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Design, Synthesis, and Biological Evaluation of Pyrazolo[1,5-*a*]pyridine-3-carboxamides as Novel Antitubercular Agents

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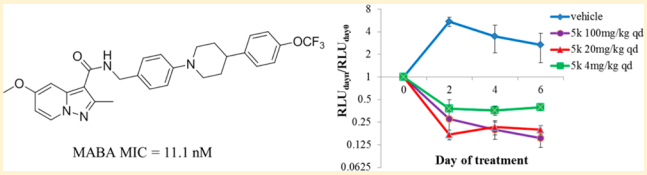
Supporting Information

ABSTRACT: A series of pyrazolo[1,5-*a*]pyridine-3-carboxamide derivatives were designed and synthesized as new anti-*Mycobacterium tuberculosis* (Mtb) agents. The compounds exhibit promising *in vitro* potency with nanomolar MIC values against the drug susceptive H37Rv strain and a panel of clinically isolated multidrug-resistant Mtb (MDR-TB) strains. One of the representative compounds (**5k**) significantly reduces the bacterial burden in an autoluminescent H37Ra infected mouse model, suggesting its promising potential to be a lead compound for future antitubercular drug discovery.



KEYWORDS: *Antitubercular agent, H37Rv, pyrazolo[1,5-a]pyridine, structure−activity relationship, tuberculosis*

Tuberculosis (TB) remains one of the world's deadliest pandemic diseases with over 9.0 million new cases and 1.5 million deaths estimated by the World Health Organization (WHO) in 2013.[1] Despite the forty-year success of the inexpensive quadruple-drug therapy [a combination of isoniazid (INH), rifampicin (RIF), pyrazinamide, and ethambutol], development and dissemination of multidrug-resistant (MDR) and extensively drug-resistant (XDR) *Mycobacterium tuberculosis* (Mtb) strains, together with coinfection with Human Immunodeficiency Virus (HIV), have intensified an urgent need for new anti-TB drug discovery.[2,3] Encouragingly, for the first time since 1970s, an ATP synthase inhibitor bedaquiline[4] (**1**, also known as TMC207) was approved by the US FDA as a novel active ingredient of combinational therapies for clinical management of adult patients with MDR pulmonary TB in 2012.[5] However, the drug possesses serious adverse effects such as cardiac arrhythmias[5] and displayed higher death rates than that of the placebo group in a clinical investigation,[6] which may limit its wide application in clinical practice. Several other anti-TB molecules were also identified with different modes of action.[2] For instance, a bicyclic nitroimidazofuran pro-drug PA-824 (**2**) was reported to kill both replicating and hypoxic nonreplicating Mtb through a Ddn-mediated activation and has been advanced to phase II clinical trial.[7,8] Imidazo[1,2-*a*]pyridine amide (IPA)[9−13] analogues [e.g., Q203 (**3**)[9,10] and compound **4**[11]] were also discovered to demonstrate strong inhibitory potencies against a panel of drug-susceptible and drug-resistant Mtb strains by targeting the QcrB subunit of the menaquinol cytochrome c oxidoreductase (bc1 complex), which is a critical component of mycobacterial energy metabolism.[14] Clinical outcomes of the compounds, particularly their capability against MDR and XDR Mtb strains in patients, are eagerly awaited. However, given the fact of only one singular FDA approval in 40 years and the high attrition rate of drug development, it is still highly valuable to identify new molecules with alternative scaffolds as effective anti-TB drug candidates.

Pyrazolo[1,5-*a*]pyridine moiety is a drug-like scaffold that is frequently observed in FDA approved or clinically investigating drugs including antiallergic agent Ibudilast,[15] platelet aggregation inhibitor KC-764,[16] dopamine D4 antagonist FAUC213,[17] etc. (Supporting Information). It shares highly similar 3-dimensional conformation and electronic property to that of imidazo[1,2-*a*]pyridine, which is the pharmaceutical core of Q203 (**3**) and compound **4**. Therefore, a series of pyrazolo[1,5-*a*]pyridine-3-carboxamide derivatives (**5a−5v**) were designed as new anti-TB agents by using a scaffold hopping strategy in which 2,5-dimethyl groups were introduced at first because of

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the synthetic feasibility and keeping the geometric similarity to that of compound **4** (Figure 1).

