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FULL PAPER

Synthesis and in vitro characterization of trehalose-based inhibitors of mycobacterial trehalose 6-phosphate phosphatases

Sunayana Kapil‡[a], Cecile Petit‡[b], Victoria N. Drago[a], Donald R. Ronning*[a] and Steven J. Sucheck*[a]

Dedicated to Professor Chi-Huey Wong on his 70th birthday

Abstract: α,α'-Trehalose plays roles in the synthesis of several cell wall components involved in pathogenic mycobacteria virulence. Its absence in mammalian biochemistry makes trehalose-related biochemical processes potential targets for chemotherapy. The trehalose-6-phosphate synthase (TPS)/trehalose-6-phosphate phosphatase (TPP) pathway, also referred to as the OtsA/OtsB2 pathway, is the major pathway involved in the production of trehalose in *Mycobacterium tuberculosis* (*Mtb*). In addition, TPP is essential for *Mtb* survival. We describe the synthesis of 6-phosphonic acid **4** (**TMP**), 6-(methylene)phosphonic acid **5** (**TEP**) , and 6-*N*-phosphonamide **6** (**TNP**) derivatives of α,α'-trehalose. These non-hydrolyzable substrate analogs of TPP were examined as inhibitors of *Mtb*, *M. lentiflavum* (*Mlt*), and *M. triplex* (*Mtx*) TPP. In all cases the compounds inhibited *Mtx* TPP most strongly, with **TMP** (IC_{50} = 288 ± 32 μM) inhibiting most strongly, followed by **TNP** (IC_{50} = 421 ± 24 μM) and **TEP** (IC_{50} = 1959 ± 261 μM). The results also indicate significant differences in the analog binding profile when comparing *Mtb* TPP, *Mlx* TPP, and *Mtx* TPP homologs.

Introduction

α,α'-Trehalose (**1**) is an essential disaccharide present in plants,[1] some bacteria,[2] fungi,[3] parasitic nematodes,[4] and insects;[5] however, it is absent in mammals. Depending on context, trehalose can serve as an energy source, metabolic regulator, anti-desiccant, and cell wall component.[2] The lack of mammalian trehalose synthesis, import, or processing pathways makes those pathways potential targets for chemotherapy in *Mycobacterium tuberculosis* (*Mtb*).[6] In *Mtb*, three pathways for synthesizing trehalose are known,[6b] they are the TreY-TreZ, TreS and the OtsA/OtsB pathways (Figure 1). The OtsA/OtsB pathway is considered most prevalent in replicating *Mtb*.[7] OtsA catalyzes the formation of trehalose-6-phosphate (**2,** T6P) from UDP-glucose or ADP-glucose and glucose-6-phosphate.[8] OtsB has two homologues, OtsB1 and OtsB2, and only OtsB2 has phosphatase activity and is essential for *Mtb* growth.[7, 9] The phosphate moiety of T6P is removed by OtsB2 to yield trehalose. The OtsA/OtsB pathway is also referred to as the trehalose-6-phosphate synthase (TPS)/trehalose-6-phosphate phosphatase (TPP) pathway and TPP is used in place of OtsB2 hereafter.

Trehalose has several fates related to virulence and cell wall synthesis. These include conversion into intracellular α-glucan, sulfolipid-1, diacyltrehalose, and polyacyltrehalose. [10] Trehalose is also modified with mycolic acids by polyketide synthase 13 (Pks13) to form mono-α-alkyl β-ketoacyl trehalose (TMM*k*).[11] TMM*k* is reduced to trehalose monomycolate (TMM) by CmrA[12] which is transported to the cell wall by MmpL3 (Figure 1).[13] The Antigen 85 Complex (Ag85) transfers[14] the mycolic acids to the cell wall arabinogalactan (AG) or to a second molecule of TMM to form trehalose dimycolate (TDM) a key virulence factor.[15] Free trehalose produced by the action of Ag85s on TMM is salvaged by the action of the ABC transporter LpqY-SugABC.[6a] It is also known that *Mtb* can enter an antibiotic resistant state under the stress of low oxygen.[16] Intriguingly, trehalose has been linked to the ability of *Mtb* to adapt its metabolism under these conditions.[17] Thus, compounds interfering with the various trehalose-producing pathways may offer new approaches for more effective treatments against *Mtb*.

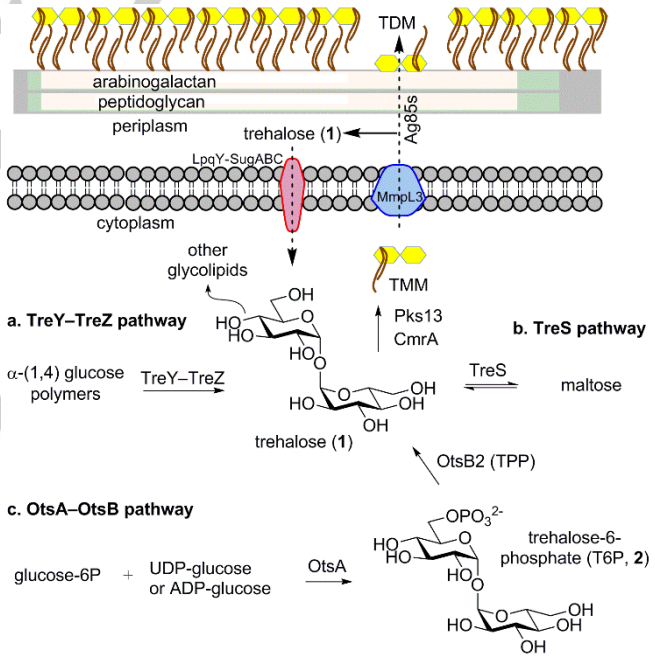


Figure 1. Routes for the synthesis and recycling of trehalose, as well as its conversion to TMM/TDM in *Mtb*. a. TreY–TreZ pathway; b. TreS pathway; c. OtsA/OtsB pathway.

Recently, 6-sulfate, 6-(methylene)phosphonate, and 6-(fluoromethylene)fluorophosphonate derivatives of α,α'-trehalose were reported active (K_i =130-480 μM) against *Mtb* TPP[18] while aryl-D-glucopyranoside 6-sulfates were also reported as mimics of T6P with activity against TPP.[19] Based on the activity of these α,α'-trehalose derivatives against *Mtb* TPP, we developed heptabenzyl α,α'-trehalose derivative **3** and synthetic routes to access 6-phosphonic acid **4** (**TMP**), 6-(methylene)phosphonic acid **5** (**TEP**), 6-*N*-phosphonamide **6** (**TNP**), and a 6-oxirane **7** derivatives of 6-deoxy-α,α'-trehalose (Figure 2). The library of 6-

deoxy-α,α'-trehalose derivatives was evaluated for inhibition against TPP homologs encoded by *Mtb*, *Mlt* and *Mtx*. Validamycin A (**8**) was included due to topological similarity with α,α'-trehalose and for known inhibitory activity against *E. coli* TPP.[20] Therefore, the *Mlt* and *Mtx* TPP homologs were included in this study because of the high sequence identity with the *Mtb* TPP, 72% and 71%, respectively, and the 100% identity in catalytic residues. Additionally, the relative paucity of available *Mtb* TPP structural information suggested protein structural dynamics that hinder crystallization. The *Mlt* and *Mtx* TPP homologs lack two large loops indicated in the *Mtb* TPP sequence and are being pursued as TPP surrogates to afford structural determination of mycobacterial TPP enzymes. Finally, the *Mtb*, *Mlt*, and *Mtx* TPP enzymes in this study exhibit K_M values of 640,[7] 130,[21] and 82 μM,[21] respectively.

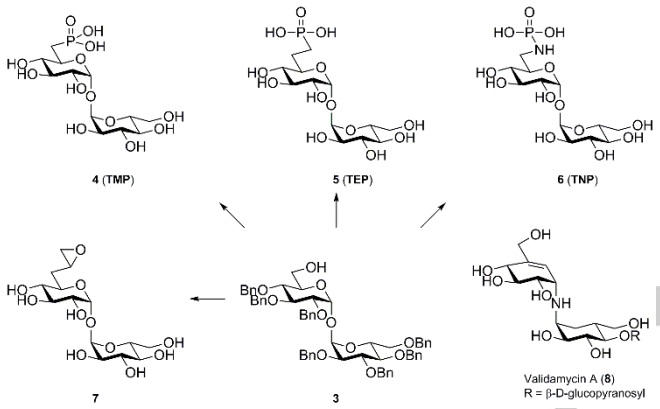
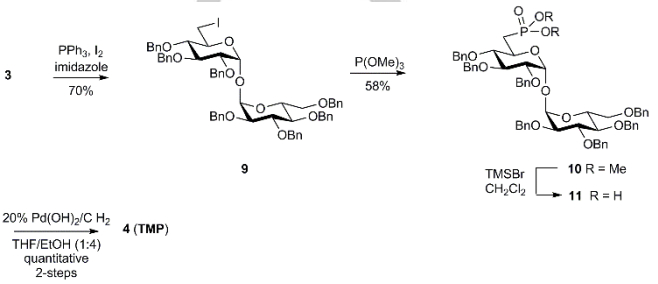


Figure 2. 6-deoxy-α,α'-trehalose derivatives prepared from heptabenzyl α,α'-trehalose derivative **3** and Validamycin A (**8**) which shows topological similarities with trehalose.

