Published in final edited form as:

Org Biomol Chem. 2016 July 07; 14(25): 6119–6133. doi:10.1039/c6ob00821f.

Synthesis and evaluation of new 2-aminothiophenes against *Mycobacterium tuberculosis*

Sandeep Thanna^{#a}, Susan E. Knudson^{#b}, Anna Grzegorzewicz^{#b}, Sunayana Kapil^a, Christopher M. Goins^a, Donald R. Ronning^a, Mary Jackson^{b,*}, Richard A. Slayden^{b,*}, and Steven J. Sucheck^{a,*}

^aDepartment of Chemistry and Biochemistry, The University of Toledo, 2801 W. Bancroft Street, Toledo, OH, USA 43606

^bMycobacteria Research Laboratories, Department of Microbiology, Immunology and Pathology, Colorado State University, Fort Collins, CO 80523

[#] These authors contributed equally to this work.

Abstract

Tuberculosis (TB) and its drug resistant forms kills more people than any other infectious disease. This fact emphasizes the need to identify new drugs to treat TB. 2-Aminothiophenes (2AT) have been reported to inhibit Pks13, a validated anti-TB drug target. We synthesized a library of 42 2AT compounds. Among these, compound **33** showed remarkable potency against *Mycobacterium tuberculosis* (*Mtb*) H37R_V (MIC = 0.23 μ M) and showed an impressive potency (MIC = 0.20-0.44 μ M) against *Mtb* strains resistant to isoniazid, rifampicin and fluoroquinolones. The site of action for the compound **33** is presumed to be Pks13 or an earlier enzyme in the mycolic acid biosynthetic pathway. This inference is based on structural similarity of the compound **33** with known Pks13 inhibitors, which is corroborated by mycolic acid biosynthesis studies showing that the compound strongly inhibits the biosynthesis of all forms of mycolic acid in *Mtb*. In summary, these studies suggest **33** represents a promising anti-TB lead that exhibits activity well below toxicity to human monocytic cells.

Introduction

Mycobacterium tuberculosis (*Mtb*) is the causative agent of tuberculosis (TB). TB ranks first for the number of deaths in humans caused by an infectious agent.¹ In 2013 there were 9.0 million active cases of TB. Of these, 0.48 case million where multidrug-resistant TB (MDR-TB). It is notable that the number of MDR-TB cases tripled from 2009 to 2013. This increase in MDR-TB and extensively drug-resistant TB (XDR-TB) has drawn worldwide attention. Furthermore, HIV-TB co-infection is a serious problem in countries where TB is

* mary.jackson@colostate.edu, richard.slayden@colostate.edu, steve.suchek@utoledo.edu.

†Electronic supplementary information (ESI) available: Detailed experimental procedures, and ¹H and ¹³C NMR spectra available for new compounds

Author contribution

Contributed compound design and synthesis: S.J.S., T.S. and S.K. Performed growth inhibition studies: S.E.K. and R.A.S. Performed mycolic acid biosynthesis studies and analysis: A.G. and M.J. Performed Ag85C inhibition studies: C.M.G. and D.R.R.

endemic. HIV positive patients are 26-31 times more likely to develop active TB in comparison to an HIV negative person. Overall, 1.5 million people die every year from this disease.

It has been difficult to eradicate TB even though it is treatable. Treatment for TB takes at least 6 months and sometimes up to 2 years. The basic 6 month therapy involves two months of an intensive phase using the quadruple drug regimen consisting of Isoniazid (INH), Rifampicin (RIF), Ethambutol (EMB) and Pyrazinamide (PZA) followed by a 4 month continuous phase consisting of INH and RIF. Often, patients do not comply with the entire regimen due to the high numbers of pills/day and side-effects. The discontinuation of treatment allows resistance to develop. Therefore, there is an immediate need for drugs with improved efficacy and reduced toxicity.

Mtb has an unusual cell envelope that contains a thick hydrophobic layer which differs from typical Gram positive (G+) and Gram negative (G-) bacteria making it resistant to many broad-spectrum drugs that normally act on G+ and G- bacteria. The main constituents of the *Mtb* cell envelope include: a plasma membrane, a cell wall core consisting of peptidoglycan in covalent attachment with arabinogalactan and mycolic acids, outer membrane lipids referred to as the mycomembrane and a loosely attached capsule.²⁻⁴ The outer membrane gives *Mtb* a cell wall architecture similar to Gram negative bacteria. The arabinogalactan and trehalose are found in the cell wall as esters of mycolic acids (MAs). Mycolic acids (MAs) are α -alkyl- β -hydroxy fatty acids with total carbon chain lengths ranging from C₆₀ to C₉₀, with an α -side chain with carbon lengths ranging from C₂₄ to C₂₆.⁵ They esterify both the cell wall arabinogalactan and extractable glycolipids of the cell particularly, trehalose monomycolate (TMM) and trehalose dimycolate (TDM, Cord Factor).

Biosynthesis of MAs involves two fatty acid synthases (FASs) FAS-I and FAS-II. FAS-I is considered “eukaryotic-like” and produces two acyl-CoA fatty acids. One fatty acid has a carbon chain length of C₂₄-C₂₆ which is carboxylated by ACCase/AccD4 to form a carboxyacyl-CoA derivative that becomes the α -branch of MAs. The second fatty acid has a chain length of C₁₆-C₁₈ and is elongated by FAS-II. FAS-II is considered a “bacterial-like” fatty acid synthase that participates in the production of the meromycolic acid chain (C₄₂-C₆₂). The product of FAS-II is a C₄₂-C₆₂ carboxylic acid which is converted to meromycoloyl-AMP by FadD32.^{5, 6} Pks13 is a polyketide synthase that catalyzes a Claisen-type condensation between the meromycoloyl-AMP and carboxyacyl-CoA to produce a mycolic β -ketoester. This product is transferred to trehalose⁷ and the β -ketoester reduced by the *Mtb* homolog of *Corynebacterineae* mycolate reductase A (CmrA) to form TMM.⁸ TMM is utilized by the Ag85 complex, a homologous family of enzymes (Ag85 A, B and C) that catalyzes a transesterification reaction that transfers MAs onto arabinogalactan (AG), trehalose and TMM forming mAG, TMM, and TDM, respectively. Pks13 and Ag85s are well-established anti-tuberculosis drug targets.^{9, 10}

Pks13 is essential for *Mtb*^{9, 11} and is encoded by the *fadD32-Pks13-accD4* gene cluster. It belongs to the type-1 polyketide synthase gene family. Pks13 has a phosphopantetheinyl (P-pant) arm on acyl carrier protein domains at the N-terminus (N-ACP) and the C-terminus (CACP) at Ser-55 and Ser-1266, respectively. The condensation reaction catalyzed by Pks13

proceeds according to Figure 1. Meromycoloyl-AMP (**2**) produced by FadD32 is transferred to the ketosynthase (KS) (**4**) domain from the P-pant of N-ACP (**3**) domain. Carboxylacyl-CoA (**5**) is transferred to P-pant of the CACP (**7**) via the acyltransferase domain (AT) (**6**). The meromycoloyl on the KS domain (**4**) and carboxyacyl on C-ACP domain (**7**) undergo Claisen-type condensation and forms an α -alkyl- β -keto thioester attached to the P-pant of C-ACP domain (**8**) which is transferred to Ser-1533 of thioesterase domain (TE) to form **9**. The TE domain shows acyltransferase activity and transfers **9** onto trehalose forming TMM keto form (TMMk,, **10**) which is further reduced by CmrA to produce trehalose monomycolate (TMM, **11**).⁷ The TMM produced is transported to the periplasmic space by a translocation machinery involving the RND-type transporter MmpL3,¹² and is subsequently utilized by Ag85 complex to produce the major cell components mAG, TMM and TDM.¹³

2-Aminothiophenes are found in a wide range of natural and synthetic products and include compounds with anti-tuberculosis activity.¹⁴⁻¹⁹ Some 2-aminothiophenes are reported to inhibit either Pks13 or Ag85s.^{9, 20} The compound I3-AG85 (**12**) has been reported to be an inhibitor of Ag85s (Figure 2).^{20, 21} Based on these reports Sanki *et al.* designed and synthesized an ethyl 2-amino-4,5,6,7-tetrahydrobenzo[*b*]thiophene-3-carboxylate derivative (**13**) as an Ag85C inhibitor with moderate activity.²² Wilson *et al.* screened 1,113 publicly available *Mtb* growth inhibiting compounds identified by NIH/MLSCN,⁹ and identified several compounds that act as Pks13 inhibitors. They found compounds **14** and **15** had low MICs against *Mtb* H37Rv (Figure 2).⁹ Herein, we report new 2-aminothiophenes which show potent activity on multiple drug resistant strains of *Mtb* and describe their effects on mycolic acid biosynthesis.

Results

Chemistry

We synthesized and evaluated a series of 2-aminothiophenes (2AT). The 2AT we prepared are of two types: i) FRT compounds formed from cores **22-25** and ii) 5PT compounds formed from core **26** (Figure 2, box). Four FRT cores were synthesized from ketones containing an active methylene group. The ketones were: benzyl 4-oxopiperidine-1-carboxylate **17**, *tert*-butyl 4-oxopiperidine-1-carboxylate **18**, 1-ethylpiperidin-4-one **19**, and cyclohexanone **20**. The 5PT core was synthesized from 2-phenylacetaldehyde **21**, (Scheme 1). 2-Alkylamido compounds **27-39** and **54-57** were synthesized from **22-26** using acid chlorides (AC). The ACs used were: acetyl chloride, benzoyl chloride, 2,3,4,5,6-pentafluorobenzoyl chloride, and 3,5-bis(trifluoromethyl)benzoyl chloride. The 3-carboxamide compounds **44-53** and **59-63** were synthesized using alkylamines (AA): butylamine, benzylamine, 4-methylbenzylamine, 2-methoxybenzylamine, and adamantylamine (Scheme 1). The core compounds **22-26** (Scheme 1) were synthesized using Gewald chemistry.²⁴ This reaction involves the use of aldehyde or ketone **17-21** with the activated nitrile, ethyl 2-cyanoacetate (**16**), in presence of elemental sulfur (S₈) and diethylamine to obtain the respective cores.²⁴ The cores **22-26**, on reacting with ACs in presence of DMAP and Et₃N, vary the R² group and afford 2-alkylamido compounds **27-39** and **54-57**. The 3-carboxamides compounds labelled **44-53** and **59-63** were accessed by deprotection of the ethyl esters **22-26** with LiOH to obtain compounds labeled **40-43** and

58,²⁵ which on further treating with AAs using coupling reagent 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC.HCl), hydroxybenzotriazole (HOBt) and DMAP afforded 3-carboxamide compounds (**44-53** and **59-63**) (Figure 3).²⁶ **Growth inhibition and cytotoxicity studies.** The library of 42 2ATs (Figure 3) was subjected to growth inhibition studies using a modified 96-well microplate Alamar blue assay (MABA). Among these compounds **33** and **38** showed MIC < 50 μ M whereas compound **29** and **30** showed an MIC < 100 μ M. The remainder of the compounds showed MIC > 200 μ M. The most potent compound was **33** MIC = 0.69 μ M (Table 1).

