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PAPER

The first C-glycosidic analogue of a novel galactosyltransferase inhibitor†

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Structural analogues and mimics of the natural sugar-nucleotide UDP-galactose (UDP-Gal) are sought after as chemical tools for glycobiology and drug discovery. We have recently developed a novel class of galactosyltransferase (GalT) inhibitors derived from UDP-Gal, bearing an additional substituent at the 5-position of the uracil base. Herein we report the first C-glycosidic derivative of this new class of GalT inhibitors. We describe a practical convergent synthesis of the new UDP-C-Gal derivative, including a systematic study into the use of radical chemistry for the preparation of galactosyl ethylphosphonate, a key synthetic intermediate. The new inhibitor showed activity against a bacterial UDP-Gal 4′-epimerase at micromolar concentrations. This is the first example of a base-modified UDP-sugar as an inhibitor of a UDP-sugar-dependent enzyme which is not a glycosyltransferase, and these results may therefore have implications for the design of inhibitors of these enzymes in the future.

Introduction

UDP-Galactose (UDP-Gal, Fig. 1) is the natural donor substrate for a range of carbohydrate-active and glycoprocessing enzymes of biological and biomedical interest, including the galactosyltransferases (GalTs),[1] UDP-Gal 4′-epimerases,[2] and UDP-galactopyranose mutases.[3] These enzymes are critically involved in the biosynthesis of glycan components of the cell wall in plants, bacteria and mycobacteria.[4] Derivatives and mimics of UDP-Gal are therefore sought after as chemical tools for glycobiology[5] and as potential enzyme inhibitors for drug discovery.[6] In the past, the development of UDP-Gal-based enzyme inhibitors has focused predominantly on the modification of the sugar moiety[7] or the pyrophosphate linkage.[8] We have recently developed a novel class of UDP-Gal derivatives modified at position 5 of the uracil base,[9] as exemplified by compound **1** (Fig. 1). 5-FT UDP-Gal **1** inhibits Gal transfer by a range of different GalTs with K_i values in the low to sub-micromolar range.[9] Evidence from structural and enzymological studies with a representative GalT shows that **1** binds at the UDP-Gal binding site, locking the target enzyme in a catalytically inactive conformation.[9] For a GalT inhibitor, this is a unique and unprecedented mode of action. These results also raise the intriguing possibility that as a close structural analogue of the natural sugar-nucleotide UDP-Gal, **1** and related molecules may also have inhibitory activity towards other UDP-Gal-dependent enzymes.

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Encouraged by the exciting biological activity of **1**, and with a view towards potential cellular applications, we became interested in the development of a hydrolytically stable analogue of **1**. In particular, we were keen to replace the scissile galactosyl phosphate bond in **1** with a chemically stable surrogate. These considerations informed the design of the new C-glycoside 5-FT UDP-C-Gal **2** (Fig. 1), which combines structural features of 5-FT UDP-Gal **1** and UDP-C-Gal **3**, a previously reported C-glycosidic UDP-Gal mimetic.[10] The replacement of the β-phosphate group in sugar-nucleotides with a phosphonate group is a common strategy for the generation of hydrolytically stable analogues.[11] Interestingly, in the case of UDP-Gal the best surrogate for the glycosyl phosphate linkage, with regard to bioactivity, is a non-isosteric C2 fragment. UDP-C-Gal **3** inhibited a representative GalT with an IC_{50} value of 40 μM, similar to the K_m value of the natural donor UDP-Gal,[10] while the corresponding isosteric C-1 phosphonate analogue of UDP-Gal[11g] showed no biological activity against a pig α-1,3-GalT.[11f] In view of these previous findings we chose a C2-linker between the galactose and phosphonate moieties in our design of 5-FT UDP-C-Gal **2**, the first C-glycosidic derivative of **1**.

Herein, we report the chemical synthesis of the new 5-FT UDP-C-Gal **2** and its biological evaluation against a UDP-Gal 4′-epimerase. The key step in our synthesis is the installation of the 5-formylthienyl substituent in position 5 under aqueous Suzuki–Miyaura conditions. This flexible synthetic approach allows the introduction of the 5-substituent in the final step of the synthesis. We also describe our findings from a systematic study into the use of radical chemistry[10,12] for the preparation of galactosyl phosphonate **5a** (Scheme 1), a central intermediate in our synthesis of **2**. Finally, we present enzymological data which show that the new 5-FT UDP-C-Gal **2** has inhibitory activity against a UDP-Gal 4′-epimerase (GalE) from *Streptococcus thermophilus*. GalE catalyses the interconversion of UDP-Gal and UDP-Glc

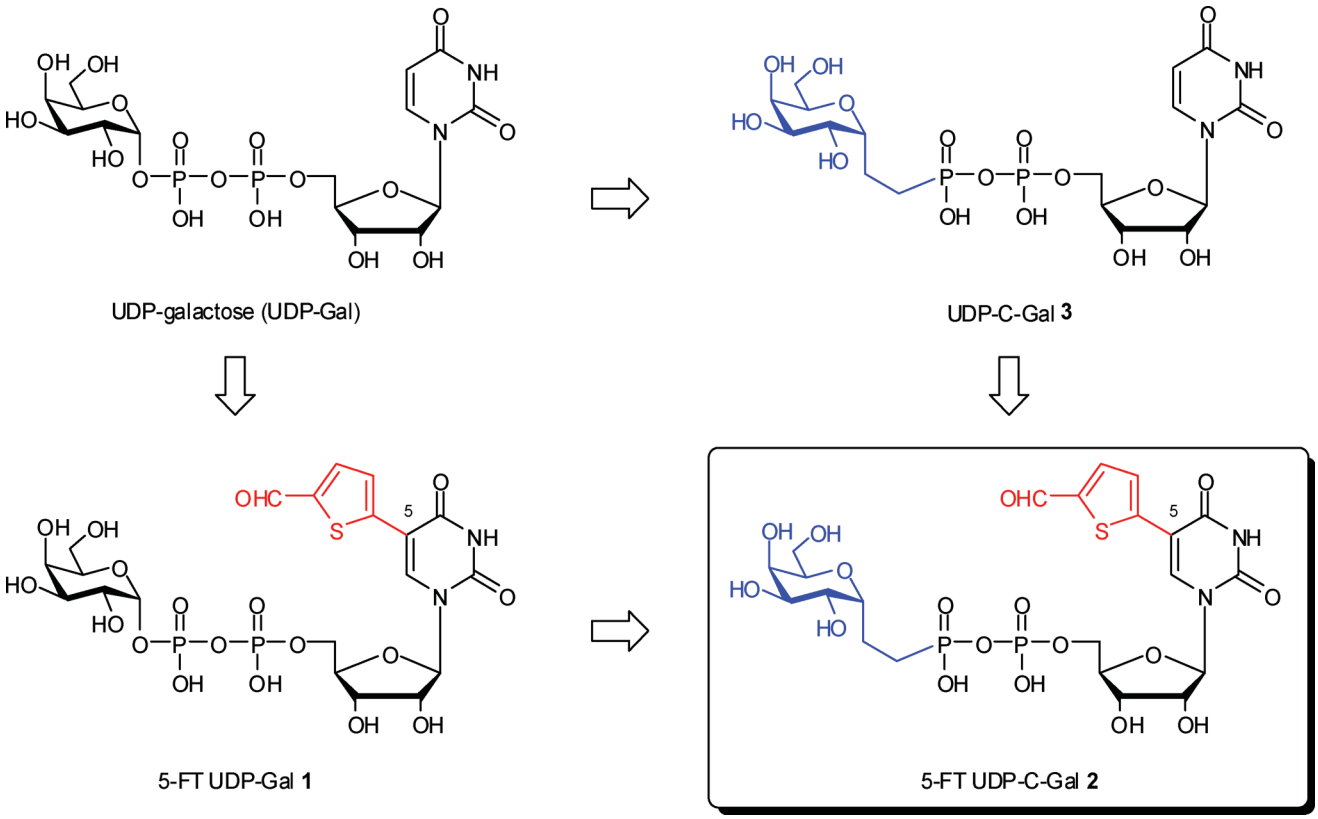


Fig. 1 Chemical structures of UDP-Gal, 5-FT UDP-Gal **1** (ref. 9), UDP-C-Gal **3** (ref. 10), and the synthetic target 5-FT UDP-C-Gal **2**.