Results

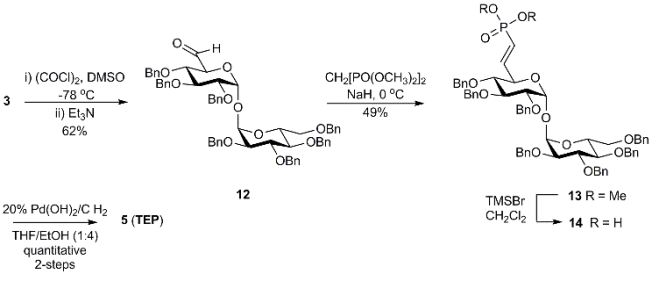
Chemistry studies. Prior research has shown that 6-sulfate, 6-(methylene)phosphonate **TEP**, and 6-(fluoromethylene)phosphonate derivatives of α,α'-trehalose possessed inhibitory activity against *Mtb* TPP;[18] however, no microbiological evaluation was reported. That data, in addition to the know growth inhibition data of 6-modified derivatives of α,α'-trehalose against mycobacteria,[14b, 22] prompted us to investigate the synthesis of additional 6-modified derivatives. In the current work we focused on synthesis and study of 6-phosphonate α,α'-trehalose **TMP**, an alternative route to 6-(methylene)phosphonate analogue **TEP**, a 6-*N*-phosphonamide analogue **TNP**, and an 6-oxirane analogue **7** due to similarity with reported *Mtb* TPP inhibitors (Figure 2).[18]

The target 6-phosphonate analog **TMP** was accessible through a heptabenzyl α,α'-trehalose intermediate **3**[22c] prepared by our reported route.[22d] The route allows gram scale access to unsymmetrical 6-modified α,α'-trehalose analogues. In order to access 6-phosphonate **TMP**, heptabenzyl α,α'-trehalose derivative **3** was converted to the 6-iodo-6-deoxy-α,α'-trehalose derivative **9** in 70% yield by treatment with triphenylphosphine in the presence of iodine and imidazole (Scheme 1). Iodide **9** was subjected to Michaelis–Arbuzov conditions by treatment with trimethyl phosphite to afford 6-phosphonate derivative **10** in 58% yield. The phosphonate ester **10** was deprotected with bromotrimethylsilane (TMSBr) to afford the 6-phosphonic acid derivative **11**. The latter was debenzylated by hydrogenolysis with 20% $Pd(OH)_2$ on carbon under 1 atm. of H_2 to afford 6-phosphonate **TMP**.



Scheme 1. Synthesis of 6-phosphonic acid-α,α'-trehalose derivative **TMP**.

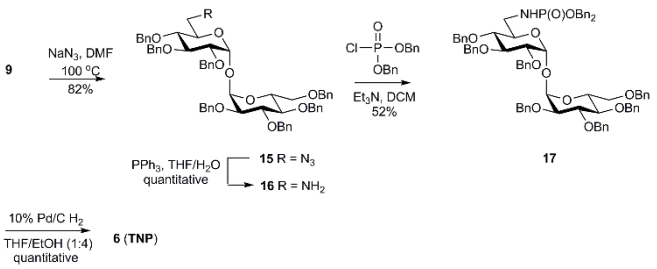
The target 6-(methylene)phosphonic acid analog **TEP** was also accessible through intermediate **3**. Intermediate **3** was first subjected to a Swern oxidation to afford aldehyde **12** in 62% yield (Scheme 2).[18] Aldehyde **12** was converted to the phosphonate **13** in 49% yield via a Horner–Wadsworth–Emmons reaction utilizing tetramethyl methylenediphosphonate and sodium hydride. The phosphonate ester **13** was deprotected with bromotrimethylsilane (TMSBr) to afford the 6-(vinylphosphonic acid) derivative **14**. The latter was debenzylated by hydrogenolysis with 20% $Pd(OH)_2$ on carbon under 1 atm. of H_2 to afford 6-(methylene)phosphonic acid **TEP**. The route is a minor modification of reported work[18] which used tetraethyl methylenediphosphonate in the Horner–Wadsworth–Emmons reaction along with other modifications in reagents and reaction sequence.



Scheme 2. Synthesis of 6-(methylenephosphonic acid)-α,α'-trehalose derivative **TEP**.

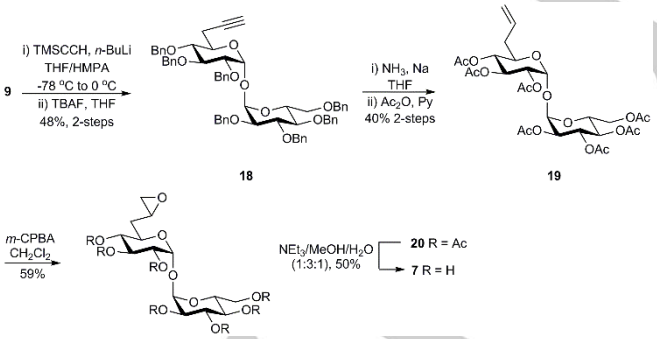
The target 6-phosphoramidic acid analog **TNP** was accessible through iodide intermediate **9**. Iodide **9** was subjected nucleophilic substitution with sodium azide to afford azide **15** in

82% (Scheme 3). Azide **15** was reduced to amine **16** via a Staudinger reaction reduction in good yield. The 6-amino-α,α'-trehalose derivative **16** was treated with dibenzylphosphoryl chloride to afford dibenyzl phosphoramidate **17**. Global deprotection of the benzyl groups with 10% Pd on carbon under 1 atm. of H_2 yielded 6-phosphoramidic acid analog **TNP**.



Scheme 3. Synthesis of 6-(phosphoramidic acid)-α,α'-trehalose derivative **TNP**.

The target oxirane analog **7** was likewise accessible through iodide intermediate **9**. Iodide **9** was subjected to nucleophilic substitution with trimethylsilylacetylene followed by deprotection of the silyl group with tetrabutylammonium fluoride (TBAF) to afford compound **18** in 48% yield over 2 steps (Scheme 4). Compound **18** was subjected to Birch reduction to reduce the benzyl groups and convert the alkyne to an alkene. The resulting hydroxyl groups were protected by acetylation to afford compound **19** in 40% yield.[23] The alkene **19** was treated with *meta*-chloroperoxybenzoic acid (mCPBA) to afford epoxide **20** as a mixture of diastereomers in 59% yield. Deprotection of the acetyl groups of **20** was achieved with NEt_3/MeOH/H_2O (1:3:1) to afford oxirane analog **7**.[24]



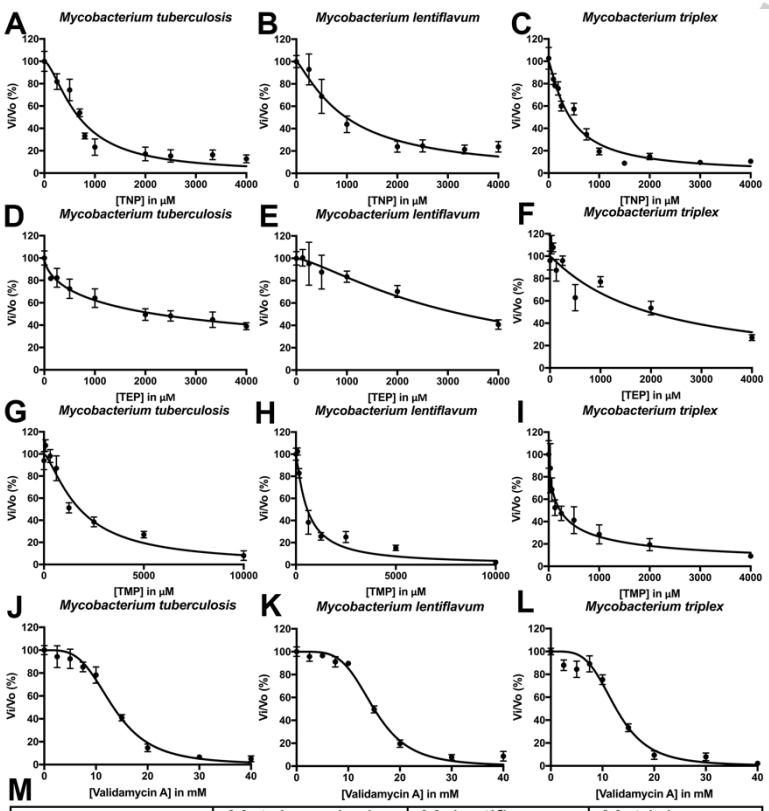
Scheme 4. Synthesis of 6-oxiranyl-α,α'-trehalose derivative **7**.

