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A new modification of anti-tubercular active molecules

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Abstract—The connection of two active molecules across an easily released bridge as a new type of potentially active molecule has been studied. The synthesis is based on derivatives that originate from isonicotinoyl hydrazide, pyrazinamide, *p*-aminosalicylic acid (PAS), ethambutol, and ciprofloxacin. The lipophilicity, hydrolysis (stability of the compounds), and antituberculotic activity as well as the structure–lipophilicity and structure–activity relationships are discussed.

1. Introduction

According to alarming data from the World Health Organisation, tuberculosis (TB) has spread to every corner of the globe.[1] As much as one-third of the world's population is currently infected, and more than 5000 people die from TB every day. A large number of the infected people are carriers of the latent form, which creates a potentially dangerous source of the illness for the future. The HIV pandemic has led to the rapid growth of the TB epidemic and increased the likelihood of people dying from TB. Another factor contributing to the rise in TB infections, and consequently to the increased number of deaths, is the emergence of multiple drug-resistance (MDR). As a result of this there is an urgent need to develop new anti-TB agents.[2–5]

Isoniazid (INH) and pyrazinamide (PZA) are widely applied as first-line drugs for the treatment of tuberculosis, usually in combination with other drugs. Modifying either of these molecules has been a challenge taken up by several research groups.[6–9] As INH is a prodrug, its therapeutic activity requires activation by mycobacterial catalase-peroxidase. INH prevents a mycolic acid biosynthesis by inhibiting a 2-*trans*-enoyl-acyl carrier protein reductase (InhA) that belongs to the FAS-II (fatty acid synthetase II) system.[10] The second example, pyrazinamide, is a prodrug that requires activation or conversion to its active form, pyrazinoic acid (POA), by the PZase/nicotinamidase enzyme. The target of POA appears to be the membrane.[5]

In the quest for biologically more potent anti-tuberculosis compounds we have designed and synthesized new derivatives that contain isoniazid, pyrazinamide, and some other moieties linked by the CH group. This new type of molecule can be regarded as a 'double active' molecule that can play the role of a prodrug with a prolonged liberation. In addition, the components involved in it may act synergetically.[11,12]

2. Chemistry

At the beginning of our studies, two derivatives, originating from either INH or PZA, were selected. The idea was to introduce an NR^1R^2 group derived from the second anti-tuberculous molecule or, eventually, from any other nucleophile capable of improving the activity of

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the new product (Fig. 1) in comparison with well-known drugs.

Following this plan, we treated INH with *N*,*N*-dimethylformamide dimethyl acetal (**A**) to obtain *N*′-4-isonicotinoyl-*N*,*N*-dimethylhydrazonoformamide (**1**) as a starting compound to obtain other INH derivatives (Scheme 1).

Unfortunately, the dimethylamino group turned out to be a poor leaving group for our purposes. Therefore, we prepared hydrazone **2**, which was easily available from the INH by employing diethoxymethyl acetate (**B**)[13,14] as a good synthon of the CHOEt fragment (Scheme 1). Namely, hydrazone **2** and similar compounds are known as good sources of the C_1 fragment that can serve for the formation of either a C–N or a C–C bond.[13,15] Thus, the treatment of ethoxymethylene hydrazones with primary or secondary amines resulted in the formation of the corresponding formamidrazones.[13,14] When heterocyclic hydrazines, possessing the hydrazino group at the vicinal position to the ring nitrogen, were allowed to react with ethoxymethylene hydrazones, the process proceeded further, leading to the construction of a new five-membered ring.[13,15] In the present study, the ethoxy group of the compound **2** was substituted with nitrogen nucleophiles (morpholine, 2-aminomethylpyridine, benzylamine, PAS, ciprofloxacin, and INH) to afford the products **3**–**8** (Scheme 2), where the INH and the nitrogen nucleophile are connected with the CH group. The structures of the products are shown in Figure 2.

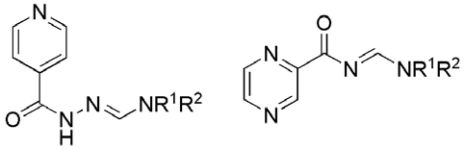
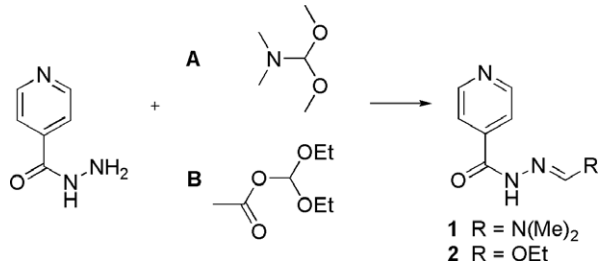
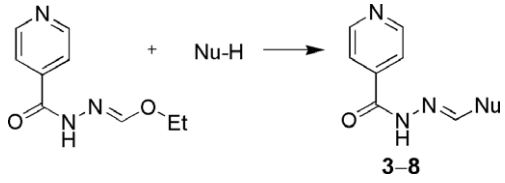


Figure 1. General structures of the target compounds.



Scheme 1. Synthesis of intermediates to obtain the INH derivates.



Scheme 2. Formation of the INH derivates.

Out of six compounds, obtained on the basis of Scheme 2, two of them (**6** and **7**) were given special attention. This is because they can be considered as prodrugs that contain two conventional drugs connected by a CH fragment. In addition, the product **8** binds two identical fragments, that is, two INH molecules. These products were stable in the solid state but they hydrolyzed in aqueous solutions, in the presence of an acid or enzymatically, leading to both TB drugs or to one drug and the formylamino derivative of the other drug.

A similar approach has been used to functionalize the pyrazinamide molecule. An activated pyrazine derivative, *N*-(dimethylaminomethylene)pyrazine-2-carboxamide (**9**), was prepared using a standard procedure from pyrazinamide and *N*,*N*-dimethylformamide dimethyl acetal (Scheme 3).