(Fig. 2a) and plays an important role in the biosynthesis of bacterial cell-surface carbohydrates such as the O-antigen in gram-negative species.[13] In thermophilic bacteria, GalE is involved in the biosynthesis of exopolysaccharides, and overexpression of GalE in *T. thermophilus* resulted in an increased capacity of biofilm production.[14] In addition, the corresponding enzyme in trypanosoma species is a validated anti-parasitic drug target.[15] GalE inhibitors are therefore of interest as potential anti-bacterial and anti-parasitic agents. To the best of our knowledge, this is the first example of a base-modified UDP-sugar as an inhibitor of a UDP-sugar-dependent enzyme which is not a glycosyltransferase, and these results may therefore have implications for the design of inhibitors of these enzymes in the future.

Results and discussion

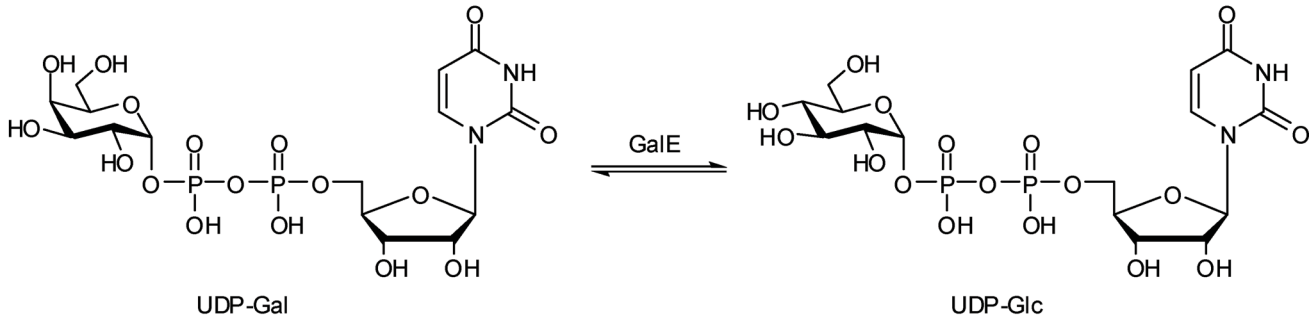
Chemistry

Our synthetic strategy for the target 5-FT UDP-C-Gal **2** was centred around the formation of the phosphate–phosphonate bond. To implement this strategy, galactosyl-1-phosphonate **7** was required as a key synthetic building block. For the preparation of **7**, we adopted the synthetic approach of Vidal and Praly.[10] These authors successfully reacted peracetylated galactopyranos-1-yl bromide **4** with diethyl vinylphosphonate under radical chemistry conditions to obtain the fully protected galactosyl-1-phosphonate **5a**, a direct synthetic precursor of **7** (Scheme 1). Among the various methods that have been described in the literature for the preparation of glycosylphosphonates,[16] advantageously, this single-step procedure affords predominantly the α-anomer, as required in our case.

Starting from galactose, glycosyl bromide **4** was obtained in two synthetic steps in 95% overall yield using standard carbohydrate chemistry (Scheme 1). Previously, the best results for the radical addition of **4** to diethyl vinylphosphonate had been achieved by the use of AIBN (azobisisobutyronitrile) as the radical initiator in combination with photochemical activation.[12,17] In order to have access to sufficient quantities of **5a**, and ultimately **7**, we decided to revisit this radical reaction, and to systematically investigate the experimental conditions for this critical step in our synthesis (Table 1). We were particularly keen to avoid photochemical activation and to replace AIBN, which can be difficult to source commercially in the UK, with an alternative radical initiator. We therefore explored different combinations of radical initiators and solvents, parameters that had not previously been investigated, while adopting other experimental conditions from Praly and co-workers.[12] In particular, to promote efficient C–C bond formation we carried out all radical reactions of glycosyl bromide **4** with an excess of diethyl vinylphosphonate, and in the presence of catalytic amounts of *n*-Bu_3SnCl and 1.5 equivalents of sodium cyanoborohydride as the reducing agent. These conditions help minimize the competing hydrogen abstraction from the intermediate glycosyl radical, a potential side reaction leading to 1-deoxysugar **5b**.[12]

We found that, interestingly, the radical reaction is relatively tolerant to changes in radical initiator and solvent (Table 1). With both AIBN and ABCN (1,1′-azobiscyclohexanecarbonitrile), which is known to be a more efficient radical initiator than

(a)



(b)

