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Published in:
Biochemistry

DOI:
[10.1021/acs.biochem.8b00231](https://doi.org/10.1021/acs.biochem.8b00231)

Publication date:
2018

Document Version
Peer reviewed version

[Link to publication in Discovery Research Portal](#)

Citation for published version (APA):

Simithy, J., Fuanta, N. R., Alturki, M., Hobrath, J. V., Wahba, A. E., Pina, I., Rath, J., Hamann, M. T., Deruiter, J., Goodwin, D. C., & Calderón, A. I. (2018). Slow-Binding Inhibition of Mycobacterium tuberculosis Shikimate Kinase by Manzamine Alkaloids. *Biochemistry*, 57(32), 4923-4933. <https://doi.org/10.1021/acs.biochem.8b00231>

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Slow-binding inhibition of *Mycobacterium tuberculosis* shikimate kinase by manzamine alkaloids

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KEYWORDS: *Mycobacterium tuberculosis*, shikimate kinase, manzamine alkaloids, protein-ligand interactions, mass spectrometry, slow-binding inhibitor, jump dilution experiments, time-dependent inhibition, molecular docking.

The abbreviations used are: ESI, electrospray ionization; LC-MS, liquid chromatography-mass spectrometry; *MtSK*, *Mycobacterium tuberculosis* shikimate kinase; EI, enzyme-inhibitor complex; EI*, isomerized enzyme-inhibitor complex; S3P, shikimate-3-phosphate.

ABSTRACT

Tuberculosis (TB) represents a significant public health crisis. There is an urgent need for novel molecular scaffolds against this pathogen. We screened a small library of marine-derived compounds against shikimate kinase from *Mycobacterium tuberculosis* (*MtSK*), a promising target for antitubercular drug development. Six manzamines previously shown to be active against *M. tuberculosis* were characterized as *MtSK* inhibitors: manzamine A (**1**), 8-hydroxymanzamine A (**2**), manzamine E (**3**), manzamine F (**4**), 6-deoxymanzamine X (**5**), and 6-cyclohexamidomanzamine A (**6**). All six showed mixed-noncompetitive inhibition of *MtSK*. The lowest K_I 's were obtained for **6** across all *MtSK*-substrate complexes. Time-dependent analyses revealed two-step, slow-binding inhibition. The behavior of **1** was typical; initial formation of an enzyme-inhibitor complex (EI) obeyed an apparent K_I of $\sim 30 \mu\text{M}$ with forward (k_5) and reverse (k_6) rate constants for isomerization to an EI* complex of 0.18 min^{-1} and 0.08 min^{-1} , respectively. In contrast, **6** showed a lower K_I for the initial encounter complex ($\sim 1.5 \mu\text{M}$), substantially faster isomerization to EI* ($k_5 = 0.91 \text{ min}^{-1}$), and slower back conversion of EI* to EI ($k_6 = 0.04 \text{ min}^{-1}$). Thus, the overall inhibition constants, K_I^* , for **1** versus **6** were $10 \mu\text{M}$ and $0.06 \mu\text{M}$, respectively. These findings were consistent with docking predictions of a favorable binding mode and a second, less tightly bound pose for **6** at *MtSK*. Our results suggest that manzamines, in particular **6**, constitute a new scaffold from which drug candidates with novel mechanisms of action could be designed for the treatment of tuberculosis by targeting *MtSK*.

INTRODUCTION

Despite many advances in both diagnosis and treatment, tuberculosis (TB) continues to be a major global health concern. In terms of mortality due to a single infectious agent, it ranks second.¹ Complicated by the emergence of HIV-TB co-infection, multi-drug resistant (MDR) and extensively drug-resistant (XDR) strains of *Mycobacterium tuberculosis*, there is an urgent need for new therapeutics. In 2012, a new drug (SIRTURO™ Bedaquiline) that targets *M. tuberculosis* ATP synthase was approved by the Food and Drug Administration (FDA) exclusively for the treatment of MDR-TB.^{2,3} It was the first drug approved in over four decades for the treatment of TB. Unfortunately, Bedaquiline has already been associated with an increased risk of potentially fatal heart conditions, further stressing the unmet need for safer antitubercular drugs with novel targets and mechanisms of action to treat resistant forms of the disease.^{4,5}

Recently identified targets essential for *M. tuberculosis* survival and persistence include shikimate kinase (*MtSK*) from the shikimate pathway.⁶ This pathway is responsible for the biosynthesis of aromatic compounds in microorganisms and higher plants, and gene knock-out studies have established that it is an essential enzyme and a valid target.⁷ *MtSK*, the fifth enzyme of the pathway, catalyzes the phosphorylation of shikimate (SA) using ATP as a phosphoryl donor to form shikimate-3-phosphate (S3P) and ADP.⁸ With no mammalian counterpart, *MtSK* represents a favorable target for the design of drugs specific to the *M. tuberculosis* pathogen with reduced risk of toxicity in the human host.^{9,10}

Some of the strategies for the development of new drugs for the treatment of TB include the phenotypic and target-based screening of libraries of small molecules and natural products to identify novel chemical entities, the repurposing of existing antibiotics, and the modification of

existing scaffolds with known antimicrobial activities.¹¹⁻¹³ Although optimization of existing drugs can shorten the length and reduce the cost of the drug discovery process, more efforts are being directed towards the combination of whole-cell screening followed by target-based screenings. Using this integrated approach, compounds that can penetrate the mycobacterial cell wall and also inhibit the activity of a validated target can be identified.¹⁴⁻¹⁶

For millennia, natural products have played a pivotal role in the treatment and prevention of many diseases.¹⁷ Some first- and second-line anti-TB drugs (e.g., rifampicin, polypeptides, cycloserine, and aminoglycosides) are derivatives of natural products, supporting the role of natural products in generating novel chemical entities and new drug classes.^{14,18} In the past, more attention was directed towards plants as sources for bioactive lead compounds and antibacterials.¹⁹ However, more recently, marine organisms have come into focus as potential sources for new therapeutic agents against infectious diseases including TB.²⁰ In particular, manzamine alkaloids, a novel structural class of compounds isolated from Indo-Pacific marine sponges, have been reported to possess anti-inflammatory, antiparasitic, insecticidal, and antibacterial activities with potential for their development against malaria and TB.²¹⁻²⁵

