Substituted $N$-benzylpyrazine-2-carboxamides, Their Synthesis, Hydro-lipophilic Properties and Evaluation of Their Antimycobacterial, and Photosynthesis-inhibiting Activity

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Abstract: This communication deals with the synthesis and evaluation of some pyrazinamide (PZA) analogues/prodrugs derived from substituted $N$-benzylpyrazine-2-carboxamides or substituted 3-(benzyl-amino)pyrazine-2-carboxamides. A series of sixteen pyrazinamide analogues with the $-\text{CO-}\text{NH-CH}_2$ - or $-\text{NH-CH}_2$ - linkers connecting the pyrazine and benzene rings was prepared by using the microwave assisted coupling reaction of substituted methylpyrazinecarboxylate with ring-substituted benzylamines and characterized. The results of $in$ $vitro$ antimycobacterial screening indicated some interesting antimycobacterial activity in the series of $N$-benzylpyrazine-2-carboxamides. From the second series, 3-(3,4-dichlorobenzylamino)-pyrazine-2-carboxamide was the most active in the inhibition of photosynthetic electron transport (PET) in spinach $(Spinacia$ $oleracea$ $L.)$ chloroplasts $(IC_{50} = 2.2 \text{ μmol/L})$.

Keywords: Pyrazinecarboxamides; Lipophilicity; $In$ $vitro$ antimycobacterial activity; Spinach chloroplasts; PET inhibition; Structure-activity relationships.

Introduction

Pyrazine is a 6π-electron-deficient heteroaromatic compound, in which the inductive effects of the nitrogen atoms induce a partially positive charge on the carbon atoms. Pyrazines are a class of compounds that occur almost ubiquitously in nature. Their effectiveness at very low concentrations and the ever increasing application of synthetic pyrazines in the flavour and fragrance industry and in the food and pharmaceutical industries are responsible for the high interest in these compounds [1]. Pyrazines are found in such heated foods as bread, different meats, baked potatoes and coffee [2], and are formed from serine and threonine [3]. Synthetic pyrazine derivatives are also useful as drugs (antiviral, anticancer, antimycobacterial, etc.), fungicides, and herbicides [1, 4]. Furthermore, pyrazine derivatives have been used as antioxidants [5, 6]. This article deals with pyrazinecarboxamide (PZA) and its derivatives. PZA was introduced to clinical practice in 1952, and it has been among the first-line antituberculosis (anti-TB) drugs. PZA is an important component in the intensive phase of short-course treatment of TB owing to its sterilizing effect, ability to act in acidic environments and excellent synergy with rifampicin. Our research is focused on the study of binuclear analogues with the $-\text{CONH}$ - bridge connecting the pyrazine and benzene rings with antimycobacterial activity. This moiety can form centro symmetric dimer pairs with the peptidic carboxamido group of some peptides, needed for binding to the receptor site, possibly by forming of hydrogen bond (see Fig. 1).
All substituted amides of pyrazinecarboxylic acid studied can be interpreted as more lipophilic aza-analogues of nicotinamide (see Fig. 2, compd. 1)[7-10]. Substituted quinoxalinecarboxylic acid amides (with a visible pyrazinamide fragment in a molecule) were identified as the active antimycobacterial derivatives with the Minimal Inhibition Concentration (MIC) on the same order as rifampicin [11]. The report that 5-chloropyrazin-2-carboxamide has different mode of action to pyrazinamide itself makes this direction in research more than relevant [12]. This study deals with the pyrazinamide structure modifications and is based on rich substitution both in pyrazine (R1 and R2) and benzene (R3) part of the target molecule (see Fig. 2). Primary in vitro screening of the synthesized compounds was performed against four mycobacterial strains. The compounds were also tested for their photosynthesis-inhibiting activity (the inhibition of photosynthetic electron transport in spinach chloroplasts (Spinacia oleracea L.). The aim of this work is to find the structure-activity relationship (SAR) in the series of substituted N-benzylpyrazine-2-carboxamides, i.e. (i) to study the influence of incorporated methylene moiety in the connecting bridge (see Fig. 2, compd. 2, blue colour); (ii) to continue in the study of the substituent variability influence on the biological activity; (iii) to compare the properties of mentioned series with the previously studied substituted N-phenylpyrazine-2-carboxamides (see Fig. 2, compd. 1)[7-10, 13]; and (iv) to determine the importance of increased lipophilic properties for biological effect of the newly prepared substituted pyrazinecarboxamides.