The treatment of **9** with ciprofloxacin gave the desired 1-cyclopropyl-6-fluoro-4-oxo-7-{[4-(pyrazin-2-ylcarbonyl)iminomethyl]piperazin-1-yl}-1,4-dihydroquinoline-3-carboxylic acid (**10**). Furthermore, the reaction of the pyrazine derivative **9** with PAS led to the 4-(2-pyrazinecarbonyliminomethyl)aminosalicylic acid (**11**) (Scheme 4). The substitution of the dimethylamino group in **9** with INH required the presence of pyridinium 4-methylbenzenesulfonate polymer bound (PTTS) to afford the appropriate product, that is, *N*-(2-isonicotinoylhydrazino)methylidenepyrazine-2-carboxamide (**12**). For the structures of the prepared compounds, see Figure 2.

An attempt to perform a similar substitution in formamidine **9** with ethambutol as a nucleophile was not successful and resulted in a completely different outcome. First, we noticed again that the presence of PTTS was essential in this transformation. However, a careful examination of the reaction product revealed that it was not a hydrazone, and it became clear that the connection of pyrazinamide with ethambutol via the CH fragment was not successful. In addition, the isolated compound was not a salt of ethambutol. This was ruled out by comparing it with the salt obtained in a separate experiment directly from ethambutol and PTTS. It turned out that the examined reaction actually led to the formation of 1,3-bis(1-hydroxybutan-2-yl)imidazolidin-2-yl 4-methylbenzenesulfonate (**13**) (Scheme 5).

3. Lipophilicity properties of the prepared compounds

The lipophilicities ($\log P$/$C\log P$ values) of the studied compounds **1** and **3**–**13** were calculated using two commercially available programs (ChemDraw Ultra 10.0 and ACD/$\log P$) and measured by means of an RP-HPLC determination of the capacity factors K with a subsequent calculation of $\log K$.[16] The results are shown in Table 1 and illustrated in Figure 3.

These results show that the experimentally determined $\log K$ values correlate relatively poorly with all the com-

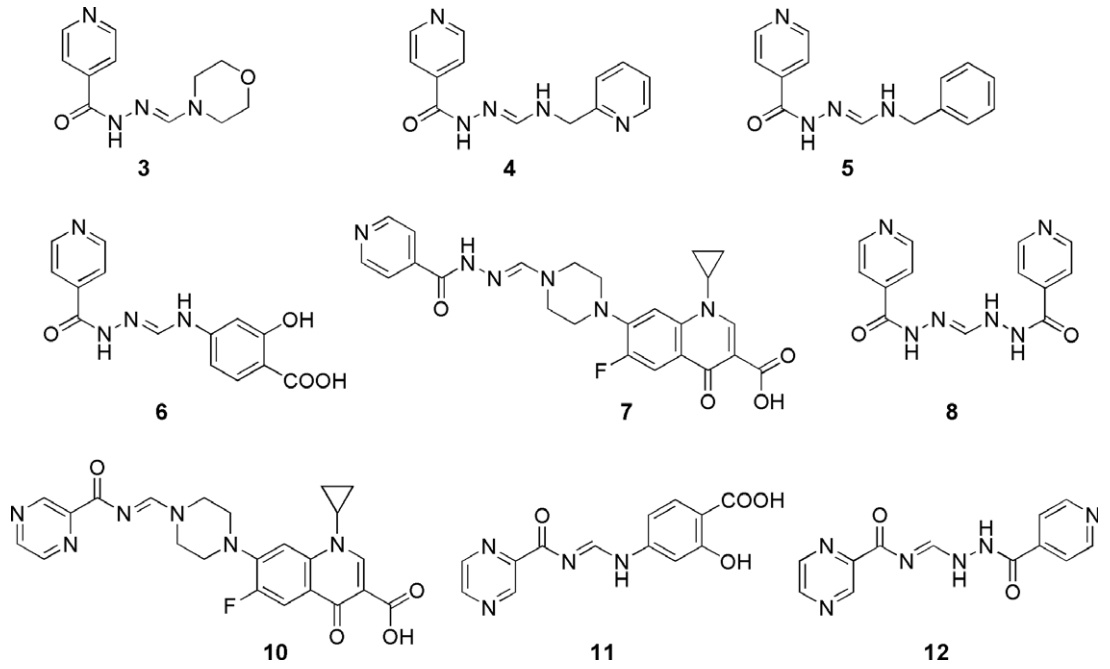
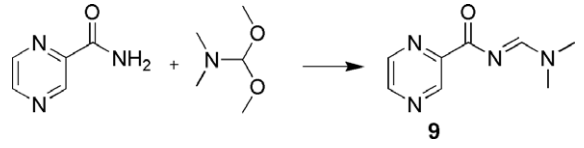
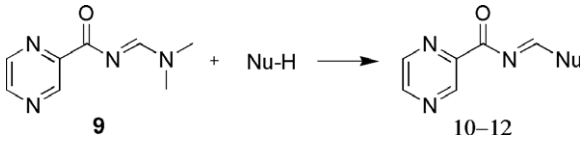


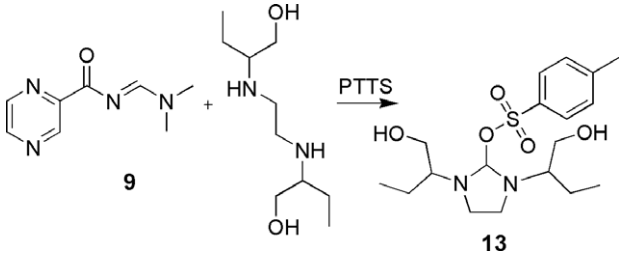
Figure 2. Structures of the prepared compounds.



Scheme 3. Preparation of an activated molecule of PZA.



Scheme 4. Formation of PZA derivates. NuH, ciprofloxacin; PAS; INH.



Scheme 5. Reaction of **9** with ethambutol—the formation of an unexpected product.

puted lipophilicity data. This observation is graphically demonstrated in Figure 3.

To compare the lipophilicities of INH, PZA, *p*-aminosalicylic acid, and ciprofloxacin versus **6–8** and **10–12** we performed the measurements for all the compounds under the same conditions. The retention times (T_R) of the above-mentioned drugs were found to be lower than the dead-time (T_D) of the potassium iodide, which was used as an analyte. This means that their lipophilicities cannot be determined under these conditions, but are evidently lower than those of **6–8** and **10–12**.

