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Study of Uridine 5′-Diphosphate (UDP)-Galactopyranose Mutase Using UDP-5-Fluorogalactopyranose as a Probe: Incubation Results and Mechanistic Implications

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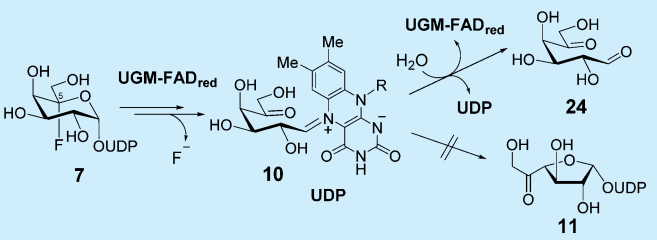
Supporting Information

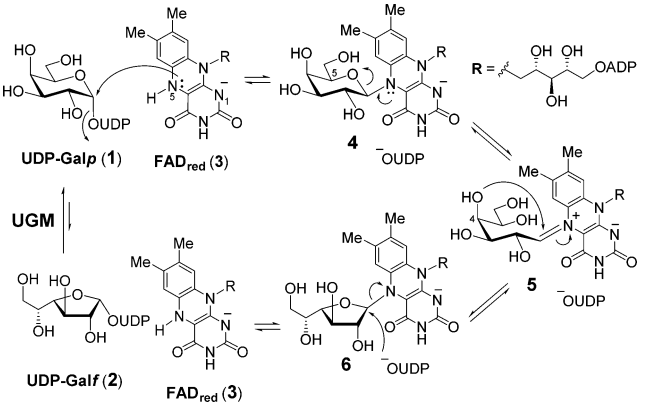
ABSTRACT: Uridine 5′-diphosphate-5-fluorogalactopyranose (UDP-5F-Gal*p*, **7**) was synthesized, and its effect on UDP-Gal*p* mutase (UGM) was investigated. UGM facilitated the hydrolysis of **7** to yield UDP and 5-oxogalactose (**24**), but no **11** was detected. ^{19}F NMR and trapping experiments demonstrated that the reaction involves the initial formation of a substrate−cofactor adduct followed by decomposition of the resulting C5 *gem*-fluorohydrin to generate a 5-oxo intermediate (**10**). The results support the current mechanistic proposal for UGM and suggest new directions for designing mechanism-based inhibitors.



Uridine 5′-diphosphate (UDP)-galactopyranose mutase (UGM) is a flavoenzyme that catalyzes the redox-neutral interconversion of UDP-galactopyranose (UDP-Gal*p*, **1**) and UDP-galactofuranose (UDP-Gal*f*, **2**).[1] This is an important enzyme for many pathogenic bacteria, including *Mycobacterium tuberculosis*, the causative agent of tuberculosis, since UDP-Gal*f* (**2**) is the precursor of Gal*f* residues found in their cell surfaces.[2] The emergence of multidrug-resistant strains of *M. tuberculosis* has prompted the search for new biomedical approaches to combat this life-threatening disease.[3] The absence of UGM in mammalian cells has made inhibition of UGM to disrupt this biosynthetic pathway a promising target in the development of new antimicrobial agents. Indeed, the inhibition of UGM has been demonstrated to adversely affect mycobacterial cell growth.[4]