The microwave assisted coupling reaction of methylester of substituted pyrazinecarboxylic acids with ring-substituted benzylamines yielded series of substituted N-benzylpyrazine-2-carboxamides 2a-j (see Fig. 2, compd. 2, and Table 1). We have chosen quite hydrophobic electron-withdrawing (halogens), and alkyl substituents on the pyrazine (methyl, tert-butyl), and their combination of substituents (alkyl, alkoxy, acetyl, OH, halogens) on benzene part. 2-Aminopyrazine-3-carboxamide (see Fig. 3) was the starting compound for the other structural modification (see Fig. 2, compd. 3, and Table 1), forming the second series, i.e. substituted 3-(benzylamino)pyrazine-2-carboxamides 3a-f.
Results and discussion

The aim of this work was to test all prepared compounds as potential antimycobacterial agents and inhibitors of the photosynthetic electron transport in spinach chloroplasts. The results are summarized in Table 1.

<table>
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<tr>
<th>Comp.</th>
<th>R¹</th>
<th>R²</th>
<th>R³</th>
<th>m.w.</th>
<th>log P/Log P (ChemOffice)</th>
<th>Antibiotic evaluation MIC (µg/mL)</th>
<th>PET inhibition in spinach chloroplasts IC₅₀ (µmol/L)</th>
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<td>H</td>
<td>H</td>
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<td>CH₃</td>
<td>H</td>
<td>227.26</td>
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<td>CH₃</td>
<td>3-CF₃</td>
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- not tested; * precipitation in medium
The antimycobacterial evaluation of all prepared compounds is not completely finished; only some the discovery of weak antimycobacterial activity in the series of $N$-benzylpyrazine-2-carboxamides 2 could be mentioned. Dependence of PET-inhibiting activity and antimycobacterial activity of the studied compounds on log $P$ is presented in Fig. 4. The PET-inhibiting activity of compounds from series 2 as well as series 3 showed linear increase with increasing lipophilicity of the compounds. Lower activity of compound 3f could be explained by possible noncoplanar conformation. In general, it can be concluded that from the aspect of herbicidal activity compounds 3 are more effective than compounds 2, whereby the activity of compound 3-(3,4-dichlorobenzyl-amino)-pyrazine-2-carboxamide 3c (2.2 µmol/L) is comparable with that of the standard DCMU (1.9 µmol/L). The obtained results are in agreement with our previous findings [14].

**Fig. 4 Dependence of PET-inhibiting activity (A) of the studied compounds on log $P$**

Acknowledgement
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Experimental

**Instrumentation and chemicals**
All organic solvents used for the synthesis were of analytical grade. The solvents were dried and freshly distilled under argon atmosphere. Melting points were determined using a Melting Point Apparatus SMP 3 (BIBBY Stuart Scientific, UK) and are uncorrected. The reactions were monitored and the purity of the products was checked by TLC (Merck UV 254 TLC plates, Darmstadt, Germany) using developing solvents petroleum ether / EtOAc (9 : 1). Purification of compounds was made using Flash Master Personal chromatography system from Argonaut Chromatography (Argonaut Technologies, Redwood City, CA, USA). As sorbent, Merck Silica Gel 60 (0.040–0.063 mm) was used (Merck). Elemental analyses were performed on an automatic microanalyser CHNS-O CE instrument (FISONS EA 1110, Milano, Italy). Infrared spectra were recorded in Nicolet Impact 400 spectrometer in KBr
pellets. $^1$H and $^{13}$C NMR Spectra were recorded on a Varian Mercury — Vx BB 300 (300 MHz for $^1$H and 75 MHz for $^{13}$C), Varian (Palo Alto CA, USA) in DMSO-d$_6$ solutions at ambient temperature. The chemical shifts were recorded as $\delta$ values in ppm and were indirectly referenced to tetramethylsilane (TMS) via the solvent signal (2.49 for $^1$H and 39.7 for $^{13}$C in DMSO-d$_6$).

