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Communication

Preference of Bacterial Rhamnosyltransferases for 6-Deoxysugars Reveals a Strategy To Deplete O-Antigens

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ABSTRACT: Bacteria synthesize hundreds of bacteria-specific or "rare" sugars that are absent in mammalian cells and enriched in 6-deoxy monosaccharides such as L-rhamnose (L-Rha). Across bacteria, L-Rha is incorporated into glycans by rhamnosyltransferases (RTs) that couple nucleotide sugar substrates (donors) to target biomolecules (acceptors). Since L-Rha is required for the biosynthesis of bacterial glycans involved in survival or host infection, RTs represent potential antibiotic or antivirulence targets. However, purified RTs and their unique bacterial sugar substrates have been difficult to obtain. Here, we use synthetic nucleotide rare sugar and glycolipid analogs to examine substrate recognition by three RTs that produce cell envelope components in diverse species, including a known pathogen. We find that bacterial RTs prefer pyrimidine nucleotide-linked 6-deoxysugars, not those containing a C6-hydroxyl, as donors. While glycolipid acceptors must contain a lipid, isoprenoid chain length, and stereochemistry can vary. Based on these observations, we demonstrate that a 6-deoxysugar transition state analog inhibits an RT *in vitro* and reduces levels of RT-dependent O-antigen polysaccharides in Gram-negative cells. As O-antigens are virulence factors, bacteria-specific sugar transferase inhibition represents a novel strategy to prevent bacterial infections.

Bacterial cell envelopes are rich in glycans that provide structural integrity and mediate intercellular interactions required for pathogenicity.[1−8] These glycans are unique, as bacteria produce sugars absent in mammalian glycomes known as "rare" or "bacteria/prokaryote-specific".[5,9−11] Among the ∼700 rare monosaccharides, L-rhamnose (L-Rha) and other 6-deoxysugars that lack a C6-hydroxyl are enriched in bacteria compared to mammals.[12] Across microbes, L-Rha is required for the construction of different cell envelope glycoconjugates.[9,12−15] In the Gram-positive, Acid-fast *Mycobacterium* genus, which includes the pathogen *Mycobacterium tuberculosis* (Mtb), an α-L-Rha-(1→3)-α-D-GlcNAc linker between the peptidoglycan and arabinogalactan layers is essential for viability (Figure 1A, *left*).[16−20] In many strains of the Gram-negative *Escherichia coli*, the same disaccharide is found in O-antigen (O-Ag) polysaccharides in lipopolysaccharide (LPS) (Figure 1A, *right*). O-Ags are required for virulence, and their sugar sequences distinguish serotypes.[9,13,21,22] While the cell envelopes of *E. coli* and Mtb differ, L-Rha is incorporated by a dedicated rhamnosyltransferase (RT) called WbbL in both species.

WbbL transfers a sugar from the dTDP-β-L-Rha donor to an acceptor, lipid pyrophosphate-GlcNAc (Figure 1B).[23,24] *E. coli* utilizes the lipid carrier undecaprenyl pyrophosphate (C55-PP, Und-PP), while Mtb uses decaprenyl pyrophosphate (C50-PP). Despite the presence of *wbbL* in diverse bacterial species, little is known about the mechanisms by which glycolipid RTs select donors among the vast pool of cellular sugar metabolites.[17,25−28] Like many O-Ag glycosyltransferases, WbbL is localized proximal to the cytoplasmic membrane, which complicates purification.[7,29,30] Additionally, discrete donor and acceptor substrates are not readily accessible, as syntheses of activated β-L-sugars[31−38] and glycolipids with long hydrophobic tails[39−43] are limited.

Since WbbL is essential for virulence (*E. coli*) or survival (Mtb), obtaining a better understanding of substrate preferences would have implications in the design of both antibiotic and antivirulence agents. Resulting antivirulence strategies may avoid selective pressures that drive antibiotic resistance mechanisms.[44] Here, we study three glycolipid RTs from different species, two from Gram-negatives, and one from mycobacteria. A collection of synthetic (deoxy)nucleoside diphosphate-sugar ((d)NDP-sugar) donor and glycolipid acceptor analogs are used to examine molecular recognition of substrates. These findings lead to a tactic to inhibit *E. coli* WbbL *in vitro* and in cells.

