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Synthesis of pyrazinamide Mannich bases and its antitubercular properties

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Abstract—A series of pyrazinamide (PAZ) Mannich bases has been synthesized by reacting PAZ, formaldehyde, and various substituted piperazines using microwave irradiation with the yield ranging from 46% to 86%. The synthesized compounds were evaluated for antimycobacterial activity in vitro and in vivo against *Mycobacterium tuberculosis* H37Rv (MTB). Among the synthesized compounds, 1-cyclopropyl-6-fluoro-1,4-dihydro-8-methoxy-7-(3-methyl-4-((pyrazine-2-carboxamido)methyl)piperazin-1-yl)-4-oxoquinoline-3-carboxylic acid (**17**) was found to be the most active compound in vitro with MIC of 0.39 and 0.2 μg/mL against MTB and multidrug-resistant MTB, respectively. In the in vivo animal model **17** decreased the bacterial load in lung and spleen tissues with 1.86 and 1.66-log10 protections, respectively.

Tuberculosis, which is caused by *Mycobacterium tuberculosis* (MTB), was a much more prevalent disease in the past than it is today, and it was responsible for the deaths of about one billion people during the last two centuries.[1] MTB is particularly a successful pathogen that latently infects about 2 billion people (about one-third) of the world population.[2] Each year, there are about 8 million new TB cases and 2 million deaths worldwide. TB is on the increase in recent years, largely owing to HIV infection, immigration, increased trade, and globalization.[2] The increasing emergence of drug-resistant TB, especially multidrug-resistant TB (MDR-TB, resistant to at least two frontline drugs such as isoniazid and rifampin), is particularly alarming. MDR-TB has already caused several fatal outbreaks[2,3] and poses a significant threat to the treatment and control of the disease in some parts of the world, where the incidence of MDR-TB can be as high as 14%.[2] There is much concern that the TB situation may become even worse with the spread of HIV worldwide, a virus that weakens the host immune system and allows latent TB to reactivate and make the person more susceptible to re-infection with either drug-susceptible or drug-resistant strains. The lethal combination of drug-resistant TB and HIV infection is a growing problem that presents serious challenges for effective TB control. In view of this situation, the World Health Organization (WHO) in 1993 declared TB a global emergency.[4] Pyrazinamide (PZA) is one of the frontline agents prescribed for the treatment of MTB. Although PZA has been used clinically since the 1950s, a proposed mechanism of action has only recently been reported to be the inhibition of the eukaryotic-like fatty acid synthetase I (FASI) of MTB.[5] PZA is considered to be a prodrug of pyrazinoic acid (POA), which is believed to be the active inhibitor of MTB.[6] Activation of PZA to POA is regulated by an enzyme pyrazinamidase present in all PZA-sensitive strains of MTB. Morphazinamide (MZA), a Mannich base derivative of PZA, has essentially the same activity against MTB as PZA, with a MIC of 8–16 μg/ml.[7] Herein, we report the microwave assisted synthesis of several Mannich bases and their in vitro and in vivo activity against MTB and MDR-TB.

The general procedures for the preparation of target compounds **1**–**17** (Tables 1 and 2) are described in Scheme 1. PAZ reacts with formaldehyde and secondary amino function of substituted piperazines to form the required Mannich bases of PAZ in 46–86% yield. Unlike conventional methods (duration 5 h),[8] microwave assisted reactions were very facile (2–3 min), and the products do not require any further purification. The purity of the synthesized compounds was checked by thin layer chromatography (TLC) and elemental analyses and the structures were identified by spectral data.[9] In general,

Keywords: Synthesis; Pyrazinamide; Mannich bases; Antitubercular.
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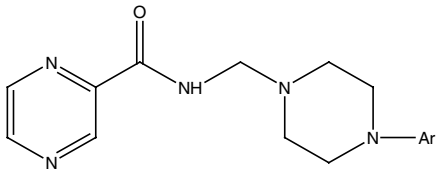
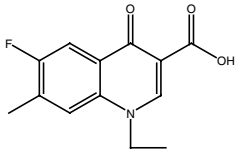
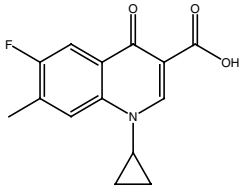
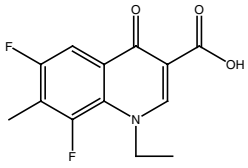
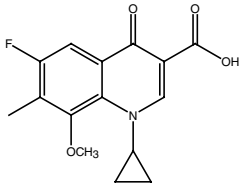
Table 1. Physical constants and in vitro antimycobacterial activity