The activity of **33** was independently validated using a National Institutes of Health screening contract. The IC₅₀ and IC₉₀ values were determined against *Mtb* H37Rv grown under both aerobic and low oxygen conditions. These MIC assays were based on measurement of growth in liquid medium of a fluorescent (mCherry) reporter strain of *Mtb* H37Rv where the readout is either optical density (OD) or fluorescence.²⁷⁻³⁰ In addition, the antimicrobial activity of compounds against *Mtb* H37Rv grown under hypoxic conditions was assessed using a low oxygen recovery assay (LORA). In this assay, bacteria are first adapted to low oxygen conditions and then exposed to compounds under hypoxia; this is followed by a period of outgrowth in aerobic conditions and growth is measured using luminescence.³¹⁻³³ The mCherry assay confirmed the initial screening result and showed an MIC = 0.23 μ M (Table 2). The LORA showed an MIC > 200 μ M, IC₅₀ = 0.27 μ M, and IC₉₀ = 15 μ M. This result is considered activity under low oxygen; however, this activity is far less than the control compound rifampicin (RIF) which shows an MIC = 0.048 μ M under the same conditions. The protocol used was amenable to HTS. We note, this assay may be prone to false-positives. A false positive can occur when a potent molecule active only in replicating conditions registers as an active candidate in non-replicating conditions by virtue of their carryover from the non-replicating phase to the outgrowth phase.³⁴

The bactericidal activity of **33** was assessed against *Mtb* H37Rv grown in aerobic conditions in 7H9-Tw-OADC medium. Viable cell counts were measured over 3 weeks of exposure to determine the rate of kill. Compounds were tested at fixed concentrations, as no MIC data were available at the time of the experiment 200, 100, 20 and 5 μ M **33** were used. The MBC was found to be < 5 μ M (Figure 4).

The cytotoxicity of **33** towards eukaryotic cells was determined using the THP-1 human monocytic cell line. THP-1 cells were differentiated into macrophage-like cells using phorbol 12-myristate 13-acetate (PMA) and incubated with compounds for 3 day after which time cell viability is measured. For **33** the IC₅₀ = 6.2 μ M which represents the concentration of compound causing a 50% loss in viability.

The intracellular activity of **33** was measured using THP-1 cells infected with an autoluminescent *Mtb* strain derived from *Mtb* H37Rv.³² THP-1 cells were again differentiated into macrophage-like cells using PMA and infected with bacteria. Infected cells were exposed to compounds for 72 hours. Viable bacterial counts are measured using luminescence as a measure of growth. The result is shown in Table 2 and is reported as an IC₅₀ = 5.6 μ M which compares to an IC₅₀ = 0.24 μ M for INH.

The activity of **33** against five resistant isolates of *Mtb* strains was assessed under aerobic conditions by determining the MIC. Strains tested were two isoniazid resistant strains (INH-R1 and INH-R2), two rifampicin resistant strains (RIF-R1 and RIF-R2) and a fluoroquinolone resistant strain (FQ-R1). The assay is based on measurement of growth in liquid medium of each strain where the readout is OD.^{333435 36} INH-R1 was derived from H37Rv and is a *katG* mutant (Y155* = truncation). INH-R2 is strain ATCC 35822. RIF-R1 was derived from H37Rv and is an *rpoB* mutant (S522L). RIF-R2 is strain ATCC 35838. FQ-R1 is a fluoroquinolone-resistant strain derived from H37Rv and is a *gyrB* mutant (D94N). Compound **33** showed potent activity MIC = 0.20-0.44 μ M against the five resistance isolates tested (Table 2). Control data for INH, RIF and Levofloxacin is also reported (Table 2) for comparison.

Effect of **33** on mycolic acid synthesis

To assess the potential inhibitory effect of **33** on mycolic acid biosynthesis, *Mtb* cultures exposed to different concentrations of **33** were metabolically labeled with [1,2-¹⁴C]acetate and the levels of TMM, TDM, and mAG-attached mycolates were determined following TLC analysis and autoradiography.²³ The studies clearly revealed that **33** strongly inhibits the synthesis of all forms of mycolic acids in the cell envelope (Figure 5).

Inhibition of Ag85C by 2-ATs

To determine whether or not any of our compounds inhibited Ag85s we performed a high throughput screening of the compound library against Ag85C using a fluorescence-based assay.²³ None of the compounds in our library showed inhibition below 200 μ M (SI-Figure 1) against Ag85C.

Discussion

Structure activity relationships (SAR)

The structures of FRT and 5PT compounds were divided into R¹, R² and R³ for FRTs and R² and R³ for 5PT, respectively, Figure 2. The most obvious relation was the introduction of COC₆F₅ at the R² position which resulted in a sharp increase in potency. It could be seen that **38** (MIC = 30 μ M) was significantly more potent than the non-fluorinated homolog **37** which showed no activity. This increase in activity is not surprising in light of the work reported by Wilson *et al.* which reported comparing compound **14** (Figure 2) who had already shown the importance of 1) 2-(perfluorobenzamido) group at R² position, 2) ethyl ester at R³ position and 3) 2-aminothiophene core.⁹

The significant finding of this study is the observation that a NEt group in aliphatic ring at the R¹ position of compound **33** showed 40-fold increase in potency compared to compound **38** using the MABA. The R = NEt group was not a random selection; but rather, it designed to be isosteric with compounds related to **12** (Figure 2). Early in the design phase we were focused on thiophenes shown to inhibit Ag85s which had similar substitution, thus we saw this as way to access a similar topology, avoid introduction of stereogenic center, and introduce a pKa modifying group. While clearly none of these compounds inhibited Ag85C to a significant degree **33** is the most potent anti-*Mtb* 2-aminothiophene reported to date.

Other observations were that replacement of the NEt with a bulky groups like N-Boc at R¹ had detrimental effect making compound **29** inactive even with a COC₆F₆ substituent at the R².

The activity requirement for the ethyl ester at R³ position is less well defined and merits further investigation. There was slight activity observed for **35** (MIC = 350 µM) which bears an R³ benzamide moiety. Thus, the fact that any activity at all was observed is somewhat intriguing to us and suggests compounds with further improvements in potency may be obtainable. Previous studies have shown substituting the ethyl ester with an amide at R³ resulted in a significant increase in MIC.⁹ The structural similarity of **33** to compounds **14** and **15** reported by Wilson, *et al.* suggest the compound may inhibit Pks13.⁹ The 5PT compounds all showed poor to no potency, as all the MICs were > 200 µM. This result indicates that six membered ring attached to thiophene is important for growth inhibition activity as even compound **56** with an R² 2-(perfluorobenzamido) group showed an MIC > 200 µM.

Compound **33** was then selected for further evaluation using *Mtb* H37Rv grown under aerobic conditions using a fluorescent reporter strain *Mtb* H37Rv. The activity under aerobic conditions was confirmed and found to be 0.23 µM. The MBC of **33** was then assessed and found to be below 5 µM, this suggests that **33** is potentially bactericidal.

The antimicrobial activity of **33** against *Mtb* H37Rv grown under hypoxic conditions was assessed using the LORA assay. The purpose of the latter assay was to evaluate **33** on *Mtb* placed in a state of non-replicating persistence (NRP). Bacteria were first adapted to low oxygen conditions and then exposed to **33** under hypoxia; this is followed by a period of outgrowth in aerobic conditions and growth is measured using luminescence. The MIC₅₀ = 0.27 µM and MIC₉₀ = 15 µM under LORA conditions indicated limited activity under low oxygen conditions (the MIC₉₀ is 65 times higher than under aerobic conditions). However, rather than seeing this as a discouraging result, it is more valuable to use the data for the insight it provides into the mechanism of action. It has been reported by Franzblau *et al.* that agents targeting the cell wall of *Mtb* (eg, INH, ethambutol, and four others evaluated) show greatly reduced activity against NRP *Mtb*, whereas drugs hitting other cellular targets have a range of activities.³¹ The authors view this result as validation that **33** targets cell wall biosynthesis.

Mtb can escape the action of antibiotics by hiding inside of macrophages. Therefore, it is important to evaluate the intracellular activity of key compounds in a human macrophage-like infection model. Compound **33** was active on *Mtb* infected macrophage-like cells showing an IC₉₀ = 9.75 µM. The data indicated a loss of potency in comparison to INH which does not lose activity (based on IC₉₀ values) in *Mtb* infected macrophage-like cells. This data suggests compound permeability can be further improved. The same human macrophage-like cell line was also used to evaluate the cytotoxicity of compound **33**. The assay revealed an IC₅₀ = 6.2 µM for viability. This value is 27 times higher than the extracellular MIC and provides a potential starting point for optimizing the therapeutic window of the compound class.

Multidrug-resistant tuberculosis (MDR-TB) is a growing problem and is due to a resistance to INH and RIF, two of the most powerful antituberculosis drugs.³⁶ Fluoroquinolones (FQs) have emerged as the most important drugs in second-line treatment of MDR-TB.³⁶ However, extensively drug-resistant TB (XDR-TB) has emerged and is defined as MDR-TB with additional resistance to fluoroquinolones and at least one of the injectable drugs amikacin, capreomycin, and kanamycin. New antitubercular agents should have activity against major and emerging drug resistant strains of *Mtb*. The panels of resistance strains evaluated includes INH-R1 which contains a mutation in the catalase-peroxidase enzyme KatG protein which activates the prodrug INH to its active form.^{37, 38} The RIF-R1 strain contains a S522L mutation in the *rpoB* encoded protein. Mutation in that region of the selected region of the gene are responsible for most rifamycin resistance in *Mtb*. To cover XDR-TB the panel contains FQ-R1 which has an amino acid substitution within the GyrB subunit of DNA gyrase.³⁹ The most exciting findings of our studies were that **33** is highly active against *Mtb* strains resistant to these three classes of antitubercular agents.

Experimental

General methods

All starting materials were purchased from Acros Organics or Sigma-Aldrich. Unless specified all the reactions were performed under nitrogen atmosphere. Reactions were monitored by thin-layer chromatography (TLC, silica gel 60 F₂₅₄) and spots were observed by UV-lamp. All solvents used for reactions were purchased from Sigma-Aldrich and Fisher Scientific, which were purified and dried by distillation and other standard procedures. Solvents for flash column chromatography were used as purchased. Purification of compounds was performed by flash chromatography on silica gel (porosity 60Å, 230-400 mesh) from Sorbent Technologies. ¹H NMR, ¹³C NMR, G-COSY and HMQC spectra were carried out using a Bruker Avance III 600 spectrometer in Fourier transformation mode. ¹H-NMR and ¹³C- NMR were referenced to residual CDCl₃ peak at 7.27 ppm, and 77.16 ppm respectively. High resolution mass spectroscopy (HRMS) was performed on a micro mass Q-TOF2 instrument.

General procedure for synthesis of 2-aminothiophene-3-carboxylate (thiophene cores: **22**, **23**, **25** and **26**)

The ethyl cyanoacetate (0.53 g, 4.71 mmol), sulfur (0.18 g, 5.68 mmol), α-methylene carbonyl compound (1.0 g, 4.28 mmol) were suspended in EtOH (10.0 mL). Diethylamine (0.62 g, 8.57 mmol) was added and the mixture was heated to 70 °C for 3 h. After reaction was complete, EtOH was evaporated under reduced pressure resulting in a brown solid, which was dissolved in CH₂Cl₂. The CH₂Cl₂ layer was washed with sat. NH₄Cl (20.0 mL), deionized H₂O (20.0 mL), and sat. NaCl (20.0 mL). After drying with anhydrous NaSO₄ and filtration, the solvent was evaporated and the crude product was purified by crystallization from cold MeOH to obtain a lime yellow product. Yield: 79% (1.21 g); silica gel TLC *R_f*= 0.50 (40% ethyl acetate in hexanes).

General procedure for 2-aminothiophene-3-carboxylate formation with 1-ethylpiperidin-4-one 24

The ethyl cyanoacetate (4.89 g, 43.24 mmol), sulfur (1.66g, 51.10 mmol), and 1-ethylpiperidine (5.0 g, 39.31 mmol) were suspended in EtOH (50.0 mL). Diethylamine (5.74 g, 78.62 mmol) was added and the mixture was heated to 70 °C for 12 h. After the reaction was complete, the EtOH was evaporated under reduced pressure to afford a brown solid. To the brown solid was added diethyl ether (30.0 mL) followed by sonication for 5 mins. The ether layer was decanted and this process was repeated twice to remove unreacted starting material. The solid compound was dissolved in ethyl acetate and filtered. The filtrate was concentrated under reduced pressure and crystallized from cold MeOH to obtain brown crystals. Yield: 75% (7.52 g); silica gel TLC R_f = 0.50 (7% methanol in dichloromethane).

