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Potent Ligands for Prokaryotic UDP-Galactopyranose Mutase That Exploit an Enzyme Subsite

Emily C. Dykhuizen[†] and Laura L. Kiessling*[,†,‡]

Departments of Chemistry and Biochemistry, University of Wisconsin−Madison, Madison, Wisconsin, 53706

kiessling@chem.wisc.edu

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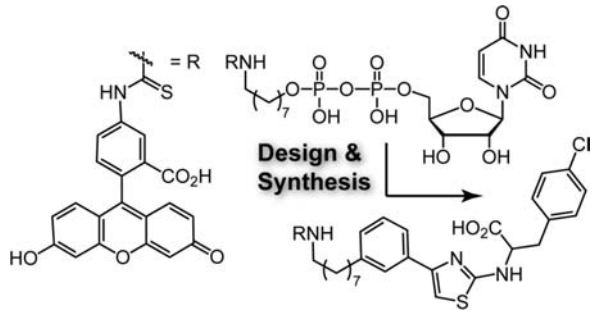
ABSTRACT



UDP-Galactopyranose mutase (UGM or Glf), which catalyzes the interconversion of UDP-galactopyranose and UDP-galactofuranose, is implicated in the viability and virulence of multiple pathogenic microorganisms. Here we report the synthesis of high-affinity ligands for UGM homologues from *Klebsiella pneumoniae* and *Mycobacterium tuberculosis*. The potency of these compounds stems from their ability to access both the substrate binding pocket and an adjacent site.

Galactofuranose (Galf) residues are present in many pathogenic microorganisms.[1] Perhaps the most notorious of these is *Mycobacterium tuberculosis*, which depends upon Galf residues as essential cell wall components.[2] The biogenesis of Galf-containing glycoconjugates requires the precursor uridine 5′-diphosphogalactofuranose (UDP-Galf). This building block is produced from UDP-galactopyranose (UDP-Galp) by the action of UDP-galactopyranose mutase (UGM) (Figure 1). The gene encoding UGM (glf) is essential for mycobacterial viability,[3] indicating that Galf-containing glycoconjugates are necessary components of the mycobacterial cell wall. Moreover, Galf residues are absent from mammalian glycoconjugates, and UGM inhibitors can block mycobacterial cell growth.[4] These observations underpin the appeal of UGM as a therapeutic target.

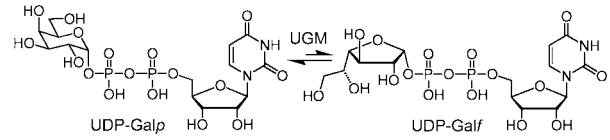


Figure 1. The reaction catalyzed by UGM.

Most inhibitors of UGM mimic the natural ligand, UDP-Gal. Simple sugar derivatives, including Gal*p* or Gal*f* analogs, can serve as inhibitors, but their activities are

[†] Department of Chemistry.
[‡] Department of Biochemistry.

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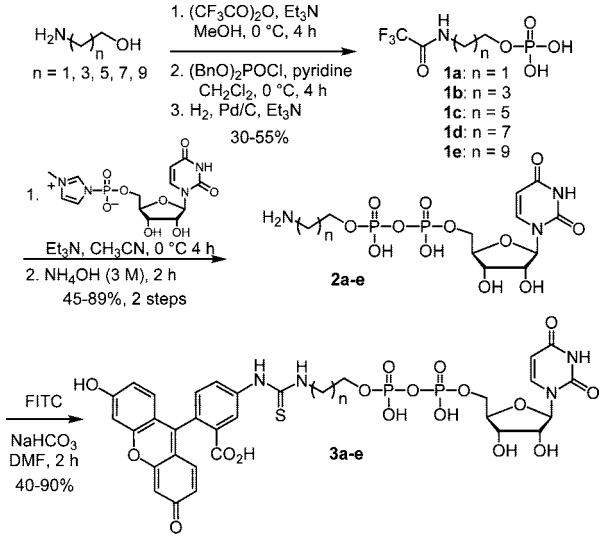
relatively modest.[5] Inhibitors that incorporate the uridine portion of the substrate bind substantially better, with affinities that approximate that of UDP-Gal*p* (K_d = ~50 μM).[6,7] Recently, we and others have identified several non substrate-based molecules as ligands.[4,8−10] These compounds bind much more tightly (K_d = 2−3 μM). Moreover, we showed that UGM inhibitors can block mycobacterial growth. Insight into the factors that lead to effective UGM ligands could guide the development of yet more potent inhibitors.

