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Sugar Nucleotide Recognition by *Klebsiella pneumoniae* UDP-D-Galactopyranose Mutase: Fluorinated Substrates, Kinetics and Equilibria

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Abstract

A series of selectively fluorinated and other substituted UDP-D-galactose derivatives have been evaluated as substrates for *Klebsiella pneumoniae* UDP-D-galactopyranose mutase. This enzyme, which catalyses the interconversion of the pyranose and furanose forms of galactose as its UDP adduct, is a prospective drug target for a variety of microbial infections. We show that none of the 2"-, 3"- or 6"-hydroxyl groups of UDP-D-galactopyranose are essential for substrate binding and turnover. However, steric factors appear to play an important role in limiting the range of substitutions that can be accommodated at C-2" and C-6" of the sugar nucleotide substrate. Attempts to invert the C-2" stereochemistry from equatorial to axial, changing D-galacto- to D-talo-configuration, in an attempt to exploit the higher percentage of furanose at equilibrium in the talo-series, met with no turnover of substrate.

Keywords

Fluorosugar nucleotides; UDP-D-galactopyranose mutase; Mechanism; Equilibrium

Introduction

The rise in the incidence of tuberculosis worldwide needs urgent attention. Resistance to current drugs is a major issue and very few new candidates are currently in clinical trials.¹ Considerable effort is being made to identify new actives for development,^{2,3} but, given the

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issues of drug resistance, novel drug targets for the treatment of tuberculosis are also being sought.⁴ Given the precedent for effective drugs acting on mycobacterial cell wall biosynthesis,^{5,6} we were attracted by the notion of targeting cell wall biosynthetic enzymes that have not been the subject of drug development programmes to date. In particular, the central role played by D-galactofuranose (*GalF*) in cell wall integrity,⁵ and the absence of this 5-membered ring form of D-galactose in man, prompted us to consider D-galactofuranose biochemistry.

Originally confirmed in *Escherichia coli*,⁷ the key enzyme for the incorporation of galactofuranose into cell surface glycan is UDP-D-galactopyranose mutase, a novel flavoprotein that is capable of reversibly interconverting UDP-D-galactopyranose (UDP-D-Galp) and UDP-D-galactofuranose (UDP-D-GalF). The latter in turn serves as a donor substrate for a galactofuranosyltransferase-mediated incorporation of *GalF* into growing oligosaccharide chains.⁸ An orthologous UDP-D-galactopyranose mutase was subsequently identified in *Klebsiella pneumoniae*⁹ and in mycobacterial species.¹⁰ More recent studies have shown that the UDP-D-galactopyranose mutase-encoding *glf* gene also occurs in a wide variety of eukaryotic microbial and metazoal pathogens, including the human parasites *Leishmania major* and *Trypanosoma cruzi*, and the fungus *Cryptococcus neoformans*, among others.¹¹ Hence, UDP-D-galactopyranose mutase may represent a potential drug target for a number of human microbial pathogens.

UDP-D-galactopyranose mutase has both a novel structure¹² and a unique catalytic mechanism (Scheme 1).¹³ It has been proposed that the reduced form of flavin co-factor¹⁴ attacks the anomeric position of the UDP-D-galactose substrate displacing UDP, which is supported by positional isotope exchange experiments.¹⁵ The flavin is then able to drive cleavage of the O5-C1 bond resulting in opening of the sugar ring. Subsequent attack of O4 on C1 achieves formation of a furanose intermediate, which is intercepted by UDP with concomitant release of the flavin (Scheme 1). Other studies have shown that 1,4-anhydrogalactopyranose is not an intermediate¹⁶ in the mutase-catalysed reaction, but there is support for oxocarbenium ion character to the reaction mechanism.¹⁷ STD-NMR spectroscopy^{18,19} and site-directed mutagenesis studies²⁰ have been employed in attempts to define the mode of substrate binding and to identify key substrate-binding residues. Numerous attempts have been made to develop inhibitors of UDP-D-galactopyranose mutase,²¹ with recent success leading not only to in vitro enzyme inhibition but also inhibition of the growth of *Mycobacterium smegmatis* (a non-pathogenic, fast-growing relation of *M. tuberculosis* that is often used for initial whole organism screening).²²

Despite the wealth of information that is now available about UDP-D-galactopyranose mutase, we still lack definitive information about the molecular basis of substrate binding and specificity. A better understanding of the substrate tolerance of this enzyme could be exploited for the generation of potential inhibitors of therapeutic value. Building on our interest in the use of synthetic and unnatural sugar nucleotides to assess enzyme specificity,²³ herein we report the generation of sugar nucleotides to probe substrate specificity of UDP-D-galactopyranose mutase. We set out to extend the observations of Barlow and Blanchard²⁴ on the effect of turnover and equilibrium position of substituting fluorine for either the 2- or 3-OH groups on UDP-D-galactopyranose (similar studies in the furanose series have been reported by Zhang and Liu²⁵). Specifically, we report investigations of 2- or 6-substitution of the galactopyranose unit of UDP-D-Galp on the kinetics of mutase action and the equilibrium position of the enzyme-catalysed reaction. These studies have relied on fluorosugar nucleotides, in particular, given that the C-F bond has interesting properties^{26,27} that have attracted considerable attention from the pharmaceutical sector.²⁸