Enzyme inhibition studies. To assess the ability of non-hydrolysable mimics of the natural TPP substrate, T6P, to inhibit TPP, **TMP**, **TEP**, **TNP**, **7** and Validamycin A (**8**) were evaluated. These non-hydrolysable mimics were tested against the TPP homologs encoded by *Mtb, Mlt* and *Mtx.* All of the inhibitory curves and resulting IC_{50} values are given in Figure 3. Few general trends of inhibition for the three homologs were observed. **TEP** exhibited the weakest inhibition for each enzyme, which is consistent with the expected enzyme mechanism. The presence of an ethyl moiety between the phosphonate and trehalose moiety is sufficiently large as to prevent appropriate simultaneous coordination of these two components of the inhibitor. The inhibitory activity of **TMP** and **TNP** exhibited IC_{50} values lacking a consistent pattern. **TNP** was the most potent inhibitor against the Mtb TPP, while **TMP** exhibited the lowest IC_{50} values for both the *Mlt* and *Mtx* TPP homologs. Compound **7** (data not shown) showed no inhibition. For comparison, Validamycin A (**8**) was also tested as an inhibitor of the mycobacterial TPPs as it had been previously shown to completely inhibit *E. coli* TPP at a concentration of 25 μM.[20] Interestingly, Validamycin A exhibited unexpectedly high IC_{50} values for the mycobacterial TPPs (Figure 3, J, K, and L). Neither the structure of the *E. coli* TPP nor the T6P-bound *Mtb* TPP have been deposited in the PDB, so it is difficult to rationalize the observed difference between the *E. coli* TPP and mycobacterial TPPs in response to Validamycin A. One difference, which merits further investigation, may lie in the lack of an N-terminal domain in *E. Coli* TPP. The structure of an inactive *Cryptococcus neoformans* TPP with T6P bound shows how both the essential Mg^{2+} is coordinated by the conserved Aspartate residues and how the phosphate moiety of T6P interacts with the Mg^{2+} as well as conserved Histidine and Lysine active site residues.[25] However, the residues in both the catalytic domain and the cap domain of *C. neoformans* TPP that interact directly with the glucose moieties of trehalose are not highly conserved in TPP homologs. It is also shown that structural changes in *C. neoformans* TPP are stimulated by binding of T6P. Therefore, it is likely that the sequence differences and structural dynamics lead to differential inhibition by Validamycin A.

Discussion

TPP is an essential enzyme for survival and virulence of *Mtb*. It catalyzes the second reaction in the *de novo* trehalose biosynthesis pathway. Since trehalose is not produced by mammals, they possess no homologues of TPP rendering it an intriguing drug target. Since enzymes evolve to strongly bind their transition-states, initial inhibitory studies on any enzyme typically attempt to mimic the expected transition states. In the case of TPP, formation of trehalose occurs through an associative-two-step mechanism (Figure 4).[26]

During the first step of the reaction, the nucleophilic aspartate, Asp153/120 (numbering corresponds with *Mtb* TPP/*Mlt* TPP residue numbering: Asp_n in Figure 4), launches an attack on the phosphoryl group of T6P, resulting in the formation of a penta-coordinate trehalose-phosphoaspartyl enzyme intermediate. Then acting as a general acid, Asp155/122 (Asp_{n+2} in Figure 4) protonates trehalose promoting formation of the phosphoaspartyl intermediate and release of free trehalose. The proposed pentacoordinate phosphorous transition states and intermediates are challenging to emulate in stable organic compounds. Therefore, we have employed phosphonate compounds as substrate analogues for the initial inhibitory studies.



M

	M. tuberculosis	*M. lentiflavum*	*M. triplex*
IC_{50} (TNP) (µM)	685 ± 41	1031 ± 87	421 ± 24
IC_{50} (TEP) (µM)	2198 ± 183	3287 ± 381	1959 ± 261
IC_{50} (TMP) (µM)	1795 ± 141	569 ± 55	228 ± 32
IC_{50} (ValA) (µM)	13,390 ± 314	15,000 ± 254	12,630 ± 367

Figure 3. TPP Inhibition studies. A, B and C; TPP inhibition curves for **TNP**. D. E, F; TPP inhibition curves for **TEP**. G, H, and I; TPP inhibition curves for **TMP**. J, K, L; TPP inhibition curves for Validamycin A. M; IC_{50} values of **TNP**, **TEP**, **TMP** and Validamycin A against *Mtb* TPP, *Mlt* TPP and *Mtx* TPP.

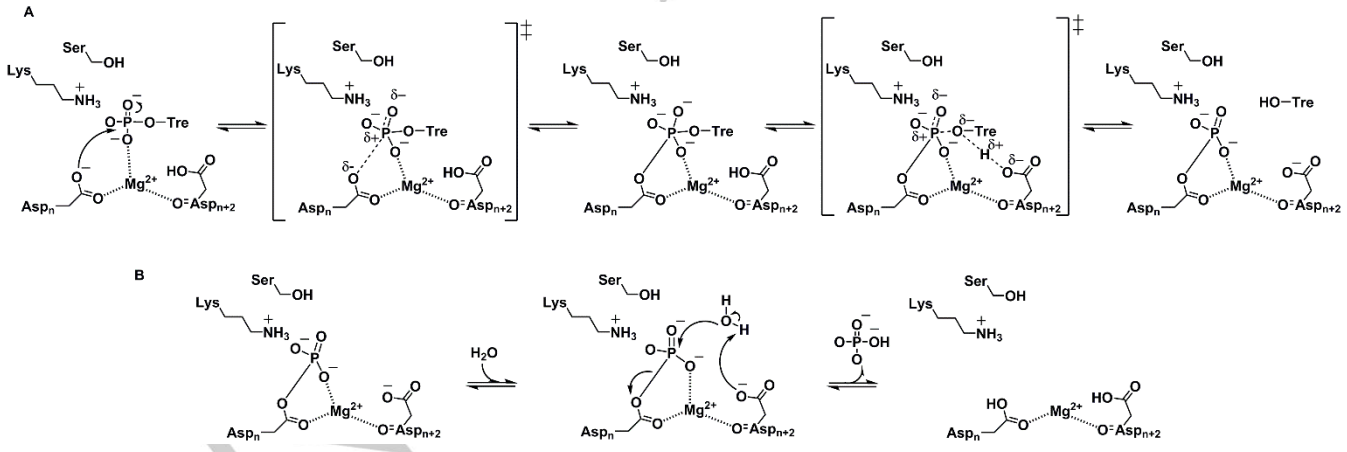


Figure 4. Associative-two-step mechanism of TPP. A. First step of the reaction. B. Second step of the reaction. Asp_n corresponds to the nucleophile. Asp_{n+2} corresponds to the general acid/general base.

A magnesium co-factor, required for this reaction to occur, affords the correct positioning of the substrate phosphoryl group and for neutralization of the negative charges forming on the transition-state. The three non-hydrolysable phosphorus-based compounds were made to mimic the T6P substrate and assess the impact of length and atom character of the chain connecting trehalose and the phospho-mimic. While the **TMP** IC_{50} against the *Mtx* TPP and *Mtb* TPP homologs was only 1.8- to 2.8-fold worse than the determined T6P K_M, respectively, **TEP** exhibited an IC_{50} that was 3.4- to 135-fold worse than the determined T6P K_M. The third inhibitor, **TNP**, was synthesized to test the role for the O-6 oxygen of T6P binding by TPP. The IC_{50} of **TNP** suggests binding affinity similar to the *Mtb* TPP binding of the T6P substrate. When compared to the inhibitory activities of **TMP** and **TEP** against *Mtb* TPP, this suggests enhanced affinity due to the presence of a hydrogen bond acceptor and donor at position 6 which has an important role in enzyme recognition. Compound **7**, on the other hand, appeared unable to interact with any of the enzymes. The moderate inhibition data is consistent with other studies that describe T6P analogs designed to mimic phosphate.[18-19] One potential path forward to improve inhibition may be to mimic phosphate with α-hydrodroxy- or α-amino- phosphonates. These bidentate moieties may have the potential to bind magnesium ions and improve inhibitory potential. In addition, α-hydrodroxy- or α-amino- groups may make up for the loss of the putative H-bond acceptor absent in the current phosphonate series. It is expected that the compounds in the current series could potentially be imported in *Mtb* by LpqY-SugABC (Figure 1) which recognizes trehalose and some of its derivatives.[14b, 22] For example, it has been shown that *Mtb* could take-up extracellular trehalose including a fluorescein-containing trehalose probe and incorporate the materials into growing bacilli.[22a] The same investigators showed that 6-fluoro-6-deoxy-α,α'-trehalose and 6-bromo-6-deoxy-α,α'-trehalose possessed activity (MIC 200 μg/mL) against *Mtb.*[22a]

