Journal of Medicinal Chemistry

Article

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# Identification of Less Lipophilic Riminophenazine Derivatives for the Treatment of Drug-Resistant Tuberculosis

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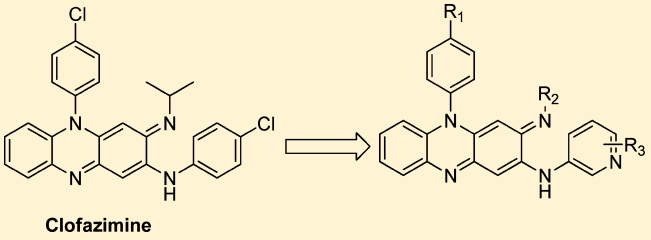
Supporting Information

**ABSTRACT:** Clofazimine (CFZ), a member of the riminophenazine class, has been studied in clinical trials for the treatment of multidrug-resistant tuberculosis (MDR-TB). CFZ has several side effects which can be attributed to its extremely high lipophilicity. A series of novel riminophenazine analogues bearing a C-2 pyridyl substituent was designed and synthesized with the goal of maintaining potent activity against *Mycobacterium tuberculosis* (*M. tuberculosis*) while improving upon its safety profile by lowering the lipophilicity. All compounds were evaluated for their in vitro activity and cytotoxicity. The results demonstrated that many new compounds had potent activity against *M. tuberculosis* with MICs of less than 0.03 μg/mL and low cytotoxicity with $IC_{50}$ values greater than 64 μg/mL. Some compounds were tested for in vivo efficacy against MDR-TB in an experimental mouse infection model. Two compounds demonstrated equivalent or better efficacy than CFZ in this model with significantly reduced skin discoloration potential.




## INTRODUCTION

The spread of multidrug-resistant tuberculosis (MDR-TB) and the emergence of extensively drug-resistant tuberculosis (XDR-TB) pose a challenge for the treatment and control of this devastating disease, one of the leading causes of death in the world.[1] The treatment of MDR-TB is lengthy, expensive, and extremely difficult, requiring daily administration of a combination of 5−7 drugs, some of them injectable, for a period of 18−24 months. Some of these existing drugs have shown significant toxicities.[2] As a consequence, fewer than 3% of MDR-TB patients worldwide are adequately treated, which contributes to the further spread of MDR-TB and the emergence of XDR-TB.[3] Therefore, there is a significant unmet medical need for safer, more effective therapy against MDR-TB.

Despite the increased effort over the past decade in searching for new drugs against TB, compounds with a novel mode of action against *M. tuberculosis* are still scarce, which presents a significant barrier for the development of a completely new regimen for the treatment of MDR-TB. It has been recognized that repurposing or optimizing existing antibiotics could be an effective approach in identifying new TB drugs.[4] Clofazimine (CFZ, Figure 1), an antimycobacterial agent first introduced in

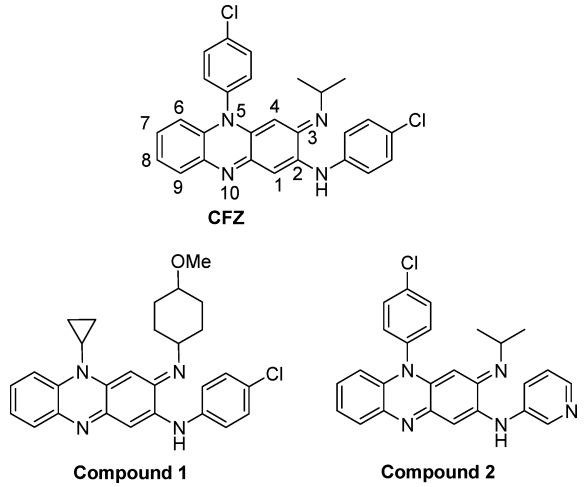
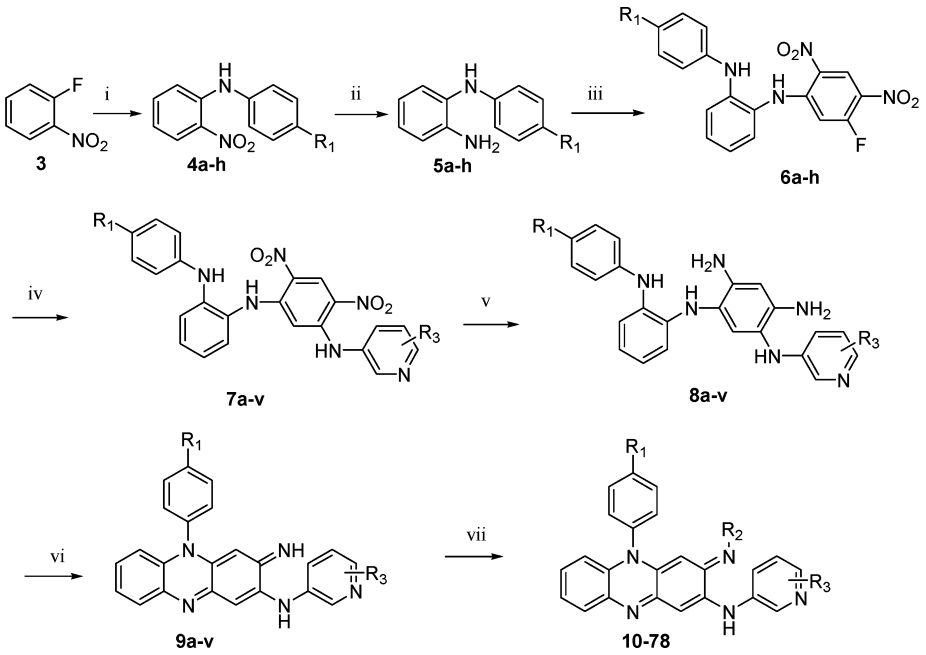



**Figure 1.** Structures of CFZ and its analogues.

**Received:** June 12, 2012
**Published:** August 29, 2012

**Scheme 1. Synthesis of the Target Compounds 10−78**[a]




4a-6a: $R_1$=H;
4b-6b: $R_1$=Me;
4c-6c: $R_1$=SMe;
4d-6d: $R_1$=F;
4e-6e: $R_1$=$CF_3$;
4f-6f: $R_1$=$OCF_3$;
4g-6g: $R_1$=Br;
4h-6h: $R_1$=Cl;

7a-9a: $R_1$=H, $R_3$=H;
7b-9b: $R_1$=Me, $R_3$=H;
7c-9c: $R_1$=SMe, $R_3$=H;
7d-9d: $R_1$=F, $R_3$=H;
7e-9e: $R_1$=$CF_3$, $R_3$=H;
7f-9f: $R_1$=$OCF_3$, $R_3$=H;
7g-9g: $R_1$=Br, $R_3$=H;
7h-9h: $R_1$=Cl, $R_3$=H;