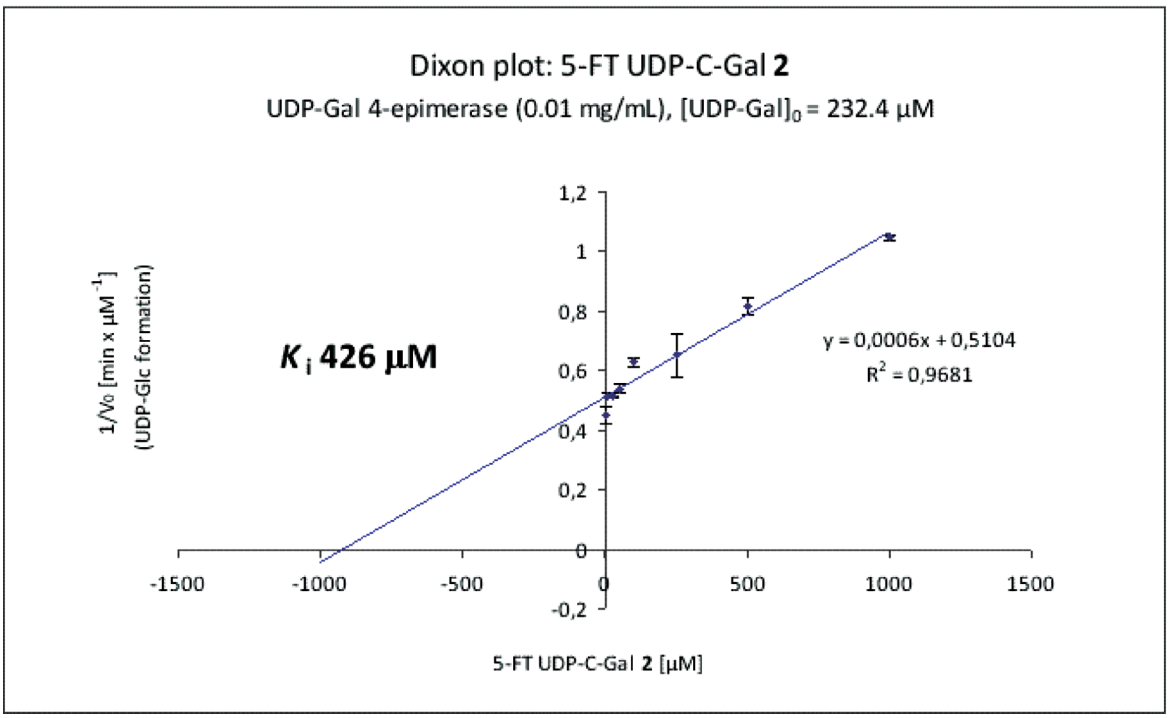
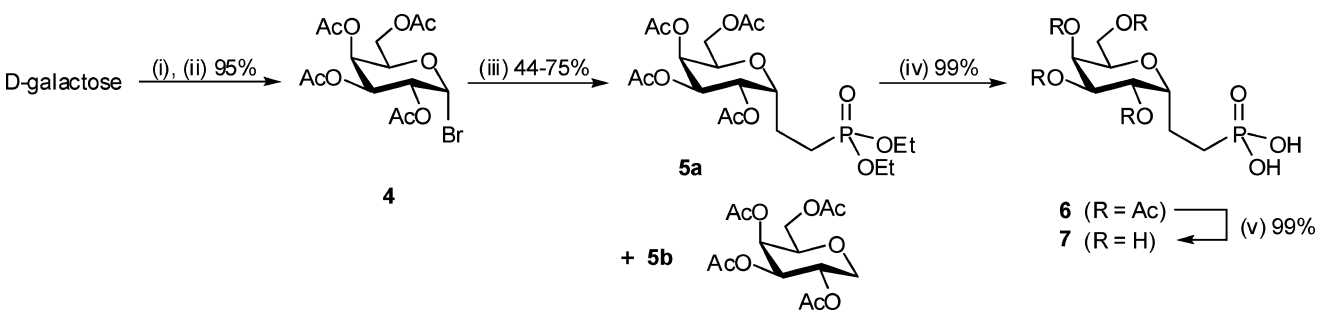


Fig. 2 (a) GalE-catalysed interconversion of UDP-Gal and UDP-Glc; (b) Dixon plot for GalE-inhibition by 5-FT UDP-C-Gal **2**.



Scheme 1 Synthesis of C-glycoside **7**. *Reagents and conditions*: (i) Ac_2O, pyridine, rt, overnight; (ii) HBr (33% in AcOH), 0 °C, 2 h; (iii) diethyl vinylphosphonate, *n*-Bu_3SnCl, $NaCNBH_3$, reflux, radical initiator, solvent, 1 to 4 days (see Table 1); (iv) Me_3SiBr, pyridine, CH_3CN, 0 °C, 3 h; (v) $H_2O/MeOH/Et_3N$, rt, 16 h.

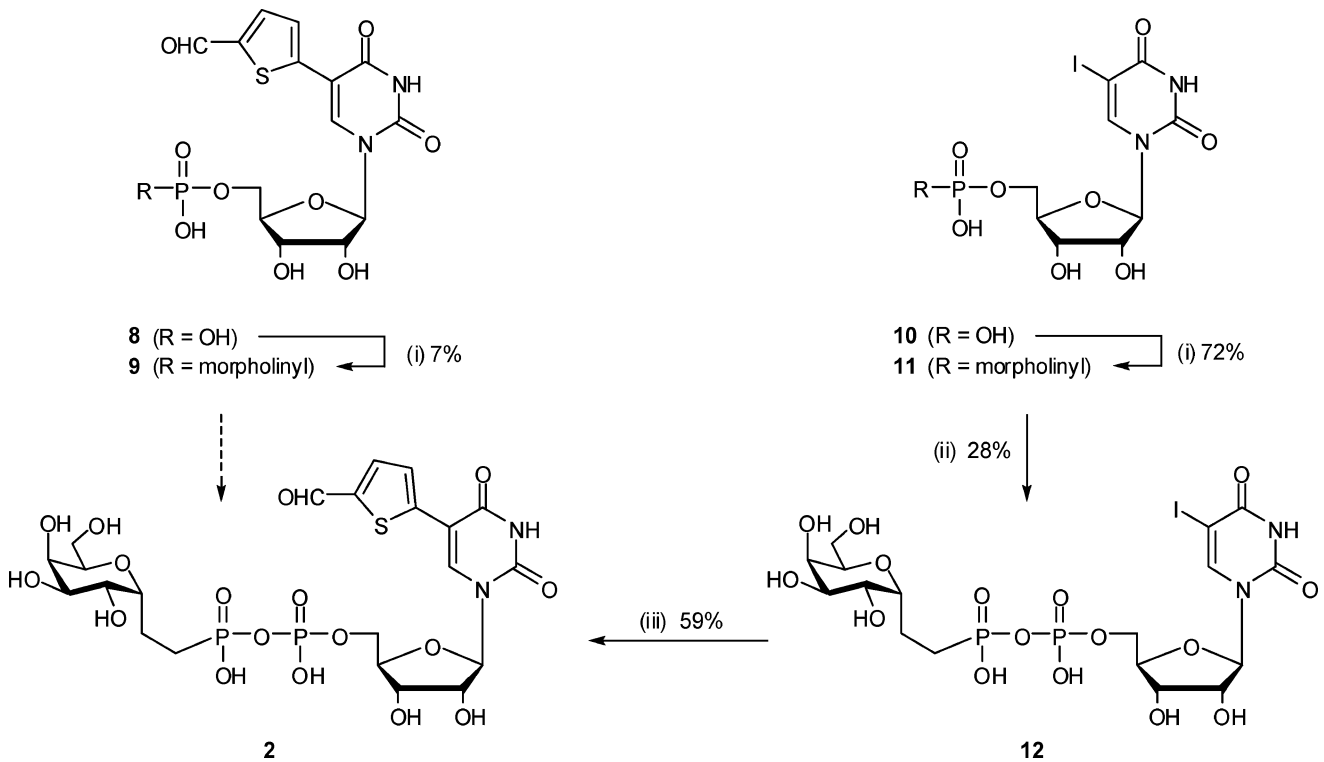
Table 1 Radical chemistry conditions for the preparation of C-glycoside **5a** (see Scheme 1 for other conditions)

Entry	Radical initiator	Equiv.	Solvent	Yield[a]
1	AIBN/photoactivation[b]	0.7	^{t}BuOH	76%[b]
2	AIBN	1	diethylether/^{t}BuOH	62%
3	AIBN	0.7	diethylether/^{t}BuOH	55%
4	AIBN	0.7	^{t}BuOH	75%
5	ABCN	1	diethylether/^{t}BuOH	44%
6	ABCN	0.7	diethylether/^{t}BuOH	71%
7	ABCN	0.7	^{t}BuOH	64%
8	Benzoylperoxide	0.7	diethylether/^{t}BuOH	53%
9	Benzoylperoxide	0.7	^{t}BuOH	54%

[a] Isolated yields, corrected for residual diethyl vinylphosphonate (0.1–0.5 molar equivalents by ^{1}H-NMR). [b] Ref. 12.