The manzamines contain a complex polycyclic ring system coupled to a β -carboline moiety (Fig. 1). Manzamine A, first isolated in 1986, has been followed by more than 80 manzamine and manzamine-related alkaloids obtained from more than 16 different species of marine sponges.^{26,27} Manzamine A, the archetype of the group, has shown promising antitubercular activity *in vitro* with an MIC₅₀ value of 1.5 μ g/mL (*M. tuberculosis* strain H37Rv), an activity comparable to the first-line drug for TB treatment, rifampicin (MIC₅₀ 0.5 μ g/mL).^{27,28}

Despite their promising anti-tubercular activity, the cytotoxicity of the manzamines has been a major drawback for their development into drugs. One proposed mechanism for their

cytotoxicity posits the planar β -carboline moiety as a DNA intercalating agent.²⁹ Consistent with this notion, amidation of the β -carboline ring to produce 6-cyclohexamidomanzamine A (Fig. 1) eliminated the cytotoxicity observed for manzamine A but produced only a modest reduction in activity against *M. intracellulare* ($IC_{50} = 1.26 \mu M$) relative to manzamine A ($IC_{50} = 0.640 \mu M$).³⁰

To date, mechanisms underlying the potent biological activities of these alkaloids remain unclear. In this report, we have screened a library of 26 marine-derived alkaloids (Table S1) against *MtSK*. From them, six inhibited *MtSK*, including manzamine A (**1**), 8-hydroxymanzamine A (**2**), manzamine E (**3**), manzamine F (**4**), 6-deoxymanzamine X (**5**), and 6-cyclohexamidomanzamine A (**6**) (Fig. 1). Initial kinetic evaluations showed all six to be mixed non-competitive inhibitors of the enzyme, with **6** showing the lowest K_I values against all forms of *MtSK* (free enzyme, each enzyme-substrate binary complex, and the enzyme-substrate ternary complex). Further analyses revealed that each compound also showed time-dependent inhibition of *MtSK*, consistent with isomerization of an initial EI complex to a more tightly-bound EI* complex. Once again, **6** showed the most favorable properties as an inhibitor with the highest forward and lowest reverse rate constants for the isomerization step. Inhibitor jump-dilution experiments confirmed that **6** was distinct in its potency from the other manzamines tested. Indeed, the K_I^* value obtained for **6** was at least 40-fold lower than that obtained for any of the other manzamines. Docking simulations of **6** with *MtSK* predicted two possible binding modes for this compound in the substrate binding pockets of *MtSK*. In the most favorable pose obtained, the 6-cyclohexamido moiety occupied the shikimate binding site, while in an alternative pose overlapped with the co-crystallized ADP. Our results suggest that manzamine alkaloids may be viable lead compounds for the development of antimycobacterial drugs and that

MtSK may be a fruitful target for manzamines and other compounds to exert their antitubercular activity.

MATERIALS AND METHODS

Chemicals. Manzamine A (**1**), 8-hydroxymanzamine A (**2**), manzamine E (**3**), manzamine F (**4**), and 6-deoxymanzamine X (**5**), were isolated from a common Indo-Pacific sponge, *Acanthostrongylophora* sp., collected from Manado, Indonesia through an optimized isolation procedure.²⁸ 6-cyclohexamidomanzamine A (**6**) was synthesized by amidation of aminomanzamines.³⁰ All compounds evaluated on this study were supplied with a minimum purity of 90% as analyzed by HPLC or H-NMR (See *Supporting Information*, Fig S1-S6). Dimethyl sulfoxide (DMSO), shikimate-3-phosphate (S3P) used for the calibration curve, the substrates adenosine-5'-triphosphate (ATP) and shikimate (SA) were purchased from Sigma-Aldrich (St. Louis, MO). All organic solvents were HPLC or LC-MS grade and were purchased from Thermo Fisher (Hanover Park, IL). All buffers and media were prepared using water purified by a Milli-Q purification system (Millipore, Billerica, MA). *MtSK* was expressed and purified following procedures described in a previous report.³¹ The purity of the protein after Ni²⁺ affinity chromatography was determined by SDS-PAGE and LC-ESI-MS, and aliquots were stored at -80 °C in 50 mM Tris-HCl, pH 7.4; 0.5 M NaCl.

***MtSK* inhibitor screening by LC-MS.** The inhibitory effect of the small library of marine-derived compounds was evaluated by an end-point LC-MS enzymatic activity assay based on the quantitative measurement of the reaction product shikimate-3-phosphate (S3P) (m/z = 253.0117 [M - H]⁻). All assays were performed at 25 °C as described previously, using 100 mM ammonium acetate, pH 7.6, 50 mM KCl and 5 mM MgCl₂ in a reaction volume of 500 μ L.³¹

Primary screening was carried out by measuring the activity of *MtSK* in the presence of each compound (50 μ M) dissolved in DMSO at saturating concentrations of the substrates ATP (1.2 mM) and SA (5 mM). Reactions were initiated by the addition of 0.2 μ M of *MtSK*, for 30 seconds and quenched by the addition of 2 μ L of 98% formic acid and vortexing. Negative control experiments were carried out in the same manner in the presence of DMSO. For mass spectrometric analyses, 5 μ L of each reaction mixture were injected onto the HPLC system.

LC-MS analysis. Reversed-phase HPLC separation of the reaction mixtures was performed on an Agilent 1200 RRLC system, using a Zorbax Eclipse Plus Phenyl-hexyl (4.6 x 100 mm 3.5 μ m, Agilent Technologies, Inc.) column. The mobile phase consisted of water with 0.1% (v/v) formic acid (A) and acetonitrile 100% (B) with a gradient elution as follows: 0 min, 2% B held for 4 min, to 30% B in the next 2 min. Each elution was followed by a 1 min post-elution with 2% B. The total elution-time analysis was 7 min at a flow rate of 0.4 mL/min and column temperature of 45 °C. Mass spectrometry was carried out in an Agilent (Little Falls, DE) 6520 Accurate-Mass Q-TOF under negative ionization mode using a narrow m/z range (m/z 100-300). Nitrogen was used as the nebulizing gas (25 psig) and drying gas (10 L/h, 350 °C). A capillary voltage of 3200 V was used for the ESI source, and the fragmentor voltage was set to 175 eV. For quantification of S3P, extracted ion chromatograms (EIC) of m/z 253.0117 [$M - H$]⁻ were integrated using Agilent MassHunter Workstation Qualitative Analysis software (version B.02.00), and the peak area was used to evaluate enzymatic activity.