General procedure for 2-amidothiophene-3-carboxylate compounds 27-39 and 54-57

Compound **24** (115.0 mg, 0.452 mmol), was dissolved in dry CH_2Cl_2 (1.0 mL) and cooled to 0 °C. *N,N*-(dimethylamino)pyridine (22.10 mg, 0.180 mmol), and Et_3N (137.0 mg, 1.350 mmol) were added to the solution followed by the dropwise addition of acid chloride (135.6 mg, 0.580 mmol). The resulting solution was allowed to warm to room temperature and allowed to stir for 12 h. After the reaction was complete the CH_2Cl_2 was evaporated under reduced pressure to obtain a crude solid which was purified by flash column chromatography on silica gel (methanol: CH_2Cl_2) to obtain a lime yellow product **33**. Yield: 45% (101.19 mg); silica gel TLC R_f = 0.38 (10% methanol in CH_2Cl_2).

General procedure for deprotection of ethyl 2-aminothiophene-3-carboxylate ester cores (Compounds 40 to 43 and 58 (exception compound 42))

The ethyl ester compounds (1.0g, 2.77 mmol) were dissolved in THF:H₂O:MeOH (3:1:1) to obtain a clear solution. LiOH (0.349 g, 8.33 mmol) was added to the solution and then the solution was heated to 70 °C and maintained at that temperature for 12 h. The solution was concentrated to obtain a solid and then the solid was taken up in water and extracted with Et₂O (20.0 mL) 3 times. The aqueous layer was acidified to pH ~ 3-4 using 6N HCl. The acidified H₂O layer was extracted with EtOAc (20.0 mL × 3). The combined EtOAc extracts were dried over anhydrous Na₂SO₄, filtered and concentrated to obtain a solid which was crystallized from CH_2Cl_2 and hexanes to obtain a crystalline brown-yellow product **40**. Yield: 69% (687.7 mg); silica gel TLC R_f = 0.26 (40% ethyl acetate in hexanes).

General procedure for 3-carboxamides formation (Compound labelled with 44-53 and 59-63)

A solution of carboxylic acid (2-amino-6-ethyl-4,5,6,7-tetrahydrothieno[2,3-*c*]pyridine-3-carboxylic acid, (**42** 400 mg, 1.77 mmol) dissolved in CH_2Cl_2 (3.6 mL) was cooled to 0 °C. A solution of the appropriate alkylamine (*p*-tolylmethanamine, 236 mg, 1.95 mmol), and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (339 mg, 1.77 mmol), 1-hydroxybenzotriazole hydrate (271 mg, 1.77 mmol) and *N,N*-(dimethylamino)pyridine (361 mg, 1.77 mmol) dissolved in CH_2Cl_2 (3.6 mL) was added. The solution was allowed to warm to room temperature and stirred for 18 h. The solution was diluted with CH_2Cl_2 (15.0 mL), washed with brine (20.0 mL), and dried over anhydrous Na₂SO₄, and concentrated to

obtain a crude solid. The product was purified by flash column chromatography (methanol:CH₂Cl₂) to obtain a light brown product **46** Yield: 40% (235.6 mg); silica gel TLC R_f = 0.41 (9% methanol in DCM). **Exception** synthesis of compound **35**: The product **46** obtained by using above general procedure was further reacted with 3,5-bis(trifluoromethyl)benzoyl chloride using general procedure for 2-amidothiophene-3-carboxylate compounds to yield product **35**.

6-(*Tert*-butyl) 3-ethyl 2-benzamido-4,7-dihydrothieno[2,3-*c*]pyridine-3,6(5*H*)-dicarboxylate (28)

Yield: 23% (61.5 mg); silica gel TLC R_f = 0.73 (7 % methanol in CH₂Cl₂); ¹H-NMR (600 MHz, CDCl₃): δ 12.30 (s, 1H, H-10), 8.03 (m, 2H, H-22,25), 7.59 (m, 1H, H-24), 7.54 (m, 2H, H-23,25) 4.55 (s, 2H, H-8), 4.40 (q, 2H, ³ $J_{15,16}$ 7.15 Hz, H-15), 3.68 (t, 2H, ³ $J_{6,5}$ 5.69 Hz, H-6), 2.92 (s, 2H, H-5), 1.50 (s, 9H, H-20,29,30), 1.43 (t, 3H, ³ $J_{16,15}$ 7.15 Hz, H-16); ¹³C-NMR (150 MHz, CDCl₃): δ 166.66 (C-2), 163.62 (C-11), 154.66 (C-17), 148.94 (C-13), 132.69 (C-24), 130.08 (C-4), 129.01 (C-25, 23), 127.50 (C-26, 22), 123.62 (C-9), 111.50 (C-3), 80.17 (C-19), 60.90 (C-15), 42.89 (C-8), 40.60 (C-6), 28.47 (C-30, 29, 20), 26.58 (C-5), 14.36 (C-16); mass spectrum (HRMS), m/z = 453.1358 (M+23)⁺ C₂₂H₂₆N₂NaO₅S requires 453.1458 (M+23)⁺.

6-(*Tert*-butyl) 3-ethyl 2-(perfluorobenzamido)-4,7-dihydrothieno[2,3-*c*]pyridine-3,6(5*H*)-dicarboxylate (29)

Yield: 82% (263.0 g); silica gel TLC R_f = 0.89 (7 % methanol in CH₂Cl₂); ¹H-NMR (600 MHz, CDCl₃): δ 12.55 (s, 1H, H-10), 4.56 (s, 2H, H-8), 4.33 (q, 2H, ³ $J_{15,16}$ 7.15 Hz, H-15), 3.66 (t, 2H, ³ $J_{6,5}$ 5.5 Hz, H-6), 2.92 (t, 2H, H-5), 1.49 (s, 9H, H-30,29,20), 1.32 (t, 3H, ³ $J_{16,15}$ 7.15 Hz, H-16); ¹³C-NMR (150 MHz, CDCl₃): δ 160.76 (C-2), 159.62 (C-11), 154.44 (C-17), 143.44 (¹ $J_{24,F}$ 251.99 Hz, C-24), 143.17 (¹ $J_{26,22-F}$ 259.69 Hz, C-26, 22), 137.65 (¹ $J_{25,23,F}$ 256.39 Hz, C-25,23), 136.94 (C-13), 135.38 (C-21), 130.72 (C-4), 125.29 (C-9), 110.67 (C-3), 80.75 (C-19), 61.86 (C-15), 42.61 (C-8), 40.05 (C-6), 28.50 (C-30, 29, 20), 26.53 (C-5) 13.87 (C-16); mass spectrum (HRMS), m/z = 521.1091 (M+H)⁺ C₂₂H₂₁F₅N₂O₅S requires 521.1091 (M+H)⁺.

6-(*Tert*-butyl) 3-ethyl 2-(3,5-bis(trifluoromethyl)benzamido)-4,7-dihydrothieno[2,3-*c*]pyridine-3,6(5*H*)-dicarboxylate (30)

Yield: 39% (134.0 g); silica gel TLC R_f = 0.82 (7 % methanol in CH₂Cl₂); ¹H-NMR (600 MHz, CDCl₃): δ 12.56 (s, 1H, H-10), 8.45 (s, 2H, H-22, 26), 8.11 (s, 1H, H-24), 4.57 (s, 2H, H-8), 4.43 (q, 2H, ³ $J_{15,16}$ 7.15 Hz, H-15), 3.70 (t, 2H, ³ $J_{6,5}$ 5.69 Hz, H-6), 2.94 (t, 2H, H-5), 1.51 (s, 9H, H-30, 33, 34), 1.43 (t, 3H, ³ $J_{16,15}$ 7.15 Hz, H-16); ¹³C-NMR (150 MHz, CDCl₃): δ 166.59 (C-2), 160.61 (C-11), 154.61 (C-17), 147.66 (C-13), 134.55 (C-21), 132.59 (C-28,29), 127.71 (C-22,26), 126.01 (C-24), 123.70 (C-9), 121.89 (C-25,23), 112.64 (C-3), 80.28 (C-29), 61.28 (C-15), 42.67 (C-8), 40.59 (C-6), 28.46 (C-34, 33, 30), 26.54 (C-5), 14.32 (C-16); mass spectrum (HRMS), m/z = 589.1216 (M+Na)⁺ C₂₄H₂₄F₆N₂NaO₅S requires 589.1202 (M+23)⁺.

Ethyl 2-benzamido-6-ethyl-4,5,6,7-tetrahydrothieno[2,3-c]pyridine-3-carboxylate (32)

Yield: 35% (39.6 mg); silica gel TLC R_f = 0.35 (10 % methanol in CH_2Cl_2); $^1\text{H-NMR}$ (600 MHz, CDCl_3): δ 12.27 (s, 1H, H-10), 8.01 (m, 2H, H-20,24), 7.58 (m, 1H, H-22), 7.52 (m, 2H, H-21,23), 4.39 (q, 2H, $^3J_{17,18}$ 7.15 Hz, H-17), 3.63 (s, 2H, H-8), 2.97 (t, 2H, $^3J_{5,6}$ 5.87 Hz, H-5), 2.80 (t, $^3J_{6,5}$ 5.63 Hz, 2H, H-6), 2.66 (q, 2H, $^3J_{15,16}$ 6.97 Hz, H-15), 1.40 (t, 3H, $^3J_{18,17}$ 7.15 Hz, H-18), 1.20 (t, 3H, $^3J_{16,15}$ 7.34 Hz, H-16); $^{13}\text{C-NMR}$ (150 MHz, CDCl_3): δ 166.86 (C-2), 163.63 (C-11), 148.69 (C-13), 132.69 (C-19), 132.38 (C-22), 129.75 (C-4), 129.07 (C-23,21), 127.58 (C-24,20), 124.36 (C-9), 111.58 (C-3), 60.86 (C-17), 51.69 (C-15), 51.10 (C-8), 50.24 (C-6), 26.94 (C-5), 14.46 (C-18), 12.56 (C-16); mass spectrum (HRMS), m/z = 359.1418 ($\text{M}+\text{H}$) $^+$ $\text{C}_{19}\text{H}_{23}\text{N}_2\text{O}_3\text{S}$ requires 359.1424 ($\text{M}+\text{H}$) $^+$.

Ethyl 6-ethyl-2-(perfluorobenzamido)-4,5,6,7-tetrahydrothieno[2,3-c]pyridine-3-carboxylate (33)

Yield: 16% (34.0 mg); silica gel TLC R_f = 0.38 (10 % methanol in CH_2Cl_2); $^1\text{H-NMR}$ (600 MHz, CDCl_3): δ 12.02 (s, 1H, H-10), 4.36 (q, 2H, $^3J_{17,18}$ 7.15 Hz, H-17), 3.65 (s, 2H, H-8), 2.97 (t, 2H, H-5), 2.82 (t, 2H, H-6), 2.68 (q, 2H, $^3J_{15,16}$ 6.79 Hz, H-15), 1.38 (t, 3H, $^3J_{18,17}$ 7.15 Hz, H-18), 1.22 (t, 3H, $^3J_{16,15}$ 7.15 Hz, H-16); $^{13}\text{C-NMR}$ (150 MHz, CDCl_3): δ 166.39 (C-2), 154.13 (C-11), 146.35 (C-13), 145.14 ($^1J_{22,\text{F}}$ 256.39 Hz, C-22), 143.27 (md, $^1J_{22,\text{F}}$ 259.69 Hz, C-20,24), 137.99 (md, $^1J_{22,\text{F}}$ 255.29 Hz, C-23,21), 130.13 (C-4), 125.47 (C-9), 112.97 (C-3), 109.77 (C-19), 81.18 (C-17), 51.65 (C-15), 50.96 (C-8), 50.10 (C-6), 26.78 (C-5), 14.37 (C-18), 12.48 (C-16); mass spectrum (HRMS), m/z = 449.0971 ($\text{M}+\text{H}$) $^+$ $\text{C}_{19}\text{H}_{18}\text{F}_5\text{N}_2\text{O}_3\text{S}$ requires 449.0953 ($\text{M}+\text{H}$) $^+$; Anal calcd for $\text{C}_{19}\text{H}_{18}\text{F}_5\text{N}_2\text{O}_3\text{S}$ C, 50.89; H, 3.82; N, 6.25; found: C, 51.11; H, 3.36; N, 6.48.