AIBN,[18] **5a** was obtained in fair to good isolated yields. In the case of AIBN the best results were obtained in ^{t}BuOH (entry 4), while for ABCN the addition of diethylether as co-solvent was beneficial (entry 6). The composition of the solvent had almost no influence when benzoylperoxide was used as the radical initiator (entries 8 and 9). Importantly, our best yields are comparable to the yield previously reported by Praly under UV-activation,[12] although longer reaction times were required under these thermal conditions. The exclusive formation of the desired α-anomer under these conditions was evident from the ^{1}H NMR spectrum of **5a**. The vicinal coupling constant $^3J_{H3,H4}$ of 5.3 Hz is indicative of the α configuration at the anomeric center of C-galactosides and in agreement with the previously reported value for α-configurated **5a**.[12] As expected, 1-deoxysugar **5b** was observed as a side product in several experiments, resulting from the *in situ* reduction of the intermediate glycosyl radical. Depending on the experimental conditions, **5b** was isolated in 10–40% yield. **5b** was formed to a substantial degree particularly in those cases where the reactants had not been specially dried prior to the reaction. This suggests that meticulously dry reagents and solvents are a prerequisite to favour radical addition over radical reduction under these conditions. On the other hand, we never observed the corresponding 2-deoxysugar resulting from the rearrangement of the peracetylated glycopyranosyl radical as described by Praly.[12] Following the successful preparation of C-glycoside **5a**, we sequentially removed the various protecting groups (Scheme 1). First, the phosphonate ester groups were readily cleaved with bromotrimethylsilane in a mixture of acetonitrile/pyridine, followed by *in situ* hydrolysis of the resulting bis(trimethylsilyl)-phosphonosugar intermediate.[19] Finally, deacetylation of **6** and purification of the reaction product by C18 reverse phase chromatography afforded the fully deprotected galactosyl phosphonate **7** in high yield.

With sufficient quantities of **7** in hand, we approached the synthesis of target 5-FT UDP-C-Gal **2** by joining the C-glycoside and UMP building blocks (Scheme 2). In principle, the installation of the 5-formylthien-2-yl substituent in position 5 of the uracil base can be carried out before or after the formation of the phosphate/phosphonate linkage. In both cases, we planned to activate the respective 5-substituted UMP derivative in the form of its phosphoromorpholidate, a well established methodology in our laboratory.[9,20,21] However, the conversion of the cross-coupled UMP derivative **8** into the corresponding phosphoromorpholidate **9** under standard Mukaiyama conditions[22] proved unexpectedly problematic (Scheme 2). Although TLC indicated the formation of morpholidate **9**, this material was unusually difficult to isolate



Scheme 2 Synthesis of target 5-FT UDP-C-Gal **2**. *Reagents and conditions*: (i) morpholine, dipyridyldisulfide, Ph_3P, DMSO, rt, 2 h; (ii) Gal-1-phosphonate **7**; tetrazole (5 equiv.), DMF, rt, 4 days; (iii) 5-formylthien-2-ylboronic acid, Cs_2CO_3, TPPTS, Na_2Cl_4Pd, H_2O, 17 h/40 °C or 2 h/55 °C.

with our standard work-up protocol (precipitation with 0.1 M NaI in acetone). During the work-up, the reaction mixture turned bright red immediately upon addition of the NaI solution, with TLC indicating the formation of side products. As a result, phosphoromorpholidate **9** was isolated in only 7% yield *via* this route. In view of this disappointing yield, we decided to explore the alternative strategy, coupling **7** to the morpholidate of 5-iodo UMP **10**[21] followed by Suzuki–Miyaura installation of the 5-substituent in the final step of the synthesis. Advantageously, this strategy also allows greater flexibility regarding the installation of different 5-substituents.

No practical problems were encountered upon conversion of 5-iodo UMP **10** into the corresponding phosphoromorpholidate **11**, which was isolated in quantitative yield. With this phosphoromorpholidate in hand, the formation of the phosphate–phosphonate linkage was carried out under the same conditions previously developed for the preparation of the parent 5-FT UDP-Gal **1**, using tetrazole as the catalyst.[9] However, compared to galactosyl-1-phosphate during the synthesis of **1**, galactosyl-1-phosphonate **7** reacted only relatively sluggishly in this transformation. This reduced reactivity necessitated the use of 5 equivalents of tetrazole and longer reaction times (4 days instead of 5 h). Fortuitously, despite the longer reaction time only relatively limited decomposition was observed, although some loss of material did occur through hydrolysis of 5-iodo UMP phosphoromorpholidate **11** to the parent nucleoside monophosphate **10**. Once formed, the product 5-iodo UDP-C-Gal **12** proved to be chemically stable under these conditions, as was expected. Unfortunately, the removal of the contaminant **10** and excess tetrazole required the sequential purification of the crude product by C-18 reverse phase and anion-exchange chromatography. Due to these repeated chromatographic purification steps, 5-iodo UDP-C-Gal **12** was obtained in only 28% isolated yield, in addition to 18% 5-iodo UMP **10**. The successful formation of the P–O–P linkage in **12** was confirmed by ^{31}P NMR spectroscopy, which showed two doublets at $\delta \sim -9$ and ~22 ppm with a coupling constant of $^{2}J_{P,P} = 27$ Hz for this phosphonophosphate derivative.

In the final step of the synthesis, the 5-formylthienyl substituent was introduced at the uracil base using Suzuki–Miyaura chemistry previously developed in our group for the cross-coupling of UDP-sugars.[21] With Na_2Cl_4Pd as the Pd source and TPPTS (triphenylphosphine trisulfonate, sodium salt) as the water-soluble ligand, no conversion was observed at room temperature, and only starting material was recovered after 3 h. However, running the reaction overnight at 40 °C under otherwise identical conditions successfully produced the cross-coupled 5-FT UDP-C-Gal **2**. In the case of the corresponding diphosphate analogue, the onset of decomposition had limited the cross-coupling time to 10 min.[9] However, the greater chemical stability of phosphonate derivative **12** permitted a significantly longer reaction time, which helped drive the cross-coupling reaction to completion at 40 °C. Importantly, no decomposition was observed even after 17 h at this temperature. Due to the pronounced chemical stability of phosphonate **12**, the cross-coupling reaction could also be carried out successfully at slightly elevated temperature, which allowed a shorter reaction time. In both cases, the cross-coupling product **2** was purified by ion-pair and/or anion-exchange chromatography and converted into its disodium salt form by eluting the purified material through a short column of Dowex-XW20 (Na^+ form). This protocol provided 5-FT UDP-C-Gal **2** in isolated yields of 35% (17 h/40 °C) and 59% (2 h/55 °C), respectively.

The relatively modest cross-coupling yields can be attributed to the formation of UDP-C-Gal **3** as a major side product, due to hydrodehalogenation in position 5 under the cross-coupling conditions. Such hydrodehalogenations are a frequently observed side reaction in heterocyclic couplings.[23] They are usually the result of an inefficient transmetallation step,[23] and the nature of the leaving group, the nature of the Pd ligand and the catalyst loading can all influence the degree of hydrodehalogenation. We found that at higher catalyst loadings, UDP-C-Gal **3** became the main reaction product, with complete conversion of starting material **12** after 3 h at room temperature, yet only traces of the cross-coupled product **2** were observed, as indicated by TLC. After work-up and purification, the ^{1}H NMR spectrum of the reaction product displayed two doublets resonating at $\delta \sim 7.53$ and 5.67 ppm, with a coupling constant of $^{3}J_{H5,H6} = 7.9$ Hz. These signals are characteristic for the two heteroaromatic protons at the uracil base, proving unambiguously the formation of the dehalogenated parent compound **3** rather than the desired cross-coupled product **2**.

