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 –CO-NH-CH_2- or -NH-CH_2- linkers connecting the pyrazine and benzene rings was prepared by using the microwave assisted coupling reaction of substituted methyl-pyrazinecarboxylate 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-dichlorobenzyl-amino)-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 μmol/L).

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

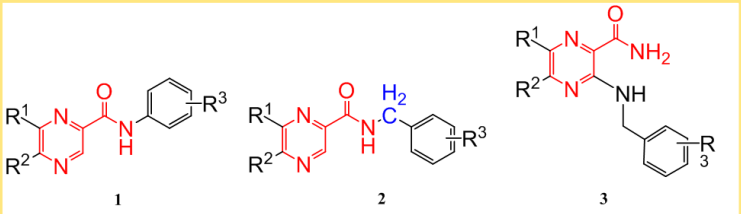
Introduction

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 -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**).

Fig. 1 Formation of centrosymetric dimer pairs of carboxamido group.

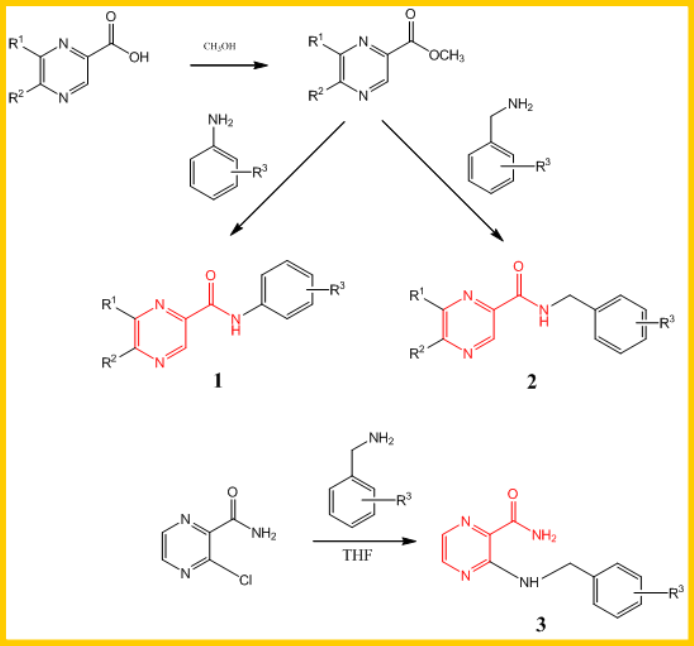
All substituted amides of pyrazinecarboxylic acid studied can be interpreted as more lipophilic aza-analogues of nicotinamide (see **Fig. 2**, compd. **1**)[7-10]. Substituted quinoxalinecarboxylic acid amides (with a visible pyrazinamide fragment in a molecule) were identified as the active antimycobacterial derivatives with the Minimal Inhibition Concentration (MIC) on the same order as rifampicin [11]. The report that 5-chloropyrazine-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 (R^1 and R^2) and benzene (R^3) 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.

Fig. 2 Structure modifications of PZA (the PZA fragment - red colour)



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 substitutents 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**.

Fig. 3 Synthesis and structures of the title compounds.



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**.

Tab. 1. Structure, lipophilicity (log *P*, Clog*P*), antimycobacterial (MIC) and PET inhibition in spinach chloroplasts (IC_{50}) of compounds **2a-j** and **3a-f** in comparison with the standards pyrazinamide (PZA) and 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU, Diurone ®).

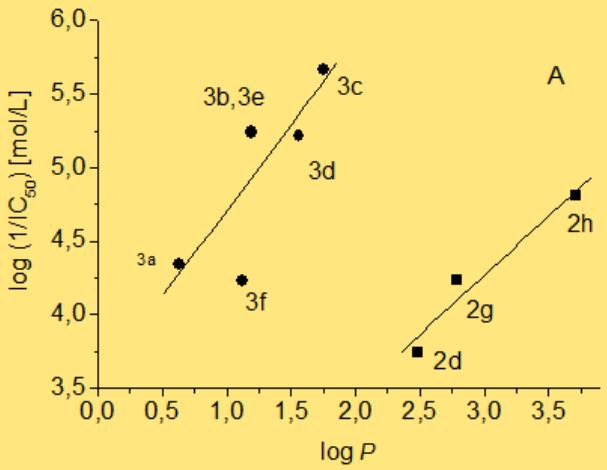
Comp.	R^1	R^2	R^3	m.w.	log *P*/Clog*P* (ChemOffice)	Antitbc evaluation **MIC** (ug/mL)	**PET** inhibition in spinach chloroplasts IC_{50} (umol/L)
2a	H	H	H	213.24	0.66/1.64348	100	*
2b	H	CH_3	H	227.26	1.36/2.14248	100	*
2c	H	CH_3	3-CF_3	295.26	2.28/3.02548	25	*
2d	H	CH_3	3,4-Cl	296.15	2.48/3.44848	100	179.5
2e	H	CH_3	4-Cl	261.71	1.92/2.85548	100	*
2f	H	H	4-OCH_3	243.26	0.53/1.56248	100	*
2g	H	$(CH_3)_3C$	H	269.15	2.79/3.46948	> 100	58.0
2h	H	$(CH_3)_3C$	3-CF_3	337.30	3.71/4.35248	50	15.6
2i	Cl	$(CH_3)_3C$	H	303.10	3.69/4.21119	> 100	*
2j	Cl	$(CH_3)_3C$	3-CF_3	371.10	4.61/5.09419	> 100	*
3a	H	H	H	228.10	0.63/1.64464	-	45.9
3b	H	H	3-Cl	262.10	1.19/2.35764	-	5.8
3c	H	H	3,4-Cl	296.04	1.75/2.95064	-	2.2
3d	H	H	3-CF_3	296.10	1.56/2.52764	-	6.1
3e	H	H	4-Cl	262.10	1.19/2.35764	-	5.7
3f	H	H	2-CH_3	242.30	1.12/2.09364	-	58.5
PZA	-	-	-	107.02	-1.31/-0.67632	6.25	-
DCMU	-	-	-	232.02	2.76/ 2.69124	-	1.9

