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

Sri Kumar Veleti, Jared J. Lindenberger, Sandeep Thanna, Donald R. Ronning, and Steven J. Sucheck*

Department of Chemistry and Biochemistry, School of Green Chemistry and Engineering, The University of Toledo, 2801 West Bancroft Street, Toledo, Ohio 43606, United States

ABSTRACT: Long treatment times, poor drug compliance, and natural selection during treatment of Mycobacterium tuberculosis (Mtbt) have given rise to extensively drug-resistant tuberculosis (XDR-TB). As a result, there is a need to identify new antituberculosis drug targets. Mtbt GlgE is a maltosyl transferase involved in α-glucan biosynthesis. Mutation of GlgE in Mtbt 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-α-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 Mtbt GlgE and a variant of Streptomyces coelicolor (Sco) GlgE 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 Mtbt GlgE. In conclusion, we designed a lead transition state inhibitor of GlgE, which will be instrumental in further elucidation of the enzymatic mechanism of Mtbt GlgE.

INTRODUCTION

Tuberculosis (TB) is a dreadful infectious disease whose causative agent is Mycobacterium tuberculosis (Mtbt).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 periplasmic 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 anomic 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 anomic oxygen on the phosphate moiety is protonated by...
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-hydroxypyrrolidines 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 glucan 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 \(K_i = 0.18\) \(\mu M\) and \(K_i = 0.33\) \(\mu M\), respectively, than 6-membered aza-sugars 1 and 2, \(K_i = 6.3\) \(\mu M\) and \(K_i = 12.6\) \(\mu M\), respectively, against yeast \(\alpha\)-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 \(\alpha\)-L-rhamnosidase (glycoside hydrolase family) from \textit{Penicillium decumbens}.\(^{10}\) For example, compounds 5–8 are good inhibitors of pyranose-based \(\alpha\)-L-rhamnosidase with \(K_i\) values 0.14, 5.5, 11.5, and 46 \(\mu M\), respectively. Keeping in mind that five-membered ring poly-hydroxypyrrolidine have been shown to exhibit very good activity against \(\alpha\)-glucosidases, we became interested in other modified analogues of these molecules as transition-state inhibitors for GlgE and designed inhibitor 9.

#### 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-\(\beta\)-D-glucopyranoside (14) (Scheme 2) and 5-azido-3-O-benzyl-5-deoxy-1,2-O-isopropyliden-\(\beta\)-D-fructopyranose (23) (Scheme 3) separately. Subsequently, the two glycosides 14 and 23 were coupled together to afford disaccharide 24, which was subjected
to global deprotection, rearrangement, and reductive amination to afford poly-hydroxypropyridine 9 as shown in (Scheme 4).

Synthesis of 2,5-Dideoxy-3-O-α-D-glucopyranosyl-2,5-imino-o-mannitol (9).16 Coupling of compound 14 and 23 using classical glycosylation conditions with N-iodosuccinimide 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 dial 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 H2 gas at 100 psi also helped to afford a polyhydroxypropyridine 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). 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.
using multiple sequence alignments to better emulate the GlgE and the Sco GlgEI-V279S variant, which was designed concerning the development of more potent transition-state inhibitors for Mtb GlgE. Compound 9 has been successfully cocrystallized 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) 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.

**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, E60) was visualized under UV light or by charring (5% H2SO4–MeOH). Flash column 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 dH2O 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 MgCl2, pH 7.5, containing 2 mM sodium azide), glycogen as maltosyl acceptor, 50 mM 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 (Vf / Vi), where Vf and Vi are steady-state rates with and without inhibitor, respectively. The equilibrium dissociation constant (Ki) 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, 13C NMR and optical rotation data are in accordance with the literature values. We evaluated the inhibitory activity of 9 against 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.
chromatography was performed on silica gel (230–400 mesh) using solvents as received. 1H NMR were recorded either on 600 MHz spectrometer in CDCl3 using residual CHCl3 as internal references, respectively. 13C NMR were recorded on the 600 MHz in CDCl3 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 mass spectrometer. EnzChek Phosphate Assay Kit was purchased for use in the MiTe GlgE inhibition assay.