To evaluate donor recognition, we assessed binding of RT to dTDP-β-L-Rha analogs obtained by our synthetic and chemoenzymatic routes.[31,45] WbbL is predicted to be membrane associated;[18,30,46] hence, *E. coli* and Mtb WbbL membrane fractions were isolated. To broaden the scope of our study and obtain higher protein quantities, we identified a putative similar protein, RfbF, in the Gram-negative thermophile *Thermus thermophilus* using sequence-based analyses (Tables S1−S2, Figures S1−S2).[23,24,47−50] Soluble RfbF isolation enabled isothermal titration calorimetry (ITC) experiments

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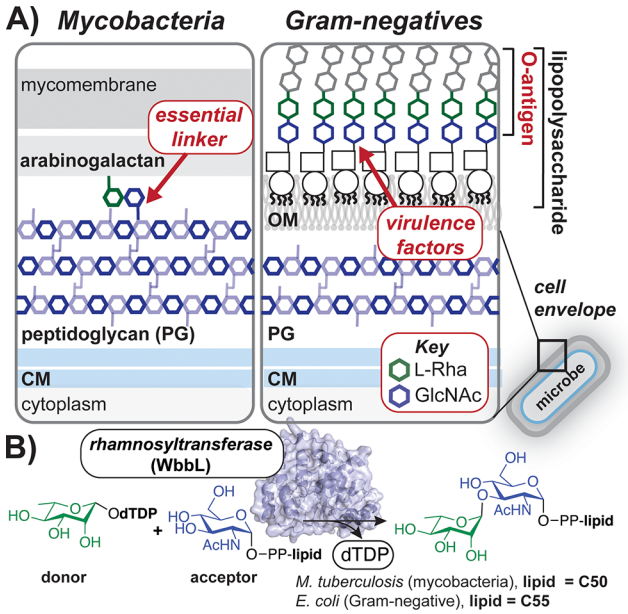
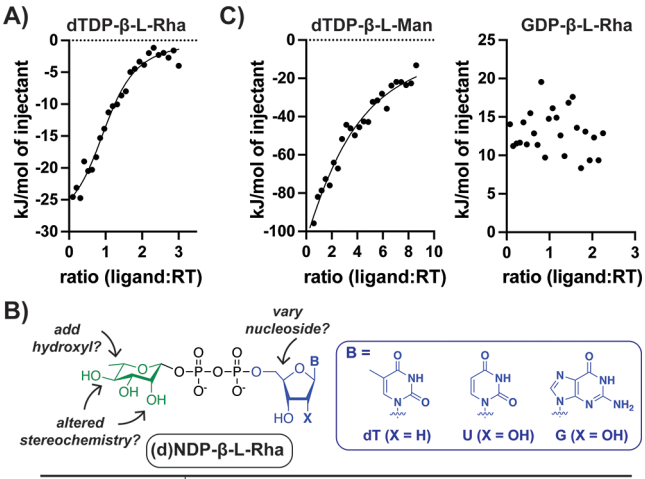


Figure 1. L-Rha is required for glycan assembly in diverse bacteria. (A) Schematic of mycobacterial and Gram-negative cell envelopes with the roles of L-Rha-GlcNAc linkages highlighted. (B) Rhamnosyltransferase-mediated synthesis of these linkages with a model of *E. coli* WbbL shown. (CM = cytoplasmic membrane; OM = outer membrane.)

