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Synthesis of a Poly-hydroxypyrolidine-Based inhibitor of *Mycobacterium tuberculosis* GlgE

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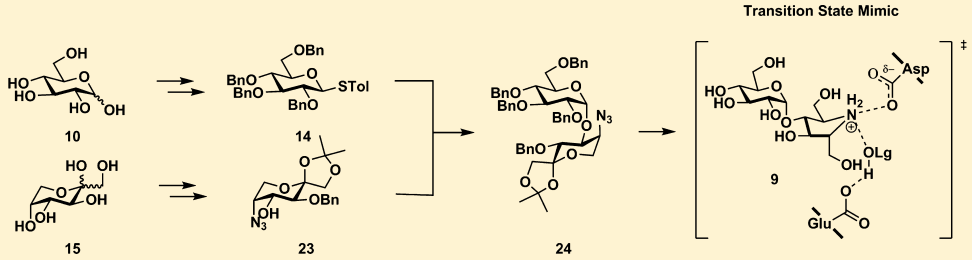
Supporting Information



ABSTRACT: Long treatment times, poor drug compliance, and natural selection during treatment of *Mycobacterium tuberculosis* (*Mtb*) have given rise to extensively drug-resistant tuberculosis (XDR-TB). As a result, there is a need to identify new antituberculosis drug targets. *Mtb* GlgE is a maltosyl transferase involved in α-glucan biosynthesis. Mutation of GlgE in *Mtb* increases the concentration of maltose-1-phosphate (M1P), one substrate for GlgE, causing rapid cell death. We have designed 2,5-dideoxy-3-*O*-α-D-glucopyranosyl-2,5-imino-D-mannitol (**9**) to act as an inhibitor of GlgE. Compound **9** was synthesized using a convergent synthesis by coupling thioglycosyl donor **14** and 5-azido-3-*O*-benzyl-5-deoxy-1,2-*O*-isopropylidene-β-D-fructopyranose (**23**) to form disaccharide **24**. A reduction and intramolecular reductive amination transformed the intermediate disaccharide **24** to the desired pyrolidine **9**. Compound **9** inhibited both *Mtb* GlgE and a variant of *Streptomyces coelicolor* (*Sco*) GlgEI with K_i = 237 ± 27 μM and K_i = 102 ± 7.52 μM, respectively. The results confirm that a *Sco* GlgE-V279S variant can be used as a model for *Mtb* GlgE. In conclusion, we designed a lead transition state inhibitor of GlgE, which will be instrumental in further elucidation of the enzymatic mechanism of *Mtb* GlgE.

INTRODUCTION

Tuberculosis (TB) is a dreadful infectious disease whose causative agent is *Mycobacterium tuberculosis (Mtb)*.[1] Streptomycin was first used to cure TB in the late 1940s. For a short time, streptomycin gave the world hope that TB could be completely eradicated. However, TB has reemerged as a major global health threat due to poverty, co-infection with HIV, long treatment times, and poor drug compliance practices. This has led to the emergence of extensively drug-resistant TB (XDR-TB), which is considered almost untreatable.[1] Hence, the discovery of novel antitubercular agents is urgently needed.

Trehalose (α-D-glucopyranosyl-(1 → 1)-α-D-glucopyranoside) is a pivotal metabolite in mycobacteria used for carrying mycolic acid during biosynthesis of the mycolyl arabinogalactan and trehalose-6,6′-dimycolate (TDM).[2] Following the mycolyl transfer reaction, trehalose is transferred back into the cytoplasm from the periplamic space and is modified in the production of various trehalolipids and sulfolipids.[3,4] Trehalose is also converted into a branched α-glucan. The conversion process involves four enzymes: trehalose synthase (TreS), maltokinase (Pep2), GlgE, and GlgB. The process involves the isomerization of trehalose to maltose (4-*O*-α-D-glucopyranosyl-D-glucose) by TreS, and then α-maltose-1-phosphate (M1P) is produced by phosphorylation of maltose by Pep2.[5] GlgE uses M1P to generate linear α-glucans. Subsequently, α-1,6 branches are introduced into the α-glucan by GlgB.[6]

Kalscheuer et al. reported that absence of GlgE led to self-poisoning by the accumulation of phosphosugar M1P, directed by a self-amplifying feedback response leading to cell death.[6] Cell death was not due to absence of glucan in the cell wall and capsule, but rather it was due to accumulation of M1P to a lethal concentration, which caused cell death. GlgE is essential for survival of the pathogen, and the absence of a human homologue substantiates GlgE as a new drug target.[6]

Syson et al. reported the structure of *Streptomyces coelicolor* (*Sco*) GlgE isoform-I, which indicated that GlgE belongs to the glycosyl hydrolase family GH13.[7] Recently, Syson et al. trapped *Sco* GlgE with 2-deoxy-2-fluoro-α-maltosyl fluoride, which provided additional evidence for a double-displacement mechanism proceeding through a transition state as shown in Scheme 1.[8] The first step of the reaction is nucleophilic attack of Asp418 on the anomeric center of M1P to generate a β-maltosyl enzyme intermediate. The enzyme intermediate is attacked by a glucan to extend the chain. It is predicted that the anomeric oxygen on the phosphate moiety is protonated by

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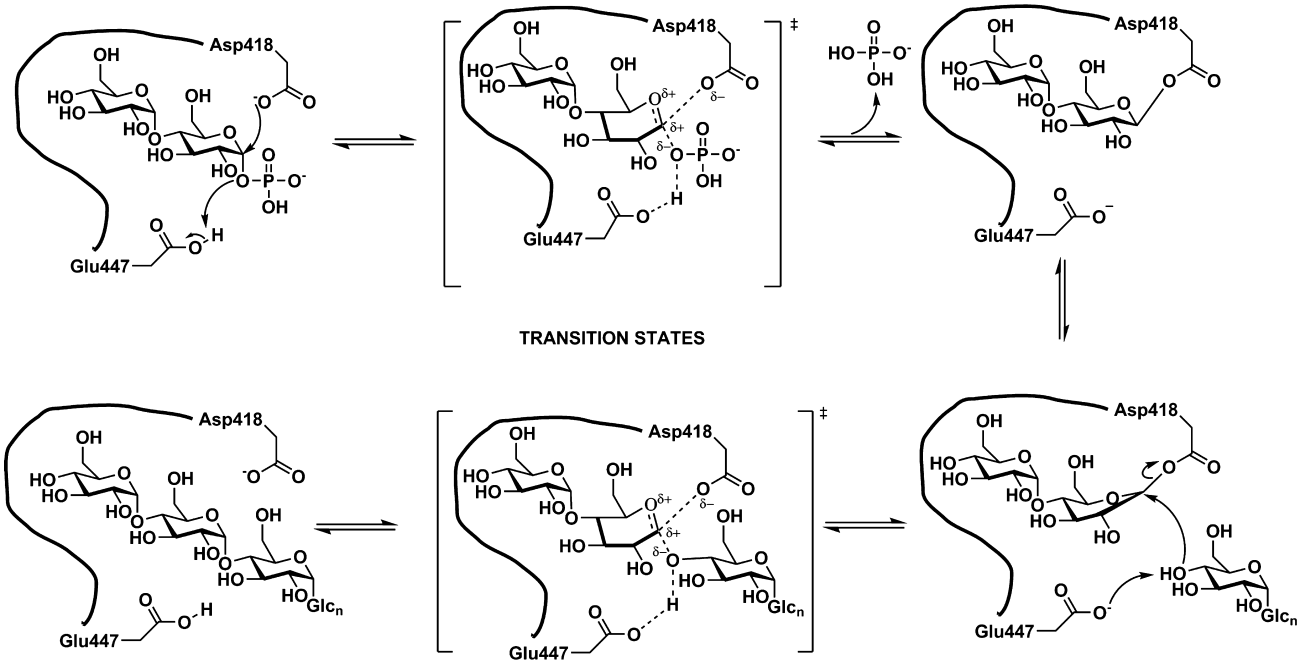
Scheme 1. Proposed α-Retaining Double-Displacement Mechanism of GlgE (*Mtb* GlgE Numbering)



