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Evaluation of NTF1836 as an inhibitor of the mycothiol biosynthetic enzyme MshC in growing and non-replicating *Mycobacterium tuberculosis*

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ABSTRACT

The mycothiol biosynthesis enzyme MshC catalyzes the ligation of cysteine with the pseudodisaccharide GlcN-Ins and has been identified as an essential enzyme in *Mycobacterium tuberculosis*. We now report on the development of NTF1836 as a micromolar inhibitor of MshC. Using commercial libraries, we conducted preliminary structure–activity relationship (SAR) studies on NTF1836. Based on this data, NTF1836 and five structurally related compounds showed similar activity towards clinical strains of *M. tuberculosis*. A gram scale synthesis was developed to provide ample material for biological studies. Using this material, we determined that inhibition of *M. tuberculosis* growth by NTF1836 was accompanied by a fall in mycothiol and an increase in GlcN-Ins consistent with the targeting of MshC. We also determined that NTF1836 kills non-replicating *M. tuberculosis* in the carbon starvation model of latency.

1. Introduction

Tuberculosis (TB), resulting from infection by *Mycobacterium tuberculosis*, continues to be a serious health concern. In 2009, there were an estimated 9.4 million cases worldwide, resulting in an estimated 1.7 million deaths.[1] Current estimates suggest that one-third of the world population harbors latent TB that emerges upon immune system compromise. TB continues to be a major cause of death among HIV-positive individuals.[2] Currently, the six to nine month multidrug protocol used in the treatment of TB is highly effective with drug-susceptible TB but deviant protocols and poor patient compliance promotes development of drug-resistance.[3] The emergence of multidrug-resistant and extensively drug-resistant TB has exacerbated the problem of tuberculosis control.[4] There is a recognized need for new faster acting drugs that are effective against drug-resistant TB while remaining compatible with other TB and HIV drugs, as they are often used in combination.[5] Ideally, there is an immediate need for drugs that can terminate the non-replicating bacilli of latent TB.[6] After a long lull in the introduction of new TB candidates a number of promising leads are now in clinical trials.[5,6] However, the drug pipeline needs continual expansion in order to successfully meet the challenges of treating drug-resistant and latent infections.[5]

Metabolic pathways unique to bacteria, and their associated enzymes, offer excellent targets for drug development. Mycobacteria are unusual in that they do not synthesize glutathione (GSH) but produce a different low-molecular-weight thiol, mycothiol (MSH, **1**, Fig. 1). In *M. tuberculosis*, MSH (**1**) acts in a similar manner as GSH.[7,8] The biosynthesis of MSH arises from UDP-GlcNAc and 1L-myo-inositol-1-phosphate (Ins-P) by five enzymes as outlined in Scheme 1.[9] Analogous to GSH, MSH is involved in detoxification. It is responsible for the removal of select antibiotics (RX, Scheme 1) through the production of mycothiol-*S*-conjugates (MSR) that are cleaved by mycothiol-*S*-conjugate amidase (Mca). This process generates a mercapturic acid (AcCySR, Scheme 1) that can be excreted. The resulting GlcN-Ins is used to regenerate MSH.[10] The mycothiol deacetylase (MshB),[11] and Mca,[10,12] are homologous enzymes and have overlapping activities.

MshB was the first mycothiol biosynthesis enzyme identified.[13] A *mshB* gene knockout in *M. tuberculosis* produced MSH, albeit at a lower level than wild-type cells during exponential growth, and exhibited increased sensitivity to rifampin and cumene hydroperoxide.[14] Since Mca also displays deacetylase activity, it may contribute to MSH production in the absence of MshB.[12] *Mycobacterium smegmatis* mutants defective in MshC have been isolated that are devoid in MSH production,[15] however, attempts to produce a *mshC* knockout in *M. tuberculosis* were unsuccessful unless a second copy of the gene was present.[16] This evidence suggested that MshC was essential in *M. tuberculosis*.

Based on this evidence, MSH biosynthesis has been suggested as a target for therapeutic development for TB. Compounds that inhibit both MshB and Mca have been synthesized and display activities in the low micromolar range,[17] but have so far failed to ablate MSH biosynthesis. No inhibitors of MshA or MshD have been reported and the phosphatase remaining enzyme, MshA2, has yet

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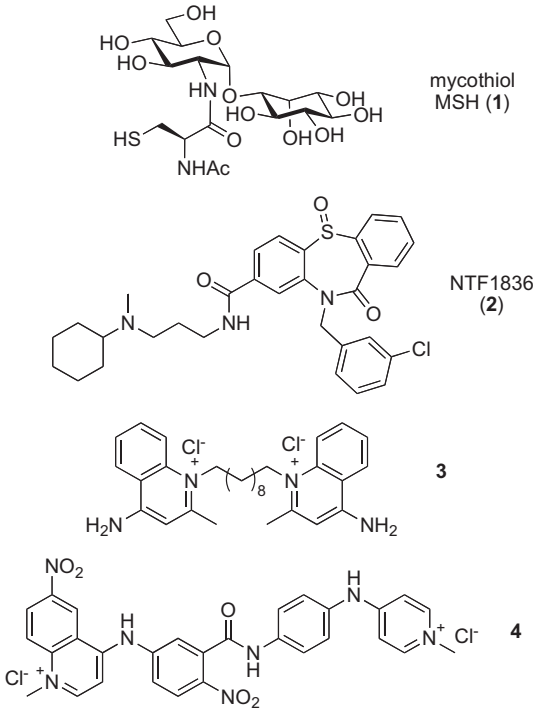


Figure 1. Structures of mycothiol (MSH, **1**) and MshC inhibitors NTF1836 (**2**), quinaldinium salt **3** and quinaldinium salt **4**.

to be identified. On the other hand, MshC has been the most studied enzyme involved in MSH biosynthesis. Based on this, we focused our efforts to identify inhibitors of MshC.

Efforts to study *M. tuberculosis* MshC have been hindered by difficulties in efficient purification of the active cloned protein via conventional His_6 affinity chromatography. The native untagged *M. tuberculosis* protein was successfully cloned and expressed in *M. smegmatis* but the purification was tedious.[18] Successful expression and affinity purification of three fusion protein constructs of *M. tuberculosis* MshC in *M. smegmatis* has been reported.[19] To date, the best results have been obtained using a maltose binding protein (MBP) fusion to generate low milligram quantities of the tagged *M. tuberculosis* MBP-MshC. Expression and purification of active His_6-tagged *M. smegmatis* MshC in *Escherichia coli* is less troublesome and high milligram amounts of this protein have been generated.[20]

Steady state kinetic studies[20] and positional isotopic exchange analysis[21] of *M. smegmatis* MshC were consistent with a Bi-Uni Uni-Bi ping-pong mechanism in which GlcN-Ins binds after formation of the bound cysteinyl-adenylate intermediate. Crystallization efforts with *M. smegmatis* MshC have so far been unsuccessful.[22] However, when the protein was treated with the potent inhibitor 5′-*O*-[*N*-(L-(cysteinyl)-sulfamoyl]adenosine (CSA) and then partially-proteolyzed with trypsin, crystallization was possible. In 2008, a 1.6 Å crystal structure was reported indicating that residues 289–293 containing the conserved KMSKS motif had been proteolyzed.[22] The structure identifies interactions of CSA with the protein, including binding of the thiolate group to the active site zinc residue.

