SUPPORTING INFORMATION

Discovery of Pyrazolopyridones as a Novel Class of Non-covalent DprE1 Inhibitor with Potent Anti-Mycobacterial Activity

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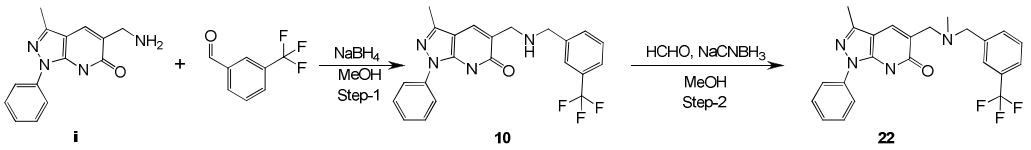
Chemistry: Synthetic procedure for Compound 22-26

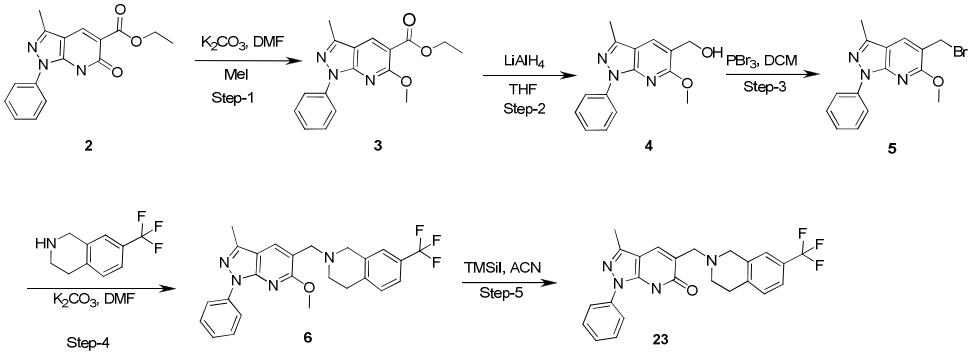
Synthesis of 3-methyl-5-((methyl(3-(trifluoromethyl)benzyl)amino)methyl)-1-phenyl-1,7-dihydro-6H-pyrazolo[3,4-b]pyridin-6-one (22):



3-methyl-1-phenyl-5-(((3-(trifluoromethyl)benzyl)amino)methyl)-1,7-dihydro-6H-pyrazolo[3,4-b]pyridin-6-one, 10: To a solution of 5-(aminomethyl)-3-methyl-1-phenyl-1,7-dihydro-6H-pyrazolo[3,4-b]pyridin-6-one **i** (0.15 g, 0.59 mmol) and Et_3N (0.164 ml,1.18 mmol) in methanol (10 ml) was added 2-(trifluoromethyl)benzaldehyde (0.102 g, 0.59 mmol) and acetic acid (catalytic) under nitrogen. The reaction mixture was stirred for 2 h, then upon cooling at 0 ^{0}C was added and $NaBH_4$ (44 mg, 1.18 mmol) in portionwise. The resulting mixture was stirred at RT for 16 h. Then the solvent was removed under reduced pressure and the crude was partition between CH_2Cl_2 and H_2O. The organic extract was washed with water, brine and concentrated under pressure. The crude was further purified through silica gel chromatography using 2% MeOH in DCM to afford 3-methyl-1-phenyl-5-(((3-(trifluoromethyl)benzyl)amino)methyl)-1,7-dihydro-6H-pyrazolo[3,4-b]pyridin-6-one **3** as a white solid. Yield: 0.1 g 41.6%.

3-methyl-1-phenyl-5-(((3-(trifluoromethyl)benzyl)amino)methyl)-1,7-dihydro-6H-pyrazolo[3,4-b]pyridin-6-one (22): To a solution of 3-methyl-1-phenyl-5-(((3-(trifluoromethyl)benzyl)amino)methyl)-1,7-dihydro-6H-pyrazolo[3,4-b]pyridin-6-one **3** (0.1 g, 0.29 mmol) in methanol (10 ml) was added formalin (0.2 ml) and sodium cyanoborohydride (27 mg, 0.435 mmol) and acetic acid (catalytic) under nitrogen at 0 ^{0}C. Then the mixture was stirred for overnight at RT. Then the solvent was removed under reduced pressure and the crude was partition between ethyl acetate and H_2O. The organic layer was washed with H_2O, brine, dried under Na_2SO_4 and concentrated under reduced pressure. The crude was purified by purified through silica gel chromatography using 1% MeOH in DCM as a eluent to afford 3-methyl-1-phenyl-5-(((3-(trifluoromethyl)benzyl)amino)methyl)-1,7-dihydro-6H-pyrazolo[3,4-b]pyridin-6-one **22** as a white solid. Yield: 35 mg, 35%.