For future studies, it would be interesting to investigate how these new compounds might effect trehalose production, *Mtb* growth or recovery from dormancy, Ag85 activity, TMM/TDM production, or biofilm formation. Related compounds, like 6-azido-6-deoxy-α,α'-trehalose, are known to inhibit growth of *M. aurum* (MIC 200 μg/mL) as well as inhibit Ag85C activity, synthesis of TMM, TDM, and reduced cell wall–bound mycolic acids and TMM export. [14b] While a library of *N*,*N*-dialkylamino and 6,6-bis(sulfonamido) analogs of α,α'-trehalose was shown active (MIC 16-128 μg/mL) against *Mtb* $H_{37}Ra$.[22b] A second 6,6-bisalkyl library of α,α'-trehalose derivatives was found active against *M. smegmatis.*[22c] In addition, 6,6-bis(α-ketoesters/amides) analogs of α,α'-trehalose have been reported to be active against Ag85C[22d] and 6-deoxy-, 6-fluoro-6-deoxy-, and 6-azido-6-deoxy-α,α'-trehalose were shown to inhibit (MIC 50-100 μM) *M. smegmatis* biofilm formation.[22e] Collectively, these studies suggest several possible applications of the target compounds.

Conclusions

The inhibitory results show that the **TEP** analogue with the longer linker and lacking a hydrogen bond acceptor significantly weakens affinity by any TPP homologue. In contrast, the lone pair of electrons on the nitrogen atom of the aminophosphonate moiety in the **TNP** analogue may interact with the magnesium co-factor to enhance binding. In contrast, the *Mlt* TPP and *Mtx* TPP exhibited a different pattern of response to the inhibitors, particularly in regards to the inhibition by **TMP**. While this lack of correlation is troubling, it is well documented that TPP enzyme active sites undergo a conformational change upon binding substrate and structural dynamics can have a profound effect on substrate or inhibitor affinity.[25] In this context, and in regards to our results regarding the lack of inhibitory effect of Validamycin A on mycobacterial TPP, in contrast to *E. coli* TPP, suggest that the N-terminal domain of mycobacterial TPP might play an important role in substrate binding. In this prospect, the shorter N-terminal domains of both *Mlt* TPP and *Mtx* TPP, in comparison to the N-terminal domain of *Mtb* TPP, would explain such discrepancies. This suggests that structural changes in *Mtb* TPP due to substrate analogue binding may be distinct from changes occurring in the *Mlx* TPP and *Mtx* TPP homologues, particularly in the case of **TMP** binding. Resolution of this discrepancy will follow structural determination of TPP/inhibitor complexes and complementary molecular dynamic studies.

Experimental Section

General Methods

All chemicals and solvents were purchased from Fisher Scientific, Acros Organics, Alfa Aesar or Sigma-Aldrich. Solvents were dried by distillation, other standard procedures and through a solvent purification system by passing HPLC grade solvent through activated alumina and copper columns. All reactions were carried out at room temperature under nitrogen atmosphere using a nitrogen balloon unless mentioned in procedure. Reactions were monitored by TLC (silica gel, f_{254}) under UV light or by charring (5% sulfuric acid-methanol) and the purification was performed by flash column chromatography on silica gel (230-400 mesh) using the solvent system specified, solvents were used without purification for chromatography. ^1H NMR and ^{13}C NMR were recorded on a Bruker Avance III 600 MHz spectrometer using $CDCl_3$ or D_2O as internal references. ^{31}P NMR were recorded on a VXRS 400 MHz using $CDCl_3$ or D_2O as solvents. High resolution mass spectrometry was performed on a Waters SYNAPT HRMS nano ESI-MS instrument and low-resolution mass spectrometry was performed on an ESquire-LC-MS.

*2,2',3,3',4,4',6'-Hepta-O-benzyl-6-iodo-6-deoxy-α-D-glucopyranosyl-(1→1)-α-D-glucopyranoside (**9**).* To a solution of **3** (0.950 g, 0.977 mmol, 1eq.) in tetrahydrofuran (10 mL) at 0 °C was added triphenylphosphine (0.307 g, 1.17 mmol, 1.2 eq.), imidazole (0.166 g, 2.44 mmol, 2.5 eq.) and iodine (0.297 g, 1.17 mmol, 1.2 eq.). The reaction was refluxed for 3 h. After completion, water (10 mL) was added to the reaction flask and organic layer was extracted with ethyl acetate (15 mL). The organic layer was washed successively with $Na_2S_2O_3$ (10 mL X 3) and water (10 mL

X 3). The ethyl acetate was dried over anhydrous Na_2SO_4 and filtered. Purification was performed by flash column chromatography on silica gel (5% ethyl acetate-hexanes) to afford a colorless viscous liquid (0.768 g, 70% yield): R_f = 0.35 (20% ethyl acetate-hexanes); ^{1}H NMR (600 MHz, $CDCl_3$): δ 7.38-7.24 (m, 33H, aromatic), 7.13 (dd, 2H, *J* = 7.4, 1.9 Hz, aromatic), 5.27 (d, 1H, *J* = 3.5 Hz, H-1), 5.23 (d, 1H, *J* = 3.5 Hz, H-1'), 4.98 (m, 3H, benzylic), 4.85 (m, 3H, benzylic), 4.72 (m, 5H, benzylic), 4.56 (d, 1H, *J* = 12.1 Hz, benzylic), 4.47 (d, 1H, *J* = 10.8 Hz, benzylic), 4.40 (d, 1H, *J* = 12.1 Hz, benzylic), 4.14 (m, 1H, H-5'), 4.09 (t, 1H, *J* = 9.3 Hz, H-3), 4.03 (t, 1H, *J* = 9.4 Hz, H-3'), 3.69 (m, 2H, H-4', H-5), 3.62 (dd, 1H, *J* = 9.6, 3.6 Hz, H-2'), 3.52 (m, 2H, H-6a', H-4), 3.41 (m, 2H, H-6b', H-4), 3.25 (dd, 1H, *J* = 10.9, 4.5 Hz, H-6a), 3.12 (dd, 1H, *J* = 10.8, 2.9 Hz, H-6b); ^{13}C NMR (150 MHz, $CDCl_3$): δ 139.03, 138.81, 138.52, 138.44, 138.35, 138.30, 138.02, 128.74, 128.68, 128.63, 128.62, 128.62, 128.60, 128.22, 128.20, 128.14, 128.14, 128.11, 127.95, 127.89, 127.86, 127.80, 127.60, 94.61, 94.24, 82.06, 81.89, 81.40, 79.81, 79.68,77.92, 75.90, 75.85, 75.68, 75.35, 73.74, 73.34, 73.01, 70.97, 68.92, 68.30, 66.12, 9.59; HRMS (ESI): calculated for $C_{61}H_{63}IO_{10}$, 1105.3364 $[M+Na]^+$; observed, *m/z* = 1105.3331 $[M+Na]^+$.

*2,2',3,3',4,4',6'-Hepta-O-benzyl-6-methylphosphonate-6-deoxy-α-D-glucopyranosyl-(1→1)-α-D-glucopyranoside (**10**).* Trimethyl phosphite (10 mL) was added to compound **9** (0.500 g, 2.16 mmol) and the reaction was refluxed for 36 h. After the completion, excess trimethyl phosphite was evaporated under reduced pressure. The product was purified by flash column chromatography on silica gel (5% ethanol-choroform) to afford the product as a colorless solid (0.202 g, yield 58%): R_f = 0.30 (8% ethanol-chloroform); ^{1}H NMR (600 MHz, $CDCl_3$): δ 7.38-7.14 (m, 35H, aromatic), 5.47 (d, 1H, *J* = 3.5 Hz, H-1), 5.26 (d, 1H, *J* = 3.5 Hz, H-1'), 4.97 (m, 4H, benzylic), 4.84 (d, 3H, *J* = 10.8 Hz, benzylic), 4.71 (m, 3H, benzylic), 4.65 (d, 1H, *J* = 11.2 Hz, benzylic), 4.55 (d, 1H, *J* = 12.3 Hz, benzylic), 4.49 (d, 1H, *J* = 11 Hz, benzylic), 4.43 (d, 1H, *J* = 12.1 Hz, benzylic), 4.30 (m, 1H, H-5), 4.11 (m, 3H, H-3, H-3', H-5'), 3.66 (m, 2H, H-2, H-2'), 3.61 (m, 6H, 2X-OCH_3), 3.55 (m, 1H, H-4'), 3.42 (t, 1H, H-4), 2.12 (m, 1H, H-6a), 1.88 (m 1H, H-6b); ^{13}C NMR (150 MHz, $CDCl_3$): δ 139.11, 138.87, 138.84, 138.68, 138.58, 138.33, 138.14, 128.62, 128.61, 128.58, 128.55, 12853, 128.48, 128.17, 128.10, 128.01, 127.76, 127.73, 92.55, 92.14, 82.18, 82.09,82.03, 81.50, 81.49, 79.81, 79.73, 78.01, 75.82, 75.71, 75.17, 75.15, 73.70, 73.27, 72.92, 70.83, 68.66, 66.92, 66.88, 66.11, 52.46, 52.41, 52.32, 52.28, 15.52; ^{31}P NMR (162 MHz, $CDCl_3$): δ 32.13 (s, 1-P); LRMS (ESI): calculated for $C_{63}H_{69}PO_{13}$, 1087.4 $[M+Na]^+$; observed, *m/z* = 1087.5 $[M+Na]^+$.