The first MSH biosynthesis inhibitor identified, NTF1836 (**2**), was found in a screen for inhibitors of the native *M. tuberculosis* MshC.[18] Recently, a screen using *M. tuberculosis* MPB-MshC identified two quinaldinium derivatives **3** and **4** (Fig. 1) as micromolar inhibitors of MshC.[23] We now report on the optimization, synthesis and biological evaluation of **2**. These studies validate MshC as the primary target of **2** in *M. tuberculosis* as well as demonstrate efficacy of **2** against non-replicating *M. tuberculosis* using the phosphate buffered saline (PBS) carbon starvation model of dormancy.[24]

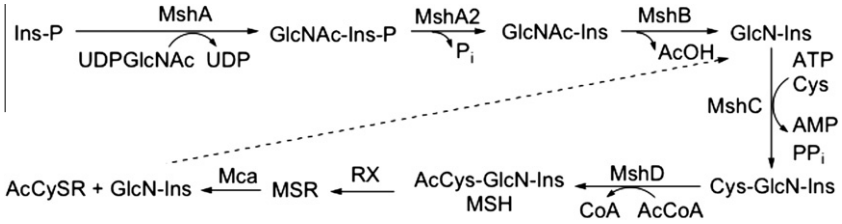
2. Results and discussion

2.1. Structure–activity relationship (SAR) studies

Our studies began by examining the structure–activity relationship (SAR) requirements of **2**. This began by obtaining a panel of 39 analogs, compounds **5**–**43** (Tables 1 and 2) that contained modifications within three positions R_1, R_2 and X.

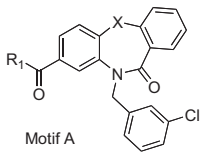
Two motifs were examined. The first (motif A, Table 1) focused on variation with the R_1 and X groups. The presence of a tri-substituted amine at the terminus of R_1 played a key role in the activity. Replacement of the amine with a sulfide as given in the comparison of **2** with **6** or **7** with **8** (Table 1) led to a complete loss of measurable activity. All of the compounds exhibiting measurable inhibition contained this amine terminus in R_1. The oxidation state of the sulfur atom X was also critical to activity. Sulfoxides (X = SO) were 5- to 15-fold better inhibitors than their corresponding sulfones (X = SO_2) as evident in the comparison of **7** with **9**, **10** with **11**, **12** with **13** or **14** with **16** (Table 1). All of the sulfoxides employed in this study were racemic mixtures of the two sulfoxide enantiomers and the reported IC_{50} values are composites of the contribution by each individual enantiomer. Only a limited number of the corresponding sulfides (X = S) were sufficiently soluble for study, however those that were, were less active than their corresponding sulfoxides (compare **5** with **2**, **15** with **14** or **18** with **17**, Table 1). In contrast, there was only a minor modification by the choice of the terminal alkyl groups in R_1. For instance, the activity of compounds containing common 3 carbon linked amine at R_1 and sulfoxide at X including **7**, **12**, **17**, **19**, and **21** displayed IC_{50} values that were within a factor of 1.5 from that for **2**. Compound **10** was less effective, possibly reflecting steric limit of the motif. Finally, compound **14** was similarly ineffective which may reflect the effect of the ether oxygen on the ionization state of the amino group.

The effect of the side chain group R_2 was also examined within the second motif (motif B, Table 2). Removal of the chloro-substituent from R_2 increased the IC_{50} value about fourfold in the

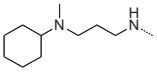
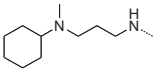
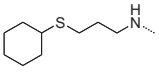
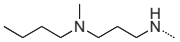
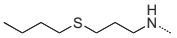
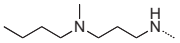
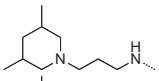
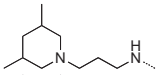
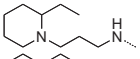
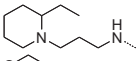
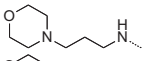
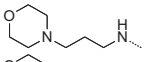
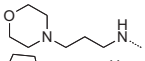
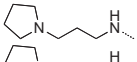
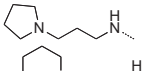
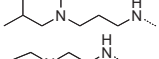
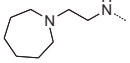
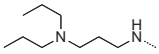
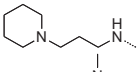
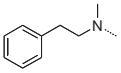
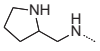


Scheme 1. Biosynthesis of mycothiol (AcCys-GlyN-Ins, MSH) arises through five steps catalyzed by the biosynthetic enzymes: MshA, MshA2 MshB, MshC and MshD. Once formed, MSH can react with select antibiotics (RX) to form mycothiol-*S*-conjugates (MSR). Cleavage of MSR by mycothiol-*S*-conjugate amidase (Mca) releases GlnN-Ins, which is recycled, and an equivalent of antibiotic modified as a mercapturic acid (AcCysSR).

Table 1
Variation of IC_{50} values for inhibition of MshC with modifications at X and R_1 in Motif A



Motif A

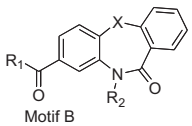
No.	R_1	X	IC_{50} (mM)
2		SO	0.085 ± 0.010
5		S	0.28 ± 0.03
6		SO	⩾1[a]
7		SO	0.11 ± 0.02
8		SO	>3[a]
9		SO_2	1.7 ± 0.3
10		SO	0.50 ± 0.06
11		SO_2	2.3 ± 0.7
12		SO	0.08 ± 0.02
13		SO_2	1.1 ± 0.3
14		SO	0.5 ± 0.1
15		S	0.8 ± 0.1
16		SO_2	~3
17		SO	0.10 ± 0.03
18		S	0.13 ± 0.03
19		SO	0.15 ± 0.03
20		SO	0.10 ± 0.02
21		SO	0.10 ± 0.05
22		S	0.32 ± 0.05
23		SO_2	>3
24		SO_2	2.5 ± 0.5

[a] From measurements at 50 μM and 100 μM.

sulfoxide series (compare **2** with **25**, Table 2) while comparable effects were not seen in the corresponding sulfones (compare **25** with **26**, Table 1). This observation suggests that the effects of X, R_1 and R_2 may not be cumulative.