Synthesis of 3-methyl-1-phenyl-5-((7-(trifluoromethyl)-3,4-dihydroisoquinolin-2(1H)-yl)methyl)-1,7-dihydro-6H-pyrazolo[3,4-b]pyridin-6-one (23):



Ethyl 6-methoxy-3-methyl-1-phenyl-1H-pyrazolo[3,4-b]pyridine-5-carboxylate 3: To a solution of ethyl 3-methyl-6-oxo-1-phenyl-6,7-dihydro-1H-pyrazolo[3,4-b]pyridine-5-carboxylate **2** (200 mg, 0.67 mmol) in DMF (5 ml) was added K_2CO_3 (190 mg, 1.34 mmol) under nitrogen and the reaction mixture was stirred for 30 mim. Upon cooling at 0 ^{0}C was added MeI (195 mg, 1.34 mmol) and the resulting mixture was stirred at RT for 16 h. The

progress of the reaction was monitored by TLC and the solvent was removed under reduced pressure. The crude was partition between CH_2Cl_2 and H_2O. The organic extract was washed with water, brine, dried under Na_2SO_4 and concentrated under pressure. The crude was purified by column chromatography using 15% EtOAC in petether) as an eluent to afford ethyl 6-methoxy-3-methyl-1-phenyl-1H-pyrazolo[3,4-b]pyridine-5-carboxylate **3** as a colorless solid. Yield: 110 mg, 52.6%.

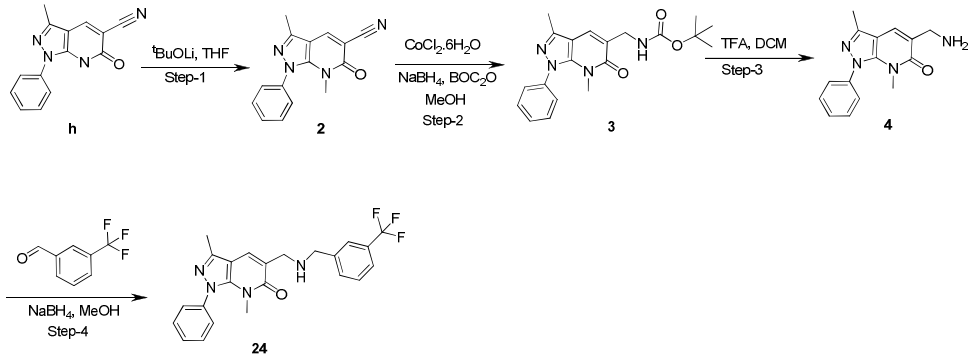
(6-Methoxy-3-methyl-1-phenyl-1H-pyrazolo[3,4-b]pyridin-5-yl)methanol 4: To a stirred suspension of LiAlH4 (27 mg, 71 mmol) in dried THF, was added dropwise a solution of ethyl 6-methoxy-3-methyl-1-phenyl-1H-pyrazolo[3,4-b]pyridine-5-carboxylate **2** (110 mg, 0.35 mmol) in dried THF (3 mL) at 0 ^{0}C. The resulting mixture stirred for another 30 minutes until the reaction was completed, which monitored by TLC. Then the reaction mixture was quenched by adding ice water (0.5 mL) and filtered through celite. The filtrate was dried under Na2SO4 and concentrated under reduced pressure to afford (6-Methoxy-3-methyl-1-phenyl-1H-pyrazolo[3,4-b]pyridin-5-yl)methanol **4** as a gummy solid. Yield: 70 mg, 77.7%.

5-(Bromomethyl)-6-methoxy-3-methyl-1-phenyl-1H-pyrazolo[3,4-b]pyridine 5: To a stirred solution of (6-Methoxy-3-methyl-1-phenyl-1H-pyrazolo[3,4-b]pyridin-5-yl)methanol **3** (70 mg, 0.26 mmol) in DCM (10 mL) was added PBr_3 (141 mg, 52 mmol) in dropwise at 0 ºC and the mixture was stirred for 1 h at RT. The solvent was evaporated under the reduced pressure and the reaction mixture was poured in to water (20 mL) and extracted with ethyl acetate. The combined organic layer was washed with water, brine, dried over sodium sulfate, and concentrated at reduced pressure to give 5-(Bromomethyl)-6-methoxy-3-methyl-1-phenyl-1H-pyrazolo[3,4-b]pyridine **5**. Yield: 60 mg (crude). The crude material was taken as such for next step without purification.

2-((6-Methoxy-3-methyl-1-phenyl-1H-pyrazolo[3,4-b]pyridin-5-yl)methyl)-7-(trifluoromethyl)-1,2,3,4-tetrahydroisoquinoline 6: To a suspension of 7-(trifluoromethyl)-1,2,3,4-tetrahydroisoquinoline (72.7 mg, 0.36 mmol) and K_2CO_3 (75 mg, 0.54 mmol) in DMF (3 ml) was added 5-(Bromomethyl)-6-methoxy-3-methyl-1-phenyl-1H-pyrazolo[3,4-b]pyridine **5** (60 mg, 0.18 mmol)under nitrogen and the reaction mixture was stirred for 5 h. The progress of the reaction was monitored by TLC and the solvent was removed under reduced pressure and the crude was partition between CH_2Cl_2 and H_2O. The organic extract was washed with water, brine, dried under Na_2SO_4 and concentrated under pressure. The crude was triturated with EtOAC and petether solvent mixture to afford 2-((6-methoxy-3-methyl-1-phenyl-1H-pyrazolo[3,4-b]pyridin-5-yl)methyl)-7-(trifluoromethyl)-1,2,3,4-tetrahydroisoquinoline **6**. The solid was taken as such for next step. Yield: 50 mg (crude).