Results and discussion

Preparation of sugar nucleotides

The enzymatic synthesis of uridine 5'-diphospho- α -D-galactopyranose (UDP-Gal) using galactokinase (GalK; E.C. 2.7.1.6) and galactose-1-phosphate uridylyltransferase (GalPUT; E.C. 2.7.7.12) was first reported by Whitesides and coworkers.²⁹ Subsequent use of these enzymes in recombinant form by Wang and coworkers enabled straightforward immobilisation of these catalysts.³⁰ Adopting the Wang approach, we demonstrated the utility of GalK and GalPUT, acting together and GalPUT alone, for effecting transformation of a range of natural and unnatural reducing sugars and sugar-1-phosphates, respectively, to their UDP-sugar adducts.³¹ In particular, the catholic nature of GalPUT enables its use to prepare UDP-D-galactofuranose from synthetic α -D-galactofuranose-1-phosphate.³²¹ In the current study, a range of sugar nucleotides (*vide infra*) were prepared using the GalK/GalPUT procedure, as described previously.³¹ In the case of UDP-6-deoxy-6-fluoro-D-galactose, both commercial 6-deoxy-6-fluoro-D-galactose and racemic 6-deoxy-6-fluoro-galactose from *de novo* synthesis³⁴ were efficiently processed to give UDP-6-deoxy-6-fluoro-D-galactose.

Evaluation of sugar nucleotides as substrates for UDP-galactopyranose mutase

Fluorinated UDP-D-galactose derivatives—Using selectively 2''- and 3''-fluorinated substrates, the work of Barlow and Blanchard²⁴ clearly shows that the UDP-D-galactopyranose mutase-catalysed reaction reaches an equilibrium position that reflects that of the corresponding reducing sugar. In addition, fluorine impacts on the rate of reaction in a manner that is dependent on its position with respect to the anomeric centre, as one would expect for a reaction mechanism that possesses carbenium ion character. We have extended this study to the corresponding 6''-fluorinated substrate (for consistency, we also report data produced in parallel for the 2''- and 3''-fluorinated substrates) (Table 1). Overall, these studies show that none of the 2'', 3''- or 6''-hydroxyl groups of UDP-D-Galp are essential for substrate binding and turnover. As with the Barlow and Blanchard work, as one 'walks' the fluorine atom around the galactose ring the rate of mutase-catalysed reaction is affected: a drop of over 50,000 fold for the 2''-fluoro compound, nearly 400 fold for the 3''-fluoro compound and only a 10 fold drop for the 6''-fluoro compound. The equilibrium position reached for the 6''-fluoro compound reflects that of the corresponding reducing sugar, as previously reported for the 2''- and 3''-fluorinated substrates.²⁴

2''-Modified UDP-D-galactose derivatives—Clearly 2''-fluorination of UDP-D-Galp has a profound impact on substrate turnover. We therefore looked further at 2''-substituted substrate analogues, focussing on the azide, amine and acetamide series (Table 2). The 2''-azido and 2''-acetamido compounds were not mutase substrates in our hands, whereas the 2''-amino compound proved to be a weak substrate (slightly increased K_m ; k_{cat} reduced ~14 fold). It is conceivable that the lack of observed turnover for the azide and acetamide is due to a complete lack of furanose at equilibrium for these sugars. The older literature³⁶ suggests that galactosamine and *N*-acetyl galactosamine exist exclusively in the pyranose form at equilibrium in aqueous solution (although this probably reflects the accuracy of available techniques at the time). However, this is not the case, with around 5% furanose being measurable in both cases by 600 MHz ¹H NMR spectroscopy (Table 2). We therefore conclude that steric constraints around the 2''-position of the substrate impact on recognition.

¹Using this procedure hundreds of mgs of UDP-Gal can be prepared (T. L. Lowary, personal communication), a key step forward for the characterisation of mycobacterial galactofuranosyltransferase activities.³³

6''-Variants of UDP-D-galactose derivatives—6''-Modified substrates for the mutase also produced intriguing results. Replacement of -OH by -F is permissible (the 6''-OH group is not essential for substrate binding), but -OH to -H (giving D-fucose) apparently renders the molecule a non-substrate (Table 3).^{II} In contrast, whilst removal of the C-6 hydroxymethyl group altogether (giving L-arabinose) increases K_m ~4 fold, k_{cat} is also increased slightly (Table 3).^{III} These data suggest a need for hydrogen bonding at the 6''-position. Removal of the hydroxymethyl group altogether, giving L-arabinose, results in an active substrate. It is plausible that the loss of the bulky hydroxymethyl group could allow space for the inclusion of a water molecule, providing potential for hydrogen bonding. In contrast, steric restrictions would disallow hydrogen bonding to occur on going from galactose to the 6''-deoxy compound.

From galactose to talose

UDP-D-galactopyranose mutase is an awkward enzyme to assay. In the forward direction, one only has an 8% conversion (to furanose) to play with. Despite the accessibility of UDP-D-Gal^β¹ to assay the reverse reaction (to pyranose), the 1,2-cis arrangement of UDP and the 2-hydroxyl on the furanose ring renders UDP-D-Gal^f prone to degradation via 1,2-cyclic phosphate intermediates. Hence a more robust pyranose-based substrate that on exposure to UDP-D-galactopyranose mutase gives a substantial proportion of the UDP-D-Gal^f equivalent would be highly desirable. Given the need to leave the pyranose C-4 axial in order for the mutase to act, D-talose attracted our attention. D-Talose was of interest to us due to the fact that a substantial amount (29%) of the reducing sugar is in the furanose form at equilibrium (Table 4). We were therefore motivated to prepare and evaluate UDP-D-talopyranose. In addition, considering the potential electronic demands that might be made by placing the 2-hydroxyl group axial, we also considered the corresponding fluoro compound,²⁶ UDP-2-deoxy-2-fluoro-D-talopyranose, where the corresponding reducing sugar shows 13% furanose at equilibrium³⁹ (Table 4).