While the trends observed in Table 1 were maintained, the second panel (Table 2) provided additional SAR data. For instance, removal of the aromatic ring in R_2 as in compounds **29**–**31** led to complete loss in activity. The set with R_1 = 4-chlorobenzyl **32**–**34** displayed very similar activities, being about three- to fourfold less effective than **2**. Here again, we observed a non-cumulative effect, as analogs **33**–**34** were screened as sulfones (X = SO_2). From

Table 2
Variation of IC_{50} values for inhibition of MshC with modifications at X, R_1, and R_2 in motif B



Motif B

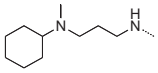
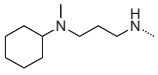
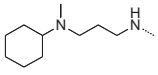
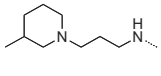
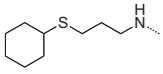
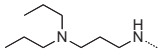
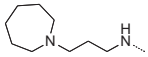
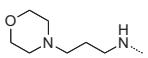
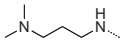
No.	R_1	R_2	X	IC_{50} (mM)
2			SO	0.085 ± 0.010
25			SO	0.3 ± 0.1
26			SO_2	0.5 ± 0.1
27			SO_2	1.7 ± 0.4
28			SO	>3
29			SO	>3
30			SO	⩾3
31			SO	>3
32			SO	0.34 ± 0.02
33			SO_2	0.33 ± 0.04
34			SO_2	0.25 ± 0.10
35			SO	0.71 ± 0.02
36			SO	>3
37			SO	>3
38			SO_2	0.8 ± 0.1
39			SO	>3
40			SO	~2
41			SO	>3
42			SO_2	1.4 ± 0.2

Table 2 (*continued*)

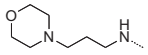
No.	R_1	R_2	X	IC_{50} (mM)
43			SO_2	>3

Table 1, one would expect that these materials would be 5- to 15-fold less active than **32**, however this was not the case (compounds **32**–**34** shared comparable activity).

Further screening, indicated that the 4-fluorobenzyl group in **35** was less active (fivefold) than the corresponding 3-chlorobenzyl derivative **19**. A larger (30-fold) loss was obtained when comparing derivatives **36** and **21**. Substitution of 3-chlorobenzyl by 2-fluorobenzyl in **37** resulted in a >35-fold loss in activity when compared to **2**. Incorporation of the 2,5-dimethylbenzyl group as R_1 in **41** and **43** resulted in no measurable inhibition whereas for **40** and **42** it led to just detectable inhibition.

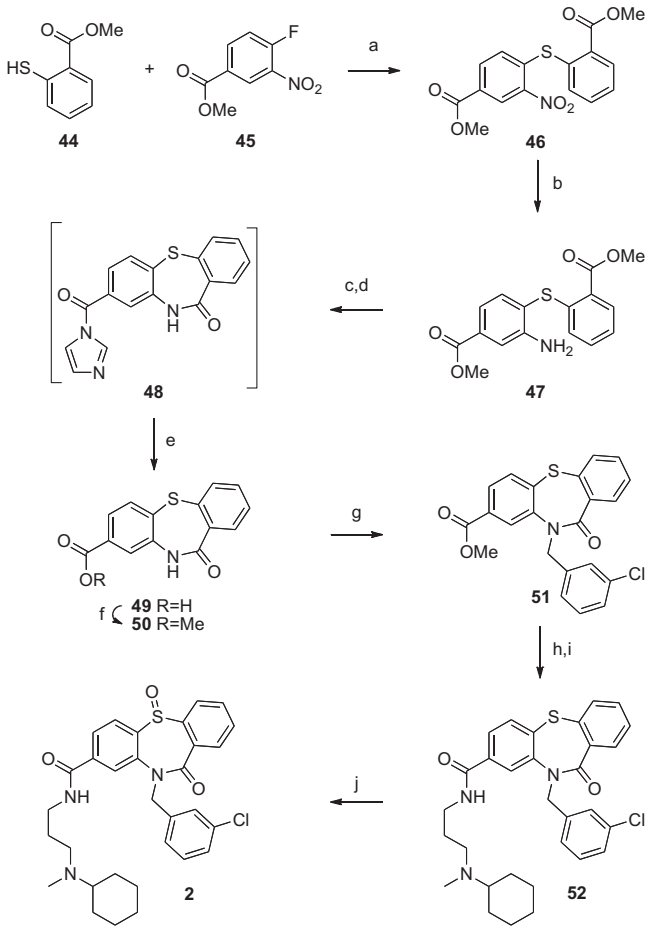
While we have yet to identify a substantially more active analog than **2**, these SAR studies indicate a clear reliance on the inclusion of a sulfoxide (X = SO) and presence of a amine functionality at the terminus of R_1 and an aryl group in R_2. Fortuitously, our initial screening efforts returned an analog whose activity was well defined. While these studies do not preclude the development of more active analogs, via more refined analog screening efforts, the observed SAR indication provide strong support for further evaluating NTF1836 (**2**). We turned our efforts to critically evaluate our ability to prepare **2**, validate its mode of action and examine its potential at treating resistant and non-replicating TB infections.

2.2. Chemical synthesis of NTF1836 (2)

While our SAR studies indicated a clear motif, two concerns remained. First, we had not independently verified the structure of **2** (compounds were commercially obtained and screened) and therefore we wanted to prepare synthetic material in house as a means of structure validation. Second, our planned biological studies required access to gram quantities of **2**. Based on these concerns, we developed an effective synthetic route to NTF1836 (**2**). After examining several approaches, the route depicted in Scheme 2 provided facile access to gram quantities of **2** in nine steps from commercial materials.

The synthesis began with a SNAr nucleophilic substitution to couple thiol **44** with arylfluoride **45** and provides yellow needles of mercaptan **46** after recrystallization. Reduction of **46** provided amine **47** in quantitative yield, which was, without purification, converted to thiazepinone **48** through a three-step process that began with hydrolysis of two methyl esters. Treatment of the resulting diacid with 1,1′-carbonyldiimidazole resulted in cyclization to acylimidazole intermediate **48**, which upon workup with 4-dimethylaminopyridine (DMAP) in dry MeOH afforded methyl ester **50** with traces of acid **49**. After optimization, purification using a combination of Dry Column Vacuum Chromatography (DCVC) and recrystallization provided gram quantities of **50** as a pure white powder. As of note, acid **49** obtained in this process could be converted to **50** by treatment with trimethylsilyldiazomethane.

At this stage ester **50** was ready for incorporation of the R_2 group. The protection of carboxylic acid **49** as its methyl ester **50** was key to facilitating this transformation. First, this conversion provided an effective means of purification as compound **50** was readily purified by recrystallization (the corresponding acid **49** could not be recrystallized). Second, it provided a more cost effective conversion to **2** by reducing the amount of 3-chlorobenzylbromide and *N*-1-cyclohexyl-*N*-1-methylpropane-1,3-diamine required in subsequent steps.