*6-Deoxy-6-(phosphonic acid)-α,α'-trehalose (**4, TMP**).* To a solution of **10** (0.080 g, 0.075 mmol) in dichloromethane (4 mL) was added bromotrimethylsilane (129 µL, 0.977 mmol, 13 eq.) dropwise and the resulting solution stirred for 2 h at room temperature. A solution of methanol-water (1.1:1.8 mL) was added followed by concentration to dryness to afford phosphonicacid **11**. The residue was dissolved in 3 mL of tetrahydrofuran-ethanol (1:4) and 20% $Pd(OH)_2$/C (80 mg) was added. The suspension was stirred overnight at room temperature under 1 atm. of hydrogen. The catalyst was filtered away through a plug of Celite® 545 that was washed with 20% methanol-dichloromethane. The filtrate and washings were concentrated to dryness to afford product **TMP** as a colorless solid (0.030 g, quantitative yeild): ^{1}H NMR (600 MHz, D_2O): δ 5.24 (d, 1H, *J* = 3.5 Hz, H-1), 5.02 (d, 1H, *J* = 3.8 Hz, H-1'), 3.94 (m, 1H, H-5), 3.75-3.53 (m, 7H, H-2, H-2', H-4, H-4', H-5', H-6a', H-6b'), 3.33 (t, 1H, *J* = 9.4 Hz, H-3'), 3.15 (t, 1H, *J* = 9.4 Hz, H-3), 2.12 (m, 1H, H-6a), 1.75 (m, 1H, H-6b); ^{13}C NMR (150 MHz, D_2O): δ 92.93, 92.88, 74.32, 74.22, 72.25, 72.05, 71.11, 70.91, 69.59, 68.03, 34.82; ^{31}P NMR (162 MHz, D_2O): δ 30.78 (s, 1-P); HRMS (ESI): calculated for $C_{12}H_{23}PO_{13}$, 429.0774 $[M+Na]^+$; observed, *m/z* = 429.0774 $[M+Na]^+$.

*2,2',3,3',4,4',6'-Hepta-O-benzyl-6-carbaldehyde-α-D-glucopyranosyl-(1→1)-α-D-glucopyranoside (**12**).* Oxalyl chloride (0.07 mL, 0.80 mmol, 4 eq.) was slowly added to a solution of dimethyl sulfoxide (0.11 mL, 1.60 mmol, 8 eq.) in dichloromethane (5 mL) maintained at -78 °C. The solution was allowed to stir at that temperature for 20 min. To this solution was added **3** (0.20 g, 0.020 mmol, 1eq.) dissolved in 2 mL of dichloromethane. This solution was allowed to stir for 1 hour at -78 °C. Triethylamine (0.45 mL, 3.2 mmol, 16 eq.) was added and the solution was stirred and additional 20 min. The reaction was allowed to warm to room temperature over 30 min. dichloromethane (5 mL) was added to the solution followed by successive washings with saturated NH_4Cl solution (10 mL X 3) and saturated NaCl solution (10 mL X 3). The organic layer was dried over anhydrous Na_2SO_4 and concentrating under reduced pressure. The residue was purified by flash column chromatography on silica gel (40% ethyl acetate-hexanes) to afford the product as a viscous liquid (0.12 g, 62% yeild): R_f = 0.37 (30% ethyl acetate-hexanes); ^{1}H NMR (600 MHz, $CDCl_3$): δ 9.36 (s, 1H, H-6), 7.40-7.13 (m, 35H, aromatic), 5.23 (d, 1H, *J* = 3.5 Hz, H-1), 5.15 (d, 1H, *J* = 3.7 Hz, H'-1), 5.02 (dd, 2H, *J* = 10.9, 5.6 Hz, benzylic), 4.87 (m, 5H, benzylic), 4.73 (d, 1H, *J* = 11.7 Hz, benzylic), 4.67 (m, 3, H-5, benzylic), 4.57 (m, 3H, benzylic), 4.48 (d, 1H, *J* = 10.6 Hz, benzylic), 4.41 (d, 1H, *J* = 12.1 Hz, benzylic), 4.15 (m, 1H, H-5'), 4.10 (t, 1H, *J* = 9.6 Hz, H-3), 4.02 (t, 1H, *J* = 9.4 Hz, H-3'), 3.70 (t, 1H, *J* = 9.6 Hz, H-4'), 3.60 (dd, 1H, *J* = 9.7, 3.7 Hz, H-2'), 3.57-3.51 (m, 3H, H-2, H-4, H-6a'), 3.40 (dd, 1H, *J* = 10.6, 1.8 Hz, H-6b'); ^{13}C NMR (150 MHz, $CDCl_3$): δ 197.98, 138.93, 138.60, 138.35, 137.99, 137.95, 137.91, 137.55, 128.71, 128.64, 128.61, 128.58, 128.54, 128.51, 128.38, 128.32, 138.23, 128.20, 128.10, 128.05, 127.92, 127.87, 127.83, 127.78, 127.66, 95.29, 94.66, 81.97, 81.73, 79.50, 78.85, 78.51, 77.78, 76.00, 75.81, 75.38, 74.85, 73.70, 73.40, 72.90, 71.05, 68.20; LRMS (ESI): calculated for $C_{61}H_{62}O_{11}$, 993.4 $[M+Na]^+$; observed, *m/z* = 993.4 $[M+Na]^+$.

*2,2',3,3',4,4',6'-Hepta-O-benzyl-6-deoxy-6E-(dimethyl vinylphosphonate)-α,α'-trehalose (**13**).* Sodium hydride, 60% dispersion in oil, (7 mg, 0.30 mmol, 3 eq.) was added to a solution of tetramethyl methylenediphosphonate (0.03 mL, 0.15 mM, 1.5 eq.) in tetrahydrofuran (3 mL) maintained at 0 °C. The mixture was stirred for 30 min. A solution of aldehyde **12** (0.10 g, 0.10 mmol, 1eq.) in 1 mL of tetrahydrofuran was added dropwise to the anion. The solution was allowed to warm to room temperature and stirred for another 2 h. The solvent was evaporated and the residue purified by flash chromatography on silica gel (5%

methanol-dichloromethane) to afford **13** as a colorless viscous liquid (0.054 g, 49% yeild): R_f = 0.48 (10% methanol-dichloromethane); ^{1}H NMR (600 MHz, $CDCl_3$): δ 7.38-7.23 (m, 33H, aromatic), 7.24-7.13 (m, 2H, aromatic), 6.96-6.88 (m, 1H, H-6), 5.94 (m, 1H, H-7), 5.23 (d, 1H, *J* = 3.7 Hz, H-1), 5.1 (d, 1H, *J* = 3.7 Hz, H-1'), 5.0 (dd, 2H, *J* = 10.8, 2.2 Hz, benzylic), 4.86 (m, 4H, benzylic), 4.75 (m, 1H, H-5), 4.72-4.76 (m, 2H, benzylic), 4.57 (m, 4H, benzylic), 4.47 (d, 1H, *J* = 10.8 Hz, benzylic), 4.39 (d, 1H, *J* = 12.1 Hz, benzylic), 4.15 (m, 1H, H-5'), 4.09 (t, 1H, *J* = 9.06 Hz, H-3), 4.03 (t, 1H, *J* = 9.06 Hz, H-3'), 3.66 (m, 6H, H-4', 2X-OCH_3), 4.56 (m, 2H, H-2 H-2'), 3.51 (m, 1H, H-6a'), 3.38 (dd, 1H, *J* = 10.6, 1.8 Hz, H-6b'), 3.25 (dd, 1H, *J* = 10.1, 9.2 Hz, H-4); ^{13}C NMR (150 MHz, $CDCl_3$): δ 149.17, 138.57, 138.35, 138.01, 137.76, 137.67, 137.50, 137.36, 128.28, 128.18, 127.75, 127.39, 127.15, 116.68, 115.42, 94.30, 93.63, 81.64, 81.58, 81.19, 78.98, 78.60, 77.41, 75.48, 75.40, 75.31, 74.87, 73.27, 72.71, 72.59, 70.45, 70.31, 70.17, 67.86, 52.10; ^{31}P NMR (162 MHz, $CDCl_3$): δ 32.42 (s, 1-P); HRMS (ESI): calculated for $C_{64}H_{69}PO_{13}$, 1099.4373 $[M+Na]^+$; observed, *m/z* = 1099.4370 $[M+Na]^+$.