Ethyl 2-(3,5-bis(trifluoromethyl)benzamido)-6-ethyl-4,5,6,7-tetrahydrothieno[2,3-c]pyridine-3-carboxylate (34)

Yield: 43% (168.9 mg); silica gel TLC R_f = 0.41 (10 % methanol in CH_2Cl_2); $^1\text{H-NMR}$ (600 MHz, CDCl_3): δ 12.53 (s, 1H, H-10), 8.45 (s, 2H, H-20, 24), 8.10 (s, 2H, H-22), 4.43 (q, 2H, $^3J_{17,18}$ 7.15 Hz, H-17), 3.76 (s, 2H, H-8), 3.05 (t, 2H, H-5), 2.92 (t, 2H, H-6), 2.77 (q, 2H, H-15), 1.42 (t, 3H, $^3J_{18,17}$ 7.15 Hz, H-18), 1.29 (t, 3H, H-16); $^{13}\text{C-NMR}$ (150 MHz, CDCl_3): δ 166.67 (C-2), 160.73 (C-11), 147.67 (C-13), 134.69 (C-19), 132.70 (C-28, 29), 120.01 (C-4), 127.83 (C-20, 24), 126.10 (C-22), 123.83 (C-9), 122.02 (C-23, 21), 112.67 (C-3), 61.37 (C-17), 51.55 (C-15), 50.69 (C-8), 49.96 (C-6), 26.14 (C-5), 14.45 (C-18), 12.13 (C-16); mass spectrum (HRMS), m/z = 517.0994 ($\text{M}+\text{H}$) $^+$ $\text{C}_{21}\text{H}_{20}\text{F}_6\text{N}_2\text{O}_3\text{S}$ requires 517.0991 ($\text{M}+\text{H}$) $^+$.

2-(3,5-Bis(trifluoromethyl)benzamido)-6-ethyl-N-(4-methylbenzyl)-4,5,6,7-tetrahydrothieno[2,3-c]pyridine-3-carboxamide (35)

Yield: 22% (18.0 mg); silica gel TLC R_f = 0.55 (10 % methanol in CH_2Cl_2); $^1\text{H-NMR}$ (600 MHz, CDCl_3): δ 13.65 (s, 1H, H-10), 8.47 (s, 2H, H-22,18), 8.08 (s, 1H, H-20), 7.23 (d, 2H, $J_{33,35}$ 6 Hz, H-33,28), 7.19 (d, 2H, $J_{35,33}$ 6Hz, H-35,29), 6.23 (s, 1H, H-23), 4.63 (d, 2H, $J_{26,23}$ 5Hz, H-26), 3.71 (s, 2H, H-8), 2.87 (m, 4H, H-5,6), 2.70 (q, 2H, $J_{15,16}$ 6 Hz, H-15), 2.36 (s, 3H, H-34), 1.23 (t, 3H, $J_{16,15}$ 6Hz, H-16); $^{13}\text{C-NMR}$ (150 MHz, CDCl_3): δ 166.20

(C-2), 160.74(C-13), 145.91 (C-11), 136.03(C-30), 134.67(C-17, 27), 132.52 (C-19, 21), 129.75 (C-35, 29), 127.78 (C-22, 18), 126.13(C-4), 125.84(C-20), 123.90, 120.29(C-9), 114.65(C-3), 51.74(C-15), 51.10(C-8), 49.93(C-6), 43.64 (C-26), 26.96 (C-5), 21.27 (C-34), 12.35 (C-16); mass spectrum (HRMS), $m/z = 592.1467$ (M+23)⁺ C₂₇H₂₅F₆N₃NaO₂S requires 592.1464 (M+23)⁺.

Ethyl 2-(perfluorobenzamido)-4,5,6,7-tetrahydrobenzo[*b*]thiophene-3-carboxylate (38)

Yield: 50.5% (188.0 mg); silica gel TLC $R_f = 0.86$ (7 % methanol in CH₂Cl₂); ¹H-NMR (600 MHz, CDCl₃): δ 12.05 (s, 1H, H-10), 4.35 (q, 2H, ³*J*_{15,16} 7.14 Hz, H-15), 2.80 (t, 2H, ³*J*_{8,7} 6.06 Hz H-8), 2.70 (t, 2H, ³*J*_{5,6} 5.94 Hz, H-5), 1.85-1.78 (m, 4H, H-6,7), 1.80 (t, 3H, ³*J*_{16,15} 7.1,4 H-16); ¹³C-NMR (150 MHz, CDCl₃): δ 166.76 (C-2), 153.97 (C-11), 145.11 (md, ¹*J*_{20,F} 253.09 Hz, C-20), 145.81 (C-13), 143.14 (md, ¹*J*_{18,22,F} 259.69 Hz, C-18, 22), 137.98 (md, ¹*J*_{19,21,F} 254.19 Hz, C-19, 21), 131.57 (C-4), 128.43 (C-9), 113.47 (C-17), 110.10 (C-3), 61.03 (C-15), 26.48 (C-8), 24.56 (C-5), 23.04(C-7), 22.88 (C-6), 14.39 (C-16); mass spectrum (HRMS), $m/z = 420.0690$ (M+H)⁺ C₁₈H₁₄F₅NO₃S requires 420.0687 (M+H)⁺.

Ethyl 2-(3,5-bis(trifluoromethyl)benzaimdo)-4,5,6,7-tetrahydrobenzo[*b*]thiophene-3-carboxylate (39)

Yield: 14.5% (60.0 mg); silica gel TLC $R_f = 0.86$ (7 % methanol in CH₂Cl₂); ¹H-NMR (600 MHz, CDCl₃): δ 12.58 (s, 1H, H-10), 8.44 (s, 2H, H-18,22), 8.08 (s, 1H, H-20), 4.40 (q, 2H, ³*J*_{15,16} 7.14 Hz, H-15), 2.82 (t, 2H, ³*J*_{8,7} 6.06 Hz, H-8), 2.71 (t, 2H, ³*J*_{5,6} 5.82 Hz, H-5), 1.84-1.81 (m, 4H, H-6, 7), 1.42 (t, 3H, ³*J*_{16,15} 7.14, H-16); ¹³C-NMR (150 MHz, CDCl₃): δ 167.11 (C-2), 160.52 (C-11), 146.88 (C-13), 135.03 (C-17), 132.71 (q, 2C, ¹*J*_{C,F} 132 C-26, 27), 131.60 (C-4), 128.23 (C-9), 127.50 (d, 2C, ³*J*_{C,F} 12.06 C-22, 18), 125.89 (m, C-20), 123.87 (C-19), 122.06 (C-21), 113.21 (C-3), 61.10 (C-15), 26.49 (C-8), 24.60 (C-5), 23.06 (C-7), 22.88 (C-6), 14.44 (C16); mass spectrum (HRMS), $m/z = 466.0833$ (M+H)⁺ C₂₀H₁₇F₆NO₃S requires 466.0906 (M+H)⁺.

Ethyl 2-(perfluorobenzamido)-5-phenylthiophene-3-carboxylate (56)

Yield: 45% (161 mg); silica gel TLC $R_f = 0$. (30% ethyl acetate in hexanes); ¹H-NMR (600 MHz, CDCl₃): δ 11.81 (s, 1H, H-6), 7.62 (dd, 2H, ³*J*_{14/18,15/17} 8.44 Hz, ³*J*_{14/18,16} 1.28 Hz, H-14, 18), 7.48 (s, 1H, H-4), 7.41 (m, 2H, H-15, 17), 7.32 (m, 1H, H-16), 4.41 (q, 2H, ³*J*_{11,12} 7.15 Hz, H-11), 1.43 (t, 3H, ³*J*_{12,11} 7.15 Hz, H-12); ¹³C-NMR (150 MHz, CDCl₃): δ 165.65 (C-2), 154.28 (C-7), 146.21 (C-9), 145.29 (d, ¹*J*_{C,F} 256.39 Hz, C-22), 143.68 (d, ¹*J*_{C,F} 260.79 Hz, C- 24, 20), 138.05 (d, ¹*J*_{C,F} 255.29 Hz, C-21, 23), 135.34 (C-13), 133.45 (C-5), 129.19 (C-15, 17), 127.97 (C-16), 125.73 (C-14, 18), 119.51 (C-14), 115.59 (C-3), 109.44 (C-19), 61.10 (C-11), 14.46 (C-12); mass spectrum (HRMS), $m/z = 464.0352$ (M+H)⁺ C₂₀H₁₂F₅NO₃S requires 464.0350 (M+H)⁺.

Ethyl 2-(3,5-bis(trifluoromethyl)benzamido)-5-phenylthiophene-3-carboxylate (57)

Yield: 67% (66.8 mg); silica gel TLC $R_f = 0$. (30% ethyl acetate in hexanes); ¹H-NMR (600 MHz, CDCl₃): δ 12.27 (s, 1H, H-6), 8.47 (s, 2H, H-24, 20), 8.12 (s, 2H, H-22), 7.64 (dd, 2H, ³*J*_{14/18,15/17} 8.25 Hz, ³*J*_{14/18,16} 1.28 Hz, H-14, 18), 7.50 (s, 1H, H-4), 7.42 (m, 2H, H-15,

17), 7.32 (m, 1H, H-16), 4.46 (q, 2H, $^3J_{11,12}$ 7.15 Hz, H-11), 1.46 (t, 3H, $^3J_{12,11}$ 7.15 Hz, H-12); ^{13}C -NMR (150 MHz, CDCl_3): δ 166.06 (C-2), 160.75 (C-7), 147.32 (C-9), 135.18 (C-19), 134.55 (C-13), 133.55 (C-5), 133.22-132.54 (q, $^2J_{\text{C,F}}$ 34.11 Hz, C-23, 21), 129.20 (C-17, 15), 127.95 (C-16), 127.84 ($^3J_{\text{C,F}}$ 3.30 Hz, C-24, 20), 126.21 (C-22), 125.73 (C-14, 18), 122.92 (d, $^1J_{\text{C,F}}$ 272.90 Hz, C-28, 29), 119.56 (C-4), 115.37 (C-3), 61.49 (C-11), 14.53 (C-12); mass spectrum (HRMS), m/z = 488.0756 ($\text{M}+\text{H}$) $^+$ $\text{C}_{22}\text{H}_{15}\text{F}_6\text{NO}_3\text{S}$ requires 488.0750 ($\text{M}+23$) $^+$.

2-Amino-6-((benzyloxy)carbonyl)-4,5,6,7-tetrahydrothieno[2,3-c]pyridine-3-carboxylic acid (40)

Yield: 69% (617.0 mg); silica gel TLC R_f = 0.55 (7 % methanol in CH_2Cl_2); ^1H -NMR (600 MHz, CDCl_3): δ 7.37 (m, 4H, H-18, 17, 21, 20), 7.32-7.31 (m, 1H, H-19), 5.10 (s, 2H, H-15), 4.31 (m, 2H, H-8), 3.59 (s, 2H, H-6), 2.68 (s, 2H, H-5); ^{13}C -NMR (150 MHz, CDCl_3): δ 166.49 (C-2), 163.51 (C-11), 154.51 (C-13), 136.86 (C-9), 130.75 (C-4), 128.43 (C-18, 20), 127.86 (C-21, 17), 127.55 (C-19), 111.56 (C-3), 42.47 (C-15), 41.13 (C-8), 40.73 (C-6), 26.77 (C-5); mass spectrum (HRMS), m/z = 355.0732 ($\text{M}+23$) $^+$ $\text{C}_{16}\text{H}_{16}\text{N}_2\text{O}_4\text{S}$ requires 355.0723 ($\text{M}+23$) $^+$.