Scheme 2. Gram-scale synthesis of NTF1836 (**2**). Reagents and conditions: (a) Cs_2CO_3, DMF, 87%; (b) PtO_2, Pd–C, EtOAc, MeOH, 98%; (c) LiOH, THF, H_2O, rt; (d) 1,1-carbonydimidazole, DMF; (e) DMAP, MeOH, 83% over three steps from **47**; (f) trimethylsilyldiazomethane, THF, rt, 1 h; (g) NaH, 3-chloro-benzylbromide, DMF, 0 °C to rt, 79%; (h) LiOH, THF, H_2O, rt; (i) *N*-1-cyclohexyl-*N*-1-methylpropane-1,3-diamine, HATU, $EtN(^iPr)_2$, DMF, rt, 16 h; 81% over two steps from **49**; (j) *m*-CPBA, $NaHCO_3$, CH_2Cl_2, 65%.

After screening, we identified an effective three-step procedure to install R_1 and R_2. This began by treating **50** with NaH and 3-chlorobenzylbromide. The resulting methyl ester **51** was then hydrolyzed, treated with HATU, and coupled to *N*-1-cyclohexyl-*N*-1-methylpropane-1,3-diamine to deliver sulfide **52**. The latter process was optimally conducted in one operation. After screening a variety of conditions, the oxidation of sulfide **51** to the desired sulfoxide **2** was most effectively conducted by treatment with $NaHCO_3$ buffered *m*-CPBA. Using this procedure, we were able to provide gram quantities of **2** (detailed synthetic procedures are provided in Section 4.2) with a 29% overall yield from **45** using a process that required the use of one DCVC column and two Flash chromatographic purifications.

2.3. NTF1836 (2) kills growing *M. tuberculosis*

The sensitivity of actively growing *M. tuberculosis* to **2** was initially tested with the Erdman strain. A culture of mid-log phase *M. tuberculosis* Erdman was diluted to an OD_{600} of 0.08 and aliquots were incubated at 37 °C for 7 d with dilutions of **2**. In three experiments, **2** inhibited the growth of *M. tuberculosis* in a dose dependent manner as measured by both change in optical density and by plating for viable bacteria (Fig. 2). Inhibition of growth was apparent at 20 μM **2** and increased at higher concentrations.

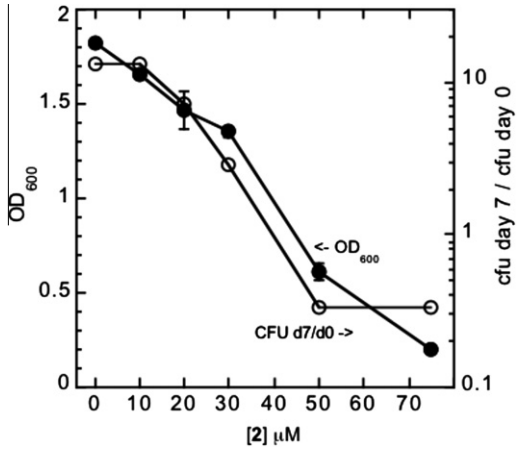


Figure 2. Inhibition of exponentially growing *M. tuberculosis* Erdman as monitored by a 7-day change in optical density at 600 nm (●; initial value 0.08) and change in viability (cfu day 7/cfu day 0; ○) as a function of the concentration of **2**. Error bars show mean and standard deviation of three determinations.

A greater sensitivity to **2** was noted in the plating results with 50% loss of viability occurring at 20 μM **2** whereas a 50% decline in OD_{600} occurred between 30 μM and 50 μM **2**. At higher concentrations of **2** (50 or 75 μM) the plating results demonstrated a 97% loss in viability compared with the zero drug control, the viable cells amounting to only one-third of those present in the day 0 inoculum.

Using a 96 well plate format, the capacity of **2** to inhibit patient isolates of *M. tuberculosis* was analyzed using four patient strains. A 95% inhibition was observed with a 110 μM concentration of **2** (Table 3). The activity of five other analogs of **2** was also tested and 4 of the 5 analogs (**12**, **18**, **19** and **20** inhibited the growth *M. tuberculosis* at similar dosages to that of **2** (Table 3). Toxicity toward Vero cells was determined as an indicator of potential human toxicity (Table 3). The results indicate that these compounds were as toxic to mammalian cells as to *M. tuberculosis*. The MIC_{95} values for these compounds were more than two orders of magnitude higher than that of rifampin.

2.4. NTF1836 (2) inhibits MSH biosynthesis in *M. tuberculosis*

If NTF1836 (**2**) functions by inhibiting MshC, addition of **2** to *M. tuberculosis* should prevent the production of Cys-GlcN-Ins and MSH, and lead to the accumulation of GlcN-Ins in cells (Scheme 1). In order to test this, samples were prepared from *M. tuberculosis* Erdman cultured for 7 d with varying dilutions of **2** and the cells were analyzed for MSH and GlcN-Ins content. The cellular level of Cys-GlcN-Ins was below detectable limits in *M. tuberculosis* Erdman[25] and was not quantitated. In two independent experiments, we observed that at 20–75 μM **2** resulted in a fourfold decline in MSH level accompanied by a ninefold increase in the level of GlcN-Ins (Fig. 3). Since **2** was toxic to *M. tuberculosis* in this concentration range (Fig. 2), the decrease in MSH level might be due to cell death rather than inhibition of MshC. However, the accumulation of GlcN-Ins was expected for MshC inhibition and was unexpected in killed cells. The pattern of GlcN-Ins and MSH changes with increasing concentration of **2** was consistent with inhibition at the MshC step of mycothiol biosynthesis and validates MshC as a target for **2** (Scheme 1).

2.5. NTF1836 (2) kills nonreplicating *M. tuberculosis*

In order to examine the effect of **2** on nonreplicating *M. tuberculosis*, we used a nutrient starvation model to generate *M. tuberculosis* in the nonreplicating state.[24] Bacteria were cultured for 6 weeks in PBS at which time dilutions of **2** were added and the cultures were incubated for an additional 15 d. Samples were then assessed for viable bacteria by plating. There was a 99% loss in viability at a dose of approximately 90 μM **2**, indicating that **2** was capable of killing nonreplicating *M. tuberculosis* (Fig. 4). Two other experiments produced similar degrees of killing of the nonreplicating bacteria.