Kinetics of inhibition of manzamine alkaloids. Initial assessment of compounds **1 - 6** was carried out to determine the mechanism of rapid-reversible inhibition exerted by each. A range of concentrations (0 - 250 μ M in DMSO) for compounds **1 – 5** and (0- 50 μ M in DMSO) for compound **6** was evaluated to determine the effect on the linear initial rates of *MtSK* activity.

For these experiments, there was no pre-incubation of enzyme with the manzamines (**1** – **6**). For each concentration of **1** – **6**, five ATP concentrations varying from 0.05 to 1.2 mM were evaluated, while keeping the SA concentration constant at saturating concentrations (i.e., 5 mM SA against an apparent K_M with respect to SA of 0.53 mM). Likewise, five different concentrations of SA ranging from 0.2 to 5 mM were used for each concentration of **1** – **6**, while the concentration of ATP was kept constant at a saturating concentration (i.e., 1.2 mM ATP against an apparent K_M with respect to ATP of 0.20 mM).³¹ Each reaction was initiated by the addition of *MtSK* (0.2 μ M), allowed to proceed for 30 seconds, and then quenched as described above.

The initial rate data were evaluated using GraphPad Prism 5.02 software (Mountain View, CA) in two ways. In the first, all data obtained from varying SA against a constant concentration ATP were fit to one of four common models of inhibition (competitive, uncompetitive, non-competitive, and mixed-noncompetitive). The same was done for all data obtained by varying ATP against a constant concentration of SA. As described below, both data sets were best fit by a mixed-noncompetitive model where compounds show unequal affinities for the free enzyme (E) *versus* the enzyme-substrate (ES) complexes. That is, the ratio of $K_{I(ES)}/K_{I(E)} \neq 1$, and in all cases reported here $K_{I(ES)}/K_{I(E)}$ values were ≥ 1 ³². In the second approach, each hyperbolic curve in a set (e.g., varied ATP, constant saturating SA, and a chosen concentration of **1** – **6**) was fit individually to a Michaelis-Menten equation (**1**), where S represents the substrate whose concentration was varied in order to obtain an individual

$$\frac{v_o}{[E]_T} = \frac{(k_{cat})_{app} [S]}{(K_M)_{app} + [S]} \quad (1)$$

apparent k_{on} (i.e., $[k_{cat}/K_M]_{app}$) with respect to the varied substrate and an apparent k_{cat} in the presence of a given concentration of inhibitor and saturating concentrations of the second

substrate. The effects of inhibitor concentration on these two parameters were then evaluated in secondary plots to make assessments regarding the mechanism of inhibition and then determine K_I 's involved.

Given the random sequential kinetic mechanism of *MtSK* catalysis,³³ we interpreted our results in the context of a cubic model (Fig. 2).³⁴ In this model, the term “ α ” accounts for the ratio of K_{ATP} for the free enzyme (E) versus the enzyme bound to shikimate (E-SA), and the complementary situation applies for K_{SA} . Since each binding sequence establishes a thermodynamic square, α applies to both substrates (i.e., $K_{SA} * \alpha K_{ATP} = \alpha K_{SA} * K_{ATP}$). Previous reports indicate that the value of α is close to 1.³³ The cubic model allows that the inhibitor may bind to any form of the enzyme (E, E-SA, E-ATP, and E-SA-ATP) where each form of the enzyme has its own K_I for a given inhibitor. These are, respectively, K_I , βK_I , γK_I , and $\beta\gamma K_I$.

From fitting the mixed-noncompetitive inhibition model to experiments where ATP concentration is varied, the Prism-designated “ K_I ” corresponds to βK_I , and the Prism-designated “ αK_I ” corresponds to $\beta\gamma K_I$ (i.e., the “ α ” returned by the software corresponds to γ in the cubic model). Similarly, when SA is the varied substrate, the K_I corresponds to γK_I and the Prism-designated “ αK_I ” corresponds to $\beta\gamma K_I$ (i.e., now “ α ” refers to β in the cubic model). For a more detailed description of this approach including the derivation of equations used to evaluate the kinetic data, the reader is referred to the *Supporting Information* document that accompanies this paper. The dissociation constant for the inhibitor binding to the free enzyme (K_I) can be estimated by dividing βK_I (obtained from experiments where ATP was varied), β (obtained from experiments where SA was varied). The same was determined using γK_I and γ from the complementary experiments (i.e., where SA was varied, and ATP was varied, respectively). Importantly, there was good agreement between both estimations of K_I .

To ensure that the mixed-noncompetitive model was a model well supported across the data we collected, we also used an approach where each curve was individually fit to a Michaelis-Menten equation. Briefly, a hyperbolic decrease in apparent k_{cat} with increasing inhibitor concentration was fit to obtain $\beta\gamma K_I$ (Equation 2). In principle,

$$(k_{cat})_{app} = \frac{k_{cat}}{\left(1 + \frac{[I]}{\beta\gamma K_I}\right)} \quad (2)$$

the same value should be obtained whether SA or ATP is the substrate varied to obtain the individual curves. For individual curves where SA is the varied substrate, the hyperbolic decrease in apparent k_{cat}/K_{SA} with increasing inhibitor concentration can be fit to obtain βK_I (Equation 3). Likewise, when ATP is the varied substrate, the decrease in apparent k_{cat}/K_{ATP} with increasing concentration of inhibitor can be fit to obtain γK_I (Equation 4). For a more detailed

$$\left(k_{cat}/K_{SA}\right)_{app} = \frac{k_{cat}}{\alpha K_{SA} \left(1 + \frac{[I]}{\beta K_I}\right)} \quad (3)$$

$$\left(k_{cat}/K_{ATP}\right)_{app} = \frac{k_{cat}}{\alpha K_{ATP} \left(1 + \frac{[I]}{\gamma K_I}\right)} \quad (4)$$

description of our approach and a derivation of Equations 2 – 4, the reader is referred to the *Supporting Information* which accompanies this paper. Importantly, where there was overlap in determined dissociation constants across both methods (e.g., γK_I and βK_I), there was close agreement, indicating the mixed-noncompetitive inhibition model and the equilibrium dissociation constants obtained from it provided a reasonable description of the initial encounter of *MtSK* with these two inhibitors. As described below, time-dependent inhibition was investigated and observed with the manzamines. However, the rate of onset of time-dependent inhibition was sufficiently slow so as to have no discernable impact within the time frame of the

assays and analyses used to diagnose mixed noncompetitive inhibition in the initial encounter of *MtSK* with manzamine alkaloids (see *Supporting Information*, Fig. S13).