7i-9i: $R_1$=Cl, $R_3$=2-Me;
7j-9j: $R_1$=Cl, $R_3$=6-Me;
7k-9k: $R_1$=Cl, $R_3$=2-OMe;
7l-9l: $R_1$=Cl, $R_3$=5-Br;
7m-9m: $R_1$=Br, $R_3$=2-Me;
7n-9n: $R_1$=Br, $R_3$=6-Me;
7o-9o: $R_1$=Br, $R_3$=2-OMe;
7p-9p: $R_1$=F, $R_3$=2-Me;

7q-9q: $R_1$=F, $R_3$=6-Me;
7r-9r: $R_1$=F, $R_3$=2-OMe;
7s-9s: $R_1$=$CF_3$, $R_3$=2-Me;
7t-9t: $R_1$=$CF_3$, $R_3$=6-Me;
7u-9u: $R_1$=$CF_3$, $R_3$=2-OMe;
7v-9v: $R_1$=$OCF_3$, $R_3$=2-OMe

[a]Reagents and conditions: (i) KF, $R_1$-aniline, 160 °C; (ii) 10% Pd/C, $H_2$ or Zn/$CH_3COOH$, rt; (iii) DFDNB, $Et_3N$, ethanol, rt; (iv) 3-aminopyridine or substituted 3-aminopyridine, $Et_3N$, THF, reflux; (v) 10% Pd/C, $H_2$, rt or Zn/$CH_3COOH$, 0 °C−rt; (vi) air, methanol, rt; (vii) $R_2NH_2$, $CH_3COOH$, dioxane, reflux or in sealed tube, 110 °C.

the 1960s for the treatment of leprosy,[5] has demonstrated excellent activity against various drug-resistant *M. tuberculosis* strains and is currently in clinical trials as a component of new regimens for the treatment of MDR-TB.[6] CFZ is a member of the riminophenazine structural family.[7,8] Its unique mechanism of action (MOA)[9] precludes it from rapid development of drug resistance. However, this compound is highly lipophilic (log *P* 5.39)[10] and accumulates extensively in skin and fat tissues, leading to an extremely long terminal half-life and undesirable side effects such as skin discoloration.[11] The undesirable properties of CFZ may preclude its widespread use in the future for the treatment of TB. Structural modification of CFZ to address the above concerns is therefore an important approach to fully utilizing the novel mode of action that this drug class could offer. The goal of our ongoing effort in this area is to identify less lipophilic derivatives of CFZ that are at least as potent as CFZ in vitro and in vivo but which exhibit improved pharmacokinetic (PK) profiles and reduced potential for skin discoloration.[7,12]

We have previously conducted extensive structural modification on CFZ to address the skin discoloration problem, a major obstacle for its clinical use. Replacement of the N-5 phenyl ring with an alkyl substituent (e.g., compound **1**, Figure 1)[13] and the C-2 phenyl ring with pyridyl (e.g., compound **2**, Figure 1)[14,15] led to analogues with reduced lipophilicity. Compound **2** demonstrated potent activity against *M. tuberculosis* both in vitro and in vivo and an improved PK profile as compared to CFZ. However, the safety profile of compound **2** as measured by its acute toxicity in mice appeared less desirable as compared to CFZ ($LD_{50}$ 422 mg/kg vs >3000 mg/kg). In addition to addressing the skin discoloration issue, the present work aimed to identify additional C-2 pyridyl analogues with improved therapeutic windows while maintaining a favorable PK and efficacy profile.

Because of the high lipophilicity and redox nature of CFZ (redox potential −0.18 V at pH 7), Barry et al. have hypothesized intracellular redox cycling as a potential mechanism of CFZ-mediated antimicrobial activity.[5] This hypothesis was further supported by the work conducted by Yano et al.[16] On the basis of these findings, we hypothesized that the substituents at the C-2, C-3, and N-5 positions in a riminophenazine molecule might modulate the redox potential of the phenazine pharmacophore and at the same time influence the physicochemical properties of the molecule. Hence we designed the following three target compound series to further study the impact of each of the three substituent patterns:

- In the first series, we focused on the N-5 modification while keeping the C-3 isopropylimino and C-2 pyridyl group intact. When the 4-chloro group in compound **2** was replaced by various substituents, a series of new N-5 derivatives **10**−**16** was obtained.
- In the second series, we focused on the C-3 modification by replacing the isopropyl group at this position with a nitrogen or oxygen-containing alkyl group. Compounds in this series (**17**−**38**) contain one of the following


dx.doi.org/10.1021/jm300828h | *J. Med. Chem.* 2012, 55, 8409−8417

substitutions: either the 4-tetrahydropyranyl, 4-methoxycyclohexyl, *N*-methyl-4-piperidyl, or *N*-isobutyl-4-piperidyl group.

- In the third series, we focused on the C-2 modification by introducing various substituents, including both electron-donating and electron-withdrawing groups, to the pyridyl ring (compound **39**–**78**).

This report details our findings regarding the structure–activity relationships of riminophenazine derivatives bearing a C-2 pyridyl substituent. We further report additional in vitro and in vivo data for selected compounds in the series to illustrate the potential of this novel riminophenazine series for the treatment of MDR- and XDR-TB.

## CHEMISTRY

The synthetic route for target compounds **10**–**78** is outlined in Scheme 1. Aromatic nucleophilic substitution of commercially available 2-fluoronitrobenzene (**3**) with aryl amines afforded compounds **4a**–**h**.[17,18] The nitro group in compounds **4a**–**f** was reduced via catalytic hydrogenation. For compounds **4g** and **4h**, zinc powder and glacial acetic acid were used to reduce the nitro group, as the halogen on the phenyl ring could be cleaved under catalytic hydrogenation conditions. Compounds **5a**–**h** were subsequently reacted with 1,5-difluoro-2,4-dinitrobenzene (DFDNB) under ambient temperature to give the corresponding **6a**–**h** as red or orange solids in good yields. The remaining fluorine group in compounds **6a**–**h** was substituted by conventional aromatic nucleophilic displacement with unsubstituted or substituted 3-aminopyridines, such as 3-amino-2-methylpyridine, 3-amino-6-methylpyridine, 3-amino-2-methoxypyridine, and 3-amino-5-bromopyridine, in ethanol, to give key intermediates **7a**–**v**. Reduction of both nitro groups in **7a**–**f** and **7p**–**v** by catalytic hydrogenation gave tetra-amines **8a**–**f** and **8p**–**v**, which underwent spontaneous oxidative cyclization to afford riminophenazines **9a**–**f** and **9p**–**v**. Accordingly, **7g**–**o** were subjected to reduction using zinc powder and glacial acetic acid similar to the conditions used for the reduction of compounds **4g** and **4h**. The crude products **8g**–**o** were washed with water or dilute ammonia to remove any remaining acetic acid for spontaneous oxidative cyclization to afford imine products **9g**–**o**.[19] Finally, replacement of the imine (**9a**–**v**) with commercially available amines or amines (*N*-isobutyl-4-aminopiperidine, *N*-methyl-4-aminopiperidine, 4-aminotetrahydropyrane, and 4-methoxycyclohexylamine)[20−22] prepared by known methods provided target compounds (**10**–**78**),[23,24] which were further purified via column chromatography or recrystallization. We found that a catalytic amount of glacial acetic acid could facilitate the final reaction and shorten the reaction time significantly. For example, for target compound **10**, the reaction was complete in about 13 h in the presence of a catalytic amount glacial acetic acid, while it took 20 h in the absence of acetic acid.