*6-Deoxy-6-(methylenephosphonic acid)-α,α'-trehalose (**5, TEP**)*. Bromotrimethylsilane (83.4 µL, 0.652 mmol, 13 eq.) was added dropwise to a solution of **13** (0.09 g, 0.05 mmol) in dichloromethane (4 mL) and the resulting solution was stirred for 2 h at room temperature. The solution was quenched with 2 mL of methanol-water (1.1:1.8) followed by concentration to dryness. To the solution of benzylated disaccharide **14** was taken up in 3 mL of tetrahydrofuran-ethanol (1:4) and 20% $Pd(OH)_2$/C (50 mg) was added. The mixture was stirred overnight under hydrogen (1 atm.). The catalyst was filtered off through Celite® 545 and washed with 20% methanol-dichloromethane. The combined filtrate and washings were concentrated to dryness to afford **TEP** as viscous syrup (0.019 g, quantitative yield): ^{1}H NMR (600 MHz, D_2O): δ 5.04 (s, 2H, H-1, H-1'), 3.74-3.64 (m, 6H, H-6a', H-6b', H-5, H-5', H-3, H-3'), 3.55-3.50 (m, 2H, H-2, H-2'), 3.32 (t, 1H, *J* = 9.6 Hz, H-4'), 3.16 (t, 1H, *J* = 9.4 Hz, H-4), 1.98 (m, 1H, H-6a), 1.84 (m, 1H, H-6b), 1.59 (m, 2H, H-7a, H-7b); ^{13}C NMR (600 MHz, D_2O): δ 93.39, 93.11, 73.14, 72.43, 72.36, 72.08, 71.61, 71.50, 71.11, 70.88, 69.60, 60.41, 24.33; ^{31}P NMR (162 MHz, D_2O): δ 27.29 (s, 1-P); HRMS (ESI): calculated for $C_{13}H_{29}PO_{13}$, 443.0930 $[M+Na]^+$; observed, *m/z* = 443.0946 $[M+Na]^+$.

*2,2',3,3',4,4',6'-Hepta-O-benzyl-6-deoxy-6-azido-α,α'-trehalose (**15**)*. Sodium azide (0.114 g, 1.76 mol, 5 eq.) was added to a solution of iodide **9** (0.380 g, 351 mmol, 1eq.) in DMF (8 mL). The mixture was heated to reflux for 24 h. The solution was diluted with ethyl acetate and washed with saturated NaCl solution (10 mL X 3). The organic layer was dried over anhydrous Na_2SO_4, filtered, and the solvent evaporated under reduced pressure. The residue was purified by flash column chromatography on silica gel (8% ethyl acetate-hexanes) to afford **15** as a colorless viscous syrup (0.300 g, 86% yield): R_f = 0.33 (20% ethyl acetate:hexanes); ^{1}H NMR (600 MHz, $CDCl_3$): δ ^{1}H NMR (600 MHz, $CDCl_3$): 7.32 (m, 33H, aromatic), 7.12 (m, 2H, aromatic), 5.22 (d, 2H, *J* = 3.5 Hz, H-1, H-1'), 4.99 (dd, *J* = 10.8, 7.3 Hz, 2H, benzylic), 4.85 (m, 4H, benzylic), 4.71 (m, 4H, benzylic), 4.62 (s, 1H, benzylic), 4.55 (m, 2H, benzylic), 4.46 (d, 1H, *J* = 10.6 Hz,, benzylic), 4.38 (d, 1H, *J* = 12.1 Hz, benzylic), 4.16 (m, 1H, H-5', H-5), 4.02 (m, 2H, H-3', H-3), 3.68 (m, 1H, H-4'), 3.61 (m, 1H, H-2), 3.56 (m, 1H, H-2'), 3.49 (m, 2H, H-6a', H-6b'), 3.37 (m, 1H, H-4), 3.18 (m, 2H, H-6a, H-6b); ^{13}C NMR (150 MHz, $CDCl_3$): δ 138.83, 138.66, 138.29, 138.16, 138.06, 137.79, 128.49, 128.43, 128.38, 128.37, 128.03, 128.01, 127.93, 127.90, 127.89, 127.73, 127.66, 127.62, 127.57, 127.47, 127.38, 94.50, 94.01, 81.82, 81.46, 79.50, 79.36, 78.30, 77.69, 77.26, 77.04, 76.83, 75.62, 75.21, 75.13, 73.53, 72.96, 72.78, 70.74, 70.35, 68.10, 51.08; HRMS (ESI): calculated for $C_{61}H_{63}IO_{10}$, 1020.4411 $[M+Na]^+$; observed, *m/z* = 1020.4424 $[M+Na]^+$.

*2,2',3,3',4,4',6'-Hepta-O-benzyl-6-deoxy-6-amino-α,α'-trehalose (**16**).* Triphenylphosphine (0.236 g, 0.900 mmoL, 3 eq.) and water (0.027 g, 1.50 mmoL, 5 eq.) were added to compound **15** (0.300 g, 0.300 mmol, 1 eq.) dissolved in tetrahydrofuran (8 mL). The solution was stirred for 12 h and concentrated under reduced pressure. Ethyl acetate (10 mL) and water (10 mL) were added to the residue and the organic layer separated and dried over anhydrous Na_2SO_4. The solvent was removed under reduced pressure and the residue subjected to flash column chromatography on silica gel (10% acetone-hexanes) to afford **16** as a white solid (0.290 g, quantitative yield): R_f = 0.21 (30% acetone:hexanes); ^{1}H NMR (600 MHz, $CDCl_3$): δ 7.67 (m, 9H, aromatic), 7.52 (m, 4H, aromatic), 7.44 (m, 9H, aromatic), 7.37-7.29 (12H, aromatic), 7.14 (dd, 2H, *J* = 7.7, 1.5 Hz, aromatic), 5.22 (d, 1H, *J* = 3.7 Hz, H-1), 5.20 (d, 1H, *J* = 3.7 Hz, H-1'), 5.01 (t, 2H, *J* = 10 Hz, benzylic), 4.88 (m, 3H, benzylic), 4.83 (d, 1H, *J* = 10.8 Hz, benzylic), 4.72 (m, 5H, benzylic), 4.64 (m, 1H, *J* = 11.2 Hz, benzylic), 4.55 (d, 1H, *J* = 12.3 Hz, benzylic), 4.48 (d, 1H, *J* = 10.8 Hz, benzylic), 4.39 (d, 1H, *J* = 12.1 Hz, benzylic), 4.18 (dd, 1H, *J* = 12.1, 2.2 Hz, H-5'), 4.08 (td, 2H, *J* = 9.3, 7.1 Hz, H-3, H-3'), 4.00 (m, 1H, H-5), 3.7 (t, 1H, *J* = 9.7 Hz, H-4'), 3.63 (dd, 1H, *J* = 9.7,3.7 Hz, H-2'), 3.53 (m, 2H, H-6a', H-4), 3.41 (m, 2H, H-6b', H-4), 2.81 (dd, 1H, *J* = 13.7, 2.8 Hz, H-6a), 2.66 (dd, 1H, *J* = 13.8, 5.3 Hz, H-6b); ^{13}C NMR (150 MHz, $CDCl_3$): δ 138.66, 138.63, 138.16, 138.04, 138.01, 137.62, 132.67, 131.98, 131.93, 131.87, 131.79, 131.76, 128.37, 128.29, 128. 21, 128.16, 127.97, 127.81, 127.77, 127.77, 127.70, 127.51, 127.47, 127.36, 127.33, 127.29, 127.27, 127.19, 93.94, 93.57, 81.67, 81.56, 79.54, 79.28, 78.08, 77.58, 75.41, 75.34, 74.87, 74.70, 73.32, 72.81, 72.60, 71.71, 70.46, 67.97, 42.34; LRMS (ESI): calculated for $C_{61}H_{63}IO_{10}$, 972.5 $[M+H]^+$; observed, *m/z* = 972.9 $[M+H]^+$.