Time-dependent inhibition of *MtSK* activity. Initial kinetic experiments which included a pre-incubation of *MtSK* and a given manzamine (**1** – **6**) produced greater *MtSK* inhibition, suggesting the inhibition involved a time-dependent mechanism. Therefore, two methods were used to characterize the slow-binding component of the inhibition. In the first, preincubation times of *MtSK* (0.2 μ M) with various fixed concentrations of inhibitors (0 – 250 μ M) were varied (0 – 60 min). The *MtSK* activity remaining following incubation was measured by initial rates of S3P production as described above. For the second approach, *MtSK* (2.5 nM) was used to initiate S3P formation in reactions containing both substrates and some concentration (0 – 150 μ M) of **1** – **6**. The progressive decrease in rate of S3P formation by *MtSK* was monitored for 20 min. All reactions were performed using the same buffer and conditions as described above. Data generated using the first approach were analyzed by Equation 6 for time-dependent inhibition:

$$v_t = v_i \exp(-k_{obs}t) \quad (6)$$

where v_t is the measured steady-state velocity after preincubation time t , and v_i represents the steady-state velocity measured at preincubation $t = 0$. k_{obs} is the apparent first-order rate constant for the loss of activity with preincubation time. Progress curves generated using the second approach were analyzed by Equation 7 for slow-binding inhibition:

$$[P] = v_s t + \left(\frac{v_i - v_s}{k_{obs}} \right) * (1 - \exp(-k_{obs}t)) \quad (7)$$

where $[P]$ represents the amount of S3P generated by *MtSK* at time t , v_i and v_s represent the initial and final steady-state rates, respectively, in the presence of inhibitors, and k_{obs} is the rate constant for the transition from the initial to final steady-state rates of *MtSK* reaction.

Scheme 1 below describes a two-step mechanism that can account for the slow binding of inhibitors. The first step involves the formation of a loose initial encounter complex (EI), and the second step involves the slow isomerization of the enzyme to form a higher affinity complex (EI*):

Scheme 1. Two-step mechanism for slow binding inhibition

In this type of inhibition, the value of k_{obs} is a saturable function of $[I]$ conforming to Equation 8. Therefore, plots of k_{obs} versus inhibitor concentration yield a rectangular hyperbola, where the y-intercept is nonzero and indicates the rate constant k_6 . The maximum value of k_{obs} approached as an asymptote with increasing concentration of inhibitor is equal to $k_6 + k_5$. The concentration of inhibitor yielding a half-maximal value of k_{obs} is equal to the apparent dissociation constant for the initial enzyme-inhibitor complex (K_I) and the true affinities (K_I^*) or dissociation constants of the high-affinity enzyme-inhibitor complex were calculated according to Equation 9.³⁵

$$k_{obs} = k_6 + \left(\frac{k_5 [I]}{K_I^{app} + [I]} \right) \quad (8)$$

$$K_I^* = \frac{K_I}{1 + \left(\frac{k_5}{k_6} \right)} \quad (9)$$

Jump dilution experiments. *MtSK* (20 μ M) was incubated with 1 mM of a given manzamine (**1 – 6**) for 1 h. The reaction was initiated by diluting the EI complex in 1.2 mM ATP and 5 mM SA in 100 mM ammonium acetate, 50 mM KCl and 5 mM MgCl₂, pH = 7.6 to a final enzyme concentration of 0.2 μ M. Reactions were quenched with formic acid at times ranging from 0 - 60 min. Quenched reactions were analyzed by LC-MS as described above to quantify S3P. Concentration of S3P produced was plotted against reaction time. Data were fit to Equation 10:

$$[P] = [A]_0 \left\{ 1 + \frac{1}{k_1 - k_2} [k_2 \exp(-k_1 t) - k_1 \exp(-k_2 t)] \right\} \quad (10)$$

where $[A]$ is the initial concentration of S3P generated by *MtSK* at time t , and k_1 and the k_2 are the rates of formation and dissociation of the EI* complex, respectively.

Computational Methods. The *MtSK* crystal structure (PDB code 2DFT)⁸ was refined by applying the Prime preparation and refinement tools of the Protein preparation wizard implemented in the Schrödinger software package (Schrödinger, LLC). After the addition of hydrogen and detection of disulfide bonds, the structure was optimized by applying default parameters of the Impref utility using the OPLS2001 force field. The maximum allowed root-mean-square deviation between the refined structure and the input crystal structure was 0.3. Ligand structures were prepared using the LigPrep utility at pH = 7.4. The Induced Fit program of the Schrödinger software applies the Glide docking method combined with Prime structural refinement tools to account for the flexibility of protein side chains within 5 Å of the ligand during docking. Three docking set-ups were used in the docking runs, defining the center of the docking grid based on the centroid position of ligands present in the *MtSK* crystal structures as follows: a. SA, b. ADP and c. the center of mass of SA and ADP. The latter site definition allowed docking into the region that included both the SA and the ATP binding site. Induced Fit

settings were at default values except the size of the box enclosing the targeted site, which was set to 30 Å.

RESULTS

Mixed-type inhibition kinetics of manzamine alkaloids. For all the manzamines examined (**1** – **6**), the effects of their concentrations on the *MtSK* reaction kinetics with varying concentrations of substrates were best fit by a mixed-type inhibition model. Our results with **6** are shown in Fig. 3. Data obtained for **1** are shown in *Supporting Information* (Fig. S7) as are data for the other manzamines (**2** – **6**) (Figs. S8 – S12, respectively). A decrease in apparent k_{cat} with increasing inhibitor concentration (observed for **1** – **6**) suggested that the mechanism of inhibition could not be exclusively competitive. However, a decrease in apparent $k_{\text{cat}}/K_{\text{M}}$ (with respect to either substrate) with increasing concentrations of **1** – **6** indicated that all six compounds bound to the free enzyme. As described below, values for β and γ in all cases were ≥ 1 , indicating the preference of the inhibitors for the free enzyme. Visual inspection of double reciprocal plots (e.g., Figs. 3C and D for **6**) confirmed these conclusions.