Enzymology

Against the bacterial α-1,4-GalT LgtC[24], the new 5-FT UDP-C-Gal **2** has shown similar activity as the parent UDP-sugar **1**.[25] In the present study, **2** was evaluated as a potential inhibitor of UDP-Gal 4′-epimerase (GalE, E.C. 5.1.3.2), an enzyme of the Leloir pathway of galactose metabolism.[26] For these experiments, we used a commercial GalE from *Streptococcus thermophilus* as a model enzyme. First, we developed an assay protocol that allowed us to follow the GalE-catalysed conversion of UDP-Gal into UDP-Glc by HPLC. For this protocol, we adapted previously published ion-pair conditions for the separation of sugar-nucleotides.[27] Despite the minimal structural difference between UDP-Gal and UDP-Glc, our optimized HPLC conditions allowed us to clearly separate the two UDP-sugars (retention times: UDP-Gal 7.7 min, UDP-Glc 8.2 min). With this protocol in hand, we incubated GalE with the natural substrate UDP-Gal at different concentrations, monitoring the reaction progress by HPLC. From these experiments, we determined a K_m for UDP-Gal of 233 ± 15 μM. Finally, we co-incubated GalE and UDP-Gal with inhibitor **2**. In the presence of the UDP-C-Gal derivative **2**, the conversion of UDP-Gal into UDP-Glc was significantly reduced, and we determined a K_i of 426 μM for **2**, based on the formation of UDP-Glc (Fig. 2b).

In principle, two explanations are conceivable for the inhibitory effect of **2** on GalE-catalysed UDP-Gal epimerization: **2** might act either as an alternative substrate of the enzyme, leading to the formation of the corresponding 5-substituted UDP-C-Glc derivative, or as an inhibitor. During the co-incubation experiments, no new peak was observed in the HPLC chromatograms of the enzymatic reactions. This suggests that GalE does not use **2** as a substrate and that **2** may indeed be a true GalE inhibitor. An intriguing question concerns the contribution of the C-glycoside motif to the GalE inhibitory activity of **2**. To answer this question, a direct comparison of the biological activity of **1** and **2** towards GalE would provide an important clue. Unfortunately, all attempts to separate 5-FT UDP-Gal **1** and the corresponding 5-FT UDP-Glc

derivative under the conditions of our HPLC assay have so far failed, precluding the determination of K_m (GalE) for **1**. A direct comparison of **1** and **2** with regard to GalE inhibition is therefore not possible at present, preventing any conclusion on the effect of replacing oxygen by carbon in this particular system. Addressing this important question will be a priority in future studies on this new class of GalE inhibitors.

Conclusion

Structural analogues and mimics of the natural sugar-nucleotide UDP-Gal are sought after as chemical tools for glycobiology and drug discovery. Starting from 5-FT UDP-Gal **1**, a novel type of GalT inhibitor,[9] we have now developed the new C-glycoside 5-FT UDP-C-Gal **2**. We have devised a practical convergent synthesis for **2** which provides access to this new inhibitor, in 6 synthetic steps from D-galactose, in sufficient quantities for biological studies. During the preparation of **2** we successfully circumvented several synthetic pitfalls. Thus, we identified suitable conditions that allow, for the first time, the preparation of C-galactoside **5a**, a key synthetic building block, under thermal conditions in practically useful yields.

In initial enzymological experiments, **2** showed inhibitory activity against the UDP-Gal 4′-epimerase GalE. This is the first example that a base-modified UDP-sugar derivative can act as an inhibitor of a carbohydrate-active enzyme that is not a glycosyltransferase. While the GalE inhibitory activity of **2** is relatively modest, our results suggest that the additional substituent at the uracil base is tolerated by this glycoprocessing enzyme. Modification of the donor at the nucleobase has recently been introduced as a novel strategy for inhibition of glycosyltransferases.[9] Our results suggest that base-modification may also represent a promising strategy for the development of inhibitors for carbohydrate-active enzymes other than glycosyltransferases. While results from our initial enzymological experiments indicate that **2** is not used as a substrate by GalE, but rather acts as an inhibitor, further biological studies will be required to clarify the precise mode of action of this new sugar-nucleotide analogue. Importantly, the chemical stability of C-glycoside **2** is significantly enhanced compared to the parent UDP-sugar **1**. This pronounced chemical stability will greatly facilitate such biological studies and the application of **2** as a novel chemical tool in enzymology and crystallography, as well as in cellular studies.

Experimental section

General methods. All reagents were obtained commercially and used as received, including anhydrous solvents over molecular sieves, unless otherwise stated. Galactose pentaacetate and galactosyl bromide **4** were prepared as previously reported (see the ESI†). Anhydrous acetonitrile was obtained after distillation over CaH_2 under nitrogen atmosphere. All moisture-sensitive reactions were carried out under an atmosphere of nitrogen in oven-dried glassware. TLC was performed on precoated slides of Silica Gel 60 F_{254} (Merck). Spots were visualised under UV light (254/280 nm) and/or by charring in anisaldehyde stain. Reaction products were characterised by low- and high-resolution mass spectrometry (LR/HR-MS) as well as ^{1}H, ^{13}C and, in the case of phosphorus-containing molecules, ^{31}P NMR spectroscopy. NMR spectra were recorded at 25 °C on a Varian VXR 400 S spectrometer (400 MHz for ^{1}H, 100 MHz for ^{13}C, 161.9 MHz for ^{31}P). Chemical shifts (δ) are reported in ppm (parts per million). Assignments of ^{1}H signals were made by first-order analysis of 1D spectra, as well as analysis of 2D ^{1}H–^{1}H correlation maps (COSY). The ^{13}C NMR assignments are supported by 2D ^{13}C–^{1}H correlations maps (HSQC). Preparative chromatography was performed on Silica Gel 60 (particle size 0.063–0.200 mm). Ion-pair and ion-exchange chromatography was performed on a Biologic LP chromatography system equipped with a peristaltic pump and a 254 nm UV Optics Module under the following conditions:

Purification method 1. Ion-pair chromatography was performed using Lichroprep RP-18 resin and a standard gradient (unless stated otherwise) of 0–15% MeOH against 0.05 M TEAB (triethylammonium bicarbonate) over a total volume of 480 mL (flow rate: 5 mL min^{-1}). Product-containing fractions were combined and reduced to dryness. The residue was co-evaporated repeatedly with methanol to remove residual TEAB.

Purification method 2. Anion exchange chromatography was performed on Bioscale™ Mini Macro-Prep High Q cartridges and a gradient of 0–100% 1 M TEAB (pH 7.3) against H_2O over a total volume of 480 mL (flow rate: 3 mL min^{-1}. Product-containing fractions were combined and reduced to dryness. The residue was co-evaporated repeatedly with methanol to remove residual TEAB.