3-methyl-1-phenyl-5-((7-(trifluoromethyl)-3,4-dihydroisoquinolin-2(1H)-yl)methyl)-1,7-dihydro-6H-pyrazolo[3,4-b]pyridin-6-one (23): To a solution of 2-((6-methoxy-3-methyl-1-phenyl-1H-pyrazolo[3,4-b]pyridin-5-yl)methyl)-7-(trifluoromethyl)-1,2,3,4-tetrahydroisoquinoline **6** (50 mg, 0.11 mmol) in ACN (5 ml) was added trimethylsilyl iodide (110 mg, 0.55 mmol) at 0 ^{0}C.The resulting reaction mixture was heated to 80°C for 1h. The reaction mixture was concentrated and diluted with water and extracted with DCM. The combined organic extract was washed with brine dried over anhydrous Na_2SO_4 and concentrated reduced pressure. The crude was purified through silica gel chromatography using (0 -5% MeOH in DCM) to yield **3**-methyl-1-phenyl-5-((7-(trifluoromethyl)-3,4-dihydroisoquinolin-2(1H)-yl)methyl)-1,7-dihydro-6H-pyrazolo[3,4-b]pyridin-6-one **23** as a white solid. Yield: 15 mg 31.25%.

Synthesis of 3,7-dimethyl-1-phenyl-5-(((3-(trifluoromethyl)benzyl)amino)methyl)-1,7-dihydro-6H-pyrazolo[3,4-b]pyridin-6-one (24):



3,7-Dimethyl-6-oxo-1-phenyl-6,7-dihydro-1H-pyrazolo[3,4-b]pyridine-5-carbonitrile 2: To a stirred solution of 3-methyl-6-oxo-1-phenyl-6,7-dihydro-1H-pyrazolo[3,4-b]pyridine-5-carbonitrile **h** (1.3 g, 5.19 mmol) in THF (10 mL) was added lithium tert-butoxide (0.42 g, 5.194 mmol) at 0 ^{0}C. To this solution CH_3I (0.737g 5.194 mmol) was added and the mixture was stirred for 16 h at RT in a sealed tube. Then solvent was evaporated under the reduced pressure and the reaction mixture was poured in to water and extracted with ethyl acetate. The organic layer was washed with H2O, brine, dried under Na_2SO_4 and concentrated under reduced pressure. The crude was further purified through silica gel chromatography 50% EA in pet ether to yield**,** 7-Dimethyl-6-oxo-1-phenyl-6,7-dihydro-1H-pyrazolo[3,4-b]pyridine-5-carbonitrile **2** as a white solid. Yield: 0.5 g, 36.4%.

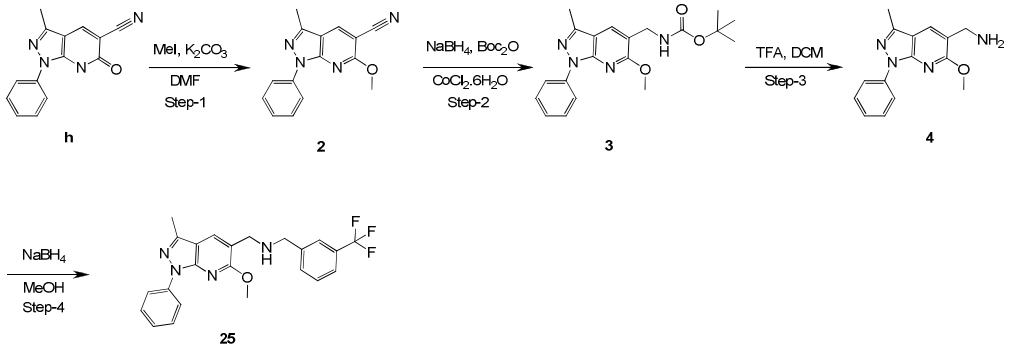
Tert-butyl ((3,7-dimethyl-6-oxo-1-phenyl-6,7-dihydro-1H-pyrazolo[3,4-b]pyridin-5-yl)methyl)carbamate 3: To a stirred solution of 7-Dimethyl-6-oxo-1-phenyl-6,7-dihydro-1H-pyrazolo[3,4-b]pyridine-5-carbonitrile **2** (0.5 g, 1.89 mmol) in methanol (10 mL), to this added were added cobalt chloride hexahydrate (0.675 g, 2.83 mmol) and di-t-butyl dicarbonate (0.824 g, 3.78 mmoles) and stirred for 2 h at rt. Subsequently was NaBH4 (0.14 g, 3.78 mmoles) at 0 ^{0}C and the mixture was stirred for 16 h at RT. Then the reaction mixture was filtered through celite and the filtrate was quenched with saturated NH_4Cl. The organic layer was separated, washed with H_2O, brine and dried under pressure. The crude was purified by column chromatography (15% EtOAc in petether) to afford tert-butyl ((3,7-dimethyl-6-oxo-1-phenyl-6,7-dihydro-1H-pyrazolo[3,4-b]pyridin-5-yl)methyl)carbamate **3** as a white solid. Yield: 0.15 g, 21.7%.

