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Discovery of novel isoxazolines as anti-tuberculosis agents

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Abstract—Nitrofuranyl isoxazolines with increased proteolytic stability over nitrofuranyl amides were designed and synthesized leading to discovery of several compounds with potent in vitro anti-tuberculosis activity. However, their in vivo activity was limited by high protein binding and poor distribution. Consequently, a series of non-nitrofuran containing isoxazolines were prepared to determine if the core had residual anti-tuberculosis activity. This led to the discovery of novel isoxazoline **12** as anti-tuberculosis agent with a MIC_{90} value of 1.56 μg/mL.

Mycobacterium tuberculosis is a very successful pathogen that infects one-third of the world's population.[1] The emergence of multi-drug resistant tuberculosis and extensively drug resistant tuberculosis coupled with an increasing number of tuberculosis patients due to the overlap between tuberculosis and AIDS epidemics has created an urgent need to develop novel therapeutics to treat this deadly disease.[1] In order to develop a better chemotherapeutic regime, it is believed that drugs are most needed to treat the latent phase of this disease. Unfortunately, latent bacteria are intrinsically more difficult to treat.[2] The nitroaromatic class of antibiotics is one of the few classes of antibiotics that have shown activity against latent *M. tuberculosis*, and nitroimidazoles PA-824 and OPC-67683 are in current clinical trials to treat tuberculosis.[3] We chose to investigate a related class, the nitrofurans. Previously, we discovered and developed a series of nitrofuranyl amides with excellent in vitro activity against *M. tuberculosis* (Fig. 1).[4] However, this series of compounds did not perform well during in vivo studies due to a short biological half life and rapid elimination. The amide linkage (shown in green, Fig. 1) was thought to be the major reason for the observed metabolic instability. Thus in this current study we evaluated the replacement of the amide linker with an isoxazoline linker (shown in pink, Fig. 1). The isoxazoline ring system represents a stable bioisosteric replacement for the amide bond that is found among many biologically active molecules and drugs.[5]

The synthesis of the nitrofuranyl isoxazoline compounds is shown in Scheme 1. First, the olefin **2** was prepared in good yield (88%) by a palladium-catalyzed aromatic

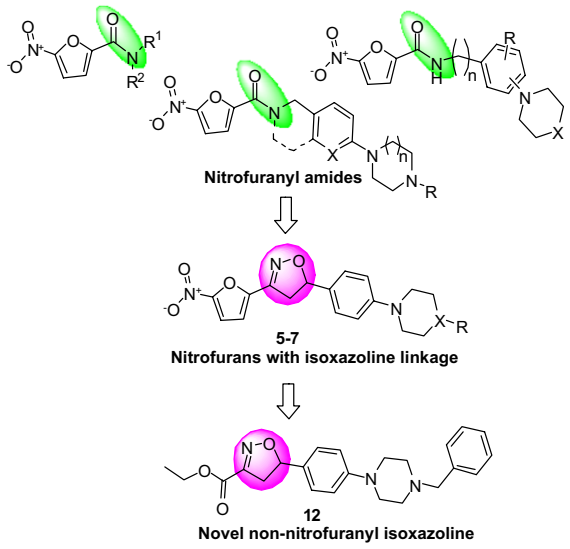

Figure 1. Discovery of novel isoxazoline compound in the course of developing nitrofuran anti-tuberculosis agents.

Keywords: Isoxazolines; Nitrofurans; Anti-tuberculosis agents; Anti-microbial agent; Tuberculosis.
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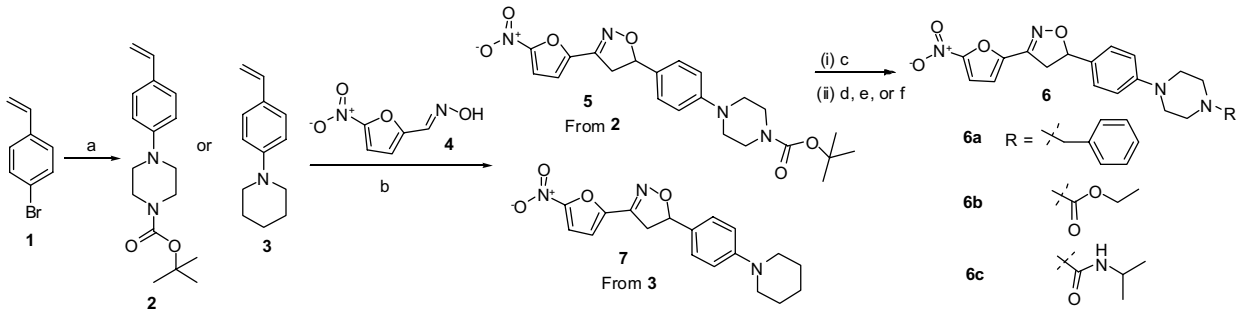


