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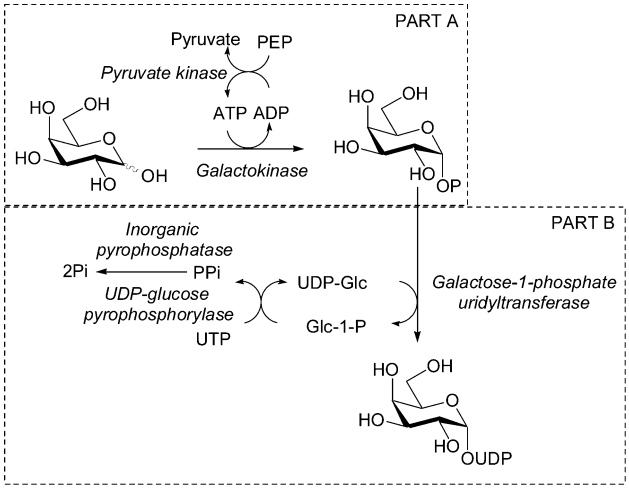
Enzymatic and chemo-enzymatic approaches provide straightforward access to uridine diphospho sugars irrespective of the relative and absolute configuration of the sugar, its ring size and substitution pattern.

The rise of glycobiology leaves an increasing need for new tools and probes to investigate the biological function of carbohydrates.[1] For instance, the widespread occurrence of glycosides in medicinal natural products[2] has attracted particular attention in relation to whole organism glycosylation engineering to modify secondary metabolite biological activity.[3] Other genetic engineering approaches have reassembled biosynthetic pathways for large-scale carbohydrate synthesis.[4] *In vitro* glycosylation engineering overcomes issues of sugar uptake, processing and/or dilution into an assortment of biochemical pathways, but requires a supply of activated sugar nucleotide building blocks.[5] In this study we set out to investigate enzymatic and chemo-enzymatic approaches to UDP-sugars in the search for generic methods that can be applied widely.

In connection with studies on microbial LPS biosynthesis, we had a need to prepare a range of UDP-sugars. Morpholidate-based pyrophosphate syntheses are notoriously unreliable, often giving only moderate yields after long reaction times and generating by-products that can be problematic to remove.[6] Alternatively, enzymatic approaches provide attractive routes to sugar nucleotides, as demonstrated by Whitesides and co-workers a while ago.[7] More recent work by Thorson and co-workers[8] with native and mutant RmlA enzymes shows how these enzymes can catalyse the formation of a wide range of dTDP- and UDP-based α-D-pyranose sugar nucleotides from the respective sugar-1-phosphates. In addition, Wang and co-workers[9] have developed immobilised multi-enzyme systems—"superbeads"—for the wholly enzymatic generation of UDP-galactose, for instance, from the corresponding reducing sugar. We chose to work with native enzymes (but possessing a His_6 tag) rather than engaging in potentially complicated, but effective, structure-based redesign[10] or forced evolution studies.[11] Based on the "superbeads" approach,[4,9] a one-pot multi-enzyme approach to UDP-galactose analogues from the corresponding reducing sugar was explored (Scheme 1, parts A and B). The use of key enzymes in immobilised form allows their use in excess, facilitates enzyme recovery and dramatically simplifies product purification. As is clear from Table 1, this approach shows a good deal of flexibility in the reducing sugars that can be transformed into the corresponding UDP-adducts in practical yields.

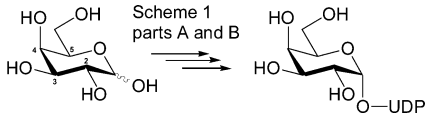
The one-pot, wholly enzymatic approach is attractive due to its simplicity, although in some cases (*e.g.* 2- and 3-azidodeoxygalactose) this approach proved ineffective. This raised the question: was the initial phosphorylation step rate limiting, or was the uridylation reaction the limiting step? Wild-type galactokinase from a number of sources has been shown to tolerate only a limited range of substrates,[14] suggesting that the initial phosphorylation step may be the problem.



Scheme 1 One-pot enzymatic synthesis of UDP-galactose.[4,9]

Following on from the work outlined in Scheme 1 (parts A and B) and Table 1, and hampered by the inflexibility of native galactokinase,[14,19,20] an alternative approach to UDP-sugars was also explored. A logical chemo-enzymatic approach to sugar nucleotides involves the chemical synthesis of sugar-1-phosphates, followed by enzymatic transformation to the nucleotide adduct (Scheme 1 part B). For a number of sugars for which

Table 1 One-pot enzymatic synthesis of UDP-D-galactopyranose analogues from reducing sugars according to Scheme 1, parts A and B[12]



Modified position	Substituent[a]	Yield (%)[13]
—	D-Galactose	98
C-2	–H	98
C-2	–F	97
C-2	$–NH_2$	95
C-2	–NHAc	19
C-2	$–N_3$	—[b]
C-3	–F	64
C-3	$–N_3$	—[b]
C-5	$–CH_3$ (D-fucose)	57
C-5	$–CH_2F$	37
C-5	–H (L-arabinose)	47

[a] Substituents replace –OH or $–CH_2OH$ groups with retention of configuration. [b] Production of the UDP-sugar adduct was not detectable by RP ion-pair HPLC.

