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Research Article

Synthesis and Evaluation as Antitubercular Agents of 5-Arylethenyl and 5-(Hetero)aryl-3-Isoxazolecarboxylate

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Strategy, Management and Health Policy				
Enabling Technology, Genomics, Proteomics	Preclinical Research	Preclinical Development Toxicology, Formulation Drug Delivery, Pharmacokinetics	Clinical Development Phases I-III Regulatory, Quality, Manufacturing	Postmarketing Phase IV

ABSTRACT A new series of 5-aryl and 5-arylethenylisoxazole carboxylate derivatives was synthesized and evaluated for in vitro activity against *Mycobacterium tuberculosis* $H_{37}R_v$. Several compounds exhibited minimum inhibitory concentrations in the low micromolar range (2.3–11.4 μM). A variety of substituents introduced around the isoxazole ring allowed the delineation of preliminary SARs for this new series of compounds. Drug Dev Res 74 : 162–172, 2013. © 2012 Wiley Periodicals, Inc.

Key words: *Mycobacterium tuberculosis*; antituberculosis agents; 3-isoxazolecarboxylic acid alkyl esters

INTRODUCTION

Tuberculosis (TB) is a chronic and often deadly infectious disease caused by various species of *Mycobacterium*, mainly *Mycobacterium tuberculosis* (*Mtb*) [Russell et al., 2010]. The World Health Organization (WHO) has estimated that one-third of the world's population is infected with *Mtb*, resulting in 9.4 million new TB cases and 1.7 million deaths from TB in 2009, for the most part in developing countries [World Health Organization, 2010; World Health Organization, 2010/2011].

Despite the severe worldwide impact of TB, it remains a neglected disease as demonstrated by the evidence that no new anti-TB drugs have been introduced in therapy over the past 40 years [Janin, 2007; Spigelman, 2007] with rifampin (RMP), discovered in 1966, the most recent drug in this field [Maggi et al., 1966]. At present, only a few drug candidates are in Phase II clinical trials, e.g., PA-824 [Stover et al., 2000] and TMC-207 [Andries et al., 2005] (Fig. 1).

For many years, TB was considered a poverty-related disease, but it now occurs in the developed world. This is due to several reasons, the most important being the development of new virulent strains, resistant to antitubercular drugs, as well as the stream of immigrants from countries where TB is endemic. In addition, the recurrence of TB is directly connected to the explosive spread of HIV as the number of HIV-positive patients coinfected with *Mtb* is constantly rising [Brooks et al., 2009]. WHO reports that one third of HIV-infected people are coinfected with TB [World

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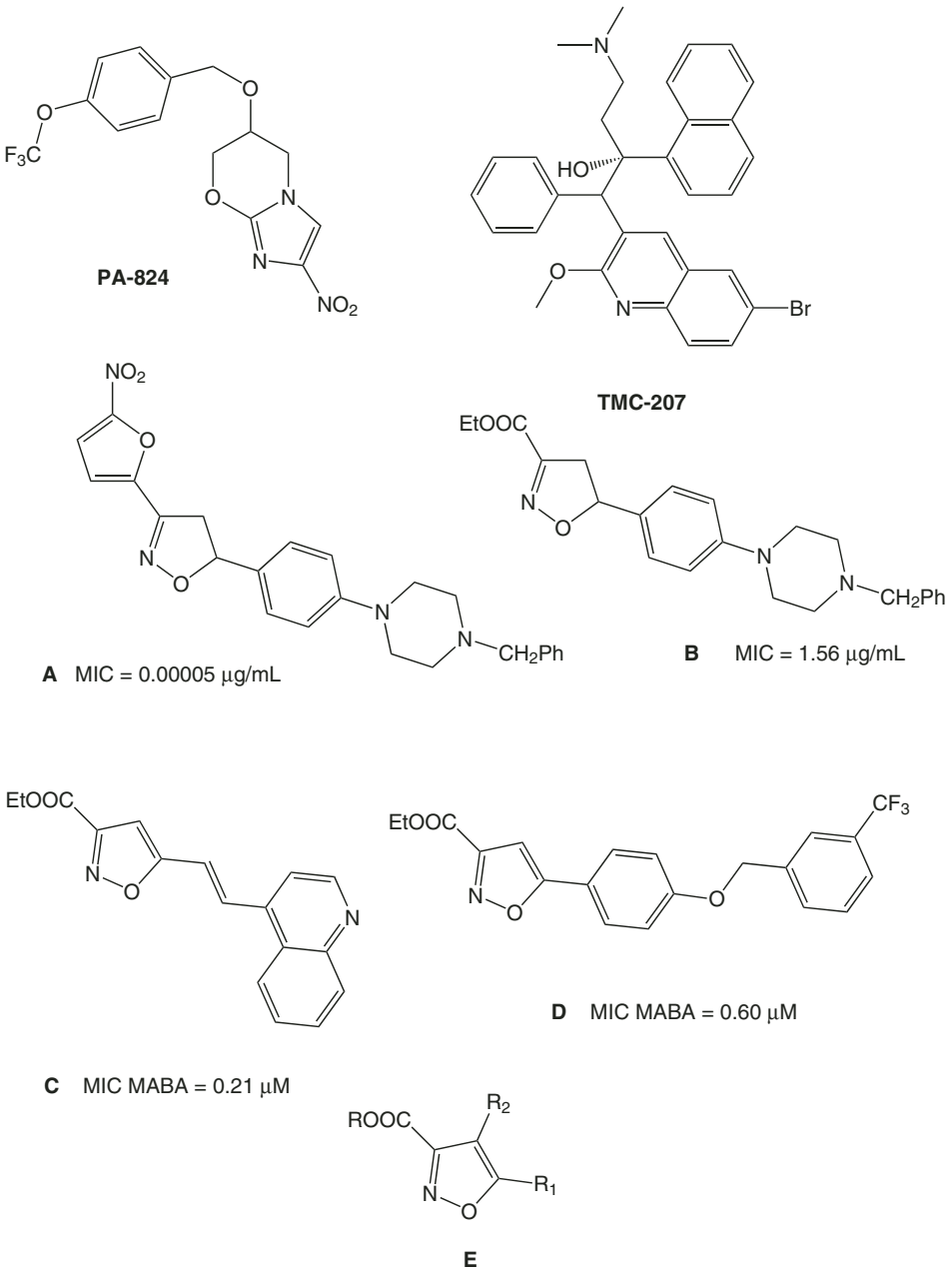


Fig. 1. Examples of anti-TB agents.

