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Synthesis and Analysis of Substrate Analogues for UDP-Galactopyranose Mutase: Implication for an Oxocarbenium Ion Intermediate in the Catalytic Mechanism

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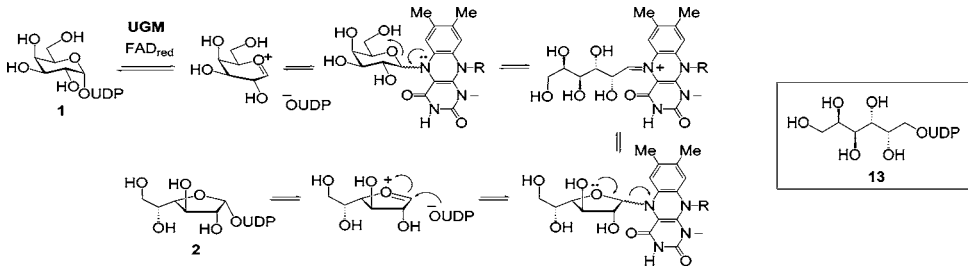
ABSTRACT



UDP-D-galactofuranose (2), which is essential for both cell growth and virulence in many pathogenic microorganisms, is converted from UDP-D-galactopyranose (UDP-Gal*p*, 1) by the flavin adenine dinucleotide (FAD)-dependent enzyme UDP-galactopyranose mutase (UGM). Here, we report the synthesis of UDP-GalOH (13) and show it as an inhibitor for UGM with a binding affinity similar to that of 1. These results are more consistent with a mechanism involving an oxocarbenium ion intermediate in UGM catalysis.

The emergence of multidrug resistant strains of human pathogens is a major concern. The prevalence of these resistant strains has compelled researchers to investigate new targets/approaches for antimicrobial drug design. The pathway for D-galactofuranose (Gal*f*) biosynthesis is one potential target for chemotherapeutic development.[1] Gal*f* is an essential component in the glycoconjugates of many pathogenic microorganisms[1] but has not been found in mammals. In the Gal*f* biosynthetic pathway, UDP-D-galactopyranose (UDP-Gal*p*, **1**) is converted to UDP-D-galactofuranose (UDP-Gal*f*, **2**) by UDP-galactopyranose mutase (UGM) (Scheme 1), and Gal*f* is subsequently transferred from **2** to various glycoconjugates by the appropriate galactofuranosyl transferase.[2] Because UGM is pivotal for mycobacterial cell growth and infection, UGM inhibitors may be potential antimicrobial agents.[2a]

UGM is a homodimeric enzyme, which binds 1 equiv of flavin adenine dinucleotide (FAD) per monomer and catalyzes the reversible conversion of UDP-Gal*p* (**1**) and UDP-Gal*f* (**2**).[3] The mechanism for the reaction catalyzed by UGM has been the subject of much discussion, and several

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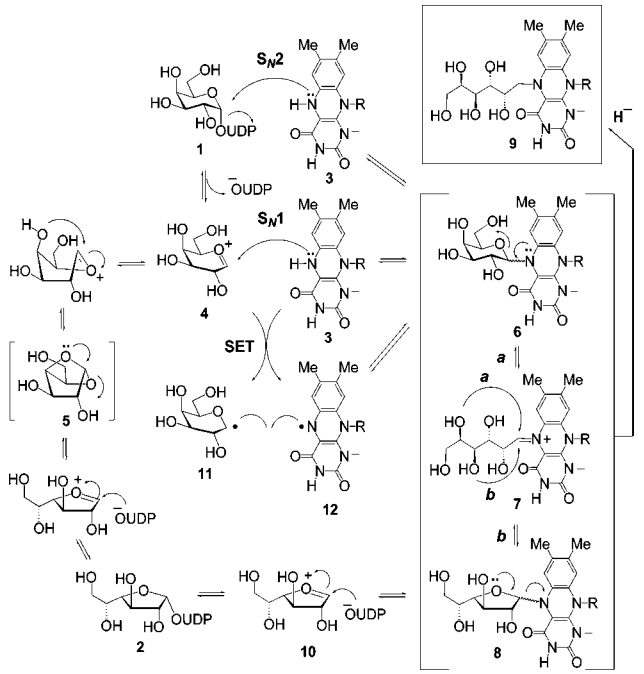
Scheme 1



possibilities have been proposed.[4] In an early report, a pathway involving a 1,4-anhydrogalactopyranose intermediate (**5**) was postulated (Scheme 1).[4a,b] This mechanism was based on a positional isotope exchange experiment which indicated cleavage of the anomeric C−O bond of UDP-Gal*p* occurred during turnover.[4a] However, this mechanism is not consistent with recent experiments showing that incubation of synthetic **5** with UGM and UDP does not yield **1** and **2** as products.[4d,5]