acid/base Glu447. Glutamate447 subsequently deprotonates the incoming glucan. Literature supports the mechanism showing retention of configuration of M1P and its conversion to α-1,4-glycosidic linkage.[6] However, until now attempts to prepare a transition-state inhibitor have not been reported.

The replacement of the endocyclic oxygen atom in carbohydrates with a nitrogen atom has been found in natural products that inhibit glycosyl hydrolases in the micromolar range.[9] Nojirimycin (5-amino-5-deoxy-D-glucose, **1**) and 1-deoxynojirimycin (1, 5-dideoxy-1, 5-imino-D-glucitol, **2**) (Figure 1) are classical examples of such compounds. Poly-hydroxypyrolidines **3** and **4** represent 5-membered ring compounds related to monosaccharides that also act as potent inhibitors for many glucosidases or glycoside hydrolases.[9] The hydroxyl groups present on these compounds resemble the glycan moiety of the substrates. In all of these examples, the nitrogen atom has the ability to become protonated to maintain a positive charge at physiological pH that permits strong interactions with a carboxylate group found in the active site of this enzyme class. The carboxylate is predicted to stabilize the positive charge that develops in the transition state, and it is notable that the 5-membered aza-sugars **3** and **4** are more potent inhibitors, with K_i = 0.18 μM and K_i = 0.33 μM, respectively, than 6-membered aza-sugars **1** and **2**, K_i = 6.3 μM and K_i = 12.6 μM, respectively, against yeast α-glucosidase.[9] According to Wong et al., nojirimycin-type azasugars can mimic only the transitory charge generated during the hydrolysis of glucosides, while the pyrolidine-type aza sugars also mimic the shape of the postulated flattened half-chair transition state (Figure 1).[10,11] Wong et al. also show that five-membered aza sugars were potent inhibitors of α-L-rhamnosidase (glycoside hydrolase family) from *Penicillium decumbens*.[10] For example, compounds **5**–**8** are good inhibitors of pyranose-based α-L-rhamnosidase with K_i values 0.14, 5.5, 11.5, and 46 μM, respectively. Keeping in mind that five-membered ring poly-hydroxypyrolidine have been shown to exhibit very good activity against α-glucosidases, we became interested in other modified analogues of these molecules as transition-state inhibitors for GlgE and designed inhibitor **9**.

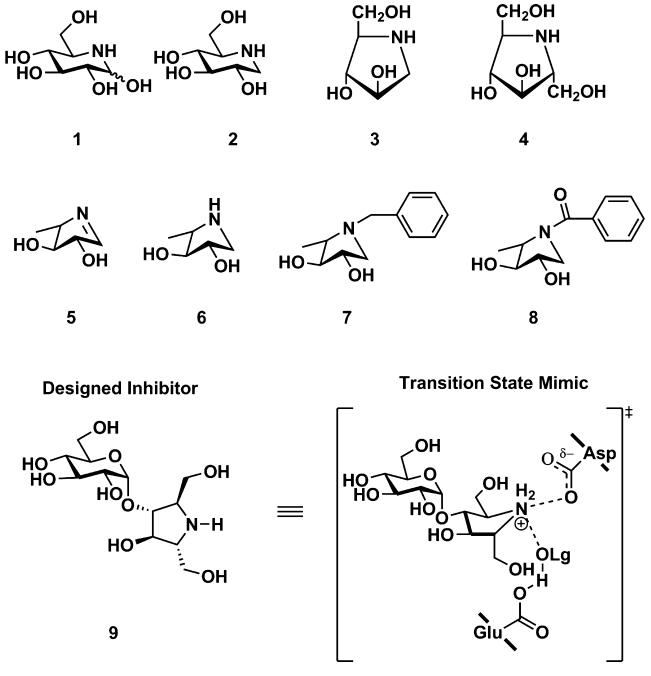


Figure 1. Poly-hydroxypyrrolidine-based inhibitor **9** and illustration of expected binding interactions in the enzyme active site.

RESULTS

Synthesis. In order to obtain the target molecule **9**, we designed a convergent synthesis. We synthesized the compounds *p*-methylphenyl 2,3,4,6-tetra-*O*-benzyl-1-thio-β-D-glucopyranoside (**14**) (Scheme 2) and 5-azido-3-*O*-benzyl-5-deoxy-1,2-*O*-isopropylidene-β-D-fructopyranose (**23**) (Scheme 3) separately. Subsequently, the two glycosides **14** and **23** were coupled together to afford disaccharide **24**, which was subjected

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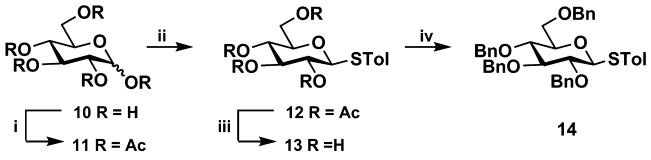
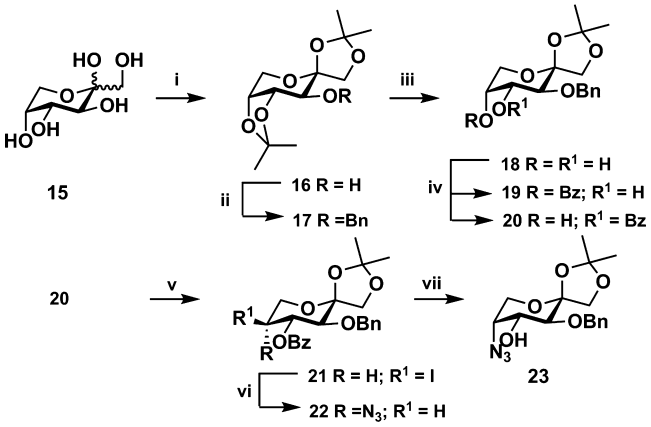
Scheme 2[a]



[a]Reagents and conditions: (i) Ac_2O, pyridine, 4-(dimethylamino)-pyridine, rt (86%); (ii) $BF_3.Et_2O$, *p*-thiocresol, CH_2Cl_2, 0 °C – rt (85%); (iii) NaOMe, MeOH, rt (95%); (iv) NaH, BnBr, *N*,*N*-dimethylformamide, rt (85%).