Health Organization, 2002], and 90% of these die within a few months of the appearance of clinical symptoms. This situation has prompted the WHO to declare TB as a global emergency [Janin, 2007].

The current standard therapy, Directly Observed Therapy Short-course [World Health Organization, 2009] is based on a combination of isoniazide (INH), Rifampin (RMP), pyrazinamide, and ethambutol (or streptomycin) for 2 months followed by further treatment with INH and RMP for an additional 4–7 months. This long treatment regimen is necessary because of the presence of a nonreplicating persistent *Mtb* phenotype (NRP-TB) [Wayne and Sohaskey, 2001] is often associated with poor patient compliances that contribute to the development of multidrug resistance. There are two types of drug resistance: multidrug resistant TB (MDR-TB) and extensively drug resistant TB (XDR-TB) [Johnson et al., 2006; Dorman and Chaisson, 2007]; the former defined as resistance to at least INI and RMP (MDR-TB) and the latter including concomitant resistance to any fluoroquinolone and at least one second-line drug, e.g.

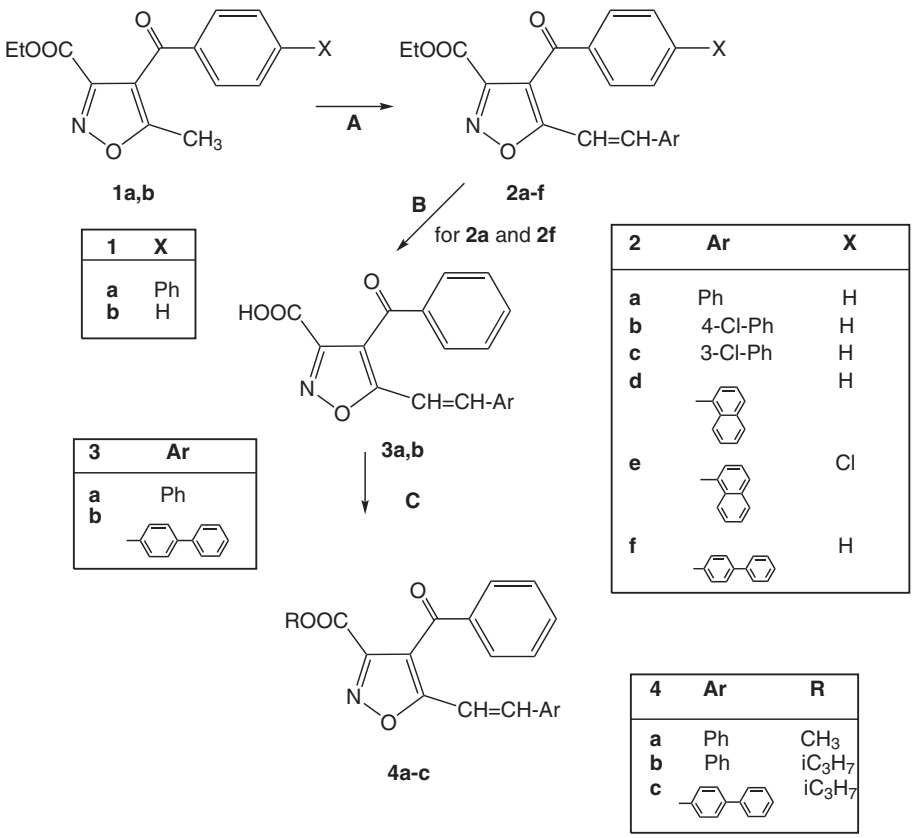


Fig. 2. Reagent and conditions: (**A**) ArCHO, EtONa, EtOH, 0°C, 2–20 h; (**B**) NaOH, EtOH, r.t., 1 h; (**C**) ROH, H_2SO_4, 80°C, 30–90 min.

kanamycin, amikacin, or capreomycin (XDR-TB). These data highlight the need for new, safer, and more potent anti-TB agents with novel mechanisms of action.

Many classes of organic compounds have been synthesized and tested as anti-TB agents, and in particular nitrogen heterocycles derivatives such as isoxazolines or isoxazoles have been described as good in vitro antitubercular agents [Johar et al., 2005; Janin, 2007; Eswarana et al., 2010]. Some representative compounds with this scaffold are shown in Figure 1. The isoxazolines **A** and **B** [Tangalapally et al., 2007] and the isoxazole derivatives **C** [Pieroni et al., 2009] and **D** [Lilienkampf et al., 2010] had interesting levels of activity against *Mtb* with minimum inhibitory concentration (MIC) values in the low or submicromolar range.

In this article, we describe the synthesis and the biological activity of a new series of anti-TB compounds with an isoxazole scaffold (Fig. 1, structure **E**), structurally related to compounds **C** and **D**. Various substituents were introduced around the isoxazole core in order to find the best substitution pattern and to define the importance of different substituents.

MATERIALS AND METHODS

Chemistry

All the final compounds were synthesized as reported in Figures 2 and 3. Figure 2 shows the synthetic procedure leading to the 4-benzoyl-5-styrylisoxazoles **2a–f** and **4a–c**. The precursors were represented by previously described isoxazoles **1a,b** [Renzi et al., 1968; Dal Piaz et al., 1991], which were condensed with the appropriate arylaldehydes to give the corresponding vinyl derivatives **2a–f**. Hydrolysis of **2a** and **2f** gives intermediate carboxylic acids **3a,b** which, in turn, were transformed into the ester derivatives **4a–c** using the appropriate alcohol and conc. H_2SO_4 at 80°C. Figure 2 shows the synthesis of the 4-unsubstituted derivatives **8a–g** and **10 a–e**. The key intermediates **7a–g** (**7a** [Fatutta and Balestra, 1958]; **7b** [Thormann et al., 2008]; **7c** [Deavegowda et al., 2010]; **7f** [Janculev and Podolesov, 1961]; **7g** [Patil et al., 2007] were obtained by condensation of commercially available **5a–g** and diethyl oxalate **6** [Fatutta and Balestra, 1958] and transformed into the final isoxazoles **8a–g** by treatment with hydroxylamine hydrochloride in ethanol and H_2SO_4. Finally, for compounds **8a–e,** a