Our efforts to generate high-affinity UGM ligands were informed by our previous design of a fluorescent UGM ligand.[9] We concluded that the UDP moiety of the substrate contributes the majority of the binding energy,[9] and subsequent studies provide additional support.[6,11] Accordingly, we tethered a fluorophore to UDP through the diphosphoryl group. UDP binds to the UGM from *K. pneumoniae* (UGM_{kleb}) with an affinity of 26 μM and to the homologue from *M. tuberculosis* (UGM_{myco}) with an affinity of 15 μM; therefore, we expected the UDP-fluorescein probe to bind with an affinity in the same range. In contrast, the probe is a potent ligand, with an affinity approximately 100-fold higher than that of UDP. Specifically, its K_d for UGM_{kleb} is 0.10 μM and that for UGM_{myco} is 0.16 μM. The finding that the addition of a fluorescein group enhances affinity suggests that the fluorophore can access a secondary binding site on the enzyme.

If the fluorescein moiety occupies an adjacent subsite, the linker separating it and the UDP moiety should influence binding. To test this hypothesis, we synthesized a panel of UDP-fluorescein derivatives in which the linker was varied systematically. We tethered the fluorophore to the nucleotide using alkyl linkers composed of two, four, six, eight, and ten methylene units (Scheme 1). The linker units were

Scheme 1. Synthesis of UDP-Fluorescein Conjugates



assembled from commercially available amino alcohol building blocks. Each amino alcohol was first protected as a trifluoroacetamide[12] and then converted into a dibenzyl phosphotriester.[12] Hydrogenolysis of the benzyl groups afforded the phosphates **1a**−**1e** as the triethylamine salts, and these were coupled to uridine 5′-monophosphate (UMP)-*N*-methylimidazolide.[13] The trifluoroacetamide group was removed and UDP derivatives **2a**−**2e** were treated with fluorescein isothiocyanate (FITC) to yield conjugates **3a**−**3e**.

The affinities of the UDP-fluorescein conjugates for UGM_{kleb} and UGM_{myco} were determined using fluorescence polarization (Table 1, Figure 2).[9] The dissociation constants

Table 1. Dissociation Constants for the Complexes of UGM and UDP-Fluorescein Conjugates **3a**−**3e** (Scheme 1)

compound	UGM_{kleb} K_d (μM)	UGM_{myco} K_d (μM)
UDP	26	15
3a	>30	>30
3b	1.9	2.5
3c	0.19	0.17
3d	0.045	0.054
3e	0.070	0.064

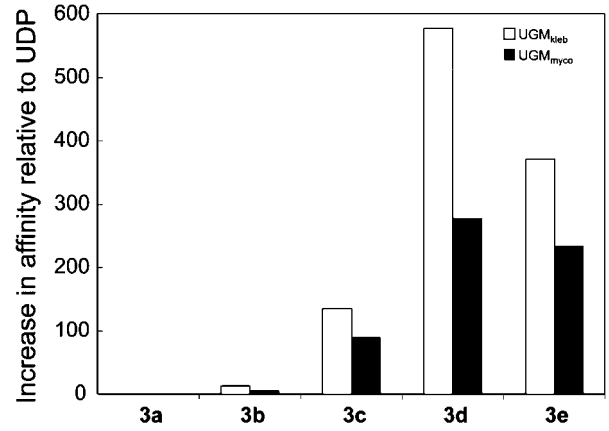


Figure 2. Relative affinity of conjugates **3a**−**3e** for UGM. The affinities are shown relative to that of UDP (**1**). An increase is observed as the alkyl linker separating the UDP and fluorescein moieties is extended.

of derivatives **3a**−**3e** for either UGM_{kleb} or UGM_{myco} vary with linker length. Compound **3a**, with the short two methylene linker, binds poorly (K_d > 30 μM); the next compound in the series, **3b**, is slightly more potent (5- to 10-fold) than UDP. Conjugate **3c**, which was used in our initial fluorescence polarization-based screen, binds about 100-fold better. The compound with the eight-methylene