Diethyl 2-(2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranosyl)-ethylphosphonate (5a). *Representative procedure* (*ABCN, diethylether/^{t}BuOH system*): under a nitrogen atmosphere, a solution of **4** (274 mg, 0.67 mmol), *n*-Bu_3SnCl (57 μL, 0.21 mmol, 0.3 equiv.), $NaBH_3CN$ (95% grade, 65 mg, 1.04 mmol, 1.5 equiv.), diethyl vinylphosphonate (1.1 mL, 6.91 mmol, 10 equiv.), ABCN (110 mg, 0.45 mmol, 0.67 equiv.), and *tert*-butanol (0.65 mL, 6.91 mmol, 10 equiv.) in diethylether (5 mL) was stirred at reflux temperature (35 °C). After 4 days, TLC (cyclohexane/EtOAc 1 : 1) showed complete consumption of the starting material and the formation of two new species, the desired diethyl 2-(2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl)-ethylphosphonate **5a** (R_f 0.1) and side product 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranose **5b** (R_f 0.47). The reaction was concentrated *in vacuo*, and the oily residue was dissolved in CH_2Cl_2 (30 mL). The organic solution was washed with water (3× 30 mL), dried over $MgSO_4$, and reduced to dryness. The residue was purified by chromatography on a silica gel column, which was eluted first with cyclohexane/EtOAc (1 : 1) and then with EtOAc/EtOH (20 : 1), to afford **5a** as a syrup (257 mg, 71%): R_f 0.5 (EtOAc/EtOH 20 : 1); δ_H (400 MHz, $CDCl_3$) 5.37 (dd, 1H, $J_{5,6}$ 3.0 Hz, $J_{6,7}$ 2.8 Hz, H-6), 5.24 (dd, 1H, $J_{3,4}$ 5.2 Hz, $J_{4,5}$ 9.3 Hz, H-4), 5.14 (dd, 1H, $J_{5,6}$ 3.3 Hz, $J_{4,5}$ 9.5 Hz, H-5), 4.22–4.00 (m, 7H, H-3, H-8a, H-8b, 2 OCH_2CH_3), 3.97 (m, 1H, H-7), 2.09, 2.04, 2.01, 1.98 (all s, 12H, 4× $C(O)CH_3$), 2.98–1.56 (m, 4H, H-1a, H-1b, H-2a, H-2b), 1.29 (dt, 6H, $^4J_{H,P}$ 2.8 Hz, $J_{H,H}$ 7.0 Hz, 2 OCH_2CH_3); δ_C (100 MHz, $CDCl_3$) 170.4, 170.0, 169.9, 169.8 (4× C═O), 72.9 (d, $J_{C,P}$ 17.0 Hz, C-3), 68.3 (C-7), 68.2 (C-4), 67.9 (C-5), 67.6 (C-6), 61.8 (d, $^2J_{C,P}$ 6.0 Hz, OCH_2CH_3), 61.7 (d, $^2J_{C,P}$ 5.0 Hz, OCH_2CH_3), 61.5 (C-8), 22.1 (C-1), 20.7 (4× $C(O)CH_3$), 19.3 (C-2), 16.4 (d, $J_{C,P}$ 6.0 Hz, 2× OCH_2CH_3); δ_P (161.9 MHz, $CDCl_3$) 32.0. *m/z* (ESI) 497.1772 M+H$^+$, $C_{20}H_{33}O_{12}P$ requires 497.1782. **5b**: R_f 0.47 (cyclohexane/EtOAc 1 : 1); δ_H (400 MHz, $CDCl_3$) 5.35 (dd, 1H,

$J_{3,4}$ 3.4 Hz, $J_{6,7}$ <1.0 Hz, H-4), 5.11 (dd, 1H, $J_{1a,2}$ 5.5 Hz, $J_{1b,2}$ 10.3 Hz, $J_{2,3}$ 10.3 Hz, H-2), 4.95 (dd, 1H, H-3), 4.10 (dd, 1H, $J_{1a,1b}$ 11.1 Hz, H-1a), 4.00 (d, 2H, $J_{5,6}$ 6.4 Hz, H-6a, H-6b), 3.74 (t, 1H, H-5), 3.20 (dd, 1H, H-1b), 2.06, 1.97, 1.96, 1.91 (all s, 12H, 4× C(O)CH_3); δ_C (100 MHz, $CDCl_3$) 170.6, 170.4, 170.3, 170.1 (4× C═O), 75.0 (C-5), 71.6 (C-3), 67.9 (C-4), 67.2 (C-1), 66.5 (C-2), 62.2 (C-6), 20.9, 20.8 (4× C(O)CH_3). *m/z* (ESI) 350.1445 M+NH_4^+, $C_{14}H_{20}O_9$ requires 350.1446.

Bis(triethylammonium) 2-(2,3,4,6-tetra-*O*-acetyl-α-D-galacto-pyranosyl)-ethylphosphonate (6). To a solution of **5a** (260 mg, 0.53 mmol) in CH_3CN (5 mL) at 0 °C, pyridine (450 µL, 5.52 mmol, 10.5 equiv.) was added, followed by Me_3SiBr (730 µL, 5.52 mmol, 10.5 equiv.). The solution was stirred at 0 °C for 3 h. The reaction was quenched with H_2O/C_5H_5N (9 : 1, 5 mL) and the solution was evaporated to dryness. The residue was purified by Purification Method 1 (0–50% MeOH against 0.05 M TEAB) to afford the triethylammonium salt of phosphonic acid **6** in quantitative yield (230 mg, 0.52 mmol): R_f 0.1 (EtOAc/EtOH 20 : 1); δ_H (400 MHz, $CDCl_3$) 5.25 (dd, 1H, $J_{5,6}$ and $J_{6,7}$ 2.4 Hz, H-6), 5.15–5.05 (m, 2H, H-4, H-5), 4.14–3.95 (m, 4H, H-3, H-7, H-8a, H-8b), 2.98 (q, 6H, *J* 7.3 Hz, NCH_2CH$_3$), 1.85–1.30 (m, 4H, H-1a, H-1b, H-2a, H-2b), 1.06 (t, 7.6H, *J* 7.3 Hz, NCH$_2$CH_3); δ_C (100 MHz, $CDCl_3$) 72.3 (d, $J_{C,P}$ 14.3 Hz, C-3), 68.4, 68.3, 68.2 (C-4/C-5/C-6/C-7), 62.0 (C-8), 46.7 (NCH$_2$CH$_3$), 22.5 (d, $J_{C,P}$ 136.4 Hz, C-1), 20.2, 20.0 (4× C(O)CH$_3$), 19.3 (C-2), 8.3 (NCH$_2$$CH_3$). *m/z* (ESI) 439.0999 M-H$^-$, $C_{16}H_{24}O_{12}P$ requires 439.1011.