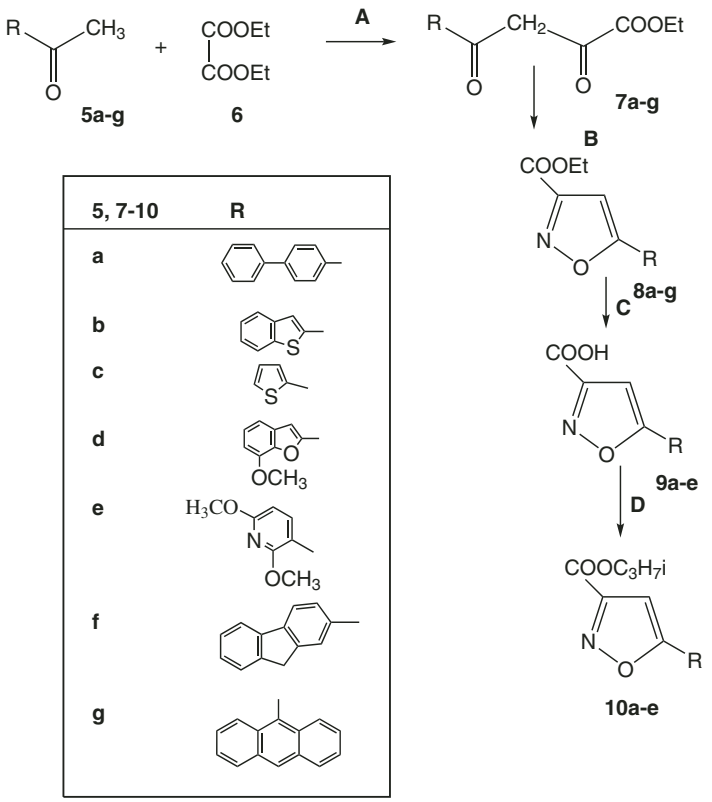


Fig. 3. Reagent and conditions: (**A**) Na, Et_2O, r.t-40°C; (**B**) $NH_2OH.HCl$, H_2SO_4, EtOH, reflux; (**C**) 1 N NaOH, EtOH, rt-60°C; (**D**) iC_3H_7OH, H_2SO_4, reflux.

further transformation in the corresponding isopropyl esters (**10a–e**) was performed following the same procedure described for compounds **4a–c**.

Experimental

All melting points were determined on a Büchi apparatus and are uncorrected. ^{1}H-NMR spectra were recorded with an Avance 400 instrument (Bruker Biospin Version 002 with SGU, Bruker AXS Inc., Madison, WI USA). Chemical shifts are reported in ppm, using the solvent as internal standard. Extracts were dried over Na_2SO_4, and the solvents were removed under reduced pressure. Merck F-254 commercial plates (Merck-Gruppe, Darmstadt, Germany) were used for analytical thin layer chromatography (TLC) to follow the reaction course. Silica gel 60 (Merck 70–230 mesh) was used for column chromatography. Microanalyses were performed with a Perkin-Elmer 260 elemental analyzer (Perkin-Elmer, Waltham, MA USA) for C, H, and N. Results were within ±0.4% of the theoretical values unless otherwise stated. Reagents and starting materials were commercially available.

General Procedures for 2a–f

To a cooled (0°C) and stirred mixture of **1a,b** [Renzi et al., 1968; Dal Piaz et al., 1991] (3.86 mmol) and the appropriate arylaldehyde (3.86 mmol) in 4–6 ml of anhydrous EtOH, a solution of sodium ethoxide, obtained from sodium (7.72 mmol) and anhydrous EtOH (5 ml), was slowly added. The mixture was stirred at 0°C for 2–20 h, then it was acidified with 6 N HCl and diluted with 30 ml of cold water. The final compounds were recovered by suction and purified by crystallization.

4-Benzoyl-5-Styryl-Isoxazole-3-Carboxylic Acid Ethyl Ester, 2a

Yield = 67%; mp = 56°C (EtOH); ^{1}H-NMR (DMSO-d_6) δ 0.97 (t, 3H, $\underline{CH_3}CH_2$, J = 7.2 Hz), 4.07 (q, 2H, $CH_3\underline{CH_2}$, J = 7.2 Hz), 7.13 (d, 1H, $\underline{CH}$ = CH, J = 16.8 Hz), 7.45–7.49 (m, 3H, Ar), 7.58 (m, 2H, Ar), 7.62–7.78 (m, 4H: 3H, Ar; 1H, CH = $\underline{CH}$), 7.82 (m, 2H, Ar). MS *m/z* 348 [M^+]. Anal. Calcd for $C_{21}H_{17}NO_4$: C, 72.61; H, 4.93; N, 4.03. Found C, 7.40; H, 4.92; N, 4.04.

4-Benzoyl-5-[2-(4-Chlorophenyl)-Vinyl]-Isoxazole-3-Carboxylic Acid Ethyl Ester, 2b

Yield = 75%; mp = 75–77°C (Et_2O); ^{1}H-NMR (DMSO-d_6) δ 0.96 (t, 3H, $\underline{CH_3}CH_2$, J = 7.2 Hz), 4.07 (q, 2H, $CH_3\underline{CH_2}$, J = 7.2 Hz), 7.15 (d, 1H, $\underline{CH}$ = CH, J = 16.8 Hz), 7.48 (m, 2H, Ar), 7.58 (m, 2H, Ar), 7.70–7.78 (m, 4H: 3H, Ar; 1H, CH = $\underline{CH}$), 7.82 (m, 2H, Ar). MS *m/z* 382 [M^+]. Anal. Calcd for $C_{21}H_{16}ClNO_4$: C, 66.06; H, 4.22; N, 3.67. Found C, 66.22; H, 4.23; N, 3.68.

4-Benzoyl-5-[2-(3-Chlorophenyl)-Vinyl]-Isoxazole-3-Carboxylic Acid Ethyl Ester, 2c

Yield = 67%; mp = 68–70°C (Et_2O); ^{1}H-NMR ($CDCl_3$) δ 1.08 (t, 3H, $\underline{CH_3}CH_2$, J = 7.2 Hz), 4.13 (q, 2H, $CH_3\underline{CH_2}$, J = 7.2 Hz), 7.05 (d, 1H, $\underline{CH}$ = CH, J = 16.4 Hz), 7.30–7.40 (m, 3H, Ar), 7.50–7.60 (m, 4H: 3H, Ar; 1H, CH = $\underline{CH}$), 7.65 (m, 1H, Ar), 7.82 (m, 2H, Ar). MS *m/z* 382 [M^+]. Anal. Calcd for $C_{21}H_{16}ClNO_4$: C, 66.06; H, 4.22; N, 3.67. Found C, 66.20; H, 4.21; N, 3.67.