Scheme 3[a]

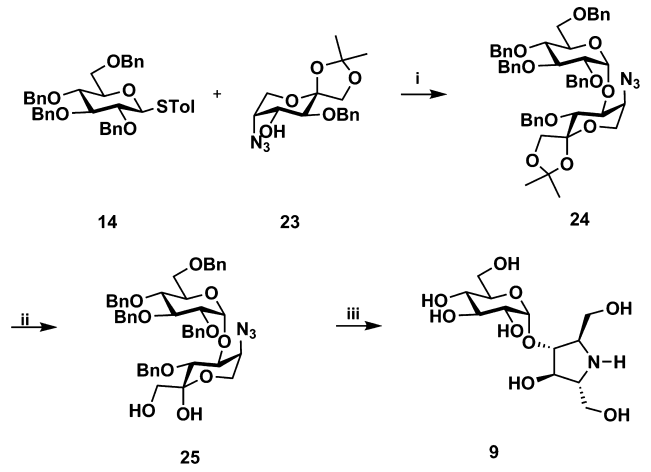


[a]Reagents and conditions: (i) 0.5% H_2SO_4, acetone, rt (59%); (ii) NaH, BnBr, DMF, rt (86%); (iii) 60% aq AcOH, 55 °C (76%); (iv) (a) *n*-Bu_2SnO, MeOH; (b) BzCl, Et_3N, dioxane, 0 °C (**19** = 38%) (**20** = 52%) (1:1.3); (v) I_2, Ph_3P, imidazole, toluene (90%); (vi) NaN_3, *N*,*N*-dimethylformamide, 100 °C (91%); (vii) NaOMe, MeOH, rt (98%).

to global deprotection, rearrangement, and reductive amination to afford poly-hydroxypyrolidine **9** as shown in (Scheme 4).

Synthesis of p-Methylphenyl 2,3,4,6-Tetra-O-benzyl-1-thio-β-D-glucopyranoside (14).[12] To synthesize the thioglycosyl donor **14**, D-glucose (**10**) was peracetylated with acetic anhydride followed by glycosylation with *p*-thiocresol and boron trifluoride diethyl etherate to generate a peracetylated thioglycosyl donor **11** with 85% yield (Scheme 2). Subsequent deacetylation under Zemplén conditions afforded tetraol **13**, which was then benzylated to afford the desired *p*-methylphenyl-2,3,4,6-tetra-*O*-benzyl-1-thio-β-D-glucopyranoside (**14**).

Scheme 4[a]



[a]Reagents and conditions: (i) *N*-iodosuccinamide, TMSOTf, 0 °C, CH_2Cl_2 (76%); (ii) 60% aq TFA, rt (85%); (iii) 5% palladium on activated carbon, toluene/water (1:1), 1 N HCl, 100 psi, quantitative.

Synthesis of 5-Azido-3-O-benzyl-5-deoxy-1,2-O-isopropylidene-β-D-fructopyranose (23).[13] Yáñez et al. and Tatibouët et al. have reported the generation of key intermediate **23** by following a protection and deprotection strategy on D-(+)-fructose **15**.[13,14] D-Fructose (**15**) was protected with acetone to give 1,2:4,5-di-*O*-acetonide (**16**). Alcohol **16** was benzylated at the OH-3 position to afford protected D-(+)-fructose (**17**). Selective deprotection of the 4,5-*O*-isopropylidene group was achieved with 60% acetic acid to produce diol **18**. Initially, controlled Garegg conditions were employed on **18** to give selective iodination at OH-5 as reported by Rollin et al. However, the major product was eliminated product as shown in the cited literature.[15] Therefore, as reported by Yáñez and co-workers, **18** was subjected to selective benzoylation using dibutyltin oxide to afford products **19** and **20** in 38% and 52% yields, respectively. The reaction of **20** with modified Garegg's conditions caused the substitution of the hydroxyl group at C-5 with iodine by inversion of configuration to give the corresponding 4-*O*-benzoyl-3-*O*-benzyl-5-deoxy-5-iodo-1,2-*O*-isopropylidene-α-L-sorbopyranose (**21**).[13] Compound **21** was subjected to an S_N2 substitution reaction with sodium azide in *N*,*N*-dimethylformamide to give product **22** with 90% yield. Finally, the deprotection of the benzoyl group at OH-4 was achieved using Zemplén conditions to afford **23** (Scheme 3).

Synthesis of 2,5-Dideoxy-3-O-α-D-glucopyranosyl-2,5-imino-D-mannitol (9).[16] Coupling of compound **14** and **23** using classical glycosylation conditions with *N*-iodosuccinamide and trimethylsilyl trifluoromethanesulfonate yielded disaccharide **24** with 76% yield with the α-anomer as a single product.[17] The deprotection of 1,2-*O*-acetonide was achieved using 60% trifluoroacetic acid to afford diol **25**. Compound **25** was first subjected to reduction and intramolecular reductive amination using methanol, ethanol, and ethyl acetate as solvents in 1 N hydrochloric acid (HCl) and yielded the desired product mixed with methyl- and ethyl-substituted products, respectively, as confirmed by ESI-MS. Presumably, aldehydes formed from trace oxidation of the respective solvents participated in a tandem reductive amination process to produce the observed masses. These undesired reductive-amination products were avoided by replacing the alcoholic solvents with a solution of toluene/water/HCl (1:1:1). The use of H_2 gas at 100 psi also helped to afford a polyhydroxypyrolidine **9** in a clean transformation (Scheme 4). To access M1P for working on inhibition studies we followed our reported procedure.[18]

Inhibition Studies. Two different GlgE homologues were studied using the EnzChek Phosphate Assay Kit (Molecular Probes). This assay couples the phosphate produced from GlgE activity to the phosphorylase activity of purine nucleoside phosphorylase, which is based on work originally described by Webb.[19] Briefly, purine nucleoside phosphorylase (PNP) converts the substrate 2-amino-6-mercapto-7-methylpurine riboside (MESG) to ribose-1-phosphate and 2-amino-6-mercapto-7-methylpurine (Figure 2).[19] Conversion of MESG to 2-amino-6-mercapto-7-methylpurine shifts absorbance from 330 to 360 nm, respectively. The assay is described in Figure 2.