5-(Aminomethyl)-3,7-dimethyl-1-phenyl-1,7-dihydro-6H-pyrazolo[3,4-b]pyridin-6-one 4: To a solution of tert-butyl ((3,7-dimethyl-6-oxo-1-phenyl-6,7-dihydro-1H-pyrazolo[3,4-b]pyridin-5-yl)methyl)carbamate **3** (0.15 g,0.407 mmol) in DCM (10 ml) was added TFA (0.15 ml) at 0 ^{0}C.The resulting reaction mixture was stirred 4 h at rt. . Then the solvent was evaporated under the reduced pressure and washed with diethyl ether to afford 5-(Aminomethyl)-3,7-dimethyl-1-phenyl-1,7-dihydro-6H-pyrazolo[3,4-b]pyridin-6-one **4** as an off-white solid. Yield: 0.13 g (100%).

3,7-dimethyl-1-phenyl-5-(((3-(trifluoromethyl)benzyl)amino)methyl)-1,7-dihydro-6H-pyrazolo[3,4-b]pyridin-6-one (24): To a solution of 5-(Aminomethyl)-3,7-dimethyl-1-phenyl-1,7-dihydro-6H-pyrazolo[3,4-b]pyridin-6-one **4** (0.130 g, 0.48 mmol) and Et_3N (0.133ml, 0.96 mmol) in methanol (10 ml) was added 2-(trifluoromethyl)benzaldehyde (83 mg, 0.48 mmol) and acetic acid (catalytic) under nitrogen. The reaction mixture was stirred for 2 h, then the reaction mixture was cooled to 0 ^{0}C and was then added $NaBH_4$ (36 mg, 0.96 mmol) in portionwise. The resulting mixture was stirred at RT for 16 h. Then the solvent was removed under reduced pressure and the crude was partition between CH_2Cl_2 and H_2O. The organic extract was washed with water, brine and concentrated under pressure which was further purified through silica gel chromatography using 1% MeOH in DCM as a eluent to give 3,7-

dimethyl-1-phenyl-5-(((3-(trifluoromethyl)benzyl)amino)methyl)-1,7-dihydro-6H-pyrazolo[3,4-b]pyridin-6-one **24** as a white solid. Yield: 20 mg (10%)

Synthesis of 1-(6-methoxy-3-methyl-1-phenyl-1H-pyrazolo[3,4-b]pyridin-5-yl)-N-(3-(trifluoromethyl)benzyl)methanamine (21):



6-Methoxy-3-methyl-1-phenyl-1H-pyrazolo[3,4-b]pyridine-5-carbonitrile 2: To a solution of 3-methyl-6-oxo-1-phenyl-6,7-dihydro-1H-pyrazolo[3,4-b]pyridine-5-carbonitrile **h** (500 mg, 2.0 mmol) in DMF (8 ml) was added K_2CO_3 (552 mg, 4.0 mmol) under nitrogen and the reaction mixture was stirred for 30 mim. Upon cooling at 0 ^{0}C was added MeI (568 mg, 4.0 mmol) and the resulting mixture was stirred at RT for 16 h. The progress of the reaction was monitored by TLC and the solvent was removed under reduced pressure and the crude was partition between CH_2Cl_2 and H_2O. The organic extract was washed with water, brine, dried under Na_2SO_4 and concentrated under pressure. The crude was purified by column chromatography using (30% EtOAC in petether) as an eluent to afford 6-methoxy-3-methyl-1-phenyl-1H-pyrazolo[3,4-b]pyridine-5-carbonitrile **2** as a colorless solid. Yield: 400 mg, 75.4%.

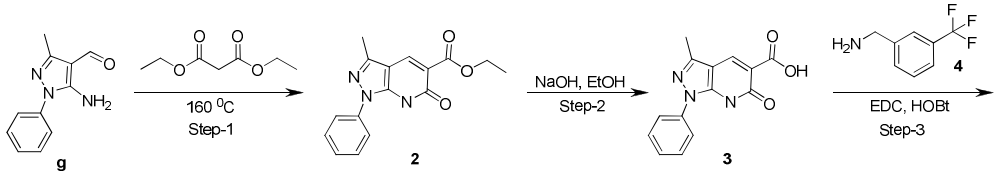
Tert-butyl((6-methoxy-3-methyl-1-phenyl-1H-pyrazolo[3,4-b]pyridin-5-yl)methyl) carbamate 3: To a stirred solution of afford 6-methoxy-3-methyl-1-phenyl-1H-pyrazolo[3,4-b]pyridine-5-carbonitrile **2** (500 mg, 1.88 mmol) in MeOH (20 mL) was subsequently treated with di-t-butyl dicarbonate (1.22 g, 5.66 mmol) and $CoCl_2.6H_2O$ (0.22 g, 0.94mmol). To the reaction mixture was then added $NaBH_4$ (0.25 g, 6.60mmol) at 0 ^{0}C and continued stirring for 16 h. Then the reaction mixture was filtered through celite and the filtrate was quenched with saturated NH_4Cl. The organic layer was separated, washed with H_2O, brine and dried under pressure. The crude was purified by column chromatography (15% EtOAc in pet ether) to afford tert-butyl((6-methoxy-3-methyl-1-phenyl-1H-pyrazolo[3,4-b]pyridin-5-yl)methyl) carbamate **3** as pale yellow solid. Yield: 250 mg, 36.2%.