2.6. Mycothiol biosynthesis as a drug target in *M. tuberculosis*

The essentiality of MSH and MSH biosynthesis in *M. tuberculosis* is controversial and likely depends on the specific growth conditions. The earliest data was obtained with saturating transposon mutagenesis, which indicated that *mshC* and *mtr* (*Rv2855*, mycothiol disulfide reductase) are essential for in vitro growth of *M. tuberculosis* H37Rv,[26] indicating that MSH itself was essential. We have constructed two MSH biosynthesis mutants in *M. tuberculosis*

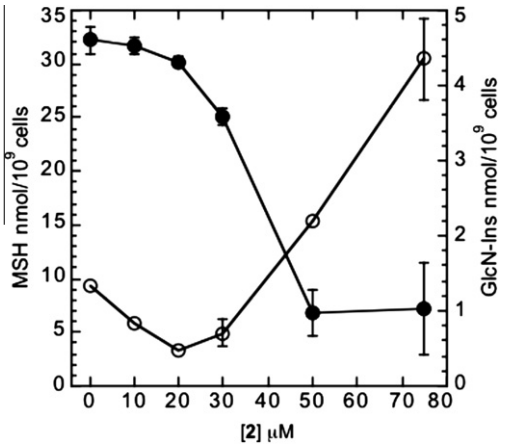


Figure 3. Concentration of MSH (●) and GlcN-Ins (○) following 7 day culture of *M. tuberculosis* Erdman in Middlebrook 7H9 medium containing varying concentrations of **2**. Error bars show mean and range of duplicate samples.

Table 3
Concentration (μM) of compounds producing 95% inhibition (MIC_{95}) of *M. tuberculosis* human isolates, or 90% (MIC_{90}) Vero cell toxicity

M. tuberculosis isolate	**2**	**12**	**17**	**18**	**19**	**20**	Rifampin
CDC1551	110	32	220	110	55	110	—
VA1215[a]	110	110	220	110	55	110	—
UCSD14768J[a]	110	55	220	110	55	110	—
VA1345[a]	110	55	220	110	55	110	—
Erdman (control strain)	28–55	55	55	55	55	28	0.24
Vero Cell toxicity[b] (MIC_{90})	50	100	50	50	100	100	—

[a] Two passages in vitro after isolation from patient.
[b] Vero Cell toxicity determined at 48 h using the neutral red assay.

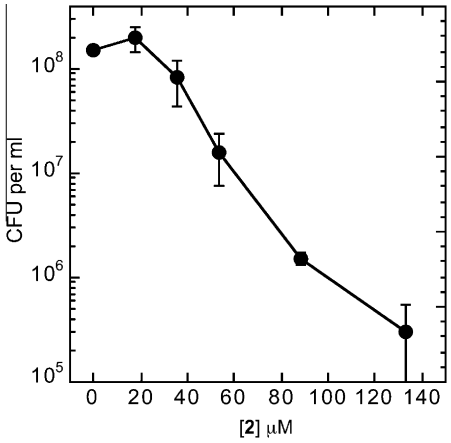


Figure 4. Viability of nonreplicating, persistent *M. tuberculosis* Erdman generated by 6 week incubation in PBS followed by addition of **2** to various concentrations, subsequent incubation for an additional 15 d, and determination of viability by plating. Error bars show mean and range of duplicate samples.

Erdman, both of which were very growth inhibited unless exogenous catalase was supplied.[14,25] The *mshB* and *mshD* mutants did produce limited amounts of MSH or related compounds and were not complete MSH knockout mutants. Studies of *M. smegmatis* indicated that *mshA* and *mshC* mutants would be complete MSH knockout mutants if produced in *M. tuberculosis*. We were unable to produce an *mshC* mutant in *M. tuberculosis* Erdman unless a second copy of the gene was present.[16] More recently Jacobs and colleagues have produced an *mshA* mutant in *M. tuberculosis* which is reported to produce no MSH and requires exogenous catalase for growth, indicating a sensitivity to oxygen.[27] Paradoxically, despite the in vitro growth fault this *mshA* mutant was not attenuated for growth in mice. Recently, a MshA mutant in *M. smegmatis* was found to produce very large amounts of organic hydroperoxide reductase (Ohr) and also to produce ~20-fold higher levels of ergothioneine.[28] A similar overproduction of ergothioneine was found in other MSH mutants in *M. smegmatis*. Although Ohr is not encoded by the *M. tuberculosis* genome the ergothioneine biosynthesis genes are present and enhanced production of ergothioneine could partially compensate for the loss of MSH in an *M. tuberculosis mshA* mutant.

This study provides chemical support for the essentiality of MshC and mycothiol in *M. tuberculosis*. MshC inhibitor **2** was found to kill actively growing and non-replicating *M. tuberculosis* in the same concentration range as the inhibition of MshC. Inhibitor **2** was also shown to specifically inhibit MshC in a concentration dependent manner in actively growing *M. tuberculosis* although other essential targets for **2** cannot be ruled out. This study indicates that MshC is a druggable and essential protein target for *M. tuberculosis*.

3. Conclusion

A survey of compounds structurally related to **2** identified several features important for the activity of **2** to inhibit MshC but did not identify inhibitors with substantially greater potency. A chemical synthesis of **2** was reported which provided material for more detailed testing. The present study establishes that **2** was lethal to both growing and non-replicating persistent *M. tuberculosis*. Cell killing by **2** was accompanied by a fall in cellular MSH level and an increase in the cellular level of GlcN-Ins, the substrate of MshC, supporting the proposed mechanism of action. While the present results support the validity of MshC as a target, a more potent and less toxic inhibitor of MshC than **2** was clearly required. The general synthetic route described can provide the basis for production of a second generation of MshC inhibitors based upon **2** and structural modeling based upon the MshC structure[22] may provide the basis for design of new MshC inhibitors.

4. Experimental

4.1. General methods

Samples (1–10 mg) of compounds **2** and **6**–**43** were purchased from ChemDiv Inc. for screening purposes. Conventional conditions were used for the chemical synthesis of **2**. Unless stated otherwise, reactions were performed in flame-dried glassware under a positive pressure of Ar using freshly distilled solvents. Tetrahydrofuran (THF), CH_2Cl_2, MeOH, and toluene were freshly purified using a Seca Solvent System (GlassContour, Laguna Beach, CA) and transferred under an Ar atmosphere. $EtN(N^i\text{-}Pr)_2$ and Et_3N were distilled from ninhydrin, dried with Na_2SO_4, redistilled from Na, and stored under Ar over 4 Å mol sieves. Thin-layer chromatography (TLC) was accomplished using EMD 60 F254 pre-coated plates (0.25 mm). Visualization was performed by UV absorbance (254 or 365 nm), iodine on silica gel, or by charring after treatment with *p*-anisaldehyde or phosphomolybdic acid. All solvent mixtures were reported as (v/v) unless noted otherwise. Flash chromatography and dry column vacuum chromatography (DCVC) were performed using EMD silica gel with 40–63 μm particle size, 40 or 60 Å pore size and 25–40 μm particle size, 60 Å pore size, respectively. NMR spectra were recorded on JOEL ECA 500 or Varian VX 500 MHz instruments. Chemical shifts were reported in parts per million (ppm) on δ scale relative to solvent $CDCl_3$ (δ 7.26 ppm), CD_3OD (δ 4.89 ppm), or D_2O (δ 4.80 ppm) for ^1H and $CDCl_3$ (δ 77.00 ppm) or CD_3OD (δ 49.15 ppm) for ^{13}C. Data for ^1H NMR were reported as follows: chemical shift, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, br = broad), integration, and coupling constants. All ^{13}C NMR spectra were recorded with complete proton decoupling. When applicable, 1D spectra were recorded using the standard protocols with proton integrations measured at decreased 30° pulse angles and increased 5 s relaxation times. Spectral assignments were confirmed by homonuclear COSY, gradient gCOSY and heteronuclear gradient HSQC experiments. Spectra were displayed and plotted using MestraNova (MestreLab Research). Low-resolution mass spectral analyses were conducted by LC–MS on a Waters model 600 HPLC with a Waters model 486 UV–vis detector and a Waters-Micromass ZMD monoquad mass detector. High-resolution mass spectral analyses were conducted on a ThermoFinnigan MAT900XL with reference to perfluorokerosene. Biological methods and materials were denoted within each study.