(Figures 2, S3–S13). dTDP-β-L-Rha bound RfbF with a stoichiometry of ~1 and ~8 μM affinity (Figure 2A–B, Table S3), which is in the concentration range of cellular nucleotide-sugars and the measured K_M (~35 μM) with crude Mtb WbbL.[30] An L-Rha-1-phosphate fragment demonstrated binding, but beyond a measurable K_D (>1 mM).[51] Unlike reported RTs that glycosylate glycans, natural products or proteins,[46,52−55] RfbF did not bind nucleotide alone (dTDP) at concentrations tested (Figures S3–S4). Stereochemical inversion at the C4-position using dTDP-6-deoxy-β-L-talose (dTDP-β-L-6dTal) led to comparable affinity as the native substrate, while altering the C2- and C4-positions with dTDP-β-L-fucose (dTDP-β-L-Fuc) produced an ~15-fold enhancement in K_D, indicating the C2-hydroxyl conformation is important for recognition. The consequence of C6-hydroxylation was assessed with dTDP-β-L-mannose (dTDP-β-L-Man) and the bacterial metabolite dTDP-α-Glc, which showed 60- and ~75-fold increases in K_D, respectively, compared to the native donor (Figure 2C, left). Similarly, alteration of the anomeric position in dTDP-α-L-Rha resulted in no detectable binding. Hence, changes to the anomeric or C6 position may cause steric clashes in the active site.

We then tested GDP- and UDP-sugars as ligands, since these nucleotides commonly activate cellular sugars.[56−58] GDP-β-L-Rha showed no binding to RfbF, likely due to the bulky purine (Figure 2C, right). UDP-β-L-Rha bound with an ~4.5-fold increase in K_D relative to dTDP-β-L-Rha; however, the cellular metabolite UDP-α-GlcNAc did not bind at μM concentrations, indicating that a C2′-hydroxyl on ribose is accommodated, but additional changes in the sugar moiety are not. These observations suggested that a pyrimidine nucleotide-β-6-deoxysugar could serve as an RT donor.

We next sought to assay WbbL and RfbF using glycolipid acceptors. Inspired by observations that O-Ag biosynthetic enzymes accept substrates with varying lipid structures,[59−61]



NDP-sugars	K_D (μM)	ΔH (kcal/mol)	ΔS (cal/mol·K)	ΔG (kcal/mol)
dTDP-β-L-Rha	**8.31** (3.66)	**-8.68** (0.59)	**-6.43** (0.68)	**-6.77** (.38)
dTDP-β-L-6dTal	**16.5** (5.5)	**-8.17** (2.70)	**-28.8** (3.7)	**0.415** (2.847)
dTDP-β-L-Fuc	**126** (1)	N/A	N/A	N/A
dTDP-β-L-Man	**496** (56)	N/A	N/A	N/A
dTDP-α-D-Glc	**637** (96)	N/A	N/A	N/A
dTDP-α-L-Rha	N/B	N/B	N/B	N/B
GDP-β-L-Rha	N/B	N/B	N/B	N/B
UDP-β-L-Rha	**36.1** 10.7	**-6.72** (1.01)	**-51.2** (14.7)	**8.55** (3.65)
UDP-α-D-GlcNAc	N/B	N/B	N/B	N/B

Figure 2. A bacterial RT prefers nucleotide-6-deoxysugars. (A) ITC binding curve of RT (RfbF) with the canonical donor demonstrating micromolar affinity. (B) Summary of ITC results for (d)NDP-sugars indicates that pyrimidine nucleotide-6-deoxy-L-sugars bind with the highest affinities. (C) Representative binding curves for (d)NDP-sugars that show weak (left) or no (right) binding.

we synthesized the following diverse glycolipids to broadly assess the promiscuity of RTs: GlcNAc-pyrophosphate-farnesyl (GlcNAc-PP-C15, **1a**), -geranylgeranyl (-C20, **1b**), -heptaprenyl (-C35, **1c**), and -undecaprenyl (-C55, **1d**) (Figure 3A). Each was accessed based on related glycolipid routes,[41−43,62] beginning with phosphorylation of peracetylated GlcNAc to form 3,4,6-tri-*O*-acetyl-α-GlcNAc-1-phosphate. The phosphosugar was activated with *N*,*N*-carbonyldiimidazole, and monophosphorylated-isoprenoid coupling was optimized to give the indicated conditions (Figure 3A, table).