4. Stability of the compounds

The decompositions of the studied compounds were measured by means of RP-HPLC methods in a water–acetonitrile solution. This means that the compounds **6–8** and **10–12** were dissolved in a 20% aqueous solution of acetonitrile and the temperature of the sample solvents was 37 °C. Both methods were validated and selected parameters met the criteria: (i) precision–repeatability (six independent analyses of the sample solutions), (ii) linearity, and (iii) accuracy (recovery). The limits of detection (LOD) and the limits of quantification (LOQ) were also found.

The experimental kinetic data, determined by means of HPLC methods, were subsequently processed using the commercially available software MATLAB (version R2006a). The decompositions as well as the decomposition half-times ($\tau_{1/2}$) of the individual compounds are shown in Supplementary data. The half-time of the reaction refers to a 50% decrease in the amount of compound (%).

The reaction order of the decomposition of the compounds investigated in an 80% aqueous solution followed pseudo-first-order degradation kinetics.[17–21] The reaction rate constants were calculated from the slopes of the plots of the natural logarithm of the residual

Table 1. Comparison of the determined log*K* values with the calculated lipophilicities (log*P*/*C*log*P*) of the prepared compounds and a comparison of the calculated lipophilicities (log*P*/*C*log*P*) of the clinically used drugs: isoniazide (INH), pyrazinamide (PZA), *p*-aminosalicylic acid (PAS), and ciprofloxacin (CPF)

Compound[a]	log*K*	log*P*/*C*log*P* ChemOffice	log*P* ACD/log*P*
1	0.2520	0.09/−0.113	−0.12 ± 0.58
3	0.2653	−0.31/0.3047	−0.89 ± 0.62
4	0.3343	0.67/−0.088	0.20 ± 0.57
5	0.5426	1.59/1.409	1.70 ± 0.57
6	0.3660	0.54/1.1212	2.33 ± 0.59
7[b]	0.9159	1.54/2.257	1.05 ± 0.86
8	0.3027	−0.56/−1.034	−0.15 ± 0.61
9	0.1718	−0.34/−1.07	−0.34 ± 0.59
10[b]	0.9700	1.11/1.300	0.82 ± 0.87
11	0.3695	0.11/0.1642	2.10 ± 0.61
12	0.3562	−0.99/−1.991	−0.38 ± 0.62
13	0.2430	3.04/2.0543	2.01 ± 0.59
INH	—	−0.60/−0.668	−0.89 ± 0.24
PZA	—	−1.31/−0.67632	−0.37 ± 0.35
PAS	—	0.88/1.0562	0.32 ± 0.34
CPF	—	1.32/−0.7252	1.33 ± 0.75

[a] Solvent: MeOH/H_2O (30/70).
[b] Solvent: MeOH/H_2O (50/50).

reactant fraction versus time. For the compounds **6**–**8** and **10**–**12** the following rate constants and half-times of the decompositions were determined: $k_6 = 0.5\ h^{-1}$ and $(\tau_{1/2})_6 = 1.4$ h, $k_7 = 0.007\ h^{-1}$ and $(\tau_{1/2})_7 = 99$ h, $k_8 = 0.01\ h^{-1}$ and $(\tau_{1/2})_8 = 69.3$ h, $k_{10} = 0.2\ h^{-1}$ and $(\tau_{1/2})_{10} = 3.5$ h, $k_{11} = 0.014\ h^{-1}$ and $(\tau_{1/2})_{11} = 49.5$ h, $k_{12} = 0.04\ h^{-1}$ and $(\tau_{1/2})_{12} = 17.3$ h. The courses and half-times of the hydrolysis are graphically illustrated.

The compounds **6**, **10**, and **12** exhibit significant degradation under the chosen experimental conditions. In fact they decompose completely within 7 days. The compounds **7**, **8**, and **11** show about 30%, 19%, and 10% of their initial concentration after 7 days, about 10%, 4%, and 1% of their initial concentration after 14 days, and about 3%, 1%, and 0.1% of their initial concentration after 21 days.

5. Antimycobacterial activity of the prepared compounds

All the described compounds were tested in vitro for their antitubercular activity at Hansen's Disease Center (Colorado State University) as part of the Tuberculosis Antimicrobial Acquisition and Coordinating Facility (TAACF) screening program for the discovery of novel drugs for the treatment of tuberculosis. The lowest MIC was found for the compounds **12** (0.1 μg/mL), **6** (0.39 μg/mL), **8** (0.39 μg/mL), and **10** (0.78 μg/mL). They have a selectivity index (SI) higher than 100, with only one exception (**10**; SI = 12.8). For more details, see Part A of Table 2. The majority of the compounds were evaluated in the National Reference Laboratory for *Mycobacterium kansasii*, also against some non-tuberculous strains. This measurement was made in concentrations of μmol/L. The results of the in vitro evaluation show an interesting activity for all compounds. The ciprofloxacin derivative **10** exhibited the highest activity against *M. kansasii* 235/80 (MIC 1 μmol/L), while the starting INH and PZA are inactive (see Part B of Table 2).

6. Conclusion

We have designed and prepared several derivatives where either INH or PZA was linked with another conventional drug by the CH fragment. The lipophilicity, as the capacity factor log*K*, was determined by RP-HPLC and the results were compared by employing two commercially available programs. The higher lipophilicity of the new compounds with respect to INH, PZA, *p*-aminosalicylic acid, and ciprofloxacin signifies a more effective transport of the molecule through cellular membranes. The hydrolysis in a water–acetonitrile solution of the most active compounds, **6–8** and **10–12**, was investigated. The half-time of the degradation was determined from the hydrolysis curve. The rate of hydrolysis of the new products was found to depend on the fragments involved in the molecule. The anti-tuberculosis activity was evaluated against *Mycobacterium tuberculosis* $H_{37}Rv$ and three non-tuberculous strains. The compounds **7**, **10**, and