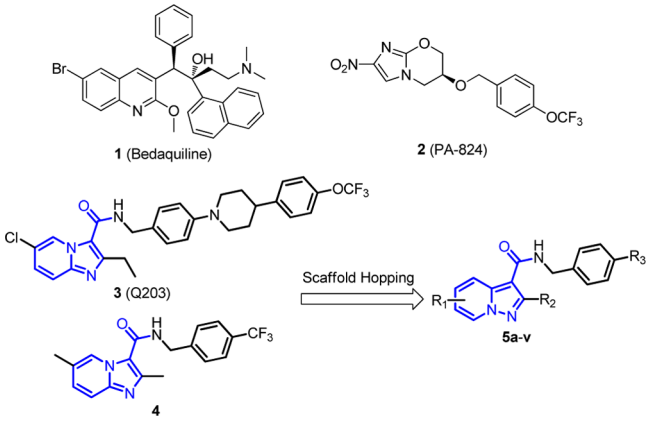
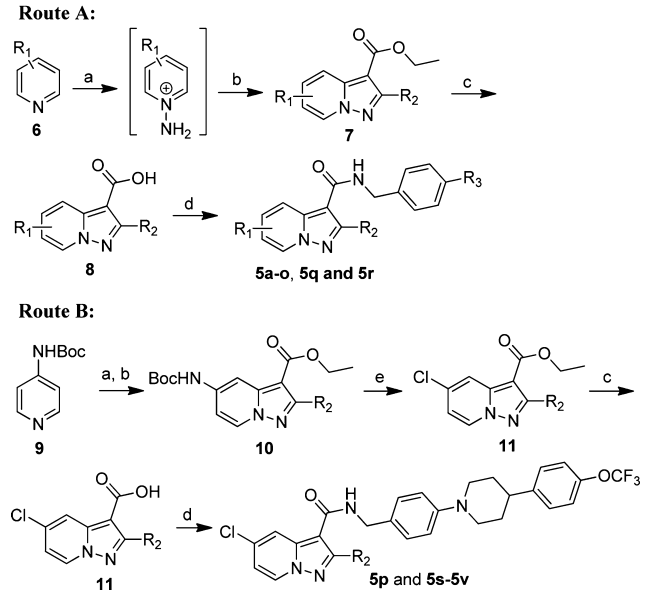


Figure 1. Representatives of the antitubercular agents and the new designed molecules.

Compounds **5a−5v** were readily synthesized using a straightforward amidation of pyrazolo[1,5-*a*]pyridine-3-carboxylic acids **8** or **11** with different primary amines (Scheme 1).

Scheme 1. Synthesis of Compounds 5a−5v[a]



[a]Reagents and conditions: (a) DNPH, MeCN, 40 °C, 18 h for all compounds expect **5q**, or MSH, DCM, 0 °C, 2 h for compound **5q**; (b) K_2CO_3, DMF, rt, 18 h, 32−57%; (c) NaOH, EtOH, H_2O, 60 °C, 100%; (d) amines, EDCI, HOBt, Et_3N, DMF, 80 °C, overnight, 40−87%; (e) (i) TFA, DCM, rt, 2 h, 100%; (ii) CuCl, con. HCl, $NaNO_2$ (aq, 0.4 M), 0−80 °C, 45 min, 46−86%.

Briefly, an N-amination of substituted pyridines **6** or **9** with O-(2,4-dinitrophenyl)hydroxylamine (DNPH) or O-mesitylenesulfonylhydroxylamine (MSH), followed by a 1,3-bipolar cycloaddition with substituted ethyl propiolate, produced the pivotal intermediate pyrazolo[1,5-*a*]pyridine-3-carboxylates **7** or **10**.[18] Compounds **7** were hydrolyzed to yield the carboxylic acids **8**, which were coupled with different commercially available or self-prepared amines (Supporting Information) to produce the designed compounds **5a−5o**, **5q**, and **5r** with good yields (Route A). While due to the difficulty of direct N-amination of halogen-substituted pyridine, additional removal of Boc protecting group and diazotization reaction of compounds **10** were needed to convert NHBoc group to chlorine substitution for the synthesis of compounds **5p** and **5s−5v** (Route B).