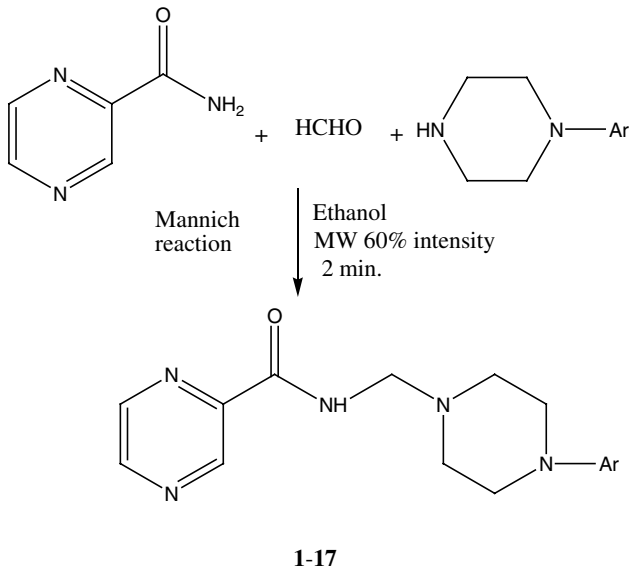
Compound	Ar	Yield (%)	Mp (°C)	log *P*[a]	MIC MTB[b]	MIC MDRTB[c]	CC_{50}[d]	SI[e]
1	Benzyl	56	170	0.69	6.25	>25.0	ND	—
2	Phenyl	52	107	1.03	6.25	>25.0	ND	—
3	4-Chloro phenyl	85	126	1.59	3.12	12.5	ND	—
4	3-Chloro phenyl	57	168	1.59	3.12	6.25	ND	—
5	2-Pyrimidinyl	49	178	−0.39	>12.5	>25.0	ND	—
6	2-Pyridyl	44	157	0.41	12.5	>25.0	ND	—
7	2-Methoxy phenyl	64	176	0.91	12.5	>25.0	ND	—
8	3-Methoxy phenyl	55	188	0.91	12.5	>25.0	ND	—
9	4-Methoxy phenyl	51	182	0.91	12.5	>25.0	ND	—
10	4-Nitro phenyl	45	142	−0.89	6.25	6.25	ND	—
11	Methyl	64	181	−1.04	12.5	>25.0	ND	—
12	4-Fluoro phenyl	68	132	1.19	1.76	1.76	>62.5	>40
13	4-Trifluoromethyl phenyl	80	169	1.95	0.78	1.76	>62.5	>80

[a] log *P* values calculated with Chem office 2004 software.
[b] Minimum inhibitory concentration (in μg/mL) required to inhibit 90% inhibition against *Mycobacterium tuberculosis*.
[c] Minimum inhibitory concentration (in μg/mL) required to inhibit 90% inhibition against multi drug resistant *M. tuberculosis*.
[d] Cytotoxic concentration of drugs in μg/mL.
[e] Ratio between MIC and CC_{50}.

Table 2. Physical constants and in vitro antimycobacterial activity

Compound	Ar	Yield (%)	Mp (°C)	log *P*[a]	MIC MTB[b]	MIC MDRTB[c]	CC_{50}[d]	SI[e]
14		63	183	0.7	1.76	0.78	>62.5	>40
15		45	231	0.66	3.13	0.78	>62.5	>20
16		46	226	1.18	0.78	0.78	>62.5	>80
17		60	123	0.85	0.39	0.2	>62.5	>160
PAZ	—	—	—	−1.31	12.5	>25.0	ND	—

[a] log *P* values calculated with Chem office 2004 software.
[b] Minimum inhibitory concentration (in μg/mL) required to inhibit 90% inhibition against *Mycobacterium tuberculosis*.
[c] Minimum inhibitory concentration (in μg/mL) required to inhibit 90% inhibition against multi drug resistant *M. tuberculosis*.
[d] Cytotoxic concentration of drugs in μg/mL.
[e] Ratio between MIC and CC_{50}.



1-17

Scheme 1. Synthetic protocol of pyrazinamide Mannich bases.

Infrared spectra (IR) revealed CH_2 (Mannich methylene) peak at 2860 and 2846 cm^{-1}. In the Nuclear Magnetic resonance spectra (^1H NMR), the signals of the respective protons of the prepared PAZ derivatives were verified on the basis of their chemical shifts, multiplicities, and coupling constants. The spectra showed a singlet at δ 4.1 ppm corresponding to $-NCH_2N-$ group; multiplet at δ 2.46–3.45 ppm for piperazine proton; multiplet at δ 8.76–9.25 ppm for aromatic pyrazine proton; and D_2O exchangeable broad singlet at 9.75 for NH proton of amide. The elemental analysis results were within ±0.4% of the theoretical values.