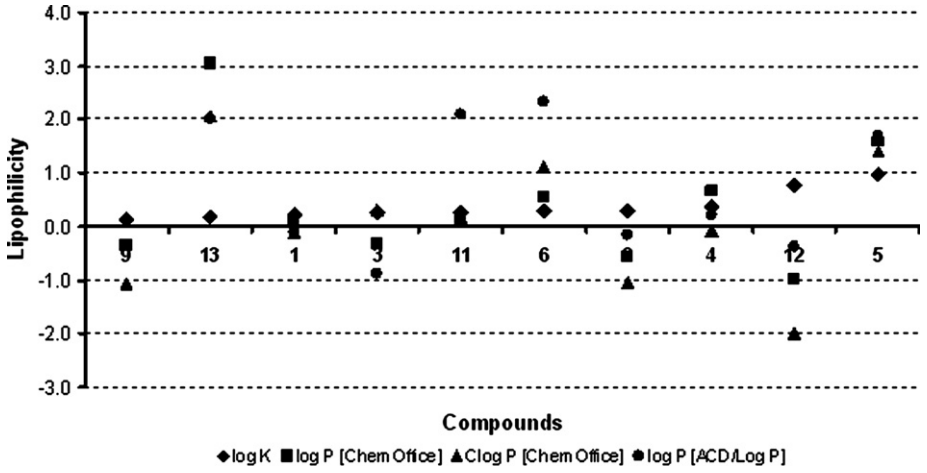


Figure 3. Experimentally determined log*K* values of the compounds **1**, **3**–**6**, **8**, **9**, and **11**–**13** and the calculated log*P*/*C*log*P* data, using the programs mentioned above.

Table 2. In vitro antimycobacterial activity of the prepared compounds

(A) Antimycobacterial activities against *Mycobacterium tuberculosis* $H_{37}Rv$, selectivity index SI, and inhibition (%). The inhibition is shown for the concentration 6.25 μg/mL

Compound	TAACF (*M. tbc* $H_{37}Rv$)			
	Inh. (%)	MIC (μg/mL)	IC_{50} (μg/mL)	SI[b]
1	1	>6.25	—	—
3	74	>6.25	—	—
4	92	6.25	>10	>1.6
5	93	>6.25	>10	—
6	96	0.39	>62.5	>160
7	99	3.13	—[a]	—[a]
8	96	0.39	>62.5	>160
9	1	>6.25	—	—
10	100	0.78	>10	>12.8
11	98	3.13	>10	>3.2
12	94	0.1	>10	>100
13	0	>6.25	—	—
INH	—	0.025–0.2	>100	—
PZA	—	6–60	—	—
CPF	98	2.00	>10	>5.0

(B) Antimycobacterial activities against *M. tuberculosis* and atypical strains *M. kansasii* and *M. avium* in comparison with isoniazid (INH) and pyrazinamide (PZA) as standards

Compound	National Reference Laboratory—MIC (μmol/L)									
	M. tbc 331/88		*Mycobacterium avium* 330/88		*Mycobacterium kansasii* 235/80			*M. kansasii* 6509/96		
	14 d	21 d	14 d	21 d	7 d	14 d	21 d	7 d	14 d	21 d
1	16	16	1000	>1000	250	500	1000	125	250	500
3	—	—	—	—	—	—	—	—	—	—
4	16	32	1000	>1000	1000	1000	1000	62.5	125	125
5	32	65.5	1000	>1000	500	1000	1000	125	250	250
6	4	8	125	125	500	1000	1000	125	250	250
7	2	4	125	250	2	4	8	2	4	8
8	—	—	—	—	—	—	—	—	—	—
9	—	—	—	—	—	—		—	—	—
10	2	4	62.5	125	1	2	4	2	2	4
11	8	16	32	62.5	16	32	32	8	16	32
12	4	8	125	125	500	1000	1000	8	8	16
13	250	500	500	1000	500	1000	1000	250	500	1000
INH	1	1	>250	>250	>250	>250	>250	2	4	4
PZA	8	8	>128	>128	>128	>128	>128	—	—	—

[a] Insoluble in DMSO at 1 μg/mL. Unable to determine IC_{50}.
[b] $SI = IC_{50}/MIC_{M.tbc.}$

11 have higher activity against non-tuberculous strains than INH or PZA as internal standards. The higher activity seems to be connected with an increase in the lipophilicity factor. In addition, the hydrolysis of new compounds ensures a prolonged liberation of the active components (INH, CPF, PZA or PAS).

7. Experimental

7.1. Chemistry

7.1.1. Instrumentation and chemicals. The chemicals were purchased from commercial sources (Aldrich, Merck, Fluka). Melting points (uncorrected) were determined on a Kofler micro-hot-stage. Elemental analyses (C, H, N) were performed with a Perkin–Elmer 2400 CHNS/O analyzer. Infrared spectra were recorded on a Bio-Rad FTS 3000 MX spectrometer in KBr pellets or NaCl disk. NMR spectra were measured in $CDCl_3$ or DMSO-d_6 solutions (if not specified otherwise, they were measured at ambient temperature) on a Bruker Avance 300 (300 MHz for ^1H and 75.5 MHz for ^{13}C). The chemical shifts, δ, are given in ppm, related to tetramethylsilane (TMS) as an internal standard. The coupling constants (J) are reported in Hertz. The reactions were monitored and the purity of the products was checked by TLC (Fluka silica gel/TLC cards 60 PF_{254}). The plates were visualized using UV light. Mass spectra were recorded with a VG-Analytical AutospecQ instrument.

7.1.2. Procedures and data of the prepared target compounds

7.1.2.1. *N′*-Isonicotinoyl-*N*,*N*-dimethylhydrazonoformamide (1).[22,23]. *N*,*N*-Dimethylformamide dimethyl acetal (1.6 mL, 11.5 mmol) was added dropwise to a stirred solution of isoniazid (1.37 g, 10 mmol) in acetonitrile (115 mL) at 50 °C. The reaction mixture was stirred

for 10 min at the same temperature. The solvent was evaporated in vacuo and the crude product was washed with diethyl ether (2 × 20 mL). The product was dried in a vacuum at room temperature and recrystallized from acetonitrile.

White solid; yield 88.5%; mp 181.5–182.5 °C (CH_3CN). IR (KBr) 3443, 3200, 3025, 2867, 1659, 1639, 1600, 1549, 1366, 1323, 1265, 1114, 1060, 916, 839, 693 cm^{-1}. ^1H NMR (DMSO-d_6, 300 MHz): δ 2.85 (s, 6H), 7.71 (d, J = 4.3 Hz, 2H), 7.90 (s, 1H), 8.70 (d, J = 4.3 Hz, 2H), 10.83 (s, 1H). ^{13}C NMR (DMSO-d_6, 75 MHz): δ 37.1, 121.0, 141.6, 150.0, 155.8, 160.0. MS (EI): 192 (m/z M^+). Anal. Calcd for $C_9H_{12}N_4O$ (192.22): C, 56.24; H, 6.29; N, 29.15. Found: C, 56.29; H, 6.53; N, 28.98.