4-Benzoyl-5-(2-Naphthalen-1-yl-Vinyl)-Isoxazole-3-Carboxylic Acid Ethyl Ester, 2d

Yield = 48%; mp = 137–139°C (Et_2O); ^{1}H-NMR (DMSO-d_6) δ 1.00 (t, 3H, $\underline{CH_3}CH_2$, J = 7.2 Hz), 4.10 (q, 2H, $CH_3\underline{CH_2}$, J = 7.2 Hz), 7.24 (d, 1H, $\underline{CH}$ = CH, J = 16.4), 7.50–7.65 (m, 5H, Ar), 7.73 (m, 1H, Ar), 7.90 (m, 2H, Ar), 7.95 (m, 1H, Ar), 7.98–8.04 (m, 2H, Ar), 8.20 (m, 1H, Ar), 8.40 (d, 1H, $\underline{CH}$ = CH, J = 16.4 H). MS *m/z* 398 [M^+]. Anal. Calcd for $C_{25}H_{19}NO_4$: C, 75.55; H, 4.82; N, 3.52. Found C, 75.31; H, 4.81; N, 3.51.

4-(4-Chlorobenzoyl)-5-(2-Naphthalen-1-yl-Vinyl)-Isoxazole-3-Carboxylic Acid Ethyl Ester, 2e

Yield = 28%; mp = 125–126°C (EtOH); ^{1}H-NMR ($CDCl_3$) δ 1.20 (t, 3H, $\underline{CH_3}CH_2$, J = 7.2 Hz), 4.24 (q, 2H, $CH_3\underline{CH_2}$, J = 7.2 Hz), 7.12 (d, 1H, $\underline{CH}$ = CH, J = 16.4 Hz), 7.50 (d, 2H, Ar, J = 8.4 Hz), 7.57–7.64 (m, 3H, Ar), 7.77 (m, 1H, Ar), 7.80–7.90 (m, 2H, Ar), 7.92 (d, 2H, Ar, J = 8.4 Hz), 8.17 (m, 1H, Ar), 8.48 (d, 1H, $\underline{CH}$ = CH, J = 16.4 Hz). MS *m/z* 432 [M^+]. Anal. Calcd for $C_{25}H_{18}ClNO_4$: C, 69.53; H, 4.20; N, 3.24. Found C, 69.69; H, 4.21; N, 3.23.

4-Benzoyl-5-(2-Biphenyl-4-yl-Vinyl)-Isoxazole-3-Carboxylic Acid Ethyl Ester, 2f

Yield = 47%; mp = 128–129°C (EtOH); ^{1}H-NMR ($CDCl_3$) δ 1.10 (t, 3H, $\underline{CH_3}CH_2$, J = 7.2 Hz), 4.15 (q, 2H, $CH_3\underline{CH_2}$, J = 7.2 Hz), 7.10 (d, 1H, $\underline{CH}$ = CH, J = 16.4 Hz), 7.40 (m, 1H, Ar), 7.46–7.55 (m, 5H, Ar), 7.60–7.65 (m, 6H, Ar), 7.70 (d, 1H, $\underline{CH}$ = CH, J = 16.4 Hz), 7.85 (m, 2H, Ar). MS *m/z* 424 [M^+]. Anal. Calcd for $C_{27}H_{21}NO_4$: C, 76.58; H, 5.00; N, 3.31. Found C, 76.77; H, 4.99; N, 3.32.

4-Benzoyl-5-(2-Biphenyl-4-yl-Vinyl)-Isoxazole-3-Carboxylic Acid, 3b

To a suspension of **2f** (0.28 mmol) in 2.5 ml of EtOH, 1 ml of 1N NaOH was added, and the mixture was stirred at room temperature for 1 h. After cooling, the mixture was acidified with 2N HCl and extracted with ethyl acetate (3 x 10 ml). Evaporation of the solvent under vacuum afforded the desired final compound. Yield = 73%; mp = 187–190°C (EtOH); ^{1}H-NMR ($CDCl_3$) δ 6.70 (d, 1H, $\underline{CH}$ = CH, J = 16.4 Hz), 7.36–7.68 (m, 11H: 10H, Ar; 1H, CH = $\underline{CH}$), 7.70–7.87 (m, 3H, Ar), 8.05 (m, 2H, Ar). MS *m/z* 396 [M^+]. Anal. Calcd for $C_{25}H_{17}NO_4$: C, 75.94; H, 4.33; N, 3.54. Found C, 75.73; H, 4.34; N, 3.54.

General Procedure for Compounds 4a–c

To a mixture of **3a** [Renzi et al., 1968] and **3b** (0.313 mmol) in 1.5–2 ml of the appropriate alcohol (methanol or isopropyl alcohol), 0.5 ml of conc. H_2SO_4 was added. The suspension was refluxed for 30–120 min, and after cooling, cold water was added, and the mixture was extracted with CH_2Cl_2 (3 × 15 ml). The solvent was evaporated in vacuum affording final compounds **4a** and **4b**. For compound **4c**, after dilution with water, the precipitate was recovered by suction and recrystallized with ethanol.

4-Benzoyl-5-Styryl-Isoxazole-3-Carboxylic Acid Methyl Ester, 4a

Yield = 94%; oil; ^{1}H-NMR ($CDCl_3$) δ 3.70 (s, 3H, CH_3), 7.03 (d, 1H, $\underline{CH}$ = CH, J = 16.4 Hz), 7.36–7.40 (m, 3H, Ar), 7.45–7.55 (m, 4H, Ar), 7.60–7.70 (m, 2H: 1H, Ar; 1H, CH = $\underline{CH}$), 7.82 (d, 2H, Ar). MS *m/z* 334 [M^+]. Anal. Calcd for $C_{20}H_{15}NO_4$: C, 72.06; H, 4.54; N, 4.20. Found C, 72.27; H, 4.55; N, 4.21.

4-Benzoyl-5-Styryl-Isoxazole-3-Carboxylic Acid Isopropyl Ester, 4b

Yield = 92%; oil; ^{1}H-NMR ($CDCl_3$) δ 1.06 (d, 6H, $CH(\underline{CH_3})_2$, J = 6.4 Hz), 5.03 (m, 1H, $\underline{CH}(CH_3)_2$), 7.05 (d, 1H, $\underline{CH}$ = CH, J = 16.4 Hz), 7.35–7.40 (m, 3H, Ar), 7.42–7.55 (m, 4H, Ar), 7.60–7.70 (m, 2H: 1H, Ar; 1H, CH = $\underline{CH}$), 7.85 (d, 2H, Ar). MS *m/z* 362 [M^+]. Anal. Calcd for $C_{20}H_{15}NO_4$: C, 73.12; H, 5.30; N, 3.88. Found C, 72.91; H, 5.29; N, 3.89.