A mixed-type mechanism of inhibition with respect to both substrates implies that the manzamine alkaloids can bind to *MtSK* forming four different complexes, but their affinities for these forms can be different. Thus, there are likely four different dissociation constants within the system: one for inhibitor interaction with the binary complex E-SA (γK_{I}), one for inhibitor interaction with the alternative binary complex, E-ATP (βK_{I}), one corresponding to the ternary complex E-SA-ATP ($\beta\gamma K_{\text{I}}$), and one for the interaction of the inhibitor with the free enzyme E (K_{I}) (Fig. 2).

There was good general agreement on the values of β , γ , βK_I , γK_I , and $\beta\gamma K_I$ across our methods of data analysis, and this extended to our estimates of K_I . Across all complexes, inhibition constants for **6** were 4- to 60-fold lower than those determined for the other manzamines **1** – **5** (Tables 1 and 2). For example, for **1**, β and γ were relatively small values (between 1 and 2), such that the range of inhibition constants observed for **1** was from ~140 μM for free *MtSK* (i.e., K_I) to ~250 μM for the ternary *MtSK*-ATP-SA complex (i.e., $\beta\gamma K_I$). Conversely, for **6**, values for β and γ were both determined to be about three. Here, the inhibition constants ranged from ~30 μM for the ternary complex (i.e., $\beta\gamma K_I$) down to ~3 μM for the free enzyme (i.e., K_I). Thus, overall, **6** is a far more potent inhibitor of *MtSK* than **1**, and with its greater preference for free *MtSK* over the *MtSK*-substrate complexes, **6** shows an apparent affinity for free *MtSK* that exceeds **1** by about 50-fold.

Time-dependent inhibition of *MtSK* by manzamine alkaloids. Pre-incubation of *MtSK* with a fixed concentration of any of the manzamines (**1** – **6**) for 60 min produced increased inhibition with time. Time-dependent inhibition is suggestive that a given inhibitor operates by either a slow-binding or irreversible mechanism of inhibition, wherein an initial EI complex undergoes a relatively slow transition (e.g., isomerization, conformational change, and/or covalent modification) to EI* (Scheme 1). Here, the rate constant k_6 is either very small or zero, respectively. In either case, determination of inhibition constants using typical rapid-equilibrium methods could result in an underestimation of the true potency of active compounds.³⁶ In the presence of either **1** or **6**, *MtSK* activity decreased exponentially with pre-incubation time (Fig. 4). Notably, there was also decrease in *MtSK* activity at preincubation $t = 0$ with increasing concentrations of **1** and **6**. This confirmed a two-step inhibition mechanism where formation of the initial EI complex is reflected in the decrease along the y-axis with increasing inhibitor

concentration, and the exponential decrease in activity thereafter reflecting the transition to EI*.³⁷ Similar results were obtained with compounds **2** – **5** (data not shown).

Because of the long pre-incubation times (≥ 1 h) required for the experiments above, enzyme stability became a factor. Therefore, we instead monitored the burst-exponential decrease in rates of product formation over time in the presence increasing inhibitor concentration to determine the inhibition mechanism rate constants for **1** – **6** (see *Materials and Methods*). Using this approach, the progress curve in the presence of **1** displayed an initial burst followed by a linear steady-state generation of product (Fig. 5A), diagnostic of a slow-onset of inhibition.³⁸ For **6**, product formation appeared to be nearly linear but the rates were visibly reduced (Fig. 5B) as compared to the uninhibited reaction, suggesting a more rapid conversion of EI to EI* than that of **1**. For **1**, k_{obs} increased hyperbolically with inhibitor concentration. From the fit of the data to equation 8, the rate constants k_5 and k_6 were estimated to be 0.18 min^{-1} and 0.08 min^{-1} , respectively, with a $^{app}K_I$ of $33\text{ }\mu\text{M}$ (Table 3). For **6**, the data revealed an upper limit for the sum k_5 and k_6 of 0.95 min^{-1} and indicated that the $^{app}K_I$ must be $\leq 2\text{ }\mu\text{M}$. This type of time-dependent inhibition was also observed for each of the other manzamines as well (**2** – **5**). Whereas **2** and **3** behaved in a manner similar to **1**, the time-dependence of inhibition by **4** and **5** showed weak dependence on inhibitor concentration. As such, we were unable to reach the limit set by the sum of k_5 and k_6 , and consequently, we were unable to determine $^{app}K_I$ or K_I^* for these compounds.

Analyses of intact MtSK by ESI-MS following its incubation with **1** or **6** did not reveal any covalent modification of the MtSK protein (data not shown), eliminating one major mechanism of irreversible inhibition. We carried out a jump-dilution experiment for pre-formed complexes of MtSK with each manzamine (**1** – **6**) in order to distinguish whether the

manzamines were irreversible or reversible, slow-binding inhibitors, and if the latter, make a more definitive determination of k_6 . Upon a 1:100 dilution into assay solution, recovery of activity as monitored by production of S3P was observed for *MtSK* preincubated with all six compounds (Fig. 6). This confirmed that none of the manzamines act as irreversible inhibitors. Interestingly, only recovery of activity for *MtSK* reacted with **6** was substantially delayed, and a fit of the data to a double-exponential function (Equation 10) returned 0.04 min^{-1} as the value for the slower of the two exponentials. We interpret this as an estimate for k_6 in the inhibition mechanism of **6**. In contrast, a delay in the return of activity upon dilution was not evident for **1** – **5**, and the activity recovered was between 39 and 59% of the no-inhibitor control. It was not immediately apparent why the expected progressive increase in activity was not observed with **1** – **5**, but kinetic modeling suggested that interference from product accumulation may have contributed to a muted response (The reader is referred to the *Supporting Information* for a more complete description of this kinetic modeling, its results, and interpretation)³⁹.

In any case, our data showed that EI* more readily reverted to active enzyme with compounds **1** - **5** compared to **6**. Accordingly, the potency of **6** was enhanced by nearly 25-fold upon isomerization of the first initial enzyme-inhibitor (EI) complex to form a more stable complex (EI*) (i.e., $[1 + k_5/k_6] = 23.8$; Table 3). We obtained much more modest values for **1**, **2**, or **3** (i.e., $[1 + k_5/k_6] = 3.3, 6.3, \text{ and } 7.9$, respectively; Table 3). Ultimately, K_I^* values estimated were 40-fold lower for **6** than any of the other manzamine inhibitors. Our results indicate that this resulted from a combination of a substantially lower K_I for formation of the initial EI complex with **6** as well as a more facile isomerization of the initial complex to EI*.