7.1.2.2. *N′*-(Morpholine-4-ylmethylene)isonicotinohydrazide (3). A solution of morpholine (90.6 mg, 1.03 mmol) in absolute ethanol (2 mL) was added to a stirred solution of ethyl isonicotinoylhydrazonoformate **2** (193 mg, 1 mmol) in absolute ethanol (5 mL) at room temperature. After 24 h of stirring, the mixture was evaporated in vacuo and the residue was washed with diethyl ether (5 mL). The crude product was purified by crystallization from absolute ethanol.

White solid; yield 85%; mp 176–177.5 °C (EtOH). IR (KBr): 3433, 3199, 3017, 2867, 1653, 1621, 1601, 1549, 1445, 1408, 1362, 1327, 1274, 1235, 1189, 1113, 1016, 959, 909, 658 cm^{-1}. ^1H NMR (DMSO-d_6, 300 MHz): δ 3.29 (t, J = 4.9 Hz, 4H), 3.61 (t, J = 4.9 Hz, 4H), 7.71 (m, 2H), 7.97 (s, 1H), 8.70 (m, 2H), 10.95 (s, 1H). ^{13}C NMR (DMSO-d_6, 75 MHz): δ 45.1, 65.2, 120.6, 140.9, 149.6, 153.9, 159.6. MS (EI): 234 (m/z M^+). Anal. Calcd for $C_{11}H_{14}N_4O_2$ (234.25): C, 56.40; H, 6.02; N, 23.92. Found: C, 56.36; H 6.25; N, 23.96.

7.1.2.3. *N*-Isonicotinoyl-*N*-(pyridine-2-ylmethyl)hydrazonoformamide (4). A solution of pyridin-2-ylmethanamine (178 mg, 1.65 mmol) in acetonitrile (1 mL) was added to a stirred solution of ethyl isonicotinoylhydrazonoformate **2** (289 mg, 1.5 mmol) in acetonitrile (15 mL) at 50 °C. The mixture was stirred for 6 h at the same temperature and for 16 h at room temperature. The precipitate was collected by filtration and recrystallized from acetonitrile.

White solid; yield 37%; mp 151–153 °C (CH_3CN). IR (KBr): 3306, 3225, 3046, 2930, 2880, 1660, 1624, 1578, 1545, 1516, 1469, 1413, 1357, 1314, 1246, 1062, 896, 845, 757, 667, 592 cm^{-1}. ^1H NMR (DMSO-d_6, 300 MHz): δ 4.45 (d, J = 5.3 Hz, 2H), 7.7 (d, J = 6.0 Hz, 2H), 7.8 (m, 3H), 8.11 (d, J = 4.5 Hz, 1H), 8.53 (m, 2H), 8.68 (d, J = 6.0 Hz, 2H), 10.85 (s, 1H). ^{13}C NMR (DMSO-d_6, 75 MHz): δ 50.9, 122.0, 122.2, 123.0, 137.5, 142.5, 149.7, 150.9, 153.2, 159.11, 160.9. MS (EI): 255 (m/z M^+). Anal. Calcd for $C_{13}H_{13}N_5O$ (255.27): C, 61.16; H, 5.13; N, 27.43. Found: C, 61.30; H, 5.33; N, 27.40.

7.1.2.4. *N*-Benzyl-*N′*-isonicotinoylhydrazonoformamide (5). A solution of benzylamine (178 mg, 1.65 mmol) in acetonitrile (2 mL) was added to a stirred solution of ethyl isonicotinoylhydrazonoformate **2** (289 mg, 1.5 mmol) in acetonitrile (15 mL) at 50 °C. The mixture was stirred for 2.5 h at 50 °C and for 2.5 h at room temperature. After that the precipitate was collected by filtration and crystallized from acetonitrile.

White solid; yield 41%; mp 169.5–171 °C (CH_3CN). IR (KBr): 3342, 3217, 3038, 2927, 2867, 1655, 1621, 1600, 1575, 1543, 1516, 1452, 1424, 1353, 1312, 1291, 1256, 1065, 995, 895,745, 706, 456 cm^{-1}. ^1H NMR (DMSO-d_6, 300 MHz, 353 K): δ 4.37 (s, 2H), 6.86 (broad s, 1H), 7.29 (m, 5H), 7.71 (broad s, 2H), 8.05 (broad s, 1H), 8.63 (s, 2H), 10.82 (s, 1H). ^{13}C NMR (DMSO-d_6, 75 MHz, 353 K): δ 44.3, 121.1, 121.6, 126.8, 127.4, 128.2, 139.3, 150.0, 152.3, 160.0. MS (EI): 254 (m/z M^+). Anal. Calcd for $C_{14}H_{14}N_4O$ (254.28): C, 66.13; H, 5.55; N, 22.03. Found: C, 66.01; H, 5.75; N, 21.89.

7.1.2.5. 2-Hydroxy-4-{[(isonicotinoylhydrazono)methyl]amino}benzoic acid (6). *Procedure A.* *p*-Aminosalicylic acid (153 mg, 1 mmol) in acetonitrile (5 mL) was added to a stirred solution of ethyl isonicotinoylhydrazonoformate **2** (193 mg, 1 mmol) in acetonitrile (13 mL) at 50 °C. The mixture was stirred for 4 h at 50 °C and for 20 h at room temperature. The yellow precipitate was collected by filtration and washed with acetonitrile. The isolated crystals were suspended in methanol at room temperature, filtered off, and dried in vacuo.

Procedure B. Diethoxymethylacetate (162 mg, 1 mmol) was added to a stirred solution of INH (137 mg, 1 mmol) and *p*-aminosalicylic acid (153 mg, 1 mmol) in acetonitrile (20 mL) at 50 °C. Precipitation of yellow crystals followed. The mixture was stirred for 1 h without heating and the yellow precipitate was collected by filtration and washed with acetonitrile. The isolated crystals were suspended in methanol at room temperature, filtered, and dried in vacuo.