Chemoenzymatic synthesis of UDP-2-deoxy-2-fluoro-D-talose

The literature synthesis of 2-deoxy-2-[¹⁸F]fluoro-D-talose from 1,2-*O*-isopropylidene-α-D-galactofuranose⁴⁰ employing nucleophilic fluorination (K¹⁸F) proceeded in 7 steps and 24% overall yield.³⁹ We were motivated to develop a shorter synthesis of 2-deoxy-2-fluoro-D-talose (**4**) that avoided using a galactofuranose system since, beyond the parent compound which is accessible from commercial galactono-1,5-lactone,³¹ such derivatives can be complicated to prepare. In our hands, attempts to introduce the fluorine by an S_N2 reaction using TBAF or DAST on an α-D-galactopyranose substrate were unsuccessful, with either no reaction occurring, or the formation of elimination products. We previously experienced similar issues when attempting to synthesise 3-deoxy-3-thio-galactopyranose derivatives.⁴¹ This prompted us to explore a strategy involving electrophilic fluorination of glycols using SelectfluorTM, which has been extensively studied.^{42,43} Treatment of protected D-galactal with SelectfluorTM gives solely the D-galacto-configured product⁴² whilst D-glucal gives a mixture of the D-manno- and D-gluco-isomers.^{44,45} Accordingly, our approach involved the preparation of a 2-deoxy-2-fluoro-D-mannose derivative from appropriately protected D-glucal and subsequent C-4 inversion to give the desired D-talo-configured compound. The previously reported 1-*O*-acetyl-3,6-di-*O*-benzyl-2-deoxy-2-fluoro-α,β-D-mannopyranose (**1**)⁴⁶, which was prepared from 3,6-di-*O*-benzyl-D-glucal, was converted to the C-4 triflate ester followed by inversion of stereochemistry using potassium nitrite in DMF⁴⁷ to give fluorotalose derivative (**2**) (Scheme 1). Of the reaction conditions investigated, the optimum

^{II} Again, in contrast to our observations (Table 3), the recent literature claims that "L-fucose mainly exists as the α- and β-pyran forms in solution with trace amounts of the furan forms."³⁷

^{III} The reverse biotransformation of UDP-Araf to UDP-Arap by UDP-galactopyranose mutase has been reported by Zhang and Liu.³⁸

temperature proved to be 50 °C where complete conversion was achieved after 4 days: at room temperature no reaction occurred, while at 80 °C substrate decomposition was observed. In the ^1H NMR spectrum of the product, coupling between both H-3 and H-4 ($J_{3,4}$ 3.0 Hz) and between H-4 and H-5 ($J_{4,5} < 1$ Hz) were small, as expected for the C-4 axial compound (**2**). The benzyl protecting groups were removed by catalytic hydrogenation with palladium on carbon. Attempted sodium hydroxide-mediated de-*O*-acetylation of (**3**) resulted in decomposition, presumably due to elimination of HF. However, de-*O*-acetylation was successfully achieved under acidic conditions using Dowex 50WX8 (H) resin in water at 80 °C. The ^1H NMR spectrum of the product was consistent with that reported previously for 2-deoxy-2-fluoro-D-talose (**4**).³⁹ Thus, our approach yielded 2-deoxy-2-fluoro-D-talose (**4**) in 4 steps and in 27% overall yield from (**1**).

Both 2-deoxy-2-fluoro-D-talose (**4**) and commercially available D-talose were tested in parallel as substrates for the GalK/GalPUT system.³¹ As can be seen in Table 5, in the conversion to the corresponding UDP adduct 2-deoxy-2-fluoro-D-talose was a good substrate for the GalK/GalPUT system – comparable to the natural substrate galactose and much better than talose. This might be attributable to the presence of a bulky C-2 axial substituent (hydroxyl) in talose, compared with the slightly smaller C-2 axial substituent (i.e. fluorine) in 2-deoxy-2-fluoro-talose, although replacement of O by F is a relatively conservative change in terms of both electronegativity and size.²⁶

Unfortunately, despite repeated attempts, we were unable to demonstrate by HPLC the turnover of either UDP-D-talose or UDP-2-deoxy-2-fluoro-D-talose by UDP-D-galactopyranose mutase. At this stage it is unclear whether this is due to steric constraints associated with recognition of the 2''- position of the sugar nucleotide (*vide supra*) or if the C-OH/C-F dipole impacts on attack on the substrate by the reduced flavin.

Conclusions

With the aid of a range of sugar nucleotide analogues we have demonstrated that none of the 2'', 3''- or 6''-hydroxyl groups of UDP-D-Galp are essential for substrate binding and turnover by UDP-D-galactopyranose mutase. However, there are clear constraints regarding permissible substitutions at C-6'' of UDP-Galp. At C-2'', whether in the equatorial D-galacto- or axial D-talo-configuration, again steric constraints come into play. As with correlating UDP-D-galactopyranose mutase and co-factor structure with mechanism of action, the precise detail of how and why substrate substitution impacts on binding and turnover remains to be resolved. Intriguingly, the recently reported plant UDP-L-arabinopyranose mutase does not require flavin for activity.⁴⁸ Further work is clearly required in order to understand these enigmatic ring contraction processes.