Modeling of compound 6 into the *MtSK* binding site. Compound **6** was docked into *MtSK* (PDB 2DFT)⁸ targeting both the shikimate and ATP binding site regions, as presented in

more detail under *Computational methods*. Figure 7A illustrates that this compound may be accommodated in two alternative docked poses, which differ in the position and conformation of pyridoindole-cyclohexamide while the rest of the molecule shows close overlap. Of the two poses the binding model with more favorable contacts (pose 1) places the pyridoindole-hexamide moiety in a deeper cavity, where its cyclohexamide group occupies the shikimate binding pocket. In the alternative binding model (pose 2) the pyridoindole-hexamide moiety binds close to the surface, and the cyclohexamide group overlaps with the co-crystallized ADP. The latter pose may correspond to a weaker bound complex compared to pose 1. The most favorable binding model obtained in our docking study is pose 1, accommodated in a deep region of the *MtSK* active site (Fig. 7B). This pose predicts the following interactions between compound **6** and *MtSK* (Fig. 7C). Thr 115 participates in two, near optimal hydrogen bonding interactions with compound **6**, while Glu 38 forms a salt bridge with a protonated amine of the ligand. The carbonyl oxygen of compound **6** coordinates the co-crystallized Mg^{2+} . The guanidinium group of Arg117 forms positive charge – aromatic stacking interactions with the pyridoindol ring system. Arg 20 is hydrogen bonding with the N of the pyridine ring. Residues involved in favorable non-polar contacts with the ligand are Pro 11, Val 35, Ile 45, Phe 49, Val 116, Pro 118, Leu 119 and Leu 132.

Docking results suggested that compound **6** may adopt two alternative poses, blocking the binding of either substrate. Of these, the pose interfering with shikimate binding may represent the more tightly bound complex. These hypotheses are consistent with the mixed-type mechanism of inhibition suggested by our kinetic analyses. The presented model may be tested in future experimental designs elucidating interactions between manzamine A derivatives and *MtSK* residues. Furthermore, the active compounds in this study have been previously evaluated

for their *in vitro* antibacterial activity against *Mycobacterium tuberculosis* (R37Rv). MIC values along with cytotoxicity in mammalian cells are summarized in Table 4. It is noteworthy that the *in vitro* activity against *M. intracellulare* of the best candidate, compound **6** (1.8 μ M), is comparable to that of the second drug of treatment for TB, ciprofloxacin (1.05 μ M).

DISCUSSION

Kinetic profiling has demonstrated that several manzamines (**1** – **6**) inhibit *MtSK* through a two-step mechanism where an initial EI complex undergoes a slow isomerization, resulting in a higher affinity EI* complex. Of the six manzamines evaluated, the 6-cyclohexamido derivative of manzamine A (**6**) was clearly the superior inhibitor of *MtSK*, a property that was evident in both steps of the inhibition mechanism. Apparent K_I values corresponding to mixed inhibition were lower for **6** than the other five manzamines regardless of the presence of either or both *MtSK* substrates. Similarly, a higher rate constant for conversion of EI to EI* (i.e., k_5) along with the largest quotient between on and off rate constants (i.e., k_5/k_6) combined to yield a considerably more potent inhibitor with an overall inhibition constant (K_I^*) in the 60 nM range, at least 40-fold lower than the other manzamines evaluated.

Docking simulations of compound **6** at *MtSK* predicted two binding poses that differ in the pyridoindole-cyclohexamide region while the rest of the ligand structure closely overlaps between the two poses (Fig. 8). The most favorable binding mode is pose 1, in which the pyridoindole-cyclo-hexamide occupies a deep lying region of the binding site. Its 6-cyclohexamide group binds favorably in the shikimate binding pocket through non-polar contacts of the cyclohexyl group while the carbonyl is anchored through Mg^{2+} coordination. In pose 2 this moiety is accommodated less favorably in a polar, solvent exposed region near the

surface. Comparison of docked poses suggests that pose 1 may correspond to a more tightly bound complex than pose 2. We hypothesize that **6** may first bind in a mode similar to pose 2, then transition to adopt the more favorable pose 1. Re-orientation of pyridoindole-cyclohexamide from a region close to the surface into a deeper site may require induced conformational changes of the structure over time. This hypothesis derived from docking predictions is a possible model consistent with the kinetic profile of **6** presented in this study. The favorable contribution of the cyclohexamide group to ligand binding also provides a possible explanation to why manzamine A (**1**) as well as **2** – **5**, all of which lack the 6-cyclohexamido- modification are inferior as *MtSK* inhibitors at all stages of the mechanism. Notably, we have previously evaluated the potential of 6-nitroharmane and ircinol A, two compounds containing the biogenetic precursors of the manzamine alkaloids, to act as inhibitors of *MtSK*,⁴⁰ and no significant inhibition was observed for these compounds. These data suggest that the entire manzamine structure is required to observe inhibition, a property that is enhanced by the 6-cyclohexamido addition.

Interestingly, other observed biological activities of the manzamines point toward a synergistic function in fighting TB infection. For example, the manzamine alkaloids have been reported to inhibit the activity of glycogen synthase kinase-3 beta (GSK-3 β).^{41,42} GSK-3 β is a serine/threonine protein kinase involved in the regulation of several cellular pathways including insulin signaling, glycogen synthesis, neurotrophic factor signaling, Wnt signaling, neurotransmitter signaling and microtubule dynamics.⁴³ Most importantly, GSK-3 β has been implicated in mycobacterial-induced interleukin-10 (IL-10) production in human peripheral blood monocytes.⁴⁴ IL-10 is an anti-inflammatory cytokine mainly produced by macrophages, and it plays an important role in the regulation of immune response against infectious pathogens.