Yellow solid; yield 33% *procedure A*, 40% *procedure B*; mp 191.5–193 °C. IR (KBr): 3212, 3088, 1943, 1636, 1257, 1414, 1325, 1260, 1167, 1102, 1063, 1013, 996, 913, 841, 785, 677 cm^{-1}. ^1H NMR (DMSO-d_6, 300 MHz): δ 6.66 (dd, J_1 = 8.65 Hz, J_2 = 2.1 Hz, 1 H), 6.85 (d, J = 2.1 Hz, 1H), 7.68 (d, J = 8.65 Hz, 1H), 7.76 (m, 3H), 8.59 (br s, 1H), 8.75 (m, 3H) (2 protons not observed, exchanged). ^{13}C NMR (DMSO-d_6, 75 MHz): δ 102.0, 105.5, 107.4, 121.2, 131.5, 141.1, 146.1, 147.2, 150.2, 160.2, 162.8, 171.8. MS (EI): 300 (m/z M^+). Anal. Calcd for $C_{14}H_{12}N_4O_4$ (300.27): C, 56.00; H, 4.03; N, 18.66. Found: C, 55.91; H, 4.25; N, 18.44.

7.1.2.6. 1-Cyclopropyl-6-fluoro-7-{4-[(isonicotinoylhydrazono)methyl]piperazin-1-yl}-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (7). Ethyl isonicotinoylhydrazonoformate **2** (193 mg, 1 mmol) in acetonitrile (15 mL) was added to a stirred solution of ciprofloxacin (331 mg, 1 mmol) in acetonitrile (15 mL) and glacial acetic acid (2.2 mL) at 40 °C. The mixture was stirred at laboratory temperature for 20 h, the precipitate was collected by filtration, and the crystals refluxed in acetonitrile (5 mL) for 15 min. The suspension was cooled, the product was filtered and dried in vacuo.

White solid; yield 49%; mp 257–258 °C. IR (KBr): 3437, 3298, 3046, 1722, 1677, 1624, 1551, 1500, 1468, 1240, 1388, 1337, 1282, 1261, 1239, 1200, 1016, 945, 912, 892, 843, 808, 749, 711, 657, 623, 540 cm^{-1}. ^1H NMR (DMSO-d_6, 300 MHz): δ 1.20 (m, 2H), 1.33(m, 2H), 3.28 (br s, 1H), 3.39 (m, 4H), 3.55 (m, 4H), 3.84 (m, 1H), 7.63 (d, J = 7.5 Hz, 1H), 7.72 (m, 2H), 7.94 (d, J = 13.2 Hz, 1H), 8.07 (br s, 1H), 8.68 (s, 1H), 8.71 (m, 2H), 10.99 (s, 1H). MS (EI): 478 (*m*/*z* M^+). Anal. Calcd for $C_{24}H_{23}N_6O_4$ (478.48): C, 60.24; H, 4.85; N, 17.56. Found: C, 60.29; H, 5.08; N, 17.68.

7.1.2.7. *N*′,*N*″-Diisonicotinoylhydrazonoformic hydrazide (8). Diethoxymethyl acetate (81 mg, 0.5 mmol) was added to a stirred solution of INH (137 mg, 1 mmol) in acetonitrile (15 mL) at 50 °C. The reaction mixture was stirred for 3 h at 50 °C and then refluxed for 2 h. Stirring was continued for another 15 h at room temperature. The precipitate was filtered off, washed with acetonitrile, and dried in vacuo at room temperature.

White solid; yield 15.1%; mp 192–193 °C. Lit.[24] mp 184–185 °C. IR (KBr): 3198, 2922, 1690, 1634, 1549, 1488, 1412, 1360, 1317, 1262, 1062, 1023, 906, 849, 753, 682 cm^{-1}. ^1H NMR (DMSO-d_6, 300 MHz): δ 7.77 (m, 4H), 8.16 (s, 1H), 8.74 (m, 4H), 11.00 (br s, 1H) (2 protons not observed, exchanged). MS (FAB): 285 (*m*/*z* MH^+). Anal. Calcd for $C_{13}H_{12}N_6O_2$ (284.27): C, 54.93; H, 4.25; N, 29.56. Found: C, 54.70; H, 4.58; N, 29.18.

7.1.2.8. *N*-(Dimethylaminomethylene)pyrazine-2-carboxamide (9). *N*,*N*-Dimethylformamide dimethyl acetal (358 mg, 3 mmol) was slowly added to pyrazine-2-carboxamide (123 mg, 1 mmol) with stirring at room temperature. The suspension was stirred for 4 h, then another part of *N*,*N*-dimethylformamide dimethyl acetal (178 mg, 1.5 mmol) was added. After 2 h of stirring the reagents were removed by decantation and then washed with diethyl ether (3 × 10 mL). The crystals were filtered off and dried in vacuo.

White solid; yield 45%; mp 117–118 °C. Lit.[8] mp 124–125.5 °C. IR (KBr): 3443, 3385, 2927, 2361, 1647, 1598, 1566, 1486, 1423, 1337, 1160, 1111, 1023, 918, 786, 623 cm^{-1}. ^1H NMR (DMSO-d_6, 300 MHz): δ 3.26 (s, 3H), 3.28 (s, 3H), 8.70 (m, 2H), 8.75 (s, 1H), 9.52 (d, J = 1.5 Hz, 1 H). ^{13}C NMR (DMSO-d_6, 75 MHz): δ 35.5, 41.5, 144.0, 146.0, 146.5, 148.7, 161.5, 174.7. MS (EI): 178 (*m*/*z* M^+). Anal. Calcd for $C_8H_{10}N_4O$ (178.19): C, 53.92; H, 5.66; N, 31.44. Found: C, 54.04; H, 5.88; N, 31.36.