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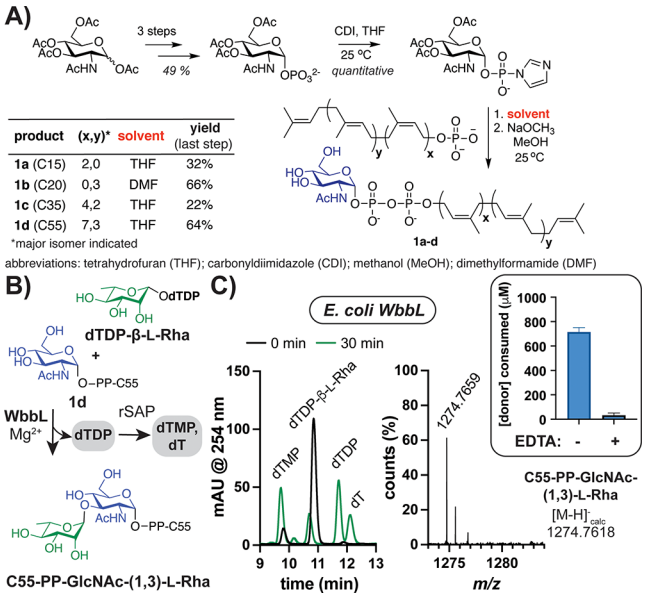

Figure 3. Synthetic glycolipids serve as RT acceptors. (A) Scheme with key steps to GlcNAc-PP-lipid analogs. (B) *E. coli* WbbL utilizes canonical substrates (1 mM each), releasing dTDP that is digested by phosphatase (rSAP). (C) HPLC (left) and HRMS (right) analyses show coupled product formation is metal-dependent (∓ 5 mM EDTA). Error bars represent standard deviation (SD) (n = 9). Some dTMP is present in the dTDP-sugar stock.

With putative acceptors in hand, we reconstituted RT activity. Commercial kits for UDP release do not detect dTDP (Figure S14). Hence, we developed HPLC- and high-resolution mass spectrometry (HRMS)-based assays using *E. coli* WbbL and the canonical acceptor (**1d**) with phosphatase (rSAP) to drive the reaction forward (Figures 3B–C, S15).[42] Unlike previously reported, additional reducing agent was not required for activity.[30] Since WbbL is a predicted metal-dependent GT-A (Figure S1),[30,63] reaction and binding buffers contained Mg^{2+}. Accordingly, the addition of EDTA inhibited RT reactions (Figures 3C, S16, S17). Heat treatment of WbbL or removal of acceptor resulted in no dTDP release as well (Figure S18a–b). When the noncanonical acceptors Ser or UDP-α-GlcNAc were used with the native donor, no turnover resulted (Figures 4A and S18c), suggesting a glycolipid is needed.[60] Evaluation of glycolipids **1a–d** with dTDP-β-L-Rha revealed that *T. thermophilus* RfbF, and *E. coli* and Mtb WbbL use acceptors with variable lipid chain length, degree of unsaturation, and stereochemistry (Figures 4B, S17, Tables S4–S5). Surprisingly, Mtb WbbL showed optimal turnover with the predominantly *trans*-prenyl C20 substrate, which differs from the canonical acceptor containing *cis* alkenes proximal to the pyrophosphate.[40,64] Conversely, *E. coli* WbbL preferred its native C55 acceptor. Overall, RTs could utilize glycolipids with chain lengths as short as C15.

Due to the improved solubility of C35 versus C55 glycolipid, we next analyzed noncanonical donors using **1c**. ITC analysis suggested that C4 inversion (dTDP-β-L-6dTal) does not affect RT binding, while addition of a C6-hydroxyl or purine nucleotide weakens binding. In the presence of **1c**, turnover of dTDP-β-L-6dTal was comparable to dTDP-β-L-Rha across RTs; however, dTDP-β-L-Man, GDP-β-L-Rha, dTDP-α-Glc, and UDP-α-GlcNAc demonstrated little to no coupling (Figures 4C, S19, Tables S4–S5). RT activity with various