All target compounds were characterized by $^{1}$H NMR, $^{13}$C NMR, and HRMS spectra. One-dimensional NOE study performed on compound **50** showed that the imino double bond in this molecule had an E configuration. Moreover, the Py-4 H on the pyridyl ring is in close proximity to 1-H on the phenazine ring, deduced from the fact that a positive NOE was observed at Py-4 H upon irradiation 1-H (Figure 2). This interesting NOE phenomenon led us to further study the configuration of this novel C-2 pyridyl substituted riminophenazine series. The single-crystal X-ray structure of CFZ was

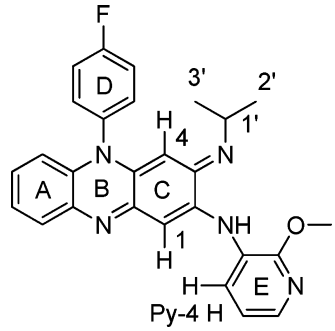



**Figure 2.** Structure of compound **50**.

reported by Hodgson in 1985.[25] The literature data showed that the value of the bond angle $\alpha$ at N(3) [defined by C(3)–N(3)–C(21) in CFZ] may be indicative of in vitro antileprosy activity in these systems (Figure 3). Intramolecular hydrogen

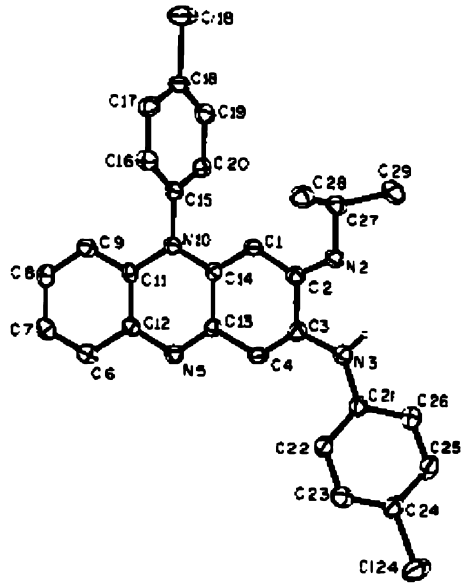



**Figure 3.** X-ray crystallographic structure of CFZ.

bonding involves N(3)-H···N(2) interaction, leading to a new five-membered ring approximately coplanar with the phenazine core. The authors hypothesized that compounds with an expanded $\alpha$ angle (131 ± 1°) induced by an intramolecular hydrogen bonding would be active in vitro. We also hypothesized that the five-membered ring formed through this intramolecular hydrogen bonding would increase the planarity of the molecules and promote intermolecular $\pi$–$\pi$ stacking interactions, which might be partially responsible for CFZ's extremely low solubility, causing crystallization in fat tissues and skin discoloration.

Single crystals of compound **50** were obtained from a solution of acetone and characterized by X-ray diffraction. The crystal structure of compound **50** is depicted in Figure 4. The lattice parameters were $a$ = 8.031(3), $b$ = 10.835(4), and $c$ = 13.656(1) Å, and the lattice volume was 2404.3(1) Å$^3$. There were four molecules in one lattice. The dihedral angles between rings were **A** (C1–C2–C3–C4–C5–C6)/**B** (N1–C1–C6–N2–C7–C12): 1.0°, **B** (N1–C1–C6–N2–C7–C12)/**C** (C11–C12–C7–C8–C9–C10): 0.3°, **B** (N1–C1–C6–N2–C7–C12)/**D** (C13–C14–C15–C16–C17–C18): 85.6°, and


dx.doi.org/10.1021/jm300828h | *J. Med. Chem.* 2012, 55, 8409–8417

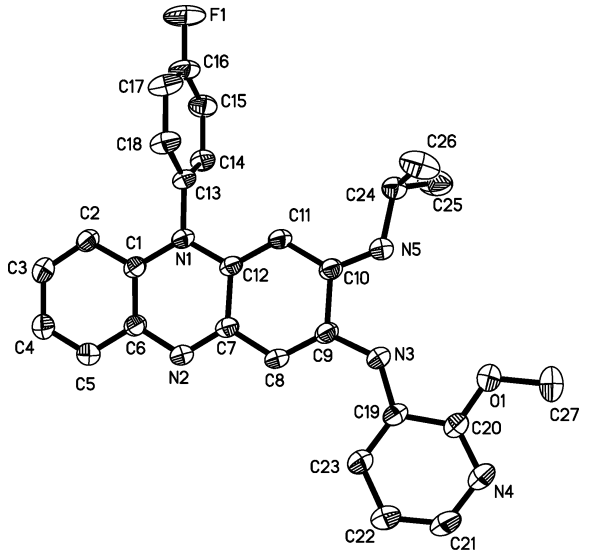



**Figure 4.** X-ray crystallographic structure of compound **50**.

**C** (C11−C12−C7−C8−C9−C10)/**E** (C19−C20−N4−C21−C22−C23): 18.4°. The configuration of the imin0 double bond is an E configuration, as already indicated by the 1D NOE data. Interestingly, the only intramolecular hydrogen bonding formed in this molecule is by N(3)-H···O(1) within the pyridyl group. This hydrogen bonding prevents the formation of the same intramolecular hydrogen bonding between N(3)-H···N(2) as observed in CFZ (Figure 3), and the important $\alpha$ angle C(9)−N(3)−C(19) is 132.8°, which was very similar to the $\alpha$ angle of 131 ± 1° as observed in CFZ. Although the phenazine core in this new series of compounds is the same as that in CFZ, the absence of the five-membered ring formed by the intramolecular hydrogen bonding between N(3)-H···N(2) could potentially reduce the extent of the molecular planarity, suggesting that the intermolecular $\pi-\pi$ stacking might be interrupted in this new series of riminophenazine derivatives. We hoped that this may translate into better solubility and reduced potential for skin discoloration. In fact, we found that the solubility of compound **50** in pH 1 buffer solution is 0.70 g/100 mL, but CFZ is practically insoluble.[15]

