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Synthesis and antimycobacterial evaluation of pyrazinamide derivatives with benzylamino substitution

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ABSTRACT

A series of 19 new compounds related to pyrazinamide were synthesized, characterized with analytical data and screened for in vitro whole cell antimycobacterial activity against *Mycobacterium tuberculosis* H37Rv, *Mycobacterium kansasii* and two types of *Mycobacterium avium*. The series consisted of 3-(benzylamino)-5-cyanopyrazine-2-carboxamides and 3-(benzylamino)pyrazine-2,5-dicarbonitriles with various substituents on the phenyl ring. RP-HPLC method was used to determine the lipophilicity of the prepared compounds. Nine compounds exerted similar or better activity against *Mycobacterium tuberculosis* compared to pyrazinamide (MIC = 6.25–12.5 μg/mL). 3-(Benzylamino)pyrazine-2,5-dicarbonitrile inhibited all of the tested mycobacterial strains with MIC within the range 12.5–25 μg/mL. Although not the most active, 4-NH_2 substituted compounds possessed the lowest in vitro cytotoxicity (hepatotoxicity), leading to selectivity index SI = 5.5 and SI >21.

In 2011 at least two studies proposing a specific subcellular target of pyrazinamide (PZA) and/or its metabolite pyrazinoic acid (POA) were published in prestigious journals. Firstly, PZA and POA were shown to bind to fatty acid synthase I (FAS I).[1] This supports the previous studies which suggested that the inhibition of this enzyme system was the mode of action of some PZA/POA derivatives.[2–5] Secondly, POA (but not PZA) was shown to bind to ribosomal protein RpsA, leading to the blockage of *trans*-translation (the process of removing ribosomes stalled upon mRNA).[6] This renewed interest in PZA can be attributed to its unique therapeutic properties, for example the ability to kill metabolically low-active mycobacteria which leads to shortening the course of therapy. Although structural modifications of PZA have not yielded any clinical agents thus far, the validation of a specific subcellular target of PZA/POA could lead to modern strategies of structure-based drug design which could enhance future analogue development.

As a part of our continuous research of new PZA derivatives with antimycobacterial activity, we report a series of 19 compounds derived from 3-chloro-5-cyanopyrazine-2-carboxamide (**4**) and 3-chloropyrazine-2,5-dicarbonitrile (**6**) by nucleophilic substitution by various benzylamines. Similar compounds, derived from **4** and **6** by reaction with anilines (therefore missing the $-CH_2-$ bridge), were prepared before and possessed moderate to good antimycobacterial activity.[7,8]

Parent compounds **4** and **6** were prepared as described in literature[7–10]—see Scheme 1. Briefly, PZA was treated with peroxoacetic acid to yield the mixture of pyrazinecarboxamide-*N*-oxides. This unseparated mixture was chlorinated with $POCl_3$ to yield three position isomers of chloropyrazine-2-carbonitrile (**1**–**3**). The main product 6-chloropyrazine-2-carbonitrile (**1**) was separated by flash chromatography and underwent homolytic amidation to produce 3-chloro-5-cyanopyrazine-2-carboxamide (**4**), which was dehydrated by $POCl_3$ to 3-chloropyrazine-2,5-dicarbonitrile (**6**). Alternatively, compound **6** can be prepared by dehydration of 6-chloro-5-cyanopyrazine-2-carboxamide (**5**) derived by amidation of 3-chloropyrazine-2-carbonitrile (**2**) as described in our previous paper.[12]

Final compounds **7a**–**7i** and **8a**–**8j** were prepared by nucleophilic substitution by various benzylamines (Scheme 2). The substitution of chlorine proceeded well at mild conditions (mostly at RT, reflux in THF for benzylamines with electron withdrawing substituents –CF_3 or –NO_2). Potassium carbonate was used as a base. The prepared compounds (all of them yellow to pale yellow solid or crystalline) were characterized with ^{1}H NMR, ^{13}C NMR, FT-IR spectroscopy, melting point and elementary analysis. The analytical data were fully consistent with proposed structures and are included in the Supplementary data.

Lipophilicity is a very important physicochemical property with great impact on biological activity and in our previous studies we have observed that antimycobacterial activity of trisubstituted pyrazines was strongly dependent on their lipophilicity.[8,11,12]

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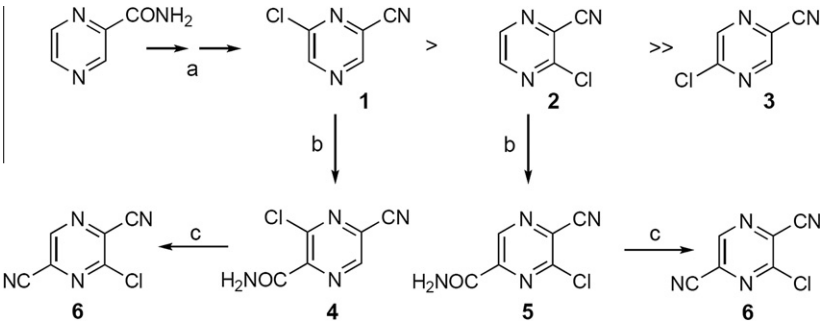
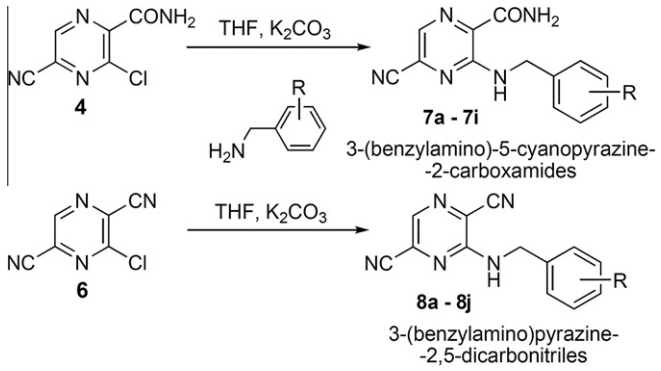


Scheme 1. Synthetic routes to parent compounds **4** and **6**. Reagents: (a) (1) H_2O_2, AcOH; (2) $POCl_3$; (b) $(NH_4)_2S_2O_8$, $HCONH_2$; (c) $POCl_3$.