(6-Methoxy-3-methyl-1-phenyl-1H-pyrazolo[3,4-b]pyridin-5-yl)methanamine 4: To a stirred solution of tert-butyl((6-methoxy-3-methyl-1-phenyl-1H-pyrazolo[3,4-b]pyridin-5-yl)methyl) carbamate **3** (250 mg, 0.67 mmol) in DCM (10 mL) was added TFA at 0 ^{0}C. The mixture was stirred at RT for 3 h. Then the solvent was removed under reduced pressure and the residue was triturated with diethyl ether to afford **(**6-methoxy-3-methyl-1-phenyl-1H-pyrazolo[3,4-b]pyridin-5-yl)methanamine **4** (200 mg, 66.6%).

1-(6-Methoxy-3-methyl-1-phenyl-1H-pyrazolo[3,4-b]pyridin-5-yl)-N-(3-(trifluoromethyl) -benzyl)methanamine (25): To a solution of (6-Methoxy-3-methyl-1-phenyl-1H-pyrazolo[3,4-b]pyridin-5-yl)methanamine **4** (200 mg, 0.74 mmol) and Et_3N (0.21 ml, 1.49mmol) in methanol (10 ml) was added 3-(trifluoromethyl)benzaldehyde (0.103 g, 0.59 mmol) and acetic acid (catalytic) under nitrogen. The reaction mixture was stirred for 2 h, then the reaction mixture was cooled to 0 ^{0}C and was added $NaBH_4$ (56 mg, 1.49mmol) in portionwise. The resulting mixture was stirred at RT for 16 h. Then the solvent was removed under reduced pressure and the crude was partition between CH_2Cl_2 and H_2O. The organic

extract was washed with water, brine, and concentrated under pressure. The crude product was purified by crystallization using CH_2Cl_2 and petether mixture to yield 1-(6-methoxy-3-methyl-1-phenyl-1H-pyrazolo[3,4-b]pyridin-5-yl)-N-(3-(trifluoromethyl)benzyl)methanamine **25** as a off-white solid. Yield: 105 mg, 33.8%.

Synthesis of 3-methyl-6-oxo-1-phenyl-N-(3-(trifluoromethyl)benzyl)-6,7-dihydro-1H-pyrazolo[3,4-b]pyridine-5-carboxamide (26):



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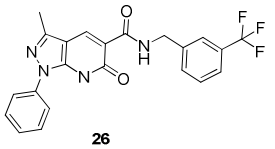

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Ethyl 3-methyl-6-oxo-1-phenyl-6,7-dihydro-1H-pyrazolo[3,4-b]pyridine-5-carboxylate 2: The a suspension of 5-amino-3-methyl-1-phenyl-1H-pyrazole-4-carbaldehyde **g** (3.5 g, 17.39 mmol) and diethylmalonate (4.17 g, 26.08 mmol) was heated at 160 °C for 8 h. The reaction mixture was diluted with diethyl ether (100 mL) and stirred for 15 min. The precipitated yellow solid was filtered and dried under vacuum to afford ethyl 3-methyl-6-oxo-1-phenyl-6,7-dihydro-1H-pyrazolo[3,4-b]pyridine-5-carboxylate **2** as a yellow solid. Yield: 0.66 g, 12.9%.

3-Methyl-6-oxo-1-phenyl-6,7-dihydro-1H-pyrazolo[3,4-b]pyridine-5-carboxylic acid 3: A solution of ethyl 3-methyl-6-oxo-1-phenyl-6,7-dihydro-1H-pyrazolo[3,4-b]pyridine-5-carboxylate **2** (0.3 g, 1.01 mmol) in ethanol (10 mL) was added NaOH (80.7 mg, 2.01 mmol) and water (5 mL) stirred at 23 °C for 3h. The Reaction mixture was concentrated under reduced pressure and diluted with water and washed with diethyl ether. Subsequently aqueous layer was acidified with 10%HCl and extracted in 10%MeOH in MDC. The organic layer was concentrated under reduced pressure to furnish 3-methyl-6-oxo-1-phenyl-6,7-dihydro-1H-pyrazolo[3,4-b]pyridine-5-carboxylic acid **3** as off white solid. Yield: 0.20 g, 73.8%.