Anti-TB activities of compounds **5a−5v** were preliminarily screened by using a cost-efficient *in vitro* assay against a selectable marker-free avirulent autoluminescent H37Ra Mtb strain in which the bacteria growth was conveniently monitored by the bioluminescence intensity without adding any substrates.[19,20] The minimum inhibitory concentration (MIC) values of the active compounds were further determined by a well-established microplate alamar blue assay (MABA) against H37Rv.[21] The MICs are interpolated values obtained by using an in-house curve-fitting program. The VERO cell growth inhibition was also tested using the Cell Counting Kit-8 (CCK-8) assay to assess the compounds' potential cytotoxicity. All of the three reference compounds (i.e., INH, RIF, and Q203) displayed comparable MIC values to the reported data,[10] supporting the reliability of our screening conditions (Table 1).

We were pleased to find the first designed molecule **5a**, which carries an identical *p*-trifluoromethylbenzyl group to that in compound **4**, exhibited strong antimycobacterial activity against Mtb strain H37Rv and the avirulent Mtb strain H37Ra with MIC values of 69.1 and 287.9 nM, respectively. Further investigation revealed the 4-CF_3 group could be replaced by a dimethylamino moiety (**5b**) without obviously affecting the anti-TB potency. When a 1-piperidinyl (**5c**) or 4-(trifluoromethyl)piperidin-1-yl (**5d**) was introduced at the R_3 position, the anti-H37Rv activity was improved 5−6-fold. The investigation also suggested that R_3 position is well tolerated with large hydrophobic substituent. For instance, trifluoromethoxyphenyl (**5e**) and 4-(4-fluorophenyl)piperazin-1-yl (**5f**) derivatives displayed comparable anti-TB activities to that of **5d**. Encouragingly, compound **5g**, which harbors the identical lipophilic tail to that of Q203, demonstrated the strongest antimycobacterial activity against both H37Rv and H37Ra Mtb strains, with MIC values of 7.7 and 5.7 nM, respectively. Moreover, compound **5g** did not display obvious cytotoxicity with an IC_{50} value of >100 μM in a VERO cell growth inhibition assay, supporting it as a new promising starting point for further structure−activity relationship study.

We first investigated the potential impact of R_1 substituent on the anti-TB activity by altering the substituted position of a methyl group on the pyridine ring (**5g−5j**). It was shown that the 5-position is optimal for substitution (**5g**). When the methyl group was merged to 4-, 6-, or 7-position, the resulting compounds (**5h**, **5i**, and **5j**) were 2−149-fold less potent against Mtb H37Rv strain. Further investigation also revealed that the 5-methyl group (**5g**) could be replaced by a methoxyl (**5k**), ethyl (**5m**), or chloride (**5p**) moiety without obviously affecting the anti-TB potency. However, a large hydrophobic substituent at this position is detrimental. For instance, 5-isopropyl (**5n**) and 5-phenyl (**5r**) displayed MIC values of 47.4 and 478.9 nM against Mtb H37Rv strain, which are 6.2- and 62.2-fold less potent than **5g**, respectively. Interestingly, a removal of the 5-substitutent group (**5l**) barely affected the suppressing function against H37Rv strain. The impact of the R_2 substituent was also investigated. It was clear that this position was well tolerated by a small hydrophobic group such as a methyl (**5g**), ethyl (**5t**), or cyclopropyl (**5u**) moiety. However, when it was unsubstituted (**5s**) or phenyl substituted

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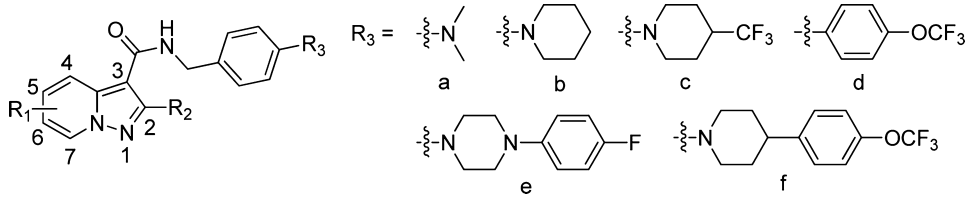
Table 1. *In Vitro* Antitubercular Activity of Compounds 5a−5v against the Mtb Strains H37Rv and H37Ra and VERO Cellular Toxicity