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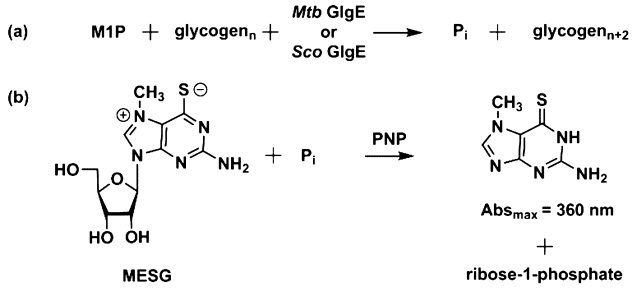

Figure 2. GlgE-coupled enzyme assay utilizing PNP; (a) M1P and glycogen (n = a number of maltose units) serve as substrates for GlgE; (b) PNP consumes P_i and MESG to form 2-amino-6-mercapto-7-methylpurine.

Using this assay, we determined a K_i for compound **9** for *Mtb* GlgE and the *Sco* GlgEI-V279S variant, which was designed using multiple sequence alignments to better emulate the *Mtb* GlgE maltosyl donor site (Figures 3 and 4). Assays were

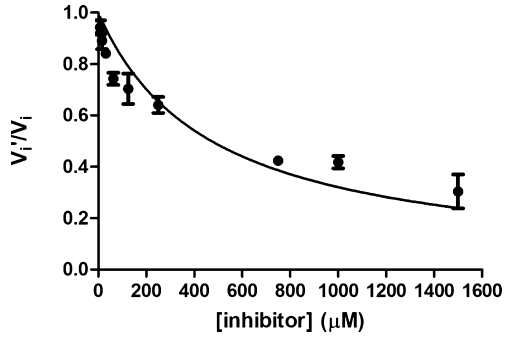

Figure 3. K_i determination of **9** with *Mtb* GlgE. V_i'/V_i are steady-state rates with and without inhibitor. Error bars are calculated from the result of reactions performed in triplicate.

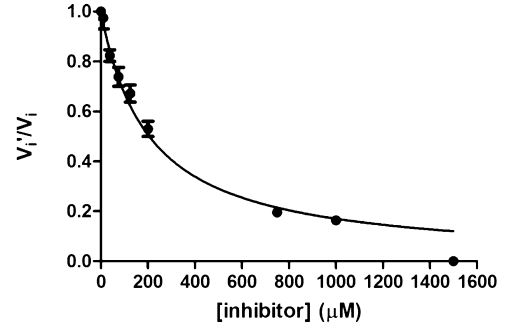

Figure 4. K_i determination of **9** with *Sco* GlgEI-V279S. V_i'/V_i are steady-state rates with and without inhibitor. Error bars are calculated from the result of reactions performed in triplicate.

performed under atmospheric pressure at 22 °C in a 96 well format on a Spectra max 340PC (Molecular Devices). All 1 mM MESG stock solutions were prepared and stored in dH_2O at −20 °C. Phosphate release, catalyzed by *Mtb* GlgE, was monitored by the production of 2-amino-6-mercapto-7-methylpurine in a coupled assay catalyzed by purine nucleoside phosphorylase. The reaction was monitored at 5 s intervals for 30 min. Each reaction consisted of 1 mM MESG, 0.2 U of PNP, 20X reaction buffer (1.0 M Tris−HCl, 20 mM $MgCl_2$, pH 7.5, containing 2 mM sodium azide), glycogen as maltosyl acceptor, 50 nM GlgE (0.01 U), 250 μM M1P, and varied concentration of inhibitor **9**. Inhibition was determined by comparing the relative rate of the reaction performed with inhibitor against a reaction that contained no inhibitor (V_i'/V_i), where V_i' and V_i are steady-state rates with and without inhibitor, respectively. The equilibrium dissociation constant (K_i) for inhibitor **9** for *Mtb* GlgE found to be 237 μM ± 27 and *Sco* GlgEI-V279S found to be 102 μM ± 7.52. These values are near the reported *Mtb* GlgE K_m of 250 μM ± 5.[6]

DISCUSSION

We have initiated efforts to develop transition-state inhibitors for GlgE by replacing of the endocyclic oxygen atom of glycosides with a nitrogen atom by considering the structures of natural products that inhibit glycoside hydrolases. We anticipate the resulting secondary ammonium moiety will mimic that partial positive charge accumulation at the anomeric carbon of the true substrate and form a strong interaction with the Asp418 nucleophile conserved in this enzyme class. The confluence of these interactions would therefore promote reasonable inhibition of all GlgE homologues. We were able to synthesize 2,5-dideoxy-3-*O*-α-D-glucopyranosyl-2,5-imino-D-mannitol (**9**) by following a convergent glycosylation strategy followed by in situ deprotection and intramolecular reductive amination. The ^1H, ^{13}C NMR and optical rotation data are in accordance with the literature values.[16] We evaluated the K_i for both *Mtb* GlgE and *Sco* GlgEI-V279S using a PNP-based coupled enzyme assay we developed. This assay has the advantage of being performed in continuous mode. We also tested whether 2,5-dideoxy-3-*O*-α-D-glucopyranosyl-2,5-imino-D-mannitol (**9**) would inhibit the coupling enzyme PNP and no inhibition was observed (Supporting Information, Figure S-21). Since compound **9** inhibited both *Mtb* GlgE and *Sco* GlgEI-V279S to a similar degree, this confirms that *Sco* GlgEI-V279S can be used as a surrogate for *Mtb* GlgE due to its similarity to the *Mtb* GlgE maltosyl donor site, enzymatic activity, laboratory stability and ease of crystallization. We are currently in the process of addressing the detailed mode of interaction of 2,5-dideoxy-3-*O*-α-D-glucopyranosyl-2,5-imino-D-mannitol (**9**) with *Sco* GlgEI-V279S via X-ray crystallography. The data from those ongoing studies are expected to provide valuable insight concerning the development of more potent transition-state inhibitors for *Mtb* GlgE.

CONCLUSION

We have synthesized a 2,5-dideoxy-3-*O*-α-D-glucopyranosyl-2,5-imino-D-mannitol (**9**). We evaluated the inhibitory activity of **9** against *Mtb* GlgE and *Sco* GlgEI-V279S. The K_i values were 237 ± 27 μM for *Mtb* GlgE and K_i 102 ± 7.52 μM for *Sco* GlgEI-V279S, respectively. We have identified a lead transition state inhibitor against GlgE enzyme. Compound **9** has been successfully cocrystallized with *Sco* GlgEI-V279S and the X-ray diffraction data will be published elsewhere. The data will help us develop an improved class of transition state-based GlgE inhibitors which may lead to the discovery of new therapies to treat TB.