## RESULTS AND DISCUSSION

All new compounds synthesized through this program were tested for their activity against *M. tuberculosis* H37Rv using the Microplate Alamar Blue Assay (MABA) in 96-well plate format; the minimum inhibitory concentration (MIC) effecting >90% growth inhibition was recorded. The compounds were further tested for mammalian cell cytotoxicity using Vero cells measured as a concentration inhibiting 50% growth ($IC_{50}$) as compared to a no-treatment control.[15] Table 1 summarizes the biological data for 69 new riminophenazine derivatives. CFZ and compound **2** were used as reference compounds. The lipophilicity of these compounds was estimated using their ClogP values, calculated using the MedChem Studio prediction software (ADMET Predictor 5.5, Simulations Plus Inc.). Generally, all compounds bearing a C-2 pyridyl group have lower ClogP values as compared to CFZ. Additional replacement of the isopropyl group on the imino group at the C-3 position by an alkyl group with a heteroatom such as N or O, with the exception of the 4-methoxycyclohexyl and *N*-isobutyl-4-piperidyl groups, further reduced ClogP values as exemplified by compound **17** (ΔClogP = −0.41), **19** (ΔClogP = −0.04) and **21** (ΔClogP = −0.72) as compared with compound **2**. However, all compounds with a substituent on the C-2 pyridyl group, regardless the nature of the substituent (2-$OCH_3$, 2-$CH_3$, 6-$CH_3$ or 5-Br), showed slightly increased ClogP as compared with their parents.

Interestingly, most of the compounds bearing a substituted pyridyl group exhibited potent in vitro activity against *M. tuberculosis*, with MIC values less than 0.03 $\mu$g/mL as compared with compound **2**. This observation seems consistent with general SAR knowledge for the riminophenazine class, that lipophilic compounds tend to be more potent.[11] *M. tuberculosis* cells have an exceptionally lipophilic cell wall and require lipophilic molecules to penetrate through passive uptake.[26] Therefore, striking a balance between reducing lipophilicity and retaining potent antimicrobial activity has been one of the major challenges in searching for new riminophenazine derivatives.

The effect of replacing the chloro atom on the N-5 phenyl ring with an electron-donating or electron-withdrawing group was first examined as shown in Table 1. Compounds **11** and **12**, with an electron-donating substituent, showed slightly weaker activity than compound **2**, whereas compounds **13**, **14**, **15**, and **16**, with a F, $CF_3$, $OCF_3$, and Br substituents, respectively, retained potent activity.

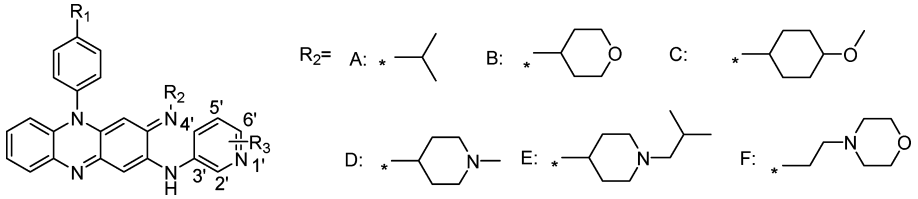
Compounds **17**−**38** possess a heterocyclic $R_2$ group attached to the imino nitrogen at the C-3 position. All compounds with a nitrogen-containing $R_2$ group showed relatively weaker potency as compared with compound **2**, with MICs ranging from 0.06 to 0.5 $\mu$g/mL. Compounds with an oxygen-containing group at this position displayed potent activity. This observation is consistent with the results obtained from our previous study.[13] 4-Tetrahydropyranyl and 4-methoxycyclohexyl substituents at this position provide both potent activity and reduce lipophilicity as measured by ClogP.

Although most compounds with an unsubstituted pyridyl group at the C-2 position had potent antimycobacterial activity, many compounds (**10**−**38**) showed a certain level of cytotoxicity, with the exception of compounds **14**, **23**, **27**, **32**, and **33**. We decided to explore the impact of a substituent $R_3$ on the potency and cytotoxicity of this promising series by keeping an electron-withdrawing substituent attached to the phenyl group at $R_1$ and an isopropyl, 4-tetrahydropyranyl, or 4-methoxycyclohexyl group at $R_2$. A larger set of substituted pyridyl analogues, such as 2-$OCH_3$, 2-$CH_3$, 6-$CH_3$, and 5-Br, were synthesized and evaluated. The majority of the compounds bearing a substituted pyridyl group showed potent antimycobacterial activity with MIC values less than 0.03 $\mu$g/mL. More importantly, many compounds in the series had low cytotoxicity, with $IC_{50}$ values greater than 64 $\mu$g/mL.

Prior to further conducting in vivo efficacy studies, a preliminary in vivo tolerability study was carried out in mice for 16 compounds which demonstrated potent activity against *M. tuberculosis* while maintaining low cytotoxicity. On the basis of the previous data obtained for compound **2** ($LD_{50}$ = 422 mg/kg), all compounds were screened in vivo with a single dose at 500 mg/kg. Table 2 summarizes the tolerability data for selected compounds by recording the number of mice which survived after an oral administration of a single dose of compounds at 500 mg/kg, followed by a 7-day observation. We found that compounds with a 2-methylpyridyl group displayed relatively high acute lethal toxicity in mice. On the other hand,


dx.doi.org/10.1021/jm300828h | *J. Med. Chem.* 2012, 55, 8409−8417

**Table 1. Physicochemical Property (ClogP), Antimycobacterial Potency (MIC), Cytotoxicity (VERO $IC_{50}$), and Selectivity Index (SI) Values for Target Compounds**