**General procedure for the synthesis of the title compounds**
The synthesis was carried out under microwave irradiation using a CEM Corp. Discover laboratory microwave with an automated Explorer unit. The microwave assisted coupling reaction of methylester of substituted pyrazinecarboxylic acids with ring-substituted benzylamines yielded series of substituted N-benzylpyrazine-2-carboxamides (see Fig. 3). We have chosen quite hydrophobic electron-withdrawing (halogens), and alkyl substitutents on the pyrazine (methyl, tert-butyl), and their combination of substituents (alkyl, alkoxy, acetyl, OH, halogens) on benzene part (see Table 1). More synthetic details will be published in separated article.

**Lipophilicity calculations**
Log $P$, *i.e.* the logarithm of the partition coefficient for n-octanol/water, was calculated using the programmes CS ChemOffice Ultra ver. 10.0 (CambridgeSoft, Cambridge, MA, U.S.A.). Clog $P$ values (the logarithm of n-octanol/water partition coefficient based on established chemical interactions) were generated by means of the CS ChemOffice Ultra ver. 10.0 (CambridgeSoft, Cambridge, MA, U.S.A.) software. The results are shown in Table 1.

**Evaluation of in vitro antimycobacterial activity**
The antimycobacterial assay was provided by University Hospital in Hradec Kralove (Czech Republic). Five strains were used: *M. tuberculosis* H37Rv CNCTC My 331/88, *M. kansasii* CNCTC My 235/80, *M. avium* CNCTC My 80/72 and *M. avium* CNCTC My 152/73 (Czech National Collection of Type Cultures, National Institute of Public Health, Prague, Czech Republic). Tested compounds were dissolved in DMSO, diluted with Šula's semisynthetic medium (Trios, Prague, Czech Republic) and placed into microdilution panel. Tested species were added in the form of suspension in isotonic saline solution. Compounds were tested at final concentrations of 200, 100, 50, 25, 12.5, 6.25 and 3.125 μg/mL. The final concentration of DMSO did not exceed 1% (v/v). It was confirmed that at this concentration DMSO itself did not affect the growth of mycobacteria. PZA was used as a standard. The cultures were grown in Šula's semisynthetic medium at pH 5.5 and 37 °C. The antimycobacterial activity was determined visually after 14 days (6 days for *M. kansasii*) of incubation as MIC [μg/mL], *i.e.* the lowest used concentration of tested substance which inhibited the growth of mycobacteria. The preliminary results (against *M. tuberculosis*) are shown in Table 1.

**Study of inhibition photosynthetic electron transport (PET) in spinach chloroplasts**
Chloroplasts were prepared from spinach (*Spinacia oleracea* L.) according to Masarovičová and Kráľová [15]. The inhibition of oxygen evolution rate (inhibition of photosynthetic electron transport, PET) in spinach chloroplasts was determined spectrophotometrically (Genesys 6, Thermo Scientific, USA) using the artificial electron acceptor 2,6-dichlorophenol-indophenol (DCIPP) according to Kráľová et al. [16] and the rate of photosynthetic electron transport was monitored as a photoreduction of DCPIP. The measurements were carried out in phosphate buffer (0.02 mol/L, pH 7.2) containing sucrose (0.4 mol/L), MgCl$_2$ (0.005 mol/L) and NaCl (0.015 mol/L). The chlorophyll content was 30 mg/L in these experiments and the samples were irradiated (~100 W/m$^2$) from 10 cm distance with a halogen lamp (250 W) using a 4 cm water filter to prevent warming of the
samples (suspension temperature 22 °C). The studied compounds were dissolved in DMSO due to their limited water solubility. The applied DMSO concentration (up to 4%) did not affect the photochemical activity in spinach chloroplasts. The inhibitory efficiency of the studied compounds was expressed by IC\textsubscript{50} values, \textit{i.e.} by molar concentration of the compounds causing 50% decrease in the oxygen evolution rate relative to the untreated control. The comparable IC\textsubscript{50} value for a selective herbicide 3-(3,4-dichlorophenyl)-1,1-dimethylurea, DCMU (Diuron®) is 1.9 μmol/L. The results are summarized in Table 1.

References: