Supporting Information

Rational Design of 5-Phenyl-3-isoxazolecarboxylic Acid Ethyl Esters as Growth Inhibitors of *Mycobacterium tuberculosis* – A Potent and Selective Series for Further Drug Development

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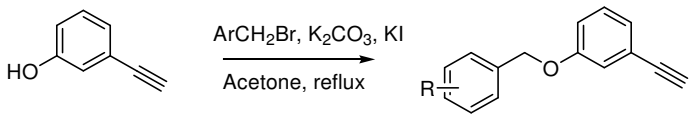
1. Synthesis of intermediates 10a–10f



General procedure. To 3-hydroxyphenylacetylene (1 eq) in acetone (12 mL/mmol, HPLC grade) was added anhydrous K_2CO_3 (6 eq) and the mixture was refluxed for 15 min. Subsequently, KI (0.5 eq) and an appropriate benzylhalide (1.2 eq) were added and the reaction mixture was refluxed for 0.5–3 h until disappearance of the starting material on TLC (EtOAc–hexane 1:4 as an eluent). The reaction mixture was cooled, filtered, and the filtrate was evaporated *in vacuo*. The crude product was purified by flash chromatography using gradient elution from hexane to 10%–30% EtOAc–hexane. The reactions were typically carried out in 200–800 mg quantities.

1-Ethynyl-3-[[(3-trifluoromethyl)phenyl]methoxy]benzene (10a). Yield 88% (colorless oil); ^{1}H NMR ($CDCl_3$) δ 3.07 (1H, s), 5.09 (2H, s), 6.98 (1H, m), 7.12 (2H, m), 7.25 (1H, m), 7.51 (1H, m), 7.60 (2H, m), 7.70 (1H, br s).

1-Ethynyl-3-(phenylmethoxy)benzene (10b). Yield 84% (colorless oil); ^{1}H NMR ($CDCl_3$) δ 3.07 (1H, s), 5.07 (2H, s), 6.99 (1H, m), 7.12 (2H, m), 7.25 (1H, m), 7.33–7.45 (5H, m).

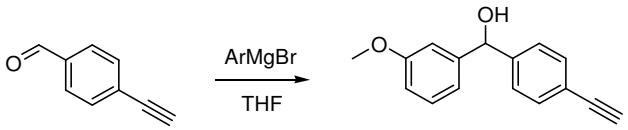
1-Ethynyl-3-[[(2-trifluoromethyl)phenyl]methoxy]benzene (10c). Yield 81% (colorless oil); ^{1}H NMR ($CDCl_3$) δ 3.06 (1H, s), 5.26 (2H, s), 6.97 (1H, m), 7.11 (2H, m), 7.24 (1H, m), 7.42 (1H, m), 7.57 (1H, m), 7.71 (2H, m).

1-Ethynyl-3-[[(4-trifluoromethyl)phenyl]methoxy]benzene (10d). Yield 91% (colorless oil); ^{1}H NMR ($CDCl_3$) δ 3.08 (1H, s), 5.13 (2H, s), 6.98 (1H, m), 7.13 (2H, m), 7.26 (1H, m), 7.55 (2H, m), 7.66 (2H, m)

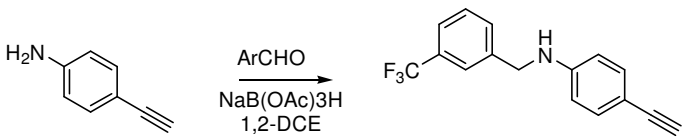
4-[(3-Ethynylphenoxy)methyl]benzoic Acid Ethyl Ester (10e). Yield 84% (colorless oil); ^{1}H NMR ($CDCl_3$) δ 1.41(3H, t, J = 7.2 Hz), 3.07 (1H, s), 4.40 (2H, q, J = 7.2 Hz), 5.13 (2H, s), 6.97 (1H, m), 7.05–7.25 (3H, m), 7.50 (2H, d, J = 8.1 Hz), 8.08 (2H, d, J = 8.1 Hz)

1-Ethynyl-3-[(3,4-difluorophenyl)methoxy]benzene (10f). Yield 59% (white solid); ^{1}H NMR ($CDCl_3$) δ 3.07 (1H, s), 5.00 (2H, s), 6.95 (1H, m), 7.07 (1H, m), 7.11–7.28 (5H, m)