All compounds were screened for their antimycobacterial activity against MTB and MDR-TB by the agar dilution method similar to that recommended by the National Committee for Clinical Laboratory Standards[10] for the determination of minimum inhibitory concentration (MIC). The MDR-TB clinical isolate was obtained from Tuberculosis Research Center, Chennai, India, and was resistant to isoniazide, rifampicin, pyrazinamide, and ofloxacin. The MIC was defined as the minimum concentration of compound required to inhibit 90% of bacterial growth and MIC's of the compounds are reported in Tables 1 and 2. Among the synthesized compounds eleven compounds (**1**–**4**, **10**, and **12**–**17**) (MIC < 12.5 μg/mL) were more active and five compounds (**6**–**9** and **11**) were equipotent (MIC: 12.5 μg/mL) to that of PAZ against MTB. Compound 1-cyclopropyl-6-fluoro-1,4-dihydro-8-methoxy-7-(3-methyl-4-((pyrazine-2-carboxamido) methyl)piperazin-1-yl)-4-oxoquinoline-3-carboxylic acid (**17**) was found to be the most active compound in vitro with MIC of 0.39 μg/mL against MTB. The aryl ring with electron-withdrawing substituents enhanced the activity (**2** vs **3**, **4**, **12**, and **13**), and with electron-donating substituents/properties decreased the activity (**2** vs **5**–**9**). The lesser/inactivity of PAZ might be due to the pH of the medium because PAZ demonstrated in vitro activity against MTB only in acidic media (pH ⩽ 5.6).[11] Against MDR-TB, when compared to PAZ (MIC >25.0 μg/mL), nine compounds were more active with a MIC ⩽12.5 μg/mL. Compound **17** was found to be the most potent (MIC = 0.20 μg/mL), and was >125 times more potent than that of the parent drug PAZ and >7 times more potent than isoniazid (MIC = 1.56 μg/mL) against MDR-TB.

Table 3. In vivo activity data of **17** against *Mycobacterium tuberculosis* in mice

Compound	Lungs (log CFU ± SEM)	Spleen (log CFU ± SEM)
Control	7.88 ± 0.22	8.84 ± 0.21
11 (100 mg/kg)	6.02 ± 0.16	7.18 ± 0.21

PAZ Mannich bases were found to be active in neutral pH, and the pH independence of these derivatives might be due to the generation of formaldehyde upon hydrolysis.[12] The lipophilicity of the drug is well known to play an important role in the penetration of these compounds into bacterial cells.[13] Our results demonstrated that simply increasing the lipophilic character of PAZ by preparing Mannich bases increased the activity, as the log *P* values of the synthesized compounds (−1.04 to 01.95) (Tables 1 and 2) were much more than the parent compound PAZ (−1.31).

Selected compounds (**12**–**17**) were further examined for toxicity (IC_{50}) in a mammalian VERO cell line at a concentration of 62.5 μg/mL. After 72 h exposure, viability was assessed on the basis of cellular conversion of MTT into a formazan product using the Promega Cell Titer 96 non-radioactive cell proliferation assay.[14] The compounds were found to be non-toxic at 62.5 μg/mL. Compound **17** showed a selectivity index (IC_{50}/MIC) of more than 160.

Subsequently, compound **17** was tested in vivo for efficacy against MTB at a dose of 100 mg/kg (Table 3) in six-week-old female CD-1 mice. In this model,[14] the mice were infected intravenously through caudal vein approximately with 10^7 viable *M. tuberculosis* ATCC 35801. Drug treatment began after inoculation of the animal with microorganism and continued for 10 days by intraperitoneal route. After 35 days of post-infection, the spleens and right lungs were aseptically removed and ground in a tissue homogenizer, and the number of viable organisms was determined by serial 10-fold dilutions and subsequent inoculation onto 7H10 agar plates. Cultures were incubated at 37 °C in ambient air for 4 weeks prior to counting. Bacterial counts were measured and compared with the counts from negative (untreated) controls (Mean culture forming units (CFU) in lung: 7.88 ± 0.22 and in spleen: 8.84 ± 0.21). Compound **17** decreased the bacterial load in lung and spleen tissues with 1.86 and 1.66-log10 protections, respectively, and was considered to be promising in reducing bacterial count in lung and spleen tissues.

Conclusion

This study has revealed that on increasing the lipophilic nature of PAZ improved the antimycobacterial activity.

Compound **17** was found to be the most promising compound and the enhanced activity might be due to the inhibition of both MTB enzymes FASI and DNA gyrase.

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