				MIC [nM (μg/mL)]		IC_{50} (μM)
compd	R_1	R_2	R_3	H37Rv[a]	H37Ra[b]	VERO[c]
5a	5-Me	Me	-CF_3	69.1 (0.024[d])	287.9 (0.1)	>100
5b	5-Me	Me	a	86.8 (0.028)	310.2 (0.1)	>100
5c	5-Me	Me	b	16.6 (0.006)	27.6−82.8 (0.01−0.03)	>100
5d	5-Me	Me	c	13.9 (0.006)	2.3 (0.001)	>100
5e	5-Me	Me	d	13.7 (0.006)	6.8 (0.003)	50.74
5f	5-Me	Me	e	13.1 (0.006)	6.6 (0.003)	>100
5g	5-Me	Me	f	7.7 (0.004)	5.7 (0.003)	>100
5h	4-Me	Me	f	1148 (0.60)	>19136 (>10.0)	n.d.[e]
5i	6-Me	Me	f	13.4 (0.007)	574.1 (0.3)	>100
5j	7-Me	Me	f	114.8 (0.06)	5741 (3.0)	n.d.
5k	5-OMe	Me	f	11.1 (0.006)	5.6 (0.003)	>100
5l	H	Me	f	9.8 (0.005)	589.9 (0.3)	>100
5m	5-Et	Me	f	11.2 (0.006)	18.6−55.9 (0.01−0.03)	>100
5n	5-*i*-Pr	Me	f	47.2 (0.026)	1816−5448 (1.0−3.0)	n.d.
5o	5-*t*-butyl	Me	f	>1771 (>1.0)	>17710 (>10)	n.d.
5p	5-Cl	Me	f	7.4 (0.004)	18.4 (0.01)	>100
5q	5-CF_3	Me	f	52.0 (0.03)	1734 (1.0)	>100
5r	5-phenyl	Me	f	478.9 (0.28)	>17105 (>10)	n.d.
5s	5-Cl	H	f	358.7 (0.20)	18905 (10.0)	n.d.
5t	5-Cl	Et	f	10.8 (0.006)	53.8 (0.03)	>100
5u	5-Cl	*cyclo*-Pr	f	17.6 (0.01)	175.7 (0.1)	>100
5v	5-Cl	phenyl	f	1547 (0.94)	>16528 (>10)	n.d.
3				10.8 (0.006)	5.4 (0.003)	>100
INH				2989 (0.41)	729 (0.10)	n.d.
RIF				36.4 (0.03)	3.64 (0.003)	n.d.

[a]Anti-TB activity assays against H37Rv were performed using MABA. [b]Anti-TB activity assays against H37Ra were performed using the autoluminescent assay. [c]VERO: African green monkey kidney cell line. The cell growth inhibition was evaluated using the CCK-8 assay. [d]The activity data in the brackets are reported in μg/mL. [e]Not determined. Values are means of two or more independent experiments, and the variation is <20%.

(**5v**), the resulting compounds were significantly less potent with MIC values of 358.7 and 1547 nM against H37Rv strain, respectively.

The antimycobacterial activities of compounds **5f**, **5g**, **5k**, and **5t** were further validated by determining their MIC, IC_{50}, and IC_{90} values against the replication of a fluorescent reporter strain of Mtb H37Rv in liquid medium under aerobic conditions (Table 2).[22] It was shown that compounds **5f** and **5k** displayed comparable antitubercular effects against H37Rv to that of RIF, whereas compounds **5g** and **5t** were moderately less potent. The IC_{50} and IC_{90} values of compound **5k** are 2.5 and 6.7 nM, respectively.

Table 2. *In Vitro* MIC, IC_{50}, and IC_{90} Values of Compounds 5f, 5g, 5k, and 5t against Fluorescent Reporter Strain of Mtb H37Rv

compd	MIC (nM)	IC_{50} (nM)	IC_{90} (nM)
5f	6.3	2.5	6.5
5g	11.0	5.1	12.0
5k	5.6	2.5	5.7
5t	129	31.3	150
RIF	6.5	4.1	6.9

Encouraged by their strong potencies against the drug-sensitive Mtb H37Rv strain, compounds **5g**, **5k**, **5p** and **5t** were further evaluated against a panel of clinical isolated 3495, P71, 9804, 4768, P163 MDR strains[23] by using an autoluminescent assay.[19] It was shown that all of the compounds displayed excellent potencies against the resistant Mtb strains with MIC values ranged from 11.1 to 1914 nM (Table 3). Particularly, compound **5k** exhibited superior inhibition against five resistant strains with similar MIC values (11.1−223 nM) to that of the wild-type Mtb H37Rv, suggesting its promising potential for both drug-sensitive and resistant Mtb strains.