*2,2',3,3',4,4',6'-Hepta-O-benzyl-6-deoxy-6-(dibenyzl phosphoramidate)-α,α'-trehalose (**17**).* Dibenzylphosphoryl chloride (0.045 g, 0.154 mmol, 3 eq.) dissolved in 2 mL of dichloromethane was added to compound **16** (0.050 g, 0.051 mmoL, 1eq.) dissolved in 5 mL of dichloromethane. Triethylamine (0.051 g, 0.51 mmoL, 10 eq.) was added and the solution stirred for 6 h. The solution was concentrated under reduced pressure and the residue subjected to flash column chromatography on silica gel (8% acetone-hexanes) to afford a **17** as aviscous syrup (0.033 g, 52% yield): R_f =0.32 (30% acetone:hexanes); ^{1}H NMR (600 MHz, $CDCl_3$): δ 7.3 (m, 43H, aromatic), 7.14 (m, 2H, aromatic), 5.12 (d, 1H, *J* = 3.7 Hz, H-1), 5.10 (d, 1H, *J* = 3.7 Hz, H-1'), 4.97 (m, 6H, benzylic), 4.81 (m, 5H, benzylic), 4.63 (m, 6H, benzylic), 4.53 (m, 1H, benzylic), 4.45 (m, 1H, benzylic), 4.38 (m, 1H, benzylic), 4.12 (m, 1H, H-5'), 4.01 (m, 3H, H-3, H-3', H-5'),

3.65 (m, 1H, H-4'), 3.56 (m, 1H, H-2'), 3.50 (m, 2H, H-5, H-6a'), 3.44 (m, 2H, H-2), 3.37 (m, 1H, H-6b'), 3.08 (m, 1H, H-4), 2.93 (m, 1H, H-6a), 2.70 (m, 1H, H-6b); ^{13}C NMR (150 MHz, $CDCl_3$): δ 138.96, 138.91, 138.46, 138.35, 138.23, 138.21, 137.96, 128.70, 128.65, 128.62, 128.56, 128.54, 128.51, 128.43, 128.40, 128.23, 128.14, 128.06, 128, 127.96, 127.91, 127.86, 127.72, 127.59, 127.56, 127.52, 94.38, 93.91, 79.55, 79.32, 77.87, 77.83, 75.73, 75.66, 75.25, 75.03, 73.66, 72.97, 72.93, 70.83, 68.30, 68.24, 68.20, 68.14, 68.11, 41.53; ^{31}P NMR (162 MHz, D_2O): δ 10.89 (s, 1-P); HRMS (ESI): calculated for $C_{61}H_{63}IO_{10}$, 1232.5289 $[M+H]^+$; observed, *m/z* = 1232.5276 $[M+H]^+$.

*6-(phosphoramidic acid)-α,α'-trehalose (**6, TNP**).* A catalytic amount of 20% $Pd(OH)_2$/C was added to a solution of benzylated disaccharide derivative **17** (0.020 g, 0.016 mmol) dissolved in 3 mL of tetrahydrofuran-ethanol (1:4). The mixture was stirred overnight under hydrogen (1 atm.). The catalyst was filtered away through a plug of Celite® 545 and washed with 20% methanol-dichloromethane. The combined filtrate and washings were concentrated to dryness afford **TNP** as a viscous syrup (0.006 g, quantitative yield): ^{1}H NMR (600 MHz, D_2O): δ 5.12 (d, 1H, *J* = 3.5 Hz, H-1), 5.07 (d, 1H, *J* = 3.5 Hz, H-1'), 3.87 (m, 1H, H-5), 3.82 (m, 1H, H-6a'), 3.75 (m, 4H, H-3, H-3', H-5', H-6b'), 3.64 (m, 1H, H-4'), 3.55 (m, 2H, H-2, H-2'), 3.33 (m, 1H, H-4), 3.25 (m, 1H, H-6a), 3.03 (m, 1H, H-6b); ^{13}C NMR (150 MHz, D_2O): δ 93 .52, 93.35, 72.46, 72.14, 71.40, 70.99, 70.66, 69.53, 68.09, 60.36, 40.44; ^{31}P NMR (162 MHz, D_2O): δ 4.62 (s, 1-P); HRMS (ESI): calculated for $C_{12}H_{23}PO_{13}$, 422.1064 $[M+H]^+$; observed, *m/z* = 422.1052 $[M+H]^+$.

2,2',3,3',4,4',6'-Hepta-*O*-benzyl-6-deoxy-6-ethynyl-α,α'-trehalose (**18**). *n*-Butyllithium (0.415 mL, 2 *M*) was slowly added to a solution of trimethylsilylacetylene (118 µL, 0.831 mmol, 3 eq.) in tetrahydrofuran (3 mL) at -78 °C. The solution was allowed to warm to 0 °C and was strirred for 30 min. Afterward, the solution was allowed to warm to room temperature over 50 min. The solution of anion was added to another round bottom flask containing iodide **9** (0.300 g, 0.277 mmol, 1 eq). To the resulting solution was added hexamethylphosphoramide (0.768 mL, 4.43 mmol, 16 eq.). The solution changed to brown in color and was stirred for 12 h at room temperature. The solution was concentrated under reduced pressure and the residue subjected to flash column chromatography on silica gel (5% ethyl acetate-hexanes) to afford **18** as a colorless viscous syrup (0.095 g, 35% yield): R_f = 0.73 (30% ethyl acetate-hexanes); ^{1}H NMR (600 MHz, $CDCl_3$): δ 7.26-7.15 (m, 33H, aromatic), 7.06 (dd, 2H, *J* = 7.5 Hz, 1.8, aromatic), 5.17 (dd, 2H, *J* = 8.9, 3.6 Hz, H-1, H-1'), 4.93 (dd, 2H, *J* = 10.8, 6.2 Hz, benzylic), 4.8 (m, 4H, benzylic), 4.63 (m, 5H, benzylic), 4.48 (d, 1H, *J* = 12.1 Hz, benzylic), 4.39 (d, 1H, *J* = 10.6 Hz, benzylic), 4.32 (d, 1H, *J* = 12.1 Hz, benzylic), 4.09 (m, 2H, H-5, H-5'), 3.97 (td, 2H, *J* = 9.3, 7.7 Hz, H-3, H-3'), 3.62 (t, 1H, *J* = 9.7 Hz, H-3), 3.53 (ddd, 3H, *J* = 9.5, 7.6, 3.6 Hz, H-4', H-2, H-2'), 3.45 (dd, 1H, *J* = 10.7, 3.2 Hz, H-6a'), 3.31 (dd, 1H, *J* = 10.6, 1.8 Hz, H-6b'), 2.37 (m, 1H, H-6a), 2.25 (dt, 1H, *J* = 17, 3.3 Hz, H-6b), 1.95 (t, 1H, *J* = 2.6 Hz, H-7 alkyne); ^{13}C NMR (150 MHz, $CDCl_3$): δ 139.03, 138.90, 138.49, 138.45, 138.30, 137.97, 128.59, 128.53, 128.17, 128.13, 127.59, 127.54, 94.54 (anomeric), 94.46 (anomeric), 81.96, 81.67, 80.46, 80.20, 79.72, 79.52, 77.82, 75.83, 75.80, 75.59, 75.28, 73.66, 72.95, 72.87, 71.05, 70.82, 68.63, 68.24, 21.24; LRMS (ESI): calculated for $C_{63}H_{64}O_{10}$, 1003.4 $[M+Na]^+$; observed, *m/z* = 1003.9 $[M+Na]^+$.

*2,2',3,3',4,4',6'-Hepta-O-acetyl-6-deoxy-6-vinyl-α,α'-trehalose (**19**).* Compound **18** (0.245 g, 0.250 mmol) was dissolved in 2 mL of tetrahydrofuran in a three-neck flask equipped with a dry ice condenser. The flask wwas then set in a dry ice-acetone bath. Ammonia gas at was passed through the condenser until several milliliters of ammonia collected. A small piece of sodium metal was added to the solution, which immediately turned turquois in color. The ammonia was refluxed at -33 °C for 15 minutes. The reaction was quenched with 5 mL of methanol. The ammonia was evaporated and the remaining liquids removed under reduced pressure and the residue further dried under high vacuum for 12 h. To the dried residue was added pyridine (3 mL), acetic anhydride (3 mL) and a catalytic amount of DMAP. The solution was stirred for 12 h and then the solvent was concentrated under reduced pressure. The residue was subjected to flash column chromatography on silica gel (5% ethyl acetate-hexanes) to afford **19** as an amorphous solid (0.095 g, 59% yield): R_f = 0.33 (30% ethyl acetate:hexanes); ^{1}H NMR (600 MHz, $CDCl_3$): δ 5.72 (m, 1H, H-7), 5.48 (m, 2H, H-3, H-3'), 5.27 (d, 2H, *J* = 3.7 Hz, H-1, H-1'), 5.01 (m, 7H, H-2, H-2', H-4, H6a,b, H8a,b), 4.23 (m, 1H, H-6a'), 4.04 (m, 3H, H-4', H-5', H-6b'), 3.38 (m, 1H, H-5), 2.06 (m, 21H, $7XCH_3$); ^{13}C NMR (150 MHz, $CDCl_3$): δ 170.67, 170.06, 169.95, 169.70, 169.69, 169.65, 169.61, 132.39, 118.48, 91.94, 71.78, 70.10, 69.74, 69.10, 68.56, 68.07, 61.78, 35.45, 29.72, 20.75, 20.64; HRMS (ESI): calculated for $C_{61}H_{63}IO_{10}$, 647.2187 $[M+H]^+$; observed, *m/z* = 647.2193 $[M+H]^+$.