Experimental

Unless stated all chemical and biochemical reagents were supplied by Sigma Chemicals, Poole, Dorset, UK. Thin-layer chromatography (TLC) was performed on aluminium-backed, pre-coated silica gel plates (Silica Gel 60 F₂₅₄, Merck). Detection was typically effected under ultraviolet (UV) light, where applicable, followed by treatment with H₂SO₄ in EtOH (5% v/v) and charring at ~180 °C. Column chromatography was performed on silica gel (40-70 μm, BDH-Merck). Optical rotations were measured at 18 °C using a Perkin-Elmer 141 polarimeter. ^1H and ^{13}C NMR spectra were recorded on a Bruker Avance spectrometer at 600 MHz or on a Varian Unity Plus spectrometer at 400 and 100.6 MHz, respectively. Chemical shifts are expressed as parts per million (ppm, δ) and are relative to the solvent as an internal reference [CDCl_3 : δ 7.27 (s) for ^1H ; δ 77.0 (t) for ^{13}C ; D_2O : δ 4.67 (s) for ^1H]. Resonance assignments were made with the aid of COSY and HSQC experiments when necessary. ^{31}P NMR spectra were recorded on a Bruker Avance DPX-300 spectrometer at

121.5 MHz using 85% H₃PO₄ (δ 0.00) as an external reference. ¹⁹F NMR spectra were recorded on a JEOL Lambda spectrometer at 376 MHz using CFCl₃ (δ 0.00) as an external reference. Low-resolution (LR) electrospray-ionisation (ESI) mass spectra (MS) were obtained on a Waters ZQ4000 mass spectrometer. High-resolution (HR) electrospray-ionisation mass spectra were obtained on a Finnigan MAT 900 XLT mass spectrometer. Anion-exchange HPLC was performed on a BioCAD™ SPRINT™ system (PerSeptive Biosystems). Anhydrous CH₂Cl₂, THF and pyridine were distilled under N₂ according to Armarego and Chai.⁴⁹

Production of sugar nucleotides

Over-expression and purification of GalK and GalPUT were performed essentially as described previously.^{30,31} Purified GalK and GalPUT were dialysed against HEPES buffer pH 8.0 prior to use. The 2-, 3- and 6-mono-fluorinated variants of UDP-D-galactopyranose, UDP-D-fucopyranose and UDP-L-arabinopyranose were prepared as described previously.³¹

Synthesis of 2-deoxy-2-fluoro-D-talose

1-O-Acetyl-3,6-di-O-benzyl-2-deoxy-2-fluoro- α , β -D-mannopyranose (1)⁴⁶: Compound (1) was prepared, on a gram scale, according to the literature⁴⁶ as predominantly the α -anomer: ¹H NMR (CDCl₃): α -anomer; δ 7.25-7.39 (m, 10H, 2 \times OCH₂Ph), 6.24 (dd, $J_{1,2}$ 2.0, $J_{1,F}$ 6.4 Hz, H-1), 4.78 (d, 1H, J_{AB} 11.6 Hz, OCH₂Ph), 4.69 (d, 1H, J_{AB} 11.6 Hz, OCH₂Ph), 4.66 (ddd, 1H, $J_{1,2}$ 2.0, $J_{2,3}$ 2.4, $J_{2,F}$ 48.8 Hz, H-2), 4.62 (d, 1H, J_{AB} 12.0 Hz, OCH₂Ph), 4.55 (d, 1H, J_{AB} 12.0 Hz, OCH₂Ph), 4.08 (dd, 1H, $J_{3,4} = J_{4,5} = 9.6$ Hz, H-4), 3.80-3.86 (m, 1H, H-5), 3.64-3.79 (m, 3H, H-3, H-6a, H-6b), 2.71 (br s, 1H, 4-OH), 2.08 (s, 3H, OAc); ¹³C NMR (CDCl₃): α -anomer; δ 168.4 (OC(O)Me), 137.7, 137.4 (2 \times C_{ipso}), 128.5, 128.3, 127.9, 127.7 (2 \times OCH₂Ph), 90.9 (d, $J_{1,F}$ 31.1 Hz, C-1), 84.9 (d, $J_{2,F}$ 179.8 Hz, C-2), 77.0 (d, $J_{3,F}$ 16.7 Hz, C-3), 73.6 (OCH₂Ph), 73.4 (C-5), 72.0 (OCH₂Ph), 69.6 (C-6), 67.2 (C-4), 20.8 (OC(O)Me); HRESIMS found m/z 422.1977 [M + NH₄]⁺. Calcd for C₂₂H₂₉FNO₆ 422.1973.