| compd | $R_1$ | $R_2$ | $R_3$ | ClogP[a] | MIC ($\mu$g/mL) | $IC_{50}$(Vero) ($\mu$g/mL) | SI[b] |
|---|---|---|---|---|---|---|---|
| CFZ | | | | 6.80 | 0.12 | 68.6 | 572 |
| **2** | Cl | A | H | 5.12 | 0.03 | 8.2 | 273 |
| **10** | H | A | H | 4.64 | 0.06 | 3.93 | 66 |
| **11** | $CH_3$ | A | H | 5.04 | 0.06 | 2.71 | 45 |
| **12** | $SCH_3$ | A | H | 5.41 | 0.05 | 1.83 | 37 |
| **13** | F | A | H | 4.74 | 0.03 | 6.25 | 208 |
| **14** | $CF_3$ | A | H | 5.18 | 0.03 | 19.66 | 655 |
| **15** | $OCF_3$ | A | H | 4.79 | 0.03 | 4.93 | 164 |
| **16** | Br | A | H | 5.22 | 0.02 | 2.3 | 115 |
| **17** | Cl | B | H | 4.71 | 0.03 | 2.5 | 83 |
| **18** | Cl | C | H | 5.46 | 0.03 | 12.03 | 401 |
| **19** | Cl | D | H | 5.08 | 0.125 | 5.58 | 45 |
| **20** | Cl | E | H | 6.15 | 0.25 | 15.06 | 60 |
| **21** | Cl | F | H | 4.40 | 0.25 | 4.02 | 16 |
| **22** | F | B | H | 4.33 | 0.06 | 7.36 | 123 |
| **23** | F | C | H | 5.09 | 0.12 | >64 | 533 |
| **24** | F | D | H | 4.71 | 0.25 | 6.44 | 26 |
| **25** | F | E | H | 5.81 | 0.125 | 30.26 | 242 |
| **26** | F | F | H | 4.03 | 0.5 | 15.09 | 30 |
| **27** | $OCF_3$ | B | H | 4.39 | 0.03 | >64 | 2133 |
| **28** | $OCF_3$ | C | H | 5.18 | 0.03 | 4.52 | 151 |
| **29** | $OCF_3$ | D | H | 4.76 | 0.25 | 4.48 | 18 |
| **30** | $OCF_3$ | E | H | 5.86 | 0.06 | 5.6 | 93 |
| **31** | $OCF_3$ | F | H | 4.09 | 0.125 | 16.12 | 129 |
| **32** | $CF_3$ | B | H | 4.75 | 0.125 | >64 | 512 |
| **33** | $CF_3$ | C | H | 5.55 | 0.03 | 28.35 | 945 |
| **34** | $CF_3$ | D | H | 5.15 | 0.125 | 10.38 | 83 |
| **35** | $CF_3$ | E | H | 6.26 | 0.06 | 27 | 450 |
| **36** | $CF_3$ | F | H | 6.26 | 0.125 | 19.86 | 159 |
| **37** | Br | B | H | 4.79 | 0.163 | 4.48 | 27 |
| **38** | Br | C | H | 5.53 | 0.062 | 1.88 | 30 |
| **39** | Cl | A | 2′-$OCH_3$ | 5.34 | 0.03 | >64 | 2133 |
| **40** | Cl | B | 2′-$OCH_3$ | 4.92 | 0.016 | >64 | 4000 |
| **41** | Cl | C | 2′-$OCH_3$ | 5.70 | 0.016 | >64 | 4000 |
| **42** | Cl | A | 2′-$CH_3$ | 5.43 | 0.055 | >64 | 1164 |
| **43** | Cl | B | 2′-$CH_3$ | 5.02 | 0.168 | 27.43 | 163 |
| **44** | Cl | C | 2′-$CH_3$ | 5.78 | 0.011 | 35.14 | 3195 |
| **45** | Cl | A | 6′-$CH_3$ | 5.54 | 0.06 | 7.09 | 118 |
| **46** | Cl | B | 6′-$CH_3$ | 5.13 | 0.03 | 9.73 | 324 |
| **47** | Cl | C | 6′-$CH_3$ | 5.79 | 0.016 | 50.88 | 3180 |
| **48** | Cl | B | 5′-Br | 5.27 | 0.036 | >64 | 1778 |
| **49** | Cl | C | 5′-Br | 5.99 | 0.052 | >64 | 1231 |
| **50** | F | A | 2′-$OCH_3$ | 4.98 | 0.03 | >64 | 2133 |
| **51** | F | B | 2′-$OCH_3$ | 4.57 | 0.039 | >64 | 1641 |
| **52** | F | C | 2′-$OCH_3$ | 5.33 | 0.016 | >64 | 4000 |
| **53** | F | A | 2′-$CH_3$ | 5.06 | 0.032 | 14.36 | 449 |
| **54** | F | B | 2′-$CH_3$ | 4.67 | 0.026 | >64 | 2462 |
| **55** | F | C | 2′-$CH_3$ | 5.41 | 0.019 | >64 | 3368 |
| **56** | F | A | 6′-$CH_3$ | 5.17 | 0.06 | 7.88 | 131 |
| **57** | F | B | 6′-$CH_3$ | 4.75 | 0.125 | 14.03 | 112 |
| **58** | F | C | 6′-$CH_3$ | 5.43 | 0.06 | >64 | 1067 |
| **59** | $CF_3$ | A | 2′-$OCH_3$ | 5.39 | 0.029 | >64 | 2207 |
| **60** | $CF_3$ | B | 2′-$OCH_3$ | 4.97 | 0.016 | >64 | 4000 |


dx.doi.org/10.1021/jm300828h | J. Med. Chem. 2012, 55, 8409−8417

**Table 1. continued**

| compd | $R_1$ | $R_2$ | $R_3$ | ClogP[a] | MIC ($\mu$g/mL) | $IC_{50}$(Vero) ($\mu$g/mL) | SI[b] |
|---|---|---|---|---|---|---|---|
| **61** | $CF_3$ | C | 2′-$OCH_3$ | 5.77 | 0.016 | >64 | 4000 |
| **62** | $CF_3$ | A | 2′-$CH_3$ | 5.48 | 0.014 | >64 | 4571 |
| **63** | $CF_3$ | B | 2′-$CH_3$ | 5.06 | 0.011 | >64 | 5818 |
| **64** | $CF_3$ | C | 2′-$CH_3$ | 5.87 | 0.011 | >64 | 5818 |
| **65** | $CF_3$ | A | 6′-$CH_3$ | 5.59 | 0.03 | 34.42 | 1147 |
| **66** | $CF_3$ | B | 6′-$CH_3$ | 5.17 | 0.032 | 35.12 | 1098 |
| **67** | $CF_3$ | C | 6′-$CH_3$ | 5.89 | 0.016 | 33.43 | 2089 |
| **68** | Br | A | 2′-$OCH_3$ | 5.43 | 0.049 | >64 | 1306 |
| **69** | Br | B | 2′-$OCH_3$ | 4.98 | 0.020 | >64 | 3200 |
| **70** | Br | C | 2′-$OCH_3$ | 5.76 | 0.031 | >64 | 2065 |
| **71** | Br | A | 2′-$CH_3$ | 5.53 | 0.062 | 39.4 | 635 |
| **72** | Br | C | 2′-$CH_3$ | 5.84 | 0.015 | 41.82 | 2788 |
| **73** | Br | A | 6′-$CH_3$ | 5.64 | 0.049 | 3.12 | 64 |
| **74** | Br | B | 6′-$CH_3$ | 5.21 | 0.044 | 21.70 | 493 |
| **75** | Br | C | 6′-$CH_3$ | 5.85 | 0.097 | 39.69 | 409 |
| **76** | $OCF_3$ | A | 2′-$OCH_3$ | 5.05 | 0.028 | >64 | 2286 |
| **77** | $OCF_3$ | B | 2′-$OCH_3$ | 4.67 | 0.016 | >64 | 4000 |
| **78** | $OCF_3$ | C | 2′-$OCH_3$ | 5.40 | 0.016 | >64 | 4000 |