Because the catalytic efficiency (k_{cat}/K_m) of UGM is increased by more than 2 orders of magnitude under reducing conditions,[3b,6] several alternative mechanisms involving the reduced FAD cofactor (**3**) have since been proposed (Scheme 1). One possible route involving the formation of a FAD−substrate adduct (**7**)[4d] is supported by experiments trapping the adduct (**7**) by chemical reduction and verification of the reduced product (**9**) using mass spectroscopy.[7] The adduct formation may be initiated by an S_N2 attack of N5 of the reduced FAD (**3**) on C-1 of the substrate (**1** in the forward direction) to give **6**/**7**/**8**. It is also possible that the attack proceeds in an S_N1 fashion at C-1 where an oxocarbenium ion intermediate (**4** in the forward direction) is generated after the cleavage of the UDP group (**1** → **4** → **6**/**7**/**8**).[7] A redox process involving a single-electron transfer (SET) from the reduced FAD (**3**) to the oxocarbenium ion intermediate (**4**) is also feasible.[4c,d] In this mechanism, a SET is followed by a radical recombination between the resulting substrate radical (**11**) and the FAD semiquinone (**12**) to give the FAD−substrate adduct (**6**/**7**/**8**).[4d] As delineated in Scheme 1, this FAD−substrate adduct may play a central role to facilitate the opening and recyclization of the galactose ring.

Formation of an oxocarbenium ion intermediate has been implicated in the mechanism of numerous chemical and enzymatic reactions.[8] The intermediacy of this ionic species has prompted the design of oxocarbenium mimics as transition-state inhibitors for many enzymes.[9] Although such an oxocarbenium ion is not an intermediate in the S_N2 mechanism for UGM catalysis, it must exist if the reaction proceeds via either an S_N1 or SET mechanism. To design effective inhibitors for this promising drug target, detailed knowledge about UGM reaction intermediates and/or transition states is essential. Herein, we report the chemical synthesis and enzymatic analysis of a linear substrate analogue, UDP-galactitol (UDP-GalOH, **13**), to probe the possible involvement of an oxocarbenium ion intermediate in UGM catalysis. Galactose (Gal), galactose-1-phosphate (Gal*p*-P), UMP, and UDP were also used, along with **13**, to study the binding of a substrate/inhibitor to UGM.

UDP-GalOH (**13**) was synthesized according to Scheme 2.[10] Methyl α-D-galactopyranoside (**14**) was used as the starting material and was converted to 2,3,4,6-tetra-*O*-benzyl-galactopyranose (**16**) following a literature procedure in two steps.[11] The linear alcohol **17** was readily obtained by reduction of **16** with $NaBH_4$. Selective protection of the primary alcohol by a *tert*-butyldimethylsilyl group followed by blocking of the 5-OH by a benzyl group gave **19**. Partial deprotection of the *tert*-butyldimethylsilyl group occurred under the benzylation conditions, which resulted in the low yield (33%) of **19**. After deprotection of the *tert*-butyldimethylsilyl group, compound **20** was treated with dibenzyl chlorophosphonate[12] to give the dibenzyl phosphate derivative **21**. The benzyl groups of **21** were then removed by hydrogenolysis on catalytic amounts of palladium hydroxide to give the phosphonate derivative **22**. The final product UDP-GalOH (**13**) was prepared by incubating **22** and uridine phosphomorpholidate in anhydrous pyridine in the presence of 1*H*-tetrazole for 4 days.[13] The desired product was purified

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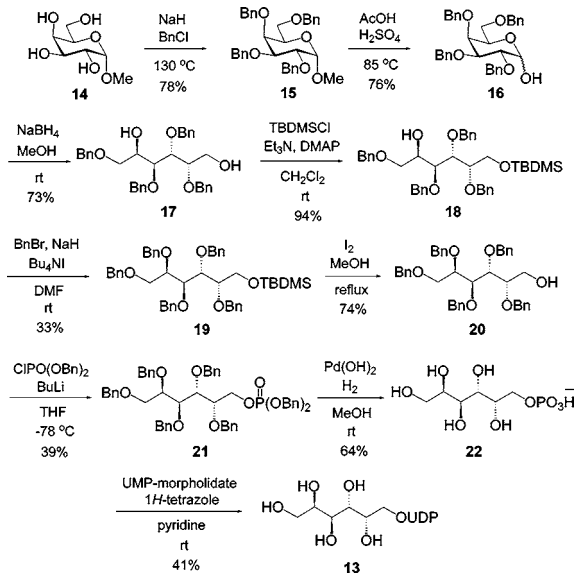
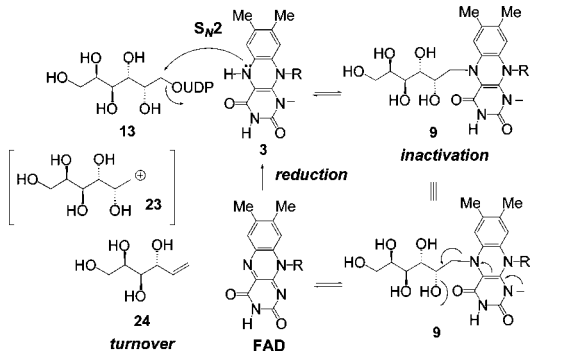
Scheme 2