4.2. Chemical synthesis of NTF1836 (2)

4.2.1. Methyl-4-((2-(methoxycarbonyl)phenyl)thio)-3-nitrobenzoate (46)

A mixture of methyl 4-fluoro-3-nitrobenzoate **45** (14. 1 g, 0.0706 mol) and methyl 2-mercaptobenzoate **44** (10 mL, 0.0727 mol) was dissolved in 120 mL of anhydrous DMF. Finely ground Cs_2CO_3 (30.9 g, 0.950 mol) was added at rt. After mixing for 30 min, the suspension was stirred at 40 °C for 2 h. After cooling to rt, the mixture was diluted with CH_2Cl_2 (200 mL) and washed with water (50 mL) and brine (50 mL) and dried (Na_2SO_4). The crude product was recrystallized from EtOAc/hexanes to afford 21.42 g (87%) of long yellow needles **46**, mp 177–178 °C; ^1H NMR (500 MHz, $CDCl_3$) δ 9.22 (s, 1H); 8.12 (dt, J = 3.8, 5.2 Hz, 1H); 8.09 (d, J = 10.6 Hz, 1H); 7.71–6.59 (m, 3H), 6.81 (d, J = 10.7 Hz, 1H), 3.08 (s, 3H), 2.89 (s, 3H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 189.1,

187.0, 162.1, 161.3, 152.8, 150.9, 147.5, 147.0, 145.1, 144.4, 143.9, 142.7, 140.1, 139.5, 41.0, 40.9; HREIMS $[M+Na]^+$ *m/z*: 370.0389 (calcd for $C_{16}H_{13}NNaO_6S$, 370.0361).

4.2.2. Methyl 3-amino-4-((2-(methoxycarbonyl)phenyl)thio)-benzoate (47)

PtO_2 (0.3 g) and Pd on carbon (5.0 g) were suspended in a mixture of compound **46** (21.42 g, 0.0669 mmol) in EtOAc (300 mL) and MeOH (300 mL). The flask was degassed, charged with H_2 and stirred under a constant atmosphere of H_2 (ballon of H_2). After 12 h, the reaction was complete as evident by TLC analyses. The reaction degassed, filled with N_2 and filtered through a 200 g plug of silica gel eluting with 1:10 MeOH/EtOAc (600 mL). The elutant was concentrated on a rotary evaporator to afford 19.12 g (98%) of an off-white powder **47**, mp 92–95 °C (dec), ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.19 (dd, J = 1.9, 9.8 Hz, 1H); 7.56 (d, J = 10.0 Hz, 1H); 7.51 (d, J = 2.1 Hz, 1H), 7.42 (dd, J = 2.2, 1.0 Hz, 1H), 7.24 (m, 1H), 7.10 (dd, J = 5.1, 14.2 Hz, 1H), 6.55 (d, J = 10.0 Hz, 1H), 3.70 (br s, 2H, NH), 3.12 (s, 3H), 3.06 (s, 3H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 189.5, 189.4, 167.4, 156.3, 153.2, 147.1, 146.8, 145.3, 139.0, 138.3, 136.7, 130.1, 129.7, 125.9, 46.3; HREIMS $[M+H]^+$ *m/z*: 318.0870 (calcd for $C_{16}H_{16}NO_4S$, 318.0800).

4.2.3. Methyl 11-oxo-10,11-dihydrodibenzo[*b,f*][1,4]-thiazepine-8-carboxylate (50)

Amine **47** (19.12 g, 0.0602 mol) was dissolved in THF (425 mL) containing H_2O (75 mL). The mixture was cooled to 0 °C and $LiOH{\cdot}H_2O$ (7.58 g, 0.181 mol) was added in one portion. After 1 h, the reaction was warmed to rt. HPLC or TLC monitoring indicated that hydrolysis was complete after 36 h at rt. The pH was adjusted to 4.0 by the addition of 2 M HCl. The remaining mixture was dried to an off white powder by rotary evaporation followed by lyophilization with MeOH (6 × 300 mL). The resulting powder was further dried by rotary evaporation with toluene (3 × 300 mL) and suspended in DMF (400 mL). After cooling to −20 °C, 1,1′-carbonyl-diimidazole (48.84 g, 0.301 mol) was added in portions over 3 h. The flask was equipped with a drying tube filled Drierite. The reaction was warmed to rt over 2 h. After 24 h of vigorous stirring at rt, reaction was cooled to 0 °C and methanol (200 mL) was added. The reaction was warmed over 1 h to rt and stirred for an addition 12 h. The contents of the flask were then diluted in EtOAc (200 mL) and filtered with 1:10 MeOH/EtOAc (2 L) through a DCVC column charged with 250 g of Celite (packed at top of column) and 500 g of silica gel (packed at bottom of column). The elutant was concentrated on a rotary evaporator. Recrystallization from a minimal amount hot toluene followed by slow cooling to rt and storage at 4 °C for 24 h provided 14.21 g (83%) of methyl ester **50** as a white powder. The remaining mother liquor contained a mixture of **50** and its corresponding acid **49** that could be converted to **50** (1.23 g, 6% yield) by treatment with 0.5 M $TMSCH_2N_2$ in THF. This recycled material was not tabulated in the yield. Mp 179–181 °C, ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.48 (s, 1H, NH), 7.85 (dd, J = 1.9, 7.4 Hz, 1H); 7.79 (d, J = 1.7 Hz, 1H); 7.77 (dd, J = 1.7, 8.0 Hz, 1H), 7.63 (d, J = 8.0 Hz, 1H), 7.49 (dd, J = 1.5, 7.5 Hz, 1H), 7.40 (ddd, J = 1.6, 8.3, 11.4 Hz, 1H), 7.39 (ddd, J = 1.6, 8.4, 8.7 Hz, 1H), 3.89 (s, 3H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 170.2. 166.3, 140.1, 137.2, 136.8, 136.0, 133.5, 133.0, 132.5, 132.5, 132.0, 129.6, 127.3, 124.2, 53.1; HREIMS $[M+Na]^+$ *m/z*: 308.0489 (calcd for $C_{15}H_{11}NNaO_3S$, 308.0357).