EXPERIMENTAL SECTION

Materials and Methods. All fine chemicals such as D-(+)-glucose monohydrate, benzyl bromide, acetic anhydride, *p*-thiocresol, benzoyl chloride, dibutyltin oxide, 5% palladium on activated carbon, trifluoroacetic acid, sodium azide, and anhydrous solvents such as anhydrous methanol and *N*,*N*-dimethylformamide were purchased and used without purification. TLC (silica gel 60, f_{254}) was visualized under UV light or by charring (5% H_2SO_4−MeOH). Flash column

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chromatography was performed on silica gel (230−400 mesh) using solvents as received. ^{1}H NMR were recorded either on 600 MHz spectrometer in $CDCl_3$ using residual $CHCl_3$ as internal references, respectively. ^{13}C NMR were recorded on the 600 MHz in $CDCl_3$ using the triplet centered at δ 77.27 as internal reference. GCOSY method was used to confirm the NMR peak assignment. High resolution mass spectrometry (HRMS) was performed on a micro mass Q-TOF2 instrument. EnzChek Phosphate Assay Kit was purchased for use in the *Mtb* GlgE inhibition assay.

1,2,3,4,6-Penta-*O*-acetyl-D-glucopyranose (11).[20,21] D-(+)-Glucose (**10**) (3.00 g, 16.6 mmol) was suspended in dry pyridine (6.71 mL, 83.2 mmol) and acetic anhydride (7.8 mL, 83.0 mmol), and a catalytic amount of 4-(dimethylamino)pyridine was added. The solution was stirred at ambient temperature for 16 h. The reaction mixture was diluted with ethyl acetate and washed successively with 1 N HCl (30 mL) and saturated aq $NaHCO_3$ (60 mL). The resulting organic phase was dried (anhydrous Na_2SO_4) and filtered, and the filtrate concentrated under reduced pressure to give product **11**: yield 86% (5.6 g); silica gel TLC R_f = 0.5 (1:1 acetone−hexane); ^{1}H NMR ($CDCl_3$, 600 MHz) δ 2.02 (d, J = 12 Hz, 9H), 2.10 (d, J = 12 Hz, 6H), 3.83 (dd, J = 1.8 Hz, 6 Hz, 1H), 4.10 (d, J = 6 Hz, 1H), 4.28 (d, J = 12 Hz, 1H), 5.11−5.26 (m, 3H), 5.70 (d, J = 12 Hz, 1H) ppm.

4-Methylphenyl 2,3,4,6-Tetra-*O*-acetyl-β-D-thioglucopyranoside (12).[20,21] Peracetylated glucose (**11**) (2.0 g, 5.1 mmol) was dissolved in dichloromethane (20 mL). To the solution were added by *p*-thiocresol (955 mg, 7.60 mmol) and boron trifluoride−diethyl etherate (0.7 mL, 5.6 mmol) at 0 °C. The resulting solution was stirred at ambient temperature under nitrogen atmosphere. The reaction was monitored by TLC and appeared to be complete after 16 h. The reaction mixture was diluted with dichloromethane (14 mL) and successively washed with saturated aq $NaHCO_3$ (16 mL), 10% aq NaCl (8 mL), and water (16 mL). The resulting organic phase was dried (anhydrous Na_2SO_4) and filtered, and the filtrate was concentrated under reduced pressure to give product **12**. The product was purified by silica gel flash column chromatography by eluting with 1:9 acetone−hexane. The product fractions were combined, concentrated, and dried in vacuum to afford a white crystalline product to give product **12**: yield 85% (1.9 g); silica gel TLC R_f = 0.61 (2:8 acetone−hexane); ^{1}H NMR ($CDCl_3$, 600 MHz) δ 2.0 (d, J = 12 Hz, 6H), 2.09 (s, 6H), 2.35 (s, 3H), 3.69 (dd, J = 1.8 Hz, 6 Hz, 1H), 4.19 (m, 2H), 4.63 (d, J = 6 Hz, 1H), 4.92−5.03 (m, 2H), 5.21 (d, J = 12 Hz, 1H), 7.12−7.40 (m, 4H) ppm.

4-Methylphenyl 1-Thio-β-D-glucopyranoside (13).[20,21] Compound **12** (800 mg, 1.76 mmol) was deacetylated by dissolving in dry methanol followed by addition of a catalytic amount of sodium metal until the solution reached pH 9. The reaction was monitored for completion using TLC. The reaction was neutralized by adding Amberlite IRA-118H H^+ resin until the pH reached 7. Then resin was filtered away and the filtrate was concentrated under reduced pressure to afford the compound **13**: yield 95% (0.48 g); ^{1}H NMR ($CDCl_3$, 600 MHz): δ 2.42 (s, 3H), 3.31−3.52(m, 4H), 3.79 (dd, J = 6 Hz, 12 Hz, 1H), 3.98 (d, J = 12 Hz, 1H), 4.64 (d, J = 10.2 Hz, 1H), 7.23−7.59 (m, 4H) ppm.

***p*-Methylphenyl 2,3,4,6-Tetra-*O*-benzyl-1-thio-β-D-glucopyranoside (14).**[12] A solution of compound **13** (462 mg, 1.61 mmol) in dry *N*,*N*-dimethylformamide (10 mL) was cooled to 0 °C. The solution was treated dropwise with a suspension of sodium hydride (60% dispersion in mineral oil) (452 g, 11.3 mmol). Benzyl bromide (1.14 mL, 9.66 mmol) was added dropwise over 30 min and the solution stirred at room temperature for 16 h. The reaction was poured over ice and extracted with diethyl ether (10 mL). The combined organic layers were washed with brine. The resulting organic phase was dried (anhydrous Na_2SO_4) and filtered, and the filtrate concentrated under reduced pressure to obtain a product. The product was purified by silica gel flash column chromatography by eluting with 1:9 acetone−hexane. The product fractions were combined, concentrated, and dried in vacuum to afford a yellow solid product **14**: yield 85% (1.87g); silica gel TLC R_f = 0.61 (8:2 hexane−ethyl acetate); ^{1}H NMR ($CDCl_3$, 600 MHz) δ 2.32 (s, 3H), 3.50 (t, J = 12 Hz, 2H), 3.64−3.81 (m, 4H), 4.55−4.92 (m, 8H), 7.04−7.52 (m, 20H) ppm.

1,2:4,5-Di-*O*-isopropylidene-β-D-fructopyranose (16).[22] To a suspension of D-(+)-fructose (**15**) (10.0 g, 55.5 mmol) and acetone (200 mL) was added concentrated sulfuric acid (0.97 mL). The reaction mixture was stirred for 2 h at room temperature. The reaction was cooled to 0 °C and was quenched with NaOH (3.0 g) in H_2O (28 mL). After evaporation of the acetone, the residue was extracted with dichloromethane. The organic phase was separated, washed with water and brine, and then dried over anhydrous $MgSO_4$. After filtration and removal of solvents under reduced pressure, the residue was recrystallized with ether/hexane to afford the product as white solid **16**: yield 59% (8.5 g); silica gel TLC R_f = 0.41 (1:1 ethyl acetate−hexane); ^{1}H NMR ($CDCl_3$, 600 MHz) δ 1.36 (s, 3H), 1.41 (s, 3H), 1.49 (s, 3H), 1.56 (s, 3H), 2.11 (d, J = 6.0 Hz, 1H), 3.71 (dd, J = 6.0, 18 Hz, 2H), 3.79 (d, J = 12 Hz, 1H), 3.92 (dd, J = 1.8 Hz,13.2 Hz, 1H), 4.25 (dd, J = 1.2 Hz, 7.8 Hz, 1H), 4.35 (d, J = 2.4 Hz, 1H), 4.62 (dd, J = 3 Hz, 8.4 Hz, 1H) ppm.