[a]ClogP values, calculated using the MedChem Studio prediction software. (ADMET Predictor 5.5, Simulations Plus Inc.). [b]SI = selectivity index $IC_{50}$/MIC (For $IC_{50}$ values of >64 $\mu$g/mL, a value of 64 $\mu$g/mL was used for the SI calculation).

**Table 2. Preliminary Acute Toxicity Study (Single Oral Dose in Mice at 500 mg/kg) for Selected Compounds**

| compd | no. of animals that survived/total no. of animals |
|---|---|
| **40** | 6/6 |
| **41** | 6/6 |
| **44** | 0/6 |
| **47** | 6/6 |
| **50** | 6/6 |
| **52** | 6/6 |
| **54** | 0/6 |
| **55** | 0/6 |
| **59** | 6/6 |
| **60** | 6/6 |
| **61** | 6/6 |
| **63** | 5/6 |
| **69** | 6/6 |
| **70** | 6/6 |
| **72** | 1/6 |
| **78** | 6/6 |

compounds with a 2-methoxypyridyl group showed good tolerance in mice at 500 mg/kg.

On the basis of the acute toxicity results in mice, complemented by measured log *P* and pharmacokinetic data (Table 3),[15] we selected six compounds that represent significant structural diversity to evaluate in vivo efficacy in a mouse model of MDR-TB infection. Compounds were given orally at a dose of 20 mg/kg, once daily for 5 days a week for a total of 20 days in BALB/c mice, as described in published protocols.[15] Table 4 indicates that all tested compounds, including the positive control CFZ, demonstrated excellent efficacy in mice by reducing lung colony forming units (CFU) by between 3.5 logs and 5.1 logs units; the log CFU counts following treatment with these compounds were significantly lower than those for the untreated control group ($P < 0.01$). During the efficacy study, we observed discoloration of the ears, internal organs, and fat tissues of the mice that were treated with riminophenazines. The test compounds caused a wide range of discolorations, with color intensity appearing in the following descending order: CFZ = **50** = **52** > **47** > **41** = **40** > **78**. This study illustrates that the tissue discoloration observed following treatment with compounds of the riminophenazine class could be significantly reduced while maintaining potent efficacy in vivo. Further evaluation of this promising compound series is currently under way to fully assess its potential for further development for the treatment of MDR- and XDR-TB.

**Table 3. Log *P* and Pharmacokinetic Parameters of Selected Compounds Dosed Orally in Mice at 20 mg/kg (Mean ± SD)[15]**

| compd | $t_{1/2}$ (h) | $C_{max}$ ($\mu$g/mL) | AUC ($\mu$g·h/mL) | Log *P* |
|---|---|---|---|---|
| CFZ | 29.74 ± 10.27 | 0.38 ± 0.24 | 6.96 ± 4.35 | 5.34 |
| **40** | 6.62 ± 3.26 | 2.01 ± 1.16 | 33.09 ± 10.81 | 4.15 |
| **41** | 7.00 ± 2.44 | 3.01 ± 0.97 | 43.86 ± 14.31 | 3.74 |
| **47** | 16.08 ± 8.53 | 1.56 ± 0.73 | 22.09 ± 9.18 | 4.11 |
| **50** | 17.5 ± 8.45 | 3.02 ± 1.57 | 52.42 ± 14.28 | 4.07 |
| **52** | 7.90 ± 4.56 | 3.65 ± 2.47 | 45.36 ± 24.12 | 4.82 |
| **78** | 41.25 ± 10.23 | 1.30 ± 0.77 | 24.06 ± 8.48 | 4.52 |

**Table 4. Efficacy of CFZ and Its Analogues against MDR-TB after 20 Days of Treatment in BALB/C Mice Infected with Clinical Isolated MDR-TB (Mean ± SD) Dosed Orally at 20 mg/kg**

| compd | log CFU/lung |
|---|---|
| untreated (day 3) | 1.74 ± 0.24 |
| untreated (day 10) | 3.69 ± 0.20 |
| untreated (day 30) | 8.35 ± 0.19 |
| CFZ | 3.54 ± 0.53 |
| **40** | 4.81 ± 0.24 |
| **41** | 4.04 ± 0.28 |
| **47** | 3.25 ± 0.14 |
| **50** | 3.91 ± 0.31 |
| **52** | 4.35 ± 0.41 |
| **78** | 4.67 ± 0.19 |


dx.doi.org/10.1021/jm300828h | *J. Med. Chem.* 2012, 55, 8409−8417

## CONCLUSIONS

A series of novel riminophenazine derivatives bearing a C-2 pyridyl substituent was designed and synthesized. Many compounds in the series exhibited potent in vitro activity against *M. tuberculosis*, with improved physicochemical properties as compared to CFZ. Electron-withdrawing substituents at the N-5 phenyl position appear important for antimycobacterial activity. 4-Tetrahydropyranyl and 4-methoxycyclohexyl are the preferred substituents at the C-3 imino nitrogen position for providing both potent antimycobacterial activity and excellent pharmacokinetic profiles. The majority of compounds with a substituted pyridyl group at the C-2 position demonstrate potent activity against *M. tuberculosis* with low mammalian cell cytotoxicity. A preliminary acute toxicity study demonstrated that compounds with a 2-methoxypyridyl group at the C-2 position were well tolerated in mice, whereas compounds with a 2-methylpyridyl appeared less tolerated. As lipophilic compounds tend to be more active against *M. tuberculosis*, one of the major challenges in identifying an improved riminophenazine compound is to balance the needs for reducing lipophilicity and retaining potent activity. Information obtained from the X-ray crystal structure of compound **50** suggested a potential solution to addressing the problem of skin discoloration which has been one of the key limiting factors for widespread clinical use of CFZ. Compounds with a 2-methoxy substituent attached to the C-2 pyridyl group demonstrated reduced potential for skin discoloration in mice, which could be attributed to the unique intramolecular hydrogen bonding and reduced intermolecular $\pi-\pi$ stacking of this structural series.