Given its promising antimycobacterial activity against both drug-susceptible and drug-resistant Mtb strains, the *in vivo* antitubercular efficacy of **5k** was evaluated using a modified real-time monitoring noninvasive mouse model infected with the selectable marker-free autoluminescent Mtb strain H37Ra.[19,20] The animals were infected with log-phase autoluminescent Mtb H37Ra via intravenous injection (2×10^6 CFU per mouse) and then were repeatedly administrated

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Table 3. Antitubercular Activity of Compounds 5g, 5k, 5p, and 5t against Drug-Resistant Clinical Mtb Isolates

		MIC (nM)[b]			
strains	resistance[a]	5g	5k	5p	5t
3495	HRSZ	1914	22.3−223	n.d.[c]	n.d.
P71	HRZ	<383	11.1−223	<184	71.8−1795
9804	HRZ	<383	<11.1	<184	<35.9
4768	EHRSZ	<383	11.1−223	<184	35.9−71.8
P163	EHSZ	<383	11.1−223	<184	<35.9

[a]H, isoniazid; R, rifampicin; S, streptomycin; E, ethambutol; Z, pyrazinamide. [b]Values are means of two independent experiments, and the variation is <20%. [c]Not determined.

with agent **5k** once daily via oral gavage for 6 consecutive days. The bacterial burden was measured by monitoring the bioluminescence intensity (relative light unit, RLU) from the same batch of live mice every other day. As shown in Figure 2,

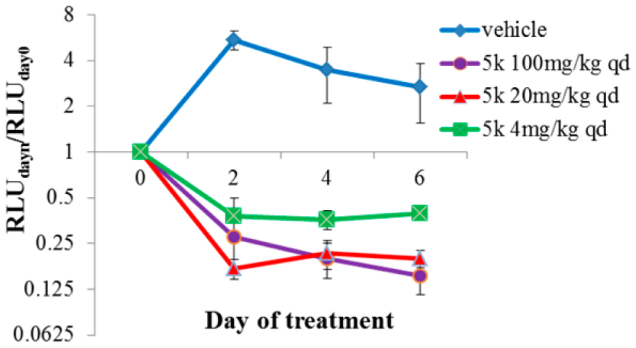

Figure 2. Mean RLU count (±SD) assessed every other day in live, anesthetized mice and normalized to the baseline RLU value. Days post-initial treatment (*x*-axis) is plotted against the corresponding RLU$_{dayn}$/RLU$_{day0}$ ratio (*y*-axis). Blue, vehicle; purple, **5k** 100 mg/kg qd; red, **5k** 20 mg/kg qd; green, **5k** 4 mg/kg qd.

compound **5k** exhibited dose-dependent *in vivo* antitubercular activity and was well tolerated in all of the tested groups with no mortality (data not shown) observed during treatment. Administration with CMC-Na (vehicle) permitted a nearly 2.8-fold increase in RLU over 6 days; whereas compound **5k** with the three tested doses prevented any increase over baseline, with RLU$_{dayn}$/RLU$_{day0}$ ratios of 0.40, 0.19, and 0.15 in 4, 20, and 100 mg/kg/day treated groups, respectively. Especially, with 100 mg/kg/day, compound **5k** exhibited a sustained bactericidal activity against Mtb H37Ra, resulting in a 3.6-fold, 5.0-fold, and 6.5-fold decrease in RLU from baseline on day 2, day 4, and day 6, respectively. These results strongly suggest the promising potential of compound **5k** to serve as a lead compound for further anti-TB drug discovery.

In summary, a series of pyrazolo[1,5-*a*]pyridine-3-carboxamide derivatives were designed as new antitubercular agents by using a scaffold hopping strategy. The compounds exhibit excellent *in vitro* inhibitory activities with low nanomolar MIC values against both drug-sensitive Mtb strain H37Rv and drug-resistant clinical isolates. One of the most promising compounds, **5k**, displayed significant bacterial burden reduction in the Mtb H37Ra infected mouse model. This compound may serve as a new promising lead compound for further antitubercular drug discovery.

■ ASSOCIATED CONTENT

Supporting Information

Experimental procedures for the synthesis of **5a−5v** and self-prepared amines, 1H NMR and 13C NMR for final compounds, and details of *in vitro* and *in vivo* assays. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsmedchem-lett.5b00176.

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Notes

The authors declare no competing financial interest.

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