3-Methyl-6-oxo-1-phenyl-N-(3-(trifluoromethyl)benzyl)-6,7-dihydro-1H-pyrazolo[3,4-b]pyridine-5-carboxamide (26): To a solution of 3-methyl-6-oxo-1-phenyl-6,7-dihydro-1H-pyrazolo[3,4-b]pyridine-5-carboxylic acid **3** (0.2 g, 0.74 mmol) in DMF (5 mL) was added 3-(trifluoromethyl)phenyl)methanamine **4** (195 mg, 1.11 mmol) followed by EDC.HCl (213.5 mg, 1.11 mmol) and HOBT (0.051 g, 0.37 mmol) and stirred at rt for 16 h. The progress of the reaction was monitored by TLC and the resulting mixture was diluted with water and extracted in ethyl acetate. The organic layer was washed with H2O, brine, dried under Na_2SO_4 and concentrated under reduced pressure. The crude was purified by column chromatography using 50% EtoAc in petether as an eluent to afford 3-methyl-6-oxo-1-phenyl-N-(3-(trifluoromethyl)benzyl)-6,7-dihydro-1H-pyrazolo[3,4-b]pyridine-5-carboxamide **26** as a white solid. Yield: 130 mg, 41.1%.

M.tuberculosis (Mtb) strains

M. tuberculosis H37Rv ATCC 27294 used for MIC determination was grown as reported earlier (Jayaram *et. al. Antimicrob. Agents Chemother*. **2003**, *47*, 2118-2124). The inocula used for all the experiments were derived from a single seed lot that had been maintained at -70°C. Briefly, *M. tuberculosis* was grown in roller bottles at 37°C for 7 to 10 days in Middlebrook 7H9 broth supplemented with 0.2% glycerol, 0.05% Tween 80 (Sigma), and 10% albumin dextrose catalase (7H9) (Difco Laboratories, Detroit, Mich.). The cells were

harvested by centrifugation, washed twice in 7H9 broth, and resuspended in fresh 7H9 broth. Aliquots of 0.5 ml were dispensed, and the seed-lot suspensions were stored at -70°C.

Determination of minimal inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

All test compound stocks and dilutions were prepared in DMSO. Mtb MICs of test compounds were determined in 7H9 medium by the standard microdilution method (Franzblau, et al *J. Clin. Microbiol*. **1998**, *36*, 362 and Balganesh *et. al. Antimicrob. Agents Chemother*. **2010**, *54*, 5167) with some modifications. Briefly, 1µl of serial two-fold dilutions of test compound were dispensed in a 384 well micro titre plate (Corning 3702), at final concentrations ranging from 100µM- 0.19µM. 40 µl (3-7 x 10^5 CFU/ml) of the bacterial culture was added to all the wells except the media control wells. Control wells included media and culture controls. The plates were packed in gas permeable polythene bags and incubated at 37°C for 5 days. Following this incubation period, 8 µl of a freshly prepared 1:1 mixture of Resazurin (0.02% in water), and 10% Tween 80 was added to all the wells. The plates were re-incubated for an additional 24 hours at 37°C and the colour conversion of all wells recorded. MIC is defined as the lowest drug concentration which prevented the colour change from blue to pink. In order to determine the MICs, absorbance was monitored at 575 nm & 610nm and their ratio calculated. Using the ratio values, the media control and the no inhibitor / culture controls are assigned as equivalent to 100% and 0% inhibition, respectively. Within the concentrations screened with the test compounds, the least concentration which yielded 80% inhibition was considered as MIC. In all the assays, Isoniazid MIC values was always generated in parallel to ensure the validity of the assay conditions. Aliquots from sample wells (MIC and higher) from the MIC plates were diluted 1:10 and plated on 7H10 agar plates. Plates were incubated at 37ºC for 3-4 weeks and the resultant CFUs were enumerated. The least compound concentration that resulted in a reduction of two $\log_{10}$CFU from the start CFU was considered as MBC.

MIC for drug sensitive and single drug resistant *M. tuberculosis* isolates

This assay was set up using the same protocol as above, however the incubation period was extended up to 2-3 weeks. Cell growth was monitored turbidometrically and the least concentration which showed no growth was identified as MIC. With single drug resistant strains, the respective resistance marker drug was included as positive control.

Killing kinetics in 7H9 Broth

The killing kinetics assay in 7H9 broth was performed in a 200 µL volume using 96-well plates with Middlebrook 7H9 medium as described (Shirude et al. *J. Med. Chem*. **2013**, *56*, 9701-9708). Serial two-fold dilutions of compounds were made in DMSO separately, with the concentrations ranging from 128 to 0.25 mg/L. From each of these dilutions, 4µL was added respective wells in a 96-well plate which contained approximately 10^6-10^7 CFU/mL of Mtb H37Rv. The plates were incubated at 37ºC and on days 0, 3, 7, 10, 14 aliquots were diluted in Middlebrook 7H9 broth and plated on Middlebrook 7H11 agar plates. Bacterial colonies were enumerated after 21-28 days. Data was expressed as the log10 CFU/mL for each drug treatment.