Bis(triethylammonium) 2-(α-D-galactopyranosyl)-ethylphosphonate (7). A solution of **6** (49.3 mg, 0.11 mmol) in H_2O/MeOH/Et_3N (7 : 3 : 1, 11 mL) was stirred for 16 h at room temperature. The reaction was evaporated to dryness and the residual white powder was purified by Purification Method 1 (100% 0.05 M TEAB) to afford the triethylammonium salt of **7** as a colorless foam (30 mg, 99%): R_f 0.2 (^{i}PrOH/H_2O/aq. NH_4OH 6 : 3 : 1); δ_H (400 MHz, D_2O) 3.82–3.70 (m, 3H, H-3, H-6, H-7), 3.59 (dd, 1H, $J_{1,2}$ 3.4 Hz, $J_{2,3}$ 9.5 Hz, H-4), 3.54–3.43 (m, 3H, H-5, H-8a, H-8b), 2.97 (q, *J* 7.3 Hz, NCH_2CH$_3$, 1.4 equiv.), 1.70–1.20 (m, 4H, H-1a, H-1b, H-2a, H-2b), 1.04 (t, *J* 7.3 Hz, NCH$_2$CH_3, 1.4 equiv.); δ_C (100 MHz, D_2O) 76.2 (d, $J_{C,P}$ 16.3 Hz, C-3), 71.6 (C-7), 69.6, 69.2 (C-5/C-6), 68.3 (C-4), 61.3 (C-8), 46.7 (NCH$_2$CH$_3$), 23.9 (d, $J_{C,P}$ 134.2 Hz, C-1), 18.4 (C-2), 8.3 (NCH$_2$$CH_3$); δ_P (161.9 MHz, D_2O) 24.9. *m/z* (ESI) 271.0590 M-H$^-$, $C_8H_{16}O_8P$ requires 271.0588.

5-(5-Formylthien-2-yl) UMP phosphoromorpholidate (9). 5-(5-Formylthien-2-yl) UMP **8** (134 mg, 0.31 mmol) was dissolved in DMSO and co-evaporated (3×) with DMF to remove residual water. The residue was dissolved in DMSO (1 mL) and morpholine (100 µL, 1.66 mmol) was added to the solution. The mixture was stirred at room temperature for 5 min. Dipyridyl disulfide (221 mg, 1.0 mmol) and triphenylphosphine (263 mg, 1.0 mmol) were added in 5 min intervals, and the reaction was stirred for 60 min at room temperature. The reaction product was precipitated by addition of NaI in acetone (0.1 M). The supernatant was removed with a pipette. The bright red residue was filtered off, washed with cold acetone, and purified by Purification Method 1 (0–20% MeOH against 0.05 M TEAB) to afford phosphoromorpholidate **9** in 7% yield (17 mg, 0.02 mmol): R_f 0.78 (^{i}PrOH/H_2O/aq. NH_4OH 6 : 3 : 1); δ_H (400 MHz, D_2O) 9.72 (s, 1H, CHO), 8.25 (s, 1H, H-6), 7.93 (d, 1H, *J* 4.2 Hz, $H_{thienyl}$), 7.60 (d, 1H, *J* 4.2 Hz, $H_{thienyl}$), 6.02 (d, 1H, $J_{1',2'}$ 4.9 Hz, H-1′), 4.40 (1H, t, *J* 5.1 Hz, H-2′), 4.32 (1H, t, *J* 4.8 Hz, H-3′), 4.24–4.15 (1H, m, H-4′), 4.09– 4.04 (2H, m, H-5′), 3.47 (m, 4H, 2× CH_2), 3.04 (q, *J* = 7.3 Hz, NCH_2CH$_3$, 3 equiv.), 2.88 (m, 4H, 2× CH_2), 1.17 (t, 28.4H, *J* 7.3 Hz, NCH$_2$CH_3, 3 equiv.); δ_P (161.9 MHz, D_2O) 10.6.

5-Iodo UMP phosphoromorpholidate (11). 5-Iodo UMP **10** [18] (292 mg, 0.65 mmol) was dissolved in DMSO and co-evaporated (3×) with DMF to remove residual water. The residue was dissolved in DMSO (0.5 mL) and morpholine (400 µL, 4.6 mmol) was added to the solution. The mixture was stirred at room temperature for 5 min. Dipyridyl disulfide (500 mg, 2.3 mmol) and triphenylphosphine (600 mg, 2.3 mmol) were added in 5 min intervals, and the reaction was stirred for 60 min at room temperature. The reaction product was precipitated by addition of NaI in acetone (0.1 M). The supernatant was removed with a pipette. The colourless residue of crude **11** was filtered off, washed with cold acetone, and used in the next reaction step without further purification.

Bis(triethylammonium) 2-(α-D-galactopyranosyl)-ethylphosphono-5-iodouridin-5′-yl phosphate (12). **11** (129 mg, 0.25 mmol) and **7** (135 mg, 0.50 mmol, 2 equiv.) were repeatedly co-evaporated with pyridine (3 mL). The residue was dried under high vacuum and dissolved in anhydrous DMF (2 mL). To this solution, tetrazole (0.45 M in CH_3CN, 2.8 mL, 1.25 mmol, 5 equiv.) was added under a nitrogen atmosphere. The reaction was stirred at room temperature for 4 days. The reaction was evaporated to dryness and the yellow powder was purified sequentially by Purification Methods 1 and 2 to afford the triethylammonium salt of **12** in 28% yield (87.5 mg, 0.07 mmol): R_f 0.5 (^{i}PrOH/H_2O/aq. NH_4OH 6 : 3 : 1); δ_H (400 MHz, D_2O) 8.24 (s, 1H, H-6), 5.90 (d, 1H, $J_{1',2'}$ 4.6 Hz, H-1′), 4.37–4.31 (m, 2H, H-2′, H-3′), 4.27–4.12 (m, 3H, H-4′, H-5′a, H-5′b), 4.03–3.88 (m, 3H, H-3″, H-4″, H-6″), 3.80 (dd, 1H, $J_{5'',6''}$ 3.3 Hz, $J_{4'',5''}$ 9.6 Hz, H-5″), 3.77–3.62 (m, 3H, H-7″, H-8″a, H-8″b), 3.16 (q, *J* 7.4 Hz, NCH_2CH$_3$, 1.7 equiv.), 2.00–1.58 (m, 4H, H-1″a, H-1″b, H-2″a, H-2″b), 1.24 (t, *J* 7.4 Hz, NCH$_2$CH_3, 1.7 equiv.); δ_C (150.9 MHz, D_2O) 163.2 (C-4), 151.6 (C-2), 145.9 (C-6), 89.7 (C-1′), 83.4 (d, $J_{C,P}$ 9.0 Hz, C-4′), 75.8 (d, $J_{C,P}$ 16.5 Hz, C-3″), 73.8 (C-2′), 71.5 (C-7″), 69.6 (C-2′), 69.1, 68.6, 68.3 (C-4″/C-5″/C-6″), 64.7 (C-5′), 61.2 (C-8″), 58.6 (C-5), 46.6 (NCH$_2$CH$_3$), 23.9 (d, $J_{C,P}$ 138.0 Hz, C-1″), 18.3 (d, $J_{C,P}$ < 5 Hz, C-2″), 8.2 (NCH$_2$$CH_3$); δ_P (161.9 MHz, D_2O) 22.3 (d, $J_{P,P}$ 26.8 Hz, CPOPO), –8.7 (d, $J_{P,P}$ 26.8 Hz, CPOPO). *m/z* (ESI) 702.9808 M-H$^-$, $C_{17}H_{27}IN_2O_{16}P_2$ requires 702.9808.