3-*O*-Benzyl-1,2:4,5-*O*-isopropylidene-β-D-fructopyranose (17).[22] To a solution of **16** (4.0 g, 15.7 mmol) in dry *N*,*N*-dimethylformamide (10 mL) was added sodium hydride (524 mg, 31.5 mmol) at room temperature. The solution was stirred for 0.5 h, and then benzyl bromide (1.3 mL, 31.5 mmol) was added. The reaction was further stirred for 1 h at room temperature, diluted with water, and then extracted with diethyl ether. The combined extracts were washed with water and brine, dried over anhydrous $MgSO_4$, and concentrated under reduced pressure. The residue was purified by flash chromatography on silica gel with 1:9 acetone−hexane to give 3-*O*-benzyl-1,2-*O*-isopropylidene-β-D-fructopyranose (**17**) as an oily product: yield 86% (4.76 g); silica gel TLC R_f = 0.34 (3:7 acetone−hexane); ^{1}H NMR ($CDCl_3$, 600 MHz) δ 1.33 (s, 3H), 1.41 (s, 3H), 1.43 (s, 3H), 1.55 (s, 3H), 3.61 (dd, J = 12.0 Hz, 30 Hz, 2H), 3.74 (d, J = 12 Hz, 1H), 3.91 (d, J = 6 Hz, 1H), 4.25 (d, J = 6 Hz, 1H), 4.45−4.68 (m, 2H), 7.28−7.34 (m, 5H) ppm.

3-*O*-Benzyl-1,2-*O*-isopropylidene-β-D-fructopyranose (18).[22] A solution of benzyl ether **17** (1.0 g, 3.2 mmol) in 60% acetic acid in water was stirred overnight at 55 °C. After evaporation of solvent, the residue was purified by flash chromatography on silica gel with 1:9 acetone−hexane to give the diol **18** as a white solid: yield 76% (675 mg); silica gel TLC R_f = 0.25 (3:7 ethyl acetate−hexane); ^{1}H NMR ($CDCl_3$, 600 MHz) δ 1.30 (s, 3H), 1.51 (s, 3H), 3.18 (d, J = 6 Hz, 1H), 3.48 (d, J = 6 Hz, 1H), 3.72 (d, J = 12.0 Hz, 1H), 3.73−4.05 (m, 2H), 4.57 (d, J = 12 Hz,1H), 4.67 (d, J = 12 Hz, 1H), 7.31−7.38 (m, 5H) ppm.

5-*O*-Benzoyl- and 4-*O*-Benzoyl-3-*O*-benzyl-1,2-*O*-isopropylidene-β-D-fructopyranose (19 and 20).[13] To a stirred solution of **18** (500 mg, 1.61 mmol) in anhydrous methanol (5 mL) was added dibutyltin oxide (441 mg, 1.77 mmol). The suspension was heated for 7 h under reflux and then concentrated to afford the 3,4-dibutylstannylene derivative as a solid foam. The material was dried overnight under vacuum. The foam was taken up in triethylamine (0.24 mL, 1.77 mmol) in anhydrous dioxane (5 mL) and cooled (0 °C). To the solution was added a solution of benzoyl chloride (0.21 mL, 1.77 mmol) in the same solvent (1 mL) by dropwise addition. The reaction was allowed to warm to room temperature and was stirred for an additional 4 h. TLC revealed the presence of two new compounds of higher mobility. Methanol (5 mL) was added, and after 30 min the mixture was concentrated and the residue was subjected to a flash chromatography with 1:9 acetone-hexane to afford first pure **19**: yield 38% (253 mg); silica gel TLC R_f = 0.26 (3:7 acetone−hexane); ^{1}H NMR ($CDCl_3$, 600 MHz) δ 1.47 (s, 3H), 1.51 (s, 3H), 3.79 (d, J = 12.0 Hz, 1H), 3.90 (dd, J = 1.8 Hz, 12 Hz, 1H), 4.0 (dd, J = 1.8 Hz, 13 Hz, 1H), 4.12−4.14 (m, 2H), 4.32 (dd, J = 6 Hz, 12 Hz, 1H), 4.78 (d, J = 12 Hz, 1H), 4.93 (d, J = 12 Hz, 1H), 5.44 (m, 1H), 7.36−8.08 (m, 10H) ppm; a second elution gave **20**; yield: 52% (350 mg); silica gel TLC R_f = 0.36 (3:7 acetone-hexane); ^{1}H NMR ($CDCl_3$, 600 MHz) δ 1.44 (s, 3H), 1.54 (s, 3H), 3.81 (dd, J = 2 Hz, 12.0 Hz, 1H), 4.01 (m, 2H), 4.13 (m, 1H), 4.66 (d, J = 12 Hz, 1H), 4.87 (d, J = 12 Hz, 1H), 5.54 (d, J = 6 Hz, 1H), 7.29−8.11 (m, 10H) ppm.

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4-*O*-Benzoyl-3-*O*-benzyl-5-deoxy-5-iodo-1,2-*O*-isopropylidene-α-L-sorbopyranose (21).[13] To a solution of triphenylphosphine (430 mg, 1.64 mmol), imidazole (223 mg, 3.28 mmol), and iodine (416 mg, 1.64 mmol) in dry toluene was added compound **20** (340 mg, 0.82 mmol). The solution was heated at 110 °C for 2 h. TLC revealed a compound higher mobility than starting material. The reaction was cooled, washed successively with 10% aq sodium thiosulfate and brine, and then concentrated. The concentrated residue was subjected to a flash chromatography with 1:9 acetone−hexane to afford compound **21**: yield 90% (0.38 g); silica gel TLC R_f = 0.5 (3:7 acetone−hexane); ^{1}H NMR ($CDCl_3$, 600 MHz) δ 1.42 (s, 3H), 1.52 (s, 3H), 3.61 (d, J = 6 Hz, 1H), 3.86 (d, J = 12 Hz, 1H), 3.90−3.94 (m, 2H), 4.11 (m, 2H), 4.47 (d, J = 12 Hz, 1H), 4.65 (d, J = 6 Hz, 1H), 5.83 (m, J = 9 Hz, 1H), 7.13−8.11 (m, 10H) ppm.