by Bio-Gel P2 size exclusion chromatography and MonoQ ion-exchange FPLC. The structure of the final product **13** was confirmed by ^{1}H, ^{13}C, and ^{31}P NMR spectroscopy as well as high-resolution FABMS.[10]

The design of UDP-GalOH (**13**) as a mechanistic probe is based on the fact that it carries a good leaving group, UDP, at C-1, and is a mimic of the putative linear intermediate **7** formed during catalysis. Thus, it is reasonable to assume that UDP-GalOH will be recognized by UGM. Subsequent reaction of **13** with the reduced flavin (**3**) in the enzyme active site will lead to the elimination of UDP and the formation of a covalent adduct **9** (Scheme 3). For this

Scheme 3



substitution reaction to proceed through the S_N1 or SET mechanism, formation of a cation intermediate (such as **23**) is required. However, the corresponding primary cation **23** is much less stable than **4** and **10**, where the positive charge can be delocalized across both the ring oxygen and the anomeric carbon (C-1). Hence, if UGM recognizes and processes UDP-GalOH, the reaction more likely proceeds via an S_N2 mechanism where no cation intermediate is involved. As shown in Scheme 3, formation of **9** may result in UGM inactivation due to covalent modification of the flavin coenzyme. A subsequent C_1-N_5 bond scission to eliminate compound **24** as a turnover product, however, could regenerate the oxidized flavin coenzyme, which may be readily reduced in the presence of excess dithionite and made available for another round of catalysis.

To test the validity of UDP-GalOH (**13**) as a mechanistic probe for UGM, the binding affinity of UDP-GalOH to UGM_{red}, the active form of UGM, was first determined. Because the bound FAD_{red} of UGM_{red} gave emission at 527 nm upon excitation at 373 nm in 100 mM KH_2PO_4 buffer (pH 7.5),[14] monitoring the change of fluorescence upon substrate binding should enable us to determine if UGM_{red} binds various substrate analogues. Consequently, fluorescence titrations of UGM_{red} with the natural substrate UDP-Gal*p* (**1**) and the synthesized UDP-GalOH (**13**) were carried out in the presence of 5 mM sodium dithionite. In both cases, the intensity of the fluorescence increases and shows saturation at high substrate concentrations.[10] Clearly, both UDP-Gal*p* and UDP-GalOH can bind to the active site of UGM_{red}. These titration curves were fitted to eq 1 to give dissociation constants (K_d) of 52 μM for UDP-Gal*p* (**1**) and of 46 μM for UDP-GalOH (**13**).[10]

$$F = F_0 + Fc\frac{([E]_0 + [S] + K_d) - \sqrt{([E]_0 + [S] + K_d)^2 - 4[E]_0[S]}}{2[E]_0} \quad (1)$$

The K_d values for UMP, UDP, Gal*p*-P, and Gal were also determined under identical conditions. As summarized in Table 1, the binding affinities of UMP and UDP are

Table 1. Dissociation Constants of Substrate Analogues

compound	K_d (μM)[a]	% relative activity[b]
UDP-Gal*p* (**1**)	52 ± 9	100
UDP-GalOH (**13**)	46 ± 8	45.9
UDP	14 ± 3	49.4
UMP	27 ± 7	50.6
Gal*p*-P	730 ± 80	100
Gal	680 ± 60	97.5

[a] Determined by fluorescence titration in the presence of $Na_2S_2O_4$. [b] Determined by dividing the % conversion of **2** to **1** in the presence of vs in the absence of the compound listed.

comparable to those of UDP-Gal*p* and UDP-GalOH. In contrast, Gal*p*-P and Gal, which lack a uridine moiety, bind UGM_{red} poorly. These observations indicate that the uridine moiety plays a significant role in binding, whereas the sugar moiety makes only a minimal contribution. The fact that UDP-arabinose and UDP-*N*-acetylgalactose are substrates for

(14) The fluorescence spectrum of UGM_{red} is similar to that of other reduced flavoenzymes reported in the literature (Ghisla, S.; Massey, V.; Lhoste, J. M.; Mayhew, S. G. *Biochemistry* **1974**, *13*, 589−597). See Figure 1 in Supporting Information.