2-Amino-6-ethyl-4,5,6,7-tetrahydrothieno[2,3-c]pyridine-3-carboxylic acid (42)

The ethyl ester compound **24** (1.18g, 4.64 mmol) was dissolved in 10.0 mL of THF:H₂O:MeOH (3:1:1) to obtain a clear solution. LiOH (0.779 g, 18.58 mmol) was added to the solution and the solution was heated to 70 °C and maintained at that temperature for 12 h. The solution was concentrated to obtain a solid. The solid was taken in Et₂O (20.0 mL) and the solution was subjected to sonication for 5 mins. The Et₂O layer was decanted and this process was repeated two times. The solid obtained was dissolved in EtOAc and the mixture was filtered to remove LiOH. The filtrate was concentrated and the solid was crystallized from methanol to a pure brownish-yellow product **42**. Yield: 66% (883.1 mg); silica gel TLC R_f = 0.14 (10 % methanol in CH_2Cl_2); ^1H -NMR (150 MHz, DMSO-*d*₆): δ 6.80 (s, 1H, H-10), 3.25 (s, 2H, H-8), 2.75 (m, 2H, H-6), 2.52 (m, 2H, H-5), 2.43 (q, 2H, $J_{13,14}$ 6Hz, H-13), 1.03 (t, 3H, $J_{14,13}$ 6Hz, H-14); ^{13}C -NMR (150 MHz, DMSO-*d*₆): δ 171.23 (C-2), 157.27 (C-11), 133.51 (C-9), 112.59 (C-4), 111.66 (C-3), 51.86 (C-8), 51.48 (C-6), 51.05 (C-13), 28.25 (C-5), 12.95 (C-14); mass spectrum (ESI), m/z = 249.07 ($\text{M}+23$) $^+$ $\text{C}_{10}\text{H}_{14}\text{N}_2\text{O}_2\text{S}$ requires 249.06 ($\text{M}+23$) $^+$.

2-Amino-*N*-butyl-6-ethyl-4,5,6,7-tetrahydrothieno[2,3-c]pyridine-3-carboxamide (44)

Yield: 59% (140.0 mg); silica gel TLC R_f = 0.59 (7 % methanol in CH_2Cl_2); ^1H -NMR (600 MHz, CDCl_3): δ 5.96 (s, 2H, H-10), 5.59 (s, 1H, H-15), 3.51 (s, 2H, H-8), 3.36 (q, 2H, H-16), 2.81 (m, 4H, H-5, 6), 2.65 (q, 2H, $^3J_{13,14}$ 7.14 Hz, H-13), 1.54 (quin, 2H, $^3J_{17,16,18}$ 7.14 Hz, H-17), 1.38 (sex, 2H, $^3J_{18,17,19}$ 7.5 Hz H-18), 1.20 (t, 3H, $^3J_{14,13}$ 7.14 Hz H-14), 0.94 (t, 3H, $^3J_{19,18}$ 7.5 Hz, H-19); ^{13}C -NMR (150 MHz, CDCl_3): δ 166.88 (C-2), 166.66 (C-11), 147.65 (C-13), 130.65 (C-4), 126.59 (C-9), 111.23 (C-3), 60.46 (C-15), 26.39 (C-8), 24.36 (C-5), 23.74 (C-17), 23.00 (C-7), 22.88 (C-6), 14.34 (C-16); mass spectrum (HRMS), m/z = 282.1647 ($\text{M}+\text{H}$) $^+$ $\text{C}_{14}\text{H}_{24}\text{N}_3\text{O}_3\text{S}$ requires 282.1635 ($\text{M}+23$) $^+$.

2-Amino-N-benzyl-6-ethyl-4,5,6,7-tetrahydrothieno[2,3-c]pyridine-3-carboxamide (45)

Yield: 59% (140.0 mg); silica gel TLC R_f = 0.57 (7 % methanol in CH_2Cl_2); $^1\text{H-NMR}$ (600 MHz, CDCl_3): δ 7.36-7.28 (m, 5H, H-18, 19, 20, 21, 22), 6.04 (s, 2H, H-10), 5.91 (s, 1H, H-15), 4.59 (d, 2H, $^3J_{16,15}$ 5.58 Hz, H-16), 3.46 (s, 2H, H-8), 2.74 (s, 4H, H-6, 5), 2.58 (q, 2H, $^3J_{13,14}$ 7.14, H-13), 1.16 (t, 3H, $^3J_{14,13}$ 7.14, H-14); $^{13}\text{C-NMR}$ (150 MHz, CDCl_3): δ 166.26 (C-2), 159.70 (C-11), 138.69 (C-9), 128.76 (C-21, 19), 127.45 (C-18, 20, 22, 4), 116.34 (C-17), 108.52 (C-3), 51.56 (C-16), 51.27 (C-8), 50.08 (C-6), 43.20 (C-13), 27.73 (C-5), 12.58 (C-14); mass spectrum (HRMS), m/z = 316.1486 ($\text{M}+\text{H}$) $^+$ $\text{C}_{17}\text{H}_{21}\text{N}_3\text{OS}$ requires 316.1478 ($\text{M}+23$) $^+$.

2-Amino-6-ethyl-N-(4-methylbenzyl)-4,5,6,7-tetrahydrothieno[2,3-c]pyridine-3-carboxamide (46)

Yield: 40% (235.6 mg); silica gel TLC R_f = 0.55 (7 % methanol in CH_2Cl_2); $^1\text{H-NMR}$ (600 MHz, CDCl_3): δ 7.20 (m, 2H, H-21,19), 7.16 (m, 2H, H-22,18), 6.03 (s, 2H, H-10), 5.87 (s, 1H, H-15), 4.53 (d, 2H, $J_{16,15}$ 6Hz, H-16), 3.50 (s, 2H, H8), 2.75 (m, 4H, H-5,6), 2.61 (q, 2H, $J_{13,14}$ 6 Hz H-13), 2.35 (s, 3H, H-23), 1.17 (t, 2H, $J_{14,13}$ 6 Hz H-14); $^{13}\text{C-NMR}$ (150 MHz, CDCl_3): δ 166.19 (C-11), 159.63 (C-2), 137.08 (C-20, 9), 135.57 (C-17), 129.43 (C-21, 19), 127.49 (C-22, 18), 116.01 (C-4), 51.52 (C-8), 51.17(C-6), 50.02 (C-13), 43.03 (C-16), 27.54 (C-23), 21.13(C-5), 12.47 (C-14); mass spectrum (HRMS), m/z = 352.1460 ($\text{M}+23$) $^+$ $\text{C}_{18}\text{H}_{23}\text{N}_3\text{OS}$ requires 352.1460 ($\text{M}+23$) $^+$

2-Amino-6-ethyl-N-(2-methoxybenzyl)-4,5,6,7-tetrahydrothieno[2,3-c]pyridine-3-carboxamide (47)

Yield: 59% (140.0 mg); silica gel TLC R_f = 0.55 (10% methanol in CH_2Cl_2); $^1\text{H-NMR}$ (600 MHz, CDCl_3): δ 7.30-7.25 (m, 2H, H-20, 19), 6.92 (t, 1H, $^3J_{21,20}$ 7.44 Hz, H-21), 6.87 (d, 1H, $^3J_{22,21}$ 8.22 H-22), 6.29 (s, 1H, H-15), 6.01 (s, 2H, H-10), 4.54 (d, 2H, $^3J_{16,15}$ 5.88 Hz, H-16), 3.87 (s, 3H, H-24), 3.47 (s, 2H, H-8), 2.76 (s, 4H, H-6, 5), 2.61 (q, 2H, $^3J_{13,14}$ 7.14 Hz, H-13), 1.18 (t, 3H, $^3J_{14,13}$ 7.14 Hz, H-14); $^{13}\text{C-NMR}$ (150 MHz, CDCl_3): δ 165.96 (C-2), 159.32 (C-11), 157.55 (C-13), 129.66 (C-4), 128.76 (C-9), 127.60 (C-22), 126.66 (C-20), 120.76 (C-21), 110.20 (C-3), 108.52 (C-19), 55.28 (C-24), 51.59 (C-16), 51.07 (C-8), 50.20 (C-6), 39.34 (C-13), 27.54 (C-5), 12.38 (C-14); mass spectrum (HRMS), m/z = 346.1587 ($\text{M}+\text{H}$) $^+$ $\text{C}_{18}\text{H}_{24}\text{N}_3\text{O}_2\text{S}$ requires 346.1584 ($\text{M}+\text{H}$) $^+$.

N-(Adamantan-1-yl)-2-amino-6-ethyl-4,5,6,7-tetrahydrothieno[2,3-c]pyridine-3-carboxamide (48)

Yield: 59% (140.0 mg); silica gel TLC R_f = 0.7 (10 % methanol in CH_2Cl_2); $^1\text{H-NMR}$ (600 MHz, CDCl_3): δ 5.84 (s, 2H, H-10), 5.37 (s, 1H, H-15), 3.50 (s, 2H, H-8), 2.78 (d, 2H, $^3J_{6,5}$ 4.8 Hz, H-6), 2.75 (d, 2H, $^3J_{5,6}$ 4.8 Hz, H-5), 2.63 (q, 2H, $^3J_{13,14}$ 7.14 Hz, H-13), 2.09-2.06 (m, 10H, H-17, 22, 21, 18, 23, 20), 1.70-1.68 (m, 5H, H-19, 24, 25), 1.86 (t, 3H, $^3J_{14,13}$ 7.14 H-14); $^{13}\text{C-NMR}$ (150 MHz, CDCl_3): δ 165.61 (C-2), 158.42 (C-11), 127.51 (C-4), 115.70 (C-9), 109.95 (C-3), 51.99 (C-13), 51.50 (C-8), 51.09 (C-6), 50.08 (C-16), 42.18 (C-17, 21, 22), 36.43 (C-19, 24, 25), 29.49 (C-18, 20, 23), 27.41 (C-5), 27.41 (C-5), 12.34 (C-14); mass spectrum (HRMS), m/z = 360.2104 ($\text{M}+\text{H}$) $^+$ $\text{C}_{20}\text{H}_{30}\text{N}_3\text{OS}$ requires 360.2104 ($\text{M}+23$) $^+$.

2-Amino-*N*-butyl-4,5,6,7-tetrahydrobenzo[*b*]thiophene-3-carboxamide (49)

Yield: 16.13% (45.0 mg); silica gel TLC R_f = 0.45 (30 % ethyl acetate in hexanes); $^1\text{H-NMR}$ (600 MHz, CDCl_3): δ 5.66 (s, 1H, H-13), 3.36 (m, 2H, H-14), 2.60 (m, 2H, H-8), 2.53 (m, 2H, H-5), 1.80 (m, 4H, H-6,7), 1.54 (m, 2H, H-15), 1.38 (sex, 2H, $^3J_{16,17}$ 7.38 Hz, H-16), 0.94 (t, 3H, $^3J_{17,16}$ 7.38 Hz, H-17); $^{13}\text{C-NMR}$ (150 MHz, CDCl_3): δ 166.53 (C-2), 158.53 (C-11), 128.80 (C-9), 118.90 (C-4), 108.95 (C-3), 38.92 (C-14), 31.88 (C-15), 27.18 (C-8), 24.53 (C-5), 22.99 (C-7), 22.90 (C-6), 20.29 (C-16), 13.81 (C-17); mass spectrum (HRMS), m/z = 275.1194 ($\text{M}+23$) $^+$ $\text{C}_{13}\text{H}_{20}\text{N}_2\text{OS}$ requires 275.1194 ($\text{M}+23$) $^+$.