1-O-Acetyl-3,6-di-O-benzyl-2-deoxy-2-fluoro- α , β -D-talopyranose (2): To a stirred solution of a 10:1 α/β mixture of compound (1) (140 mg, 0.35 mmol) and pyridine (0.5 mL) in CH₂Cl₂ (5 mL) under N₂ at -78 °C, Tf₂O (70 μ L, 0.42 mmol) was added dropwise. The reaction was allowed to warm to 0 °C and stirred at this temperature for 1 h. The reaction was diluted with CH₂Cl₂ (20 mL), washed with cold satd NaHCO₃ (25 mL), cold 0.5 M HCl (25 mL), cold water (25 mL), cold satd NaCl (25 mL), dried (MgSO₄), filtered and concentrated. To the residue in DMF (4 mL) was added KNO₂ (317 mg, 3.7 mmol) and the reaction was stirred at 50 °C under N₂ and monitored by TLC analysis (EtOAc/hexane 1:2). After 4 d, the reaction mixture was filtered and the solid was washed with CH₂Cl₂. The combined filtrate and washings were concentrated under reduced pressure. Purification of the crude product by flash chromatography (EtOAc/hexane 1:2) gave predominantly the α -anomer of (2) as a colourless gum (61 mg, 44%): R_f = 0.13 (EtOAc/hexane 1:2); ¹H NMR (CDCl₃): α -anomer; δ 7.30-7.38 (m, 10H, 2 \times OCH₂Ph), 6.32 (dd, 1H, $J_{1,2}$ 2.0, $J_{1,F}$ 8.0 Hz, H-1), 4.76 (d, 1H, J_{AB} 11.6 Hz, OCH₂Ph), 4.67 (d, 1H, J_{AB} 11.6 Hz, OCH₂Ph), 4.62 (ddd, 1H, $J_{1,2}$ 2.0, $J_{2,3}$ 3.0, $J_{2,F}$ 48.4 Hz, H-2), 4.59 (d, 1H, J_{AB} 12.0 Hz, OCH₂Ph), 4.55 (d, 1H, J_{AB} 12.0 Hz, OCH₂Ph), 4.10-4.16 (m, 1H, H-4), 3.98 (ddd, 1H, $J_{4,5} < 1$, $J_{5,6a}$ 6.4, $J_{5,6b}$ 5.2 Hz, H-5), 3.83 (dd, 1H, $J_{5,6a}$, $J_{6a,6b}$ 10.0 Hz, H-6a), 3.71 (dd, 1H, $J_{6a,6b}$, $J_{5,6b}$ 5.2 Hz, H-6b), 3.63 (ddd, 1H, $J_{2,3} = J_{3,4} = 3.0$, $J_{3,F}$ 29.6 Hz, H-3), 2.48 (br s, 1H, 4-OH), 2.06 (s, 3H, OAc); ¹³C NMR (CDCl₃): α -anomer; δ 168.3 (OC(O)Me), 137.8, 136.9 (2 \times C_{ipso}), 128.6, 128.3, 128.2, 127.9, 127.8 (2 \times OCH₂Ph), 91.2 (d, $J_{1,F}$ 32.6 Hz, C-1), 86.2 (d, $J_{2,F}$ 179.8 Hz, C-2), 73.7 (OCH₂Ph), 72.4 (C-5), 71.4 (d, $J_{3,F}$ 15.1 Hz, C-3), 70.1 (OCH₂Ph), 68.7 (C-6), 66.0 (C-4), 20.8 (OC(O)Me); HRESIMS found m/z 422.1977 [M + NH₄]⁺. Calcd for C₂₂H₂₉FNO₆ 422.1973.

1-O-Acetyl-2-deoxy-2-fluoro- α , β -D-talopyranose (3): To a solution of (2) (50 mg, 0.12 mmol) in MeOH (5 mL) was added AcOH (1 drop). The solution was flushed with Ar, and then Pd on C (10% w/w, 5 mg) was added. The reaction was flushed with H₂ and stirred at room temperature and atmospheric pressure overnight. The reaction was filtered and the filtrate was concentrated under reduced pressure. Purification of the crude product by flash chromatography (EtOAc \rightarrow EtOAc/MeOH 5:1) gave a 10:1 α/β mixture of (3) as a colourless amorphous mass (23 mg, 82%): R_f = 0.10 (EtOAc); ¹H NMR (CD₃OD): α -anomer; δ 6.13 (dd, 1H, $J_{1,2}$ 2.0, $J_{1,F}$ 9.2 Hz, H-1), 4.44 (ddd, 1H, $J_{1,2}$ 2.0, $J_{2,3}$ <2, $J_{2,F}$ 48.4 Hz, H-2), 3.76-3.83 (m, 2H, H-3, H-4), 3.61-3.74 (m, 1H, H-3), 3.65 (dd, 1H, $J_{5,6a}$ 4.8, $J_{6a,6b}$ 11.6 Hz, H-6a), 3.61 (dd, 1H, $J_{6a,6b}$, $J_{5,6b}$ 5.2 Hz, H-6b), 1.99 (s, 3H, OAc); ¹³C NMR (CD₃OD): α -anomer; δ 170.3 (OC(O)Me), 92.7 (d, $J_{1,F}$ 33.4 Hz, C-1), 89.2 (d, $J_{2,F}$ 178.3 Hz, C-2), 75.3, 69.2 (C-4, C-5), 67.0 (d, $J_{3,F}$ 16.7 Hz, C-3), 62.6 (C-6), 20.7 (OC(O)Me); LRESIMS 247 ([M + Na]⁺, 16%), 205 (100%). HRESIMS found m/z 242.1033 [M + NH₄]⁺. Calcd for C₈H₁₇FNO₆ 242.1034.