In addition to its therapeutic potential, the unique catalytic mechanism of UGM has also attracted much attention. It has been shown that UGM is catalytically active only under reducing conditions, where its flavin adenine dinucleotide (FAD$_{red}$, **3**) coenzyme remains reduced throughout this overall redox-neutral reaction.[5] The reduced FAD **3** acts as a nucleophile to displace the UDP moiety from **1** or **2** to form a covalent linkage between N5 of FAD and C1 of Gal*p* (**1** → **4**) or Gal*f* (**2** → **6**).[6] Subsequent scission of the C1−O5 or C1−O4 bond is assisted by the lone pair on N5 of FAD to yield acyclic iminium ion intermediate **5**, which was first detected by trapping with hydride reagent[7] and was observed in a recent crystal structure of a UGM mutant.[8] Recyclization of **5** produces the furanosyl ring of Gal*f* (**5** → **6**) or the pyranosyl ring of Gal*p* (**5** → **4**). This recyclization reaction is followed by the elimination of reduced FAD, which may occur concurrently with nucleophilic attack at C1 by UDP, leading to the formation of UDP-Gal*f* (**6** → **2**) or UDP-Gal*p* (**4** → **1**) as the product (Scheme 1). At equilibrium, the ratio of UDP-Gal*p* to UDP-Gal*f* is approximately 10 to 1.[5] A series of FAD analogues were used to verify the role of FAD$_{red}$ in this isomerization reaction. The data supported a chemical mechanism for UGM involving an S_N2-type displacement of UDP from UDP-Gal*p*/Gal*f* by N5 of FAD$_{red}$.[6c,i]

Scheme 1. Current Mechanistic Model of UGM Catalysis



In an effort to learn more about the catalytic properties of UGM and to develop new mechanism-based inhibitors targeting UGM, UDP-5F-Gal*p* (**7**) was recognized as a promising core structure. It was expected that **7** would react

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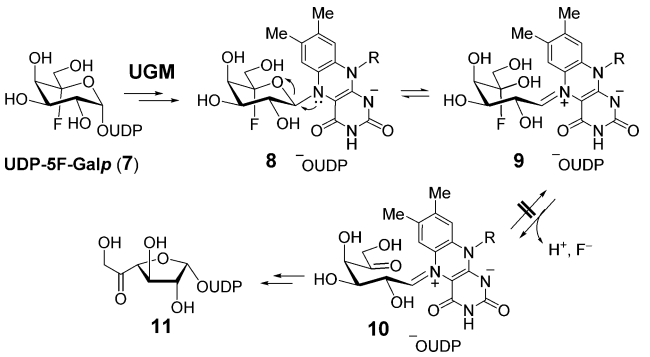
Org. Lett. XXXX, XXX, XXX−XXX

with UGM to form cofactor−substrate adduct **8**. Subsequent ring opening of **8** to form the iminium ion intermediate would result in a *gem*-fluorohydrin moiety at C5 (**9**) that should undergo rapid dehydrofluorination[9] to afford **10** (Scheme 2).

Scheme 2. Predicted Reaction of UGM with UDP-5F-Gal*p* (7) Based on the Working Mechanistic Model



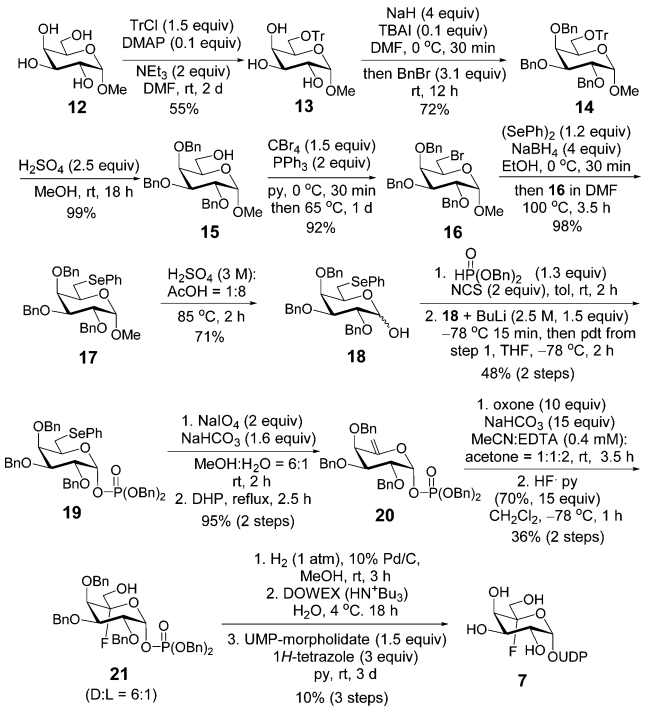
The absence of C5−OH in **10** would prevent cyclization of **10** to regenerate the pyranosyl ring but would still allow C1−O4 bond formation to yield UDP-5-oxo-Gal*f* (**11**). The 5-oxo group in **11** may react with a nucleophilic residue in the active site to form a covalent adduct and thus inhibit the enzyme. In addition, further modification of the C6 hydroxyl group to a better leaving group in **7** could enhance the nucleophilic susceptibility at C6 in **10** or **11** and promote enzyme modification and inactivation.