4-Benzoyl-5-(2-Biphenyl-4-yl-Vinyl)-Isoxazole-3-Carboxylic Acid Isopropyl Ester, 4c

Yield = 65%; mp = 123–125°C (EtOH); ^{1}H-NMR ($CDCl_3$) δ 1.07 (d, 6H, CH($\underline{CH_3}$)$_2$, J = 6.4 Hz), 5.03 (m, 1H, $\underline{CH}(CH_3)_2$), 7.10 (d, 1H, $\underline{CH}$ = CH, J = 16.4 Hz), 7.35–7.56 (m, 6H, Ar), 7.60–7.77 (m, 6H: 5H, Ar, 1H, CH = $\underline{CH}$), 7.82 (m, 1H, Ar), 7.85 (d, 2H, Ar). MS *m/z* 438 [M$^+$]. Anal. Calcd for $C_{28}H_{23}NO_4$: C, 76.87; H, 5.30; N, 3.20. Found C, 76.85; H, 5.29; N, 3.21.

General Procedure for Compounds 7d and 7e

To a stirred mixture of the commercially available **5d** or **5e** (5,26 mmol) and diethyl oxalate (5–15 mmol) in anhydrous ether (50 ml), 5.25–7.40 mmol of Na was added in a dropwise manner. The suspension was stirred at room temperature for 4 h and then heated at 40°C for 4–7 h. After cooling, the precipitate was filtered off and washed with anhydrous ether. The solid was dissolved in water and acidified with 6N HCl to afford a crude precipitate that was collected by suction and purified by crystallization from ethanol.

4-Hydroxy-4-(7-Methoxybenzofuran-2-yl)-2-oxo-but-3-Enoic Acid Ethyl Ester, 7d

Yield = 85%; mp = 108–111°C (EtOH); ^{1}H-NMR ($CDCl_3$) δ 1.45 (t, 3H, $\underline{CH_3}CH_2$, J = 7.2 Hz), 4.07 (s, 3H, OCH_3), 4.42 (q, 2H, $CH_3\underline{CH_2}$, J = 7.2 Hz), 7.00 (m, 1H, Ar), 7.20 (s, 1H, Ar), 7.25–7.33 (m, 2H, Ar), 7.65 (s, 1H, Ar). MS *m/z* 291 [M$^+$]. Anal. Calcd for $C_{15}H_{14}O_6$: C, 62.07; H, 4.86. Found C, 62.22; H, 4.85.

4-(2,6-Dimethoxypyridin-3-yl)-4-Hydroxy-2-oxo-but-3-Enoic Acid Ethyl Ester, 7e

Yield = 84%; mp = 86–89°C (EtOH); ^{1}H-NMR ($CDCl_3$) δ 1.43 (t, 3H, $\underline{CH_3}CH_2$, J = 7.2 Hz), 4.03 (s, 3H, OCH_3), 4.12 (s, 3H, OCH_3), 4.40 (q, 2H, $CH_3\underline{CH_2}$, J = 7.2 Hz), 6.45 (d, 1H, Ar, J = 8.8 Hz), 7.41 (s, 1H, Ar), 8.28 (d, 1H, Ar, J = 8.8 Hz). MS *m/z* 282 [M$^+$]. Anal. Calcd for $C_{13}H_{15}NO_6$: C, 55.51; H, 5.38; N, 4.98. Found C, 55.68; H, 5.37; N, 4.99

General Procedure for Compounds 8b, 8d-g

To a suspension of **7b** [Thormann et al., 2008] or **7d-g** [Janculev and Podolesov, 1961; Patil et al., 2007; Deavegowda et al., 2010] (0.51 mmol) and hydroxylamine hydrochloride (2.5–3.02 mmol, dissolved in 0.5 ml of water) in 2–6 ml of EtOH, 0.3–0.4 ml of conc. H_2SO_4 was added. The mixture was heated at 80°C for 15–45 min. After cooling, the solvent was evaporated under vacuum to afford a residue which, after treatment with cold water (15 ml), gave rise to a crude precipitate that was recovered by suction. The final compounds **8b**, **8e**, and **8g** were purified by crystallization, whereas compounds **8d** and **8f** were purified by column chromatography using toluene/ethyl acetate 9.5:0.5 as eluent.

5-Benzo[b]thiophen-2-yl-Isoxazole-3-Carboxylic Acid Ethyl Ester, 8b

Yield = 69%; mp = 130–131°C, dec. (EtOH); ^{1}H-NMR ($CDCl_3$) δ 1.47 (t, 3H, $\underline{CH_3}CH_2$, J = 7.2 Hz), 4.51 (q, 2H, $CH_3\underline{CH_2}$, J = 7.2 Hz), 6.92 (s, 1H, isoxazole), 7.45 (m, 2H, Ar), 7.85 (s, 1H, Ar), 7.90 (m, 2H, Ar). MS *m/z* 274 [M$^+$]. Anal. Calcd for $C_{14}H_{11}NO_3S$: C, 61.52; H, 4.06; N, 5.12. Found C, 61.64; H, 4.07; N, 5.11.

5-(7-Methoxybenzofuran-2-yl)-Isoxazole-3-Carboxylic Acid Ethyl Ester, 8d

Yield = 40%; mp = 119–121°C; purified by column chromatography (toluene/ethyl acetate 9.5:0.5); ^{1}H-NMR ($CDCl_3$) δ 1.45 (t, 3H, $\underline{CH_3}CH_2$, J = 7.2 Hz), 4.07 (s, 3H,OCH_3), 4.50 (q, 2H, $CH_3\underline{CH_2}$, J = 7.2 Hz), 6.93 (m, 1H, Ar), 7.14 (s, 1H, isoxazole), 7.24–7.30 (m, 2H, Ar), 7.36 (s, 1H, Ar). MS *m/z* 288 [M$^+$]. Anal. Calcd for $C_{15}H_{13}NO_5$: C, 62.72; H, 4.56; N, 4.88. Found C, 62.94; H, 4.57; N, 4.87.

5-(2,6-Dimethoxypyridin-3-yl)-Isoxazole-3-Carboxylic Acid Ethyl Ester, 8e

Yield = 82%; mp = 131–133°C, (Ether); ^{1}H-NMR ($CDCl_3$) δ 1.47 (t, 3H, $\underline{CH_3}CH_2$, J = 7.2 Hz), 4.01 (s, 3H, OCH_3), 4.13 (s, 3H, OCH_3), 4.50 (q, 2H, $CH_3\underline{CH_2}$, J = 7.2 Hz), 6.48 (d, 1H, Ar, J = 8.4 Hz), 7.05 (s, 1H, isoxazole), 8.19 (d, 1H, Ar, J = 8.4 Hz). MS *m/z* 279 [M$^+$]. Anal. Calcd for $C_{13}H_{14}N_2O_5$: C, 56,11; H, 5,07; N, 10,07. Found C, 56.24; H, 5.06; N, 10.04.