4.2.4. Methyl 10-(3-chlorobenzyl)-11-oxo-10,11-dihydrodibenzo[*b,f*][1,4]thiazepine-8-carboxylate (51)

NaH washed free of oil (1.49 g, 0.0622 mol) was added carefully in portions to ester **50** (14.21, 0.0498 mol) dissolved in DMF (200 mL) under Ar at −20 °C. After warming to 5 °C over 1 h, the resulting suspension was cooled to −20 °C and 3-chlorobenzylbromide (12.79 g, 0.0622 mmol) in DMF (40 mL) was added drop wise over 30 min. The reaction was then warmed over 2 h to 0 °C, stirred at 0 °C for 3 h, warmed to rt over 2 h, then stirred at rt for 12 h. Care was taken to stir vigorously and warm the reaction slowly. The reaction was cooled to 0 °C and terminated by the addition of 5% aq MeOH (100 mL). The mixture was then filtered with EtOAc (800 mL) through DCVC column charged with 100 g of Celite (packed at top of column) and 150 g of silica gel (packed at bottom of column). The eluted material was concentrated on a rotary evaporator and recrystallization from toluene to afford 16.21 g (79%) of **51** as a white powder. Mp 168–171 °C, ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.00 (d, J = 1.7 Hz, 1H); 7.76 (dd, J = 1.7, 7.6 Hz, 1H); 7.73 (ddd, J = 0.6, 1.8, 8.1 Hz, 1H), 7.63 (d, J = 8.1 Hz, 1H), 7.44 (m, 1H), 7.41 (br s, 1H), 7.35 (dddd, J = 0.6, 1.6, 5.2, 12.8 Hz, 1H), 7.32 (dddd, J = 0.6, 1.6, 7.5, 14.8 Hz, 1H), 7.27 (m, 2H), 7.20 (m, 2H), 5.80 (d, J = 15.2 Hz, 1H), 4.91 (d, J = 15.2 Hz, 1H), 3.89 (s, 3H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 169.9, 165.8, 143.5, 141.8, 138.9, 138.3, 137.7, 134.6, 133.6, 131.8, 131.7, 131.4, 131.4, 129.9, 129.2, 128.3, 127.8, 127.5, 126.8, 126.3, 54.2, 52.7; HREIMS $[M+Na]^+$ *m/z*: 432.0527 (calcd for $C_{22}H_{16}ClNNaO_3S$, 432.0437).

4.2.5. 10-(3-Chlorobenzyl)-*N*-(3-(cyclohexyl(methyl)amino)-propyl)-11-oxo-10,11 dihydrodibenzo[*b,f*][1,4]thiazepine-8-carboxamide (52)

Ester **51** (16.12 g, 0.0393 mol) was dissolved in THF (170 mL) containing H_2O (30 mL). After cooling to 0 °C, $LiOH{\cdot}H_2O$ (4.95 g, 0.117 mol) was added in one portion. After 2 h at 0 °C, the reaction was warmed to rt. HPLC or TLC indicated that hydrolysis was complete after 23 h at which point the pH was adjusted to 7.0 by the addition of 2 M HCl. The remaining mixture was dried by sequential rotary evaporation followed by lyophilization with MeOH (6 × 100 mL) resulting in yellow wax. The wax was further dried by rotary evaporation of toluene (3 × 120 mL). The resulting material was suspended in $EtN(^iPr)_2$ (20.55 mL, 0.117 mmol) in DMF (150 mL). After cooling to 0 °C, HATU (22.42 g, 0.0590 mol) was in three portions over 15 min. The reaction was warmed to rt over 1 h. After an additional 2 h at rt, *N*-1-cyclohexyl-*N*-1-methylpropane-1,3-diamine (7.00 g, 0.0411 mmol) was added in 10 mL DMF. After 18 h of vigorous stirring at rt, the mixture was diluted in EtOAc (200 mL) and filtered with a mixture of 1:5 MeOH/EtOAc (1 L) through a DCVC column charged with 100 g of Celite (packed at top of column) and 100 g of silica gel (packed at bottom of column). The elutant was concentrated on a rotary evaporator. Flash chromatography (2:1 Hx/EtOAc to 10:1 MeOH/EtOAc) afforded 5.68 g (81%) of clear wax **52**. ^{1}H NMR (500 MHz, CD_3OD) δ 7.96 (d, J = 1.9 Hz, 1H), 7.65 (m, 2H); 7.56 (dd, J = 1.9, 8.1 Hz, 1H), 7.48 (m, 1H), 7.40 (m, 1H), 7.38 (m, 2H), 7.29 (dd, J = 1.5, 7.4 Hz, 1H), 7.22 (t, J = 7.6 Hz, 1H), 7.18 (m, 1H) 5.84 (d, J = 15.5 Hz, 1H), 4.95 (d, J = 15.5 Hz, 1H), 3.44 (td, J = 1.7, 6.4 Hz, 2H), 3.20 (ddt, J = 3.4, 11.9, 14.3 Hz, 2H), 3.13 (t, J = 7.5 Hz, 2H), 2.76 (s, 3H), 1.98 (m, 4H), 1.88 (m, 2H), 1.67 (m, 1H), 1.47 (dq, J = 2.8, 12.9 Hz, 2H), 1.34 (tq, J = 2.8, 12.1 Hz, 2H), 1.17 (tq, J = 3.6, 13.0 Hz, 2H 1H); ^{13}C NMR (125 MHz, CD_3OD) δ 171.1, 169.1, 144.4, 141.7, 140.5, 139.7, 138.9, 137.0, 135.4, 134.6, 132.8, 132.5, 132.4, 131.1, 130.4, 129.2, 128.7, 127.5, 126.6, 126.4, 66.5, 54.6, 52.5, 38.9, 37.1, 36.6, 31.8, 27.8, 26.3, 26.2, 26.1; HREIMS $[M+Na]^+$ *m/z*: 570.1917 (calcd for $C_{31}H_{34}ClN_3NaO_2S$, 570.1958).