To test these premises, we prepared the targeted compounds and investigated their effects on the activity of UGM. Reported herein are the chemical syntheses of **7** along with its 6-deoxy-6-fluoro derivative (**26**), characterization of their reactions with UGM, and the mechanistic implications of the incubation outcomes.

The epoxide fluoridolysis strategy developed by Coward and co-workers[10] was applied to synthesize **7**. As depicted in Scheme 3, the reaction was initiated by derivatization of the C6 hydroxyl group of methyl α-D-galactopyranoside (**12**) with triphenylmethyl chloride (**12** → **13**). Benzyl protection of the remaining hydroxyl groups followed by acid hydrolysis selectively exposed the C6 hydroxyl (**13** → **15**),[11] which was then phenylselenylated via bromination and substitution (**15** → **17**). Deprotection of the anomeric hydroxyl group of **17** and subsequent reaction with freshly prepared dibenzyl phosphorochloridate gave α-phosphate **19** exclusively. Oxidation of **19** and thermal decomposition of the resulting selenoxide produced *exo*-olefin **20**.[12] Epoxidation using dimethyldioxirane (DMDO) generated in situ[13] and subsequent ring opening using hydrogen fluoride[10] gave the desired fluorohydrin **21** as the major product along with its L isomer (section S2 in the Supporting Information (SI)). Global benzyl deprotection and coupling with uridine 5′-monophosphate (UMP)[14] provided UDP-5F-Gal*p* (**7**).

Incubation of **7** (200 μM) with UGM (less than 1 μM) was carried out at 37 °C for 5 min in 50 μL of 100 mM potassium phosphate (KP_i) buffer (pH 7.5) in the presence of 20 mM $Na_2S_2O_4$.[5] No consumption of **7** was apparent as monitored by high-performance liquid chromatography (HPLC) (see the SI for HPLC methods). However, depletion of **7** (200 μM) was observed when the enzyme concentration and reaction time were increased to 20 μM and 1 h, respectively. Meanwhile, the

Scheme 3. Synthesis of UDP-5F-Gal*p* (7)



appearance of two new peaks in the HPLC traces of the reaction workups, one at 24.1 min and the other at 30.5 min, was also noted (Figure 1, trace c). The species responsible for

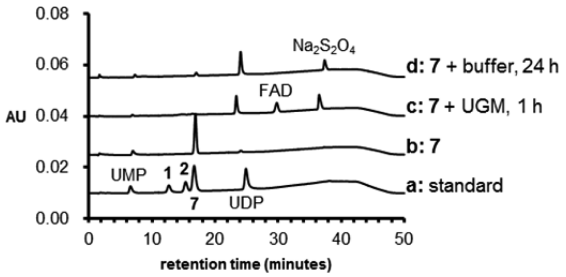


Figure 1. HPLC traces (method A; see the SI) of the incubation of UDP-5F-Gal*p* (**7**) with UGM: (a) standards of related uridine-containing species; (b) synthetic **7**; (c, d) reactions of 200 μM **7** with (c) 20 μM UGM for 1 h and (d) buffer for 24 h.

these new peaks were determined to be UDP and FAD on the basis of coelution with standards and the mass of each species as verified by mass spectrometry (section S3). While UDP was derived from **7**, FAD was detected as a result of its dissociation from UGM during the workup. In the absence of enzyme, the formation of UDP and consumption of **7** were also observable, but only over an extended period of time (24 h; Figure 1, trace d). No reaction product consistent with **11** was detected under any of the HPLC conditions tested.