Despite IL-10 protective role in the host, its immunosuppressive properties have been shown to be exploited by mycobacteria to facilitate immune evasion and long-term infections in the lungs.^{45,46} BCG vaccine, widely used for vaccination against *M. tuberculosis* has been reported to inhibit GSK-3 β activity resulting in increased production of IL-10, consistent with that inhibition of this protein kinase is beneficial to the host in the development of protective immunity against *M. tuberculosis*.⁴⁷ Interestingly, manzamine A and some of its analogs showed selective binding for human GSK-3 β .²⁸ When tested in a cell-based assay that measures GSK-3 β dependent tau phosphorylation, manzamine A significantly inhibited tau phosphorylation within cells at concentrations as low as 5 μ M with no cytotoxicity.²⁸

Despite their structural complexity, the manzamine alkaloids in this study constitute a novel scaffold for the potential development of new leads for the treatment of tuberculosis. The time-dependent component of the inhibition exerted by these compounds on *MtSK* represents a significant clinical advantage, as a prolonged residence time of a drug on a particular target may result in an enhanced duration of the desired pharmacological effect *in vivo*.^{48,49} Since manzamine A and some of its analogs can inhibit both GSK-3 β and *MtSK*, we suggest that these compounds are promising candidates for further studies leading to the development of new drug candidates specific to the *M. tuberculosis* pathogen. This is most particularly so for the 6-cyclohexamido derivative, compound **6**, due to its inhibition profile, *in vitro* antibacterial activity, and lack of cytotoxicity.

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ACKNOWLEDGMENTS

This work was supported by an Auburn University Intramural Grants Program (AU-IGP) grant to AC and DG, through the Office of the Vice President for Research (OVPR). JS is grateful to the Secretaría Nacional de Ciencia y Tecnología” (SENACYT) in collaboration with the “Instituto para la Formación de Recursos Humanos” (IFARHU) of the Panamanian government for Ph.D. scholarship. MTH thanks NCCIH Grant #RO1AT007318, The US-Egypt Fellowship program, The China Scholarship Council, Kraft Foods, The Cooper Family and SC SmartState program for financial support.

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Table 1. Inhibition constants determined by varied [ATP], fixed [shikimate]

Compound	Inhibition Parameter ^a				
	β^b	γK_i (μM) ^c	γK_i (μM) ^d	$\beta\gamma K_i$ (μM) ^e	est K_i (μM) ^f
1	1.0 $\pm$ 0.4	210 $\pm$ 60	210 $\pm$ 20	205 $\pm$ 30	120 $\pm$ 60
2	2 $\pm$ 1	76 $\pm$ 25	83 $\pm$ 22	180 $\pm$ 80	11 $\pm$ 7
3	1.8 $\pm$ 1.0	180 $\pm$ 60	175 $\pm$ 44	312 $\pm$ 96	63 $\pm$ 46
4	6.6 $\pm$ 3.6	121 $\pm$ 23	130 $\pm$ 15	1100 $\pm$ 660	40 $\pm$ 15
5	6.6 $\pm$ 3.7	96 $\pm$ 18	105 $\pm$ 24	1000 $\pm$ 400	21 $\pm$ 8
6	3.7 $\pm$ 1.0	8 $\pm$ 1	9 $\pm$ 2	32 $\pm$ 7	3.2 $\pm$ 0.9

^aShikimate held constant 5.0 mM; ATP was varied (0.05 – 1.2 mM) against a series of fixed inhibitor concentrations.

^bDetermined as “ α ” from fitting to a mixed-type inhibition model.

^cDetermined as “ K_i ” from fitting to a mixed-type inhibition model.

^dDetermined from replotting of $(k_{\text{cat}}/K_{\text{ATP}})_{\text{app}}$ vs. [inhibitor].

^eDetermined from replotting of $(k_{\text{cat}})_{\text{app}}$ vs. [inhibitor].

^f K_i (i.e., dissociation constant for inhibitor binding to free *MtSK*) was estimated by dividing γK_i from mixed-type fitting (this table) by γ from mixed-type fitting (Table 2).

Table 2. Inhibition constants determined by varied [shikimate], fixed [ATP]

Compound	Inhibition Parameter ^a				
	γ^b	βK_i (μM) ^c	βK_i (μM) ^d	$\beta\gamma K_i$ (μM) ^e	est K_i (μM) ^f
1	1.7 $\pm$ 0.8	150 $\pm$ 50	160 $\pm$ 50	290 $\pm$ 60	151 $\pm$ 67
2	7 $\pm$ 4	31 $\pm$ 10	22 $\pm$ 9	187 $\pm$ 46	15 $\pm$ 9
3	2.9 $\pm$ 1.9	105 $\pm$ 39	139 $\pm$ 76	112 $\pm$ 20	58 $\pm$ 39
4	3 $\pm$ 1	142 $\pm$ 32	141 $\pm$ 24	442 $\pm$ 68	22 $\pm$ 13
5	4.5 $\pm$ 1.5	77 $\pm$ 15	44 $\pm$ 18	272 $\pm$ 73	12 $\pm$ 7
6	2.5 $\pm$ 0.6	9.0 $\pm$ 1.5	7.6 $\pm$ 1.7	25 $\pm$ 2	2.4 $\pm$ 0.8

^aATP held constant at 1.2 mM; shikimate was varied (0.2 – 5.0 mM) against a series of fixed inhibitor concentration.

^bDetermined as “ α ” from fitting to a mixed-type inhibition model.

^cDetermined as “ K_i ” from fitting to a mixed-type inhibition model.

^dDetermined from replotting of $(k_{\text{cat}}/K_{\text{SA}})_{\text{app}}$ vs. [inhibitor].

^eDetermined from replotting of $(k_{\text{cat}})_{\text{app}}$ vs. [inhibitor].

^f K_i (i.e., dissociation constant for inhibitor binding to free *MtSK*) was estimated by dividing βK_i from mixed-type fitting (this table) by β from mixed-type fitting (Table 1).

Table 3. Inhibition constants from time-dependent inhibition.

Compound	$^{app}K_I$ μM	K_I^* μM	k_5 (min^{-1})	k_6 (min^{-1})
1	33 ± 3	10 ± 2	0.18 ± 0.01	0.08 ± 0.01
2	15 ± 11	2.4 ± 0.01	0.37 ± 0.09	0.07 ± 0.1
3	55 ± 15	6.9 ± 0.9	0.09 ± 0.01	0.013 ± 0.004
4	ND ^a	ND	ND	ND
5	ND ^a	ND	ND	ND
6	1.4 ± 0.9	0.06 ± 0.03	0.91 ± 0.03	0.04 ± 0.02^b

^aTime-dependent inhibition evident, but k_5 could not be reached, thus, $^{app}K_I$, K_I , and k_6 , could not be determined.
^bDetermined from jump dilution experiments (see Fig. 6)

Table 4. *In vitro* anti *Mycobacterial* activities and cytotoxicities of manzamine compounds.