UGM is consistent with this conclusion.[15] A previous X-ray crystal structure and modeling studies showed that the uridine moiety is close to a conserved Trp156 residue, suggesting that hydrophobic stacking with UDP is a crucial binding interaction between UGM and UDP-sugars.[3b,16] Binding and modeling studies performed by STD-NMR[17] also showed that UDP binds to the active site of UGM in a manner analogous to the natural substrate UDP-Gal*p*. Taken together, these results strongly indicated that UMP, UDP, UDP-Gal*p* (**1**), and UDP-GalOH (**13**) all bind to the same site of UGM_{red} and are likely positioned similarly within this site.

Having the proper binding of UDP-GalOH (**13**) in the active site of UGM established, we investigated the catalytic competence of UGM on **13**. UDP-GalOH was incubated with UGM_{red} in 100 mM KH_2PO_4 buffer (pH 7.5) at 37 °C for 2 min, and the reaction mixture was analyzed by HPLC (Figure 1). The analysis showed that UDP-GalOH is stable under

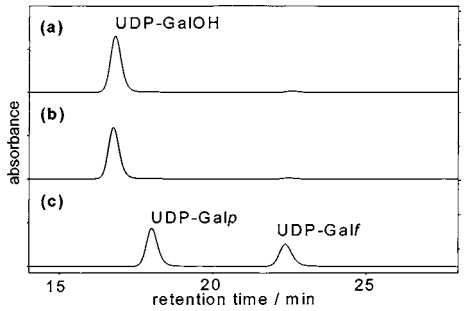


Figure 1. HPLC analysis of UGM reactions.[10] (a) UDP-GalOH (**13**) in the absence of UGM_{red}, (b) UGM_{red} + UDP-GalOH, (c) UGM_{red} + UDP-Gal*f* (**2**).

the reaction conditions (Figure 1a), but it is not a substrate for UGM_{red} (Figure 1b). As a control, almost 60% of UDP-Gal*f* (**2**) was converted to UDP-Gal*p* (**1**) by UGM_{red} under the identical conditions (Figure 1c). A longer incubation period (1 h) at 37 °C did not produce any product either.

UDP-GalOH (**13**) was then examined as a potential inhibitor for UGM by incubating it (2.5 mM) with UGM_{red} (8.2 nM) for 10 min at room temperature prior to the addition of the natural substrate UDP-Gal*f* (**2**) (50 μM). After that, the reaction was continued for an additional 2 min at 37 °C. The competence of UDP, UMP, Gal*p*-P, and Gal as substrates or inhibitors was also tested under identical conditions. The extent of inhibition by these compounds was evaluated by comparing UGM_{red} activities in the absence and presence of the inhibitors (Table 1). Among the analogues examined, UDP is known to be an inhibitor for UGM.[18] As expected, we observed inhibition of UGM_{red} to 49% by UDP under our assay conditions. Because the previous STD−NMR study had demonstrated competition between UDP and UDP-Gal*p* (**1**) in binding to UGM_{red},[17] the observed inhibition of UGM_{red} by UDP based on the activity assay is most likely competitive. Gal*p*-P and Gal exhibited no/little detectable inhibition, whereas UDP-GalOH (**13**) and UMP exhibited levels of inhibition comparable with UDP. Because only compounds containing a UDP/UMP group are inhibitors for UGM_{red}, these results once again demonstrate that the uridine moiety plays a major role in substrate recognition and binding. More importantly, the correlation between the extent of inhibition and the binding affinity determined by fluorescence titration strongly suggests that UDP-GalOH and UMP, behaving the same as UDP, are also competitive inhibitors for UGM_{red}.

In summary, the above results demonstrated that UDP-GalOH (**13**) binds to the active site of UGM in a manner similar to that of UDP and UDP-Gal*p* (**1**). The inability of UDP-GalOH (**13**) to be processed by UGM_{red} is likely a reflection of its inability to form a reactive oxocarbenium ion intermediate or to stabilize an oxocarbenium transition state. The fact that UDP-GalOH binds closely to the reduced flavin (**3**) but fails to react with it to form a covalent adduct (**9**) argues against an S_N2 mechanism for UGM catalysis. Thus, the available evidence, although indirect, is more consistent with a mechanism involving an oxocarbenium ion intermediate (**4** or **10**) which is susceptible to attack by a nucleophile,[8b,e,19] such as the reduced flavin (**3**), to form the FAD−substrate adduct (**6**/**7**/**8**). The electron-deficient nature of the oxocarbenium ions could also facilitate electron transfer from FAD_{red} to form a radical pair, such as **11** and **12**, followed by covalent bond formation. Attempts to resolve whether the interconversion catalyzed by UGM proceeds via an S_N1 or an SET pathway are in progress.

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Supporting Information Available: Preparation of **13** and analysis of binding of various substrate analogues to UGM (PDF) are presented. This material is available free of charge via the Internet at http://pubs.acs.org.

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