2-Deoxy-2-fluoro- α , β -D-talose (4)³⁹: Dowex 50WX8 (H) ion-exchange resin (25 mg) was added to the acetate compound (3) (10 mg, 0.04 mmol) in water (5 mL). The reaction was stirred overnight at 80 °C after which time TLC analysis (CH₂Cl₂/MeOH 8:1) showed complete consumption of the starting material. The reaction was filtered, the resin washed with water and the combined filtrate and washings were concentrated under reduced pressure to give 2-deoxy-2-fluoro-D-talose (4) as a colourless amorphous mass (6.0 mg, 74%): R_f = 0.16 (CH₂Cl₂/MeOH 8:1); LRESIMS m/z 205 ([M + Na]⁺, 100%). HRESIMS found m/z 200.0927 [M + NH₄]⁺. Calcd for C₆H₁₅FNO₅ 200.0929. ¹H NMR (D₂O): δ 5.50 (dd, $J_{1,2}$ 3.0, $J_{1,F}$ 13 Hz, H-1 β furanose), 5.45 (d, $J_{1,2}$ 9.0 Hz, H-1 α), 4.88 (d, $J_{1,F}$ 21.0 Hz, H-1 β pyranose), 4.75 (d, $J_{2,F}$ 52.0 Hz, H-2 β pyranose), 4.63 (d, $J_{2,F}$ 48.0 Hz, H-2 α pyranose). The ¹H NMR spectroscopic data for compound (4) are consistent with those reported previously.³⁹

General protocol for the synthesis of sugar nucleotides using GalK/GalPUT

A typical procedure for the enzymatic synthesis of sugar nucleotides from the corresponding reducing sugars ran as follows: Uridine 5'-triphosphate (UTP, 11 mg, 20 μ mol), adenosine 5'-triphosphate (ATP, 0.03 mg, 0.05 μ mol), phospho(enol)pyruvic acid (PEP, 4 mg, 20 μ mol), uridine 5'-diphosphoglucose (0.03 mg, 0.05 μ mol) and monosaccharide (3 mg, 16 μ mol) were dissolved in buffer (300 μ L, sodium phosphate pH 8.0, 5 mM KCl, 10 mM MgCl₂). The pH of the reaction mixture was re-adjusted to 8.0, as required, and GalU (2 U), pyruvate kinase (20 U, 20 μ L), inorganic pyrophosphatase (2 U, 20 μ L), GalK (2.4 mg, approx. 100 U) and GalPUT (2.4 mg, approx. 100 U) were added, giving a total reaction volume of 600 μ L. The reaction was flushed with N₂ and incubated at 30 °C and monitored by HPLC analysis.

HPLC analysis was carried out using a PorosHQ50 anion exchange column (3.9 mL, Applied Biosystems) at a flow rate of 12 mL min⁻¹ with detection at 265 nm. Aliquots of reaction mixture (10–50 μ L) were applied to the column, which had been equilibrated with water (2 column volumes). Elution with water (2 column volumes) was followed sequentially by a linear gradient of NH₄HCO₃ (5 mM \rightarrow 54 mM, over 20 column volumes), a linear gradient of NH₄HCO₃ (54 mM \rightarrow 189 mM, over 2 column volumes), washing with NH₄HCO₃ (1 M, 3 column volumes), followed by water (2 column volumes). Under these conditions retention times for sugar nucleotides were typically 3-4 min.

After 24 h incubation, addition of MeOH (400 μ L) to the reaction mixture was employed to precipitate protein, which was removed by centrifugation. The supernatant was filtered through a 0.45 μ m syringe filter and the filtrate was applied to a Poros HQ20 anion

exchange column (2.1 mL, Applied Biosystems) which had been equilibrated with water (2 column volumes). The column was eluted, at 30 mL min⁻¹ with detection at 265 nm, with water (3 column volumes) followed by a linear gradient of NH₄HCO₃ (30 mM→79 mM over 6 column volumes), and then washed with NH₄HCO₃ (1 M, 3 column volumes), followed by water (2 column volumes). Appropriate fractions were combined and freeze-dried to give the desired sugar nucleotides as their triammonium salts.

Uridine 5'-diphospho-2-deoxy-2-fluoro- α -D-talopyranose

The title compound was prepared using the standard procedure detailed above. ¹H NMR (D₂O): δ 7.64 (d, 1H, $J_{5,6}$ 8.0 Hz, H-6), 5.80 (d, 1H, $J_{1',2'}$ 3.6 Hz, H-1'), 5.66 (m, 1H, H-5), 5.57 (br dd, 1H, $J_{1'',P} = J_{1'',F} = 7.8$ Hz, H-1''), 4.48-4.62 (m, 1H, H-2''), 4.12-4.16 (m, 2H, H-2', H-3'), 3.92-4.08 (m, 4H, H-4', H-5'a, H-5'b, H-5''), 3.82 (ddd, 1H, $J_{2',3'} = J_{3'',4''} = 3.0$, $J_{3'',F}$ 34.0 Hz, H-3''), 3.72-3.75 (m, 1H, H-4''), 3.61 (dd, 1H, $J_{5'',6''a}$ 7.6, $J_{6''a,6''b}$ 11.6 Hz, H-6''a), 3.54 (dd, 1H, $J_{5'',6''b}$ 4.8 Hz, $J_{6''a,6''b}$, H-6''b); ¹⁹F NMR (D₂O): δ -202.32 (ddd, J 49.3, 33.8, 8.0 Hz, 2-F); ³¹P NMR (D₂O): δ -7.69 (d, 1P, J 20.2 Hz, P β), -10.17 (d, 1P, J 20.2 Hz, P α); LRESIMS 567 ([M - H]⁻, 21%), 61 (100%). HRESIMS found m/z 567.0437 [M - H]⁻. Calcd for C₁₅H₂₂FN₂O₁₆P₂ 567.0434.