2-Amino-*N*-(4-methylbenzyl)-4,5,6,7-tetrahydrobenzo[*b*]thiophene-3-carboxamide (51)

Yield: 25% (83.0 mg); silica gel TLC R_f = 0.46 (7 % methanol in CH_2Cl_2); $^1\text{H-NMR}$ (600 MHz, CDCl_3): δ 7.22 (d, 2H, $^3J_{17,16}$ 7.98 Hz, H-17, 19), 7.15 (d, 2H, $^3J_{16,17}$ 7.86 Hz, H-16, 20), 5.95 (s, 1H, H-13), 4.54 (d, 2H, $^3J_{14,13}$ 5.46 Hz, H-14), 2.58 (m, 2H, H-8), 2.54 (m, 2H, H-5), 2.34 (s, 3H, H-21), 1.77 (m, 4H, H-6, 7); $^{13}\text{C-NMR}$ (150 MHz, CDCl_3): δ 166.38 (C-2), 158.62 (C-11), 137.03 (C-9), 135.65 (C-18), 129.40 (C-19, 17), 128.82 (C-5), 127.56 (C-16, 20), 119.21 (C-4), 108.90 (C-3), 43.04 (C-14), 27.21 (C-8), 24.55 (C-5), 22.92 (C-7), 22.85 (C-6), 21.12 (C-21); mass spectrum (HRMS), m/z = 301.1377 ($\text{M}+\text{H}$) $^+$ $\text{C}_{17}\text{H}_{21}\text{N}_2\text{OS}$ requires 301.1369 ($\text{M}+\text{H}$) $^+$.

2-Amino-*N*-(2-methoxybenzyl)-4,5,6,7-tetrahydrobenzo[*b*]thiophene-3-carboxamide (52)

Yield: 12% (40.0 mg); silica gel TLC R_f = 0.35 (30 % ethyl acetate-hexanes); $^1\text{H-NMR}$ (600 MHz, CDCl_3): δ 7.31-7.25 (m, 2H, H-17,20), 6.94-6.88 (m, 2H, H-19,18), 6.36 (s, 1H, H-13), 4.55 (d, 2H, $^3J_{14,13}$ 5.58 Hz, H-14), 3.88 (s, 3H, H-22), 2.60 (m, 2H, H-5), 2.53 (m, 2H, H-8), 1.79 (m, 4H, H-6,7); $^{13}\text{C-NMR}$ (150 MHz, CDCl_3): δ 166.19 (C-2), 158.75 (C-11), 157.53 (C-16), 129.67 (C-9), 128.95 (C-20), 128.71 (C-18), 126.75 (C-15), 120.77 (C-4), 118.73 (C-19), 110.17 (C-17), 108.92 (C-3), 55.20 (C-22), 39.25 (C-14), 27.01 (C-8), 24.54 (C-5), 23.04 (C-7), 22.92 (C-6); mass spectrum (HRMS), m/z = 317.1316 ($\text{M}+\text{H}$) $^+$ $\text{C}_{17}\text{H}_{21}\text{N}_2\text{O}_2\text{S}$ requires 317.1316 ($\text{M}+\text{H}$) $^+$.

***N*-(Adamantan-1-yl)-2-amino-4,5,6,7-tetrahydrobenzo[*b*]thiophene-3-carboxamide (53)**

Yield: 13% (45.0 mg); silica gel TLC R_f = 0.53 (30 % ethyl acetate-hexanes); $^1\text{H-NMR}$ (600 MHz, CDCl_3): δ 5.43 (s, 1H, H-13), 2.58 (m, 2H, H-8), 2.53 (m, 2H, H-5), 2.05 (m, 9H, H-15, 16, 18, 19, 20, 21), 1.78 (m, 4H, H-6, 7), 1.70 (m, 6H, H-17, 22, 23), $^{13}\text{C-NMR}$ (150 MHz, CDCl_3): δ 165.89 (C-2), 157.87 (C-11), 128.84 (C-9), 118.95 (C-4), 110.31 (C-3), 51.94 (C-14), 42.19 (C-15, 19, 20), 36.47 (17, 23, 22), 29.52 (C-16, 18, 21), 27.32 (C-8), 24.57 (C-5), 23.09 (C-7), 22.94 (C-6); mass spectrum (HRMS), m/z = 331.1843 ($\text{M}+\text{H}$) $^+$ $\text{C}_{19}\text{H}_{27}\text{N}_2\text{OS}$ requires 331.1839 ($\text{M}+23$) $^+$.

2-Amino-*N*-benzyl-5-phenylthiophene-3-carboxamide (60)

Yield: 59% (166.4 mg); silica gel TLC R_f = 0.33 (30% ethyl acetate in hexanes); $^1\text{H-NMR}$ (600 MHz, CDCl_3): δ 7.41 (dd, 2H, $^3J_{10/14,11/13}$ 8.25 Hz, $^3J_{10/14,12}$ 1.28 Hz, H-10, 14), 7.37 (m, 4H, H-21, 19, 22, 18), 7.32 (m, 3H, H-12, 11, 13), 7.21 (m, 1H, H-20), 6.95 (s, 1H, H-4), 6.04 (s, 1H, H-15), 4.60 (d, 2H, $^3J_{16,15\text{NH}}$ 5.69 Hz, H-16); $^{13}\text{C-NMR}$ (150 MHz, CDCl_3): δ 165.73 (C-2), 160.65 (C-7), 138.68 (C-5), 133.99 (C-17), 128.99 (C-11, 13),

128.90 (C-21, 19), 127.97 (C-10, 14), 127.66 (C-12), 126.81 (C-20), 124.79 (C-22, 18), 118.08 (C-4), 109.56 (C-3), 43.42 (C-16); mass spectrum (HRMS), $m/z = 331.0876$ ($M + 23$)⁺ C₁₈H₁₆N₂OS requires 331.0876 ($M + 23$)⁺.

2-Amino-*N*-(4-methylbenzyl)-5-phenylthiophene-3-carboxamide (61)

Yield: 57% (167 mg); silica gel TLC $R_f = 0.35$ (30% ethyl acetate in hexanes); ¹H-NMR (600 MHz, CDCl₃): δ 7.41 (dd, 2H, ³ $J_{10/14,11/13}$ 8.44 Hz, ³ $J_{10/14,12}$ 1.10 Hz, H-10, 14), 7.32 (m, 2H, H-11, 13), 7.27 (d, 2H, H-22, 18), 7.22 (m, 1H, H-12), 7.19 (d, 2H, H-19, 21), 6.92 (s, 1H, H-4), 2.36 (s, 3H, H-23); ¹³C-NMR (150 MHz, CDCl₃): δ 165.55 (C-7), 160.45 (C-2), 137.31 (C-5), 135.47 (C-20), 133.89 (C-17), 129.46 (C-11, 13), 128.87 (C-21, 19), 127.92 (C-9), 125.40 (C-12), 124.66 (C-10, 14), 117.94 (C-4), 117.94 (C-4), 109.51 (C-3), 43.11 (C-16), 21.14 (C-23); mass spectrum (HRMS), $m/z = 323.1213$ ($M + H$)⁺ C₁₉H₁₈N₂OS requires 323.1227 ($M + 23$)⁺.

2-Amino-*N*-(2-methoxybenzyl)-5-phenylthiophene-3-carboxamide (62)

Yield: 68% (210.9 mg); silica gel TLC $R_f = 0.3$ (30% ethyl acetate in hexanes); ¹H-NMR (600 MHz, CDCl₃): δ 7.41 (dd, 2H, ³ $J_{10/14,11/13}$ 8.44 Hz, ³ $J_{10/14,12}$ 1.10 Hz, H-10, 14), 7.35-7.31 (m, 3H, H-11, 12, 13), 7.29 (m, 1H, H-18), 7.21 (m, 1H, H-19), 6.97-6.94 (m, 2H, H-20, 4), 6.92 (d, 1H, ³ $J_{21,20}$ 8.25 Hz, H-21), 6.29 (s, 1H, H-15), 4.59 (d, 2H, ³ $J_{16,15NH}$ 2.75 Hz, H-16), 3.90 (s, 3H, H-23); ¹³C-NMR (150 MHz, CDCl₃): δ 165.53 (C-7), 157.73 (C-2), 133.99 (C-22), 129.88 (C-9), 129.02 (C-5), 128.99 (C-11, 13), 126.89 (C-12), 126.48 (C-17, 19), 124.88 (C-10, 14), 120.88 (C-18, 20), 118.21 (C-4), 111.13 (C-21), 110.54 (C-3), 55.56 (C-16), 39.24 (C-23); mass spectrum (HRMS), $m/z = 339.1159$ ($M + H$)⁺ C₁₉H₁₈N₂O₂S requires 339.1162 ($M + H$)⁺.

N-(adamantan-1-yl)-2-amino-5-phenylthiophene-3-carboxamide (63)

Yield: 44% (140.4 mg); silica gel TLC $R_f = 0.62$ (30% ethyl acetate in hexanes); ¹H-NMR (600 MHz, CDCl₃): δ 7.44 (dd, 2H, ³ $J_{10/14,11/13}$ 8.44 Hz, ³ $J_{10/14,12}$ 1.28 Hz, H-10, 14), 7.33 (m, 3H, H-13, 11), 7.22 (m, 1H, H-12), 6.90 (s, 1H, H-4), 2.16 (s, 9H, H-23, 20, 21, 18, 17, 22), 1.76 (m, 6H, H-25, 19, 24); ¹³C-NMR (150 MHz, CDCl₃): δ 165.44 (C-7), 158.84 (C-2), 134.07 (C-5), 128.97 (C-11, 13), 126.81 (C-12), 125.82 (C-9), 124.85 (C-10, 14), 118.39 (C-4), 111.64 (C-3), 52.27 (C-16), 42.16 (C-20, 21, 22, 17), 36.53 (C-25, 19, 24), 29.65 (18, 23); mass spectrum (HRMS), $m/z = 353.1697$ ($M + H$)⁺ C₂₁H₂₅N₂OS requires 353.1682 ($M + H$)⁺.

In vitro Mtb Alamar Blue assay (MABA)

Mtb H37Rv is a drug sensitive laboratory reference strain used in the MIC studies.⁴⁰ The bacteria were grown at 37 °C in Difco™ 7H9 Middlebrook liquid media (BD Biosciences, 271310) supplemented with 10% Middlebrook OADC Enrichment, 0.05% Tween (G-Biosciences, 786-519) and 0.2% Glycerol. A modified 96-well microplate Alamar Blue assay (MABA) was used to establish MIC values.⁴¹ Briefly, compounds were solubilized in DMSO and were two-fold serially diluted in 7H9 media (DMSO < or equal to 0.02% DMSO). Bacteria were grown to mid-log growth, diluted and added to the wells with compounds. Cell and media controls were included in each plate. The plates were incubated

at 37 °C for 6 days when Alamar blue was added to each well and then the plate was incubated overnight (Alamar blue final concentration 0.001%). On day seven, the last well that showed no sign of growth and remained blue was considered the MIC for that compound. Standard calculations were used to calculate the IC₅₀ for the compounds.

***In vitro* dual readout MIC assay**

The MIC of compound was determined by measuring bacterial growth after 5 days in the presence of test compounds. Compounds were prepared as 10-point two-fold serial dilutions in DMSO and diluted into 7H9-Tw-OADC medium in 96-well plates with a final DMSO concentration of 2 %. The highest concentration of compound was 200 µM where compounds were soluble in DMSO at 10 mM. Each plate included assay controls for background (medium/DMSO only, no bacterial cells), zero growth (100 µM rifampicin) and maximum growth (DMSO only), as well as a rifampicin dose response curve. Plates were inoculated with *Mtb* and incubated for 5 days; growth was measured by OD₅₉₀. To calculate the MIC, the 10-point dose response curve was plotted as % growth and fitted to the Gompertz model using GraphPad Prism 5. The MIC was defined as the minimum concentration at which growth was completely inhibited and was calculated from the inflection point of the fitted curve to the lower asymptote (zero growth). In addition dose response curves were generated using the Levenberg-Marquardt algorithm and the concentrations that resulted in 50% and 90% inhibition of growth were determined (IC₅₀ and IC₉₀ respectively)

MIC Under Low Oxygen (LORA Assay)

Test compounds were prepared as 20-point two-fold serial dilutions in DMSO and diluted into Dubos-Tw-Albumin (DTA) medium in 96-well plates with a final DMSO concentration of 2%. The highest concentration of compound was 200 µM where compounds were soluble in DMSO at 10 mM. Control compounds (metronidazole and rifampicin) were prepared as two-fold serial dilutions in DMSO and diluted into DTA medium in 96-well plates with a final DMSO concentration of 2%. *M. tuberculosis* constitutively expressing the *luxABCDE* operon was inoculated into DTA medium in gas-impermeable glass tubes and incubated for 18 days to generate hypoxic conditions (Wayne model of hypoxia). At this point, bacteria are in a non-replicating state (NRP stage 2) induced by oxygen depletion. Oxygen-deprived bacteria were inoculated into compound assay plates and incubated under anaerobic conditions for 10 days followed by incubation under aerobic conditions (outgrowth) for 28 h. Growth was measured by luminescence. Oxygen-deprived bacteria were also inoculated into compound assay plates and incubated under aerobic conditions for 5 days. Growth was measured by luminescence. Rifampicin was included in each plate and metronidazole was included in each run as positive controls for aerobic and anaerobic killing of *M. tuberculosis*, respectively.