5-Azido-4-*O*-benzoyl-3-*O*-benzyl-5-deoxy-1,2,-*O*-isopropylidene-β-D-fructopyranose (22).[13] A stirred solution of **21** (275 mg, 0.52 mmol) and sodium azide (171 mg, 2.62 mmol) in dry *N*,*N*-dimethylformamide (10 mL) was heated at 100 °C for 24 h. TLC revealed a slightly slower running compound in comparison to starting material. The reaction was cooled, diluted with water, and then extracted with diethyl ether. The combined extracts were washed with water and brine, dried over anhydrous $MgSO_4$, and concentrated under reduced pressure. The residue was purified by flash chromatography on silica gel with 1:9 acetone−hexane to give compound **22** as a crystalline product: yield 91% (0.23 g); silica gel TLC R_f = 0.73 (3:7 acetone−hexanes); ^{1}H NMR ($CDCl_3$, 600 MHz) δ 1.44 (s, 3H), 1.53 (s, 3H), 3.78 (dd, J = 6 Hz, 12 Hz, 1H), 3.99 (dd, J = 12 Hz, 18 Hz, 2H), 4.05 (d, J = 6 Hz, 1H), 4.15 (dd, J = 6 Hz, 12 Hz, 1H), 4.27 (m, 1H), 4.67 (d, J = 12 Hz, 1H), 4.88 (d, J = 12 Hz, 1H), 5.66 (dd, J = 6 Hz, 12 Hz, 1H), 7.29−8.14 (m, 10H) ppm.

5-Azido-3-*O*-benzyl-5-deoxy-1,2-*O*-isopropylidene-β-D-fructopyranose (23).[15] The compound **22** (0.21 g, 0.47 mmol) was debenzoylated by being dissolved in dry methanol followed by addition of a catalytic amount of sodium metal until the solution reached pH 9. The reaction was monitored for completion using TLC. The reaction was neutralized by adding Amberlite IRA-118H H^+ resin until the pH reached 7. The resin was filtered away, and the filtrate concentrated under reduced pressure and purified by flash chromatography on silica gel with 1:9 acetone−hexane to afford the compound **23**: yield 98% (0.15 g); silica gel TLC R_f = 0.42 (3:7 acetone−hexanes); ^{1}H NMR ($CDCl_3$, 600 MHz) δ 1.43 (s, 3H), 1.48 (s, 3H), 3.67 (d, J = 12 Hz, 1H), 3.94−4.05 (m, 5H), 4.19 (dd, J = 6 Hz, 12 Hz, 1H), 4.76 (d, J = 12 Hz, 1H), 4.84 (d, J = 12 Hz, 1H), 7.31−7.38 (m, 5H) ppm.

2,3,4,6-Tetra-*O*-benzyl-1-(5-azido-3-*O*-benzyl-5-deoxy-1,2-*O*-isopropylidene-β-D-fructopyranose)-α-D-glucopyranoside (24). A mixture of *p*-methylphenyl 2,3,4,6-tetra-*O*-benzyl-1-thio-β-D-glucopyranoside (**14**) (185 mg, 0.280 mmol) and 5-azido-3-*O*-benzyl-5-deoxy-1,2,-*O*-isopropylidene-β-D-fructopyranose (**23**) (80.0 mg, 0.23 mmol) was dissolved in dichloromethane (5 mL). Powdered 4 Å molecular sieves were added (200 mg) to the solution, and the mixture was stirred under N_2 at 0 °C 1 h. *N*-Iodosuccinamide (80.3 mg, 0.350 mmol) and trimethylsilyl triflate (0.017 mL, 0.097 mmol) were added. After being stirred for 10 min, the reaction mixture was neutralized with triethylamine, diluted with dichloromethane, and filtered through Celite. The filtrate was washed successively with saturated sodium thiosulfate, water, and brine. The filtrate was dried over sodium sulfate (anhydrous), filtered, concentrated to dryness, and purified by flash chromatography by eluting with 1:9 acetone−hexane to give compound **24**: yield 76% (0.35 g); $[\alpha]^{23}_D$ = −14.0 (c = 1, $CHCl_3$); silica gel TLC R_f = 0.45 (3:7 ethyl acetate−hexanes); ^{1}H NMR ($CDCl_3$, 600 MHz) δ 1.44 (s, 3H, CH_3), 1.48 (s, 3H, CH_3), 3.49 (dd, 1H, J = 3.6, 9.6 Hz, H-2′), 3.57 (t, 1H, J = 9.6 Hz, H-6a′), 3.67 (m, 1H, H-4′), 3.86 (dd, 2H, J = 9.0, 29.0 Hz, H_a, H_b), 3.97 (dd, 2H, J = 12, 84 Hz, H-$6_{a,b}$), 4.06 (m, 2H, H-3′, H-6′b), 4.14 (m, 1H, H-5′), 4.44−5.15 (m, 10H, $-CH_2Ph$), 4.97 (d, 1H, J = 3.6 Hz, H-1′), 7.15−7.31 (m, 25H, aromatic H's), 4.85−4.37 (m, 14H, $-CH_2Ph$), 5.40 (d, J = 3.6 Hz, H-1′), 7.32- 7.39 (m, 35H, aromatic H's); ^{13}C NMR (150 MHz, $CDCl_3$) δ 26.2, 27.2, 61.4, 61.9, 69.0, 71.6, 71.9, 73.5, 73.7, 74.3, 75.3, 75.8, 79.9, 79.9, 80.7, 81.9, 100.4, 106.0, 112.3, 127.5, 127.6, 128.1, 128.5, 138.0, 138.3, 138.7 ppm; mass spectrum (HRMS), m/z = 880.3793 $(M + Na)^+$, $C_{50}H_{55}N_3O_{10}$ requires 880.3785.

2,3,4,6-Tetra-*O*-benzyl-1-(5-azido-3-*O*-benzyl-5-deoxy-β-D-fructopyranose)-α-D-glucopyranoside (25). A solution of compound **24** in 60% aqueous trifluoroacetic acid was stirred at room temperature for 30 min. The solution was concentrated and repeatedly coevaporated with toluene. The residue was purified by silica gel flash column chromatography by eluting with 1:9 ethyl acetate−hexanes. The product fractions were combined, concentrated, and dried in vacuum to afford a yellow solid product **25**: yield 85% (1.87 g); $[\alpha]^{23}_D$ = 10.1 (c = 1, $CHCl_3$); silica gel TLC R_f = 0.61 (8:2 hexanes−ethyl acetate); ^{1}H NMR ($CDCl_3$, 600 MHz) δ 3.36 (dd, 2H, J = 6, 72 Hz, H-6a, H-6b), 3.51 (dd, 1H, J = 3.6, 7.2 Hz, H-2′), 3.62 (m, 4H's, H-5, H-4, H-a, H-6′), 3.70 (d, 1H, J = 18 Hz, H-3′), 3.96 (d, 1H, J = 18 Hz, Ha), 4.05 (m, 3H, H-3′, H-4, H-6′), 4.12 (m, 1H, H-5′), 4.4−5.0 (m, 10H's, $-CH_2Ph$), 5.01 (d, 1H, J = 3.6 Hz, H-1′), 7.07- 7.33 (m, 25H, aromatic H's); ^{13}C NMR (150 MHz, $CDCl_3$) δ 55.3, 57.1, 60.9, 61.5, 65.2, 65.2, 68.1, 69.0, 71.5, 71.6, 73.1, 73.4, 73.6, 73.7, 74.2, 74.9, 75.2, 76.0, 77.0, 77.2, 77.9, 78.9, 79.7, 80.2, 81.8, 82.3, 96.9, 98.1, 100.4, 127.9, 128.5, 128.8, 137.0, 137.3, 137.8, 137.9, 138.5, 138.7 ppm; mass spectrum (HRMS), m/z = 840.3472 $(M + Na)^+$, $C_{47}H_{51}N_3O_{10}$ requires 840.3469.