Uridine 5'-diphospho- α -D-talopyranose⁵⁰

The known title compound was prepared using the standard procedure detailed above. ¹H NMR (D₂O): δ 7.77 (d, 1H, $J_{6,5}$ 8.0 Hz, H-6), 5.85 (d, 1H, $J_{1',2'}$ 4.0 Hz, H-1'), 5.80 (d, 1H, $J_{5,6}$ 8.0 Hz, H-5), 5.47 (dd, 1H, $J_{1'',2''}$ 1.6, $J_{1'',P}$ 8.0 Hz, H-1''), 4.19-4.22 (m, 2H, H-2', H-3'), 4.19-4.22 (m, 2H, H-2', H-3'), 4.11-4.14 (m, 1H, H-4'), 4.02-4.09 (m, 2H, H-5'a, H-5'b), 3.96-4.00 (m, 1H, H-5''), 3.78-3.85 (m, 3H, H-2'', H-3'', H-4''), 3.68 (dd, 1H, $J_{5'',6''a}$ 7.2, $J_{6''a,6''b}$ 12.0 Hz, H-6''a), 3.61 (dd, 1H, $J_{5'',6''b}$, $J_{6''a,6''b}$ 4.8 Hz, H-6''b); ³¹P NMR (D₂O): δ -7.84 (d, 1P, J 20.4 Hz, P β), -10.13 (d, 1P, J 20.4 Hz, P α); LRESIMS 565 ([M - H]⁻, 1%), 75 (100%). HRESIMS found m/z 565.0473 [M - H]⁻. Calcd for C₁₅H₂₃N₂O₁₇P₂ 565.0478. The ¹H NMR spectroscopic data for this compound are consistent with those reported previously.⁵⁰

Production of UDP-galactopyranose mutase

Recombinant *K. pneumoniae* UDP galp mutase was purified from *Escherichia coli* as described previously.¹⁴

Determination of mutase reaction kinetic parameters

The kinetic parameters of those compounds that could serve as substrates were determined by preparing a series of samples containing the purified mutase and the specific compound being tested over a range of appropriate substrate concentrations in a total volume of 100 μ L of mutase buffer (50 mM MOPS, 10 mM sodium dithionite, 2 mM MgCl₂ adjusted to pH 7.4). Each sample was incubated at 37 °C with the amount of enzyme and the length of incubation used to control conversion to be within 20%. The reaction mixture was then quenched by placing the reaction mixture in boiling water for 30 seconds. A sample of the reaction mixture was then applied to a Phenomenex Luna 5 μ C18 HPLC column (4.6 \times 250 mm), eluting in an isocratic manner at 1 mL min⁻¹ with 6 column volumes of potassium phosphate buffer (50 mM potassium phosphate and 2.5 mM TBAMS adjusted to pH 6.9)⁵¹ with detection at 265 nm. Kinetic parameters were deduced by fitting data to the Michaelis-Menten equation using GraFit version 5 (Erithacus Software Ltd).

Determination of mutase reaction equilibria

The equilibrium constant, K_{eq} , was calculated from the ratio of product to substrate at equilibrium. The ratios were determined by integration of the corresponding pyranose and

furanose peaks from HPLC chromatograms. Specifically, a 100 μ L incubation mixture containing an appropriate amount of the mutase enzyme and 50 μ M of substrate in mutase buffer was allowed to reach equilibrium, as determined by the constant product/substrate ratio judged by HPLC.

Acknowledgments

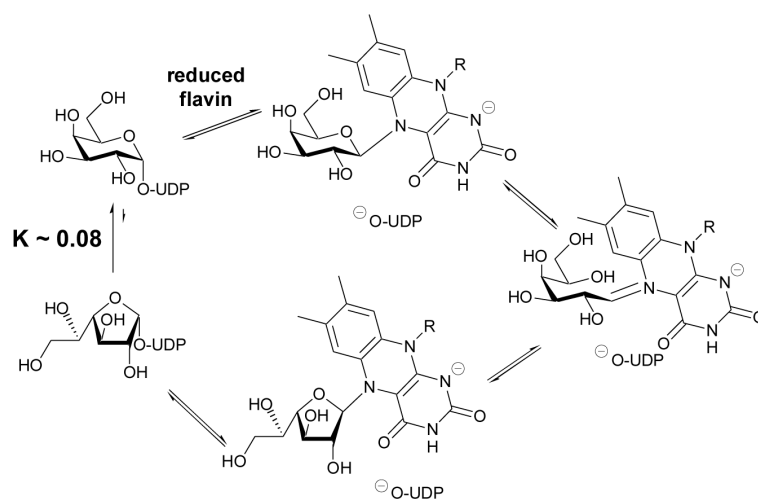
We thank Dr Monica Tello for assistance with protein expression, Dr Martin Rejzek for advice and assistance with HPLC analyses and Professor Andrea Vasella for helpful suggestions. This work was supported by the UK Engineering and Physical Sciences Research Council (Grant ref: GR/S82053/02), the Biotechnology and Biological Sciences Research Council (core support grant to the John Innes Centre), and through a Wellcome Trust International Research Collaboration grant.