† Electronic supplementary information (ESI) available: cloning and over-expression details; protein production and purification; collated spectroscopic information for all compounds reported. See http://www.rsc.org/suppdata/cc/b4/b410184g/

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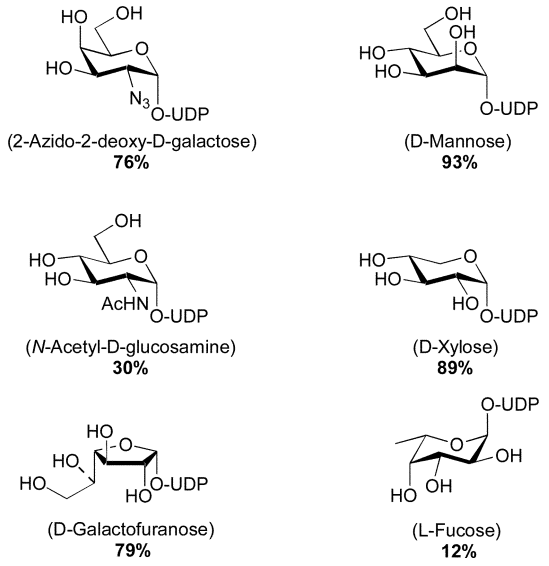

Fig. 1 UDP-sugars prepared from the corresponding sugar-1-phosphates according to Scheme 1, part B only.[21]

phosphorylation by galactokinase was not detectable (as judged by TLC; 2-azido-2-deoxy-D-galactose, D-mannose, D-xylose, L-fucose) the corresponding anomeric phosphates were either obtained from commercial suppliers or prepared by chemical synthesis, and subjected to the action of galactose-1-phosphate uridyltransferase (Scheme 1, part B only; Fig. 1).

In stark contrast to galactokinase, galactose-1-phosphate uridyltransferase appears to possess a remarkably relaxed substrate specificity (Fig. 1). For instance, not only will it accept D-galactopyranose-1-phosphate as a substrate, but it will also take the geometrically unrelated D-galactofuranose-1-phosphate, providing straightforward access to UDP-galactofuranose[22] (the latter is a substrate for a novel mutase enzyme involved in galactose metabolism in pathogenic microorganisms[23]). Further, native galactose-1-phosphate uridyltransferase will also accept L-fucose-1-phosphate as a substrate, providing access to UDP-adducts of the antipodal L-series of sugars.[20]

In summary, we have identified straightforward enzymatic and chemo-enzymatic approaches to UDP-sugars. The latter is seemingly unaffected by the relative and absolute configuration of the sugar, its ring size and substitution pattern. The apparently relaxed substrate specificity of native galactose-1-phosphate uridyltransferase noted in this study serves to emphasise the need to be cautious in making use of sequence-based gene function assignments in the search for new chemically competent biocatalysts (as opposed to those that are kinetically competent to effect physiological processes). In general, even at low efficiency ($<1\%$ of the rate of reaction with the natural substrate), biotransformations can still provide straightforward access to biomolecules that would be laborious to prepare by chemical synthesis. This study also highlights the ease with which flexible catalysts can be accessed directly from nature without recourse to elaborate protein engineering.

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13 *Typical 1-pot enzymatic procedure* (adapted from Wang *et al.*[49]): UTP (20 mg 36 μmol), ATP (0.1 mg 0.18 μmol), PEP (14 mg, 52 μmol), UDP-glucose (0.1 mg 0.16 μmol) and monosaccharide (10 mg 55 μmol) were dissolved in buffer (500 μl, 50 mM HEPES, pH 8.0, 5 mM KCl, 10 mM $MgCl_2$). Pyruvate kinase (50 U), glucose-1-phosphate uridyltransferase (5 U), inorganic pyrophosphatase (5 U), immobilised galK and galPUT were added (0.5 ml resin containing 100 U of galK and 75 U of galPUT; *for weak substrates additional units were used—see ESI*†). The reaction was flushed with nitrogen and stirred at 30 °C for 8 hours whilst being monitored by HPLC. The immobilised enzymes were removed by centrifugation and the remaining solution was spin-filtered (Amicon YM10). The flow through was applied to a Poros HQ50 anion exchange column (3 ml; Perceptive Biosystems) and eluted with a linear gradient of NH_4HCO_3 (5 to 250 mM over 45 column volumes; 20 ml min^{-1}). Sugar nucleotides, which typically eluted at around 50 mM bicarbonate, were detected at 265 nm. Relevant samples were concentrated and NH_4HCO_3 was removed by lyophilisation to give required compounds as their diammonium salts.

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