***5-FT UDP-C-Gal*: Bis(sodium) 2-(α-D-galactopyranosyl)-ethylphosphono-5-(5-formylthien-2-yl)uridin-5′-yl phosphate (2).** To a 2-necked round bottom flask charged with **12** (56.7 mg, 2.3 equiv. TEA salts, 0.07 mmol), 5-formylthien-2-ylboronic acid (20 mg, 0.13 mmol, 1.8 equiv.) and Cs_2CO_3 (39 mg, 0.16 mmol, 2.3 equiv.) was added degassed H_2O (5 mL). The flask was purged with N_2. TPPTS (2.5 mg, 0.004 mmol, 0.06 equiv.) and Na_2Cl_4Pd (0. 5 mg, 0.002 mmol, 0.025 equiv.) were added, and the reaction was stirred under N_2 for 2 h at 55 °C. The reaction was cooled to room temperature and filtered through a Millipore syringe filter (0.22 µm, 33 mm). The filtrate was concentrated *in vacuo* to give a white powder, which was purified sequentially by Purification Methods 1 and 2. Side product **3**, resulting

from dehydrohalognation, eluted first (17.3 mg, 2.0 equiv. TEA, 0.02 mmol, 32% yield), followed by cross-coupling product **2** (38 mg, 2.3 equiv. TEA, 0.04 mmol, 59% yield). Purified **2** was converted into its sodium salt form by elution from a Dowex-Na^+ column: R_f 0.6 (^{i}PrOH/H_2O/aq. NH_4OH 6 : 3 : 1); δ_H (400 MHz, D_2O) 9.76 (s, 1H, C*H*O), 8.44 (s, 1H, H-6), 7.98 (d, 1H, *J* 4.2 Hz, $H_{thienyl}$), 7.73 (d, 1H, *J* 4.2 Hz, $H_{thienyl}$), 6.01 (d, 1H, $J_{1',2'}$ 4.8 Hz, H-1′), 4.46–4.39 (m, 2H, H-2′, H-3′), 4.33–4.23 (m, 3H, H-4′, H-5′a, H-5′b), 3.95–3.89 (m, 2H, H-3″, H-4″), 3.85 (dd, 1H, $J_{6'',7''} < 1$ Hz, H-6″), 3.71 (dd, 1H, $J_{5'',6''}$ 3.4 Hz, $J_{4'',5''}$ 9.4 Hz, H-5″), 3.67–3.55 (m, 3H, H-7″, H-8″a, H-8″b), 1.95–1.53 (m, 4H, H-1″a, H-1″b, H-2″a, H-2″b); δ_C (150.9 MHz, D_2O) 187.1 (*C*HO), 163.5 (C-4), 151.2 (C-2), 144.5 (C-6), 141.3, 139.6, 138.3, 125.2, 108.9 (C5, 4× $C_{thienyl}$), 89.1 (C-1′), 83.5 (d, $J_{C,P}$ 8.9 Hz, C-4′), 75.6 (d, $J_{C,P}$ 18.2 Hz, C-3″), 74.2 (C-2′), 71.4 (C-7″), 69.5, 69.5, 69.0 (C-3′/C-5′/C-6″), 68.3 (C-4″), 64.7 (C-5′), 61.1 (C-8″), 23.8 (d, $J_{C,P}$ 140.0 Hz, C-1″), 18.2 (d, $J_{C,P} < 5$ Hz, C-2″); δ_P (161.9 MHz, D_2O) 22.2 (d, $J_{P,P}$ 27.4 Hz, C*P*OPO), –8.4 (d, $J_{P,P}$ 27.4 Hz, CPO*P*O). *m*/*z* (ESI) 687.0651 M-H$^-$, $C_{22}H_{29}N_2O_{17}P_2S_1$ requires 687.0668.

***UDP-C-Gal*: Bis(triethylammonium) 2-(α-D-galactopyranosyl)-ethylphosphonouridin-5′-yl phosphate (3)[10].** R_f 0.44 (^{i}PrOH/H_2O/aq. NH_4OH 6 : 3 : 1); δ_H (400 MHz, D_2O) 7.95 (d, 1H, *J* 8.2 Hz, H-5), 5.94 (m, 2H, H-1′, H-6), 4.36–4.32 (m, 2H, H-2′, H-3′), 4.26–4.12 (m, 3H, H-4′, H-5′a, H′5′b), 4.02–3.91 (m, 3H, H-3″, H-4″, H-6″), 3.82–3.77 (m, 1H, H-5″), 3.76–3.53 (m, 3H, H-7″, H-8″a, H-8″b), 3.16 (q, *J* 7.4 Hz, N*CH*$_2$CH$_3$, 3.2 equiv.), 2.00–1.57 (m, 4H, H-1″a, H-1″b, H-2″a, H-2″b), 1.24 (t, *J* 7.4 Hz, NCH$_2$C*H*$_3$, 3.2 equiv.); δ_P (161.9 MHz, D_2O) 22.2 (d, $J_{P,P}$ 26.8 Hz, C*P*OPO), –8.4 (d, $J_{P,P}$ 26.8 Hz, CPO*P*O)

Enzymology. *Streptococcus thermophilus* UDP-Gal 4′-epimerase (GalE) was purchased from Calbiochem. For the determination of K_m UDP-Gal, GalE (15 μL, 2.5 mg L^{-1}), UDP-Gal (15 μL, final concentrations: 10 μM–1mM) and $MnCl_2$ (15 μL, 1mM) in Tris/HCl buffer (pH 7) were incubated for 15 min at 30 °C (total volume 150 μL, all concentrations are final concentrations). For the determination of K_i [**2**], GalE (15 μL, 10 mg L^{-1}), UDP-Gal (15 μL, 232 μM), **2** (15 μL, final concentrations: 10 μM–1mM) and $MnCl_2$ (15 μL, 1mM) in Tris/HCl buffer (pH 7) were incubated for 15 min at 30 °C (total volume 150 μL, all concentrations are final concentrations). The reactions were stopped by cooling in dry ice. Samples (injection volume 80 μL) were analyzed immediately by HPLC on a PerkinElmer Series 200 machine equipped with a Supelcosil™ LC-18-T column (5 μm, 25 cm × 4.6 mm), a column oven (set to 35 °C), and a diode array detector. The following buffers were used for HPLC analysis[25]: buffer A—potassium phosphate (100 mM), tetrabutylammonium bisulfate hydrogen sulfate (8 mM), pH 6.5; buffer B—buffer A/methanol (70/30), pH 6.5. All buffers were filtered through 0.2 μm filters prior to use. The elution gradient was as follows (flow rate: 1.5 mL min^{-1}): 5% buffer B for 2 min, 5 to 50% buffer B linearly for 15 min, 50% B for 1 min, 50 to 100% A for 2 min, and 95% A for 5 min. The depletion of UDP-Gal and the formation of UDP-Glc, the product of the epimerization reaction, were monitored at 254 nm. The formation of UDP-Glc was quantified based on peak area, in reference to a UDP-Glc calibration curve, and used for the calculation of kinetic parameters. K_m and v_{max} values were determined by fitting data points to a Michaelis–Menten curve using GraFit 5.0.10. The K_i value of **2** was determined by linear regression analysis (Dixon plot) using 0, 10, 25, 50, 100, 250, 5000 and 1000 μM of inhibitor with 232 μM UDP-Gal in Tris/HCl buffer. All experiments were carried out in triplicate. Control experiments carried out in the absence of enzyme showed no significant degree of chemical hydrolysis (<2% after 3 h).

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