4.2.6. *N*-[3-(Cyclohexylmethylamino)propyl]-10-[(3-chlorophenyl)methyl]-10,11-dihydro-11-oxo-dibenzo-[*b,f*][1,4]-thiazepine-8-carboxamide-5-oxide (2)

Sulfide **52** (2.25 g, 4.09 mmol) was dissolved in CH_2Cl_2 (200 mL) containing $NaHCO_3$ (1.72 g, 20.43 mmol). *m*-CPBA was purified by dissolving commercial material in CH_2Cl_2 and washing repetitively with a 10% w/v solution of $NaHCO_3$, brine, and then drying by rotary evaporation. A sample of freshly purified *m*-CPBA (0.78 g,

4.50 mmol) was added as a solid to the cooled mixture of 50 and stirred for 2 h at −78 °C. The solution was then warmed over 2 h to 0 °C at which point 0.05 M NaOH (100 mL) was added, and the mixture was extracted with CH_2Cl_2 (3 × 200 mL). The combined organic layers were then washed with 0.05 M NaOH (50 mL) and brine (100 mL), dried with Na_2SO_4, and concentrated by rotary evaporation. Flash chromatography (2:1 Hx/EtOAc to 10:1 MeOH/EtOAc) afforded 1.50 g (65%) as an off-white powder. Mp 141–143 °C, ^{1}H NMR (500 MHz, CD_3OD) δ 8.05 (m, 1H); 7.85 (dt, J = 1.7, 8.1 Hz, 1H); 7.81 (d, J = 7.6 Hz, 1H); 7.75–7.66 (m, 3H), 7.57 (m, 1H), 7.40 (br s, 1H), 7.33–7.26 (m, 3H), 5.86 (d, J = 15.3 Hz, 1H), 4.93 (d, J = 15.5 Hz, 1H), 3.47 (m, 2H), 3.24 (m, 2H), 3.08 (m, 1H), 2.78 (s, 3H), 2.05 (m, 4H), 1.89 (d, J = 13.0 Hz 2H), 1.69 (d, J = 14.1, Hz, 1H), 1.55–1.34 (m, 3H), 1.19 (m, 2H); ^{13}C NMR (125 MHz, CD_3OD) δ 168.7, 167.2, 149.4, 149.0, 140.3, 138.9, 136.9, 135.6, 133.9, 132.5, 132.3, 131.5, 132.42, 129.7, 129.6, 129.2, 128.0, 127.8, 126.8, 122.4, 120.4, 66.5, 53.7, 52.5, 38.1, 36.5, 36.5, 28.8, 26.7, 26.2, 26.1, 26.0; HREIMS $[M+Na]^+$ m/z: 586.1929 (calcd for $C_{31}H_{34}ClN_3NaO_3S$, 586.1907).

4.3. Biological evaluation

4.3.1. Measurement of MSH and GlcN-Ins levels

MSH from *M. tuberculosis* cell extracts was determined by reaction with monobromobimane (Invitrogen) and quantitation by HPLC analysis with fluorescent detection as previously described.[14] GlcN-Ins was determined in *M. tuberculosis* cell extracts using the AccQ Tag amine reagent (Waters) as previously described.[14]

4.3.2. Determination of IC_{50} values for MshC

The MshC activity was assayed by the production of Cys-GlcN-Ins from 100 μM Cys, 100 μM ATP, 50 μM GlcN-Ins and recombinant *M. tuberculosis* MshC. The Cys-GlcN-Ins was quantitated by HPLC with fluorescence detection after reaction with monobromobimane, as previously described.[29] Inhibitors were dissolved in DMSO and were added at 50, 100, 200 and 400 μM to the substrate mix prior to the addition of MshC. The DMSO level was limited to 5% below which the activity of MshC was unaffected. Assays with visible inhibitor precipitation were discarded. The IC_{50} values were determined from a linear fit of the ratio of MshC activity without inhibitor to that with inhibitor.

4.3.3. Mycobacterium strains and culture

M. tuberculosis Erdman (ATCC 35801) and *M. tuberculosis* CDC1551 were obtained from the American Type Culture Collection (ATCC) and the Centers for Disease Control (CDC), respectively. Human isolates of *M. tuberculosis* were obtained from UC San Diego Medical Center and the Veterans Administration Hospital in La Jolla, CA via Don Guiney. Unless otherwise stated *M. tuberculosis* strains were cultured in Middlebrook 7H9 media with 0.05% Tween 80 and ADS supplement as described previously.[14]

4.3.4. Inhibition of *M. tuberculosis* growth by 2

M. tuberculosis Erdman and patient isolates were grown in Middlebrook 7H9 + 10% ADS (albumin–dextrose–saline) to mid-log phase and the bacteria were adjusted to an OD_{600} of 0.08 in fresh 7H9 + ADS.[14] Varying amounts of compound **2** from 10 μM to 75 μM from a 23 mM stock solution in DMSO were added to 8 mL aliquots of the bacteria that were then incubated in vented T-25 flasks for 7 d at 37 °C. Duplicate samples were prepared including control samples that contained an equivalent amount of DMSO as was present in the 75 μM sample. After 7 d, samples were taken for measurement of growth by OD_{600}, of viability by plating on Middlebrook 7H9 + OADC agar plates and quantitation of MSH and GlcN-Ins as described above.[14] The effect of each compound on growth was also analyzed using a 96 well plate assay. Dilutions of drug were incubated with 200 μL of bacteria that had been adjusted to an OD_{600} of 0.100. The plate was incubated at 37 °C until the wells containing the medium plus DMSO control were completely confluent with bacteria, usually 8–10 d. The dilution of drug that inhibited approximately 95% growth as determined visually was noted.

4.3.5. Inhibition of non-replicating *M. tuberculosis* by 2

In order to produce non-replicating bacteria, *M. tuberculosis* were cultured under nutrient starvation conditions as described by Betts[24] with the addition of 0.05% Tween 80 to the PBS to limit the sticking and clumping of bacteria. A culture of *M. tuberculosis* Erdman was grown in Middlebrook 7H9 plus ADS to mid-log phase and was then washed twice with 0.05% Tween 80 in PBS. After washing, the bacteria were re-suspended in their original volume with 0.05% Tween 80 in PBS (OD_{600} = 0.6–0.8) and 15 mL aliquots were incubated at 37 °C in 50 mL polypropylene tubes (Fisher) with the cap tightened. The tubes were opened weekly for cell viability counts. After 6 weeks, dilutions of **2** were added to the tubes, which were incubated for an additional 15 d. The number of viable bacteria was determined by plating serial dilutions on Middlebrook 7H9 agar medium with OADC (Difco).

4.3.6. Cytotoxicity of compounds to Vero cells

The toxicity of the compounds in Table 3 was tested using the neutral red assay for Vero cell cytotoxicity as previously described[30] with minor modifications. African green monkey Vero-E6 cells were seeded at 10^4 cells in 200 μL of DMEM medium with 10% fetal calf serum. Compounds were added to the medium at 25, 50, 100, and 200 μM (DMSO <2%) and the cells incubated for 2 d at 37 °C. The medium was removed and replaced with medium containing 40 μg/mL neutral red and then incubated for 3 h for uptake of the dye. The medium was removed and the cells quickly washed with 0.5% formaldehyde with 1% $CaCl_2$. The wells were filled with 200 μL of 1% HOAc in 50% aq EtOH. After 10 min, the microtiter plate was read at 540 nm. The results were expressed as the test compound concentration required to reduce the cell number by 1 log (MIC_{90}).

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Supplementary data

Supplementary data (copies of NMR spectra on NTF1836 (**2**) and select synthetic intermediates have been provided) associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2011.05.028.

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