Compound	<i>Mtb</i> (H37Rv) MIC ₅₀ ($\mu\text{g/mL}$)	<i>M. intracellulare</i> IC ₅₀ (μM)	Cytotoxicity (Vero) IC ₅₀ ($\mu\text{g/mL}$)
1	1.5	0.64	1.2
2	0.9	0.17	1.1
3	3.8	NT	NC
4	2.6	NT	NC
5	1.8	NT	4.7
6	NT	1.26	NC
Rifampin	0.5	NT	NT
Ciprofloxacin	NT	1.05	NT
Doxorubicin	NT	NT	5.0

Values were obtained from the literature (23, 27, 28 and 30). MIC₅₀ values for the first and second line of treatment drugs rifampin and ciprofloxacin are reported as a reference, as well the cytotoxicity of the drug doxorubicin against Vero cells. NC = no cytotoxicity up to 4.76 $\mu\text{g/mL}$. NT = not tested.

FIGURE LEGENDS

FIGURE 1. Structures of manzamines investigated in this study. Manzamine A (**1**), 8-hydroxy manzamine A (**2**), manzamine E (**3**), manzamine F (**4**), 6-deoxymanzamine X (**5**), and 6-cyclohexamidomanzamine A (**6**).

FIGURE 2. Kinetic model for the mixed-type inhibition of *MtSK* reaction with shikimate (SA) and ATP by manzamine compounds.

FIGURE 3. Effect of compound **6** on *MtSK* activity. The concentration of ATP was varied relative to a constant 5 mM shikimate concentration (A) or the concentration of shikimate was varied against a constant 1.2 mM ATP concentration (B) in the presence of 0 μM ($\bullet$), 3.12 μM ($\blacksquare$), 6.25 μM ($\blacktriangle$), 12.5 μM ($\blacktriangledown$), 25 μM ($\blacklozenge$), and 50 μM ($\bullet$) of compound **6**. The data of each panel were globally fit to a mixed inhibition model (GraphPad Prism 5.02 software). Double-reciprocal analyses corresponding to panels A and B are also presented (C and D, respectively). All reactions contained 0.2 μM *MtSK* and were carried out at 25°C in 100 mM ammonium acetate, pH 7.6. Points are the average of duplicate injections, and error bars show the range of values.

FIGURE 4. Effect of inhibitor concentration and preincubation time on the activity of *MtSK*. *MtSK* was preincubated for the times indicated with 0 μM ($\bullet$), 10 μM ($\blacksquare$), 25 μM ($\blacktriangledown$), 50 μM ($\blacktriangle$), 100 μM ($\bullet$), and 150 μM ($\blacksquare$) of compound **1** (A), or 0 μM ($\bullet$), 3.12 μM ($\blacksquare$), 6.25 μM ($\blacktriangledown$), 12.5 μM ($\blacktriangle$), 25 μM ($\bullet$), and 50 μM ($\blacksquare$) of compound **6** (B). All activity assays contained 0.2 μM *MtSK*, 5 mM shikimate, and 1.2 mM ATP and were carried out at 25°C in 100 mM ammonium acetate, pH 7.6. Shown is one representative of duplicate experiments. Points are the average of duplicate injections, and error bars show the range of values.

FIGURE 5. Effect of compounds **1** and **6** on production of shikimate-3-phosphate (S3P) by *MtSK* over time. Product (i.e., S3P) generation by *MtSK* (2.5 nM) was monitored in reactions containing 0 μM ($\bullet$), 10 μM ($\blacksquare$), 25 μM ($\blacktriangledown$), 50 μM ($\blacktriangle$), 100 μM ($\bullet$), and 150 μM ($\blacksquare$) of compound **1** (A) or compound **6** (B). Reactions were initiated by the addition of *MtSK* 2.5 nM to solutions containing the indicated concentration of inhibitor as well as fixed concentrations of shikimate (5 mM) and ATP (1.2 mM). Reactions were carried out at 25°C in 100 mM ammonium acetate, pH 7.6. Insets show the re-plots of k_{obs} values against inhibitor concentration. Shown is one representative of duplicate experiments. Points are the average of duplicate injections, and error bars show the range of values.

FIGURE 6. Recovery of *MtSK* activity following dilution of *MtSK*-inhibitor complexes. *MtSK* (20 μM) was pre-incubated for 1 hour without any inhibitor ($\bullet$) or with 1 mM **1** ($\blacksquare$), **2** ($\blacktriangledown$), **3** ($\blacktriangle$), **4** ($\bullet$), **5** ($\blacklozenge$), or **6** ($\blacksquare$) to achieve significant target inactivation. Enzyme-inhibitor complexes were then diluted 1:100 into a reaction buffer containing 5.0 mM shikimate, 1.2 mM ATP, and 100 mM ammonium acetate, pH 7.6, to follow enzyme reactivation. Points are the average of duplicate injections.

FIGURE 7. Two docked poses of compound **6** are shown in the *MtSK* crystal structure, PDB code 2DFT (panel A). Positions of shikimate (SA), ADP and Mg^{2+} are also included for comparison. Carbon atoms in docked poses are colored as indicated; carbons in shikimate and ADP are colored dark and light purple, respectively. All other atoms are colored by atom type

(oxygen red, nitrogen blue, hydrogen white, phosphorus magenta, Mg^{2+} pink). The electrostatic potential surface of the *MtSK* binding site with docked poses 1 and 2 is shown in panel B, with analogous color scheme for the ligands as in A. A close-up view of docked pose 1 with *MtSK* residues closest to the ligand is displayed in panel C. Carbon atoms of compound 6 are colored green and of *MtSK* residues in light yellow. All other atoms are colored by atom type. Hydrogen bonding and salt bridging interactions are indicated with dashed lines.

Figure 1

1 manzamine A ($R^1 = R^2 = H$)

2 8-hydroxymanzamine A ($R^1 = OH$; $R^2 = H$)

6 6-cyclohexamido-
manzamine A ($R^1 = H$;

3 manzamine E ($R = H$)

4 manzamine F ($R = OH$)

5 6-deoxy manzamine X

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7

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**Slow, tight-binding inhibition of *Mycobacterium tuberculosis* shikimate kinase by
manzamine alkaloids**

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