Six compounds with potent activity against *M. tuberculosis* in vitro, low acute toxicity in mice, and excellent pharmacokinetic profiles were selected and evaluated in a mouse model of acute MDR-TB infection. All compounds showed significant efficacy, producing 3−5 logs of CFU reduction in the lungs as compared with the untreated group after 20 days of treatment. Compounds **41** and **47** exhibited equal or better in vivo efficacy against MDR-TB than CFZ with reduced discoloration potential in fat tissues and organs. Further evaluation of these promising compounds is under way to fully assess their potential for further development.

## EXPERIMENTAL SECTION

**1. General Information and Methods.** *1.1. Chemistry.* $^1$H NMR spectra were obtained on Varian mercury-300 at 300 MHz. $^{13}$C NMR spectra were obtained on Varian-400 at 100 MHz or Inova-500 at 125 MHz in $CDCl_3$,. DMSO-$d_6$, or acetone-$d_6$. Chemical shifts and coupling constants are recorded in units of ppm and Hz, respectively. Melting points were determined on Yanaco MP-J3 melting point apparatus. High-resolution mass spectra were measured on an Agilent 1100 series LC/MSD trap mass spectrometer (ESI-TOF). All target compounds were purified by chromatography or recrystallization and have purity of ≥95% as determined by HPLC/MS analysis conducted on an Agilent 1100 system, using a reversed-phase C18 column with 75% $CH_3CN$ in water (0.1% HCOOH) with a flow rate of 0.4 mL·min$^{-1}$.

All reagents and solvents were purchased from commercial sources unless otherwise indicated. Thin layer chromatography was carried out on silica gel plates ($GF_{254}$) with visualization of components by UV light (254 nm) or exposure to $I_2$. Column chromatography was carried out on silica gel (200−300 mesh) or neutral alumina (100−200 mesh).

*1.2. Minimum Inhibitory Concentration and Cytotoxicity Assays.* These were carried out according to published protocols.[15]

*1.3. In Vivo Acute MDR-TB Infection Assay.* SPF BALB/c male mice weighing 18−20 g were used in this study. Mice were infected via aerosol with a suspension of $5 \times 10^6$ CFU/mL multidrug-resistant *Mycobacterium tuberculosis* (the MDR clinical isolate (040), known to be resistant to INH, RFP, PTO, RPT, OFX, and LVFX) using a Glas-Col inhalation system, to deposit 50−100 bacilli into the lungs of each animal. The course of infection was followed by plating homogenates of harvested organs [$n$ = 3] on 7H11agar plates (7H11 plates containing 10% oleic acid−albumin−dextrose−catalase (OADC) enrichment and 50 μg/mL cycloheximide, 200 U/mL polymyxin B, 50 μg/mL carbenicillin, and 20 μg/mL trimethoprim) and determining CFU on days 3, 10, and 30 postinfection. Drugs and compounds were dissolved or suspended in 0.5% CMC and administered by oral gavage in a maximum volume of 200 μL such that a dose of 20 mg/kg body weight was achieved. Mice were treated 5/7 days per week during the acute phase of infection, from day 10 until day 30. Each treated group was composed of 5 or 6 mice, while the control group, which received only CMC, was composed of 7−10 mice. Mice were sacrificed the day after the last day of treatment, organ weights determined, lungs removed, homogenized, and serially diluted in 10-fold steps in HBSS. One hundred μL were spread on 7H11 agar in duplicate. The plates were incubated at 37 °C for 2−3 weeks. Data are expressed as the $\log_{10}$ (and as $\log_{10}$ reduction) provided by a given dose of the compound against the growth of the organism in the untreated control group. Mean $\log_{10}$ values were calculated from bacterial burden counts. Student's $t$ test was used to compare means between test and control groups. A $P$ value of ≤0.05 was considered significant.

**2. Procedure A for Synthesis of the Intermediate 5g and 5h.** *2-(4-Chloroanilino)-aniline (**5h**).* Zinc powder (9 g, 13.8 mol) was added portionwise into a mixture of **4h** (2.5 g, 10 mmol) in 50 mL of $CH_2Cl_2$ and 3 mL of glacial acetic acid and stirred at room temperature for 4 h. After filtration, the filtrate was concentrated to dryness. Water was added and filtered, and the resulting material was washed with water to give a brown solid. This solid was used directly in the next step without further purification.

In a similar manner, **5g** was synthesized according to procedure A.

**3. Procedure B for Synthesis of the Intermediate 9g−o.** *5-(4-Chlorophenyl)-3-imino-2-(3-pyridylamino)-3,5-dihydrophenazine (**9h**).* Zinc powder (71 g, 1.09 mol) was added portionwise into a mixture of **7h** (28.6 g, 60 mmol) in 150 mL of glacial acetic acid cooled with an ice−water bath. The mixture was stirred until the color turned to light green and then filtered and washed with glacial acetic acid and anhydrous methanol. The filtrate was concentrated, and the residue was treated with water and made alkaline with ammonia. The solid was filtered, washed with water, and then dissolved in anhydrous methanol. The solution was stirred under air overnight. The solid formed was filtered to give crude product 23.1 g, which was taken to the next step without further purification.

In a similar manner, **9g** and **9i−o** were synthesized according to procedure B.

**4. Procedure C for Synthesis of the Target Compounds.** *2-Anilino-nitrobenzene (**4a**).* A mixture of 2-fluoronitrobenzene (21.2 g, 150 mmol), aniline (16.8 g, 180 mmol), and anhydrous potassium fluoride (8.7 g, 150 mmol) was stirred at 160 °C for 8 h. The mixture was then cooled, water and ethyl acetate were added, the water layer was extracted with ethyl acetate, and the organic layer was washed with 2N HCl and dried with anhydrous sodium sulfate. After filtration, the filtrate was concentrated in vacuo, and 32 g of crude product was recrystallized in 100 mL of 95% ethanol to give 24.6 g **4a** as a red solid in 76.6% yield; mp 69−70 °C. $^1$H NMR (300 MHz, acetone-$d_6$) $\delta$: 9.43 (s, 1 H), 8.16 (d, $J$ = 7.2 Hz, 1 H), 7.35−7.51 (m, 5 H), 7.21−7.27 (m, 2 H), 6.86 (t, $J$ = 7.2 Hz, 1 H).