To assess whether the hydrolysis of UDP-5F-Gal*p* to release UDP is catalyzed by UGM, UGM at various concentrations (0.0, 0.8, 2, and 5 μM) was incubated with 200 μM UDP-5F-Gal*p* (**7**) anaerobically at 37 °C. The consumption of **7** and formation of UDP were followed up to 24 h, as shown in Figure S5. Except for UDP, no other uridine-containing product was detected in the reaction (section S4). The apparent first-order hydrolysis rate of **7** increased from 0.112 ± 0.003 h^{-1} in the

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Org. Lett. XXXX, XXX, XXX−XXX

absence of UGM to 1.108 ± 0.008 h^{-1} with 5 μM UGM. It is thus clear that UGM can accelerate the hydrolysis of **7**. A comparison was also made using assay mixtures containing **7** and *apo*-UGM or *apo*-UGM reconstituted with either FAD or 5-deaza-FAD. Only incubation with the FAD-reconstituted UGM showed significant hydrolysis activity compared with the no-enzyme control (section S5). A reductant such as $Na_2S_2O_4$ is also required for the hydrolysis (section S5). These results demonstrate that reduced FAD (**3**) plays a direct role in UGM-catalyzed hydrolysis of **7**.

To study the catalytic function of **3**, the reaction mixture was treated with $NaBH_3CN$ in order to trap the putative Schiff base adduct formed between the reduced FAD and **7**.[7] Two new species were indeed detected by liquid chromatography–electrospray ionization mass spectrometry (LC-ESI-MS): **22** (m/z 948.2 for $[M - H]^-$ and 473.6 for $[M - 2H]^{2-}$) and **23** (m/z 950.2 for $[M - H]^-$ and 474.6 for $[M - 2H]^{2-}$) (Figure 2A and section S6). Hence, the reduced FAD (**3**) acts as a

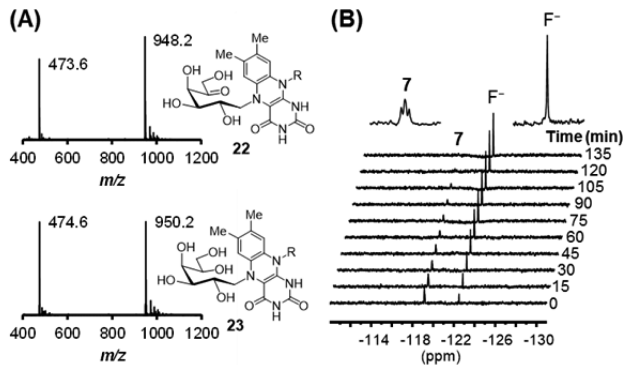
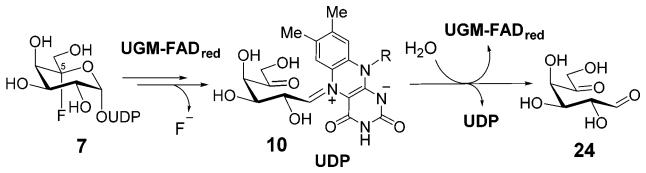


Figure 2. (A) ESI-MS (negative-ion mode) of the adducts **22** and **23** trapped from reactions of UGM with UDP-5F-Gal*p* (**7**) in the presence of $NaBH_3CN$. (B) ^{19}F NMR spectra of the reaction of **7** with UGM acquired every 15 min for 12 h. Shown here are the spectra for the first 135 min.

nucleophile to displace UDP of UDP-5F-Gal*p* (**7**) as it does during the catalysis of the UDP-Gal*p*/UDP-Gal*f* isomerization reaction. When the reaction of **7** and UGM in KP_i buffer (in D_2O) was monitored using ^{19}F NMR spectroscopy, a time-dependent reduction of the 5-F triplet signal of **7** (at −119 ppm) was observed, while a new singlet signal appeared at −122 ppm (Figure 2B and section S7). The chemical shift of the latter peak is consistent with the reported value for free fluoride.[15] These results suggest that the reaction between **7** and UGM proceeds at least up to **9**, followed by its decomposition to **10** as shown in Scheme 2. However, the reaction ensues no further than **10** since UDP-5-oxo-Gal*f* (**11**), the predicted product of the reaction of **7** with UGM, was not detected under the HPLC conditions examined. The fact that UGM does not lose activity during incubation with **7** (data not shown) indicates that the reduced FAD could somehow be regenerated from **10**.