5-(9H-Fluoren-2-yl)-Isoxazole-3-Carboxylic Acid Ethyl Ester, 8f

Yield = 68%; mp = 158–161°C, dec.; purified by column chromatography (toluene/ethyl acetate 9.5:0.5); ^{1}H-NMR ($CDCl_3$) δ 1.46 (t, 3H, $\underline{CH_3}CH_2$, J = 7.2 Hz), 4.02 (s, 2H, Ph$\underline{CH_2}$Ph), 4.51 (q, 2H, $CH_3\underline{CH_2}$, J = 7.2 Hz), 6.98 (s, 1H, isoxazole), 7.38–7.47 (m, 2H, Ar), 7.61 (m, 1H, Ar), 7.85–7.92 (m, 3H, Ar), 8.03 (s, 1H, Ar). MS *m/z* 306 [M$^+$]. Anal. Calcd for $C_{19}H_{15}NO_3$: C, 74.74; H, 4.95; N, 4.59. Found C, 74.56; H, 4.96; N, 4.58.

5-Anthracen-9-yl-Isoxazole-3-Carboxylic Acid Ethyl Ester, 8g

Yield = 74%; mp = 139–141°C, (EtOH); ^{1}H-NMR ($CDCl_3$) δ 1.53 (t, 3H, $\underline{CH_3}CH_2$, J = 7.2 Hz), 4.60 (q, 2H, $CH_3\underline{CH_2}$, J = 7.2 Hz), 7.09 (s, 1H, isoxazole), 7.51–7.57 (m, 4H, Ar), 7.80 (m, 2H, Ar), 8.10 (m, 2H, Ar), 8.67 (s, 1H, Ar). MS *m/z* 318 [M^+]. Anal. Calcd for $C_{20}H_{15}NO_3$: C, 75.70; H, 4.76; N, 4.41. Found C, 75.88; H, 4.76; N, 4.42.

General Procedure for 9b and 9d,e

A mixture of **8b** or **8d,e** (0.91 mmol) and 1N NaOH (1–1.5 ml) in 3–4 ml of EtOH was stirred at room temperature for 20–30 min. For compound **9b**, the suspension was heated at 50°C for 30 min. After evaporation of the solvent under vacuum, water was added, and the suspension was acidified with 6N HCl (1–2 ml). The crude precipitate was recovered by suction (compounds **9b** and **9d)**, whereas compound **9e** was extracted with ethyl acetate (3 × 20 ml).

5-Benzo[b]thiophen-2-yl-Isoxazole-3-Carboxylic Acid, 9b

Yield = 47%; mp = 192–195°C, dec.; (EtOH); ^{1}H-NMR (DMSO-d_6) δ 7.41 (s, 1H, isoxazole), 7.49 (m, 2H, Ar), 7.99 (m, 1H, Ar), 8.10 (m, 1H, Ar), 8.18 (s, 1H, Ar). MS *m/z* 246 [M^+]. Anal. Calcd for $C_{12}H_7NO_3S$: C, 58.77; H, 2.88; N, 5.71. Found C, 58.94; H, 2.87; N, 5.73.

5-(7-Methoxybenzofuran-2-yl)-Isoxazole-3-Carboxylic Acid, 9d

Yield = 76%; mp = 179–182°C, dec.; (EtOH); ^{1}H-NMR (DMSO-d_6) δ 3.99 (s, 3H, OCH_3), 7.09 (m, 1H, Ar), 7.27–7.35 (m, 3H: 2H, Ar; 1H, isoxazole), 7.72 (s, 1H, Ar). MS *m/z* 260 [M^+]. Anal. Calcd for $C_{13}H_9NO_5$: C, 60.24; H, 3.50; N, 5.40 Found C, 60.36; H, 3.51; N, 5.41.

5-(2,6-Dimethoxypyridin-3-yl)-Isoxazole-3-Carboxylic Acid, 9e

Yield = 56%; mp = 200–203°C, dec.; (EtOH); ^{1}H-NMR (DMSO-d_6) δ 3.96 (s, 3H, OCH_3), 4.08 (s, 3H, OCH_3), 6.59 (d, 2H, Ar, J = 8.4 Hz), 6.97 (s, 1H, isoxazole), 8.21 (d, 2H, Ar, J = 8.4 Hz). MS *m/z* 251 [M^+]. Anal. Calcd for $C_{11}H_{10}N_2O_5$: C, 52.80; H, 4.03; N, 11.20. Found C, 52.94; H, 4.02; N, 11.17.

General Procedure for 10a–e

Compounds **10a–e** were obtained starting from **9a–e** (compounds **9a** [Fatutta and Balestra, 1958] and **9c** [Schneider et al., 2008]) following the general procedure described for compounds **4a–c**. For compounds **10b–d**, after dilution with cold water, the precipitate was recovered by suction and purified by crystallization, whereas compound **10e** was purified by column chromatography using cyclohexane/ethyl acetate 1/1 as eluent.

5-Biphenyl-4-yl-Isoxazole-3-Carboxylic Acid Isopropyl Ester, 10a

Yield = 55%; mp = 101–103°C; (EtOH); ^{1}H-NMR ($CDCl_3$) δ 1.46 (d, 6H, $CH(\underline{CH_3})_2$, J = 6.4 Hz), 5.32–5.42 (m, 1H, $\underline{CH}(CH_3)_2$), 6.97 (s, 1H, isoxazole), 7.43 (m, 1H, Ar), 7.50 (m, 2H, Ar), 7.67 (m, 2H, Ar), 7.75 (d, 2H, Ar, J = 8.4 Hz), 7.92 (d, 2H, Ar, J = 8.4 Hz). MS *m/z* 308 [M^+]. Anal. Calcd for $C_{19}H_{17}NO_3$: C, 74.25; H, 5.58; N, 4.56. Found C, 74.50; H, 5.56; N, 4.57.