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linker, **3d**, had the highest affinity: it is 300-fold more potent than UDP. The separation afforded by the eight-methylene linker seems to be optimal for binding. The affinity of **3e** (ten-methylene linker) for UGM is similar to that of **3d**. Given the influence of linker length on affinity, we determined UGM inhibition constants (IC_{50} values) for the ligand series. A similar trend was observed for **3b**−**3d**, with **3d** displaying the greatest inhibitory potency against UGM_{myco} and **3b** the least (see Supporting Information). The dependence of binding affinity on linker length suggests that there is a subsite that the fluorophore occupies and that ligands that can exploit this subsite are highly potent.

Because the activites of compounds **3a**−**3e** are influenced dramatically by the linker, we explored its importance. First, we tested the contribution of the linker alone. Uridine diphosphate derivative **2d**, with the eight-methylene linker but no fluorophore, has an affinity for UGM that is comparable to that of UDP. These data indicate that the linker has little influence on binding in the absence of the fluorophore. To test whether the chemical composition of the linker contributes to affinity, we synthesized conjugates in which the UDP and fluorescein groups were tethered using oligo(ethylene glycol) linkers. The amino alcohols derived from tri(ethylene glycol) and tetra(ethylene glycol)[14] were converted to UDP-fluorescein conjugates **4a** and **4b** using the route outlined in Scheme 1 (Figure 3). For UGM_{myco}, compound **4a**, with an eight-atom linker, bound with affinity 20-fold greater than that of UDP. Still, its affinity was more than 10-fold lower than that of the conjugate with the eight-methylene linker (compound **3d**). A similar trend was observed with UGM_{kleb}. The affinity difference for the oligo(ethylene glycol) and the corresponding alkyl conjugate could arise from altered conformational preferences.[15] Specifically, the conformation of an oligo(ethylene glycol) linker will be influenced by the gauche effect. Nevertheless, conjugate **4b**, with a longer oligo(ethylene glycol) linker, did not exhibit improved binding to either UGM_{kleb} or UGM_{myco}. Thus, the affinity differences cannot be attributed solely to alterations in conformational preferences. We hypothesize that the alkyl linker gives rise to more favorable binding because it can pack against the fluorophore.[16] Whatever the mechanism, the data indicate that the linker plays a role in the binding of UDP-fluorescein compounds to UGM.

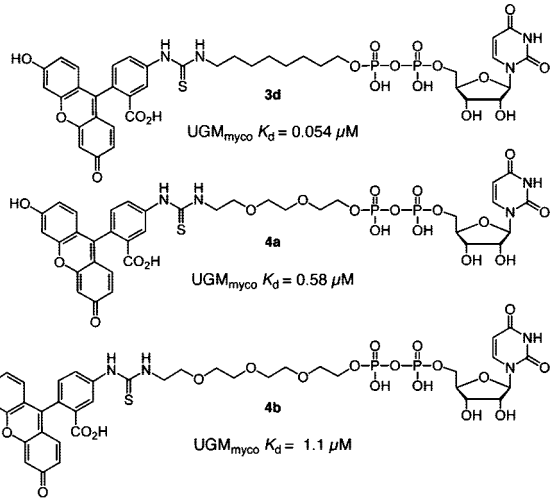

Figure 3. Oligo(ethylene glycol)-containing conjugates bind to UGM with decreased affinity compared to that of alkyl-containing conjugates.

To investigate whether binding of the fluorophore alone could be detected, we synthesized fluorescein derivative **5** (Figure 4). Interestingly, no binding to UGM_{myco} or UGM_{kleb} was observed, even in the presence of high concentrations of UDP. This result indicates that the UDP moiety and the fluorophore must be linked.

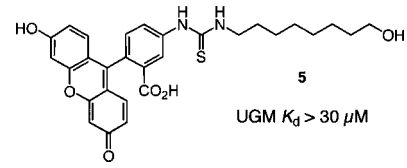

Figure 4. No UGM binding is detectable for the simple fluorescein derivative **5**.