*2,2',3,3',4,4',6'-Hepta-O-acetyl-6-deoxy-6-oxiranyl-α,α'-trehalose (**20**).* Compound **19** (0.050 g, 0.077 mmol, 1 eq.) was dissolved in dichloromethane (3 mL) and 3-chloroperoxybenzoic acid (0.066 g, 0.387 mmol, 5 eq.) was added. The solution was stirred for an hour at room temperature and the solvent was was evaporated under reduced pressure. The reside was subjected to flash column chromatography on silica gel (10% ethyl acetate-hexanes) to afford **20** as an amorphous solid (0.030 g, 59% yield): R_f = 0.42 (30% ethyl acetate:hexanes); ^{1}H NMR (600 MHz, $CDCl_3$): δ 5.51 (m, 3H), 5.32 (m, 4H), 5.08 (m, 7H), 4.92 (t, 1H, *J* = 9.7 Hz), 4.23 (dt, 2H, *J* = 12.6, 6.3 Hz), 4.06 (m, 5H), 2.98 (m, 1H), 2.78 (m, 2H) 2.43 (td, 2H, *J* = 5.5, 2.7 Hz), 2.17 (m, 6H, $2XCH_3$), 2.08 (m, 36H, $12XCH_3$); ^{13}C NMR (150 MHz, $CDCl_3$): δ 170.69, 170.11, 170.10, 170.02, 169.98, 169.95, 169.94, 169.87, 169.87, 169.79, 196.73, 169.65, 169.64, 169.62, 92.29 (anomeric), 92.11 (anomeric), 91.75 (anomeric), 91.34 (anomeric), 72.20, 71.85, 70.24, 70.13, 70.05, 70.00, 69.95, 69.92, 69.40, 69.22, 68.62, 68.17,68.09, 67.49, 67.38, 61.85, 48.27, 48.18, 20.65; LRMS (ESI): calculated for $C_{61}H_{63}IO_{10}$, 685.2 $[M+Na]^+$; observed, *m/z* = 685.3 $[M+Na]^+$.

*6-deoxy-6-oxiranyl-α,α'-trehalose (**7**).* Compound **20** (0.044 g, 0.066 mmol) was dissolved in 9 mL of a mixture of triethylamine-methanol-water (2:6:1). The solution was stirred for an hour in dark. The solvent was evaporated under reduced pressure and subjected to flash column chromatography on silica gel using ethyl acetate-isopropanol-water (6:3:1) as an eluent to afford **7** as an viscous syrup (0.012 g, 50% yield): R_f = 0.45 (6:3:1 ethyl

acetate-isopropanol-water); ^{1}H NMR (600 MHz, D_2O): δ 5.05 (m, 2H, anomeric), 3.87 (m, 1H), 3.72 (m, 5H), 3.54 (m, 2H), 3.33 (m, 2H), 3.19 (m, 1H), 3.09 (m, 2H), 1.79 (m, 2H). ^{13}C NMR (150 MHz, D_2O): δ 93.54, 93.38, 93.21, 93.04, 73.24, 73.19, 72.53, 72.50, 72.41, 72.11, 71.97, 71.38, 71.07, 71.05, 70.92, 70.29, 69.62, 69.59, 69.52, 69.16, 60.42, 51.44, 50.44, 48.28, 46.71, 46.59, 33.63, 23.19, 20.40, 8.15; HRMS (ESI): calculated for $C_{61}H_{63}IO_{10}$, 369.1397 $[M+H]^+$; observed, *m/z* = 369.1219 $[M+H]^+$.

Protein expression and purification. The *otsB2* gene, encoding for the *Mtb* (UniProt code: P9WFZ5) trehalose-6-phosphate phosphatase, TPP, was amplified using *Mtb* genomic DNA and inserted into a modified pET-28 plasmid. Codon optimized genes encoding the TPP orthologs from *Mlt* (UniProt code: A0A0E4GVV3) and *Mtx* (UniProt code: A0A024JUE5) were purchased from IDT Inc., amplified and inserted into modified pET-32 plasmids. The resulting pDR28-*otsB2,* pDR32-*Mlt-otsB2* and pDR32-*Mtx-otsB2* plasmids were used in this study to encode and produce recombinant TPP possessing N-terminal polyhistidine tags. The sequences of the expression plasmids mentioned above were confirmed by DNA sequencing (MWG Operon). These plasmids were used to transform *E. coli* T7 Rosetta. The cells were then cultured at 37 °C in Luria Broth to an $O.D._{600\ nm}$ of 0.6. Gene expression was then induced by adding IPTG to a final concentration of 1 mM followed by a 24-hour incubation at 16 °C. The bacteria were harvested by centrifugation and resuspended with buffer A (20 mM Tris pH 7.5, 150 mM NaCl, 5 mM $MgCl_2$ and 5 mM β-mercaptoethanol). Following bacterial resuspension, 10 µM of lysozyme and 100 µM of DNase I were added to the resuspended cells, which were then incubated for 30 minutes on ice before sonication. The lysate was then centrifuged at 15,000 x *g* for 45 min and the resulting supernatant 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 step gradient. First, one column volume of 12% buffer B (150 mM imidazole, 150 mM NaCl, 5 mM $MgCl_2$ and 5 mM β-mercaptoethanol) was applied followed by eight column volumes of 100% buffer B. The purity of the protein was assessed using SDS-PAGE. The purified proteins were then dialyzed into 20 mM Tris pH 8.0 for enzymatic assays.

Steady-state kinetics and inhibition studies. The EnzChek® Phosphate Assay Kit (E-6646) (Molecular Probes) was used to monitor phosphate production from the hydrolysis of T6P by TPP in a continuous coupled assay. The phosphate released in solution is then used by purine nucleoside phosphorylase (PNP) to convert 2-amino-6-mercapto-7-methylpurine riboside (MESG) into ribose-1-phosphate and 2-amino-6-mercapto-7-methylpurine shifting the absorbance from 330 nm for MESG to 360 nm.

All kinetic and inhibitory studies were performed using the EnzChek® Phosphate Assay Kit (Molecular Probes) at 37 °C and the absorbance was measured at 360 nm using a Synergy™ H4 Hybrid Multi-Mode Microplate Reader (BioTek). The inhibition data were measured using 15 nM *Mtb* TPP-WT, 5 nM *Mlt* TPP-WT and 20 nM *Mtx* TPP-WT varying the concentration of **TNP** (31 µM to 4 mM), **TEP** (31 µM to 4 mM), **TMP** (78 µM to 10 mM), and Validamycin A (3.75 mM to 40 mM) in 25 µL reactions. Normalized data were fitted using the equation $Y=100/(1+(X^{HillSlope})/(IC50^{HillSlope}))$ as implemented in Prism 7.0 (GraphPad). All measurements were performed in quadruplicate.

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Notes and references

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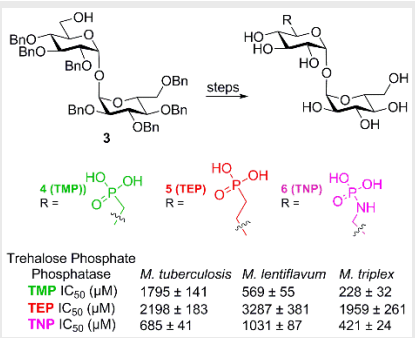

Trehalose Phosphate Phosphatase

	M. tuberculosis	*M. lentiflavum*	*M. triplex*
TMP IC_{50} (μM)	1795 ± 141	569 ± 55	228 ± 32
TEP IC_{50} (μM)	2198 ± 183	3287 ± 381	1959 ± 261
TNP IC_{50} (μM)	685 ± 41	1031 ± 87	421 ± 24

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Synthesis and in vitro characterization of trehalose-based inhibitors of mycobacterial trehalose 6-phosphate phosphatases