Scheme 1. Synthesis of nitrofuranyl derivatives with an isoxazoline linker. Reagents and conditions: (a) *N*-Boc piperazine or piperidine, $PdCl_2[P(o\text{-}tol)_3]$, NaO^tBu, toluene, 100 °C, 3 h; (b) *N*-chlorosuccinimide, pyridine, dry Et_3N, $CHCl_3$, 60 °C–rt, 2 h; (c) CF_3COOH–H_2O, THF, rt; (d) BnBr, K_2CO_3, DMF, rt, 6 h; (e) EtOCOCl, Et_3N, THF, rt, 6 h; (f) ^{i}PrNCO, Et_3N, THF, rt, 6 h.

amination reaction on *p*-bromo styrene **1** with *N*-Boc-piperazine.[6] Second, to establish the isoxazoline bridge, the nitrile oxide was generated in situ from oxime **4** following Torsell's procedure, which upon treatment with olefin **2**, underwent a [3 + 2] regioselective cycloaddition[7] to give isoxazoline **5** in 67% yield.[8] Boc-deprotection of **5** was achieved by aqueous trifluoroacetic acid treatment to yield the free amine. The free amine was treated with benzyl bromide in the presence of K_2CO_3 to afford **6a** (59%). Compounds **6b** and **6c** were obtained by reacting the free amine with ethyl chloroformate and isopropyl isocyanate (82% and 86%), respectively.[9] Compound **7** was synthesized in a similar manner to compound **5** starting by reacting **1** and piperidine to form **3** (86% yield) and then reacting **3** with **4** to give isoxazoline **7** (63% yield).

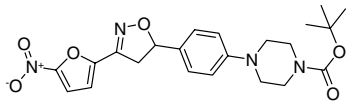
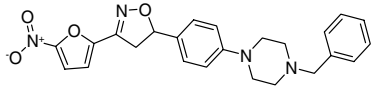
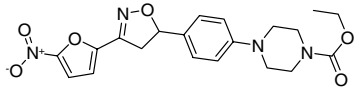
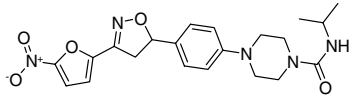
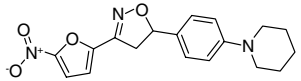
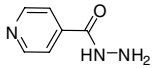
The anti-tuberculosis activity of compounds **5**, **6a**–**c**, and **7** were tested using microbroth dilution (Table 1).[10] All compounds in this series demonstrated outstanding MIC activity and compounds **6a**–**c** were advanced for in vivo testing in a short term mouse model of tuberculosis infection.[11] Unfortunately, only modest reduction in the bacterial load was observed after a 9 day treatment regime (Table 1) ($P < 0.05$). This led us to more closely examine the biopharmaceutic and pharmacokinetic properties of the series. Solubility was determined at two different pH values using a miniaturized shake-flask method.[12] Metabolic stability of the compounds was assessed in pooled rat liver microsomal preparations by monitoring disappearance of the compound. The percentage of intact parent compound was estimated using an LC–MS/MS assay. Plasma protein binding was determined by equilibrium dialysis using RED® devices (Pierce Biotechnology Inc., Rockford, IL). The results of these studies are also included in Table 1. Compound **6a** was selected for further in vivo evaluation of pharmacokinetic properties in rats. Compound **6a** was found to have an oral bioavailability of about 35%, an acceptable elimination half-life about 2.6 h but a relatively small volume of distribution of 2.0 L/kg.[13] The in vivo efficacy of anti-infective agents is usually dictated by their intrinsic antimicrobial activity and their free, unbound concentration in the target tissue, as only free, non-protein bound drug is pharmacologically active. Thus these results seem to suggest that the limiting factor for these highly protein bound compounds is tissue penetration.