Intracellular efficacy of pyrazolopyridones in THP-1 macrophages

THP-1 cells (ATCC) were cultured in 75cm^2 flask to confluence using RPMI 1640 with 10% fetal calf serum (Sigma, St. Louis, Mo.) supplemented with 2mM L-glutamine and 2mM pyruvate. The cells were grown in a 37ºC incubator with 5% CO2 and 95% air till they reach a density of 500,000 cells/mL. The assay was set up as given in Shirude et al. *J. Med. Chem*. **2013**, *56*, 9701-9708. Briefly,from the culture, cells at a density of 1-2 x 10^5 cells/mL were infected with M. tuberculosis H37Rv at a multiplicity of infection (MOI) of 1:10 (macrophage: bacteria) for 1 hour at 37ºC (batch infection). After 1 hour, the cells were washed twice with prewarmed phosphate buffered saline to remove extracellular bacteria and then resuspended in complete RPMI1640. Phorbol myristate acetate (Sigma) at 40 nM concentration was used to differentiate the cells to macrophage and were allowed adhere to 96-well plate for 24 hours at 37ºC. After 24 hours, varying concentrations of the test compounds are added to the monolayers and incubated for 7 days. The macrophage monolayers were periodically observed under a microscope to monitor adverse changes in the cell morphology due to drug toxicity. At the start of drug treatment and at 7 days post-treatment, the monolayers were gently washed and lysed with 0.04% SDS and plated on Middlebrook 7H11 agar plates. Bacterial colonies were enumerated after 21-28 days. Data were expressed as the $\log_{10}$CFU for each drug treatment.

MIC in hypoxic condition

The protocols used by Shirude et al. (*J. Med. Chem*. 2013, *56*, 9701) were used for performing the MIC experiments in hypoxic conditions.

MIC determined from non-replicating model

Table S1

Compound #	MIC in Hypoxia model (μM)
15	100
16	50
17	>200
18	100
19	>200

MIC modulation studies

BTZ043 resistant mutants were raised as described (Makarov et al. *Science*, **2009**, *324*, 5928). Rv3790 (DprE1) over-expression (OE) strain of Mtb was generated by cloning the 1.3kb fragment amplified using the primer pair and *M. tuberculosis* H37Rv ATCC 27294 genomic DNA as template.

- 3790Fmt : TATTGGATCCATTGAGCGTGGGAGCTAC
- 3790Rmt : AATAAAGCTTCTACAGCAGCTCCAAGCGTC

BamHI and HindIII digested amplicon was cloned into BamHI and HindIII digested pMV261, downstream of hsp60 promoter. The recombinant plasmid was electroporated into Mtb *and* the transformants were screened by colony PCR for the presence of Rv3790 using the primer pair

- MV261F: AGCGAGGACAACTTGAGCCGTCMV261R: CCTGGCAGTCGATCGTACGCTAG.

Identified recombinant strains were verified for over-expression of Rv3790 (DprE1) by measuring the up-shift in MIC for BTZ043 in a resazurin based MIC assay. MIC determination for Mtb strains over-expressing DprE1 or strains resistant to BTZ to ascribe the MOA of the compounds were carried out using the same methodology as given above. As a positive control we used BTZ043 for the MIC modulation studies.

Determination of Resistance Frequency

A single step selection method was used to generate spontaneous resistant mutants against Compound 10 as described earlier (Shirude et al., 2012). Briefly, a mid-logarithmic phase culture of *M. tuberculosis* H37Rv was centrifuged and concentrated 100-fold to achieve a bacterial number of ~10^{10} CFU/mL. The bacterial culture was diluted and plated onto compound 10 containing plates corresponding to 4X and 8X MBC concentrations of compound 10. Appropriate dilutions of the bacterial culture were also plated on drug-free Middlebrook 7H11 agar to enumerate the bacterial numbers in the start culture. Plates were incubated for 4 weeks at 37^{o}C and the CFUs in drug-free plates were enumerated. The drug-containing plates were incubated for up to 6 weeks at 37^{o}C to enumerate the number of spontaneously resistant colonies.

The spontaneous rate of resistance was calculated by dividing the number of colonies on drug -containing plates (at a given concentration) divided by the total number of viable bacteria estimated on drug-free plates. In order the determine the level of cross resistance to the parent compound as well as standard TB drugs, resistant colonies were randomly picked from the drug containing plates and grown in complete 7H9 broth and their MIC determined as described in the material and methods.

Genetic Mapping of mutations conferring resistance to pyrazolopyridones

In order to determine the genetic basis for reduced susceptibility to pyrazolopyridones, resitant mutants were analyzed for genetic changes in the *Rv3790* gene encoding the DprE1 protein. Chromosomal DNA was isolated from characterized resistant clones by boiling the cultures for 20 minutes. The boiled supernatants were subjected to PCR analysis using specific *M. tuberculosis Rv3790* primers to amplify the entire DprE1 gene. The PCR was performed with cycling parameters of 94°C for 30 s, 67.5°C for 30 s, and 72°C for 2 min for 30 cycles in a DNA Engine Dyad cycler (Bio-Rad). PCR products were cleaned (PCR purification kit, Qiagen), quantitated and sequenced (Microsynth, Switzerland). The DNA sequence from the resistant clones were aligned against the wild-type H37Rv *Rv3790* gene using Vector NTI software to detect mutations in the target gene.