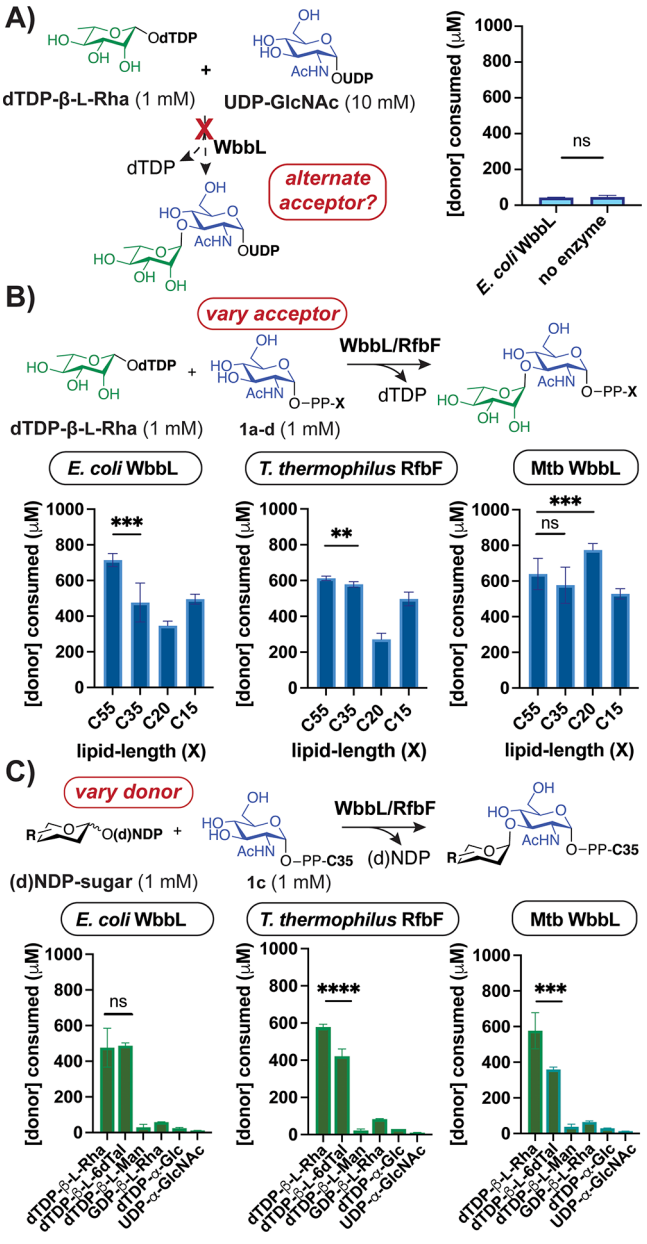

Figure 4. Diverse RTs accept a range of substrates. (A) Comparison of reactions ± *E. coli* WbbL indicates UDP-α-GlcNAc is not used as an acceptor (t = 1 h). (B) Evaluation of glycolipid acceptors shows various lipids are tolerated. (C) Analysis of donor substrates validates that bacterial RTs prefer dTDP-6-deoxysugars, not GDP-sugars or (d)NDP-sugars with a C6-hydroxyl (t = 30 min for B and C). **** p < 0.0001, *** p < 0.0002, ** p < 0.0021, ns: nonsignificant, paired t test used; error bars represent SD (n = 3–9).

donors reflects ITC results and indicates RfbF functions similarly to WbbL.

In *E. coli*, WbbL is required to synthesize oligosaccharide units (O-units), which are flipped, polymerized, and ligated to Lipid A's core sugars in a model O-Ag (O16) pathway, as well as others[7,13,65] (Figure 5A). Since O-Ag are virulence factors,[21] we next aimed to evaluate the cellular consequences of WbbL inhibition. As L-Rha-1-phosphate weakly bound to RfbF, we hypothesized that a reported 6-deoxy-iminosugar (**2**)[66,67] resembling L-Rha could block the donor site. We synthesized[67] and then titrated **2** into *E. coli* WbbL reactions containing the native acceptor with the donor present around cellular