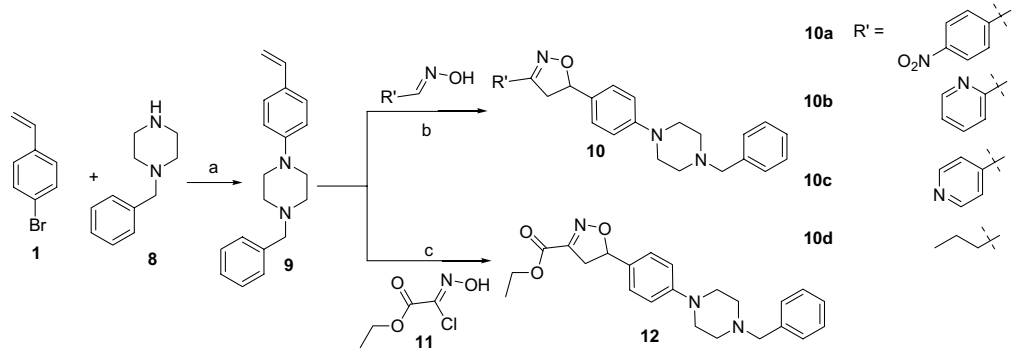
The outstanding anti-tuberculosis potency of **5**–**7** led us to question if the core isoxazoline scaffold itself had any intrinsic anti-tubercular activity. To test this hypothesis, a subsequent set of isoxazoline compounds was synthesized keeping the main core but altering the nitrofuran portion (Scheme 2) using a similar synthetic strategy. First, the *p*-bromostyrene was subject to a palladium catalyzed amination reaction with benzylpiperazine under similar conditions described earlier to give the olefin intermediate **9** in 79% yield. Then **9** was reacted with different oximes in the presence of NaOCl and catalytic triethylamine to give corresponding 3 + 2 cycloaddition products **10a**–**d** in 45–60% yields. The ester analog was created by reacting **9** with the commercially available building block **11** in the presence of base triethylamine to afford isoxazoline ethyl ester derivative **12** in 71% yield.[14]

The anti-tuberculosis activity of this series was determined and is shown in Table 2. Compound **12** was most active with a MIC_{90} of 1.56 μg/mL against *M. tuberculosis*. The remainder of the compounds **10a**–**d** did not show any appreciable activity. Importantly, **12** represents a novel isoxazoline chemotype for which anti-tuberculosis properties have not been previously noted.

In conclusion, isoxazoline linked nitrofurans were synthesized. These compounds had better anti-tuberculosis activity in vitro and had improved serum half lives over corresponding compounds in the previous nitrofuranyl amide series, demonstrating that the strategy of replacing the amide bond with isoxazoline ring was successful.[4] However, the series still possessed limited in vivo efficacy. A detailed pharmacokinetic analysis of these agents showed them to be limited by low solubility, high serum protein binding, and a low volume of distribution. When these results are combined, it strongly suggests that in vivo efficacy is limited by poor tissue penetration and low concentrations of free drug at the site of infection. These factors are now being addressed in the design of the next generation of compounds in this series.

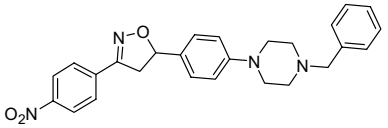
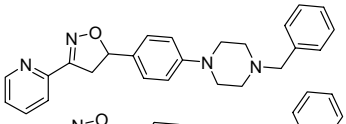
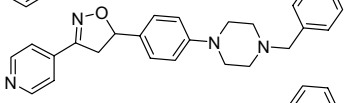
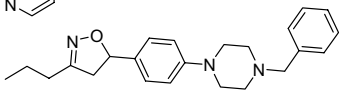
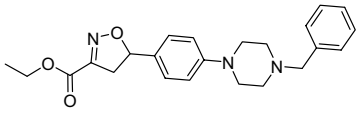
Table 1. Anti-tuberculosis activity and in vitro data of nitrofuran compounds with isoxazoline linkage (NT, not tested)

Compound	Structure	*M. tb* MIC_{90} (μg/mL)	Solubility (mg/L)		MW (g/mol)	*c*log *P*	% Protein bound	Microsomal stability % remaining at 90 min	Log_{10} reduction in *M. tb* CFU in lung versus untreated controls ±SEM
			pH 6.0	pH 7.4					
5		0.0001	NT	NT	442.5	3.99	NT	NT	NT
6a		0.00005	3.34	0.236	432.5	4.29	99.9	31	0.64 ± 0.21
6b		0.0001	4	3.6	414.4	3.28	99.0	12	0.59 ± 0.22
6c		0.0002	27.7	20	427.5	2.58	97.3	26	0.83 ± 0.20
7		0.00156	0.017	0.014	341.4	4.48	98.8	6	NT
Isoniazid		0.025	NT	NT	137.1	−0.67	NT	NT	3.98 ± 0.26