Minimum bactericidal concentration (MBC)

Mtb was grown aerobically to logarithmic phase and inoculated into liquid 7H9-Tw-OADC medium containing four different compound concentrations with a final maximum concentration of 2% DMSO. Compounds were tested at fixed concentrations, as no MIC data were available at the time. (200, 100, 20 and 5 µM). Cultures were exposed to

compounds for 21 days and cell viability measured by enumerating colony forming units on agar plates on day 0, 7, 14 and 21. MBC was defined as the minimum concentration required to achieve a 2-log kill in 21 days. For compounds with > 1-log kill, an assessment of time- and/or concentration-dependence was determined from the kill kinetics. DMSO was used as a positive control for growth.

Intracellular activity and cytotoxicity

The cytotoxicity of compounds was determined by measuring THP-1 cell viability after 3 days in the presence of test compounds. Compounds were prepared as 10-point three-fold serial dilutions in DMSO. The highest concentration of compound tested was 50 μ M where compounds were soluble in DMSO at 10 mM. THP-1 cells were cultured in complete RPMI and differentiated into macrophage-like cells using 80 nM PMA overnight at 37°C, 5% CO₂. Cells were inoculated into assay plates and cultured for 24h before compound dilutions were added to a final DMSO concentration of 0.5%. Each run included staurosporine as a control. Assay plates were incubated for 3 days at 37°C, 5% CO₂; growth was measured using the CellTiter-Glo[®] Luminescent Cell Viability Assay (Promega) which uses ATP as an indicator of cell viability. Relative luminescent units (RLU) were measured using a Biotek Synergy 4 plate reader. The dose response curve was fitted using the Levenberg–Marquardt algorithm. The IC₅₀ was defined as the compound concentration that produced 50% inhibition of growth.

The activity of compounds against intracellular bacteria was determined by measuring viability in infected THP-1 cell after 3 days in the presence of test compounds. Compounds were prepared as 10-point three-fold serial dilutions in DMSO. The highest concentration of compound tested was 50 μ M where compounds were soluble in DMSO at 10 mM.

THP-1 cells were cultured in RPMI 1640 complete medium and differentiated into macrophage-like cells using 80 nM PMA overnight at 37°C, 5% CO₂. THP-1 cells were infected with a luminescent strain of H37Rv (which constitutively expresses *luxABCDE*) at a multiplicity of infection of 1 and incubated overnight at 37°C, 5% CO₂. Infected cells were recovered using Accutase/EDTA solution, washed twice with PBS to remove extracellular bacteria and seeded into assay plates. Compound dilutions were added to a final DMSO concentration of 0.5%. Assay plates were incubated for 72 h at 37°C, 5% CO₂. Each run included isoniazid as a control. Relative luminescent units (RLU) were measured using a Biotek Synergy 2 plate reader. The dose response curve was fitted using the Levenberg–Marquardt algorithm. The IC₅₀ and IC₉₀ were defined as the compound concentrations that produced 50% and 90 % inhibition of growth respectively.

Effect of 33 on lipid synthesis

Mtb H37Rv mc²6206 grown in 7H9-OADC- 0.05% Tyloxapol supplemented with 0.2% casaminoacid, 48 μ g/mL pantothenate and 50 μ g/mL L-leucine (OD₆₀₀ =0.5) was treated with either no drug (control) or 0.90 μ M **33**, 2.23 μ M **33**, 4.46 μ M **33**, or 8.93 μ M **33** for 16 h. [1,2-¹⁴C]-acetate was added to the culture at the same time as the inhibitor. Total lipids were extracted from bacterial cells, and mycolic acid methyl esters (MAMEs) and fatty acid methyl esters (FAMEs) were prepared and previously described.¹² Total lipids, FAMEs and

MAMEs were analyzed by TLC and revealed by autoradiography. The MIC of **33** against *Mtb* H37Rv mc²6206 grown in 7H9-OADC-Tyloxapol medium was determined as 0.4 µg/ml.

Conclusion

In this study we have prepared a new 2AT compound with excellent activity, **33** MIC = 0.23 µM, which inhibits mycolic acid biosynthesis in *Mtb*. This is the most potent 2AT reported to date. The 2AT library was screened against Ag85C however, no significant activity was observed below 200 µM. Based on structural similarities with reported compounds **33** is presumed to be Pks13 inhibitor or targets an earlier enzyme in the mycolic acid biosynthetic pathway. The loss of antibacterial activity under NRP culture conditions supports the notion that the compounds are targeting mycolic acid biosynthesis. Importantly, the compounds are active against laboratory generated and reference INH, RIF and FQ resistant *Mtb* strains. This suggests the compounds will be active against MDR and XDR strains of *Mtb*. Compound **33** was active at concentrations significantly below concentrations required to effect the viability of human macrophage-like cells; however, the reduced activity in *Mtb* infected macrophage-like cells suggests the physical properties of the lead requires improvement. Taken together, these studies suggest **33** represents a promising preclinical lead for further optimization.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

This work was supported in part by a DeArce Memorial Fund grant to S. J. S. from The University of Toledo, a grant from the National Institutes of Health (grant number AI105084) to S.J.S and D. R. R., and with Federal funds from the Division of Microbiology and Infectious Diseases, National Institute of Allergy and Infectious Diseases (Dr. Jim Boyce), National Institutes of Health, Department of Health and Human Services, under Contract No. HHSN2722011000091/HHSN27200002 A14.

Notes and references

1. WHO. Global Tuberculosis Report 2015. 20 edn.. World Health Organization France; 2015.
2. Dmitriev BA, Ehlers S, Rietschel ET, Brennan PJ. Int. J. Med. Microbiol. 2000; 290:251–258. [PubMed: 10959727]
3. Umesiri FE, Sanki AK, Boucau J, Ronning DR, Sucheck SJ. Med. Res. Rev. 2010; 30:290–326. [PubMed: 20099253]
4. Foley HN, Stewart JA, Kavunja HW, Rundell SR, Swarts BM. Angew Chem Int Ed Engl. 2016; 55:2053–2057. [PubMed: 26757001]
5. Marrakchi H, Laneelle MA, Daffe M. Chem. Biol. 2014; 21:67–85. [PubMed: 24374164]
6. Slayden RA, Barry CE 3rd. Tuberculosis. 2002; 82:149–160. [PubMed: 12464486]
7. Sabine G, Fabienne B, Françoise L, Cécile B, Wladimir M, Christian C, Christophe G, Lionel M, Mamadou D, Annaïk Q. Chem. Biol. 2014; 21:16601669.
8. Lea-Smith DJ, Pyke JS, Tull D, McConville MJ, Coppel RL, Crellin PK. J Biol Chem. 2007; 282:11000–11008. [PubMed: 17308303]

9. Wilson R, Kumar P, Parashar V, Vilcheze C, Veyron-Churlet R, Freundlich JS, Barnes SW, Walker JR, Szymonifka MJ, Marchiano E, Shenai S, Colangeli R, Jacobs WR Jr, Neiditch MB, Kremer L, Alland D. *Nat. Chem. Biol.* 2013; 9:499–506. [PubMed: 23770708]
10. Mary J, Catherine R, Marie-Antoinette L, Christophe G, Christine LW, Danielle E, Brigitte G, Mamadou D. *Mol. Microbiol.* 1999; 31:1573–1587. [PubMed: 10200974]
11. Portevin D, De Sousa-D'Auria C, Houssin C, Grimaldi C, Chami M, Daffé M, Guilhot C. *Proc. Natl. Acad. Sci. U.S.A.* 2004; 101:314–319. [PubMed: 14695899]
12. Grzegorzewicz AE, Pham H, Gundi VA, Scherman MS, North EJ, Hess T, Jones V, Gruppo V, Born SE, Kordulakova J, Chavadi SS, Morisseau C, Lenaerts AJ, Lee RE, McNeil MR, Jackson M. *Nat Chem Biol.* 2012; 8:334–341. [PubMed: 22344175]
13. Belisle JT, Vissa VD, Sievert T, Takayama K, Brennan PJ, Besra GS. *Science.* 1997; 276:1420–1422. [PubMed: 9162010]
14. Grundner C, Perrin D, Hooft van Huijsduijnen R, Swinnen D, Gonzalez J, Gee CL, Wells TN, Alber T. *Structure.* 2007; 15:499–509. [PubMed: 17437721]
15. Balamurugan K, Perumal S, Reddy ASK, Yogeewari P, Sriram D. *Tetrahedron Lett.* 2009; 50:6191–6195.
16. Lu X, Wan B, Franzblau SG, You Q. *Eur. J. Med. Chem.* 2011; 46:3551–3563. [PubMed: 21641695]
17. Nallangi R, Samala G, Sridevi JP, Yogeewari P, Sriram D. *Eur. J. Med. Chem.* 2014; 76:110–117. [PubMed: 24583350]
18. Silva Lourenco MC, Rodrigues Vicente F, de Oliveira Henriques M, Peixoto Candea AL, Borges Goncalves RS, Nogueira TC, de Lima Ferreira M, Nora de Souza MV. *Bioorg. Med. Chem. Lett.* 2007; 17:6895–6898. [PubMed: 17967538]
19. Kantevari S, Patpi SR, Sridhar B, Yogeewari P, Sriram D. *Bioorg. Med. Chem. Lett.* 2011; 21:1214–1217. [PubMed: 21237641]
20. Warriar T, Tropis M, Werngren J, Diehl A, Gengenbacher M, Schlegel B, Schade M, Oschkinat H, Daffe M, Hoffner S, Eddine AN, Kaufmann SHE. *Antimicrob. Agents Chemother.* 2012; 56:1735–1743. [PubMed: 22290959]
21. Scheich C, Puetter V, Schade M. *J. Med. Chem.* 2010; 53:8362–8367. [PubMed: 21073150]
22. Ibrahim DA, Boucau J, Lajiness DH, Veleti SK, Trabbic KR, Adams SS, Ronning DR, Sucheck SJ. *Bioconjugate Chem.* 2012; 23:2403–2416.
23. Favrot L, Grzegorzewicz AE, Lajiness DH, Marvin RK, Boucau J, Isailovic D, Jackson M, Ronning DR. *Nat. Commun.* 2013; 4:1–10.
24. Masakazu F, Taketsugu S, Haruaki I, Naoko I. *Bioorg. Med. Chem. Lett.* 2002; 12:1607–1611. [PubMed: 12039573]
25. Takeji E, Hitomi S, Tetsushi W, Teruhisa H, Yoshiharu H. *Environ. Sci. Technol.* 1997; 31:2772–2776.
26. Nikolakopoulos G, Figler H, Linden J. *Bioorg. Med. Chem.* 2006; 14:2358–2365. [PubMed: 16314104]
27. Ollinger J, Bailey MA, Moraski GC, Casey A, Florio S, Alling T, Miller MJ, Parish T. *Plos One.* 2013; 8:e60531. [PubMed: 23593234]
28. Zelmer A, Carroll P, Andreu N, Hagens K, Mahlo J, Redinger N, Robertson BD, Wiles S, Ward TH, Parish T, Ripoll J, Bancroft GJ, Schaible UE. *J Antimicrob Chemoth.* 2012; 67:1948–1960.
29. Carroll P, Schreuder LJ, Muwanguzi-Karugaba J, Wiles S, Robertson BD, Ripoll J, Ward TH, Bancroft GJ, Schaible UE, Parish T. *Plos One.* 2010; 5:e9823. [PubMed: 20352111]
30. Lambert RJW, Pearson J. *J Appl Microbiol.* 2000; 88:784–790. [PubMed: 10792538]
31. Cho SH, Warit S, Wan BJ, Hwang CH, Pauli GF, Franzblau SG. *Antimicrob. Agents Chemother.* 2007; 51:1380–1385. [PubMed: 17210775]
32. Andreu N, Zelmer A, Fletcher T, Elkington PT, Ward TH, Ripoll J, Parish T, Bancroft GJ, Schaible U, Robertson BD, Wiles S. *Plos One.* 2010; 5:e10777. [PubMed: 20520722]
33. Wayne LG. *Methods Mol. Med.* 2001; 54:247–269. [PubMed: 21341080]
34. Gold B, Roberts J, Ling Y, Quezada LL, Glasheen J, Ballinger E, Somersan-Karakaya S, Warriar T, Warren JD, Nathan C. *Antimicrob Agents Ch.* 2015; 59:6521–6538.