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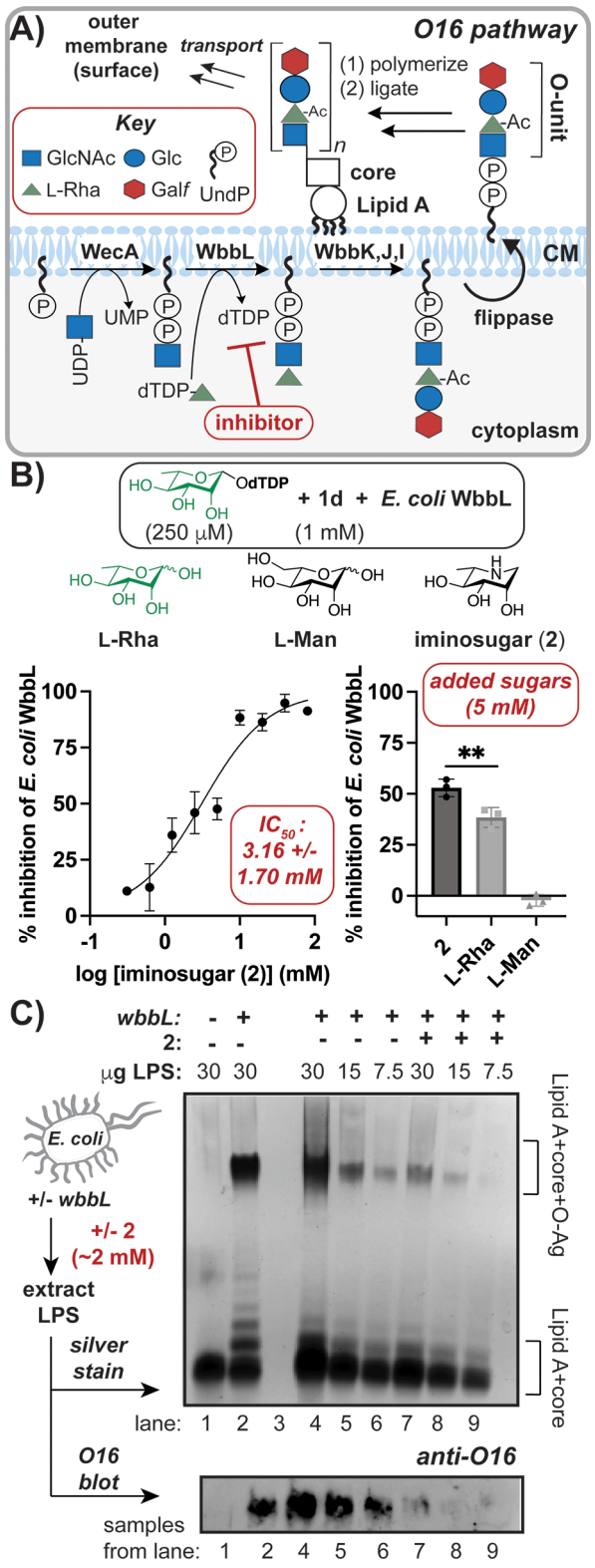

Figure 5. A 6-Deoxy-L-iminosugar inhibits *E. coli* WbbL-dependent O-Ag synthesis. (A) Schematic of O16 O-Ag model pathway, which requires WbbL. (B) *In vitro* inhibition assays indicate iminosugar **2** inhibits WbbL, while L-Man does not (IC_{50} with 95% confidence interval). (C) Addition of **2** to *E. coli* expressing *wbbL* reduces O-Ag synthesis as demonstrated by silver staining (top) and anti-O16 blotting (bottom) of purified LPS; Lipid A standard was utilized (Figure S26). Paired *t* test used; error bars represent SD (n = 3).

concentrations.[56] The resulting half maximal inhibitory concentration (IC_{50}) was ~3 mM (Figure 5B). Direct binding of **2** to RfbF was not detected by ITC, indicating a K_D > 1 mM (Figure S20). **2** may instead bind the acceptor complex more tightly, as observed for other iminosugars with added nucleotide.[68] Compared to structurally related L-monosaccharides (Figure 5B), **2** was a more potent WbbL inhibitor than L-Rha (~53% versus 38% inhibition, respectively), likely because it acts as a transition state mimic.[69] Lack of inhibition by L-Man reinforced the importance of a C6-deoxy in binding. *E. coli* WbbL docking experiments suggested that **2** binds like the native sugar in the donor site, while L-Man does not (Figure S21).