2. Synthesis of intermediates 20, 29, and 32

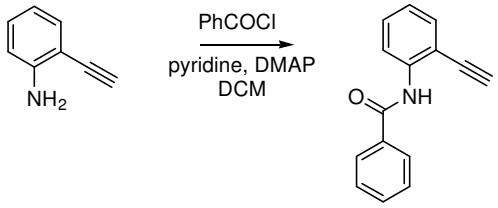


***α*-(4-Ethenylphenyl)-(3-methoxybenzene)methanol (20).** 4-Ethynylbenzaldehyde (0.20 g, 1.5 mmol) in anhydrous THF (15 mL) was cooled to 0 °C. 1M solution of 3-methoxyphenylmagnesium bromide (0.39 g, 1.8 mmol) in THF was added drop wise and the reaction mixture was stirred 4 h at room temperature. The reaction was quenched with aqueous sat. NH_4Cl (50 mL), extracted with ether (2 × 50 mL), washed with brine (30 mL), and dried with Na_2SO_4. After evaporation, the crude product was purified by flash chromatography using gradient elution from hexane to 50% EtOAc–hexane to give the acetylene intermediate **20** in 78% yield. ^{1}H NMR δ 2.25 (1H, br s), 3.80 (3H, s), 5.82 (1H, s), 6.83 (1H, m), 6.93 (2H, m), 7.27 (1H, m), 7.36 (2H, d, J = 8.1 Hz), 7.36 (2H, d, J = 8.1 Hz).



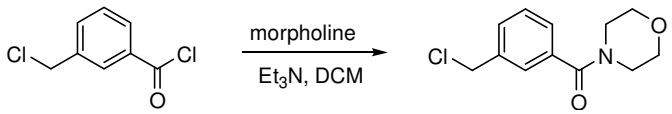
***N*-(4-Ethynylphenyl)-3-(trifluoromethyl)benzenemethanamine (29).** To a solution of 4-(trifluomethyl)benzaldehyde (0.30 g, 1.7 mmol) in anhydrous 1,2 dichloroethane (13 mL) were added 4-ethynylaniline (0.20 g, 1.7 mmol), $NaB(OAc)_3H$ (0.43 g, 2.0 mmol), and acetic acid (0.11 g, 0.11 mL, 1.9 mmol). The mixture was stirred at room temperature for 3 h. The reaction mixture was poured into water (40 mL) and extracted with CH_2Cl_2 (3 × 20 mL). The combined organic layers were washed with saturated $NaHCO_3$ (20 mL) and brine (20 mL), and dried with $MgSO_4$. After filtration, the solvent was

evaporated and the crude product was purified first by flash chromatography (gradient elution from hexane to 15% EtOAc–hexane). Yield 53% (colorless oil). ^{1}H NMR ($CDCl_3$) δ 2.96 (1H, s), 4.30 (1H, broad s), 4.42 (2H, d, *J* = 5.7 Hz), 6.53 (2H, m), 7.31 (2H, m), 7.46 (1H, m), 7.54 (2H, m), 7.61 (1H, m).



***N*-(2-Ethynylphenyl)benzamide (32)**. Pyridine (0.41 g, 5.1 mmol, 0.41 mL) was added slowly to a cold solution of 2-ethynylaniline (0.30 g, 2.6 mmol, 0.29 mL), benzoyl chloride (0.43 g, 3.1 mmol, 0.36 mL), and 4-DMAP (0.03 g, 0.26 mmol) in CH_2Cl_2 (30 mL), and the reaction mixture was stirred at room temperature for overnight. The reaction was quenched with 3% HCl (30 mL), extracted with CH_2Cl_2 (3 × 15 mL), washed with brine (20 mL) and water (20 mL), and dried with $MgSO_4$. After filtration, the solvent was evaporated to give crude *N*-(2-ethynylphenyl)benzamide as a beige solid in 98% yield. Intermediate **32** was used for the subsequent step without further purification. ^{1}H NMR (CDCl3) δ 3.6 (1H, s), 7.09 (1H, dt, J = 1.0 Hz, J = 7.6 Hz), 7.44 (1H, m), 7.50–7.60 (4H, m), 7.93 (2H, m), 8.61 (1H, d, J = 8.4 Hz), 8.80 (1H, br s).

3. Synthesis of [3-(chloromethyl)phenyl]-4-morpholinylmethanone



[3-(Chloromethyl)phenyl]-4-morpholinyl-methanone. 3-(Chloromethyl)benzoyl chloride (0.27 g, 0.2 mL, 1.4 mmol) in anhydrous CH_2Cl_2 was cooled to 0 °C, followed by drop wise addition of Et_3N (0.43 g, 0.59 mL, 4.2 mmol)and morpholine (0.18 g, 0.18 mL, 2.1 mmol), respectively. The reaction mixture was stirred at room temperature for 2 h, quenched with water (50 mL) and extracted with CH_2Cl_2 (2 x 15 mL).