Scheme 2. Synthesis of Isoxazoline compounds by altering the nitrofuran motif. Reagents and conditions: (a) $PdCl_2[P(o\text{-tol})_3]$, NaO^tBu, toluene, 100 °C, 3 h; (b) oxime, 5% NaOCl, cat. Et_3N, CH_2Cl_2, rt, (c) Et_3N, CH_2Cl_2, rt.

Table 2. Anti-tuberculosis activity of isoxazolines **10a–d** and **12**

Compound	Structure	MIC_{90} (μg/mL)
10a		>200
10b		>200
10c		50
10d		>200
12		1.56

As the nitrofuranyl isoxazole series was so potent in vitro, we explored if the core isoxazoline had any intrinsic anti-tuberculosis activity. This led to the discovery of a novel isoxazoline compound **12** with MIC_{90} value of 1.56 μg/mL. This is a new chemotype though less potent than the nitrofurans it does offer some significant potential advantages including increased solubility as the compounds are less crystalline and lower potential side effects as no nitro group is present. Further optimization of this series is ongoing and will be reported subsequently.

Acknowledgment

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9. Representative analytical data of compound **6a**. ^{1}H NMR (500 MHz, $CDCl_3$): δ 2.5–2.62 (4H, broad s), 3.1–3.23 (4H, m), 3.32 (1H, dd, J = 9.0, 17.3 Hz), 3.53 (2H, s), 3.66 (1H, dd, J = 11.2, 17.3 Hz), 5.66 (1H, dd, J = 9.0, 11.2 Hz), 6.83 (2H, d, J = 8.7 Hz), 6.95 (1H, d, J = 3.9 Hz), 7.14–7.24 (3H, m), 7.25–7.33 (5H, m); ^{13}C NMR (300 MHz, $CDCl_3$): ppm 29.14, 40.59, 48.05, 52.27, 62.35, 83.56, 111.82, 112.47, 115.39, 126.59, 127.82, 128.79, 128.97, 146.84, 147.27, 151.08; ESI MS: 455.2 (M+23). HPLC purity: 100%, t_R = 5.23 min.
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13. Pharmacokinetic studies of compound **6a**.

Route	$t_{1/2}$ (h)	AUC_∞ (μg h/L)	Volume of distribution (L/kg)	Clearance (L/h/kg)	Bio-availability
IV (10 mg/kg)	2.6	19,091	2.0	0.53	N/A
Oral (100 mg/kg)	4.2	65,931	9.3	1.58	34.5%

14. Synthetic procedure for compound **12**. To a stirred mixture of olefin **9** (0.2 g, 0.719 mmol) and Et_3N (0.2 mL, 1.438 mmol) in anhydrous CH_2Cl_2 (10 mL), ethyl chloroximido acetate (0.163 g, 1.079 mmol) was added in portions at 0 °C. The reaction mixture was stirred at rt for 8 h and washed with water (2 × 10 mL), dried (anhyd. Na_2SO_4), and concentrated under reduced pressure. The crude product was purified by flash chromatography to give **12** (0.2 g, 71%) as a yellow solid. ^{1}H NMR (500 MHz, $CDCl_3$): δ 1.37 (3H, t, J = 7.0 Hz), 2.6 (4H, t, J = 4.8 Hz), 3.17–3.25 (5H, m), 3.5–3.51 (3H, m), 4.36 (2H, q, J = 7.3 Hz), 5.7 (1H, dd, J = 9.2, 11.4 Hz), 6.89 (2H, d, J = 8.7 Hz), 7.21 (2H, d, J = 8.7 Hz), 7.25–7.29 (2H, m), 7.31–7.37 (3 H, m); ^{13}C NMR (500 MHz, $CDCl_3$): δ 14.24, 40.84, 48.73, 52.98, 62.07, 63.06, 85.33, 115.79, 127.22, 127.31, 128.35, 129.23, 129.56, 138.01, 151.28, 151.69, 160.78; MS: 394.4 (M + 1); HPLC purity: 100% t_R = 5.13 min.