Scheme 2. Synthesis of final compounds.

Therefore much attention was paid to correct determination of the lipophilicity of the prepared compounds. Lipophilicity parameters *C*log*P* (ChemOffice) and ACD/log*P* of the prepared compounds were calculated using software routinely engaged in our laboratory.[13] Calculated values indicated that the compounds from series **8** (dicarbonitrile derivatives) were less lipophilic than corresponding compounds from series **7** (cyanocarboxamides) with equal substitution on the phenyl ring (see Table 1). This was in contrast both with our expectations and to the R_f values observed during TLC. Therefore the lipophilicity was measured experimentally by means of chromatographic separation on RP-HPLC C_{18} column and expressed as log*k* value derived from retention time of individual compounds.[14]

Lipophilicity substituent constants π for substituents *R* were calculated both from measured (log*k*) and calculated (*C*log*P*, ACD/log*P*) values (Table 2). In both series **7** and **8** the measured π values for individual substituents R were very similar—compare $\pi(\log k)_7$ versus $\pi(\log k)_8$ columns in Table 2. This indicates the capability of our method to measure correct values. The plot $\pi(\log k)$ on π(ACD/log*P*)—see Figure 1—showed a good linear correlation ($R^2 = 0.985$, $n = 17$) with regression line running very close to the point [0;0] (which would be intersected if the correlation was ideal). Similarly, the plot $\pi(\log k)$ on π(*C*log*P*) showed correlation $R^2 = 0.966$, $n = 17$ (not shown). This correlation indicates that both ChemOffice *C*log*P* and ACD/log*P* algorithms were accurate in calculating π increments based on the substituents of the phenyl ring. According to experimentally determined log*k*, all dicarbonitriles **8a**–**8i** were little more lipophilic compared to matching cyanocarboxamides **7a**–**7i**. The virtual exchange of 5-$CONH_2$ group to 5-CN conferred δ (log*k*) = 0.034 ± 0.016 (mean ± SD).

Prepared compounds were screened for antimycobacterial activity against *M. tuberculosis* H37Rv, *M. kansasii* and two strains of *M. avium* by microdilution panel method.[15] For results see Table 1. In series **7** there were four compounds active against *M. tuberculosis*, but none of them was active against tested nontuberculous mycobacteria (NTM; namely *M. kansasii* and two strains of *M. avium*). On contrary, series **8** yielded six active compounds and most of them exerted at least some activity against NTM as well. Most significantly, compound **8a** inhibited all of the tested mycobacterial strains with MIC within the range 12.5–25 μg/mL (53–106 μM). The fact that pyrazinedicarbonitriles possessed better activity against NTM in comparison to analogous cyanopyrazinecarboxamides is in concordance with observations in our previous series, for example compare Ref. 11,12. Compounds with electron-donating substituents *R* = (4-CH_3; 4-NH_2) were active in both series, whereas strongly electron-withdrawing substituents *R* = (3-CF_3; 3-NO_2) produced active compounds only within series **7**. Chlorine substituents *R* = (3-Cl; 4-Cl) were active in series **8**, but not in series **7**. No direct correlation was found between antimycobacterial activity (log (1/MIC)) and lipophilicity (log*k*). Also application of electronic Hammett constants σ of substituents *R* on the aromatic ring did not produce a universal rule describing the SAR applicable for both series.

Regarding antimycobacterial activity against *M. tbc* H37Rv, the most active compounds (**7b**, **7d**, **8a**, **8b**, **8e**) exerted similar or slightly better activity compared to PZA standard, that is MIC = 6.25–12.5 μg/mL. Taking into consideration significantly higher molecular weights of prepared compounds in comparison with PZA, compound **8b** (MIC = 25 μM) scored two times better then PZA (MIC = 51–102 μM) against *M. tbc* H37Rv. For MICs in (μM) see Table 1—values in parentheses.

As mentioned before, the final compounds reported in this Letter are methylene homologs of 3-(phenylamino)-5-cyanopyrazine-2-carboxamides and 3-(phenylamino)pyrazine-2,5-dicarbonitriles published before, whose antimycobacterial activity against *M. tbc* H37Rv was MIC = 12.5–25 μg/mL and MIC = 8–16 μg/mL, respectively.[7,8] Although the comparison is limited by minor differences in the testing method and sometimes different substituents *R* on the aromatic ring, we conclude that the insertion of $–CH_2–$ bridge is not primarily connected with the loss of antitubercular activity, for at least some of the prepared methylene homologs possess activity comparable with phenylamino predecessors.

Drug-induced hepatotoxicity is a well-known side effect of many of the first line antituberculosis agents (PZA, isoniazid, rifampicin)[16,17] and unfortunately there is a synergy effect which increases the toxicity when these drugs are administered in combination. Human liver carcinoma cell line (Hep G2) has been used as a model to study this toxicity synergism in vitro.[18,19] According to WHO treatment guidelines,[20] the basic regime for the treatment of non-complicated tuberculosis lasts at least 6 months and combines all of the potentially hepatotoxic antituberculosis agents mentioned above. Due to this multidrug-treatment approach it is very probable that a newly developed antitubercular drug would be used in combination with at least some of these hepatotoxic drugs; therefore the hepatotoxicity of new drugs should be considered very cautiously.