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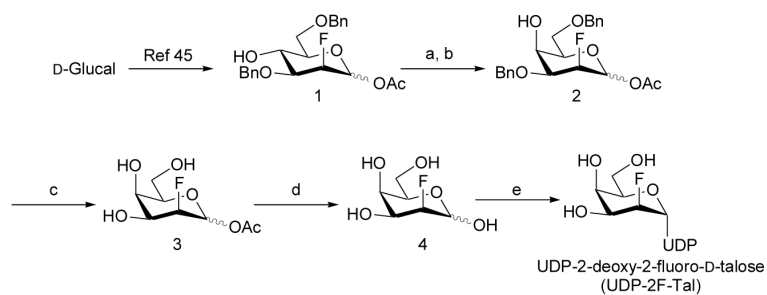
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Scheme 1.
Proposed role of flavin in the mechanism of action of UDP-D-galactopyranose mutase

**Scheme 2.**

Reagents: (a) Tf_2O , pyr, CH_2Cl_2 ; (b) KNO_2 , DMF, $50\text{ }^\circ\text{C}$, 4 days, [44% over 2 steps]; (c) Pd-C, H_2 , MeOH [82%]; (d) Dowex 50WX8 (H^+), H_2O , $80\text{ }^\circ\text{C}$, 16 h [74%]; (e) GalK/GalPUT, UTP (see Table 5).

Table 1

Kinetic parameters for turnover of fluorinated *galacto*-nucleotides by *Klebsiella pneumoniae* UDP-D-galactopyranose mutase.^a

Compound*	K_m μM	k_{cat} min^{-1}	Relative $k_{\text{cat}}(\text{UDP-Galp})/k_{\text{cat}}$	Mutase reaction: furanose observed %	Reducing sugar: furanose observed %
UDP-D-Galactopyranose ^{24,31}	805	2120	1	8.1	7.7 ²⁴
UDP-D-Galactofuranose ³¹	16	2210	0.96	8.1	7.7 ²⁴
UDP-2-Deoxy-2-fluoro-D-galactopyranose ^{21a,24,31}	203	0.04	53100	2.3	3.2 ²⁴
UDP-3-deoxy-3-fluoro-D-galactopyranose ^{24,31}	280	5.5	386	1.9	2.3 ²⁴
UDP-6-deoxy-6-fluoro-D-galactopyranose ^{24,31}	200	211	10.1	12.5	13.0 ³⁵

^aThe error in the data is typically 5-10%, or less

* For UDP-4-deoxy-4-fluoro-D-galactopyranose see ref 21a

Table 2

Kinetic parameters for turnover of 2''-substituted *D*-galacto-nucleotides by *Klebsiella pneumoniae* UDP-*D*-galactopyranose mutase.^a

Compound	K_m μM	k_{cat} min^{-1}	Relative $k_{cat}(\text{UDP-Galp})/k_{cat}$	Mutase reaction: furanose observed %	Reducing sugar: ³⁶ furanose observed %
UDP- <i>D</i> -Galactopyranose ^{24,31}	805	2120	1	8.1	7.7
UDP-2-Deoxy-2-fluoro- <i>D</i> -galactopyranose ^{24,31}	203	0.04	53100	2.3	3.2
UDP-2-Azido-2-deoxy- <i>D</i> -galactopyranose ³¹	-	-	-	ND	1.2 [*]
UDP-2-Amino-2-deoxy- <i>D</i> -galactopyranose ³¹	1140	83	13.8	4.8	5.0 [*]
UDP-2-Acetamido-2-deoxy- <i>D</i> -galactopyranose ³¹	-	-	-	ND	5.1 [*]

^aThe error in the data is typically 5-10%, or less

ND - not detectable

^{*} This study

Table 3

Kinetic parameters for turnover of 6''-variants of *D*-galacto-nucleotides by *Klebsiella pneumoniae* UDP-galactopyranose mutase.^a

Compound	K_m μM	k_{cat} min^{-1}	Relative $k_{\text{cat}}(\text{UDP-Galp})/k_{\text{cat}}$	Mutase reaction: furanose observed %	Reducing sugar: ³⁶ furanose observed %
UDP- <i>D</i> -Galactopyranose ^{24,31}	805	2120	1	8.1	7.7
UDP-6-Deoxy-6-fluoro- <i>D</i> -galactopyranose ³¹	200	211	10.1	12.5	13.0
UDP-6-Deoxy- <i>D</i> -galactopyranose (<i>D</i> -Fuc) ³¹	-	-	-	ND	3.7*
UDP- <i>L</i> -Arabinopyranose ³¹	3150	2530	0.84	6.4	4.5

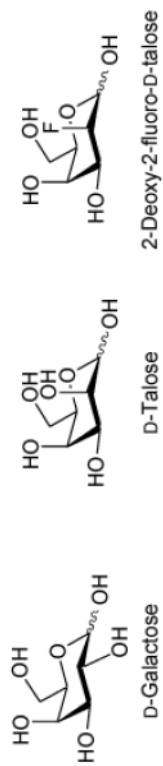
^aThe error in the data is typically 5-10%, or less

ND - not detectable

* This study

Table 4

Reducing sugar anomeric composition at equilibrium in aqueous solution



Compound	α -pyranose	β -pyranose	α -furanose	β -furanose	Reducing sugar: furanose observed % ³⁶	Mutase reaction: furanose observed %
D-Galactose	31.8	60.5	3.1	4.6	7.7	8.1
D-Talose	42.0	29.0	16.0	13.0	29.0	0 [*]
2-Deoxy-2-fluoro-D-talose	50.0	37.0	8.0	5.0	13.0 ³⁹	0 [*]

* this study

Table 5One-pot enzymatic synthesis catalysed by GalK and GalPUT.³¹

Substrate	Product	Yield (%) ^a
D-Galactose	UDP-D-Galp	78
D-Talose	UDP-D-Talp	15
2-Deoxy-2-fluoro-D-talose	UDP-2F-D-Talp	71

^aAfter 24 h incubation