*1-[2-(Anilino)anilino]-3-fluoro-4,6-dinitrobenzene (**6a**).* A mixture of **4a** (24.6 g, 115 mmol) and 10% Pd/C (2.4 g) in ethanol was shaken at room temperature under a hydrogen atmosphere (40 psi) for 1.5 h. After filtration, DFDNB (23.4 g, 115 mmol) and triethylamine (11.6 g, 115 mmol) were added to the filtrate (**5a**); the mixture was stirred at room temperature for 3 h, filtered, and washed with ethanol to give 38.1 g **6a** as a red solid in 89.9% yield; mp 165−167 °C. $^1$H NMR (300 MHz, acetone-$d_6$) $\delta$: 9.93 (s, 1 H), 9.00 (d, $J$ = 8.1 Hz, 1 H),


dx.doi.org/10.1021/jm300828h | J. Med. Chem. 2012, 55, 8409−8417

7.32−7.42 (m, 3 H), 7.22 (t, $J$ = 7.5 Hz, 2 H), 7.02−7.08 (m, 3 H), 6.91 (t, $J$ = 7.2 Hz, 1 H), 6.69 (d, $J$ = 14.1 Hz, 1 H).

*1-[2-(Anilino)anilino]-3-(3-pyridyl)amino-4,6-dinitrobenzene (**7a**).* A mixture of **6a** (14.73 g, 40 mmol), 3-aminopyridine (3.76 g, 40 mmol), triethylamine (4.04 g, 40 mmol), and THF was refluxed for 18 h. After being cooled to room temperature, the mixture was concentrated in vacuo, $CH_2Cl_2$ was added to the residue, and the solid obtained was filtered to give 15.95 g of product as a red solid in 90.1% yield; mp 212−213 °C. $^1$H NMR (300 MHz, DMSO-$d_6$) $\delta$: 9.71 (s, 1 H), 9.49 (s, 1 H), 9.00 (s, 1 H), 8.37−8.41 (m, 2 H), 7.60−7.63 (m, 2 H), 7.32−7.36 (m, 1 H), 7.08−7.20 (m, 5 H), 6.82−6.85 (m, 4 H), 5.96 (s, 1 H).

*5-Phenyl-3-imino-2-(3-pyridyl)amino-3, 5-dihydrophenazine (**9a**).* A mixture of **7a** (15 g, 34 mmol) and 10% Pd/C in methanol and THF was shaken at room temperature under a hydrogen atmosphere (40 psi) for 10 h. After filtration, the filtrate was concentrated in vacuo and the residue (**8a**) was dissolved in methanol, and the solution stirred at room temperature under air for 10 h. The mixture was filtered to give 7.69 g of product as a red solid in 62.5% yield; mp 216−218 °C. $^1$H NMR (300 MHz, DMSO-$d_6$) $\delta$: 8.54 (d, $J$ = 2.1 Hz, 1 H), 8.25 (d, $J$ = 5.7 Hz, 1 H), 7.76−7.81 (m, 3 H), 7.71 (d, $J$ = 7.5 Hz, 1 H), 7.60−7.63 (m, 1 H), 7.50−7.52 (m, 2 H), 7.38−7.42 (m, 1 H), 7.15−7.23 (m, 2 H), 6.63 (s, 1 H), 6.42 (d, $J$ = 7.2 Hz, 1 H), 5.40 (s, 1 H). ESI/MS: $m/z$ $[M + H]^+$ 364.1534.

*5-Phenyl-3-isopropylimino-2-(3-pyridyl)amino-3,5-dihydrophenazine (**10**).* To a solution of **9a** (0.182 g, 0.5 mmol) and isopropylamine (1 mL, 11.7 mmol) in dioxane was added 0.06 mL of glacial acetic acid, and the mixture was stirred and heated at 110 °C in a sealed tube for 13 h. The mixture was concentrated in vacuo, and the residue was purified by column chromatography on silica gel, using ethyl acetate; 170 mg black oil was obtained, which was purified by column chromatography on neutral alumina, using petro ether:ethyl acetate = 2:1 to give 125 mg of product as a red solid in 61.6% yield; mp 183−185 °C. $^1$H NMR (300 MHz, $CDCl_3$) $\delta$: 8.59 (d, $J$ = 2.4 Hz, 1 H), 8.32 (d, $J$ = 3.9 Hz, 1 H), 7.62−7.80 (m, 5 H), 7.29−7.35 (m, 3 H), 7.10−7.19 (m, 2 H), 6.85 (s, 1 H), 6.49 (d, $J$ = 7.5 Hz, 1 H), 5.28 (s, 1 H), 3.38−3.46 (q, $J$ = 6.0 Hz, 1 H), 1.07 (d, $J$ = 6.0 Hz, 6 H). $^{13}$C NMR (100 MHz, $CDCl_3$) $\delta$: 150.9, 150.6, 144.2, 143.9, 143.7, 137.6, 136.9, 135.6, 135.1, 131.7, 131.3, 129.7, 128.8, 128.2, 127.8, 127.6, 123.6, 122.8, 114.2, 99.4, 89.1, 49.3, 23.5. HRMS (ESI-TOF$^+$): $m/z$ $[M + H]^+$ calcd for $C_{26}H_{24}N_5$, 406.2031; found, 406.2041.

In a similar manner, other target compounds (**11**−**78**) were synthesized according to procedure C.

## ■ ASSOCIATED CONTENT

### Ⓢ Supporting Information

Additional characterizations for compounds; NOE spectra and crystal data of compound **50**. This material is available free of charge via the Internet at http://pubs.acs.org.

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### Notes

The authors declare no competing financial interest.

## ■ ACKNOWLEDGMENTS

We thank the Global Alliance for TB Drug Development and the National Science and Technology Project of China (no. 2009ZX09102-054) for financial support of this project.

## ■ ABBREVIATIONS USED

CFZ, clofazimine; TB, tuberculosis; *M. tuberculosis*, *Mycobacterium tuberculosis*; MDR-TB, multidrug-resistant tuberculosis; XDR-TB, extensively drug-resistant tuberculosis; MOA, mechanism of action; $LD_{50}$, dose that is lethal in 50% of test subjects; DFDNB, 1,5-difluoro-2,4-dinitrobenzene; NMR, nuclear magnetic resonance; HRMS, high-resolution mass spectrometry; NOE, nuclear overhauser effect; MABA, microplate alamar blue assay; MIC, minimum inhibitory concentration; $IC_{50}$, half-maximum inhibitory concentration; PK, pharmacokinetic; CFU, colony forming unit; INH, isoniazid; RFP, rifampicin; PTO, protionamid; RPT, rifapentine; OFX, ofloxacin; LVFX, levofloxacin; HBSS, hank's balanced salt solution; SI, selectivity index; $T_{1/2}$, half-life; $C_{max}$, maximum plasma concentration; AUC, area under curve

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