5-Benzo[b]thiophen-2-yl-Isoxazole-3-Carboxylic Acid Isopropyl Ester, 10b

Yield = 85%; mp = 101–103°C, (cicloexane); ^{1}H-NMR ($CDCl_3$) δ 1.46 (d, 6H, $CH(\underline{CH_3})_2$, J = 6.4 Hz), 5.32–5.41 (m, 1H, $\underline{CH}(CH_3)_2$), 6.91 (s, 1H, isoxazole), 7.45 (m, 2H, Ar), 7.85 (s, 1H, Ar), 7.89 (m, 2H, Ar). MS *m/z* 288 [M^+]. Anal. Calcd for $C_{15}H_{13}NO_3S$: C, 62.70; H, 4.56; N, 4.87. Found C, 62.87; H, 4.57; N, 4.86.

5-Thiophen-2-yl-Isoxazole-3-Carboxylic Acid Isopropyl Ester, 10c

Yield = 59%; mp = 53–56°C, (EtOH); ^{1}H-NMR ($CDCl_3$) δ 1.44 (d, 6H, $CH(\underline{CH_3})_2$, J = 6.4 Hz), 5.35 (m, 1H, $\underline{CH}(CH_3)_2$), 6.79 (s, 1H, isoxazole), 7.17 (m, 1H, Ar), 7.52 (m, 1H, Ar), 7.59 (m, 1H, Ar). MS *m/z* 238 [M^+]. Anal. Calcd for $C_{11}H_{11}NO_3S$: C, 55.68; H, 4.67; N, 5.90. Found C, 55.89; H, 4.67; N, 5.88.

5-(7-Methoxybenzofuran-2-yl)-Isoxazole-3-Carboxylic Acid Isopropyl Ester, 10d

Yield = 82%; mp = 96–100°C; purified by column chromatography (cicloexane/ethyl acetate 1:1); ^{1}H-NMR ($CDCl_3$) δ 1.45 (d, 6H, $CH(\underline{CH_3})_2$, J = 6.4 Hz), 4.07 (s, 3H, OCH_3), 5.35 (m, 1H, $\underline{CH}(CH_3)_2$), 6.93 (m, 1H, Ar), 7.13 (s, 1H, isoxazole), 7.26 (m, 2H, Ar), 7.36 (s, 1H, Ar). MS *m/z* 302 [M^+]. Anal. Calcd for $C_{16}H_{15}NO_5$: C, 63.78; H, 5.02; N, 4.65. Found C, 63.59; H, 5.04; N, 4.66.

5-(2,6-Dimethoxypyridin-3-yl)-Isoxazole-3-Carboxylic Acid Isopropyl Ester, 10e

Yield = 86%; mp = 119–121°C, (EtOH); ^{1}H-NMR ($CDCl_3$) δ 1.45 (d, 6H, $CH(\underline{CH_3})_2$, J = 6.4 Hz), 4.00

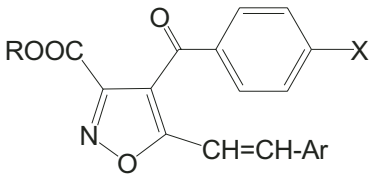
(s, 3H, OCH_3), 4.13 (s, 3H, OCH_3), 5.35 (m, 1H, $\underline{CH}(CH_3)_2$), 6.48 (d, 1H, Ar, J = 8.4 Hz), 7.04 (s, 1H, isoxazole), 8.19 (d, 1H, Ar, J = 8.4 Hz). MS *m/z* 293 [M^+]. Anal. Calcd for $C_{14}H_{16}N_2O_5$: C, 57.53; H, 5.52; N, 9.58. Found C, 57.82; H, 5.53; N, 9.61.

Biological Assays

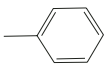
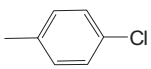
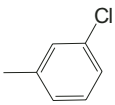
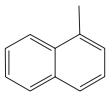
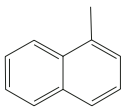
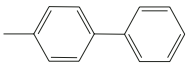
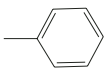
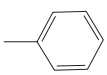
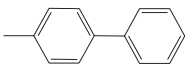
Microplate Almar blue assay (MABA) [Franzblau et al., 1998]

Test compound MICs against *Mtb* $H_{37}RV$ (ATCC27294) were assessed by the MABA using RMP and INH as positive controls. Compound stock solutions were prepared in DMSO at concentration of 12.8 mM, and the final test concentrations ranged from 128 μM to 0.5 μM. Twofold dilutions of compounds were prepared in Middlebrook 7H12 medium (7H9 broth containing 0.1% w/v casitone, 5.6 μg/ml palmitic acid, 5 mg/ml bovine serum albumin, 4 mg/ml catalase, filter-sterilizes) in a volume of 100 μl in 96-well microplates (BD Optilux™, 96-well Microplates, black/clear flat bottom; Becton,-Dickenson, Franklin Lakes, NJ USA). *Mtb* cultures (100 μl inoculum of 2×10^5 cfu/ml) were added, yielding a final test volume of 200 μl. The

TABLE 1. Anti-TB Activity of Compounds 2a–f and 4a–c



2a-f, 4a-c

Compound	*R*	Ar	X	MABA[a] MIC (μM)	LORA[a] MIC (μM)	Vero cell IC_{50} (μM)
2a	C_2H_5		H	58.2	96.6	95.17
2b	C_2H_5		H	35.5	109.1	>128
2c	C_2H_5		H	81.6	62.3	108.4
2d	C_2H_5		H	26.9	54.7	>128
2e	C_2H_5		Cl	>128	>128	ND[b]
2f	C_2H_5		H	114.6	101.3	111.5
4a	CH_3		H	26.8	54.1	88.07
4b	iC_3H_7		H	61.2	>128	100.6
4c	iC_3H_7		H	64.0	82.3	56.74
INH				0.4	>128	>128
RMP				0.1	1.9	110
PA-824				0.4	3.0	>128

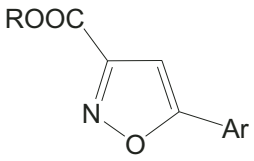
[a]Mtb strain $H_{37}R_v$.
[b]Not determined.
INH, isoniazide; LORA, low oxygen recovery assay; MABA, microplate Alamar blue assay; MIC, minimum inhibitory concentration; RMP, rifampin.

plates were incubated at 37°C. On the seventh day of incubation, 12.5 µl of 20% Tween 80 and 20 µl of Alamar Blue (Invitrogen BioSource™; Life Technologies, Grand Island, NY USA) were added to the wells. After incubation at 37°C for 16–24 h, well fluorescence was measured (ex 530, em 590 nm). MICs were defined as the lowest concentration effecting a reduction in fluorescence of ≥ 90% relative to the mean of replicate bacteria-only controls.