- 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*



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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 δ 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_{50} values, *i.e.* by molar concentration of the compounds causing 50% decrease in the oxygen evolution rate relative to the untreated control. The comparable IC_{50} value for a selective herbicide 3-(3,4-dichlorophenyl)-1,1-dimethylurea, DCMU (Diurone®) is 1.9 μmol/L. The results are summarized in **Table 1**.

References:

1. Doležal, M. Biologically active pyrazines of natural and synthetic origin. *Chem. Listy* 2006, 100, 959-966.
2. Buchbauer, G.; Klein, C. Th.; Wailzer, B.; Wolschann, P. Quantitative Structure-Activity-Relationships of Pyrazines with Bell Pepper Flavor. *J. Agric. Food Chem.* 2000, 48, 4273-4278.
3. Chi-Kuen Shu Pyrazine Formation from Serine and Threonine. *J. Agric. Food Chem.* 1999, 47, 4332-4335.
4. Gezginci, M. H.; Martin, A. R.; Franzblau, S. G. Antimycobacterial activity of substituted isosteres of pyridine and pyrazinecarboxylic acids. *J. Med. Chem.* 2001, 44, 1560-1563.
5. Jeanjot, P.; Bruyneel, F.; Arrault, A.; Gharbi, S.; Cavalier, J.-F.; Abels, A.; Marchand, C.; Touillaux, R.; Rees, J.-F.; Marchand-Brynaert, J. *N*-(Alkyl)-2-amino-1,4-pyrazine Derivatives: Synthesis and Antioxidative Properties of 3- and 3,5-p-hydroxyphenyl-substituted Compounds. *Synthesis* 2003, 4, 513-522.
6. Cavalier, J.-F.; Burton, M.; De Tollenaere, C.; Dussart, F.; Marchand, C.; Rees, J.-F.; Marchand-Brynaert, J. 2,6-Diamino-3,5-diaryl-1,4-pyrazine Derivatives as Novel Antioxidants. *Synthesis* 2001, 5, 768-772.
7. Doležal, M.; Hartl, J.; Miletín, M.; Macháček, M.; Kráľová, K. Synthesis and photosynthesis-inhibiting activity of some anilides of substituted pyrazine-2-carboxylic acids. *Chem. Pap.* 1999, 53, 126-128.
8. Doležal, M.; Vičík, R.; Miletín, M.; Kráľová, K. Synthesis and antimycobacterial, antifungal, and photosynthesis-inhibiting evaluation of some anilides of substituted pyrazine-2-carboxylic acids. *Chem. Pap.* 2000, 54, 245-248.
9. Doležal, M.; Palek, L.; Vinšová, J.; Buchta, V.; Jampílek, J.; Kráľová, K. Substituted Pyrazinecarboxamides; Synthesis and Their Biological Evaluation. *Molecules* 2006, 11, 242-256.
10. Doležal, M.; Čmedlová, P.; Palek, L.; Vinšová, J.; Kuneš, J.; Buchta, V.; Jampílek, J.; Kráľová, K. Synthesis and antimycobacterial evaluation of substituted pyrazinecarboxamides. *Eur. J. Med. Chem.* 2008, 43, 1105-1113.
11. Ancizu, S.; Moreno, E.; Torres, E.; Burguete, A.; Pérez-Silanes, S.; Benítez, D.; Villar, R.; Solano, B.; Marín, A.; Aldana, I.; Cerecetto, H.; González, M.; Monge, A. Heterocyclic-2-carboxylic Acid (3-Cyano-1,4-di-*N*-oxidequinoxalin-2-yl)amide Derivatives as Hits for the Development of Neglected Disease Drugs. *Molecules* 2009, 14, 2256-2272.
12. Cynamon, M.H.; Speirs, R.J.; Welch, J.T. In vitro antimycobacterial activity of 5-chloropyrazinamide. *Antimicrob Agents Chemother.* 1998, 42, 462-463.
13. Doležal, M.; Zitko, J.; Kešetovičová, D.; Kuneš, J.; Svobodová, M. Substituted N-Phenylpyrazine-2-carboxamides: Synthesis and Antimycobacterial Evaluation. *Molecules* 2009, 14, 4180-4189.
14. Doležal, M.; Kráľová, K. Synthesis and evaluation of pyrazine derivatives with herbicidal aktivity. Chapter 27, p. 581–610. In "*Herbicides, Theory and Applications*" by Sonia Soloneski and Marcelo L. Larramendy (eds.), 27 chapters, 1st ed., Vienna, InTech, 2011, 610 p.
15. Masarovičová, E.; Kráľová, K. Approaches to measuring plant photosynthesis activity. In *Handbook of Photosynthesis* 2nd Ed.; Pessarakli M. Eds.; Taylor & Francis Group: Boca Raton, London-New York-Singapore, 2005; pp. 617–656.
16. Kráľová, K.; Šeršeň, F.; Sidóová, E. Photosynthesis inhibition produced by 2-alkylthio-6-R-benzothiazoles. *Chem. Pap.* 1992, 46, 348-350.