7.1.2.9. 1-Cyclopropyl-6-fluoro-4-oxo-7-{[4-(pyrazin-2-ylcarbonyl)iminomethyl]piperazin-1-yl}-1,4-dihydroquinoline-3- carboxylic acid (10). *N*-[(Dimethylamino)methylene]pyrazine-2-carboxamide **9** (178 mg, 1 mmol) in acetonitrile (5 mL) was added to a stirred solution of ciprofloxacin (331 mg, 1 mmol) as a mixture of acetonitrile (15 mL) and glacial acetic acid (2.2 mL) at 50 °C. The reaction mixture was stirred at room temperature for 20 h. A yellow precipitate was collected by filtration and the crystals refluxed in acetonitrile (5 mL) for 15 min. The suspension was cooled, and light-yellow crystals were filtered and dried in vacuo at room temperature.

Lightly yellow solid; yield 58%; mp 261–264 °C. IR (KBr): 3442, 2926, 2851, 1714, 1616, 1628, 1586, 1562, 1473, 1343, 1264, 1245, 1165, 1119, 1017, 941, 903, 835, 809, 782, 724, 614, 481, 438 cm^{-1}. ^1H NMR (DMSO-d_6, 300 MHz) δ 1.20 (m, 2H), 1.34 (m, 2H), 3.50 (m, 4H), 3.83 (m, 1H), 3.93 (m, 2H), 4.10 (m, 2H), 7.64 (d, J = 7.5 Hz, 1H), 7.96 (d, J = 13.2 Hz, 1H), 8.68 (s, 1H), 8.77 (s, 2H), 8.88 (s, 1H), 9.39 (s, 1H), 15.13 (br s, 1H). MS (FAB): 465 (*m*/*z* MH^+). Anal. Calcd for $C_{23}H_{21}FN_6O_4$ (464.4): C, 59.48; H, 4.56; N, 18.09. Found: C, 59.40; H, 4.59; N, 18.05.

7.1.2.10. 4-(2-Pyrazinecarbonyliminomethyl)aminosalicylic acid (11). To the stirred solution of *N*-[(dimethylamino)methylene]pyrazine-2-carboxamide **9** (267 mg, 1.5 mmol) in acetonitrile (5 mL) was added a solution of *p*-aminosalicylic acid in acetonitrile (15 mL) at room temperature. The mixture was stirred for 4 h. The precipitate was collected by filtration and washed with acetonitrile. The isolated crystals were suspended in methanol, filtered, and dried in vacuo at room temperature.

White-gray solid; yield 30%; mp 222–225 °C. IR (KBr) 3179, 2362, 1721, 1682, 163, 1487, 1340, 1302, 1268, 1241, 1180, 1159, 1105, 1021, 989, 899, 864, 788, 766, 701, 594, 489, 461 cm^{-1}. ^1H NMR (DMSO-d_6, 300 MHz): δ 3.16 (br s, 1H), 3.17 (br s, 1H), 5.97 (d, J = 2.1 Hz, 1H), 6.08 (dd, J_1 = 8.7 Hz, J_2 = 2.3 Hz, 1H), 7.41 (d, J = 8.7 Hz, 1H), 8.67 (s, 1H), 8.82 (m, 2H), 9.25 (d, J = 9.24 Hz, 1H), 11.63 (br s, 1H) (2 protons not observed, exchanged). MS (EI): 286 (*m*/*z* M^+). Anal. Calcd for $C_{15}H_{12}N_2O_4$ (284.26): C, 54.55; H, 3.52; N, 19.57. Found: C, 54.39; H, 3.72; N, 19.24.

7.1.2.11. *N*-[(2-Isonicotinoylhydrazino)methylidene]-2-pyrazinecarboxamide (12). To the stirred solution of *N*-(dimethylaminomethylene)pyrazine-2-carboxamide **9** (400 mg, 2.25 mmol) in acetonitrile (5 mL) pyridinium toluene-4-sulfonate polymer bound (643 mg, 2.25 mmol) was added at 50 °C. After 3 min a solution of isoniazid (310 mg, 2.25 mmol) in acetonitrile (22 mL) was added. The mixture was stirred for 1 h at the same temperature then cooled and the solid was collected by filtration, dried, and extracted with methanol (40, 30, and 20 mL). Extracts were collected and evaporated to dryness. The crude product was washed with methanol and dried in vacuo at room temperature.

White solid; yield 36%; mp 230–232 °C. IR (KBr): 3437, 3262, 1682, 1659, 1624, 1546, 1501, 1407, 1339, 1282, 1231, 1170, 1124, 1065, 1022, 922, 845, 756, 712, 680 cm^{-1}. ^1H NMR (DMSO-d_6, 300 MHz): δ 7.82 (m, 2H), 8.80 (m 3H), 8.94 (d, J = 2.4 Hz, 1H), 9.08 (br s, 1H), 9.27 (s, 1H), 11.39 (br s, 1H), 11.75 (br s, 1H). ^{13}C NMR (DMSO-d_6, 75 MHz): δ 109.1, 121.3, 140.4, 141.7, 143.7, 144.1, 148.2, 150.3, 160.9, 163.0. MS (EI): 270 (*m*/*z* M^+). Anal. Calcd for $C_{12}H_{10}N_6O_2$ (270.25): C, 53.33; H, 3.73; N, 31.10. Found: C, 53.30; H, 3.82; N, 30.90.

7.1.2.12. 1,3-Bis(1-hydroxybutan-2-yl)imidazolidin-2-yl 4-methylbenzenesulfonate (13). Pyridinium 4-methylbenzenesulfonate polymer bound (PTTS, 428 mg, 1.5 mmol) was added to a stirred solution of *N*-(dimethylaminomethylene)pyrazine-2-carboxamide **9** (267 mg, 1.5 mmol) in acetonitrile (5 mL) at 50 °C. After 5 min a solution of ethambutol free base (306 mg, 1.5 mmol) in acetonitrile (15 mL) was added. The reaction mixture was stirred for 6 h at 50 °C, the precipitate was collected by filtration and washed with diethyl ether. The crude product was recrystallized from ethanol–diethyl ether.

White solid; mp 109–111 °C ($C_2H_5OH/(C_2H_5)_2O$). IR (KBr): 3348, 3273, 2967, 1650, 1514, 1461, 1403, 1305, 1211, 1126, 1067, 1037, 1011, 820, 683, 569 cm^{-1}.^{1}H NMR (DMSO-d_6, 300 MHz) δ 0.98 (t, J = 7.5 Hz, 6H), 1.62 (m, 4H), 2.35 (s, 3H), 3.63 (m, 4H), 3.83 (m, 2H), 4.08 (s, 4H), 5.27 (br s, 2H), 7.18 (d, J = 7.8 Hz, 2H), 7.76 (d, J = 8.1 Hz, 2H), 8.38 (s, 1H).