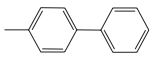
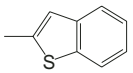
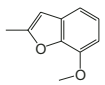
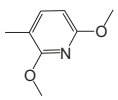
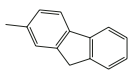
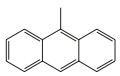
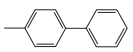
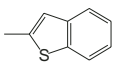
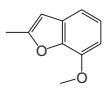
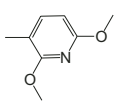
Low-Oxygen-Recovery Assay (LORA) [Cho et al., 2007]

A low-oxygen adapted culture of recombinant $H_{37}Rv$ (pFCA-luxAB), expressing a *Vibrio harveyii*

TABLE 2. Anti-TB Activity of Compounds 8a–g and 10a–e



8a-g, 10a-e

Compound	*R*	Ar	MABA[a] MIC (µM)	LORA[a] MIC (µM)	Vero cell IC_{50} (µM)
8a	C_2H_5		3.3	30.1	102
8b	C_2H_5		2.7	25.3	>128
8c	C_2H_5		3.5	58.9	>128
8d	C_2H_5		2.2	13.1	>128
8e	C_2H_5		>128	>128	ND[b]
8f	C_2H_5		>128	>128	ND[b]
8g	C_2H_5		>128	>128	ND[b]
10a	iC_3H_7		2.3	63.5	>128
10b	iC_3H_7		11.4	26.6	>128
10c	iC_3H_7		2.4	14.0	>128
10d	iC_3H_7		5.6	7.3	>128
10e	iC_3H_7		>128	>128	ND[b]
INH			0.4	>128	>128
RMP			0.1	1.9	>128
PA-824			0.4	3.0	>128

[a]Mtb strain $H_{37}R_v$.
[b]Not determined.
INH, isoniazide; LORA, low oxygen recovery assay; MABA, microplate Alamar blue assay; MIC, minimum inhibitory concentration; RMP, rifampin.

luciferase gene with an acetamidase promoter, was grown in a BiostatQ fermentor (Sartorius Group, Gottingen, Germany). Cells were collected on ice, washed in PBS, and stored at –80°C. Approximately 10^5 cfu/ml of thawed NRP cells were exposed to twofold serial dilutions of test compound in 7H9 broth in 96-well plates, incubated for 10 days anaerobically at 37°C. Luminescence readings were obtained following a 28-h recovery in an aerobic environment (5% CO_2). The data were analyzed graphically, and the lowest concentration of test compound preventing metabolic recovery (90% reduction relative to untreated cultures) was determined as described previously.

Cytotoxicity Assay [Falzari et al., 2005]

Cytotoxicity was determined by exposing different concentrations of samples to Vero cells. Briefly, samples were dissolved at 12.8 mM in DMSO. Six threefold dilutions were performed in growth medium MEM (Gibco, Grand Island, NY, USA), containing 10% fetal bovine serum (HyClone, Logan, UT, USA), 25 mM N-(2-hydroxyethyl)-piperazine-N′-2-ethanesulfonic acid (HEPES, Gibco), 0.2% $NaHCO_3$ (Gibco), and 2 mM glutamine (Irvine Scientific, Santa Ana, CA, USA). Final DMSO concentrations did not exceed 1% v/v. Compound dilutions were assayed in duplicate in 96-well tissue culture plates (Becton Dickinson Labware, Lincoln Park, NJ, USA) in a volume of 50 µl per well. An equal volume containing 5×10^5 log phase Vero cells (CCL-81; ATTC, Rockville, MD, USA) was added to each well, and cultures were incubated at 37°C in 5% CO_2 atmosphere. After 72 h, cell viability was measured using the CellTiter 96 aqueous nonradioactive cell proliferation assay (Promega Corp. Madison, WI, USA) according to the manufacturer's instructions. Absorbance at 490 nm was read in a Victor^2 multilabel reader (Perkin Elmer). The IC_{50} values were determined using a curve-fitting program.

RESULTS AND DISCUSSION

The activity of compounds **2a–f**, **4a–c**, **8a–g**, and **10a–e** was evaluated against *Mtb* TB strain $H_{37}Rv$ in a MABA [Franzblau et al., 1998] and against NRP-TB in a LORA [Cho et al., 2007] with the activity expressed as MIC values (minimum inhibitory concentrations). Furthermore, toxicity to *Mtb* an in vitro cytotoxicity test was assessed using Vero cells. (Tables 1 and 2). INH, RMP, and PA-824 were used as reference compounds.

Starting from 5-vinylisoxazoles **2a–f** and **4a–c** (Table 1), compounds show a low activity against *Mtb* with the only noteworthy observation being the difference of activity between compounds **2d** and **2e**, which differ only by a chlorine in the para–position of benzoyl fragment. Compound **2d** was one of the more active in this series (MIC = 26.9 µM) with **2e** being inactive (MIC > 128 µM), suggesting that the chlorine is detrimental to activity. For most compounds, there is a correspondence between MABA and LORA values and with the exception of compounds **2b** and **2d** (Vero cell IC_{50} > 128 µM), they also showed little in vitro cytotoxicity (Vero cell 56.74 < IC_{50} < 128 µM)

Data on the series of 5-(hetero)aryl-4-unsubstituted isoxazoles **8a–g** and **10a–e** are shown in Table 2. With the exception of compounds **8e**, **8f, 8g**, and **10e** improved activity was observed as compared with the previous compounds. The residue *R* of the ester function can be changed as the 3-ethyl and the 3-isopropyl ester derivatives show comparable activity. However, there is a crucial role for the substituent at position 5. Compounds with a diphenyl, thiophene, benzothiophene, or a methoxybenzofurane had MIC values in the low micromolar range (MIC = 2.2–5.6 µM), whereas the introduction of aromatic portions endowed with major bulk (fluorene and anthracene nucleus) resulted to inactive compounds (**8f** and **8g**, MIC > 128 µM). The same inactivity was observed for the two 5-(2,6-dimethoxypiridine) derivatives, **8e** and **10e**, that contain a strongly basic nitrogen. An analogous activity trend occurred in the LORA, whereas MIC values were approximately an order of magnitude higher than in MABA. Moreover, with the exception of **8a,** all active compounds had no toxicity in Vero cell with IC_{50} values > 128 µM. These initial results suggest that position 4 of the isoxazole ring must be unsubstituted and the ethylenic spacer must be deleted from position 5 and the aromatic portion, with an appropriate steric hindrance directly linked to C-5 isoxazole. Further studies are in progress to better define the requirements in the isoxazole scaffold to improve antitubercular activity.

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