Because the fluorescein group is an extended aromatic system, we investigated whether the attachment of other aryl substituents to UDP also would afford potent UGM ligands. Specifically, we appended UDP-octanolamine **2d** to naphthyl isothiocyanate to form conjugate **6** (Figure 5). The affinity of compound **6** for UGM was 25-fold greater than that of UDP. These results indicate that the naphthyl group can occupy the subsite we identified.

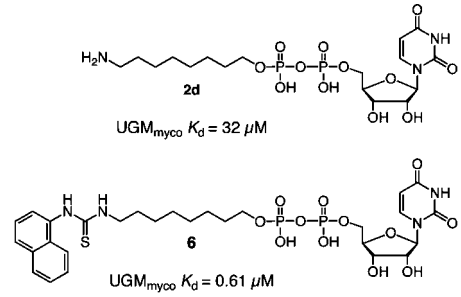

Figure 5. Aryl substituents, such as naphthyl, are able to occupy the subsite occupied by the fluorescein group.

Our findings led us to test whether we could exploit the putative subsite by elaborating UGM ligands that are not

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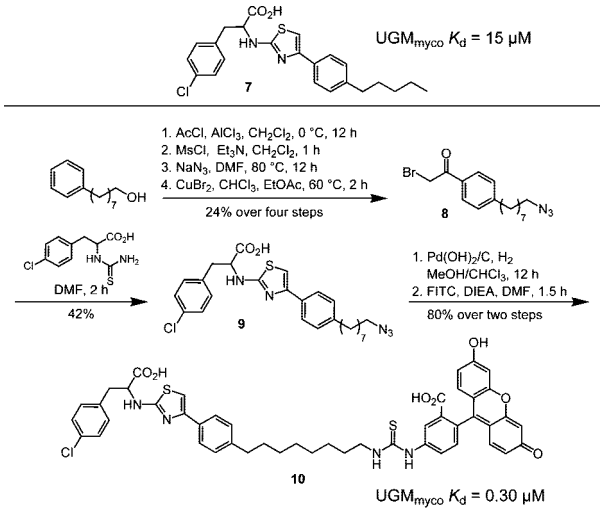
direct substrate mimics. We recently showed that 2-aminothiazoles function as competitive inhibitors of UGM.[9] We postulated that appending a fluorophore to this core could afford an extremely potent UGM ligand. For modification, we selected 2-aminothiazole **7** with an affinity of 15 μM[4] as a starting point. On the basis of our model for inhibitor binding,[4] we reasoned that a fluorescein group could be introduced by converting the pentyl group into a tether.

As a precursor to an aminothiazole with the desired attributes, we synthesized compound **9**, which bears an eight-methylene linker terminating in an azide (Scheme 2), and converted it into fluorescein derivative **10**. The synthesis of aminothiazole **10** began with a Friedel−Crafts acylation of 8-phenyl-1-octanol to produce the acetophenone.[17] The alcohol was converted to the azide through the mesylate followed by α-bromination with $CuBr_2$ to provide **8**.[18] The α-bromoketone **8** was cyclized with the thiourea derived from 4-chlorophenylalanine to generate 2-aminothiazole **9**.[4] The azide was reduced with catalytic hydrogenation to afford the amine, which was modified with fluorescein isothiocyanate (FITC). The resulting fluorescein conjugate **10** has a K_d of 0.38 μM for UGM_{kleb} and 0.30 μM for UGM_{myco}. Thus, substituted aminothiazole **10** binds 40- to 50-fold more tightly than does thiazole **7**. The IC_{50} value of 3.5 μM for **10** with UGM_{myco} (Supporting Information) indicates it is one of the most potent inhibitors described to date.[4]

Scheme 2. Synthesis of a Non-Substrate-Based UGM Inhibitor That Incorporates Fluorescein



In summary, we have identified a subsite present on the UGM from *K. pneumoniae* and *M. tuberculosis*. This subsite can be exploited to afford potent UGM inhibitors. Indeed, the compounds described herein are the most effective UGM ligands reported; their dissociation constants approach 50 nM. We anticipate that our findings will facilitate the design and optimization of potent, cell permeable UGM inhibitors for use as antimycobacterial agents and probes of the role of UGM in a wide range of pathogenic microorganisms.

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Supporting Information Available: Detailed experimental procedures, including binding curves, and compound synthesis. This information is available free of charge via the Internet at http://pubs.acs.org.

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