^{13}C NMR (DMSO-d_6, 75 MHz): δ 10.4, 21.2, 21.4, 44.8, 60.2, 62.6, 125.7, 128.6, 13.8, 142.5, 158.4. MS (EI): 215 (*m*/*z* M^+–4-$CH_3C_6H_4SO_3$). Anal. Calcd for $C_{18}H_{30}N_2O_5S$ (386.51): C, 55.93; H, 7.82; N, 7.25. Found: C, 56.14; H, 8.01; N, 7.24.

7.2. Lipophilicity HPLC determination (capacity factor *K*/calculated log *K*)

The HPLC separation module Waters Alliance 2695 XE and Waters Photodiode Array Detector 2996 (Waters Corp., Milford, MA, USA) were used. The chromatographic column Symmetry® C_{18} 5 μm, 4.6 × 250 mm, Part No. WAT054275 (Waters Corp., Milford, MA, USA) was used. The HPLC separation process was monitored by Millennium32® Chromatography Manager Software, Waters 2004 (Waters Corp., Milford, MA, USA). As a mobile phase the mixture of MeOH p.a. (30.0%) and H_2O-HPLC—Milli-Q grade (70.0%) was used for all the compounds, except the ciprofloxacin derivates **7** and **10**. In these cases a mixture of MeOH p.a. (50.0%) and H_2O-HPLC—Mili-Q grade (50.0%) was applied. The total flow of the column was 0.7 ml/min, injection 30 μl, column temperature 22 °C, respectively, 25 °C for **7** and **10**; the sample temperature was 10 °C. The detection wavelength was 265 nm. The KI methanolic solution was used for the dead-time (T_D) determination. Retention times (T_R) were measured in minutes.

The capacity factors K were calculated using the Millennium32® Chromatography Manager Software according to the formula $K = (T_R - T_D)/T_D$, where T_R is the retention time of the solute, whereas T_D denotes the dead time obtained via an unretained analyte. Log$K$, calculated from the capacity factor K, is used as the lipophilicity index converted to the log$P$ scale. The log$K$ values of the individual compounds are shown in Table 1.

7.3. Lipophilicity calculations

Log$P$, that is, the logarithm of the partition coefficient for *n*-octanol/water, was calculated using the programs CS ChemOffice, ChemDraw Ultra ver. 10.0 (CambridgeSoft, Cambridge, MA, USA) and ACD/Log$P$ ver. 1.0 (Advanced Chemistry Development Inc., Toronto, Canada). The *C*log$P$ values (the logarithm of *n*-octanol/water partition coefficient based on established chemical interactions) were generated by means of CS ChemOffice Ultra ver. 7.0 (CambridgeSoft, Cambridge, MA, USA) software. The results are shown in Table 1.

7.4. Stability of compounds

The HPLC separation module Waters Alliance 2695 XE and Waters Photodiode Array Detector 2996 (Waters Corp., Milford, MA, USA) were used. The HPLC separation process was monitored by Millennium32® Chromatography Manager Software, Waters 2004 (Waters Corp., Milford, MA, USA).

7.4.1. Chromatographic conditions for 6–8. The chromatographic column Discovery® HS F5 5 μm, 4.6 × 150 mm, (Supelco, Bellefonte, PA, USA) was used. The mixture of phosphate buffer 0.05 M (pH 2.0) and MeCN p.a. (65:35) was applied as a mobile phase and 20% MeCN was chosen as a sample solvent. The total flow of the column was 1.0 mL/min, injection 20 μL, column temperature 30 °C and sample temperature 37 °C. The detection wavelength was 265 nm. Time of analysis was 10 min.

7.4.2. Chromatographic conditions for 10–12. The chromatographic column Phenomenex Synergi™ Polar-RP 4 μm, 4.6 × 250 mm, (Phenomenex, Torrance, CA, USA) was used. The mixture of phosphate buffer 0.05 M (pH 6.0) and MeCN p.a. (55:45) was applied as a mobile phase and 20% MeCN was chosen as a sample solvent. The other parameters were the same as described for the compounds **6**–**8**.

7.5. Physico-chemical calculations

Reaction orders and rate constants were calculated using the program MATLAB ver. R2006a (The Math Works, Natick, MA, USA).

7.6. Antimycobacterial evaluation

The compounds were screened for antituberculotic activity under the direction of the US National Institute of Health, NIAD Division (TAACF). Primary screening was conducted at a single concentration 6.25 μg/mL against *M. tuberculosis* $H_{37}Rv$ (ATCC2729) in BACTEC 12B medium using a broth microdilution assay, the Microplate Almar Blue Assay (MABA). Compounds demonstrating at least 90% inhibition in the primary screening (MIC < 6.25 μg/mL) were tested at a lower concentration against *M. tuberculosis* $H_{37}Rv$ to determine the MIC testing by MABA. The MIC is defined as the lowest concentration effecting a reduction in fluorescence of 99% relative to the controls.[25]

The in vitro antimycobacterial activity of all the compounds was further evaluated against *M. tuberculosis*

CNCTC My 331/88, *M. kansasii* CNCTC My 235/80, *M. kansasii* 6509/96, and *Mycobacterium avium* CNCTC My 330/88 in The National Reference Laboratory for *Mycobacterium kansasii*, Regional Institute of Hygiene, Ostrava, Czech Republic. All strains were obtained from the Czech National Collection of Type Cultures (CNCTC), except for *M. kansasii* 6509/96, which was clinically isolated. The antimycobacterial activities were determined in a Sula semisynthetic medium (SEVAC, Prague, Czech Republic). The compounds were added to the medium as dimethylsulfoxide solutions. The following concentrations were used: 250, 125, 62, 32, 16, 8, 4, 2, and 1 μmol/L. The MIC values were determined after incubation at 37 °C for 7, 14, and 21 days. The MIC was the lowest concentration of a substance at which the inhibition of the growth of mycobacteria occurred, see Table 2.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2007.01.051.

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