To evaluate the cellular effect of the iminosugar, an *E. coli* strain expressing *wbbL* (Figure S22) was grown with and without **2**. Following LPS extraction, *E. coli* cells exposed to **2** produced >50% less LPS than untreated samples when an equivalent number of cells was analyzed (Figures S23–S24), even though **2** was not toxic (Figure S25).[70−72] To assess if the loss of LPS was due to diminished O-Ag, we analyzed extracted LPS by SDS-PAGE followed by silver staining and blotting for O16. As seen in Figure 5C, O-Ag levels decreased in samples containing **2** (lanes 7−9) versus untreated cells (lanes 4−6), with an ~40% reduction calculated by densitometry (Figure S26).[73] However, there was not complete loss of O-Ag as in *E. coli* lacking functional *wbbL* (lane 1) compared to cells with native *wbbL* (lane 2). Hence, the iminosugar is not an antibacterial, but impairs WbbL-dependent O-Ag synthesis, which represents a potential antivirulence strategy to impair host infection.[3,44,70,74]

In conclusion, we found that donor recognition by WbbL/RfbF is driven by three factors: the absence of a C6-hydroxyl, and the presence of a β-anomeric center and pyrimidine nucleotide. Binding was enthalpy-driven, with negative free energy only for the canonical donor. We hypothesize that as K_D increases, an unfavorable entropic contribution results from less desolvation and/or conformational freedom of non-native ligands upon binding.[52,75−82] Exclusion of activated α-sugars and C6-hydroxyl sugars prevents "incorrect" sugar incorporation into glycans, as cellular UDP-α-GlcNAc and dTDP-α-Glc concentrations (~200 μM) are below measured K_D values.[56,58,83−86] Notably, a natural product RT utilized dTDP-α-Glc as a donor, demonstrating different preferences exist.[87] WbbL proteins transferred dTDP-β-L-6dTal[88] to glycolipids, implying that bacterial polysaccharides can be engineered, as explored by others.[7,89−93] Acceptor analysis indicated that membrane fractions containing endogenous lipids could be used to assess preferences. While RTs can recognize short lipids, *E. coli* WecA, the first transferase in the O-Ag pathway (Figure 5A), requires at least a C35 lipid substrate; hence, shorter lipid lengths are sufficient for later stages of assembly.[59,60,94] Based on predicted structural similarity across these RTs, it is perhaps unsurprising that consistent substrate recognition trends were observed.

In contrast to well-studied bacterial glycosyltransferases, including the peptidoglycan precursor synthase MurG,[95,96] few RT inhibitors exist.[66,67] Since MurG uses the donor UDP-α-GlcNAc, methods to modulate MurG are not transferable to WbbL. Further, while the toxin colicin M depletes O-Ag by targeting carrier lipid modification, it also affects essential biosynthetic pathways.[97] We found that **2** reduces cellular O-Ag levels and is nontoxic; however, like other iminosugars, **2** requires improvement before assessing virulence effects in a

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host.[66,98] Our findings will inform substrate analogue design to discover more potent inhibitors, and probe surface glycan biosynthesis mechanisms and host−pathogen interactions.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/jacs.3c03005.

Tables S1−S8, Figures S1−S26; description and schemes of experimental methods for nucleotide sugar and glycolipid syntheses; compound characterization (NMR, mass spectrometry); methods for plasmid construction, protein expression and purification (Tables S6−S8 contain information on plasmids, primers and strains that were utilized); and additional analyses of enzymatic activities of WbbL and RfbF, confirmation of coupled products (HPLC and mass spectrometry analyses), and bioinformatic analyses (PDF)

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Notes

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