Determination of DprE1 IC_{50}

DprE1 assays were performed based on the principle described previously (Neres *et. al., Sci. Transl. Med.* **2012**, *4*, 150ra121 and Shirude et al. *J. Med. Chem*. **2013**, *56*, 9701-9708). The 50 µl reaction was carried out in 384-well black plates (Catalogue # 3573, Corning and Costar, NY) at 25°C in assay buffer containing 50 mM Glycyl-Glycine pH 8.5, 200 mM potassium glutamate, 10 µM FAD and 0.002% Brij-35, 2% DMSO and 75 nM purified DprE1 enzyme. The enzyme was pre-incubated in the FAD containing assay mix for 30 minutes prior to the start of the reaction. The reactions were started with a mix containing the substrate; 300 µM farnesyl-phosphoryl-beta-D-ribofuranose, the coupling enzyme; 0.01

mg/mL horse radish peroxidase (Sigma-Aldrich P-6782) and 50 µM amplex red (Invitrogen A-22177). The conversion of amplex red to resorufin was monitored by measuring fluorescence (excitation wavelength of 563 nm and emission wavelength of 585 nm) in kinetic mode in Tecan Saffire II. This complete reaction was the positive control and the rate of reaction in presence of 10 µM BTZ043 was taken as the background. The background rate was subtracted from all reactions to get the background subtracted rate of reaction. For IC_{50} measurements, compounds were dissolved in DMSO and serially diluted to a concentration which was 50X the desired test concentration. 1 µL of the stock was used in 50 µL reaction volume.
The mutant DprE1 enzymes of Msm, Y321H and C394G corresponded to mutant Mtb DprE1 Y314H and C387G respectively. These were purified with the same protocol as used for wild type DprE1 enzyme.

In-vitro DMPK and cytotoxicity assays

The protocols used by Shirude et al. (*J. Med. Chem*. 2013, *56*, 9701) were used for performing the assays reported in Table 5.

Modeling

The DprE1 complex structures (PDB ID: 4KW5 and 4FDO) were analyzed and refined in maestro using protein preparation wizard from Schrodinger suite of programs prior to docking. The grid files were generated for the substrate binding site. Glide XP module was used for docking without applying any constraints.
The ligand overlay was performed using ROCS (Rapid overlay of chemical structures) from Openeye Suite of programs. The co-ordinates of minimized structure from docking of pyrazolopyridone were used as reference.
The druggablity assessment for the active sites was performed using Sitemap from Schrodinger. The bound ligands (CT319 in the case of pdb ID 4FDO and TCA1 in the case of 4KW5) were used to define the active site. The region covering 6 Å radius were examined during the evaluation. The site points, hydrophoblic (Yellow) and hydrophilic surface area (blue and red) were shown below (Figure S1). The corresponding ligands are overlaid. The drugscore calculated by sitemap for closed site (pdb ID 4FDO) and open site (4KW5) were found to be 1.23 and 1.07, respectively. These drug scores when compared to the values derived for a list of well validated targets (Halgreen, T. J. *Chem. Inf. Model.* **2009**, *49*, 377-389) suggest that DprE1 active site is highly druggable.

Sitemap analyses of active sites of DprE1

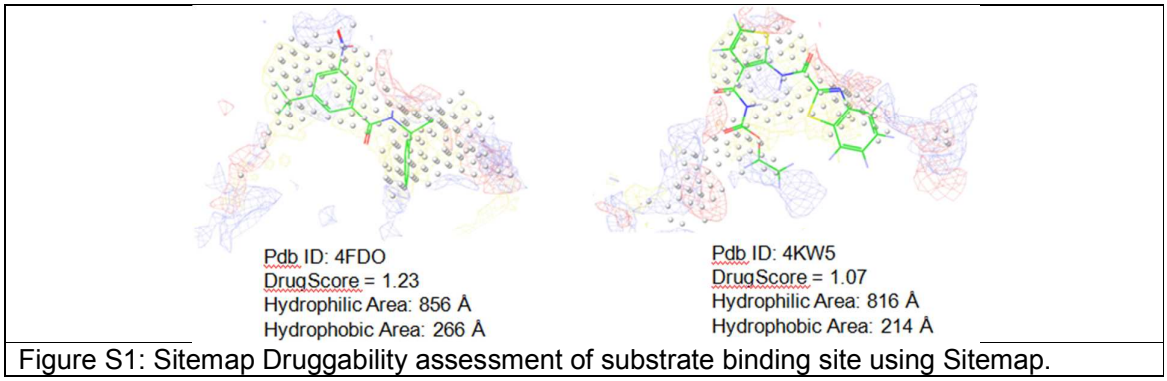

Figure S1: Sitemap Druggability assessment of substrate binding site using Sitemap.

Results from analog screening from corporate library

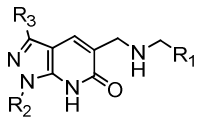

Table S2

cmpd	R1	R2	R3	MtbMIC (uM)
S1				**30**
S2				**8**
S3				**6.2**