To further characterize the turnover product from the reaction of **7** with UGM, the reaction mixture after lyophilization was incubated with *O*-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine (PFBHA) in pyridine, followed by the treatment with acetic anhydride.[16] LC-ESI-MS analysis of the workup revealed the occurrence of peaks at m/z 564.1 and 759.1, consistent with mono- and di-*O*-pentafluorobenzyl oxime acetates of 5-oxo-D-galactose (**24**), respectively (section S8). The identification of **24** as the turnover product in this experiment suggested that **10** was hydrolyzed to regenerate the reduced FAD and the active enzyme (Scheme 4).

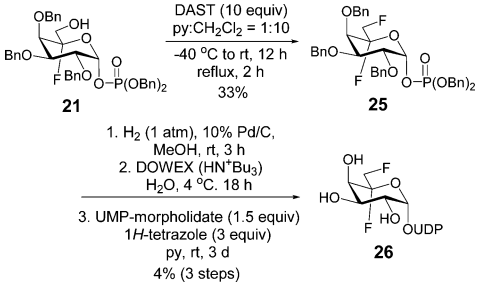
Scheme 4. Proposed Hydrolysis of 7 by UGM through the Intermediacy of 10



The reason that **10** cannot cyclize to form the furanosyl ring via the C4–OH is not clear. One possible scenario is that the C5 carbonyl group of **10** may be involved in a hydrogen-bonding network that hinders the proper alignment of the C4 hydroxyl group to reach C1 of **10** in the active site. The extended lifetime of **10** would result in its hydrolysis. In fact, we did observe that UGM could catalyze the hydrolysis of UDP-Gal*p* (**1**) at high enzyme concentration and extended incubation time (section S9). It is thus likely that hydrolysis of the Schiff base intermediate (**5** or **10**) is an inherent side activity of UGM but is typically suppressed by minimizing the lifetime of the intermediate.

The formation of the C5-oxo-bearing intermediate **10** during the enzymatic reaction prompted the design and synthesis of UDP-[5,6-F_2]-Gal*p* (**26**), which is expected to react with UGM similarly to generate 6-deoxy-6-fluoro-**10**, whose α-fluoro carbonyl functionality might be susceptible to modification by an active-site residue. As shown in Scheme 5, the hydroxyl

Scheme 5. Synthesis of UDP-[5,6-F_2]-Gal*p*



group at C6 of **21** was subjected to fluorination using diethylaminosulfur trifluoride (DAST) to generate **25**. The resulting product was hydrogenated to remove the benzyl protecting groups followed by coupling with UMP to give **26**. Although facilitated hydrolysis of **26** at C1 to yield UDP and release of fluoride were noted in the presence of UGM as observed for **7**, no apparent decrease in the activity of UGM was observed when 2 μM enzyme was preincubated with 200 μM **26** for up to 24 h (data not shown).

In summary, the C5-fluorinated substrate analogue **7** was prepared, and its reaction with UGM was fully characterized. Release of UDP from **7** is UGM-dependent, and compound **24** was identified as the turnover product. Our results clearly revealed the intermediacy of **5** (or **9**/**10**) in the catalytic mechanism of UGM and lend further credence to the currently accepted mechanism of UGM. They also suggest a more associative mechanism (S_N2-like) during substrate–FAD

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Org. Lett. XXXX, XXX, XXX–XXX

adduct formation. In addition, the inherent hydrolytic activity of UGM was also unraveled. These findings, in conjunction with the observation that a C5-oxo intermediate is generated from the C5−F substrate analogue during turnover, may be of use in the design of mechanism-based inhibitors for UGM. Although our first attempt (**26**) was not successful, exploration of the chemical space at C6 of **7** is nevertheless a promising direction for future research.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.orglett.6b01618.

Experimental procedures regarding chemical synthesis and enzymatic assays, assay results, and complete spectroscopic characterization of all new compounds (PDF)

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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