The combined organic layers were washed with 5% HCl (10 mL), sat. $NaHCO_3$ (10 mL), and brine (10 mL), and dried with Na_2SO_4. After filtration, the solvent was evaporated to give the title compound as colorless oil in 99% yield, which was used for the next step without further purification. ^{1}H NMR ($CDCl_3$) δ 3.40–3.85 (8H, m), 4.60 (2H, s), 7.34–7.47 (4H, m).

3. Description of the biological assays

Microplate Alamar Blue assay (MABA).[1] Briefly, the test compound MICs against *Mtb* H_{37}RV (ATCC 27294) were assessed by the MABA using rifampin and isoniazid as positive controls. Compound stock solutions were prepared in DMSO at a concentration of 12.8 mM, and the final test concentrations ranged from 128 μM to 0.5 μM. Two fold 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-sterilized) in a volume of 100 μL in 96-well microplates (BD Optilux™,96-well Microplates , black/clear flat bottom). *Mtb* cultures (100 μL inoculum of 2×10^5 cfu/mL) were added, yielding a final testing volume of 200 μL. The 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™) were added to the wells. After incubation at 37 °C for 16–24 h, fluorescence of the wells was measured (ex 530, em 590 nm). The MICs ware defined as the lowest concentration effecting a reduction in fluorescence of $\geq$ 90% relative to the mean of replicate bacteria-only controls.

Low-oxygen recovery assay (LORA).[2] Briefly, a low-oxygen adapted culture of recombinant H_{37}Rv (pFCA-luxAB), expressing a *Vibrio harveyii* luciferase gene with an acetamidase promoter, was grown in a BiostatQ fermentor. Cells were collected on ice, washed in PBS, and stored at –80 °C. Circa 10^5 cfu/mL of thawed NRP cells were exposed to 2-fold serial dilutions of test compound in 7H9 broth in white 96-well plates, which were incubated 10 days anaerobically at 37 °C. Luminescence readings were obtained

(1) Franzblau, S. G.; Witzig, R. S.; Mclaughlin, J. C.; Torres, P.; Madico, G.; Hernandez, A.; Degnan, M. T.; Cook, M. B.; Quenzer, V. K.; Ferguson, R. M.; Gilman, R. H. Rapid, low-technology MIC determination with clinical Mycobacterium tuberculosis isolates by using the microplate Alamar Blue assay. *J. Clin. Microbiol*. **1998**, 36, 362–366.

(2) Cho, S. H.; Warit, S.; Wan, B.; Hwang, C. H.; Pauli, G. F. Franzblau S. G. Low-Oxygen-Recovery Assay for High-Throughput Screening of Compounds against Nonreplicating Mycobacterium tuberculosis. *Antimicrob. Agents Chemother.* **2007**, *51*, 1380–1385.

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. [3] Cytotoxicity was determined by exposing different concentrations of samples to Vero cells. Briefly, samples were dissolved at 12.8 mM in DMSO. Six 3-fold dilutions were performed in growth medium MEM (Gibco, Grand Island, NY), containing 10% fetal bovine serum (HyClone, Logan, UT), 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). Final DMSO concentrations did not exceed 1% v/v. Drug dilutions were distributed in duplicate in 96-well tissue culture plates (Becton Dickinson Labware, Lincoln Park, NJ) at a volume of 50 μL per well. An equal volume containing 5×10^5 log phase Vero cells (CCL-81; American Type Culture Collection, Rockville, MD) was added to each well and the cultures were incubated at 37 °C in an atmosphere containing 5% of CO_2. After 72 h, cell viability was measured using the CellTiter 96 aqueous non-radioactive cell proliferation assay (Promega Corp., Madison, WI) according to the manufacturer's instructions. Absorbance at 490 nm was read in a Victor^2 multilabel reader (PerkinElmer). The IC_{50}s were determined using a curve-fitting program.

(3) Falzari, K.; Zhu, Z.; Pan, D.; Liu, H.; Hongmanee, P.; Franzblau., S. G. In vitro and in vivo activities of macrolide derivatives against Mycobacterium tuberculosis. *Antimicrob Agents Chemother*. **2005** *4*9, 1447–1454.