35. Ollinger J, Bailey M, Casey A, Florio S, Alling T, Moraski GC, Miller MJ, Parish T. Plos One. 2013; 8:e60531. [PubMed: 23593234]
36. Johnston JC, Shahidi NC, Sadatsafavi M, Fitzgerald JM. Plos One. 2009; 4:e6914. [PubMed: 19742330]
37. Johnsson K, King DS, Schultz PG. J Am Chem Soc. 1995; 117:5009–5010.
38. Johnsson K, Schultz PG. J Am Chem Soc. 1994; 116:7425–7426.
39. Pantel A, Petrella S, Veziris N, Brossier F, Bastian S, Jarlier V, Mayer C, Aubry A. Antimicrob. Agents Chemother. 2012; 56:1990–1996. [PubMed: 22290942]
40. Knudson SE, Kumar K, Awasthi D, Ojima I, Slayden RA. Tuberculosis. 2014; 94:271–276. [PubMed: 24746463]
41. Kumar K, Awasthi D, Lee SY, Zanardi I, Ruzsicska B, Knudson S, Tonge PJ, Slayden RA, Ojima I. J. Med. Chem. 2011; 54:374–381. [PubMed: 21126020]

Figure 1.
Mechanism of Pks13. FadD32 is a long-chain acyl-AMP ligase (**FAAL32**)

Figure 2.

Selected structures of previously reported Ag85C and Pks13 inhibitors with a 2-aminothiophene core. FRT = fused ring 2-aminothiophenes, 5PT = 2-amino-5-phenylthiophenes. A medium sized library of FRT and 5PT has been synthesized as antitubercular agents in this work. R^1 = NCbz, NBoc, NEt, CH_2 , R^2 = acyl, R^3 = alkylamino or alkyloxy.

Scheme 1.

Synthesis scheme for 2-aminothiophene cores **22** to **63**. Reagents and conditions: i) S_8 , Et_2N , EtOH , **22** (86%), **23** (63%), **24** (75%), **25** (83%), **26** (61%); (ii) Acid chloride (AC), Et_3N , DCM , DMAP (15-82%); (iii) LiOH , THF : H_2O : MeOH (3:1:1) (44-89%); (iv) Alkylamine (AA), EDC , HCl , HOBt , DMAP , dry CH_2Cl_2 (21-61%). Diversity of substrates used in this investigation.

27, 41%; R¹ = NBoc, R² = CH₃CO, R³ = OEt

28, 23%; R¹ = NBoc, R² = C₆H₅CO, R³ = OEt

29, 82%; R¹ = NBoc, R² = C₆F₅CO, R³ = OEt

30, 39%; R¹ = NBoc, R² = 3,5-(CF₃)₂C₆H₃CO, R³ = OEt

31, 45%; R¹ = NEt, R² = CH₃CO, R³ = OEt

32, 35%; R¹ = NEt, R² = C₆H₅CO, R³ = OEt

33, 45%; R¹ = NEt, R² = C₆F₅CO, R³ = OEt

34, 43%; R¹ = NEt, R² = 3,5-(CF₃)₂C₆H₃CO, R³ = OEt

35, 22%; R¹ = NEt, R² = 3,5-(CF₃)₂C₆H₃CO, R³ = 4-(CH₃)BnNH

36, 59%; R¹ = CH₂, R² = CH₃CO, R³ = OEt

37, 57%; R¹ = CH₂, R² = C₆H₅CO, R³ = OEt

38, 50%; R¹ = CH₂, R² = C₆F₅CO, R³ = OEt

39, 15%; R¹ = CH₂, R² = 3,5-(CF₃)₂C₆H₃CO, R³ = OEt

40, 69%; R¹ = NCbz, R² = H, R³ = OH

41, 89%; R¹ = NBoc, R² = H, R³ = OH

42, 66%; R¹ = NEt, R² = H, R³ = OH

43, 44%; R¹ = CH₂, R² = H, R³ = OH

44, 36%; R¹ = NEt, R² = H, R³ = BuNH

45, 25%; R¹ = NEt, R² = H, R³ = BnNH

46, 40%; R¹ = NEt, R² = H, R³ = 4-(CH₃)BnNH

47, 30%; R¹ = NEt, R² = H, R³ = 2-(OCH₃)BnNH

48, 29%; R¹ = NEt, R² = H, R³ = AdamantyINH

49, 26%; R¹ = CH₂, R² = H, R³ = BuNH

50, 24%; R¹ = CH₂, R² = H, R³ = BnNH

51, 36%; R¹ = CH₂, R² = H, R³ = 4-(CH₃)BnNH

52, 23%; R¹ = CH₂, R² = H, R³ = 2-(OCH₃)BnNH

53, 21%; R¹ = CH₂, R² = H, R³ = AdamantyINH

54, 58%; R² = CH₃CO, R³ = OEt

55, 94%; R² = C₆H₅CO, R³ = OEt

56, 45%; R² = C₆F₅CO, R³ = OEt

57, 45%; R² = 3,5-(CF₃)₂C₆H₃CO, R³ = OEt

58, 83%; R² = H, R³ = OH

59, 61%; R² = H, R³ = BuNH

60, 59%; R² = H, R³ = BnNH

61, 57%; R² = H, R³ = 4-(CH₃)BnNH

62, 68%; R² = H, R³ = 4-(CH₃)BnNH

63, 44%; R² = H, R³ = AdamantyINH

Figure 3.

2-Aminothiophenes prepared. Yield, written after compound number

Figure 4. The bactericidal activity of **33** assessed against *Mtb* H37Rv grown in aerobic conditions in 7H9-Tw-OADC medium. Viable cell counts are measured over 3 weeks of exposure to determine the rate of kill.

Figure 5.

(A) Effect of **33** on TMM and TDM synthesis. The same volume of total lipid samples was loaded per lane and the TLC plate was developed in chloroform:methanol:water (20:4:0.5, by vol.). The cells treated with **33** showed the significant decrease in TMM and TDM contents concomitant with a slight increase in free mycolates (FM). The synthesis of other lipids (phosphatidylethanolamine, PE; cardiolipin, CL) is not affected by **33**. Mycolic acid methyl esters (MAMEs) were prepared from extractable lipids (B) and delipidated cells (C) so as to analyze separately the populations of mycolates esterified in the cell wall arabinogalactan and extractable lipids; the same volume of samples was loaded per lane. The TLC plate was developed three times in the solvent system n-hexanes:ethyl acetate (95:5, by vol.). The cells treated with **33** showed the significant decrease in all forms of mycolic acids (alpha-, methoxy- and keto-) isolated either from extractable lipids or cell wall. The results are indicative of a general, **33**-concentration dependent, decrease in mycolic acid biosynthesis in the treated cells.

Table 1MICs and IC₅₀s (μM) of the 2AT Library *Mtb* H37Rv

Comp No	NV/CLogP	MIC	IC ₅₀	Comp No	NV/CLogP	MIC	IC ₅₀
22	0/2.80	> 200	N/A	43	0/1.88	507	N/A
23	0/2.39	> 300	N/A	44	0/2.15	> 300	> 300
24	0/1.73	> 300	N/A	45	0/2.11	> 300	N/A
25	0/2.52	> 400	N/A	46	0/2.56	> 300	N/A
26	0/3.26	> 400	N/A	47	0/2.12	> 200	N/A
27	0/2.12	> 200	N/A	48	0/3.48	> 200	N/A
28	0/3.79	> 200	N/A	49	0/2.94	> 300	N/A
29	1/4.33	> 100	N/A	50	0/2.90	> 300	N/A
30	2/5.51	> 100	N/A	51	0/3.35	333	N/A
31	0/1.46	> 300	N/A	52	0/2.91	316	N/A
32	0/3.50	279	N/A	53	0/4.27	> 300	N/A
33	0/4.03	0.69	0.65	54	0/3.00	> 300	N/A
34	1/5.22	> 200	N/A	55	0/4.67	> 200	N/A
35	2/5.69	350	N/A	56	1/5.20	> 200	N/A
36	0/2.25	> 300	N/A	57	1/6.39	> 200	N/A
37	0/3.95	> 600	N/A	58	0/2.62	228	129
38	0/4.45	30	13	59	0/3.68	> 300	N/A
39	1/5.64	> 200	N/A	60	0/3.64	325	N/A
40	0/2.16	> 300	N/A	61	0/4.09	> 300	N/A
41	0/1.75	> 300	N/A	62	0/3.65	> 200	N/A
42	0/1.09	> 400	N/A	63	1/5.01	> 200	N/A

NV = Number of violations for Lipinski rule of five, CLogP = Calculated LogP value.

Table 2

Evaluation of 33 MICs and ICs (μM) against *Mtb* under aerobic, low oxygen, and intracellular conditions.
Evaluation of 33 against drug resistant strains of *Mtb*.

H37RV (aerobic)	H37RV (low oxygen)	H37RV (intracellular)	INH-R1	INH-R2	RIF-R1	RIF-R2	FQ-R1
MIC = 0.23	MIC > 200		MIC = 0.44	MIC = 0.20	MIC = 0.30	MIC = 0.44	MIC = 0.37
MIC ₅₀ = 0.12	IC ₅₀ = 0.27	IC ₅₀ = 5.6	IC ₅₀ = 0.16	IC ₅₀ = 0.053	IC ₅₀ = 0.095	IC ₅₀ = 0.11	IC ₅₀ = 0.14
MIC ₉₀ = 0.23	IC ₉₀ = 15	IC ₉₀ = 9.75	IC ₉₀ = 0.45	IC ₉₀ = 0.20	IC ₉₀ = 0.35	IC ₉₀ = 0.48	IC ₉₀ = 0.39
Controls							
RIF	MIC = 0.048, n=8		MIC = 0.012	MIC = 0.0070	MIC = 3.5	MIC > 50	MIC = 0.020
INH		IC ₅₀ = 0.24	MIC > 200	MIC > 200	MIC = 0.18	MIC > 0.60	MIC = 0.32
Levofloxacin			MIC = 1.3	MIC = 1.4	MIC = 1.2	MIC = 1.2	MIC = 24

INH-R1 was derived from H37Rv and is a katG mutant (Y155* = truncation). INH-R2 is strain ATCC 35822. RIF-R1 was derived from H37Rv and is an rpoB mutant (S522L). RIF-R2 is strain ATCC 35838. FQ-R1 is a fluoroquinolone-resistant strain derived from H37Rv and is a gyrB mutant (D94N).