1,2,3,4,6-Penta-O-acetyl-β-D-glucopyranose (11). 19,20 1H-δ-glucose (10) (3.0 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 saturated aq NaHCO3 (16 mL), 10% aq NaOH, and water, and then dried with anhydrous Na2SO4. The resulting organic phase was washed with saturated aq NaHCO3 (60 mL). The resulting organic phase was dried (anhydrous Na2SO4) and concentrated under reduced pressure to give product 11: yield 86% (5.6 g); 1H NMR (CDCl3, 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 suspended in dry acetone (20 mL) and saturated aq NaHCO3 (5.6 mL, 5.6 mmol) at 0 °C. The solution was stirred at ambient temperature under nitrogen atmosphere. The reaction was allowed to warm to room temperature and was further stirred for 1 h at room temperature, diluted with water, and then extracted with ethyl ether. The combined extracts were washed with water and brine, dried over anhydrous MgSO4, and concentrated under reduced pressure. The residue was recrystallized with ether/hexane to afford the product as a white solid (2.0 g, 5.1 mmol). 1H NMR (CDCl3, 600 MHz): δ 1.43 (s, 3H), 1.55 (s, 3H), 2.11 (d, J = 6 Hz, 1H), 3.61 (d, J = 12 Hz, 1H), 3.90 (d, J = 12 Hz, 1H), 4.25 (d, J = 12 Hz, 1H), 4.87 (d, J = 3 Hz, 8.4 Hz, 1H) ppm.

3-O-Benzyl-1,2,4,5-Tri-O-isopropylidene-β-D-fructopyranose (16). 22 To a solution of 16 (40 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 ethyl ether. The combined extracts were washed with water and brine, dried over anhydrous MgSO4 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-1,2,4,5-Tri-O-isopropylidene-β-D-fructopyranose (17) as an oily product: yield 86% (4.76 g); GCOSY method: yield 95% (0.48 g); 1H NMR (CDCl3, 600 MHz): δ 1.33 (s, 3H), 1.41 (s, 3H), 1.43 (s, 3H), 1.55 (s, 3H), 1.61 (s, 3H), 3.61 (d, J = 12 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-1,2,4,5-Tri-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); 1H NMR (CDCl3, 600 MHz): δ 0.25 (3:7 acetone–hexane); 1H NMR (CDCl3, 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 Hz, 1H), 3.73–4.05 (m, 2H), 4.25 (d, J = 6 Hz, 1H), 4.67 (d, J = 12 Hz, 1H), 7.31–7.38 (m, 5H) ppm.