2,5-Dideoxy-3-*O*-α-D-glucopyranosyl-2,5-imino-D-mannitol (9).[16] Palladium (5%) on carbon (catalytic amount) was added to a solution of compound **25** (90.0 mg, 0.08 mmol) in toluene (0.5 mL) and water (0.5 mL). The reaction mixture was stirred at ambient temperature under hydrogen atmosphere (98 psi) for 24 h. The catalyst was filtered away, and reaction mixture was washed with methanol. The filtrate was concentrated and the residue purified by gel permeation column chromatography on Sephadex LH-20 (H_2O) to obtain pure compound **9** as a crystalline solid: yield quantitative (27 mg); $[\alpha]^{23}_D$ = +48.5 (c = 0.1, H_2O) [lit.[16] $[\alpha]^{20}_D$ = +49.5 (c = 0.1, H_2O)]; ^{1}H NMR ($CDCl_3$, 600 MHz) δ 3.32−3.93 (m, 12 H, H_a, H_b, H-1, H-4, $H_{a'}$, $H_{b'}$, H-2′, H-3′, H-4′, H-5′, H-$6_{a,b}$), 4.20 (t, 1H, J = 6 Hz, H-2), 4.30 (t, 1H, J = 6 Hz, H-3), 5.19 (d, 1H, J = 3.84 Hz, H-1′); ^{13}C NMR (150 MHz, $CDCl_3$) δ 57.1, 58.3, 60.3, 62.0, 63.1, 69.2, 70.8, 72.4, 72.5, 73.5, 80.4, 98.1 ppm; mass spectrum (HRMS), m/z = 326.1463 $(M + H)^+$, $C_{12}H_{23}NO_9$ requires 326.1451.

Protein Expression and Purification. The *glgE* (Rv1327c) gene from *M. tuberculosis* strain H37Rv was PCR amplified using the following primers: 5′- CAC CAT ATG AGT GGC CGG GCA AT-3′ and 5′- AAA GGA TCC TCA CCT GCG CAG CA- 3′. The product was placed between the NdeI and *Bam*HI cut sites of a modified pET-28 plasmid (EMD Biosciences). The resulting pDR28-glgE encodes a recombinant GlgE enzyme possessing an N-terminal polyhistidine tag. The gene encoding the Sco GlgEI-V279S variant was placed between the NdeI and XhoI cut sites of pET32 (EMD Biosciences) resulting in plasmid pET32-*Sco*-V279S. The recombinant protein expressed by this plasmid possesses a C-terminal polyhistidine tag. The sequences of both plasmids were confirmed by DNA sequencing.

The pDR28-glgE or pET32-Sco-V279S plasmid was used to transform T7 Rosetta cells. The bacterial cells were cultured at 37 °C in Luria Broth to an O.D. of 1.2−1.6 at 600 nm. Protein expression was induced by the addition of IPTG to a final concentration of 1 mM. Cells were harvested by centrifugation after incubating for 24 h at 16 °C. Pelleted cells were resuspended in buffer A containing 5 mM imidazole, 500 mM NaCl, 20 mM Tris pH 7.5, 10% glycerol, and 5 mM β-mercaptoethanol. Lysozyme (10 μM) and DNaseI (100 μM) were added to the cell resuspension and incubated for 1 h on ice prior to lysis by sonication. The resulting suspension was centrifuged at 15000*g* for 30 min. The supernatant was applied to a 5 mL HiTrap Talon Crude column (GE Healthcare) previously equilibrated with buffer A. Proteins were eluted from the column by applying a linear gradient of imidizole from 5−500 mM over 20 column volumes. Fractions containing GlgE were pooled and applied to a Hi-Load Superdex 200 size-exclusion column for further purification. GlgE was eluted from the column isocratically with a buffer containing 150 mM NaCl, 20 mM Tris pH 7.5, and 0.3 mM TCEP. Fractions containing only GlgE were subsequently pooled. The purity of the protein was

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confirmed using SDS-PAGE and concentrated using ultrafiltration. *Sco* GlgEI-V279S was purified using the same protocol.

Inhibition Studies. Assays were performed under atmospheric pressure at 22 °C in a 96-well format on a Spectra max 340PC (Molecular Devices). All 1 mM MESG stock solutions were prepared and stored in dH_2O at −20 °C. Phosphate release catalyzed by *Mtb* GlgE was monitored using a coupled assay utilizing purine nucleoside phosphorylase at 5 s intervals for 10 min. For each reaction, a master mix was made consisting of 1 mM MESG (20 μL), 0.2 U of PNP (1 μL), 20× reaction buffer (1.0 M Tris−HCl, 20 mM $MgCl_2$, pH 7.5, containing 2 mM sodium azide) (5 μL), glycogen (0.5 μL), 50 nm GlgE (5 μL), 250 μM M1P (3.125 μL), and 40 mM stock solution of 2,5-dideoxy-3-*O*-α-D-glucopyranosyl-2,5-imino-D-mannitol (**9**) inhibitor in dH_2O and was added for each corresponding stock solution to reach the desired inhibitor concentration (0.0−1.5 mM) and varied volumes of dH_2O for an overall reaction volume of 100 μL. Inhibitor **9** was not added to the positive control, and the negative control lacked GlgE. Inhibition was determined by comparing the relative rate of the reaction performed with inhibitor against a reaction that contained no inhibitor (V_o'/V_o), where V_o' and V_o are steady-state rates with and without inhibitor, respectively. The equilibrium dissociation constant (K_i) for inhibitor was obtained by fitting the data into the following equation using Prism

$$\frac{V_i'}{V_i} = \frac{K_m + [S]}{K_m + [S] + \frac{K_m[I]}{K_i}}$$

where K_m is the Michaelis−Menten constant for M1P and [S] and [I] are the concentrations of M1P and inhibitor, respectively.

■ ASSOCIATED CONTENT

Supporting Information

^1H and ^{13}C NMR spectra for new compounds are available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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