3-O-Benzyl- and 4-O-Benzyl-3-O-benzyl-1,2-O-isopropylidene-β-D-fructopyranose (19 and 20). 19 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-dibutyldithiane 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). The solution was added a solution of benzyl 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 Rf = 0.26 (3:7 acetone–hexane); 1H NMR (CDCl3, 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 (d, J = 1.8 Hz, 13 Hz, 1H), 4.12–4.14 (m, 2H), 4.22 (d, 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 Rf = 0.36 (3:7 acetone–hexane); 1H NMR (CDCl3, 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.
4-O-Benzoyl-3-O-benzyl-5-deoxy-5-ido-1,2-O-isopropylidene-α-L-sorbofuranose (21). 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, a reaction was cooled, washed successively with 10% aqueous sodium thiosulfate and brine, and then concentrated. The concentrated residue was subjected to a flash chromatography with 1:9 acetonitrile–hexane to afford compound 21: yield 90% (0.38 g); silica gel TLC Rf = 0.5 (3:7 acetonitrile–hexane); 1H NMR (CDCl3, 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), 5.85 (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-fructofuranose (22). A stirred solution of 21 (275 mg, 0.52 mmol) and sodium azide (171 mg, 2.62 mmol) in dry N,N-dimethylaniline (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 MgSO4, and concentrated under reduced pressure. The residue was purified by flash chromatography on silica gel with 1:9 acetonitrile–hexane to give compound 22 as a crystalline product: yield 91% (0.23 g); silica gel TLC Rf = 0.13 (9:1 acetonitrile–hexane–hexane); 1H NMR (CDCl3, 600 MHz) δ 1.14 (s, 3H), 1.53 (s, 3H), 3.78 (dd, J = 6 Hz, 12 Hz, 1H), 4.05 (d, J = 6 Hz, 1H), 4.15 (d, J = 6 Hz, 12 Hz, 1H), 4.27 (m, 1H), 4.67 (d, J = 12 Hz, 1H), 4.88 (d, J = 6 Hz, 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-fructofuranose (23). The compound 22 (0.21 g, 0.47 mmol) was denbenzyolated 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-118 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 acetonitrile–hexane to afford the compound 23: yield 98% (0.15 g); silica gel TLC Rf = 0.42 (3:7 acetonitrile–hexane–hexane); 1H NMR (CDCl3, 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 (d, J = 6 Hz, 12 Hz, 1H), 4.76 (d, J = 12 Hz, 1H), 4.84 (d, J = 6 Hz, 12 Hz, 1H), 7.31–7.38 (m, 5H) ppm. 2,3,4,6-Tetra-O-benzyl-1-(5-azido-3-0-benzyl-5-deoxy-1,2-O-isopropylidene-β-D-fructofuranosyl)-α-D-glucopyranoside (24). A mixture of p-methylphenylboronic acid, triethylamine, and potassium carbonate in dioxane was placed between the NdeI and SmaI sites of the plasmid pET32-Sco-V279S. The recombinant protein expressed by this plasmid possesses a C-terminal polyhistidine tag. The sequences of the primers used for the cloning were: 5′-GCGAATTCATGCATGGCCGAGCCGGTAC-3′ and 5′-AAAAGGATCCCTCACTGGCAGCAGA-3′. The product was used as template and amplified by PCR using the following primers: 5′-GACAATATTGCTGCTCTC-3′ and 5′-AAAAAGGATCCCTCACTGGCAGCAGA-3′. The PCR product was cloned into the pUC19 vector, and the resulting plasmid was transformed into E. coli JM109. The recombinant GlgE enzyme possessing an N-terminal polyhistidine tag was expressed in the transformed 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 suspension and incubated for 1 h on ice prior to lysis by sonication. The resulting suspension was centrifuged at 15000g for 30 min. The supernatant was applied to a 5 mL HiTrap TALON metal affinity column (GE Healthcare) previously equilibrated with buffer A containing 5 mM imidazole, 500 mM NaCl, 20 mM Tris pH 7.5, and 0.3 mM TCEP. 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 isotropically 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 determined by SDS-PAGE.
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 dH2O at −20 °C. Phosphate release catalyzed by MtGlcE 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 μM Tris- HCl, 50 mM MgCl2, pH 7.5, containing 2 mM sodium azide) (5 μL), glycogen (50 μL), 50 nm GlgE (5 μL), 250 μM M1P (3.125 μL), and 40 mM stock solution of 2.5-dideoxy-3-O-arabinofuranosyl-2,5-imino-D-mannitol (9) inhibitor in dH2O and was added for each corresponding stock solution to reach the desired inhibitor concentration (0.0–1.5 mM) and varied volumes of dH2O 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 (V9/o) and V0 are steady-state rates with and without inhibitor, respectively. The equilibrium dissociation constant (K) for inhibitor was obtained by fitting the data into the following equation using Prism

\[
\frac{V'_{i}}{V_{i}} = \frac{K_{m} + [S]}{K_{m} + [S] + K_{i}[I] / K}
\]

where \(K_{i}\) is the Michaelis–Menten constant for M1P and [S] and [I] are the concentrations of M1P and inhibitor, respectively.

**ASSOCIATED CONTENT**

Supporting Information

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

**AUTHOR INFORMATION**

**Corresponding Author**

*E-mail: steve.sucheck@utoledo.edu.*

**Notes**

The authors declare no competing financial interest.

**ACKNOWLEDGMENTS**

This work was supported in part by a DeArce Memorial Fund grant to S.J.S. from the University of Toledo and a grant from the National Institutes of Health (Grant No. AI105084) to S.J.S. and D.R.R. We thank Dr. Clifton E. Barry III at the NIH-NIAID and EM technician Elizabeth Fischer at the Rocky Mountain Laboratories in Hamilton, Montana, for providing the TEM of Mycobacterium tuberculosis. We also thank Prof. Donald R. Ronning at the University of Toledo for creating the cover image and providing a docked structure of the iminosugar-based inhibitor bound to the active site of Sco GlgEI-V279S.

**REFERENCES**


9450